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Metabolites from the Mangrove Endophytic Fungus *Phomopsis* sp. (#zsu-H76)

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Three unique metabolites, namely, phomopsis-H76 A (**1**), B (**2**), and C (**3**), were isolated from the mangrove endophytic fungus *Phomopsis* sp. (#zsu-H76). Structures were determined by spectroscopic methods, mainly 1D and 2D NMR spectroscopy. Compound **1**–**3** are all dimers. Compounds **2** and **3** possesses a pyrano[4,3-*b*]pyran-5(2*H*)-one ring system that is unprecedented in nature. Primary bioassays showed that **1** accelerated the growth of subintestinal vessel plexus (SIV) branch markedly, whereas **3** showed inhibition of SIV. Compounds **1**–**3** showed no activity in antibacterial and cytotoxic tests.

Introduction

It is known that fungi of the genus *Phomopsis* produce a rich variety of metabolites. Over the last 40 years, more than 40 unique compounds have been found from this genus, including peptides, lactones, xanthones, mycoepoxydienes, and so on.[1] In our ongoing research on the bioactive metabolites from marine fungi,[2] the endophytic fungus *Phomopsis* sp. (#zsu-H76) was isolated from mangrove tree *Excoecaria agallocha* of the South China Sea coast and its metabolites were analyzed In a previous study, three new large ring lactones were isolated from this fungus.[3] Recently we further isolated three metabolites, namely, phomopsis-H76 A (**1**), B (**2**) and, C (**3**), which belong to new series of compounds. It is interesting that compounds **1**–**3** all possess dimeric structures and unique ring systems. In this paper, we describe their isolation, structural elucidation, and bioactivity.

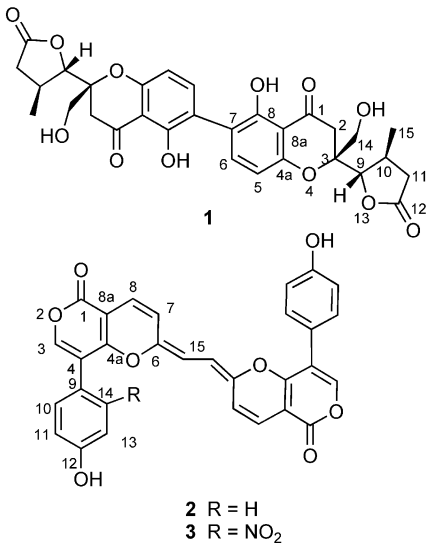

Figure 1. Molecular structures of **1**–**3**.

The ethyl acetate extract of a fermentation broth of the fungus was subjected to silica gel column chromatography, which led to the isolation of **1**–**3** (Figures 1 and 2).

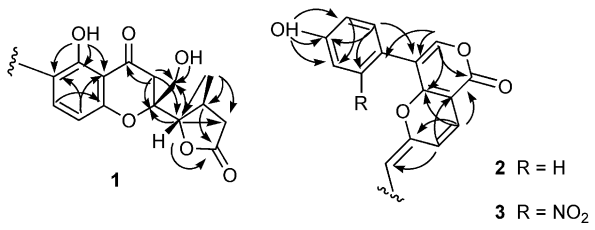

Figure 2. The key HMBC correlations of **1**–**3**.

Results and Discussion

Compound **1** was obtained as light-yellow powder. HRMS (EI) analysis provided the molecular formula $C_{30}H_{30}O_{12}$ (obsd. *m*/*z* = 582.1731 $[M]^+$, calcd. *m*/*z* = 582.1732). In the ^{13}C NMR and DEPT spectra, there were 15 signals, which were assigned to 1 methyl group, 3 methylenes, 4 methines, 5 quaternary carbon atoms, 1 carbonyl carbon (δ_C = 200.2 ppm), and one γ-lactone carbonyl carbon (δ_H = 178.7 ppm, IR: $\tilde{\nu}$ = 1777 cm^{-1}). In the ^1H NMR spectrum, a total of 15 protons were observed, including an exchangeable proton at δ_H = 5.41 ppm (likely

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an aliphatic hydroxy group), and another chelated phenol hydroxy proton (δ_H = 11.97 ppm). On the basis of the analysis of the NMR spectra, the elemental composition was deduced as $C_{15}H_{15}O_6$, which is just half the molecular formula. Thus, compound **1** should be a dimer.

