

Synthesis and Biochemical Analysis of Complex Chain-Elongation Intermediates for Interrogation of Molecular Specificity in the Erythromycin and Pikromycin Polyketide Synthases

Jonathan D. Mortison, Jeffrey D. Kittendorf, and David H. Sherman*

*Life Sciences Institute, Department of Chemistry and Department of Medicinal Chemistry,
210 Washtenaw Avenue, University of Michigan, Ann Arbor, Michigan 48109*

Received July 20, 2009; E-mail: davidhs@umich.edu

Abstract: The 6-deoxyerythronolide B synthase (DEBS) and pikromycin (Pik) polyketide synthase (PKS) are unique multifunctional enzyme systems that are responsible for the biosynthesis of the erythromycin and pikromycin 14-membered ring 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. In these studies, the native pentaketide intermediate for DEBS was synthesized and employed for *in vitro* chemoenzymatic synthesis of macrolactone products in engineered monomodules Ery5, Ery5-TE, and Ery6. A comparative analysis was performed with the corresponding Pik module 5 (PikAIII) and module 6 (PikAIV), 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.

Introduction

Biosynthetic assembly of polyketide natural products occurs on multienzyme megasynthases termed polyketide synthases (PKSs).¹ 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. These domains are uniquely arranged in bacterial type I PKSs, where they are organized into covalently bound collinear modules. 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)^{2,3} or exchange of full modules to form hybrid PKS systems.^{4–6} 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.^{5–7} These studies

highlight the need for detailed knowledge regarding the fundamental mechanistic underpinnings of PKS catalytic processes.

Currently, the most well studied modular type I PKSs are the 6-deoxyerythronolide B synthase (DEBS, Figure 1)^{8,9} and the pikromycin PKS¹⁰ (Pik, Figure 2). 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.

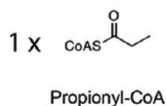
In the DEBS system, many pioneering experiments have carved out an understanding of modular PKS catalytic efficiency and substrate specificity. Probing of individual DEBS modules, including 5 and 6, with short chain diketide model substrates

- (1) Staunton, J.; Weissman, K. J. *Nat. Prod. Rep.* **2001**, *18*, 380–416.
- (2) Donadio, S.; McAlpine, J. B.; Sheldon, P. J.; Jackson, M.; Katz, L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7119–7123.
- (3) McDaniel, R.; Kao, C. M.; Fu, H.; Hevezi, P.; Gustafsson, C.; Betlach, M.; Ashley, G.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **1997**, *119*, 4309–4310.
- (4) Tang, L.; Fu, H.; McDaniel, R. *Chem. Biol.* **2000**, *7*, 77–84.
- (5) Yoon, Y. J.; Beck, B. J.; Kim, B. S.; Kang, H. Y.; Reynolds, K. A.; Sherman, D. H. *Chem. Biol.* **2002**, *9*, 203–214.

- (6) Menzella, H. G.; Reid, R.; Carney, J. R.; Chandran, S. S.; Reisinger, S. J.; Patel, K. G.; Hopwood, D. A.; Santi, D. V. *Nat. Biotechnol.* **2005**, *23*, 1171–1176.
- (7) McDaniel, R.; Thamchaipenet, A.; Gustafsson, C.; Fu, H.; Betlach, M.; Betlach, M.; Ashley, G. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1846–1851.
- (8) Cortes, J.; Haydock, S. F.; Roberts, G. A.; Bevitt, D. J.; Leadlay, P. F. *Nature* **1990**, *348*, 176–178.
- (9) Donadio, S.; Staver, M. J.; McAlpine, J. B.; Swanson, S. J.; Katz, L. *Science* **1991**, *252*, 675–679.
- (10) Xue, Y.; Zhao, L.; Liu, H. W.; Sherman, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12111–12116.

DEBS PKS

Starter unit:



Extender units:

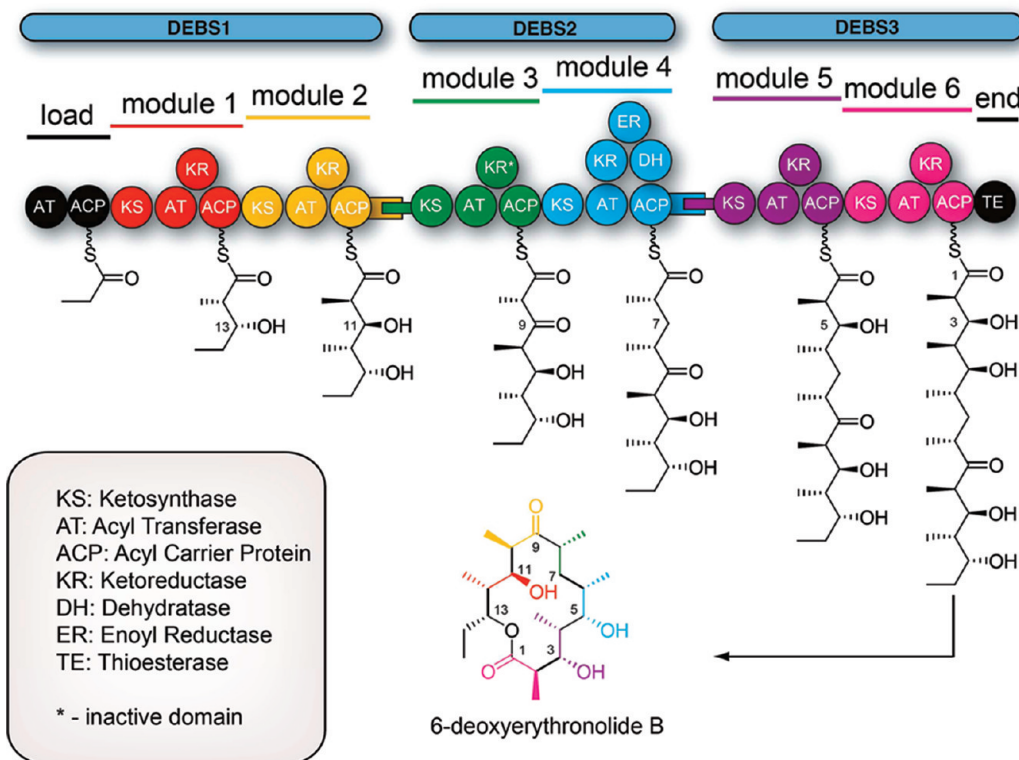
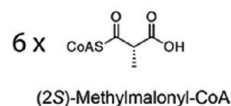


