

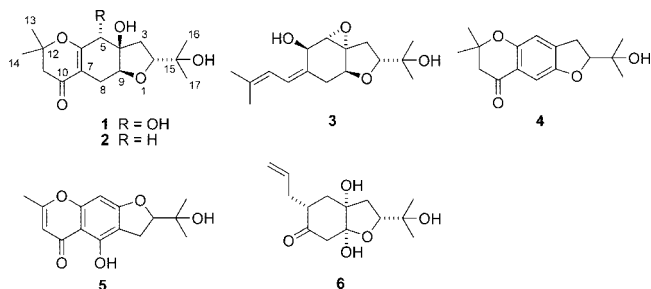
Pestalotheols A–D, Bioactive Metabolites from the Plant Endophytic Fungus *Pestalotiopsis theae*Erwei Li,[†] Renrong Tian,[‡] Shuchun Liu,[†] Xulin Chen,^{*,‡} Liangdong Guo,[†] and Yongsheng Che^{*,†}

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Pestalotheols A–D (**1–4**), four new metabolites, have been isolated from cultures of an isolate of the plant endophytic fungus *Pestalotiopsis theae*. The structures of these compounds were determined by NMR spectroscopy, and **1** is further confirmed by X-ray crystallography. The absolute configurations of compounds **1** and **3** were assigned by application of the modified Mosher method. Pestalotheol C (**3**) displayed an inhibitory effect on HIV-1_{LAI} replication in C8166 cells with an EC₅₀ value of 16.1 μ M.

Plant endophytic fungi are well-known as sources of bioactive secondary metabolites.^{1,2} *Pestalotiopsis* species are very common in their distribution, occurring on a wide range of substrata, and many are saprobes, while others are either pathogenic or endophytic on living plant leaves and twigs.³ Chemical investigations of some *Pestalotiopsis* spp. have afforded a variety of bioactive natural products.^{4–14} *Pestalotiopsis theae* is known as a causal fungus for tea gray blight disease, and chemical studies of this fungus have led to the identification of phytotoxins and plant growth regulators.^{15–17} During an ongoing search for new bioactive metabolites from plant endophytic fungi, a subculture of an isolate of *P. theae* (LN560), obtained from branches of an unidentified tree on Jianfeng Mountain, Hainan Province, People's Republic of China, was grown in solid-substrate fermentation culture. Its organic solvent extract displayed an inhibitory effect on HIV-1_{LAI} replication in C8166 cells. Bioassay-guided fractionation of this extract afforded four new compounds, which have been named pestalotheols A–D (**1–4**). Details of the isolation, structure elucidation, and biological activity of these compounds are reported herein.



Results and Discussion

The molecular formula of pestalotheol A (**1**) was determined to be C₁₆H₂₄O₆ (five degrees of unsaturation) by analysis of its HRESIMS (*m/z* 335.1466 [M + Na]⁺; Δ +0.4 mmu) and NMR data (Table 1). Analysis of the ¹H, ¹³C, and HMQC NMR spectroscopic data of **1** revealed the presence of three exchangeable protons, four methyl groups, three methylene units, three oxymethines, three oxygenated quaternary carbons, two olefinic carbons, and one α,β -conjugated carbonyl carbon (δ_C 192.6). These data accounted for all ¹H and ¹³C NMR resonances and required the compound to be tricyclic. Analysis of the ¹H–¹H COSY NMR data

