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Dibrefeldins A and B, A pair of epimers representing the first brefeldin A dimers with cytotoxic activities from *Penicillium janthinellum*

Fanrong Zeng[1], Chunmei Chen[1], Ali Adhab Al Chnani, Qun Zhou, Qingyi Tong, Wenjing Wang, Yi Zang, Jiaojiao Gong, Zhaodi Wu, Junjun Liu, Jianping Wang, Hucheng Zhu*, Yonghui Zhang*

Hubei Key Laboratory of Natural Medicinal Chemistry and Resource Evaluation, School of Pharmacy, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, People's Republic of China

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ABSTRACT

Dibrefeldins A and B (**1** and **2**), two unexpected brefeldin A (BFA) dimers, as well as brefeldin F (**3**), brefeldin G (**4**), and 14-hydroxy-BFA (**5**), three new BFA derivatives, together with three new naturally occurring BFA derivatives (**6**–**8**) and four known analogues (**9**–**12**), were isolated from the fungus *Penicillium janthinellum*. Dibrefeldins A and B (**1** and **2**) represent the first examples of BFA dimers formed by an esterification between two BFA monomer units. Brefeldin F (**3**) has an *α*,*β*-unsaturated γ-lactone ring, and this moiety was first discovered in naturally occurring BFA derivatives. The structures and relative/absolute configurations of these derivatives were elucidated by extensive spectroscopic methods, ^{13}C NMR calculations, and single-crystal X-ray diffraction. Compounds **1**, **2**, **8**, and **9** showed excellent cytotoxic activities against six cancer cell lines with IC_{50} values ranging from 0.01 to 4.45 μM.

1. Introduction

Cancer is a leading cause of death around the world. According to the statistics of the World Health Organization (WHO), cancer was responsible for 8.8 million deaths in 2015 [1]. Current methods for treating cancer can prolong the survival of the patient but cannot completely cure cancer, and the main challenge is killing cancer cells selectively [2]. This situation will become even more severe in the future with the rapid aging of the global population [3]. Therefore, developing new anticancer agents remains critical.

Brefeldin A (BFA), a secondary metabolite of several Ascomycetes fungi, was first isolated in 1958 from *Penicillium decumbens* [4,5]. BFA shows a variety of biological activities, such as antitumor, antifungal, antiviral, and antimitotic [4,6,7], and it can induce apoptotic cell death in ovarian carcinoma cells [3,8]. Although BFA is a potential chemotherapeutic against cancer, it still has many limitations, mainly due to its low selectivity between tumor and normal cells [9,10]. Therefore, many efforts have been made by pharmaceutical chemists and pharmacologists to develop higher efficiency and lower toxicity analogues of BFA [3,11].

To date, most naturally occurring BFA derivatives have been isolated from the genus *Penicillium* derived from soil and marine sediments. Previous investigations on *Penicillium janthinellum* have suggested that the main metabolites of this fungus are indole diterpenoid alkaloids [12–14], azaphilones [15], epipolythiodioxopiperazine (ETP) alkaloids [16], and BFA derivatives [2,17]. In our ongoing search for bioactive secondary metabolites from the genus *Penicillium* [18,19], *P. janthinellum*, isolated from soil collected in Youyang County of Chongqing, was phytochemically investigated. This study lead to the isolation of 12 BFA derivatives (Fig. 1): dibrefeldins A and B (**1** and **2**), two unexpected brefeldin A (BFA) dimers; brefeldin F (**3**), brefeldin G (**4**), and 14-hydroxy-BFA (**5**), three new BFA derivatives; three new naturally occurring BFA derivatives [BFA *seco*-acid (**6**) [20], *seco*-BFA methyl ester (**7**) [10,20], and 10,11-epoxy-BFA (**8**) [20,21]]; and four known analogues [brefeldin A (**9**) [6], 7-dehydro-BFA *seco*-acid (**10**) [22], 4-*epi*-BFA (**11**) [23], and 6α-hydroxy-brefeldin C (**12**) [2]]. Dibrefeldins A and B (**1** and **2**) represent the first examples of BFA dimers formed by the esterification of two monomeric BFA units, and brefeldin F (**3**) features a new *α*,*β*-unsaturated γ-lactone ring. In this paper, we present the details of the isolation, structure elucidation, and bioactivity evaluation of these isolated compounds.

* Corresponding authors.
E-mail addresses: zhuhucheng@hust.edu.cn (H. Zhu), zhangyh@mails.tjmu.edu.cn (Y. Zhang).
[1] These authors contributed equally to this work.

