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内生真菌叶下珠生拟茎点霉的次级代谢产物研究

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摘要 从广藿香内生真菌叶下珠生拟茎点霉(*Phomopsis phyllanthicola* A658)中分离纯化得到 6 个聚酮类化合物, 分别鉴定为 dothiorelone O (**1**), 2-[3,5-二羟基-2-(7-羟基辛酰基)苯基]乙酸 (**2**), dothiorelone M (**3**), dothiorelone K (**4**), cytosporone B (**5**), 和 cytosporone C (**6**). 其中化合物 **1** 为新化合物, 化合物 **2** 为新天然产物, 新化合物的结构进一步通过 HR-ESIMS, 1D NMR 和 2D NMR 等光谱技术确定. 此外, 对分离所得的化合物进行了体外细胞毒测试, 结果表明化合物 **3**～**5** 显示出不同程度的细胞毒活性.

关键词 广藿香; 内生真菌; 叶下珠生拟茎点霉; 细胞毒活性

Cytotoxic Secondary Metabolites from an Endophytic Fungal Strain of *Phomopsis phyllanthicola*

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Abstract The chemical investigation of the broth extract of the endophytic fungus *Phomopsis phyllanthicola* A658, which was isolated from the medicinal plant *Pogostemon cablin*, resulted in the isolation of one new compound, named dothiorelone O (**1**) and one new natural product 2-(3,5-dihydroxy-2-(7-hydroxyoctanoyl)phenyl)acetic acid (**2**), along with four known derivatives. Their structures were fully characterized by means of detailed spectroscopic analysis for new structures, and in comparison with published data for known compounds. Moreover, all of the compounds were evaluated for *in vitro* cytotoxic activities against SF-268, MCF-7, HepG-2, and A549 tumor cell lines.

Keywords *Pogostemon cablin*; endophytic fungus; *Phomopsis phyllanthicola*; cytotoxicity

1 Introduction

Phomopsis is an important phytopathogenic genus, which contains more than 900 species named from a wide range of hosts.[1] The previous investigation of the fungi belonging to the genus *Phomopsis* has successfully disclosed many secondary metabolites, such as xanthone derivatives,[2~7] dihydronaphthalenones,[8] polyketides,[9] and lactones.[10~12] Owing to our continuing interest in the discovery and development of new constituents with bioactive potentials from endophytic fungal strains,[13~16] a phytochemical study on the EtOAc extract of the fungus *Phomopsis phyllanthicola* A658, which was derived from medicinal plant *Pogostemon cablin*, resulted in the isolation of one new compound, named dothiorelone O (**1**) and one new natural product 2-(3,5-dihydroxy-2-(7-hydroxyoctanoyl)phenyl)acetic acid (**2**), along with four known compounds, dothiorelone M (**3**),[17] dothiorelone K (**4**),[17] cytosporone B (**5**),[18] and cytosporone C (**6**)[18] (Figure 1). Herein, the details of the isolation, structural elucidation, and cytotoxic activity of these compounds were described.

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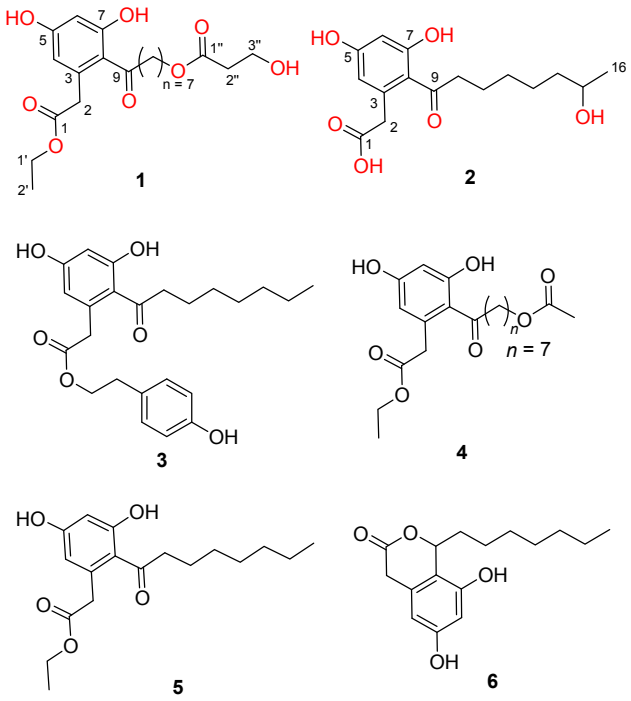

