Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 16 (2006) 391–394
Bioorganic & Medicinal Chemistry Letters

Macrolactonization to 10-deoxymethynolide catalyzed by the recombinant thioesterase of the picromycin/methymycin polyketide synthase

Weiguo He,[a] Jiaquan Wu,[a] Chaitan Khosla[b] and David E. Cane[a,*]

[a]*Department of Chemistry, Box H, Brown University, Providence, RI 02912-9108, USA*
[b]*Departments of Chemical Engineering, Chemistry and Biochemistry, Stanford University, Stanford, CA 94305-5025, USA*

Received 8 September 2005; accepted 26 September 2005
Available online 24 October 2005

Abstract—The recombinant thioesterase (TE) domain of the picromycin/methymycin synthase (PICS) catalyzes the macrolactonization of **3**, the *N*-acetylcysteamine thioester of *seco*-10-deoxymethynolide to generate 10-deoxymethynolide (**1**) with high efficiency. By contrast, **4**, the 7-dihydro derivative of *seco*-thioester **3**, undergoes exclusive hydrolysis by PICS TE to *seco*-acid **5**. The recombinant TE domain of 6-deoxyerythronolide B synthase (DEBS TE) shows the same reaction specificity as PICS TE, but with significantly lower activity.

Methymycin and picromycin are homologous 12- and 14-membered ring macrolide antibiotics produced by *Streptomyces venezuelae*.[1] The responsible picromycin/methymycin synthase (PICS), a typical modular polyketide synthase (PKS), has the unusual property of catalyzing the multi-step biosynthesis of both 10-deoxymethynolide (**1**)[2] and narbonolide (**2**), the parent macrolide aglycones of methymycin and picromycin, respectively (Fig. 1).[3] The final step in the biosynthesis of each aglycone is the release from PICS of the respective ACP-(acyl carrier protein) bound acyclic hexaketide and heptaketide acyl-thioester intermediates with concomitant macrolactonization, a reaction catalyzed by a dedicated thioesterase (TE) domain located at the C-terminus of PICS module 6.[3,4]

Recently, we have functionally expressed the PICS TE domain in *Escherichia coli* and characterized the purified recombinant enzyme mechanistically and kinetically.[5] The crystal structures of the TE domains of both PICS and the closely related 6-deoxyerythronolide B synthase (DEBS TE)[6] show that each protein, which belongs to the α,β-hydrolase family, possesses a large substrate binding tunnel suitable for lactonization of polyketide

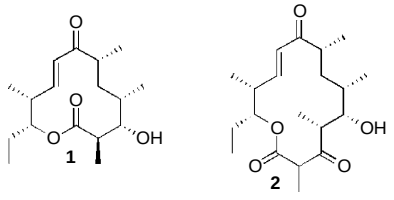


Figure 1. 10-Deoxymethynolide (**1**) and narbonolide (**2**).

acyl thioester substrates.[7] The reaction itself involves a classical Ser-His-Asp catalytic triad and a covalent acyl-enzyme intermediate.[6,7]

Both TE domains have considerable catalytic flexibility, as evidenced by their ability to support the formation of 6-, 8-, 12-, 14-, and 16-membered ring lactones when artificially fused to the C-termini of appropriate PKS modules.[8] On the other hand, the corresponding recombinant excised TE domains have to date only been shown to mediate the hydrolysis of model acyl *N*-acetylcysteamine acylthioester (SNAC) substrates, including long-chain ω-1-hydroxyacyl-SNAC analogues.[6] By contrast, the recombinant TE domain of the related epothilone synthase can catalyze the macrolactonization of the SNAC-thioester of *seco*-epothilone C to give the 14-membered lactone epothilone C, accompanied by significant levels of competing substrate hydrolysis.[9]

Keywords: Antibiotics; Biosynthesis; Polyketides; Synthase; Thioesterase; Macrolactonization.
* Corresponding author. E-mail: david_cane@brown.edu

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.09.077

Similarly, recombinant TE domains from the nonribosomal peptide synthetases for tyrocidine, gramicidin, and pristinamycin synthetase, can also catalyze efficient macrolactamization and macrolactonization of a wide variety of acyclic peptide and depsipeptide substrates, again accompanied by varying proportions of hydrolysis.[10]

We now report that both recombinant PICS TE and DEBS TE can rapidly and efficiently catalyze the cyclization of the *seco*-SNAC-thioester **3** to 10-deoxymethynolide (**1**), without any detectable competing hydrolysis. By contrast, the same enzymes mediate the exclusive hydrolysis of the reduced analogue, *seco*-7-dihydro-10-deoxymethynolide (**4**), to the corresponding *seco*-acid **5**.

