Natural Products

DOI: 10.1002/anie.201000032

Biochemical and Structural Characterization of the Tautomycetin Thioesterase: Analysis of a Stereoselective Polyketide Hydrolase**

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Tautomycetin (TMC) is a polyketide metabolite produced by *Streptomyces* sp. CK4412 and *Streptomyces griseochromogenes*.[1] This intriguing molecule was previously shown to possess activated T-cell-specific immunosuppressive activity with a novel mode of pharmacological action, both in vivo and in vitro.[2] More recent studies have also revealed promising anticancer activity in a variety of models.[3–5]

The biosynthesis of complex polyketide compounds in bacteria often occurs by an assembly-line type mechanism that is catalyzed by type I modular polyketide synthases (PKSs). In a key final step, the characteristic macrolactone scaffold is generated for the macrolide antibiotics erythromycin and pikromycin.[6] This termination process is catalyzed by a thioesterase (TE) domain that is located at the carboxy-terminus of the final PKS elongation module. The activity of this domain results in cleavage of the acyl chain from the adjacent ACP, followed (typically) by macrocyclization.[7,8] The macrolactone is often modified further to give the final bioactive compound.[9]

The structure of TMC is highly unusual;[10] it is one of the few known examples of a polyketide natural product that bears a terminal alkene group (Figure 1). The TMC biosynthetic gene cluster (*tmc*) has recently been characterized, revealing two putative type I PKSs (TmcA and TmcB), along with 16 additional gene products that are presumably involved in chain construction, tailoring, and regulation (Figure 2).[11,12]

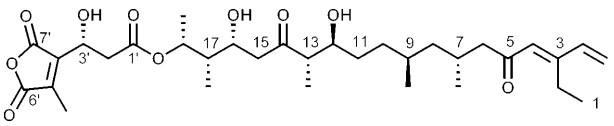

Figure 1. TMC, produced by *Streptomyces* sp. CK4412.

Based on pathway annotation and biosynthetic principles, we hypothesized that the TMC TE catalyzes termination of chain assembly through generation of the free acid, as it contains the highly conserved sequence GxSxG and GdH motifs, as well as the Ser-His-Asp catalytic triad, characteristic of the α,β-hydrolase class of serine hydrolases. Installation of the terminal olefin is presumed to occur through decarboxylative dehydration during the post-PKS maturation of the polyketide to form the final TMC product. DNA-sequence analysis of open reading frames downstream of TMC reveals several potential candidate enzymes for catalyzing this transformation: two putative decarboxylases and a dehydratase.[11,12] These biosynthetic steps remain unclear, thus motivating us to elucidate the biochemical details of this process.

Based on our efforts to understand chain termination and terminal alkene formation in the biosynthesis of TMC, we report herein the cloning, biochemical characterization, and the 2.0 Å crystal structure of the TE domain for this pathway; the first high-resolution structural analysis of a linear chain-terminating TE.

The TMC TE was amplified from cosmid pTMC2290 and inserted into the vector pMCSG7 (see the Supporting Information).[11] To assess enzyme function, two short-chain enantiomerically pure TMC substrate mimics were synthesized in two steps (Scheme 1).[13,14] We first evaluated the hydrolysis of model substrates **4** and **5** by the TMC TE. After overnight incubation and LCMS analysis, we found that the enzyme was greater than 350 times more active toward the (*R*)-isomer **4** than the (*S*)-isomer **5** (Figure 3). This observation was surprising, as previously characterized TEs from macrolactone-forming PKSs exhibit a high degree of substrate and stereochemical tolerance.[15,16] However, this selectivity is consistent with the predicted stereochemistry of the β-hydroxy group (eliminated during decarboxylative dehydration) based on sequence analysis of the preceding module 9 KR domain (see the Supporting Information, Figure S2).[17] Mutation of the TMC TE active site Ser132 to Ala completely abrogated hydrolysis of the substrate (not shown) and confirmed its key role in catalysis.

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[**] This work was supported by the NIH (grant GM076477) and the Hans W. Vahlteich Professorship (D.H.S.), UL1RR024986 (J.B.S), by NIH grant DK042303 (J.L.S.), and by KOSEF grant (MEST 2009-0078663; E.-S.K.) GM/CA CAT is supported by the NIH National Institute of General Medical Sciences and the National Cancer Institute at the APS, which is supported by the US Department of Energy Office of Science.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201000032.

