Cembrane type diterpenoids from the leaves of *Croton oblongifolius* Roxb. and their bioactivities

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Abstract

Current study focused on the isolation, characterization and evaluation of biological activities of secondary metabolites from *C. oblongifolius*. Two cembrane type diterpenes; crotocembranal and crotocembraneic acid were isolated. Structures of the compounds were elucidated by analyzing spectroscopic data. Cytotoxicity, cancer chemopreventive properties antibacterial activities were tested. Both compounds showed mild cytotoxicity towards the cancer cell line tested. Crotocembraneic acid exhibited minimum inhibitory concentration of (MIC) of 25 μg/mL for *Mycobacterium tuberculosis H37Ra and* 100 μg/mL for *Staphylococcus epidermidis*. Crotocembranal was inactive as an anti-infective agent.

Introduction

Croton is a genus which belongs to the family Euphorbiaceae covering over 1300 species widespread in tropical regions of the world. Several species have an extended role in the traditional medicine systems in Asia, Africa and South America.

The plant *C. oblongifolius* Roxb., is a middle-sized tree and grows in India, Sri Lanka and Thailand. It has been used in the traditional Thai medicine to alleviate dysmenorrhea (fruits), as a purgative (seeds), and to treat dyspepsia (bark) and dysentery (roots). Even though Seshadri *et al* extensively investigated the metabolites of *C. oblongifolius, Roengsumran et al* reported that the same plant when collected in Thailand contains different constituents, possibly due to geographical variation-. Two cembrane type diterpenoids namely, crotocembranal and crotocembraneic acid were isolated from the current study. Hundreds of cembranoids from plants, insects as well as of marine origin have been isolated to date. The notable biological property of this group of compounds is the cytotoxicity. In addition, they have also been reported to have numerous biological activities such as anti-inflammatory, neuroprotective, calcium-antagonistic, anti-arthritic and antimicrobial effects. Herein report the isolation, characterization and biological activities of two cembrane type diterpenoids crotocembranal and crotocembraneic acid which were isolated from the leaves of *C. oblongifolius* Roxb.

Materials and Methods

General experimental procedures

UV spectra were obtained from a Shimadzu UV-1700 PharmaSpect UV–Vis spectrophotometer. IR spectra were acquired with a Perkin Elmer Spectrum ONE spectrophotometer using Attenuated Total Reflectance (ATR) technique. NMR spectra were recorded on a Bruker Avance 300 (^{1}H at 300 MHz, ^{13}C at 75 MHz). HRESI-MS spectra were obtained from a Bruker Micro-TOF mass spectrometer. Column chromatography was packed with Sephadex LH-20 (GE Health Care Bio-Sciences AB).

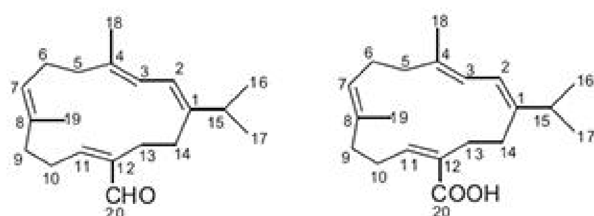
Plant material

Leaves of *C. oblongifolius* were collected from Nakhonsawan province, Thailand, in October 2014. The plant, *C. oblongifolius,* was previously authenticated by Panarat Charoenchai, and the specimen (no. CRI 285) was deposited at the Laboratory of Natural Products Chulabhorn Research Institute, Bangkok, Thailand.

