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Substrate Spectrum of Tyrocidine Thioesterase Probed with Randomized Peptide *N*-Acetylcysteamine Thioesters

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Abstract—Apparent kinetic constants k_{cat} and K_m were determined for tyrocidine thioesterase (TycC TE) using randomized peptide *N*-acetylcysteamine thioesters as substrate analogues. The enzyme has been found to be adequately active for the synthesis of positional-scanning libraries for novel antibiotic screening with reduced k_{cat}/K_m in the range of 2 to 82 folds lower than that of the wild-type sequence

Introduction

Widespread antibiotic resistance in pathogenic microbes has become a serious health problem worldwide and has impelled researchers to explore new sources of antibiotics.[1] One of the more attractive approaches that promise to generate new antibiotics utilizes strategies in combinatorial biosynthesis by taking advantage of numerous naturally occurring enzymes involved in the biosynthesis of natural products, such as polyketides and polypeptides.[2] These enzymes, called polyketide synthase (PKS) or non-ribosomal peptide synthases (NRPS), are large and multifunctional proteins modularly organized in the same order of ketide or peptide sequences in natural products.[3] By functional domain swapping, deletion or addition, and 're-programming' of these synthases, natural product-like compounds have been successfully obtained.[4] As an alternative, enzyme-catalyzed synthesis of these natural product analogues can be achieved using thioesterases (TE) at the carboxyl terminus of NRPSs which have been recently found to retain cyclization activity and possess a broad substrate profile in their putative forms.[5] The latter approach may be advantageous since only one enzyme is involved and linear substrate precursors are easily accessible with conventional chemical methods.

In the biosynthesis of polyketide- and polypeptide-based antibiotics, thioesterases are ubiquitously integrated at the carboxyl terminus of synthases that cyclize or hydrolyze the growing linear chains to afford the desired natural products. It has been shown that most of these enzymes, if not all, use a conserved catalytic triad consisting of a serine, a histidine, and an acidic residue (Asp or Glu).[6] The catalysis involves sequential acylation and deacylation of the active-site serine residue at a conserved GXSXG motif.[7] The acylation step involves the transfer of an acyl group from a thiol on an acyl-carrier protein to the active site serine. Deacylation of the resulting acyl-*O*-TE complex occurs either by hydrolysis or intramolecular cyclization to form a macrolactone- or macrolactam-based natural product. In the isolated form, a thioesterase, such as that in 6-deoxyerythronlide B synthase,[7b,8] may lose its cyclization capability and therefore demonstrates only hydrolytic activity towards the corresponding substrate.

Recently, putative thioesterases in NRPSs such as that of tyrocidine and gramicidin were found to be active towards their linear substrate analogues possessing variable chain length (6–14 aa residues) and backbone rigidity and to correctly cyclize them into the expected products.[5] In addition, it has been shown that the N-terminal D-Phe and the penultimate ornithine (Orn) at the C-terminus of the wild-type substrate are critical for the cyclization to occur efficiently.[5] These findings suggest that it is possible to utilize these enzymes for the

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synthesis of natural product analogues as a means of antibiotics engineering. However, effects of the sequence variation at remaining positions of the wild-type sequence over the cyclization rate of these enzymes remain unclear. In order to address this issue, and to further explore the general feasibility of using these enzymes for combinatorial synthesis of peptide-based macrocyclic library for novel antibiotics screen, we have thus far synthesized positional-scanning peptide libraries based on the wild-type substrate for tyrocidine thioesterase (Fig. 1), and used them to establish the substrate specificity as well as the cyclization activity of this enzyme.

Since *N*-acetylcysteamine (NAC) has been shown in previous studies to be a good mimic of the wild-type phosphopantetheine tether that links the growing peptide chain to the peptidyl carrier protein (PCP) in NRPS,[5,8] the positional-scanning libraries were therefore designed to possess a general structure containing D-Phe-XXXXXXX-Orn-Leu-SNAC as shown in Figure 1, with NAC linked with the C-terminus of the peptide via a thioester bond. The N-terminal D-Phe and C-terminal Orn-Leu, present in the wild-type tyrocidine biosynthetic precursor at their corresponding positions, were retained in the libraries since the two residues, D-Phe and Orn, had been previously shown to be critical for enzymatic recognition and catalysis,[5] whereas the terminal Leu was kept intact for convenient solid-phase synthesis. Each of the remaining seven positions (**O_1**–**O_7**) in the wild-type sequence of tyrocidine was positionally scanned, one position at a time, with all 20 proteingenic amino acids. In all, a total of 140 different libraries were synthesized and tested against TycC TE.

