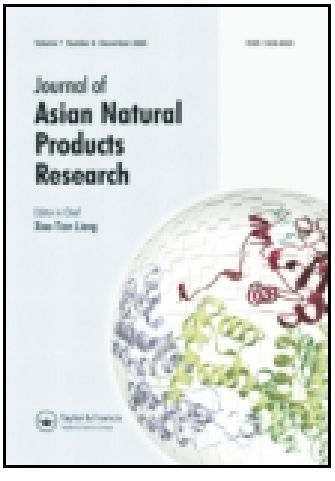

Journal of Asian Natural Products Research

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ganp20

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Published online: 10 Apr 2014.

To cite this article: Zi-Ming Chen, Wen-Bin Dong, Zheng-Hui Li, Tao Feng & Ji-Kai Liu (2014) New chlamydosporol derivatives from the endophytic fungus Fusarium sp. #001, Journal of Asian Natural Products Research, 16:5, 465-470, DOI: 10.1080/10286020.2014.904853

To link to this article: http://dx.doi.org/10.1080/10286020.2014.904853

Journal of Asian Natural Products Research, 2014
Vol. 16, No. 5, 465–470, http://dx.doi.org/10.1080/10286020.2014.904853

New chlamydosporol derivatives from the endophytic fungus *Fusarium* sp. #001

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(*Received 12 December 2013; final version received 12 March 2014*)

Two new chlamydosporol derivatives, fusarilactone A (**1**) and fusarilactone B (**2**), together with nine known compounds (**3**–**11**), have been isolated from the crude extract of endophytic fungus *Fusarium* sp. #001. The structures of new compounds **1** and **2** were elucidated on the basis of extensive spectroscopic methods. Compound **1** showed mild cytotoxicities against three tumor cell lines (SMMC-7721, A-549, and MCF-7).

Keywords: endophytic fungi; chlamydosporol derivatives; *Fusarium* sp; *Eupatorium adenophorum*; cytotoxicity

1. Introduction

Endophytic fungi are eukaryotic organisms that live in apparently healthy plants and cause no apparent symptoms of disease for the host plant [1]. They have proven to be a rich source of novel organic compounds with interesting biological activities and a high level of chemical diversity [2,3]. *Fusarium* is a large genus of filamentous fungi with a wide distribution in soil and in association with plants. These fungi produce various secondary metabolites such as enniatins [4], trichothecenes [5], alkaloids [6,7], and lactones [8]. In our efforts to discover bioactive natural products of endophytic fungi, about 200 strains of endophytic fungi were isolated from *Eupatorium adenophorum*. Among these strains, *Fusarium* sp. #001, attracted our attention because a crude extract of this fungal culture was lethal to brine shrimp (*Artemia salina*). This fungal strain was fermented on rice solid medium on a large scale. The bioactive metabolites were isolated by bioassay-guided fractionation, resulting in the purification of two new compounds fusarilactones A and B (**1** and **2**), together with nine known compounds, *O*-methylisochlamydosporol (**3**) [9], oxysporidinone (**4**) [10], enniatin A (**5**) [11], enniatin B (**6**) [12], enniatin A_1 (**7**) [11], enniatin B_1 (**8**) [11], enniatin D (**9**) [4], and a mixture of enniatin E_1 (**10**) and enniatin E_2 (**11**) [4] (Figure 1). The new compounds were evaluated for their cytotoxicities against five human cancer cell lines. In this paper, the isolation, structure elucidation, and cytotoxic activities of the new compounds are described.

2. Results and discussion

Compound **1** was obtained as an amorphous solid and its molecular formula was determined to be $C_{12}H_{16}O_5$ by HR-EI-MS at *m/z* 240.0998 $[M]^+$, suggesting five degrees of unsaturation. The ^1H NMR spectrum showed signals for an olefinic proton at δ_H 5.43 (s, H-3), two oxymethylene protons at δ_H 4.22 (dd, $J = 14.8$,

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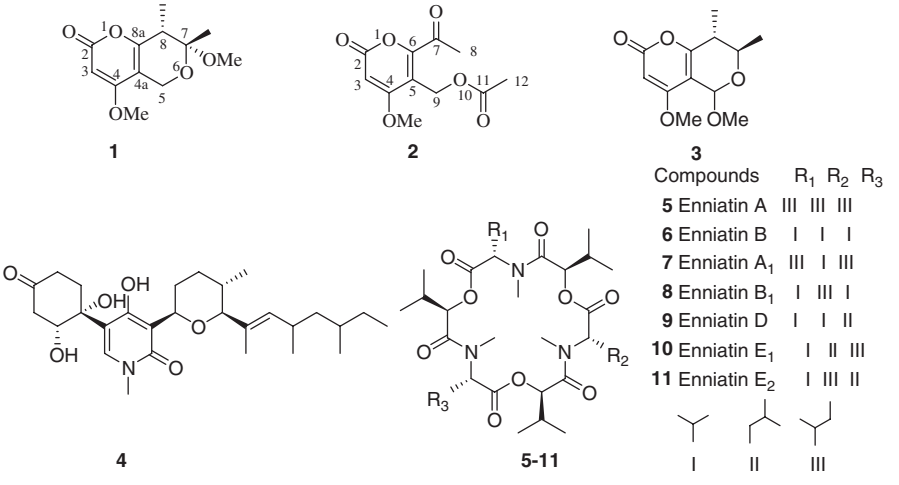

