Chemo- and Regioselective Peptide Cyclization Triggered by the N-Terminal Fatty Acid Chain Length: The Recombinant Cyclase of the Calcium-Dependent Antibiotic from *Streptomyces coelicolor*†

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ABSTRACT: Here we report the first biochemical characterization of a recombinant nonribosomal peptide cyclase of a streptomycete, the model actinomycete *Streptomyces coelicolor* A3(2). This bacterium produces the calcium-dependent antibiotic (CDA), which is a branched cyclic macrolactone belonging to the group of acidic lipopeptides. The recombinant CDA3 cyclase from CDA synthetase efficiently catalyzes ring formation of linear peptidyl thioester substrates based on a sequence analogous to natural CDA. Four leaving groups were attached to the C-terminus of the undecapeptide: coenzyme A (CoA), phosphopantetheine, *N*-acetylcysteamine (SNAC), and thiophenol. The best rates for cyclization were determined for the thiophenol substrate, revealing that chemical reactivity is more important than cofactor recognition. The cyclase catalyzes the formation of two regioisomeric macrolactones, which arise from simultaneous nucleophilic attack of the two adjacent Thr_2 and Ser_1 residues onto the C-terminus of the acyl-enzyme intermediate. This relaxed regioselectivity has not been observed for any other recombinant NRPS or PKS cyclases so far. Substitution of either Ser_1 or Thr_2 by alanine led to selective formation of a decapeptide or undecapeptide lactone ring. In contrast to that, CDA3 cyclase strictly retains stereoselectivity for both nucleophiles, accepting only L-configured Ser_1 and Thr_2 for cyclization. Further, our studies provide evidence for the crucial role of N-terminal fatty acyl groups of lipopeptides in controlling the regio- and chemoselectivity of enzyme-catalyzed macrocyclization. Elongation of the fatty acyl group of our thioester substrate from C_2 to C_6 as in CDA turned the relaxed regioselectivity into a strict regioselectivity, yielding solely the decapeptide lactone ring with a significantly improved cyclization-to-hydrolysis ratio.

Macrocyclization represents an important pathway in nature to obtain highly bioactive compounds with important pharmaceutical applications. Among these natural products are antibiotics, such as daptomycin, friulimicin, amphomycin, and the calcium-dependent antibiotic (CDA)[1] (Figure 1), as well as immunosuppressive agents, such as cyclosporin and rapamycin (*1*). The macrocyclic structure constrains these compounds to a biologically active conformation by enhancing the rigidity of the ring skeleton and increases its stability against the proteolytic digest (*2*). The biosynthesis of such macrocyclic natural products is accomplished by nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS), and hybrid NRPS/PKS systems (*3*−*5*). These modularly organized enzymes comprise all catalytic units necessary for the assembly of linear peptide/polyketide precursors and their subsequent macrocyclization. The final step of this assembly line is usually catalyzed by a C-terminal thioesterase (TE) domain, which is about 28−35 kDa in size (*6*). TE domains are highly specialized cyclization catalysts, as indicated by their low overall identity ranging between 10% and 15%, which is the lowest among NRPS domains (*7*). They catalyze the formation of cyclic or branched cyclic natural products, as macrolactams (tyrocidine, iturin) or macrolactones (CDA, pristinamycin). Recent research on excised TE domains of the tyrocidine and surfactin synthetases revealed information of their substrate tolerance and cyclization mechanism (*3*, *8*, *9*). The observed high substrate tolerance of the tyrocidine cyclase led to the generation of comprehensive libraries of new macrocyclic compounds, which were screened for improved therapeutic activity (*10*).

Here we report the first biochemical characterization of an excised cyclase of a streptomycete, the TE domain of the calcium-dependent antibiotic (CDA3 TE). CDA is one of four antibiotics produced by *Streptomyces coelicolor* A3-

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[1] Abbreviations: CDA, calcium-dependent antibiotic; CoA, coenzyme A; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; ESI, electron spray ionization; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethaniminium hexafluorophosphate *N*-oxide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; HP, His patch; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; nd, not detected; NRPS, nonribosomal peptide synthetase; NTA, nitrilotriacetic acid; OD, optical density; PCP, peptidyl-carrier protein; PCR, polymerase chain reaction; PKS, polyketide synthase; ppant, 4′-phosphopantetheine; PyBOP, benzotriazol-1-yloxotris(pyrrolidino)phosphonium hexafluorophosphate; RP-LCMS, reverse-phase liquid chromatography−mass spectroscopy; Sfp, PPTase involved in surfactin production; SNAC, *N*-acetylcysteamine; TCEP, tris(carboxyethyl)phosphine; TE, thioesterase; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TIPS, triisopropylsilane; t_R, retention time.

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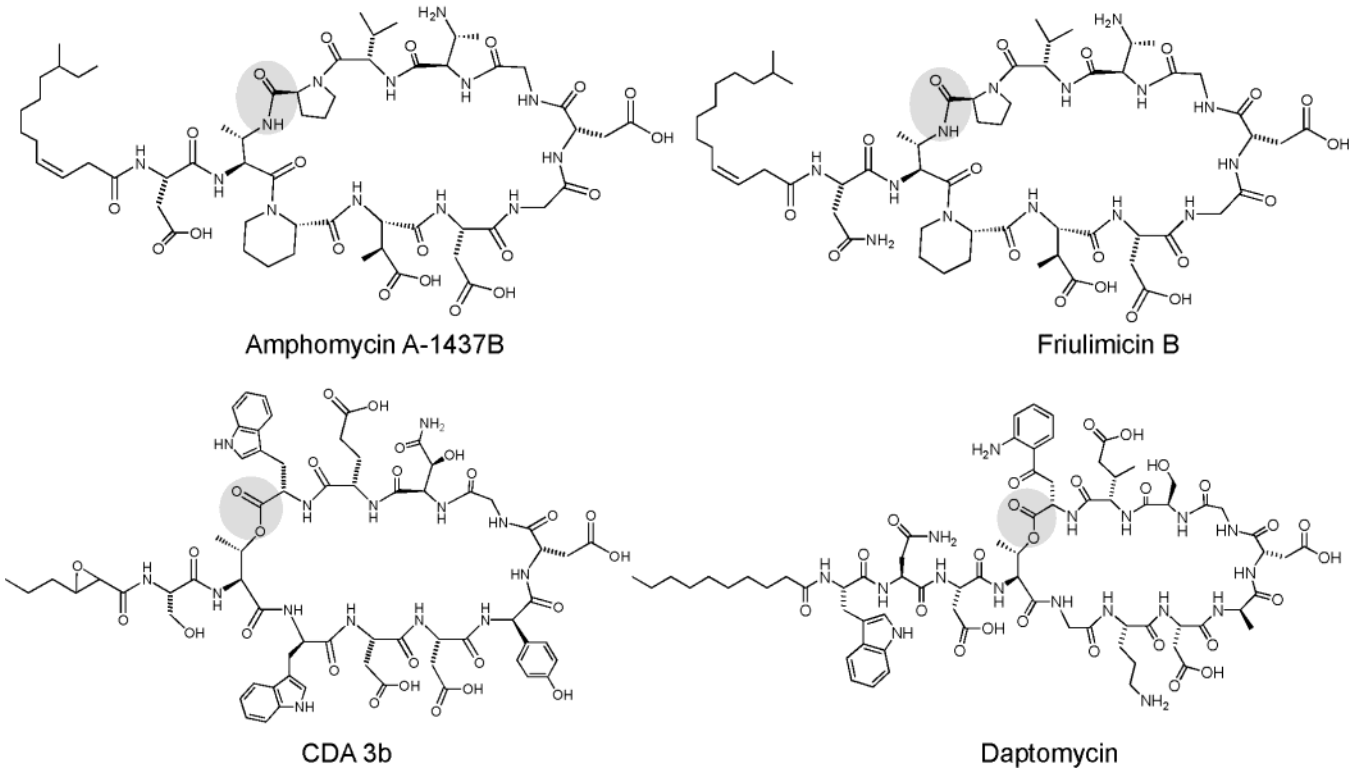


FIGURE 1: The group of the acidic lipopeptide antibiotics. All structures are comprised of a decapeptide lactone or lactam ring (ester or amide linkage highlighted by shading), including several acidic residues probably important for calcium binding and antibiotic activity as well as several D-configured and nonproteinogenic residues. CDA is produced by *S. coelicolor*, friulimicin B and amphomycin A-1437B are produced by by *Actinoplanes friuliensis*, and daptomycin is derived from A21978C isolated from *Streptomyces roseosporus*.

