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Biochemical Investigation of Pikromycin Biosynthesis Employing Native Penta- and Hexaketide Chain Elongation Intermediates

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Abstract: The unique ability of the pikromycin (Pik) polyketide synthase to generate 12- and 14-membered ring macrolactones presents an opportunity to explore the fundamental processes underlying polyketide synthesis, specifically the mechanistic details of chain extension, keto group processing, acyl chain release, and macrocyclization. We have synthesized the natural pentaketide and hexaketide chain elongation intermediates as *N*-acetyl cysteamine (NAC) thioesters and have used them as substrates for in vitro conversions with engineered PikAIII+TE and in combination with native PikAIII (module 5) and PikAIV (module 6) multifunctional proteins. This investigation demonstrates directly the remarkable ability of these monomodules to catalyze one or two chain extension reactions, keto group processing steps, acyl-ACP release, and cyclization to generate 10-deoxymethynolide and narbonolide. The results reveal the enormous preference of Pik monomodules for their natural polyketide substrates and provide an important comparative analysis with previous studies using unnatural diketide NAC thioester substrates.

Introduction

The rapid rise in resistance to MLS (macrolide−lincomycin−streptomycin) antibiotics underscores an urgent need to develop alternatives to current clinical antimicrobial agents.[1] Semisynthetic modifications of erythromycin have provided the ketolide class of antibiotics, such as telithromycin (Ketek) and cethromycin (ABT-773), which are characterized in part by a 3-keto group with activity against MLS-resistant pathogens.[2,3] Continued advances toward effective redesign of polyketide biosynthesis suggest a growing role for metabolic engineering in providing new ketolide templates for next-generation macrolide antibiotics capable of overcoming antibiotic resistant microbial pathogens.

Polyketide biosynthesis is catalyzed by modular multifunctional proteins termed polyketide synthases (PKSs) that catalyze the repetitive condensation of malonyl- or methylmalonyl coenzyme-A thioester monomers. Bacterial type I PKSs are organized into modules each of which catalyzes one cycle of elongation. A prototypical module minimally consists of three domains: an acyl carrier protein (ACP), an acyltransferase (AT) that loads the ACP with a malonyl or methylmalonyl extender unit, and a β-ketosynthase (KS) domain that catalyzes the decarboxylative condensation between extender units (linked to the ACP domain) and the growing polyketide chain (linked via the KS active site Cys residue) to afford a β-ketoacyl-ACP. Each elongation cycle results in a β-keto-group that can undergo additional reductive processing steps. Catalytic domains that perform these reactions include a ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER). The absence of β-keto processing domains results in retention of a carbonyl group, a KR alone gives rise to β-hydroxyl functionality, and a KR and DH generate an alkene, while a KR, DH, and ER combination leads to complete reduction to an alkane. Processing of the growing polyketide through the PKS enzyme complex leads to the fully elaborated chain elongation product that is released either as a carboxylic acid or as macrolactone by the thioesterase (TE) domain located on the C-terminus of the last module. The collinearity between the genetic architecture and biochemical organization of polyketide biosynthesis, coupled with increasingly powerful tools to manipulate modular PKSs presents opportunities to create novel structures based on a ketolide core.[4,5]

Currently, our knowledge of type I PKSs is largely based on the analysis of 6-deoxyerythronolide B polyketide synthase (DEBS),[6,7] which appears to be unusually tolerant and can accept

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a variety of nonnatural structural and stereochemical variations to the polyketide chain.[8] This tolerance has been partly inferred through in vivo experiments where *N*-acetylcysteamine (NAC) thioester activated di- and triketide substrates supplemented in the fermentation medium are incorporated onto early stage PKS modules. These precursor directed biosynthetic approaches have produced new 14- and 16-membered ring macrolides and provided latent functionality at the C-13 position of the macrolactone ring, enabling further synthetic modifications.[4,9−13] Moreover, in vitro experiments using di- and triketide NAC thioester substrates with purified modules from the DEBS and Pik PKS systems have provided comparative information on the inherent substrate specificity (k_{cat}/K_M) and channeling of these PKS enzymes. Biochemical analyses of engineered monomodules from DEBS3 (modules 5 and 6) revealed that the substrate specificities were partly dependent on the stereochemistry and functionality at the α and β position of the polyketide chain substrate and revealed that they have increased flexibility relative to analogous Pik monomodular PKS enzymes.[14−17] Specifically, the calculated k_{cat} values for extension and lactonization of diketide substrates by the terminal Pik PKS monomodules were approximately 3 orders of magnitude lower than the in vivo rates of antibiotic production consistent with a strict substrate specificity for these enzymes.[17]

The use of diketide and triketide NAC thioester substrates has proven invaluable for investigating fundamental mechanisms of polyketide biosynthesis. However, their relevance in determining the inherent selectivity of a non-native cognate module remains unclear, as these substrates do not adequately represent the natural chain elongation intermediates processed by non-native modules. It is apparent that analysis of substrate specificity of PKS modules with natural chain elongation substrates would provide a more accurate measure of the catalytic and kinetic capacity of these systems and accelerate acquisition of knowledge required to design modular PKS systems with novel specificities and processing characteristics.

Thus, generating fully elaborated polyketide chain elongation intermediates is a fundamental requirement for elucidating the complex biochemical processes involved in polyketide biosynthesis. In some cases, previously developed synthetic methodologies can be applied directly or with little modification to facilitate biosynthetic investigations. For example, in vitro lactonization and hydrolysis rates for the isolated TE domain of epothilone PKS, a microbial secondary metabolite with derivatives in clinical evaluation, have been examined using a full-length NAC thioester substrate generated by direct ring opening of the natural product to the corresponding *seco*-acid.[18] A previously developed synthetic strategy[19] provided the advanced acyl chain (as the corresponding NAC thioester), presumed to be a natural substrate in the epothilone pathway. The goal of that in vivo study was to test the ability of the final three modules of the epothilone PKS to extend and lactonize the molecule in *Escherichia coli* cells.[19] The need to employ natural substrate advanced chain elongation intermediates is critical for advancing our understanding of these systems. Thus, combining versatile synthetic strategies toward studies of isolated purified modular PKSs promises to unveil the complex properties of these multifunctional enzymes. This concept has motivated the current study, and its clear outcome demonstrates the power of this approach for dissecting modular PKS function and specificity and for expanding the potential for chemoenzymatic-based drug discovery approaches.

Streptomyces venezuelae ATCC 15439 produces the 12- and 14-membered ring macrolides, methymycin (**3**), and pikromycin (**4**), respectively, through the activity of the pikromycin PKS (Pik) (Figure 1). Initiation of polyketide biosynthesis by PikAI and successive elongation through PikAIII provides an ACP_5-bound hexaketide thioester that can undergo two possible fates. Transfer to PikAIV KS_6 and subsequent elongation provides a heptaketide-bound S-ACP_6 intermediate that is cyclized by the C-terminal thioesterase domain to provide the 14-membered ring macrolactone narbonolide (**2**). Alternatively, the hexaketide can be cyclized by the C-terminal thioesterase of PikAIV (by skipping of the chain from ACP_5 to ACP_6 in the absence of a final chain extension step)[20] to provide the 12-membered ring macrocycle 10-deoxymethynolide (**1**). The Pik PKS provides an ideal starting point for chemoenzymatic synthesis of macrocyclic lactones, since narbonolide is analogous to 6-deoxyerythronolide B but possesses the critical 3-keto group found in ketolides, contains an alkene functionality at C10−C11 enabling subsequent chemical modifications, and is not limited by protection of the C-6 hydroxyl group characteristic of the erythromycin-derived semisynthetic ketolides.[21]

Herein we report the synthesis of the native pentaketide and hexaketide chain elongation intermediates of pikromycin biosynthesis, including a kinetic analysis involving the final two modules (Pik module 5 and Pik module 6) of the Pik PKS. This work represents the first biochemical investigation of modular PKS substrate specificity employing advanced polyketide chain intermediates. This versatile synthetic approach to advanced polyketide intermediates and their analysis as substrates for modular PKSs represents a significant step toward exploring mechanistically the basis for sequential chain extension, processing, and macrolactone ring formation by the Pik PKS.

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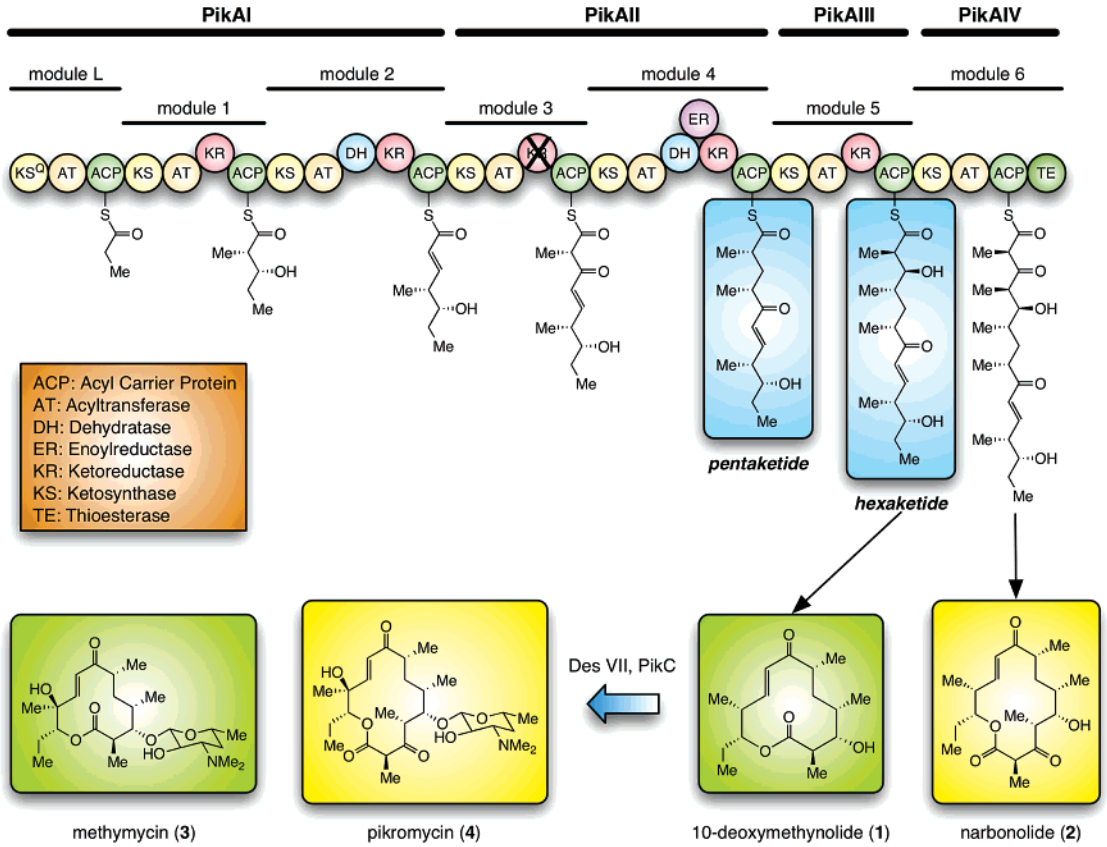
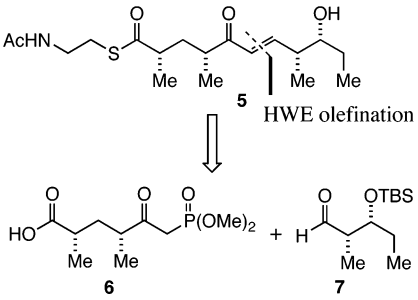
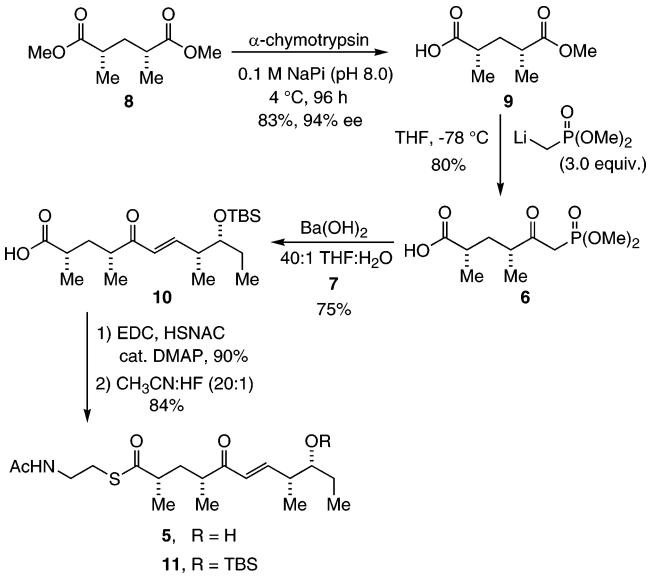


