Type I Polyketide Synthases: Methodology, Biocatalysis, and Evaluation of Substrate Promiscuity

by

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This report, by its very length, defends itself against the risk of being read.

- Sir Winston Churchill

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Abstract

The gram-positive prokaryotes of the *Streptomyces* genus are prolific producers of secondary metabolites including a plethora of complex polyketide compounds. These natural products are constructed through decarboxylative Claisen condensations of simple malonic acids from primary metabolism by multidomain, modular enzymes called polyketide synthases (PKS) in a manner analogous to an industrial assembly line. A prominent example of one such pathway is the pikromycin (Pik) cluster from *S. venezuelae* ATCC 15439, which biosynthesizes a suite of 12- and 14-membered macrolide antibiotics. This pathway has been a workhorse in the Sherman lab for in vivo work, in vitro biochemistry, and more recently, biocatalysis and in depth structural analysis.

This dissertation describes synthetic chemistry, in vitro biochemistry, and in vitro biocatalysis focused on the final two PKS modules from the Pik cluster, PikAIII and PikAIV. First, the native pentaketide from the Pik pathway was chemically synthesized and employed to optimize in vitro biochemistry/biocatalysis with PikAIII-TE and PikAIII/PikAIV, culminating in a biocatalytic platform for macrolide production in 13 linear steps. Next, the native hexaketide from the Pik pathway was synthesized from fermentation derived 10-deoxymethynolide and employed to optimize in vitro biochemistry of PikAIV and excised Pik thioesterase (TE) domain, revealing the ability to control the catalytic cycle of PikAIV and gain entry to 12- or 14-membered macrolactones with greater than 10:1 selectivity for either ring size. Finally, we simulated "combinatorial biosynthesis" in a controlled in vitro environment with PikAIII-TE and PikAIII/PikAIV to identify catalytic bottlenecks using unnatural pentaketide substrates that mimic engineering early in the pathway. Analyses of results generated to date indict the TE domain as the bottleneck in combinatorial biosynthesis, and a crucial target for protein engineering.

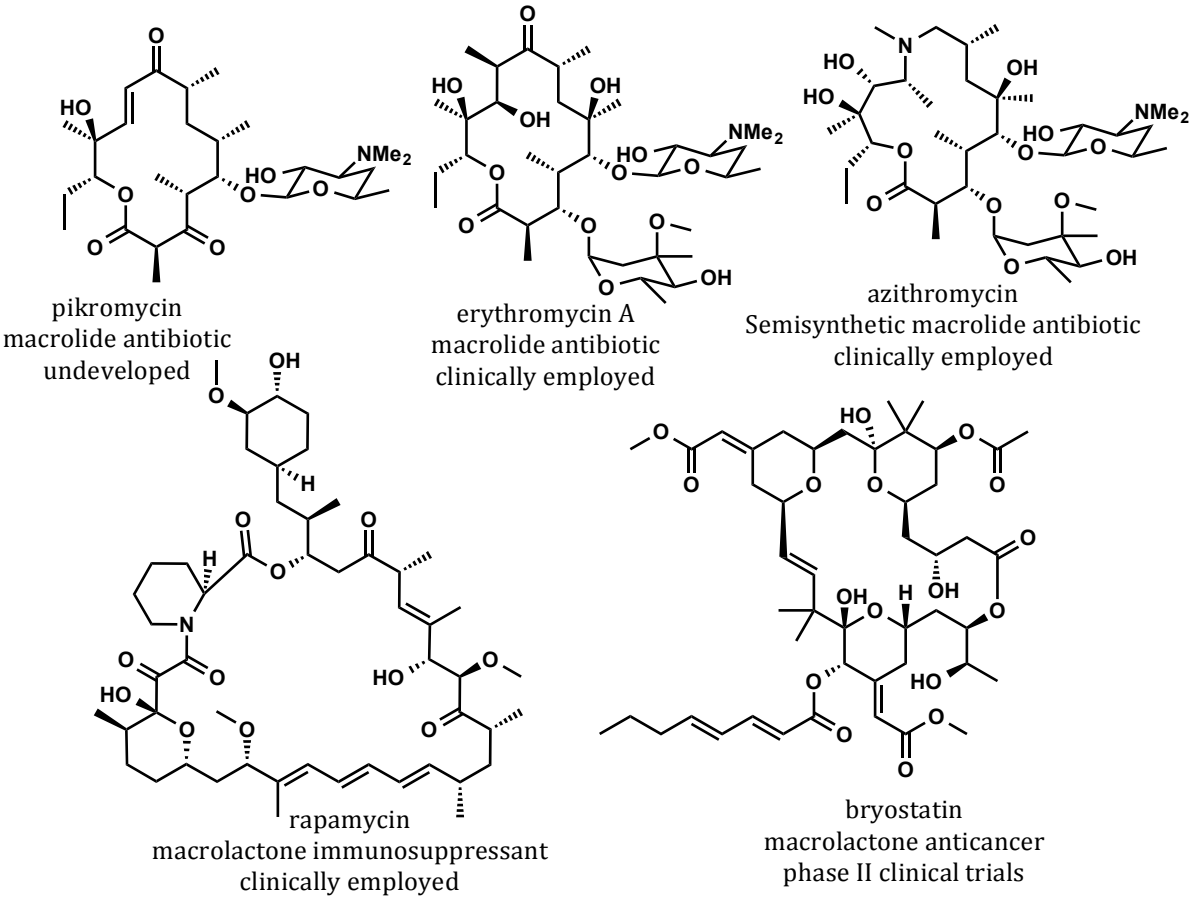
Chapter 1

Type I Polyketide Synthases and Polyketide Natural Products

1.1 Introduction

Polyketide natural products have been clinical mainstays for over sixty years, with prominent examples employed in the treatment of an impressively diverse range of diseases (Figure 1.1). Pharmacological properties ranging from antimicrobial, antiparasitic, anticancer, to immunosuppressive activities are attributed to polyketides, and many are indispensible to human and veterinary medicine.[1] These intricate natural products have garnered wide spread attention from medical, chemical, pharmacological, and biological scientific communities, each attracted to different facets of these compounds; therapeutic potential, structural complexity, synthetic construction (total synthesis and method development), pharmacological target elucidation, biosynthesis, and analog generation.[2]

Figure 1.1 Polyketide natural products

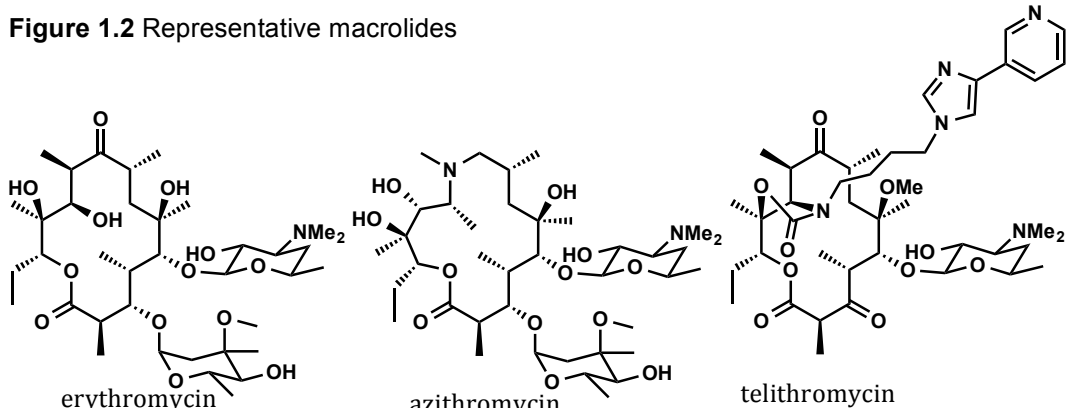


The story of the macrolide antibiotics begins in 1950 with the isolation of pikromycin,[3] which was quickly overshadowed by the more efficacious erythromycin A[4] entering the clinic in the mid 1950s. R.B. Woodward coined the term macrolide in 1957 as a portmanteau of macrolactone glycoside, the essential components of this class of antibiotics.[4a] Macrolides are potent antibacterial agents that disrupt protein synthesis by selectively binding to the 50S subunit of the prokaryotic ribosome.[5]

Recent studies implicate macrolides as sequence selective allosteric modulators of the ribosomal peptidyl transferase center (PTC), and as physical impediments for the growing peptide chain in the nascent peptide exit tunnel (NPET).[5] In addition, macrolides are known to disrupt the process of ribosome assembly by binding to the 50S subunit before full particle assembly.[6] While erythromycin is still commonly prescribed 60 years after its introduction, the macrolide class has evolved substantially over time to afford dramatically improved properties.[4a]

Erythromycin, fermented industrially from improved strains of *Saccharopolyspora erythraea,* is considered a first generation macrolide. While erythromycin is a potent antibiotic, it suffers from a number of shortcomings, including acid catalyzed degradation in the human stomach to a spiroketal motilin agonist, yielding painful stomach cramps.[4a] As such, erythromycin is administered orally with an enteric coating, which allows the drug be released in more hospitable intestinal environment.

Figure 1.2 Representative macrolides

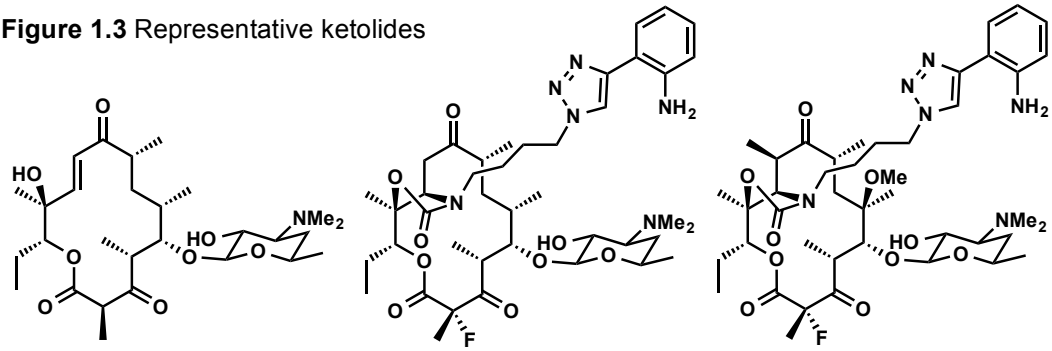


The class evolved to second generation macrolides with the introduction of clarithromycin and azithromycin (Figure 1.1) which enjoy improved pharmacokinetic (PK) and pharmacodynamic (PD) properties. Clarithromycin differs from erythromycin by a single methyl group installed on the C-6 hydroxyl group, which improves the acid stability of the drug. Azithromycin is an expanded 15-membered macrolide, which is synthesized from erythromycin though an oxime mediated Beckmann rearrangement and subsequent methylation of the installed secondary amine. Azithromycin, distributed as the "Z-Pak," is one of the most prescribed drugs of all time as it is able to treat many types of infections when taken just once daily for 3-5 days. As with all classes of antibiotics to date, resistance arose to both erythromycin and the second generation

macrolides. While the second generation mainly sought to improve PK/PD properties, the third generation addressed acquired resistance.

Macrolide resistance is conferred through methylation of the RNA bases within the ribosome to decrease binding efficacy, efflux of the macrolide out of the cell, and less commonly, by direct modification of the macrolide through hydrolysis of the macrolactone, phosphorylation, or glucosylation.[4a] MLS_B (macrolide, lincosamides, streptogramin B) resistance is conferred through mono- or dimethylation of A2058 in 23S ribosomal RNA. MLS_B is most commonly encountered though induction of erm (erythromycin resistance methylase) genes, though there are examples of constitutively expressed erm genes. Induction of ermC occurs when mRNA coding for a 19-amino acid peptide preceding the ermC gene stalls after 9-amino acids have been translated (in the presence of erythromycin.)[7] This leads to a change in the mRNA secondary structure and allows the ribosome to bind to the previously inaccessible ermC ribosome binding site (RBS) and translate ermC. This process requires the presence of the *L*-cladinose sugar of erythromycin, as such, third generation macrolides are known as "ketolides" where *L*-cladinose has been cleaved and the resulting C3 hydroxyl group is oxidized to a ketone.

Figure 1.3 Representative ketolides



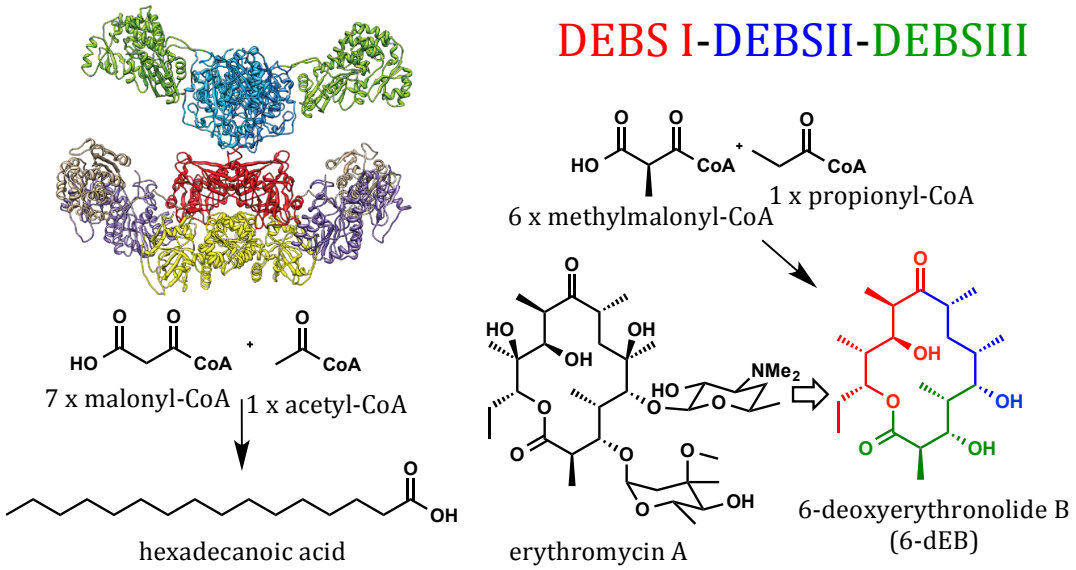
Pikromycin is naturally a ketolide, and while pikromycin and ketolides derived from erythromycin display only weak antibacterial activity, the induction of MLS_B genes is greatly diminished. Screening of heterocyclic side chains to improve binding efficacy generated telithromycin (approved 2004), cethromycin, and solithromycin and conferred another benefit, evasion of efflux pumps. Telithromycin and other ketolides are able to bind to two or more sites on the ribosome, and have been observed to overcome even constitutively expressed erm genes in some cases.[8] While the evolution of the macrolide class is a triumph of modern medicine, further exploration of macrolide chemical space is extremely limited.

While briefly mentioned above, all clinically employed macrolides are furnished through semi-synthesis of fermentation-derived erythromycin limiting modification of deep-seated functionality. Furthermore, performing chemistry on a complex natural product requires intensive synthetic efforts. Installing a single methyl group onto the C6 hydroxyl group requires a multi-step

sequence to generate clarithromycin, and conversion of clarithromycin to telithromycin requires 8 more steps.[9] Total synthesis of macrolides is achievable[10] but is constrained to academic interest, as the complexity of these compounds requires understandably long synthetic schemes and low overall yields. Though total synthesis is unlikely to provide metric tons of designer macrolides in the foreseeable future, the biosynthetic machinery used to satisfy clinical demand for macrolides could yield macrolide libraries through direct fermentation if the natural products community can understand and manipulate these complex pathways. Furthermore, following identification of a lead unnatural macrolide, industrial fermentation to produce the API (or starting material thereof) would fall within existing workflows.

Isolation and utilization of polyketide natural products predates identification of the biosynthetic machinery responsible for the biogenesis of these compounds by several decades. The early 1990's enjoyed a polyketide renaissance, with identification and subsequent cloning of type I polyketide synthases.[11] The genes coding for PKS proteins were aligned with domains known in type I modular fatty acid synthases (FAS, Figure 1.4).[12] The homology between PKS and FAS genes supported the long held hypothesis that polyketides were biosynthesized in a manner analogous to fatty acids,[12] with a few key differences.

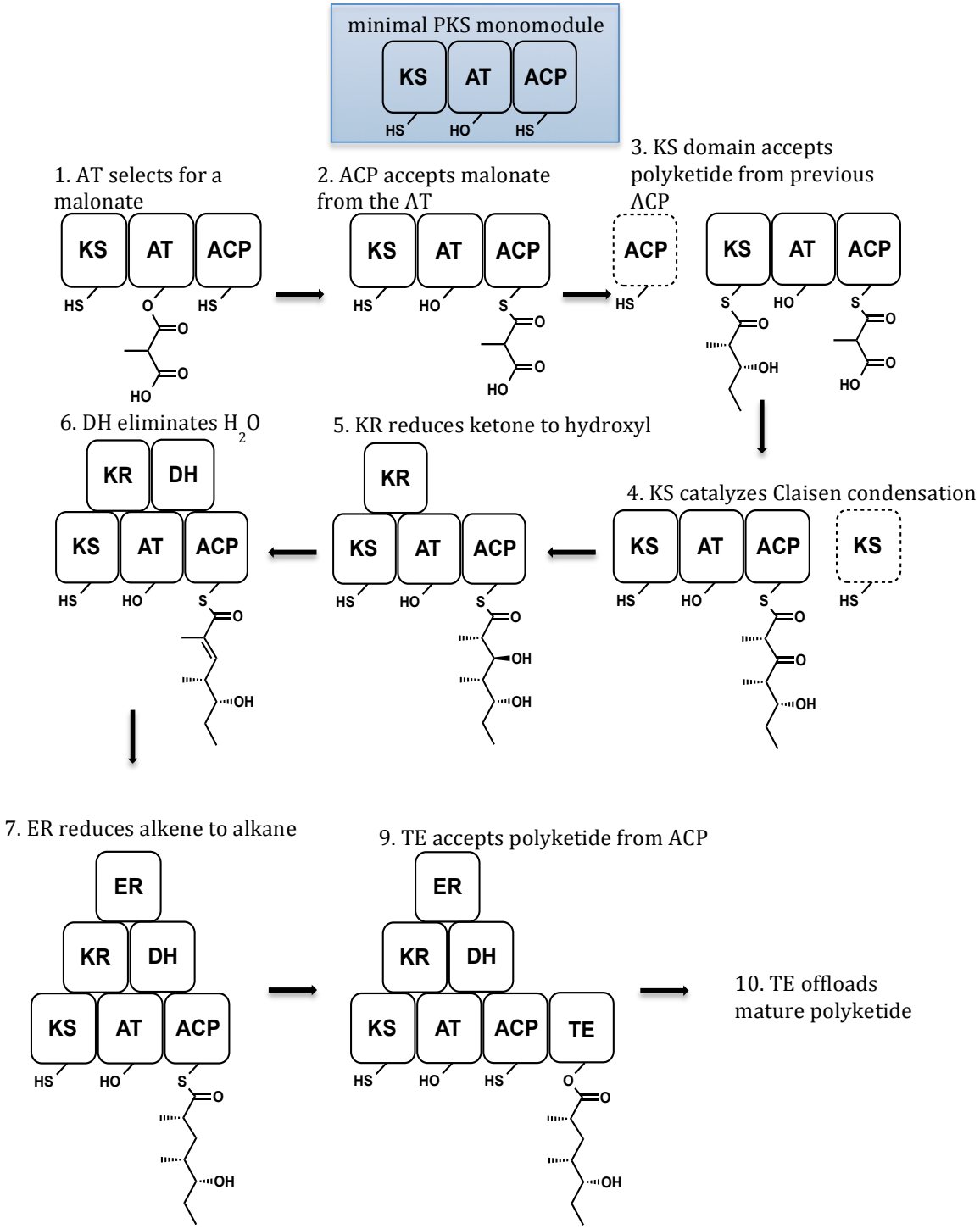
Figure 1.4 Fatty acid and polyketide synthases



Type I FAS enzymes biosynthesize fatty acids in an iterative manner, where malonyl-Coenzyme A (M-CoA) delivers malonate and iterative rounds of decarboxylative Claisen condensations and β-keto tailoring yield a mature chain, which is then released from the synthase. PKS modules, on the other hand, only perform a single decarboxylative Claisen condensation from a specified malonate, and β-keto tailoring domains need not reduce to the alkane (though possible), before transfer to the next module. Through this process, total control is exerted over the growing

polyketide chain, allowing for precise biosynthesis of natural products.

Scheme 1.1 Type 1 polyketide synthase catalytic cycle(s)

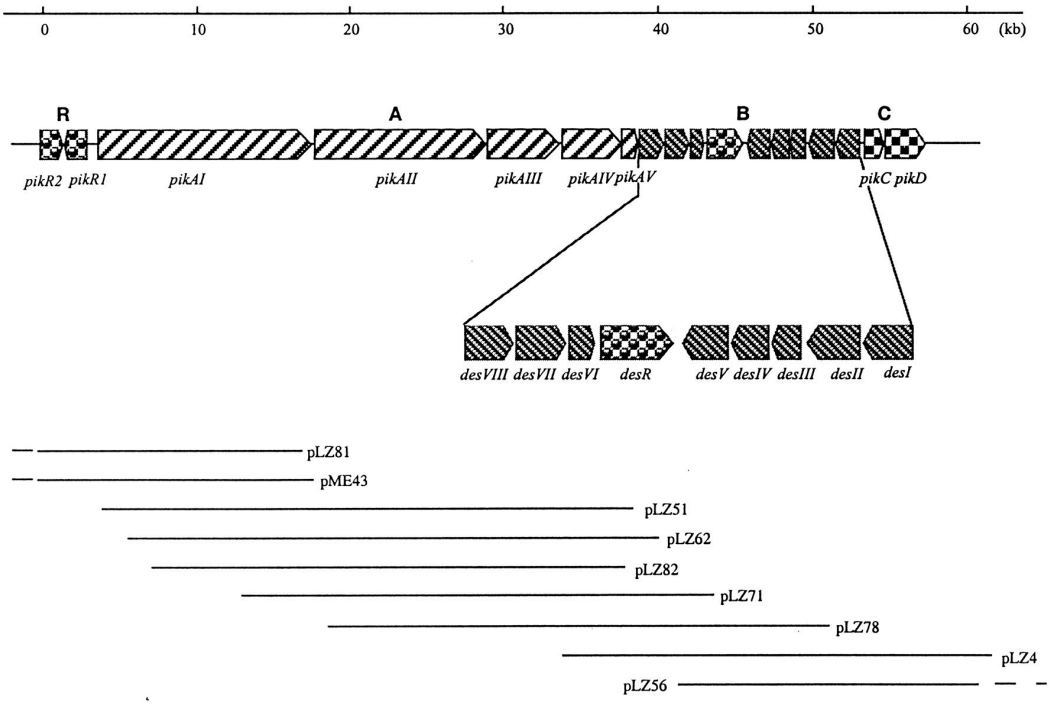


The catalytic cycle of a generic type I PKS module (Scheme 1.1) can involve as little as three requisite domains.[13] The smallest functional module must contain ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains, where the AT is first acylated by

malonyl(or methylmalonyl, ethylmalonyl, etc.)CoA. The ACP accepts the malonate from the AT domain, and the KS domain accepts a growing polyketide from the upstream ACP and catalyzes a stereospecific Claisen condensation to extend the chain by two carbons. If the module lacks domains responsible for β-keto tailoring then the chain will be transferred to the next module, if ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains are present, then the β-keto will be processed to a hydroxyl group (KR), alkene (DH), or alkane (ER), respectively.

The final module of a type I pathway will posses a thioesterase (TE) domain at the C-terminus, and this domain is responsible for off loading the mature polyketide as a hydrolyzed carboxylic acid or lactonized as a ring. In the case of macrolide antibiotics such as methymycin, erythromycin, pikromycin, and methymycin, the TE offloads a 12- or 14- membered macrolactone ring. Such macrolactonization events are commonly performed in biomimetic total syntheses,[14] though such reactions are notoriously difficult to perform[10b] requiring high dilution to prevent dimerization/polymerization and often elevated temperatures and extended reactions times to overcome entropic barriers. Biomimetic ring closings have lost favor in recent years to ring closing metathesis (RCM) and other metal-based methods that are able to coordinate distal functionality and promote efficient cyclization. In contrast, TE domains excel at macrolactizations[15] employing extremely mild reaction conditions (buffered H_2O as solvent, room temperature, high dilution not required).

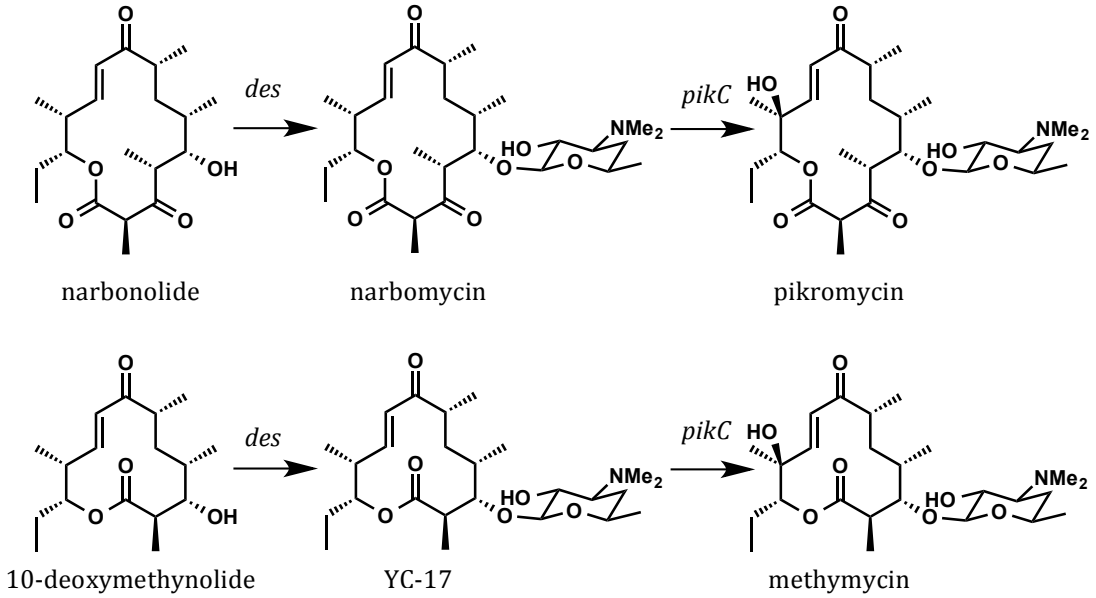
Figure 1.5 The pikromycin (Pik) biosynthetic pathway[24]



The pikromycin biosynthetic cluster (Pik) was first reported in 1998 from *S. venezuelae* ATCC 15439 (Figure 1.5).[16] A generic type I PKS probe revealed two PKS pathways, though a more specialized DNA probe *tylAI* involved in dososamine biosynthesis from the tylosin pathway

was used to locate the Pik pathway. Genetic knockouts of the other Type I PKS pathway had no effect on the biosynthesis of pikromycin or methymycin. A cosmid library was generated, resulting in complete coverage of the ~60 kilobase Pik pathway. Analysis of the assembled DNA sequences revealed 18 clustered genes: two ribosomal methyl transferases (*pikR1*, *pikR2*), four polyketide synthases (*pikAI, pikAII, pikAIII, pikAIV*), a type II thioesterase (*pikAV*), nine genes involved in desosamine biosynthesis and appendage (*desI-desVII* and *desR*), a p450 (*pikC*), and a pathway regulator (*pikD*) .

Scheme 1.2 Products of the Pik pathway

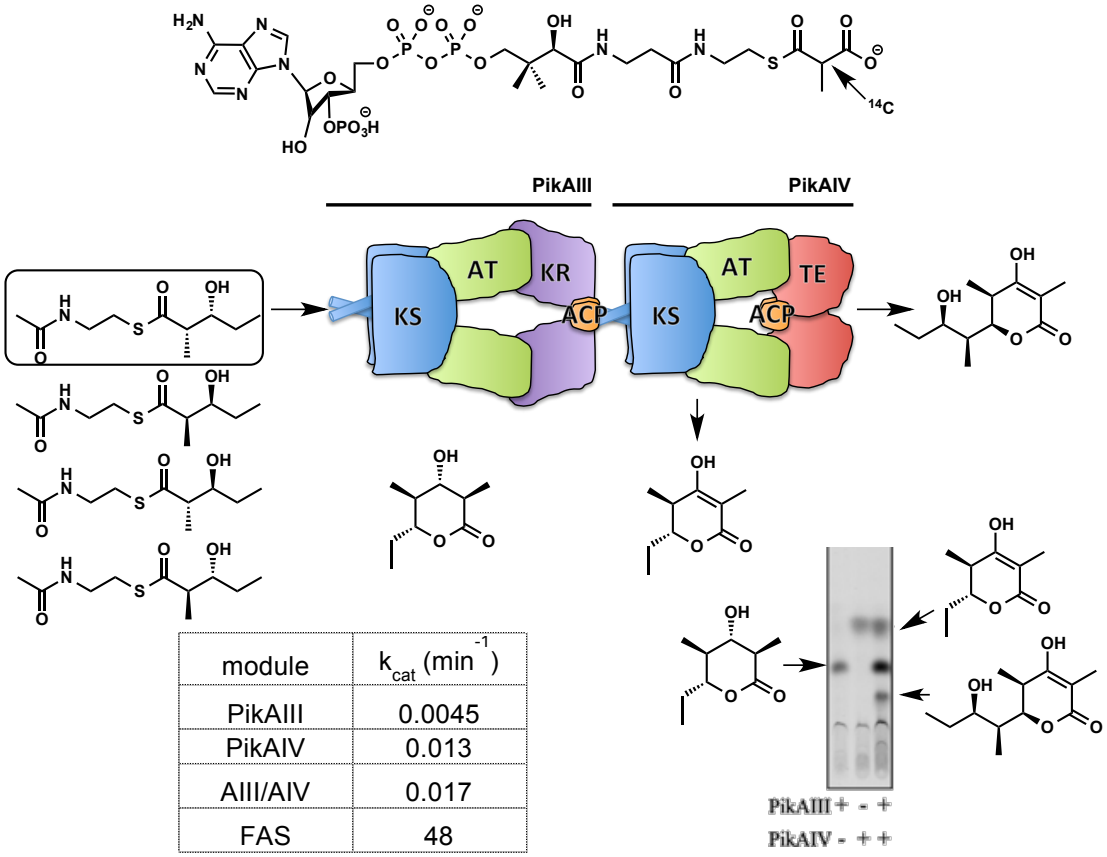


A series of knockout experiments verified that this pathway did indeed produce two classes of macrolides, the 12-membered methymycins and the 14-membered pikromycins. Disruption of *pikAI* resulted in a mutant that produced neither class of macrolactones or macrolides, while disruption of desosamine biosynthesis (*desVI and desV*) produced macrolactones narbonolide and Cthynolide but not macrolides. Disruption of the type II thioesterase (*pikAV*) resulted in dramatically decreased titers though macrolide production remained detectable. Disruption of the *pikC* p450 led to accumulation of reduced macrolides YC-17 and narbomycin (Scheme 1.2).

The results of this study were groundbreaking as this was the first time a single type I PKS pathway demonstrated to produce two different classes of polyketides (12-memebered methymycin and 14-membered pikromycin), additionally, the substrate promiscuity of tailoring enzymes desVII and PikC were able to accommodate either macrolactone, marking this pathway

as a near ideal model system for combinatorial biosynthesis efforts.

Scheme 1.3 In vitro analysis of PikAIII and PikAIV with diketide substrates

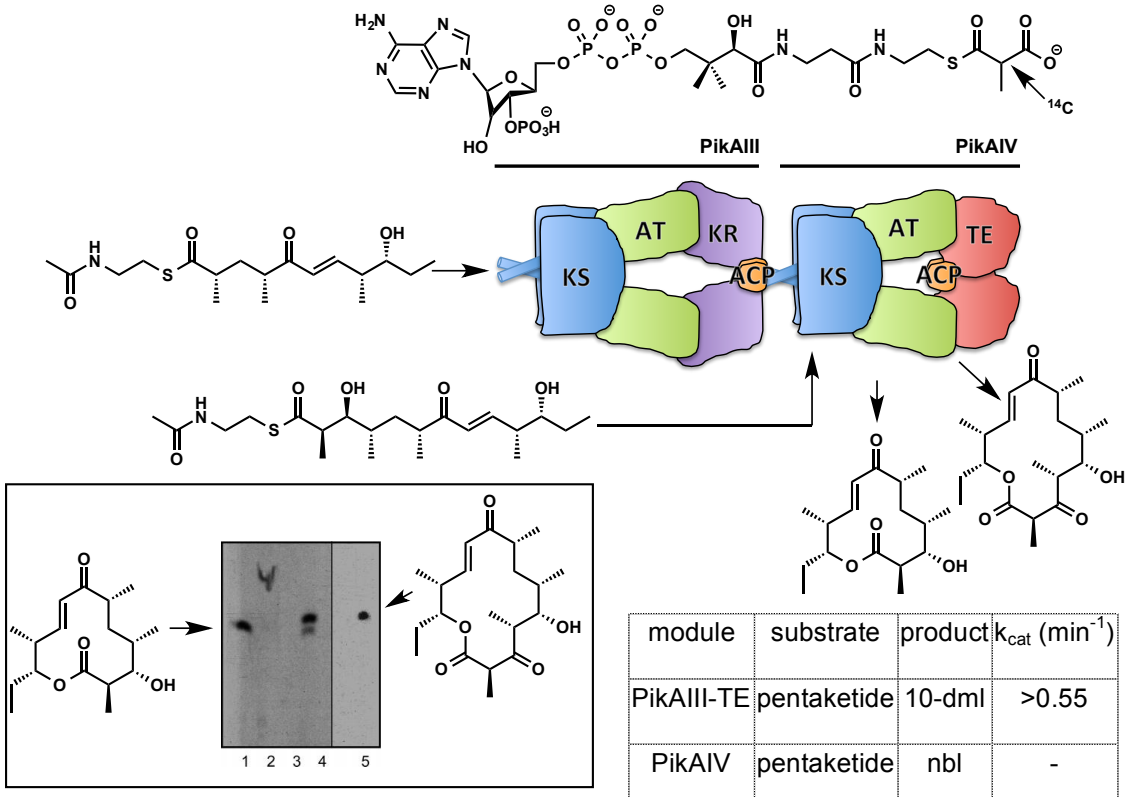


module	k_{cat} (min^{-1})
PikAIII	0.0045
PikAIV	0.013
AIII/AIV	0.017
FAS	48

To further elucidate the unique ability of PikAIII and PikAIV to catalyze the formation of two different macrolactones, the Sherman lab moved to in vitro analysis.[17] Briefly, PikAIII and PikAIV as 6xhis constructs were heterologously expressed in *E. coli* BL21 (DE3) cells co-expressing an *sfp* gene from *B. subtilis* (for post-translational modification of phosphopantetheine onto the ACP domain) and purified through nickel affinity chromatography. Four diketide substrates possessing the four possible stereochemical configurations were synthesized as *N*-acetylcysteamine thioesters and incubated with purified protein, NADPH, and methylmalonyl-coenzyme A (MM-CoA), where the C2 position of the malonate was radiolabeled with ^{14}C. After incubation, the reactions were analyzed by radio-TLC (with verification from authentic standards). Multiple outcomes could be envisioned when incubating unnatural substrates with PKS modules including 1) substrate not accepted 2) substrate hydrolyzed 3) substrate accepted by PikAIII and cyclized spontaneously yielding a reduced triketide product 4) substrate accepted by PikAIII and cyclized by PikAIV TE yielding a reduced triketide product 5) substrate accepted by PikAIII and passed to PikAIV yielding a tetraketide product 6) substrate accepted by PikAIV and cyclized yielding an oxidized triketide product. The *(2S,3R) syn*-substrate was preferentially accepted under all conditions (PikAIII, PikAIV, and PikAIII/PikAIV), though PikAIV could also accept the

(2R,3S) syn-configuration. Neither *anti*-configured provided detectable conversion to tri- or tetraketide products indicating preference for *syn* diketide substrates. The *(2S,3R) syn*-substrate yielded single (radioactive) products when incubated with PikAIII or PikAIV monomodules, but gave all three possible products when incubated with PikAIII/PikAIV with the reduced triketide as the predominant product. Of note is the glacial rate (0.0045-0.017 k_{cat}/min) of conversion with diketide substrates when compared to chicken liver fatty acid synthase (FAS, 48 k_{cat}/min), a difference of four orders of magnitude.[18] This incredible difference is rate between the two megasynthases could be an inherent (primary vs. secondary metabolism), or perhaps due to suboptimal catalysis when employing unnatural diketide substrates.

Scheme 1.4 In vitro analysis of PikAIII and PikAIV with native substrates



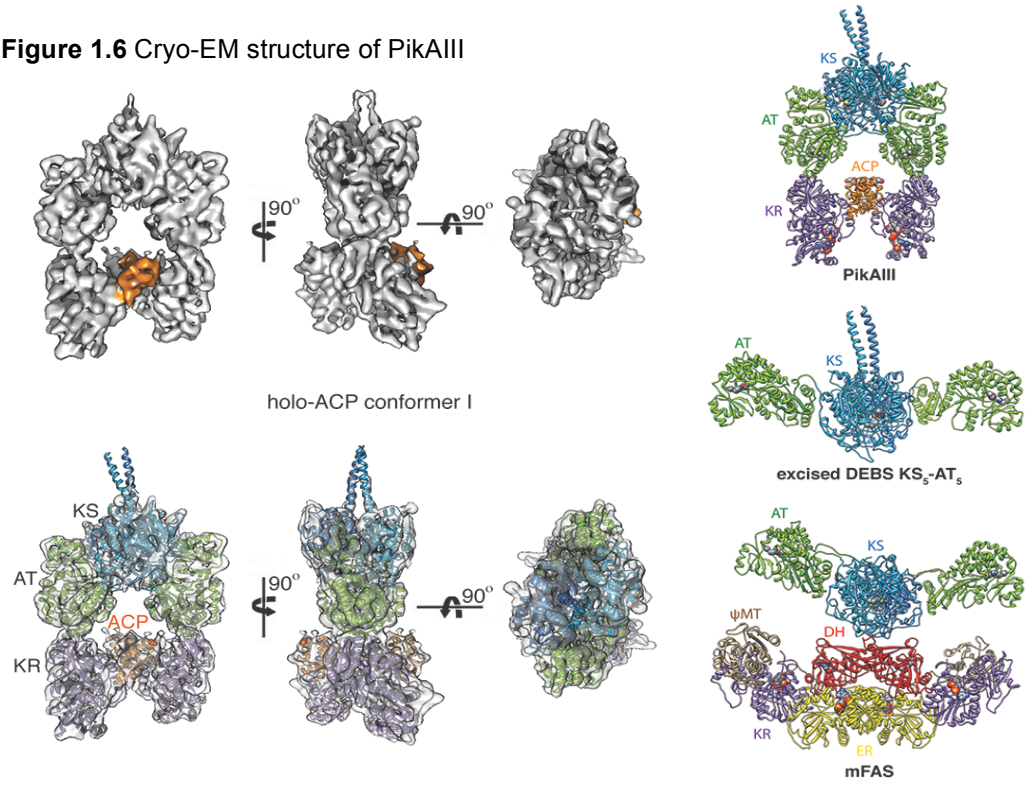
module	substrate	product	k_{cat} (min^{-1})
PikAIII-TE	pentaketide	10-dml	>0.55
PikAIV	pentaketide	nbl	-
AIII/AIV	pentaketide	10-dml	3
AIII/AIV	pentaketide	nbl	3.3
PikAIV	hexaketide	nbl	>4.4

To test the hypothesis that catalysis is poor with diketide substrates, native substrates were synthesized[19] and tested in conditions identical to that of aforementioned diketide substrates (Scheme 1.4.) While native polyketide substrates represent a significant investment in synthetic chemistry, the stark contrast in enzymatic catalysis left no doubt to the necessity of employing native substrates when studying these complex enzymes. Measured rates with native substrates

were 2-3 orders of magnitude faster than with diketide model compounds, approaching that of chicken liver FAS [48 vs. 4.4 k_{cat} (min^{-1})], dispelling the notion that secondary metabolite synthases are substantially slower than those from primary metabolism.

Recent work from a four-group collaboration (Håkansson, Sherman, Smith, and Skinitotis laboratories) provided new insights to both PKS structure and catalytic cycle.[20] PikAIII from the Pik pathway was selected for in depth electron cryo-microscopy, ultimately providing sub-nanometer-resolution and capture of multiple conformations within the catalytic cycle. Briefly, a PikAIII 6xhis construct was expressed in *E. coli* Bap1 cells[21] (genome incorporation of the *sfp* gene from *B. subtilis*) and purified through nickel affinity chromatography, and two subsequent rounds of gel filtration chromatography. Purified PikAIII was absorbed onto mesh grids followed by blotting and vitrification. Vitrified samples were imaged with a transmission electron microscope equipped with a field emission gun. Imaging was conducted at ~20 electrons per $Å^2$ at a magnification of x66,964. Particles were selected and refined from a sphere-like initial reference to provide a structure of PikAIII with a final resolution of 7.3-9.5Å (Figure 1.5). This model was fit with X-ray crystal data from homologous domains from the 6-deoxyerythronolide B synthase,[22] providing a first glimpse into the overall architecture of a PKS module for the first time. PikAIII forms a 328 kilodalton dimer that revealed an internal reaction chamber where the ACP is free shuttle the growing polyketide chain to the next catalytic domain as processing

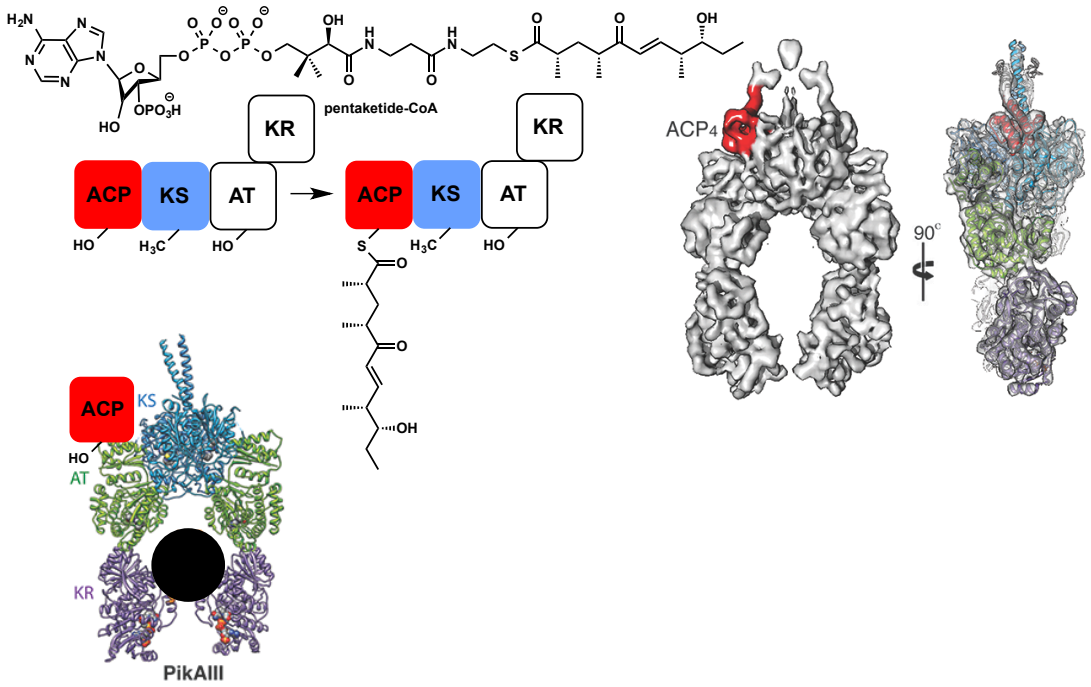
Figure 1.6 Cryo-EM structure of PikAIII



occurs. This structure is quite different from the anticipated FAS architecture (Figure 1.5). A fused construct of PikAIII where ACP_4 was fused to the N-terminus of the KS domain(KS active

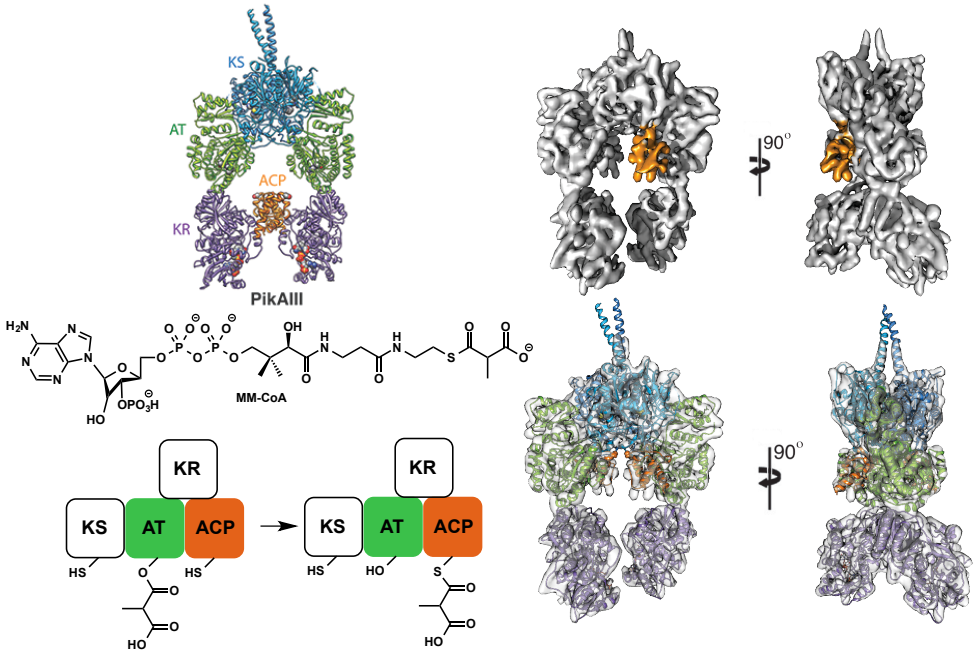
site cysteine was mutated to alanine and ACP_5 was deleted) elucidated how modules are able to pass growing polyketides to the next module. ACP_5 was loaded with pentaketide-CoA using SFP and Cryo-EM of this construct observed ACP_4 docked on top of KS_5, where the ACP is poised to deliver substrate to the KS domain (Figure 1.6).

Figure 1.7 Cryo-EM structure of ACP_4-PikAIIIΔACP_5 with Pik pentaketide



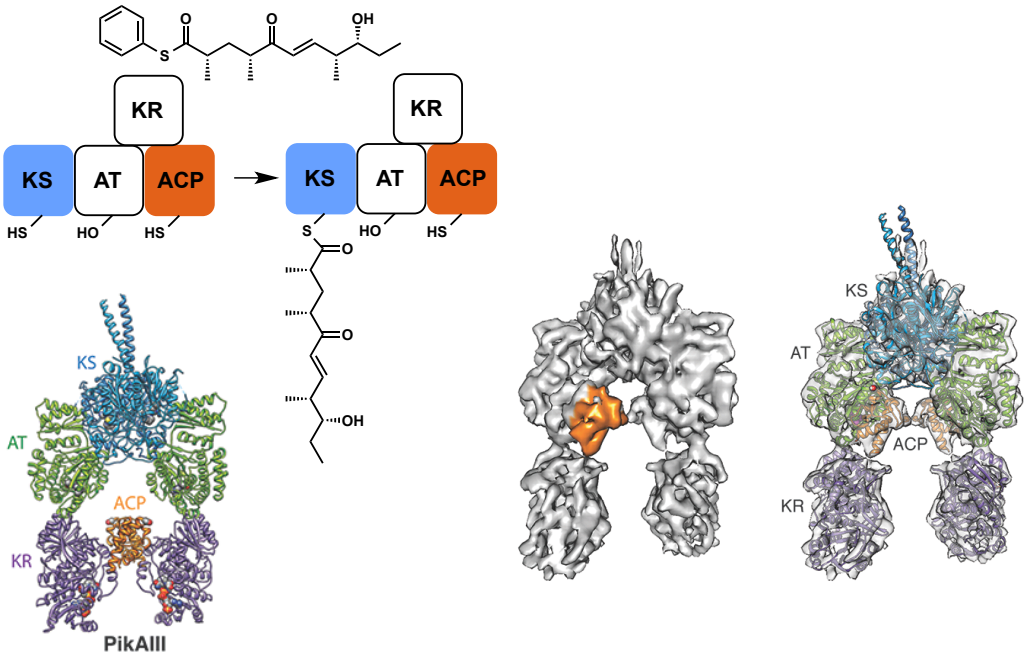
Next, the original construct was used to observe how ACP_5 oriented relative to the KS domain when loaded with MM-CoA. When loaded with methyl-malonate, ACP_5 oriented itself far from where pentaketide loaded ACP_4 positioned itself to KS_5. This observation, supported with mutagenesis coupled with biochemical assays, suggests a second entrance tunnel into the KS, where the Claisen condensation occurs (Figure 1.7).

Figure 1.8 Cryo-EM structure of PikAIII with methyl malonate



Incubation of PikAIII with the thiophenol-thioester of the Pik pentaketide (see chapter 2) loaded the active site cysteine 209 of the KS domain with high fidelity leading to global conformational shifts including AT moving closer to the KS domain,

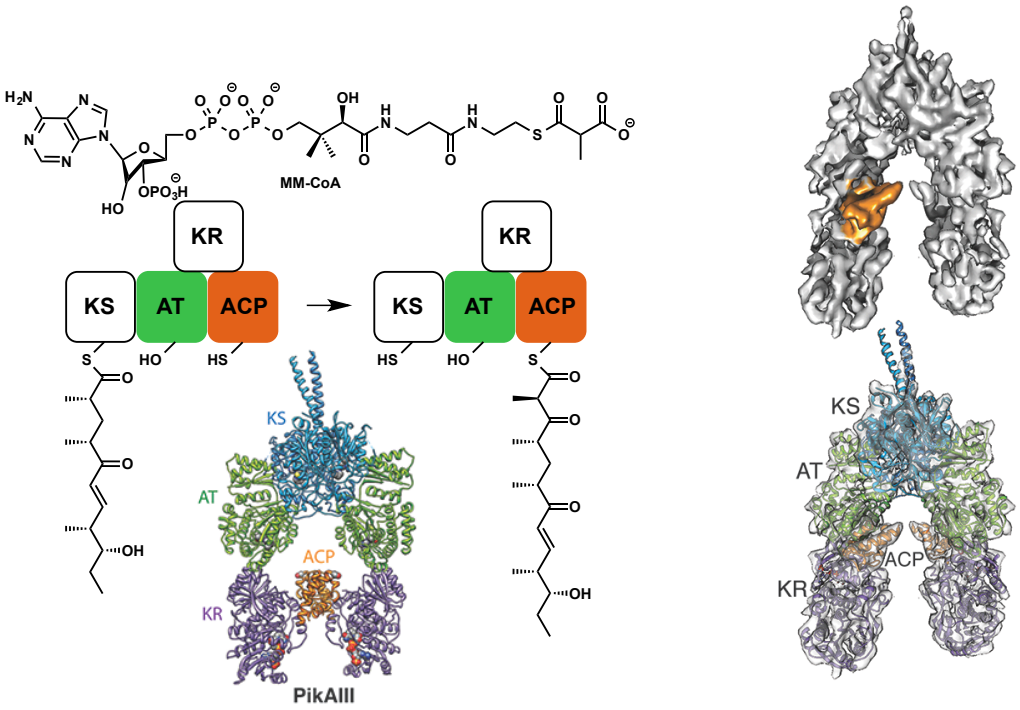
Figure 1.9 Cryo-EM structure of PikAIII with Pik pentaketide



several loops shifiting in the KS domain, and repositioning of the ACP. Most dramatically, the KR has undergone an end-to-end flip of roughly 180° (Figure 1.8.) To observe the module after the

Claisen condensation has occurred, PikAIII was incubated with MM-CoA and the thiophenol thioester pentaketide (Figure 1.9.)