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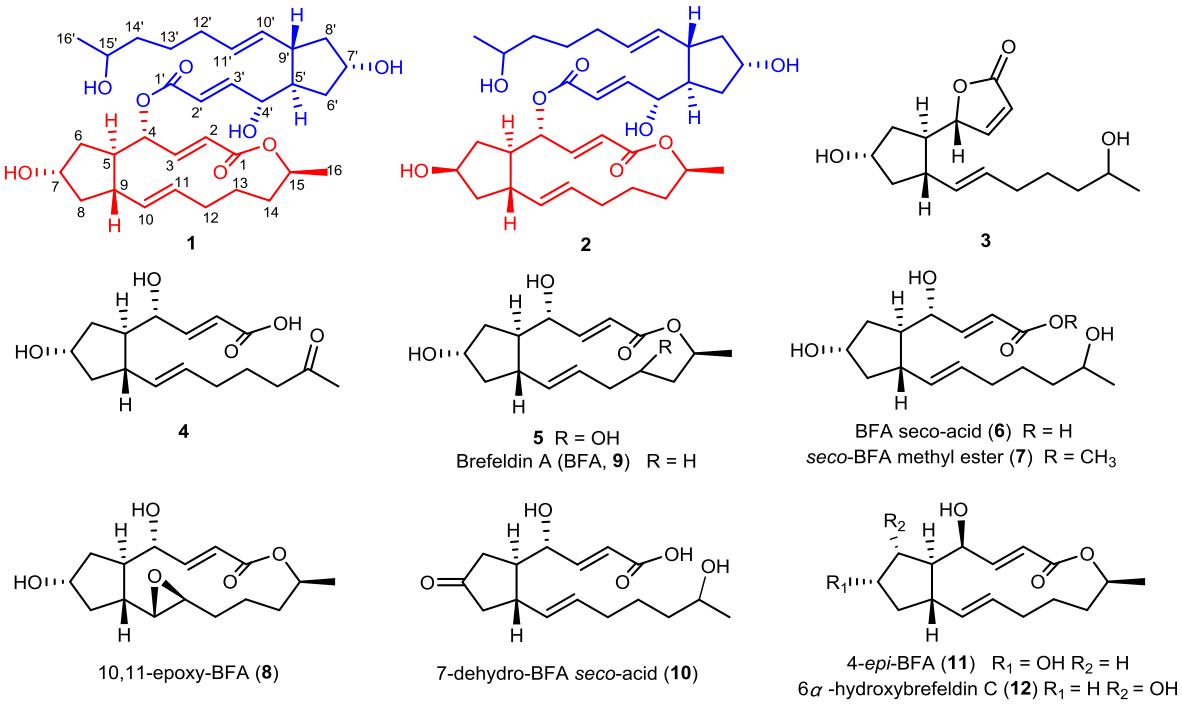

Fig. 1. Chemical structures of compounds **1–12**.

2. Experimental section

2.1. General experimental procedures

Optical rotations were obtained in a 0.7 mL cell on a Rudolph Autopol IV automatic polarimeter (Rudolph Research Analytical, Hackettstown, NJ, U.S.A.). UV spectra were recorded with a PerkinElmer Lambda 35 spectrophotometer (PerkinElmer, Inc., Fremont, California, U.S.A.). ECD data were acquired with a JASCO-810 instrument (JASCO Co., Ltd., Tokyo, Japan). IR spectra were measured by a Bruker Vertex 70 FT-IR spectrophotometer (Bruker, Karlsruhe, Germany). NMR spectra were determined on Bruker AM-400 and 600 NMR spectrometers (Bruker, Karlsruhe, Germany) by using solvent signals (DMSO-d_6 (δ_H 2.50/δ_C 39.52) and CD_3OD (δ_H 3.31/δ_C 49.0)) as references. HRESIMS data were performed on a Bruker microTOF II spectrometer. Compounds were acquired using an Agilent 1260 semipreparative HPLC system outfitted with a DAD. Column chromatography was conducted on silica gel (100–200 mesh, 200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, People's Republic of China), ODS (50 μm, YMC, Japan), and Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden). Thin-layer chromatography was performed on precoated TLC plates (200–250 μm thickness, silica gel 60 F_{254}, Qingdao Marine Chemical Inc.). Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH.

2.2. Fungal material

In February 2017, the fungus was isolated from soil obtained in Youyang, County, Chongqing, China, which is well known as the hometown of Artemisia apiacea. The internal transcribed spacer (ITS) region was amplified by PCR using primers ITS1 (50-TCCGTAGGTGA ACCTGCGG-30) and ITS4 (50-TCCTCCGCTTATTGATATGC-30), then submitted to GenBank and identified as *Penicillium janthinellum* by ITS sequence homology. A voucher sample numbered as ZFR20170213 has been preserved in the culture collection center of Tongji Medical College, Huazhong University of Science and Technology.

The strain was incubated on potato dextrose agar (PDA) at 28 °C for 7 days, and the agar was then cut into small pieces (approximately $1 \times 1 \times 1\ cm^3$) and inoculated into sterilized Erlenmeyer flasks (250×1 L) each containing 200 g of rice and 220 mL of water.

2.3. Fermentation and extraction

Approximately 2 g of soil was diluted to 2–10 g/ml with sterile water. Then, 100 μl of the solution was removed with a pipette and sprayed on potato dextrose agar with chloramphenicol (0.1 g/L). When the above steps were completed, the plate was incubated at 28 °C. Single colonies were not transferred to another plate until the strain grew. The strain was incubated on potato dextrose agar (PDA) at 28 °C for 7 days, and the agar was then cut into small pieces (approximately $1 \times 1 \times 1\ cm^3$) and inoculated into sterilized Erlenmeyer flasks (250×1 L) each containing 200 g of rice and 220 mL of water. After incubating at 28 °C for 23 days, the fermentation broth was thoroughly mixed with alcohol (CH_3CH_2OH) and then extracted three times with ethyl acetate (EtOAc).

