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Biocatalytic Synthesis of Pikromycin, Methymycin, Neomethymycin, Novamethymycin, and Ketomethymycin

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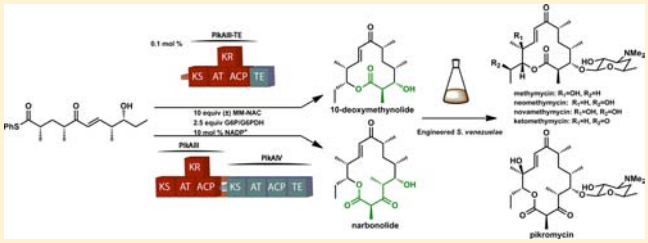
Supporting Information

ABSTRACT: A biocatalytic platform that employs the final two monomodular type I polyketide synthases of the pikromycin pathway in vitro followed by direct appendage of D-desosamine and final C−H oxidation(s) in vivo was developed and applied toward the synthesis of a suite of 12- and 14-membered ring macrolide natural products. This methodology delivered both compound classes in 13 steps (longest linear sequence) from commercially available (*R*)-Roche ester in >10% overall yields.



INTRODUCTION

Biocatalysis offers tremendous potential to complement conventional methods in synthetic chemistry.[1] Traditionally used for discrete chemical reactions, recent chemoenzymatic advances have enabled cascades that streamline synthesis by reducing step counts, increasing yields, and minimizing waste generation.[2,3] While such processes are becoming increasingly prevalent, they are typically employed in the production of relatively simple synthons for starting material or later incorporation into mature molecules. Conversely, the biosynthetic machinery involved in the generation of complex secondary metabolites could theoretically be leveraged at late stages of a synthesis to achieve similar improvement in step counts and overall yields. Specifically, modular type I polyketide synthases (PKSs) are of interest as a single polypeptide chain is capable of performing multiple regio- and stereospecific reactions while stabilizing otherwise labile oligoketide substrates.

Pikromycin (**1**) is a PKS-derived macrolide antibiotic belonging to the ketolide subclass, originally isolated in 1950.[4,5] The subsequent discovery of erythromycin A (**2**, Figure 1) and its success as a broad-spectrum antibiotic initiated drug discovery efforts through isolation and semisynthetic modification,[6] with third-generation macrolides treating front line infections almost 60 years later. Through ongoing strain improvement programs, fermentation-derived erythromycin provides the starting material for a number of clinically relevant antibiotics, including second-generation clarithromycin and azithromycin and the third-generation ketolides telithromycin (**4**), cethromycin, and solithromycin. Despite the availability of erythromycin as a commodity fine chemical, rigorous structure−activity relationship (SAR) studies of the ketolide class are constrained by the inherent limitations of semisynthesis using this complex natural product. For example, methods to directly remove, add, or modify alkyl groups in the macrolactone ring remain elusive with current synthetic technology. In contrast, de novo production of ketolides though total synthesis offers the opportunity to

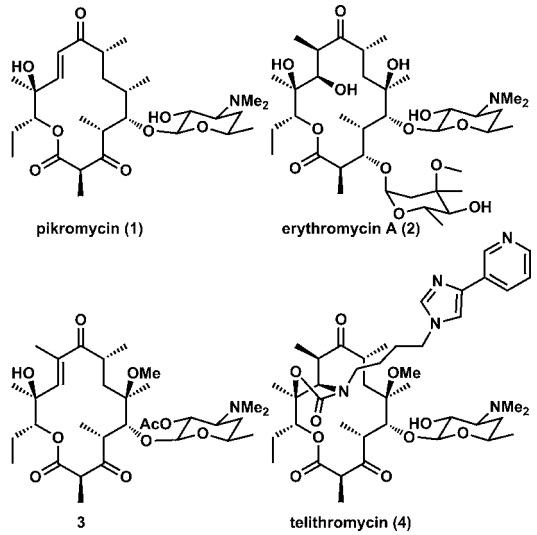


Figure 1. Macrolide antibiotics including ketolide **3**,[7] an intermediate en route to telithromycin from erythromycin A.

