Cyclization of Backbone-Substituted Peptides Catalyzed by the Thioesterase Domain from the Tyrocidine Nonribosomal Peptide Synthetase†

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ABSTRACT: The excised C-terminal thioesterase (TE) domain from the multidomain tyrocidine nonribosomal peptide synthetase (NRPS) was recently shown to catalyze head-to-tail cyclization of a decapeptide thioester to form the cyclic decapeptide antibiotic tyrocidine A [Trauger, J. W., Kohli, R. M., Mootz, H. D., Marahiel, M. A., and Walsh, C. T. (2000) *Nature 407*, 215−218]. The peptide thioester substrate was a mimic of the TE domain's natural, synthetase-bound substrate. We report here the synthesis of modified peptide thioester substrates in which parts of the peptide backbone are altered either by the replacement of three amino acid blocks with a flexible spacer or by replacement of individual amide bonds with ester bonds. Rates of TE domain catalyzed cyclization were determined for these substrates and compared with that of the wild-type substrate, revealing that some parts of the peptide backbone are important for cyclization, while other parts can be modified without significantly affecting the cyclization rate. We also report the synthesis of a modified substrate in which the N-terminal amino group of the wild-type substrate, which is the nucleophile in the cyclization reaction, is replaced with a hydroxyl group and show that this compound is cyclized by the TE domain to form a macrolactone at a rate comparable to that of the wild-type substrate. These results demonstrate that the TE domain from the tyrocidine NRPS can catalyze cyclization of depsipeptides and other backbone-substituted peptides and suggest that during the cyclization reaction the peptide substrate is preorganized for cyclization in the enzyme active site in part by intramolecular backbone hydrogen bonds analogous to those in the product tyrocidine A.

A highly diverse array of medicinally useful natural products are biosynthesized by modular nonribosomal peptide synthetases (NRPSs),[1] polyketide synthases (PKSs), and hybrid NRPS/PKS systems (*1*−*3*). Many such natural products have a macrocyclic structure, including the antibiotics erythromycin and daptomycin, the immunosuppressants rapamycin and cyclosporin, the antifungal agent echinocandin, and the antitumor agent epothilone. Characterizing the specificity of the macrocyclization step during natural product biosynthesis is important for enzymatic approaches to the synthesis of novel compounds (*3*−*7*).

The macrocyclization step during the biosynthesis of nonribosomal peptides and polyketides is generally catalyzed by a 28−35 kDa thioesterase (TE) domain, which is present as the C-terminal domain of a multidomain enzymatic assembly line (*1*−*3*). The TE domain's natural substrate is the full-length linear peptide/polyketide chain attached to a phosphopantetheine tether in an adjacent carrier protein domain by a thioester linkage. The mechanism of TE catalysis, which is similar to that of serine proteases, involves transfer of the full-length acyl chain from phosphopantetheine to the TE active site serine residue, followed by deacylation of the resulting acyl-enzyme intermediate (*8*, *9*). Deacylation of the acyl enzyme can occur either by hydrolysis to form a linear product or by intramolecular reaction with a nucleophile in the acyl chain to form a cyclic product.

Biosynthesis of the cyclic decapeptide antibiotic tyrocidine A is catalyzed by the tyrocidine NRPS, which consists of three protein subunits, TycA (123 kDa), TycB (405 kDa), and TycC (724 kDa) (*10*). The C-terminal TE domain from the tyrocidine NRPS (TycC TE, 28 kDa) catalyzes head-to-tail cyclization of a decapeptide thioester intermediate and product release (Figure 1A). We recently reported that the isolated TycC TE, obtained by expression of a single domain construct, catalyzes efficient cyclization of the decapeptide thioester substrate D-Phe-Pro-Phe-D-Phe-Asn-Gln-Tyr-Val-Orn-Leu-SNAC (**TLP**, Orn = ornithine, SNAC = *N*-acetylcysteamine) to form tyrocidine A (*11*) (Figure 1B). By alanine scanning mutagenesis of the peptide thioester substrate, we found that recognition of only two amino acid side chains, D-Phe1 and Orn9, is critical for cyclization, while the other side chains can be changed without significantly affecting the maximum cyclization rate (*11*). This result suggested that TE domains could be used in engineered enzymatic assembly lines to form a wide variety of products

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[1] Abbreviations: NRPS, nonribosomal peptide synthetase; Orn, ornithine; PCP, peptidyl carrier protein domain; SNAC, *N*-acetylcysteamine; TE, thioesterase domain; TycC TE, TE domain from the tyrocidine NRPS.

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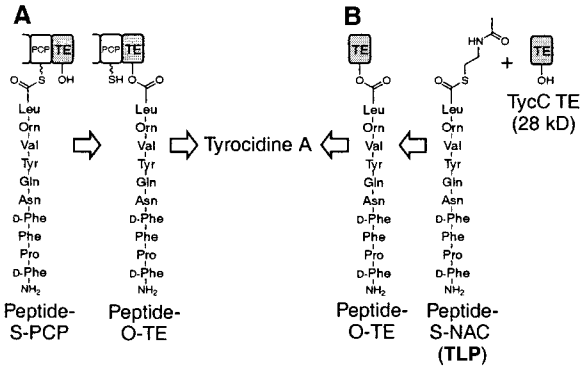

FIGURE 1: Peptide macrocyclization catalyzed by the thioesterase domain from the tyrocidine NRPS. (A) Mechanism of the macrocyclization step during tyrocidine A biosynthesis. Boxes represent individual protein domains from the C-terminal part of the TycC subunit of the tyrocidine NRPS: PCP, peptidyl carrier protein domain; TE, thioesterase domain. The thiol (SH) and hydroxyl (OH) groups represent phosphopantethiene and the TE active site serine residue, respectively. (B) Experimental system for probing the substrate specificity of the TycC TE-catalyzed macrocyclization reaction. The full-length TycC subunit (724 kDa) is replaced with the excised TycC TE domain (28 kDa), and the natural substrate peptide-S-PCP is replaced with peptide-S-NAC (**TLP**) (S-NAC = *N*-acetylcysteamine).

