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Carboxyl Formation from Methyl via Triple Hydroxylations by XiaM in Xiamycin A Biosynthesis

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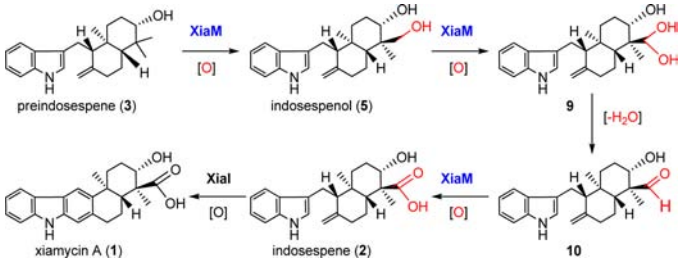
ABSTRACT



The P450 enzyme XiaM was identified as a candidate to form the C-24 carboxyl group in xiamycin A (1). Alteration of medium composition led to the discovery of four new compounds from the Δ*xiaM* and the Δ*xiaK* (encoding an aromatic ring hydroxylase) mutants. Biotransformation experiments revealed that XiaM was capable of converting a methyl group to a carboxyl group through diol and aldehyde intermediates.

Indolosesquiterpene alkaloids, primarily found as plant metabolites, are only recently isolated from actinomycetes, for example, oridamycins,[1] xiamycin A (**1**, Figure 1), and its analogues.[2] We also reported the reisolation of **1** from a marine-derived *Streptomyces* sp. SCSIO 02999, accompanied by four new members, oxiamycin, chloroxiamycin, and dixiamycins A and B (two unusual N−N-coupled atropo-diastereomeric dimers of **1**).[3] By partially genome sequencing *S.* sp. SCSIO 02999, we identified and characterized the **1** biosynthesis gene cluster, which represented the first example of the bacterial biosynthesis of indolosesquiterpenes.[4] The **1** gene cluster was also recently identified from an endophytic *Streptomyces* sp. HKI0576, the heterologous expression of which led to the discovery of a new C−N-coupled dimer of **1**.[5] Based on our experimental data from the metabolite analysis of 13 *xia*-gene inactivation mutants, feeding of putative biosynthetic intermediates to nonproducing mutants, and *in vitro* biochemical characterizations, we proposed a model for **1** biosynthesis in *S.* sp. SCSIO 02999.[4] Notably, an unprecedented oxidative cyclization strategy was revealed to form the pentacyclic ring system of **1** from indosespene (**2**, Figure 1), which was catalyzed by the indole oxygenase

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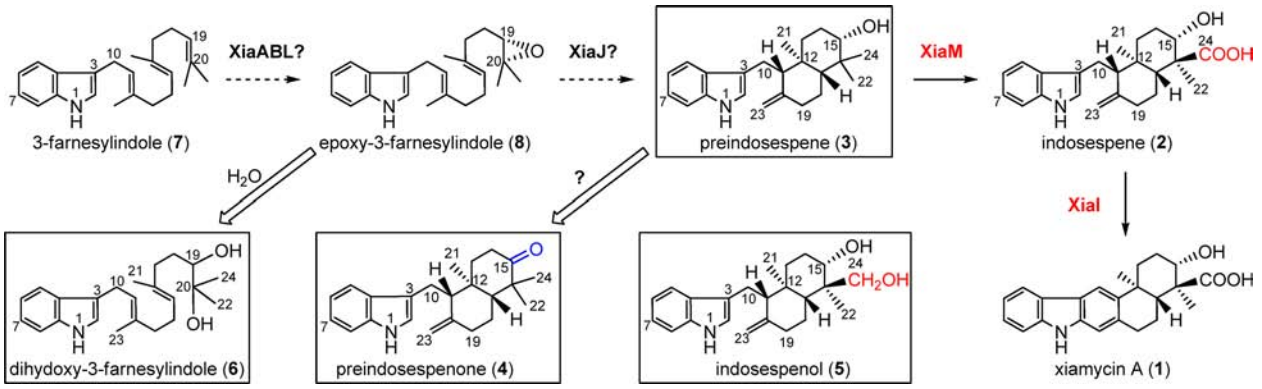

Figure 1. New products (boxed) isolated from the Δ*xiaM* (**3** and **4**) and Δ*xiaK* (**5** and **6**) mutants and the proposed model for biosynthesis of **1** in *Streptomyces* sp. SCSIO 02999. Solid arrows, confirmed steps in this study or our previous study;[4] dashed arrows, deduced biosynthetic steps; bar arrows, deduced shunt steps. The absolute configurations of compounds **3** and **4** were not experimentally established yet. They were drawn on the basis of biosynthetic implications.

