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New secondary metabolites from the endophytic fungus *Fusarium* sp. HP-2 isolated from "Qi-Nan" agarwood

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ABSTRACT

A novel pyrone derivative (**1**) bearing two fused five-member rings, together with two new naphthalenone derivatives (**2**, **3**), as well as two known compounds (**4**, **5**) were obtained from the endophytic fungus *Fusarium* sp. HP-2, which was isolated from "Qi-Nan" agarwood. The structures of the new compounds were elucidated by analysis of 1D and 2D NMR, and by HRESIMS spectra, as well as by comparison with the literature. Bioactivity results indicated that compound **3** showed weak acetylcholinesterase inhibitory activity.

1. Introduction

Agarwood, known as a sedative, analgesic, and digestive in traditional medicine, is the fragrant resinous heartwood formed by *Aquilaria* or *Gyrinops* species of the family Thymelaeaceae in response to injury [13]. Natural agarwood formation is occurred only under special conditions, such as lightning strike, animal grazing and insect attack [21]. With the increasement of commercial demand of agarwood recently years, while low yield from the nature, not only their constituents are studied [12,20], but also the formation mechanism have received widely attention [5,19]. It is generally accepted that physical injury is company with fungi infection, and a number of endophytic fungi, such as *Menanotus flavolives* [11], *Penicillium polonicum* [2], *Aspergillus* sp., *Cladosporium* sp., and *Mucor* sp. [9,14] have been reported to be in favor of agarwood production. Tunstall was firstly reported the fungi-inoculation method in 1929 [1], and later Gibson found that the endophytic fungus *Cytosphaera mangiferae* was able to induce the formation of agarwood when inoculated to the healthy trees [1]. For a better understanding of the agarwood endophytic fungi, "Qi-Nan", the superior quality of agarwood, was collected for endophytic fungi isolation, which led to the identification of *Fusarium* sp. HP-2. Thereby the cultivation of *Fusarium* sp. HP-2 on solid rice medium was carried out, and previous phytochemical studies led to the isolation and identification of eight compounds [17]. Herein, the ongoing studies contributed a novel pyrone derivative together with two new naphthalenone derivatives and two known compounds. Meanwhile, their antimicrobial activities, acetylcholinesterase inhibitory activity, and cytotoxicities were evaluated.

2. Experimental

2.1. General

Optical rotations were measured on a Rudolph Autopol III polarimeter (Rudolph Research Analytical, America). IR absorptions were obtained on a Nicolet 380 FT-IR instrument (Thermo, America) using KBr pellets. 1D and 2D NMR spectra were recorded on Bruker Avance 500 NMR spectrometers (Bruker, Germany), using TMS as an internal standard. HRESIMS were measured with an API QSTAR Pulsar mass spectrometer (Bruker, Germany). Column chromatography was performed with silica gel (60–80, 200–300 mesh, Qingdao Haiyang Chemical Co. Ltd., China), ODS gel (20–45 μm, Fuji Silysia Chemical Co. Ltd., America) and Sephadex LH-20 (Merck, Germany). TLC was carried out on silica gel G precoated plates (Qingdao Haiyang Chemical Co. Ltd.), and spots were detected by spraying with 5% H_2SO_4 in EtOH followed by heating.

2.2. Fungal material

The endophytic fungus *Fusarium* sp. HP-2 was isolated from Chinese agarwood "Qi-Nan", collected in Ledong County, Hainan Province, PR China in 2011. The fungus was identified as *Fusarium* sp. on the basis of

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its macroscopic appearance and molecular characteristics. The voucher sample was deposited at the Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences.

2.3. Fermentation and isolation

The endophytic fungus *Fusarium* sp. HP-2 was inoculated and cultured on a PDA agar for 7 days. A piece of mycelial agar plugs (0.5 cm × 0.5 cm) were inoculated into 250 mL Erlenmeyer flask containing potato dextrose broth (PDB) (80 mL). The culture was shaked at 120 rpm at room temperature for three days. Production medium of solid rice in 1000 mL Erlenmeyer flasks was inoculated with 10 mL seed solution for each one. Erlenmeyer flasks were incubated at room temperature under static conditions for 45 days, and cultures from 200 Erlenmeyer flasks were harvested for the isolation of substances.

Following incubation, the culture was diced and extracted with EtOH for three times through cheesecloth. Subsequently, the filtrate was dissolved in water and then extracted successively with petroleum ether (PE), EtOAc and *n*-BuOH. By TLC analysis, the EtOAc and *n*-BuOH fractions were combined for further separation. The mixture was separated into ten fractions (Fr.1–10) on a silica gel column using a stepwise gradient elution of $CHCl_3$/MeOH (100:0–0:100). Fr.5 (5.2 g) was sent to an ODS gel column eluted with MeOH-H_2O to give 11 fractions (Fr.5.1-Fr.5.11). Fr.5.2 was subjected to Sephadex LH-20 column eluting with MeOH, and then chromatographed on silica gel column eluting with PE/EtOAc (*v*/*v*, 12:1) to afford **1** (4.4 mg). Fr.5.10 was repeatedly subjected to Sephadex LH-20 column eluting with MeOH followed by acetone, and then chromatographed on silica gel column eluting with PE/EtOAc (*v*/*v*, 25:1) to afford **2** (1.5 mg). Fr.7 (15.9 g) was chromatographed on an ODS gel column eluted with MeOH-H_2O to give ten fractions (Fr.7.1-Fr.7.10). Fr.7.3 was subjected to Sephadex LH-20 eluting with MeOH and then separated by a silica gel column eluting with $CHCl_3$/MeOH (*v*/*v*, 200:1) to obtain **3** (6.5 mg). By the same procedure, compounds **4** (5.2 mg) and **5** (3.4 mg) were isolated from fraction 5.

