

ORIGINAL ARTICLE

Cytochalasins from cultures of endophytic fungus *Phoma multirostrata* EA-12

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Two new cytochalasins, multirostratin A (1) and 20-oxo-deoxaphomin (2), together with five known analogues (3–7), were obtained from the endophytic fungus *Phoma multirostrata* EA-12. The structures of 1 and 2 were elucidated by MS and 1D- and 2D-NMR spectroscopic data analyses, as well as by comparison of data with those of analogues reported in the literature. Compounds 1 and 2 showed moderate cytotoxicity against five tumor cell lines (HL-60, A-549, SMMC-7721, MCF-7 and SW-480).

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INTRODUCTION

Endophytic fungi have been proved to be a productive source of structurally diverse and biologically active secondary metabolites.^{1–3} In our effort to search for structurally interesting and bioactive natural products, >200 strains of endophytic fungi isolated from *Eupatorium adenophorum* were preliminarily screened for their toxicity against brine shrimp (*Artemia salina*). In these investigated fungi, one strain, named EA-12, identified as *Phoma multirostrata* was found lethal to brine shrimp. Chemical investigation on cultures of *P. multirostrata* led to the isolation of two new cytochalasins, multirostratin A (1) and 20-oxo-deoxaphomin (2), together with five known analogues, deoxaphomin (3),⁴ cytochalasin A (4),⁵ cytochalasin B (5),⁵ cytochalasin Z₂ (6)⁶ and cytochalasin F (7) (Figure 1).⁷ The new structures were elucidated by extensive spectroscopic methods, while the known analogues were identified by comparison data with those reported in the literature. Compound 1 was the first cytochalasin with a macrocyclic ring including a furan moiety. The new compounds 1 and 2 showed significant cytotoxicities against five tumor cell lines of HL-60, A-549, SMMC-7721, MCF-7 and SW-480.

RESULTS AND DISCUSSION

Compound 1 was obtained as white, amorphous solid, which was assigned the molecular formula C₂₉H₃₅NO₃ from its HR-EI-MS (*m/z* 445.2622 [M]⁺, calcd for C₂₉H₃₅NO₃, 445.2617), indicating 13° of unsaturation. Its IR spectrum exhibited absorption bands for a hydroxy (3424 cm⁻¹), amide carbonyl (1700 cm⁻¹), olefinic bond (1641 cm⁻¹) and phenyl (1606, 1023, 701 cm⁻¹) groups. 1D NMR and HSQC spectra of 1 revealed 29 carbon signals, including 2 methyls, 6 methylenes (one exo-methylene), 15 methines (one oxymethine) and 6 quaternary carbons (including three carbonyls) (Table 1). The ¹H NMR spectrum showed two methyl signals at δ_H

0.92 (3H, d, *J* = 6.9 Hz, H-24) and 0.96 (3H, d, *J* = 6.7 Hz, H-11), two exocyclic CH₂= group at δ_H 5.10 (1H, s, H-12a) and 5.28 (1H, s, H-12b), a double bond group at δ_H 5.43 (1H, m, H-14) and 6.07 (1H, ddd, *J* = 15.4, 9.8, 1.5 Hz, H-13) and a monosubstituted phenyl group at δ_H 7.12 (2H, d, *J* = 7.0 Hz, H-2', 6'), 7.23 (1H, t, *J* = 7.0 Hz H-4') and 7.30 (2H, d, *J* = 7.0 Hz, H-3', 5'). In addition, the presence of a 2,5-disubstituted furan moiety in 1 was assumed by the NMR signals at δ_H 5.93 (1H, d, *J* = 3.0 Hz, H-21), 6.10 (1H, d, *J* = 3.0 Hz H-22) and at δ_C 106.2 (C-22), 107.2 (C-21), 153.2 (C-23) and 156.2 (C-20).

Analysis of the ¹H–¹H COSY spectrum gave two C-linkage fragments: C-10/C-3/C-4/C-5/11-Me and C-7/C-8/C-13/C-14/C-15/C-16/C-17 or 24-Me/C-18/C-19, as shown in bold in Figure 2. In the HMBC spectrum (Figure 2), the correlation of H-4 and H-8 to C-9, C-1 and C-23 indicated that C-1, C-4, C-8 and C-23 were all connected to C-9, and the correlation of H-12 to C-5, C-6 and C-7, together with H-3 to C-3, C-4 and C-5, established an isoindolone moiety in 1. Furthermore, the HMBC correlation of H-21 and H-22 to C-8, C-9, C-19 and C-20, indicating a furan moiety located between C-19 and C-23 (Figure 2). The monosubstituted phenyl group was connected to the isoindolone moiety via C-10 on the correlations of aromatic proton signal at δ_H 7.12 (H-2'), with the carbon signal at δ_C 44.2 (C-10). Thus the planar structure of 1 was established.

