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Secondary Metabolites of *Aspergillus* sp. CM9a, an Endophytic Fungus of *Cephalotaxus mannii*

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Abstract: Eleven compounds belonging to eight structure types, namely cyclopentenedione (**1**), diketopiperazines (**2**, **9**, **10**), lactone (**3**), benzophenone (**4**, **5**), terpene (**6**), anthraquinone (**7**), diphenyl ethers (**10**), and alkaloid (**11**) were isolated from the cultivation extract of the strain *Aspergillus* sp. CM9a, which was isolated from the stems of *Cephalotaxus mannii*. Among them, compounds **1**, **2**, and **3** were determined to be new ones on the basis of spectroscopic data including 1D- and 2D- NMR experiments and HR Q-TOF MS. The structures of the eight known compounds were characterized based on their NMR data and by comparison with those reported.
Keywords: *Aspergillus* sp. CM9a; diketopiperazine; anthraquinone; *Cephalotaxus mannii.*

1. Introduction

Plant endophytes are a group of microorganisms living within plant internal tissues or organs without causing any apparent symptoms or diseases in the hosts. They can serve as important sources of bioactive compounds, presumably due to the symbiotic relationship with their hosts [1]. Since endophytes constitute a valuable source of secondary metabolites for the discovery of new pharmaceuticals and lead compounds [2], we have started to investigate endophytic fungi as a source for biologically active natural products, and isolated a series of new compounds from endophytic microorganisms [3–7]. In this work, we report the isolation and structure elucidation of three new compounds **1** – **3**, together with eight known ones **4** – **11**, from *Aspergillus* sp. CM9a, an endophytic strain of the medicinal plant *Cephalotaxus mannii*.

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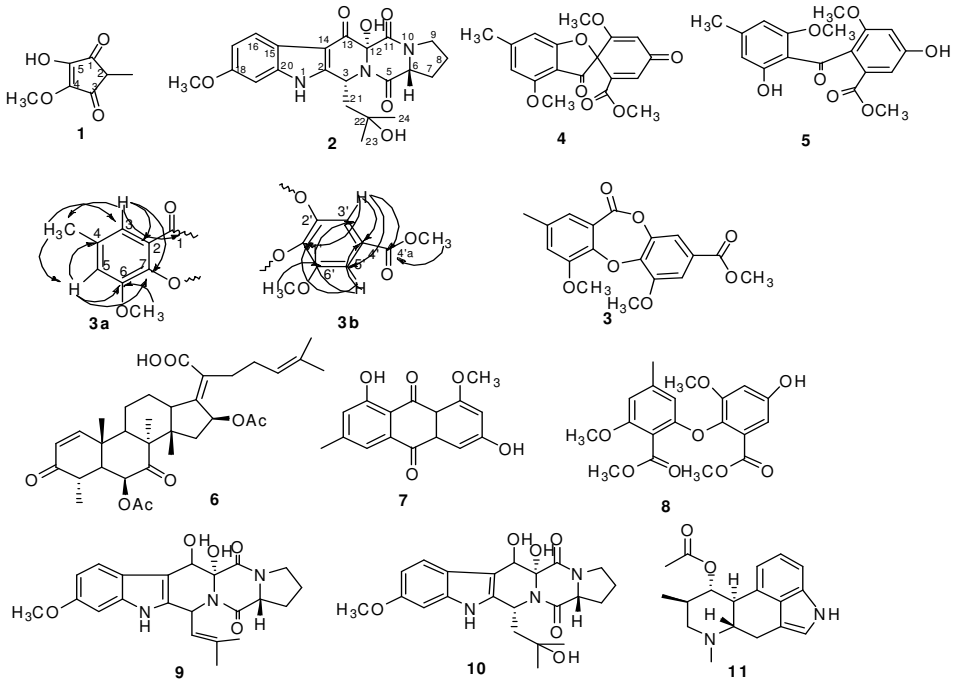

2. Materials and Methods

2.1. Microorganism Material

The fungal stain (CM9a) was isolated from the current-year stems of *Cephalotaxus mannii* collected in Xishuangbanna, Yunnan, China. The strain CM9a was found to grow well on potato dextrose agar (PDA) medium, with white hyphal and green spores. According to its ITS sequence of rDNA (ITS1-5.8S-ITS2), the stain was identified as *Aspergillus* sp.

