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Pestalofones A–E, bioactive cyclohexanone derivatives from the plant endophytic fungus *Pestalotiopsis fici*

Ling Liu [a,b], Shuchun Liu [a], Xulin Chen [c], Liangdong Guo [a], Yongsheng Che [a,*]

[a] Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences, No. 13 Beiyitiao, Zhongguancun, Beijing 100080, People's Republic of China
[b] Graduate School of Chinese Academy of Sciences, Beijing 100039, People's Republic of China
[c] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, People's Republic of China

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ABSTRACT

Pestalofones A–E (**1**–**5**), five new cyclohexanone derivatives, have been isolated from cultures of the plant endophytic fungus *Pestalotiopsis fici*, along with the known compounds, isosulochrin (**6**), isosulochrin dehydrate (**7**), and *iso*-A82775C (**8**). The structures of **1**–**5** were determined by NMR spectroscopy, and the absolute configuration of **1** was assigned using the modified Mosher method. Compounds **1**, **2**, and **5** displayed inhibitory effects on HIV-1 replication in C8166 cells, whereas **3** and **5** showed significant antifungal activity against *Aspergillus fumigatus*.

1. Introduction

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.[4–11] Our prior studies of the fungus *Pestalotiopsis fici* (W106-1) grown in different solid-substrate fermentation cultures have led to the isolation of different types of new bioactive metabolites.[12,13] In the course of this work, a subculture of this fungal strain was subjected to scale-up fermentation on rice in order to identify minor active components. The resulted crude extract from scale-up fermentation showed potent antifungal activity against *Aspergillus fumigatus* (ATCC 10894) and weak inhibitory effects on HIV-1 replication in C8166 cells, and its HPLC finger-print revealed the presence of different types of secondary metabolites from those isolated previously. On the basis of above results, bioassay-guided fractionation of this extract was performed, leading to the isolation of five new metabolites, which we named pestalofones A–E (**1–5**), along with the known compounds, isosulochrin (**6**),[14] isosulochrin dehydrate (**7**),[14] and *iso*-A8277C (**8**).[13] Details of the isolation, structure elucidation, and biological activities of these metabolites are reported herein.

2. Results and discussion

Pestalofone A (**1**) was obtained as colorless oil. It was assigned the molecular formula $C_{16}H_{22}O_3$ (six degrees of unsaturation) on the basis of its HRESIMS [*m/z* 285.1468 $(M+Na)^+$; Δ −0.7 mmu] and NMR data (Table 1), which is the same as that of *iso*-A8277C (**8**).[13] Interpretation of the ^1H, ^{13}C, and HMQC NMR spectroscopic data of **1** revealed the presence of one exchangeable proton, four methyl groups, two methylene units, two oxymethines, one oxygenated sp^3 quaternary carbon, six olefinic carbons (three of which are protonated), and one α,β-unsaturated ketone carbon (δ_C 195.0). These data accounted for all ^1H and ^{13}C NMR resonances and required the compound to be bicyclic. Analysis of the ^1H–^1H COSY NMR data led to the identification of three isolated proton spin-systems corresponding to the C-4–C-5, C-7–C-9 (including OH-8), and C-11–C-12 subunits of structure **1**. The structure of the two prenoids in **1** was established by HMBC correlations from H_3-1/2 to C-3 and C-4, and H_3-14/15 to C-12 and C-13. HMBC correlations from H_2-5 to C-6, C-7, and C-16, and from H-7 to C-5, C-6 and C-16 led to the connection of C-6 to C-5, C-7, and C-16. Other correlations from the olefinic proton H-11 to C-9, C-10, and C-16, and from H_2-9 to C-10, C-11, and C-16 completed the cyclohexanone ring with C-10 attached to C-11 via an *exo*-cyclic double bond. Key HMBC correlations from the exchangeable proton (OH-8) to

* Corresponding author. Tel./fax: +86 10 82618785.
E-mail address: cheys@im.ac.cn (Y. Che).

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Table 1
NMR spectroscopic data of pestalofone **A** (**1**) in acetone-d_6

Position	δ_H[a] (J in Hz)	δ_C[b]	HMBC (H → C#)
1	1.63, s	18.0	2, 3, 4
2	1.67, s	25.9	1, 3, 4
3		135.2	
4	5.09, t (7.5)	118.7	1, 2, 5
5a	2.40, dd (16, 7.5)	28.3	3, 4, 6, 7, 16
5b	2.66, dd (16, 7.5)		3, 4, 6, 7, 16
6		63.2	
7	3.53, d (1.5)	64.6	5, 6, 8, 9, 16
8	4.16, dddd (11, 6.0, 5.5, 1.5)	66.0	7, 9
9a	2.43, dd (15, 11, 2.5)	29.9	7, 8, 10, 11, 16
9b	2.86, dd (15, 5.5)		7, 8, 10, 11, 16
10		128.3	
11	7.39, dd (13, 2.5)	133.6	9, 10, 13, 16
12	6.16, br d (13)	121.1	10, 11, 14, 15
13		148.6	
14	1.91, s	18.9	12, 13, 15
15	1.92, s	27.0	12, 13, 14
16		195.0	
OH-8	4.47, d (6.0)		7, 8, 9

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

C-7, C-8, and C-9 indicated that the hydroxy group was attached to C-8. Considering the ^{13}C NMR chemical shifts of C-6 (δ_C 63.2) and C-7 (δ_C 64.6), as well as the bicyclic nature of **1**, these two carbons have to be connected to the remaining oxygen to form an epoxide moiety. On the basis of these data, the planar structure of pestalofone A was established as shown in **1** (Fig. 1).

