Chemistry & Biology, Vol. 12, 873–881, August, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.05.019

Fluorescence Resonance Energy Transfer as a Probe of Peptide Cyclization Catalyzed by Nonribosomal Thioesterase Domains

Jan Grünewald, Florian Kopp, Christoph Mahlert, Uwe Linne, Stephan A. Sieber,[1] and Mohamed A. Marahiel*
Fachbereich Chemie/Biochemie
Philipps-Universität Marburg
Hans-Meerwein-Strasse
D-35032 Marburg
Germany

Summary

Macrocyclization of synthetic peptides by thioesterase (TE) domains excised from nonribosomal peptide synthetases (NRPS) has been limited to peptides that contain TE-specific recognition elements. To alter substrate specificity of these enzymes by evolution efforts, macrocyclization has to be detected under high-throughput conditions. Here we describe a method to selectively detect cyclic peptides by fluorescence resonance energy transfer (FRET). Using this method, picomolar detection limits were easily realized, providing novel entry for kinetic studies of catalyzed macrocyclization. Application of this method also provides an ideal tool to track TE-mediated peptide cyclization in real time. The general utility of FRET-assisted detection of cyclopeptides was demonstrated for two cyclases, namely tyrocidine (Tyc) TE and calcium-dependent antibiotic (CDA) TE. For the latter cyclase, this approach was combined with site-directed affinity labeling, opening the possibility for high-throughput enzymatic screening.

Introduction

A variety of peptides, glycopeptides, lipopeptides, lipoglycopeptides, and polyketidyl-peptidyl hybrids have macrocyclic structures in which the rigidity of the ring skeletons constrains these natural products to biologically active conformations [1–4]. In the biosynthesis of these natural products, such as the macrolactone antibiotic daptomycin [5], the macrolactone antifungal fengycin [6], and the macrolactam antibiotics tyrocidine [7] and friulimicin [8], by nonribosomal peptide synthetases (NRPS), C-terminal 30–40 kDa thioesterase domains (TE) introduce this conformational rigidity by acting as macrocyclization catalysts [9]. Recent research on recombinant TE domains provided insights into their substrate selectivity and catalytic mechanism and allowed the chemoenzymatic generation of novel macrocyclic compounds [10, 11].

The chemoenzymatic approach combines the strength of synthetic peptide synthesis with the strength of stereoselective and regioselective TE domain cyclization. However, a limitation of this strategy is the high enzymatic selectivity of TE domains for cognate substrates, which can be a disadvantage when the cyclization of substrate analogs is desired. Exchanges of C- and N-terminal residues of the linear peptide substrate often abolish formation of the macrocyclic product [2]. In other cases, the yield of enzymatically generated ring formation dramatically suffers from linear hydrolytic byproducts due to nucleophilic attack of water molecules onto the acyl-enzyme intermediate [12]. Recently, it was shown that the addition of nonionic detergent significantly improves the cyclic to hydrolyzed product ratio of the excised TE domain from the tyrocidine synthetase [13]. Nevertheless, since the utility of TE domains strongly depends on substrate tolerance, efficient turnover, and high product yield, there is a constant need for engineered TE domains. This could be achieved by directed protein evolution, as previously shown for lipases [14]. However, such an approach requires reliable detection of cyclized peptides under high-throughput conditions.

Herein, we present the design and utility of fluorescent probes capable of accurately indicating enzyme-mediated peptide cyclization via fluorescence resonance energy transfer (FRET) [15]. These probes are available via simple solid phase peptide synthesis and are characterized by picomolar detection limits. Fluorescence occurs by a process that comprises the excitation of a tryptophan (Trp) residue that subsequently transfers its energy to a nonproteinogenic kynurenine (Kyn) residue; this excited chromophore then relaxes via emission of blue light [16]. These two fluorophores are originally found in the lipopeptide antibiotic daptomycin, which was clinically approved under the trade name Cubicin (Figure 1A). The Trp residue was previously shown to have a low fluorescence yield due to energy transfer to the Kyn residue. Further, the relative quantum yield of daptomycin-incorporated Kyn is 7.6 times greater than Kyn as a free amino acid, thereby allowing Kyn fluorescence (emission wavelength $\lambda_{Em} \approx$ 455 nm) to be a sensitive probe of daptomycin-phospholipid membrane interactions [16]. This efficient energy transfer results from the spectral overlap between the Trp donor emission ($\lambda_{Em} \approx$ 330 nm) and Kyn acceptor absorption (excitation wavelength $\lambda_{Ex} \approx$ 350 nm) [16, 17].

We recently showed that the C-terminal TE domain excised from the nonribosomal CDA synthetase retains autonomous macrocyclization activity with linear peptide thioester substrates based on a primary sequence analogous to daptomycin [18]. Interestingly, the linear peptide precursor adopts a conformation that spatially separates these two fluorophores, limiting the Kyn emission process (Figure 1B). However, the cyclized daptomycin analog adopts a conformation in which the Kyn fluorophore is in close spatial proximity to the Trp residue, allowing FRET to occur (Figure 1A). Therefore, the Kyn fluorescence difference between the linear and cyclized peptide can be exploited to reliably detect cy-

*Correspondence: marahiel@chemie.uni-marburg.de
[1]Present address: The Scripps Research Institute, Cravatt Laboratory, 10550 North Torrey Pines Road, La Jolla, California 92037.

