Peptide Cyclization

Peptidyl Thiophenols as Substrates for Nonribosomal Peptide Cyclases**

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Natural small peptide molecules such as cyclosporin, vancomycin, and penicillin have attracted much attention in the past because of their exceptional pharmacological and biological activities. A common feature of all these compounds is their constrained structure, which ensures the precise functionality that is important for interaction with the dedicated molecular target. In many cases, rigidity is achieved by cyclization of the peptide backbone, which leads to diverse macrolactams and macrolactones. Since peptide cyclization can be a difficult task in chemical synthesis, there has been significant interest in exploring enzymatic cyclization mechanisms to develop new synthesis routes for the production of modified natural cyclic peptides.[1] Nature achieves the synthesis of linear peptide precursors and their subsequent cyclization by using large multienzyme complexes referred to as nonribosomal peptide synthetases (NRPS).[2] These multimodular enzymes contain all the catalytic units necessary for the assembly of the linear peptide sequence. In addition, a peptide cyclase domain referred to as the thioesterase domain (TE) is present at the end of the assembly line and catalyzes the release of the cyclic product. Recent research on excised tyrocidine and surfactin peptide cyclases provided insights into their substrate tolerance and cyclization mechanism, and allowed a chemoenzymatic approach to be used to generate a library of novel products.[3–5]

Characterization of these enzymes requires translation between the biological and chemical languages. Linear peptide precursors were produced by solid-phase peptide synthesis and subsequently coupled to an analogue of the natural cofactor. In the multienzyme complex, the linear peptide is tethered to a phosphopantetheine (ppan) arm on the peptidyl carrier protein (PCP) through a thioester bond. To retain the enzymatic recognition elements associated with this natural cofactor, the last part of the phosphopantetheine arm, *N*-acetylcysteamine thioester (SNAC), was used as a mimic for the natural PCP ppan arm and attached to the C-terminal end of the synthetic linear peptide substrate.[6] In contrast to the surfactin and tyrocidine peptide cyclases, many cyclases from other NRPS systems, such as fengycin, mycosubtilin, and syringomycin, were found to be inactive with peptidyl SNAC substrates (data not shown). We recently overcame this limitation for the fengycin cyclase by using 4′-phosphopantetheine transferase (Sfp) to catalyze the loading of peptidyl coenzyme A (CoA) onto the invariant serine residue of the PCP domain of a recombinant apo PCP–TE didomain.[7] Cyclization and hydrolysis products were observed when this approach was used, which indicates that precise presentation of the substrate by ppan-PCP is essential for recognition and catalysis by the cyclase. After product release, the cofactor ppan remains attached to the PCP–TE didomain and blocks Sfp-catalyzed transfer of additional peptidyl CoAs onto ppan-PCP and limits this approach to a single turnover. We have now developed a new strategy to force multiple turnover cycles and expand the utility of this method. This strategy is based on a thioester exchange reaction between the free ppan-PCP thiol group and a soluble thioester peptide substrate that should ensure chemical reloading of the substrate onto the ppan-PCP–TE didomain.[8,9]

We report herein a new chemoenzymatic route to cyclic compounds through activity-based TE enzyme acylation with reactive thioester leaving groups (thiophenol). This method has allowed biochemical characterization of fengycin, mycosubtilin, and syringomycin peptide cyclases, which are not active with SNAC substrates. The potency of reactive thioester leaving groups attached to surfactin in cyclization reactions was evaluated in comparison with data obtained previously for SNAC substrates.

Our initial aim was to achieve a thioester exchange reaction between the free thiol group of ppan in the fengycin bidomainal fragment ppan-PCP–TE[7] and synthetic fengycin thiophenol (FLP-tp; Scheme 1). Surprisingly, control reactions of FLP-tp with apo PCP–TE, which lacks the free thiol group, revealed hydrolysis and cyclization activity not observed with SNAC substrates (Scheme 2 and Figure 1 A). A control reaction with a fengycin cyclase containing a Ser-to-Ala mutation in the active site showed that this mutation abolishes the activity. The identities of all substrates (Table 1) and products (Table 2) were verified by HPLC-MS. No increase in product turnover was observed with holo PCP–TE, which indicates that the thiophenol substrate efficiently directly acylates the serine residue in the active-site of the TE enzyme. Thiophenol has excellent leaving group properties as a result of delocalization of the thiolate electrons throughout the aromatic ring. In contrast, no such stabilization is possible with the mimic SNAC. The results emphasize that, for these peptidyl thioester substrates, the chemical reactivity of the