Figure 1. Illustration of the DEBS PKS from the erythromycin biosynthetic pathway. DEBS is responsible for generation of the aglycone 6-deoxyerythronolide B (6-DEB).

revealed that they each have an inherent molecular selectivity profile and a high degree of catalytic competence.^{11–13} However, 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 that must accept and process increasingly complex polyketide intermediates in the growing chain. Comparative study of late modules of the pikromycin PKS^{14,15} showed that extension and processing of model diketides by Pik monomodules were nearly 3 orders of magnitude less efficient than DEBS. More in-depth experiments,¹⁶ though, showed that these diketide model compounds do not effectively probe the molecular recognition features of these PKS modules. Rather, utilizing full-length native chain elongation substrates in biochemical studies of PKS modules yields a more valuable

assessment of kinetic parameters and modular specificity, as 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.

To further our assessment of the molecular recognition features in late-stage DEBS modules, we were motivated to synthesize the native pentaketide substrate and employ it for *in vitro* biochemical analysis of engineered monomodules from the DEBS3 polypeptide. To establish an effective experimental system, the bimodular DEBS3 was separated into Ery5 (module 5), Ery5-TE (module 5 + engineered TE fusion), Ery6 (module 6 + native TE fusion), and DEBS TE (excised TE domain). The catalytic competence and substrate specificity profiles of these modules were evaluated and compared to corresponding late monomodules (module 5 and module 6) from the Pik PKS.

Results

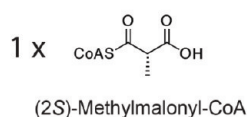
Total Synthesis of DEBS SNAC Pentaketide Substrate.

Retrosynthetic analysis of the native DEBS pentaketide (**1**) revealed an aldol disconnection between C6–C7, to give keto acid **2** and aldehyde **3** as the required fragments (Scheme 1). Selectivity for the desired anti-Felkin *syn* diastereomer could be achieved in the aldol coupling via a chelation-control

- (11) Gokhale, R. S.; Tsuji, S. Y.; Cane, D. E.; Khosla, C. *Science* **1999**, *284*, 482–485.
- (12) Wu, N.; Kudo, F.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **2000**, *122*, 4847–4852.
- (13) Wu, N.; Tsuji, S. Y.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **2001**, *123*, 6465–6474.
- (14) Beck, B. J.; Aldrich, C. C.; Fecik, R. A.; Reynolds, K. A.; Sherman, D. H. *J. Am. Chem. Soc.* **2003**, *125*, 12551–12557.
- (15) Yin, Y.; Lu, H.; Khosla, C.; Cane, D. E. *J. Am. Chem. Soc.* **2003**, *125*, 5671–5676.
- (16) Aldrich, C. C.; Beck, B. J.; Fecik, R. A.; Sherman, D. H. *J. Am. Chem. Soc.* **2005**, *127*, 8441–8452.

Pik PKS

Starter unit:



Extender units:

