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Dissecting modular synthases through inhibition: A complementary chemical and genetic approach

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ABSTRACT

Modular synthases, such as fatty acid, polyketide, and non-ribosomal peptide synthases (NRPSs), are sophisticated machineries essential in both primary and secondary metabolism. Various techniques have been developed to understand their genetic background and enzymatic abilities. However, uncovering the actual biosynthetic pathways remains challenging. Herein, we demonstrate a pipeline to study an assembly line synthase by interrogating the enzymatic function of each individual enzymatic domain of BpsA, a NRPS that produces the blue 3,3'-bipyridyl pigment indigoidine. Specific inhibitors for each biosynthetic domain of BpsA were obtained or synthesized, and the enzymatic performance of BpsA upon addition of each inhibitor was monitored by pigment development in vitro and in living bacteria. The results were verified using genetic mutants to inactivate each domain. Finally, the results complemented the currently proposed biosynthetic pathway of BpsA.

Modular synthases make up the core biosynthetic machinery that produce essential small molecule metabolites such as fatty acids, polyketides, and non-ribosomal peptides. Besides their involvement in fatty acid anabolism throughout all kingdoms of life, they are responsible for the production of bioactive compounds crucial for cell survival in bacteria, fungi, and plants. These molecules often possess antibiotic, immunosuppressive, cytostatic, and cytotoxic activities and have gained considerable attention in modern medicine and agriculture.1-3

Biosynthesis by modular synthases resembles an assembly-line strategy unique among metabolic pathways. Each synthase consists of individual domains possessing distinctive functions, such as the recognition of the substrate (amino acid, acyl-coenzyme A (acyl-CoA), or acyl-acyl carrier protein (acyl-ACP) species), propagation of the growing chain, tailoring and condensation of intermediates, and finally, release of the product.2,4 For non-ribosomal peptide synthetases (NRPSs), the basic components include adenylation (A), peptidyl carrier protein (PCP), condensation (C) and thioesterase (TE) domains.5,6 Due to their size and complexity, the study of modular synthases remains challenging, including mechanism, structure, and sometimes even identification of the final product. With this report, we aim to demonstrate the dissection of a modular NRPS to probe the activity of each domain, as well as the overall activity of the ensemble. Here, we provide a case study of the blue pigment synthetase A (BpsA), the bacterial source of the blue pigment indigoidine (Fig. 1).

Although the blue pigment indigoidine was first observed in 1890,7 the indigoidine synthase was only discovered in 2002 as IndC in the plant pathogen Dickeya daccartii.8 IndC is conserved in many organisms, and is annotated as IndC, BpsA, IgiD, or as putative synthase (Figs. S1 and S2).9 IndC is a single protein multi-domain NRPS module, that utilizes two molecules of L-glutamine as building blocks to produce the indigoidine product.8 In 2007, a homologous synthase was identified and characterized in Streptomyces lavendulae, called blue pigment synthase A (BpsA). The biological role of these blue pigment synthases remains speculative, even so many years after their discovery. They appear to be present in many different organisms, ranging from bacteria to eukaryotes, and their genes are often cryptic, such as in the bacterial insect pathogen Photobacterium luminescens (Figs. S1 and S2).10

In vitro experiments showed that BpsA can only accept L-Gln over all proteinogenic amino acids, and that the synthase oxidizes and dimerizes the amino acid.11 BpsA is a single module NRPS, containing an oxidation (Ox) domain integrated into the A domain, a peptidyl carrier protein (PCP), and a terminal thioesterase (TE) domain (Fig. 2). The Ox domain harbors a flavin cofactor, as shown by UV absorbance...
measurements, and purified protein has the characteristic yellow coloration of flavoproteins. Like all modular synthases, BpsA requires post-translational modification by a 4′-phosphopantetheinyl transferase (PPTase). The PPTase transfers the 4′-phosphopantetheine moiety of the cofactor CoA onto a conserved serine residue of the PCP, enabling the PCP to tether the natural product via a labile thioester linkage. BpsA produces the blue pigment indigoidine and this enzymatic transformation was utilized for the development of novel reporter systems, blue-white screens in *Streptomyces* species, a glutamine sensor, a potential semiconductor component, and other microbiology applications.

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**Fig. 1.** Pipeline for the biochemical characterization of a modular pigment-producing synthase. After heterologous expression of the synthase gene and purification of the protein, the activity of each individual domain is inhibited by specific small molecules and analyzed in vitro and in vivo by pigment formation. Additionally, mutant proteins of each individual domain are generated and analysed subsequently. Phylogenetic analysis provides further information on the diversity and versatility of the synthase.