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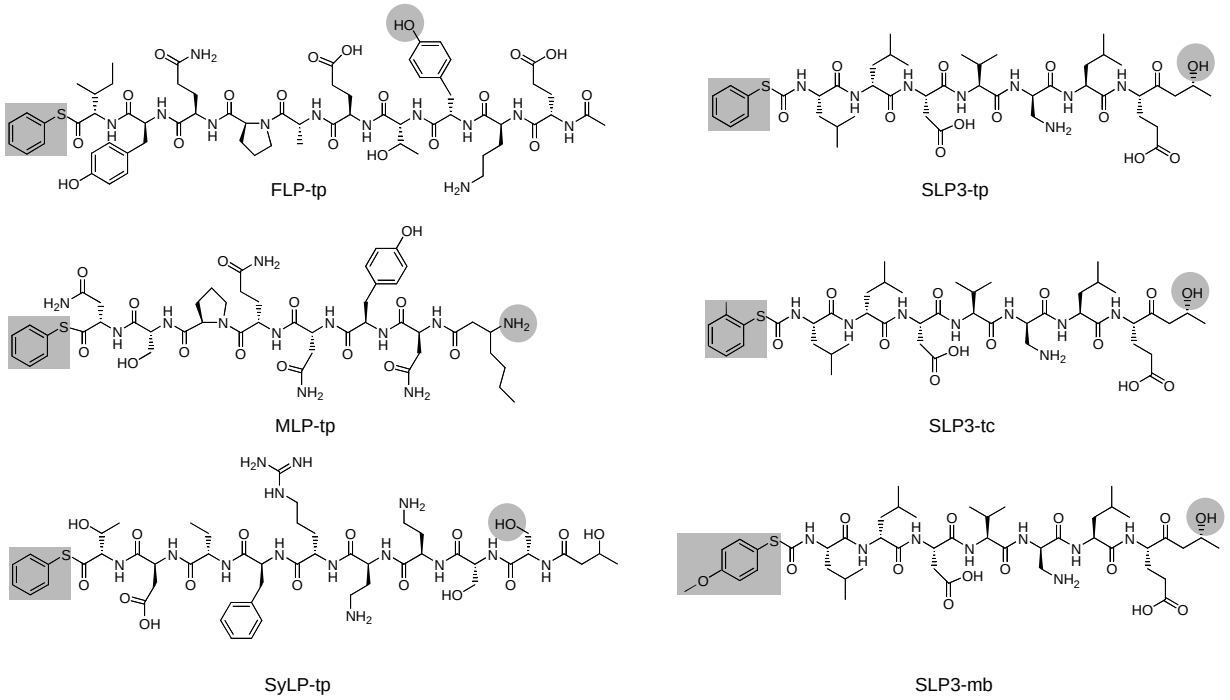
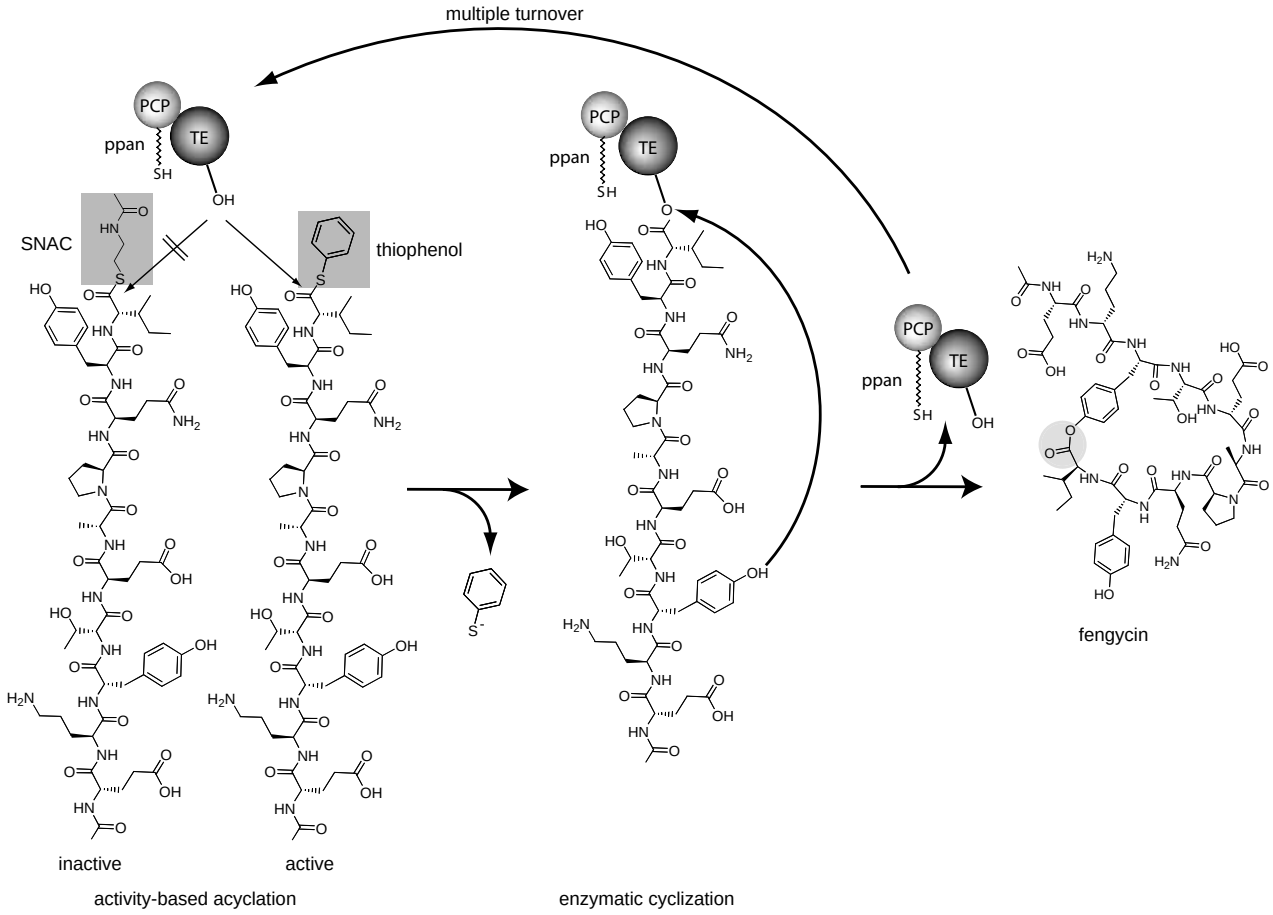
[**] We gratefully acknowledge the Studienstiftung des deutschen Volkes (S.A.S.), Deutsche Forschungsgemeinschaft (M.A.M.), and National Institutes of Health (grant no. GM20011; C.T.W.). We thank Dirk Schwarzer for helpful comments, Uwe Linne for MS/MS sequencing, Christina Dauth for her help with the synthesis, and Antje Schäfer for technical assistance. J.T. thanks Kim Albizati, Van Martin, and Shanghui Hu for their generous support of his Pfizer secondment.

