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Polyketide synthase thioesterases catalyze rapid hydrolysis of peptidyl thioesters

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ABSTRACT

Polyketide synthase (PKS) thioesterases (TEs) catalyze the macrocyclization of linear acyl chains into macrolactones. Herein we show that peptide based substrates are processed by PKS TEs with greater catalytic efficiency than more native like acyl substrates. This result strengths the link between PKS and non-ribosomal peptide synthetase systems and provides a new tool for studying PKS TEs.

The thiotemplate-directed biosynthesis strategy is shared by modular polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs).[1,2] Numerous important pharmaceutical agents are produced by PKSs or NRPSs, such as erythromycin (**1**), [3–5] pimaricin (**2**), [6,7] epothilone (**3**), [8–10] tyrocidine,[11,12] and gramicidin S.[13] Both classes of enzymes have a modular architecture and function like an assembly-line, incorporating simple building blocks such as malonyl-CoA (PKS) or amino acid (NRPS) into a growing linear chain.

The final step in both PKS and NRPS biosynthesis is often macrocyclization, which is catalyzed by thioesterases (TE) present at the C-terminus of PKSs and NRPSs. Macrocyclization is crucial for the bioactivity and the pharmaceutical utility of the compounds produced (Fig. 1).[14] These striking similarities in the biosynthetic strategies for the formation of polyketides and non-ribosomal peptides have led us to study the homology between PKS and NRPS TEs.

PKS and NRPS TEs are mechanistically homologous, catalyzing macrocyclization via a two-step mechanism.[15] The first step, which is rate determining in vitro,[16–18] is the acylation of an active site serine by a linear thioester substrate. This is followed by either intramolecular nucleophilic attack leading to macrocyclization[14] or hydrolysis leading to the linear carboxylate.[19,20]

PKS and NRPS TEs are structurally and functionally homologous. Both show high primary sequence homology and high-resolution crystallographic analysis shows that both classes of TE belong to α/β hydrolase family.[21–24] In vitro kinetic characterization of TEs has shown them to be substrate specific with specificity constants varying over orders of magnitude. Recent studies show that substrate selectivity in both NRPS and PKS TEs is mediated through

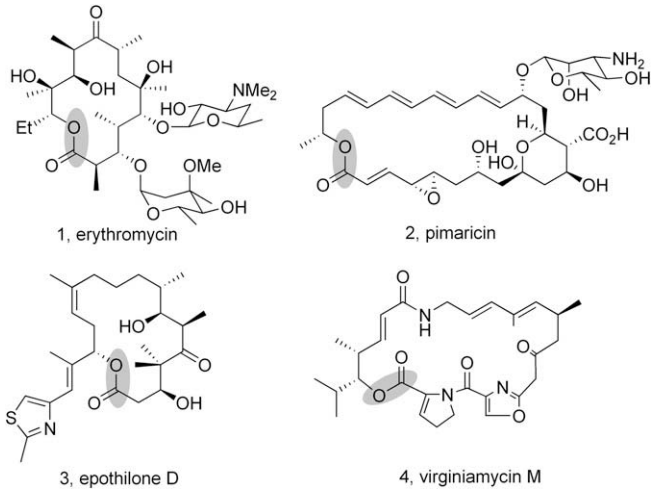

Figure 1. Structures of erythromycin, pimaricin, epothilone D, and virginiamycin M. The bonds generated by the thioesterase domains are highlighted.

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hydrophobic interactions between the substrates and the enzyme binding pocket.[25–28]

Many natural products are produced by hybrid PKS/NRPS system.[19,29,30] TE substrates produced by these hybrid systems possess functional groups typical of both polyketide and peptide. For example, the DKxanthene,[19] and virginiamycin M[31] (**4**) biosynthetic pathways, which are predominantly comprised of PKS modules, also contain multiple NRPS modules. To cyclize these natural products, the respective TEs need to accommodate functional groups from both polyketides and peptides.

While highly homologous, some data has been emerging suggesting differences between PKS and NRPS TEs. In general it appears that NRPS TEs are substantially more kinetically competent than PKS TEs. Specificity constant (k_{cat}/K_M) and turnover rate (k_{cat}) for in vitro substrate processing are generally much greater for NRPS TEs. Additionally the Michaelis constants (K_M) for NRPS TEs tend to be much lower than those seen for PKS TEs.

To further probe these two key macrocyclizing enzymes, we set out to investigate the substrate tolerance of the PKS TEs with NRPS-like peptidyl substrates. The TE-mediated hydrolysis of simple amino acid and dipeptide thioesters was kinetically characterized for the TEs from the pimaricin (Pim TE),[32] epothilone (Epo TE),[33] and 6-deoxyerythronolide B (DEBS TE)[16] biosynthetic pathways. We show that PKS TEs can efficiently process these amide-containing peptide-like substrates and that these substrates are processed with much higher specificity constants than any other PKS TE substrates characterized to date. This observation further strengthens the link between PKS and NRPS systems.

