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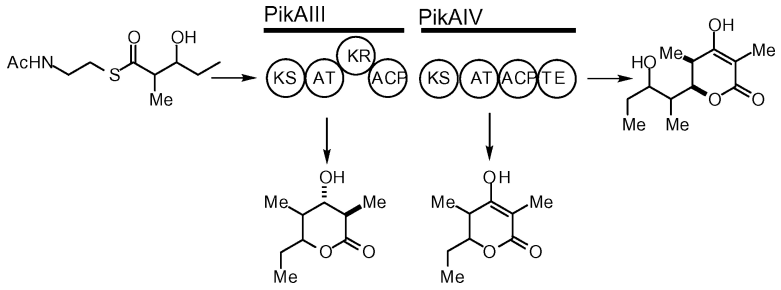

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Substrate Recognition and Channeling of Monomodules from the Pikromycin Polyketide Synthase

Brian J. Beck,[†,||] Courtney C. Aldrich,[†,||] Robert A. Fecik,[‡] Kevin A. Reynolds,[§] and David H. Sherman*[,†,||]

Contribution from the Department of Microbiology and Biotechnology Institute, University of Minnesota, Minneapolis, Minnesota 55455; Department of Medicinal Chemistry, University of Minnesota, Minneapolis, Minnesota 55455; and Department of Medicinal Chemistry and Institute for Structural Biology and Drug Discovery, Virginia Commonwealth University, Richmond, Virginia 23219

Received February 24, 2003; E-mail: davidhs@umich.edu

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 the chain extension process. We have overexpressed and purified PikAIII (module 5) and PikAIV (module 6) and assessed the ability of these proteins to generate tri- and tetraketide lactone products using *N*-acetylcysteamine-activated diketides and ^{14}C-methylmalonyl-CoA as substrates. Comparison of the stereochemical specificities for PikAIII and PikAIV and the reported values for the DEBS modules reveals significant differences between these systems.

Introduction

Polyketides are a diverse class of natural products that possess antibiotic, anticancer, and immunosuppressive bioactivities as well as others.[1] Their biosynthesis shares a common mechanism where the repetitive condensation of simple malonic acid derivatives are catalyzed by modular, multifunctional proteins, the polyketide synthases (PKSs). A prototype type I PKS enzyme is organized into modules each of which catalyzes one cycle of chain elongation. A module minimally consists of three domains: a ketosynthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP). Additionally, a module may include optional reductive domains (ketoreductase (KR), dehydratase (DH), and enoylreductase (ER)) that are responsible for adjusting the oxidation state of the β-position. The terminal module of the PKS assembly line contains a thioesterase (TE) domain which releases the fully elongated polyketide chain either as a macrolactone or as a carboxylic acid.[1]

Much of our current knowledge of PKS systems is largely based from the study of the 6-deoxyerythronolide B polyketide synthase (DEBS). DEBS is organized into three large bimodular proteins (DEBS1, 2, and 3) that direct the biosynthesis of the clinically important drug Erythromycin. The chain elongation and processing functions of DEBS modules have been studied in-vitro using simple diketide *N*-acetylcysteamine (NAC) thioesters to mimic the natural polyketide chain intermediates. Specifically, the four diastereomeric NAC thioesters of 2-methyl-3-hydroxypentanoic acid have been investigated with native bimodular DEBS proteins, as well as DEBS proteins that have been reengineered and purified as non-native monomodular enzymes.[2−6] The DEBS modules tolerate these short diketide chain lengths and have a universal specificity for the (2*S*, 3*R*) stereochemistry. "Docking" of the domains at the NH_2 and COOH ends of these bimodular proteins (DEBS 1, 2, and 3) has been proposed to promote the sequential interaction of PKS modules and the channeling of polyketide chain elongation intermediates.[4−6] Generalization and utilization of these observations require validation by a comparative analysis with other PKS systems.

Streptomyces venezuelae ATCC 15439 produces the 12- and 14-membered ring aglycones, 10-deoxymethynolide (10-Dml, **1**), and narbonolide (Nbl, **2**), respectively, through the activity of the pikromycin (Pik) PKS (Figure 1).[7] Initiation of polyketide biosynthesis by PikAI and successive elongation through PikAII and PikAIII provide a hexaketide leading to 10-Dml, whereas Nbl production requires an additional extension of the hexaketide chain intermediate with methylmalonyl-CoA catalyzed by PikAIV. In vivo expression studies have led to alternative models for the partitioning of the hexaketide chain produced by PikAIII. Initially, an N-terminally truncated form of PikAIV lacking its docking domain was proposed to exclusively catalyze

[†] Department of Microbiology and Biotechnology Institute, University of Minnesota.
[‡] Department of Medicinal Chemistry, University of Minnesota.
[§] Virginia Commonwealth University.
[||] Current address: Department of Medicinal Chemistry, Department of Chemistry, and Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48109-1065.

(1) Katz, L. *Chem. Rev.* **1997**, *97*, 2557−2575.
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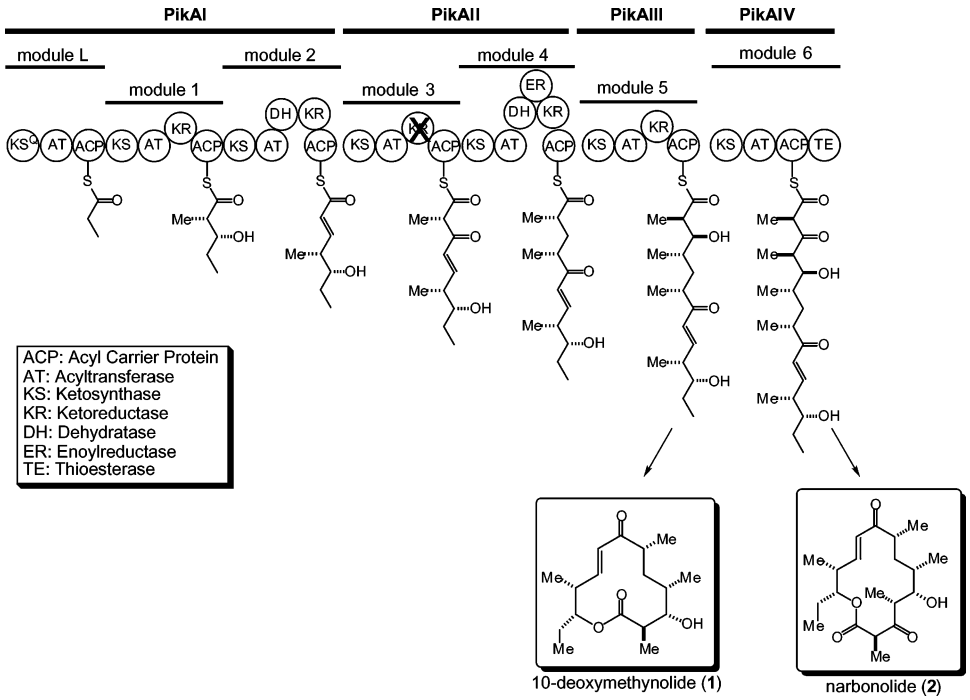

Figure 1. Biosynthesis of 10-deoxymethynolide and narbonolide by the Pikromycin (Pik) PKS in *S. venezuelae*.

