The Williams Lab

Medicinal Microbes: Making Drugs Using Synthetic Biology

chemsynthbiol Williams Lab

synthetic biology • enzyme engineering
• drug discovery + production
• natural products

NC STATE UNIVERSITY

Current Graduate Students

Miles Calzini
Anuran Gayen
Jessie Garcia
Rachael Hall
Yiwei Li
Lexie Malico
Melissa Mitchler
Lindsay Nichols
Zhongtian Zhang

Current Undergraduate Students

Jenay Brown
Christian McRoberts
Monique Reid

Join a dynamic, collaborative, and inter-disciplinary group

- Our research is at the interface of chemistry, biology, & engineering
- Diverse projects to suit your needs
- Attend conferences
- Try your own ideas
- Collaborators on campus and world-wide
- Graduate in 5 years
- Students are prepared for success!

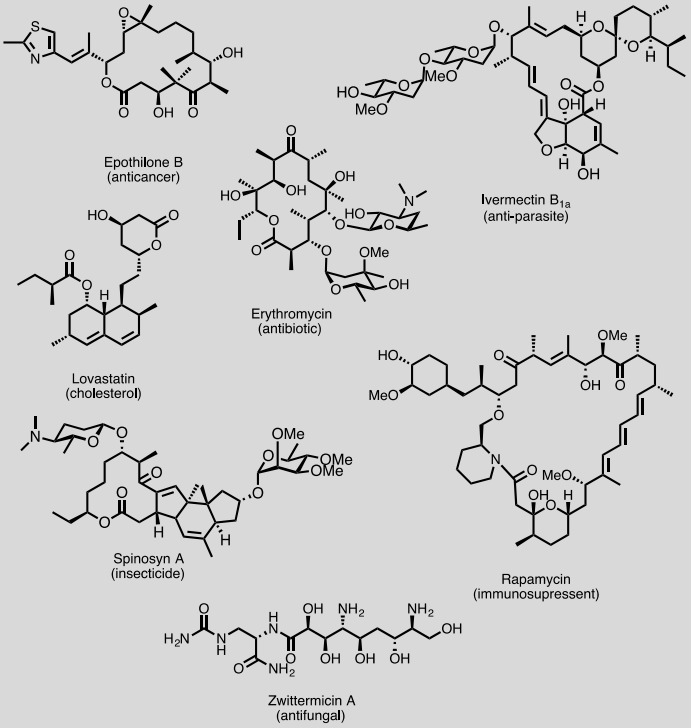
Proven history of graduate student success

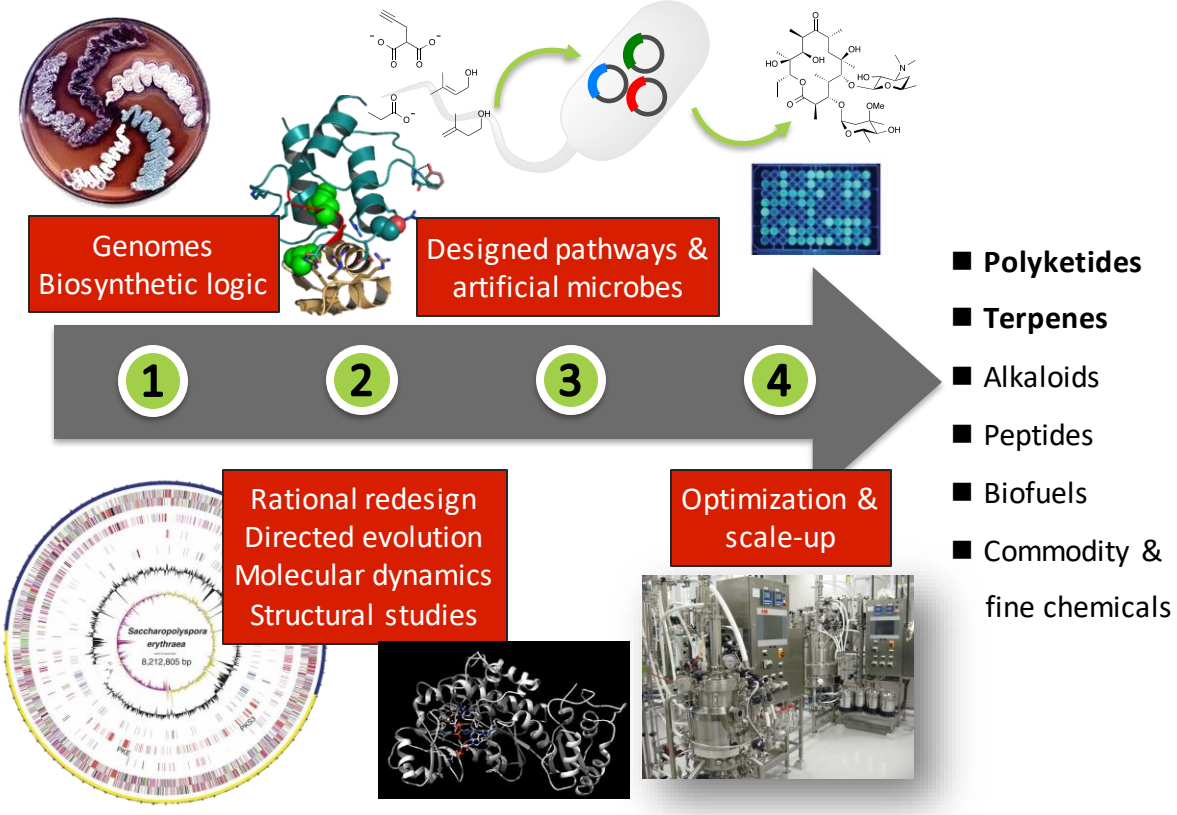
- Percy Lavon Julian Award for Research
- Miles Anderson Award for Teaching
- Chemistry Graduate Scholars Research Fellowship
- NIH Molecular Biotechnology Fellowships
- Internships at Novozymes, GSK etc.
- Involved with patent submission, tech transfer, NSF i-CORPS
- Collaboration with MBA program
- Numerous local, regional and national poster prizes

ACCESSING NEW DRUGS VIA NATURAL PRODUCT BIOSYNTHESIS

We are developing a "genes-to-molecules" synthetic biology pipeline for the synthesis, diversification, and discovery of natural products for drug discovery.

