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Pestalachlorides A–C, antifungal metabolites from the plant endophytic fungus *Pestalotiopsis adusta*

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ABSTRACT

Pestalachlorides A–C (**1**–**3**), three new chlorinated benzophenone derivatives, have been isolated from cultures of an isolate of the plant endophytic fungus *Pestalotiopsis adusta*. The structures of these compounds were determined mainly by NMR spectroscopy, and the structures of **1** and **3** were further confirmed by X-ray crystallography. Pestalachloride A (**1**) was obtained as a mixture of two inseparable atropisomers (**1a** and **1b**), whereas pestalachloride C (**3**) was found to be a racemic mixture. Compounds **1** and **2** displayed significant antifungal activities against three plant pathogens.

1. Introduction

Endophytic fungi are microorganisms living within the tissues of host plants, and have proven to be rich sources of bioactive natural products.[1] As one class of the most widely distributed endophytic fungi, *Pestalotiopsis* spp. have attracted much attention in recent years for their ability to produce a variety of bioactive secondary metabolites.[2–9] During an ongoing search for new bioactive compounds from plant endophytes, a subculture of an isolate of *Pestalotiopsis adusta* (L416), obtained from the stem of an unidentified tree in Xinglong, Hainan Province, People's Republic of China, was grown in solid-substrate fermentation culture. Its organic solvent extract displayed significant antifungal activities against three plant pathogenic fungi, *Fusarium culmorum* (CGMCC 3.4595), *Gibberella zeae* (CGMCC 3.2873), and *Verticillium aibo-atrum* (CGMCC 3.4306). Bioassay-directed fractionation of this extract afforded three new chlorinated benzophenones, which we named pestalachlorides A–C (**1**–**3**). Pestalachloride A (**1**) was obtained as an inseparable mixture of two atropisomers (**1a** and **1b**), and pestalachloride C (**3**) was found to be a naturally occurring racemic mixture as demonstrated by the X-ray data. Pestalachlorides A (**1**) and B (**2**) displayed potent antifungal activities against the above three plant pathogens. Details of the isolation, structure elucidation, and biological activities of these compounds are reported herein.

2. Results and discussion

Pestalachloride A (**1**) was obtained as a mixture of two atropisomers in a 5:4 ratio, as determined by integration of some well-resolved ^{1}H NMR resonances for each isomer. Exhaustive efforts to separate this mixture employing column chromatography and HPLC using various stationary and mobile phases were unsuccessful in even partially resolving them. Therefore, the structure elucidation of **1** was performed on the mixture. The molecular formula of **1** was determined as $C_{21}H_{21}Cl_2NO_5$ (11 degrees of unsaturation) by analysis of its HRESIMS [m/z 436.0686 $(M-H)^-$] and NMR data (Table 1). Interpretation of the ^{1}H, ^{13}C, and HMQC NMR spectroscopic data of **1** revealed the presence in each component of three exchangeable protons, four methyl groups (one of which is a methoxy), one methylene unit, one methine, 12 aromatic carbons (one of which is protonated), one trisubstituted olefin, and one carbonyl carbon (δ_C 173.6). These data accounted for all ^{1}H and ^{13}C NMR resonances except one exchangeable proton (OH-3), and required **1** to be tricyclic with two aryl rings.

The NMR data for the major and minor isomers were considered separately for structure elucidation purpose. In the major isomer (**1a**), two isolated proton spin-systems corresponding to the *N*–C-8 and C-1′–C-5′ (H_3-4′/H_3-5′ showed allylic couplings to H-2′) subunits of structure **1** were established on the basis of ^{1}H–^{1}H COSY data and confirmed by HMBC correlations. HMBC correlations from H-8 to C-1, C-2, C-6, and C-7, the amide proton (δ_H 7.60) to C-2, C-7, and C-8, and from H-4 to C-2, C-3, C-5, and C-6 permitted completion of the isoindolin-1-one moiety. Correlations from H_2-1′ to C-5,

