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A New *α*-Pyrone Derivative from Endophytic Fungus *Pestalotiopsis microspora*

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Abstract: A new *α*-pyrone derivative **1**, along with four known congeners **2**, **3**, **4** and **5**, were isolated from the solid-substrate fermentation medium of the endophytic fungus *Pestalotiopsis microspore* isolated from the branch of *Taxus chinensis*. Their structures and relative configurations were elucidated by extensive spectroscopic analysis. The cytotoxic activities of the isolated *α*-pyrone derivatives against two tumor cell lines as well as compound **1**'s antimicrobial activity against three bacteria and three plant-pathogenic strains were evaluated. No antimicrobial activity was observed for compound **1**. The cytotoxicity against Caski and HeLa tumor cell lines was insignificant. To our surprise, the known compound **4** showed significant gibberellin synergistic activity towards *Distylium chinense* seeds.

Keywords: pyrone; endophytic fungus; *Pestalotiopsis microspora*; gibberellin synergistic activity.

1. Introduction

Endophytic fungi, which inhabit normal tissues of host plants without causing apparent symptoms of pathogenesis, are novel and rich sources of bioactive natural products including alkaloids, steroids, terpenoids, isocoumarins, quinones, flavonoids, phenyl propanoids, lignans, phenols and aliphatics [1-6]. In 1993, since Strobel isolated the endophytic fungus *Taxomyces andreanae* from the bark of Pacific yew, which could produce the anti-cancer drug paclitaxel such as its host plant, the medicinal and organic chemists have focused on the endophytic fungi to find new and feasible sources of taxol [7]. *Pestalotiopsis* species are widely distributed all over the world, and most of them are plant pathogens, and many are saprobes in soil or in plant debris [8-9]. Previous investigations on this genus have led to the isolation of numerous novel and active metabolites [10-17].

During an ongoing search for novel bioactive natural products from species of this genus, a strain of *Pestalotiopsis microspora* was isolated from the branch of *Taxus chinensis*, collected from Yichang, and it was grown on solid-substrate fermentation culture which led to the isolation of five *α*-

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pyrone derivatives, including a new compound. In this paper, the isolation and structural identification of compounds **1-5** and their bioactivities were reported.

2. Materials and Methods

2.1. General

HPLC spectra were recorded on DIONEX Ultimate 3000 HPLC (America Dionex) and Waters series HPLC 1525EF (America Waters). 1D and 2D NMR spectra were obtained on a Bruker Avance III 400 MHz instruments with TMS as internal standard. The ionization method was ESI and operated in positive ion mode (Thermo). UV and CD spectra were measured on a Jasco-810 polarimeter (JASCO Corporation, Japan). Single crystal diffraction spectra was obtained on a X-ray diffractometer (Science instrument Corporation, Japan). Thin layer chromatography (TLC) was performed on plates precoated with silica gel GF254 (10-40 μm, Qingdao Marine Chemical Inc.). Column chromatography (CC) was performed over silica gel (200-300 mesh, Qingdao Marine Chemical Inc.) and Sephadex LH-20 (GE Healthcare Co.). Petroleum ether, ethyl acetate, acetone, chloroform, methanol, dimethyl sulfoxide, all used reagents used were analytical grade (Tianjin chemical reagent. Co., Ltd.).

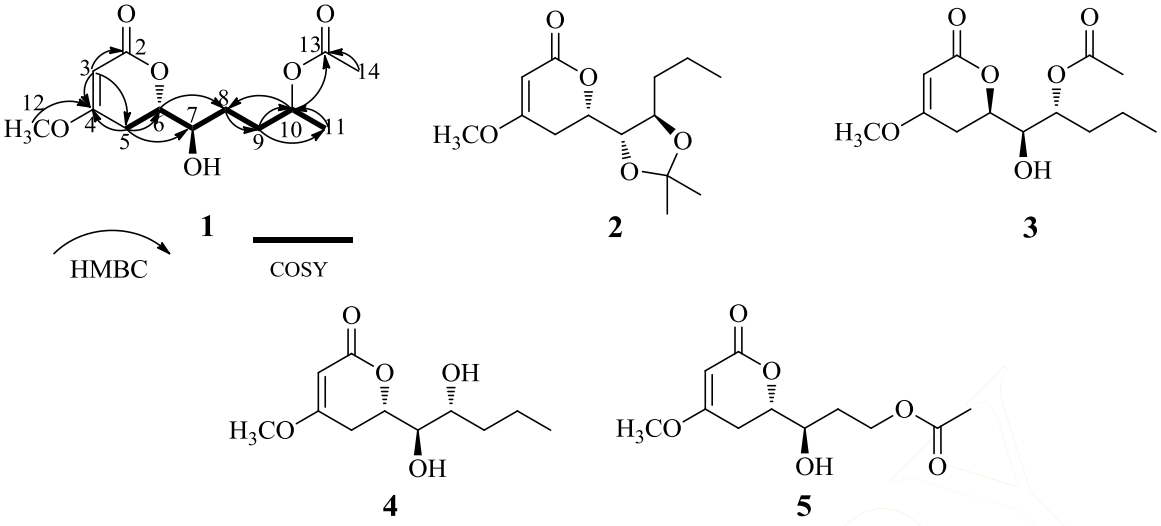
2.2. Microorganism Material

The strain HDS-CZP-1-1-1 was isolated from the branch of *Taxus chinensis* collected from Yichang, Hubei Province, China, in 2012. Based on the morphological assessment by Dr. Tu. Both, and 16S rDNA sequencing (GenBank accession number: KM657341), the strain was identified as *Pestalotiopsis microspora*.