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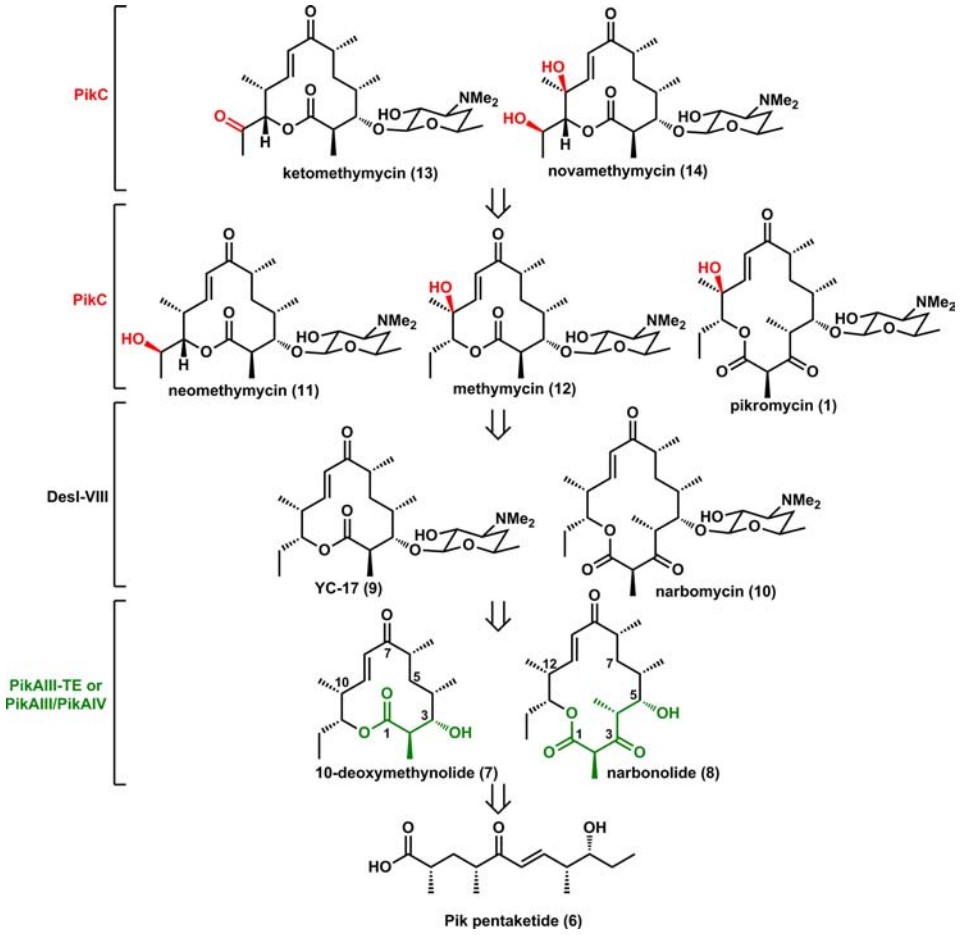
Scheme 1. Retrosynthetic Analysis[a]



[a](red) PikC: P450 catalyzing mono- and dihydroxylation of YC-17 (**9**) and narbomycin (**10**) macrolides. DesI-VIII: D-desosamine biosynthetic pathway catalyzing glycosylation of 10-deoxymethynolide (**7**) and narbonolide (**8**) macrolactones. (green) PikAIII-TE or PikAIII/PikAIV: PKS modules capable of converting Pik pentaketide (**6**) to macrolactones 10-deoxymethynolide (**7**) or narbonolide (**8**).

explore previously inaccessible chemical space for discovery of new anti-infective agents.[8−11] At present, this remains a formidable goal as conventional synthetic approaches understandably suffer from practical limitations, even in the case of natural macrolides or their simplified macrolactone[12,13] skeletons. Total syntheses have been previously reported for erythromycin[14−16] and pikromycin;[17] however, the innate complexity of these compounds requires high step counts, resulting in low overall yields. An underexplored route to macrolactone scaffolds employs PKS enzymes in a controlled in vitro environment. Development of biocatalytic approaches using modular PKSs has the potential to provide ready access to natural macrolactones and increase our functional understanding of these complex proteins toward creating libraries of novel macrolides in an efficient manner.

