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Pestaloficiols A–E, bioactive cyclopropane derivatives from the plant endophytic fungus *Pestalotiopsis fici*

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Abstract—Pestaloficiols A–E (**1**–**5**), five new cyclopropane derivatives, have been isolated from cultures of the plant endophyte *Pestalotiopsis fici*. The structures of these compounds were determined by NMR spectroscopy, and the absolute configuration of **1** was assigned using the modified Mosher method. Compounds **1**, **2**, and **4** displayed inhibitory effects on HIV-1 replication in C8166 cells.

1. Introduction

The fungal species of the genus *Pestalotiopsis* have been demonstrated to be rich sources of bioactive secondary metabolites with diverse structural features.[1–10] Our prior chemical studies of three different species of this genus have led to the isolation of a variety of new bioactive metabolites,[11–13] such as chloropupukeananin, the first chlorinated pupukeanane derivative possessing the highly functionalized tricyclo-[4.3.1.0^{3,7}]-decane skeleton, isolated as an anti-HIV-1 component from *Pestalotiopsis fici* (W106-1).[13] In the course of this work, a subculture of the same fungal strain was grown in another fermentation culture to examine its metabolic profile under different growing conditions. Although its organic solvent extract displayed similar magnitude of inhibitory effects to that of chloropupukeananin on HIV-1 replication in C8166 cells, HPLC analysis revealed the presence of totally different secondary metabolites in the crude extract. Bioassay-guided fractionation of this extract afforded five new metabolites, which have been named pestaloficiols A–E (**1**–**5**). Details of the isolation, structure elucidation, and biological activity of these compounds are reported herein.

2. Results and discussion

Pestaloficiol A (**1**) was obtained as colorless oil. It was assigned as a molecular formula of $C_{16}H_{22}O_4$ (six degrees of unsaturation) on the basis of its HRESIMS [*m*/*z* 301.1402 $(M + Na)^+$; Δ + 0.8 mmu] and NMR data (Table 1). Detailed analysis of the ^{1}H, ^{13}C, and HMQC NMR spectroscopic data of **1** revealed the presence of one exchangeable proton, four methyl groups, three methylene units, three methines (two of which are oxymethines), three sp^3 quaternary carbons (two of which are oxygenated), one tetra substituted olefin unit, and one α,β-unsaturated ketone carbon (δ_C 190.0). These data accounted for all ^{1}H and ^{13}C NMR resonances and required the compound to be tetracyclic. Interpretation of the ^{1}H–^{1}H COSY NMR data led to the identification of two isolated proton spin-systems corresponding to the C-5–C-7 (including OH-6) and C-11–C-12 subunits of structure **1**. HMBC correlations from H-5b to C-4, C-4a, and C-8a led to the connection of C-4a to C-4, C-5, and C-8a. In turn, correlations of H-7 with C-8, C-11, and C-12, as well as H-11 and H_2-12 with C-7, C-8, and C-8a indicated that C-8 was attached to both C-7 and C-8a, leading to the identification of a

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Table 1. NMR spectroscopic data of pestaloficiol (**1**) in acetone-d_6

Position	δ_H[a] (J in Hz)	δ_C[b]	HMBC (H → C#)
2		81.0	
3	2.39, d (17); 2.45, d (17)	47.8	2, 4, 4a, 13, 14
4		190.0	
4a		107.0	
5a	2.16, dd (16, 2.0)	26.6	4a, 7, 8a
5b	2.80, dd (16, 4.1)		4, 4a, 6, 7, 8a
6	3.93, dddd (4.1, 4.0, 3.5, 2.0)	65.8	
7	4.06, d (3.5)	84.4	6, 8, 10, 11, 12
8		33.3	
8a		167.8	
10		82.9	
11	2.07, dd (8.5, 5.5)	39.6	7, 8, 8a, 10, 12, 16
12a	0.90, dd (5.5, 4.0)	17.9	7, 8, 8a, 10, 11
12b	1.44, dd (8.5, 4.0)		7, 8, 8a, 10, 11
13	1.34, s	26.5	2, 3, 14
14	1.35, s	26.0	2, 3, 13
15	1.19, s	26.8	10, 11, 16
16	1.48, s	29.6	10, 11, 15
OH-6	3.49, d (4.0)		5, 6

