Organic LETTERS

Letter

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Elucidating Hydroxylation and Methylation Steps Tailoring Piericidin A1 Biosynthesis

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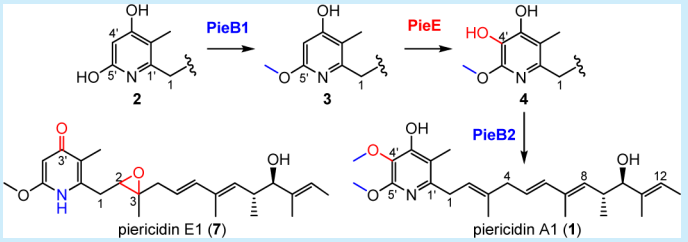
Supporting Information



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 ^{15}N-labled aspartate and serine.[5] Recently, the biosynthetic locus of **1** has been successfully identified from *Streptomyces piomogeues* 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*

Received: November 25, 2013
Published: January 10, 2014

dx.doi.org/10.1021/ol4034176 | *Org. Lett.* 2014, 16, 736−739

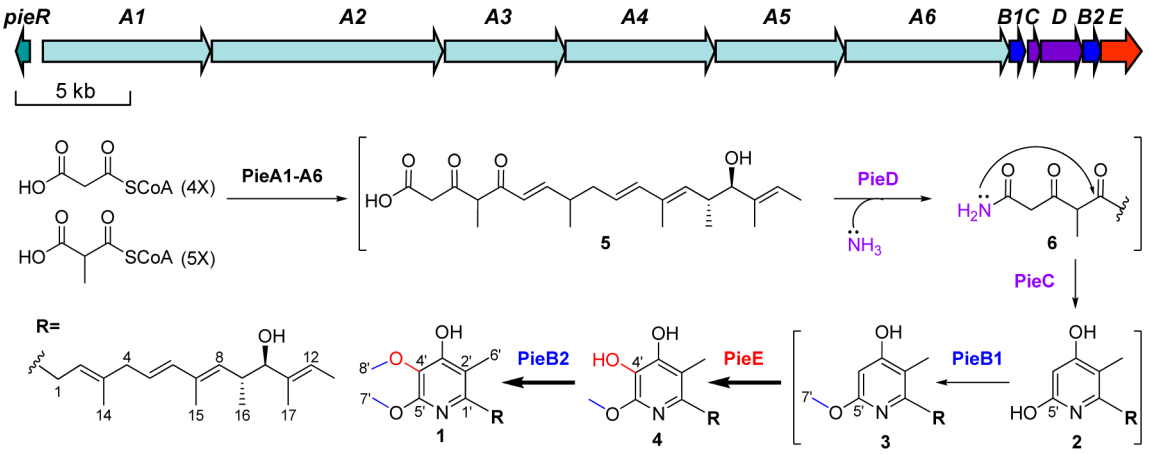


Figure 1. Genetic organization of the *pie* gene cluster in *Streptomyces* sp. SCSIO 03032 and the proposed **1** biosynthetic pathway, focusing on the post-PKS modification steps. Bold arrows indicate biosynthetic steps that were confirmed in this study.

sp. SCSIO 03032 displays the same genetic organization as that in *S. piomogeues*,[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 C_{18} 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 ^{1}H and ^{13}C NMR spectroscopic data (Tables S1 and S2, Figure S5, Supporting Information). The other compound **7** presented a molecular formula of $C_{24}H_{35}NO_4$ (m/z [M + H]$^+$ 402.2637, calcd 402.2639) deduced by HR-ESI-MS (Figure S6,

