Identification of the First Diphenyl Ether Gene Cluster for Pestheic Acid Biosynthesis in Plant Endophyte Pestalotiopsis fici

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Identification of the First Diphenyl Ether Gene Cluster for Pestheic Acid Biosynthesis in Plant Endophyte *Pestalotiopsis fici*

Xinxin Xu,[a] Ling Liu,[a] Fan Zhang,[a] Wenzhao Wang,[a] Jinyang Li,[a] Liangdong Guo,[a] Yongsheng Che,*[b] and Gang Liu*[a]

The diphenyl ether pestheic acid was isolated from the endophytic fungus *Pestalotiopsis fici*, which is proposed to be the biosynthetic precursor of the unique chloropupukeananes. The pestheic acid biosynthetic gene (*pta*) cluster was identified in the fungus through genome scanning. Sequence analysis revealed that this gene cluster encodes a nonreducing polyketide synthase, a number of modification enzymes, and three regulators. Gene disruption and intermediate analysis demonstrated that the biosynthesis proceeded through formation of the polyketide backbone, cyclization of a polyketo acid to a benzophenone, chlorination, and formation of the diphenyl ether skeleton through oxidation and hydrolyzation. A dihydrogeodin oxidase gene, *ptaE*, was essential for diphenyl ether formation, and *ptaM* encoded a flavin-dependent halogenase catalyzing chlorination in the biosynthesis. Identification of the *pta* cluster laid the foundation to decipher the genetic and biochemical mechanisms involved in the pathway.

Introduction

Endophytic fungi are microorganisms that reside in the internal tissues of living plants or animals without causing apparent disease.[1] They have been demonstrated to be a rich source of bioactive metabolites with diverse structural features.[2] Over the past decades, endophytic fungi have attracted increasing attention, due to the pharmaceutical and ecological significance of their secondary metabolites.[3] The widely prescribed anticancer drug paclitaxel (taxol), one of the most notable natural products initially isolated from the Pacific yew tree *Taxus brevifolia*,[4] was later found in the endophytic fungus *Taxomyces andreanae*[5] and several species of the genus *Pestalotiopsis*.[6] A loline alkaloid produced by the endophytic fungus *Epichloe festucae* could increase the resistance of grasses to insect herbivores and environmental stresses.[7]

The endophytic fungus *Pestalotiopsis fici* CGMCC3.15140 was isolated from the branches of *Camellia sinensis* (Theaceae) in a suburb of Hangzhou, China. Chemical investigations of this strain led to the discovery of an array of novel metabolites including chloropupukeananin, the first chloropupukeanane derivative with a highly functionalized tricyclo-[4.3.1.0³,⁷]-decane skeleton,[8] and related compounds showing significant antimicrobial, antitumor, and anti-HIV activities.[9] Pestheic acid (**1**), which is a plant growth regulator first isolated from the pathogenic plant fungus *Pestalotiopsis theae*,[10] was co-isolated with chloropupukeananes and proposed to be one of the precursors of chloropupukeananes.[8]

Pestheic acid (**1**) possesses a diphenyl ether structure. Its analogues, isolated from fungi, include asterric acid, geodin hydrate,[11] and RES-1214-1.[12] Mutation studies in *Aspergillus terreus*[13] and in vitro synthetic efforts[14] established the biosynthetic relationship of a group of phenolic compounds, including asterric acid, sulochrin, dechlorogeodin, and their corresponding chlorinated series: dihydrogeodin, geodin, geodin hydrate, and geodoxin. The diphenyl ether structure of asterric acid was derived from the benzophenone sulochrin, which was converted to the intermediate dechlorogeodin by a phenol oxidative coupling reaction. The spiran structure of dechlorogeodin then underwent hydrolysis to form asterric acid. Radioactive tracer studies demonstrated that sulochrin was derived from condensation of acetate and malonate units.[15] Further, anthraquinone emodin and questin were demonstrated to be the precursors of sulochrin and dihydrogeodin in the biosynthetic pathway of geodin.[16,17] Enzymes involved in the biosynthesis of geodin, including dihydrogeodin oxidase,[18] emodin *O*-methyltransferase,[19] emodinanthrone oxygenase,[20] and questin oxygenase[21] have been also identified in *A. terreus* by Ushio Sankawa's group. Recently, the biosynthetic gene cluster of monodictyphenone and endocrocin, which are also biosynthetic related compounds of emodin, were respectively identified in *Aspergillus nidulans*[22] and *Aspergillus fumigatus*.[23] However, no report regarding the complete biosynthetic pathway

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for asterric acid or asterric acid-like diphenyl ethers (i.e., pestheic acid) at a genetic level has been documented.

In the current work, we focus on the biosynthesis of pestheic acid (**1**). The biosynthetic gene (*pta*) cluster of **1** was identified in *P. fici* through genome scanning. The biosynthetic pathway was elucidated through gene disruption, intermediate detection, and enzymatic analysis. To our knowledge, this is the first report of the gene cluster responsible for the biosynthesis of a diphenyl ether. This study also shed light on the genetic and biochemical mechanisms involved in the pathway, which is vital to unveil the biosynthetic pathway of chloropupukeananes in future work.

Results and Discussion

Identification of the pestheic acid gene cluster from *P. fici*

The carbon skeleton of **1** (Scheme 1) is identical to that of other fungal diphenyl ethers, including asterric acid and geodin hydrate from *A. terreus*[13] and RES-1214-1 (**2**) from *Pestalotiopsis* sp.,[12] and is similar to those of anthraquinones including geodin from *A. terreus*,[24] emodin from *Aspergillus wentii*,[25] questin, sulochrin, and dihydrogeodin from *Penicillium frequentans*,[26] and griseofulvin from *Penicillium patulum*.[27] It has been demonstrated that the backbone of emodin was synthesized by a nonreducing polyketide synthase (PKS), MdpG, containing the typical ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains but lacking the thioesterase/Claisen cyclase (TE/CLC) domain.[22] Considering their close structural and biosynthetic relationship, a similar nonreducing PKS synthase could be involved in the biosynthesis of **1**.

