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Chemistry and weak antimicrobial activities of phomopsins produced by mangrove endophytic fungus *Phomopsis* sp. ZSU-H76

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Abstract

Three metabolites named phomopsin A (**1**), B (**2**) and C (**3**), together with two known compounds cytosporone B (**4**) and C (**5**), were isolated from the mangrove endophytic fungus, *Phomopsis* sp. ZSU-H76 obtained from the South China Sea. Their structures were elucidated by spectroscopic methods, mainly by 1D and 2D NMR spectroscopic techniques. The medium-sized cyclic phenol ether based on **1** or **2** is rare in natural products. In bioassays, compounds **1**, **2**, and **3** had no significant antibiotic activities, but compounds **4** and **5** inhibited two fungi *Candida albicans* and *Fusarium oxysporum* with an MIC ranging from 32 to 64 μg/ml.

Keywords: Mangrove; *Excoecaria agallocha*; *Euphorbiaceae*; Endophytic fungus; Metabolites; *Phomopsis* sp.; Antimicrobial activity

1. Introduction

Marine-derived fungi have proven to be a rich source of structurally unique and biologically active secondary metabolites (Bugni and Ireland, 2004). In our search for new metabolites from marine-derived mangrove fungi from the South China Sea, we have isolated many significant new bioactive metabolites (Yang et al., 2006; Chen et al., 2005, 2003; Lin et al., 2002, 2001a,b). This paper reports the isolation, structural elucidation and biological activities of three new metabolites, phomopsin A (**1**), B (**2**) and C (**3**), and two known compounds cytosporone B (**4**) and C (**5**) from the mangrove endophytic fungus, *Phomopsis* sp. ZSU-H76 isolated from the stem of mangrove tree *Excoecaria agallocha* from Dong Zai, Hainan, China.

2. Results and discussion

The fungus ZSU-H76 is an endophytic fungus, which was isolated from the stem of the mangrove tree *E. agallocha*. It is apospory and was identified as *Phomopsis* sp. by DNA internal transcribed spacer (ITS) region (see Section 4). The photograph of its mycelium is shown in Fig. 1. *Phomopsis* sp. could well grows well in a GPY liquid medium.

Compound **1** was obtained as a white powder, with molecular formula $C_{18}H_{24}O_5$ as determined by HR-EIMS at *m*/*z* 320.1610 (calc. 320.1618). The IR spectrum (KBr) showed absorption bands for hydroxyl (3292 cm^{-1}), conjugated carbonyl (1658 cm^{-1}), ester carbonyl (1714 cm^{-1}) and aromatic (1611, 1586, and 1464 cm^{-1}) functional groups. In the ^1H NMR spectrum for **1**, one D_2O-exchangeable proton was observed downfield, which was shown to be a phenolic OH signal at δ 11.32 ppm. The presence of a 1,2,3,5-tetrasubstituted benzene ring was deduced from the following proton signals at δ 6.25