 DOI: 10.1002/ange.200352787 © 2004 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 1. Structures of peptidyl aryl thioester substrates. Leaving groups are indicated by boxes and nucleophiles for enzymatic intramolecular cyclization are indicated by circles.



Scheme 2. Activity-based enzyme acylation. The active-site serine residue of the fengycin peptide cyclase (TE) is selectively acylated by a reactive peptidyl thiophenol substrate. The acyl-enzyme intermediate is then captured by an intramolecular nucleophilic attack by Tyr3 to give the cyclic peptidolactone fengycin. No activity is observed with a peptidyl SNAC substrate previously believed to be important for enzyme recognition.

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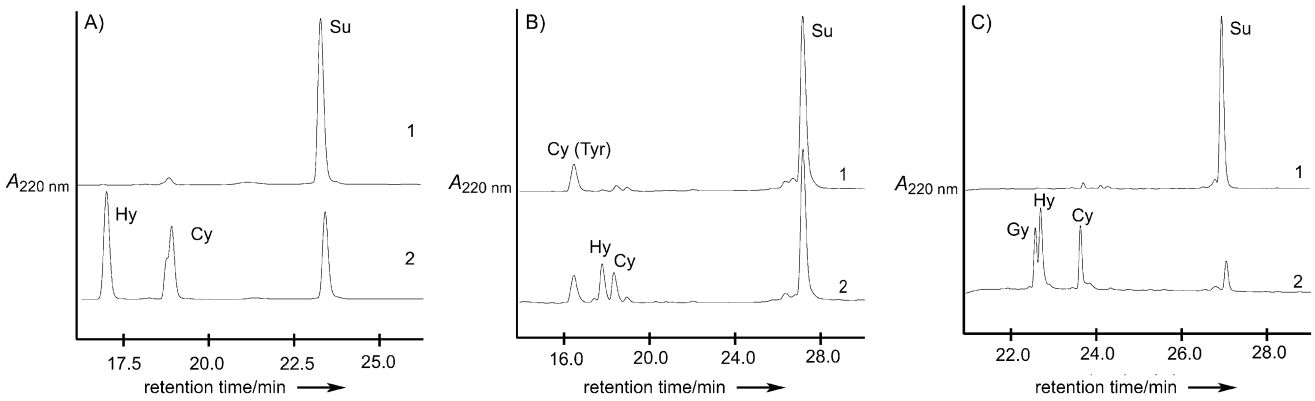