The ^1H–^1H COSY correlation between the oxymethine proton 9-H (δ_H = 4.37 ppm) and methine proton 10-H (δ_H = 2.79 ppm), and correlations of 10-H with the methylene proton 11-H (δ_H = 2.75, 2.22 ppm) and the methyl proton 15-H (δ_H = 1.15 ppm), combined with the HMBC correlations from 11-H to C-9, C-10, and C-12, established the γ-lactone substructure. In the ^{13}C NMR, there were six aromatic carbon signals, and the ^1H NMR showed *ortho*-aromatic protons 6-H (δ_H = 7.38 ppm, J = 8.5 Hz) and 5-H (δ_H = 6.48 ppm, J = 8.5 Hz). These signals were assigned to a tetrasubstituted benzene ring. The HMBC correlations from OH (δ_H = 11.97 ppm) to C-7, C-8, and C-8a; from 6-H to C-5, C-7, and C-4a; and from 5-H to C-7 and C-8a defined the substituted positions. Signals for a chelated phenolic hydroxy group with the corresponding ketone carbonyl group and an oxygen bearing *meta*-carbon (4$_a$ δ_C = 161.5 ppm) in the benzene ring suggested the presence of a chromone unit in the monomer. The HMBC spectrum assembled the intact molecular structure (see Table 1). The key correlations from 9-H to C-2 and C-3 connected the chromone unit and the γ-lactone unit at C-3 and C-9. According to the correlations from 14-H to C-3, C-2, and C-9, the C-14 atom was also attached at C-3. In the ROESY spectrum, the correlation between 9-H and CH_3-15 revealed that they were on the same side of the γ-lactone ring. The ROESY spectrum showed correlation between 9-H and 2-H, between 9-H and 15-H, and between 14-H and 2-H, thus implying that 9-H, 2-H, 15-H, and 14-H were in the same plane. Meanwhile, the moiety of **1** identical to Blennolide E was reported by Krohn,[4] except that the hydromethyl group was changed into an ester group. Comparing the correlations of Blennolide E with **1**, the stereochemistry of **1** was tentatively deduced to be the same of that of Blennolide E. Compound **1** is a dimer containing a chromone and a γ-lactone unit.

Compound **2** was isolated as a yellow powder. The molecular formula was determined from HRMS (ESI), which gave the molecular ion peak at m/z = 505.0915 $[M - H]^-$ (calcd. m/z = 505.0923), and the MS (ESI) showed a peak at m/z = 530 $[M + H + Na]^+$. The HRMS (EI) spectrum fragment at m/z = 253.0521 (calcd. for $C_{15}H_9O_4$, 253.0495) suggested that **2** was also a dimer (Table 2). This deduction was supported by the following data. In the ^{13}C and ^1H NMR spectra, there were only 15 carbon and 9 proton signals, respectively, including the carbon atoms of 7 double bonds and a δ-lactone carbonyl carbon (δ_C = 174.8 ppm, IR: $\tilde{\nu}$ =1711 cm^{-1}); 8 olefinic protons and 1 hydroxy proton (δ_H = 9.60 ppm). The above data accounted for 8 of the 11.5 unsaturation equivalents required for the monomer of **2**, which indicated that this monomer had three rings.

In the ^1H NMR spectrum, one AA′XX′ spin systems at δ_H = 7.35 ppm (d, J = 8.4 Hz, 2 H) and δ_H = 6.79 ppm (d, J = 8.4 Hz, 2 H) revealed a *para*-disubstituted benzene ring. The HMBC multiple correlations of 3-H with C-1, C-4, and C-4a were used to define the δ-lactone ring system. The COSY correlation between 8-H to 7-H, and the long-range correlation from 7-H to 15-H, together with the HMBC correlations of 8-H to C-1 and C-6 and 7-H to C-8a and C-15, showed a contiguous sequence from C-8a to C-15. Both downfield carbon atoms C-4a (δ_C = 157.5 ppm) and C-6 (δ_C = 162.5 ppm) required bonding of an oxygen atom. In the monomer, only one oxygen atom was unassigned; therefore, it had to be attached at C-4a and C-6 to form a cyclic ether. Finally, the position linking two monomers could only be at C-15.

