Chemoenzymatic Design of Acidic Lipopeptide Hybrids: New Insights into the Structure−Activity Relationship of Daptomycin and A54145†

Florian Kopp, Jan Grünewald,‡ Christoph Mahlert, and Mohamed A. Marahiel*

Philipps-Universität Marburg, Fachbereich Chemie/Biochemie, Hans-Meerwein-Strasse, D-35032 Marburg, Germany

Received May 12, 2006; Revised Manuscript Received July 4, 2006

ABSTRACT: The acidic lipopeptides, including the clinically approved antibiotic daptomycin, constitute a class of structurally related branched cyclic peptidolactones and peptidolactams synthesized by nonribosomal peptide synthetases (NRPSs). In this study, the excised peptide cyclases from A54145 and daptomycin NRPSs were shown to be able to catalyze the macrocyclization of peptide thioester substrates, which were chemically produced by solid phase peptide synthesis. Applying this chemoenzymatic strategy, we generated derivatives of A54145 and daptomycin as well as hybrid molecules of both compounds. Bioactivity determination of the derived cyclic molecules revealed new insights into the structure−activity relationship of the acidic lipopeptide family. The general importance of several amino acid positions, including two conserved aspartic acid residues, was confirmed to be substantial for antibiotic potency. As a robust macrocyclization catalyst, the peptide cyclase excised from A54145 synthetase is the first cyclase of a branched cyclic lipopeptide, which catalyzes both macrolactonization and macrolactamization. The results presented herein illustrate the advantages of combining organic synthesis with natural product biosynthetic enzymes to explore the interplay between structural features and biological activity.

The acidic lipopeptide family constitutes an emerging class of antibiotics, including daptomycin, A54145, the calcium-dependent antibiotic (CDA),[1] friulimicin, amphomycin, and laspartomycin (Figure 1) (*1*). All of these cyclic lipopeptides originate from soil-inhabiting streptomycetes, which produce more than two-thirds of naturally derived antibacterial agents (*2*). Significantly, in 2003, daptomycin was approved in the United States under the trade name Cubicin as the first member of this new structural class of compounds. Cubicin is clinically used for the treatment of severe skin and skin structure infections caused by Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) (*3*).

As for many natural products that are pharmacologically relevant, the biosynthesis of the acidic lipopeptides is accomplished by nonribosomal peptide synthetases (NRPSs). In principle, these modularly organized megaenzymes comprise all catalytic units necessary for the assembly of the linear peptide chain and the release of the mature product. As a key determinant, nonribosomal peptides often contain unique structural features such as nonproteinogenic amino acids, D-amino acids, and C- and N-methylated residues (*4*). This extremely high degree of structural diversity is impressively exemplified by daptomycin which includes the unusual amino acids kynurenine, ornithine, and the C-methylated 3-methylglutamate. Additionally, several D-configured amino acids such as D-alanine, D-serine, and D-asparagine are found in the daptomycin peptide backbone. A second acidic lipopeptide antibiotic, A54145, contains the N-methylated sarcosine and occurs as a complex of various individual compounds that differ in their peptide sequence, namely, glutamate/3-methylglutamate (position 12) and valine/isoleucine (position 13) (*5*).

However, considering the acidic lipopeptides as Ca^{2+}-dependent antibiotics, these compounds share intriguing structural similarities, suggesting a general importance of some conserved motifs for antibacterial activity. All acidic lipopeptide antibiotics are branched cyclic decapeptide lactones or lactams, and similar amino acids can be found at common positions of their macrocyclic scaffold. Most notably, two aspartic acid residues are strictly conserved at ring positions 7 and 9, followed by glycine at position 10, using the daptomycin numbering convention. Moreover, D-configured and achiral amino acids commonly occupy positions 5 and 8 of the acidic peptide core, while position 11 is strictly reserved for D-configured amino acids (Figure 1).

Recently, the biosynthetic gene clusters of daptomycin and A54145, encoding the NRPS complexes of these valuable secondary metabolites, have been sequenced (*6*, *7*). The final step in nonribosomal peptide synthesis is the cleavage of the covalently attached linear peptide product from the

† This work has been supported by the Deutsche Forschungsgemeinschaft (M.A.M.) and the Fonds der Chemischen Industrie (F.K. and M.A.M.).

* To whom correspondence should be addressed. Phone: +49-6421-2825722. Fax: +49-6421-2822191. E-mail: marahiel@chemie.uni-marburg.de.

‡ Present address: Schultz Laboratory, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037.

[1] Abbreviations: CDA, calcium-dependent antibiotic; DAB, diaminobutyrate; DAP, diaminopropionate; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethaniminium hexafluorophosphate *N*-oxide; Hepes, 2-*N*′-[*N*-(2-hydroxyethyl)piperazinyl]ethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; nd, not detected; NRPS, nonribosomal peptide synthetase; NTA, nitrilotriacetic acid; OD, optical density; Orn, ornithine; PCP, peptidyl carrier protein; PCR, polymerase chain reaction; RP-LC−MS, reverse phase liquid chromatography and mass spectroscopy; SAR, structure−activity relationship; TE, thioesterase; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; t_R, retention time.

10.1021/bi0609422 CCC: $33.50
Published on Web 08/09/2006

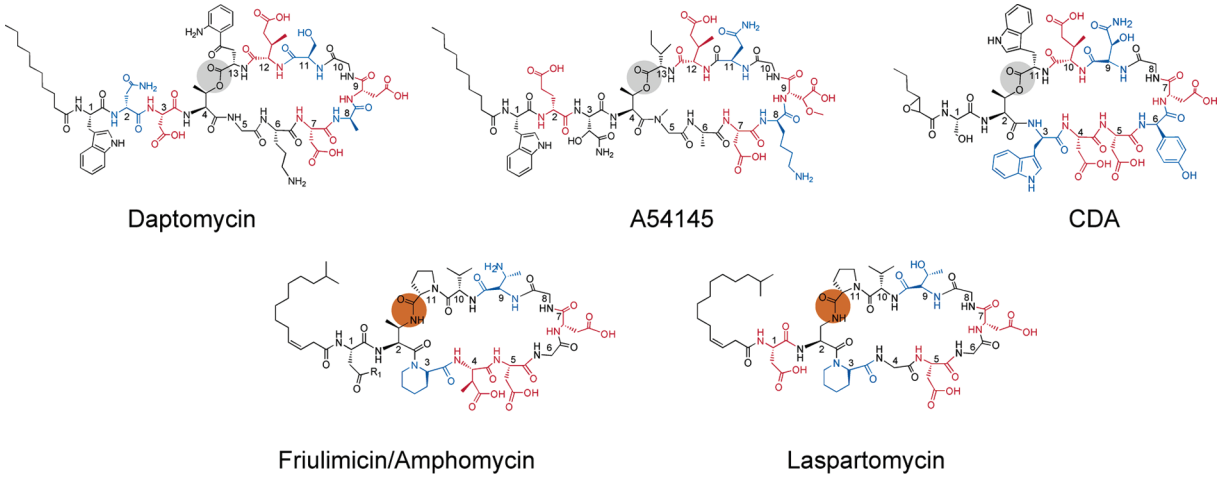

FIGURE 1: Structures of the acidic lipopeptide family. The peptide backbone of these nonribosomally synthesized peptides contains several aspartate and glutamate residues colored red, leading to the overall acidic nature of these compounds. Additionally, at least two D-configured amino acids, colored blue, can be found for each member. The conformation of the shown undeca- and tridecapeptides is constrained by macrocyclization. Lactones are highlighted in gray and lactams in orange. $R_1 = NH_2$ for friulimicin, and $R_1 = OH$ for amphomycin.