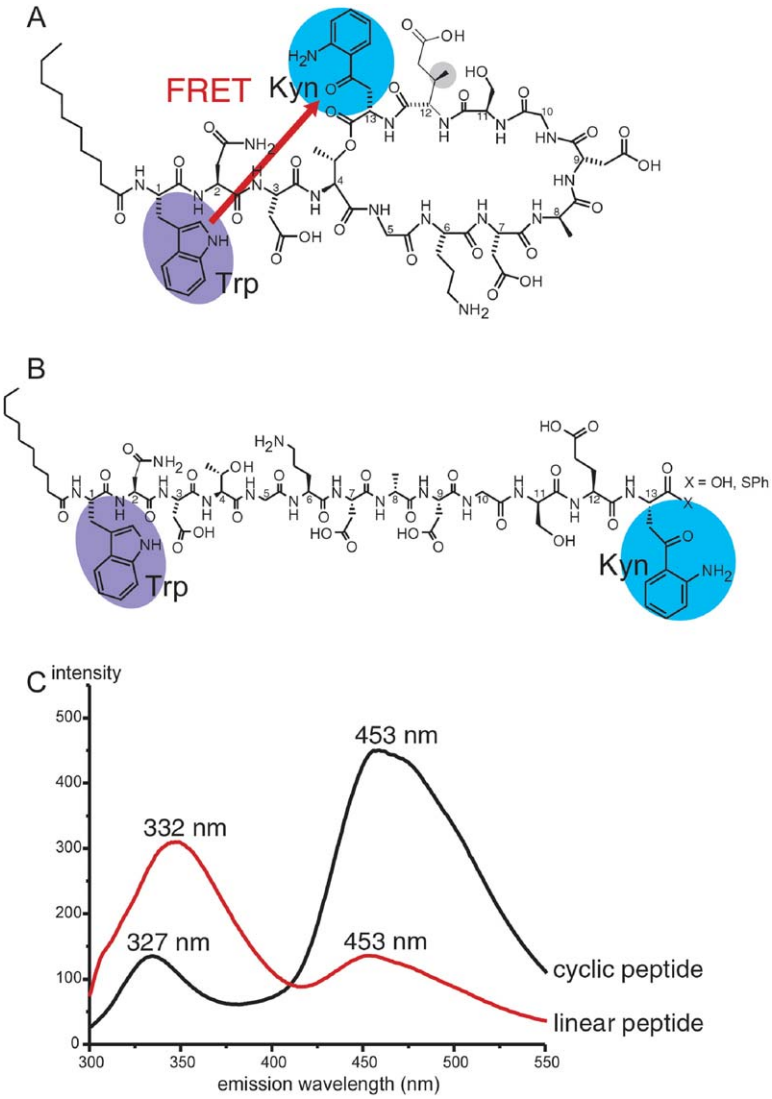

Figure 1. Fluorescence Resonance Energy Transfer in Daptomycin

(A) Daptomycin is a branched cyclic nonribosomally assembled acidic lipopeptide produced by *Streptomyces roseosporus*. This decapeptide lactone adopts a conformation in which the kynurenine (Kyn) fluorophore is in close spatial proximity to the tryptophan (Trp) residue, allowing fluorescence resonance energy transfer (FRET) to occur. The methyl group of nonproteinogenic L-3-methylglutamate is indicated by shading.

(B) The structure of a linear daptomycin derivative is shown. The fluorescent residues Kyn and Trp are highlighted.

(C) Emission spectra of the linear and cyclic daptomycin derivatives in methanol and DMSO 9:1 (v/v): red line, linear daptomycin derivative; black line, cyclic daptomycin derivative; excitation wavelength = 280 nm.

clized peptide under high-throughput conditions to greatly facilitate protein evolution efforts.

Results and Discussion

Synthesis and Fluorescence Characteristics of Linear and Cyclic Daptomycin Peptides

The cyclic lipopeptide antibiotic daptomycin is a tridecapeptide that contains two fluorophores: tryptophan (Trp, W) at the N terminus and kynurenine (Kyn, U) at the C terminus (Figure 1A). The poor fluorescence yield of the Trp residue situated between the C_{10}-fatty acid and the peptide headgroup was reported earlier [16]. It was assumed that this poor Trp emission is due to fluorescence resonance energy transfer (FRET), which results from the combination of the proximity of the two fluorophores and the overlap of the Trp emission spectrum ($\lambda_{Em} \approx 330$ nm) and Kyn absorption spectrum ($\lambda_{Ex} \approx 350$ nm). The proximity of these fluorophores mainly arises from the lactone bond, which connects threonine at position 4 with the C-terminal Kyn residue. We thus reasoned that the energy transfer might be less efficient in the linear peptide derived from daptomycin by opening of the decapeptide lactone ring (Figure 1B). Given this assumption, we chose to compare the fluorescence characteristics of cyclic to linear daptomycin. The latter was prepared by solid-phase peptide synthesis, followed by treatment with trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and water in a ratio of 95:2.5:2.5 (v/v) to yield the linear peptide acid. For the generation of cyclic daptomycin (Figure 1A), linear peptidyl thiophenol was converted to the macrolactone in the presence of CDA cyclase [18]. In the linear and cyclic daptomycin sequence, we replaced L-3-methylglutamate at position 12 by glutamate for synthetic reasons. To investigate the assumed distance-dependent energy transfer between the two fluorophores Trp and Kyn, the linear and cyclic daptomycin analogs were excited at 280 nm, followed by measurement of the emission spectra. Fluorescence measurements were carried out in methanol and dimethylsulfoxide (DMSO) in a ratio of 9:1 (v/v) due to 2.1-fold

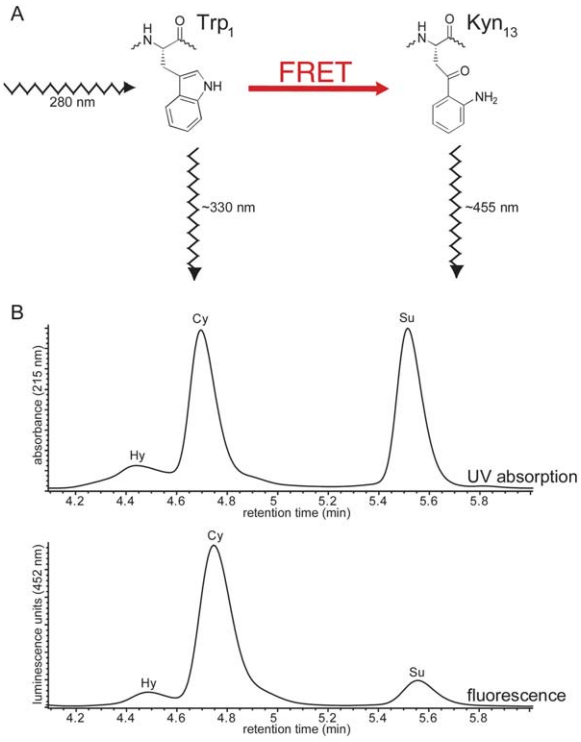

Figure 2. Proposed Model for the Energetic Interactions between Trp and Kyn

(A) In case of the linear daptomycin derivative (Figure 1B), excitation at 280 nm preferentially leads to emission of Trp. After cyclization (Figure 1A), this emission at ~330 nm is efficiently quenched due to fluorescence resonance energy transfer (FRET), which induces fluorescence of Kyn in the visible region of light.

(B) Fluorescence enhancement by TE-mediated peptide cyclization is shown. An assay containing 200 μM linear peptidyl thioester (**InDap-U_1W_{13}**) and 5 μM CDA TE was quenched by aqueous TFA and analyzed by HPLC: upper HPLC trace, absorbance at 215 nm; lower HPLC trace, fluorescence at 452 nm (excitation wavelength = 280 nm). Su, substrate; Hy, hydrolyzed product; Cy, cyclized product. The fluorescence signal is slightly shifted to higher retention times, because the solvent reaches the fluorescence detector after passing the UV-detection cell.

