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The Methymycin/Pikromycin Biosynthetic Pathway: A Model for Metabolic Diversity in Natural Product Biosynthesis

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Abstract

The methymycin/pikromycin (Pik) macrolide pathway represents a robust metabolic system for analysis of modular polyketide biosynthesis. The enzymes that comprise this biosynthetic pathway display unprecedented substrate flexibility, combining to produce six structurally diverse macrolide antibiotics in *Streptomyces venezuelae*. Thus, it is appealing to consider that the pikromycin biosynthetic enzymes could be leveraged for high throughput production of novel macrolide antibiotics. Accordingly, efforts over the past decade have focused on the detailed investigation of the six-module polyketide synthase, desosamine sugar assembly and glycosyl transfer, and the cytochrome P450 monooxygenase that is responsible for hydroxylation. This review summarizes the advances in understanding of pikromycin biosynthesis that have been gained during the course of these investigations.

Introduction

The ability of microorganisms to rapidly evolve resistance mechanisms towards clinically utilized antibiotic therapies has placed an urgent demand on the discovery and development of novel antibacterial agents¹. The macrolide antibiotics, which are chemically characterized by a glycoside-linked macrolactone ring, remain the most effective natural product anti-infective agents to date and as such continue to be featured in ongoing efforts toward the development of new pharmaceuticals that target drug-resistant pathogens. The success of the macrolide antibiotics is attributed to their ability to bind to the 23S bacterial rRNA, an interaction that blocks bacterial protein synthesis by interfering with the channeling of the nascent peptide product². Within this therapeutic class, the polyketide-derived natural product erythromycin was the first to be introduced into human clinical use, and its chemical structure continues to serve as a template for the generation of novel, semi-synthetic antibacterial agents including the 14-membered ring macrolide clarithromycin and the 15-membered ring macrolide azithromycin (Figure 1). Most recently, the erythromycin scaffold has inspired the ketolide class of antibiotics, of which telithromycin (Figure 1) was the first approved for clinical applications. This class of compounds offers a marked advantage over previous generations of macrolide antibiotics, as it does not induce the macrolide-lincosamide-streptogramin B (MLS_B) family of resistance and has thus proven to be effective at combating previously resistant respiratory pathogens³. Based on this success, the continued pursuit of the discovery

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of structurally diverse ketolides remains an attractive strategy for combating the continuous emergence of multi-drug resistant pathogens.

The structural complexity displayed by the macrolide class of antibiotics in general, and the ketolides in particular, presents substantial challenges for the synthesis of analog libraries of target compounds. For example, telithromycin is generated by a 12-step semi-synthesis starting with the 14-membered ring macrolide erythromycin A⁴. To reduce this synthetic complexity, there is a strong interest to leverage the catalytic power and specificity of biosynthetic enzymes to increase the structural diversity of polyketide natural products, an approach often referred to as “combinatorial biosynthesis”^{5,6}.

The biosynthesis of macrolide polyketides relies on type I modular polyketide synthases (PKSs) to catalyze the step-wise condensation of simple carboxylic acid derivatives. Organizationally, type I PKSs are arranged into modules, wherein each module is comprised of a set of catalytic activities that is responsible for a single elongation of the polyketide chain and the appropriate reductive processing of the β -keto functionality (Figure 2)⁷. A minimal elongation module contains an acyl transferase (AT) domain, an acyl-carrier protein (ACP) domain, and a ketosynthase (KS) domain. The AT domain is responsible for loading the appropriate coenzyme A extender unit, typically malonyl-CoA or methylmalonyl-CoA, onto the phosphopantetheinylated ACP domain. Subsequently, the KS domain decarboxylates and then condenses the ACP-bound extender unit with the growing polyketide chain obtained from the preceding module to yield an ACP-bound β -ketoacyl intermediate. In addition to the three core domains, each elongation module may contain up to three additional domains [ketoreductase (KR), dehydratase (DH), enoyl reductase (ER)] that are responsible for the reductive processing of the β -keto functionality prior to the next extension step. These reductive steps contribute greatly to the overall structural diversity that is observed among polyketide natural products. The presence of a KR domain alone gives rise to a β -hydroxyl functionality, the presence of both a KR and a DH domain generates an alkene, while the combination of KR, DH, and ER results in complete reduction to the alkane. Finally, a thioesterase (TE) domain, typically found at the terminus of the last elongation module, catalyzes the termination of polyketide biosynthesis. The activity of this domain results in the cleavage of the acyl chain from the adjacent ACP and formation of the macrocyclic ring⁸.

Further structural variability is imparted into the nascent macrolactone by the action of post-PKS tailoring enzymes. These enzymes, such as glycosyltransferases, monooxygenases, and methyl transferases, catalyze the regio- and stereospecific glycosylation, hydroxylation, epoxidation, and methylation reactions that give rise to the final macrolide antibiotic. Often, the additional functionalities incorporated by these enzymatic reactions are critical for the observed bioactivity of the resultant macrolide antibiotics.

Pikromycin and the pikromycin PKS: Pathway characterization & metabolic diversity

For more than a decade, research in the Sherman laboratory has been focused on investigating a modular type I PKS system in *Streptomyces venezuelae* known to produce the ketolide antibiotic, pikromycin **1** (Figure 3). While the naturally occurring metabolite is not a clinically useful anti-infective agent, **1** does provide a chemical scaffold that has potential utility as raw material in the semi-synthetic production of novel ketolide compounds. Moreover, exploitation of the biosynthetic enzymes responsible for the synthesis of **1** offers an attractive biosynthetic strategy for generating a diverse library of ketolide antibiotics. To this end, the Sherman laboratory has been engaged in a cross-disciplinary and collaborative research program aimed at developing a detailed understanding of pikromycin biosynthesis with the long-term goal of

manipulating the enzymes involved in pikromycin assembly and tailoring for production of novel macrolide antibiotics.

