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# Elucidating the Cyclization Cascades in Xiamycin Biosynthesis by Substrate Synthesis and Enzyme Characterizations

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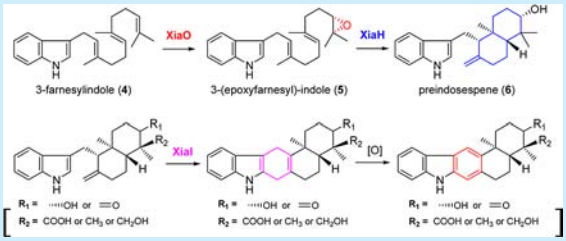
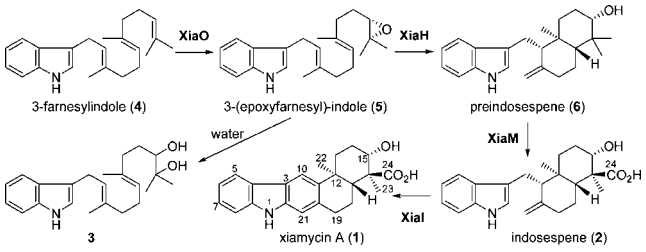
Supporting Information

**ABSTRACT:** Indolosesquiterpene xiamycin A features a pentacyclic core structure. The chemical synthesis of two key precursors, 3-farnesylindole and 3-(epoxyfarnesyl)-indole, allowed elucidation of the enzymatic cascades forming the pentacyclic ring system of xiamycin A by XiaO-catalyzed epoxidation and the membrane protein XiaH-catalyzed terpene cyclization. The substrate flexibility of XiaI, an indole oxygenase for assembly of the central ring, was also demonstrated.




Indolosesquiterpenes are a growing class of natural products that display a variety of biological properties such as antibacterial, anticancer, and antiviral activities.[1] Recently, indolosesquiterpenes have been isolated from actinomycetes,[2] which is complementary to the primary plant and fungi producers.[3] The intriguing structures of actinomycete-generated indolosesquiterpenes have inspired great interest from both the chemical and biosynthetic perspective. Total syntheses of a number of natural products from the xiamycin and oridamycin families have recently been achieved by the groups of Baran[4] and Li,[5] respectively. The xiamycin A (**1**, Scheme 1) biosynthetic gene cluster was independently reported by us from a marine-derived *Streptomyces* sp. SCSIO 02999[6] and by the Hertweck group from a mangrove endophyte *Streptomyces* sp. HKI0576.[7] We have previously identified XiaI, an indole oxygenase, as a cyclase that catalyzes the C-3 hydroxylation of the biosynthetic intermediate indosespene, which triggers a cyclization to form the central ring of **1** (Scheme 1).[6] We have also reported that the P450 enzyme XiaM is responsible for forming the C-24 carboxyl of xiamycin A via consecutive triple hydroxylations of a methyl group (Scheme 1).[8] Very recently, the Hertweck group illustrated the flavoenzyme XiaK (named XiaH by them[7]) as a regiodivergent catalyst for promoting the C,N and N,N coupling reactions to form dixiamycins through a radical-based mechanism.[9] However, the enzymatic steps prior to preindosespene (**6**, Scheme 1) formation in xiamycin A biosynthesis remain unresolved.

**Scheme 1. Proposed Biosynthetic Pathway for Xiamycin A (1)**




It has been reported that the biosynthesis of fungal metabolites, the meroterpenoid pyripyropene A[10] and the indole-diterpene paxilline,[11] involve an epoxidation and a subsequent cyclization in their ring construction.[12] In this study, by utilizing a biomimetic synthesis of biosynthetic intermediates, 3-farnesylindole (**4**) and 3-(epoxyfarnesyl)-indole (**5**), we were able to demonstrate the oxidoreductase XiaO as an epoxidase and the membrane protein XiaH as a cyclase. Furthermore, we reported the substrate flexibility of another cyclase XiaI and the generation of xiamycin analogues. Thus, the biosynthetic machinery for the construction of the pentacyclic core structure in xiamycin A was reconstituted.