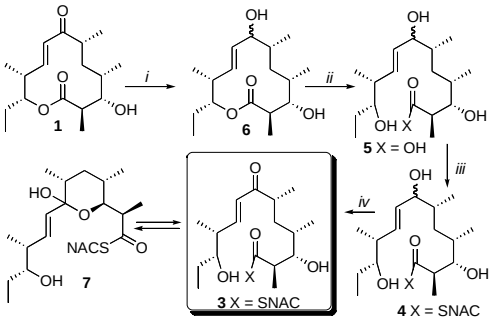
In designing an appropriate substrate for TE-catalyzed cyclization, we speculated that the highly substituted *seco*-ester of a natural macrolide aglycone might be intrinsically prone to macrolactonization. The majority of known macrolide aglycones, however, carry a hydroxyl group at C-5, which would favor non-enzyme-catalyzed conversion of the derived *seco*-SNAC-thioesters to the corresponding δ-lactones. We therefore chose to prepare **3**, the *seco*-SNAC-ester of 10-deoxymethynolide (**1**),[2] rather than that of narbonolide (**2**).

Treatment of **1** with $NaBH_4/CeCl_3$ afforded 7-dihydro-10-deoxymethynolide (**6**) as a ~3:1 mixture of 7*S*/7*R* diastereomers (50% yield), as determined by ^1H NMR (Scheme 1).[11,12] Hydrolysis of **6** with 0.5 M LiOH (1:1 MeOH–H_2O, reflux, 2 days) afforded the corresponding reduced *seco*-acid **5** in 50% yield, without detectable epimerization of the C-2 methyl group. The *seco*-acid **5** was converted to the 7-dihydro-SNAC-thioester **4** ($(PhO)_2PON_3$, HSNAC, Et_3N; 55% yield), which in turn could be selectively oxidized to *seco*-SNAC-thioester **3** by treatment with MnO_2 (CH_2Cl_2; 35% yield).[12] ^1H and ^{13}C NMR analysis of **3** indicated that it was in equilibrium with 50–60% of the cyclic hemiacetal **7** in $CDCl_3$.[12,13]

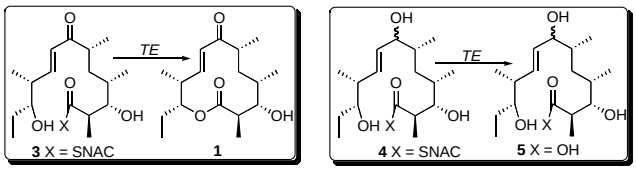
Incubation of thioester **3** with purified recombinant PICS TE[5] resulted in exclusive macrolactonization to give 10-deoxymethynolide (**1**), with no detectable competing hydrolysis to the corresponding *seco*-acid, as monitored by RP-HPLC with UV detection (Scheme 2).[12] In preparative scale reactions, **3** was completely consumed over 18 h, indicating that the hemiacetal form **7** had been cyclized as well, presumably by prior reversion to the ketone **3**. The formation of macrolactone **1** was confirmed by direct ^1H NMR and MS comparison with an authentic sample. By contrast, under the same conditions PICS TE did not cyclize the corresponding 7-dihydro-*seco*-thioester **4**, instead yielding only the hydrolysis product **5**. Addition of the non-ionic detergent Brij58, previously reported to enhance the proportion of TE-catalyzed cyclization relative to hydrolysis of acyclic substrates,[10b] did not lead to any detectable formation of macrolactone **6**. These observations were rigorously confirmed by radio-HPLC analysis of the reaction conducted using [1,3,5,7,9,11-^{14}C]-**4**, [^{14}C]-**5**, and [^{14}C]-**6** standards.[12,14]

We also found that the closely related thioesterase, DEBS TE, which normally mediates the biosynthesis of the 14-membered ring macrolide aglycone 6-deoxyerythronolide B and which resembles PICS TE in structure and mechanism of action,[7,8] showed the same pattern of reaction specificity as PICS TE, catalyzing the cyclization of **3** to **1** but exclusively hydrolyzing the reduced *seco*-NAC-thioester **4** to the *seco*-acid **5**, albeit at reduced rates compared to PICS TE (Scheme 2 and Table 1).

The rate of macrolactonization of *seco*-ester **3** is pH-dependent, with a maximum at pH 7.7. The steady-state kinetic parameters for the lactonization and hydrolysis reactions of **3** and **4**, respectively, were therefore determined at pH 7.7 for both PICS TE and DEBS TE (Table 1), varying the concentration of each substrate and directly fitting the data to the Michaelis–Menten equation. Although both TE domains catalyzed the exclusive macrolactonization of **3** to 10-deoxymethynolide (**1**), PICS TE was by far the superior cyclization catalyst, with observed values of k_{cat} and k_{cat}/K_m for **3** that were ~150-fold greater than those displayed by DEBS TE. Interestingly, the K_m for the acyclic hexaketide **3** was about the same for both thioesterases, in spite of the fact that the natural substrate for DEBS TE is a heptaketide thioester. The maximum rate of hydrolysis by PICS TE of the reduced substrate **4** was ~8-fold lower than the k_{cat} for cyclization of the natural substrate **3**, while the corresponding K_m for hydrolysis of **4** was actually ~4-fold lower, resulting in only a net ~2-fold decrease in specificity constant k_{cat}/K_m for the two substrates. Intriguingly, while PICS TE had a k_{cat}/K_m for cyclization of **3** that was twice the measured k_{cat}/K_m for hydrolysis of **4**, DEBS TE showed a ~20-fold preference in k_{cat}/K_m for hydrolysis of **4** over cyclization of **3**.