(2) (*11*). Its activity against a wide range of Gram-positive bacteria depends strongly on the presence of calcium ions. CDA acts as a ionophore capable of promoting the transport of monovalent ions across lipid bilayer membranes. It is assumed that calcium promotes the aggregation of several CDA molecules to create a transmembrane channel due to its interaction with the acidic residues of CDA (*12*).

The CDA NRPS contains three protein subunits, CDA1 (798.6 kDa), CDA2 (394.7 kDa), and CDA3 (258.5 kDa), which are encoded on an 82 kb region of the genome (*13*). The protein subunits are further subdivided into 11 modules catalyzing the incorporation of the same number of amino acids into the peptide backbone. The undecapeptide CDA contains three D-configured amino acids as well as nonproteinogenic amino acids, including D-4-hydroxyphenylglycine, D-3-phosphohydroxyasparagine or D-3-hydroxyasparagine, and L-3-methylglutamate. Recently, (*Z*)-dehydrotryptophan was discovered to be another nonproteinogenic amino acid of several CDA variants (*14*). CDA is termed as an acidic lipopeptide due to its high content of acidic amino acids and the occurrence of an N-terminal 2,3-epoxyhexanoyl side chain as the lipid part.

CDA is cyclized by the C-terminal TE domain of the synthetase. This cyclase mediates the nucleophilic attack of L-2-threonine onto the C-terminal carboxyl group, which leads to the formation of a branched cyclic macrolactone. The structure of CDA is very similar to that of the antibiotic daptomycin, which is of great pharmacological importance due to its possible activity against methicillin-resistant *Staphylococcus aureus* strains (MRSA) and vancomycin-resistant enterococci (VRE) (*14*). Daptomycin is a decapeptide lactone comprising six identical residues of CDA that are located in the same ring positions. Hence the CDA cyclase may play a significant role in an attempt to synthesize this structurally related antibiotic in vitro. Further, the CDA cyclase can be employed in a chemoenzymatic approach for the generation of comprehensive libraries of derivatives of CDA, which can be screened for elevated microbiological activity.

EXPERIMENTAL PROCEDURES

Cloning and Expression of the CDA3 TE Domain. The *cda3 te* gene fragment was amplified by PCR using the chromosomal DNA of *S. coelicolor* A3(2) as template. The PCR reaction was carried out with Turbo DNA polymerase (Stratagene). Amplification of *cda3 te* [containing CDA3 residues 2113−2417 (*15*)] was performed using the oligonucleotides 5′-C ACC ATG CGC GGC GGC CGG GAG CC and 5′-GGC GAC CTC GGT CGA ATC G. The blunt-end PCR product was directionally cloned into a pBAD202/D-TOPO vector (Invitrogen) using the pBAD directional TOPO expression kit (Invitrogen). The pBAD202/D-TOPO vector appends an N-terminal His-patch (HP) thioredoxin leader and a C-terminal His_6 tag to the expressed protein. DNA sequencing of the yielded vector pBAD202/D-TOPO-TE(CDA3) was performed by GATC Biotech on an ABI prism 310 genetic analyzer (Applied Biosystems). For expression, the cloning product was transformed into *Escherichia coli* BL21 (Amersham Biosciences). The transformed cells were grown to OD = 0.4 (600 nm), induced with 0.01% arabinose, and again grown at 25 °C for 2.5 h. The recombinant protein was purified by Ni-NTA affinity chromatography (Amersham Pharmacia Biotech). Dialysis into 25 mM Hepes and 50 mM NaCl, pH 7.0, was carried

Table 1: Characterization of Substrates by MS

compound	species	ionization method	obsd mass (calcd mass) (Da)
1−CoA	$[M + H]^+$	ESI	2176.4 (2176.6)
1−ppant	$[M + H]^+$	ESI	1767.3 (1767.6)
1−SNAC	$[M + H]^+$	ESI	1528.4 (1528.6)
1−thiophenol	$[M + H]^+$	ESI	1519.3 (1519.5)
2	$[M + H]^+$	ESI	1575.4 (1575.6)
3	$[M + H]^+$	ESI	1473.4 (1473.5)
4	$[M + H]^+$	ESI	1503.2 (1503.5)
5	$[M + H]^+$	ESI	1489.3 (1489.5)
6	$[M + H]^+$	ESI	1489.3 (1489.5)
7	$[M + H]^+$	ESI	1503.4 (1503.5)
8	$[M + H]^+$	ESI	1519.4 (1519.5)
9	$[M + H]^+$	ESI	1519.3 (1519.5)
10	$[M + H]^+$	ESI	1519.4 (1519.5)

out using HiTrap desalting columns (Amersham Pharmacia Biotech). The concentration of the purified protein was determined spectrophotometrically using the estimated extinction coefficient at 280 nm. After being flash frozen in liquid nitrogen, the protein was stored at −80 °C over several months.

Synthesis of Peptidyl-SNAC and Peptidyl-Thiophenol Substrates. All linear peptides were synthesized by solid-phase peptide synthesis as described earlier (*3*). Protected amino acids were purchased from Novabiochem and Bachem Biosciences. All other compounds were purchased from Sigma-Aldrich, except HBTU and HOBt·H_2O (IRIS). The preparation and purification of peptidyl-thiophenol substrates and peptidyl-SNAC substrates were described previously (*16*). The identities of peptidyl-SNAC and peptidyl-thiophenol substrates were determined by reverse-phase liquid chromatography−mass spectroscopy (RP-LCMS) (Table 1).

Synthesis of Peptidyl-CoA and Peptidyl-ppant Substrates. The synthesis of phosphopantetheine (ppant) was described previously (*17*). One equivalent of coenzyme A, 0.2 equiv of TCEP·HCl, and 0.5 unit/μmol nucleotide pyrophosphatase (Sigma) were dissolved in Hepes buffer (50 mM, pH 7.5). The mixture was incubated at 30 °C for 18 h and then lyophilized to dryness.

The preparation of peptidyl-ppant substrates is based on the synthesis of peptidyl-CoA substrates described elsewhere (*18*). To 1 equiv of protected peptide were added 1.5 equiv of ppant, 1.5 equiv of PyBOP, and 4 equiv of potassium carbonate, dissolved in a 1:1 THF/water mixture. The mixture was stirred for 2 h at room temperature which was followed by the removal of the solvent. Subsequent cleavage of the side-chain protecting groups was carried out using a mixture containing trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and water in a ratio of 95:2.5:2.5. The deprotected peptidyl-ppant substrate was precipitated in ice-cold diethyl ether and purified by preparative high-performance liquid chromatography (HPLC) on a Äkta purifier (Pharmacia) HPLC system with a reverse-phase C_{18} Nucleodur (Macherey and Nagel) column. Identification of the peptidyl-CoA and peptidyl-ppant substrates was verified by liquid chromatography−mass spectroscopy (RP-LCMS) (Table 1).

Assays. Reactions were carried out in 25 mM Hepes and 50 mM NaCl, pH 7.0, in a total volume of 50 μL. Dissolution of **2** (Figure 4) was facilitated by the addition of 5% DMSO (v/v). In standard reactions the substrate concentration was 250 μM. For kinetic studies the substrate concentration was varied in the range 25 μM−1 mM. Reactions were initiated by addition of enzyme to a final concentration of 5 μM. Reactions were quenched by addition of 35 μL of 4% TFA/H_2O. All assays were analyzed by RP-LCMS on a C_{18} Nucleodur column (Macherey and Nagel, 250/3, pore diameter 100 Å, particle size 3 μm) except for assays with **1**−SNAC (Figure 4), which were analyzed on a C_{18} Nucleosil column (Macherey and Nagel, 250/3, pore diameter 120 Å, particle size 3 μm) with the following gradients: all thiophenol thioesters (except **2**), **1**−CoA, and **1**−SNAC, 0−40 min, 15−45% acetonitrile/0.1% TFA in water/0.1% TFA, 0.4 mL/min, 45 °C; **1**−ppant, 0−40 min, 5−60% acetonitrile/0.1% TFA in water/0.1% TFA, 0.4 mL/min, 45 °C; and **2**, 0−40 min, 15−60% acetonitrile/0.1% TFA in water/0.1% TFA, 0.4 mL/min, 45 °C.

