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Flavoenzyme-Catalyzed Atropo-Selective *N,C*-Bipyrrole Homocoupling in Marinopyrrole Biosynthesis

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Supporting Information

ABSTRACT: Axially chiral biaryl compounds are frequently encountered in nature where they exhibit diverse biological properties. Many are biphenols that have C−C or C−O linkages installed by cytochrome P450 oxygenases that control the regio- and stereoselectivity of the intermolecular coupling reaction. In contrast, bipyrrole-coupling enzymology has not been observed. Marinopyrroles, produced by a marine-derived streptomycete, are the first 1,3′-bipyrrole natural products. On the basis of marinopyrrole's unusual bipyrrole structure, we explored its atropo-selective biosynthesis in *Streptomyces* sp. CNQ-418 in order to elucidate the *N,C*-bipyrrole homocoupling enzymology. Through a series of genetic experiments involving the discovery and heterologous expression of marinopyrrole biosynthesis genes, we report that two flavin-dependent halogenases catalyze the unprecedented homocoupling reaction.

Cross-coupling and homocoupling reactions are important organic transformations that conjoin two hydrocarbon fragments through the aid of a catalyst. While a myriad of synthetic approaches have been developed to construct axially chiral biaryl compounds inspired by naturally occurring organic molecules,[1] our mechanistic understanding of enzyme-catalyzed oxidative coupling reactions is limited largely to cytochrome P450 oxygenases, laccases, and peroxidases that biosynthesize biphenols, alkaloids, and plant-derived aromatics.[2] Nature's capacity to biosynthesize biaryl compounds is far greater and includes pyrrole-containing aromatics such as hexabromo-2,2′-bipyrrole,[3] pentabromopseudiline,[4] and marinopyrrole[5] for which pyrrole coupling enzymology has not been reported. Herein, we describe the discovery of a pair of $FADH_2$-dependent halogenases involved in marinopyrrole biosynthesis that catalyze an atropo-selective *N,C*-biaryl homocoupling reaction.

The densely halogenated bipyrrole natural products marinopyrroles A−F (**1**−**6**, Figure 1) were recently reported from the bacterium *Streptomyces* sp. CNQ-418 isolated from ocean sediments.[5,6] These marine microbial metabolites are the first natural 1,3′-bipyrroles and possess potent antibiotic activity against methicillin-resistant *Staphylococcus aureus*.[6,7] Because of their axially chiral structures, the biosynthesis of the

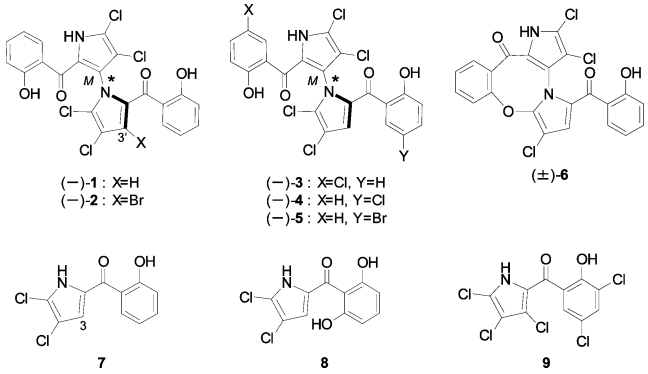

Figure 1. Structures of marinopyrroles A−F (**1**−**6**) and related compounds.

marinopyrroles may proceed through an unprecedented bipyrrole homocoupling reaction operating in an atropo-selective manner. The fact that monomeric monodeoxypyoluteorin (**7**) is coproduced by strain CNQ-418[5] strongly suggests its intermediacy in the biosynthesis of the dimeric marinopyrroles. Such a strategy was recently executed in the biomimetic total synthesis of racemic, marinopyrrole A via an intermolecular Ullman coupling reaction.[8] We thus set out to explore marinopyrrole biosynthesis in *S.* sp. CNQ-418 in order to elucidate how nature has solved this atropo-selective, bipyrrole homocoupling reaction that could influence the future development of new biaryl coupling reagents.

