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# Enzymatic Macrolactonization in the Presence of DNA Leading to Triostin A Analogs

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## SUMMARY

**Excised thioesterase domains are versatile catalysts for macrocyclization. However, thioesterase-catalyzed cyclization is often precluded due to the occurrence of hydrolysis and product inhibition. To circumvent these obstacles, we devised an unprecedented strategy: coincubation with DNA to capture the cyclic products possessing DNA-binding properties. In experiments involving echinomycin thioesterase-catalyzed macrolactonization leading to the cyclic triostin A analog TANDEM, we found that the addition of DNA drastically improved the yield of TANDEM (19% → 67%), with a complete reversal of the cyclization:hydrolysis ratio (1:2 → 18:1). Furthermore, the applicability of this protocol was demonstrated for a variety of substrates. The results described herein provide insight into the mechanism of echinomycin thioesterase-catalyzed conversions and also pave the way for chemoenzymatic synthesis of the quinoxaline antibiotics and their analogs.**

## INTRODUCTION

In nature, a variety of small molecules with rich structural diversity are assembled by modular megaenzymes such as nonribosomal peptide synthetases (NRPSs) (Sieber and Marahiel, 2005), polyketide synthases (PKSs), and NRPS/PKS hybrid systems (Fischbach and Walsh, 2006). A common structural feature of medicinally important natural products, including the immunosuppressant cyclosporine (NRPS), the antibiotic erythromycin (PKS), and the antitumor agent epothilone (NRPS/PKS), is a constrained macrocyclic skeleton. The rigidified cyclic skeleton allows these compounds to adopt a biologically active conformation for interaction with the dedicated biomacromolecular target and also increase their stability against proteolytic digestion. In the biosynthesis of nonribosomal peptides and polyketides, the macrocyclization step is generally catalyzed by a thioesterase (TE) domain embedded at the C terminus of a multidomain enzymatic assembly line (Kohli and Walsh, 2003). Since construction of the macrocyclic skeleton is intrinsically disfavored entropically due to the loss of conformational freedom, chemoenzymatic approaches with the excised TE domains have been explored to provide an alternative and efficient method for accessing macrocyclic natural products in place of conventional chemical synthesis.

Among the cyclic peptides biosynthesized by NRPSs, some members having $C_2$- or $C_3$-symmetric cyclic skeletons are thought to be constructed via oligomerization and subsequent cyclization (Kohli and Walsh, 2003). Studies on the TE domain of the iterative enterobaction synthase (EntF) suggested that the C-terminal TE domain catalyzes not only the cyclization, but also the ligation, of linear peptides (Shaw-Reid et al., 1999; Zhou et al., 2006). In a similar fashion, the iterative mechanism has been assumed for the biosynthesis of gramisidin S (Stoll et al., 1970; Hori et al., 1989; Krätzschmar et al., 1989), thiocoraline (Lombo et al., 2006), and valinomycin (Cheng, 2006). Recently, in vitro experimentation with an excised gramicidin TE (GrsB TE) has deciphered the detailed mechanism of the iterative TE domain (Hoyer et al., 2007).

Echinomycin (**1**) is isolated from various species of streptomycete (Dell et al., 1975) and is the best known member of the quinoxaline/quinoline antibiotic group, which includes triostin A (**2**) (Otsuka et al., 1976), SW-163C (Kurosawa et al., 2001), thiocoraline (Romero et al., 1997), and BE-22179 (Okada et al., 1994) (Figure 1). The common structural feature of these $C_2$-symmetric compounds is the crossbridged cyclic octapeptide dilactone core linked by twin quinoxaline/quinoline 2-carbonyl chromophores (Dawson et al., 2007). Echinomycin (**1**) shows potent antitumor activity by inhibition of DNA replication and RNA synthesis. Both **1** and **2** bind strongly to double-stranded DNA by bisintercalation, in which twin chromophores are inserted into the duplex sandwiching 5′-CG pairs (Warning and Wakelin, 1974; Vandyke and Dervan, 1984; Wang et al., 1984). In contrast, triostin A tetra-*N*-demethyl analog (TANDEM, **3**) (Ciardelli et al., 1978) exhibits distinct sequence selectivity for 5′-TA steps, despite the small structural difference between **2** and **3** (Lavesa and Fox, 2001). In a similar fashion, the structurally related quinoxaline/quinoline antibiotics show a range of DNA-binding affinities and sequence selectivities (Dawson et al., 2007; Boger et al., 2001).

We recently identified the entire gene cluster responsible for the formation of **1** (Figure 2A) and achieved total biosynthesis of **1** and **2** in *Escherichia coli* (Watanabe et al., 2006). During echinomycin biosynthesis, quinoxaline-2-carboxylic acid (QC), a starter unit of peptide chain elongation, is biosynthesized from L-tryptophan via a (2*S*, 3*S*)-β-hydroxytryptophan intermediate (Figure 2B) (Koketsu et al., 2006). Ecm1-mediated adenylation of QC conducts the loading of the quinoxaline-2-carbonyl group onto FabC (Schmoock et al., 2005). Subsequent peptide

 

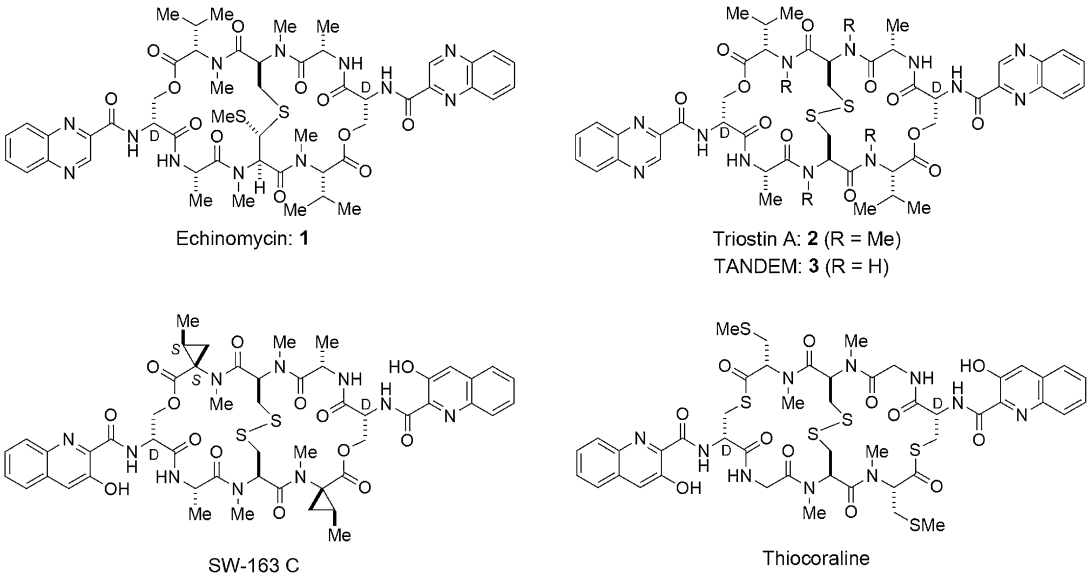


**Figure 1. Structures of Selected Quinoxaline/Quinoline Antibiotics**

elongation (D-Ser → L-Ala → *N*-Me-L-Cys → *N*-Me-L-Val) is catalyzed by modular NRPSs (Ecm6 and Ecm7), affording a PCP-bound tetrapeptidyl thioester intermediate that is then transferred to a serine residue in the active site of the terminal TE domain. Without detailing the precise reaction mechanisms, the TE domain is assumed to mediate dimerization of the tetrapeptides on the PCP and TE domains and subsequent macrolactonization entailing the release of the octapeptidyl dilactone framework, **4**. Oxidation of the twin thiols by Ecm17 forms the disulfide bridge and produces **2**. Finally, the disulfide of **2** is converted into the thioacetalbridge of echinomycin, **1**, via a unique rearrangement mediated by SAM-dependent methyltransferase (Ecm18).