A variety of projects are available that focus on different aspects of natural product biosynthesis, enzyme engineering, metabolic engineering, synthetic biology, and drug discovery.

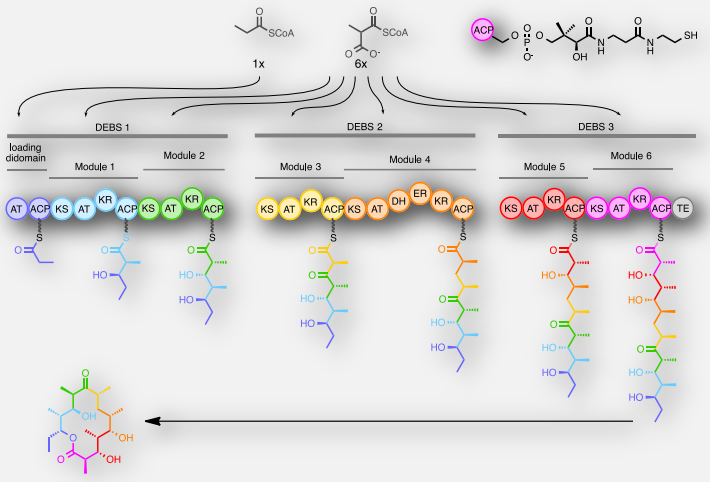




EXAMPLES OF AVAILABLE PROJECTS

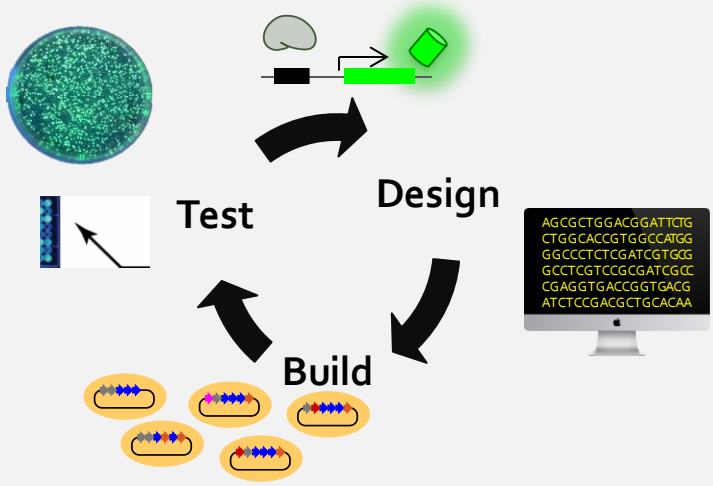
Engineering mega-enzyme assembly lines

Many natural products are constructed via the controlled assembly of small molecule building blocks catalyzed by long sequences of enzymes. We are probing the synthetic capabilities of these enzymes and learning how to manipulate these assembly lines to make new molecules.



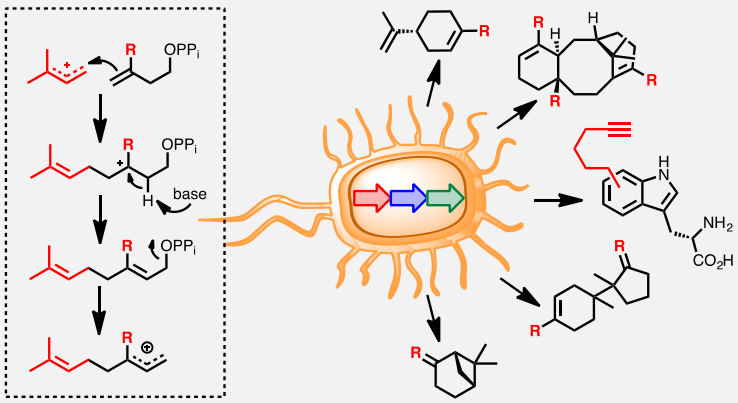
Biosensor-guided high-throughput engineering of biosynthetic pathways

We are creating genetically-encoded biosensors that turn on fluorescent signals in the presence of target small molecules. These are leveraged to screen the ability of large libraries of pathway and enzyme variants to make non-natural small molecules.



Design and optimization of artificial enzymatic pathways for terpene diversification

In Nature, terpenes are constructed from just two building blocks, limiting their structural diversity. We have constructed an artificial pathway for generation of a wide variety of terpene building blocks and their installation into complex scaffolds.