Although pikromycin **1** was first isolated in 1951⁹, the details of its biosynthesis were not elucidated until almost five decades later, when Xue et al. reported the cloning and subsequent genetic sequence of the pikromycin biosynthetic gene cluster from *Streptomyces venezuelae* ATCC 15439¹⁰. Analysis of the approximately 60 kb of DNA sequence led to the identification of 18 discrete genes that could readily be divided into five separate loci: PKS (*pikA*), desosamine biosynthesis (*des*), cytochrome P450 hydroxylase (*pikC*), a transcriptional activator (*pikD*), and a resistance locus (*pikR*) (Figure 3). As highlighted throughout this review, a majority of work has been directed at investigating and exploiting the biosynthetic enzymes encoded by *pikA*, *des*, and *pikC* for the production of novel ketolide compounds.

The *pikA* gene locus (Figure 3) encodes five polypeptide chains (PikAI-PikAV) that together comprise a type I modular polyketide synthase (Pik PKS) (Figure 4). The first four polypeptides, PikAI-PikAIV, consist of a single loading module and six PKS elongation modules that are responsible for directing macrolactone assembly. Notably, the final two elongation modules are monomodular, as each is contained on a separate polypeptide (PikAIII & PikAIV). A similar modular organization is also observed in the highly related tylosin PKS system¹¹. Finally, PikAV is a type II thioesterase. *In vitro* characterization of this discrete enzyme suggests that it functions to remove aberrantly decarboxylated extender units from PKS ACP domains, thus preventing blockage of the PKS assembly line¹².

Shortly after determination of the complete DNA sequence of the *pikA* locus, genetic disruption studies unambiguously demonstrated that the pikromycin PKS is unique from other known PKS systems due to its capacity to efficiently generate two distinct macrolactone products^{10, 13}. Based on the known co-linear nature of polyketide biosynthesis¹⁴, it is readily apparent that pre-mature termination of polyketide biosynthesis at PikAIII results in the production of the 12-membered ring macrolactone, 10-deoxymethynolide **2**, while continued elongation through PikAIV, followed by TE catalyzed cyclization results in the synthesis of the 14-membered ring macrolactone narbonolide **3** (Figure 4). Remarkably, both macrolactones **2** and **3** are appended at the C5 position with the deoxyamino sugar, desosamine, via the activity of the *desVII*-encoded glycosyltransferase. This glycosylation reaction yields the 12-membered ring macrolide YC-17 **4** and the 14-membered ring macrolide narbomycin **5** (Figure 3). Furthermore, both macrolide compounds **4** and **5** are either singly or doubly hydroxylated by the *pikC*-encoded cytochrome P450 to yield a suite of antibiotic compounds that consists of the 12-membered ring macrolides, methymycin **6**, neomethymycin **7**, and novamethymycin **8**, as well as the 14-membered ring macrolides pikromycin **1**, novapikromycin **9**, and neopikromycin **10** (Figure 3). Together, the unique biosynthetic capabilities of the pikromycin PKS, coupled with the substrate flexibility of the respective tailoring enzymes position the pikromycin biosynthetic system as a powerful tool for the generation of novel macrolide antibiotic compounds.

Desosamine biosynthesis: Implications for sugar pathway engineering

The earliest attempts to leverage the pikromycin biosynthetic system for the production of novel compounds involved manipulating genes that are responsible for biosynthesis of the deoxyamino sugar, desosamine (3-dimethylamino-3,4,6-trideoxyhexose). Due to its involvement in key anchoring interactions with the 23S rRNA subunit, the glycosidic component of macrolide antibiotics is essential to their observed bioactivity². Thus, strategies aimed at altering the chemical structure of glycosidic moieties attached to macrolactone scaffolds would be expected to affect the biological activity of macrolides by altering binding affinities or overcoming resistance mechanisms.

The initial sequencing of the pikromycin biosynthetic pathway identified a cluster of genes (Figure 3) that was predicted to encode all enzymes (DesI-DesVI) required for transformation of the common primary metabolite *D*-glucose-1-phosphate to thymidine diphosphate *D*-desosamine (TDP-desosamine) (Figure 5)¹⁰. In addition, the desosamine biosynthetic gene locus includes a gene, *desVII*, whose product is the glycosyltransferase responsible for subsequent attachment of the aminosugar to the polyketide backbone¹⁰. *DesVIII* encodes an enzyme of unknown function; however, it has recently been shown that the *desVIII* gene product is absolutely required for DesVII activity *in vitro*¹⁵. Finally, a ninth open reading frame, *desR*, encodes a putative β -glucosidase¹⁰. Interestingly, deletion of the *desR* gene in *S. venezuelae* led to accumulation of macrolides that contain a desosaminyl-glucosyl dissaccharide moiety¹⁶. Based on this, it has been proposed that β -glucosylation of the 2'-desosaminyl hydroxyl moiety serves as part of the self-resistance mechanism employed by *S. venezuelae*. The presumed activity of DesR is expected to reactivate the antibiotic during or after cellular secretion by removal of the glucose moiety¹⁶. A similar mechanism of self-protection has been implicated in the production of oleandomycin by *S. antibioticus*¹⁷.