Compound **3** was a yellow powder. HRMS (ESI) data showed $[M + H]^+$ at m/z = 597.0781 (calcd. for $C_{30}H_{16}N_2O_{12}$ m/z = 597.0781), and the MS (ESI) showed [M + 1] at m/z = 597. The HRMS (EI) spectrum showed a

Table 1. NMR spectroscopic data (DMSO) of **1**.[a,b]

Atom	δ_C [ppm]	δ_H [ppm] (multiplicity, J [Hz])	COSY[b]	HMBC[b]
1	200.2			
2	39.9	3.11 (d, 17.5) 2.93 (d, 17.5)		C-1, C-3, C-8a, C-9, C-14
3	86.6			
4a	161.5			
5	109.4	6.48 (d, 8.5)	6-H	C-7, C-8a
6	142.7	7.38 (d, 8.5)	11-H	C-7, C-4a, C-5
7	118.8			
8	160.6			
8a	109.6			
9	89.3	4.37 (d, 4.5)	10-H	C-3, C-2, C-10, C-12, C-15, C-14
10	31.8	2.79 (m)	9-H,15,11	C-3, C-12, C-15
11	38.6	2.75 (dd, 9.5, 12.5) 2.22 (d, 12.5)	10-H	C-9, C-10, C-12, C-15
12	178.7			
14	65.0	3.68 (s)	14-OH	C-3, C-2, C-9
15	22.7	1.15 (d, 6.5)	10-H	C-9, C-10, C-11
8-OH		11.97 (s)		C-8, C-7, 8a
14-OH		5.41 (s)	14-H	C-3, C-14

[a] Measured at 500 MHz (for ^1H) and 125 MHz (for ^{13}C). [b] For the HMBC and COSY spectra, see the Supporting Information.

Table 2. NMR spectroscopic data (DMSO) of **2**.[a,b]

Atom	δ_C [ppm]	δ_H [ppm] (mult., *J* [Hz])	COSY[b]	HMBC[b]
1	174.8			
3	152.8	8.24 (s)		C-1, C-4, C-4a, C-9
4	123.5			
4a	157.5			
6	162.5			
7	115.2	6.92 (dd, 2.4,8.4)	8-H	C-8a, C-15
8	127.3	7.95 (d, 8.4)	7-H	C-1, C-6, C-4a
8a	116.6			
9	122.6			
10	130.1	7.35 (d, 8.4)	11-H	C-4, C-12
11	115.0	6.79 (d, 8.4)	10-H	C-9, C-12, C-13
12	157.2			
13	115.0	6.79 (d, 8.4)	14-H	C-9, C-12, C-11
14	130.1	7.35 (d, 8.4)	13-H	C-4, C-12
15	102.1	6.84 (d, 2.4)	7-H	C-7, C-6, C-4a
12-OH		9.60 (s)		C-13, C-12,C-11

[a] Measured at 500 MHz (for ^{1}H) and 125 MHz (for ^{13}C). [b] For the HMBC and COSY spectra, see the Supporting Information.

fragment at *m*/*z* = 298.0337 for $C_{15}H_8NO_6$ (calcd. 298.0346) to be half of the molecular formula, with one nitrogen and two oxygen atoms more than that of the monomer of **2**. Therefore it was supposed that **3** was also dimer. The NMR spectra were similar to those of **2**, except for the absence of an aromatic methine but with the addition of an aromatic quaternary carbon and two doublets (2 H each) of aromatic protons changed to two doublets (1 H each) and one singlet (1 H). These findings indicated that the structure of **3** was identical to that of **2**, except that one nitro group had replaced one aromatic proton of **2**. On the basis of the HMBC correlations from OH to C-11, C-12, and C-13, the NO_2 group was located at C-14. Finally, the overall structure of **3** was proved by analysis of the HMBC and COSY experiments (see Table 3).