2.4. Purification

Under reduced pressure, the EtOAc was evaporated to afford a brown total extract (25 g), which was then subjected to a silica gel column chromatography (CC, 100–200 mesh) eluting with petroleum ether/EtOAc (100:0–0:1, v/v) to afford seven fractions (Fr.1–Fr.7) based on TLC analysis. Fr.5 (3 g) was further separated by reversed-phase MPLC ($MeOH/H_2O$, 20:80–100:0) to obtain nine fractions (Fr.5.1–Fr.5.9). Fr.5.5 (800 mg) was purified by Sephadex LH-20 (CH_2Cl_2/MeOH = 1:1) and silica gel CC (200–300 mesh) eluting with CH_2Cl_2/MeOH (120:1–1:1, v/v) to afford four fractions (Fr.5.5.1–Fr.5.5.4). Fr.5.5.3 was further purified by semipreparative HPLC (MeOH–H_2O, 58:42, v/v) to yield compounds **1** (3.0 mg, t_R = 28.0 min), **2** (1.5 mg, t_R = 31.0 min), and **3** (2.8 mg, t_R = 35.0 min). Compounds **4** (2.0 mg, t_R = 25.0 min), **5** (2.0 mg, t_R = 28.0 min), **6** (3.0 mg, t_R = 32.0 min), and **7** (2.8 mg, t_R = 37.0 min) were obtained from Fr.5.5.4 by semipreparative HPLC (MeOH–H_2O, 45:55, v/v). Compounds **8** (6.0 mg, t_R = 25.0 min), **9** (2.5 mg, t_R = 29.0 min), **10** (5.0 mg, t_R = 35.0 min), **11** (3.0 mg, t_R = 40.0 min), and **12** (2.8 mg, t_R = 45.0 min) were obtained from Fr.5.5.2 by semipreparative HPLC (MeOH–H_2O, 54:46, v/v).

Table 1
NMR Data of Compounds **1–5** in CD_3OD (δ in ppm).

	1[a]		**2**[b]		**3**[a]		**4**[a]		**5**[a]	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	167.5		167.5		175.8		169.4		168.0	
2	118.5	5.59 dd (15.7, 1.9)	118.5	5.56 dd (15.7, 1.8)	121.7	6.13 dd (5.8, 2.0)	120.5	5.97 m	117.6	5.81 dd (15.6, 2.0)
3	150.0	7.36 dd (15.7, 3.3)	150.1	7.39 dd (15.7, 3.3)	159.2	7.69 dd (5.8, 1.5)	150.3	6.88 dd (15.6, 4.7)	155.2	7.39 dd (15.6, 2.9)
4	78.0	5.33 m	78.8	5.38 m	85.8	5.13 dt (4.4, 1.8)	70.2	4.21 m	76.5	4.04 dt (9.6, 2.5)
5	51.0	2.14 m	50.9	1.90 m	47.4	2.19 m	48.8	1.97 m	53.3	1.85 m
6	41.6	1.91 m; 1.63 m	41.0	2.15 m; 1.55 m	36.2	1.53 m	34.9	1.59 m; 1.81 m	42.0	1.82 m; 2.03 m
7	72.8	4.20 m	73.3	4.22 m	72.2	4.20 m	71.4	4.20 m	72.9	4.22 m
8	43.9	2.17 m; 1.46 m	44.3	1.86 m; 1.56 m	43.6	2.20 m; 1.43 m	42.3	1.42 m; 2.16 m	44.8	1.46 m; 2.15 m
9	45.2	2.53 quint (8.7)	45.5	2.81 m	45.6	2.46 quint (9.0)	43.2	2.43 d (8.2)	45.4	2.41 m
10	137.8	5.31 m	136.5	5.22 dd (15.1, 9.6)	134.5	5.41 dd (15.2, 8.7)	129.6	5.39 dd (6.3, 4.3)	141.1	5.38 dd (15.3, 9.4)
11	131.8	5.79 ddd (14.9, 10.1, 4.5)	132.6	5.88 ddd (15.1, 10.3, 4.5)	132.8	5.54 dt (15.2, 6.6)	134.6	5.40 m	127.2	5.50 ddd (14.9, 9.8, 4.9)
12	32.9	2.02 m; 1.87 m	33.0	2.01 m	33.5	2.04 m	31.5	2.01 m	42.3	2.02 m; 2.17 m
13	27.9	1.88 m; 0.91 m	28.0	1.88 m; 0.89 m	26.8	1.52 m; 1.43 m	23.2	1.60 m; 1.64 m	73.8	3.19 dddd (11.1, 5.3, 3.3, 1.5)
14	35.0	1.76 m; 1.61 m	34.9	1.78 m; 1.60 m	39.7	1.43 m	42.2	2.48 t (7.3)	45.2	1.57 ddd (15.2, 6.0, 1.5); 2.00 m
15	73.5	4.81 m	73.5	4.82 m	68.4	3.72 m	210.7		71.9	5.06 qdd (12.5, 6.3, 1.4)
16	21.0	1.24 d (6.2)	21.0	1.25 d (6.2)	23.5	1.14 d (6.2)	28.1	2.13 s	21.1	1.28 d (6.3)
1′	167.0		167.0							
2′	119.8	6.10 dd (15.6, 1.8)	119.8	6.10 dd (15.6, 1.8)						
3′	154.2	7.07 dd (15.6, 4.4)	154.2	7.06 dd (15.6, 4.4)						
4′	71.7	4.25 m	71.6	4.24 m						
5′	50.2	1.99 m	50.1	1.99 m						
6′	36.5	1.81 m; 1.60 m	36.4	1.81 m; 1.58 m						
7′	72.7	4.20 m	72.7	4.20 m						
8′	43.8	2.16 m; 1.43 m	43.8	2.16 m; 1.41 m						
9′	44.8	2.44 quint (9.0)	44.8	2.44 m						
10′	135.3	5.41 m	135.3	5.39 m						
11′	132.0	5.44 m	132.0	5.46 dt (15.2, 6.5)						
12′	33.6	2.02 m	33.6	2.01 m						
13′	26.9	1.43 m; 1.39 m	26.9	1.45 m; 1.39 m						
14′	39.7	1.43 m	39.7	1.41 m						
15′	68.4	3.71 m	68.4	3.71 m						
16′	23.5	1.14 d (6.2)	23.5	1.14 d (6.3)						

[a] ^1H (400 MHz) and ^{13}C (100 MHz).
[b] ^1H (600 MHz) and ^{13}C (150 MHz).