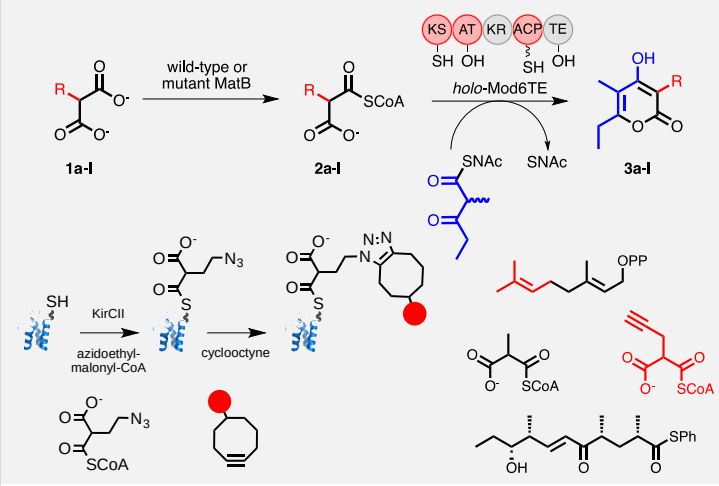
METHODS & TECHNOLOGY

Organic Chemistry

Synthesis of small molecules as potential substrates for enzymes

Synthesis of probes/inhibitors to study enzymes

Utilization of “click” chemistry to rapidly diversity natural products



Molecular Biology & Biochemistry

Gene cloning, mutagenesis, library construction, site-directed mutagenesis, CRISPR-Cas9, genome engineering

Protein expression & purification, enzyme assays, high-performance liquid chromatography

In silico protein analysis

Homology modeling, sequence analysis, molecular dynamic simulations

High-throughput tools

Biosensor screening, microplate assays, genetic selections, flow cytometry, robotic liquid handling

DSM

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zymergen

Destinations of recent graduates

- Intrexon, San Francisco
- Amyris, Bay Area
- Bayer Crop Science, RTP
- Locus Biosciences, RTP
- Novozymes, NC
- BASF (Professional Development Program)
- Scripps, postdoc
- Emory, postdoc
- UC-Davis, postdoc
- Duke, postdoc

Graduates are well-suited to careers in the synthetic biology & protein engineering industry and academia

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Engineering the Substrate Specificity of a Modular Polyketide Synthase for Installation of Consecutive Non-Natural Extender Units

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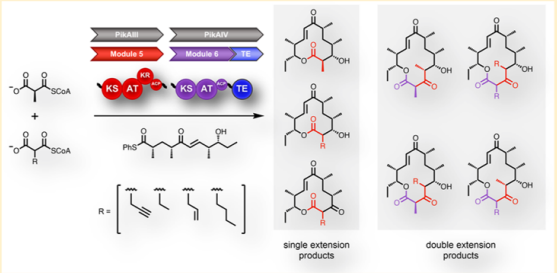
Supporting Information

ABSTRACT: There is significant interest in diversifying the structures of polyketides to create new analogues of these bioactive molecules. This has traditionally been done by focusing on engineering the acyltransferase (AT) domains of polyketide synthases (PKSs) responsible for the incorporation of malonyl-CoA extender units. Non-natural extender units have been utilized by engineered PKSs previously; however, most of the work to date has been accomplished with ATs that are either naturally promiscuous and/or located in terminal modules lacking downstream bottlenecks. These limitations have prevented the engineering of ATs with low native promiscuity and the study of any potential gatekeeping effects by domains downstream of an engineered AT. In an effort to address this gap in PKS engineering knowledge, the substrate preferences of the final two modules of the pikromycin PKS were compared for several non-natural extender units and through active site mutagenesis. This led to engineering of the methylmalonyl-CoA specificity of both modules and inversion of their selectivity to prefer consecutive non-natural derivatives. Analysis of the product distributions of these bimodular reactions revealed unexpected metabolites resulting from gatekeeping by the downstream ketoreductase and ketosynthase domains. Despite these new bottlenecks, AT engineering provided the first full-length polyketide products incorporating two non-natural extender units. Together, this combination of tandem AT engineering and the identification of previously poorly characterized bottlenecks provides a platform for future advancements in the field.



INTRODUCTION

Type I polyketide synthases (PKSs) are responsible for the biosynthesis of some of the most clinically important bioactive compounds in Nature, including the blockbuster drugs erythromycin A (antibiotic), rapamycin (immunosuppressant/anticancer), and avermectin (anthelmintic).[1] These PKSs are giant assembly line pathways that can be broken down into individual modules (Figure 1), each of which is responsible for incorporation of a single extender unit, often a coenzyme A (CoA)-linked malonate derivative. The acyltransferase (AT) within each module acts as the "gatekeeper" domain due to its innate ability to select a specific extender unit for priming of its cognate acyl carrier protein (ACP). Despite the structural diversity of polyketides, the AT domains responsible for selecting the extender units for each module typically include only three substrates: malonyl-CoA, methylmalonyl-CoA, and, to a lesser degree, ethylmalonyl-CoA.[2] Thus, except in a few rare cases, the selected substrates account for relatively narrow chemical diversity.[3−7] Instead, polyketide diversity in Nature comes from varying oxidations, cyclization patterns, or post-PKS modifications. This is represented by the four final products that result from the pikromycin (Pik) PKS (Figure 1). The development of chemoenzymatic approaches that employ non-natural malonyl-CoA analogues affords the opportunity to increase the structural diversity of polyketides by engineering PKSs.[8−10]

Traditionally, PKS engineering has focused on exchanging modules or domains to alter the final product structure, but there are three critical limitations: (1) most PKS modules incorporate natural extenders that lack useful chemical handles, (2) non-native protein−protein interactions often result in chimeras with poor catalytic efficiencies,[11,12] and (3) to achieve site-selective installation of a given non-natural extender unit into a polyketide, the specificity of the domain/module chimera must be orthogonal to that of the