2.3. Fermentation and Isolation

A slant culture of the strain was inoculated in 500 mL Erlenmeyer flasks containing 200 mL of PDB as seed medium, and the flasks were incubated for 7 days at 28 ^{o}C on a rotary shaker at 180 rpm. A 3 mL seed culture was used to inoculate one hundred 500 mL Erlenmeyer flasks containing 35 g rice and 80 mL water, and these solid-substrate fermentation mediums were incubated at 28 ^{o}C for 30 days.

The culture medium were extracted four times with ethyl acetate and the filtrate was concentrated to dryness in vacuum to afford a dark crude extract (80 g), and the crude extract was degreased with petroleum ether, at last the EtOAc crude extract (20 g) was obtained. The extract was fractionated by column chromatography (CC) using normal-phase silica gel (6 cm×40 cm) and eluted with a petroleum ether/acetone gradient from 1:0 to 0:1 (*v/v*) to afford Fr. 1–Fr. 13. Compound **4** (30 mg) was precipitated during isolation. The Fr. 7 (4 g) was fractionated by Sephadex LH-20 column chromatography eluting with $CHCl_3$-MeOH (1:1) to afford eight fractions. Fraction 3 (300 mg) was successively separated by semipreparative reversed-phase HPLC (250 ×10 mm, AQ-C18), eluting with 25% MeCN in H_2O for 5 min, 25%-80% for 60 min at a flow rate 2.5 mL/min with UV detection at 240 nm) to afford **1** (6 mg), **2** (4 mg), **3** (2 mg), **4** (50 mg) and **5** (4 mg).



1 **2** **3** **4** **5**

Figure 1. ^{1}H-^{1}H COSY correlations and the selected HMBC correlations of compound **1** and the structures of compounds **1**-**5**.

3. Results and Discussion

3.1. Structure elucidation

Compound **1** was obtained as yellowish oil. The molecular formula of **1** was determined as $C_{13}H_{20}O_6$ from the HRESIMS of the molecular ion peak (*m/z* 273.1332 $[M+H]^+$, calcd. 273.1333), indicating four degrees of unsaturation. The ^{1}H-NMR spectrum (Table 1) displayed the presence of three methyl proton signals [δ_H 1.16 (3H, d, 6.4), 1.98 (3H, s), 3.72 (3H, s)], three methylene proton signals [δ_H 1.47 (2H, m), 2.71 (1H, dd, 1.6, 12), 2.23 (1H, dd, 4, 16.8), 1.67 (1H, m), 1.50 (1H, m)], and four methine proton signals [δ_H 3.43 (1H, m), 4.79 (1H, m), 4.26 (1H, m), 5.14 (1H, d, 1.6)]. The ^{13}C-NMR and DEPT spectra exhibited 13 carbon resonances, which were ascribable to three methyls including one methoxyl group, three methylenes, four methines and three quaternary carbons. Two carbonyl groups and a pair of double bond were due to three degrees of unsaturation, and the remaining unsaturation indicated that there was a cyclic system in compound **1**. Further interpretation of HMBC spectrum showed the following long-range correlations (Figure **1**): H-3 to C-2 and C-4, H-5 to C-4 and C-6. The above mentioned HMBC evidence, along with the proton spin system: H-5/H-6 observed in ^{1}H-^{1}H COSY (Figure **1**), led to the establishment of *α*-pyrone core unit in **1**. Similarly, a long-chain substituted alkyl group was deduced from the correlations of H-6/H-7/H-8/H-9/H-10/H-11 observed in ^{1}H-^{1}H COSY, coupling with HMBC correlations from H-8 to C-9, from H-9 to C-10 and C-11, from H-10 and H-14 to C-13, from H-11 to C-10. The linkage between a 2H-pyran-2-one core unit and a long-chain substituted alkyl group was clearly detected from C-7 to C-6, since HMBC (Figure **1**) correlations from H-5 to C-6 and C-7, from H-6 to C-8 were observed. Moreover, the HMBC (Figure **1**) correlations from H-10 to C-13, and also C-10 were observed in the downfield (δ_C 70.5), indicating that the acetyl group was present at C-10(*O*) position. Therefore, the planar structure of **1** was tentatively assigned as shown in Figure **1**. The absolute configuration of C-7 was deduced as (7*S*) by comparing the CD spectral data with those of compound **4**, but the relative configuration of C-10 in compound **1** was not by CD or Mosher method, because of acetylation of the hydroxyl group. Thus, the compound **1** was named as Pestalopyrone A.