To identify the biosynthetic gene cluster of **1**, the genome of *P. fici* was sequenced by using the 454 sequencing platform. Analysis of the genomic data (unpublished) revealed a 50 kb contig containing a putative nonreducing PKS gene, *ptaA*. The deduced protein of PtaA closely resembled the nonreducing PKS GsfA involved in griseofulvin biosynthesis.[28] A closer analysis of the nearby genes showed the presence of a putative gene, *ptaM*, downstream of *ptaA* in the contig, which encoded

a halogenase that could catalyze the chlorination in the biosynthesis of **1**. Further analysis identified a gene cluster including *ptaA* in the contig, which was designated as the *pta* cluster (Figure 1A). The annotated genes and the deduced protein functions, along with similarities to their homologues, were listed in Table 1. Transcriptional analysis of the *pta* cluster was
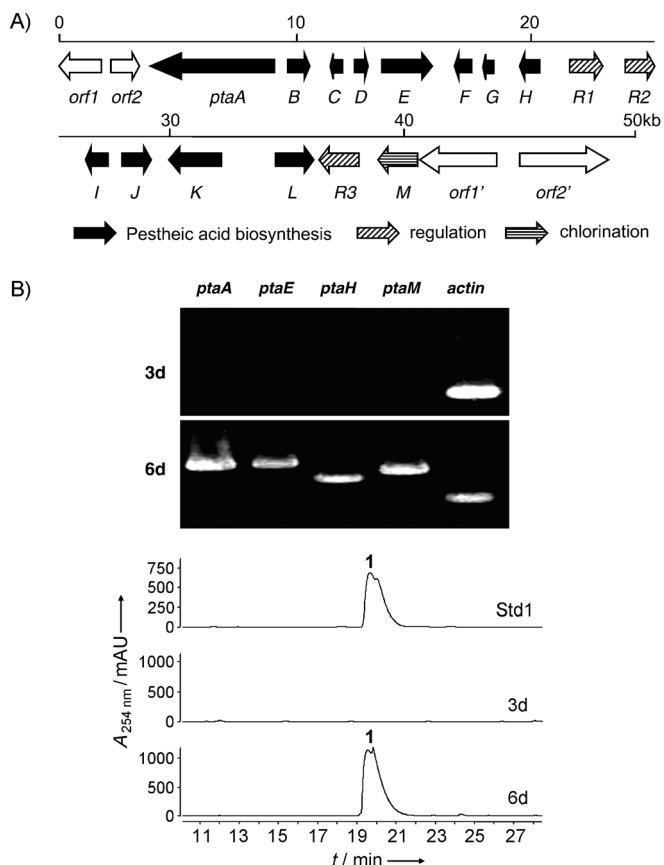

Figure 1. Organization of the pestheic acid biosynthetic gene cluster in *P. fici*. A) The gene cluster spans a 50 kb DNA fragment and contains 20 ORFs (see Table 1). B) RT-PCR analysis of the transcriptional level of the genes within the pestheic acid biosynthetic gene cluster, and LC/MS analysis of pestheic acid produced by *P. fici* which was cultured on PDA plates for 3 or 6 days. Std1, standard compound of pestheic acid (**1**).
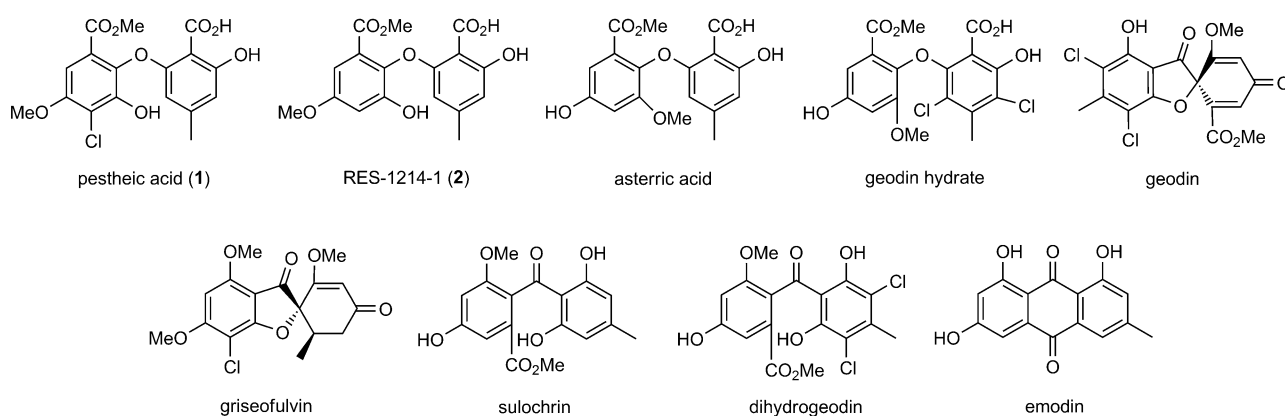
Scheme 1. Diphenyl ethers and their congeners from fungi. The chemical structures of pestheic acid (**1**) from *P. fici*,[8] RES-1214-1 (**2**) from *Pestalotiopsis* sp.,[12] asterric acid, geodin hydrate, geodin, sulochrin, and dihydrogeodin from *A. terreus*,[13] emodin from *A. wentii*,[25] and griseofulvin from *P. patulum*.[27]

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Table 1. Putative genes within the *pta* cluster.

| Gene	Size (bp/aa)	Blastp homologue	Identity [%]	Conserved domain	E value
orf1	1928/182	no significant similarity found		no putative conserved domains have been detected	
orf2	1290/379	no significant similarity found		no putative conserved domains have been detected	
ptaA	5614/1746	*Penicillium aethiopicum*, GsfA	43	SAT-KS-MAT-PT-ACP	
ptaB	1007/268	*Talaromyces stipitatus* ATCC 10500, metallo-β-lactamase domain protein, putative	58	lactamase B	8.47×10^{-14}
ptaC	426/142	*Aspergillus ochraceoroseus*, HypB2	39	DUF1772, domain of unknown function	5.88×10^{-18}
ptaD	613/138	*Penicillium chrysogenum*, Pc16g10800	53	EthD domain	2.95×10^{-15}
ptaE	2288/631	*A. terreus*, dihydrogeodin oxidase	58	Cu oxidase 3	7.06×10^{-49}
				Cu oxidase	1.90×10^{-25}
				Cu oxidase 2	4.16×10^{-32}
ptaF	786/261	*A. flavus*, NRRL3357 AflX	41	NADH(P) binding domain	1.36×10^{-4}
ptaG	494/144	*Metarhizium anisopliae*, ARSEF 23, cytochrome P450 monooxygenase, putative	47	no putative conserved domains have been detected	
ptaH	918/305	*Grosmannia clavigera* kw1407, methyltransferase type 11	44	*S*-adenosylmethionine-dependent methyltransferases	1.52×10^{-9}
ptaR1	1533/418	*A. fumigatus*, Af293, toxin biosynthesis regulatory protein AflJ	40	no putative conserved domains have been detected	
ptaR2	1335/444	*A. flavus*, AflR	30	GAL4-like Zn_2Cys_6 binuclear cluster DNA-binding domain	1.92×10^{-7}
				aflatoxin regulatory protein	1.51×10^{-4}
ptaI	1020/339	*Candida tenuis* ATCC 10573, S-adenosyl-L-methionine-dependent methyltransferase	29	*S*-adenosylmethionine-dependent methyltransferases	5.66×10^{-6}
ptaJ	1335/444	*Aspergillus paraciticus*, AflY	35	DUF4243, protein of unknown function	1.58×10^{-58}
ptaK	2384/585	*Botryotinia fuckeliana*, similar to extracellular dihydrogeodin oxidase/laccase	50	Cu oxidase 3	2.07×10^{-46}
				Cu oxidase	8.81×10^{-22}
				Cu oxidase 2	2.21×10^{-32}
ptaL	1728/372	*T. stipitatus* ATCC 10500, disulfide oxidoreductase, putative	52	pyridine nucleotide-disulfide oxidoreductase	6.43×10^{-6}
ptaR3	1811/564	*T. stipitatus* ATCC 10500, C6 transcription factor, putative	30	GAL4-like Zn2Cys6 binuclear cluster DNA-binding domain	5.68×10^{-8}
				fungal specific transcription factor domain	2.50×10^{-41}
ptaM	1789/540	*Chaetomium chiversii*, RadH flavin-dependent halogenase	52	pfam01494, FAD binding domain	6.88×10^{-7}
orf1'	3487/707	*Methanospirillum hungatei* JF-1, PAS/PAC sensor signal transduction histidine kinase	25	no putative conserved domains have been detected	
orf2'	4016/908	*Macrophomina phaseolina* MS6, ATPase AAA+ type core	43	the AAA+ (ATPases associated with a wide variety of cellular activities) superfamily	2.50×10^{-10}

