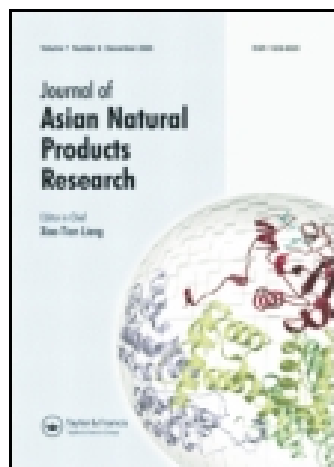


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Two new compounds from an endophytic fungus *Pestalotiopsis heterocornis*

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Two new compounds, 7-hydroxy-5-methoxy-4,6-dimethyl-7-*O*- α -L-rhamnosyl-phthalide (**2**) and 7-hydroxy-5-methoxy-4,6-dimethyl-7-*O*- β -D-glucopyranosyl-phthalide (**3**), along with one known and related metabolite 7-hydroxy-5-methoxy-4,6-dimethylphthalide (**1**) were isolated from the EtOAc extract of fermentation broth of an endophytic fungus *Pestalotiopsis heterocornis*. The structures of these compounds were elucidated on the basis of spectroscopic methods (UV, IR, HR-ESI-MS, 1D NMR, and 2D NMR).

Keywords: *Pestalotiopsis heterocornis*; endophytic fungus; phthalide derivatives

1. Introduction

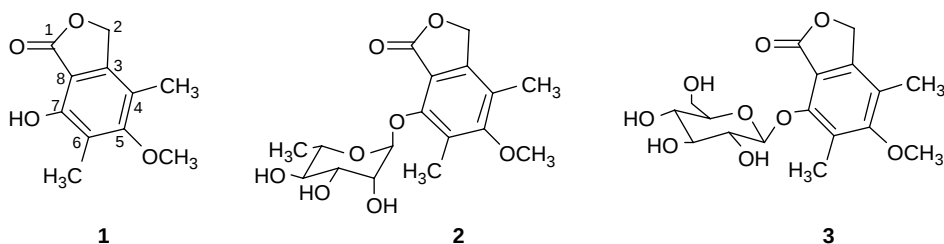
Endophytic fungi that inhabit normal tissues of the host plants without causing apparent pathogenic symptoms have been demonstrated to be rich sources of bioactive natural products [1–3]. As one class of the most widely distributed endophytic fungi, *Pestalotiopsis* spp. have attracted much attention in recent years for their ability to produce a variety of natural products such as triterpenoids, polyketides, meroterpenoids and so on [4–15]. *Pestalotiopsis heterocornis* (L421) was isolated from the stem of *Bruguiera gymnorrhiza* collected in Dongzhai, Hainan Province of China. Chemical investigation on the ethyl acetate extract of the fermentation broth of L421 afforded two new compounds 7-hydroxy-5-methoxy-4,6-dimethyl-7-*O*- α -L-rhamnosyl-phthalide (**2**) and 7-hydroxy-5-methoxy-4,6-dimethyl-7-*O*- β -D-glucopyranosyl-phthalide (**3**), together with one known compound, 7-hydroxy-5-methoxy-4,6-dimethylphthalide (**1**; Figure 1). Here,

we report the isolation and structural elucidation of these compounds.

2. Results and discussion

Compound **2** was obtained as a yellow oil, and its molecular formula was determined to be $C_{17}H_{22}O_8$ by HR-ESI-MS at m/z 377.1215 $[M + Na]^+$. The IR spectrum indicated the presence of hydroxyl (3374 cm^{-1}), ester carbonyl (1744 cm^{-1}), and benzene ring (1605 and 1441 cm^{-1}). The absorption maxima in the UV spectrum (216, 247, and 292 nm) also exhibited the presence of a benzene ring. The ^1H NMR spectrum showed one methoxyl at δ_{H} 3.79 (3H, s) and three methyls at δ_{H} 2.27 (3H, s), 2.21 (3H, s), and 1.13 (3H, d, $J = 6.2\text{ Hz}$). In addition, five proton signals were found in the range of δ_{H} 3.5–6.0, which suggested the existence of a sugar moiety. On acid hydrolysis using 0.5 mol l^{-1} HCl at 70°C for 3 h, rhamnose was detected in the water solution. The specific rotation ($[\alpha]_{\text{D}}^{22} - 64$) demonstrated its L-configuration. The

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Figure 1. Structures of compounds **1–3**.

