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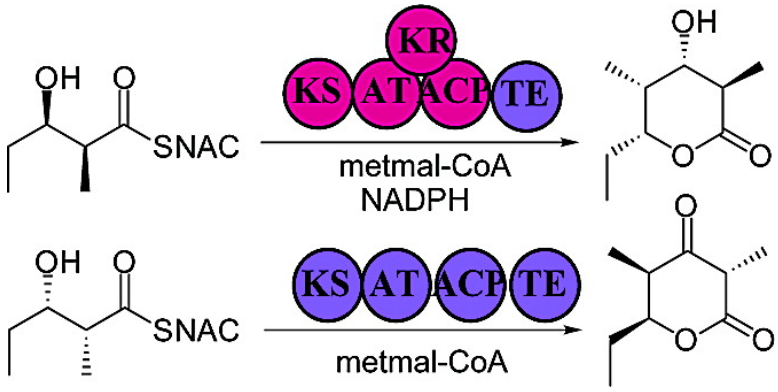

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Expression and Kinetic Analysis of the Substrate Specificity of Modules 5 and 6 of the Picromycin/Methymycin Polyketide Synthase

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Abstract: Picromycin synthase (PICS) is a multifunctional, modular polyketide synthase (PKS) that catalyzes the conversion of methylmalonyl-CoA to narbonolide and 10-deoxymethynolide, the macrolide aglycone precursors of the antibiotics picromycin and methymycin, respectively. PICS modules 5 and 6 were each expressed in *Escherichia coli* with a thioesterase domain at the C-terminus to allow release of polyketide products. The substrate specificity of PICS modules 5+TE and 6+TE was investigated using *N*-acetylcysteamine thioesters of 2-methyl-3-hydroxy-pentanoic acid as diketide analogues of the natural polyketide chain elongation substrates. PICS module 5+TE could catalyze the chain elongation of only the syn diketide (2*S*,3*R*)-**4**, while PICS module 6+TE processed both syn diastereomers, (2*S*,3*R*)-**4** and (2*R*,3*S*)-**5**, with a 2.5:1 preference in k_{cat}/K_m for **5** but did not turn over either of the two anti diketides. The observed substrate specificity patterns are in contrast to the 15−100:1 preference for **4** over **5** previously established for several modules of the closely related erythromycin PKS, 6-deoxyerythronolide B synthase (DEBS).

Complex polyketides, such as the macrolide antibiotics methymycin, erythromycin, picromycin, and tylosin, are derived from the simple 2- and 3-carbon building blocks acetyl-, propionyl-, and butyryl-CoA, activated as the corresponding malonyl-, methylmalonyl-, and ethylmalonyl-CoA derivatives and linked together by a series of reactions closely related to the well-understood chain-building reactions of fatty acid biosynthesis.[1] The more than 100 known macrolides differ from one another in the number and choice of building blocks, leading to 12-, 14-, and 16-membered ring lactones, and in the precise mix of fatty acid synthase-like reactions that are used at each stage of polyketide chain elongation. It is now well-established that the parent macrolide aglycones are assembled by large, polyfunctional proteins, known as polyketide synthases (PKSs) that are organized into groups of independently folded enzyme domains known as modules, each of which is responsible for a single round of polyketide chain elongation and functional group modification. Each module has a minimal core set of biochemical activities corresponding to an acylcarrier protein (ACP) domain on which the growing chain is assembled, an acyltransferase (AT) domain that primes the ACP domain with the appropriate malonyl- or methylmalonyl chain extender unit and a β-ketoacyl-ACP synthase (ketosynthase or KS) domain that accepts a preformed polyketide acyl chain from either a loading domain or an upstream donor module and catalyzes the actual polyketide chain extension by means of a decarboxylative condensation with the malonyl- or methylmalonyl-ACP (Figure 1). Modules may also carry additional activities such as a β-ketoacyl-ACP reductase (KR) domain, a dehydratase (DH) domain, and an enoylacyl-ACP reductase (ER) domain. At the C-terminus of the last module is a dedicated thioesterase (TE) domain which releases the mature polyketide chain by lactonization to generate the corresponding macrolide aglycone.