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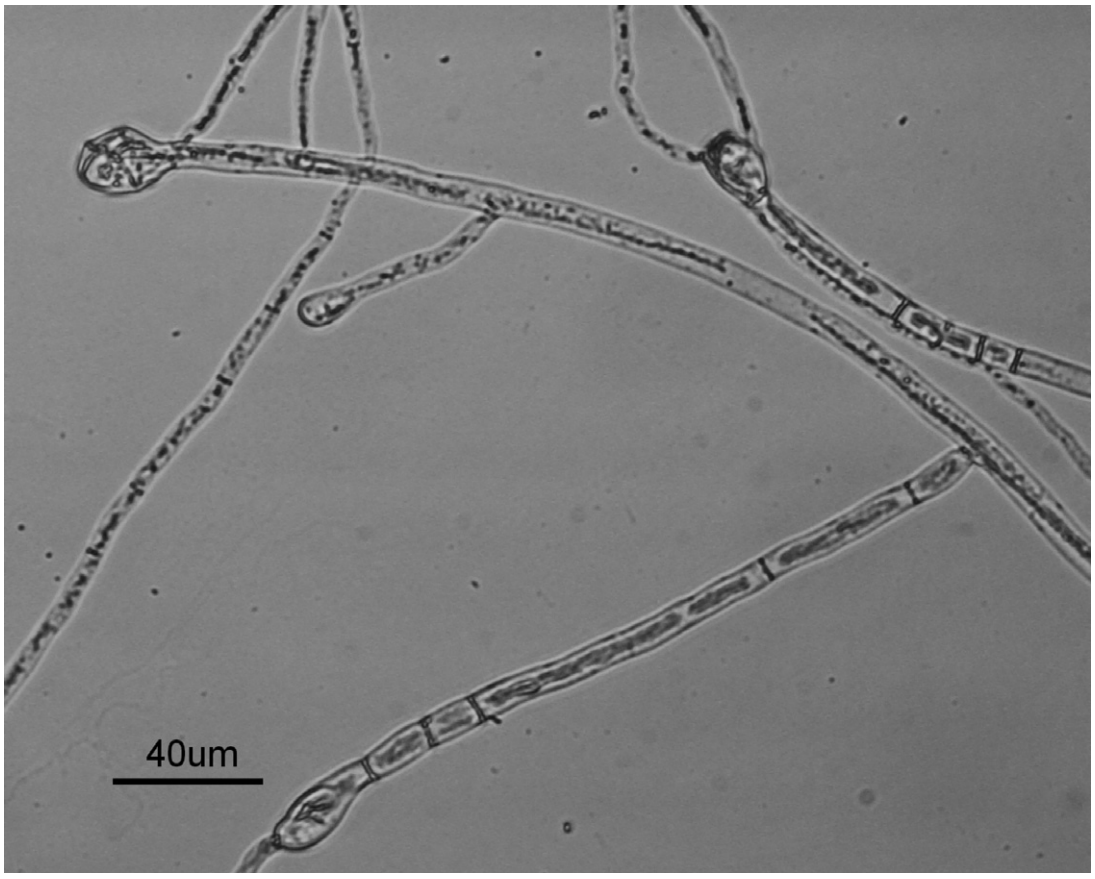


Fig. 1. Light microscopic picture of mycelium of *Phomopsis* sp. ZSU-H76.

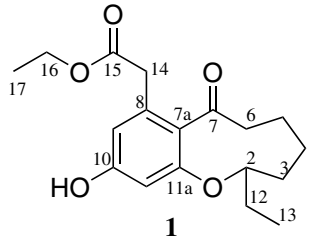
(1 H, *d*, $J = 2.4$ Hz) and δ 6.24 (1 H, *d*, $J = 2.4$ Hz). The remaining proton signals in upfield were attributed to an aliphatic cycle or chain in **1**. The ^{13}C NMR and DEPT spectra indicated there were two methyl groups, seven methylenes, three methines, and six quaternary carbons including one carbonyl carbon (δ 206.7) and an ester carbonyl carbon (δ 171.7). In ^{1}H–^{1}H COSY spectrum of **1**, the correlations of H-16 and H-17, H-12 and H-2, H-13, H-2 and H-3, H-12, H-3 and H-2, H-4, H-4 and H-3, H-5, H-5 and H-4, H-6 showed existence of two partial structures of C-16–C-17 and C-13–C-12–C-2–C-3–C-4–C-5–C-6. The signals of δ 4.18 (2H, *q*, $J = 7.2$ Hz, H-16) and δ 1.26 (3H, *t*, $J = 7.2$ Hz, H-17) were attributed to an ethoxy group. The ethoxy group was linked to the carbonyl group at δ 171.7 (C-15) by the HMBC correlations from H-16 to C-15 and C-17. The HMBC correlations from H-14 to C-7a, C-8, C-9, and C-15, showed that the methylene of the sharp singlet at δ 3.76 (H-14) was located between the carbonyl group (C-15) and the benzene ring (C-8). Correlations in HMBC spectrum, H-2–C-11a, and H-6–C-4, C-5, C-7, C-7a suggested C-2, C-6 connecting to C-11a, C-7a through an ether bond and one carbonyl group (C-7), respectively. The hydroxyl group (δ 11.32) was placed at C-10 (δ 160.44) by the HMBC correlations from OH-10 to C-9, C-10, and C-11. Thus, the structure of compound **1** was determined to be 2-ethyl-10-hydroxy-2,3,4,5,6,7-hexahydro-7-oxo-benzoxonin-8-acetic acid ethyl ester (**1**), named phomopsin A.