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

Position	1a			1b	
	δ_H[a] (*J* in Hz)	δ_C[b]	HMBC[c] (H → C#)	δ_H[a] (*J* in Hz)	δ_C[b]
1		173.6			173.8
2		110.2			110.6
3		156.2			156.0
4	6.38, s	101.8	1, 2, 3, 5, 6	6.33, s	101.6
5		164.4			161.1
6		115.2			115.2
7		148.7			148.1
8	6.27, s	53.2	1, 2, 6, 7, 9, 10, 14	6.13, s	53.8
9		119.9			119.2
10		156.4			155.1
11		121.0			120.4
12		135.9			135.8
13		118.1			118.7
14		151.1			152.2
1′	3.02, m; 3.09, m	24.9	5, 6, 7, 2′, 3′	2.85, m; 3.02, m	24.8
2′	4.60, td (6.3, 1.5)	123.3	6, 1′, 4′, 5′	4.48, td (5.4, 1.5)	123.3
3′		131.2			131.0
4′	1.38, s	25.5	2′, 3′, 5′	1.36, s	25.5
5′	1.45, s	17.7	2′, 3′, 4′	1.41, s	17.7
1″	2.44, s	18.3	10, 11, 12, 13, 14	2.45, s	18.3
NH	7.60, s		2, 7, 8	7.60, s	
OH-5	8.90, s		6	8.80, s	
OH-10	7.88, s		9, 10,11	7.88, s	
OCH_3-14	3.30, s	60.9	14	4.00, s	62.3

[a] Recorded at 400 MHz.
[b] Recorded at 100 MHz.
[c] Recorded at 300 MHz.

C-6, and C-7 led to the connection of the prenyl group to C-6. Other correlations from H_3-1″ to C-11, C-12, and C-13, the phenolic proton at 7.88 ppm to C-9, C-10, and C-11, and from H-8 to C-9, C-10, and C-14 established the persubstituted aryl ring that is directly attached to C-8. An HMBC correlation of OCH_3-14 with C-14 enabled assignment of the methoxy group to C-14; therefore, the two chlorine atoms presented in **1** must be attached to C-11 and C-13 to complete the 2,4-dichloro-5-methoxy-3-methylphenol moiety.[10] HMBC cross-peak of the phenolic proton at 8.90 ppm with the aryl carbon C-6 indicated that its neighboring carbon C-5 is attached to a hydroxy group, leaving the remaining exchangeable proton (OH-3) in **1** being attached to C-3, and this assignment was partially supported by the downfield ^{13}C NMR chemical shift for C-3 (δ_C 156.2). Analysis of the 1D and 2D NMR data for the minor isomer (**1b**) established the same planar structure as the major one. On the basis of these data, the gross structure of pestalachloride A was established as shown in **1** (**1a** and **1b**) (Fig. 1).

The structure of pestalachloride A (**1**) was finally confirmed by X-ray crystallographic analysis, and a perspective ORTEP plot is shown in Figure 2. Pestalachloride A was found to crystallize as a mixture of 8*S* and 8*R* enantiomers. To our knowledge, two natural products, isopestacin and pestacin, also isolated from *Pestalotiopsis microspora* as antifungal agents, have been previously reported as racemic mixtures of the *S* and *R* enantiomers.[4,5] Even though the specific rotation values of the synthetic 3*S*- and 3*R*-phenyl-2,3-dihydro-1*H*-isoindolin-1-ones have been reported before,[11–13] it was not feasible to assign the NMR chemical shifts for each diastereomer simply by comparing the specific rotation value of pestalachloride A (**1**) with those reported for the synthetic isoindolinones mentioned above.

The observation of two isomers in the ^1H NMR spectrum of **1** was likely due to the hindered rotation around the C-8–C-9 bond. To assess the energy barrier for the atropisomers of **1**, variable-temperature ^1H NMR spectra were recorded at 298, 337, 353, and 373 K. The ^1H NMR spectra acquired at different temperatures all displayed signals for a mixture of two isomers with the same 5:4 ratio, indicating that the rotation of the persubstituted aryl ring around the C-8–C-9 bond was restricted for both isomers, partially due to the bulky prenyl substituent at C-6.[14] To account for the dramatic differences in NMR chemical shifts for the methoxy group in two diastereomers (δ_H/δ_C 3.30/60.9 vs 4.00/62.3), the solution structure of **1** was examined using NOESY data. For the major isomer, the methoxy protons (δ_H 3.30) showed correlation to the olefinic proton H-2′ (δ_H 4.60), suggesting that in this conformation the methoxy group might be in or closer to the shielding zone originated from the isoindolinone ring system. For the minor isomer, the methoxy protons (δ_H 4.00) were correlated to the methine proton H-8 (δ_H 6.13) in the NOESY spectrum, indicating that the methoxy group is likely in the deshielding zone of the isoindolinone ring

