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Identification of the Biosynthetic Pathway for the Antibiotic Bicyclomycin

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Abstract

Diketopiperazines (DKPs) are a large group of natural products with diverse structures and biological activities. Bicyclomycin is a broad-spectrum DKP antibiotic with unique structure and function: it contains a highly oxidized bicyclic [4.2.2] ring and is the only known selective inhibitor of the bacterial transcription termination factor, Rho. Here, we identify the biosynthetic gene cluster for bicyclomycin containing six iron-dependent oxidases. We demonstrate that the DKP core is made by a tRNA-dependent cyclodipeptide synthase, and hydroxylations on two unactivated sp³ carbons are performed by two mononuclear iron, α -ketoglutarate-dependent hydroxylases. Using bioinformatics, we also identify a homologous gene cluster prevalent in a human pathogen *Pseudomonas aeruginosa*. By observing bicyclomycin production from heterologously expression of the pseudomonas *bcm* gene cluster, we establish *P. aeruginosa* as a new producer of bicyclomycin. Our work uncovers the biosynthetic pathway for bicyclomycin and sheds light on the intriguing oxidation chemistry that converts a simple DKP into a powerful antibiotic.

Graphical Abstract

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Notes

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information contains supplemental methods, figures, and NMR spectra. It is available free of charge on the ACS Publications website.

Diketopiperazines (DKPs) are important scaffolds for synthetic drugs and natural products. DKP natural products are widespread and display diverse structures and biological activities.¹ The structural diversity results from incorporation of various amino acids and numerous modifications to the DKP core,² which yield potent activities such as signaling (cyclo(L-Phe-L-Pro)),³ antimicrobial (albonoursin),⁴ and anticancer (gliotoxin) (Figure 1A).⁵

Bicyclomycin (**1**) is a structurally and functionally unique DKP.⁶ Isolated in 1972 from multiple *Streptomyces*, it contains a DKP bridged by a cyclic ether forming a distinctive [4.2.2] bicyclic fused ring system (Figure 1A).⁷ This bicyclic core is decorated by a terminal olefin and a trihydroxy unit. Bicyclomycin has a unique mode of action: it interferes with bacterial transcription by selectively inhibiting the prokaryotic transcription termination factor, Rho.^{8,9} Because Rho is ubiquitous in many bacteria, bicyclomycin exhibits broad spectrum activity against multidrug-resistant, Gram-negative pathogens such as *Escherichia coli* and *Klebsiella pneumoniae*.⁷ Synthetic efforts have shown that the bicyclic ring and the trihydroxy unit are essential for antibiotic activity, while the terminal olefin can be modified to moderately increase potency.¹⁰ The pharmacological profile of bicyclomycin has led to its use in veterinary medicine and clinical investigations for its use to treat gastrointestinal tract infections.^{11,12}

Despite the extensive synthetic and mode of action studies of bicyclomycin spanning five decades, very little is known about its biosynthesis. Early feeding studies indicated that leucine and isoleucine are precursors to the DKP core,¹³ but the enzymes responsible for their incorporation are unknown. Additionally, the DKP core undergoes formal oxidation at eight out of ten sp³ carbons. One oxidation has been proposed to involve a cytochrome P450-type enzyme,¹⁴ but the responsible P450 has yet to be identified. It is also unclear what additional enzymes are involved in these oxidations. Known biosyntheses of DKPs use one of two primary enzyme families: non-ribosomal peptide synthetases (NRPSs) and cyclodipeptide synthases (CDPSs).⁴ NRPSs are large, multimodular megasynthases that activate free amino acids,¹⁵ whereas CDPSs condense activated amino acids in the form of aminoacyl-tRNAs.^{16,17} We set out to identify which family of DKP-forming enzymes are required and what tailoring enzymes are responsible for the highly oxidized structure of bicyclomycin.

