NOTE

Pyrenocines N–O: two novel pyrones from *Colletotrichum* sp. HCCB03289

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The Journal of Antibiotics (2014) 67, 791–793; doi:10.1038/ja.2014.59; published online 28 May 2014

Pyrenocines are better known as phytotoxic metabolites been found in *Alternaria*, *Curvularia*, *Penicillium* and *Phomopsis* strains. The class of compounds showed several biological activities, reportedly being phytotoxic, antifungal, cytotoxic, antitrypanosomal and antimalarial.^{1–3} Fungi of the genus *Colletotrichum*, a class of the widely distributed endophytic fungi, were reported as prolific producers of bioactive metabolites.^{4–7} However, there are few reports on pyrenocines found in genus *Colletotrichum*. During our ongoing chemical investigations of endophytic fungi as sources of new cytotoxic natural products, the organic solvent extract of solid substrate fermentation culture of a *Colletotrichum* sp. HCCB03289 strain displayed cytotoxicity against cancer cell lines. Bioassay-guided fractionation of the extract led to the isolation of two novel pyrenocines N–O (1–2), and five known pyrenocines macommelin-9-acetate (3),⁸ pyrenocine A (4),⁹ pyrenocine B (5),⁹ pyrenocine E (6)¹⁰ and novae-zelandin A (7).¹¹ Details of the isolation, structure elucidation and the cytotoxic activities of these compounds are presented here.

The culture of *Colletotrichum* sp. HCCB03289 was isolated from traditional Chinese medicinal plant *Ludwigia prostrata* Roxb specimen collected from Tianmu mountain, Zhejiang Province of China, in July 2008. The isolation and identification methods of endophytic fungal strain HCCB03289 were carried out as reported.¹² The ITS (internal transcribed spacer) sequence data were submitted to GenBank with accession no. KF594420. Its ITS sequence was similar to that of *Colletotrichum gloeosporioides* strain HGUP0020 (maximal identities: 100%), but due to the lack of a sexual structure in the culture, the endophytic fungus was then identified at the genus level only as *Colletotrichum* sp. The fungal strain was cultured on slants of potato dextrose agar at 28 °C for 5 days. Then, 5.0 ml of spore suspension (spore/cell 1×10^6 ml⁻¹) was inoculated into 500 ml Erlenmeyer flask ($\times 100$) each with 80 g of rice medium at 28 °C for 30 d. The fermented rice substrate was freeze-dried and extracted with ethyl acetate (10 litres) subsequently. The organic solvent was evaporated under vacuum to obtain a crude extract (20.0 g), which was fractionated by silica gel column chromatography (2.6 $\times$ 40 cm) using petroleum ether/ethyl acetate (7:3, 6:4, 3:7, 1:9) gradient

elution to afford four fractions, Frs. 1–4. The Fr. 1 (1.0 g) was further separated by Sephadex LH-20 (Pharmacia, Uppsala, Sweden) eluted with CHCl₃/CH₃OH (10:1) to yield a subfraction of 200 mg, which was then purified by semi-prep. RP-HPLC (YMC-Pack RP-C18 column (YMC, Kyoto, Japan), 10 $\times$ 250 mm, 65% CH₃OH in H₂O for 40 min, 2.5 ml min⁻¹) to yield novae-zelandin A (7, 6.5 mg; t_R 28.3 min). The Fr. 2 (1.5 g) was further separated by Sephadex LH-20 eluted with CHCl₃/CH₃OH (5:1) to yield a subfraction of 150 mg. Purification of the subfraction by semi-prep. RP-HPLC (10 $\times$ 250 mm, 45% CH₃OH in H₂O for 30 min, 2.5 ml min⁻¹) afforded pyrenocine E (6, 11.6 mg; t_R 23.5 min). The Fr. 3 (1.2 g) was further separated by semi-prep. RP-HPLC (YMC-Pack RP-C18, 20 $\times$ 250 mm, 50% CH₃OH in H₂O for 15 min, gradient to 100% in 30 min, 7 ml min⁻¹) to afford two subfractions, Frs. 2.1–2.2. The Fr. 2.1 (12.3–13.2 min, 90 mg) was then purified by semi-prep. RP-HPLC (10 $\times$ 250 mm, 35% MeOH in H₂O for 30 min, 2 ml min⁻¹) to yield pyrenocine B (5, 8.7 mg; t_R 14.5 min). The Fr. 2.2 (15.2–16.5 min, 0.12 g) was then purified by semi-prep. RP-HPLC (10 $\times$ 250 mm, 35% MeOH in H₂O for 40 min, 2 ml min⁻¹) to yield pyrenocine A (4, 15.0 mg; t_R 31.5 min) and macommelin-9-acetate (3, 10.6 mg; t_R 36.5 min). The Fr. 4 (200 mg) was purified by semi-prep. RP-HPLC (10 $\times$ 250, 20% MeOH in H₂O for 40 min, 2 ml min⁻¹) to afford pyrenocine N (1, 12.5 mg; t_R 32.1 min) and pyrenocine O (2, 23.6 mg; t_R 30.6 min). UV data were recorded on a Shimadzu Biospec-1601 spectrometer (Shimadzu, Kyoto, Japan). IR data were recorded using a Nicolet 6700 FT-IR spectrometer (Thermo-Fisher, Madison, WI, USA) with KBr disks. Optical rotations were measured by JASCO P-2000 spectropolarimeter (Jasco, Tokyo, Japan). ¹H and ¹³C NMR data were acquired with a Bruker Avance-500 (Bruker, Fällanden, Zurich, Switzerland) and Bruker Avance-400 spectrometer using solvent signals (CDCl₃; δ_H 7.26/ δ_C 77.6, CD₃OD; δ_H 3.31/ δ_C 49.0) as references. HRESIMS data were acquired using a Waters QTOFMS Premier spectrometer (Waters, Milford, MA, USA).