performed by RT-PCR for compound **1**-producing and -nonproducing conditions. The results showed a clear association between the production of **1** and the expression of the genes (*ptaA*, *ptaE*, *ptaH*, and *ptaM*) in the cluster (Figure 1 B), suggesting that the *pta* cluster is very likely to be involved in the biosynthesis of **1** in *P. fici*.

Production of pestheic acid requires a nonreducing PKS coding gene, *ptaA*

To prove the function of the *pta* cluster in **1** biosynthesis, a transformation system for *P. fici* was developed based on *Agrobacterium tumefaciens*-mediated transformation (ATMT) previously used in other fungal strains.[29] As the strain *P. fici* was sensitive to hygromycin B in PDA medium, a hygromycin B phosphotransferase gene, *hph*, was used as the selection marker. Upon replacement by *hph*, the nonreducing PKS coding gene *ptaA* was deleted by double crossover homologous recombination. The hygromycin B-resistant transformants were selected and screened by PCR using primers inside and outside the *ptaA* gene. Three *ptaA* deletion mutants were further confirmed by Southern blot analysis to exclude ectopic integrations of the deletion cassette into the genome. All of the

mutants showed the expected band size for correct homologous integration of deletion cassette into the corresponding genomic locus of *ptaA* (Figure 2 A). LC/MS analysis of the *ptaA* disruption mutants showed that production of **1** was totally abolished (Figure 2 B), indicating that *ptaA* is essential for the biosynthesis of **1** in *P. fici*. Additionally, the production of chloropupukeananes in the *ptaA* disruption mutant was totally abolished (Figure S1), whereas the production of iso-A82775C (**5**), another precursor of chloropupukeananes, was unaffected (Figure 2 B). These results confirmed that pestheic acid is one of the precursors of chloropupukeananin and congeners.

As the genes responsible for secondary metabolite biosynthesis are generally clustered in fungi,[30] we created deletions of *orf2* and *orf1'*, which are flanked by *ptaA* and *ptaM*, to determine the minimal contiguous gene cluster involved in **1** biosynthesis (Figure S6 E and F). Metabolite analysis revealed that the deletion mutants of *orf2* and *orf1'* still produced **1** (Figure 2 B), indicating that they were located outside the *pta* cluster. In contrast, the knockout experiments of *ptaA* and *ptaM* confirmed their essential role in biosyn-
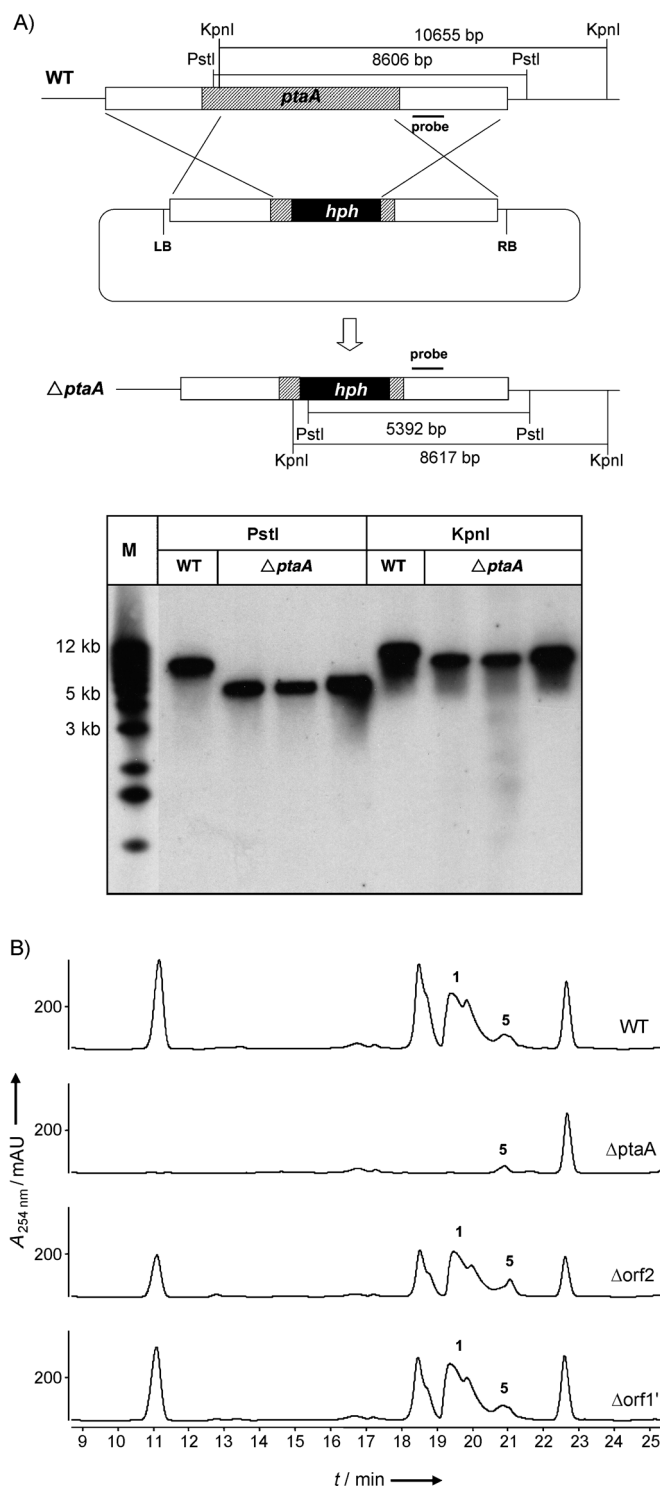

iso-A82775C (**5**)

