JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Subscriber access provided by UNIV OF YORK

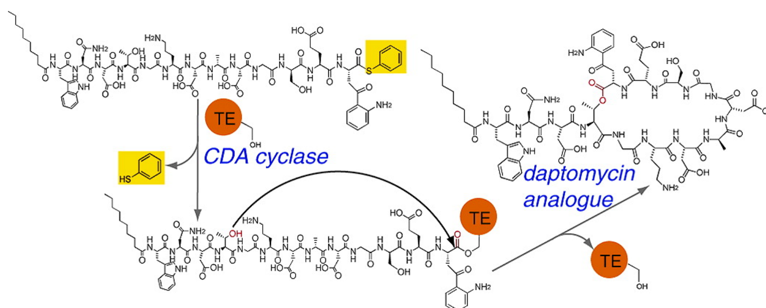
Article

Synthesis and Derivatization of Daptomycin: A Chemoenzymatic Route to Acidic Lipopeptide Antibiotics

Jan Grnewald, Stephan A. Sieber, Christoph Mahlert, Uwe Linne, and Mohamed A. Marahiel

J. Am. Chem. Soc., **2004**, 126 (51), 17025-17031• DOI: 10.1021/ja045455t • Publication Date (Web): 03 December 2004

Downloaded from http://pubs.acs.org on April 5, 2009



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 2 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

ACS Publications
High quality. High impact.

Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

J|A|C|S
ARTICLES
Published on Web 12/03/2004

Synthesis and Derivatization of Daptomycin: A Chemoenzymatic Route to Acidic Lipopeptide Antibiotics

Jan Grünewald, Stephan A. Sieber,† Christoph Mahlert, Uwe Linne, and Mohamed A. Marahiel*

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

Received July 28, 2004; E-mail: marahiel@chemie.uni-marburg.de

Abstract: Daptomycin is a branched cyclic nonribosomally assembled acidic lipopeptide, which is the first clinically approved antibiotic of this class. Here we show that the recombinant cyclization domain of the *Streptomyces coelicolor* calcium-dependent antibiotic (CDA) nonribosomal peptide synthetase (NRPS) is a versatile tool for the chemoenzymatic generation of daptomycin derivatives. Linear CDA undecapeptide thioesters with single exchanges at six daptomycin-specific residues were successfully cyclized by CDA cyclase. Simultaneous incorporation of all six of these residues into the peptide backbone and elongation of the N-terminus of CDA by two residues yielded a daptomycin derivative that lacked only the β-methyl group of L-3-methylglutamate. Bioactivity studies with several substrate analogues revealed a significant role of nonproteinogenic constituents for antibacterial potency. In accordance with acidic lipopeptides, the bioactivity of the chemoenzymatic assembled daptomycin analogue is dependent on the concentration of calcium ions. Single deletions of the four acidic residues in the peptide backbone suggest that only two aspartic acid residues are essential for antimicrobial potency. These two residues are strictly conserved among other nonribosomal acidic lipopeptides and the EF-motif of ribosomally assembled calmodulin. Based on these findings CDA cyclase is a versatile catalyst that can be used to generate novel daptomycin derivatives that are otherwise difficult to obtain by chemical modification of the parental tridecapeptide to improve further its therapeutic activity.

Introduction

Nonribosomal peptides constitute a large class of highly diverse natural products with extraordinary pharmacological importance.[1] The bioactivity of these complex secondary metabolites reaches from antibiotic over immunosuppressive, cytostatic to toxic. They are therefore interesting targets for the development of new drugs with improved or altered activities. Many of these nonribosomal peptides have a macrocyclic structure which constrains these compounds to a biologically active conformation.[2] This structural feature complicates the synthesis of novel variants as chemical methods are mostly inefficient for regio- and stereospecific cyclization of peptides.[3] In nature the macrocyclization of nonribosomal peptides is mostly mediated by thioesterase domains (TE, cyclase), which are the C-terminal catalytic units of the corresponding biosynthetic clusters, the nonribosomal peptide synthetases (NRPS). It was shown that TE domains excised from the NRPS assembly lines retain cyclization activity with artificial peptidyl thioester substrates.[4−6] Since these autonomous catalytic units are very permissive for alterations of the peptide backbone, there is a utility in applying these enzymes for the generation of new variants in a chemoenzymatic approach.[7]

Here we show that this strategy is suitable for the production of daptomycin and novel derivatives of this cyclic nonribosomal peptide in vitro (Figure 1A).

Daptomycin is a semisynthetic compound derived from the fermentation of *Streptomyces roseosporus*.[9,10] This antibiotic belongs to the group of acidic lipopeptides which require physiological levels of calcium ions for their activity.[11] Daptomycin, under the trade name Cubicin, is the first member of this class to have gained approval for clinical use.[12] Recently, its three-dimensional structure was determined.[13] Daptomycin

† Present address: The Scripps Research Institute, Cravatt Laboratory, 10550 North Torrey Pines Road, La Jolla, CA 92037.