Among some 20 PKSs that have been sequenced to date, by far the most thoroughly studied has been the 6-deoxyerythronolide B synthase (DEBS) of *Saccharopolyspora erythraea*, which consists of 3 homodimeric megaproteins of two modules each that mediate the 6 rounds of polyketide chain elongation with release of the 14-membered macrolide aglycone 6-deoxyerythronolide B (**1**) (Figure 1A).[2−4] Closely related to DEBS is the picromycin/methymycin synthase (PICS) of *Streptomyces venezuelae*,[5] which has the unusual property of mediating the

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(1) For recent comprehensive reviews of modular PKS genetics and biochemistry, see: (a) Cane, D. E. (Guest Editor) *Chem. Rev.* **1997**, *97* (7). (b) Khosla, C.; Gokhale, R. S.; Jacobsen, J. R.; Cane, D. E. *Annu. Rev. Biochem.* **1999**, *68*, 219−253. (c) Staunton, J.; Wilkinson, B. In *Comprehensive Natural Products Chemistry, Polyketides and Other Secondary Metabolites Including Fatty Acids and Their Derivatives*; Sankawa, U., Ed.; Elsevier: Oxford, 1999; Vol. 1, pp 495−532. (d) Ikeda, H.; Omura, S. In *Macrolide Antibiotics. Chemistry, Biology, and Practice*; Omura, S., Ed.; Academic Press: San Diego, CA, 2002; pp 286−326.

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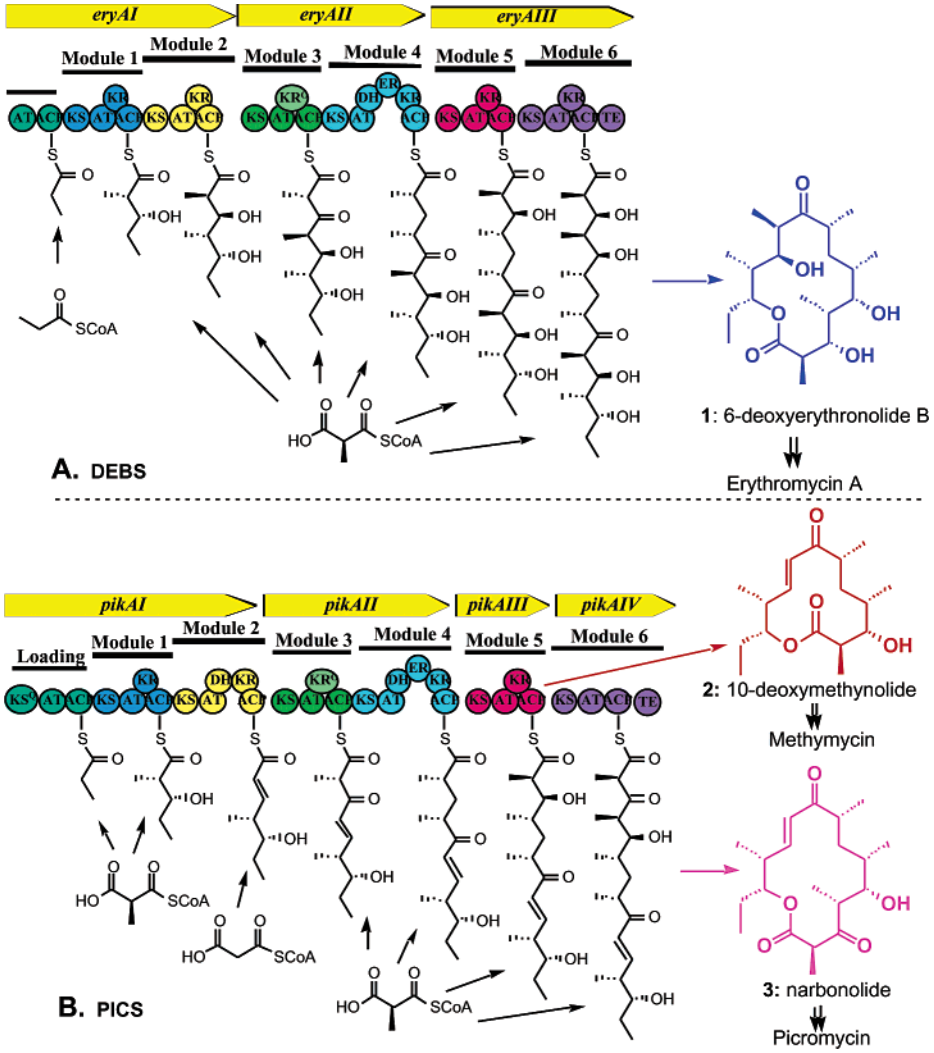

Figure 1. Modular organization and biochemical function of PKS gene clusters. (A) 6-Deoxyerythronolide B synthase (DEBS). (B) Picromycin/methymycin synthase (PICS).