Here, we report two approaches for chemoenzymatic synthesis employing the excised echinomycin TE domain (Ecm TE) expressed in *E. coli*. First, we explored in vitro dimerization-cyclization of a tetrapeptidyl thioester substrate not only to gain insight into the reaction mechanism, but also to achieve chemoenzymatic synthesis of **2**. Second, we investigated macrolactonization of octapeptidyl substrates to produce **3** and its analogs. To suppress hydrolysis of the ester linkages as well as product inhibition, we developed an unprecedented, to our knowledge, protocol: coincubation with double-stranded DNA to capture cyclized products that possess bisintercalating properties. The efficiency and applicability of Ecm TE-catalyzed cyclization in the presence of DNA have been demonstrated.

## RESULTS

### Cloning and Expression of the Ecm TE Domain

An expression plasmid was constructed by subcloning of the gene fragment encoding the TE domain region of the NRPS gene (*ecm7*) into pET22b vector. The border of the Ecm TE domain of Ecm7 was defined on the basis of alignment with the characterized TE domains tyrocidine TE, surfactin TE, gramicidin TE, and calcium-dependent antibiotic CDA3 TE (Trauger et al., 2000; Kohli et al., 2001; Grunewald et al., 2004) (see Figure S1 available online). The recombinant Ecm TE was successfully expressed in *E. coli* BL21 (DE3) at 24°C as a soluble protein and was purified by Ni-chelating affinity chromatography. The recombinant protein was obtained with a yield of 18 mg/l. SDS-PAGE analysis indicated that the protein purity was >95% (Figure S2).

### Ecm TE-Mediated Conversion of Tetrapeptidyl-SNAC Substrate 5 to Triostin A, 2

To explore the potential of the excised Ecm TE domain to catalyze dimerization of the tetrapeptidyl linear substrate and subsequent lactonization leading to the $C_2$-symmetric octapeptidyl dilactone core, we synthesized a tetrapeptidyl thioester, **5**, as the native monomer substrate based on a solution-phase procedure (Figure 3 and Supplemental Data). The C-terminal carboxylic acid of the peptide chain was condensed with *N*-acetylcysteamine (SNAC), which is a mimic of the phosphopantetheinyl moiety of the holo-PCP domain. Since substrate **5** contains a free thiol group, a nonenzymatic thiol/thioester exchange reaction would compete with the desired dimerization and subsequent cyclization. A control experiment upon incubation of **5** without Ecm TE resulted in nonenzymatic formation of thioesterified and thiolactonized products **14** and **15**, respectively (Figure S3, traces 1A–1C), under reducing conditions in the presence of 1 mM tris(2-carboxyethyl)phosphine (TCEP) to retain the thiol in its reduced form (Burns et al., 1991). In consideration of the competitive reactions, Ecm TE-catalyzed conversion of **5** was conducted under reducing conditions. LC-ESI-MS analysis revealed the formation of the linear octapeptidyl dimeric product, **7**, as well as the desired dilactone, **4** (Figure S3, traces 2A–2C). The identity of the desired **4**, with twin thiol groups, was validated by comparison with the authentic sample in two ways (Figure S3, traces 3 and 4). First, the authentic **4** was prepared by reductive cleavage of the disulfide bond of naturally occurring **2** isolated from *Streptomyces triostinicus* (Otsuka et al., 1976). Furthermore, isolation of the resulting dilactone, **4**, from the Ecm

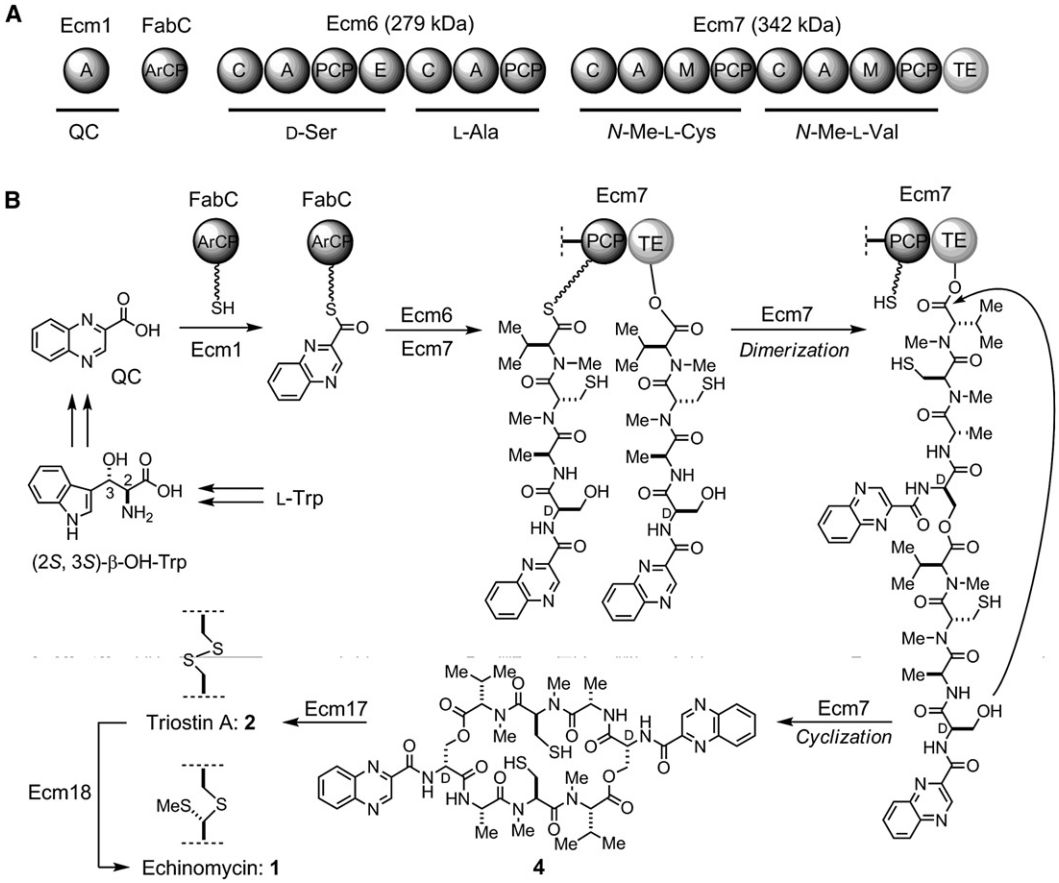


**Figure 2. Echinomycin Biosynthesis by Modular Nonribosomal Peptide Synthetases**

(A) Modular organization of echinomycin NRPSs. Bars indicate the positions of modules within the protein, whereas individual domains and proteins are shown in spheres. Ecm1 is quinoxaline-2-carboxylic acid (QC) activating enzyme. FabC is an aryl carrier protein. Ecm6 contains two modules consisting of seven domains. Ecm7 contains two modules consisting of nine domains, including thioesterase. A, adenylation domain; ArCP, aryl carrier protein; C, condensation domain; E, epimerization domain; PCP, peptidyl carrier protein; TE, thioesterase domain.

(B) The proposed pathway for echinomycin biosynthesis. QC is transformed from L-tryptophan via a (2*S*,3*S*)-β-hydroxyl tryptophan intermediate. QC is activated by Ecm1 and then tethered to FabC through a phosphopantetheinyl arm. Peptide chain elongation is catalyzed by Ecm6 and Ecm7. The C-terminal TE domain mediates dimerization and cyclorelease. Subsequent transformations to afford echinomycin (**1**) through disulfide formation and thioacetalization are mediated by Ecm17 and Ecm18, respectively.

TE-catalyzed reaction mixture and subsequent aerobic oxidation to form a disulfide bridge allowed for the chemoenzymatic synthesis of **2**. These results indicate that Ecm TE can recognize the native monomer tetrapeptidyl-SNAC substrates and catalyze dimerization and subsequent lactonization, albeit with low efficiency.

