



A New Benzophenone Derivative from the Endophyte *Shiraia* sp. BYJB-1

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Abstract: A new benzophenone derivative, shiraone A (**1**), along with seven known compounds, linchenxantone (**2**), 4-hydroxymellein (**3**), hypocrellin A (**4**), hypocrellin B (**5**), 9,11-dehydroergosterol peroxide (**6**), ergosterol peroxide (**7**), and lactariolide I (**8**), were isolated from the cultures of *Shiraia* sp. BYJB-1, a fungus from the leaves of the medicinal plant *Selaginella delicatula*. The structures of these compounds were established via extensive spectroscopic techniques, including 1D/2D NMR, HRESIMS and through comparison with data reported in the literatures. In addition, the cytotoxic activities and antibacterial activities of compounds **1-8** were evaluated, and compounds **6** and **7** exhibited moderate cytotoxic activities against SMMC7721 cell line with IC₅₀ values of 25.9 and 45.8 μ M respectively.

Keywords: Endophytic fungus; *Shiraia* sp.; benzophenone; cytotoxic activity; antibacterial activity. ©2022 ACG Publications. All right reserved.

1. Introduction

As a class of microbial community symbiotic with host plants, a number of endophytic fungi could synthesize bioactive secondary metabolites which assist the host plants in protection and survival against various biotic and abiotic factors (i.e., pest, drought) [1]. Furthermore, some of these metabolites are very valuable as lead compounds for novel drug research [2, 3]. As part of our continuous commitment to discover structurally unique and biologically active natural products from medicinal plants and endophytic fungi, *Shiraia* sp. BYJB-1 was isolated for the first time from the *Selaginella delicatula*, a traditional medicinal plant documented in Chinese Pharmacopoeia [4] that showed have a variety of medicinal values such as treat amenorrhea, dysmenorrhea, cancer defect, falling and fluttering injury [5]. Plant-derived fungi of the genus *Shiraia* are reported to be producers of various secondary metabolites including perylenequinones, polysaccharides, sterols and so on,

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which have been shown to have antibacterial, antitumor and antioxidant activities [6-10]. A chemical investigation on the ethyl acetate extract of the endophytic fungus *Shiraia* sp. BYJB-1 led to the isolation of one new benzophenone derivative (**1**), and seven known compounds (**2-8**). To our knowledge, compounds **1-3** and **8** were obtained from the genus *Shiraia* for the first time. Herein, we describe the fermentation, isolation, structure elucidation, *in vitro* cytotoxic and antibacterial activities of these compounds.

2. Materials and Methods

2.1. General Experimental Procedures

Exact mass and molecular formulas were obtained with Agilent 6500 series Q-TOF mass spectrometer (Agilent, Singapore) analyser with positive ion mode. 1D and 2D NMR spectra were measured on a Bruker AVIII-600 spectrometer (Bruker, Karlsruhe, Germany) and using TMS as an internal standard. The reversed-phase preparative HPLC was conducted using an Agilent 1100 (Agilent Technologies, California, USA) instrument connected to a Innoval ODS-2 column (5 μ m, 10 mm \times 250 mm) with a VWD detector. Silica gel (80-100 and 200-300 mesh, Qingdao Marine Chemical Factory, Qingdao, China), macroporous resin (HPD-100, Hebei, China), Sephadex LH-20 (Toyopearl Tosoh, Tokyo, Japan) and C₁₈ reversed-phase silica gel (50 μ m, Fuji, Kasugai, Japan) were used for column chromatography. All solvents were of analytical grade.

2.2. Fungal Material

The fungal strain *Shiraia* sp. BYJB-1 was isolated from the leaves of *Selaginella delicatula*, collected in Huangsang nature reserve in Shaoyang city, Hunan Province of China, in July 2019. The fungus was identified as *Shiraia* sp. BYJB-1 through the sequence analysis of rDNA ITS region, and the sequence of its rDNA region had been submitted to GenBank (Accession No. 7X7M35MH016). Moreover, the strain was deposited in Xiangya School of Pharmaceutical Sciences, Central South University.

2.3. Fermentation, Extraction and Isolation

The fungus was cultured in solid medium, which was prepared by autoclaving each 500 mL conical flask with 250 g of rice and 300 mL of ultrapure water in it. The fermentation was performed in 20 flasks under static conditions at a constant temperature of 28 °C for a month. The fungal culture was extracted with ethyl acetate added to each flask, and the extract was subsequently dried under vacuum to afford 60.2 g.