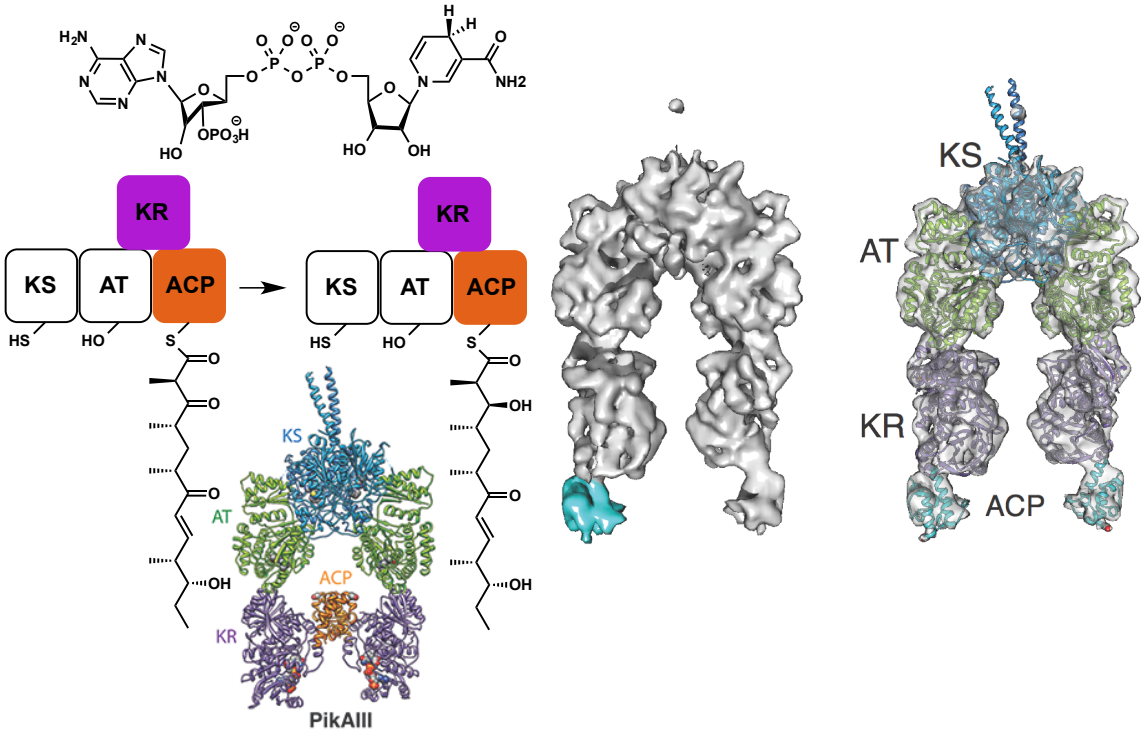
Figure 1.10 Cryo-EM structure of PikAIII with Pik pentaketide and methyl malonate



In this structure, the KR remains proximal to the AT, and the AT is shifted toward the KS by 8Å, possibly preventing intermediate transfer from the upstream PikAII ACP_4 to the KS_5 active site. Given the long, 43 amino acid linker between the KR and ACP, it is unclear whether the KR acts on the same or opposite monomer. In any event, this conformational change allows the β-ketohexaketide access to a KR domain for the final reduction before being passed to PikAIV.

For the final catalytic step, PikAIII was incubated with the thiophenol pentaketide, MM-CoA, and NADPH to generate the reduced β-hydroxyhexaketide (Figure 1.10) which generated three independent conformational states for the final round of catalysis. In all conformers, the catalytic domains are identically positioned, with the KS side entrance occluded and the KR domain oriented with its active site proximal to the AT. Strikingly, the ACP domains are below the KR domain and completely outside the catalytic chamber is each conformer. In all conformers, Ser 1438 is pointing away from PikAIII, appearing poised to transfer the fully processed polyketide to PikAIV. This elucidates how PKS modules maintain directional fidelity, where the substrate is sequested within the internal reaction chamber until it has been fully processed. Only after complete processing is the polyketide ejected from the reaction chamber allowing transfer to the downstream PKS module.

Figure 1.11 Cryo-EM structure of PikAIII with Pik pentaketide, methyl malonate, and NADPH



1.2 Thesis Outline

Based on the strong foundation of prior PKS enzymology established in the Pik pathway, we sought to build upon existing knowledge and develop PikAIII-TE, PikAIII and PikAIV beyond considerable obstacles such as radioactive assays and requirement of cost prohibitive cofactors. Ideally, we sought to improve the throughput and cost-per-reaction of in vitro biochemistry to levels amenable for studying complex facets of PKS function that would potentially require thousands of reactions to elucidate.

Chapter 2 focuses on developing PikAIII-TE, and PikAIII/PikAIV in vitro biochemistry with the synthetic Pik pentaketide through optimizing reaction parameters. During the course of this work, nearly every component of in vitro PKS biochemistry was optimized, including 1) thioester handle to load the enzyme and initiate catalysis 2) replacement of expensive MM-CoA with inexpensive synthetic MM-NAC 4) replacement of stoichiometric NADPH to NADP+ recycling requiring just 10 mol % of the cofactor 5) deployment of PKS modules as purified or crude cell preparations 6) deployment of thiol scavengers to improve product yield through minimizing conjugate addition to the starting material or macrolactone products 7) Improved conversion enabled abandoning antiquated radioactive assays and introduction of routine, medium throughput HPLC analysis. Ultimately, the optimized system(s) enabled preparative production of either 10-deoxymethynolide or narbonolide in just 12 linear steps. With synthetically useful PKS catalysis achieved, we developed a macrolide production platform by developing a whole cell

biotransformation using engineered variants of ATCC 15439 *S. venezuelae*. Through the combination of synthetic chemistry, PKS catalysis, and whole cell biotransformations, we synthesized a suite of five Pik macrolides in 13 linear steps, and reported these findings in 2013 (Hansen, D. A.; Rath, C. M.; Eisman, E. B.; Narayan, A. R.; Kittendorf, J. D.; Mortison, J. D.; Yoon, Y. J.; Sherman, D. H. *J. Am. Chem. Soc.* **2013**, *135*, 11232). These advances have been leveraged in three other publications since then, (Whicher, J. R.; Smaga, S. S.; Hansen, D. A.; Brown, W. C.; Gerwick, W. H.; Sherman, D. H.; Smith, J. L. *Chem. Biol.* **2013**, *20*, 1340. Whicher, J. R.; Dutta, S.; Hansen, D. A.; Hale, W. A.; Chemler, J. A.; Dosey, A. M.; Narayan, A. R. H.; Hakansson, K.; Sherman, D. H.; Smith, J. L.; Skiniotis, G. *Nature* **2014**, *510*, 560. Dutta, S.; Whicher, J. R.; Hansen, D. A.; Hale, W. A.; Chemler, J. A.; Congdon, G. R.; Narayan, A. R. H.; Hakansson, K.; Sherman, D. H.; Smith, J. L.; Skiniotis, G. *Nature* **2014**, *510*, 512.) highlighting the dividends paid by investing in reaction optimization and assay development.

Chapter 3 revisits standalone PikAIV using a panel of Pik hexaketide substrates to elucidate the influence of substrate ester in determining the outcome of PikAIV catalysis. The Pik hexaketide substrates were accessed through chemical degradation of fermentation derived 10-deoxymethynolide from an engineered variant of ATCC 15439 *S. venezuelae.* As the Pik hexaketide is prone to degradation during routine procedures such as SiO_2 chromatography or storage at -20 °C, we developed protection strategies to alleviate this experimental bottleneck. We pursued two distinct protective groups 1) a small methyl ether that would remain attached throughout the catalytic cycle, and 2) a 2-nitrobenzyloxymethyl ether that could be cleaved by irradiation with ultraviolet light immediately before use to provide the native hexaketide on demand. The advances in substrate stabilization enabled evaluation of substrate esters, where we observed dramatic variation in the product distribution provided though PikAIV catalysis, with greater than 10:1 selectivity for either the 14 membered macrolactone narbonolide through full module catalysis or the 12 membered macrolactone 10-deoxymethyonolide through direct macrolactonization. The findings of this work have been submitted for publication (Hansen, D. A.; Koch, A. A.; Sherman, D. H.submitted).

Chapter 4 explores the complex topic of combinatorial biosynthesis in Type I PKS pathways. The linear, modular nature of PKS enzymes could, at least in theory, lead to production of natural product analog libraries or direct fermentation of a specific unnatural product through rational enzyme engineering or directed evolution efforts. However, combinatorial biosynthesis has been largely unsuccessful, where no methods or products from such efforts have yet to reach commercial viability. While some unnatural products have been reported in the literature, the overwhelming majority of combinatorial pathways suffer from greatly diminished titers relative to WT due to numerous complicating factors when engineering these complex pathways. As such, we chose to explore the tractable Pik pathway and “simulate combinatorial biosynthesis” early in the pathway (PikAI) and evaluate how WT PikAIII-TE or PikAIII/PikAIV PKS modules were able to process combinatorial polyketides. Accordingly, we synthesized a panel of unnatural pentaketides

bearing modifications that would have occurred if the loading module or PikAI possessed a mutant AT domain accepting different extender units (malonate vs. methyl malonate) or mutant KS domain to construct all possible stereochemical configurations derived from the Claisen condensation. By directly assaying these combinatorial pentaketides with WT PikAIII-TE or PikAIII/PikAIV we can evaluate how a downstream PKS module can handle early pathway engineering *while avoiding protein-centric complications arising from protein engineering or directed evolution.* Work described in Chapter 4 is ongoing at present.

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1.1

Chapter 2

Development of a Biocatalytic Platform for production of Pik Macrolides

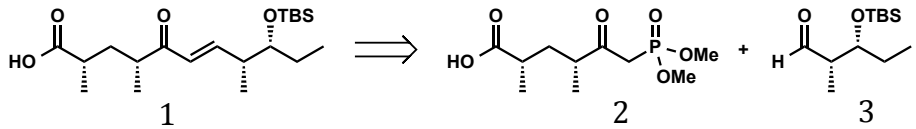
2.1 Introduction

Total synthesis of macrolide natural products presents a formidable challenge to the synthetic chemist. While total synthesis is capable of furnishing minute quantities of macrolides,[1] this approach is at present constrained to academic interest, as the complexity of these compounds requires understandably involved synthetic schemes and low overall yields. Although total synthesis will not provide metric tons of designer macrolides in the foreseeable future, the biosynthetic machinery used to satisfy clinical demand could conceivably furnish designer macrolides at industrial scale and within existing workflows *if the natural products community can understand and manipulate these complex pathways.* Toward this end, we sought to optimize in vitro PKS catalysis of the final two modules of the Pik macrolide pathway (PikAIII and PikAIV) to facilitate higher-throughput analysis and dispense of antiquated radioactivity based assays. While PKS optimization was initially envisioned simply as a means to streamline biochemical characterization for basic study of these complex enzymes, advances in PKS catalysis ultimately allowed for biocatalytic synthesis of Pik macrolides.

2.2 Synthesis of the Pik pentaketide seco-acid

In order to study the final two modules of the Pik pathway, PikAIII and PikAIV, we first needed to synthesize the native pentaketide substrate. This substrate has been synthesized previously during initial characterization of PikAIII (Scheme 2.1).[2]

Scheme 2.1 – 1st Generation synthesis of the Pik pentaketide

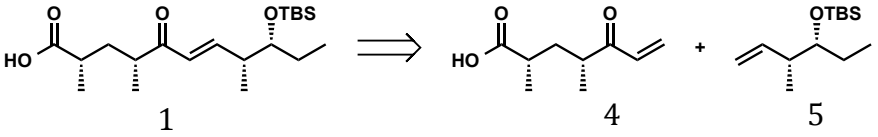


1 2 3

Aldrich et al. recognized a disconnection of the central α,β-unsaturated ketone allowing for a convergent coupling of two fragments via a barium hydroxide mediated Horner-Wadsworth-Emmons olefination.[3] This approach proved highly effective at constructing the Pik pentaketide,

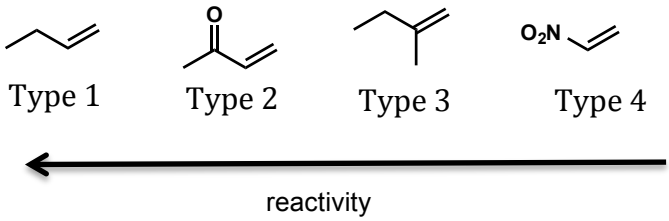
and, as such, we chose to utilize the same central disconnection in a manner more suitable for analog synthesis (*vide infra*, Chapter 4). We envisioned a cross-metathesis union of the two fragments using olefin metathesis catalysts such as Grubbs 2nd generation[4] or Hoveyda-Grubbs 2nd generation.[5] This would allow for utilizing common α,β-unsaturated ketone left fragment, and a variety of monosubstituted olefin right fragments(Scheme 2.2).

Scheme 2.2 2nd Generation synthesis of the Pik pentaketides (**2**)



According to Grubbs,[6] alkenes can be categorized intro four different types based upon reactivity under cross-metathesis conditions with 2nd generation catalysts (Figure 2.1). Type I alkenes will homodimerize rapidly and the subsequent dimers are still accessible to metathesis catalysts and can go on to react with other alkenes. Type II alkenes homodimerize slowly, and homodimers are sluggish to react further. Type III alkenes do not homodimerize, but will react participate in metathesis reactions with type I and II alkenes. Type IV alkenes are inert to cross-metathesis but do not deactivate metathesis catalysts.

Figure 2.1 Four types of olefins based on cross-metathesis reactivity

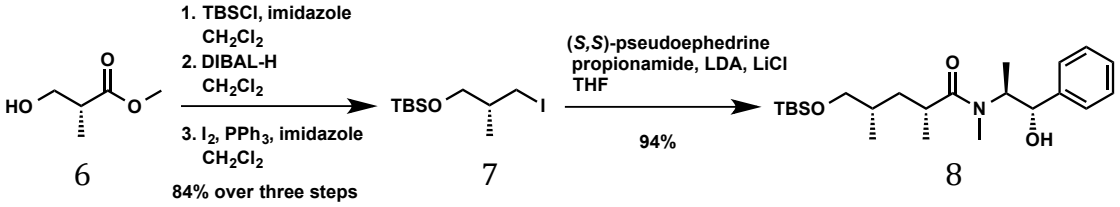


When attempting cross-metathesis with two type I alkenes, a statistical mixture of products would be expected unless one partner is used in great excess, and E/Z selectivity is often poor. However, cross metathesis between a type I and type II alkenes, such as a type II α,β-unsaturated ketone **4**, and type I mono-substituted olefin **5** allow high yielding metathesis to occur without considerable excess of either partner and typically greater than 20:1 E/Z selectivity. With this straightforward approach in mind, we envisioned a scalable synthesis of **4** through a routine sequence involving Myers alkylation,[7] which has been successfully employed in the synthesis of related DEBS pentaketide.[8] Other options were considered, the most attractive being desymmetrization of *meso*-dimethyl glutaric anhydride via $[Rh(COD)Cl]_2$/*t*-Bu-PHOX controlled alkylzinc addition.[9] Type I silyl-ether **5** could be accessed through robust Evans aldol methodology.[10]

The synthesis of **4** began with commercially available (*R*)-Roche ester (**6)**, which is a product of enantioselective microbial oxidation of isobutyric acid,[11] a common starting material for polyketide natural products where the initial stereocenter is "bought."[12] A rapid three step

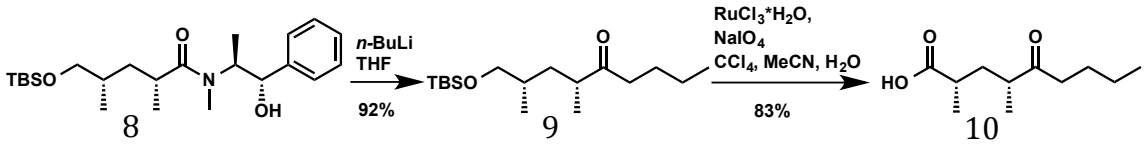
procedure prepares **6** for the Myers alkylation, TBS protection of the primary hydroxyl group, DIBAL-H reduction of the ester to a hydroxyl group which is then iodinated to provide **7** under Appel like conditions (Scheme 2.3). This three-step sequence was optimized to avoid chromatography until after iodination, and was performed in a single day on several occasions.[13]

Scheme 2.3 Conversion of *(R)*-Roche ester (**6**) to amide **8**



7 was alkylated initially using (*S,S*)-pseudoephedrine propionamide to **8**,[7] though later efforts employed (*S,S*)-pseudoephenamine propionamide[14] under similar conditions with identical results (dr >20:1) as pseudoephedrine became harder to acquire from commercial suppliers (Scheme 2.3). While the Myers alkylation is a robust and scalable reaction,[15] displacement of the auxiliary with ethyllithium (EtLi) toward **4** proved to be unexpected bottleneck. EtLi is commercially available though expensive, dilute (0.5 M) in benzene:cyclohexane, and often hard to acquire, compared to *n*-BuLi which is widely available as inexpensive, concentrated (2.5-10 M) solutions in hexanes. While initial displacement of the pseudoephedrine auxiliary with EtLi was indeed successful, we decided to move to *n*-BuLi displacement to generate **9**, which would ultimately provide butyl ketoacid **10** (Scheme 2.4) instead of the originally targeted ethyl ketoacid towards **4**. Upon oxidation to α,β-unsaturated ketone from either either **4** or **10**, we expected similar reactivity during cross-metathesis to a type I right fragment.

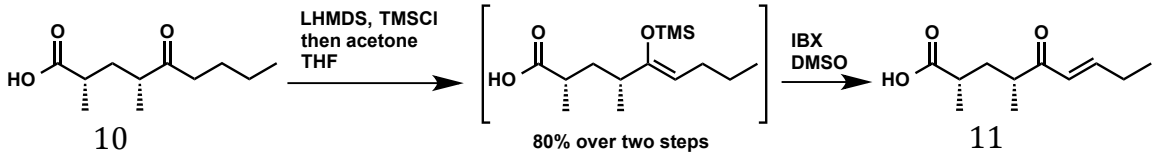
Scheme 2.4 Conversion of amide **8** to amide **10**



Transformation of silyl ether **9** to ketoacid **10** was accomplished under in a single step with a RuO_4 oxidation under Sharpless conditions[16] which served to both oxidize the silyl ether to a silyl ester with concomitant loss of the TBS group and without epimerization of the α stereocenter(Scheme 2.4). This direct oxidation contributes to the step economy of this scheme when compared to a three step deprotection, alcohol to aldehyde oxidation, and final aldehyde to acid oxidation. More importantly, this approach bypasses the unprotected alcohol, which would be expected to form a thermodynamically favorable pyran hemiketal[17] posing multiple potential complications. With a reliable route to **10** in hand we began to evaluate methods to dehydrogenate the saturated ketone. A number of methods exist for such dehydrogenations, the most prominent being the Saegusa-Ito oxidation[18] and derivatives there-of. The Saegusa-Ito

reaction involves oxidation of silyl-enol ethers with stoichiometric $Pd(OAc)_2$ or 50 mol % $Pd(OAc)_2$ using *p*-benzoquinone as a co-oxidant. Of primary concern is exclusively trapping the kinetic enolate of **10** (at C6) without concomitant deprotonation of the C2, which would become reasonably acidic after the acid is transiently protected as a silyl-ester. Initial attempts were met with considerable resistance, where attempting to trap the kinetic enolate with LDA/TMSCl at -78 °C and subsequent oxidation with $Pd(OAc)_2$ or IBX[19] provided variable product mixtures of partially epimerized α,β-unsaturated ketone **11**,

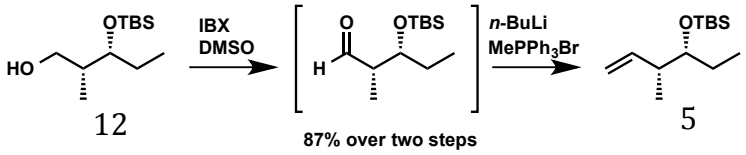
Scheme 2.5 Oxidation of saturated **10** to α,β-unsaturated ketone **11**



and partially epimerized recovered starting material **10**. Switching from LDA to LHMDS eliminated epimerization,[20] and implementation of an acetone quench to scavenge excess LHMDS/TMSCl prior to aqueous workup improved reproducibility of the process(Scheme 2.5). Dehydrogenation to **11** with stoichiometric $Pd(OAc)_2$ resulted in difficulties during purification, while employing the inexpensive alternative IBX could be removed more readily by column chromatography. Trace quantities of **10** observed from hydrolysis of the intermediate silyl-enol ether were somewhat separable via SiO_2 chromatography, though readily separable via $AgNO_3$:SiO_2 chromatography. With a scalable route to **11** secured we turned our attention to the right fragment **5**.

Type I olefin **5** was easily secured through routine Evans aldol chemistry,[21] reductive displacement of the oxazolidinone auxiliary with $LiBH_4$,[22] followed by IBX oxidation and Wittig olefination (Scheme 2.6).

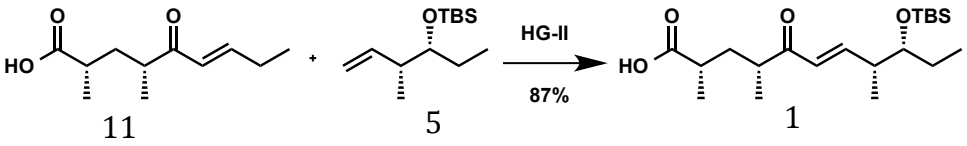
Scheme 2.6 Synthesis of olefin **5**



With both fragments completed, we began to evaluate cross-metathesis conditions to forge compound **1**. We screened Grubbs 2nd generation[4] or Hoveyda-Grubbs 2nd generation[5] beginning in CH_2Cl_2 at room temperature with 3 mol % of either catalyst, 1 equiv **11** (0.1 M) and 1.5 equiv **5** (0.15 M). Only trance conversion was observed at room temperature, and raising the temperature reflux (40 °C) improved the rate somewhat but it the reaction was still too slow to be synthetically useful. Moving to PhMe and increasing the temperature to 60°C, 70°C, 80°C improved the initial rate but led to variable yields with incomplete conversion. Additional optimization ultimately led us to run the reaction neat at 50 °C with vigorous stirring. When run neat, catalyst initiation is easily

observed through visible etheylene gas evolution. After 12 h at 50 °C, only trace **11** can be observed by TLC and crude ^{1}H NMR indicates complete conversion to **1**(Scheme 2.7).

Scheme 2.7 Cross metathesis of **5** and **11** to yield

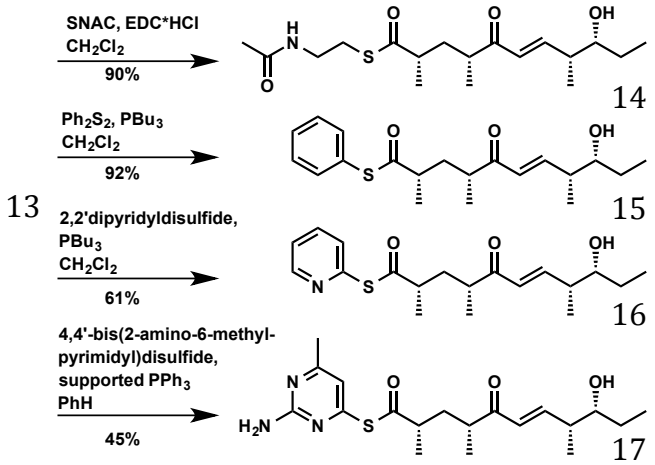


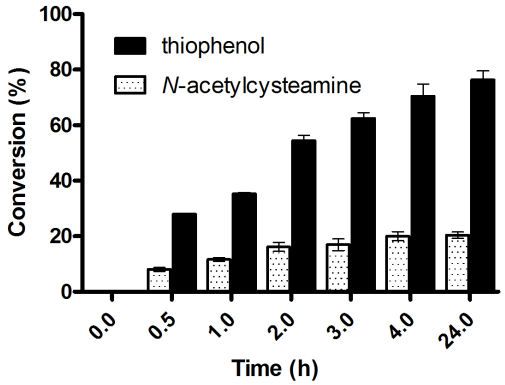
2.3 Optimization of PKS biochemistry

Just 2 steps are required to prepare the seco-acid **1** for in vitro enzymatic reactions: esterification and deprotection. We considered the (thio)ester of the substrate required to load a PKS enzyme as a logical place to start our optimization efforts. *N*-acetylcysteamine (NAC) thioesters have been used exclusively (save rare acyl-ACP examples) for studying PKS enzymes in vitro. The first instances of using NAC thioesters to study PKS enzymes predates knowledge of the enzymes themselves, where isotopically labeled natural product precursors were employed as NAC thioesters[23] with the aim of penetrating the cell wall of the producing organism before uptake by the biosynthetic pathway. These pioneering studies laid the foundation for future in vitro PKS biochemistry, and the use of NAC thioesters became universal without evaluation of other means to acylate a given module. With a seemingly obvious experiment is left unexplored in the literature, we were suspicious that perhaps deviation from NAC esters was met with failure and thus unreported. The use of NAC could be justified as this moiety mimics the terminal portion of the phosphopantetheine (ppant) arm that polyketide substrates are tethered to in vitro, however, this logic doesn't withstand scrutiny; there is no evidence that the KS domain recognizes the ppant arm of the upstream ACP. Interaction of two PKS modules is mediated by C and N terminal docking domains[24] placing the ACP in close proximity to the KS for chain transfer. When performing PKS biochemistry with a purified PKS module, this protein-protein interaction is absent requiring the substrate to diffuse onto the active site cysteine KS domain. If we assumed no recognition of the NAC moiety, a more reactive ester could potentially enhance the rate of acylation, and, in turn improve PKS catalysis. Such effects have been studied in detail within the context of native chemical ligation,[25] where transthioesterification between a terminal cysteine and a synthetic thioester must occur prior to rearrangement to the native amide backbone. Proper selection of a thioesters ester can provide complete ligation in a matter a minutes, while improper selection can leave a ligation incomplete after multiple days. Aryl thioesters undergo transthioesterification more readily than (most) alkyl thioesters,[25] so we sought to generate a small panel Pik pentaketide aryl thioesters to assay against the NAC thioester. Aqueous hydrogen fluoride deprotection of silyl-ether **1** provided alcohol **13** which was reductively thioesterified using commercial aryl disulfides(Scheme 2.8). We synthesized NAC thioester as

described previously,[2] and thiophenol thioester **15** from phenyl disulfide and tributylphosphine. Initial attempts using triphenylphospine as the reducing agent were sluggish,[26] suggesting that the nucleophilicity of the phosphine plays a key role in promoting reductive thioesterification.[27]

Scheme 2.8 Thioesterification of **13**





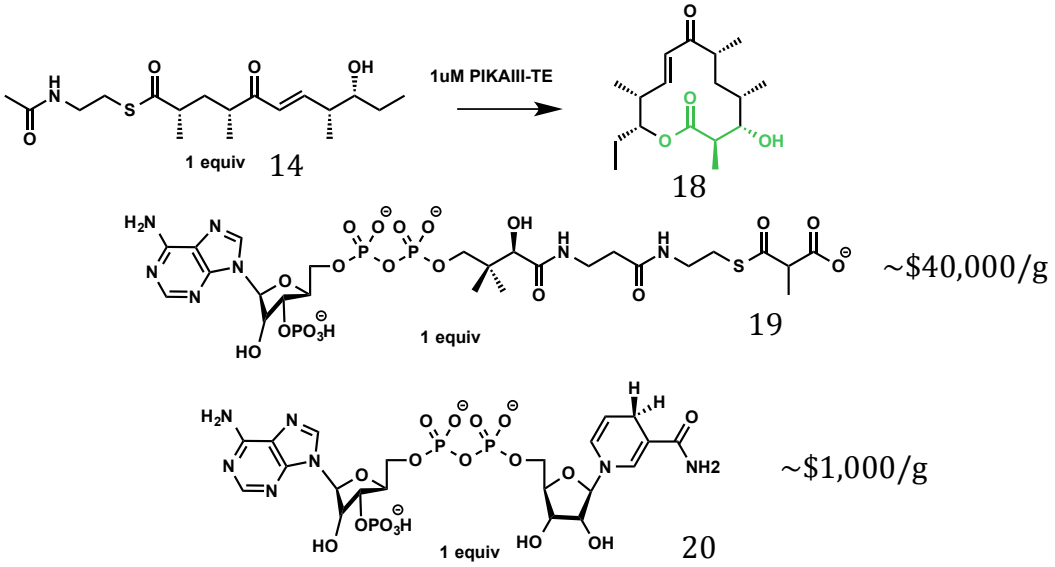
16 was synthesized in a similar manner by employing 2,2'-dipyridyldisulfide, though **17** required more creative conditions due to the insolubility of 4,'4-bis(2-amino-6-methyl-pyrimidyl)disulfide. When attempting to synthesize **17** PhH at 100 °C using PBu_3, the second equivalent of 4-(2-amino-6-methyl-pyrimidyl)sulfide underwent conjugate addition into the α,β-unsaturated ketone. Substituting PPh_3 for PBu_3 provided **17** as an inseparable mixture of $O=PPh_3$, requiring the use of solid supported PPh_3.

With pentaketides in hand we incubated **14-17** with purified PikAIII-TE, methylmalonyl extender unit **19** or **23** (vide infra, Schemes 2.9 and 2.10), and biological hydride donor **20** or (vide infra, Schemes 2.9) under nonoptimized conditions to get some sense of efficacy. After initial frustrations with ultra-low throughput radio-TLC, we attempted more modern analysis via sensitive quadrapole time of flight liquid chromatography/mass spectrometry (QTOF LC/MS). One-hour incubation of **14-17** with PikAIII-TE revealing significant differences in conversion depending on the type of ester employed. Thiophenol thioester **15** produced ~4 times more 10-dml (**18**) relative to traditionally employed NAC thioester **14**, while **16** and **17** enjoyed ~2-fold increases relative to **14**. As the thiophenol thioester **15** was superior in terms of both enzymatic conversion and ease of synthesis, we sought to attain absolute quantification along the reactions time course. While high-end LC/MS analysis could potentially replace radio-TLC to analyze PKS biochemistry, concerns of variation in ionization efficiency from run-to-run, expense, and limited availability of instrument time led us to consider other analysis methods. 10-dml (**18**) has a weak chromophore (α,β-unsaturated ketone) which absorbs at ~236nm making simple HPLC analysis a potential option, though the low conversion of **14** to 10-dml (**18**) made product detection

challenging. However, the dramatically improved conversion of **15** to 10-dml (**18**) allowed us to develop a medium-throughput HPLC based work-flow for future optimization(Scheme 2.8).

Scheme 2.9 illustrates how an in vitro PKS reaction is typically performed,[2,8] where a synthetic substrate is incubated with a purified module and requisite cofactors. While aforementioned considerations of low conversion were improved through thioester optimization thus enabling HPLC based workflow, we next sought to address cofactor considerations of poor atom economy and exorbitant expense.

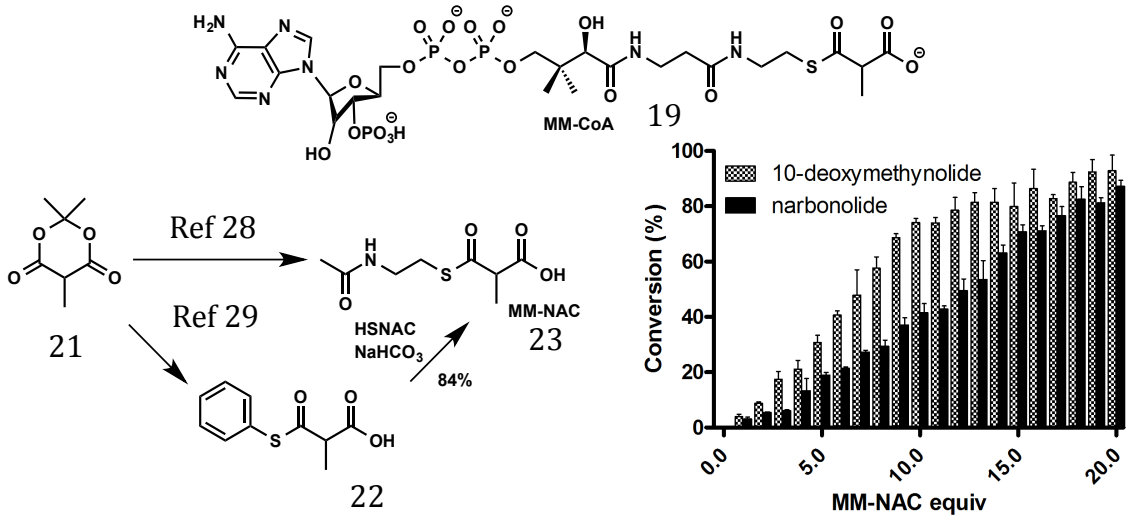
Scheme 2.9 Traditional PKS biochemistry with PikAIII-TE



The first cofactor examined was methylmalonyl-coenzyme A (MM-CoA, **19**), which is the endogenous cofactor responsible for delivering methylmalonate to the AT domain. **19** is commercially available and universally used in a stoichiometric manner to study PKS modules in vitro. Testing a defined hypothesis with a small number of microscale PKS reactions can justify the cost of **19** as only a few milligrams would be required, however, we expected to run thousands of reactions where the cost of **19** would quickly become unsustainable. A lone report in the literature suggested truncation of the CoA moiety can still function as a viable methylmalonate cofactor[28] with DEBS PKS modules, albeit at a higher concentrations. In the case of MM-NAC (**23**), it is likely that the AT domain does indeed recognize the ppant arm of CoA as this transfer is not mediated by protein:protein interactions and the use of a NAC thioester can be justified in this manner. As such, we sought to reproduce this result with the Pik modules. We were able to reproduce the reported synthesis of **23** at small scale though the one step synthesis from methyl meldrum's acid **21** was hindered by difficulties in seperation of extremely hydrophillic **23** from side products formed during the reaction. As such, we devloped a two step route through **22**[29] which is a crystaline solid that can made at decagram scale without chromatography from **21.** With **22** in hand, we were able to access **23** by simple transthioesterification with *N*-

acetylcysteamine in aqueuous sodium bicarbonate. Purification continued to be a problem as the extreme polarity **23** prevents extraction into organic solvent or chromatography on SiO_2. Ultimately, we devised a scheme to acidify the reaction mixture upon completion with a solid supported sulfonic acid resin G26, which is both commercially available and inexpensive. The resin is filtered off along with complexed Na^+ ions, and organic purities are extracted from the aqueous layer with CH_2Cl_2 leaving pure **23** in the aqueous layer. Lyophilization then provided pure **23**, and this two-step sequence is amenable to decagram synthesis without requiring chromatographic purification. With MM-NAC (**23**) in hand, we evaluated the concentration dependence of this cofactor anaolog with PikAIII-TE and PikAIII/PikAIV in vitro using thiophenol pentaketide **15** analyzed by HPLC (Scheme 2.10).

Scheme 2.10 Synthesis and evaluation of methylmalonyl *N*-acetylcysteamine (**23**)

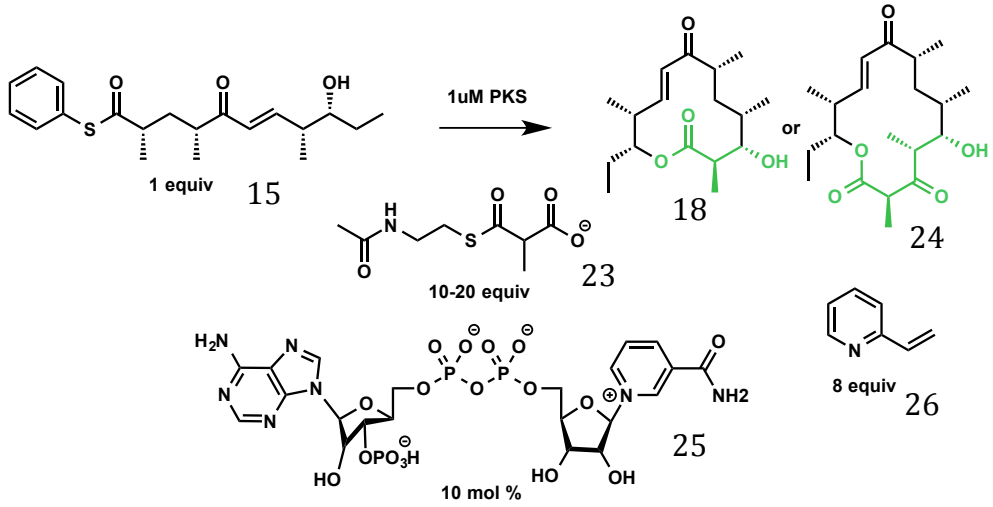


As the Khosla group had observed in the DEBS pathway, we observed a concentration dependence of **23** well above what is required with MM-CoA (**19**) or when considering required requisite methyl malonate stoichiometry, suggesting that the AT domain does not recognize the NAC moiety with the same affinity as CoA. A control experiment using thiophenol **22** in place of **23** provided only trace conversion, further supporting that AT domain in Pik modules is recognizing CoA or truncations thereof. Nevertheless, the incredible improvement in cost per reaction and the synthetically accessibility of **23** motivated us to forgo the use of expensive **19** in downstream optimization. We next explored NADPH recycling systems, beginning with the use of less expensive and more stable $NADP^+$ (**25**). The first tested was the Wong/Whitesides glucose-6-phosphate/glucso-6-phosphate dehydrogenase recycling system,[30] which proved so efficient we were able to decrease $NADP^+$ (**25**) down to 10 mol % relative to penaketide **15** further reducing costs.

With cofactor and thioesters optimization completed, we sought one last improvement to scavenge thiols liberated during the reaction (thiophenol or NAC). This was motivated by a few

reasons 1) to boost yields by preventing conjugate addition of thiols into the α,β-unsaturated ketone of the substrate or products 2) sequester liberated thiophenol to improve upon the horrific odor of the reactions 3) prevent formation of mixed disulfides that can complicate HPLC analysis. A screen of electrophiles was largely uninteresting, as mildly electrophilic (α,β-unsaturated acids and amides) were poor scavengers and offered little improvement, while stronger electrophiles (maleimides, nitrostyrenes) completely inhibited catalysis likely through alkylating the active site cysteine of the KS domain. One compound stood about above the rest, 2-vinylpyridine (**26**).[31] 2-vinylpyridine has been successfully employed as a glutathione scavenger when studying glutathione reductase, which, like PKS modules, contains active site cysteine(s) required for catalysis. In our hands, 2-vinylpyridine had no detectable inhibition of PKS catalysis at 8 mM, and near instantaneous scavenging of thiophenol. In fact, the odor of thiophenol could no longer be detected during PKS reactions despite the ppb detection threshold of the human nose.

Scheme 2.11 Optimized PKS biocatalysis with PikAIII-TE or PikAIII/PikAIV



entry	scale (mmol)	conc (mM)	MM-SNAC (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

With biochemical parameters began to consider the potential synthetic utility of PikAIII-TE and PikAIII/AIV as biocatalysts. As the pentaketide **15** is synthesized in 11 linear steps, PKS processing would yield 10-dml (**18**) or narbonolide (**24**) in just 12 linear steps. As such we purified larger quantities of PikAIII-TE and PikAIII/PikAIV and tested the scalability of these reactions. Initial iterations were scaled up 2000-fold, from 50 μL to 200 mL converting 0.2 millimoles of pentaketide **15** (~70 mg) to a theoretical ~60 mg of 10-dml (**18**) or ~70 mg narbonolide (**24**).

Enzymatic reactions were treated as if they were a normal chemical reaction, monitored by TLC, extracted when completed, and then macrolactone products 10-dml (**18**) or narbonolide (**24**) were purified by SiO_2 flash chromatography (scheme 2.11, entry 1[a]) 10-dml (**18**) tolerated this treatment well, though narbonolide (**24**) appeared to decompose slightly (through possible C5-C9 hemiketal formation) creating problems for spectroscopic analysis. We hypothesized that protecting the hydroxyl group prior to chromatography would prevent degradation. Esterification with Ac_2O/NEt_3 in the presence of catalytic DMAP worked well and provided stable acetyl-narbonolide (**32**) facilitating product characterization.

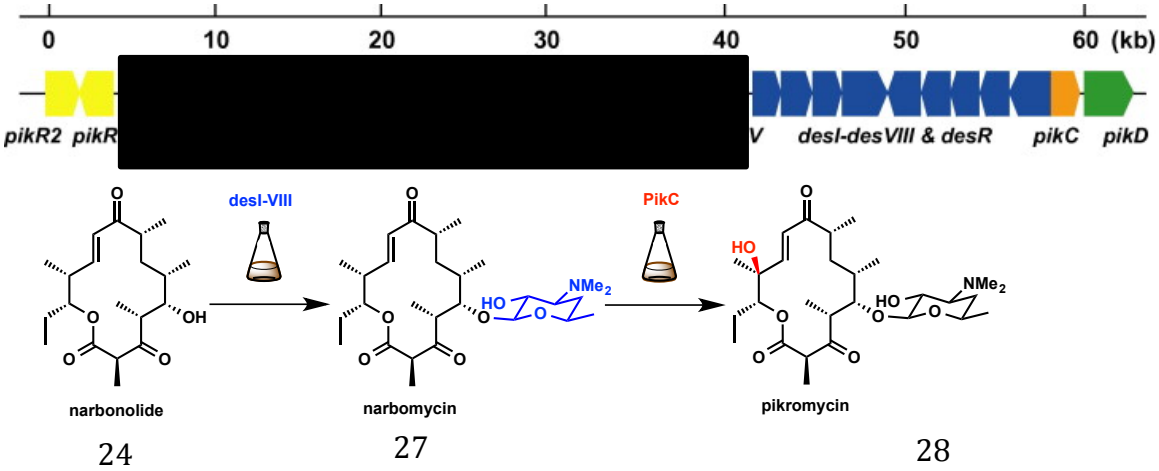
During the course of reactions with purified[a] PKS modules (scheme 2.11, entry 1[a]) we observed protein precipitation (presumably though denaturation or aggregation), and considered the use of PKS modules as crude cell lysate,[b] a common approach in industrial biocatalysis. Complications can arise if the protein of interest is hydrolyzed by proteases, if other cellular enzymes can catalyze side reactions with the starting material or product, and direct extraction becomes difficult often requiring an initial miscible organic solvent precipitation, filtration, and evaporation before product extraction. Furthermore, accurate quantification of enzymatic quantification in crude cell lysate is no small task, typically resulting in an approximation. However, many proteins display improved stability, activity, and reproducibility in crude cell lysate, and by removing the burden of protein purification catalyst preparation becomes a significantly more efficient and scalable process. As such, we evaluated PKS reactions employing the modules in crude cell lysate (scheme 2.11, entry 2[b]-4[b]) with a slight improvement in macrolactone yield. Attempts to increase the reaction concentration to 4 μM PKS with purified modules led to erratic, lower conversion of **15** to either macrolactone with increased protein precipitation (suggesting aggregation), though crude cell lysate was operable under these more concentrated conditions(scheme 2.11, entry 3[b],4[b]). Entry 4[b] was intended to demonstrate the scalability of PKS catalysis, employing 0.5 g of pentaketide **15** generating ~250 mg of either macrolactone.

2.4 Biotransformation of macrolactones to macrolides

While the majority of total synthesis efforts towards macrolides terminate with achieving the macrolactone core,[32] we opted to go a step further and evaluate biotansformations of macrolactones to mature macrolide natural products. Abiotic synthetic efforts often stop at macrolactones as the post-PKS tailoring steps are extraordinarily hard to perform without enzymatic assistance. In the Pik pathway, the amino-sugar desosamine is biosynthesized from glucose and appended onto hindered a hydroxyl group on the macrolactone core. The final tailoring step is a C-H oxidation of the macrolactone core mediated by the P450 PikC, where the oxidation is directed by the desosamine sugar.[33] Without utilizing the desaosamine biosynthetic pathway, desosamine requires 9 chemical steps to construct,[1d,34] followed by glycosylation and

deprotection. The P450 oxidation mediated by PikC presents an even greater synthetic challenge as no current C-H method exists to perform such a transformation. When targeting oxidized macrolactones or macrolides through total synthesis the hydroxyl group is embedded into the ring at an early stage.[1a-c,35]

Scheme 2.12 Biotransformation of narbonolide (**24**) to pikromycin (**28**)



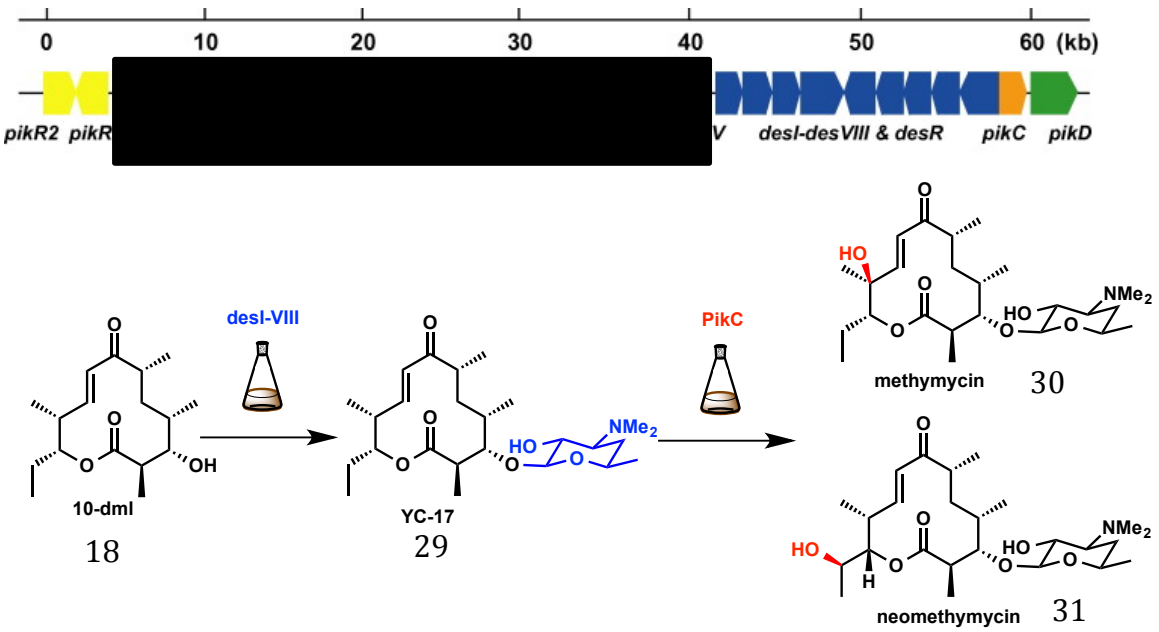
24 27 28

Biotransformation of either macrolactone to macrolides was examined with engineered variants of *S. venezuelae* ATCC 15439 DHS2001[36] and YJ112.[37] Both strains have had the Pik PKS genes knocked out through iterative insertions of a hygromycin marker and produce no macrolactone or macrolide secondary metabolites, and YJ112 posses an extra copy of pathway regulator pikD. The tailoring genes are intact and biotransformation of both 10-dml (**18**) and narbonolide (**24**) are known[37] though unoptimized for macrolactone to macrolide mass balance. Small-scale inoculation of narbonolide (**24**) to DHS2001 or YJ112 cultures experienced variation in initial rate and total conversion pikromycin (**28**), motivating studies to isolate and evaluate growth phase dependence. Addition of narbonolide (**24**) to high OD_{600} cultures resulted in rapid, but incomplete conversion, while addition to prelog phase cultures were initially slow but afforded complete conversion with either strain. Addition of acetyl-narbonolide (**32**, 5 μM, vide infra) to cultures accelerated the biotransformation, completing the biocatalytic synthesis of pikromycin (**28**) in 13 linear steps from commercially available (*R*)-Roche ester (Scheme 2.12).

Biotransformations of 10-dml (**18**) to methymycin macrolides proved to be more interesting. Under identifical conditions and in parallel with narbonolide (**24**) biotransformations, neither glycosylation nor oxidation of 10-dml (**18**) was observed. Perplexed, this experiment was repeated multiple times with the same outcome. To confirm that the problem wasn't with synthetic 10-dml (**18**), we fermented natural 10-dml (**18**, vide infra, chapter 3) and the purified the compound to homogeneity via prep-HPLC. Attempted biotransformation with naturally derived 10-dml (**18**) gave the same result, no conversion observed. We next attempted a mixed biotransformation with 10-dml (**18**) and narbonolide (**24**) and at last observed conversion of 10-

dml (**18**) to YC-17 (**29**) and methymycins (**30, 31**), though 10-dml (**18**) did not covert fully while narbonolide (**24**) did. Additional experiments where successive doses of narbonolide (**24**) were added provided more complete conversion to (**29**) and methymycins. The hypothesis developed from these experiments was as follows: the PKS product narbonolide (**24**) is responsible for inducing Pik tailoring genes, while the PKS product 10-dml (**18**) cannot induce Pik tailoring genes. While an interesting insight into complex pathway regulation, this phenomenon was restricting access to the methymycin macrolides and required a solution.

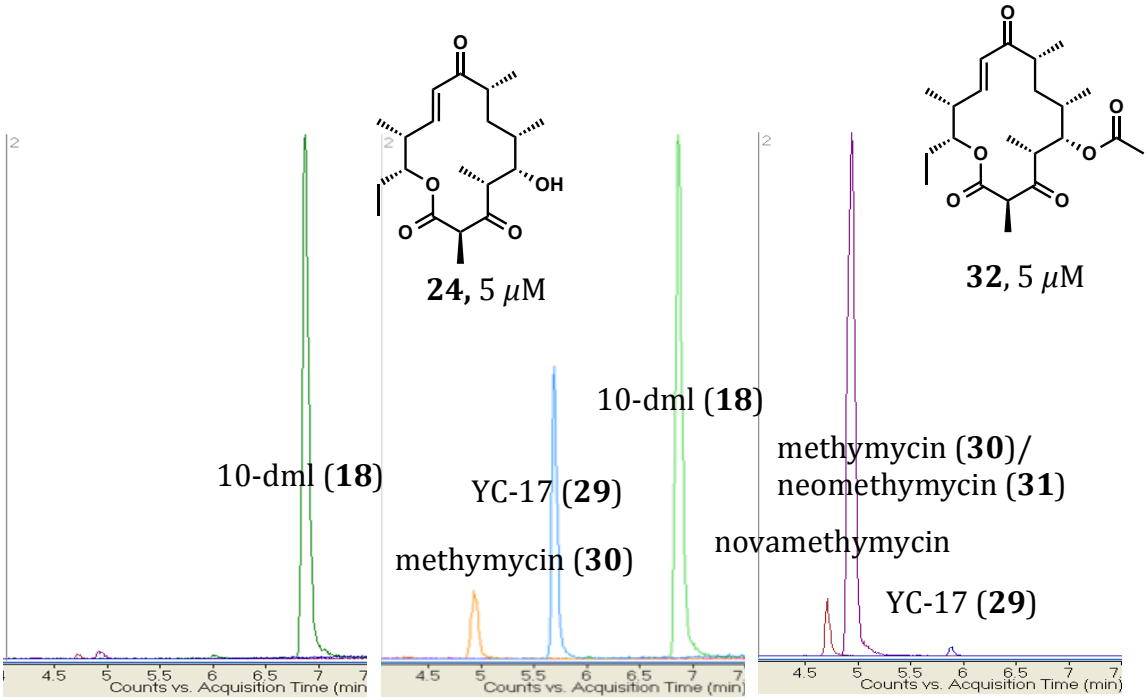
Scheme 2.13 Biotransformation of 10-dml (**18**) to methymycins (**28**)



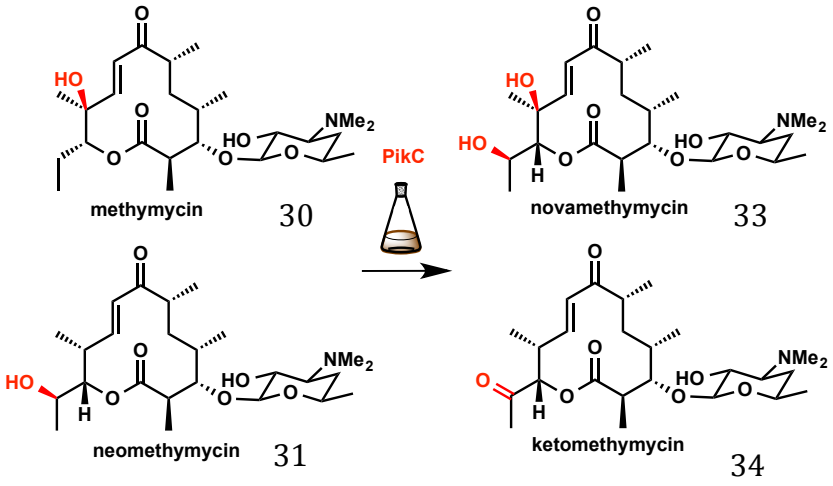
Iterative doses of narbonolide (**24**) was an option but a poor one, as the tailoring genes **24** induced quickly glycosylated **24** to narbomycin (**27**) which appeared to have little ability to keep the *des* genes functional. As such, we considered using acetyl-narbonolide (**32**) as non-consumable inducer, where the hydroxyl group required for glycosylation is protected. If the acetate did not interfere with binding to whatever cellular sensor was recognizing narbonolide (**24**), then perhaps only a low concentration would be required to affect complete conversion of 10-dml (**18**) to methymycin macrolides. To our surprise, this strategy not only worked, but worked at very low concentrations of acetyl-narbonolide (**32**), only 5 μM was required to affect complete transformation of 10-dml (**18**) to methymycin macrolides. Figure 2.2 shows a 24 h time point LC/MS analysis of DHS2001 cultures, with the trace on the right displaying 10-dml (**18**) conversion without induction, the trace in the middle 10-dml (**18**) conversion without induced with 5 μM narbonolide (**24**), and the trace on the right with 5 μM acetyl-narbonolide (**32**). The difference in biotransformation efficiency was striking, and allowed effective biotransformation of 10-dml (**18**) to methymycins (Scheme 2.13).