Bacterial PKS pathways mediate the extension and processing of polyketide chains in a stepwise, stereospecific manner, typically adding two (acetate) or three (propionate) carbon atoms per module.[18] In the case of propionate extension, methylmalonyl-coenzyme A (MM-CoA, **5**) serves as a masked enolate of the extender unit. The acyl transferase (AT) domain selects for methylmalonate and transfers this subunit to the phosphopantetheine (Ppant) arm of the acyl carrier protein (ACP), where the ketosynthase (KS) domain accepts a growing chain elongation intermediate from the previous module and catalyzes a decarboxylative Claisen condensation placing the extended chain onto the ACP for reductive tailoring. The ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains modify the chain at the β-position (depending on the module and the presence of processing domains), while the terminal thioesterase domain (TE) catalyzes cyclization of the macrolactone product.[19−22]

Streptomyces venezuelae ATCC 15439 produces a suite of 12- and 14-membered ring macrolide antibiotics with late-stage PKS modules (e.g., PikAIII and PikAIV) in the biosynthetic pathway demonstrating substrate flexibility.[23,24] The unnatural PikAIII-TE fusion protein produces the 12-membered ring macrolactone 10-deoxymethynolide (10-dml, **7**) from the *N*-acetylcysteamine thioester (NAC) (**21**) of the natural Pik pentaketide (**6**) (Scheme 1). Incubation with both PikAIII and PikAIV monomodules results in two propionate extensions followed by macrocyclization to yield the 14-membered ring aglycone narbonolide (nbl, **8**).[25] Although considerable progress has been made in microscale analysis of modular PKS systems using advanced polyketide chain elongation intermediates,[20,24,25] we were motivated to explore the possibility of a more efficient and scalable biocatalytic system.

From a practical perspective, preparative scale (>1 mmol) biocatalysis by type I modular PKSs has been impeded by a number of technical challenges including (i) slow loading of

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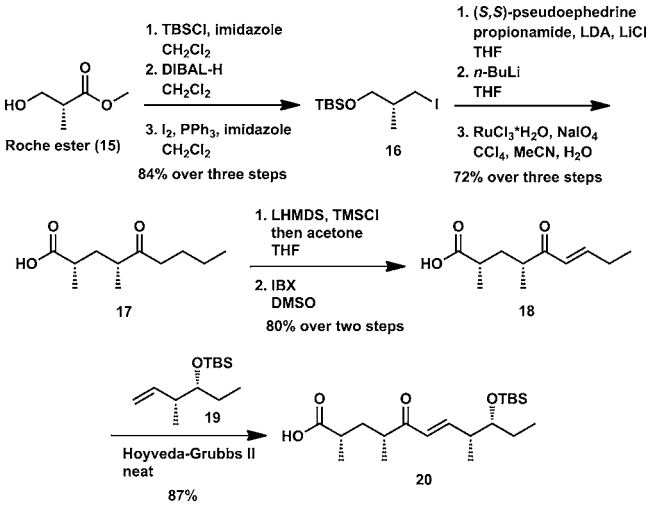
substrate onto the KS, (ii) stoichiometric and cost-prohibitive cofactors, and (iii) the large, fragile nature of PKS proteins. Accordingly, we sought to address these challenges and develop multifunctional PKS enzymes as options in the toolbox of synthetic chemistry. Finally, tailoring of the macrolactones to macrolides was envisioned through whole cell biotransformation to append D-desosamine and perform C−H oxidation(s) by the PikC monooxygenase (Scheme 2).