Figure 1. Modular organization of the pikromycin PKS. The extension of the polyketide chain through PikAI−II produces a pentaketide that is elongated by PikAIII (module 5) to produce a hexaketide intermediate. PikAIV (module 6) lactonizes this intermediate to 10-deoxymethynolide (**1**) or catalyzes its extension and subsequent cyclization to narbonolide (**2**). Both aglycones undergo further tailoring to afford methymycin (**3**) and pikromycin (**4**), respectively.

Scheme 1



Scheme 2



Results

Synthesis of Pentaketide and Hexaketide NAC Thioester Substrates. The natural pentaketide and hexaketide substrates for Pik modules 5 and 6 were obtained by total synthesis as well as by degradation chemistry of 10-deoxymethynolide (**1**). While the pentaketide may be obtained through degradation of 10-deoxymethynolide (**1**), a more versatile total synthesis approach was developed on the basis of the potential to generate analogues for future mechanistic studies. Retrosynthetically, dissection of the desired pentaketide **5** across the olefin is via a Horner−Wadsworth−Emmons olefination between β-keto ester **6** and known aldehyde **7**[22] (Scheme 1). The requisite phosphonate building block was prepared by desymmetrization of meso-diester **8** employing α-chymotrypsin to afford monoester **9** in 94% ee (Scheme 2).[23] The stereoselective hydrolysis of the *pro-S* methyl ester was designed to allow protection of the carboxylic acid during the subsequent phosphonate formation. Thus, addition of 3 equivalents of the lithium anion of dimethyl methylphosphonate to **9** afforded β-ketophosphonate **6**, wherein the first equivalent deprotonated the carboxy function that served to protect it from over addition while the second equivalent added to the methyl ester to provide the correspond-

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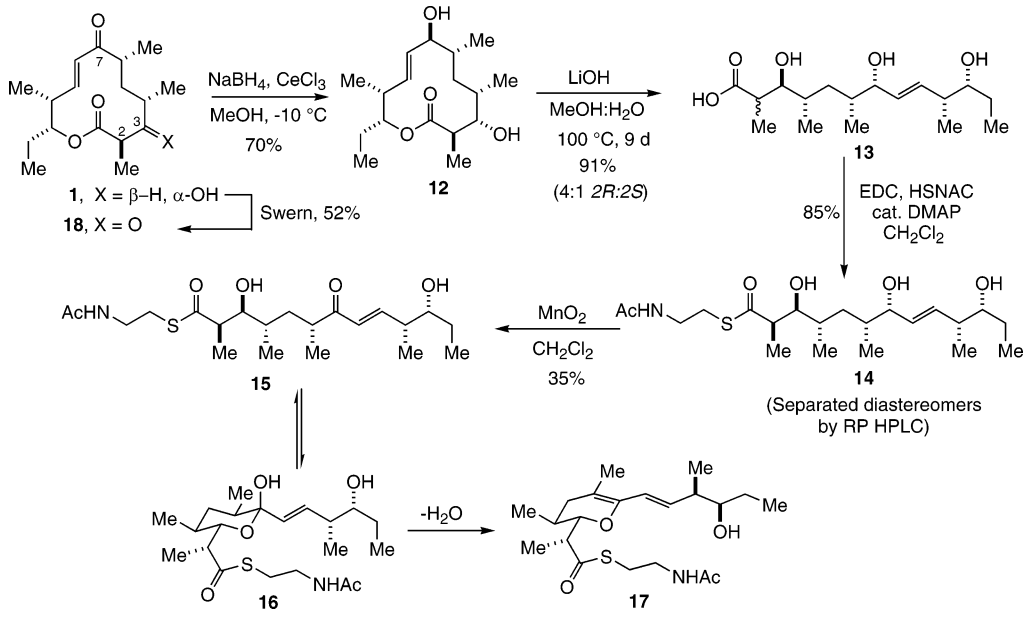
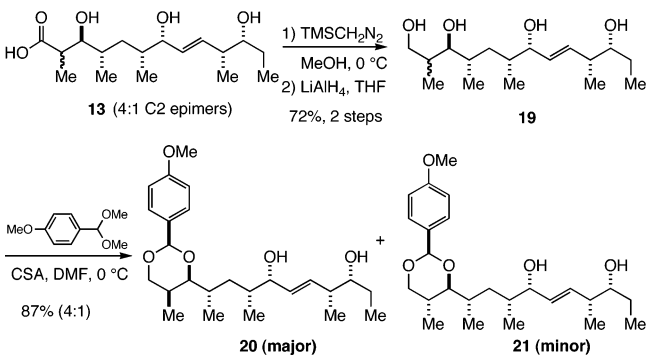
Scheme 3



ing β-ketophosphonate functionality. Next, Horner−Wadsworth−Emmons olefination of **6** with aldehyde **7** was found to optimally occur utilizing barium hydroxide as base to provide enone **10** (E:Z > 20:1).[24] The acid was converted to the NAC thioester **11** employing EDC and catalytic DMAP without any observed competitive Michael addition of the thiol to the enone.[25] Deprotection of the TBS ether with hydrofluoric acid in acetonitrile provided the pentaketide NAC thioester **5** in only five steps from **8**. The convergent and efficient chemoenzymatic synthesis of the pentaketide **5** also enables direct access to additional analogue structures.

10-Deoxymethynolide (**1**) represents an excellent starting material for degradation work, since direct hydrolysis yields the natural hexaketide substrate. To obtain sufficient amounts of 10-deoxymethynolide required for this approach, an engineered mutant strain of *S. venezuelae* that produces 10-deoxymethynolide (25 mg/L) as the exclusive product was used.[26] The titer was further improved by incubating with XAD-16 resin (500 g, 55 L incubation), which served to absorb the 10-deoxymethynolide and led to production levels (∼4-fold improvement) of approximately 100 mg/L. Unfortunately, direct hydrolysis of the 10-deoxymethynolide (LiOH, 50 °C, 5 h, 100% consumption of 10-deoxymethynolide) was plagued by a number of side reactions, which precluded isolation of the desired *seco*-acid (not shown). Protection of the ketone prevented the undesired epimerization of the C-6 stereocenter and hemiketalization with the C-3 alcohol. This was accomplished by Luche reduction of 10-deoxymethynolide with $NaBH_4$ and $CeCl_3$ to furnish allylic alcohol **12** (Scheme 3).[27] Hydrolysis of **12** required significant forcing conditions[28] (as compared to 10-deoxymethynolide) to afford **13** as a 4:1 mixture of C-2 epimers.

Scheme 4



To confirm the stereochemistry of the C-2 stereocenter, the mixture of epimeric hexaketide acids **13** was reduced to tetraols **19**, which were selectively protected as *p*-methoxybenzylidene acetals **20** and **21** (Scheme 4). The stereochemistry of the major isomer was correlated with a related benzylidene acetal fragment.[29] Conversion of the C-7 epimeric mixture of **13** to the NAC thioesters **14** was achieved using standard conditions, and the diastereomers were separated by preparative reverse-phase HPLC (Scheme 3). Chemoselective oxidation of the allylic alcohol of **14** was accomplished with MnO_2 to afford **15**.[30] The native hexaketide **15** was in equilibrium with a mixture of both α and β anomeric hemiacetals **16** (only the β-anomer is shown) and was prepared directly before use due to its propensity to undergo dehydration, leading to dihydropyran derivative **17**. Additionally, an authentic standard of 3-oxo-10-deoxymethynolide **18** was required, since this is the putative product resulting

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(27) The resultant C-7 alcohol was assigned the 7*S* stereochemistry on the basis of the most accessible face of the macrocycle. 10-Deoxymethynolide was modeled using a Monte Carlo search in Macromodel, and the minimum energy conformation was further optimized at the AM1 level with Gaussian 98.
(28) Minimization of both 10-deoxymethynolide (**1**) and **12** at the AM1 level does not reveal any structural change that could account for this great difference in reactivity. The greater reactivity of 10-deoxymethynolide versus **12** may be due to the enone function, which may undergo reversible Michael addition of methoxide, providing a more conformationally flexible structure due to the reduced torsional strain as a result of the sp^2 to sp^3 change in hybridization of the C-8 and C-9 carbons. However, the putative Michael adduct is not observable within the detection limit (<1%) of ^1H NMR when taken in CD_3ONa/CD_3OD.
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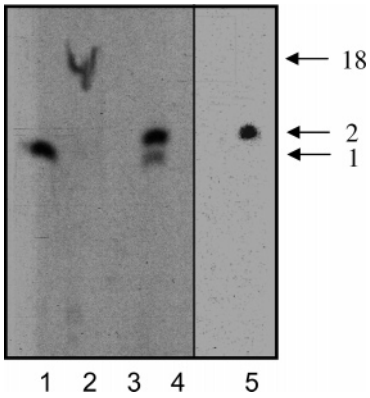

Figure 2. Radio-TLC of in vitro reaction products produced using the pentaketide and hexaketide NAC thioester substrates with recombinant pikromycin modules. TLC autoradiography of ^{14}C-lableled 10-deoxymethynolide (**1**), narbonolide (**2**), and 3-oxo-10-deoxymethynolide (**18**). 2-[^{14}C]Methylmalonyl-CoA was used as the radiolabeled substrate; concentrations of all substrates and Pik monomodule concentrations are described in the Experimental Section. Developed with 5% MeOH/$CHCl_3$. Lanes 1−4 were acquired together, while the data for lane 5 was obtained separately. Lane 1, PikAIII+TE incubated with pentaketide **5** to afford 10-deoxymethynolide (**1**); lane 2, PikAIII-TE incubated with **5** in the absence of NADPH to afford 3-oxo-10-deoxymethynolide (**18**); lane 3, PikAIII incubated with **5**, no products were observed; lane 4, PikAIII and PikAIV incubated with **5** to afford both narbonolide (**2**) as the upper spot and 10-deoxymethynolide (**1**) as the lower spot; lane 5, PikAIV incubated with hexaketide **15** to afford narbonolide (**2**).

from incubation of the native pentaketide with PikAIV or of the pentaketide with PikAIII-TE in the absence of NADPH. This authentic standard was prepared by Swern oxidation of 10-deoxymethynolide (**1**) (Scheme 3).

