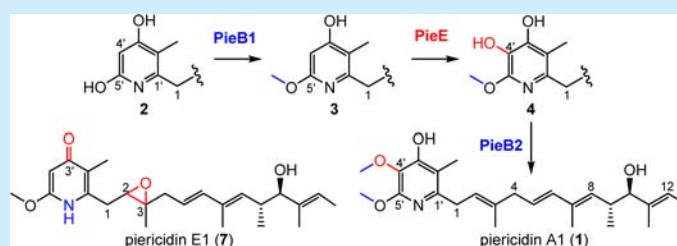


Elucidating Hydroxylation and Methylation Steps Tailoring Piericidin A1 Biosynthesis

Yaolong Chen,^{†,‡,§} Wenjun Zhang,^{†,§} Yiguang Zhu,^{*,†} Qingbo Zhang,[†] Xinpeng Tian,[†] Si Zhang,[†] and Changsheng Zhang^{*,†}[†]Key Laboratory of Tropical Marine Bio-resources and Ecology, RNAM Center for Marine Microbiology, Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China[‡]University of the Chinese Academy of Sciences, Beijing 100049, China

S Supporting Information



ABSTRACT: The piericidin A1 (**1**) gene cluster was identified from the deep-sea derived *Streptomyces* sp. SCSIO 03032. Our in vivo and in vitro experiments verified PieE as a 4'-hydroxylase and PieB2 as a 4'-O-methyltransferase, allowing the elucidation of the post-PKS modification steps involved in **1** biosynthesis. In addition, the shunt metabolite piericidin E1 (**7**) was identified as a novel analogue featuring a C-2/C-3 epoxy ring.

Piericidins are a family of α -pyridone antibiotics that are isolated mainly from various *Streptomyces* species of terrestrial, marine, and symbiotic origins.¹ Structurally, piericidins feature a pyridone core attached with variable polyene side chains. Due to the high structure similarity to ubiquinone, piericidin A1 (**1**, Figure 1) is known as a potent inhibitor of both mitochondrial and bacterial NADH-ubiquinone oxidoreductase (complex I).² In addition to broad antimicrobial and insecticidal activities, **1** is shown as a GRP78 down-regulator and displays cytotoxic activity for etoposide resistant cancer cells during glucose deprivation, thus being identified as an anticancer agent.³

The total synthesis of **1** has been achieved by several groups.⁴ Previous feeding studies demonstrated that the carbon backbone of **1** is derived from a linear polyketide that is formed from four acetates and five propionates and revealed the low incorporation of ¹⁵N-labeled aspartate and serine.⁵ Recently, the biosynthetic locus of **1** has been successfully identified from *Streptomyces piumogues* var. Hangzhouwanensis, revealing that **1** originates from a modular polyketide synthase (PKS) pathway.⁶ In contrast to the use of a hybrid polyketide synthase–nonribosomal peptide synthetase (PKS–NRPS) pathway to incorporate amino acids as the nitrogen source to construct the α -pyridone ring in the biosynthesis of other well-studied α -pyridone natural products kirromycin and tenellin,⁷ an ATP-dependent amidotransferase PieD plays a key role in introducing the nitrogen into the α -pyridone ring in piericidin.⁶ In addition, the terminal thioesterase (TE) of the piericidin A1

PKS module was demonstrated to hydrolyze a β,δ -diketo thioester polyketide product.⁶ However, the enzymatic consequences of post-PKS modifications, involving a hydroxylation and two methylations to furnish the biosynthesis of **1**, have not been elucidated. In this study, we report the characterization of PieE as a 4'-hydroxylase and PieB2 as a 4'-O-methyltransferase, allowing the dissection of the tailoring steps for the biosynthesis of **1** in the deep-sea derived *Streptomyces* sp. SCSIO 03032.