The relative configuration of pestalofone A (**1**) was assigned by analysis of the ^1H–^1H coupling constants and NOESY correlations (Fig. 2). The large *trans*-diaxial-type coupling constant of 11 Hz observed between H-8 and H-9a indicated that H-8 and H-9a are pseudoaxial-oriented, and a small coupling constant of 1.5 Hz

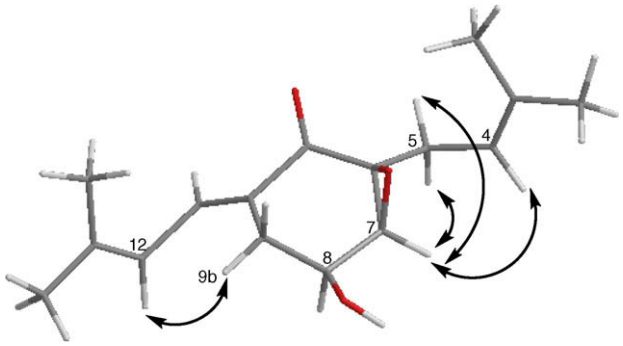


Figure 2. Key NOESY correlations for pestalofone A (**1**).

between H-7 and H-8 places H-7 in a pseudoequatorial orientation with respect to the corresponding cyclohexanone ring. NOESY correlations of H-7 with H-4 and H_2-5 indicated that these protons are all on the same face of the ring system. Other correlation of H-12 with H-9b was used to assign the *E*-geometry for the C-10/C-11 double bond. Therefore, the relative configuration of pestalofone A was proposed as shown in **1** (Fig. 1).

The absolute configuration of pestalofone 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** was calculated in order to assign the absolute configuration at C-8 (Fig. 3). Calculations for all of the relevant signals except H_3-14 suggested the 8*S* absolute configuration, which is the same as that of its known precedent **8**.[13] Therefore, all relevant stereogenic centers in **1** were assigned the 6*S*, 7*S*, and 8*S* absolute configurations on the basis of the $\Delta\delta$ results summarized in Figure 3.

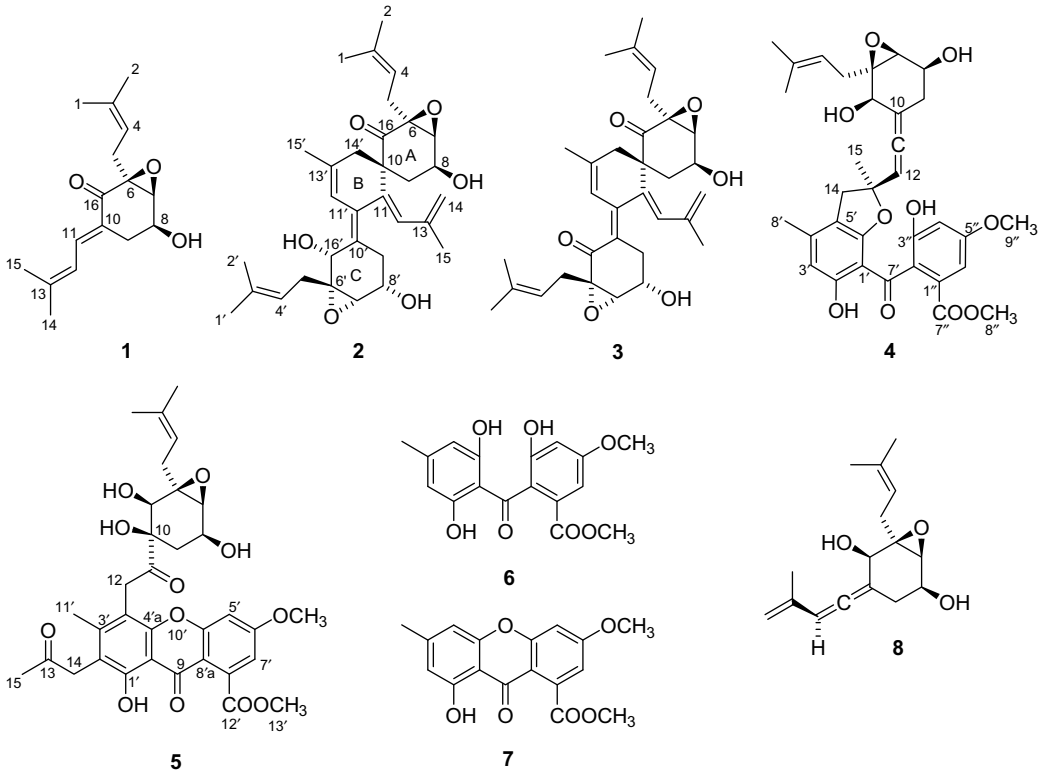
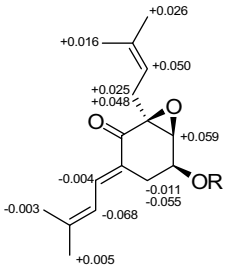


Figure 1. Structures of pestalofones A–E (**1**–**5**), isosulochrin (**6**), isosulochrin dehydrate (**7**), and *iso*-A82775C (**8**).



1a R = (*S*)-MTPA
1b R = (*R*)-MTPA

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

The molecular formula of pestalofone B (**2**) was determined to be $C_{32}H_{42}O_6$ (12 degrees of unsaturation) on the basis of HRESIMS [*m/z* 545.2861 $(M+Na)^+$; Δ +1.3 mmu] analysis and the NMR data (Table 2). Interpretation of the ^1H, ^{13}C, and HMQC NMR spectroscopic data of **2** revealed the presence of three exchangeable protons, six methyl groups, five methylene units, five oxymethines, three sp^3 quaternary carbons (two of which are oxygenated), 12 olefinic carbons (five of which are protonated including one for a terminal olefin), and one ketone carbon (δ_C 205.9). Comparison of the NMR data of **2** with those of **1** and the known compound **8** indicated that **2** could be a dimerization product originating from either **1** or **8**. Detailed analysis of the HMBC data for **2** established the partial structures for an epoxycyclohexanone (ring A) and an epoxycyclohexane (ring C), with a prenoid attached to C-6 and C-6′, respectively. HMBC correlations from H_2-9 to C-10, C-11, and C-14′, H-12′ to C-11, C-11′, C-14′, and C-15′, and from H_3-15′ to C-12′, C-13′, and C-14′ allowed assignment of the cyclohexene moiety (ring B) that was joined spirally to ring A at C-10. While correlations from H_3-15 to C-12, C-13, and C-14 established the C-12–C-15 substructure of **2**, and those from the olefinic proton H-12 to C-10, C-11, and C-11′ led to the connection of this subunit to ring B at C-11. Key HMBC correlations from H-12′ to C-10′ and from H_2-9′ to C-11′ enabled the connection of rings B and C via the C-10′/C-11′ double bond. The three hydroxys in **2** were assigned to corresponding methine carbons by relevant ^1H–^1H COSY correlations. On the basis of these data, the gross structure of pestalofone B was established as shown in **2** (Fig. 1).