Figure 1. HPLC traces of peptide cyclases (SNAC inactive) incubated with peptidyl thiophenol substrates. A) Fengycin cyclase incubated with FLP-tp for 1.5 h (trace 2). Trace 1 was obtained after incubation of the substrate with a cyclase in which the active-site Ser residue is mutated to Ala. B) Mycosubtilin cyclase incubated with MLP-tp for 5 min (trace 2). Trace 1 shows incubation of the substrate without enzyme. Uncatalyzed cyclization through Tyr3 [Cy(Tyr)] is observed. C) Syringomycin cyclase incubated with SyLP-tp for 30 min (trace 2). Trace 1 shows substrate incubation without enzyme. Su = substrate, Hy = hydrolyzed product, Cy = cyclized product, Gy = glycerol adduct.

Table 1: Characterization of substrates by ESI MS.

Compound	Observed (calculated) mass for $[M+H]^+$ [Da]
FLP-tp	1361.4 (1361.6)
SLP-tp	965.4 (965.5)
MLP-tp	1055.5 (1055.5)
SyLP-tp	1175.6 (1175.5)
SLP-tc	979.4 (979.5)
SLP-mb	995.4 (995.5)

Table 2: Characterization of products by ESI MS.

Compound	Observed (calculated) mass for $[M+H]^+$ [Da] cyclized product	hydrolyzed product	Cy/Hy ratio
FLP-tp	1251.4 (1251.6)	1269.4 (1269.6)	0.85/1
SLP-tp	855.4 (855.5)	873.4 (873.5)	0.78/1
MLP-tp	945.4 (945.4)	963.4 (963.5)	0.85/1
SyLP-tp	1065.3 (1065.5)	1083.6 (1083.5)	0.65/1
SLP-tc	855.4 (855.5)	873.4 (873.5)	0.73/1
SLP-mb	855.4 (855.5)	873.4 (873.5)	0.64/1

thiophenol leaving group is more important for enzyme acylation than recognition of SNAC, the cofactor analogue. Kinetic data for the cyclization of FLP-tp (catalytic rate constant $k_{cat} = 0.33\ min^{-1}$, Michaelis constant $K_M = 461\ \mu M$, Cy/Hy ratio = 0.85/1) indicate saturation kinetics and suggest sufficient catalytic efficiency for preparative-scale reactions.

Surfactin cyclase (Srf TE), which was previously characterized with SNAC substrates,[4] was incubated with surfactin thiophenol to evaluate the general utility of thiophenol leaving groups in substrates for other peptide cyclases (SLP3-tp; Scheme 1). HPLC analysis revealed enzyme-catalyzed cyclization and hydrolysis activity, with $K_M = 126\ \mu M$, and $k_{cat} = 5.66\ min^{-1}$ and $11.33\ min^{-1}$ for cyclization and hydrolysis, respectively (Figure 2A). Comparison of the catalytic cyclization efficiency for SLP3-tp ($k_{cat}/K_M = 44.9\ mM^{-1}min^{-1}$) with that for SLP3-SNAC ($2.9\ mM^{-1}min^{-1}$)[4] revealed a 15-fold higher activity for the thiophenol substrate, with a Cy/Hy ratio of 0.78/1 for thiophenol and 0.54/1 for SNAC. These results are consistent with a common acyl enzyme intermediate for the two