(*3*−*7*) and that isolated TE domains could be useful for the synthesis of macrocyclic compounds from linear thioester substrates. A key remaining question, which is relevant both to these potential applications and to the mechanism of TE catalyzed peptide cyclization, was whether all or part of the substrate's peptide backbone is necessary for cyclization.

Tyrocidine A has an antiparallel β-sheet structure stabilized by four hydrogen bonds, as evidenced by NMR data (*12*) and a crystal structure of the closely related compound gramicidin S (*13*) (Figure 2A). An NMR study of gramicidin S suggests that, in tyrocidine A, there may also be a hydrogen bond between the D-Phe1 carbonyl group and the ornithine δ-amino group (*14*) (Figure 2A). The well-defined structure of tyrocidine A suggested that "product-like" intramolecular hydrogen bonds may contribute to preorganization of the substrate (and perhaps also stabilization of the tetrahedral intermediate) for cyclization (Figure 2B).

We report here the synthesis and rates of TycC TE catalyzed cyclization of backbone-substituted analogues of the wild-type sequence substrate **TLP** (or the Pro2 to Ala mutant of the wild-type sequence, **P2A**, in two cases). To substitute the peptide backbone, we either replaced three amino acid blocks with a flexible spacer amino acid (substrates **S1**, **S2**, and **S3**) (Figure 3A) or replaced individual amide bonds with ester bonds (substrates **D1**, **D2**, **D3**, and **D4**) (Figure 5A). Replacement of amide bonds with ester bonds, which potentially eliminates two hydrogen bonds, has been used to probe the role of backbone hydrogen bonds in protein folding and catalysis and in protein−protein interactions (*15*−*22*). In addition, we report the synthesis and TycC TE catalyzed cyclization of the substrate **D5**, in which the N-terminal amino group in **TLP** is replaced with a hydroxyl group.

MATERIALS AND METHODS

Materials. Standard Fmoc-protected amino acid monomers were purchased from NovaBiochem. L-Lactic acid, L-phenyllactic acid, D-phenyllactic acid, and (*S*)-2-hydroxy-3-methylbutyric acid were purchased from Aldrich. Fmoc-8-amino-3,6-dioxaoctanoic acid was purchased from Neosystem (Strasbourg, France).

Synthesis of Peptide-SNAC and Depsipeptide-SNAC Substrates. Peptide-SNAC substrates were prepared using a Perseptive Biosystems 9050 synthesizer (0.3 mmol scale) on 2-chlorotrityl resin using Fmoc-protected monomers (side chain protecting groups used were trityl for Asn and Gln, *tert*-butyl for Tyr, and Boc for Orn) except for the N-terminal monomer, which was Boc-protected, and hydroxy acids, which had no protecting groups. Amino acid and hydroxy acids were incorporated using diisopropylcarbodiimide (DIPCDI)/1-hydroxybenzotriazole (HOBt) activation, except for couplings to hydroxy acids, for which DIPCDI/4-(dimethylamino)pyridine (DMAP) (3.0 equiv of amino acid, 0.15 equiv of DMAP) activation was used. The peptide was cleaved from the resin with 1:1:3 acetic acid/trifluoroethanol/dichloromethane (3 h, 24 °C) and then precipitated with *n*-hexane, and the solvent was removed by rotary evaporation. The protected peptide was then dissolved in tetrahydrofuran (THF), a solution of dicyclohexylcarbodiimide (DCC) (1.2 equiv) and HOBt (1.2 equiv) in THF and *N*-acetylcysteamine (2.5 equiv) added, and the reaction stirred (35 min, 24 °C). Potassium carbonate (0.6 equiv) was then added and the reaction stirred (3 h, 24 °C), filtered, and concentrated. Deprotection of the peptide-SNAC was carried out using 16:3:1 trifluoroacetic acid (TFA)/dichloromethane/*N*-acetylcysteamine (3 h, 24 °C) and precipitated with ether. Reverse-phase (C_{18}) HPLC purification (20−50% acetonitrile in 0.1% TFA/water over 30 min) afforded the peptide-SNAC TFA salt (10−25% yield from the protected peptide) in >95% purity (by analytical HPLC) as a white solid. The identities of the peptide-SNACs were verified by MALDI-TOF mass spectrometry carried out on a Perseptive Biosystems Voyager DE instrument (Table 1).

Protein Expression. TycC TE DNA was cloned, expressed with a C-terminal hexahistidine purification tag, and purified as described previously (*11*).