dedicated NRPSs by a so-called thioesterase (TE) domain (*8*). TE domains, also known as peptide cyclases, act as macrocyclization catalysts to release cyclic or branched cyclic peptides such as daptomycin and A54145. Through macrocyclization, the biologically active conformation of these molecules is constrained and the resulting structural rigidity ensures a precise orientation required for the specific interaction with their cellular target.

TE domains excised from different NRPSs and expressed as isolated enzymes have been capable of serving as versatile enzymatic tools in terms of cyclizing chemically synthesized peptide thioester analogues of their natural substrates. Pioneering work from Trauger et al. was followed by more recent publications that utilized the chemoenzymatic approach to generate various derivatives of streptogramin B antibiotics (*9*, *10*). Specifically, CDA and daptomycin derivatives have been prepared through enzyme-mediated peptide cyclization employing CDA TE, and the significance of single amino acids for daptomycin bioactivity was tested (*11*). During this first study of acidic lipopeptide structure−activity relationship (SAR), the importance of the conserved endocyclic aspartate residues and the β-methyl group of glutamate 12 for daptomycin bioactivity became evident. Unfortunately, chemoenzymatic peptide cyclization can be limited by low yields due to competing hydrolysis or the fact that specific recognition elements intrinsic to cognate substrates can be needed for efficient substrate cyclization (*12*, *13*). To overcome this drawback, suitable NRPS cyclases are needed for a given synthetic problem to minimize this undesired side reaction. Thereby, macrocyclic peptides become available with excellent yields.

Herein, we report the biochemical characterization of two new peptide cyclases from daptomycin and A54145 NRPSs. On the basis of new sequence information from genetic studies, it was possible to clone and express these TE domains together with their associated peptidyl carrier proteins (PCPs): daptomycin and A54145 PCP TE. By utilizing these enzymes, derivatives of daptomycin and A54145 as well as hybrid molecules of the two compounds became available for exploitation and allowed a better understanding of the acidic lipopeptide SAR. Further, these two enzymes hold great potential for the development of novel molecules related to daptomycin with an improved spectrum of pharmaceutical properties.

EXPERIMENTAL PROCEDURES

Cloning and Expression of A54145 PCP TE, CDA PCP TE, and Daptomycin PCP TE Didomains. The *a54145 pcp-te* gene fragment [containing Lpt residues 93072−94047 (*7*)] was amplified by PCR from chromosomal DNA of *Streptomyces fradiae*. Amplification was carried out with Pfu Turbo DNA polymerase (Stratagene) using the oligonucleotides 5′-AAA AAG GAT CCG GCC GTC CGC CGC GCG AC-3′ and 5′-AAA AAA GCG GCC GCT CAT ACC TCT CGT TTC TTC GTG GTG GCT CC-3′. After digestion with *Kpn*I and *Hind*III, the DNA fragment was ligated into a *Kpn*I- and *Hind*III-digested, derivatized pQE60 vector (Qiagen), which appends an N-terminal heptahistidine tag to the expressed protein. The *cda pcp-te* gene fragment was cloned, expressed, and purified as previously described (*14*). The daptomycin *pcp-te* gene fragment [containing Dpt residues 1384−2665 (*6*)] was amplified with the oligonucleotides 5′-AAA AAA GGT ACC GGC GCG CAC CCC AGT CGC-3′ and 5′-AAA AAA AAG CTT TCA GGT GCC GGC GCC CAG CC-3′ using chromosomal DNA of *Streptomyces roseosporus* as a template, digested with *Bam*HI and *Not*I, and ligated into the above-mentioned vector, previously digested with *Bam*HI and *Not*I. DNA sequencing of the derived plasmid 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 an OD of 0.5 (600 nm), induced with 1 mM IPTG, and again grown at 30 °C for 2.5 h. The recombinant proteins were purified by 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 proteins was determined spectrophotometrically using the estimated extinction coefficient at 280 nm. After being flash-frozen in liquid nitrogen, the proteins were stored at −80 °C.

Synthesis of Peptidyl Thiophenol Substrates. All linear peptides were produced by solid phase peptide synthesis

(SPPS) on an Advanced Chem Tech APEX 396 synthesizer (0.1 mmol scale) as described previously (*13*). Protected amino acids were purchased from Novabiochem and Bachem Biosciences. The synthesis of *N*-(9-fluorenylmethoxycarbonyl)-L-kynurenine was described previously (*11*). All other compounds were purchased from Sigma-Aldrich, except HBTU and HOBt·H_2O (IRIS biotech). The preparation and purification of peptidyl thiophenol substrates were described previously (*15*). The identities of peptidyl thiophenol substrates were determined by reverse phase liquid chromatography and mass spectroscopy (RP-LC−MS) and MALDI-TOF (Supporting Information).

Assays. Enzymatic reactions were carried out in a total volume of 50 μL, containing 25 mM Hepes, 50 mM NaCl, and 5% DMSO (v/v) at pH 7.0. The mixture containing 250 μM thiophenol substrate and 5 μM enzyme was incubated at 25 °C for 2.5 h.

For kinetic studies, the substrate concentration was varied from 50 μM to 1 mM, and reactions were quenched via addition of 35 μL of a 4% TFA/H_2O solution. All assays were analyzed by RP-LC−MS on a C_{18} Nucleodur column (Macherey and Nagel, 250/3, pore diameter of 100 Å, particle size of 3 μm) with the following gradient: 15−60% acetonitrile, 0.1% TFA in water, 0.1% TFA from 0 to 40 min at 0.3 mL/min and 45 °C.

Identities of the products were verified by ESI-MS (Supporting Information). Connection regiospecificity of cyclic products was determined by MS/MS analysis on an API Qstar Pulsar I device (Applied Biosystems) (Supporting Information).

