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Pestalotiolide A, a New Antiviral Phthalide Derivative from a Soft Coral-derived Fungus *Pestalotiopsis* sp.

Yan-Lai Jia, Fei-Fei Guan, Jie Ma, Chang-Yun Wang, and Chang-Lun Shao*

Key Laboratory of Marine Drugs, The ministry of Education of China, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, People's Republic of China

Abstract – Chemical investigation of the fermentation broth of a Soft Coral-Derived fungus *Pestalotiopsis* sp., led to the isolation of a new phthalide derivative, pestalotiolide A (**1**), three known analogues (**2**, **3** and **4**), along with 5'-O-acetyl uridine (**5**) first isolated as a natural product. The structure of the new compound (**1**) was established by comprehensive spectroscopic analysis and chemical methods. Compounds **1 - 4** possessed varying degrees of antiviral activities, which was reported for the first time. Compared to the positive control ribavirin (IC_{50} = 418.0 μM), pestalotiolide A (**1**) exhibited significant anti-EV71 activity *in vitro*, with an IC_{50} value of 27.7 μM. Furthermore, the preliminary structure-activity relationship of antiviral activities was also discussed.
Keywords – Marine Fungus, *Pestalotiopsis* sp., Structure-Activity Relationship, Antiviral Activity

Introduction

The fungal species of the genus *Pestalotiopsis* have been demonstrated to be rich sources of bioactive secondary metabolites with diverse structural features.[1-10] In the course of a search for structurally novel and bioactive natural compounds, chromatographic separation of the fungus *Pestalotiopsis* sp. (ZJ-2009-7-6), isolated from a soft coral *Sarcophyton* sp. collected from Yongxing Island in the South China Sea, has afforded a new phthalide derivative,pestalotiolide A (**1**), three known phthalide derivatives **2 - 3**[14] and **4**[13,14] , along with 5'-O-acetyl uridine (**5**) [11,12]. Herein, details of the evaluation of the isolation, structure elucidation, antiviral activity of these compounds and the preliminary structure-activity relationship of antiviral activity are described in the study.

Experimental

General experimental procedures – Melting points were recorded on a CBI021-X-6 precision micro melting point apparatus. Optical rotations were measured on a JASCO P-1020 digital polarimeter. UV spectra were recorded on a HITACHI UH 5300 spectrophotometer. IR spectra were recorded on a Nicolet-Nexus-470 spectrometer using KBr pellets. NMR spectra were recorded on an Agilent DD2 500 MHz NMR spectrometer (500 MHz for ^1H and 125 MHz for ^{13}C), using TMS as internal standard. ESIMS and HRESIMS spectra were obtained from a Micromass Q-TOF spectrometer and Thermo Scientific LTQ Orbitrap XL spectrometer. Semipreparative HPLC was performed on a Waters 1525 system using a C_{18} (Kromasil, 5 μm, 10 × 250 mm) column coupled with a Waters 2996 photodiode array detector. Silica gel (Qing Dao Hai Yang Chemical Group Co.; 200 - 300 mesh), octadecylsilyl silica gel (Unicorn; 45 - 60 μm), and Sephadex LH-20 (Amersham Biosciences) were used for column chromatography. Precoated silica gel plates (Yan Tai Zi Fu Chemical Group Co.; G60, F-254) were used for thin-layer chromatography.

Fungal material – The fungal strain *Pestalotiopsis* sp. (ZJ-2009-7-6) was isolated from a piece of fresh tissue from the inner part of a soft coral *Sarcophyton* sp., collected from Yongxing Island in the South China Sea in November, 2009. The strain was deposited at the Key Laboratory of Marine Drugs, the Ministry of Education of China, School of Medicine and Pharmacy, Ocean University of China, Qingdao, China. The fungus was identified as a *Pestalotiopsis* sp. whose 617 base pair ITS sequence had 99% sequence identity to that of *Pestalotiopsis* sp. DFFW (EF055190). The sequence data have been submitted to GenBank, accession number HM486429.[10]

*Author for correspondence
Chang-Lun Shao, Key Laboratory of Marine Drugs, The ministry of Education of China, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266003, People's Republic of China
Tel: +82-; E-mail: shaochanglun@163.com

