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Insights into Caerulomycin A Biosynthesis: A Two-Component Monooxygenase CrmH-Catalyzed Oxime Formation

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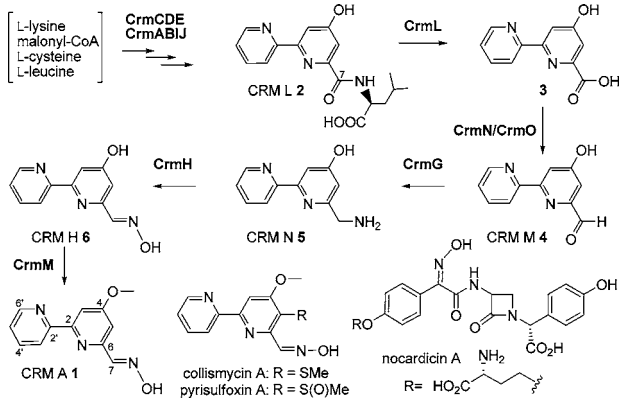
Supporting Information

ABSTRACT: The immunosuppressive agent caerulomycin A features a unique 2,2′-bipyridine core structure and an unusual oxime functionality. Genetic and biochemical evidence confirms that the oxime formation in caerulomycin A biosynthesis is catalyzed by CrmH, a flavin-dependent two-component monooxygenase that is compatible with multiple flavin reductases, from a primary amine via a *N*-hydroxylamine intermediate. Structure homologue-guided site-directed mutagenesis studies identify four amino acid residues that are essential for CrmH catalysis. This study provides the first biochemical evidence of a two-component monooxygenase that catalyzes oxime formation.

Caerulomycins (CRMs) were first discovered from *Streptomyces caeruleus*[1] and were recently reisolated from a marine-derived *Actinoalloteichus cyanogriseus* WH1-2216-6 with numerous new analogues.[2] Caerulomycin A (CRM A **1**, Scheme 1) bears a unique 2,2′-bipyridine core structure and an unusual oxime functionality, with high structure similarity to collismycins[3] and pyrisulfoxins (Scheme 1).[4] CRM A **1** exhibited antimicrobial, antiamoebic, and antitumor activities[5] and was under development as an immunosuppressive agent.[6] The unique structure and remarkable pharmaceutical potential of **1** have attracted significant attention for biosynthetic studies. The recent elucidations of **1** and collismycin A biosynthetic gene clusters suggested a common mechanism for 2,2′-bipyridine ring formation from a hybrid polyketide synthase (PKS)/nonribosomal peptide synthase (NRPS) machinery.[7] Although the exact mechanism for the 2,2′-bipyridine ring formation in **1** and collismycin A was still in controversy,[7a−c] we unequivocally demonstrated that the maturation of **1** involved an unusual amidohydrolase CrmL, capable of removing the L-leucine at C-7 of an early biosynthetic intermediate CRM L **2** (Scheme 1).[7a] Similar amidohydrolase activity was also indicated in the collismycin A biosynthesis.[7c] On the basis of bioinformatic analysis and experimental evidence, we proposed a post-PKS/NRPS biosynthetic pathway for **1** (Scheme 1).[7a]

Scheme 1. Proposed Post-PKS/NRPS Biosynthetic Pathway for CRM A 1 and Chemical Structures for Collismycin A, Pyrisulfoxin A, and Nocardicin A



The formation of an oxime moiety is rarely found in the biosynthesis of microbial secondary metabolites. The only reported case is the cytochrome P450 monooxygenase NocL-catalyzed oxime formation during nocardicin A (Scheme 1) biosynthesis.[8] Recently, an FAD-dependent monooxygenase ClmM was suggested to be involved in the oxime formation in the collismycin A pathway.[7d] However, the biosynthetic origin of the oxime in **1** remains elusive. In this study, we demonstrate that the monooxygenase CrmH (also called CaeB5[7b]) is responsible for catalyzing the oxime formation in **1**. Our in vivo gene disruption and in vitro biochemical experiments reveal that (i) CrmH functions as a flavin-dependent two-component monooxygenase, requiring an additional NAD(P)H-dependent flavin reductase to provide reduced flavin cofactors, to convert a primary amine to an oxime via a *N*-hydroxylamine intermediate; (ii) CrmH is compatible with eight flavin reductases, either from *E. coli* or from *A. cyanogriseus* WH1-

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2216-6; and (iii) four amino acid residues M95, S165, F251, and H377 in CrmH are essential for its catalytic activity.