Concentrations of various peptidyl thioesters were calculated using experimentally determined extinction coefficients at a wavelength of 220 nm. The extinction coefficient of peptide thiophenol substrates was assumed to be identical to that of cyclized and hydrolyzed products. Kinetic characterization of the cyclization and hydrolysis reactions was performed by determining initial rates at 5−10 substrate concentrations using two time points at each concentration within the linear region of the enzyme verified by time courses.

Preparation of Cyclic Acidic Lipopeptides for Bioactivity Assays. For the semipreparative scale preparation of cyclic acidic lipopeptides, the reactions were carried out in a total volume of 3−6 mL with 5 μM purified TE, 250 μM peptide substrate activated as thiophenol, 25 mM Hepes, 50 mM NaCl, and 5% DMSO (v/v) at pH 7.0 and 25 °C. The reaction was monitored by analytical HPLC. After 3−5 h, the reaction mixture was directly run over a 250/21 Nucleodur 100-5 C18 reverse phase column (Macherey and Nagel) by applying a gradient from 35 to 55% acetonitrile, 0.1% TFA in water, 0.1% TFA over 30 min at a flow rate of 20 mL/min. The purity of the obtained products was analyzed by analytical HPLC (≥90% pure).

Determination of Bioactivity. The biological activity of the cyclized derivatives was assayed against *Bacillus subtilis* PY79. For the determination of MIC values, 2-fold serial dilutions of the cyclic peptides and authentic daptomycin were prepared in microtiter plates as previously described (*16*), using LB medium containing 74 mg of Ca^{2+}/L. Therefore, 80 μL of an overnight culture diluted 1/10000 was added to each well and incubated at 37 °C for 18 h prior to visual determination of MICs.

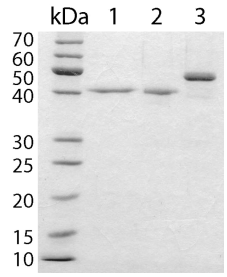

FIGURE 2: Purified recombinant cyclases used in this study: lane 1, A54145 PCP TE (41.9 kDa); lane 2, daptomycin PCP TE (41.2 kDa); and lane 3, CDA PCP TE (44.5 kDa). Enzymes were overexpressed in *E. coli* BL21 and purified by Ni−NTA affinity chromatography. Protein samples were resolved by SDS−PAGE (10%) and visualized with Coomassie stain.

Table 1: Kinetic Parameters of the NRPS Cyclases of the Acidic Lipopeptides

enzyme	substrate	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} mM^{-1})
A54145 PCP TE	**A54145(Ile)**	80[a]	0.22[a]	2.80[a]
daptomycin PCP TE	**Dap**	50[a]	0.18[a]	3.59[a]
CDA TE	**CDA**	40[b]	0.10[b]	2.44[b]

[a] Standard Deviation of ±15%. [b] From ref *14*.

RESULTS

A54145 PCP TE Catalyzes Cyclization of Various Synthetic A54145 Analogues. Recombinant A54145 PCP TE was solubly expressed in *E. coli* at 30 °C containing a N-terminal heptahistidine tag and purified by Ni−NTA affinity chromatography. The recombinant protein was obtained with a yield of 7 mg/L (Figure 2). To examine the ability of A54145 PCP TE to catalyze macrolactonization, we synthesized two peptide thioester analogues of the natural A54145 substrate, **A54145(Val)** and **A54145(Ile)**, with regard to the fact that A54145 was isolated as a mixture of two major compounds containing either valine or isoleucine at position 13 of the peptide sequence (*5*). For synthetic reasons, the chemically synthesized substrates had the following modifications compared to authentic A54145: L-3-*O*-methylaspartic acid at position 9 replaced with L-aspartic acid and L-3-hydroxyasparagine at position 3 substituted with the proteinogenic amino acid L-asparagine.

The reaction of A54145 PCP TE with **A54145(Val)** led to the formation of the expected cyclic product (t_R = 33.0 min) which was identified by ESI-MS (Figure 3A). The flux toward hydrolysis (t_R = 31.9 min) was remarkably low, revealing a cyclization-to-hydrolysis ratio of 12/1. After incubation of **A54145(Ile)** with A54145 cyclase, the cyclization product was detected (t_R = 33.9 min) and the occurrence of the undesired hydrolysis product (t_R = 32.9 min) was as low as that for **A54145(Val)** with a cyclization-to-hydrolysis ratio of 12/1 (Figure 3B). MS/MS sequencing of the derived cyclic species confirmed that cyclization occurs through threonine at position 4, leading to the 10-amino acid macrolactone (Supporting Information). The kinetic data obtained for A54145 PCP TE with **A54145(Ile)** revealed a K_M value of 80 μM and a k_{cat} of 0.22 min^{-1} (Table 1).

Next, we addressed the question of whether A54145 PCP TE is able to catalyze the formation of macrolactones with different ring sizes. Therefore, three peptide thioester sub-

