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New Monoterpenoid Coumarins from *Clausena anisum-olens*

Yun-Song Wang [1,2], Hong-Pin He [2], Jing-Hua Yang [1], Ying-Tong Di [2] and Xiao-Jiang Hao [2,*]

[1] Key Laboratory of Medicinal Chemistry for Natural Resource, Ministry of Education, School of Chemical Science and Technology, Yunnan University, Kunming 650091, P.R. China; E-mails: wangys@ynu.edu.cn (Yun-Song Wang); yangjh@ynu.edu.cn (Jing-Hua Yang)

[2] State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, P. R. China; E-mails: hehongping@mail.kib.ac.cn (Hong-Pin He), diyt@mail.kib.ac.cn (Ying-Tong Di)

* Author to whom correspondence may be addressed; E-mail: haoxj@mail.kib.ac.cn; Tel: +86-871-5223263/ Fax: 86-871-5219684

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Abstract: Two new monoterpenoid coumarins: anisucumarin A (**1**) and B (**2**), a pair of epimers, were isolated from *Clausena anisum-olens*. Their structures were established based on extensive spectroscopic analyses.

Keywords: Rutaceae; *Clausena anisum-olens*; anisucumarin A/B; monoterpenoid coumarins

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Introduction

The plants of the Rutaceae family are one of the richest sources of coumarins [1-6]. In this family, plants of *Clausena* genus are widely distributed in the south of China and many are used in Chinese traditional medicine [7]. Phytochemical studies on *Clausena* species have mainly focused on coumarins and carbazole alkaloids [4-9]. Some of the isolated coumarins showed interesting biological

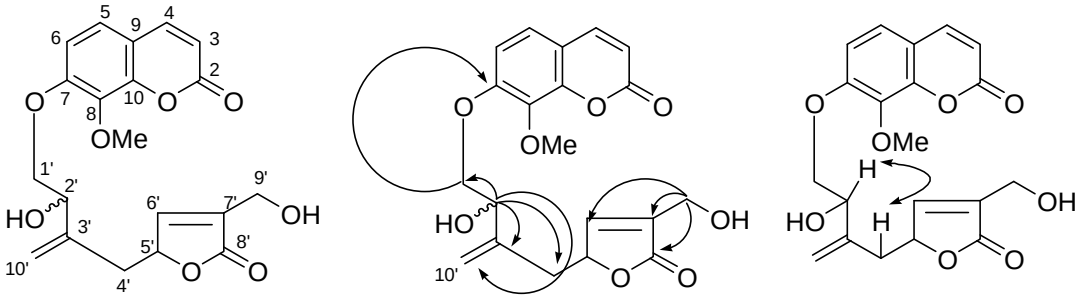
activity, for example, nordentatin displayed strong antibacterial activities [6] and the furanone-coumarin clauslatones A-J exhibited tumor-promotion inhibitory effects [5].

Clausena anisum-olens is a shrub found growing in Hekou County of the Yunnan Province and the leaves and twigs of this plant are used for the treatment of dysentery and arthritis [7]. In a preliminary pharmacological study, the EtOH extract of the leaves and twigs of *Clausena anisum-olens* exhibited antifungal activities against three *Candida* species: *C. albicans*, *C. tropicalis,* and *C. krusei*. Previous studies on *Clausena anisum-olens* resulted in the isolation of a novel cyclopeptide [10]. In the present study, an epimer pair of new monoterpenoid coumarins anisucumarin A (**1**) and B (**2**) were isolated. Herein, we report the isolation and structural elucidation of these two new coumarins.

Results and Discussion

The powdered leaves and twigs of *Clausena anisum-olens*, collected from Hekou County, Yunnan province, were extracted with 90% ethanol. The concentrated extract suspended in water was successively extracted with petroleum ether, AcOEt and n-BuOH. The AcOEt extract was subjected to chromatography on silica gel, Sephadex LH-20 and RP C-18 to yield compounds **1** and **2** as a pair of inseparable epimers.