Extraction and isolation – The fungal strain was cultivated in solid medium (each containing 80 g of rice, 120 mL of H_2O, and 3.6 g of natural sea salt from Yangkou saltern, China) in 1000 ml Erlenmeyer flasks (30 flasks) at 28 °C for 9 weeks. The fermented solid medium was extracted three times with EtOAc and three times with CH_2Cl_2/MeOH (v/v, 1 : 1). The combined EtOAc layers were evaporated to dryness under reduced pressure to give an EtOAc extract. The EtOAc extract (6.0 g) was subjected to vacuum liquid chromatography (VLC) on silica gel using step gradient elution with EtOAc-petroleum ether (PE) (0 - 100%) and then with MeOH-EtOAc (0 - 100%) to afford eight fractions (Fr.1 - Fr.8). Fr.3 was first subjected to repeated column chromatography (CC) on silica gel (EtOAc-PE,v/v, 3 : 7) and separated by Sephadex LH-20 CC (PE-CH_2Cl_2-MeOH, v/v/v, 2 : 1 : 1), then further purified on HPLC with 40% MeOH-H_2O to yield **1** (3.5 mg). Fr.5 was fractionated on silica gel CC (CH_2Cl_2-MeOH, v/v, 30 : 1), then purified by Sephadex LH-20 CC (MeOH) to obtain two subfractions, Fr.5-1 - Fr.5-2. Fr.5-1 was purified on HPLC with 55% MeOH-H_2O to give **3** (4.2 mg). Fr.5-2 was purified with 30% MeOH-H_2O to yield **2** (5.1 mg). Fr.2 was subjected to repeated CC on silica gel (EtOAc-PE, v/v, 3 : 7) and separated by Sephadex LH-20 CC (PE-CH_2Cl_2-MeOH, v/v/v, 2 : 1 : 1), then further purified on HPLC with 70% MeOH-H_2O to yield **4** (12.1 mg). Fr.4 was applied to repeated CC on silica gel (EtOAc-PE, v/v, 2 : 8) and separated by Sephadex LH-20 CC (PE-CH_2Cl_2-MeOH, v/v/v, 2 : 1 : 1) and further purified on HPLC with 15% MeOH-H_2O to afford **5** (3.1 mg).

Pestalotiolide A (1) – Colorless crystal. mp 170 - 173 °C; $[\alpha]_D^{25}$: +12.0 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.05), 254 (3.71), 294 (3.57) nm; IR (KBr) ν_{max} cm^{-1}: 3420, 2920, 1737, 1512, 1456, 1085; ^1H and ^{13}C NMR data: see Table 1.; ESI-MS *m/z* 413 $[M+H]^+$; HRESIMS *m/z* 413.1466 $[M+H]^+$; (calcd for $C_{19}H_{25}O_{10}$, 413.1488).

7-Hydroxy-5-methoxy-4,6-dimethyl-7-O-β-D-glucopyranosyl-phthalide (2) – Colorless crystal, ^1H NMR (CD_3OD, 500 MHz): δ_H 5.27 (2H, s, H-2), 5.15 (1H, d, *J* = 7.9 Hz, H-1'), 3.79 (3H, s, CH_3O-5), 3.76 (1H, m, H-6a), 3.64 (1H, m, H-6b), 3.53 (1H, t, 8.4, H-2'), 3.45 (1H, t, 8.4, H-5'), 3.39 (1H, m, H-4'), 3.22 (1H, m, H-3'), 2.32 (3H, s, CH_3-6), 2.22 (3H, s, CH_3-4); ^{13}C NMR (CD_3OD, 125 MHz) δ_C 172.1 (C-1), 164.4 (C-5), 154.1 (C-7), 147.7 (C-3), 128.4 (C-6), 122.5 (C-4), 113.1 (C-8), 106.0 (C-1'), 78.0 (C-5'), 77.9 (C-3'), 75.3 (C-2'), 70.9 (C-4'), 70.0 (C-2), 62.3 (C-6'), 60.7 (MeO-5), 11.5 (Me-4), 10.9 (Me-6). ESIMS *m/z*: 393.05 $[M+Na]^+$.