**Fig. 2. A)** Genomic organization of BpsA (gene cluster: AB295063, protein: BAE93896) in *Streptomyces lavendulae* subsp. lavendulae including domain-specific inhibitors. **orfA** is a putative ribose-phosphate pyrophosphokinase, **bpsA** codes for indigoidine synthase, **bpsB** for a regulator, and **bpsC** for a putative *S*-adenosylmethionine synthase. **bpsA** = 3,849 bp = 1,282 aa. **B)** 1 is a L-Gln-sulfonylfluoride-containing inhibitor of A-domain, 2 a thio-carbonate inhibitor of flavin-dependent OX-domain, 3 a Gln-pantetheinamide substrate for *in situ* transformation into a CoA analog and subsequent loading onto a carrier protein using a PPTase, 4 a sulfonyl-fluoride-containing inhibitor of proteases and thioesters, and 5 is 6-NOBP, a general PPTase inhibitor.
Recent engineering efforts have led to genetically modified roses with a blue color,22 as well as bioproduction of indigoidine at titers approaching 100 g/L.23 Additionally, within an International Genetically Engineered Machine (iGEM) synthetic biology project, NRPS domain swapping led to production of novel indigoidine-containing peptides.24 The iGEM project also swapped the PCP domain with various other PCPs, resulting in large variability in the amount of product. However, Owen et al.25 had previously co-expressed a PCP replacement of BpsA with various carrier proteins showing no (or severely reduced) indigoidine formation. In contrast to this split system, Beer et al.26 found that many of the PCP replacement mutants do produce indigoidine. Here we tested three BpsA mutants from the Walsh lab that encode for the carrier protein domain of TdiA,26 and observed no activity (Fig. S11).24 It appears that the linker regions between domains (here between Ox-A-PCP and PCP-TE) are crucial for productive catalysis and stable protein expression.27

**Fig. 3.** Inhibition of individual BpsA domains. A) Inhibition of the A domain by 5′-O-[N-(L-phenylalanyl)sulfamoyl] adenosine 1. B) Inhibition of the PPTase Sfp by 6-NOBPS. C) Inhibition of TE domain by PMSF 4 and D) AEBSF. E) Activity of BpsA by varying the ratio of CoA to the non-hydrolysable LGln- pantetheinamide analog 3 during preincubation of BpsA with Sfp and CoaA/D/E.
We expressed and purified flavin-bound apo-BpsA and post-translationally modified the protein using CoA and the promiscuous PPTase Sfp. Holo-BpsA showed robust activity in vitro, and E. coli BL21 cells co-expressing Sfp and BpsA produced indigo dine when grown with liquid or solid media. Supplying the double transformant with L-Gln in the growth media boosts the production of the insoluble blue pigment, and Mn2+ supplementation is superior to Mg2+, the latter presumably involved in PPTase activity (Fig. S3).30

To confirm the bioinformatic prediction of domains, we set out to design and synthesize individual inhibitors for each domain within BpsA. The A-domain is an adenylate-forming enzyme that loads L-Gln onto the PCP.31 BpsA contains an interrupted A-domain, in which the Ox domain separates a large N-terminal from a smaller C-terminal portion of the A-domain (Fig. 2B).31 Adenylate-forming enzymes, including aminoacyl-tRNA synthetases, are inhibited by sulfamoyl-containing analogs (abbreviated as AMS).12,32-39 AMS compounds are effective inhibitors of adenylating enzymes because they closely mimic the short-lived aminoacyl-intermediate formed when the enzyme catalyzes the condensation of an amino acid and AMP (derived from ATP). We synthesized and analyzed 5′-O-(L-glutamyl)-sulfamoyladenosine 1 as an inhibitor of BpsA. Only addition of millimolar amounts of this inhibitor resulted in a substantial decrease in the formation of blue pigment in vitro, with an IC50 of 3.8 mM (Fig. 3A). The high concentrations required suggest a competitive, non-covalent mechanism of inhibition, as previously observed in inhibition of the biosynthesis of mycobactin with salicyl-amino-sulfonamide-adenosine.39 However, why 1 does not inhibit BpsA as efficiently as other sulfonamide inhibitors is unclear. Salicylic acid-sulfonamide (Sal-AMS) inhibits the A-domains MbtA, BasE, EntE and VibE at 9 nM, 90 nM, 0.2 and 0.1 µM, respectively.3334 Luciferase is inhibited by dehydrocucurileryl-AMS at 0.3 µM,35 and acyl-CoA ligase MenE by O-succinylbenzoic acid-AMS at 6 µM.36 Thus, it remains a question why we observe poor inhibition of BpsA by L-Gln-AMS. We hypothesize that due to its polarity, it has low affinity for the A-domain of BpsA and perhaps uptake is poor. Alternatively, interrupted A-domains might bind this class of inhibitor differently compared to classical A-domains.