XiaI.[4] However, most gene knockouts failed to produce detectable intermediates. Only **2** was isolated from the Δ*xiaK* (encoding an aromatic ring hydroxylase) mutant as a major product, along with a minor amount **1**, and XiaK was shown to be pivotal in biosynthesizing oxiamycin, an unusual metabolite containing a seven-membered oxa-ring.[4] In contrast, no intermediates were isolated from the Δ*xiaM* (encoding a cytochrome P450 enzyme) mutant. Given the ease of the OSMAC (One Strain - Many Compounds) approach to enhance the diversity of microbial natural products by alteration of easily accessible cultivation parameters,[6] changing of the media composition resulted in the discovery of new products from the Δ*xiaM* and Δ*xiaK* mutants. We report herein (i) the isolation and structural elucidation of four new products from these two mutants and (ii) the characterization of XiaM as a triple-hydroxylating P450 oxygenase to transform a methyl to a carboxyl group in **1** biosynthesis.

Because the Δ*xiaM* mutant produced no detectable **1**-related biosynthetic intermediates in the AM6 medium,[4] we then cultivated this mutant in the modified AM6-4 medium.[7] This approach led to the detection of two new products, which were isolated from a 15 L culture and characterized to be preindosespene (**3**, 1.2 mg) and preindosespenone (**4**, 2.7 mg) (Figure 1). The molecular formula of **3** was established to be $C_{23}H_{31}NO$ through HRESIMS (*m*/*z* 360.2290 $[M + Na]^+$). A comparison of its ^1H and ^{13}C NMR data with those of **2** (Table S1, Figure S1) revealed that the carboxyl carbon (δ_C 181.3, C-24) in **2** was replaced by a methyl (δ_H/δ_C 1.02/28.4, C-24) in **3**. Consequently, the chemical shift of C-16 shifted from δ_C 54.9 in **2** to δ_C 39.8 in **3**. The presence of a Me-24 in **3** was further supported by HMBC correlations from H-24 to C-15/C-16/C-17/C-22 (Figure 2). The NOE correlations were observed between H-11 and H-17 (Figure 2), indicating that both protons were *cis*-oriented. However, no NOE correlations were observed for H-15 and Me-21 (Figure S1). We assumed an absolute configuration of **3** as (11*S*, 12*R*, 15*S*, 17*R*) according to that of **2** (Figure 1), given the similar CD spectra of **3** and **2** (Figure S2), and the putative biosynthetic intermediacy of **3**. Preindosepenone (**4**) was assigned a molecular formula of $C_{23}H_{29}NO$ through HRESIMS (*m*/*z* 358.2127 $[M + Na]^+$). The ^1H and ^{13}C NMR data of **4** were very similar to those of **3**, except for the main difference in the case of C-15 (Table S1, Figure S3). The hydroxyl methine (δ_H/δ_C 3.31/78.9, C-15) in **3** was oxidized to a keto-carbonyl carbon (δ_C 216.7, C-15) in **4**; meanwhile the chemical shifts of C-14 and C-16 in **4** shifted downfield 6.8 and 8.1 ppm, respectively, for the deshielding effect from the keto-carbonyl carbon. The location of the keto group at C-15 in **4** was supported by HMBC correlations from H-13/H-14/H-22/H-24 to C-15 (Figure 2). The absolute structure of **4** was assumed as (11*S*, 12*R*, 17*R*), according to that of **2** from a biosynthetic perspective.