(1) Schwarzer, D.; Finking, R.; Marahiel, M. A. *Nat. Prod. Rep.* **2003**, *20*, 275−287.
(2) Walsh, C. T. *Science* **2004**, *303*, 1805−1810.
(3) Bordusa, F. *ChemBioChem* **2001**, *2*, 405−9.
(4) Trauger, J. W.; Kohli, R. M.; Mootz, H. D.; Marahiel, M. A.; Walsh, C. T. *Nature* **2000**, *407*, 215−218.
(5) Kohli, R. M.; Walsh, C. T. *Chem. Commun. (Cambridge)* **2003**, *3*, 297−307.
(6) Sieber, S. A.; Marahiel, M. A. *J. Bacteriol.* **2003**, *185*, 7036−7043.
(7) Kohli, R. M.; Walsh, C. T.; Burkart, M. D. *Nature* **2002**, *418*, 658−661.
(8) 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.; Micklefield, J. *Chem. Biol.* **2002**, *9*, 1175−1187.
(9) Debono, M.; Abbott, B. J.; Molloy, R. M.; Fukuda, D. S.; Hunt, A. H.; Daupert, V. M.; Counter, F. T.; Ott, J. L.; Carrell, C. B.; Howard, L. C.; Boeck, L. D.; Hamill, R. L. *J. Antibiot. (Tokyo)* **1988**, *41*, 1093−1105.
(10) Tally, F. P.; DeBruin, M. F. *J. Antimicrob. Chemother.* **2000**, *46*, 523−526.
(11) Richter S. S.; Kealey, D. E.; Murray, C. T.; Heilmann, K. P.; Coffman, S. L.; Doern, G. V. *J. Antimicrob. Chemother.* **2003**, *52*, 123−127.
(12) Raja, A.; LaBonte, J.; Lebbos, J.; Kirkpatrick, P. *Nat. Rev. Drug Discovery* **2003**, *2*, 943−944.
(13) Ball, L.-J.; Goult, C. M.; Donarski, J. A.; Micklefield, J.; Ramesh, V. *Org. Biomol. Chem.* **2004**, *2*, 1872−1878.

10.1021/ja045455t CCC: $27.50 © 2004 American Chemical Society

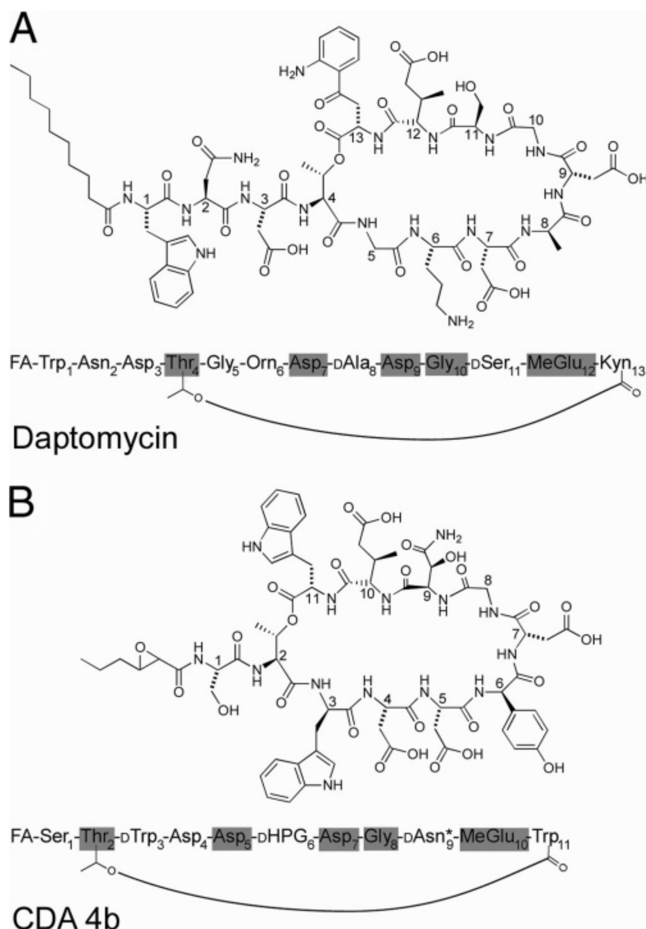


Figure 1. Structures of two members of the class of acidic lipopeptides are shown in two different fashions. Common amino acids which are found at equivalent positions in the lactone rings are indicated by shading. MeGlu = L-3-methylglutamate; DAsn* = D-3-hydroxyasparagine; DHPG = D-4-hydroxyphenylglycine. (A) The branched cyclic tridecapeptide daptomycin derived from the fermentation of *Streptomyces roseosporus*. (B) The calcium-dependent antibiotic (CDA) produced by *Streptomyces coelicolor* consists of 11 residues. CDA 4b represents one example of this family of compounds.[8]

exhibits rapid in vitro bactericidal activity against a number of clinically significant Gram-positive bacteria. These include resistant pathogens, such as vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), glycopeptide intermediately susceptible *S. aureus* (GISA), coagulase-negative staphylococci (CNS), and penicillin-resistant *Streptococcus pneumoniae* (PRSP).[10] Daptomycin has a unique mechanism of action appearing to bind to the Gram-positive cell membrane by its lipid moiety, followed by calcium-dependent insertion and oligomerization. The daptomycin oligomers are forming ion channels disrupting the functional integrity of the membrane and triggering a release of intracellular ions. This causes disruption of bacterial membrane potential leading to rapid cell death.[14−16]

Structurally, daptomycin is a depsipeptide containing 13 amino acids which, in addition to a decanoyl fatty acid (FA) attached to the N-terminus, contains two D-configured as well as nonproteinogenic residues, including L-kynurenine (Kyn), L-ornithine (Orn), and L-3-methylglutamate. It is comprised of a decapeptide lactone ring derived from cyclization of the L-threonine side chain onto the C-terminal carboxyl group.[17] Daptomycin shares this structural feature to the calcium-dependent antibiotic (CDA), which also belongs to the group of acidic lipopeptides (Figure 1B). Remarkably, five of the amino acids in the daptomycin lactone ring are found at the same positions in CDA.[8] We have recently shown that the C-terminal TE domain excised from the nonribosomal CDA synthetase retains autonomous macrocyclization activity with synthetic peptide thioester substrates based on a primary sequence analogous to natural CDA. It was shown that the CDA TE is a quite permissive cyclization catalyst, which allows the creation of macrolactones with various ring sizes.[18]

Here we report, that this cyclization catalyst is also very permissive for amino acid exchanges allowing the in vitro synthesis of the closely related antibiotic daptomycin. Furthermore, we present the viability of this cyclase in producing derivatives of daptomycin, which are not accessible by the chemical modification of the parental compound. This enables us to explore the calcium-dependence of this important antibiotic by subsequent deletion of the four acidic residues, which are likely to be important for the interaction with calcium ions.[13] We have also performed an evaluation of the function of the nonproteinogenic residues for bioactivity.

