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A NEW DIHYDROISOCOUMARIN FROM THE STRAIN *Aspergillus* sp. CMM, AN ENDOPHYTIC FUNGUS OF *Cephalotaxus mannii*

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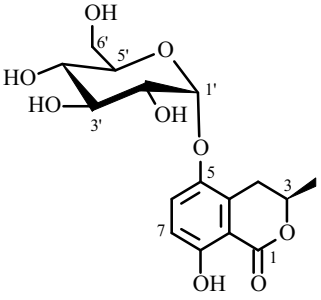
From the branch tissue of Cephalotaxus mannii*, the endophytic fungal strain CMM was isolated and determined to belong to the genus Aspergillus according to its internal transcribed spacer (ITS) sequence of rDNA (ITS1-5.8S-ITS2). From the extracts of the fermentation broth of CMM, a new compound, namely 5-*O*-α-D-glucopyranosyl-5-hydroxymellein (**1**), was isolated, together with a known compound 4-hydroxymellein (**2**). Their structures were elucidated on the basis of 1D and 2D NMR data.*

Key words: *Cephalotaxus mannii,* 5-*O*-α-D-glucopyranosyl-5-hydroxymellein, 4-hydroxymellein.

Endophytic fungi are found in almost all plants and have been shown to be a rich source of biologically active metabolites [1]. *Cephalotaxus mannii*, an important medicinal plant widely distributed in southern Asia, contains harringtonine and related cytotoxic substances [2]. During our search for new bioactive natural products from the endophytic microorganisms, a series of compounds has been isolated [3, 4]. In continuing our studies, we investigated the secondary metabolites produced by the fungal strain CMM isolated from the sterilized twigs of *C. mannii*. In this work, we report the isolation and structure elucidation of 5-*O*-α-D-glucopyranosyl-5-hydroxymellein (**1**) and 4-hydroxymellein (**2**), which were obtained by chromatographic purification of the extracts from the fermentation products of the fungal strain CMM.

Compound **1** was obtained as a colorless crystal. The molecular formula of **1** was determined to be $C_{16}H_{20}O_9$ by analysis of the NMR spectroscopic data and ESI-MS measurement of the quasi-molecular ion at *m/z* 379.2 $[M+Na]^+$. Inspection of the ^1H and ^{13}C NMR, HMQC, and HMBC data (Table 1) determined the structure of 1 as 5-*O*-α-D-glucopyranosyl-5-hydroxymellein. The ^1H NMR spectra of **1** exhibited signals for dihydroisocoumarin lactone at δ 4.74 (m), 2.79 (m) and 3.83 (m) assigned to H-3 and H_2-4, respectively (Table 1) [5–7]. Two aromatic protons at δ 6.83 (d, J = 9.1) and 7.49 (d, J = 9.3), seven sugar protons at δ 5.34 (d, J = 3.2), 3.59 (dd, J = 3.3, 9.8), 3.72 (t, J = 9.8), 3.83 (m), 3.41 (m) 3.80 (m) and 3.69 (m), and a methyl group at δ 1.52 (d, J = 6.2) were observed as well.



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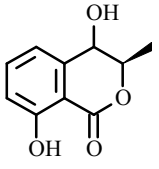

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TABLE 1. ^{1}H NMR (500 MHz), ^{13}C NMR (DEPT, 125 MHz), and HMBC Spectral Data of **1** (CD_3OD) and **2** (CD_3OD)

C atom	**1**			2	
	δ_H (multiplicity, J/Hz)	δ_C	HMBC (C/H)	δ_H (multiplicity, J/Hz)	δ_C
1	-	167.0 s		-	171.0 s
2	-	-		-	-
3	4.74 m	77.7 d		4.73 m	80.0 d
4	3.83 m 2.79 m	29.7 t	C-11, C-3, C-10, C-9, C-5	4.56 (d, J = 1.6)	67.7 d
5	-	147.1 s		6.98 (d, J = 8.1)	119.8 d
6	6.83 (d, J = 9.1)	116.8 d	C-10, C-8	6.98 (d, J = 8.1)	118.5 d
7	7.49 (d, J = 9.3)	127.6 d	C-5, C-8, C-9	7.56 (t, J = 8.1)	137.7 d
8	-	158.7 s		-	162.9 s
9	-	130.7 s		-	143.2 s
10	-	109.4 s		-	108.4 s
11	1.52 (d, J = 6.2)	21.0 q	C-3, C-4	1.53 (d, J = 6.5)	16.4 q
1′	5.34 (d, J = 3.2)	101.0 d	C-2′, C-3′, C-5		
2′	3.59 (dd, J = 3.3, 9.8)	73.4 d	C-3′, C-4′		
3′	3.72 (t, J = 9.8)	74.9 d	C-3′		
4′	3.83 m	75.0 d	C-5′		
5′	3.41 m	71.2 d	C-4′, C-6′		
6′	3.80 3.69	62.6 t	C-4′		

