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 Graduates
Miles Calzini, Anuran Gayen, Jessie Garcia, Lexie Malico, Melissa Mitchler, Nichole Montero, Lindsay Nichols, Thaddeus Paulsel, Zhongtian Zhang

Current Undergraduates
Jenay Brown, Christian McRoberts, Breck Wcisel

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- Diverse projects to suit your needs
- Attend conferences
- Try your own ideas
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- Miles Anderson Award for Teaching
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- Internships at Novozymes, GSK etc.
- Involved with patent submission, tech transfer, NSF i-CORPS
- Collaboration with MBA program
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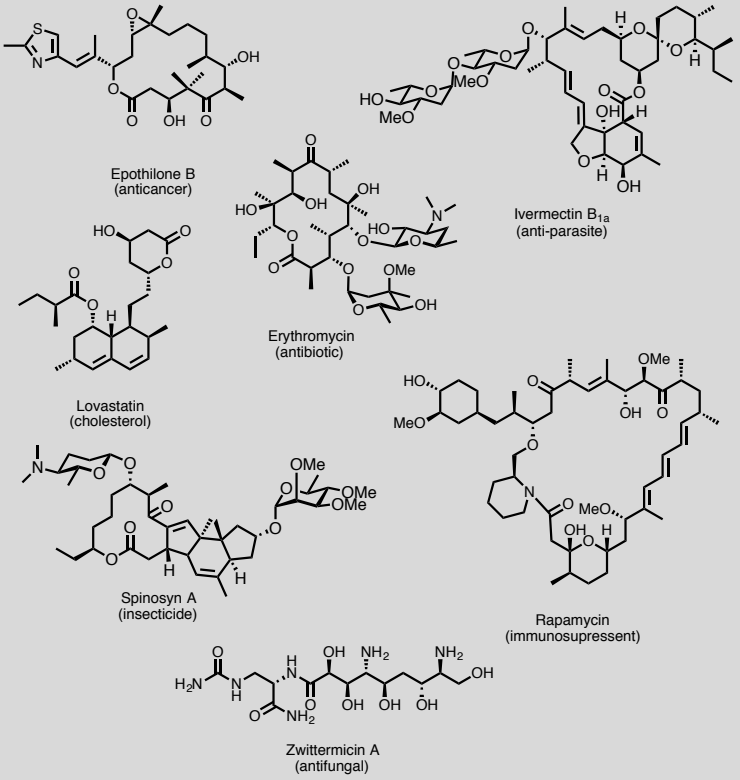
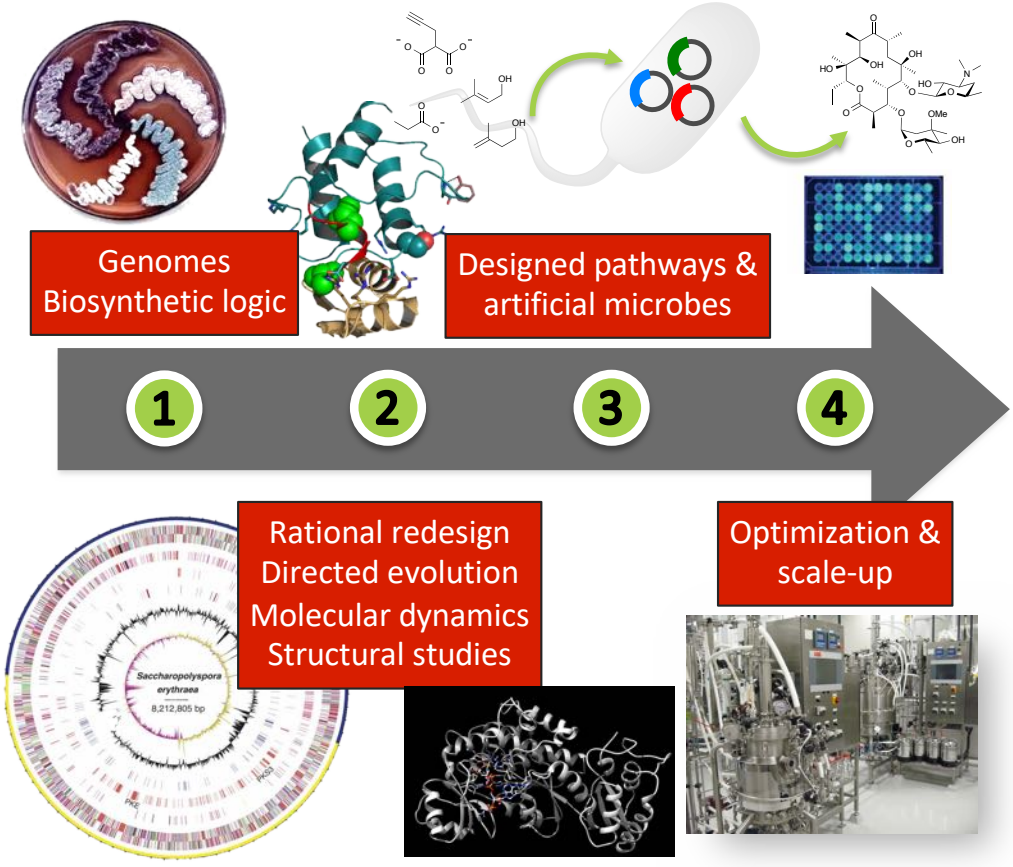
ACCESSING NEW DRUGS VIA NATURAL PRODUCT BIOSYNTHESIS