formation of both the 12-membered ring macrolide aglycone 10-deoxymethynolide (**2**),[6] the precursor of methymycin,[7] and the 14-membered ring narbonolide (**3**), the precursor of picromycin (Figure 1B).[7b,8] The precise proportions of methymycin and picromycin are responsive to fermentation conditions.[7a,8] Intriguingly, Sherman has shown that formation of the 12-membered macrolactone **2** is a consequence of the expression of an N-terminal truncated form of PICS module 6, which has a defective KS domain but whose thioesterase (TE) domain catalyzes the lactonization of the hexaketide produced by PICS module 5.[9] Sherman and his collaborators have also provided evidence that generation of **2** involves a domain-skipping mechanism, in which the acylic hexaketide produced by PICS module 5 must first be transferred to the ACP of module 6 via the truncated KS6 domain, prior to macrolactonization mediated by the TE located at the C-terminus of PICS module 6.[10]

The fidelity and efficiency of intermodular transfer of growing polyketide chains have been shown to be subject to three levels of molecular recognition: (1) protein−protein recognition involving preferential interaction of cognate ACP_n−KS_{n+1} pairs in which the ACP of the upstream donor module interacts with the KS domain of the appropriate downstream acceptor module;[11,12] (2) specific linker−linker recognition in which the C-terminal peptide region of the donor module pairs with a short N-terminal peptide of the acceptor module by an apparent coiled-coil interaction;[12] and (3) the intrinsic substrate specificity of the acceptor KS domain for the structure and stereochemistry of the incoming polyketide.[13]

To address the issue of the intrinsic substrate specificity and tolerance of individual modules, we have been examining the

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ability of PKS modules to recognize and process a wide variety of model diketides and triketides. Our experiments to date, which have largely focused on the well-characterized DEBS system, have established that individual DEBS modules have a remarkable tolerance for variations in substrate chain length and substitution but frequently exhibit strict stereochemical preferences.[14] For example, DEBS module 2, fused at the C-terminus to the DEBS TE (DEBS module 2+TE), showed an ~100:1 preference in relative k_{cat}/K_m for the syn (2*S*,3*R*)-diketide **4** over the diastereomeric syn (2*R*,3*S*)-**5** while strictly discriminating against either of the anti diastereomers **6** and **7**. Similar substrate specificity patterns were shown by DEBS module 3+TE, module 5+TE, and module 6+TE, with **4** being favored over **5** by factors of ~15−30:1. Thus, while the *natural* polyketide elongation substrates of the various DEBS modules may differ in structure and stereochemistry, all four of the modules examined have comparable stereochemical and structural preferences.

To understand better the programming of modular PKSs, it is essential to extend such studies beyond the prototype DEBS PKS. While DEBS has served as a conceptual and mechanistic model for the organization and function of modular polyketide synthases in general, there have been no prior reports of the in vitro investigation of other intact PKS modules. PICS modules 5 and 6 each catalyze polyketide chain elongation steps in the biosynthesis of the 14-membered macrolide aglycone narbonolide (**3**) that are analogous to those mediated by DEBS modules 5 and 6, respectively, in the formation of 6-deoxyerythronolide B (**1**) (Figure 1). One significant organizational difference between the two sets of PKS modules, however, is that DEBS modules 5 and 6 are covalently linked in a single, bimodular PKS subunit, DEBS3, encoded by *eryAIII*,[2,3] while the analogous PICS modules 5 and 6 function as independent proteins encoded by distinct open reading frames *pikAIII* and *pikAIV*, respectively.[5] While PICS module 5 carries out a set of biochemical reactions completely analogous to those mediated by DEBS module 5, the biochemical functions of PICS module 6 differ from those of the corresponding DEBS module 6: PICS module 6, which generates an unreduced 3-ketoacylthioester product lacks a KR domain such as that present in DEBS module 6 which normally generates a 3-hydroxyacylthioester product. In this respect, PICS module 6 also differs from modules 3 of both PICS and DEBS, each of which also generates a 3-ketoacylthioester product but contains a functionally inactive KR domain, as distinguished from no KR domain at all.

In extending our inquiries to an investigation of the substrate specificity and tolerance of individual PICS modules, we have focused on two central questions: (1) Do individual PICS modules exhibit substrate preferences analogous to those of the corresponding DEBS modules? (2) Do differing PICS modules show similar substrate preferences to one another, as has been observed for DEBS modules, or does the substrate preference vary from one PICS module to another? The answers to these questions have important implications, not only for the understanding of PKS programming but for attempts to engineer the production of novel polyketides by using PKS modules in new combinations.