Figure 1. Structures of compounds **1**–**11**.

3.1 Hz, H-5α) and 4.41 (d, $J = 14.8$ Hz, H-5β), two methyls at δ_H 1.26 (d, $J = 7.0$ Hz, 8-Me) and 1.45 (s, 7-Me), two methoxy moieties at δ_H 3.31 (s, 7-OMe) and 3.80 (s, 4-OMe), a methine proton at δ_H 2.77 (q, $J = 7.0$ Hz, H-8) (Table 1). The ^{13}C NMR spectrum of **1** showed 12 carbon resonances ascribable for 2 methyls, 2 methoxyls, 1 oxymethylene, 2 methines, and 5 quaternary carbons (Table 1), as supported by DEPT and HSQC experiments. The NMR data of **1** had the general structural features of chlamydosporol [13], except that a hydroxy proton [δ_H 6.24 (1H, s, 7-OH)] in chlamydosporol was replaced with a methoxy group [δ_H 3.31 (1H, s, 7-OMe), δ_C 49.2 (CH_3, 7-OMe)] in **1**. In the HMBC spectrum (Figure 2), the correlation from 7-OMe at δ_H 3.31 (1H, s) to C-7 at δ_C 100.0 (s) further confirmed this assignment. The relative configuration of **1** was

Table 1. ^{1}H and ^{13}C NMR spectral data for compounds **1** and **2** in $CDCl_3$.

	1			**2**	
Position	δ_C[a], mult.	δ_H[a] (J in Hz)	Position	δ_C[b], mult.	δ_H[b] (J in Hz)
2	164.7, s		2	161.1, s	
3	87.8, d	5.43, s	3	93.1, d	5.74, s
4	168.5, s		4	169.1, s	
4a	105.9, s		5	112.4, s	
5α	56.2, t	4.22, dd (14.8, 3.1)	6	153.8, s	
5β		4.41, d (14.8)			
7	100.0, s		7	193.6, s	
8	41.2, d	2.77, q (7.0)	8	28.3, q	2.56, s
8a	157.6, s		9	54.8, t	5.20, s
4-OMe	56.5, q	3.80, s	11	170.7, s	
7-Me	21.1, q	1.45, s	12	21.0, q	2.05, s
7-OMe	49.2, q	3.31, s	4-OMe	57.1, q	3.90, s
8-Me	11.2, q	1.26, d (7.0)			

[a] ^{1}H at 500 MHz and ^{13}C at 100 MHz.
[b] ^{1}H at 600 MHz and ^{13}C at 125 MHz.

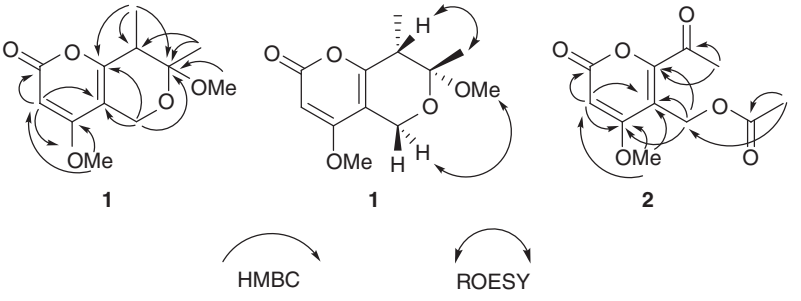

Figure 2. Key 2D correlations of compounds **1** and **2**.

determined by the ROESY correlation of 7-Me/H-8, which implied that 7-Me and H-8 should be in the same side (β-orientation). Therefore, **1** was identified to be 7-*O*-methyl chlamydosporol, and named fusarilactone A.