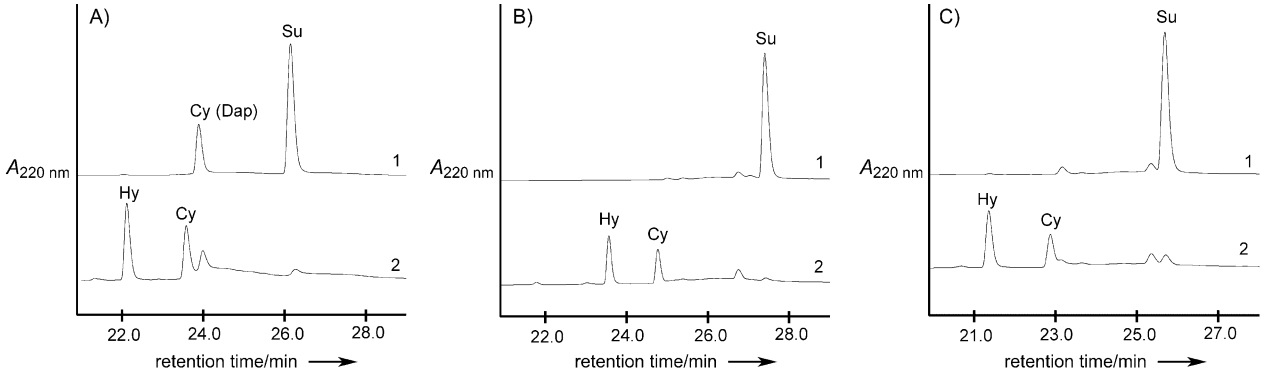

Figure 2. HPLC traces of surfactin cyclase (SNAC active) incubated with aromatic thioester substrates. A) Incubation of surfactin cyclase with SLP-tp for 10 min (trace 2). Trace 1 shows incubation of the substrate without enzyme. Uncatalyzed cyclization through a diaminopropionic acid residue at position 3 [Cy(Dap)] is observed. B) Incubation of surfactin cyclase with SLP-tc for 10 min (trace 2). Trace 1 shows substrate incubation without enzyme. C) Incubation of surfactin cyclase with SLP-mb for 10 min (trace 2). Trace 1 shows substrate incubation without enzyme. Su = substrate, Hy = hydrolyzed product, Cy = cyclized product.

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substrates, and breakdown of this intermediate may be rate-determining for the SLP3-tp reaction. However, the greater thermodynamic activation of the peptide effected by thiophenol seems to facilitate nonenzymatic cyclization, as observed in the surfactin control reaction without enzyme (Figure 2A). MS/MS sequencing of the cyclic species obtained (minus Srf TE) showed that the uncatalyzed cyclization occurs through a diaminopropionic acid (Dap) residue at position three (Table 3). Such nonenzymatic cyclization of peptides is an intrinsic property of some peptide sequences, as has been reported before for the tyrocidine series.[10] To suppress unspecific nonenzymatic cyclization of the thiophenol peptides through their side chains, the reactivity of the leaving group was decreased by substitution of the aromatic ring. Substituents with a positive inductive or mesomeric effect (methyl and methoxy groups) were introduced onto the aromatic thiophenol ring at the *ortho*, *meta*, or *para* positions. Each of the six new aryl thiol leaving groups was coupled to the free carboxylic acid moiety of the surfactin peptide, which was subsequently incubated with surfactin cyclase. Replacement of thiophenol with *o*-thiocresol (SLP3-tc) or *p*-methoxybenzenethiol (SLP3-mb) gave the best suppression of unspecific background cyclization, while enzyme-catalyzed substrate cyclization and hydrolysis rates remained constant (Table 2, Scheme 1, Figure 2B and C). The attachment of substituents to the aromatic ring can therefore adjust the activity as desired for any peptidyl thioester substrate. Substitution in the *ortho* and *para* positions seems to be preferred, probably because of the destabilizing impact on the thiophenolate anion. Srf TE directs cyclization towards the β-OH group of the fatty acyl chain rather than the DAP residue, which undergoes amide formation in nonenzymatic cyclization.

Table 3: MS/MS fragmentation data for autocatalytic cyclization products.

Compound	Species	Observed mass of fragments (calculated mass) [Da]		
		cyclization through Dap3	cyclization through Tyr2	cyclization through Ser6
SLP3 cycle[a]	$[M+H]^+$	527.316 (527.319)		
SLP3 cycle[b]	$[M+H]^+$	n.d. (510.293)		
MLP cycle[a]	$[M+H]^+$		704.278 (704.300)	n.d. (202.083)
MLP cycle[b]	$[M+H]^+$		687.254 (687.274)	n.d. (185.056)

[a] Fragmentation of the peptide bond at the proposed cyclization position. [b] Fragmentation of the adjacent NH–CH bond. N/D = not detected.