Extraction and isolation

Powdered, air-dried leaves (0.8 kg) of *C. oblongifolius* were macerated with dichloromethane to yield a crude extract of 37.6 g. The crude extract was subjected to silica gel column chromatography (SGCC) (10×56 cm), a stepwise gradient elution was performed with hexane:CH_2Cl_2 and CH_2Cl_2:MeOH stepwise gradients and yielded 19 fractions (A-1 to A-19). Fractions A7 and A8 which had similar TLC patterns and proton nuclear magnetic resonance (^{1}H NMR) spectra were combined (658.8 mg) and separated by SGCC (2x44

cm), eluted with hexane:CH_2Cl_2 (4:6) to obtain 8 fractions (B1-B8). Fractions B4 and B5 which had similar TLC patterns and ^1H NMR spectra were combined (57.1 mg), and further separated by SGCC (2x44 cm) eluted with a gradient solvent system of hexane:CH_2Cl_2 giving crotocembranal (compound **1**) (20.2 mg). Fraction A11 (840.2 mg) was separated by Sephadex LH-20 column chromatography (3x100 cm), to obtain 11 fractions (C1-C11). Fractions C-10 and C-11 were combined (395 mg) and further separated using SGCC (1.5×17 cm), eluted with a gradient solvent system of hexane:CH_2Cl_2, to yield 14 fractions (D-1 to D-14). Fraction D5 to D7 gave crotocembraneic acid (compound 2) (77.7 mg).



Biological activity

Biological activities of the isolated compounds were evaluated at the Central Research Facility for Bioassays-Chulabhorn Research Institute, Natural Products Laboratory-Chulabhorn Research Institute, and Ramathibodi Hospital, Mahidol University, Thailand. Procedures for the determination of respective biological activities are briefly describe below.

Cytotoxic activity

Cytotoxic activities against adhesive cell lines; HepG2 (human hepatocellular carcinoma cell line), HUCCA-1 (cholangiocarcinoma cell line), and A549 (human adenocarcinomic alveolar epithelial cell line) were evaluated using the MTT assay. XTT assay was used for the assessment of cytotoxicity of non-adhesive cell line MOLT-3 (human acute lymphoblastic leukemia-T cell type). Etoposide and doxorubicin were used as the reference drugs.

Chemoprevention activity

Scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals

The potential for scavenging free radicals was determined photometrically by reacting the test samples (dissolved in DMSO) with DPPH, as described by Gerhauser *et al* . Ascorbic acid (vitamin C) was used as the reference compound, exhibiting an IC_{50} value of 21.2 µM.

Measurement of oxygen radical absorbance capacity (ORAC)

Peroxyl or hydroxyl radical absorbance capacity of test sample was tested by a modified ORAC assay, following the method previously described by Gerhauser *et al* . Results were expressed as ORAC units, where 1 ORAC unit equals the net protection of β-phycoerythrin (β-PE) produced by 1 µM trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a water soluble vitamin E analog. Only scavenging capacities of more than 1 ORAC unit were considered as positive.

Aromatase inhibitory assay (AIA)

The inhibition of aromatase was performed according to the method previously described by Stresser *et al.* using a CYP19/methoxy-4-trifloromethyl-coumar-in (MFC) high throughput inhibition screening kit. Ketoconazole, which typically has IC_{50} value of 2.4 µM, was used as the reference compound.

Inhibition of superoxide anion radical formation by xanthine/xanthine oxidase (X/XO assay)

The formation of superoxide anion radicals by X/XO was performed by following the method described by Gerhauser *et al* . Superoxide dismutase (30 U/mL) was used as the control. Allopurinol, a known inhibitor of xanthine oxidase , was used as a positive control with an IC_{50} value of 3.0 µM.

Antibacterial activity

Minimum inhibitory concentration (MIC) of the compound was tested on gram positive (*Bacillus cereus*, *Enterococcus faecalis, Staphylococcus aureus*, and *Staphylococcus epidermidis*), and gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Shigella flexneri*) bacteria by broth microdilution method described by Andrews *et al* . The compound was dissolved in dimethyl sulfoxide (DMSO), and a two-fold serial dilution in 96-well plates was carried out. Bacterial suspensions were prepared in normal saline solution and adjusted to a turbidity of the 0.5 McFarland standard. Final concentration of the DMSO did not exceed 0.5% (v/v). The plates were incubated at 36 °C (±1) for 20 h, and the absorbance was measured at 600 nm to determine the MICs of the tested compounds. Chloramphenicol, tetracycline and vancomycine were used as the standard drugs. The MIC is defined as the lowest concentration of the compound that inhibits the growth of microorganism.