TycC TE-Catalyzed Macrocyclization. Reactions were carried out in 25 mM MOPS, pH 7.0, in a total volume of 400 μL. Reactions were initiated by addition of TycC TE, quenched after 1 min by the addition of 25 μL of 1.7% TFA/water, and stored at −80 °C. The reactions were then thawed, 85 μL of acetonitrile was added, and the products were analyzed by analytical HPLC (Beckman System Gold) with monitoring at 220 nm (20−100% acetonitrile in 0.1% TFA/water over 35 min, Vydac protein and peptide C_{18} column). Product formation was observed to be linear with time to 2 min with the tyrocidine linear thioester precursor **TLP**. For all other substrates, rates were assumed to be in the linear range at 1 min. Initial rates for all substrates were calculated using 1 min time points. No product formation was observed under these conditions with any of the substrates in the absence of TycC TE, with the exception of the substrate DPhe-Pro-O-Phe-DPhe-Asn-Gln-Tyr-Val-Orn-Leu-SNAC, for which spontaneous self-cleavage by DPhe-Pro diketopiperazine formation was observed (see Results section). The standard deviations for reported k_{obs}, k_{cat}, and K_M values are approximately ±15% (see ref *11*). Concentrations of enzymatic reaction products were determined by assuming that the peptide-SNAC and the products have equal extinction

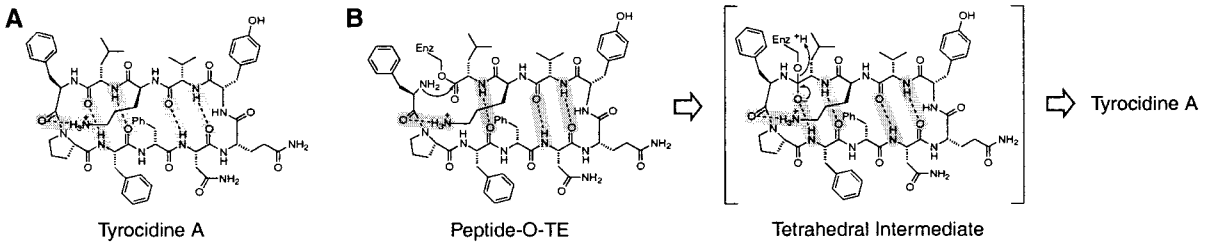

FIGURE 2: Models of tyrocidine A and macrocyclization reaction intermediates. (A) Model of tyrocidine A structure, with intramolecular hydrogen bonds highlighted with shading. (B) Models of the peptide-O-TE acyl enzyme intermediate and the tetrahedral intermediate, with the peptide shown in a product-like conformation. Putative product-like hydrogen bonds are highlighted with shading.

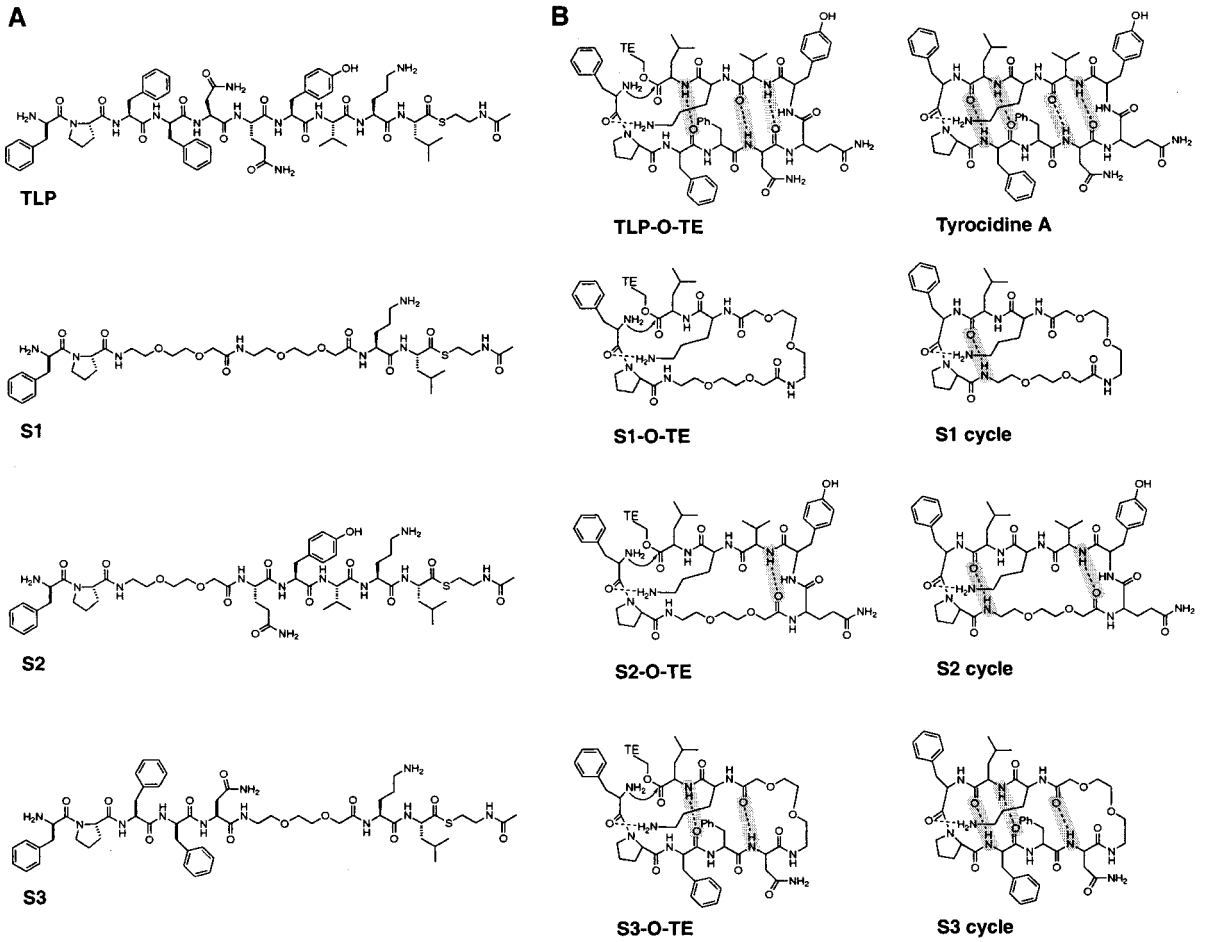

FIGURE 3: Structures of peptide-SNAC substrates and models of their cyclization intermediates and cyclic products. (A) Structures of the wild-type sequence substrate **TLP** and the backbone-substituted analogues **S1**, **S2**, and **S3**. The structure and purity of all substrates were verified by MALDI-TOF MS and analytical HPLC. (B) Models of the acyl-enzyme intermediates and the cyclic products corresponding to the adjacent peptide-SNAC substrates. Putative tyrocidine A-like hydrogen bonds are highlighted with shading.

coefficients at 220 nm. The identities of the cyclic products were verified by MALDI-TOF mass spectrometry (Table 1).

