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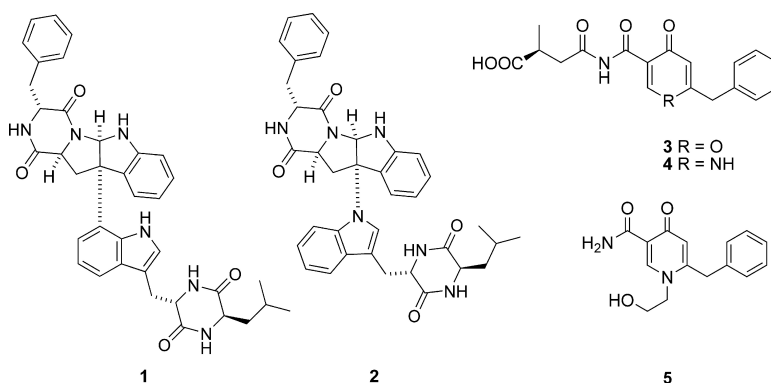
Article

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J. Nat. Prod., **2008**, 71 (11), 1861-1865 • Publication Date (Web): 15 October 2008

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Pestalazines and Pestalamides, Bioactive Metabolites from the Plant Pathogenic Fungus *Pestalotiopsis theae*

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Received June 17, 2008

Pestalazines A (**1**) and B (**2**), two new diketopiperazine heterodimers, and pestalamides A–C (**3**–**5**), three new amides, have been isolated from cultures of the plant pathogenic fungus *Pestalotiopsis theae*. The structures of these compounds were elucidated mainly by NMR spectroscopy. The absolute configurations of **1** and **2** were determined using Marfey's method on their acid hydrolysates and by comparison of their CD spectra with that of a model compound. Compounds **1**, **3**, and **6** displayed inhibitory effects on HIV-1 replication in C8166 cells. Compound **3** also showed potent antifungal activity against *Aspergillus fumigatus*.

Many species of the fungal genus *Pestalotiopsis* are either endophytic or pathogenic on living plant leaves and twigs. *Pestalotiopsis theae* is normally known as a causal fungus for tea gray blight disease, and chemical studies of metabolites of this fungus have led to the identification of phytotoxins and plant growth regulators.^{1–3} Our prior chemical investigations of some of the plant endophytic *Pestalotiopsis* spp. have afforded a variety of new bioactive natural products,^{4–7} including four new metabolites recently discovered from an endophytic strain (LN560) of *P. theae*.⁵ During an ongoing search for new bioactive metabolites from plant endophytic and/or pathogenic fungi, a pathogenic isolate of *P. theae* (W148), obtained from branches of *Camellia sinensis* at Hangzhou Botanical Garden, Hangzhou, Zhejiang Province, People's Republic of China, was grown in a solid-substrate fermentation culture. Its ethyl methyl ketone extract displayed inhibitory effects on HIV-1 replication in C8166 cells and antifungal activity against *Aspergillus fumigatus*. A follow-up fractionation of this extract led to the isolation of two new diketopiperazine heterodimers, pestalazines A (**1**) and B (**2**), and three new amides, pestalamides A–C (**3**–**5**), along with the known compounds asperazine (**6**),⁸ aspernigrin A (**7**),⁹ and carbonarone A (**8**).¹⁰ Details of the isolation, structure elucidation, and bioactivities of these new metabolites are presented herein.

Results and Discussion

Pestalazine A (**1**) was assigned a molecular formula of C₃₇H₃₈N₆O₄ on the basis of HRESIMS analysis (*m/z* 653.1249 [M + Na]⁺; Δ −0.9 mmu) and NMR data (Table 1). Analysis of the ¹H, ¹³C, and HMQC NMR spectroscopic data of **1** revealed the presence of two methyl groups, four methylene units, six methines, one quaternary carbon, 20 aromatic/olefinic (13 of which are protonated) carbons, and four amide/carboxyl carbons. These data, together with five exchangeable protons, accounted for all ¹H and ¹³C NMR resonances for **1** and are consistent with the molecular formula C₃₇H₃₈N₆O₄. Interpretation of the ¹H, ¹³C, and 2D NMR spectra, especially HMBC, revealed the peptidic nature of **1**. Four ¹³C NMR resonances at δ_C 169.8, 170.5, 170.6, and 171.3 ppm were characteristic of amide carbonyls. In addition, four methine peaks at δ_H/δ_C at 3.25/56.5, 4.68/58.6, 3.43/56.3, and 3.80/57.2 ppm

were indicative of four amino acid α-CH groups. A molecular formula search on C₃₇H₃₈N₆O₄ rapidly identified a known compound, WIN 64745,¹¹ which has the same elemental composition as **1** and is also a diketopiperazine heterodimer isolated from *Aspergillus* sp.¹¹ However, detailed comparison of the NMR spectroscopic data of **1** with those of WIN 64745 revealed significant differences and precluded **1** from being the known compound.