Results

Construction of the expression vector for PICS module 6+TE was facilitated by the fact that this single module naturally carries the PICS thioesterase domain at its C-terminus, just downstream of ACP6. PCR was used to amplify the entire PICS module 6+TE coding region, using as a template plasmid pKOS039-86 harboring the complete PICS gene cluster,[5b] and the amplified DNA was inserted after appropriate subcloning into the T7-based expression vector pET21 to give plasmid pHL5-07. Engineering of the expression vector for PICS module 5+TE required the fusion of DNA segments corresponding to PICS module 5 (*pikAIII*) as well as the PICS TE domain from the 3′-end of module 6 (*pikAIV*). We have previously reported the heterologous expression, substrate specificity, and protein structure of the PICS TE domain.[15] Module 5 was first obtained from pKOS039-86 using PCR primers to amplify bp 1-4434 of *pikAIII* and the amplified DNA introduced into pET-21 to give plasmid pHL5-20. Although the intrinsic N-terminal boundary of the PICS TE domain in the ~100 amino acid region linking the consensus C-terminus of PICS ACP6 and the consensus N-terminus of the TE domain is not known, previous studies carried out in our laboratory had established that PICS thioesterase of apparently optimum activity could be obtained by retaining an upstream linker peptide, starting 25 amino acids downstream of the ACP6 C-terminus.[15] Accordingly, the PICS TE domain was PCR amplified from bp 3145-4038 of *pikAIV*, and the resultant DNA was ligated into pHL5-20 downstream of the module 5 insert to give plasmid pHL6-76 harboring the gene encoding PICS module 5+TE.

Heterologous expression in *E. coli* of functional PKS modules requires posttranslational modification of the apo-ACP domain by addition of the 4′-phosphopantetheinyl side chain under control of the *sfp* gene product.[11,12,16] Accordingly, plasmids pHL6-76 and pHL5-07 were used in separate experiments to transform *E. coli* BAP1, a derivative of the standard *E. coli* expression host BL21(DE3) that has been modified to carry a chromosomally integrated copy of the *sfp* gene.[17] After induction of protein expression with 1 mM IPTG, cultures were allowed to incubate for a further 12 h at the reduced temperature of 20 °C so as to minimize the generation of insoluble inclusion bodies. After disruption of the cells by passage through a French press and removal of contaminating nucleic acids from the resulting lysate, successive anion exchange on Q-sepharose and hydrophobic interaction chromatography through phenyl sepharose gave PICS modules 5+TE and 6+TE, each of >95% purity, as judged by SDS-PAGE and densitometry, and in yields of 24 mg and 14 mg protein/L culture, respectively (Figure 2).

PKS modules normally acquire their physiological polyketide chain elongation substrate from the appropriate upstream donor module. We have shown that DEBS modules can also readily

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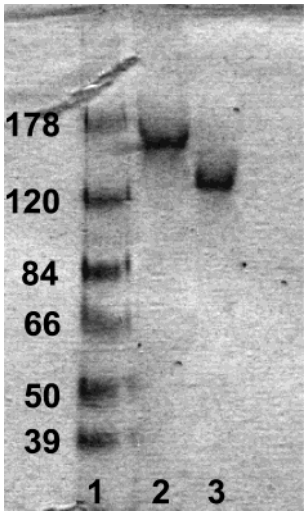
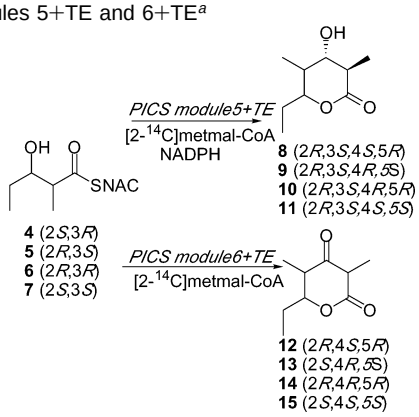


Figure 2. SDS-PAGE gel of purified PICS modules 5+TE and 6+TE. Lane 1, prestained protein molecular weight markers; Lane 2, PICS module 5+TE; Lane 3, PICS module 6+TE.

Scheme 1. Incubation of Diketide-SNAC Substrates **4**−**7** with PICS Modules 5+TE and 6+TE[a]



[a] Triketide lactones **9**−**11**, **14**, and **15** could not be detected by TLC phosphoimaging.

process *N*-acetylcysteamine (-SNAC) thioesters of simple analogues of the natural diketide substrate of DEBS module 2, (2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-SACP.[12,13] We therefore prepared the four diastereomeric SNAC esters **4**−**7**, corresponding to this natural diketide intermediate, and tested each as substrates for both PICS modules 5+TE and 6+TE (Scheme 1). Incubation of PICS module 5+TE with (2*S*,3*R*)-**4** in the presence of [2-^{14}C]methylmalonyl-CoA and NADPH gave the predicted triketide lactone product **8**, which was identical by comparison with an authentic sample. On the other hand, PICS module 5+TE failed to convert any of the three diketide diastereomers **5**, **6**, and **7** to the corresponding triketide lactones **9**−**11**.[18] Interestingly, PICS module 6+TE converted both syn diketide diastereomers, (2*S*,3*R*)-**4** and (2*R*,3*S*)-**5**, plus [2-^{14}C]-methylmalonyl-CoA to the corresponding enantiomeric triketide ketolactones **12** and **13**, respectively, but failed to catalyze elongation of either of the anti diastereomers, **6** and **7**[19] (Scheme 1, Figure 3).

