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A New Halimane Diterpenoid from *Croton crassifolius*

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Croton is a large genus of the Euphorbiaceae family, comprising approximately 1200 species of trees, shrubs and herbs distributed throughout tropical and subtropical regions of the world.[1] The plants of the genus *Croton* are well-known for producing a variety of diterpenoids with a wide range of biological activities.[2-6] *Croton crassifolius* Geisel. is a monecious undershrub widely distributed throughout the south and southeast China, Thailand, Vietnam, and Laos.[7] The roots of *C. crassifolius* has been used in folk and traditional Chinese medicine for treatment of stomachache, sore throat, and rheumatism.[8] In Thailand, it is used by indigenous people for treatment of cancer.[9] Previous chemical investigation on *C. crassifolius* revealed that the main constituents were sesquiterpoids and bicyclic diterpenoids.[9-13] As part of our continuing studies on the constituents of the traditional herbal medicines,[14,15] the present paper deals with the isolation and structural elucidation of a new halimane diterpenoid, named crassifoliusin A (**1**), along with a known halimane diterpenoid, chettaphanin I (**2**),[9,16] from the 95% aqueous EtOH extract of the roots of *C. crassifolius*. The cytotoxicity of compound **1** was evaluated against HepG2, SGC-7901, and K562 cell lines.

Compound **1** was obtained as yellow crystals from acetone and exhibited a quasi-molecular ion peak at *m/z* 357.1688 $[M + H]^+$ (calcd for $C_{21}H_{25}O_5$, 357.1697) in the HR-ESI-MS. Taking together with the analysis of the ^{1}H- and ^{13}C-NMR spectra, the molecular formula of **1** was deduced as $C_{21}H_{24}O_5$. The absorption bands in its IR spectrum suggested the presence of hydroxyl (3467 cm^{-1}), ester carbonyl (1722 cm^{-1}), conjugated carbonyl (1682 cm^{-1}), and furan ring moiety (1505, 870 cm^{-1}),[17,18] respectively. The ^{1}H-NMR spectrum displayed typical signals of one secondary methyl group at δ_H 1.15 (d, J = 5.4 Hz, H_3-17), two tertiary methyl groups at δ_H 1.51 (s, H_3-19), and 1.28 (s, H_3-20), the methoxyl of an ester at δ_H 3.79 (s), three ethylenic protons attributed to a β-substituted furan ring at δ_H 6.60 (brs, H-14), 7.37 (brs, H-15), and 8.17 (brs, H-16),[19] one olefinic proton at δ_H 6.51 (s, H-11), and a characteristic AB coupling system attributed to an isolated methylene group at δ_H 2.93 (d, J = 16.2 Hz, H-3a) and 2.69 (d, J = 16.2 Hz, H-3b). The ^{13}C-NMR and DEPT spectra of **1** showed 21 carbon signals, of which 20 were from the diterpene skeleton and one from the esterified methyl group. Signals for a β-substituted furan ring at δ_C 142.8 (C-16), 142.5 (C-15), 119.5 (C-13), and 110.1 (C-14) were observed. Other salient features of these spectra include signals assigned to an α,β-unsaturated ketone group at δ_C 193.8 (C-2), an ester carbonyl group at δ_C 175.6 (C-18), one tertrasubstituted double bond group at δ_C 132.3 (C-1), and 170.4 (C-10), one trisubstituted double bond group at δ_C 142.3 (C-11), and 130.2 (C-12), one oxygenated quaternary carbon at δ_C 75.8 (C-5). The remaining signals were due to three methyls, three methylenes, one methines, two quarternary carbons (Table 1). The ^{1}H-^{1}H COSY spectrum of **1** enabled extensive systems to be declineated. Vicinal

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of **1** in $CDCl_3$ (δ in ppm, J in Hz)

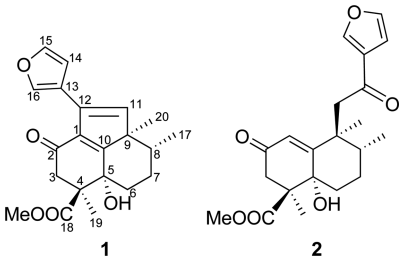
Position	δ_H	δ_C
1		132.3 (s)
2		193.8 (s)
3	2.93 (1H, d, 16.2) 2.69 (1H, d, 16.2)	47.7 (t)
4		52.3 (s)
5		75.8 (s)
6	1.75 (1H, m) 1.48 (1H, m)	38.2 (t)
7	1.97 (1H, m) 1.67 (1H, m)	26.7 (t)
8	1.61 (1H, m)	45.9 (d)
9		58.6 (s)
10		170.4 (s)
11	6.51 (1H, s)	142.3 (d)
12		130.2 (s)
13		119.5 (s)
14	6.60 (1H, brs)	110.1 (d)
15	7.37 (1H, brs)	142.5 (d)
16	8.17 (1H, brs)	142.8 (d)
17	1.15 (3H, d, 5.4)	18.4 (q)
18		175.6 (s)
19	1.51 (3H, s)	20.6 (q)
20	1.28 (3H, s)	12.5 (q)
OMe	3.79 (3H, s)	52.4 (q)
5-OH	2.75 (1H, s)	