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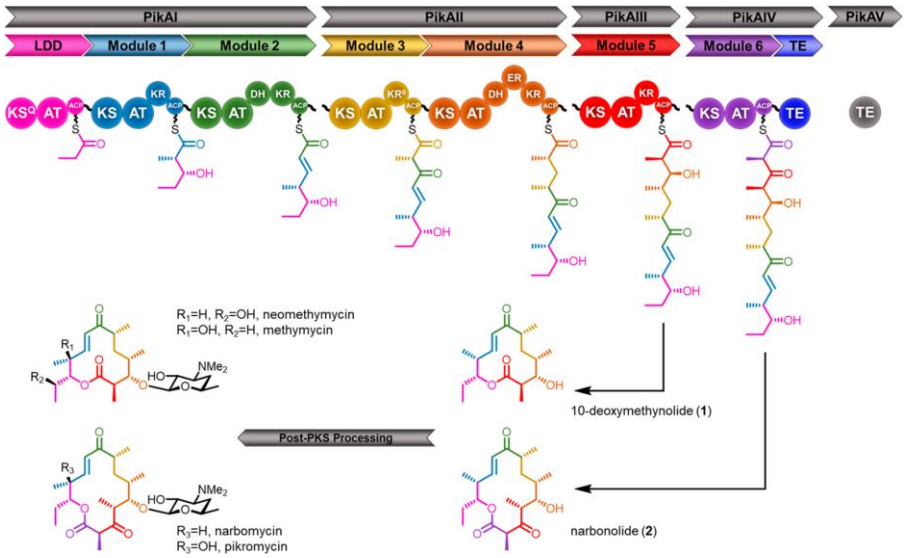

Figure 1. Pikromycin polyketide synthase and its products. ACP = acyl carrier protein; AT = acyltransferase; DH = dehydratase; ER = enoylreductase; KR = ketoreductase; KS = ketosynthase; KS^{Q} = ketosynthase-like decarboxylase; TE = thioesterase.

native, intact extension modules. In order to produce non-natural extender units, we and others have utilized and engineered malonyl-CoA synthetases or similar enzymes to create a panel of PKS substrates bearing a variety of useful chemical moieties.[8−10,13−15] The second issue has been approached through introduction of AT active site mutations, with varying levels of success.[9,16−19] For example, replacing the conserved YASH motif that is found in methylmalonyl-specific ATs with motifs from other natural ATs (e.g., HAFH from malonyl-CoA-specific ATs) can lead to changes in AT specificity. However, these changes alone have not completely inverted AT specificity between natural substrates and therefore do not provide the requisite orthogonality for site-selective modification of the polyketide structure.[20] In contrast, we and others have demonstrated that inherent extender unit promiscuity of some ATs provides a platform for creating new substrate specificities via site-directed mutagenesis. For example, the methylmalonyl-CoA-utilizing EryAT6 and corresponding terminal extension module (Ery6) of the 6-deoxyerythronolide B synthase (DEBS) from erythromycin A biosynthesis has significant promiscuity toward larger non-natural extender units.[21] These non-natural substrates were utilized by an engineered Ery6 module with the YASH to RASH variant, resulting in a switch from 92% methylmalonyl-CoA incorporation (wild-type enzyme) to 88% propargylmalonyl-CoA (non-natural) incorporation into the polyketide chain.[18] The ability to manipulate the substrate preference of another AT from DEBS (EryAT2) toward longer alkyl chains via a VASH motif (found in some natural ethylmalonyl-CoA-specific ATs) was also demonstrated, albeit to a lesser extent.[9] These shifts in substrate selectivity are notable and rely on the inherent promiscuity of the AT as an opportunity for redesigning substrate specificity in PKSs. Additionally, most AT engineering is accomplished with terminal extension modules that are at the end of the assembly line lacking downstream bottlenecks and involve installation of only one non-natural extender unit into the final product structure.

Herein, the ability of site-specific mutagenesis to manipulate the extender unit specificity of ATs that do not display inherent promiscuity was explored. To this end, the Pik PKS, responsible for the biosynthesis of two core macrolactones, a 12-membered 10-deoxymethynolide (10-dML, **1**) and a 14-membered narbonolide (**2**) in *Streptomyces venezuelae* ATCC 15439, was selected as a target for mutagenesis.[22] We proposed that the extension modules of this pathway would be less promiscuous toward larger extenders than the prototypical DEBS modules due to its evolution in a host that also produces ethylmalonyl-CoA and to hydrolytic proof-reading by the AT and PikAV (TEII).[23−25] Using the final two modules from this PKS, the native promiscuity of each module was first compared with a panel of natural and non-natural extender units and via a series of domain exchanges. Next, the substrate selectivity of each module was successfully engineered toward non-natural extender units via site-directed mutagenesis. Finally, a hitherto unrecognized bottleneck in PKS engineering was highlighted. To our knowledge, this is the first example of successful substrate selectivity inversion in an AT that does not belong to the prototypical DEBS assembly line. Moreover, and to the best of our knowledge, it represents the first example of two non-natural extender units being incorporated into a single full-length polyketide product.

RESULTS

Characterization of the PikAIII/PikAIV System. To date, the majority of AT substrate selectivity engineering work has been conducted in DEBS Ery6, a terminal module chosen at least in part because the fully extended non-natural chains do not need to be passed through other modules.[16−18,21,26] The Pik PKS provides a unique opportunity to probe the specificity of two adjacent monomodules that control formation of a 12- or 14-membered ring macrolactone, 10-dML (**1**) and narbonolide (**2**), respectively (Figure 1). These two enzymes are evolutionarily related, with 74% amino acid identity over two-thirds of their sequences (PikAIV lacks a KR

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Letter
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An Artificial Pathway for Isoprenoid Biosynthesis Decoupled from Native Hemiterpene Metabolism