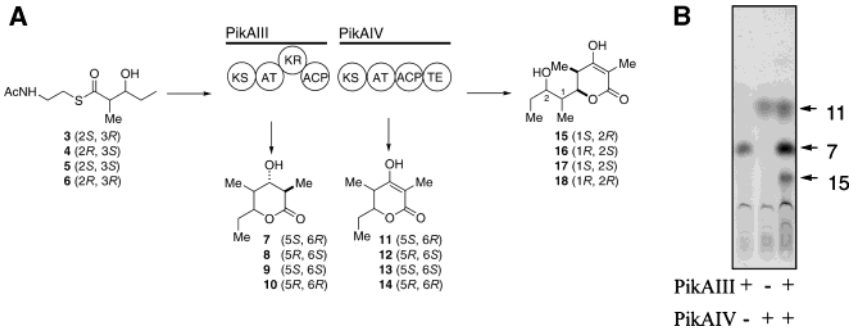

Figure 2. Synthesis of multiple lactones by PikAIII and PikAIV using *N*-acetylcysteamine (NAC)-activated thioesters **3**−**6**. (A) Synthesis of **7**−**10** and **11**−**14** requires the extension of the diketide by PikAIII and PikAIV, respectively, whereas synthesis of **15**−**18** requires elongation of the diketide through PikAIII and PikAIV. (B) Radio-TLC identification of reaction products **7**, **11**, and **15** by incubation with NAC diketide **3**.

the synthesis of 10-Dml.[8] Subsequently, site-directed mutants of PikAIV indicated that the hexaketide chain may "skip" through the domains of PikAIV leading to macrolactonization by the thioesterase.[9] To understand the mechanistic details of polyketide chain extension in this system, we sought to identify the substrate specificity and the interaction between these monomodular proteins using diketide substrates. We reasoned that acceptance of a diketide by either PikAIII or PikAIV would produce a triketide that could be cyclized to generate triketide lactones (TKLs) **7**−**10** and **11**−**14**, respectively. Accordingly, PikAIV extension of the triketide chain produced by PikAIII would yield tetraketides **15**−**18**[10,11] (Figure 2A).

Results

Protein Purification and Assays for Substrate Incorporation. PikAIII and PikAIV holoenzymes were overexpressed and purified as previously described.[12] Purified PikAIII and PikAIV were individually reacted with 2-[^{14}C]-methylmalonyl-CoA and each of the diketide NAC thioesters **3**−**6**.[13] The tri- and tetraketide products were identified using synthetic standards and quantified by radio-TLC (Figure 2B). A time-course analysis of both PikAIII and PikAIV demonstrated that enzyme activity remained constant during the first 120 min of reaction (data not shown). The initial rates, v, at a given [S] were determined by single time-point stopped-time incubations at 90 min. All reactions were run in triplicate employing a fresh enzyme preparation. Apparent steady-state kinetic values (k_{cat} and K_m)[14] were determined for enzyme−substrate pairings that yielded a detectable product by fitting the normalized v versus [S] plots to the Michaelis−Menten equation. (The normalized v versus

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(9) Beck, B. J.; Yoon, Y. J.; Reynolds, K. A.; Sherman, D. H. *Chem. Biol.* **2002**, *9*, 575−583.
(10) TKL **8**−**10**, **13**, and **14** and tetraketides **16**−**18** were not observed by radio-TLC.
(11) The C-2 stereocenters of compounds **11**, **12**, **15**, and **16** are configurationally labile and, for simplicity, are represented as the enol tautomers. In aqueous and methanolic solutions, β-keto-δ-lactones **11**−**12** and **15**−**16** exist exclusively as the enol tautomers. In chloroform, TKL **11** and **12** exist as a ratio of keto:enol tautomers which varied from 10:1 to 2:1. Tetraketide **15** is only sparingly soluble in chloroform but exists as a 5.5:1 mixture of keto:enol tautomers. The insolubility of tetraketide **16** in chloroform prevented evaluation of its tautomeric ratio.
(12) Beck, B. J.; Aldrich, C. C.; Fecik, R. A.; Reynolds, K. A.; Sherman, D. H. *J. Am. Chem. Soc.* **2003**, *125*, 4682−4683.
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Table 1. Steady-State Kinetic Parameters for the Formation of Triketide Lactones by PikAIII and PikAIV

module	substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($min^{-1}\,mM^{-1}$)
PikAIII	(2*S*,3*R*)-**3**	0.0045 ± 0.0003	3.1 ± 0.8	0.0015
	(2*R*,3*S*)-**4**	na[a]	na	na
	(2*S*,3*S*)-**5**	na	na	na
	(2*R*,3*R*)-**6**	na	na	na
PikAIV	(2*S*,3*R*)-**3**	0.013 ± 0.002	10.5 ± 3.1	0.0012
	(2*R*,3*S*)-**4**	0.0076 ± 0.0007	5.7 ± 1.6	0.0013
	(2*S*,3*S*)-**5**	na	na	na
	(2*R*,3*R*)-**6**	na	na	na

[a] na, no activity detected.

[*S*] plots of these reactions are available in the Supporting Information.)

Verification of Reaction Products. Reaction products were identified by comigration with authentic standards, and GC−MS or LC−MS analysis was employed to rigorously confirm these assignments. PikAIII only reacted with *syn*-diketide (2*S*,3*R*)-**3** while PikAIV only accepted *syn*-diketides (2*S*,3*R*)-**3** and (2*R*,3*S*)-**4**. To provide sufficient product for detection, PikAIII and PikAIV were incubated for 16 h with the appropriate diketide NAC esters. Incubation of PikAIII with (2*S*,3*R*)-**3** produced TKL **7** (retention time (t_{ret}), 7.73 min) as the only diastereomeric product as determined by GC−MS analysis detecting at *m*/*z* 171 $(M-H)^+$ in single-ion mode employing chemical ionization (see Supporting Information for GC−MS traces and conditions). Incubation of PikAIV with (2*S*,3*R*)-**3** and (2*R*,3*S*)-**4** afforded the enantiomeric keto-lactones **11** and **12** (t_{ret}, 21.00 min) as confirmed by LC−MS analysis detecting at *m*/*z* 169 $(M-H)^-$ in negative ion mode using electrospray ionization (ESI). Tetraketide **15** (t_{ret}, 21.90 min) was similarly analyzed by LC−MS detecting at *m*/*z* 227 $(M-H)^-$ in negative ion mode (ESI). (See Supporting Information for LC−MS traces and HPLC conditions.)

