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NOTE

Two new antitumor constituents from a soil fungus *Curvularia inaequalis* (strain HS-FG-257)

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Fungi have been recognized as a prolific source of secondary metabolites and 38% biologically active compounds originated from microbes are produced by fungi, which have yielded some of the most important products of the pharmaceutical industry, such as penicillins, cephalosporins, mevastatin and lovastatin.[1,2] Nevertheless, fungi are still one of the most promising microbiotic sources for new lead compounds because only a small part of the mycota is known.[3] To find more pharmaceutically active metabolites from fungi, we carried out a chemical investigation on dematiaceous hyphomycetes. During our research, we found that the crude extract from *Curvularia inaequalis* strain HS-FG-257 exhibited cytotoxicity against certain tumor cell lines. The further work led to the discovery of two new substances, curvularone A (**1**) and 4-hydroxyradianthin (**2**). In this paper, we report the fermentation, isolation, chemical characterization and the bioactivities of the two new compounds.

The producing strain HS-FG-257 was isolated from a soil sample collected from woodland of Heilongjiang province, China. It was provided and identified as *Curvularia inaequalis* Shear by the Professor Tianyu Zhang at the Shandong Agricultural University, China. The strain HS-FG-257 has been deposited in the Pharmaceutical Research Culture Collection, Zhejiang Hisun Group Co., Ltd. with accession No: HS-FG-257.

The strain was grown and maintained on potato dextrose agar slant and incubated for 6–7 days at 24 °C. The stock culture was transferred into 1-l erlenmeyer flasks containing 250 ml of the seed medium and incubated at 24 °C for 24 h, shaken at 150 r.p.m. Then, 1 l of the culture was transferred into a 50-l fermentor containing 30-l of the producing medium consisting of peptone 0.5%, potato starch 0.5%, yeast extract 0.2%, NaCl 0.4%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.2% (pH 6.2–6.4). The fermentation was carried out at 24 °C for 7 days stirred at 100 r.p.m. min^{-1} with an aeration rate of 900 l of air per hour.

The final 30 l of broth from 50-l fermentor was filtered and the resulting cake was washed with water (3 l) and subsequently extracted with MeOH (3 l). The supernate and the wash water were subjected to a Diaion HP-20 resin column eluting with 95% EtOH (5 l). The MeOH extract and the EtOH eluents were evaporated under reduced pressure to ~1 l at 50 °C and the resulting concentrate was extracted three times using an equal volume of EtOAc. The combined EtOAc phase was concentrated under reduced pressure to yield a mixture (15 g). The mixture was subjected to a Sephadex LH-20 gel (GE Healthcare, Glies, UK) column eluted with $CHCl_3$/MeOH (1:1, v/v) and detected by TLC to give five fractions (Fr.1 to Fr.5). The Fr.4 was chromatographed on a silica gel (Qingdao Haiyang Chemical Group, Qingdao, Shandong, China; 100–200 mesh) column and successively eluted with a stepwise gradient of $CHCl_3$/MeOH (100:0–50:50, v/v) to obtained three fractions Fr.4-1 to Fr.4-3 based on the TLC profiles. The Fr.4-2 was subjected to another silica gel column eluted with $CHCl_3$/MeOH (90:10–70:30, v/v) to give three fractions (Fr.4-2-1, Fr.4-2-2 and Fr.4-2-3). Fr.4-2-3 was further isolated by semi-preparative HPLC (Agilent 1100, Zorbax SB-C18, 5 μm, 250 × 9.4 mm i.d.; 1.5 ml · min^{-1}; 220 nm; Agilent, Palo Alto, CA, USA) eluting with CH_3CN/H_2O (25:75, v/v) to obtain compound **1** (t_R 8.6 min, 11 mg). Fr.4-1 was purified by semi-preparative HPLC eluting with CH_3CN/H_2O (40:60, v/v) to yield compound **2** (t_R 14.0 min, 21 mg). ^1H and ^{13}C NMR spectra were measured with a Bruker DRX-400 (400 MHz for ^1H and 100 MHz for ^{13}C) spectrometer (Rheinstetten, Germany). The ESIMS and HRESIMS spectra were taken on a Q-TOF Micro LC-MS-MS mass spectrometer (Milford, MA, USA).

