Chemical and Biochemical Interrogation of Molecular Specificity in Modular Polyketide Synthases

by

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Table of Contents

List of Figures

List of Schemes

ABSTRACT

Chemical and Biochemical Interrogation of Molecular Specificity in Modular Polyketide Synthases

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Polyketide natural products represent a diverse set of chemical entities that are prized by organic and medicinal chemists for their complex molecular architectures and unique pharmacological properties. These natural products are generated as secondary metabolites in a variety of marine and terrestrial microbial sources through the decarboxylative condensation of coenzyme A (CoA) esters of simple malonic acids. These condensation reactions occur on large, modular enzyme complexes called polyketide synthases (PKSs). Two prime examples of these unique multifunctional enzyme systems are the 6-deoxyerythronolide B synthase (DEBS) and pikromycin (Pik) PKS, which are responsible for the biosynthesis of the erythromycin and pikromycin aglycones, respectively. Together, these natural product biosynthetic systems provide excellent platforms to examine the fundamental structural and catalytic elements that govern polyketide assembly, processing and macrocyclization.

Due to the modular architecture of bacterial type I PKSs, rational bioengineering of these enzymes has opened up an avenue toward the rapid generation of polyketide chemical diversity through chemoenzymatic synthesis and combinatorial biosynthesis. Realization of this goal, however, requires a detailed understanding of molecular specificity in the catalytic domains of these PKS enzymes. Toward this goal, this dissertation describes the development of synthetic methodologies to access late-stage polyketide chain-elongation intermediates from the DEBS and Pik systems and their subsequent employment in biochemical studies with engineered PKS modules. Specifically, the native pentaketide intermediate for the DEBS system was synthesized and employed for *in vitro* chemoenzymatic synthesis of macrolactone products in the final engineered monomodules Ery5, Ery5-TE and Ery6 as well as bimodular DEBS3. A comparative analysis was performed with the corresponding Pik module 5 (PikAIII) and module 6 (PikAIV), utilizing native chain Pik chain-elongation intermediates, and dissecting key similarities and differences between these highly related PKSs. The data revealed that individual modules in the DEBS and Pik PKSs possess distinctive molecular selectivity profiles, and suggest that substrate recognition has evolved unique characteristics in each system. Additional work has been put forth to establish a general methodology to access DEBS and Pik penta- and hexaketide analogues that encompass a number of stereochemical, structural and functional group variations.

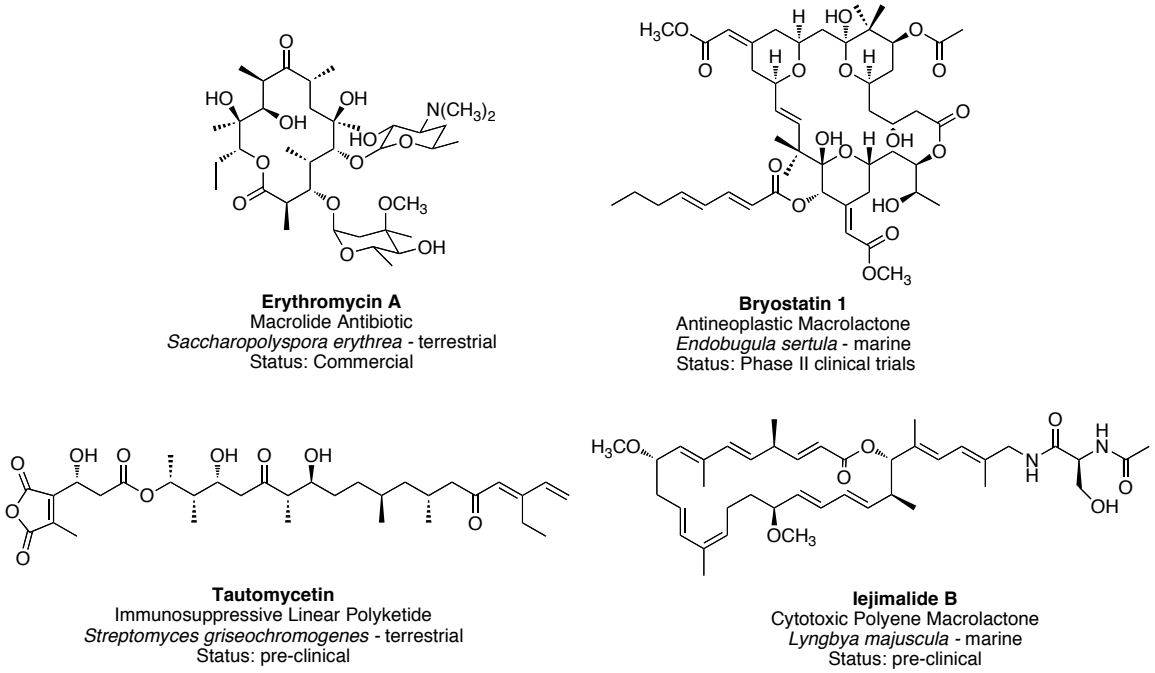
Chapter 1

Polyketide Natural Products and Type I Modular Polyketide Synthases

1.1 Introduction

Polyketides constitute a large class of structurally diverse natural products (**Figure 1.1**) that possess a wealth of pharmacological activities including antimicrobial, antimycotic, antiparasitic, antitumor, and immunosuppressive, making them extremely valuable lead compounds in drug discovery and development. In fact, over two-thirds of newly introduced drugs worldwide in the past two decades have been natural products (many of which are partially or completely polyketide-based) or derivatives thereof.[1]

Figure 1.1 – Some representative polyketide-based natural products.



Erythromycin A
Macrolide Antibiotic
Saccharopolyspora erythrea - terrestrial
Status: Commercial

Bryostatin 1
Antineoplastic Macrolactone
Endobugula sertula - marine
Status: Phase II clinical trials

Tautomycetin
Immunosuppressive Linear Polyketide
Streptomyces griseochromogenes - terrestrial
Status: pre-clinical

Iejimalide B
Cytotoxic Polyene Macrolactone
Lyngbya majuscula - marine
Status: pre-clinical

However, clinical development of many of these promising candidate drugs is currently bottlenecked by difficulties in large scale compound production, as natural product isolates are often low-yielding, and their structural complexities typically makes total synthesis a limited option. Furthermore, difficulty in generating analogue libraries of a parent structure limits the effectiveness of SAR studies to modulate the desired properties of the lead compound.

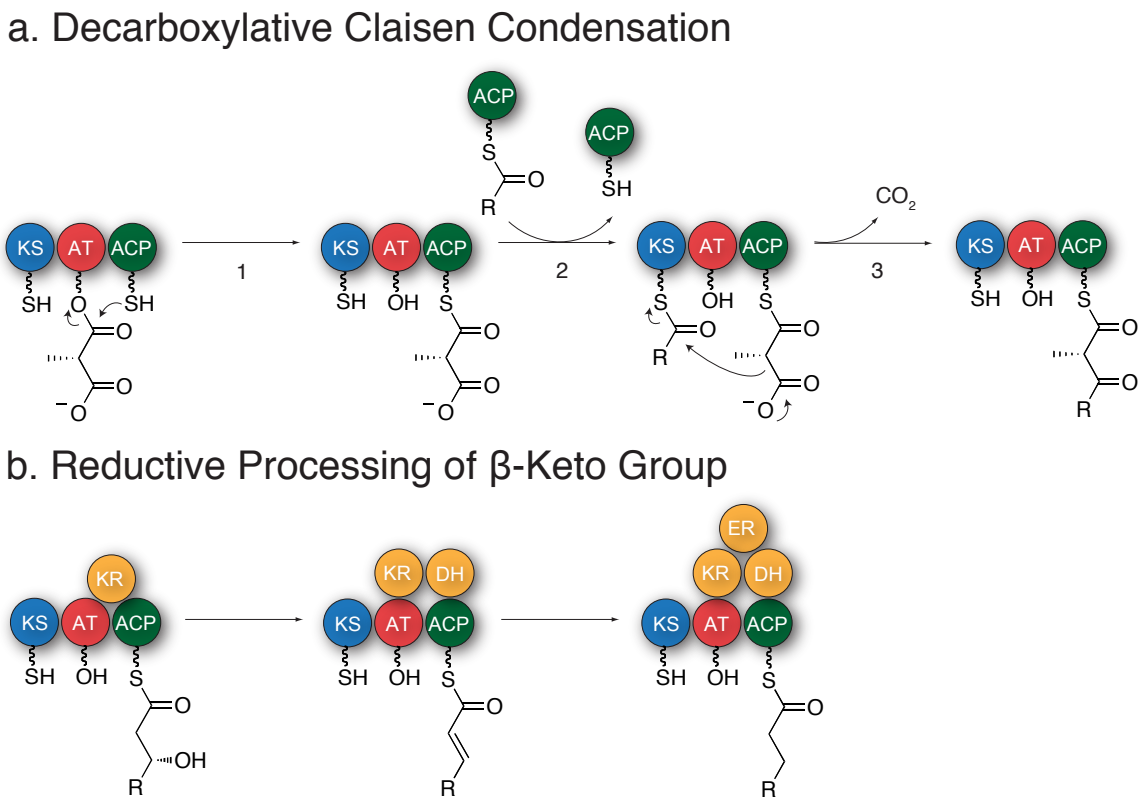
This difficulty has been acutely felt in the development efforts toward macrolides, a large family of antibiotics that bind to the 50S ribosomal subunit of both gram-positive and negative bacteria and block peptidyl transferase activity.[2] The representative member of this family, erythromycin, has been in clinical use for nearly sixty years to combat a variety of common infections and has served as a template for the production of new antibiotics in this class. Despite the success of the macrolides, however, there is a critical need for rapid access to new antibiotics due to the evolution of macrolide-resistant pathogen strains that pose a growing threat to human health. Currently, the complex structural and functional features of macrolides make them particularly difficult targets for synthetic efforts, and semisynthesis has been the only reliable but limited avenue for next generation macrolides such as the azalides and ketolides. Recent total syntheses of erythromycin[3] and azithromycin[4] have required in excess of 40 synthetic operations and are not readily amenable to scaffold diversification for the generation of analogues. As a result, focused study has been centered on engineered biosystems for the chemoenzymatic synthesis of new macrolide scaffolds.

Modern attempts to expand access to chemical diversity have increasingly employed multi-disciplinary tools from the scientific arsenal. From these tools,

chemoenzymatic approaches to generate natural products has emerged as a promising strategy toward this goal, particularly through the manipulation of enzymes from biosynthetic pathways of polyketide secondary metabolites. These strategies leverage the unique selectivity and catalytic prowess of these enzymes, which are responsible for producing countless natural products in marine and terrestrial organisms. Biosynthetic assembly of polyketide natural products occurs on multi-enzyme megasynthases termed polyketide synthases (PKSs).[5] In these systems, natural product scaffolds are built from CoA esters of simple organic acid monomers by an assembly line of enzymatic domains that catalyze their condensation into more complex polyketide chains (**Figure 1.2**). These domains are uniquely arranged in bacterial type I PKSs, where they are organized into covalently bound collinear modules, and each module is responsible for a single elongation and processing step in the PKS enzyme pathway.

The unique architecture of type I modular PKS systems affords the opportunity for rational bioengineering of PKS enzymes for the generation of novel chemotypes. Predictable chemical permutations in a polyketide scaffold can be achieved by a variety of engineering strategies such as the modification of individual PKS catalytic domains (i.e. inactivation, substitution, addition, deletion)[6,7] or exchange of full modules to form hybrid PKS systems.[8-10] To date, hundreds of novel polyketides have been produced utilizing these methods. However, in many cases, engineered PKS proteins have disrupted or significantly attenuated polyketide production, precluding the efficient generation of large libraries of new compounds.[9-11] These studies highlight the need for detailed knowledge regarding the fundamental mechanistic underpinnings of PKS catalytic processes.

Figure 1.2 – (a) PKS condensation mechanism 1) Loading of the acyl carrier protein (ACP) by the acyltransferase (AT) with a malonyl extender unit 2) Loading of the KS (ketosynthase) domain with a polyketide unit from an upstream ACP domain 3) KS-catalyzed decarboxylation and condensation to give a β-keto acyl ACP intermediate (b) Processing of the β-keto group by the ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) reductive domains: KR alone yields a hydroxyl group, KR + DH yields an alkene, or KR + DH + ER yields a saturated carbon backbone.



1.2 The Erythromycin and Pikromycin/Methymycin Polyketide Synthases

Currently, the most well studied modular type I PKSs are the 6-deoxyerythronolide B synthase (DEBS, **Figure 1.3**)[12,13] and the pikromycin PKS[14] (Pik, **Figure 1.4**). These two systems share many similar features, yet contain substantive differences in modular organization, substrate specificity and product distribution. Specific key differences lie in the final two modules (5 and 6) of these PKSs, which are contained on a single bimodular polypeptide in DEBS to produce one 14-membered ring macrolactone 6-deoxyerythronolide B (6-DEB), but are on individual monomodular

polypeptides in Pik and produce both the 12-membered ring macrolactone 10-deoxymethynolide (10-Dml) and the 14-membered ring macrolactone narbonolide (Nbl). As a result, the "late" modules of these systems have been the subjects of significant detailed analysis.

Figure 1.3 – Illustration of the PKS from erythromycin biosynthesis (DEBS). DEBS is responsible for the generation of the aglycone 6-deoxyerythronolide B (6-DEB).

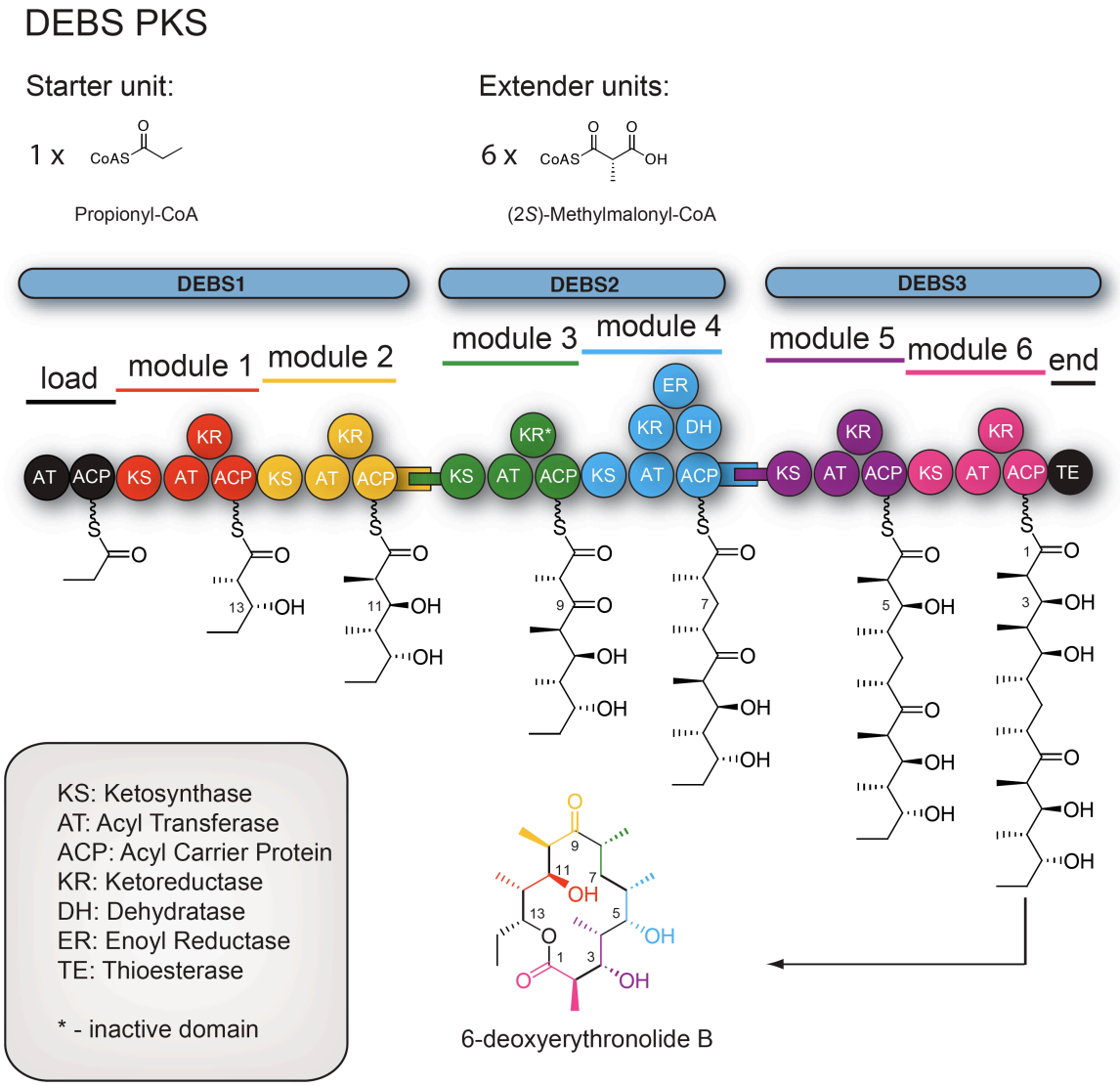
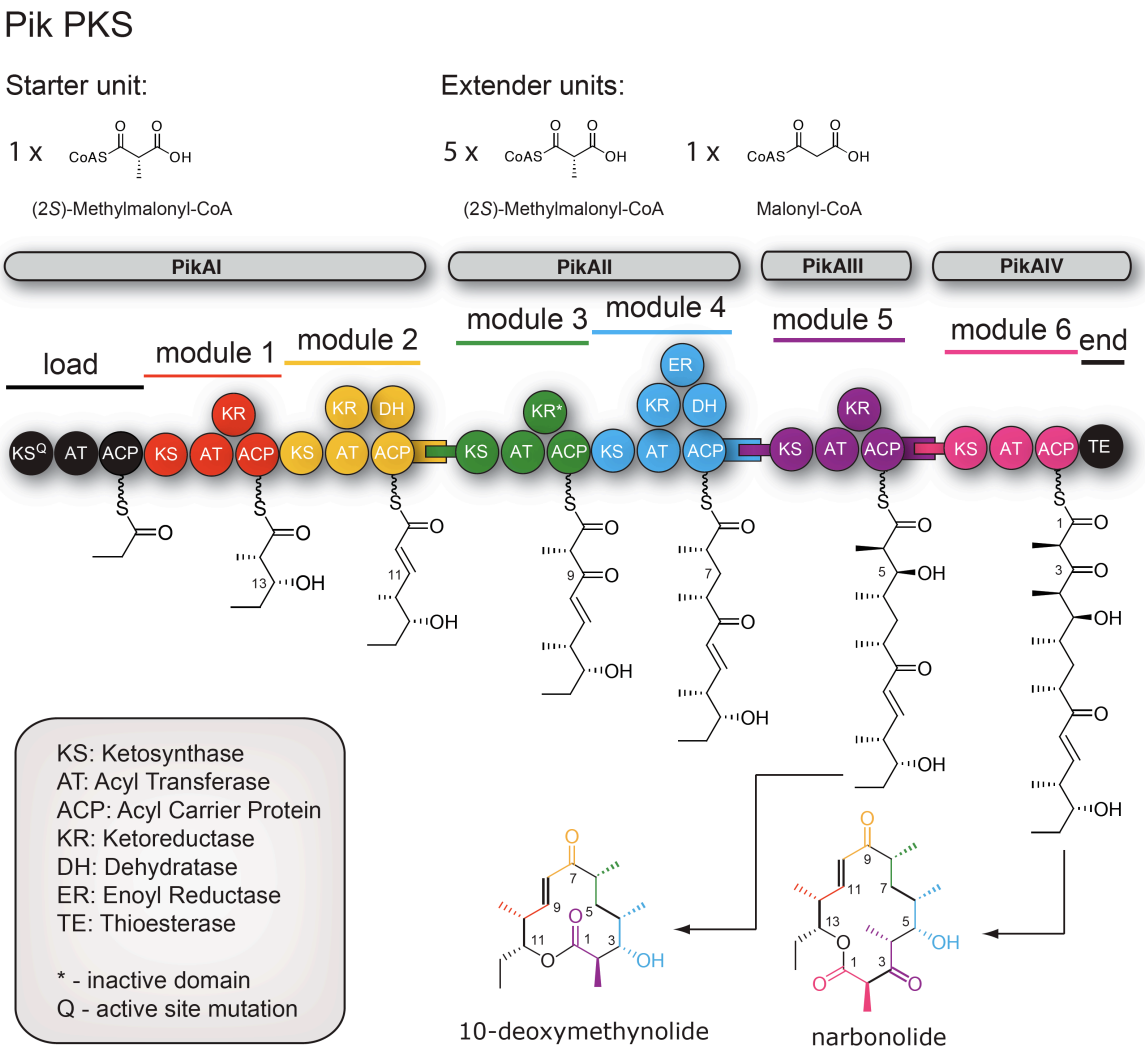


Figure 1.4 – Illustration of the PKS from methymycin/pikromycin biosynthesis (Pik). Pik is responsible for generation of the aglycones 10-deoxymethynolide (10-Dml) and narbonolide (Nbl).

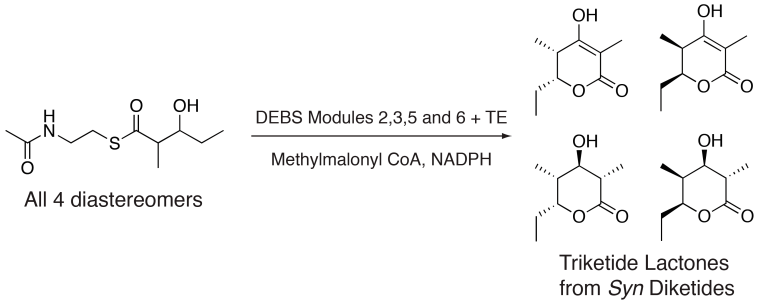


1.3 Previous Work in the DEBS and Pik PKS Systems

In the DEBS system, many pioneering experiments have carved out a fundamental understanding of modular PKS catalytic efficiency and substrate specificity. Probing of individual DEBS modules, including 5 and 6, with short chain diketide model substrates (**Scheme 1.1**) gave triketide lactone products and revealed that they each have an inherent molecular selectivity profile and a high degree of catalytic competence.[15-17] In these studies, only *syn* diketide substrates were accepted, with module 6 demonstrating the highest catalytic efficiency and substrate flexibility. On the other hand, of the

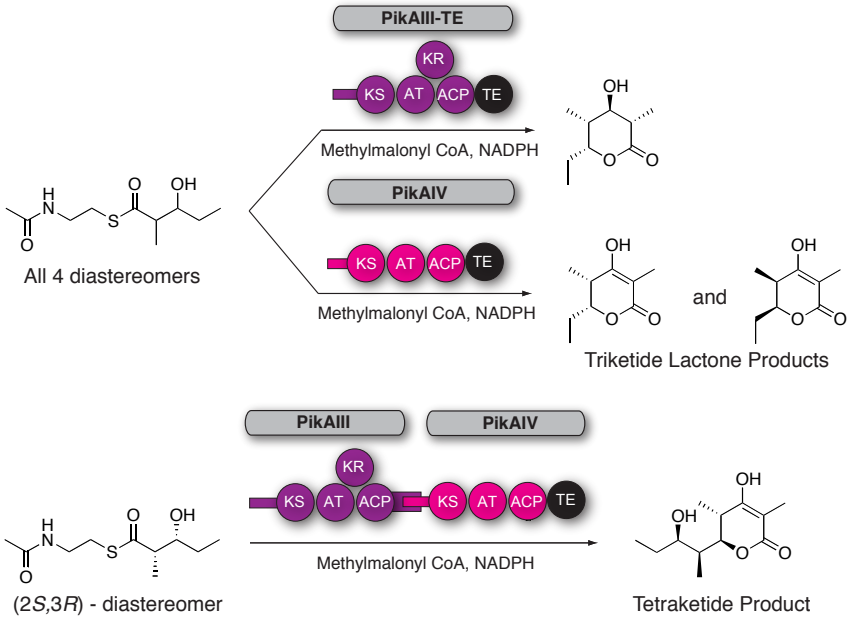
modules surveyed, module 5 demonstrated the lowest efficiency and tightest molecular specificity. While these experiments laid an important foundation for understanding the catalytic elements of individual DEBS modules, the optimal substrates in these *in vitro* experiments correlate poorly to the native substrates that are normally accepted and processed by these modules *in vivo*. This is especially true of the downstream modules (i.e., modules 5 and 6) that must accept and process increasingly complex polyketide intermediates in the growing chain, raising questions regarding the contributions of more distal functionality in molecular recognition of polyketide chain-elongation intermediates.

Scheme 1.1 – Probing Molecular Selectivity in DEBS Modules with Diketides



Comparative study of the analogous late modules of the pikromycin PKS[18, 19] showed that extension and processing of model diketides by Pik modules 5 (PikAIII) and 6 (PikAIV) (**Scheme 1.2**) were nearly three orders of magnitude less efficient than in DEBS, though the catalytic efficiencies of PikAIII and PikAIV were comparable. Similar to what was seen in the DEBS system, PikAIII and PikAIV accepted only *syn* diketide SNAC substrates with PikAIII exhibiting a higher molecular stringency. These studies established an important groundwork for understanding PKS catalytic events in Pik.

Scheme 1.2 – *In Vitro* Analysis of PikAIII and PikAIV with Diketide Substrates

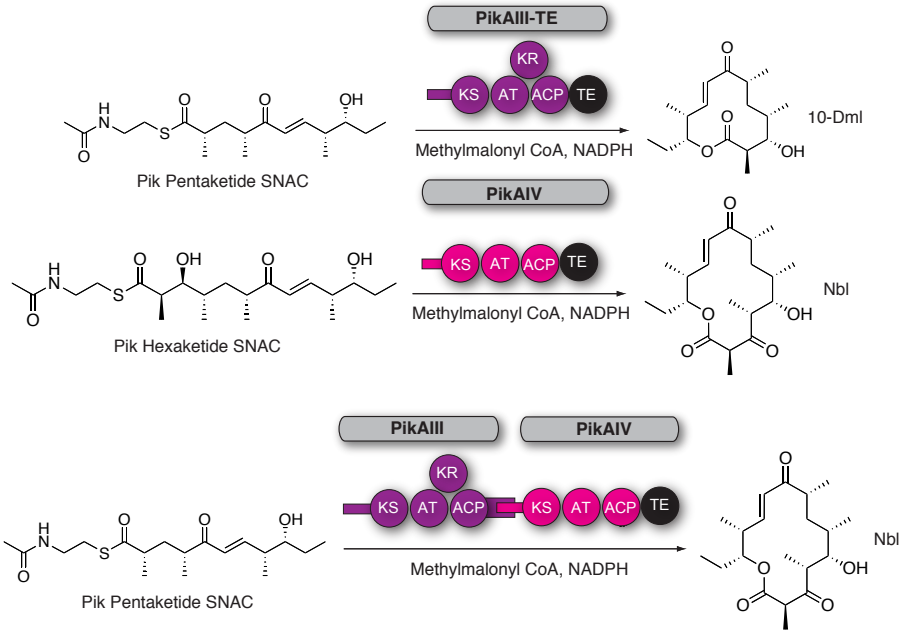


However, they again raised questions regarding the effectiveness of diketides as molecular probes in late-stage PKS modules. This limitation was borne out in later experiments,[20] which clearly showed that diketide model compounds do not effectively probe the molecular recognition features of these PKS modules. Rather, utilizing full-length native chain elongation substrates (**Scheme 1.3**) in biochemical studies of PKS modules yields a more valuable assessment of kinetic parameters and modular specificity. This was evidenced by the fact that native penta- and hexaketide substrates for Pik modules 5 (PikAIII) and 6 (PikAIV) were elongated, processed and cyclized 2-3 orders of magnitude more efficiently than the corresponding diketide model substrates.

These experiments also represent the first time that such advanced polyketide chain-elongation intermediates had been synthesized from a PKS biosynthetic pathway and highlight the inherent difficulties in utilizing a biotic strategy for accessing polyketides. For example, during synthetic attempts to access the chemically sensitive

Pik hexaketide substrate for module 6 of the Pik PKS, it was seen that that this substrate readily undergoes hemiketalization of the C3 hydroxyl on the electrophilic C7 ketone, followed by dehydration when removed from its native PKS environment.[20] Many salient examples of novel synthetic strategies toward functionally and stereochemically diverse polyketide fragments have emerged from the Tan,[21] Leighton,[22] and Carreira[23] laboratories; however, these methods employ standard abiotic approaches to polyketide synthesis where sensitive functionalities are protected and not revealed until an opportune time in the synthesis. Accessing complex PKS intermediates, however, requires a rethinking of these strategies, as many of these intermediates are inherently unstable outside of the PKS complexes and cannot be protected through typical synthetic means.