Scheme 2. Second-Generation[25] Approach to Pik Pentaketide



RESULTS

Synthesis of Pik Pentaketide. Retrosynthetically, we envisioned disconnection at the enone double bond, simplifying the necessary building blocks to α,β-unsaturated ketone **18**, a type II olefin,[26] matched to undergo cross metathesis with type I fragment **19**. Synthesis of enone **18** began with TBS protection of (*R*)-Roche ester (**15**),[27,28] which was subsequently reduced and iodinated to yield **16**. Alkylation with (*S*,*S*)-pseudoephedrine propionamide,[29] displacement of the auxiliary with *n*-BuLi, and RuO_4 oxidation[30] furnished saturated acid **17**. Conversion of **17** to enone **18** was envisioned through oxidation of the corresponding silyl enol ether.[31] Trapping the kinetic enolate of **17** with LDA/TMSCl at −78 °C proved problematic, as epimerization was observed through a presumed silyl ketene intermediate. Employment of LHMDS/TMSCl at −78 °C facilitated smooth trapping of the enolate,[32] and subsequent IBX oxidation of the crude silyl enol ether yielded compound **18**.[31] This two-step sequence initially demonstrated poor reproducibility but was improved through quenching excess LHMDS/TMSCl with a dropwise addition of acetone prior to an aqueous quench to provide **18** in good yield.[33] Common metathesis conditions were evaluated with 1.5 equiv of silyl ether **19** and 3 mol % of HG-II,[34] where the highest yields were achieved when the reaction was run neat at 50 °C for 12 h. Cleavage of the silyl ether with hydrogen fluoride in acetonitrile afforded the unactivated pentaketide **5**. With the desired substrate **20** in hand, we considered alternatives to NAC thioesters in an effort to increase efficiency of loading target KS domains to initiate PKS chain extension.

Synthesis and Evaluation of Pentaketide Thioesters. We envisioned four Pik pentaketide substrates of varying reactivity and aqueous solubility for direct PKS loading. NAC **21** was synthesized as described previously,[25] while **22**−**24** were generated from corresponding disulfides.[35] Activated pentaketides **21**−**24** were incubated with purified PikAIII-TE, extender unit, and hydride donor under nonoptimized conditions (Figure 2). QTOF-LC/MS analysis of 50 μL reaction mixtures after 1 h revealed significant differences in conversion between aryl thioesters and traditionally employed NAC thioesters. Thiophenol-activated **22** showed a 4-fold increase in conversion to 10-dml, while **23** and **24** demonstrated 2-fold increases. Furthermore, **22** showed no detectable hydrolysis in the absence of enzyme or in the presence of denatured enzyme, while both **23** and **24** underwent detectable hydrolysis. Compounds **21** and **22** were further analyzed via HPLC calibrated against a 10-dml standard curve providing absolute quantification along the reaction time course. Having demonstrated the superiority of thiophenol-activated pentaketide **22** to the previously employed **21**, attention was turned toward exploring alternatives to superstoichiometric quantities of MM-CoA (**5**) and NADPH as essential cofactors.

Evaluation of Superstoichiometric Cofactors. Replacement of MM-CoA is requisite to attain a practical chemoenzymatic platform due to its cost (>$1000/25 mg)[36] and poor