Experimental Section

The identity of compounds was verified by analytical HPLC, ESI-MS, MALDI-TOF MS, MS−MS sequencing, or ^{1}H NMR as outlined in the Supporting Information.

Synthesis of Peptide Thioester Substrates. Peptide synthesis was carried out on an Advanced ChemTech APEX 396 synthesizer (0.1 mmol scale) by using 2-chlorotrityl resin (IRIS biotech) as described earlier.[19] Nonprotected kynurenine sulfate, hexanoic acid, and decanoic acid were purchased from Sigma-Aldrich. N_α-acetylated serine and glutamate were obtained from Bachem. All 9-fluorenylmethoxycarbonyl (Fmoc) amino acids and coupling reagents were purchased from IRIS biotech and Novabiochem. The following side chain protection groups were employed: *t*-Bu for Asp, Glu, D-Ser, and Thr; Boc for Orn and Trp; Aloc for Orn; and Trt for Asn and Gln. Solution-phase thioester formation and purification of thiophenol thioesters were performed as described elsewhere.[20]

Synthesis of *N*-(9-Fluorenylmethoxycarbonyl)-L-kynurenine. N^α-Fmoc-protection of kynurenine was conducted with 9-fluorenylmethyl succinimidyl carbonate (Fmoc-OSu) according to a previous protocol.[21] Kynurenine sulfate (306 mg, 1 mmol) and sodium bicarbonate (252 mg, 3 mmol) were dissolved in a mixture of acetone (2.5 mL) and water (2.5 mL). After addition of Fmoc-OSu (Novabiochem) (337.3 mg, 1 mmol), the solution was stirred overnight. The mixture was acidified to pH 2 with concentrated hydrochloric acid, and the acetone was removed by rotary evaporation. The product was extracted with chloroform and washed with 0.1 N HCl and water. After drying over anhydrous sodium sulfate, the combined organic phases were evaporated.

Assays of Peptide Thiophenol Substrates. The expression and purification of CDA TE were described earlier.[18] Enzymatic reactions

(14) Lakey, J. H.; Ptak, M. *Biochemistry* **1988**, *27*, 4639−4645.
(15) Silverman, J. A.; Perlmutter, N. G.; Shapiro, H. M. *Antimicrob. Agents Chemother.* **2003**, *47*, 2538−2544.
(16) Alborn, W. E., Jr.; Allen, N. E.; Preston, D. A. *Antimicrob. Agents Chemother.* **1991**, *35*, 2282−2287.
(17) Debono, M.; Barnhart, M.; Carrell, C. B.; Hoffmann, J. A.; Occolowitz, J. L.; Abbott, B. J.; Fukuda, D. S.; Hamill, R. L.; Biemann, K.; Herlihy, W. C. *J. Antibiot. (Tokyo)* **1987**, *40*, 761−777.
(18) Grünewald, J.; Sieber, S. A.; Marahiel, M. A. *Biochemistry* **2004**, *43*, 2915−2925.
(19) Kohli, R. M.; Trauger, J. W.; Schwarzer, D.; Marahiel, M. A.; Walsh, C. T. *Biochemistry* **2001**, *40*, 7099−7108.
(20) Sieber, S. A.; Tao, J.; Walsh, C. T.; Marahiel, M. A. *Angew. Chem., Int. Ed.* **2004**, *43*, 493−498.
(21) Paquet, A. *Can. J. Chem.* **1982**, *60*, 976−980.

were carried out in a total volume of 50 μL with 25 mM 2-*N*′-[*N*-(2-hydroxyethyl)piperazinyl]ethansulfonic acid (Hepes), 50 mM NaCl, and 2−10% DMSO (v/v) at pH 7.0 and 25 °C. Reactions were initiated by addition of enzyme to a final concentration of 5 μM. Quenching of the assays was accomplished by addition of 35 μL of 4% trifluoroacetic acid (TFA)/water. Product analysis was conducted by analytical HPLC on a C_{18} Nucleodur column (Macherey and Nagel, 250/3, pore diameter 100 Å, particle size 3 μm) using a gradient from 15 to 60% of 0.1% TFA/acetonitrile in 0.1% TFA/water over a time period of 40 min (0.4 mL/min, 45 °C). The regiochemistry of cyclic products was determined by MS−MS analysis on an API Qstar Pulsar I Q-q-TOF mass spectrometer (Applied Biosystems). Kinetic characterization of the cyclization reactions was performed by determining initial rates at varying substrate concentrations. This was done by using two time points at each concentration within the linear region of the enzyme. Separate k_{cat} and K_M values could not be determined due to severe substrate inhibition at concentrations greater than 250 μM, which might be related to micelle formation of the amphiphilic substrates.[22]

Preparation of Linear and Cyclic Peptides for Bioassays. Semipreparative scale production of linear and cyclic peptide products was performed to obtain enough material for bioactivity studies. For the semipreparative scale generation of cyclic products, the reactions were carried out in a total volume of 3−12 mL with 5 μM CDA TE, 250 μM peptide thiophenol, 25 mM Hepes, 50 mM NaCl, and 5% DMSO (v/v), at pH 7.0 and room temperature. Substrate turnover into cyclic product and linear peptide acid was monitored by analytical HPLC. After a time period of 2−5 h 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 solid was redissolved in 500 μL of 35% acetonitrile/water. Purification of the cyclic products was achieved on a 250/10 Nucleodur 100-7 C18 reversed-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}. The purity of the products was more than 95% as determined by analytical HPLC.

Semipreparative scale generation of the linear peptide acid was performed by treatment of the peptide loaded 2-chlorotrityl resin with a mixture containing trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and water in a ratio of 95:2.5:2.5 (v/v). The peptide acid was precipitated in ice cold diethyl ether and purified by semipreparative HPLC as described for the cyclic products.