The relative configuration of rings A and C of pestalofone B (**2**) was assigned by comparison of the ^1H–^1H coupling constants and NOESY data (Fig. 4) with those of compounds **1** and **8**. NOESY correlations of H-9a with H-14′a, and H-8 with H-14a revealed their proximity in space, whereas correlations of H-12 with H_2-9′ and of H-12′ with H_3-15′ and H-16′ established the *Z*-geometry of the C-11/C-12 and the *E*-geometry of the C-10′/C-11′ olefins. The absolute configuration of **2** was deduced on the basis of biosynthetic considerations (Fig. 5) and by analogy to **1** and **8**. Collectively, these data permitted assignment of structure **2** to the new natural product, pestalofone B.

The molecular formula of pestalofone C (**3**) was established as $C_{32}H_{40}O_6$ (13 degrees of unsaturation) by analysis of its HRESIMS [*m/z* 543.2742 $(M+Na)^+$; Δ –2.1 mmu] and NMR data (Table 2), which is two mass unit less than that of **2**. Analysis of the ^1H, ^{13}C, and HMQC NMR data of **3** revealed the presence of nearly identical structural features to those found in **2**, except that the resonances for an oxygenated methine unit (δ_H/δ_C 4.71/65.2) and the attached hydroxy proton (δ_H 3.76; OH-16′) were replaced by that for a ketone carbon (δ_C 197.6) in the NMR spectra of **3**, which was consistent with the HRESIMS data for **3**. This observation was further confirmed by HMBC correlations from H_2-5′ and

Table 2
NMR spectroscopic data of pestalofones B (**2**) and C (**3**) in acetone-d_6

Position	Pestalofone B (**2**)		Pestalofone C (**3**)	
	δ_H[a] (*J* in Hz)	δ_C[b]	δ_H[a] (*J* in Hz)	δ_C[b]
1	1.61, s	18.1	1.61, s	18.1
2	1.68, s	25.9	1.67, s	25.9
3		135.5		135.7
4	4.99, t (7.5)	118.0	5.00, t (7.5)	118.0
5a	2.41, dd (16, 7.5)	27.5	2.42, dd (16, 7.5)	27.5
5b	2.77, dd (16, 7.5)		2.82, dd (16, 7.5)	
6		62.8		62.8
7	3.43, d (1.5)	61.9	3.46, d (1.5)	61.9
8	4.11, dddd (10, 6.0, 4.5, 1.5)	65.7	4.16, dddd (10, 6.0, 4.5, 1.5)	65.6
9a	1.66, dd (14, 10)	35.4	1.70, dd (14, 10)	35.2
9b	2.01, dd (14, 4.5)		1.98, dd (14, 4.5)	
10		52.0		52.8
11		136.6		135.9
12	5.71, s	132.3	5.92, s	135.1
13		144.7		144.5
14a	4.74, s	115.4	4.78, s	115.6
14b	4.88, s		4.93, s	
15	1.62, s	22.5	1.65, s	22.2
16		205.9		205.3
1′	1.66, s	18.1	1.63, s	18.0
2′	1.69, s	25.9	1.69, s	25.9
3′		135.3		135.5
4′	5.11, t (7.5)	119.4	5.06, t (7.5)	118.6
5′a	2.24, dd (15, 7.5)	33.1	2.39, dd (15, 7.5)	28.4
5′b	2.74, dd (15, 7.5)		2.72, dd (15, 7.5)	
6′		64.5		63.6
7′	3.19, d (1.5)	62.3	3.49, d (1.5)	62.8
8′	3.81, dddd (12, 6.5, 5.5, 1.5)	69.0	4.12, dddd (12, 6.3, 5.5, 1.5)	67.5
9′a	2.33, dd (15, 12)	31.4	2.58, dd (15, 12)	34.2
9′b	2.94, dd (15, 5.5)		3.06, dd (15, 5.5)	
10′		133.9		127.5
11′		137.2		148.6
12′	6.43, s	120.5	7.12, s	122.2
13′		136.6		143.1
14′a	1.74, d (18)	44.7	1.84, d (18)	44.8
14′b	3.04, d (18)		3.11, d (18)	
15′	1.77, s	23.6	1.80, s	24.0
16′	4.71, d (9.0)	65.2		197.6
OH-8	4.27, d (6.0)		4.52, d (6.0)	
OH-8′	4.18, d (6.5)		4.34, d (6.3)	
OH-16′	3.76, d (9.0)			

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

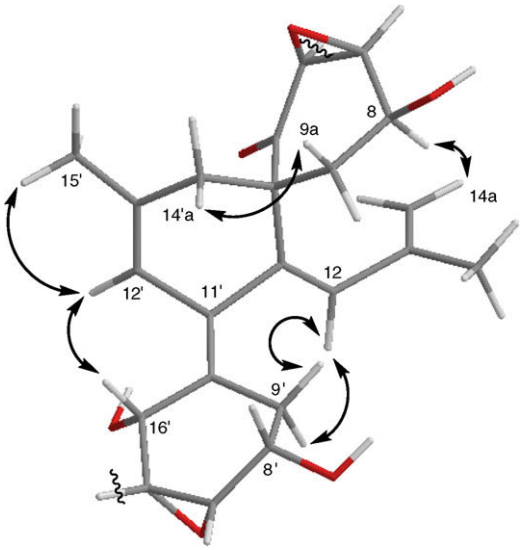


Figure 4. Key NOESY correlations for pestalofone B (**2**).