Antimycobacterial activity

Microplate alamar blue assay (MABA) was used to determine the antimycobacterial activity of compounds and Mycobacterium tuberculosis H37Ra (non-virulent strain) was used as the test organism. Compounds were individually dissolved in DMSO, and they were diluted two folds in 100 μL of Middlebrook 7H9GC medium in 96-well plates. A mycobacterial suspension was prepared in 0.04 % Tween 80 and diluted with sterile distilled water to a turbidity of the 1.0 McFarland standard. The mycobacterial suspension was subsequently diluted 1:50 with 7H9G medium, and 100 μL was added to the wells. The highest final concentration of DMSO was 0.156 % (v/v). The plates were incubated at 37 ºC (±1) for approximately 7 days. Then 12.5 μL of 20 % Tween 80 and 20 μL of alamar blue (SeroTec Ltd., Oxford, United Kingdom) were added to all wells. The growth of the mycobacteria was determined after reincubation at 37 ºC for 16 to 24 h by visual determination of a color change from blue to pink. The MIC is defined as the lowest concentration of the compound which prevents the color change. Rifampicin was used as the reference drug.

Results

Structure elucidation and characterization of compounds 1 and 2

Compound **1** was a yellow coloured oil. The high resolution electrospray ionization mass spectrometry (HRESI-MS) showed a pseudomolecular ion peak at *m/z* of 287.2309 $(M+H)^+$, calcd. m/z 287.2312 for $(C_{20}H_{30}O+H)^+$, suggesting the molecular formula of the compound **1** as $C_{20}H_{30}O$. The infra-red (IR) spectrum of the compound exhibited strong absorption peaks for C-H stretching at 2962 and 2929 cm^{-1}, and a carbonyl peaks at 1713 cm^{-1}. The ultraviolet (UV) spectrum of the compound showed absorption peaks at 368, 228.5 and 204.5 nm.

The proton nuclear magnetic resonance (^{1}H NMR spectrum) (300 MHz, acetone-d_6) (**Figure 1**) of compound **1** indicated the presence of four methyl protons at δ *1.05* (s, H_3-16 and 17) and 1.72 (s, H_3-18 and 19), six methylene protons which resonate between δ 2.16- 2.53, one sp^3 methine protons that resonate at δ 2.42 (m, H-15), four sp^2 methine protons that resonate at δ 5.16 (t, J = 6.4, H-7), 5.89 (d, J = 11.0, H-3), 5.97 (d, J = 11.0, H-2), and 6.43 (t, J = 7.7, H-11) ppm. Moreover, proton signal of an aldehyde was observed at δ *9.28 (s, H-20).*