Biological Activity Assays. Two-fold serial dilutions of 1 linear peptide acid and 10 peptidolactones including authentic daptomycin as reference compound were prepared in microtiter plates as described elsewhere.[7] All tests were performed with *B. subtilis* PY79. Incubation was carried out at 37 °C for 18 h prior to visual determination of MICs. The bactericidal activity was examined in LB media as well as in LB media supplemented with 50 mg of Ca^{2+}/L. The calcium content of LB media was determined by elementary analysis to be 23.6 mg/L.

For determination of hemolytic activity sheep blood agar plates were used. Sterile disks (6 mm diameter) were soaked with 2.5−10 μL of solutions of cyclic peptides in methanol varying in their concentrations from 0.64 μg/mL to 640 μg/mL. Plates were incubated at 37 °C for 16 h. Surfactin was used as a positive control. The hemolytic zone diameters were determined by visual assay.

Results

Assaying the Substrate Tolerance of CDA Cyclase to Produce Daptomycin. To explore the ability of CDA TE to catalyze cyclization of a linear daptomycin precursor peptide in principle, we first synthesized four peptide thioester substrates that differ from a CDA sequence analogue by only one single amino acid substitution. Specifically, we incorporated residues specific for daptomycin into the peptide backbone of the CDA lactone ring. Therefore, we changed D-Trp_3 to Gly_3 (**AcCDA-G3**), L-Asp_4 to L-Orn_4 (**AcCDA-O4**), D-Phe_6 to D-Ala_6 (**AcCDA-DA6**), and D-Asn_9 to D-Ser_9 (**AcCDA-DS9**) (Figure 2).

Additionally, the 2,3-epoxyhexanoyl fatty acid moiety of native CDA was replaced with a shorter acetyl chain to increase solubility. Assaying for CDA TE mediated cyclization revealed that three of four substrates were completely converted to macrocycles after 3 h. The only exception was **AcCDA-G3**, where only traces of cyclized product were detected within this period of time (data not shown). In all cases, the CDA cyclase catalyzed the formation of two regioisomeric macrolactones, which arise from simultaneous nucleophilic attack of the two adjacent Thr_2 and Ser_1 residues onto the C-terminus of the acyl enzyme intermediate as described earlier.[18]

To better approximate natural CDA, we elongated the N-terminal acetyl chains of **AcCDA-G3**, **AcCDA-O4**, **AcCDA-DA6**, and **AcCDA-DS9** by four methylene groups through replacement by hexanoic side chains (**HexCDA-G3**, **HexCDA-O4**, **HexCDA-DA6**, and **HexCDA-DS9**) (Figure 2). The poor water solubility of the peptide thioester substrates was improved by the addition of 5% DMSO (v/v). In accordance with our previous results, the elongated acyl side chain dramatically increased the regioselectivity of the enzyme mediated macrocyclization yielding exclusively the decapeptide lactone ring derived from nucleophilic attack of L-threonine onto the C-terminus[18] (see Supporting Information). Remarkably, the conversion to cyclic product was substantial for all substrates. This includes also **HexCDA-G3**, which is significantly converted to the macrocyclic product. In addition, the two peptide thioester substrates **HexCDA-U11** and **HexCDA-D1** were designed in which the C-terminal nonproteinogenic amino acid L-Kyn (U) and the exocyclic residue L-Asp of daptomycin were introduced to the CDA backbone (Figure 2). Incubation of CDA TE with the corresponding peptidyl thiophenol substrates displays substantial formation of the corresponding peptidolactones. In conclusion, all single residue exchanges of the CDA backbone were tolerated by CDA cyclase, when hexanoic acid was fused to the N-terminus.

Testing the Effect of Simultaneous Changes and Branch Point Movement. The single residue scan described above suggests that CDA TE is a permissive cyclization catalyst and raises the question of the effect on cyclization of simultaneous changes in the linear peptide precursor. Therefore, we synthesized a peptide thioester substrate (**Dap**) which was based on a sequence analogous to daptomycin.[9] L-3-Methylglutamic acid in position twelve was replaced by L-glutamic acid for synthetic ease (Figure 2). In contrast to the substrates derived from CDA, **Dap** incorporates a longer N-terminal extension including two additional amino acids fused to a decanoyl fatty acid moiety, which moves the branch point of the peptidolactone from position two to position four. Incubation of **Dap** with CDA cyclase revealed the formation of two products that were identified by ESI-MS as linear peptide acid (t_R = 30.2 min) and decapeptide lactone (t_R = 30.8 min) (Figure 3).

MS−MS sequencing of the cyclic species confirmed that cyclization is mediated through nucleophilic attack of L-Thr at position four as fragment ions containing the predicted linkage from Thr_4 to Kyn_{13} can be identified (see Supporting Information). Cyclization occurred with a k_{cat}/K_M value of 22.2 mM^{-1}

(22) Tseng, C. C.; Bruner, S. D.; Kohli, R. M.; Marahiel, M. A.; Walsh, C. T.; Sieber, S. A. *Biochemistry* **2002**, *41*, 13350−13359.