Compound **2** was a white powder. Its molecular formula was identical to that of **1** by HR-EIMS at *m*/*z* 320.1613 (calc. 320.1618). The ^{1}H NMR spectrum of **2** was very similar to that of **1**, except for a sharp doublet instead of a triplet at about δ 1.10. In a ^{13}C NMR spectrum of **2**, the two signals of δ 68.2 (CH, C-2) and δ 14.1 (CH_3, C-13), differed from the resonances of δ 73.2 (CH, C-2) and δ 9.9 (CH_3, C-13) in **1**, which suggested that the two compounds shared a similar structure and were isomeric. The methine at δ 68.2 (C-2) attached to the methyl group (C-13) was shown to be connected to C-12a through an ether bond by HMBC correlations from H-2 (δ 3.78) to C-12a (δ 164.1). Therefore, the structure of compound **2** was established as 2-methyl-11-hydroxy-2,3,4,5,6,7,8-heptahydro-8-oxo-benzoxonin-9-acetic acid ethyl ester (**2**), named phomopsin B.

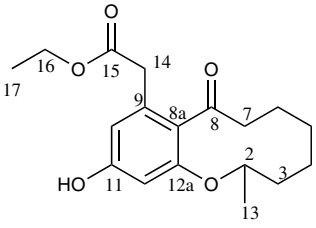
Compound **3** was a white powder. Its molecular formula was determined to be $C_{19}H_{28}O_5$ by HR-EIMS at *m*/*z* 336.1925 (calc. 336.1931). In the ^{13}C NMR and DEPT spectra, **3** has one carbon signal at δ 55.4 (–OCH_3) more than that of the known compound **4** (cytosporone B, $C_{18}H_{26}O_5$) (Brady et al., 2000). The ^{1}H NMR spectrum of **3** was very similar to that of **4**, except for the presence of a sharp singlet at δ 3.81 (–OCH_3) and absence of a broad singlet signals at δ 7.34 (–OH) in **4**. This demonstrated that

the hydroxyl group (–OH) in **4** was replaced by a methoxyl group (–OCH_3) in **3**. The methoxyl group (δ 3.81, δ 55.4) was placed at C-5 (δ 163.6) by HMBC correlations. The HMBC correlations of the hydroxyl proton at δ 12.53 with C-2, C-3, and C-4 indicated the hydroxyl group locating at C-3 (δ 159.2). According to correlations of HMBC and ^1H–^1H COSY, compound **3** was identified as 3-hydroxy-5-methoxy-2-(1-oxooctyl)-benzenacetic acid ethyl ester (**3**), named phomopsin C.

In addition, two known compounds **4** and **5** were identified as cytosporone B and C, respectively, by comparison of their spectroscopic data with those in the literature (Brady et al., 2000). They were isolated for the first time from an endophytic fungus, *Cytospora* sp. CR200, and were inactive in antibacterial tests (Singh et al., 2000; Janso et al., 2000).