The chemical structure of the monomer **7** is closely related to that of two phenylpyrrole antibiotics, pyoluteorin (*plt*, **8**) and pyrrolomycin D (*pyr*, **9**), produced by *Pseudomonas fluorescens* Pf-5[9] and *Streptomyces vitaminophilum* ATCC31673 (*formerly Actinosporangium vitaminophilum*),[10] respectively. Based on the biosynthetic studies of **8** and **9**,[9,10] we were able to clone and sequence the 36-kb putative marinopyrrole biosynthetic gene (*mpy*) cluster spanning two cosmid clones (Figure 2a, Table S3). Sequence analysis revealed 20 open reading frames (ORFs) that showed clear relationships with most ORFs associated with **8** and **9** biosynthesis, thereby suggesting that **7** is biosynthesized from L-proline and three

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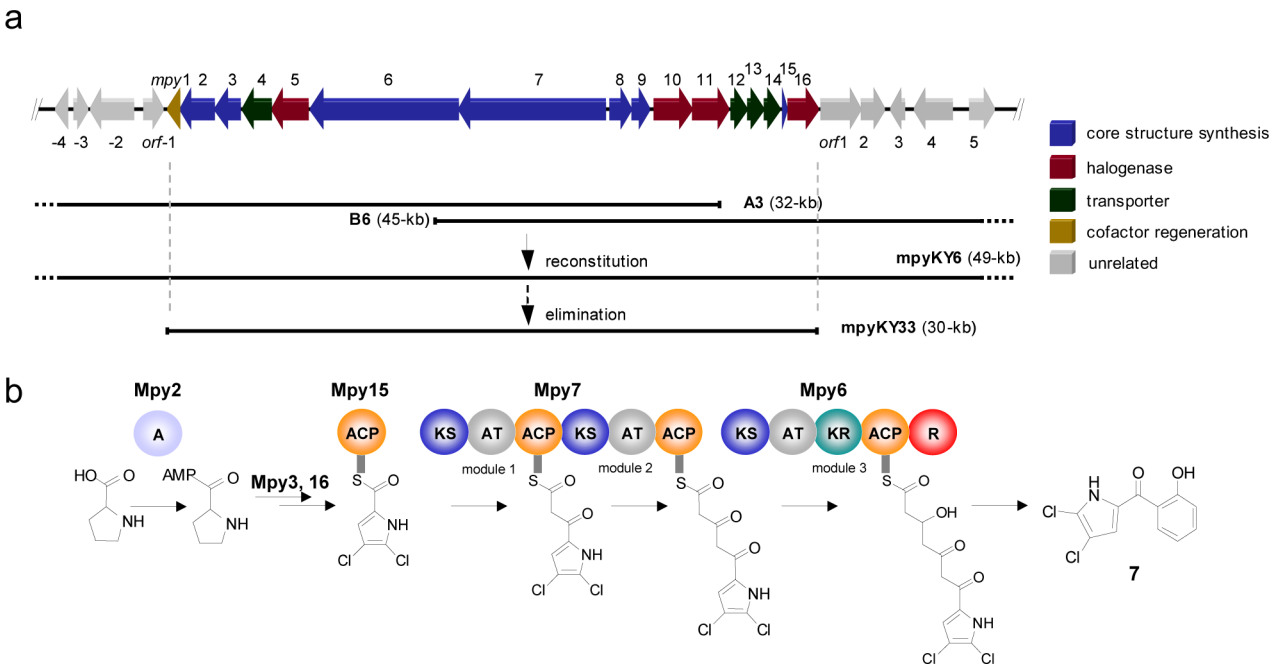

Figure 2. (a) Gene organization of marinopyrrole biosynthetic gene (*mpy*) cluster in *Streptomyces* sp. CNQ-418 and schematic representation of cosmids used in this study. Each arrow represents the direction of transcription of an ORF. See the Supporting Information for the deduced functions of the ORFs. (b) Proposed biosynthetic pathway of monomeric monodeoxypyoluteorin (**7**). Abbreviations: A, adenylation domain; ACP, acyl carrier protein; KS, ketosynthase; AT, acyltransferase; KR, ketoreductase; R, thioester reductase.