We used whole genome sequencing and bioinformatics to identify the bicyclomycin biosynthetic gene cluster. A reported bicyclomycin producer, *Streptomyces cinnamoneus* ATCC 21532 (a type strain for *S. saproonensis*) was obtained, and its ability to produce bicyclomycin was confirmed by liquid chromatography coupled-high resolution mass spectrometry (LC-HRMS) analysis. The retention time of bicyclomycin from culture extracts was identical to a synthetic standard (Figure S1). The sequence of this strain was determined by PacBio sequencing and assembled to 10 large contigs (accession No. NHZO00000000). We mined the genome for NRPSs and CDPSs that could catalyze DKP formation and identified a single, putative CDPS homolog using known CDPSs as queries. The identified CDPS gene resides just upstream of six putative oxidase genes: five non-heme mononuclear iron, α -ketoglutarate (α -KG)-dependent oxidases (Fe/ α -KG oxidases) and a cytochrome P450 oxidase (Figure 1B, Table S1). On the opposite strand, an eighth gene encodes a putative permease, which is homologous to multidrug resistance proteins of the major facilitator superfamily.¹⁸ We name this 9 kb candidate gene cluster *bcm*.

We evaluated the gene cluster assignment using a combination of biochemistry and bioinformatics. First, the permease *bcmH* was overexpressed in an *E. coli* strain sensitive to bicyclomycin. Overexpression increased the minimal inhibitory concentration of bicyclomycin against *E. coli* from 30 μ g/mL to >500 μ g/mL, suggesting that *bcmH* plays a role in self-resistance to bicyclomycin (Figure S2). Second, phylogenetic analysis of *bcmA* with the known CDPSs showed that *bcmA* is most related to a CDPS from *Pseudomonas aeruginosa* (48% sequence identity, 65% similarity, Figure S3), suggesting these two genes perform similar function. Interestingly, the *P. aeruginosa* CDPS has been shown to synthesize cyclo(L-Ile-L-Leu) (cIL, **2**, Figure 2A),¹⁹ supporting the role of BcmA in synthesizing the cIL precursor to bicyclomycin. Third, conversion of **2** to bicyclomycin entails seven formal oxidations, which can be catalyzed by the six oxidases encoded in the *bcm* pathway, BcmB–G (Figure 1B). Taken together these data support the gene cluster assignment, and we propose that CDPS BcmA generates the DKP **2**, and BcmB–G oxidize **2** to produce bicyclomycin.

To validate our hypothesis and gain further insight into bicyclomycin biosynthesis, we tested whether BcmA synthesizes DKP **2** (Figure 2A). The gene *bcmA* was expressed in *E. coli* and LC-HRMS analysis of the supernatant revealed a species with identical mass and retention time to the synthetic standard of **2** with L,L configuration (Figure 2B). A minimal amount of **2**, near the detection limit of LC-HRMS, was observed in controls carrying the empty vector; this is presumably a protein degradation by-product. Expression of *bcmA* in a 1 L culture of *E. coli* expressing BcmA enabled purification of **2** and structural confirmation by NMR (Figures S9 and S10). The heterologously expressed **2** has a similar specific rotation to that of the synthetic **2**, suggesting that it also has the L, L configuration (Supporting Information). Interestingly, we also identified a second cyclodipeptide from *E. coli* expressing *bcmA*, and characterized the structure as cyclo(Val-Leu) (cVL) (Figures S4A, S11, and S12). This result suggests that BcmA can use both *E. coli* Ile-tRNA and Val-tRNA as substrates, consistent with the reported promiscuity of other CDPSs.¹⁹

To further confirm the function of BcmA, we purified recombinant BcmA and reconstituted its activity *in vitro* (Figure 2C, Figure S4B). Incubation of BcmA with *E. coli* lysates

generated **2** and cVL, but pretreatment of the lysate with RNase A abolished production (Figure 2C, Figure S4B). When RNasin was subsequently added to inhibit RNase activity and fresh tRNAs were introduced, production of **2** and cVL was restored (Figure 2C, Figure S4B). These results show that BcmA is an aminoacyl-tRNA dependent CDPS and likely catalyzes the first step in bicyclomycin biosynthesis.