2.5. Spectroscopic data of compounds

Dibrefeldins A (**1**): Colorless, transparent oil; $[\alpha]_D^{25}$ −42.1 (*c* 0.47, MeOH); UV (MeOH) λ_{max} (log ε) 202 (2.8) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 208 (+17.52), 250 (−0.79); IR (KBr) ν_{max} 3343, 2931, 2859, 1702, 1650, 1265, 1164, 1128, 11073, and 977 cm^{-1}; ^1H and ^{13}C NMR data, see (Table 1); HRESIMS *m/z* 583.3234 $[M+Na]^+$ (calcd for $C_{32}H_{48}O_8Na$, 583.3247).

Dibrefeldins B (**2**): Colorless, transparent oil; $[\alpha]_D^{25}$ −32.0 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 202 (2.8) nm; ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 207 (+20.15), 260 (−1.46); IR (KBr) ν_{max} 3432, 2926, 2850, 1633, 1488, 1386, 1025, 716, and 691 cm^{-1}; ^1H and ^{13}C NMR data, see (Table 1); HRESIMS *m/z* 583.3235 $[M+Na]^+$ (calcd for $C_{32}H_{48}O_8Na$, 583.3247).

Brefeldin F (**3**): Colorless, transparent oil; $[\alpha]_D^{25}$ −43.5 (*c* 0.26, MeOH); UV (MeOH) λ_{max} (log ε) 206 (2.9); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 243 (+16.65), 214 (−9.51); IR (KBr) ν_{max} 3408, 2961, 2931, 2859, 1746, 1173, 1028, 976, and 824 cm^{-1}; ^1H and ^{13}C NMR data, see (Table 1); HRESIMS *m/z* 303.1552 $[M+Na]^+$ (calcd for $C_{16}H_{24}O_4Na$, 303.1572).

Brefeldin G (**4**): Colorless, transparent oil; $[\alpha]_D^{25}$ –17.0 (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ε) 206 (2.88); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 214 (+19.02), 253 (−1.13); IR (KBr) ν_{max} 3427, 2928, 2853, 1703, 1638, 1567, 1388, 1275, 1027, and 573 cm^{-1}; ^1H and ^{13}C NMR data, see (Table 1); HRESIMS *m/z* 319.1512 $[M+Na]^+$ (calcd for $C_{16}H_{24}O_4Na$, 319.1521).

14-Hydroxy-BFA (**5**): Colorless, transparent oil; $[\alpha]_D^{25}$ –41.4 (*c* 1.32, MeOH); UV (MeOH) λ_{max} (log ε) 206 (2.86); ECD (MeOH) λ_{max} ($\Delta\varepsilon$) 207 (+17.41), 244 (−1.66); IR (KBr) ν_{max} 3431, 2931, 1697, 1641, 1501, 1449, 1383, 1268, 1119, 1068, and 984 cm^{-1}; ^1H and ^{13}C NMR data, see (Table 1); HRESIMS *m/z* 319.1513 $[M+Na]^+$ (calcd for $C_{16}H_{24}O_4Na$, 319.1521).

2.6. Computational details

The conformations of **3A** and **3B** generated by BALLOON [24,25] were subjected to semiempirical PM3 quantum mechanical geometry optimizations using the Gaussian 09 program. Duplicate conformations were identified and removed when the root-mean-square (RMS) distance was less than 0.5 Å for any two geometry-optimized conformations. The remaining conformations were further optimized at the B3LYP/6-31G(d) level in methanol with the IEFPCM solvation model using Gaussian 09, and the duplicate conformations emerging after these calculations were removed according to the same RMS criteria mentioned above. The harmonic vibrational frequencies were calculated to confirm the stability of the final conformers. The NMR chemical shifts were calculated for each conformer at the B3LYP/6-311++G(d,p)//B3LYP/6-31G(d) level with methanol as solvent by the IEFPCM solvation model in Gaussian 09, and the values were then combined using Boltzmann weighting according to their population contributions. The comparison of the experimental and calculated chemical shifts was realized using the DP4+ algorithm published by Sarotti at https://sarotti-nmr.weebly.com/.