malonate molecules via a modular polyketide synthase (PKS) (Figure 2b). We, however, were not able to probe marinopyrrole biosynthesis in the native strain CNQ-418 since it did not survive long-term storage. Therefore, to connect the *mpy* locus with marinopyrrole biosynthesis, we assembled the entire *mpy* cluster from two overlapping cosmid clones (A3 and B6) into a single cosmid (mpyKY6) by λ-RED recombination (Figure S1)[12] in order to express the complete cluster in the host *Streptomyces coelicolor* M512. The heterologous expression of the reconstituted *mpy* cluster resulted in the production of dimeric marinopyrroles **1**–**4** (Figure 3a) with the productivity of the major metabolite **1** at 10–20 mg/L, thus providing clear evidence that all genes required for marinopyrrole biosynthesis were present on the reconstituted cluster. Furthermore, chirality analysis of heterologously biosynthesized **1** confirmed that it maintained its *M*-configuration as in the natural product. We thus

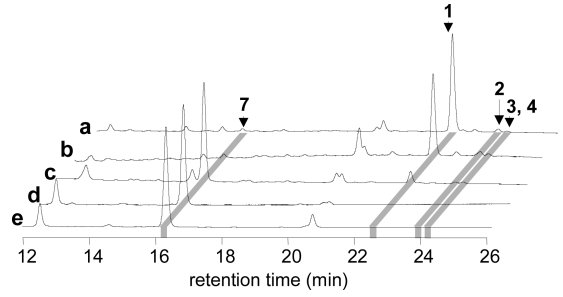

Figure 3. Comparative HPLC analysis of heterologously produced marinopyrroles extracted from (a) *S. coelicolor* M512/mpyKY8 (reconstituted); (b) *S. coelicolor* M512/mpyKY33 (*mpy1*–*16*); (c) *S. coelicolor* M512/mpyKY15 (Δ*mpy1*); (d) *S. coelicolor* M512/mpyKY28 (Δ*mpy10*); and (e) *S. coelicolor* M512/mpyKY29 (Δ*mpy11*). HPLC was monitored at 320 nm.

employed this heterologous expression system in all later experiments to probe the *in vivo* function of the *mpy* genes.

We first deleted the *mpy6* gene encoding the putative offloading PKS module-3. As expected, this mutant completely lost the ability to produce monomeric and dimeric pyrroles (Figure S2a), thereby providing an opportunity to explore the intermediacy of monomer **7** in marinopyrrole biosynthesis. Chemical complementation of the mutant with **7** restored dimeric marinopyrrole synthesis (**1**–**4**) (Figure S2b), which confirmed it as a precursor and allowed for the concerted search for the desired marinopyrrole coupling enzyme gene. We next constructed several minimized *mpy* gene clusters by eliminating peripheral genes from the reconstituted cluster in order to determine the exact boundaries of the marinopyrrole locus (Table S4). While we originally considered that the hypothetical protein encoded by *orf4* participated in the coupling reaction, the heterologous expression of *mpy1*–*16* resulted in the production of **1**–**4** (Figure 3b), demonstrating that all genes involved in *mpy* biosynthesis are harbored on the minimized cosmid mpyKY33 (Figure 2a). Among the 16 genes, most have homologues in the *plt* cluster[13] except for the *mpy1* flavin reductase-encoding gene and the *mpy4* putative membrane protein-encoding gene. While elimination of the *mpy4* gene resulted in unaltered marinopyrrole production, deletion of the *mpy1* gene dramatically reduced production of dimeric **1**–**4** with concomitant production of monomeric **7** (Figure 3c), thereby clearly suggesting that the desired coupling enzyme is a flavoprotein.