Identities of the products were verified by ESI-MS (Table 2). Connection regiospecificity of cyclic products was determined by MS-MS analysis on an API Qstar Pulsar I (Applied Biosystems) (Table 3). Concentrations of various peptidyl thioesters were calculated using experimentally determined extinction coefficients at a wavelength of 220 nm. The extinction coefficient of **1**−SNAC was assumed to be identical to cyclized and hydrolyzed products. Kinetic characterization of the cyclization and hydrolysis reactions was performed by determining initial rates at five to seven substrate concentrations using two time points at each concentration within the linear region of the enzyme verified by time courses.

The cyclic products were isolated using analytical HPLC. After lyophilization to dryness the separated cyclic product was incubated with enzyme (5 μM) in a total volume of 50 μL (25 mM Hepes, 50 mM NaCl, pH 7.0). The assay was quenched after 3 h by addition of 35 μL of 4% TFA/H_2O and analyzed by LCMS.

RESULTS

Thioredoxin−CDA3 TE Fusion. CDA3 TE is a recombinant cyclase of the model actinomycete *S. coelicolor* A3(2). The high GC content (ca. 75%) of this soil-dwelling, filamentous bacterium complicated the expression of this cyclase. Initial attempts to express CDA3 TE in a pQE-70 vector failed. Hence a different cloning strategy was employed for the expression of this first recombinant cyclase of a streptomycete. The *cda3 te* fragment was cloned into the pBAD202/D-TOPO vector (Invitrogen) using a one-step cloning strategy without ligase and restriction enzymes. This expression system appends an N-terminal His-patch thioredoxin domain (11.7 kDa) to the recombinant cyclase, which facilitates solubility and translation efficiency of the expressed protein (49.4 kDa) (*19*) (Figure 2).

CDA3 Cyclase Catalyzes Ring Formation of a Synthetic CDA Analogue. To examine the ability of CDA3 cyclase to catalyze macrolactonization, we synthesized an peptide thioester analogue of the natural CDA substrate. So far seven CDA variants have been isolated, which differ in their incorporation of nonproteinogenic amino acids (*14*). For synthetic reasons these nonproteinogenic amino acids were substituted by similar proteinogenic amino acids. D-4-Hydroxyphenylglycine at position 6 was substituted by D-phenylalanine, and D-3-phosphohydroxyasparagine/D-3-hydroxyasparagine at position 9 was replaced by D-aspar-

Table 2: Characterization of Products by MS

compound	species	ionization method	obsd mass (calcd mass) (Da)		Cy/Hy ratio
			cyclized product	hydrolyzed product	
1−CoA	$[M + H]^+$	ESI	1409.4 (1409.5)	1427.4 (1427.5)	8:1
1−ppant	$[M + H]^+$	ESI	1409.3 (1409.5)	1427.3 (1427.5)	2:1
1−SNAC	$[M + H]^+$	ESI	1409.3 (1409.5)	1427.4 (1427.5)	9:1
1−thiophenol	$[M + H]^+$	ESI	1409.4 (1409.5)	1427.3 (1427.5)	5:1
2	$[M + H]^+$	ESI	1465.4 (1465.6)	1483.4 (1483.6)	10:1
3	$[M + H]^+$	ESI	nd[a] (1363.5)	1381.4 (1381.5)	
4	$[M + H]^+$	ESI	1393.4 (1393.5)	1411.4 (1411.5)	3:1
5	$[M + H]^+$	ESI	1379.4 (1379.5)	1397.3 (1397.5)	1:3
6	$[M + H]^+$	ESI	nd (1379.5)	1397.3 (1397.5)	
7	$[M + H]^+$	ESI	nd (1393.5)	1411.4 (1411.5)	
8	$[M + H]^+$	ESI	1409.4 (1409.5)	1427.4 (1427.5)	2:1
9	$[M + H]^+$	ESI	1409.4 (1409.5)	1427.2 (1427.5)	1:5
10	$[M + H]^+$	ESI	nd (1409.5)	1427.4 (1427.5)	

[a] nd = not detected.

agine (Figure 3). At positions 10 and 11 the proteinogenic amino acids L-glutamate and L-tryptophan were incorporated into the peptide backbone as in the case of the CDA variants CDA1b and CDA3b. The natural occurring glycine at position 8 was substituted by alanine in order to avoid aspartimide formation (*20*). For solubility reasons the N-terminus of the synthetic CDA analogue was acetylated instead of attaching the 2,3-epoxyhexanoyl fatty acid of the natural occurring CDA. Hence the sequence of the synthesized **1** was chosen as follows: acetyl-L-Ser_1-L-Thr_2-D-Trp_3-L-Asp_4-L-Asp_5-D-Phe_6-L-Asp_7-L-Ala_8-D-Asn_9-L-Glu_{10}-L-Trp_{11} (Figure 4). The C-terminus of **1** was attached to the SNAC leaving group (Figure 5), which mimics the last part of the ppant arm of the natural cofactor. Assaying for CDA3 TE mediated cyclization revealed that the peptide−SNAC is cyclized. Remarkably, two products with the expected mass for cyclization were observed (Figure 6B). Taking into account that **1** possesses two adjacent nucleophiles, L-Ser_1 and L-Thr_2, it was assumed that both nucleophiles are involved in ring formation. To ensure that no other nucleophiles except L-Ser_1 and L-Thr_2 contribute to cyclization, we synthesized a thioester substrate **3** (Figure 4), where both nucleophiles of **1** are replaced by alanine. As expected, upon incubation with CDA3 cyclase no cyclization product was detected. Instead, the substrate was fully hydrolyzed (data not shown).

The identities of both cyclic products were confirmed by MS-MS sequencing (Table 3). For the peak with the lower retention time (t_R = 28.0 min) four fragments were detected, which did not appear in the MS-MS-spectrum of the peak with t_R = 28.6 min. These fragments were characteristic for an undecapeptide lactone, where Ser_1 contributes as internal nucleophile. In theses cases simultaneous fragmentation occurred between Ser_1 and Thr_2 as well as between Glu_{10} and Trp_{11}, leading to fragments, where the former lactone bond was still maintained. If cyclization would occur via Thr_2, no such fragments would have been obtained with the apparent masses of Ser_1 cyclization. Therefore, the peak with t_R = 28.6 min can be assigned to the lactone cyclizing via Thr_2, because this is the only remaining nucleophile in our CDA analogue as evidenced with **3**. The ratio between these two regioisomeric macrolactones was determined as 1:4 (undecapeptide lactone:decapeptide lactone). The flux toward hydrolysis was very low, revealing a cyclization-to-hydrolysis ratio of 9:1.

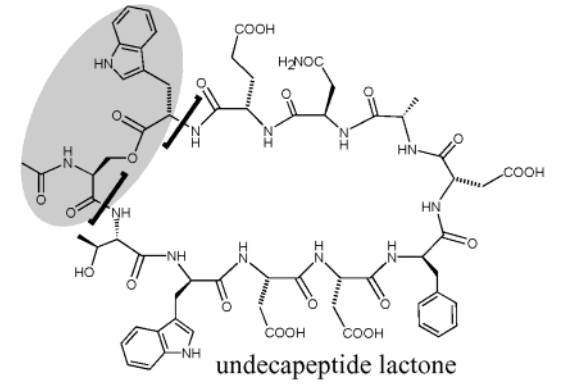
CDA3 cyclase was also probed with structurally unrelated peptidyl thioesters. The sequences of the linear peptide substrates were derived from surfactin, fengycin, bacitracin, and syringomycin. Incubation of CDA3 cyclase with the corresponding peptidyl-thiophenol substrates did not show any enzyme-mediated cyclization. Identities of all substrates and products were verified by HPLC-MS (data not shown).

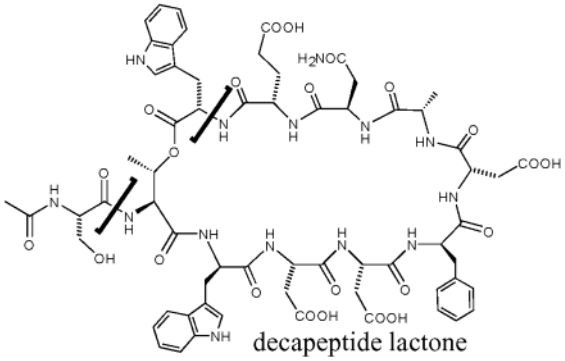
Selecting the Best Leaving Group for Macrolactonization Mediated by the CDA3 Cyclase. To determine which leaving group is best suited to support CDA3 TE catalyzed macrolactonization, four different leaving groups were attached to the C-terminus of **1** (Figure 5). Besides the SNAC leaving group, which was successfully employed for in vitro cyclization of tyrocidine A, gramicidin S, and surfactin (*3*, *8*, *21*), three other leaving groups were tested for their ability to support ring formation of **1**. Phosphopantetheine is structurally identical to the prosthetic group of the peptidyl-carrier protein, which is derived from coenzyme A. Coenzyme A was successfully employed for loading chemically synthesized peptidyl-coenzyme A substrates onto apoPCP using the phosphopantetheinyl transferase Sfp. Using this experimental approach led to the characterization of fengycin cyclase, which formerly displayed no activity with peptidyl-SNAC substrates (*22*). The recombinant CDA cyclase was also probed with the **1** tethered to the thiophenol leaving group, which possesses no structural analogy to the phosphopantetheine arm of the PCP. Nevertheless peptidyl-thiophenol thioesters allowed biochemical characterization of fengycin, mycosubtilin, and syringomycin, which did not show any activity with SNAC substrates (*16*). A probable reason for these results might be the higher chemical reactivity of thiophenol due to efficient delocalization of thiolate electrons throughout the aromatic ring system.