## Ecm TE-Catalyzed Macrolactonization of Octapeptidyl-SNAC Substrates to Produce Triostin A Analogs

To evaluate the activity of Ecm TE in catalyzing macrolactonization, the linear octapeptidyl thioester, **9**, was designed (Figure 3). Substrate **9** contains an ester linkage between D-Ser and L-Val residues as well as a SNAC thioester at the C terminus. Furthermore, we envisioned chemoenzymatic synthesis of **3** by a combination of solid-phase synthesis of **9** (the tetra-*N*-demethylated analog of **7**) and enzymatic macrolactonization. First of all, the octapeptidyl thioester, **9**, containing twin thiol groups, was incubated with Ecm TE under reducing conditions in the presence of TCEP. However, nonenzymatic thiol/thioester exchange reactions generated byproducts, and only a trace amount of the desired dilactone, **11**, was detected (data not shown).

To circumvent the experimental difficulties caused by the free thiol groups, substrate **12**, containing a disulfide bond, was synthesized. Inspired by the critical effects of intramolecular backbone hydrogen bonds on TE-mediated cyclization (Trauger et al., 2001), the disulfide bridge of **12** was thought to facilitate peptide preorganization into a product-like conformation for macrocyclization. Incubation of **12** with Ecm TE for 8 hr provided cyclized **3** and hydrolyzed **13** in a 19% and 43% yield, respectively, with a cyclization:hydrolysis ratio of 1:2 (Figure 4A). The identities of the products were confirmed by LC-MS analysis (Table S1). The $k_{cat}/K_M$ value for cyclization was 0.751 $mM^{-1}$ $min^{-1}$ (Table 1, entry 1).

## Substrate Tolerance of Ecm TE

Having developed a chemoenzymatic protocol to produce $C_2$-symmetrical cyclic peptides, the substrate tolerance of Ecm TE was investigated by using nine octapeptidyl-SNAC

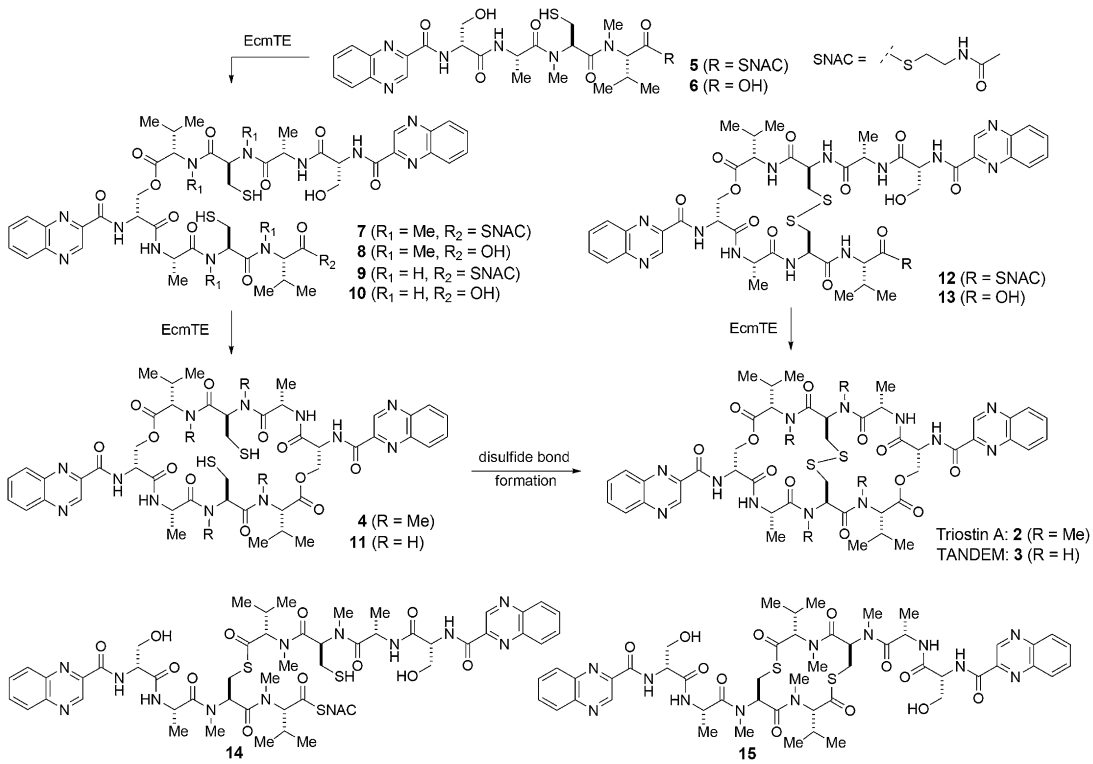


**Figure 3. Schematic Presentation of Chemoenzymatic Synthesis of Triostin A and TANDEM by Using Ecm TE**

Chemically synthesized peptides **6** and **13** were condensed with *N*-acetyl cycteamine (SNAC) to produce tetrapeptidyl-SNAC thioester, **5**, and octapeptidyl-SNAC thioester, **12**, respectively. Thioester **9**, containing two thiols, was synthesized from **12** by reductive cleavage of the disulfide. Aerobic oxidation of **4** allowed for installation of the disulfide bond, affording **2**. Thioester **14** and thiolactone **15** were formed via nonenzymatic thiol/thioester exchange reactions.

thioester analogs, **16**–**24**, with differing amino acid compositions, and their kinetic parameters were compared with those of **12** (Figure 5 and Figure S4). First, replacement of the L-Cys residues of **12** into either L-Ala or L-Ser (**12** → **16**/**17**) was examined in order to verify the effects of the disulfide bridge in facilitating peptide preorganization into product-like conformations. As expected, loss of the disulfide bridge reduced the yield of cyclic products (Table 1, entries 2 and 3). Second, participation of the side chain at the position of the L-Ala residues in **12** was investigated. All changes that include modification of steric factors (**18**, **19**) as well as installation of hydroxyl groups (**20**) were found to hinder enzymatic cyclization (entries 4–6). Next, the L-Val residues of **12** were replaced by L-Phe or L-Ala (**21** and **22**) to assess the steric influence of an adjacent amino acid side chain in the substitution of the thioester group. The change to sterically demanding L-Phe in **21** had little influence on cyclization (entry 7), but it led to suppression of hydrolysis. On the other hand, the presence of the less hindered L-Ala in **22** resulted in acceleration of enzymatic cyclization by approximately four times ($k_{cat}/K_M$ value) (entry 8), although there was considerable competition from hydrolysis of the SNAC thioester (Figure 4D[ii]). Finally, analogs **23** and **24**, containing twin pyrazine or naphthalene rings, respectively, in place of the quinoxaline rings, were synthesized to investigate substrate tolerance at the aromatic rings (entries 9 and 10). Whereas Ecm TE-catalyzed cyclization of **23** resulted in slow conversion ($k_{cat}/K_M$ = 0.046 $mM^{-1}$ $min^{-1}$), **24** exhibited ~5-fold higher reactivity ($k_{cat}/K_M$ = 3.87 $mM^{-1}min^{-1}$) than **12** ($k_{cat}/K_M$ = 0.751 $mM^{-1}$ $min^{-1}$). These results indicate that the presence of nitrogen atoms in the chromophore is not essential, but the ring size of the chromophore plays an important role in molecular recognition by Ecm TE.

## Time Course of Ecm TE-Catalyzed Conversion of Octapeptidyl-SNAC Substrate 12

As described above, enzymatic cyclization of a variety of octapeptidyl-SNAC substrates was realized. However, Ecm TE-mediated conversions in vitro inevitably entailed hydrolysis, producing carboxylic acids. In an attempt to analyze the reaction profile of Ecm TE-mediated conversion of **12**, the proportions of substrate **12**, the cyclized product **3**, and the hydrolyzed products **13** and **25** were monitored during the course of the reaction (Figure 4B). The proportion of the desired product, **3**, was increased at the initial stage of the reaction and reached a maximum of 23% after 5 hr. However, the yield of **3** was then decreased to 12% after 12 hr. In contrast, the amount of carboxylic acid **13**, formed by the hydrolysis of the thioester group of **12**, increased proportionally to 25% and 56% at 5 hr and 12 hr, respectively. These results indicated that product inhibition and Ecm TE-mediated hydrolysis of **3** are possible major obstacles in the production of dilactone **3**. Besides hydrolysis of the thioester to yield **13**, a small quantity of **25** was formed by hydrolysis of the ester group of **12**.