[a] Recorded at 400 MHz.
[b] Recorded at 100 MHz.

cyclopropane ring and a cyclohexene unit that are joined spirally at C-8. Other correlations from H_3-15 and H_3-16 to C-10 and C-11 indicated that C-11, C-15, and C-16 are all connected to C-10. HMBC correlations from H_2-3 to C-4 and C-4a and H_3-13 and H_3-14 to C-2 and C-3 permitted completion of the C-2–C-4a substructure of **1**, with C-13 and C-14 directly attached to C-2. A key HMBC correlation from H-7 to C-10 established the tetrahydrofuran subunit of **1**. Considering the NMR chemical shifts of C-2 (δ_C 81.0) and C-8a (δ_C 167.8), as well as the tetracyclic nature of **1**, these two carbons have to be connected to the remaining oxygen atom to form a dihydropyranone moiety. On the basis of these data, the planar structure of pestaloficiol A was established as shown in Figure 1.

The relative configuration of pestaloficiol A (**1**) was assigned by analysis of ^1H–^1H coupling constants and NOESY correlations (Fig. 2). The small coupling constants observed between H_2-5 and H-6 (2.0 and 4.1 Hz, respectively) indicated that H-6 is in a pseudoequatorial orientation with respect to the corresponding cyclohexene ring. The large coupling constant of 8.5 Hz between H-11 and H-12b of the cyclopropane ring revealed that these protons have a *syn*-relative orientation.[14] NOESY correlations of H-7 with H-5a and H-12a, and H-12a with H_3-15 indicated that these protons are all on the same face of the ring system, with H-7 adopting a pseudoaxial orientation. Other correlations from H_3-16 to H-11 and the exchangeable proton OH-6 were used to place these protons on the opposite face of the molecule, thereby establishing the relative configuration of pestaloficiol A (**1**).

The absolute configuration of pestaloficiol A (**1**) was assigned by application of the modified Mosher method.[15,16] Treatment of **1** with (*S*)-MTPA Cl and (*R*)-MTPA Cl afforded the *R*-MTPA ester (**1a**) and *S*-MTPA ester (**1b**), respectively. The difference in chemical shift values ($\Delta\delta = \delta_S - \delta_R$) for the diastereomeric esters **1b** and **1a** were calculated in order to assign the absolute configuration at C-6 (Fig. 3). Calculations for all the relevant signals except H-11 suggested the *S*absolute configuration at C-6. Therefore, all relevant chiral centers in pestaloficiol A (**1**) were assigned the 6*S*, 7*R*, 8*S*, and 11*S* absolute configurations on the basis of the $\Delta\delta$ results summarized in Figure 3.

The molecular formula of pestaloficiol B (**2**) was determined to be $C_{16}H_{24}O_5$ (five degrees of unsaturation) on the basis of HRESIMS [*m*/*z* 319.1524 $(M + Na)^+$; Δ – 0.8 mmu] analysis and the NMR data (Table 2), which is 18 mass units higher than that of compound **1**. Analysis of the ^1H, ^{13}C, and HMQC NMR data of **2** revealed the presence of structural features similar to those found in **1**, except that resonances for two more exchangeable protons were observed in the ^1H NMR

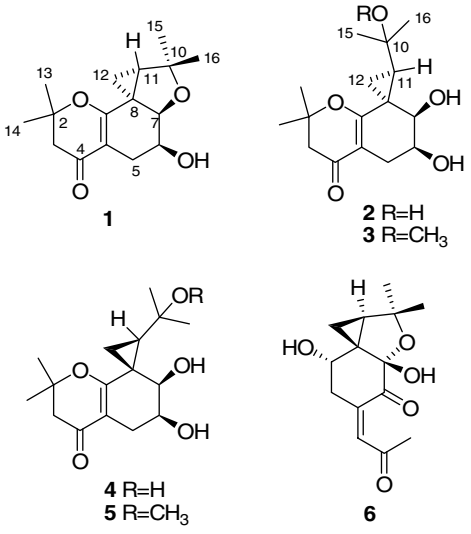

Figure 1. Structures of pestaloficiols A–E (**1**–**5**) and papyracon A (**6**).