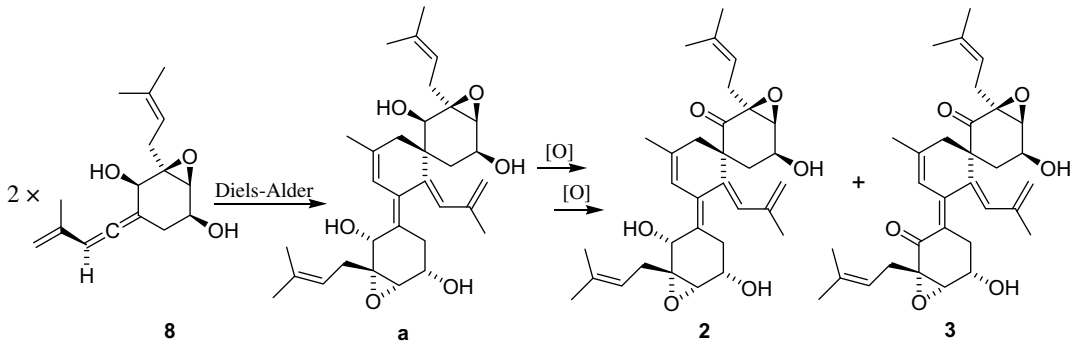


Figure 5. Proposed biosynthetic pathways for pestalofones B (**2**) and C (**3**).

H_2-9′ to the ketone carbon at δ_C 197.6 ppm. On the basis of these considerations, the gross structure of pestalofone C was proposed, and its absolute configuration was deduced as shown by analogy to **2**.

The elemental composition of pestalofone D (**4**) was determined as $C_{33}H_{36}O_{10}$ (16 degrees of unsaturation) by analysis of its HRESIMS [*m/z* 615.2189 $(M+Na)^+$; Δ +1.2 mmu] and NMR data (Table 3). Interpretation of the ^1H, ^{13}C, and HMQC NMR spectroscopic data of **4** revealed the presence of four exchangeable protons, six methyl groups (two *O*-methyls), three methylene units, three oxymethines, two oxygenated sp^3 quaternary carbons, 17 olefinic/aromatic carbons (five of which are protonated), one carboxyl carbon, and one ketone carbon. These data accounted for all the ^1H and ^{13}C NMR resonances and required the compound to be pentacyclic. Comparison of the ^1H and ^{13}C NMR spectra of **4** with those of the known metabolites isosulochrin (**6**) and *iso*-A82775C (**8**) indicated that **4** contained similar partial structures, suggesting that it could be a heterodimeric metabolite derived from **6** and **8**. In addition, the molecular formula of **4** ($C_{33}H_{36}O_{10}$) is equivalent to the summation of those for **6** ($C_{17}H_{16}O_7$) and **8** ($C_{16}H_{22}O_3$) subtracting two hydrogen atoms. Analysis of the HMBC NMR spectroscopic data for **4** confirmed the presence of the partial structures that are nearly identical to the core structures of **6** and **8**, with structural variations needed to be identified by further interpretation of its 2D NMR data. Specifically, HMBC cross-peaks from H_3-15 to C-12, C-13, and C-14 indicated that the sp^3 quaternary carbon C-13 was connected to C-12, C-14, and C-15. Considering the ^{13}C NMR chemical shifts of C-13 (δ_C 89.3) and C-6′ (δ_C 160.1), as well as the pentacyclic nature of **4**, these two carbons have to be connected to the remaining oxygen atom to form a dihydrofuran moiety, thereby completing the gross structure of **4** as shown. The relative configuration of the newly formed stereogenic center C-13 was deduced based on NOESY correlations of H-12 with H-14b and H_3-15, and of H-14a with H_3-15 (Fig. 6). Since pestalofone D (**4**) could be derived from **6** and **8** via a series of reactions (Fig. 7), and in this presumed reaction cascade, the hydroxy group at C-6′ could attack C-13 from both faces, leading to the formation of two possible stereoisomers at C-13 (**4** and **4′**; Fig. 6). However, NOESY correlation of the pseudoequatorial proton H-9a with H_3-15 indicated that **4** is the observed product (Fig. 6). Considering the absolute configuration established for **8**, the stereogenic center C-13 in **4** was assigned the *S* absolute configuration.

Table 3
NMR spectroscopic data of pestalofone D (**4**) in acetone-d_6

Position	δ_H[a] (*J* in Hz)	δ_C[b]	HMBC (H → C#)
1	1.61, s	18.0	2, 3, 4
2	1.66, s	25.9	1, 3, 4
3		135.6	
4	5.07, t (7.5)	118.9	1, 2, 5
5a	2.07, dd (15, 7.5)	33.0	3, 4, 6, 7, 16
5b	2.61, dd (15, 7.5)		3, 4, 6, 7, 16
6		65.7	
7	3.22, d (1.5)	62.9	5, 6, 8, 9, 16
8	3.96, dddd (9.5, 5.5, 6.2, 1.5)	68.4	
9a	2.09, dd (15, 5.5)	31.0	7, 8, 10, 11, 16
9b	2.27, ddd (15, 9.5, 2.5)		8, 10, 11, 16
10		105.0	
11		201.4	
12	5.11, d (2.5)	97.4	10, 16
13		89.3	
14a	2.77, d (15)	39.5	4′, 5′, 6′, 12, 13, 15
14b	2.96, d (15)		4′, 5′, 6′, 12, 13, 15
15	1.20, s	26.2	12, 13, 14
16	4.03, d (8,5)	68.4	10
1′		107.7	
2′		162.9	
3′	6.23, s	109.5	1′, 2′, 5′, 8′
4′		143.3	
5′		117.9	
6′		160.1	
7′		199.4	
8′	2.14, s	19.7	3′, 4′, 5′
1″		130.9	
2″		125.7	
3″		156.0	
4″	6.71, d (2.0)	106.4	2″, 3″, 5″, 6″
5″		161.4	
6″	7.05, d (2.0)	106.4	2″, 4″, 5″, 7″
7″		166.6	
8″	3.67, s	52.4	7″
9″	3.85, s	56.0	5″
OH-8	4.10, d (6.2)		
OH-16	3.85, d (8.5)		
OH-2′	12.4, s		1′, 2′, 3′
OH-3″	8.12, br s		