We next examined the CDA3 cyclase catalyzed product formation with the CoA (Figure 6A), ppant, and thiophenol thioester substrates of **1**. The observed products were identical to that observed for **1**−SNAC. Notably, the enzyme-catalyzed flux toward hydrolysis was bigger for **1**−ppant than for the three other thioester substrates.

To determine the best leaving group for CDA3 TE mediated cyclization of **1**, the kinetics of cyclization for all four peptidyl thioesters were measured. The cyclization reaction follows Michaelis−Menten kinetics with the K_M and k_{cat} values reported in Table 4. Although **1**−ppant is structurally identical to the ppant arm of the PCP, its K_M

Table 3: MS-MS Sequencing of Macrolactones Derived from CDA Analogues **1** and **2**[a]





compound	molecular formula	species	obsd mass of fragments (calcd mass) (Da) (cyclization via Ser_1)
undecapeptide lactone	$C_{15}H_{18}N_3O_3^+$	$[M + H]^+$	288.118 (288.135)
	$C_{16}H_{17}N_2O_4^+$		301.126 (301.119)
	$C_{17}H_{19}N_4O_5^+$		359.138 (359.136)
	$C_{17}H_{21}N_4O_5^+$		361.172 (361.151)
decapeptide lactone	$C_{15}H_{18}N_3O_3^+$	$[M + H]^+$	nd[b] (288.135)
	$C_{16}H_{17}N_2O_4^+$		nd (301.119)
	$C_{17}H_{19}N_4O_5^+$		nd (359.136)
	$C_{17}H_{21}N_4O_5^+$		nd (361.151)
decapeptide lactone with hexanoic acid	$C_{15}H_{18}N_3O_3^+$	$[M + H]^+$	nd (344.197)
	$C_{16}H_{17}N_2O_4^+$		nd (357.181)
	$C_{17}H_{19}N_4O_5^+$		nd (415.198)
	$C_{17}H_{21}N_4O_5^+$		nd (417.214)

[a] The molecular part highlighted by shading represents a fragment (301.119 Da) characteristic for the undecapeptide lactone, where Ser_1 contributed as the internal nucleophile. If cyclization would occur via Thr_2, no such fragment would have been obtained, because simultaneous fragmentation of the same two bonds would only result in an opening of the corresponding lactone ring. Fragmentation of C−C bonds in the fatty acyl chain of **2** is not possible under experimental conditions.[b] nd = not detected.

value is at least 10-fold higher than those measured for **1**−thiophenol or **1**−SNAC (Table 4). We also discovered that the k_{cat} value for cyclization of the **1**−thiophenol substrate [k_{cat}(undecapeptide lactone) = 0.097 min^{-1}; k_{cat}(decapeptide lactone) = 0.323 min^{-1}] was approximately 3-fold higher than observed for the other three CDA thioesters. The highest flux to cyclization mediated by the CDA3 cyclase was observed for the **1**−thiophenol substrate. Its catalytic efficiency for cyclization [k_{cat}/K_M(undecapeptide lactone) = 2.44 mM^{-1} min^{-1}; k_{cat}/K_M(decapeptide lactone) = 8.13 mM^{-1} min^{-1}] was approximately 10−16-fold the

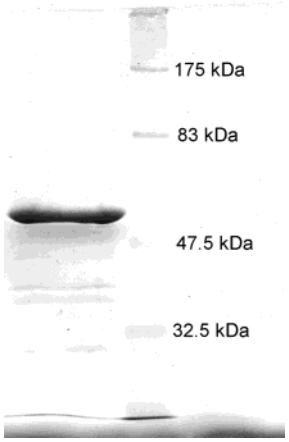


FIGURE 2: Purified recombinant CDA3 cyclase examined in this study. CDA3 cyclase (49.4 kDa) was overproduced in *E. coli* BL21 and purified by Ni-NTA affinity chromatography. The concentrated protein was resolved by SDS−PAGE (12.5%) and visualized by Coomassie stain.

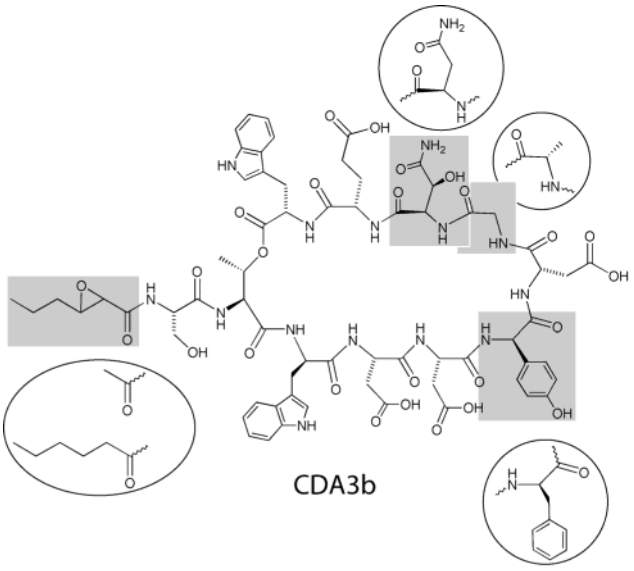


FIGURE 3: Comparison between peptide analogues used in this work and wild-type CDA3b. Regions highlighted by shading represent the molecular parts of CDA3b, which were replaced in the CDA analogues **1** and **2**. The deviating parts of these analogues are indicated by circles.

value of the SNAC substrate [k_{cat}/K_M(undecapeptide lactone) = 0.156 mM^{-1} min^{-1}; k_{cat}/K_M(decapeptide lactone) = 0.799 mM^{-1} min^{-1}]. Surprisingly, the k_{cat}/K_M value for CDA3 TE mediated cyclization was the second lowest for **1**−ppant [k_{cat}/K_M(undecapeptide lactone) = 0.023 mM^{-1} min^{-1}; k_{cat}/K_M-(decapeptide lactone) = 0.115 mM^{-1} min^{-1}]. This result clearly indicates that the structural identity to the ppant arm of the PCP is not important for recognition by the dissected enzyme.

The cyclization-to-hydrolysis ratios for **1**−SNAC and **1**−CoA in the presence of CDA3 TE were determined to be 9:1 and 8:1, respectively (Table 2, right column). Those values confirm the extraordinarily high chemoselectivity of this cyclase, which catalyzes almost exclusively macrolactonization of **1**. The lower cyclization-to-hydrolysis ratio of 5:1 for **1**−thiophenol confirms the higher background hydrolysis of this substrate due to the high chemical reactivity of the attached thiophenol leaving group. Surprisingly, the