Figure 1. The key HMBC and ROESY correlations of compounds **1**/**2.**



1/2 Key HMBC of **1/2** Selected ROESY of major one

Structural elucidation of the new coumarins was mainly determined by spectroscopic 1D- and 2D-NMR experiments (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HMQC and HMBC; see Table 1), HR-ESI-MS, UV and IR. The molecular formula of compounds **1**/**2** was determined to be $C_{20}H_{20}O_8$ by HRESI-MS exhibiting the quasimolecular ion at *m/z* 389.1249 $[M+H]^+$, which indicated eleven degrees of unsaturation. The UV spectra of **1**/**2** displayed typical absorption bands at λ_{max} 211, 256, and 318 nm, respectively, accompanied with some minor bands. This feature was similar to that of a 7,8-dioxygenated coumarin with a C-10 terpenoid side chain containing a γ-lactone [5]. The IR bands at 3439 and 1730 cm^{-1} indicated the presence of hydroxyl groups and α,β-unsaturated-γ-lactone group in these molecules. The EI-MS spectra showed fragment ion at *m/z* 100, which was characteristic of 8-OMe coumarin and prominent fragment ion at *m/z* 370 corresponding to loss of H_2O [11].

Through careful analysis of ^{1}H-NMR spectra the presence of a 7,8-dioxygenated coumarin

backbone as a common structural unit in **1**/**2** was further deduced by a methoxy singlet signal at δ 3.95 and two sets of ^{1}H AB doublets at δ_H 6.26 and 7.86 (each d, J = 9.6Hz) and δ_H 7.31 and 7.08 (each d, J = 8.7 Hz), which were easily assignable to H-3 and H-4 and to H-5 and H-6 on the coumarin skeleton, respectively (Table 1). Analysis of the ^{1}H- and ^{13}C-NMR spectra, including COSY, HMQC and HMBC, suggested the presence of a C_{10} terpenoid side chain in **1**/**2**. Two olefinic protons on a terminal methylene at δ 5.24, 5.42 (each d, J = 6.3Hz) were attributed to H-10′ according to signal complexity and chemical shift. The other olefinic proton at δ 7.55 (1H, d, J = 1.7Hz) and a lone 2H-broad singlet at δ 4.31 were observed in the ^{1}H spectrum, and the long-distance correlations between a 2H-broad singlet at δ 4.31 and δ 57.0 (t, C-9′), 137.3 (s, C-7′), 151.7 (d, C-6′), 174.3 (s, C-8′) indicated the presence of a 3-hydroxymethyl-3,4-unsaturated-γ-lactone moiety in the molecules. Two nonequivalent *O*-benzylic protons at δ 4.16, 4.21 (each 1H, m) were assigned to C-1′ according to HMBC correlations. In the monoterpenoid side chain, the proton at δ 4.59 (m) correlated with a methine carbon at δ 73.7 (d) in an HMQC experiment. The observation of HMBC cross peaks between this proton and four carbons at δ_C 37.1 (t), 73.4 (t), 116.2 (t) and 144.9 (s) suggested that a hydroxyl group was attached to C-2′ (Figure 1).

The difference between **1** and **2** was due to the stereochemistry of hydroxyl group at C-2′. The NMR peaks of C-1′, C-2′, C-3′ and C-10′ appeared as pairs (Table 1), indicating the presence of **1** and its C-2′ stereoisomer **2**. The ^{1}H- and ^{13}C-NMR spectra established that **1** and **2** consisted of two epimers in a 3:2 ratio. The compound pair resulted in a single spot by multiple solvent systems HPLC. Attempts in the separation of the epimers by HPLC, however, failed to split the products. A reason for this might be a small difference in the interactions between a pair of epimers and the column material for achieving their separation. An analysis of ROESY experiments showed significant NOE correlations between H-2′ and H-4b′ in the major epimers (Figure 1). However, the same NOE correlation was not observed in the minor epimers. The evidences support the presence of a pair of epimers **1**/**2** instead of different conformations of one compound.