led to the identification of three isolated proton spin-systems corresponding to the C-2–C-3, C-5–C5–OH, and C-8–C-9 fragments of structure **1**. HMBC correlations from H-2 to C-3 and C-9, from H-3a to C-2 and C-4, and from H-3b to C-4 and C-9 established the tetrahydrofuran subunit. In turn, correlations from H-5 to C-4, C-6, C-7, and C-9, the exchangeable proton at 4.95 ppm to C-4, C-5, and C-6, H₂-8 to C-4, C-6, C-7, and C-9, and H-9 to C-4 and C-5 led to the identification of a reduced furanocyclohexene moiety with a hydroxy group attached to C-5. Other correlations of H₃-16 and H₃-17 with C-2 and C-3 indicated that C-2, C-16, and C-17 are all connected to C-15, while those from H₃-13 and H₃-14 to C-11 and C-12, H₂-11 to C-7 and C-10, and H₂-8 to C-10 completed the C-10–C-14 substructure of **1**, with C-10 directly attached to C-7. Key HMBC correlations from the second exchangeable proton (δ_H 3.86) to C-3, C-4, C-5, and C-9 and the third exchangeable proton (δ_H 3.16) to C-2, C-15, C-16, and C-17 indicated that C-4 and C-15 are attached to free hydroxy groups. Considering the chemical shifts of C-6 (δ_C 167.5) and C-12 (δ_C 80.6), as well as the unsaturation requirement for **1**, C-6 and C-12 have to be connected to the same oxygen atom to form a dihydropyranone moiety. On the basis of these data, the gross structure of pestalotheol A was established as **1**.

The relative configuration of pestalotheol A (**1**) was assigned by analysis of ¹H–¹H coupling constants and NOESY data (Table 1). The large *trans*-diaxial-type coupling constant observed between H-8b and H-9 (11 Hz) indicated that both protons are in pseudoaxial orientations with respect to the corresponding cyclohexene ring. NOESY correlations from the exchangeable proton OH-4 with H-2 and H-8b indicated that these protons are all on the same face of the ring system, whereas correlations from H-9 to OH-5 were used to place them on the opposite face of the molecule, thereby establishing the relative configuration of pestalotheol A as **1**. Ultimately, the structure of **1** was confirmed by X-ray crystallographic analysis, and a perspective ORTEP plot is shown in Figure 1.

The absolute configuration of pestalotheol A (**1**) was assigned by application of the modified Mosher method.^{18,19} 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** was calculated in order to assign the absolute configuration at C-5. Calculations for all of the relevant signals except one (H₃-16) suggested the *R* absolute configuration at C-5. Therefore, the 2*R*, 4*R*, 5*R*, and 9*S* absolute configuration was proposed for compound **1** on the basis of the $\Delta\delta$ results summarized in Figure 2.

Pestalotheol B (**2**) was assigned a molecular formula of C₁₆H₂₄O₅ (five degrees of unsaturation) on the basis of its HRESIMS (*m/z*

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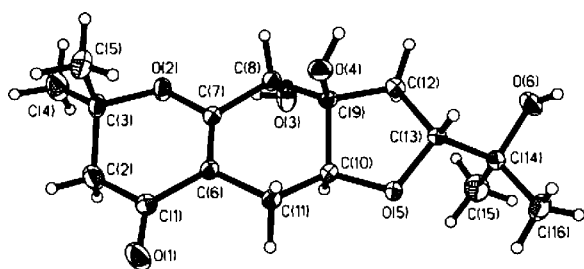
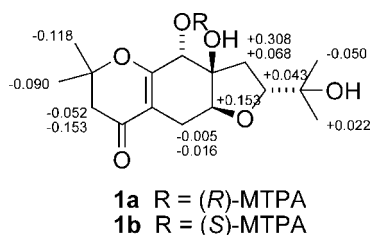
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Table 1. NMR Spectroscopic Data of Pestalothel A (**1**) in Acetone-*d*₆