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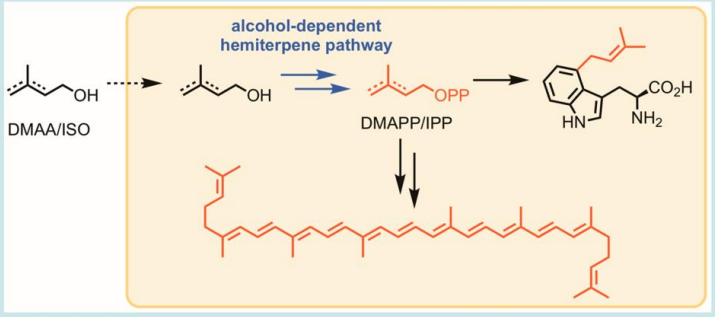
Supporting Information



ABSTRACT: Isoprenoids are constructed in nature using hemiterpene building blocks that are biosynthesized from lengthy enzymatic pathways with little opportunity to deploy precursor-directed biosynthesis. Here, an artificial alcohol-dependent hemiterpene biosynthetic pathway was designed and coupled to several isoprenoid biosynthetic systems, affording lycopene and a prenylated tryptophan in robust yields. This approach affords a potential route to diverse non-natural hemiterpenes and by extension isoprenoids modified with non-natural chemical functionality. Accordingly, the prototype chemo-enzymatic pathway is a critical first step toward the construction of engineered microbial strains for bioconversion of simple scalable building blocks into complex isoprenoid scaffolds.

KEYWORDS: *isoprenoids, terpenoids, hemiterpene, kinase, lycopene, prenyltransferase*

Isoprenoids comprise >55 000 natural products for which methods to access and diversify their structures are in high demand.[1−3] Ultimately, the isoprene motif plays a critical role in modulating the biological activity of isoprenoids, determines their utility as tools to study and treat human diseases, and provides the basis to develop new fuels and chemicals.[4−7] Notably, although several valuable isoprenoids have been accessed *via* heterologous expression,[8−11] our ability to diversify isoprenoids is extremely limited largely due to critical limitations imposed by native isoprenoid biosynthesis. First, only the mevalonate (MEV) and 1-deoxy-D-xylulose-5-phosphate (DXP) pathways (Figure 1A) are known to produce the universal hemiterpene isoprenoid diphosphate building blocks, dimethylallyl pyrophosphate (DMAPP), and isopentenyl pyrophosphate (IPP).[12,13] The negatively charged hemiterpenes are not cell permeable, thus preventing feeding them or analogues thereof into cultures. The MEV and DXP pathways involve at least six enzymatic steps[13] each with stringent substrate specificity and therefore offer little opportunity to diversify the structures of isoprenoids through feeding in non-natural precursors.[14] As a result, while precursor-directed biosynthesis has proven a powerful approach to access diverse structures of natural products[15,16]—especially polyketides[17−21]—by feeding non-natural building blocks, this approach has not yet been applied to isoprenoids. Furthermore, late-stage biosynthetic modification of isoprenoid scaffolds is typically limited to oxidations, often catalyzed by P450s.[11,22] Second, terpene metabolism is highly regulated and is a burden to the carbon supply on the cell.[23] For example, the MEV pathway uses three molecules of phosphate donor (ATP) and two reducing equivalents (NADPH) for each DMAPP/IPP, while the DXP pathway requires two phosphate donors (ATP and CTP) and two reducing equivalents (NADPH) (Figure 1A).[13] Third, given that native terpenes are typically essential for maintenance of the cell, genetic modification of native hemiterpene pathways would likely be lethal.[24] Together, these limitations could be overcome by supplying a membrane-permeable carbon building block dedicated for a designer pathway that would function independent of native isoprenoid metabolism. A potential strategy for hemiterpene biosynthesis could start with isopentenol (ISO) and dimethylallyl alcohol (DMAA) which are converted to the required diphosphates *via* stepwise

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ACS chemical biology

Articles

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Extender Unit Promiscuity and Orthogonal Protein Interactions of an Aminomalonyl-ACP Utilizing Trans-Acyltransferase from Zwittermicin Biosynthesis

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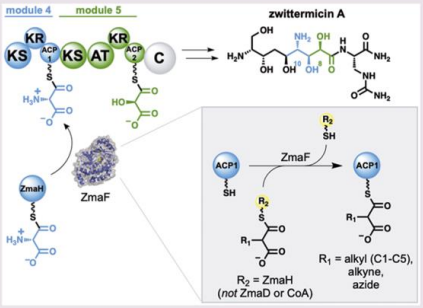
Supporting Information

ABSTRACT: Trans-acting acyltransferases (trans-ATs) are standalone enzymes that select and deliver extender units to polyketide synthase assembly lines. Accordingly, there is interest in leveraging trans-ATs as tools to regioselectively diversify polyketide structures. Yet, little is known regarding the extender unit and acyl carrier protein (ACP) specificity of trans-ATs, particularly those that utilize unusual ACP-linked extender units. For example, the biosynthesis of the antibiotic zwittermicin involves the trans-AT ZmaF, which is responsible for installing a rare ACP-linked aminomalonyl extender unit. Here, we developed a method to access a panel of non-natural and non-native ACP-linked extender units and used it to probe the promiscuity of ZmaF, revealing one of the most promiscuous ATs characterized to date. Furthermore, we demonstrated that ZmaF is highly orthogonal with respect to its ACP specificity, and the ability of ZmaF to trans-complement noncognate PKS modules was also explored. Together, these results set the stage for further engineering ZmaF as a tool for polyketide diversification.