Initial evidence of the role of DesVII in glycosylation of the Pik PKS derived macrolactone products was established via the generation of a *desVII* deletion strain of *S. venezuelae*¹⁰. Removal of *desVII* effectively halted production of all macrolides known to be produced by the pikromycin biosynthetic system; however, accumulation of macrolactones **2** and **3** was observed, strongly implicating involvement of the *desVII*-encoded glycosyltransferase in appending desosamine to the macrolactone core. This result offered the first insight into the acceptor substrate specificity of this desosaminyl transferase, as it clearly demonstrated that DesVII is responsible for glycosylating both macrolactones generated by the pikromycin PKS. This finding also correlates well with the fact that a single set of desosamine biosynthetic genes, and in particular a single glycosyltransferase, is encoded within the pikromycin biosynthetic pathway (Figure 3).

The putative roles of the enzymes involved in the assembly of TDP-desosamine were initially assigned based on amino acid homology. A series of gene disruption experiments was performed to confirm these assignments and to illuminate the precise sequence of biosynthetic events that give rise to production of the nucleotidyl-amino sugar (Figure 5). Interestingly, several of these experiments resulted in *S. venezuelae* mutants that produced novel macrolide compounds containing desosamine analogs, thus highlighting the flexibility of the desosaminyl transferase to recognize and attach unnatural sugars onto the polyketide scaffold. For example, deletion of the predicted *N,N*-dimethyltransferase encoding gene, *desVI*, resulted in a 12-membered ring macrolide appended with a 3-*N*-acetylated desosamine analog¹⁸, while deletion of *desV* yielded macrolide compounds containing 4,6-dideoxyhexose¹⁹. It is interesting to note that although each of these genetic deletion experiments produced novel macrolides, the appended sugar groups were not always the structurally predicted desosamine analogs. For example, *N*-acetylation of the deoxyamino sugar produced by the Δ *desVI* mutant was unforeseen and, given that the resultant macrolide lacked anti-bacterial activity, has been hypothesized to serve a role in host cell self-resistance¹⁸. Likewise, in the Δ *desV* mutant, an unanticipated reduction of the C3-keto group, presumably by an extra-pathway reductase, results in the observed 4,6-dideoxy sugar attached to the 12-membered ring macrolactone¹⁹. Although the exact mechanism remains unclear, this reductive activity has been exploited in a Δ *desI* mutant to successfully replace the desosaminyl moiety with *D*-quinovose, offering yet another example of substrate flexibility of DesVII²⁰.

The successful replacement of desosamine with quinovose prompted Liu and co-workers to further engineer the desosamine biosynthetic pathway for synthesis and incorporation of unnatural sugars onto the Pik PKS generated macrolactones. Accordingly, a hybrid glycosylation pathway was constructed whereby the 4-aminotransferase gene from the

calicheamicin biosynthetic pathway²¹, *calH*, was introduced into the Δ *desI* mutant strain²². As expected, the CalH gene product was able to convert the accumulated TDP-4-keto-desosamine intermediate into the corresponding TDP-4-amino-hexose derivative. Indeed, novel 12- and 14-membered ring macrolide compounds were identified in fermentation broth extracts from the genetically engineered *S. venezuelae* strain, each possessing the unnatural aminodeoxy-hexose; however, similar to what was observed in the Δ *desVI* mutant, the incorporated 4'-amino functionality was modified by acetylation. Furthermore, neither of the novel 12- or 14-membered ring macrolides demonstrated antibacterial activity in a disk diffusion assay, again implying that the acetyl modification may be involved in host self-protection.

Within the desosamine biosynthetic pathway, the DesVII glycosyltransferase has clearly demonstrated extraordinary substrate flexibility with its capacity to accept various unnatural sugar substrates produced in the *S. venezuelae des*-deletion strains. In addition, this glycosyltransferase is also unique in its inherent ability to accept different sized macrolactone acceptor substrates, as it is responsible for glycosylating both macrolactones **2** and **3**. Interestingly, this macrolactone flexibility does not appear constrained to these two naturally occurring examples. In an impressive *in vivo* study, Tang and McDaniel demonstrated that the desosaminyl transferase, DesVII, was able to recognize and glycosylate over 20 structural analogs of the 14-membered ring macrolactone, 6-deoxyerythronolide B (6-DEB)²³. Furthermore, Yoon et al. observed the *in vivo* DesVII mediated glycosylation of novel macrolactone compounds produced by hybrid modular polyketide synthases that were constructed from the Pik, DEBS, and tylosin PKSs²⁴.

Although the feasibility of producing unnatural macrolides via sugar pathway engineering has certainly been demonstrated *in vivo*, several issues with this production method have motivated development of an *in vitro* system for accessing novel compounds. For example, product yields from fermentation are generally low. Moreover, the large-scale production of macrolide antibiotics within bacterial hosts can be limited due the effect of the accumulated compound (s) on cell growth. To overcome these limitations, Liu and co-workers have cloned and heterologously expressed DesVII in an *E. coli* host in order to investigate the possibility of performing macrolactone glycosylation *in vitro*¹⁵. Surprisingly, initial glycosylation attempts with the purified enzyme were unsuccessful; however, DesVII, in the presence of DesVIII, efficiently converted 10-deoxymethynolide **2** into the corresponding macrolide, YC-17 **4**. Although the exact role of DesVIII remains unknown, this *in vitro* study demonstrated that it is essential for DesVII-mediated glycosylation. In a subsequent investigation, Borisova et al. leveraged the combined catalytic power of DesVII and DesVIII for *in vitro* production of novel macrolides²⁵. Results from this work further established the potential of an *in vitro* glycosylation technology to produce novel macrolide antibiotics. This study also provided enhanced details regarding DesVII substrate specificity. Specifically, the data reconfirmed that DesVII has a stringent requirement for a 6-deoxyhexose substrate. However, with few exceptions, broad structural variation within the 6-deoxyhexose moiety as well as the macrolactone acceptor, including 16-membered rings, is well tolerated. In addition, Kao et al. have shown that the DesVII/DesVIII catalyzed glycosylation is not restricted to macrolactone acceptors, as this pair of enzymes can also glycosylate linear polyketide intermediates *in vitro*²⁶. In sum, the remarkable substrate flexibility of the desosaminyl transferase from *S. venezuelae* appears to be well suited for use in future metabolic engineering strategies aimed at the production of novel macrolide compounds.

