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New Cytotoxic Metabolites from Pathogenic Fungus *Cylindrocarpon destructans* Associated with *Meconopsis grandis*

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Abstract: Phytochemical investigation on pathogenic fungus *Cylindrocarpon destructans* isolated form *Meconopsisgrandis* plant led to the isolation of two new isochromene derivatives namely, 6,8-dimethoxy-3-methyl-3,4-dihydrobenzoisochromene-4,9,10-triol (**1**) and 3,5,6-trihydroxy-4-methylbenzoisochromene-9,11-dione (**2**) along with four known compounds (**3-6**). The structures of these compounds were elucidated by 1D and 2D NMR and mass spectroscopic data analysis. The isolated compounds were evaluated for cytotoxic activity. The compounds **1-4** showed good inhibition against the growth of cell lines MCF-7 and PC-3. Compounds **5-6** showed minimum inhibitory effect of cancerous cell lines growth.

Keywords: Pathogenic fungus; *Cylindrocarpon destructans; Meconopsis grandis;*cytotoxic activity

1. Introduction

Species of *Cylindrocarpon* Wollenw. are common and may be isolated as soil inhabitants, saprobes on dead plant material, root colonizers or pathogens, or weak pathogens of various herbaceous and woody plants [1]. *Cylindrocarpon* destructans (Zinnsm.) [anamorph of Neonectriaradicicola] and *C. obtusisporum* have been reported to cause the root rots of various hosts [2, 3], and a black foot disease of grapevines [4-6]. *C. destructans* (*C. radicicola*) has frequently been reported to cause decay of woody seedlings, especially conifers, and many other hosts as well [2]. Generally this fungus is not severe in its pathogenicity and has been regarded in many cases as the wound infectious fungus or the secondary invader.

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Cylindrocarpon species have been rarely associated with human disease. They are known to cause post traumatic keratitis [7, 8] and, have been implicated in mycetoma following injury [9, 10], athlete's foot [11], peritonitisina case of continuous ambulatory peritoneal dialysis [10], localized invasive lesion in a case of AML [12], disseminate disinfection in neutropenic patients [13]. The human infecting species include *C. cyanescens, C. destructans, C. lichenicola* and *C. vaginae* [14].

Previous phytochemical studies on *Cylindrocarpon* species have resulted in the isolation of Orsenol, Orsellinic acid, Ilicicolin (A-F), Ascochlorin, fatty acids [15], Cylindrols [16], Cylindrocyclin [17], Colletorine, Colletochlorin and Curvularine derivatives [18].

Only few metabolites such as radicicol and radicicolin [19] have been reported from *Cylindrocarpon destructans* (*C. radicola*). Herein we reported the isolation and structure elucidation of two new compounds (**1-2**) and four known compounds (**3-6**) from *Cylindrocarpon*destructan (*C. radicicola*). The isolated compounds were screened for cytotoxic activities.

2. Materials and Mehods

2.1. General

Optical rotations were measured with an Abbemat 300 spectrometer. NMR spectra were recorded with a Bruker Avance (400 MHz for ^{1}H and 100 MHz for ^{13}C spectrometer; Bruker Corporation, Switzerland). HRESIMS spectra were recorded using an LTQ-Orbitrap LC-MS spectrometer (Thermo Corporation, USA). UV spectra were recorded on a Blue Star A spectrophotometer. Thin layer chromatography silica gel GF_{254} (Qingdao Marine Chemical, Factory PR China) were used for TLC. Sephadex LH-20 (Amersham Pharmacia) and Silica gel (100-200, 200-300, and 300-400 mesh, Qingdao Marine Chemical Factory, Qingdao, PR China) were used for column chromatography. The reagents in the research process were analytical grade from Guangzhou chemical reagent factory.

2.2. Fungal Material

The fungal strain *Cylindrocarpon destructans* (or *C. radicicola*) (Gen accession number KC904953) was isolated from fresh roots of Meconopsisgrandis (Tibetan Blue puppy) in Tibetan Plateau near to Damxung, Tibet, China. The fungus was identified as using morphological characteristics and ITS region. A voucher specimen (DH 29) has been preserved on PDA at 4°C at the school of marine science, Sun Yat-Sen University.