The deep-sea derived *Streptomyces* sp. SCSIO 03032 was previously shown to produce spiroindimicins, an unusual group of spiro-containing bisindole alkaloids.⁸ In addition, we found that this strain could also produce piericidin A1 (**1**), the mass and NMR spectroscopic data of which are consistent with those reported for **1** (Figure S1 and Tables S1 and S2, Supporting Information). From the draft genome sequence of *Streptomyces* sp. SCSIO 03032 (data not shown), we identified the biosynthetic gene cluster of **1** (GenBank accession no. KF874660), which comprises 12 biosynthetic genes that encode putative proteins involved in the biosynthesis of **1** (Figure 1), including a transcription regulator PieR, six typical modular PKSs PieA1–A6, a putative cyclase PieC, an amidotransferase PieD, two methyltransferases PieB1 and PieB2, and a monooxygenase PieE (Table S3, Supporting Information). The biosynthetic gene cluster of **1** in *Streptomyces*

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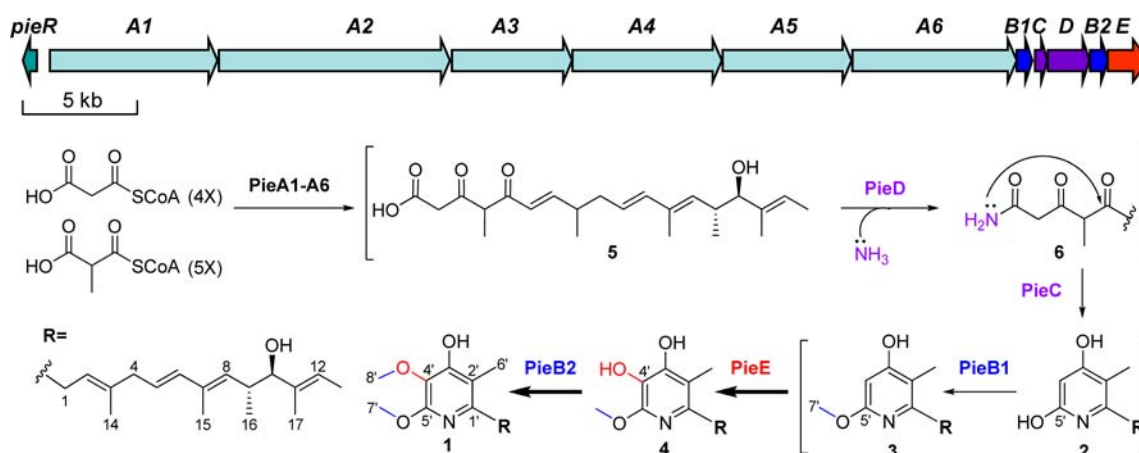


Figure 1. Genetic organization of the *pie* gene cluster in *Streptomyces* sp. SCSIO 03032 and the proposed **1** biosynthetic pathway, focusing on the post-PKS modification steps. Bold arrows indicate biosynthetic steps that were confirmed in this study.

sp. SCSIO 03032 displays the same genetic organization as that in *S. piomogeus*,⁶ despite their distinct terrestrial and marine origins. Given the high amino acid sequence similarities and identities for their corresponding biosynthetic enzymes encoded in the two biosynthetic loci of **1** (Table S4, Supporting Information), we propose that **1** was biosynthesized in a similar manner in both strains (Figure S2, Supporting Information). Briefly, the six modular PKSs PieA1–A6 function to incorporate four molecules of malonyl-CoA and five molecules of methylmalonyl-CoA under the colinearity rule to construct a nascent polyketide chain, which is released by the terminal TE domain to form a C₁₈ linear carboxylic acid **5** (Figure 1). Subsequently, the ATP-dependent amidotransferase PieD, belonging to the class II glutamine amidotransferases,⁹ transfers a nitrogen group from amine donors (ammonia or glutamine) to the carboxylic acid group in **5**, yielding **6** (Figure 1). Although annotated as a hypothetical protein with distant similarity to the well-characterized polyketide cyclase TcmF2,¹⁰ PieC is proposed to catalyze an intramolecular condensation between the amine and the δ -keto group in **6**, leading to the cyclized α -pyridone product **2** (Figure 1).⁶ Finally, a hydroxylation by PieE and two methylations by PieB1 and PieB2 complete the biosynthesis of **1**.