Determination of the Dissociation Constant Between PikAIII and PikAIV. The apparent dissociation constant, K_D, of the PikAIII and PikAIV interaction was determined using the analysis reported by Tsuji et al.[4] When PikAIII and PikAIV were reacted together with (2*S*,3*R*)-**3**, a product mixture of TKLs **7** and **11** and tetraketide **15** was observed by radio-TLC (Figure 2B). In the present system, PikAIV partitions the incoming triketide into two products, TKL **7** and tetraketide **15**; therefore, the formation of both products is a measure of the interaction of PikAIII with PikAIV. However, as **7** is also produced by PikAIII, albeit at a reduced rate, this contribution was subtracted from the overall observed production of **7**. Variation of PikAIV (P4) at a constant concentration of PikAIII (P3) under saturating concentrations of all substrates (MMCoA, NADPH, and (2*S*,3*R*)-**3**) provided the saturation curve in Figure 3[15] which is described by eq 1, where v is the sum of the velocities of both tetraketide **15** and the corrected velocity of triketide **7**. The maximal velocity, v_{max}, is equal to $k_{cat}[P3]_0$, where $[P3]_0$ is the total concentration of PikAIII. Nonlinear regression analysis of eq 1 provided a K_D of 95 ± 32 nM.[16]

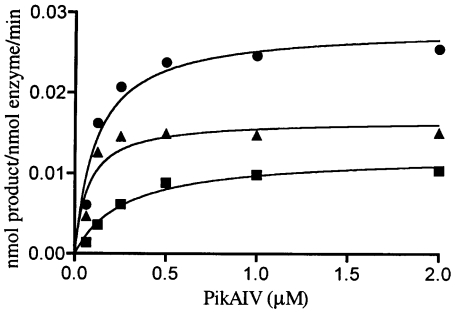
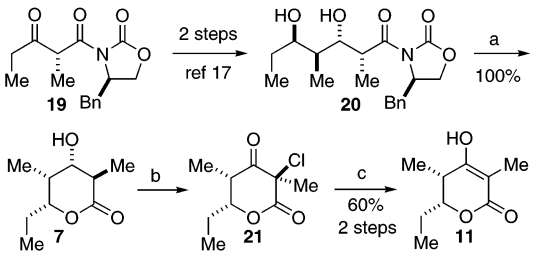


Figure 3. Production of TKL **7** and tetraketide **15** by PikAIII and PikAIV interaction. (▲) TKL **7**; (■) tetraketide **15**; (●) total production.

Scheme 1 [a]



[a] Key: (a) 1 N aq HCl:THF (4:1), 60 °C, 40 h; (b) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −78 °C → −20 °C; (c) Zn dust, AcOH, CH_2Cl_2, 0 °C.

$$v = \frac{v_{max}[P4]}{K_D + [P4]} \quad (1)$$

Synthesis of Tri- and Tetraketide Standards. A flexible synthetic strategy was desired that could efficiently provide all diastereomeric tri- and tetraketide products employing a common intermediate. This was most effectively accomplished utilizing Evans diketide synthon **19**.[18] Aldol reaction of β-ketoimide **19** with simple aldehydes provides a rapid entry to triketides wherein the choice of Lewis acid ($TiCl_4$, $Sn(OTf)_2$, or $(c\text{-hex})_2BCl$) allows access to three of the four possible diastereomeric triketide products.[18,19] Additionally, the choice of reducing agent allows the stereochemistry of the β-keto function to be adjusted as desired. Triketide **20**[17] was prepared according to Evans; subsequent δ-lactonization proceeded in quantitative yield under aqueous acidic conditions to afford lactone **7** (Scheme 1).[20] Ketolactone standard **11**[21] is available directly by oxidation of **7**; however initial attempts employing the Dess−

(14) Since these are complex multifunctional systems reacting with non-natural substrates, the rate-determining step remains to be determined. Thus, the presented k_{cat} values represent a composite of individual rate constants for the loading of diketide substrates, the Claisen condensation performed by the KS domain, reductive processing of the keto function, and lactonization.

(15) TKL **7** production shown in Figure 3 results from PikAIV stimulation only. These data were obtained by subtracting the contribution due to spontaneous lactonization ($k_{cat} = 0.0045\ min^{-1}$) from the observed production of **7**.

(16) The apparent K_D can be taken as an upper limit; thus, the binding interaction may actually be stronger than calculated employing this analysis. The dissociation constant K_D is equal to $[P3]_L[P4]/[P3_L{\cdot}P4]$. Also, $[P3]_0 = [P3]_L + [P3]_F$, where $P3_L$ is the concentration of PikAIII ACP_5-loaded triketide and $P3_F$ is equal to concentration of free-PikAIII without a loaded triketide. If interaction between $P3_L$ and P4 results in channeling of the triketide to P4 resulting in the formation of **7** and **15**, then we can write $v = k_{cat}[P3_L{\cdot}P4]$. This is alternatively expressed by eq 1 in terms of the known parameters, [P4] and $[P3]_0$. However, this analysis neglects the effect of $[P3]_F$ on the overall process. In fact, $[P3]_F$ will act as a competitive inhibitor of P4. The net effect is to underestimate K_D by a factor of $(1 + [P3]_F/K_i)$.

(17) Evans, D. A.; Kim, A. S.; Metternich, R.; Novack, V. J. *J. Am. Chem. Soc.* **1998**, *120*, 5921−5942.

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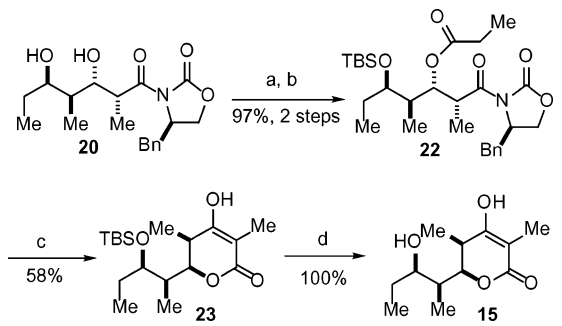
(19) Evans, D. A.; Ng, H. P.; Clark, S.; Rieger, D. L. *Tetrahedron Lett.* **1992**, *48*, 2127−2142.

(20) For an alternative synthesis of TKL **7**, see: Wilkinson, A. L.; Hanefeld, U.; Wilkinson, B.; Leadlay, P. F.; Staunton, J. *Tetrahedron Lett.* **1998**, *39*, 9827−9830.

(21) Luo, G.; Pieper, R.; Rosa, A.; Khosla, C.; Cane, D. E. *Bioorg. Med. Chem.* **1996**, *4*, 995−999.

Scheme 2 [a]



[a] Key: (a) TBSOTf, 2,6-lutidene, CH_2Cl_2, 0 °C, 5 min; (b) $(EtCO)_2O$, DMAP, CH_2Cl_2, 50 °C, 7 h; (c) KHMDS (4.0 equiv), THF, −78 °C, 2 h; (d) Dowex 50WX2-H^+, THF:H_2O (1:1), 50 °C, 20 h.