2.3. Fermentation, Extraction and Fractionation

The fungal strain was cultured for 6 days at 28 ^{0}C in Petri dishes containing Potato dextrose agar. The agar supported mycelia were then cut and transferred to 1000 mL Erlenmeyer flasks containing 500 mL potato dextrose broth (12 gm of PDB dissolved in 500 mL of 3% saline water) and then incubated at 28 ^{0}C for 5 days with continuous shaking on shaker at 150 rpm. Then 10 mL of the fungal broth were added into rice medium (110 bottles each 1000 mL Erlenmeyer flasks, each containing 60 g rice in 80 mL of 3% saline water) and were incubated for 30 days under static conditions and light. After incubation, the mycelia cultivated rice medium were crushed and extracted three times with methanol. The methanol extract was concentrated with rotary evaporator to get methanolic crude which was then suspended in 20 % MeOH-H_20. The suspension was then fractionated with n-hexane, chloroform, ethyl acetate to get the corresponding n-hexane (15 g), Chloroform (26 g) and ethyl acetate (44 g) fractions. The chloroform extract was separated into 7 sub-fractions (J-P) by silica gel column chromatography by gradient elution of petroleum ether/CH_2Cl_2 (from 90:10 to 00:100) and then CH_2Cl_2/MeOH (from 100:00 to 00:100). Ethyl acetate fraction was further fractionated over silica gel column chromatography with gradient elution of pet ether Ether/EtOAc (100:00 – 00:100) and then EtOAc/MeOH (100:00 to 00:100) to get 9 fractions (Q-Y).

3.4. Purification of the Compounds

Fraction **M** was further separated over silica gel CC eluted with pet ether/CH_2Cl_2 (90:10 to 00:100) and then CH_2Cl_2/MeOH (5 and 10% MeOH in CH_2Cl_2) to afford 5 subfractions (M-1 to M-5). Fraction M-4 was purified by sephadex LH-20 with CH_2Cl_2: MeOH (v/v; 1:1) and then by semipreparative HPLC (65 % MeOH-H_2O flow rate 1.5 ml /min; $C_{18,}$ 10×250 mm, 5 um) to afford two compounds (**5** and **6**). Fraction **P** was further separated over silica gel CC eluted with pet ether/CH_2Cl_2 (90:10 to 00:100) and then CH_2Cl_2/MeOH (5 and 15% MeOH in CH_2Cl_2) to afford 4 subfractions (P-1 to P-4). Fraction P-3 was purified by HPLC (70 % MeOH-H_2O flow rate 1.5 ml /min; $C_{18,}$ 10×250 mm, 5 um) to afford compounds **2** (13 mg).

Fraction **S** was subjected to series of silica gel chromatographic separation by gradient elution with pet ether/EtOAc and then EtOAc/MeOH and then to HPLC (70% MeOH-H_2O, flow rate 1.0 ml/min; C18; 10×250 mm, 5 um) to get a pure compound **1**. Fraction **T** was rechromatographed over Silica gel CC by gradient elution with EtOAc/MeOH (100:00 to 00:100) to get 7 sub-fractions (T-1 to T-7). Fraction T-6 was applied to reverse phase silica gel column MeOH/H_2O (70:30) and then to semipreparative HPLC with (80% CH_3CN-H_2O flow rate 1.5 ml /min; $C_{18,}$ 10×250 mm, 5 um) to yield two pure compounds (**3** and **4**).

*3.4.1. 6,8-dimethoxy-3-methyl-3,4-dihydrobenzoisochromene-4,9,10-triol (**1**):* Yellow powder; UV (MeOH)λ_{max} (logε): 230 (4.78), 284 (4.12), 304 (3,98), 338 (3.86) nm; HRESIMS *m/z:* 307.1140 $[M+H]^+$ (calcd for $C_{16}H_{16}O_{6,}$ 307.1136). ^{1}H NMR and ^{13}C NMR data see Table 1.