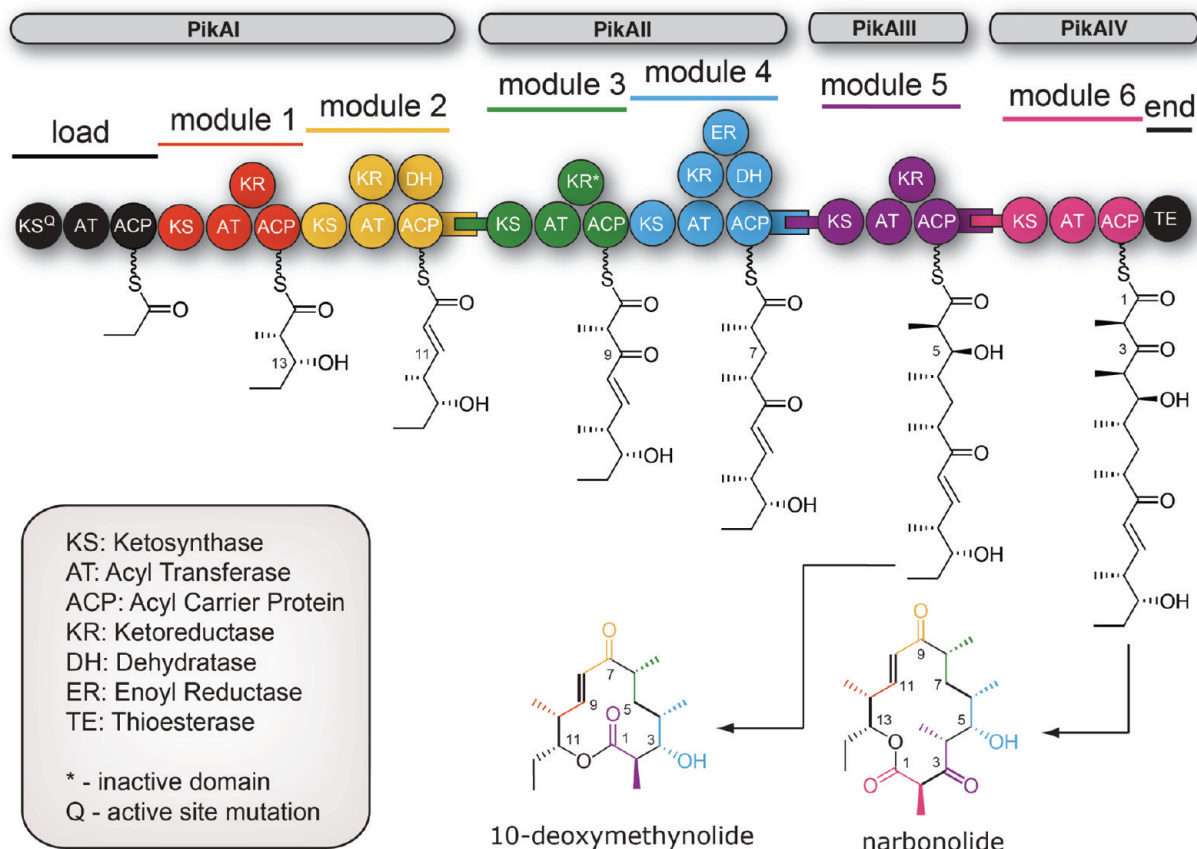
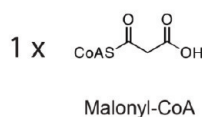
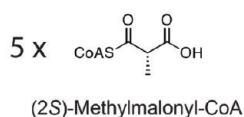
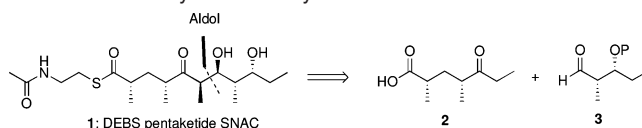


Figure 2. Illustration of the Pik PKS from the methymycin/pikromycin biosynthetic pathway. Pik produces the aglycones 10-deoxymethynolide (10-Dml) and narbonolide (Nbl).

element.^{17,18} 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 2). Diastereoselective Myers alkylation¹⁹ of **4** with the enolate of (*S,S*)-pseudoephedrine propionamide gave amide **5** with high selectivity for the desired 2,4-*syn* product.²⁰ The amide was then readily ethylated with EtLi to furnish ketone **6**. Originally, ketone **6** was employed as the intended aldol-coupling partner, but complications arose in subsequent steps, making the strategy untenable. This was circumvented by instead using the C1-oxidized keto acid **2** as the direct coupling partner. Attempts to oxidize C1 via two-step deprotection/oxidation procedures were

Scheme 1. Retrosynthetic Analysis



problematic due to varying degrees of epimerization; however, one-pot Sharpless oxidation of the TBS ether using RuO₄²¹ proved to be a clean and facile route to **2**.

Synthesis of the right fragment **3** began with known Evans aldol product **7**,^{22,23} which was protected with a benzyl formacetal group (Scheme 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 BOM ether protecting group was utilized since its extended linker had been thought to allow chelation at

(17) Masamune, S.; Ellingboe, J. W.; Choy, W. *J. Am. Chem. Soc.* **1982**, *104*, 5526–5528.

(18) Martin, S. F.; Lee, W. C.; Pacofsky, G. J.; Gist, R. P.; Mulhern, T. A. *J. Am. Chem. Soc.* **1994**, *116*, 4674–4688.

(19) Myers, A. G.; Yang, B. H.; Chen, H.; McKinstry, L.; Kopecky, D. J.; Gleason, J. L. *J. Am. Chem. Soc.* **1997**, *119*, 6496–6511.

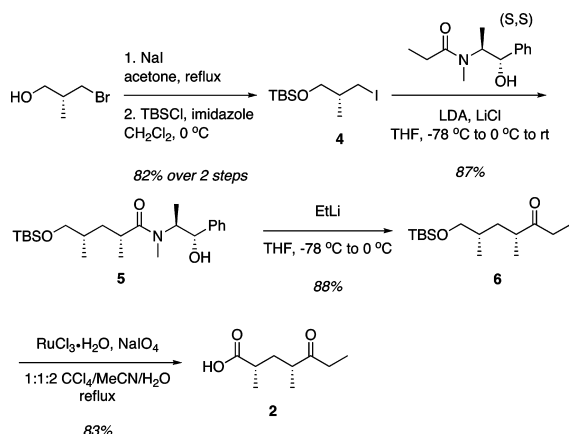
(20) Amide **5** was isolated as a single diastereomer. The 2,4-*syn* diastereomer could be confirmed by reductive removal of the pseudoephedrine auxiliary (see ref 19) and TBS deprotection to give the *meso* 1,5-diol.