1



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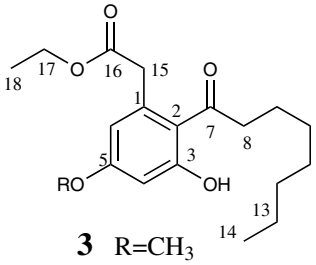
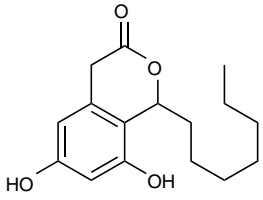


3 R=CH_3
4 R=H



5

In the preliminary bioassays, compounds **1**, **2**, and **3** had no significant antibiotic activity against three bacterial and two fungal strains. Compounds **4** and **5** inhibited two fungi *Candida albicans* and *Fusarium oxysporum* with an MIC ranging from 32 to 64 μg/ml, however, they had no significant antibacterial activity (Table 3).

3. Concluding remarks

The five compounds isolated from this fungus are biogenetic octaketides. They differ in that **3**, **4**, and **5** have long aliphatic chains, while **1** and **2** are a closed ring in different positions. The structures of **1** and **2**, with medium-sized cyclic phenol ether, are rare in natural products.

4. Experimental

4.1. General experimental procedures

Melting points were detected on a Fisher–Johns hot-stage apparatus and were uncorrected. NMR data were recorded on a Varian Inova-500 NB spectrometer, using $CDCl_3$ as solvent and TMS as internal standard. Mass spectra were acquired on a VG-ZAB mass spectrometer. IR spectra were obtained on a Nicolet 5DX-FTIR spectrophotometer, and UV spectra were measured on a Shimadzu UV-240 spectrophotometer. Column chromatography (CC) was carried out on silica gel (200–300 mesh; Qingdao haiyang chemicals).

4.2. Fungus isolation and growth conditions

The fungus was isolated from the stem of mangrove tree *E. agallocha* from Dong Zai, Hainan, China, and deposited in The Department of Applied Chemistry, the number is ZSU-H76. The nucleotide sequences obtained in this study have been submitting to GenBank and assigned accession numbers EU236702. Analysis of sequences: highly similar sequences are EF423532.2 (Max ident 98%, E value 0.0, Query coverage 93%), AY577815.1 (Max ident 97%, E value 0.0, Query coverage 95%), EF026104.1 (Max ident 97%, E value 0.0, Query coverage 95%), AF000207.1 (Max ident 97%, E value 0.0, Query coverage 95%). The fungus was cultivated in a GPY the liquid medium (glucose 10 g/l, peptone 2 g/l, yeast extract 1 g/l, NaCl 3 g/l). Starter cultures were maintained on cornmeal seawater agar. Plugs of agar supporting mycelia growth were cut and transferred aseptically into a 250 ml Erlenmeyer flask containing 100 ml of liquid medium, and incubated at 30 °C on a rotary shaker for 5–7 days. The mycelium was aseptically transferred into 500 ml Erlenmeyer flasks containing the liquid medium (200 ml), and incubated at 30 °C at 200 rpm for 25 days. Fig. 1 shows the light microscopic picture of the mycelium of *Phomopsis* sp. ZSU-H76.

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data for compounds **1** and **2** in $CDCl_3$ (*J* values in Hz)

