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XANTHONES FROM THE FERMENTATION PRODUCTS OF AN ENDOPHYTIC FUNGUS *PHOMOPSIS* SP.

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Abstract – Three new xanthones, dalienxanthones A-C (**1-3**), together with three known analogues (**4-6**), were isolated from the fermentation products of an endophytic fungus *Phomopsis* sp. cultivated on *Paris daliensis*. Their structures were identified by extensive spectroscopic techniques, especially 2D-NMR. The cytotoxicity activities of all compounds were evaluated against five cancer cell lines including NB4, A549, SHSY5Y, PC3, and MCF-7. Compounds **3** and **4** showed cytotoxicities against SHSY5Y cells with IC_{50} values of 3.8 and 3.5 μM, respectively. The other compounds also showed cytotoxicity for some tested cell lines with IC_{50} values between 4.6 - 9.2 μM.

The endophytes fungus have become an important source of natural products with novel structures or biologically activities.[1] The genus *Phomopsis*, belonging to Sphaerosidales, was an important endophyte resource with variety of species and wide distribution.[2] The previous study on endophytic fungus *Phomopsis* sp. reported the secondary metabolites of pyrone derivatives, xanthones, polyketides, alkaloids, sesquiterpenes, et al. Some of them showed significant antimicrobial, antimalarial, antitubercular, and anticancer cell lines activities.[3-9] In order to explore new bioactive constituents from the fermentation products of this genus, one of endophytic fungus *Phomopsis* sp. was isolated from *Paris daliensis*, and the chemical constituents of its fermentation products were investigated. As a result, three new (**1-3**) and three known (**3-6**) xanthones were isolated. All compounds were evaluated for their cytotoxicity activities against five cancer cell lines. Here we describe the isolation and structure elucidation of new compounds and anticancer cell lines activities of these xanthones.

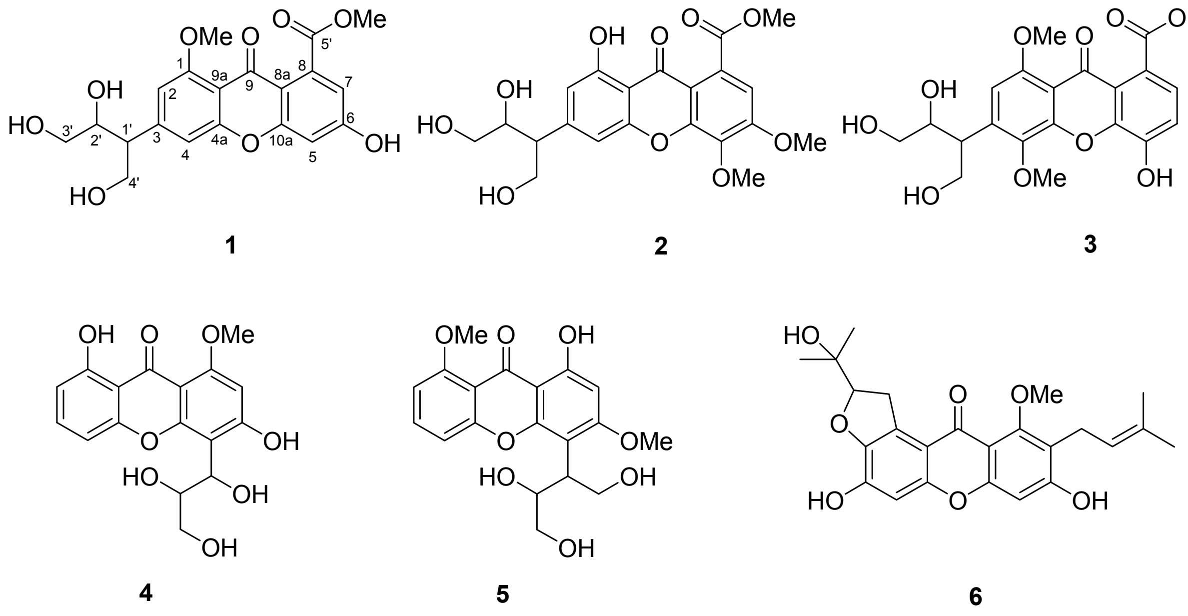

Figure 1. The structures of compounds **1** - **6**

The fermented substrate was extracted with 70% aqueous acetone. The extract was subjected repeatedly to column chromatography on silica gel, RP-18, and semi-preparative RP-HPLC separation to afford compounds **1**-**6**. Their structures were shown in Figure 1, and the ^{1}H and ^{13}C NMR data of the compounds **1**-**3** were listed in Table 1. By comparing with the literature, the known compounds were identified as, 3,8-dihydroxy-4-(2,3-dihydroxy-1-hydroxymethylpropyl)-1-methoxyxanthone (**4**),[10] oliganthins E (**5**),[11] and cratoxylumxanthone D (**6**).[12]