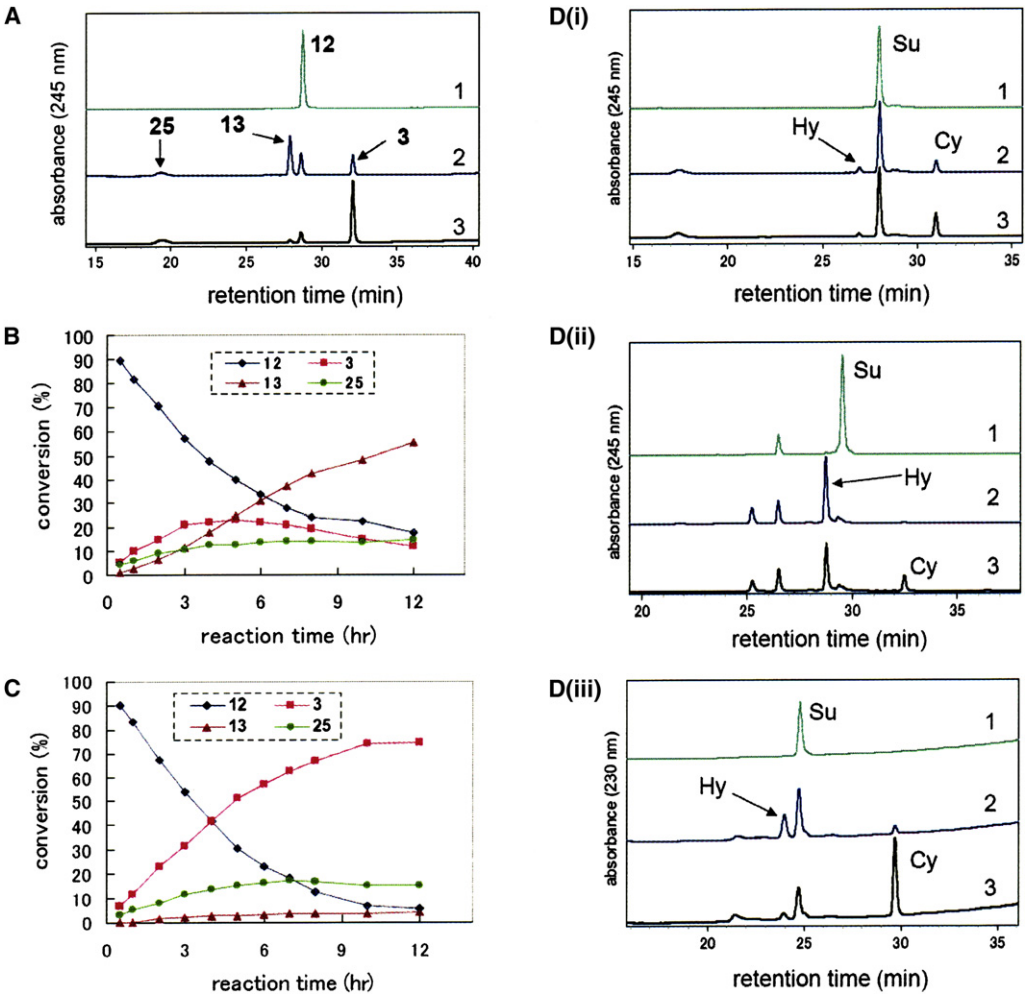


**Figure 4. Cyclization of Octapeptidyl-SNAC Substrates Mediated by Ecm TE**

(A) HPLC traces of reactions (after 8 hr) with initial contents of 5 μM Ecm TE, 25 μM **12**, 25 mM HEPES (pH 7.5, 24°C), <1% DMSO. Trace 1: negative control without enzyme. Trace 2: conversion of **12** by Ecm TE without DNA. Trace 3: coincubation with 25 μM DNA (5′-GATATCGATATC-3′).

(B) Time course of Ecm TE-mediated conversion of substrate **12** in the absence of DNA. The chemical structure of **25**, formed by hydrolysis of the ester group, is shown in Figure 6.

(C) Time course of Ecm TE-mediated conversion of **12** in the presence of DNA.

(D) HPLC traces from Ecm TE-mediated conversion of various peptide analogs in the presence of 25 μM DNA. Trace 1: negative control without enzyme. (i) Incubation of **18** with Ecm TE for 8 hr at 24°C and pH 7.5 in the absence (Trace 2) or presence (Trace 3) of DNA. (ii) Incubation of **22** with Ecm TE for 2 hr at 24°C and pH 7.0 in the absence (Trace 2) or presence (Trace 3) of DNA. (iii) Incubation of **24** with Ecm TE for 2 hr at 24°C and pH 7.5 in the absence (Trace 2) or presence (Trace 3) of DNA. Su, substrate; Hy, thioester-hydrolyzed product; Cy, cyclized product.

## Improvements of Ecm TE-Catalyzed Macrolactonization Exploiting the DNA-Binding Abilities of Cyclic Peptides

It has been reported that recombinant TEs are inhibited by cyclic products and also cause the hydrolysis of cyclized products (Grunewald et al., 2004; Tseng et al., 2002). The addition of detergent (Yeh et al., 2004) and the use of the organic solvent (Wagner et al., 2006) have been carried out in an attempt to enhance cyclic product formation. In this study, we focused on the DNA-intercalating properties of the cyclized product **3** and devised an alternative approach involving the use of DNA. TANDEM **3** is known as a synthetic DNA-intercalating agent and shows a preference for 5′-TA steps, particularly 5′-ATAT (Lavesa and Fox, 2001). We thus envisioned that capture of the cyclic product by DNA containing ATAT sequences could prevent hydrolysis as well as product inhibition and thereby enhance the enzymatic production of cyclic **3** (Figure 6). In fact, conversion of **12** (25 μM) by 5 μM Ecm TE in the presence of DNA (25 μM; sequence: 5′-GATATCGATATC-3′; the underlined sequences represent the DNA-binding sites of TANDEM) proceeded successfully to afford the cyclic product **3** in 67% yield with substantial suppression of hydrolysis (**13**: 4% yield) after 8 hr of incubation (Figure 4A, trace 3). Whereas the cyclization:hydrolysis ratio in the absence of DNA was 1:2, the addition of the DNA dramatically reversed the ratio to 18:1 and significantly enhanced production of the cyclic product (19% → 67% yield).

Next, the time course of the reaction in the presence of DNA was monitored (Figure 4C). The cyclized product was formed in 52% and 75% yield after incubation for 5 hr and 12 hr,

**Table 1. $k_{cat}/K_M$ Values for Cyclization with Ecm TE**

| Entry | Substrate | $k_{cat}/K_M$ Cyclization Values ($mM^{-1}$ $min^{-1}$) |
|---|---|---|
| 1 | **12**[a] | 0.751 ± 0.150 |
| 2 | **16** | 0.028 ± 0.003 |
| 3 | **17** | 0.045 ± 0.002 |
| 4 | **18** | 0.311 ± 0.080 |
| 5 | **19** | 0.194 ± 0.036 |
| 6 | **20** | 0.141 ± 0.008 |
| 7 | **21**[b] | 0.647 ± 0.055 |
| 8 | **22**[b] | 3.13 ± 0.24 |
| 9 | **23** | 0.046 ± 0.007 |
| 10 | **24** | 3.87 ± 0.39 |

[a] A separate evaluation of kinetic parameters for the cyclization of **12** with Ecm TE was performed to determine the $k_{cat}$ and $K_M$ values as 0.0315 ± 0.0053 $min^{-1}$ and 42.2 ± 7.3 μM, respectively.
[b] Substrates **21** and **22** were incubated at pH 7.0 due to their lability toward hydrolysis, whereas the other reactions were conducted at pH 7.5.

respectively. In contrast, formation of the hydrolyzed product **13** was suppressed considerably to 3% and 4% after incubation for 5 hr and 12 hr, respectively. Furthermore, upon incubation for 12 hr, substrate **12** was almost entirely consumed (recovered **12**: <6%), whereas, in the absence of DNA, more than 18% of **12** remained (Figure 4B). In an attempt to verify the critical role of DNA in Ecm TE-catalyzed conversions, DMSO, a conventional DNA-denaturing agent, was added to the above-described reaction cocktail (5% v/v), and this was found to abolish the effects of DNA on Ecm TE-catalyzed conversion (data not shown). In addition to this, coincubation of DNA (5′-AACGTGCACGTT-3′) without the specific binding site (5′-ATAT-3′) of TANDEM **3** failed to bring about the positive effects of DNA on the macrolactonization and resulted in the formation of a significant amount of the hydrolyzed product **13** (Figure S5). Thus, these experimentations demonstrated the importance of the sequence-specific interaction between the cyclic product **3** and DNA.