Compound **6** was readily identified as the known metabolite asperazine by comparison of its NMR and MS data with those reported.⁸ Comparison of the ¹H and ¹³C NMR spectroscopic data of **1** with those of **6** revealed their structural similarities. In the downfield region of their ¹³C NMR spectra, 20 sp² carbon resonances were observed for **1** versus 26 for **6**, suggesting that **1** had one less aromatic ring than **6**. The molecular weight of **1** is 34 mass units less than that of **6**, and this number corresponds to the difference in mass between the phenylalanine (Phe) and the leucine (Leu) residues, suggesting that **1** is a diketopiperazine heterodimer equivalent to **6** with a Phe unit replaced by Leu in one of the monomeric subunits. Analysis of the ¹H–¹H COSY and HMBC data of **1** further confirmed the presence of a Leu residue. Further interpretation of the ¹H–¹H COSY, HMQC, and HMBC NMR spectra of **1** allowed assignment of all ¹H and ¹³C NMR resonances, and these data were fully consistent with the presence of one unit each of tryptophan (Trp), Phe, and Leu in **1**. Key HMBC correlations from H-2 and H-12 to C-24 and from H-25 to C-3 established the linkage between C-3 and C-24. On the basis of these data, the gross structure of pestalazine A was established as shown in **1**.

The multiplet patterns and the NOESY data for the α-protons are often analyzed to assign the relative configuration of diketopiperazine rings.^{8–12} In compounds WIN 64745¹¹ and ditryptophenalanine,¹² a ⁵J_{HH} ≈ 1.0 Hz has been observed between the *cis* α-protons of the diketopiperazine rings, but this small coupling constant was not found between H-11 and H-15 in pestalazine A (**1**) and the known compound asperazine (**6**), suggesting a *trans* configuration for these protons.⁸ The orientation of H-2, H-11, and the C-23 indole moiety was assigned by NOESY data (Table 1). NOESY correlations of H-11 with H-2 and H-25 indicated that these protons were close in space. However, the orientation of H-34 and H-37 could not be determined on the basis of ¹H NMR coupling constants.

Marfey's method¹³ was applied to determine the absolute configuration of Leu (C-37) and Phe (C-15) residues in pestalazine

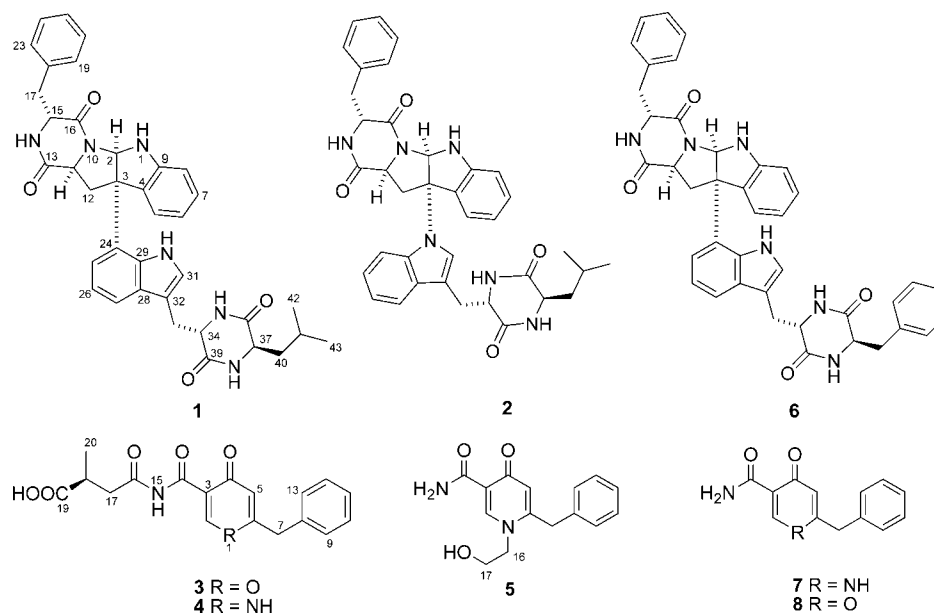
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Chart 1

