SUPPLEMENTARY MATERIAL

New analogues of brefeldin A from sediment-derived fungus *Penicillium* sp. DT-F29

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ABSTRACT

Four new analogues of brefeldin A named 7, 7-dimethoxybrefeldin C (**3**), 6 α -hydroxybrefeldin C (**4**), 4-*epi*-15-*epi*-brefeldin A (**5**), 4-*epi*-8 α -hydroxy-15-*epi*-brefeldin C (**6**), together with four known analogues (**1**, **7–9**) were isolated from a fermentation of the sediment-derived fungus *Penicillium* sp. DT-F29. The structures of these compounds were elucidated on the basis of extensive spectroscopic and chemical methods. In the bioactivity assays, only compounds **1** and **8** showed significant inhibitory activities against human lung adenocarcinoma cell. In addition, compound **1** was first reported for the potent ability to reactivate latent HIV with EC₅₀ value of 0.03 μ M.

Keywords: sediment-derived fungus; *Penicillium*; brefeldin A; anti-tumour; latent HIV

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Experimental

General experimental procedures

The NMR spectra were recorded at 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR on Bruker Ascend TM 600 in DMSO-d_6 or CDCl_3 using TMS as internal standard. LC-MS analyses were performed on Agilent 6230 TOF LC/MS system and Zobax SB-C18 (5 μm , 4.6 $\times$ 150 mm) column with gradient elution. IR spectrum was recorded on JASCO FT-IR4100, optical rotation was measured on JASCOP-1010. HPLC analysis was performed on Shimadzu High Performance Liquid Chromatography (DGU-20A5 Degasser, LC-20AT Liquid Chromatograph, SIL-20AC Auto Sampler, SPD-M20A Diode Array Detector, CTO-20AC Column Oven) using Inerstil ODS-SP (5 μm , 4.6 $\times$ 250 mm) column. UPLC analyses were performed by Acquity UPLC system (Waters, Milford, MS, USA) and a BEH C18 column (2.1 mm $\times$ 100 mm, 1.7 μm). Preparative HPLC was performed on Beijing Chuangxintongheng

LC3000 Semi-preparation Gradient HPLC System using Sepax Amethyst C-18(5 μ m, 21.2 $\times$ 250 mm) column. Column chromatography was performed with silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Qingdao, China).

Fungal Material

Penicillium sp. DT-F29 (deposited in China General Microbiological Culture Collection Center and publically available) was isolated from marine sediments of Dongtou County (Zhejiang province, China, N 27°49'04.44" E 121°07'55.33") and identified through morphology characters and 16S rDNA sequencing (GenBank accession number KT443922).

Fermentation, Extraction and Isolation of main compounds (1, 3-9)

Culture mediums used were PDB/PDA. The PDB medium contained 100g/L potato, 10 g/L glucose and dissolved in sea water; PDA medium contained another 20 g/L agar compared with PDB medium. The fungus was cultivated in 500-mL Erlenmeyer flasks containing 250 mL culture medium under 28 °C for 30 days, and a pre-experimental was carried out for repeatability before large-scale fermentation, and the result indicated a good reproducibility (Figure S3). The 100L broth were extracted with EtOAc three times, and then solvent were evaporated to dryness under reduced pressure to yield 20 g crude extract. The crude extract was subjected to a silica gel column, eluting with a gradient of CH₂Cl₂–CH₃OH(60:1 to 0:1, v/v) to afford seven bioactive fractions containing Brefeldin A analogues (Fr.1–Fr.7). Fr.1 was fractionated on a silica column eluting with PE (petroleum ether) –EtOAc (5:1 to 1:1, v/v) to give eleven subfractions (Fr.1.1–Fr.1.13). Then, Fr.1.7 was finally purified by preparative

HPLC (MeOH–H₂O, 65:35) to afford compounds 3 (10.4 mg) and 9 (29.9 mg). Fr.2 was separated into eight subfractions (Fr.2.1–Fr.2.8) by silica column eluting with PE–EtOAc (4:1 to 1:1, v/v). Fr.2.3 was further purified by preparative HPLC eluting with Fr.2.3 with 65% MeOH–H₂O to afford 5 (11.7 mg) and Fr.2.4 with 35% CH₃CN–H₂O to give 6 (2.7 mg). Fr.3 was subjected to silica gel column using gradient elution with PE–EtOAc (3:1 to 1:1, v/v) to afford nine subfractions (Fr.3.1–Fr.3.9). Fr.3.5 was further purified by preparative HPLC eluting with 50% MeOH–H₂O to give 4 (6.3 mg). Fr.4 was separated into eight subfractions (Fr.4.1–Fr.4.8) by silica gel column using gradient elution with CH₂Cl₂–CH₃OH (25:1 to 10:1, v/v). Fr.4.3 was further purified by preparative HPLC eluting with 55% MeOH–H₂O to give 8 (6.6 mg). Fr.5 was subjected to silica gel column using gradient elution with PE–EtOAc (3:1 to 1:1, v/v) to afford seven subfractions (Fr.5.1–Fr.5.7). Fr.5.5 was further purified by preparative HPLC eluting with 55% MeOH–H₂O to give 1 (200 mg). Fr.7 was fractionated on a silica column eluting gradient elution with CH₂Cl₂–CH₃OH (15:1 to 5:1, v/v) to afford eleven subfractions (Fr.7.1–Fr.7.11). Fr.7.7 was further purified by preparative HPLC eluting with 40% MeOH–H₂O to give 7 (2.4 mg).