Figure 1 Structures of compounds **1**～**6** isolated from *Phomopsis phyllanthicola* A658

2 Results and discussion

2.1 Structural elucidation of new compounds

Compound **1** was isolated as a yellow oil. Its molecular formula was determined as $C_{21}H_{30}O_8$ based on the HRESIMS with pseudo-molecular peak at *m*/*z* 411.2105 $[M+H]^+$, implying seven degrees of unsaturation. The ^1H NMR spectrum of **1** revealed that an aromatic ring system was present at δ_H 6.34 (d, J=2.5 Hz, 1H, H-4) and 6.30 (d, J=2.5 Hz, 1H, H-6), one methyl signal at δ_H 1.29 (t, J=7.1 Hz, 3H, H-2'), three oxygenated methylenes at δ_H 4.14 (t, J=6.7 Hz, 2H, H-16), 4.21 (t, J=7.1 Hz, 2H, H-1') and 4.68 (t, J=6.2 Hz, 2H, H-3"). The ^{13}C NMR combined with HSQC data (Table 1) of **1** exhibited 21 carbon signals, including one methyl, eleven methylenes, two methines, and seven quaternary carbons (δ_C 170.8, 136.9, 160.1, 164.6, 116.6, 169.4, 206.5, 169.4).

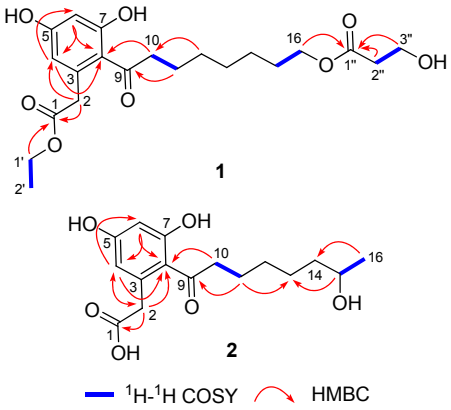

Figure 2 Key ^1H-^1H COSY and HMBC correlations for compounds **1** and **2**

The ^1H-^1H COSY spectrum indicated the presence of four continues fragments C-9/C-10, C-15/C-16, H-1'/H-2', and H-2"/H-3". The HMBC correlations from H-4 to C-6

Table 1 ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for **1** and **2** in CD_3OD

No.	**1**		**2**	
	δ_H (J/Hz)	δ_C	δ_H (J/Hz)	δ_C
1		170.8, C		174.2[a], C
2	3.86, s	41.8, CH_2	3.58, s	38.6, CH_2
3		136.9, C		136.1, C
4	6.30, d (2.5)	112.4, CH	6.23, d (2.2)	110.3, CH
5		160.1, C		160.1, C
6	6.34, d (2.5)	103.2, CH	6.27, d (2.2)	101.2, CH
7		164.6, C		158.5, C
8		116.6, C		119.9, C
9		206.5, C		208.3, C
10	2.86, t (7.3)	43.2, CH_2	2.90～2.93, m	43.2[a], CH_2
11	1.34～1.36, m	29.0, CH_2	1.41～1.44, m	25.3, CH_2
12	1.72～1.74, m	24.8, CH_2	1.33～1.35, m	29.1, CH_2
13	1.34～1.36, m	25.6, CH_2	1.64～1.66, m	24.0, CH_2
14	1.34～1.36, m	28.9, CH_2	1.41～1.44, m	39.2, CH_2
15	1.63～1.65, m	28.4, CH_2	3.69～3.71, m	67.2, CH
16	4.14, t (6.7)	65.6, CH_2	1.13, d (6.2)	22.1, CH_3
1'	4.21, q (7.1)	61.5, CH_2		
2'	1.29, t (7.1)	14.2, CH_3		
1"		169.4, C		
2"	3.00, t (6.2)	31.1, CH_2		
3"	4.68, t (6.2)	69.8, CH_2		

[a] Detected by HMBC.

and C-8, H-6 to C-4 and C-8 further confirmed the 1,2,3,5-tetrasustituted phenyl group in the molecule. A closer comparison of its ^{1}H NMR and ^{13}C NMR data with those of the co-isolated known compound **4** suggested that it should share a dothiorelone skeleton due to the similarity of their NMR profiles in most cases,[17] whereas the obvious difference between compounds **1** and **4** was the replacement of the acetate moiety by an hydroxypropanoate (δ_C 169.4, 31.1, 69.8) functionality in **1**. This deduction was further confirmed by the HMBC correlations (Figure 2) from the H-3" and H-2" to C-1", and H-16 to C-1" and the COSY fragment C-2"/C-3". Therefore, compound **1** was determined and named as dothiorelone O.