position	δ_{H}^a (J in Hz)	δ_{C}^b , mult.	HMBC (H \rightarrow C#)	NOESY
2	4.12, dd (10, 6.4)	85.8, CH	3, 9, 15, 16, 17	4-OH, 16, 17
3a	2.49, dd (13, 10)	35.2, CH ₂	2, 4, 15	5, 5-OH, 9, 16, 17
3b	1.76, dd (13, 6.4)		4, 9	4-OH, 5
4		80.1, qC		
5	4.08, d (5.7)	72.8, CH	4, 6, 7, 9	3a, 3b
6		167.5, qC		
7		108.6, qC		
8a	2.63, dd (15, 5.7)	22.7, CH ₂	4, 6, 7, 9, 10	
8b	2.12, dd (15, 11)		4, 6, 7, 9, 10	4-OH
9	3.90, dd (11, 5.7)	77.0, CH	4, 5	3a, 5-OH, 16, 17
10		192.6, qC		
11a	2.54, d (16)	47.7, CH ₂	7, 10, 12, 13, 14	
11b	2.37, d (16)		7, 10, 12, 13, 14	
12		80.6, qC		
13	1.41, s	27.8, CH ₃	10, 11, 12, 14	
14	1.33, s	24.5, CH ₃	11, 12, 13	
15		72.1, qC		
16	1.18, s	26.7, CH ₃	2, 15, 17	2, 3a, 9
17	1.09, s	25.6, CH ₃	2, 15, 16	2, 3a, 9
OH-4	3.86, s		3, 4, 5, 9	2, 3b, 8b
OH-5	4.95, d (5.7)		4, 5, 6	3a, 9
OH-15	3.16, s		2, 15, 16, 17	3a

^a Recorded at 400 MHz. ^b Recorded at 100 MHz.

**Figure 1.** Thermal ellipsoid representation of **1**.**Figure 2.** $\Delta\delta$ values (in ppm) = $\delta_S - \delta_R$ obtained for (*S*)- and (*R*)-MPTA esters **1a** and **1b**.

319.1520 [M + Na]⁺; Δ +0.1 mmu) and NMR data (Tables 2 and 3) Analysis of the ¹H and ¹³C NMR data for **2** revealed the presence of structural features similar to those found in **1**, except that the oxymethine ($\delta_{\text{H}}/\delta_{\text{C}}$ 4.08/72.8) was replaced by signals for a methylene unit ($\delta_{\text{H}}/\delta_{\text{C}}$ 2.13, 2.07/41.2) in the NMR spectra of **2**, and this observation was supported by HMBC correlations from these newly observed methylene protons to C-4 and C-7. Therefore, the gross structure of pestalothel B was proposed as **2**. The relative configuration of **2** was assigned by analysis of ¹H–¹H coupling constants, NOESY data, and comparison of its ¹H NMR data with those of pestalothel A (**1**). The absolute configuration of pestalothel B (**2**) was assigned as shown by analogy to pestalothel A (**1**).

The molecular formula of pestalothel C (**3**) was established as C₁₆H₂₄O₄ (five degrees of unsaturation) on the basis of HRESIMS analysis (*m/z* 303.1569 [M + Na]⁺; Δ +0.3 mmu) and the NMR data (Tables 2 and 3). The ¹H and ¹³C NMR data for **3** differed significantly from those of **1** and **2**, suggesting a substantial structural change. Upon analysis of COSY and HMBC data, the same partial structure of a tetrahydrofuran ring with a 2-hydroxy-

Table 2. ¹H NMR Spectroscopic Data of Pestalothels B–D (**2–4**) in Acetone-*d*₆

position	pestalothel B (2)	pestalothel C (3)	pestalothel D (4)
	δ_{H}^a (J in Hz)	δ_{H}^a (J in Hz)	δ_{H} (J in Hz)
2	4.10, dd (8.6, 7.5)	4.07, dd (9.4, 6.7)	4.59, dd (8.8, 8.6)
3a	2.53, dd (13, 8.6)	2.54, dd (13, 9.4)	3.28, dd (17, 8.6)
3b	2.00, dd (13, 7.5)	1.71, dd (13, 6.7)	3.18, dd (17, 8.8)
4			
5a	2.13, d (10)	3.74, d (2.7)	6.79, s
5b	2.07, d (10)		
6		4.63 br, d (8.5)	
7			
8a	2.64, dd (15, 5.6)	2.49, t (12)	6.95, s
8b	2.12, dd (15, 11)	2.21, dd (12, 4.8)	
9	3.67, dd (11, 5.6)	3.85, dd (12, 4.8)	
10		6.14, d (11)	
11a	2.49, d (16)	6.24, d (11)	2.66, s
11b	2.37, d (16)		
12			
13	1.39, s	1.78, s	1.40, s
14	1.34, s	1.73, s	1.40, s
15			
16	1.19, s	1.08, s	1.19, s
17	1.06, s	1.23, s	1.25, s
OH-4	3.75, s		
OH-6		3.87, d (8.7)	
OH-15	3.19, s	3.31, s	3.62, s