Acetyl-narbonolide induction was more pronounced with strain YJ112, providing increased conversion to the doubly oxidized novamethymycin (**33**), and a previously unknown methymycin was detected by LC/MS as well. Preparative biotransformation and HPLC purification yielded a methymycin we named ketomethymycin, presumably arising from a second hydroxylation of neomethymycin at the C13 position, generating a transient germinal di-hydroxylated compound that eliminated water to form the ketone.

Figure 2.2 LC/MS analysis of 10-dml (**18**) to methymycins (**28**)



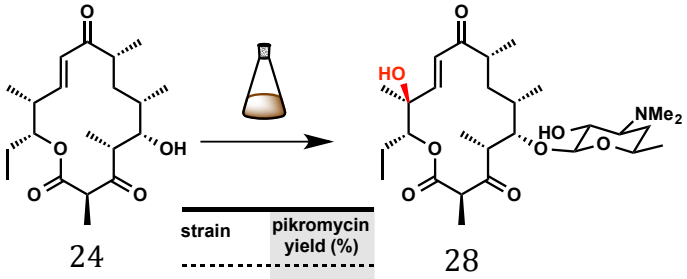
Scheme 2.14 Second oxidation of methymycins



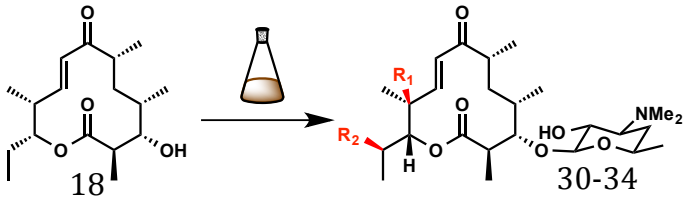
LC/MS analysis of WT *S. venezuelae* ATCC 15439 reveled trace quantities of a compound with identical mass and retention time to ketomethymycin (**34**) indicating that this macrolide is indeed

a trace secondary metabolite made under normal laboratory culture conditions. With functional biotransformation conditions in hand, preparative scale conversion smoothly converted macrolactones to macrolides, completing the biocatalytic synthesis in 13 steps with divergence at the 11th step.

Scheme 2.15 Biotransformation of macrolactones to macrolides



strain	pikromycin yield (%)
YJ112	72

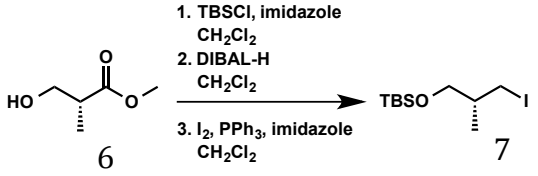


strain	combined yield (%)	methymycin		neomethymycin		novamethymycin		ketomethymycin
YJ112	71	3.5	:	5	:	3	:	1
DHS2001	66	3.5	:	7	:	1	:	trace

2.5 Chemistry Experimental

Reactions were performed in evacuated (<0.05 torr) flame dried glassware containing PFTE coated magnetic stir bars fitted with rubber septa backfilled with dry N_2 and run under a positive pressure of dry N_2 provided by a mineral oil bubbler unless stated otherwise (open flask). Reactions at elevated temperatures were controlled by IKA RET Control Visc (model RS 232 C), room temperature (RT) reactions were conducted at ~23 °C, reactions run cooler than room temperature were performed in a cold room (4 °C), an ice bath (0 °C), dry ice/acetone (-78 °C), or isopropanol/ThermoNESLAB (model CC100) for all other temperatures. Commercial purification system MBraun-MB-SPS # 08-113 provided all dry solvents unless stated otherwise (technical grade). Analytical thin-layer chromatography (TLC) was performed with EMD 60 F_{254} pre-coated glass plates (0.25 mm) and visualized using a combination of UV, *p*-anisaldehyde, $KMnO_4$, and Bromocresol green stains. Flash column chromatography was performed using EMD 60 Gerduran® (particle size 0.04-0.063) silica gel. NMR spectra were recorded on a Varian 600 MHz spectrometer. ^{1}H NMR spectra were recorded relative to residual solvent peak ($CDCl_3$ δ_H 7.26 ppm, D_6-DMSO δ_H 2.50 ppm) and reported as follows: chemical shift (ppm), multiplicity,

coupling constant (Hz), and integration. Multiplicity abbreviations are as follows: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, h = hextet, ovlp = overlap, br = broad signal. ^{13}C NMR spectra were recorded relative to residual solvent peaks ($CDCl_3$ δ_C 77.0 ppm, D_6-DMSO δ_c 39.5 ppm). High resolution mass spectrometry was performed on an Agilent quadrapole time-of-flight spectrometer (Q-TOF 6500 series) by electrospray ionization (ESI).



6 7

7: An open 500-mL flask was charged with (*R*)-Roche ester **6** (TCI, 10.00 g, 84.65 mmol, 1.00 equiv), imidazole (Fisher, 6.34 g, 93.12 mmol, 1.10 equiv), technical grade CH_2Cl_2, (170 mL, 0.5M) and cooled to 0 °C. Tertbutyldimethylsilyl chloride (Oakwood, 14.03 g, 93.12 mmol, 1.10 equiv) was added in 5 portions. The resulting solution was stirred for 1 h at 0 °C and became cloudy with white precipitate. A half-saturated NH_4Cl solution was added until the precipitate was completely dissolved. The organic layer was separated and the aqueous layer extracted 2x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with CH_2Cl_2. Concentration and subsequent high vacuum yielded the silyl-ether as colorless oil (19.65 g, 84.55 mmol) that was carried onto the next step without further purification.

A 500-mL flask containing the silyl-ether (19.65 g, 84.55 mmol) was added CH_2Cl_2 (170 mL, 0.5M) and cooled to -78 °C. A second bath was prepared and cooled to -42 °C. DIBAL-H (Sigma, 25.25 g, 31.64 mL, 177.56 mmol, 2.10 equiv) was added slowly down the side of the flask and stirred for 5 min at -78 °C. The flask was placed in the -42 °C bath for 1 h and then recooled to -78 °C. Methanol (100 mL) was added slowly and the solution was stirred for 15 min at -78 °C. The reaction was decanted into of vigorously stirring CH_2Cl_2 (200 mL) at RT, layered with saturated Na/K tartrate (300 mL) and stirred until the layers became clear. The organic layer was separated and the aqueous layer was extracted 2x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was then rinsed 2x with CH_2Cl_2. Concentration and subsequent high vacuum yielded the alcohol as acolorless oil (16.66 g, 81.51 mmol) that was carried onto the next step without further purification.

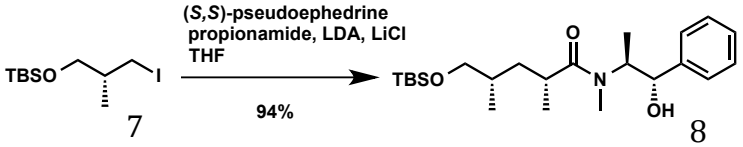
To an open 500-mL flask wrapped in foil containing the alcohol (16.66 g, 81.51 mmol) was added technical grade CH_2Cl_2 (163 mL, 0.5 M) and cooled to 0 °C. Imidazole (Fisher, 8.33 g, 122.27 mmol, 1.50 equiv) and triphenylphospine (AK, 27.79 g, 105.96 mmol, 1.30 equiv) were added in single portions and stirred until dissolved. Iodine (Fisher, 27.93 g, 110.03 mmol, 1.35 equiv) was added in 10 portions while maintaining internal temperature <5 °C. Complete addition of iodine provided a purple/brown solution that was stirred at 0 °C for 5 min, and an additional 1 h at RT.

The reaction was quenched by addition of a cold saturated sodium thiosulfate solution resulting in a colorless bi-phasic mixture. The organic layer was separated and washed once with saturated sodium thiosulfate, and filtered through a sodium sulfate plug, which was then rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography: Et_2O/hexanes (5:95) gave **7** as a colorless oil (22.50 g, 71.60 mmol, 84% yield over three steps).

^{1}H NMR ($CDCl_3$, 599 MHz) δ 3.52 (dd, *J* = 10.0, 5.0 Hz, 1H), 3.40 (dd, *J* = 10.0, 6.9 Hz, 1H) 3.30 (dd, *J* = 9.5, 5.1 Hz, 1H), 3.25 (dd, *J* = 9.5, 5.7 Hz, 1H), 1.67-1.60 (m, 1H), 0.95 (d, *J* = 6.7 Hz, 3H), 0.90 (s, 9H), 0.06 (s, 6H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 66.7, 37.4, 25.9, 18.2, 17.2, 13.7, -5.4.

HRMS: Calculated $[M+H]^+$ 315.0636, found 315.0656.



8: To a 500-mL flask containing (*S,S*)-pseudoephedrine propionamide[7] (25.34 g, 114.66 mmol, 1.60 equiv) was added THF (300 mL) and stirred at RT until the solid dissolved completely. The flask was then cooled to -78 °C.

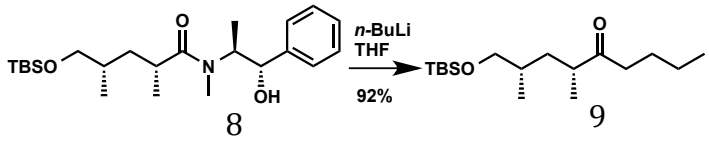
A 1000-mL flask containing LiCl (Fisher, 36.45 g, 859.92 mmol, 12.00 equiv) was flame dried under vacuum iteratively until the inline monometer no longer responded to the flame. THF (150 mL) was added by cannula, followed by diisopropylamine (Sigma, distilled from activated sieves (4 Å), 33.15 mL, 23.93 g, 236.48 mmol, 3.30 equiv) and the flask was cooled to -78 °C. Slow addition of *n*-BuLi (Sigma, 2.46M in hexanes, 90.30 mL, 221.15 mmol, 3.10 equiv) at -78 °C, warmed to 0 °C and held for five minutes before recooling to -78 °C.

The entire contents of the 500-mL flask containing (*S,S*)-pseudoephedrine propionamide solution was added to the 1000 mL flask by cannula. The resulting solution was stirred for 1 h at -78 °C, 30 min at 0 °C, and 5 min at RT before it was recooled to 0 °C. **7** (22.50 g, 71.66 mmol) was dissolved in THF (20 mL) and added dropwise to the solution, which was allowed to warm to RT and stirred for 12 h. The reaction was quenched with saturated NH_4Cl (300 mL), and poured into a 2 L separatory funnel containing H_2O (500 mL), and the aqueous layer was extracted 3x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was then rinsed 2x with CH_2Cl_2. Concentration and flash chromatography: EtOAc/Hexanes (30:70) afforded pale yellow solid **8** (27.46 g, 67.36 mmol, 94% yield).

^{1}H NMR ($CDCl_3$, 599 MHz): δ 7.37-7.20 (m, 5H), 4.62-4.52 (m, 2H), 4.35 (s, 1H), 3.43 (dd, *J* = 9.8, 4.8 Hz, 2H), 3.36 (dd, *J* = 9.9, 5.9 Hz, 2H), 2.83 (s, 3H), 2.78-2.70 (m, 1H), 1.70-1.62 (m, 1H), 1.60-1.52 (m, 1H), 1.14 (d, *J* = 7.1 Hz, 3H) 1.15-1.04 (m, 1H), 1.07 (d, J= 6.7 Hz, 3H), 0.87 (s, 9H), 0.81 (d, *J* = 6.6 Hz, 3H), 0.01 (s, 6H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 179.1, 142.6, 128.7, 128.2, 127.4, 126.9, 126.2, 76.5, 67.9, 37.6, 34.2, 33.1, 25.9, 18.3, 17.5, 17.3, 14.4, -5.5, -5.4.

HRMS: Calculated $[M+Na]^+$ 430.2748, found 430.2740.



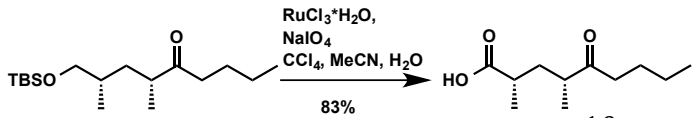
8 9

9: To a 1000-mL flask containing **8** (27.46 g, 67.36 mmol) was added THF (275 mL, final concentration ~0.2M) and stirred at RT until the solid dissolved, then cooled to -78°. A second flask charged with *n*-BuLi (Sigma, 2.46M in hexanes, 60.24 mL, 148.19 mmol, 2.20 equiv) was cooled to -78 °C, and added to the solution of **8** via cannula. The reaction was stirred for 10 min at -78 °C, and 30 min at 0 °C before addition of diisopropylamine (Sigma, 20.77 mL, 15.00 g, 148.19 mmol, 2.20 equiv), and stirred for 15 min at 0 °C. The reaction was quenched with AcOH/Et_2O (20:80, 200 mL). The organic layer was carefully washed 2x with saturated sodium bicarbonate, brine, subsequently filtered through a sodium sulfate plug, which was then rinsed 2x with Et_2O. Concentration and flash chromatography: EtOAc/Hexanes (5:95) afforded colorless oil **9** (18.62 g, 61.97 mmol, 92% yield).

^{1}H NMR ($CDCl_3$, 599 MHz) δ 3.40 (dd, *J* = 9.7, 5.6 Hz, 1H), 3.37 (dd, *J* = 9.8, 6.0 Hz, 1H), 2.64 (h, *J* = 6.9 Hz, 1H), 2.48-2.36 (m, 1H), 1.80-1.74 (m, 1H), 1.59- 1.50 (m, 4H), 1.29 (h, *J* = 7.4 Hz, 2H), 1.06 (d, *J* = 6.8 Hz, 3H), 1.07-1.00 (ovlp m, 1H), 0.92-0.86 (ovlp m, 6H), 0.8 (s, 9H), 0.03 (s, 6H).

^{13}C NMR ($CDCl_3$, 151 MHz): δ 214.9, 67.9, 44.1, 40.4, 36.8, 33.5, 25.9, 25.8, 22.4, 18.3, 17.2, 17.1, 13.9, -5.46, -5.48.

HRMS: Calculated $[M+Na]^+$ 323.2377, found 323.2378.



9 10

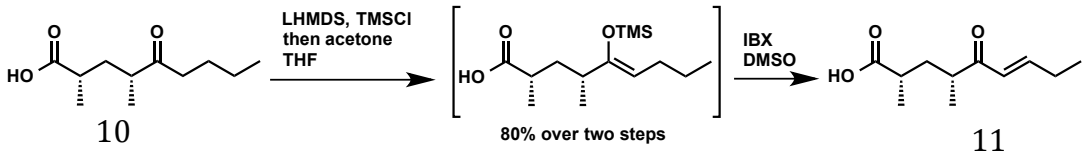
10: To an open 1000 mL flask containing **9** (18.62 g, 61.97 mmol) was added $CCl_4/CH_3CN/H_2O$ (1:1:2, 300 mL, 0.2M), $NaIO_4$ (AK, 66.27 g, 309.85 mmol, 5.00 equiv), and $RuCl_3*H_2O$ (Fisher, 0.13 mg, 0.62 mmol, 1 mol%). The flask was fitted with a reflux condenser and heated to 70 °C with vigorous stirring. The solution turned yellow and vigorous stirring at 70 °C was contiued until the solution turned black (~2 d), cooled to RT and filtered through celite (celite washed 2x with MeCN). The biphasic solution was concentrated and resuspended in hexanes. The organic layer was carefully extracted with saturated sodium bicarbonate until gas evolution ceased (~5x), and back washed with 1x with hexanes. The aqueous layer was carefully brought to pH 3 with phosphoric acid and extracted 5x with CH_2Cl_2. Subsequent filtration through a sodium sulfate

plug then rinsed 2x with CH_2Cl_2 and concentration provided **9** as a pale yellow oil (10.30 g, 51.44 mmol, 83% yield).

^{1}H NMR ($CDCl_3$, 599 MHz) δ 2.61 (h, *J* = 7.0 Hz, 1H), 2.55- 2.38 (m, 3H), 2.08 (ddd, *J* = 14.5, 8.8, 6.1 Hz, 1H), 1.54 (p, *J* = 7.4 Hz, 2H), 1.40-1.32 (m, 1H), 1.29 (h, *J* = 7.3 Hz, 2H), 1.19 (d, *J* = 7.0 Hz, 3H), 1.09 (d, *J* = 7.0 Hz, 3H), 0.89 (t, *J* = 7.3 Hz, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 214.0, 182.4, 44.00, 40.6, 37.2, 36.0, 25.7, 22.3, 17.5, 16.4, 13.8.

HRMS: Calculated $[M+H]^+$ 201.1485, found 201.1485.



10 11

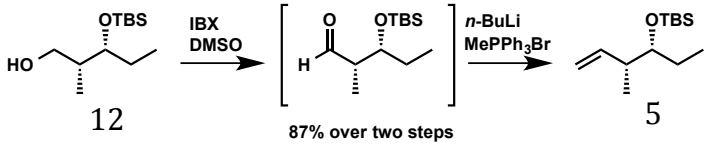
11: To a 500 mL flask containing **10** (6.01 g, 30 mmol) in THF (30 mL) at -78 °C was added TMSCl (Sigma, 15.23 mL, 13.00 g, 120.00 mmol, 4 equiv) down the side of the flask. A second flask was charged with LHMDS solution (Sigma, 1M in THF, 120.00 mL, 120.00 mmol, 4 equiv) and cooled to -78 °C, then added to the **10** solution by cannula and stirred at -78 °C for 30 min, followed by dropwise addition of acetone (10 mL) with 10 min additional stirring. The solution was quenched at -78 °C with phosphate buffer (1M, pH 7, 120 mL) and layered with Et_2O. The aqueous layer was extracted 3x Et_2O, combined organic layers washed with brine. Filtration through a sodium sulfate plug, which was then rinsed 2x with Et_2O and concentration gave the crude trimethylsilyl enol ether of **10** (contaminating (isopropenyloxy)trimethylsilane was mostly removed under subsequent high vacuum).

IBX[38] (0.4M in DMSO, 150 mL, 60.00 mmol, 2.00 equiv) was added to the crude silyl enol ether and stirred for 12 h (the solution turns yellow and a white precipitate forms). The reaction was diluted with H_2O (300 mL) and extracted 3x Et_2O, with combined organic extracts washed 1x with brine, subsequently filtered through a sodium sulfate plug, which was then rinsed 2x with Et_2O. Concentration gave crude silyl enol ether. The crude material was suspended in hexanes (allowing a white precipitate to settle) before transfer onto the flash column: AcOH/EtOAc/Hexanes (1:10:89) to yield **11** as a pale yellow oil (4.80 g, 24.21 mmol, 81% yield).

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.96 (dt, *J* = 15.7, 6.4 Hz, 1H), 6.16 (d, *J* = 15.7 Hz, 1H), 2.86 (h, *J* = 7.0 Hz, 1H), 2.54 (h, *J* = 7.0 Hz, 1H), 2.25 (p, *J* = 6.8 Hz, 1H), 2.12 (ddd, *J* = 14.4, 8.3, 6.6 Hz, 1H), 1.44-1.38 (m, 1H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.13 (d, *J* = 6.9 Hz, 3H), 1.08 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz): δ 203.3, 182.0, 149.5, 127.6, 41.4, 37.1, 36.3, 25.6, 17.5, 16.5, 12.2.

HRMS: Calculated $[M+H]^+$ 199.1329, found 199.1382.



The following two procedures were performed concurrently:

5: A 250 mL flask containing $MePPh_3Br$ (AK, 8.25 g, 23.10 mmol, 1.10 equiv) was placed in an oil bath, and heated to 110 °C under high vacuum for 4 h. The flask was cooled to RT and backfilled with N_2, THF (100 mL, 0.2M) and cooled to 0 °C. *n*-BuLi (Sigma, 2.35M, 9.83 mL, 23.10 mmol, 1.10 equiv) was added dropwise and the reaction was allowed to warm to RT and stirred for a minimum 1 h.

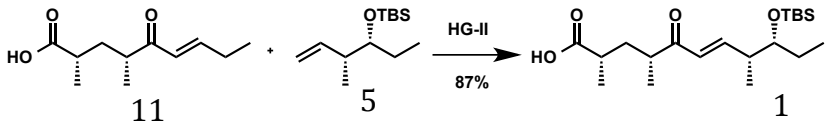
To an open 500 mL flask was added **12**[21-22] (4.88 g, 21.00 mmol), DMSO (80 mL, 0.25M) and IBX (8.82 g, 31.50 mmol, 1.50 equiv) in a single portion. The reaction was monitored by TLC, and after consumption of **12** (~4 h) Et_2O (100 mL) was added. The reaction was quenched with a cold solution of sodium thiosulfate (100 mL), and stirred for 30 min. The aqueous layer was separated and the organic layer was washed 2x with saturated thiosulfate, brine, dried with sodium sulfate, filtered, rinsed 2x with Et_2O and concentrated to give crude aldehyde of **S12**, which was dissolved in THF (20 mL) and used immediately.

Both flasks were cooled to -78 °C and crude **12** was added by cannula to the prepared ylide. The solution was stirred at -78 °C for 30 min, warmed to RT and stirred for an additional 30 min. The reaction was quenched with half saturated NH_4Cl (200 mL) and extracted 3x with pentane, filtered through a sodium sulfate plug, rinsed 2x with CH_2Cl_2 and concentrated to give the crude alkene product. Flash chromatography: Et_2O/pentane (2:98) afforded **5** as a clear oil (4.20 g, 18.38 mmol, 87% over two steps.)

^{1}H NMR ($CDCl_3$, 599 MHz) δ 5.84 (ddd, *J* = 17.5, 10.5, 7.2 Hz, 1H), 5.03-4.95 (m, 2H), 3.46 (q, *J* = 5.5 Hz, 1H), 2.31 (h, *J* = 6.7 Hz, 1H), 1.49-1.36 (m, 2H), 0.97 (d, *J* = 6.8 Hz, 3H), 0.90 (s, 9H), 0.86 (t, *J* = 7.4 Hz, 3H), -0.14 (s, 6H).

^{13}C NMR ($CDCl_3$, 151 MHz): 141.7, 113.6, 77.00, 42.28, 26.49, 25.91, 18.16, 14.97, 9.44, -4.34, -4.46.

EI MS: Calculated $[M\text{-}C(CH_3)_3]^+$ 171.1, found 171.1.



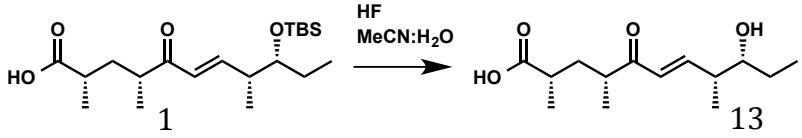
S11: A 25 mL recovery flask was charged with **11** (0.99 g, 5.00 mmol), **5** (1.71 g, 7.50 mmol, 1.50 equiv.) and HG-II (Sigma, 94 mg, 0.15 mmol, 3 mol%) under a stream of N_2. An 18 gauge needle was placed into the septum, venting to the atmosphere (in addition to positive pressure of N_2) and

the flask was heated to 50 °C for 12 h. After cooling to RT, the solution was dissolved in CH_2Cl_2 (20 mL) and transferred to a 250 mL flask, further diluted with CH_2Cl_2 (20 mL) and cooled to 0 °C. Remaining catalyst was destroyed by careful addition of H_2O_2 (15 % by volume, 20 mL) and vigorous stirring for 1 h at 0 °C.[39] The organic layer was separated and the aqueous layer was extracted 2x with CH_2Cl_2, filtered through a sodium sulfate plug, rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography: AcOH/EtOAc/hexanes (1:10:89) to yield **1** (1.62 g, 4.37 mmol, 87% yield).

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.94 (dd, *J* = 15.9, 7.5 Hz, 1H), 6.14 (dd, *J* = 16.0, 1.3 Hz, 1H), 3.59-3.53 (m, 1H), 2.87 (h, *J* = 6.9 Hz, 1H), 2.58-2.44 (m, 2H), 2.17-2.07 (m, 1H), 1.52-1.44 (m, 1H), 1.43-1.34 (m, 2H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.12 (d, *J* = 6.9 Hz, 3H), 1.03 (d, *J* = 6.8 Hz, 3H), 0.88 (s, 9H), 0.86 (ovlp t, *J* = 7.0Hz, 3H), 0.04 (s, 3H), 0.03 (s, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz): δ 203.0, 181.5, 150.8, 127.9, 76.4, 41.5, 41.3, 37.0, 36.2, 26.8, 25.8, 21.3, 18.1, 17.5, 16.7, 14.2, 9.6, -4.4, -4.9.

HRMS: Calculated $[M+H]^+$ 371.2616, found 371.2641.



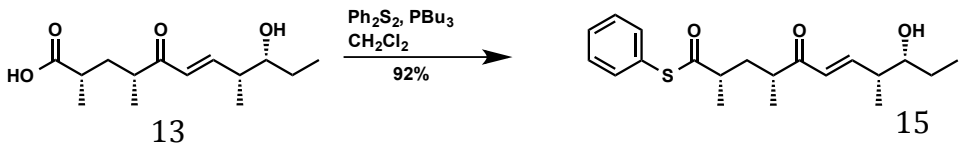
1 13

13: To an open 25 mL polyethylene bottle was added **1** (0.20 g, 0.54 mmol) and MeCN (0.44 mL, 0.5M) and aq. HF (48%, 0.1 mL). The reaction was monitored by TLC and diluted with H_2O/CH_2Cl_2 upon completion. The organic layer was separated and the aqueous layer (plastic separatory funnel) was extracted 2x with CH_2Cl_2 followed by filtration through a sodium sulfate plug, then rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography: AcOH/EtOAc/Hexanes (1:25:74) to yield **13** as a colorless oil (0.12 g, 0.47 mmol, 87% yield). The aqueous layer was brought to pH=10 before disposal.

^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.94 (dd, *J* = 16.0, 7.3 Hz, 1H), 6.17 (dd, *J* = 16.0, 1.1 Hz, 1H), 3.55 (p, *J* =5.4 1H), 2.90 (h, *J* = 6.9 Hz, 1H), 2.60-2.44 (m, 2H), 2.15-2.06 (m, 1H), 1.59-1.50 (m, 1H), 1.49-1.32 (m, 2H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.13 (d, *J* = 6.9 Hz, 3H), 1.09 (d, *J* = 6.8 Hz, 3H), 0.98 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR ($CDCl_3$, 101 MHz): δ 203.1, 180.8, 149.8, 128.5, 76.05, 42.2, 41.4, 37.31, 36.9, 27.0, 17.8, 16.4, 13.6, 10.4.

HRMS: Calculated $[M+H]^+$ 257.1747, found 257.1750.



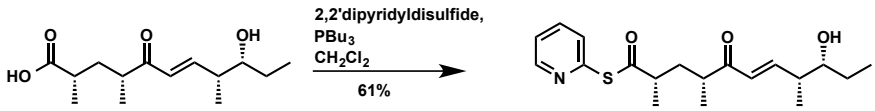
13 15

15: To a 25 mL flask was added **13** (20 mg, 0.078 mmol) and Ph_2S_2 (Fisher, 19 mg, 0.086 mmol, 1.10 equiv.) dissolved in CH_2Cl_2 (0.78 mL, 0.1M) and cooled to 0 ^oC. PBu_3 (Sigma, distilled neat, 21 µL, 17 mg, 0.085 mmol, 1.10 equiv) was added slowly while keeping the solution <5 ^oC, the reaction mixture was stirred for 10 min before it was quenched with $CuSO_4$ impregnated silica gel and concentrated. Flash chromatography: EtOAc/hexanes (25:75) to yield **15** (25 mg, 24.2 mmol, 92%).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.41 (s, 5H), 6.87 (dd, *J* = 15.9, 7.6 Hz, 1H), δ 6.17 (dd, *J* = 15.9, 1.2 Hz, 1H), 3.50-3.42 (m, 1H), 2.93-2.80 (m, 2H), 2.48-2.39 (m, 1H), 2.20 (ddd, *J* = 14.4, 8.8, 5.9 Hz, 1H), 1.59-1.31 (m, 3H), 1.25 (d, *J* = 6.9 Hz, 3H), 1.16 (d, *J* = 6.9 Hz, 3H), 1.08 (d, *J* = 6.8 Hz, 3H), 0.95 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR ($CDCl_3$, 101 MHz): δ 202.6, 201.3, 149.8, 134.4, 129.4, 129.2, 128.3, 127.6, 75.9, 46.0, 42.3, 41.3, 36.7, 27.4, 18.5, 16.4, 13.9, 10.33.

HRMS: Calculated $[M+H]^+$ 349.1832, found 349.1853.



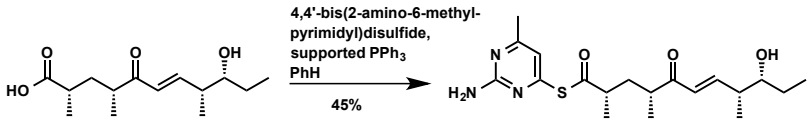
13 16

16: To a 25 mL flask was added **13** (24 mg, 0.094 mmol) and 2,2'-dipyridyldisulfide (TCI, 22 mg, 0.10 mmol, 1.10 equiv.) dissolved in CH_2Cl_2 (0.94 mL, 0.1M) and cooled to 0 ^oC. PBu_3 (Sigma, distilled neat, 26 µl, 21 mg, 0.10 mmol, 1.10 equiv) was added dropwise keeping the solution <5 ^oC, the reaction mixture was stirred for 10 min before it was quenched with $CuSO_4$ impregnated silica gel and concentrated. Flash chromatography: EtOAc/hexanes (25:75) to yield colorless oil **16** (20 mg, 0.057 mmol, 61% yield).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.62 (d, *J* = 4.9, 1H), 7.75 (td, *J* = 7.7, 2.0 Hz, 1H), 7.61 (d, *J* = 7.8 Hz, 1H), 7.30 (dd, *J* = 7.6, 4.8 Hz, 1H), 6.91 (dd, *J* = 15.9, 7.4 Hz, 1H), 6.16 (d, *J* = 15.9 Hz, 1H), 3.55-3.45 (m, 1H), 2.99-2.80 (m, 2H), 2.51-2.38 (m, 1H), 2.21 (ddd, *J* = 14.3, 9.0, 5.8 Hz, 1H), 1.60-1.30 (m, 3H), 1.28 (d, *J* = 6.9 Hz, 3H), 1.15 (d, *J* = 6.9 Hz, 3H), 1.08 (d, *J* = 6.8 Hz, 3H), 0.95 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 202.7, 200.4, 151.3, 150.4, 150.0, 137.1, 130.3, 128.4, 123.6, 75.8, 46.6, 42.3, 41.0, 37.0, 27.3, 18.4, 16.4, 13.7, 10.4.

HRMS: Calculated $[M+H]^+$ 350.1784, found 350.1788.



13 17

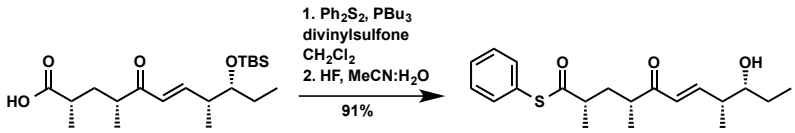
17: To a 20 mL sealed tube was added **13** (30 mg, 0.117 mmol) and 4,4'-Bis(2-amino-6-methylpyrimidyl) disulfide (TCI, 49 mg, 0.1755 mmol, 1.5 equiv) suspended in benzene (3 mL,

0.04M), followed by solid supported PPh_3 (Sigma, 1.36 mmol/g, 130 mg, .1755 mmol, 1.50 equiv.). The reaction was heated to 100 °C for 15 min, cooled to RT, filtered through glass wool, then rinsed 2x with CH_2Cl_2 and concentrated: flash chromatography: EtOAc/hexanes (40:60 to 100:0) to yield **17** (20 mg, 0.052 mmol, 45% yield).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.93 (s, 1H) 6.92 (dd, *J* = 7.6 Hz, 1H), 6.16 (dd, *J* = 16.0, 1.3 Hz, 1H), 5.18 (br s, 2H), 3.53 (p, *J* = 6.6 Hz, 1H), 2.91 (h, *J* = 6.8, 1H), 2.98-2.74 (m, 1H), 2.54-2.40 (m, 1H), 2.36 (s, 3H), 2.32-2.13 (m, 1H), 1.60-1.31 (m, 3H), 1.26 (d, *J* = 6.9 Hz, 3H), 1.14 (d, *J* = 6.9 Hz, 3H), 1.09 (d, *J* = 6.8 Hz, 3 H), 0.97 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 202.5, 199.1, 179.2, 168.9, 150.0, 128.4, 114.8, 76.7, 75.9, 47.1, 42.3, 40.9, 36.9, 27.3, 24.0, 18.3, 16.6, 13.6, 10.4.

HRMS: Calculated $[M+H]^+$ 380.2002, found 380.2003.



13 15

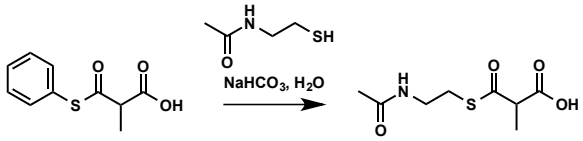
15: To a 100 mL flask containing **15** (1.59 g, 4.29 mmol) was added Ph_2S_2 (Fisher, 1.03 g, 4.72 mmol, 1.10 equiv) and divinylsulfone (Oakwood, 0.50 mL, 0.56 g, 4.72 mmol, 1.10 equiv) dissolved in CH_2Cl_2 (22 mL, 0.2M) and cooled to 0 °C. PBu_3 (Sigma, distilled neat, 1.38 mL, 1.13 g, 5.58 mmol, 1.30 equiv.) was added dropwise keeping the solution <5 °C. The reaction was stirred for 20 min and quenched with saturated sodium bicarbonate (50 mL). The aqueous layer was extracted 2x with CH_2Cl_2, filtered through a sodium sulfate plug, which then rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography: EtOAc/hexanes (5:95) gave the crude thioester of **15,** which was used immediately in the following step.

To an open 25 mL polyethylene bottle was added crude thioester of **15** and MeCN (4 mL, 1M) and aq. HF (48%, 1 mL). The reaction was monitored by TLC and upon completion it was diluted with CH_2Cl_2 (10 mL) and carefully quenched with saturated sodium bicarbonate. The aqueous layer was extracted 2x with CH_2Cl_2. Filtration through a sodium sulfate plug then rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography: EtOAc/hexanes (15:85) gave **15** (1.37 g, 3.93 mmol, 91% yield over two steps).

^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.41 (s, 5H), 6.87 (dd, *J* = 15.9, 7.6 Hz, 1H), δ 6.17 (dd, *J* = 15.9, 1.2 Hz, 1H), 3.50-3.42 (m, 1H), 2.93-2.80 (m, 2H), 2.48-2.39 (m, 1H), 2.20 (ddd, *J* = 14.4, 8.8, 5.9 Hz, 1H), 1.59-1.31 (m, 3H), 1.25 (d, *J* = 6.9 Hz, 3H), 1.16 (d, *J* = 6.9 Hz, 3H), 1.08 (d, *J* = 6.8 Hz, 3H), 0.95 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR ($CDCl_3$, 101 MHz): δ 202.6, 201.3, 149.8, 134.4, 129.4, 129.2, 128.3, 127.6, 75.9, 46.0, 42.3, 41.3, 36.7, 27.4, 18.5, 16.4, 13.9, 10.33.

HRMS: Calculated $[M+H]^+$ 349.1832, found 349.1853.



23: To an open 500 mL flask containing H_2O (220 mL, 1M) sparged with N_2 for 30 minutes (sparging maintained through course of reaction) was added **22**[29] (60 g, 285 mmol, 1.3 equiv.) followed by $NaHCO_3$ (177.4 g, 2 mol, 2.5 equiv.). HSNAC[40] (23.264 mL, 26 g, 219 mmol) was added dropwise over 30 min and stirred for 4 h and monitored by TLC for the loss of **22**. The solution was carefully acidified with G26 resin (Sigma, washed 2x 1M HCl prior to use) until a pH of 2-3 was achieved, the solution was filtered through glass wool, and the aqueous layer was washed 3x with CH_2Cl_2 [organic layer was placed in an Erlenmeyer and stirred with bleach (added slowly) before disposal]. The aqueous layer was flash frozen and lyophilized to yield **23** (40.4 g, 184.26 mmol, 84% yield) as a white solid.

For use in enzymatic reactions, **23** was suspended in H_2O (500mM) aliquots, the pH was raised to 7.2 through careful addition of $NaHCO_3$, flash frozen and stored at -20 °C.

Note: **23** rapidly decarboxylates in DMSO-D_6. MeCN-D_3 proved acceptable for both ^1H and ^{13}C NMR, though complete decarboxylation was observed after ~7 days when stored on the bench at RT.

^{1}H NMR (CD_3CN, 400 MHz) δ 6.71 (s, 1H), 3.67 (q, *J* = 7.1 Hz, 1H), 3.38-3.23 (m, 2H), 3.06-2.91 (m, 2H), 1.85 (s, 3H), 1.34 (d, *J* = 7.1 Hz, 3H).

^{13}C NMR (CD_3CN, 101 MHz) δ 197.9, 172.2, 171.5, 55.3, 39.4, 29.5, 22.9, 14.7.

HRMS: Calculated $[M+H]^+$ 220.0638, found 220.0646.

2.6 Polyketide Synthase Experimental

PKS Protein Preparation

All H_2O was obtained from a Millipore Milli-Q system (serial P3MNO3809A) using Millipore Q-Gard 2/Quantum Ex Ultrapure organex cartridges. LB broth Miller was obtained from EMD and autoclaved before use. Glycerol was obtained from EMD, HEPES was obtained from Calbiochem (Omnipur grade), Isopropyl-b-D-thiogalactopyranoside (IPTG) was obtained from Gold Biotechnology. Kanamycin Sulfate (Kan) was obtained from Amresco. ACS grade imidazole and NaCl were obtained from Fisher Scientific. pH was determined on a Symphony SB70P pH meter (serial SN005695) calibrated according to manufacturer's specifications. Ni-NTA agarose was purchased from Qiagen and pre-equilibrated with five column volumes of lysis buffer. PD-10 columns were purchased from GE and pre-equilibrated with five column volumes

of storage buffer. Cells were lysed using a 550 Sonic Dismembrator purchased from Fisher Scientific. Optical density (OD_{600}) was determined using an Eppendorf Biophotometer.

Cloning, expression and purification of all proteins (PikAIII, PikAIV, PikAIII-TE) has been previously reported,[2,41] and expression and purification optimized for activity and reproducibility. Buffers: (lysis) HEPES (50 mM), NaCl (300 mM), imidazole (10mM), glycerol (10% v/v), pH 8.0 (wash) HEPES (50 mM), NaCl (300 mM), imidazole (30mM), glycerol (10% v/v), pH 8.0 (elution) HEPES (50 mM), NaCl (300 mM), imidazole (300mM), glycerol (10% v/v), pH 8.0 (storage) HEPES (50 mM), NaCl (150 mM), EDTA (1 mM), glycerol (20% v/v), pH 7.2. Bap[42] cells bearing plasmids for expression of respective PKS modules were taken from glycerol cell stocks stored at -80°C and grown in LB (10mL) with Kan (50 mg/L), and grown overnight at 37°C. The following morning, LB (1L) containing Kan (50 mg/L) was inoculated with the entire overnight culture, and shaken at 37°C until they reached an OD_{600} of 0.25 - 0.3 at which point they were removed and allowed to cool to RT. When an OD_{600} of 0.4 was reached, the cultures were induced with IPTG (300μM) and shaken at 180RPM at 20°C for 18 hours. Cells were pelleted at 5000g (4 °C) for 10 minutes.

Purification of PKS Proteins

The following steps were conducted in <2 hours for maximum and reproducible enzymatic activity. Cells were suspended in 15 mL of lysis buffer per liter of culture broth *via* vortex, and sonicated on ice at 60% power 6 x 10s with 60s rest periods. Cellular debris was pelleted in a precooled (4°C) centrifuge at 40,000g for 10 min, and the supernatant was applied to 3 mL of Ni-NTA resin and allowed to drip through. 15 mL of wash buffer was added, the column was gently pressurized with a syringe, and the enzyme of interest was eluted with 15 mL of elution buffer with gentle syringe pressure. Protein containing fractions were determined via Bradford assay and pooled. Buffer exchange was performed using a PD-10 column, and protein containing fractions were determined via Bradford assay and pooled, aliquoted, flash frozen in liquid N_2, and stored at -80 °C.

Analytical PKS Reactions

Glucose-6-phosphate was purchased from Biosynth, glucose-6-phosphate dehydrogenase (yeast) was purchased from Alpha Aesar, and NADP+ was obtained from Amresco. 2-vinylpyridine (Sigma) was added as a thiol scavenger to reactions monitored by HPLC without discernible loss of enzymatic activity. All reactions were conducted in triplicate at 50 μL scale, initiated by addition of PKS enzyme(s), quenched when designated by addition of MeOH (3x by

vol, 150 µL) and clarified by centrifugation at 20800g for 2 minutes. The resulting solution was analyzed without further manipulation.

QTOF-LC/MS analysis

Analytical liquid chromatography/mass spectrometry (LC/MS) was performed on an Agilent LC system (1290 series) coupled to an Agilent QTOF mass spectrometer (6500 series) using a Phenomenex Synergi 4µ Hydro RP 100 x 2 mm column (serial 48836-5) at 50°C. Method: 0.4 mL/min, A: H_2O 0.1% formic acid, B: MeCN 0.1% formic acid, 0% B 0-2 min, 0-100% B linear gradient 1-9 min, 100% 9-10 min, 100-0% B 10-10.5 min linear gradient re-equilibration, 0-4 min were diverted to waste. The mass spectrometer was operated in profile mode in the positive ion mode with automatic lock mass infusion at 121.0508 and 922.0098 m/z using Agilent HP-Mix. The source temperature was 325°C with drying gas at 5 L/min and nebulizing gas at 30 Psig. The capillary was set to 3500 V, the fragmenter was set to 175 V, the skimmer was set to 65 V, and the octapole RF was set to 750 V peak to peak. Spectra were measured from 100-1,500 m/z with 500 ms/spectrum.

HPLC analysis

Analytical high performance liquid chromatography (HPLC) was performed on a Beckman Coulter system (model 366 serial 385-1160) using a Phenomenex Luna 5µ C18 250 x 4.6mm column (serial 466013-1) monitoring at 250nM. Method: 1.5mL/min, A: H_2O 0.1% formic acid, B: MeCN 0.1% formic acid, 5% B 0-1 min, 5-100% B linear gradient 1-12 min, 100% B 12-15 min, 5% 15-17.5 min re-equilibration.

Standard curves were constructed in triplicate at five concentrations, with concentration (0.5mM-0.03125mM) corresponding to conversion to macrolactone products (200%-12.5% conversion), for example: 0.25mM corresponds to 100% theoretical conversion. (0.5mM/200%, 0.25mM/100%, 0.125mM/50%, 0.0625mM/25%, 0.03125mM/12.5%) immediately before or after reaction analysis.

Crude Cell Lysate Preparation

Expression of all modules was performed as described in the **Protein Purification** section. Cells were pelleted at 5000g (4°C) for 10 minutes, and resuspended in 10mL of storage buffer [HEPES (50 mM), NaCl (150 mM), EDTA (1 mM), glycerol (20% v/v), pH 7.2] per liter of pelleted culture broth *via* vortex and sonicated on ice at 60% power 6 x 10s with 60s rest periods. Cellular

debris was pelleted in a precooled (4 °C) centrifuge at 40,000g for 10 min. Crude cell lysate was either used immediately or flash frozen in N_2 and thawed on ice without discernible loss in activity. Protein concentration was crudely normalized to that of purified protein though densitometry, and used without further manipulation.

Representative semi-preparative and preparative PKS reactions

All reactions were performed once, initiated by addition of PKS enzyme(s), quenched after 4 h by addition of 2x volume of acetone, placed in a -20 °C freezer for one hour and filtered through a celite plug. Remaining insoluble material was suspended in acetone and this solution was used to rinse the celite plug. Acetone was removed through rotary evaporation and the aqueous layer was 3x with CH_2Cl_2. Filtration through a sodium sulfate plug was performed then rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography: EtOAc/Hexanes (30:70) afforded 10-dml. Flash chromatography: EtOAc/Hexanes (30:70) afforded narbonolide though yields varied as did complexity of spectra associated with the isolated macrolactone. Acetylation of narbonolide after workup but before chromatography stabilized yields and purity of the product. Acetylation conditions: CH_2Cl_2 (0.1M) at 0°C for 20 min with Ac_2O (10 equiv), NEt_3 (12 equiv), DMAP (cat). The reaction was quenched with half saturated NH_4Cl, the organic layer was separated and the aqueous layer was 2x extracted with CH_2Cl_2. Filtration through a sodium sulfate plug, which was then rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography: EtOAc/Hexanes (20:80) afforded acetyl-narbonolide.

Representative semi-preparative and preparative PKS reactions

All reactions were performed once, initiated by addition of PKS enzyme(s), quenched after 4 h by addition of 2x volume of acetone, placed in a -20 °C freezer for one hour and filtered through a celite plug. Remaining insoluble material was suspended in acetone and this solution was used to rinse the celite plug. Acetone was removed through rotary evaporation and the aqueous layer was 3x with CH_2Cl_2. Filtration through a sodium sulfate plug was performed then rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography: EtOAc/Hexanes (30:70) afforded 10-dml. Flash chromatography: EtOAc/Hexanes (30:70) afforded narbonolide though yields varied as did complexity of spectra associated with the isolated macrolactone. Acetylation of narbonolide after workup but before chromatography stabilized yields and purity of the product. Acetylation conditions: CH_2Cl_2 (0.1M) at 0°C for 20 min with Ac_2O (10 equiv), NEt_3 (12 equiv), DMAP (cat). The reaction was quenched with half saturated NH_4Cl, the organic layer was separated and the aqueous layer was 2x extracted with CH_2Cl_2. Filtration through a sodium sulfate plug, which was

then rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography: EtOAc/Hexanes (20:80) afforded **acetyl-narbonolide (32)**.

Reaction 1: semi-preparative scale up of analytical reactions

Conditions: sodium phosphate buffer (400mM, 20% v/v glycerol, 200mL, pH = 7.2), Pik pentaketide **15** (70 mg, 0.2 mmol, 1mM), MM-NAC (20 equiv, 20mM), $NADP^+$ (0.1 equiv, 0.1mM), glucose-6-phosphate (2.5 equiv, 2.5mM), glucose-6-phosphate dehydrogenase (0.5 unit/mL), 2-vinylpyridine (8mM), purified PikAIII-TE or PikAIII/PikAIV (1 uM, 0.1 mol%), 4 hours, stationary, RT. The aforementioned purification protocol gave **10-dml (18)** (31.5 mg, 0.11 mmol, 53%) as a viscous colorless oil that foams under high vacuum, or **acetyl-nbl (32)** (37.5 mg, 0.094 mmol, 47%), that crystalizes upon standing.

Reaction 2: semi-preparative crude cell lysate evaluation

Conditions: sodium phosphate buffer (400mM, 20% v/v glycerol, 200mL, pH = 7.2), Pik pentaketide **15** (70 mg, 0.2 mmol, 1mM) MM-SNAC (20 equiv, 20mM), $NADP^+$ (0.1 equiv, 0.1mM), glucose-6-phosphate (2.5 equiv, 2.5mM), glucose-6-phosphate dehydrogenase (0.5 unit/mL), 2-vinylpyridine (8mM), cell free PikAIII-TE or PikAIII/PikAIV (1 uM, 0.1 mol%), 4 hours, stationary, RT. The aforementioned purification protocol gave **10-dml (18)** (36.8 mg, 0.124 mmol, 62%) as a viscous colorless oil, which foams under high vacuum or **acetyl-nbl (32)** (43.4 mg, 0.11 mmol, 55%) that crystalizes upon standing.

Reaction 3: semi-preparative crude cell lysate buffer/concentration evaluation
Conditions: sodium phosphate buffer (50mM, 2.5% v/v glycerol, 50mL, pH = 7.2), pentaketide **15** (70 mg, 0.2 mmol, 4mM) MM-SNAC (10 equiv, 40mM), $NADP^+$ (0.1 equiv, 0.4mM), glucose-6-phosphate (2.5 equiv, 10mM), glucose-6-phosphate dehydrogenase (2 unit/mL), 2-vinylpyridine (8mM), cell free PikAIII-TE or PikAIII/PikAIV (4μM, 0.1 mol%), 4 hours, stationary, RT. The aforementioned purification protocol gave **10-dml (18)** (39.2 mg, 0.124 mmol, 66%) as a viscous colorless oil that foams under high vacuum, or **acetyl-nbl (32)** (43 mg, 0.11 mmol, 55%) that crystalizes upon standing.

Reaction 4: preparative PKS catalysis, and scale up of reaction 3.

Conditions: sodium phosphate buffer (50mM, 2.5% v/v glycerol, 375mL, pH = 7.2), pentaketide **15** (500 mg, 1.43 mmol, 4mM) MM-SNAC (10 equiv, 40mM), $NADP^+$ (0.1 equiv, 0.4mM), glucose-6-phosphate (2.5 equiv, 10mM), glucose-6-phosphate dehydrogenase (2 unit/mL), 2-vinylpyridine

(8mM), cell free PikAIII-TE or PikAIII/PikAIV (4μM, 0.1 mol%), 4 hours, stationary, RT. The aforementioned purification protocol gave **10-dm(18)** (256 mg, 0.86 mmol, 60%) as a viscous colorless oil that foams under high vacuum, or **acetyl-nbl(31)** (277 mg, 0.7 mmol, 49%) which crystalizes upon standing.

10-dml (18)

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.74 (dd, *J* = 15.7, 5.4 Hz, 1H), 6.42 (d, *J* = 15.7 Hz, 1H), 5.00 (ddd, *J* = 8.2, 5.4, 2.1 Hz, 1H), 3.55 (d, *J* = 10.4 Hz, 1H), 2.68-2.48 (m, 3H), 1.76-1.50 (m, 4H), 1.36-1.24 (ovlp m, 2H), 1.30 (d, *J* = 6.8 Hz, 3H), 1.22 (d, *J* = 7.0 Hz, 3H), 1.11 (d, *J* = 6.8 Hz, 3H), 1.00 (d, *J* = 6.2 Hz, 3H), 0.91 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 205.1, 174.9, 147.1, 125.6, 78.0, 73.7, 45.1, 43.3, 38.0, 33.2, 33.2, 25.1, 17.6, 17.4, 16.4, 10.3, 9.5.

HRMS: Calculated $[M+H]^+$ 297.2060, found 297.2055

Acetyl-narbonolide (31)

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.75 (dd, *J* = 15.8, 6.0 Hz, 1H), 6.14 (d, *J* = 15.8 Hz, 1H), 5.36 (d, *J* = 8.2 Hz, 1H), 4.91 (ddd, *J* = 8.3, 5.9, 2.9 Hz, 1H), 3.77 (q, *J* = 7.0 Hz, 1H), 2.91 (p, *J* = 7.5 Hz, 1H), 2.74-2.62 (m, 2H), 2.07 (s, 3H), 2.00 (s, 1H), 1.71-1.65 (m, 1H), 1.64-1.57 (m, 3H), 1.35 (d, *J* = 7.0 Hz, 3H), 1.16 (d, *J* = 7.4 Hz, 3H), 1.13 (d, *J* = 6.8 Hz, 3H), 1.11 (d, *J* = 7.0 Hz, 3H), 0.97 (d, *J* = 6.8 Hz, 3H), 0.91 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 206.0, 202.6, 170.3, 169.0, 147.8, 126.5, 78.5, 74.98, 50.5, 47.7, 43.0, 38.3, 35.5, 34.7, 23.1, 20.7, 17.4, 16.4, 14.7, 13.5, 12.1, 10.4.