(21) Carlsen, P. H. J.; Katsuki, T.; Martin, V. S.; Sharpless, K. B. *J. Org. Chem.* **1981**, *46*, 3936–3938.

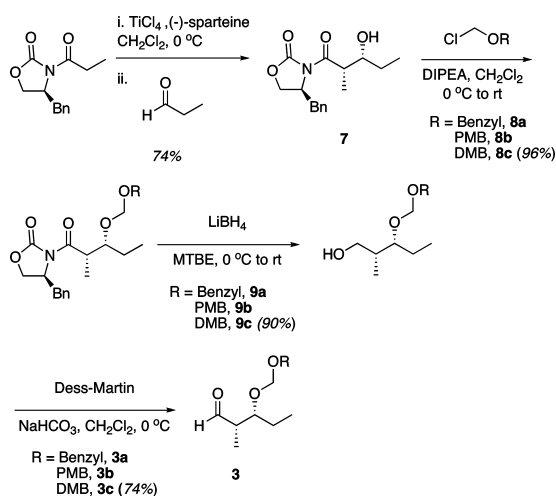
(22) Crimmins, M. T.; King, B. W.; Tabet, E. A.; Chaudhary, K. *J. Org. Chem.* **2001**, *66*, 894–902.

(23) Pilli, R. A.; de Andrade, C. K. Z.; Souto, C. R. O.; de Meijere, A. *J. Org. Chem.* **1998**, *63*, 7811–7819.

Scheme 2. Synthesis of Left Fragment



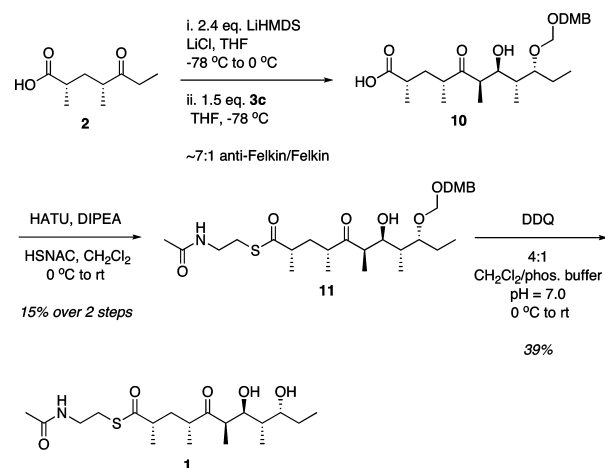
Scheme 3. Synthesis of Right Fragment



the C9 hydroxyl, thus enabling good anti-Felkin selectivity in analogous aldol couplings.¹⁷ However, attempts to remove this group at the end of the synthesis either failed or resulted in degradation of the desired pentaketide; therefore, 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. The *p*-methoxybenzyl (PMBOM) formacetal derivative also proved difficult to remove at the end of the synthesis, but the 3,4-dimethoxybenzyl (DMBOM) derivative was readily deprotected. Therefore, Evans-syn aldol product **7** was protected using DMBOMCl to give **8c**, followed by reductive removal of the Evans auxiliary with LiBH₄ to give alcohol **9c**. Oxidation to aldehyde **3c** was then best accomplished using Dess-Martin periodinane.

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 selectivity²⁴ and modest yield (Scheme 4). This aldol step was followed by subsequent thioesterification with *N*-acetyl cysteamine (SNAC). Due to the exceptional acid sensitivity of **1**, buffered conditions were imperative in the final

Scheme 4. Aldol Coupling and Deprotection



oxidative deprotection of the DMBOM group with DDQ, and deprotection yields suffered somewhat due to competing oxidation to form the DMB orthoester. 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.

Assay of DEBS Monomodule Activity. Engineered Ery5, Ery5-TE, and Ery6 were incubated with the synthetic DEBS pentaketide SNAC substrate and 2-[¹⁴C]-methylmalonyl CoA, and the products were visualized by radio-TLC. In addition, duplicate reactions were performed utilizing unlabeled methylmalonyl CoA, and reaction products were analyzed and confirmed by LC-MS/MS. As demonstrated previously *in vivo*,²⁵ the Ery5-TE fusion protein also generates an unnatural 12-membered ring macrolactone (**12**) *in vitro* (Figures 3 and 4; Scheme 5). This confirms that DEBS TE is capable of producing 12-membered macrocycles, similar to previous observations using PikAIII-TE. 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,²⁶ 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,²⁵ suggesting that its formation *in vitro* might be caused by suboptimal enzymatic processing from the unnatural Ery5-TE fusion protein.

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 S1, Supporting Information) but required further

(24) The anti-Felkin *syn* diastereomer could be confirmed by conversion and correlation to a known lactone derivative: Crimmins, M. T.; Slade, D. J. *Org. Lett.* **2006**, *8*, 2191–2194.