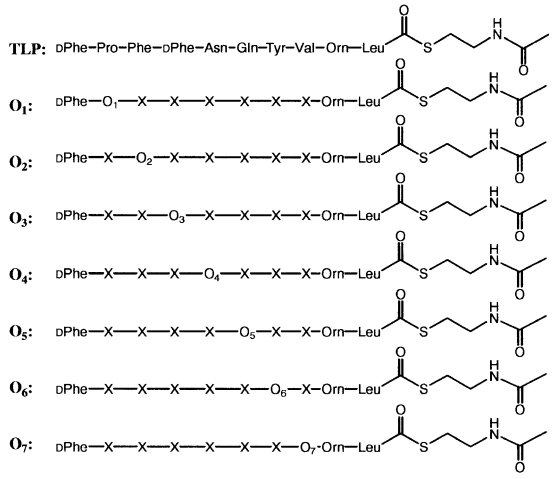

Figure 1. Design of the positional-scanning libraries to probe the substrate specificity of tyrocidine thioesterase (TycC TE). The libraries consist of seven separate sub-libraries (**O_1-O_7**), each composed of 20 different peptide mixtures having a single position defined with one of the 20 natural amino acids (represented as O), and the remaining six positions are composed of an isokinetic mixture of 19 amino acids (represented as X; cysteine is omitted). All libraries contain a D-Phe N-terminus and an Orn-Leu-NAC C-terminus. The wild-type Tyrocidine sequence is denoted as **TLP**.

Results and Discussion

Using a standard positional-scanning protocol as previously described,[9] the 140 peptide libraries were synthesized on a 2-chlorotrityl resin (derivatized with Fmoc-Leu-OH) using protocols developed by Walsh et al. for the synthesis of the wild-type tyrocidine substrate.[5] After cleavage with 1:4 acetic acid/dichloromethane (3 h, 24 °C), the protected peptides were precipitated with *n*-hexane, followed by drying in vacuo. The resulting products (1 equiv) were redissolved in tetrahydrofuran (THF) or dimethylformamide (DMF), followed by addition of DIC (5 equiv), HOBt (5 equiv) and *N*-acetylcysteamine (10 equiv). After 30 min incubation at 24 °C, DIEA (0.6 equiv) was added and the reaction was continued overnight. Upon concentration in vacuo, the protected peptide–SNAC libraries were treated with reagent R (90% TFA, 5% thioanisol, 3% anisole, 2% Ethanedithiol; 3 h, 24 °C), and precipitated with cold ether. Extensive washing (at least six times) with cold ether was applied to remove residual thiol impurities from the libraries in order to minimize background readings in subsequent experiments. The wild-type tyrocidine substrate was similarly synthesized as a positive control to ensure the quality of the peptide libraries. Accurate quantitation of the resulting peptide libraries was achieved through determination of the thioester content (peptide–SNAC) in each peptide mixture. This was done, with Ellman's reagent,[10] by measuring the release of free thiol (NAC) from the peptide thioester following complete hydrolysis (0.1 N NaOH, 1 h).

The tyrocidine thioesterase (TycC TE) was amplified from *Bacillus brevis* (ATCC8185) genomic DNA using primers 5′-CGCGAATTGGCCCAGCCGGCCATGGC CCGCTTTGAGAGCAGATACGGC-3′ and 5′-GGCCA ATTCGGCCGCTTTCAGGATGAACAGT-TCTTGC-3′ and Deep Vent DNA polymerase (New England Biolabs). The amplification product was digested with *Sfi* I and *Eag* I and subsequently cloned into pFAB5c. His vector[11] digested with the same enzymes. The cloned TycC TE gene was verified by DNA sequencing and expressed with a 6×His tag at the C-terminus in *Escherichia coli XL1-Blue* (Stratagene) in Luria broth at 30 °C. The 29 kD thioesterase was purified from the periplasm with metal-chelating affinity chromatography in high yield (5 mg L^{-1}) and high purity (>95% pure by SDS-PAGE).