Assay and Measurement of Kinetic Parameters. PikAIII, PikAIV, and engineered PikAIII-TE holoenzymes were purified as previously described[15,17] and individually reacted with 2-[^{14}C]-methylmalonyl-CoA and each of the pentaketide **5** and hexaketide **15** NAC thioesters. The resulting products were identified using synthetic standards and quantified by radio-TLC. The initial rates, v, at a given [S] were determined by single time point stopped-time incubations at 20 minutes. All reactions were run in triplicate employing a fresh enzyme preparation and identified by comigration with authentic standards and HPLC or preparative-TLC in conjunction with ESI(+) analysis to rigorously confirm these assignments. Apparent steady-state kinetic values were determined for enzyme-substrate pairings that yielded a detectable product by fitting the normalized v vs [S] plots to the Michaelis−Menten equation.

We first investigated the engineered PikAIII-TE fusion protein to accept and process pentaketide **5** to produce 10-deoxymethynolide (**1**). In vitro studies of pentaketide **5** with PikAIII-TE provided 10-deoxymethynolide as the sole product (Figure 2, lane 1). Initial velocities as a function of substrate concentration were determined under saturating concentrations of methylmalonyl-CoA (800 μM) and NADPH (1 mM) and are plotted in Figure 3A. The limited solubility of pentaketide substrate [**5**] < 1 mM in aqueous buffer did not allow saturation to be achieved; however, fitting of the data by linear regression analysis to the reduced Michaelis−Menten equation, where [S] < K_M, allowed determination of the specificity constant. The specificity constant k_{cat}/K_M for this reaction is 0.55 ± 0.029 mM^{-1} min^{-1}, which is approximately 150-fold greater than the value obtained when utilizing the simple (2*S*,3*R*)-diketide NAC thioester substrate analogue.[15,17] The limited solubility of

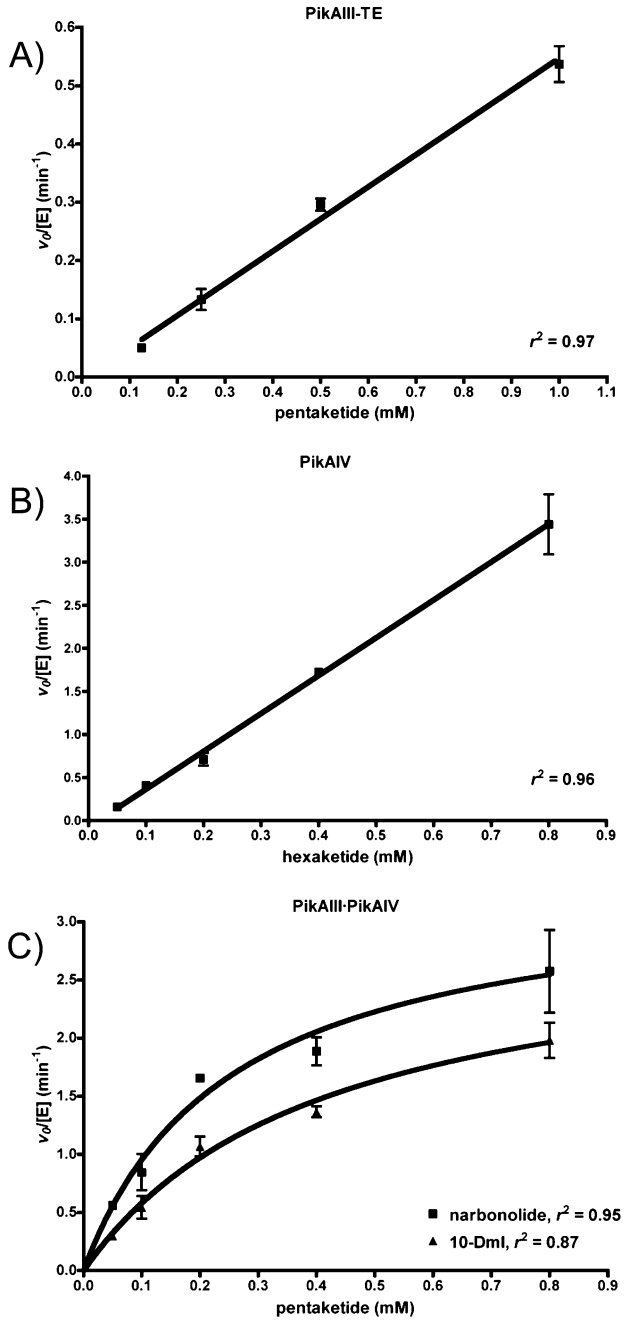

Figure 3. Normalized plots used to determine the steady-state kinetic parameters of Pik monomodules with native penta- and hexaketide substrates. (A) PikAIII-TE + pentaketide **5** as substrate to afford 10-deoxymethynolide (**1**), k_{cat}/K_M = 0.55 ± 0.029 mM^{-1} min^{-1}; (B) PikAIV + hexaketide **15** as substrate to afford narbonolide (**2**), k_{cat}/K_M = 4.4 ± 0.24 mM^{-1} min^{-1}; (C) PikAIII·PikAIV + pentaketide **5** to afford 10-deoxymethynolide (**1**), $k_{cat(10-dml)}$ = 3.0 ± 0.33 min^{-1} and $K_{M(10-dml)}$ = 0.25 ± 0.073 mM^{-1} min^{-1}, and narbonolide (**2**), $k_{cat(nbl)}$ = 3.3 ± 0.40 min^{-1} and $K_{M(nbl)}$ = 0.41 ± 0.094 mM^{-1} min^{-1}.

pentaketide substrate **5** prevented determination of individual k_{cat} and K_M parameters, but the K_M is clearly greater than 1 mM. Likely, the increase in the specificity constant is due exclusively to an enhancement of the catalytic efficiency (k_{cat}), since the K_M (>1 mM) appears to be comparable to that of the corresponding (2*S*,3*R*)-diketide substrate (K_M = 7.3 mM).[15,17] When NADPH was excluded from the reaction mixture, PikAIII-TE readily converted the pentaketide substrate **5** to 3-oxo-10-deoxymethynolide (**18**), indicating some flexibility in

Table 1. Steady-State Kinetic Parameters for the Extension and Lactonization of Penta- and Hexaketide Substrates by PikAIII, PikAIII-TE, and PikAIV

	pentaketide (**5**)			hexaketide (**15**)		
module(s)	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} min^{-1})	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} min^{-1})
PikAIII	–[a]	–	–	nd	nd	nd
PikAIII-TE	nd[b]	>1[c]	0.55 ± 0.029[c]	–	–	–
PikAIV	–	–	–	nd	>1[d]	4.4 ± 0.24[d]
PikAIII + PikAIV	3.0 ± 0.33[c]	0.25 ± 0.07[c]	12 ± 0.31[c]	nd	nd	nd
	3.3 ± 0.40[d]	0.41 ± 0.09[d]	8.1 ± 0.26[d]			

[a] No activity detected. [b] Not determined. [c] This value represents the steady-state kinetic parameter for 10-deoxymethynolide (**1**) formation. [d] This value represents the steady-state kinetic parameter for narbonolide (**2**) formation.

the TE domain (Figure 2, lane 2). No 3-oxo-10-deoxymethynolide (**18**) was observed in reactions with NADPH, indicating rapid reduction of the β-keto group before TE catalyzed lactonization. This is in contrast to earlier experiments with PikAIII and PikAIV using diketide substrates, where an apparent competition between ketoreduction by the KR_5 domain of PikAIII and lactonization by PikAIV resulted in a mixture of triketide lactones with different oxidation states.[17] Additionally, we incubated hexaketide **15** with PikAIII-TE in both the presence and absence of NADPH, but no product formation was observed (see Supporting Information for radio-TLC). As expected, incubation of the pentaketide **5** with native PikAIII protein (e.g. lacking a TE domain) did not produce detectable levels of 10-deoxymethynolide (**1**) (Figure 2, lane 3). In contrast, incubation of simple diketides with the native PikAIII afforded triketide lactone products due to spontaneous release. However, in this case a hydroxyl group was appropriately located to facilitate a slow intramolecular lactonization.[17] The hexaketide-bound S-ACP_5 intermediate does not contain an analogous hydroxyl group and thus is not released, and presumably remains linked to the ACP_5 domain.

Next, we examined the ability of PikAIV to process the native hexaketide substrate. Incubation of PikAIV with hexaketide **15** and 2-[^{14}C]methylmalonyl-CoA resulted in chain extension to the final heptaketide, release, and cyclization to afford narbonolide (**2**) as detected by radio-TLC (Figure 2, Lane 5). Since 10-deoxymethynolide (**1**) does not incorporate radiolabeled methylmalonyl-CoA, we were unable to observe it through radio-TLC analysis and thus only report the kinetic activity for the formation of narbonolide (**2**). In addition to a low solubility value (<1 mM), the hexaketide substrate **15** is in equilibrium with a mixture of anomeric hemiacetals **16** (only the β-anomer shown). The apparent specificity constant for hexaketide **15** with PikAIV as determined by analogy to the pentaketide **5** with PikAIII-TE was found to be 4.4 ± 0.24 mM^{-1} min^{-1}, which is approximately 3000-fold greater than the values obtained utilizing simple diketide substrates (Figure 3B).[15,17] Additionally, the ability of PikAIV to accept the pentaketide substrate **5** to afford 3-oxo-10-deoxymethynolide (**18**) was examined, but no product formation was observed, in accordance with the apparent strict substrate specificity of the Pik monomodules.