Understanding hydroxylation in pikromycin biosynthesis: Investigations of PikC

Within macrolide antibiotic biosynthetic pathways, cytochrome P450 monooxygenases are found to catalyze the regio- and stereospecific installation of hydroxyl and epoxide substituents that commonly decorate macrolide natural products. In addition to further enhancing the biological activities of the macrolide antibiotics, these functionalities serve to increase the chemical diversity of the polyketide natural products and also to provide reactive sites that are amenable for additional chemical modification. Thus, monooxygenases with broad substrate specificities would be useful for facilitating the creation of novel macrolide analogs. As such, the *pikC*-encoded cytochrome P450 hydroxylase (PikC) (Figure 3) functions as yet another tool for the generation of structurally diverse macrolide antibiotics ¹⁰.

PikC, the sole P450 hydroxylase present in the pikromycin gene cluster, displays unparalleled flexibility towards the macrolactone core of its macrolide substrates. This P450 catalyzes hydroxylation at C10 of the 12-membered ring macrolide YC-17 **4** to yield methymycin **6** or the C12 position to yield neomethymycin **7** ²⁷. Hydroxylation at both the C10 and C12 positions yields the doubly hydroxylated macrolide novamethymycin **8** ²⁸. With respect to 14-membered ring macrolide substrates, PikC is responsible for C12 hydroxylation of narbomycin **5** to give rise to the macrolide antibiotic pikromycin **1** ²⁷. Recently, Lee et al. have identified two additional 14-membered ring macrolide antibiotics, neopikromycin **9** and novapikromycin **10**, from extracts of *S. venezuelae* fermentation broth ²⁹. Subsequently, it has been shown that PikC is able to catalyze *in vitro* hydroxylation at C14 of narbomycin **5** to yield ketolide **9** as well as dihydroxylation at both C12 and C14 to give ketolide **10** ²⁹.

Further evidence of the unusual substrate flexibility of PikC was uncovered during deletion experiments involving the desosamine biosynthetic genes. As described above, several mutant *S. venezuelae* strains produced novel macrolide antibiotics containing desosamine analogs. Subsequent structural determination of isolated 12-membered ring macrolides positively identified hydroxylation patterns at C10 and C12, indicating that despite the presence of the unnatural sugar moiety, PikC maintained the ability to catalyze hydroxylation of the macrolactone core ^{18, 20, 22}. However, PikC was not able to modify all newly identified macrolide compounds. Interestingly, it appears that hydroxylation of the macrolactone correlates with functionality at the glycosyl C4' position. Specifically, when C4' is functionalized with a hydroxyl or an *N*-acetyl moiety, the macrolactone ring fails to be hydroxylated ^{20, 22}. In contrast, the expected hydroxylation pattern is observed when the C4' was unmodified ^{20, 22}. The specific factors involved in the sensitivity of PikC to C4' modification remain unclear.

The diverse hydroxylation patterns that result from PikC raise important questions regarding the substrate recognition and binding properties of this monooxygenase. How is it that a single enzyme can not only recognize macrolide antibiotics containing variability with respect to macrolactone size, but also hydroxylate at different positions around each macrolactone ring? To address these issues, Podust and co-workers solved the x-ray crystal structure of PikC bound with the 12-membered ring macrolide, YC-17 **4**, and the 14-membered ring macrolide, narbomycin **5** (Figure 6) ³⁰. Interestingly, despite the differences in size, the macrolactone portions of both macrolides occupy the same binding pocket in the PikC active site. Within this pocket, macrolactone binding is largely mediated via hydrophobic interactions with side chain active site residues. In contrast to the macrolactone, the desosamine sugar is able to occupy one of two different binding pockets, depending on the size of the macrolactone ring system (Figure 6). Within each desosamine binding pocket, the most critical interaction involves sandwiching of the positively charged desosaminyl-C3'-dimethylamino group by a pair of carboxylate-containing residues (Glu85 and Asp50 for narbomycin & Glu94 and Glu85

for YC-17). Based on calculated distances from the dimethylamino group, Glu85 and Glu94 are proposed to form salt bridges with the positively charged amine found in narbomycin **5** and YC-17 **4**, respectively, whereas the more distal carboxylate side chains are hypothesized to compensate for the positively charged functional group ³⁰.

Further insight into the importance of these three acidic active site amino acids was gained through mutagenesis experiments in which Asp50, Glu94, and Glu85 were individually replaced with alanine, or the corresponding amino side-chain residue, asparagine or glutamine. Subsequent biochemical experiments revealed that the side chains involved in the putative salt bridge, Glu85 for narbomycin **5** and Glu94 for YC-17 **4**, with the dimethylamino group are essential for catalytic activity, whereas the distal carboxylate side chains, Asp50 and Glu85, involved in charge stabilization for narbomycin and YC-17, respectively, are dispensable. Taken together, the co-crystal structures and biochemical data suggest that the diverse hydroxylation patterns catalyzed by PikC can be attributed to two specific anchoring orientations of the desosamine sugar, as opposed to an induced fit model of binding. Undoubtedly, this enhanced insight into PikC substrate binding offers a tremendous opportunity to design unnatural glycosylated substrates for PikC hydroxylation. In addition, this structural detail might enable engineering of PikC site-directed mutants that favor one desosamine binding orientation, thereby shifting reaction products to a single, desired bioactive metabolite.