7, 7-dimethoxybrefeldin C (**3**), white powder, $[\alpha]_D^{25} +52.1$ (c 0.10, MeOH); UV (MeOH) $\lambda_{\max}$ 206, 225 nm; IR (CH₃OH) $\nu_{\max}$ 3444, 2975, 2935, 1709, 1645, 1451, 1257, 1129, 1048, 1007, 981, 832 cm⁻¹; HRESIMS m/z 671.3766 [2M+Na]⁺, (calcd for C₃₆H₅₆O₈ Na, 671.3771).

6 α -hydroxybrefeldin C (**4**), colorless needles (crystallised in CH₂Cl₂), $[\alpha]_D^{25} +85.6$ (c 0.10, MeOH); UV (MeOH) $\lambda_{\max}$ 204, 223 nm; IR (CH₃OH) $\nu_{\max}$ 3403, 2938, 1706, 1645, 1565,

1449, 1354, 1260, 1127, 1075, 1006, 977, 901, 831 cm^{-1} ; HRESIMS m/z 303.1567 $[\text{M}+\text{Na}]^+$, (calcd for $\text{C}_{16}\text{H}_{24}\text{O}_4$ Na 303.1572).

4-*epi*-15-*epi*-brefeldin A (**5**), colorless needles (crystallised in CH_2Cl_2), $[\alpha]^{25}_{\text{D}}$ -79.4 (c 0.10, MeOH); UV (MeOH) λ_{max} 205, 221 nm; IR (CH_3OH) ν_{max} 3365, 2939, 1794, 1704, 1644, 1562, 1451, 1353, 1265, 1196, 1156, 1091, 1055, 1024, 986, 902, 858 cm^{-1} ; HRESIMS m/z 303.1572 $[\text{M}+\text{Na}]^+$, (calcd for $\text{C}_{16}\text{H}_{24}\text{O}_4$ Na 303.1572).

4-*epi*-8 α -hydroxy-15-*epi*-brefeldin C (**6**), colorless needles (crystallised in CH_2Cl_2), $[\alpha]^{25}_{\text{D}}$ -17.0 (c 0.05, MeOH); UV (MeOH) λ_{max} 208, 221 nm; IR (CH_3OH) ν_{max} 3424, 2980, 1694, 1645, 1562, 1451, 1356, 1273, 1126, 1091, 1056, 985, 898 cm^{-1} ; HRESIMS m/z 303.1572 $[\text{M}+\text{Na}]^+$, (calcd for $\text{C}_{16}\text{H}_{24}\text{O}_4$ Na 303.1572).

Cytotoxicity assays and reactivating latent HIV assays

The cytotoxicity against Human lung adenocarcinoma cell line H1975 was evaluated using cell counting kit-8 (DOjinDo, Japan) assay. H1975 cells were grown in 1640 medium with 10% FBS and seeded at a density of 6×10^3 cells per 200 μL per well in a 96 well plate, and incubated for 24 h. After that, cells were treated with the indicated concentration of compounds for 24 h. Then 10 μL of CCK-8 was added to each well of the plate and incubated for 1–4 h, the absorbance at 450 nm was measured using Varioskan Flash Multimode Reader (Thermo Scientific, USA).

The activity to reactivate latent HIV was investigated on J-Lat clones C11 cells. J-Lat clones C11 cells were washed with phosphate-buffered saline (PBS) and incubated with the

indicated concentrations of agent at different points in time. Cells were washed and resuspended in PBS containing 2% paraformaldehyde. GFP expression was measured by FACSscan (Becton Dickinson FACSscan Flow Cytometer) and FACS data were analyzed with FLOWJO software (Tree Star, CA). GFP-associated fluorescence was differentiated from background fluorescence by gating of live cells (10,000 events total) and two-parameter analysis.