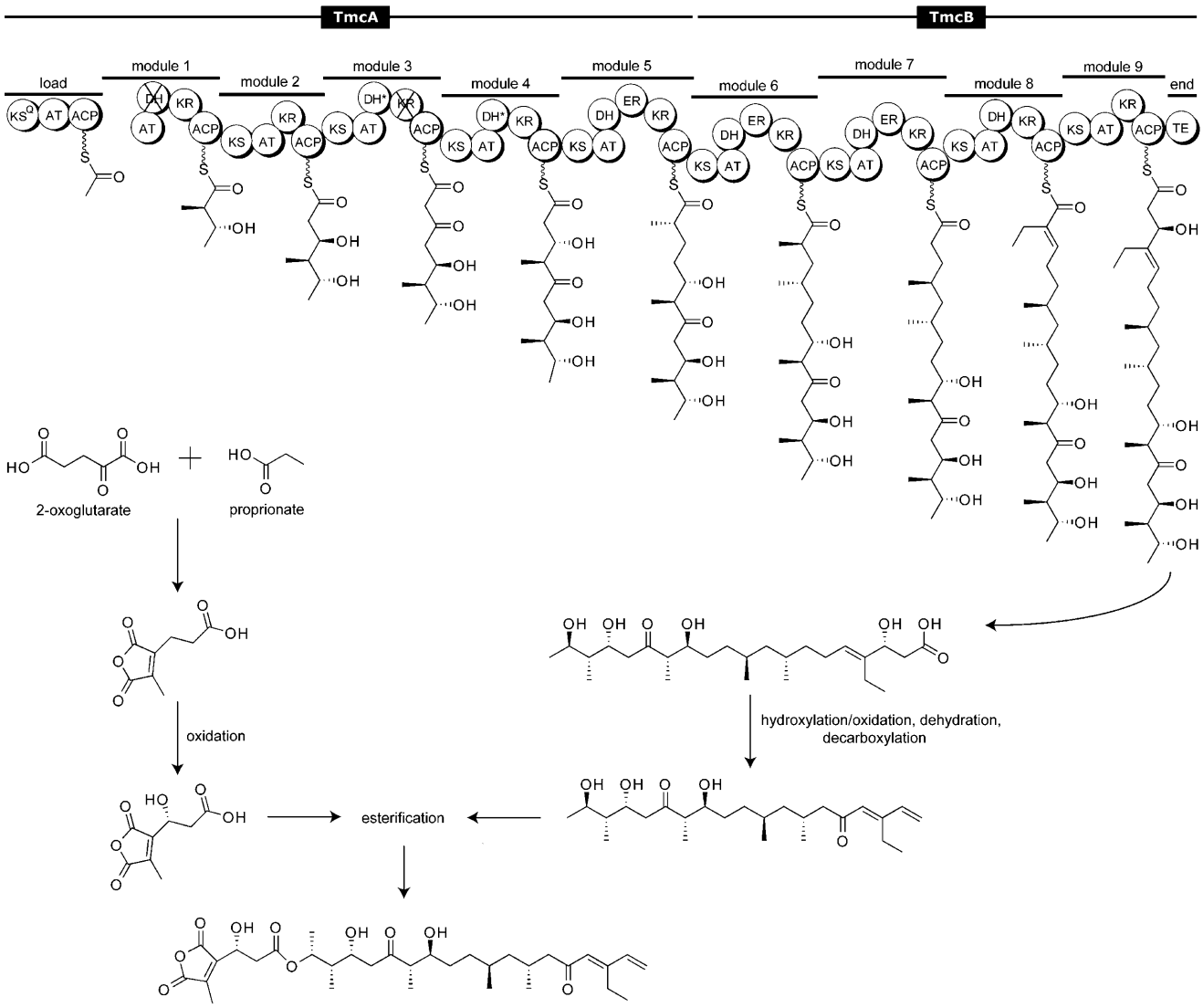
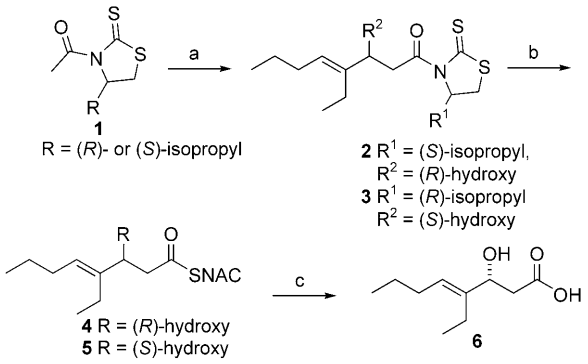


Figure 2. Proposed steps in the TMC biosynthetic pathway. KS = ketosynthase, AT = acyltransferase, ACP = acyl carrier protein, DH = dehydratase, KR = ketoreductase, ER = enoylreductase, TE = thioesterase. Domains predicted to be inactive are crossed out. Domains that are active but not utilized are indicated with a star. The ordering of post-PKS tailoring reactions is unknown. KS^Q = malonyl-ACP decarboxylase.