Enzymatic activity of TycC TE towards the positional-scanning peptide libraries was assayed in 500 μL of 25 mM MOPS buffer (pH 7.0) at room temperature. Reactions were initiated by addition of TycC TE and quenched at various time points by addition of 200 μL of DMSO followed by flash freezing in liquid nitrogen. Aliquots (250 μL) were then thawed, added another 200 μL of DMSO and the total thiol amount was determined by addition of 1 μL of 0.5 M 2,2′-dithiodipyridine and measurement of the saturated absorbance at 350 nm ($\varepsilon = 1.3\times10^4$ M^{-1} cm^{-1} under given conditions).[12] For each peptide mixture, a pilot reaction, without addition of the thioesterase, was run in parallel with the

enzymatic reaction as a control to account for the background thioester hydrolysis. After subtracting the background (A_{350}) from that of the total thiol, the enzyme-catalyzed release of thiol was plotted against reaction time to determine the initial reaction rate ($t=0$). Initial rates at various substrate concentrations were subsequently determined and used to derive apparent constants, k_{cat} and K_m, with Lineweaver–Burke plot. A simplified version of this method was also used to determine kinetic constants for some substrate analogues. Briefly, by using an excessive amount of the substrate relative to that of the enzyme, the rate of the enzyme-catalyzed reaction at the initial 2 min remained constant and therefore could be determined from the released thiol using one data point. This simplified method was found to give practically identical results.

Using the assay developed above, k_{cat} and K_m for the wild-type substrate sequence were determined to be 44 min^{-1} and 9.2 μM, respectively. In comparison with results obtained from HPLC-based assay reported by others,[5a] and independently confirmed by us, k_{cat} is comparable (60 min^{-1} by HPLC) while K_m is 3-fold larger (3 μM by HPLC). The standard deviation of this method is ±15%, consistent with that of the HPLC assay.

Every member in a given positional-scanning sub-library (**O_1–O_7**) contains a large number of sequences, and is mostly similar except for the particular amino acid at the scanned position. To avoid the laborious determination of kinetic constants for each substrate analogue mixture in all libraries, we randomly chose one sub-library (**O_3**, Fig. 1) and determined the apparent k_{cat}, K_m for each library member. To assess the positional effect of one particular amino acid in the substrate analogue on the kinetic constants, library members containing a randomly chosen amino acid, asparagine (N), at all seven scanned positions (**O_1-Asn** to **O_7-Asn**, Table 1) were determined for TycC TE activity. To further ensure the comprehensive representation of the whole library by data obtained from the above chosen library members, nine other members were randomly chosen for determination of the apparent k_{cat} and K_m. Kinetic data obtained from all chosen peptide mixtures are summarized in Table 1 and graphically represented in Figure 2.

Comparison of kinetic constants obtained from the positional-scanning libraries and from the wild-type sequence (**TLP**) shows that, discrete amino acids at individually scanned positions indeed have a significant effect on the k_{cat} and K_m. The Michaelis constant of all library members tested ranges from 0.5- to 26-fold of the wild-type sequence **TLP**, whereas the rate constant, decreased by 1.5- to 7-fold compared to that for **TLP**, is less affected by variations in the amino acid side chain.

In comparison with the k_{cat}/K_m for **TLP**, the apparent catalytic efficiency of TycC TE is reduced by 2 to 82-fold for the positional-scanning libraries. The relatively mild reduction in k_{cat}/K_m is consistent with the previous

Table 1. Kinetic constants of enzymatic reactions catalyzed by TycC TE towards the randomly chosen positional-scanning library members