HRMS: Calculated $[M+H]^+$ 395.2428, found 395.2436

Narbonolide (24)

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.89 (dd, *J* = 16.3, 4.9 Hz, 1H), 6.10 (dd, *J* = 16.4, 1.8 Hz, 1H), 5.16-5.11 (m, 1H), 3.91-3.83 (m, *J* = 4.9 Hz, 1H), 3.71 (q, *J* = 7.0 Hz, 1H), 3.05-2.97 (m, 1H), 2.73-2.65 (m, 2H), 2.61 (s, 1H), 1.73-1.58 (m, 4H), 1.53-1.44 (m, 1H), 1.35 (d, *J* = 7.0 Hz, 3H), 1.25 (s, 2H), 1.14 (d, *J* = 6.8 Hz, 3H), 1.09 (d, *J* = 6.8 Hz, 3H), 0.92 (t, *J* = 7.4 Hz, 3H) 1.40-0.78 (ovlp m, 4 H)

^{13}C NMR ($CDCl_3$, 151 MHz) δ 207.6, 204.9, 170.9, 148.5, 128.9, 78.1, 72.6, 50.3, 50.2, 39.8, 38.8, 36.4, 35.1, 24.2, 18.6, 18.2, 14.3, 10.9, 10.8, 10.4.

HRMS: Calculated $[M-H_2O+H]^+$ 335.2217, found 335.2225

2.7 Biotransformation Experimental

Spore stock preparation from *Streptomyces venezuelae* strains

All H_2O used was obtained from a Millipore Milli-Q system (serial P3MNO3809A) using Millipore Q-Gard 2/Quantum Ex Ultrapure organex cartridges. Yeast extract, meat extract, glucose, agar was obtained from EMD. N-Z amine was obtained from Sigma Aldrich. Soytone and soluble starch were obtained from BD. MOPS was purchased from AK Scientific. $CaCl_2$ and $NaCl_2$ were obtained from Fisher Scientific. pH was determined on a Symphony SB70P pH meter (serial SN005695) calibrated according to the manufacturer's specifications. Optical density (OD_{600}) was determined using an Eppendorf Biophotometer. All solutions are autoclaved unless stated otherwise and manipulations occurred in a sterile laminar flow hood.

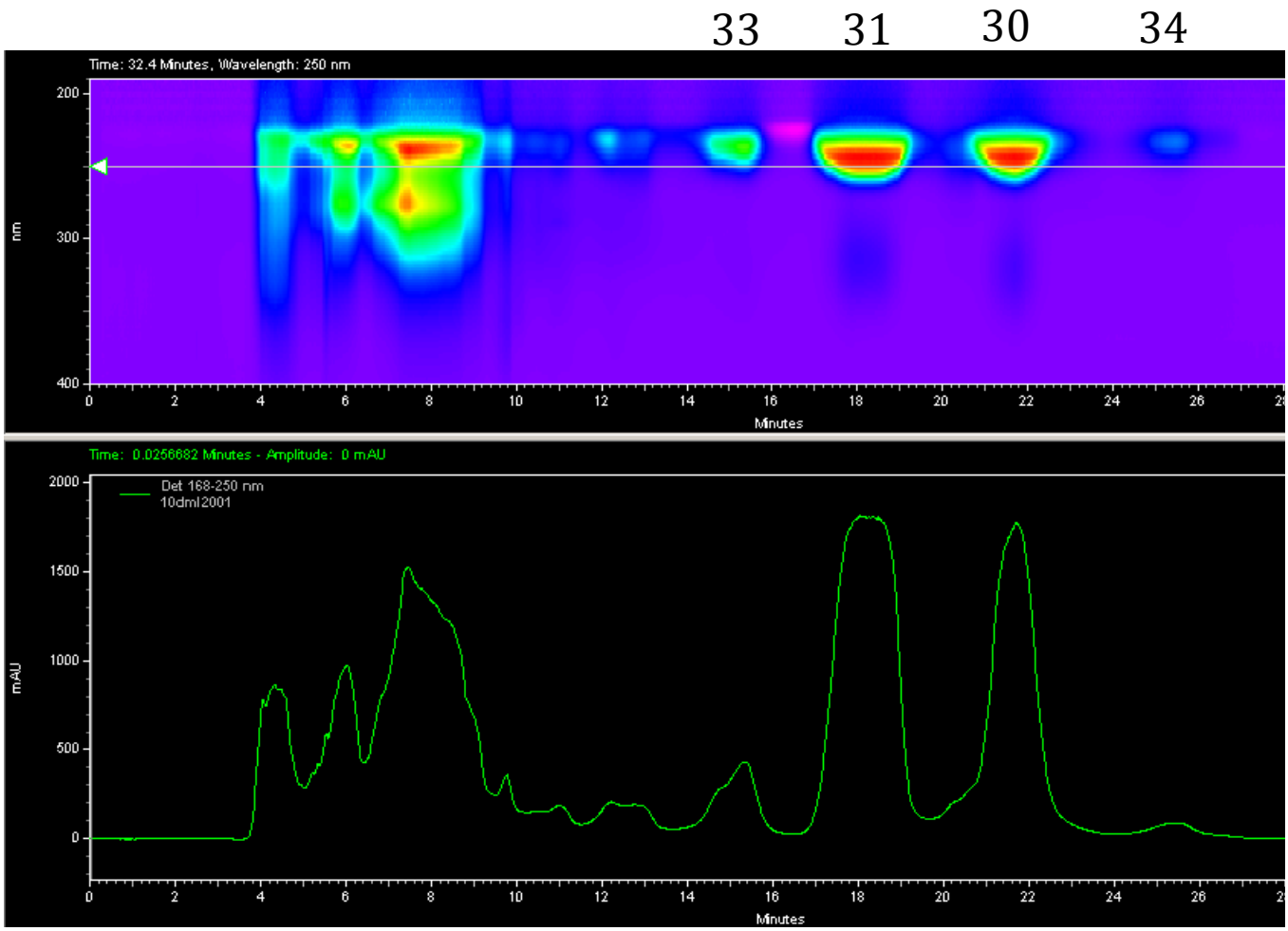
Engineered variants of *Streptomyces venezuelae* ATCC 15439 designated DHS2001 and YJ112 were grown on Bennett's agar (1L = 1 g yeast extract, 1 g meat extract, 2 g N-Z amine, 10 g glucose, 15 g agar, pH 7.3) plates (30 mL) at 28°C until reaching a high spore density (~4-6 days). H_2O (9 mL) was added to the plate, and the spores were suspended by scraping with an inoculation loop. This solution was added to a 15 mL sterile tube, vortexed vigorously for 1 min, and filtered through cotton into another 15 mL sterile tube. The spores were pelleted by centrifugation (2000g, 5 min), the supernatant decanted, and the spores were resuspended in 20% glycerol solution (1 mL), transferred to a sterile screw top vial and flash frozen in N_2.

Biotransformation of 10-dml to methymycins

Twelve baffled 250 mL flasks containing SCM media (1 L = 15 g soluble starch, 20 g soytone, 0.1 g calcium chloride, 1.5 g yeast extract, 10.5 g MOPS, pH 7.2) (100 mL), each inoculated with spore stock (10 µl) and incubated at 28°C, 180 RPM until reaching an OD_{600} = 0.1 (~12-15 h). 10-dml (120 mg, 0.405 mmol, 0.34mM, 10 mg per flask, 100 mg/L) was added as a DMSO solution (50 mg/mL) followed by acetyl-nbl (5uM, ~2.5 mg/L), and the flasks continued incubation at 28°C, 180 RPM for 48 h. Combined culture broth was concentrated by rotary evaporation to 1/3 of the original volume followed by 2x volume of acetone, placed in a -20°C freezer for one hour and filtered through a celite plug. Remaining insoluble material was suspended in acetone and used to rinse the celite plug. Acetone was removed through rotary evaporation and the aqueous layer was saturated with NaCl, pH was adjusted to 11, and the solution 3x extracted with EtOAc. The organic layer was dried with sodium sulfate, filtered, and concentrated. Macrolides were purified directly by preparatory HPLC using a Phenomenex Luna 5u C18 250 x 21.2mm column (serial 444304-4) at a flow rate of 9 mL/min with an isocratic mobile phase of H_2O/MeCN (45/55) and a 0.1% NEt_3 modifier.

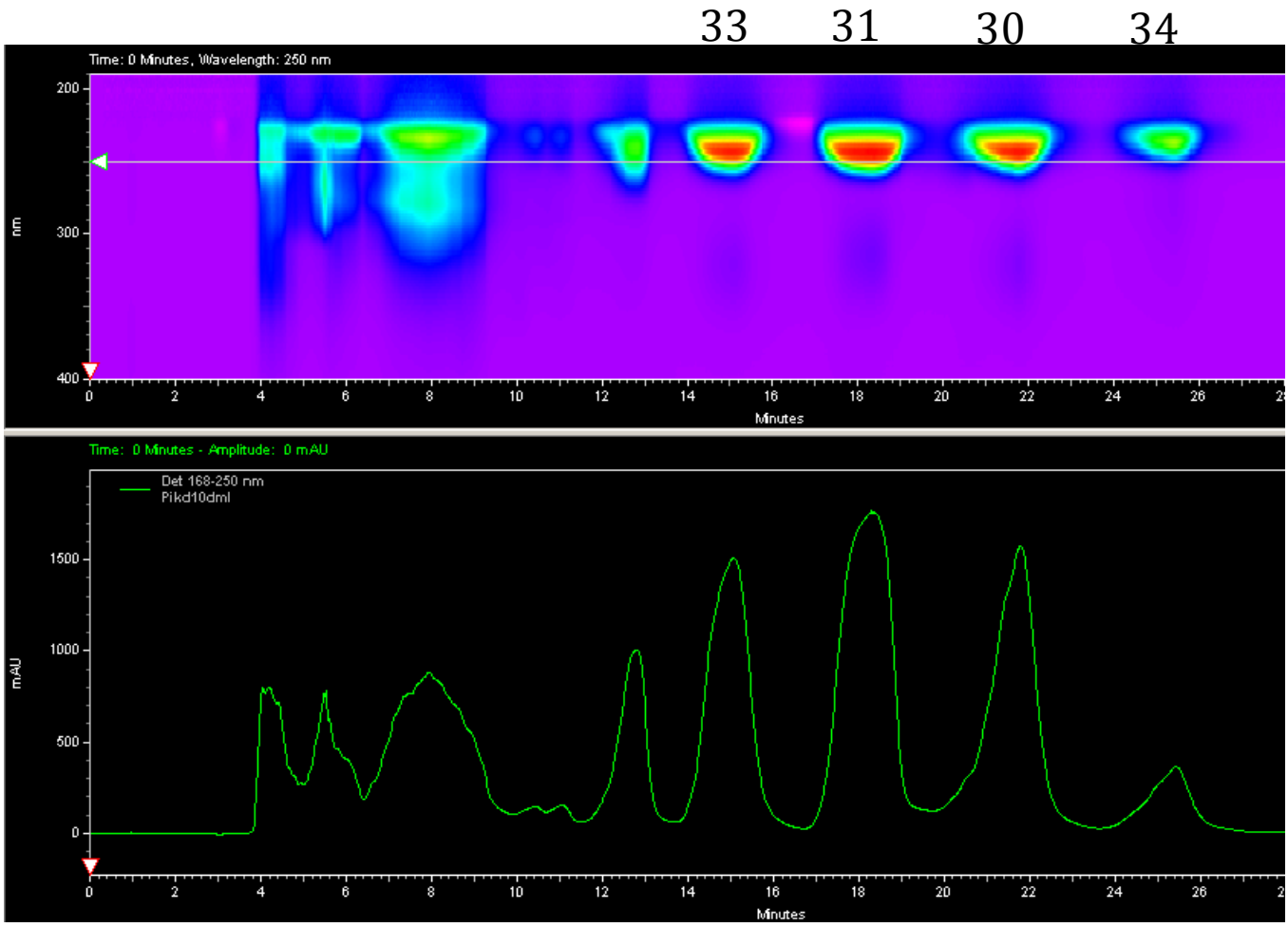
DHS2001: **Methymycin** (**30**) (39 mg, 0.083 mmol, 20.5% yield) **Neomethymycin**(**31**) (75 mg, 0.160 mmol, 39.5% yield) **Novamethymycin** (**33**) (11 mg, 0.023 mmol, 5.7%) **Ketomethymycin** (**34**) (2 mg, 0.004 mmol, 1%)

Total: **methymycins** (0.27 mmol, 66%, Methymycin:Neomethymycin:Novamethymycin:Ketomethymycin = 3.5:7:1:trace)



YJ112: **Methymycin** (**30**) (37 mg, 0.079 mmol, 19.5% yield) **Neomethymycin** (**31**) (58 mg, 0.123 mmol, 30% yield) **Novamethymycin** (**33**) (32 mg, 0.065 mmol, 16%) **Ketomethymycin** (**34**) (11 mg, 0.023 mmol, 6%)

Total: **methymycins** (0.29 mmol, 71%, Methymycin:Neomethymycin:Novamethymycin:Ketomethymycin = 3.5:5:3:1)



Methymycin

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.58 (d, *J* = 15.9 Hz, 1H), 6.32 (d, *J* = 15.9 Hz, 1H), 4.75 (dd, *J* =10.8, 2.2 Hz, 1H), 4.23 (d, *J* = 7.3 Hz, 1H), 3.59 (d, *J* = 10.4 Hz, 1H), 3.51-3.43 (m, 1H), 3.40 (br s, 1H), 3.20 (dd, *J* = 10.2, 7.3 Hz, 1H), 2.85 (dq, *J* = 13.8, 6.9 Hz, 1H), 2.60-2.50 (m, 1H), 2.50-2.43 (m, 1H), 2.25 (s, 6H), 1.98-1.89 (m, 1H), 1.72-1.61 (m, 2H), 1.56-1.39 (m, 2H), 1.42 (d, *J* = 6.9 Hz, 3H), 1.34 (s, 3H), 1.28-1.19 (ovlp m, 2H) 1.21 (d, *J* = 6.1 Hz, 3H), 1.16 (d, *J* = 6.9 Hz, 3H), 1.00 (d, *J* = 6.7 Hz, 3H), 0.89 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 204.5, 175.2, 148.9, 125.6, 105.1, 85.5, 76.3, 74.3, 70.3, 69.5, 65.8, 45.1, 44.2, 40.2, 33.9, 33.6, 28.2, 21.2, 21.1, 19.4, 17.6, 17.4, 16.1, 10.7.

HRMS: Calculated $[M+H]^+$ 470.3112, found 470.3152.

Neomethymycin

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.73 (dd, *J* = 15.7, 5.5 Hz, 1H), 6.42 (dd, *J* = 15.7, 1.3 Hz, 1H), 4.77 (dd, *J* = 9.0, 2.0 Hz, 1H), 4.21 (d, *J* = 7.3 Hz, 1H), 3.86 (dq, *J* = 12.3, 6.1 Hz, 1H), 3.57 (d, *J* = 10.5 Hz, 1H), 3.50-3.42 (m, 1H), 3.41 (ovlp br s, 1H), 3.19 (dd, *J* = 10.2, 7.3 Hz, 1H), 3.07-3.0 (m, 1H), 2.86 (dq, *J* = 11.0, 6.9 Hz, 1H), 2.56-2.46 (m, 1H), 2.49-2.41 (m, 1H), 2.24 (s, 6H), 1.71-1.60 (m, 2H), 1.48-1.37 (m, 1H), 1.39 (d, *J* = 6.9 Hz, 3H), 1.28-1.19 (ovlp m, 2H), 1.21 (d, *J* = 6.1 Hz, 3H), 1.18 (d, *J* = 7.0 Hz, 3H), 1.16 (d, *J* = 7.4 Hz, 3H), 1.13 (d, *J* = 6.8 Hz, 3H), 1.00 (d, *J* = 6.6 Hz, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 205.2, 174.8, 147.1, 126.2, 105.0, 85.6, 75.4, 70.3, 69.5, 66.2, 65.8, 45.1, 43.9, 40.2, 35.4, 34.1, 33.4, 28.2, 21.1, 21.0, 17.6, 17.4, 15.8, 9.8.

HRMS: Calculated $[M+H]^+$ 470.3112, found 470.3137.

Novamethymycin

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.63 (d, *J* = 16.0 Hz, 1H), 6.31 (d, *J* = 16.0 Hz, 1H), 4.65 (d, *J* = 9.2 Hz, 1H), 4.22 (d, *J* = 7.3 Hz, 1H), 4.12 (dq, *J* = 12.3, 6.1 Hz, 1H), 3.59 (d, *J* = 10.4 Hz, 1H), 3.47 (dq, *J* = 12.5, 6.6 Hz, 2H), 3.20 (dd, *J* = 9.9, 7.4 Hz, 1H), 2.85 (dq, *J* = 13.9, 6.9 Hz, 1H), 2.60-2.51 (m, 1H), 2.50-2.43 (m, 1H), 2.25 (s, 6H), 1.71-1.62 (m, 2H), 1.49 (s, 3H), 1.48-1.40 (ovlp m, 1H), 1.40 (d, *J* = 6.9 Hz, 3H), 1.28-1.18 (ovlp m, 2H), 1.22 (d, *J* = 6.0 Hz, 3H), 1.17 (ovlp d, *J* = 7.5 Hz, 3H), 1.16 (ovlp d, *J* = 7.4 Hz, 3H), 1.00 (d, *J* = 6.6 Hz, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 204.3, 174.1, 148.2, 125.4, 105.1, 85.3, 75.5, 74.2, 70.3, 69.6, 67.7, 65.8, 45.2, 44.0, 40.2, 33.8, 33.6, 28.2, 21.1, 20.9, 20.1, 17.5, 17.4, 15.8.

HRMS: Calculated $[M+H]^+$ 486.3061, found 486.3074.

Ketomethymycin

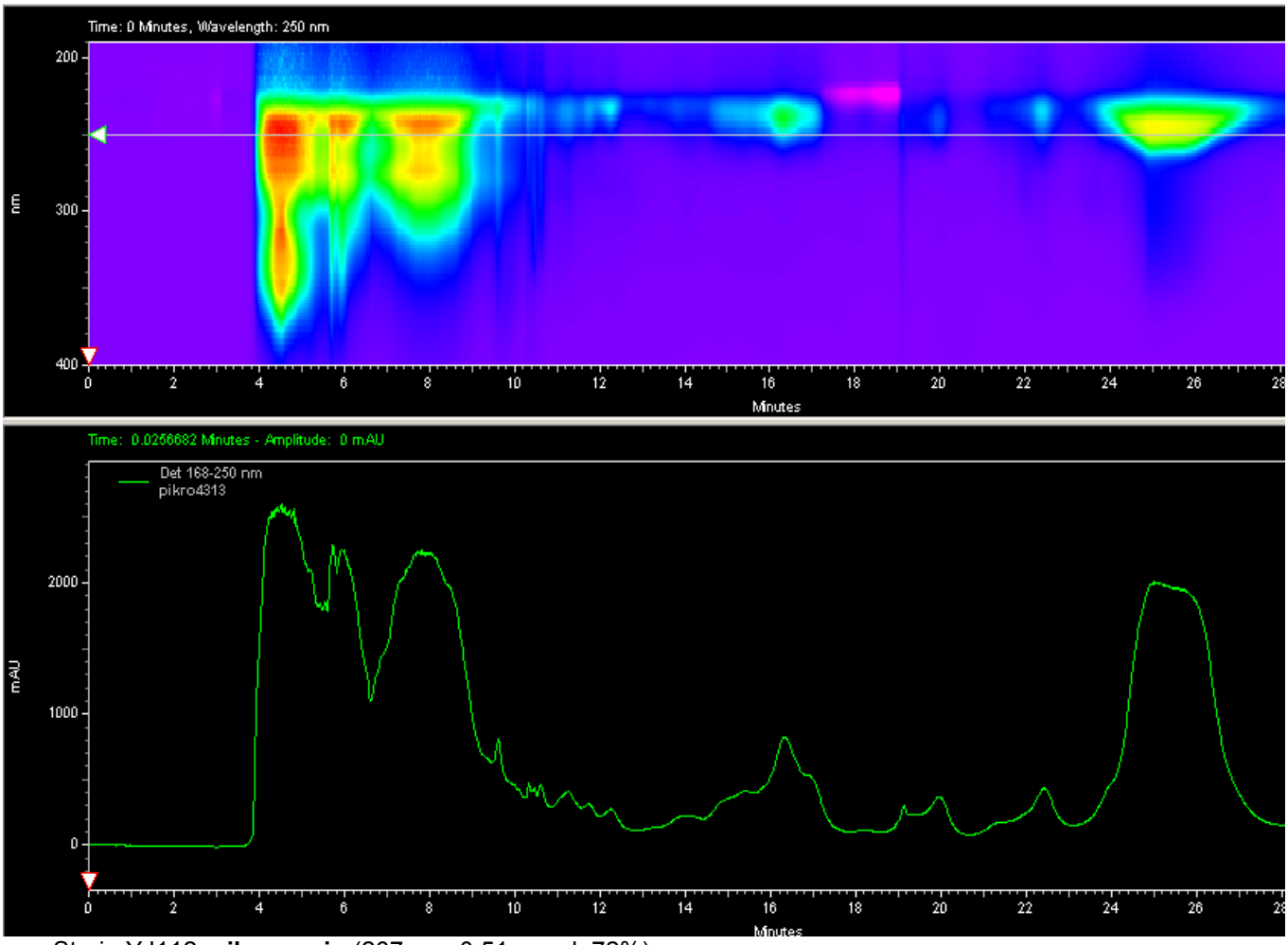
^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.73 (dd, *J* = 15.7, 5.6 Hz, 1H), 6.51 (d, *J* = 15.7 Hz, 1H), 5.27 (d, *J* = 2.5 Hz, 1H), 4.26 (d, *J* = 7.3 Hz, 1H), 3.61 (d, *J* = 10.5 Hz, 1H), 3.49 (dq, *J* = 12.0, 6.4 Hz, 1H), 3.43 (br s, 1H), 3.28-3.19 (m, 2H), 3.04 (dq, *J* = 13.1, 6.5, 6.1 Hz, 1H), 2.60-2.51 (m, 1H), 2.51-2.44 (m, 1H), 2.27 (s, 6H), 2.17 (s, 3H), 1.73 (br t, *J* = 13.3 Hz, 1H), 1.68-1.62 (m, 1H), 1.57-1.47 (ovlp m, 1H), 1.50 (d, *J* = 6.9 Hz, 3H), 1.30-1.21 (ovlp m, 1H), 1.23 (d, *J* = 6.2 Hz, 3H), 1.18-1.12 (m, *J* = 13.0, 6.8 Hz, 1H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.06 (d, *J* = 6.8 Hz, 3H), 1.02 (d, *J* = 6.7 Hz, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 204.9, 204.6, 174.6, 144.6, 127.2, 105.1, 85.5, 77.1, 70.3, 69.6, 65.9, 45.0, 43.9, 40.2, 36.7, 33.9, 33.7, 28.1, 27.4, 21.1, 17.7, 17.3, 15.6, 10.5.

HRMS: Calculated $[M+H]^+$ 468.2956, found 468.2962.

Biotransformation of narbonolide to pikromycin

Twenty five baffled 250 mL flasks containing SCM media (1L = 15 g soluble starch, 20 g soytone, 0.1 g calcium chloride, 1.5 g yeast extract, 10.5 g MOPS, pH 7.2) (100mL), were inoculated with spore stock (*S. venezuelae* strain YJ112, 10 µl) and incubated at 28°C, 180 RPM until OD_{600} = 0.1 (~12-15 h). Crude narbonolide (250 mg, 0.71 mmol, 0.28mM, 10 mg per flask, 100 mg/L, 200 µl per flask) was added in a DMSO solution (50 mg/mL), followed by acetyl-narbonolide in a 50mg/mL DMSO solution (5µM, ~2.5 mg/L) and the flasks were incubated at 28°C, 180 RPM for 48 h. Combined culture broth was concentrated by rotary evaporation to 1/3 original volume followed by 2x volume of acetone, placed in a -20°C freezer for one hour and filtered through a celite plug. Remaining insoluble material was suspended in acetone and this solution was used to rinse the celite plug. Acetone was removed through rotary evaporation and the aqueous layer was saturated with NaCl, the pH was adjusted to 11 and the solution was 3x extracted with EtOAc. The organic layer was dried with sodium sulfate, filtered, and concentrated. Pikromycin was purified directly by preparatory HPLC using a Phenomenex Luna 5u C18 250 x 21.2mm column (serial 444304-4) at a flow rate of 9 mL/min with an isocratic mobile phase of H_2O/MeCN (45/55) and a 0.1% NEt_3 modifier. After evaporation, pikromycin was dissolved in CH_2Cl_2 and flash chromatography through a short plug of silica gel: MeOH/CH_2Cl_2 (10/90).



Strain YJ112: **pikromycin** (267 mg, 0.51 mmol, 72%)

Note: ^{1}H and ^{13}C NMR experiments conducted at 50°C.

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.63 (d, *J* = 15.7 Hz, 1H), 6.30 (d, *J* = 15.7 Hz, 1H), 5.00 (dd, *J* = 11.0, 2.1 Hz, 1H), 4.34 (d, *J* = 7.2 Hz, 1H), 3.98-3.92 (ovlp m, 1H), 3.97-3.88 (ovlp m, 1H), 3.95 (ovlp q, *J* = 7.3 Hz, 1H), 3.59-3.52 (m, 1H), 3.23 (dd, *J* = 10.1, 7.4 Hz, 1H), 3.19 (t, *J* = 3.6, 1H), 2.75-2.65 (m, 1H), 2.48 (m, 1H), 2.28 (s, 6H), 2.18-2.09 (m, 1H), 1.77-1.69 (m, 1H), 1.70-1.64 (m, 1H), 1.57-1.50 (m, 1H), 1.57-1.50 (m, 1H), 1.47-1.38(ovlp m, 1H), 1.45 (d, *J* = 7.3 Hz, 3H), 1.32 (s, 3H), 1.30 (d, *J* = 7.1 Hz, 3H), 1.24 (d, *J* = 6.1 Hz, 3H), 1.10 (d, *J* = 6.5 Hz, 3H), 1.05 (d, *J* = 6.9 Hz, 3H), 1.05-0.96 (m, 2H), 0.89 (t, *J* = 7.3 Hz, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 212.6, 203.5, 170.4, 145.4, 129.2, 104.9, 83.5, 75.1, 70.0, 69.7, 65.9, 53.2, 46.6, 43.0, 40.2, 37.7, 35.8, 28.4, 23.3, 23.0, 21.1, 17.5, 14.7, 13.2, 10.6.

HRMS: Calculated $[M+H]^+$ 526.3374, found 526.3395

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(13) However, such rapidity was not required, as the intermediates could be stored without concern of decomposition.

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Chapter 3

Substrate Controlled Divergence in PikAIV Catalysis with Stabilized Hexaketide Substrates

The work described in this chapter was conducted alongside fellow Sherman lab graduate student Aaron A. Koch, who contributed to many aspects of the research herein.

Terminal type I PKS modules typically catalyze the formation of a single product where the ketosynthase (KS) domain accepts the growing polyketide chain from an upstream acyl carrier protein (ACP), final processing is performed, and the terminal thioesterase (TE) domain releases the product. PikAIV is unique in the ability to accept the Pik hexaketide onto either the KS or TE domain, to generate the 14-membered macrolactone narbonolide(**16**) or 12-membered macrolactone 10-dml(**1**) respectively.

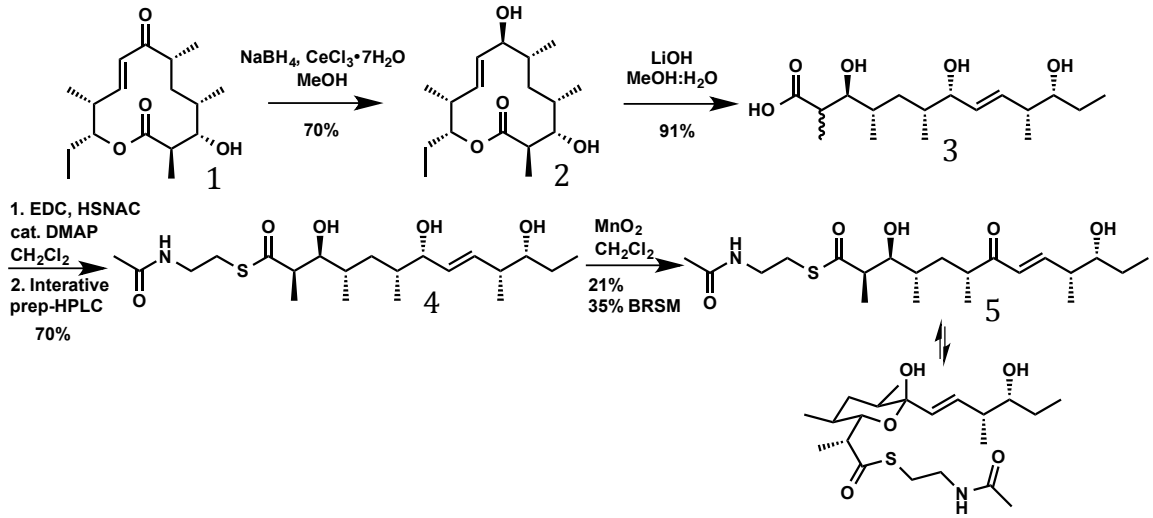
Advances made in PKS biochemistry (chapter 2) were applied to evaluate the terminal Pik module, PikAIV, with a panel of native Pik hexaketide substrates. The Pik hexaketide substrates were accessed through chemical degradation of fermentation derived 10-dml(**1**) from an engineered variant of *S. venezuelae* ATCC 15439. As the Pik hexaketide is inherently unstable due to intramolecular hemiketalization subsequent dehydration,[1] we developed protection strategies to alleviate this experimental bottleneck. We pursued two distinct protective groups 1) a small methyl ether that would remain attached throughout the catalytic cycle, and 2) a 2-nitrobenzyloxymethyl ether that could be photolyzed to provide the Pik hexaketide on demand. The advances in substrate protection enabled thorough evaluation of substrate control on domain loading. We observed dramatic variation in the product distribution dictated by the type of ester employed, with greater than 10:1 selectivity for either the 14-membered macrolactone narbonolide (**16**) through full module catalysis or the 12-membered macrolactone 10-deoxymethynolide (**1**) through direct macrolactonization.

3.1 Synthesis of the Pik hexaketide seco-acid

We considered two options for accessing the Pik hexaketide, 1) fully synthetic[2] or 2) degradation of fermentation derived 10-dml(**1**).[1] Either strategy was viable as intermediates from Pik pentaketide (chapter 2) could be diverted to synthesize the Pik hexaketide and 10-dml(**1**)

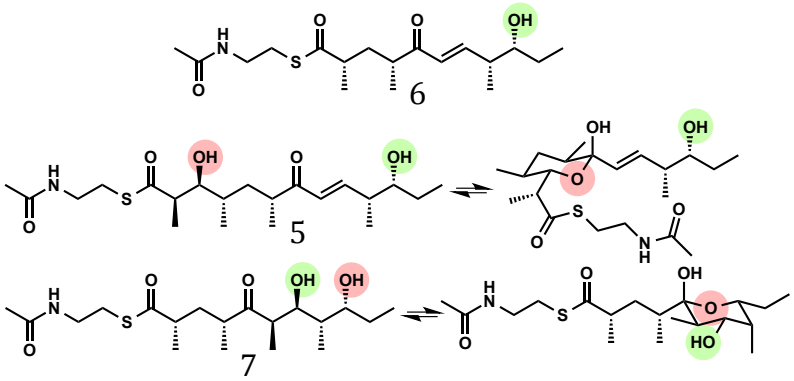
fermentation is a practical method to provide grams of the macrolactone. Ultimately, we decided to pursue a second-generation degradation strategy toward the Pik hexaketide. The first generation degradation of 10-dml (Scheme 3.1) exploited the most intuitive disconnection to the hexaketide. As the hexaketide is the seco-acid of 10-dml(**1**), hydrolysis of the macrolactone and subsequent esterification could theoretically yield hexaketide ready for PKS biochemistry in just two steps. Direct hydrolysis fails for two reasons 1) the hexaketide seco-acid is unstable and decomposes rapidly through hemiketalization and dehydration pathways and 2) 10-dml(**1**) is exceedingly difficult to hydrolyze requiring forcing conditions, destroying any product formed.

Scheme 3.1 First generation synthesis of the NAC Pik hexaketide **5**



As such, a Leuche reduction of the α,β-unsaturated ketone to an allylic alcohol provided **2**, which, when hydrolyzed would be stable as the hemiketalization degradation pathway would be prevented(Scheme 3.1). **2** was then refluxed in LiOH solution for 9 d resulting in partial epimerization at the C2 position of **3**. Thioesterification and prep-HPLC was sufficient to separate the C2 epimers yielding **4** then just one oxidation away from targeted hexaketide **5**.

Figure 3.1 Previously synthesized polyketide substrates



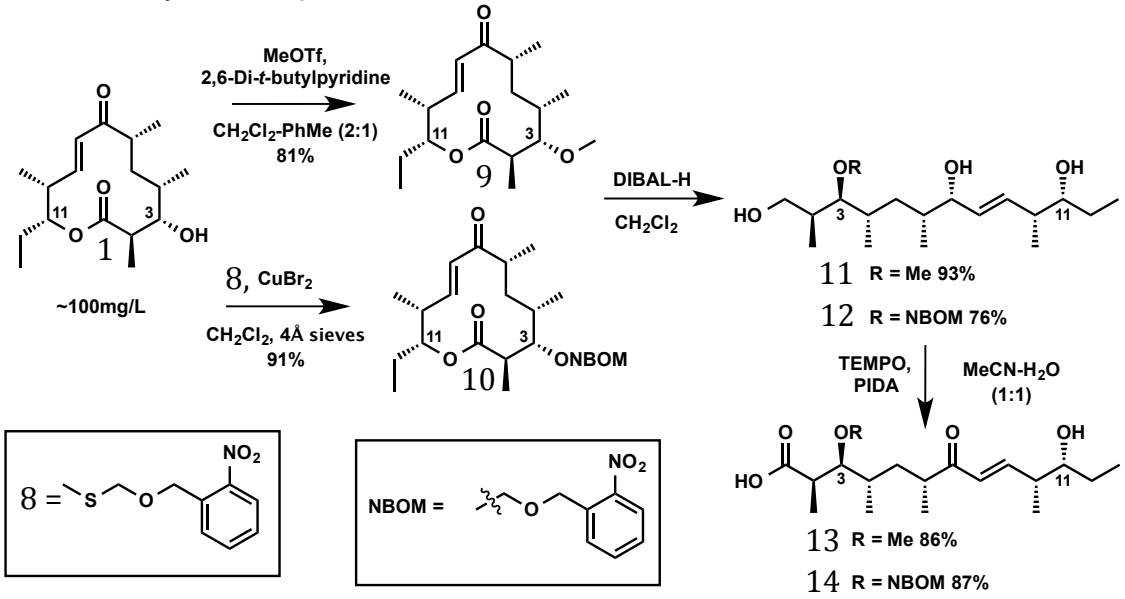
The allylic alcohol was returned to the requisite α,β-unsaturated ketone oxidation state with MnO_2 providing **5** after prep-HPLC as a mixture of linear chain and closed hemiketals.[1] While this route was able to provide a few milligrams of **5** for functional studies of PikAIV or excised TE domain, we sought to develop countermeasures against hemiketal formation, and, in turn, a scalable route to Pik hexaketides that avoided prep-HPLC.

Total synthesis of polyketide natural products routinely employs protecting group arrays where the last step is often a deprotection to unveil the final target molecule. While mature natural products possess some measure of implicit stability having survived purification from natural sources, polyketide intermediates often degrade rapidly through intramolecular hemiketalization and dehydration pathways. Although the structural basis remains unclear, polyketide elongation intermediates that are covalently attached to the ACP domain during biosynthesis are likely stabilized through sequestration within the PKS module(Figure 3.1).[3] Unsurprisingly, instability of polyketide substrates needed to study PKS modules in vitro is a commonly encountered experimental bottleneck where the substrate rapidly decomposes once synthesized. Pik pentaketide **6** (chapter 2) has been widely utilized[1,4] in the Sherman lab and this can be attributed, at least in part, to the inherent stability of the compound. The lone hydroxyl group (highlighted in green) is inert because the α,β-unsaturated ketone renders intramolecular hemiketalization disfavorable, allowing for facile final deprotection and long term storage of **6**. Removing the α,β-unsaturated ketone results in concomitant loss of stability in the case of related DEBS pentaketide **7**, whose construction was plagued by a problematic final deprotection.[4b] The Pik hexaketide **5** suffers from similar stability problems,[1-2] due to similar hemiketalization and dehydration pathways. While we were specifically targeting the Pik hexaketide, we sought to develop general strategies to overcome this common problem. Thus, to address the instability the Pik hexaketide, we considered two distinct stabilization strategies: (i) a sterically undemanding protecting group that would remain attached throughout the catalytic cycle, and (ii) a protecting group that could be removed in a controlled manner to provide the native hexaketide immediately before use in reactions with PikAIV or the excised Pik TE domain. Ultimately, a methyl ether protecting group was chosen to satisfy (i) and a photocleavable 2-nitrobenzyloxymethyl ether (NBOM)[5] was explored to address objective (ii).

To synthesize protected Pik hexaketides, we first needed to ferment a 10-dml(**1**). We utilized an engineered strain of *Streptomyces venezuelae* ATCC 15439 designated DHS8708 where desosamine biosynthesis was knocked out through disruption of *desI*. DHS8708 no longer produces macrolides but does produce either 10-dml (**1**) or narbonolide (**16**) depending on media employed.[6] Soy based media biases fermentation heavily towards 10-dml(**1**), and fermentation optimization indicated aeration to be intimately tied to resulting macrolactone titer, prompting use of heavily baffled Fernbach flasks and/or a bioreactor. While the bioreactor typically provided 100-120 mg/L, shake flasks also performed well (~90-100 mg/L) meaning that 34 L runs would

typically provide over 3 g of 10-dml (**1**). With an adequate amount of 10-dml (**1**) secured, we initiated the synthetic route to Pik hexaketides by protecting the C3 hydroxyl group. Methylation of the C3 hydroxyl group proved extremely challenging, presumably due to steric hindrance arising from the two vicinal methyl groups. Only trace etherification of the hindered C3 hydroxyl group was detected in neat MeI/Ag_2O at room temperature or when heated in a pressure tube, and decomposition to a complex mixture of products was observed with excess Me_3OBF_4/1,8-bis(dimethylamino)naphthalene in CH_2Cl_2 at all temperatures investigated.[13] MeOTF and 2,6-Di-*t*-butylpyridine provided initial promise, though some decomposition was observed when run at RT in CH_2Cl_2 or when cooled to 4 °C, and attempts to run the reaction at colder temperatures led extremely slow conversion. Switching to less polar PhMe suppressed decomposition but the rate was glacial at 4 °C, and epimerization was observed when the reaction mixture was warmed to RT. Ultimately a concentrated, mixed solvent system of CH_2Cl_2/PhMe (2:1, 2 M) with 1.2 equiv of MeOTf and 2,6-Di-*t*-butylpyridine in CH_2Cl_2/PhMe (2:1, 2 M) at 4 °C furnished the desired product **9** in good yield, albeit at an extended reaction time (72 h).

Scheme 3.2 Synthesis of protected Pik hexaketides



Initially, we attempted to install a 2-nitrobenzyl group onto the C3 hydroxyl group directly though this too proved challenging. Attempts to install the 2-nitrobenzyl ether with 2-nitrobenzyl bromide, 2-nitrobenzyl trifluoromethanesulfonate, or 2-nitrobenzyl trichloroacetimidate were met with met with failure under all surveyed conditions. As such, we moved to a 2-nitrobenzyloxymethyl (NBOM)[5] group which could be appended smoothly by employing **8** with stoichiometric $CuBr_2$ to furnish **9**.[7] Attention was then focused on opening the macrolactone ring known to be particularly recalcitrant towards hydrolysis.[2,8] We considered a number of alternatives including oxidative cleavage of α,β-unsaturated ketone,[9] or cross-metathesis with ethylene and a 2nd generation metathesis catalyst to open the α,β-unsaturated ketone. We

pursued the cross-metathesis approach briefly, though the α,β-unsaturated ketone proved largely unreactive, and changing the electronics (reduction/silyl protection of the resulting allylic alcohol) faired little better (~15% yield.) Accordingly, we considered that a two-step global reduction and selective oxidation would neatly side-step problematic hydrolysis procedures. A strong reducing agent could provide a linear triol, where each hydroxyl group would differ in reactivity: primary, secondary allylic, and secondary homoallylic alcohols. From this triol, we could theoretically oxidize in a chemoselective manner to give the hexaketide seco-acid in one or two additional steps.

Excess $LiAlH_4$ in THF at RT proved sluggish and gave a mixture of diastereomers, whereas reduction with DIBAL-H proceeded smoothly to provide single stereoisomers. Chemoselective oxidation of triols **11** and **12** with TEMPO/PIDA adjusted the oxidation state of the primary hydroxyl group to a carboxylic acid, and the allylic alcohol to the desired α,β-unsaturated ketone without oxidizing the homoallylic hydroxyl group at C11 (Scheme 3.2). With desired *seco*-acids **12** and **14** in hand, we esterified both hexaketides with a variety of alcohols and thiols.

3.2 Evaluation of Pik hexaketide Esters

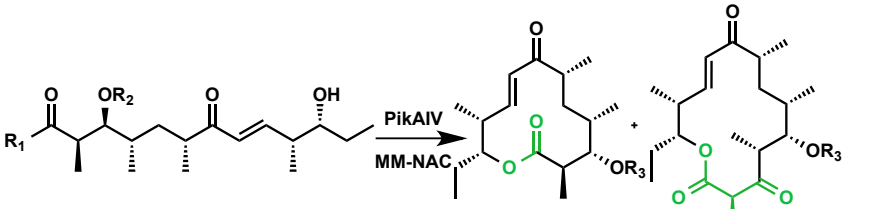
In vitro studies of PikAIV with its native substrate have raised interesting questions about studying PKS enzymes in vitro. For example, when incubated directly with *N*-acetylcysteamine Pik hexaketide **5** PikAIV afforded a 4:1 ratio of macrolactones 10-dml (**1**) and narbonolide (**15**).[10] This result contrasts sharply with reaction schemes pairing PikAIII/PikAIV[2,4a,11] with Pik pentaketide **6** where PikAIII performs an extension and delivers the hexaketide to PikAIV via an ACP_5 thioester; narbonolide (**16**) is the major product. These results suggest that the traditionally employed *N*-acetylcysteamine thioester might be a poor choice for loading the KS domain with high fidelity, and motivated exploratory studies of substrate ester influence with PikAIV. We had some confidence in this approach as optimization of PikAIII (as an unnatural TE fusion[12] or when paired with the final module, PikAIV) demonstrated improved catalysis with thiophenol thioesters[4a] over *N*-acetylcysteamine thioesters (chapter 2).

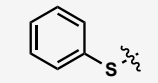
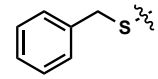
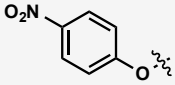
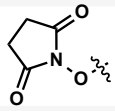
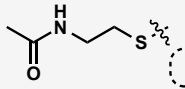
We intended to synthesize a series of NBOM and methyl protected hexaketide esters, incubate them PikAIV or excised Pik TE, and then analyze the catalytic outcome in terms of conversion to 10-dml (**1**):narbonolide (**15**) [or methyl-10-dml (**9**):methyl-narbonolide (**16**)] and the ratio there-of to empirically evaluate substrate ester influence on in vitro catalysis. For the first round of experiments, we employed a panel of 10 hexaketides (five different esters for both methyl and NBOM protected hexaketides) with PikAIV and MM-NAC[4a,13] (Table 3.1).

We had initially hoped to achieve deprotection in situ where an NBOM protected substrate could be photolyzed in the presence of enzyme, though we observed pH dependence

on photolysis[14] requiring a two step procedure where photolysis occurs before the deprotected hexaketide is administered to PikAIV or the excised Pik TE domain. For NBOM protected substrates, 4-nitrophenol (Table 3.1, entry 6) and *N*-hydroxysuccinimide (entry 8) substrates decomposed rapidly upon photolysis and subsequently gave generally low conversion to macrolactones. In contrast, the corresponding hexaketide thiophenol, benzyl mercaptan and *N*-acetylcysteamine thioesters photolyzed smoothly, though benzyl mercaptan thioesters (entry 4) gave lower overall conversion to either macrolactone. Remarkably, we observed significant selectivity in product formation depending on the type of ester employed, where the thiophenol thioester (entry 2) demonstrated greater than 10:1 selectivity for narbonolide (**15**). On the other hand, the corresponding hexaketide *N*-acetylcysteamine thioester (entry 10) showed greater than 10:1 selectivity for 10-dml (**1**).

Table 3.1 Evaluation of stabilized Pik hexaketides with PikAIV and MM-NAC



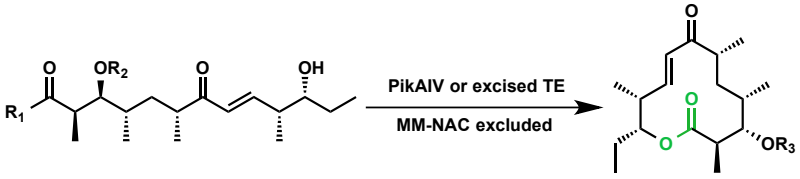
Entry	R_1	R_2	$h\nu$	Conv (%)		Conv (%)
1		Me	-	4 ± 0.1	:	6 ± 0.2
2		NBOM	+	3 ± 0.3	:	41 ± 0.5
3		Me	-	trace	:	ND
4		NBOM	+	trace	:	trace
5		Me	-	4 ± 0.2	:	ND
6		NBOM	+	trace	:	15 ± 0.6
7		Me	-	18 ± 3.3	:	3 ± 0.4
8		NBOM	+	3 ± 1.3	:	trace
9		Me	-	28 ± 2.1	:	ND
10		NBOM	+	52 ± 2.9	:	4 ± 0.3

Initial results clearly demonstrated that the type of ester employed is of utmost importance.[15] In our hands, NAC had a preference for direct TE loading to generate 10-dml (**1**), as did NHS (entries 7 and 8.) Unfortunately, benzyl mercaptan was ineffective at loading either domain though we had anticipated it to be a competent handle for enzyme acylation.

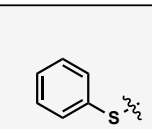
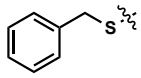
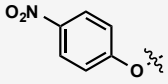
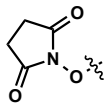
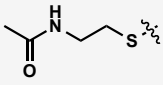
In parallel experiments (Table 3.1), methylated substrates were converted to methyl protected 10-dml (**9**) or methyl protected narbonolide (**16**) albeit with selectivity shifted toward methyl 10-dml (**9**) and reduced overall conversions relative to native substrates furnished through

NBOM photolysis. To further elucidate the macrocycle product distribution imparted by the thio- or oxoester employed, we altered the reaction conditions by excluding MM-NAC in PikAIV reactions,[10] and also by examining the excised Pik TE domain, eliminating the possibility of narbonolide (**15**) or methyl protected narbonolide (**16**) formation (Table 3.2).

Table 3.2 Evaluation of stabilized Pik hexaketides with PikAIV and Pik TE



1 R_3=H
9 R_3=Me

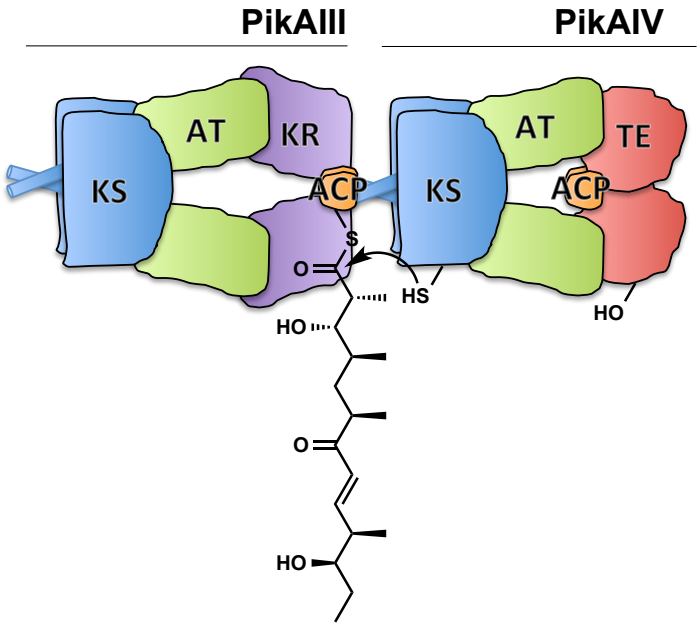
Entry	R_1	R_2	$h\nu$	Enzyme	Conv to 2 or 7(%)
1		Me	-	PikAIV	4 ± 0.2,[a] 6 ± 0.15[b]
2		Me	-	TE	10 ± 0.6
3		NBOM	+	PikAIV	17 ± 0.8,[a] 29 ± 3.5[b]
4		NBOM	+	TE	36 ± 0.8
5		Me	-	PikAIV	trace,[a] 3 ± 0.5[b]
6		Me	-	TE	4 ± 0.3
7		NBOM	+	PikAIV	5 ± 0.5[a],4 ± 0.5[b]
8		NBOM	+	TE	14 ± 0.5
9		Me	-	PikAIV	4 ± 0.2,[a] 12 ± 0.7[b]
10		Me	-	TE	11 ± 0.3
11		NBOM	+	PikAIV	15 ± 0.5,[a] 11± 0.6[b]
12		NBOM	+	TE	16 ± 0.6
13		Me	-	PikAIV	22 ± 4.2,[a] 35 ± 1.3[b]
14		Me	-	TE	55 ± 6.2
15		NBOM	+	PikAIV	5 ± 0.5 [a], 4 ± 0.7[b]
16		NBOM	+	TE	6 ± 0.18
17		Me	-	PikAIV	29 ± 2.7,[a] 66 ± 4.7[b]
18		Me	-	TE	66 ± 4.7
19		NBOM	+	PikAIV	61 ± 5.6,[a] 90 ± 2.1[b]
20		NBOM	+	TE	90 ± 1.9

Incubation of hexaketides with PikAIV in the absence of MM-NAC or with the excised TE domain demonstrated variation in macrolactonization efficiency to 10-dml (**1**) or methyl 10-dml (**15**) dictated by the ester employed (Table 3.2). Consistent with PikAIV reactions where MM-NAC was present, the *N*-acetylcysteamine thioester (Table 3.2, entries 16-20) gave the highest conversion to 10-dml (**1**) or methyl protected 10-dml (**9**) under all conditions tested, with *N*-hydoxysuccinimide esters providing moderate conversion to methyl protected 10-dml (**15**) (Table 3.2, entries 13-14). These experiments demonstrate thiophenol thioesters to be a poor choice for

direct macrolactonization utilizing either PikAIV or the excised TE domain (Table 3.2, entries 1-4). The slow conversion of thiophenol thioesters with the TE domain explains, at least in part, the superiority of this handle over traditionally employed NAC with Pik PKS modules.

In vivo, chain transfer is mediated through C and N terminal docking domains[16] placing the ACP in close proximity to the downstream KS for chain transfer. The Pik pathway is unusual in the ability to make two macrolactone through differential modes of catalysis with PikAIV.[11] Narbonolide (**15**) is the major product when PikAIII and PikAIV are incubated with Pik pentaketide **6,** where chain transfer from PikAIII ACP to PikAIV KS is the predominant pathway. 10-dml (**1**) can be detected as a minor product, presumably though yet undefined protein:protein interaction between PikAIII ACP and PikAIV TE. Mutagenesis of the KS or ACP domains of PikAIV exclusively gives 10-dml (**1**), indicating direct transfer from PikAIII KS to the TE domain.