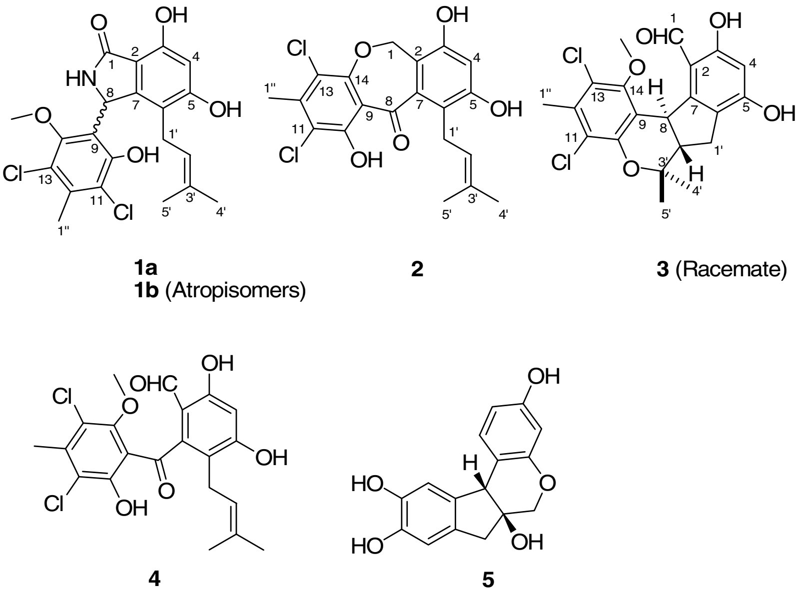

Figure 1. Structures of pestalachlorides A–C (**1**–**3**), pestalone (**4**), and brazilin (**5**).

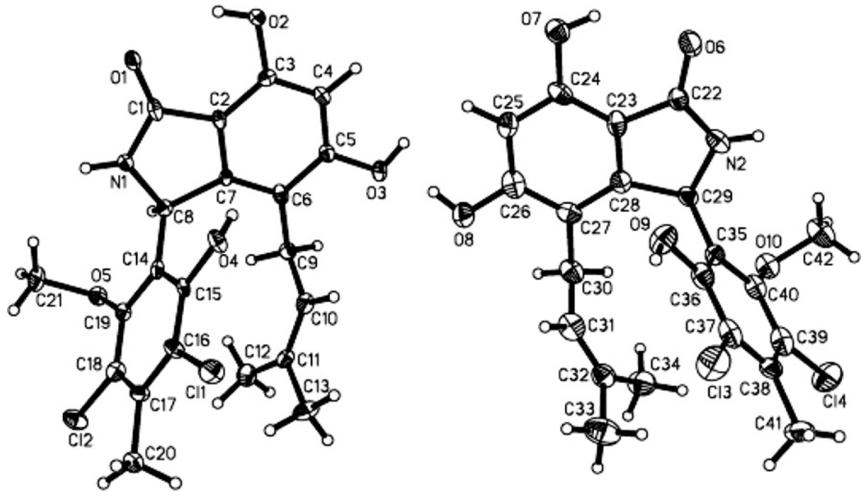

Figure 2. Thermal ellipsoid representation of pestalachloride A (**1**).

system. From steric hindrance point of view, the first conformation could be slightly preferred to the second one, leading to the formation of an atropdiastereomeric mixture in a 5:4 ratio as detected by ^{1}H NMR, rather than a racemic mixture as demonstrated by X-ray crystallography.[14,15]

The molecular formula of pestalachloride B (**2**) was assigned as $C_{20}H_{18}Cl_2O_5$ (11 degrees of unsaturation) by analysis of its HRESIMS [m/z 407.0447 $(M-H)^-$; Δ +0.6 mmu] and NMR data (Table 2). Analysis of the ^{1}H, ^{13}C, and HMQC NMR spectroscopic data of **2** revealed the presence of three exchangeable protons, three methyl groups, two methylene carbons (one of which is oxygenated), one trisubstituted olefin unit, 12 aromatic carbons (one of which is protonated), and one conjugated carbonyl carbon (δ_C 199.8). These data suggested the tricyclic feature of **2**. Interpretation of the 1D and 2D NMR data of **2** revealed the presence of the same pentasubstituted aryl ring with a prenyl group attached to C-6, and the same persubstituted benzene with two chlorines attached, as those found in **1**. HMBC correlations from H_2-1 to C-2, C-3, C-7, and C-14 led to the connection of the two aryls via an oxymethylene unit, with C-1 attached to C-2. The remaining conjugated carbonyl carbon C-8 in **2** must be connected to both C-7 and C-9 based on unsaturation requirement, and the downfield chemical shift for the phenolic proton attached to C-10 (δ_H 12.74) is supportive for this assignment, due to the formation of an intramolecular hydrogen-bond between the phenolic proton and the oxygen of the carbonyl carbon. Therefore, the gross structure of pestalachloride B was established as **2**.