The relative configuration of 1 was established by a combination of coupling constant and ROESY data. In the ¹H NMR spectrum, the large coupling constant (*J* = 10.2 Hz) for H-7 and H-8 suggested the axial–axial orientation of the proton pair. The *E*-geometry of the Δ¹³-double bond was deduced from the large coupling constant observed for H-13 (*J* = 14.7 Hz). In the ROESY spectrum, the correlations of H-3/H₃-11, H-5/H-4 and H-5/H-8 were observed, which indicated that H-3, H₃-11 and H-7 were in the same orientation (α), whereas H-4, H-5 and H-8 were in the opposite

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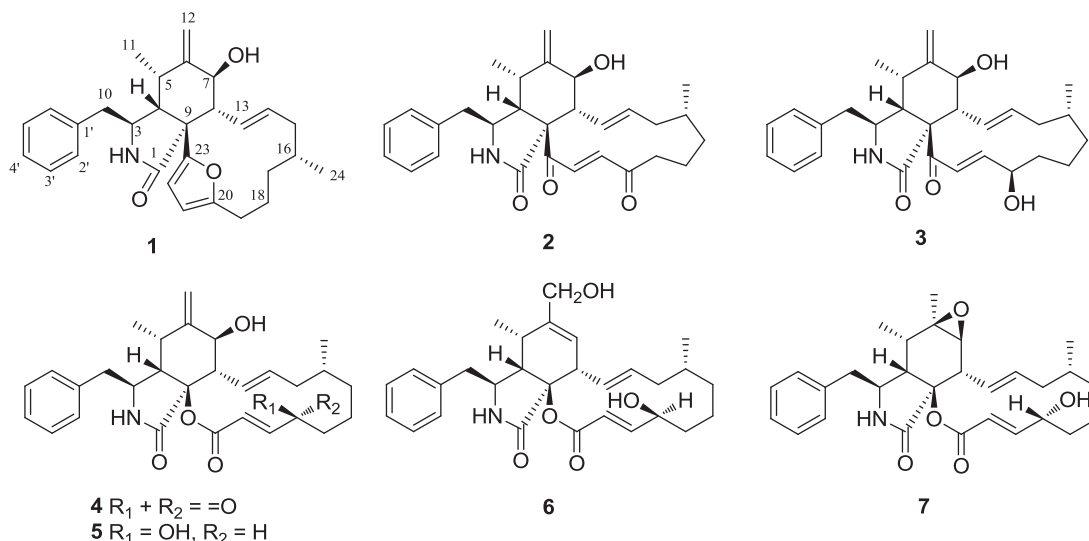


Figure 1 Structures of cytochalasins (1–7) from the endophytic fungus *Phoma multirostrata*.

direction (β) (Figure 2). The correlations of H-7/H-13, H-14/H-15b, H-15b/H-14, H-15b/H-16 and H-15a/H₃-24 indicated that H-13 and H₃-24 were α -oriented, while H-14 and H-16 were β -oriented. Consequently, the structure of **1** was established and given the name multirostratin A.

Compound **2** was obtained as white, amorphous solid. Its molecular formula was determined as C₂₉H₃₅NO₃ from the positive HREIMS molecular ion peak at m/z 461.2587 [M]⁺, indicating 13° of unsaturation. The NMR spectral data of **2** (Table 1) resembled to the known analogue deoxaphomin (**3**) except that the ¹³C signal at 74.0 ppm (C-20) in **3** was changed to 204.2 p.p.m. in **2**, suggesting that a keto carbon atom instead of an oxygen-bearing methine existed in **2**. In addition, the ¹H signal of H-21 was split into a doublet ($J = 16.2$ Hz) instead of a double doublet ($J = 15.3, 8.1$ Hz) in **3**. This was in accordance with the HMBC correlations from H-21 (δ_H 7.70 (1H, d, $J = 16.2$ Hz)) and H-22 (δ_H 6.85 (1H, d, $J = 16.2$ Hz)) to C-20 (δ_C 204.2) and C-19 (δ_C 41.5) (Figure 2). The other correlations in the HMBC and ¹H–¹H COSY spectra as shown in Figure 2 also confirmed the connectivities in compound **2**. The relative configuration of **2** was established by a combination of coupling constant and ROESY experiment. In the ¹H NMR spectrum, the large coupling constant ($J = 10.5$ Hz) for H-7 and H-8 suggested the axial–axial orientation of the proton pair. The *E*-geometry of the Δ^{13} and Δ^{21} -double bonds was deduced from the large coupling constant observed for $J_{H-13,H-14} = 15.2$ Hz and $J_{H-21,H-22} = 16.2$ Hz. The ROESY spectrum of **2** displayed similar patterns to those of **1**, which suggested that the relative configurations of the chiral centers in **2** were the same to those of **1** (Figure 2). Consequently, compound **2** was identified as 20-oxo-deoxaphomin.

