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# Diterpenoids from the Stem Barks of *Croton robustus*

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Three compounds were isolated from the stem barks of *Croton robustus*. Their structures were elucidated as trachyloban-19-oic acid, trachyloban-19-ol and poilaneic acid by spectroscopic analysis. Among them, trachyloban-19-ol and methyl trachyloban-19-oate exhibited weak cytotoxic activity against gastric carcinoma and colon carcinoma with $ED_{50}$ of 9.2, 9.6 and 8.3, 9.1 μg/mL, respectively.

**Key words:** *Croton robustus*, Trachyloban-19-oic acid, Trachyloban-19-ol, Poilaneic acid, Cytotoxic activity

## INTRODUCTION

*Croton robustus* Kurz. or *Croton siamensis* Craib. (Euphorbiaceae) was known as "Plao Lueat" in Thailand. It is scarcely distributed in the northern part of Thailand. *C. robustus* is a medium-sized tree and very similar to *Croton oblongifolius*. The characteristic of this plant is the red resinous material that flows out of the branches when they were cut off. In Thai Ethnopharmacopoeia, wood of *C. robustus* is used as antianemic agent, and the barks and leaves are used to stop bleeding and to treat skin diseases. Up to date, there is no report on the chemical constituents and biological activity of this plant in the literature. This paper describes the isolation and structural determination of the chemical constituents of this plant and their cytotoxic activity.

## MATERIALS AND METHODS

### General procedure

Melting points were determined with a Fisher Johns melting point apparatus and were uncorrected. IR spectra were recorded on KBr disc with Nicolet Impact 410 Spectrophotometer. $^1$H- and $^{13}$C-NMR were recorded with Bruker ACF 200 and JEOL JNM-A500 spectrometers. MS spectra were measured with Fisons Instruments Trio 2000 mass spectrometer. The optical rotation was measured with Perkin Elmer 241 polarimeter.

### Plant material

The stem barks of *C. robustus* was collected from Amphur Nakorn Thai, Pitsanuloke province, Thailand in December 1999. The sample was authenticated by comparison with the herbarium collection number BKF 022771 in the Royal Forest Department, Bangkok, Thailand.

### Extraction and isolation

The powdered, sun-dried stem barks of *C. robustus* (6 Kg) was extracted with hexane (2×10 L). The hexane extract was filtered and evaporated *in vacuo* to obtain a dark-yellow oil (60 g). The plant residue was then extracted with chloroform (2×10 L) and methanol (2×8 L) successively to yield crude chloroform extract as dark red oil (80 g) and crude methanol extract as dark-red gummy residue (8 g). The TLC analysis revealed that crude hexane extract and crude $CHCl_3$ extract contained basically the same components. The *n*-hexane extract (40 g) was separated by silica gel column chromatography (200 g) and eluted with *n*-hexane-chloroform mixture with stepwise increasing polarity. Compounds **1**, **4** and **2** were obtained from 5%, 15% and 50% $CHCl_3$ in *n*-hexane, respectively.

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**Trachyloban-19-oic acid (1)**

White solid, mp: 128-131 °C; $[\alpha]_D^{20}$-73.1° ($CHCl_3$, *c* 1.0); $^1$H-NMR (500 MHz, $CDCl_3$) $\delta_H$ (ppm): 0.58 (1H, dt, *J* = 2.4, 7.9 Hz, H-12), 0.78 (1H, dd, *J* = 4.0, 13.1 Hz, H-1), 0.82 (1H, dd, *J* = 3.3, 7.9 Hz, H-13), 0.88 (3H, s, $CH_3$-20), 0.99 (1H, ddd, *J* = 4.3, 13.4, 13.4 Hz, H-3), 1.00 (1H, dd, *J* = 2.8, 11.0 Hz, H-5), 1.08 (1H, br., H-9), 1.14 (3H, s, $CH_3$-17), 1.19 (1H, m, H-14), 1.21 (3H, s, $CH_3$-18), 1.23 (1H, d, *J* = 11.3 Hz, H-15), 1.32 (1H, dt, *J* = 4.9, 13.1 Hz, H-7), 1.39 (1H, d, *J* = 11.3 Hz, H-15), 1.45 (1H, dt, *J* = 3.4, 6.7 Hz, H-7), 1.67 (1H, ddd, *J* = 2.4, 7.3, 14.7 Hz, H-11), 1.72 (1H, ddd, *J* = 1.2, 9.2, 12.4 Hz, H-6), 1.75 (1H, dt, *J* = 2.7, 6.1 Hz, H-6), 1.84 (1H, ddd, *J* = 6.4, 10.1, 13.7 Hz, H-2), 1.87 (1H, ddd, *J* = 3.1, 3.1, 11.4 Hz, H-11), 2.12 (1H, ddd, *J* = 3.4, 3.4, 7.3 Hz, H-3); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta_C$ (ppm): 184.5 (C-19), 57.0 (C-5), 52.8 (C-9), 50.3 (C-15), 43.7 (C-4), 40.7 (C-8), 39.4 (C-1), 39.2 (C-7), 38.9 (C-10), 37.8 (C-3), 33.1 (C-14), 28.9 (C-18), 24.3 (C-13), 22.4 (C-16), 21.7 (C-6), 20.5 (C-12), 20.5 (C-17), 19.7 (C-11), 18.7 (C-2), 12.4 (C-20); IR $\nu_{max}$ (KBr) cm$^{-1}$: 3300-2400 (br, OH), 1695 (s, C=O), 1460 (m), 1265 (m); EIMS m/z: 302 [M$^+$] (100), 287 (30), 260 (12), 257 (10), 246 (80), 231 (30).