Functions of PieC and PieD have been investigated by *in vivo* genetic experiments to support the proposed pathway for **1**.⁶ Disruption of *pieD* abolished the production of **1** and the inactivation of *pieC* led to the decrease of the yield of **1**.⁶ However, the modification steps after the α -pyridone ring formation remain elusive. To this end, we are interested in probing the functions of PieE, PieB1, and PieB2. A PCR approach was then utilized to screen a SuperCos 1-based genomic library of *Streptomyces* sp. SCSIO 03032 to yield four overlapping cosmids that cover the entire biosynthetic gene cluster of **1** (Figure S3, Supporting Information). Next, the PCR-targeted gene inactivation of *pieE* was carried out according to the reported REDIRECT strategy (Figure S4, Supporting Information).¹¹ HPLC analysis of the Δ *pieE* mutant revealed the production of two compounds that were distinct from **1** (Figure 2). One was characterized to be identical to previously reported Mer-A2026B (**3**, Figure 1),¹² by HR-ESI-MS and ¹H and ¹³C NMR spectroscopic data (Tables S1 and S2, Figure S5, Supporting Information). The other compound **7** presented a molecular formula of C₂₄H₃₅NO₄ (*m/z* [M + H]⁺ 402.2637, calcd 402.2639) deduced by HR-ESI-MS (Figure S6,

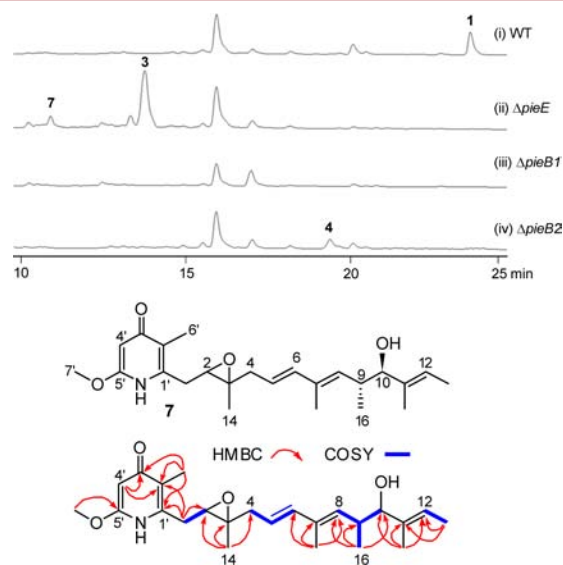


Figure 2. HPLC traces for metabolite profiling of different strains and the structure of pteridin E1 (**7**): (i) *Streptomyces* sp. SCSIO 03032, (ii) Δ *pieE* mutant, (iii) Δ *pieB1* mutant, (iv) Δ *pieB2* mutant, UV detection at 238 nm.

Supporting Information). The comparison of the ¹H and ¹³C NMR spectroscopic data of **7** (Tables S1 and S2, Figure S6, Supporting Information) and **3** revealed that two olefinic carbon signals (δ_C 123.5 C-2, 134.4 C-3) in **3** were replaced by an oxymethine (δ_H 4.26 H-2, δ_C 73.4 C-2) and an oxygenated quaternary carbon (δ_C 75.3 C-3) in **7**, indicating a C-2/C-3 epoxy ring structure, which was supported by HMBC correlations from H₃-14 to C-2/C-3/C-4, and from H-1 to C-2/C-3 (Figure S6, Supporting Information). In addition, a carbonyl group (δ_C 181.2 C-3') signal was observed in **7**, which was located at C-3' by key HMBC correlations from both H-4' and H₃-6' to C-3'. Thus the planar structure of **7** was established (Figure 2). The relative configuration of **7** was assigned by analyzing the ¹H–¹H coupling constants (Table S1, Supporting Information) and NOESY correlations (Figure S6, Supporting Information). The absolute configuration at C-10 of **7** was determined as 10*R* by the modified Mosher's method (Table S5 and Figure S7, Supporting Information) and thus also established the 9*R* configuration (Figure S7, Supporting Information). However, the absolute configuration at C-2/C-3

in **7** remained unassigned. Different from the presence of the C-11/C-12 epoxy ring in ptericidin C and D series,¹³ **7** is novel to feature a C-2/C-3 epoxy ring, and was designated as ptericidin E1. Given that **7** was isolated from the $\Delta pieE$ mutant and PieE was the only oxygenase found in the gene cluster of **1**, we hypothesized that **7** was a shunt metabolite from the pathway of **1** and the formation of the C-2/C-3 epoxy ring in **7** was probably catalyzed by an unknown enzyme encoded in the genome of *Streptomyces* sp. SCSIO 03032.