Compounds **1** and **2** showed a certain cytotoxic activities against HL-60, A-549, SMMC-7721, MCF-7 and SW-480, the IC₅₀ values varied between 7.7 and 15.8 μ M, as listed in Table 2.

In summary, cytochalasins are a group of polyketide synthases-non ribosomal peptide synthetases (PKS-NRPS) hybrid fungal metabolites that have been isolated from different genera of fungi, such as *Phomopsis*,⁸ *Aspergillus*,⁹ *Spicaria*,¹⁰ *Xylaria*,¹¹ *Periconia*,¹² etc. They display a wide range of biological activities, including cytotoxic,^{11,12} antibiotic,¹³ inhibition of HIV-1 protease¹⁴ and phytotoxic activities;¹⁵ their most unusual property is the ability to cause cells

to extrude their nuclei, leading to the formation of nuclei free cells. In general, cytochalasins are structurally characterized by a highly substituted isoindolone fused with an 11- to 13-membered carbocyclic or 12- to 14-membered lactone rings, and most recently, an unprecedented 9-membered carbocyclic rings.¹² To date, >100 cytochalasins and derivatives have been isolated from a range of fungi. To the best of our knowledge, multirostratin A (**1**) was the first example with a macrocyclic ring including a furan moiety.

METHODS

General experimental procedures

Optical rotations were taken on a Horiba SEAP-300 polarimeter (Horiba, Kyoto, Japan). UV spectra were obtained on a Hitachi UV 210A spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were measured on a Bio-Rad FTS-135 spectrometer with KBr pellets (SPECTRO, Kleve, Germany). 1D and 2D NMR spectra were recorded on a Bruker AM-400 or DRX-500 NMR spectrometer (Bruker, Karlsruhe, Germany). HR-EI-MS were recorded on a Waters Auto Premier P776 spectrometer (Waters, Milford, MA, USA). Preparative HPLC was performed on an Agilent 1100 series with a Zorbax SB-C18 (5 μ m, 9.4 \times 150 mm) column (Agilent Technologies, Santa Clara, CA, USA). Preparative MPLC was performed on a Buchi apparatus equipped with a Buchi fraction collector C-660, a Buchi pump module C-605 and a manager C-615 (Büchi Labortechnik AG, Flawil, Switzerland). Column chromatography (CC) was performed on silica gel (200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, People's Republic of China) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden). Fractions were monitored by TLC, and spots were visualized by heating silica gel plates sprayed with 10% H₂SO₄ in EtOH.

Fungal material

The fungus *P. multirostrata* EA-12 was isolated from fresh leaves of *E. Adenophorum* collected in Kunming Institute of Botany, Kunming, Yunnan province, PR 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 (100%) as that for the sequence of *P. multirostrata* (compared with EF585392.1). The strain is preserved at the Kunming Institute of Botany, Chinese Academy of Sciences (No. F2012076).

Fermentation, extraction and isolation

This fungal strain was cultured on potato dextrose agar medium at 25 °C for 10 days. The agar plugs were inoculated in 500-ml Erlenmeyer flasks, each