It is evident that the native pikromycin pathway pentaketide and hexaketide chain elongation intermediates are processed 2−3 orders of magnitude more efficiently by both PikAIII-TE and PikAIV relative to diketide substrates. We therefore proceeded to examine the pentaketide **5** and its ability to undergo sequential chain extension, processing, and release using in vitro pairing of native PikAIII and PikAIV monomodules. Both 10-deoxymethynolide (**1**) and narbonolide (**2**) were observed by radio-TLC (Figure 2, lane 4). Thus, loading of the pentaketide onto KS_5 of PikAIII, extension, and keto group reduction afforded an ACP bound hexaketide thioester. Intermodular transfer to a full-length PikAIV and subsequent elongation, release, and cyclization provided narbonolide (**2**), while TE-mediated release in the absence of a second methylmalonate extension afforded 10-deoxymethynolide (**1**).

A steady-state kinetic analysis of the sequential chain elongation process employing the pentaketide **5** with both PikAIII and PikAIV was performed. Thus, variation of the pentaketide concentration utilizing 1 μM of PikAIII in the presence of NADPH (1 mM), methylmalonyl-CoA (800 μM), and PikAIV (1 μM), each experimentally determined to be at saturating concentrations, provided the reaction rates shown in Figure 3C that were fit by nonlinear regression analysis to the Michaelis−Menten equation. The calculated k_{cat} for 10-deoxymethynolide (**1**) and narbonolide (**2**) synthesis were determined to be 3.0 ± 0.33 and 3.3 ± 0.40 min^{-1} respectively. Since PikAIV produces both 10-deoxymethynolide (**1**) and narbonolide (**2**), the composite k_{cat} = 6.3 min^{-1} ± 0.52 min^{-1} provides a measure of the total flux through the PikAIII·PikAIV complex. This value is approximately 100-fold greater than the highest value determined with diketide substrates and monomodular PikAIII-TE or PikAIV proteins.[15,17] Similarly, the K_M values for pentaketide **5** were found to be 0.25 ± 0.073 and 0.41 ± 0.094 mM for 10-deoxymethynolide (**1**) and narbonolide (**2**) formation, respectively. The individual K_M values are approximately 10-fold lower than the best diketide substrate with Pik monomodules.[15,17] The specificity constant for the PikAIII·PikAIV modular pairing with the natural pentaketide substrate **5** using the k_{cat} and K_M values for 10-deoxymethynolide (**1**) production is 12 ± 0.31 mM^{-1} min^{-1}, which exceeds by more than 3000-fold the best reported value (k_{cat}/K_M = 0.0038 mM^{-1} min^{-1}) obtained using a diketide analogue.[15,17] A comparison of these kinetic values for individual modules reacting with pentaketide **5** or hexaketide **15** is shown in Table 1. Additionally, hexaketide **15** was incubated with a mixture of PikAIII and PikAIV to afford narbonolide as the exclusive product (see Supporting Information for radio-TLC). The efficient processing of the natural pentaketide **5** by Pik modules 5 and 6 in vitro indicates that this chain elongation intermediate represents an excellent reference point for comparative studies using modified chain elongation substrates.

Discussion

Chiral Recognition of Diketide and Native Substrates. A primary challenge of studying modular PKS systems has been the availability of natural chain elongation intermediates for investigation of substrate specificity in late-stage extension, processing, and macrocyclization. To begin addressing the specificity and catalytic efficiency of late-stage biosynthetic modular PKS enzymes, short chain substrate mimics such as diketide NAC thioesters were used to probe the specificity of non-native monomodules of the DEBS PKS system.[14,31−34] Results from these model studies showed that all modules efficiently processed the *syn*-diketide substrates with k_{cat} values on the order of 1 min^{-1}, whereas *anti*-diketide substrates were not accepted. Since the 6-deoxyerythronolide (DEBS) chain elongation intermediates do not possess relative anti-stereochemistry, these results supported the notion that such substrates adequately mimicked the native advanced polyketide chain intermediates. We have previously shown that PikAIII and PikAIV process such diketide substrates almost 1000-fold less efficiently than the corresponding DEBS modules, providing the first comparative analysis of a PKS system against the DEBS PKS.[17] Our current observations show clearly that the native pentaketide **5** and hexaketide **15** NAC thioester substrates are processed at 2−3 orders of magnitude more efficiently than simple diketide analogues, suggesting that more remote structural features of the polyketide chain play an important role in substrate recognition.

The pentaketide and hexaketide chain intermediates used in this study serve as powerful molecular probes to investigate the substrate recognition features employed by late stage PKS modules; however, several potential limitations are evident. First is their relatively poor aqueous solubility, which may be problematic for even longer and more lipophilic substrates.[47] A second important consideration regarding more highly functionalized substrates is the propensity for polyketide chains to undergo additional reorganizations through hemiacetalizations, spiroacetalizations, and conjugate additions. These and other potential intramolecular modifications leading to heterocyclic ring structures may not be efficiently processed or may be unstable. Although many polyketides undergo spontaneous rearrangements, the timing of these events during polyketide biosynthesis is not known. Recent crystallographic analysis of a KS domain has suggested that PKS modules are passed down a narrow protein channel.[35] Such a channel may prevent intramolecular reactions of the processed polyketide chain by maintaining the elongation intermediate in an extended conformation. In solution, the native hexaketide **15** investigated in this study was found to exist in equilibrium with a mixture of anomeric hemiacetals **16**. Not only was this compound extremely labile, decomposing through dehydration to afford a dihydropyran derivative **17**, but PikAIV was found to possess a high K_M value (>1 mM) toward it, even though it is the native substrate for this enzyme. By contrast, the K_M for the pentaketide **5** with PikAIII·PikAIV pairing is 0.33 mM. The increased K_M value may instead reflect the lower concentration of the open chain tautomer, suggesting that the hemiacetal tautomer is not recognized. Thus, the apparent K_M value for the open-chain tautomer assuming this scenario is related as follows: $K_M = K_{eq}K_{M(obs)}$, where K_{eq} represents the equilibrium constant between the open and closed chain tautomers of the hexaketide. While the observed K_M value of the pentaketide **5** with PikAIII·PikAIV pairing is certainly lower than hexaketide **15** with PikAIV, this value still appears quite high for a native substrate. However, it should be recognized that the present substrate relies on simple diffusion to the KS domain, whereas under normal in vivo conditions this substrate is channeled from the ACP_5 domain of PikAIII. This channeling is kinetically much more efficient, and a steady-state kinetic analysis using ACP-bound diketide analogues and DEBS-TE monomodules quantified the kinetic enhancement as being as much as 100 times faster than simple diffusion.[33] Thus, the substrate affinity for the KS domain is programmed in part through protein−protein interactions between cognate KS and ACP domains.

Our investigation of the non-native fusion protein PikAIII-TE wherein the C-terminal TE domain from PikAIV was engineered onto the C-terminus of PikAIII employing the pentaketide **5** led to the formation of 10-deoxymethynolide (**1**). The specificity constant is 0.55 mM^{-1} min^{-1}, while the K_M is greater than 1 mM. For comparison, the calculated specificity for the pentaketide with the PikAIII·PikAIV pairing is 12 mM^{-1} min^{-1}. Thus, the pentaketide is processed approximately 22-fold less efficiently by the non-native fusion PikAIII-TE protein versus the native proteins. This attenuation cannot be partitioned into individual kinetic parameters, since only the specificity constant of PikAIII-TE was measured. Likely, the decrease is due to improper channeling of the elongated ACP_5-hexaketide substrate to the TE domain, which in turn may be caused by a nonoptimal linker region between the ACP_5 and TE domains of the non-native fusion protein. We were intrigued about the possibility of producing narbonolide by PikAIII-TE employing the hexaketide as an unnatural substrate. Successful processing of the hexaketide by PikAIII-TE in the absence of NADPH could potentially generate narbonolide and provide another mechanism for the formation of this polyketide. However, hexaketide **15** was not elongated by PikAIII-TE either in the presence or absence of NADPH, suggesting that PikAIII-TE is highly evolved to process its native pentaketide substrate. Further studies are required to establish whether failure of

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reaction occurs at the substrate loading, chain extension, or cyclization/release stage.

PikAIII and PikAIV Interaction Leading to 10-Deoxymethynolide. The formation of 10-deoxymethynolide (**1**) by PikAIII and PikAIV is of great interest biochemically as the elongated hexaketide chain bound to the ACP_5 domain of PikAIII must be channeled to the C-terminal TE domain of PikAIV.[20,36] A full mechanistic understanding of this process may allow the effective redesign of the Pik PKS assembly line to afford exclusively the more valuable 14-membered ring ketolide template narbonolide (**2**). On the other hand, it would also provide a means to redesign modular PKSs for creation of additional structural diversity in other complex macrolide pathways. Although we have not systematically investigated each potential model for generation of 10-deoxymethynolide and narbonolide by the Pik PKS, we can already discount some potential mechanisms from the data obtained in the current analysis. First, direct spontaneous cyclization of the Pik ACP_5-bound elongated hexaketide does not occur, since PikAIII lacking a TE domain did not produce 10-deoxymethynolide. In a second model, an N-terminal truncated form of PikAIV lacking its linker domain was proposed to exclusively catalyze the synthesis of 10-deoxymethynolide.[36] The observation that full-length PikAIV could provide 10-deoxymethynolide when paired with PikAIII is not fully consistent with the initial truncation model, although it does not rigorously disprove it either. A third model, termed the "skipping" model, proposes that the hexaketide is channeled from the ACP_5 domain of PikAIII to the KS_6 domain of PikAIV, but rather than undergoing a final elongation, it can be transferred to the ACP_6 domain, if this is unoccupied by the usual methylmalonyl extender unit.[20] Alternatively, direct transfer from ACP_5 of PikAIII to ACP_6 of PikAIV is possible, effectively bypassing the KS_6 domain.[37] Subsequent channeling to the TE domain provides a mechanism whereby the hexaketide effectively "skips" through PikAIV to the TE domain. An in vivo study employing site-directed mutants provided support for this model, since site-directed inactivation of the KS_6, AT_6, or ACP_6 domains of PikAIV completely abolished 10-deoxymethynolide production, suggesting that these active sites have a role in the process.[20] Currently, we are exploring the overexpression of site-directed mutant forms of PikAIV to verify the skipping model using the current in vitro system with pentaketide **5**. A fourth model suggests that the TE domain can directly accept the ACP-bound hexaketide. While PikAIII and PikAIV interact through the respective C- and N-terminal docking domains, the linker regions between individual domains may impart flexibility to the overall protein, allowing repositioning of the TE domain to contact the ACP_5 domain. Alternatively, the monomodular arrangement would allow for direct interaction between ACP_5 and the TE domain independent of this interaction. The conformational flexibility and dynamics of intact PKS modules are poorly understood at the present, and further investigation will likely provide new insights into novel mechanisms of polyketide assembly.