The crude extract was subjected to macroporous resin column chromatography eluting with EtOH-H₂O (v/v, 20:80-0:100) to give three fractions (Fr. A to Fr. C). Fr. B (25.7 g) was separated on a silica gel CC eluting with PE-EtOAc-MeOH (v/v/v, 100:0:0-0:0:100) to obtain 11 fractions (Fr. B-1 to Fr. B-11). Fr. B-5 (0.9 g) was separated by Sephadex LH-20 column to provide 6 subfractions, and Fr. B-5-4 (0.2 g) was further purified by prep-HPLC with MeCN-H₂O (0-25 min, 50-70%, 3 mL/min) to obtain compound **3** (3.6 mg, t_R = 17.5 min). Fr. B-5-5 was purified by prep-HPLC with MeCN-H₂O (0-20 min, 70-95%, 3 mL/min) to obtain compound **8** (4.3 mg, t_R = 18 min). Fr. B-2 (1.2 g) was separated on a reversed-phase ODS column with a gradient of MeOH-H₂O (v/v, 20:80-100:0) to give four subfractions (Fr. B-2-1 to Fr. B-2-4). Fr. B-2-2 (0.3 g) was further purified by prep-HPLC with MeCN-H₂O (0-30 min, 30-60%, 3 mL/min) to obtain compounds **1** (3.5 mg, t_R = 15.5 min) and **2** (5.6 mg, t_R = 18.7 min). Fr. B-2-4 was purified by prep-HPLC with MeCN-H₂O (0-30 min, 80-95%, 3 mL/min) to obtain compounds **6** (6.9 mg, t_R = 22.8 min) and **7** (7.6 mg, t_R = 27.5 min). Fr. B-11 was performed on Sephadex LH-20 column to provide 3 subfractions, and Fr. B-11-1 (0.2 g) was then purified by prep-HPLC with MeCN-H₂O (0-30 min, 70-95%, 3 mL/min) to give compounds **4** (4.8 mg, t_R = 19.2 min) and **5** (4.2 mg, t_R = 24.1 min).

2.4. Cytotoxicity and Antimicrobial Activity

Cytotoxicity of compounds **1-8** were tested against two human hepatoma cell lines (HepG2 and SMMC7721) using a microplate 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin at 37 °C. The suspended cells (100 μ L) with an initial density of 1×10^5 cells/mL were seeded into each well of a 96-well microplate and put in 37 at 5 % CO₂ condition to incubate for 24h. After that, the cells were treated with the tested compounds of concentrations ranging from 6.25 to 100 μ M. The cells were further cultured for 48h, and then 50 μ L of MTT (2mg/mL) was added to each well. After 4 hour later, DMSO (200 μ L/well) was added to dissolve the formazan crystals, and the absorbance was measured at 567 nm. The inhibition rates were calculated by the formula: Inhibition rate = $1 - \frac{\text{the mean OD of the medicated group}}{\text{the mean OD of the solvent control group}}$. Sorafenib and DMSO were used as positive and negative control. The experiments were carried out in triplicate.

2.5. Cytotoxicity and Antimicrobial Activity

Antimicrobial activities of compounds **1-8** were evaluated against MRSA and *E. coli*. The determinations of MIC values were carried out by the Microbroth dilution method in Mueller-Hinton broth medium (MHB) [11]. The clinical drugs Vancomycin and Kanamycin were used as the positive controls. Optical density values for cell concentrations were recorded at 600 nm using a UV-6000 spectrophotometer (MEYASH, Shanghai, China).