The Δ*xiaK* mutant was previously shown to produce **1** and **2** in the AM6 medium.[4] When it was cultured in the AM6-4 medium, two additional products were detected, which were isolated from a 15 L culture and characterized as indosespenol (**5**, 3.7 mg) and dihydroxyl-3-farnesylindole (**6**, 1.4 mg) (Figure 1). The molecular formula of **5** was determined as $C_{23}H_{31}NO_2$ on the basis of HRESIMS (*m*/*z* 376.2240, $[M + Na]^+$). The inspection of 1D and 2D ^1H and ^{13}C NMR spectral data suggested high structural similarities of **5** and **2** (Table S1, Figure S4). The main difference was that the carbonyl carbon (δ_C 181.3, C-24) in **2** was replaced by a hydroxymethyl (δ_H/δ_C 3.54, 3.30/67.5, C-24), and the chemical shifts of C-15, C-16, and C-17 in **5** were shifted upfield 2.5, 10.9, and 3.4 ppm, respectively. The structure of **5** was further supported by HMBC correlations from H-24 to C-15/C-16/C-17/C-22 (Figure 2).

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(7) For medium composition: (a) AM6-4: glycerin 10 g/L, bacterial peptone 5 g/L, $CaCO_3$ 5 g/L, sea salt 30 g/L, pH 7.0; (b) AM6: soluble starch 20 g/L, yeast extract 5 g/L, glucose 10 g/L, bacterial peptone 5 g/L, $CaCO_3$ 5 g/L, sea salt 30 g/L, pH 7.0.

The absolute configuration of **5** was determined to be the same as that of **2** (11*S*, 12*R*, 15*S*, 16*S*, 17*R*), for their almost identical CD spectra (Figure S2). Dihydroxyl-3-farnesylindole (**6**) was isolated as a gray powder. Its molecular formula $C_{23}H_{33}NO_2$ was derived from the HRESIMS (*m*/*z* 378.2424 $[M + Na]^+$). Comparison of its ^1H and ^{13}C NMR spectroscopic data (Figure S5) with those of 3-farnesylindole (**7**, Figure 1) from *Uvaria pandensis*[8] revealed that the two sp^2 vinyl carbons (δ_C 124.0, C-19 and 130.7, C-20) in **7** were replaced by two oxygenated sp^3 carbons (δ_C 73.9, C-19; 79.2, C-20) in **6**. The structure of **6** was further supported by the observed HMBC correlations (Figure 2).

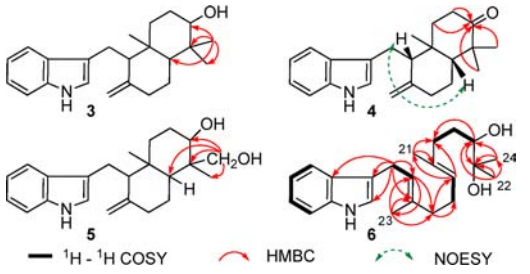

Figure 2. Selected key COSY, HMBC, and NOESY correlations for compounds **3**–**6**.

The natural occurrence of **3** and **5** indicated that XiaM might function as an oxygenase to confer **2** from **3** via **5** during **1** biosynthesis. To validate this hypothesis, we made a construct pCSG2605 for the expression of *xiaM* in *E. coli* (see Supporting Information). However, only insoluble XiaM could be detected, even at 16 °C (Figure S6), preventing the in vitro characterization of XiaM. Alternatively, we fed **3** to *E. coli* BL21(DE3)/pCSG2605. Surprisingly, four additional products with UV spectra characteristic of **3** were detected (Figure 3A, trace i), which were absent in the control *E. coli* BL21(DE3)/pET28a feeding with **3** (Figure 3A, trace ii). Upon LC-MS analyses, products with t_R = 13.9 and 14.5 min were confirmed to be **2** and **5**, respectively, on the basis of the same retention times and the same molecular masses (Figure S7) as the standards **2** and **5** (Figures 3A, traces i-iv). The product (t_R = 11.1 min) had a molecular mass of 369 (*m*/*z* 370.3 $[M + H]^+$, 368.5 $[M - H]^-$), consistent with that of the proposed structure **9** (Figure 3A, trace i; Figure 3B; Figure S7). The formula of the product (t_R = 15.3 min) was determined as $C_{23}H_{29}NO_2$ on the basis of HRESIMS (*m*/*z* 352.2272 $[M + H]^+$, 374.2093 $[M + Na]^+$), consistent with the proposed structure of **10** (Figure 3A, trace i; Figure 3B; Figure S7). Given the instability of the diol structure of **9**, we suppose that **10** was a spontaneous dehydration product of **9**. When **5** was fed to *E. coli* BL21(DE3)/pCSG2605, three additional products were observed (Figure 3A, trace v), identical to the products **9**, **2**, and **10** from biotransformation of **3** upon HPLC analyses. Again, the control strain *E. coli* BL21-(DE3)/pET28a could not process **5** (Figure 3A, trace iv). Cumulatively, these biotransformation experiments suggested that XiaM was capable of catalyzing triple hydroxylations (Figure 3B): (i) **3** was first hydroxylated at C-24 to form **5**; (ii) a second hydroxylation at C-24 converted **5** to **9**, which was spontaneously dehydrated to afford **10**; (iii) a third hydroxylation at C-24 transformed **10** to the final product **2**.