To assess more quantitatively the relative substrate specificity of the two PICS modules, the steady-state kinetic parameters

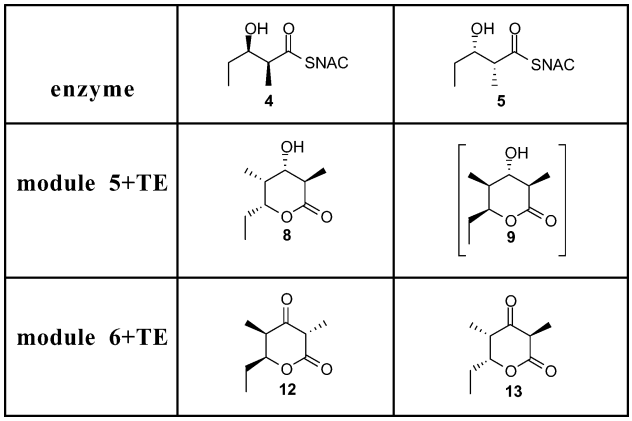


Figure 3. Formation of triketide lactones by incubation of diketide-SNAC substrates with PICS modules. Triketide lactone **9** was not detected.

Table 1. Steady-State Kinetic Parameters for the Formation of Triketide Lactones by PICS Modules 5+TE (mod5+TE) and 6+TE (mod6+TE)

substrate	module	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
(2*S*,3*R*)-**4**	mod5+TE	0.028 ± 0.001	7.3 ± 0.8	0.064
	mod6+TE	0.0038 ± 0.002	5.9 ± 0.9	0.011
(2*S*,3*R*)-**5**	mod5+TE	na[a]	na[a]	na[a]
	mod6+TE	0.057 ± 0.003	35 ± 4	0.027

[a] na = no activity detected.

were determined for each active substrate. In each case, the k_{cat} and K_m values were calculated by direct fitting of the plots of v versus [S] to the Michaelis−Menten equation. The data, including the calculated values of k_{cat}/K_m, are presented in Table 1. Based on comparison of k_{cat}/K_m values, PICS module 6+TE showed a modest 2.5:1 preference for the (2*R*,3*S*)-diketide-SNAC substrate **5** over the syn enantiomer (2*S*,3*R*)-**4**, while the latter was the only substrate turned over by PICS module 5+TE. Comparison of relative k_{cat} values for PICS module 6+TE revealed a more pronounced 15:1 preference for (2*R*,3*S*)-**5** over (2*S*,3*R*)-**4**, but this was offset by a 6-fold greater K_m for **5**. (2*S*,3*R*)-diketide **4** was turned over by PICS module 5+TE with a k_{cat} that was 7-fold greater than that of the same substrate with PICS module 6+TE and with essentially the same value of K_m.

Discussion

We have previously described the heterologous expression and kinetic analysis of several single DEBS modules.[11,12] We have now extended these studies to two structurally and biochemically closely related PKS subunits, modules 5 and 6 of the picromycin synthase (PICS). Previous experience with DEBS modules had shown that, in the absence of a downstream acceptor module to process the polyketide chain elongation product, a TE domain is required at the C-terminus in order to catalyze the release of the newly generated triketide product as either the triketide lactone or the corresponding triketide ketolactone. Expression vectors for both PICS modules 5+TE and 6+TE were engineered using native *pikAIII* and *pikAIV* as the source of DNA. Although PICS module 6 carries a natural

(18) None of the corresponding unreduced triketide ketolactones **13**−**15** were detected in these incubations, indicating that the discrimination against diketides **5**−**7** by PICS module 5+TE is due to the KS5 domain and not to the associated ketoreductase (KR5) domain.

(19) Due to facile enolization of the 3-ketolactones **12**−**15**, the methyl group at C-2 adopts the more stable equatorial configuration in each diastereomer. The (2*S*)-configuration in triketide ketolactones **13** and **15** thus does not reflect the intrinsic *D*-stereochemistry at this position generated by PICS module 6.