RESULTS

Spacer-Substituted Substrates. We reported previously that recognition of only two of the ten amino acid side chains, D-Phe1 and Orn9, in the wild-type sequence substrate **TLP** (Figure 3A) is critical for TycC TE catalyzed cyclization. This result suggested that some or all of the central six amino acids could be replaced by a flexible spacer. To test this, we synthesized substrates in which blocks of three amino acids are replaced with an 8-amino-3,6-dioxaoctanoic acid residue: **S1** in which all six central residues are replaced, **S2** in which Phe3−Asn5 are replaced, and **S3** in which Gln6−Val8 are replaced (Figure 3A). In an initial screen of the substrates, comparison of observed rates (k_{obs}) of TycC TE catalyzed cyclization (35 μM peptide-SNAC, 50 nM TycC TE, 25 mM MOPS, pH 7.0, 1 min reaction time) revealed that **S3** is cyclized at a rate comparable to that of **TLP**, while the cyclization rates for **S1** and **S2** are significantly reduced relative to **TLP** (Figure 4A). When the kinetics of cyclization for the single-spacer molecules were studied more closely, the substrates were seen to show a similar increase in K_M but marked difference in k_{cat} (Figure 4B). The increased K_M values for **S2** ($K_M = 22$ μM) and **S3** ($K_M = 27$ μM) relative to **TLP** ($K_M = 3$ μM) likely reflect that both substrates have similar conformational entropy which is greater than that of **TLP**. Only **S3** cyclizes efficiently ($k_{cat} = 53$ min^{-1}), with a rate similar to that of the wild-type substrate **TLP** ($k_{cat} = 60$ min^{-1}). The slow

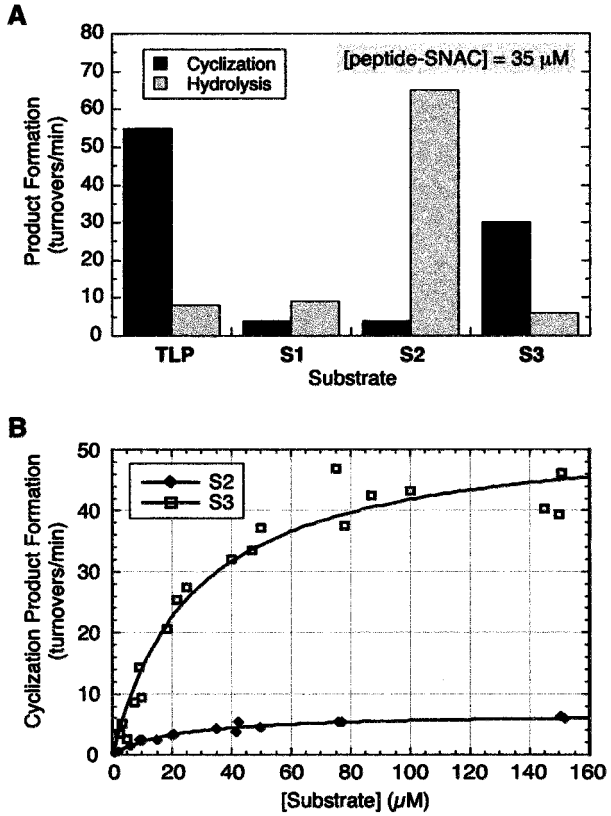

FIGURE 4: Cyclization of backbone-substituted peptide-SNAC substrates **S1**, **S2**, and **S3** catalyzed by the TE domain from tyrocidine synthetase. (A) Observed rates (k_{obs}) of peptide-SNAC cyclization and hydrolysis. Reactions contained 35 μM peptide-SNAC, 50 nM TycC TE, and 25 mM MOPS, at pH 7.0 and 24 °C (reaction time = 1 min). Data were determined by HPLC assay, and the identities of the products were verified by MALDI-TOF MS. (B) Kinetic profile of spacer substrates **S2** (closed diamonds) and **S3** (open squares). Reactions contained variable peptide-SNAC concentrations, 50 nM TycC TE, and 25 mM MOPS, pH 7.0 and 24 °C. Turnover rates were calculated by quantifying product formation at 1 min. The fit to the Michaelis−Menten curve shown was used to determine k_{cat} and K_M.

rate of cyclization of **S2** (k_{cat} = 7 min^{-1}) indicates that the amide bonds replaced in **S2** (those between Phe3 and D-Phe4 and between D-Phe4 and Asn5) are important for cyclization.