Compound **2** was isolated as an amorphous solid. Its molecular formula was established as $C_{11}H_{12}O_6$ by HR-EI-MS which displayed a quasimolecular peak at *m/z* 240.0643 $[M]^+$. The ^1H NMR spectrum of **2** was characterized by signals corresponding to two methyls at δ_H 2.05 (s, H-12) and δ_H 2.56 (s, H-8), a methoxy moiety at δ_H 3.90 (s, 4-OMe), an oxygen-bearing methylene singlet at δ_H 5.20 (s, H-9), and an olefinic proton at δ_H 5.74 (s, H-3) (Table 1). The ^{13}C NMR and DEPT spectra showed 11 carbon resonances including 1 methoxyl, 2 methyls, 1 methylene, 1 methine, and 6 quaternary carbons (including 3 carbonyls and 3 olefinic carbons) (Table 1). The long-range $^1H-^{13}C$ correlations observed in the HMBC spectrum established a 4,5,6-trisubstituted α-pyrone (Figure 2). The presence of this structural element was also supported by the observed UV absorption maximum at 304 nm and strong IR absorption bands at 1725 and 1562 cm^{-1}. The connectivity of functional groups in **2** was elucidated on the basis of HMBC techniques. The key HMBC correlations from H-8 (δ_H 2.56, s) to C-7 (δ_C 193.6) and C-6 (δ_C 153.8) showed that the acetyl moiety was attached at C-6; from H-9 (δ_H 5.20, s) to C-5 (δ_C 112.4), C-4 (δ_C 169.1), and C-6 (δ_C 153.8), and H-12 (δ_H 2.05, s) to C-11 (δ_C 170.7) and C-9 (δ_C 54.8), which revealed the presence of a $-CH_2OOCCH_3$ moiety and its attachment to C-5 of α-pyrone ring. Thus, the structure of **2** was established as fusarilactone B, as shown in Figure 1.

Compounds **1** and **2** were evaluated for their cytotoxicities against five human cancer cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as reported previously [14]. Compound **1** showed cytotoxic activities against SMMC-7721, A-549, and MCF-7 cell lines, with the IC_{50} values varying between 17.5 and 35.1 μmol, as listed in Table 2.

Table 2. Cytotoxicities of compounds **1** and **2** against five cancer cell lines (IC_{50}, μmol).

Compounds	HL-60	SMMC-7721	A-549	MCF-7	SW480
1	>40	35.1	17.5	17.7	>40
2	>40	>40	>40	>40	>40
Cisplatin[a]	1.1	4.3	5.1	15.4	15.4

[a] Positive control.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a Horiba SEPA-300 polarimeter (Horiba, Kyoto, Japan). UV spectra were obtained by using a Shimadzu UV-2401A spectrometer (Shimadzu, Kyoto, Japan). IR spectra were obtained on a Bruker Tensor 27 FTIR spectrometer (Bruker, Karlsruher, Germany) with KBr pellets. NMR spectra were obtained on Bruker AV-400 and DRX-500 instruments, and a Bruker Avance III 600 spectrometer with tetramethylsilane as an internal standard at room temperature (Bruker, Karlsruher, Germany). Mass spectra (HR-EI-MS) were recorded on a Waters Autospec Premier p776 spectrometer (Waters, Milford, MA, USA). Preparative high pressure liquid chromatography (HPLC) was performed on an Agilent 1100 series with a Zorbax SB-C18 (5 mm, 9.4 mm × 150 mm) column (Agilent Technologies, Santa Clara, CA, USA). Column chromatography (CC) was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Ltd, Qingdao, China) and Sephadex LH-20 (Amersham Biosciences, Upssala, Sweden). Fractions were monitored by TLC (GF_{254}, Qingdao Haiyang Chemical Co., Ltd, Qingdao, China), and spots were visualized by heating silica gel plates immersed in vanillin–H_2SO_4 in EtOH.

3.2 Fungal material

The fungus *Fusarium* sp. #001 was isolated from the leaves of *E. adenophorum* collected in Kunming Institute of Botany, Kunming, Yunnan province, China in July 2012. The fungus was identified by observing the morphological characteristics and analysis of the internal transcribed spacer regions. The result from the BLAST search indicated that the sequence was the same (99%) as that for the sequence of *Fusarium* sp. (compared to HQ166539.1). A voucher specimen has been deposited in the herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (No. F2012016).

The strain *Fusarium* sp. #001 was cultured on PDA medium at 25°C for a week. The agar plugs containing strain #001 were inoculated into Erlenmeyer flasks containing 50 ml potato dextrose liquid broth. Flask cultures were incubated at 25°C on a rotary shaker at 160 rpm for 2 days as seed culture. Then, 10 ml of the seed culture was inoculated to autoclaved solid medium (100 g rice, 100 ml H_2O). Incubation was carried out in twenty 500 ml Fernbach flasks each containing solid medium at 25°C for 21 days.