The configuration of these *O*-terpenoidal coumarins **1** and **2** remained to be determined. So far, the stereochemistry of this type of *O*-terpenoidal coumarins reported previously has not been resolved [5, 12-14]. Further structure elucidation on the stereochemistry pertaining to the C-2′ and C-5′ of **1/2** is in progress. In summary, the ^{1}H-NMR and ^{13}C-NMR spectra (Table 1), HMQC, HMBC data established the structures of **1** and **2** as a pair of epimers of monoterpenoid coumarins (Figure 1).

In a preliminary study, the EtOH extract of *Clausena anisum-olens* and the two isolated compounds were screened for antifungal activity against *C. albicans*, *C. tropicalis* and *C. krusei*, using the broth microdilution method described in [15]. To validate the MIC end points for antifungal testing of plant extracts, a classification of MIC values used is as follows: strong inhibitors – MIC up to 0.5 mg/mL; moderate inhibitors – MIC between 0.6 and 1.5 mg/mL and weak inhibitors – MIC above 1.6 mg/mL [16]. The EtOH extract of *C. anisum-olens* exhibited *in vitro* antifungal activities against *C. albicans*, *C. tropicalis* and *C. krusei*, with MIC values of 1.0, 0.25, 0.5 mg/mL. However, the new compound pair **1** and **2** didn't show antifungal activities *in vitro* in this bioassay. The fractionation of *Clausena anisum-olens* EtOH extract guided by the bioassays may lead to the isolation of the inhibitor compounds.

Table 1. The ^{1}H- and ^{13}C-NMR data for compounds **1** and **2** (in CD_3OD, δ in ppm, J in Hz).

No.	**1** (major epimer)		**2** (minor epimer)	
	δ_H	δ_C	δ_H	δ_C
2	/	162.7 (s)	/	162.7 (s)
3	6.26 (d, 9.5 Hz)	113.8 (d)	6.26 (d, 9.5 Hz)	113.8 (d)
4	7.86 (d, 9.5 Hz)	146.0 (d)	7.86 (d, 9.5 Hz)	146.0 (d)
5	7.31(d, 8.7 Hz)	124.7 (d)	7.31 (d, 8.7 Hz)	124.7 (d)
6	7.08 (d, 8.7 Hz)	111.5 (d)	7.08 (d, 8.7 Hz)	111.5 (d)
7	/	156.3 (s)	/	156.3 (s)
8	/	137.3 (s)	/	135.2 (s)
9	/	115.3 (s)	/	115.3 (s)
10	/	149.1 (s)	/	145.2 (s)
1'a	4.21 (m)	73.4 (t)	4.22 (m)	73.4 (t)
1'b	4.16 (m)	73.4 (t)	4.15 (m)	73.4 (t)
2'	4.59 (m)	73.7 (d)	4.57 (m)	73.6 (d)
3'	/	144.9 (s)	/	145.2 (s)
4'a	2.64 (dd, 14.2, 7.2 Hz)	37.1 (t)	2.71 (dd, 14.6, 5.1 Hz)	37.1 (t)
4'b	2.53 (dd, 14.2, 6.3 Hz)	37.1 (t)	2.62 (dd, 14.6, 8.1 Hz)	37.1 (t)
5'	5.38 (m)	82.4 (d)	5.34 (m)	82.8 (d)
6'	7.55 (d, 1.7 Hz)	151.7 (d)	7.55 (d, 1.7 Hz)	151.5 (d)
7'	/	137.3 (s)	/	137.3 (s)
8'	/	174.3 (s)	/	174.3 (s)
9'	4.31 (s)	57.0 (t)	4.31 (s)	57.0 (t)
10'a	5.42 (d, 6.3 Hz)	116.2 (t)	5.42 (d, 6.3 Hz)	115.9 (t)
10'b	5.24 (d, 6.3 Hz)	116.2 (t)	5.24 (d, 6.3 Hz)	115.9 (t)
OMe	3.95 (s)	61.9 (q)	3.95 (s)	61.9 (q)

^{1}H- and ^{13}C-NMR spectra were obtained at 500 and 125 MHz, respectively, and assigned by the ^{1}H-^{1}H COSY, HMQC and HMBC experiments.