Scheme 1. Syntheses of **3** and **4**. Reagents: (i) $NaBH_4$, $CeCl_3$; (ii) LiOH, Δ, MeOH/H_2O; (iii) (a) $(PhO)_2PON_3$, (b) HSNAC; (iv) MnO_2, CH_2Cl_2.



Scheme 2. PICS and DEBS TE-catalyzed macrolactonization and hydrolysis of **3** and **4**.

Table 1. Steady state kinetic parameters for PICS and DEBS TE

Substrate	TE	Reaction	k_{cat} (min^{-1})	K_m (mM)[a]	k_{cat}/K_m (M^{-1} s^{-1})[a]
3	PICS	Cyclization	54.4 ± 5.8	4.1 ± 0.8	221 ± 50
	DEBS	Cyclization	0.36 ± 0.09	3.9 ± 1.7	1.5 ± 0.8
4	PICS	Hydrolysis	6.6 ± 0.9	1.1 ± 0.4	100 ± 44
	DEBS	Hydrolysis	4.5 ± 1.0	2.5 ± 1.3	31 ± 19

[a] The values of K_m and k_{cat}/K_m for **3** are uncorrected for the presence of hemiacetal **7** since the fraction of **7** present under the incubation conditions is unknown and the two forms are rapidly interconverted.

As previously reported,[5] 2-methyl-3-ketopentanoyl-SNAC, a diketide analogue of the natural acyclic 3-keto-heptaketide precursor of narbonolide, has a k_{cat} for hydrolysis of 56 min^{-1}, a value that is essentially equal to the k_{cat} determined for PICS TE-catalyzed macrolactonization of **3**, while the corresponding k_{cat}/K_m for hydrolysis of this 3-ketodiketide is 3.5-fold lower than that for cyclization of **3** by PICS TE. Notably, the k_{cat}/K_m previously determined for hydrolysis of the reduced diketide, (2*R*,3*S*)-2-methyl-3-hydroxypentanoyl-SNAC,[5] is >20-fold lower than that determined here for cyclization of (2*R*,3*S*)-2-methyl-3-hydroxy-**3**.

PICS TE is unique among recombinant PKS and NRPS TE domains characterized to date, in that it can catalyze exclusive in vitro macrolactonization of its natural substrate without competing hydrolysis.[9,10] The observed enhancement in the rates of cyclization as well as the previously noted active site flexibility observed for helices 6 and 7 of PICS TE[7] makes PICS TE an attractive component of the tool kit for engineering chimeric PKS modules with superior catalytic properties.[15]

Acknowledgments

This research was supported by NIH Grants GM22172 (D.E.C.) and CA66736 (C.K.).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.09.077.