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

Position	Pestalachloride B (**2**)		Pestalachloride C (**3**)	
	δ_H[a] (J in Hz)	δ_C[b]	δ_H[a] (J in Hz)	δ_C[c]
1	5.34, s	66.6	9.76, s	194.2
2		114.5		113.2
3		153.2		165.2
4	6.76, s	106.5	6.34, s	102.5
5		157.6		161.4
6		120.5		124.2
7		141.9		146.0
8		199.8	4.41, d (12)	45.9
9		112.7		129.0
10		156.6		150.8
11		114.7		123.0
12		143.8		133.8
13		114.7		121.6
14		156.3		150.2
1′a	3.39, d (6.5)	24.5	3.06, dd (15, 8.0)	29.0
1′b			2.64, ddd (15, 11, 1.5)	
2′	4.90, t (6.5)	124.3	2.42, ddd (12, 11, 8.0)	57.9
3′		132.1		83.5
4′	1.46, s	25.5,	1.58, s	29.6
5′	1.58, s	17.9	1.44, s	23.8
1″	2.51, s	18.9	2.45, s	17.9
OH-3	8.96, s		11.92, s	
OH-5	8.78, s			
OH-10	12.74, s			
OCH_3-14			3.11, s	61.5

[a] Recorded at 500 MHz.
[b] Recorded at 150 MHz.
[c] Recorded at 100 MHz.

Pestalachloride C (**3**) was assigned a molecular formula of $C_{21}H_{20}Cl_2O_5$ (11 degrees of unsaturation) by analysis of its HRESIMS [m/z 445.0599 $(M+Na)^+$; Δ −1.4 mmu] and NMR data (Table 2). The ^{1}H and ^{13}C NMR spectra of **3** displayed signals corresponding to the same partial structures of a 2,4-dichloro-5-methoxy-3-methylphenol moiety and a pentasubstituted benzene, like those appearing in **1** and **2**. However, the two olefinic carbons (C-2′/C-3′; δ_C 123.3/131.2 in **1a**; 124.3/132.1 in **2**) of the prenoid were replaced by signals for a methine (δ_C 57.9) and an oxygenated sp^3 quaternary (δ_C 83.5) carbons in the ^{13}C NMR spectra of **3**. In addition, interpretation of the NMR data for **3** revealed the presence of an aldehyde unit (δ_H/δ_C 9.76/194.2), and it was connected to the pentasubstituted aromatic ring at C-2 on the basis of HMBC correlations from the aldehyde proton H-1 to C-2 and C-3. The downfield chemical shift for the phenolic proton (OH-3; δ_H 11.92) is in agreement with this assignment, due to the intramolecular hydrogen-bonding between the phenolic proton and the oxygen of the aldehyde carbon. Analysis of the ^{1}H–^{1}H COSY NMR data led to the identification of one isolated proton spin-system corresponding to the C-8/C-2′/C-1′ substructure of **3**. HMBC correlations from H-1′a to C-6, C-7, C-8, and C-2′ and from H-1′b to C-6, C-7, and C-2′ established the cyclopentane moiety that is fused to the pentasubstituted aryl ring at C-6/C-7. The remaining ring in **3** was established as a dihydropyran by a weak, but distinct four-bond HMBC correlation from H_3-4′ to C-10, and the unaccounted exchangeable proton in **3** was attached to C-5 by default to complete the planar structure of pestalachloride C.