Table 1. ^1H and ^{13}C NMR data of **1** in CD_3OD. (δ in ppm, *J* in Hz, 500 MHz for ^1H and 125 MHz for ^{13}C)

No.	δ_C (*J* in Hz)	δ_H (*J* in Hz)
1	172.1	–
2	70.2	5.27 (2H, s)
3	148.1	–
4	122.8	–
5	164.5	–
6	128.5	–
7	153.9	–
8	113.4	–
1'	105.9	5.12 (1H, d,7.8)
2'	75.7	3.54 (1H, t, 8.5)
3'	78.1	3.45 (1H, t, 8.4)
4'	71.5	3.37 (1H, m)
5'	75.6	3.37 (1H,m)
6'	64.2	4.33 (1H, d, 11.8)
		4.19 (H, dd, 11.8,5.5)
1"	172.4	
2"	20.6	1.94 (3H, s)
5-OMe	60.8	3.79 (3H, s)
4-Me	11.4	2.22 (3H, s)
6-Me	11.0	2.26 (3H, s)

7-Hydroxy-5-methoxy-4,6-dimethyl-7-O-α-L-rhamnosyl-phthalide (3) – Yellow oil, ^1H NMR (CD_3OD, 500 MHz): δ_H 5.54 (1H, d, *J* = 1.5 Hz, H-1'), 5.23 (2H, d, *J* = 6.3 Hz, H-2), 4.48 (1H, m, H-2'), 4.00 (1H, m, H-5'), 3.89 (1H, dd, *J* = 9.6 Hz, *J* = 3.2 Hz, H-3'), 3.79 (3H, s, CH_3O-5), 3.50 (1H, t, *J* = 9.5 Hz, H-4'), 2.28 (3H, s, CH_3-6), 2.21 (3H, s, CH_3-4), 1.24 (3H, d, *J* = 6.5 Hz, H-6'); ^{13}C NMR (CD_3OD, 125 MHz) δ_C 171.3 (C-1), 164.4 (C-5), 154.9 (C-7), 148.5 (C-3), 126.6 (C-6), 122.2 (C-4), 113.1 (C-8), 106.4 (C-1'), 73.4 (C-4'), 72.3 (C-3'), 72.2 (C-5'), 72.0 (C-2'), 69.8 (C-2), 60.9 (MeO-5), 18.0 (Me-6'), 11.3 (Me-4), 10.6 (Me-6). ESIMS *m/z*: 377.2 $[M+Na]^+$.

7-Hydroxy-5-methoxy-4,6-dimethylphthalidc (4) – Colorless crystal, ^1H NMR ($CDCl_3$, 500 MHz): δ_H 7.68 (1H, s,7-OH), 5.19 (2H, s, H-2), 3.76 (3H, s, 5-OMe), 2.19 (3H, s, CH_3-6), 2.14(3H, s, CH_3-4); ^{13}C NMR ($CDCl_3$,125 MHz) δ_C 173.0 (C-1), 163.8 (C-5), 153.7 (C-7), 143.4 (C-3), 118.6 (C-6), 116.5(C-4), 106.1 (C-8), 70.1 (C-2), 60.3 (5-OMe), 11.4 (4-Me), 8.5 (6-Me). ESIMS *m/z*: 209.15 $[M+H]^+$.

5'-*O*-acetyl uridine (5) – Colorless crystal, ^1H NMR (CD_3OD/Acetone-d_6, 500 MHz): δ_H 8.78 (1H, d, *J* = 8.1 Hz, H-6), 7.02 (1H, d, *J* = 2.4 Hz,.4, H-1'), 6.89 (1H, d, *J* = 8.1 Hz, H-5), 5.57 (2H, m, H-5'), 5.46 (1H, m, H-2'), 5.41 (2H, m, H-3', H-4'), 2.45 (3H, s, CH_3CO); ^{13}C NMR

(CD_3OD/Acetone-d_6, 125 MHz) δ_C 169.8 (C-1''), 162.7 (C-4), 150.1 (C-2), 139.8 (C-6), 101.7 (C-5), 90.6 (C-1'), 81.4 (C-4'), 73.8 (C-2'), 69.7 (C-3'), 63.1 (C-5'), 20.1 (C-2''). ESIMS *m/z*: 309.02 $[M+Na]^+$.