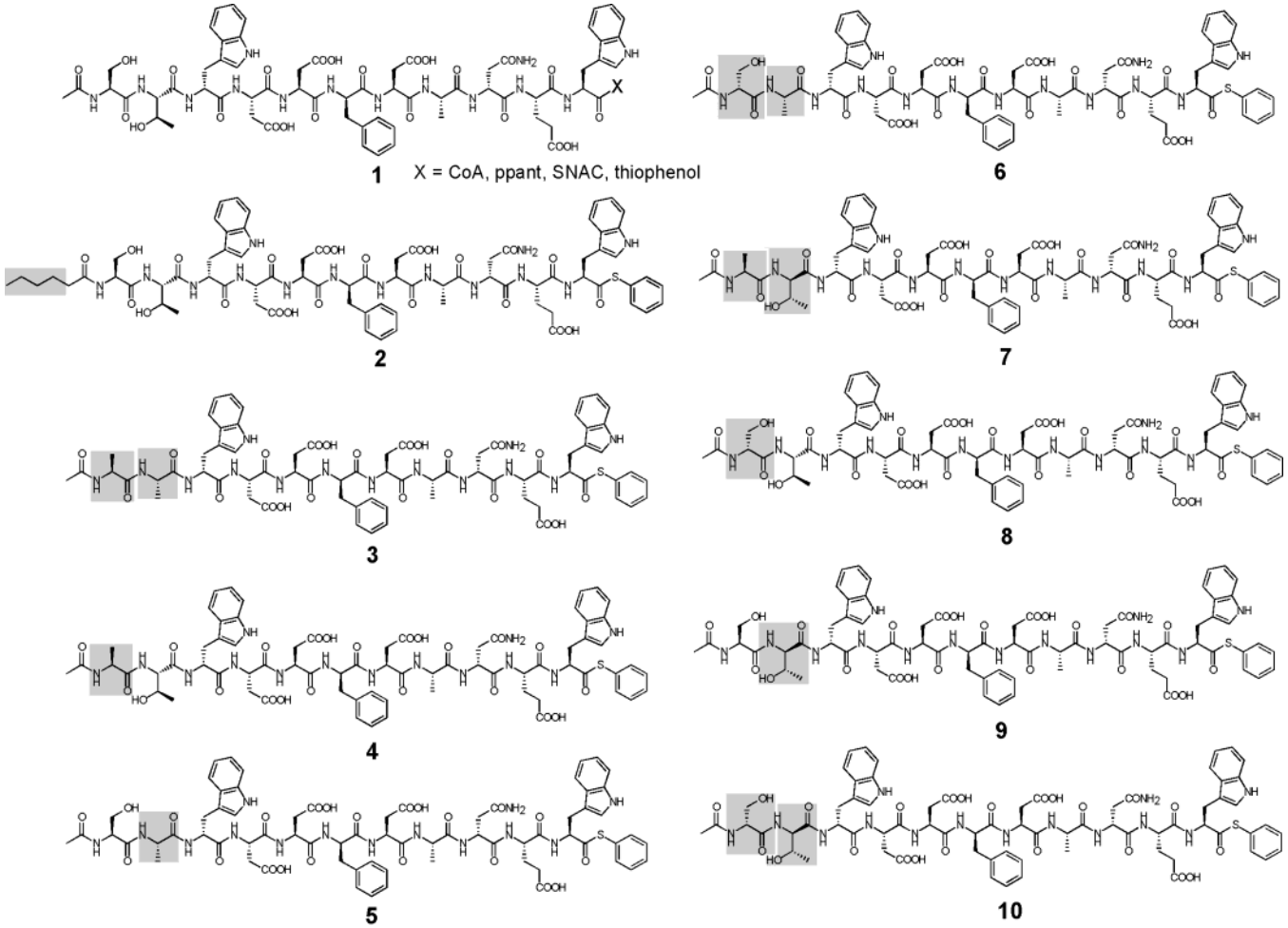


FIGURE 4: Peptides synthesized for study of the regio-, stereo-, and chemoselectivity of the CDA3 cyclase mediated macrolactonization. Regions highlighted by shading represent differences in substrates with respect to **1**. Compound **2** is a linear CDA thioester analogue, where the C-terminal acyl chain is elongated to its natural length. Compounds **3**−**5** are characterized by the replacement of the nucleophilic residues Ser_1 and/or Thr_2 with L-alanine. Compounds **6** and **7** each contain only one internal nucleophile, Ser_1 or Thr_2, with a stereochemistry opposite of the natural configuration. Compounds **8**−**10** possess both internal nucleophiles, Ser_1 and Thr_2, where either one or both are changed in their configuration. CoA = coenzyme A, ppant = phosphopantetheine, and SNAC = *N*-acetylcysteamine.

cyclization-to-hydrolysis ratio of **1**−ppant was even lower than for **1**−thiophenol.

Regioselectivity of CDA3 Cyclase. Our results with the thioester substrates of **1** showed that the recombinant CDA3 cyclase catalyzes the simultaneous formation of two regioisomeric cyclic products. To determine whether our cyclase can be employed for the regioselective formation of peptidolactones with defined ring sizes, two additional thiophenol substrates, **4** and **5**, were synthesized (Figure 4). Compound **4** lacks Ser_1 as a cyclization nucleophile, whereas **5** lacks Thr_2, which was described to be the only cyclization nucleophile in naturally occurring CDA (*12*). Probing CDA3 cyclase with these two CDA analogues revealed the formation of only one cyclization product in each case (Figure 7). Reaction profiles showed that **5** was primarily converted into the peptide acid with a cyclization-to-hydrolysis ratio of 1:3 (Table 2). Conversely, **4** led primarily to the formation of the peptidolactone, yielding a cyclization-to-hydrolysis ratio of 3:1, which indicates that Thr_2 is the preferred nucleophile for cyclization.

Stereoselectivity of CDA3 Cyclase. We reported previously that syringomycin cyclase does not tolerate a change in stereochemistry of the cyclization nucleophile Ser_1 (*16*). To test if CDA3 cyclase retains its stereoselectivity as well, we synthesized two thiophenol CDA derivatives, **6** and **7** (Figure 4). These peptidyl thioesters allow the selective examination of the stereochemistry of only one nucleophilic amino acid due to the replacement of the second one by alanine. Incubation of these CDA derivatives with CDA3 cyclase resulted only in the formation of hydrolysis product, indicating the importance of L-configured serine and threonine in positions 1 and 2 for the enzyme-mediated macrolactonization (data not shown).

We also synthesized the substrates **8** and **9** (Figure 4), in which either serine or threonine is replaced by its corresponding D-isomer. We find that **8** and **9** generated only one cyclization peak on the HPLC trace in the presence of CDA3 cyclase (Figure 8). Remarkably, cyclization was observed to be the main product for **8** (cyclization-to-hydrolysis ratio = 2:1), while, on the other hand, **9** was converted primarily into the peptide acid (cyclization-to-hydrolysis ratio = 1:5) (Table 2). These results show that cyclization selectively occurs via the L-configured amino acids serine and threonine, while the latter is the preferred cyclization nucleophile of the CDA3 cyclase. The role of CDA3 cyclase as a stereoselective macrolactonization catalyst could be further emphasized by the thiophenol substrate **10** (Figure 4), which possesses only D-configured nucleophiles. Incubation of this

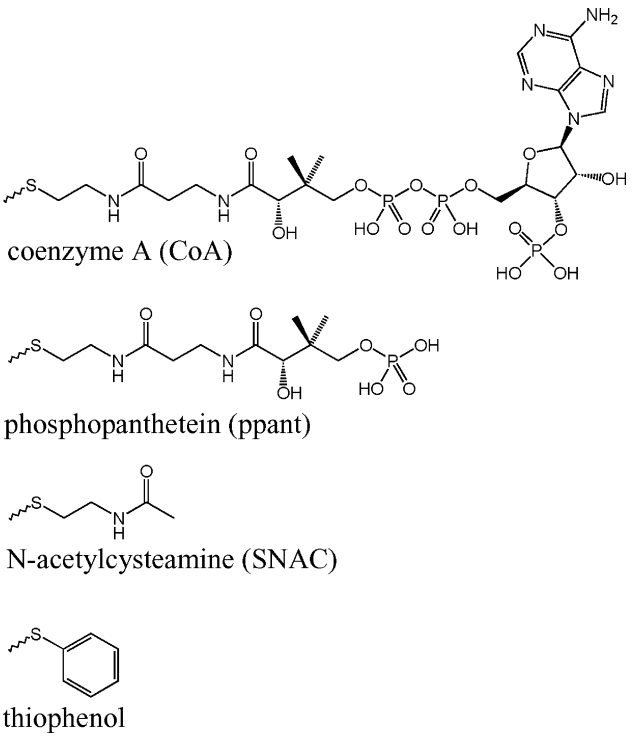


FIGURE 5: Structures of the leaving groups appended to the C-terminus of **1**. Coenzyme A (CoA), phosphopantetheine (ppant), and *N*-acetylcysteamine (SNAC) are mimics of the phosphopantetheine cofactor covalently bound to an invariant Ser residue of the peptidyl-carrier protein (PCP). In contrast to that, thiophenol has no structural similarity to this prosthetic group.

substrate with CDA3 cyclase revealed only hydrolysis (data not shown), thereby emphasizing the need for a correct stereochemistry for the enzyme-mediated cyclization at positions 1 and 2, respectively.