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.





Polyketides

Terpenes

Alkaloids

Peptides

Biofuels

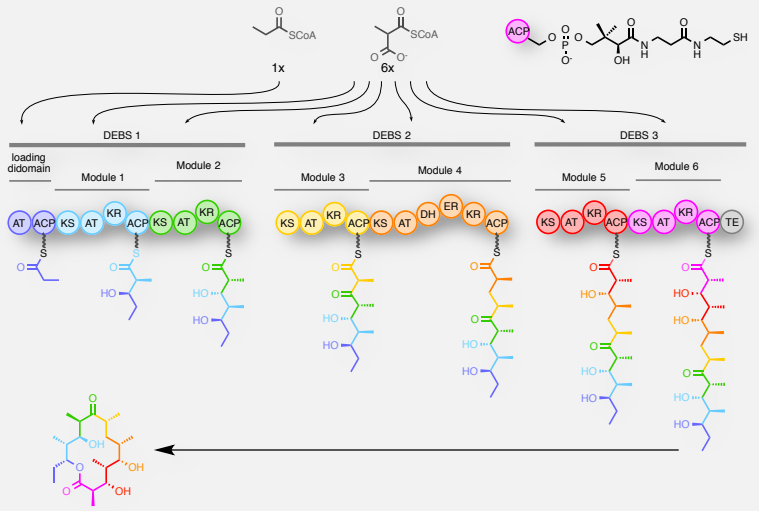
Commodity &

fine chemicals

EXAMPLES OF AVAILABLE PROJECTS

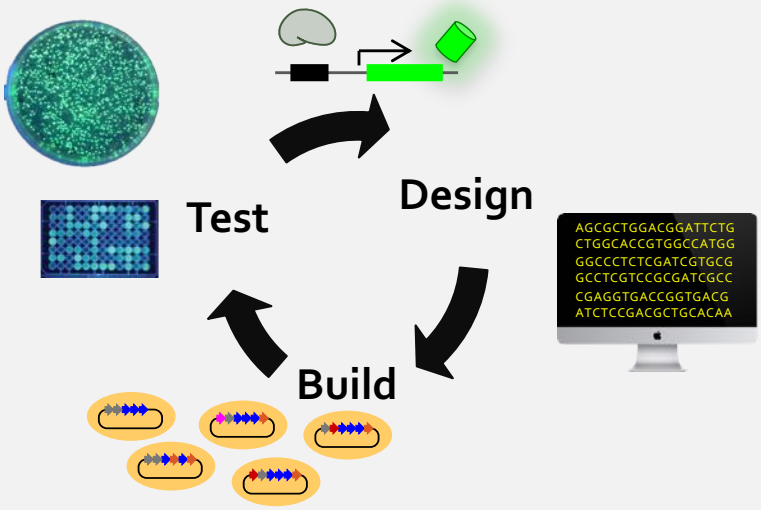
Engineering mega-enzyme assembly lines

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.



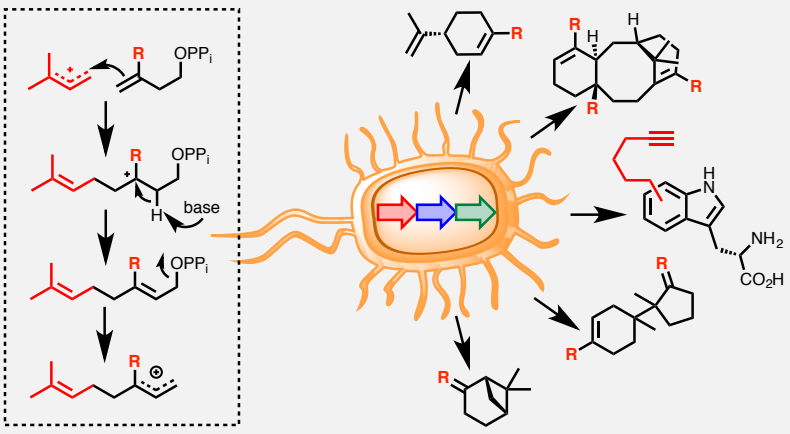
Biosensor-guided high-throughput engineering of biosynthetic pathways

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.