References and notes

1. (a) Omura, S.; Takeshima, H.; Nakagawa, A.; Miyazawa, J. *J. Antibiot.* **1976**, *29*, 316; (b) Hori, T.; Maezawa, I.; Nagahama, N.; Suzuki, M. *J. Chem. Soc. Chem. Commun.* **1971**, 304.
2. Lambalot, R. H.; Cane, D. E. *J. Antibiot.* **1992**, *45*, 1981.
3. (a) Xue, Y.; Zhao, L.; Liu, H.-w.; Sherman, D. H. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 12111; (b) Tang, L.; Fu, H.; Betlach, M. C.; McDaniel, R. *Chem. Biol.* **1999**, *6*, 553; (c) Yin, Y.; Lu, H.; Khosla, C.; Cane, D. E. *J. Am. Chem. Soc.* **2003**, *125*, 5671; (d) Beck, B. J.; Aldrich, C. C.; Fecik, R. A.; Reynolds, K. A.; Sherman, D. H. *J. Am. Chem. Soc.* **2003**, *125*, 12551.
4. Beck, B. J.; Yoon, Y. J.; Reynolds, K. A.; Sherman, D. H. *Chem. Biol.* **2002**, *9*, 575.
5. Lu, H.; Tsai, S. C.; Khosla, C.; Cane, D. E. *Biochemistry* **2002**, *41*, 12590.
6. Gokhale, R. S.; Hunziker, D.; Cane, D. E.; Khosla, C. *Chem. Biol.* **1999**, *6*, 117.
7. (a) Tsai, S.-C.; Lu, H.; Cane, D. E.; Khosla, C.; Stroud, R. M. *Biochemistry* **2002**, *41*, 12598; (b) Tsai, S.-C.; Miercke, L. J. W.; Krucinski, J.; Gokhale, R.; Chen, J. C. H.; Foster, P. G.; Cane, D. E.; Khosla, C.; Stroud, R. M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14808.
8. (a) Khosla, C.; Gokhale, R. S.; Jacobsen, J. R.; Cane, D. E. *Annu. Rev. Biochem.* **1999**, *68*, 219; (b) 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, p 495.
9. Boddy, C. N.; Schneider, T. L.; Hotta, K.; Walsh, C. T.; Khosla, C. *J. Am. Chem. Soc.* **2003**, *125*, 3428.
10. (a) Trauger, J. W.; Kohli, R. M.; Walsh, C. T. *Biochemistry* **2001**, *40*, 7092; (b) Kohli, R. M.; Trauger, J. W.; Schwarzer, D.; Marahiel, M. A.; Walsh, C. T. *Biochemistry* **2001**, *40*, 7099; (c) Yeh, E.; Lin, H.; Clugston, S. L.; Kohli, R. M.; Walsh, C. T. *Chem. Biol.* **2004**, *11*, 1573; (d) Mahlert, C.; Sieber, S. A.; Grunewald, J.; Marahiel, M. A. *J. Am. Chem. Soc.* **2005**, *127*, 9571.
11. Attempted enzymatic hydrolysis of 10-deoxymethynolide (**1**) or transesterification with HSNAC using PICS TE or DEBS TE resulted in either recovery of unreacted starting material or decomposition due to competing conjugate addition of *N*-acetylcysteamine. Treatment of **1** with aq acid or base caused substantial decomposition.
12. See Supplementary Data for experimental details.
13. After completion of this work and while this manuscript was in preparation, Sherman reported an essentially equivalent preparation of *seco*-SNAC-thioester **3** from 10-deoxymethynolide (**1**). See: Aldrich, C. C.; Beck, B. J.; Fecik, R. A.; Sherman, D. H. *J. Am. Chem. Soc.* **2005**, *127*, 8441. In the same report, Sherman also observed that incubation of **3** with recombinant PICS module 6+TE in the presence of [2-^{14}C]methylmalonyl-CoA resulted in polyketide chain elongation and formation of narbonolide (**2**). They were unable, however, to monitor the competing direct cyclization of unlabeled **3** to unlabeled 10-deoxymethynolide (**1**) using a radio-TLC-based assay. We have independently carried out analogous incubations with PICS module 6+TE and have observed the formation of a ~4:1 mixture of both 10-deoxymethynolide (**1**) and narbonolide (**2**), with a k_{cat} of 18 min^{-1} for the formation of **1** at 20 °C (K_m **3**: 3.3 mM; k_{cat}/K_m **3**: 90 $M^{-1}s^{-1}$). (Wu, J.; He, W.; Khosla, C.; Cane, D.E. Angew. Chemie **2005**, *44*, in press.) Not only is 10-deoxymethynolide (**1**) the major product of the action of PICS module 6+TE on the *seco*-hexaketide thioester **3**, but, as reported here, the free recombinant TE domain actually catalyzes the direct cyclization of **3** with a k_{cat} three times *faster* than that of the complete PICS module 6+TE. These combined observations appear to be at odds with earlier

reports[4] that in vivo formation of 10-deoxymethynolide in engineered strains of *S. venezuelae* from the natural hexaketide substrate requires an intact PICS module 6 with active ketosynthase-6, acyl transferase-6, and acyl carrier protein-6 domains.

14. [1,3,5,7,9,11-^{14}C]-**1** was isolated from a culture of *S. venezuelae*, grown in the presence of the inhibitor xanthotoxin, as previously described,[3] and supplemented with [1-^{14}C]propionate. The purified [^{14}C]-**1** was converted to labeled **3**, **4**, **5**, and **6** by the procedure described.
15. While this manuscript was in preparation, Sherman independently described the cyclization of **3** and hydrolysis of **5** catalyzed by PICS TE. Although they reported that they were unable to measure directly the individual k_{cat} and K_m parameters for the cyclization, using the linear portion of the v versus S plot at low substrate concentrations they estimated the k_{cat}/K_m value to be 1.67 M^{-1} min^{-1}, nearly an order of magnitude less than the value of 13.5 M^{-1} min^{-1} reported here (cf. Table 1). See: Aldrich, C. C.; Venkatraman, L.; Sherman, D. H.; Fecik, R. A. *J. Am. Chem. Soc.* **2005**, *127*, 8910.