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

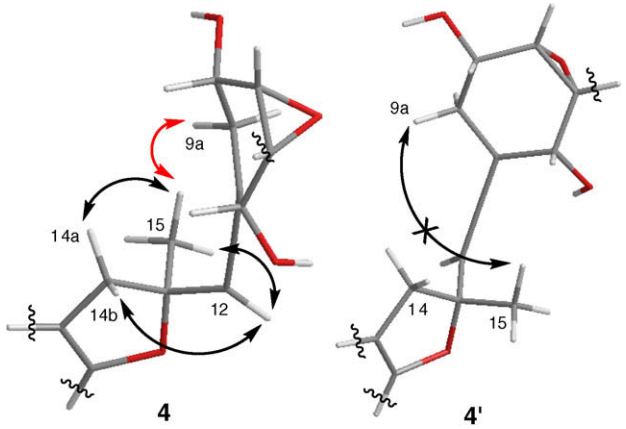


Figure 6. Key NOESY correlation of H-9a with H_3-15 for **4**, but such correlation was not observed for the other possible product **4′**, indicating that the absolute configuration of C-13 in pestalofone D was *S* as depicted in **4**.

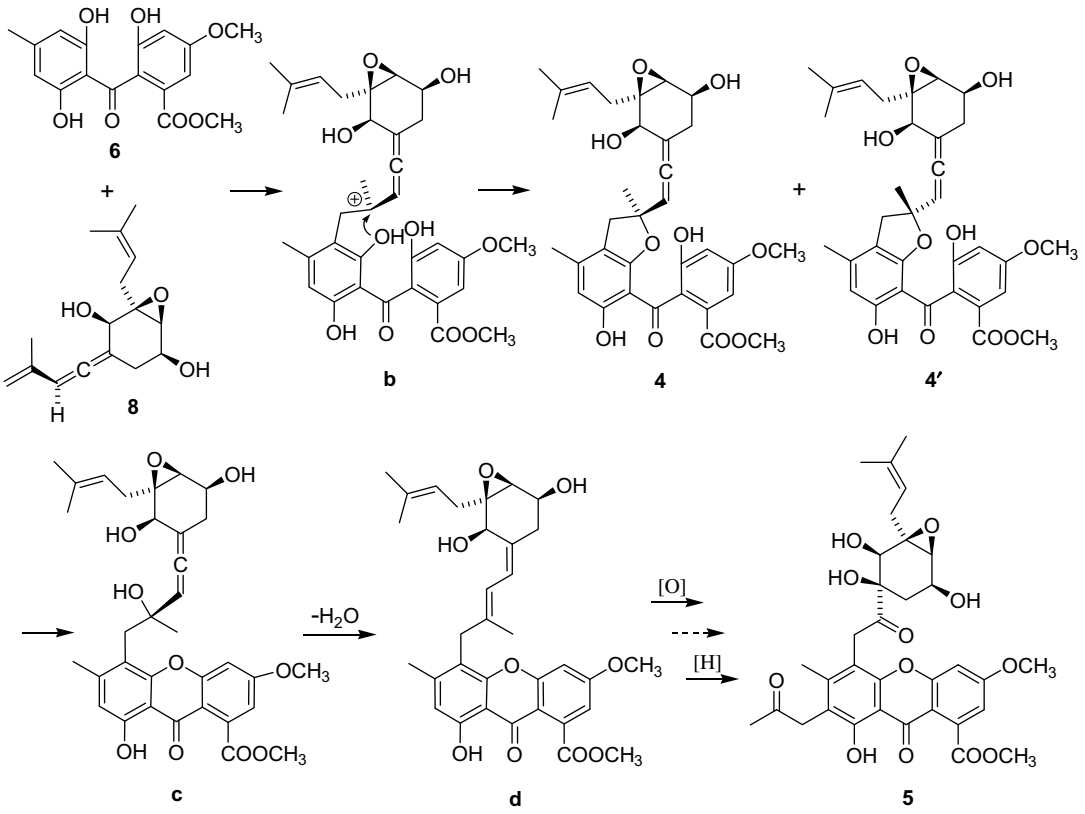


Figure 7. Proposed biosynthetic pathways for pestalofones D (**4**) and E (**5**).

Pestalofone E (**5**) was assigned a molecular formula of $C_{33}H_{36}O_{12}$ (16 degrees of unsaturation) by HRESIMS [*m/z* 627.2105 $(M+Na)^+$; *Δ −0.6* mmu] analysis and the NMR data (Table 4). Interpretation of the ^1H, ^{13}C, and HMQC NMR spectroscopic data of **5** revealed the presence of four exchangeable protons, six methyl groups (two *O*-methyls), four methylene units, three oxymethines, two oxygenated sp^3 quaternary carbons, 14 olefinic/aromatic carbons (three of which are protonated), one carboxyl carbon, and three ketone carbons. Comparison of the ^1H and ^{13}C NMR data of **5** with those of **7** and **8** revealed the presence of common structural features for these metabolites, again suggesting that **5** could be derived from **7** and **8** via a series of reactions. The HMBC data for **5** revealed the presence of partial structures similar to the core structures of **7** and **8**. The structure of a 2-oxo-propyl moiety was established based on HMBC correlations from H_3-15 to C-13 (δ_C 205.4) and C-14, and it was attached to C-2′ by HMBC cross-peaks from H_2-14 to C-1′, C-2′, and C-3′. Correlations from H_2-12 to C-3′, C-4′, C-4′a, C-10, and C-11 (δ_C 208.4) led to the connection of C-11 to C-10 and C-12, and C-12 to C-4′. All exchangeable protons in **5** were assigned to corresponding carbons based on relevant ^1H–^1H COSY and HMBC correlations. Therefore, the gross structure of pestalofone E was established as **5**.