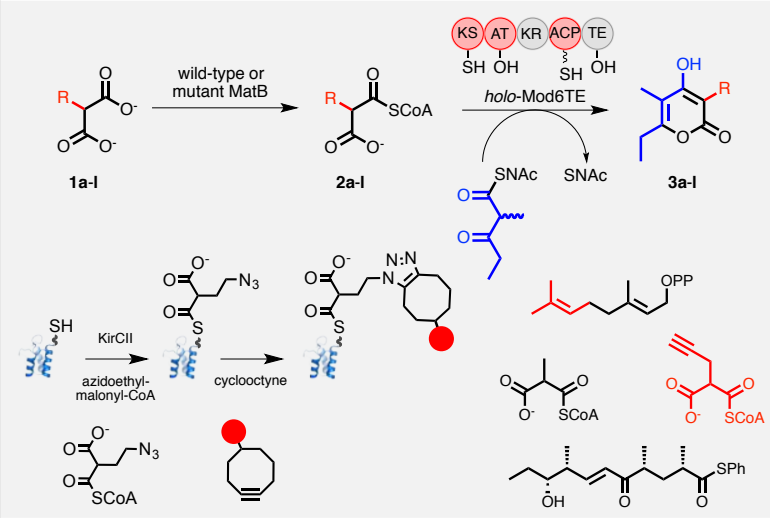
METHODS & TECHNOLOGY

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

Bristol-Myers Squibb

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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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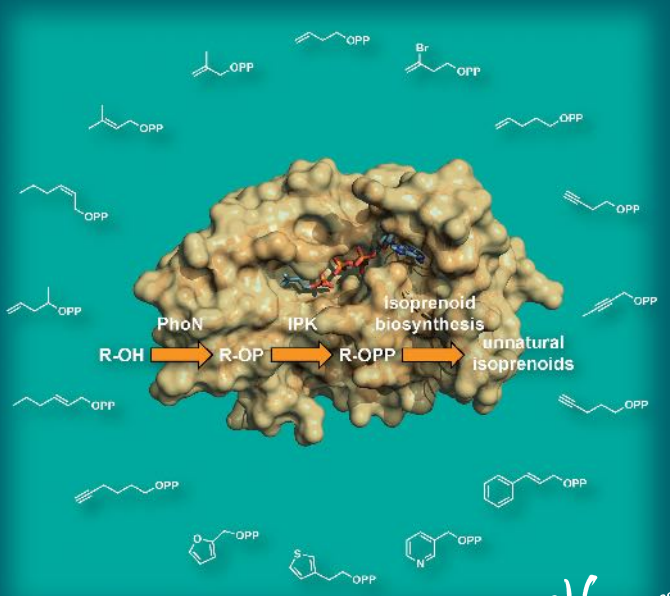
SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY



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G. J. Williams et al.
Probing the Substrate Promiscuity of Isopentenyl Phosphate Kinase as a Platform for Hemiterpene Analogue Production

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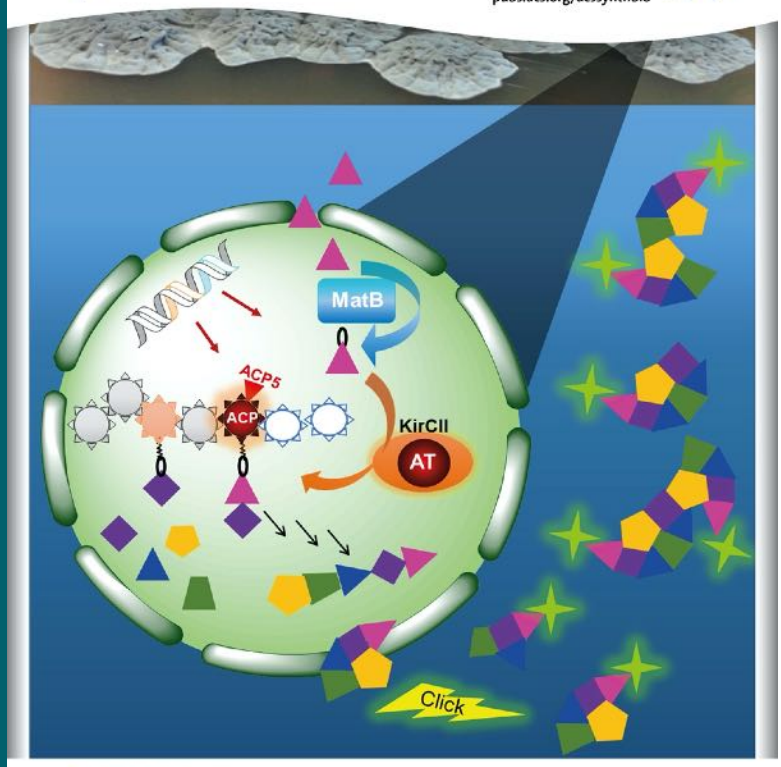