Bioinformatic analysis of the **1** biosynthetic gene cluster retrieved two putative monooxygenases, CrmF and CrmH.[7a] Given that the inactivation of *crmF* did not affect **1** production, while the Δ*crmH* mutant failed to produce **1**,[7a] CrmH was proposed as the most likely candidate responsible for the oxime formation. HPLC analysis of the Δ*crmH* mutant revealed the production of two compounds that were distinct from **1** (Figure 1). The minor compound **7** was characterized to be

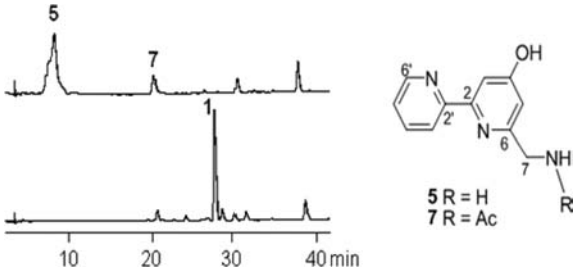

Figure 1. HPLC analysis of metabolite profile of the Δ*crmH* mutant (i) and WT (ii) with UV detection at 313 nm, and chemical structures of CRM N **5** and CRM J **7**.

identical to previously reported CRM J (Figure 1),[2a] by ESIMS as well as ^{1}H and ^{13}C NMR spectroscopic data (Table S1, Figure S1). The major one was characterized to be CRM N **5** (Figure 1). The molecular formula of **5** was established as $C_{11}H_{11}N_3O$ through HRESIMS (*m*/*z* 202.0976 $[M + H]^+$, calcd 202.0980). The comparison of ^{1}H and ^{13}C NMR spectroscopic data revealed that **5** differed from **7** only by the absence of signals of an acetyl group (δ_H/δ_C, 1.93/22.5, C-10; δ_C, 169.4, C-9) in **7** (Table S1, Figure S2). We hypothesized that compound **7** was an acetylated shunt product of the real intermediate **5**.

The accumulation of **5** in the Δ*crmH* mutant suggested that **5** should be a CrmH substrate. For in vitro characterizations, soluble *N*-His_6-tagged CrmH proteins were produced in *E. coli* BL21(DE3) harboring the plasmid pCSG2209 (Table S2) and were purified to near-homogeneity by Ni-NTA chromatography (Figure S3). The purified CrmH proteins were colorless and did not release any flavin cofactors (FAD or FMN) upon boiling (Figure S4). Incubation of **5** with CrmH yielded no detectable products, even in the presence of cofactors FAD (or FMN), NAD(P)H. In contrast, compound **5** was indeed biotransformed into a new product in *E. coli* BL21 (DE3)/pCSG2209 (Figure S5). The new product displayed the same retention time and molecular mass as those of CRM H **6** (Scheme 1, Figure S5), previously isolated from *A. cyanogriseus* WH1-2216-6.[2a] These results suggested that certain unknown cofactors or proteins in *E. coli* BL21(DE3) were required for the conversion of **5** to **6**. Further bioinformatic analysis showed that CrmH exhibited distant similarity with the *N*-hydroxylase VlmH (30% identity), a two-component monooxygenase family relying on a flavin reductase for amine oxidation in valanimycin biosynthesis.[9] To our delight, three products with UV spectra similar to those of **5** were detected (Figure 2A, trace iii; Figure S6), when Fre, a well-characterized *E. coli* flavin reductase,[10] was added to a CrmH assay containing **5**, FAD, and NADH. No such conversions were detected in control assays lacking either CrmH, Fre, or NADH (Figure 2A, traces iv–vi). LC-MS revealed the products with t_R = 26.1 and 22.6 min were **6** and