The observed catalytic activity of the fengycin and surfactin peptide cyclases on peptidyl thiophenol substrates encouraged us to investigate other peptide cyclases with this new technology. Mycosubtilin PCP–TE and syringomycin TE were cloned from their multimodular synthetases, overexpressed, and purified as soluble proteins.[11,12] Both enzymes failed to show any activity with peptidyl SNAC substrates (data not shown). Thiophenol was therefore attached to peptide sequences derived from those of mycosubtilin (MLP-tp) and syringomycin (SyLP-tp; Scheme 1). The peptide analogue of syringomycin differs from the wild-type peptide[13] for synthetic reasons: the 4-Cl-threonine residue at position 9 was replaced by threonine, dehydrobutyrine by aminobutyric acid, and β-hydroxy aspartate by aspartate, and the long fatty acid moiety was replaced by a shorter β-hydroxy fatty acyl chain to improve the solubility. In syringomycin, cyclization occurs through a serine side chain, whereas the fatty acid chain seems to be important in the mycosubtilin sequence, since macrolactamization occurs through the β-amino fatty acid moiety (Scheme 1). The natural fatty acid chain of mycosubtilin was replaced by a C_4-β-aminoacyl chain, which was synthesized chemically (see the Experimental section). Incubation of mycosubtilin peptide cyclase with MLP-tp led to enzyme-catalyzed hydrolysis and cyclization (Figure 1B). Cyclization occured with a k_{cat} value of 2.16 min^{-1}, a K_M value of 3991 μM, and a Cy/Hy ratio of 0.85/1. The high K_M value could be the result of the absence of enzymatic recognition elements in the artificial cofactor thiophenol. As was observed for surfactin, autocatalytic self-cyclization of MLP-tp ocurred in a control reaction without enzyme. The identity of the new species was investigated by MS/MS sequencing and cyclization was shown to occur through the tyrosine residue at position two (Table 3), again a distinct regiospecificity from that imposed during enzymatic cyclization. Incubation of syringomycin peptide cyclase with SyLP-tp produced both cyclized and hydrolyzed products (Figure 1C). A glycerol adduct was also formed because 5% glycerol had to be added to stabilize syringomycin peptide cyclase. Saturation behavior was observed and the k_{cat} and K_M values of the cyclization determined ($k_{cat} = 0.32$ min^{-1} and $K_M = 28$ μM, Cy/Hy ratio = 0.65/1). Previously recorded data show that in vivo cyclization of syringomycin occurs through the L-serine residue at position one[13] and not at the adjacent D-serine residue at position two, which raised the question of whether a change in stereochemistry at positions one and two could alter the product formed. Two new thiophenol substrates were made, one with D-serine at position one and L-serine at position two, and the other with L-serine at positions one and two. Incubation of these substrates with syringomycin cyclase only led to hydrolysis in both cases, which demonstrates the importance of Ser1,2 stereochemistry for enzymatic cyclization (data not shown).

Activity-based enzyme acylation with soluble thiophenol-based peptide substrates is a powerful method to obtain enzymatic catalysis by NRPS peptide cyclases. This methodology led to the formation of cyclic peptidolactone or -lactam products of the fengycin, mycosubtilin, and syringomycin cyclases, enzymes that were inactive when peptidyl SNACs were used as potential substrates. Typical yields of macrocyclic products ranged from 96–40%. As expected, undesired hydrolytic by-products are formed in this in vitro system because the substrate presentation and enzyme environment are unnatural. The ratio of cyclization to hydrolysis could be increased by providing natural unmodified thiophenol substrates that fit precisely into the active site of the enzyme.

While we and others have previously used SNAC leaving groups as ppan surrogates on the assumption that the structure is important for enzyme recognition and activity, we report herein that the potential of the leaving group for

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chemical acylation seems to be a more crucial determinant of reactivity than cofactor recognition. Peptides with thiophenol and other aromatic leaving groups will allow the characterization of new NRPS peptide cyclases and will therefore overcome a major limitation of past research in this area. Libraries of peptide and hybrid polyketide–peptide[14] analogues can be synthesized easily and screened for altered or increased biological activity. Selective acylation of enzymes by titration with aromatic thioesters could also be important for many other enzymatic systems, such as polyketide synthases or serine hydrolases.

Experimental Section

Cloning, expression, and purification of mycosubtilin PCP–TE and syringomycin TE: The mycosubtilin (ATCC 6633) and syringomycin (DSMZ B-301D) gene fragments encoding Myc PCP–TE and Syr TE were amplified from chromosomal DNA by the polymerase chain reaction (PCR) with Pfu polymerase (Stratagene) and the following oligonucleotides: Myc PCP–TE, 5′-AAA AAC ATA TGG CTG CTC CCC GAA CGT TGA TT-3′ and 5′-AAA AAC TCG AGT GTT CGA TCA GAT TTT GTG CGA CTC-3′; Syr TE, 5′-AAA AAC ATA TGG GCC AGG CAC GCC CC-3′ and 5′-AAA AAC TCG AGC TCA GCT GGC GCG GTT ATC-3′. The PCR products were cloned into the *NdeI*/*XhoI* site of a pET37b vector (Novagene). *Escherichia coli* TOP 10 (Novagen) was used for preparation of recombinant plasmids. The identities of all constructs were confirmed by sequencing. The plasmids were used to produce proteins with a C-terminal hexahistidine tag.