The relative configuration of pestalachloride C (**3**) was assigned by analysis of ^{1}H–^{1}H coupling constants and NOESY data (Fig. 3). The large *trans*-diaxial-type coupling constant observed between H-2′ with H-8 (12 Hz) suggested a *trans*-relationship between these protons.[16] NOESY correlations of H-8 with H-1 and H_3-4′, and of H_3-4′ with H-1′b indicated that these protons are all on the same face of the ring system, whereas those of H_3-5′ with H-

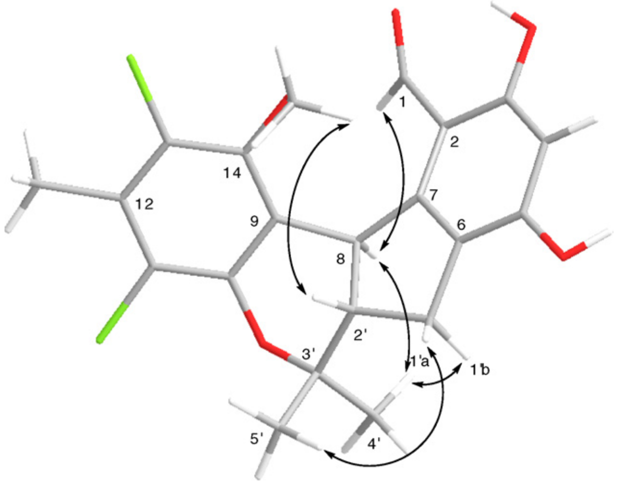

Figure 3. Key NOESY correlations for pestalachloride C (**3**).

1′a were used to place them on the opposite face of the molecule, thereby establishing the relative configuration of pestalachloride C as depicted. NOESY correlation of the OCH_3-14 with H-2′ suggested that the methoxy group is in or closer to the shielding zone originated from the cyclopentane-dihydropyan ring system in **3**.

Ultimately, the stereochemistry of pestalachloride C (**3**) was determined by single-crystal X-ray crystallographic analysis, and a perspective ORTEP plot is shown in Figure 4. The X-ray data indicated that pestalachloride C is a naturally occurring racemic mixture, which is also consistent with its zero optical rotation value.

Pestalachlorides A–C (**1**–**3**) were evaluated for antifungal activities against a small panel of the plant pathogenic fungi, and the results are shown in Table 3. Pestalachloride A (**1**) displayed potent antifungal activity against *F. culmorum*, with an IC_{50} value of 0.89 μM, while pestalachloride B (**2**) exhibited remarkable activity against *G. zeae*, with an IC_{50} value of 1.1 μM. In our assays, pestalachloride C (**3**) did not show noticeable in vitro antifungal activities against *F. culmorum*, *G. zeae*, and *V. aibo-atrum* ($IC_{50} > 100$ μM).

Pestalachlorides A–C (**1**–**3**) are new members of the relatively rare chlorinated benzophenone type of metabolites. Biogenetically, these compounds could be derived from one unit of prenoid and a polyketide. Compounds **1**–**3** are structurally related to the known compound pestalone (**4**) and its desmethyl analogue,[10] and could be considered as lactamized or cyclized products from these precedents. Pestalachloride A (**1**) could be derived from the known compound pestalone (**4**), and the racemization of **1** was probably due to the presence of a stabilized cationic intermediate (Fig. 5), as described in the literature for pestacin.[5] However, due to the hindered rotation around the C-8/C-9 bond in **1**, two atropisomers (**1a** and **1b**) were observed in the ^{1}H NMR spectrum of **1** instead of a racemic mixture. Natural products containing an isolated isoindolin-1-one moiety as core structure have been previously reported, such as fungal metabolites cichorine,[17] duricaulic acid,[18] xylactam,[19] the memnobotrins,[20] and the plant metabolite fumaramidine.[21] However, pestalachloride A (**1**) differs significantly from those known metabolites by having a relatively rare 2,4-dichloro-5-methoxy-3-methylphenol moiety connected to the isoindolin-1-one core structure. Pestalachloride C (**3**) occurs naturally as a racemic mixture, and it possesses the same 7,11*b*-dihydrobenz[*b*]indeno[1,2-*d*]pyran ring system as the plant metabolites brazilin (**5**) and the haematoxylins.[22] In all probability, the racemization of **3** proceeds in a manner similar to that of **1** and **4** through cationic intermediates. The discovery of these new bioactive secondary metabolites indicates that systematic chemical studies of *Pestalotiopsis* spp. are necessary to fully explore their potential for the production of new antifungal natural products.

Table 3
Antifungal activities of pestalachlorides A–C (**1**–**3**)

Compound	IC_{50} (μM)			MIC (μM)		
	F. culmorum	*G. zeae*	*V. aibo-atrum*	*F. culmorum*	*G. zeae*	*V. aibo-atrum*
1	0.89 ± 0.10	54.4 ± 0.8	58.3 ± 1.4	7.2	114.4	114.4
2	4.7 ± 0.2	1.1 ± 0.08	7.9 ± 0.2	49.0	12.2	12.2
3	>118.5	>118.5	>118.5	>236.9	>236.9	>236.9

Plant pathogenic fungi: *Fusarium culmorum*, *Gibberella zeae*, and *Verticillium aibo-atrum*.