**Trachyloban-19-ol (2)**

White solid, mp: 130-131°C; $[\alpha]_D^{20}$-42.2° ($CHCl_3$, *c* 1.0); $^1$H-NMR (500 MHz, $CDCl_3$) $\delta_H$ (ppm): 0.54 (1H, dt, *J* = 2.4, 7.9 Hz, H-12), 0.72 (1H, dd, *J* = 3.7, 13.4 Hz, H-1), 0.78 (1H, dd, *J* = 3.1, 7.9 Hz, H-13), 0.86 (1H, dd, *J* = 1.5, 3.7 Hz, H-5), 0.88 (1H, m, H-3), 0.89 (3H, s, $CH_3$-20), 0.91 (3H, s, $CH_3$-18), 1.08 (1H, m, H-9), 1.10 (3H, s, $CH_3$-17), 1.12 (1H, m, H-14), 1.13 (1H, m, H-6), 1.21 (1H, d, *J* = 11.6 Hz, H-15), 1.28 (1H, dd, *J* = 3.4, 9.2 Hz, H-7), 1.31 (1H, m, H-2), 1.35 (1H, d, *J* = 11.3 Hz, H-15), 1.40 (1H, dd, *J* = 3.7, 6.7 Hz, H-7), 1.48 (1H, m, H-2), 1.54 (1H, m, H-6), 1.61 (1H, ddd, *J* = 2.4, 7.0, 14.6 Hz, H-11), 1.73 (1H, ddt, *J* = 1.5, 1.8, 11.9 Hz, H-3), 1.85 (1H, ddd, *J* = 3.1, 11.3, 1.6 Hz, H-11), 2.00 (1H, d, *J* = 11.9 Hz, H-14), 3.39 (1H, dd, *J* = 0.9, 10.9 Hz, H-19), 3.69 (1H, d, *J* = 10.7 Hz, H-19); $^{13}$C-NMR (125 MHz, $CDCl_3$) $\delta_C$ (ppm): 65.6 (C-19), 56.8 (C-5), 53.4 (C-9), 50.3 (C-15), 40.7 (C-8), 39.4 (C-7), 39.3 (C-1), 38.3 (C-4), 38.2 (C-10), 35.7(C-3), 33.4 (C-14), 26.8 (C-18), 24.2 (C-13), 22.4 (C-16), 20.6 (C-12), 20.5 (C-17), 20.3 (C-6), 19.9 (C-11), 17.8 (C-2), 15.1 (C-20); IR $\nu_{max}$ (KBr) cm$^{-1}$: 3450 (s, OH), 2930 (s), 2858 (s), 1640 (m), 1440 (m), 1370 (m), 1035 (m); EIMS *m/z*: 288 [M+] (100), 273 (20), 271 (5), 257 (80), 217 (30).

**Poilaneic acid (4)**

Transparent oil; $[\alpha]_D^{20}$-128.9° ($CHCl_3$, *c* 1.0); $^1$H-NMR (200 MHz, $CDCl_3$) $\delta_H$ (ppm): 5.0-6.2 (4H), 3.05 (1H, m), 1.5-2.5 (11H, m), 1.82 (3H, s), 1.62 (3H, s), 0.80-0.84 (6H); $^{13}$C-NMR (50 MHz, $CDCl_3$) $\delta_C$ (ppm): 173.3 (C-20), 147.6 (C-19), 135.1 (C-18), 131.3 (C-17), 130.9 (C-16), 130.4(C-15), 128.8 (C-14), 127.9 (C-13), 125.7 (C-12), 47.8 (C-11), 38.8 (C-10), 32.7(C-9), 32.1 (C-8), 28.9 (C-7), 26.2 (C-6), 25.8 (C-5), 20.9 (C-4), 19.9 (C-3), 19.3 (C-2), 14.4 (C-1); IR $\nu_{max}$ (neat) cm$^{-1}$: 3450 (s), 2930 (s), 2858 (s), 1640 (m), 1440 (m), 1370 (m), 1035 (m), 1460 (m), 1265 (m); EIMS m/z: 302 [M$^+$] (100), 259 (22), 213 (10), 119 (22), 107 (22), 105 (85), 93 (80).