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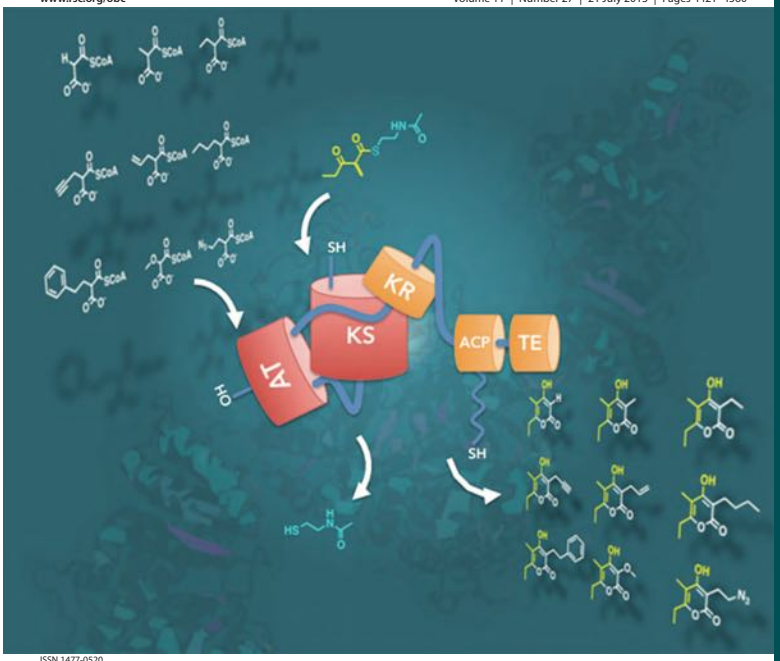

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RSC Publishing

PAPER
Gavin J. Williams *et al.*
Promiscuity of a modular polyketide synthase towards natural and non-natural extender units

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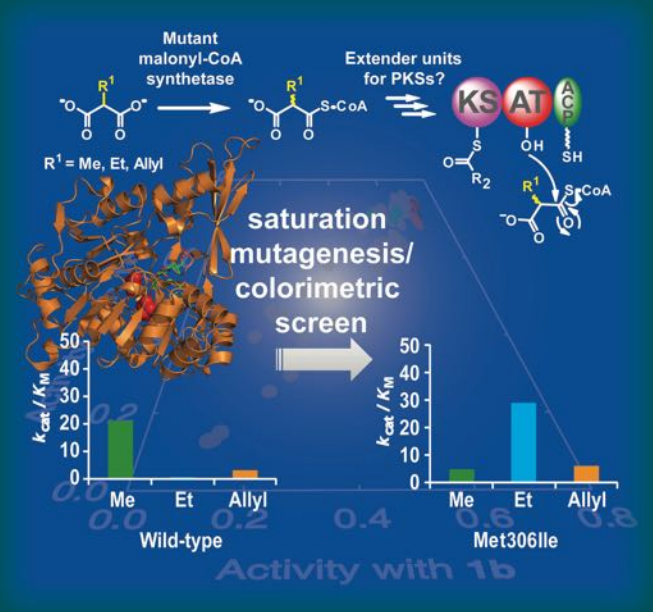

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The inside cover picture shows the shift in substrate specificity of malonyl-CoA synthetase towards non-native malonate analogues afforded by active-site saturation mutagenesis followed by colorimetric screening for improved activity. Mutant synthetases could provide extender units for probing the activity of polyketide synthases. For further details, see the paper by G. J. Williams and I. Koryakina on p. 2289 ff.

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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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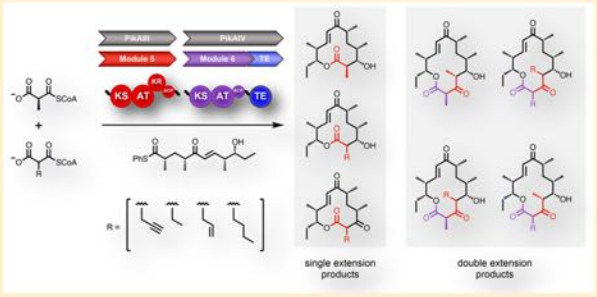
Supporting Information