Scheme 1.3 – *In Vitro* Analysis of Pik Modules with Native Chain-Elongation Intermediates



1.4 Experimental Plan

Based on the results of these previous studies in DEBS and Pik, we sought to further our assessment of the molecular recognition features in late-stage DEBS modules

utilizing native chain-elongation intermediates. We were thus motivated to synthesize the native pentaketide and hexaketide substrates from DEBS and employ them for *in vitro* biochemical analysis of engineered monomodules from the DEBS3 polypeptide. The development and implementation of a synthetic plan to access these substrates is laid out in Chapters 2 and 3 of this dissertation and are published in part in Mortison et al. *J. Am. Chem. Soc.* **2009**, *131*, 15784-15793.

To establish an effective experimental system for our proposed biochemical experiments, we sought to access the bimodular DEBS3 protein as well as separate it into into monomodular enzymes Ery5 (module 5), Ery5-TE (module 5 + engineered TE fusion), Ery6 (module 6 + native TE fusion), and DEBS TE (excised TE domain). With native DEBS chain-elongation intermediates in hand, we envisioned performing a detailed biochemical analysis of both the catalytic competence and substrate specificity profiles of DEBS modules 5 and 6, as well as a comparative analysis of the corresponding late monomodules (module 5 and module 6) from the Pik PKS. The details of this work are described in Chapter 4 of this dissertation and published in Mortison et al. *J. Am. Chem. Soc.* **2009**, *131*, 15784-15793. The described work with the DEBS3 protein is currently being developed for a future publication, the details of which are laid out in Chapter 6.

Another goal of our research is to establish a convergent and diversity-oriented strategy to access not only native chain-elongation intermediates from diverse PKS systems, but also valuable analogues thereof, encompassing a number of structural, stereochemical and functional group variations. These analogues could then be employed in biochemical studies to continue our rigorous assessment of molecular specificity in

modular PKSs. Chapter 5 of this dissertation describes the current progress toward that goal, which will be featured in a future publication.

Finally, Chapter 6 of this dissertation describes the future direction of our work in modular PKS systems, outlining key experiments planned with DEBS3 and proposed work with novel chain-elongation intermediate analogues. At the same time, this chapter describes the growing potential for utilizing PKS enzymes in combinatorial biosynthesis and chemoenymatic synthesis. This information is laid out in the manuscript for an invited *Perspective* to the Journal of Organic Chemistry, which is currently being prepared for submission.

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Chapter 2

Preliminary Efforts Toward the Synthesis of the DEBS Pentaketide Chain-Elongation Intermediate

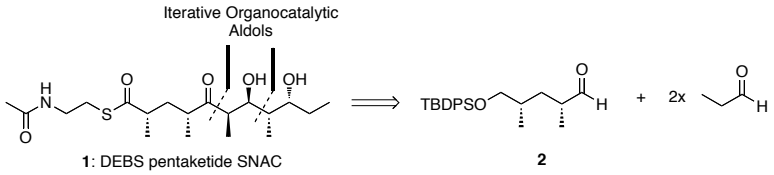
Toward the goal of utilizing native chain elongation intermediates as chemical probes to study the final modules from the DEBS PKS system, our initial work sought to access the native DEBS penta- and hexaketide intermediates by total synthesis. Approaches toward the DEBS chain-elongation intermediates, encompassed their synthesis as *N*-acetylcysteamine thioesters to mimic the native phosphopantetheine arm of the enzymes and facilitate diffusive loading of the ketosynthase active site of the PKS modules.

2.1. Iterative Organocatalytic Aldol Strategy

In the first generation synthesis, it was envisioned that the C6-C8 stereoarray of the DEBS pentaketide could be constructed using chemistry developed by the MacMillan group (Princeton University). Here, two iterations of a mild and selective organocatalytic cross-aldol reaction[1] could generate the C6-C8 *syn-anti* stereotriad. Retrosynthesis revealed disconnections between C5-C6 and C7-C8, with known aldehyde **2**[2] as the key fragment along with two molecules of propionaldehyde (**Scheme 2.1**). The first iteration of the cross-aldol between aldehyde **2** and propionaldehyde could be accomplished with L-proline, followed a second iteration utilizing D-proline and another equivalent of

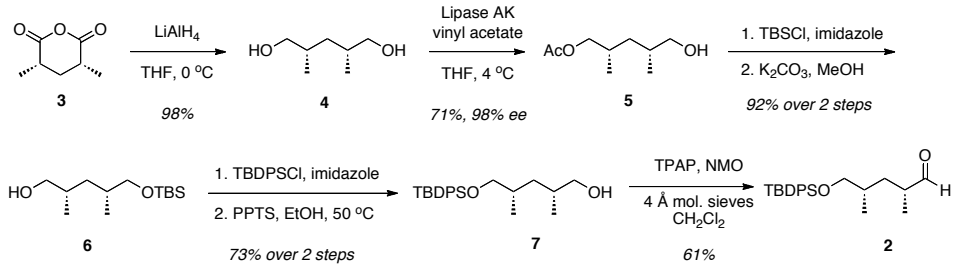
propionaldehyde. It was rationalized that this strategy could not only yield an efficient method for synthesizing the native pentaketide, but also provide flexible access to C6-C9 analogues containing new functional or stereochemical features. This could be accomplished by utilizing different aldehyde donors and altering the enantiosense of the proline catalyst in the two aldol iterations.

Scheme 2.1 – Retrosynthetic Analysis of the DEBS Pentaketide Via Iterative Organocatalytic Aldol.



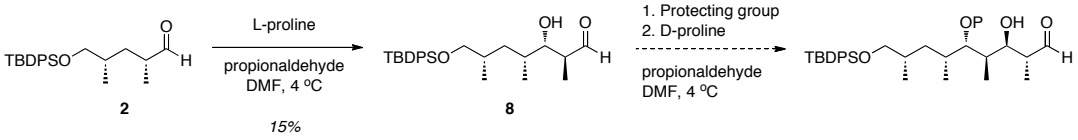
Aldehyde **2** was synthesized on a multigram scale, starting with *meso*-anhydride **3**[3] (**Scheme 2.2**). Anhydride **3** was reduced with lithium aluminum hydride to give the *meso*-2,4-dimethyl-1,5-pentanediol **4**, which was then subject to an enzymatic desymmetrization with Lipase AK (Amano) with vinyl acetate as the acetate donor to give the mono-acetate **5** in 98% *ee*. Following a number of functional group manipulations, including the introduction of TBS and TBDPS groups, alcohol **7** was obtained following the selective deprotection of the bis-silyl ether. Ley oxidation of alcohol **7** with TPAP/NMO gave the desired aldehyde **2** in 7 steps from the starting *meso*-anhydride **3**.

Scheme 2.2 – Synthesis of Key Aldehyde Fragment **2**



Cross-aldol of **2** with propionaldehyde was accomplished using an L-proline catalyst to give aldol product **8**, albeit in a low yield of 15% (**Scheme 2.3**). While observed diastereoselectivity was high, attempts to optimize reaction yields disappointingly did not produce significant improvement. Changes in temperature, catalyst loading, concentration and rate of addition of donor aldehyde were explored. Unfortunately, it appeared that aldehyde **4** was a poor acceptor in this cross-aldol, and competing homodimerization of propionaldehyde could not be suppressed. This was surprising since α-branched aldehydes had been employed successfully as acceptors in these organocatalytic reactions.[1] Since these methods were suboptimal for efficient synthesis of our target substrates, we began evaluating new synthetic options.

Scheme 2.3 – Iterative Organocatalytic Cross-Aldol Reactions.

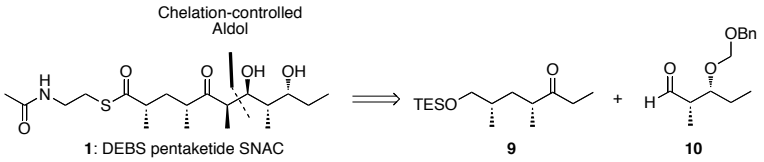


2.2 Chelation-Controlled Aldol Strategy

Despite these earlier setbacks, we found success with a synthetic strategy that was both efficient and flexible. After re-evaluation of the literature, it was found that a chelation-controlled aldol reaction[4, 5] between ethyl ketone **9** and known aldehyde **10**

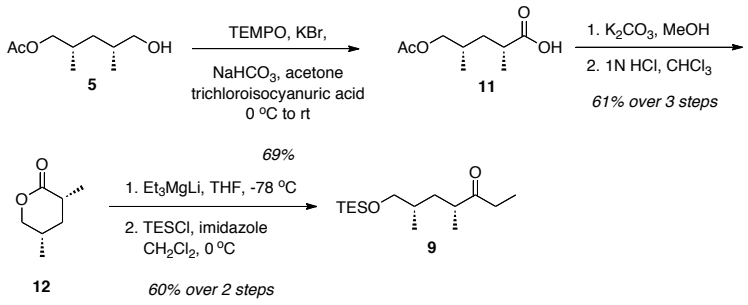
could serve as the key bond forming step in generating entire carbon framework and desired stereochemistry of the DEBS pentaketide (**Scheme 2.4**). Literature precedent rationalized the use of a BOM ether group on aldehyde **10** to mediate chelation in the aldol transition state to give the desired anti-Felkin *syn* stereochemistry at C6-C7.

Scheme 2.4 – Retrosynthetic Analysis of DEBS Pentaketide Via Chelation-Controlled Aldol.



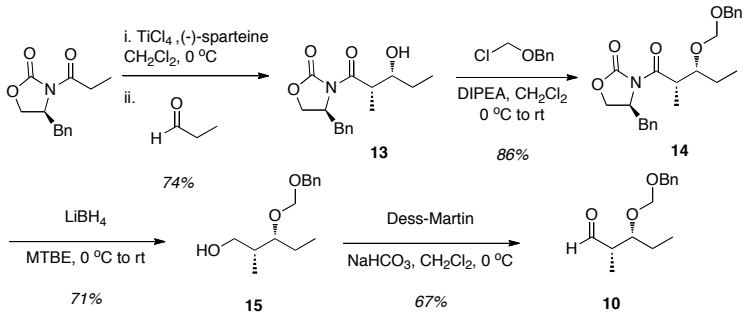
The left fragment consisting of ethyl ketone **9** was synthesized on a multigram scale from the enantiopure δ-valerolactone **12**[6] (**Scheme 2.5**). Lactone **12** was made starting from our previously desymmetrized *meso*-2,4-dimethyl-1,5-pentanediol **5**, followed by mild oxidation of the free alcohol to the carboxylic acid **11**.[7] The acetyl group was deprotected by basic methanolysis, and **12** was formed by vigorous stirring of the acid in a biphasic system of 2 N HCl and chloroform. Attempts to directly add EtMgBr or EtLi to the lactone gave poor conversion to the desired lactol. However, addition of the "ate" complex Et_3MgLi[8] proceeded to smoothly convert the lactone to the intermediate lactol, which then yielded desired ethyl ketone **9** after subsequent protection of the primary alcohol as the TES ether.

Scheme 2.5 – Synthesis of the Left Fragment



Synthesis of the right fragment proceeded via known Evans aldol adduct **13**,[9, 10] (**Figure 2.6**) assembled using the Crimmins procedure. Protection of the free secondary alcohol was then accomplished using BOMCl to give oxazolidinone **14**. The choice of the BOM protecting group was based on literature precedent,[4, 5] indicating that the extended linker of the benzyl formacetal allows for chelation in the transition state, thus providing selectivity for the desired anti-Felkin addition. The Evans auxiliary could then be removed with $LiBH_4$ to give the alcohol **15**. The alcohol was readily oxidized to known aldehyde **10**,[5] with Dess-Martin periodinane providing the most reliable and consistent results.

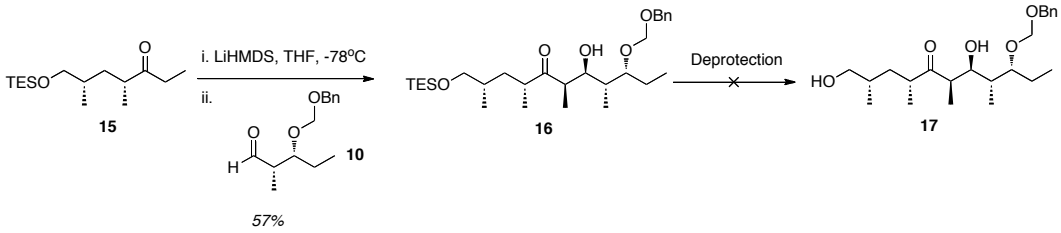
Scheme 2.6 – Synthesis of the Right Fragment



The *Z*(*O*)-enolate of ketone **9** was formed with lithium hexamethydisilazide and aldol coupling to aldehyde **10** was accomplished with good diastereoselectivity (~7:1) for the desired anti-Felkin product **16**, containing the entire pentaketide carbon framework (**Scheme 2.7**). Attempts to deprotect the triethylsilyl group, however, failed under a variety of conditions, and deprotected product **17** could not be isolated. The main decomposition product appears to arise from hemiketalization of the C1 alcohol with the C5 ketone, followed by dehydration. Due to this facile degradation, a new protecting group at C1 was sought that could be removed under more mild conditions. For that

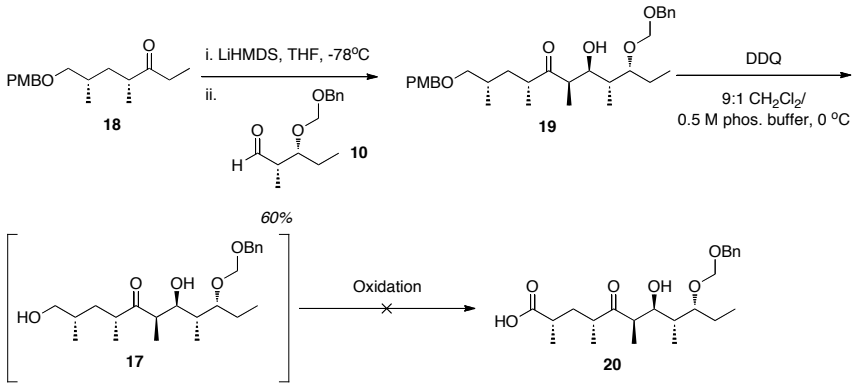
purpose, a *p*-methoxy benzyl (PMB) group was chosen, due to its ready deprotection under mild oxidative conditions.

Scheme 2.7 – Aldol Coupling and C1 TES Ether Deprotection



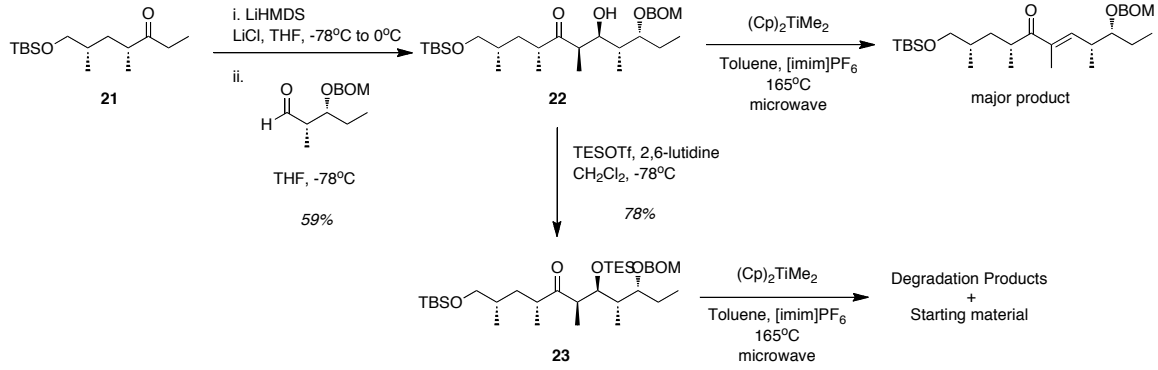
Coupling of the new C1 PMB-protected ketone **18** with aldehyde **10** could be accomplished in good yield and selectivity for the anti-Felkin product **19**. Deprotection with DDQ gave the free primary alcohol **17**, however, purification by silica gel chromatography resulted in product degradation. As a result, the isolated crude alcohol was subjected directly to oxidation conditions by chemoselective oxidation of the primary alcohol to the aldehyde,[11] followed by Pinnick oxidation to the carboxylic acid. Unfortunately, no conditions could be found that could give the C1 oxidized product **20**. Only degradation products were observed. Protecting the secondary C7 alcohol first as the TES ether and utilizing other oxidation methods also failed to give the desired result.

Scheme 2.8 – Aldol Coupling and C1 PMB Ether Deprotection



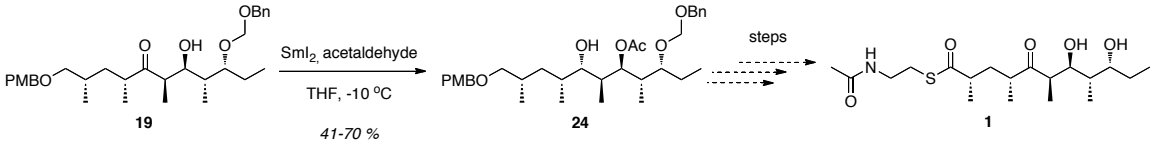
Due to the problems associated with hemiketalization at the electrophilic C5 ketone, we sought to transform this group into another one that could be unmasked near the end of the synthesis. One approach toward this was to mask the C5 keto group as an olefin, operating under the rationale that this functional group could be readily converted to the ketone by oxidative cleavage of the olefin at the end of the synthesis under mild conditions. At the same time, precedent from the Ley group during their synthesis of Spongistatin had shown successful olefination of an electronically and sterically similar keto group with Petasis reagent under microwave conditions.[12] The substrate for the olefination was **22**, generated by aldol coupling of TBS-protected ketone **21** (**Scheme 2.9**). Direct olefination of **22** with Petasis reagent at 165 °C in the microwave, resulted in primarily elimination of the C7 alcohol. As a result, he alcohol was protected as the TES ether **23** and re-subjected to the olefination conditions. Unfortunately, none of the desired product was obtained. Instead, most of the starting ketone was recovered along with some minor degradation products. Use of other olefination reagents such as Tebbe or Lombardo gave similar results, and so this strategy was discontinued.

Scheme 2.9 – C5 Olefination Strategy with Petasis Reagent



Alternatively, another method for this was to generate the protected secondary alcohol at C5. It was envisioned that an Evans-Tischenko reduction[13] would provide both a stereoselective 1, 3-reduction as well as provide the requisite differential protection of the C7 alcohol as the acetate (**Scheme 2.10**). Reaction of β-hydroxy ketone **19** with samarium (II) iodide and acetaldehyde gave stereospecific reduction of the C5 alcohol along with the C7 acetate. Results of this transformation, however, were disappointingly variable, especially during larger scale reactions, giving significant amounts of retro-aldol products. Yields of **24** ranged from 41-70%, but they were deemed acceptable for our current efforts.

Scheme 2.10 – Evans-Tischenko Reduction of β-Hydroxy Ketone **19**



Nonetheless, despite the promise of the work accomplished thus far, parallel experiments resulted in a new and more efficient strategy toward the DEBS pentaketide. This new approach circumvented many of the difficulties and complexities of the current approach, which required multiple protection/deprotection steps and oxidation state manipulations to complete the DEBS pentaketide. This work is detailed in the forthcoming chapter.

2.3 References

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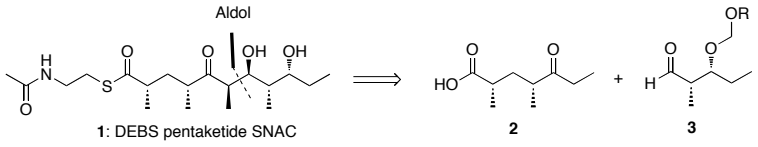
Chapter 3

Synthesis of the DEBS Pentaketide Chain-Elongation Intermediate

Based on the previous success obtaining the full carbon framework of the DEBS pentaketide utilizing a chelation-controlled aldol strategy (Chapter 2), we continued to pursue this strategy as the primary route to the DEBS pentaketide chain-elongation intermediate. Due to the propensity for hemiketalization and dehydration from the deprotected C1 alcohol of the pentaketide seen earlier, it was envisioned that changing the oxidation state at C1 to the carboxylic acid could circumvent this problem. Thus, retrosynthetic analysis of the native DEBS pentaketide (**1**) instead gave keto acid **2** and aldehyde **3** as the required fragments (**Scheme 3.1**). At the same time, a shortening of the synthesis of the left fragment was devised utilizing a via a Myers alkylation strategy[1] (*vide infra*).

Scheme 3.1 – Retrosynthetic Analysis of the DEBS Pentaketide Chain-Elongation Intermediate

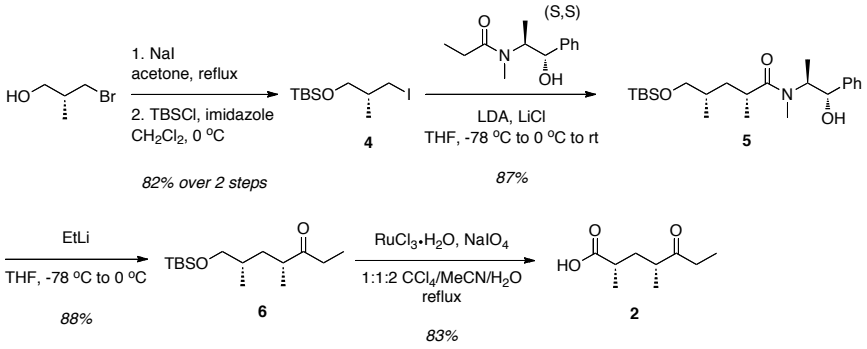


3.1 Synthesis of the Left Fragment

Synthesis of the left fragment started with commercially available (*R*)-3-bromo-2-methyl-1-propanol, which was readily converted to iodide **4** by halide displacement and TBS protection of the primary alcohol (**Scheme 3.2**). Diastereoselective Myers alkylation[1] of **4** with the enolate of (*S,S*)-pseudoephedrine propionamide gave amide **5**

with high selectivity for the desired 2,4-*syn* product.[2] The amide was then readily ethylated with EtLi to furnish ketone **6**. Attempts to oxidize C1 via 2-step deprotection/oxidation procedures were problematic due to varying degrees of epimerization. However, one pot Sharpless oxidation of the TBS ether using RuO_4[3] proved to be a clean and facile route to **2**. This reaction is thought to proceed by oxidation of the TBS ether directly to the TBS ester, which is then hydrolyzed to give the carboxylic acid. By contrast to earlier strategies toward the left fragment requiring nine synthetic steps, as laid out in Chapter 2, this route provided the left fragment in only five steps.

Scheme 3.2 – Synthesis of the Left Fragment

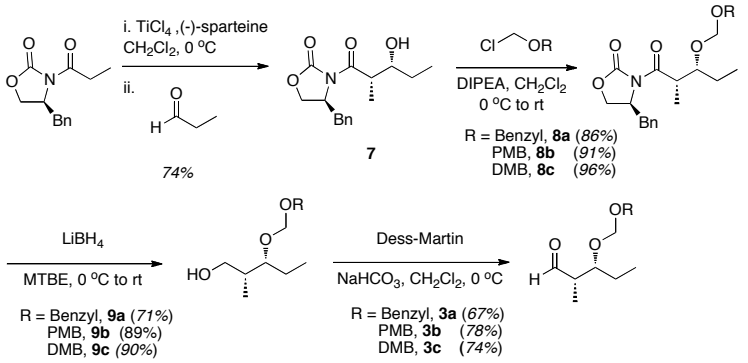


3.2 Synthesis of the Right Fragment

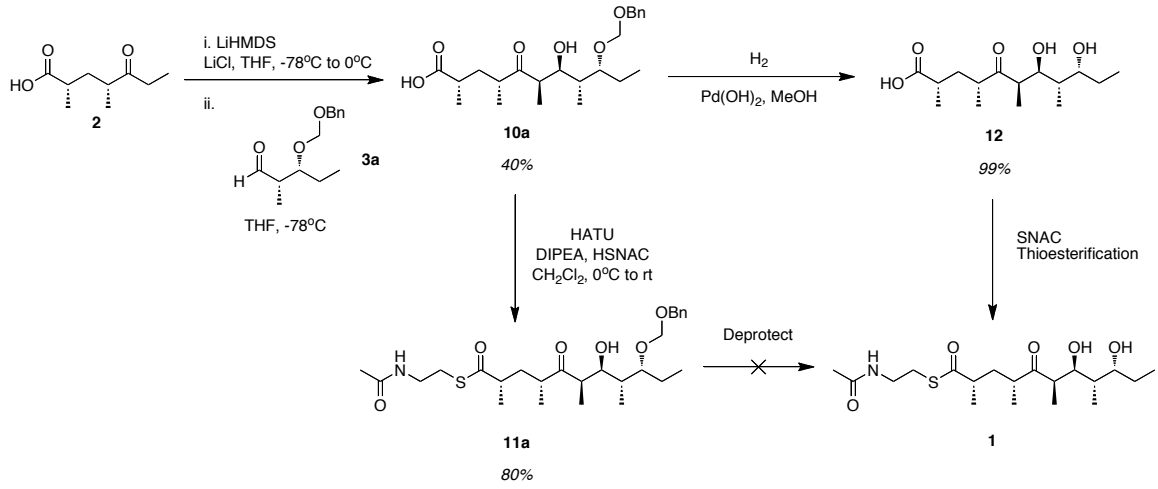
Synthesis of the right fragment **3** began with known Evans aldol product **7**[4,5], which was protected with a benzyl formacetal group (**Scheme 3.3**). The protecting group strategy applied at the C9 hydroxyl proved crucial for selectivity in the aldol coupling and for final deprotection of the pentaketide substrate. Initially, the aldol product was protected as the benzyloxymethyl (BOM) ether with BOMCl to give protected oxazolidinone **8a**. The Evans auxiliary was then removed with $LiBH_4$ to give alcohol **9a**, followed by Dess-Martin oxidation to aldehyde **3a**. Subsequent aldol coupling and

thioesterification with *N*-acetyl cysteamine proceeded smoothly to give products **10a** and **11a**, respectively (**Scheme 3.4**). Attempts to remove the BOM protecting group at the end of the synthesis, however, failed with a variety of hydrogenation catalysts, even with very high catalyst loadings and use of free thiol scavengers. Use of protic or Lewis acids for deprotection resulted in degradation of the desired pentaketide. The BOM ether could be removed quantitatively from **10a** by catalytic hydrogenolysis with Pearlman's catalyst to give the pentaketide *seco*-acid **12**, indicating that the NAC thioester is problematic for the deprotection step.

Scheme 3.3 – Synthesis of the Right Fragment



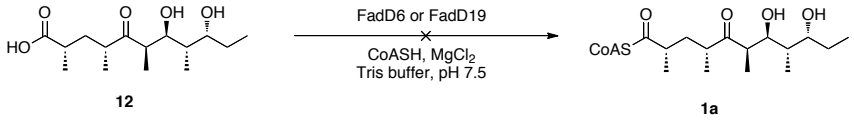
Scheme 3.4 – Aldol Coupling and Preliminary Deprotection Attempts



3.3 Chemoenzymatic Thioesterification Strategies

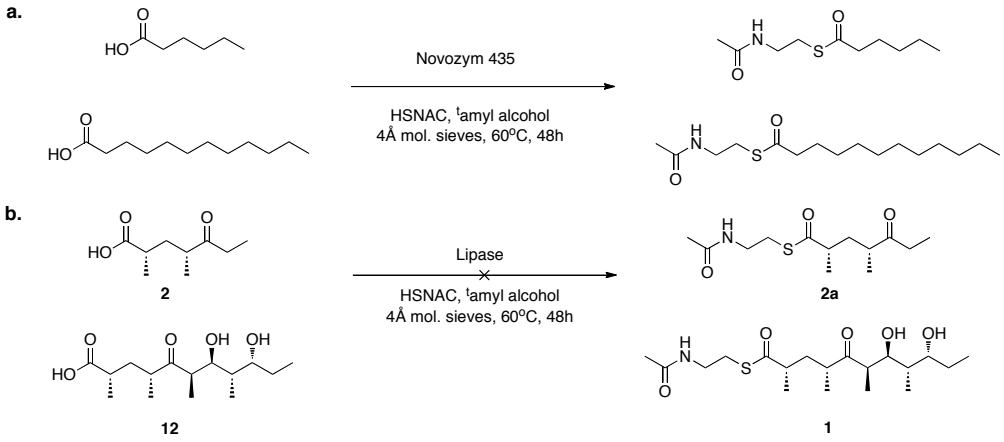
Seco-acid **12** was subjected to a number of chemical thioesterification conditions to install the SNAC moiety, but pentaketide degradation was observed in all attempts. As a result, chemoenzymatic options were also explored. The first strategy employed promiscuous fatty acid CoA ligases reported by the Gokhale group (National Institute of Immunology, New Delhi, India)[6, 7] from *Mycobacterium tuberculosis*. These CoA ligases had been reported to accept long chain fatty acids containing methyl, hydroxyl and olefin substitution on the carbon backbone, even at the α and β positions. The CoA ligases FadD6 and FadD19[8] were expressed and shown to be active by their ability to produce lauroyl CoA from lauric acid and free coenzyme A. Unfortunately, incubation of *seco*-acid **12** with either FadD6 and FadD19 did not show detectable levels of the DEBS pentaketide CoA **1a**[9] (**Scheme 3.5**).