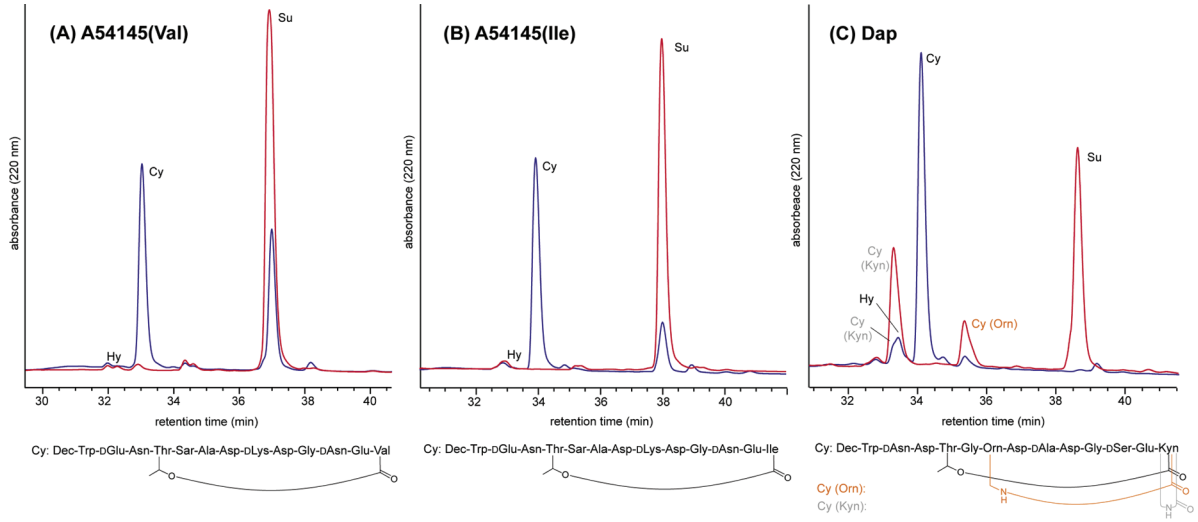

FIGURE 3: Cyclization of peptide thioester analogues of the acidic lipopeptides by A54145 and daptomycin PCP TE followed by HPLC and MS. Red traces are the negative controls with the heat-denatured enzyme. Blue traces are the result of the assay with the native enzyme. (A) HPLC trace of the **A54145(Val)** macrocyclization mediated by A54145 PCP TE. (B) HPLC trace of the macrocyclization assay of **A54145(Ile)** with A54145 PCP TE. (C) HPLC trace of the **Dap** macrocyclization reaction mediated by daptomycin PCP TE. Su is the chemically synthesized thioester substrate analogue and Cy the cyclized product. Hy indicates hydrolysis; Cy(Kyn) and Cy(Orn) are side products due to the reaction of the activated C-terminus with the side chain nucleophile of L-ornithine and L-Kyn, respectively.

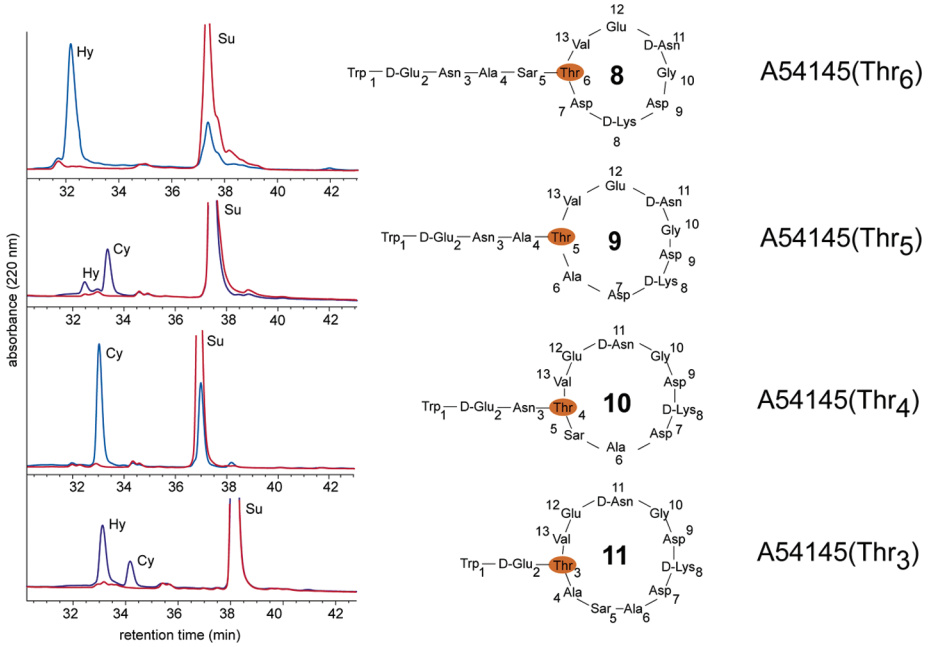

FIGURE 4: Formation of 9−11-amino acid cyclic macrolactones mediated by A54145 cyclase. At the left is the HPLC analysis of the cyclization reaction including 250 μM peptide thioester substrate, 5 μM A54145 PCP TE, 50 mM NaCl, and 25 mM Hepes (pH 7.0) incubated at 25 °C for 2.5 h. The products were characterized by MS/MS fragmentation (Supporting Information). At the right are peptide sequences of the assayed thioester substrates; the ring size and the cycle-forming threonine are highlighted. Su is the chemically synthesized thioester substrate analogue and Cy the cyclized product. Hy indicates hydrolysis.

strates, **A54145(Thr$_3$)**, **A54145(Thr$_5$)**, and **A54145(Thr$_6$)**, were synthesized. The cycle-forming L-threonine, originally found at position 4 of the A54145 peptide backbone, was placed at amino acid position 3, 5, or 6, while alanine occupied position 4 of those peptide thioester substrates. Consequently, after cyclization, a macrolactone consisting of 11, 9, or 8 amino acids should be formed.

As shown in Figure 4, **A54145(Thr$_3$)** was cyclized (t_R = 34.2 min), but hydrolysis (t_R = 33.1 min) was favored (cyclization-to-hydrolysis ratio of 1/3). Branch point movement of one amino acid position was also tolerated in the case of **A54145(Thr$_5$)** in forming the nonapeptide lactone (t_R = 33.4 min) after incubation with A54145 PCP TE. Interestingly, the occurrence of hydrolysis (t_R = 32.5 min) was decreased compared to that of **A54145(Thr$_3$)**, revealing the cyclization-to-hydrolysis ratio of 4/1. In contrast to the reaction with **A54145(Thr$_3$)** and **A54145(Thr$_5$)**, attempts to cyclize **A54145(Thr$_6$)** with A54145 PCP TE resulted exclusively in hydrolysis of the peptide thioester substrate (t_R = 32.3 min) and did not lead to the eight-membered lactone. MS/MS sequencing confirmed the identity of the derived species as shown in the Supporting Information.

Daptomycin PCP-TE Catalyzes Cyclization of Various Synthetic Daptomycin Analogues. Recombinant daptomycin PCP TE was expressed in a manner analogous to that of A54145 PCP TE, yielding 8 mg/L of culture medium after Ni−NTA purification (Figure 2). To test the ability of daptomycin PCP TE to catalyze the cyclization of daptomycin thioester substrates, we chemically synthesized peptide thioester **Dap** based on the daptomycin peptide sequence. For synthetic reasons, the artificial substrate contained the proteinogenic amino acid L-glutamate at position 12 instead of L-3-methylglutamate, which can be found in the peptide backbone of authentic daptomycin. The reaction of daptomycin PCP TE with **Dap** generated four products which were analyzed by ESI-MS (Figure 3). The expected cyclic product (t_R = 34.1 min) was formed along with the hydrolysis product (t_R = 33.3 min) (cyclization-to-hydrolysis ratio of 9/1). Additionally, two minor peaks (t_R = 35.4 min) and (t_R = 33.4 min) were observed due to nonenzymatic cyclization via an alternative side chain nucleophile, namely, the amino group of L-Orn_6 or L-Kyn_{13}. These spontaneously formed products, the octapeptide lactam (t_R = 35.4 min) via L-Orn_6, and the seven-ring lactam (t_R = 33.4 min), generated through the nucleophilic attack of the amino group from the kynurenine side chain on the C-terminal carboxyl function, became more apparent in the negative control with the heat-denatured enzyme (Figure 3). The identity of these species was previously confirmed by MS/MS fragmentation during studies with CDA TE (*11*). The cyclization reaction of **Dap** catalyzed by daptomycin PCP TE revealed a K_M value of 50 μM and a k_{cat} of 0.18 (Table 1).