TE domain at its C-terminus, the PICS TE domain had to be ligated to the C-terminus of PICS module 5. In designing the PICS module 5+TE construct, we were guided by our earlier observation that retention at the N-terminus of the recombinant TE domain of ~75 amino acids of the natural ACP6-TE interdomain peptide resulted in maximum thioesterase activity.[15] Although both PICS modules 5+TE and 6+TE could each be expressed in satisfactory yield and activity using *E. coli* BL21-(DE3)-CodonPlus-RP cells harboring a separate plasmid expressing the *sfp* gene for the requisite acyl carrier protein synthetase activity (data not shown), we found it was more convenient to use *E. coli* BAP1. This engineered expression strain, derived from *E. coli* BL21(DE3), and which contains a chromosomally integrated copy of *sfp*, gave protein of comparable yield and activity without the need for additional antibiotic selection. Use of reduced temperature (20 °C) optimized the production of properly folded soluble protein while reducing the competing formation of insoluble inclusion bodies. Similar strategies have also been used for the heterologous expression of other high molecular weight (subunit MW >150 kDa) PKS[12] and nonribosomal peptide synthetase (NRPS) modules.[20]

Purification of both PICS modules 5+TE and 6+TE was readily achieved by a two-step sequence of anion exchange and hydrophobic interaction chromatography. Although the parent expression vector harboring each PICS module construct, pET21, appends an His6-Tag to the C-terminus of each module, we found that the purification efficiency of the Ni^{2+}-affinity columns was very low in both cases, at least for the Ni^{2+} resins examined (data not shown).

Despite the close similarity in the respective biochemical reactions that they catalyze, PICS modules 5 and 6 show marked differences in substrate preferences compared to the those of the corresponding DEBS modules 5 and 6.[14a] In comparing substrate preferences between individual modules within a single PKS or between different PKLS systems, the absolute values of k_{cat}/K_m, which provide a quantitative measure of enzyme specificity, are much less important than the *relative* values of this steady-state kinetic parameter. Direct comparison of k_{cat} values of individual substrates for modules derived from different PKS systems is of a more limited significance, due to inherent differences in PKS domain boundaries, protein sequence, and intrinsic biochemistry, as well as vector composition and expression conditions. Nonetheless, a number of simple generalizations emerge: (1) PICS module 5+TE processes only the syn (2*S*,3*R*)-diketide substrate **4**, in contrast to DEBS module 5+TE, which turns over both syn diketides, (2*S*,3*R*)-**4** and (2*R*,3*S*)-**5**, albeit with a significant 16:1 k_{cat}/K_m preference for **4**. The phosphoimaging method used for detection of triketide lactones in the PICS module 5+TE assays could easily have detected the turnover of (2*R*,3*S*)-**5** at a level 1−2% of that for (2*S*,3*R*)-**4**. Earlier experiments using the more nearly physiological diketide-ACP thioesters instead of the -SNAC analogues lowers the observed K_m values for the diketide diastereomers without significantly altering the *relative* k_{cat}/K_m specificity for pairs of substrates.[11] For this reason, the significant preference of PICS module 5+TE for the (2*S*,3*R*)-diketide diastereomer (**4** or its -ACP thioester equivalent) is unlikely to be reversed or even attenuated significantly even if PICS module 5+TE were able to process the corresponding ACP thioester of **5** at some measurable rate. (2) The maximum rate of turnover, k_{cat} 0.028 min^{-1}, of **4** by PICS module 5+TE is ~9-fold lower than that measured for the same substrate with DEBS module 5+TE. (3) PICS module 6+TE exhibits an ~2.5:1 k_{cat}/K_m preference for (2*R*,3*S*)-**5** over (2*S*,3*R*)-**4**, in striking contrast to DEBS module 6+TE, which exhibits a nearly 20:1 preference for (2*S*,3*R*)-**4** over (2*R*,3*S*)-**5**, corresponding to a net 50-fold reversal in substrate specificity. The differences in relative k_{cat} values are even more pronounced, with PICS module 6+TE displaying an ~15:1 preference for (2*R*,3*S*)-**5** over (2*S*,3*R*)-**4** in comparison to the opposite >7-fold preference previously determined for DEBS module 6+TE. (4) The k_{cat} of 0.057 min^{-1} for **5**, the preferred substrate of PICS module 6+TE, is more than 40-fold less the k_{cat} 2.4 min^{-1} measured for the same (less preferred) substrate with DEBS module 6+TE.

Interestingly, the substrate preferences of PICS modules 5 and 6 apparently reflect the stereochemical features of their natural pentaketide and hexaketide substrates. Thus, the pentaketide intermediate generated by PICS module 4 carries an L-methyl group at C-2, analogous to that found at C-2 of the (2*S*,3*R*)-2-methyl-3-hydroxy diketide **4**. (Figure 1B) In like manner, the hexaketide produced by PICS module 5 that serves as the natural substrate for PICS module 6 has the same 2-D-methyl, 3-L-hydroxy substitution pattern as the (2*R*,3*S*)-diketide **5** preferred by PICS module 6+TE. Curiously, this stereochemical correlation is not shown by DEBS module 6, which strongly prefers the unnatural (2*S*,3*R*)-diketide **4** as a substrate analogue.

