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6-Deoxyerythronolide B Synthase Thioesterase-Catalyzed Macrocyclization Is Highly Stereoselective

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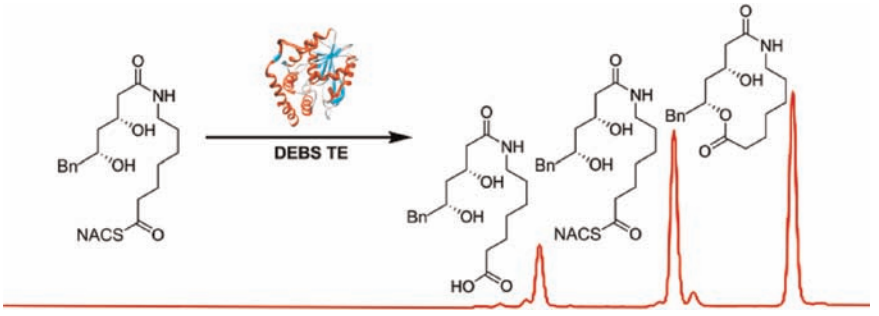
ABSTRACT



Macrocyclic polyketide natural products are an indispensable source of therapeutic agents. The final stage of their biosynthesis, macrocyclization, is catalyzed regio- and stereoselectively by a thioesterase. A panel of substrates were synthesized to test their specificity for macrocyclization by the erythromycin polyketide synthase TE (DEBS TE) in vitro. It was shown that DEBS TE is highly stereospecific, successfully macrocyclizing a 14-member ring substrate with an *R* configured *O*-nucleophile, and highly regioselective, generating exclusively the 14-member lactone over the 12-member lactone.

Macrocyclic polyketide natural products have been developed into successful pharmaceuticals in nearly all therapeutic areas.[1] The macrocyclic nature of these compounds is critical to their potent and selective biological activities as it reduces their conformational flexibility, effectively lowering the entropic cost of binding, which leads to high affinity and selectivity of binding.[2]

During the biosynthesis of macrocyclic polyketides, the macrolactone linkage is formed by a thioesterase (TE)[3] found at the C-terminus of the last modular type I polyketide synthase (PKS) in the biosynthetic pathway.[4] This step is essential for turnover of the mature polyketide from the PKS machinery to which it has been covalently bound. While essential for successful biosynthesis, TE-catalyzed macrocyclization is still very poorly understood.[5]

TEs catalyze macrolactone formation by a two-step mechanism (Figure 1). The active site serine is first acylated by a linear polyketide thioester. This acyl-enzyme intermediate then undergoes an intramolecular attack leading to macrolactone formation and its release from the enzyme. The acylation step is well-characterized. Based on kinetic and high-resolution structural data, it is clear that

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this step is rate-determining in vitro and highly substrate tolerant with hydrophobic enzyme–substrate interactions controlling substrate specificity.[6]

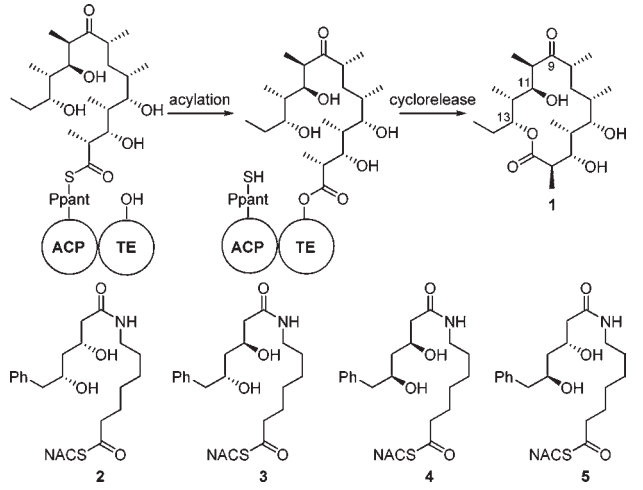

Figure 1. DEBS TE-catalyzed macrocyclization of 6-deoxyerythronolide B (**1**) and synthetic analogs **2**–**5** which were designed to probe the stereo- and regioselectivity of DEBS TE macrocyclization.