Depsipeptide Substrates. Our results with the spacer-substituted substrates **S1**, **S2**, and **S3** indicated that some parts of the peptide backbone are necessary for efficient cyclization. To probe the importance of individual amide bonds for cyclization, we synthesized depsipeptide substrates **D1**, **D2**, **D3**, and **D4** (Figure 5). Substrates **D1** and **D2** are based on the wild-type substrate **TLP**, while **D3** and **D4** are based on a Pro2 to Ala mutant of the wild-type substrate **P2A**. We reported earlier that **TLP** and **P2A** have similar kinetic parameters (k_{cat} and K_M) for TycC TE catalyzed cyclization (*11*). Use of **P2A** as a reference compound allowed us to probe with **D3** the amide bond between Pro2 and Phe3 and with **D4** the amide bond between D-Phe1 and Pro2. For **D3**, the analogous compound based on **TLP** in which Phe3 is changed to L-phenyllactate (DPhe-Pro-O-Phe-DPhe-Asn-Gln-Tyr-Val-Orn-Leu-SNAC) was first synthesized but undergoes both spontaneous and TycC TE catalyzed self-cleavage via formation of D-Phe-Pro diketopiperazine, which interferes with determination of the cyclization rate. The K_M values for all of the depsipeptide substrates were determined to be

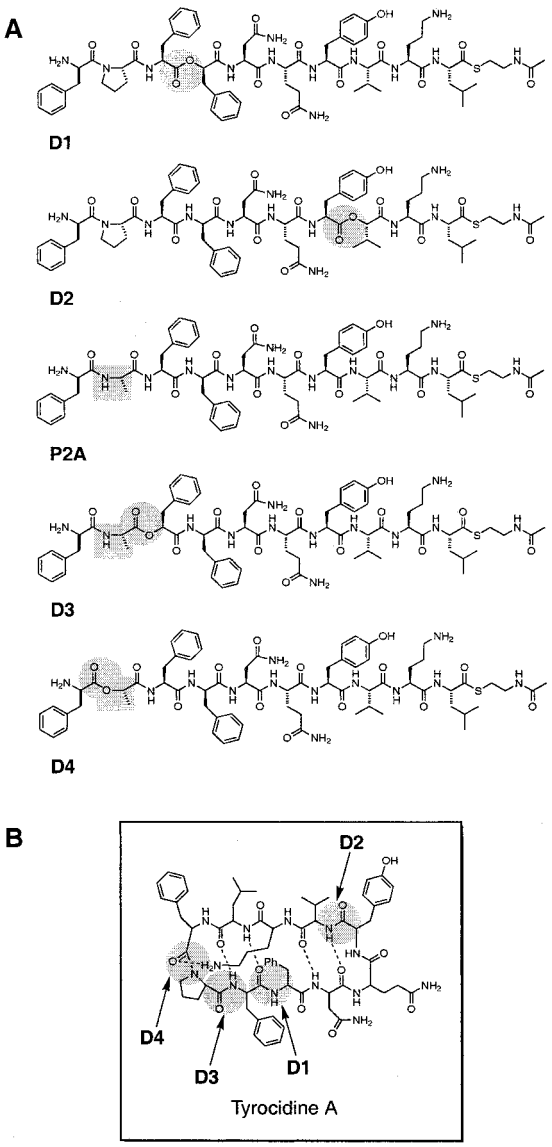

FIGURE 5: (A) Structures of peptide-SNAC substrates. Differences from the wild-type substrate **TLP** are highlighted with shading. The identity and purity of the compounds were verified by MALDI-TOF MS and analytical HPLC. (B) Model of tyrocidine A, with amide bonds that are replaced by ester bonds in the depsipeptide-SNAC substrates **D1**, **D2**, **D3**, and **D4** indicated with shading.

less than 10 μM. Comparison of observed rates of TycC TE catalyzed cyclization at 35 μM peptide-SNAC (50 nM TycC TE, 25 mM MOPS, pH 7.0, 1 min reaction time) was therefore studied and assumed to be revealing of the saturation rate (k_{cat}). Reaction profiles of the depsipeptide substrates revealed that **D1** and **D2** are efficiently cyclized at rates comparable to that of **TLP**, while **D3** and **D4** cyclize at significantly lower rates (Figure 6). These results show that TycC TE can catalyze efficient cyclization of some depsipeptide substrates, while the amide bonds replaced in other depsipeptides appear to be important for cyclization.

Cyclization via Macrolactone Formation. We also synthesized the substrate **D5**, in which the N-terminal amino group in **TLP** is replaced with a hydroxyl group, and measured its cyclization rate. We find that **D5** is cyclized by the TycC TE to form a macrolactone analogue of tyrocidine A (Figure 7B) with k_{cat} = 17 min^{-1} (versus 59 min^{-1} for **TLP**) and K_M = 2 μM (versus 3 μM for **TLP**). In

Table 1: Characterization of Peptide-SNAC Substrates and Cyclic Products by MALDI-TOF Mass Spectrometry

	[M + H]	
compound	calculated	observed
S1	881.5	881.5
S2	1126.6	1126.6
S3	1144.6	1144.7
S1 cycle	762.4	762.5
S2 cycle	1007.6	1007.6
S3 cycle	1025.5	1025.5
D1	1390.7	1390.7
D2	1390.7	1390.7
D3	1364.7	1364.8
D4	1364.7	1364.7
D1 cycle	1271.7	1271.8
D2 cycle	1271.7	1271.8
D3 cycle	1245.7	1245.8
D4 cycle	1245.7	1245.8
D5	1390.7	1390.8
D5 cycle	1271.7	1271.8

contrast to all of the other peptide thioester substrates we have tested, substrate inhibition (by an unknown mechanism) was observed with **D5** at concentrations greater than 6 μM, with complete inhibition of cyclization observed at or above 35 μM. The reported k_{cat} and K_M values were determined using substrate concentations up to 6 μM.

DISCUSSION

Cyclization of Backbone-Substituted Peptides by the TycC TE Domain. In a previous study, we found that the isolated TE domain from the tyrocidine NRPS can catalyze efficient cyclization of a peptide-SNAC substrate that mimics its natural, protein-bound substrate. Alanine scanning mutagenesis of the peptide-SNAC substrate showed that recognition of only two of the ten amino acid side chains is critical for cyclization. Our results here expand upon these results by showing that certain backbone-substituted substrates, namely, **S3** and the depsipeptide substrates **D1** and **D2**, cyclize at rates similar to that of the wild-type substrate **TLP**. This finding is potentially important for the synthesis of backbone-substituted cyclic peptides catalyzed either by engineered NRPSs or by isolated TE domains.