The indole diterpene paxilline has been reported to be biosynthesized from the intermediate 3-geranylgeranyl-indole which undergoes a flavin-dependent monooxygenase PaxM-catalyzed epoxidation followed by a membrane protein PaxB-

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catalyzed cyclization.[11] In the biosynthetic gene cluster of xiamycin A, the monooxygenase XiaO resembles PaxM and the putative membrane XiaH is similar to PaxB, indicating that they may catalyze similar reactions. We have previously reported that the Δ*xiaO* insertional mutant XM46 abolished the production of xiamycin and its analogues, without accumulation of any related biosynthetic intermediates.[6] To avoid any polar effects putatively caused by insertional mutation, we constructed a Δ*xiaO* mutant XM46i by in-frame deletion (Figure S1). Again, no intermediates were detected in this mutant (Figure 1). Bioinformatics analysis predicts that XiaH belongs

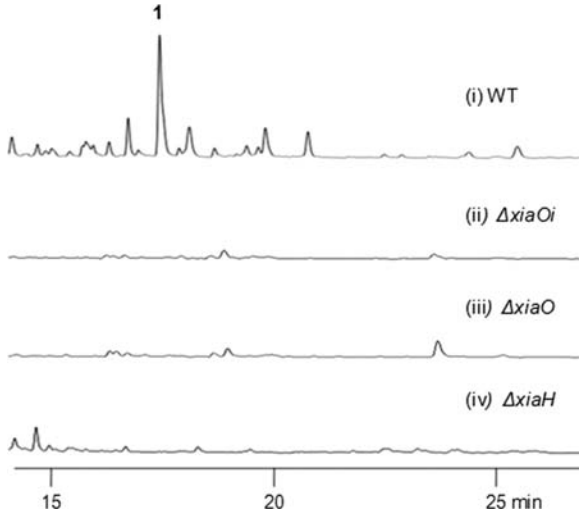


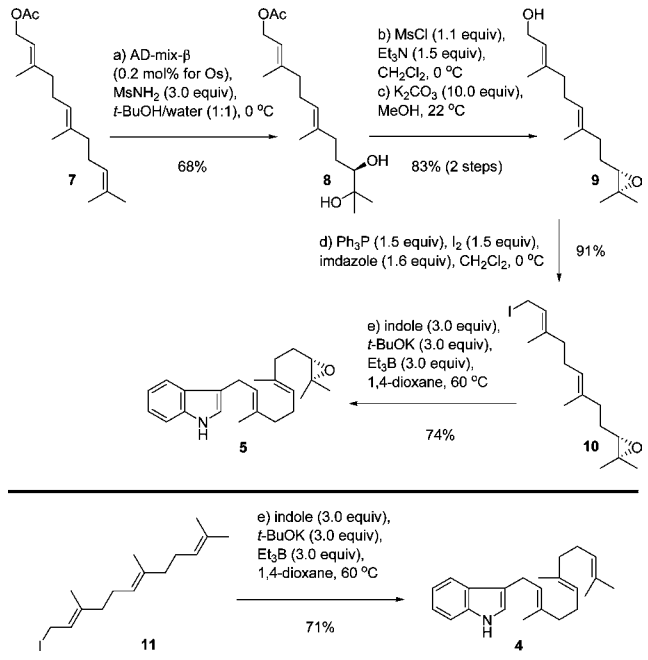

**Figure 1.** HPLC analysis of metabolite profile of the *xia* gene knockout mutants. (i) *Streptomyces* sp. SCSIO 02999 wild type; (ii) Δ*xiaO* in-frame deletion mutant XM46i; (iii) Δ*xiaO* insertional mutant XM46; (iv) Δ*xiaH* insertional mutant XM39.

to membrane proteins with multiple transmembrane domains (Figure S2). Insertional mutation of *xiaH* led to the mutant XM39 (Figure S3) that produced none of the xiamycin-related metabolites (Figure 1). In a previous study by Hertweck et al.,[7] inactivation of the *xiaO* homologue (named *xiaL*,[7] 97% identity) or *xiaH* homologue (named *xiaK*,[7] 95% identity) also abolished the production of xiamycin analogues in the corresponding mutants of *Streptomyces* sp. HKI0576. These experiments demonstrated that XiaO and XiaH are essential for xiamycin A biosynthesis.