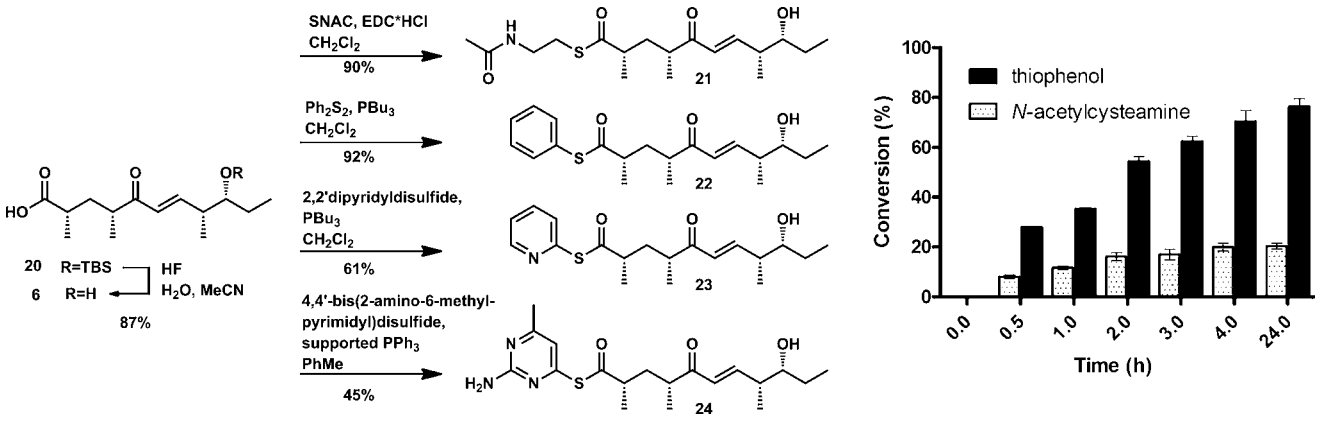


Figure 2. Synthesis and evaluation of activated Pik pentaketides. Enzymatic reaction conditions: 1 mM Pik pentaketide, 10 mM MM-NAC (vide infra), 0.5 mM $NADP^+$, 2.5 mM glucose-6-phosphate, 0.5 unit/mL glucose-6-phosphate dehydrogenase, 8 mM 2-vinylpyridine, and 1 μM purified PikAIII-TE, 1−24 h. Conversion of **21** and **22** to 10-deoxymethynolide (**7**) was monitored (HPLC) with data represented as mean ± standard deviation. n = 3.

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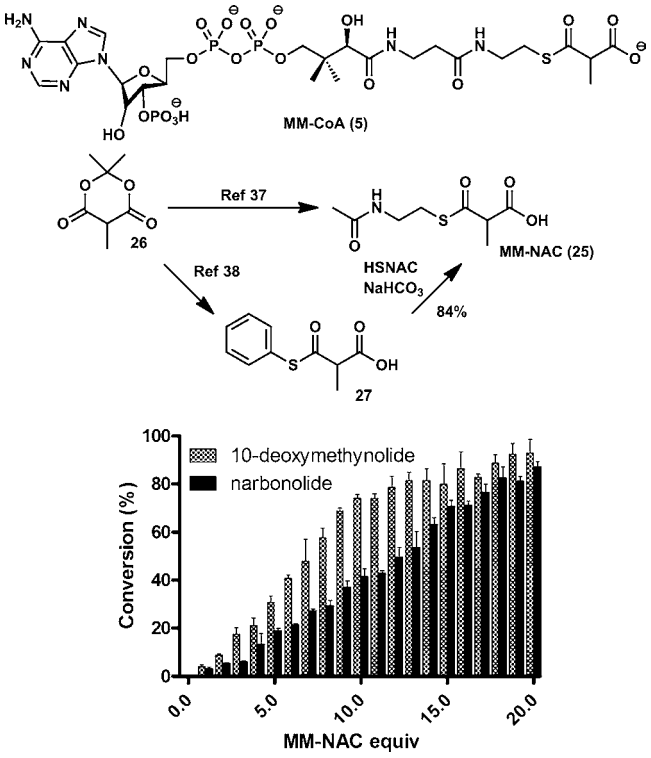
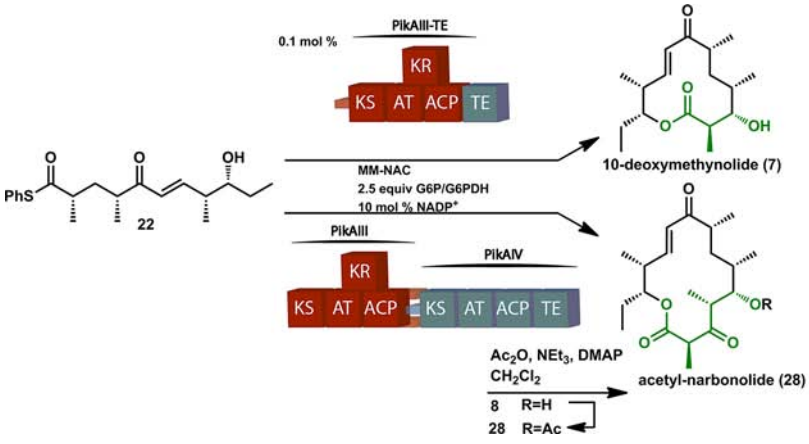


Figure 3. Synthesis and evaluation of methylmalonyl *N*-acetylcysteamine (MM-NAC, **25**). Enzymatic reaction conditions: 1 mM Pik pentaketide **22**, 1−20 mM MM-NAC, 0.5 mM $NADP^+$, 2.5 mM glucose-6-phosphate, 0.5 unit/mL glucose-6-phosphate dehydrogenase, 8 mM 2-vinylpyridine, and 1 μM purified PikAIII-TE or PikAIII/PikAIV, 4 h. Conversion of pentaketide **22** to 10-deoxymethynolide (**7**) or narbonolide (**8**) was monitored (HPLC) with data represented as mean ± standard deviation. n = 3.