Scheme 3.5 – Chemoenzymatic Installation of CoA on the DEBS Pentaketide



In collaboration with the Dordick group, we also sought to generate the DEBS pentaketide SNAC utilizing a number of commercially available lipases.[10] Early results from the Dordick group had indicated that immobilized *Candida antarctica* lipase B (CALB, Novozyme 435) was capable of catalyzing the thioesterification of simple carboxylic acids in the presence of *N*-acetylcysteamine (**Scheme 3.6**). This methodology was applied to both the triketide acid **2** as well as our pentaketide *seco*-acid **12**, but installation of the SNAC moiety was unsuccessful. Screening a number of other immobilized lipases as well as varying temperatures and solvents did not yield the desired results.

Scheme 3.6 – Chemoenzymatic Thioesterification of a) Fatty Acids with Novoym 435 and b) Attempted Thioesterfication of Triketide **2** and Pentaketide *Seco*-Acid **12**.



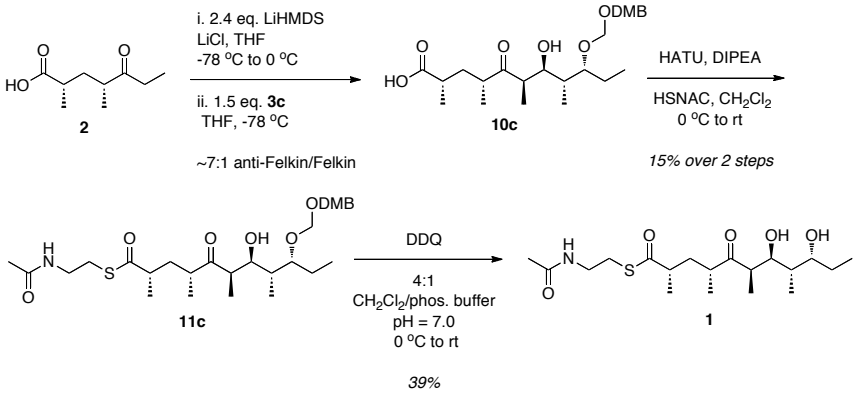
3.4 C9 Protecting Group Optimization

Concurrent with our chemoenzymatic thioesterification methods, other synthetic strategies were explored, including new protecting groups for the C9 hydroxyl. Of the protecting groups surveyed, only related benzyl formacetals did not abolish aldol selectivity. Simpler benzyl-derived protecting groups, for example, gave a complex diastereomeric mixture of products in the aldol step. Analogous work utilizing a p-methoxybenzyl (PMBOM) formacetal group (**Scheme 3.4**) also created difficulties with its removal at the end of the synthesis. Oxidative deprotection of the PMBOM moiety with DDQ or CAN surprisingly did not give detectable amounts of the desired pentaketide product. Ultimately, the electron rich 3,4-dimethoxybenzyl (DMBOM) formacetal proved to be the only viable protecting group candidate that could be readily deprotected to give the final pentaketide intermediate. Therefore, Evans-*syn* aldol product **7** was protected using DMBOMCl to give **8c**, followed by reductive removal of the Evans auxiliary with $LiBH_4$ to give alcohol **9c**. Oxidation to aldehyde **3c** was then best accomplished using Dess-Martin periodinane.

3.5 Final Aldol Coupling and Deprotection

With the pendant C9 protecting group optimized, coupling of the two fragments proceeded first by enolization of keto acid **2** with LiHMDS, followed by an aldol reaction with the DMBOM-protected aldehyde **3c** to give the full carbon chain of the DEBS pentaketide with good (approximately 7:1) anti-Felkin/Felkin selectivity[11] and modest yield (**Scheme 3.7**). Selectivity for the desired anti-Felkin *syn* diastereomer could be achieved in the aldol coupling via a chelation-control element,[12,13] presumably through either coordination of the lithium cation with one of the formacetal linkages or a cation-π interaction with the electron-rich aryl ring of the 3,4-dimethoxybenzyl group. This aldol step was followed by subsequent thioesterification to give the protected DEBS pentaketide SNAC. Due to the exceptional acid-sensitivity of **1**, buffered biphasic conditions (4:1 CH_2Cl_2/0.5 M phosphate buffer, pH 7) were imperative in the final oxidative deprotection of the DMBOM group with DDQ. Deprotection yields suffered somewhat due to competing oxidation to form a DMB orthoester side product. Nonetheless, this convergent synthetic strategy provided the target pentaketide SNAC substrate in a sequence of only eight linear steps, avoiding excessive protecting group and functional group manipulations.

Scheme 3.7 – Completion of the DEBS Pentaketide SNAC Synthesis



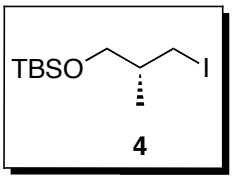
3.6 Discussion

Synthesis of the DEBS pentaketide revealed some of the inherent challenges present in the total synthesis of linear polyketides, which often suffer from facile degradation pathways. This was seen in the final deprotection step of the pentaketide, where the intermediate readily yielded to acid-catalyzed hemiketalization and dehydration under most conditions. The synthesis thus required the sparsely used DMBOM group, which enabled mild deprotection conditions and provided the necessary compatibility with the chelation requirements of the earlier aldol step. In addition, similar degradation pathways thwarted our effort toward the desired DEBS hexaketide intermediate, which contains two hydroxyl groups disposed for hemiketalization at C5. These challenges highlight an important, yet often under-appreciated aspect of the PKS catalytic mechanism, whereby the linear polyketide chain elongation intermediates remain covalently linked to the enzyme complex throughout the PKS extension process, rather than being diffusively off-loaded after each catalytic cycle. This mechanism protects the chemically sensitive and transient intermediates against numerous potential degradation pathways until they are released as more stable products.

3.7 Experimental

Materials and General Procedures. Commercially available reagents were purchased from Sigma-Aldrich, Acros Organics or Fluka, or prepared from established literature procedures. $RuCl_3 \cdot H_2O$ was obtained from Strem Chemicals, Inc. Anhydrous solvents were obtained either using an MBRAUN MB-SPS solvent purification system or purchased in septum-sealed bottles from Acros Organics (AcroSeal) or EMD (DriSolv). Diisoproylamine and *N, N*-diisopropylethylamine were distilled over CaH_2 and stored

under argon. Alkyllithiums were titrated prior to use with diphenylacetic acid. Air- and moisture-sensitive reactions were carried out under an inert argon atmosphere in flame-dried glassware. Flash chromatography was carried out using Merck Silica Gel 60 (230-400 mesh) and thin layer chromatography was performed on Merck TLC plates pre-coated with Silica Gel 60 F_{254}. ^{1}H and ^{13}C NMR spectra were obtained on either Varian MR400 or Inova 500 spectrometers. Proton chemical shifts are reported in ppm relative to TMS with the residual solvent peak as an internal standard. Proton NMR data is reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz) and integration. High-resolution mass spectra were obtained on a Waters Micromass AutoSpec Ultima Magnetic Sector mass spectrometer.



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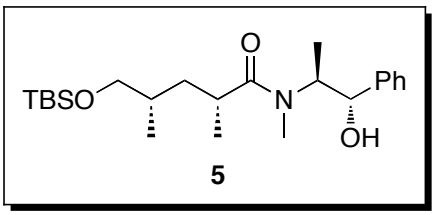
Product 4. A suspension of (*R*)-(-)-3-bromo-2-methyl-1-propanol (4.995 g, 32.64 mmol) and NaI (19.571 g, 130.57 mmol) in 60 mL acetone was brought to reflux at 70 °C. After 18 h, the reaction mixture was diluted with 50 mL H_2O and the volatiles were removed *en vacuo*. The mixture was then extracted with 3 × 50 mL CH_2Cl_2, and the combined organics were washed with 20 mL saturated $Na_2S_2O_3$, dried over $MgSO_4$, filtered and concentrated.

The crude iodide was re-dissolved in 100 mL CH_2Cl_2 and cooled to 0 °C. Imidazole (2.445 g, 35.91 mmol) and TBSCl (5.412 g, 35.91 mmol) were then added sequentially. A white precipitate formed immediately, and the reaction was monitored by TLC. After 1 h, the reaction was filtered and concentrated. The crude mixture was re-suspended in 100

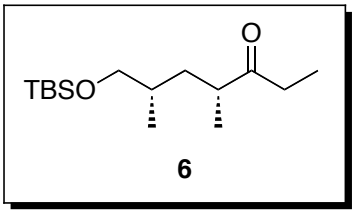
mL pentane and filtered, rinsing the filter with an additional 50 mL pentane. The filtrate was concentrated and the crude oil was purified by flash chromatography (100% hexanes) to give 8.358 g (81.5%) of a colorless oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 3.53 (dd, *J* = 10.0, 5.0 Hz, 1H), 3.40 (dd, *J* = 10.0, 6.9 Hz, 1H), 3.30 (dd, *J* = 9.5, 5.2 Hz, 1H), 3.24 (dd, *J* = 9.5, 5.6 Hz, 1H), 1.79 – 1.53 (m, 1H), 0.95 (d, *J* = 6.7 Hz, 3H), 0.90 (s, 9H), 0.06 (s, 3H), 0.06 (s, 3H); ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ -5.4, 13.7, 17.2, 18.2, 25.9, 37.4, 66.7; HRMS CI$^+$ (*m/z*): 315.0656 (Predicted $[M+H]^+$ for $C_{14}H_{24}OSiI$ is 315.0641).



5

Product 5. *n*-Buli (2.35 M in hexanes, 33.58 mL, 78.91 mmol) was added dropwise at -78 °C to a stirring suspension of flame-dried LiCl (12.948 g, 305.45 mmol) and diisopropylamine (11.871 mL, 84.00 mmol) in 50 mL THF. The reaction was warmed to 0 °C briefly for 5 min, then re-cooled to -78°C. (*S,S*)-(+)-pseudoephedrine propionamide (9.013 g, 40.73 mmol) in 100 mL THF was added dropwise by cannula, and the reaction was stirred for 1 h at -78 °C, 30 min at 0 °C, and 5 min at room temperature. Iodide **4** (8.000 g, 25.45 mmol) in 10 mL THF was added dropwise at 0 °C, then the reaction was allowed to warm to room temperature and stirred for 22 h. The reaction was quenched with 100 mL saturated NH_4Cl and diluted with 100 mL EtOAc. The layers were separated and the aqueous layer was extracted with 2 × 100 mL EtOAc. The combined organics were dried over $MgSO_4$, filtered and concentrated. The crude oil was purified by flash chromatography (30% EtOAc/Hexanes) to give 8.994 g (86.7%) of a colorless solid. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 7.43 – 7.10 (m, 5H), 4.64 – 4.51 (m,

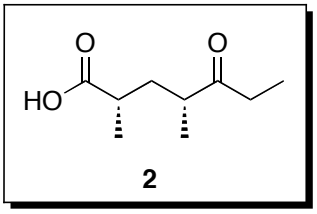
2H), 4.34 (s, 1H), 3.43 (dd, J = 9.8, 4.8 Hz, 1H), 3.36 (dd, J = 9.8, 5.9 Hz, 1H), 2.83 (s, 3H), 2.80 – 2.64 (m, 1H), 1.70 – 1.60 (m, 1H), 1.58 – 1.47 (m, 1H), 1.13 (d, J = 7.1 Hz, 3H), 1.20 – 1.04 (m, 1H), 1.07 (d, J = 6.7 Hz, 3H), 0.86 (s, 9H), 0.81 (d, J = 6.6 Hz, 3H), 0.01 (s, 6H); ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ -5.5, -5.4, 14.4, 17.3, 17.5, 18.3, 25.9, 33.1, 34.1, 37.6, 67.9, 76.5, 76.7, 77.0, 77.3, 126.2, 126.9, 127.4, 128.3, 128.7, 142.6, 179.1; HRMS ESI$^+$ (*m/z*): 430.2740 (Predicted $[M+Na]^+$ for $C_{23}H_{41}NO_3Si$ is 430.2753). As expected from literature precedent,[1] amide **5** was isolated as a mixture of amide rotamers. The reported NMR data only denotes peaks arising from the major rotamer.



6

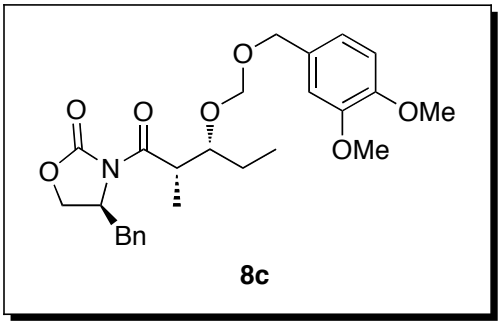
Product 6. To a stirring suspension of amide **5** (1.866 g, 4.58 mmol) in 50 mL THF was added EtLi (0.46 M in 90/10 benzene:cyclohexane, 22.89 mL, 10.53 mmol) at -78 °C. The reaction was stirred 10 min, then warmed to 0 °C and stirred an additional 30 min. Subsequently, 1 equivalent of diisopropylamine was added to scavenge excess EtLi and the mixture stirred for 15 min. The reaction was quenched with 20% AcOH/Et_2O, diluted with H_2O and EtOAc, and the layers were separated. The aqueous was extracted with 1 × 50 mL EtOAc, and the combined organics were dried over $MgSO_4$, filtered and concentrated. The crude oil was purified by flash chromatography (2% EtOAc/Hexanes) to give 1.101 g (88.3%) of a colorless oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 3.39 (dd, J = 10.3, 6.4 Hz, 1H), 3.35 (dd, J = 10.2, 6.5 Hz, 1H), 2.70 – 2.60 (m, 1H), 2.52 – 2.35 (m, 2H), 1.77 (ddd, J = 13.8, 7.9, 6.1 Hz, 1H), 1.60 – 1.44 (m, 2H), 1.06 (d, J = 6.9 Hz, 3H), 1.02 (t, J = 7.3 Hz, 3H), 0.87 (s, 9H), 0.86 (d, J = 6.4 Hz, 3H), 0.01 (s, 6H); ^{13}C NMR

($CDCl_3$, 100.5 MHz) δ -5.5, -5.5, 7.7, 17.1, 17.3, 18.2, 25.8, 33.6, 33.6, 36.9, 43.9, 67.9, 215.2; HRMS ESI^+ (*m/z*): 295.2076 (Predicted $[M+Na]^+$ for $C_{15}H_{32}O_2Si$ is 295.2069).



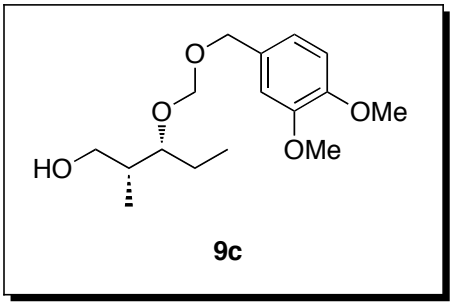
2

Product 2. Ketone **6** (1.081 g, 3.97 mmol) was dissolved in 20 mL 1:1:2 $CCl_4/CH_3CN/H_2O$ and then $RuCl_3 \cdot H_2O$ (0.082 g, 0.397 mmol) and $NaIO_4$ (4.242 g, 19.84 mmol) were added sequentially. The reaction was brought to reflux at 70 °C and heated for 16 h overnight. The mixture was cooled to room temperature, diluted with 20 mL of CH_3CN, and filtered through a plug of Celite. The plug was washed with an additional 100 mL of CH_3CN and the filtrate volatiles were removed *en vacuo*. The mixture was taken up in 50 mL EtOAc and extracted with 3 × 50 mL half saturated $NaHCO_3$. The combined aqueous portion was then back extracted with 1 × 50 mL Et_2O. The aqueous layer was then carefully acidified with dropwise addition of concentrated HCl to pH 2, then extracted with 4 × 50 mL CH_2Cl_2. The organic extracts were dried over $MgSO_4$, filtered and concentrated to give 0.564 g (82.6%) of a colorless oil. ^1H NMR ($CDCl_3$, 499.9 MHz) δ 11.86 (br s, 1H), 2.62 – 2.54 (m, 1H), 2.51 – 2.37 (m, 3H), 2.03 (ddd, *J* = 14.0, 8.9, 6.4 Hz, 1H), 1.32 (ddd, *J* = 13.7, 7.7, 5.7 Hz, 1H), 1.14 (d, *J* = 7.0 Hz, 3H), 1.05 (d, *J* = 7.0 Hz, 3H), 0.98 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.7 MHz) δ 7.7, 16.5, 17.5, 34.0, 36.1, 37.2, 43.7, 182.5, 214.6; HRMS ESI^+ (*m/z*): 195.1002 (Predicted $[M+Na]^+$ for $C_9H_{16}O_3$ is 195.0997).



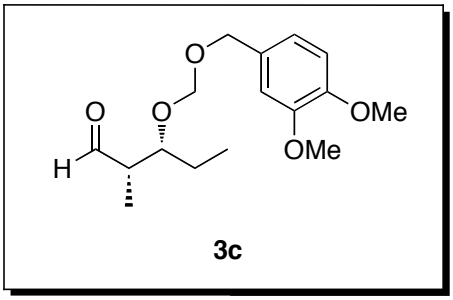
8c

Oxazolidinone 8c. To a solution of Evans aldol product **7** (2.000 g, 6.87 mmol), TBAI (0.254 g, 0.687 mmol), and DIPEA (5.98 mL, 34.32 mmol) in 50 mL CH_2Cl_2 was added DMBOMCl[14] (7.437 g, 34.32 mmol) in 20 mL CH_2Cl_2 dropwise at 0 °C. The reaction was warmed to room temperature and stirred for 16 h. The reaction was quenched with 50 mL H_2O and the layers were separated. The aqueous was extracted with 2 × 25 mL CH_2Cl_2, and the combined organics were dried over $MgSO_4$, filtered and concentrated. The crude oil was purified by flash chromatography (20% to 30% EtOAc/Hexanes) to give 3.106 g (95.9%) of a pale yellow oil that slowly solidified. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 7.32 – 7.16 (m, 3H), 7.14 – 7.06 (m, 3H), 6.88 – 6.80 (m, 2H), 6.73 (d, J = 7.9 Hz, 1H), 4.73 (d, J = 7.6 Hz, 1H), 4.71 (d, J = 7.9 Hz, 1H), 4.50 (ABq, ν_A = 31.7, ν_B = 11.5 Hz, 2H), 4.42 – 4.33 (m, 1H), 3.99 (dd, J = 9.0, 2.0 Hz, 1H), 3.96 (dd, J = 6.9, 3.9 Hz, 1H), 3.90 – 3.82 (m, 2H), 3.82 (s, 3H), 3.78 (s, 3H), 3.23 (dd, J = 13.3, 3.1 Hz, 1H), 2.67 (dd, J = 13.3, 9.9 Hz, 1H), 1.63 (qd, J = 7.7, 4.6 Hz, 2H), 1.23 (d, J = 7.0 Hz, 3H), 0.96 (t, J = 7.5 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ 10.1, 10.8, 25.6, 37.6, 41.2, 55.8, 66.0, 69.8, 80.1, 94.3, 110.7, 110.8, 119.9, 127.2, 128.8, 129.3, 130.5, 135.4, 148.5, 148.9, 153.3, 174.9; HRMS ESI$^+$ (*m/z*): 494.2160 (Predicted $[M+Na]^+$ for $C_{26}H_{33}NO_7$ is 494.2155).



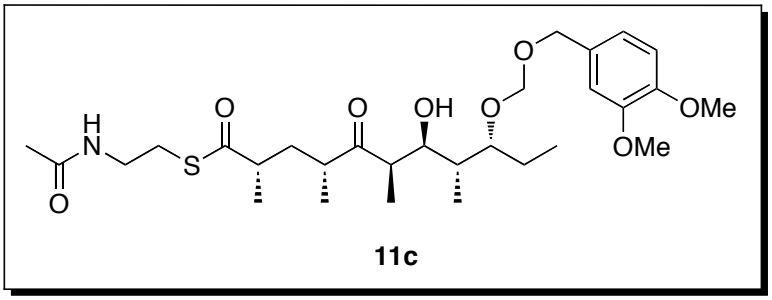
9c

Product 9c. $LiBH_4$ (2.0 M in THF, 3.83 mL, 7.66 mmol) was added dropwise to a solution of oxazolidinone **8** (3.010 g, 6.38 mmol) and MeOH (0.341 mL, 7.66 mmol) in 30 mL MTBE at 0 °C. The reaction was stirred for 20 min, then warmed to room temperature and monitored by TLC. After 3 h, the reaction was quenched with 30 mL saturated Na/K tartrate and stirred until the layers became clear. The layers were separated and the aqueous was extracted with 2 × 25 mL EtOAc. The combined organics were dried over $MgSO_4$, filtered and concentrated. The crude oil was purified by flash chromatography (30% EtOAc/Hexanes) to give 1.716 g (90.1%) of a pale yellow oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 6.91 – 6.75 (m, 3H), 4.75 (ABq, ν_A = 17.4, ν_B = 6.9 Hz, 2H), 4.55 (ABq, ν_A = 31.9, ν_B = 11.5 Hz, 2H), 3.85 (s, 3H), 3.83 (s, 3H), 3.71 – 3.58 (m, 1H), 3.58 – 3.39 (m, 1H), 2.69 (br s, 1H), 2.06 – 1.86 (m, 1H), 1.68 – 1.54 (m, 1H), 1.54 – 1.41 (m, 0H), 0.90 (t, J = 7.4 Hz, 3H), 0.82 (d, J = 7.0 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ 10.5, 10.8, 24.1, 37.6, 55.8, 55.9, 65.3, 69.9, 81.2, 94.3, 110.9, 111.1, 120.4, 130.0, 148.7, 149.0; HRMS ESI$^+$ (*m/z*): 321.1672 (Predicted $[M+Na]^+$ for $C_{16}H_{26}O_5$ is 321.1678).



3c

Product 3c. To a suspension of alcohol **9** (1.251 g, 4.19 mmol) and $NaHCO_3$ (1.761 g, 20.96 mmol) in 40 mL CH_2Cl_2 was added Dess-Martin periodinane (2.133 g, 5.03 mmol) at 0 °C. The reaction was stirred 5 min, then warmed to room temperature and monitored by TLC. After 1 h, the reaction was quenched by addition of 20 mL of half saturated $Na_2S_2O_3$ and stirred until the layers became clear. The layers were separated and the aqueous was extracted with 2 × 25 mL CH_2Cl_2. The combined organics were dried over $MgSO_4$, filtered and concentrated. The crude oil was purified by flash chromatography (20% EtOAc/Hexanes) to give 0.941 g (75.7%) of a pale yellow oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 9.78 (s, 1H), 7.10 – 6.41 (m, 1H), 4.75 (ABq, ν_A =18.1, ν_B = 7.2 Hz, 2H), 4.47 (ABq, $\nu_A = \nu_B$ = 11.4 Hz, 2H), 4.01 (td, J = 6.8, 3.4 Hz, 1H), 3.87 (s, 3H), 3.85 (s, 3H), 2.56 (qd, J = 7.0, 3.4 Hz, 1H), 1.73 – 1.64 (m, 1H), 1.63 – 1.50 (m, 1H), 1.11 (d, J = 7.0 Hz, 3H), 0.94 (t, J = 7.4 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ 7.6, 10.3, 24.8, 49.4, 55.8, 55.9, 69.7, 78.6, 93.8, 110.9, 111.3, 120.5, 130.1, 148.7, 149.0, 204.5; HRMS ESI$^+$ (*m/z*): 319.1521 (Predicted $[M+Na]^+$ for $C_{16}H_{24}O_5$ is 319.1527).

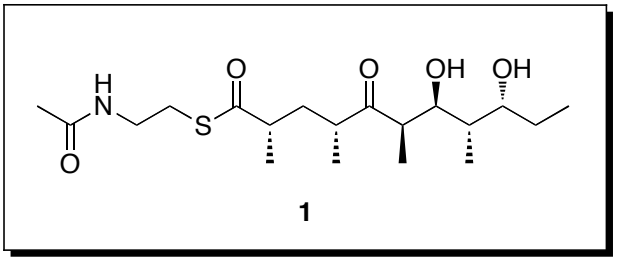


11c

Product 11c. LiHMDS (1.0 M in THF, 4.85 mL, 4.85 mmol) was added dropwise to a stirring solution of keto acid **2** (0.348 g, 2.03 mmol) and flame-dried LiCl (0.343 g, 8.10 mmol) in 10 mL THF at -78 °C. The reaction was stirred for 1 h, then warmed to 0 °C for 30 min. The reaction was then re-cooled to -78 °C, and the aldehyde **3c** (0.901 g, 3.04 mmol) in 5 mL THF was added dropwise. Following addition, the reaction was stirred for 90 min, then quenched with 20 mL saturated NH_4Cl. The mixture was diluted with 20 mL EtOAc, then extracted with 3 × 25 mL half saturated $NaHCO_3$. The aqueous extracts were back extracted with 1 × 25 mL Et_2O, then carefully acidified to pH 2 with 1 N HCl. The aqueous layer was then extracted with 4 × 25 mL CH_2Cl_2. The combined organics were dried over $MgSO_4$, filtered and concentrated. The crude oil (containing a ~7:1 anti-Felkin/Felkin mixture of *seco*-acids by ^{1}H NMR) was carried on to the next step without further purification.

The crude acid was dissolved in 20 mL CH_2Cl_2, followed by addition of HATU (1.152 g, 3.03 mmol), DIPEA (1.06 mL, 6.06 mmol) and HSNAC (0.258 mL, 2.43 mmol). The reaction became clear, and the dark yellow solution was stirred for 18 h at room temperature. The reaction was quenched with 20 mL saturated $NaHCO_3$, and the resulting layers were separated. The aqueous layer was then extracted with 2 × 25 mL CH_2Cl_2. The combined organics were dried over $MgSO_4$, filtered and concentrated. The crude oil was partially purified by flash chromatography (2% MeOH/CH_2Cl_2), then purified by preparative HPLC on a Phenomenex Luna C18(2) column (5 μm, 21.2 × 250 mm) using a CH_3CN/H_2O + 0.1% TFA gradient (10% to 100%, 60 min, 10 ml/min). The desired compound eluted between 45.5 and 46.5 (detecting at 254 nm) min to give 0.168 g (14.6%) of a colorless oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 6.90 – 6.66 (m, 1H), 6.05

(br s, 1H), 4.78 (ABq, ν_A = 23.0, ν_B = 6.8 Hz, 2H), 4.55 (ABq, $\nu_A = \nu_B$ = 11.5 Hz, 2H), 3.95 (dd, *J* = 9.5, 2.5 Hz, 1H), 3.89 – 3.85 (m, 1H), 3.87 (s, 3H), 3.85 (s, 3H), 3.51 – 3.30 (m, 2H), 3.10 – 2.89 (m, 2H), 2.82 – 2.73 (m, 2H), 2.69 – 2.62 (m, 1H), 2.09 (ddd, *J* = 14.5, 8.6, 6.2 Hz, 1H), 1.97 (s, 3H), 1.82 – 1.65 (m, 2H), 1.56 – 1.44 (m, 1H), 1.37 – 1.27 (m, 1H), 1.16 (d, *J* = 6.8 Hz, 3H), 1.08 (d, *J* = 7.0 Hz, 3H), 1.06 (d, *J* = 6.8 Hz, 3H), 0.91 (t, *J* = 7.4 Hz, 3H), 0.83 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ 8.6, 10.3, 10.7, 16.8, 18.6, 22.8, 24.6, 28.2, 36.8, 37.7, 39.8, 42.0, 46.4, 46.4, 55.8, 55.9, 69.8, 72.0, 80.6, 94.6, 110.9, 111.1, 120.4, 130.0, 148.6, 149.0, 171.4, 203.7, 217.7; HRMS ESI$^+$ (*m/z*): 592.2933 (Predicted $[M+Na]^+$ for $C_{29}H_{47}NO_8S$ is 592.2920).