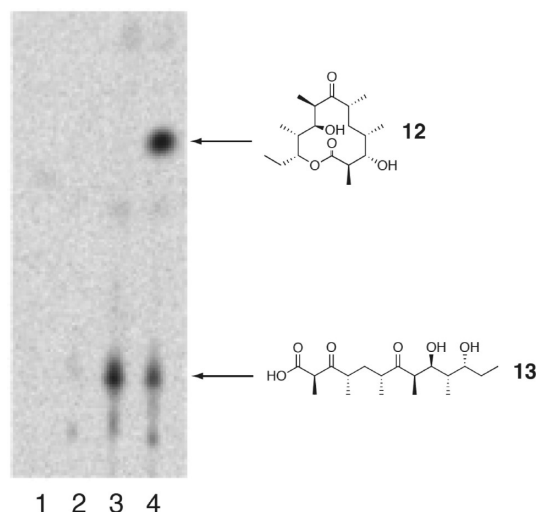


Figure 3. Radio-TLC of reaction products for Ery5-TE incubated with DEBS pentaketide SNAC. Assay conditions are described in the Experimental Section. Lane 1: No substrate (negative control). Lane 2: No enzyme (negative control). Lane 3: Ery5-TE w/o NADPH. Lane 4: Ery5-TE w/ NADPH.

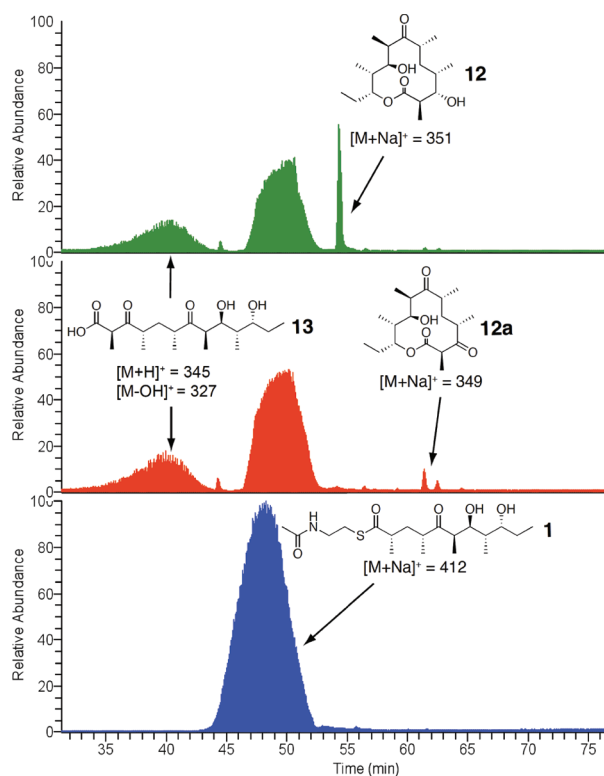
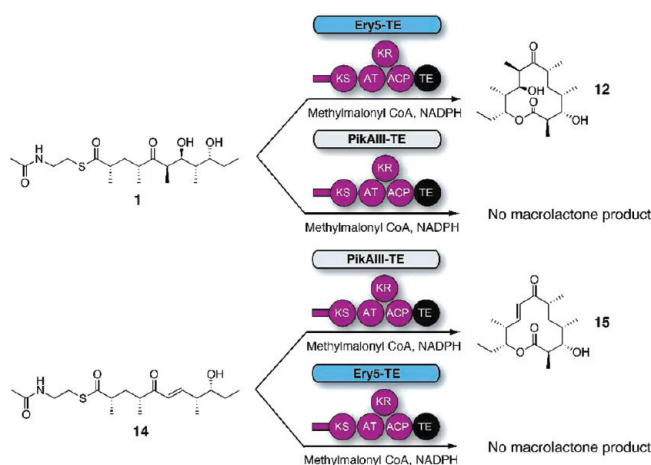


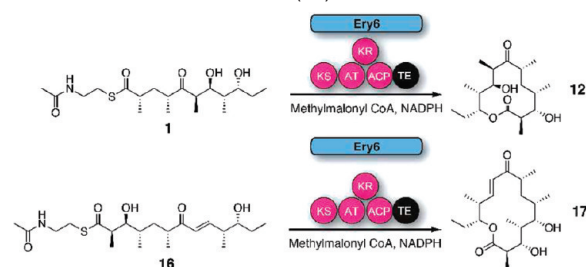
Figure 4. LC-MS chromatogram in selective ion mode (SIM) of Ery5-TE incubated with DEBS pentaketide SNAC. Top: Ery5-TE (green). Middle: Ery5-TE w/o NADPH (red). Bottom: No enzyme control (blue).

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 domains²⁷ (unlike in mono-

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



Scheme 6. Interrogation of Ery6 with Non-Native DEBS Pentaketide and Pik Hexaketide (16) Substrates



modules 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. 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*²⁸ and *in vitro*.²⁹ Remarkably, we observed that Ery6 was able to accept the DEBS pentaketide resulting in low levels of macrolactone **12** and hydrolysis product **13** (Scheme 6; Figure 5). This demonstrates an unexpected flexibility in this module toward incoming substrates bearing noncognate functionality and chain length.