2.7. Cytotoxic assay

The growth inhibitory effects of compounds **1–12** on six cancer cell lines human promyelocytic leukemia cells (HL-60), glioma cells (U87MG), human breast cancer cells (MDA-MB-231), human non-small-

cell lung cancer cells (A549), human hepatocellular carcinoma cell lines (HEP-3B), human colorectal cancer cell (SW480) and normal colorectal mucosa cell line (NCM460), were measured. All cell lines were cultured in DMEM or RPMI-1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (HyClone) at 37 °C in a humidified atmosphere with 5% CO_2. For the cell viability assay, cells were plated into 96-well plates in 50 µl of medium, and the compounds were serially diluted in the medium and delivered to the cells as 2× solutions in 50 µl of medium (adhered cells were grown for 24 h before adding the compounds). After 48 h, the cell viability was detected using a CCK-8 Kit (Dojindo, Japan) according to the manufacturer's instructions. Growth relative to untreated cells was calculated, and these data were used to generate dose-response curves. The IC_{50} values (50% inhibition concentration) of the compounds for each cell line were calculated using SPSS.

3. Results and discussion

3.1. Structural elucidations of armochaetoglosins a–c (1–5)

Compound **1** was obtained as a colorless and transparent oil. It has a molecular formula of $C_{32}H_{48}O_8$ as established by its $[M+Na]^+$ ion peak at *m/z* 583.3234 in its HRESIMS spectrum, and this formula requires nine degrees of unsaturation. Analysis of the ^1H NMR data (Table 1) of **1** together with an examination of its HSQC data showed eight olefinic protons [δ_H 7.36 (dd, J = 15.7, 3.3 Hz), 7.07 (dd, J = 15.6, 4.4 Hz), 6.10 (dd, J = 15.6, 1.8 Hz), 5.79 (ddd, J = 14.9, 10.1, 4.5 Hz), 5.59 (dd, J = 15.7, 1.9 Hz), 5.44 (m), 5.41 (m), and 5.31 (m)], six oxygenated methine protons [δ_H 5.33 (m), 4.81 (m), 4.25 (m), 4.20 (m), 4.20 (m), and 3.71 (m)], and two methyl groups [δ_H 1.24 (d, J = 6.2 Hz) and 1.14 (d, J = 6.2 Hz)]. The ^{13}C NMR and DEPT spectra of **1** revealed 32 carbon resonances that could be ascribed to two ester carbonyl groups, eight olefinic carbons, 10 methines including six oxygenated carbons, 10 aliphatic methylenes, and two methyl groups. These analyses suggested compound **1** was an unexpected BFA dimer. Because there are no previous reports of BFA dimers, it is suspected that it is a mixture of two BFA monomers. However, further HPLC analyses with different solvent systems and retested HRESIMS spectra supported the fact that compound **1** is a pure dimeric compound.

The gross structure of unit A of **1** was established by comparison of its 1D NMR with those of **9** and by elucidation of its 2D NMR spectra, including $^1H-^1H$ COSY and HMBC (Fig. 2). The first fragment of C-1-C-2-C-3-C-4 was deduced by the obvious $^1H-^1H$ COSY correlations of H-2/H-3/H-4 and the HMBC correlations from H-2 (δ_H 5.59) and H-3 (δ_H 7.36) to C-1 (δ_C 167.5) and C-4 (δ_C 78.0). The second chain, from Me-16 to C-10, was also elucidated by carefully analyses of its $^1H-^1H$ COSY correlations (H-9/H-10/H-11/H-12/H-13/H-14/H-15/Me-16) as well as the HMBC correlations from Me-16 to C-14 and C-15, from H-15 to C-13, and from H-10 and H-11 to C-12. In addition, the five-membered carbon ring constructed by C-5-C-6-C-7-C-8-C-9 was elucidated by the $^1H-^1H$ COSY correlations of H-6/H-5/H-9/H-8 and the HMBC correlations from H-7 to C-5 and C-9 and from H-8 to C-6. The connections of these fragments were determined from the $^1H-^1H$ COSY cross-peaks of H-5/H-4 and H-9/H-10 and the HMBC interactions from H-4 to C-9, from H-6 to C-4, and from H-11 to C-9. Finally, the HMBC correlation from H-15 to C-1 indicated the presence of a thirteen-membered lactone ring. Therefore, the planar structure of unit A was elucidated, and it was identical to **9**. The ^1H and ^{13}C NMR data of unit B of **1** were similar to unit A. However, the chemical shift of C-15′ (δ_C 68.4) was dramatically shifted upfield compared to that of C-15 (δ_C 73.5) in unit A, which together with the absence of an HMBC correlation from H-15′ to C-1′ suggested the opening of the lactone of unit B. Finally, the HMBC interaction from H-4 to C-1′ revealed units A and B were connected via an ester group. Therefore, the planar structure of compound **1** was finally determined.

The relative configurations of unit A were determined to be identical to those of **9** by a NOESY experiment (Fig. 3). The NOESY correlation of H-7 with H-9 suggested these groups were cofacial, and they were assigned to be *β*-oriented according to compound **9**. Consequently, the NOESY correlation of H-10/H-5 indicated that H-5 should be *α*-oriented. In addition, based on the conformation of the lactone ring as deduced from the NOESY correlations of H-9/H-3, H-9/H-11, and H-11/H-3, the NOESY correlations of H-4/H-9 and H-4/H-3 revealed that H-4 should be *β*-oriented. Similarly, the relative configuration of unit B was determined from its NOESY data to be as shown (Fig. 3). A literature investigation revealed that the chemical shift of C-16 is δ_C 21 when Me-16 is *β*-oriented and δ_C 18 when Me-16 is *α*-oriented (Table S1). Thus, Me-16 in unit A of **1** was deduced to be *β*-oriented, however, due to the opening of the lactone ring in unit B, C-15′ could not be determined here. Thus, the relative configuration of **1** except C-15′ was elucidated.