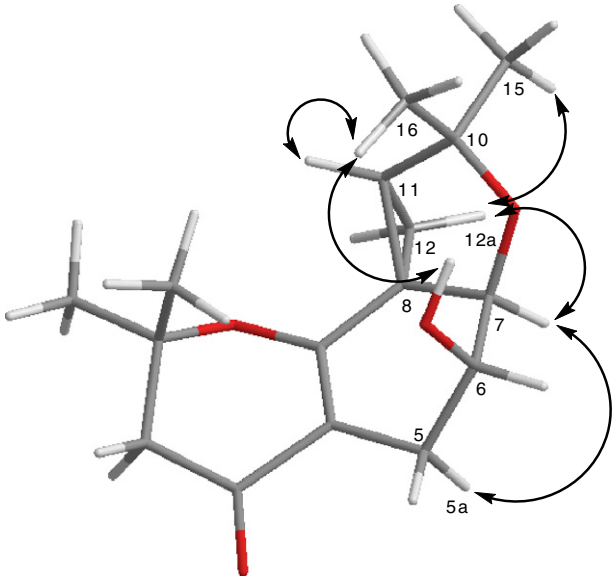

Figure 2. Key NOESY correlations for pestaloficiol A (**1**).

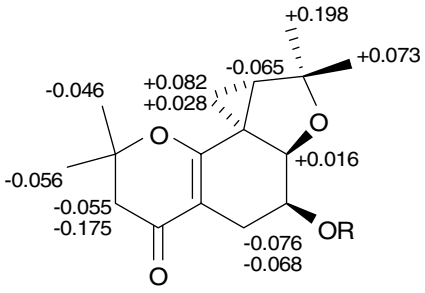

Figure 3. $\Delta\delta$ values (in ppm) = $\delta_S - \delta_R$ for (*S*)- and (*R*)-MPTA esters **1a** and **1b**.

spectrum of **2**. COSY correlation of one exchangeable proton (δ_H 3.89) with H-7 led to the connection of a hydroxy group to C-7; therefore, the other exchangeable proton (δ_H 3.20) was assigned to the hydroxy group attached to C-10. This conclusion was indirectly supported by lack of HMBC correlation from H-7 to C-10. On the basis of these considerations, the gross structure of pestaloficiol B was proposed as shown. The relative configuration of **2** was also assigned by analysis of ^1H–^1H coupling constants and NOESY data (Fig. 4). The large *trans*-diaxial-type coupling constant observed between H-5a and H-6 (10 Hz) indicated that H-5a and H-6 are in pseudoaxial orientations, and a small coupling constant of 2.0 Hz between H-6 and H-7 places H-7 in a pseudoequatorial orientation with respect to the corresponding cyclohexene ring. NOESY

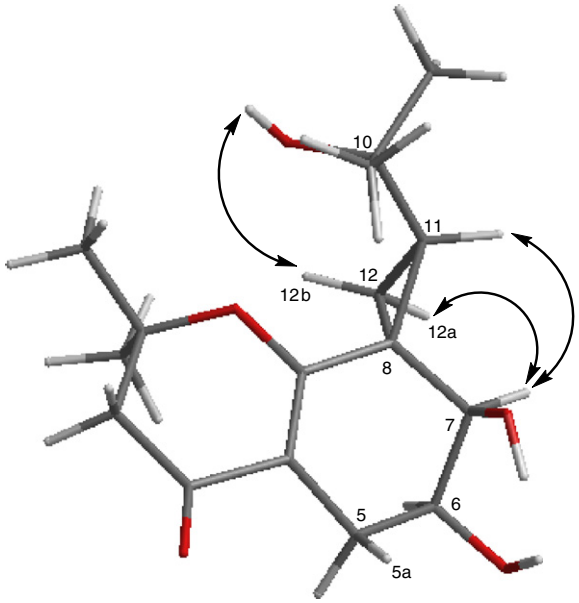

Figure 4. Key NOESY correlations for pestaloficiol B (**2**).

correlations of H-7 with H-11 and H-12a revealed their proximity in space. Therefore, the relative configuration of pestaloficiol B was proposed as shown. The absolute configuration of **2** was deduced on the basis of biosynthetic considerations and by analogy to **1**.