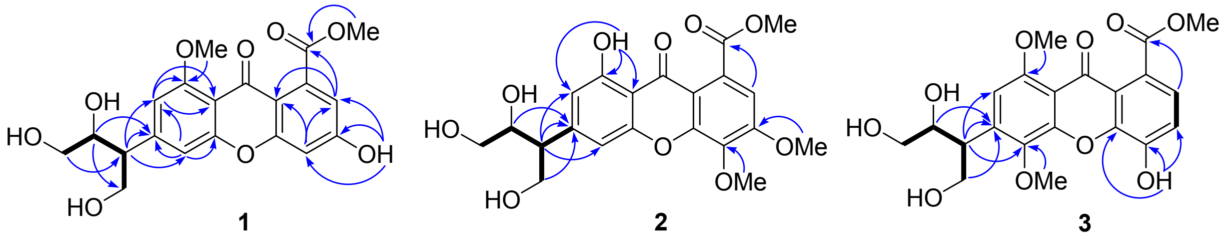

Figure 2. Selected HMBC (↷) and ^{1}H-^{1}H COSY (—) correlations of **1**, **2** and **3**

Compound **1** was obtained as a yellow gum. Its molecular formula was determined to be $C_{20}H_{20}O_9$ on the basis of positive HRESIMS *m/z* 427.1012 $[M+Na]^+$, (calcd $C_{20}H_{20}O_9Na$ for 427.1005), with 11 degrees of unsaturation. The UV absorptions at 305, 242, and 210 nm showed an extended chromophore and a substituted benzene ring. Its IR spectral data showed the presence of hydroxy groups (3386 cm^{-1}), carbonyl groups (1722 and 1659 cm^{-1}) and phenyl groups (1598, 1542, 1462 cm^{-1}). The ^{1}H-NMR spectrum (Table 1) displayed four aromatic methines (δ_H 6.65 1H s, H-2; 6.97 1H s, H-4; 6.82 1H s, H-5;

and 6.78 1H s, H-7), two methoxy group (δ_H 3.80 3H s, OMe-1 and 4.00 3H, s, OMe-5'), two oxygenated methylenes (δ_H 4.53 and 4.60, 2H, m, H-3'; 4.11 and 4.14, 2H, m, H-4'), two methines, including one oxygenated (δ_H 5.02, 1H, m, H-2') and one non-oxygenated (δ_H 4.35, 1H, m, H-1'), and one phenolic proton (δ_H 12.81, 1H, s, Ar-OH-6). Further analysis of its ^{13}C-NMR (DEPT) data (Table 1) revealed the presence of one ketone and one ester carbonyls, twelve aromatic carbons (four of which were protonated) indicative of two substituted phenyl rings, two methoxy group, two methylenes, and two methines. By careful analysis of the above data, we concluded that **1** was a xanthone analogue (C1 ~ C-9, C-4a, C-8a ~ C-10a; H-2, H-4, H-5, H-7). The additional carbons account for the remaining substituents, [a 1-hydroxymethyl-2,3-dihydroxypropyl structure unit (C1' ~ C-4'), a methoxycarbonyl group (C-5', OMe-5'), a methoxy group (OMe-1), and a phenolic hydroxy group (Ar-OH-6)]. The ^{1}H-^{1}H COSY correlations between H-4'/H-1'/H-2'/H-3' also supported the existence of 1-hydroxymethyl-2,3-dihydroxypropyl unit. This structure unit connected at C-3 was supported by the HMBC correlations from H-1' to C-2, C-3, and C-4, and from H-2 and H-4 to C-1' (Figure 2). The HMBC correlation of methoxy proton signal (δ_H 3.80, 3H, s) with C-1 (δ_C 163.9) supported the methoxy group located at C-1. The hydroxy group located at C-6 position was deduced from the HMBC correlations of hydroxy proton (δ_H 11.54) with C-5, C-6, and C-7. HMBC correlations from the methoxy proton (δ_H 4.00) and H-7 (δ_H 6.78)

Table 1. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) data for **1-3** (δ in ppm and J in Hz)