The relative configuration of **5** was also determined by analogy to **8**, except that for C-10, which was assigned by NOESY correlation of H_2-12 with H-16. The absolute configuration of **5** was presumed to be analogous to that of **8**.

The known metabolites, isosulochrin (**6**), isosulochrin dehydrate (**7**), and *iso*-A8277C (**8**) were isolated as the major components from the crude extract, and their structures were readily identified by comparison of the NMR and MS data with those reported in the literature.[13,14] Pestalofones A–E (**1**–**5**) were tested for in vitro activity against HIV-1. Compounds **1**, **2**, and **5** showed inhibitory effects on HIV-1 replication in C8166 cells, with EC_{50} values of 90.4, 64.0, and 93.7 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). Pestalofones A–E (**1**–**5**) were also evaluated for activities against *Candida albicans* (ATCC 10231), *Geotrichum candidum* (AS2.498), and *A. fumigatus* (ATCC 10894). Pestalofones C (**3**) and E (**5**) showed significant antifungal activity against *A. fumigatus*, with IC_{50}/MIC values of 1.10/35.3, 0.90/31.2 μM, respectively (the positive control fluconazole showed IC_{50}/MIC values of 7.35/163.4 μM).

Pestalofone A (**1**) is a new analog of *iso*-A82775C (**8**), and biogenetically, these compounds could be derived from two units of prenoids and a polyketide. Pestalofones B (**2**) and C (**3**) could be derived from two units of *iso*-A82775C (**8**), first via Diels–Alder reaction,[17] to form an intermediate with a cyclohexene moiety spirally joined to the epoxycyclohexane ring at C-10, and then followed by selective oxidation of the hydroxy groups (Fig. 4). Compounds **2** and **3** possess a previously undescribed, unique highly functionalized skeleton with the presence of two polyoxygenated cyclohexanes, one is spirally joined to the cyclohexene moiety, and the other is linked by an *exo*-cyclic double bond. Pestalofone D (**4**) could be the heterodimeric metabolite derived from the known precursors isosulochrin (**6**) and *iso*-A82775C (**8**) via a series of reactions, whereas pestalofone E (**5**) was presumed to be derived from **4** through further reactions (Fig. 5). The discovery of these unique bioactive secondary metabolites from *P. fici* suggested that the fermentation conditions for this fungus should be further explored and optimized to maximize its potential for the production of new bioactive metabolites.

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

Table 4
NMR spectroscopic data of pestalofone E (**5**) in acetone-d_6

Position	δ_H[a] (J in Hz)	δ_C[b]	HMBC (H → C#)
1	1.66, s	18.1	2, 3, 4
2	1.72, s	25.9	1, 3, 4
3		135.4	
4	5.22, t (6.5)	118.8	1, 2
5a	2.35, dd (15, 6.5)	34.5	3, 4, 6, 7, 16
5b	2.69, dd (15, 6.5)		3, 4, 6, 7, 16
6		64.9	
7	3.39, d (1.5)	63.9	5, 6, 8, 9, 16
8	4.30, dddd (10, 5.5, 5.0, 1.5)	65.7	
9a	1.92, dd (16, 5.5)	30.4	7, 8, 10, 11, 16
9b	2.04, dd (16, 10)		7, 8, 10, 11
10		80.8	
11		208.4	
12a	4.30, d (19)	35.9	3′, 4′, 4′a, 10, 11
12b	4.58, d (19)		3′, 4′, 4′a, 10, 11
13		205.4	
14	3.91, s	40.9	1′, 2′, 3′, 13
15	2.18, s	29.0	13, 14
16	3.96, d (10)	72.5	5, 9, 10
1′		158.4	
2′		117.5	
3′		148.9	
4′		113.4	
4′a		153.4	
5′	7.17, d (2.0)	102.7	6′, 7′, 8′a, 10′a
6′		165.9	
7′	6.96, d (2.0)	113.0	5′, 8′a, 12′
8′		136.2	
8′a		110.9	
9′		181.0	
9′a		106.7	
10′a		159.0	
11′	2.19, s	17.3	2′, 3′, 4′
12′		169.1	
13′	3.91, s	53.0	12′
14′	4.00, s	56.9	6′
OH-8	4.03, d (5.0)		8
OH-10	4.71, s		16
OH-16	3.82, d (10)		6, 16
OH-1′	12.81, s		1′, 2′, 9′a

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

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; pyridine-d_5: δ_H 7.21, 7.58, 8.73) 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 3000^{plus} spectrometer, and HRESIMS data were obtained using a Bruker APEX III 7.0 T spectrometer.

3.2. Fungal material and fermentation

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. Scale-up fermentation was carried out in twelve 500-mL Erlenmeyer flasks each containing 80 g of rice. Spore inoculum was prepared by suspension in sterile, distilled H_2O to give a final spore/cell suspension of 1×10^6/mL. Distilled H_2O (100 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 15 lb/in.^{2} for 30 min.[18] 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.

3.3. Extraction and isolation

The fermented material was extracted with EtOAc (4×1.0 L), and the organic solvent was evaporated to dryness under vacuum to afford 10 g of crude extract. The extract was fractionated by Silica gel VLC using petroleum ether–EtOAc gradient elution. The fraction eluted with 10% EtOAc were purified by reversed-phase HPLC (Kramosil C_{18} column; 10 μm; 10 × 250 mm; 2 mL/min; 40–70% CH_3OH in water over 25 min) to afford the known compounds isosulochrin (**6**; 40.0 mg, t_R 12.0 min) and *iso*-A82775C (**8**; 32.0 mg, t_R 16.5 min). The fraction eluted with 13% EtOAc was subsequently fractionated by Sephadex LH-20 column chromatography using 1:1 $CHCl_3/CH_3OH$ as eluents, and one subfraction (30 mg) was further purified by reversed-phase HPLC (65–78% CH_3OH in water over 35 min) to afford the known compound isosulochrin dehydrate (**7**; 30.0 mg; t_R 32.5 min) and pestalofone A (**1**; 4.0 mg; t_R 20.8 min). The fractions eluted with 20%, 30%, 35%, and 48% EtOAc were individually separated by Sephadex LH-20 column chromatography eluted with 1:1 $CHCl_3/CH_3OH$. Purification of resulted subfractions using different gradients afforded pestalofones B (**2**; 10.0 mg, t_R 14.7 min; 85–100% CH_3OH in water over 20 min), C (**3**; 5.0 mg, t_R 18.0 min; 70–80% CH_3OH in water over 20 min), D (**4**; 6.0 mg, t_R 37.7 min; 50–70% CH_3OH in water over 40 min), and E (**5**; 10.0 mg, t_R 30.1 min; 65–80% CH_3OH in water over 30 min).