As for A54145 PCP TE, we tested the ability of daptomycin PCP TE to cyclize peptide thioester substrates **Dap(Thr$_3$)**, **Dap(Thr$_5$)**, and **Dap(Thr$_6$)** with the cycle-forming L-threonine at positions 3, 5, and 6, respectively. Assaying for cyclization revealed that **Dap(Thr$_3$)** and **Dap(Thr$_5$)** substrates were cyclized, indicating that branch point movement of one amino acid was tolerated by Dap PCP TE (data not shown). Incubation of **Dap(Thr$_6$)** with Dap PCP TE led only to the hydrolysis product. In contrast to the results with A54145 PCP TE, the formation of the macrolactones derived from **Dap(Thr$_3$)** and **Dap(Thr$_5$)** was accompanied by a stronger occurrence of hydrolysis.

A54145 Catalyzes both Macrolactonization and Macrolactamization. All members of the acidic lipopeptide family have macrocyclic structures to constrain their biological active form. Interestingly, this structural rigidity is not always achieved via the formation of a macrolactone as for daptomycin and A54145 since friulimicin, amphomycin, and laspartomycin contain a 10-membered macrolactam (Figure 1). To explore the ability of the recombinant cyclases of A54145, CDA, and daptomycin to catalyze the formation of macrolactams, the three peptide thioester substrate analogues, **Dap(DAP)**, **A54145(DAP)**, and **CDA(DAP)**, were synthesized carrying the cyclization nucleophile diaminopropionate (DAP) at position 4 of their peptide sequence. Remarkably, **A54145(DAP)** was cyclized highly efficiently by A54145 PCP TE (t_R = 32.3 min) as shown in Figure 5. The cyclization reaction revealed a very low flux toward hydrolysis (t_R = 30.9 min) (cyclization-to-hydrolysis ratio of 12/1) in analogy to the macrolactonization of peptide thioester substrate analogues **A54145(Val)** and **A54145(Ile)**. To confirm the predicted amide linkage between DAP and the C-terminal amino acid of A54145, MS/MS sequencing clearly identified that the observed macrolactam is formed via DAP_4 and Kyn_{13} (Supporting Information). In contrast to this result, no cyclization of **Dap(DAP)** or **CDA(DAP)** was observed after incubation with the dedicated cyclization catalyst. Additional attempts to cyclize **Dap(DAP)** and **CDA(DAP)** with A54145 PCP TE also failed.

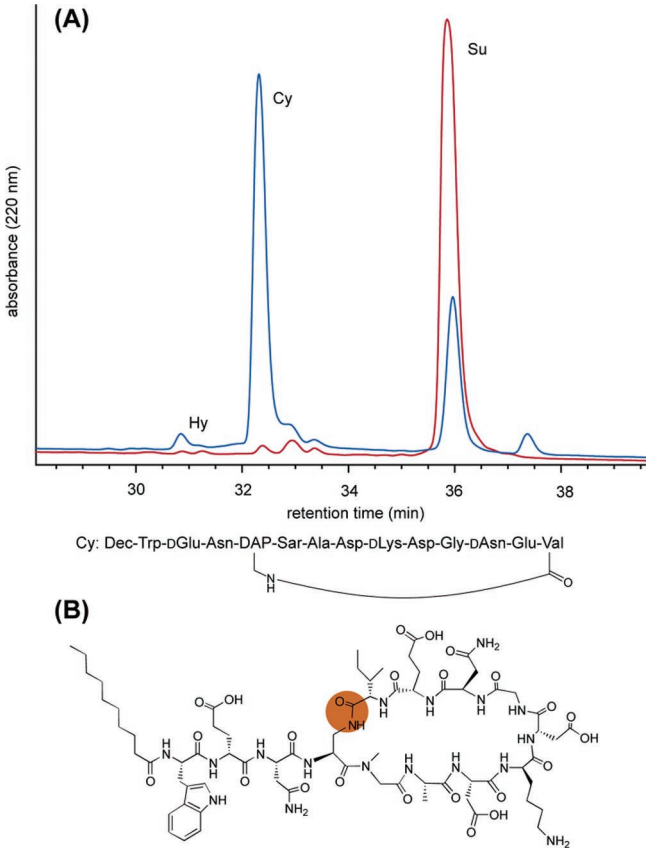

FIGURE 5: Cyclization of **A54145(DAP)** leads to the formation of a macrolactam analogue of A54145. (A) HPLC chromatogram of the A54145 PCP TE-mediated reaction. The blue trace shows product formation after incubation with A54145 PCP TE [250 μM **A54145(DAP)**, 5 μM A54145 PCP TE, 50 mM NaCl, and 25 mM Hepes at pH 7.0 and 25 °C for 2 h]; the red trace shows the negative control with the heat-denatured enzyme. (B) Chemical structure of the generated macrolactam. The amide linkage is highlighted in orange. DAP is diaminopropionate, Su the chemically synthesized thioester substrate analogue, and Cy the cyclized product. Hy indicates hydrolysis.

Table 2: Substrate Specificity of the Acidic Lipopeptide NRPS Cyclases with a Cyclization-to-Hydrolysis Ratio

	daptomycin PCP TE	A54145 PCP TE	CDA PCP TE
Dap	9/1	10/1	2/1
A54145(Val)	5/1	12/1	no turnover
CDA	hydrolysis	hydrolysis	6/1

Enzymatic Cross Reactions of A54145, Daptomycin, and CDA Cyclase. To gain further information about the substrate specificity of A54145 PCP TE, Dap PCP TE, and CDA PCP TE, we examined the ability of these enzymes to cyclize the peptide thioester substrate analogues of daptomycin (**Dap**), A54145 [**A54145(Val)**] and CDA (**CDA**). Table 2 summarizes the results, including cyclization-to-hydrolysis ratios. As shown before, each recombinant cyclase was able to catalyze its associated substrate analogue. Interestingly,