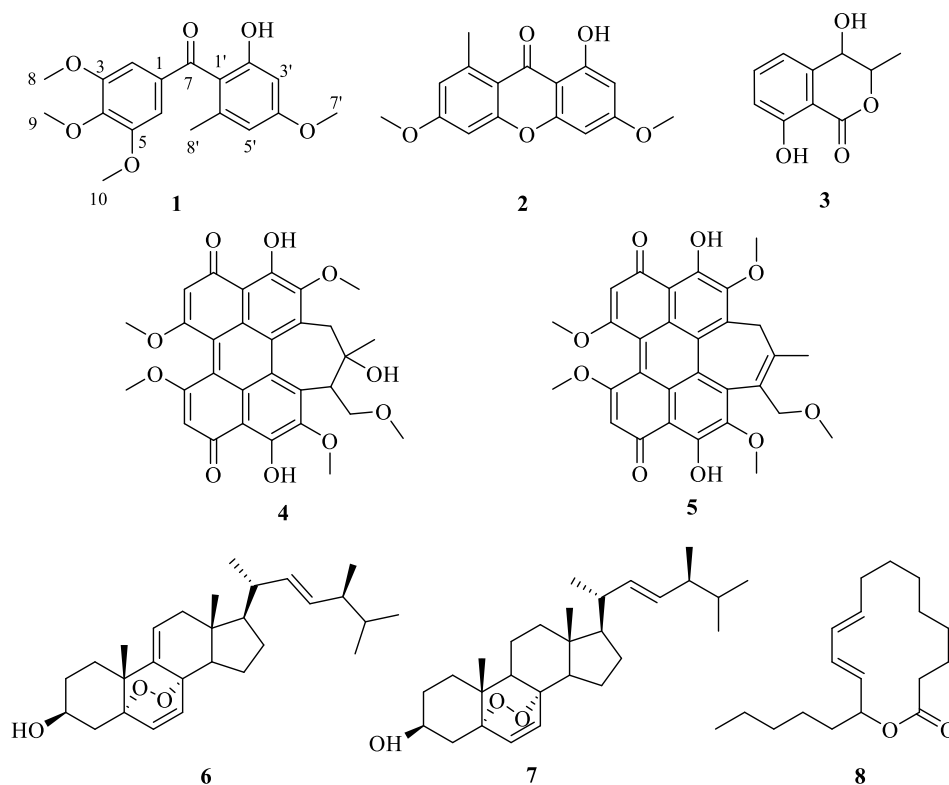


Figure 1. Structures of compounds **1-8**

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was obtained as a light yellow powder, and its molecular formula was determined as $C_{18}H_{20}O_6$ based on HRESIMS ion peaks at m/z 333.1340 $[M + H]^+$ and 355.1152 $[M + Na]^+$ (calcd for $C_{18}H_{20}O_6$, 333.1338 $[M + H]^+$, 355.1158 $[M + Na]^+$), indicating 9 degrees of unsaturation. The 1H -NMR spectrum (Table 1) of **1** showed two pairs of meta coupling aromatic protons at δ_H 6.33 (1H, d, $J = 2.4$ Hz, H-3'), 6.30 (2H, s, H-2 and H-6) and 6.26 (1H, d, $J = 2.4$ Hz, H-5'), one exchangeable proton at δ_H 13.10 (1H, s, 2'-OH), four methoxyl protons signal at δ_H 3.82, 3.78, 3.68 (12H, s, H-9, H-7', H-8, and H-10), and one methyl protons signal at δ_H 1.89 (3H, s, H-8'). The ^{13}C -NMR spectrum (Table 1) of **1** exhibited 15 carbon resonances (includes three sets of overlapping carbon signals) consist of one ketone carbonyl carbon signal at δ_C 199.2 (C-7), two benzene ring carbon signals at δ_C 166.5, 164.9, 163.0, 157.9, 143.5, 117.2, 114.9, 111.6, 100.0, and 92.1 (C-1 to C-6, and C-1' to C-6'), four methoxyl group carbon signals at δ_C 56.8, 56.8, 56.4 and 56.4 (C-8 to C-10, and C-7'), and one methyl carbon on the benzene ring signal at δ_C 22.4 (C-8'). Based on the 1D NMR data, compound **1** is suggested to be a benzophenone derivative, in which there is a symmetrical benzene ring.

Construction of the structure for **1** was accomplished by interpretation of 2D NMR data (Figure 2). In the HMBC spectrum of **1**, the aromatic protons at δ_H 6.30 (H-2 and H-6) showed cross-peaks with the carbonyl carbon at δ_C 199.2 (C-7) and three *O*-linked carbon signals at δ_C 163.0 and 157.9 (C-4, C-3, and C-5), while the aromatic protons at δ_H 6.33 and 6.26 (H-3' and H-5') were correlated with a quaternary at δ_C 143.5 (C-1') and a *O*-linked carbon at δ_C 166.5 (C-2'). The H-5' was correlated with a benzyl carbon signal at δ_C 164.9 (C-4'). The benzyl carbon at δ_C 22.4 (C-8') showed cross-peaks with the C-5' and quaternary C-1' as well. According to the HMBC correlations of methoxy protons with *O*-linked carbons, the methoxy groups were placed at C-3, C-4, C-5, and C-4', and the hydroxyl was placed at C-2'. Therefore, the compound **1** was identified as (2-hydroxy-4-methoxy-6-methylphenyl)(3,4,5-trimethoxyphenyl) methanone and named as shiraone A.