Table 1 ^1H and ^{13}C NMR spectroscopic data of 1–3

	1^a		2^a		3^b	
	δ_{H} (J in Hz)	δ_{Cmult}	δ_{H} (J in Hz)	δ_{Cmult}	δ_{H} (J in Hz)	δ_{Cmult}
1		174.7s		172.7 s		176.5 s
3	3.39 t (7.3)	53.5 d	3.35 m	53.6 d	3.38 m	54.2 d
4	2.72 m	49.2 d	3.11 t (4.4)	45.8 d	2.98 dd (6.2, 1.4)	46.5 d
5	2.93 m	31.8 d	2.84 m	32.3 d	2.72 m	32.4 d
6		147.8 s		148.1 s		151.0 s
7	4.03 d (10.2)	70.6 d	3.96 d (10.5)	70.1 d	3.94 d (9.8)	73.0 d
8	2.58 t (10.2)	53.0 d	2.45 m	50.9 d	2.55 t (9.8)	51.6 d
9		50.2 s		62.5 s		63.8 s
10a	2.73 m	44.2 t	2.48 m	44.5 t	2.40 dd (13.1, 8.3)	43.9 t
10b			2.82 m		2.72 dd (13.1, 6.7)	
11	0.96 d (6.7)	13.2 q	1.13 d (6.7)	14.3 q	0.73 d (6.7)	13.2 q
12a	5.10 s	115.1t	5.16 s	114.3 t	5.03 s	114.1 t
12b	5.28 s		5.37 s		5.18 s	
13	6.41 dd (15.4, 10.0)	128.7 d	6.07 ddd (15.4, 9.8, 1.5)	127.3 d	6.06 ddd (15.2, 9.6, 1.8)	128.1 d
14	5.23 m	134.8 d	5.43 m	137.6 d	5.26 m	137.5 d
15a	1.98 m	39.1 t	1.90 m	40.2 t	1.80 m	40.5 t
15b	2.15 m		2.13 dd (14.2, 2.3)		2.08 dd (14.1, 2.5)	
16	1.65 m	31.5 d	1.54 m	31.9 d	1.54 m	34.2 d
17a	0.87 m	30.5 t	1.17 m	32.8 t	1.10 m	35.1 t
17b	0.98 m		1.38 ddd (14.1, 9.8, 4.0)		1.28 m	
18a	1.48 m	21.0 t	1.59 m	20.9 t	1.11 m	19.8 t
18b	1.85m		1.88 m		1.40 m	
19a	2.41 m	27.4 t	2.47 m	41.5 t	1.93 m	36.3 t
19b	2.72 m		2.76 m		1.44 m	
20		156.2 s		204.2 s	4.16 m	74.0 d
21	5.93 d (3.0)	107.2 d	7.70 d (16.2)	133.1 d	6.68 dd (16.2, 8.1)	149.8 d
22	6.10 d (3.0)	106.2 d	6.85 d (16.2)	138.2 d	6.91 d (16.2)	125.9 d
23		153.2 s		197.4 s		198.8 s
24	0.92 d (6.9)	21.0 q	0.92 d (6.6)	20.9 q	0.91 d (6.8)	20.9 q
1'		137.6 s		137.0 s		138.2 s
2',6'	7.12 d (7.0)	129.2 d	7.12 d (7.0)	129.3 d	7.14 d (7.0)	130.8 d
3',5'	7.30 t (7.0)	128.9 d	7.30 t (7.0)	129.1 d	7.30 t (7.0)	129.6 d
4'	7.23 t (7.0)	127.0 d	7.23 t (7.0)	127.3 d	7.23 t (7.0)	127.8 d
NH	5.63 s		5.66 s			

^aRecorded in CDCl_3 at 500 MHz for ^1H and 100 MHz for ^{13}C .^bRecorded in methanol- d_4 at 400 MHz for ^1H and 100 MHz for ^{13}C .

containing 100 ml of potato dextrose media. Flask cultures were incubated at 28 °C on a rotary shaker at 160 r.p.m. for 2 days as seed culture. Sixty 500-ml Erlenmeyer flasks each containing 150 ml of potato dextrose broth were individually inoculated with 25 ml of seed culture and incubated at 25 °C on a rotary shaker at 160 r.p.m. for 15 days.

The fermented broth was filtered through cheesecloth to separate supernatant and mycelia. The former was extracted with EtOAc (3 × 15 liter), and the organic solvent was evaporated to dryness under vacuum to afford the extract of the supernatant. The mycelia was extracted with acetone (3 × 1 liter) and then concentrated under reduced pressure to afford an aqueous solution. The aqueous solution was extracted with EtOAc (3 × 1 liter), and the EtOAc solution was evaporated to dryness under vacuum to obtain the crude extract of the mycelia. Both extracts were combined to yield a residue (11.0 g). The residue was subjected to silica gel CC with a gradient elution system of $\text{CHCl}_3/\text{MeOH}$ (from 100:0 to 0:100, V/V) to obtain seven fractions. Fraction B was separated to six subfractions (B1~B6) by MPLC eluted with $\text{MeOH}/\text{H}_2\text{O}$ (from 0:100 to 100:0, V/V). Fraction B2 (1.8 g) was separated by silica gel CC eluted with $\text{CHCl}_3/\text{MeOH}$ (from 100:1 to 96:4, V/V) to afford **5** (1.4 g). Fraction B3 was further separated by preparative HPLC ($\text{MeCN}/\text{H}_2\text{O}$, from 30:70 to 60:40, V/V, 10 ml min⁻¹ in 30 min) to yield **4** (8.0 mg, t_{R} = 16.2 min) and **6** (24.5 mg, t_{R} = 14.8 min). Fraction B5 (1.7 g) was separated by silica gel CC eluted with $\text{CHCl}_3/\text{Me}_2\text{CO}$ (from 20:1 to 3:1, V/V) to obtain five