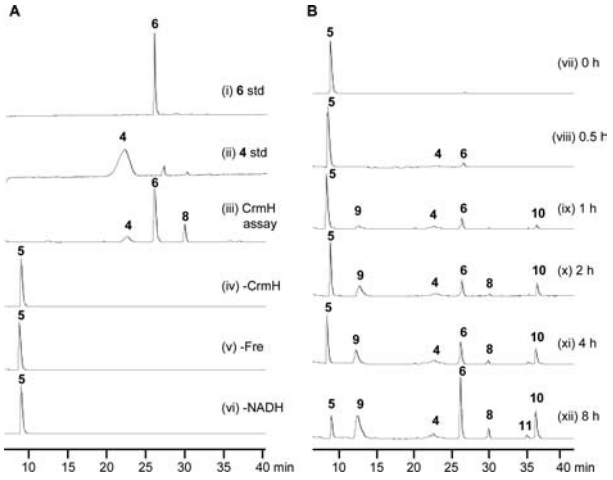

Figure 2. HPLC analysis of CrmH enzyme assays. (A) Reconstitution of CrmH in vitro activity. Standard **6** (i); standard **4** (ii); a complete CrmH assay containing **5**, CrmH, Fre, FAD, and NADH (iii); assay in (iii) minus CrmH (iv); assay in (iii) minus Fre (v); assay in (iii) minus NADH (vi). (B) A time course of a CrmH assay in (iii) minus FAD for 0 h (vii), 0.5 h (viii), 1 h (ix), 2 h (x), 4 h (xi), and 8 h (xii). A general CrmH assay was performed in Tris-HCl buffer (50 mM, pH 8.0) at 28 °C for 8 h, using 400 μM **5**, 5 μM CrmH, 2 μM Fre, 0.1 mM FAD, and 2 mM NADH.

CRM M **4** (Scheme 1), respectively, on the basis of the same retention times and molecular masses (Figure S7) as standards **6** and **4** (Figure 2A, traces i and ii). The standard **4** was obtained through chemical degradation of **6**[7b] and was confirmed by ^{1}H NMR analysis (Figure S8). The formula of the product **8** (t_R = 29.8 min) was determined as $C_{11}H_9N_3O_2$ (*m*/*z* 216.0769 $[M + H]^+$, calcd 216.0773) (Figure S7). Compound **8** was unstable and was transformed into **6** and **4** upon purifying attempts (Figure S9). Thus, we assumed that **8** (Scheme 2) was a *Z*-configured isomer of **6**. In the CYP79A1-catalyzed oxime formation during the plant biosynthesis of dhurrin, both *E*- and *Z*-oxime products were detected.[11]

Given that purified Fre contains noncovalently bound FAD (Figure S10), we assayed the CrmH activity in the absence of flavin cofactors. Surprisingly, a CrmH time course assay comprising **5**, Fre, and NADH allowed the detection of three more products **9**–**11**, along with **6**, **4**, and **8** (Figure 2B). The yields of **9** and **10** increased with longer incubation times, and compound **11** was only detectable in a tiny amount (Figure 2B). The product **9** was determined to be *N*-hydroxy-**5** (Scheme 2), on the basis of the same UV spectra of **9** and **5**, and the chemical formula $C_{11}H_{11}N_3O_2$ of **9** that was derived from HRESIMS (*m*/*z* 218.0925 $[M + H]^+$, calcd 218.0930, Figure S7). In attempts to purify **9** for NMR characterization, **9** was found to be unstable and was spontaneously turned into multiple products under vacuum (**4**, **6**, and **10**), or upon freeze-drying (**4**, **6**, **8**, and **10**), or by incubation at room temperature (**4** and **6**) (Figure S11). Similar to our observation, the true enzymatic intermediate *p*-hydroxylaminobenzoate from AurF-catalyzed nitro formation in aureothin biosynthesis[12] could spontaneously become a nitroso species in the absence of AurF, putatively arising from nonenzymatic oxidation.[12c] Recently, a hydroxylamine intermediate in the MbaC reaction was also shown to be readily transformed nonenzymatically into nitroso- and nitro-containing products in malleobactin biosynthesis.[13] The products **10** and **11** displayed the same chemical formula of $C_{22}H_{17}N_5O_3$ on the basis of LC-HRESIMS (Figure S7). The