The second domain of BpsA is a flavin-dependent oxidation domain. The Ox domain is most likely responsible for oxidizing either glutamine tethered to the PCP or a cyclized glutamine intermediate. Takahashi et al. demonstrated by point-mutational analysis of the Ox domain that binding of the cofactor flavin mononucleotide FMN is essential for indigo dine production.11 Other flavin-dependent oxidation domains in NRP s have been studied by the Walsh lab, including EpoB, the oxidation domain involved in epothilone biosynthesis.36 EpoB oxidizes the dihydro heterocyclic thiazolin ring to a heteroaromatic oxidation state, forming methylthiazoloylcarboxy-5-EpoB. The Ox domain of BpsA shares 29% identity with EpoB (AAF62884) (Table S1). Although some NRP Ox-domains have been characterized, few inhibitors of these oxidases are known. Inspired by the work of Walsh and co-workers, we recently synthesized 4H-benzo[d][1,3]oxathin-2-one (BOTO) 2 as Ox-domain inhibitor to investigate flavin-dependent oxidation domains.43 Here, we found that micromolar amounts are sufficient to fully inhibit the enzyme when applied to E. coli expressing Sfp and BpsA (Fig. 4A).

The third domain of BpsA is a PCP, which is not an enzyme but a carrier protein, making traditional enzymatic inhibition impossible. However, our lab has developed a methodology to attach non-hydrolyzable analogs to thioester bound substrates to carrier proteins through the preparation of pantetheine analogs elaborated by CoA biosynthetic enzymes and the promiscuous PPTase Sfp.42 Facile synthesis of L-Grnatheineamide 3 allowed for the labeling of the BpsA PCP with a non-hydrolyzable substrate mimic. This crypto-BpsA showed no activity in vitro (Fig. S4), and in direct competition assay between CoA and L-Grnatantheineamide -CoA, decreased BpsA activity was clearly observed when as the ratio of CoA to L-Grnatantheineamide decreased (Fig. 3E).

In order to tether substrates to NRP s for catalysis, all PCPs require post-translational modification by a PPTase. PPTases have only recently been recognized and investigated as potential anti-microbial targets.43,44 In our work on the discovery of inhibitors for the B. subtilis PPTase, Sfp, we found the general PPTase inhibitor 6-NOBP 5 shows modest inhibition against a variety of PPTases.33,45 Based on these findings Owen et al.14 used 5 as PPTase inhibitor as demonstrated by indigoidine production with BpsA. Indeed, 5 inhibited BpsA with an IC50 of 25 µM. (Fig. 3B and SI Experimental Methods).

The fourth domain of BpsA is the TE, which presumably releases the product (or an intermediate) from the PCP. While the exact mechanism is unknown, release of 5-amino-pyridinedione, followed by spontaneous dimerization of two products, forming either colorless leuco-indigoidine or blue indigoidine, is a proposed mechanism.11,28 Numerous TE inhibitors have been developed over the past decades that rely on the nucleophilicity of the active site serine or cysteine.36,47 To inhibit the TE of BpsA, we used the general protease inhibitor phenylmethylsulfonl fluoride (PMSF) 4 and a derivative of the reactive phenylsulfonyl fluoride moiety, 4-(2-aminoethyl) benzensulfonyl fluoride (AEBSF). Blue pigment formation was abrogated by micromolar concentrations of either inhibitor, suggesting that although its function is speculative, the TE domain is critical for catalysis (Fig. 3C, D). Interestingly, 4 showed ten-fold greater inhibition of blue pigment production than AEBSF, indicating that steric effects or polarity of the substrates that enter the TE is important.

A BpsA mutant lacking the TE domain exhibits no turnover when compared to wild type (Fig. S5A). In an effort to discern the importance of the TE domain, we incubated the BpsA mutant lacking the TE domain with a previously described type II thioesterase, TycF.46 TycF is known to remove substrates from the phophoshapteethene arm of PCPs. However, the TE mutant was unable to produce blue pigment with or without TycF (Fig. S5B), suggesting that the TE domain of BpsA directly participates in the formation of indigoidine, and does not simply hydrolyze the tethered substrate off of the PCP.

