A NEW NAPHTHO-γ-PYRONE FROM MANGROVE ENDOPHYTIC FUNGUS ZSU-H26

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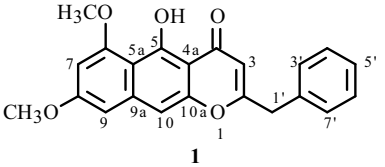
*A new naphtho-γ-pyrone, 5-hydroxy-6,8-dimethoxy-2-benzyl-4H-naphtho[2,3-b]-pyran-4-one (**1**), together with three known compounds 5,7-dihydroxy-2-methylbenzopyran-4-one (**2**), 3,5-dihydroxy-2,7-dimethylbenzopyran-4-one (**3**) and cyclo(Tyr-Tyr) (**4**) were isolated from the mangrove endophytic fungus* Phomopsis *sp. ZSU-H26 obtained from the South China Sea. Their structures were elucidated by spectroscopic methods, mainly 1D and 2D NMR spectroscopic techniques. Primary bioassays showed that **1** exhibited cytotoxicity against HEp-2 and HepG2 cells with IC_{50} values of 10 and 8 μg/mL, respectively.*

Keywords: mangrove endophytic fungus, naphtho-γ-pyrone, metabolites.

Mangrove endophytic fungi have proven to be a rich source of structurally unique and biologically active secondary metabolites. In our search for new metabolites from marine mangrove endophytic fungi from the South China Sea, we have isolated many significant new bioactive metabolites [1–7]. This paper reports the isolation and characterization of a new naphtho-γ-pyrone, 5-hydroxy-6,8-dimethoxy-2-benzyl-4H-naphtho[2,3-b]-pyran-4-one (**1**), together with three known compounds 5,7-dihydroxy-2-methylbenzopyran-4-one (**2**), 3,5-dihydroxy-2,7-dimethylbenzopyran-4-one (**3**), and cyclo(Tyr-Tyr) (**4**) from the mangrove endophytic fungus *Phomopsis* ZSU-H26 isolated from the stem of the mangrove tree *Excoecaria agallocha* from Dong Zai, Hainan, China. The cytotoxic effects of compound **1** against HEp-2 cells and HepG2 cells were first measured.

The ethyl acetate extract of a fermentation broth of the fungus was repeatedly chromatographed on silica gel using gradient elution from petroleum ether to ethyl acetate to give compound **1** from the 20% ethyl acetate/petroleum ether fraction as pale yellow needles. Compound **1** has the molecular formula $C_{22}H_{18}O_5$, as determined by HR-EI-MS (*m/z* 362.1148 $[M]^+$; calcd for $C_{22}H_{18}O_5$, 362.1153) and NMR spectra (see Table 1), being indicative of 14 degrees of unsaturation.



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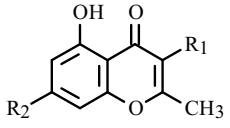

2, 3

2: R_1 = H, R_2 = OH
3: R_1 = OH, R_2 = CH_3

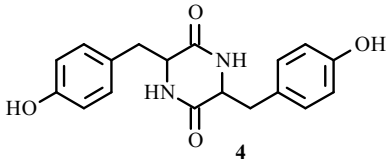

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TABLE 1. NMR Data of **1** ($CDCl_3$, δ, ppm, J/Hz)