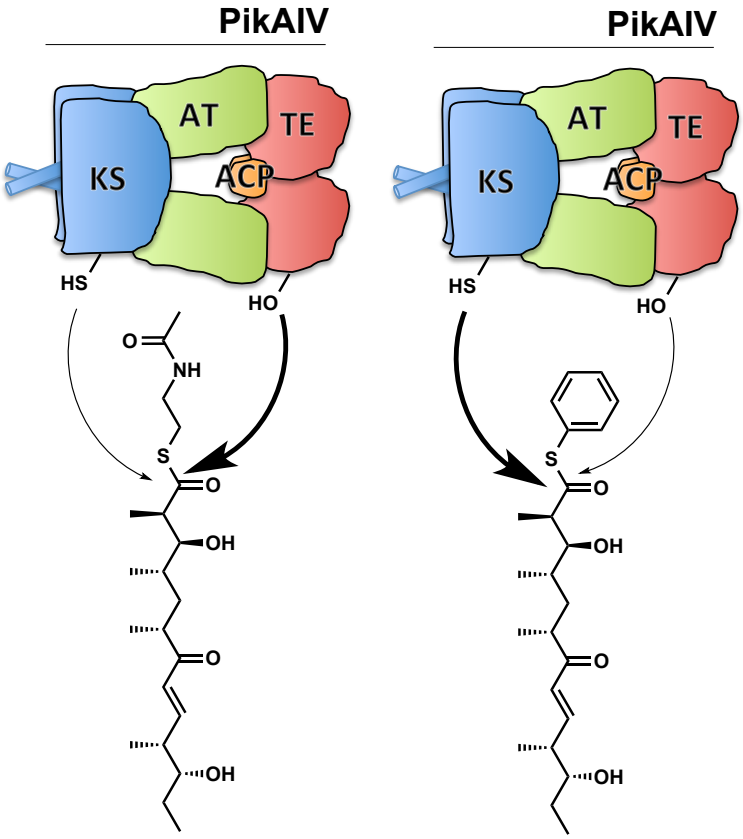
Figure 3.2 Transfer of the Pik hexaketide from PikAIII ACP to PikAIV KS



When performing PKS biochemistry with a purified, standalone PKS module, this protein:protein interaction between modules is absent requiring the substrate to diffuse onto the active site cysteine KS domain. An apparently universal assumption that runs through the PKS literature is that substrate will exclusively load the KS domain despite studies demonstrating that excised PKS TE domains function as promiscuous hydrolases.[17] While PikAIV is an unusual terminal PKS module as direct cyclization is possible if the native KS substrate loads the TE domain,[11] a more common outcome when studying other PKS modules would entail hydrolysis of starting material and decreased conversion to desired product. An additional complication of directly acylating the TE would be hijacking the catalytic cycle, possibly resulting in conformational change[18] suppressing desired full module catalysis. Ultimately, understanding the

effect of direct TE acylation will require structural studies to see what conformational changes are occurring, if any, and subsequent effect on catalysis.

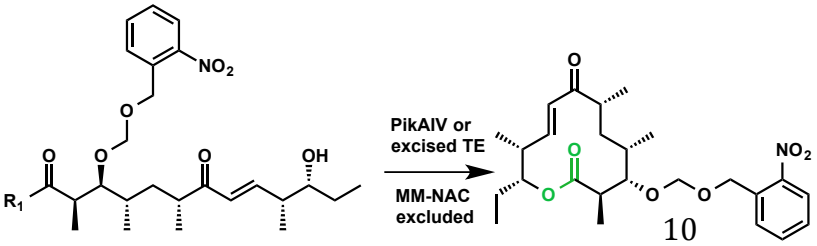
Figure 3.3 Acylation pathways of PikAIV with Pik hexaketides



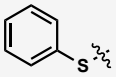
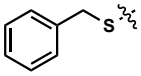
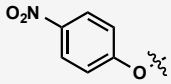
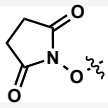
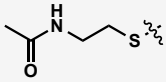
An explanation as to why the thiophenol hexaketide is poor at loading the TE domain compared to the NAC hexaketide despite enhanced electrophilicity may lie in substrate preference of the TE. The Pik TE domain appears to preferentially accept alkyl thioesters (NAC) over aryl thioesters (thiophenol), though the reasonably small substrate panel examined here is not sufficient to empirically conclude that the Pik TE prefers alkyl esters, and substrate preference from amino acid sequence is problematic even with well studied esterases/lipases.[19] Whatever the origin of this shift in catalysis, the implications cannot be overstated *if general to other type I pathways*. Up until very recently (the beginning of the authors graduate studies), PKS enzymology was studied using ^{14}C labeled extender units, meaning that potential side reactions not incorporating ^{14}C, such as rapid TE hydrolysis, could not be observed. As such, the PKS community as a whole might be reporting artificially slow enzymatic rates and conversions (% conversion is rarely if ever reported) due to utilization of NAC thioesters in virtually every study to date. Indeed, this excerpt from two preeminent PKS enzymologists when studying PikAIV with NAC hexaketide **5** highlights this concern: “The observed 4:1 ratio of lactonization to chain elongation for the processing of **5** may represent the intrinsic ratio of these two processes that

leads to the characteristic formation of 12- and 14-membered-ring macrolides"[10] where this ratio was attributed to intrinsic protein function and not an artifact arising from poor emulation of in vivo PKS function. The Sherman lab itself has made the same error in the past, in fact, when we studied PikAIV previously using **5** and ^{14}C labeled extender units we did not even know that 10-dml(**1**) was the major product, or even made at all![2] Whatever experimental oversights can be observed in the literature, it is our hope that the work describe here can help remedy past pitfalls when studying these complex enzymes.

Table 3.3 Flexibility of the TE domain with NBOM protected substrates



10

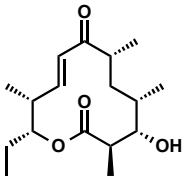
Entry	R_1	*hν*	Enzyme	(%)
1		-	PikAIV	trace
2		-	TE	**3** ± 0.1
3		-	PikAIV	trace
4		-	TE	trace
5		-	PikAIV	**3** ± 0.1
6		-	TE	**5** ± 0.15
7		-	PikAIV	**16** ± 1.5
8		-	TE	**19** ± 3.7
9		-	PikAIV	**34** ± 6.7
10		-	TE	**41** ± 2.8

A series of reactions to explore substrate flexibility were conducted with NBOM protected hexaketides and PikAIV (excluding MM-NAC) or Pik TE without photolysis, and yielded surprising conversion to NBOM protected 10-dml **10** (Table 3.3). The same general trends were observed with *N*-acetylcysteamine giving the highest levels of conversion, followed by *N*-hydroxysuccinimide, with aryl and benzyl thio- and oxoesters giving uniformly low levels of product formation. Further exploration with PikAIV and (MM-NAC included) failed to generate NBOM protected narbonolide, and heat inactivated enzymes also failed to produce either NBOM protected macrolactone.

The results described in Table 3.3 are surprising; we did not expect such a large protecting group to be accommodated within the Pik TE. This highlights the utility of performing seemingly absurd control experiments, as surprises do occur from time to time.

Based on the dramatic selectivity in catalytic outcome observed between esters in this chapter and chapter 2, we conclude that substrate engineering is a previously unappreciated but critical component of in vitro PKS biochemistry and biocatalysis. Further exploration of these strategies will certainly assist in downstream work with PikAIV of the Pik TE. While structural studies of PikAIV with native hexaketides will further elucidate the basis for substrate selectivity, perhaps a more important future direction is to apply this same approach to other type I PKS pathways. If similar outcomes are observed in related pathways (DEBS, Tyl, etc.), then future biocatalytic development could be optimized, at least in part, though substrate engineering approaches described in this chapter and chapter 2.

3.3 Chemistry Experimental



10-dml (**1**)

***Streptomyces venezuelae* ATCC 15439 Δ*des1* (DHS8708, aph kanamycin resistance gene insertion) spore stock and fermentation**

Purified H_2O from a Millipore Milli-Q system with Millipore Q-Gard 2/Quantum Ex Ultrapure organex cartridges was used for spore stock generation and subsequent fermentation. Fermentation was conducted in a New Brunswick BioFlo 3000 fermenter (10 L vessel fitted with stainless steel baffles with temperature maintained by a Neslab RTE-111 circulator) and Corning Fernbach flasks (2.8 L) with deep baffles (3x) fitted with 16" stainless steel springs. Agar, meat and yeast extracts, and glucose were purchased from EMD. Soluble starch and soytone were obtained from BD. N-Z amine, soybean flour, antifoam 204, XAD-16 resin, and $CoCl_2 \cdot 6H_2O$ were obtained from Sigma Aldrich. MOPS was purchased from AK Scientific. NaCl and $CaCl_2$ were obtained from Fisher Scientific. A Symphony SB70P pH meter was calibrated according to the manufacturer's specifications and used to monitor the pH of all solutions during adjustment. Optical density (OD_{600}) was determined using an Eppendorf Biophotometer. All solutions were autoclaved and manipulations were carried out in a UV sterilized laminar flow hood.

An engineered variant of *Streptomyces venezuelae* ATCC 15439 designated DHS8708 was grown on Bennett's agar (1 L = 1 g meat extract, 1 g yeast extract, 10 g glucose, 2 g N-Z amine, 15 g agar, pH 7.3) plates (~30 mL) at 28 °C until reaching a high spore density (~4-6 days). Spores were suspended by addition of H_2O (9 mL) to the plate followed by scraping with an inoculation loop. This solution was decanted into a 15 mL sterile tube, vortexed vigorously for 1 min, and then filtered through a sterile cotton plug into another 15 mL sterile tube. The spores

were pelleted by centrifugation (2000 x g, 5 min) and the supernatant was discarded. The pelleted spores were resuspended in 20% glycerol solution (1 mL), transferred to a sterile screw top vial, flash frozen in N_2 and stored at -80 °C.

Seed culture media: 1 L = 20 g soytone, 15 g soluble starch, 1.5 g yeast extract, 0.1 g calcium chloride, 10.5 g MOPS, pH 7.2.

10-dml production media: 1 L = 20 g glucose, 15 g soybean flour, 5 g $CaCl_2$, 1 g NaCl, 0.002 g $CoCl_2 \bullet 6H_2O$, (0.5 mL antifoam 204 was added to media used in the fermenter). Note: the fermenter was autoclaved at 1/3 volume with 2/3 volumes of H_2O (containing 0.5 mL/L antifoam 204) added after sterilization.

Seed culture: A 250 mL Erlenmeyer flask with deep baffles (3x) and a 6" stainless steel spring was inoculated with DHS8708 spore stock (10 µL) and incubated at 28 °C, 180 RPM until OD_{600} = ~1 (~13-16 h). Note: Inoculation of production media with high OD_{600} seed culture results in decreased titers of **1**.

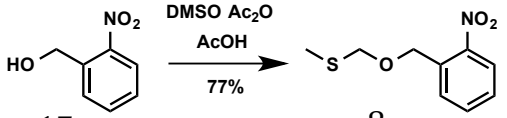
1: 24 Fernbach flasks (2.8 L) containing 1 L production media and capped with a milk filter were inoculated with 1/500 v/v of the seed culture and incubated at 28 °C, 180 RPM. 2.5 cm orbit, for 60 h. Concurrently, the fermenter containing 10 L production media was inoculated with 1/500 v/v of the seed culture and incubated at 28 °C, 400 RPM, 15 L/min aeration (air passed through a 0.2 µm inline filter) for 60 h. Cells were pelleted at 5000 x g (4 °C) for 10 min and subsequently discarded. The supernatant was extracted with XAD-16 resin (Sigma, 15 g/L, used as received) with gentle agitation. XAD-16 extraction efficiency was analyzed by extracting 5 mL of resin-free supernatant with 5 mL CH_2Cl_2, which was evaporated and resuspended in 250 µL MeOH. TLC (30% EtOAc/hexanes, visualized with *p*-anisaldehyde) indicated complete extraction after 4-6 h. The resin was collected by vacuum filtration though a porous polyethylene filter before loading into a glass flash column previously fitted with a plug of glass wool. The resin was washed with H_2O (~1 L) and allowed to dry under air pressure for 1 h. The resin was extracted with acetone (1x 500 mL) followed by EtOAc (500 mL) until no **1** could be detected in eluting EtOAc by TLC (~2-3x). The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed with EtOAc (2x) and concentrated. Flash chromatography: EtOAc/hexanes (20:80) gave **1** as an oil (3.56 g, 12.01 mmol, 0.105 g/L) that foamed under high vacuum, and slowly crystalized upon standing.

Spectroscopic data matched that reported previously.[4a]

Chemistry

Reactions were performed in evacuated (<0.05 torr) flame dried glassware containing PFTE coated magnetic stir bars fitted with rubber septa backfilled with dry N_2 and run under a positive pressure of dry N_2 provided by a mineral oil bubbler unless stated otherwise (open flask).

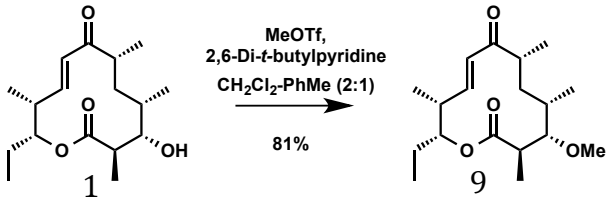
Reactions at elevated temperatures were controlled by IKA RET Control Visc (model RS 232 C), room temperature (RT) reactions were conducted at ~23 °C, reactions run cooler than room temperature were performed in a cold room (4 °C), an ice bath (0 °C), dry ice/acetone (-78 °C), or isopropanol/ThermoNESLAB (model CC100) for all other temperatures. Commercial purification system MBraun-MB-SPS # 08-113 provided all dry solvents unless stated otherwise (technical grade). Analytical thin-layer chromatography (TLC) was performed with EMD 60 F_{254} pre-coated glass plates (0.25 mm) and visualized using a combination of UV, *p*-anisaldehyde, $KMnO_4$, and Bromocresol green stains. Flash column chromatography was performed using EMD 60 Gerduran® (particle size 0.04-0.063) silica gel. NMR spectra were recorded on a Varian 600 MHz spectrometer. ^{1}H NMR spectra were recorded relative to residual solvent peak ($CDCl_3$ δ_H 7.26 ppm, D_6-DMSO δ_H 2.50 ppm) and reported as follows: chemical shift (ppm), multiplicity, coupling constant (Hz), and integration. Multiplicity abbreviations are as follows: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, h = hextet, ovlp = overlap, br = broad signal. ^{13}C NMR spectra were recorded relative to residual solvent peaks ($CDCl_3$ δ_C 77.0 ppm, D_6-DMSO δ_c 39.5 ppm). High resolution mass spectrometry was performed on an Agilent quadrapole time-of-flight spectrometer (Q-TOF 6500 series) by electrospray ionization (ESI).



17 8

8: Adapted from literature procedure,[20] an open 1 L roundbottom flask was charged with **17** (AK, 20 g, 131 mmol, 1 equiv), acetic acid (Fisher, glacial, 314 g, 299 mL, 5220 mmol, 40 equiv) and stirred at RT until dissolved. DMSO (EMD, technical grade, 204 g, 185 mL, 2612 mmol, 20 equiv) was added in one portion, followed by slow addition of Ac_2O (Fisher, 267 g, 246 mL, 2612 mmol, 20 equiv). The flask was capped with a rubber septum and flushed with N_2, with a positive pressure of N_2 maintained thereafter. The reaction was monitored by loss of starting material (TLC) indicating completion at ~72 h. The solution was decanted into an addition funnel and added dropwise into a stirring solution of 10 M KOH (1.2 L, 90 equiv) at 0 °C. After complete addition, the solution was warmed to RT and allowed to stir for 2 h before it was extracted Et_2O (3x). The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed with EtOAc (2x) and concentrated. Flash chromatography: EtOAc/hexanes (5:95) and subsequent rotary high vacuum (4 h) gave **8** as a bright yellow oil (21.6 g, 101 mmol, 77% yield).

Spectroscopic data matched that of Banerjee and colleagues.[20]

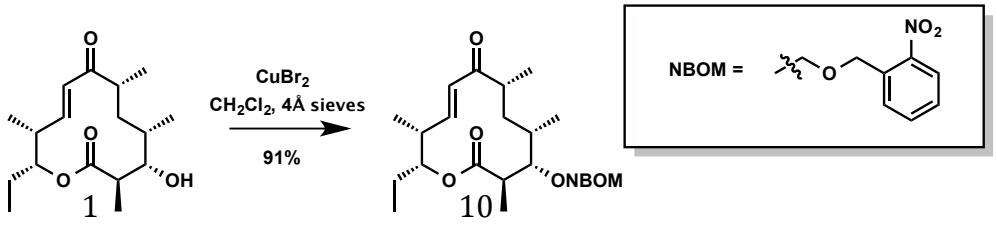


9: A 4 dram vial was charged with **1** (3 x PhMe azeotrope, 0.40 g, 1.35 mmol, 1.00 equiv), CH_2Cl_2:PhMe (2:1, 0.69 mL, 2 M) and cooled to 4 °C. 2,6-di-*t*-butylpyridine (TCI, 0.31 g, 0.36 mL, 1.62 mmol, 1.20 equiv) was added followed by methyl trifluoromethanesulfonate (Oakwood, fractionally distilled neat [collected ~100 °C at atmospheric pressure], 0.27 g, 0.18 mL, 1.62 mmol, 1.20 equiv) and stirred for 72 h at 4 °C. The reaction was quenched with a saturated NH_4Cl solution and extracted with CH_2Cl_2 (3x). The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed with CH_2Cl_2 (2x) and concentrated. Flash chromatography: EtOAc/hexanes (5:95 to 25:75) gave **9** (0.34 g, 1.09 mmol, 81%) as a colorless crystalline solid.

^{1}H NMR (599 MHz; $CDCl_3$): δ 6.65 (dd, *J* = 15.7, 5.5 Hz, 1H), 6.35 (dd, *J* = 15.7, 1.1 Hz, 1H), 4.91 (ddd, *J* = 8.5, 5.8, 2.5 Hz, 1H), 3.45 (s, 3H), 3.08 (dd, *J* = 10.5, 0.9 Hz, 1H), 2.61-2.55 (m, 2H), 2.46 (dqd, *J* = 12.9, 6.6, 3.9 Hz, 1H), 1.68-1.61 (m, 1H), 1.56 (t, *J* = 13.2 Hz, 1H), 1.53-1.46 (m, 1H), 1.31-1.26 (m, 1H), 1.23 (d, *J* = 6.9 Hz, 3H), 1.18-1.13 (ovlp m, 1H), 1.14 (d, *J* = 7.0 Hz, 3H), 1.05 (d, *J* = 6.8 Hz, 3H), 0.97 (d, *J* = 6.7 Hz, 3H), 0.85 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 204.8, 174.8, 146.7, 125.6, 88.4, 73.5, 62.8, 45.0, 43.4, 37.8, 33.8, 33.7, 25.0, 17.9, 17.5, 16.0, 10.2, 9.5.

HRMS: Calculated $[M+H]^+$ 311.2217, found 311.2223.



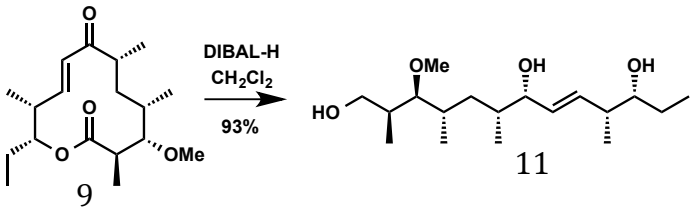
10: Adapted from literature procedure,[7] a 100 mL round bottom flask was charged with **1** (0.80 g, 2.70 mmol, 1.00 equiv) and **10** (2.30 g, 10.80 mmol, 4.00 equiv) and CH_2Cl_2 (27.0 mL, 0.1 M). 4Å molecular sieves (Sigma, powdered, activated at 180 °C under high vacuum overnight, 8.00 g) were added in a single portion and the solution was cooled to -20 °C. $CuBr_2$ (Sigma, 2.41 g, 10.80 mmol, 4.00 equiv) was added in a single portion and the solution was stirred vigorously at -20 °C for 12 h, warmed to RT and stirred 2 h, and quenched by the addition of glycerol (6.21 g, 4.92 mL, 67.48 mmol, 25.0 equiv) and 4 h additional stirring. The solution was diluted with EtOAc and vacuum filtered though a fritted glass filter. The solid was subsequently washed with EtOAc (3x). The organic layer was washed with a saturated EDTA (disodium salt) solution (2x), filtered

through a sodium sulfate plug, which was subsequently rinsed with EtOAc (2x) and concentrated. Flash chromatography: EtOAc/hexanes (15:85) afforded **10** (1.14 g, 2.47 mmol, 91%) as a pale yellow oil.

^{1}H NMR (599 MHz; $CDCl_3$): δ 8.08 (d, *J* = 8.2 Hz, 1H), 7.80 (d, *J* = 7.8 Hz, 1H), 7.64 (t, *J* = 7.5 Hz, 1H), 7.44-7.42 (m, 1H), 6.72 (dd, *J* = 15.7, 5.5 Hz, 1H), 6.41 (d, *J* = 15.7 Hz, 1H), 5.03 (q, *J* = 12.3 Hz, 2H), 4.96 (ddd, *J* = 8.5, 5.7, 2.5 Hz, 1H), 4.87 (q, *J* = 10.1 Hz, 2H), 3.53 (d, *J* = 10.5 Hz, 1H), 2.71 (dq, *J* = 10.5, 6.9 Hz, 1H), 2.64-2.60 (m, 1H), 2.50 (dqd, *J* = 12.9, 6.6, 3.9 Hz, 1H), 1.73-1.64 (m, 2H), 1.57-1.50 (m, 1H), 1.36-1.31 (m, 1H), 1.27-1.23 (ovlp m, 1H), 1.25 (d, *J* = 6.9 Hz, 3H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.10 (d, *J* = 6.8 Hz, 3H), 0.97 (d, *J* = 6.6 Hz, 3H), 0.89 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR: (151 MHz; $CDCl_3$): δ 204.8, 174.8, 146.9, 146.9, 134.7, 133.8, 128.4, 127.9, 125.7, 124.7, 98.0, 87.3, 73.7, 67.0, 45.0, 43.1, 37.9, 33.9, 33.6, 25.1, 17.8, 17.6, 16.4, 10.3, 9.5.

HRMS: Calculated $[M+Na]^+$ 484.2306, found 484.2322.



9 11

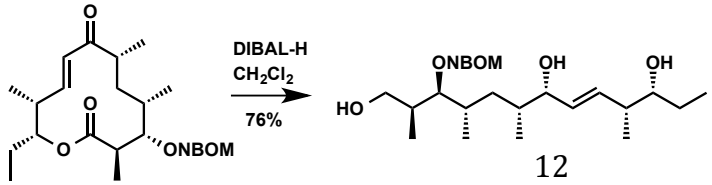
11: A 100 mL round bottom flask was charged with **9** (0.51 g, 1.64 mmol, 1.00 equiv), CH_2Cl_2 (16.4 mL, 0.1 M) and cooled to -78 °C. DIBAL-H (Sigma, 1.03 g, 1.29 mL, 7.20 mmol, 4.40 equiv) was added down the side of the flask. The solution was stirred for 1 h before it was warmed to RT for 10 min, and recooled to -78 °C. MeOH (5 mL) was added dropwise and the mixture was stirred for 15 min before removal of the cooling bath and addition of a saturated Na/K tartrate solution. Stirring was continued until the layers became clear, followed by extraction with EtOAc (3x). The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed with EtOAc (2x) and concentrated. Flash chromatography: EtOAc/hexanes (70:30 to 100:0) afforded **11** (0.49 g, 1.53 mmol, 93%) as a colorless gum.

^{1}H NMR (599 MHz; D_6-DMSO): δ 5.47-5.37 (m, 2H), 4.44 (t, *J* = 5.1 Hz, 1H), 4.36 (d, *J* = 4.7 Hz, 1H), 4.27 (d, *J* = 5.7 Hz, 1H), 3.77 (q, *J* = 4.9 Hz, 1H), 3.32-3.29 (ovlp m, 1H), 3.29 (s, 3H), 3.26-3.22 (m, 1H), 3.10 (dtd, *J* = 8.7, 5.9, 2.9 Hz, 1H), 2.95 (dd, *J* = 6.3, 3.4 Hz, 1H), 2.06 (h, *J* = 7.0 Hz, 1H), 1.83-1.76 (m, 1H), 1.74-1.67 (m, 1H), 1.67-1.62 (m, 1H), 1.56-1.50 (m, 1H), 1.44 (dqd, *J* = 14.0, 7.1, 3.4 Hz, 1H), 1.24 (m, 1H), 0.94 (d, *J* = 6.8 Hz, 3H), 0.84 (t, *J* = 7.4 Hz, 3H), 0.81 (m, 6H), 0.76 (d, *J* = 6.9 Hz, 3H).

^{13}C NMR (151 MHz; D_6-DMSO): δ 133.0, 132.2, 84.8, 75.0, 73.4, 64.3, 59.7, 42.2, 37.2, 36.3, 36.1, 32.5, 27.1, 17.0, 16.4, 15.8, 11.5, 10.2.

HRMS: Calculated $[M+Na]^+$ 339.2506, found 339.2517.

Note: For configuration of the allylic hydroxyl group see the **X-Ray Crystallography** section.



10 12

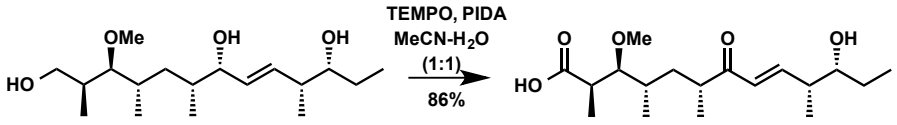
12: A 250 mL round bottom flask was charged with **10** (1.66 g, 3.60 mmol, 1.00 equiv), CH_2Cl_2 (36 mL, 0.1 M) and cooled to -78 °C. DIBAL-H (Sigma, 2.25 g, 2.82 mL, 15.86 mmol, 4.40 equiv) was added down the side of the flask and the solution was stirred for 1 h, warmed to 0 °C briefly (~1 min), and recooled to -78 °C. MeOH (5 mL) was added dropwise and stirring was continued for 15 min before removal of the cooling bath and addition of a saturated Na/K tartrate solution. Stirring was continued until the layers became clear, followed by extraction with CH_2Cl_2 (3x). The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed with CH_2Cl_2 (2x) and concentrated. Flash chromatography: EtOAc/hexanes (50:50 to 100:0) afforded **12** (1.28 g, 2.73 mmol, 76%) as a light yellow gum.

^{1}H NMR (599 MHz; D_6-DMSO): δ 8.09 (m, 1H), 7.79-7.75 (m, 2H), 7.59-7.55 (m, 1H), 5.44-5.32 (m, 2H), 4.93 (q, *J* = 18.9 Hz, 2H), 4.79 (s, 2H), 4.48 (t, *J* = 5.1 Hz, 1H), 4.36 (d, *J* = 4.6 Hz, 1H), 4.26 (d, *J* = 5.6 Hz, 1H), 3.73 (q, *J* = 4.8 Hz, 1H), 3.46 (dd, *J* = 5.7, 3.0 Hz, 1H), 3.30-3.23 (m, 2H), 3.09 (dtd, *J* = 8.6, 5.9, 2.8 Hz, 1H), 2.04 (h, *J* = 7.0 Hz, 1H), 1.87-1.81 (m, 1H), 1.77-1.71 (m, 1H), 1.65-1.59 (m, 1H), 1.54-1.47 (m, 1H), 1.42 (dqd, *J* = 14.0, 7.1, 3.4 Hz, 1H), 1.23-1.15 (m, 1H), 0.93 (d, *J* = 6.8 Hz, 3H), 0.85-0.82 (m, 6H), 0.80 (d, *J* = 6.8 Hz, 3H), 0.78-.75 (ovlp m, 1H), 0.73 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (151 MHz; D_6-DMSO): δ 147.5, 134.5, 134.3, 133.5, 132.5, 129.2, 128.9, 124.9, 96.2, 82.4, 75.4, 73.8, 66.3, 64.7, 42.7, 37.0, 36.6, 36.4, 33.6, 27.6, 17.3, 16.8, 16.0, 12.2, 10.6.

HRMS: Calculated $[M+Na]^+$ 490.2775, found 490.2790.

Note: Configuration of the allylic hydroxyl group was assigned by analogy with **11**.



11 13

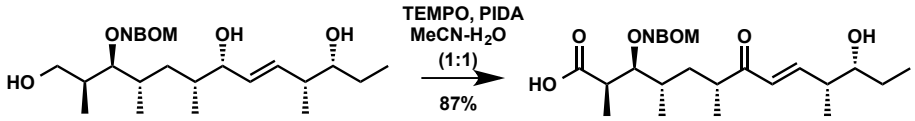
13: A 9 dram vial was charged with **11** (0.59 g, 1.86 mmol, 1.00 equiv) and H_2O:MeCN (1:1, 18.6 mL, 0.1 M) and cooled to 4 °C. TEMPO (Sigma, 0.29 g, 1.86 mmol, 1.00 equiv) and PIDA (AK scientific, 2.39 g, 7.43 mmol, 4.00 equiv) were added in single portions. The reaction was monitored by ^{1}H NMR spectroscopy after 12 h (small aliquot added to excess MeOH, concentrated, and dissolved in $CDCl_3$) for loss of the intermediate aldehyde. After 20 h, the solution was decanted into MeOH (100 mL) and concentrated. The oil was dissolved in Et_2O and

extracted with half saturated sodium bicarbonate solution (3x). The combined aqueous layers were backwashed with Et_2O:hexanes (1:1), carefully acidified to pH 2-3 with phosphoric acid and extracted with EtOAc (3x). The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed with EtOAc (2x) and concentrated. Flash chromatography: AcOH/EtOAc/hexanes (1:25:75 to 1:50:50) to yield **13** (0.53 g, 1.61 mmol, 86%) as a pale yellow oil.

^{1}H NMR (599 MHz; $CDCl_3$): δ 6.95 (dd, *J* = 15.8, 6.5 Hz, 1H), 6.26 (dd, *J* = 15.7, 1.4 Hz, 1H), 3.68 (dt, *J* = 8.3, 4.2 Hz, 1H), 3.44 (s, 3H), 3.23 (dd, *J* = 7.4, 4.0 Hz, 1H), 2.84-2.78 (m, 1H), 2.67 (quint, *J* = 7.1 Hz, 1H), 2.50-2.45 (m, 1H), 1.88 (ddd, *J* = 13.9, 9.9, 4.1 Hz, 1H), 1.62-1.46 (ovlp m, 3H), 1.27-1.22 (ovlp m, 1H), 1.21 (d, *J* = 6.9 Hz, 3H), 1.13 (d, *J* = 6.9 Hz, 3H), 1.08 (d, *J* = 6.9 Hz, 3H), 0.97 (ovlp m, 6H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 203.8, 178.5, 149.6, 126.8, 87.1, 76.0, 61.1, 42.7, 42.0, 41.2, 34.8, 34.2, 27.4, 17.6, 17.0, 13.3, 11.9, 10.4.

HRMS: Calculated $[M+Na]^+$ 351.2142, found 351.2146.



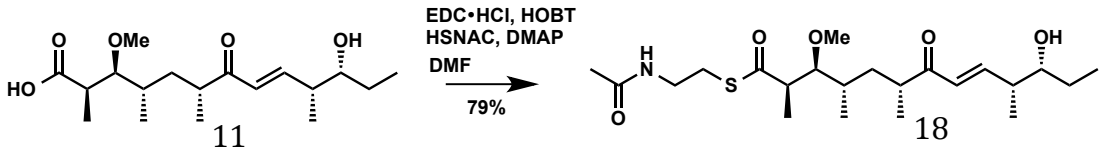
12 14

14: A 9 dram vial was charged with **12** (0.81 g, 1.74 mmol, 1.00 equiv) and H_2O:MeCN (1:1, 17.4 mL, 0.1 M) and cooled to 4 °C. TEMPO (Sigma, 0.27 g, 1.74 mmol, 1.00 equiv) and PIDA (AK scientific, 2.24 g, 6.96 mmol, 4.00 equiv) were added in single portions. The reaction was monitored by ^{1}H NMR spectroscopy (small aliquot added to excess MeOH, concentrated, and dissolved in $CDCl_3$) after 12 h for loss of the intermediate aldehyde. After 17 h, the solution was decanted into MeOH (100 mL) and concentrated. Flash chromatography: AcOH/EtOAc/hexanes (1:25:75 to 1:50:50) to yield **14** (0.73 g, 1.52 mmol, 87%) as a pale yellow oil.

^{1}H NMR (599 MHz; $CDCl_3$): δ 8.08 (dd, *J* = 8.2, 1.1 Hz, 1H), 7.79 (d, *J* = 7.8 Hz, 1H), 7.64 (td, *J* = 7.6, 0.8 Hz, 1H), 7.45-7.42 (m, 1H), 6.96 (dd, *J* = 15.8, 6.4 Hz, 1H), 6.23 (dd, *J* = 15.8, 1.4 Hz, 1H), 5.06-5.01 (m, 2H), 4.87 (s, 2H), 3.71-3.67 (m, 2H), 2.86-2.80 (m, 1H), 2.71 (quint, *J* = 7.1 Hz, 1H), 2.50-2.46 (m, 1H), 1.87 (ddd, *J* = 14.0, 9.4, 4.7 Hz, 1H), 1.69-1.63 (m, 1H), 1.57-1.44 (m, 2H), 1.24-1.19 (ovlp m, 1H), 1.21 (d, *J* = 7.0 Hz, 3H), 1.10 (d, *J* = 6.9 Hz, 3H), 1.07 (d, *J* = 6.9 Hz, 3H), 0.98-0.95 (ovlp m, 6H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 204.0, 179.1, 149.8, 147.0, 134.6, 133.6, 128.6, 127.9, 127.4, 124.6, 96.7, 84.2, 76.0, 67.0, 41.9, 41.7, 41.4, 35.2, 34.4, 27.1, 17.5, 16.6, 13.2, 12.4, 10.4.

HRMS: Calculated $[M+Na]^+$ 502.2411, found 502.2421.

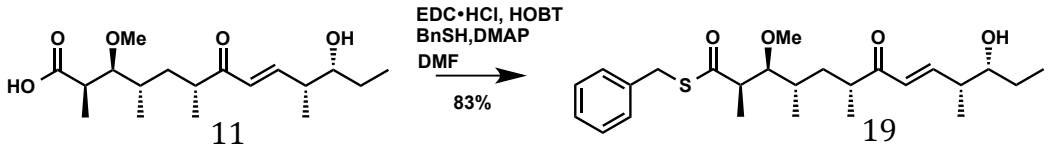


17: A 4 dram vial was charged with **11** (0.030 g, 0.091 mmol, 1.000 equiv), EDC•HCl (Chem-Impex, 0.026 g, 0.137 mmol, 1.500 equiv), and HOBT (Sigma, 0.015 g, 0.109 mmol, 1.200 equiv). The solids were dissolved in DMF (0.9 mL, 0.1 M) and stirred 30 min at RT. HSNAC (0.013 g, 0.012 mL, 0.109 mmol, 1.200 equiv) was added, stirred 10 min, followed by DMAP (Sigma, ~ 1 mg, cat) and stirred 12 h. The solution was diluted with EtOAc, washed with H_2O, and the aqueous layer was extracted with EtOAc (2x). The combined organic extracts were filtered through a sodium sulfate plug, which was subsequently rinsed with EtOAc (2x) and concentrated. Flash chromatography: EtOAc to yield **18** (0.031 g, 0.072 mmol, 79%) as a colorless oil.

^{1}H NMR (599 MHz; $CDCl_3$): δ 7.14 (br s, 1H), 6.87 (dd, *J* = 15.9, 7.7 Hz, 1H), 6.15 (d, *J* = 15.9 Hz, 1H), 3.65-3.58 (m, 1H), 3.50 (dt, *J* = 8.8, 4.4 Hz, 1H), 3.44 (s, 3H), 3.25 (dd, *J* = 8.6, 2.9 Hz, 1H), 3.20-3.15 (m, 2H), 2.95-2.88 (m, 2H), 2.86-2.81 (m, 1H), 2.47-2.41 (m, 1H), 1.95 (s, 3H), 1.90 (ovlp ddd, *J* = 13.7, 10.4, 3.5 Hz, 1H), 1.54-1.47 (m, 2H), 1.42-1.35 (m, 1H), 1.24 (d, *J* = 6.9 Hz, 3H), 1.24-1.20 (ovlp m, 1H), 1.12 (d, *J* = 7.0 Hz, 3H), 1.08 (d, *J* = 6.8 Hz, 3H), 0.96 (ovlp t, *J* = 7.5 Hz, 3H), 0.93 (ovlp d, *J* = 6.9 Hz, 3H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 204.8, 202.8, 170.6, 150.2, 128.7, 87.5, 75.7, 61.9, 51.5, 42.2, 40.9, 39.0, 33.8, 33.6, 29.2, 27.4, 23.0, 18.9, 17.6, 15.2, 13.7, 10.3.

HRMS: Calculated $[M+Na]^+$ 452.2441, found 452.2459.



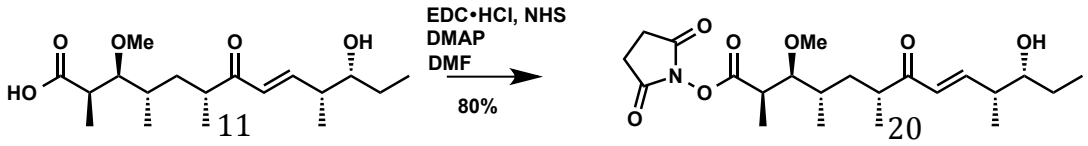
19: A 4 dram vial was charged with **11** (0.030 g, 0.091 mmol, 1.000 equiv), EDC•HCl (Chem-Impex, 0.026 g, 0.137 mmol, 1.500 equiv), and HOBT (Sigma, 0.015 g, 0.109 mmol, 1.200 equiv). The solids were dissolved in DMF (0.9 mL, 0.1 M) and stirred 30 min at RT. Benzyl mercaptan (Sigma, 0.014 g, 0.013 mL, 0.109 mmol, 1.200 equiv) was added, stirred 10 min, followed by DMAP (Sigma, ~ 1 mg, cat) and stirred 12 h. The solution was diluted with EtOAc, washed with H_2O, and the aqueous layer was extracted with EtOAc (2x). The combined organic extracts were filtered through a sodium sulfate plug, which was subsequently rinsed with EtOAc (2x) and concentrated. Flash chromatography: EtOAc/hexanes (20:80 to 30:70) to yield **19** (0.033 g, 0.076 mmol, 83%) as a colorless oil.

^{1}H NMR (599 MHz; $CDCl_3$): δ 7.28-7.27 (m, 4H), 7.22 (dq, *J* = 8.7, 4.3 Hz, 1H), 6.85 (dd, *J* = 15.8, 7.7 Hz, 1H), 6.20 (d, *J* = 15.8 Hz, 1H), 4.10 (s, 2H), 3.50-3.46 (m, 1H), 3.32-3.31 (ovlp m, 1H), 3.31 (s, 3H), 2.87-2.80 (ovlp m, 2H), 2.42 (dq, *J* = 12.8, 6.4 Hz, 1H), 1.92 (ddd, *J* = 13.6, 9.4,

4.1 Hz, 1H), 1.60-1.48 (ovlp m, 2H), 1.38 (m, 1H), 1.21 (d, *J* = 7.0 Hz, 3H), 1.13 (ddd, *J* = 14.0, 9.3, 4.9 Hz, 1H), 1.09-1.07 (ovlp m, 6H), 0.95 (t, *J* = 7.4 Hz, 3H), 0.89 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 203.7, 202.1, 149.2, 137.4, 128.8, 128.5, 128.3, 127.2, 86.7, 75.8, 60.6, 50.7, 42.3, 42.0, 35.6, 34.5, 33.1, 27.4, 18.0, 17.0, 13.9, 12.6, 10.3.

HRMS: Calculated $[M+Na]^+$ found 457.2383, found 457.2380.



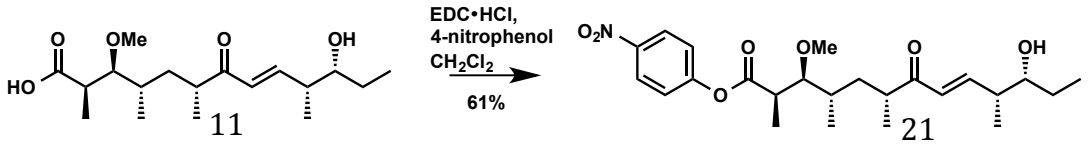
11 20

20: A 4 dram vial was charged with **11** (0.030 g, 0.091 mmol, 1.000 equiv), EDC•HCl (Chem-Impex, 0.026 g, 0.137 mmol, 1.500 equiv). The vial was cooled to 0 °C and solids were dissolved in DMF (0.9 mL, 0.1 M) and stirred 10 min at 0 °C. *N*-hydroxysuccinimide (Sigma, 0.013 g, 0.109 mmol, 1.200 equiv) was added, stirred 10 min at 0 °C and 10 min at RT, followed by addition of DMAP (Sigma, ~ 1 mg, cat). The reaction mixture was stirred 4 h. The solution was diluted with EtOAc, washed with H_2O, and the aqueous layer was extracted with EtOAc (2x). The combined organic extracts were filtered through a sodium sulfate plug, which was subsequently rinsed with EtOAc (2x) and concentrated. Flash chromatography: EtOAc/hexanes (40:60 to 50:50) to yield **20** (0.031 g, 0.073 mmol, 80%) as a colorless oil.

^{1}H NMR (599 MHz; $CDCl_3$): δ 6.86 (dd, *J* = 15.8, 7.7 Hz, 1H), 6.20 (dd, *J* = 15.8, 1.0 Hz, 1H), 3.49 (dt, *J* = 8.8, 4.4 Hz, 1H), 3.43 (s, 3H), 3.40 (dd, *J* = 5.8, 5.0 Hz, 1H), 2.99-2.94 (m, 1H), 2.91-2.85 (m, 1H), 2.81 (br d, *J* = 7.3 Hz, 4H), 2.42 (dq, *J* = 12.2, 6.3 Hz, 1H), 2.01 (ddd, *J* = 13.6, 9.5, 3.9 Hz, 1H), 1.68-1.61 (m, 1H), 1.54-1.47 (m, 1H), 1.42-1.36 (m, 1H), 1.28 (d, *J* = 7.0 Hz, 3H), 1.16 (ddd, *J* = 14.0, 9.3, 4.8 Hz, 1H), 1.11 (d, *J* = 6.9 Hz, 3H), 1.06 (d, *J* = 6.8 Hz, 3H), 0.96-0.92 (ovlp m, 6H).

13C NMR (151 MHz; $CDCl_3$): δ 203.6, 170.8, 169.1, 149.3, 128.4, 85.9, 75.7, 60.6, 42.1, 42.0, 39.4, 35.8, 34.7, 27.3, 25.6, 18.1, 16.9, 13.6, 11.2, 10.3.

HRMS: Calculated $[M+Na]^+$ 448.2306, found 448.2315.



11 21

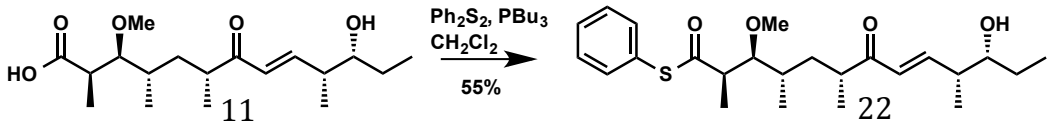
21: A 4 dram vial was charged with **11** (0.030 g, 0.091 mmol, 1.000 equiv), EDC•HCl (Chem-Impex, 0.026 g, 0.137 mmol, 1.500 equiv). The vial was cooled to 0 °C and solids were dissolved in CH_2Cl_2 (0.9 mL, 0.1 M) and stirred for 30 min at 0 °C. 4-nitrophenol (0.015 g, 0.109 mmol, 1.200 equiv) was added and stirred for 10 min at 0 °C. The mixture was warmed to RT and stirred an additional 12 h. The solution was added directly onto a column of silica for purification. Flash

chromatography: EtOAc/hexanes (20:80) to yield **21** (0.025 g, 0.056 mmol, 61%) as a colorless oil.

^{1}H NMR (599 MHz; $CDCl_3$): δ 8.28-8.26 (m, 2H), 7.35-7.32 (m, 2H), 6.86 (dd, *J* = 15.8, 7.7 Hz, 1H), 6.20 (dd, *J* = 15.8, 1.1 Hz, 1H), 3.50-3.47 (ovlp m, 1H), 3.47 (s, 3H), 3.39 (dd, *J* = 6.3, 5.2 Hz, 1H), 2.97 (quint, *J* = 6.9 Hz, 1H), 2.91 (dqd, *J* = 10.5, 6.9, 3.8 Hz, 1H), 2.45-2.39 (m, 1H), 2.03 (ddd, *J* = 13.6, 10.3, 3.3 Hz, 1H), 1.67-1.60 (m, 2H), 1.51 (dqd, *J* = 14.3, 7.3, 3.9 Hz, 1H), 1.41-1.32 (ovlp m, 1H), 1.35 (d, *J* = 7.0 Hz, 3H), 1.20 (ddd, *J* = 13.8, 10.0, 3.9 Hz, 1H), 1.14 (d, *J* = 7.0 Hz, 3H), 1.07 (d, *J* = 6.8 Hz, 3H), 0.98 (d, *J* = 6.8 Hz, 3H), 0.95 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 203.6, 173.2, 155.5, 149.5, 145.3, 128.5, 125.2, 122.6, 86.7, 75.8, 61.1, 42.4, 42.2, 41.7, 35.0, 34.4, 27.4, 18.6, 17.1, 13.8, 12.6, 10.3.

HRMS: Calculated $[M+Na]^+$ 472.2306, found 472.2315.

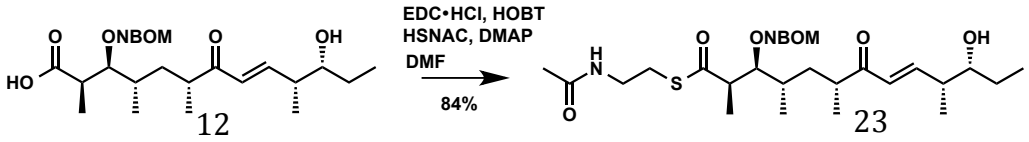


22: A 9 dram vial was charged with **11** (0.050 g, 0.152 mmol, 1.000 equiv) and Ph_2S_2 (Sigma, 0.037 g, 0.167 mmol, 1.100 equiv). The solids were dissolved in CH_2Cl_2 (1.52 mL, 0.1 M) and cooled to -42 °C. PBu_3 (Sigma, 0.040 g, 0.049 mL, 0.198 mmol, 1.300 equiv) was added dropwise and the solution was stirred 20 min at -42 °C. The reaction was quenched at -42 °C with a saturated $CuSO_4$ solution and allowed to warm to RT. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (2x). The organic layer was washed with saturated EDTA (disodium salt, 2x) and filtered through a sodium sulfate plug, which was subsequently rinsed with CH_2Cl_2 (2x) and concentrated. Flash chromatography: EtOAc/hexanes (25:75) afforded **22** (0.035 g, 0.083 mmol, 55%) as a colorless oil.

^{1}H NMR (599 MHz; $CDCl_3$): δ 7.40 (s, 5H), 6.85 (dd, *J* = 15.8, 7.7 Hz, 1H), 6.22 (d, *J* = 15.8 Hz, 1H), 3.46 (dt, *J* = 8.9, 4.4 Hz, 1H), 3.41 (s, 3H), 3.36 (t, *J* = 5.6 Hz, 1H), 2.95 (quint, *J* = 6.7 Hz, 1H), 2.92-2.86 (m, 1H), 2.43-2.37 (m, 1H), 1.98 (ddd, *J* = 13.7, 9.6, 4.0 Hz, 1H), 1.68-1.60 (m, 2H), 1.50 (dqd, *J* = 14.3, 7.3, 3.9 Hz, 1H), 1.40-1.33 (m, 1H), 1.29 (d, *J* = 7.0 Hz, 3H), 1.22 (ddd, *J* = 14.0, 9.5, 4.7 Hz, 1H), 1.13 (d, *J* = 6.9 Hz, 3H), 1.06 (d, *J* = 6.8 Hz, 3H), 0.96-0.93 (ovlp m, 6H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 203.7, 200.7, 149.2, 134.5, 129.3, 129.1, 128.4, 127.6, 86.7, 75.8, 60.9, 50.8, 42.3, 41.9, 35.4, 34.5, 27.4, 18.2, 17.0, 13.8, 13.2, 10.3.

HRMS: Calculated $[M+Na]^+$ 443.2227, found 443.2234.

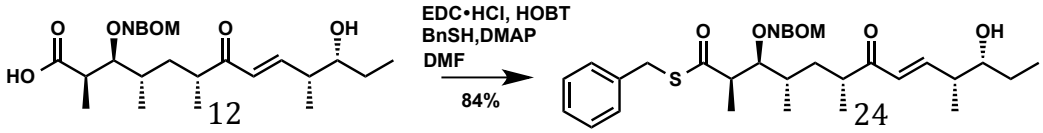


23: A 4 dram vial was charged with **12** (0.050 g, 0.104 mmol, 1.000 equiv), EDC•HCl (Chem-Impex, 0.030 g, 0.156 mmol, 1.500 equiv), and HOBT (Sigma, 0.017 g, 0.125 mmol, 1.200 equiv). The solids were dissolved in DMF (1 mL, 0.1 M) and stirred 30 min at RT. HSNAC (0.015 g, 0.014 mL, 0.125 mmol, 1.200 equiv) was added, stirred 10 min, followed by DMAP (Sigma, ~ 1 mg, cat) and stirred 12 h. The solution was diluted with EtOAc, washed with H_2O, and the aqueous layer was extracted with EtOAc (2x). The combined organic extracts were filtered through a sodium sulfate plug, which was subsequently rinsed with EtOAc (2x) and concentrated. Flash chromatography: EtOAc/hexanes (70:30 to 80:20) to yield **23** (0.051 g, 0.088 mmol, 84%) as a pale yellow oil.

^{1}H NMR (599 MHz; $CDCl_3$): δ 8.08 (dd, *J* = 8.2, 1.1 Hz, 1H), 7.78 (d, *J* = 7.8 Hz, 1H), 7.66-7.63 (m, 1H), 7.46-7.43 (m, 1H), 6.99 (br s, 1H), 6.88 (dd, *J* = 15.9, 7.7 Hz, 1H), 6.15 (dd, *J* = 15.9, 1.1 Hz, 1H), 5.03 (q, *J* = 14.0 Hz, 2H), 4.84 (s, 2H), 3.71 (dd, *J* = 8.0, 3.0 Hz, 1H), 3.60-3.55 (m, 1H), 3.51 (dq, *J* = 8.6, 4.3 Hz, 1H), 3.21-3.12 (m, 2H), 2.98-2.87 (ovlp m, 2H), 2.86-2.81 (m, 1H), 2.46-2.41 (m, 1H), 1.94 (s, 3H), 1.93-1.90 (ovlp m, 1H), 1.58 (m, *J* = 10.3, 6.9, 3.5 Hz, 1H), 1.54-1.46 (m, 1H), 1.42-1.35 (m, 1H), 1.25-1.20 (ovlp m, 4H), 1.11 (d, *J* = 7.0 Hz, 3H), 1.07 (d, *J* = 6.8 Hz, 3H), 0.95 (t, *J* = 7.4 Hz, 3H), 0.92 (d, *J* = 6.9 Hz, 3H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 204.5, 202.6, 170.6, 150.3, 147.1, 134.6, 133.7, 128.6, 128.0, 124.7, 97.1, 85.1, 75.7, 67.1, 51.0, 42.2, 40.8, 38.9, 34.0, 33.8, 29.1, 27.4, 23.0, 18.8, 17.3, 15.2, 13.7, 10.3.

HRMS: Calculated $[M+Na]^+$ calculated 603.2711, found 603.2736.

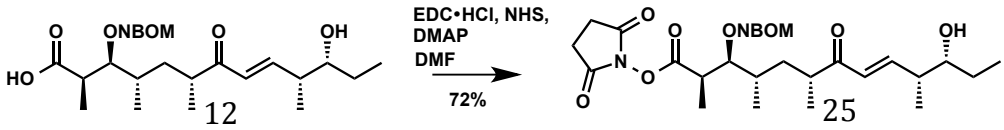


24: A 4 dram vial was charged with **12** (0.030 g, 0.063 mmol, 1.000 equiv), EDC•HCl (Chem-Impex, 0.018 g, 0.094 mmol, 1.500 equiv), and HOBT (Sigma, 0.010 g, 0.076 mmol, 1.200 equiv). The solids were dissolved in CH_2Cl_2 (0.63 mL, 0.1 M) and stirred for 30 min at RT. Benzyl mercaptan (Sigma, 0.009 g, 0.076 mmol, 1.200 equiv) was added and stirred for 10 min, followed by addition of DMAP (Sigma, ~ 1 mg, cat). The reaction mixture was stirred for 2 h. The solution was diluted with EtOAc, washed with H_2O, and the aqueous layer was extracted with EtOAc (2x). The combined organic extracts were filtered through a sodium sulfate plug, which was subsequently rinsed with EtOAc (2x) and concentrated. Flash chromatography: EtOAc/hexanes (30:70) to yield **24** (0.031 g, 0.053 mmol, 84%) as a pale yellow oil.