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A)

B)
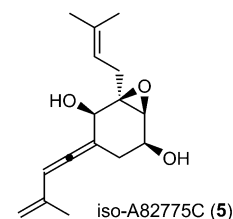
Figure 2. A) Construction and Southern Blot analysis of the *ptaA* disruption mutant of *P. fici*. M, 1 kb ladder; WT, the wild-type strain; Δ*ptaA*, the *ptaA* disruption mutant. B) LC/MS analysis of pestheic acid (**1**) produced by the *ptaA*, *orf2*, and *orf1′* disruption mutants (Δ*ptaA*, Δ*orf2*, Δ*orf1′*) and the wild-type strain (WT) of *P. fici*. **5**, iso-A82775C.

thesis of **1** (Figure 2B; Figure 4A). These results suggest that the minimal pestheic acid biosynthetic gene cluster contains 16 genes, from *ptaA* to *ptaM*.

Bioinformatic analysis of the *pta* gene cluster

Identification of the *pta* cluster is an important step en route to elucidate the enzymatic basis for the biosynthesis of **1**. A BLAST search was performed to analyze the putative functions of the genes within and flanking the cluster (Table 1). As demonstrated above, the *ptaA* gene encoding the nonreducing PKS without the TE/CLC domain is responsible for condensation of acetate and malonate to form the carbon skeleton of **1**. The deduced product of *ptaB* showed high similarity to β-lactamase-type atrochrysone carboxyl ACP thioesterase (ACTE)[31] and metallo-β-lactamase (VrtG).[28] Similar proteins include EncB from *A. fumigatus*[23] and AdaB from *Aspergillus niger*.[32] Both proteins are responsible for hydrolysis and Claisen cyclization of the polyketide endocrocin anthrone[23] and TAN-1612[32] instead of the TE/CLC domain of PKS.

The *ptaC* gene encodes a protein with the DUF1772 domain, showing 36% identities and 56% positives to the anthrone oxidase HypC, which catalyzes the oxidation of norsolorinic acid anthrone (NAA) during aflatoxin production in *A. flavus*.[33] The deduced protein, PtaJ, is a Baeyer–Villiger oxidase highly simi-
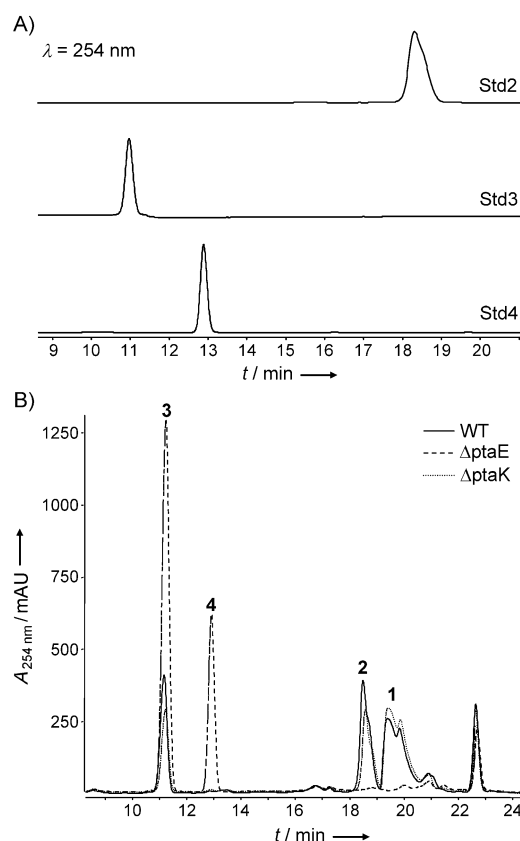
A)

B)

Figure 3. LC/MS analysis of the metabolites produced by the *ptaE* and *ptaK* disruption mutants and the wild-type stain of *P. fici*. A) LC/MS analysis of the standard compounds (Std2–4) of RES-1214-1 (**2**), isosulochrin (**3**), and chloroisosulochrin (**4**). B) Production of RES-1214-1 (**2**) and pestheic acid (**1**) was abolished in the *ptaE* disruption mutant (Δ*ptaE*), but accumulation of isosulochrin (**3**) and chloroisosulochrin (**4**) was observed. The production of pestheic acid did not obviously change in the *ptaK* disruption mutant (Δ*ptaK*). Std2–4: standard compound of RES-1214-1 (**2**), isosulochrin (**3**), and chloroisosulochrin (**4**).

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lar to AflY from *Aspergillus parasiticus*[34] and MdpL from *A. nidulans*.[35] MdpL and AflY were proposed to be involved in the oxidative cleavage of emodin to form the benzophenone skeleton and the conversion of versicolorin A (VA) to sterigmatocystin (ST), respectively. Two *S*-adenosyl-ʟ-methionine-dependent methyltransferases are encoded by *ptaH* and *ptaI* in this cluster. Methylation of the hydroxy groups has been observed in aflatoxin,[36] elloramycin,[37] hatomarubigin,[38] and others. The *ptaE* gene encodes a putative dihydrogeodin oxidase (DHGO), which catalyzes the regio- and stereospecific phenol oxidative coupling in the last step of geodin biosynthesis in *A. terreus*.[39] In addition to *ptaE*, the deduced protein of *ptaK* in the cluster also contains a multi-copper oxidase domain, sharing 51% similarity to DHGO from *A. terreus*. DHGO catalyzes phenol oxidative coupling in dihydrogeodin more effectively than in sulochrin,[39] suggesting that chlorination is important for the phenol oxidative coupling reaction. In agreement, a flavin-dependent halogenase coding gene *ptaM* is located in this cluster. The deduced protein PtaF is similar to the NADH-dependent oxidoreductase AflX from *A. flavus*,[40] whereas PtaG is similar to the cytochrome P450 monooxygenase but lacks the conserved domain. AflX is responsible for the conversion of VA to demethylsterigmatocystin (DMST) during aflatoxin biosynthesis, but its enzymatic function remains poorly understood.[40]

Three genes in the *pta* cluster encode regulators. The deduced protein of *ptaR1* showed high similarity to AflJ, a coactivator in aflatoxin biosynthesis,[41] whereas PtaR2 is similar to AflR, a positive regulator controlling transcription of most genes involved in aflatoxin biosynthesis.[42] The *ptaR3* gene encodes a functionally unknown protein having a GAL4-like Zn$_2$Cys$_6$ binuclear cluster DNA-binding domain and a fungal specific transcription factor domain. Three regulatory genes are located in the cluster, suggesting that the production of **1** is controlled by a complex regulatory mechanism.