and long-range coupling correlations from the methyl (H_3-17) to the methine (H-8), with the spin system continuing from H-8 through H_2-7 to H_2-6, indicated the presence of the structural unit CH_3(17)–CH(8)–CH_2(7)–CH_2(6). In the HMBC experiment, long-range correlations between the following protons and carbons were observed: H_3-19 and C-3, C-4, C-5, C-18; H_2-3 and C-1, C-2, C-4, C-5, C-18; H_2-6 and C-4, C-5, C-7, C-8, C-10; H_3-17 and C-7, C-8, C-9; H_3-20 and C-8, C-9, C-10, C-11; H-15 and C-13, C-14, C-16. These spectral data suggested the presence of a halimane diterpenoid skeleton with a *β*-substituted furan ring in **1**. The ^{13}C-NMR spectrum was similar to chettaphanin I (**2**),[9,16] except for the lack of a C-12 keto carbonyl group, instead of the presence of olefinic carbons at 142.3 (C-11), and 130.2 (C-12). The HMBC spectrum also showed correlations between H-11 and C-1, C-8, C-9, C-10, suggested that the presence of a cyclopentadiene ring formed by C-9, C-10, C-1, C-12 and C-11 in **1**.

The relative configuration of **1** was established by analysis of NOEs detected in difference NOE experiments and X-ray crystallographic diffraction. Difference NOE experiment irradiating H_3-17 enhanced the intensity of H_3-20. Additionally, irradiation of H_3-19 enhanced the intensities of OH-5 and H-3α (δ_H 2.69). Therefore, H_3-17 and H_3-20, OH-5, H_3-19 and H-3α should have a *cis* relationship, respectively. The structure and relative configuration of **1** were finally confirmed by a single-crystal X-ray diffraction (Fig. 3). Thus, compound **1** was assigned as 5α-hydroxy-15,16-epoxy-2-oxo-halima-1(10),11,13(16),14-tetraene-18-oic acid methyl ester, and named crassifoliusin A.

In addition, one known compound was isolated. By comparing its NMR spectroscopic data with those in the literature, its structure was determined to be chettaphanin I (**2**)[9,16] (Fig. 1).

The new compound **1** was tested for its cytotoxicity against three human cancer cell lines (HepG2, SGC-7901, and K562) using MTT method with Doxorubicin as the positive control. It showed no cytotoxicity against tested cell lines (IC_{50} > 50 μg/mL).



1 **2**

Figure 1. Structures of compounds **1** and **2**.

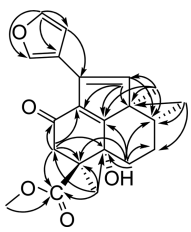


Figure 2. Key HMBC correlations (H→C) of compound **1**.

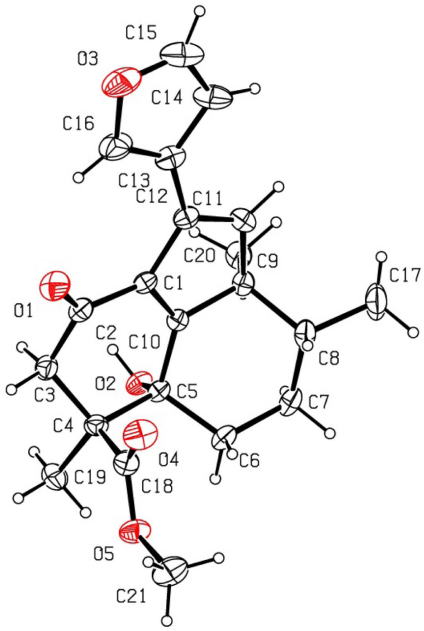


Figure 3. X-ray crystal structure of compound **1**.