3.3 Extraction and isolation

The fermented rice substrate was extracted with EtOAc (3 × 4 l) to afford a crude residue (20.0 g). This extract was subjected to CC on silica gel eluted with a gradient of $CHCl_3$–MeOH (v/v, 100:0 to 0:100) to afford fractions 1–5. Fraction 2 (3.55 g, lethal rate to brine shrimp, 100% at 100 μg/ml and 83% at 10 μg/ml) was first separated by silica gel CC eluted with $CHCl_3$–MeOH (95:5) and further subjected to Sephadex LH-20 eluting with MeOH to yield three subfractions 2A–2C. Fraction 2A (200 mg) was further separated by preparative HPLC (CH_3CN–H_2O; 65:35) to yield **6** (5.0 mg, t_R 20.6 min). Fraction 2B (170 mg) was purified by preparative HPLC (CH_3CN–H_2O; 7:3) to give **8** (12.2 mg, t_R 22.3 min) and **9** (8.0 mg, t_R 26.5 min). Fraction 2C (350 mg) was further separated by preparative HPLC (CH_3CN–H_2O; 3:1) to afford **5** (70.0 mg, t_R 28.9 min), **7** (15.0 mg, t_R 25.6 min), and a mixture of **10** and **11** (10.0 mg, t_R 23.1 min). Fraction 3 (1.6 g; lethal rate to brine shrimp, 93% at 100 μg/ml, and 53% at 10 μg/ml) was separated by silica gel eluted with $CHCl_3$–MeOH (9:1), then was further purified by silica gel CC eluted with a gradient of $CHCl_3$–MeOH (v/v, 98:2 to 94:6) to give five subfractions

3A–3E. Fraction 3C (180 mg) was further separated by preparative HPLC (CH_3CN–H_2O, 15:85 → 35:65, 20 min) to yield **1** (2.3 mg, t_R 17.5 min), **2** (5.7 mg, t_R 9.9 min), and **3** (7.2 mg, t_R 13.5 min). Fraction 3D (70 mg) was then subjected to Sephadex LH-20 (MeOH) and silica gel CC (petroleum ether–Me_2CO, 3:7) to afford **4** (5.2 mg).

3.3.1 *Fusarilactone A (**1**)*

An amorphous solid; $[\alpha]_D^{25}$ − 25.05 ($c = 0.30$, MeOH); UV (MeOH) λ_{max} (log ε): 204 (4.27), 282 (3.72) nm; IR (KBr) ν_{max} 3429, 2934, 1726, 1653, 1569, 1460 cm^{-1}; for ^1H (500 MHz) and ^{13}C NMR (100 MHz) spectral data ($CDCl_3$), see Table 1; HR-EI-MS: *m/z* 240.0998 $[M]^+$ (calcd for $C_{12}H_{16}O_5$, 240.0998).

3.3.2 *Fusarilactone B (**2**)*

An amorphous solid; $[\alpha]_D^{25}$ − 3.42 ($c = 0.23$, MeOH); UV (MeOH) λ_{max} (log ε): 197 (3.70), 224 (4.04), 304 (3.38) nm; IR (KBr) ν_{max} 3430, 3074, 1725, 1704, 1635, 1562, 1406 cm^{-1}; for ^1H NMR (600 MHz) and ^{13}C NMR (125 MHz) spectral data ($CDCl_3$), see Table 1; HR-EI-MS: *m/z* 240.0643 $[M]^+$ (calcd for $C_{11}H_{12}O_6$, 240.0634).

3.4 *Cytotoxicity assay*

Five human cancer cell lines: breast cancer SK-BR-3, hepatocellular carcinoma SMMC-7721, human myeloid leukemia HL-60, pancreatic cancer PANC-1, and lung cancer A-549 cells were used in the cytotoxic assay. Cells were cultured in Roswell Park Memorial Institute-1640 medium or in Dulbecco's modified Eagle medium (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (Hyclone) in 5% CO_2 at 37°C. The cytotoxicity assay was performed according to the MTT method in 96-well microplates. Briefly, 100 μl of adherent cells was seeded into each well of 96-well cell culture plates and allowed to adhere for 12 h before addition of test compounds, while suspended cells were seeded just before drug addition with initial density of 1×10^5 cells/ml. Each tumor cell line was exposed to the test compound at concentrations of 0.0625, 0.32, 1.6, and 8 μM in triplicates for 48 h, with cisplatin (Sigma-Aldrich, St Louis, MO, USA) as positive control (for IC_{50} values, see Table 2). After compound treatment, cell viability was detected and a cell growth curve was graphed. IC_{50} values were calculated by Reed and Muench's method [15].

Acknowledgments

This project was financially supported by National Natural Science Foundation of China (U1132607) and Youth Innovation Promotion Association of CAS (2011312D11019).

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