^{1}H NMR (599 MHz; $CDCl_3$): δ 8.07-8.06 (m, 1H), 7.77 (d, *J* = 7.7 Hz, 1H), 7.65-7.61 (m, 1H), 7.42 (t, *J* = 7.6 Hz, 1H), 7.27-7.19 (m, 5H), 6.83 (dd, *J* = 15.8, 7.7 Hz, 1H), 6.16 (dd, *J* = 15.8, 0.8 Hz, 1H), 4.98 (q, *J* = 15.0 Hz, 2H), 4.76-4.71 (m, 2H), 4.04 (s, 2H), 3.78 (t, *J* = 5.2 Hz, 1H), 3.46 (dt, *J* = 8.8, 4.4 Hz, 1H), 2.88-2.79 (ovlp m, 2H), 2.39 (dq, *J* = 12.7, 6.4 Hz, 1H), 1.92 (ddd, *J* = 13.7, 9.5, 4.1 Hz, 1H), 1.66-1.59 (m, 2H), 1.49 (dqd, *J* = 14.3, 7.2, 3.9 Hz, 1H), 1.40-1.32 (m, 1H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.10 (ddd, *J* = 14.1, 9.4, 4.9 Hz, 1H), 1.05 (d, *J* = 6.8 Hz, 3H), 1.03 (d, *J* = 6.9 Hz, 3H), 0.93 (t, *J* = 7.4 Hz, 3H), 0.90 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 203.5, 201.8, 149.4, 147.1, 137.3, 134.8, 133.6, 128.8, 128.8, 128.7, 128.5, 128.3, 127.9, 127.2, 124.7, 96.3, 83.7, 75.8, 66.9, 50.3, 42.3, 41.7, 35.7, 34.7, 33.1, 27.4, 18.0, 16.6, 13.8, 13.1, 10.3.

HRMS: Calculated $[M+Na]^+$ calculated 608.2652, found 608.2658.

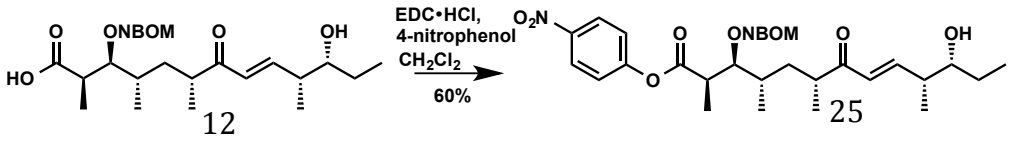


25: A 4 dram vial was charged with **12** (0.030 g, 0.063 mmol, 1.000 equiv), EDC•HCl (Chem-Impex, 0.018 g, 0.094 mmol, 1.500 equiv). The vial was cooled to 0 °C and the solids were dissolved in DMF (0.63 mL, 0.1 M) and stirred 10 min at 0 °C. *N*-hydroxysuccinimide (Sigma, 0.011 g, 0.094 mmol, 1.500 equiv) was added, stirred 10 min at 0 °C and an additional 10 min at RT, followed by DMAP (Sigma, ~ 1 mg, cat) and stirred 4 h. The solution was diluted with EtOAc, washed with H_2O, and the aqueous layer was extracted with EtOAc (2x). The combined organic extracts were filtered through a sodium sulfate plug, which was subsequently rinsed with EtOAc (2x) and concentrated. Flash chromatography: EtOAc/hexanes (40:60 to 50:50) to yield **25** (0.026 g, 0.045 mmol, 72%) as a pale yellow oil.

^{1}H NMR: (599 MHz; $CDCl_3$): δ 8.08 (dd, *J* = 8.2, 0.9 Hz, 1H), 7.82 (d, *J* = 7.7 Hz, 1H), 7.66-7.63 (m, 1H), 7.43 (t, *J* = 7.5 Hz, 1H), 6.86 (dd, *J* = 15.8, 7.6 Hz, 1H), 6.17 (dd, *J* = 15.8, 0.9 Hz, 1H), 5.11-5.00 (m, 2H), 4.90 (dd, *J* = 25.3, 6.9 Hz, 2H), 3.85 (t, *J* = 5.1 Hz, 1H), 3.49 (dt, *J* = 8.8, 4.4 Hz, 1H), 3.07-3.02 (m, 1H), 2.90-2.76 (ovlp m, 5H), 2.44-2.39 (m, 1H), 2.02 (ddd, *J* = 13.7, 9.7, 3.9 Hz, 1H), 1.80-1.68 (m, 2H), 1.50 (dqd, *J* = 14.2, 7.2, 4.0 Hz, 1H), 1.42-1.35 (m, 1H), 1.32 (d, *J* = 7.0 Hz, 3H), 1.15 (ddd, *J* = 14.0, 9.5, 4.7 Hz, 1H), 1.06-1.05 (ovlp m, 6H), 0.96-0.93 (ovlp m, 6H).

^{13}C NMR: (151 MHz; $CDCl_3$): δ 203.4, 170.6, 169.1, 162.0, 149.4, 147.0, 134.9, 133.7, 128.6, 128.4, 127.8, 124.7, 96.5, 83.1, 75.7, 67.0, 42.1, 41.7, 39.4, 35.7, 34.8, 29.7, 27.2, 25.6, 18.1, 16.6, 13.5, 12.1, 10.4.

HRMS: Calculated $[M+Na]^+$ calculated 599.2575, found 599.2595.

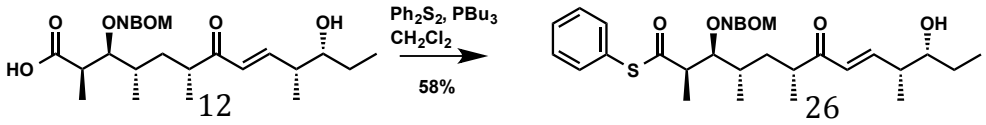


25: A 4 dram vial was charged with **12** (0.030 g, 0.063 mmol, 1.000 equiv), EDC•HCl (Chem-Impex, 0.018 g, 0.094 mmol, 1.500 equiv). The vial was cooled to 0 °C and the solids were dissolved in CH_2Cl_2 (0.63 mL, 0.1 M) and stirred for 30 min at 0 °C. 4-nitrophenol (0.011 g, 0.0756 mmol, 1.200 equiv) was added, stirred 10 min at 0 °C, and an additional 12 h at RT. The solution was added directly onto a flash column. Flash chromatography: EtOAc/hexanes (30:70) to yield **25** (0.023 g, 0.038 mmol, 60%) as a pale yellow oil.

^{1}H NMR (599 MHz; $CDCl_3$): δ 8.17-8.15 (m, 2H), 8.01 (dd, *J* = 8.1, 1.0 Hz, 1H), 7.62 (d, *J* = 7.7 Hz, 1H), 7.54 (td, *J* = 7.5, 1.0 Hz, 1H), 7.43-7.40 (m, 1H), 7.27-7.25 (m, 2H), 6.87 (dd, *J* = 15.8, 7.7 Hz, 1H), 6.20 (dd, *J* = 15.8, 1.0 Hz, 1H), 4.97 (s, 2H), 4.88 (q, *J* = 6.5 Hz, 2H), 3.85 (t, *J* = 5.4 Hz, 1H), 3.49 (dt, *J* = 8.8, 4.4 Hz, 1H), 3.04-2.98 (m, 1H), 2.94-2.88 (m, 1H), 2.45-2.40 (m, 1H), 2.07 (ddd, *J* = 13.6, 10.4, 3.3 Hz, 1H), 1.69-1.48 (ovlp m, 3H), 1.41-1.35 (m, 1H), 1.32 (d, *J* = 7.0 Hz, 3H), 1.16 (ddd, *J* = 13.8, 10.0, 3.9 Hz, 1H), 1.12 (d, *J* = 7.0 Hz, 3H), 1.07 (d, *J* = 6.8 Hz, 3H), 0.98 (ovlp m, 6H).

^{13}C NMR: (151 MHz; $CDCl_3$): δ 203.4, 172.8, 155.4, 149.7, 147.6, 145.2, 133.9, 133.3, 129.1, 128.4, 128.2, 125.0, 124.7, 122.6, 97.2, 84.9, 75.8, 67.4, 42.2, 42.1, 41.7, 35.6, 34.6, 27.4, 18.6, 16.7, 13.8, 11.7, 10.3.

HRMS: Calculated $[M+Na]^+$ calculated 623.2575, found 623.2573.



26: A 9 dram vial was charged with **12** (0.055 g, 0.115 mmol, 1.00 equiv) and Ph_2S_2 (Sigma, 0.028 g, 0.121 mmol, 1.100 equiv). The solids were dissolved in CH_2Cl_2 (1.15 mL, 0.1 M) and cooled to -42 °C. PBu_3 (Sigma, 0.030 g, 0.037 mL, 0.150 mmol, 1.300 equiv) was added dropwise, and the solution was stirred for 20 min at

- 42 °C. The reaction was quenched at -42 °C with a saturated $CuSO_4$ solution and allowed to warm to RT. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (2x). The organic layer was washed with a saturated EDTA solution (disodium salt, 2x) and filtered through a sodium sulfate plug, which was subsequently rinsed with CH_2Cl_2 (2x). The organic layers were combined and concentrated. Flash chromatography: EtOAc/hexanes (30:70) afforded **26** (0.038 g, 0.056 mmol, 58%) as a pale yellow oil.

^{1}H NMR (599 MHz; $CDCl_3$): δ 8.08-8.07 (m, 1H), 7.77 (d, *J* = 7.8 Hz, 1H), 7.63-7.61 (m, 1H), 7.44-7.42 (m, 1H), 7.38 (s, 5H), 6.85 (dd, *J* = 15.8, 7.7 Hz, 1H), 6.19 (dd, *J* = 15.8, 0.8 Hz, 1H), 5.03 (q, *J* = 15.7 Hz, 2H), 4.85 (s, 2H), 3.83 (t, *J* = 5.3 Hz, 1H), 3.46 (dt, *J* = 8.8, 4.4 Hz, 1H), 3.01

(quint, *J* = 6.6 Hz, 1H), 2.91-2.85 (m, 1H), 2.42-2.36 (m, 1H), 1.99 (ddd, *J* = 13.8, 9.6, 4.1 Hz, 1H), 1.71-1.56 (m, 2H), 1.49 (dqd, *J* = 14.3, 7.3, 3.9 Hz, 1H), 1.40-1.32 (m, 1H), 1.30 (d, *J* = 7.0 Hz, 3H), 1.20 (ddd, *J* = 14.0, 9.5, 4.7 Hz, 1H), 1.09 (d, *J* = 6.9 Hz, 3H), 1.04 (d, *J* = 6.8 Hz, 3H), 0.96 (d, *J* = 6.8 Hz, 3H), 0.93 (t, *J* = 7.4 Hz, 3H).

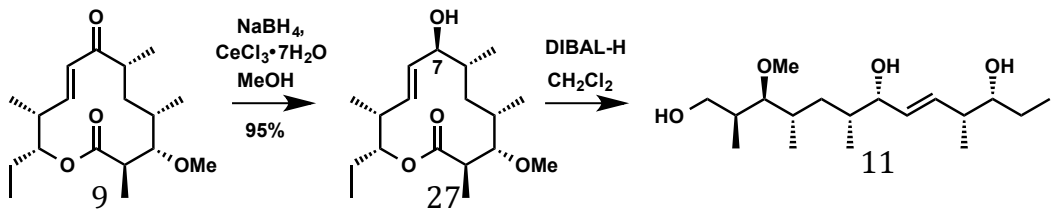
^{13}C NMR (151 MHz; $CDCl_3$): δ 203.5, 200.4, 149.4, 147.1, 134.7, 134.5, 134.7, 133.7, 129.3, 129.1, 128.8, 128.4, 127.9, 127.5, 124.7, 96.6, 83.9, 75.8, 67.1, 50.4, 42.3, 41.6, 35.5, 34.7, 27.4, 18.2, 16.7, 13.8, 13.7, 10.3.

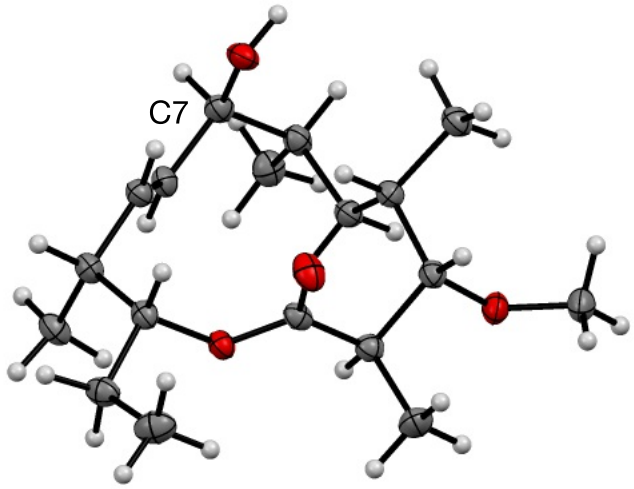
HRMS: Calculated $[M+Na]^+$ 594.2496, found 594.2499.

X-Ray Crystallography: 27 was dissolved in a minimum volume of Et_2O, diluted with hexanes (~5x v/v) and concentrated to half volume under a stream of N_2. Colorless block-like crystals of **S20** were grown from the resulting hexanes solution at -30 °C. A crystal of dimensions 0.14 x 0.06 x 0.04 mm was mounted on a Rigaku AFC10K Saturn 944+ CCD-based X-ray diffractometer equipped with a low temperature device and Micromax-007HF Cu-target micro-focus rotating anode (λ = 1.54187 A) operated at 1.2 kW power (40 kV, 30 mA). The X-ray intensities were measured at 85(1) K with the detector placed at a distance 42.00 mm from the crystal. A total of 3842 images were collected with an oscillation width of 1.0° in ω. The exposure time was 1 sec. for the low angle images, 5 sec. for high angle. The integration of the data yielded a total of 24668 reflections to a maximum 2θ value of 136.46° of which 3403 were independent and 3309 were greater than 2σ(I). The final cell constants (Table 1) were based on the xyz centroids 17310 reflections above 10σ(I). Analysis of the data showed negligible decay during data collection; the data were processed with CrystalClear 2.0 and corrected for absorption. The structure was solved and refined with the Bruker SHELXTL (version 2008/4) software package, using the space group P2(1) with Z = 2 for the formula C18H32O4. All non-hydrogen atoms were refined anisotropically with the hydrogen atoms placed in a mix of idealized and refined positions. Full matrix least-squares refinement based on F2 converged at R1 = 0.0295 and wR2 = 0.0771 [based on I > 2sigma(I)], R1 = 0.0305 and wR2 = 0.0781 for all data. Acknowledgement is made for funding from NSF grant CHE-0840456 for X-ray instrumentation.

Sheldrick, G.M. SHELXTL, v. 2008/4; Bruker Analytical X-ray, Madison, WI, 2008.

CrystalClear Expert 2.0 r12, Rigaku Americas and Rigaku Corporation (2011), Rigaku Americas, 9009, TX, USA 77381-5209, Rigaku Tokyo, 196-8666, Japan.





Alternative preparation of 27: An open 25mL round bottom flask was charged with **9** (0.100 g, 0.322 mmol, 1.000 equiv), $CeCl_3 \cdot 7H_2O$ (0.120 g, 0.322 mmol, 1.00 equiv) and MeOH (technical grade, 3.2 mL, 0.1 M). The solution was stirred at RT until the solids had dissolved completely, and then cooled to -78 °C. $NaBH_4$ (0.012 g, 0.322 mmol, 1.00 equiv) was added in a single portion and stirred for 10 min. The solution was decanted cold into 1 M HCl, and the aqueous solution was extracted with CH_2Cl_2 (3x). The combined organic extracts were filtered through a sodium sulfate plug, which was subsequently rinsed with CH_2Cl_2 (2x) and concentrated. Flash chromatography: EtOAc/hexanes (10:90 to 15:85) afforded **27** (0.096 g, 0.307 mmol, 95%) as a colorless oil that crystalized upon standing.

^{1}H NMR: (599 MHz; $CDCl_3$): δ 5.66 (ddd, *J* = 15.8, 4.7, 1.7 Hz, 1H), 5.48 (ddd, *J* = 15.8, 3.5, 1.6 Hz, 1H), 4.97 (ddd, *J* = 8.9, 5.3, 3.5 Hz, 1H), 4.09 (br s, 1H), 3.49 (s, 3H), 3.13 (dd, *J* = 10.3, 1.6 Hz, 1H), 2.58 (ovlp m, 2H), 1.92 (ttd, *J* = 10.6, 7.1, 3.4 Hz, 1H), 1.87-1.81 (m, 1H), 1.68-1.58 (ovlp m, 2H), 1.58-1.51 (m, 1H), 1.33 (ddd, *J* = 13.7, 11.0, 2.8 Hz, 1H), 1.24 (d, *J* = 6.8 Hz, 3H), 1.05 (ovlp m, 6H), 0.96 (d, *J* = 7.1 Hz, 3H), 0.90 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 175.5, 130.8, 128.5, 88.9, 75.8, 62.8, 43.5, 37.7, 35.3, 33.3, 32.6, 24.3, 20.4, 17.5, 16.2, 10.8, 10.4

HRMS: Calculated $[M\text{-}H_2O+H]^+$ 295.2268, found 295.2274.

27 was converted to **11** using conditions identical to direct reduction of **9** to **11**, where **11** produced by either method was identical.

3.4 PKS and TE Biochemistry Experimental

Purified H_2O from a Millipore Milli-Q system with Millipore Q-Gard 2/Quantum Ex Ultrapure organex cartridges was used for all cell culture, protein purification, and enzymatic reactions. *E. coli* seed culture was grown in 15 mL sterile tubes, and subsequently grown in Corning Fernbach flasks (2.8 L) with 3x deep baffles. LB broth (Miller) and glycerol were obtained from EMD. Isopropyl-β-D-thiogalactopyarnoside (IPTG) and Kanamycin (Kan) sulfate were obtained from Gold Biotechnology. Streptomycin sulfate (Strep) was obtained from AK scientific. NaCl, $CaCl_2$ and imidazole were obtained from Fisher Scientific. Lysozyme was purchased from RPI. Benzonase was purchased from Sigma Aldrich. PD-10 colums were purchased from GE scientific and equilibrated with 5 column volumes of storage buffer before use. Ni-NTA agarose resin was purchased from Qiagen and pre-equilibrated with five column volumes of lysis buffer before use.

A Symphony SB70P pH meter was calibrated according to the manufacturer's specifications and used to monitor the pH of all solutions during adjustment. Cells were lysed using a 550 Sonic Dismembrator purchased from Fisher Scientific. Optical density (OD_{600}) was determined using an Eppendorf Biophotometer. All solutions were autoclaved or sterile filtered through a 0.2 μm filter.

Buffers: lysis: HEPES (50 mM), NaCl (300 mM), imidazole (10 mM), glycerol (10% v/v), pH 8.0.
wash: HEPES (50 mM), NaCl (300 mM), imidazole (30 mM), glycerol (10% v/v), pH 8.0.
elution HEPES (50 mM), NaCl (300 mM), imidazole (300 mM), glycerol (10% v/v), pH 8.0.
storage: HEPES (50 mM), NaCl (150 mM), EDTA (1 mM), glycerol (20% v/v), pH 7.2.
PikAIV reactions: sodium phosphate (400 mM), glycerol (20% v/v), 2-vinylpyridine (8 mM), pH 7.2.
Pik TE reactions: sodium phosphate (400 mM), 2-vinylpyridine (8 mM), pH 7.2.

Stock solutions: hexaketide substrates (50 mM in DMSO), 2-vinylpyridine (500 mM in DMSO), ascorbic acid (500 mM in H_2O), sodium metabisulfite (100 mM in H_2O), PikAIV reaction buffer [2x, sodium phosphate (800 mM), glycerol (40% v/v), pH 7.2], Pik TE reaction buffer [2x, sodium phosphate (800 mM), pH 7.2], MM-SNAC (500 mM in H_2O, neutralized to pH 7.2 with $NaHCO_3$).

Protein Expression

The cloning, expression and purification of PikAIV[21] and the Pik TE[22] has been reported previously. A starter culture of *E. coli* (BAP1)[23] cells containing the corresponding plasmids for

expression of respective proteins was generated by inoculating LB broth Miller (10 mL) containing Kan (50 mg/L) and Strep (50 mg/L) with frozen glycerol stocks and grown overnight at 37 ˚C. The following morning, LB (1.5 L) containing Kan (50 mg/L) and Strep (50 mg/L) was inoculated with the entire overnight culture and grown at 37 ˚C to an OD_{600} of 0.3-0.4. The cells were then cultured at 20 ˚C until an OD_{600} of 0.7-0.8 was reached; at which point protein expression was induced via addition of IPTG (300 μM) and the cultures were incubated at 200RPM, 2.5 cm orbit, at 20 ˚C for a minimum of 18 hours.

Protein Purification

To retain maximum enzymatic activity, the following purification procedure was performed at 4 ˚C in less than 2 hours. Overexpression cultures were harvested by centrifugation (5,500 x g, 10 min, 4 ˚C) and cell pellets were suspended in 5 mL of lysis buffer per gram of cells via vortex. Cell lysis was accomplished by gentle agitation at 4 ˚C with 0.4 mg/ml lysozyme and 8 units/ml benzonase for 30 min followed by sonication on ice (6 x 10s with 50s rest periods). Cellular debris was pelleted by centrifugation (40,000 x g, 15 min, 4 ˚C), and the supernatant applied to 3 mL of Ni-NTA resin. After binding, the column was washed with 25 mL of wash buffer under gentle syringe pressure and the target protein was eluted with 15 mL of elution buffer. Protein containing fractions were assessed via their absorption at 280 nm, pooled, and buffer exchanged into storage buffer using a PD-10 column. Finally, protein containing fractions were determined via their absorption at 280 nm, pooled, flash frozen in liquid N_2, and stored at -80 ˚C.

Analytical Enzymatic Reactions

All enzymatic reactions were performed in triplicate at a volume of 50 μL and were initiated via the addition of enzyme. 2-vinylpyridine (Sigma) was employed as a thiol scavenger (8mM final concentration) in all reactions. After 4 h stationary incubation at RT, the reactions were quenched with 3 volumes of MeOH (150 μL), clarified by centrifugation (17,000 x g, 15 min, 4˚C) and analyzed for macrolactone production. In all cases, the reactions were carried out in PikAIV or Pik TE reaction buffers.

Methyl Protected Substrates:

Reactions employing methylated substrates were performed as one-pot reactions containing phosphate buffer, methylated hexaketide (1 mM), with or without MM-SNAC (20 mM). Catalysis was initiated via the addition of enzyme, either TE (10 μM) or PikAIV (2.5 μM and 10 μM). Conversion to macrolactones was monitored by Method A (**HPLC analysis** section).

NBOM Protected Substrates:

Enzymatic reactions utilizing NBOM protected substrates were performed over two steps. First, a solution of ascorbic acid (25mM final concentration), sodium metabisulfite (1mM final concentration), NBOM protected substrate (1mM final concentration), and H_2O (requisite dead volume) was irradiated under a consumer facial tanning lamp at a height of 14 cm (Verseo #AH129c) for 20 min to furnish the deprotected Pik hexaketide. NOTE: Irradiation through the

side of the microtubes employed (Axygen #MCT-175-C) did not interfere with photolysis, and this process was reproducible over the course of this study. After photolysis, the solution was diluted with either PikAIV of Pik TE reaction buffer, MM-NAC (20 mM final concentration when included). Catalysis was initiated via the addition of enzyme, TE (10 μM) or PikAIV (2.5 μM and 10 μM), and incubated for 4 hours. Conversion to macrolactones was monitored by Method B (**HPLC analysis** section).

HPLC analysis

Macrolactone production was monitored via analytical high performance liquid chromatography (HPLC) using a Beckman Coulter instrument (model 366 serial 385-1160) and a Zorbax SB-Phenyl 3.5 μM 4.6 x 150 mm column (part number 863953-912) at a wavelength of 250 nm.

Method A: For reactions employing methylated substrates, separation was accomplished by the following method: 3.0 mL/min, solvent A: H_2O, solvent B: MeCN, 20% B 0-1 min, 20-60% B linear gradient 1-10 min, 100% B 10-11 min, 20% B 11-12 min.

Method B: For reactions employing NBOM protected substrates, separation was accomplished by the following method: 3.0 mL/min, solvent A: H_2O, solvent B: MeCN, 10% B 0-1 min, 10-40% B linear gradient 1-10 min, 100% B 10-11 min, 10% B 11-12 min.

Samples were quantified by linear regression using equations derived from fitting the peak areas of the corresponding standard curves. Standard curves were generated by analyzing macrolactone standards in triplicate at a range of concentrations from 1.0-0.0156 mM, representing a range in percent conversions from 400-6.5%, and were linear in all cases. Conversions below 6.5% were quantified via extrapolation of the standard curve.

3.5 References

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Chapter 4

Exploratory Simulated Combinatorial Biosynthesis in the Pik pathway

This chapter explores the complex topic of combinatorial biosynthesis in Type I PKS pathways. As PKS pathways assemble natural products in a manner analogous to an industrial assembly line,[1] biosynthetic engineers have attempted to manipulate the assembly line to get unnatural products through 1) swapping in homologous domains or even whole modules from other pathways 2) mutagenesis of KS domains to alter stereochemistry of the Claisen condensation 3) mutagenesis of the AT domain to select for various malonates 4) mutagenesis of KR domains to change the stereochemistry of the resulting β-hydroxyl group or α-stereocenter 5) and mutagenesis of ER domains to change the stereochemistry of the α-stereocenter amongst a plethora of other approaches. These modifications, while seemingly minor and localized if one employs an enzyme-centric viewpoint,[2] must be considered holistically in terms of the whole pathway when one considers the downstream chemistry necessary to yield a final unnatural product. Consider a theoretical macrolide biosynthetic pathway consisting of six PKS monomodules, which would ultimately produce a 14-membered macrolactone. Suppose we could mutate the KS domain in module I to WT levels of efficiency where a previously (*R)*-selective Claisen was exchanged for a (*S)*-selective Claisen without any modifications in the rest of the pathway. Let module I also contains a KR domain; what effect would the unnatural (*S)*-Claisen product have on the stereoselectivity and rate of the reduction? As KR domains can epimerize α-stereocenters in some cases, would the KR simply restore the natural (*R*)-configuration? How would the unnatural (*S*)-stereocenter affect the rate of the transfer to and the subsequent rate of the KS domain in module II? As the unnatural polyketide moved farther down the pathway and the unnatural stereocenter became more distal would perturbation of catalytic rate decrease? If the whole pathway processed the unnatural polyketide effectively, would the TE domain be able to catalyze macrocyclization to offload the final product? If so, at what rate? Let us suppose that the unnatural polyketide imposed no rate penalties throughout the entire pathway save for the final TE domain, how would this affect global pathway flux? If we are studying the effect in vivo, how does any one of these scenarios affect the titer of the unnatural product with respect to WT?

These and many more questions aside, enzyme engineering and directed evolution efforts could, in theory, lead to production of unnatural product analog libraries or direct

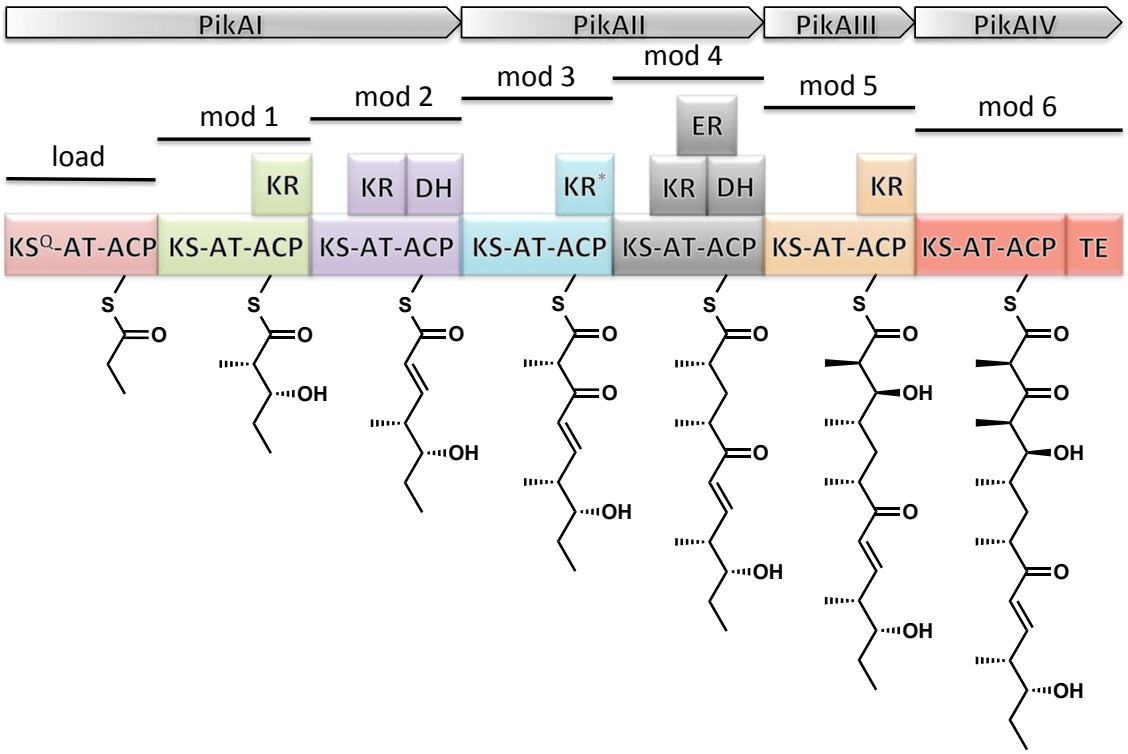
fermentation of a specific unnatural product. If combinatorial biosynthesis could be fully realized the reward would be immense; so this intricate web of interlaced problems is worth parsing.

Combinatorial biosynthesis has been largely unsuccessful, where no methods or products from such efforts have yet to reach commercial viability despite 25 years of inquiry. While some unnatural products have been reported in the literature, the overwhelming majority of combinatorial pathways suffer from greatly diminished titers relative to WT.[3] As such, we chose to explore the tractable Pik pathway and "simulate combinatorial biosynthesis" early in the pathway (PikAI) and evaluate how WT PikAIII-TE or PikAIII/PikAIV PKS modules were able to process unnatural (i.e. combinatorial) polyketides. Accordingly, we synthesized a panel of unnatural pentaketides bearing modifications that would have occurred if the loading domain or module 1 possessed a mutant AT domain accepting different extender units (malonate vs. methyl malonate) and mutant KS or KR domains to construct all possible stereochemical configurations derived from the Claisen condensation and subsequent reduction/epimerization. By directly assaying these unnatural pentaketides with WT PikAIII-TE or PikAIII/PikAIV we can evaluate how a downstream PKS module can handle early pathway engineering *while avoiding protein-centric complications arising from protein engineering or directed evolution.* In this chemistry-centric approach, we are examining substrate flexibility of the final modules of a pathway to perform final processing and macrolactonization of unnatural substrates.

Work described in Chapter 4 is ongoing at present.

4.1 Design of unnatural Pik pentaketides

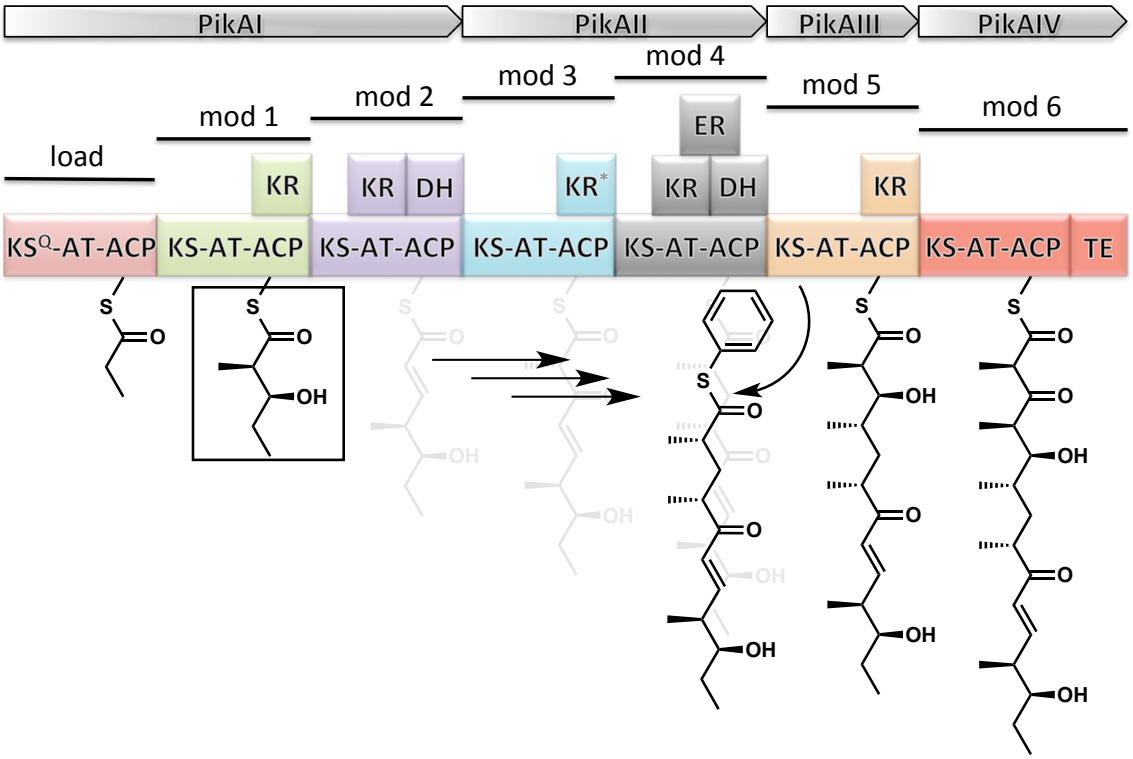
Figure 4.1 The Pik PKS pathway



The Pik pathway has been studied extensively by the Sherman laboratory and others both in vivo and in vitro and serves as an ideal pathway for combinatorial efforts as it naturally makes two classes of macrolactones, where the terminal TE is a rare example of a bifunctional macrolactone-forming domain. Furthermore, subsequent tailoring (glycosylation and hydroxylation) steps have demonstrated wide substrate flexibility, where unnatural macolactones generated from combinatorial PKS modules stand a reasonable chance of being tailored with WT glycosyltransferase (desVII/desVIII) and p450 (PikC) enzymes.[4] Additionally, *S. venezuelae* ATCC 15439 is a rare example of a fast growing macrolide producer that biosynthesizes during log phase, meaning maximum titer is achieved in just over 48 hours as opposed to more common stationary phase production resulting in 1-2 week fermentations with related organisms. Finally, the titer of WT *S. venezuelae* ATCC 15439 is roughly 0.25 g/L, exceedingly high for an

unimproved natural producer, especially when considering the short fermentation time.

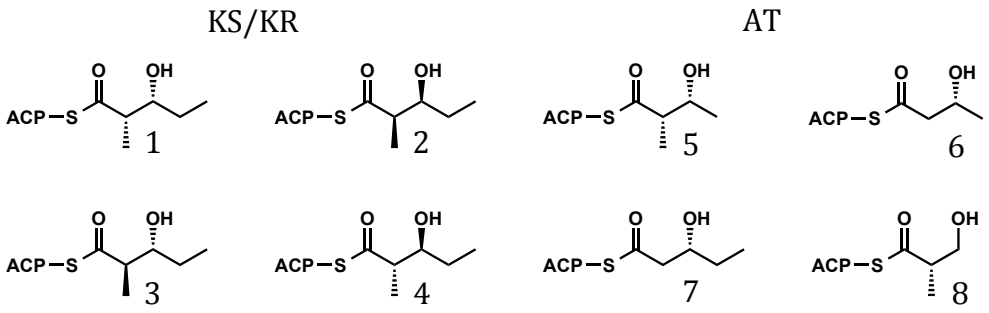
Figure 4.2 Simulated combinatorial biosynthesis in the Pik PKS pathway



As such we decided to synthesize a panel of unnatural Pik pentaketides using an established synthetic route designed for convergent diversification at a late stage (chapter 2). Once in hand, unnatural pentaketides would then be intercepted by the Pik pathway (either PikAIII-TE or PikAIII/PikAIV) using previously optimized enzymatic reactions (chapter 2); and the outcome of catalysis would be determined by isolating products (if any) and standard NMR (and X-ray diffraction if applicable) based structural characterization.

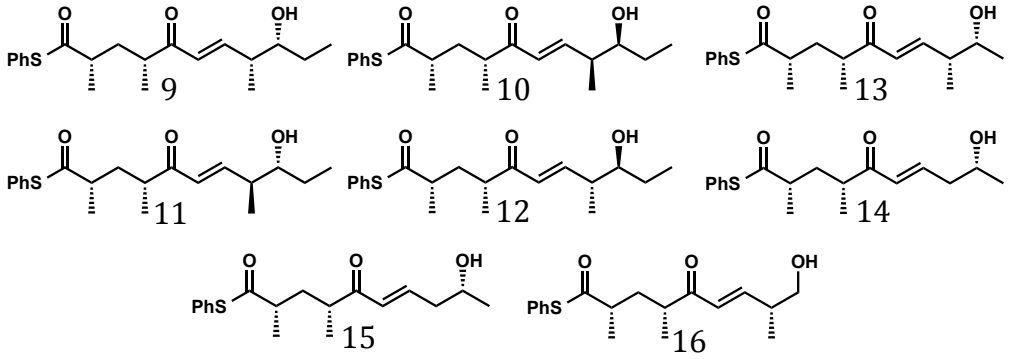
The panel of targeted unnatural pentaketides was designed to "simulate combinatorial biosynthesis" early in the pathway if modifications occurred in the loading module or module 1 of PikAI (Figure 4.3). **1** is the natural diketide from the WT Pik pathway, while **2-4** encompass all possible stereoisomers arising from mutated or swapped KS and/or KR domain(s). **5** and **7** would arise from an acetate starter unit derived from a mutated or swapped AT domain in the loading module or PikAI that accepts malonyl-CoA, respectively. **6** would require both acetate starter and extender units derived from two malonyl-CoA specific AT domains. **8** would arise from loading module capable of utilizing a formyl starter unit.

Figure 4.3 Combinatorial polyketides on ACP_1 from early pathway engineering



These unnatural diketides would then be processed to unnatural pentaketides before being passed from ACP_4 of PikAII to the KS of PikAIII, which is where we intended to intercept the pathway with synthetic unnatural pentaketides (Figure 4.4).

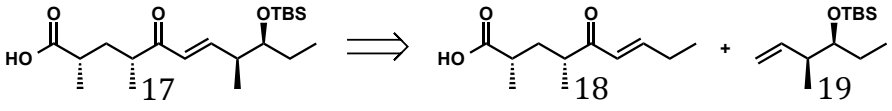
Figure 4.4 Combinatorial synthetic pentaketides as substrates for PikAIII



4.2 Synthesis of unnatural Pik pentaketides

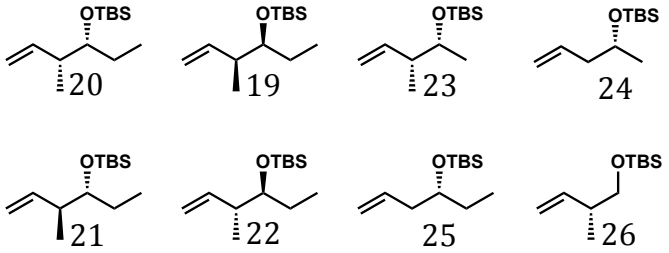
The synthesis of unnatural Pik pentaketides follows an identical synthetic scheme to that described in chapter 2. A common α,β-unsaturated ketone **18** is joined with a variety of analogs of the natural type I olefin.

Scheme 4.1 Central disconnection for Pik pentaketide analogs



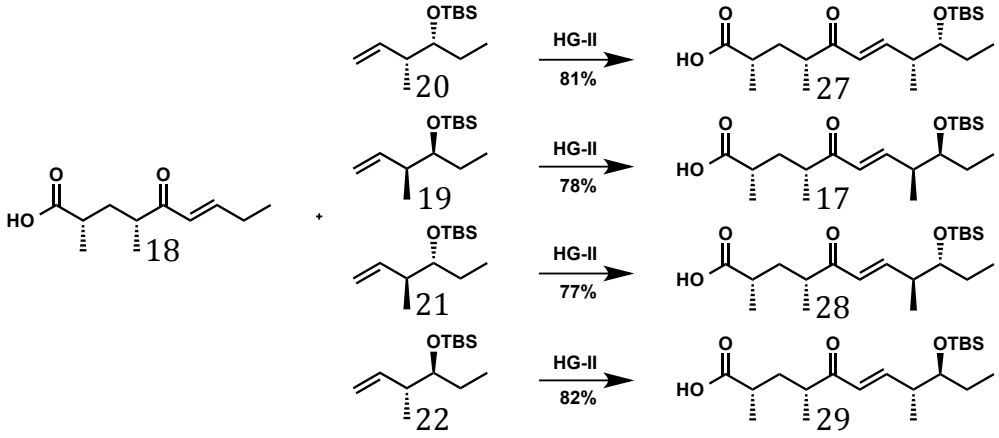
Since we already had a scalable route to **18** secured, we simply needed to secure relatively simple type I fragments, perform cross metathesis and final esterification/deprotection to provide analogs **10-16** (Figure 4.5.)

Figure 4.5 Combinatorial analogs of WT right fragment **20**



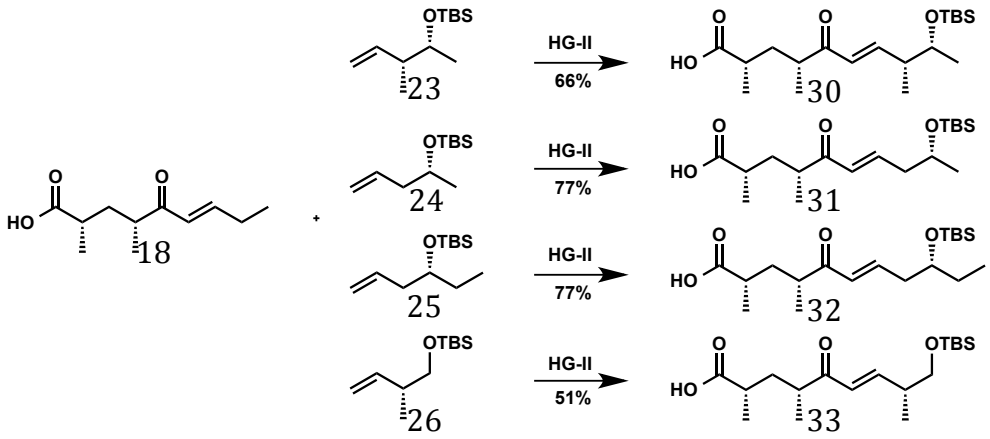
WT fragment **20** was synthesized as previously described (chapter 2) using Evans aldol methodology,[5] as were fragments **19** and **23**. A Krische crotylation followed by immediate TBS protection provided fragments **21** and **22**.[6] The homo-allylic alcohol precursor of **24** is commercially available, requiring only TBS protection. Opening (*R*)-1,2- epoxybutane with vinylmagnesium bromide and catalytic CuCl provided the linear homo-allylic alcohol,[7] which was then converted to the silyl-ether **25**.

Scheme 4.2 Synthesis of Pik pentaketides stereoisomer seco-acid analogs



A three step route to **26** began with (*S*)-Roche ester, which was TBS protected, DIBAL-H reduced the ester to an aldehyde which was directly subjected to Wittig olefination.

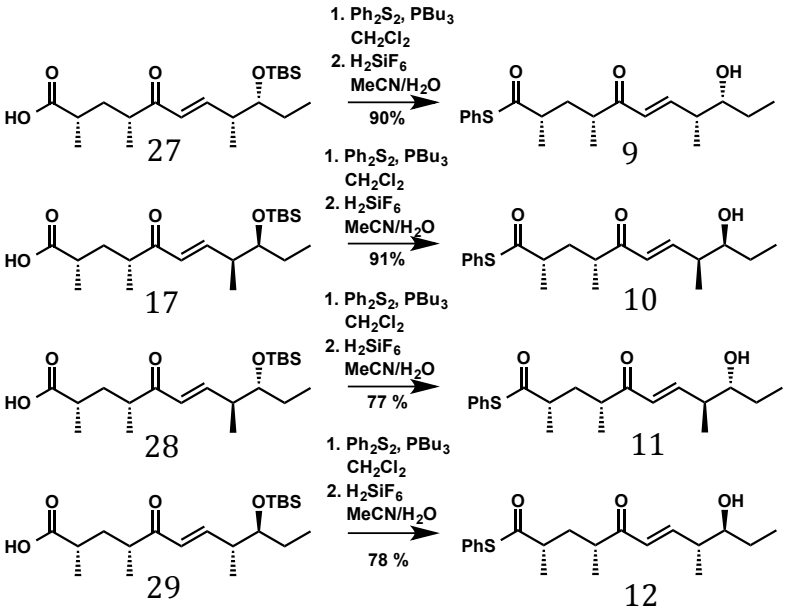
Scheme 4.3 Synthesis of Pik pentaketides truncation analogs



With all desired right fragments in hand, we employed slightly modified cross-metathesis conditions (chapter 2) to generate all targeted Pik pentaketide analogs. We found that increasing the temperature from 50 °C to 60 °C gave slightly improved yields at smaller scales with the additional benefit of near complete catalyst decomposition after 12 h (remaining catalyst was occasionally observed by TLC after 12 h, though it could be destroyed by raising the temperature to 80 °C for a brief period of time). Cross-metathesis employing **19-22** was uneventful, with isolated yields of ~80% seemingly unaffected by the stereochemical configuration of the right fragment (Scheme 4.2). Cross metathesis with **23-26** was slightly more interesting (Scheme 4.3), with the highest yields (77%) observed with those fragments lacking α-methyl substitution. Fragments **23** and **26** afforded slightly worse yields (66% and 51%, respectively) presumably due to α-methyl sterics in combination with the increased volatility relative to **19-22** (Scheme 4.3).

With seco-acids in hand, we first sought to prepare the stereoisomer panel for enzymatic reactions with PikAIII-TE and PikAIII/PikAIV through thioesterification and final deprotection (Scheme 4.4). Anti-analogs **11** and **12** proved acid sensitive requiring milder conditions than those previously employed (some elimination observed with excess HF in MeCN/H_2O). Common deprotection methods employing TBAF or TASF were unsatisfactory due to observed hydrolysis of the thioester. Moving to substoichiometric H_2SiF_6[8] minimized competitive elimination of the homo-allylic alcohol, though yields were still lower with *anti*-pentaketides **11** and **12** than with *syn*-pentaketides **9** and **10**.

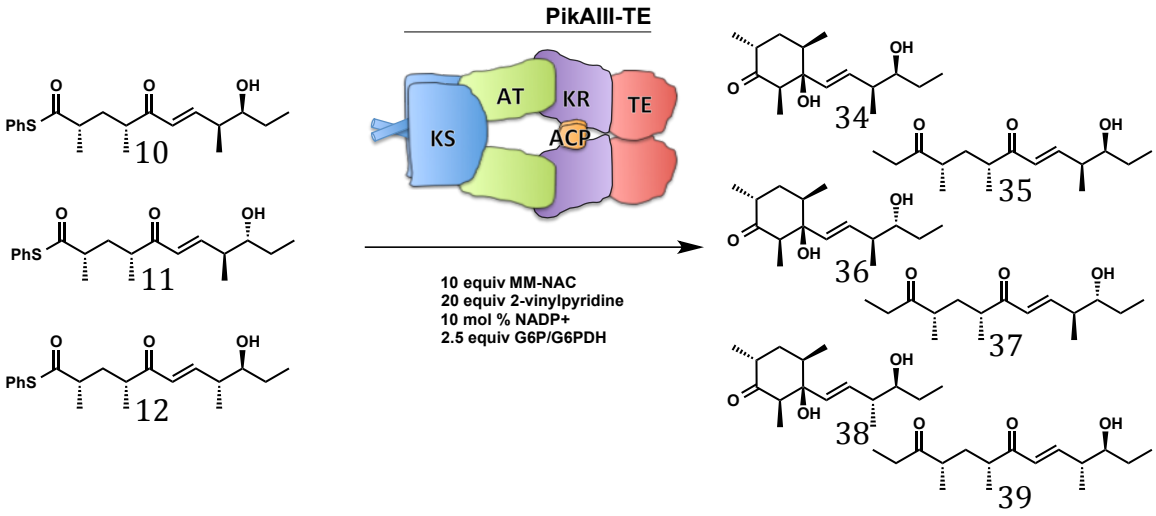
Scheme 4.4 Synthesis of Pik pentaketides stereoisomer analogs



With this first set of analogs in hand, we decided to explore the substrate flexibility of PikAIII-TE with **9-12**. Initial runs with PikAIII-TE as previously described (chapter 2) led to complete consumption (by TLC) of WT pentaketide **9** as expected, but incomplete consumption of **10-12** after 4 h along with formation of low levels of possible macrolactone products. Doubling the reaction time to 8 h resulted in complete consumption of **10-12** but the yield of 10-dml with **9** decreased slightly as a NAC conjugate adduct was observed. As such, we increased the concentration of 2-vinylpyridine (thiol scavenger) from 8 mM to 20 mM, which resorted the ~65% yield of 10-dml from **9** and perhaps more importantly, we began to observe a second product

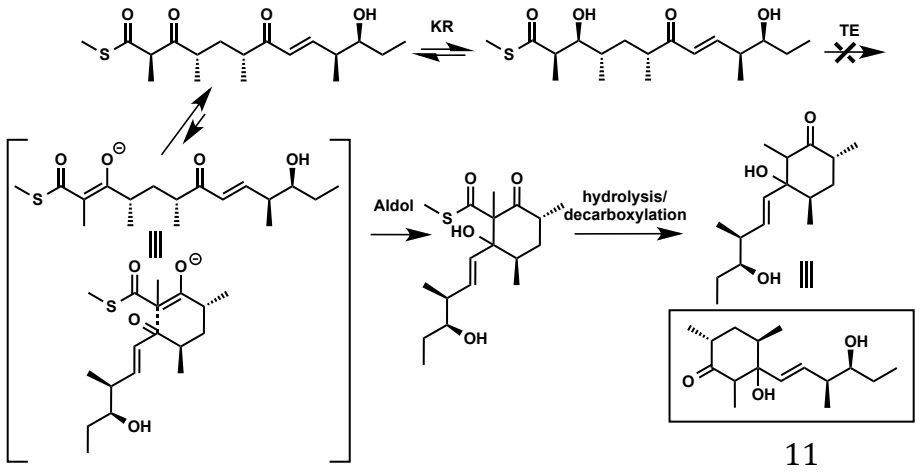
forming with **10-12**.

Scheme 4.5 Incubation of stereoisomer panel with PikAIII-TE



As **10-12** were being accepted and elongated by the enzyme but not macrolactonized, we hypothesized these substrates were mechanism based inhibitors of the TE domain, resulting in substrate stalling in the TE domain and, in turn, increasing residency in the upstream KR domain (Scheme 4.6). As such, we turned our attention to studying the Pik TE domain and neglected **30-33** for the time being. We chose to pursue a two-prong approach, first with synthesis of the C11 epi-hexaketide **46**, and finally with a heptaketide affinity label **49**.