**Table 1.** NMR Spectroscopic Data of Pestalazine A (**1**) in CD₃OD

position	δ_H^a (J in Hz)	δ_C^b , mult.	HMBC (H \rightarrow C#)	NOESY ^a
2	5.80, s	84.9, CH	4, 9, 12, 24	11
3		59.1, qC		
4		133.6, qC		
5	6.73, d (8.0)	124.6, CH	3, 4, 6, 7, 9	
6	6.60, t (8.0)	120.9, CH	4, 5, 7, 8	
7	7.03, t (8.0)	129.4, CH	5, 6, 8, 9	
8	6.72, d (8.0)	111.8, CH	4, 6, 7, 9	
9		148.5, qC		
11	4.68, dd (10, 7.0)	58.6, CH	3, 12, 13, 16	2, 25
12	2.45, dd (13, 10); 3.42, dd (13, 7.0)	39.4, CH ₂	2, 4, 11, 13, 24	
13		171.3, qC		
15	3.25, (m)	56.5, CH	13, 16, 18	
16		169.8, qC		
17	2.73, dd (14, 5.0); 2.93, dd (14, 5.0)	39.3, CH ₂	15, 16, 19, 23	
18		136.6, qC		
19, 23	6.97, m	131.1, CH	17, 18, 20, 21, 22	
20, 22	7.15, m	129.4, CH	18, 19, 21, 23	
21	7.15, m	128.2, CH	19, 20, 22, 23	
24		125.0, qC		
25	7.28, d (8.0)	120.2, CH	3, 24, 26, 27, 29	11
26	7.05, t (8.0)	120.4, CH	24, 25, 27, 28	
27	7.48, d (8.0)	119.9, CH	25, 26, 28, 29, 32	
28		130.0, qC		
29		135.1, qC		
31	6.86, s	126.2, CH	28, 29, 32	
32		109.8, qC		
33	2.95, dd (14, 4.5); 3.15, d (14, 4.5)	30.1, CH ₂	28, 31, 32, 34, 39	
34	3.46, br t (4.5)	56.3, CH	33, 36, 39	
36		170.6 qC		
37	3.80, dd (10, 5.5)	57.2, CH	36, 39, 40	
39		170.5, qC		
40	1.43, m; 1.56, m	42.8, CH ₂	37, 42, 43	
41	1.68, m	25.6, CH	40, 42, 43	
42	0.89, d (6.5)	23.3, CH ₃	40, 41, 43	
43	0.85, d (7.0)	21.7, CH ₃	40, 41, 42	

^a Recorded at 600 MHz. ^b Recorded at 150 MHz.

A (**1**). HPLC analysis of the 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) derivatives of the acid hydrolyzates of **1** gave the same retention times as those prepared from samples of authentic D-Leu and D-Phe. Therefore, C-15 and C-37 were both assigned the *R* absolute configuration. Considering the relative configuration established for **1** by NOESY data, the remaining

stereogenic centers were assigned 2*R*, 3*S*, and 11*S* absolute configuration. The absolute configuration of C-34 was assigned by application of the CD exciton chirality method. The CD spectrum of **1** showed negative Cotton effects at 224 ($\Delta\epsilon -27$) and 282 ($\Delta\epsilon -0.7$) nm and positive Cotton effects at 240 ($\Delta\epsilon +7.1$) and 297 ($\Delta\epsilon +5.1$) nm, which was nearly identical to that of **6**,⁸ suggesting the 34*S* absolute configuration for **1**. Collectively, these data permitted assignment of structure **1** to the new natural product pestalazine A.

The molecular formula of pestalazine B (**2**) was established as C₃₇H₃₈N₆O₄ by analysis of its HRESIMS (m/z 653.2840 [M + Na]⁺; $\Delta +0.7$ mmu) and NMR data (Table 2). The ¹H, ¹³C, and HMQC NMR spectra of **2** showed the presence of similar structural fragments to those found in **1**, except that the chemical shift of C-3 (δ_C 59.1) was shifted significantly downfield (δ_C 74.2), and the resonance for one additional aromatic proton was observed in the ¹H NMR spectrum of **2**. These suggested that C-3 could be directly connected to the nitrogen (N-30) of the Trp moiety. An HMBC correlation from the aromatic proton H-31 to the sp³ quaternary carbon C-3 confirmed the presence of this C–N linkage, thereby completing the gross structure of pestalazine B as shown.

The relative configuration of **2** was assigned by comparison of the ¹H and ¹³C NMR spectroscopic data, especially the ¹H–¹H coupling constants and NOSEY data with those of **1**. Pestalazine B (**2**) possesses the same planar structure as an analogue of the known compound Q20547-A,¹⁴ which contains L-Leu and L-Phe units. The CD spectrum of **2** showed negative Cotton effects at 219 ($\Delta\epsilon -1.5$) and 277 ($\Delta\epsilon -0.80$) nm and positive Cotton effects at 241 ($\Delta\epsilon +0.65$) and 298 ($\Delta\epsilon +0.83$) nm, which was nearly identical to those of compounds **1** and **6**, indicating that **2** also has D-Leu and D-Phe, and this observation was further confirmed by Marfey's analysis of the acid hydrolyzates.¹³