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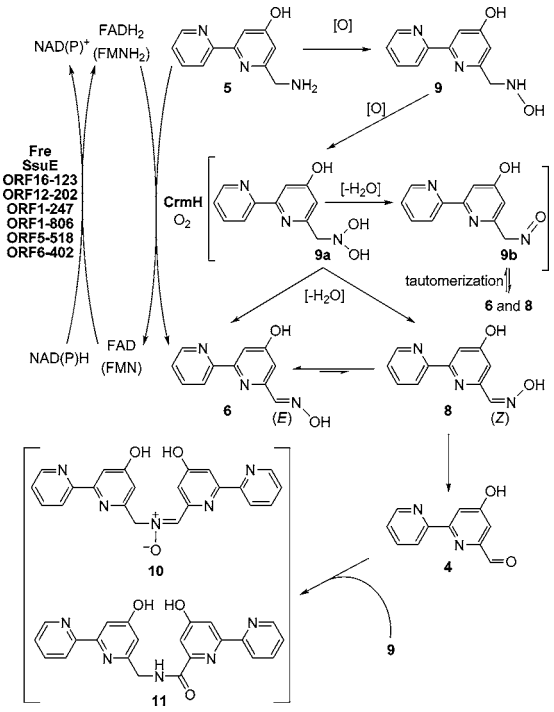
Scheme 2. A Proposed CrmH Catalytic Cycle[a]



[a]Structures of compounds **10** and **11** were not yet determined and were deduced from LC-MS/MS analysis (Figure S12).

UV spectrum of **10** was slightly different from the almost identical UV spectra of **4** and **11** (Figure S6). The product **10** underwent quick and spontaneous decomposition into **4** and **9**, while **11** was rather stable (Figure S9). Given the labile degradation of **10** into **4** and **9**, and the tiny amount of compound **11**, the elucidation of their exact structures was impractical. On the basis of MS/MS fragmentation analysis (Figure S12), structures of **10** and **11** were tentatively assigned as shown in Scheme 2. Both **10** and **11** were likely derived from the condensation of **4** and **9** (Figure S12).

Many flavin-dependent two-component monooxygenases have been identified in natural product biosynthetic pathways,[14] using reduced flavins provided either by a heterologous[15] or a pathway-specific flavin reductase.[16] The genes encoding a flavin reductase and a monooxygenase in the flavin-dependent two-component enzyme system are usually clustered in the same operon.[14] However, no reductase partner for CrmH was found in the **1** biosynthetic gene cluster.[7a] Bioinformatic analysis of the draft genome sequence of *A. cyanogriseus* WH1-2216-6 (data not shown) retrieved eight genes that were annotated to encode NAD(P)H-dependent flavin reductase family proteins, six of which were overproduced and purified from *E. coli* (Figure S13). The yellow color of purified ORF12-202 and ORF16-123 proteins indicated the presence of flavin cofactors. Upon boiling, ORF12-202 released FAD and a minor amount of FMN, while ORF16-123 released only FMN (Figure S13). The other four proteins, ORF1-247, ORF1-806, ORF5-518, and ORF6-402, were nearly colorless (Figure S13). These six flavin reductases, together with another *E. coli* flavin reductase SsuE,[17] were assayed for their ability to provide reduced flavins for CrmH catalytic activity. HPLC analysis revealed that CrmH could utilize both $FADH_2$ and $FMNH_2$ for its amine oxidation activity (Figure S14). The flavin reductases Fre, SsuE, ORF1-247, ORF1-806, ORF5-518, and ORF6-402 could reduce both FAD and FMN (Figure S14). Intriguingly, ORF16-123 could only reduce FAD, even though FMN was detected to be bound to ORF16-123. As expected, no exogenously provided flavins (FAD or FMN) were required for ORF12-202 in assisting the CrmH catalysis, given that it bound with both FAD and FMN. When NADH was replaced by NADPH in the CrmH assays, we also observed the efficient conversion of **5** to **6** in combination with either of the eight flavin reductases to provide reduced flavins (Figure S15). These data are consistent with the CrmH catalytic cycle shown in Scheme 2 and support the prior findings that the monooxygenase does not form a complex with the reductase and that the reduced flavins for oxygenase activity are diffused from the reductase.[16] Given the nonenzymatic conversion rate of **9** to other products including **6** was slow (Figure S11), we hypothesize that **9** is an intermediate of CrmH catalysis and undergoes a second CrmH-catalyzed *N*-hydroxylation to form **9a**, which was spontaneously dehydrated to directly form the enantiomer pair of **8** and **6** (Scheme 2). Alternatively, the dehydration of **9a** would lead to a nitroso compound **9b**, which was tautomerized into **6** and **8** (Scheme 2). The *Z*-configured **8** was easily transformed into **6** and **4**. The condensation of **4** and **9** would lead to the formation of **10** and **11** (Scheme 2). Similar dimerized byproducts were also identified in the *N*-oxygenases AurF- and MbaC-catalyzed reactions, with proposals of dimers being derived from a nonenzymatic coupling of a nitroso species and a hydroxylamine intermediate.[12a,13]