Antiviral activity assays – The antiviral activities of **1 - 5** against respiratory syncytial virus (RSV), enterovirus 71 (EV71), herpes simplex virus (HSV-1), influenza virus (H1N1) and coxsackie virus (Cox-B3) were determined by the CPE inhibition assay, according to established procedures[15]. Ribavirin was used as a positive control.

Hydrolysis of Pestalotiolide A (1) – An aliquot (1.0 mg) of pestalotiolide A (**1**) in anhydrous methanol (2.0 mL) and potassium carbonate (2.0 mg) were stirred at 37 °C for 6 h on a magnetic stirrer. The reaction mixture was then extracted with methanol (3 mL × 3), and the MeOH extract was evaporated to dryness. The residue was purified on a C_{18} reversed-phase column, eluted with MeOH-H_2O (3 : 7), to give **1a** as a colorless crystal [0.5 mg, $[\alpha]_D^{25}$: +15.3 (c 0.2, MeOH)].

Hydrolysis derivative of 1 (1a) – Colorless crystal; ^1H NMR (CD_3OD, 500 MHz): δ_H 5.27 (2H, s, H-2), 5.15 (1H, d, *J* = 7.8 Hz, H-1'), 3.79 (3H, s, CH_3-5), 3.76 (1H, m, H-6a), 3.65 (1H, dd, *J* = 11.8, 5.3 Hz, H-6b), 3.54 (1H, t, *J* = 8.5 Hz, H-2'), 3.45 (1H, t, *J* = 8.5 Hz, H-5'), 3.39 (1H, t, *J* = 8.6 Hz, H-4'), 3.22 (1H, m, H-3'), 2.32 (3H, s, CH_3-6), 2.22 (3H, s, CH_3-4). ESIMS *m/z*: 393.05 $[M+Na]^+$.

Result and Discussion

Pestalotiolide A (**1**) was obtained as a colorless crystal and exhibited a $[M+H]^+$ peak at *m/z* 413.1466, corresponding to a molecular formula of $C_{19}H_{24}O_{10}$ in the positive HRESIMS. The IR spectrum of **1** indicated the presence of hydroxy (3420 cm^{-1}), ester carbonyl (1737 cm^{-1}), and benzene ring (1512 and 1456 cm^{-1}). The NMR spectra of **1** were very similar to those of the known compound, 7-hydroxy-5-methoxy-4,6-dimethyl-7-O-*β*-D-gluco-pyranosyl-phthalide **(2)**[14], except for one more acetyl group supported by the signal of the carbon (C-6') moving downfield, one more ester carbonyl carbon (C-1'') and methyl carbon (C-2'') (Fig. 1). The key HMBC correlations from H-6' to C-1'', from H-2'' to C-1'', and from H-1' to C-7 revealed that the acetyl group moiety was linked to C-6' position and the glucosyl moiety was connected to C-7 position (Fig. 2), respctively. The NMR signals of the anomeric proton and carbon at $\delta_{H/C}$ 5.12 (d, *J* = 7.8)/105.9 (CH) suggested the *β*-configuration of the glucoside. Moreover, methanolysis of **1** gave **1a** [$[\alpha]_D^{25}$: +15.3 (c 0.2, MeOH)], which was proved to be compound **2** [$[\alpha]_D^{25}$: +19.0 (c 0.2, MeOH)] by comparing their ESIMS and ^1H NMR data.Thus, the structure of pestalotiolide A (**1**) was determined to be 6'-O-acetyl-7-hydroxy-5-methoxy-4,6-dimethyl-7-O-β-D-glucopyranosyl-phthalide. Pestalotiolide A (**1**), detected in the fermentation broth by using HPLC analysis, was a natural product, which was proved by the fact that pestalotiolide A (**1**) cound not be detected by using HPLC analysis after reacting the compound **2** with methanol and EtOAc used in the procedure of extraction and isolation for twenty days at room temperature.

Compounds **3 - 4** are known compounds reported in the literatures[13,14]. Compound **5** was isolated as a natural product for the first time[12], which was enzymatically synthesized by an essential CALB lipase (lipase B from *Candida antarctica*).