Bioinformatic analysis shows that PieE is a putative FAD-dependent monooxygenase. PieE shares the highest identity (74%) to EME97622, a protein encoded by a putative locus of **1** in the genome of *S. mobaraensis* (Table S4, Supporting Information), the first described **1** producer.^{1a} In addition, PieE exhibits similarity to 2,4-dichlorophenol 6-monooxygenase TfdB and methylhydroquinone oxygenase MhqA encoded in diverse bacteria genomes.¹⁴ The bioinformatic analysis of PieE, together with the accumulation of **3** in the $\Delta pieE$ mutant, indicated PieE as the 4'-hydroxylase modifying the α -pyridone ring in **3**. To validate this hypothesis, soluble N-His₆-fused PieE protein was produced in *E. coli* BL21(DE3) harboring the pET28a-based expression plasmid pCSG601 (Table S6, Supporting Information), and was purified to near homogeneity via Ni-NTA chromatography (Figure S8, Supporting Information). Consistent with the yellow color of the purified PieE, noncovalently bound FAD was released from heat-denatured PieE (Figure S9, Supporting Information). The subsequent incubation of 100 μ M **3** and 5 μ M PieE in the presence of 2 mM NADH (or NADPH) at 28 °C led to the conversion of **3** to a new product **4** (Figure 3A, traces i and ii), the yield of

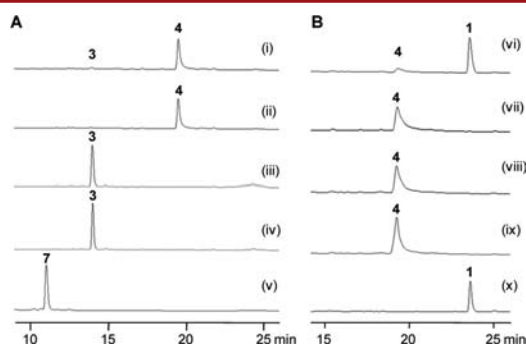


Figure 3. (A) HPLC traces for analyzing *in vitro* assays of PieE: (i) a PieE assay containing 100 μ M **3**, 5 μ M PieE, and 2 mM NADH; (ii) PieE assay in which NADH was replaced by NADPH; (iii) assay in (i) minus PieE; (iv) assay in (i) minus NAD(P)H; (v) PieE assay containing 100 μ M **7**, 5 μ M PieE, and 2 mM NADH. A standard PieE assay was performed in Tris-HCl buffer (50 mM, pH 8.0) at 28 °C for 2 h. (B) HPLC traces for *in vitro* assays of PieB2 and PieB1: (vi) PieB2 assay containing 100 μ M **4**, 10 μ M PieB2, and 2 mM SAM; (vii) assay in (vi) minus PieB2; (viii) assay in (vi) minus SAM; (ix) PieB1 assay containing 100 μ M **4**, 10 μ M PieB1, and 2 mM SAM; (x) standard **1**. The PieB2 (or PieB1) assays were conducted in Tris-HCl buffer (50 mM, pH 8.0) at 28 °C for 2 h.

which increased with a longer incubation time (Figure S10, Supporting Information). Compound **3** remained unchanged in control assays lacking either PieE or NAD(P)H (Figure 3A, traces iii, iv). To determine its chemical structure, 10 mg of **4** was isolated and purified from a 15 mL reaction mixture containing 15 mg of **3**, 5 μ M PieE, and 2 mM NADH. The chemical formula of **4** was established as C₂₄H₃₅NO₄ by HR-ESI-MS (m/z [M + H]⁺ 402.2647, calcd 402.2639, Figure S11,