Pyrenocine N (1) was obtained as yellow oil. The molecular formula C₁₀H₁₂O₄ was determined by positive HR-ESI-MS at m/z 197.0808 [M + H]⁺ requiring five degrees of unsaturations. The IR

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Received 29 December 2013; revised 18 March 2014; accepted 14 April 2014; published online 28 May 2014

spectrum suggested the presence of hydroxyl group (3405 cm^{-1}) and carbonyl group (1700 cm^{-1}). The ^1H , ^{13}C and HSQC spectra of **1** revealed the presence of two methyl groups, one methylene unit, two oxygenated methines, four olefinic carbons and one carboxyl carbon. These data (Table 1 and Supplementary Fig. S1–S5) accounted for all ^1H and ^{13}C resonances except one exchangeable proton and suggested **1** contained two rings. The presence of the C-4 oxygenated pyrone moiety was evident from δ_{H} 5.36 (s, H-5) in the ^1H NMR spectrum, and δ_{C} 176.0 (C-4), 168.5 (C-6), 157.3 (C-2), 113.4 (C-3) and 85.0 (C-5) in the ^{13}C NMR spectrum.¹³ Analysis of the ^1H , ^1H -COSY NMR data led to the identification of one isolated ^1H spin-system corresponding to the C-7 to C-10 subunit of structure **1**. HMBCs (Figure 1) of H₂-7 to C-2, C-3 and C-4 and of H-8 with C-4 permitted completion of the dihydrofuran subunit. Correlations from H₃-11 to C-2 and C-3 indicated the connection of C-11 to C-2. The remaining hydroxyl could be located at C-9 corresponding the ^{13}C -NMR chemical shift 68.6. On the basis of these data, the gross structure of **1** was characterized (Figure 2).

Pyrenocine O (**2**) was obtained as yellow oil, which assigned the same molecular formula $\text{C}_{10}\text{H}_{12}\text{O}_4$ as pyrenocine N (**1**) by positive HR-ESI-MS at m/z 197.0812 $[\text{M} + \text{H}]^+$. Analysis of the ^1H and ^{13}C NMR data (Table 1 and Supplementary Fig. S9–S13) for **2** revealed the presence of nearly identical structural features to those found in **1**, except that the chemical shifts for the two oxymethines C-8 ($\delta_{\text{H}}/\delta_{\text{C}}$ 4.78/92.4 in **1**; 4.75/92.3 in **2**) and C-9 ($\delta_{\text{H}}/\delta_{\text{C}}$ 4.12/68.6 in **1**; 3.86–3.90/69.6 in **2**), as well as the ^1H – ^1H coupling constant

observed between H-8 and H-9 were different. These data implied that **2** was a stereoisomer of **1**, and this conclusion was supported by analysis of its COSY and HMBC data.

The relative configuration of compounds **1** and **2** was assigned by NOED data (Supplementary Fig. S6). Upon irradiation of H₃-10, enhancement was observed for H-8 in the NOE difference spectrum of **1**, suggesting a *cis* relationship between H₃-10 and H-8, whereas enhancement was not observed for H-8 in the spectrum of **2**, indicating a *trans* relationship between H₃-10 and H-8.

The absolute configuration of pyrenocine N (**1**) was assigned by application of the modified Mosher method.^{14,15} Treatment of **1** with (S)-MTPA Cl and (R)-MTPA Cl afforded the R-MTPA ester (**1a**) and S-MTPA ester (**1b**), respectively. The difference in chemical shift values ($\Delta\delta = \delta_{\text{S}} - \delta_{\text{R}}$) for the diastereomeric esters **1b** and **1a** were calculated in order to assign the absolute configuration at C-9 (Figure 3 and Supplementary Fig. S7, S8).