The ^{13}C NMR spectral signals at δ 101.0, 73.4, 74.9, 75.0, 71.2, and 62.6 and an anomeric proton at δ 5.34 (d, J = 3.2) in the ^{1}H NMR spectra suggested the presence of an α-D-glucopyranosyl moiety in **1**. Besides the signals for the α-D-glucopyranosyl moiety, the ^{13}C NMR and DEPT spectra further revealed the presence of a C_{10}-unit including one methyl, one methylene, three methines, and five quaternary carbons. In the HMBC spectra, the proton at δ 6.83 (H-6) showed ^{1}H–^{13}C long-range correlations to C-10 and C-8, 7.49 (H-7) to C-5, C-8, and C-9, and the methylene protons (at δ 3.83 and 2.79) to C-11, C-3, C-9, C-10, and C-5, indicating the presence of a dihydroisocoumarin moiety. The attachment site of the glucose unit was indicated by the ^{1}H–^{13}C long-range correlation between the anomeric proton at δ 5.34 and C-5 (δ 147.1) in the HMBC spectra. Therefore, compound **1** was determined to be 5-*O*-α-D-glucopyranosyl-5-hydroxymellein.

Compound **2** was obtained as a colorless crystal. The molecular formula of **2** was determined to be $C_{10}H_{10}O_4$ by analysis of the NMR spectroscopic data and ESI-MS measurement of the quasi-molecular ion at *m/z* 195.1 $[M+H]^+$. Inspection of the ^{1}H NMR, ^{13}C NMR, and DEPT spectra revealed 10 carbon signals for one methyl, five methines, and four quaternary carbons, including one carbonyl at δ 171.0 (C-1). The ^{1}H NMR showed three olefinic protons at δ 7.56 (t, 8.1) and 6.98 (d, 8.1, 2H). Comparison of the proton and carbon NMR signals of **2** with those of mellein [8, 9] indicated that 2 is a derivative of dihydroisocoumarin. The hydroxyl group at C-4 was confirmed by the low-field shift of the carbon at δ 67.7 and the proton at δ 4.56 (H-4). The HMBC and ^{1}H–^{1}H COSY further supported this substitution. Therefore, compound **2** was determined to be 4-hydroxymellein [10].

EXPERIMENTAL

General Experimental Procedures. Column chromatography (CC): silica gel (200–300) mesh; *Qingdao Marine Chemical Factory*, Qingdao, P. R. China), silica gel GF_{254} (Merck), RP-18 (Merck), and Sephadex LH-20 (Amersham Biosciences) were used. TLC: precoated silica gel GF_{254} plates (0.20–0.25 mm, *Qingdao Marine Chemical Factory*). ^{1}H and ^{13}C NMR spectra: Bruker DRX-500 spectrometer, at 500/125 MHz, in MeOD; δ in ppm rel. to Me_4Si, J in Hz. ESI-MS: Thermo-Finnigan Advantage LCQ mass spectrometer; in *m/z*.

Microbial Material. Branches of *Cephalotaxus mannii* collected at Xishuangbanna (Yunnan Province, P. R. China) in March 2004. The material was washed under running tap water, then sterilized successively with 75% aq. EtOH (1 min) and 1.2% sodium hypochlorite (8 min), and then rinsed with sterile H_2O. The sterilized plant material was cut into small pieces and

incubated at 28°C on PDA medium. During cultivation, the hyphal tips of the growing fungi were removed and inoculated onto fresh PDA media and incubated for at least two weeks at 28°C. After being purified by the hyphal tip method [11], the pure isolates were transferred to PDA slant tubes as deposit. The strain CMM was inoculated on a slope of PDA media in a test tube and cultivated for 5 d at 25°C to afford seed cultures. Solid-state fermentation was performed with PDA media for 10 days at 28°C.

The CMM strain was identified by amplification of the ITS sequence of rDNA (ITS1-5.8S-ITS2), and its sequence was submitted to GenBank (accession No.EF62400). Blast search showed that the sequence of CMM was highly homologous to other Aspergillus species, indicating that this strain is a member of the genus *Aspergillus*.

Extraction and Isolation. The fungal strain *Aspergillus* sp. CMM, was cultured on PDA media (10 L) for 10 days. The culture was extracted three times with an equal volume of AcOEt–MeOH–AcOH 80:15:5 (v/v/v) at room temperature. The organic solutions were collected by filtration and removed under vacuum at 40°C to yield the crude extract (19.5 g).

The extract was subjected to MPLC (170 g RP-18) and eluted with H_2O and 30, 50, 70, and 100% MeOH (1.5 L each) to yield 5 fractions: Fr. D1–D5. Fraction D2 (2.02 g) was subjected to CC (140 g Sephadex LH-20; MeOH). All fractions were analyzed by TLC ($CHCl_3$–MeOH 10:1) and pooled accordingly into eleven portions (Fr. D21–D211). Fraction D24 (190 mg) was subjected to CC (40 g Sephadex LH-20; MeOH) to afford D24a (172 mg). Fraction D24a was subjected to CC (7g SiO_2; $CHCl_3$–MeOH 50:1, 10:1) to afford **1** (5 mg). Fraction D25 (400 mg) was subjected to CC (40 g Sephadex LH-20; MeOH) to afford D25a (370 mg). Fraction D25a was subjected to CC (9g SiO_2; $CHCl_3$–MeOH 50:1, 20:1, 10:1) to afford **2** (30 mg).

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