Position	**1**				**2**			
	^{1}H (*J*)	^{13}C	HMBC	COSY	^{1}H (*J*)	^{13}C	HMBC	COSY
2	3.55 *m*	73.2	C-11a	H-3, H-12	3.78 *m*	68.2	C-12a	H-3, H-13
3	1.47 *m*	36.3	C-2, C-4	H-2, H-4	1.44 *m*	39.0	C-2	H-2, H-4
4	1.46 *m*	25.1	C-2, C-3, C-5	H-3, H-5	1.65 *m*	25.3	C-3, C-5	H-3, H-5
5	1.73 *m*	24.6	C-3, C-4, C-6	H-4, H-6	1.33 *m*	29.0	C-4, C-6	H-6
6	2.85 *t* (7.2)	43.2	C-4, C-5, C-7, C-7a	H-5	1.71 *m*	24.8	C-5, C-7, C-8	H-5, H-7
7		206.7			2.84 *t* (7.5)	43.1	C-5, C-6, C-8, C-8a	H-6
7a		117.1						
8		136.4				206.6		
8a						116.8		
9	6.24 *d* (2.4)	112.5	C-7a, C-10, C-11, C-14			136.6		
10		160.4			6.26 *d* (1.5)	112.4	C-8a, C-11, C-12, C-14	
11	6.25 *d* (2.4)	103.0	C-7a, C-9, C-10, C-11a			160.3		
11a		163.0						
12	1.48 *m*	30.1	C-2, C-13	H-2, H-13	6.28 *d* (1.5)	103.2	C-8a, C-10, C-11, C-12a	
12a						164.1		
13	0.93 *t* (7.2)	9.8	C-2, C-12	H-12	1.18 *d* (6.5)	14.1	C-2, C-3	H-2
14	3.76 *s*	41.4	C-7a, C-8, C-9, C-5		3.79 *s*	41.6	C-8a, C-9, C-10, C-15	
15		171.7				171.1		
16	4.18 *q* (7.2)	61.5	C-15, C-17	H-17	4.18 *q* (7.5)	61.4	C-15	H-17
17	1.26 *t* (7.2)	14.1	C-16	H-16	1.26 *t* (7.5)	23.4	C-16	H-16
10–OH	11.32 *s*		C-9, C-10, C-11					
11–OH					11.80 *s*		C-10, C-11, C-12	

4.3. Antimicrobial assay

The compounds were tested against five aerobic reference strains for their inhibitory activity. Antimicrobial assays were performed using a modified version of the 2-fold serial dilutions method as Fromtling et al. (1993). Experimental results are presented in Table 3.

4.4. Extraction and isolation

The cultures (80 l) were separated into mycelium and filtrate. The filtrate was concentrated to 3.5 l below 50 °C, and extracted five times by shaking with an equal volume of EtOAc. Collection and evaporation of EtOAc *in vacuo* yielded the EtOAc extract. The EtOAc extracts were subjected to silica gel CC using gradient elution with petroleum ether–EtOAc (90:10–60:40), to give five fractions (A–E). Fraction D was purified by silica gel CC with a gradient of petroleum ether–EtOAc (80:20–60:40) to give four subfractions (B1–B4). Subfraction B3 was further purified by precoated TLC (silica gel F_{254}) using petroleum ether–EtOA (80:20) as mobile phase to give compound **1** (3.5 mg). Fraction C was purified by silica gel CC with petroleum ether–EtOAc (85:15) to give compound **2** (4 mg). The mycelium was immersed in MeOH (4 l) for 20 days. Evaporation of the MeOH extract gave brown oil. The oil was applied to a silica gel column, eluted with a gradient of petroleum ether–EtOAc (5:95–50:50) affording four fractions (A–D). Fraction B was applied to silica gel column elutal with petroleum ether–EtOAc (90:10) to give compound **3** (6 mg). Fraction C was separated by silica gel CC (petroleum ether–EtOAc, 80:20) and preparative TLC (petroleum ether–EtOAc, 70:30) to give compound **4** (5 mg). Fraction D was purified by preparative TLC (petroleum ether–EtOAc, 80:20) to give compound **5** (4 mg).

4.5. 2-Ethyl-10-hydroxy-2,3,4,5,6,7-hexahydro-7-oxo-benzoxonin-8-acetic acid ethyl ester (*1*)

White powder, m.p. 88–90 °C; $[\alpha]_D^{22}$ −76.1 (c 0.05, MeOH). UV λ_{max} (MeOH) nm (logε): 221(2.64),

Table 2
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data for compound **3** in $CDCl_3$ (*J* values in Hz)