Converting **2** to bicyclomycin requires oxidation of several sp³ carbons. Such reactions generally require metalloenzymes with high-valent metal-oxo intermediates.^{20–22} The *bcm* gene cluster harbors six iron-dependent oxidases, which could carry out this chemistry. We used synthetic **2** with L,L configuration for *in vitro* reconstitutions of iron-dependent enzymes. We found that BcmC converts **2** to hydroxylated cIL (**3**) in the presence of α-KG, Fe(II), and ascorbate (Figure 3A and B). Activity was abolished when α-KG was omitted or boiled BcmC was used (Figure 3A&B, Figure S6). We enzymatically synthesized and isolated milligram quantities of pure **3** for 1D (¹H, ¹³C, ¹³C APT) and 2D (¹H-¹H COSY, and HSQC) NMR analysis. The C₈ proton signal is no longer present in the ¹H NMR spectrum of **3**, and the doublets for the diastereotopic methyl groups (C₉ and C₁₀) in **2** appear as distinct singlets in **3** (Figures S15–S19). These changes indicate that **3** corresponds to 8-hydroxy-cIL (Figure 3A). We conclude that BcmC catalyzes C₈ hydroxylation in bicyclomycin biosynthesis.

We found that BcmG modifies **3**, quantitatively hydroxylating **3** in the presence of α-KG, Fe(II), and ascorbate to the dihydroxylated product, **4** (Figure 3C). Several milligrams of compound **4** was isolated from enzymatic reactions for ¹H, ¹H-¹H COSY, ¹³C, HSQC, and HMBC NMR analysis in *d*₆-DMSO (Figures S20–S24). ¹H NMR depicts one methyl singlet signal was lost from the leucine. Two doublets of doublets appeared at 3.19 and 3.23 ppm, corresponding to a hydroxylation on C₉. This result, in conjunction with other NMR analysis indicates that **4** is 8,9-dihydroxy-cIL (Figure 3C, Figures S20–24).

Using LC-HRMS, we observed that BcmG completely converted **3** to **4** in 10 minutes (Figure 3D). Surprisingly, longer incubation times (20–40 minutes) led to the disappearance of **4** and the formation of two new products **5a** and **5b** (Figure 3C&D). The masses for **5a** and **5b** are two Da less than compound **4**. The ¹H NMR spectra for **5a** and **5b** are similar with both showing only one proton on C₉ and a single amide proton at N₄ (Figures S25, S31, and S33). These observations are consistent with the formation of a C-N bond from C₉ to N₁ of **4** to produce diastereomers **5a** and **5b** containing an unusual γ,δ-dihydroxy-γ-methyl-proline. Further NMR analyses of **5a** (¹H-¹H COSY, ¹³C, HSQC, HMBC, and NOESY) support these structural assignments (Figures S26–S37). Stereochemistry assignments are inconclusive at C₈ of **5a** and **5b** and by extension **4**, due to the lack of NOE signals between the C₆-hydrogen and C₈-substituents (Figures S30 and S37).

We propose that prolonged incubation of **4** with BcmG led to further oxidization at C₉ to produce an α-hydroxy aldehyde intermediate, which undergoes addition by amide N₁ to form **5a** and **5b** (Figure 3C). In support of this proposal, BcmG is required for conversion of **4** to **5a** and **5b** (Figure S7). This mechanism is consistent with the formation of δ-hydroxy-proline postulated in the biosynthesis of thiopeptide GE37468.²³ Because neither **5a** or **5b**

were detected in the culture extract of *S. cinnamomeus*, these compounds are likely *in vitro* overoxidation products by BcmG, instead of *in vivo* off-pathway products.

Based on these results, we propose the biosynthetic pathway for bicyclomycin depicted in Figure 4A. In the first step, BcmA constructs the cIL backbone from charged leucyl- and isoleucyl-tRNAs. BcmC and BcmG subsequently modify the leucine half of cIL, catalyzing hydroxylations at C₈ and C₉, respectively. BcmG can catalyze a second oxidation at C₉, generating an unusual shunt product that contains a unique nonproteinogenic amino acid γ,δ -dihydroxy- γ -methyl-proline. Oxidases BcmB, D, E and F are likely responsible for the remaining transformations needed to generate bicyclomycin: three additional oxygenations, cyclic ether formation, and desaturation. The precise order of these modifications will require further characterization.