We have recently explored the substrate specificity and tolerance of a variety of PKS modules belonging to the erythromycin, picromycin, and rifamycin synthases.[21] Experiments with PICS module 6+TE revealed a relatively broad substrate tolerance, consistent with the findings reported here. For example, PICS module 6+TE could process the (2*S*,3*R*)-diketide, either generated in vivo by the fused DEBS loading domain+module 1 or provided in vitro as the -SNAC ester. PICS module 6+TE also elongated the natural erythromycin triketide, (2*R*,3*S*,4*S*,5*R*)-2,4-dimethyl-3,5-hydroxyheptanoyl-ACP, generated in vivo by coexpressed DEBS1 carrying the appropriate C-terminal linker to the corresponding tetraketide ketolactone.

The results reported here demonstrate the intrinsic substrate specificity of modules 5+TE and 6+TE of the picromycin synthase. Despite clear similarities in biochemistry and underlying module organization, PICS and DEBS modules show clear differences in substrate specificity and tolerance. These results emphasize the importance of extending the study of PKS specificity and function beyond the now well-studied DEBS system and suggest a note of caution in extrapolating from studies of a single PKS system.

Experimental Section

Materials and General Methods. The four diketide-SNAC diastereomers used in this study (**4**−**7**) as well as reference samples of the derived triketide lactones **8**, **9**, and **12** were each prepared as previously described.[13,14,22] DL-[2-^{14}C]-Methylmalonyl coenzyme A (54

(20) For example, see: Chen, H.; O'Connor, S.; Cane, D. E.; Walsh, C. T. *Chem. Biol.* **2001**, *8*, 899−912.

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mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Oligonucleotide primers were purchased from Integrated DNA Technologies. DNA sequencing of PCR products was performed on double-stranded plasmids by the HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory at the Yale University School of Medicine (New Haven, CT). Reagents for kinetic assays and chemical synthesis were purchased from Sigma−Aldrich Chemical Co. and were of the highest available grade. Culture medium components were obtained from Difco.

Ligations, restriction endonuclease digestions, transformations of competent cells, preparation of buffers and media, as well as other standard molecular biological procedures were carried out as previously described.[23] All PCR reactions were carried out using *pfu* turbo polymerase (Stratagene), using conditions recommended by the manufacturer, and were supplemented by the addition of 10% (v/v) DMSO. Protein concentration was determined by the method of Bradford[24] using the BioRad kit. SDS PAGE gels were calibrated with the Invitrogen prestained protein ladder.

Construction of Expression Plasmids for PICS Modules 5+TE and 6+TE. Plasmid pKOS039-86 containing the complete PICS gene cluster,[5b] *pikAI-pikAIV* (GenBank accession no. AF079138),[5a] which was generously provided by R. McDaniel of Kosan Biosciences, Inc., served as the template for PCR amplification of PICS module 5, PICS module 6, and PICS TE DNA. *E. coli* GM2163 was the cloning host for construction of both PICS expression vectors. The forward primer for amplification of PICS module 5 was 5′-AGTGAGT**CATATG**GCGAACAACGAAGACAAGCTCCGCGACTAC-3′ having an engineered *Nde*I site (bold) and the native ATG (underlined) start codon. The reverse primer was 5′-AGTGAGT**AAGCTT**GTACGCCTCGTGGA-3′ which introduced an *Hind*III site (bold) at the C-terminal end of the amplicon. The resultant PCR product, corresponding to bp 1-4434 of *pikAIII*, was ligated into the *Nde*I and *Hind*III sites of pHL4-71, a derivative of pBluescript II KS(+) (Stratagene) in which an *Nde*I site had been introduced into the multiple cloning site, and the resultant construct was used to transform *E. coli* GM2163. To avoid any potential errors that might result from infidelity in PCR amplification and minimize resequencing, the *Bst*I−*Msc*I fragment (bp 129-3970) within the PCR-amplified PICS module 5 was replaced with its counterpart in pKOS039-86. The entire PICS module 5 gene was then excised by digestion with *Nde*I and *Hind*III and ligated into the corresponding sites of pET-21c(+) to produce plasmid pHL5-20. To append the PICS TE domain, the corresponding TE gene from bp 3145-4038 of *pikAIV* was amplified by PCR using the forward primer 5′-GTGAGT**AAGCTT**TCCGGGGCCGACACCGGCG-3′ and the reverse primer 5′-AGTGAGT**CTCGAG**CTTGCCCGCCCCCTCGATGCC-3′ to introduce *Hind*III and *Xho*I restriction sites (bold) at the N- and C-terminal ends of the insert, respectively. The resulting PCR product was subsequently cloned into the *Hind*III and *Xho*I sites of pHL5-20 to produce plasmid pHL6-76 encoding PICS module 5+TE.