C atom	δ_C (DEPT)	δ_H	COSY	HMBC
2	166.8 (C)			H-1′, 3
3	112.2 (CH)	6.26 (s)		H-1′
4	182.9 (C)			H-3
4a	104.7 (C)			H-3, 10, 5-OH
5	159.4 (C)			5-OH
5a	109.1 (C)			H-7, 9, 10, 5-OH
6	158.4 (C)			H-7, 6-OCH_3
7	97.1 (CH)	6.38 (d, J = 2.4)		H-9
8	161.6 (C)			H-7, 9, 8-OCH_3
9	97.9 (CH)	6.57 (d, J = 2.4)		H-7, 10
9a	141.0 (C)			H-9, 10
10	102.8 (CH)	6.86 (s)		H-9
10a	153.6 (C)			H-10
5-OH		13.29 (s)		
6-OCH_3	56.1 (CH_3)	3.99 (s)		
8-OCH_3	55.4 (CH_3)	3.91 (s)		
1′	40.6 (CH_2)	3.74 (s)		H-3, 3′, 7′
2′	135.3 (C)			H-1′, 3′, 4′, 6′, 7′
3′	128.5 (CH)	7.15 (m)	H-4′	H-1′, 4′, 5′, 7′
4′	128.9 (CH)	7.23 (m)	H-3′, 5′	H-3′, 5′, 6′
5′	127.6 (CH)	7.18 (m)	H-4′, 6′	H-3′, 4′, 6′, 7′
6′	128.9 (CH)	7.23 (m)	H-5′, 7′	H-4′, 5′, 7′
7′	128.5 (CH)	7.15 (m)	H-6′	H-1′, 3′, 5′, 6′

The IR spectrum (KBr) of compound **1** showed absorption bands for hydroxyl (3460 cm^{-1}), conjugated carbonyl (1690 cm^{-1}), and aromatic (1615, 1589, and 1419 cm^{-1}) functional groups. The ^{13}C NMR and DEPT spectra showed 22 carbon signals, including two methoxyl groups, one sp^3 methylene, nine sp^2 methines, and 10 quaternary sp^2 carbons (one of which is carbonyl), indicating the compound to be tetracyclic. In the ^1H NMR spectrum of **1**, the presence of a one-substituted benzene ring was deduced from the following proton signals at δ_H 7.15–7.23 (5H, m). The ^1H NMR spectrum also displayed signals for two methoxyls (δ_H 3.99, 3.91), two *meta*-coupled aromatic protons (δ_H 6.57, 6.38, J = 2.4 Hz), two singlet aromatic or olefinic protons (δ_H 6.86, 6.26), and one phenolic hydroxyl proton (δ_H 13.29). These data indicated that **1** is a rubrofusarin B-type naphtho-γ-pyrone [8]. In the ^1H–^1H COSY spectrum of **1**, the correlations of H-3′ and H-4′, H-4′ and H-3′, 5′, H-5′ and H-4′, 6′, and H-6′ and H-5′, 7′ showed the existence of a one-substituted benzene ring.

The HMBC correlations from H-1′ to C-2, C-3, C-2′, C-3′, and C-7′ showed that the methylene of the sharp singlet at δ 3.74 (H-1′) was located between the naphtho-γ-pyrone group (C-2) and the benzene ring (C-2′). Two methoxyl groups (δ_H 3.99, 3.91) were placed at C-6 (δ_C 158.4) and C-8 (δ_C 161.6) respectively by HMBC correlations. The HMBC correlations of the hydroxyl proton at δ_H 13.29 with C-4a, C-5, and C-5a indicated that the hydroxyl group was located at C-5 (δ_C 159.4). Finally, the structure of compound **1** was determined to be 5-hydroxy-6,8-dimethoxy-2-benzyl-4H-naphtho[2,3-b]-pyran-4-one.

Furthermore, three known compounds **2**, **3**, and **4** were identified as 5,7-dihydroxy-2-methylbenzopyran-4-one, 3,5-dihydroxy-2,7-dimethylbenzopyran-4-one, and cyclo(Tyr-Tyr), respectively, by comparison of their spectroscopic data with the literature [9–11].

Primary bioassays showed that **1** exhibited cytotoxicity against HEp-2 and HepG2 cells with IC_{50} values of 10 and 8 μg/mL, respectively.

EXPERIMENTAL

NMR data were recorded on a Varian Inova-500 NB spectrometer, with 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 was carried out on silica gel (200–300 mesh; Qingdao haiyang chemicals).

Fungus Material and Culture Conditions. The fungus ZSU-H26 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. Starter cultures were maintained on cornmeal seawater agar. Plugs of agar supporting mycelium growth were cut and transferred aseptically to a 250 mL Erlenmeyer flask containing 100 mL of liquid medium (glucose 10 g/L, peptone 2 g/L, yeast extract 1 g/L, NaCl 3 g/L). The flask was incubated at 30°C on a rotary shaker for 5–7 days. The mycelium was aseptically transferred to 500 mL Erlenmeyer flasks containing culture liquid (200 mL).The flasks were then incubated at 30°C for 25 days.