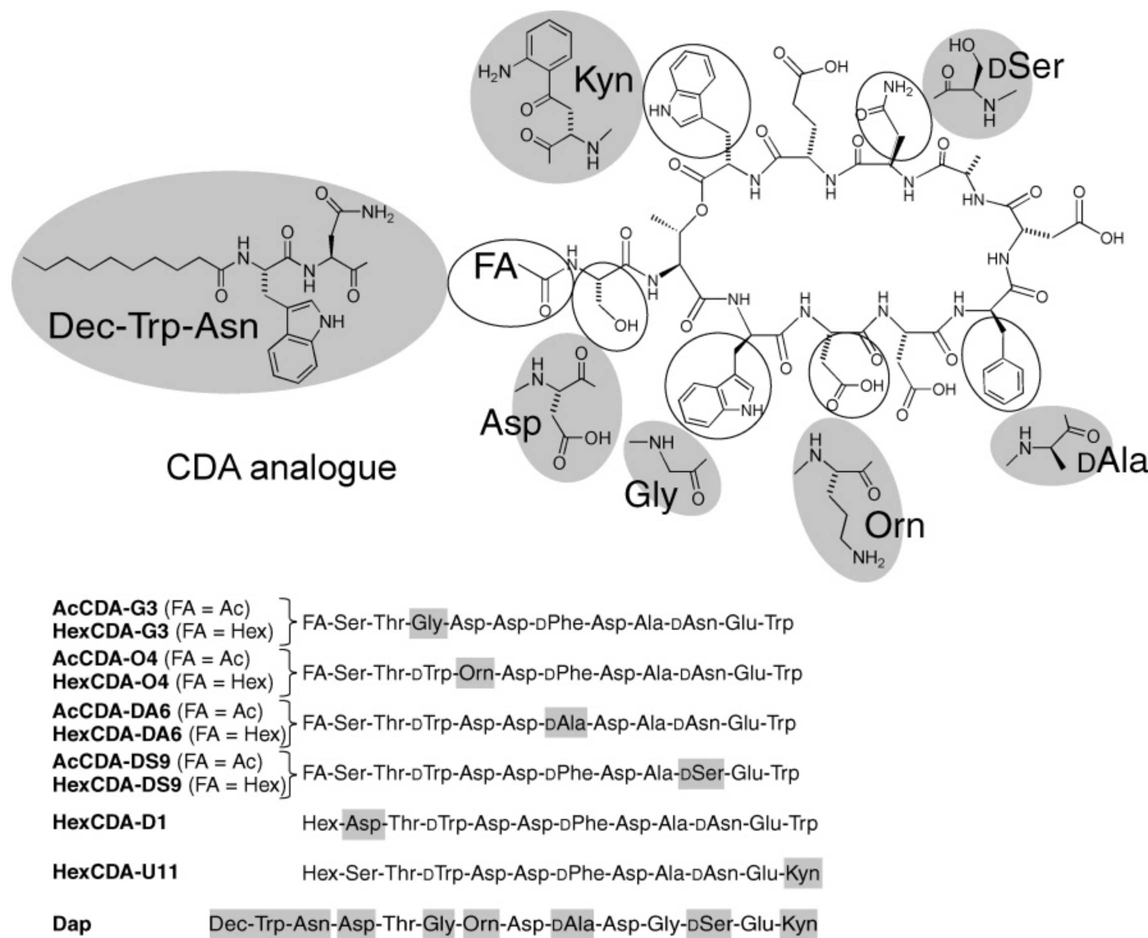


Figure 2. Structure of a CDA analogue is shown. Due to synthetic ease some unusual constituents of native CDA were replaced as described earlier.[18] Single amino acid substitution experiments were carried out to probe the ability of CDA TE to generate branched cyclic daptomycin. The incorporation of daptomycin-specific residues into the peptide backbone is indicated by shading. In **Dap** all substitutions were performed simultaneously and the N-terminus was extended by two additional amino acids fused to a decanoyl fatty acid moiety. Ac = acetic acid, Dec = decanoic acid, Hex = hexanoic acid.

$\min^{-1}$ and a ratio of cyclization to hydrolysis of 3:1. A control reaction without enzyme showed that the formation of this macrolactone was abolished. Instead, two new cyclic products were observed at retention times of 30.0 and 32.5 min, respectively. The identities of these two species were investigated by MS−MS fragmentation (see Supporting Information). For the product with the lower retention time, cyclization was shown to occur through the L-Kyn residue at position 13 leading to a seven-membered lactam. In the other case nonenzymatic cyclization yielded an octapeptide lactam ring derived from nucleophilic attack of the L-Orn residue at position six onto the C-terminal carboxyl group. The formation of this macrolactam was abolished by orthogonal Aloc-protection of the L-Orn residue (**Dap-Aloc**) (see Supporting Information).

Derivatization of Daptomycin and Bioactivity Studies. To explore the significance of selected amino acid side chains for the bioactivity of the antibiotic daptomycin, we synthesized eight peptide thioesters (**Dap-N3**, **Dap-N7**, **Dap-N9**, **Dap-Q12**, **Dap-DD11**, **Dap-Aloc**, **Dap-W13**, and **Dap-W13K6**) in addition to the already mentioned linear precursor peptide **Dap** (Figure 4).

All compounds were tested as substrates for enzymatic transformation by CDA cyclase. In each case the conversion to cyclic product was sufficient to allow semipreparative-scale reactions (data not shown). The purified cyclic products were then tested for their bactericidal activity against *B. subtilis* PY79 (Table 1).