atom economy. Khosla and co-workers reported methylmalonyl *N*-acetylcysteamine (MM-NAC, **25**) as a surrogate for MM-CoA in the erythromycin pathway (DEBS), albeit at higher concentrations.[37] In our hands, gram-scale synthesis of MM-NAC was hindered by difficulties in separation from side products due to the extreme hydrophilicity of the compound. An alternative two-step route proceeded through compound **27**, a crystalline solid available in a single step from methyl Meldrum's acid (**26**).[38] Transthioesterification[39] in aqueous sodium bicarbonate followed by acidification with cation exchange resin, washing of the aqueous phase with CH_2Cl_2, and subsequent lyophilization provided rapid access to decagrams of **25**. The stoichiometric dependence of MM-NAC was examined in a stop point assay after 4 h with both PikAIII-TE and PikAIII/PikAIV (Figure 3). While enzymatic NADPH recycling was potentially problematic in the presence of electrophilic thioesters, the glucose-6-phosphate/glucose-6-phosphate dehydrogenase system[40] proved sufficiently robust to be employed without modification. Ultimately, $NADP^+$ could be recycled effectively at 10 mol % relative to pentaketide substrate.

Semipreparative and Preparative PKS Catalysis. Following cofactor optimization, we examined the scalability of the in vitro macrolactone-forming PKS reactions. Scale-up of analytical 50 μL reactions to semipreparative 200 mL reactions (0.2 mmol **22**) while identical concentrations and stoichiometry were maintained provided 10-dml (**7**) in 53% yield and acetyl-narbonolide (**28**) in 47% yield, after silica gel chromatography (Figure 4). The observed precipitation of modular PKS proteins during the reaction spurred a final evaluation of conditions, and transitioning from purified protein to crude cell lysate while identical concentrations and stoichiometry were maintained demonstrated modest improvement, generating 10-dml in 62% yield or acetyl-narbonolide in 55% yield. Increased enzymatic stability enabled subsequent reactions to be conducted at a 4-fold greater concentration while the stoichiometry of MM-NAC decreased by a factor of 2, affording 10-dml in 66% yield or acetyl-narbonolide in 55% yield. With final reaction conditions in hand, preparative-scale catalysis with 0.5 g of Pik pentaketide **22** (1.43 mmol) generated 10-dml in 60% yield and acetyl-narbonolide in 49% yield.

Biotransformation of Macrolactones to Macrolides. As the final step toward targeted macrolides, we evaluated whole cell biocatalysis for introduction of D-desosamine and the final p450-mediated C−H oxidation(s). Engineered variants of *S. venezuelae* ATCC 15439 designated strains DHS2001[41] and YJ112[42] were assayed for their ability to transform macrolactones to macrolides (Figure 5). Observed variation in levels



entry	scale (mmol)	conc (mM)	MM-NAC (equiv)	PikAIII-TE Yield (%)	PikAIII/PikAIV Yield (%)
1[a]	0.2	1	20	53	47
2[b]	0.2	1	20	62	55
3[b]	0.2	4	10	66	55
4[b]	1.43	4	10	60	49

Figure 4. Semipreparative and preparative PKS cascade to macrolactones. Enzymatic reaction conditions: 1−4 mM Pik pentaketide **22**, 10−20 equiv of MM-NAC, 0.1 mM $NADP^+$, 2.5−10 mM glucose-6-phosphate, 0.5−2 unit/mL glucose-6-phosphate dehydrogenase, 1−4 μM purified[a] or cell free[b] PikAIII-TE or PikAIII/PikAIV, 4 h. Narbonolide was acetylated to **28** prior to chromatography to prevent degradation. Yields are calculated from isolated macrolactone after chromatography.