subfractions (B5a~B5e). Fraction B5b (150 mg) was further separated by preparative HPLC ($\text{MeCN}/\text{H}_2\text{O}$, from 25:75 to 60:40, V/V, 10 ml/min in 35 min) to afford **1** (5.2 mg, t_{R} = 29.2 min), **2** (4.8 mg, t_{R} = 19.0 min) and **7** (3.1 mg, t_{R} = 25.6 min). Fraction B5c (88 mg) was subjected to sephadex LH-20 ($\text{CHCl}_3/\text{MeOH}$, 1:1, V/V) and silica gel CC ($\text{CHCl}_3/\text{MeOH}$, 20:1) to yield **3** (11.2 mg).

Physico-chemical properties

Multirostratin A (**1**): white, amorphous solid; $[\alpha]_{\text{D}}^{25}$ +118.0 (c 0.20 MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (4.38) nm; IR (KBr) ν_{max} 3424, 2949, 1700, 1641, 1453, 1023, 978, 701 cm⁻¹. ^1H and ^{13}C NMR data (see Table 1); HR-ESI-MS (pos.) m/z 445.2622 (calcd for $\text{C}_{29}\text{H}_{35}\text{NO}_3$, 445.2617).

20-Oxo-deoxaphomin (**2**): white, amorphous solid; $[\alpha]_{\text{D}}^{25}$ +61.1 (c 0.21 MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (4.35), 233 (3.93) nm; IR (KBr) ν_{max} 3423, 2923, 1691, 1662, 1454, 1275, 1024, 973 cm⁻¹. ^1H and ^{13}C NMR data (see Table 1); HR-ESI-MS (pos.) m/z 461.2566 (calcd for $\text{C}_{29}\text{H}_{35}\text{NO}_4$, 461.2578).

Cytotoxic activity

Cytotoxicities of multirostratin A (**1**) and 20-oxo-deoxaphomin (**2**) were assayed against five tumor cell lines, including SK-BR-3 (breast cancer cell

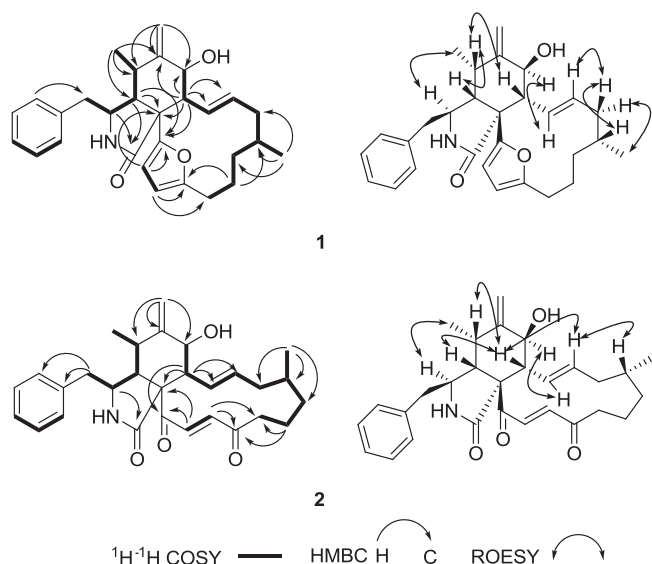


Figure 2 Selected 2D NMR correlations of **1** and **2**.

Table 2 Cytotoxicity of compounds **1** and **2** against five cancer cell lines (IC_{50} μM)

Compound	HL-60	SMMC-7721	A-549	MCF-7	SW480
1	7.8	9.8	10.8	11.6	15.8
2	14.2	10.4	7.7	9.6	11.6
Cisplatin ^a	1.0	4.3	5.1	15.4	15.4

^aPositive control.

line), SMMC-7721 (hepatocellular carcinoma cell line), HL-60 (human myeloid leukemia cell line), PANC-1 (pancreatic cancer cell line) and A-549 (lung cancer cell line). Assays were performed as described previously.¹⁶

Supplementary

1D and 2D NMR and MS spectra of compounds **1** and **2** are available in Supplementary Information.

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

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