The antiviral activities of **1 - 5** against respiratory syncytial virus (RSV), enterovirus 71 (EV71), herpes simplex virus (HSV-1), influenza virus (H1N1), and coxsackie virus (Cox-B3) were determined by the CPE inhibition assay, according to established procedures[15].

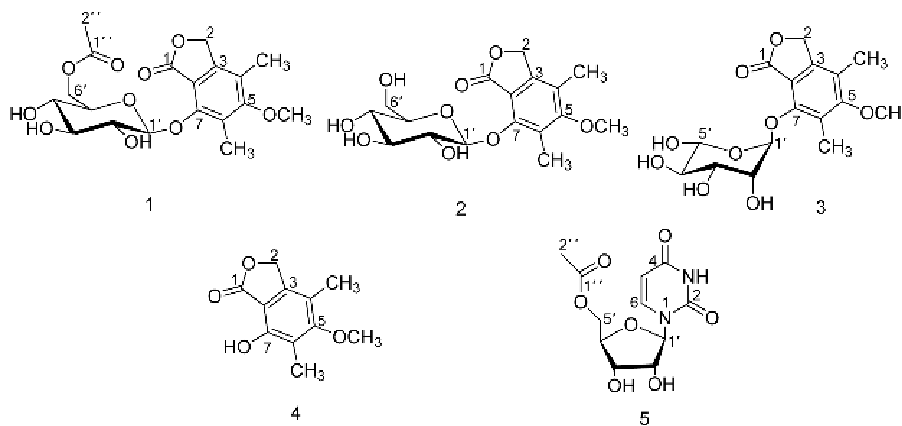

Fig. 1. The structures of **1 - 5** isolated from *Pestalotiopsis* sp.

Table 2. Antiviral activities of the tested compounds **1 - 5** [a]

virus	TC_{50}/IC_{50} (μM)					
	1	**2**	**3**	**4**	**5**	Ribavirin
EV71	254.9/27.7	104.6/51.6	316.4/111.0	–	262.2/110.0	A/418.0
RSV	303.4/80.9	337.8/25.6	–	360.1/21.0	–	A/78.0
HSV-1	–	337.8/63.9	–	–	–	A/313.0
H1N1	–	–	–	–	–	625/156.0
Cox-B3	–	–	353.1/95.9	360.6/19.6	297.2/127.5	A/39.0

[a] the symbol "–" means no antiviral activities, A > 4000. Cytotoxicity (TC_{50}) and antiviral activity (IC_{50}) are located in the left and right side of the symbol "/", respectively.

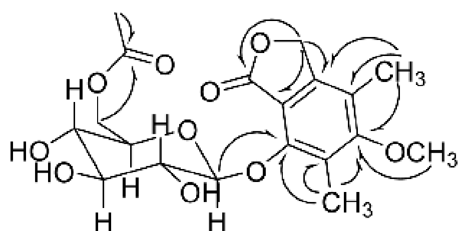

Fig. 2. Key HMBC (—→) correlations of **1**.

Compounds **1 - 5** possessed varying degrees of antiviral activities (Table 2). Compared to ribavirin (IC_{50} 418.0 μM), pestalotiolide A (**1**) exhibited potent anti-EV71 activity, with an IC_{50} value of 27.7 μM. Compound **2** showed strong antiviral activities against EV71, RSV, and HSV-1 with IC_{50} values of 51.6 μM, 25.6 μM and 63.9 μM, respectively. Compound **4** displayed pronounced antiviral activities against Cox-B3 and RSV with IC_{50} values of 19.6 μM and 21.0 μM, which were stronger than those of the positive control ribavirin, with IC_{50} values of 39.0 μM and 78.0 μM, respectively. Compound **5** and **3** shared similar antiviral activities with pronounced antiviral activities against EV71.

Furthermore, compounds **1 - 4** were a series of phthalide derivatives, which had close activity-structure relationship in antiviral activities. The glycosidation of 7-OH significantly increased anti-EV71 activity and the acetylation of 6'-OH increased anti-EV71 activity. The acetoxy group at C-6' had a positive contribution to anti-EV71 activity.

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