Based on these encouraging results, nine peptidyl-SNAC analogs, **16**–**24**, were subjected to Ecm TE-mediated cyclization in the presence of DNA. No information on the DNA-binding properties of the cyclic products of these analogs had been available until we carried out the enzymatic conversions. Improvement of enzymatic cyclization by the addition of DNA would indicate that the resulting cyclic peptide had DNA-intercalating activity. Out of the ten octapeptidyl-SNAC substrates tested, in six cases, including **16**–**17**, **19**–**21**, and **23**, the addition of DNA did not cause a substantial change in the production of the cyclic product. However, for three analogs, **18**, **22,** and **24**, the presence of DNA resulted in an increase in the yield of the desired cyclic products (**18**: 8% → 25%; **22**: 3% → 13%; and **24**: 8% → 58%), as shown in Figure 4D. These results indicate that the addition of DNA promotes Ecm TE-catalyzed enzymatic cyclization without loss of enzymatic activity and is applicable to a variety of substrates.

### Binding of Cyclic Peptides to DNA with Bisintercalation

To substantiate the effects of DNA on Ecm TE-mediated cyclization, the interaction between DNA and cyclic peptides was verified based on the ability of the peptides to induce unwinding of negatively supercoiled DNA (Boger et al., 1996, 2001; Huang et al., 1982) (Figure S6). The unwinding of DNA by echinomycin, **1**, was carried out as a positive control, as it was previously reported that the addition of **1** gradually decreased the agarose gel electrophoresis mobility of supercoiled ΦX174 DNA (unwinding) at increasing concentrations, followed by a return to normal mobility (rewinding) at even higher concentrations. When the experiment was carried out with TANDEM **3** and its analogs, cyclized products of **18** and **24**, which were obtained in improved yields by the addition of DNA, the mobility of ΦX174 DNA was reduced in a dose-dependent manner, as expected, although changes indicating conversion to unwinding/rewinding states were not observed. However, the linear octapeptidyl-SNAC thioester, **12**, as well as the cyclized product of **22** did not induce apparent changes in DNA mobility. Interestingly, the DNA-binding abilities correlate with the yields of the cyclized products in the presence of DNA. Thus, these results substantiate the finding that DNA can selectively capture cyclized products via bisintercalation in the presence of its linear octapeptidyl-SNAC thioester precursor.

## DISCUSSION

Recently, the chemoenzymatic construction of macrocyclic skeletons by using a variety of TE domains has been investigated for the synthesis of complex natural products and its analogs (Kohli and Walsh, 2003; Kohli et al., 2002). In the biosynthesis of the antitumor antibiotic echinomycin, the modular NRPSs Ecm6 and Ecm7 mediate the formation of the first tetrapeptide on the PCP domain of Ecm7 (Figure 2), and the C-terminal TE domain awaits the assembly of the second tetrapeptide and catalyzes dimerization and subsequent macrolactonization to liberate the $C_2$-symmetric cyclic depsipeptide (Kohli and Walsh, 2003; Shaw-Reid et al., 1999; Hoyer et al., 2007). Here, we present chemoenzymatic syntheses of triostin A, **2**, and its analogs, in which peptidyl-SNAC thioesters were prepared by chemical synthesis in place of the modular NRPSs (Ecm6 and Ecm7) and were then treated with the excised Ecm TE domain in vitro to cause dimerization and subsequent macrolactonization.

First of all, tetrapeptidyl thioester, **5**, which contains a free thiol, was synthesized and subjected to conversion mediated by excised Ecm TE under reducing conditions to maintain the thiol groups in their reduced form. Although the nonenzymatic thiol/thioester exchange reaction was predominant, yielding **14** and **15** as the major products, formation of the desired triostin A precursor, **4**, and subsequent aerobic oxidation to form the disulfide bridge allowed for chemoenzymatic synthesis of triostin A, **2** (Figure 3). Apart from the products of the nonenzymatic thiol/thioester exchange reactions, dilactone **4** and linear octapeptidyl-SNAC ester, **7**, were obtained. In addition, hydrolysis leading to carboxylic acid **6** was observed. These results suggest that intermolecular condensation of the tetrapeptidyl-*O*-TE intermediate with free **5** is sluggish, and, instead, nucleophilic attack of a thiol group or a water molecule to the intermediate occur preferentially. While Ecm TE-mediated dimerization in vitro relies on intermolecular condensation, the dimerization of tetrapeptides tethered to PCP and TE domains in vivo can proceed efficiently. It is important to note that the NRPS (Ecm7)

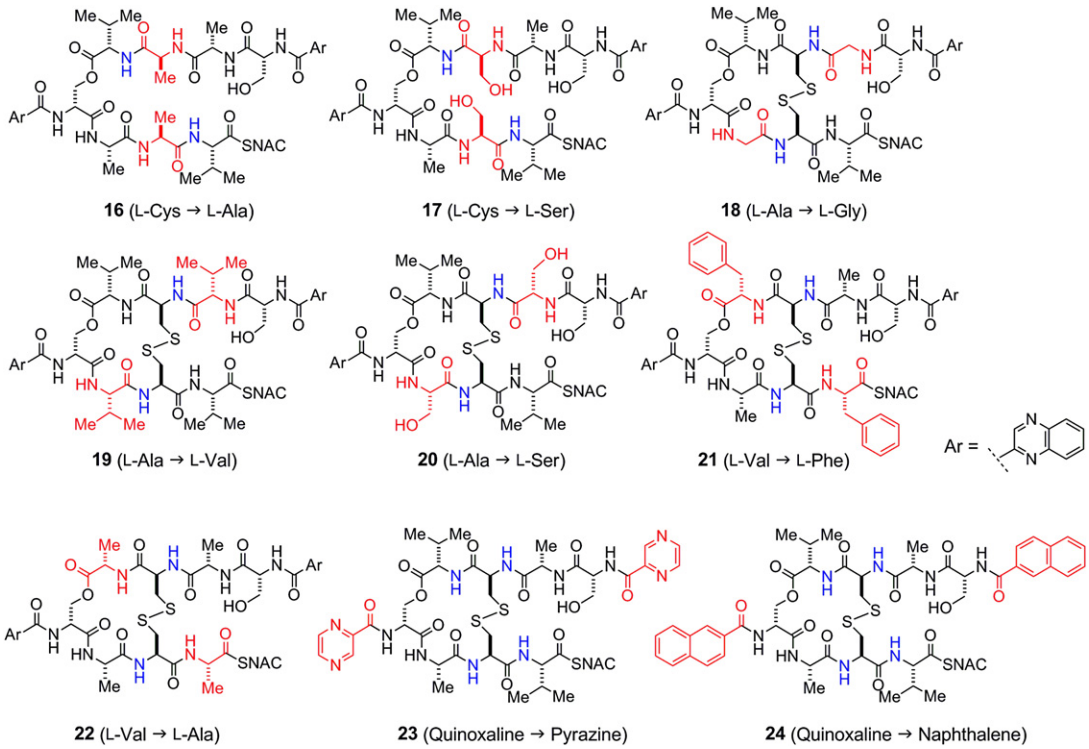


**Figure 5. Structures of Octapeptidyl-SNAC Thioester Substrates**

Regions highlighted by color represent differences in the substrates with respect to triostin A, **2**. Modified amino acid compositions are shown in red, and *N*-demethylated amide nitrogens are shown in blue.

responsible for echinomycin biosynthesis successfully regulates chemical reactivity by modulating the effective molarity of the reactive groups to form ester bond linkages in the presence of free thiols and a thioester.