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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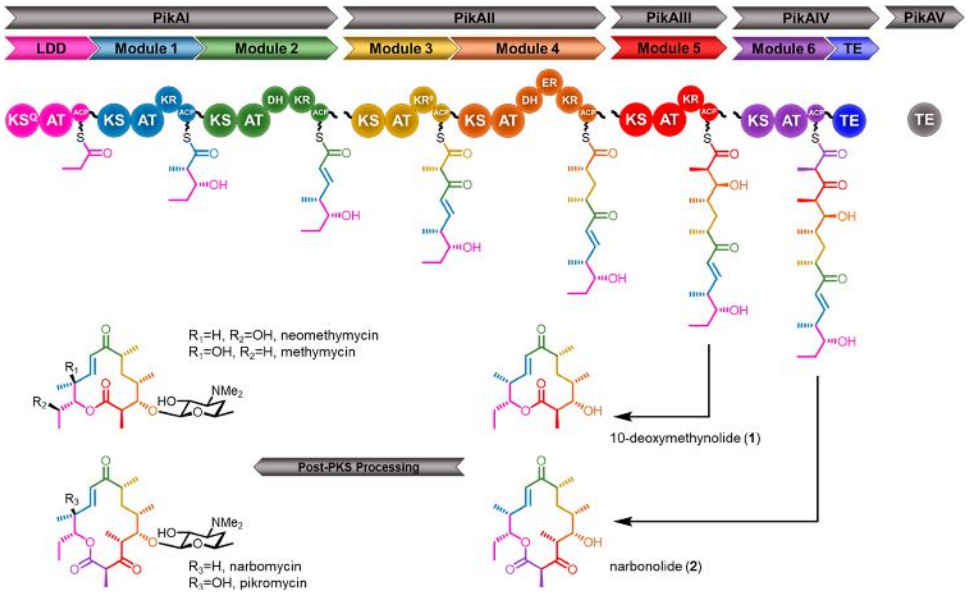

Figure 1. Pikromycin polyketide synthase and its products. ACP = acyl carrier protein; AT = acyltransferase; DH = dehydratase; ER = enoylreductase; KR = ketoreductase; KS = ketosynthase; KS^Q = ketosynthase-like decarboxylase; TE = thioesterase.

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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An Artificial Pathway for Isoprenoid Biosynthesis Decoupled from Native Hemiterpene Metabolism

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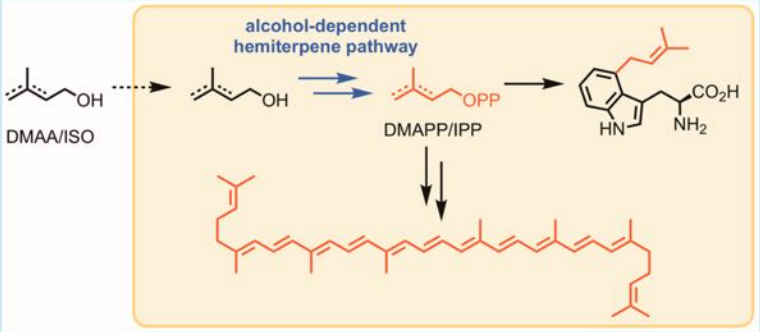
Supporting Information



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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CHEMBIOCHEM Communications

Probing the Substrate Promiscuity of Isopentenyl Phosphate Kinase as a Platform for Hemiterpene Analogue Production**

Sean Lund,[a, b] Taylor Courtney,[a, c] and Gavin J. Williams*[a, d]

Isoprenoids are a large class of natural products with wide-ranging applications. Synthetic biology approaches to the manufacture of isoprenoids and their new-to-nature derivatives are limited due to the provision in nature of just two hemiterpene building blocks for isoprenoid biosynthesis. To address this limitation, artificial chemo-enzymatic pathways such as the alcohol-dependent hemiterpene (ADH) pathway serve to leverage consecutive kinases to convert exogenous alcohols into pyrophosphates that could be coupled to downstream isoprenoid biosynthesis. To be successful, each kinase in this pathway should be permissive of a broad range of substrates. For the first time, we have probed the promiscuity of the second enzyme in the ADH pathway—isopentenyl phosphate kinase from *Thermoplasma acidophilum*—towards a broad range of acceptor monophosphates. Subsequently, we evaluate the suitability of this enzyme to provide unnatural pyrophosphates and provide a critical first step in characterizing the rate-limiting steps in the artificial ADH pathway.