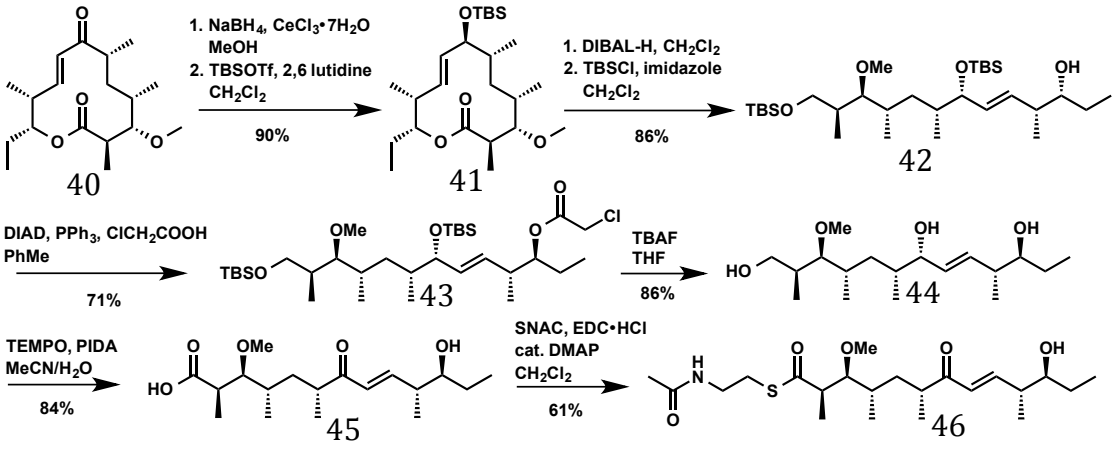
Scheme 4.6 Proposed mechanism for formation of **34** from **11**



4.3 Synthesis and Evaluation of the Pik C11 epi-hexaketide

Given the success of synthesizing Pik hexaketide substrates (chapter 3) from 10-dml, we hoped that we could simply invert the C11 hydroxyl group of **47** through a Mitsunobu inversion or related reaction. Unfortunately, the homo-allylic hydroxyl group was predominantly eliminated under all conditions examined, prompting a lengthier synthetic scheme where the α,β-unsaturated ketone was subjected to 1,2 reduction under Leuche conditions to raise the pKa of the C10 position (Scheme 4.6). C3 methyl protected 10-dml **40** was reduced and protected to yield **41**, which was further reduced and again protected to provide **42**. A number of Mitsunobu conditions were examined, where DIAD outperformed DEAD in all cases examined and chloroacetic acid[9] was superior to all other acids examined,[10] including 4-nitrobenzoic acid.[11] While some elimination was observed during the synthesis of **43**, the inversion proceeded with an acceptable yield (71%). In exploratory deprotections, compound **43** was found to unexpectedly sensitive to mildly acidic deprotection (HF) typically used with related compounds, prompting use of TBAF, which was able to deprotect both silyl-ethers and hydrolyze chloroacetate[12] in a one pot reaction. The final oxidation and thioesterification were operational under previously described conditions (chapter 3) to yield compound **46**.

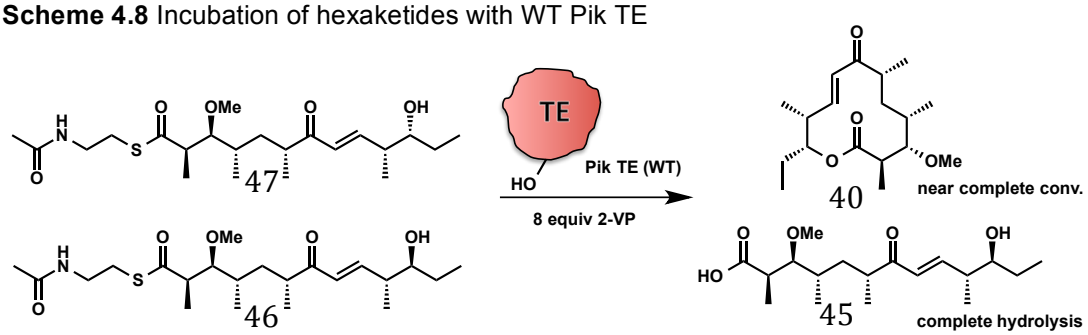
Scheme 4.7 Synthesis of the Pik C11 epi-hexaketide **46**



With the C11 epi-hexaketide **46** in hand, we sought to compare this compound to the natural hexaketide to see what the affect of this epimerized stereocenter had on macrolactonization. An initial 12 h incubation of **46** and **47** with WT Pik TE resulted in substantial NAC conjugate addition, resulting in employing the now familiar thiol scavenger 2-vinylpyridine (2-VP). With 2-VP, no conjugate addition product was observed, and we observed near complete

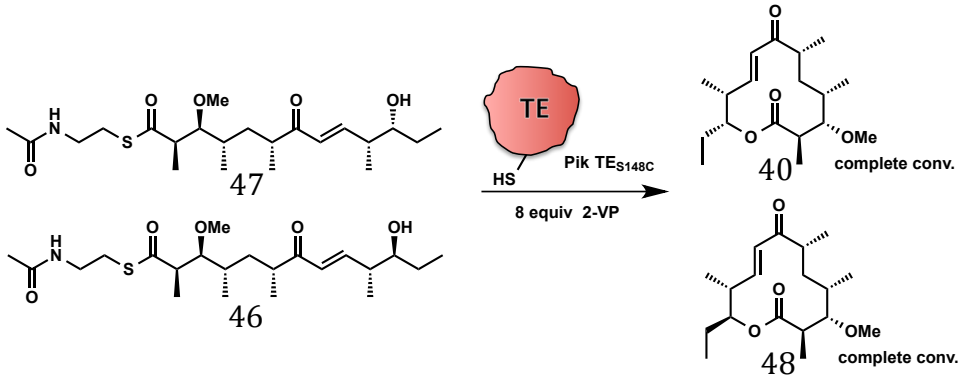
conversion of **47** to **40**, while **46** was quantitatively hydrolyzed, supporting the TE hypothesis.

Scheme 4.8 Incubation of hexaketides with WT Pik TE

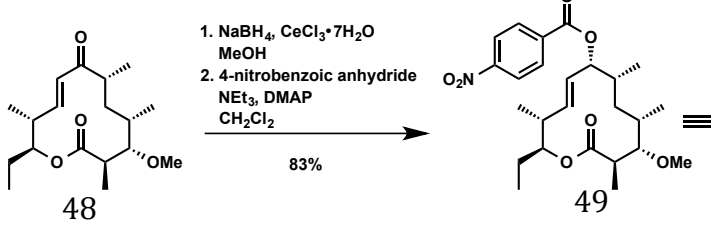


An identical experiment using the improved Pik TE_{S148C}[13] yielded surprising results. In this construct, the nucleophilic serine of the catalytic triad has been replaced with cysteine in a manner reminiscent of cysteine protease. This mutation was originally designed to accelerate the rate of acylation with little regard to the subsequent macrolactonization, though the mutation appears to improve both acylation and subsequent macrolactonization. A 12 h incubation of **47** to resulted in quantitative conversion to **40**, and more surprisingly, a 12 h incubation of **46** resulted in quantitative conversion to macrolactone hypothesized to be **48**, though a homodimer was not outside the realm of possibility as neither NMR methods nor ESI-HRMS (detecting masses corresponding to both monomer and dimer) conclusively solved the structure of **48**.

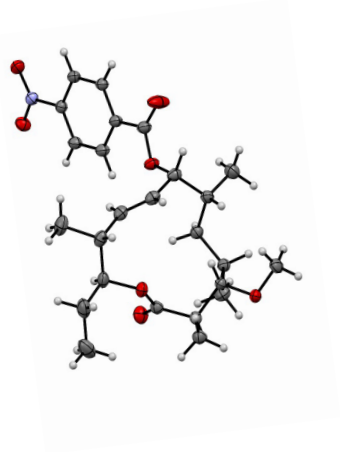
Scheme 4.9 Incubation of hexaketides with mutant Pik TE_{S148C}



Scheme 4.10 Derivatization of **48** to **49**



48 49



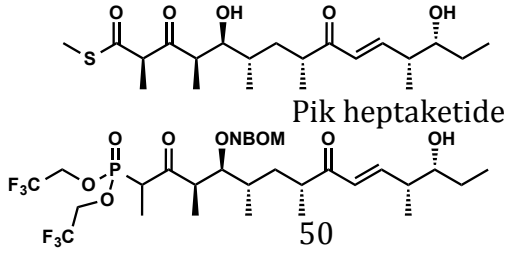
Initial attempts to crystalize **48** directly were met with failure under all attempted conditions, though Leuche reduction and acylation with 4-nitrobenzoic anhydride did provide crystals suitable for X-ray diffraction, indicating the product was indeed a monomer. Interestingly, an attempted time course of **46** converting to **48** at hour increments failed as complete conversion was observed after only 1 h, where conversion of **47** to **40** with WT Pik TE were not yet complete after 12 h! These preliminary observations of catalysis with Pik TE_{S148C} certainly encourage further study into this truly amazing point mutation.

4.3 Synthesis of a Pik heptaketide affinity label

PKS enzymologists have published a number of excised studies examining excised TE domains. Early work attempted biochemical characterization,[14] though difficulty in accessing native substrates required the use of non-native thioesters that were simply hydrolyzed rather than macrolactonized. Structural work followed,[15] which answered some questions but raised many more. DEBS TE X-Ray structures clearly showed a substrate channel running through the enzyme, a Ser-His-Asp catalytic triad commonly observed in esterases, and a wide, concave active site. The active site was surprisingly sparse, with only a few residues capable of participating in hydrogen bonding. The Sherman, Smith, and Fecik groups initiated a collaboration to examine the Pik TE using combined structural, biochemical, and chemical biology approaches.[16] This work marks the first time a PKS TE was biochemically characterized with a native substrate or structurally characterized with affinity label mimics of a native substrates. A key structural finding with affinity labels was that the compound was observed to curl toward the orientation required for macrolactonization while forming only one hydrogen bond with the enzyme. Apparently, a combination of a low energy substrate conformer in combination with a seemingly nonspecific active site template is enough to orient the linear chain towards functional macrolactonization, providing valuable insight into how these enzymes function. However, the affinity labels used were fragments of the native substrate and did not provide a complete picture

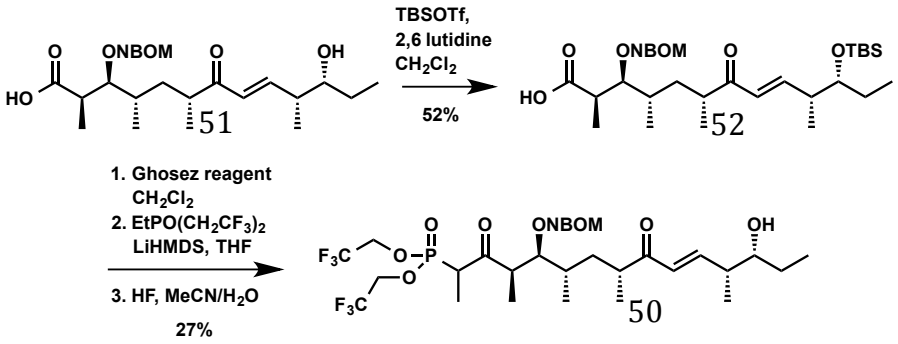
into the intricacies of the Pik TE. To rectify this shortcoming, we decided to synthesize a full-length affinity label mimic of the Pik heptaketide. This affinity label **50** could potentially be captured in two states, one where the active site serine has been labeled and the chain remains linear, and a further step where a tetrahedral intermediate could be captured if the terminal hydroxyl group cyclizes and forms a narbonolide mimic. While previous affinity labels where diphenyl phosphonates, we hypothesized we could improve inhibition though using alkyl leaving groups over aryl(chapter 3).

Figure 4.6 Heptaketide based affinity label **50**



A short four step sequence from compound **51** (three steps from 10-dml, chapter 3) provided compound **50.** TBS protection of the homo-allylic hydroxyl group provided silyl-ether **52**, which was converted to an acyl chloride with the Ghosez reagent, alkylated,[17] and deprotected to provide **50** in poor yield (27%) over a three step sequence.[18] Compound **50** is currently under evaluation.

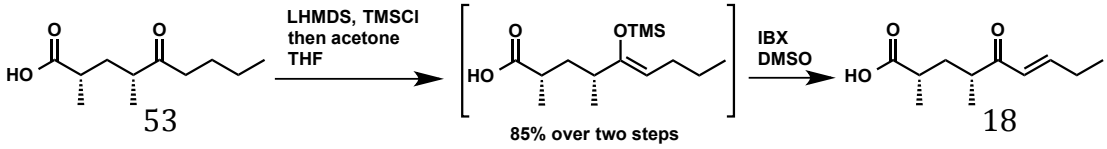
Scheme 4.11 Synthesis of heptaketide based affinity label **49**



4.4 Chemistry Experimental

Reactions were performed in evacuated (<0.05 torr) flame dried glassware containing PFTE coated magnetic stir bars fitted with rubber septa backfilled with dry N_2 and run under a positive pressure of dry N_2 provided by a mineral oil bubbler unless stated otherwise (open flask). Reactions at elevated temperatures were controlled by IKA RET Control Visc (model RS 232 C), room temperature (RT) reactions were conducted at ~23 °C, reactions run cooler than room

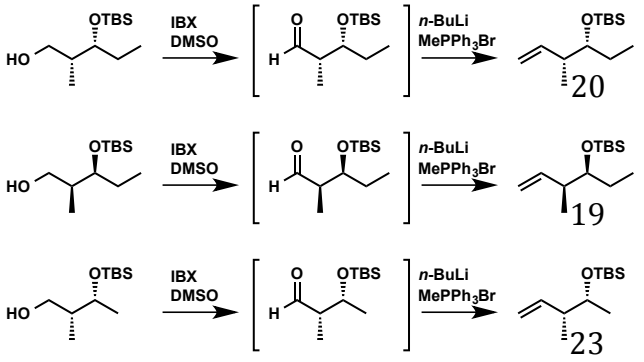
temperature were performed in a cold room (4 °C), an ice bath (0 °C), dry ice/acetone (-78 °C), or isopropanol/ThermoNESLAB (model CC100) for all other temperatures. Commercial purification system MBraun-MB-SPS # 08-113 provided all dry solvents unless stated otherwise (technical grade). Analytical thin-layer chromatography (TLC) was performed with EMD 60 F_{254} pre-coated glass plates (0.25 mm) and visualized using a combination of UV, *p*-anisaldehyde, $KMnO_4$, and Bromocresol green stains. Flash column chromatography was performed using EMD 60 Gerduran® (particle size 0.04-0.063) silica gel. NMR spectra were recorded on a Varian 600 MHz spectrometer. ^1H NMR spectra were recorded relative to residual solvent peak ($CDCl_3$ δ_H 7.26 ppm, D_6-DMSO δ_H 2.50 ppm, D_6-acetone δ_c 2.05 ppm) and reported as follows: chemical shift (ppm), multiplicity, coupling constant (Hz), and integration. Multiplicity abbreviations are as follows: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, h = hextet, ovlp = overlap, br = broad signal. ^{13}C NMR spectra were recorded relative to residual solvent peaks ($CDCl_3$ δ_C 77.0 ppm, D_6-DMSO δ_c 39.5 ppm, D_6-acetone δ_c 29.8 ppm). High resolution mass spectrometry was performed on an Agilent quadrapole time-of-flight spectrometer (Q-TOF 6500 series) by electrospray ionization (ESI).



18 (modified from chapter 2): To a 500 mL flask containing **53** (8.98 g, 44.8 mmol, 1 equiv) in THF (30 mL) at -78 °C was added TMSCl (freshly distilled, Sigma, 22.8 mL, 19.5 g, 179.3 mmol, 4 equiv) down the side of the flask. A second flask was charged with LHMDS solution (Sigma, 1 M in THF, 179.3 mL, 179.3 mmol, 4 equiv) and cooled to -78 °C. LHMDS solution was added dropwise to the **53** solution via by cannula then stirred at -78 °C for 30 min, followed by dropwise addition of acetone (11.71 g, 14.8 mL, 201.6 mmol, 4.5 equiv) with 10 min additional stirring. The solution was allowed to warm to RT and concentrated. The crude solid was suspended in hexanes and filtered through a fritted funnel, the solid was then rinsed 2x with hexanes. The filtrate was poured through a sodium sulfate plug, which was then rinsed 2x with hexanes and concentration gave the crude trimethylsilyl enol ether of **53** (contaminating (isopropenyloxy)trimethylsilane was mostly removed under subsequent high vacuum).

IBX[19] (0.4 M in technical grade DMSO, 224 mL, 89.6 mmol, 2.00 equiv) was added to the crude silyl enol ether and stirred for 12 h (the solution turns yellow and a white precipitate forms) in an open flask. The reaction was diluted with H_2O (2 volumes) and extracted with Et_2O:hexanes (4:1, 3x) Combined organic extracts washed 1x with brine, subsequently filtered through a sodium sulfate plug, which was then rinsed 2x with Et_2O. Concentration and flash chromatography:

EtOAc/Hexanes (10:90) to AcOH/EtOAc/Hexanes (1:10:89) gave **18** as a pale yellow oil (7.55 g, 38.10 mmol, 85% yield). Matched spectral data from chapter 2.



General two-step oxidation/olefination procedure[5d] for **19, 20,** and **23** from Evans aldol products[5c,20]

A flask containing $MePPh_3Br$ (AK, 1.10 equiv) was placed in an oil bath, and heated to 110 °C under high vacuum for 4 h. The flask was cooled to RT and backfilled with N_2, THF (0.2 M) and cooled to 0 °C. *n*-BuLi (Sigma, 1.10 equiv) was added dropwise (solution turns colorless to red and solid $MePPh_3Br$ dissolves completely) and the reaction was allowed to warm to RT and stirred for a minimum 1 h.

To an open flask was added **silyl-ether alcohol** (1 equiv), technical grade DMSO (80 mL, 0.25 M) and IBX (1.50 equiv) in a single portion. The reaction was monitored by TLC, and after consumption of starting material (~4 h) Et_2O was added. The reaction was quenched with a cold solution of sodium thiosulfate, and stirred for 30 min. The aqueous layer was separated and the organic layer was washed 2x with saturated thiosulfate, brine, dried with sodium sulfate, filtered, rinsed 2x with Et_2O and concentrated to give the crude aldehyde, which was dissolved in THF (20 mL) and used immediately.

Both flasks were cooled to -78 °C and the crude aldehyde was added by cannula to the prepared ylide. The solution was stirred at -78 °C for 30 min, warmed to RT and stirred for an additional 30 min. The reaction was quenched with half saturated NH_4Cl and extracted 3x with pentane, filtered through a sodium sulfate plug, rinsed 2x with pentane and carefully concentrated (rotovap bath cooled to 0°C) to give the crude alkene product. Flash chromatography: pentane afforded **19, 20, or 23** as a clear oil.

Note: **19, 20,** and **23** are volatile under high vacuum.

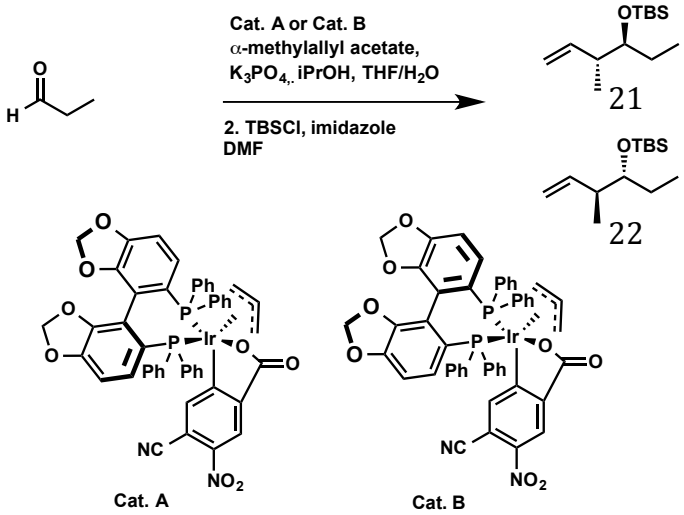
19: 87% at 18 millimole scale

^{1}H NMR ($CDCl_3$, 599 MHz) δ 5.84 (ddd, *J* = 17.5, 10.4, 7.3 Hz, 4H), 5.07 – 4.91 (m, 2H), 3.47 (dd, *J* = 11.0, 5.4 Hz, 4H), 2.31 (h, 6.7Hz, 1H), 1.49 – 1.36 (m, 2H), 0.97 (d, *J* = 6.8 Hz, 3H), 0.90 (s, 9H), 0.86 (t, 7.4 Hz, 3H), 0.04 (s, 6H).

^{13}C NMR ($CDCl_3$, 151 MHz): δ 141.8, 113.2, 77.00, 42.3, 26.5, 25.9, 18.2, 15.0, 9.5, -4.3, -4.4.
EI HRMS: Calculated $[M\text{-}CH_3]^+$ 213.1669, found 213.1683.

20: 88% at 6.5 millimole scale
^{1}H NMR ($CDCl_3$, 599 MHz) δ 5.84 (ddd, *J* = 17.5, 10.4, 7.3 Hz, 1H), 5.07 – 4.91 (m, 2H), 3.46 (q, *J* = 5.5 Hz, 1H), 2.31 (h, *J* = 6.7 Hz, 1H), 1.49-1.36 (m, 2H), 0.97 (d, *J* = 6.8 Hz, 3H), 0.90 (s, 9H), 0.86 (t, *J* = 7.4 Hz, 3H), 0.04 (s, 6H).
^{13}C NMR ($CDCl_3$, 151 MHz): δ 141.8, 113.6, 77.00, 42.3, 26.5, 25.9, 18.2, 15.0, 9.5, -4.3, -4.4.
EI HRMS: Calculated $[M\text{-}CH_3]^+$ 213.1669, found 213.1678.

23: 82% yield at 10 millimole scale.
^{1}H NMR ($CDCl_3$, 599 MHz) δ 5.80 (ddd, *J* = 17.7, 10.5, 7.5 Hz, 1H), 5.03 – 4.95 (m, 2H), 3.63 (p, *J* = 6.1 Hz, 1H), 2.16 (h, *J* = 6.6 Hz, 1H), 1.07 (d, *J* = 6.2 Hz, 3H), 0.98 (d, *J* = 6.8 Hz, 3H), 0.89 (s, 9H), 0.04 (d, *J* = 2.9 Hz, 6H).
^{13}C NMR ($CDCl_3$, 151 MHz): δ 141.6, 113.9, 71.9, 45.5, 25.9, 21.0, 18.1, 15.4, -4.4, -4.8.
EI HRMS: Calculated $[M\text{-}CH_3]^+$ 199.1513, found 199.1526.



General two-step crotylation[6]/silylation procedure for **21** and **22**

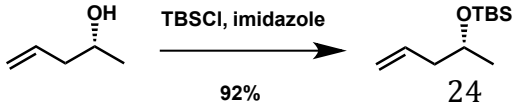
An oven dried pressure tube was charged with catalyst **A or B**[10] (5 mol %), α-methylallyl acetate (TCI, 2 equiv), K_3PO_4 (EMD, 0.5 equiv), iPrOH (EMD, 2 equiv), H_2O (5 equiv), propionaldehyde (Sigma, distilled neat, 1 equiv), THF (2M) under a stream of nitrogen. The tube was sealed and placed in a 60 °C oil bath, stirred for 48 hours. After cooling to RT, 5 volumes of *n*-pentane were added (relative to THF) and the heterogenous mixture was filtered through a plug of sodium sulfate, then rinsed 2x with *n*-pentane. (Crude catalyst was subsequently recovered by rinsing 3x with CH_2Cl_2.)[21] Pentane carefully removed (rotovap bath cooled to 0°C).

To the crude crotylation product was added DMF (0.5M) followed by imidazole (Fisher, 5 equiv) and tertbutyldimethylsilyl chloride (Oakwood, 5 equiv). The solution was warmed to 60 °C and stirred for 12 hours. After cooling to RT, the solution was diluted with H_2O and extracted 2x n-pentane, filtered through a plug of sodium sulfate, then rinsed 2x with n-pentane. Careful concentration (rotovap bath cooled to 0 °C) and flash chromatography: pentane yields **21** or **22** as a clear oil.

Note: **21** and **22** are volatile under high vacuum

21: 64% over two steps at 38 millmole scale (using catalyst **B,** from (*R*)-SEGPHOS).
(**dr** >**20:1***;* only one diasteromer is observed by **^{1}H-NMR**)
(only one diasteromer is observed by **^{1}H-NMR** after cross-metathesis with **S3,** suggesting **er >15:1**)
^{1}H NMR ($CDCl_3$, 599 MHz) δ 5.84 – 5.75 (m, 1H), 5.03 – 4.96 (m, 2H), 3.46 (td, *J* = 6.1, 4.0 Hz, 1H), 2.39 – 2.23 (m, 1H), 1.45 – 1.37 (m, 1H), 1.12 – 0.94 (m, 4H), 0.94 – 0.77 (m, 13H), 0.03 (d, *J* = 15.6 Hz, 6H). 0.98 (d, *J* = 6.9 Hz, 1H), 0.89 (s, 9H), 0.04 (s, 6H).
^{13}C NMR ($CDCl_3$, 151 MHz): δ 141.1, 114.1, 77.2, 42.7, 26.4, 25.9, 18.2, 15.5, 10.1, -4.3, -4.5.
EI HRMS: Calculated $[M\text{-}CH_3]^+$ 213.1669, found 213.1679.

22: 53% over two steps at 19 millimole scale. (Using catalyst **A**, from (*S*)-SEGPHOS)
(**dr** >**20:1***;* only one diasteromer is observed by **^{1}H-NMR**)
(only one diasteromer is observed by **^{1}H-NMR** after cross-metathesis with **S3,** suggesting **er >15:1**)
^{1}H NMR ($CDCl_3$, 599 MHz) δ 5.84 – 5.74 (m, 1H), 5.02 – 4.95 (m, 2H), 3.46 (td, J = 6.0, 4.2 Hz, 1H), 2.43 – 2.26 (m, 1H), 1.43 – 1.37 (m, 1H), 0.98 (d, J = 6.9 Hz, 1H), 0.89 (s, 9H), 0.84 (t, J = 7.5 Hz, 1H), 0.04 (s, 6H).
^{13}C NMR ($CDCl_3$, 151 MHz): δ 141.1, 114.1, 77.2, 42.7, 26.4, 25.9, 18.2, 15.6, 10.1, -4.3, -4.5.
EI HRMS: Calculated $[M\text{-}CH_3]^+$ 213.1669, found 213.1681.



24

A 50-mL flask was charged with (*R*)-4-penten-2-ol (Sigma, 1 g, 11.61 mmol, 1 equiv), DMF (11.6 mL, 1M), followed by imidazole (Fisher, 0.95 g, 13.96 mmol, 1.2 equiv) and tertbutyldimethylsilyl chloride (Oakwood, 4.21 g, 27.94 mmol, 1.1 equiv). The reaction was stirred for 12 h at RT

before the solution was loaded onto a flash column: pentane to yield **24** (2.14 g, 10.67 mmol, 92% yield) as a colorless oil.

Note: **24** is volatile under high vacuum.

^{1}H NMR ($CDCl_3$, 599 MHz) δ 5.81 (ddt, *J* = 17.3, 10.2, 7.2 Hz, 1H), 5..06 – 4.99 (m, 2H), 3.84 (h, *J* = 6.1 Hz, 1H), 2.30 – 2.09 (m, 2H), 1.13 (d, *J* = 6.1 Hz, 3H), 0.89 (s, 9H), 0.05 (s, 3H), 0.05 (s, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 135.6, 116.5, 68.4, 44.3, 25.8, 23.38, 18.14, -4.5, -4.7.

EI HRMS: Calculated $[M\text{-}CH_3]^+$ 185.1356, found 185.1363.

25

S10: Adapted from literature procedure[7]

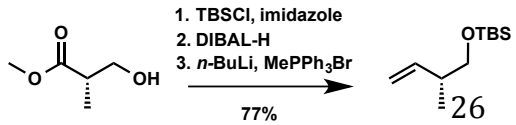
To a 250mL flask was added CuCl (Sigma, flame dried under vacuum, 0.55 g, 5.45 mmol, 20 mol %), vinylmagnesium bromide (Sigma, 1M in THF, 55.5 mL, 55.5 mmol, 2 equiv), then cooled to -10 °C. (*R*)-1,2-epoxybutane (Sigma, 2 g in 8 mL THF, 27.7 mmol, 1 equiv) was added via syringe drive over the course of 1 h. After complete addition, the solution was allowed to warm to 0 °C followed by the addition of solid imidazole (Fisher, 3.96 g, 58.2 mmol, 2.1 equiv) and tertbutyldimethylsilyl chloride (Oakwood, 8.77 g, 58.2 mmol, 2.1 equiv). The flask was fitted with a reflux condenser and heated to 70 °C for 18 h. After cooling to RT the solution was diluted with pentane, washed 2x with H_2O, 1x saturated sodium thiosulfate and filtered through a sodium sulfate plug then rinsed 2x with pentane. Careful concentration (rotovap bath cooled to 0°C) and flash chromatography: pentane yields **25 (**5.16 g, 24.11 mmol, 87% over two steps) as a colorless oil.

Note: **25** is volatile under high vacuum.

^{1}H NMR ($CDCl_3$, 599 MHz) δ 5.81 (ddt, *J* = 17.3, 10.1, 7.2 Hz, 1H), 5.08 – 4.98 (m, 1H), 3.63 (p, *J* = 5.8 Hz, 1H), 2.26 – 2.15 (m, 2H), 1.54 – 1.38 (m, 2H), 1.34 – 0.98 (m, 1H), 0.89 (s, 9H), 0.87 (ovlp t, *J* = 8.0 Hz, 3H), 0.05 (s, 6H).

^{13}C NMR ($CDCl_3$, 151 MHz): δ 135.5, 116.4, 73.1, 41.4, 29.4, 25.9, 18.1, 9.6, -4.5, -4.6.

EI HRMS: Calculated $[M\text{-}CH_3]^+$ 199.1513, found 199.1521.



26

26: An open 250-mL flask was charged with (*S*)-Roche ester (TCI, 3.0 g, 25.40 mmol, 1.00 equiv), imidazole (Fisher, 1.90 g, 27.94 mmol, 1.10 equiv), technical grade CH_2Cl_2, (51 mL, 0.5M) and cooled to 0 °C. Tertbutyldimethylsilyl chloride (Oakwood, 4.21 g, 27.94 mmol, 1.1 equiv) was added in 5 portions. The resulting solution was stirred for 1 h at 0 °C and became cloudy with white precipitate. A half-saturated NH_4Cl solution was added until the precipitate was completely dissolved. The organic layer was separated and the aqueous layer extracted 2x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with CH_2Cl_2. Concentration and subsequent high vacuum yielded colorless oil that was carried onto the next step without further purification.

The following two steps were performed concurrently:

To a 250-mL flask containing $MePPh_3Br$ (AK, 9.98 g, 27.94 mmol, 1.10 equiv) was placed in an oil bath, and heated to 110 °C under high vacuum for 4 h. The flask was cooled to RT and backfilled with N_2, THF (128 mL, 0.2M) and cooled to 0 °C. *n*-BuLi (Sigma, 2.48M, 11.26 mL, 1.10 equiv) was added dropwise (solution turns colorless to red and solid $MePPh_3Br$ dissolves completely) and the reaction was allowed to warm to RT and stirred for a minimum 1 h.

To a 250-mL flask containing crude silyl ether (~25.4 mmol, 1 equiv) was added CH_2Cl_2 (51 mL, 0.5M) and cooled to -78 °C. DIBAL-H (Sigma, 3.79 g, 4.75 mL, 26.67 mmol, 1.05 equiv) was added slowly down the side of the flask and stirred for 1 h at -78 °C. Methanol (30 mL) was added slowly and the solution was stirred for 15 min at -78 °C. The reaction was decanted into of vigorously stirring CH_2Cl_2 (50 mL) at RT, layered with saturated Na/K tartrate (100 mL) and stirred until the layers became clear. The organic layer was separated and the aqueous layer was extracted 2x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was then rinsed 2x with CH_2Cl_2. Concentration and subsequent high vacuum yielded crude aldehyde which was dissolved in THF (10 mL) and used immediately.

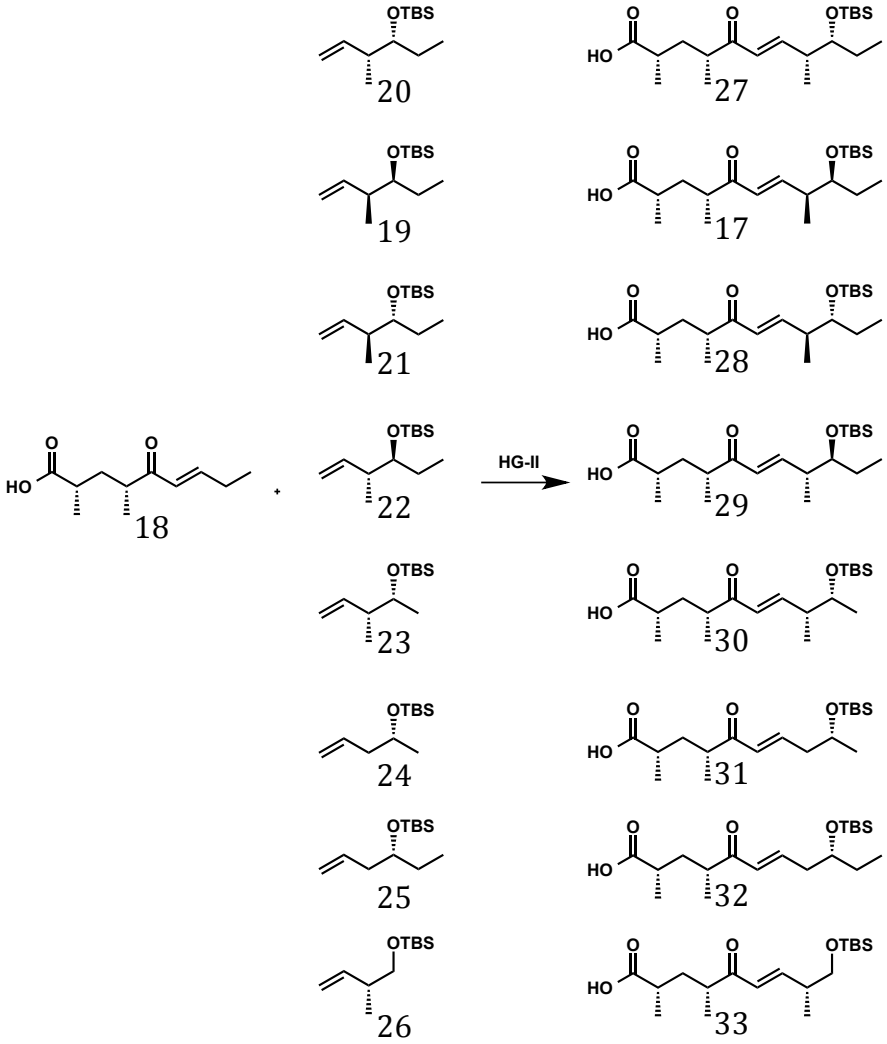
Both flasks were cooled to -78 °C and crude aldehyde was added by cannula to the prepared ylide. The solution was stirred at -78 °C for 30 min, warmed to RT and stirred for an additional 30 min. The reaction was quenched with half saturated NH_4Cl (50 mL) and extracted 3x with pentane, filtered through a sodium sulfate plug, rinsed 2x with pentane and carefully concentrated to give the crude alkene product. Flash chromatography: pentane afforded **26** as a clear oil (3.91 g, 19.51 mmol, 77% over three steps.)

Note: **26** is volatile under high vacuum.

^{1}H NMR ($CDCl_3$, 599 MHz) δ 5.77 (ddd, *J* = 17.3, 10.4, 6.9 Hz, 1H), 5.07 – 4.96 (m, 2H), 3.51 (dd, *J* = 9.8, 6.2 Hz, 1H), 3.41 (dd, *J* = 9.7, 7.0 Hz, 1H), 2.37 – 2.27 (m, 1H), 1.00 (d, *J* = 6.8 Hz, 3H), 0.89 (s, 9H). 0.04 (s, 6H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 141.4, 113.9, 67.9, 40.3, 25.9, 18.3, 16.0, -5.3, -5.4.

EI HRMS: Calculated $[M\text{-}CH_3]^+$ 185.1356, found 185.1366.



17, 27-33: General cross metathesis of **18** and silyl ethers **19-26**

A 10 mL recovery flask was charged with **18** (1 equiv), **silyl-ether** (**19-26**, 1.5 equiv) and Hoveyda-Grubbs 2nd generation (Sigma, 24 mg, 0.04 mmol, 3 mol%) under a stream of N_2. An 18 gauge needle was placed into the septum, venting to the atmosphere (in addition to positive pressure of N_2) and the flask was heated to 60 °C for 12 h. Flash chromatography: AcOH/EtOAc/hexanes (1:10:89) to yield **17-33**. Note: Typically, Hoveyda-Grubbs 2nd generation

catalyst had decomposed completely (TLC) after 12 h, though it remained detectable on occasion. Raising the temperature to 80 °C for an additional 2 h resulted in complete catalyst decomposition.

S27: 81% yield at a 1.26 mmol scale.
^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.94 (dd, *J* = 15.9, 7.5 Hz, 1H), 6.14 (dd, *J* = 15.9, 1.1 Hz, 1H), 3.59-3.53 (m, 1H), 2.87 (h, *J* = 6.9 Hz, 1H), 2.58-2.44 (m, 2H), 2.17-2.07 (m, 1H), 1.52-1.44 (m, 1H), 1.43-1.34 (m, 2H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.12 (d, *J* = 6.9 Hz, 3H), 1.03 (d, *J* = 6.8 Hz, 3H), 0.88 (s, 9H), 0.86 (ovlp t, *J* = 7.0Hz, 3H), 0.04 (s, 3H), 0.03 (s, 3H).
^{13}C NMR ($CDCl_3$, 151 MHz) δ 203.0, 181.5, 150.8, 127.9, 76.4, 41.5, 41.3, 37.0, 36.2, 26.8, 25.8, 21.3, 18.1, 17.5, 16.7, 14.2, 9.6, -4.4, -4.5.
HRMS: Calculated $[M+H]^+$ 371.2612, found 371.2619.

17: 78% yield at a 1.26 millimole scale.
^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.94 (dd, J = 15.9, 7.4 Hz, 1H), 6.14 (dd, J = 15.9, 1.0 Hz, 1H), 3.59-3.53 (m, 1H), 2.87 (h, *J* = 6.9 Hz, 1H), 2.58-2.44 (m, 2H), 2.17 – 2.08 (m, 2H), 1.52-1.44 (m, 1H), 1.19 (d, *J* = 7.0 Hz, 3H), 1.12 (d, *J* = 6.9 Hz, 3H), 1.03 (d, *J* = 6.8 Hz, 3H), 0.88 (s, 9H), 0.86 (ovlp t, *J* = 7.0Hz, 3H), 0.04 (s, 3H), 0.03 (s, 3H).
^{13}C NMR ($CDCl_3$, 151 MHz) δ 202.9, 181.8, 150.8, 127.9, 76.4, 41.6, 41.3, 37.0, 36.2, 26.8, 25.8, 18.1, 17.5, 16.7, 14.3, 9.6, -4.4, -4.5.
HRMS: Calculated $[M+H]^+$ 371.2612, found 371.2614.

28: 77% yield at a 1.26 millimole scale.
^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.90 (dd, *J* = 15.9, 7.9 Hz, 1H), 6.14 (d, *J* = 15.9 Hz, 1H), 3.56 – 3.52 (m, *J* = 1H), 2.12 (h, *J* = 7.2 Hz, 1H), 2.56 – 2.43 (m, 2 H), 2.12 (ddd, 14.4, 7.8, 6 Hz, 1H), 1.50 – 1.33 (m, 2H), 1.19 (d, *J* = 7.0 Hz, 3H), 1.12 (d, *J* = 6.9 Hz, 3H), 1.05 (d, *J* = 6.8 Hz, 3H), 0.88 (s, 9H), 0.85 (t, *J* = 7.4 Hz, 3H), 0.04 (s, 3H), 0.03 (s, 3H).
^{13}C NMR ($CDCl_3$, 151 MHz) δ 203.0, 182.1, 150.2, 128.3, 76.6, 41.5, 41.2, 37.9, 36.2, 27.1, 25.9, 25.8, 18.1, 17.4, 16.7, 15.4, 9.6, -4.3, -4.6.
HRMS: Calculated $[M+H]^+$ 371.2612, found 371.2612.

29: 82% yield at a 1.26 millimole scale.
^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.91 (dd, *J* = 16.0, 7.9 Hz, 1H), 6.14 (d, *J* = 15.9 Hz, 1H), 3.58 - 3.51 (m, 1H), 2.87 (h, *J* = 7.2 Hz, 1H), 2.58 – 2.44 (m, 2H), 2.13 (ddd, 14.4, 7.8, 6 Hz, 1H), 1.52 – 1.35 (m, 2H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.13 (d, *J* = 7.0 Hz, 3H), 1.06 (d, *J* = 6.8 Hz, 3H), 0.89 (s, 9H), 0.86 (t, *J* = 7.4 Hz, 3H), 0.05 (s, 3H), 0.04 (s, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 203.0, 181.7, 150.2, 128.3, 76.6, 41.5, 41.2, 36.9, 36.2, 27.1, 25.8, 18.1, 17.5, 16.7, 15.4, 9.6, -4.3, -4.6.

HRMS: Calculated $[M+H]^+$ 371.2612, found 371.2609.

30: 66% yield at a 1.26 millimole scale.

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.91 (dd, *J* = 15.9, 7.7 Hz, 1H), 6.14 (d, *J* = 15.9 Hz, 1H), 3.76 (p, *J* = 6.0 Hz, 1H), 2.87 (h, *J* = 7.2 Hz, 1H), 2.53 (h, *J* = 7.8 Hz, 1H), 2.35 (h, *J* = 6.6 Hz, 1H), 2.12 (ddd, *J* = 14.3, 8.2, 6.5 Hz, 1H), 1.41 (ddd, *J* = 13.8, 7.2, 6.5 Hz, 1H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.12 (d, *J* = 6.9 Hz, 3H), 1.08 (d, *J* = 6.2 Hz, 3H), 1.04 (d, *J* = 6.8 Hz, 3H), 0.88 (s, 9H), 0.04 (s, *J* = 3H), 0.03 (s, *J* = 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) 203.9, 182.0, 150.4, 128.2, 71.3, 44.5, 41.2, 37.0, 36.3, 25.8, 21.0, 18.0, 17.4, 16.6, 14.8, -4.4, -4.9.

31: 77% yield at a 1.26 millimole scale.

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.90 (dt, *J* = 15.2, 7.4 Hz, 1H), 6.18 (d, *J* = 15.7 Hz, 1H), 3.95 (h, *J* = 15.7 Hz, 1H), 2.85 (h, *J* = 7.2 Hz, 1H), 2.57 - 2.49 (m, 1H), 2.38 – 2.30 (m, 1H), 2.12 (ddd, *J* = 14.4, 7.8, 6 Hz, 1H), 1.41 (ddd, *J* = 13.9, 7.6, 6.2 Hz, 11H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.16 (d, *J* = 6.1 Hz, 3H), 1.12 (d, *J* = 6.9 Hz, 3H), 0.87 (s, 9H), 0.05 (s, 3H), 0.04 (s, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 202.7, 181.9, 144.7, 130.4, 77.19, 67.6, 42.7, 41.3, 37.0, 36.2, 25.8, 23.8, 18.0, 17.5, 16.6, -4.5, -4.8.

HRMS: Calculated $[M+H]^+$ 357.2456, found 357.2457.

32: 77% yield at a 1.26 millimole scale.

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.91 (dt, *J* = 15.3, 7.5 Hz, 1H), 6.18 (dt, *J* = 15.8, 1.2 Hz, 1H), 3.73 (p, *J* = 5.8 Hz, 1H), 2.85 (h, *J* = 7.8 Hz, 1H), 2.59 – 2.45 (m, 1H), 2.41 (m, 2H), 2.12 (ddd, *J* = 14.4, 7.4, 5.3 Hz, 4H), 1.51 – 1.44 (m, 1H), 1.41 (ddd, *J* = 13.9, 7.7, 6.2 Hz, 1H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.12 (d, *J* = 6.9 Hz, 3H), 0.88 (ovlp t, *J* = 7.4 Hz, 3H), 0.88 (s, 9H), 0.04 (s, 3H), 0.03 (s, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 202.6, 181.9, 144.5, 130.4, 72.4, 41.3, 40.0, 37.0, 36.2, 29.9, 25.8, 18.0, 17.5, 16.6, 9.5, -4.5, -4.5.

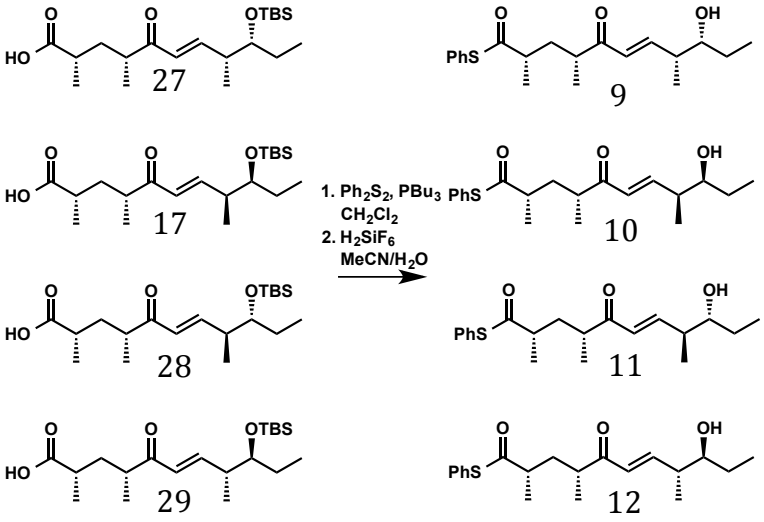
HRMS: Calculated $[M+H]^+$ 357.2456, found 357.2452.

33: 51% yield at a 1.26 millimole scale.

^{1}H NMR ($CDCl_3$, 599 MHz) δ 6.86 (dd, *J* = 15.9, 7.3 Hz, 1H), 6.18 (dd, *J* = 15.9, 1.2 Hz, 1H), 3.54 (d, *J* = 6.4 Hz, 1H), 2.86 (h, *J* = 7.2 Hz, 1H), 2.60 – 2.47 (m, 1H), 2.12 (ddd, *J* = 13.8, 7.8, 6 Hz, 1H), 1.41 (ddd, *J* = 13.9, 7.7, 6.1 Hz, 1H), 1.20 (d, *J* = 7.0 Hz, 3H), 1.12 (d, *J* = 6.9 Hz, 3H), 1.06 (d, *J* = 6.8 Hz, 3H), 0.88 (s, 9H), 0.04 (s, 3H), 0.03 (s, 3H).

^{13}C NMR ($CDCl_3$, 151 MHz) δ 203.0, 182.2, 150.2, 128.00, 66.9, 41.4, 39.4, 37.0, 36.2, 25.8, 18.2, 17.5, 16.6, 15.6, -5.4, -5.4

HRMS: Calculated $[M+H]^+$ 343.2299, found 343.2301.



9-12: General thioesterification and TBS deprotection of **17, 27-29.**

A round bottom flask was charged with seco-acid (**17, 27-29**, 1 equiv), Ph_2S_2 (Sigma, 1.1 equiv), and CH_2Cl_2 (0.1 M) and cooled to - 78 °C. PBu_3 (Sigma, distilled neat, 1.3 equiv) was added dropwise and the reaction was stirred for 45 min at - 78 °C before being removed from the cooling bath and immediate quench with a saturated aq. $CuSO_4$ solution and warming to RT. The organic layer was separated, and the aq. layer was extracted further with CH_2Cl_2 (2x), CH_2Cl_2 layers combined and filtered through a sodium sulfate plug, rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography (silica topped with 1:1 SiO_2:$CuSO_4$): EtOAc/Hexanes (2:98 to 4:96) afforded crude thioesters as a clear oils, which were used immediately in the subsequent step.

An open polyethylene bottle was charged with crude thioester, MeCN (1 M) and cooled to 0 °C. H_2SiF_6 (Fisher, 25% in H_2O, 0.8 equiv) was added and the reaction was stirred at 0 °C until complete by TLC. The reaction was monitored by TLC and upon completion it was diluted with CH_2Cl_2 (10 mL) and carefully quenched with saturated sodium bicarbonate. The aqueous layer was extracted 2x with CH_2Cl_2. Filtration through a sodium sulfate plug then rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography: EtOAc/hexanes (15:85) gave **17, 27-29** as colorless oils.

9: 90% at a 4 millimole scale.

^{1}H-NMR (599 MHz; d_6-acetone): δ 7.46-7.43 (m, 5H), 6.93 (dd, *J* = 15.9, 8.0 Hz, 1H), 6.22 (dd, *J* = 16.0, 0.8 Hz, 1H), 3.68 (d, *J* = 5.7 Hz, 1H), 3.46-3.42 (m, 1H), 2.98 (h, *J* = 6.9 Hz, 1H), 2.84 (h, *J* = 7.1 Hz, 1H), 2.45-2.40 (m, 1H), 2.18-2.14 (m, 1H), 1.50 (dqd, *J* = 14.1, 7.2, 3.6 Hz, 1H), 1.43 (dt, *J* = 13.7, 6.8 Hz, 1H), 1.39-1.31 (m, 1H), 1.22 (d, *J* = 6.9 Hz, 3H), 1.13 (d, *J* = 6.9 Hz, 3H), 1.09 (d, *J* = 6.8 Hz, 3H), 0.93 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (151 MHz; d_6-acetone): δ 202.4, 200.7, 151.1, 135.3, 130.07, 129.95, 128.89, 128.79, 75.9, 46.8, 43.6, 41.6, 37.7, 28.3, 18.3, 17.2, 14.9, 10.7

HRMS: Calculated $[M+H]^+$ 349.1832, found 349.1837.

10: 91% at a 1.92 millimole scale.

^{1}H-NMR (599 MHz; d_6-acetone): δ 7.46-7.43 (m, 5H), 6.93 (dd, *J* = 15.9, 8.0 Hz, 1H), 6.22 (dd, *J* = 15.9, 1.0 Hz, 1H), 3.68 (d, *J* = 5.7 Hz, 1H), 3.45 (dtt, *J* = 11.5, 5.8, 2.9 Hz, 1H), 2.98 (h, *J* = 6.9 Hz, 1H), 2.84 (h, *J* = 6.9 Hz, 1H), 2.45-2.40 (m, 1H), 2.16 (dt, *J* = 14.0, 7.1 Hz, 1H), 1.51 (dqd, *J* = 14.1, 7.2, 3.6 Hz, 1H), 1.43 (dt, *J* = 13.7, 6.9 Hz, 1H), 1.39-1.31 (m, 1H), 1.22 (d, *J* = 6.9 Hz, 3H), 1.13 (d, *J* = 6.9 Hz, 3H), 1.09 (d, *J* = 6.8 Hz, 3H), 0.93 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (151 MHz; d_6-acetone): δ 202.4, 200.8, 151.1, 135.3, 130.09, 129.98, 128.92, 128.81, 76.0, 46.8, 43.7, 41.6, 37.7, 28.4, 18.4, 17.2, 14.9, 10.7.

HRMS: Calculated $[M+H]^+$ 349.1832, found 349.1835.

11: 77% at a 1.62 millimole scale.

^{1}H-NMR (599 MHz; d_6-acetone): δ 7.43-7.39 (m, 5H), 6.93 (dd, *J* = 15.9, 8.3 Hz, 1H), 6.17 (dd, *J* = 15.9, 0.9 Hz, 1H), 3.63 (d, *J* = 5.5 Hz, 1H), 3.42 (dq, *J* = 8.9, 4.5 Hz, 1H), 2.95 (hex, *J* = 6.9 Hz, 1H), 2.80 (dq, *J* = 14.3, 7.0 Hz, 1H), 2.43-2.38 (m, 1H), 2.13 (dt, *J* = 14.0, 7.1 Hz, 1H), 2.01 (dt, *J* = 4.4, 2.2 Hz, 1H), 1.47-1.32 (m, 1H), 1.19 (d, *J* = 6.9 Hz, 3H), 1.09 (d, *J* = 6.9 Hz, 3H), 1.07 (d, *J* = 6.9 Hz, 3H), 0.89 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (151 MHz; d_6-acetone): δ 202.3, 200.8, 150.3, 135.3, 130.1, 123.0, 129.6, 128.8, 76.3, 46.8, 43.3, 41.5, 37.7, 28.6, 18.3, 17.3, 16.7, 10.6

HRMS: Calculated $[M+H]^+$ 349.1832, found 349.1829.

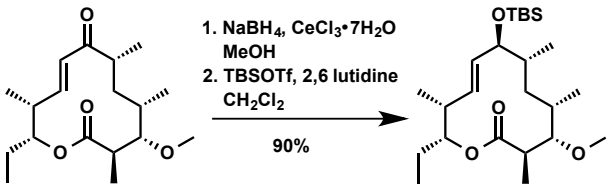
12: 78% at a 1.76 millimole scale.