^a Recorded at 400 MHz.

isopropyl side chain attached at C-2, like that appearing in **1** and **2**, was established. Four isolated proton spin-systems corresponding to the C-2–C-3, C-5–C-6 (including C-6–OH), C-8–C-9, and C-10–C-11 subunits of structure **3** were established by analysis of its COSY NMR data. HMBC correlations from H-5 to C-3 and C-4, H-6 to C-7 and C-8, and H-8b to C-4, C-6, C-7, and C-9 permitted completion of a cyclohexane unit. Key HMBC correlations from H₃-13 and H₃-14 to C-11 and C-12 and from the olefinic proton H-10 to C-6 and C-8 enabled the completion of the C-7–C-14 partial structure of **3**, with C-7 directly attached to C-10. Correlations from the exchangeable proton (δ_{H} 3.87) to C-5, C-6, and C-7 established the connectivity between C-6 and a hydroxy group. Considering the chemical shifts of C-4 (δ_{C} 69.5) and C-5 (δ_{C} 60.1), as well as the unsaturation requirement for **3**, C-4 and C-5 have to be connected to one oxygen atom to form an epoxide moiety. On the basis of these data, the planar structure of pestalothel C was established as depicted in **3**.

Table 3. ¹³C NMR Spectroscopic Data of Pestaloths B–D (2–4) in Acetone-*d*₆

position	pestalothol B (2)	pestalothol C (3)	pestalothol D (4)
	δ_{C}^a , mult.	δ_{C}^a , mult.	δ_{C}^a , mult.
2	85.9, CH	86.0, CH	90.2, CH
3	39.8, CH ₂	31.1, CH ₂	31.6, CH ₂
4	81.3, qC	69.5, qC	139.5, qC
5	41.2, CH ₂	60.1, CH	115.6, CH
6	167.4, qC	64.8, CH	155.7, qC
7	108.4, qC	134.3, qC	119.9, qC
8	22.4, CH ₂	35.4, CH ₂	103.5, CH
9	76.8, CH	77.8, CH	155.3, s qC
10	191.3, qC	128.7, CH	191.9, qC
11	47.7, CH ₂	121.9, CH	49.0, CH ₂
12	80.6, qC	136.2, qC	79.5, qC
13	27.4, CH ₃	26.4, CH ₃	26.7, CH ₃
14	25.2, CH ₃	18.0, CH ₃	26.5, CH ₃
15	72.0, qC	71.9, qC	71.4, qC
16	26.9, CH ₃	25.8, CH ₃	26.0, CH ₃
17	25.6, CH ₃	26.7, CH ₃	25.6, CH ₃

^a Recorded at 100 MHz.

The relative configuration of pestalothol C (**3**) was assigned by analysis of ¹H–¹H coupling constants and NOESY correlations (Figure 3). The large coupling constant observed between H-8a and H-9 (12 Hz) and between H-2 and H-3a (9.4 Hz) suggested a *trans* relationships between adjacent protons. NOESY correlations from H-8a to H-2 and OH-6 and from H-5 to H-3b placed these protons on the same face of the ring system, and correlations of H-9 with H-3a and H-6 indicated that they are on the opposite face of the ring system. NOESY correlations of H-6 with H-11 and of H-8b with H-10 established the *Z*-geometry of the C-7/C-10 double bond. On the basis of these data, the relative configuration of pestalothol C was established as shown in **3**.

The absolute configuration of pestalothol C (**3**) was also assigned by application of the modified Mosher method. The differences in chemical shift values ($\Delta\delta = \delta_{\text{S}} - \delta_{\text{R}}$) for the diastereomeric esters **3b** and **3a** were calculated in order to assign the absolute configuration at C-6. Calculations for all of the relevant signals suggested the *R* absolute configuration at C-6, and the 2*R*, 4*R*, 5*S*, 6*R*, and 9*S* absolute configuration was proposed for compound **3** on the basis of the results summarized in Figure 4.