**Cytotoxic assays by MTT method (Twentyman *et al.*, 1987)**

Bioassay of cytotoxic activity against human cell cultures *in vitro* was performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] colorimetric method. Each isolate was evaluated in a test for cytotoxicity against BT474 (breast carcinoma), CHAGO (lung carcinoma), HEP-G2 (hepatocarcinoma), KATO-3 (gastric carcinoma), and SW620 (colon carcinoma). Doxorubicin hydrochloride was used as a positive control.

## RESULTS AND DISCUSSION

The chromatographic separation of the *n*-hexane soluble fraction from *C. robustus* led to the isolation of trachyloban-19-oic acid (**1**), trachyloban-19-ol (**2**) and poilaneic acid (**4**).

Compound **1** was obtained as white solid from $CHCl_3$-hexane (300 mg, 0.005% wt/wt of dried stem bark). The IR spectrum of **1** revealed the presence of carboxylic group according to the broad absorption band between 3300 to 2400 cm$^{-1}$ and the strong absorption band at 1965 cm$^{-1}$ due to the carboxylic acid carbonyl stretching. The $^1$H-NMR spectrum of **1** showed the presences of doublets at 1.23 and 1.39 ppm, doublet of doublets at 0.82 and 1.00 ppm, doublet of doublet of doublets at 0.99, 1.67, 1.72, 1.84, 1.87 and 2.12 ppm, doublet of triplets at 0.58, 1.32, 1.45 and 1.75 ppm and three tertiary methyl singlets [$\delta_H$ 0.88 (s, 3H-20), 1.14(s, 3H-17) and 1.21(s, 3H-18)].

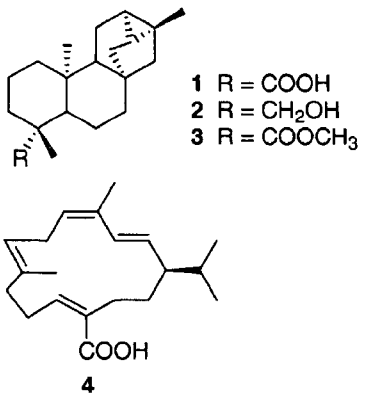


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**Fig. 1.** Structures of compounds **1-4**

The $^{13}$C-NMR spectrum showed 20 signals. One signal at 184.5 ppm belonged to a carboxyl group. The DEPT experiments indicated the presence of four saturated methines at 57.0, 52.8, 24.3 and 20.5 ppm, three methyl carbons at 28.8, 20.5 and 12.4 ppm, eight methylene carbons at 50.3, 39.4, 39.2, 37.8, 33.1, 19.7 and 18.7 ppm. Therefore, the carbon signals at 184.5, 43.7, 40.7, 38.9 and 22.4 ppm were quaternary carbons.

Compound **1** showed a molecular ion at m/z 302 ($C_{20}H_{30}O_2$) which indicated the double bond equivalence (DBE) of 6. The NMR data precluded the possibility of unsaturation, therefore, **1** must have a pentacyclic skeleton. The presences of doublet of doublet signal at $\delta_H$ 0.82 ($J$= 3.36 and 7.94 Hz) and doublet of triplet signal at $\delta_H$ 0.58 ($J$ = 2.4, 7.9 Hz) were typical of a cyclopropane system and suggested that Compound **1** belongs to the trachylobane series of diterpenes. The information from 2D-NMR techniques including HMQC, HMBC, COSY and NOESY correlation (data not shown) were used to identify the structure of Compound **1** as trachyloban-19-oic acid (Pyrek J., 1970).

Compound **2** was obtained as white solid from $CHCl_3$-hexane (40 mg, 0.0006% wt/wt of dried stem bark). The IR spectrum of **2** showed the presence of a hydroxyl group according to the broad and strong absorption band at 3500 to 3200 cm$^{-1}$. The molecular formula of **2** was assigned to be $C_{20}H_{32}O$ from EIMS [M$^+$] (m/z = 288) which indicated 5 DBE. The $^{13}$C-NMR spectrum of **2** was similar to that of **1** except for the downfield position of C-19 (65.6 ppm) compared with that of **1** (184.5 ppm). $^1$H-NMR spectrum showed doublet of doublet signals ($\delta_H$ 3.39, $J$ = 0.9, 11.0 Hz) and doublet signal ($\delta_H$ 3.69, $J$ = 10.7 Hz) of $H_2$-19. Comparison of spectral data including $^1$H-NMR, $^{13}$C-NMR, HMQC, HMBC, COSY and NOESY correlation, of this compound with those of **1** demonstrated that **2** differed from **1** only in having a hydroxyl group attached to C-19. Based on the spectral data discussed above the structure of **2** was assigned to be trachyloban-19-ol (da Costa *et al.*, 1996).