To elucidate the exact functions of XiaO and XiaH, we synthesized 3-farnesyl indole (**4**) and its monoepoxy derivative **5** (Scheme 2), respectively, as substrates for XiaO and XiaH enzymatic studies. Farnesyl acetate **7** was subjected to the conditions of Sharpless asymmetric dihydroxylation (AD-mix-*β*, $MsNH_2$)[13] to give diol **8** in 68% yield, which underwent mesylation (MsCl, $Et_3N$) followed by ring closure ($K_2CO_3$, MeOH) to afford epoxide **9** in 83% yield. The acetyl group was also removed in one pot. Treatment of this primary alcohol with $PPh_3$ and $I_2$ provided iodide **10**. Using the protocol developed by Yang et al.,[14] we performed the regioselective alkylation of indole at C3 with **10** to obtain compound **5** (Figure S4 for NMR data) in 74% yield. 3-Farnesyl indole (**4**, Figure S5 for NMR data) was also prepared from farnesyl iodide **11** with good efficiency in 71% yield.[15]

Having the putative substrates in hand, we then carried out the functional studies of XiaO and XiaH. To characterize the function of XiaO, we first overexpressed *xiaO* in *E. coli* using

**Scheme 2. Chemical Synthesis of 3-Farnesyl Indole (4) and Its Mono-Epoxy Derivative (5)**




pET28a as a vector, resulting in an insoluble XiaO. The *E. coli* mediated biotransformation also failed to convert **4** into **5**. Alternatively, we cloned the *xiaO* gene into a pPWW50A vector to afford the plasmid pCSG2705. Unfortunately, no expression of XiaO was observed, either, in this *Stretomyces* expression system. To our delight, **4** was biotransformed into two products **5** and **3** by *S. coelicolor* YF11[16] carrying pCSG2705 (Figure 2A, traces ii–v), while no conversion of **4** was detected in the control strain *S. coelicolor* YF11/pPWW50A (Figure 2A, trace i). The identities of compounds **5** and **3** were confirmed by comparison with standards and LC–MS analysis (Figure S6). Compound **3** was previously isolated from the Δ*xiaK* mutant and was assumed to be derived from the hydrolysis of **5**.[8] Indeed, synthesized **5** also spontaneously decomposed to **3** when dissolved in water (Figure 2A, trace iv). Thus, our biotransformation experiments validated that XiaO functioned to catalyze the epoxidation of **4** to afford **5**.

XiaO is a rather large protein with a size of 718 amino acids containing two distinct domains: the N-terminus belongs to the tryptophan 2,3-dioxygenase family, while the C-terminus is an FAD-dependent oxidoreductase (Figure 2B). The N-terminal domain in XiaO is absent in its functionally analogous proteins for fungal synthesis of indole terpenes. The phylogenetic tree showed that even the FAD-dependent oxidoreductase domain of XiaO (257–718 aa) had very low identity (<20%) with indole terpene epoxidases and was more closely related to epoxidases MonCI and Lsd18 involved in polyether formation (Figure S7).[17] Intriguingly, the truncated t-XiaO (257–718 aa, removing the N-terminal domain) was still functional to biotransform **4** to **5** (Figure 2A, trace vi), with high conversion comparable to that of the wild type XiaO (Figure 2A, trace ii). This observation indicated that the N-terminal domain (tryptophan 2,3-dioxygenase family) in XiaO was not essential for its catalytic activity and was probably a redundant domain during evolution.

To find out whether the membrane protein XiaH functioned as a terpene cyclase, similar to Pyr4 in the pyripyropene A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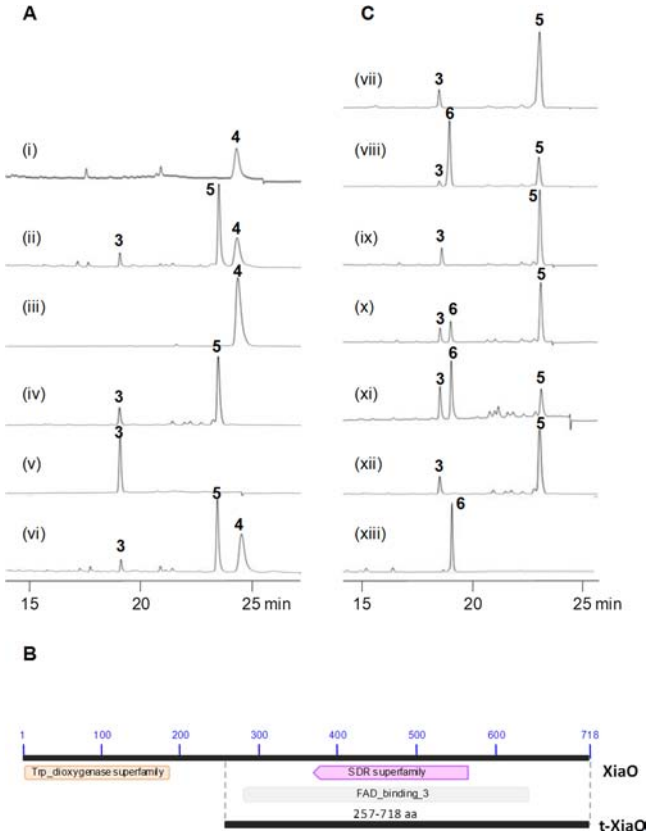