Position	^{1}H (*J*)	^{13}C	HMBC	COSY
1		136.2		
2		115.9		
3		165.2		
4	6.38 *d* (2.0)	100.4	C-2, C-3, C-5, C-6	
5		163.6		
6	6.33 *d* (2.0)	112.5	C-2, C-4, C-5, C-15	
7		206.6		
8	2.83 *t* (7.2)	43.2	C-2, C-7, C-9, C-10	H-9
9	1.70 *m*	25.0	C-7, C-8, C-10	H-8, H-10
10	1.26 *m*	29.1	C-9, C-11, C-12	H-9
11	1.26 *m*	29.0	C-10, C-12	
12	1.23 *m*	31.6	C-13	
13	1.24 *m*	22.5	C-14	H-14
14	0.88 *t* (7.2)	14.2	C-12, C-13	H-13
15	3.86 *s*	42.0	C-1, C-2, C-6, C-16	
16		170.7		
17	4.18 *q* (7.2)	61.3	C-16, C-18	H-18
18	1.24 *t* (7.2)	14.0	C-17	H-17
5–OCH_3	3.81 *s*	55.4	C-5	
3–OH	12.53		C-2, C-3, C-4	

Table 3
Tests of MIC (μg/ml) of the compounds against three bacterial and two fungal strains[a]

Strains	Compounds						
	1	**2**	**3**	**4**	**5**	Gen[b]	Nys[b]
Staphylococcus aureus ATCC27154	>128	>128	>128	>128	>128	2	NT
Escherichia coli ATCC 25922	>128	>128	>128	>128	>128	4	NT
Salmonella enteritidis ATCC 13076	>128	>128	>128	>128	>128	2	NT
Candida albicans ATCC 10231	>128	>128	>128	64	32	NT	2
Fusarium oxysporum f.sp.cubense	>128	>128	>128	64	32	NT	4

[a] Results are expressed as the minimum inhibitory concentration (MIC).
[b] Gentamicin (Gen), Nystatin (Nys): positive control; NT, not tested.

266(2.42). For ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectra, see Table 1. IR (KBr) υ_{max} cm^{-1}: 3292, 2935, 2875, 1714, 1658, 1611, 1586, 1464, 1326, 1272, 1164. EIMS *m/z* (rel. int.): 320(13, $[M]^+$), 292(5, $[M–H_2O]^+$), 248(25) 205(31), 196(45), 167(100), 150(17). HR-EIMS $[M]^+$ found 320.1610 $C_{18}H_{24}O_5$, calc. for 320.1618.

4.6. 2-Methyl-11-hydroxy-2,3,4,5,6,7,8-heptahydro-8-oxo-benzoxonin-9-acetic acid ethyl ester (**2**)

White powder, m.p. 112–114 °C; $[\alpha]_D^{22}$ −150.0 (c 0.09, MeOH). UV λ_{max} (MeOH) nm (log ε): 223(2.97), 268(2.71). For ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectra, see Table 1. IR (KBr) υ_{max} cm^{-1}: 3281, 2930, 2856, 1688, 1640, 1616, 1593, 1474, 1325, 1298, 1273, 1168. EIMS *m/z* (rel. int.): 320(10, $[M]^+$), 223(27), 205(17), 195(39), 167(100), 150(18). HR-EIMS $[M]^+$ found 320.1613 $C_{18}H_{24}O_5$, calc. for 320.1618.

4.7. 3-Hydroxy-5-methoxy-2-(1-oxooctyl)-benzenacetic acid ethyl ester (**3**)

White powder, m.p. 73–75 °C; UV λ_{max} (MeOH) nm (log ε): 230(3.58), 266(3.33), 296(3.17). For ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) spectra, see Table 2. IR (KBr) υ_{max} cm^{-1}: 3229, 2925, 2854, 1703, 1659, 1612, 1590, 1440, 1312, 1201, 1157. EIMS *m/z* (rel. int.): 336(24, $[M]^+$), 318(10, $[M–H_2O]^+$), 237(50) 210(62), 181(100), 164(32). HR-EIMS $[M]^+$ found 336.1925, $C_{19}H_{28}O_5$, calc. for 336.1931.

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