Given its broad substrate specificity, PikC is an attractive candidate to be developed into a useful industrial biocatalyst for the hydroxylation of novel macrolide compounds containing an appropriately linked desosamine sugar. While much work remains, preliminary experiments have begun to develop the tools necessary for achieving this goal. For example, PikC has been recently shown to be robust enough to be used in high-throughput biocatalytic technologies involving the incorporation of biomolecules onto functional surfaces of microfluidic devices ³¹. Nickel-NTA immobilized PikC was able to convert 90% of the macrolide YC-17 **4** to methymycin **6** and neomethymycin **7** when packed in a microfluidic channel. Interestingly, the half-life of PikC is increased from 3 hours in solution to 9 hours when immobilized within this microfluidic platform ³¹. In a related development, Li et al. have described the engineering of PikC into a self-sufficient hydroxylation catalyst whereby the P450 is fused with the RhFRED reductase domain ³². Because the native redox partner of PikC remains unknown, *in vitro* assays have historically relied on a costly reduction system comprised of spinach ferredoxin reductase and ferredoxin. The fusion of PikC with the RhFRED reductase domain has effectively eliminated the requirement for this cumbersome three-component reaction system, as RhFRED is capable of transferring electrons from NADPH to the heme domain of the P450. Impressively, this hybrid form of PikC demonstrates considerably enhanced catalytic efficiency in hydroxylating YC-17 **4** and narbomycin **5** compared to wild type enzyme.

Investigating chain elongation and macrolactone distribution: Analysis of PikAIII and PikAIV

The modular nature of type I PKS systems presents an appealing strategy for the generation of structurally diverse polyketide natural products. For example, inactivation of individual keto group processing domains (KR, DH, ER) can produce predicted structural alterations in the final product ^{5, 6}. Likewise, addition, deletion, or substitution of intact elongation modules can impart structural variety into polyketide metabolites ^{5, 6}. It has been estimated that, using these combinatorial techniques, modular PKSs have the potential to generate hundreds of millions of polyketide compounds ³³. Despite this promise, successful re-engineering of PKS systems has been met with only limited success ^{34–36} suggesting that much remains to be learned regarding the intricacies of domain substrate specificities, modular interactions, and reaction mechanisms. Given its intrinsic ability to generate two unique macrolactone products, the

pikromycin PKS is an attractive candidate for detailed *in vitro* investigation in order to gain key insight into the subtleties of polyketide synthesis by PKS systems.

The modular PKS responsible for generating the erythromycin macrolactone core structure, 6-deoxyerythronolide B (6-DEB), has proven particularly amenable to *in vitro* study; thus, much of what is known regarding PKS catalysis has been gained from the DEBS PKS system³⁷. Given the large size of the PKS polypeptide products (typically >100 kDa per monomer, depending on the number of catalytic domains), detailed study of additional modular PKSs has been limited in scope, largely due to problems associated with protein expression in *E. coli*. In addition, generation of natural chain elongation substrates presents a significant challenge to the synthetic chemist, due to the complex structure of polyketide intermediates, which typically include multiple asymmetric centers. As such, most *in vitro* studies to date have utilized simple, unnatural diketide chain elongation intermediates as substrates. Although they have proven useful in PKS investigations, the data obtained from their use is limited, as precise details regarding reaction kinetics and substrate specificities cannot be fully elucidated.

Recent efforts in the Sherman laboratory have focused on unraveling the details of the chain elongation and termination events that are catalyzed by the final two elongation modules of the pikromycin PKS. These two protein targets were initially selected for *in vitro* work due to their involvement in mediating the partitioning of the different sized macrolactone products (Figure 4). Additionally, given that PikAIII and PikAIV are both monomodular proteins, they were predicted to be the most accessible by heterologous overexpression in a bacterial host. Indeed, despite their large size (~140 kDa & ~160 kDa, respectively) this proved to be the case. Both PikAIII and PikAIV have each been individually co-expressed with the phosphopantetheinyl transferase from *B. subtilis* (*sfp*) in *E. coli* BL21 (DE3)^{38, 39}. The inclusion of this promiscuous phosphopantetheinyl transferase ensured that the ACP domains would be sufficiently modified with the phosphopantetheinyl prosthetic group. FT-MS analysis of the purified recombinant modules has verified that the apo to holo conversion of ACP domains is greater than 95%⁴⁰.

In the first comparative study to those performed initially on the DEBS PKS system, a series of diketide *N*-acetylcysteamine (NAC) thioesters was synthesized and used to investigate initiation, elongation, and termination reactions that lead to triketide and tetraketide lactone structures from PikAIII and the combination of PikAIII and PikAIV^{39, 41}. During *in vivo* PKS catalyzed polyketide biosynthesis, the KS domain accepts its substrate from the upstream ACP domain, where it is attached as a thioester to the phosphopantetheine moiety. Because the *in vitro* studies relied on diffusive loading of the substrate onto the KS domain of the elongation module of interest, the presence of the *N*-acetylcysteamine was included on the synthetic diketide substrates in order to mimic the phosphopantetheine chain that extends from ACP domains. An important discovery made during these initial *in vitro* studies was that the non-natural diketide substrates, routinely used during the *in vitro* characterizations of the DEBS PKS, do not adequately substitute for the natural polyketide substrates of the pikromycin PKS system. Specifically, the measured k_{cat} values for PikAIII and PikAIV extension and termination were approximately three orders of magnitude lower than the *in vivo* rates of pikromycin **1** production that have been observed during *S. venezuelae* fermentation⁴¹.