**Figure 2.** (A) Functional analyses of XiaO by *S. coelicolor* YF11-mediated biotransformation. (i) *S. coelicolor* YF11/pPWW50A feeding with **4**; (ii) *S. coelicolor* YF11/pCSG2705 (for *xiaO* expression) feeding with **4**; (iii) authentic **4**; (iv) synthesized **5** dissolved in water; (v) authentic **3**; (vi) *S. coelicolor* YF11/pCSG2712 (for truncated t-*xiaO* expression) feeding with **4**. (B) Conserved domains in XiaO and depiction of truncated XiaO (t-XiaO, 257−718 aa). (C) Functional analyses of XiaH *in vivo* and *in vitro*. (vii) *E. coli* BL21(DE3)/pET28a feeding with **5**; (viii) *E. coli* BL21(DE3)/pCSG2613 (harboring *xiaH*) feeding with **5**; (ix) cell free extracts of *E. coli* BL21(DE3)/pET28a incubated with **5**; (x) supernatants of cell-free extracts of *E. coli* BL21(DE3)/pCSG2613 after ultracentrifugation incubated with **5**; (xi) "membrane fraction" of *E. coli* BL21(DE3)/pCSG2613 incubated with **5**; (xii) synthesized **5** dissolved in water; (xiii) authentic **6**.

pathway,[10] and PaxB in the paxilline pathway,[11] we cloned *xiaH* into pET28a to afford the plasmid pCSG2613. Unfortunately, no expression of XiaH was detected in *E. coli* BL21(DE3) harboring pCSG2613. Nonetheless, we fed **5** to *E. coli* BL21(DE3)/pCSG2613. After inducing *xiaH* expression with IPTG, **5** was found to be converted to a product displaying the same retention time and molecular mass as those of preindosespene (**6**, Figure S8), previously isolated from the Δ*xiaM* mutant,[8] together with the decomposed shunt product **3** from **5** (Figure 2C, trace viii). When the control strain *E. coli* BL21(DE3)/pET28a was treated in the same manner, only the shunt product **3** was detected (Figure 2C, trace vii). This observation indicated that XiaH was a *bona fide* terpene cyclase and was functional in *E. coli* BL21(DE3)/pCSG2613. Considering the nature of XiaH as a membrane protein, we then collected the pellet fraction (membrane fraction) by ultracentrifugation of the cell-free extracts of *E. coli* BL21-(DE3)/pCSG2613. In an *in vitro* assay performed with the "membrane fraction" resuspended in reaction buffer, the efficient formation of preindosespene (**6**) from **5** was detected, which was absent in the control assay of incubating **5** with cell-free extracts of *E. coli* BL21(DE3)/pET28a (Figure 2C, traces ix−xi). The supernatants of *E. coli* BL21(DE3)/pCSG2613 after ultracentrifugation was also capable of converting **5** into **6** (Figure 2C, trace x). We also constructed an *S. coelicolor* YF11 strain coexpressing *xiaO* and *xiaH* and found out that **4** was biotransformed into **6** with high efficiency in this strain (Figure S9).