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 Bruker Avance-300, -400, and Varian Inova-500, -600 spectrometers 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 3000^{plus} spectrometer, and HRESIMS data were obtained using a Bruker APEX III 7.0 T spectrometer.

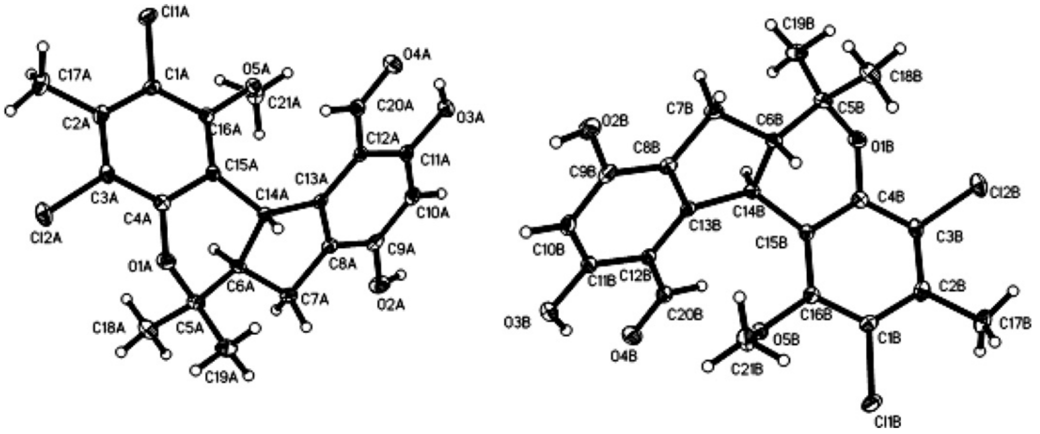

Figure 4. Thermal ellipsoid representation of pestalachloride C (**3**).

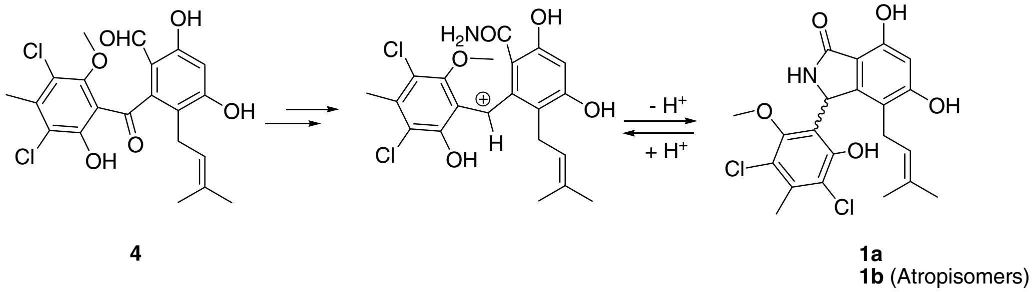

Figure 5. Proposed racemization mechanism in pestalachloride A (**1**).

3.2. Fungal material

The culture of *P. adusta* was isolated by one of the authors (L.G.) from the stem of an unidentified tree in Xinglong, Hainan Province, People's Republic of China, in April, 2005. The isolate was identified and assigned the Accession No. L416 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 (6% dextrin, 2% maltose, 0.75% cotton-seed meal, 0.7% peptone, 0.25% $CaCO_3$, 0.25% $MgSO_4{\cdot}7H_2O$, 0.1% $FeSO_4{\cdot}7H_2O$, 0.001% $ZnSO_4$; final pH 6.0) and 30 g of vermiculite were individually inoculated with 15 mL of the seed culture, and were incubated at 25 °C under static conditions for 40 days.