Kyn-fluorescence enhancement compared to 10% DMSO in water (data not shown). Interestingly, the Kyn emission at 453 nm of the linear daptomycin analog is minimal, resulting in a 4-fold difference between linear and cyclic peptide (Figure 1C). According to our model (Figure 2A), two different energetic pathways for the linear and cyclic daptomycin analogs are proposed. In case of the linear peptide, excitation at 280 nm produces preferentially UV light ($\lambda_{em} \approx 330$ nm). Conversely, excitation of Trp in the cyclic peptide generates a strong Kyn emission in the visible region of light ($\lambda_{em} \approx 455$ nm) due to FRET. This shows that the linear peptide adopts random coil conformation that spatially separates the two fluorophores, whereas the cyclized peptide adopts a conformation in which Trp and Kyn are in close spatial proximity, allowing FRET to occur. Further proof was obtained by analytical HPLC in combination with fluorescence studies (Figure 2B). An assay with 200 μM linear (ln) daptomycin-like peptide precursor **InDap-U_1W_{13}** (comprising Kyn at position 1 and Trp at position 13; Figure 3 shows the corresponding cyclic peptide **cyDap-U_1W_{13}**) and 5 μM CDA cyclase was quenched after a time period of 30 min by the addition of aqueous trifluoroacetic acid (TFA). Analytical HPLC with monitoring at 215 nm revealed identical concentrations of peptidyl thiophenol substrate and its macrolactone product (Figure 2B). Remarkably, determination of the emission at 452 nm using a fluorescence detector at an excitation wavelength of 280 nm significantly enhanced signal intensity of the decapeptide lactone. Peak area integration exhibited a 5.5-fold amplification of visible fluorescence in comparison to the linear precursor, indicating a greatly improved energy transfer between Trp and Kyn in the cyclic peptide.

Examination of Distance-Dependent Interactions between Donor and Acceptor

We reasoned that the above FRET experiments could be transferred to a variety of peptides with varying spatial proximity of donor and acceptor. To investigate the feasibility of FRET-detected peptide cyclization, we chose to create a small library of linear and cyclic daptomycin-like peptides (Figure 3). In contrast to authentic daptomycin, the positions of the fluorophores Trp and Kyn in the peptide backbone were exchanged. This strategy maximizes the yield of cyclic peptide generated by CDA TE because Trp is the C-terminal amino acid of cognate CDA [19]. Therefore, in all cases the conversion to cyclic product was sufficient to allow fluorescence studies (data not shown). To vary the distance between both fluorophores, Trp was kept at the C terminus, whereas the position of Kyn was systematically altered. All of the HPLC-purified linear and cyclic peptides were dissolved in a mixture of methanol and DMSO 9:1 to a final concentration of 200 μM, followed by fluorescence measurements.

Since the distances between the fluorophores were systematically altered in the above-mentioned library of linear and cyclic (cy) daptomycin derivatives (Figure 3), we expected a distance-dependent relationship of integrated acceptor fluorescence to integrated donor fluorescence (Em_{Kyn}/Em_{Trp} ratio). As shown in Figure 4A, the Em_{Kyn}/Em_{Trp} ratio changes in the order **cyDap-U_1W_{15}** < **cyDap-U_1W_{14}** < **cyDap-U_1W_{13}** < **cyDap-U_2W_{13}** < **cyDap-U_3W_{13}** < **cyDap-U_5W_{13}** > **cyDap-U_7W_{13}**. Therefore, decreasing distance between both fluorophores clearly correlates with more effective energy transfer. Indeed, the compounds **cyDap-U_3W_{13}** and **cyDap-U_5W_{13}**, the latter one with the fluorescent Kyn moved to a ring position, show the highest Em_{Kyn}/Em_{Trp} ratios of this study because the FRET pair is separated only by the cyclization nucleophile threonine.

In general, excitation at 280 nm revealed 3- to 5-fold increased Em_{Kyn}/Em_{Trp} ratios, when the linear peptide precursors were converted into the corresponding macrolactones by CDA cyclase, suggesting that the Trp-Kyn pair may be employed as a reliable FRET probe to monitor enzyme-catalyzed peptide cyclization. The only exceptions were **InDap-U_1W_{15}** and **InDap-U_1W_{14}**, where peptide cyclization yielded less than 2-fold increases of the Em_{Kyn}/Em_{Trp} ratios (Figure 4A), indicating that more than three amino acid residues in between

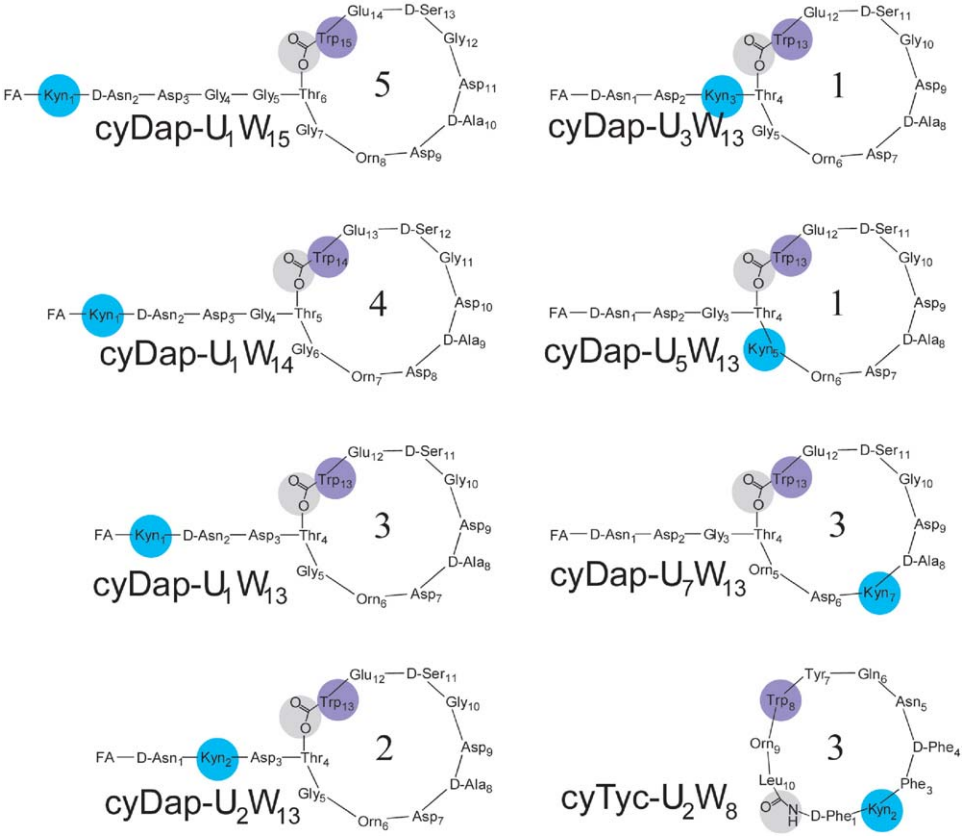

Figure 3. Macrocyclic Peptides Used in This Study

All fluorophore-tagged cyclopeptides are shown using 3 letter amino acid codes. The FRET pair in each compound is highlighted. The figures in the middle of the macrocycles denote the number of amino acid residues between Trp and Kyn. Macrolactonization/macrolactamization is indicated by gray shading. Kyn, kynurenine; Orn, ornithine; FA, fatty acid.