Four flavoprotein-encoding genes (*mpy5*, *mpy10*, *mpy11*, and *mpy16*) are encoded in the minimized *mpy* cluster, and all four annotate as $FADH_2$-dependent halogenases by BLAST analysis (Figure 2a, Table S3). Among them, Mpy5 lacks the motifs GXGXXG and WXWXI associated with other characterized $FADH_2$-dependent halogenases,[13] suggesting that Mpy5 may not be a functional flavoprotein. We therefore generated three

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halogenase gene deletion mutants in order to individually interrogate their *in vivo* functions. As anticipated, the *mpy16* gene deletion completely abolished production of the marinopyrroles, supporting our biosynthetic proposal that Mpy16 catalyzes the initiating dichlorination of the pyrrolyl-*S*-carrier protein as previously demonstrated for the homologous PltA in pyoluteorin biosynthesis (Figure 1b).[11] Deletion of *mpy10*, on the other hand, completely abolished production of dimeric **1**–**4** with alternative overproduction of **7** (Figure 3d). We similarly observed the same outcome for the deletion of *mpy11* (Figure 3e), suggesting that both Mpy10 and Mpy11 halogenases are responsible for the coupling reaction. To exclude the possibility of unintended polar effects on gene expression in these mutants, we reintroduced the deleted genes back to the coupling-deficient mutants through conjugative integration and under control of the strong constitutive *ermE** promoter (Figure S3). As expected, dimeric **1**–**4** formation was restored only when the mutants had both the *mpy10* and *mpy11* genes (Figure 4), clearly demonstrating that two

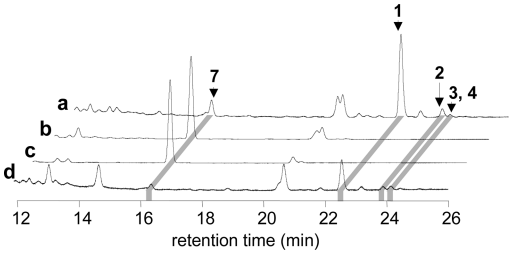

Figure 4. Comparative HPLC analysis of heterologously produced marinopyrroles extracted from (a) *S. coelicolor* M512/mpyKY28 (Δ*mpy10*)/pKY01-*mpy10*; (b) *S. coelicolor* M512/mpyKY28 (Δ*mpy10*)/pKY01-*mpy11*; (c) *S. coelicolor* M512/mpyKY29 (Δ*mpy11*)/pKY01-*mpy10*; and (d) *S. coelicolor* M512/mpyKY29 (Δ*mpy11*)/pKY01-*mpy11*. HPLC was monitored at 320 nm.

$FADH_2$-dependent halogenases Mpy10 and Mpy11 are involved in the atropo-selective *N,C*-bipyrrole homocoupling in the presence of the pathway specific flavin reductase Mpy1.

We further corroborated this observation by constructing a new expression system harboring only the three genes *mpy1*, *mpy10*, and *mpy11* (Figure S4). To our surprise, however, we did not observe dimerization of exogenously added **7** by the engineered *S. coelicolor* mutant. Dimerization was only achieved upon coexpression of the adjacently transcribed ABC transporter gene cassette *mpy12*–*14* that presumably facilitates the transmembrane transport of the marinopyrroles in a similar manner to the *plt* ABC transporters.[14] In fact, deletion of the transporter gene cassette from the minimized cluster resulted in no secretion of marinopyrroles, thereby supporting this observation (data not shown). Once again, both *mpy10* and *mpy11* were required for monomer homocoupling, as elimination of either gene prevented dimerization.