The absolute configuration of **1** was determined by comparison of its ECD with that of **9** (Fig. 4), whose absolute configuration was determined by single-crystal X-ray diffraction analysis with Cu Kα radiation in this study (Fig. 5, CCDC 1864959).

Compound **2** has the same molecular formula ($C_{32}H_{48}O_8$) as that of

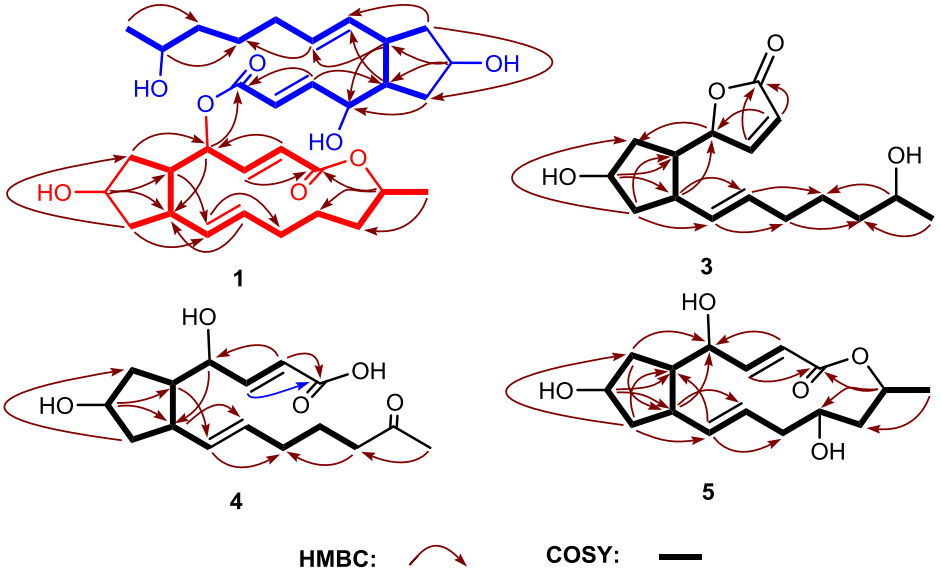

Fig. 2. Key ^1H-^1HCOSY and HMBC correlations of compounds **1** and **3–5**.

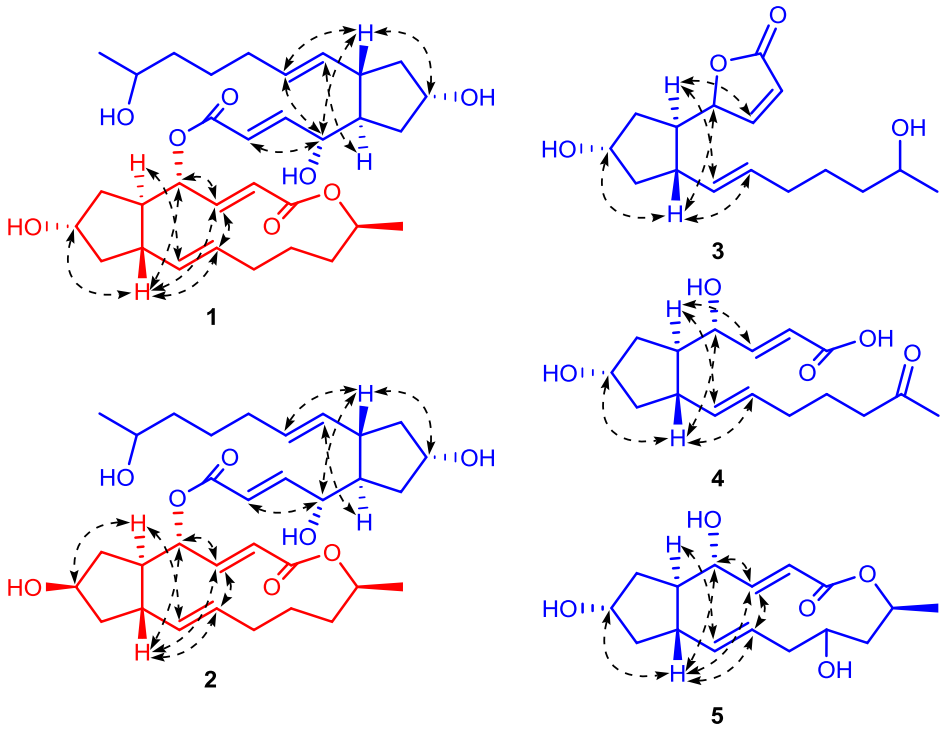

Fig. 3. Key NOESY correlations of compounds **1**–**5**.

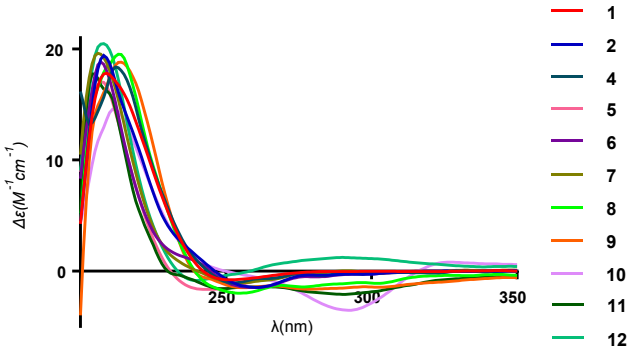

Fig. 4. Experimental ECD spectra of **1**, **2**, and **4**–**12** in MeOH.