Table S1. ^1H NMR spectroscopic data (δ in ppm, J in Hz) for **1**, **3-6** analogues in CDCl_3

position	1	3	4	5	6
2	5.91, dd (15.7, 1.9)	5.89, dd (15.7, 1.9)	5.93, dd (15.6, 2.0)	6.11, dd (15.5, 2.3)	5.99, dd (15.7, 2.3)
3	7.36, dd (15.7, 3.1)	7.34, dd (15.7, 3.1)	7.25, dd (15.6, 3.2)	6.89, dd (15.5, 2.0)	6.90, dd (15.7, 2.1)
4	4.10, ddd (9.9, 3.1, 1.9)	4.14, ddd (10.1, 3.1, 1.9)	4.32, ddd (9.9, 3.2, 2.0)	4.51, ddd (2.3, 2.2, 2.0)	4.61, ddd (4.9, 2.3, 2.1)
5	1.95, quint(9.9)	1.74, m (overlap)	1.51, m (overlap)	2.20, m	2.26, m
6	2.08, m (overlap)	2.15, m	4.17, q (7.1)	2.09, m	2.15, m
	1.53, m (overlap)	1.95, m		1.84, m (overlap)	1.75, m (overlap)
7	4.37, m		1.93, m 1.65, m	4.29, m	1.75, m (overlap)
8	2.20, m 1.83, m (overlap)	2.10, m 1.58, dd (13.2, 9.6)	1.84, m (overlap) 1.58, m	1.84, m (overlap) 1.39, m	4.13, m

9	2.36, quint (9.9)	2.43, quint (9.6)	2.29, qd (9.3, 6.6)	2.60, dtd (11.7, 9.4, 7.0)	2.37, td (8.1, 4.6)
10	5.28, dd (15.2, 9.9)	5.18, dd (15.2, 9.6)	5.26, dd (15.1, 9.3)	5.17, dd (14.8, 9.4)	5.41, dd (15.3, 8.1)
11	5.70, ddd (15.2, 10.3, 4.6)	5.74, ddd (15.2, 10.3, 4.5)	5.66, ddd (15.1, 10.1, 4.7)	5.30, ddd (14.8, 10.6, 4.1)	5.42, ddd (15.3, 8.4, 4.6)
12	2.02, m (overlap)	2.00, m (overlap)	2.00, m	2.01, m	2.08, m
	1.83, m (overlap)	1.84, m (overlap)	1.84, m (overlap)	1.84, m (overlap)	1.94 (m, 1H)
13	1.83, m (overlap)	1.84, m (overlap)	1.84, m (overlap)	1.69, m (overlap)	1.57, m (overlap)
	0.94, m	0.92, m	0.94, m	1.44, m	
14	1.73, m (overlap)	1.74, m (overlap)	1.73, m	1.69, m (overlap)	1.57, m (overlap)
	1.53, m (overlap)	1.52, m	1.51, m (overlap)	1.58, m	
15	4.86, dqd (12.5, 6.3, 1.7)	4.84, dqd (12.6, 6.2, 1.7)	4.86, dqd (11.0, 6.3, 1.7)	5.24, qt (6.6, 3.4)	5.21, qt (6.6, 3.5)
16	1.26, d (6.3)	1.25, d (6.2)	1.25, d (6.3)	1.21, d (6.6)	1.22, d (6.6)
17		3.22, s			
18		3.20, s			

Table S2. ^{13}C NMR spectroscopic data (δ in ppm, J in Hz) for **1**, **3-6** analogues in CDCl_3

position	1	3	4	5	6
1	166.3, C	166.4, C	166.5, C	166.2, C	166.0, C
2	117.8, CH	117.9, CH	117.8, CH	120.1, CH	119.4, CH
3	151.6, CH	151.6, CH	151.5, CH	151.4, CH	151.3, CH
4	76.1, CH	75.8, CH	75.8, CH	71.6, CH	77.6, CH
5	52.2, CH	50.8, CH	60.6, CH	47.8, CH	47.7, CH
6	41.4, CH_2	38.6, CH_2	79.8, CH	38.2, CH_2	33.2, CH_2
7	72.7, CH	109.4, C	33.7, CH_2	73.5, CH	35.2, CH_2
8	43.4, CH_2	41.1, CH_2	29.9, CH_2	44.1, CH_2	72.1, CH
9	44.4, CH	43.5, CH	43.9, CH	40.4, CH	46.6, CH
10	136.7, CH	135.3, CH	136.5, CH	134.5, CH	130.3, CH
11	130.6, CH	131.4, CH	130.4, CH	133.1, CH	135.6, CH

12	32.0, CH ₂	32.0, CH ₂	31.9, CH ₂	32.8, CH ₂	26.8, CH ₂
13	26.9, CH ₂	26.8, CH ₂	26.9, CH ₂	23.4, CH ₂	23.7, CH ₂
14	34.3, CH ₂	34.2, CH ₂	34.3, CH ₂	32.0, CH ₂	32.9, CH ₂
15	71.9, CH	71.9, CH	71.9, CH	70.2, CH	70.6, CH
16	21.0, CH ₃	21.0, CH ₃	21.0, CH ₃	18.0, CH ₃	18.4, CH ₃
17		49.8, CH ₃			
18		48.8, CH ₃			