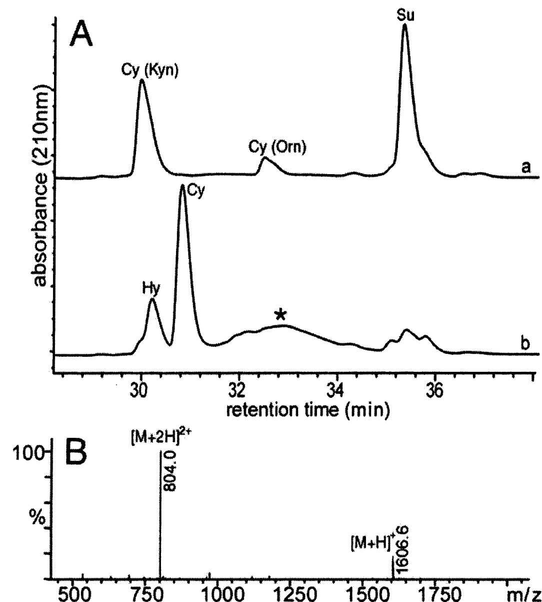
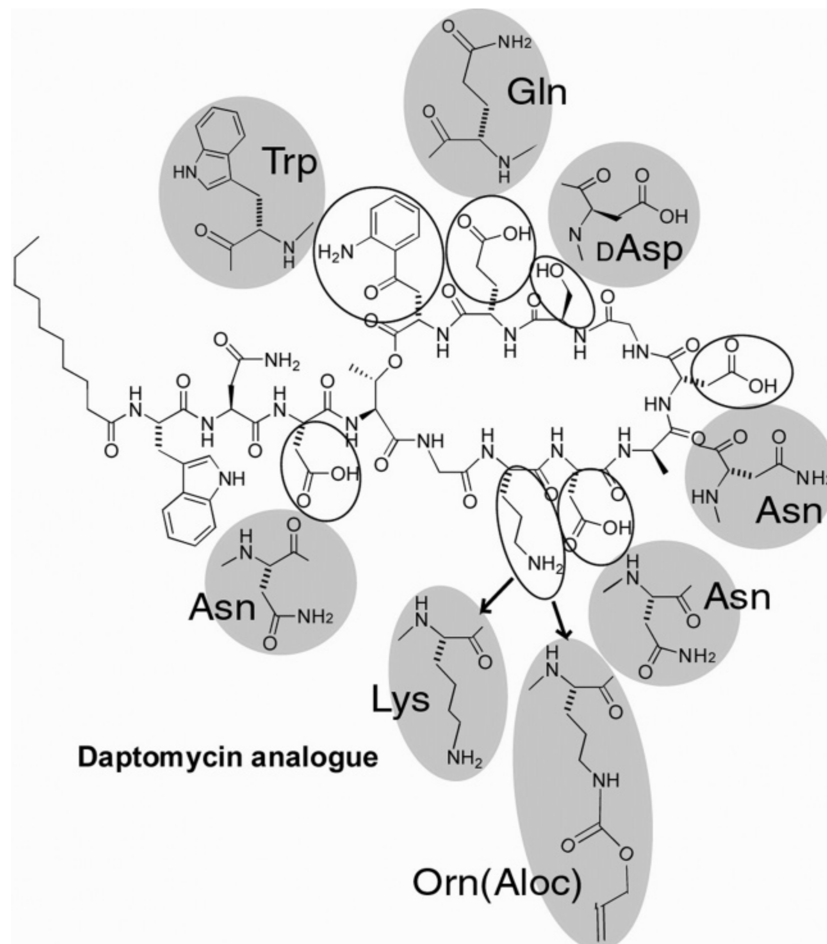


Figure 3. Cyclization of a daptomycin analogue mediated by CDA TE. (A) Reactions of the linear precursor peptide Dap followed by HPLC. Trace a displays the negative control without enzyme (250 μM substrate, 25 mM Hepes, 50 mM NaCl, pH 7.0 at 25 °C for 2 h). Trace b shows the same reaction in the presence of 5 μM CDA cyclase. Enzyme is indicated with an asterisk. (B) Shown is the ESI mass spectrum of the peptidolactone (t_R = 30.9 min) generated by CDA TE.



Dap	Dec-Trp-Asn-Asp-Thr-Gly-Orn-Asp-DAla-Asp-Gly-DSer-Glu-Kyn
Dap-N3	Dec-Trp-Asn-Asn-Thr-Gly-Orn-Asp-DAla-Asp-Gly-DSer-Glu-Kyn
Dap-N7	Dec-Trp-Asn-Asp-Thr-Gly-Orn-Asn-DAla-Asp-Gly-DSer-Glu-Kyn
Dap-N9	Dec-Trp-Asn-Asp-Thr-Gly-Orn-Asp-DAla-Asn-Gly-DSer-Glu-Kyn
Dap-Q12	Dec-Trp-Asn-Asp-Thr-Gly-Orn-Asp-DAla-Asp-Gly-DSer-Gln-Kyn
Dap-Aloc	Dec-Trp-Asn-Asp-Thr-Gly-Orn(Aloc)-Asp-DAla-Asp-Gly-DSer-Glu-Kyn
Dap-DD11	Dec-Trp-Asn-Asp-Thr-Gly-Orn-Asp-DAla-Asp-Gly-DAsp-Glu-Kyn
Dap-W13	Dec-Trp-Asn-Asp-Thr-Gly-Orn-Asp-DAla-Asp-Gly-DSer-Glu-Trp
Dap-W13K6	Dec-Trp-Asn-Asp-Thr-Gly-Lys-Asp-DAla-Asp-Gly-DSer-Glu-Trp

Figure 4. Chemoenzymatic derivatization of daptomycin. The daptomycin analogue Dap cyclized by CDA TE only lacks the β-methyl group of L-3-methylglutamate (position 12) of authentic daptomycin. The residues that were incorporated into the backbone of Dap are indicated by shading. The corresponding sequences of the daptomycin derivatives are shown below. The differences to Dap are highlighted by gray boxes. Aloc = allyloxycarbonyl, Dec = decanoic acid.

At first we compared the bioactivity of the cyclic peptide product of **Dap** to authentic daptomycin. Both compounds differ only by the β-methyl group of glutamic acid in position twelve. The macrolactone of **Dap** has a MIC of 20 μg/mL, whereas the reference compound has an MIC of 3 μg/mL (Table 1). This result indicates that the β-methyl group of glutamic acid is crucial for bioactivity. Further, when L-Kyn_{13} was replaced by L-Trp_{13} (**Dap-W13**), the MIC increased 5-fold to 100 μg/mL, displaying the importance of this nonproteinogenic amino acid for the bactericidal activity of daptomycin. In contrast to that, successive substitution of the third nonproteinogenic residue L-Orn_6 by L-Lys_6 in **Dap-W13K6** did not cause a further increase of the MIC (Table 1).

In accordance to authentic daptomycin, the antimicrobial behavior of **Dap** strongly depends on the presence of physiological concentrations of calcium ions.[11] The MIC increased at least 12-fold, when the concentration of free calcium ions was reduced from 73.6 mg/L to 23.6 mg/L (Table 1). Recent results with the closely related CDA suggest that the acidic residues are crucial for antimicrobial potency.[23] To prove that, we tested the cyclic peptides **Dap-N3**, **Dap-N7**, **Dap-N9**, and **Dap-Q12** for antibiotic activity. In each peptidolactone one acidic residue of daptomycin is deleted by substitution with either L-Asn or L-Gln(Figure 4). Single deletion of the aspartic

(23) Uguru, G. C.; Milne, C.; Borg, M.; Flett, F.; Smith, C. P.; Micklefield, J. *J. Am. Chem. Soc.* **2004**, *126*, 5032−5033.