3.3.1. Pestalofone A (1)

Colorless oil; $[\alpha]_D$ +25 (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{max} 309 (ε 9800) nm; IR (neat) ν_{max} 3418 (br), 2973, 2915, 2859, 1679,1619, 1576, 1442, 1136, 1034 cm^{-1}; ^{1}H, ^{13}C NMR, and HMBC data, see Table 1; NOESY correlations (acetone-d_6, 400 MHz) H-4 ↔ H-7; H_2-5 ↔ H-7; H-7 ↔ H-4, H_2-5; H-9b ↔ H-12; H-12 ↔ H-9b; HRESIMS obsd *m/z* 285.1468 (M+Na)$^+$, calcd for $C_{16}H_{22}O_3Na$, 285.1461.

3.3.2. Pestalofone B (2)

Colorless oil; $[\alpha]_D$ −29 (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{max} 252 (ε 14,100) nm; IR (neat) ν_{max} 3397 (br), 2970, 2927, 1705, 1645, 1439, 1378, 1254, 1029 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; HMBC data (acetone-d_6, 400 MHz) H_3-1 → C-2, 3, 4; H_3-2 → C-1, 3, 4; H-4 → C-1, 2, 5; H_2-5 → C-3, 4, 6, 16; H-7 → C-5, 6, 8, 9; H_2-9 → C-7, 8, 10, 11, 14′, 16; H-12 → C-10, 11, 11′, 14, 15; H_2-14 → C-12, 13, 15; H_3-15 → C-12, 13, 14; H_3-1′ → C-2′, 3′, 4′; H_3-2′ → C-1′, 3′, 4′; H-4′ → C-1′, 2′, 5′; H_2-5′ → C-3′, 4′, 6′, 7′, 16′; H-7′ → C-5′, 6′, 8′, 9′; H_2-9′ → C-7′, 8′, 10′, 11′, 16′; H-12′ → C-10′, 11, 11′, 14′, 15′; H-14′a → C-9, 10, 11, 12′, 13′, 15′, 16; H-14′b → C-9, 10, 11, 12′, 13′; H_3-15′ → C-12′, 13′, 14′; H-16′ → C-5′, 9′, 10′, 11′; NOESY correlations (acetone-d_6, 400 MHz) H-4 ↔ H-7; H_2-5 ↔ H-7; H-7 ↔ H-4, H_2-5; H-8 ↔ H-14a; H-9a ↔ H-14′a; H-12 ↔ H_2-9′, H-14a, H_3-15; H-14a ↔ H-8, H-12; H_3-15 ↔ H-12; H-4′ ↔ H-7′; H_2-5′ ↔ H-7′, H-16′; H-7′ ↔ H-4′, H_2-5′; H_2-9′ ↔ H-12; H-12′ ↔ H_3-15′, H-16′; H-14′a ↔ H-9a; H_3-15′ ↔ H-12′; H-16′ ↔ H_2-5′, H-12′; HRESIMS obsd *m/z* 545.2861 (M+Na)$^+$, calcd for $C_{32}H_{42}O_6Na$, 545.2874.

3.3.3. Pestalofone C (3)

Colorless oil; $[\alpha]_D$ −139 (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{max} 319 (ε 8300) nm; IR (neat) ν_{max} 3423 (br), 2971, 2929, 1709, 1674, 1440, 1378, 1233, 1028 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; HMBC data (acetone-d_6, 400 MHz) H_3-1 → C-2, 3, 4; H_3-2 → C-1, 3, 4; H-4 → C-1, 2; H_2-5 → C-3, 4, 6, 7, 16; H-7 → C-5, 6, 8, 9; H-

9a → C-7, 8, 10, 11, 14′; H-9b → C-7, 8, 10, 11, 14′, 16; H-12 → C-10, 11, 11′, 14, 15; H_2-14 → C-12, 13, 15; H_3-15 → C-12, 13, 14; H_3-1′ → C-2′, 3′, 4′; H_3-2′ → C1′, 3′, 4′; H-4′ → C-1′, 2′; H_2-5′ → C-3′, 4′, 6′, 7′, 16′; H-7′ → C-5′, 6′, 8′, 9′; H-9′a → C-8′, 10′, 11′; H-9′b → C-7′, 8′, 10′, 11′, 16′; H-12′ → C-10′, 11, 11′, 14′, 15′; H_2-14′ → C-9, 10, 11, 12′, 13′, 15′, 16; H_3-15′ → C-12′, 13′, 14′; NOESY correlations (acetone-d_6, 400 MHz) H-4 ↔ H-7; H_2-5 ↔ H-7; H-7 ↔ H-4, H_2-5; H-8 ↔ H-14a; H-9a ↔ H-14′a; H-12 ↔ H_2-9′, H-14a, H_3-15; H-14a ↔ H-8, H-12; H_3-15 ↔ H-12; H-4′ ↔ H-7′; H_2-5′ ↔ H-7′; H-7′ ↔ H-4′, H_2-5′; H_2-9′ ↔ H-12; H-12′ ↔ H_3-15′; H-14′a ↔ H-9a; H_3-15′ ↔ H-12′; HRESIMS obsd *m/z* 543.2742 $(M+Na)^+$, calcd for $C_{32}H_{40}O_6Na$, 543.2721.