The elemental composition of pestalamide A (**3**) was established as C₁₈H₁₇NO₆ by analysis of its HRESIMS (m/z 366.0950 [M + Na]⁺; $\Delta -0.2$ mmu) and NMR data (Table 3). Interpretation of the ¹H, ¹³C, and HMQC NMR spectroscopic data of **3** revealed the presence of two exchangeable protons, one methyl group, two methylene units, one methine, two trisubstituted olefinic moieties, one monosubstituted phenyl ring, and four carbonyl carbons. These data accounted for all the ¹H and ¹³C NMR resonances and suggested that compound **3** was monomeric. Analysis of the ¹H–¹H COSY NMR data identified two isolated proton spin-systems corresponding to the C-17–C-18–C-20 fragment and one monosubstituted aryl ring. HMBC correlations of H₂-17 and H-18 with

Table 2. NMR Spectroscopic Data of Pestalazine B (**2**) in Acetone-*d*₆

position	δ_H^a (J in Hz)	δ_C^b , mult.	HMBC (H \rightarrow C#)	NOESY ^a
1	6.83, d (3.0)			
2	6.03, d (3.0)	83.4 CH	4, 9, 3, 12	31
3		74.2, qC		
4		129.8, qC		
5	6.90, d (7.2)	123.3, CH	3, 7, 9	
6	6.62, t (7.2)	119.4, CH	4, 8	
7	7.14, m	130.5, CH	5, 9	
8	6.83, d (7.8)	110.9, CH	4, 6, 9	
9		148.9, qC		
11	4.82, dd (12, 6.0)	57.2, CH	3, 13, 16	31
12	2.42, dd (14, 12), 3.68, dd (14, 6.0);	41.2, CH ₂	2, 4, 11, 13	
13		168.8, qC		
14	7.32, br.s		13, 15	
15	3.47, br.t (4.8)	56.1, CH	13, 16, 18	
16		168.4, qC		
17	2.88, overlapped; 3.06, dd (14, 4.8)	38.9, CH ₂	15, 16, 19, 23	
18		137.1, qC		
19, 23	7.14, m	130.9, CH	17, 21	
20, 22	7.20, m	130.9, CH	18, 19, 21, 23	
21	7.20, m	127.4, CH	19, 23	
24	6.75, d (7.8)	112.8, qC	26, 28	
25	6.95, t (7.8)	122.2, CH	27, 29	
26	6.98, t (7.8)	120.3, CH	24, 28	
27	7.53, d (7.8)	120.3, CH	25, 29, 32	
28		130.8, qC		
29		136.3, qC		
31	7.64, s	126.4, CH	28, 29, 32, 33	2, 11
32		109.9, qC		
33	3.15, dd (14, 4.8); 3.21, dd (14, 5.4)	30.2, CH ₂	28, 31, 39	
34	3.62, dd (5.2, 4.8)	55.8, CH	32, 33, 36, 39	
35	7.38, br s		34, 36	
36		168.9 qC		
37	3.85, m	56.7, CH	36, 39, 40	
38	8.05, d (3.5)			
39		168.6, qC		
40	1.50, m; 1.73, m	42.8, CH ₂	37, 42, 43	
41	1.80, m	24.9, CH	40, 42, 43	
42	0.95, d (6.6)	23.2, CH ₃	40, 41, 43	
43	0.90, d (6.0)	21.7, CH ₃	40, 41, 42	

^a Recorded at 600 MHz. ^b Recorded at 150 MHz.

Table 3. NMR Spectroscopic Data of Pestalamide A (**3**) in Acetone-*d*₆

position	δ_H^a (J in Hz)	δ_C^b , mult.	HMBC (H \rightarrow C#)
2	8.88, s	164.4, CH	3, 6, 14
3		119.2, qC	
4		178.8, qC	
5	6.44, s	116.5, CH	3, 4, 6, 7, 14
6		170.7, qC	
7	4.06, s	39.6, CH ₂	5, 6, 8, 9, 13
8		135.8, qC	
9, 13	7.38, m	129.7, CH	7, 8, 10, 11
10, 12	7.38, m	130.1, CH	8, 9, 11, 13
11	7.38, m	128.3, CH	9, 10, 12, 13
14		162.1, qC	
16		173.7, qC	
17	3.29, dd (18, 8.0); 2.96, (m)	42.5, CH ₂	16, 18, 19, 20
18	2.90, (m)	39.3, CH	16, 17, 19, 20
19		176.7, qC	
20	1.24, d (7.0)	17.4, CH ₃	17, 18, 19
NH-15	11.74, br s		
COOH-19	10.6, br s		

^a Recorded at 500 MHz. ^b Recorded at 125 MHz.