Negative $\Delta\delta$ values were observed for H-10 (-0.08) and H-8 (-0.03), while positive $\Delta\delta$ values were observed for H α -7 ($+0.08$), H β -7 ($+0.07$), H-5 ($+0.02$) and H-11 ($+0.01$), indicating the *R* absolute configuration at C-9 (Figure 2). Accordingly the configurations at C-8 and C-9 were assigned as 8*S* and 9*R* for **1**. Therefore, the absolute configuration of **2** was deduced as 8*S* and 9*S*.

Pyrenocine N (1): Yellow oils. $[\alpha]_{\text{D}}^{22} = +28.2$ (c 0.7, CH_3OH). UV (CH_3OH): 238 (log ϵ 2.43), 285 (log ϵ 3.66). IR (neat) ν_{max} 3405 (br), 2975, 2925, 1700, 1656, 1602, 1440, 1272, 1222 cm^{-1} . ^1H - and ^{13}C -NMR: see Table 1. HRESI-MS (positive) m/z : 197.0808 $[\text{M} + \text{H}]^+$, $\text{C}_{10}\text{H}_{13}\text{O}_4^+$; calcd 197.0814).

Pyrenocine O (2): Yellow oils. $[\alpha]_{\text{D}}^{22} = +63.0$ (c 0.20, CH_3OH). UV (CH_3OH): 238 (log ϵ 2.17), 285 (log ϵ 3.40). IR (neat) ν_{max} 3423

Table 1 ^1H (500MHz, CDCl_3) and ^{13}C NMR (100MHz, CD_3OD) spectra data of **1** and **2** (δ in p.p.m., J in Hz)

Position	1		2	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
2		157.3		157.2
3		113.4		113.4
4		176.0		176.1
5	5.36 (s, 1H)	85.0	5.44 (s, 1H)	85.2
6		168.5		168.6
7 α	2.99 (dd, 15.1, 6.0, 1H)	25.0	3.00 (dd, 15.2, 8.8, 1H)	27.0
7 β	2.87 (dd, 15.3, 8.9, 1H)		2.84 (dd, 15.3, 6.6, 1H)	
8	4.78 (ddd, 9.0, 6.5, 3.5, 1H)	92.4	4.75 (ddd, 8.7, 6.7, 5.3, 1H)	92.3
9	4.12 (qd, 6.5, 3.9, 1H)	68.6	3.86–3.90 (m, 1H)	69.6
10	1.20 (d, 6.5, 3H)	18.5	1.32 (d, 6.5, 3H)	18.9
11	2.16 (s, 3H)	17.6	2.19 (s, 3H)	17.6

Figure 2 Structures of compounds of **1**–**7**.

Figure 1 ^1H – ^1H COSY and key HMBCs of **1**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Figure 3 $\Delta\delta_{\text{S-R}}$ values of MTPA esters of **1**.

Table 2 Cytotoxic activities of compounds 1–7

Compound	Cytotoxicity, CC ₅₀ (μg ml ⁻¹)		
	A549	MDA-MB-231	PANC-1
1	> 500	> 500	> 500
2	> 500	> 500	> 500
3	> 500	> 500	> 500
4	1.2	3.9	3.2
5	7.2	25.8	14.1
6	15.6	26.3	19.1
7	175.6	373.2	150.0
5-Fluorouracil	75	47	65

(br), 2977, 2929, 1706, 1602, 1434, 1216 cm⁻¹. ¹H- and ¹³C-NMR: see Table 1. HRESI-MS (positive) *m/z*: 197.0812([M + H]⁺, C₁₀H₁₃O₄⁺; calcd 197.0814).

Compounds 1–7 were evaluated for cytotoxic activities against three human cancer cell lines: A549, MDA-MB-231 and PANC-1 *in vitro* using the MTT method (Table 2). Compound 4 showed most potent antitumor activities with CC₅₀ values less than 4.0 μg ml⁻¹ against cancer cell lines, which was stronger than that of 5-fluorouracil (the positive control). Compounds 5–6 displayed significant cytotoxicity, whereas compounds 1–3 have no cytotoxic activities. Based on the analysis of cytotoxicity potencies and the structural characteristics of 4–6 and 1–3 or 7, it was found that 3-ketone group in branched chain (4–6) would significantly increase the cytotoxic activities. Moreover, α-, β-unsaturated ketone in branched chain of compound 4 could further strengthen cytotoxicity than that of 5 or 6.

ACKNOWLEDGEMENTS

This work was supported by the National S&T Major Special Project on Major New Drug Innovation (2012ZX09301002-003(-007)).

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)