3.3.4. Pestalofone D (4)

Colorless oil; $[\alpha]_D$ −16 (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{max} 214 (ε 25,600), 281 (10,900) nm; IR (neat) ν_{max} 3396 (br), 2970, 2853, 1973, 1722, 1640, 1610, 1496, 1444, 1338, 1048 cm^{-1}; ^1H, ^{13}C NMR, and HMBC data, see Table 3; NOESY correlations (acetone-d_6, 400 MHz) H-4 ↔ H-7, H-16; H_2-5 ↔ H-7, H-16; H-7 ↔ H-4, H_2-5; H-9a ↔ H_3-15; H-12 ↔ H-14b, H_3-15; H-14a ↔ H_3-15, H_3-8′; H-14b ↔ H-12, H_3-8′; H_3-15 ↔ H-9a, H-12, H-14a; H-16 ↔ H-4, H_2-5; H-3′ ↔ H_3-8′; H_3-8′ ↔ H-3′, H_2-14; H-4″ ↔ H_3-9″; H-6″ ↔ H_3-9″; H_3-9″ ↔ H-4″, H-6″; HRESIMS obsd *m/z* 615.2189 $(M+Na)^+$, calcd for $C_{33}H_{36}O_{10}Na$, 615.2201.

3.3.5. Pestalofone E (5)

Colorless oil; $[\alpha]_D$ −12 (*c* 0.1, CH_3OH); UV (CH_3OH) λ_{max} 237 (ε 20,800), 273 (16,600) nm; IR (neat) ν_{max} 3467 (br), 2930, 2858, 1717, 1644, 1573, 1425, 1386, 1237, 1149, 1032 cm^{-1}; ^1H, ^{13}C NMR, and HMBC data, see Table 4; NOESY correlations (acetone-d_6, 400 MHz) H-4 ↔ H-7; H_2-5 ↔ H-7, H-16; H-7 ↔ H-4, H_2-5; H_2-12 ↔ H_3-11′, H-16; H_2-14 ↔ H_3-11′, H_3-15; H_3-15 ↔ H_2-14; H-16 ↔ H_2-5, H_2-12; H_3-11′ ↔ H_2-12, H_2-14; H-5′ ↔ H_3-14′; H-7′ ↔ H_3-14′; H_3-14′ ↔ H-5′, H-7′; OH-8 ↔ OH-10; OH-10 ↔ OH-8, OH-16; OH-16 ↔ OH-10; HRESIMS obsd *m/z* 647.2105 $(M+Na)^+$, calcd for $C_{33}H_{36}O_{12}Na$, 647.2099.

3.3.6. Isosulochrin (6)

^1H, ^{13}C NMR, and the ESIMS data were fully consistent with literature values.[14]

3.3.7. Isosulochrin dehydrate (7)

^1H, ^{13}C NMR, and the ESIMS data were fully consistent with literature values.[14]

3.3.8. *iso*-A82775 (8)

^1H, ^{13}C NMR, and the ESIMS data were fully consistent with literature values.[13]

3.3.9. 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 7.74 (1H, dd, *J* = 12, 3.0 Hz, H-11), 6.14 (1H, d, *J* = 12 Hz, H-12), 6.01 (1H, ddd, *J* = 9.6, 6.0, 1.5 Hz, H-8), 5.26 (1H, t, *J* = 7.5 Hz, H-4), 4.05 (1 H, d, *J* = 1.5 Hz, H-7), 2.97 (1H, dd, *J* = 15, 6.0 Hz, H-9b), 2.86 (1H, ddd, *J* = 15, 9.6, 3.0 Hz, H-9a), 2.85 (1H, dd, *J* = 16, 7.5 Hz, H-5b), 2.73 (1H, dd, *J* = 16, 7.5 Hz, H-5a), 1.68 (3H, s, H_3-15), 1.67 (3H, s, H_3-14), 1.57 (3H, s, H_3-1), 1.57 (3H, s, H_3-2).

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 7.73 (1H, dd, *J* = 12, 3.0 Hz, H-11), 6.07 (1H, d, *J* = 12 Hz, H-12), 6.02 (1H, ddd, *J* = 9.6, 6.0, 1.5 Hz, H-8), 5.31 (1H, t, *J* = 7.5 Hz, H-4), 4.21 (1H, d, *J* = 1.5 Hz, H-7), 2.92 (1H, dd, *J* = 15, 6.0 Hz, H-9b), 2.90 (1H, dd, *J* = 16, 7.5 Hz, H-5b), 2.85 (1H, ddd, *J* = 15, 9.6, 3.0 Hz, H-9a), 2.75 (1H, dd, *J* = 16, 7.5 Hz, H-5a), 1.67 (3H, s, H_3-15), 1.68 (3H, s, H_3-14), 1.58 (3H, s, H_3-1), 1.59 (3H, s, H_3-2).

3.4. Anti-HIV bioassays

Anti-HIV assays included cytotoxicity and HIV-1 replication inhibition evaluations.[12] 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 four days 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. C8166 cells were exposed to HIV-1 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 four 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 five 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.

3.5. Antifungal bioassays

Antifungal bioassays were conducted in triplicate by following the National Center for Clinical Laboratory Standards (NCCLS) recommendations.[19] The yeasts, *Candida albicans* (ATCC 10231) and *Geotrichum candidum* (AS2.498), were grown on Sabouraud dextrose agar, and the fungus, *A. fumigatus* (ATCC 10894), was grown on potato dextrose agar. Targeted microbes (3–4 colonies) were prepared from broth culture (28 °C for 48 h), and the final spore suspensions of yeasts (in SDB medium) and *A. fumigatus* (in PDB medium) were 10^5 cells/mL and 10^4 mycelial fragments/mL, respectively. Test samples (10 mg/mL as stock solution in DMSO and serial dilutions) were transferred to 96-well clear plate in triplicate, and the suspension of the test organisms was added to each well achieving a final volume of 200 μL (fluconazole was used as the positive control). After incubation, the absorbance at 595 nm was measured with a microplate reader (TECAN), and the inhibition rate was calculated and plotted versus test concentrations to afford the IC_{50}. The MIC was defined as the lowest test concentration that completely inhibited the growth of the test organisms.[20,21]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.11.066.

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