C-16 led to the connection of C-16 with C-17, and those from H₂-17, H-18, and H₃-20 to C-19 indicated that C-17, C-19, and C-20 were all connected to C-18. The presence of a *para*-disubstituted 4*H*-pyran-4-one moiety was established on the basis of HMBC correlations from H-2 (δ_H 8.88) and H-5 (δ_H 6.44) to C-3, C-4,

Table 4. NMR Spectroscopic Data of Pestalamides B (**4**) in Acetone-*d*₆ and C (**5**) in CD₃OD

position	pestalamide B (4)		pestalamide C (5)	
	δ_H^a (J in Hz)	δ_C^b , mult.	δ_H^a (J in Hz)	δ_C^b , mult.
2	8.52, s	164.4, CH	8.54, (s)	149.0, CH
3		119.2, qC		118.8, qC
4		178.8, qC		179.1, qC
5	6.36, s	116.5, CH	6.28, (s)	122.4, CH
6		170.7, qC		154.6, qC
7	4.06, s	39.6, CH ₂	4.13, (s)	38.9, CH ₂
8		135.8, qC		137.0, qC
9, 13	7.34, m	129.7, CH	7.17, d (7.5)	130.1, CH
10, 12	7.37, m	130.1, CH	7.32, t (7.5)	130.2, CH
11	7.20, m	128.3, CH	7.25, t (7.5)	128.5, CH
14		162.1, qC		168.4, qC
16		173.7, qC	4.11, t (4.5)	56.6, CH ₂
17	3.29, dd (18, 8.0); 2.96, (18, 5.0)	42.5, CH ₂	3.68, t (4.5)	61.8, CH ₂
18	2.90, (8.0, 7.0, 5.0)	39.3, CH		
19		176.7, qC		
20	1.12, d (7.0)	17.4, CH ₃		

^a Recorded at 500 MHz. ^b Recorded at 150 MHz.

and C-6 and by comparison of its ¹H and ¹³C NMR chemical shifts with those of the closest related known analogue, microsphaerone A.¹⁵ Other correlations from H-2 to C-14 and from H₂-7 to C-5, C-6, C-8, and C-9/13 indicated that the carbonyl carbon C-14 was directly attached to C-3, and the disubstituted 4*H*-pyran-4-one moiety and the monosubstituted aryl ring were both connected to C-7. Although no HMBC correlation was observed from the amide proton (NH-15) to any of the relevant carbons, comparison of the ¹H and ¹³C NMR data of **3** with those of microsphaerone A¹⁵ led to the connection of this amide proton to both C-14 and C-16. The remaining exchangeable proton in **3** was assigned to the carboxylic acid moiety (C-19) to complete the gross structure of pestalamide A. The absolute configuration of C-18 in **3** was assigned as *S* by comparison of its specific rotation ($[\alpha]_D -12$) with that ($[\alpha]_D -8.9$) of the model compound microsphaerone A.¹⁵

Pestalamide B (**4**) was assigned the molecular formula C₁₈H₁₈N₂O₅ by analysis of its HRESIMS (*m/z* 365.1109 [M + Na]⁺; $\Delta -0.1$ mmu) data, and this formula has one more NH and one less oxygen than that of **3**. The ¹H and ¹³C NMR spectroscopic data of **4** were nearly identical to those of **3**, except that the chemical shift of H-2 (δ_H 8.88) was shifted slightly upfield (δ_H 8.52) in the ¹H NMR spectrum of **4**, indicating that the oxygen atom of the 4*H*-pyran-4-one ring presented in **3** was replaced by an NH to form a pyridin-4(1*H*)-one moiety in **4**. Relevant ¹H–¹H COSY and HMBC correlations confirmed the proposed structure of **4**. The stereogenic carbon C-18 in **4** was also assigned the *S* absolute configuration by comparison of its specific rotation ($[\alpha]_D -8.0$) with that of **3** ($[\alpha]_D -12$).

The molecular formula of pestalamide C (**5**) was determined to be C₁₅H₁₆N₂O₃ (nine degrees of unsaturation) on the basis of HRESIMS analysis (*m/z* 295.1065 [M + Na]⁺; $\Delta -1.2$ mmu) and the NMR data (Table 4). Analysis of the ¹H and ¹³C NMR spectroscopic data of **5** revealed the presence of the same partial structures of a monosubstituted aryl ring and a *para*-disubstituted pyridin-4(1*H*)-one moiety with a carbonyl carbon attached to C-3, similar to the arrangement in **4**. However, signals for the 2-methylsuccinic acid moiety present in **3** and **4** were absent in the NMR spectra of **5**. Additional signals for two mutually coupled broad triplets (δ_H/δ_C 4.11/56.6 and 3.68/61.8, respectively) were observed, corresponding to a –CH₂CH₂– subunit connected to two heteroatoms. HMBC correlations of H₂-16 with C-2 and C-6 led to the connection of C-16 to the nitrogen of the disubstituted pyridin-4(1*H*)-one ring. Considering the chemical shift value for C-17 (δ_C 61.8), the only hydroxy group in **5** was attached to this methylene carbon, and the remaining exchangeable protons were assigned as

amide protons. On the basis of these data, the structure of pestalamide C was determined as shown in 5.