Extraction and Separation of Metabolites. The cultures (100 L) were filtered through cheesecloth. The filtrate was concentrated to 3 L below 60°C and extracted five times by shaking with an equal volume of ethyl acetate. Collection and evaporation of ethyl acetate in vacuo yielded the extracts (50 g). The extracts were chromatographed on silica gel using gradient elution with petroleum ether–ethyl acetate (90:10 to 60:40) to give five fractions (A–E). Fraction A was purified by column chromatography on silica gel with petroleum ether–ethyl acetate (80:20) to give compound **1** (8 mg). Fraction B was purified by column chromatography on silica gel with petroleum ether–ethyl acetate (60:40) and separated by preparative TLC (petroleum ether–ethyl acetate, 50:50) to give compound **2** (9 mg), **3** (11 mg) and **4** (25 mg), respectively.

5-Hydroxy-6,8-dimethoxy-2-benzyl-4H-naphtho[2,3-b]-pyran-4-one (1). Pale yellow needles, mp 223–225°C. UV spectrum (CH_3OH, λ_{max}, nm) (log ε): 230 (4.15), 256 (4.02), 290 (2.89). IR spectrum (KBr, ν, cm^{-1}): 3460 (OH), 2930, 2856, 1690 (C=O), 1615, 1589, 1419, 1265, 1210, 1020. EI-MS (*m/z*, I_{rel}, %): 362 (100) $[M]^+$, 344 $[M–H_2O]$ (25), 333 (60), 240 (14), 215 (10). ^1H, ^{13}C NMR see Table 1.

5,7-Dihydroxy-2-methylbenzopyran-4-one (2). Colorless crystals, mp 248–250°C. ^1H NMR (300 MHz, acetone-d_6, δ, ppm, J/Hz): 11.11 (1H, br.s), 9.79 (1H, br.s), 6.36 (1H, d, J = 2.1), 6.35 (1H, d, J = 2.1), 6.37 (1H, s), 2.23 (3H, s). ^{13}C NMR (75 MHz, acetone-d_6, δ, ppm): 222.9 (C), 166.2 (C), 165.6 (C), 163.8 (C), 154.5 (C), 140.3 (C), 104.4 (CH), 102.5 (CH), 101.5 (CH), 18.7 (CH_3).

3,5-Dihydroxy-2,7-dimethylbenzopyran-4-one (3). Yellow oil. ^1H NMR (300 MHz, acetone-d_6, δ, ppm): 11.50 (1H, s), 6.69 (1H, br.s), 6.58 (1H, br.s), 5.76 (1H, br.s), 2.46 (1H, s), 2.41 (3H, s). ^{13}C NMR (75 MHz, acetone-d_6, δ, ppm): 175.8 (C), 159.3 (C), 155.6 (C), 149.8 (C), 146.7 (C), 136.5 (C), 111.0 (CH), 107.6 (CH), 107.2 (C), 22.5 (CH_3), 15.2 (CH_3).

Cyclo(Tyr-Tyr) (4). Colorless solid, mp 276–278°C. ^1H NMR (300 MHz, pyridine-d_5, δ, ppm, J/Hz): 8.12 (NH, br.s), 7.33 (1H, dd, J = 6.5, 2.0), 7.10 (1H, dd, J = 6.5, 2.0), 3.28 (1H, dd, J = 13.5, 7.5), 2.73 (1H, dd, J = 13.5, 7.5), 2.43 (1H, dd, J = 7.5, 7.5). ^{13}C NMR (75 MHz, pyridine-d_5, δ, ppm): 167.5 (C), 158.0 (CH), 131.8 (CH), 127.6 (C), 116.3 (CH), 44.8 (CH), 40.7 (CH_2).

Bioasays. The cytotoxic assays were performed using the MTT assay method [12]. Compound **1** exhibited cytotoxicity against HEp-2 and HepG2 cells with IC_{50} values of 10 and 8 μg/mL, respectively.

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