Table 1. ^{1}H NMR (400MHz, *J* in Hz) and ^{13}C NMR Data (100 MHz) for compound **1** in DMSO-d_6

NO.	**1**	
	δ_C	δ_H
2	166.3 (C)	-
3	89.7 (CH)	5.14 (1H, *d*, 1.6)
4	173.5 (C)	-
5	28.6 (CH_2)	2.71 (1H, *dd*, 1.6, 12) 2.23 (1H, *dd*, 4, 16.8)
6	77.9 (CH)	4.26 (1H, *m*)
7	70.2 (CH)	3.43 (1H, *m*)
8	28.2 (CH_2)	1.47 (2H, *m*)
9	31.9 (CH_2)	1.67 (1H, *m*) 1.50 (1H, *m*)
10	70.5 (CH)	4.79 (1H, *m*)
11	19.9 (CH_3)	1.16 (3H, *d*, 6.4)
12	56.3 (CH_3)	3.72 (3H, *s*)
13	170.0 (C)	-
14	21.1 (CH_3)	1.98 (3H, *s*)

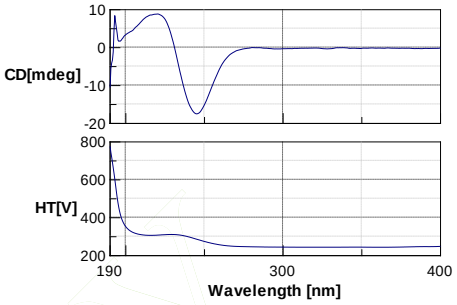


Figure 2. CD Spectrum of compound **1**

Compound **2** was a known compound, which was synthesized by Meyer [18]. Compound **3** was another known metabolite, isolated from *Penicillium sp*. [19]. The structures of LL-P880γ (**4**) and Scirpyrone C (**5**) were established by comparison of their spectroscopic data with literatures. Compound **4** was first isolated from *Penicillium citreo-viride* as a pestalotin analogue, which was a gibberellin synergist, and subsequently isolated from *Penicillium sp.* [20]. Compound **5** was previously isolated from solid-substrate fermentation culture of the plant pathogenic *Pestalotiopsis scirpina* [12]. Herein, the X-ray crystallographic structure of compound **4** was presented for the first time.

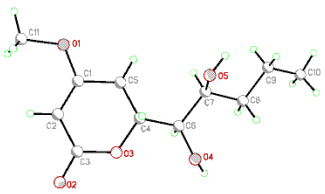


Figure 3. X-ray crystallographic structure of **4**

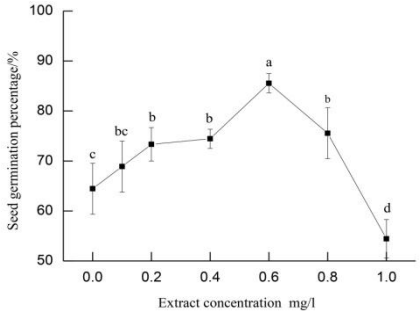


Figure 4. Effects of different concentrations extract on *Distylium chinense* germination rate about gibberellin synergies activity ($\bar{x}\pm s$)

*Crystal data and structure refinement for compound **4***: Compound **4** was obtained as a colorless monoclinic crystal. The molecular formula is $C_{11}H_{18}O_5$, fw=230.25, monoclinic space group P2(1), unit cell dimensions a=5.103(6) Å, b=11.466(14) Å, c=20.89(3) Å, V=1222(3) Å3, $\alpha=\beta=\gamma=90°$, Z=4, d_{calcd}=1.251 mg/m3, crystal size 0.16×0.15×0.12 mm, T=296(2) K, μ=0.098 mm^{-1}, F(000)=496. A total of 1665 unique reflections were collected on a CCD area detector diffractometer with graphite-

monochromated Mo Kα radiation (λ=0.71073 Å). The structure was solved by direct methods (SHELXS-97) and expanded using Fourier techniques (SHELXS-97). The final cycle of full-matrix least-squares refinement was based on 1665 unique reflections and 147 variable parameters and converged with unweighted and weighted agreement factors of R1 = 0.0716 and wR2 = 0.1899 [I$>2\sigma$(I)].

3.2 Cytotoxicity, Antimicrobial and Gibberellin synergistic activity

The biological activity of compound **1** was examined by cytotoxicity [21] and antimicrobial [22] bioassays. The cytotoxicity against Caski and HeLa tumor cell lines was determined. Compound **1** showed no activity (with IC_{50} values of 2300 μM and 2500 μM). In the antimicrobial assay, no activity was observed for against three bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Micrococcus luteus*) and three plant pathogens (*Xanthomonas campestris pv. Oryzae*, *Erwinia carotovora subsp. Carotovora* and *Pseudomnas syringae pv. actinidae*) (the inhibition zone was 0).

Compound **4** (LL-P880γ), as pestalotin analogue, showed significant gibberellin synergistic activity [23] towards *Distylium chinense* seeds, with the substrate LL-P880γ concentration of 0.6 mg/L. The germination rate of the seeds was 85.56%.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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