Recombinant proteins were overproduced in *E. coli* BL21 cells (Novagen) by using standard protocols.[15] Purification of the proteins was carried out as described previously[16] and the products were analyzed by electrophoresis on sodium dodecylsulfate polyacrylamide gel. After dialysis against standard assay buffer (25 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) and 50 mM NaCl, pH 7.0), all proteins were shock-frozen in liquid nitrogen. 5% Glycerol was added to the Syr TE buffer. The concentrations ($mg\,mL^{-1}$) of the purified proteins were determined photometrically at 280 nm by using calculated extinction coefficients (Myc PCP–TE, $0.83\ mol^{-1}\,dm^3\,cm^{-1}$; Syr TE, $0.80\ mol^{-1}\,dm^3\,cm^{-1}$). Cloning and expression of Srf TE,[17] Fen PCP–TE, and the Ser-to-Ala mutant Fen PCP–TE[7] was described previously.

Synthesis of *N*-*tert*-butoxycarbonyl-3-amino-heptanoic acid: General:[18] All commercially available reagents were used without further purification unless otherwise noted. All organic solvents used were anhydrous, were bought from Aldrich, and were of the highest purity available. All reactions were performed in a dry N_2 or Ar atmosphere unless otherwise indicated. All reactions were monitored by analytical thin-layer chromatography performed with the indicated solvent onsilica gel 60 F_{254} plates (E. Merck; 0.25 mm) unless otherwise indicated. Compounds were visualized under UV light (λ = 254 nm). Flash column chromatography was performed in the indicated solvent on silica gel 60 (E. Merck, 40–63 m). NMR spectra (^1H, ^{13}C) were recorded on a Bruker spectrometer (300 MHz). LC-MS was carried out by using ESI positive and negative modes. LC chromatography runs were performed on a Tuna C18 column (3μ, 30 × 4.6 mm) with ACN/H_2O (containing 0.1% formic acid) as the mobile phases, a gradient of 10–90% ACN in 3.5 min then isocratic for 1 min, a flow rate of $0.8\ mL\,min^{-1}$, and detection at 220 nm and 254 nm.

Synthesis of 3-amino ester **2:** β-ketoester **1** (1 g, 5.8 mmol) was dissolved in MeOH (15 mL) and ammonium acetate (4.7 g, 60 mmol) was added. The mixture was stirred at RT for 17 h and LC-MS showed that the the reaction was complete. After rotary evaporation of MeOH, the crude product was dissolved in EtOAc (50 mL), and 1N NaOH (30 mL) was added. The organic layer was separated from the mixture, washed with brine (50 mL), and dried over Na_2SO_4. Rotary evaporation gave the desired product **2** (943 mg, 95%): ESI MS: *m*/*z*: $[M+H]^+$ 172.20. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 0.91$ (t, $J = 7.1$ Hz, 3H), 1.25 (t, $J = 7.1$ Hz, 3H), 1.37 (m, 2H), 1.51 (m, 2H), 2.12 (t, $J = 7.9$ Hz, 2H), 4.09 (q, $J = 7.1$ Hz, 2H), 4.53 ppm (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 14.0$, 14.9, 22.5, 30.3, 36.4, 58.8, 83.6, 164.3, 170.8 ppm.

Synthesis of 3-amino ester **3**: Compound **2** (400 mg, 2.34 mmol) was added to a mixture of HOAc (5 mL) and $NaBH(OAc)_3$ (992 mg, 4.68 mol) at 10°C. The reaction was monitored by LC-MS. After 1 h, the mixture was dissolved in methyl *tert*-butyl ether (10 mL) and washed with 10% Na_2CO_3 (10 mL × 2). The organic layer was separated from the mixture and dried over Na_2SO_4. Rotary evaporation gave the desired product **3** (304 mg, 75%): ESI MS: *m*/*z*: $[M+H]^+$ 174.20. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 0.89$ (t, $J = 7.6$ Hz, 3H), 1.25 (t, $J = 7.0$ Hz, 3H), 1.28–1.60 (m, 6H), 2.19–2.47 (m, 2H), 3.14–3.19 (m, 1H), 4.13 ppm (q, $J = 7.6$ Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 14.3$, 14.6, 23.0, 28.3, 37.7, 43.1, 48.7, 60.6, 173.0 ppm.