Compound **2** was obtained as a white powder. Its molecular formula was established as $C_{16}H_{22}O_6$ with six indices of hydrogen deficiency based on the molecular ion $[M+H]^+$ determined at *m*/*z* 311.1468 with the aid of the high-resolution electrospray ionization mass spectroscopy (HRESIMS). The ^{1}H NMR spectrum of **2** revealed that an aromatic ring system was present at δ_H 6.23 (d, J=2.2 Hz, 1H, H-4) and 6.27 (d, J=2.2 Hz, 1H, H-6), one methyl signal at δ_H 1.13 (d, J=6.2 Hz, 3H, H-16). The ^{13}C NMR combined with HSQC data (Table 1) of **2** exhibited 16 carbon signals, including one methyl, six methylenes, three methines, and six quaternary carbons. The ^{1}H-^{1}H COSY spectrum indicated the presence of two continues fragments C-9/C-10 and C-15/C-16. A closer comparison of the ^{1}H NMR and ^{13}C NMR profiles of **2** with those of **1** indicated that compound **2** was also a dothiorelone derivative. Compound **2** was previously reported as a biotransformation compound,[19] which was isolated as a natural product and fully characterized by spectral methodology including NMR and HRESIMS at the first time in our study.

2.2 *In vitro* cytotoxicity assay

Compounds **1**～**6** were preliminary evaluated *in vitro* for their cytotoxicities against SF-268, MCF-7, HepG-2 and A549 cell lines with adriamycin as the positive control. Compounds **3**～**5** exhibited weak growth inhibitory activities against the tumor cell lines SF-268, MCF-7, HepG-2 and A549 with IC_{50} values of 38.9, 73.3, 31.4, and 32.5 μmol/L, 38.8, 87.3, 36.1, and 35.2 μmol/L, 23.9, 25.9, 17.8, and 17.6 μmol/L, respectively.

Table 2 Cytotoxic activity of compounds **1**～**6**

Compd.	IC_{50}/(μmol•L^{-1})			
	SF-268	MCF-7	HepG-2	A549
1	⩾100	⩾100	⩾100	⩾100
2	⩾100	⩾100	⩾100	⩾100
3	38.9±0.38	73.3±5.00	31.4±5.00	32.5±3.98
4	38.8±3.11	87.3±0.82	36.1±1.46	35.2±0.41
5	23.9±0.33	25.9±0.54	17.8±1.04	17.6±0.76
6	⩾100	⩾100	⩾100	⩾100
Doxorubicin	0.41±0.05	1.93±0.13	0.16±0.01	0.26±0.04

3 Experimental

3.1 General experimental material

UV spectra were measured on a Shimadzu UV-2600 UV-visible spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were performed on a Shimadzu IR Affintiy-1 spectrometer (Shimadzu, Kyoto, Japan). 1D and 2D NMR spectra were recorded on a Bruker Avance-500 spectrometer (Bruker, Fällanden, Switzerland) with TMS as internal standard. HREIMS was measured on a Thermo MAT95XP high resolution mass spectrometer and EIMS on a Thermo DSQ EI mass spectrometer (Thermo Scientific, Massachusetts, USA). All solvents were analytical grade (Guangzhou Chemical Plant, Guangzhou, China). Silica gel (200～300 mesh) was used for column chromatography, and precoated silica gel GF_{254} plates (Qingdao Marine Chemical Inc., Qingdao, China) were used for TLC spotting. C_{18} reversed-phase silica gel (40～63 μm, Merck, German), and Sephadex LH-20 gel (Pharmacia Fine Chemical Co. Ltd., Uppsala, Sweden) were also used for column chromatography. TLC spots were visualized under UV light and by dipping into 10% H_2SO_4 in alcohol followed by heating.

3.2 Fungal material

The strain A658 was isolated from the plant *Pogostemon cablin*, which was collected from Yangchun city of Guangdong Province, China, in October 2012. The strain was identified by sequence analysis of rDNA ITS (internal transcribed spacer) region. The sequence of ITS region of the fungal strain A658 has been submitted to GenBank (Accession No. KF498871). By using BLAST (nucleotide sequence comparison program) to search the GenBank database, A658 has 99.80% similarity to *Phomopsis phyllanthicola* A6 (Accession No. EF488373). The strain is preserved at the Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Institute of Microbiology.