Compounds **7** and **8** were also isolated from the crude extract and identified as the known metabolites aspernigrin A and carbonarone A, respectively, by comparison of their NMR and MS data with those reported.^{9,10}

Compounds **1–8** were tested for *in vitro* activity against HIV-1. Compounds **1**, **3**, and **6** showed inhibitory effects on HIV-1 replication in C8166 cells, with EC₅₀ values of 47.6, 64.2, and 98.9 μ M, respectively (the CC₅₀ values for these compounds are all greater than 100 μ M; the positive control indinavir sulfate showed an EC₅₀ value of 5.5 nM). Compounds **1–8** were also evaluated for activity against *Candida albicans* (ATCC 10231), *Geotrichum candidum* (AS2.498), and *Aspergillus fumigatus* (ATCC 10894). Pestalamide A (**3**) displayed potent antifungal activity against *A. fumigatus*, with IC₅₀/MIC values of 1.50/57.8 μ M (the positive control fluconazole showed IC₅₀/MIC values of 7.35/163.4 μ M).

Pestalazine A (**1**) is closely related to the known compound asperazine (**6**), which was initially isolated from a sponge-derived *Aspergillus* sp. and was the second example of this type of diketopiperazine heterodimers.⁸ Both metabolites contain the relatively rare D-amino acid residues. Pestalazine B (**2**) possesses the same planar structure as that of an analogue of the known compound Q20547-A,¹⁴ but contains one unit each of the D-Leu and D-Phe, instead of the L-Leu and L-Phe residues. Pestalamide A (**3**) is closely related to microsphaerone A¹⁵ and berkeleyamide B,¹⁶ but differs from microsphaerone A by having a benzyl group attached to C-7 instead of a C-11 aliphatic chain, and from berkeleyamide B by having a 2-methylsuccinic acid moiety attached to N-15, instead of a 3-methylbutanoic acid unit. In addition, the disubstituted 4H-pyran-4-one moiety is connected to the benzene ring via a methylene unit in **3–5**, rather than an acylated oxymethine carbon, like that found in berkeleyamides B and C.¹⁶ Pestalamides B (**4**) and C (**5**) possess the same disubstituted pyridin-4(1H)-one as in the known compound aspernigrin A (**7**),⁹ but with different substituents at either N-1 or C-3. Comparing to those metabolites discovered from previous investigation of the endophytic strain of *P. theae* (LN560),⁵ our current work on this pathogenic strain led to the isolation of a completely different class of metabolites, indicating that the habitat for the fungus and/or the fungus–host relationship may play a major role in regulating the production of secondary metabolites.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and UV data were recorded on a Shimadzu Biospec-1601 spectrophotometer. CD spectra were recorded on a JASCO J-815 spectropolarimeter, using CH₃OH as solvent. IR data were recorded using a Nicolet Magna-IR 750 spectrophotometer. ¹H and ¹³C NMR data were acquired with Bruker Avance-400, -500, and -600 spectrometers using solvent signals (CD₃OD; δ_{H} 3.35/ δ_{C} 49.9; acetone-*d*₆; δ_{H} 2.05/ δ_{C} 29.8, 206.0) 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.

Fungal Material. The culture of *P. theae* was isolated by one of authors (L.G.) from the branches of *Camellia sinensis* at Hangzhou Botanical Garden (118°11' E, 30°27' N), Hangzhou, Zhejiang Province, in May 2005. The isolate was identified and assigned the accession number W148 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 5 days. Fermentation was carried out in four 500 mL Fernbach flasks each containing 75 g of rice. Spore inoculum was prepared by suspension in sterile, distilled H₂O to give a final spore/