Evidence That Product-like Hydrogen Bonds Facilitate Peptide Preorganization. As discussed above, our observation that **S3** cyclizes efficiently, while **S2** is a poor cyclization substrate, indicates that the part of the peptide backbone replaced in **S2**, namely, D-Phe3−Asn5, is important for cyclization. We attempted to rationalize these results by modeling the peptide-O-TE acyl-enzyme intermediates for these substrates and for **TLP** with the peptides in product-like conformations and with product-like intramolecular hydrogen bonds. In these models, the **TLP-O-TE** intermediate is stabilized by three backbone-to-backbone hydrogen bonds (Figure 3B). In **S3-O-TE**, which cyclizes efficiently, two of these putative hydrogen bonds (the ones closest to the cyclization junction) are retained, while in **S2-O-TE**, which cyclizes poorly, only one of these hydrogen bonds (the one farthest from the cyclization junction) is retained (Figure 3B). Thus, the experimental results are consistent with a model in which product-like backbone-to-backbone hydrogen bonds facilitate preorganization of the peptide chain for cyclization. Alternatively, it is possible that the backbone segment replaced in **S2** removes substrate backbone to enzyme hydrogen bonds that are important for substrate recognition.

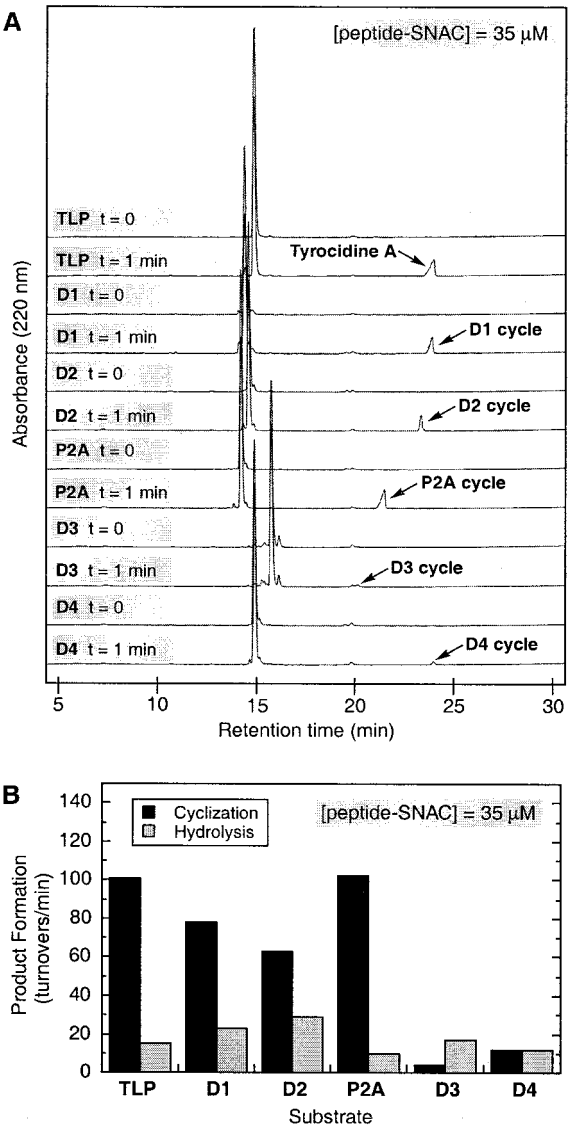

FIGURE 6: Cylization of depsipeptide-SNAC substrates catalyzed by the TycC TE domain. (A) HPLC traces and (B) rates (k_{obs}) of cyclization and hydrolysis for reactions that initially contained 35 μM peptide-SNAC, 50 nM TycC TE, and 25 mM MOPS, at pH 7.0 and 24 °C (reaction time = 1 min). Cyclic products are labeled on the HPLC traces. The identities of the cyclic products were verified by MALDI-TOF MS.

We also examined the results obtained with depsipeptide-SNAC substrates by modeling their corresponding acyl-enzyme intermediates in a product-like conformation (Figure 5B). The amide to ester substitutions in these substrates potentially eliminate two hydrogen bonds: one from the amide NH group and one to the amide carbonyl group due to the decreased electron-donating ability of the ester oxygen relative to the amide nitrogen (*15*−*17*). The ester bond, like the amide bond, exists primarily in the trans conformation (*23*). Substrate **D1** cyclizes at a rate comparable to that of the wild-type substrate **TLP** (Figure 6B) despite the elimination of one of the central β-sheet hydrogen bonds in the model (Figure 5B). In constrast, substrate **S2**, which removes both central β-sheet hydrogen bonds in the model, is a poor cyclization substrate. The difference suggests that one, but