Trp and Kyn significantly limits the energy transfer. These results indicate that the fluorophores can be positioned at various sites of the peptide backbone to allow efficient FRET, clearly demonstrating that FRET-assisted detection of peptide cyclization is not limited to the special case of daptomycin. Detection of peptide cyclization by FRET may also be applicable to many different cyclopeptides by simple incorporation of the fluorophores Trp and Kyn into the N- and C-terminal parts of the peptide backbone.

Real-Time Monitoring of Peptide Cyclization

Cyclase-catalyzed macrolactonization/macrolactamization is usually monitored by quenching of enzyme assays at various time points, followed by HPLC-MS analysis on reverse-phase columns [20]. Quenching can be conducted either by addition of diluted TFA or by addition of organic solvents to irreversibly denature the enzyme. To this end, the FRET-assisted detection of peptide cyclization provides a useful means to follow reactions in real time without disrupting enzymatic integrity. By incorporating fluorescent amino acids into linear peptide precursors, adding the respective recombinant cyclization enzyme, and measuring the visible fluorescence intensity (λ_{Ex} = 280 nm; λ_{Em} = 452 nm) at defined time intervals, one can easily estimate the progression of the peptide cyclization reaction. We demonstrated this technique with the daptomycin derivative **InDap-U_3W_{13}** in which the fluorescent residues Trp and Kyn were placed at positions 13 and 3, respectively (Figure 3). After addition of 5 μM CDA cyclase, fluorescence intensity at 452 nm followed a time-dependent hyperbolic function, reaching a maximum after approximately 50 min (Figure 5A). HPLC studies revealed total conversion to cyclic product within this period of time. Also, a control reaction in the presence of the corresponding heat-denatured enzyme indicated no significant change in fluorescence emission.

The real-time monitoring of CDA cyclase-mediated peptide cyclization raised the question of whether this approach may be applicable to other peptide cyclization catalysts. To answer this question, we chose the well-characterized tyrocidine synthetase thioesterase domain (Tyc TE), which efficiently catalyzes head-to-tail cyclization with decapeptidyl thioesters. It was previously shown that Tyc TE tolerates replacement of residues 2–8 of peptidyl thioesters [7, 21]. To follow Tyc TE-catalyzed macrolactamization by FRET, we incorporated the Trp/Kyn pair into the wild-type tyrocidine A sequence. Specifically, we replaced Pro_2 by Kyn and Val_8 by Trp (Figure 3 shows the corresponding cyclic

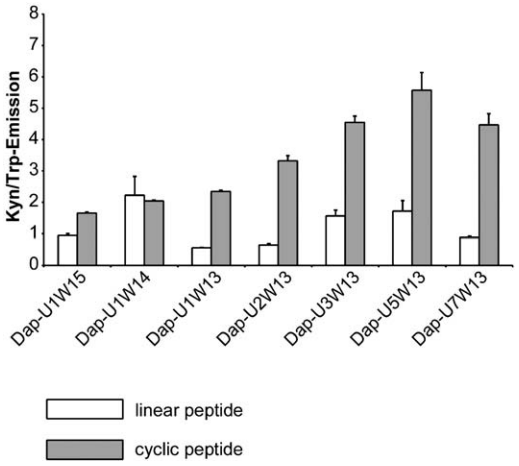

Figure 4. Fluorescence Studies of Linear and Cyclic Daptomycin Derivatives

Determination of Em_{Kyn}/Em_{Trp} ratios of linear and cyclic daptomycin derivatives with systematically altered distance between Trp and Kyn: linear daptomycin derivatives, white bars; cyclic daptomycin derivatives, gray bars. All bars represent mean values of three measurements. Excitation wavelength = 280 nm. U, kynurenine; W, tryptophan. The error bars represent the standard error of the mean.

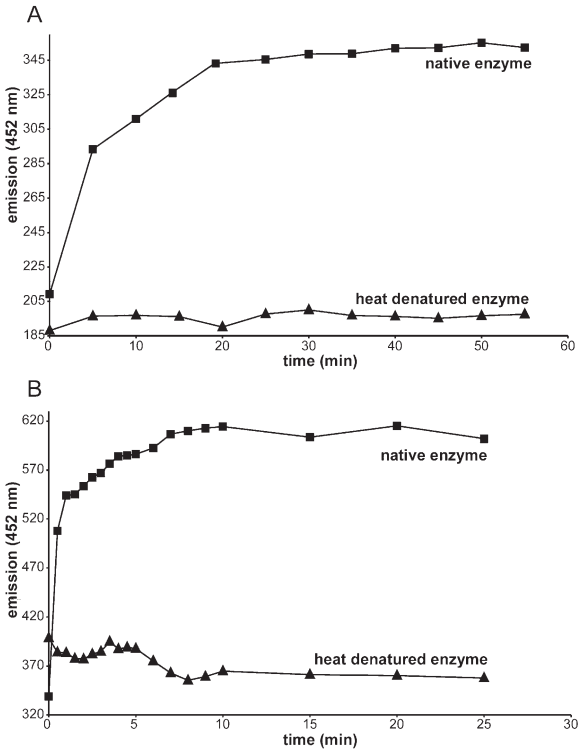

Figure 5. Real-Time Monitoring of Peptide Cyclization

(A) Sample contained 75 μM **InDap-U$_3$W$_{13}$** and 5 μM CDA TE (square). The negative control was conducted in the presence of 5 μM heat-denatured CDA cyclase (triangle).

(B) The cuvette contained 50 μM **InTyc-U$_2$W$_8$** and 0.5 μM Tyc TE (square). The negative control was done in the presence of 0.5 μM heat-denatured Tyc TE (triangle). Excitation wavelength = 280 nm, emission wavelength = 452 nm.

peptide **cyTyc-U$_2$W$_8$**). Incubation of the resulting substrate **InTyc-U$_2$W$_8$** with Tyc TE indicated a time-dependent increase in fluorescence emission (λ_{Em} = 452 nm) (Figure 5B), although the cyclization-to-hydrolysis ratio was quite low (0.28) as determined by analytical HPLC. Finally, no change in fluorescence was observed for heat-denatured Tyc TE.

In conclusion, this study offers a simple means to evaluate reaction progression of catalyzed macrolactonization/macrolactamization by FRET. The results also provide evidence that the position of the Trp fluorophore is not restricted to the C terminus of the linear peptide precursor, as demonstrated by the substrate **InTyc-U$_2$W$_8$**. Therefore, this observed high flexibility in primary sequence makes FRET-assisted detection of cyclopeptides a general tool for peptide cyclases.