Experimental Section

General Experimental Procedures. Melting points were determined on an X-4 digital display micromelting point apparatus, and are uncorrected. UV spectra were recorded on a Shimadzu UV-2550 UV-Vis spectrophotometer. Optical rotations were measured on a Perkin Elmer 341 polarimeter. IR spectra were taken on a Nicolet NEXUS 670 FT-IR spectrometer. NMR spectra were recorded on a Varian Mercury-600BB NMR spectrometers with TMS as internal standard. HR-ESI-MS data were recorded on a Thermo LTQ Orbitrap Elite mass spectrometer. Sephadex LH-20 were supplied by Amersham Pharmacia Biotech. Silica gel (200-300 mesh) used for column chromatography and silica gel GF_{254} (10-40 μM) used for TLC were supplied by the Qingdao Marine Chemical Factory, Qingdao, China. Spots were detected on TLC under UV light or by heating after spraying with 5% H_2SO_4 in C_2H_5OH (v/v).

Plant Material. The roots of *C. crassifolius* were purchased from Hebei Anguo Medicine Market, and were originally collected from Fujian province of China in September 2012. A voucher specimen (No. 201209CC) was deposited at the School of Pharmacy, Lanzhou University and was identified by Dr. Jian-Yin Li.

Extraction and Isolation. The air-dried and powdered roots of *C. crassifolius* (9.5 kg) were extracted four times with 95% aqueous EtOH at room temperature to give a residue (962 g) after evaporation. The residue was suspended in H_2O and extract with EtOAc and *n*-BuOH. The EtOAc partition (731 g) was applied to silica gel CC, eluting with petroleum ether-acetone (40:1–0:1 gradient system). Then

six crude fractions (A-F) were obtained by TLC analysis. Fraction B was subjected to silica gel CC eluting with petroleum ether-acetone (50:1 to 10:1) to give two subfractions (Fr.B1 and Fr.B2). The subfration Fr.B2 (6.5 g) was further applied to silica gel CC, and eluted with petroleum ether-EtOAc (20:1, 10:1, 5:1, 3:1) to give Fr.B2.1–Fr.B2.4. The Fr.B2.2 was subjected to CC on Sephadex LH-20 ($CHCl_3$-MeOH, 1:1) and silica gel (petroleum ether-acetone, 15:1 to 2:1) to yield compound **1** (5 mg). Fraction D was subjected to silica gel CC eluting with petroleum ether-acetone (20:1 to 3:1) to give two subfractions (Fr.D1 and Fr.D2). The subfration Fr.D2 (55 g) was further applied to silica gel CC, and eluted with petroleum ether-acetone (10:1, 5:1, 3:1) to give compound **2** (10 g).

Crassifoliusin A (1): Yellow crystals (acetone); mp 126-129 ºC; $[\alpha]_D^{26}$ –20º (*c* 0.1, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 216.2 (3.69) nm; IR (KBr) ν_{max} cm^{-1}: 3467, 2932, 2873, 1722, 1682, 1505, 1458, 1257, 1231, 1123, 870, 757; HR-ESI-MS *m/z*: 357.1688 $[M+H]^+$ (calcd for $C_{21}H_{25}O_5$, 357.1697); ^{1}H-NMR (600 MHz, $CDCl_3$) and ^{13}C-NMR (150 MHz, $CDCl_3$) spectral data are listed in Table 1.

X-ray Crystallographic Analysis of Compound 1. $C_{21}H_{24}O_5$, molecular weight (*M*w) = 356.40, orthorhombic, space group $P2_12_12_1$, a = 8.8900 (12) Å, b = 9.7794 (8) Å, c = 21.473 (3) Å, $\alpha = b = \gamma = 90^o$, V = 1866.9 (4) $Å^3$, Z = 4, Dc = 1.268 mg/m^3, μ(Mo Kα) = 0.090 mm^{-1}, F(000) = 760, and T = 292 K, crystal dimensions 0.35 × 0.31 × 0.11 mm was selected for X-ray analysis. The reflection data were collected on an Agilent Technologies SuperNova, Dual source, EOS CCD with mirror optics, using graphite-monochromated Mo-K*a* radiation (λ = 0.7107 Å). A total of 3310 reflections were collected in the range $3.0^o \leq \theta \leq 25.7^o$, of which 2108 unique reflections with $I > 2\sigma$ (I) were collected for the analysis. The structure was solved by direct methods using SHELXS97 and refined by full matrix least-squares on F^2. The final R and R_W factors were 0.060 and 0.128, respectively.

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Supporting Information. The spectral data of compound **1** are available on request from the correspondence author.

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