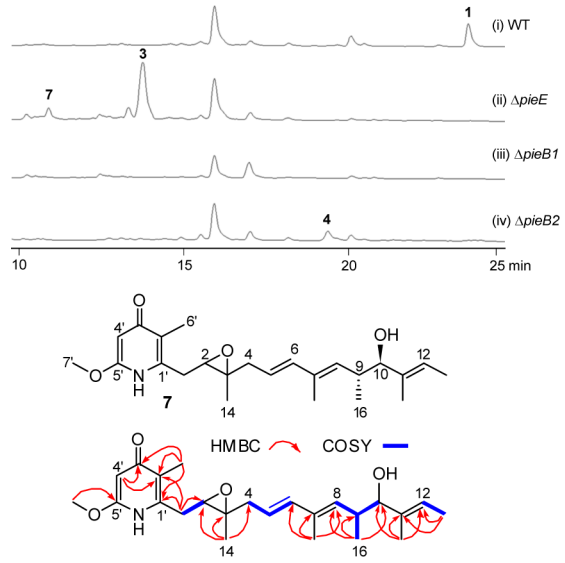


Figure 2. HPLC traces for metabolite profiling of different strains and the structure of piericidin E1 (**7**): (i) *Strptomyces* sp. SCSIO 03032, (ii) Δ*pieE* mutant, (iii) Δ*pieB1* mutant, (iv) Δ*pieB2* mutant, UV detection at 238 nm.

Supporting Information). The comparison of the ^{1}H and ^{13}C 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 H_3-14 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 H_3-6′ to C-3′. Thus the planar structure of **7** was established (Figure 2). The relative configuration of **7** was assigned by analyzing the ^{1}H−^{1}H 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

dx.doi.org/10.1021/ol4034176 | *Org. Lett.* 2014, 16, 736−739

in **7** remained unassigned. Different from the presence of the C-11/C-12 epoxy ring in piericidin C and D series,[13] **7** is novel to feature a C-2/C-3 epoxy ring, and was designated as piericidin E1. Given that **7** was isolated from the Δ*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 Δ*pieE* mutant, indicated PieE as the 4′-hydroxylase modifying the α-pyridone ring in **3**. To validate this hypothesis, soluble *N*-His_6-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 μM **3** and 5 μM 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

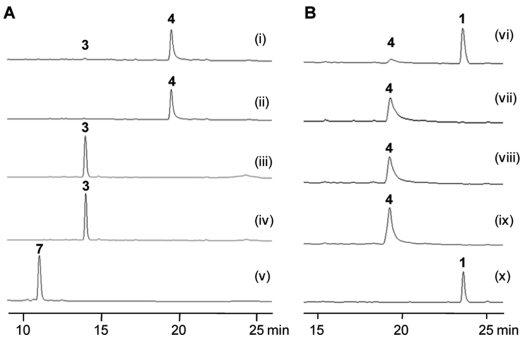


Figure 3. (A) HPLC traces for analyzing in vitro assays of PieE: (i) a PieE assay containing 100 μM **3**, 5 μM PieE, and 2 mM NADH; (ii) PieE assay in which NADH was replaced by NADPH; (iii) assay in (i) minus PieE; (iv) assay in (i) minus NAD(P)H; (v) PieE assay containing 100 μM **7**, 5 μM PieE, 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 μM **4**, 10 μM PieB2, and 2 mM SAM; (vii) assay in (vi) minus PieB2; (viii) assay in (vi) minus SAM; (ix) PieB1 assay containing 100 μM **4**, 10 μM 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 μM PieE, and 2 mM NADH. The chemical formula of **4** was established as $C_{24}H_{35}NO_4$ 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-piericidin 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 neoaureothin and aureothin 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 Δ*pieB1* mutant (Figure 2, trace iii), while the Δ*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 Δ*pieB1* mutant suggested that PieB1 was indeed involved in the biosynthesis of **1**, probably responsible for forming the 5′-*O*-methyl group in piericidins **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]

dx.doi.org/10.1021/ol4034176 | *Org. Lett.* 2014, 16, 736−739

■ 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.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work is supported in part by MOST (2012AA092104 and 2010CB833805), CAS (XDA11030403, KSCX2-EW-G-12), NSFC (31125001, 31290233), and the Administration of Ocean and Fisheries of Guangdong Province (GD2012-D01-001, GD2012-D01-002). We are grateful to analytical facility at SCSIO for recording spectroscopic data.

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