2.2. Fermentation and Isolation

The strain was fermented twice, the first time it was cultivated 14 d at 28°C with 3 L of PDA (Potato Dextrose Agar) media, the second time, 10 L of PDA under the same culture conditions were used. The culture media were chopped, diced, and extracted with AcOEt/MeOH/AcOH 80 : 15 : 5 at room temperature. The organic solution was collected through filtration. The remaining agar residue was extracted until the filtrate was colorless. The filtrates were concentrated under vacuum to remove organic solvents. The aqueous solution was extracted several times with ethyl acetate to yield a crude mixture as a syrup (4.2 g and 26.5 g, *resp.*).

Isolation:

a) For the first fermentation, the crude extract (4.2 g) was separated into nine fractions (Fr. A-H) by column chromatography (RP-18, 80 g), eluted with MeOH/H_2O (0 : 100, 40 : 60, 60 : 40, and 100 : 0). These fractions were further purified by repeated column chromatography over Sephadex LH-20, RP-18 silica gel and silica gel.

Fr. C (699 mg) was separated by CC (RP-18, 80 g, MeOH/H_2O 30 : 70; 40 : 60; 50 : 50) to give four fractions (*Fr. C1-C4*). Fr. *C2* was separated into four fractions (*Fr. C2a-C2d*) by CC (*Sephadex* LH-20, MeOH). *Fr. C2b* was subjected to RP-18 silica gel (80 g, MeOH/H_2O 35 : 65; 40 : 60) and *Sephadex* LH-20 (acetone and MeOH) to give two fractions *Fr. C2b2c3* and *Fr. C2b2c4*. *Fr. C2b2c3* was purified by CC (SiO_2, petroleum ether(PE)/acetone 9 :1), to yield **4** (5.0 mg), *Fr. C2b2c4* was purified by CC (SiO_2, PE/acetone 8 : 1), to yield **5** (6.0 mg). *Fr. C2d* was subjected to repeated CC (*Sephadex* LH-20, acetone) to afford **2** (9.0 mg).

Compound **7** (3.0 mg) was isolated by repeated CC (*Sephadex* LH-20, MeOH) from Fr. *E* (176 mg).

b) For the second fermentation, the crude extract (26.5 g) was separated into thirteen fractions (*Fr. A-M*) by CC (RP-18, 170 g), eluted with MeOH/H_2O (0 : 100, 30 : 70, 50 : 50, 70 : 30 and 100 : 0). These fractions were further purified by repeated CC on *Sephadex* LH-20, RP-18 silica gel and normal silica gel.

Fr. A (133 mg) was separated by CC (RP-18, 30 g, 30 : 70; 10 : 90; 20 : 80) to afford five fractions (*Fr. A1-A5*). *Fr. A2* was subjected to CC (*Sephadex* LH-20, MeOH) and recrystallization (acetone/MeOH), to afford **1** (5.8 mg).

One part of *Fr. K* (485 mg) was something insoluble in MeOH, and was determined as **6** by TLC comparison after filtration. The soluble part *Fr. K1* (367 mg) was subject to RP-18 silica gel (80 g, MeOH/H_2O 35 : 65; 45 : 55; 55 : 45), CC (*Sephadex* LH-20, acetone), repeated CC (*Sephadex LH-20*, MeOH) and CC (SiO_2, PE/EtOAc 8 : 1), to afford **8** (3.8 mg).

Fr. L (197 mg) was separated by CC (*Sephadex* LH-20, MeOH) and CC (RP-18, 30 g, MeOH/H_2O 55 : 45; 65 : 35; 75 : 25), to give five subfractions (*Fr. L2a-L2e*). *Fr. L2b* was subjected to CC (*Sephadex* LH-20, acetone) and recrystallization (acetone/H_2O) to afford **9** (4.6 mg).

Fr. I (499.3 mg) was separated by CC (*Sephadex* LH-20, MeOH) to give five subfractions (*Fr. I1-I5*). *Fr. I4* was subjected to CC (*Sephadex* LH-20, acetone), CC (Sephadex LH-20, MeOH), repeated CC (RP-18, 30 g, MeOH/H_2O 35 : 65; 45 : 55; 55 : 45) and recrystallization (acetone/H_2O) to afford **10** (3.5 mg).

Fr. I3 was subjected to repeated CC over RP-18, *Sephadex* LH-20, and normal silica gel to afford **3** (5.0 mg). *Fr. I2* was subjected to CC (*Sephadex* LH-20, acetone) and repeated CC (RP-18, 30 g, MeOH/H_2O 30 : 70; 40 : 60), CC (SiO_2, PE/EtOAc 14 : 1)and HPLC (Agilent 1200 series, column: 250 × 4.6; 1.0 mL/min; MeOH/H_2O 38 : 62) to afford **11** (3.3 mg).