The molecular formula of pestaloficiol C (**3**) was established as $C_{17}H_{26}O_5$ (five degrees of unsaturation) by analysis of its HRESIMS [*m*/*z* 333.1680 $(M + Na)^+$;

Table 2. NMR spectroscopic data of pestaloficiols B–E (**2**–**5**)

Position	Pestaloficiol B (**2**)		Pestaloficiol C (**3**)		Pestaloficiol D (**4**)		Pestaloficiol E (**5**)	
	δ_H[a] (*J* in Hz)	δ_C[b]	δ_H[a] (*J* in Hz)	δ_C[c]	δ_H[a] (*J* in Hz)	δ_C[c]	δ_H[a] (*J* in Hz)	δ_C[c]
2		81.8		80.6		80.2		80.0
3	2.45, d (17); 2.59, d (17)	47.6	2.34, d (17); 2.45, d (17)	47.2	2.31, d (17); 2.42, d (17)	47.6	2.30, d (17); 2.44, d (17)	47.0
4		190.0		190.6		190.3		191.0
4a		112.0		109.7		107.7		106.4
5a	2.35, dd (16, 10)	26.9	2.35, dd (16, 11)	26.5	2.29, dd (16, 10)	25.4	2.27, dd (16, 11)	24.9
5b	2.65, dd (16, 6.5)		2.66, dd (16, 6.5)		2.61, dd (16, 6.5)		2.64, dd (16, 5.5)	
6	3.94, ddd (10, 6.5, 2.0)	68.5	4.04, dddd (11, 7.0, 6.5, 2.0)	67.9	3.94, dddd (10, 6.5, 6.0, 2.0)	69.8	3.90, dddd (11, 6.5, 5.5, 2.0)	68.6
7	3.10, br d (2.0)	80.3	3.09, dd (3.5, 2.0)	80.0	4.46, dd (3.0, 2.0)	69.6	4.22, dd (3.0, 2.0)	68.5
8		32.4		30.7		31.8		29.6
8a		165.7		165.7		168.3		167.8
10		68.9		74.0		69.2		73.8
11	1.39, dd (9.5, 7.5)	41.7	1.28, dd (10, 8.0)	39.4	1.56, dd (9.5, 7.8)	37.3	1.59, dd (9.0, 8.0)	35.1
12a	1.13, dd (9.5, 5.0)	13.1	1.11, dd (10, 5.0)	13.8,	1.25, dd (9.5, 5.5)	12.9	1.25, dd (9.0, 5.5)	12.1
12b	1.64, dd (7.5, 5.0)		1.57, dd (8.0, 5.0)		1.33, dd (7.8, 5.5)		1.28, dd (8.0, 5.5)	
13	1.36, s	25.6	1.33, s	24.0	1.30, s	25.0	1.27, s	24.9
14	1.41, s	26.8	1.39, s	25.5	1.33, s	27.3	1.30, s	26.0
15	1.23, s	30.0	1.17, s	26.7	1.29, s	33.0	1.26, s	26.3
16	1.24, s	31.5	1.21, s	27.1	1.34, s	31.0	1.33, s	26.1
OH-6	3.68, br s		3.60, d (7.0)		3.59, d (6.0)		3.62, d (6.5)	
OH-7	3.89, br s		3.82, d (3.5)		3.69, d (3.0)		3.58, d (3.0)	
OH-10	3.20, s				3.71, s			
OMe-10			3.17, s	49.7			3.16, s	49.3

[a] Recorded at 400 MHz in acetone-d_6.
[b] Recorded at 100 MHz in acetone-d_6.
[c] Recorded at 100 MHz in $CDCl_3$.

Δ − 0.8 mmu] and NMR data (Table 2). The ^{1}H and ^{13}C NMR data of **3** closely resembled those of **2**, except that the resonance for a hydroxy proton at 3.20 ppm (OH-10) was replaced by the resonances for a methyl group (δ_H/δ_C 3.17/49.7) in its NMR spectra, which was consistent with the HRESIMS data for **3**. Therefore, pestaloficiol C (**3**) was identified as the methyl ether of **2**, and its absolute configuration was deduced as shown by analogy to **2**.