To date PKS TE-catalyzed macrocyclization has been observed only with native substrates and very close analogs.[5,7] For example in vivo the TE from the 6-deoxyerythronolide B biosynthetic pathway (DEBS TE) catalyzes the formation of its native 14-member ring, **1**, as well as the non-native but highly homologous 16-, 12-, and 8-member rings.[7]

In vitro macrolactonization has only been observed in four studies.[5] The TEs from the pikromycin and epothilone biosynthetic pathways (PIK TE and Epo TE) have successfully generated their native products, 10-deoxymethonolide and epothilone C respectively.[5a−c] The DEBS TE has also been shown to macrocyclize the precursor to 10-deoxymethonolide,[5c] and the TE from the zearalenone pathway has successfully generated a close analog of zearalenone.[5d] All other substrates investigated in vitro have failed to yield detectable macrocyclic products, demonstrating rigorous substrate specificity for macrocyclization.[8] Identifying the molecular basis controlling TE-catalyzed macrocyclization is a current and serious gap in our understanding of polyketide biosynthesis, limiting our ability to design TEs with tunable substrate tolerance and regioselectivity for the macrocyclization of new polyketides generated by both de novo engineering of polyketide synthases and chemical synthesis.

Characterization of the macrocyclization activity of PKS TEs requires substrate analogs that vary the stereochemistry and position of the natural product's functional groups. Due to the synthetic complexity of these substrates,[5b] these essential studies have not progressed. We thus focused on identifying synthetically tractable substrates that can be used to systematically probe TE macrocyclization activity. Herein we disclose the first non-native TE substrate that undergoes macrocyclization by a type I PKS TE. With the complete stereoisomer library of this substrate, our study shows macrocyclization to be exquisitely stereoselective and unpredictable in its selectivity, highlighting the need for high-resolution characterization of the enzyme–substrate interactions in this enzyme.

Having demonstrated the ability of DEBS TE to rapidly hydrolyze short amide-containing thioester substrates,[9] we designed four amide-based stereoisomeric substrates for the DEBS TE, **2**–**5** (Figure 1). The amide was selected to mimic the ketone found in the native substrate. This ketone has been proposed to play a role in controlling the substrate's conformation and helping to properly position the nucleophilic intramolecular hydroxy group in the active site channel.[5b,c] High-resolution structural characterization of a nonhydrolyzable acyl-enzyme intermediate of the related PIK TE suggests that the ketone interacts with a hydrophilic barrier consisting of bulk water and a glutamine at the exit from the enzyme's substrate channel, inducing a turn in the substrate.[6c] This turn is proposed to place the intramolecular nucleophilic alcohol in close proximity to both the catalytic base and the electrophilic carbonyl, favoring macrocyclization over hydrolysis.[6c] The presence of the ketone in TE substrates however has been shown to lead to hemiketal formation, complicating in vitro analysis.[5b,c] Replacement of the ketone with an amide preserves the carbonyl for interaction with the proposed hydrophobic barrier, prevents hemiketal formation, and facilitates fragment coupling during substrate synthesis. The presence of the C11, C13 diol in the substrate further enabled the possibility of 14- and 12-member macrolactone formation. Both of these ring sizes have been generated by DEBS TE.[5b,c,7] Lastly, the native ethyl substituent at C13 was replaced with a benzyl chromophore. This modification was not anticipated to substantially impact TE activity because the benzyl substituent, along with other sterically more demanding groups, has been shown to be tolerated at this position in vivo by the DEBS TE.[7b,10]

This panel of substrates was thus been designed to provide insight into the role of stereochemistry on macrocyclization activity and the regiochemistry of macrocyclization.