To more accurately mimic the naturally occurring chain elongation reaction that is catalyzed by the combination of PikAIII and PikAIV, the natural pentaketide chain elongation substrate of PikAIII was synthesized⁴². As with the diketide substrates, the pentaketide was generated as the NAC-thioester in order to mimic the ACP bound intermediate. *In vitro*, combination of PikAIII and PikAIV effectively converted the NAC-pentaketide thioester substrate into both 10-deoxymethynolide **2** and narbonolide **3**, the first reported chemoenzymatic synthesis of either of these macrolactone products⁴². Remarkably, kinetic analysis of macrolactone

assembly catalyzed by PikAIII and PikAIV with this natural length substrate yielded an apparent k_{cat} that was approximately 50-fold higher than the diketide substrates. Furthermore, the apparent K_M value for the pentaketide chain elongation intermediate was approximately 25-fold lower than that of the best diketide substrate⁴². The results of these kinetic studies strongly support the use of natural chain length polyketide intermediates for obtaining a detailed mechanistic understanding of these multifunctional proteins.

Together, the ability to produce recombinant PikAIII and PikAIV, coupled with the ready availability of the *N*-acetylcysteamine pentaketide chain elongation intermediate, provided the unique opportunity to perform detailed interrogations into the mechanism that drives formation of multiple macrolactone products. It has previously been demonstrated that the TE domain of PikAIV is responsible for generating both 10-deoxymethynolide **2** and narbonolide **3** from their respective linear chain elongation intermediates⁴³; hence, the PikAIII bound linear intermediate must be presented to the TE domain of PikAIV for 10-deoxymethynolide **2** generation. However, direct evidence of the molecular mechanism of 10-deoxymethynolide **2** production has been limited to *in vivo* observations^{43,44}.

Initial *in vitro* studies focused on the roles of the individual domains of PikAIV in 10-deoxymethynolide **2** synthesis. Site-directed mutagenesis experiments, in which the KS, AT, and ACP domains of PikAIV were individually inactivated via alanine substitution of key amino acid residues, strongly indicated that these domains are not essential for 10-deoxymethynolide **2** production⁴⁰. Subsequent *in vitro* kinetic analyses suggested that the hexaketide intermediate does not “skip” through PikAIV to reach the terminal TE domain⁴⁰. Thus, efforts focused on examining the role of the docking domain mediated protein-protein interaction that occurs between PikAIII and PikAIV in partitioning of the two macrolactone products. Within PKS systems, docking domains are appended to both the carboxy-terminus and amino-terminus of interacting polypeptides in order to facilitate intermodular communication and transfer of polyketide intermediates^{45–47}. To determine if the intermodular interaction between PikAIII and PikAIV was important for 10-deoxymethynolide **2** synthesis, both the carboxy-terminal docking domain of PikAIII and the amino-terminal docking domain of PikAIV were truncated to prevent productive interaction with their corresponding partner module. Results obtained from *in vitro* biochemical assays clearly demonstrated that PikAIII and PikAIV most efficiently generate the 12-membered ring macrolactone when engaged in their cognate docking domain interaction⁴⁰. These data suggest a model whereby PikAIV, when interacting with the upstream monomodule, can adopt a conformation that enables the TE domain to directly interact with ACP domain of PikAIII to offload the hexaketide intermediate (Figure 7). This enhanced understanding of multiple macrolactone production by the Pik PKS system could aid re-engineering efforts aimed at controlling the ratio of 12- and 14-membered ring macrolactones. Moreover, it is intriguing to speculate that genetic engineering might enable incorporation of this mechanism into a variety of PKS systems, thus increasing the potential pool of macrolide antibiotics that can be generated via manipulation of PKS biosynthetic pathways.

Thioesterase mediated macrocyclization: Development of a general macrolactonization catalyst?

While the importance of glycosidic moieties that decorate macrolide antibiotics is structurally understood², an often-underappreciated structural feature of this therapeutic class is the characteristic macrolactone ring. A probable role for the macrocyclic core structure is to reduce the number of possible conformations that can be adopted by the final compound. It is likely that this imposed conformational restraint favors the bioactive conformation of the macrolide antibiotic, resulting in the sugar subunits being appropriately positioned for interaction with the bacterial ribosome. Whether or not this is a correct hypothesis, it is clearly apparent that

the macrolactone moiety of macrolide antibiotics is critical for their biological activity, and therefore must be considered during development of next generation therapeutics. Although several synthetic strategies have proven capable of generating macrolactones of eight atoms or more⁴⁸, these methods can suffer from poor yields due to factors such as complex protecting group strategies, poor regioselectivity, and intermolecular oligomerizations. To overcome these synthetic challenges, nature has employed a thioesterase catalytic domain to terminate polyketide biosynthesis. Thioesterases, typically found at the terminus of final elongation modules in PKS biosynthetic systems, utilize a simple Ser-His-Asp catalytic triad to efficiently catalyze cleavage and subsequent regiospecific cyclization of the fully extended linear intermediate. The relative efficiency at which these catalysts can generate macrolactone scaffolds from linear intermediates makes them attractive candidates to be developed into a useful chemoenzymatic tool. It has previously been established that a single thioesterase domain is responsible for cyclizing both macrolactone compounds that are produced by the Pik PKS⁴³. As such, it was anticipated that a detailed *in vitro* investigation of the Pik TE could offer insight into its inherent substrate flexibility, which could aid in the transformation of this enzymatic domain into a general macrolactonization catalyst.