Isoprenoids comprise >80 000 natural products for which methods to access and diversify their structures are in high demand given their broad applications as pharmaceuticals, additives, fragrances, cosmetics, biofuels, and platform chemicals to produce value-added products.[1] Notably, in nature all isoprenoids are built through the controlled assembly of two hemiterpenes: isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP).[2] Aside from the impressive product promiscuity and diversity of reactivities catalyzed by terpene cyclases and P450s, the use of only two building blocks by isoprenoid metabolism severely restricts the diversity of possible biosynthetically derived isoprenoid structures. To address this need, our group[3] and another[4] have recently demonstrated the ability of an artificial two-step biosynthetic pathway to produce the hemiterpenes IPP and DMAPP. Our prototype alcohol-dependent hemiterpene (ADH) pathway uses PhoN, a nonspecific acid phosphatase from *Shigella flexneri*,[5] in conjunction with isopentenyl phosphate (IP) kinase (IPK)[6] from *Thermoplasma acidophilum* (Figure 1 A). On simple provision of exogenous isopentenol (ISO) and dimethylallyl alcohol (DMAA), the ADH pathway was able to support hemiterpene production sufficient to drive lycopene production and the prenylation of an aromatic amino acid through the action of a promiscuous dimethylallyltryptophan synthase (DMATS).[3]

The short ADH pathway is therefore highly portable between microbial hosts. It could be easily optimized by enzyme and metabolic engineering, and also likely reduces the metabolic burden of the host strain. Furthermore, previous studies have shown that PhoN displays broad substrate specificity,[7] thus suggesting that a variety of non-native and unnatural alcohols could be processed by the prototype or engineered ADH pathway if IPK were also permissive. The substrate specificity of IPK is less well understood, although natural long-chain allylic isoprenoids are very poor substrates (this has been improved by enzyme engineering[8]), and two unnatural monophosphates have also been used, but with poor results.[6] Interestingly, the crystal structure of IPK reveals a hydrophobic pocket for the isopentenyl phosphate alkyl tail that could also accommodate substrate analogues (Figure 1 B).[9] However, the ability of IPK to utilize a broad array of diverse alkyl monophosphates has yet to be probed, so the full scope and utility of the ADH and similar pathways remains unknown. Accordingly, we set out to construct a panel of diverse monophosphates with which to probe the promiscuity of IPK and to begin to describe and understand its requirements for catalysis.

A codon-optimized IPK gene from *T. acidophilum*[6,9] (Table S1 in the Supporting Information) was synthesized and subcloned into pET28a. After overexpression in *Escherichia coli* BL21(DE3), the enzyme was purified by metal-chelate affinity chromatography. A panel of diverse monophosphates was synthesized in single-step fashion from their corresponding alcohols by using a previously reported synthetic procedure that provides mixtures of phosphorylated products (Scheme 1 A).[10] The panel was designed to include various alkyl chain lengths (C_4–C_{15}), degrees of branching (e.g., straight chain, α/β-branching), diverse substituents (e.g., bromo, aryl, and ester functions), and

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[**] A previous version of this manuscript has been deposited on a preprint server (https://doi.org/10.26434/chemrxiv.7765241.v1)

Supporting information and the ORCID identification numbers for the authors of this article can be found under https://doi.org/10.1002/cbic.201900135.