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
TLP	9.2	44	4.78	**O_3-Ser**	14	7.8	0.57	**O_5-Asn**	112	80	0.72
O_3-Val	145	27	0.19	**O_3-Thr**	31	8.1	0.26	**O_6-Asn**	7.6	20	2.63
O_3-Leu	29	8.3	0.28	**O_3-Lys**	15	14	0.92	**O_7-Asn**	55	12	0.22
O_3- Ile	32	11	0.33	**O_3-Arg**	24	12	0.51	**O_1-Asp**	96	10	0.11
O_3-Met	20	16	0.80	**O_3-Tyr**	65	11	0.17	**O_1-Ala**	31	7.0	0.23
O_3-Phe	4.8	6.3	1.30	**O_3-His**	82	12	0.15	**O_4-Val**	25	7.2	0.29
O_3-Asp	238	14	0.058	**O_3-Pro**	132	15	0.11	**O_4-Leu**	42	10	0.23
O_3-Asn	19	8.0	0.42	**O_3-Cys**	27	6.6	0.25	**O_5-Val**	54	9.6	0.18
O_3-Glu	12	6.6	0.57	**O_3-Trp**	7.5	7.2	0.95	**O_5-Leu**	135	43	0.32
O_3-Gln	16	7.0	0.43	**O_1-Asn**	82	7.0	0.084	**O_5-Ile**	82	7.4	0.090
O_3-Gly	22	8.0	0.36	**O_2-Asn**	172	14	0.083	**O_7-Gln**	62	14	0.22
O_3-Ala	19	11	0.60	**O_4-Asn**	13	33	2.65	**O_7-Arg**	33	9.0	0.28

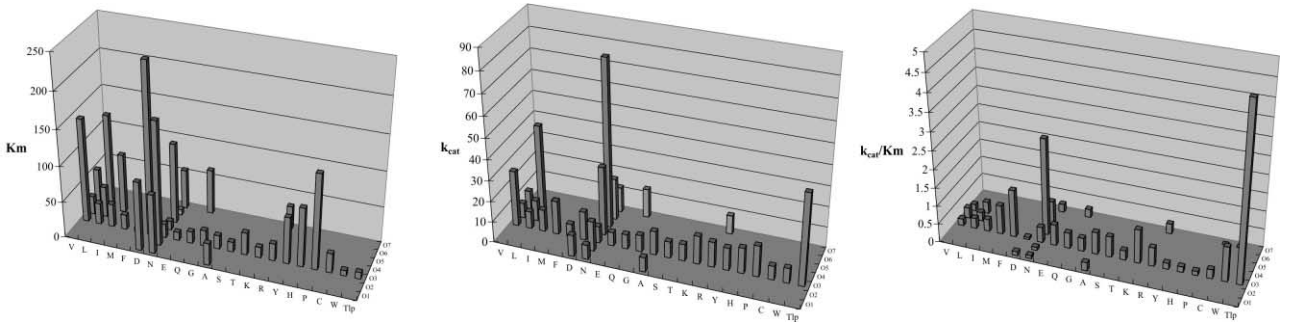

Figure 2. Graphic representation of the kinetic constants of tyrocidine thioesterase-catalyzed reactions towards randomly selected positional-scanning library members: (A) Michaelis constant (K_m); (B) rate constant (k_{cat}); (C) catalytic efficiency (k_{cat}/K_m). Structures of the sub-library **O_1–O_7** are shown in Figure 1.

observations that the N-terminal D-Phe and the C-terminus Orn play a significant role in determining the substrate recognition by the thioesterase.[5] However, a factor of up to 82-fold reduction in k_{cat}/K_m indicates that the side chain combination at remaining positions is also important for the thioesterase activity. In addition, in view of the favorable kinetic data, the fact that **O_3** position is a D-amino acid in the wild-type substrate indicates that D-configuration is not a determining structural factor for the thioesterase's activity.

For library members with the chosen Asn at all seven randomized positions and other nine randomly chosen mixtures, their kinetic constants fall within the same range as the **O_3** sub-library, showing little or no apparent correlation between the catalytic efficiency of the enzyme and the structure of different amino acid side chains. This result supports that the obtained data is likely to be truly representative of the entire positional scanning libraries.

Conclusion

By determining apparent kinetic constants for the enzymatic reaction catalyzed by tyrocidine thioesterase (TycC TE) towards positional-scanning peptide libraries derived from the wild-type tyrocidine substrate, it has been found that side chain structures and sequence combinations of the nonessential residues in the wild-type substrate moderately reduced the catalytic efficiency of the putative enzyme. No apparent correlation was found between the variation in enzyme activity and the property or location of the side chain in the substrate analogue. Specifically, tyrocidine thioesterase was found to be less efficient by a factor of 2- to 82-fold in catalytic efficiency towards the positional-scanning libraries in comparison with that for the wild-type substrate sequence **TLP**.