*Extending the N-Terminal Acyl Chain of **1***. The observation that CDA3 cyclase catalyzes the generation of two regioisomeric macrolactones on incubation with **1** (Figure 6) contradicts the findings of Kempter et al., who characterized naturally occurring CDA to be cyclized over Thr_2 (*12*). Therefore, we tried a better approximation of the natural interaction between the linear peptide precursor and the CDA cyclase. This was achieved by loading **1**−CoA onto the PCP domain of a recombinant CDA3 PCP−TE fusion protein catalyzed by the phosphopantetheinyl transferase Sfp. Nevertheless, the relaxed regioselectivity was still maintained, producing two peptidolactones with different ring sizes (data not shown). Hence, we concluded that the nonregioselective cyclization in vitro may be due to the structural deviation of **1** from naturally occurring CDA. Therefore, we synthesized **2** as a new CDA analogue (Figure 4), which was structurally closer to its natural counterpart. We replaced the N-terminal acetyl residue of **1** by a hexanoyl fatty acid. The 2,3-epoxy group of natural CDA was omitted for synthetic reasons. Surprisingly, the reaction profile of **2** revealed that this compound was efficiently transformed into only one macrocycle (Figure 9). MS-MS sequencing revealed that solely the decapeptide lactone was formed (Table 3), because none of the four fragments characteristic for the macrolactone cyclizing via Ser_1 were detected. This result indicates that the N-terminal hexanoyl fatty acid appears to be important for regioselective cyclization of **2**. The kinetic parameters for the CDA3 TE catalyzed cyclization were determined as $K_M = 65\ \mu M$ and $k_{cat} = 1.92\ min^{-1}$ (Table 4). In fact, the cyclization-to-hydrolysis ratio of 10:1 (Table 2, right column) was significantly higher than for the corresponding thiophenol−**1** with the N-terminal acetyl residue (cyclization-to-hydrolysis ratio of 5:1). This result provides evidence for the substantial contribution of the hexanoyl fatty acid in shifting the chemoselectivity of the CDA3-catalyzed reaction toward cyclization.

Reopening of Regioisomeric Macrolactones by CDA3 Cyclase. The regioisomeric cyclic products derived from **1** (Figure 4) on incubation with CDA3 cyclase were probed for the enzyme-mediated reverse reaction, where the ester bond is hydrolyzed to form the linear peptide acid. Hydrolysis of both macrolactones was solely detected in the presence of CDA3 cyclase, indicating an enzyme-mediated reopening of the cycles. Additionally, the CDA lactone ring cyclizing via Thr_2 was slightly converted into the regioisomeric peptidolactone cyclizing via Ser_1, confirming the existence of an acyl-enzyme intermediate, which can be captured either by water or by one of the two internal nucleophiles (data not shown).

DISCUSSION

In nature the C-terminal CDA3 cyclase of CDA synthetase catalyzes the formation of a branched cyclic macrolactone through the nucleophilic attack of the L-Thr_2 residue onto the C-terminal L-Trp_{11} of the bound acyl-undecapeptidyl oxoester. The released CDA macrolactone is structurally related to other antibiotics, including daptomycin, friulimicins, and amphomycins (Figure 1) (*14*). To gain a deeper understanding of the regio-, stereo-, and chemoselective cyclization mechanism of this class of acidic lipopeptides, we expressed the CDA3 thioesterase domain as excised cyclase from the CDA synthetase. This CDA3 cyclase has been successfully assayed in a chemoenzymatic approach for the in vitro cyclization of various synthetic peptidyl thioesters based on a modified CDA sequence. This finding is potentially important for engineering the synthesis of novel peptides based on CDA, which can be screened for altered biological activity.

Streptomycetes are a group of soil bacteria, which possess an important role in modern medicine as they produce over two-thirds of the naturally derived antibiotics in current use (*23*). The excised CDA3 TE domain is the first recombinant cyclase of this group of microorganisms. It was probed with four different peptidyl thioesters based on a sequence similar to CDA, including the leaving groups SNAC, phosphopantetheine, coenzyme A, and thiophenol (Figure 5). We observed that **1**−thiophenol (Figure 4) was the best cyclization substrate, although it has no structural similarity to the ppant cofactor. Comparison of the catalytic cyclization efficiency of **1**−thiophenol with **1**−SNAC revealed a 10−16-fold higher activity for the peptidyl-thiophenol substrate (Table 4). This result is in good agreement with recent experiments of the recombinant surfactin cyclase (Srf TE), where it was shown that the enzyme-mediated cyclization with the peptidyl-thiophenol substrate ($k_{cat}/K_M = 44.9\ mM^{-1}\ min^{-1}$) was 15 times more efficient than with the corresponding SNAC substrate ($k_{cat}/K_M = 2.9\ mM^{-1}\ min^{-1}$) (*16*). Therefore, one can conclude that the chemical reactivity of the leaving group displays a very important property for enzyme acylation in trans.

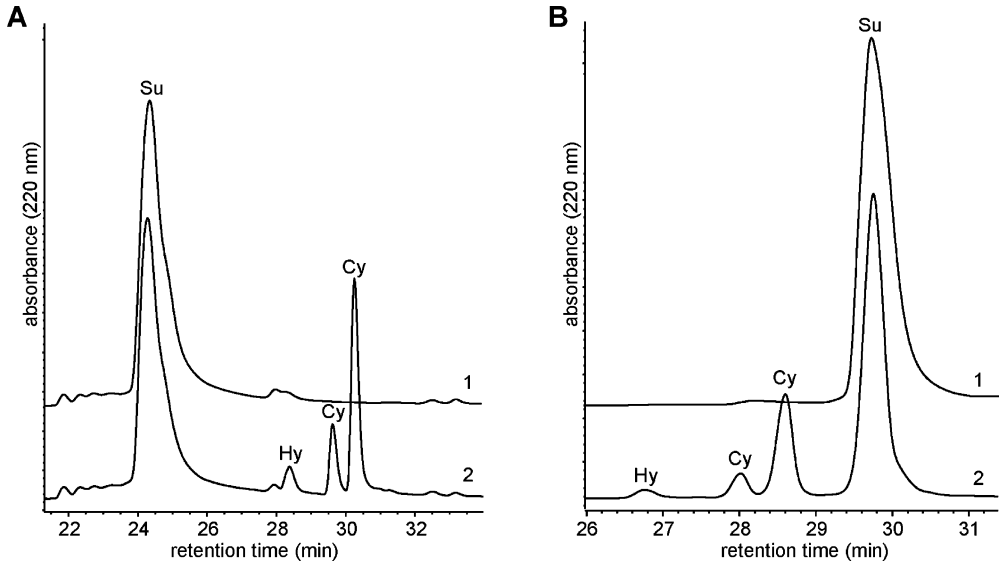


FIGURE 6: Exploring the role of the leaving group for CDA3 cyclase catalyzed ring formation. (A) HPLC trace of CDA3 cyclase incubated with **1**−CoA for 5 h at 20 °C (trace 2). Trace 1 shows incubation of substrate without enzyme. (B) CDA3 cyclase incubated with **1**−SNAC for 3 h at 20 °C (trace 2). Trace 1 shows substrate incubation in the absence of enzyme. Su = substrate, Hy = hydrolyzed product, and Cy = cyclized product.

Table 4: Kinetic Constants of **1** (Four Different Leaving Groups Attached to the C-Terminus) and **2**

		k_{cat} (min^{-1})		k_{cat}/K_M ($min^{-1}\,mM^{-1}$)	
substrate	K_M (μM)	undeca-peptide lactone	decapeptide lactone	undeca-peptide lactone	decapeptide lactone
1−CoA	8150	0.030	0.178	0.004	0.022
1−ppant	1440	0.023	0.115	0.016	0.080
1−SNAC	147	0.023	0.117	0.156	0.799
1−thiophenol	40	0.097	0.323	2.44	8.13
2	65	nd[a]	1.92	nd	29.8

[a] nd = not detected.

The observation that structural similarity to the cofactor of the PCP is not an important feature for enzyme acylation in trans was further confirmed by the phosphopantetheine leaving group (Figure 5). The structure of this compound exactly matches the prosthetic group of the PCP. Nevertheless, **1**−ppant (Figure 4) revealed a 10-fold lower catalytic efficiency for cyclization than **1**−SNAC (Table 4). The poorer leaving group properties of ppant (K_M = 1440 μM) compared to SNAC (K_M = 147 μM) may be due to additional steric repulsions of this larger ppant arm surrogate, which is reflected in a 10-fold higher K_M value (Table 4). Hence, enzymatic recognition of the ppant group by the TE domain in trans is less favored than in cis, where this structural element is properly aligned by the adjacent PCP for TE acylation. In accordance to the observed trend, coenzyme A as the biggest leaving group employed in this work revealed the highest K_M value of 8150 μM, which is probably caused by the steric hindrance of the attached 3′,5′-ADP part.