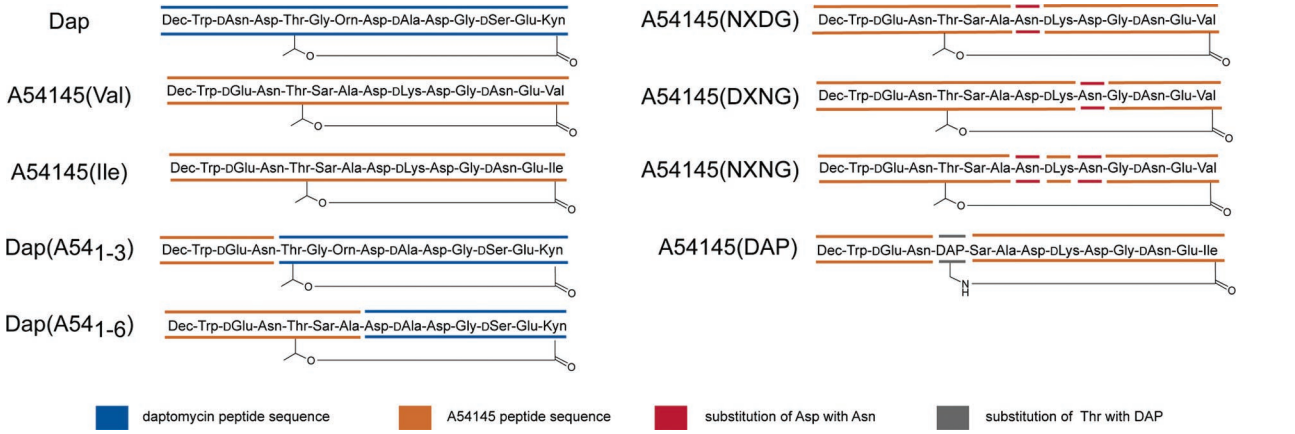

FIGURE 6: Derivatives of A54145 and daptomycin tested for bioactivity. The macrocyclic compounds were derived from peptide thioester substrates after incubation with A54145 and daptomycin cyclase. Amino acids from the daptomycin peptide sequence are colored blue and those from the A54145 peptide sequence orange. Substitution of the acidic amino acids is highlighted in red. The macrolactam analogue of A54145 contains DAP as the cycle-forming amino acid side chain instead of threonine, as marked in gray.

A54145 PCP TE cyclized **Dap** and Dap PCP TE was able to cyclize **A54145(Val)**. On the other hand, only hydrolysis was observed for the substrate analogue **CDA** after incubation with A54145 and daptomycin cyclase. As previously shown, CDA PCP TE was able to cyclize **Dap**, but **A54145-(Val)** was not converted after incubation with CDA cyclase.

Remarkably, the cyclization-to-hydrolysis ratio for **Dap** with A54145 PCP TE (cyclization-to-hydrolysis ratio of 10/1) was as high as that for the naturally associated pair of cyclase and substrate analogue (cyclization-to-hydrolysis ratio of 9/1). Further, the A54145 analogue **A54145(Val)** was cyclized with less than ~10% hydrolysis (cyclization-to-hydrolysis ratio of 12/1) by A54145 PCP TE, revealing the excellent cyclization activity of this recombinant TE domain.

Generation of Acidic Lipopeptide Hybrids and Determination of Bioactivity. To elucidate structural features that are important for antibacterial activity of the acidic lipopeptides, we synthesized nine peptide thioester substrates: **Dap**, **A54145(Val)**, **A54145(Ile)**, **Dap(A54$_{1-3}$)**, **Dap(A54$_{1-6}$)**, **A54145(DAP)**, **A54145(NXDG)**, **A54145(DXNG)**, and **A54145(NXNG)** (Figure 6). In accordance with the previously discussed results, A54145 and daptomycin PCP TE were found to be the most sufficient cyclization catalyst for these substrates. After cyclization on a semipreparative scale, the macrocyclic products were purified with reversed phase HPLC and the antibacterial activity was determined against *B. subtilis* PY79.

At first, we examined the bioactivity of the daptomycin, and A54145 approximates **Dap**, **A54145(Ile)**, and **A54145-(Val)**. In comparison to authentic daptomycin with a MIC of 2 μg/mL, the cyclic peptide **Dap** (at position 12, glutamate is incorporated instead of β-methylglutamate) had a MIC value of 11 μg/mL (Table 3). According to previously published chemoenzymatic modifications of daptomycin, the 6-fold higher MIC of **Dap** with respect to authentic daptomycin reveals the importance of 3-methylglutamate for antibacterial activity (*11*). Since naturally occurring A54145 variants differ at position 13, we generated the peptidolactone **A54145(Val)** and **A54145(Ile)**. Interestingly, **A54145(Ile)** had a MIC value of 25 μg/mL, while **A54145(Val)** was less effective against *B. subtilis*, revealing a MIC value of 200 μg/mL. With regard to the fact that both compounds differ only in one amino acid, this result impressively underlines the great importance of single amino acid substitutions for bactericidal activity.

Table 3: MIC Values for Acidic Lipopeptide Derivatives against *B. subtilis* PY79

compound	MIC$_{90}$ (μg/mL) at 73.6 mg of Ca^{2+}/L
authentic daptomycin	2
Dap	11
A54145(Val)	200
A54145(Ile)	25
Dap(A54$_{1-3}$)	20
Dap(A54$_{1-6}$)	20
A54145(DAP)	25
A54145(NXDG)	>900
A54145(DXNG)	>900
A5145(NXNG)	>900

Additionally, two hybrid molecules of A54145 and daptomycin were synthesized (Figure 5). To explore the relevance of the exocyclic amino acids, we designed a peptide thioester substrate based on the natural daptomycin peptide sequence carrying the three N-terminal amino acids of A54145**, Dap(A54$_{1-3}$)**. Surprisingly, the exchange of the amino acid tail did not crucially affect bioactivity, indicating that these residues are not substantial for antimicrobial behavior. To better approximate A54145, the bioactivity of cyclized **Dap(A54$_{1-6}$)**, a daptomycin/A54145 hybrid containing the amino acids 1−6 of A54145 and 7−13 of daptomycin peptide sequence, was further examined. Interestingly, the MIC for **Dap(A54$_{1-6}$)** did not change compared to **Dap(A54$_{1-3}$)**.

In contrast to daptomycin, A54145, and CDA, friulimicin, amphomycin, and laspartomycin are comprised of a decapeptide lactam, which is formed via an amide bond linkage between DAP/DAB and the C-terminal amino acid proline. Since macrolactams are more stable against hydrolysis than macrolactones, we addressed the antibacterial activity of **A54145(DAP)**, which has been cyclized by A54145 PCP TE. The derived MIC value of 25 μg/mL was in good agreement with the MIC value of **A54145(Ile)**.

Finally, to prove the significance of acidic amino acid residues for the antibacterial behavior of A54145, three peptidolactones, **A54145(NXDG)**, **A54145(DXNG)**, and **A54145(NXNG)**, was tested. Single deletion of aspartic acid

as well as the double deletion of both aspartate residues led to the total loss of antibacterial activity as shown in Table 3.