Lu et al. first reported the cloning, heterologous expression and purification of the excised thioesterase domain from the Pik PKS system in 2002⁴⁹. Subsequently, a series of NAC-diketide thioesters was utilized to preliminarily assess substrate specificity⁴⁹. Because these substrates do not accurately represent the atomic length of the natural chain elongation intermediates that are cyclized by the TE domain, it is impossible to truly predict specificity trends from this initial characterization. Moreover, due to the size of the unnatural substrate, the assay used in these studies simply measures the hydrolytic cleavage rate of the NAC functional group from the diketide substrate rather than the natural cyclization reaction. Nonetheless, these results clearly demonstrated that the Pik TE has broader substrate specificity relative to the DEBS TE, as it is tolerant of all diastereomeric configurations of the 2-methyl and 3-hydroxyl functional groups⁴⁹.

Advances in the chemical synthesis of natural chain elongation intermediates have enabled a more complete understanding of the kinetics and substrate specificity of Pik TE catalysis. Specifically, Aldrich et al. have described two separate methods for generating the natural pikromycin NAC-hexaketide chain elongation intermediate, which is the linear precursor to 10-deoxymethynolide **2**^{42, 50}. *In vitro*, excised Pik TE has demonstrated the ability to catalyze complete conversion of this linear polyketide intermediate to the corresponding 12-membered ring macrolactone 10-deoxymethynolide **2**. Poor aqueous solubility of the substrate prevented the determination of kinetic parameters; however, the specificity constant (k_{cat}/K_M) was estimated to be $1.7 \text{ mM}^{-1} \text{ min}^{-1}$ ⁵⁰. Interestingly, preliminary substrate specificity investigations revealed that the enzyme was sensitive to structural changes at the C7 position of the hexaketide substrate⁵⁰. The native substrate containing an enone carbonyl at the C7 position is exclusively cyclized by Pik TE; however, reduction of the C7-carbonyl to the allylic alcohol resulted in a hexaketide substrate analog that was exclusively hydrolyzed by Pik TE to yield the corresponding *seco*-acid. This interesting and unexpected discovery was the first indication that, despite its natural substrate tolerance for chain length variation (ie, hexaketide & heptaketide), Pik TE is sensitive to minor functional group modifications of its natural substrates.

Further interrogation of the substrate specificity requirements of the Pik TE utilized high-resolution x-ray crystallography to provide an enhanced appreciation the active site architecture of this PKS catalytic domain. Subsequent to the initial biochemical characterization⁴⁹, the x-ray crystal structure of the Pik TE was solved by Tsai et al. in 2002⁵¹. Comparison of this structure to DEBS TE revealed overall similarities in both the dimer interfaces and geometry of the substrate channel. Notably, unique features were observed within the active site

composition of the two related thioesterases, likely reflecting differences in substrate specificity. However, the absence of a bound ligand in the active site of the Pik TE active site prevented an accurate assessment of the specificity determinants of this unique PKS catalytic domain.

Most recently, Fecik and co-workers have demonstrated that diphenylphosphonate triketides can effectively serve as affinity labels for the Pik TE active site, offering a valuable tool for the examination of the substrate specificity and catalytic mechanism of this PKS domain^{52, 53}. Co-crystallization studies with these compounds revealed the presence of a non-classical oxyanion hole in which only a single hydrogen bond is donated from an active site residue⁵². It has been hypothesized that the absence of a second hydrogen bond donor, which is typically found in related serine hydrolases, is due to the need to accommodate both ends of the polyketide during cyclization of the acyl-serine intermediate. Once proof-of-concept was established that the diphenylphosphonate compounds enabled trapping of the acyl-enzyme intermediate within the active site, an extended chain diphenylphosphonate pentaketide was synthesized and co-crystallized with Pik TE⁵³. This investigation led to remarkable insight into Pik TE catalysis (Figure 8)⁵³. Specifically, the resulting co-crystal structure revealed that in the absence of direct protein-substrate interactions, substrate macrocyclization is directed by two well-ordered water molecules near the exit of the substrate channel that serve to re-route the hydrophobic substrate back towards the active site. Furthermore, computational studies in which the missing atoms of both the hexaketide and heptaketide substrates were modeled onto the trapped intermediate indicated that the presence of an α/β unsaturated ketone effectively constrains the conformation of the linear substrate, thereby positioning the distal hydroxyl for direct attack of the acyl-enzyme intermediate⁵³. To be sure, such a “substrate-assisted” mechanism would limit the utility of Pik TE as a general macrolactonization catalyst, due to the fact that an appropriately positioned enone functionality would be a required component of any linear intermediate. Despite this, work continues to understand, explore and expand the substrate tolerance of this PKS catalytic domain.

Summary

Manipulation of modular PKS systems offers an exciting strategy for the generation of structurally diverse libraries of novel natural products that have potential medicinal value, especially for anti-microbials⁵. However, due to limited knowledge of PKS molecular mechanisms, catalytic activities, kinetic parameters, and protein-protein interactions, this potential remains largely unrealized. Over the last decade, our laboratory, as well as those of our collaborators, has been pioneering an understanding of these issues through the detailed investigation of the pikromycin PKS system from *Streptomyces venezuelae*. As highlighted in this review, this work has provided fundamental insights into all stages of pikromycin biosynthesis. It is with great anticipation that the cumulative knowledge gained through these studies will serve as the foundation for future efforts aimed at leveraging the catalytic power and specificity of the pikromycin biosynthetic system for production of novel ketolide anti-infective agents.