Cross Reactivity between DEBS and Pik Systems. To probe the substrate specificity of the DEBS and Pik late modules, both DEBS pentaketide (**1**) and Pik pentaketide (**14**) substrates³⁰ 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 5). 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 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. PikAIII-TE incubated with the DEBS pentaketide SNAC revealed low

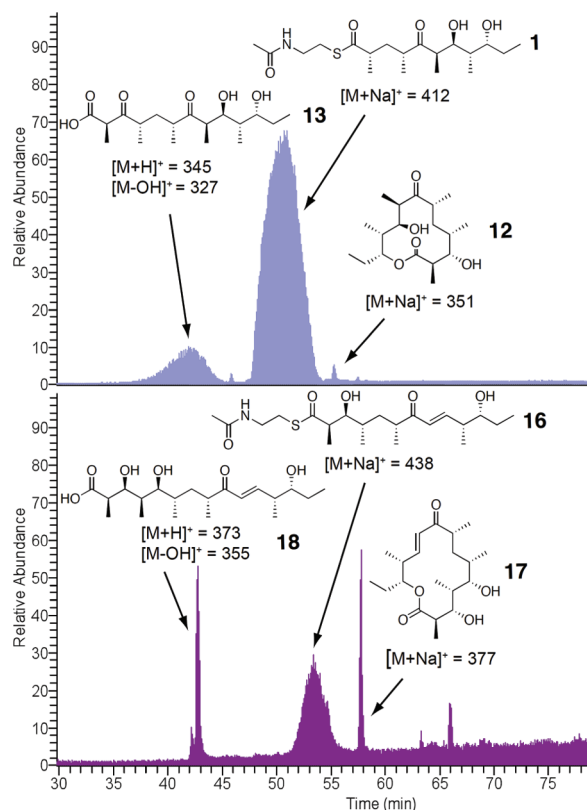


Figure 5. SIM LC-MS chromatogram of Ery6 reactions. Top: Ery6 w/ DEBS pentaketide SNAC (light blue). Bottom: Ery6 w/ Pik hexaketide SNAC (purple).

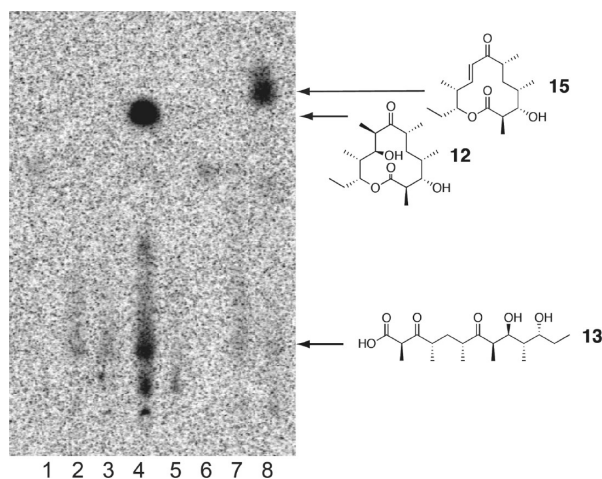


Figure 6. Radio-TLC of noncognate pairing of Pik/DEBS pentaketide SNACs incubated with Ery5-TE and PikAIII-TE. Lane 1: No enzyme w/ Pik pentaketide (negative control). Lane 2: Ery5-TE w/ Pik pentaketide (No NADPH). Lane 3: Ery5-TE w/ Pik pentaketide. Lane 4: Ery5-TE w/ DEBS pentaketide (positive control).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

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, noncognate 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.

Unlike the experiments involving interrogation of noncognate 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 hexaketide³¹ (Scheme 6), though not the Pik pentaketide. When Ery6 was incubated with Pik hexaketide (**16**), both 3-hydroxy-narbornolide (**17**) and hydrolyzed linear heptaketide (**18**) were observed in the radio-TLC (Figure S2, Supporting Information) and LC-MS/MS assay (Figure 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.²⁶ As had been observed previously *in vitro*,¹⁶ 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 7), the result of direct loading, extension, and hydrolysis on the more flexible PikAIV monomodule.

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 earlier aldol step. In addition, similar degradation pathways thwarted current efforts toward the desired DEBS hexaketide intermediate. These challenges highlight an important, yet often underappreciated 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

- (25) Kao, C. M.; Luo, G. L.; Katz, L.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **1995**, *117*, 9105–9106.
- (26) Aldrich, C. C.; Venkatraman, L.; Sherman, D. H.; Fecik, R. A. *J. Am. Chem. Soc.* **2005**, *127*, 8910–8911.
- (27) Buchholz, T. J.; Geders, T. W.; Bartley, F. E.; Reynolds, K. A.; Smith, J. L.; Sherman, D. H. *ACS Chem. Biol.* **2009**, *4*, 41–52.
- (28) Xue, Y.; Sherman, D. H. *Nature* **2000**, *403*, 571–575.
- (29) Kittendorf, J. D.; Beck, B. J.; Buchholz, T. J.; Seufert, W.; Sherman, D. H. *Chem. Biol.* **2007**, *14*, 944–954.
- (30) The Pik pentaketide was synthesized as previously described. See ref 16.
- (31) The Pik hexaketide was synthesized from degradation of 10-DML as previously described. See ref 16.