No.	**1**		**2**		**3**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	163.9 s		161.0 s		154.0 s	
2	108.9 d	6.65 s	108.9 d	6.60 (d) 1.8	107.0 d	6.77 s
3	149.0 s		148.4 s		130.1 s	
4	104.5 d	6.97 s	105.1 d	6.90 (d) 1.8	144.8 s	
5	103.1 d	6.82 s	146.8 s		155.7 s	
6	165.1 s		152.7 s		121.8 d	7.41 (d) 8.8
7	113.6 d	6.78 s	106.8 d	7.19 s	125.4 d	7.73 (d) 8.8
8	136.8 s		130.7 s		126.6 s	
9	177.9 s		182.0 s		177.7 s	
4a	155.9 s		154.7 s		146.9 s	
8a	109.9 s		111.1 s		119.2 s	
9a	107.7 s		108.0 s		107.8 s	
10a	158.3 s		145.3 s		147.6 s	
1'	41.9 d	4.35 m	43.2 d	4.50 m	41.3 d	4.42 m
2'	73.0 d	5.02 m	72.2 d	5.10 m	72.5 d	5.11 m
3'	66.2 t	4.53, 4.60 m	65.9 t	4.71, 4.80 m	66.1 t	4.62, 4.70 m
4'	62.6 t	4.11, 4.14 m	62.4 t	4.19, 4.24 m	62.0 t	4.16, 4.22 m
5'	169.1 s		169.7 s		169.2 s	
OMe-1	55.9 q	3.80 s			56.0 q	3.80 s
OMe-4					61.0 q	3.80 s
OMe-5			61.0 q	3.80 s		
OMe-6			56.2 q	3.81 s		
OMe-5'	53.0 q	4.00 s	52.7 q	4.00 s	52.8 q	4.02 s
Ar-OH-1				13.19 s		
Ar-OH-5						11.60 s
Ar-OH-6		11.54 s				

to the ester carbonyl (δ_C 169.1) suggested that the methoxycarbonyl group was connected at C-8. From above information, the gross structure of **1** was established as shown in Figure 1.

Both compounds **2** and **3** showed similar NMR data to these of **1**, which suggested that they have the similar skeleton. The molecular formula of **2** was assigned to $C_{21}H_{22}O_{10}$ on the basis of its HRESIMS data at *m*/*z* 457.1106 $[M+Na]^+$ (calcd 457.1111). The obvious chemical shift differences between compounds **1** and **2** were resulted from the substituents variation on the aromatic rings. In compound **2**, the 1-hydroxymethyl-2,3-dihydroxypropyl structure unit located at C-3 was supported by the HMBC correlations from H-1' to C-2, C-3, and C-4, and from H-2 and H-4 to C-1'. The methoxycarbonyl group at C-8 was supported by the HMBC correlations from the methoxy proton (δ_H 4.00) and H-7 (δ_H 7.19) to the ester carbonyl (δ_C 169.7). Two methoxy groups located C-5 and C-6 were supported by the HMBC correlations of two methoxy protons (δ_H 3.80 and 3.81) with C-5 and C-6, respectively. The phenolic hydroxy group located at C-1 was supported by the HMBC ccorrelations of phenolic hydroxy proton with C-1, C-2, and C-9a. Accordingly, the structure of **2** was determined as shown (Figure 1).

The obvious chemical shift differences between compounds **2** and **3** were also resulted from the substituents variation on the aromatic rings. By analysis of its HMBC correlations, the correlations from H-1' to C-2, C-3 and C-4, and from H-2 and H-4 to C-1' supported the 1-hydroxymethyl-2,3-dihydroxypropyl structure unit located at C-3; the correlations from H-7 to the ester carbonyl supported the methoxycarbonyl group at C-8; the correlations from phenolic hydroxy proton to C-5, C-6, and C-10a supported the phenolic hydroxy group located at C-5; the correlations from two methoxy protons to C-1 and C-4 supported two methoxy groups located C-1 and C-4, respectively. Thus, compound **3** was finally identified as shown (Figure 1).

Compounds **1-6** were tested for their cytotoxic activities against NB4, A549, SHSY5Y, PC3, and MCF7 tumor cell lines using a previously reported procedure with taxol as the positive control).[13] The results were shown in Table 2. Compounds **3** and **4** showed cytotoxicities against SHSY5Y cells with IC_{50} values of 3.8 and 3.5 μM, respectively. The other compounds also showed moderate cytotoxicity for some tested cell lines with IC_{50} values between 4.6 -9.2 μM.

Table 2. Cytotoxic activity of compounds **1 - 6**

Compounds	NB4	A549	SHSY5Y	PC3	MCF7
1	8.1	>10	6.4	5.2	>10
2	>10	5.3	8.2	>10	7.6
3	5.7	>10	3.8	7.5	6.0
4	6.8	8.4	3.5	6.7	5.6
5	7.8	8.0	>10	>10	9.2
6	5.8	7.6	4.6	7.8	6.9
Taxol	0.03	0.02	0.2	0.2	0.1

NB4, human leukemia cell; A549, carcinomic human alveolar basal epithelial cell; SHSY5Y, human neuroblastoma cell; PC3, Human prostate cancer cell; MCF7, Human breast adenocarcinoma cell.