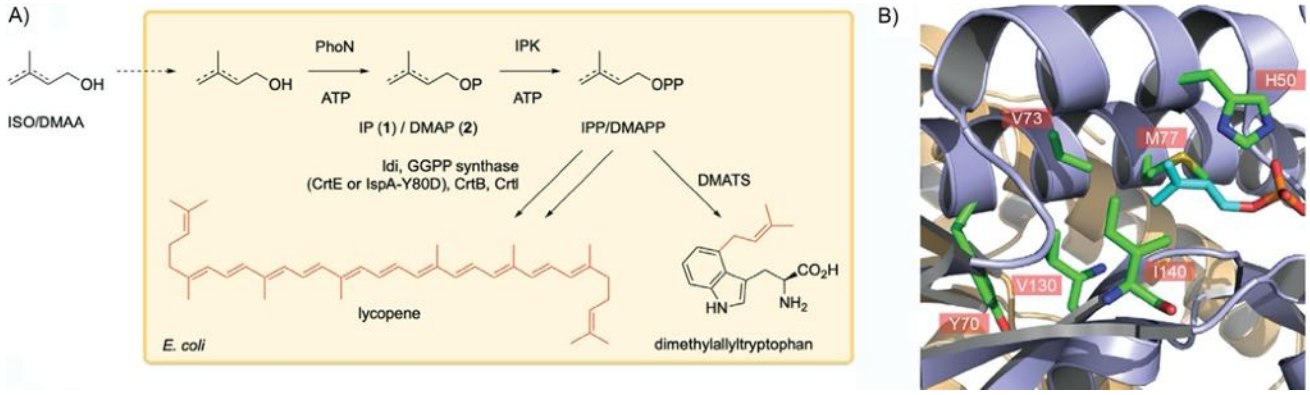
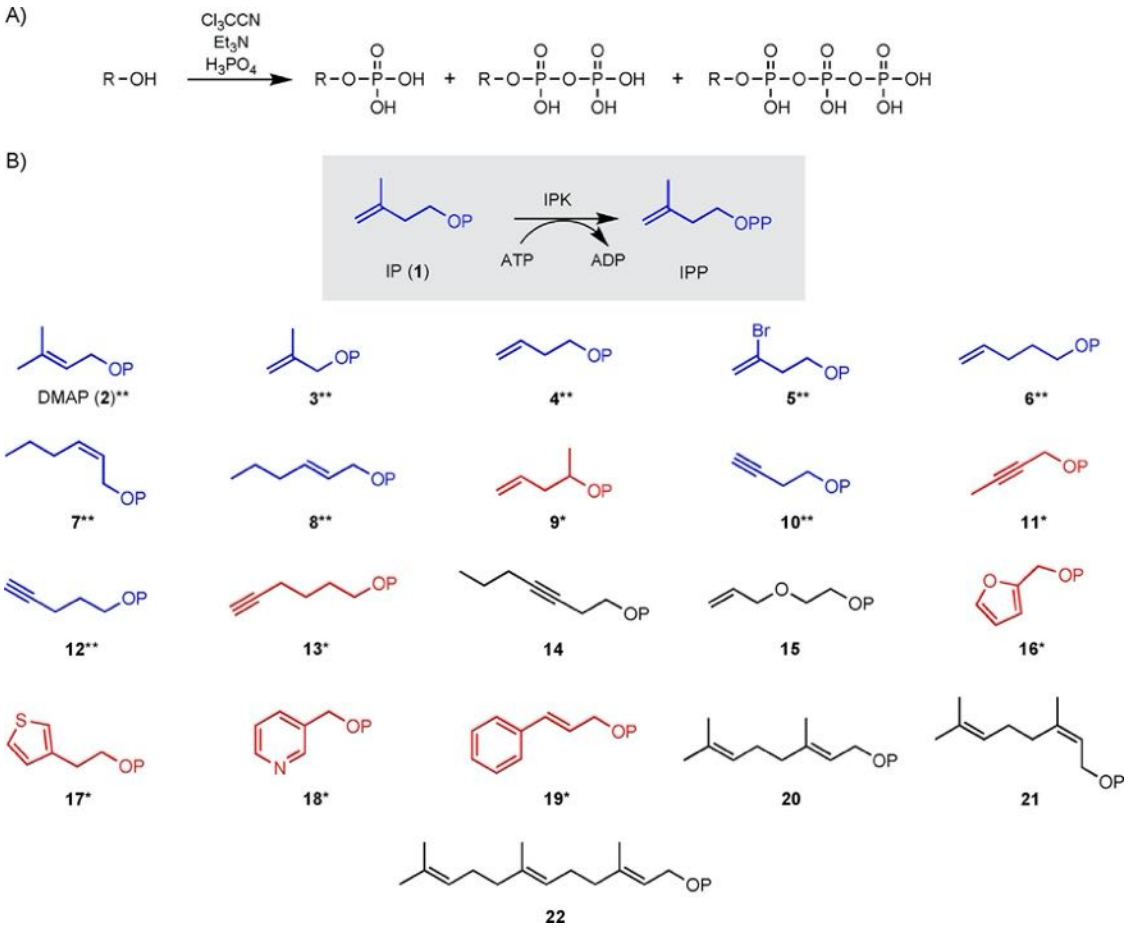


Figure 1. The two-enzyme "alcohol-dependent hemiterpene" (ADH) pathway. A) Scheme illustrating the roles of PhoN and IPK in converting ISO and DMAA into IPP and DMAPP. B) Active site of wild-type IPK (PDB ID: 3LKK). Residues surrounding the isopentenyl phosphate ligand (cyan sticks) are shown as green sticks.



Scheme 1. Substrate promiscuity of wild-type IPK. A) General synthetic procedure illustrating the preparation of a panel of alcohol monophosphates. B) Substrate scope of wild-type IPK determined by MS analysis; Blue (**): > 50% conversion by MS analysis. Red (*): < 25% conversion. Black: no conversion detected.

assorted degrees of unsaturation (alkenes, alkynes, aromatics) in order to characterize the substrate flexibility of IPK. After isolation of each alcohol monophosphate and confirmation of the product by HRMS analysis (Table S2), the substrate specificity of the enzyme was initially assessed in vitro by determination of the degrees of conversion to the corresponding pyrophosphates in the presence of ATP by low-resolution MS analysis of the IPK-catalyzed reaction mixtures (Scheme 1 B).