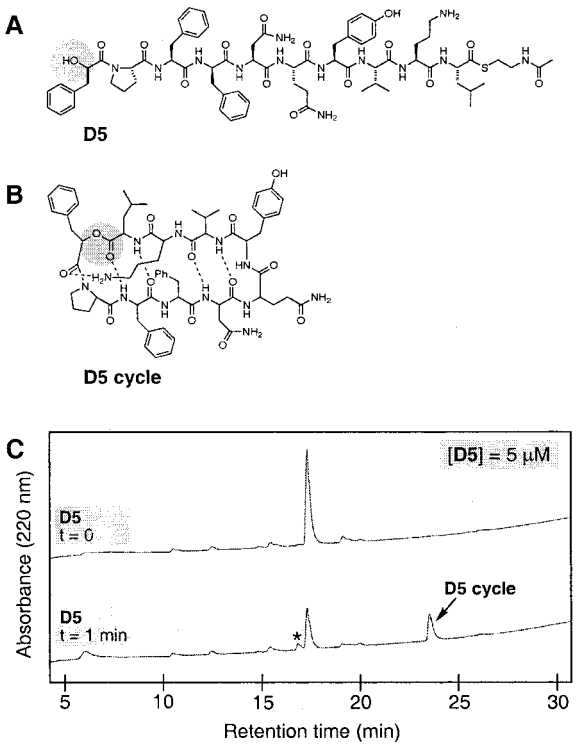

FIGURE 7: Cyclization via ester bond formation catalyzed by the TycC TE domain. (A) Structure of the peptide-SNAC substrate **D5**, in which the N-terminal D-Phe residue of the wild-type substrate **TLP** is replaced with D-phenyllactate. (B) Structure of the macrolactone product **D5 cycle**. (C) HPLC traces of reactions that initially contained 5 μM **D5**, 100 nM TycC TE, and 25 mM MOPS, at pH 7.0 and 24 °C (reaction time = time 0 or 1 min). The peak corresponding to the cyclic product is labeled, and the peak corresponding to hydrolyzed **D5** is marked with an asterisk. The structures of the substrate and the cyclic product were verified by MALDI-TOF MS.

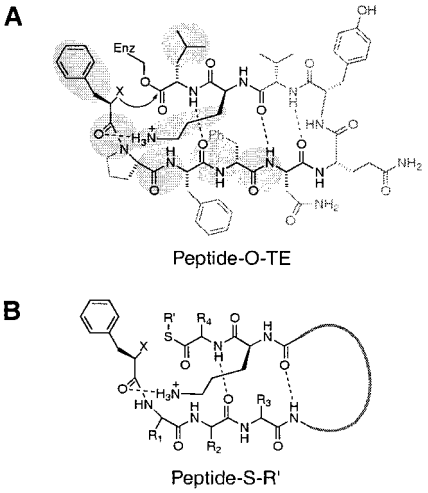

FIGURE 8: Model of substrate recognition by the TycC TE domain. (A) The acyl-enzyme intermediate that forms during cyclization to tyrocidine A is modeled in a product-like conformation. Parts of the molecule that, when altered, significantly affect the maximum cyclization rate (≥4-fold drop in k_{cat}) of either the wild-type substrate **TLP** or the substrate **P2A** are highlighted with shading: (i) D-Phe1, Orn9, and Leu10 side chains (based on ref 11) and (ii) the amide bonds between D-Phe1 and Pro2/Ala2 (based on **D3**, this study), between Pro2/Ala2 and Phe3 (based on **D4**, this study), and between Phe3 and Asn5 (based on **S2**, this study). Parts of the molecule that can be changed without significantly affecting the maximum cyclization rate are shown in gray: (i) side chains of residues Pro2, Phe3, D-Phe4, and Asn5 (based on ref 11) and (ii) residues Gln6 through Val8 (based on **S2**, this study). (B) Minimal cyclization substrate for the TycC TE domain based on the experimental results summarized in part A. The curved gray line represents a variable linker, R_1, R_2, R_3, and R_4 represent variable amino acid side chains, and X is either NH_2 or OH.

not both, of the central hydrogen bonds can be removed without strongly affecting the cyclization rate. Substrate **D2**, which eliminates the putative β-sheet hydrogen bond farthest from the cyclization junction (Figure 5B), also cyclizes at a rate comparable to that of **TLP** (Figure 6B), consistent with the observation that removal of the same hydrogen bond in **S3** has little effect on the cyclization rate. Substrate **D3**, in which the amide bond between Ala2 and Phe3 in **P2A** is replaced (Figure 5B), is a poor cyclization substrate (k_{obs} = 4 min^{-1}) (Figure 6B). According to the models in Figure 2B, elimination of this amide bond is not expected to affect any hydrogen bonds in the peptide-O-TE intermediate but could eliminate a hydrogen bond that stabilizes the tetrahedral intermediate, analogous to the "oxyanion hole" in serine proteases (*24*) (alternatively, the substitution could affect a substrate to enzyme hydrogen bond). However, the analogous depsipeptide substrate based on **TLP** (DPhe-Pro-O-Phe-DPhe-Asn-Gln-Tyr-Val-Orn-Leu-SNAC) was cyclized by TycC TE at a faster rate (k_{obs} = 17 min^{-1}) despite competition from rapid enzyme-catalyzed self-cleavage by diketopiperazine formation (k_{obs} = 109 min^{-1}) (data not shown). Thus, the importance of the amide bond between Ala2 and Phe3 for cyclization was unpredictably sequence dependent. Substrate **D4**, which potentially eliminates a hydrogen bond between the carbonyl group of D-Phe1 and the δ-amino group of ornithine (Figure 5B), cyclizes at a significantly reduced rate compared to **P2A**. This result suggests that a D-Phe1 to ornithine δ-amino group hydrogen bond helps to orient the N- and C-terminal ends of the substrate for cyclization (see Figure 2B) (alternatively, the substitution could affect a substrate to enzyme hydrogen bond). In sum, there is evidence that product-like hydrogen bonds that cannot form with substrates **D3** and **D4** may facilitate substrate cyclization. A more detailed and conclusive understanding of substrate−substrate and substrate−enzyme hydrogen-bonding interactions must await high-resolution structural analysis.