cell suspension of 1×10^6 /mL. Distilled H₂O (100 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 15 lb/in² for 30 min.¹⁷ 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 freeze-dried and extracted with ethyl methyl ketone (3 \times 500 mL), and the organic solvent was evaporated to dryness under vacuum to afford 4.01 g of crude extract, which was chromatographed on a Si gel column (5 \times 25 cm) using CH₂Cl₂–CH₃OH gradient elution to afford seven fractions. The fraction (40 mg) eluted with 92:8 CH₂Cl₂–CH₃OH was separated by Sephadex LH-20 column chromatography using CH₃OH as eluent, and the subfractions were further purified by semipreparative reversed-phase HPLC (Agilent Zorbax SB-C₁₈ column; 5 μ m; 9.4 \times 250 mm; 2 mL/min) to afford pestalazine (**1**; 2.0 mg, *t*_R 18.4 min; 65% CH₃OH in H₂O over 5 min, 65% to 80% over 40 min), the known compound asperazine (**6**; 3.2 mg, *t*_R 22.5 min; same gradient as in purification of **1**), pestalazine B (**2**; 2.0 mg, *t*_R 26.0 min; same gradient as in purification of **1**), and pestalamide C (**5**; 1.2 mg, *t*_R 18.0 min; 30% CH₃OH in H₂O over 2 min, 30% to 70% over 30 min). The fractions (100 mg) eluted with 95:5 and 94:6 CH₂Cl₂–CH₃OH were combined and separated again by Sephadex LH-20 column chromatography using CH₃OH as eluent. Purification of the resulting subfractions by semipreparative reversed-phase HPLC with different gradients afforded the known compound aspernigrin A (**7**; 1.4 mg, *t*_R 15.0 min; 30% CH₃CN in H₂O over 2 min, 30% to 45% over 20 min, 45% to 80% over 20), pestalamide A (**3**; 5.0 mg, *t*_R 26.0 min; same gradient as in purification of **7**), the known compound carbonarone A (**8**; 1.2 mg, *t*_R 18.1 min; 30% CH₃OH in H₂O over 2 min, 30% to 90% over 40 min), and pestalamide B (**4**; 1.3 mg, *t*_R 22.9 min; same gradient as in purification of **8**).

Pestalazine A (1): amorphous, pale yellow powder; [α]_D +203 (c 0.1, MeOH); UV (MeOH) λ_{max} 223 (ϵ 11 600), 275 (ϵ 8400), 285 (ϵ 9900) nm; CD (c 1.0×10^{-4} M, CH₃OH) λ_{max} ($\Delta\epsilon$) 224 (–27), 240 (+7.1), 282 (–0.7), 297(+5.1) nm; IR (neat) ν_{max} 3442, 3366 (br), 2926, 1676, 16087, 1484, 1440 cm^{–1}; ¹H, ¹³C, HMBC, and NOESY data, see Table 1; HRESIMS obsd *m/z* 653.2856 [M + Na]⁺ (calcd for C₃₇H₃₈N₆O₄Na, 653.2847).

Pestalazine B (2): amorphous, pale yellow powder; [α]_D +199 (c 0.1, MeOH); UV (MeOH) λ_{max} 230 (ϵ 8600), 292 (ϵ 19 600) nm; CD (c 1.0×10^{-4} M, CH₃OH) λ_{max} ($\Delta\epsilon$) 219 (–1.5), 241 (+0.7), 277 (–0.8), 298 (+0.8) nm; IR (neat) ν_{max} 3256 (br), 2957, 1678, 1611, 1484, 1436 cm^{–1}; ¹H, ¹³C, HMBC, and NOESY data, see Table 2; HRESIMS obsd *m/z* 653.2840 [M + Na]⁺ (calcd for C₃₇H₃₈N₆O₄Na, 653.2847).

Pestalamide A (3): amorphous, pale brown powder; [α]_D –12 (c 0.1, MeOH); UV (MeOH) λ_{max} 244 (ϵ 12 800), 312 (ϵ 11 100) nm; IR (neat) ν_{max} 3367 (br), 2968, 2932, 1696, 1652, 1606, 1571, 1507, 1413 cm^{–1}; ¹H, ¹³C, and HMBC data, see Table 3; HRESIMS obsd *m/z* 366.0950 [M + Na]⁺ (calcd for C₁₈H₁₇NO₆Na, 366.0948).

Pestalamide B (4): pale yellow oil; [α]_D –8.0 (c 0.1, MeOH); UV (MeOH) λ_{max} 246 (ϵ 11 300), 310 (ϵ 10 600) nm; IR (neat) ν_{max} 3383 (br), 2969, 1694, 1652, 1605, 1571, 1498, 1414 cm^{–1}; ¹H, and ¹³C data, see Table 4; HMBC data (acetone-*d*₆, 600 MHz) H-2 \rightarrow C-4, 6, 14; H-5 \rightarrow C-3, 6, 7; H₂-7 \rightarrow C-5, 9, 13; H-9/13 \rightarrow C-7, 8, 11; H-10/12 \rightarrow C-8, 9, 11, 12, 13; H-11 \rightarrow C-9/13, 10/12; H₂-17 \rightarrow C-18, 19, 20; H-18 \rightarrow C-16, 17, 19, 20; H₃-20 \rightarrow C-17, 18, 19; HRESIMS obsd *m/z* 365.1109 [M + Na]⁺ (calcd for C₁₈H₁₈N₂O₅Na, 365.1108).