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erythromycin A

clarithromycin

azithromycin

telithromycin

Figure 1.
Chemical structures of clinical macrolide antibiotics erythromycin A, clarithromycin, azithromycin, and telithromycin.

Figure 2. Model of a dimeric type I PKS elongation module

At a minimum, a PKS elongation module is comprised of a ketosynthase (KS) domain, acyl transferase (AT) domain, and an acyl-carrier protein (ACP) domain. In addition, any given PKS elongation may include a ketoreductase (KR) domain, dehydratase (DH) domain, and enoyl reductase (ER) domain. These additional domains act to reductively process the β -keto functionality. Inter-polypeptide communication is mediated by docking domains that are appended at the N-terminus of KS domains and the C-terminus of ACP domains. The terminal elongation module of PKS systems typically contains a thioesterase (TE) domain that is responsible for terminating biosynthesis via the formation of a macrocycle. This figure was adapted from Buchholz et al.⁵⁴.

Figure 3. The methymycin/pikromycin biosynthetic pathway

The methymycin/pikromycin biosynthetic pathway is comprised of 18 discrete genes, clustered into 5 separate loci, which are responsible for the generation of 6 different macrolide antibiotics. The *pikA* locus (shown in red) encodes a type I PKS that directs the biosynthesis of the 12- and 14-membered ring macrolactones, 10-deoxymethynolide **2** and narbonolide **3**. The *des* locus (shown in blue) encodes all proteins responsible for the generation of the desosamine sugar moiety and attachment to macrolactones **2** and **3** to give the macrolides YC-17 **4** and narbomycin **5**. *PikC* (shown in orange) is a cytochrome P450 monooxygenase that serves to hydroxylate macrolides **4** and **5** to give the macrolide antibiotics methymycin **6**, neomethymycin **7**, novamethymycin **8**, pikromycin **1**, neopikromycin **9** and novapikromycin **10**. The *pikR* locus (shown in yellow) contains two macrolide-lincosamide-streptogramin B type resistant genes (*pikR1* and *pikR2*) that provide self-protection to the *S. venezuelae* host. The *pikD* gene (shown in green) encodes a transcriptional regulatory factor⁵⁵.

Figure 4. The pikromycin type I polyketide synthase

The pikromycin polyketide synthase is comprised of a single loading module and six elongation modules that span four polypeptide chains (PikAI-PikAIV). Chain elongation through PikAIII followed by thioesterase catalyzed termination results in the 12-membered ring macrolactone product, 10-deoxymethynolide **2**, while continued elongation of the polyketide chain produces the 14-membered ring macrolactone, narbonolide **3**. Both products undergo further processing via a glycosyltransferase and P450 hydroxylase to yield the methymycin and pikromycin suite of antibiotics. PikAV is a type II thioesterase that functions to remove prematurely decarboxylated extender units from ACP domains. Abbreviations: KS, ketosynthase; AT, acyl transferase; ACP, acyl carrier protein; KR, ketoreductase; DH, dehydratase; ER, enoyl reductase; TE, thioesterase.

Figure 5. Biosynthetic pathway of the desosamine deoxyamino sugar

The des locus of the pikromycin PKS system encodes all enzymes (DesI-DesVI) that are required to transform TDP-glucose into TDP-desosamine. The activity of DesVII and DesVIII transfers the desosamine sugar to 10-deoxymethynolide **2** and narbonolide **3** to yield the macrolide antibiotics YC-17 **4** and narbomycin **5**. Genetic manipulation of the desosamine biosynthetic pathway has resulted in the generation of several novel macrolide structures 18-20, 22.

Figure 6. Alternative anchoring of desosamine observed within the PikC active site
The PikC active site bound with narbomycin **5** (orange) and YC-17 **4** (yellow) is shown. For the 14-membered ring macrolide, the positively charged desosaminyl-C3'-dimethylamine (blue atom) is anchored between Glu85 and Asp50, whereas in the 12-membered ring macrolide, it is sandwiched between Glu85 and Glu94. PDB coordinates 2C6H and 2C7X were used to generate this figure ³⁰.

Figure 7. Proposed model of multiple macrolactone formation by the monomodules PikAIII & PikAIV

Production of both 10-deoxymethynolide **2** and narbonolide **3** by the pikromycin PKS system requires that PikAIII and PikAIV engage in their docking domain mediated protein-protein interaction. Subsequent to this interaction, the hexaketide intermediate can follow one of two paths. As shown in **path A**, the hexaketide intermediate that extends from the ACP domain of PikAIII can be offloaded and cyclized to 10-deoxymethynolide **2** by the terminal TE domain of PikAIV. Alternatively, the hexaketide intermediate can follow **path B**, in which it is transferred from the ACP domain of PikAIII to the KS domain of PikAIV. The hexaketide intermediate then undergoes an additional round of elongation and β -keto processing prior to being offloaded and cyclized by the TE domain to give narbonolide **3**.

Figure 8. Snapshot of the pikromycin thioesterase acyl intermediate

The active site residue of the pikromycin thioesterase, serine 148, is covalently modified with the extended chain diphenylphosphonate affinity label. Two well-ordered water molecules coordinated between Gln183 and Ala217 form a “hydrophilic barrier” near the exit of the substrate channel and are proposed to redirect the linear polyketide intermediate back into the active site. This results in the observed “curled” conformation of the bound ligand. This figure was generated from PDB coordinates 2HFK⁵³.