A New Dihydroxanthone from the Fermentation Products of Endophytic Fungus of *Phomopsis amygdali*

LIMEI LI, HUAN WANG, JUANXIA YANG, JIE LOU, GUIYOU LIU, XUEMEI GAO, QIUFEN HU and YANQING YE*

Key Laboratory of Chemistry in Ethnic Medicinal Resources, State Ethnic Affairs Commission & Ministry of Education, Yunnan University of Nationalities, Kunming 650031, P.R. China

*Corresponding author: E-mail: yey-qing@163.com

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A new dihydroxanthone, (1R,2R)-2-hydroxy-6-(hydroxymethyl)-8-methoxy-9-oxo-2,9-dihydro-1H-xanthene-1-carboxylic acid (**1**) was isolated from the fermentation products of a *Phomopsis* fungus. Its structure was elucidated by spectroscopic methods. Compound **1** was tested for its cytotoxicity and it showed high cytotoxicity against NB4 and PC3 cell with IC₅₀ values of 4.5 and 3.8 μM.

Keywords: Dihydroxanthone, *Phomopsis* fungus, Fermentation products, Cytotoxicity.

INTRODUCTION

The *Phomopsis* species known as an important phytopathogenic genus contains more than 900 species named from a wide range of hosts¹. These microorganisms produce a number of secondary metabolites with various biological activities, including antimicrobial^{2,3}, antifungal^{4,5}, antimalarial^{6,7}, antitumor^{7,8}, etc. The xanthone derivatives are important metabolites isolated from the *Phomopsis* genus and they appeals to medicinal chemists because of their pronounced pharmacological effects^{9,10}.

With the aim of multipurpose utilization endophytic fungus of isolated from the rhizome of *Paris polyphylla* var. *yunnanensis* and identify bioactive natural products, the phytochemical investigation on fermentation products of the endophytic fungus *Phomopsis amygdali*. was carried out. As a result, a new dihydroxanthone (**1**) (Fig. 1) was isolated. In addition, the cytotoxicity of compound **1** was evaluated. This article reported the isolation, structure elucidation and cytotoxicity of the new compound.

Fig. 1. Structure of **1**

EXPERIMENTAL

Optical rotations were measured with a Horiba SEPA-300 polarimeter. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. IR spectra were obtained in KBr disc on a Bio-Rad Wininfrared spectrophotometer. ESI-MS were measured on a VG Auto Spec-3000 MS spectrometer. ¹H, ¹³C and 2D NMR spectra were recorded on Bruker DRX-500 instrument with TMS as internal standard. Column chromatography was performed on silica gel (200-300 mesh), or on silica gel H (10-40 mm, Qingdao Marine Chemical Inc., China). Second separate was used an Agilent 1100 HPLC equipped with ZORBAX-C18 (21.2 mm × 250 mm, 7 mm) column and DAD detector.

The culture of *Phomopsis* sp. was isolated from the rhizome of *Paris polyphylla* var. *yunnanensis* collected from Shizhong, Yunnan, People's Republic of China, in 2011. The strain was identified by Gang Du based on the analysis of the ITS sequence. It was cultivated at room temperature for 7 days on potato dextrose agar at 28 °C. Agar plugs were inoculated into 250 mL Erlenmeyer flasks each containing 100 mL potato dextrose broth and cultured at 28 °C on a rotary shaker at 180 rpm for 5 days. Large scale fermentation was carried out in 50 Fernbach flasks (500 mL) each containing 100 g of rice and 120 mL of distilled H₂O. Each flask was inoculated with 5 mL of cultured broth and incubated at 25 °C for 45 days.

Extraction and isolation: The fermentation products were extracted four times with 70 % acetone (4 × 5 L) at room temperature and filtered. The crude extract (42.5 g) was applied

TABLE-1
¹H- AND ¹³C-NMR DATA OF COMPOUND **1** (500 AND 125 MHz, IN CD₃OD)

No.	δ _c (m)	δ _H (m, J, Hz)	No.	δ _c (m)	δ _H (m, J, Hz)
1	47.5 d	4.22 d (3.8)	9	183.2 s	
2	65.2 d	4.68 dd (3.8, 4.8)	4a	158.4 s	
3	137.6 d	6.45 d (4.8, 9.9)	8a	110.9 s	
4	123.8 d	6.68 dd (9.9)	9a	112.2 s	
5	106.2 d	6.95 s	10a	156.4 s	
6	149.5 s		1'	176.4 s	10.24 brs
7	108.5 d	6.74 s	2'	65.1 t	4.65 s
8	162.8 s		8-OMe	55.9 q	3.81 s

to silica gel (200-300 mesh) column chromatography, eluting with a CHCl₃-acetone gradient system (20:1, 9:1, 8:2, 7:3, 6:4, 5:5), to give six fractions A-F. The further separation of fraction D (7:3, 6.53 g) by silica gel column chromatography and preparative HPLC (32 % MeOH, flow rate 12 mL/min) to give **1** (5.4 mg).