Biocatalysis with PKS Modules. The use of recombinant PKS modules as synthetically useful biocatalysts is an unexplored area for the chemoenzymatic synthesis of natural product templates. In the present system, a pentaketide was converted through the action of PikAIII and PikAIV into the significantly more complex heptaketide narbonolide (**2**) bearing three additional chiral centers. By contrast, the chemical synthesis of narbonolide from the pentaketide intermediate would require approximately a dozen additional chemical operations. In order for PKS modules to be truly useful as biocatalysts, however, they must display a large total turnover number (TTON), which is the total moles of substrate produced per mole of enzyme over the lifetime of the biocatalyst.[38] Additionally, the biocatalyst should be readily available and have good stability. Currently, the optimal overexpression conditions afford approximately 10 mg/L of PikAIII and PikAIV, which translates to about 100 nmol/L of culture. To be considered synthetically relevant, a TTON of at least 10^4 is required, since 100 nmol of enzyme would produce at least 1 mmol of product, a synthetically tractable amount given the costs required to obtain the required biocatalysts. Previously, we reported the use of simple diketide NAC thioester substrates with PikAIII and PikAIV and observed a maximal TTON of 10; however, this low value was due to the extraordinarily low k_{cat} values, $\sim$0.01 min^{-1}. The use of the native pentaketide substrate **5** with PikAIII and PikAIV by contrast displays a TTON of approximately 300 (data not shown), primarily as a result of the increased turnover number of the native substrate. While this represents a significant improvement over our earlier results, the TTON still requires considerable optimization. Several factors may contribute to the observed TTON, including product inhibition of the enzymes, enzyme deactivation by aberrant decarboxylation of methylmalonyl S-ACP, and hydrolysis of substrate by the TE domain. Enzyme deactivation through aberrant decarboxylation of ACP-bound methylmalonyl leading to the formation of propionyl-ACP is well-documented.[39] The resulting stalled PKS assembly line is unable to undergo subsequent Claisen condensation; however, the rate of this spontaneous decarboxylation is not known. A second mechanism that may cause poor TTON involves hydrolysis of the substrate by the TE domain. In fact, we observed significant hydrolysis of the substrate NAC thioesters **5** during extended reaction times by the isolated Pik TE domain (data not shown). Diffusive loading can occur to either the KS or TE domain. Under normal physiological conditions, this competitive mechanism is not operative, since the TE receives its substrate by channeling from the antecedent ACP domain; however, in vitro the substrates can diffusively load onto either domain. Utilization of PKS modules to mediate the in vitro chemoenzymatic synthesis of substrates represents a powerful means to prepare ketolide analogues; however, the current limitations identified here will need to be overcome to apply PKS modules as synthetically useful biocatalysts.

Although, polyketide derivatives (e.g. the avermectins[40] and epothilones[41,42]) with improved bioactivity have been developed through genetic modifications of host strains and tailoring enzymes, molecular genetic engineering of altered polyketide biosynthetic pathways remains slow and tedious. Successful manipulation of PKS modules to produce macrolactone derivatives requires an understanding of the protein−protein interactions between modules that allows channeling of substrates as well as any substrate recognition features inherent to the extending module. Alteration of the polyketide chain through molecular genetic manipulation of the PKS (i.e. domain exchanges or module fusions) can produce chains with varied reduction or alkyl group functionalization, as well as the

introduction of amino acids into the elongating chain. Efficient processing of these derivatives requires the accommodation by downstream modules. Prescreening of late-stage PKS modules with synthetic substrates would likely identify limitations that can be addressed by additional genetic modifications so that in vivo generated intermediates are channeled and efficiently processed.

Significance. The highly favorable reactions between pentaketide **5** and hexaketide **15** NAC thioester substrates and the final two modules [PikAIII (module 5) and PikAIV (module 6)] of the pikromycin PKS show that these enzymes have evolved to optimally process their native substrates. Our results also suggest that not only do PKS modules recognize vicinally located structural features (α and β positions), but that more remote structural features are decisively involved in proper substrate recognition and efficient processing by the polyketide synthases. A systematic investigation employing both structural and stereochemical substrate analogues is necessary to understand the recognition features and catalytic properties of these modules and direct the development of bioactive structures. Screening of downstream modules with substrate analogues may identify specificity limitations that must be overcome through protein reengineering before scaled synthesis. Eventually, these downstream modules may be used biocatalytically or expressed in a suitable host for biotransformation of synthetic substrates to generate macrolactones that cannot be efficiently produced by synthetic or biochemical methods alone.

Experimental Section

Protein Overexpression and Purification. Cloning, overexpression, and purification of PikAIII and PikAIV have been previously described.[17] The PikAIII−TE protein was constructed as described by Cane and co-workers[15] but expressed and purified using the same conditions reported for PikAIII and PikAIV. For this work all proteins were coexpressed in *E. coli* cotransformed with a compatible plasmid that expresses *Bacillus subtilus sfp* under the control of the T7 promoter to enhance the phosphopantetheine modification of these proteins. Protein purity was confirmed to be greater than 95% by SDS−PAGE. Proteins were stored at −80 °C in storage buffer (100 mM sodium phosphate, pH 7.2, 1 mM EDTA, 1 mM TCEP, 20% glycerol) for months without significant loss of activity.

In Vitro Polyketide Production. The reactivity of PikAIII, PikAIII-TE, and PikAIV with SNAC pentaketide **5** and hexaketide **15** was determined by measuring the incorporation of 2-[^{14}C]methylmalonyl-CoA into the resulting narbonolide and 10-deoxymethynolide products. 2-[^{14}C]Methylmalonyl-CoA (54.3 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc., and all other chemicals were purchased from Sigma. Enzymatic reactions were run in 400 mM sodium phosphate buffer (pH 7.2) containing 5 mM NaCl, 1 mM EDTA, 1 mM TCEP, 1 mM NADPH (for reactions with PikAIII and PikAIII-TE), 20% glycerol, and 5% DMSO in a final volume of 50 μL. We have previously demonstrated that the enzyme activity rate remained linear for up to 1 h using these conditions.[17] In reactions designed to determine the individual reactivity of each protein with pentaketide **5** and hexaketide **15**, protein was added to a concentration of 1 μM, and pentaketide and hexaketide concentrations ranged from 50 μM to 1 mM. In reactions designed to determine the reactivity of PikAIII and PikAIV together with pentaketide **5**, 1 μM of both PikAIII and PikAIV were used. In all reactions, enzyme was equilibrated in buffer for 5 min at 30 °C and the reaction was initiated with the addition of 2-[^{14}C]methylmalonyl-CoA (diluted for a specific activity = 0.515 mCi/mmol) for a final saturating concentration of 800 μM. The reaction was maintained at 30 °C for 20 min then quenched by vortexing with EtOAc (250 μL), and the products were extracted (2 × 250 μL, EtOAc). The extracts were dried using a Speed-vac and the resulting oil was dissolved in CH_2Cl_2 (30 μL) and spotted onto a silica gel TLC plate (Merck, 20 × 20 cm with 2.5 cm concentration zone) and developed using 5% MeOH/$CHCl_3$. The silica gel TLC plates were placed on a phosphorimager screen and analyzed using a Typhoon (Molecular Dynamics) phosphoimager. Product formation was quantified using ImageQuant (Molecular Dynamics) using a standard curve generated with 2-[^{14}C]methylmalonyl-CoA. Apparent steady-state kinetic values (k_{cat} and K_M) were determined for enzyme−substrate pairings that yielded a detectable product by fitting the normalized v vs [S] plots to the Michaelis−Menten equation using GraphPad Prism software.

Confirmation of Reaction Products. Reaction products were identified by comigration with authentic standards and ESI(+)-MS analysis was employed to rigorously confirm these assignments. A large-scale enzymatic reaction (2 mL) with pentaketide **5** and both PikAIII and PikAIV using the assay conditions described above (except using cold MMCoA) was performed to provide sufficient 10-deoxymethynolide (**1**) and narbonolide (**2**) for detection. The reaction was quenched at 2 h and extracted with EtOAc (3 × 2 mL). The extracts were dried using a Speed-vac, and the resulting oil was dissolved in CH_2Cl_2 (100 μL) and spotted onto a preparative silica gel TLC plate and developed using 5% MeOH/$CHCl_3$. The overlapping band corresponding to 10-deoxymethynolide (**1**) and narbonolide (**2**) was excised and the semipurified reaction products recovered by extraction with 10% MeOH/$CHCl_3$ (15 mL). The extracts were concentrated and dissolved in 70:25:5 H_2O:CH_3CN:DMSO (200 μL) and then purified by analytical reverse-phase HPLC (Econosil C-18, 250 mm × 4.6 mm) with a gradient from 30% to 70% CH_3CN:H_2O containing 0.1% TFA over 50 min at a flow rate of 1 mL/min detecting at 233 nm. 10-Deoxymethynolide (**1**, t_R = 32 min) and narbonolide (**2**, t_R = 25 min) were manually collected and the fractions concentrated and then redissolved in MeOH (1 mL) containing 200 μM NaCl and analyzed by ESI(+).[43] 10-Deoxymethynolide (**1**): (ESI+) *m*/*z* 319.1 ($C_{17}H_{28}O_4$ + Na^+ requires 319.2). Narbonolide (**2**): (ESI+) *m*/*z* 375.1 ($C_{20}H_{32}O_5$ + Na^+ requires 375.2). (See Supporting Information for HPLC traces.)

Synthesis of Substrates and Standards. General Procedures. THF and ether were distilled from Na/benzophenone, and CH_2Cl_2 was distilled from CaH_2. Flash chromatography was performed with Fisher grade silica gel 60 (230−400 mesh) with the indicated solvent system.[44] All reactions were performed under an inert atmosphere of dry argon in oven-dried (150 °C) glassware. Optical rotations were determined on an Rudolph Research Autopol III polarimeter using the sodium D line (λ = 589 nm) at 23 °C and are reported as follows: $[\alpha]_D$, concentration (c = g/100 mL), and solvent. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Mercury 300 spectrometer or Bruker 500 MHz spectrometer. Proton and carbon assignments when given are based on COSY, HMQC, and TOCSY analysis. Proton chemical shifts are reported in ppm from an internal standard of residual chloroform (7.26 ppm) or methanol (3.31 ppm), and carbon chemical shifts are reported using an internal standard of residual chloroform (77.0 ppm) or methanol (49.1 ppm). Proton chemical data are reported as follows: chemical shift, multiplicity (ovlp = overlapping, s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, br = broad), coupling constant, and integration. High-resolution mass spectra were obtained at the University of Michigan Mass Spectrometry Laboratory on a Waters Ultima magnetic sector mass spectrometer equipped with an electrospray interface.