1

Product 1. DDQ (0.018 g, 0.079 mmol) was added to **11** (0.009 g, 0.016. mmol) in a biphasic mixture of 4:1 CH_2Cl_2/0.5 M phosphate buffer, pH 7.0 at 0 °C. The reaction was warmed to room temperature and monitored by TLC. The reaction was quenched with 5 mL saturated $NaHCO_3$, and diluted with 10 mL CH_2Cl_2. The layers were separated and the aqueous was extracted with 2 × 10 mL CH_2Cl_2. The combined organics were dried over $MgSO_4$, filtered and concentrated. The crude material was resuspended in 1 mL CH_3CN, run through a 0.22 μm hydrophobic syringe filter (Millipore), and then purified by preparative HPLC on a Phenomenex Luna C18(2) column (5 μm, 21.2 × 250 mm) using a CH_3CN/H_2O gradient (10% to 100%, 60 min, 10 ml/min). The desired compound eluted at 33 and 37.5 min (detecting at 240 nm) to give 2.4 mg (39%) of a

colorless oil as a ~2:1 equilibrium mixture of open linear pentaketide and closed hemiketal. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 5.91 (br s, 0.35H), 5.85 (br s, 0.65H), 3.91 (dt, *J* = 8.8, 2.8 Hz, 0.65H), 3.87 – 3.73 (m, 1.35H), 3.58 – 3.27 (m, 3H), 3.10 – 2.97 (m, 2H), 2.85 (ddd, *J* = 14.2, 7.1, 2.9 Hz, 1H), 2.81 – 2.61 (m, 2H), 2.12 (ddd, *J* = 14.4, 8.7, 5.8 Hz, 1H), 1.97 (s, 2H), 1.96 (s, 1H), 1.90 – 1.76 (m, 2H), 1.73 (ddd, *J* = 9.0, 7.0, 2.0 Hz, 0.65H), 1.64-1.50 (m, 1.65H), 1.48 – 1.31 (m, 2H), 1.23 (d, *J* = 7.0 Hz, 1H), 1.20 (d, *J* = 6.9 Hz, 2H), 1.14 (d, *J* = 7.2 Hz, 2H), 1.11 (d, *J* = 6.9 Hz, 2H), 0.98 (t, *J* = 7.4 Hz, 2H), 0.96 (d, *J* = 5.8 Hz, 1H), 0.95 (d, *J* = 6.5 Hz, 1H), 0.89 (t, *J* = 7.4 Hz, 1H), 0.84 (d, *J* = 7.0 Hz, 2H), 0.82 (d, *J* = 6.7 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ 4.5, 9.5, 10.4, 11.0, 11.0, 15.0, 16.3, 16.4, 18.6, 19.8, 23.2, 25.2, 26.4, 28.4, 28.5, 29.7, 34.7, 35.9, 36.3, 37.9, 38.9, 42.7, 45.6, 46.3, 47.4, 71.8, 73.0, 73.6, 74.4, 76.7, 100.4, 170.4, 203.6, 218.5; HRMS ESI$^+$ (*m/z*): 412.2119 (Predicted $[M+Na]^+$ for $C_{19}H_{35}NO_5S$ is 412.2134).

3.8 References

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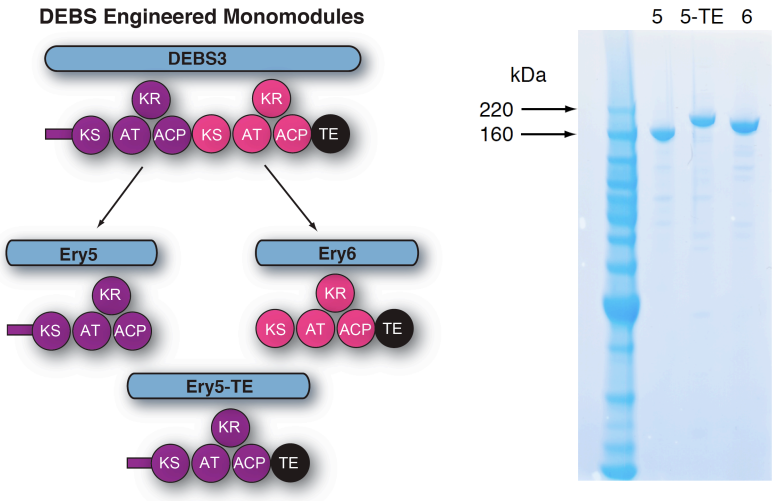
Chapter 4

Biochemical Interrogation of Molecular Specificity in the Erythromycin and Pikromycin PKSs

4.1 Biochemical Analysis of DEBS Modules 5 and 6

With the native DEBS pentaketide substrate in hand, we first focused our attention toward a rigorous biochemical study of the final DEBS modules 5 (Ery5) and 6 (Ery6). Recombinant Ery5 and Ery6 monomodules were cloned, expressed and purified, as well Ery5-TE containing an unnatural C-terminal fusion of the terminal DEBS thioesterase (TE) to allow for cyclization of extended polyketide products (**Figure 4.1**).

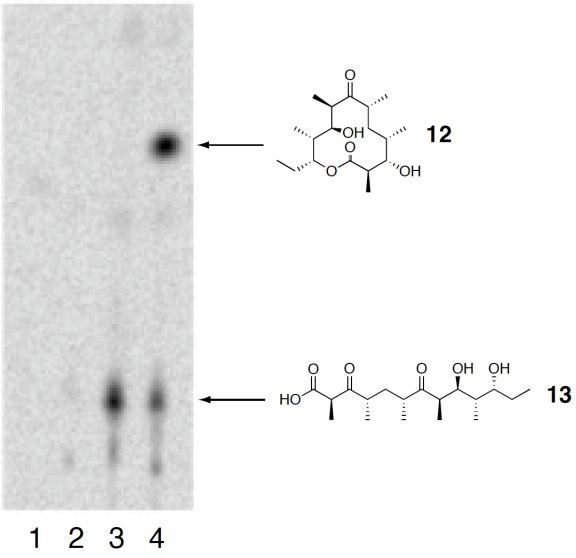
Figure 4.1 – Illustration of the engineering of DEBS monomodules from the DEBS3 bimodular protein (left) and SDS-PAGE of purified monomodules Ery5, Ery5-TE and Ery6 (right).



The engineered Ery5, Ery5-TE and Ery6 were incubated with the synthetic DEBS pentaketide SNAC substrate and 2-[^{14}C]-methylmalonyl CoA, and the products were

visualized by radio-TLC. Duplicate reactions were also performed utilizing unlabeled methylmalonyl CoA, and reaction products were analyzed and confirmed by LC-MS/MS. As demonstrated previously *in vivo,*[1] the Ery5-TE fusion protein also generates an unnatural 12-membered ring macrolactone (**12**) *in vitro* (**Figure 4.2** and **4.3**; **Scheme 4.1**). This confirms that DEBS TE is capable of producing 12-membered macrocycles, analogous to previous observations of flexibility in the Pik TE.

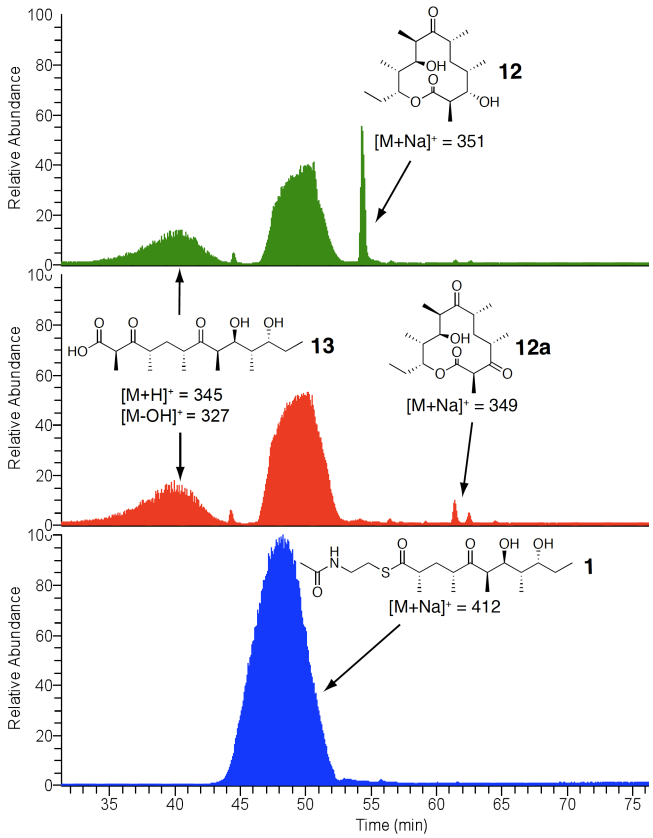
Figure 4.2 - Radio-TLC of reaction products for Ery5-TE incubated with DEBS pentaketide SNAC. Assay conditions are described in the Experimental Section. Lane 1: No substrate (negative control); Lane 2: No enzyme (negative control); Lane 3: Ery5-TE w/o NADPH; Lane 4: Ery5-TE w/ NADPH.



However, macrolactone **12** occurs with significant formation of linear hydrolysis products. Interestingly, in the absence of NADPH cofactor the reaction showed little production of the predicted 3-oxo macrocyclic derivative (**12a**), but instead converted intermediates to an unreduced hexaketide *seco*-acid **13**. This suggests the possibility of a critical hydrogen-bonding interaction in the DEBS TE active site involving the β-position of the substrate. Thus, in comparison to the Pik TE where reduction of the enone functionality in the substrate to the allylic C-7 alcohol abrogated cyclization,[2] subtle functional group changes can also significantly affect the ability of DEBS TE to catalyze

macrocyclization. Also of note was the formation of some **13** even when NADPH was present in excess with Ery5-TE. This suggests a kinetic competition between reduction of the ACP-bound hexaketide by the Ery5 ketoreductase (KR) domain followed by TE-mediated macrolactonization, or premature transfer of unreduced hexaketide to the TE active site prior to reduction resulting in hydrolytic release. *Seco*-acid **13** was not previously reported from *in vivo* studies using an engineered DEBS1-DEBS2-M5-TE system,[1] suggesting that its formation *in vitro* might be caused by suboptimal enzymatic processing from the unnatural Ery5-TE fusion protein.

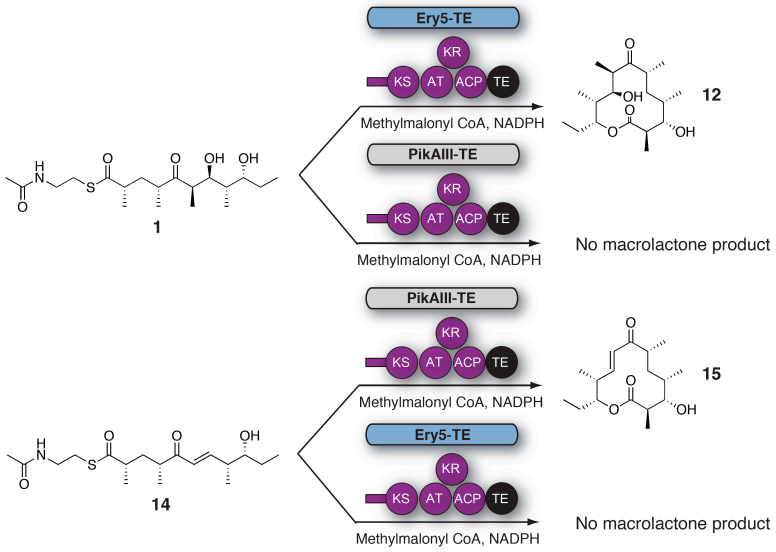
Figure 4.3 - LC-MS chromatogram in selective ion mode (SIM) of Ery5-TE incubated with DEBS pentaketide SNAC. Top: Ery5-TE (green); Middle: Ery5-TE w/o NADPH (red); Bottom: No enzyme control (blue).



Next, further probing of the DEBS monomodules was carried out in Ery5 lacking the terminal thioesterase fusion and the final module Ery6. Radioassay of Ery5 and Ery6

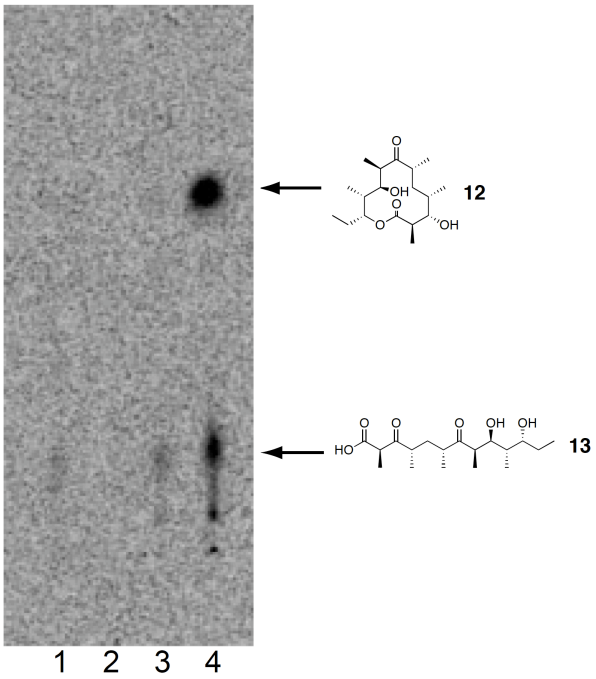
showed that both modules appeared to generate low, but detectable levels of product when diffusively loaded with the DEBS pentaketide (**Figure 4.4**) but required further structural confirmation to confirm their identity (*vide infra*). A low level or lack of product formation was expected since Ery5 does not have a thioesterase domain to catalyze chain release, and the pentaketide is not the native substrate for Ery6. Since the total synthesis of the DEBS hexaketide chain elongation intermediate proved problematic due to its facile degradation, Ery6 could not be evaluated with its native substrate at this time. As expected, the pairing of Ery5-Ery6 did not generate 6-DEB, since the engineered monomodules are now physically distinct and lack native docking domains[3] (unlike in monomodules PikAIII and PikAIV) to mediate polyketide chain transfer between the separated polypeptides. LC-MS/MS analysis confirmed that despite lacking a TE domain, detectable amounts of unreduced hexaketide *seco*-acid **13** are released from Ery5 presumably through adventitious hydrolysis of the intermediate from the Ery5 ACP.

Scheme 4.1 - Illustration of *In Vitro* Enzymatic Reactions for Cognate and Non-Cognate Pentaketide/Module 5 Pairs from the Pik and DEBS PKSs.



Addition to the reaction mixture of either the DEBS or Pik TE *in trans* with Ery5, however, did not restore production of **12**. This contrasts with the pikromycin system where Pik TE is able to function *in trans* to catalyze formation of 10-Dml from PikAIII both *in vivo*[4] and *in vitro*.[5] Remarkably, we observed that Ery6 was able to accept the DEBS pentaketide resulting in low levels of macrolactone **12** and hydrolysis product **13** (**Figure 4.5**). This demonstrates an unexpected flexibility in this module toward incoming substrates bearing non-cognate functionality and chain length.

Figure 4.4 - Radio-TLC analysis of Ery5 and Ery6 with DEBS pentaketide SNAC. Lane 1: Ery5 + Ery6; Lane 2: Ery5; Lane 3: Ery6; Lane 4: Ery5-TE (positive control).

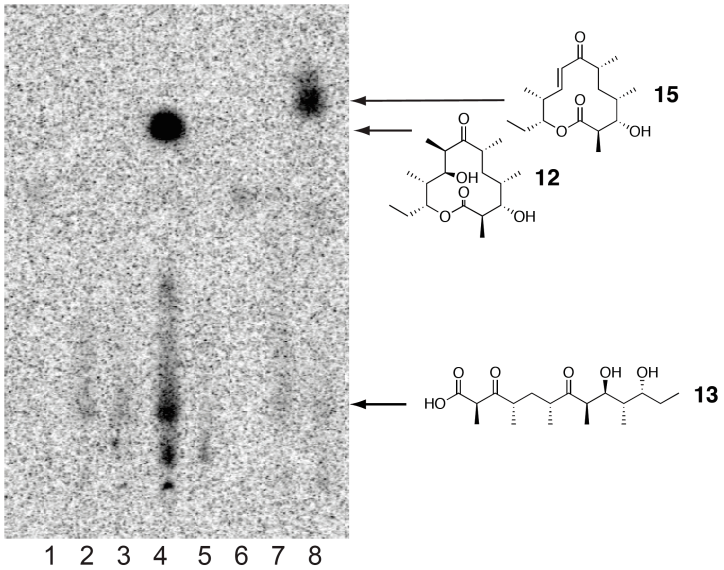


4.2 Cross Reactivity Between the DEBS and Pik Systems

In order to probe the substrate specificity of the DEBS and Pik late modules, both DEBS pentaketide (**1**) and Pik pentaketide (**14**) substrates[6] were assessed for their ability to be loaded, extended, processed and cyclized in unnatural substrate/PKS combinations (e.g. DEBS pentaketide SNAC against PikAIII-TE, Pik pentaketide SNAC against Ery5-

TE) (**Scheme 4.1**). Radio-TLC of the reaction products did not show detectable levels of macrolactones **12** or 10-Dml (**15)** relative to controls with either PikAIII-TE or Ery5-TE in reactions containing the respective non-native pentaketide substrate (**Figure 4.6**). LC-MS/MS analysis of the reactions also did not provide detectable levels of these macrolactones, instead showing the major products to be the pentaketide *seco*-acids, most likely arising from hydrolysis of the parent SNAC compounds by the terminal thioesterase domains. With PikAIII-TE, this hydrolytic activity is especially pronounced, resulting in essentially complete hydrolysis of the SNAC substrate.

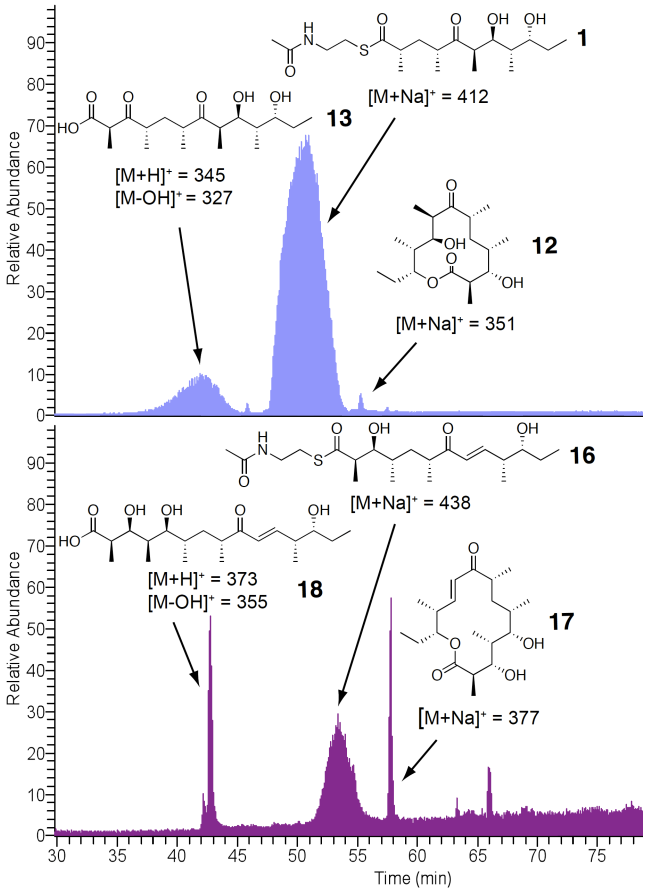
Figure 4.5 - Radio-TLC of non-cognate pairing of Pik/DEBS pentaketide SNACs incubated with Ery5-TE and PikAIII-TE. Lane 1: No enzyme w/ Pik pentaketide (negative control); Lane 2: Ery5-TE w/ Pik pentaketide (No NADPH); Lane 3: Ery5-TE w/ Pik pentaketide; Lane 4: Ery5-TE w/ DEBS pentaketide (positive control); Lane 5: No enzyme w/ DEBS pentaketide (negative control); Lane 6: PikAIII-TE w/ DEBS pentaketide (No NADPH); Lane 7: PikAIII-TE w/ DEBS pentaketide; Lane 8: PikAIII-TE w/ Pik pentaketide (positive control).



PikAIII-TE incubated with the DEBS pentaketide SNAC revealed low levels of hexaketide hydrolysis product **13** by LC-MS/MS, indicating that some substrate can be accepted and extended by the enzyme, albeit very inefficiently. To assess the influence of the TE domain in these cross-PKS assays, non-cognate modules/substrates were

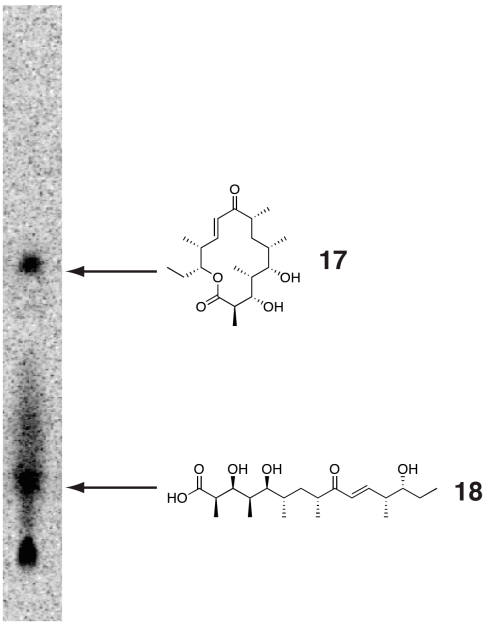
incubated together, but native TEs were added *in trans* to determine if turnover could be enhanced. Ery5 was thus incubated with Pik pentaketide and the Pik TE, and PikAIII was incubated with the DEBS pentaketide and the DEBS TE. Neither reaction, however, showed detectable levels of macrolactones by radio-TLC or LC-MS/MS.

Figure 4.6 - SIM LC-MS chromatogram of Ery6 reactions. Top: Ery6 w/ DEBS pentaketide SNAC (light blue) Bottom: Ery6 w/ Pik hexaketide SNAC (purple).



Unlike the experiments involving interrogation of non-cognate pentaketide/module 5 pairs, the module 6 PKSs from Pik and DEBS demonstrated some flexibility toward non-native substrates. Ery6 was able to accept, extend, process and cyclize the non-native Pik hexaketide[7] (**Scheme 4.2**), though not the Pik pentaketide.

Figure 4.7 - Radio-TLC of Ery6 incubated with Pik hexaketide SNAC.

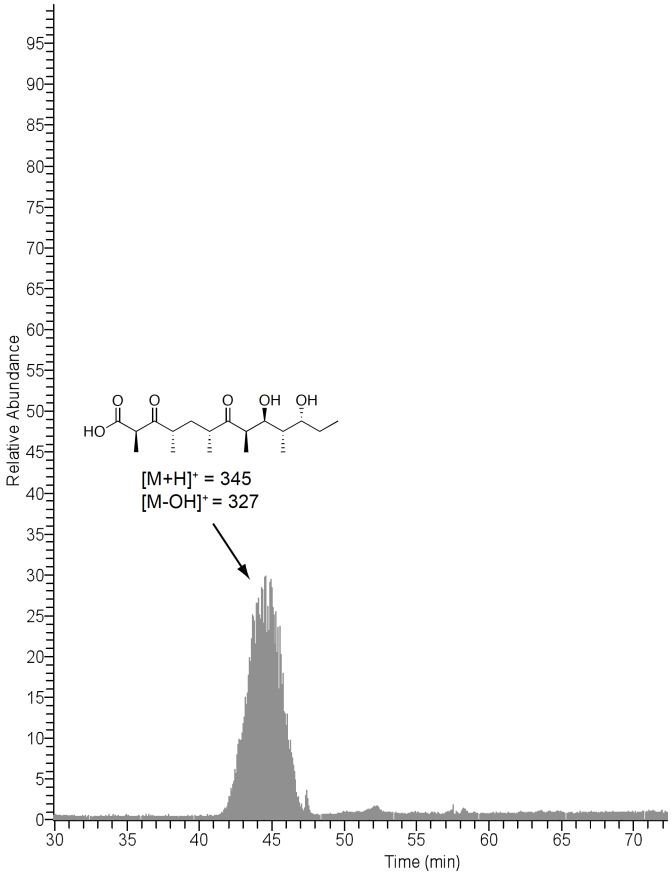


When Ery6 was incubated with Pik hexaketide (**16**), both 3-hydroxy-narbonolide (**17**) and hydrolyzed linear heptaketide (**18**) were observed in the radio-TLC (**Figure 4.7**) and LC-MS/MS assay (**Figure 4.5**). In this case, no competition between ketoreduction and cyclization was detected. Since DEBS hexaketide currently remains unavailable, PikAIV tolerance could not be evaluated in an analogous experiment. Interestingly, when the DEBS pentaketide was incubated with PikAIV, detectable levels of hexaketide *seco*-acid **13** were generated, though the predicted cyclized product **12a** was absent. This observation conforms to the current hypothesis regarding ring-formation requirements of the Pik TE, where a rigid enone at C7-C9 was revealed to be essential for cyclization of the Pik hexaketide intermediate by the thioesterase.[2] As had been observed previously *in vitro*,[8] the Pik pentaketide was not accepted by PikAIV to produce 3-oxo-10-Dml.

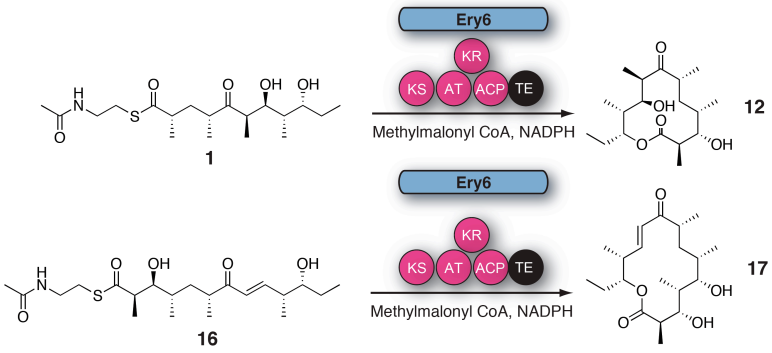
Finally, bimodular pairing of PikAIII-PikAIV did not result in production of predicted macrolactones **12** or 3-oxo-6-DEB when incubated with the DEBS pentaketide. This is consistent with the rigid molecular specificity exhibited by PikAIII toward the

DEBS pentaketide. Nonetheless, hydrolyzed hexaketide **13** was observed in this bimodular context (**Figure 4.8**), the result of direct loading, extension and hydrolysis on the more flexible PikAIV monomodule.

Figure 4.8 - SIM LC-MS chromatogram for DEBS pentaketide SNAC incubated with PikAIII-PikAIV.



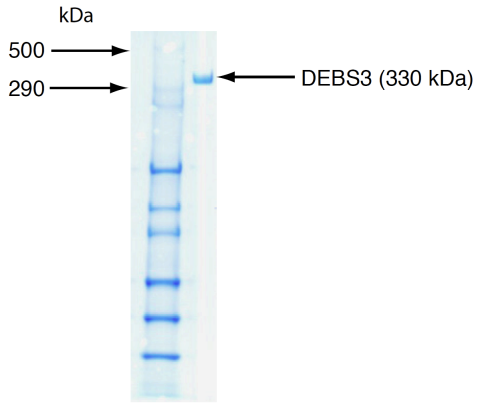
Scheme 4.2 - Interrogation of Ery6 with Non-Native DEBS Pentaketide and Pik Hexaketide (**16**) Substrates.



4.3 Chemoenzymatic Synthesis of 6-DEB

Following our biochemical analysis of the individual DEBS monomodules 5 and 6, we sought to further our modular PKS work with the native bimodular DEBS3 protein. Despite extensive efforts in the DEBS system, until recently,[9, 10] active DEBS3 had not been expressed and purified recombinantly as a stand alone enzyme. Rather, previous work with DEBS3 had primarily relied on utilizing cell-free preparations of DEBS3 from heterologous expressions in *Streptomyces coelicolor*[11] or *E. coli*.[12] As a result, we sought to clone and express recombinant DEBS3 in *E. coli* as a C-terminally His-tagged protein that could be purified and used for *in vitro* assays.

Figure 4.9 – SDS-PAGE analysis of purified DEBS3.



After successful cloning, expression and purification of DEBS3 (**Figure 4.9**), we assessed its ability to chemoenzymatically generate 6-DEB (**19**) from our synthetic DEBS pentaketide substrate (**Scheme 4.3**). We were pleased to observe clear formation of **19** by LC-MS analysis when DEBS3 was incubated with the DEBS pentaketide intermediate, with no detectable levels of non-cyclized hydrolysis products (**Figure 4.10**). As expected, formation of putative product **17** was not detected when DEBS3 was

incubated the Pik pentaketide, which is in line with our previous observations of molecular stringency in DEBS module 5.

Scheme 4.3 – Incubation of DEBS and Pik Pentaketide Intermediates with the DEBS3 Bimodular Protein.