Pysarone A (**1**): yellow gum; $[\alpha]_D^{26}$ +34.8 (*c* 1.0, MeOH); ^{1}H and ^{13}C NMR data: Table 1; HRESIMS *m/z* 253.1073 $[M + H]^+$ (calcd for $C_{13}H_{17}O_5$, 253.1073).

3-Demethoxyl-fusarnaphthoquinone B (**2**): red crystal; $[\alpha]_D^{26}$ –56.7 (*c* 1.0, $CHCl_3$); ^{1}H and ^{13}C NMR data: Table 1; HRESIMS *m/z* 245.0813 $[M - H]^-$ (calcd for $C_{14}H_{13}O_4$, 245.0813).

(2*S*, 3*S*, 4*S*)-8-Dehydroxy-8-methoxyl-dihydronaphthalenone (**3**): red solid; $[\alpha]_D^{26}$ –28.7 (*c* 1.0, $CHCl_3$); ^{1}H and ^{13}C NMR data: Table 1; HRESIMS *m/z* 307.1176 $[M - H]^-$ (calcd for $C_{16}H_{17}O_6$, 307.1176).

2.4. Activities assay

The antimicrobial and cytotoxicity activities *in vitro* of the compounds were evaluated by the filter paper disc agar diffusion method [18] and MTT method [10], respectively. Moreover, the compounds were also tested for their AChE inhibitory activity using the Ellman colorimetric method [4].

3. Results and discussion

Compound **1** was isolated as a yellow gum. The molecular formula was established as $C_{13}H_{16}O_5$ by the pseudomolecular ion peak at *m/z* 253.1073 $[M + H]^+$(calcd for $C_{13}H_{17}O_5$, 253.1073) as observed from the HRESIMS spectrum, requiring six degrees of unsaturation. The IR spectrum of **1** showed characteristic absorptions of hydroxyl (3435 cm^{-1}) and *α*, *β*-unsaturated carbonyl (1632 cm^{-1}) functionalities. The ^{1}H and ^{13}C NMR spectra (Table 1) revealed two methyls (including one secondary methyl δ_H 1.35/ δ_C 18.2, and one methoxyl δ_H 3.50/ δ_C 60.3 groups), two methylenes (δ_H 2.30, 2.15/ δ_C 39.2; δ_H 2.00, 2.14/ δ_C 25.3), five methines (including three oxygen-bearing methines at δ_H 4.80/ δ_C 89.1; δ_H 4.47/ δ_C 82.5; δ_H 4.23/ δ_C 68.4), and four quaternary carbons (including two carbonyls at δ_C 217.2 and 164.4, and two olefinic carbons at δ_C 157.5 and 133.7), which were supported by DEPT and HSQC spectra (Table 1). The inferred double bond and two carbonyls accounted for three degrees of unsaturation, and the remaining three degrees of unsaturation supported the existence of three rings. The 5,6-dihydro-*α*-pyrone (ring A) was constructed by the

Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of compounds **1**–**3** (δ in ppm).

position	**1**[a]		**2**[b]		**3**[c]	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	164.4C		206.0C		197.2C	
2			33.9 CH_2	3.11, ddd (17.5, 11.4, 4.5) 2.56, ddd (17.5, 3.7, 3.7)	56.7 CH	2.97, br d (5.5)
3	82.5 CH	4.47, dq (6.5, 6.5)	31.7 CH_2	2.27, m (2H, overlapping)	43.3 CH	2.85, dd (8.6, 5.0)
4	68.4 CH	4.23, d (6.5)	61.9 CH	5.40, dd (3.3, 3.3)	74.5 CH	5.44, br s
4a	157.5C		124.7C		131.2C	
5	89.1 CH	4.80, d (8.5)	141,1C		135.4C	
5a	53.0 CH	3.17, dd (8.5, 8.5)				
6	217.2C		139.1C		156.6C	
7	39.2 CH_2	2.30, m 2.15, m	116.4C		96.0 CH	6.46, s
8	25.3 CH_2	2.14, m 2.00, m	156.8C		152.1C	
8a	45.5 CH	3.59, m	111.8C		112.1C	
8b	133.7C					
9	18.2 CH_3	1.35, d (6.5)	103.2 CH	6.52, s	44.1 CH_2	2.73, dd (17.9, 8.6) 2.45, dd (17.9, 5.0)
10	60.3 CH_3	3.50, s	162.8C		206.3C	
11			14.3 CH_3	2.47, s	30.5 CH_3	2.18, s
12			11.5 CH_3	2.26, s	66.1 CH_2	4.13, dd (9.2, 5.5) 3.74, d (9.2)
13			–		56.4 CH_3	3.89, s
14			–		56.3 CH_3	3.95, s

[a] Measured in MeOD.
[b] Measured in MeOD: $CDCl_3$ = 1:1.
[c] Measured in $CDCl_3$.