Many polyketides are constructed via the action of modular assembly lines called type I polyketide synthases (PKSs) whereby each module is responsible for the installation and tailoring of an extender unit building block that forms the polyketide scaffold. Each extender unit is selected by an acyltransferase (AT) domain that in most cases is embedded within each module of the assembly line. Given that such cis-ATs define large portions of polyketide structure, there continues to be much interest in utilizing them to diversify the structures of natural products.[1] In particular, ATs are often targeted for enzyme engineering to introduce non-native or non-natural extender units into polyketides.[2,3] Recently, the promiscuity of ATs has proven to be a useful platform for engineering ATs with new and orthogonal extender unit specificities,[4−8] thus providing a strategy for potential regioselective modification of polyketide structure. An emerging class of ATs differs from their canonical cis-counterparts in that they are standalone "discrete" enzymes. These "trans-ATs" utilize malonyl-CoA (M-CoA) and transfer the malonyl unit to their cognate polyketide synthase module(s).[9,10] Yet, a small number of trans-ATs natively installs more unusual extender units and can in principle be leveraged to diversify the structures of polyketide natural products. For example, the trans-AT KirCII in the biosynthesis of the antibiotic kirromycin utilizes ethylmalonyl-CoA (EM-CoA) and has been shown to display activity with several non-natural extender units.[11,12] This polyspecificity has been used to produce regioselectively modified kirromycin derivatives via precursor-directed biosynthesis, demonstrating the remarkable plasticity of the kirromycin assembly line.[13] All other trans-ATs that use unusual (i.e., nonmalonyl) extender units require ACP-linked extender units instead of CoA-linked substrates. However, nothing is known regarding the ability of trans-ATs to accept non-natural ACP-linked extender units. Indeed, the substrate scope of canonical cis-ATs that depend on ACP-linked extenders is also poorly described, while only a few examples of cis-ATs that use multiple extender units are known. For example, the cis-AT from the FK506 PKS accepts both ACP-linked and CoA-linked extender units in its natural pathway.[14] The module 4 cis-AT of the FkbB PKS incorporates an allylmalonyl unit through an ACP-linked substrate, while an ethylmalonyl unit is incorporated by the same AT through a CoA-linked substrate.[15]

Interestingly, the biosynthesis of zwittermicin A (ZMA) includes two ATs that each use relatively rare and distinct ACP-linked extender units.[16] Notably, the C10-amino group in ZMA is installed via the action of the trans-AT ZmaF, which transfers an aminomalonyl unit from the ACP-linked substrate aminomalonyl-ZmaH (AmM-ZmaH) to a single carrier protein within the ZMA assembly line, ZmaA-ACP1 (ZACP1) (Figure 1).[17] The next module in the ZMA assembly line incorporates

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Research Article

pubs.acs.org/synthbio

Development of Transcription Factor-Based Designer Macrolide Biosensors for Metabolic Engineering and Synthetic Biology

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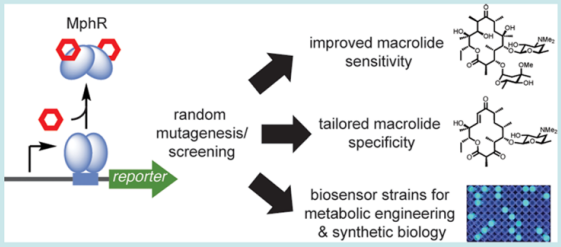
Supporting Information

ABSTRACT: Macrolides are a large group of natural products that display broad and potent biological activities and are biosynthesized by type I polyketide synthases (PKSs) and associated enzymatic machinery. There is an urgent need to access macrolides and unnatural macrolide derivatives for drug discovery, drug manufacture, and probe development. Typically, efforts to engineer the biosynthesis of macrolides and macrolide analogues in various microbial hosts are hampered by the complexity of macrolide biosynthetic pathways and our limited ability to rationally reprogram type I PKSs and post-PKS machinery. High-throughput approaches based on synthetic biology and directed evolution could overcome this problem by testing the function of large libraries of variants. Yet, methods that can identify mutant enzymes, pathways, and strains that produce the desired macrolide target are not generally available. Here we show that the promiscuous macrolide sensing transcription factor MphR is a powerful platform for engineering variants with tailored properties. We identified variants that displayed improved sensitivity toward erythromycin, tailored the inducer specificity, and significantly improved sensitivity to macrolides that were very poor inducers of the wild-type MphR biosensor. Designer macrolide biosensors should find broad utility and enable applications related to high-throughput synthetic biology and directed evolution of macrolide biosynthesis.



Macrolides are a group of diverse natural products that display broad and potent biological activities, including antibacterial, anticancer, antifungal, antiprotozoal, and immunomodulating activities.[1,2] Access to large quantities of macrolides and analogues thereof is critical for the discovery of new biological activities, optimization of pharmacological properties, and probe discovery and development.[3,4] Biosynthetic approaches to macrolide production offer enormous potential and numerous benefits compared to traditional chemical approaches. The scaffolds of macrolides are constructed by type I polyketide synthases (PKSs). These are large multifunctional protein complexes organized in a modular fashion.[5] Each module is responsible for the selection and installation of a ketide unit into the polyketide, while a terminal cyclase is responsible for regiospecific macrocyclization and/or hydrolysis of the polyketide chain. The number, identity, order of modules, and specificity of the cyclase describes the structure of the corresponding polyketide. These scaffolds are often further elaborated by tailoring enzymes to afford the mature, biologically active natural product.