Table 1. MIC Determination of Daptomycin Derivatives against *B. subtilis* PY79

compound	MIC_{90} (μg/mL) at 73.6 mg/L Ca^{2+} [at 23.6 mg/L Ca^{2+}]
Dap	20 [>240]
authentic daptomycin	3 [20]
Dap-Hyd	>960
Dap-N3	80
Dap-N7	>960
Dap-N9	>960
Dap-Q12	30
Dap-DD11	>320
Dap-Aloc	80
Dap-W13	100
Dap-W13K6	100

acid residues in the lactone ring (**Dap-N7** and **Dap-N9**) resulted in a total loss of bioactivity (Table 1). Surprisingly, replacement of the two remaining acidic residues (**Dap-N3** and **Dap-Q12**) did not abolish bactericidal potency, indicating that these residues are not essential for calcium binding. Interestingly, the bioactivity of **Dap** was also abolished, when an additional acidic residue was incorporated into the peptide backbone of the lactone ring (**Dap-DD11**) (Table 1).

The biological function of the positively charged Orn_6 remains unclear.[14] Nevertheless, masking of the side chain by Aloc-protection (**Dap-Aloc**) significantly increased the MIC 4-fold in comparison to **Dap**, indicating the importance of this residue for antibiotic activity. In addition, the lactone ring of **Dap** was shown to be important for bioactivity as the corresponding linear peptide acid (**Dap-Hyd**) did not show antimicrobial properties. Further, the cyclic peptide **Dap** completely lacked hemolytic activity up to a concentration 4-fold above the MIC even after the addition of 50 mg/L Ca^{2+} (data not shown). This demonstrates its specific interaction with prokaryotic membranes.

Discussion

Natural products play an important role in drug development, which is exemplified by the finding that most antibiotics and anticancer drugs in human use were derived from such compounds.[24,25] Among these complex compounds is the group of acidic lipopeptides, which includes the recently approved antibiotic daptomycin (Figure 1A). To our best knowledge, modifications of this nonribosomal lipopeptide have been performed at only two sites so far: the α-amino group of L-Trp_1 and the δ-amino group of L-Orn_6.[9,26,27] To make this cyclic tridecapeptide more accessible to derivatization, we report herein a new chemoenzymatic strategy which allows the production of novel variants of this important antibiotic.

The approach described here combines solid phase peptide synthesis with the excised nonribosomal CDA TE domain as a versatile macrocyclization catalyst. Recently we demonstrated that this cyclase catalyzes ring formation of linear peptide thioesters based on a sequence analogous to natural CDA.[18] This acidic lipopeptide is very similar in structure to daptomycin. Both compounds are decapeptide lactones, which share five common amino acid side chains in identical ring positions. This striking similarity encouraged us to carry out single amino acid substitution experiments. Instead of performing an alanine scan, we incorporated daptomycin-specific residues into the peptide backbone of CDA (Figure 2). Applying this strategy revealed that CDA TE is a very permissive cyclase. When hexanoic acid was fused to the N-terminus of the thioester substrates, CDA cyclase accepted all six amino acid side chains characteristic of daptomycin, including one exocyclic position. This is quite remarkable, considering that the side chain alterations in some positions resulted in dramatically different substituents, such as Gly for D-Trp_3 and L-Orn for L-Asp_4. Interestingly, substitution of the hexanoyl fatty acid by acetic acid decreased the substrate tolerance of CDA cyclase. Specifically, for the substrate thioester **AcCDA-G3** only trace amounts of cyclic product were detected under standard assay conditions. Recently we reported that the regio- and chemoselectivity of the CDA TE catalyzed cyclization reaction depends on the chain length of the N-terminal acyl residue.[18] The results of this study provide evidence that this feature also influences the substrate tolerance of TE mediated macrocyclization.

The results of the single amino acid substitution experiments raised the question of the effect on cyclization of simultaneous changes in potentially substitutable positions. Assaying the linear precursor peptide **Dap** for CDA TE mediated cyclization revealed that this cyclase behaves very tolerantly toward multiple residue substitutions in the peptide backbone. Macrolactonization of **Dap** occurred with a k_{cat}/K_M only 1.3-fold reduced from that of the linear CDA analogue[18] and the ratio of cyclization to hydrolysis significantly dropped from 10:1 to 3:1. However, the cyclization to hydrolysis ratio of **Dap** is still remarkable in comparison to studies with TycC TE, where simultaneous side chain alterations led to the predominance of hydrolysis over cyclization.[28] The efficient conversion to cyclic product may be caused by the Asp-D-Ala-Asp-Gly motif of the linear peptide precursor. It is known that in the closely related acidic lipopeptide amphomycin the similar Asp-Gly-Asp-Gly motif induces a type II β-turn, which may preorganize the substrate in a productlike conformation.[29] Furthermore, CDA TE is capable of maintaining a macrolactone ring size of 10 residues by tolerating a branch point movement from Thr_2 to Thr_4 as proved by MS−MS sequencing. This result suggests that the active site cavity of the CDA cyclase is large enough to accommodate the N-terminal extension of daptomycin, which is composed of two additional amino acids linked to a decanoyl fatty acid residue. Hence, the cyclase of the calcium-dependent antibiotic is a permissive cyclization catalyst allowing the synthesis of daptomycin in vitro. Although the methyl group of L-3-methylglutamate (L-3-MeGlu) was omitted, it is very likely that the TE tolerates this nonproteinogenic amino acid for catalytic activity due to its occurrence in four natural variants of cognate CDA at the same position. It is assumed that a putative glutamate-3-methyltransferase catalyzes the stereospecific β-methylation of glutamate prior to activation by a specific adenylation (A) domain which differs from conventional