3.3 Fermentation, extraction and compound isolation

A658 was cultured in potato dextrose broth (potato 20%, glucose 2%, K_2HPO_4 0.3%, $MgSO_4$•$7H_2O$ 0.15%, vitamin B 10 mg/L). The fungus A658 was maintained on potato dextrose agar (PDA) medium at 28 ℃ for 5 d, and then three pieces (0.5 cm×0.5 cm) of mycelial agar plugs were inoculated into 20×500 mL Erlenmeyer flasks, each containing 250 mL potato dextrose broth. After 4 d of incubation at 28 ℃ on a rotary shaker at 120 r/min, 25 mL seed cultures was aseptically transferred into each of a total of 125 flasks (1000 mL) containing 500 mL of potato dextrose broth. The liquid cultivation that followed was kept for 7 d at 28 ℃ and 120 r/min on a rotary shaker.

The culture (75 L) was centrifuged to give the broth and mycelia. The broth was exhaustively extracted with EtOAc for four times, and then the EtOAc layers were combined and evaporated under reduced pressure at a temperature

not exceeding 40 ℃ to yield a dark brown gum (23 g). The crude EtOAc extract was subjected to silica gel (*n*-hexane/EtOAc, *V*∶*V*=10∶1→1∶2) column chromatography to afford 6 fractions (Fr.1～Fr.6).

Fr.2 was subjected to CC on Sephadex LH-20 (CH_2Cl_2/MeOH, *V*∶*V*=1∶1) to yield three sub-fractions of Fr.2-1～Fr.2-3. Fr.2-1 was purified by silica gel flash column chromatography and semiprep-HPLC (MeOH/H_2O, *V*∶*V*=80∶20, 3 mL/min) to give compounds **3** (2.0 mg, t_R=11.5 min), **4** (2.0 mg, t_R=8.0 min), and **6** (10.0 mg, t_R=10.5 min). Fr.2-2 was further purified by silica gel flash column chromatography (*n*-hexane/EtOAc, *V*∶*V*=5∶1→1∶1) to yield compound **5** (100.0 mg). Fr.2-3 was further purified by silica gel flash column chromatography (*n*-hexane/EtOAc, *V*∶*V*=5∶1→1∶1) to yield compound **1** (5.0 mg) and Fr.2-3-1. Fr.2-3-1 was further purified by semiprep-HPLC (MeOH/H_2O, *V*∶*V*=60∶40, 3 mL/min) to give three subfration Fr.2-3-1～Fr.2-3-3. Fr.2-3-2 was further purified by semiprep-HPLC (MeOH/H_2O, *V*∶*V*=46∶54, 3 mL/min) to afford compound **2** (2.5 mg, t_R=22.0 min).

3.4 Spectroscopic data

Dothiorelone O (**1**): Yellow oil; UV (MeOH) λ_{max} [log ε/(L•mol^{-1}•cm^{-1})]: 206 (5.30), 268 (4.90), 299 (4.77) nm; ^{1}H NMR and ^{13}C NMR see Table 1; IR ν_{max}: 3308, 2934, 1607, 1458, 1271, 1200, 1148, 1024, 1003, 934, 845, 801, 762, 721, 648 cm^{-1}; HRESIMS calcd for $C_{21}H_{31}O_6$ $[M+H]^+$ 411.2019, found 411.2105.

2-(3,5-Dihydroxy-2-(7-hydroxyoctanoyl)phenyl)acetic acid (**2**): White powder; $[\alpha]_D^{20}$ 0 (*c* 0.03, MeOH); UV (MeOH) λ_{max}: 204 (5.22), 218 (5.15), 272 (4.75) nm; ^{1}H NMR and ^{13}C NMR see Table 1; IR ν_{max}: 3268, 2932, 1732, 1714, 1609, 1593, 1464, 1397, 1373, 1327, 1260, 1028, 847 cm^{-1}; HRESIMS calcd for $C_{16}H_{23}O_6$ $[M+H]^+$ 311.1489, found 311.1468.

3.5 *In vitro* cytotoxicity assay

The cytotoxicities of compounds **1**～**6** were assayed against four tumor cell lines, including SF-268 (human glioma cell line), MCF-7 (human breast adenocarcinoma cell line), HepG-2 (human liver cell line), and A549 (human lung cancer cell line). Assays were performed by the SRB method.[20]

Supporting Information 1D and 2D NMR, UV, HRESIMS spectra of compounds **1** and **2**. These materials can be downloaded for free from the website (http://sioc-journal.cn/).

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