3.3. Extraction and isolation

The fermented material was extracted with MEK (3× 500 mL), and the organic solvent was evaporated to dryness under vacuum to afford 3.67 g of crude extract. The extract was fractionated by Silica gel VLC using petroleum ether–EtOAc gradient elution. The fraction eluted with 30% EtOAc was further fractionated by Sephadex LH–20 column chromatography using 1:1 $CHCl_3/CH_3OH$ as eluents, and one subfraction (50 mg) was purified again by Sephadex LH–20 column chromatography using CH_3OH as eluent to afford pestalachloride A (**1**; 20.0 mg). The fraction eluted with 20% EtOAc was also fractionated by Sephadex LH-20 column chromatography using 1:1 $CHCl_3/CH_3OH$ as solvents, one subfraction (10 mg) was purified by semipreparative reversed-phase HPLC (Kramosil C_{18} column; 10 μm; 10 × 250 mm; 2 mL/min) to afford pestalachloride B (**2**; 2.0 mg, t_R 18.6 min; 90–100% CH_3OH in water over 25 min). Another fraction eluted with 8% EtOAc was subjected to semipreparative reversed-phase HPLC purification (Kramosil C_{18} column; 10 μm; 10 × 250 mm; 2 mL/min) to afford pestalachloride C (**3**; 3.5 mg, t_R 32.0 min;70–100% CH_3OH in water over 40 min).

3.3.1. Pestalachloride A (1)

Colorless platelet, mp 205–207 °C; $[\alpha]_D$ +5.0 (*c* 0.4, CH_3OH); UV (CH_3OH) λ_{max} 211 (ε 100,100), 253 (ε 17,600), 296 (ε 12,200) nm; IR (neat) ν_{max} 3338 (br), 1662, 1404, 1281, 1177, 1092 cm^{-1}; ^1H, ^{13}C NMR, and HMBC data, see Table 1; NOESY correlations for **1a** (acetone-d_6, 600 MHz) H-8 ↔ H_2-1′, H-2′; OCH_3-14 ↔ H-2′; H_2-1′ ↔ H-8, H_3-5′; H-2′ ↔ OCH_3-14, H_3-4′; H_3-4′ ↔ H-2′; H_3-5′ ↔ H_2-1′; NOESY correlations for **1b** (acetone-d_6, 600 MHz) H-8 ↔ OCH_3-14, H_2-1′; OCH_3-14 ↔ H-8; H_2-1′ ↔ H-8, H_3-5′; H-2′ ↔ H_3-4′; H_3-4′ ↔ H-2′; H_3-5′ ↔ H_2-1′; HRESIMS obsd *m*/*z* 436.0686 $(M-H)^-$, calcd for $C_{21}H_{20}Cl_2NO_5$, 436.0718.

3.3.2. Pestalachloride B (2)

Yellow gum; UV (CH_3OH) λ_{max} 211 (ε 28,000), 224 (ε 19,000), 301 (ε 8800), 376 (ε 4900) nm; IR (neat) ν_{max} 3390 (br), 2928, 1618, 1593, 1404, 1378, 1351, 1227, 1165, 1098 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HMBC data (acetone-d_6, 600 MHz) H_2-1 → C-2, 3, 7, 14; H-4 → C-2, 3, 5, 6; H_2-1′ → C-5, 6, 7, 2′, 3′; H-2′ → C-4′, 5′; H_3-4′ → C-2′, 3′, 5′; H_3-5′ → C-2′, 3′, 4′; H_3-1″ → C-11, 12, 13; OH-3 → C-2, 3; OH-5 → C-4, 5, 6; OH-10 → C-9, 10, 11; HRESIMS obsd *m*/*z* 407.0447 $(M-H)^-$, calcd for $C_{20}H_{17}Cl_2O_5$, 407.0453.

3.3.3. Pestalachloride C (3)

Colorless platelet, mp 280–282 °C; $[\alpha]_D$ 0 (*c* 0.10, CH_3OH); UV (CH_3OH) λ_{max} 229 (ε 34,000), 284 (ε 15,000), 328 (ε 7800) nm; IR (neat) ν_{max} 3140 (br), 2928, 1642, 1621, 1448, 1373, 1306, 1276, 1173, 1138, 1029 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HMBC data (acetone-d_6, 400 MHz) H-1 → C-2, 3, 4; H-4 → C-2, 3, 5, 6, 1′; H-8 → C-6, 7, 9, 10, 14, 2′, 3′; H-1′a → C-5, 6, 7, 8, 2′; H-1′b → C-6, 7, 2′, 3′; H-2′ → C-8, 9, 3′, 4′, 5′; H_3-4′ → C-10, 2′, 3′, 5′; H_3-5′ → C-2′, 3′, 4′; H_3-1″ → C-10, 11, 12, 13, 14; OH-3 → C-2, 3, 4, 5; OCH_3-14 → C-14; NOESY correlations (acetone-d_6, 400 MHz) H-1 ↔ H-8; H-8 ↔ H-1, H_3-4′; H-1′a ↔ H_3-5′; H-1′b ↔ H_3-4′; H-2′ ↔ OCH_3-14; H_3-4′ ↔ H-8, H-1′b; H_3-5′ ↔ H-1′a; OCH_3-14 ↔ H-2′; HRESIMS obsd *m*/*z* 445.0599 $(M+Na)^+$, calcd for $C_{21}H_{20}Cl_2O_5Na$, 445.0585.