(1R,2R)-2-hydroxy-6-(hydroxymethyl)-8-methoxy-9-oxo-2,9-dihydro-1H-xanthene-1-carboxylic acid (1): yellow gum; [α]_D^{24.5} -8.21 (c 0.20, MeOH); UV (CH₃OH), λ_{max} (log ε) 340 (3.82), 272 (4.14), 210 (4.38) nm; IR (KBr, ν_{max}, cm⁻¹): 3420, 2945, 2850, 1752, 1654, 1602, 1538, 1455, 1416, 1274, 1158, 1072, 966, 857; ¹³C NMR and ¹H NMR data (500 and 125 MHz, in CD₃OD) see Table-1; positive ESIMS *m/z* 341 [M + Na]⁺; positive HRESIMS *m/z* 341.0632 [M + Na]⁺ (calcd for C₁₆H₁₄NaO₇, 341.0637).

RESULTS AND DISCUSSION

A 70 % aq. acetone extract prepared from fermentation products of the endophytic fungus *Phomopsis amygdali* was subjected repeatedly to column chromatography on Silica gel, Sephadex LH-20, RP-18 and Preparative HPLC to afford compound **1**. The structure of **1** was shown in Fig. 1 and its ¹H and ¹³C NMR data were listed in Table-1.

Compound 1 was obtained as a yellow powder: The molecular formula was established as C₁₆H₁₄NaO₇ *m/z* 341.0632 [M + Na]⁺ (calcd. for 341.0637), which indicated 10 degrees of unsaturation. Its UV spectrum showed the maximum absorption at 340, 272 and 210 nm. Strong absorption bands accounting for hydroxy (3420 cm⁻¹), carbonyl (1752, 1654 cm⁻¹) and aromatic groups (1602, 1538, 1455 cm⁻¹) could also be observed in its IR spectrum. The ¹³C and DEPT spectra of **1** revealed 16 carbon, composed of one methoxy group (δ_c 55.9), one methine group (δ_c 47.5), one oxidized methine group (δ_c 65.2), one oxidized methylene group (δ_c 65.1), four aromatic methines (δ_c 137.6, 123.8, 106.2 and 108.5), six aromatic quatern (δ_c 149.5, 162.8, 158.4, 110.9, 112.2 and 156.4) and two carbonyl carbons (δ_c 183.2 and 176.4). The ¹³C NMR signal 176.4 was assigned to a carbonyl group. The ¹H NMR spectrum revealed four aromatic protons [δ_H (6.74 s), (6.95 s), (6.45 d, *J* = 9.9, 4.8 Hz) and (6.68 dd, *J* = 9.9 Hz)]. Two aromatic protons at δ_H 6.45 (d, *J* = 9.9, 4.8 Hz) and 6.68 (dd, *J* = 9.9 Hz) were connected to those at δ_H 4.68 (dd *J* = 3.8, 4.8 Hz) and 4.22 (d, *J* = 3.8 Hz).

This connectivity was confirmed by correlation of these four protons ¹H-¹H COSY spectrum (Fig. 2). In addition, one methoxy and one hydroxymethyl protons appeared at δ_H 3.81

(s) and 4.65 (s), respectively. The precise connectivities between proton and carbon signals were established by interpretation of HMBC data. In HMBC spectrum of **1**, the proton at δ_H 4.22 (H-1) was correlated with C-2 (δ_c 65.2), C-3 (δ_c 137.6), C-4a (δ_c 158.4), C-9 (δ_c 183.2), C-9a (δ_c 112.2). These correlations indicated the existence of dihydroxanthene skeleton¹¹. The HMBC correlations (Fig. 2) of the methoxy proton signal (δ_H 3.81) with C-8 (δ_c 162.8) suggested the methoxy group should be located at C-8. The hydroxymethyl group located at C-6 was supported by the HMBC correlations of H-2' (δ_H 4.65) with C-5 (δ_c 106.2), C-6 (δ_c 149.5) and C-7 (δ_c 108.5). Furthermore, a hydroxy group should be located at C-2 to support the existence of an oxidized methylene. The configurations of 1R, 2R in **1** were deduced from the comparison of ¹H and ¹³C NMR data, coupling constants and ROESY correlations with these of AGI-B4,¹¹ of which configuration was unambiguously established by an X-ray structure analysis. According to above informations, compound **1** was assigned as (1R, 2R)-2-hydroxy-6-(hydroxymethyl)-8-methoxy-9-oxo-2,9-dihydro-1H-xanthene-1-carboxylic acid.

Fig. 2. Key HMBC (—) and ¹H-¹H COSY (—) correlations of **1**

Xanthenes are known to exhibit cytotoxic effects^{6,9,12}. Compound **1** were tested for its cytotoxicity against five tumor cells line (NB4, A549, SHSY5Y, PC3 and MCF7) using a previously reported procedure¹³. The results showed that **1** exhibited high cytotoxicity against NB4 and PC3 cell with IC₅₀ values of 4.5 and 3.8 μM.

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