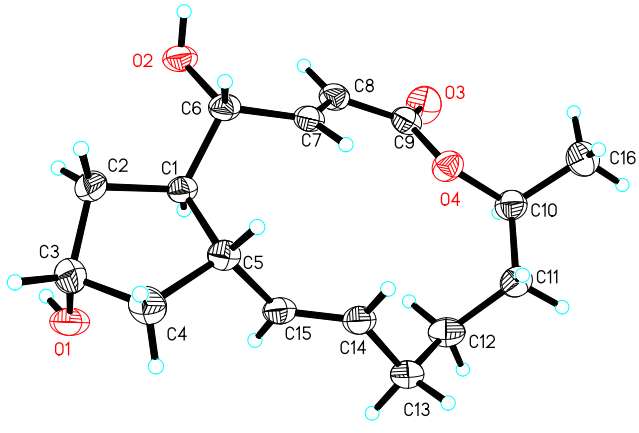

Fig. 5. X-ray structure of **9**.

1 based on its HRESIMS ion peak detected at *m/z* 583.3235 $[M+Na]^+$. A comparison of their ^1H and ^{13}C NMR data (Table 1) indicated that the structures of these two compounds are closely related. Further analyses of their 2D NMR data, particularly the HMBC and $^1H-^1H$ COSY correlations, indicated that compound **2** shared a common planar structure with **1**. A detailed comparison of the ^1H and ^{13}C NMR data of **2** and **1** revealed slight differences in C-4-C-11, suggesting they were likely stereoisomers. Further analysis of the NOESY spectrum of **2** revealed the key NOESY correlation of H-7/H-5 for **2** rather than H-7/H-9 for **1**, suggesting the opposite configuration of H-7 in **2**. The other NOESY correlations, including H-9/H-3, H-9/H-4, H-9/H-11, H-11/H-3, and H-5/H-10, were the same as those of **1**. Therefore, compound **2** was determined to be the C-7 epimer of **1**. The absolute configuration of **2** was determined based on the similarities between the ECD curves of **2** and **1** (Fig. 4).

Compound **3** gave an $[M+Na]^+$ ion peak at *m/z* 302.1552 in its HRESIMS spectrum, which indicated a molecular formula of $C_{16}H_{24}O_4$ with five degrees of unsaturation. The ^1H NMR data (Table 1) of **3** revealed the presence of four olefinic protons [δ_H 7.69 (dd, J = 5.8, 1.5 Hz), 6.13 (dd, J = 5.8, 2.0 Hz), 5.54 (dt, J = 15.2, 6.6 Hz), and 5.41 (dd, J = 15.2, 8.7 Hz)], three oxygenated methines [δ_H 5.13 (dt, J = 4.4, 1.8 Hz), 4.20 (m), and 3.72 (m)], one methyl group [δ_H 1.14 (d, J = 6.2 Hz)]. The ^{13}C NMR and DEPT spectra of **3** displayed 16 carbon resonances, which could be attributed to an ester carbonyl, four olefinic methines, five aliphatic methines (including three oxygenated carbons), five aliphatic methylenes, and a methyl carbon. Based on these NMR data, compound **3** was speculated to be an analogue of **9**. Careful comparison of the ^{13}C NMR data of **3** with those of **9** disclosed that the main differences between these compounds were the downfield shifted resonances of C-1 (δ_C 175.8), C-2 (δ_C 121.7), C-3 (δ_C 159.2), and C-4 (δ_C 85.8) and the upfield shifted resonances of C-5 (δ_C 47.4), C-6 (δ_C 36.2), and C-15 (δ_C 68.4). These changes suggested the opening of the 13-membered lactone and the generation of a new α,β-unsaturated γ-lactone ring between C-1 and C-4. Followed inspection of the HMBC and $^1H-^1H$ COSY spectra confirmed this putative structure. The NOESY correlations of H-7/H-9 and H-5/H-10 revealed that the five-membered ring of compound **3** had the same relative configuration as that of **9**. The configuration of C-15 was preliminarily assigned as shown based on biosynthetic considerations. Based on the small coupling constant ($J_{4,5}$ = 4.4 Hz) between H-4 and H-5, it was deduced that they should be cofacial. However, due to the free rotation of the single

Table 2
Cytotoxic Activities of Compounds **1–12** Against Seven Cell Lines (IC_{50}, μM).

Comd	HL-60	U87MG	MDA-MB-231	A549	HEP-3B	SW480	NCM460
1	2.67 ± 0.14	0.1 ± 0.00	1.11 ± 0.34	0.68 ± 0.08	0.54 ± 0.10	0.83 ± 0.12	0.97 ± 0.07
2	2.55 ± 0.12	0.3 ± 0.00	1.05 ± 0.26	0.75 ± 0.10	0.63 ± 0.10	0.77 ± 0.01	0.88 ± 0.09
3	> 40	> 40	> 40	> 40	> 40	> 40	> 40
4	> 40	> 40	> 40	> 40	> 40	> 40	> 40
5	> 40	> 40	> 40	> 40	> 40	> 40	> 40
6	> 40	> 40	> 40	> 40	> 40	> 40	> 40
7	> 40	> 40	> 40	> 40	> 40	> 40	> 40
8	4.45 ± 0.05	3.75 ± 0.01	3.82 ± 0.03	3.98 ± 0.06	3.91 ± 0.09	4.10 ± 0.01	4.10 ± 0.03
9	0.11 ± 0.02	0.01 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.00
10	> 40	> 40	> 40	> 40	> 40	> 40	> 40
11	> 40	> 40	> 40	> 40	> 40	> 40	> 40
12	> 40	> 40	> 40	> 40	> 40	> 40	> 40