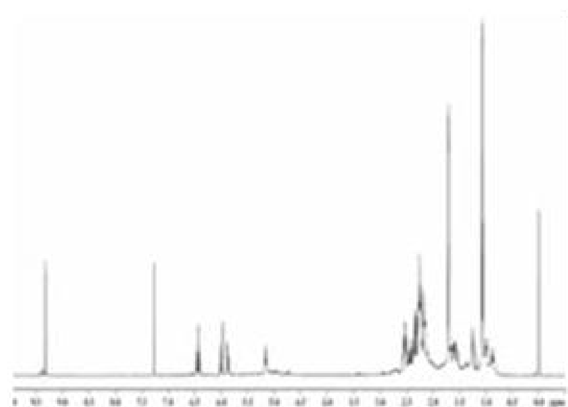


Figure 1 ^{1}H NMR spectrum of compound **1**

The carbon-13 NMR (^{13}C NMR) spectrum (75 MHz) (**Figure 2**) of the compound displayed signals for 19 carbons. Distortionless enhancement by polarization transfer (DEPT) spectral data classified these carbons as six methine carbons, six methylene carbons, four methyl carbons, and four non-protonated carbons. Analysis of the chemical shifts of ^{13}C NMR spectrum indicated the presence of five sp^2 methine carbons, one sp^3 methine carbons, six sp^3 methylene carbons, four methyl carbons, and four sp^2 non-protonated carbons. The carbons resonance at δ *118.4 (C-2), 119.9 (C-1), 129.0 (C-7), and 155.0 (C-1) were* sp^2 methine carbons. Carbon resonances at δ 134.1 (C-8), 135.5 (C-4), 143.9 (C-12), and 146.3 (C-1) were sp^2 non-protonated carbons. Moreover, a carbons resonance at δ *196.0* (C-20) was a carbonyl carbon of an aldehyde. The heteronuclear single quantum coherence (HSQC) spectral data were used to assign the protons being attached to carbon (**Table 1**). The core structure of the compound **1** was established by analyzing the proton correlation spectroscopy (^{1}H-^{1}H COSY), heteronuclear multiple bond correlation (HMBC), and nuclear overhauser enhancement spectroscopy (NOESY) spectral data.

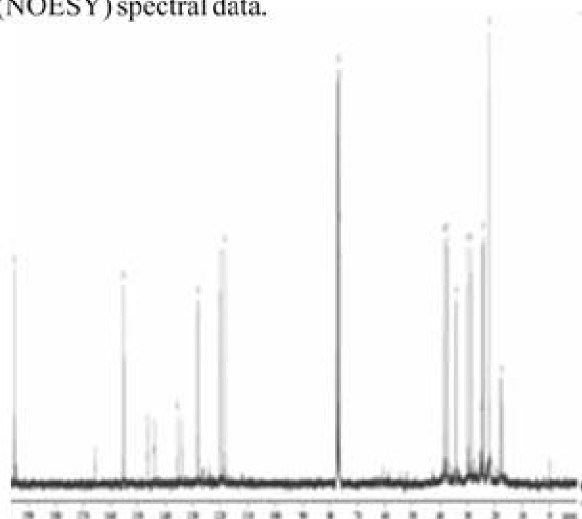


Figure 2 ^{13}C NMR of compound **1**

Establishment of a core structure of compound 1

The ^{1}H-^{1}H COSY spectrum of 1 established the fragments of H-2/H-3; H-5/H-6/H-7; H-9/H-10/H-11; H-13/H-14; and H-15/H-16/H-17. The cyclic core structure of compound 1 was established by analyzing the HMBC correlations including H-2 toC-1, C-4, and C-15; H-3 to C-5 and C-18; H_2-6 to C-4and C-7; H-7 to C-5, C-9 and C-19; H_2-10 to C-8, C-11, and C-12;H-11 to C-10,C-13 and C-20;and H_2-14 to C-1 and C-15. The position of the aldehyde group was determined by the HMBC correlations of both H-11 and H_2-13 to C-20 and an aldehyde proton H-20 to C-11, C-12, and C-13.

The ^{1}H NMR signal of H-2 was a doublet, coupling with H-3. The coupling constant 11.0 Hz indicated a *trans* double bond (*E* geometry) of the H-2/H-3 double bond. This was further confirmed by the NOESY correlations between H-2 andH_3-16, H_3-17, and H_3-18. Moreover, the NOESY correlations H-3 and H_2-5 and H-7 and H_2-9 suggested an *E* geometry of C-3/ C-4 and C-7/C-8 double bonds, while a correlation between H-11 and H_2-13 suggested a *Z* geometry of C-11/C-12 double bond. On the basis of these spectral data core structure of compound **1** was established.