Martin periodinane[22] or PCC resulted in decomposition to unidentifiable products. It was found that Swern conditions[23,24] successfully oxidized the alcohol of TKL **7** to the desired keto function but additionally resulted in chlorination to afford α-chloroketone **21**.[25] The decomposition pathway observed with other oxidation reagents is likely due to overoxidation of the initially formed keto-lactone.[26] The success of the Swern oxidation thus lies in the protection afforded by chlorination at the C-2 position which serves to prevent further oxidation at this position.[27] Dechlorination was achieved by reduction with zinc and acetic acid to provide the desired ketolactone **11**.[11,28,29] TKL standards **8** and **12** were prepared in an analogous fashion from β-ketoimide **19**.[30]

Triketide **20** was rapidly elaborated to the desired tetraketide target **15** by taking advantage of a novel Claisen-like cyclization developed by Brandänge and Leijonmarck.[31−33] First, regioselective protection of triketide **20**,[17] followed by acylation of the remaining secondary alcohol with propionic anhydride, provided **22** in nearly quantitative yield (Scheme 2). Treatment of **22** with potassium bis(trimethylsilyl)amide in THF at −78 °C resulted in regioselective deprotonation of the side chain propionate group and intramolecular Claisen-like cyclization with expulsion of the oxazolidinone auxiliary to furnish tetraketide **23** with no competing α,β-elimination of propionic acid.[34] Deprotection of tetraketide **23** with Dowex cationic exchange resin yielded the desired tetraketide **15** in quantitative yield and analytical purity after filtration from the resin. Synthetic tetraketide **15** was identical to a biosynthetically derived sample by comparison of ^1H NMR, ^{13}C NMR, and HRMS spectral data.[35] This short six step sequence starting from **19** allowed a very efficient synthesis of the tetraketide target, and an analogous series of reactions provided tetraketide **16**.[30]

Discussion

Comparison of the specificity constants (k_{cat}/K_m) reveals that PikAIV does not distinguish between *syn*-diketides (2*S*,3*R*)-**3** and (2*R*,3*S*)-**4**. In contrast, DEBS 3 (module 6) was reported to accept both *syn*-diketide substrates with a 20-fold preference for (2*S*,3*R*)-**3**.[6] The specificity constants for PikAIV with (2*S*,3*R*)-**3** and (2*R*,3*S*)-**4** are, respectively, 890-fold and 45-fold less than those reported for DEBS 3 (module 6) with the same substrates. Thus, PikAIV displays extraordinary specificity and processes the synthetic non-natural diketides at levels 2 to 3 orders of magnitude lower than the corresponding DEBS system. Additionally, PikAIV does not accept *anti*-diketides (2*S*,3*S*)-**5** and (2*R*,3*R*)-**6** which is consistent with the behavior of DEBS modules.[6,36]

We have also characterized native PikAIII, which lacks a C-terminal TE domain. This represents the first example where a monomodule has been evaluated without such a domain. Previously, unnatural DEBS monomodules were investigated with a TE domain fused to the C-terminal end of the protein. We found that PikAIII produces a TKL product in the absence of a TE domain. In this case, lactonization is not enzymatically mediated but is due to spontaneous cyclization. PikAIII discriminates between the *syn* diastereomers elongating only the (2*S*,3*R*)-**3** diketide substrate, while no TKL product was observed with (2*R*,3*S*)-**4**. Since PikAIII has been observed to extend (2*R*,3*S*)-**4** generated through an intramolecular priming reaction,[12] the KS_5 domain likely discriminates against diffusive loading of this enantiomer. This stereochemical selectivity distinguishes PikAIII from the reported activities of individual DEBS modules that accept both *syn* configurations albeit with a preference for (2*S*,3*R*)-**3**.[6] Without enzymatic release, the observed k_{cat} value cannot be directly compared to DEBS values; however, the relative k_{cat} observed for PikAIII is still insightful. Cane and co-workers have recently reported the activity of a PikAIII-TE hybrid and observed nearly identical results with a k_{cat} 4-fold greater than our value.[37] Last, PikAIII did not accept *anti*-diketides (2*S*,3*S*)-**5** and (2*R*,3*R*)-**6** as similarly reported for the DEBS modules.[6,36]

To extend this work further, the channeling of a non-natural substrate and protein−protein interactions between PikAIII and

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(23) Omura, K.; Swern, D. *Tetrahedron* **1978**, *34*, 1651−1660.
(24) Oxidation to β-keto-δ-lactones is reported to be problematic; however, modified Swern conditions employing TFAA/DMSO have been successfully employed. See: Sakai, N.; Ohfune, Y. *J. Am. Chem. Soc.* **1992**, *114*, 998−1010.
(25) A single diastereomer was observed; however, the stereochemistry depicted for α-chloroketone **21** has not been rigorously confirmed. An NOE enhancement was not observed between the C-2 methyl group and H-4 which suggests that it should reside in the equatorial position indicated.
(26) When 1 equiv of oxalyl chloride was employed in the Swern oxidation, the reaction went to approximately 50% conversion to α-chloroketone **21**, indicating that chlorination of the initially formed keto-product proceeds faster than oxidation of the starting alcohol.
(27) Interestingly, the reported oxidation by Cane and co-workers[21] of triketide lactone **1** under Swern conditions afforded a pyrone product (30%), along with recovered starting material (40%) and unidentified material (30%). We found that α-chloroketones **21** and **23** were unstable to silica gel chromatography which may explain the discrepancy in the reported results.
(28) For an alternative synthesis of β-keto-δ-lactones **11** and **12**, see refs 12 and 21.
(29) Interestingly, in chloroform, the keto-tautomer of **11** exists as a 16:1 mixture of 2*R*:2*S* epimers. Gas phase calculations at the AM1 level indicate that both epimeric keto-lactones exist in a twist-boat conformation and are approximately equienergetic. GC−MS analysis in fact reveals two peaks at m/z= 168 $(M-2H)^+$ in a ratio of 58:42 in support of the semiempirical calculations; thus, the epimeric ratio in chloroform reflects a difference in solvation.
(30) See Supporting Information.
(31) Brandänge, S.; Leijonmarck, H. *Tetrahedron Lett.* **1992**, *33*, 3025−3028.
(32) Vanderwal, C. D.; Vosburg, D. A.; Weiler, S.; Sorenson, E. *J. Org. Lett.* **1999**, *1*, 645−648.
(33) Hinterding, K.; Singhanat, S.; Oberer, L. *Tetrahedron Lett.* **2001**, *42*, 8643−8645.
(34) Sorenson and co-workers[32] have demonstrated the importance of the potassium counterion in this reaction as lithium and sodium enolates reportedly[31] afforded an 11-membered ring due to a competing endo-attack on the oxazolidinone carbonyl.
(35) Kao, C. M.; Luo, G.; Katz, L.; Cane, D. E.; Khosla, C. *J. Am. Chem. Soc.* **1996**, *118*, 9184−9185.
(36) During the review of this manuscript, a complementary report by Cane and co-workers[37] reported a modest preference for the (2*S*,3*R*)-**3** NAC diketide by PikAIV and the same diastereoselectivity with PikAIII modified with a thioesterase from the pikromycin cluster.
(37) Yin, Y.; Lu, H.; Khosla, C.; Cane, D. E. *J. Am. Chem. Soc.* **2003**, *125*, 5671−5676.