10-Deoxymethynolide (1). A concentrated spore suspension (100 μL) of SC1016[26] was used to inoculate 1 L of SCM medium consisting of 15 g of soluble starch, 20 g of soytone (Difco), 0.1 g of $CaCl_2$, 1.5 g of yeast extract, 0.3 g of Mazu 204, and 10.5 g of MOPS (pH 7.2, adjusted with 6 N KOH) in a 2 L baffled flask. This primary inoculum was grown at 30 °C on a rotary shaker (200 rpm) for 24 h. The entire volume was used to inoculate a 55 L of SCM media in a 75 L bioreactor containing XAD-16 resin (10 g/L, 500 g, prewashed with MeOH prior

to addition to the reactor). During fermentor cultivation, the temperature was maintained at 30 °C and the pH at 7.2 via addition of 1.0 N NaOH or 1 N H_2SO_4. The aeration rate was set at 25 L/min and the agitation was controlled at 400 rpm such that the dissolved oxygen tension was maintained above 40%. The culture was grown for 66 h, and the XAD-16 resin was collected by filtering through a 60-mesh screen. The resin was washed with H_2O (500 mL) and then 10-deoxymethynolide was eluted with MeOH (500 mL). The MeOH rinse was concentrated onto Celite (20 g) to afford a brown cake. Flash chromatography (SiO_2, 200 g, 1% MeOH/CH_2Cl_2) afforded 10-deoxymethynolide (5.17 g, 94 mg/L) as a light yellow oil that slowly solidified at −20 °C, the ^{1}H NMR and ^{13}C NMR of which matched literature data.[45]

(*E*)-(2*S*,4*R*,8*R*,9*R*)-*S*-2-Acetamidoethyl 9-Hydroxy-2,4,8-trimethyl-5-oxoundec-6-enethioate (5). To a solution of TBS-pentaketide **11** (44.5 mg, 0.0944 mmol) in CH_3CN (5 mL) at 23 °C in a 15 mL plastic vial was added 48% aqueous HF (0.25 mL). After 16 h, the reaction was quenched with saturated aqueous $NaHCO_3$ (5 mL) and then extracted with CH_2Cl_2 (3 × 30 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Flash chromatography (SiO_2, 2 g, 3% MeOH/CH_2Cl_2) afforded (28 mg, 84%) the title compound as a colorless oil: TLC R_f = 0.16 (3% MeOH/$CHCl_3$); $[\alpha]_D$ +36.5 (c = 0.203, MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.90 (dd, J = 15.9, 7.8 Hz, 1H), 6.15 (dd, J = 15.9, 1.2 Hz, 1H), 6.00 (br s, 1H), 3.51−3.57 (m, 1H), 3.35−3.49 (m, 2H), 2.99−3.04 (m, 2H), 2.82 (hext, J = 6.9 Hz, 1H), 2.70−2.79 (m, 1H), 2.42−2.52 (m, 1H), 2.10−2.20 (m, 1H), 1.98 (s, 3H), 1.35−1.58 (m, 3H), 1.18 (d, J = 6.9 Hz, 3H), 1.11 (app t, J = 6.9 Hz, 6H), 0.98 (t, J = 7.5 Hz, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 203.8, 202.6, 170.2, 150.0, 128.1, 75.8, 46.7, 42.3, 41.5, 39.5, 37.2, 28.5, 27.3, 23.3, 18.7, 17.0, 13.6, 10.5; HRMS (ESI+) m/z 380.1871 ($C_{18}H_{31}NO_4S$ + Na^+ requires 380.1872).

(2*S*,4*R*)-6-(Dimethoxyphosphoryl)-2,4-dimethyl-5-oxohexanoic Acid (6). To a solution of dimethyl methylphosphonate (236 μL, 2.20 mmol, 3.3 equiv) in THF (3 mL) at −78 °C was added a solution of *n*-BuLi (2.5 M in hexanes, 1.38 mL, 2.20 mmol, 3.3 equiv) to afford a white heterogeneous mixture, which was stirred for an additional 15 min. A solution of ester **9** (119 mg, 0.67 mmol, 1.0 equiv, azeotropically dried with PhH, 2 × 5 mL) in THF (3 mL) was added slowly down the side of the flask to the lithium phosphonate solution at −78 °C to afford a clear solution. After stirring for 25 min, the cooling bath was removed and the solution was allowed to warm to 0 °C. The reaction was partitioned between EtOAc (25 mL) and 1 N aqueous HCl (10 mL) and saturated aqueous NaCl (10 mL). The organic layer was separated and the aqueous layer was extracted with EtOAc (2 × 25 mL). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated under reduced pressure to a colorless oil. Flash chromatography (SiO_2, 20 g, 5% MeOH/CH_2Cl_2) afforded (142 mg, 80% yield) a colorless oil: TLC R_f = 0.20 (5% MeOH/CH_2Cl_2); $[\alpha]_D$ −0.63 (c = 2.2, MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 9.22 (br s, 1H, OH), 3.80 (s, 3H, OMe), 3.77 (s, 3H, OMe), 3.21−3.26 (m, 1H, one H-6), 3.15−3.19 (m, 1H, one H-6), 2.80 (hext, J = 6.9 Hz, 1H, H-4), 2.47−2.54 (m, 1H, H-2), 2.10 (ddd, J = 13.8, 8.9, 5.9 Hz, 1H, H-3), 1.32 (ddd, J = 13.8, 7.8, 5.7 Hz, 1H, H-3), 1.19 (d, J = 6.9 Hz, 3H, C-4 Me), 1.12 (d, J = 6.9 Hz, 3H, C-2 Me); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 204.7 (d, $^3J_{C-P}$ = 6.5 Hz, C-5), 179.9 (C-1), 53.2 (d, $^3J_{C-P}$ = 3.8 Hz, OMe), 53.1 (d, $^3J_{C-P}$ = 3.8 Hz, OMe), 45.2 (C-4), 39.0 (d, $^2J_{C-P}$ = 131 Hz, C-6), 37.1 (C-2), 35.7 (C-3), 17.7 (C-4 Me), 15.8 (C-2 Me); ^{31}P NMR ($CDCl_3$) δ 24.8; HRMS (ESI+) m/z 289.0813 ($C_{10}H_{19}O_6P$ + Na^+ requires 289.0817).

(2*S*,4*R*)-4-(Methoxycarbonyl)-2-methylpentanoic Acid (9). α-Chymotrypsin (EC 3.4.21.1, 500 mg, 25,500 units) was added to a vigorously stirring suspension of **8** (5.16 g, 27.4 mmol, 1.0 equiv) in 100 mM sodium phosphate (pH 8.0, 150 mL) at 4 °C. A 1.0 *N* aqueous NaOH solution (27.4 mL, 27.4 mmol, 1.0 equiv) was added dropwise over 96 h to maintain the pH between 7.0 and 8.0. During this time the substrate slowly dissolved to form a cloudy solution. Additional α-chymotrypsin was added (500 mg) at 48 h. After 96 h, the reaction was filtered using a Centriprep YM-10 MW membrane filter to remove the enzyme. The filtrate was then acidified to pH 2.0 by the addition of concentrated HCl and extracted with EtOAc (3 × 150 mL). The organic extracts were concentrated and chromatographed (SiO_2, 2% MeOH/CH_2Cl_2) to afford (3.99 g, 83%) of the title compound as a light yellow oil, the ^{1}H and ^{13}C NMR of which agreed with reported values.[23] $[\alpha]_D$ −3.94 (c = 7.18, $CHCl_3$) corresponds to 94% ee.[46]

(*E*)-(2*S*,4*R*,8*R*,9*R*)-9-(*tert*-Butyldimethylsilanyloxy)-2,4,8-trimethyl-5-oxoundec-6-enoic Acid (10). To a solution of phosphonate **6** (194 mg, 0.73 mmol, 1.0 equiv) in THF (3.0 mL) was added finely ground $Ba(OH)_2 \cdot 8H_2O$ (437 mg, 1.38 mmol, 1.9 equiv) and the reaction stirred 5 min. A solution of aldehyde **7**[22] (202 mg, 0.88 mmol, 1.2 equiv) in 20:1 THF:H_2O (3 mL) was added and the reaction stirred for 16 h. The reaction was partitioned between EtOAc (30 mL) and 1.0 *N* aqueous HCl (30 mL). The aqueous layer was extracted with EtOAc (2 × 20 mL), and the combined organic extracts were dried (Na_2SO_4) and concentrated under reduced pressure to a colorless oil. Flash chromatography (SiO_2, 5 g, 2% MeOH/$CHCl_3$) afforded the title compound (203 mg, 75%) as a colorless oil: TLC R_f = 0.18 (1% MeOH + 0.5% glacial AcOH/CH_2Cl_2); $[\alpha]_D$ +27 (c = 0.90, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.94 (dd, J = 15.9, 7.5 Hz, 1H), 6.14 (dd, J = 15.9, 1.5 Hz, 1H), 3.55 (q, J = 5.9 Hz, 1H), 2.86 (hext, J = 7.0 Hz, 1H), 2.44−2.57 (m, 2H), 2.07−2.17 (m, 1H), 1.44−1.52 (m, 1H), 1.34−1.43 (m, 2H), 1.18 (d, J = 6.9 Hz, 3H), 1.11 (d, J = 7.0 Hz, 3H), 1.02 (d, J = 6.8 Hz, 3H), 0.88 (ovlp s, 9H), 0.86 (ovlp t, J = 7.4 Hz, 3H), 0.03, (s, 3H), 0.02 (s, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 203.0, 182.4, 150.8, 127.9, 76.4, 41.5, 41.2, 37.0, 36.2, 26.8, 25.8, 18.1, 17.5, 16.6, 14.2, 9.6, −4.3, −4.5; HRMS (ESI+) m/z 393.2433 ($C_{20}H_{38}O_4Si$ + Na^+ requires 393.2437).