Compound 2 was a white solid, and had a molecular formula of $C_{20}H_{30}O_2$ as determined by atmospheric pressure chemical ionization coupled time of flight mass spectrometry (APCI-TOF MS). Its IR spectrum showed absorption bands at 1713 cm^{-1} and 3422 cm^{-1} of carbonyl and hydroxyl functionalities, respectively. The ^{1}H NMR spectrum of compound **2** was similar to compound 1 except the missing signal for an aldehyde proton (Figure 3).

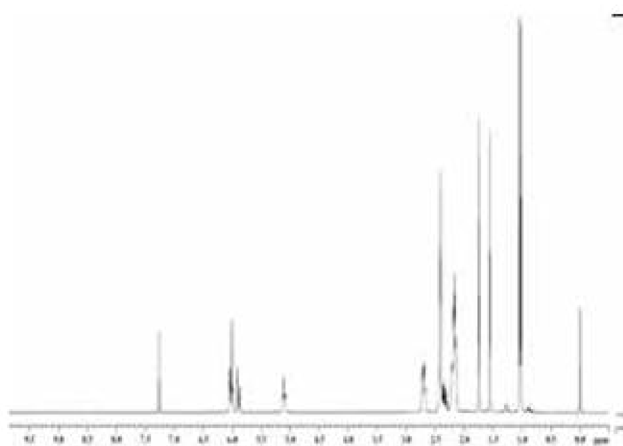


Figure 3 ^{1}H NMR spectrum of compound **2**

Moreover, ^{13}C NMR spectral data revealed that the carbonyl carbon (δ 194.0) of an aldehyde group in compound 1 has been replaced by a carboxylic acid group in compound 2 (δ 174.7) (Figure 4).

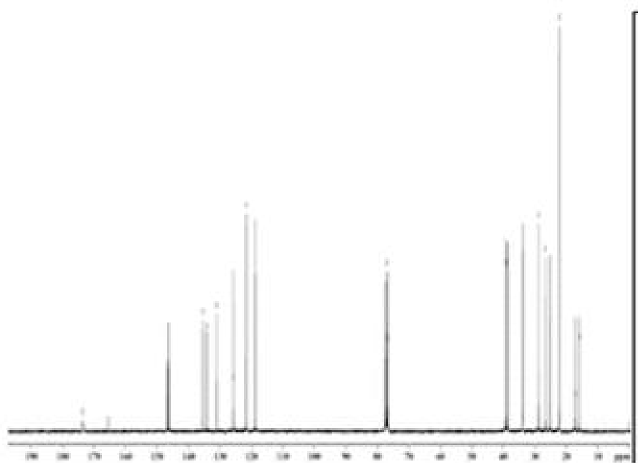


Figure 4 ^{13}C NMR of compound 1

Similar to compound 1, the core structure of compound 1 was established with the aids of ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY spectral data (**Table 1**).

Table ^{1}H and ^{13}C NMR data of compounds 1 and 2 in deuterated chloroform ($CDCl_3$)

Position	1		2	
	δ_C (ppm)	δ_H (ppm), multiplicity (*J* in Hz)	δ_C (ppm)	δ_H (ppm), multiplicity (*J* in Hz)
1	146.3, C	-	146.4, C	-
2	118.4, CH	5.97 (d, *J* = 11.0)	118.8, CH	6.02, d (10.5)
3	119.9, CH	5.89 (d, *J* = 11.0)	121.6, CH	5.89, d (11.3)
4	135.5, C	-	135.2, C	-
5	38.5, CH_2	2.28 (m)	39.2, CH_2	2.15, m
6	37.4, CH_2	2.16 (m)	25.1, CH_2	2.20, m
7	129.0, CH	5.16 (t, *J* = 6.4)	125.7, CH	5.10, t (5.9)
8	134.1, C	-	134.0, C	-
9	24.8, CH_2	2.25 (m)	38.6, CH_2	2.15, m
10	30.0, CH_2	2.53 (m)	26.5, CH_2	2.70, m
11	155.0, CH	6.43 (t, *J* = 7.7)	146.7, CH	6.01, t (5.2)
12	143.9, C	-	130.9, C	-
13	24.1, CH_2	2.34 (m)	28.7, CH_2	2.39, m
14	28.7, CH_2	2.23 (m)	33.6, CH_2	2.40, m
15	34.0, CH	2.42 (m)	33.8, CH	2.35, m
16	22.1, CH_3	1.05 (d, *J* = 3.2)	22.1, CH_3	1.03, d (6.8)
17	22.1, CH_3	1.05 (d, *J* = 3.2)	22.1, CH_3	1.03, d (6.8)
18	18.0, CH_3	1.72 (s)	17.0, CH_3	1.74, s
19	17.3, CH_3	1.73 (s)	15.8, CH_3	1.54, s
20	194.0, C=O	9.32 (s)	174.7, C=O	-