^{1}H-NMR (599 MHz; d_6-acetone): δ 7.46-7.44 (m, 5H), 6.97 (dd, *J* = 15.9, 8.4 Hz, 1H), 6.20 (dd, *J* = 16.0, 0.9 Hz, 1H), 3.70-3.69 (m, 1H), 3.48-3.44 (m, 1H), 3.00 (h, *J* = 6.9 Hz, 1H), 2.87-2.81 (m,

1H), 2.47-2.41 (m, 1H), 2.16 (ddd, *J* = 14.0, 7.6, 6.6 Hz, 1H), 1.50-1.35 (m, 3H), 1.22 (d, *J* = 6.9 Hz, 3H), 1.13 (d, *J* = 6.9 Hz, 3H), 1.11 (d, *J* = 6.9 Hz, 3H), 0.93 (t, *J* = 7.4 Hz, 3H).

13C NMR (151 MHz; d_6-acetone): δ 202.4, 200.7, 150.3, 135.3, 135.3, 130.1, 129.0, 129.9, 129.6, 128.8, 76.3, 46.8, 43.4, 41.4, 37.8, 28.6, 18.3, 17.2, 16.7, 10.6.

HRMS: Calculated $[M+H]^+$ 349.1832, found 349.1851.



40 41

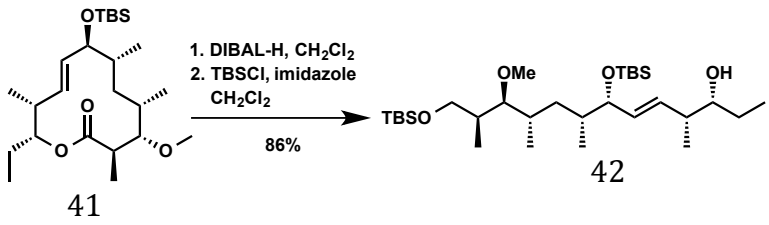
41: An open 100-mL flask was charged with **40** (1.18 g, 3.6 mmol, 1.00 equiv), $CeCl_3$•$7H_2O$ (Fisher, 1.342 g, 3.6 mmol, 1.0 equiv), technical grade MeOH (36 mL, 0.1 M), stirred until dissolved and cooled to -78 °C. $NaBH_4$ (Fisher, 0.14 g, 3.6 mmol, 1 equiv). The resulting solution was stirred for 10 min at -78 °C and decanted into aq. HCl (1 M), the organic layer was separated and the aqueous layer extracted 2x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with CH_2Cl_2. Concentration and subsequent high vacuum yielded colorless oil that was carried onto the next step without further purification.

A 100-mL flask was charged with the crude allylic-alcohol, CH_2Cl_2 (36 mL, 0.1 M) and cooled to -78 °C. 2,6-lutidine (Sigma, 0.50 g, 0.54 mL, 4.68 mmol, 1.3 equiv) was added followed by dropwise addition of TBSOTf (Sigma, 1.14 g, 0.99 mL, 4.32 mmol, 1.2 equiv) and the resulting solution was stirred -78 °C for 1 h before aq. NH_4Cl (sat.) quench. The organic layer was separated and the aqueous layer extracted 2x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with CH_2Cl_2. Flash chromatography: EtOAc/hexanes (5:95) gave **41** as a colorless oil (1.38 g, 3.234 mmol, 90%)

^{1}H-NMR (599 MHz; $CDCl_3$): δ 5.69-5.66 (m, 1H), 5.39 (dt, *J* = 15.4, 2.3 Hz, 1H), 5.00 (ddd, *J* = 8.8, 5.4, 3.2 Hz, 1H), 4.06 (s, 1H), 3.49 (s, 3H), 3.12 (dd, *J* = 10.3, 1.7 Hz, 1H), 2.61-2.55 (m, 1H), 2.51-2.48 (m, 1H), 2.02-1.96 (m, 1H), 1.89-1.85 (m, 1H), 1.68-1.63 (m, 1H), 1.57-1.51 (m, 1H), 1.35-1.30 (m, 1H), 1.25 (d, *J* = 6.8 Hz, 3H), 1.04 (ovlp m, 6H), 1.01-0.96 (m, 1H), 0.93-0.90 (m, 15H), 0.03 (s, 3H), -0.01 (s, 3H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 175.2, 131.3, 127.5, 89.1, 77.3, 75.5, 62.7, 43.5, 37.6, 35.7, 32.63, 32.59, 26.0, 24.6, 20.5, 18.3, 17.7, 16.3, 10.34, 10.22, -4.9, -5.2.

HRMS: Calculated $[M+Na]^+$ 449.3058, found 449.3056.



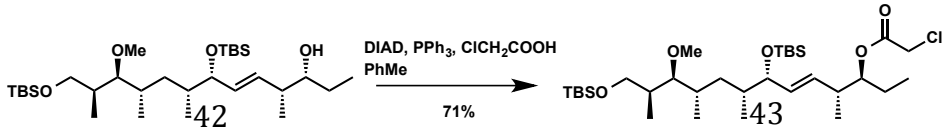
42: An 100-mL flask was charged with **41** (1.48 g, 3.45 mmol, 1.00 equiv), CH_2Cl_2 (35 mL, 0.1 M) and cooled to -78 °C. DIBAL-H (2.16 g, 15.21 g, 4.4 equiv) was added dropwise, and the solution was stirred at -78 °C for 5 min, warmed to 0 °C for ~ 1 min, and then recooled to -78 °C. The reaction was partially quenched by dropwise addition of MeOH (5 mL) followed by an additional 15 min at -78 °C, followed by aq. Na/K tartrate (sat.) and warming to RT. The biphasic solution was stirred until the layers became clear. The organic layer was separated and the aqueous layer extracted 2x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with CH_2Cl_2. Concentration and subsequent high vacuum yielded a colorless oil that was carried onto the next step without further purification.

An open 100-mL flask was charged with the crude diol and CH_2Cl_2 (7 mL, 0.5 M), and cooled to 0 °C. Imidazole (Fisher, 0.28 g, 4.15 mmol, 1.2 equiv) was added followed by TBSCl (Oakwood, 0.57 g, 3.79 mmol, 1.1 equiv), and the resulting solution was stirred for 1 h at 0 °C before aq. NH_4Cl (sat.) quench. The organic layer was separated and the aqueous layer extracted 2x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with CH_2Cl_2. Flash chromatography: EtOAc/hexanes (5:95) gave **42** as a colorless oil (1.69 g, 3.1 mmol, 90%)

^{1}H-NMR (599 MHz; $CDCl_3$): δ 5.45 (t, *J* = 2.8 Hz, 2H), 3.86 (d, *J* = 4.6 Hz, 1H), 3.50 (t, *J* = 9.0 Hz, 1H), 3.41 (dd, *J* = 9.6, 6.0 Hz, 1H), 3.37 (s, 3H), 3.00 (dd, *J* = 7.7, 2.2 Hz, 1H), 2.25 (dd, *J* = 11.2, 6.4 Hz, 1H), 2.00 (s, 1H), 1.83 (dt, *J* = 13.1, 6.8 Hz, 2H), 1.72 (dt, *J* = 13.0, 6.2 Hz, 1H), 1.61 (dt, *J* = 12.2, 6.1 Hz, 1H), 1.52 (dtd, *J* = 14.2, 7.2, 3.8 Hz, 1H), 1.37 (dt, *J* = 14.8, 7.5 Hz, 1H), 1.00 (d, *J* = 6.9 Hz, 3H), 0.96 (t, *J* = 7.4 Hz, 3H), 0.89 (t, *J* = 7.6 Hz, 21H), 0.82 (d, *J* = 6.7 Hz, 3H), 0.78 (d, *J* = 6.9 Hz, 3H), 0.02 (d, *J* = 27.6 Hz, 12H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 133.4, 132.9, 85.1, 77.8, 65.9, 60.3, 42.1, 37.40, 37.3, 36.9, 33.6, 26.9, 25.9, 18.2, 18.2, 17.5, 16.5, 14.4, 10.52, 10.36, -3.9, -4.8, -5.3, -5.41.

HRMS: Calculated $[M-(HOTBS)+H]^+$ 413.3445, found 413.3440.

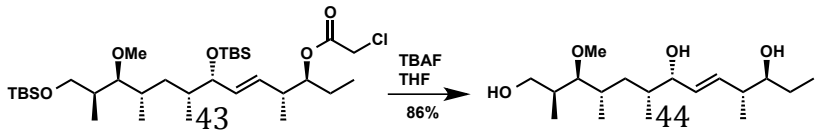


43: A 100-mL round bottom flask was charged with **42** (0.93 g, 1.70 mmol, 1 equiv), PPh_3 (AK, 2.23 g, 8.5 mmol, 5 equiv), chloroactic acid (Sigma, 0.80 g, 8.5 mmol, 5 equiv), PhMe (17 mL, 0.1 M), and then cooled to 0 °C. DIAD (Sigma, 1.78 g, 1.67 mL, 8.5 mmol, 5 equiv) was added dropwise over 10 min, and the resulting solution was stirred at 0 °C for 10 min, RT for 12 h. The solution was concentrated and purified by flash chromatography: Et_2O/hexanes (1:99 to 4:96) gave **43** as a colorless oil (0.75 g, 1.207 mmol, 71%)

^{1}H-NMR (599 MHz; $CDCl_3$): δ 5.49 (dd, *J* = 15.5, 6.6 Hz, 1H), 5.41 (dd, *J* = 15.5, 8.2 Hz, 1H), 4.84 (q, *J* = 5.6 Hz, 1H), 4.04 (s, 2H), 3.95 (dd, *J* = 6.5, 3.5 Hz, 1H), 3.51 (t, *J* = 8.8 Hz, 1H), 3.42 (dd, *J* = 9.6, 6.0 Hz, 1H), 3.37 (s, 3H), 3.03 (dd, *J* = 7.0, 2.7 Hz, 1H), 2.45-2.42 (m, 1H), 1.93-1.87 (m, 1H), 1.86-1.80 (m, 1H), 1.73-1.68 (m, 1H), 1.68-1.62 (m, 1H), 1.59 (dq, *J* = 14.0, 6.8 Hz, 2H), 1.01 (d, *J* = 6.9 Hz, 3H), 0.89 (d, *J* = 7.7 Hz, 18H), 0.87-0.84 (m, 6H), 0.81 (d, *J* = 6.9 Hz, 3H), 0.04 (s, 6H), 0.03 (s, 3H), -0.02 (s, 3H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 167.1, 133.9, 130.6, 84.8, 80.9, 76.5, 66.2, 60.1, 41.0, 39.9, 37.4, 36.6, 33.0, 25.95, 25.91, 24.6, 18.25, 18.17, 16.80, 16.73, 16.1, 10.8, 9.7, -3.9, -4.8, -5.33, -5.40.

HRMS: Calculated $[M+Na]^+$ 643.3951, found 643.3949.

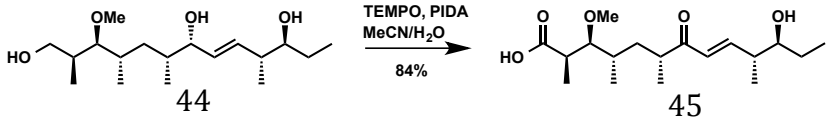


44: A 100-mL round bottom flask was charged with **43** (1.16 g, 1.86 mmol, 1 equiv), THF (9 mL, 0.05 M), and cooled to 0 °C. TBAF (Sigma, 1 M in THF, 27.92 mL, 15 equiv) was added dropwise over 30 min, the resulting solution was warmed to RT and stirred for 48 h. The reaction was mostly complete after 48 h (TLC), and quenched with $CaCO_3$ (5.6 g, 55.8 mmol, 30 equiv) and H_2O/EtoAC with 5 min additional stirring. The organic layer was separated and the aqueous layer extracted 2x with EtoAc. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with EtOAC. Flash chromatography: acetone/hexanes (30:70) gave **44** as a colorless oil (0.51 g, 1.61 mmol, 86%).

^{1}H-NMR (599 MHz; d_6-DMSO): δ 5.49 (dd, *J* = 15.5, 7.9 Hz, 1H), 5.37 (dd, *J* = 15.6, 6.2 Hz, 1H), 4.42 (t, *J* = 5.1 Hz, 1H), 4.34 (d, *J* = 4.6 Hz, 1H), 4.24 (d, *J* = 5.3 Hz, 1H), 3.78 (q, *J* = 4.9 Hz, 1H), 3.34-3.31 (m, 1H), 3.30 (s, 3H), 3.26-3.19 (m, 2H), 2.96 (dd, *J* = 6.2, 3.4 Hz, 1H), 2.16 (ttd, *J* = 6.7, 6.3, 5.9 Hz, 1H), 1.81 (dp, *J* = 13.4, 6.7 Hz, 1H), 1.74-1.63 (m, 2H), 1.58-1.51 (m, 1H), 1.36-1.21 (m, 2H), 0.95 (d, *J* = 6.9 Hz, 3H), 0.84 (t, *J* = 7.4 Hz, 3H), 0.82-0.80 (ovlp m, 6H), 0.77 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (151 MHz; d_6-DMSO): δ 132.7, 131.7, 84.8, 75.1, 73.6, 64.3, 59.6, 41.5, 37.2, 36.3, 36.1, 32.5, 26.7, 17.0, 16.6, 15.8, 11.5, 10.5

HRMS: Calculated $[M+Na]^+$ 339.2506, found 339.2508.



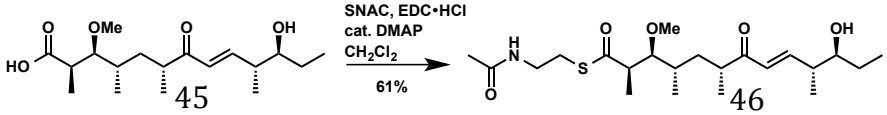
44 45

44: A 9-dram round bottom flask was charged with **44** (0.44 g, 1.4 mmol, 1 equiv), MeCN/H_2O (14 mL, 0.1 M) and cooled to 4 °C. TEMPO (Sigma, 0.22 g, 1.4 mmol, 1 equiv) and PIDA (AK scientific, 1.8 g, 5.6 mmol, 4.00 equiv) were added in single portions and the resulting solution was stirred vigorously and 4 °C. The reaction was monitored by ^{1}H NMR spectroscopy after 12 h (small aliquot added to excess MeOH, concentrated, and dissolved in $CDCl_3$) for loss of the intermediate aldehyde. After 16 h, the reaction was decanted into MeOH and concentrated. Flash chromatography: EtOAc/hexanes (50:50) to AcOH/EtOAc/hexanes (1:50:49) gave **45** as a colorless oil (0.39 g, 1.19 mmol, 84%).

^{1}H-NMR (599 MHz; $CDCl_3$): δ 6.97 (dd, *J* = 15.9, 7.6 Hz, 1H), 6.23 (d, *J* = 15.9 Hz, 1H), 3.46 (dt, *J* = 8.6, 4.4 Hz, 1H), 3.42 (s, 3H), 3.28 (dd, *J* = 7.1, 4.5 Hz, 1H), 2.87-2.81 (m, 1H), 2.57 (p, *J* = 7.0 Hz, 1H), 2.42 (h, *J* = 6.4 Hz, 1H), 1.93 (ddd, *J* = 13.7, 9.4, 4.1 Hz, 1H), 1.87-1.70 (m, 3H), 1.59-1.53 (m, 3H), 1.45 (dp, *J* = 14.6, 7.3 Hz, 1H), 1.17 (d, *J* = 6.9 Hz, 3H), 1.11-1.09 (m, 6H), 0.97-0.95 (m, 6H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 203.9, 178.9, 148.2, 128.2, 86.9, 76.9, 61.0, 42.5, 42.0, 41.6, 35.1, 34.3, 27.8, 17.8, 17.0, 16.0, 12.9, 10.1.

HRMS: Calculated $[M+Na]^+$ 351.2142, found 351.2140.



45 46

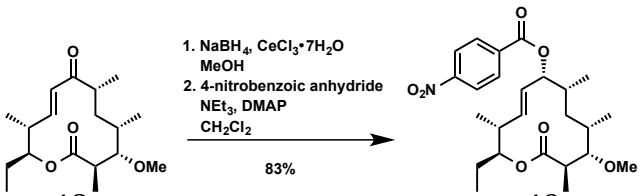
46: A 50 mL round bottom flask was charged with **45** (0.42 g, 1.27 mmol, 1 equiv), EDC•HCl (Chem-impex, 0.36 g, 1.90 mmol, 1.5 equiv), HOBT (Sigma, 0.21 g, 1.52 mmol, 1.5 equiv), and the flask was cooled to 0 °C. DMF (13 mL, 0.1 M) was added and the resulting solution was stirred at 0 °C for 30 min, followed by HSNAC (0.18 g, 1.52 mmol, 1.2 equiv), stirred at 0 °C for an additional 10 min before DMAP addition (cat., ~1 mg). The solution was stirred at at 0 °C for an additional 10 min before warming to RT and 24 h additional stirring. The reaction was diluted with EtOAc, washed with H_2O. The organic layer was separated and the aqueous layer extracted 2x with EtoAc. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with EtOAC. Flash chromatography [column topped with (SiO_2:$CuSO_4$)]: EtOAc (**46**) as a colorless oil (0.32 g, 0.77 mmol, 61%).

^{1}H-NMR (599 MHz; d_6-acetone): δ 7.34 (s, 1H), 6.96 (dd, *J* = 15.9, 8.4 Hz, 1H), 6.22 (dd, *J* = 15.9, 0.8 Hz, 1H), 3.77 (d, *J* = 5.3 Hz, 1H), 3.46 (dq, *J* = 8.6, 4.3 Hz, 1H), 3.36 (s, 3H), 3.35-3.26 (m, 3H), 3.06-2.92 (m, 3H), 2.97-2.83 (m, 2H), 2.47-2.41 (m, 1H), 1.91 (ddd, *J* = 13.6, 9.6, 4.0 Hz,

1H), 1.85 (s, 3H), 1.63-1.57 (m, 1H), 1.49-1.35 (m, 2H), 1.17 (d, *J* = 6.9 Hz, 3H), 1.20-1.15 (ovlp m, 1H), 1.11 (d, *J* = 6.9 Hz, 3H), 1.07 (d, *J* = 6.9 Hz, 3H), 0.95-0.92 (ovlp m, 6H).

^{13}C NMR (151 MHz; d_6-acetone): δ 203.9, 202.4, 170.0, 150.1, 130.0, 87.5, 76.3, 61.0, 51.8, 43.4, 41.6, 39.5, 36.1, 35.0, 29.2, 28.7, 22.8, 18.7, 17.3, 16.8, 13.7, 10.6

HRMS: Calculated $[M+H]^+$ 430.2622, 430.2628.



48 49

49: A 4 dram vial was charged with **48** (0.022 g, 0.071 mmol, 1 equiv), $CeCl_3 \bullet 7H_2O$ (0.026 g, 0.071 mmol, 1 equiv), and MeOH (0.7 mL, 0.1 M). The resulting solution was stirred at RT until the solids dissolved, and subsequently cooled to -78 °C. $NaBH_4$ (0.003 g, 0.071 mmol, 1 equiv) was added in a single portion. The reaction was stirred at -78 °C for 20 min before the solution was decanted into aq. HCl (1 M) and CH_2Cl_2. The organic layer was separated and the aqueous layer extracted 2x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with CH_2Cl_2 and concentrated. The crude allylic alcohol was used in the subsequent step without further purification.

A 4 dram vial was charged with the crude allylic alcohol (0.071 mmol, 1 equiv), CH_2Cl_2 (0.7 mL, 0.1 M) and cooled to 0 °C. NEt_3 (0.009 g, 0.013 mL, 0.092 mmol, 1.3 equiv), 4-nitrobenzoic anhydride (0.027 g, 0.085 mL, 0.085 mmol, 1.2 equiv), and DMAP (~1 mg, cat.) were added sequentially and the solution was stirred at 0 °C for 20 min, then quenched with aq. sodium bicarbonate (sat.). The organic layer was separated and the aqueous layer extracted 2x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography: EtOAc/hexanes (5:95) gave **49** as a colorless solid (0.027 g, 0.059 mmol, 83%).

^{1}H-NMR (599 MHz; $CDCl_3$): δ 8.33 (d, *J* = 8.8 Hz, 2H), 8.25 (d, *J* = 8.8 Hz, 2H), 5.57 (s, 1H), 5.43 (dd, *J* = 15.5, 2.9 Hz, 1H), 5.29-5.25 (m, 1H), 4.62-4.58 (m, 1H), 3.41 (s, 3H), 3.05 (dd, *J* = 9.6, 1.2 Hz, 1H), 2.77-2.71 (m, 1H), 2.44-2.38 (m, 1H), 2.06 (dt, *J* = 13.8, 7.1 Hz, 1H), 1.76-1.70 (m, 3H), 1.61-1.55 (m, 2H), 1.30 (d, *J* = 6.9 Hz, 3H), 1.08 (d, *J* = 7.0 Hz, 3H), 1.04 (d, *J* = 6.8 Hz, 3H), 0.94 (d, *J* = 6.7 Hz, 3H), 0.90 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 174.2, 164.0, 150.6, 135.5, 131.9, 130.8, 128.3, 123.7, 85.4, 78.7, 77.6, 59.7, 43.6, 41.4, 34.8, 34.3, 31.1, 24.6, 19.0, 17.0, 16.51, 16.40, 9.1

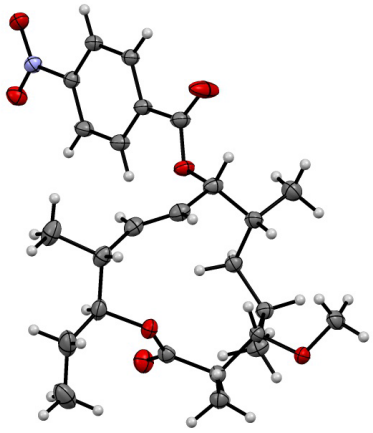
HRMS: Calculated $[M+Na]^+$ 484.2306, 484.2306.

Structure Determination.

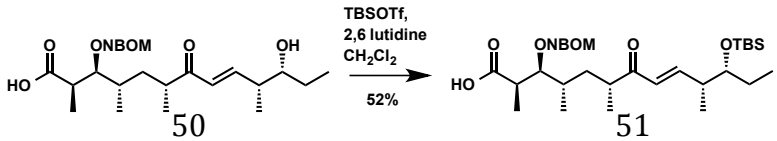
Colorless needles of **49** were grown from a hexanes solution of the compound at -30 deg. C. A crystal of dimensions 0.26 x 0.01 x 0.01 mm was mounted on a Rigaku AFC10K Saturn 944+ CCD-based X-ray diffractometer equipped with a low temperature device and Micromax-007HF Cu-target micro-focus rotating anode (λ = 1.54187 A) operated at 1.2 kW power (40 kV, 30 mA). The X-ray intensities were measured at 85(1) K with the detector placed at a distance 42.00 mm from the crystal. A total of 2633 images were collected with an oscillation width of 1.0 ° in ω. The exposure times were 15 sec. for the low angle images, 75 sec. for high angle. The integration of the data yielded a total of 98528 reflections to a maximum 2θ value of 136.48° of which 9383 were independent and 8176 were greater than 2σ(I). The final cell constants (Table 1) were based on the xyz centroids 44319 reflections above 10σ(I). Analysis of the data showed negligible decay during data collection; the data were processed with CrystalClear 2.0 and corrected for absorption. The structure was solved and refined with the Bruker SHELXTL (version 2008/4) software package, using the space group P2(1)2(1)2(1) with Z = 4 for the formula C25H35NO7. There are two crystallographically independent molecules per asymmetric unit. All non-hydrogen atoms were refined anisotropically with the hydrogen atoms placed in idealized positions. Full matrix least-squares refinement based on F2 converged at R1 = 0.0518 and wR2 = 0.1275 [based on I > 2sigma(I)], R1 = 0.0591 and wR2 = 0.1328 for all data. Additional details are presented in Table 1 and are given as Supporting Information in a CIF file. Acknowledgement is made for funding from NSF grant CHE-0840456 for X-ray instrumentation.

Sheldrick, G.M. SHELXTL, v. 2008/4; Bruker Analytical X-ray, Madison, WI, 2008.

CrystalClear Expert 2.0 r12, Rigaku Americas and Rigaku Corporation (2011), Rigaku Americas, 9009, TX, USA 77381-5209, Rigaku Tokyo, 196-8666, Japan.



49

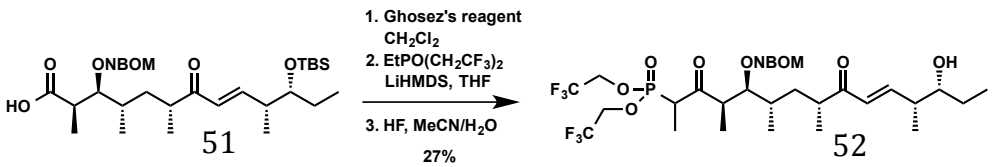


51: A 50 mL round bottom flask was charged with **50** (0.69 g, 1.45 mmol, 1 equiv), CH_2Cl_2 (15 mL, 0.1 M) and cooled to -78 °C. 2,6-lutidine (Sigma, 0.40 g, 3.77 mmol, 2.6 equiv) was added followed by dropwise addition of TBSOTf(Oakwood, 0.92 g, 3.48 mmol, 2.4 equiv) and additional stirring for 1 h at -78 °C. The reaction was quenched with aq. NH_4Cl (sat),and decanted into aq. HCl (1 M). The organic layer was separated and the aqueous layer extracted 2x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with CH_2Cl_2 and concentrated. The silyl-ester survived this work-up and was subsequently hydrolyzed in MeOH (15 mL, 0.1 M) at 0 °C with K_2CO_3 (0.22 g, 1.59 mmol, 1.1 equiv). After 45 min, the reaction was diluted with EtOAc and washed with aq. HCl (1 M). The organic layer was separated and the aqueous layer extracted 2x with EtoAc. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with EtOAC. AcOH/EtOAc/hexanes (1:10:89) gave **51** as a colorless oil (0.45 g, 0.75 mmol, 52%). Note: rotamers were observed in $CDCl_3$. Denoted in parentheses in the **^{13}C**.

^{1}H-NMR (599 MHz; $CDCl_3$): δ 8.07-8.06 (m, 1H), 7.79-7.78 (m, 1H), 7.64-7.61 (m, 1H), 7.44-7.41 (m, 1H), 6.93 (ddd, *J* = 15.9, 7.2, 3.0 Hz, 1H), 6.11 (dd, *J* = 16.0, 1.2 Hz, 1H), 5.04-4.98 (m, 2H), 4.84 (qd, *J* = 8.0, 2.8 Hz, 2H), 3.77 (t, *J* = 5.2 Hz, 1H), 3.57-3.54 (m, 1H), 2.93-2.90 (m, 1H), 2.73-2.68 (m, 1H), 2.49-2.46 (m, 1H), 2.03-1.98 (m, *J* = 3.3 Hz, 1H), 1.64-1.63 (m, 1H), 1.50-1.43 (m, 1H), 1.34 (dp, *J* = 14.1, 7.1 Hz, 1H), 1.18-1.16 (m, 3H), 1.12 (dt, *J* = 9.3, 4.6 Hz, 1H), 1.07-1.05 (m, 3H), 1.01 (d, *J* = 6.8 Hz, 3H), 0.92-0.84 (m, 15H), 0.04 (dd, *J* = 6.9, 1.8 Hz, 6H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 203.9, (150.26, 150.23), 147.1, 134.8, 133.6, (128.7, 128.36), 128.34, 127.9, 124.7, 96.6, 83.9, (76.65, 76.61), 67.0, 41.62, (41.53, 41.48), (40.98, 40.95), (35.94, 35.90), 34.5, (26.62, 26.59), 25.9, (18.27, 18.24), 18.13, 16.5, 14.3, 9.7, -4.36, -4.51.

HRMS: Calculated $[M+Na]^+$ 616.3276, found 616.3275.



52: A 25 mL flask was charged with **51** (0.050 g, 0.084 mmol, 1 equiv) and CH_2Cl_2 (0.8 mL, 0.1 M). Ghosez's reagent (Acros, 0.025 g, 0.025 mL, 0.185 mmol, 2.2 equiv) was added dropwise and the resulting solution was stirred for 90 min at RT. The solution was concentrated and placed under high vacuum.

Concurrently, a second 25 mL flask was charged with Bis(2,2,2-trifluoroethyl) ethylphosphonate (0.076 g, 0.278 mmol, 3.3 equiv) and THF (0.8 mL) and cooled to - 98 °C (a deep bath of MeOH with temperature maintained by careful addition of liquid N_2.) LHMDS (Sigma, 1 M in THF, 0.28 mL, 3.3 equiv) was added dropwise down the side of the flask and this solution was stirred at - 98 °C for 10 min. The acid chloride generated from **51** was dissolved in THF (0.4 mL, 0.2 M) and transferred dropwise to the lithiated phosphonate solution via cannula, down the side of the flask. The transfer was quantitated with additional THF (0.4 mL, 0.2 M) and subsequent dropwise cannula. The resulting solution was stirred at - 98 °C for 2 h and quenched with aq. NH_4Cl (sat.) The organic layer was separated and the aqueous layer extracted 2x with CH_2Cl_2. The combined organic extracts were washed with brine and filtered through a sodium sulfate plug, which was subsequently rinsed 2x with CH_2Cl_2. Flash chromatography: EtOAc/hexanes (10:90) provided a crude silyl-ether that was deprotected immediately in the following step.

To an open 1.5 mL epi-tube was added crude silyl-ether and MeCN (0.16 mL, 0.5 M) and aq. HF (48%, 0.1 mL). The reaction was monitored by TLC and upon completion it was diluted with CH_2Cl_2 (10 mL) and carefully quenched with saturated sodium bicarbonate. The aqueous layer was extracted 2x with CH_2Cl_2. Filtration through a sodium sulfate plug then rinsed 2x with CH_2Cl_2 and concentrated. Flash chromatography: EtOAc/hexanes (30:70) gave **52** (0.017 g, 0.023 mmol, 27%). Note: the epimeric center formed α to the phosphonate resulted in a complex ^{1}H NMR spectrum, where the annotation is apparent and unselected for a single diasteromer. Assumed epimeric carbons are denoted in parentheses in the **^{13}C**.

^{1}H-NMR (599 MHz; d_6-acetone): δ ^{1}H-NMR (599 MHz; aceton-d_6): δ 8.10-8.07 (m, 1H), 7.88-7.82 (m, 1H), 7.79-2.75 (m, 1H), 7.60-7.57 (m, 1H), 6.95-6.89 (m, *J* = 8.0 Hz, 1H), 6.21-6.18 (m, *J* = 0.9 Hz, 1H), 5.10-4.92 (m, 2H), 4.92-4.80 (m, 2H), 4.70-4.60 (m, 4H), 4.07-3.93 (m, 1H), 3.82 (t, *J* = 4.8 Hz, 0.5H), 3.72-3.66 (ovlp m, *J* = 6.6 Hz, 1.5H), 3.46-3.34 (m, 1.5H), 3.24-3.20 (m, 0.5H), 3.07-2.97 (m, *J* = 6.8, 3.8 Hz, 1H), 2.80-2.77 (m, 1H), 2.44-2.36 (m, 1H), 2.03-1.98 (m, 1H), 1.59-1.40 (m, 4H), 1.37-1.27 (m, 5H), 1.20-1.11 (m, 4H), 1.09-1.01 (m, 6H), 0.99-0.87 (m, 6H).

^{13}C NMR (151 MHz; d_6-acetone): (209.32, 209.28), (207.27, 207.25), (206.2, 205.9), (204.06, 203.95), (151.2, 150.9), (135.4, 135.1), (134.49, 134.40), (129.90, 129.73), (129.55, 129.47), (129.22, 129.09), (125.31, 125.29), (97.46, 97.37), (85.1, 83.3), (75.93, 75.80), (67.55, 67.46), (63.1, 62.87, 62.83, 62.77, 62.73), 50.1, (48.91, 48.89), (46.4, 46.1), (45.5, 45.2), (43.66, 43.63), (43.58, 43.56), (41.5, 41.3), (36.34, 36.27), (35.81, 35.71), (28.36, 28.31), (19.2, 18.9), (17.36, 17.17), (14.89, 14.73), 12.9, (12.30, 12.26), 12.12, (11.27, 11.22), 10.6

HRMS: Calculated [M+Na^+] 758.2499, found 758.2500.

4.5 Enzymatic Experimental

Protein Preparation

All H_2O was obtained from a Millipore Milli-Q system (serial P3MNO3809A) using Millipore Q-Gard 2/Quantum Ex Ultrapure organex cartridges. LB broth Miller was obtained from EMD and autoclaved before use. Glycerol was obtained from EMD, HEPES was obtained from Calbiochem (Omnipur grade), Isopropyl-b-D-thiogalactopyranoside (IPTG) was obtained from Gold Biotechnology. Kanamycin Sulfate (Kan) was obtained from Amresco. ACS grade imidazole and NaCl were obtained from Fisher Scientific. pH was determined on a Symphony SB70P pH meter (serial SN005695) calibrated according to manufacturer's specifications. Ni-NTA agarose was purchased from Qiagen and pre-equilibrated with five column volumes of lysis buffer. PD-10 columns were purchased from GE and pre-equilibrated with five column volumes of storage buffer. Cells were lysed using a model 705 Sonic Dismembrator purchased from Fisher Scientific. Optical density (OD_{600}) was determined using an Eppendorf Biophotometer.

Bap1[22] cells bearing plasmids for expression of respective PKS modules were taken from glycerol cell stocks stored at - 80°C and grown in LB (10mL) with Kan (50 mg/L), and grown overnight at 37°C. The following morning, LB (1L) containing Kan (50 mg/L) was inoculated with the entire overnight culture, and shaken at 37°C until they reached an OD_{600} of 0.6-0.7 at which point they were removed and allowed to cool to RT, then to 20 °C. When an OD_{600} of 0.8 was reached, the cultures were induced with IPTG (300μM) and shaken at 180RPM at 20 °C for 18 hours. Cells were pelleted at 5000g (4 °C) for 10 minutes.

PKS Crude Cell Lysate Preparation

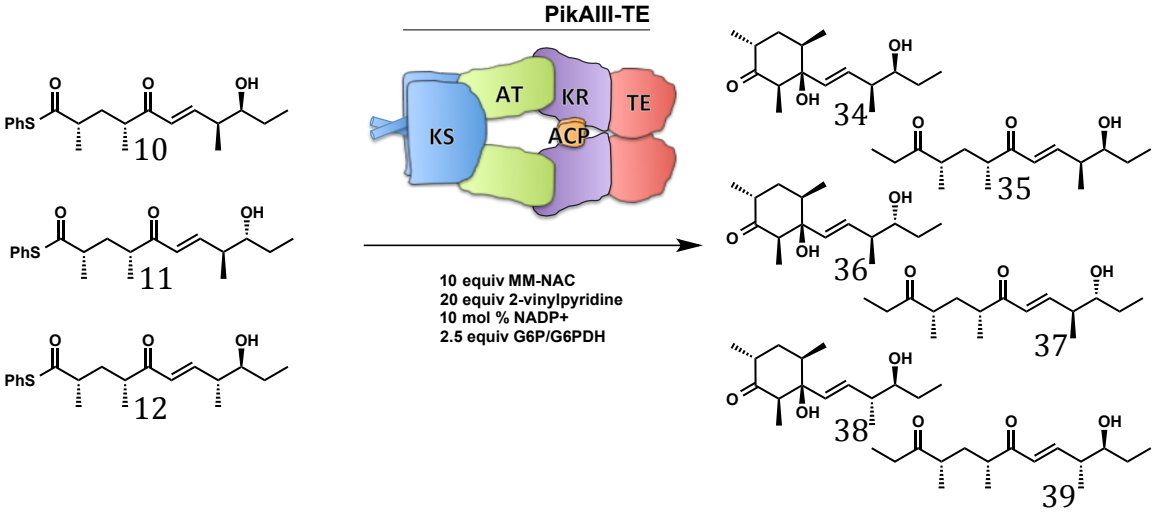
Frozen cells were resuspended in 100 mL of storage buffer [HEPES (50 mM), NaCl (150 mM), EDTA (1 mM), glycerol (20% v/v), pH 7.2] per 20 grams of pelleted culture broth via vortex. Cells were lysed by addition of 1 mg/ml lysozyme immediately before sonication in a brine/ice at 70% power 100 x 5s with 15s rest periods. Cellular debris was pelleted in a precooled (4 °C) centrifuge at 65,000g for 10 min. Crude cell lysate was either used immediately or flash frozen in N_2 and thawed on ice without discernible loss in activity. Protein concentration was crudely normalized to that of purified protein though densitometry, and used without further manipulation.

Pik TE Purification

The following steps were conducted in <2 hours for maximum and reproducible enzymatic activity. Cells were suspended in 5 mL of lysis buffer [HEPES (50 mM), NaCl (300 mM),

imidazole (10mM), glycerol (10% v/v), pH 8.0] per 20 grams of pelleted culture broth via vortex. Cells were lysed by addition of 1 mg/ml lysozyme immediately before sonication in a brine/ice at 70% power 100 x 5s with 15s rest periods. Cellular debris was pelleted in a precooled (4 °C) centrifuge at 65,000 g for 10 min. Cellular debris was pelleted in a precooled (4°C) centrifuge at 40,000 g for 10 min, and the supernatant was applied to 4 mL of Ni-NTA resin and allowed to drip through. 15 mL of wash buffer [HEPES (50 mM), NaCl (300 mM), imidazole (30mM), glycerol (10% v/v), pH 8.0] was added, the column was gently pressurized with a syringe, and the enzyme of interest was eluted with 15 mL of elution buffer [HEPES (50 mM), NaCl (300 mM), imidazole (300mM), glycerol (10% v/v), pH 8.0] with gentle syringe pressure. Protein containing fractions were determined via Bradford assay and pooled. Buffer exchange was performed using a PD-10 column, and protein containing fractions were determined via Bradford assay and pooled, aliquoted, flash frozen in liquid N_2, and stored at -80 °C.

Incubation of **10-12** with PikAIII-TE



Reaction conditions: sodium phosphate buffer (50 mM, 2.5% v/v glycerol, 50 mL total, pH = 7.2), pentaketide **10-12** (70 mg, 0.2 mmol, 4 mM) MM-SNAC (10 equiv, 40 mM), $NADP^+$ (0.1 equiv, 0.4 mM), glucose-6-phosphate (2.5 equiv, 10 mM), glucose-6-phosphate dehydrogenase (2 units/mL), 2-vinylpyridine (20 mM), cell free PikAIII-TE (Crude cell estimated conc. ~15 µM, 4 µM in reaction, 0.1 mol %), 8 hours, stationary, RT.

Workup and purification: Quenched with acetone (2x volume, 100mL), placed in a -20 °C freezer for 1 h and filtered through a celite plug. Remaining insoluble material was suspended in acetone and this solution was used to rinse the celite plug. Acetone was removed through rotary evaporation and the aqueous layer was saturated with NaCl and extracted 3x EtOAc. Combined organic layers were washed with brine and filtered through a sodium sulfate plug was performed

then rinsed 2x with EtOAc and concentrated. Flash chromatography: acetone/hexanes (8:92) afforded compounds **34-39**.

34 from pentaketide **10** (2.5 mg, 0.009 mmol, 4% yield)
^{1}H-NMR (599 MHz; $CDCl_3$): δ 5.54 (dd, *J* = 15.7, 8.0 Hz, 1H), 5.39 (d, *J* = 15.7 Hz, 1H), 3.41 (ddd, *J* = 8.7, 5.3, 3.6 Hz, 1H), 2.51 (dq, *J* = 19.0, 6.4 Hz, 2H), 2.34 (h, *J* = 6.8 Hz, 1H), 2.09-2.03 (m, 1H), 1.91 (ddd, *J* = 13.3, 5.7, 4.1 Hz, 1H), 1.60-1.53 (m, 3H), 1.42-1.33 (m, 3H), 1.05 (d, *J* = 6.8 Hz, 3H), 1.03 (d, *J* = 6.4 Hz, 3H), 0.97 (t, *J* = 7.4 Hz, 3H), 0.94 (d, *J* = 6.8 Hz, 3H), 0.87 (d, *J* = 6.8 Hz, 3H).
^{13}C NMR (151 MHz; $CDCl_3$): δ 211.9, 135.0, 131.7, 81.5, 76.7, 52.1, 44.6, 42.2, 39.8, 39.6, 27.2, 15.6, 15.0, 14.4, 10.2, 7.9.
HRMS: Calculated $[M+Na]^+$ 291.1931, found 291.1931.

35 from pentaketide **10** (2.5 mg, 0.009 mmol, 4% yield)
^{1}H-NMR (599 MHz; $CDCl_3$): δ 6.87 (dd, *J* = 15.9, 8.1 Hz, 1H), 6.18 (dd, *J* = 15.9, 0.8 Hz, 1H), 3.57-3.52 (m, 1H), 2.77 (h, *J* = 6.9 Hz, 1H), 2.60 (dq, *J* = 14.0, 7.0 Hz, 1H), 2.55-2.42 (m, 3H), 2.15-2.10 (m, 1H), 1.90 (s, 1H), 1.55 (dqd, *J* = 14.3, 7.2, 3.9 Hz, 1H), 1.47-1.39 (m, 1H), 1.24 (dt, *J* = 13.8, 6.7 Hz, 2H), 1.10 (d, *J* = 6.8 Hz, 3H), 1.08 (d, *J* = 6.9 Hz, 3H), 1.07-1.03 (ovlp m, 6H), 0.98 (t, *J* = 7.4 Hz, 3H).
^{13}C NMR (151 MHz; $CDCl_3$): δ 215.0, 203.2, 149.9, 128.4, 75.9, 43.7, 42.5, 41.4, 36.1, 34.5, 27.3, 17.2, 16.7, 13.9, 10.4, 7.7
HRMS: Calculated $[M+Na]^+$ 291.1931, found 291.1932.

36 from pentaketide **11** (2 mg, 0.007 mmol, 3.5% yield)
^{1}H-NMR (599 MHz; $CDCl_3$): δ 5.54 (dd, *J* = 15.7, 8.5 Hz, 1H), 5.42 (d, *J* = 15.7 Hz, 1H), 3.37 (dt, *J* = 7.9, 4.2 Hz, 1H), 2.55-2.48 (m, 2H), 2.30 (dq, *J* = 14.2, 7.0 Hz, 1H), 2.06 (dqd, *J* = 12.7, 6.5, 4.1 Hz, 1H), 1.91 (ddd, *J* = 13.3, 5.8, 4.0 Hz, 1H), 1.60-1.53 (m, 2H), 1.44-1.37 (m, 2H), 1.06 (d, *J* = 6.9 Hz, 3H), 1.03 (d, *J* = 6.4 Hz, 3H), 0.97 (t, *J* = 7.4 Hz, 3H), 0.94 (d, *J* = 6.8 Hz, 3H), 0.89 (d, *J* = 6.8 Hz, 3H).
^{13}C NMR (151 MHz; $CDCl_3$): δ 211.8, 136.1, 130.8, 81.5, 76.5, 52.0, 44.6, 42.4, 39.67, 39.52, 27.4, 17.3, 15.1, 14.4, 10.0, 7.9.
HRMS: Calculated $[M+Na]^+$ 291.1931, found 291.1930.

37 from pentaketide **11** (2 mg, 0.007 mmol, 3.5% yield)
^{1}H-NMR (599 MHz; $CDCl_3$): δ 6.83 (dd, *J* = 15.9, 8.7 Hz, 1H), 6.16 (d, *J* = 15.9 Hz, 1H), 3.48 (dt, *J* = 8.5, 4.4 Hz, 1H), 2.77 (h, *J* = 6.9 Hz, 1H), 2.62 (dq, *J* = 13.9, 7.0 Hz, 1H), 2.56-2.50 (m, *J* = 17.8, 7.3 Hz, 1H), 2.47-2.40 (m, 2H), 2.15-2.10 (m, 1H), 1.62-1.53 (m, 1H), 1.46 (tt, *J* = 14.7, 7.4

Hz, 1H), 1.25-1.20 (m, 2H), 1.12 (d, *J* = 6.8 Hz, 3H), 1.08 (d, *J* = 6.9 Hz, 3H), 1.06-1.03 (m, 6H), 0.98 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 215.2, 203.2, 149.1, 129.3, 76.3, 43.8, 42.7, 41.3, 36.3, 34.6, 27.7, 17.4, 16.6, 16.4, 10.1, 7.8.

HRMS: Calculated $[M+Na]^+$ 291.1931, found 291.1930.

38 from pentaketide **12** (1 mg, 0.004 mmol, 2% yield)

^{1}H-NMR (599 MHz; $CDCl_3$): δ 5.54 (dd, *J* = 15.7, 8.5 Hz, 1H), 5.41 (d, *J* = 15.7 Hz, 1H), 3.37 (dt, *J* = 8.2, 4.3 Hz, 1H), 2.54-2.48 (m, 2H), 2.33-2.27 (m, 1H), 2.04 (dqd, *J* = 12.6, 6.4, 4.1 Hz, 1H), 1.91 (ddd, *J* = 13.3, 5.9, 4.0 Hz, 1H), 1.59-1.51 (m, 3H), 1.43-1.37 (m, 2H), 1.06 (d, *J* = 6.9 Hz, 3H), 1.02 (d, *J* = 6.4 Hz, 3H), 0.97-0.95 (ovlp m, 6H), 0.87 (d, *J* = 6.8 Hz, 3H).

^{13}C NMR (151 MHz; CDCl3): δ 211.9, 136.0, 130.8, 81.5, 76.6, 52.1, 44.6, 42.3, 39.67, 39.61, 27.5, 17.3, 14.9, 14.4, 10.1, 8.1.

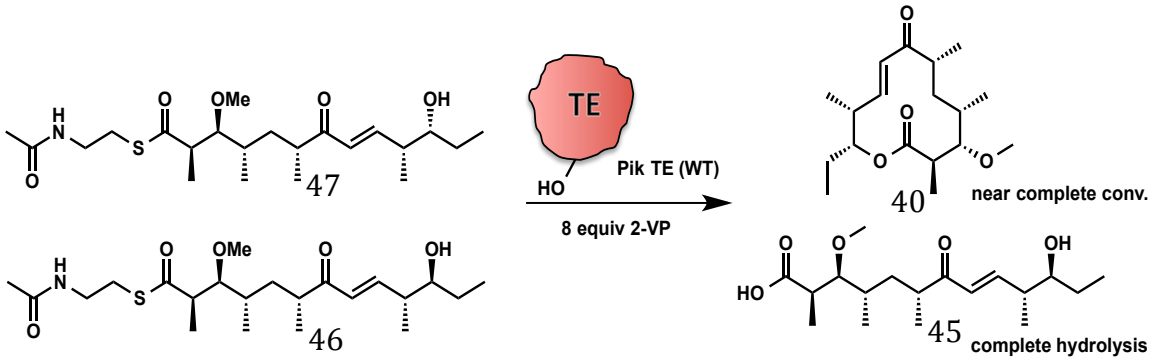
HRMS: Calculated $[M+Na]^+$ 291.1931, found 291.1931.

39 from pentaketide **12** (1 mg, 0.004 mmol, 2% yield)

^{1}H-NMR (599 MHz; $CDCl_3$): δ 6.91 (dd, *J* = 15.9, 8.0 Hz, 1H), 6.18 (d, *J* = 15.9 Hz, 1H), 3.48-3.47 (m, 1H), 2.80-2.75 (m, 1H), 2.60-2.37 (ovlp m, 4H), 2.13-2.09 (m, 1H), 1.84-1.80 (m, 1H), 1.59-1.52 (m, 1H), 1.47-1.40 (m, 1H), 1.26-1.22 (m, 1H), 1.11 (d, *J* = 6.9 Hz, 3H), 1.07 (d, *J* = 6.9 Hz, 3H), 1.06-1.02 (m, 6H), 0.97 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (151 MHz; $CDCl_3$): δ 214.9, 203.2, 149.0, 129.1, 76.3, 43.7, 42.3, 41.4, 36.0, 34.3, 27.6, 17.08, 16.94, 15.9, 10.0, 7.8.

HRMS: Calculated $[M+Na]^+$ 291.1931, found 291.1932.

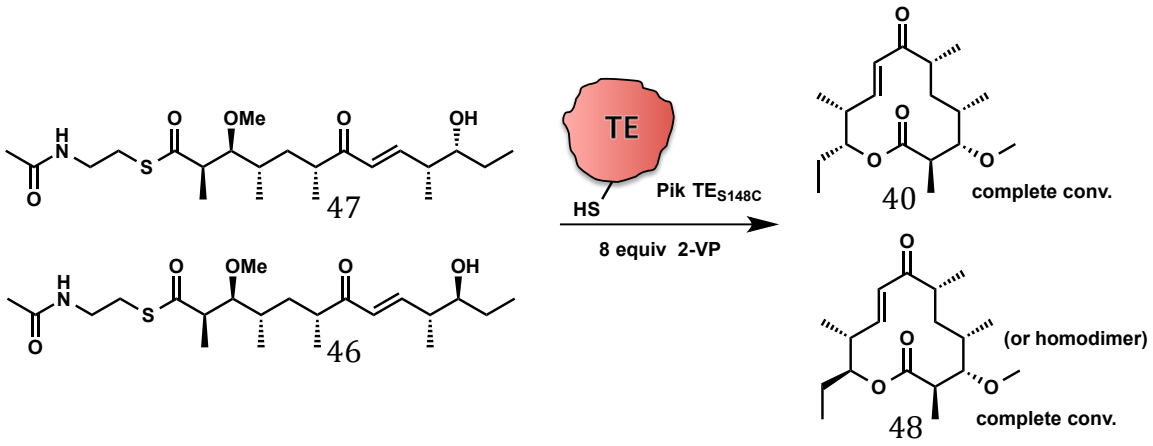


Reaction conditions: sodium phosphate buffer (400 mM, 4 mL total, pH = 7.2), hexaketide **46 or 47** (1.7 mg, 0.004 mmol, 1mM), 2-vinylpyridine (8 mM), purified Pik TE (10 μM in reaction, 1 mol %), 18 hours, stationary, RT.

Workup and analysis: After 18 h, reactions were directly extracted 3x EtOAc. Combined organic layers were washed with brine and filtered through a sodium sulfate plug was performed then

rinsed 2x with EtOAc and concentrated. Crude reaction mixtures were analyzed by ^{1}**H** NMR in d_6-acetone and correlated to known compounds **40** and **45** (chapter 3)

40: From hexaketide **47, 40** was the major product, with some **47** detectable but below reliable integration. Estimated >95% conversion.
45: From hexaketide **46**, **45** was the only detectable product.



Reaction conditions: sodium phosphate buffer (400 mM, 4 mL total, pH = 7.2), hexaketide **46 or 47** (1.7 mg, 0.004 mmol, 1mM), 2-vinylpyridine (8 mM), purified Pik TE (10 μM in reaction, 1 mol %), 18 hours, stationary, RT.

Workup and analysis: After 18 h, reactions were directly extracted 3x EtOAc. Combined organic layers were washed with brine and filtered through a sodium sulfate plug was performed then rinsed 2x with EtOAc and concentrated. Crude reaction mixtures were analyzed by ^{1}**H** NMR in d_6-acetone and correlated to known compound **40** (chapter 3)

40: From hexaketide **47, 40** was the only detectable product.
45: From hexaketide **46**, **48** was the only detectable product.

As there is no authentic standard to confirm the structure of **48**, a scaled up reaction (100 mL) under otherwise identical conditions provided **48** which was purified via flash chromatography: EtOAc/hexanes (10/90) afforded compound **48** (0.028 g, 0.09 mmol, 90%).

^{1}H-NMR (599 MHz; $CDCl_3$): δ 6.59 (dd, *J* = 16.3, 8.0 Hz, 1H), 6.06 (d, *J* = 16.3 Hz, 1H), 4.48 (ddd, *J* = 9.6, 7.1, 5.0 Hz, 1H), 3.45 (s, 3H), 3.18 (dd, *J* = 8.9, 1.8 Hz, 1H), 2.77-2.65 (m, 3H), 2.08-2.02 (m, 1H), 1.80-1.75 (m, 2H), 1.62 (ddd, *J* = 14.3, 8.1, 5.9 Hz, 1H), 1.26 (d, *J* = 7.0 Hz, 3H), 1.16-1.12 (ovlp m, 1H), 1.13 (d, *J* = 6.7 Hz, 3H), 1.09 (d, *J* = 6.7 Hz, 3H), 1.07 (d, *J* = 6.9 Hz, 3H), 0.93 (t, *J* = 7.4 Hz, 3H).

^{13}C-NMR (151 MHz; $CDCl_3$): δ 204.6, 173.5, 146.8, 128.8, 86.2, 80.8, 60.2, 42.8, 42.4, 39.7, 36.6, 34.1, 25.0, 17.4, 16.2, 15.60, 15.45, 9.5

HRMS: Calculated $[M+H]^+$ 311.2217, found 311.2216.

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