A panel of eight amide containing, *N*-acetyl cysteamine (SNAC) thioester substrates was synthesized to probe the substrate specificity of PKS TEs. For these substrates, no intramolecular nucleophiles are available to undergo TE catalyzed cyclization. Therefore, after loading the substrates onto the thioesterase to form the acyl–enzyme intermediate, only TE catalyzed hydrolysis can occur. The substrate panel can be divided into two groups. Substrates **6, 8, 10, 12, 14,** and **16** are thioesters activated *N*-*tert*-butyl carbamate protected amino acids. Substrates **6**, **8**, **10**, and **12** vary in the substitution and stereochemistry at the α-position. Substrates **14** and **16** differ in the location of the amide linkage. Substrates **19** and **21** are dipeptides and have different stereochemistry at δ-position (Fig. 2). The chemical diversity of this panel represents multiple structural elements from non-ribosomal peptide substrates, including amide bonds, epimerized proteinogenic amino acids, and non-proteinogenic amino acids. All compounds were synthesized by standard solution phase organic synthesis techniques and were purified to homogeneity as measured by ^{1}H NMR spectroscopy.

The excised recombinant thioesterase domains from the pimaricin, epothilone, and 6-deoxyerythronolide B pathways, Pim TE, Epo TE, and DEBS TE, respectively, were over-expressed in *Escherichia coli* and isolated in high purity by affinity chromatography. Steady state kinetic parameters were determined for the thioesterase catalyzed hydrolysis of substrates **6, 8, 10, 12, 14**, **16**, **19**, and **20** by quantifying the production of free thiol using Ellman's reagent.[16,34]

Due to the poor solubility of the substrates in the reaction buffer and the high K_M,[16,34] it was not possible to determine k_{cat} and the K_M independently for all substrates. For **16**, **19,** and **21** with the Epo TE and **16** with the Pim TE k_{cat} and K_M could be determined independently. The specificity constant (k_{cat}/K_M) was determined for all remaining substrates. In these cases K_M is estimated at greater than 2 mM as enzyme saturation was not reached with 4 mM substrate concentrations.

Specificity constants for TE-mediated hydrolysis of NRPS-like peptidyl substrates by PKS TEs are given in Table 1. The Epo TE was able to hydrolyze **16** with k_{cat} = 1.82 ± 0.06 s^{-1} and K_M = 0.40 ± 0.06 mM, **19** with k_{cat} = 3.1 ± 0.4 s^{-1} and K_M = 1.3 ± 0.5 mM, and **21** with k_{cat} = 19.2 ± 2.2 s^{-1} and K_M = 2.9 ± 0.6 mM. The Pim TE hydrolyzed **16** with k_{cat} = 1.2 ± 0.2 s^{-1} and K_M = 0.8 ± 0.1 mM.

Our kinetic data shows that amide containing peptidyl substrates are processed by PKS TEs. The specificity constants observed for the peptidyl substrates are greater than or equal to specificity constants determined for more native-like polyacetate and polypropionate substrates.[34] The K_Ms observed and estimated for these peptidyl substrates were comparable to the K_Ms for polyketide-like substrates.[33,35] This suggests that an increase in k_{cat} is responsible for the greater kinetic efficiency in the processing of peptidyl substrates versus polyketide-like substrates by PKS TEs.

Mono-substitution at the α-position appears to be tolerated by the DEBS and Epo TE but not the Pim TE. The *S* and *R* enantiomers of alanine, **10** and **8,** respectively, are efficiently hydrolyzed by DEBS and Epo TE. Neither of these substrates is hydrolyzed by the Pim TE. This is surprising since a previous study had shown that Pim TE can hydrolyze α-methylated polypropionate sub-