Cyclization to a Macrolactone Catalyzed by the TycC TE. Our finding that some parts of the peptide backbone in **TLP** are important for cyclization suggested that hydrogen-bonding interactions involving the peptide backbone contribute to preorganization of the substrate for cyclization. If the overall rate of cyclization depends on the rate of substrate preorganization, then an analogue of the wild-type substrate in which the peptide backbone is unchanged but the N-terminal amino group is changed to a hydroxyl group (**D5**, Figure 7A) would be expected to cyclize at a rate comparable to that of **TLP** despite the lower intrinsic nucleophilicity of hydroxyl versus amine. Indeed, we found that **D5** is cyclized with a k_{cat} reduced only 4-fold compared to **TLP**.

Model of Substrate Recognition by the Tyrocidine TE. Figure 8A provides a qualitative summary of our experimental results from this study and an earlier study (*11*) superimposed on a model of the wild-type peptide-O-TE (**TLP-O-TE**) acyl-enzyme intermediate. Parts of the substrate that, when changed in either **TLP** or **P2A**, significantly reduce the maximum cyclization rate (decreases by ≥4-fold) are highlighted with shading, while parts of the substrate

that can be changed without significantly affecting the cyclization rate are shown in gray. On the basis of these results, we created a model of a minimal cyclization substrate for the TycC TE domain, shown in Figure 8B in a product-like conformation stabilized by putative intramolecular hydrogen bonds.

Importance of TE Domain Specificity. Understanding the specificity of TE domain catalyzed peptide macrocyclization reactions is important because it illuminates the mechanism by which these enzymes bring the ends of peptide substrates together for cyclization. In addition, characterization of TE specificity is important for the engineered biosynthesis of novel compounds and for the enzymatic cyclization of synthetic substrates. For example, our studies with the TycC TE indicate that there is considerable leeway for modification of the linear substrate without affecting the cyclization rate, suggesting that this TE domain could be used to prepare cyclic peptide libraries that could be screened for useful biological activity. A key issue that is addressed in the following paper (*25*) is to investigate the generality of this enzymatic approach to macrocycle synthesis by further characterizing TycC TE specificity and by studying the TE domains from other enzymatic assembly lines.

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REFERENCES

1. Marahiel, M. A., Stachelhaus, T., and Mootz, H. D. (1997) *Chem. Rev. 97*, 2651−2674.
2. Donadio, S., Staver, M. J., McAlpine, J. B., Swanson, S. J., and Katz, L. (1991) *Science 252*, 675−679.
3. Cane, D. E., Walsh, C. T., and Khosla, C. (1998) *Science 282*, 63−68.
4. Stachelhaus, T., Schneider, A., and Marahiel, M. A. (1995) *Science 269*, 69−72.
5. Huthcinson, C. R. (1998) *Curr. Opin. Microbiol. 1*, 319−329.
6. McDaniel, R., Thamchaipenet, A., Gustafsson, C., Fu, H., Betlach, M., Betlach, M., and Ashley, G. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 1846−1851.
7. Ranganathan, A., Timoney, M., Bycroft, M, Cortes, J., Thomas, I. P., Wilkinson, B., Kellenberger, L., Hanefeld, U., Galloway, I. S., Staunton, J., and Leadlay, P. F. (1999) *Chem. Biol. 6*, 731−741.
8. Lawson, D. M., Derewenda, U., Serre, L., Ferri, S., Szittner, R., Wei, Y., Meighen, E. A., and Derewenda, Z. S. (1994) *Biochemistry 33*, 9382−9388.
9. Li, J., Szittner, R., Derewenda, Z. S., and Meighen, E. A. (1996) *Biochemistry 35*, 9967−9973.
10. Mootz, H. D., and Marahiel, M. A. (1997) *J. Bacteriol. 179*, 6843−6850.
11. Trauger, J. W., Kohli, R. M., Mootz, H. D., Marahiel, M. A., and Walsh, C. T. (2000) *Nature 407*, 215−218.
12. Kuo, M. C., and Gibbons, W. A. (1980) *Biophys. J. 32*, 807−836.
13. Hull, S. E., Karlsson, R., Main, P., Woolfson, M. M., and Dodson, E. J. (1978) *Nature 275*, 206−207.
14. Krauss, E. M., and Chan, S. I. (1982) *J. Am. Chem. Soc. 104*, 6953−6961.
15. Koh, J. T., Cornish, V. W., and Schultz, P. G. (1997) *Biochemistry 36*, 11314−11322.
16. Chapman, E., Thorson, J. S., and Schultz, P. G. (1997) *J. Am. Chem. Soc. 119*, 7151−7152.
17. Shin, I., Ting, A. Y., and Schultz, P. G. (1997) *J. Am. Chem. Soc. 119*, 12667−12668.
18. Lu, W., Randal, M., Kossiakoff, A., and Kent, S. B. H. (1999) *Chem. Biol. 6*, 419−427.
19. Beligere, G. S., and Dawson, P. E. (2000) *J. Am. Chem. Soc. 122*, 12079−12082.
20. Nakhle, B. M., Silinski, P., and Fitzgerald, M. C. (2000) *J. Am. Chem. Soc. 122*, 8105−8111.
21. Low, D. W., and Hill, M. G. (2000) *J. Am. Chem. Soc. 122*, 11039−11040.
22. England, P. M., Zhang, Y., Dougherty, D. A., and Lester, H. A. (1999) *Cell 96*, 89−98.
23. Wiberg, K. B., and Laidig, K. E. (1987) *J. Am. Chem. Soc. 109*, 5935.
24. Fersht, A. (1984) *Enzyme Structure and Mechanism*, 2nd ed., pp 405−411, W. H. Freeman, New York.
25. Kohli, R. M., Trauger, J. W., Schwarzer, D., Marahiel, M., and Walsh, C. T. (2001) *Biochemistry 40*, 7099−7108.

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