A BLAST search of the BpsA TE domain results in hits (24% homology, Table S3) in atromentin synthase49 and TdiA,50 in which TEs are involved in cyclization/dimerization and dimerization of two activated indolepyruvic acid monomers, respectively. Fascinatingly, the human FAS (FASN) thioesterase also appears as a potential hit, with 21% homology, whereas the entire synthase has low identity (Table S4). All four TEs have the conserved His residue (2481FASN), and the level of sequence similarity is further demonstrated in the TE-domain phylogenetic tree (Fig. S6). Constructing phylogenetic trees based on A, PCP, Ox and TE domains (Figs. S7–S9 and Tables S1–S4), reveals that many putative synthases have similar domains, and that homologs of the individual BpsA domains can be found in very different synthases. Combined, this bioinformatic data shows that although organisms and synthases are evolutionarily far removed from each other, individual domains show convergent evolution.

To verify our in vitro results, we characterized a set of BpsA mutants designed with selectively inactivated domains. We expressed and purified a BpsA mutant with a K597E mutation in the Ox-domain, preventing the binding of flavin, leading to a colorless-protein; a BpsA mutant with an excised TE domain; a stand-alone Ox-domain; and BpsA mutants with the TdiA PCP swapped into the synthase. We verified that each of these mutants are inactive. To assess whether two BpsA proteins can act in trans to produce indigoidine, a matrix of inactive mutants of BpsA was made (Figs. S7–S9, Table S10). No significant activity was observed for any of the mutant combinations, indicating that the L-Gln substrates loaded onto the PCP of BpsA are incapable of interacting with catalytic domains on a different monomer of BpsA to produce indigoidine.

After demonstrating systematic inhibition of each domain of BpsA, we set out to evaluate the behavior of these inhibitors in living bacteria. The inhibitor activities were evaluated using an E. coli strain co-expressing BpsA and Sfp, in which BpsA activity can be visualized by blue pigmentation of bacterial colonies on agar plates. We spotted the inhibitors on sterile filter paper discs that were placed on top of freshly
spread *E. coli* cells overexpressing both BpsA and Sfp (Fig. 4). Whereas the A-domain inhibitor 1 shows activity *in vitro*, this molecule does not show any effect in bacteria. It is known that some acyl-sulfonamides cannot efficiently enter cells, and combined with the high measured IC_{50} and high polarity, 1 is a poor *in cellulo* inhibitor. Ox-domain inhibitor 2 shows a marked effect *in cellulo*. As concentration of 2 was decreased, we observe a shift from growth inhibition (close to the center of the disk) to growth without formation of pigment to growth with blue pigment production (Fig. 4A). Pantetheinamide 3 has no effect on the formation of blue pigment when supplemented to *E. coli* overexpressing BpsA/Sfp. PPTase inhibitor 5 shows toxicity, but no clear absence of blue pigmentation, perhaps because 5 is a nonspecific PPTase inhibitor and *E. coli* harbors an essential PPTase that is also inhibited by 6-NOBP. The thioesterase inhibitors 4 and ABESF show severe toxicity to *E. coli* at high concentrations. At lower concentrations, both general inhibitors still exhibited toxicity, but the presence of white colonies could be detected (Fig. 4C, D). Although only two of the inhibitors tested here show promising results *in vivo*, this approach can be a useful addition to our toolbox for the interrogation of synthases.

Taken together, this data shows that using this small toolbox of inhibitors, we can quickly deduce the enzymatic activities of the domains of an NRPS *in vitro*, and these tools can be applied to both known and unknown pathways. From these straightforward experiments, we learned that the interrupted A-domain is functional and active, the Ox-domain is flavin-dependent, the PCP-domain can be loaded with various unnatural cargo *in vitro*, and the TE-domain is essential for blue pigment production and is inhibited by cysteine-targeting inhibitors. We, and others, can now apply these tools towards characterization of the hypothetical enzymatic and/or non-enzymatic route to the final product indigoidine. We are confident that such a systematic approach opens up many new paths to precisely dissect the individual steps of multi-domain synthases.

We have designed a systematic chemical and genetic approach for interrogating the individual activities of each domain of a large natural product synthase. Although current technology facilitates bioinformatic discovery of genes and proteins involved in biosynthesis of secondary metabolites, there is a clear need to verify predictions with experimental data. Selective inhibition of synthase domains will become a key component in the toolbox of the chemical biologist for the functional elucidation of the mechanism of assembly-line synthases.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.126820.

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