Remarkably, of the 22 monophosphates tested, 17 of them (**1**–**13**, **16**–**19**) gave ions, detected by MS, corresponding to

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Development of a Genetically Encoded Biosensor for Detection of Polyketide Synthase Extender Units in *Escherichia coli*

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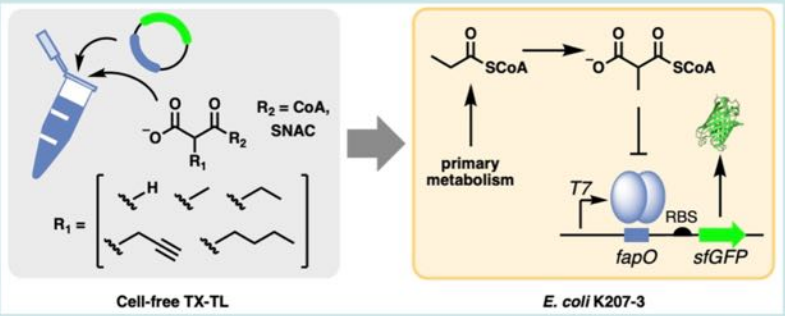
Supporting Information



ABSTRACT: The scaffolds of polyketides are constructed via assembly of extender units based on malonyl-CoA and its derivatives that are substituted at the C2-position with diverse chemical functionality. Subsequently, a transcription-factor-based biosensor for malonyl-CoA has proven to be a powerful tool for detecting malonyl-CoA, facilitating the dynamic regulation of malonyl-CoA biosynthesis and guiding high-throughput engineering of malonyl-CoA-dependent processes. Yet, a biosensor for the detection of malonyl-CoA derivatives has yet to be reported, severely restricting the application of high-throughput synthetic biology approaches to engineering extender unit biosynthesis and limiting the ability to dynamically regulate the biosynthesis of polyketide products that are dependent on such α-carboxyacyl-CoAs. Herein, the FapR biosensor was re-engineered and optimized for a range of mCoA concentrations across a panel of *E. coli* strains. The effector specificity of FapR was probed by cell-free transcription−translation, revealing that a variety of non-native and non-natural acyl-thioesters are FapR effectors. This FapR promiscuity proved sufficient for the detection of the polyketide extender unit methylmalonyl-CoA in *E. coli*, providing the first reported genetically encoded biosensor for this important metabolite. As such, the previously unknown broad effector promiscuity of FapR provides a platform to develop new tools and approaches that can be leveraged to overcome limitations of pathways that construct diverse α-carboxyacyl-CoAs and those that are dependent on them, including biofuels, antibiotics, anticancer drugs, and other value-added products.

KEYWORDS: *malonyl-CoA, polyketide, biosensor, transcription factor, synthetic biology*

Engineered bacterial transcriptional regulators have contributed immensely to chemical and synthetic biology by providing highly modular and tunable devices for sensing small molecules.[1−3] By coupling them to fluorescent or chromogenic readouts, transcription-factor-based biosensors have been applied as tools that detect key metabolites, regulate biosynthetic circuits, guide metabolic engineering, and enable directed evolution of enzymes and pathways.[4−7] Yet, biosensors for the detection of natural products and their biosynthetic precursors are not yet widely available, limiting the ability of high-throughput strategies to be applied to many important classes of molecules. For instance, malonyl-CoA (mCoA, Figure 1) plays an integral role in cellular primary and secondary metabolism as a building block for fatty acids, phenylpropanoids, polyketides, and hybrid natural products.[8] Because of this, mCoA biosynthesis has been a longstanding target of metabolic engineering efforts.[9−13] Accordingly, genetically encoded biosensors for the detection of mCoA have been constructed using FapR, a transcriptional regulator found in nearly all Gram-positive bacteria that acts as a global regulator for fatty acid biosynthesis.[14] FapR has been characterized as the only member of its regulator family owing to its unique, highly dynamic structure.[15,16] Crystal structures of FapR indicate a dimer, whereby each monomer is comprised of a C-terminal ligand-binding domain and an N-terminal domain that binds to its cognate DNA operator, *fapO*. Dependent on the promoter, FapR has been shown to act as

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

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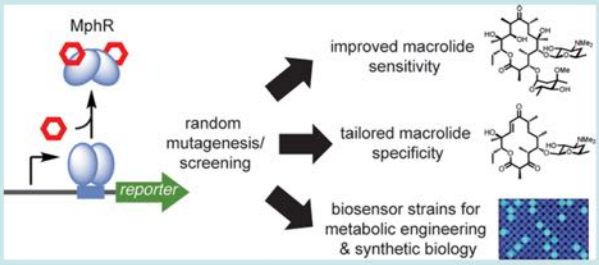
Supporting Information

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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