Generally, the k_{cat}/K_M values of the CDA thioester analogues for cyclization significantly increase in the order **1**−CoA < **1**−ppant < **1**−SNAC < **1**−thiophenol (Table 4). This indicates that the formation of the peptidyl-O-TE intermediate displays the rate-determining step in TE-mediated cyclization in vitro.

In nature the C-terminal TE domain catalyzes the release of the NRPS-tethered linear peptide precursor by macrocyclization. So far, it has been shown that the regio- and stereoselectivity of this cyclization process is retained in excised TE domains, which makes these recombinant cyclases attractive for the in vitro synthesis of new cyclic compounds with defined structure (*24*). Here, we show that the recombinant CDA3 cyclase from *S. coelicolor* A3(2) catalyzes the cyclization of a linear CDA analogue (**1**) (Figure 4) with a relaxed regioselectivity. The results show that two regioisomeric cyclic products were generated (Figure 6). MS-MS sequencing revealed that the main cyclic product was derived from nucleophilic attack of L-Thr_2 onto the C-terminus. This corresponds to naturally occurring CDA, where cyclization occurs regioselectively via the same residue (*12*). In contrast to that, the recombinant CDA3 cyclase also mediated the formation of a regioisomeric macrolactone, where L-Ser_1 was identified as the nucleophile for cyclization (Figure 10). This resulted in an increase of the ring size by one residue to a total number of 11. Therefore, CDA3 TE is the first cyclase where simultaneous formation of two macrocycles with different ring sizes was observed. The ratio between these regioisomeric products was independent of the four leaving groups (coenzyme A, ppant, SNAC, thiophenol) attached to the C-terminus of the linear peptide precursor **1**. This indicates that solely the common acyl-enzyme intermediate of all thioester substrates determines the relaxed regioselectivity of the product formation (Figure 10).

We recently reported that fengycin PCP−TE can catalyze the cyclization of the linear fengycin CoA substrate by covalent loading of the peptidyl substrate onto the PCP (*22*). It was shown that cyclization occurred regioselectively via nucleophilic attack of Tyr at position 3, despite the presence of two adjacent nucleophiles at position 2 (Orn) and position 4 (Thr), respectively. Relocation of Tyr from position 3 to position 2 resulted in the formation of a peptidolactone ring, where the ring size was expanded by one residue. This result indicates that relaxed regioselectivity presumably arises from identical or at least similar nucleophilic residues in adjacent positions, as in the case of CDA. This acidic lipopeptide is

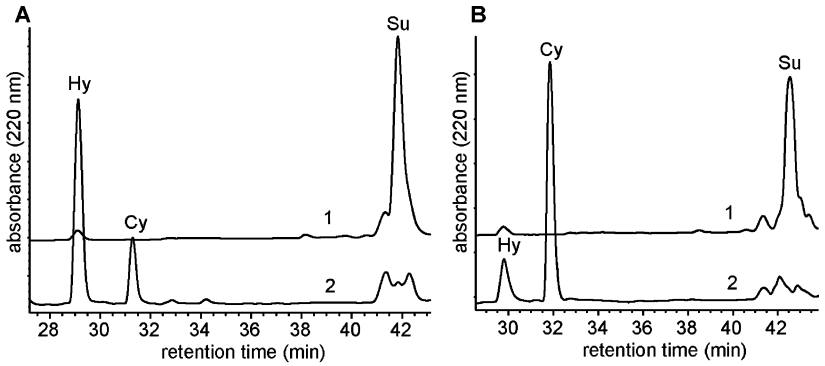


FIGURE 7: Regioselective formation of decapeptide lactone or undecapeptide lactone catalyzed by CDA3 cyclase. HPLC traces of CDA3 cyclase incubated with peptidyl-thiophenol substrates. (A) CDA3 cyclase incubated with **5** for 3 h at 20 °C (trace 2). Trace 1 shows incubation of substrate in the absence of enzyme. (B) CDA3 cyclase incubated with **4** for 3 h at 20 °C (trace 2). Trace 1 shows substrate incubation without enzyme. No uncatalyzed cyclization is observed. Su = substrate, Hy = hydrolyzed product, and Cy = cyclized product.

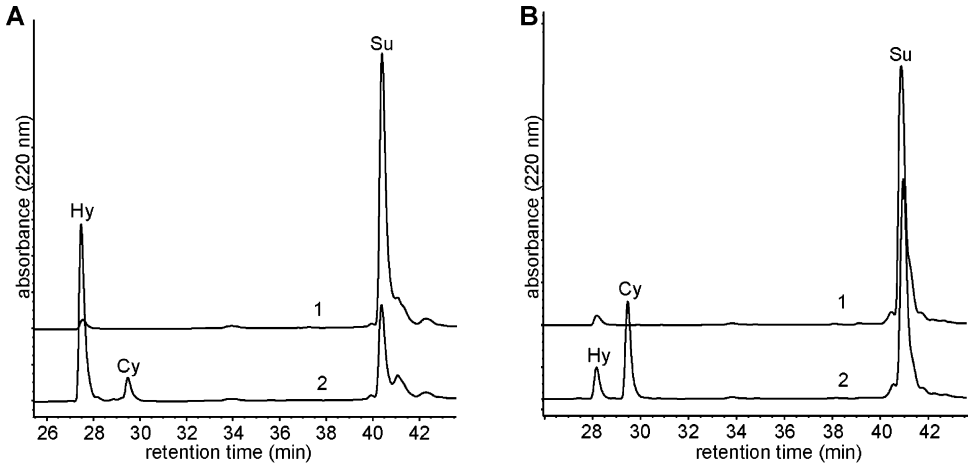


FIGURE 8: Probing the stereoselectivity of CDA3 cyclase mediated macrolactonization. HPLC traces of CDA3 cyclase incubated with peptidyl-thiophenol substrates, where either Ser_1 or Thr_2 is replaced by its D-configured isomer. (A) Incubation of CDA3 cyclase with **9** for 3 h at 20 °C (trace 2). Trace 1 shows incubation of substrate without enzyme. (B) CDA3 cyclase incubated with **8** for 3 h at 20 °C (trace 2). Trace 1 shows substrate incubation in the absence of enzyme. Su = substrate, Hy = hydrolyzed product, and Cy = cyclized product.

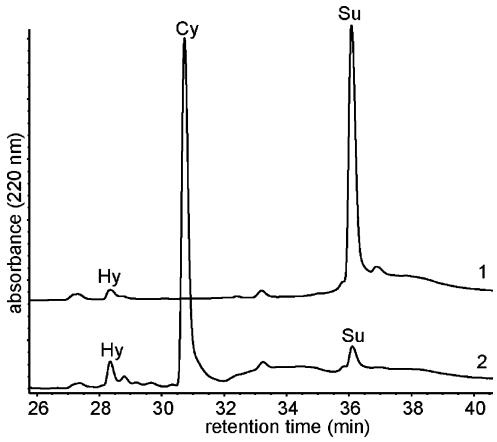


FIGURE 9: The N-terminal hexanoyl fatty acid residue ensures regioselective cyclization of the CDA thioester analogue. HPLC trace of CDA3 cyclase incubated with **2** for 3 h at 20 °C (trace 2). Trace 1 shows incubation of substrate in the absence of enzyme.

characterized by two nucleophilic residues in position 1 (Ser) and position 2 (Thr), which differ only by a methyl group in the β-position. By substitution of one of this residues by alanine, it was demonstrated that CDA3 cyclase can be forced to selectively produce one of the observed two regioisomeric peptidolactones (Figure 7). Probing CDA3 cyclase with **4** resulted in the formation of the decapeptide lactone ring derived from cyclization of Thr onto the C-terminus. Conversely, incubation with **5** led selectively to the release of the isomeric undecapeptide lactone ring where cyclization occurred via Ser. The cyclization-to-hydrolysis ratio was much smaller in the latter case, indicating that CDA3 cyclase prefers Thr_2 as the cyclization nucleophile. These results are in contrast to experiments with syringomycin cyclase, which regioselectively chooses only one of two adjacent serine residues for in vitro cyclization (*16*).