Scheme 1. Synthesis of *N*-acetylcysteamine (NAC) short chain TMC analogues. Reagents and conditions: a) *trans*-2-ethyl-2-hexenal, tin triflate, *N*-ethylpiperidine, CH_2Cl_2, −78 °C, 70%; b) NAC, imidazole (3 equiv), CH_2Cl_2, 85%; c) LiOH, H_2O_2, 10% aq. THF, 90%.

Steady-state kinetic analysis of the TMC TE was performed with compound **4** using a discontinuous coupled fluorescence-based assay.[19] The TE was found to have a catalytic efficiency of $(22 \pm 2)\ m^{-1}s^{-1}$, with a k_{cat} of $(2.2 \pm 0.2)\ min^{-1}$ and a K_m of (1.7 ± 0.3) mM, based on the non-linear regression fit to the Michaelis–Menten equation ($R^2 = 0.85$; see the Supporting Information, Figure S3). Whilst relatively slow against **4**, this efficiency is comparable to previously described kinetic parameters for the pikromycin and erythromycin TEs using model diketide substrates.[20] Interestingly, the data fit equally well to the allosteric kinetic model ($R^2 = 0.88$), yielding similar kinetic constants as the previous fit (see the Supporting Information, Figure S3). Moreover, the Hill coefficient that was calculated from this fit (1.7) is consistent with possible enzyme cooperativity. If true, this finding, to the best of our knowlege, represents the first report of allosterism demonstrated by a thioesterase; however, these results should be interpreted with care given that

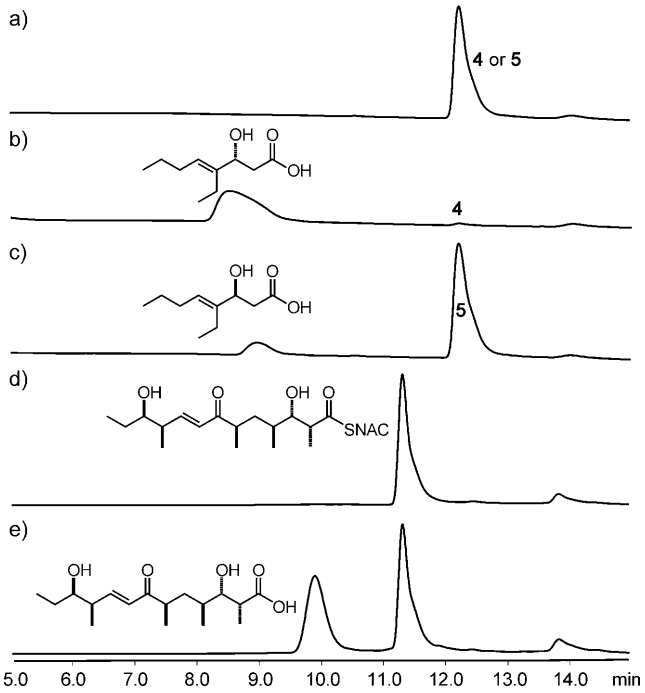


Figure 3. Hydrolysis by the TMC TE. a) **4** or **5**, no enzyme; b) **4** with TE; c) **5** with TE; d) pikromycin hexaketide, no enzyme; e) pikromycin hexaketide with TE. All substrates were at 5 mM concentrations and incubated overnight with 1 μM enzyme before analysis by LCMS. All masses were as expected and, for traces (b) and (c), the retention time of the acid matched that of the standard **6** (see the Supporting Information, Figure S1). Comparison of the shape and size of the active site tunnel in TMC TE, relative to the homologous Pik and DEBS TEs revealed why the enzyme forms a linear hydrolysis product as opposed to a macrolactone. The bulky side chains of Tyr161, Phe163, and Leu205 constrict the "exit" side of the substrate tunnel, leaving only enough space for an extended acyl chain (Figure 4c).

the recombinant TE domain was removed from its native polypeptide context for this study. No hydrolysis of the corresponding (*S*)-isomer **5** was observed at these concentrations over this time period.