Fluorescence Resonance Energy Transfer Can Be Used to Measure Kinetics of Enzyme-Mediated Peptide Cyclization

The above-mentioned results show that the close spatial proximity of donor and acceptor in the cyclic peptide induces up to 5.5-fold amplification of visible fluorescence in comparison to the linear precursor. In addition, FRET-assisted detection of peptide cyclization easily realizes picomolar detection limits using conventional fluorescence detectors without optimization. For example, the cyclic daptomycin derivative **cyDap-U$_2$W$_{13}$** was characterized by a detection limit of 7 pmol, whereas its linear counterpart **InDap-U$_2$W$_{13}$** showed a limit of 28 pmol. This high sensitivity raised the question of whether FRET can be used to accurately determine kinetics of enzyme-mediated peptide cyclization. To address this question, thioester substrates of daptomycin derivatives (**InDap-U$_1$W$_{13}$** and **InDap-U$_3$W$_{13}$**) were chosen for kinetic measurements, which were performed by analytical HPLC combined with fluorescence detection. Initial investigations revealed a linear correlation between fluorescence emission at 452 nm and the concentrations of the respective cyclic peptides (**cyDap-U$_1$W$_{13}$** and **cyDap-U$_3$W$_{13}$**) as determined by calibration curve plots, thus allowing simple quantification of the fluorophor-containing macrolactones. Kinetic characterization of the cyclization reactions was then carried out by calculating the initial reaction rates at 5–7 substrate concentrations. We found that the k_{cat}/K_M values derived from integrated fluorescence emission were in good agreement with the corresponding k_{cat}/K_M values determined by UV absorbance at 215 nm, resulting in 1.2- to 1.6-fold differences between both detection methods (Table 1).

FRET-Assisted Detection of Peptide Cyclization of Immobilized CDA Cyclase

It has recently been shown that the use of NRPS-derived peptide carrier protein (PCP) for biotin labeling is amenable to high-throughput enzymatic screening [22], which can be a powerful method for identifying and evolving biological catalysts. The PCP is a versatile tag for labeling PCP fusion proteins due to its small

Table 1. Determination of Cyclization Kinetics Including Standard Errors: UV Absorption versus Kyn Fluorescence

Compound	k_{cat}/K_M ($mM^{-1}min^{-1}$)	Method
InDap-U_1W_{13}	5.09 ± 4.25	absorbance (215 nm)
	6.13 ± 4.17	fluorescence: emission wavelength = 452 nm
InDap-U_3W_{13}	4.34 ± 1.85	absorbance (215 nm)
	6.75 ± 2.39	fluorescence: emission wavelength = 452 nm

U = kynurenine, W = tryptophan, and In = linear.

size (80 amino acids) and its good portability to various target proteins [23]. This autonomously folded domain directs the specific biotin labeling of the target protein in a complex mixture of cellular proteins, which is efficiently catalyzed by the promiscuous 4′-phosphopantetheinyl transferase Sfp from *B. subtilis* [24]. We therefore reasoned that this method could be transferred to high-throughput screening of TE domains in order to improve or alter their substrate specificity by directed evolution efforts.

Since in the native context of NRPS, the PCP domain is N-terminally associated with the TE domain, we simply excised the PCP-TE didomain from the synthetase. Specifically, we heterologously expressed CDA PCP-TE, a 44.5 kDa protein from the *S. coelicolor* CDA biosynthetic machinery that was fused to an N-terminal heptahistidine tag [19]. The yield of the didomain was around 4–5 mg/l. The purified CDA PCP-TE was then labeled with Ppant biotin in the presence of Sfp and biotin CoA as described earlier [23]. Site-directed posttranslational modification was allowed to proceed for 90 min at 30°C, followed by confirmation with an API Qstar Pulsar i Q-q-TOF mass spectrometer (measured mass *m/z* 45382, calculated mass 45379). The labeling-reaction mixture was then run over an avidin column (bed volume 1 ml). After washing the column, the immobilized CDA PCP-TE didomain was incubated with 5 mM of **InDap-U_1W_{13}** for 3 hr at 25°C. Reaction products were eluted off the column, and the mixture was characterized by HPLC/MS (Figure 6A). Interestingly, **InDap-U_1W_{13}** was significantly converted to the macrolactone product **cyDap-U_1W_{13}**. The observed cyclization-to-hydrolysis ratio was 7.0, validating that the immobilized CDA cyclase retains excellent cyclization activity. Additionally, minor amounts of an octapeptide lactam derived from cyclization via Orn_6 were detected as described previously [18]. In contrast to the desired macrolactone, this byproduct is also generated in the absence of bound CDA cyclase, indicating its nonenzymatic origin. Finally, the PCP-TE didomain was eluted with 5 mM biotin, and the protein was identified with SDS-PAGE to yield a single band of correct size (data not shown).

Remarkably, the substrate conversion into products was accompanied by a 3.5-fold increase in fluorescence emission at 452 nm (Figure 6B). This result suggests that rapid detection of peptide cyclization by FRET can be transferred to immobilized TE domains, thus making the PCP-TE-tagging approach amenable to high-throughput enzymatic screening. We also conclude that this technique can be used with a variety of NRPS peptide cyclases and will further allow evolution of their substrate selectivities. Moreover, PCP-TE tagging could in principle be used to successively cyclize multiple linear peptide precursors in a combinatorial fashion.

Significance

Here we have shown a robust system for specifically detecting macrocyclic peptides by fluorescence resonance energy transfer (FRET). In this approach, peptide cyclization catalyzed by NRPS-derived TE domains brings the donor (Trp) and the acceptor (Kyn) in sufficiently close proximity to enable efficient FRET. Trp and Kyn are readily incorporated into the peptide backbone by well-established solid-phase peptide chemistry and show excellent spectral overlap between the donor emission and acceptor absorption. A library of daptomycin-like peptides revealed that this approach allows variable positioning of the fluorophores in the N- and C-terminal parts of the peptide sequence, which is significant given that TE domains do not tolerate exchanges of specific resi-

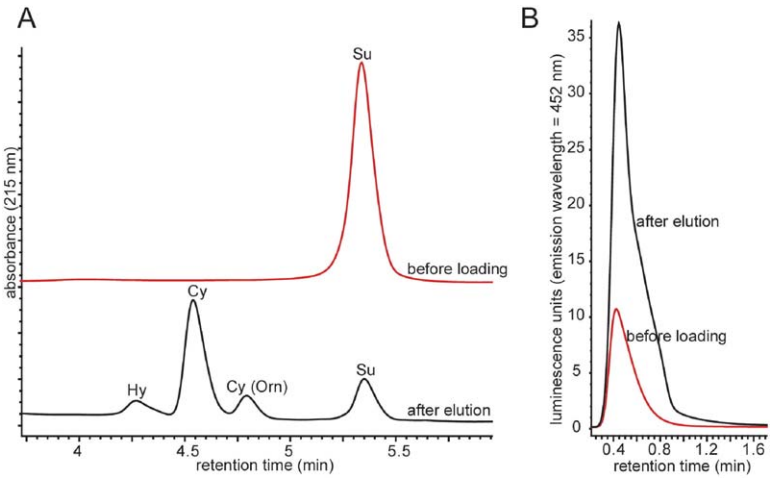

Figure 6. Immobilization of CDA Cyclase by Site-Directed Posttranslational Modification and Subsequent Detection of Reaction Products

(A) HPLC trace of **InDap-U_1W_{13}** prior to cyclization by immobilized CDA cyclase (red trace). Immobilized CDA TE was incubated with **InDap-U_1W_{13}** for 3 hr at 25°C (black trace).