PikAIV were investigated using the (2*S*,3*R*)-**3** diketide substrate. When PikAIII and PikAIV were reacted together with (2*S*,3*R*)-**3**, a product mixture of TKL **7** and **11** and tetraketide **15** was observed by radio-TLC (Figure 2B). Previous studies involving channeling of triketide chain intermediates between DEBS monomodules with natural docking domains reportedly gave exclusive formation of tetraketide **15**.[4] Introduction of a linker mismatch into the DEBS modules resulted in premature release of the chain intermediate and formation of TKL **7**. Interestingly, the rate of TKL **7** synthesis by PikAIII was accelerated approximately 4-fold ($k_{cat} = 0.017$ min^{-1}) with the addition of PikAIV. Indeed, PikAIII with a covalently attached TE domain was recently reported to form TKL **7** at a comparable rate.[37] We note that the PikAIV dependent release of the triketide chain from PikAIII resembles the natural mechanism of 10-Dml formation; however, this consequence is an artifact of the non-natural substrate and the apparent substrate specificity of these modules. Significantly, we observed that PikAIV containing an inactivated (C207A) KS_6 domain did not show this rate enhancement, while PikAIV containing an inactivated (S1196A) TE domain retained activity for TKL **7** cyclization, indicating a role for KS_6 in lactonization (see Supporting Information for radio-TLC). In contrast, the TE domain is required for macrolactonization of the hexaketide leading to 10-Dml.[8] These results indicate that the KS_6 domain can apparently function as a thioesterase when provided with a triketide chain intermediate. Presumably, the oxy anion hole[38] that mediates the Claisen condensation also stabilizes the tetrahedral intermediate formed during "spontaneous" lactonization. Thus, despite the natural interaction of PikAIII and PikAIV, PikAIV does not permit complete elongation of the triketide chain channeled by PikAIII, suggesting a reduced tolerance for the triketide intermediate that is released as TKL **7**. This reduced tolerance and the apparent lactonization activity of KS_6 likely explain the synthesis of TKL **7** (but not tetraketide **15**) with the in vivo pairing of DEBS1 and PikAIV,[39] whereas the combination of DEBS1 and DEBS Module3+TE produced a greater amount of tetraketide **15** relative to TKL **7**.[40,41] The calculated dissociation constant for the PikAIII−PikAIV interaction of 100 nM is approximately 10-fold lower than the K_D reported for the interaction between DEBS modules indicating a more specific interaction inherent to Pik module 5 and module 6.[4]

Significance. The observation that Pik modules 5 and 6 show greater specificity with di- and triketide substrates compared to DEBS modules highlights important distinctions between these systems. Comparative analysis using authentic substrates with other PKS systems will determine whether the apparent stringency of the Pik PKS or the flexibility of DEBS is more typical of these multifunctional enzymes. Thus, the mechanisms that modular PKSs have evolved to control processing of unnatural chain intermediates represents a key consideration for full development of combinatorial biosynthesis for generating novel natural products.

Experimental Section

Protein Overexpression and Purification. The construction of expression plasmids for PikAIII and PikAIV has been previously reported.[12] Proteins were individually expressed in *Escherichia coli* BL21 (DE3) using plasmids pET28b and pET24b, respectively (Novagen). The PikAIII protein was engineered with an NH_2-6×HIS tag, and PikAIV was modified to contain a COOH-6×HIS tag. Both proteins were coexpressed with the *Bacillus subtilis sfp* gene encoding a phosphopantetheine transferase for post-translational modification of the ACP domains.[42] Overexpression cultures were grown at 37 °C to an $A_{600} = 0.5-0.6$, equilibrated to 22−25 °C for 20 min, then induced using 0.1 mM of isopropyl-1-thio-D-galactopyranoside (IPTG), and incubated overnight. Cells were harvested by centrifugation at 3000 × *g* and suspended in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 150 mM NaCl, 10 mM imidazole, 1 mM DTT, 10% glycerol). After disruption of the cells using a French press, the clarified lysate was loaded onto Ni-NTA resin (Qiagen) previously equilibrated with lysis buffer. Both PikAIII and PikAIV were eluted from the Ni-NTA resin in lysis buffer containing 250 mM imidazole. Fractions containing the eluted protein were pooled, and a PD-10 column (Pharmacia) was used to exchange to storage buffer (100 mM NaH_2PO_4, pH 7.2, 1 mM EDTA, 1 mM DTT, 20% glycerol). The purity of each preparation was determined to be >90% by SDS-PAGE.

In Vitro Polyketide Production. The reactivity of PikAIII and PikAIV with NAC diketides **3**−**6** was determined by measuring the incorporation of 2-[^{14}C]-methylmalonyl-CoA into the resulting tri- and tetraketide lactone products. 2-[^{14}C]-Methylmalonyl-CoA (55 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc., and all other chemicals were purchased from Sigma. Enzymatic reactions were run in 400 mM NaH_2PO_4 buffer (pH 7.2) containing 5 mM NaCl, 1 mM EDTA, 1 mM DTT, 800 μM NADPH (for reactions with PikAIII), 20% glycerol, and 5% DMSO in a final volume of 100 μL. In reactions designed to determine the individual reactivity of each protein with each diketide diastereomer, protein was added to a concentration of 2 μM, and diketide concentrations ranged from 1 to 25 mM. For experiments investigating the interaction between PikAIII and PikAIV, assays and product detection were performed as described above with 2 μM PikAIII, 0.06−2 μM PikAIV, and 12 mM (2*S*,3*R*)-**3**. 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 = 1.35 mCi/mmol) for a final saturating concentration of 435 μM. The reaction was maintained at 30 °C for 1.5 h and then quenched, and the products were extracted with EtOAc (2 × 450 μL). The extracts were dried under a stream of N_2, and the products were resolved on silica gel TLC plates using 60% EtOAc/hexanes as the solvent; polyketide products were identified using authentic reference compounds. Separation of reaction extracts by TLC allowed visualization of radiolabeled products by autoradiography. Product formation was quantified using ImageQuant (Molecular Dynamics) using a standard curve generated with 2-[^{14}C]-methylmalonyl-CoA, and apparent kinetic values were determined using GraphPad Prism software.

Synthesis of Substrates and Reference Compounds. The diketide NAC esters **3**−**6** were synthesized essentially as described except for two minor modifications[43] and determined to have a de > 99% by HPLC.[13] See Supporting Information for general procedures.