Next, we examined the ability of the TMC TE to catalyze the intramolecular cyclization of the linear pikromycin hexaketide intermediate to form the 12-membered ring, macrolactone 10-deoxymethynolide.[18] This substrate possesses the (*R*)-β-hydroxy stereochemistry that is preferred by the TMC TE. Although cyclization of the hexaketide did not occur, partial hydrolysis to the linear carboxylic acid was observed after overnight incubation with the enzyme (Figure 3). This hydrolysis revealed some substrate flexibility of the TMC TE as its native substrate (final chain elongation intermediate, Figure 2) lacks an α-methyl group, and the distal portions of the two compounds differ considerably.

To understand the basis for this unique selectivity further, we pursued structural analysis of the TMC TE protein, which is a dimeric member of the α/β-hydrolase family that has a fold similar to other type I PKS TEs.[15,19] The protein structure consists of two discrete motifs, an α/β-hydrolase core capped by an α-helix lid domain. The α/β-hydrolase core fold is a seven-stranded, predominantly parallel β-sheet surrounded by five α helices (Figure 4a). Canonical α/β-hydrolase domains include a helix between β6 and β7 which is lacking in the macrolactone-forming Pik and DEBS TEs. This helix (α4) is present in TMC TE and is proposed to impact the processing of the product, thus directing hydrolysis as opposed to cyclization (see below). The lid domain consists of four α helices, two (αL1 and αL2) from the N-terminal thirty-three residues, and two (αL3 and αL4) from a forty-five residue region between β5 and β6. As with Pik and DEBS TEs, the dimer interface is mediated exclusively through the

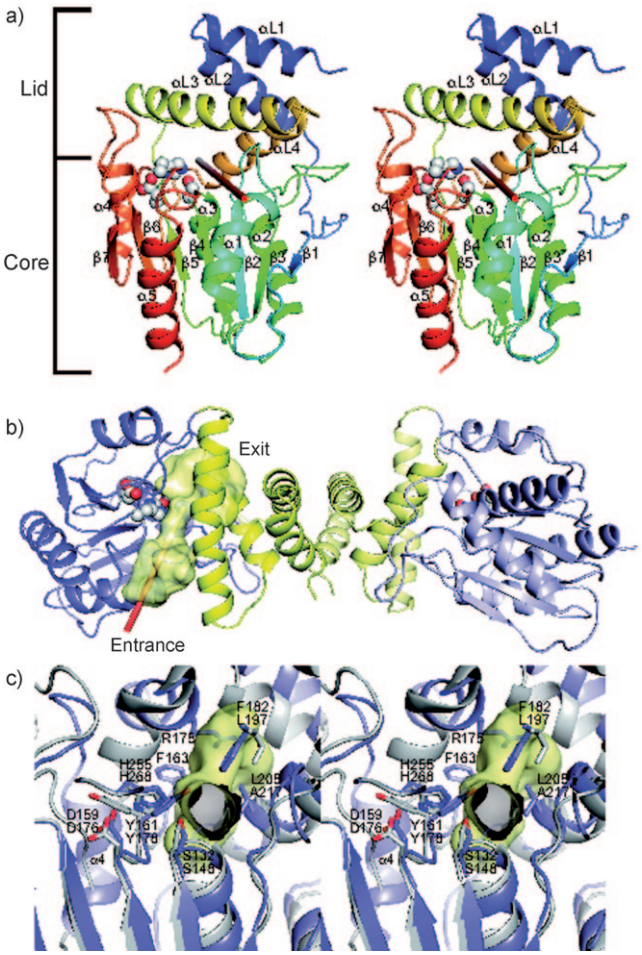


Figure 4. Structure of TMC TE. a) Stereodiagram of TMC TE monomer, rainbow colored from the N-terminus (blue) to the C-terminus (red). The active site triad is shown as spheres. Lid and α/β-hydrolase core domains are indicated. The pointer (red rod) indicates the direction of entry into the substrate tunnel. b) TMC TE dimer interactions are mediated by the lid domains (yellow). The substrate tunnel (shown for one monomer) passes through the protein with the active site (spheres) in the center. The dimer axis is vertical in this view. The pointer (red rod) is the same as for (a). c) Stereodiagram of the active site, looking from the entrance (along the red rod in part a). Pik TE (grey) is superimposed on TMC TE (blue) with TMC TE substrate channel (yellow surface). The catalytic triad and residues that constrict the active site relative to Pik TE are labeled. Equivalent residues are labeled with the TMC TE designation above the Pik TE designation. Helix α4 is present only in the TMC TE structure. Figure S7 (see the Supporting Information) shows comparison with the Pik TE substrate tunnel.

two N-terminal helices (αL1 and αL2) that form the top of the lid domain (Figure 4b).