Although the ratio of cyclic versus hydrolytic products of the thioesterase-catalyzed reaction could not be determined experimentally due to difficulties in discerning cyclic and linear peptides on HPLC because of the diverse chemical entities present in the substrate analogue mixtures, cyclization is expected to be largely favored over hydrolysis ($>10^5$ times) based on previous investigations.[5] On the other hand, although being less active by up to 82-fold, tyrocidine thioesterase should be sufficiently active to turnover the randomized substrate analogues due to its high activity towards the wild-type substrate. Therefore, it is expected that this enzyme is adequately active for the synthesis of other positional-scanning cyclic peptide libraries for future antibiotic screens.

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References and Notes

1. (a) Neu, H. C. *Science* **1992**, *257*, 1064. (b) Cohen, M. L. *Science* **1992**, *257*, 1050. (c) Cohen, M. L. *Nature* **2000**, *406*, 762.
2. (a) Khosla, C.; Zawada, R. J. *Trends Biotech*. **1996**, *14*, 335. (b) Cane, D. E.; Walsh, C. T.; Khosla, C. *Science* **1998**, *282*, 63. (c) Staunton, J.; Wilkinson, B. *Curr. Opin. Chem. Biol*. **2001**, *5*, 159.
3. (a) Hopwood, D. A. *Chem. Rev*. **1997**, *97*, 2465. (b) von Döhren, H.; Keller, U.; Vater, J.; Zocher, R. *Chem. Rev*. **1997**, *97*, 2675. (c) Marahiel, M. P.; Stachelhaus, T.; Mootz, H. D. *Chem. Rev*. **1997**, *97*, 2651.
4. (a) Jacobsen, C. J.; Hutchinson, C. R.; Cane, D. E.; Khosla, C. *Science* **1997**, *277*, 367. (b) Khosla, C. *Chem. Rev*. **1997**, *97*, 2557. (c) Carreras, C. W.; Santi, D. V. *Curr. Opin. Biotech*. **1998**, *9*, 403.
5. (a) Trauger, J. W.; Kohli, R. M.; Mootz, H. D.; Marahiel, M. A.; Walsh, C. T. *Nature* **2000**, *407*, 215. (b) Trauger, J. W.; Kohli, R. M.; Walsh, C. T. *Biochemistry* **2001**, *40*, 7092. (c) Kohli, R. M.; Trauger, J. W.; Schwarzer, D.; Marahiel, M. A.; Walsh, C. T. *Biochemistry* **2001**, *40*, 7099.
6. Lawson, D. M.; Derewenda, U.; Serre, L.; Ferri, S.; Szittner, R.; Wei, Y.; Meighen, E. A.; Derewenda, Z. S. *Biochemistry* **1994**, *33*, 9382.
7. (a) Shaw-Reid, C. A.; Kelleher, N. L.; Losey, H. C.; Gehring, A. M.; Berg, C.; Walsh, C. T. *Chem. Biol*. **1999**, *6*, 385. (b) Aggarwal, R.; Caffrey, P.; Leadlay, P. F.; Smith, C. J.; Staunton, J. *J. Chem. Soc., Chem. Comm*. **1995**, *15*, 1519.
8. Gokhale, R. S.; Hunziker, D.; Cane, D. E.; Khosla, C. *Chem. Biol*. **1998**, *6*, 117.
9. Pinilla, C.; Appel, J. R.; Houghten, R. A. *Biochem. J*. **1994**, *301*, 847.
10. Ellman, G. L. *Arch. Biochem. Biophys*. **1959**, *82*, 70.
11. Orum, H.; Andersen, P. S.; Oster, A.; Johansen, L. K.; Riise, E.; Bjørnvad, M.; Svendsen, I.; Engberg, J. *Nucleic Acids Res*. **1993**, *21*, 4491.
12. Egwim, I. O. C.; Gruber, H. J. *Anal. Biochem*. **2001**, *288*, 188.