(3*R*,4*S*,5*S*,6*R*)-6-Ethyl-4-hydroxy-3,5-dimethyl-tetrahydro-pyran-2-one (7). A solution of triketide **20**[17] (299 mg, 0.857 mmol) in THF:1

(38) Huang, W.; Jia, J.; Edwards, P.; Dehesh, K.; Schneider, G.; Lindqvist, Y. *EMBO J* **1998**, *17*, 1183−1191.
(39) Kim, B. S.; Cropp, T. A.; Florova, G.; Lindsay, Y.; Sherman, D. H.; Reynolds, K. A. *Biochemistry* **2002**, *41*, 10827−10833.
(40) Pieper, R.; Gokhale, R. S.; Luo, G.; Cane, D. E.; Khosla, C. *Biochemistry* **1997**, *36*, 1846−1851.
(41) Gokhale, R. S.; Hunziker, D.; Cane, D. E.; Khosla, C. *Chem. Biol.* **1999**, *6*, 117−125.
(42) Lambalot, R. H.; Gehring, A. M.; Flugel, R. S.; Zuber, P.; LaCelle, M.; Marahiel, M. A.; Reid, R.; Khosla, C.; Walsh, C. T. *Chem. Biol.* **1996**, *3*, 923−936.
(43) The original procedure of Evans was employed for the LiOOH-mediated hydrolysis of the oxazolidinone auxiliary. See: Evans, D. A.; Britton, T. C.; Ellman, J. A. *Tetrahedron Lett.* **1987**, *28*, 6141−6144. In the subsequent coupling reaction with *N*-acetylcysteamine, DCC was replaced by EDC and the urea byproduct was removed with an aqueous 1 N HCl wash. Also, the reported DCC coupling reaction was allowed to stir for 24−72 h; however, the reaction was complete in less than 1 h at 0 °C.

N aq HCl (4:1, 50 mL) was heated at 60 °C for 40 h. The reaction was cooled to room temperature, and the THF was removed under reduced pressure. The resulting mixture was partitioned between EtOAc and H_2O. The organic layer was separated, and the aqueous layer was extracted with EtOAc (2×). The combined organic extracts were washed with saturated aq NaCl, dried (Na_2SO_4), filtered, and concentrated under reduced pressure to an oil. Chromatography on silica gel (20 g) with 30% EtOAc/hexanes (300 mL) to 50% EtOAc/hexanes (150 mL) afforded 147 mg (0.857 mmol, 100% yield) of a colorless oil. Additionally, 150 mg (99%) of the oxazolidinone auxiliary was recovered. Lastly, 34 mg of 4-chlorobutanol was isolated as a result of decomposition of THF under the reaction conditions. mp 37−41 °C; TLC R_f = 0.45 (40% EtOAc/hexanes); $[\alpha]^{25}_D$ +122 (c = 1.29, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.95 (ovlp d, 3H, J = 6.9 Hz), 0.99 (ovlp t, 3H, J = 7.5 Hz), 1.39 (d, 3H, J = 7.5 Hz), 1.57 (dp, 1H, J = 13.2, 7.8 Hz), 1.82 (dp, 1H, J = 13.2, 7.8 Hz), 2.0 (br s, 1H), 2.12−2.20 (m, 1H), 2.46 (dq, 1H, J = 10.5, 7.2 Hz), 3.81 (dd, 1H, J = 10.2, 3.9 Hz), 4.12 (ddd, 1H, J = 8.7, 6.6, 2.7 Hz); ^{13}C NMR ($CDCl_3$) δ 4.5, 10.0, 14.4, 25.3, 36.7, 39.8, 73.9, 81.3, 173.5; IR (film) 3440 (w, br), 2972 (m), 2941 (w), 2883 (w), 1711 (s), 1458 (m), 1360 (m), 1217 (m), 1111 (m), 980 (m) cm^{-1}; HRMS (EI) m/z calcd for $C_9H_{16}O_3$ (M^+), 172.1099; found, 172.1094.

(3*R*,5*S*,6*R*)-3-Chloro-6-ethyl-3,5-dimethyl-dihydro-pyran-2,4-dione (21). To a solution of oxalyl chloride (102 μL, 1.19 mmol, 5.0 equiv) in CH_2Cl_2 (2 mL) at −78 °C was added dropwise a solution of DMSO (127 μL, 1.79 mmol, 7.5 equiv) in CH_2Cl_2 (0.5 mL), and the resulting solution was stirred for 15 min. A solution of alcohol **7** (40.7 mg, 0.238 mmol, 1.0 equiv) in CH_2Cl_2 (1 mL + 1 mL wash) was added dropwise, and the resulting solution was stirred for 30 min. Et_3N (332 μL, 2.38 mmol, 10.0 equiv) was added to the cloudy solution to afford a clear solution which was stirred for 1 h at −78 °C and warmed to 0 °C over another hour to afford a milky white heterogeneous mixture. The reaction was partitioned between Et_2O and 0.1 N aq HCl. The organic layer was separated and washed successively with H_2O and saturated aq NaCl, dried (Na_2SO_4), filtered, and concentrated under reduced pressure to 46 mg (0.225 mmol, 95% yield) of a colorless oil. The crude ^{1}H NMR spectrum indicates a purity greater than 80%. The product decomposes on silica gel; however, rapid flash chromatography on silica gel (2 g) with 50% EtOAc/hexanes afforded 12 mg (0.0588 mmol, 25% yield) of a colorless oil. TLC R_f = 0.54 (20% EtOAc/hexanes); $[\alpha]^{25}_D$ +46.0 (c = 0.135, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 1.08 (t, 3H, J = 7.8 Hz), 1.15 (d, 3H, J = 6.9 Hz), 1.60−1.80 (m, 2H), 1.85 (s, 3H), 3.01 (qd, 1H, J = 7.2, 3.3 Hz), 5.01 (ddd, 1H, J = 8.4, 5.1, 3.0 Hz); ^{13}C NMR ($CDCl_3$) δ 9.9, 10.0, 21.6, 24.5, 43.1, 59.0, 78.4 167.1, 199.9; IR (film) 2975 (w), 2929 (w), 2882 (w), 2854 (w), 1763 (s), 1732 (s), 1457 (w), 1377 (w), 1278 (m), 1126 (m), 1107 (m), 991 (w), 971 (w) cm^{-1}; HRMS (EI) m/z calcd for $C_9H_{13}{}^{35}ClO_3$ (M^+), 204.0553; found, 204.0555.