The molecular formula of pestalothol D (**4**) was determined to be C₁₆H₂₀O₄ (seven unsaturations) by analysis of its HRESIMS (*m/z* 299.1252 [M + Na]⁺; $\Delta +0.2$ mmu) and NMR data (Tables 2 and 3). Analysis of its NMR data revealed the presence of the same 2-hydroxyisopropyl side chain and dihydropyranone moiety as those present in **1** and **2**, except that the signals for four sp³ carbons, C-4, C-5, C-8, and C-9, were replaced by four aromatic resonances in the NMR spectra of **4**, indicating that the cyclohexene ring occurring in **1** and **2** was aromatized. HMBC correlations from H-5 to C-3, C-6, C-7, and C-9 and from H-8 to C-4, C-6, C-9, and C-10 further confirmed the presence of an aromatic ring in **4**. The ¹³C NMR chemical shift of C-9 (δ_{C} 155.3) indicated that it was oxygenated, and an HMBC correlation of H-2 with C-9 established the connectivity of C-2 and C-9 via an oxygen to form a dihydrofuran ring. On the basis of these data, the structure of pestalothol D was established as depicted in **4**.

Pestaloths A–D (**1**–**4**) were tested for in vitro activity against HIV-1_{LAI}. Pestalothol C (**3**) showed an inhibitory effect on HIV-1 replication in C8166 cells, with EC₅₀ and CC₅₀ values of 16.1 and 163 μ M, respectively (selectivity index, SI = 10.1; the positive control indinavir sulfate showed an EC₅₀ value of 8.18 nM). Pestaloths A–D (**1**–**4**) were also evaluated for activity against a panel of bacteria including *Staphylococcus aureus* (ATCC 6538), *Streptococcus mutans* (ATCC 25175), *Enterococcus faecalis* (ATCC 19433), and *Sarcina lutea* (CMCC B28001), and the fungi *Geotrichum candidum* (AS2.498), *Candida albicans* (ATCC 10231), and *Aspergillus fumigatus* (ATCC 10894). However, none of these

compounds showed noticeable in vitro antimicrobial or antifungal activity when tested at 100 μ g/disk.

Pestaloths A (**1**), B (**2**), and D (**4**) are new members of the chromenone type of metabolites. Biogenetically, these compounds could be derived from two units of isoprenoids and a polyketide, whereas pestalothol C (**3**) could be their biosynthetic precursor. Natural products containing a reduced tetrahydro-2*H*-furo[3,2-*g*]chromene unit have been reported previously, such as visaminol (**5**).²⁰ However, pestaloths A (**1**), B (**2**), and D (**4**) differ significantly from their naturally occurring precedents in the identity of the substituents and, most notably, in the fused pattern between the cyclohexene (aromatic in **4**) moiety and the furan ring. Pestalothol C (**3**) is closely related to illifunone E (**6**)²¹ and illicinone E,²² but differs in the identity of the substituents as well as the presence of an epoxide moiety *cis*-fused to the cyclohexane ring.

Experimental Section

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. ¹H and ¹³C 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) as references. The HMQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. ESIMS data were recorded on a Bruker Esquire 3000Plus spectrometer, and HRESIMS data were obtained using a Bruker APEX III 7.0 T spectrometer.

Fungal Material. The culture of *P. theae* was isolated by one of the authors (L.G.) from the branches of an unidentified tree near the Jianfeng Mountain, Hainan Province, in April 2005. The isolate was identified and assigned the accession number LN560 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. Fermentation was carried out in four 500 mL Fernbach flasks each containing 100 g of rice. Spore inoculum was prepared by suspension in sterile, distilled H₂O to give a final spore/cell suspension of 1 × 10⁶/mL. Distilled H₂O (100 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 15 lb/in.² for 30 min.²³ After cooling to room temperature, each flask was inoculated with 5.0 mL of the spore inoculum and incubated at 25 °C for 40 days.