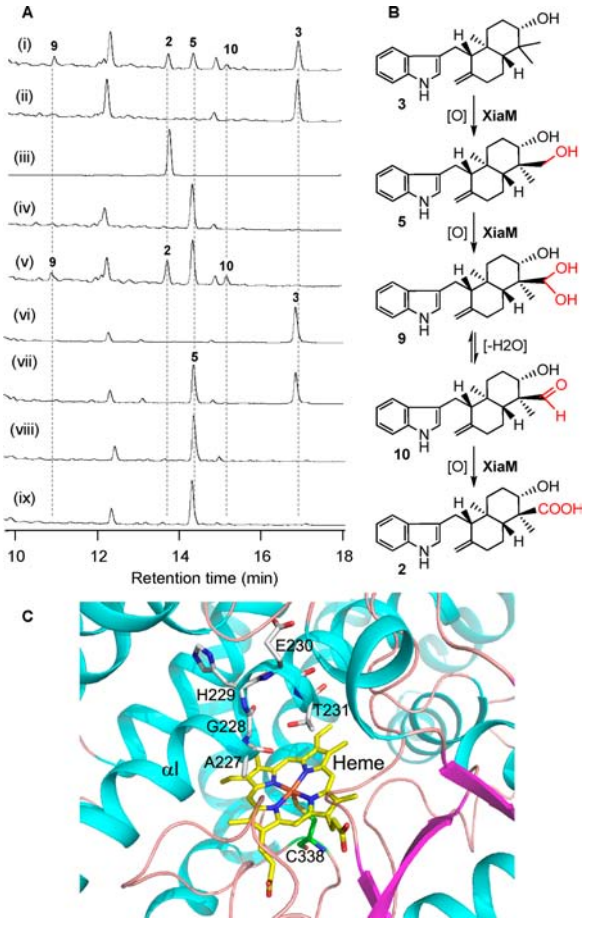

Figure 3. (A) HPLC traces of biotransformations in *E. coli*. *E. coli* BL21(DE3)/pCSG2605 feeding with **3** (i) and **5** (v), *E. coli* BL21(DE3)/pET28a feeding with **3** (ii) and **5** (iv), **2** standard (iii), *E. coli* BL21(DE3)/pCSG2641 (carrying XiaM C338A mutation) feeding with **3** (vi) and **5** (viii), *E. coli* BL21(DE3)/pCSG2640 (carrying XiaM T231A mutation) feeding with **3** (vii) and **5** (ix). Retention time (t_R): **9** (11.1 min), **2** (13.9 min), **5** (14.5 min), **10** (15.3 min), **3** (17.0 min). (B) the proposed sequential reactions catalyzed by XiaM. (C) The heme binding pocket and the I-helix (αI) of XiaM. This homology structure model was generated on the basis of PikC (PDB ID: 2BVJ).