3.3.4. X-ray crystallographic analysis of pestalachloride A (1)[23]

Upon crystallization from $MeOH/H_2O$ (10:1) using the vapor diffusion method, colorless crystals were obtained for **1**, a crystal (0.14 × 0.12 × 0.02 mm) was separated from the sample and mounted on a glass fiber, and the data were collected using a Rigaku Saturn CCD area detector with graphite-monochromated Mo Kα radiation $\lambda = 0.71070$ Å at 113(2) K. Crystal data: $C_{21}H_{21}Cl_2NO_5$, $M = 438.29$, space group triclinic, $P\bar{1}$; unit cell dimensions $a = 7.905(6)$ Å, $b = 14.768(12)$ Å, $c = 17.319(14)$ Å, $V = 1982(3)$ Å^3, $Z = 4$, $D_{calcd} = 1.469$ mg/m^3, $\mu = 0.362$ mm^{-1}, $F(000) = 912$. The structure was solved by direct methods using SHELXL-97[24] and refined using full-matrix least-squares difference Fourier techniques. All non-hydrogen atoms were refined with anisotropic displacement parameters, and all hydrogen atoms were placed in idealized positions and refined as riding atoms with the relative isotropic parameters. Absorption corrections were applied with the Crystalclear.[25] The 20,264 measurements yielded 6980 independent reflections after equivalent data were averaged and Lorentz and polarization corrections were applied. The final refinement gave $R_1 = 0.1288$ and $wR_2 = 0.3290$ $[I > 2\sigma(I)]$.

3.3.5. X-ray crystallographic analysis of pestalachloride C (3)

[23] Upon crystallization from MeOH using the vapor diffusion method, colorless crystals were obtained for **3**, a crystal (0.50 × 0.44 × 0.17 mm) was separated from the sample and

mounted on a glass fiber, and the data were collected using a Rigaku Raxis Rapid IP area detector with graphite-monochromated MoKα radiation $\lambda = 0.71073$ Å at 173(2) K. Crystal data: $C_{22}H_{24}Cl_2O_6$, $M = 455.31$, space group triclinic, $P\bar{1}$; unit cell dimensions $a = 9.0692(18)$ Å, $b = 10.129(2)$ Å, $c = 12.381(3)$ Å, $V = 1048.5(4)$ Å^3, $Z = 2$, $D_{calcd} = 1.442$ mg/m^3, $\mu = 0.347$ mm^{-1}, $F(000) = 476$. The structure was solved by direct methods using SHELXL-97[24] and refined using full-matrix least-squares difference Fourier techniques. All non-hydrogen atoms were refined with anisotropic displacement parameters, and all hydrogen atoms were placed in idealized positions and refined as riding atoms with the relative isotropic parameters. Absorption corrections were applied with the Rapid Auto.[26] The 9273 measurements yielded 4802 independent reflections after equivalent data were averaged and Lorentz and polarization corrections were applied. The final refinement gave $R_1 = 0.0469$ and $wR_2 = 0.1064$ [$I > 2\sigma(I)$].

3.4. Antifungal bioassays

Antifungal bioassays were conducted in triplicate by following National Center for Clinical Laboratory Standards (NCCLS) recommendations.[27] The plant pathogens were obtained from China General Microbial Culture Collection (CGMCC), and were grown on potato dextrose agar (PDA). The fungal inocula were prepared from broth cultures that were incubated at 25 °C for 48 h, and the final suspensions contained 1×10^4 hyphae/mL. Test samples (10 mg/mL as stock solution in DMSO and serial dilutions) were transferred to 96-well clear plate in triplicate, and the suspensions of test organisms were added to each well to achieve a final volume of 200 μL with alamar blue (10 μL of 10% solution) added to each well as indicator. After incubation at 28 °C for 48 h, the fluorescence intensity was measured at $E_x/E_m = 544/590$ nm using a microtiter plate reader. The inhibition rate was calculated and plotted versus test concentrations to afford the IC_{50} and MIC.

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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.2008.07.075.

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