PICS module 6+TE was amplified by PCR from the full-length *pikAIV* gene (bp 1-4038), excluding the normal stop codon, using a forward primer 5′−AGTGAGT**CATATG**ACGAGTTCCAACGAACAGTTGGTGGACGCT-3′ with an *Nde*I site (bold) overlapping the native ATG start codon (underlined) and the same reverse primer that had been used to amplify the TE domain alone. The PCR product was digested with *Nde*I and *Xho*I and ligated into the corresponding sites of pHL4-71, and the internal *Bsp*EI−*Blp*I fragment of pHL4-71 was then replaced by the corresponding fragment (bp 64-3408) from pKOS039-86. The *Nde*I−*Xho*I insert in the resulting plasmid was subcloned into pET-21c(+) to produce plasmid pHL5-07 harboring PICS module 6+TE. Sequencing of the PCR-derived regions of pHL6-76 and pHL5-07 through the cloning junctions confirmed the integrity of the DNA sequences.

Expression and Purification of PICS Module 5+TE and PICS Module 6+TE. Plasmids pHL6-76 and pHL5-07, harboring PICS module 5+TE and PICS module 6+TE, respectively, were used to transform *E. coli* BAP1, a derivative of the T7-based expression host *E. coli* BL21(DE3) containing a chromosomally integrated copy of the *sfp* gene allowing in vivo 4′-phosphopantetheinylation of the ACP domains of the expressed PICS modules.[17] Expression and purification procedures were similar for both modules and largely paralleled protocols previously developed for other PKS modules.[12,14] Cultures of *E. coli* BAP1/pHL6-76 and BAP1/pHL5-07 were grown in LB medium at 37 °C under standard antibiotic selection until $OD_{600} = 0.6$ and cooled to 20 °C and induced with a 1 mM final concentration of IPTG. After incubation for a further 12 h at 20 °C, cells were harvested by centrifugation, resuspended in a disruption buffer consisting of 200 mM sodium phosphate (pH 7.1), 0.2 M sodium chloride, 2.5 mM dithiothreitol, 2.5 mM EDTA, 1.5 mM benzamidine, 30% glycerol (v/v), and 2 mg/L pepstatin A and leupeptin, and then passed through a French press at 16 000 psi. The resulting cell lysate was treated with 1 mg/mL DNaseI (25 mM $MgCl_2$) for 20 min, followed by DNA precipitation with 0.15% polyethylenimine for 20 min. The cell debris and precipitated DNA were removed by centrifugation at 53 000*g*.

Protein purification procedures were performed at 4 °C. The supernatant was applied to a Q-sepharose column and purified using a gradient from 100% buffer A [100 mM sodium phosphate buffer (pH 7.1), 2.5 mM dithiothreitol, 1 mM EDTA, and 20% glycerol (v/v)] to 100% buffer B (buffer A plus 0.5 M NaCl). PICS modules eluted at ∼0.3 M NaCl. The appropriate fractions were collected, diluted with buffer C (buffer A plus 1 M $(NH_4)_2SO_4$), and loaded onto a phenyl-sepharose column. Using a gradient from 40% buffer C to 100% buffer A, the proteins of interest eluted at ∼0.2 M $(NH_4)_2SO_4$. The protein fractions containing PICS module 5+TE or 6+TE were collected and concentrated with a Centriprep YM-100 (Amicon) and then were exchanged into buffer A by passage through a PD-10 column (AmershamPharmacia).

Kinetic Assays. Kinetic determinations were performed in a total volume of 100 μL of buffer A containing 10% DMSO, variable concentrations of diketide-SNAC substrates **4** and **5**, and 100 μM DL-[2-^{14}C]-methylmalonyl coenzyme A (1 mCi/mmol). Assays of PICS module 5+TE also included 1 mM NADPH. Enzymatic incubations were carried out for 3 h at 30 °C, a period during which controls established that the enzymatic reactions were still linear. The reactions were quenched by the addition of ethyl acetate and vortex mixing. The respective triketide lactone products were extracted twice with ethyl acetate, the products were separated by TLC, and the yields were quantitated by phosphoImaging as previously described. The individual triketide lactones were identified as described previously.[12] All steady-state kinetic measurements were carried out in duplicate. The data were fit to the Michaelis−Menten equation by nonlinear least squares regression using Kaleidagraph software (version 3.05, Adelbeck Software) to calculate k_{cat} and K_m based on the calculated molecular weights for PICS module 5+TE (1786 amino acids, 187.6 kDa) and PICS module 6+TE (1354 amino acids, 143.0 kDa).

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Supporting Information Available: Normalized v versus [S] plots for the reactions of diketides **4** and **5** catalyzed by PICS modules 5+TE and 6+TE (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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