DHGO coding gene *ptaE* is responsible for conversion of chloroisosulochrin to pestheic acid

As demonstrated, the stereospecific phenol oxidative coupling catalyzed by DHGO is the most important reaction during the formation of geodin from dihydrogeodin.[39] Three analogues of pestheic acid: RES-1214-1 (**2**), isosulochrin (**3**), and chloroisosulochrin (**4**), were coisolated from the fermentation cultures of *P. fici* (Figures S2–S5; Tables S1 and S2; Figure 3A).

Considering the structural similarity of **1** to asterric acid, and of **3** to sulochrin and dihydrogeodin, the stereospecific phenol oxidative coupling reaction should be involved in **1** biosynthesis. Interestingly, two genes in the *pta* cluster (*ptaE* and *ptaK*) were predicted to encode DHGO-like proteins. To study the functions of *ptaE* and *ptaK* and clarify whether PtaE and PtaK are involved in the phenol oxidative coupling in the conversion of benzophenones **3** and **4** to diphenyl ethers **2** and **1**, the two genes were knocked out by homologous recombination. As expected, the *ptaE* mutant no longer produced **1** and **2**, but accumulated **3** and **4** (Figure 3B). In contrast, disruption of *ptaK* did not affect the production of **1** (Figure 3B). Therefore, PtaE is presumably the key enzyme to catalyze the oxidative coupling reactions of benzophenones **3** and **4**, regardless of chlorination in *P. fici*.

A flavin-dependent halogenase coding gene *ptaM* is responsible for chlorination in pestheic acid biosynthesis

The halogen group in natural products such as rebeccamycin and vancomycin is important for their biological activities.[43] Enzymatic chlorination is generally catalyzed by haloperoxidases or flavin-dependent halogenases.[44] Compounds **1** and **4** were isolated from the fermentation cultures of *P. fici*, along with their nonchlorinated precursors, **2** and **3**. Therefore, we proposed that a flavin-dependent halogenase encoded by *ptaM* catalyzed the chlorination of **3**, which could be the earliest committed intermediate in the biosynthesis of **1**. To verify this proposal, the *ptaM* gene in *P. fici* was knocked out by homologous recombination, and the secondary metabolites produced by the wild-type strain and the *ptaM* knockout mutant
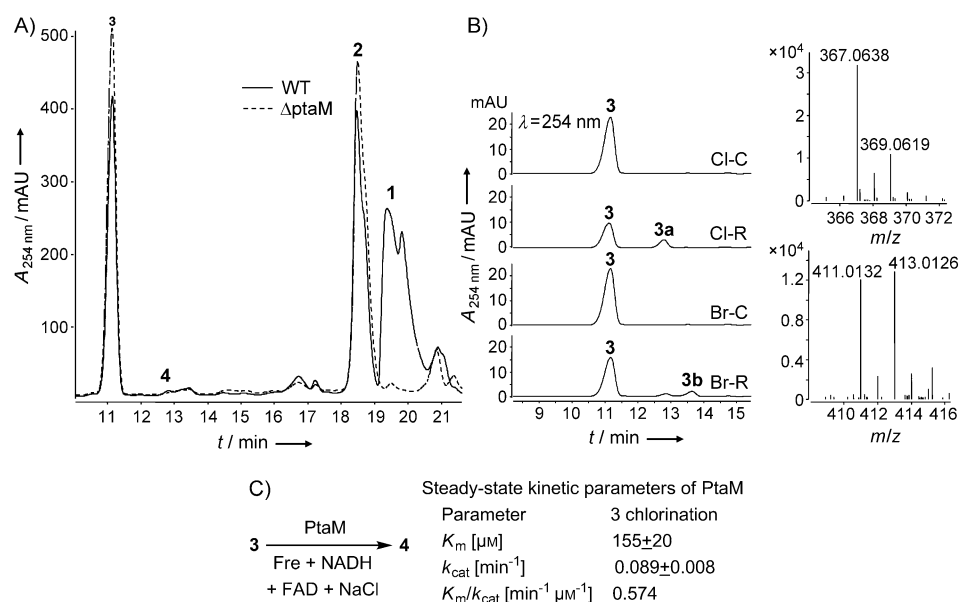

Figure 4. PtaM was responsible for cholorination in pestheic acid biosynthesis. A) LC/MS analysis of the metabolites produced by the wild-type strain (WT) and the *ptaM* disruption mutant (Δ*ptaM*). Production of chloroisosulochrin (**4**) and pestheic acid (**1**) was abolished in Δ*ptaM*. B) LC/MS analysis of chlorination and bromination of isosulochrin catalyzed by PtaM in vitro. **3**, isosulochrin; **3a**, chloroisosulochrin; **3b**, monobrominated isosulochrin; Cl-C, control of chlorination; Cl-R, chlorination of **3**; Br-C, control of bromination; Br-R, bromination of **3**. C) PtaM-catalyzed chlorination.

were detected in fermentation cultures by LC/MS (Figure 4A). Production of **4** and **1** was completely abolished in the mutant, whereas their nonchlorinated precursors, **3** and **2**, were accumulated (Figure 4A), indicating that the *ptaM* gene is essential for the cholorination step in the biosynthesis of **1**.

PtaM catalyzes the conversion of isosulochrin to chloroisosulochrin in vitro

To confirm the chlorination of **1** by a flavin-dependent halogenase, the *ptaM* gene was expressed in *E. coli* as a recombinant protein with an N-terminal His$_6$ tag (63.5 kDa). The chlorination catalyzed by a flavin-dependent halogenase requires the reduced FADH$_2$ which could be provided by a partner flavin reductase,[45] but no such gene was found in the *pta* cluster. Therefore, a flavin reductase coding gene *fre* was cloned from *E. coli* and expressed as the N-terminal His-tagged protein (29.5 kDa). The PtaM-His$_6$ and Fre-His$_6$ proteins were purified to near homogeneity on an Ni-NTA column (Figure S7). After adding the coupling Fre-His$_6$ to the reaction mixture, the enzymatic activity of PtaM-His$_6$ to transform **2** and **3** was tested as previously described.[45, 46] LC/MS analysis of the reaction mixture demonstrated that PtaM converted **3** to product **3a** (Figure 4B). The mass spectrum of **3a** showed the $[M+H]^+$ quasimolecular peaks at *m/z* 367 and 369, with a ratio of 3:1 (Figure 4B), which is a characteristic isotope pattern of monochlorinated compounds. The UV spectra, retention time, and *m/z* value of **3a** matched the authentic standard of **4**, indicating that PtaM could transform **3** to **4** both in vivo and in vitro. However, PtaM failed to convert **2** to the corresponding chlorinated product (data not shown). These results confirmed that chlorination only occurred at the earliest step in the biosynthesis and that PtaM has strict substrate specificity.