Synthesis of *N*-*tert*-butoxycarbonyl 3-amino-heptanoic acid (**4**): $(Boc)_2O$ (Boc, *tert*-butoxycarbonyl; 440 mg, 1.96 mmol, 2 equiv) was added to a solution of **3** (170 mg, 0.98 mmol) in 1N NaOH (4 mL) and dioxane (4 mL). The mixture was stirred at RT for 24 h, adjusted to pH 3.0, and extracted with CH_2Cl_2 (10 mL × 2). The organic layer was dried over Na_2SO_4. Rotary evaporation gave the desired product **4** (304 mg, 75%): ESI MS: *m*/*z*: $[M-H]^+$ 244.10. ^1H NMR (300 MHz, $CDCl_3$): $\delta = 0.81$ (t, $J = 7.5$ Hz, 3H), 1.23–1.50 (m, 6H), 1.36 (s, 9H), 2.31 (d, $J = 6.6$ Hz, 2H), 3.76 ppm (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) $\delta = 14.37$, 23.8, 29.2, 29.7, 35.9, 41.3, 80.3, 158.3, 175.6 ppm.

Synthesis of peptidyl thiophenol substrates: All linear peptides were produced by solid-phase peptide synthesis as described previously.[17] Protected amino acids were purchased from Novabiochem and Bachem Bioscience. All other compounds were purchased from Sigma–Aldrich. The general procedure for the synthesis of peptidyl thiophenol substrates is based on the synthesis of peptidyl SNACs described previously.[6] Thiophenol or a thiophenol derivative (10 equiv), *N*,*N*′-dicyclohexylcarbodiimide (2 equiv), and 1-hydroxy-1*H*-benzotriazole (2 equiv) in tetrahydrofuran were added to the protected peptide (1 equiv). The mixture was agitated for 3 h and after 30 min potassium carbonate (4 equiv) was added. The solvent was removed and acid-labile side-chain protecting groups were cleaved by incubation with triflouracetic acid (TFA)/triflourethanol/water (95:2.2:2.5). The deprotected peptidyl thiophenol was purified by preparative HPLC on an Äkta purifier (Pharmacia) HPLC system with a reversed-phase C18 Nucleodur column (Macherey and Nagel). The identities of the peptidyl thiophenol substrates were verified by HPLC-MS.

Assays: Reactions were carried out in 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES, 25 mM) and NaCl (50 mM) at pH 7.0 in a total volume of 50 μL. The substrate concentration was 100 μM for standard reactions and varied in the kinetic investigations. Reactions were initiated by addition of enzyme to final concentrations of 5 μM for Fen PCP–TE, 1 μM for Srf TE, 30 μM for Myc PCP–TE, and 15 μM for Syr TE. Reactions were quenched at various time points by addition of 4% TFA/H_2O (35 μL), and the products were analyzed by HPLC-MS on a reversed-phase C_{18} Nucleodur column (Macherey and Nagel, 250/3, pore diameter 100 Å, particle size, 3 μm) under the following conditions: Fen PCP–TE: 0–35 min, 30–60% acetonitrile/0.1% TFA in water/0.1% TFA, $0.4\ mL\,min^{-1}$, 40°C; Srf TE: 0–40 min, 20–55% acetonitrile/0.1% TFA in water/0.1% TFA, $0.4\ mL\,min^{-1}$, 40°C; Myc PCP–TE: 0–40 min, 5–50% acetonitrile/0.1% TFA in water/0.1% TFA, $0.4\ mL\,min^{-1}$, 40°C; Syr TE: 0–40 min, 10–50% acetonitrile/0.1% TFA in water/0.1% TFA, $0.4\ mL\,min^{-1}$, 40°C. The identities of the products were confirmed by ESI-MS and cyclic by-products were additionally analyzed by MS/MS to verify the connection regiospecificity (API Qstar Pulsar I, Applied Biosystems). The concentrations of the peptidyl thiophenol

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thioesters were calculated by using experimentally determined extinction coefficients at 220 nm and the coefficients were assumed to be the same for the cyclized and hydrolyzed products. Kinetic characterization of the cyclization and hydrolysis reactions was carried out by determining the initial reaction rates at 5–7 substrate concentrations. At each concentration, measurements were taken at two time points within the linear region of the enzyme-catalyzed reactions, as determined by recording the reaction time courses.

Received: September 4, 2003 [Z52787]

Keywords: antibiotics · biomimetic synthesis · cyclization · enzyme catalysis · peptides

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