*3.4.2. 3,5,6-trihydroxy-4-methylbenzoisochromene-9,11-dione (**2**):* White amorphous powder; $[\alpha]_D^{20}$ +65, (c 0.025, MeOH); UV (MeOH)λ_{max} (logε): 278 (4.12), 243 (3.41), 304 (3,98), 338 (3.86) nm; HRESIMS *m/z:* 261.0363 $[M+H]^+$ (calcd for $C_{16}H_{16}O_{6,}$ 261.0354). ^{1}H NMR and ^{13}C NMR data see Table 1.

*3.5. Cytotoxicity MTT Assa*y

3.5.1. Preparation of Cell Lines and Cell Cultures

MCF-7, PC-3 and WI-38 cells were seeded in culture plates containing MEM, DMEM Media, glutamine (2mM), penicillin (100 Units/mL) and streptomycin (100 µg/mL) accompanied with 10 % heat-inactivated fetal bovine serum under humidified atmosphere at constant temperature of 37 °C in 5% CO2 incubator. After achieving a monolayer with 80% confluence of both adherent cell lines, the cells were cultured in 96-well plates at a seeding density of 5000-10000/well to start cytotoxicity experiments.

3.5.2. MTT Assays Procedure

The method is based on the principal that was previously described by [20] with some modifications to perform cytotoxicity assay cells. The MCF-7, PC-3 and WI-38 cells were cultured for 24 thin Micro plate Elisa Reader for 96 Well Plate (ELX 800) BioTek USA. Different concentrations of test compounds (10, 1, 0.1, 0.01, 0.001 µM) were inoculated in test wells while control and blank wells were also prepared using cell with media and dimethyl sulfoxide (DMSO), respectively. The plates were then incubated for 48 h at 37 °C. After that cells were fixed with 50 µL of MTT solution at 37 °C for 1 h. The plates were washed 5 times with PBS (Phosphate-Buffered Saline). After that the MTT solution was removed and added 100 µL of DMSO The absorbance was measured at 570 nm subtracting the background measurement. Results were reported as mean of three independent experiments (± SEM) and expressed as percent inhibitions calculated by the formula. Inhibition (%) = [100 - (abs of test comp/abs of control) × 100)]. IC50 values of selected compounds exhibiting >50% activity at 0.5 mM were calculated after suitable dilutions.

3. Results and Discussion

The fungus *Cylindrocarpon* sp. DH 29 was cultured in rice solid medium for 30 days. The $CHCl_3$ and EtOAc fraction were repeatedly fractionated and purified by using silica gel column chromatography, sephadex LH-20, reverse phase silica column and HPLC to obtain two new compound (**1-2**) and four known compounds (**3-6**) Figure 1.