Compound **1** was isolated as colorless oil. Its molecular formula $C_{11}H_{12}O_4$ was assigned on the basis of HRESIMS (*m/z* 231.0634 $[M+Na]^+$), indicating 6 degrees of unsaturation. The IR spectrum showed the absorptions of hydroxyl (3366 cm^{-1}) and carbonyl

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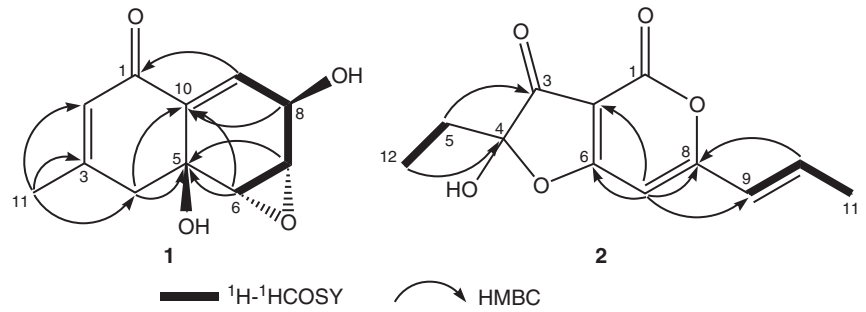

Figure 1 Structures and key ^{1}H–^{1}H COSY and HMBC correlations of **1**, **2**.

Table 1 ^{1}H and ^{13}C NMR data for compounds 1, 2

	δ_H (*J* in Hz)			δ_C	
Position	***1*** *(in CDCl$_3$)*	***1*** *(in DMSO-d$_6$)*	***2*** *(in CDCl$_3$)*	***1*** *(in CDCl$_3$)*	***2*** *(in CDCl$_3$)*
1				186.6 (s)	155.2 (s)
2	5.95 (br s)	5.94 (br s)		125.8 (d)	98.1 (s)
3				158.5 (s)	192.6 (s)
4	2.75 (d, 18.1)	2.65 (d, 18.2)		41.3 (t)	111.8 (s)
	2.53 (d, 18.1, 1.1)	2.46 (d, 18.2)			
5			1.84 (q, 7.4)	68.7 (s)	28.6 (t)
6	3.23 (d, 3.3)	3.19 (d, 3.6)		56.7 (d)	185.3 (s)
7	3.36 (br s)	3.33 (br s)	6.65 (s)	54.3 (d)	95.6 (d)
8	4.54 (d, 4.8)	4.42 (dd, 8.8, 4.9)		62.5 (d)	168.5 (s)
9	6.48 (d, 4.8, 1.7)	6.34 (dd, 4.9, 1.2)	6.39 (br d, 15.6)	130.0 (d)	123.8 (d)
10			6.89 (m)	134.2 (s)	141.4 (d)
11	2.01 (br s)	1.96 (br s)	1.96 (dd, 7.0, 1.4)	24.3 (q)	19.1 (q)
12			0.85 (t, 7.4)		7.0 (q)
5-OH		5.25 (s)			
8-OH		5.19 (d, 8.8)			
4-OH			8.35 (s)		

(1674 cm^{-1}) groups. The ^{1}H NMR spectrum showed signals corresponding to protons of one methyl at δ 2.01 (3H, br s), a methylene at δ 2.53 (1H, d, J = 18.1 Hz), 2.75 (1H, dd, J = 18.1, 1.1 Hz), an oxymethine at δ 4.54 (1H, d, J = 4.8 Hz), two olefinic methines at δ 5.95 (1H, br s), 6.48 (1H, dd, J = 4.8, 1.7 Hz), as well as two proton signals at δ 3.23 (1H, d, J = 3.3 Hz), 3.36 (1H, br s). The ^{13}C and DEPT NMR spectra of **1** showed 11 carbon signals comprising one conjugated carbonyl at δ 186.6 (s), two sp^2 quaternary carbons at δ 158.5 (s), 134.2 (s), two sp^2 methines at δ 130.0 (d), 125.8 (d), one oxygenated quaternary carbon at δ 68.7 (s), one oxymethine at δ 62.5 (d), a methylene at δ 18.1 (t), a methyl at δ 24.3 (q) in addition to two methine resonances at δ 56.7 (d), 54.3 (d). These data confirmed the molecular formula and also indicated the three-cyclic nature of the molecule. The structural unit of C-6 −C-9 was determined from the ^{1}H–^{1}H COSY spectrum (Figure 1). The HMBC correlations (Figure 1) from H_3-11 to C-2, C-3 and C-4 indicated the linkage of these four carbon signals as shown in Figure 1. The observed HMBC correlated signals from H-4 and H-6 to C-5, C-10, from H-7 to C-5, and from H-8 to C-10 established the connection of C-4, C-6 and C-10 through C-5. The left carbonyl group (C-1) and the HMBC correlation from H-9 to C-1 established the connection of C-2 and C-10 via C-1. The chemical shifts of C-6 and C-7 (Table 1) and the remained one degree of unsaturation revealed the existence of an epoxide unit. Additionally, considered the molecular formula of **1**, two hydroxy groups were attached at C-5 and C-8, respectively. Consequently, the planner structure of **1** was elucidated as shown in Figure 1. The NOESY correlations (in DMSO-d_6, Figure 2) of H-6, H-7 and 8-OH, 5-OH and H-6 established the relative stereochemistry of **1**.