(*E*)-(2*S*,4*R*,8*R*,9*R*)-*S*-2-Acetamidoethyl 9-(*tert*-Butyldimethylsilanyloxy)-2,4,8-trimethyl-5-oxoundec-6-enethioate (11). To a stirring solution of acid **10** (53.1 mg, 0.143 mmol, 1.0 equiv), EDC·HCl (34 mg, 0.215 mmol, 1.5 equiv), and DMAP (1.7 mg, 0.014 mmol, 0.1 equiv) in CH_2Cl_2 (1.5 mL) at 23 °C was added *N*-acetylcysteamine (22 μL, 0.20 mmol, 1.43 equiv). After 16 h, the reaction was diluted with EtOAc (10 mL) and washed successively with 1 *N* aqueous HCl, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl and then dried (Na_2SO_4) and concentrated under reduced pressure to a colorless oil. Flash chromatography (SiO_2, 1% MeOH/$CHCl_3$) afforded (60.3 mg, 90%) the title compound as a colorless oil. TLC R_f = 0.15 (1% MeOH/$CHCl_3$); $[\alpha]_D$ + 36.3 (c = 0.755, MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.93 (dd, J = 15.9, 7.4 Hz, 1H), 6.12 (dd, J = 15.9, 1.0 Hz), 5.99 (br s, 1H), 3.56 (q, J = 5.6 Hz, 1H), 3.35−3.47 (m, 2H), 3.01 (t, J = 6.2 Hz, 2H), 2.79 (hext, J = 7.2 Hz, 1H), 2.69 (hext, J = 6.9 Hz, 1H), 2.44−2.53 (m, 1H), 2.12−2.22 (m, 1H), 1.98 (s, 3H), 1.30−1.55 (m, 3H), 1.17 (d, J = 6.9 Hz, 3H), 1.11 (d, J = 7.0 Hz, 3H), 1.03 (d, J = 6.8 Hz, 3H), 0.88 (ovlp s, 9H), 0.87 (ovlp t, J = 7.5 Hz, 3H), 0.04 (s, 3H), 0.03 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 203.5, 202.5, 170.1, 150.7, 127.6, 76.4, 46.5, 41.6, 39.5, 36.8, 29.8, 28.5, 26.8, 25.9, 23.2, 18.4, 18.2, 17.3, 14.3, 9.8, −4.2, −4.4; HRMS (ESI+) m/z 494.2743 ($C_{24}H_{45}NO_4SSi$ + Na^+ requires 494.2736).

(7*S*)-7-Dihydro-10-deoxymethynolide (12). To a stirring solution of 10-deoxymethynolide (391 mg, 1.32 mmol, 1.0 equiv) and $CeCl_3 \cdot 7H_2O$ (492 mg, 1.32 mmol, 1.0 equiv) in MeOH (10 mL) at −10 °C was added $NaBH_4$ (50 mg, 1.32 mmol, 1.0 equiv) in three portions. After 5 min, the reaction was diluted with EtOAc (20 mL) and poured onto 1 *N* aqueous HCl (20 mL) and saturated aqueous NaCl (20 mL). The aqueous layer was extracted with EtOAc (2 × 25 mL), and the combined organic extracts were dried (Na_2SO_4) and concentrated under reduced pressure to a yellow oil. Flash chromatography (SiO_2, 40 g, 2% MeOH/$CHCl_3$) afforded (275 mg, 70%) a white foam: TLC R_f = 0.28 (5% MeOH/$CHCl_3$, vanillin stain); $[\alpha]_D$ = + 84.0 (c = 1.07, MeOH); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.70 (ddd, J = 15.9, 4.8, 1.8 Hz, 1H, H-9), 5.49 (ddd, J = 15.9, 3.6, 1.8 Hz, 1H, H-8), 5.00 (ddd, J = 8.7, 5.1, 3.0 Hz, 1H, H-11), 4.09−4.14 (br m, 1H, H-7), 3.53 (dd, J = 10.2, 1.8 Hz, 1H, H-3), 2.48−2.62 (m, 2H, H-10, H-2), 1.84−

1.98 (m, 2H, H-4, H-6), 1.50−1.72 (m, 5H, one H-5, H-12, 2 O*H*), 1.32−1.41 (m, 1H, one H-5), 1.27 (d, J = 6.9 Hz, 3H, C-10 Me), 1.05 (app t, J = 6.6 Hz, 6H, C-2 Me, C-4 Me), 0.98 (d, J = 6.9 Hz, 3H, C-6 Me), 0.91 (t, J = 7.2 Hz, 3H, H-13); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 171.2, 130.8, 128.2, 78.7. 77.2, 75.9, 43.6, 37.8, 35.4, 32.6, 31.9, 24.4, 20.7, 17.3, 16.6, 10.9, 10.5; HRMS (ESI+) m/z 321.2036 ($C_{17}H_{30}O_4$ + Na^+ requires 321.2042).

(*E*)-(2*R*,3*S*,4*S*,6*R*,7*S*,10*R*,11*R*)-3,7,11-Trihydroxy-2,4,6,10-tetramethyltridec-8-eneioic Acid (13) and (*E*)-(2*S*,3*S*,4*S*,6*R*,7*S*,10*R*,11*R*)-3,7,11-Trihydroxy-2,4,6,10-tetramethyltridec-8-eneoic Acid, Minor Isomer (C_2-*epi*-13). To a stirring solution of diol **12** (184 mg, 0.62 mmol, 1.0 equiv) in MeOH (10 mL) at 23 °C was added a 0.62 M aqueous LiOH solution (10 mL, 6.2 mmol, 10.0 equiv) to afford a slightly cloudy solution. The resulting solution was refluxed for 9 d. LiOH precipitated during the course of the reaction as a white solid; thus an additional 1:1 MeOH:0.62 M aqueous LiOH solution (5 mL) was added every 3 d. The reaction was cooled to 23 °C and quenched with 1.0 *N* aqueous HCl (20 mL). This solution was partitioned between EtOAc (30 mL) and saturated aqueous NaCl (30 mL). The aqueous phase was extracted with EtOAc (2 × 30 mL), and the combined organic extracts were dried (Na_2SO_4) and concentrated under reduced pressure to an oil. Flash chromatography (SiO_2, 20 g, 1% glacial AcOH + 5% MeOH/CH_2Cl_2) afforded (178 mg, 91%) the title compound as an inseparable 4:1 mixture of **13** and **C_2-epi-13**: TLC R_f = 0.09 (1% glacial AcOH & 5% MeOH/$CHCl_3$, vanillin stain); ^{1}H NMR (CD_3OD, 300 MHz) δ 5.47−5.61 (m, 2H, H-8, H-9), 3.95 (ovlp t, J = 5.1 Hz, 0.8H, H-7$_{major}$), 3.95−4.01 (ovlp m, 0.2H, H-7$_{minor}$), 3.61 (t, J = 6.0 Hz, 0.8H, H-3$_{major}$), 3.47−3.52 (m, 0.2H, H-3$_{minor}$), 3.25−3.29 (ovlp m, 1H, H-11), 2.58−2.68 (m, 1H, H-2), 2.20 (hext, J = 6.6 Hz, 1H, H-10), 1.86 (ddd, J = 13.5, 7.8, 3.6 Hz, 1H, one H-5), 1.61−1.73 (ovlp m, 2H, H-4, H-6), 1.52−1.61 (ovlp m, 1H, one H-12), 1.28−1.42 (m, 1H, one H-12), 1.16 (d, J = 6.9 Hz, 3H, C-2 Me), 1.04 (d, J = 6.6 Hz, 3H, C-10 Me,), 0.89−0.98 (m, 10 H, C-4 Me, C-6 Me, H-13, one H-5);^{13}C NMR (CD_3OD, 75 MHz) δ 156.8, 135.3, 133.0, 78.3, 78.0, 75.9, 44.2, 38.4, 36.9, 35.9 (2C), 28.5, 18.0, 16.6, 16.5, 12.2, 10.9; HRMS (ESI+) m/z 339.2144 ($C_{17}H_{32}O_5$ + Na^+ requires 339.2147).

(*E*)-(2*R*,3*S*,4*S*,6*R*,7*S*,10*R*,11*R*)-*S*-2-Acetamidoethyl 3,7,11-Trihydroxy-2,4,6,10-tetramethyltridec-8-enethioate (14) and (*E*)-(2*S*,3*S*,4*S*,6*R*,7*S*,10*R*,11*R*)-*S*-2-Acetamidoethyl 3,7,11-Trihydroxy-2,4,6,10-tetramethyltridec-8-enethioate, Minor Isomer (C_2-*epi*-14). To a solution of **13** and **C_2-*epi*-13** (64 mg, 0.20 mmol, 1 equiv), EDC·HCl (48 mg, 0.30 mmol, 1.5 equiv), and DMAP (2.5 mg, 0.1 mmol, 0.1 equiv) in CH_2Cl_2 (2 mL) at 23 °C was added *N*-acetylcysteamine (65 μL, 0.60 mmol, 3 equiv). After 16 h, the reaction was partitioned between 0.1 N aqueous HCl (5 mL) and EtOAc (5 mL). The aqueous layer was extracted with EtOAc (2 × 5 mL), and the organic extracts were washed with saturated aqueous NaCl (5 mL), dried (Na_2SO_4), and concentrated under reduced pressure to a colorless oil. The resulting oil was dissolved in 35% CH_3CN/H_2O (2 mL) and purified in two portions by preparative reverse-phase HPLC (Econosil C18, 22 × 250 mm, Alltech). An isocratic elution of 65:35 H_2O:CH_3CN with 0.1% TFA at a flow rate of 10 mL/min monitoring at 240 nm afforded (58 mg, 70%) **14** (t_R = 28.0 min) as a white solid after lyophilization and (12.6 mg, 15%) of **C_2-*epi*-14** (t_R = 24.1 min) as a white solid. Major isomer **14**: $[\alpha]_D$ = +7.60 (c = 0.605, MeOH); ^{1}H NMR (CD_3OD, 500 MHz) δ 5.40−5.47 (m, 2H, H-8, H-9), 3.84 (t, J = 4.4 Hz, 1H, H-7), 3.56 (t, J = 5.9 Hz, 1H, H-3), 3.22−3.26 (m, 2H, AcNHCH$_2$C*H*$_2$S), 3.17−3.20 (ddd, J = 9.3, 6.4, 3.4 Hz, 1H, H-11), 2.87−2.97 (m, 2H, AcNHC*H*$_2$CH$_2$S), 2.80 (p, J = 6.7 Hz, 1H, H-2), 2.12 (hext, J = 6.6 Hz, 1H, H-10), 1.83 (s, 3H, C*H*$_3$C═ONHCH$_2$CH$_2$S), 1.71−1.76 (m, 1H, one H-5), 1.54−1.63 (m, 2H, H-4, H-6), 1.45−1.53 (m, 1H, one H-12), 1.21−1.31 (m, 1H, one H-12), 1.11 (d, J = 6.9 Hz, 3H, C-2 Me), 0.95 (t, J = 6.8 Hz, 3H, C-10 Me), 0.86 (ovlp t, J = 7.5 Hz, 3H, H-13), 0.85 (ovlp d, J = 6.7 Hz, 3H, C-4 Me), 0.81 (ovlp d, J = 6.8 Hz, 3H, C-6 Me), 0.77−0.81 (ovlp m, 1H, one H-5); ^{13}C NMR (CD_3OD, 125 MHz) δ 203.7 (C-1), 173.4 (CH$_3$*C*═ONHCH$_2$CH$_2$S), 135.5 (C-9), 133.0 (C-8), 78.3 (C-3), 77.8 (C-11), 76.1 (C-7), 52.9 (C-2), 44.0 (C-10), 40.1 (AcNHCH$_2$*C*H$_2$S), 38.3 (C-4 or C-6), 36.3 (C-5), 35.9 (C-4 or C-6), 29.1 (AcNH*C*H$_2$CH$_2$S), 28.4 (C-12), 22.5 (*C*H$_3$C═ONHCH$_2$CH$_2$S), 18.0 (C-4 Me or C-6 Me), 16.4 (2C, C-10 and C-4 Me or C-6 Me), 13.1 (C-2 Me), 10.7 (C-13); HRMS (ESI+) m/z 440.2451 ($C_{21}H_{39}NO_5S$ + Na^+ requires 440.2447).