Accordingly, these systems offer the potential for the synthesis of large quantities of polyketides *via* microbial fermentation and combinatorial biosynthesis of analogues by mixing and matching modules and tailoring enzymes. However, the sheer size, mechanistic diversity,[6] and poor understanding of how specificity and catalysis are controlled by type I PKSs and their associated post-PKS enzymatic machinery render rational design of new pathways difficult.[5,7,8] For example, many hybrid PKSs designed to produce polyketide analogues fail or are less active than wild-type machinery.[9,10] Moreover, tailoring enzymes with the requisite regioselectivity or substrate specificity/promiscuity are often unavailable to achieve late-stage functional modification of a given target macrolide. In addition, biosynthetic pathways often require expression in heterologous hosts to overcome limitations often associated with the intractable genetic systems, unknown regulatory systems, and poorly understood molecular biology of natural product producing microbes.[11] Critically though, whereas DNA synthesis and assembly technologies can access large numbers of refactored and engineered gene clusters to address this need, methods to report their ability to produce the desired natural product in similarly high throughput are not always available. Consequently, the full synthetic potential of type I PKSs and associated biosynthetic machinery has yet to be realized. However, synthetic biology and directed evolution offer an opportunity to overcome these challenges by testing the functions of large libraries of variants. Successes in this area

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Current Opinion in Microbiology

Engineering enzymatic assembly lines for the production of new antimicrobials

Edward Kalkreuter[1,2] and Gavin J Williams[1,2]

A large portion of natural products are biosynthesized by the polyketide synthase and non-ribosomal peptide synthetase enzymatic assembly lines. Recent advancements in the study of these megasynthases has led to many new examples that demonstrate the production of non-natural natural products. The field is likely nearing the ability to design and build new biosynthetic pathways de novo. We discuss the various recent approaches taken towards this goal, focusing on the installation of new substrates, the swapping of enzymatic domains and modules, and the impact of metabolic engineering and synthetic biology. We also address the challenges remaining alongside the many successes in this area.

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Introduction

Natural products are biosynthesized by a wide variety of enzymes and pathways, each with their own unique assembly strategies. Two of the largest classes, polyketides and non-ribosomal peptides, are synthesized by polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs), respectively. Together, polyketides and non-ribosomal peptides are wide-spread in Nature (averaging more than one gene cluster per bacterial genome) and demonstrate potent antimicrobial activities highlighted by such clinically-relevant compounds as erythromycin A, avermectin, vancomycin, and daptomycin [1]. Because of the significant value of these antimicrobials (> $20 billion annually for polyketides alone) and complex molecular structures, there is intense interest in engineering their producing biosynthetic pathways to access new natural products and analogues that fine-tune the biological activity and pharmacological profile [2].

In Nature, many polyketides are constructed by PKSs that comprises a series of linear modules (modular or type I PKSs), each responsible for the incorporation of a single malonyl-derived extender unit (Figure 1). In a module, an acyltransferase (AT) domain selects the appropriate extender unit, which is usually Coenzyme A (CoA)-linked. The malonate derivative is transferred from the AT to the acyl carrier protein (ACP), which acts as a shuttle to transfer all substrates and intermediates between the other domains. The ACP brings the extender unit to the ketosynthase (KS) domain, which catalyzes a Claisen condensation between the previously-extended chain and the extender on the ACP. The chain can be further decorated by other tailoring domains like the ketoreductase (KR), dehydratase (DH), enoylreductase (ER), or methyltransferase (MT). The fully-elongated chain is then released or cyclized by a thioesterase (TE) domain [3]. Like PKSs, NRPSs are also carrier protein-mediated systems, and as such, they are often found as hybrid systems with PKS components, like the rapamycin, rifamycin, and zwittermicin pathways. NRPSs function through an analogous set of discrete domains to the PKSs. Similar to the AT, the adenylation (A) domain of NRPSs is responsible for selecting the amino acid incorporated by each module. The peptidyl carrier protein (PCP) is equivalent to the ACP, and the condensation (C) domain performs like the KS. NRPSs also have additional cyclization, methylation, and reduction domains present [4].

Although accurate structural predictions of PKS-encoded and NRPS-encoded products are not yet fully possible from a given sequence of domains, there is a collinearity between the pathways and their products that invites pathway engineering for new natural products. This is best demonstrated by the ability to regioselectively alter the final product by targeting the corresponding module. Much attention in the synthetic biology community has been focused on accessing this new chemical space by engineering existing pathways and combining multiple pathways for analogue production, improving the titers of existing products, and uncovering the rules for working with this unique and powerful set of biosynthetic enzymes (Figure 2).

Challenges associated with engineering PKSs and NRPSs

Although there have been numerous successes towards reprogramming PKSs and NRPSs, there are still many

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Inversion of Extender Unit Selectivity in the Erythromycin Polyketide Synthase by Acyltransferase Domain Engineering

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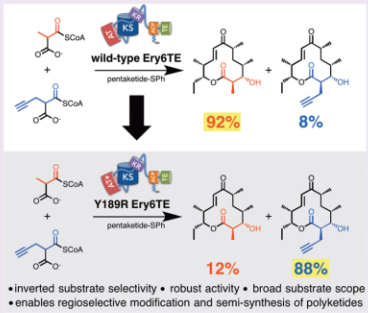
Supporting Information

ABSTRACT: Acyltransferase (AT) domains of polyketide synthases (PKSs) select extender units for incorporation into polyketides and dictate large portions of the structures of clinically relevant natural products. Accordingly, there is significant interest in engineering the substrate specificity of PKS ATs in order to site-selectively manipulate polyketide structure. However, previous attempts to engineer ATs have yielded mutant PKSs with relaxed extender unit specificity, rather than an inversion of selectivity from one substrate to another. Here, by directly screening the extender unit selectivity of mutants from active site saturation libraries of an AT from the prototypical PKS, 6-deoxyerythronolide B synthase, a set of single amino acid substitutions was discovered that dramatically impact the selectivity of the PKS with only modest reductions of product yields. One particular substitution (Tyr189Arg) inverted the selectivity of the wild-type PKS from its natural substrate toward a non-natural alkynyl-modified extender unit while maintaining more than twice the activity of the wild-type PKS with its natural substrate. The strategy and mutations described herein form a platform for combinatorial biosynthesis of site-selectively modified polyketide analogues that are modified with non-natural and non-native chemical functionality.