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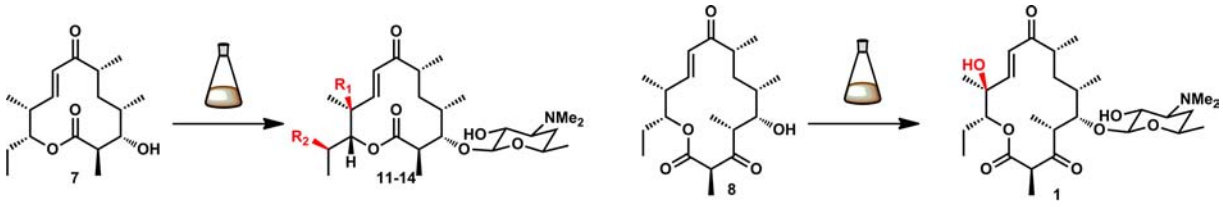

strain	combined yield (%)	methymycin (12)		neomethymycin (11)		novamethymycin (14)		ketomethymycin (13)
YJ112	71	3.5	:	5	:	3	:	1
DHS2001	66	3.5	:	7	:	1	:	trace

strain	pikromycin yield (%)
YJ112	72

Figure 5. Biotansformation of macrolactones to macrolides. Biotransformation conditions: macrolactone added to 100 mg/L with 2.5 mg/L acetyl-narbonolide (**28**) when the culture reached OD_{600} = 0.1, incubated for 48 h. Methymycin (**12**), R_1 = OH, R_2 = H; neomethymycin (**11**), R_1 = H, R_2 = OH; novamethymycin (**14**), R_1 = OH, R_2 = OH; ketomethymycin (**13**), R_1 = H, R_2 = O. Yields are calculated as the sum of differentially oxidized macrolides after HPLC purification.

of bioconversion from culture to culture (normalized by time and based on initial culture inoculation to addition of macrolactone) motivated studies to isolate and evaluate growth phase dependence. Addition of the macrolactone substrate to high OD_{600} DHS2001 cultures resulted in fast initial rates but incomplete conversions. By contrast, addition of macrolactone to prelog phase cultures resulted in the highest overall conversion to macrolide products. While conversion of 10-dml to methymycins (**11**−**14**) was undetectable with either strain, this was overcome by addition of acetyl-narbonolide (**28**)[43] to afford >99% conversion to target macrolides over 48 h. Addition of acetyl-narbonolide to narbonolide biotransformations increased rates and overall conversion (>99%) in a similar manner. Bioconversion of 10-dml with DHS2001 predominately afforded neomethymycin and methymycin, with low levels of novamethymycin isolated as well. *S. venezuelae* strain YJ112 mediated conversion of 10-dml with superior access to higher oxidation states, including increased levels of novamethymycin, and enabled discovery of the new macrolide ketomethymycin arising from a presumed dihydroxylation at the C-12 position. Addition of narbonolide to *S. venezuelae* strain YJ112 provided pikromycin in 72% yield.

DISCUSSION

Modern synthesis of polyketide natural products is routinely enabled by a chiral auxiliary methodology that predictably forms C−C bonds in a stereoselective, biomimetic manner. Prominent examples, including Myers alkylation[29,44] and Evans aldol[45] protocols and variants thereof, are commonly employed in constructing these complex secondary metabolites. Indeed, a recent synthesis of narbonolide [>19 steps (longest linear sequence) and <3% overall yield][13] and related macrolactone 6-dEB [22 steps (longest linear sequence) and 7.8% overall yield][46] relied heavily on these "practical laboratory emulations of the series of acylation/reduction reactions performed by polyketide synthases."[47] For example, an aldol reaction with *N*-propionyl-2-oxazolidone and propionaldehyde mimics one round of KS catalyzed propionate extension and KR reduction. In this approach, each new C−C bond formed requires further steps to protect the installed functionality, displace the auxiliary, and oxidize or otherwise activate the chain for the next C−C bond construction. The thiophenol-activated Pik pentaketide **22** was synthesized in this manner (11 linear steps and 38% yield), demonstrating the typical strengths (>90% yield per operation) and shortcomings (numerous redox and protecting group manipulations) of this approach. PKS catalysis offers an attractive alternative to synthetic emulators, as post C−C bond redox steps are performed on the protein with effective stabilization of labile polyketide chains. Covalent attachment of the chain elongation intermediate to the ACP eliminates the need for protective groups prior to subsequent elongation or cyclization.