Pestalamide C (5): white powder; UV (MeOH) λ_{max} 238 (ϵ 8200), 312 (ϵ 9300) nm; IR (neat) ν_{max} 3377 (br), 2967, 2930, 1695, 1652, 1605, 1571, 1500, 1414 cm^{–1}; ¹H and ¹³C data, see Table 4; HMBC data (CD₃OD, 600 MHz) H-3 \rightarrow C-4, 6, 14, 16; H-5 \rightarrow C-3, 6, 7; H₂-7 \rightarrow C-5, 9, 13; H-9/13 \rightarrow C-7, 8, 11; H-10/12 \rightarrow C-8, 9, 11, 12, 13; H-11 \rightarrow C-9/13, 10/12; H₂-16 \rightarrow C-2, 6; H₂-17 \rightarrow C-16; HRESIMS obsd *m/z* 295.1065 [M + Na]⁺ (calcd for C₁₅H₁₆N₂O₃Na, 295.1053).

Asperazine (6): ¹H NMR, ¹³C NMR, and the ESIMS data were fully consistent with literature values.⁸

Aspernigrin A (7): ¹H NMR, ¹³C NMR, and the ESIMS data were fully consistent with literature values.⁹

Carbonarone A (8): ¹H NMR, ¹³C NMR, and the ESIMS data were fully consistent with literature values.¹⁰

Absolute Configuration of Pestalamides A (1) and B (2).¹³ Separate solutions of pestalamides A (**1**; 0.3 mg) and B (**2**; 0.3 mg) in 6 N HCl (1 mL) were heated at 100 °C for 16 h. Upon removal of excess HCl under vacuum, the hydrolysates were placed in a 1 mL reaction vial

and treated with 1% solution of FDAA (200 μ L) in acetone, followed by 1.0 N NaHCO₃ (40 μ L). The reaction mixtures were heated at 45 °C for 1.5 h, cooled to room temperature, and then acidified with 2.0 N HCl (20 μ L). In a similar fashion, standard D- and L-Phe and Leu were derivatized separately. The derivatives of the hydrolysates and the standard amino acids were subjected to reversed-phase HPLC analysis (Kromasil C₁₈ column; 10 μ m, 4.6 \times 250 mm; 1.0–1.5 mL/min) at 25 °C using the following gradient program: solvent A, 50 mM (Et₃NH)₃PO₄ at pH 3.0; solvent B, acetonitrile; linear gradient, 10%–35% of B in 60 min with UV detection at 340 nm. The retention times for FDAA derivatives of standard D-Phe, L-Phe, D-Leu, and L-Leu were 62.9, 54.3, 66.2, and 49.5 min, respectively, while those for FDAA derivatives of pestalazine A (1) and B (2) hydrolysates were 62.6, 65.6, 62.7, and 65.7 min, respectively.

Antifungal Bioassays. Antifungal bioassays were conducted in triplicate by following the National Center for Clinical Laboratory Standards (NCCLS) recommendations.¹⁸ The yeasts, *Candida albicans* (ATCC 10231) and *Geotrichum candidum* (AS2.498), were grown on Sabouraud dextrose agar, and the fungus, *Aspergillus 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 *Aspergillus fumigatus* (in PDB medium) were 10⁵ cells/mL and 10⁴ mycelial fragments/mL, respectively. Test samples (10 mg/mL as stock solution in DMSO and serial dilutions) were transferred to a 96-well clear plate in triplicate, and the suspension of the test organisms was added to each well, achieving a final volume of 200 μ L (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 IC₅₀. The MIC was defined as the lowest test concentration that completely inhibited the growth of the test organisms.^{19,20}

Anti-HIV Assays. Anti-HIV assays included cytotoxicity and HIV-1 replication inhibition evaluations.^{5,7} Cells (3 \times 10⁴/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 period, 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. 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 \times 10⁴ 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 five times with PBST, 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 five 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.

Acknowledgment. We gratefully acknowledge financial support from the National Basic Research Program of China (Grant 2009CB522302), the Key Project of the National Hi-Tech Research and Development (Grant 2007AA021506), the Key Project of Chinese Academy of Sciences (Grant KSCX2-YW-G-013), and the National Natural Science Foundation of China (Grant 30670055).

Supporting Information Available: ¹H and ¹³C NMR spectra of pestalazines A (1) and B (2) and pestalamides A–C (3–5), and CD spectra of pestalazines A (1) and B (2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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NP800357G