Conclusions

Two new monoterpenoid coumarins anisucumarin A (**1**) and B (**2**), whose separation was not successfully achieved were isolated as a pair of epimers from *Clausena anisum-olens*. Their structures were established based on extensive spectroscopic studies. The EtOH extract of *Clausena anisum-olens* and the monoterpenoid coumarins anisucumarin A (**1**) and B (**2**) were screened for antifungal activity against *C. albicans*, *C. tropicalis* and *C. krusei*. The EtOH extract of *Clausena anisum-olens* exhibited *in vitro* antifungal activities against above bioassays but the monoterpenoid coumarins anisucumarin A (**1**) and B (**2**) failed to show detectable inhibitory activity against the fungus.

Experimental

General

Commercial silica-gel plates (Qing Dao Marine Chemical Group Co.) were used for TLC analyses. Melting points was measured on XRC-1 micro-melting point apparatus and uncorrected. UV/VIS Spectra was measured on Shimadzu UV-2401PC spectrophotometer; λ_{max} in nm. IR spectra were obtained on Bio-Rad FTS-135 infrared spectrophotometer, ν_{max} in cm^{-1}. ^{1}H- and ^{13}C- NMR as well as 2D-NMR spectra were recorded on Brucker DRX-500 spectrometer with TMS as internal standard, coupling constant *J* in Hz. MS spectra was performed on VG Autospec-3000 mass spectrometers.

Plant material

The leaves and twigs of *Clausena anisum-olens* were collected in Hekou County of Yunnan province, P. R. China, in May 2003 and identified by Professor De-Ding Tao of Kunming Institute of Botany. A voucher specimen (No. 02041705) is deposited in State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

Extraction and isolation

The powdered leaves and twigs of *Clausena anisum-olens* (22.5 kg) was repeatedly extracted with EtOH at room temperature. The extract was then concentrated under reduced pressure to give brown syrup, which was partitioned in H_2O and extracted with solvents into petroleum ether-fraction, AcOEt-fraction and n-BuOH-fraction fractions. The AcOEt extracts (110.5g) were subjected to silica gel column chromatography eluting with PE-AcOEt (4:1, 2:1, 1:1, 2:3), AcOEt, AcOEt–MeOH (8:2, 7:3, 6:4, 1:1), MeOH, by which nine fractions (I-IX) were obtained. Fraction III was resubmitted to silica gel column chromatography, Pharmadex LH-20 (MeOH) and RP C-18 to yield compounds **1/ 2** (11 mg).

Anisucumarin A and B (**1** and **2**, a pair of epimers). Light yellow oil; IR (KBr): 3439, 2927, 2855, 1730, 1608; UV λ_{max} (MeOH) nm: 318, 256, 211; ^{1}H-NMR (δ ppm, CD_3OD) and ^{13}C-NMR: see Table 1; EI-MS *m/z* 388 ($[M]^+$, 100), 370 (15), 358 (5), 339 (4), 205 (26), 192 (100), 164 (22); HR-ESI-MS *m/z* 389.1249 ($[M+1]^+$)(calcd for $C_{20}H_{20}O_8$ 389.1236).

Assay for biological activity

The broth microdilution test M27-A2 [15] was used for the assessment of *in vitro* antifungal activity of the EtOH extract of *Clausena anisum-olens* and the compounds against *Candida albicans* ATCC 90028, *Candida tropicalis* ATCC 750, *Candida krusei* ATCC 6258. Amphotericin B was used as a reference drug. The procedure was performed in RPMI 1640 medium buffered to pH 7.0 with 3-morpholinopropane-1-sulfonic acid (0.165mol). Drug–free controls were included. The minimal inhibitory concentrations (MICs) were determined after 24 h and 48 h of static incubation at 35 °C.

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Sample Availability: Available from the authors.