With the *bcm* cluster in hand, we queried available sequenced genomes for bacteria harboring similar clusters, and identified homologous gene clusters from *Streptomyces kanamyceticus*, *Mycobacterium chelonae*, and hundreds of *Pseudomonas aeruginosa* strains (Figure 4B). *P. aeruginosa* is an opportunistic human pathogen and a leading cause for hospital-acquired infections, especially among cystic fibrosis patients.²⁴ Its ability to produce bicyclomycin or analogs was previously unknown. To gain further insights into the distribution of the *bcm*-like cluster in the genus *Pseudomonas*, we examined 2560 genomes in the *Pseudomonas* Genome Database. We found that 449 genomes harbor *bcm*-like clusters, among which 445 are *P. aeruginosa*, accounting for 27.2% of all *P. aeruginosa* genomes in the database.²⁵ Cloning and overexpression of this gene cluster from *P. aeruginosa* ATCC 14886 resulted in high-level production of a molecule with mass, retention time, and fragmentation pattern identical to bicyclomycin, suggesting that the homologous cluster in *P. aeruginosa* is sufficient for producing this antibiotic (Figure 4C, Figure S7). These results demonstrate that the *bcm*-like cluster is widely distributed in this bacterial pathogen and suggest that bicyclomycin production is important for the physiology of the pathogen.

In summary, we have identified the gene cluster for bicyclomycin and found that bicyclomycin belongs to the growing class of CDPS-dependent DKP natural products. We uncovered six new DKP-modifying enzymes, including five Fe/ α -KG oxidases and a cytochrome P450 oxidase. We functionally characterized two Fe/ α -KG oxidases, which catalyze regioselective hydroxylations to create part of the trihydroxy unit in bicyclomycin. We have also demonstrated that a human pathogen of significant health relevance can produce bicyclomycin. Our findings shed light on the biosynthetic logic for a unique and useful antibiotic. Functional studies of the remaining oxidases and the order of these modifications will be the focus of future studies. Of particular interest are the desaturation and cyclic ether ring formation. Iron-dependent oxidases are powerful and versatile catalysts for activation of C-H bonds, which are increasingly explored for production of chemicals and catalysis of non-natural reactions.^{26,27} Understanding how the *bcm* oxidases distinguish closely related substrates and catalyze very different oxidation reactions will likely yield important insights into the mechanisms and substrate selectivity of these enzymes, and inform efforts in engineered biosynthesis of natural products and development of biocatalysts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Structure of representative diketopiperazines (DKPs). (B) Identification of the biosynthetic gene cluster *bcm*.

Figure 2.

BcmA is a tRNA-dependent cyclodipeptide synthase. (A) Reaction catalyzed by BcmA. (B) Extracted ion chromatogram (EIC) of **2** (m/z 227.1754 $[M + H]^+$) in the supernatant of *E. coli* (i) overexpressing *BcmA*, (ii) harboring empty vector, and (iii) synthetic standard of **2**. (C) *in vitro* reconstitution of BcmA activity using *E. coli* cell lysate. EICs of **2** was shown. *E. coli* cell lysate was (i) incubated with BcmA; (ii) incubated with RNase A first, then with RNasin, followed by the addition of yeast tRNA mix and BcmA. Negative controls include (iii) omission of BcmA, (iv) omission of RNasin, and (v) cell lysate alone.

Figure 3.

In vitro reconstitution of BcmC and BcmG activity. (A) Reaction catalyzed by BcmC. (B) HPLC analysis of time-point assays of BcmC converting **2** to 8-hydroxy-cIL (**3**). (C) Reactions catalyzed by BcmG. (D) LC-HRMS analysis of the BcmG reaction. EICs of substrate **3** (m/z , 243.1703 $[M + H]^+$, left), product **4** (m/z , 259.1652 $[M + H]^+$, middle), and shunt products **5a** and **5b** (m/z , 257.1496 $[M + H]^+$, right).

Figure 4.

Proposed biosynthesis of bicyclomycin and prevalence of *bcm* cluster in other bacteria. (A) Proposed biosynthesis of bicyclomycin. (B) *bcm* homologous gene clusters identified by genome mining. (C) The *bcm*-like gene cluster in *P. aeruginosa* can produce bicyclomycin. EICs of bicyclomycin (m/z , 285.1081, $[M+H-H_2O]^+$) from (i) *P. aeruginosa* ATCC 14886 harboring empty vector, (ii) *P. aeruginosa* ATCC 14886 overexpressing the *bcm*-like cluster, and (iii) a bicyclomycin standard.