Minor isomer **C_2-*epi*-14**: $[\alpha]_D$ = +47 (c = 0.095, MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.52 (dd, J = 15.6, 7.5 Hz, 1H, H-9), 5.45 (dd, J = 15.6, 5.9 Hz, 1H, H-8), 3.92 (dd, J = 5.9, 3.6 Hz, 1H, H-7), 3.55 (dd, J = 8.6, 3.4 Hz, 1H, H-3), 3.19−3.29 (part obsc m, 3H, H-11, AcNHCH$_2$C*H*$_2$S), 2.89−2.98 (m, 2H, AcNHC*H*$_2$CH$_2$S), 2.83 (dq, J = 8.6, 7.0 Hz, 1H, H-2), 2.13 (hext, J = 6.9 Hz, 1H, H-10), 1.84 (s, 3H, C*H*$_3$C═ONHCH$_2$CH$_2$S), 1.70−1.78 (m, 1H, H-4), 1.59−1.64 (ovlp m, 1H, one H-5), 1.55−1.60 (ovlp m, 1H, H-6), 1.45−1.55 (ovlp m, 1H, one H-12), 1.24−1.33 (m, 1H, one H-12), 1.07 (d, J = 7.0 Hz, 3H, C-2 Me), 0.98 (d, J = 6.8 Hz, 3H, C-10 Me), 0.93 (d, J = 6.8 Hz, 3H, C-4 Me), 0.88 (ovlp t, J = 7.4 Hz, 3H, H-13), 0.84−0.87 (ovlp m, 1H, one H-5), 0.83 (d, J = 6.7 Hz, 3H, C-6 Me); ^{13}C NMR (CD_3OD, 125 MHz) δ 204.2 (C-1), 173.5 (CH$_3$C═ONHCH$_2$CH$_2$S), 135.4 (C-9), 133.1 (C-8), 79.0 (C-3), 77.8 (C-11), 75.2 (C-7), 53.5 (C-2), 43.9 (C-10), 40.1 (AcNHCH$_2$*C*H$_2$S), 37.8 (C-6), 34.1 (C-4), 33.8 (C-5), 29.1 (AcNH*C*H$_2$CH$_2$S), 28.4 (C-12), 22.5 (*C*H$_3$C═ONHCH$_2$CH$_2$S), 18.1 (C-4 Me), 16.3 (2C, C-6 Me and C-10 Me), 15.6 (C-2 Me), 10.7 (C-13); HRMS (ESI+) m/z 440.2451 ($C_{21}H_{39}NO_5S$ + Na^+ requires 440.2447).

3-Oxo-10-deoxymethynolide (18). To a stirring solution of $(COCl)_2$ (90 μL, 1.03 mmol, 3.0 equiv) in CH_2Cl_2 (3 mL) at −78 °C was added a solution of DMSO (98 μL, 1.38 mmol, 4.0 equiv) in CH_2Cl_2 (1 mL). After 10 min, a solution of 10-deoxymethynolide (102 mg, 0.34 mmol, 1.0 equiv) in CH_2Cl_2 (1.0 mL + 1.0 mL wash) was added dropwise via cannula and the reaction stirred another 15 min at −78 °C. Et_3N (237 μL, 1.70 mmol, 5.0 equiv) was added dropwise and the resulting solution stirred 30 min at −78 °C and then the dry ice bath was removed and the reaction warmed to 23 °C. The reaction solution was washed successively with 1 *N* aqueous HCl (5 mL), H_2O (5 mL), and saturated aqueous NaCl (5 mL) and then dried (Na_2SO_4) and concentrated under reduced pressure to an oil. Flash chromatography (SiO_2, 10 g, 40% EtOAc/hexanes) afforded (52 mg, 52%) a white crystalline solid: mp 97−98 °C; TLC R_f = 0.70 (50% EtOAc/hexanes); $[\alpha]_D$ +1.9 × 10^2 (c = 1.4, MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.82 (dd, J = 15.8, 5.2 Hz, 1H), 6.48 (dd, J = 15.8, 1.2 Hz, 1H), 5.09 (ddd, J = 8.3, 5.6, 2.3 Hz, 1H), 3.56 (q, J = 7.1 Hz, 1H), 2.69−2.75 (m, 1H), 2.56−2.64 (m, 1H), 2.41−2.48 (m, 1H), 1.94 (ddd, J = 14.1, 12.0, 2.0 Hz, 1H), 1.68−1.78 (m, 1H), 1.55−1.63 (m, 1H), 1.28−1.36 (ovlp m, 1H), 1.31 (ovlp d, J = 7.1 Hz, 3H), 1.21 (d, J = 7.0 Hz, 3H), 1.14 (d, J = 6.8 Hz, 3H), 1.01 (d, J = 6.4 Hz, 3H), 0.92 (t, J = 7.4 Hz, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 207.2, 203.9, 172.3, 147.7, 125.8, 75.4, 50.0, 45.1, 41.6, 38.2, 38.1, 25.0, 17.3, 14.1, 13.5, 10.2, 9.4; HRMS (ESI+) m/z 317.1723 ($C_{17}H_{26}O_4$ + Na^+ requires 317.1729).

(*E*)-[10-(2*R*,4*S*,5*R*)-3*R*,4*R*,7*S*,8*R*,10*S*]-10-[2-(4-Methoxy-benzyl)-5-methyl-[1,3]dioxan-4-yl]-4,8-dimethylundec-5-ene-3,7-diol (20). To a 4:1 mixture of acids **13** and C_2-*epi*-**13** (50 mg, 0.158 mmol, 1.0 equiv) in MeOH (5 mL) was added TMSCH$_2$N$_2$ (2.0 *M* in Et$_2$O, 790 μL, 1.58 mmol, 10.0 equiv) at 0 °C. The reaction was stirred for 10 min at 0 °C and then concentrated under reduced pressure. Flash chromatography (SiO_2, 10 g, 2% MeOH/CH_2Cl_2) afforded 39 mg (75%) of the methyl ester as a colorless oil. The methyl ester was dissolved in THF (5 mL), and $LiAlH_4$ (10 mg, 0.25 mmol, 2.0 equiv) was added at 0 °C. The reaction was stirred for 16 h at 23 °C and then diluted with EtOAc (10 mL) and washed with 1.0 *N* aqueous sodium potassium tartrate solution (10 mL). The aqueous layer was extracted with EtOAc (2 × 10 mL), and the combined organic extracts were dried (Na_2SO_4), and concentrated under reduced pressure to a colorless oil (39.1 mg, 108%), which was taken directly onto the next step. To a mixture of tetraols **19** prepared in the previous step (azeotropically dried with PhMe, 2 × 10 mL) was added a solution of *p*-MeOC$_6$H$_4$CH(OMe)$_2$ (30 μL, 0.17 mmol,

1.5 equiv) and CSA (2.7 mg, 0.012 mmol, 0.10 equiv) in DMF (1.0 mL), and the resulting solution was stirred for 16 h at 23 °C. The reaction was diluted with EtOAc (50 mL), washed with H_2O (3 × 25 mL), dried (Na_2SO_4), and concentrated under reduced pressure to a colorless oil. Flash chromatography (SiO_2, 5 g, 2% MeOH/$CHCl_3$) afforded the major isomer **20** (26 mg, 55%) and the minor isomer **21** (7 mg, 15%). Data for major isomer **20**. TLC $R_f = 0.38$ (2% MeOH/$CHCl_3$, vanillin stain); $[\alpha]_D = -1.74$ ($c = 0.575$, MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.40 (d, $J = 8.7$ Hz, 2H, Ar-*ortho*-H), 6.88 (d, $J = 8.7$ Hz, 2H, Ar-*meta*-H), 5.52 (dd, $J = 15.7$, 7.2 Hz, 1H, H-9), 5.46 (dd, $J = 15.7$, 5.5 Hz, 1H, H-8), 5.42 (s, 1H, acetal-H), 4.00−4.04 (m, 3H, H-1, H-7), 3.79 (s, 3H, $ArOCH_3$), 3.46 (dd, $J = 10.0$, 2.1 Hz, 1H, H-3), 3.33 (ddd, $J = 8.8$, 5.1, 3.9 Hz, 1H, H-11), 2.20 (hext, $J = 6.8$ Hz, 1H, H-10), 1.89 (ddd, $J = 11.9$, 8.1, 3.7 Hz, 1H, one H-5), 1.70−1.80 (ovlp m, 2H, H-4, H-6), 1.62−1.70 (ovlp m, 1H, H-2), 1.54−1.62 (ovlp br s, 2H, C-7 OH, C-11 OH), 1.47−1.54 (ovlp m, 1H, one H-12), 1.28−1.37 (m, 1H, one H-12), 1.16 (d, $J = 6.9$ Hz, 3H, C-2 Me), 0.98−0.90 (ovlp m, 1H, one H-5), 0.97 (ovlp d, $J = 6.8$ Hz, 3H, C10-Me), 0.93 (t, $J = 7.4$ Hz, 3H, H-13), 0.87 (d, $J = 6.8$ Hz, 3H, C-4 or C-6 Me), 0.85 (d, $J = 6.8$ Hz, 3H, C-4 or C-6 Me); ^{13}C NMR (C_6D_6, 125 MHz) δ 160.4, 134.0, 132.5, 132.2, 128.3, 113.9, 102.2, 85.3, 76.4, 74.8, 73.8, 54.8, 42.5, 38.0, 37.4, 32.7, 30.5, 27.5, 16.5, 15.7, 14.8, 11.1, 10.7; HRMS (ESI+) *m*/*z* 443.2777 ($C_{25}H_{40}O_5 + Na^+$ requires 443.2773).

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Supporting Information Available: HPLC traces of compounds **1** and **2** isolated from in vitro reactions with PikAIII and PikAIV, representative radio-TLC data used to determine steady-state kinetic parameters of Pik monomodules, and ^{1}H NMR and ^{13}C NMR spectra of **5**, **6**, **9**−**14**, **18**, and **20**. This material is available free of charge via the Internet at http://pubs.acs.org.

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