Pestaloficiol D (**4**) was assigned the same molecular formula $C_{16}H_{24}O_5$ as that of pestaloficiol B (**2**) on the basis of its HRESIMS [*m*/*z* 319.1512 $(M + Na)^+$; Δ +0.4 mmu] and NMR data (Table 2). Analysis of its ^{1}H–^{1}H COSY and HMBC NMR data established the same planar structure as that of **2**, indicating that **4** could be a stereoisomer of **2**. The ^{1}H–^{1}H coupling patterns for H-5a, H-6, and H-7 indicated that H-5a and H-6 are pesudoaxial-oriented and H-7 is pseudoequatorial-oriented, and such assignments were further supported by NOESY correlation of H-5a with OH-7 (Fig. 5). The vicinal coupling constant of 9.5 Hz between H-11 and H-12a of the cyclopropane ring in **4** again suggested a *cis*-configuration.[14] Considering the significant difference in chemical shift values for H-7 and C-7 in compounds **4** and **2** (δ_H/δ_C 4.46/69.6 in **4** versus 3.10/80.3 in **2**), the relative configurations at C-8 and C-11 in **4** could be different from those in **3**. NOESY correlations of H-7 with H_3-15 and H-12b with OH-7 indicated that these protons are close to each other in space. These data indicated that **4** is a stereoisomer of **2** at C-8 and C-11. The absolute configuration of **4** was presumed to be analogous to that of **2**.

The elemental composition of pestaloficiol E (**5**) was determined as $C_{17}H_{26}O_5$ (five degrees of unsaturation) on the basis of HRESIMS and NMR data (Table 2), which is 14 mass units higher than that of **4**. Analysis of its ^{1}H and ^{13}C NMR data revealed the presence of a methoxy group (δ_H/δ_C 3.16/49.3) in **5**, rather than a hydroxy group attached to C-10 (δ_H 3.71) in **4**, and this change was also consistent with its HRESIMS data. On the basis of these considerations, the structure of **5** was established as the methyl ether of **4**. Based on the similarities of the coupling constant values with those observed for **4**, compound **5** was proposed to have the same relative configurations at C-6, C-7, C-8, and C-11 as in **4**.

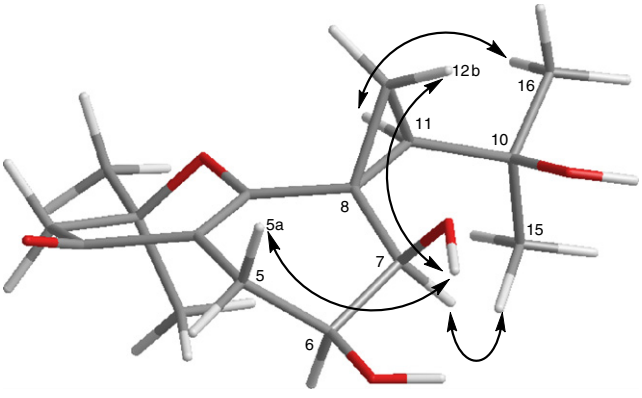

Figure 5. Key NOESY correlations for pestaloficiol D (**4**).

Pestaloficiols A (**1**), B (**2**), and D (**4**) were tested for in vitro activity against HIV-1, and they showed inhibitory effects on HIV-1 replication in C8166 cells, with EC_{50} values of 26.0, 98.1, and 64.1 μM, respectively (all three compounds showed CC_{50} values of greater than 200 μM; the positive control indinavir sulfate showed an EC_{50} value of 8.81 nM). Pestaloficiols C (**3**) and E (**5**) were not tested due to sample limitations.