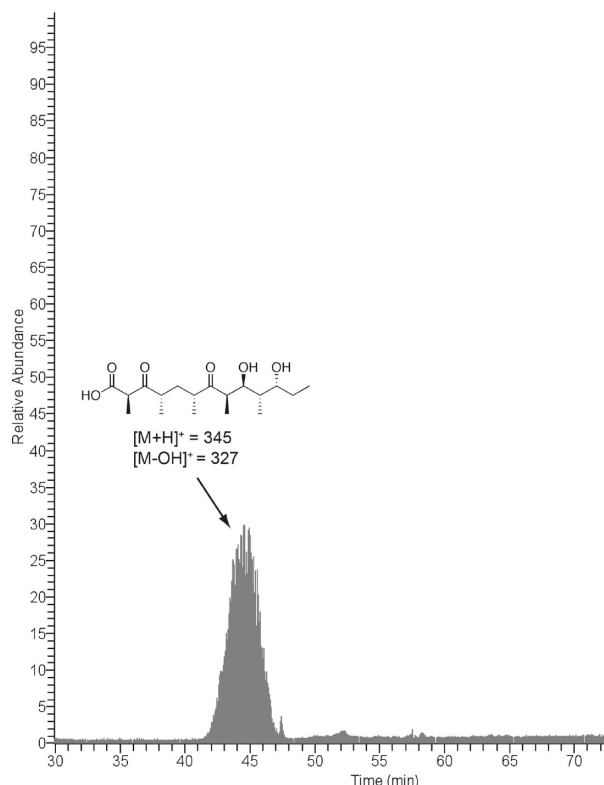


Figure 7. SIM LC-MS chromatogram for DEBS pentaketide SNAC incubated with PikAIII-PikAIV.

and transient intermediates against numerous potential degradation pathways until they are released as more stable products.

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*^{12,13} or *in vivo*²⁵ 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 diketides¹⁴ rather than native substrates. With native Pik pentaketide, no 3-oxo-10-Dml was observed with PikAIII-TE when NADPH was included.¹⁶ 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.

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-171,³² 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.³³ 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.¹⁶

While there is evidence that Pik TE has developed direct protein–protein interactions with PikAIII²⁹ 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. 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.³⁴ These modifications at the terminus of the chain elongation intermedi-

(32) Tsai, S. C.; Miercke, L. J. W.; Krucinski, J.; Gokhale, R.; Chen, J. C. H.; Foster, P. G.; Cane, D. E.; Khosla, C.; Stroud, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14808–14813.

(33) Akey, D. L.; Kittendorf, J. D.; Giraldes, J. W.; Fecik, R. A.; Sherman, D. H.; Smith, J. L. *Nat. Chem. Biol.* **2006**, *2*, 537–542.

ate, 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.¹³ 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 noncognate 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. Future efforts will seek to assess the kinetic parameters of the flux through the DEBS modules with native chain-elongation intermediates, including the contribution of channeling to molecular specificity. Concurrently, continued analysis of substrate tolerance in modules from both systems will be assessed using analogues that encompass a variety of stereochemical, structural, and functional group variations.

Experimental Section

Materials and General Procedures. Substrate Synthesis.

Commercially available reagents were purchased from Sigma-Aldrich, Acros Organics, or Fluka or prepared from established literature procedures. $\text{RuCl}_3 \cdot \text{H}_2\text{O}$ was obtained from Strem Chemicals, Inc. The pikromycin pentaketide and hexaketide SNAC substrates were prepared as previously described.¹⁶ Anhydrous solvents were obtained either by using an MBRAUN MB-SPS solvent purification system or by purchasing them in septum-sealed bottles from Acros Organics (AcroSeal) or EMD (DriSolv). Diisopropylamine and *N,N*-diisopropylethylamine were distilled over CaH_2 and stored under argon. Alkylolithiums 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 precoated with Silica Gel 60 F_{254} . ^1H and ^{13}C NMR spectra were obtained on either a Varian MR400 or an Inova 500 spectrometer. Proton chemical shifts are reported in ppm relative to TMS with the residual solvent peak as an internal standard. Proton NMR data are 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.

Iodide 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 of acetone was brought to reflux at 70 °C. After 18 h, the reaction mixture was diluted with 50 mL of 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 of saturated $\text{Na}_2\text{S}_2\text{O}_3$, dried over MgSO_4 , filtered, and concentrated.

The crude iodide was redissolved in 100 mL of 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 resuspended in 100 mL of pentane and filtered, rinsing the filter with an additional 50 mL of 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.

(34) Jacobsen, J. R.; Hutchinson, C. R.; Cane, D. E.; Khosla, C. *Science* **1997**, 277, 367–369.

^1H 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 ESI^+ (m/z): 315.0656 (Predicted $[\text{M} + \text{H}]^+$ for $\text{C}_{14}\text{H}_{24}\text{OSi}$ is 315.0641).

Amide 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 of THF. The reaction was warmed to 0°C briefly for 5 min and then recooled 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 of THF was added dropwise at 0°C , and then the reaction was allowed to warm to room temperature and stirred for 22 h. The reaction was quenched with 100 mL of saturated NH_4Cl and diluted with 100 mL of 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. ^1H 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 $[\text{M} + \text{Na}]^+$ for $\text{C}_{23}\text{H}_{41}\text{NO}_3\text{Si}$ is 430.2753). As expected from literature precedent,¹⁹ **5** was isolated as a mixture of amide rotamers. The reported NMR data only denote peaks arising from the major rotamer.

Ketone 6. To a stirring suspension of amide **5** (1.866 g, 4.58 mmol) in 50 mL of 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 for 10 min, then warmed to 0°C , and stirred an additional 30 min. Subsequently, 1 equiv of diisopropylamine was added to scavenge excess EtLi, and the mixture was stirred for 15 min. The reaction was quenched with 20% AcOH/ Et_2O and diluted with H_2O and EtOAc, and the layers were separated. The aqueous portion 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. ^1H 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 $[\text{M} + \text{Na}]^+$ for $\text{C}_{15}\text{H}_{32}\text{O}_2\text{Si}$ is 295.2069).