3. Results and Discussion

3.1. Structure elucidation

Compound **1** was isolated as a white powder and determined to have the molecular formula $C_7H_8O_4$ by HR-Q-TOF-MS and ^{13}C NMR. The molecular formula indicated four degrees of unsaturation. The ^1H and ^{13}C NMR for **1** revealed that three of the four unsaturation degrees were attributed to two carbonyls, and a double bond. Thus, the remaining unsaturation degree came from a ring. The ^{13}C NMR (DEPT) spectra of **1** revealed 7 signals: one methyl (at δ10.0), a methoxy (at δ58.3), one methine (at δ 43.4) and four quaternary carbon atoms including two keto carbonyl carbon atoms at δ 194.5 and 195.7. The HMBC correlations from the methyl protons H-C(2a) to C(1), C(3) and C(2), from H-C(2) to C(1), C(3) and C(2a), together with the ^1H-^1H COSY correlations between H-C(2) and H-C(2a), revealed the connectivities from C(1) to C(3).

The HMBC correlations from the methoxy proton H-C(4a) (δ 4.15) to C(4) indicated that the methoxy group was attached to C(4). According to the chemical shift of C(4) and C(5), the presence of a double bond was indicated. The IR absorptions indicated the presence of OH groups (3353 cm^{-1}),

carbonyl (1683 cm^{-1}) and unsaturated carbon/carbon double bonds (1648 cm^{-1}). The above data suggested the structure of compound **1** as 4-hydroxy-5-methoxy-2-methylcyclopent-4-ene-1,3-dione.

Table 1. The NMR data of compound **1**. Recorded at 600/150 MHz in acetone-d_6; δ in ppm, *J* in Hz.

position	δ_C	δ_H (mult, *J* in Hz)	HMBC
1	194.5(C)	/	/
2	43.4(CH)	2.80 (q, 7.6, 1H)	C(1), C(3), C(2a)
3	195.7(C)	/	/
4	148.8(C)	/	/
5	147.4(C)	/	/
2a	10.0(CH_3)	1.13 (d, 7.6, 3H)	C(1), C(3)
4-OMe	58.3(CH_3)	4.15 (s, 3H)	C(4)

Compound **2** was obtained as a white powder. Its molecular formula $C_{22}H_{25}N_3O_6$ was established by HR Q-TOF MS and ^{13}C NMR data, indicating twelve degrees of unsaturation. Eight of these unsaturation degrees were attributed to three carbonyls, a benzene ring and a double bond, thus, the remaining units of unsaturation came from four rings. A tri-substituted benzene was indicated by the ^1H NMR spectral data δ 7.93 (d, *J* = 8.8 Hz), δ 6.91 (dd, *J* = 8.6, 1.9 Hz) and δ 6.87 d, *J* = 2.0 Hz) (Table 2). The presence of a diketopiperazine system in **2** was suggested on the basis of the amide carbonyl absorptions at 1659 and 1640 cm^{-1} together with the absence of the amide II band near 1550 cm^{-1} in the IR spectra, which was further supported by the amide carbonyl carbon signals at δ 167.3 (C(5)) and 167.2 C(11)) in the ^{13}C NMR spectrum. The formation of this diketopiperazine was determined as a 3-oxo-tryptophan and a proline residue on the basis of HMBC correlations. An isoprenyl residue was assigned to include two tertiary methyl (δ 32.3 and 28.7), a methylene (δ 46.4(CH_2)), a methine (δ 48.4(CH)) and a quaternary carbon (δ 71.9(C)) group. Moreover, the isoprenyl methane linked C(3) with C(21) due to HMBC correlations from the proton at δ 6.24 (H-C(3)) to C(2), C(5), C(12), C(14), C(21) and C(22) (Table 2). Therefore, a pentacyclic ring skeleton was assigned for **2** including a 6-*O*-methylindole moiety, a diketopiperazine and an isoprenyl residue. Compared with literature NMR and MS data [8], the only difference between Verruculogen TR2 and **2** was the oxidation of the 13-OH to form a carbonyl in **2**.