anomeric proton signal at δ_{H} 5.53 ($\delta > 5$) and the anomeric carbon signal at δ_{C} 106.4 ($J_{\text{C1-H1}} = 166 \text{ Hz}$) further suggested the presence of α -L-rhamnose in the molecule. Seventeen carbon signals were shown in the ^{13}C NMR spectrum, including the carbon signals of hexa-substituted benzene ring at δ_{C} 164.5, 154.9, 148.5, 126.6, 122.2, and 113.2, an oxygenated methylene at δ_{C} 69.8, and an ester carbonyl at δ_{C} 171.3. With the help of the HSQC spectrum, the ^{13}C NMR spectrum showed one methoxyl carbon at δ_{C} 60.9, three methyls at δ_{C} 18.0, 11.3, and 10.6, six carbon signals belonging to the α -rhamnose moiety at δ_{C} 106.4, 73.5, 72.4, 72.2, 72.0, and 18.0. The structure assembly of **2** was mainly achieved using the HMBC experiment. The HMBC correlations between the methoxyl at δ_{H} 3.79 and C-5 at δ_{C} 164.5; the methyl at δ_{H} 2.21 and C-3 at δ_{C} 148.5, C-4 at δ_{C} 122.2, and C-5 at δ_{C} 164.5; the methyl at δ_{H} 2.27 and C-5 at δ_{C} 164.5, C-6 at δ_{C} 126.6, and C-7 at δ_{C} 154.9; the methylene at δ_{H} 5.21 and C-1 at δ_{C} 171.3 and C-3 at δ_{C} 148.5; and H-1' at δ_{H} 5.53 (1H, d, $J = 1.2 \text{ Hz}$) and C-7 at δ_{C} 154.9 suggested a methoxyl, two

methyls, a methylene, and a rhamnose moiety located at C-5, C-4, C-6, C-2, and C-7, respectively (Figure 2). Thus, the structure of **2** was characterized as 7-hydroxy-5-methoxy-4,6-dimethyl-7- O - α -L-rhamnosyl-phthalide (Figure 1). It was a new phthalide derivative, i.e. the rhamnoside of known compound **1**.

Compound **3** was obtained as a colorless crystal, and its molecular formula was determined to be $\text{C}_{17}\text{H}_{22}\text{O}_9$ by HR-ESI-MS at m/z 393.1156 $[\text{M} + \text{Na}]^+$. Its IR spectrum indicated the presence of hydroxyl (3374 cm^{-1}), ester carbonyl (1736 cm^{-1}), and benzene ring (1513 and 1452 cm^{-1}). The UV spectrum showed absorption maxima at 212, 249, and 291 nm. The ^1H NMR and ^{13}C NMR spectral data of **3** were very similar to those of **2** (Table 1), except that the methyl at δ_{C} 18.0 (C-6') in **2** was replaced by the methylene at δ_{C} 62.4 (C-6') in **3**. This difference indicated that a hydroxyl group was attached at C-6' in **3** and in another word, the rhamnosyl in compound **2** was changed to glucosyl in compound **3**. This conclusion was supported by the result of

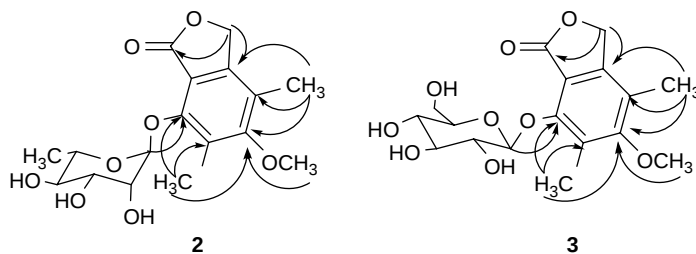
Figure 2. The key HMBC correlations of compounds **2** and **3**.

Table 1. ^1H (600 MHz) and ^{13}C (150 MHz) NMR spectral data of **2** and **3** in MeOD and pyridine- d_5 , respectively.

Position	2		3	
	δ_{C}	δ_{H} (J, Hz)	δ_{C}	δ_{H} (J, Hz)
1	171.3		170.2	
2	69.8	5.21 (2H, s)	69.5	5.02 (2H, s)
3	148.5		146.5	
4	122.2		120.8	
5	164.5		163.0	
6	126.6		126.9	
7	154.9		153.6	
8	113.2		112.5	
5-OCH ₃	60.9	3.79 (3H, s)	59.8	3.59 (3H, s)
4-CH ₃	11.3	2.21 (3H, s)	10.9	1.94 (3H, s)
6-CH ₃	10.6	2.27 (3H, s)	10.8	2.50 (3H, s)
1'	106.4	5.53 (1H, d, 1.2)	106.0	5.98 (1H, d, 7.5)
2'	72.0	4.48 (1H, dd, 3.3, 1.2)	75.9	4.40 (1H, m)
3'	72.4	3.89 (1H, dd, 9.5, 3.3)	78.7	4.34 (1H, m)
4'	73.5	3.52 (1H, dd, 9.5, 9.5)	71.2	4.31 (1H, m)
5'	72.2	4.00 (1H, m)	78.5	4.00 (1H, m)
6'	18.0	1.23 (3H, d, 6.2)	62.4	4.44 (1H, m) 4.31(1H, m)

acid hydrolysis that D-glucose was detected in the water solution of **3**. The coupling constant ($J = 7.5$ Hz) of the anomeric proton (δ_{H} 5.98) demonstrated that the glucose was in β -orientation. The HMBC correlation of H-1' at δ 5.98 with C-7 at δ 153.6 indicated that the glucosyl moiety was linked as C-7 position (Figure 2). Thus, the structure of compound **3** was established as 7-hydroxy-5-methoxy-4,6-dimethyl-7-*O*- β -D-glucopyranosyl-phthalide (Figure 1). It was also a new phthalide derivative, i.e. the glucoside of known compound **1**.