The results discussed above show that CDA3 TE is a quite permissive cyclization catalyst, which allows the creation of macrolactones with various ring sizes. This result is very encouraging, as it shows that CDA3 cyclase may be employed for the in vitro synthesis of the antibiotic daptomycin (Figure 1), which is of extraordinarily high therapeutic importance (*25*). CDA and daptomycin are both comprised of decapeptide lactones, which share five identical residues at the same positions (*14*). Remarkably, all residues in the lactone ring share a common configuration, and cyclization occurs in both cases over L-threonine, which generates a branched cyclic peptide with a fatty acid attached to the N-terminus.

We further explored the stereoselectivity of the CDA3 TE mediated ring formation by incubating this recombinant cyclase with five linear CDA thiophenol analogues. The simultaneous replacement of L-Ser_1 and L-Thr_2 from **1** by the corresponding D-configured amino acids in **10** resulted

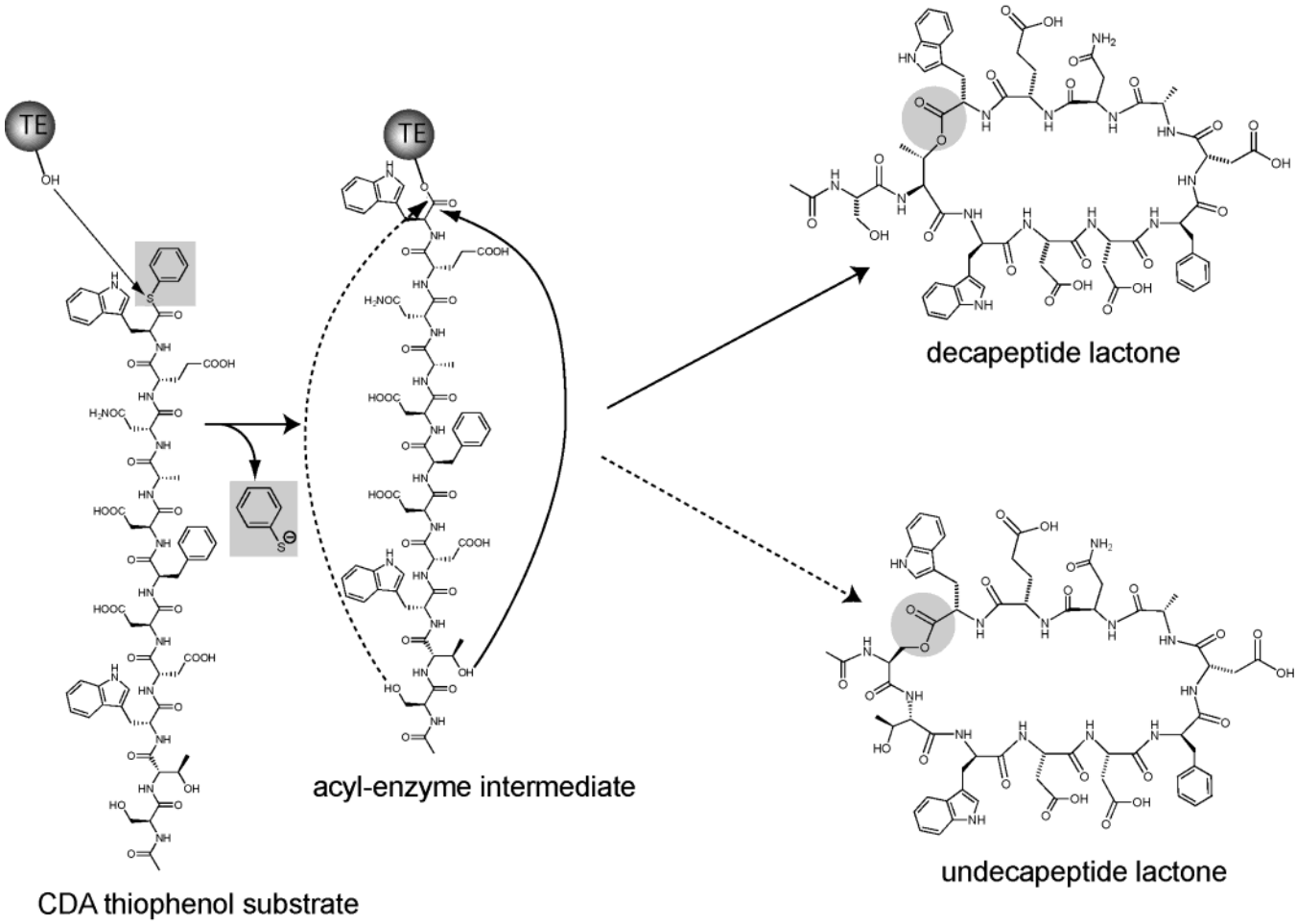


FIGURE 10: Acylation of CDA3 cyclase with the **1**−thiophenol substrate. The active site serine residue of the CDA3 cyclase (TE) is acylated by the reactive **1**−thiophenol substrate (thiophenol leaving group highlighted by shading). The generated acyl-enzyme intermediate is then captured either by Thr_2 to generate a decapeptide lactone (solid line) or by Ser_1 to release the regioisomeric undecapeptide lactone (dotted line).

in substantial hydrolysis but no cyclization in the presence of CDA3 cyclase. The same results were obtained when we probed CDA3 cyclase with substrates **6** and **7**, which permitted the selective examination of the stereochemistry at positions 1 and 2 (data not shown). Remarkably, although Ser_1 does not take part in the cyclization process of naturally occurring CDA, its involvement in the formation of the undecapeptide lactone ring mediated by CDA3 cyclase is strictly stereoselective. Therefore, CDA3 cyclase provides another example of stringent stereoselective discrimination against cyclizing nucleophiles with deviating stereochemistry. Additionally, we probed CDA3 cyclase with the thiophenol substrates **8** and **9**, where the stereochemistry of either Ser_1 or Thr_2 was changed (Figure 8). Surprisingly, CDA3 cyclase employed only the residue with the correct stereo information for the cyclization of the linear peptide precursor. Hence, it is possible to selectively generate the decapeptide lactone ring or the regioisomeric undecapeptide lactone ring without replacing either serine or threonine by a nonnucleophilic residue (e.g., alanine).

The observed relaxed regioselectivity of the CDA thioester substrates with CDA3 cyclase clearly deviates from the natural NRPS system, where cyclization occurs regioselectively by nucleophilic attack of L-Thr_2. Therefore, we tried a better approximation of the natural CDA substrate. We synthesized a new CDA thiophenol analogue (**2**) where the N-terminal acyl chain was elongated by four methylene groups to its natural length (Figure 4). Remarkably, CDA3 TE catalyzed the formation of only one macrocyclic product (Figure 9). Cyclization occurred regioselectively via L-Thr_2, producing the decapeptide lactone ring as is also observed in the natural NRPS machinery. This result suggests that the N-terminal fatty acid of CDA controls the regioselectivity of the enzyme-mediated ring formation. Further, the k_{cat} value for formation of the decapeptide lactone increased by a factor of 6 compared to **1**−thiophenol with the acetylated N-terminus (Table 4). This may be explained due to a better alignment of the attacking Thr_2 nucleophile through favorable hydrophobic interactions between the hexanoic fatty acid residue and the enzyme's active site. Finally, the elongated acyl chain induced a much better chemoselectivity of the CDA3 cyclase catalyzed reaction. The cyclization-to-hydrolysis ratio of the corresponding thiophenol substrates rose from 5:1 (**1**) to 10:1 (**2**), which is to our best knowledge the highest chemoselectivity for a recombinant cyclase observed so far (Table 2). This very selective flux toward cyclization could be due to the improved exclusion of water from the active site mediated by the hydrophobic fatty acid, which facilitates the capture of the acyl-O-TE intermediate by the internal nucleophile. Therefore, the relatively low cyclization-to-hydrolysis ratios of the in vitro cyclization of lipopeptides such as surfactin, fengycin, mycosubtilin, and syringomycin

may be due to the shortened N-terminal acyl chains of the linear peptide analogues. On the other hand, tyrocidine A as an antibiotic without a lipid part is cyclized by the corresponding recombinant cyclase with a quite high chemoselectivity (cyclization-to-hydrolysis ratio of 6:1) (*21*). Thus, the selective flux to cyclic product is presumably obtained by an alternative mechanism, which does not depend on an fatty acid attached to the N-terminus.

In summary, our results suggest that elongating the N-terminal acyl chain of linear peptide precursors improves the chemoselectivity, regioselectivity, and kinetics of recombinant TE-mediated macrocyclization. Therefore, the role of these fatty acids is not constrained to biological tasks, e.g., hydrophobic interaction with lipid bilayer membranes.

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