Pestaloficiols A–E (**1**–**5**) are new members of the chromenone type of metabolites with a cyclopropane moiety joined spirally at C-8 to a cyclohexene unit. Biogenetically, these compounds could be derived from two units of prenoids and one polyketide. Pestaloficiol A (**1**) is structurally related to the known compound papyracon A (**6**).[17] However, **1** differs significantly from its naturally occurring precedent by virtue of the presence of an additional dihydropyranone ring fused to the cyclohexene unit to form a previously undescribed skeleton among members of this class. Pestaloficiols B–E (**2**–**5**) are the ring-opening products of **1**, and the absolute configuration of C-11 in **2** and **3** (11*R*) is reversed compared to that of **1** (11*S*). The absolute configurations for C-8 (8*R*) and C-11 (11*R*) in **4** and **5** are opposite to those in **1** (8*S* and 11*S*, respectively) due to the change of orientation for the cyclopropane ring. The discovery of these new bioactive secondary metabolites further demonstrated that the plant endophytic fungi could be useful sources for new bioactive natural products.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and UV data were recorded on a Hitachi U-2800 spectrophotometer. IR data were recorded using a Bruker Vertex 70 spectrophotometer. ^{1}H and ^{13}C NMR data were acquired with a Bruker Avance-400 spectrometer using solvent signals (acetone-d_6: δ_H 2.05/δ_C 29.8, 206.1; $CDCl_3$: δ_H 7.26/δ_C 77.0; pyridine-d_5: δ_H 7.21, 7.58, and 8.73) as references. The HMQC and HMBC experiments were optimized f or 145.0 and 8.0 Hz, respectively. ESIMS data were recorded on a Bruker Esquire 3000^{plus} spectrometer, and HRESIMS data were obtained using a Bruker APEX III 7.0 T spectrometer.

3.2. Fungal material

The culture of *P. fici* was isolated by one of the authors (L.G.) from the branches of an unidentified tree in suburb of Hangzhou, in April 2005. The isolate was identified and assigned the Accession No. W106-1 in L.G.'s culture collection at the Institute of Microbiology, Chinese Academy of Sciences, Beijing. The fungal strain was cultured on slants of potato dextrose agar (PDA) at 25 °C for 10 days. The agar plugs were used to inoculate 250-mL Erlenmeyer flasks, each containing 50 mL

of media (0.4% glucose, 1% malt extract, and 0.4% yeast extract), and the final pH of the media was adjusted to 6.5 before sterilization. Flask cultures were incubated at 25 °C on a rotary shaker at 170 rpm for five days. Ten 500-mL Erlenmeyer flasks, each containing 150 mL of liquid media (2% maltose, 6% dextrin, 0.7% peptone, 0.75% cotton-seed meal, 0.25% $MgSO_4 \cdot 7H_2O$, 0.25% $CaCO_3$, 0.1% $FeSO_4 \cdot 7H_2O$, and 0.001% $ZnSO_4 \cdot 7H_2O$; final pH 6.0) and 30 g of vermiculite, were individually inoculated with 15 mL of the seed culture and incubated at 25 °C under static conditions for 40 days.

3.3. Extraction and isolation

The fermented material was extracted with EtOAc (3× 300 mL), and the organic solvent was evaporated to dryness under vacuum to afford 3.2 g of crude extract. The extract was fractionated by Silica gel VLC using CH_2Cl_2–MeOH gradient elution. The fraction eluted with 98:2 CH_2Cl_2–MeOH (69 mg) was subsequently fractionated by Sephadex LH–20 column chromatography using $CHCl_3$–CH_3OH (50:50) as solvents. One subfraction (20 mg) was further separated by semi-preparative reversed-phase HPLC (Kramosil C_{18} column; 10-μm; 10 × 250 mm; 2 mL/min) to afford pestaloficiols C–E (**3**–**5**; **3**, 2.0 mg, t_R16.5 min; **4**, 6.0 mg, t_R 15.6 min; **5**, 2.2 mg, t_R 20.6 min; 50% to 80% MeOH in water over 23 min). The Silica gel VLC fractions eluted with 97:3 (81 mg) and 95:5 (43 mg) CH_2Cl_2–MeOH were fractionated again by Sephadex LH-20 column chromatography using $CHCl_3$–CH_3OH (50:50) as eluents. Purification of obtained subfractions using different gradients afforded pestaloficiols A (**1**; 3.0 mg; t_R 15.0 min; 60% to 70% MeOH in water over 14 min) and B (**2**; 2.8 mg, t_R 14.0 min; 40% to 60% CH_3OH in water over 20 min).