Although sequence alignments revealed low identities of CrmH with other two-component monooxygenases (Figure S16), a CrmH homology structure model (Figure 3A) was constructed on the basis of crystal structures of three oxidoreductases KijD3 (3M9V),[18] *p*-hydroxyphenylacetate hydroxylase (2JBT),[19] and 2VIG. According to this model, four residues (M95, S130, F251, and H377) were predicted to be in the substrate binding pocket and might be critical for the binding of **5**, and two residues (G138 and S165) were important for flavin binding. Subsequent site-directed mutagenesis generated six soluble CrmH mutants M95A, S130A, G138C, S165V, F251A, and H377A (Figure S3). Biochemical assays revealed that the M95A, F251A, S165V, and H377A mutants completely lost the activity for oxidizing **5** (Figure 3B, traces i–iv), while the S130A mutant retained only a little activity of converting **5** to **6** (Figure 3B, trace v). In contrast, the G138C mutant displayed catalytic activity comparable to the wild type CrmH (Figure 3B, traces vi and vii). The complete loss of activities in the mutants M95A, F251A, and H377A indicated these three residues are pivotal in lining the substrate binding pocket. It is very likely that H377 contributes to stabilizing the substrate and/or intermediate molecules via a H-bond. S130 may also play a similar role by providing H-bond interactions. In addition, the H-bonding interaction between the hydroxyl group of S165 and the flavin isoalloxazine ring is essential to anchor the flavin molecule in a proper orientation within the CrmH active site as evidenced by the loss of activity in the S165 V mutant.

In summary, we have validated CrmH as a flavin-dependent, two-component monooxygenase catalyzing oxime formation in CRM A (**1**) biosynthesis. Six flavin reductases from *A. cyanogriseus* WH1-2216-6 were identified to be compatible with CrmH catalysis and might serve as new tools to characterize other enzymes requiring flavin reductases. With four residues being confirmed as essential for CrmH catalysis,

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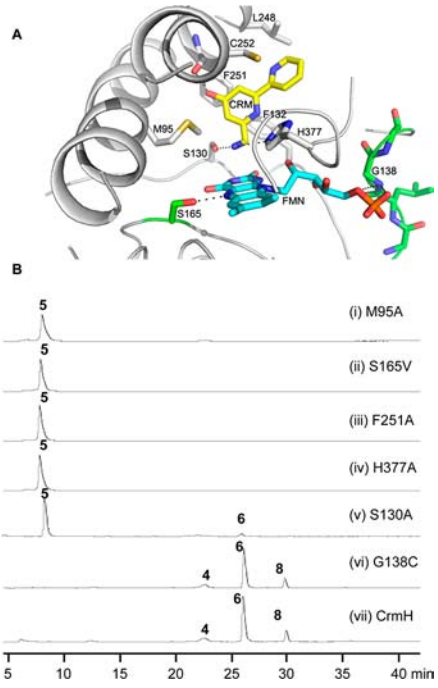

Figure 3. A structure model of the CrmH active site and HPLC analysis of assays with CrmH mutants. (A) The homology structure model was constructed based on three crystal structures (PDB IDs: 3M9V, 2JBT and 2VIG), followed by manual docking of **5** into the substrate binding pocket of the FMN-bound CrmH. (B) Assays were performed in Tris-HCl buffer (50 mM, pH 8.0) containing 400 μM **5**, 0.1 mM FAD, 2 mM NADH, 2 μM Fre with 5 μM M95A (i), S165V (ii), F251A (iii), H377A (iv), S130A (v), G138C (vi), or CrmH (vii) at 28 °C for 8 h.

further structural and mechanistic investigations on CrmH are warranted.

■ 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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Notes

The authors declare no competing financial interest.

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