Despite the clear potential for synthetic utility and more than two decades of investigation,[48,49] the development of in vitro PKS-based biocatalytic systems has been hindered by the inherent complexity of double these enzymes. Initial development was complicated by codependent factors that precluded optimizing individual reaction parameters in a stepwise manner. For example, while sensitive radioassays have been necessary to compensate for the inefficiency of most in vitro PKS reactions and the microscale at which they are conducted, their qualitative readout and low-throughput nature make them unsuitable for screening reaction conditions. Fortunately, increased accessibility to high-resolution LC/MS enabled us to avoid use of radioactive substrates and establish basic parameters (Figure 2) from which to proceed.

While evaluation of thioester leaving groups by QTOF-LC/MS illuminated striking improvement in substrate conversion and ultimately enabled the development of simple HPLC assays, initial experiments appeared less than promising. The NAC-activated Pik pentaketide **21** is sparingly water-soluble, despite the polarity imparted by NAC, and substitution to more hydrophobic thiophenol **22** led to a visible dispersion at 1 mM concentrations. Fortunately, loss of turbidity could be used to visually track the progress of the reaction, with consumption of starting material rendering a clear solution. The superior conversion of aryl thioesters compared to conventional NAC thioesters suggests that increased reactivity trumps possible KS−Ppant interactions. Of particular note is the apparent preference for the Pik pentaketide substrate to load the KS active site cysteine over the ACP Ppant arm, as concomitant ACP acylation would effectively remove the module from participating in catalysis. In retrospect, the observed fidelity in loading the PikAIII KS domain active site cysteine residue is surprising given the demonstrated efficacy of thiophenol thioesters in loading Ppant arms of PCP domains.[50−53] Ultimately, PKS-mediated conversion of Pik pentaketide proceeded with modest yield to 10-dml (60% yield, 23% overall from **15**) or acetyl-narbonolide (49% yield, 18% overall from **15**) at preparative scale, generating ∼250 mg of either macrolactone.

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Chemical glycosylation of both macrolactones with D-desosamine[54,55] and subsequent C−H oxidation through PikC monooxygenase have been described,[56] though complications obtaining and attaching D-desosamine significantly impede this approach. This amino sugar can be chemically synthesized in nine steps,[55,57] though it is more commonly harvested from degradation of erythromycin A.[58,59] Utilizing D-desosamine acquired from degradation requires a multistep sequence of protection, activation, glycosylation, and deprotection. Further, glycosylation of hindered secondary alcohols in either macrolactone has been challenging and proceeds with moderate yields.[17,55]

Alternatively, the direct biotransformation of macrolactones to macrolides presents an attractive option in converting 10-dml to methymycins (**11**−**14**) and narbonolide to pikromycin (**1**).[60] In this approach, the 10-dml and narbonolide macrolactones are natural substrates for the D-desosamine biosynthetic pathway and subsequent PikC oxidation within engineered variants of *S. venezuelae* ATCC 15439. Indeed, strain YJ122 efficiently converted crude narbonolide to pikromycin (72% yield, 13% overall from **15**). Bioconversion of 10-dml to methymycins proved to be tunable depending on the strain employed, with DHS2001 biotransformation affording primarily monohydroxylated products (combined 66% yield, 15% overall from **15**). *S. venezuelae* strain YJ112-mediated conversion of 10-dml demonstrated increased accessibility to dihydroxylated novamethymycin and provided access to previously unknown ketomethymycin (combined 71% yield, 16% overall from **15**). Careful analysis of wild-type *S. venezuelae* ATCC 15439 culture revealed production of a compound having an identical mass and retention time to synthetic ketomethymycin, suggesting that this compound is indeed a secondary metabolite made in trace quantities under normal laboratory culture conditions.

CONCLUSION

Advances in polyketide chain elongation intermediate activation and optimization of in vitro reaction parameters yielded practical type I PKS biocatalysis for scalable de novo macrolide production. The flexibility of the Pik pathway enabled divergent generation of either 10-dml or narbonolide and optimized whole cell biotransformations that effectively tailored macrolactones to macrolides, with *S. venezuelae* strain YJ112 enabling access to ketomethymycin bearing a previously unobserved oxidation state at the C-12 position. This platform offers practical entry to natural 12- and 14-membered ring macrolides from the Pik pathway, and the potential of this system to produce unnatural macrolides will be reported in due course.

ASSOCIATED CONTENT

Supporting Information

Full experimental details and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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