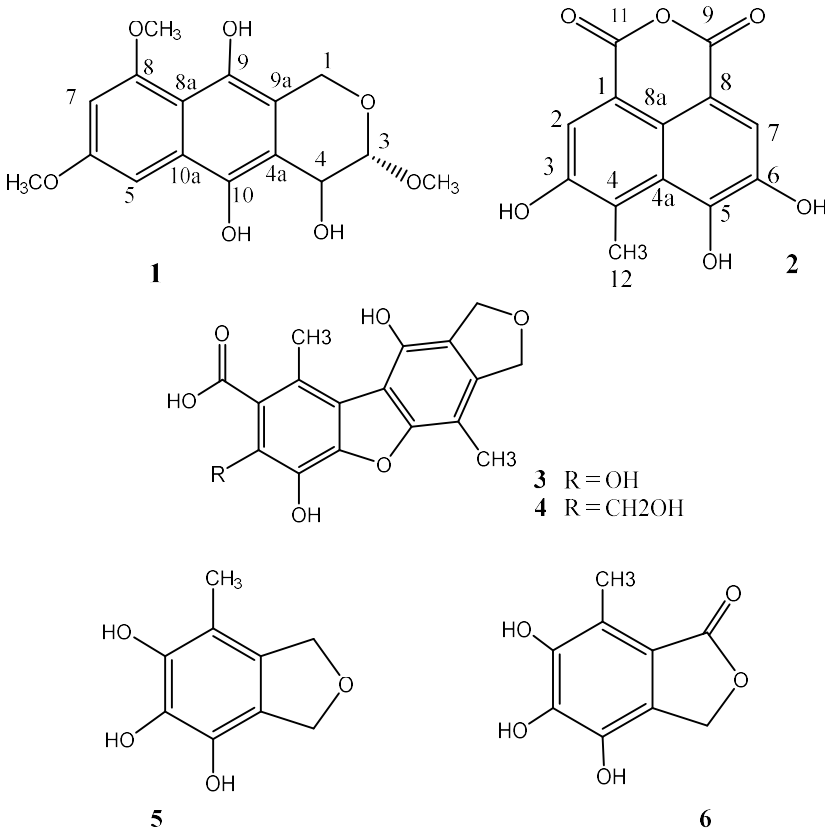

Figure 1. Structures of compounds **1-6**

Compound **1** was isolated as yellow amorphous powder. Its molecular formula was deduced on the basis of HRESIMS as $C_{16}H_{18}O_6$ (*m/z*: 307.1140 $[M+H]^+$ with eight degrees of unsaturation. The ^{1}HNMR spectrum of compound 1 displayed a couple of doublets resonated at δ 6.10 (1H, *J* = 2.1) and 6.56 (1H, *J* = 2.1) assignable to aromatic protons and a pair of doublets resonated at δ 4.78 (1H, *J* = 15.6) and 5.20 (1H, *J* = 15.6) were attributed to two geminal oxygenated methylenic protons. The quartet of doublet at δ 3.71 (1H, *J* = 6.3, 1.7) and a doublet resonated at δ 3.83 (1H, *J* = 1.7) were characteristics of two vicinal methine protons. COSY correlation (Figure **S6**) between the two H-1 proton established their geminal relationship while the correlation between H-3 and H-4 suggested their vicinal relationship. The two methoxy groups exhibited signals at H*δ* 3.43 (3H, s) and 4.09 (3H, s) and the doublet at δ 1.19 (3H, *J* = 6.3) was due to methyl protons. The ^{13}CNMR spectrum along with DEPT experiment showed the presence of four tertiary carbons that include two aromatic and two oxygenated methine carbons, one oxygenated methylene carbon, three primary carbons including two methoxy and one methyl carbons and eight quaternary carbons. The HMBC (Figure **S8**) correlations from H-5 to C-6, C-8a, C-10a and from H-7 to C-8 and C-8a were in consistent with the position of these protons. Finally the structure of compound **1** was assigned as 6,8-dimethoxy-3-methyl-3,4-dihydrobenzoisochromene-4,9,10-triol on the basis of the spectral data that share common structural unit

with the compounds available in literature [21, 22). The structure of compound **1** was further confirmed by 2D experiments and mass spectra (Figure **S6- S8**).

Table 1. ^{1}H (400 MHz) and ^{13}C (100MHz) NMR data for Compound **1** and Compound **2** (MeOD)

Position	**1**		**2**	
	δ H (*J* in Hz)	**δ C**	**δ H (*J* in Hz)**	**δ C**
1	4.78 (1H, d, *J* = 15.6), 5.20 (1H, d, *J* = 15.6)	66.52		97.0
2			6.81 (1H, s)	117.3
3	3.71 (1H, qd, *J* = 6.3, 1.7)	73.97		166.7
4	3.83 (1H, d, *J* = 1.7)	66.02		112.4
4a		134.48		151.7
5	6.56 (1H, d, *J* = 2.1)	99.13		165.4
6		149.2		164.7
7	6.10 (1H, d, *J* = 2.1)	97.62	6.42 (1H, s)	99.5
8		136.44		91.7
8a		109.95		136.9
9		157.14		167.9
9a		113.54		
10		157.10		
10a		124.88		
11	1.19 (3H, d, *J* = 6.3)	15.02		167.6
12			2.87 (3H, s)	25.05
6-OCH_3	4.09 (3H, s)	55.46		
8-OCH_3	3.43 (3H, s)	53.98		