The known compound was readily identified as 7-hydroxy-5-methoxy-4,6-dimethylphthalide (**1**) by comparing its NMR spectral data with those reported in the literature [16].

3. Experimental

3.1 General experimental procedures

Melting point (MP) was determined on an X-4 micromelting point apparatus (Cany Precision Instruments Co., Ltd., Shanghai,

China). Optical rotations were measured on a Perkin-Elmer 241 polarimeter (Perkin-Elmer, Waltham, MA, USA). UV spectra were obtained on a Shimadzu UV-210 spectrometer (Shimadzu, Kyoto, Japan). IR spectra were obtained on a Perkin-Elmer 577 spectrometer (Perkin-Elmer, Waltham, MA, USA). The NMR spectral data were recorded on Bruker AM-600 (600 MHz for ^1H and 150 MHz for ^{13}C ; Bruker, Faellanden, Switzerland) with tetramethylsilane (TMS) as an internal standard. The HR-ESI-MS data were obtained on a Bruker apexultra 7.0 T spectrometer (Bruker, Karlsruhe, Germany). HPLC was performed on Waters series (717 Plus Autosampler, 600 Controller, 2489 UV/Visible Detector; Waters, Milford, MA, USA) by using SymmetryPrepTM C18 semi-preparative column (7 μm , 7.8 mm $\times$ 150 mm; Waters, Milford, MA, USA). Column chromatography (CC) was performed on silica gel (200–300 mesh; Yantai Zhi Fu Chemical Co., Ltd., Yantai, China) and RP-18

(12 nm, S-50 μm , YMC Co., Ltd., Kyoto, Japan).

3.2 Strain isolation and cultivation

Strain L421 was isolated from the stems of *B. gymnorrhiza* collected in Dongzhai, Hainan Province of China. The strain L421 was identified as *P. heterocornis* by Prof. Jing-Ze Zhang and has been deposited in Key Laboratory of Medicinal Chemistry and Molecular Diagnosis of Ministry of Education of Hebei University.

The fungal strain was cultured on slants of comprehensive potato dextrose agar (CPDA) at 28°C for 5 days. Agar plugs were used to inoculate 1000-ml Erlenmeyer flasks each containing 600 ml of media (CPDA), and the final pH of the media was adjusted to 6.5 before sterilization. Flask cultures were incubated at 28°C on a rotary shaker at 150 rpm for 5 days. Fermentation was carried out in eighty 500-ml Erlenmeyer flasks each containing 100 g of rice. Distilled H₂O (100 ml) was added to each flask, and the contents were soaked overnight before autoclaving at 15 lb in⁻² for 30 min. After cooling to room temperature, each flask was inoculated with 10.0 ml of the spore inoculum and incubated at 28°C for 60 days.

3.3 Extraction and isolation

The fermented material was extracted with EtOAc (8 $\times$ 1.0 l), and the organic solvent was evaporated to dryness under vacuum to afford the crude extract (108.0 g). The extract was applied on a silica gel column chromatography, and eluted with petroleum ether–EtOAc–MeOH gradient to afford 10 fractions. Compound **1** (20 mg) was obtained as a colorless crystal from fraction 2 (230 mg). Fraction 6 (2.0 g) was subjected to silica gel column chromatography, eluted with CHCl₃–MeOH (100:0–0:100), yielding seven subfractions (1–7). Subfraction 3 was purified by HPLC (30% CH₃OH in H₂O for 2 min,

followed by 30–40% for 18 min; detection at 254 nm; t_R = 12 min; 2.0 ml min⁻¹) to afford compound **2** (10 mg). Fraction 9 (1.6 g) was then subjected to silica gel reverse-phase column chromatography, eluted with H₂O–MeOH (100:0–0:100), yielding 10 subfractions (1–10). Compound **3** (15 mg) was obtained as a colorless crystal from subfraction 2.

3.3.1 Compound 2

A yellow oil, $[\alpha]_D^{22}$ – 64 (c = 0.25, MeOH). UV (MeOH) λ_{max} (nm): 216, 247, and 292. IR (KBr) ν_{max} (cm⁻¹): 3374, 1744, 1605, and 1441. For ¹H and ¹³C NMR spectral data, see Table 1. HR-ESI-MS: m/z 377.1215 [M + Na]⁺ (calculated for C₁₇H₂₂O₈Na, 377.1207).

3.3.2 Compound 3

A colorless crystal, MP 169–171°C. $[\alpha]_D^{22}$ + 22.8 (c = 0.1, MeOH). UV (MeOH) λ_{max} (nm): 212, 249, and 291. IR (KBr) ν_{max} (cm⁻¹): 3374, 1736, 1513, and 1452. For ¹H and ¹³C NMR spectral data, see Table 1. HR-ESI-MS: m/z 393.1156 [M + Na]⁺ (calculated for C₁₇H₂₂O₉Na, 393.1156).

Acknowledgements

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