Table S3. Bioactivity Assays of Compounds **1**, **3-9**

Compound	IC ₅₀ (H1975)/ μ M	EC ₅₀ (J-Lat clones C11 cells) / μ M
1	<0.2	$3.3 \pm 0.3 \times 10^{-2}$
8	5.2 ± 0.6	>10
3-6, 9	>40	>10
Positive control ^a	4.8 ± 0.2	0.8 ± 0.2

^aPositive control for H1975 and J-Lat clones C11 cells were Fedratinib (Zhang et al. 2015) and Prostratin (DeChristopher et al. 2012), respectively. All results are expressed as mean $\pm$ SD; n = 3 for all groups.

Figure S1. Key HMBC and ¹H-¹H COSY correlations of compound **3-6**

Figure S2. Key NOESY correlations of compound 3-6

Figure S3. The TLC (a) and HPLC (b) analysis for repeatability. (a) PE–EtOAc=1:1 (v/v), (b) MeOH–H₂O:30-100% (0-70 min), 100%(70-85 min). 1 and 2 represent different crude extract of broth fermented in same conditions.

Figure S4. ^1H NMR (600 MHz, CDCl_3) spectra of compound **1**.

Figure S5. ^{13}C NMR(150 MHz, CDCl_3) spectra of compound **1**.

Figure S6. ^1H NMR (600 MHz, DMSO) spectra of compound **1**.

Figure S7. ^{13}C NMR (150 MHz, DMSO) spectra of compound **1**.

Figure S8. HRESIMS spectrum of compound **3**.

Figure S9. ¹H NMR (600 MHz, CDCl₃) spectra of compound **3**.

Figure S10. ^{13}C NMR(150 MHz, CDCl_3) spectra of compound **3**.

Figure S11. DEPT 135 (600 MHz, CDCl_3) spectra of compound **3**.

Figure S12. ^1H - ^1H COSY spectrum of compound **3**.

Figure S13. HSQC spectrum of compound **3**.

Figure S14. HMBC spectrum of compound **3**.

Figure S15. NOESY spectrum of compound **3**.

Figure S16. HRESIMS spectrum of compound **4**.

Figure S17. ^1H NMR (600 MHz, CDCl_3) spectra of compound **4**.

Figure S18. ^{13}C NMR(150 MHz, CDCl_3) spectra of compound **4**.

Figure S19. DEPT 135 (600 MHz, CDCl_3) spectra of compound **4**.

Figure S20. ^1H - ^1H COSY spectrum of compound **4**.

Figure S21. HSQC spectrum of compound **4**.

Figure S22. HMBC spectrum of compound **4**.

Figure S23. NOESY spectrum of compound **4**.

Figure S24. HRESIMS spectrum of compound **5**.

Figure S25. ¹H NMR (600 MHz, CDCl₃) spectra of compound **5**.

Figure S26. ^{13}C NMR(150 MHz, CDCl_3) spectra of compound **5**.

Figure S27. DEPT 135 (600 MHz, CDCl_3) spectra of compound **5**.

Figure S28. ^1H - ^1H COSY spectrum of compound **5**.

Figure S29. HSQC spectrum of compound **5**.

Figure S30. HMBC spectrum of compound **5**.

Figure S31. NOESY spectrum of compound **5**.

Figure S32. HRESIMS spectrum of compound **6**.

Figure S33. ¹H NMR (600 MHz, CDCl₃) spectra of compound **6**.

Figure S34. ^{13}C NMR(150 MHz, CDCl_3) spectra of compound **6**.

Figure S35. DEPT 135 (600 MHz, CDCl_3) spectra of compound **6**.

Figure S36. ^1H - ^1H COSY spectrum of compound **6**.

Figure S37. HSQC spectrum of compound **6**.

Figure S38. HMBC spectrum of compound **6**.

Figure S39. NOESY spectrum of compound **6**.

Figure S40. ^1H NMR (600 MHz, CDOD_3) spectra of compound **7**.

Figure S41. ^{13}C NMR (150 MHz, CDOD_3) spectra of compound **7**.

Figure S42. ^1H - ^1H COSY spectrum of compound **7**.

Figure S43. HSQC spectrum of compound **7**.

Figure S44. HMBC spectrum of compound **7**.

Figure S45. NOESY spectrum of compound **7**.

Figure S46. ^1H NMR (600 MHz, CDCl_3) spectra of compound **8**.

Figure S47. ^{13}C NMR (150 MHz, CDCl_3) spectra of compound **8**.

Figure S48. ^1H NMR (600 MHz, CDCl_3) spectra of compound **9**.

Figure S49. ^{13}C NMR (150 MHz, CDCl_3) spectra of compound **9**.

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