Investigation of similar dimerization activity by TE domains has thus far been limited to GrsB TE, EntD TE, and TyrC TE (Kohli and Walsh, 2003; Shaw-Reid et al., 1999; Hoyer et al., 2007; Kohli et al., 2001), and two possible mechanisms, either a forward or a backward reaction, have been postulated for peptide ligation by the PCP/TE domains (Hoyer et al., 2007). In the course of echinomycin biosynthesis, it is quite probable to assume the two mechanisms for dimerization: (1) nucleophilic attack of the hydroxyl group of the first TE-bound monomer on the second monomer presented by the PCP domain led to a dimer tethered to the TE domains (forward reaction), or (2) attack of the hydroxyl group of the second PCP-bound monomer on the first TE-bound monomer, forming a PCP-bound dimer that is subsequently transferred to the TE domain for macrolactonization (backward reaction) (Figure S7). In vitro conversion of tetrapeptidyl thioester, **5**, by Ecm TE revealed formation of the octapeptidyl-SNAC thioester, **7** (Figure S3). Given that **7** is equivalent to the PCP-bound intermediate of the backward reaction (Figure S7), it may be presumed that the formation of **7** is consistent with the ''backward'' mechanism. On the contrary, it is also probable that **7** could be a dead-end product, and an actual intermediate merely accumulates. To verify the intermediary of **7** in the course of the backward reaction, we intended to conduct Ecm TE-mediated conversion of **7**. However, the fragile nature of **7** possessing two disulfides and a thioester in the same molecule precluded isolation and further investigations. Alternatively, a tetra-*N*-demethyl analog of the PCP-bound intermediate for the backward reaction was then envisaged. In fact, Ecm TE-catalyzed macrolactonizations of octapeptidyl thioester analogs such as **12** have been demonstrated (Table 1). Although it requires further investigations at this stage, these findings are compatible with the ''backward'' mechanism, affording an octapeptidyl thioester intermediate in the biosynthesis of the depsipeptide core of echinomycin, **1**, and triostin A, **2**.

Aside from the mechanistic insights into peptide ligation by NRPSs, chemoenzymatic cyclization of linear octapeptidyl thioesters by excised Ecm TE to produce TANDEM **3** and its analogs was then investigated. First, cyclization by Ecm TE of the linear octapeptidyl-SNAC, **9**, which contains twin thiol groups, was examined. However, only a trace amount of the desired depsipeptide, **4**, was obtained (data not shown). In an attempt to improve the enzymatic cyclization, we then designed substrate **12**, which has a disulfide bridge that was expected to restrict the flexibility of the chain and thereby facilitate preorganization of the substrate, with close proximity of the terminal thioester and the D-Ser hydroxyl groups as well as the appropriate orientation for macrolactonization. In a similar fashion, a model for substrate recognition by TycC TE, in which product-like intramolecular backbone hydrogen bonds facilitate preorganization of the peptide chain for cyclization, has been postulated (Trauger et al., 2001). Installation of the disulfide bridge was also intended to protect the nucleophilic thiol groups to prevent undesired

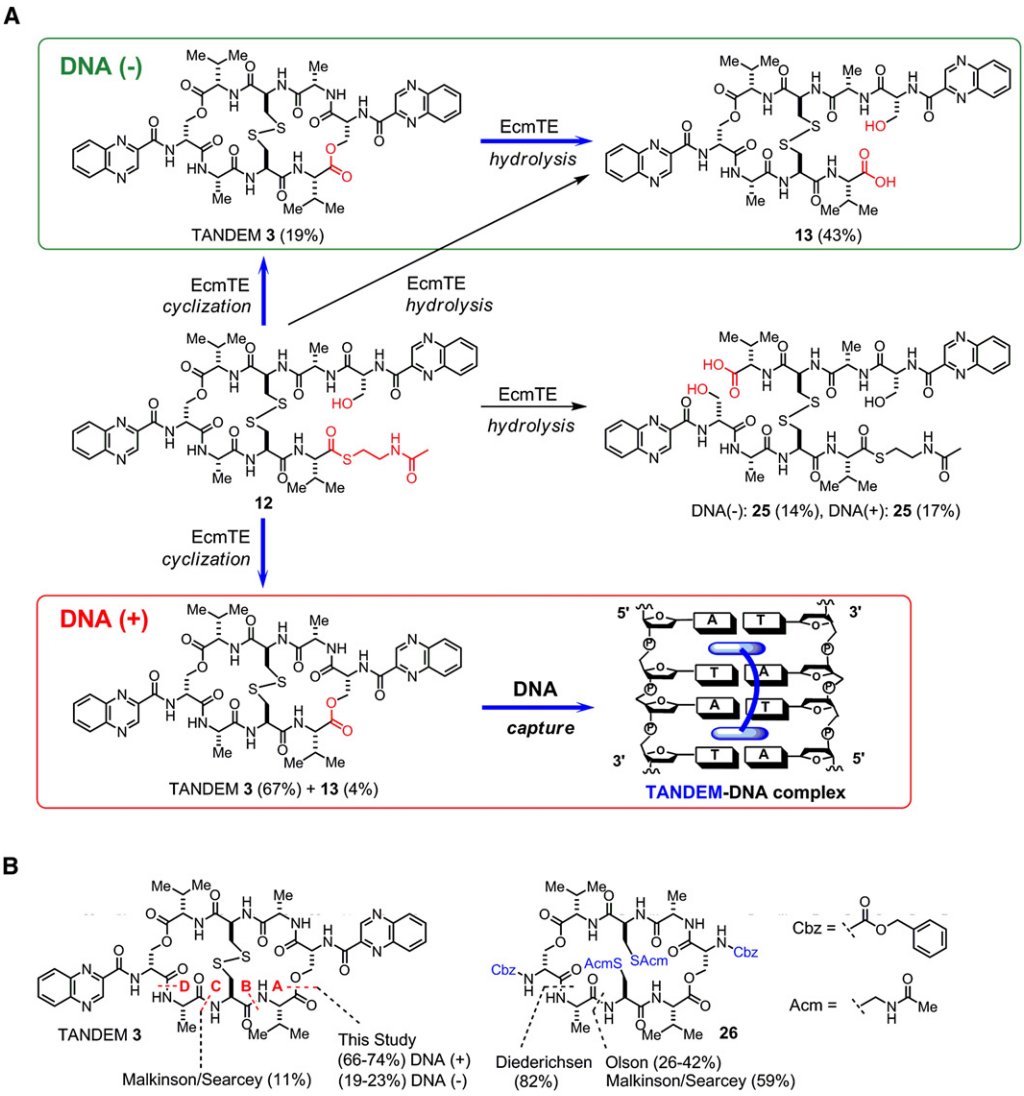


**Figure 6. Ecm TE-Catalyzed Macrocyclization in the Presence of DNA Leading to TANDEM**

(A) A diagram demonstrating the effect of DNA on the conversion of **12** by Ecm TE. Bold arrows (blue) indicate the major pathways. Upon incubation of **12** with Ecm TE for 8 hr, the results shown in green (without DNA) and red (with DNA) were obtained. In the absence of DNA, the cyclic product **3** was obtained in low yield (19%), and, instead, a significant amount of **13** (43%) was formed. Hydrolysis of **3** is assumed to be a major pathway to **13**. In the presence of DNA, the yield of cyclized **3** was dramatically improved, to 67%. Since the desired product, **3**, can be captured by DNA through bisintercalation, hydrolysis of **3** to give **13** was almost completely suppressed. In both cases, small quantities of **25** were formed by ester hydrolysis of **12**.