EXPERIMENTAL

General. Optical rotations were measured in a Horiba SEPA-300 polarimeter. UV spectra were measured on Perkin-Elmer Lambda L14 spectrometer. IR spectra were recorded on a Bio-Rad Win infrared spectrophotometer with KBr disc. ESI-MS were obtained by VG Auto Spec-3000 MS spectrometer. ^{1}H, ^{13}C and 2D NMR spectra were recorded on Bruker DRX-500 instrument. Column chromatography was performed on silica gel (200-300 mesh or 10 ~ 40 μm, Qingdao Marine Chemical Ltd, China). Separation of subfracions was used an Agilent 1100 HPLC equipped with ZORBAX-C_{18} (21.2 mm × 250 mm, 7.0 μm) column and DAD detector.

Fungal Material. The culture of *Phomopsis* sp. was isolated from the rhizome of *Paris daliensis* H. Li & V. G. Soukup collected from Heqing County, Dali prefecture, Yunnan Province, People's Republic of China, in 2011. The strain was identified by Prof. Gang Du (Yunnan University of Nationalities) based on the analysis of the ITS sequence (Genbank accession number KF609280). It was cultivated 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 20 Fernbach flasks (500 mL) each containing 100 g of rice and 120 mL of distilled H_2O. Each flask was inoculated with 5.0 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 ×5.0 L) at room temperature and filtered. The crude extract (65.8 g) was applied to silica gel (200–300 mesh) column chromatography, eluting with a $CHCl_3$-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 B (9:1, 12.2 g) by silica gel column chromatography, eluted with petroleum ether-EtOAc (9:1, 8:2, 7:3, 6:4, 1:1), yielded mixtures B1–B5. Fraction B2 (8:2, 2.2 g) was subjected to preparative HPLC (65% MeOH, flow rate 12 mL/min) to give **6** (11.8 mg), Fraction B5 (1:1, 1.8 g) was subjected to preparative HPLC (52% MeOH, flow rate 12 mL/min) to give **1** (14.2 mg), **2** (12.6 mg), **3** (13.1 mg), and **5** (15.2 mg). The further separation of fraction C (8:2, 3.51 g) by silica gel column chromatography, and preparative HPLC (50% MeOH, flow rate 12 mL/min) to give **4** (14.9 mg).

Cytotoxicity Assay. The cytotoxicity tests for the isolates were performed by against NB4, A549, SHSY5Y, PC3, and MCF7 tumor cell lines by MTT-assay (with taxol as the positive control).[13]

Dalienxanthone A (1): $C_{20}H_{20}O_9$; yellow gum; $[\alpha]_D^{24.8}$ +11.6 (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.36), 242 (3.68), 305 (3.82) nm; IR (KBr) ν_{max} 3386, 3072, 2928, 2860, 1722, 1659, 1598, 1542, 1462, 1368, 1125, 1075, 876, 763 cm^{-1}; ^{13}C NMR and ^{1}H NMR data (125 and 500 MHz, in C_5D_5N) see Table 1; ESIMS *m/z* (positive ion mode) 427 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 427.1012

$[M+Na]^+$ (calcd $C_{20}H_{20}NaO_9$ for 427.1005).

Dalienxanthone B (**2**): $C_{21}H_{22}O_{10}$; yellow gum; $[\alpha]_D^{24.5}$ +8.2 (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.32), 245 (3.63), 308 (3.93) nm; IR (KBr) ν_{max} 3402, 3082, 2915, 2874, 1728, 1653, 1600, 1548, 1449, 1378, 1120, 1057, 880, 745 cm^{-1}; ^{13}C NMR and ^{1}H NMR data (125 and 500 MHz, in C_5D_5N) see Table 1; ESIMS *m/z* (positive ion mode) 457 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 457.1106 $[M+Na]^+$ (calcd $C_{21}H_{22}NaO_{10}$ for 457.1111).

Dalienxanthone C (**3**): $C_{21}H_{22}NaO_{10}$; yellow gum; $[\alpha]_D^{24.8}$ +12.8 (c 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.30), 252 (3.74), 312 (3.87) nm; IR (KBr) ν_{max} 3395, 3076, 2922, 2853, 1719, 1650, 1596, 1537, 1459, 1357, 1238, 1132, 1084, 868, 769 cm^{-1}; ^{13}C NMR and ^{1}H NMR data (125 and 500 MHz, in C_5D_5N) see Table 1; ESIMS *m/z* (positive ion mode) 457 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 457.1113 $[M+Na]^+$ (calcd $C_{21}H_{22}NaO_{10}$ for 457.1111).

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