Table 3. NMR spectroscopic data (DMSO) of **3**.[a,b]

Atom	δ_C [ppm]	δ_H [ppm] (mult., *J* [Hz])	COSY[b]	HMBC[b]
1	174.4			
3	154.1	8.46 (s)		C-1,C-4, C-9, C-4a
4	123.2			
4a	157.6			
6	162.8			
7	115.5	6.96 (dd, 2.4, 8.8)	8-H, 15-H	C-8a, C-15
8	127.3	7.98 (d, 8.8)	7-H	C-1, C-6, C- 4a
8a	116.5			
9	121.4			
10	119.0	7.18 (d, 8.8)	11-H	C-4, C-12,C-14
11	135.5	7.75 (d, 8.8)	10-H, 13-H	C-12,C-13
12	157.2			
13	125.2	8.16 (d, 2.4)	11-H	C-9, C-11, C-12
14	136.6			
15	102.3	6.89 (d, 2.4)	7-H	C-7, C-4a
12-OH		11.00 (s)		C-11, C-12, C-13

[a] Measured at 500 MHz (for ^{1}H) and 125 MHz (for ^{13}C). [b] For the HMBC and COSY spectra, see the Supporting Information.

The bioactivity of the three metabolites was tested by zebrafish embryos collection. Zebrafish embryos were incubated with three compounds at a final concentration of 100 μM for 72 h. Data were expressed as mean ± SD. Results were obtained from three independent experiments. Results showed that compound **1** could accelerate subintestinal vessel plexus (SIV) branch markedly, whereas **3** inhibited blood vessel growth (Figure 3). In the antibacterial test, **1**–**3** showed no effect against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli*, and *Sarcina lutea* at 50 μg mL^{-1}. The IC_{50} values of cytotoxic activity against KB, KBv200, and MCF-7 cell lines were over 50 μmol mL^{-1}.

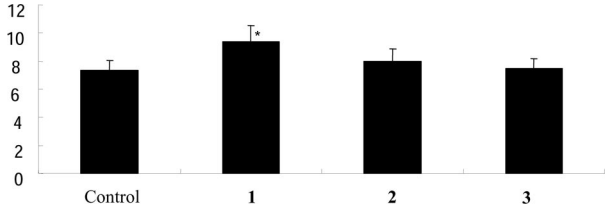

Figure 3. The effect of three compounds on embryonic angiogenesis. Asterisks: significant differences (*$p < 0.05$).

Conclusions

Phomopsis sp. (#zsu-H76) is a prolific producer of novel metabolites. Six unique compounds with bioactivities have been isolated from this fungus. Compounds **1**–**3** are all dimeric structures, and **2** and **3** contain a pyrano[4,3-*b*]pyran-5(2*H*)-one ring system. This is unprecedented in nature. In the primary bioassays, **1** markedly accelerated the growth of subintestinal vessel plexus (SIV) branch, whereas **3** inhibited blood vessel growth; however, **1**–**3** showed no activities in antibacterial and cytotoxic tests.

Experimental Section

General: Melting points were determined with an X-4 micromelting point apparatus. Optical rotations were measured with a Schmidt & Haensch Polartronic HH W5 polarimeter. UV spectra were measured with a Shimadzu UV-2501 PC spectrophotometer. IR spectra were measured with a Bruker Vector 22 spectrophotometer. ^{1}H and ^{13}C NMR spectroscopic data were recorded with a Varian Inova 500 (400) MB NMR spectrometer operating at 500(400) and 125 MHz for ^{1}H and ^{13}C, respectively (TMS as internal standard). HRMS (EI) were measured with a Thermo MAT95XP high-resolution mass spectrometer, and HRMS (ESI) were measured with a Shimadzu LC–MS (IT-TOF). MS (EI) were with a Thermo DSQ EI-mass spectrometer. LC–MS, Applied Biosystems/MDS Sciex, and ESI source. Si gel (200–300 mesh, Qingdao Haiyang Chemical Co.Ltd) was used for column chromatography (CC).