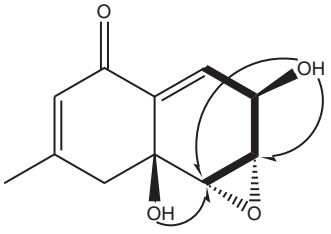

Figure 2 The main NOESY correlations observed in **1**.

Compound **2** was obtained as white powder. Its molecular formula was determined to be $C_{12}H_{12}O_5$ by the HRESIMS ion at *m/z* 259.0595 $[M+Na]^+$ (calculated for $C_{12}H_{12}NaO_5$, 259.0577), showing 7 degrees of unsaturation. The UV spectrum of **2** showed absorption maxima at 335 nm (logε 4.06), 281 nm (logε 3.73), 272 nm (logε 3.72), 218 nm (logε 4.09), suggesting the presence of conjugated system. The IR spectrum indicated the presence of hydroxy

(3419 cm^{-1}), conjugated carbonyl (1684, 1759 cm^{-1}) groups. The ^{1}H NMR spectrum (Table 1) of **2** revealed a methyl triplet at δ 0.85 (3H, t, $J=7.4$ Hz), an olefinic methyl at δ 1.96 (3H, dd, $J=7.0$, 1.4 Hz), a methylene proton signal at δ 1.84 (2H, q, $J=7.4$ Hz), a *trans* double bond at δ 6.39 (1H, br d, $J=15.6$ Hz), 6.89 (1H, m) in addition to two singlet proton signals at δ 8.35 (1H, s) 6.65 (1H, s). The ^{13}C NMR and DEPT spectra (Table 1) exhibited 12 carbon resonances, which corresponded to two methyls (δ 7.0, 19.1), a methylene (δ 28.6), three downfield methines (δ 95.6, 123.8, 141.4), one conjugated carbonyl (δ 192.6) and five downfield quaternary carbon (δ 185.3, 168.5, 155.2, 111.8, 98.1). In the ^{1}H–^{1}H COSY spectrum (Figure 1) of **2**, the correlations of H_3-12 with H-5, H-10 with H-9 and H-11 showed the presence of an ethyl group and the moiety of C-9 −C-11 as shown in Figure 1. Comparison of the UV spectrum and the NMR data of **2** with those of radianthin[4] suggested that **2** was a derivative of radianthin and the only difference between **2** and radianthin was the C-4 oxymethine group in radianthin was replaced by a quaternary carbon bearing one oxygen and one hydroxyl in **2**. Thus, the structure of **2** was established as shown in Figure 1. The observed HMBC correlations from H_3-12 to C-4, from H-7 to C-2, C-6, C-8 and C-9, from H-5 to C-3 further confirmed the structure assignment of **2**, and the stereochemistry of C-4 remained unknown.

The cytotoxicities of compounds **1** and **2** were assayed *in vitro* against the ACHN and HepG2 cell lines by the CCK8 method as described in our previous papers.[5,6] Compound **1** exhibited cytotoxic activity with IC_{50} values of 4.78 and 13.11 μg ml^{-1}, respectively. The values of **2** were 54.18 and 52.07 μg ml^{-1}.

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