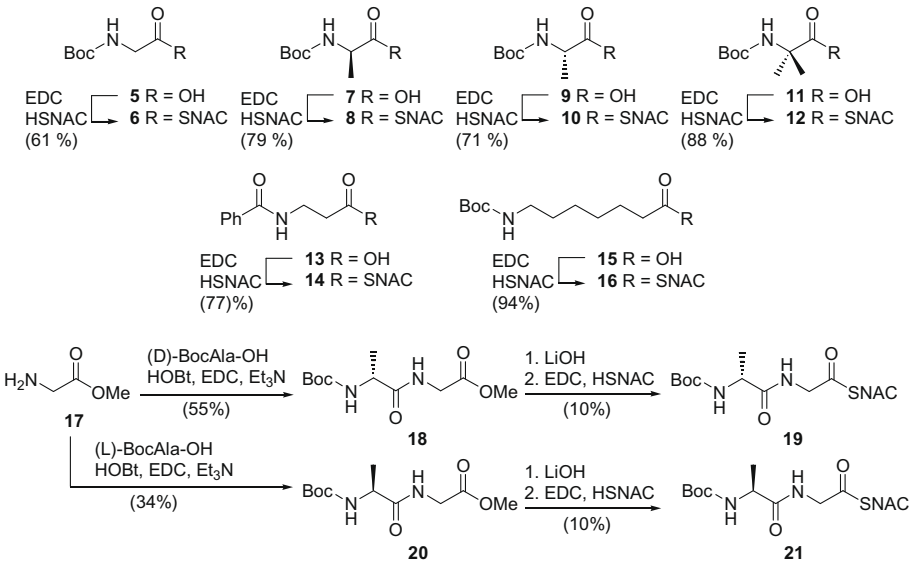

Figure 2. Synthesis of substrates used to investigate the substrate specificity of PKS thioesterase domains.

Table 1
Steady state kinetic perimeters for thioesterase catalyzed hydrolysis.

	DEBS TE k_{cat}/K_M (M^{-1} S^{-1})	Epo TE k_{cat}/K_M (M^{-1} S^{-1})	Pim TE k_{cat}/K_M (M^{-1} S^{-1})
6	7.0 ± 0.4	145 ± 10	1.29 ± 0.05
8	0.50 ± 0.01	12.5 ± 0.5	NR
10	0.88 ± 0.03	91.3 ± 2.9	NR
12	NR	NR	NR
14	7.75 ± 0.19	24.2 ± 1.2	1.14 ± 0.06
16	1.47 ± 0.07	75.1 ± 11.5	23.3 ± 3.6
19	0.80 ± 0.11	38.8 ± 14.6	NR
21	0.39 ± 0.03	110.0 ± 27.2	NR

NR, not reactive.

strates.[32] We proposed that peptidyl substrates adopt a different binding mode preventing substrates with α-substitution from loading onto the active site serine. The Epo TE shows a 10-fold preference for the *S* configuration (**10** versus **8**); however, no substituents are found at the C2 position in the epothilone family of products. The gem dimethyl compound **12** is not processed by any of the TEs, suggesting that the α-position can only tolerate a single substituent.

Our results show that the Pim TE is more substrate specific than the DEBS or Epo TEs. Only compounds without methyl substituents, **6**, **14**, and **16**, are processed by the Pim TE. Even when substitution is present only at the δ-position, such as in compounds **19** and **21**, the Pim TE is unable to catalyze hydrolysis. Interestingly, Pim TE has a high specificity constant for hydrolysis of **16** and has a relatively low K_M value (K_M 0.84 mM). These data indicate **16** binds fairly tightly to the Pim TE binding pocket, leading to an increase in enzyme–substrate complex concentration and an increase in reaction rate. This is not a surprise. Pimaricin is a highly hydrophobic polyene, suggesting that the Pim TE has evolved a hydrophobic substrate binding surface, which can also tightly bind the hydrophobic chain of **16**.

Of three TEs, Epo TE is the most robust enzyme, showing the highest hydrolytic activity and substrate tolerance. Our data suggest that Epo TE prefers no substitution at the α position such as substrates **6**, **16**, and **21**. The specificity constants observed for **6** is the highest seen for a PKS TE. Epo TE shows enantioselectivity for dipeptide hydrolysis (**21** versus **19**). The similar K_m values (2.90 mM for **19** and 1.31 mM for **21**) indicate there is no significant difference in interactions driving enzyme–substrate complex formation. The large k_{cat} difference (19.2 min^{-1} for **21** and 3.05 min^{-1} for **19**) indicates substantial interactions stabilizing the transition state for acyl–enzyme intermediate formation with **21**.

In conclusion our results demonstrate that PKS TEs can process NRPS-like peptidyl substrates efficiently. We show that in many cases the peptidyl substrates are processed more efficiently that PKS-like substrates. PKS TEs process peptidyl substrates with comparable Michaelis constants to polypropionate substrates but with greater turnover rates. Surprisingly, this suggests that the increased rates of hydrolysis are not driven by better recognition of the peptidyl substrates. Instead, we propose that new interactions are being generated in the transition states that stabilize formation of the acyl–enzyme intermediate for peptidyl substrates to a greater extent than polypropionate substrates.

The ability to load peptidyl substrates onto PKS TEs provides a powerful new tool for studying substrate specificity and macrocyclization. Since an enormous number of amino acids are commercially available, and the synthesis of small peptide libraries is facile, the substrate tolerance and specificity of PKS TEs can be probed much more rapidly. Incorporation of amide linkages into complex TE substrates will also facilitate study of TE-mediated macrocyclization by allowing easy synthetic access to libraries of substrates capable of undergoing macrocyclization. Lastly this result further strengthens the linkage between PKS and NRPS biosynthetic systems.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.01.040.

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