3.3.1. Pestaloficiol A (1). Colorless oil; $[\alpha]_D$ +69 (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{max} 293 (ε 28100) nm; IR (neat) ν_{max} 3416, 2976, 2910, 1718, 1644, 1591, 1432, 1260, 1165 cm^{-1}; ^1H, ^{13}C NMR, and HMBC data, see Table 1; NOESY correlations (acetone-d_6, 400 MHz) H-5a ↔ H-7; H-5b ↔ OH-6; H-7 ↔ H-5a, H-12a; H-11 ↔ H_3-16; H-12a ↔ H-7, H_3-15; H_3-15 ↔ H-7, H-12a; H_3-16 ↔ H-11, OH-6; OH-6 ↔ H-5b, H_3-16; HRESIMS obsd *m*/*z*301.1402 $(M + Na)^+$, calcd for $C_{16}H_{22}O_4Na$, 301.1410.

3.3.2. Pestaloficiol B (2). Colorless oil; $[\alpha]_D$ +22 (*c* 0.05, CH_3OH); UV (CH_3OH) λ_{max} 292 (ε 10300) nm; IR (neat) ν_{max} 3409, 2973, 2930, 1726, 1652, 1590, 1413, 1250, 1115 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HMBC data (acetone-d_6, 400 MHz) H-3a → C-2, 4, 4a, 13, 14; H-3b → C-2, 4, 13, 14; H-5a → C-4a, 6, 8a; H-5b → C-4, 4a, 6, 7, 8a; H-7 → C-5, 6, 8, 8a, 11, 12; H-11 → C-7, 8, 8a, 10, 12, 16; H-12a → C-7, 8, 8a, 10, 11; H-12b → C-7, 8, 8a, 10, 11; H_3-13 → C-2, 3, 14; H_3-14 → C-2, 3, 13; H_3-15 → C-10, 11, 16; H_3-16 → C-10, 11, 15; NOESY correlations (DMSO-d_6, 400 MHz) H-7 ↔ H-11, H-12a; H-11 ↔ H-7, H_3-16; H-12a ↔ H-7, 11; H-12b ↔ OH-10; HRESIMS obsd *m*/*z* 319.1524 $(M+Na)^+$, calcd for $C_{16}H_{24}O_5Na$, 319.1516.

3.3.3. Pestaloficiol C (3). Colorless oil; $[\alpha]_D$ +10 (*c* 0.08, CH_3OH); UV (CH_3OH) λ_{max} 296 (ε 5770) nm; IR (neat) ν_{max} 3394, 2976, 2934, 2827, 1651, 1586, 1419, 1256, 1163 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HRESIMS obsd *m*/*z* 333.1680 $(M + Na)^+$, calcd for $C_{17}H_{26}O_6Na$, 333.1672.

3.3.4. Pestaloficiol D (4). Colorless oil; $[\alpha]_D$ −14 (*c* 0.05, CH_3OH); UV (CH_3OH) λ_{max} 291 (ε 22900) nm; IR (neat) ν_{max} 3372, 2974, 2934, 1723, 1637, 1581, 1419, 1219, 1165 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HMBC data (acetone-d_6, 400 MHz) H-3a → C-2, 4, 13, 14; H-3b → C-2, 4, 13, 14; H-5a → C-4, 4a, 6, 7, 8a; H-5b → C-4, 4a, 6, 7, 8a; H-7 → C-5, 6, 8, 8a, 11, 12; H-11 → C-7, 8, 8a, 10, 12; H-12a → C-7, 8, 8a, 10, 11; H-12b → C-7, 8, 8a, 10, 11; H_3-13 → C-2, 3, 14; H_3-14 → C-2, 3, 13; H_3-15 → C-10, 11, 16; H_3-16 → C-10, 11, 15; NOESY correlations (DMSO-d_6, 400 MHz) H-5a ↔ OH-6, OH-7; H-6 ↔ H_3-15; H-7 ↔ OH-10, H_3-15; H-11 ↔ H_3-16; H-12b ↔ OH-7, H_3-16; HRESIMS obsd *m*/*z* 319.1512 $(M + Na)^+$, calcd for $C_{16}H_{24}O_5Na$, 319.1516.

3.3.5. Pestaloficiol E (5). Colorless oil; $[\alpha]_D$ −8 (*c* 0.05, CH_3OH); UV (CH_3OH) λ_{max} 291 (ε 13300) nm; IR (neat) ν_{max} 3395, 2974, 2936, 2831, 1732, 1651, 1587, 1413, 1250, 1161 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HRESIMS obsd *m*/*z* 333.1654 $(M + Na)^+$, calcd for $C_{17}H_{26}O_6Na$, 333.1672.