Type I polyketide synthases (PKSs) are huge mega-enzyme assembly lines that catalyze the condensation of acyl-CoA thioester building blocks to form the scaffolds of a large variety of clinically relevant polyketides.[1,2] PKSs are organized into modules of enzyme domains, whereby each discrete module is responsible for the installation and modification of a malonyl-derived extender unit into the growing polyketide chain[3] (Figure 1A). The acyltransferase (AT) domain of these modular PKSs controls the specific extender unit selected by each module, which ultimately dictates large portions of polyketide structure as these extender units are assembled into natural product scaffolds. Accordingly, ATs offer powerful potential opportunities for the synthesis of regioselectively modified analogues for optimization of pharmacological properties[4−8] and the development of molecular probes.[9]

Numerous studies have described the ability of AT domains to discriminate between extender units naturally offered to the PKS in the producing organism.[10,11] Consequently, AT-swapping and complementation of inactivated ATs by *trans*-ATs have been explored in attempts to direct the installation of alternative extender units into polyketides.[6,7,12,13] However, chimeric/hybrid PKSs are often completely inactive or display activity reduced by several orders of magnitude, compared to their wild-type counterparts.[13−15] Moreover, such approaches have been largely limited to the incorporation of naturally occurring extender units and by the narrow extender unit specificity of wild-type ATs. Thus, the ability to introduce diverse chemical functionality in a regioselective fashion by these approaches is limited by the inherent extender unit specificity of the AT.

To address these limitations, and in an effort to minimally perturb important structural features of PKSs, attempts have been made to alter the extender unit specificity of individual AT domains by site-directed mutagenesis.[14,16−18] The installation of a non-natural extender unit directed to a single position in a polyketide requires a mutant AT that no longer recognizes its natural extender unit but instead favors alternative substrates which themselves are not utilized by other ATs in the PKS (Figure 1B). To date, mutant ATs with inverted extender unit specificities have not been reported, largely due to our insufficient understanding of extender unit specificity in PKSs, and partly as a result of the difficulties associated with determining substrate specificity of mutant PKSs *in vivo*.[19]

Recently, our group reported that the terminal module from the 6-deoxyerythronolide B synthase (DEBS) displays remark-

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Polyketide Bioderivatization Using the Promiscuous Acyltransferase KirCII

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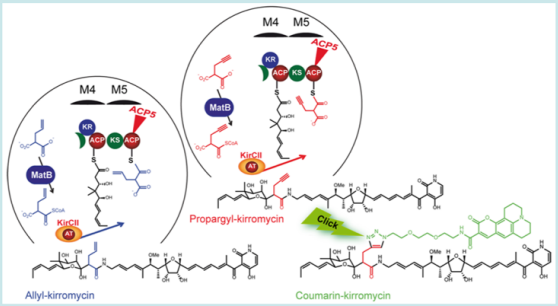
Supporting Information

ABSTRACT: During polyketide biosynthesis, acyltransferases (ATs) are the essential gatekeepers which provide the assembly lines with precursors and thus contribute greatly to structural diversity. Previously, we demonstrated that the discrete AT KirCII from the kirromycin antibiotic pathway accesses nonmalonate extender units. Here, we exploit the promiscuity of KirCII to generate new kirromycins with allyl- and propargyl-side chains *in vivo*, the latter were utilized as educts for further modification by "click" chemistry.



KEYWORDS: *antibiotic, kirromycin, polyketide synthase, trans-acyltransferase, engineering, click chemistry*

Polyketides are important secondary metabolites widely used as antibiotics, antifungals, and drugs for other clinical applications.[1,2] Erythromycin, mupirocin, rapamycin, FK506, lovastatin, and epothilone B are examples of antimicrobial, immunosuppressant, hypocholesterolemic and anticancer drugs, respectively. All these compounds are biosynthesized by complex multifunctional enzymes with modular architecture, the polyketide synthases (PKSs).

In typical modular type I PKSs, acyltransferase domains (ATs) select and load malonyl-CoA derived precursors onto acyl carrier proteins (ACPs). The AT domains are either embedded in the PKS (*cis*-ATs) or encoded by distinct genes and function *in trans* as discrete enzymes (*trans*-ATs).[3,4] After the AT-dependent loading step, ketosynthases (KSs) catalyze the condensation of ACP-linked extender units. Optional domains, such as ketoreductases (KRs), dehydratases (DHs), enoylreductases (ERs), or methyltransferases (MTs) further process the polyketide chain. In most cases, the product is released from the assembly line by a thioesterase (TE) domain and modified by post-PKS tailoring enzymes.

Notably, the AT domain determines which extender unit is incorporated into the growing polyketide chain.[5−7] Consequently, the extender unit specificity of the AT cumulatively dictates large portions of the final polyketide structure, and could be leveraged to improve the pharmacological properties of polyketide-based chemical entities through alteration of the precursors and/or ATs.[8] These features make AT engineering attractive for rational modification of polyketide assembly lines.

Directed engineering approaches such as AT domain swapping, AT site-directed mutagenesis[9] and cross-complementation of an AT-inactivated *cis*-PKS have been described for various polyketides, including erythromycin, rapamycin, tylosin, and geldanamycin.[10] However, most of the previous attempts to vary AT substrate specificity target *cis*-AT PKS pathways. Furthermore, all studies on extender unit variation involve nonalkyne precursors with the exception of 2-propargyl-erythromycin[11] and propargyl-premonensin,[12] which were

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