Kinetic parameters were determined for the conversion of **3** to **4** by PtaM in vitro. Reactions were assayed in the presence of various concentrations of **3**. PtaM catalyzed chlorination of **3** with a K_m of (155 ± 20) μм, a k_{cat} of (0.089 ± 0.008) min^{-1}, and a k_{cat}/K_m of 0.574 min^{-1} mм$^{-1}$ (Figure 4C).

To further explore the potential halogenation activity of PtaM, we also examined other halogen donors such as bromide and iodide. The results showed that PtaM could incorporate bromine into **3** to yield the corresponding monobrominated product, **3b** (Figure 4B). The mass spectrum showed that **3b** was consistent with an $[M+H]^+$ of 411 and 413 in a 1:1 ratio characteristic of a monobrominated species (Figure 4B). Chlorinated product **3a** was still observed in the bromination reaction, due to undetermined chloride contamination in the system. No iodinated products were observed in the reaction by PtaM, such as other previously reported flavin-dependent halogenases such as Rdc2[45] and RebH.[46]
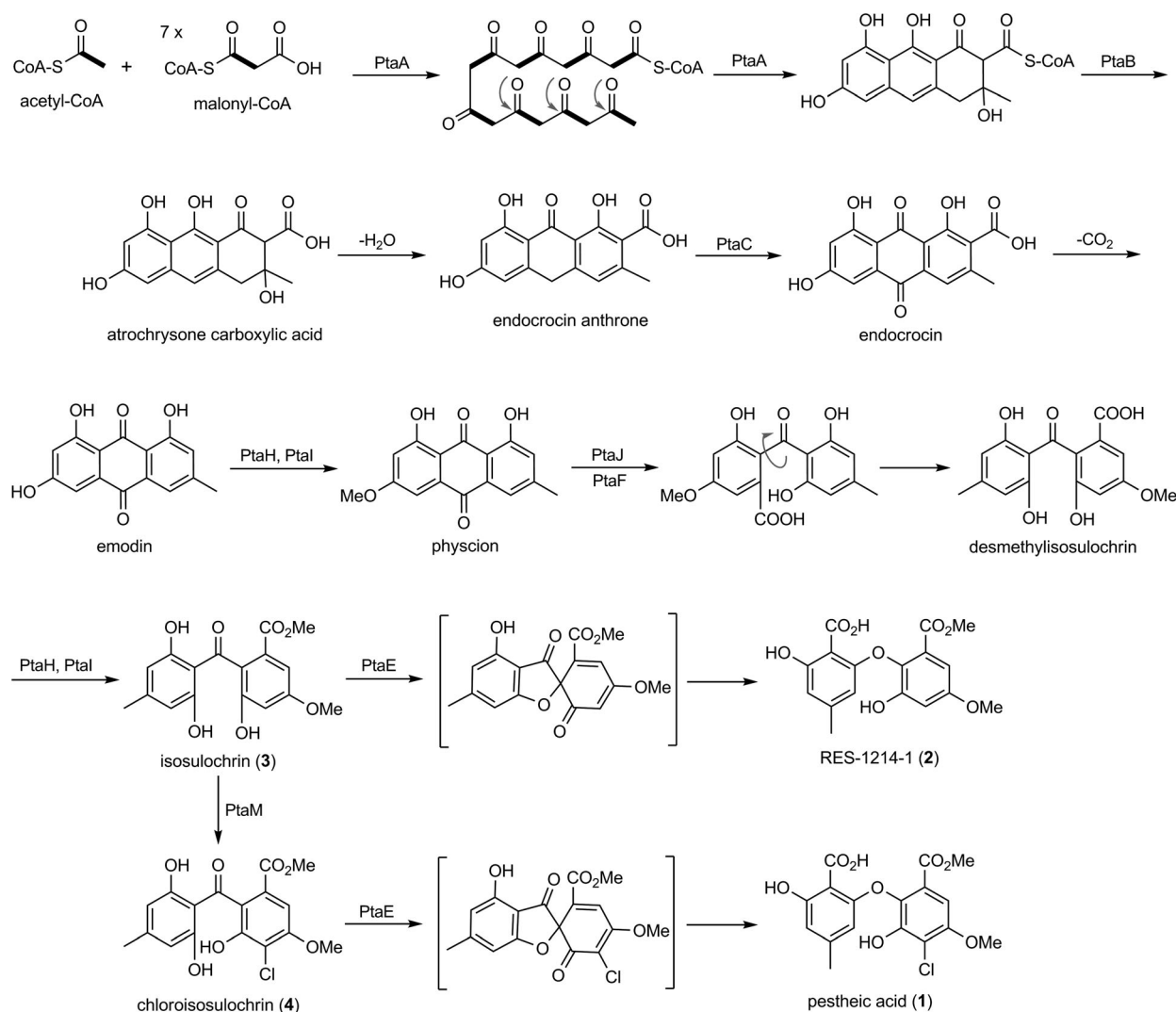
Proposed biosynthetic pathway for pestheic acid

Based on our results from genetic and chemical investigations, as well as the previously reported biosynthetic pathway of asterric acid,[13] geodin,[20] emodin,[22,47] and endocrocin,[23] the biosynthetic pathway for **1** was proposed (Scheme 2). In our study, disruption of the *ptaA* gene completely abolished the production of **1** and its congeners, indicating that the biosynthesis initiates from condensation of acetate and malonate units catalyzed by the nonreducing PKS PtaA. As the PtaA protein is TE/CLC domain-deficient, hydrolysis and Claisen cyclization of the polyketide could be catalyzed by PtaB containing a β-lactamase domain. This hypothetical function of β-lactamase has been demonstrated by Awakawa et al.[31] The PtaB protein might hydrolyze the thioester bond between the ACP of PtaA and the intermediate to release atrochrysone carboxylic acid, which is spontaneously dehydrated to form endocrocin anthrone. Endocrocin anthrone is then converted to endocrocin, catalyzed by the anthrone oxygenase PtaC. Previous studies have suggested that spontaneous decarboxylation of endocrocin occurred to generate emodin.[48] However, neither endocrocin nor emodin was detected in the fermentation cultures of *P. fici*, probably due to the fact that emodin was readily converted to **3** or degraded by some unknown enzymes in *P. fici*. An O-methyltransferase could methylate emodin to form physcion in *P. fici* as in *A. terreus*.[49] PtaJ could then catalyze the oxidative cleavage of physcion, and rotation of the intermediate could then afford desmethylisosulochrin. Such a rotation of the intermediate was also observed in aflatoxin biosynthesis.[50] PtaF, a putative NADH-dependent oxidoreductase, might also participate in the oxidative cleavage step, as found for AflX.[40] Desmethylisosulochrin will be catalyzed by another O-methyltransferase to form isosulochrin. Two methylation steps have been found in geodin biosynthesis.[39] In consistence with that, two methyltransferase coding genes *ptaH* and *ptaI* are located in the gene cluster of **1**. However, whether they play similar roles as in geodin biosynthesis remained unknown.