C—C bond between C-4 and C-5, the configuration of C-4 could not be determined from the NOESY spectrum. To determine the relative configuration of C-4, ^{13}C NMR calculations were performed on both candidates (**3A** and **3B**, Fig. S1). The experimental and theoretical data showed correlation coefficients of 0.9990 and 0.9991 and Max absolute deviations of 2.9 and 3.1 for **3A** and **3B** (Fig. S2 and Table S2), respectively. The relative configuration of C-4 of **3** could not determined. Therefore, the DP4+ probability was used to analyze the calculated data of **3A** and **3B**, and the results showed that the probability of 3A was 100% [26]. Thus, the relative configuration of **3** was determined.

Compound **4**, a colorless and transparent oil, gave an $[M+Na]^+$ ion peak at *m/z* 319.1512 in its HRESIMS spectrum, indicating a molecular formula of $C_{16}H_{24}O_5$, which corresponds to five degrees of unsaturation. The ^1H and ^{13}C NMR data of **4** closely resembled those of **9**, except for the presence of an additional carbonyl resonance at δ_C 210.7 and the absence of an oxygenated methine, which suggested the opening of the 13-membered lactone ring and further oxidation of C-15. Analyses of the HMBC and $^1H-^1H$ COSY spectra confirmed the structure of **4**. The relative configuration of **4** was determined by analysis of its NOESY spectrum and comparison of its ^1H and ^{13}C NMR data with those of compound **9**. The absolute configuration of **4** was elucidated by comparison of its ECD curve with that of **9** (Fig. 4).

A SciFinder search revealed that the structure of compound **5** has only been reported in a patent [27], but we could not find the structure or any spectral data for this compound. Therefore, compound **5** was considered to be a new compound and named 14-hydroxy-BFA (**5**). It has a molecular formula of $C_{16}H_{24}O_5$, as revealed by its HRESIMS data, which has one more oxygen atom than BFA (**9**). The ^1H and ^{13}C NMR data of **5** closely resembled those of **9**, except for the carbon resonance at δ_C 73.8 (C-13), which indicates the presence of an additional hydroxyl group. The HMBC correlations from H-15 to C-1, C-13, C-14, and C-16 and the $^1H-^1H$ COSY cross-peaks of Me-16/H-15/H_2-14/H-13 confirmed the hydroxyl group was located at C-13. The structure and relative configuration of **5** were further determined by analyses of its 2D NMR spectra and comparison of its ^1H and ^{13}C NMR data with those of compound **9**. The absolute configuration of **5** was elucidated by comparison of its ECD curve with that of **9**.

Compounds **6–8** were previously obtained by chemical synthesis, and this is the first report of them as naturally occurring BFA derivatives. The absolute configurations of compounds **6–8** and **10–12** were confirmed by comparison of their ECD spectra with that of **9** (Fig. 4). Compound **10** was previously named (2*E*,4*R**)-4-hydroxy-4-{(1*R**,2*S**)-2-[(1*E*,6*S**)-6-hydroxy-hept-1-en-1-yl]-4-oxocyclopentyl but-2-enoic acid because its absolute configuration was not determined in the original literature [1]. Therefore, based on the absolute configuration confirmed in this study, the name of **10** was revised to (2*E*,4*S*)-4-hydroxy-4-{(1*S*,2*R*)-2-[(1*E*,6*R*)-6-hydroxy-hept-1-en-1-yl]-4-oxocyclopentyl} but-2-enoic acid, and it was given the trivial name 7-dehydro-BFA *seco*-acid (**10**).

3.2. In vitro cytotoxic activity against six cancer cell lines,

Compounds **1–12** were evaluated for their cytotoxic activities against six cancer cell lines (HL-60, U87MG, MDA-MB-231, A549, HEP-3B, and SW480) and a normal cell line, NCM460. Compounds **1**, **2**, **8**, and **9** showed excellent cytotoxic activities against the six cancer cell lines with IC_{50} values ranging from 0.01 to 4.45 μM (Table 2). Based on the bioactivity data, we found that in terms of structural characteristics, the activities of BFA derivatives are closely related to the 13-membered lactone ring and the *α* configuration of the 4-OH moiety, which is consistent with previous reports [2,20,22].

4. Conclusions

In summary, our study on *P. janthinellum* lead to the isolation of 12 BFA derivatives. Dibrefeldins A and B (**1** and **2**) represent the first examples of BFA dimers formed by the esterification of two monomeric BFA units. Brefeldin F (3) has an *α*,*β*-unsaturated γ-lactone ring, which was first discovered in naturally occurring BFA derivatives. Compounds **1**, **2**, **8**, and **9** showed significant cytotoxic activities against six cancer cell lines with IC_{50} values ranging from 0.01 to 4.45 μM. Our studies disclosed that the 13-membered lactone ring and the *α* configuration of the 4-OH moiety are responsible for the cytotoxic activities of these BFA derivatives, which clearly show what groups pharmaceutical chemists should focus on during derivatizations of BFA.

5. Notes

The authors declare no competing financial interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.01.042.

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