In accord with other type I TEs, the active site triad (Ser132, Asp159, and His255) is located on loops at the C-terminal edge of the core β-sheet after β4, β5, and β7. Ser132 is positioned in a classic nucleophilic elbow within the signature sequence Gly_{130}-His_{131}-Ser_{132}-Xaa-Gly_{134}. The oxyanion hole is formed from the backbone amide groups from Ser133 and Thr66. As observed previously with Pik TE and DEBS TE,[8,15,19] the active site is located at the center of an elongated substrate tunnel, which is open at both ends, that spans the width of the enzyme (Figure 4b). Compared to Pik and DEBS TEs, the TMC TE substrate tunnel is relatively narrow and constricted in the region containing the polyketide intermediate during hydrolysis.

Despite these insights, a structural rationale for the observed chiral preference of TMC TE for (*R*)-β-OH is not readily apparent. We surmise that in the free-enzyme structure, the Ser133 side-chain (adjacent to the catalytic Ser132) blocks the presumed oxyanion hole by hydrogen bonding with the backbone NH of Thr66. The Ser133 side-chain Cα–Cβ bond must rotate in order for the substrate thioester oxygen to occupy the oxyanion hole. However, substrate modeling into the active site suggests that, owing to the restricted dimensions of the chamber, only the (*R*)-β-OH can be accommodated with the Ser133 side-chain rotated to either of the available alternative rotamer positions.

Based on previous work that revealed a strategy for converting the rat FAS type II TE from a hydrolase into an acyltransferase by double mutation of the active site Ser132 to Cys and His255 to Arg,[20] we were motivated to determine whether the corresponding mutations in the TMC TE would provide both an acyl-enzyme intermediate for use in further structural studies, as well as a valuable acyltransferase for generating new acyl-CoA (CoA = coenzyme A) species.

The double mutant was obtained and incubated overnight with either thioester **4** or **5** to form an acyl-enzyme intermediate that was observed by low-resolution mass spectrometry (see the Supporting Information, Figure S4), with no evidence of hydrolysis of the thioesters to the carboxylic acids (not shown). High-resolution Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) analysis revealed that the intact protein was labeled in a 2.5:1 ratio (see the Supporting Information, Figure S5). Trypsin digestion of the acylated TMC TE protein followed by high-resolution MS confirmed that loading occurred at the Cys132 residue (see the Supporting Information, Table S2). In contrast, no labeling of wild-type TMC TE (see the Supporting Information, Figure S4) nor the S132C or H255R single mutants were observed (not shown). Efforts to crystallize the acyl-enzyme species are ongoing.

After observation of an acyl-enzyme intermediate, we attempted to displace the acyl group with free CoA to create a new CoA thioester. This method would be useful for the formation of synthetically challenging CoA substrates and the conversion of off-loaded biosynthetic intermediates into CoAs for use in chemoenzymatic synthesis. The reaction was attempted using two different methods, but formation of a new CoA species was only observed with method 2 (see the Supporting Information). However, this formation was non-enzymatic, as the new compound was observed in the presence and absence of enzyme (see the Supporting Information, Figure S6), thereby suggesting a chemical trans-thioesterification at high pH values. Whilst this result was surprising, we expect that this method will be generally applicable for the synthesis of small quantities of novel CoA compounds when the starting thioester is not sensitive to high pH levels. The TE Ser132Cys and His255Arg double mutant also provides a general strategy for affinity labeling of thioesterase enzymes with a range of natural and unnatural substrates.

In summary, the TMC TE is a polyketide hydrolase that exhibits a high degree of stereoselectivity at the β-hydroxy position. X-ray crystallography has provided the first high-resolution structure of a linear polyketide-chain-terminating TE, which shows the enzyme to have a constrained substrate chamber relative to macrolactone-forming TEs. Although the basis for relatively low substrate tolerance remains unclear, it is now possible to assess the role of specific amino acid residues that might be important in the observed stereoselectivity toward acyl-(*R*)-β-OH. Moreover, construction of a double mutant form of select TMC TE active site residues has provided a new method for affinity labeling of the enzyme active site that should be applicable to other members of the β-hydrolase family of TEs. Finally, this work provides a path to explore a process for polyketide termination that involves initial release of the polyketide chain followed by decarboxylative elimination. This represents a unique mechanism compared to the recently elucidated curacin TE that catalyzes concomitant hydrolysis and decarboxylative elimination of sulfate leading to a terminal olefin as a final step in the pathway.[21]

Received: January 4, 2010
Published online: July 9, 2010

Keywords: acyltransferases · polyketides · structure elucidation · tautomycetin · thioesterases

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