(5*S*,6*R*)-6-Ethyl-4-hydroxy-3,5-dimethyl-5,6-dihydro-pyran-2-one (11). To a solution of oxalyl chloride (66 μL, 0.77 mmol, 5.0 equiv) in CH_2Cl_2 (1 mL) at −78 °C was added dropwise a solution of DMSO (82 μL, 1.16 mmol, 7.5 equiv) in CH_2Cl_2 (0.5 mL), and the resulting solution was stirred for 15 min. A solution of alcohol **7** (26.5 mg, 0.154 mmol, 1.0 equiv) in CH_2Cl_2 (0.5 mL + 0.5 mL wash) was added dropwise, and the resulting solution was stirred for 30 min. Et_3N (215 μL, 1.54 mmol, 10.0 equiv) was added, and the reaction was stirred for 15 min at −78 °C and then slowly warmed to −20 °C over another hour to afford a milky white heterogeneous mixture. Glacial AcOH (176 μL, 3.08 mmol, 20 equiv) and Zn dust (206 mg, 3.08 mmol, 20 equiv) were added to the heterogeneous reaction mixture, and the mixture warmed to 0 °C over 10 min. The reaction was partitioned between CH_2Cl_2 and 0.1 N aq HCl. The organic layer was separated and washed successively with H_2O, saturated aq $NaHCO_3$, and saturated aq NaCl, dried (Na_2SO_4), filtered, and concentrated under reduced pressure to an oil. Chromatography on silica gel (2 g) with 50% Et_2O/pentane afforded 30.7 mg (0.180 mmol, 117% yield) of a white solid. The product was further purified by recrystallization by dissolving the product in a minimum of hot CH_2Cl_2, followed by careful addition of a layer of pentane (approximately 5 times the volume of CH_2Cl_2) on top of the CH_2Cl_2 layer. The product recrystallized from the resulting two-layer solution at −10 °C overnight to afford 16 mg (0.0941 mmol, 60% yield) of fine white needles that had a pleasant brown sugar-molasses odor. mp 110−113 °C; TLC R_f = 0.36 (50% EtOAc/hexanes); $[\alpha]^{25}_D$ −57.0 (c = 3.05, MeOH); ^{1}H NMR ($CDCl_3$) 25:1:8 mixture of 9*R*-keto:9*S*-keto:enol isomers δ 0.98 (t, 1H, J = 7.5 Hz, enol CH_3), 1.07 (t, 3H, J = 7.8 Hz, keto CH_3), 1.11 (d, 3H, J = 7 Hz, keto CH_3), 1.35 (d, 3H, J = 6.9 Hz, keto CH_3), 1.58−1.72 (m, 1.33H, keto + enol CH_2), 1.78 (s, 1H, enol HO−C=C−CH_3), 1.78−1.94 (m, 1.33H, keto + enol CH_2), 2.38−2.48 (m, 0.3H, enol C=C−*H*), 2.62 (qd, 1H, J = 7.5, 2.7 Hz, keto CH), 4.22 (ddd, 0.3H, J = 9.6, 6, 3 Hz, enol C*H*OC=O), 4.65 (ddd, 1H, J = 8.1, 5.1, 3.0 Hz, keto C*H*OC=O); ^{13}C NMR ($CDCl_3$) δ 8.3, 9.9, 10.0, 24.1, 44.4, 50.4, 78.5, 169.9, 205.2; ^{1}H NMR (CD_3OD) δ 1.01 (t, 3H, J = 7.5 Hz), 1.08 (d, 3H, J = 6.9 Hz), 1.52−1.64 (m, 1H), 1.70 (s, 3H), 1.68−1.81 (m, 1H), 2.38 (qd, 1H, J = 6.9, 3.3 Hz), 4.23 (ddd, 1H, J = 8.7, 5.7, 3.0 Hz); ^{13}C NMR (CD_3OD) δ 8.9, 10.2, 10.9, 25.3, 37.5, 80.7, 98.0, 172.3, 173.1; IR (film) 3116 (m, br), 2975 (m), 2941 (m), 2883 (m), 2689 (w, br), 1652 (s), 1539 (w), 1456 (m), 1393 (s), 1369 (s), 1354 (s), 1128 (s), 987 (m), 768 (m) cm^{-1}; HRMS (EI) m/z calcd for $C_9H_{14}O_3$ (M^+), 170.0943; found, 170.0952.

4-(*R*)-Benzyl-3-[(2*R*,3*S*,4*S*,5*R*)-5-(*tert*-butyldimethylsilanyloxy)-3-propionyloxy-2,4-dimethylheptanoyl]-oxazolidin-2-one (22). To a solution of triketide **20** (206 mg, 0.59 mmol, 1.0 equiv) and 2,6-lutidene (77 μL, 0.66 mmol, 1.1 equiv) in CH_2Cl_2 (5 mL) at 0 °C was added TBSOTf (152 μL, 0.66 mmol, 1.1 equiv), and the reaction was stirred for 5 min. The reaction mixture was diluted with CH_2Cl_2 and washed successively with 1 N aq HCl, H_2O, saturated aq $NaHCO_3$, and saturated aq NaCl, dried (Na_2SO_4), filtered, and concentrated under reduced pressure to afford 278 mg (102% yield) of a yellow oil.[17] To a solution of the yellow oil in CH_2Cl_2 (5 mL) was added propionic anhydride (304 mL, 2.36 mmol, 4.0 equiv), Et_3N (249 mL, 2.36 mmol, 4.0 equiv), and 4-DMAP (15 mg, 0.12 mmol, 0.2 equiv), and the solution was refluxed for 7 h until complete by TLC. The reaction was cooled to room temperature, diluted with EtOAc, and washed consecutively with 1 N aq HCl, H_2O, saturated aq $NaHCO_3$, and saturated aq NaCl, dried (Na_2SO_4), filtered, and concentrated under reduced pressure to an oil. Chromatography on silica gel (25 g) with 10% EtOAc/hexanes afforded 300 mg (0.58 mmol, 97% yield) of a colorless oil. TLC R_f = 0.60 (20% EtOAc/hexanes); $[\alpha]^{25}_D$ −75.2 (c = 1.10, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.00 (s, 3H), 0.04 (s, 3H), 0.88 (t, 3H, J = 7.5 Hz), 0.92 (s, 9H), 0.98 (d, 3H, J = 6.9 Hz), 1.19 (t, 3H, J = 7.5 Hz), 1.21 (d, 3H, J = 7.2 Hz), 1.57 (p, 2H, J = 7.5 Hz), 1.85−1.90 (m, 1H), 2.37 (ddd, 2H, J = 15.3, 7.5, 2.7 Hz), 2.82 (dd, 1H, J = 13.2, 10.2 Hz), 3.33 (dd, 1H, J = 13.2, 3.3 Hz), 3.57 (td, 1H, J = 7.8, 1.2 Hz), 4.16 (dd, 1H, J = 8.4, 1.8 Hz), 4.24 (ddd, 1H, J = 13.8, 7.2, 1.8 Hz), 4.32 (t, 1H, J = 7.8 Hz), 4.48 (dddd, 1H, J = 10.2, 7.8, 3.3, 1.8 Hz), 5.21 (dd, 1H, J = 10.5, 2.1 Hz), 7.25−7.40 (m, 5H); ^{13}C NMR ($CDCl_3$) δ −5.2, −3.5, 7.9, 8.2, 9.5, 10.4, 18.2, 26.0, 27.7, 28.2, 37.4, 38.0, 39.1, 56.5, 66.4, 72.4, 74.1, 127.0, 128.7, 129.3, 135.6, 153.6, 173.9, 174.7; IR (neat) 2946 (m), 2883 (w), 2857 (w), 1783 (s), 1734 (m), 1701 (m), 1463 (w), 1381 (m), 1245 (m), 1211 (m), 1104 (m), 1045 (m), 836 (m) cm^{-1}; HRMS (EI) m/z calcd for $C_{28}H_{45}NO_6Si$ (M^+), 519.3016; found, 519.2998.