(B) Comparison of synthetic approaches to TANDEM **3**. Yields for macrocyclization to form the octapeptidyl cyclic skeleton are shown in parentheses. The four potential sites for macrocyclization, A–D, are shown in red. Protecting groups in the synthetic intermediate **26** for the total synthesis of **3** are shown in blue.

thiol/thioester exchanges. Ecm TE-catalyzed cyclization of **12** was carried out, producing the desired TANDEM **3** in 19% yield after 8 hr of incubation. However, a considerable amount of carboxylic acid **13** was obtained (43% yield) via hydrolysis of the thioester group.

Based on reaction monitoring, we deduced two obstacles for enzymatic production of the cyclic product **3**: product inhibition of Ecm TE, and hydrolysis of **3**, causing ring opening to yield **13**. To overcome these obstacles, we focused our attention on the DNA-binding ability of TANDEM **3** via bisintercalation and envisioned the addition of DNA containing TANDEM-binding sequences (5′-ATAT-3′) in order to capture the cyclic product **3** (Figure 6A). Gratifyingly, the addition of DNA with two such binding sites (5′-GATATCGATATC-3′) resulted in a dramatic improvement in the yield of the cyclic product **3** (19% → 67%), with a complete reversal of the cyclization:hydrolysis ratio (1:2 → 18:1) after 8 hr of incubation. Furthermore, coincubation of DNA increased consumption of substrate **12** (76% → 87%). Thus, the improvement in the reaction yield with the addition of DNA, which captures the cyclic product **3** detaching from Ecm TE, can be explained by prevention of product inhibition as well as by hydrolysis of the cyclic product. It is also worth mentioning that inhibition of Ecm TE by DNA has not been observed.

As mentioned above, the hydrolysis of substrates and cyclized products generally competes with the desired cyclization and often constitutes a major obstacle for enzymatic conversion of nonnatural substrates. To shift the cyclization:hydrolysis ratio in favor of cyclization, modifications such as the addition of nonionic detergent (Yeh et al., 2004) and the use of DMF as a solvent (Wagner et al., 2006) have been reported. The reported approaches aim to provide a hydrophobic environment that prevents nucleophilic attack of a water molecule on the acyl-enzyme intermediate by embedding the excised TE domain either in detergent micelles or an aprotic organic solvent. Thus, the approach described herein, which exploits the DNA-binding ability of the cyclic products, provides a hitherto unexplored, to our knowledge, protocol for improvement of TE-catalyzed cyclization. Furthermore, in vitro enzymatic synthesis, which makes use of DNA to exclude the cyclic products released from the enzymatic machinery, is strategically analogous to bacterial biosynthesis in vivo, which uses transmembrane transporters to pump out antibiotics before they accumulate to harmful concentrations within a producing cell (Walsh, 2003). According to recent genetic analysis, biosynthetic gene clusters for quinoxaline/quinoline antibiotics contain genes that encode ABC transporters for transmembrane secretion systems in this class of compounds, such as thiocoraline (Lombo et al., 2006).

Next, the applicability of the DNA coexistent protocol was investigated in the conversion of synthetic substrates **16**–**24**. Whereas the addition of DNA had a negligible effect on the conversion of substrates **16**–**17**, **19**–**21**, and **23**, the yields of cyclic products in the conversions of substrates **18**, **22**, and **24** were improved by a factor of 3–5. Furthermore, the DNA-binding abilities of **3** and the cyclic products of **18** and **24** were confirmed by induction of unwinding in supercoiled DNA based on agarose gel electrophoresis mobility (Figure S4). Thus, the DNA coexistent protocol was demonstrated to be applicable to TE domain-catalyzed conversion of a variety of synthetic substrates. Furthermore, this protocol represents an attractive possibility for the screening of DNA-binding small molecules. In the presence of DNA possessing any type of targeted sequence of small-molecule binding sites in TE-catalyzed cyclization, the production of DNA-binding cyclic peptides and the primary screening of their DNA-binding ability could be carried out simultaneously.

Finally, the features of previous syntheses of TANDEM **3** were compared with the present chemoenzymatic synthesis. The primary task in the successful synthesis of **3** is ring closure leading to a macrocyclic skeleton at the final stage of the synthesis. As shown in Figure 6B, there are four potential sites (A–D) for macrocyclization. Lactonization at site A is identical to the mode of ring closure in the biosynthesis of triostin A, **2**. However, this type of macrocyclization, with the formation of an ester linkage, has never been reported, presumably due to the poor nucleophilicity of an alcohol of the D-Ser residue. Boger and coworkers reported macrolactamization at site A to afford an aza analog of triostin A by replacing the D-Ser with D-β-aminoalanine, providing an amide versus ester linkage in the cyclic peptide skeleton (Boger and Lee, 2000). On the other hand, macrolactamization at site B has been avoided thus far, because the C-terminal L-Cys residue with a variety of protecting groups has been shown to undergo racemization upon anchoring as an ester during peptide elongation (Fujiwara et al., 1994; Han et al., 1997). Malkinson and Searcey reported macrolactamization at site C to afford triostin A, **2** (Malkinson et al., 2005). However, cyclization of the substrate without protecting groups to afford **2** directly resulted in a low yield (11%). This mode of cyclization (site C) showed an improved yield of up to 59% with the use of a protected substrate with a Cbz group for D-Ser and an Asm group for the thiol. In turn, Diederichsen reported condensation of Cbz-protected C-terminal D-Ser with an L-Ala residue to allow for macrolactamization at site D, yielding the protected **26** in 86% yield (Lorenz and Diederichsen, 2004). Thus, chemical approaches have necessitated protecting groups for efficient construction of the macrocyclic skeleton, and, consequently, additional protecting group manipulations are required to afford the desired **3**. In contrast, the chemoenzymatic approach described herein allows for direct conversion of thioester **12** to **3** in satisfactory yields (67% for a 8 hr incubation; 74% for a 10 hr incubation) without racemization. Thus, we have developed a straightforward synthetic route to TANDEM **3** and its analogs by harnessing the potential of excised TE-catalyzed macrolactonization.

## SIGNIFICANCE

**The C-terminal TE domain of modular NRPS allows for site-selective macrocyclization in the presence of multiple nucleophiles without the use of protecting groups, producing cyclic peptides with a diverse array of functional groups linked to the constrained skeletons. We showed that the recombinant Ecm TE domain can catalyze dimerization and subsequent lactonization in vitro to form the $C_2$-symmetric octadepsipeptide core of triostin A. Conversions of peptidyl thioesters with free thiol groups provide insight into the key role of the Ecm TE, which catalyzes the formation of ester linkages by activating hydroxyl groups in the presence of intrinsically more nucleophilic thiol groups, precluding thiol/thioester exchange reactions. In an effort to achieve chemoenzymatic synthesis of the synthetic derivative TANDEM and its analogs, substrate preorganization into product-like conformations by installation of a disulfide bridge was shown to be critical for Ecm TE-catalyzed cyclization. More importantly, coincubation with DNA successfully suppressed undesired hydrolysis as well as product inhibition, and it resulted in a remarkable improvement in the yield of cyclized products (greater than three-fold improvement: 19% → 67%). In vitro enzymatic synthesis employing DNA to capture and thereby exclude the cyclic product from the active site of the biosynthetic enzyme has a tactical analogy with in vivo bacterial biosynthesis that uses transmembrane transporters to pump out antibiotics. This chemoenzymatic strategy making use of DNA will be useful for other quinoxaline/quinoline antibiotics and will also be generally applicable to small molecules with DNA-binding properties.**

## EXPERIMENTAL PROCEDURES

### Cloning and Expression of the Ecm TE Domain

The echinomycin gene fragment encoding Ecm TE was amplified by PCR from cosCD4 (containing the echinomycin biosynthetic gene cluster) by using *SP-Taq* DNA polymerase (Cosmo Genetech) with the following oligonucleotides: forward primer 5′-ATACATATGGACACCGACGCCCAGTTCGA-3′, reverse primer 5′-AAAAAGCTTCTGCGACGCCCGCAGCATGC-3′. The amplified