(B) Detection of generated cyclopeptide by FRET. The red trace shows the fluorescence signal of **InDap-U_1W_{13}** prior to loading onto the column. The black trace shows the fluorescence signal after CDA TE-mediated cyclization. Excitation wavelength = 280 nm, emission wavelength = 452 nm.

dues of their peptidic substrates. This high flexibility in primary sequence makes FRET-assisted detection of cyclopeptides a general tool for peptide cyclases, as demonstrated for CDA TE and Tyc TE. Moreover, picomolar detection limits and a linear correlation between acceptor fluorescence and cyclopeptide concentration are realized, thus allowing determination of cyclization kinetics.

Rapid and sensitive detection of peptide cyclization by FRET can be transferred to avidin-immobilized cyclases using site-directed biotin labeling of PCP-TE didomains. This PCP-TE-tagging approach can serve as a valuable tool in high-throughput enzymatic screening to alter the substrate specificity of peptide cyclases, thereby allowing the synthesis of novel cyclopeptides. Alternatively, the methodology developed could be utilized for the combinatorial synthesis of these macrocyclic compounds.

Experimental Procedures

Cloning and Expression of CDA TE and CDA PCP-TE

The *cda te* and *cda pcp-te* gene fragments were amplified by PCR using the chromosomal DNA of *S. coelicolor* A3(2) as template [25]. The PCR reaction was carried out with Turbo DNA polymerase (Stratagene). Amplification of *cda te* was performed using the oligonucleotides 5′-AAA AAA GGA TCC CGC GGC GGC CGG GAG CC-3′ and 5′-AAA AAA AAG CTT GGC GAC CTC GGT CGA ATC GAG CG-3′. For cda *pcp-te*, the following oligonucleotides were used: 5′-AAA AAA GGA TCC CGC ACC GTC GAG GGC CGC TC-3′ and 5′-AAA AAA AAG CTT GGC GAC CTC GGT CGA ATC GAG CG-3′. The PCR products of *cda te* and *cda pcp-te* were cloned into the BamHI/HindIII site of a derivatized pQE60 vector (Qiagen). DNA sequencing was performed by GATC Biotech on an ABI prism 310 genetic analyzer (Applied Biosystems). The plasmids were directly used to produce proteins with an N-terminal heptahistidine tag. Overproduction of recombinant proteins was carried out in *E. coli* XL1-Blue (Stratagene). Cells were grown to OD = 0.5 (600 nm), induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and again grown at 25°C for 3 hr. The recombinant proteins were purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Amersham Pharmacia Biotech). Dialysis into 25 mM HEPES and 50 mM NaCl (pH 7.0) was carried 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.

Synthesis of Linear Peptide Thioester Substrates

Linear peptides were synthesized on an Advanced ChemTech APEX 396 peptide synthesizer (0.1 mmol scale, N-[(1H-benzotriazol-1-yl)(dimethylamino)methylene]-N-methylmethaniminium hexafluorophosphate N-oxide/1-hydroxybenzotriazole (HBTU/HOBt) activation) by using 2-chlorotrityl resin derivatized with the appropriate C-terminal amino acid using Fmoc-protected monomers except for the N-terminal monomer of the linear tyrocidine derivative, which was Boc protected. N^{α}-Fmoc protection of kynurenine was conducted with 9-fluorenylmethyl succinimidyl carbonate (Fmoc-OSu) as described previously [18]. The peptides were cleaved from the solid support using 1:2:7 acetic acid:trifluorethanol:dichloromethane (DCM), then the protected peptides were precipitated with n-hexane and the solvent was removed by rotary evaporation. To make the peptide thioesters, the peptides (1 eq.) were dissolved in DCM, and diisopropylcarbodiimide (DCC, 2 eq.), HOBt (2 eq.), thiophenol or N-acetylcysteamine (10 eq.), and potassium carbonate (2 eq.) were added [20]. The reaction was stirred at ambient temperature for 3 hr. After removal of the DCM, the protected peptide thioesters were treated with 95:2.5:2.5 trifluoroacetic acid (TFA):water:triisopropylsilane (2 ml) at room temperature for 2 hr. Precipitation of the peptide thioesters was carried out with ice-cold ether (30 ml). After centrifugation, the peptide thioesters were dissolved in 50% acetonitrile/water and purified on a semipreparative 250/21 Nucleodur 100-5 C_{18} reverse-phase column (Macherey and Nagel) by applying a gradient from 20% to 60% acetonitrile in 0.1% TFA/water over 30 min at a flow rate of 20 ml min^{-1}. The peptide thioesters were characterized by MALDI-MS or ESI-MS: **InDap-U_1W_{13}** m/z 1717.2 ([M+H]$^+$), calcd 1716.7; **InDap-U_2W_{13}** m/z 1716.3 ([M+H]$^+$), calcd 1716.7; **InDap-U_3W_{13}** m/z 1717.0 ([M+H]$^+$), calcd 1716.7; **InDap-U_5W_{13}** m/z 1716.6 ([M+H]$^+$), calcd 1716.7; **InDap-U_7W_{13}** m/z 1717.0 ([M+H]$^+$), calcd 1716.7; **InDap-U_1W_{14}** m/z 1773.9 ([M+H]$^+$), calcd 1773.7; **InDap-U_1W_{15}** m/z 1830.3 ([M+H]$^+$), calcd 1830.8; **InTyc-U_2W_8** m/z 1569.9 ([M+H]$^+$), calcd 1569.7.

Preparation of Cyclic Peptides for Fluorescence Studies

250 μM peptidyl-thiophenol thioesters were incubated for 2–5 hr with 5 μM CDA cyclase in 25 mM HEPES, 50 mM NaCl, and 5% DMSO (v/v) at pH 7.0 and room temperature. Reactions were monitored with analytical HPLC and thin-layer chromatography (TLC) on silica gel 60 F_{254} plates (Merck) and visualized under UV (365 nm). The semipreparative scale assays were quenched by adding TFA to a final concentration of 1.6% (v/v). After flash freezing in liquid nitrogen, the samples were lyophilized overnight. The resulting solids were redissolved in 1 ml of 50% acetonitrile/water. Purification of the cyclic products was carried out on a 250/10 Nucleodur 100-7 C_{18} reverse-phase column (Macherey and Nagel) by applying a gradient from 35% to 45% acetonitrile in 0.1%TFA/water over 30 min at a flow rate of 8 ml min^{-1}. Lyophilized cyclic peptides were dissolved in methanol:DMSO 9:1 to a final concentration of 200 μM. The purities were checked by analytical HPLC, and the concentrations were determined by comparing the area of absorption at 215 nm with that of a known concentration of linear peptide thioester or daptomycin. The cyclic peptides were characterized by MALDI-MS or ESI-MS: **cyDap-U_1W_{13}** m/z 1607.1 ([M+H]$^+$), calcd 1606.7; **cyDap-U_2W_{13}** m/z 1606.9 ([M+H]$^+$), calcd 1606.7; **cyDap-U_3W_{13}** m/z 1607.0 ([M+H]$^+$), calcd 1606.7; **cyDap-U_5W_{13}** m/z 1606.8 ([M+H]$^+$), calcd 1606.7; **cyDap-U_7W_{13}** m/z 1606.9 ([M+H]$^+$), calcd 1606.7; **cyDap-U_1W_{14}** m/z 1663.8 ([M+H]$^+$), calcd 1663.7; **cyDap-U_1W_{15}** m/z 1721.0 ([M+H]$^+$), calcd 1720.8; **cyTyc-U_2W_8** m/z 1450.6 ([M+H]$^+$), calcd 1450.7.