Supporting Information), which was 16 mass units higher than that of **3**, suggesting that **4** was a hydroxylated product of **3**. The ¹H NMR spectrum of **4** (Tables S1 and S2 and Figure S11, Supporting Information) was almost identical to that of **3**, except that an aromatic proton signal (δ_H 5.95 H-4') in **3** was absent in **4**, indicating the location of the hydroxyl group at C-4' in **4** (Figure 1). The structure assignment of **4** was further supported by HMBC correlations from H-1 to C-1'/C-2', from H-6' to C-1'/C-2'/C-3', and from H-7' to C-5' (Table S7 and Figure S12, Supporting Information). Thus, the product **4** was elucidated as 4'-O-demethyl-ptericidin A1 (Figure 1), confirming PieE as the 4'-hydroxylase in biosynthesis of **1**. However, no conversion of **7** by PieE was detected (Figure 3A, trace v).

There are two methyltransferase-encoding genes *pieB1* and *pieB2* in the **1** gene clusters from three different strains (Table S4, Supporting Information). PieB1 from *Streptomyces* sp. SCSIO 03032 shows high homology to S-adenosylmethionine (SAM)-dependent methyltransferases NorI (65% identity) and AurI (60% identity) involved in neoauriothrin and aureothrin pathways,¹⁵ while PieB2 exhibits the highest identity (49%) to 3-demethylubiquinone-9 3-methyltransferase in *Rhodococcus jostii* RHA1.¹⁶ To probe the exact role of PieB1 and PieB2, the genes *pieB1* and *pieB2* were inactivated by PCR-targeting strategy (Figure S4, Supporting Information). No **1**-related compounds were detected in the $\Delta pieB1$ mutant (Figure 2, trace iii), while the $\Delta pieB2$ mutant produced a compound that was identified as **4** on the basis of the same retention times and molecular masses as the standard **4** (Figure 2, trace iv; Figure S13, Supporting Information). To further probe functions of PieB1 and PieB2 *in vitro*, both *pieB1* and *pieB2* were cloned into pET28a and pGEX-6p-1 (Table S6, Supporting Information). Soluble PieB1 and PieB2 proteins were only achieved by the fusion with GST-tags and were purified using GST-Bind Purification Kits (Figure S8, Supporting Information). Subsequently, **4** was found to be converted by PieB2 to a product coeluted with **1** (Figure 3B, traces vi and x), the yield of which increased with longer incubation time in a time course assay (Figure S14, Supporting Information). No conversions of **4** to **1** were observed in control assays lacking either PieB2 or SAM (Figure 3B, traces vii, viii). No change of **4** was detected by using PieB1 instead of PieB2 (Figure 3B, traces ix). These data confirm that PieB2 is a specific 4'-O-methyltransferase that tailors **1** biosynthesis. However, the lack of the putative substrate **2** (Figure 1) prevents the *in vitro* functional characterization of PieB1. Nevertheless, the abolishment of **1** production in the $\Delta pieB1$ mutant suggested that PieB1 was indeed involved in the biosynthesis of **1**, probably responsible for forming the 5'-O-methyl group in ptericidins **3**, **7**, and **1**.

In summary, we have identified the biosynthetic gene cluster of **1** from the deep-sea derived *Streptomyces* sp. SCSIO 03032. PieE was characterized both *in vivo* and *in vitro* as the 4'-hydroxylase to provide the 4'-hydroxyl group in the α -pyridone moiety of **1**. PieB2 was verified as the 4'-O-methyltransferase by *in vivo* genetic disruptions and *in vitro* biochemical assays, and PieB1 was indicated as the requisite 5'-O-methyltransferase, in contrast to the methylation of two adjacent hydroxyl groups on ubiquinone by a single O-methyltransferase Coq3.¹⁷

■ ASSOCIATED CONTENT

■ Supporting Information

Experimental procedures and materials and characterization data of compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: zhuyiguang2003@163.com.

*E-mail: czhang2006@gmail.com.

Author Contributions

[§]These authors contribute equally.

Notes

The authors declare no competing financial interest.

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