300 MHz for ^{1}H and 75 MHz for ^{13}C, chemical shift (δ) in ppm

Crotocembranal (**1**): Yellow oil (20.2 mg); UV (EtOH) λmax (log ε): 368.0 (1.85), 228.5 (4.16), 204.5 (4.19) nm; IR (UATR) νmax: 3422, 2962, 2929, 1713, 1452, 1377, 1266, 1163, 1047, 983. 735, 703 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$), ^{13}C NMR (75 MHz, $CDCl_3$) see Table 1; ESI-TOF MS: calcd. for $C_{20}H_{31}O$, m/z 287.2312 (M+H)+, found 287.2309.

Crotocembraneic acid (**2**): White crystals (77.7 mg); melting point 105-107 °C; UV (EtOH) λmax (log ε): 204.0 (3.39) nm; IR (UATR) νmax: 3422, 2957, 2927, 1712, 1454, 1377, 1180, 1059, 736, 703 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$), ^{13}C NMR (75 MHz, $CDCl_3$) see Table 1; ESI-TOF MS: calcd. for $C_{20}H_{31}O_2$, m/z 303.2311 (M+H)+, found 303.2314.

Biological activities of the compounds 1 and 2

Compound 1 exhibited cytotoxic activity against MOLT-3, HepG2, HuCCA-1, and A549 cell lines with IC_{50} values of 16.09, 29.01, 30.60 and 48.15 µg/mL, respectively. However, compound 2 exhibited cytotoxic activity against MOLT-3 cell line with IC_{50} valueof 46.22 µg/mL, but it was not active against other cell lines.

Table 2 Cytotoxic activities of the compounds 1 and 2

Compound	IC_{50} values for cancer cell lines (µg/mL)			
	MOLT-3	HepG2	HuCCA-1	A549
1	16.09 ± 1.38	29.01 ± 2.58	30.60 ± 0.08	48.15 ± 0.04
2	46.22 ± 2.17	I	I	I
Etoposide	0.04 ± 0.01	24.32 ± 1.88	ND	ND
Doxorubicin Hydrochloride	ND	0.22 ± 0.03	0.33 ± 0.04	0.27 ± 0.02

Both compounds were inactive for cancer chemopreventive tests. Crotocembraneic acid exhibited antimycobacterial activity with a MIC of 25 µg/mL and antibacterial activity against *Staphylococcus epidermidis* with a MIC of 100 µg/mL. Crotocembranal did not show any activity for antibacterial and antimycobacterial tests.

Conclusions

The plant *Croton oblongifolius* Roxb. is a rich source of ditepene compounds with diverse structures including cembrane type diterpenoids. Compounds that belong to this class are produced by terrestrial and marine organisms and the most remarkable property is cytotoxicity. Even though crotocembranal showed mild cytotoxicity towards MOLT-3, HuCCA-1 and A549 cancer cell lines, it exhibited IC_{50} value of 29.01 for HepG2 which was in line with the standard drug etoposide. As an anti-infective agent, crotocembraneic acid is more effective compared to crotocembranal.

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