Determination of k_{cat}/K_M Values

Kinetic characterization of the cyclization reactions was carried out by determining initial rates at varying peptide thioester concentrations. This was done by using two time points at each substrate concentration within the linear region of CDA TE. The reactions were carried out in 25 mM HEPES and 50 mM NaCl (pH 7.0) with 5% DMSO (v/v) at 20°C. The enzyme concentration used was 5 μM. Quenching was accomplished by addition of 35 μl of 4% TFA/H_2O. All assays were analyzed on a reverse-phase C_{18} Nucleodur column (Macherey and Nagel, 125/3, pore diameter 100 Å, particle size 3 μm) with the following gradient: 0–10 min, 30%–90% acetonitrile/0.1% TFA in water/0.1% TFA, 0.8 ml/min, and 40°C. Concentrations of cyclic products were determined using the area of absorption at 215 nm. Alternatively, concentrations of cyclic peptides were calculated as the integrated fluorescence emission at 452 nm using a fluorescence detector (Standard FLD cell, Agilent) at an excitation wavelength of 280 nm (PMT gain 10). In both cases the concentrations were determined by comparing the area of absorption at 215 nm or the area of emission at 452 nm with that of known concentrations of the corresponding cyclic peptides (**cyDap-U_1W_{13}** and **cyDap-U_3W_{13}**). Separate k_{cat} and K_M values could not be determined due to severe substrate inhibition at concentrations greater than 200 μM, which might be related to micelle formation of the amphiphilic substrates [26].

Synthesis of Biotin CoA

Biotin CoA was prepared according to Yin et al. [22]. A solution of biotin maleimide (Pierce) (10 mg, 19 μmol) in 300 μl DMSO was added to coenzyme A lithium salt (Sigma) (18.2 mg, 23 μmol) in 2 ml MES acetate 50 mM at pH 6.0. After stirring overnight, the reaction mixture was purified on a semipreparative 250/21 Nucleodur 100-5 C_{18} reverse-phase column (Macherey and Nagel) by applying a gradient from 0% to 60% acetonitrile in 0.1% TFA/water over 35 min

at a flow rate of 18 ml min^{-1}. The purified product was characterized by MALDI-MS: Biotin CoA m/z 1293.2 ($[M+H]^+$), calcd 1293.3.

Peptide Cyclization by the Immobilized CDA PCP-TE Didomain

Sfp phosphopantetheinyl transferase-catalyzed biotin CoA labeling of the purified CDA PCP-TE protein was performed according to Clugston et al. [27]. In a total volume of 100 μl, 5 μM Sfp, 5 μM biotin CoA, 10 mM $MgCl_2$, and 1 mM tris(2-carboxyethyl)phosphine (TCEP) in 50 mM HEPES (pH 7.5) were incubated with 20 μM CDA PCP-TE didomain for 90 min at 30°C. The labeling reaction mixture was then run over a column loaded with SoftLink soft release avidin resin (Promega) (bed volume 1 ml). Incubation was carried out at 4°C prior to washing with a solution containing 5 ml 0.1 M $NaPO_4$ (pH 7.0). 5 mM peptidyl thiophenol **InDap-U_1W_{13}** dissolved in 100 μl DMSO was then added to the column. After incubation at 25°C for 3 hr, the reaction products were eluted with 1 ml phosphate buffer. After lyophilizing to dryness, the eluate was extracted with methanol:DMSO 9:1. Product analysis was conducted by analytical HPLC on a C_{18} Nucleodur column (Macherey and Nagel, 125/3, pore diameter 100 Å, particle size 3 μm) by applying a gradient from 30% to 90% acetonitrile in 0.1% TFA/water over 10 min at a flow rate of 0.8 ml min^{-1} and by fluorescence detection without HPLC column (Standard FLD cell [Agilent], emission wavelength 452 nm, excitation wavelength 280 nm, PMT gain 10, injection volume 10 μl, 100% methanol over 2 min, 0.5 ml min^{-1}). The CDA PCP-TE didomain was eluted with 5 mM biotin in the phosphate buffer. The eluate was resolved by SDS-PAGE (12.5%) and visualized by Coomassie stain.

Fluorescence Techniques

Fluorescence measurements were performed on a spectrofluorometer (FP-6500, Jasco) with slits set to 10 nm (excitation) and 5 nm (emission) at an excitation wavelength of 280 nm. The detector was set to low sensitivity. Constant temperature was achieved by circulating water through the cell holder in which the temperature was measured by a temperature controller (ETC-273T, Jasco). The fluorescence results are expressed as the relative quantum yield (ϕ) calculated as the integrated fluorescence emission between 300 and 390 nm (Trp) and between 400 and 550 nm (Kyn). All experiments were performed in 10 mm path length cuvettes containing 300 μl of linear peptide thioester or cyclic peptide dissolved in methanol:DMSO 9:1 to a final concentration of 200 μM. Fluorescence measurements were also carried out using a fluorescence detector (Standard FLD cell, Agilent) without HPLC column (emission wavelength 452 nm, excitation wavelength 280 nm, PMT gain 10, injection volume 10 μl, 100% methanol over 2 min, 0.5 ml min^{-1}). Absorptions measurements were performed on an Ultrospec 3000 UV/Visible Spectrophotometer (Pharmacia Biotech).

Real-Time Fluorescence Measurements

Reactions were carried out in 10 mm path length cuvettes in a total volume of 300 μl with 25 mM HEPES, 50 mM NaCl, and 5% DMSO (v/v) at pH 7.0 and 25°C. The enzyme concentrations were 0.5 μM for Tyc TE and 5 μM for CDA cyclase. The negative controls were conducted in the presence of the corresponding heat-denatured enzymes. Reactions were initiated by addition of 50 μM peptidyl SNAC **InTyc-U_2W_8** or 75 μM peptidyl thiophenol **InDap-U_3W_{13}**. Real-time measurements were performed using various time points with slits set to 5 nm (excitation) and 5 nm (emission) at an excitation wavelength of 280 nm and an emission wavelength of 452 nm. The detector was set to medium sensitivity. All assays were quenched by the addition of 1.6% TFA (v/v) and analyzed by analytical HPLC.