DISCUSSION

Nonribosomal peptides constitute a highly diverse array of molecules with therapeutical relevance and play an important role in modern medicine. Among these compounds, the clinically approved antibiotic daptomycin represents the new class of acidic lipopeptides. Due to the fact that the acidic lipopeptides are complex molecules to approach synthetically, the chemoenzymatic strategy described herein was shown to be a promising tool for creating hybrid derivatives of these strongly related natural products.

The chemoenzymatic strategy combines automated SPPS with enzymatic peptide cyclization mediated through excised TE domains from NRPSs. On the basis of sequence information derived from acidic lipopeptide biosynthetic gene clusters (*6*, *7*), we were able to express and purify daptomycin and A54145 cyclase together with their associated PCP domains. Notably, both isolated enzymes were active as macrocyclization catalysts when tested with peptide thioester analogues of their natural substrates, **A54145(Val)**, **A54145(Ile)**, and **Dap**. In general, enzymatic peptide cyclization is often limited by low yields due to the occurrence of hydrolysis of the enzyme-bound oxoester intermediate. Previous work on excised TE domains from the tyrocidine (*12*) and pristinamycin (*10*) synthetases revealed cyclization-to-hydrolysis ratios of 1/1 and 3/1, respectively, for their natural substrate analogues, which is typical for isolated TE domains. To enhance macrocyclization activity, several strategies have been developed reaching from the addition of nonionic detergents to the use of organic solvents (*12*, *17*). However, A54145 and daptomycin cyclase revealed extraordinary cyclization-to-hydrolysis ratios of 12/1 and 9/1 toward peptide thioester analogues of their natural substrates, allowing the synthesis of the desired macrolactones with less than 10% hydrolysis. Therefore, these two enzymes are the favorable cyclization catalysts for the rapid generation of daptomycin derivatives. To explore the utility of these versatile macrocyclization catalysts for peptide cyclization in general, peptide thioester substrate analogues of different ring sizes were tested with A54145 and daptomycin PCP TE. In conclusion, it became obvious that cycles containing 9 and 11 amino acids can be generated with A54145 and daptomycin cyclase, although for the 11-amino acid lactone hydrolysis was predominant over cyclization.

From the structural point of view, the acidic lipopeptides show several striking similarities. Daptomycin, A54145, and CDA share five common amino acids in identical ring positions, and their nonribosomal assembly is achieved by a highly similar biosynthetic pathway (*6*, *7*, *18*). The final step in biosynthesis, the release of the mature peptide product, is achieved by the TE domains of the associated NRPS systems. In comparison to the overall level of TE identity of ~15%, the level of sequence identity among the acidic lipopeptide cyclases is in the range of 40% (*19*). Because of this fact, the sequence identity among the acidic enzymatic cross reactions of A54145, daptomycin, and CDA cyclase were expected. When using the cyclases of A54145 and daptomycin to cyclize the substrate analogue **CDA**, no product formation was observed, only hydrolysis. Since no crystal structure is available for A54145 and daptomycin TE, we can only speculate that undecapeptide **CDA** does not fill the substrate pocket of A54145 and the daptomycin cyclase active site, which are optimized for the larger tridecapeptides. Therefore, the admission of water molecules and the hydrolytical cleavage of the acyl−enzyme intermediate are favored. Nevertheless, A54145 cyclase was able to cyclize the peptide thioester analogue of **Dap** and vice versa. Although in both cases the cyclization activity was found to be relaxed for internal amino acid substitutions of the peptide thioester substrates, C- and N-terminal recognition elements were found to be essential for efficient peptide cyclization. CDA and daptomycin contain aromatic amino acids at the C-terminus, and A54145 contains aliphatic amino acids isoleucine and valine. Therefore, CDA cyclase was permissive toward daptomycin substrate analogues but did not catalyze the cyclization of peptide thioester **A54145(Val)**.

Encouraged by the versatile catalytic properties of the acidic lipopeptide TE domains, we set out to employ A54145 and daptomycin cyclase for the synthesis of hybrid molecules of daptomycin and A54145. Subsequent bioactivity studies allowed further conclusions about their SAR. A major structural feature of the acidic lipopeptide family is that cyclization can be achieved by the formation of either peptide lactones or peptide lactams. For friulimicin, amphomycin, or laspartomycin, the macrocyclic ring is formed via an amide linkage between the C-terminal proline and diaminopropionic or -butyric acid. However, daptomycin and CDA cyclase did not exhibit any cyclization activity toward **Dap(DAP)** and **CDA(DAP)**, whereas **A54145(DAP)** was converted to the macrolactam after incubation with A54145 cyclase. Hence, A54145 PCP TE is the first excised cyclase of a branched lipopeptide NRPS that catalyzes both macrolactonization and macrolactamization. Tested for bioactivity, macrolactam **A54145(DAP)** had a MIC value of 25 μg/mL similar to that of **A54145(Ile)**, meaning that the substitution of an ester bond through an amide bond does not affect antimicrobial behavior primarily. Obviously, additional extensive studies would be necessary to draw a conclusion in terms of the improved in vivo stability of the macrolactam variant of A54145.

The bioactivity of parental compounds **Dap**, **A54145(Val)**, and **A54145(Ile)** is summarized in Table 3. Cyclized **Dap** revealed an MIC value that was 6-fold higher than that of authentic daptomycin, reflecting the crucial role of L-3-methylglutamate. Previous studies have shown that the antibacterial activity of the acidic lipopeptide antibiotics is Ca^{2+}-dependent and daptomycin and A54145 reach their maximum antimicrobial activity in the presence of a Ca^{2+} concentration of ~50 mg/L as found in human serum (*20*). To learn more about the significance of single amino acid residues of A54145 in the interaction with Ca^{2+} ions, peptidolactones **A54145(NXDG)**, **A54145(DXNG)**, and **A54145(NXNG)** were synthesized and tested for biological activity. Replacement of aspartic acid at positions 7 and/or 9 leads to a total loss of bioactivity for all three compounds and showed that these two amino acids are crucial elements for calcium binding. Experiments with modified CDA, derived from a mutasynthesis approach (*18*) and former chemoenzymatically synthesized daptomycin derivatives (*11*), are in good agreement with the results presented herein.

This leads us to the conclusion that Asp_7 and Asp_9 belong to a structural motif, which is generally important for the binding of Ca^{2+} ions and crucial for antimicrobial activity of all acidic lipopeptide antibiotics.

Compared to those of A54145, the three exocyclic amino acids of daptomycin are highly similar and are only subtly different. Both compounds contain tryptophan at position 1, followed by a D-amino acid at position 2. Additionally, one acidic residue and asparagine or hydroxymethylasparagine can be found. These characteristics suggest a similar conformation for this exocyclic part of the tridecapeptide molecules in aqueous solution. In accordance with this, the chemoenzymatically synthesized daptomycin−A54145 hybrid **Dap(A54$_{1-3}$)** did not show a significant change in antimicrobial potency compared to **Dap**.