*ecm TE* fragment was subcloned into pGEM-T vector by using the pGEM-T vector system (Promega). The resulting plasmid was digested with the restriction enzymes NdeI and BamHI. The digested DNA fragment was ligated into the NdeI/BamHI site of a pET22b (Novagen) by using DNA ligation kit ver. 2.1 (Takara). The construct (pET22b-*ecmTE*) was confirmed by sequencing with an ABI prism 310 genetic analyzer (Applied Biosystems). For expression, the pET22b-*ecmTE* was transformed into *E. coli* BL21(DE3). The transformed cells were grown at 37°C to OD = 0.6 (600 nm) and then induced with 0.4 mM IPTG. After additional incubation at 24°C for 8 hr, the cells were harvested by centrifugation at 2,500 × g for 15 min and resuspended in the disruption buffer (0.1 M Tris-HCl, 0.3 M NaCl, 10 mM imidazole, 10 mM $MgCl_2$, 10% v/v glycerol [pH 7.2]). Treatment with 1 mg/ml lysozyme for 5 min on ice and subsequent sonication disrupted the cells. The insoluble residues were removed by centrifugation at 18,600 × g for 30 min, and the supernatant protein was purified by Ni-NTA affinity chromatography (GE Healthcare). Buffer exchange into the reaction buffer (25 mM HEPES, 50 mM NaCl [pH 7.5]) was carried out by using a PD-10 column (GE Healthcare). Protein purity was confirmed by SDS-PAGE analysis to be greater than 95% (Figure S2). The protein concentration was estimated by using the Bradford assay for the BSA standard. The purified protein was flash frozen in liquid nitrogen and stored at −80°C for several months without significant loss of activity.

### Solid-Phase Synthesis of Linear Peptides

Peptides were synthesized manually by using a stepwise solid-phase procedure (Figure S8). Protected amino acids and reagents were purchased from Watanabe Chemical Industries, Novabiochem, and Wako Pure Chemical Industries. All peptide couplings were carried out for 30 min in DMF by using a 5 equiv. (over resin loading) of a protected amino acid activated with HBTU (5 equiv.) and HOBt (5 equiv.) in the presence of *i*-$Pr_2$NEt (10 equiv.). Each coupling reaction was repeated, and completion was detected by using the Kaiser test for free amines. If coupling was not completed, the reaction was further repeated until a negative Kaiser test was obtained. *N*-Fmoc protection was removed by using 20% (v/v) piperidine in DMF (2 × 10 min). The *o*-trityl group of the D-Ser side chain was cleaved by exposure to 2% (v/v) TFA in dry $CH_2Cl_2$ containing 5% (v/v) *i*-$Pr_3$SiH (3 × 5 min; 1 × 20 min). Esterification of the free hydroxyl group of D-Ser was performed in DMF by using *N*-Fmoc-Val (5 equiv.) and *N*,*N*′-diisopropylcarbodiimide (DIC: 5 equiv.) in the presence of a catalytic amount (10 mol%) of 4-dimethylaminopyridine (2 × 1.5 hr). *S*-Acm deprotection and simultaneous disulfide-bond formation were effected on the solid phase by treatment with iodine (10 equiv.) in DMF (1 hr), followed by thorough washing of the resin-bound depsipeptide with copious DMF, $CH_2Cl_2$, and 50% (v/v) MeOH in $CH_2Cl_2$, and then drying in vacuo (Malkinson et al., 2005). The peptides were liberated from the solid support by exposure with TFA containing 2.5% (v/v) water and 2.5% (v/v) *i*-$Pr_3$SiH for 2 hr at 25°C. After removal of TFA under reduced pressure, the crude peptides were precipitated in cold anhydrous $Et_2O$ and centrifuged. The precipitates were washed with cold anhydrous $Et_2O$, dried in vacuo, and used without further purification.

### Synthesis of Peptidyl-SNAC Thioester Substrates

The mixture of the crude peptide-*N*-acetylcysteamine (5 equiv.), PyBOP (3 equiv.), *i*-$Pr_2$NEt (3 equiv.) in THF/DMF (4:1) was stirred for 30 min at −20°C and then warmed up to room temperature. After being stirred for an additional 3 hr, the mixture was concentrated in vacuo. The residues were purified by high-performance liquid chromatography (HPLC) on a LC-10AD vp HPLC system (Shimadzu) with a Wakosil-II 5C18 column (Wako Pure Chemical Industries). The fractions were lyophilized to dryness and then dissolved in DMSO. The identities of the peptidyl-SNAC substrates were verified by LC-ESI-MS (LC: Agilent 1100 system, MS: JEOL, JMS-T100LP AccuTOF LC-plus) with a Zorbax Eclipse XDB-C18 column (Agilent, 50/2.1, particle size of 5 μm) (Table S1).

### Ecm TE-Catalyzed Dimerization and Lactonization of Tetrapeptidyl-SNAC Substrate 5

Enzymatic reactions were carried out in a buffer solution (25 mM HEPES, 50 mM NaCl, 1 mM TCEP [pH 7.5]) at 24°C in a total volume of 25 μl. The reactions were initiated by the addition of enzyme (Ecm TE: 10 μM), with a substrate concentration of 300 μM. After incubation for 2 hr, the reactions were quenched by the addition of 5% TFA/$CH_3CN$ (5 μl). The reaction products were analyzed by LC-ESI-MS under the following conditions: 0–5 min, 20% acetonitrile/0.1% HCOOH in $H_2O$/0.1% HCOOH; 5–25 min, linear gradient 20%–80% acetonitrile/0.1% HCOOH in $H_2O$/0.1% HCOOH. The cyclized product **4** was separated by HPLC and concentrated to dryness under a stream of $N_2$. The resulting residue was left aerobically at room temperature overnight to form a disulfide bond by oxidation. The resulting sample was analyzed by LC-ESI-MS to confirm the formation of **2**.

### Ecm TE-Catalyzed Macrolactonization of Octapeptidyl-SNAC Substrates

Enzymatic reactions were conducted under the same conditions as described above, except for the total volume (50 μl), the substrate concentration (25 μM), and the enzyme concentration (Ecm TE: 5 μM). The substrate concentration was 25 μM for standard reactions and varied in the kinetic experiments. The reactions were quenched at various times by the addition of 2% TFA/$H_2O$ (50 μl). The reaction products were analyzed by HPLC (Shimadzu) with an Inertsil ODS-3 column (GL Science, 150/3, pore diameter: 100 Å, particle size: 3 μm) under the following conditions: 0–5 min, 30% acetonitrile/0.05% TFA in $H_2O$/0.05% TFA; 5–35 min, linear gradient 30%–80% acetonitrile/0.05% TFA in $H_2O$/0.05% TFA; or 0–5 min, 20% acetonitrile/0.05% TFA in $H_2O$/0.05% TFA; 5–35 min, linear gradient 20%–80% acetonitrile/0.05% TFA in $H_2O$/0.05% TFA; 0.4 ml/min, 45°C. The extinction coefficients at 245 nm and 325 nm (the absorption maximum of the quinoxaline rings) were assumed to be the same for the substrates and products. The identities of the products were confirmed by LC-ESI-MS analysis (Table S1).

### Ecm TE-Catalyzed Macrolactonization in the Presence of DNA

The HPLC-purified oligonucleotide (5′-GATATCGATATC-3′) was purchased from Hokkaido System Science and used without further purification. The underlined sequences represent the sites previously reported to bind with TANDEM (Lavesa and Fox, 2001). The oligonucleotide was dissolved in the reaction buffer (25 mM HEPES, 50 mM NaCl [pH 7.5]) as a stock solution. In the standard reaction, the final concentration of the oligonucleotide was adjusted to 25 μM. The reactions were conducted under the same conditions as described above.

## SUPPLEMENTAL DATA

Supplemental Data include eight figures, one table, Supplemental Experimental Procedures and Supplemental References and can be found with the article online at http://www.chembiol.com/cgi/content/full/15/8/818/DC1/.

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