3.3.6. Preparation of (*R*)-MTPA ester (1a) and (*S*)-MTPA ester (1b). A sample of **1** (1.0 mg, 0.004 mmol), (*S*)-MPTA Cl (2.0 μL, 0.011 mmol), and pyridine-d_5 (0.5 mL) was allowed to react in an NMR tube at ambient temperature for 24 h, with the ^1H NMR data of the *R*-MTPA ester derivative (**1a**) were obtained directly on the reaction mixture: ^1H NMR (pyridine-d_5, 400 MHz) δ 5.54 (1H, ddd, J = 4.1, 4.0, 2.0 Hz, H-6), 4.40 (1H, d, J = 4.1 Hz, H-7), 2.58 (2H, s, H_2-3), 2.53 (1H, dd, J = 16, 4.0 Hz, H-5b), 2.14 (1H, dd, J = 8.5, 6.0 Hz, H-11), 1.72 (1H, dd, J = 16, 2.0 Hz, H-5a), 1.40 (1H, d, J = 8.5, 4.5 Hz, H-12b), 1.33 (3H, s, H_3-16), 1.29 (6H, s, H_3-13/H_3-14), 1.00 (1H, d, J = 6.0, 4.5 Hz, H-12a), 0.88 (3H, s, H_3-15).

Similarly, the reaction mixture from another sample of **1** (1.0 mg, 0.004 mmol), (*R*)-MPTA Cl (2.0 μL, 0.011 mmol), and pyridine-d_5 (0.5 mL) was processed as described above for **1a** to afford **1b**: ^1H NMR (pyridine-d_5, 400 MHz) δ 5.53 (1H, ddd, J = 4.1, 4.0, 2.0 Hz, H-6), 4.41 (1H, d, J = 4.1 Hz, H-7), 2.53 (1H, d, J = 17, H-3b), 2.45 (1H, dd, J = 16, 4.0 Hz, H-5b), 2.41 (1H, d, J = 17, H-3a), 2.07 (1H, dd, J = 8.5, 6.0 Hz, H-11), 1.65 (1H, dd, J = 16, 2.0 Hz, H-5a), 1.48 (1H, d, J = 8.5, 4.5 Hz, H-12b), 1.40 (3H, s, H_3-16), 1.24 (3H, s, H_3-13), 1.23 (3H, s, H_3-14), 1.03 (1H, d, J = 6.0, 4.5 Hz, H-12a), 1.08 (3H, s, H_3-15).

3.4. Anti-HIV bioassays

Anti-HIV assays included cytotoxicity and HIV-1 replication inhibition evaluations. The cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-

nyltetrazolium bromide (MTT) method as described in the literature.[18] Cells (3×10^4/well) were seeded into a 96-well microtiter plate in the absence or presence of various concentrations of test compounds in triplicate and incubated at 37 °C in a humid atmosphere of 5% CO_2. After a 4-day incubation, cell viability was measured by the MTT method. The concentration that caused the reduction of viable cells by 50% (CC_{50}) was determined. In parallel with the MTT assay, a HIV-1 replication inhibition assay was determined by p24 antigen capture ELISA.[19] C8166 cells were exposed to HIV-1 (MOI = 0.058) at 37 °C for 1.5 h, washed with PBS (phosphate-buffered saline) to remove free viruses, and then seeded into a 96-well microtiter plate at 3×10^4 cells per well in the absence or presence of test compounds (indinavir sulfate was used as positive control). After 4 days, the supernatant was collected and inactivated by 0.5% Triton X-100. The supernatant was diluted three times, added to the plate coating with anti-p24 McAb (provided by Dr. Bin Yan, Wuhan Institute of Virology, Wuhan, People's Republic of China), and incubated at 37 °C for 1 h. After washing 5 times with PBST (phosphate-buffered saline with Tween-20), the HRP (horseradish peroxidase) labeled anti-p24 antibody (provided by Dr. Bin Yan) was added and incubated at 37 °C for 1 h. The plate was washed 5 times with PBST, followed by adding OPD (ortho-phenylenediamine) reaction mixture. The assay plate was read at 490 nm using a micro plate reader within 30 min. The EC_{50} values based on p24 antigen expression level were calculated.

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