Further, successive substitution of the daptomycin peptide backbone with amino acids from A54145 as in **Dap(A54$_{1-6}$)** was possible, illustrating the opportunity to construct structural hybrids of these two compounds. As reported in Table 3, the MIC of **Dap(A54$_{1-6}$)** was similar to that of **Dap(A54$_{1-3}$)**, underlining the importance of the C-terminal halves of these molecules. Moreover, the synthesized analogues of the two major found A54145 variants displayed a significant difference in MIC values; that of **A54145(Val)** was increased 8-fold compared to that of **A54145(Ile)**. On the basis of this result, it became obvious that amino acid residues at position 13 have a crucial influence on the antimicrobial behavior of A54145. The role of this C-terminal position is currently difficult to explain, because the acidic lipopeptide mode of action is not yet fully understood and their molecular targets are discussed controversially (*21*−*23*). However, identifying this structural element that is important for biological activity makes it a promising site for further chemical modifications.

In conclusion, the "tool set" of peptide cyclases from the acidic lipopeptides has been enlarged by two new members, A54145 and daptomycin PCP TE. These viable cyclization catalysts make this class of substances more readily accessible for derivatization. The chemoenzymatic approach described herein provides new opportunities to explore the relatively unclear acidic lipopeptide mode of action and to develop novel molecules related to daptomycin with an improved or modified spectrum of activity.

ACKNOWLEDGMENT

We gratefully acknowledge CUBIST Pharmaceuticals (Dr. P. Brian) for providing chromosomal DNA of *S. roseusporu*s and *S. fradiae*. Additionally, we thank Dr. Uwe Linne for MS/MS sequencing and Katja Kräling for excellent technical assistance.

SUPPORTING INFORMATION AVAILABLE

Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Baltz, R. H., Miao, V., and Wrigley, S. K. (2005) Natural products to drugs: Daptomycin and related lipopeptide antibiotics, *Nat. Prod. Rep. 22*, 717−41.
2. Berdy, J. (2005) Bioactive microbial metabolites, *J. Antibiot. 58*, 1−26.
3. Raja, A., LaBonte, J., Lebbos, J., and Kirkpatrick, P. (2003) Daptomycin, *Nat. Rev. Drug Discovery 2*, 943−4.
4. Sieber, S. A., and Marahiel, M. A. (2005) Molecular mechanisms underlying nonribosomal peptide synthesis: Approaches to new antibiotics, *Chem. Rev. 105*, 715−38.
5. Fukuda, D. S., Du Bus, R. H., Baker, P. J., Berry, D. M., and Mynderse, J. S. (1990) A54145, a new lipopeptide antibiotic complex: Isolation and characterization, *J. Antibiot. 43*, 594−600.
6. Miao, V., Coeffet-Legal, M. F., Brian, P., Brost, R., Penn, J., Whiting, A., Martin, S., Ford, R., Parr, I., Bouchard, M., Silva, C. J., Wrigley, S. K., and Baltz, R. H. (2005) Daptomycin biosynthesis in *Streptomyces roseosporus*: Cloning and analysis of the gene cluster and revision of peptide stereochemistry, *Microbiology 151*, 1507−23.
7. Miao, V., Brost, R., Chapple, J., She, K., Gal, M. F., and Baltz, R. H. (2005) The lipopeptide antibiotic A54145 biosynthetic gene cluster from *Streptomyces fradiae*, *J. Ind. Microbiol. Biotechnol*., 1−12.
8. Kohli, R. M., and Walsh, C. T. (2003) Enzymology of acyl chain macrocyclization in natural product biosynthesis, *Chem. Commun.*, 297−307.
9. 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*, 215−8.
10. Mahlert, C., Sieber, S. A., Grünewald, J., and Marahiel, M. A. (2005) Chemoenzymatic approach to enantiopure streptogramin B variants: Characterization of stereoselective pristinamycin I cyclase from *Streptomyces pristinaespiralis*, *J. Am. Chem. Soc. 127*, 9571−80.
11. 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*, 17025−31.
12. 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*, 1573−82.
13. 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*, 7092−8.
14. Grünewald, J., Sieber, S. A., and Marahiel, M. A. (2004) 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*, *Biochemistry 43*, 2915−25.
15. Sieber, S. A., Walsh, C. T., and Marahiel, M. A. (2003) Loading peptidyl-coenzyme A onto peptidyl carrier proteins: A novel approach in characterizing macrocyclization by thioesterase domains, *J. Am. Chem. Soc. 125*, 10862−6.
16. Kohli, R. M., Walsh, C. T., and Burkart, M. D. (2002) Biomimetic synthesis and optimization of cyclic peptide antibiotics, *Nature 418*, 658−61.
17. Wagner, B., Baumann, M., Sieber, S. A., and Marahiel, M. A. (2006) Solvent engineering substantially enhances the chemoenzymatic production of surfactin, *ChemBioChem* (in press).
18. Hojati, Z., Milne, C., Harvey, B., Gordon, L., Borg, M., Flett, F., Wilkinson, B., Sidebottom, P. J., Rudd, B. A., Hayes, M. A., Smith, C. P., and Micklefield, J. (2002) Structure, biosynthetic origin, and engineered biosynthesis of calcium-dependent antibiotics from *Streptomyces coelicolor*, *Chem. Biol. 9*, 1175−87.
19. Finking, R., and Marahiel, M. A. (2004) Biosynthesis of nonribosomal peptides, *Annu. Rev. Microbiol. 58*, 453−88.
20. Eliopoulos, G. M., Thauvin, C., Gerson, B., and Moellering, R. C., Jr. (1985) In vitro activity and mechanism of action of A21978C1, a novel cyclic lipopeptide antibiotic, *Antimicrob. Agents Chemother. 27*, 357−62.
21. Silverman, J. A., Perlmutter, N. G., and Shapiro, H. M. (2003) Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*, *Antimicrob. Agents Chemother. 47*, 2538−44.
22. Laganas, V., Alder, J., and Silverman, J. A. (2003) In vitro bactericidal activities of daptomycin against *Staphylococcus aureus* and *Enterococcus faecalis* are not mediated by inhibition of lipoteichoic acid biosynthesis, *Antimicrob. Agents Chemother. 47*, 2682−4.
23. Boaretti, M., and Canepari, P. (1995) Identification of daptomycin-binding proteins in the membrane of *Enterococcus hirae*, *Antimicrob. Agents Chemother. 39*, 2068−72.

BI0609422