Extraction and Isolation. The fermented rice substrate was extracted repeatedly with MEK (3 × 500 mL), and the organic solvent was evaporated to dryness under vacuum to afford 2.51 g of crude extract. The extract was fractionated by silica gel VLC using petroleum ether–EtOAc gradient elution. The fraction (26 mg) eluted with 15% EtOAc was further separated by semipreparative reversed-phase HPLC (Kramosil C₁₈ column; 10 μ m; 10 × 250 mm; 2 mL/min) to afford pestalothol D (**4**; 4.5 mg, *t_R* 18.4 min; 60 to 80% CH₃OH in water over 30 min). The fractions eluted with 30% (50 mg), 50% (100 mg), 55% (140 mg), and 60% (150 mg) EtOAc were fractionated again by Sephadex LH-20 column chromatography using CHCl₃–CH₃OH (1:1) as eluents. Purification of these fractions with different gradients afforded pestaloths A (**1**; 30.0 mg, *t_R* 11.8 min; 30 to 60% CH₃OH in water over 35 min), B (**2**; 4.5 mg, *t_R* 16.0 min; 40 to 55% CH₃OH in water over 25 min), and C (**3**; 9.0 mg, *t_R* 17.2 min; 55 to 75% CH₃OH in water over 30 min).

Pestalothol A (1): colorless needles, mp 223–225 °C; [α]_D +122 (*c* 1.17, CH₃OH); UV (CH₃OH) λ_{max} 278 (ϵ 6500) nm; IR (neat) ν_{max} 3391 (br), 2976, 1650, 1595, 1373, 1258, 1165, 1085, 1052 cm⁻¹; ¹H, ¹³C NMR, HMBC, and NOESY data, see Table 1; HRESIMS obsd *m/z* 335.1466 [M + Na]⁺ (calcd for C₁₆H₂₄O₆Na, 335.1470).

X-ray Crystallographic Analysis of Pestalothol A (1).²⁴ Upon crystallization from MeOH–H₂O (10:1) using the vapor diffusion method, colorless crystals were obtained for **1**, a crystal (0.26 × 0.24 × 0.18 mm) was separated from the sample and mounted on a glass fiber, and data were collected using a Bruker SMART 1000 CCD diffractometer with graphite-monochromated Mo K α radiation, $\lambda = 0.71073$ Å at 294(2) K. Crystal data: C₁₆H₂₄O₆, *M* = 312.35, space group orthorhombic, *P*2₁2₁2₁; unit cell dimensions *a* = 6.465(2) Å, *b* = 10.184(4) Å, *c* = 25.525(9) Å, *V* = 1680.6(10) Å³, *Z* = 4, *D*_{calcd} = 1.234 mg/m³, $\mu = 0.094$ mm⁻¹, *F*(000) = 672. The structure was solved

were grown on Sabouraud dextrose agar, and the fungus, *Aspergillus fumigatus* (ATCC 10894), was grown on potato dextrose agar. Test compounds were absorbed onto individual paper disks (6 mm diameter) at 100 $\mu\text{g}/\text{disk}$ and placed on the surface of agar. The assay plates were incubated at 25 °C for 48 h (at 37 °C for 24 h for antimicrobial activity) and examined for the presence of a zone of inhibition.

Anti-HIV Assays. Anti-HIV assays included cytotoxicity and HIV-1 replication inhibition evaluations. The cytotoxicity was measured by the MTT method as described in the literature.²⁸ Cells ($3 \times 10^4/\text{well}$) 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% CO₂. After a 4-day incubation, cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The concentration that caused the reduction of viable cells by 50% (CC₅₀) was determined. In parallel with the MTT assay, a HIV-1 replication inhibition assay was determined by p24 antigen capture ELISA.²⁹ C8166 cells were exposed to HIV-1_{LAI} (MOI = 0.058) at 37 °C for 1.5 h, washed with PBS 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, and the HRP-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 reaction mixture. The assay plate was read at 490 nm using a microplate reader within 30 min. The inhibition rate and the EC₅₀ based on p24 antigen expression level were calculated, and the selective index was calculated as CC₅₀/EC₅₀.

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Supporting Information Available: ¹H and ¹³C NMR spectra of pestalothols A–D (1–4). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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