Keto Acid 2. Ketone **6** (1.081 g, 3.97 mmol) was dissolved in 20 mL of 1:1:2 $\text{CCl}_4/\text{CH}_3\text{CN}/\text{H}_2\text{O}$, and then $\text{RuCl}_3 \cdot \text{H}_2\text{O}$ (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 of EtOAc and extracted with 3×50 mL of 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 and 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 $[\text{M} + \text{Na}]^+$ for $\text{C}_9\text{H}_{16}\text{O}_3$ is 195.0997).

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 of CH_2Cl_2 was added DMBOMCl³⁵ (7.437 g, 34.32 mmol) in 20 mL of 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 of H_2O , and the layers were separated. The aqueous portion was extracted with 2×25 mL of 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. ^1H 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, $\nu_A = 31.7$, $\nu_B = 11.5$ 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 $[\text{M} + \text{Na}]^+$ for $\text{C}_{26}\text{H}_{33}\text{NO}_7$ is 494.2155).

Alcohol 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 of 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 of saturated Na/K tartrate and stirred until the layers became clear. The layers were separated, and the aqueous portion was extracted with 2×25 mL of 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. ^1H NMR (CDCl_3 , 399.5 MHz) δ 6.91–6.75 (m, 3H), 4.75 (ABq, $\nu_A = 17.4$, $\nu_B = 6.9$ Hz, 2H), 4.55 (ABq, $\nu_A = 31.9$, $\nu_B = 11.5$ 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 $[\text{M} + \text{Na}]^+$ for $\text{C}_{16}\text{H}_{26}\text{O}_5$ is 321.1678).

Aldehyde 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 of CH_2Cl_2 was added Dess-Martin periodinane (2.133 g, 5.03 mmol) at 0°C . The reaction was stirred for 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 $\text{Na}_2\text{S}_2\text{O}_3$ and stirred until the layers became clear. The layers were separated, and the aqueous portion was extracted with 2×25 mL of 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. ^1H NMR (CDCl_3 , 399.5 MHz) δ 9.78 (s, 1H), 7.10–6.41 (m, 1H), 4.75 (ABq, $\nu_A = 18.1$, $\nu_B = 7.2$ 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,

(35) Prepared as previously described: Trost, B. M.; Frederiksen, M. U.; Papillon, J. P. N.; Harrington, P. E.; Shin, S.; Shireman, B. T. *J. Am. Chem. Soc.* **2005**, *127*, 3666–3667.

111.3, 120.5, 130.1, 148.7, 149.0, 204.5; HRMS ESI⁺ (*m/z*): 319.1521 (Predicted [M + Na]⁺ for C₁₆H₂₄O₅ is 319.1527).

DMBOM-Protected DEBS Pentaketide SNAC 11. 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 of THF at −78 °C. The reaction was stirred for 1 h and then warmed to 0 °C for 30 min. The reaction was then recooled to −78 °C, and the aldehyde **3c** (0.901 g, 3.04 mmol) in 5 mL of THF was added dropwise. Following addition, the reaction was stirred for 90 min and then quenched with 20 mL of saturated NH₄Cl. The mixture was diluted with 20 mL of EtOAc and then extracted with 3 × 25 mL of half saturated NaHCO₃. The aqueous extracts were back extracted with 1 × 25 mL of Et₂O and then carefully acidified to pH 2 with 1 N HCl. The aqueous layer was then extracted with 4 × 25 mL of CH₂Cl₂. The combined organics were dried over MgSO₄, filtered, and concentrated. The crude oil (containing a ~7:1 anti-Felkin/Felkin mixture of *seco*-acids by ¹H NMR) was carried on to the next step without further purification.

The crude acid was dissolved in 20 mL of CH₂Cl₂, 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 of saturated NaHCO₃, and the resulting layers were separated. The aqueous layer was then extracted with 2 × 25 mL of CH₂Cl₂. The combined organics were dried over MgSO₄, filtered, and concentrated. The crude oil was partially purified by flash chromatography (2% MeOH/CH₂Cl₂) and then purified by preparative HPLC on a Phenomenex Luna C18(2) column (5 μm, 21.2 mm × 250 mm) using a CH₃CN/H₂O + 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. ¹H NMR (CDCl₃, 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, *ν*_A = *ν*_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); ¹³C NMR (CDCl₃, 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 C₂₉H₄₇NO₈S is 592.2920).

DEBS Pentaketide SNAC 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 CH₂Cl₂/0.5 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 of saturated NaHCO₃ and diluted with 10 mL of CH₂Cl₂. The layers were separated, and the aqueous portion was extracted with 2 × 10 mL of CH₂Cl₂. The combined organics were dried over MgSO₄, filtered, and concentrated. The crude material was resuspended in 1 mL of CH₃CN, 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 mm × 250 mm) using a CH₃CN/H₂O 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 an ~2:1 equilibrium mixture of open linear pentaketide and closed hemiketal. ¹H NMR (CDCl₃, 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); ¹³C NMR (CDCl₃, 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 C₁₉H₃₅NO₅S is 412.2134).

Acknowledgment. This research was supported by a Rackham Regents' Predoctoral Fellowship and a graduate research fellowship from Eli Lilly & Co. (to J.D.M.), an NIH NRSA (GM075641) Postdoctoral Fellowship (to J.D.K.), and NIH Grant GM076477 and the Hans W. Vahlteich Professorship (to D.H.S.).

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at <http://pubs.acs.org>.

JA9060596