Table 2. The NMR data for compound **2**. Recorded at 600/150MHz in $CDCl_3$ (δ in ppm, *J* in Hz)

position	δ_H (*J* in Hz)	δ_C	HMBC
2	/	150.4(C)	/
3	6.24 (d, 7.6, 1H)	48.4(CH)	C(2), C(5), C(12), C(14), C(21), C(22)
5	/	167.3(C)	/
6	4.21(dd, 6.8, 9.2,1H)	60.0(CH)	C(5),C(7)
7	2.50(m, 1H), 2.05(m, 1H)	29.4(CH_2)	C(9)
8	2.13(m, 1H), 1.98(m, 1H)	22.5(CH_2)	C(7),C(12)
9	2.59(m, 1H), 2.27(m, 21H)	48.3(CH_2)	C(8),C(7)
11	/	162.7(C)	/
12	/	84.7(C)	/
13	/	184.2(C)	/
14	/	107.6(C)	/
15	/	118.6(C)	/
16	7.93(d, 8.8,1H)	121.8(CH)	C(14),C(18),C(20)
17	6.91(dd, 8.6, 1.9,1H)	111.8(CH)	C(15),C(18),C(19)
18	/	157.5(C)	/
18-OMe	3.84(s, 3H)	55.7(CH_3)	C(18)
19	6.87(d, 2.0,1H)	95.8(CH)	C(15),C(18),C(17)
20	/	136.8(C)	/
21	3.74(m, 2H)	46.4(CH_2)	C(23),C(24)
22	/	71.9(C)	/
23	1.58(s, 3H)	32.3(CH_3)	C(8),C(24),C(21),C (22)
24	1.40(s, 3H)	28.7(CH_3)	C(23),C(21),C(22)

Compound **3** was obtained as a white powder. Its molecular formula $C_{18}H_{16}O_7$ was established by HR Q-TOF MS, indicating eleven degrees of unsaturation. Ten of the unsaturation degrees were attributed to two carbonyls and two benzene rings. Thus, the remaining one unsaturation degree came from a ring. The IR absorption at 2926, 1710 and 1610 cm^{-1} indicated the presence of Me groups and aryl ketone. The ^{13}C NMR (DEPT) spectra of **3** revealed 18 signals: one methyl, three methoxy, four methine and ten quaternary carbon atoms including two carboxyl carbon atoms at δ 165.5 and 166.5. The HMBC correlations from the protons of the aromatic methine groups (H-C(3) to C(1), C(2), C(4a), C(5) and C(7), and H-C(5) to C(3), C(4a) and C(6) and the protons of the methyl group (H-C(4a) to C(2), C(3), C(4) and C(5)), and the ^1H-^{13}C long-range correlations from the proton at δ 3.84 to C(6) indicated a tetra-substituted benzene ring (Figure 1, **3a**).

The HMBC correlations from the protons of the aromatic methine groups (H-C(3') to C(1'), C(4'), C(5') and C(6'a), H-C(5') to C(1'), C(4') and C(3')) and the protons of the methoxyl group (6'-OMe to H-C(6')), revealed a tetra-substituted benzene ring in the structure as shown in Figure 1. 3b.

Comparison the chemical shift between C(1) and C(2'), and C(7) and C(1') revealed that C(1) and C(2') and C(7), C(1') were connected via oxygen, respectively. Therefore, the structure of **3** was determined.

Table 3. The NMR data for compound **3**. Recorded at 600/150MHz in MeOD(δ in ppm, *J* in Hz)

position	δ_H (*J* in Hz)	δ_C	HMBC
1	/	168.5(C)	/
2	/	110.8(C)	/
3	5.86(s,1H)	106.1(CH)	C(1),C(2,C(4a),C(5),C(7)
4	/	141.2(C)	/
4a	2.19(s,3H)	20.6(CH_3)	C(5),C(3),C(2),C(4)
5	6.51(s,1H)	104.9(CH)	C(1) , C(2),C(3),C(4a), C(6)
6	/	157.2(C)	/
6-OMe	3.84(s,3H)	55.2(CH_3)	C(6)
7	/	156.3(C)	/
1'	/	134.9(C)	/
2'	/	155.3(C)	/
3'	6.85(d,2.8,1H)	107.5(CH)	C(5'),C(1'),C(4'),C(4'a)
4'	/	128.5(C)	/
4'a	/	166.4(C)	/
4'a-OMe	3.72(s,3H)	51.4(CH_3)	C(4'a)
5'	6.76(d, 2.8,1H)	104.5(CH)	C(3'),C(1'),C(4')
6'	/	153.9(C)	/
6'-OMe	3.74(s, 3H)	55.0(CH_3)	C(6')

Eight known compounds were elucidated as trypacidin (**4**) [9], benzoic acid (**5**) [10], helvolic acid (**6**) [11], 1,6-dihydroxy-8-methoxy-3-methylanthracene-9,10-dione (**7**) [12], diphenyl ether dimethyl 2,3'-dimethylosoate (**8**) [13], cyclotryprostatine A (**9**) [14], Verruculogen TR2 (**10**) [8] and roquefortine A (**11**) [15] based on their 1D, 2D NMR and by comparison their NMR data with those reported.

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