Methylation of compound **1** (200 mg) with diazomethane in ether gave quantitatively the corresponding methyl ester **3** (209 mg) as a white solid, mp: 98-100°C, $[\alpha]_D^{20}$-73.8° ($CHCl_3$, *c* 1.0) [(da Costa *et al.*, 1996), mp. 93-95°C; $[\alpha]_D^{25}$-68.48° ($CHCl_3$, *c* 1.55)]. Reduction of compound **3** with $LiAlH_4$ in dry $Et_2O$ and purification by $SiO_2$ column chromatography gave the corresponding alcohol with identical spectroscopic data with those of **2**.

Compound **4** was obtained as transparent oil from $CHCl_3$-*n*-hexane (60 mg, 0.001% wt/wt of dried stem bark). The IR spectrum of **4** showed the presence of a carboxylic group to the broad absorption band between 3500 to 2200 cm$^{-1}$ and the strong absorption band at 1697 cm$^{-1}$ due to the carboxylic acid carbonyl stretching.

The $^1$H-NMR spectrum of Compound **4** possessed an isopropyl group at $\delta$ 0.80 (3H), and 0.84 (3H), two olefinic methyl groups at $\delta$ 1.66 and 1.82, and five olefinic protons at $\delta$ 5.0-6.2. The $^{13}$C-NMR spectrum showed 20 signals which the carboxyl group of carboxylic acid corresponding to the signal at 173.3 ppm. The eight signals of olefinic carbons appeared at 147.6, 135.1, 131.3, 130.9, 130.4, 128.8, 127.9 and 125.7 ppm. The DEPT experiments indicated the presence of five sp$^2$ methine carbons at 147.6, 131.3, 130.4, 127.9 and 125.7 ppm, two saturated methines at 47.8 and 32.7 ppm, five methylene carbons at 38.9, 32.1, 28.9, 26.2 and 25.8 ppm, and four methyl carbons at 20.9, 19.9, 19.3 and 14.4 ppm. Therefore, the carbon signals at 173.3, 135.1, 130.9 and 128.8 ppm were quaternary carbons.

Compound **4** showed a molecular ion at *m/z* = 302 ($C_{20}H_{30}O_2$) which indicated DBE of 6. Therefore, **4** must consist of one ring in addition to the four double bonds and a carboxyl group. These data indicated that **4** could be a cembranoid possessing a 14-membered ring diterpene skeleton. By searching through the information in the literature, 4 was assigned as poilaneic acid which was previously isolated from *C. poilanei* (Sato *et al.*, 1981).

For the cytotoxic activity evaluation, only compounds **2** and **3** exhibited weak cytotoxic activity against KATO-3 and SW620 at $ED_{50}$ 9.2, 9.6 and 8.3, 9.1 µg/mL, respectively. Compounds **1** and **4** were inactive at 10 µg/mL. Under the same test condition, doxorubicin hydrochloride exhibited cytotoxic activity against KATO-3 and SW620 at $ED_{50}$ 1.7 and 1.1 µg/mL, respectively.

Compounds **1** and **2** have been reported in many plant species and they exhibit various biological activities, for example, compounds **1-3** have been shown to possess moderate trypanomicidal activity against *T. cruzi* (da Costa *et al.*, 1966). Compound **1** is reported as a larvicidal factor in the florets of a sunflower (*Helianthus annus*) cultivar resistant to sunflower moth (*Homoeosoma electellum*) (Waiss *et al.*, 1977). It also strongly inhibits the growth of *Heliothis zea* and *Pectinophora gossypiella larvae* (Elliger *et al.*, 1976). Moreover, it has been shown to possess contractile activity of uterine muscle (Ponce-Monter *et al.*, 1999). Compound **4** is shown to have inhibitory activity on cAMP phosphodiesterase (Roengsumran *et al.*, 2002). Therefore, this work reveals the chemical constituents and their weak cytotoxicity of this plant for the first time. Moreover, other biological activities could be realized from its chemical constituents. These compounds trachyloban-19-oic acid (**1**) and trachyloban-19-ol (**2**) were isolated for the first time from genus *Croton*.

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