A putative biosynthetic pathway to **1** is presented in (Scheme 1). The key intermediates 3,4,5-trimethoxybenzoic acid (**I**) was obtained by shikimic acid pathway. And the other key intermediates 2-hydroxy-4-methoxy-6-methylbenzoic acid (**II**) was yielded by acetate-malonate pathway. Finally, compound **1** was obtained through a series of biosynthesis with the 3,4,5-trimethoxybenzoic acid and 2-hydroxy-4-methoxy-6-methylbenzoic acid.

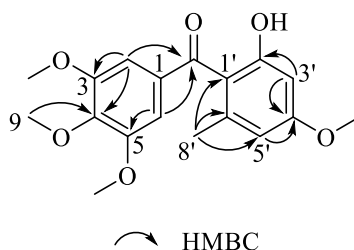
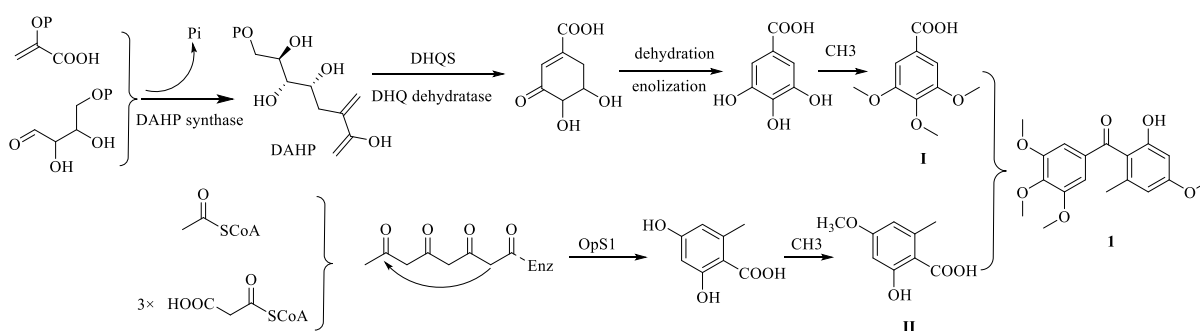
The known compounds **2-8** were isolated identified as linchenxantone (**2**) [12], 4-hydroxymellein (**3**) [13], hypocrellin A (**4**) [6], hypocrellin B (**5**) [6], 9,11-dehydroergosterol peroxide (**6**) [14], ergosterol peroxide (**7**) [15], and lactariolide I (**8**) [16] by comparing their spectroscopic data with those reported in literatures.

Compounds **1-8** were evaluated for their cytotoxic activities against SMMC7721 and HepG2 hepatoma cell lines (Table S1), and antibacterial activities against MRSA and *E. coli* (Table S2). Among them, compound **6** showed moderate cytotoxicity against SMMC7721 ($IC_{50} = 25.94 \pm 2.07 \mu M$) and HepG2 ($IC_{50} = 50.48 \pm 1.94 \mu M$). In addition, compounds **6-8** displayed weak antibacterial activities against MRSA, and the MIC values were 62.5 $\mu g/mL$.

In summary, one new benzophenone derivative, shiraone A (**1**), was isolated and identified, along with four known compounds (**2-8**), in which compounds **1-3** and **8** were isolated from this genus for the first time. The cytotoxic activities against two human hepatoma cell lines (HepG2 and SMMC7721) and antibacterial activities against MRSA and *E. coli* were evaluated. The results not only enriched the study on the chemical constituents of *Shiraia* genus, but also indicated that endophytes, especially isolated from medical plants, still play a very significant role in discovering structurally unique and biologically active natural products.

Table 1. ^1H (600 MHz) and ^{13}C (150 MHz) NMR data for compound **1** (DMSO- d_6 , δ in ppm, J in Hz)

Position (H)	δ_{H} (J in Hz)	δ_{C}	Position (H)	δ_{H} (J in Hz)	δ_{C}
1		114.9	1'		117.2
2	6.30 s	92.1	2'		166.5
3		157.9	3'	6.33 d (2.4)	100.0
4		163.0	4'		164.9
5		157.9	5'	6.26 d (2.4)	111.6
6	6.30 s	92.1	6'		143.5
7		199.2	7'	3.78 s	56.4
8	3.68 s	56.8	8'	1.89 s	22.4
9	3.82 s	56.4	2'-OH	13.10 s	
10	3.68 s	56.8			

**Figure 2.** Key HMBC correlations of compound **1****Scheme 1.** Putative biosynthetic pathway of the compound **1**

Acknowledgments

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Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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