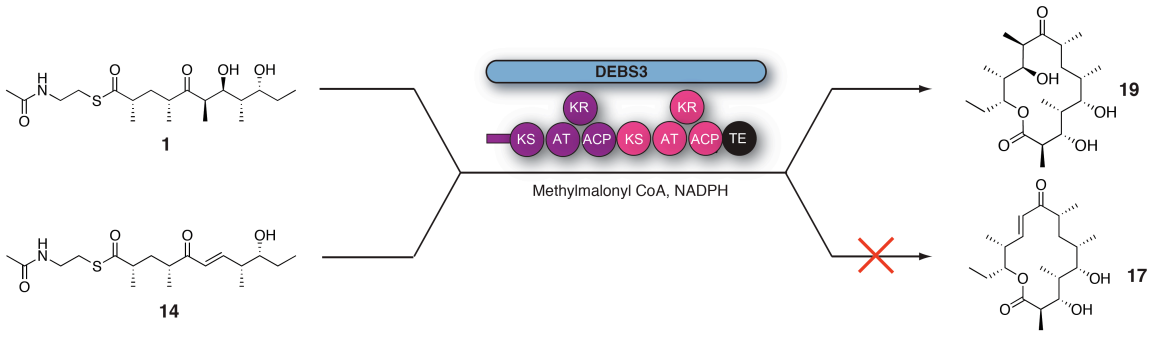
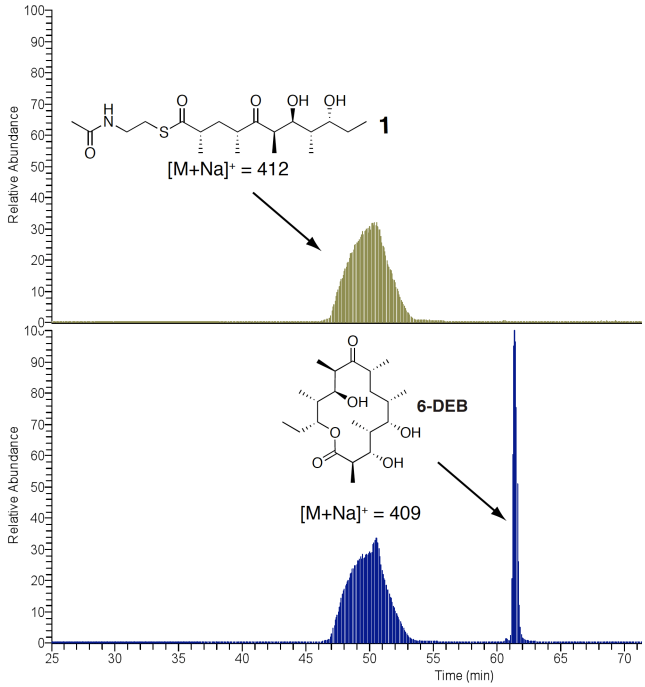


Figure 4.10 – SIM LC-MS chromatogram from DEBS pentaketide incubated with DEBS3. Top panel: No enzyme (gold). Bottom panel: DEBS3+DEBS pentaketide SNAC (blue).



4.4 Discussion

Analysis of the catalytic abilities of the dissected DEBS monomodules revealed distinctive profiles relative to their naturally occurring counterparts in the Pik system. From these studies, the macrocyclization activity of the DEBS and Pik TEs was revealed

to be a key issue, especially in the formation of 12-membered ring products. In Pik, the ability to form 12-membered macrocycles is well evolved, giving nearly exclusive formation of 10-Dml or 3-oxo-10-Dml (when NADPH cofactor is excluded) when presented with the natural hexaketide substrate. However, with the DEBS TE, the ability to generate the analogous macrolactones **12** and **12a** competes significantly with hydrolysis activity. This inefficiency appears to stem from incomplete ketoreduction by the KR of Ery5, followed by subsequent difficulty in cyclizing the resultant 3-oxo hexaketide derivative by DEBS TE. It appears that the unnatural TE fusion facilitates facile cyclization/hydrolysis of unreduced hexaketide prior to reduction by the KR. The reason for inefficient KR function is unclear, as it has not been reported in any previous studies performed *in vitro*[13,14] or *in vivo*[1] with DEBS module 5. In this case, the interplay of multiple factors could cause premature cyclization/hydrolysis of the acyl chain from Ery5-TE, though structural issues related to the ACP-TE fusion appear likely to be strong contributors. Since the TE fusion and linker region are unnatural in this engineered monomodule, this perturbation might result in premature transfer of extended hexaketide to the TE active site prior to complete processing by the ketoreductase. This possibly results from a decrease in the rate of Ery5 modular catalytic events, an increase in the rate of intermediate transfer to the thioesterase from the Ery5 ACP, or a combination of both. Similar competition between reduction and cyclization has also been reported previously with the analogous engineered PikAIII-TE, though this occurred only when the module was incubated with diketides[15] rather than native substrates. With native Pik pentaketide, no 3-oxo-10-Dml was observed with PikAIII-TE when NADPH was included.[8] Thus, unlike Ery5-TE, incomplete reduction in PikAIII-TE is likely a substrate-driven process

rather than a result of enzyme structural elements that perturb its function. In studies with the full DEBS3 protein, incubation with the synthetic DEBS pentaketide SNAC gave 6-DEB as the exclusive product by LC-MS/MS. In this case, these competing elements between reduction and cyclization were not observed. This was not surprising as DEBS3 is unaltered from its native bimodular configuration. These results highlight the critical nature of understanding modular structural elements in PKS engineering efforts.

The differing cyclization activities of the Pik and DEBS TEs suggest unique substrate binding modes within their active sites. The oxidation state at the β-position is important for TE-mediated cyclization in DEBS, but not in Pik. Thus, it can be reasoned that the β-hydroxy group in the extended DEBS hexaketide acts as a hydrogen-bond donor in the TE active site, creating an interaction that is vital for efficient cyclization. This would not be surprising, as the TE has evolved to generate its sole product 6-DEB from the native heptaketide intermediate, which also contains a β-hydroxy group. Abrogation of this interaction by replacement with a keto group could result in attenuated cyclization. It has been proposed from modeling of 6-DEB into the active site of the DEBS TE crystal structure that the 3-position hydroxyl participates in a hydrogen-bond with Asn-180 and the backbone carbonyl of Tyr-171,[16] which is consistent with this observation. This contrasts sharply with the hypothesized binding mode for Pik TE, which has been shown from structural studies using affinity-label probes to have minimal active site hydrogen-bonding interactions with incoming substrates.[17] Here, the oxidation state at the β-position does not have a critical effect on macrolactonization, as Pik TE efficiently cyclizes both a hexaketide containing a β-hydroxy and a heptaketide containing a β-keto group to give 10-Dml and Nbl, respectively.[8]

While there is evidence that Pik TE has developed direct protein-protein interactions with PikAIII[5] to enable formation of 10-Dml from this monomodule, Ery5 and the DEBS TE appear to be unable to engage in a similar type of molecular recognition. Providing DEBS TE or Pik TE *in trans* to substrate-bound Ery5 does not enhance release of macrolactone products. Similarly, excised DEBS TE provided *in trans* also failed to catalyze release of intermediates from PikAIII, strongly suggesting that PikAIII and Pik TE are capable of engaging in a unique type of molecular interaction.

When PKS substrate specificity is considered, both Pik and DEBS module 5 appear to have a strong preference for their native pentaketide chain-elongation intermediates. Both modules (with engineered TE domains) are capable of efficiently accepting and processing their respective pentaketide substrates and cyclizing them to 12-membered ring macrolactones. However, when incubated with the pentaketide from the reciprocal system, neither module was tolerant toward the non-native substrate, despite high structural similarity that differs only at C6-C7 of the respective polyketide chains. Also, when analogous biochemical analysis was performed with the full bimodular DEBS3 protein, the same level of stringency was observed, as the Pik pentaketide did not appear to be accepted to form the putative macrolactone product 3-hydroxy-narbonolide. These results suggest that even small functional changes distal to the enzyme acyl-thioester linkage are capable of precluding efficient PKS activity. This contrasts with earlier precursor-directed biosynthesis studies in DEBS, where distal functional changes from unnatural starter units were well-tolerated by downstream modules and incorporated into 6-DEB analogues.[18] These modifications at the terminus of the chain elongation intermediate, however, are located at a position more remote than those in the substrates

from the current studies. Further analysis is thus required to determine whether the substrate-loading event, chain-elongation/processing/termination or all of these steps represent potential roadblocks to catalysis. Previously, it has been shown that the PKS channeling mechanism plays a key role in substrate loading, and that diffusive loading of an acyl-SNAC may not reflect the authentic molecular recognition features of an individual module. In those studies, inherently poor diketide model substrates that could not be accepted by diffusive loading were accepted by PKS modules if presented as appropriate upstream ACP-bound intermediates.[14] Nonetheless, these suboptimal substrates were still extended and cyclized (as triketide latones) at much lower rates than optimized diketide model compounds. Since these current studies have relied on diffusive loading, it might be informative to assess the contributions of channeling to the specificity of the substrate-loading event with native chain elongation intermediates.

In contrast to Ery5, Ery6 appears to have significantly increased flexibility toward non-native substrates. Ery6 was capable of accepting, extending and cyclizing the non-cognate DEBS pentaketide and Pik hexaketide substrates, though not the Pik pentaketide intermediate. This was surprising as the DEBS pentaketide and Pik hexaketide not only have differing chain lengths (10 and 12 carbons, respectively), but also differ in their substitution and stereochemistry at the proximal α and β positions as well as at more distal positions. The fact that the Pik pentaketide was not accepted also suggests a key recognition feature at C6-C7 in Ery6, as these are the only chemically unique positions compared to DEBS pentaketide. It is possible that the rigid double bond is structurally untenable for Ery6; however, the Pik hexaketide also contains an alkene, although its location is shifted to the C8-C9 position. Thus, since the presence of the enone alone

cannot explain poor activity, the possibility remains that the position of the enone is a key consideration. To a lesser degree than Ery6, PikAIV also demonstrated flexibility in its ability to accommodate the DEBS pentaketide, catalyzing extension to the hydrolyzed linear hexaketide *seco*-acid **13**; however, the Pik TE could not cyclize the pendant DEBS hexaketide intermediate to the expected macrolactone **12a** in this reaction. At the same time, the failure of PikAIV to accept the Pik pentaketide indicates that this substrate may not be well tolerated outside of its native module PikAIII.

Taken together, the substrate selectivity profiles that have emerged from these studies for the late modules from the DEBS and Pik PKSs suggest rigidity in their fifth modules with pentaketide substrates, yet tolerance in their sixth and final module. Module 5 in these PKSs may act as stringent molecular gatekeepers, especially for diffusive loading of the KS, as module 5 must mediate intermodular transfer of intermediates from the upstream module 4, and promiscuous loading of the exposed KS active site could result in competing enzymatic pathways or domain inactivation. Module 6, on the other hand, appears to lack this level of stringency. In the native bimodular context of DEBS3, the Ery6 KS accepts chain-elongation intermediates in an intramodular fashion and is likely inaccessible to diffusive loading. Thus, increased flexibility would not be detrimental to overall modular efficiency and substrate stringency would be unnecessary. Due to its monomodular architecture, PikAIV also contains an accessible KS active site and must accept intermediates through an intermodular transfer. Thus, we might expect that PikAIV would exhibit a similar level of molecular specificity as PikAIII or Ery5. Nonetheless, PikAIV is capable of accepting

complex non-native intermediates, though more rigorous interrogation is necessary to fully assess its specificity.

Based on this comparative analysis, the late modules in the DEBS and Pik polyketide synthases have evolved unique specificity profiles and catalytic capacities. Specific insights have been gained toward understanding key recognition features necessary for TE-mediated cyclization in these systems, which is vital for efficient formation of new macrolactone scaffolds utilizing TE domains as biocatalysts. This new information also provides an additional basis for future bioengineering efforts involving a range of PKS modules. Due to their promiscuity, both Pik and DEBS module 6, especially Ery6, appear to be excellent candidates for engineering of new biosynthetic pathways.

4.5 Experimental

Substrate Synthesis. The DEBS pentaketide SNAC synthesis is detailed in Chapter 3. The pikromycin pentaketide and hexaketide SNAC substrates were prepared as previously described.[8]

Molecular Cloning. *E. coli* XL-1 Blue or DH5α was used as a bacterial host for manipulation of plasmid DNA and standard molecular cloning techniques were employed throughout the construction of the DEBS overexpression clones. DNA sequence analysis of all clones generated in this study was performed at the University of Michigan DNA sequencing core facility. PCR amplifications were performed using pFU Turbo DNA Polymerase (Stratagene), LA-Taq DNA polymerase (Takara Bio) or KOD Extreme Hot Start DNA polymerase (Novagen).

The DEBS module 5 was amplified from cosmid pDHS1178, previously identified to contain the DEBS PKS gene cluster, by PCR using the following primer pair: 5' – GGGAGGAGCATATGAGCGGTGACAACGGC – 3' and 5' – GAGGGATCCTTAGAGCCGCTCCAGGTAGTG – 3'. Following restriction digestion with *Nde*I and *Bam*HI, the purified PCR product was ligated into similarly digested pET28b to give the Ery5 overexpression plasmid pDHS1177. Similarly, the DEBS module 6 was PCR amplified from the following pair of oligonucleotides: 5' – GAGGCTAGCGACCCGATCGCGATCG – 3' and 5' – CTCCTCCTCGAGTCATGAATTCCCTCCGCCCAG – 3'. Upon restriction digest with *Nhe*I and *Hind*III, the PCR product was cloned into pET21b to give the Ery6 expression plasmid pDHS1174. The unnatural fusion protein, Ery5-TE, DEBS module 5 was constructed in two steps. First, module 5 was PCR amplified as above using primers designed with engineered *Nde*I and *Bam*HI restriction sites; however, in this case, the *Bam*HI containing reverse primer was slightly redesigned to eliminate inclusion of the stop codon. The resulting PCR fragment was ligated into pET21b following restriction digestion with *Nde*I and *Bam*HI to give plasmid pJKDEBS5. In a second PCR reaction, DNA encoding the DEBS TE was amplified from the *Bam*HI restriction site containing primer 5' – GAGGGATCCCAGCAGCTCGACAGCGGGACT – 3' and the *Hind*III restriction site containing primer 5' – CTCCTCCTCGAGTCATGAATTCCCTCCGCCCAG – 3'. The doubly digested PCR product was subsequently cloned into similary-digested pJKDEBS5 to yield the Ery5-TE overexpression plasmid pDHS1172. Cloning of PikAIII, PikAIII-TE, PikAIV, Pik TE and DEBS TE have been previously described.[19-21]

E. coli (DE3) host overexpression plasmid pDEBS3 containing *eryAIII* gene (NCBI Accession Number: NC009142) encoding module 5 – 6 – TE of erythromycin biosynthetic pathway, with an in-frame C-terminal 6xHis tag was generated as follows. In the initial step, unnatural erythromycin module 5 – TE chimeric gene fusion was PCR amplified using primers 5' – CAACATATGAGCGGTGACAACGGCATGACC – 3' and 5' – CTAAGCTTTCCTGAATTCCCTCCGCCCAGCCAG – 3', with pDHS1172 as template (Mortison et al. *J. Am. Chem. Soc.*, **2009**, *131*, 15784-15793). The PCR product and plasmid pET24b+ (Novagen) with desired C-terminal His 6x tag were doubly digested with NdeI and HindIII and ligated together to generate pET24b+DEBS3Trunk. To generate entire module 5 – 6 – TE expression plasmid, module 6 was introduced between module 5 – TE fusion in pET24+DEBS3Trunk using unique restriction sites within the module 5 – TE. Restriction sites BbvCI and EcoRI were determined to cut the *eryAIII* open reading frame at 3475 bp and 9507 bp respectively. These enzymes were also determined to not nick the backbone of the plasmid pET24+DEBS3Trunk. Using primers 5' – CTGCTGGGCAGGCGCGGTGC – 3' and 5' – CTAAGCTTTCCTGAATTCCCTCCGCCCAGCCAG – 3' and genomic DNA of *Saccharopolyspora erythraea* as a template, a 6.05 kb DNA product containing part of module 5, all of module 6 and TE was amplified. The PCR product and the pET24b+DEBS3Trunk were then digested with BbvCI and EcoRI, size-selected and purified for ligating together. The clones were confirmed by restriction analysis mapping and DNA sequencing to be the desired module 5 – 6 – TE (DEBS3) encoding clones with an in-frame C-terminal His 6x tag fusion.

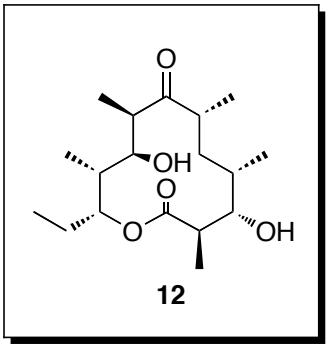
Protein Expression and Purification. The expression and purification of all proteins has been previously reported[9, 10, 19, 21, 22] and similar protocols were followed in this study. The expression of each module containing polypeptide (PikAIII, PikAIV, PikAIII-TE, Ery5, Ery6, Ery5-TE) was accomplished in the *E. coli* (BAP1) expression strain, a bacterial strain that has been genetically engineered to express type I modular PKS proteins.[12] This *E. coli* strain encodes the phosphopanteitheinyl transferase (*pptase*) gene from *B. subtilis* on its chromosome. Expression of the heterologous Pptase ensures the efficient post-translational modification of ACP domains. *E. coli* (BAP1) was generously provided to the Sherman laboratory by Stanford University Professor Chaitain Khosla. Expression of DEBS3 was achieved in BAP1 co-transformed with the pRARE plasmid, provided by Clay Brown of the University of Michigan Life Sciences Institute High Throughput Protein (HTP) Lab. This plasmid contains genes for the overexpression of tRNAs for rare codons in *E. coli*. *E. coli* BL21(DE3) was used to express Pik TE and DEBS TE. Cultures were grown at 37 °C with shaking to an OD_{600} of 0.8-1.0 in TB media. Protein expression was induced with the addition of IPTG to a final concentration of 0.4 mM. The cultures continued to incubate, with shaking, at 18° C for 16-18 hours (overnight).

Each polyhistidine-tagged protein was subsequently purified by nickel-affinity chromatography. Cells from overexpression cultures (typically 1 L) were harvested by centrifugation (5000g, 12 min, 4 ºC) and resuspended in approximately 25 mL lysis buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 10% glycerol, 20 mM imidazole). Following membrane disruption via sonication (6 x 20 s with 30 s intervening between pulses), the cellular debris was pelleted by centrifugation (40,000g, 30 minutes, 4 ºC). The cell free

lysate was sterile filtered with a 0.45 mm syringe filter after which it was directly loaded onto a 5 mL his-trap column using an AKTA FPLC (GE Healthcare). Polyhistidine-tagged proteins were eluted with a linear gradient of lysis buffer with 400 mM imidazole and fractionated into 4 mL fractions. Protein containing fractions, as identified by A280 intensities and confirmed by SDS-PAGE, were pooled, concentrated and exchanged into storage buffer (100 mM NaH_2PO_4, pH 7.2, 1 mM EDTA, 1 mM TCEP, 20% glycerol) using PD-10 desalting columns (GE Healthcare). Protein purity was confirmed to be greater than 95% by SDS-PAGE analysis. Purified proteins were dispensed into 100 μL aliquots, flash frozen in liquid nitrogen and stored at -80 °C. Final protein concentrations were determined A280 measurements, from theoretical extinction coefficients.

Biochemical Assay of DEBS and Pik monomodules. Reactivity of PikAIII, PikAIV, Ery5, Ery5-TE, Ery6 and DEBS3 with native pentaketide substrates was assessed by incorporation of 2-[^{14}C]-methylmalonyl CoA to form extended polyketide products. 2-[^{14}C]-methylmalonyl CoA (55.5 mCi/mmol) was purchased from American Radiolabeled Chemicals and diluted to give a stock solution with specific activity of 1 mCi/mmol and a final concentration of 4 mM. All other chemicals were purchased from Sigma. Enzymatic reactions were run at a final volume of 50 μL in a buffer containing 400 mM sodium phosphate (pH 7.4), 5 mM NaCl, 1 mM EDTA, 1 mM TCEP, 4 mM NADPH (when used), 20% glycerol and 5% DMSO. For radioassay experiments, enzymes and substrates were added to a final concentration of 5 μM (3 μM for reactions with DEBS3) and 1 mM, respectively. The reactions were incubated for 18 h at 30°C, then quenched by addition of 250 μL EtOAc and extracted with an additional 2 × 250 μL EtOAc. The organic extracts were concentrated under a stream of N_2, re-suspended in 30

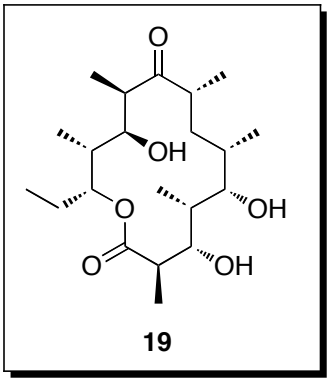
μL CH_2Cl_2, and spotted on a Merck silica gel TLC plate. The TLC plate was developed in 5% MeOH/CH_2Cl_2, exposed to a phophorimager screen for 36 h and analyzed using a Typhoon phosphorimager (Molecular Dynamics). For LC-MS/MS experiments, 100 μL reactions were used with 5 μM (3 μM for reactions with DEBS3) enzyme and 1 mM substrate, and the reactions again were incubated for 18 h at 30°C. The reactions were stopped by filtering through a Microcon YM-10 centrifugal filter device (Millipore) to remove the enzyme. 100 μL of 20% CH_3CN/H_2O was run through the filter to ensure elution of all polyketide products. LC-MS/MS analysis was performed on a ThermoElectron Finnigan LTQ Linear Ion Trap. Chromatographic separation was achieved on a Phenomenex Luna C18(2) column (3 μm, 2.0 × 250 mm) using a CH_3CN/H_2O gradient (20%, 20 min; 20% to 100%, 60 min, 0.1 mL/min).



12

Confirmation of Macrolactone 12. A large scale (2 mL) enzymatic reaction of Ery5-TE with DEBS pentaketide and cold methymalonyl-CoA was incubated for 24h followed by extraction with 3 × 4 mL EtOAc. The crude organic extracts were concentrated and dissolved in 100 μL MeOH and purified by semi-preparative HPLC on a Phenomenex Luna C18(2) column (5 μm, 4.6 × 250 mm) using a gradient of CH_3CN/10 mM NH_4OAc buffer, pH 6.0 (20 to 100%, 30 min, 1 mL/min). Fractions containing the

desired compound, as determined by ESI(+)-MS, were pooled and concentrated to give < 1 mg of **12**. ^{1}H NMR of macrolactone **12** was obtained on a Varian MR600 spectrometer fitted with a capillary flow probe, and the spectrum was correlated to that previously described.[1] HRMS ESI^{+} (*m/z*): 351.2138 (Predicted $[M+Na]^{+}$ for $C_{19}H_{32}O_5$ is 351.2147).



19

Confirmation of 6-DEB (**19**). Formation of 6-DEB by incubation of DEBS pentaketide **1** with DEBS3 was confirmed by correlating retention time in the LC-MS with an authentic standard of 6-DEB. Also, MS/MS fragmentation patterns from the LC-MS were identical between the authentic 6-DEB and that from the enzymatic assay.

4.6 References

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Chapter 5

Toward a Convergent and Diversity-Oriented Synthesis of Complex Polyketide Chain-Elongation Intermediates and Their Analogues

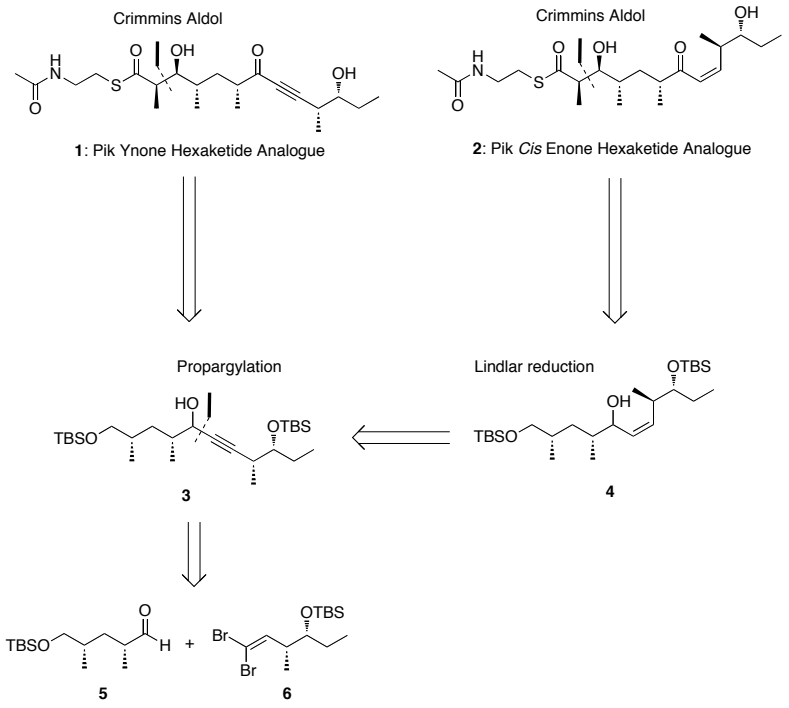
With established synthetic methods to access native chain-elongation intermediates for both the DEBS and Pik PKSs, we focused our attention on the synthesis of key analogues that would continue our work probing the molecular specificity requirements of the final modules of these systems. Of particular interest were analogues that could test both the flexibility of the final 6th modules in Pik and DEBS, as well as the structural requirements for TE-mediated cyclization.

5.1 Toward the Synthesis of Unique Pik Hexaketide Analogues

It was envisioned that ynone and *cis* enone hexaketide analogues **1** and **2** could serve these dual purposes (**Scheme 5.1**). The *sp* hybridization in ynone **1** would provide an inflexible region from C6-C7 of the hexaketide, while *cis* enone **2** would create a "kink" in the normally linear structure of the molecule. Due to the rigid geometries of both **1** and **2**, loading onto the KS of either Pik or DEBS module 6 would require a significant degree of active site flexibility to accommodate these structures. At the same time, it is unclear whether the thioesterase domains from either PKS could cyclize these structurally unique C6-C7 analogues. From molecular modeling, it is apparent that

cyclization of both of these structures is theoretically possible and should not be limited by apparent ring strain.

Scheme 5.1 – Retrosynthetic Analysis of Pik Ynone and *Cis* Enone Hexaketide Analogues

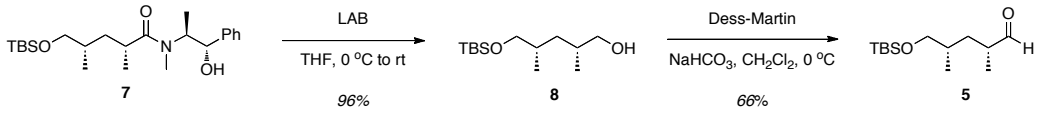


Retrosynthesis of ynone **1** begins with a Crimmins aldol[1] to generate the C2-C3 stereocenters. This disconnection provides propargyl alcohol **3** as a key fragment, which in turn can be generated by propargylation of aldehyde **5** using an acetylide synthon, generated by dibromoolefin **6**. Access to **2** was could be achieved in a similar manner, beginning with a Crimmins aldol disconnection to key intermediate **4**. Branching the synthetic scheme from propargylic alcohol **3** by simple Lindlar reduction would give access to this (*Z*)-allylic alcohol **4**.

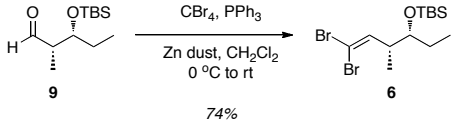
With this synthetic plan in place, we set out to make key fragments **5** and **6**. Synthesis of **5** began with known amide **7** (see Chapter 3), which was readily reduced with lithium trihydroaminoborate (LAB)[2] to give known alcohol **8** (**Scheme 5.2**). This

could then be oxidized by Dess-Martin periodinane to give the desired aldehyde. Dibromoolefin **6** could be synthesized by modification of the methods for the Corey-Fuchs alkyne synthesis. Starting with known aldehyde **9**,[3] olefination with the phosphorus ylide generated from carbon tetrabromide and triphenylphoshine lead to product **6** (**Scheme 5.3**).