Chlorination of isosulochrin by PtaM could also play an important role in the biosynthesis of **1**, considering the significantly affected activity of DHGO by chlorination of the intermediates in geodin biosynthesis.[51] Comparing the structure of geodin to **1**, it was found that chlorination of geodin occurred in the aromatic A ring, whereas that of **1** occurred in the cyclohexadienone B ring.[51] In agreement with the above results, chlorination of **1** is catalyzed by a flavin-dependent halogenase rather than a chloroperoxidase as for geodin. It has been reported that the diphenyl ether was related to spiran generated by phenol oxidative coupling from the benzophenone precursor.[11, 13] However, the spiran intermediate was not detected in the fermentation cultures of *P. fici* in the current work, possibly due to its instability or fast hydrolysis to form a diphenyl ether. Recently, Yu and Snider reported that the spiran spirofuranone was readily converted to dechlorodihydromaldoxin (RES-1214-1 (**2**)) and dihydromaldoxin (pestheic acid (**1**)) with 65% sulfuric acid.[52]

DHGO catalyzed the stereospecific phenol oxidative coupling in dihydrogeodin to form (+)-geodin in *A. terreus*.[39] The structural similarity of **3** to dihydrogeodin and that of the proposed spiran intermediate to geodin suggested involvement of a similar enzyme in the oxidative coupling reaction. Interestingly, both PtaE and PtaK have the same multi-copper oxidase domain and share high similarity to DHGO. It is possible for PtaE and PtaK to catalyze the same reaction to convert **3** to

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Scheme 2. Proposed biosynthetic pathway for pestheic acid. Proteins involved in the proposed catalytic reactions are indicated. The intermediates isolated from *P. fici* are indicated by numbers.

a spiran. However, the *ptaK* disruption mutant did not show the same phenotype as the *ptaE* mutant, which lost the ability to produce **1** and **2** (Figure 3). In the biosynthesis of geodin, dihydrogeodin was used as the substrate of DHGO.[53] Our results indicated that DHGO had substrate flexibility, as PtaE was responsible for both conversions of **3** to **2** and **4** to **1**. Disruption of *ptaK* did not remarkably affect the production of **1**, implying that PtaE is the key enzyme to catalyze the conversion of **3** to spiran, regardless of chlorination.

Geodoxin was isolated from *A. terreus*[54] and was demonstrated to be derived from diphenyl ether geodin hydrate by oxidative coupling.[13,14] Maldoxin, which was an analogue of geodoxin, was isolated from the fungus genus *Xylaria* with three diphenyl ethers: dihydromaldoxin (pestheic acid (**1**)), iso-dihydromaldoxin, and dechlorodihydromaldoxin (RES-1214-1 (**2**)).[51] In addition, Suzuki and Kobayashi suggested an alternative biosynthetic hypothesis in which pestheic acid (**1**) is first oxidized to maldoxin, which was actually involved in the Diels–Alder reaction to form chloropupukeananin in *P. fici*.[55] The new proposal was supported by subsequent synthetic efforts

of Yu and Snider.[56] Thus, we speculated that maldoxin could also be synthesized through an oxidative coupling reaction of pestheic acid (**1**) by PtaK in *P. fici*. Conversely, maldoxin was not detected in the fermentation cultures of *P. fici*, possibly because it could easily react with iso-A82775C (**5**) to form chloropupukeananes. To demonstrate this hypothesis, in vitro enzymatic activity assays of PtaK should be carried out in future work.

Conclusions

In this study, the gene cluster of **1** was cloned and characterized from *P. fici* through genome scanning and transcriptional analysis. Results from gene disruption and intermediate analysis provided insights into the biosynthesis of **1**. To our knowledge, this is the first demonstration of the biosynthetic gene cluster for diphenyl ether. The *ptaE* gene coding a dihydrogeodin oxidase is essential for converting the benzophenone intermediates to diphenyl ethers, whereas *ptaM* encoding a flavin-dependent halogenase is critical to catalyze the chlorination

step in the biosynthesis of **1**. Identification of the *pta* cluster not only provided the basis for understanding of genetic and biochemical mechanisms involved in the biosynthesis of **1**, but also laid the foundation for further exploration of the biosynthesis of chloropupukeananin and congeners.

Experimental Section

Strains, plasmids, and culture conditions: Fungal strains and plasmids used in this study are listed in Table S3. The fungus *P. fici* was isolated from the branches of *Camellia sinensis* in a suburb of Hangzhou, China, in April, 2005. The strain was identified as *P. fici* based on sequence analysis of the ITS region (GenBank Accession number DQ812914) of the ribosomal DNA and assigned as CGMCC3.15140 in the China General Microbial Culture Collection (CGMCC) center. The strain was cultured on potato dextrose agar (PDA) plates at 28 °C for 15 days to produce spores, which were inoculated in Fernbach flasks (100 mL), each containing rice medium (10 g). Fermentation was carried out at 28 °C for 20 days. Scale-up fermentation was carried out in 12 Erlenmeyer flasks (500 mL), each containing rice medium (80 g) and incubated at 28 °C for 40 days. The PDA medium was also used for pestheic acid (**1**) production and the transcriptional analysis of *pta* genes in *P. fici*. TSA medium (per liter: tryptone (17 g), soy peptone (3 g), glucose (2.5 g), NaCl (5 g), $K_2HPO_4 \cdot 3H_2O$ (2.5 g), and agar (15 g); pH 7.0) supplemented with hygromycin was used to select the transformants of *P. fici*. Liquid medium (0.4 % glucose, 1 % malt extract, and 0.4 % yeast extract; pH 6.5) was used for the growth of *P. fici*, and the collected mycelia were used for genomic DNA extraction.

E. coli TOP10 was routinely used for plasmid propagation. The rosetta strain of *E. coli*, purchased from Beijing Transgen Biotech Co. Ltd., was used for gene expression.

Molecular manipulations: The primers used in this study were listed in Table S4. DNA isolation and manipulation in *E. coli* and *P. fici* were performed as previously described.[29] PCR amplification was performed on an Eppendorf Mastercycler with KOD FX polymerase (Toyobo). Primer synthesis and DNA sequencing were performed by Life Technologies (Shanghai, China).