Compound **2** was isolated as white amorphous powder. The HREIMS spectra gave [M+H]+ peak at *m/z* 261.0363 consistent with the molecular formula of C13H8O6 corresponding to nine degrees of unsaturation. The ^{1}HNMR spectrum of compound **2** displayed a couple of singlets resonated at 6.81 and 6.42 assignable to aromatic protons. A singlet resonated at 2.87 was characteristics of methyl protons. ^{13}C NMR spectrum exhibited signals for 13 carbon that were assigned by DEPT as two aromatic methine carbons, one methyl carbon and ten quaternary carbons that include two carbonyl carbon and eight fully substituted aromatic carbons. The aromatic protons were found to show a long range HMBC correlations with the carbonyl carbons resonated at 167.9 and 167.6 indicated the connectivity of these protons at C-2 and C-7 respectively. On the basis of these analysis the structure of compound 2 was assigned as 3,5,6-trihydroxy-4-methylbenzoisochromene-9,11-dione that has close resemblance with the available literature (Lin et al. 2012).The known compounds **3-6** were identified by comparison of their spectral data with the literature available and include *Preussiafuran A* (**3**), *Preussiafuran B* (**4**) [24], *4,5,6-trihydroxy-7-methyl-1,3-dihydroisobenzofuran* (**5**), *4,5,6-trihydroxy-7-methylphthalide* (**6**) [25].

The compounds **1-6** were evaluated for their in vitro cytotoxicity (Table 2). All the compounds were analyzed against two different cancer cell lines cell lines MCF-7 and PC-3 was determined by MTT assay. Tamoxifen was used as standard drug. The compounds 1, 2, 3 and 4 inhibit MCF-7 with IC_{50} values of 05.37± 1.03, 09.02 ± 1.20, 08.04 ± 2.10, 10.04 ± 0.20 and Cell viability (%) 03.02± 2.11, 6.07 ± 0.30, 04.14± 0.81, 05.21± 0.01 (μM). The PC-3 with IC_{50} values of 04.12 ± 0.38, 12.07 ± 0.11, 09.41 ± 1.81 and 08.13± 2.31 (μM) and Cell viability (%) 2.04 ± 0.09, 07.14 ± 1.2, 05.41± 07 and 04.71 ± 0.62 respectively. The compounds **5-6** showed minimum inhibitory effect of cancerous cell lines growth.

Table 2. Cytotoxicity of compounds **1-6**, IC_{50} values (µM) and cell survival (values ± standard Deviation)*

Compounds	IC50 ± SEM[a]			
	MCF-7		PC-3	
	IC_{50} (µM)	Cell survival (%)	IC_{50} (µM)	Cell survival (%)
1	5.37± 1.03	3.02± 2.11	4.12± 0.38	2.04± 0.09
2	9.02 ± 1.20	6.07 ± 0.30	12.07 ± 0.11	7.14 ± 1.2
3	8.04 ± 2.10	4.14± 0.81	9.41 ± 1.81	5.41 ± 07
4	10.04 ± 0.20	5.21± 0.01	8.13± 2.31	4.71 ± 0.62
5	24.18± 1.08	9.02 0.05	28.07± 0.02	7.02± 0.20
6	18.04± 0.10	10.27 ± 2.47	21.04 ± 0.20	8.04 ± 0.22
Tamoxifen[b]	0.00053 ± 0.0004		0.0036 ± 0.0007	

* Values shown are mean ± SEM, no. of experiments = 3
[a] Inhibitory effect of cancerous cell lines growth
[b] standard drug

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Supplementary material

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

Conflict of interest

The authors declare no conflict of interest.

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