Scheme 5.2 – Synthesis of Aldehyde **5**



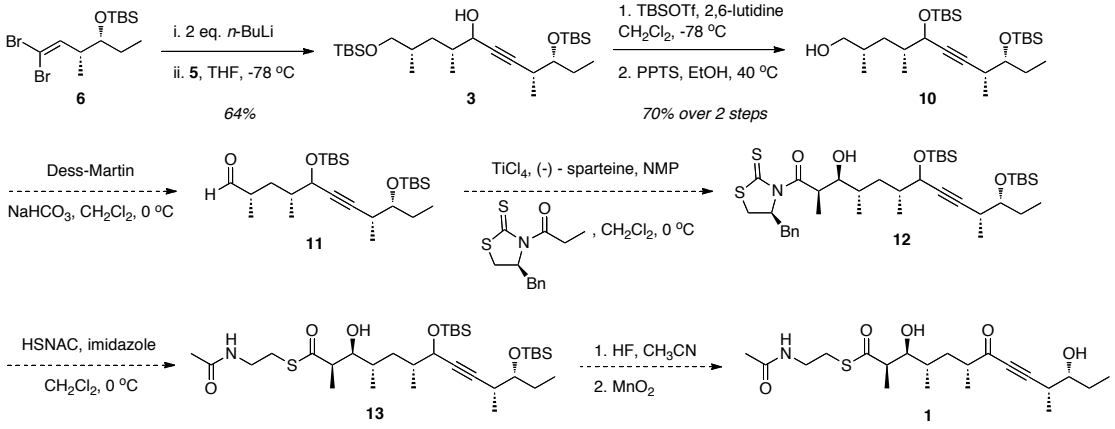
Scheme 5.3 – Synthesis of Dibromoolefin **6**.



With both fragments in hand, coupling proceeded smoothly by first generating the acetylide by addition of two equivalents of *n*-BuLi to **6**, followed by addition of the aldehyde **5** at -78 °C to give a mixture of diasteromeric propargylic alcohols **3** (**Scheme 5.4**). At the same time, direct propargylation of amide **7** was also explored using the *in situ* generated acetylide; however this failed under a number of conditions, including the addition of a variety of Lewis acids (e.g., $BF_3{\cdot}Et_2O$, $AlMe_3$, $TiCl_4$) and coordinating solvents (e.g., HMPA and DMPU) (**Scheme 5.5**). This was attributed to the poor nucleophilicity of the acetylide as well as the steric bulk of the tertiary pseudoephedrine amide.

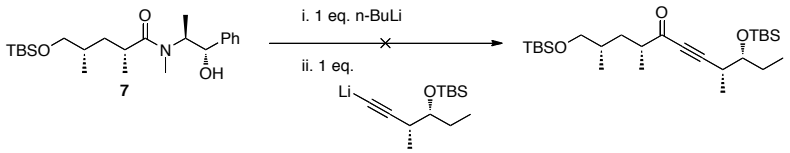
Following coupling, the free secondary alcohol of **3** was protected as the TBS ether and the primary TBS group was selectively deprotected to give the free C1 primary alcohol **10**. From this point, synthesis of ynone analogues **1** could be accomplished in 5 steps. Oxidation to aldehyde **11** with Dess-Martin periodinane would be followed by Crimmins aldol[1] coupling with the known thiazolinethione chiral auxiliary to give desired Evans-*syn* product **12**. Concomitant removal of the auxiliary and installation of the SNAC moiety to give **13** could be effected by simple stirring with *N*-acetylcysteamine and imidazole. Completion of the Pik ynone hexaketide **1** could then be completed by removal of both secondary TBS ethers, followed by chemoselective oxidation of the propargyl alcohol with MnO_2.

Scheme 5.4 – Synthesis of the Ynone Pik Hexaketide Analogue **1**

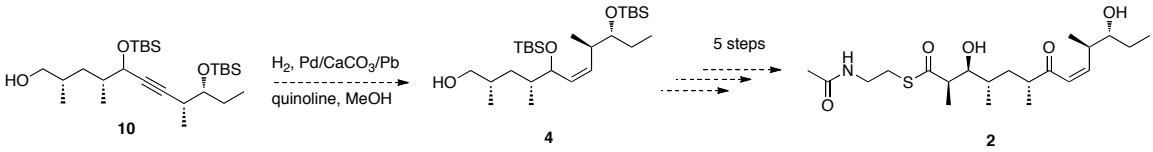


By analogy, synthesis of the *cis* enone analogue **2** could be accomplished from starting from alcohol **10**. Partial reduction of the alkyne using the Lindlar catalyst would selectively give the *cis* alkene intermediate **4**. This could then be carried through an identical sequence of transformations to give analogue **2**.

Scheme 5.5 – Unsuccessful Direct Propargylation of Pseudoephedrine Amide **7**



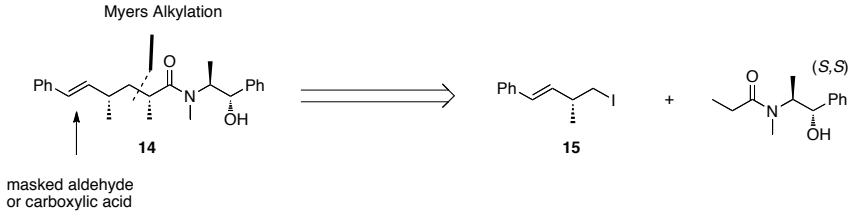
Scheme 5.6 – Proposed Synthesis of *Cis* Enone Analogue **2**



5.2 Toward a Convergent Strategy for Synthesis of PKS Intermediates

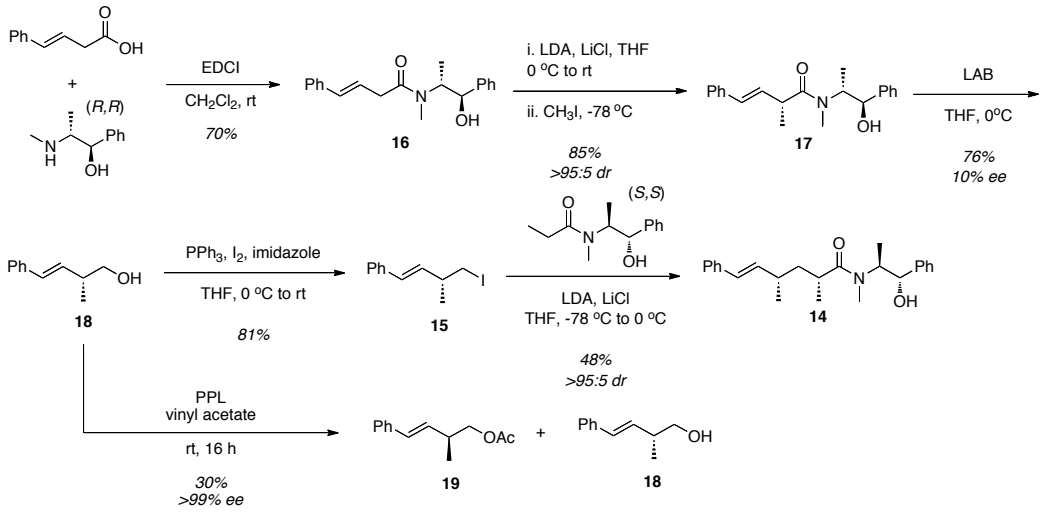
To complement our previous work in the synthesis of DEBS and Pik chain-elongation intermediates, we sought to develop a convergent methodology that would allow us to rapidly access native intermediates and non-native analogues from both systems. After careful analysis, amide **14** (**Scheme 5.7**) was chosen as a versatile new left fragment that could be used in the synthesis of both Pik and DEBS chain-elongation intermediates and their analogues. Amide **14** contains a terminal styryl group that can function as an aldehyde or carboxylic acid equivalent that can be selectively unmasked under mild oxidative methods. This circumvents the intermediacy of a free hydroxyl group that can participate in hemiketalization/degradation pathways. This fragment can be synthesized by Myers alkylation of iodide **15**.

Scheme 5.7 – Retrosynthesis for a New Left Fragment



Synthesis of **14** began by first accessing the requisite alkyl iodide **15**. It was thought that generating the desired (*S*)-stereocenter could best be accomplished by simple Myers alkylation of amide **16** (**Scheme 5.8**), which was made by coupling of *trans*-styrylacetic acid with (*R*, *R*)-pseudoephedrine. Diastereoselective alkylation of **16** with methyl iodide furnished the amide **17** with >95:5 selectivity. The diastereoselectivity could be determined by ^{1}H NMR by converting **17** to a 1,3-oxazolium triflate intermediate.[4] Complete selectivity for α-methylation was observed at -78 °C, whereas higher temperatures gave some γ-methylated product. Reductive removal of the auxiliary with lithium amidotrihydroborate[2] disappointingly resulted in nearly complete racemization (10% ee) of the resulting alcohol **18**.[5] Attempts to reduce the auxiliary under other conditions gave poor conversions and primarily tertiary amine products.

Scheme 5.8 – Synthesis of New Left Fragment **14**



Despite this disappointing result, it was thought that enrichment of the enantiopurity of alcohol **18** could be achieved via a lipase-mediated enzymatic resolution. Literature precedent indicated that a number of 2-methyl substituted alcohols could be kinetically resolved using commercially-available lipases.[6] As a result, the racemic

alcohol was subject to an enzymatic resolution, screening a number of commercial lipases (**Table 5.1**). It was found that Amano Lipase AK from *Pseudomonas fluorescens* gave good results for the enantiopurity (90% ee) and yields (32%) of the (*R*)-alcohol. Porcine pancreatic lipase (PPL), however, gave excellent results in the kinetic resolution giving (*R*)-**18** in >99% ee and 30% yield. Lipase PS from *Burkholderia cepacia* also gave enantiopure **18**, but the yields from this resolution were low due to high rate of acetylation of the (*R*)-alcohol by the lipase. Thus, the alcohol was enriched to a >99% ee with PPL using vinyl acetate as both the solvent and acetyl donor. The recovered acetate was subject to methanolysis, and the resulting (*S*)-**18** could be obtained in 55% yield and 60% ee.

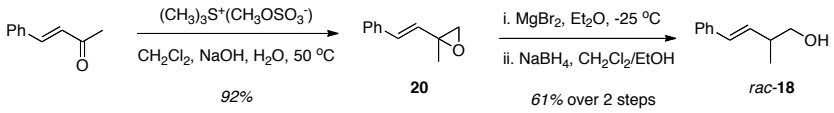
Table 5.1 – Screening of Commercial Lipases for Enzymatic Resolution of Alcohol **18**.

Lipase	Time (hours)	ee *R* alcohol[a]	Yield (%)
Amano Lipase AK (*Pseudomonas fluorescens*)[b]	112	90%	32
Porcine Pancreatic Lipase (PPL)[c]	16	>99%	30
Amano Lipase A (*Aspergillus niger*)[c]	72[d]	10%[e]	Not isolated
Amano Lipase PS (*Burkholderia cepacia*)[b]	72	>99%	5

[a]Enantiomeric excess determined by chiral HPLC analysis. Reaction conditions: [b]0.2 M in THF, 5 eq. vinyl acetate, 20% wt lipase; [c]Vinyl acetate used as solvent (0.5 M), 100% wt lipase. [d]Low conversion rate observed. [e]No appreciable change in ee from starting alcohol.

With resolution of alcohol **18** sufficiently worked out, conversion of (*R*)-**18** to iodide **15** could be accomplished under Appel conditions. Alkylation with the pseudoephedrine propionamide auxiliary then gave amide **14** (**Scheme 5.8**). At the same time, improved methods were explored to scale up the synthesis of the racemic alcohol. A concise method was developed that utilized inexpensive starting materials and required only one chromatographic purification. Starting from (*E*)-4-phenyl-3-buten-2-one ($0.1/g), epoxidation of the ketone with trimethylsulfonium methyl sulfate under phase transfer conditions could be achieved in excellent yield[7] (**Scheme 5.9**). Treatment of the oxirane with $MgBr_2$ induced rearrangement to the β,γ-unsaturated aldehyde, which was subsequently reduced to *rac*-**18**. The racemic alcohol could then subjected to lipase-mediated resolution to give the desired (*R*)-**18**.

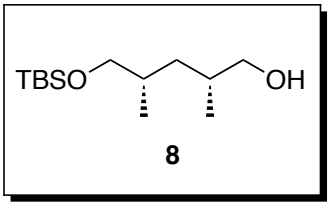
Scheme 5.9 – Synthesis of *Rac*-**18**



5.3 Experimental

Materials and General Procedures. Commercially available reagents were purchased from Sigma-Aldrich, Acros Organics, Fluka or Strem Chemicals, Inc. *Trans*-Styrylacetic acid was synthesized on a multigram scale according to the established literature procedure[8] or purchased from Sigma-Aldrich. Anhydrous solvents were obtained either using an MBRAUN MB-SPS solvent purification system or purchased in septum-sealed bottles from Acros Organics (AcroSeal) or EMD (DriSolv). Diisoproylamine and 2,6-lutidine were distilled over CaH_2 and stored under argon. Alkyllithiums were titrated prior to use with diphenylacetic acid. Air- and moisture-

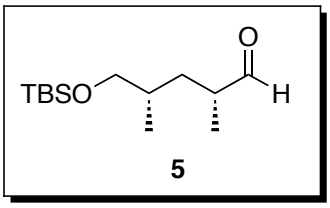
sensitive reactions were carried out under an inert argon atmosphere in flame-dried glassware. Flash chromatography was carried out using Merck Silica Gel 60 (230-400 mesh) and thin layer chromatography was performed on Merck TLC plates pre-coated with Silica Gel 60 F_{254}. ^{1}H and ^{13}C NMR spectra were obtained on either Varian MR400 or Inova 500 spectrometers. Proton chemical shifts are reported in ppm relative to TMS with the residual solvent peak as an internal standard. Proton NMR data is reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz) and integration. High-resolution mass spectra were obtained on a Waters Micromass AutoSpec Ultima Magnetic Sector mass spectrometer.



8

Product 8. *n*-BuLi (2.458 M in hexanes, 6.32 mL, 15.53 mmol) was added dropwise at -78 °C to a stirring solution of diisopropylamine (2.30 mL, 16.27 mmol) in 20 mL THF. The reaction was warmed to 0 °C for 10 min, then borane-ammonia complex (0.491 g, 15.92 mmol) was added in a single portion. The reaction was stirred for 15 min, warmed to ambient temperature, and then stirred an additional 15 min. The reaction was then cooled to 0 °C and amide **7** (1.545 g, 3.79 mmol) in 10 mL THF was added dropwise by cannula (washing the flask with an additional 1 mL THF to ensure complete transfer of the amide). After 10 min stirring at 0 °C, the reaction was allowed to warm to ambient temperature and stirred overnight (18 h). The reaction was quenched at 0 °C by dropwise addition of saturated Na/K tartrate (50 mL), warmed to ambient temperature, and stirred until the layers became clear and homogeneous. The aqueous

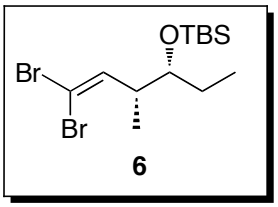
phase was extracted with 3 × 50 mL EtOAc, and the combined organics were dried over $MgSO_4$, filtered and concentrated. The crude oil was purified by flash chromatography (20% EtOAc/Hexanes) to give 0.892 g (95.5%) of a colorless oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 3.47 (dd, *J* = 10.6, 5.2 Hz, 1H), 3.41 (dd, *J* = 9.7, 5.8 Hz, 1H), 3.38 (dd, *J* = 10.6, 6.4 Hz, 1H), 3.34 (dd, *J* = 9.7, 6.3 Hz, 1H), 1.75 – 1.61 (m, 2H), 1.41 (dt, *J* = 13.6, 6.8 Hz, 1H), 0.92 (d, *J* = 6.7 Hz, 3H), 0.88 (d, *J* = 5.3 Hz, 3H), 0.88 (m, 1H), 0.87 (s, 9H), 0.02 (s, 6H). ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ -5.4, 17.7, 17.8, 18.3, 25.9, 33.2, 33.2, 37.2, 68.2, 68.2. HRMS ESI$^+$ (*m/z*): 269.1911 (Predicted $[M+Na]^+$ for $C_{13}H_{30}O_2Si$ is 269.1913).



5

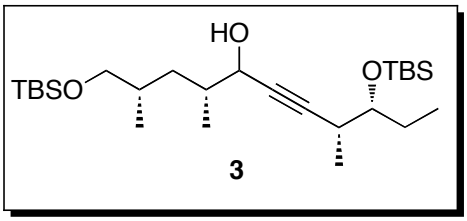
Product 5. To a suspension of alcohol **8** (0.155 g, 0.63 mmol) and $NaHCO_3$ (0.264 g, 3.15 mmol) in 6 mL CH_2Cl_2 was added Dess-Martin periodinane (0.293 g, 0.692 mmol) at 0 °C, and the reaction was monitored by TLC. After 2 h, the reaction was quenched by addition of 10 mL of half saturated $Na_2S_2O_3$ and stirred until the layers became clear. The aqueous phase was extracted with 3 × 25 mL CH_2Cl_2, and the combined organics were dried over $MgSO_4$, filtered and concentrated. The crude oil was purified by flash chromatography (5% EtOAc/Hexanes) to give 0.102 g (66.0%) of a colorless oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 9.55 (d, *J* = 2.5 Hz, 1H), 3.40 (d, *J* = 5.8 Hz, 2H), 2.49 – 2.39 (m, 1H), 1.84 (ddd, *J* = 13.9, 7.8, 6.2 Hz, 1H), 1.72 – 1.57 (m, 1H), 1.09 (ddd, *J* = 13.9, 7.7, 6.3 Hz, 1H), 1.07 (d, *J* = 7.0 Hz, 3H), 0.87 (d, *J* = 6.8 Hz, 3H), 0.86 (s, 9H), 0.01 (s, 6H). ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ -5.5, -5.5, 14.2, 17.1, 18.2,

25.9, 33.3, 34.6, 44.2, 67.8, 205.3. HRMS ESI$^+$ (*m/z*): (Predicted [M+Na]$^+$ for $C_{13}H_{28}O_2Si$ is 244.1859).



6

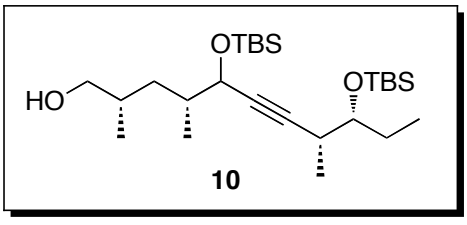
Product 6. CBr_4 (1.008 g, 3.04 mmol) was added to a slurry of PPh_3 (0.797 g, 3.04 mmol) and Zn dust (0.199 g, 3.04 mmol) in 15 mL CH_2Cl_2 at ambient temperature, stirring 15 min. Aldehyde **9** (0.350 g, 1.52 mmol) in 5 mL CH_2Cl_2 was then added and the reaction was stirred for 16 h. The reaction was poured into 100 mL pentane and filtered, washing the filter with an additional 50 mL pentane. The organics were concentrated and purified by flash chromatography (100% hexanes) to give 0.436 g (74.3%) of a colorless oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 6.30 (d, *J* = 9.5 Hz, 1H), 3.56 – 3.51 (m, 1H), 2.60 – 2.46 (m, 1H), 1.55 – 1.37 (m, 2H), 0.95 (d, *J* = 6.8 Hz, 3H), 0.88 (s, 9H), 0.86 (t, *J* = 6.3 Hz, 3H), 0.03 (s, 3H), 0.03 (s, 3H). ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ -4.7, -4.3, 9.4, 13.1, 18.1, 25.8, 27.4, 42.2, 75.2, 87.4, 142.5. HRMS EI$^+$ (*m/z*): 326.9411 (Predicted [M-Bu]$^+$ for $C_{13}H_{33}Br_2OSi$ is 326.9415).



3

Product 3. *n*-BuLi (2.458 M in hexanes, 0.37 mL, 0.92 mmol) was added dropwise at -78 °C to a stirring solution of dibromoolefin **6** (0.177 g, 0.46 mmol) in 2 mL

THF. The reaction was stirred for 5 min at that temperature, then warmed to 0 °C for 15 min, monitoring conversion to the acetylide by TLC. When the dibromoolefin was completely consumed, the reaction was re-cooled to -78 °C and the aldehyde **5** (0.102 g, 0.42 mmol) in 2 mL THF was added dropwise by cannula (washing the flask with an additional 0.5 mL THF to ensure complete transfer of the aldehyde). The reaction was stirred for 30 min, monitoring by TLC, then quenched with 5 mL saturated NH_4Cl. The aqueous phase was extracted with 3 × 20 mL CH_2Cl_2 and the combined organic extracts were dried over $MgSO_4$, filtered and concentrated. The crude oil was purified by flash chromatography (5% EtOAc/Hexanes) to give 0.126 g (64.1%) of a colorless oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 4.23 (br s, 1H), 3.53 – 3.43 (m, 2H), 3.1 (td, *J* = 9.7, 6.7 Hz, 1H), 2.60 – 2.50 (m, 1H), 1.88 – 1.62 (m, 4H), 1.61 – 1.41 (m, 2H), 1.12 (d, *J* = 7.0 Hz, 3H), 1.04 – 0.92 (m, 1H), 0.97 (d, *J* = 6.6 Hz, 1.5H), 0.96 (d, *J* = 6.8 Hz, 1.5H), 0.91 – 0.85 (m, 24H), 0.04 (s, 1.5H), 0.03 (s, 1.5H), 0.02 (s, 1.5H), 0.02 (s, 1.5H). ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ -5.4, -4.5, -4.3, 8.5, 8.5, 15.2, 15.7, 17.4, 17.4, 17.5, 17.9, 18.1, 18.2, 18.3, 25.9, 25.9, 27.1, 31.4, 31.5, 33.1, 33.3, 36.1, 36.9, 37.0, 66.8, 67.1, 68.0, 68.1, 76.0, 80.5, 81.4, 88.3, 88.7. HRMS ESI$^+$ (*m/z*): 493.3515 (Predicted $[M+Na]^+$ for $C_{26}H_{54}O_3Si_2$ is 493.3509).

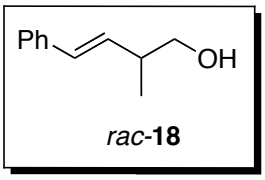


10

Product 10. To a stirring solution of **10** (0.122 g, 0.26 mmol) in 2.5 mL CH_2Cl_2 was added sequentially 2,6-lutidine (45 μL, 0.39 mmol), followed by TBSOTf (71 μL,

0.31 mmol) at -78 °C. The reaction was stirred 30 min, monitoring by TLC, followed by quenching with 5 mL saturated NH_4Cl. The aqueous phase was extracted with 3 × 10 mL CH_2Cl_2, and the combined organics were dried over $MgSO_4$, filtered and concentrated. The crude oil was purified by flash chromatography (3% EtOAc/Hexanes) to give 0.132 g (87.0%) of a colorless oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 4.19 (dd, *J* = 4.9, 1.7 Hz, 0.5H), 4.15 (dd, *J* = 4.5, 2.0 Hz, 0.5H), 3.56 – 3.41 (m, 2H), 3.24 (ddd, *J* = 12.6, 9.7, 7.4 Hz, 1H), 2.58 – 2.46 (m, 1H), 1.80 – 1.60 (m, 3H), 1.60 – 1.38 (m, 3H), 1.11 (d, *J* = 6.9 Hz, 3H), 0.96 – 0.81 (m, 36H), 0.12 – -0.05 (m, 18H). ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ -5.4, -5.2, -5.1, -4.5, -4.5, -4.4, -3.6, 8.2, 8.3, 8.3, 15.4, 15.8, 17.7, 17.9, 18.0, 18.1, 18.1, 18.2, 18.3, 25.6, 25.8, 25.8, 25.9, 25.9, 27.1, 31.5, 33.1, 33.2, 36.4, 36.8, 37.8, 37.9, 67.1, 67.4, 68.2, 68.4, 76.0, 81.5, 82.3, 87.0, 87.3. HRMS ESI$^+$ (*m/z*): 607.4374 (Predicted $[M+Na]^+$ for $C_{32}H_{68}O_3Si_3$ is 607.4374).

PPTS (0.012 g, 0.051 mmol) was added to a solution of the tri-TBS ether (0.030 g, 0.051 mmol) in 0.5 ml EtOH and stirred 18 h at ambient temperature. The next day, the reaction was stirred an additional 1 h at 40 °C, then concentrated. The crude mixture was purified by flash chromatography (10% EtOAc/Hexanes) to give 0.019 g (80.1%) of a colorless oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 4.22 – 4.15 (m, 1H), 3.56 – 3.44 (m, 2H), 3.43 – 3.35 (m, 1H), 2.61 – 2.49 (m, 1H), 1.82 – 1.66 (m, 3H), 1.66 – 1.44 (m, 3H), 1.13 (d, *J* = 7.0 Hz, 3H), 1.00 – 0.92 (m, 6H), 0.91 – 0.87 (m, 21H), 0.13 – 0.04 (m, 12H). ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ -5.1, -5.1, -4.5, -4.4, -4.4, -4.4, 8.4, 8.5, 15.9, 16.4, 17.7, 17.8, 17.9, 18.0, 18.1, 18.2, 18.2, 25.8, 25.8, 25.9, 27.0, 31.6, 31.6, 33.2, 33.3, 36.0, 36.5, 37.9, 37.9, 67.3, 67.4, 67.9, 68.0, 76.2, 81.7, 82.0, 87.3, 87.4. HRMS ESI$^+$ (*m/z*): 493.3504 (Predicted $[M+Na]^+$ for $C_{26}H_{54}O_3Si_2$ is 493.3509).

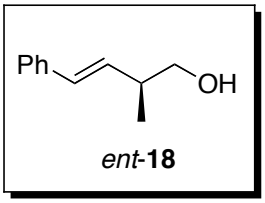


rac-**18**

Product *rac*-**18**. To a solution of $MgBr_2$ (2.542 g, 13.81 mmol) in 10 mL Et_2O was added oxirane **20**[7] (7.373 g, 46.02 mmol) in 20 mL Et_2O at -25 °C. The reaction was stirred 30 min, monitoring by TLC, followed by quenching with 25 mL saturated NH_4Cl and dilution with an additional 100 mL Et_2O. The layers were separated and the organics were washed with 100 mL 0.5 M citric acid. The combined organics were dried over $MgSO_4$, filtered and concentrated to a volume of 150 mL. MeOH (2.05 mL, 3 equivalents) was added followed by $NaBH_4$ (5.223 g, 138.1 mmol) at 0 °C. The reaction was monitored by TLC and quenched by dropwise addition of 1 N HCl and saturated Na/K tartrate at 0 °C until the layers became clear and homogeneous. The layers were separated and the aqueous phase was extracted with 2 × 50 mL Et_2O. The combined organics were dried over $MgSO_4$, filtered and concentrated. The crude oil was purified by flash chromatography (10% Et_2O/Pentane) to give 4.554 g (61.2%) of a pale yellow oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 7.38 – 7.14 (m, 5H), 6.47 (d, *J* = 15.9 Hz, 1H), 6.08 (dd, *J* = 15.9, 7.9 Hz, 1H), 3.59 (ddd, *J* = 10.5, 7.4, 5.6 Hz, 1H), 3.51 (ddd, *J* = 10.5, 7.5, 4.8 Hz, 1H), 2.64 – 2.46 (m, 1H), 1.46 (dd, *J* = 7.4, 4.8 Hz, 1H), 1.11 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ 16.5, 40.2, 67.3, 126.1, 127.3, 128.5, 130.9, 132.5, 137.2. HRMS EI$^+$ (*m/z*): 162.1052 (Predicted $[M+H]^+$ for $C_{11}H_{14}O$ is 162.1044). The enantiomers were analyzed by chiral HPLC on a Daicel Chiralpak AD column (250 × 4.6 mm; isocratic 2% Isopropanol/Hexanes, 30 min, 1 mL/min). (*R*)-isomer t_r = 14.8 min,

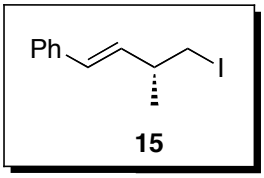
(*S*)-isomer t_r = 17.1 min. The desired (*R*)-isomer was correlated to a standard of the alcohol synthesized as previously described in the literature.[9]

Enzymatic Kinetic Resolution of ***rac*-18**. To a solution of ***rac*-18** (2.00 g, mmol) in 25 mL vinyl acetate was added porcine pancreatic lipase (Type II, 2.00 g), and the reaction was stirred vigorously for 16 h. The enzyme was filtered off and the volatiles removed *en vacuo.* The crude oil was purified by flash chromatography (20% Et_2O/pentane) to give 0.603 g (30.2%, >99% ee) of a colorless oil. The enantiomeric excess was determined by chiral HPLC on a Daicel Chiralpak AD column (250 × 4.6 mm; isocratic 2% Isopropanol/Hexanes, 30 min, 1 mL/min). (*R*)-isomer t_r = 14.8 min.