(5*R*,6*S*)-6-[(1*S*,2*R*)-2-(*tert*-Butyldimethylsilanyloxy)-1-methyl-butyl]-4-hydroxy-3,5-dimethyl-5,6-dihydro-pyran-2-one (23). A solution of propionate **22** in THF (5 mL) at −78 °C was treated with a freshly prepared solution of KHMDS (3.5 mL, 0.35 M in THF, 1.23 mmol, 4.0 equiv) and stirred for 2 h. The reaction was quenched with a saturated aq NH_4Cl/MeOH/H_2O (1:1:1, 30 mL) mixture at −78 °C and warmed to 0 °C. The reaction mixture was partitioned between EtOAc and H_2O. The aqueous layer was separated and acidified to pH 2 with 1 N aq HCl and extracted with EtOAc (3×). The combined

organic layers were washed with saturated aq NaCl, dried (Na_2SO_4), filtered, and concentrated under reduced pressure to a white solid. Chromatography on silica gel (15 g) with 10% EtOAc/hexanes (100 mL) to 15% EtOAc/hexanes (200 mL) afforded 68 mg of a white solid. The product was further purified by recrystallization from MeOH to afford 61 mg (0.17 mmol, 58% yield) of fine white needles. TLC R_f = 0.25 (20% EtOAc/hexanes); $[\alpha]^{25}_D$ −14.8 (c = 0.894, CH_2Cl_2); ^{1}H NMR (CD_3OD) δ 0.0 (s, 3H), 0.09 (s, 3H), 0.83 (d, 3H, J = 7.2 Hz), 0.87 (s, 9H), 0.88 (t, 3H, J = 7.5 Hz), 1.10 (d, 3H, J = 6.9 Hz), 1.50−1.63 (m, 2H), 1.70 (s, 3H), 1.88 (dqd, 1H, J = 10.5, 6.9, 1.5 Hz), 2.44 (qd, 1H, J = 6.9, 3.0 Hz), 4.14 (ovlp ddd, 1H, J = 8.7, 6.3, 1.8 Hz), 4.18 (dd, 1H, J = 10.8, 2.7 Hz); ^{13}C NMR (CD_3OD) δ −4.4, −3.9, 7.3, 8.7, 10.5, 10.6, 19.0, 26.5, 29.0, 36.0, 37.2, 67.9, 79.1, 98.0, 171.8, 174.2; IR (film) 2957 (s), 2930 (s), 2884 (m), 2857 (m), 1783 (m), 1733 (s), 1653 (m), 1461 (m), 1388 (s), 1257 (s), 1128 (s), 1027 (m), 835 (s), 775 (s) cm^{-1}; HRMS (EI) m/z calcd for $C_{18}H_{34}O_4Si$ (M^+), 342.2226; found, 342.2221.

(5*R*,6*S*)-4-Hydroxy-6-[(1*S*,2*R*)-2-hydroxy-1-methyl-butyl]-3,5-dimethyl-5,6-dihydro-pyran-2-one (15). Protected tetraketide **23** (23.5 mg, 0.069 mmol) was stirred with Dowex 50WX2-H^+ resin (100 mg) in THF:H_2O (1:1, 2 mL) at 50 °C for 20 h. The Dowex 50WX2 resin was prepared as follows: the resin was treated with 6 N aq HCl, washed with H_2O until the filtrate was pH 5.0, and then air-dried for 10 min. The reaction mixture was filtered through a plug of cotton and concentrated under reduced pressure to 15.7 mg (0.069 mmol, 100% yield) of a white solid. The product is sparingly soluble in $CDCl_3$ and moderately soluble in MeOH. TLC R_f = 0.23 (40% EtOAc/hexanes); $[\alpha]^{25}_D$ 68 (c = 0.66, MeOH); ^{1}H NMR ($CDCl_3$) exists as a 5.5:1 mixture of keto:enol tautomers. Data for keto tautomer δ 0.86 (d, 3H, J = 6.9 Hz), 1.00, (t, 3H, J = 7.5 Hz), 1.13 (d, 3H, J = 7.2 Hz), 1.34 (d, 3H, J = 6.6 Hz), 1.39−1.68 (m, 3H, C-8 CH_2 + OH), 1.91 (ddq, 1H, J = 10.3, 6.9, 1.8), 2.66 (qd, 1H, J = 7.5, 2.7 Hz), 3.70 (q, 1H, J = 6.6 Hz), 4.07 (ddd, 1H, J = 6.6, 4.8, 1.8 Hz), 4.84 (dd, 1H, J = 10.2, 2.1 Hz); Identifiable peaks of the enol tautomer δ 1.80 (s, 3H, H-12), 2.38−2.46 (m, 1H, H-4), 4.34 (dd, 1H, J = 10.5, 3.3 Hz, H-5), 4.62 (ddd, 1H, J = 9.6, 6.0, 3.0 Hz, H-7); ^{1}H NMR (CD_3OD) δ 0.82 (d, 3H, J = 6.9 Hz), 0.98 (t, 3H, J = 7.3 Hz), 1.09 (d, 3H, J = 7.2 Hz); 1.34−1.48 (m, 1H), 1.50−1.64 (m, 1H), 1.70 (s, 3H), 1.80 (dqd, 1H, J = 10.5, 6.9, 1.8 Hz), 2.42 (qd, 1H, J = 6.9, 3.0 Hz), 3.98 (ddd, 1H, J = 8.7, 4.8, 1.8 Hz), 4.26 (dd, 1H, J = 10.8, 3.0 Hz); ^{13}C NMR (CD_3OD) δ 7.8, 8.7, 10.5, 11.4, 28.6, 35.9, 39.0, 71.1, 79.4, 98.0, 172.1, 174.1; IR (film) 3178 (w, br), 2973 (m), 2938 (m), 2882 (w), 2690 (w, br), 1654 (s, br), 1456 (w), 1390 (m), 1368 (m), 1312 (w), 1123 (m), 993 (m), 966 (w), 766 (w) cm^{-1}; HRMS (EI) m/z calcd for $C_{12}H_{20}O_4$ (M^+), 228.1362; found, 228.1355.

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Supporting Information Available: Normalized v vesus [S] plots for the reactions of (2*S*,3*R*)-diketide **3** with PikAIII and PikAIV as well as (2*R*,3*S*)-diketide **4** with PikAIV. GC−MS trace and conditions for compound **7** isolated from in vitro reaction of (2*S*,3*R*)-diketide **3** with PikAIII. LC−MS traces and HPLC conditions of compounds **11** and **15** isolated from in vitro reactions with PikAIII + PikAIV. ^{1}H NMR and ^{13}C NMR spectra of all compounds reported in Schemes 1 and 2. Full experimental details and characterization for **8**, **12**, **16**, and intermediates required for their synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

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