Acknowledgments

We thank Christian Wirges for fluorescence measurements and Gabi Schimpff-Weiland and Antje Schäfer for technical assistance. We also acknowledge Dr. Antonio Pierik, Dr. Nadine Schracke, Dr. Henning D. Mootz, and Dr. Jun Yin for helpful comments. This work has been supported by the Fonds der Chemischen Industrie (to J.G. and M.A.M.) and the Deutsche Forschungsgemeinschaft (to S.A.S. and M.A.M.).

Received: April 28, 2005
Revised: May 19, 2005
Accepted: May 19, 2005
Published: August 26, 2005

References

1. Kahne, D., Leimkuhler, C., Lu, W., and Walsh, C. (2005). Glycopeptide and lipoglycopeptide antibiotics. Chem. Rev. *105*, 425–448.
2. Sieber, S.A., and Marahiel, M.A. (2005). Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. Chem. Rev. *105*, 715–738.
3. Walsh, C.T. (2004). Polyketide and nonribosomal peptide antibiotics: modularity and versatility. Science *303*, 1805–1810.
4. Kohli, R.M., and Walsh, C.T. (2003). Enzymology of acyl chain macrocyclization in natural product biosynthesis. Chem. Commun. (Camb.) *303*, 297–307.
5. Kirkpatrick, P., Raja, A., LaBonte, J., and Lebbos, J. (2003). Daptomycin. Nat. Rev. Drug Discov. *2*, 943–944.
6. Vanittanakom, N., Loeffler, W., Koch, U., and Jung, G. (1986). Fengycin—a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. J. Antibiot. (Tokyo) *39*, 888–901.
7. Trauger, J.W., Kohli, R.M., Mootz, H.D., Marahiel, M.A., and Walsh, C.T. (2000). Peptide cyclization catalysed by the thioesterase domain of tyrocidine synthetase. Nature *407*, 215–218.
8. Vertesy, L., Ehlers, E., Kogler, H., Kurz, M., Meiwes, J., Seibert, G., Vogel, M., and Hammann, P. (2000). Friulimicins: novel lipopeptide antibiotics with peptidoglycan synthesis inhibiting activity from *Actinoplanes friuliensis* sp. nov. II. Isolation and structural characterization. J. Antibiot. (Tokyo) *53*, 816–827.
9. Schwarzer, D., Finking, R., and Marahiel, M.A. (2003). Nonribosomal peptides: from genes to products. Nat. Prod. Rep. *20*, 275–287.
10. Bruner, S.D., Weber, T., Kohli, R.M., Schwarzer, D., Marahiel, M.A., Walsh, C.T., and Stubbs, M.T. (2002). Structural basis for the cyclization of the lipopeptide antibiotic surfactin by the thioesterase domain SrfTE. Structure (Camb) *10*, 301–310.
11. Lin, H., Thayer, D.A., Wong, C.H., and Walsh, C.T. (2004). Macrolactamization of glycosylated peptide thioesters by the thioesterase domain of tyrocidine synthetase. Chem. Biol. *11*, 1635–1642.
12. Kohli, R.M., Takagi, J., and Walsh, C.T. (2002). The thioesterase domain from a nonribosomal peptide synthetase as a cyclization catalyst for integrin binding peptides. Proc. Natl. Acad. Sci. USA *99*, 1247–1252.
13. Yeh, E., Lin, H., Clugston, S.L., Kohli, R.M., and Walsh, C.T. (2004). Enhanced macrocyclizing activity of the thioesterase from tyrocidine synthetase in presence of nonionic detergent. Chem. Biol. *11*, 1573–1582.
14. Reetz, M.T. (2002). Lipases as practical biocatalysts. Curr. Opin. Chem. Biol. *6*, 145–150.
15. Förster, T. (1948). Intermolecular energy migration and fluorescence. Ann. Phys. *2*, 55–75.
16. Lakey, J.H., and Ptak, M. (1988). Fluorescence indicates a calcium-dependent interaction between the lipopeptide antibiotic LY146032 and phospholipid membranes. Biochemistry *27*, 4639–4645.
17. Jung, D., Rozek, A., Okon, M., and Hancock, R.E. (2004). Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin. Chem. Biol. *11*, 949–957.
18. Grünewald, J., Sieber, S.A., Mahlert, C., Linne, U., and Marahiel, M.A. (2004). Synthesis and derivatization of daptomycin: a chemoenzymatic route to acidic lipopeptide antibiotics. J. Am. Chem. Soc. *126*, 17025–17031.
19. Hojati, Z., Milne, C., Harvey, B., Gordon, L., Borg, M., Flett, F., Wilkinson, B., Sidebottom, P.J., Rudd, B.A., Hayes, M.A., et al. (2002). Structure, biosynthetic origin, and engineered biosynthesis of calcium-dependent antibiotics from *Streptomyces coelicolor*. Chem. Biol. *9*, 1175–1187.

20. Sieber, S.A., Tao, J., Walsh, C.T., and Marahiel, M.A. (2004). Peptidyl thiophenols as substrates for nonribosomal peptide cyclases. Angew. Chem. Int. Ed. Engl. *43*, 493–498.
21. Trauger, J.W., Kohli, R.M., and Walsh, C.T. (2001). Cyclization of backbone-substituted peptides catalyzed by the thioesterase domain from the tyrocidine nonribosomal peptide synthetase. Biochemistry *40*, 7092–7098.
22. Yin, J., Liu, F., Li, X., and Walsh, C.T. (2004). Labeling proteins with small molecules by site-specific posttranslational modification. J. Am. Chem. Soc. *126*, 7754–7755.
23. La Clair, J.J., Foley, T.L., Schegg, T.R., Regan, C.M., and Burkart, M.D. (2004). Manipulation of carrier proteins in antibiotic biosynthesis. Chem. Biol. *11*, 195–201.
24. Lambalot, R.H., Gehring, A.M., Flugel, R.S., Zuber, P., LaCelle, M., Marahiel, M.A., Reid, R., Khosla, C., and Walsh, C.T. (1996). A new enzyme superfamily—the phosphopantetheinyl transferases. Chem. Biol. *3*, 923–936.
25. Bentley, S.D., Chater, K.F., Cerdeno-Tarraga, A.M., Challis, G.L., Thomson, N.R., James, K.D., Harris, D.E., Quail, M.A., Kieser, H., Harper, D., et al. (2002). Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature *417*, 141–147.
26. Tseng, C.C., Bruner, S.D., Kohli, R.M., Marahiel, M.A., Walsh, C.T., and Sieber, S.A. (2002). Characterization of the surfactin synthetase C-terminal thioesterase domain as a cyclic depsipeptide synthase. Biochemistry *41*, 13350–13359.
27. Clugston, S.L., Sieber, S.A., Marahiel, M.A., and Walsh, C.T. (2003). Chirality of peptide bond-forming condensation domains in nonribosomal peptide synthetases: the C5 domain of tyrocidine synthetase is a (D)C(L) catalyst. Biochemistry *42*, 12095–12104.