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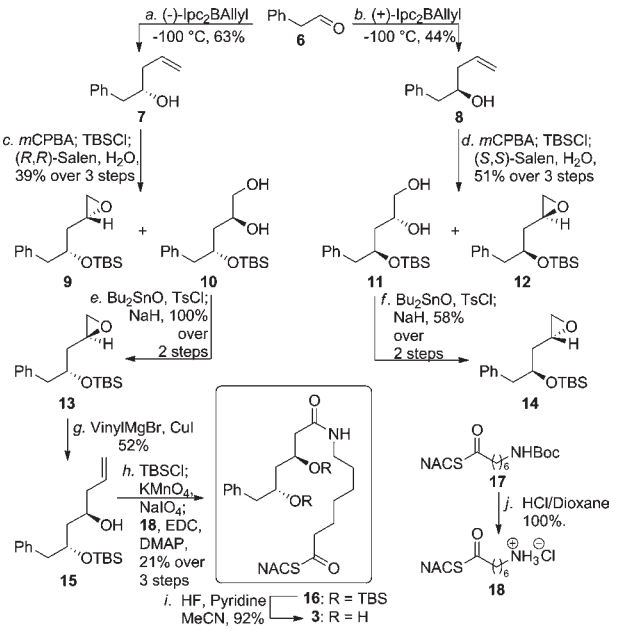
Scheme 1. Synthesis of Enantioenriched Substrate **3**



Substrates **2**–**5** were synthesized as shown in Scheme 1. The Brown allylation[11] was used to set the C13 stereocenter, generating the two enantioenriched homoallylic alcohols **7** and **8**. Following protection and epoxidation, the Jacobsen hydrolytic kinetic resolution[12] was used to set the C11 stereocenters, ultimately providing access to all four stereoisomeric protected epoxyalcohols **9**, **12**–**14**. Regioselective vinyl cuprate addition to each enantioenriched epoxide, followed by the oxidative cleavage of the olefin under $KMnO_4/NaIO_4$ conditions, generated the required carboxylic acids which were coupled with the 7-aminoheptanoate derivative **18**.[9,13] Deprotection of the silyl ethers in the presence of the thioester using HF in acetonitrile completed the synthesis of all four desired substrates **2**–**5**.

The DEBS TE proved to be highly substrate specific for macrocyclization (Figure 2). LCMS analysis of 18 h incubations of 5 μM DEBS TE (50 mM phosphate, pH 7.4) with 2.5 mM **2**–**5** showed the exclusive macrocyclization of substrate **2**. Syntheses of the authentic macrocyclization standards (see Supporting Information (SI)) enabled unambiguous identification of this enzymatic product as the 14-member ring macrocycle **19**, demonstrating that the DEBS TE-catalyzed a highly regioselective macrocyclization. The remaining three substrates, **3**–**5**, underwent hydrolysis, generating the free acids **21**–**23**.

Steady state kinetic characterization of TE-catalyzed consumption of each substrate provided the turnover number, Michaelis constant, and, when the enzyme could not be saturated, the specificity constant (**2**, k_{cat} = 0.11 ± 0.02 min^{-1}, K_m = 1.5 ± 0.6 mM, k_{cat}/K_m = 1.2 ± 0.5 M^{-1} s^{-1}; **3**, k_{cat} = 0.21 ± 0.08 min^{-1}, K_m = 2.2 ± 1.6 mM, k_{cat}/K_m = 1.6 ± 0.6 M^{-1} s^{-1}; **4**, k_{cat} = 0.6 ± 0.1 min^{-1}, K_m = 1.8 ± 1 mM, k_{cat}/K_m = 5.6 ± 3.1 M^{-1} s^{-1}; **5**, k_{cat}/K_m = 0.42 ± 0.04 M^{-1} s^{-1}). The observed specificity constants are comparable to the known range determined for DEBS TE-catalyzed hydrolysis of *N*-acetylcysteamine (NAC) thioesters (0.04–31 M^{-1} s^{-1})[6a,8c,9,14] and macrocyclization (1.5 ± 0.8 M^{-1} s^{-1}).[5b,c] They also agree well with the specificity constant for the DEBS TE-catalyzed hydrolysis of the highly analogous *N*-Boc-7-aminoheptanoate NAC thioester (1.47 ± 0.07 M^{-1} s^{-1}).[9] The observation that the pseudo-first-order rate constant for macrocyclization is