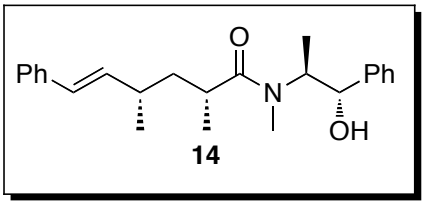
***ent*-18**

The recovered acetate **19** was dissolved in 12 mL MeOH and K_2CO_3 was added. The reaction was stirred 2 h, then quenched with 10 mL H_2O. The volatiles were removed *en vacuo*, and the remaining aqueous phase was extracted with 3 × 20 mL CH_2Cl_2. The combined organic extracts were dried over $MgSO_4$, filtered and concentrated to give 1.102 g (55.1%, 60% ee) of a pale yellow oil. The enantiomeric excess was determined by chiral HPLC on a Daicel Chiralpak AD column (250 × 4.6 mm; isocratic 2% Isopropanol/Hexanes, 30 min, 1 mL/min). (*S*)-isomer t_r = 17.1 min, (*R*)-isomer t_r = 14.8 min.



15

Product 15. PPh_3 (0.243 g, 0.92 mmol), I_2 (0.327 g, 0.92 mmol), and imidazole (0.084 g, 1.232 mmol) were added sequentially to a stirring solution of **18** (0.100 g. 0.62 mmol) at 0 °C. The reaction was stirred for 15 min and then warmed to ambient temperature for 15 min. The reaction was quenched with 15 mL saturated $Na_2S_2O_3$ and the aqueous was extracted with 3 × 15 mL CH_2Cl_2. The combined organic extracts were dried over $MgSO_4$, filtered and concentrated. The crude material was purified by flash chromatography (100 % Pentane) to give 0.136 g (81.4%) of a colorless oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 7.39 – 7.18 (m, 5H), 6.44 (d, *J* = 15.9 Hz, 1H), 6.08 (dd, *J* = 15.9, 7.5 Hz, 1H), 3.26 (dd, *J* = 9.6, 5.8 Hz, 1H), 3.20 (dd, *J* = 9.6, 6.7 Hz, 1H), 2.62 – 2.50 (m, 1H), 1.23 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ 15.0, 20.8, 39.0, 126.2, 127.4, 128.6, 130.2, 133.2, 137.1. HRMS EI$^+$ (*m/z*): 272.0056 (Predicted $[M+H]^+$ for $C_{11}H_{14}I$ is 272.0062).



14

Product 14. *n*-BuLi (2.458 M, 0.63 mL, 1.55 mmol) was added dropwise to a stirring suspension of flame-dried LiCl (0.253 g, 5.99 mmol) and diisopropylamine (0.23 mL, 1.65 mmol) in 2.5 mL THF at -78 °C. The reaction was warmed to 0 °C for 5 min, then re-cooled to -78 °C. (*S, S*) – pseudoephedrine propionamide (0.177 g, 0.80 mmol) in

2 mL THF was added dropwise by cannula. The reaction was stirred for 1 h at -78 °C, 25 min at 0 °C, and 5 min at ambient temperature. The reaction was cooled to 0°C and the iodide (0.136 g, 0.50 mmol) in 1 mL THF was added dropwise by cannula (rinsing the flask with an additional 0.5 mL THF to ensure complete transfer of the iodide). The reaction was stirred for 18 h at 4 °C and then 12 h at ambient temperature, followed by quenching with 10 mL saturated NH_4Cl. The aqueous phase was extracted with 3 × 15 mL EtOAc, and the combined organic extracts were dried over $MgSO_4$, filtered and concentrated. The crude material was purified by flash chromatography (40% EtOAc/Hexanes) to give 0.088 g (48.1%) of a pale yellow oil. ^{1}H NMR ($CDCl_3$, 399.5 MHz) δ 7.44 – 7.09 (m, 10H), 6.41 (d, *J* = 15.9 Hz, 0.4H), 6.34 (d, *J* = 15.8 Hz, 1H), 6.11 (dd, *J* = 15.8, 8.8 Hz, 0.4H), 5.97 (dd, *J* = 15.8, 8.6 Hz, 1H), 4.45 – 4.35 (m, 1H), 4.40 (br s, 1H), 4.34 – 4.24 (m, 0.4H), 4.04 – 3.92 (m, 0.4H), 3.01 – 2.92 (m, 0.4H), 2.85 (s, 1H), 2.78 (s, 3H), 2.69 – 2.58 (m, 1H), 2.48 – 2.38 (m, 0.4H), 2.37 – 2.22 (m, 1H), 1.91 – 1.81 (m, 0.4H), 1.74 – 1.63 (m, 1H), 1.63 – 1.54 (m, 0.4H), 1.45 – 1.34 (m, 1H), 1.13 (d, *J* = 6.8 Hz, 1H), 1.11 (d, *J* = 6.7 Hz, 3H), 1.09 (d, *J* = 6.7 Hz, 1H), 1.04 (d, *J* = 6.7 Hz, 3H), 0.93 (d, *J* = 4.8 Hz, 3H), 0.91 (d, *J* = 6.4 Hz, 1H).^{13}C NMR ($CDCl_3$, 100.5 MHz) δ 14.2, 15.4, 17.3, 17.4, 20.2, 21.2, 21.7, 23.5, 26.7, 33.7, 34.8, 35.6, 35.9, 40.7, 40.9, 58.1, 75.1, 76.6, 125.9, 126.1, 126.3, 126.9, 126.9, 127.0, 127.5, 128.3, 128.5, 128.6, 128.6, 128.8, 135.7, 136.3, 137.5, 140.9, 142.6, 178.1, 179.2. HRMS ESI$^+$ (*m/z*): 388.2540 (Predicted $[M+Na]^+$ for $C_{24}H_{31}NO_2$ is 388.2252).

5.4 References

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Chapter 6

Future Directions

To date, significant work toward an understanding of molecular specificity in the DEBS and Pik modular PKS systems has been accomplished. The studies presented in this dissertation have provided both synthetic methods to access advanced PKS chain-elongation intermediates, as well as a unique insight into the underpinnings of the molecular recognition features in the final modules of these PKSs. As are result, we are uniquely positioned to further our assessment of key aspects of polyketide biosynthesis in both of these systems. Our current efforts have focused on four main goals: assessing the kinetics of loading, extension, processing and cyclization in the final DEBS modules 5 and 6, utilizing our native DEBS pentaketide substrate; probing the barriers to processing non-native intermediates in module 5 of the Pik PKS; continued development of synthetic methods for efficiently accessing diverse and novel analogues of Pik and DEBS chain-elongation intermediates; and detailed probing of the structural requirements of macrocyclization by terminal PKS thioesterase domains.

6.1 Kinetic Analysis of Late-Stage Polyketide Assembly in the Erythromycin PKS

Access to the bimodular DEBS3 protein provides a unique opportunity to perform an *in vitro* kinetic analysis of the flux through the final two modules in the erythromycin PKS utilizing our native chain-elongation intermediates, thus providing a direct

comparison with previous kinetic studies on the analogous final modules in the Pik system (PikAIII and PikAIV).[1] Currently, we are aiming to assess the steady-state rate of formation of the erythromycin aglycone 6-DEB when DEBS3 is incubated with our native DEBS pentaketide intermediate, dissecting individual k_{cat} and K_M parameters. These will be directly compared with the rates of tetraketide formation when DEBS3 is incubated with short diketide model substrates. This work is being done in collaboration with Sherman lab postdoctoral fellow, Dr. Sathish Rajamani.

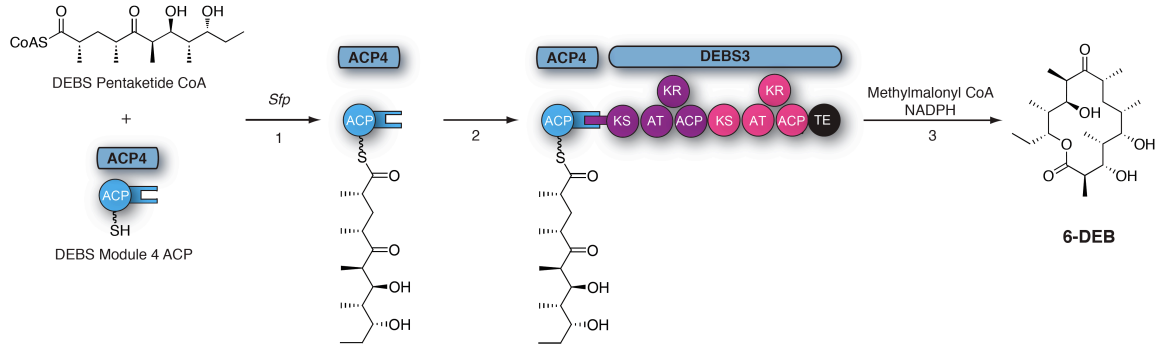
Here, a quantitative assessment of the contributions of both proximal and distal functionality to molecular recognition of PKS intermediates by the final DEBS modules can be gleaned. In the Pik system, processing of native chain-elongation intermediates occurred at rates 2-3 orders of magnitude greater than with diketide model substrates.[1] Considering the molecular specificity profiles exhibited by DEBS module 5, it is hypothesized that a similar kinetic gain may be seen in the DEBS system when the native pentaketide substrate is utilized. At the same time, these experiments provide the first opportunity to assess the native bimodular DEBS3 protein rather than the unnatural dissected and engineered monomodules Ery5 and Ery6. Unlike analogous studies in PikAIII and PikAIV, which are both natively monomodular, dissection of the DEBS modules into monomodular Ery5 and Ery6 may perturb their catalytic efficiency and thus may not present the authentic catalytic capabilities of the native enzymes in a kinetic analysis. However, these potential problems are avoided in this experimental system.

Upon completion of this analysis, it is envisioned that future kinetic studies would focus on exploration of the contribution of protein-protein interactions in the channeling of substrates between PKS modules. For example, docking domains play an important

role in mediating the interaction of modules on different polypeptides.[2] In DEBS, these interactions have been shown to provide a significant kinetic gain (approximately 3 orders of magnitude increase in the specificity constant k_{cat}/K_M) when diketides substrates were presented to downstream modules bound to the upstream ACP with the appropriate C-terminal docking domain.[3] In addition, these interactions allowed upstream modules to accept and process normally poor substrates that could not be by accepted by diffusive loading onto the ketosynthase domain.

In order to perform these studies with native intermediates, synthesis of the CoA analogues of the Pik and DEBS native chain elongation intermediates would be required, followed by their subsequent loading onto the upstream ACP (**Figure 6.1**). This would provide an *in vitro* experimental system that very closely mimics the *in vivo* PKS environment, taking into account the contributions of docking domain pairing, protein-protein interactions between KS and ACP domains, as well as the molecular recognition features of the polyketide chain-elongation intermediates.

Figure 6.1 – Channeling of PKS intermediates in the DEBS3 PKS modules 1) Loading of the upstream ACP4 with DEBS pentaketide CoA 2) Docking of ACP4 with DEBS3 3) Extension, processing and cyclization of the DEBS pentaketide to form 6-DEB. *Sfp* = phosphopantetheinyl transferase from *B. subtilis.*



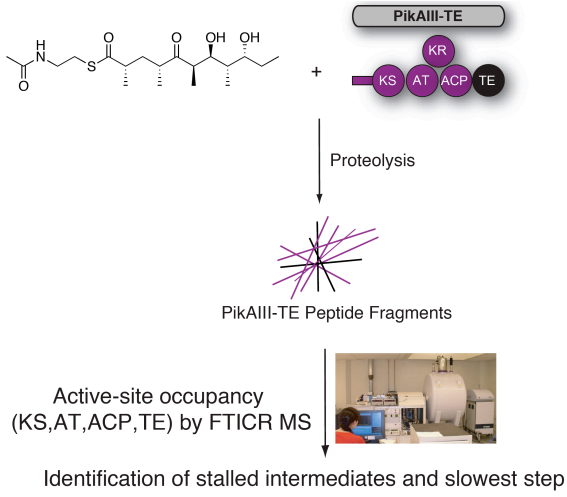
6.2 FT-ICR Mass Spectrometry Analysis of Loading, Extension and Processing of the Non-Native DEBS Pentaketide in PikAIII

The molecular stringency exhibited by the module 5s from the Pik and DEBS PKS raises interesting questions regarding the factors that prevent their efficient processing of non-native substrates. As seen in the biochemical analysis laid out in Chapter 4 of this dissertation, incubation of DEBS and Pik pentaketide substrates with non-cognate module 5s did not result in formation of the putative 12-membered macrolactones. Thus, it is a goal to understand what steps in the PKS mechanism are the primary barriers to catalysis with non-native intermediates. In collaboration with another graduate student in the Sherman lab, Chris Rath, and Professor Kristina Håkansson (University of Michigan), we will seek to utilize fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) to identify these steps.

Previous FT-ICR MS work has shown that PikAIII-TE incubated with the native Pik pentaketide substrate does not show appreciable build up of polyketide intermediates on the PKS active sites of PikAIII-TE,[4] most likely the result of rapid extension, processing and cyclization to macrolactone products. When incubated with the non-native DEBS pentaketide, it is hypothesized that an accumulation of stalled intermediates on PikAIII-TE will provide insight into the mechanistic underpinnings of molecular stringency in this module. For example, buildup of the DEBS pentaketide on the PikAIII KS domain would indicate a slowing of the PKS decarboyxlative condensation process that extends the polyketide unit. This work will be accomplished through multiple time point incubations of the synthetic DEBS pentaketide SNAC with PikAIII-TE, followed by proteolysis of the enzyme and analysis of the peptide fragments by FT-ICR mass

spectrometry (**Figure 6.2**). The reactions will be scanned for known protein fragments containing the PikAIII-TE active site residues to determine potential accumulation of stalled PKS intermediates. Reciprocal work in the DEBS system could also be performed to detect analogous accumulation of stalled intermediates when the Pik pentaketide is incubated with Ery5-TE or DEBS3.

Figure 6.2 – FT-ICR MS analysis of active site occupancy of Pik module 5 incubated with the unnatural DEBS pentaketide substrate.



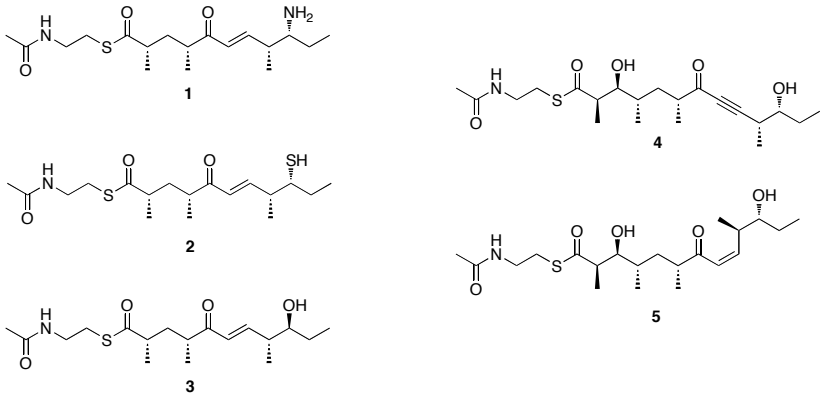
6.3 Completion of Synthetic Platform for Natural and Non-Natural Polyketide Chain-Elongation Intermediates

Continued development of synthetic methods to access to polyketide chain-elongation intermediates and their analogues remains an important goal to rigorously probe the specificity requirements of PKS modules. Targeted analogues will encompass a variety of stereochemical, structural and functional group changes that will test the limits of loading, extension, processing and cyclization of unnatural substrates by modular PKS enzymes. The work laid out in Chapters 3 and 5 of this dissertation have provided a

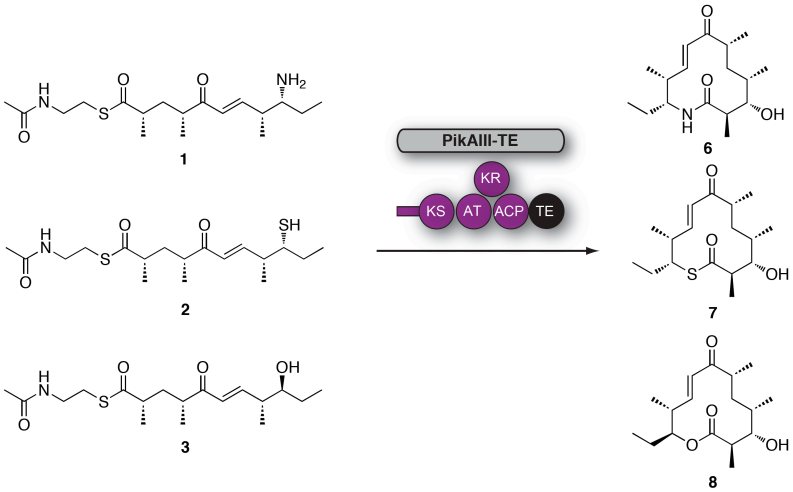
foundation for convergent access to new penta- and hexaketide analogues from both Pik and DEBS.

Some key Pik pentaketide analogues targets incorporate new functionality or stereochemistry at C9 (**Figure 6.3**). Analogues **1** and **2** change the C9 hydroxyl to an amino and thiol group, respectively, testing the Pik TE's ability to utilize non-oxygen nucleophiles during macrocyclization. Incubation of these analogues with PikAIII-TE would give the putative 12-membered ring macrolactam **6** and macrothiolactone **7** analogues of 10-Dml (**Scheme 6.1**). This type of flexibility has been shown with the TE from the tyrocidine NRPS,[5] which is capable of using both an oxygen and amino nucleophile during cyclization; however, similar flexibility has never been demonstrated in a thioesterase from a PKS system. Alternatively, analogue **3** would probe the more subtle effect of stereochemistry on the efficiency of macrolactonization to 10-Dml epimer **8**. Hexaketide analogues **4** and **5** (see Chapter 5 for synthetic progress toward these analogues) would probe the structural flexibility of loading onto PikAIV by introducing rigidity due to *sp* hybridization in the ynone **4** or the "kink" introduced by the *cis* geometry of alkene in enone **5**. At the same time, these intermediates could be directly incubated with Pik TE to test its ability to cyclize these structurally demanding intermediates to their putative 12-membered ring analogues **9** and **10** (**Scheme 6.2**).

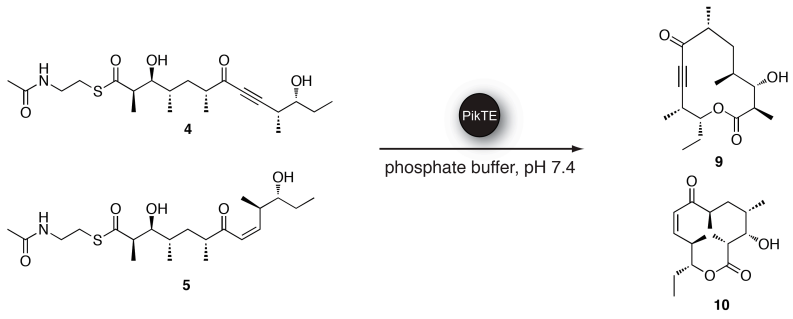
Figure 6.3 – Targeted Pik penta- and hexaketide chain-elongation analogues.



Scheme 6.1 – Loading, Extension, Processing and Cyclization of Unnatural Pik Pentaketides to Putative 10-Dml Analogues



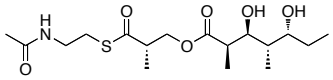
Scheme 6.2 – Direct Cyclization of Unnnatural Pik Hexaketide Analogues by Pik TE



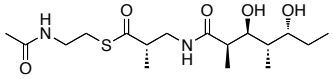
Synthesis of new DEBS pentaketide analogues will also be targeted to probe the specificity of DEBS module 5 and 6, as well as the full DEBS3 bimodule. Some

examples of targeted analogues include **11** and **12** (**Figure 6.4**), which incorporate oxygen and nitrogen heteroatom replacements for C4, respectively. These ester and amide compounds would change the electronic properties at the C5 carbonyl, as well as change the conformational dynamics of these pentaketides. Analogue **13**, on the other hand, contains a change in the stereochemistry at C9, aimed at probing its effect on cyclization to epimeric 12- and 14-membered ring macrolactones by the DEBS TE. Ultimately, these targeted analogues will aid in our efforts to build a rational basis for molecular recognition of substrates by PKS modular enzymes.

Figure 6.4 – Targeted DEBS pentaketide analogues.



11



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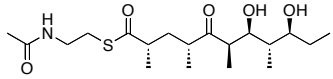


13

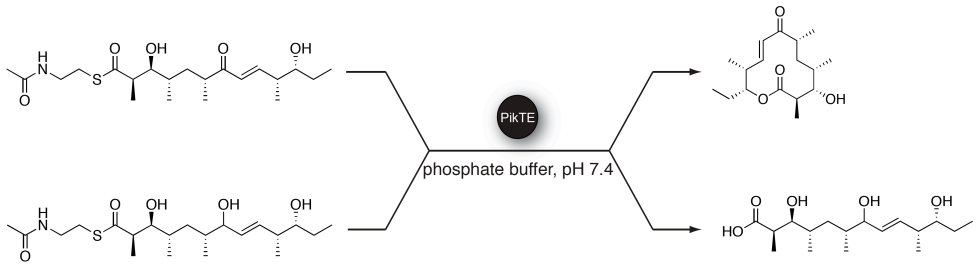
6.4 Furthering Understanding of the Structural Requirements of Macrocyclization in the Pik TE

Due to the pivotal role thioesterase domains play in natural product biosynthesis, understanding the specificity of the Pik and DEBS TEs is essential for their application both *in vitro* and *in vivo*. As a result, both TEs have been the subject of experiments exploring their chemoenzymatic potential. Detailed biochemical work laid out in Chapter 4 of this dissertation and previous work from the Sherman group[6] have demonstrated that both the Pik and DEBS TE have unique requirements for macrocyclization. Our current

efforts will seek a more in depth understanding of the structural basis for macrolactonization by these TEs, merging synthetic chemistry with structural biology.

In the Pik system, the current model suggests a primarily structure-driven process of cyclization with Pik TE. When the Pik TE was excised from its native context, it was shown to catalyze macrocyclization of the synthetic pikromycin hexaketide SNAC substrate, exclusively forming 10-Dml[6] (**Scheme 6.3**). However, when the enone of the substrate was reduced at C9 to the allylic alcohol, cyclization by Pik TE was abrogated. It was hypothesized from this work, that the rigid enone structure of the Pik hexaketide provides a favorable entropic contribution necessary for TE-mediated cyclization.

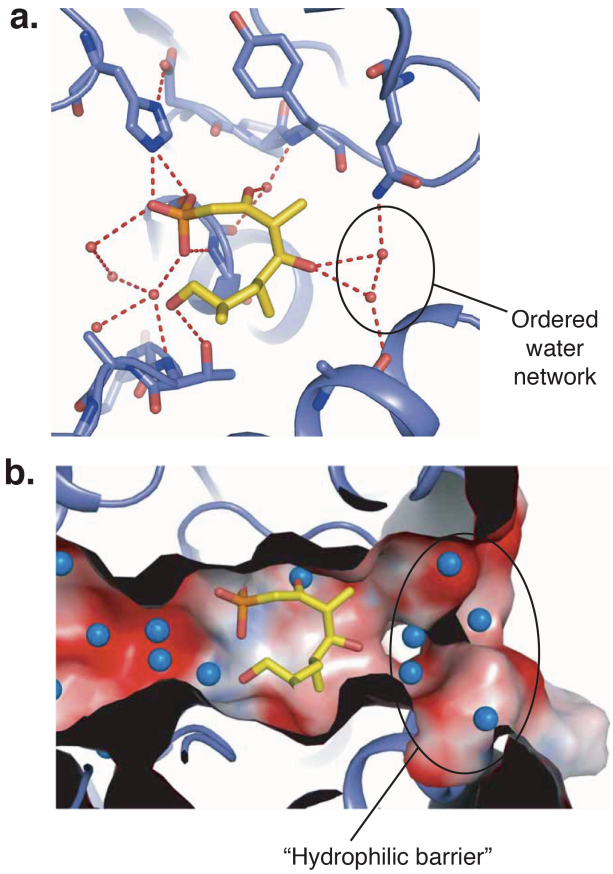
Scheme 6.3 - *In Vitro* Chemoenzymatic Synthesis of 10-Deoxymethynolide with Pik TE.



This was further supported by structural biology studies where the Pik TE was co-crystallized with bound affinity labels mimicking a portion of the pikromycin cyclization intermediate.[7] Here, it was shown that cyclization appears to be primarily a conformationally-driven process with Pik TE, as minimal contacts were seen between the affinity label and the TE active site residues. Rather, a water network on one side of the thioesterase channel appeared to form a "hydrophilic barrier" that directed curling of the intermediate toward cyclization (**Figure 6.5**). Also, modeling in the enone stucture for

the Pik hexa- and heptaketide cyclization intermediates showed a favorable disposition of the appropriate internal hydroxy nucleophile for macrocyclization.

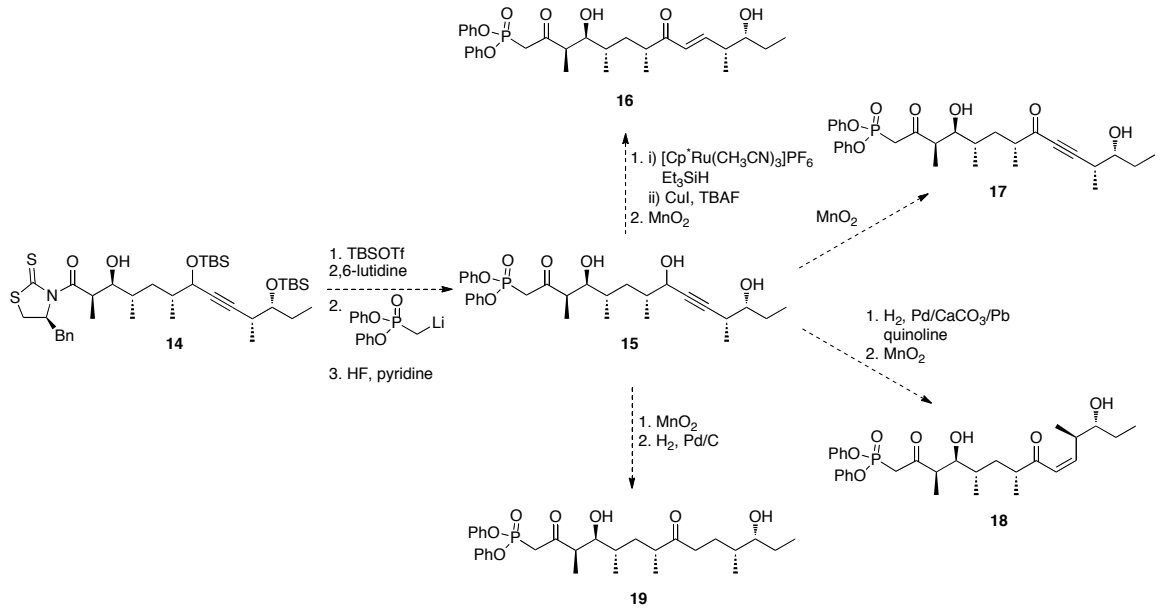
Figure 6.5 – Pik TE crystal structure (1.8 Å) a) active site with a bound affinity label mimicking the Pik cyclization intermediate. An ordered water network on right side of the channel forms a "hydrophilic barrier," inducing curl of the cyclization intermediate b) electrostatic surface representation of the Pik TE substrate channel.



To generate a more complete picture regarding the structural requirements of Pik TE-mediated cyclization, we envision synthesizing a suite of full-length diphenylphosphonate inhibitors that mimic the Pik heptaketide cyclization intermediate, incorporating novel structural changes to view the active site conformations of bound

PKS intermediates (**Scheme 6.3**). These inhibitors could potentially be accessed by a minor modification of the strategy laid out in Chapter 5 to access hexaketide analogues **4** and **5**. Beginning with Crimmins aldol product **14**, protection of the secondary alcohol as the TBS ether, followed by addition of lithiated diphenyl methylphosphonate would give the full inhibitor backbone. Global deprotection would then yield **15**, which could then be converted to a number of structurally unique inhibitors.

Scheme 6.4 – Synthesis of Novel Diphenylphosphonate Inhibitors for Pik TE



Conversion of **15** to the native (*E*)-alkene could be accomplished via selective *trans* hydrosilylation of the alkyne catalyzed by a cationic ruthenium complex, followed by protodesilylation of the vinyl silane.[8, 9] This would give a diphenylphosphonate inhibitor mimicking the heptaketide containing the C9 reduced allylic alcohol, whereas further chemoselective oxidation of the allylic alcohol with MnO_2 would yield **16** with the natural enone structure. Alternatively, direct oxidation of **15** with MnO_2 would give

propargyl ketone analogue **17** that would rigorously test the flexibility of the TE active site. Access to the *cis* enone analogue **18** would be accessed by selective Lindlar reduction to give the (*Z*)-alkene and subsequent oxidation. Finally, synthesis of the reduced alkene analogue **19** could be achieved by allylic oxidation of propargyl alcohol to the ynone, followed by complete hydrogenation of the alkyne.