Fungal Strain: The fungus *Phomopsis* sp. (#zsu-H76) 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 fungus was cultivated in a GYT medium (glucose 10 g L^{-1}, peptone 2 g L^{-1}, yeast extract 1 g L^{-1}, NaCl 3 g L^{-1}). 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 the liquid medium (100 mL) and incubated at 25 °C on a rotary shaker for 5–7 d. The mycelium was aseptically transferred into a 500-mL Erlenmeyer flask containing the liquid medium (250 mL). The mycelium was harvested and resuspended to 300 L liquid GYT medium, incubated at 25 °C for 25 d.

Extraction and Separation of Metabolites: The cultures (100 L) were separated into mycelium and filtrate. The filtrate was concentrated to 4 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 by using gradient elution with petroleum ether and EtOAc to obtain **1** (7.0 mg), **2** (5.0 mg), and **3** (3.5 mg) from the EtOAc/petroleum ether (70:30,50:50, and 60:40) fractions, respectively.

Compound 1: Light-yellow powder. M.p. 283–285 °C. $[a]_D^{25}$ = +20 (c = 0.020, MeOH). UV (MeOH): λ_{max} (logε) = 253 (0.28) nm. IR (KBr): $\tilde{\nu}$ = 3396, 1777, 1626, 1491, 1435, 1370, 1268, 1223, 1060, 1015, 895, 873 cm^{-1}. For ^{1}H, ^{13}C, and 2D NMR spectroscopic data, see Table 1. HRMS (EI): calcd. for $C_{30}H_{30}O_{15}$ 582.1732; found 582.1731.

Compound 2: Yellow powder. M.p. 266–268 °C. UV (MeOH): λ_{max} (logε) = 299 (0.62), 248 (1.52) nm. IR (KBr): $\tilde{\nu}$ = 3324, 3101, 2921, 2852, 1711, 1632, 1596, 1518, 1462, 1385, 1307, 1280, 1240, 1192, 1097, 888, 843, 791 cm^{-1}. For ^{1}H, ^{13}C, and 2D NMR spectroscopic data, see Table 2. HRMS (ESI): for $C_{30}H_{18}O_8$ [M – H]$^-$ 505.0923; found 505.0915. HRMS (EI): calcd. for $C_{15}H_9O_4$ 253.0495; found 253.0521. MS (ESI): m/z = 530 [M + H + Na]$^+$.

Compound 3: Yellow powder. M.p. 317–320 °C. UV (MeOH): λ_{max} (logε) = 251 (0.69) nm. IR (KBr): $\tilde{\nu}$ = 3403, 3101, 2922, 2584, 1629, 1582, 1526, 1464, 1425, 1376, 1345, 1315, 1270, 1106, 936, 897, 796, 690 cm^{-1}. For ^{1}H, ^{13}C, and 2D NMR spectroscopic data, see Table 3. HRMS (ESI): calcd. for $C_{30}H_{16}N_2O_{12}$ 597.0781 [M + H]$^+$; found 597.0781. HRMS (EI): calcd. for $C_{15}H_8O_6N$ 298.0346; found 298.0337. MS (ESI): m/z = 597 [M + 1].

Embryo Collection and Drug Treatment: Zebrafish embryos were used to examine the effect of different compounds on embryonic angiogenesis. Embryos were maintained in embryo water (0.2 g L^{-1} of Instant Ocean Salt in distilled water) at 27 °C, and the embryos were sorted for viability and developmental stage (shield stage) at 6 hpf. Three embryos were placed into each well of a 96-well plate containing 100 μL embryo water ± drug treatment. The embryos were examined daily for viability, gross morphological abnormalities, and blood vessel development using an inverted Olympus DP70 epifluorescence microscope (Olympus, Tokyo, Japan). At 72 hpf, the embryos were anesthetized by using 0.05% 2-phenoxyethanol in embryo water and each embryo was examined for the presence of ectopic vessels in the subintestinal vessel plexus (SIV).

Supporting Information (see footnote on the first page of this article): IR and 1D and 2D NMR spectra of **1**–**3**, HREIMS of **1**, HRESIMS of **2** and **3**.

Acknowledgments

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