Bioinformatic analysis reveals that XiaM belongs to cytochrome P450 enzymes, a large family of oxidative hemoproteins, which account for the diverse oxygenation steps involved in the secondary metabolic pathways to generate diversified natural products.[9] Comparison of XiaM with a variety of cytochrome P450 enzymes reveals

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the presence of two characteristic motifs in bacterial natural product P450s (Figure S8): (i) the "GXXXC" motif in the heme-binding loop harboring the invariant cysteine (C338 in XiaM); (ii) the (A/G)GXXT motif with the conserved glycine and theronine (T231 in XiaM), the latter of which is critical for the H-bonding network.[9] An XiaM homology model, constructed on the basis of the P450 PikC,[10] also highlighted the importance of C338 for heme binding and the highly conserved segment AGHET (Figure S8) in the vicinity of the active site located at the I-helix of these enzymes (Figure 3C). In accordance with this model, the XiaM C338A mutant lost the ability to process **3** and **5** (Figure 3A, traces vi and viii). Interestingly, the XiaM T231A mutant was only capable of catalyzing the first hydroxylation to convert **3** to **5** (Figure 3A, traces vii and ix), indicating that T231 was essential in coordinating the second hydroxylation in XiaM catalysis.

Herein we illustrated that the P450 XiaM functioned to confer the C-24 carboxyl group via consecutive triple hydroxylations of a methyl group. Such multifunctional P450 enzymes are precedent in nature, from animals,[11] plants,[12] to microorganisms (Figure S9).[13] Well studied examples include the P450 enzyme CYP27A1 (a sterol 27-hydroxylase) for bile acid biosynthesis in rabbit liver, the *Arabidopsis* kaurene oxidase CYP701A3 in plant hormone gibberellin biosynthesis, and the P450 CYP71AV1 in plant biosynthesis of the antimalarial drug artemisinin. Such transformations were also indicated in microbial natural product biosynthesis by in vivo gene disruption, for example, the P450s AmphN and FscP in the biosynthesis of amphotericin B and FR-008, and the cytochrome P450 BonL in bongkrekic acid biosynthesis.[13] Although lacking experimental support, the P450s KijA and TcaE2 were proposed to introduce the *N*-carboxyl group of the kijanose from a methyl group in the biosynthesis of kijanimicin and tetrocarcin A.[14] Remarkably, the unique heterocycle-forming P450 oxygenase AurH in aureothin biosynthesis was engineered by a single mutation to change its function to regioselectively catalyze the oxidation of a methyl group to a carboxylic acid.[15] In general, P450s require redox partners, ferredoxin reductase and ferredoxin, to support the transfer of two electrons from NAD(P)H to the heme iron in P450 catalysis.[9] In this study, XiaM appeared to be functional alone in a heterologous *E. coli* host, suggesting that XiaM might be compatible to a certain electron transfer system in *E. coli*. A similar phenomenon was observed for a P450 from *Acinetobacter* sp. OC4 that retained partial activity in *E. coli*-mediated biotransformations lacking native redox partners.[16]

In conclusion, by changing cultivation conditions of the Δ*xiaM* and Δ*xiaK* mutants, we obtained four new intermediates (or shunt products) of **1**, preindosespene (**3**), preindosespenone (**4**), indosespenol (**5**), and dihydroxy-3-farnesylindole (**6**). Compound **6** was probably a shunt product of the hydrolysis-prone biosynthetic intermediate, epoxy-3-farnesylindole (**8**) (Figure 1). A similar hydrated product of epoxide was also observed in the fungal biosynthesis of pyripyropene.[17] Compound **4** might be derived from **3** by an unknown dehydrogenase in *S.* sp. SCSIO 02999. The conversions of **3** and **5** to **2** in *E. coli* expressing *xiaM* clearly demonstrated that the function of XiaM was to catalyze three-step hydoxylations to produce **2** from **3** via the route **3**→**5**→**9**↔**10**→**2** (Figure 3B). Our studies revealed that C338 was essential for XiaM catalysis and the T231A mutant preserved only the first hydroxylating activity. The catalytic mechanism of this family of enzymes thus warrants further biochemical and structural investigations.

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Supporting Information Available. Experimental details, compounds spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.