Sequence analysis: The nucleoside sequence of the pestheic acid gene (*pta*) cluster has been submitted to the GenBank database under the accession number KC145148. The ORFs were predicted from the sequence by using the GENSCAN Web Server at MIT (http://genes.mit.edu/GENSCAN.html) and FGENESH (Softberry). The deduced proteins of corresponding genes were compared with other known proteins by on-line BLAST methods (http://www.ncbi.nlm.nih.gov/blast/). The UMA algorithm was used to predict domains in PKS.[57]

Construction of the gene disruption mutants of *P. fici*: To construct the gene disruption mutants of *P. fici*, the DNA fragments of the upstream and downstream regions of target genes were amplified from the genomic DNA by using the primers listed in Table S1. The amplified DNA fragments were respectively recombined to the KpnI site (upstream region) or PacI site (downstream region) of the plasmid pAg1-H3 by using the Fast PCR Clone kit (PUEX), according to the manufacturer's protocol. As an exception, the downstream region of *ptaA* was digested with SwaI/AscI and cloned into the corresponding sites of the plasmid pAg1-H3.

The resulting plasmids were introduced into *P. fici* through *Agrobacterium tumefaciens*-mediated transformation (ATMT) as previously described.[29] Hygromycin B-resistant colonies were selected

after being cultured on PDA at 28 °C for 5 days. The disruption mutants were confirmed by PCR using primers inside and outside the corresponding genes. Southern blot analysis was performed with the DIG-High Prime DNA labeling and detection starter kit II for further confirmation of the *ptaA* disruption mutants. The probe DNA was amplified from genomic DNA of the wild-type strain using the oligonucleotides ptaBRTS and ptaBRTA.

RNA isolation and RT-PCR: The mycelia of *P. fici* cultured on PDA plates for 3 or 6 days were collected. Pestheic acid (**1**) production was detected after 3 or 6 days of growth of *P. fici*, and the total RNA was isolated from fungal mycelia using Trizol Reagent (Invitrogen) according to the commercial manual. RNA was reverse transcribed using the PrimeScript RT reagent kit (Perfect Real Time) following the manufacturer's protocol (TaKaRa). PCR amplification was performed on an Eppendorf Mastercycler using rTaq polymerase (TaKaRa) with primers ptaARTS/ptaARTA for *ptaA*, ptaERTS/ptaERTA for *ptaE*, ptaHRTS/ptaHRTA for *ptaH*, ptaMRTS/ptaMRTA for *ptaM*, and actinF/actinR for the actin gene, respectively. An initial denaturation at 95 °C for 4 min was followed by 30 cycles of amplification (95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min) and an additional 8 min at 72 °C.

Heterologous expression and purification of PtaM and Fre: The mycelia of *P. fici* cultured in rice medium for 15 days were harvested, and the total RNA was isolated. First-strand cDNA reverse transcribed from the total RNA was used as the template. A 1620 bp DNA fragment containing the entire cDNA of *ptaM* was amplified with the primers ptaMF and ptaMR. A 699 bp DNA fragment containing the open reading frame of the *fre* gene was amplified from the genomic DNA of *E. coli* BL21 with the primers freF and freR. The amplified DNA fragments were cloned into the vector pEASY-Blunt (Transgen, Beijing, China) and sequenced to verify their accuracy. They were then digested by NdeI and EcoRI and inserted into the corresponding sites of the pET28a vector to generate the plasmids pET28a::ptaM and pET28a::fre, respectively.

To express the corresponding proteins, the recombinant plasmids were introduced into *E. coli* Rosetta strains. The Rosetta strain containing plasmid pET28a::ptaM or pET28a::fre was grown overnight at 37 °C in LB medium supplemented with kanamycin. The above overnight culture (2 mL, 1 %, *v/v*) was inoculated into LB medium (200 mL) and incubated at 37 °C on a rotary shaker to an OD_{600} of 0.6. The culture was induced by IPTG (0.1 mM) and incubated at 28 °C for an additional 4 h. The harvested cells were washed with binding buffer (20 mM Tris·HCl, 500 mM NaCl, 5 mM imidazole, and 5 % glycerol; pH 7.9). After being resuspended in the same buffer (20 mL), the cells were lysed by sonication. The lysate was centrifuged at 12 000 rpm for 20 min at 4 °C, and the soluble fraction was purified with a Ni-NTA spin column (Novagen), according to the manufacturer's manual. The concentration of the purified proteins was determined using the BAC protein assay reagent (Novagen).

In vitro enzymatic activity of PtaM: The halogenation activity of PtaM was measured as previously described.[45, 46] The typical reaction mixture (100 μL) contained substrate (0.05 mg), FAD (100 μM), NADH (10 mM), NaCl (10 mM, NaBr for bromination), Fre (16 μM), and PtaM (32 μM) in phosphate buffer (0.1 M, pH 7.0). The components were mixed and incubated at 28 °C for 30 min. The reaction mixture, including all components except for PtaM, was carried out concomitantly as the control. The reaction mixtures were extracted with ethyl acetate (EtOAc) and analyzed by LC/MS. To measure the kinetic parameters for the chlorination of isosulochrin by PtaM, a series of reaction systems were set up in the presence of FAD

(100 μm), NADH (10 mm), NaCl (10 mm), Fre (16 μm), PtaM (16 μm), and various concentrations of **3** (0.3 to 1.8 mm, in triplicate). The reaction mixtures were mixed thoroughly and maintained at 28 °C for 30 min. Product formation was quantified by extracted ion chromatogram (EIC) plots of **3a** based on the area of the peaks. The steady-state parameters k_{cat} and K_m were calculated by non-linear fitting of the Michaelis–Menten equation using Origin 7.5.

Chemical analysis and compound isolation: Metabolite analysis was performed on an Agilent 6520 Accurate-Mass QTOF LC/MS system (Agilent Technologies) equipped with an electrospray ionization (ESI) source. The fermentation cultures of *P. fici* and its transformants or reaction mixtures were extracted with equal volumes of EtOAc for LC/MS analysis (Kromasil 100-5 C_{18} column; 4.6× 250 mm; 40 % MeOH in H_2O for 2 min; 40–80 % MeOH in H_2O over 23 min; 1 mL min^{-1}; 25 °C).

Compound isolation and structural characterization were carried out as previously described.[8]

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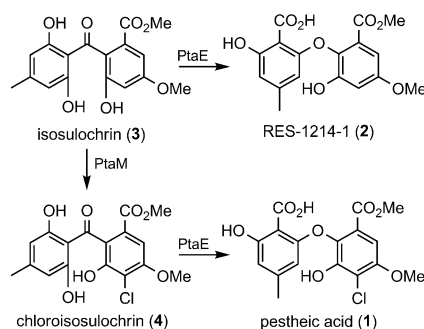
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FULL PAPERS

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■■ – ■■

Identification of the First Diphenyl Ether Gene Cluster for Pestheic Acid Biosynthesis in Plant Endophyte *Pestalotiopsis fici*

Gene identification: The pestheic acid biosynthetic gene (*pta*) cluster was identified in *Pestalotiopsis fici*. A dihydrogeodin oxidase gene, *ptaE*, was essential for diphenyl ether formation, and *ptaM* encoded a flavin-dependent halogenase catalyzing chlorination in the biosynthesis.