We have previously demonstrated that the oxygenase XiaI is responsible for converting indosespene (**2**) into prexiamycin (**2a**, Figure 3) which was spontaneously oxidized to **1** to

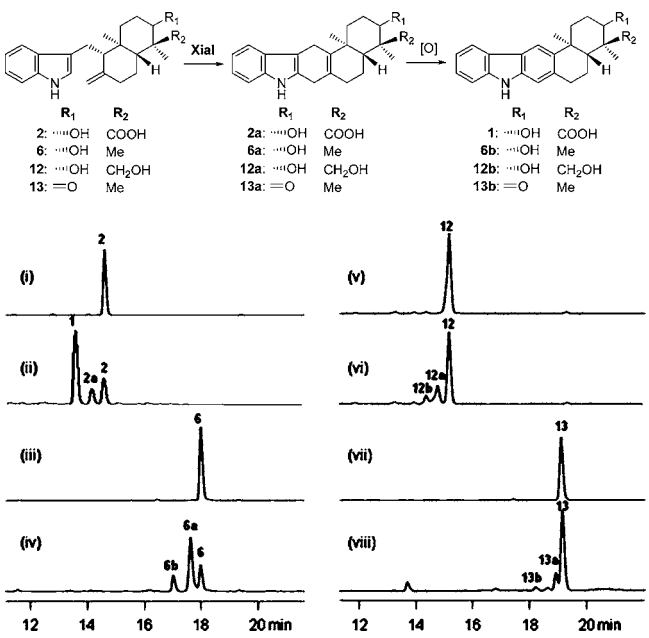


**Figure 3.** Schematic representation of XiaI-catalyzed reactions and HPLC analyses of XiaI assays. A standard XiaI assay comprising 400 μM substrate (**2**, **6**, **12**, or **13**), 1 mM FAD, 2 mM NADH, and 5 μM XiaI in 50 mM Tris-HCl (pH 8.0) buffer, incubated at 28 °C for 12 h. Control assays lacking XiaI with **2** (i), **6** (iii), **12** (v), and **13** (vii); XiaI assays with **2** (i), **6** (iii), **12** (v), and **13**.

construct the central ring.[6] Interestingly, XiaI was shown to exhibit substrate flexibility and could recognize several surrogate substrates, such as preindosespene (**6**), indosespenol (**12**), and preindosespenone (**13**), which were previously reported from the Δ*xiaM* or Δ*xiaK* mutants.[8] Similarly, **6**, **12**, and **13** were first oxidized by XiaI to the intermediates **6a**, **12a**, and **13a** and then were spontaneously aromatized to the final products **6b**, **12b**, and **13b** (Figure 3, and Figure S10 for LC-MS analysis of products). This observation placed XiaI as a useful catalyst capable of producing novel indolosesquiterpenes that were not naturally isolated from the wild type strain *Streptomyces* sp. SCSIO 02999 or from the gene-knockout mutants.

In this study, XiaH was confirmed to belong to a novel family of cyclases involved in indole terpene biosynthesis. Further bioinformatic analysis reveals the presence of XiaH homologues in sequenced actinomycete genomes. Interestingly, the XiaH homologues in 6 strains (*Streptomyces olivaceus* NRRL B-3009, *Streptomyces* sp. NRRL S-15, *Streptomyces fradiae* ATCC 19609, *Streptomyces* sp. NRRL F-2890, *Streptomyces* sp. AA0539, *Amycolatopsis nigrescens* CSC17Ta-90) are clustered with enzymes highly conserved in the xiamycin gene cluster (Figure


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S11), indicating that they have the potential to produce xiamycin or related indole terpenes. Further analysis reveals that the annotated XiaO homologues in *S. olivaceus* NRRL B-3009 and *Streptomyces* sp. NRRL F-2890 contain only a C-terminal domain in XiaO, while the annotated XiaO homologues in *Streptomyces* sp. AA0539 and *A. nigrescens* CSC17Ta-90 are split to two proteins corresponding to the N- and C-terminal domains in XiaO, respectively (Figure S11). This may indicate an evolutionary divergence of XiaO homologues in different strains, given that the C-terminal domain alone in XiaO has been confirmed to be functional.

In summary, we have validated XiaO (including the truncated version without the N-terminal tryptophan 2,3-dioxygenase domain) as an epoxidase and XiaH as a terpene cyclase in xiamycin A (**1**) biosynthesis. The biomimetic synthesis of key intermediates **4** and **5** enabled the stepwise reconstitution of the pentacyclic ring system in xiamycin A. This study suggests that the indole terpene ring construction in bacteria utilizes similar strategies as demonstrated in fungi. The substrate flexibility of another cyclase XiaI was also demonstrated, opening the door for chemoenzymatic generation of indole terpene analogues.

## ■ ASSOCIATED CONTENT

### Supporting Information

Experimental procedures, characterization data for compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

‖H.L. and Y.S. contributed equally.


### Notes

The authors declare no competing financial interest.

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