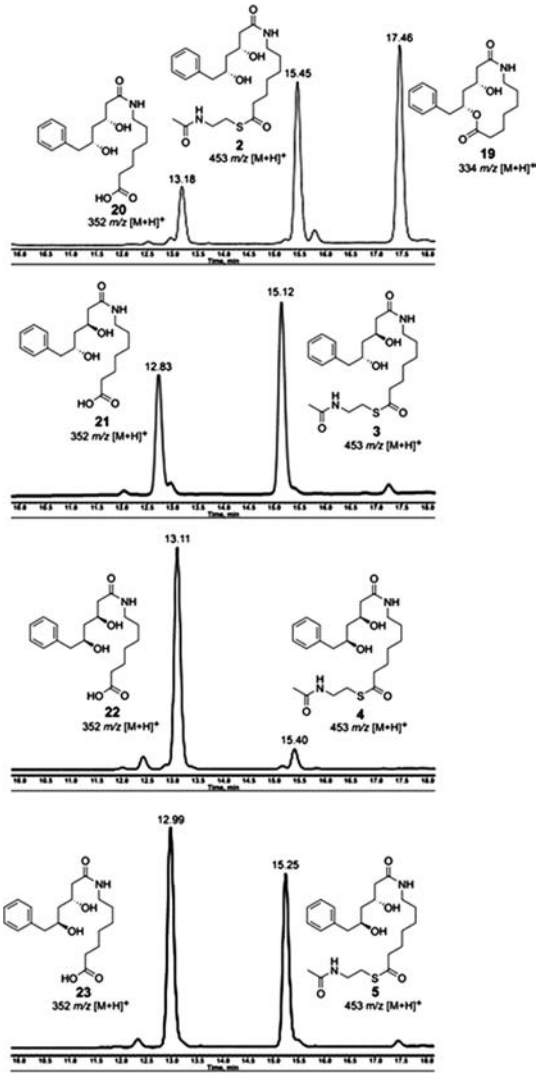


Figure 2. LCMS traces (254 nm with observed *m*/*z* values) and product distribution from 18 h incubations of DEBS TE (5 μM in 50 mM phosphate pH 7.4) with substrates **2**–**5** (2.5 mM).

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comparable to those observed for hydrolysis suggests that active site acylation is also rate determining for macrocyclization.[6a] Because the enzyme–substrate interactions governing hydrolysis versus macrocyclization are likely developed after the initial acylation step, the kinetic parameters cannot provide insight into these interactions.

A working hypothesis in the community has been that stereochemistry of the nucleophilic alcohol plays a major role in controlling macrocyclization activity. This is supported by the observation that all 16-, 14-, 12-, and 8-member rings produced by the DEBS TE have an *R* configured *O*-nucleophile.[5b,c,7] While the successful macrocyclization of **2** is in agreement with this hypothesis, the hydrolysis of **3** is not. The hydrolysis of **3** is even more surprising since its stereochemistry at C11 and C13 matches that of the native substrate. To confirm that **3** was not macrocyclized by the DEBS TE and then subsequently ring opened, as is seen with the Epo TE,[5a] we incubated the 14-member ring macrocyclic product of **3** (see SI for its synthesis) with the DEBS TE. After prolonged incubation, no ring opening of the macrocycle was observed, confirming that the DEBS TE catalyzes the direct hydrolysis of **3**.

This first systematic study of the role of stereochemistry on PKS TE-catalyzed macrocyclization reveals that the DEBS TEs can macrocyclize analogs that differ substantially from its native substrate. While this study agrees with the hypothesis that the carbonyl at C9 and the stereochemistry of the nucleophilic alcohol are critical for macrocyclization,[5b,c,7] our data show they are not sufficient. The inability of **3** to undergo macrocyclization, even though it is the most homologous of our analogs to the native substrate, demonstrates that additional key enzyme–substrate interactions play a role in macrocyclization.

Presumably since hydrophobic interactions have been shown to play a major role in substrate recognition during acyl-enzyme intermediate formation,[6] hydrophobic interactions between the enzyme and the substrate's methyl groups may play an important role in macrocyclization. As additional functional groups and side chains are added to the substrate, the role of the innate conformational preference of the varying substrates for macrocyclization will become increasingly important and may also ultimately play a role in controlling the exquisite selectivity of the macrocyclization reaction.[2b,6c] Ultimately, understanding the interactions that control TE macrocyclization versus hydrolysis will require high-resolution, structural data of nonhydrolizable analogs of substrates like **2**–**5** bound in the TE active site.

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Supporting Information Available. Experimental procedures and characterization data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.