6.5 Frontiers and Opportunities in Chemoenzymatic Synthesis

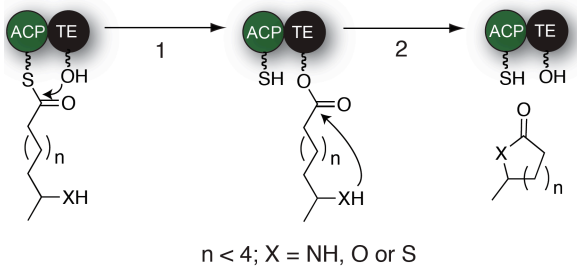
As has been demonstrated through these studies, understanding modular PKS systems for re-engineering efforts provides the exciting potential to access chemical diversity through combinatorial biosynthesis and chemoenzymatic synthesis. Here, we present examples of some of the most recent advances in chemoenzymatic synthesis that complement our studies in modular PKSs, and identify current trends and opportunities in this growing field. Particular attention has been centered on chemoenzymatic oxidation (e.g. - hydroxylation and epoxidation) and chain termination (e.g. – macrocyclization) strategies.

Macrocyclization

In their biologically relevant conformations, many PKS and non-ribosomal peptide synthetase (NRPS)-derived natural products contain macrocyclic rings that equal or exceed 8 atoms, making them challenging targets for synthetic chemists. In generating macrocyclic lactones[10] and lactams by synthetic methods, common hurdles include the need for orthogonal protecting group strategies, complex conformational effects of linear cyclization intermediates (entropic and enthalpic factors), regioselectivity, and competing intermolecular dimerizations/oligomerizations. These factors often lead to longer synthetic routes and decreased yields of target compounds.

In PKS and NRPS systems, cyclization is often carried out by a discrete terminal thioesterase (TE) domain, containing a characteristic serine, histidine, aspartate catalytic triad similar to serine hydrolases. During this process, the final biosynthetic intermediate from the last PKS or NRPS elongation module is passed to the TE active site serine residue to form an *O*-acyl TE intermediate, followed by cleavage of the acyl enzyme by regiospecific nucleophilic attack of an internal hydroxy or amino group to produce the macrolactone or lactam (**Figure 6.6**). Due to the unique ability of these TE enzymes to efficiently catalyze macrocyclization, many efforts have explored their potential as biocatalysts when excised from their native PKS or NRPS context.

Figure 6.6 - Illustration of TE-mediated macrocyclization 1) transfer of final intermediate to TE active site serine 2) regioselective intramolecular cleavage of the acyl-enzyme intermediate by an internal nuclophile.



P450 Hydroxylation and Epoxidation

Another set of exceptional challenges for organic chemists are the selective hydroxylation and epoxidation of unactivated C-H bonds and olefins, respectively, by traditional synthetic methodologies. Mild oxidation of organic compounds, however, can be accomplished biocatalytically by the P450 superfamily of enzymes, which are heme-containing proteins that couple with a reductase partner protein to activate molecular oxygen using NADPH/NADH.[11] Specifically, P450 enzymes from natural product biosynthetic pathways are capable of effecting many difficult oxidative transformations

on natural product scaffolds in both a regio- and stereospecific manner, making them attractive biocatalysts for chemoenzymatic synthesis.

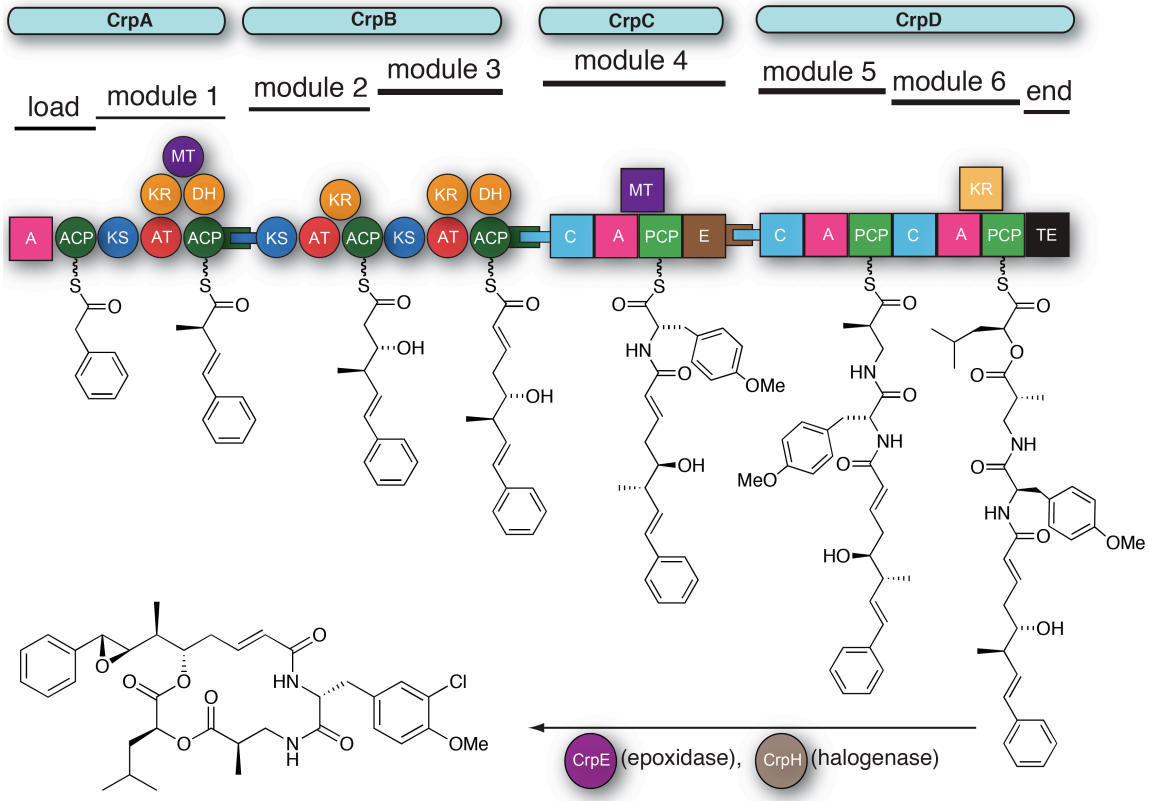
Cryptophycin

The cryptophycins are a class of hybrid PKS/NRPS natural products isolated from a pair of related lichen cyanobacterial symbionts, *Nostoc* sp. ATCC 53789 and GSV 224.[12,13] These compounds are exceptionally potent tubulin-depolymerizing agents and are not active substrates of P-glycoprotein (P-gp) or multiple drug resistance-associated protein (MRP),[14] making them viable chemotherapeutic alternatives against cancers that overexpress both of these transporters and are resistant to the *vinca* alkaloids and taxol. So, despite the discontinuation of synthetic crytophycin analogue LY355703 as a candidate in recent phase II clinical trials,[15] these natural products still garner significant interest in their development as anticancer drugs.[16]

Recent access to the cryptophycin (Crp) biosynthetic gene cluster has spurred development of embedded enzymes from this pathway as biocatalysts in the generation of new cryptophycins with improved pharmacological profiles. The peptolide core of the most abundant cryptophycin analogue, cryptophycin 1, consists of a PKS-derived phenyloctenoic acid (Unit A) and three NRPS-derived amino acids: 3-chloro-*O*-methyl-*D*-tyrosine (Unit B), methyl β-alanine (Unit C) and *L*-leucic acid (Unit D) (**Figure 6.7**). However, there is significant diversity within this class of compounds, which contains more than 25 analogues that incorporate many substitutional variations on the core scaffold. This diversity suggests a high degree of flexibility in the enzymes of the Crp biosynthetic machinery, a hypothesis that was further borne out in precursor-directed

biosynthetic studies, where a number of unnatural units were incorporated into new cryptophycin analogues by the Crp enzymes.[17]

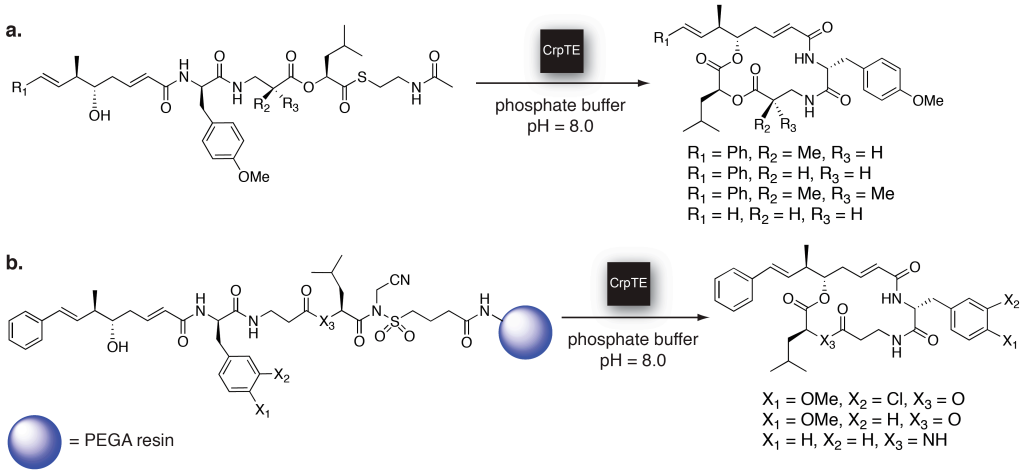
Figure 6.7 – Illustration of the Crp PKS/NRPS responsible for biosynthesis of the cryptophycin peptolide core. Epoxidation by CrpE and chlorination by CrpH gives cryptophycin 1. C = condensation domain, A = adenylation domain, PCP = peptidyl carrier protein, MT = methyltransferase, E = epimerase. Circles denote PKS domains and squares denote NRPS domains.



In generating synthetic Crp analogues, efficient macrocyclization and epoxidation represent two challenging issues that can be addressed by chemoenzymatic methods. In a recently described approach, the Crp TE was excised and heterologously overexpressed as a recombinant enzyme for cyclization of linear Crp precursors.[18] NAC thioester-activated *seco*-cryptophycins were chemically synthesized, incorporating 3 different Unit C moieties, and utilized to interrogate the *in vitro* activity and substrate specificity of Crp TE (**Figure 6.8a**). Robust macrocylization was observed in each case, suggesting changes to the β-alanine site are well tolerated by the thioesterase. Interestingly however, a linear

cryptophycin precursor lacking the phenyl ring on the styryl moiety of Unit A was poorly cyclized by Crp TE, and instead primarily hydrolyzed to the *seco*-acid. This indicated that an aryl group at that position is critical for efficient macrocyclization.

Figure 6.8 – Chemoenzymatic macrocyclization of a) solution phase and b) solid phase linear cryptophycin intermediates by Crp TE *in vitro*.

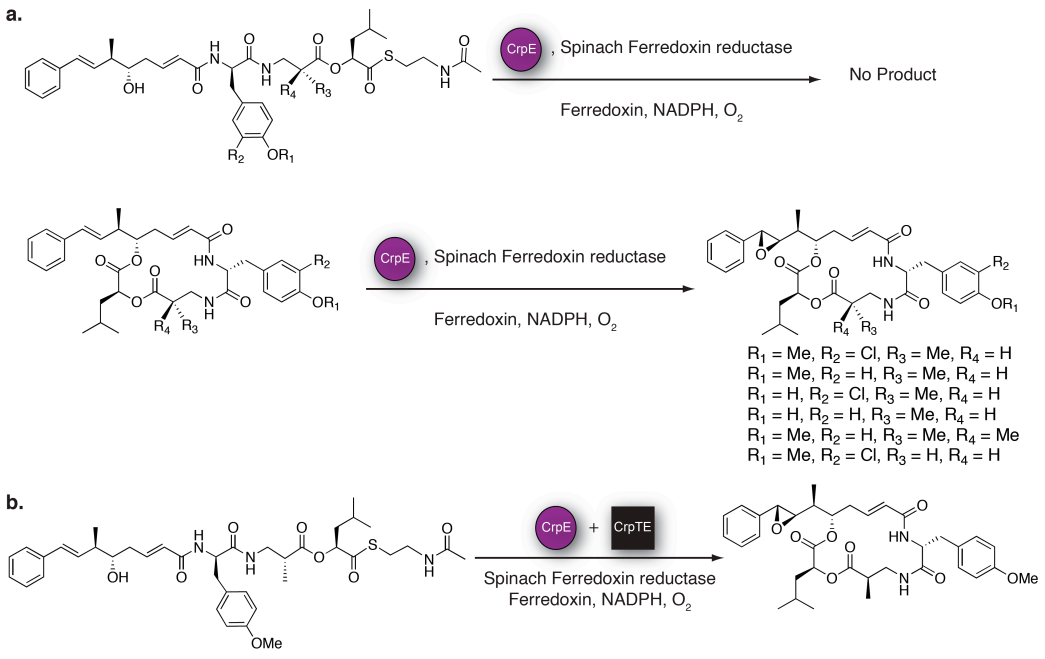


In a subsequent study, it was shown that this methodology could be expanded to solid-phase bound cryptophycin precursors.[19] Several *seco*-cryptophycin analogues were synthesized as acyl sulfonamides on safety-catch PEGA resin, which were activated with iodoacetonitrile and subsequently subjected to macrocyclization reactions with Crp TE (**Figure 6.8b**). Here, Crp TE was not only capable of cyclizing these substrates directly from the activated solid support, but also tolerated changes to the Unit B aryl ring as well as a switch from an ester to an amide linkage between Unit C and D. These results clearly demonstrate the value of the cryptophycin thioesterase as a versatile biocatalyst for the synthesis of novel cryptophycins from linear and resin-bound precursors.

With regards to the Crp β-epoxide moiety, insertion can only be accomplished with modest diastereoselectivity by chemical methods and separation of the resulting mixture of α/β diastereomers is cumbersome.[20,21] At the same time, the cryptophycin

epoxide group confers a 100-fold increase in compound potency, thus necessitating its installation.[22] Recently, the engineered P450 epoxidase CrpE from cryptophycin biosynthesis has been shown to be a viable alternative to these inefficient processes. In those studies, CrpE was expressed as a recombinant protein containing an N-terminal maltose-binding protein (MBP) tag and incubated with a small library of desepoxy cryptophycin substrates *in vitro*[23] (**Figure 6.9**). The resulting β-epoxy cryptophycins were generated efficiently as single diastereomers, though linear cryptophycins were not epoxidated, indicating that cyclization is a prerequisite for CrpE activity. At the same time, the epoxidase also demonstrated tolerance toward functional changes on Units B and C. Finally, it was also shown *in vitro* that CrpTE and CrpE can be used in tandem to cyclize and epoxidate linear cryptophycins in a single reaction.[17] This has the potential to greatly streamline the chemoenzymatic synthesis of new cryptophycins by accomplishing the two most difficult steps in an efficient and economical manner.

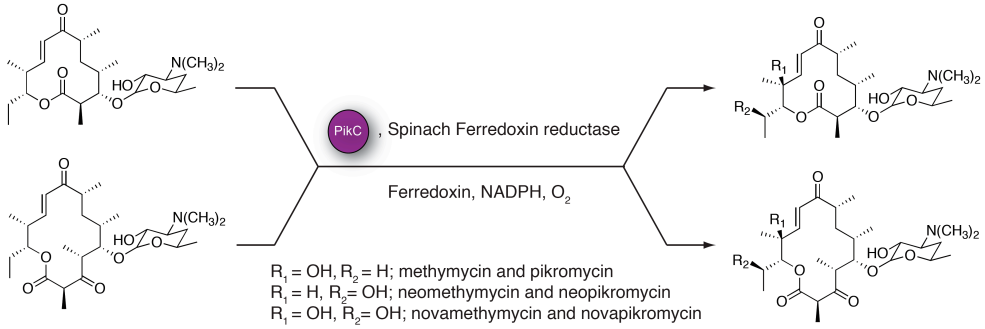
Figure 6.9 – *In vitro* chemoenzymatic a) epoxidation of cryptophycin intermediates by the CrpE epoxidase and b) tandem epoxdation and macrocyclizaiton of linear Crp intermediates by CrpE and Crp TE.



Pikromycin

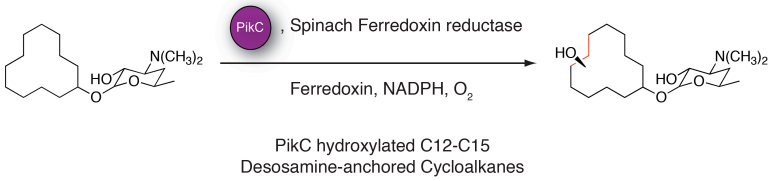
In addition to the thioesterase domain from the Pik biosynthetic pathway, the P450 hydroxylase, PikC, also represents an attractive candidate for chemoenzymatic synthesis since it exhibits broad substrate selectivity. Natively, PikC installs the difficult quaternary C10 and C12 hydroxyls in the major methymycin and pikromycin metabolites, respectively.[24] In a series of *in vitro* studies with recombinant PikC, the P450 was shown to be extraordinarily flexible, hydroxylating both the C10 and C12 positions of the 12-membered ring macrolide YC-17 (10-Dml glycoslyated with desosamine on the C-3 hydroxyl) as well as both the C12 and C14 positions of the 14-membered ring macrolide narbomycin (narbonolide glycosylated with desosamine on the C-5 hydroxyl)[24-26] (**Figure 6.10**). Here, it was determined that substrate anchoring of the macrolactones by the desosamine sugar in the PikC active site was vital for activity and selectivity.[27] Also, the alternative hydroxylation patterns seen in these *in vitro* studies were identified natively *in vivo* as minor Pik metabolites neomethymycin/novamethymycin and neopikromycin/novapikromycin.[26, 28]

Figure 6.10 – Hydroxylation patterns of YC-17 and narbomycin by the PikC P450.



Recent experiments have given further insight into PikC's inherent flexibility, where a set of relatively unsubstituted hydrocarbon macrocycles were hydroxylated by the P450 *in vitro*, regardless of ring size.[29] The only required substitution for hydroxylation by PikC was the appendage of a desosamine sugar on the macrocycle. Here, the desosamine positioned the most distal carbons on the macrocycles close to the enzyme Fe center, resulting in hydroxylation at those positions (**Figure 6.11**).

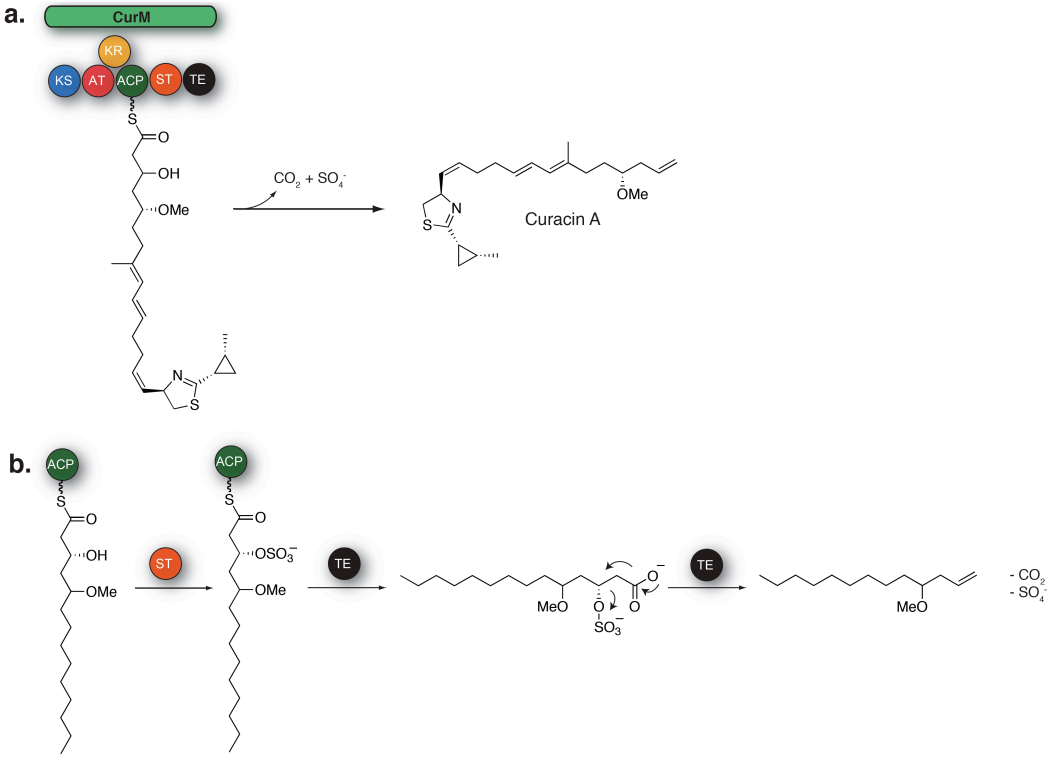
Figure 6.11 - Demonstration of substrate flexibility in the PikC P450 hydroxylase on desosamine-anchored unsubstituted cycloalkanes. Carbons distal to the desosamine were hydroxylated (shown in red).



Curacin and Tautomycetin

With the ongoing discovery of new natural products and their putative biosynthetic pathways, the biochemical toolbox for chemoenzymatic synthesis continues to expand. This has resulted in increasing levels of chemical diversity and the uncovering of enzymes that can perform unique chemical transformations. As has been discussed earlier, macrocyclization by a PKS or NRPS terminal thioesterase domain is often a typical step in forming bioactive natural products; however, some natural products defy this paradigm. This is the case with the natural products curacin A[30] (Cur) and tautomycetin[31] (Tmc), where distinctive chain termination events give rise to linear products with rare terminal olefin groups. In both cases, a series of hydrolysis, decarboxylation, and dehydration reactions appears to be required for formation of this functionality, though the mechanisms for accomplishing this are divergent in the two systems.

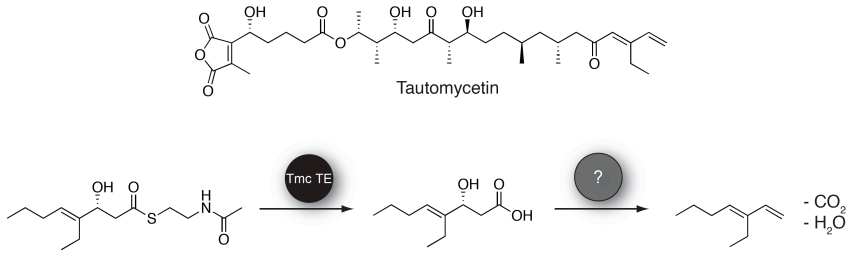
Figure 6.12 - Illustration of a) chain termination in the final CurM module, generating the terminal olefin in Curacin A. b) The sulfotransferase (ST) in CurM preferentially adds a sulfate to the (*R*)-hydroxyl of a synthetic curacin mimic, followed by sequential hydrolysis and decarboxylation by the Cur TE to give the terminal alkene.



For the Cur and Tmc systems, terminal thioesterases hydrolyze their respective intermediate metabolites from upstream ACPs to give linear *seco*-acids. In curacin biosynthesis, this TE-mediated cleavage is preceded by an unusual embedded sulfotransferase (ST) enzyme in the final CurM module that transfers a sulfonate to the β-hydroxy group of the ACP-tethered intermediate[32] (**Figure 6.12**). Following sulfonation, the TE domain catalyzes hydrolysis of the intermediate phosphopantetheinyl thioester with concomitant decarboxylation and elimination of sulfate to give the putative terminal olefin. By contrast, in tautomycetin biosynthesis the linear *seco*-acid formed by TE hydrolysis of the final intermediate is unactivated for elimination at the β-hydroxy position. Here, hydrolysis occurs in a stereospecific manner favoring the (*R*)-hydroxyl group at the β-position. At the same time, unlike in Cur biosynthesis,

decarboxylation/elimination is uncoupled from the thioesterase activity.[33] Rather, it is presumed that committed decarboxylase/dehydratase enzymes are responsible for decarboxylation and elimination of water to give the final product (**Figure 6.13**). Currently, two putative decarboxylases and a dehydratase, encoded by *tmcJ*, *tmcK*, and *tmcM* are candidate enzymes for catalyzing these reactions.

Figure 6.13 - *In vitro* assay of Tautomycetin (Tmc) TE activity. TMC TE preferentially hydrolyzes synthetic Tmc SNAC substrate mimics containing an (*R*)-hydroxyl at the β-position. Decarboxylation and dehydration of the resulting *seco*-acid are carried out by unidentified enzymes to give the terminal olefin.



Future Outlook

Realization of the promise of chemoenzymatic synthesis is rooted in a successful understanding of the fundamental mechanistic underpinnings of the enzymes of interest. Through a synergistic merging of synthetic chemistry with enzymology and structural biology, detailed knowledge can be gleaned for natural product biosynthetic systems, thus paving the way for their rational application toward generating new compounds of interest.

The ability of recombinant thioesterases to chemoenzymatically generate macrocyclic natural product scaffolds has excellent promise for medicinal chemistry, providing a robust and environmentally friendly alternative to difficult chemical macrocyclization strategies. The examples presented are representative of the broad utility of TEs as biocatalysts toward this end. Salient examples have also been

demonstrated with thioesterases in other PKS/NRPS systems such as tyrocidine[5, 34-37] and epothilone,[38, 39] further supporting this general premise. In order to leverage the catalytic power and specificity of recombinant TEs, however, future studies must first aim at detailed understanding of their many underlying facets and limitations. It can be envisioned that through structure-based protein engineering efforts, rational development of TEs with expanded substrate tolerances and increased catalytic efficiencies would be within reach.

At the same time, P450 hydroxylases and epoxidases are well positioned to address the distinctly difficult task of selectively oxidizing C-H bonds and alkenes in a stereo- and regiospecific manner. Both the CrpE epoxidase and PikC hydroxylase are powerful examples of the utility of P450s for post-PKS/NRPS tailoring of natural product scaffolds. Nonetheless, development of P450 enzymes for chemoenzymatic synthesis is not without significant hurdles. For example, P450s require reductase enzyme partners for activity, and may not be compatible with *in vivo* partners during heterologous expression. For *in vitro* work, this requires the use of an exogenous reductase partner, such as commercially available spinach ferredoxin reductase.[23, 24] This strategy, however, is not economical for large-scale chemoenzymatic synthesis and does not necessarily reflect general reductase compatibility of other natural product pathway P450s. In the case of PikC, this was hurdle overcome by making a PikC-RhFRED fusion to generate a self-sufficient reductase-coupled P450.[25] General applicability of this strategy was demonstrated in the same study, where a similar fusion was made with one of the erythromycin P450s, EryF, to again give a self-sufficient enzyme. Lastly, substrate specificity requirements of P450 enzymes, such as the desosamine anchoring mechanism

of PikC,[27, 29] must be considered in order to successfully apply them to new substrates. Nevertheless, despite these challenges, the opportunity afforded by biosynthetic P450s remains exciting.

Working in larger systems, the manipulation of complete PKS modules with multiple enzymatic domains represents a distinctly challenging goal in chemoenzymatic synthesis, but one that can pay extraordinary dividends. By harnessing the biosynthetic potential of full modules, it can be envisioned that an array of polyketide scaffolds containing myriad stereochemical and functional permuations could be accomplished in combinatorial fashion. The examples of the Pik and DEBS PKSs are a testament to the utility of intact modules for chemoenzymatic synthesis both *in vitro* and *in vivo*. At the same time, current work in other systems such as epothilone[38, 39] and soraphen[40-42] continue to expand our access to new PKS modules with unique substrate specificities and catalytic capabilities. So, while current understanding of PKS modules in many systems is still insufficient or incomplete for practical combinatorial biosynthesis, the most recent studies using native chain elongation intermediates from Pik and DEBS[1, 43] have been particularly crucial in paving the way for detailed biochemical evaluations of underlying mechanisms in those two PKSs. This suggests that future studies must continue to employ native or near-native substrates and their analogues to rigorously probe inherent molecular specificities of modular PKS domains for accepting, elongating, processing, and cyclizing substrates.

Finally, the unique and divergent strategies for rendering a terminal alkene product from the curacin A and tautomycetin biosynthetic pathways highlight the malleability of PKS and NRPS systems toward the evolution of new enzyme activities.

Indeed, amongst biosynthetic chain termination strategies, new patterns continue to emerge that depart from more conventional macrocyclization and hydrolysis strategies. In addition to terminal olefin formation, reductase-catalyzed off-loading of aldehyes[44] and TE-mediated intermolecular cyclooligomerizations[45-47] have also been reported. Still, these enzyme activities only represent a small fraction of all marine and terrestrial metabolic diversity and potential. Mechanisms for constructing other novel structures such as the cyclopropane ring and vinyl chloride moieties in curacin A and the jamaicamides[48] and the exocyclic enones on the bryostatin core[49] have also been elucidated recently. So, with the ongoing discovery and addition of new enzymes to the biochemical toolbox, the prospects for accessing valuable chemical diversity through chemoenzymatic synthesis continues to remain bright.

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