(24) Harvey, A. *Drug Discovery Today* **2000**, *5*, 294−300.
(25) Wohlleben, W.; Pelzer, S. *Chem. Biol.* **2002**, *9*, 1163−1164.
(26) Siedlecki, J.; Hill, J.; Parr, I.; Yu, X.; Morytko, M.; Zhang, Y.; Silverman, J.; Controneo, N.; Laganas, V.; Li, T.; Li, J.; Keith, D.; Shimer, G.; Finn, J. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4245−4249.
(27) Hill, J.; Siedlecki, J.; Parr, I.; Morytko, M.; Yu, X.; Zhang, Y.; Silverman, J.; Controneo, N.; Laganas, V.; Li, T.; Lai, J.-J.; Keith, D.; Shimer, G.; Finn, J. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4187−4191.
(28) Kohli, R. M.; Takagi, J.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1247−1252.
(29) Lakey, J. H.; Maget-Dana, R.; Ptak, M. *Biochem. Biophys. Res. Commun.* **1988**, *150*, 384−390.

consensus								D	X	D	G				
daptomycin	1	W	N	D	T	G	O	D	DA	D	G	DS	Z	Z	13
CDA4b	1			S	T	DW	D	D	DZ	D	G	DZ	Z	W	11
amphomycins	1			D	Z	DZ	D	D	G	D	G	DZ	V	P	11
friulimicins	1			N	Z	DZ	D	D	G	D	G	DZ	V	P	11
calmodulin	15	E	A	F	S	L	F	D	K	D	G	D	G	T	27
	51	D	M	I	N	E	V	D	A	D	G	N	G	T	63
	88	E	A	F	R	V	F	D	K	D	G	N	G	F	100
	124	E	M	I	R	E	A	D	I	D	G	D	G	Q	136

Figure 5. Alignment of nonribosomal acidic lipopeptides with the calcium binding EF-hand motif of ribosomally assembled calmodulin.[32] The conserved amino acids of the consensus sequence DXDG are highlighted by shading. O = ornithine, Z = unusual amino acid.

glutamate-activating A domains.[8] This indicates that the corresponding tailoring step occurs prior to peptide cyclization by the TE domain. Notably, β-methylation of glutamate displays a key role in the biological activity of daptomycin. Substitution of L-3-MeGlu$_{12}$ by L-Glu$_{12}$ in **Dap** yielded a 7-fold increase of the MIC against *B. subtilis*. This suggests that the more hydrophobic L-3-MeGlu induces a closer contact of daptomycin with the bacterial lipid bilayer than its nonmethylated counterpart. The importance of the nonproteinogenic constituents on the antimicrobial action of daptomycin was further shown for the tryptophan metabolite L-kynurenine. Replacement by L-tryptophan resulted in an additional 5-fold increase of the MIC in comparison to **Dap**.

The bioactivity of daptomycin is dependent on the presence of calcium ions. These divalent cations presumably trigger the oligomerization of daptomycin molecules to form ion channels, which disrupt the membrane potential of the bacterial cell.[15] Despite the 7-fold higher MIC of **Dap** in comparison to authentic daptomycin, its bactericidal activity was clearly calcium-dependent (Table 1). It is speculated that calcium interacts with the four acidic residues of daptomycin.[13] To explore this, we used our chemoenzymatic approach to delete these residues through replacement by noncharged amino acids. Significantly, only L-Asp$_7$ and L-Asp$_9$ in the lactone ring are essential for antibiotic activity. This is in agreement to recent experiments with CDA, where it was shown that deletion of L-Asp$_7$ in the cyclic part abolished antimicrobial behavior.[23] Moreover, these ring-membered aspartic acid residues are strictly conserved among the group of acidic lipopeptides including also the calcium-dependent antibiotics friulimicins and amphomycins[30,31] (Figure 5).

The consensus sequence DXDG of these nonribosomal peptides is also part of the calcium binding EF-hand motif of ribosomally assembled calmodulin.[32] Therefore, acidic lipopeptides and calmodulin use a similar language for calcium recognition despite their biosynthetic difference.

The function of L-Orn$_6$ in daptomycin is still unclear.[14] It was recently shown that the positively charged amino group of this residue is not essential for bactericidal activity but reduced the potency 8-fold compared to daptomycin.[27] We observed a 4-fold drop in efficiency when the positive charge was masked by Aloc-protection.

Conclusions

In conclusion, CDA cyclase is a viable tool for the synthesis of daptomycin and derivatives that are hardly accessible by chemical modification of the parental compound. This allowed us to explore the influence of nonproteinogenic and charged residues on the bioactivity of this approved antibiotic. In future, this chemoenzymatic strategy using the versatility of CDA TE can be employed for the combinatorial generation of comprehensive libraries of daptomycin analogues that can be screened for improved therapeutic activity.

Acknowledgment. We gratefully acknowledge the generous gift of daptomycin from Cubist Pharmaceuticals. We also thank Jonas Treutwein and Christian Wirges for their excellent help with the synthesis, Björn Wagner for helpful comments, Judita Mascarenhas for providing *B. subtilis* PY79, Daniel Garbe for assistance with bioassays, and Antje Schäfer and Christiane Bomm for technical assistance. This work has been supported by the Fonds der Chemischen Industrie (to J.G. and M.A.M.), Studienstiftung des deutschen Volkes (to S.A.S.), and the Deutsche Forschungsgemeinschaft (to M.A.M.).

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

JA045455T

(30) Vertesy, L.; Ehlers, E.; Kogler, H.; Kurz, M.; Meiwes, J.; Seibert, G.; Vogel, M.; Hammann, P. *J. Antibiot. (Tokyo)* **2000**, *53*, 816−827.
(31) Banerjee, D. K.; Scher, M. G.; Waechter, C. J. *Biochemistry* **1981**, *20*, 1561−1568.
(32) Yazawa, M.; Yagi, K. *Biochem. Biophys. Res. Commun.* **1980**, *96*, 377−381.