The distinctive gene organization of two tandem $FADH_2$-dependent halogenase genes showing significant similarity to *mpy10* and *mpy11* was previously observed in the gene cluster of the structurally related antibiotic pyrrolomycin D (**9**) where their protein products are presumed to chlorinate the phenol and pyrrole residues (Table S5).[10] In the case of marinopyrrole biosynthesis, we speculate that C-3 halogenation of **7** directs its enzymatic coupling with a second molecule of **7** (Figure 5a). This mechanistic scenario parallels the approach employed in the Kanakis and Sarli chemical synthesis of racemic **1**.[8] Alternatively, halogenation of the pyrrole nitrogen of **7** could in turn facilitate an electrophilic aromatic substitution reaction with a second equivalent of monomer **7** (Figure 5b). Furthermore, a single electron transfer mechanism to give the *N*-radical cation is also plausible. Since 3-halo-**7** and *N*-halo-**7** were not reported from the native strain *S.* sp. CNQ-418[5] nor did we observe their production in any of our *mpy* heterologous *S. coelicolor* M512 constructs, consequently, we hypothesize that a trihalogenated derivative of **7** may be formed in trans and not released by a functional complex of Mpy10-Mpy11 prior to atropo-selective dimerization with unmodified **7** (Figure 5). Additional halogenation of **1** to the chlorinated and brominated **2**–**5** may take place immediately following the homocoupling reaction. An alternative possibility of unmodified **7** serving as the directing coupling agent in which H-3 is lost as a hydride upon homocoupling is compatible for a flavoenzyme acting as a dehydrogenase, yet it is not consistent with the requirement of the flavin reductase Mpy1.

In conclusion, we have successfully characterized the molecular basis of marinopyrrole biosynthesis that includes an unprecedented *N,C*-bipyrrole homocoupling reaction. Our work supports the hypothesis that the novel chiral coupling

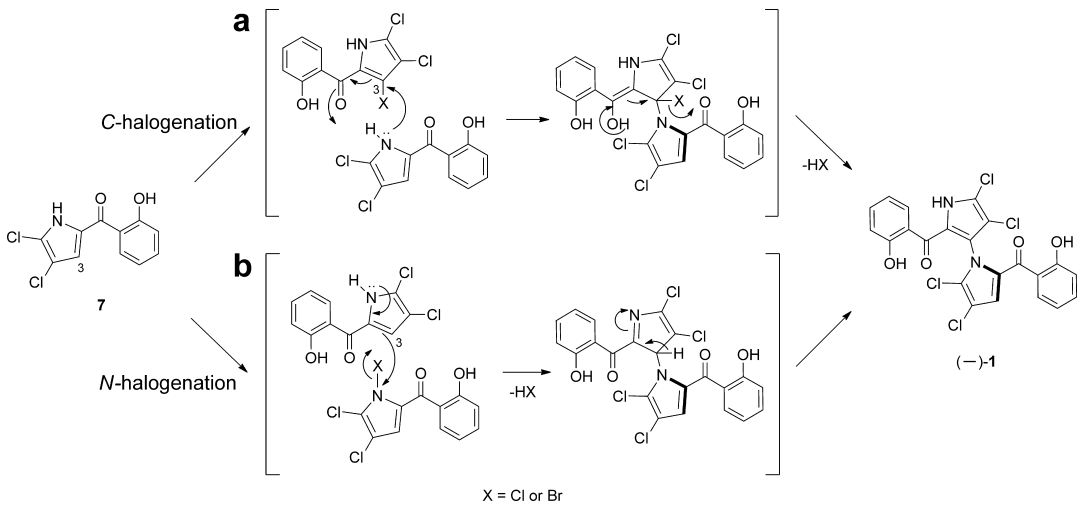

Figure 5. Proposed *N,C*-bipyrrole homocoupling mechanisms via (a) C-3 halogenation and (b) *N*-halogenation of **7**.

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reaction of two **7** molecules is catalyzed by the two $FADH_2$-dependent halogenases Mpy10 and Mpy11 in the presence of the pathway specific flavin reductase Mpy1 in which the 46% identical (78% similar) halogenases may operate as a functional complex. Such a cryptic biohalogenation strategy has only been previously observed in the biosynthesis of cyclopropyl rings.[15] Since the involvement of $FADH_2$-dependent halogenases in biaryl formation has never been observed, our discovery lays the foundation for future *in vitro* studies with recombinant proteins to explore the mechanistic details of this novel *N,C*-biaryl homocoupling enzymology.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S4, Tables S1−S5, experimental methods. 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.

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