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Rational Design of Bacitracin A Derivatives by Incorporating Natural Product Derived Heterocycles

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Abstract: Heterocycles display common structural motifs in nonribosomally produced peptides with an enormous impact on their bioactivity. In the case of the branched cyclic Bacitracin A, the thiazoline moiety is manufactured during NRPS peptide chain elongation. Here we describe a method to selectively alter the heterocyclic metal binding subunit of Bacitracin A by the synthesis of heterocyclic building blocks that were successfully coupled to the linear decapeptide and subsequently cyclized using the excised bacitracin PCP-TE bidomain. Utilization of this cyclase allowed the first generation of branched cyclic bacitracin derivatives containing thiazole and oxazoles. The generated bacitracin derivatives showed bactericidal activity, indicating the possibility of altering the biological important heterocyclic subunit and overcoming existing limitations for the application of bacitracin.

Introduction

Five-membered heterocyclic moieties such as thiazolines, oxazolines, their corresponding oxidized thiazoles, oxazoles, or reduced thiazolidines, oxazolidines, are signature metal binding motifs common to many clinically important nonribosomally derived macrocyclic peptides.[1] Biosynthetically, the incorporation of these heterocycles into the growing peptide chain results from enzymatic cyclization of cysteinyl, seryl, or threonyl side chains, respectively, onto the proceeding carbonyl group of the peptide substrate during peptide elongation. This intramolecular cyclization is catalyzed by so-called cyclization (Cy) domains, that are imbedded within the multimodular peptide synthetases that act as the assembly line temple for such macrocyclic peptides.[2] The thiazoline or oxazoline oxidative state is not always the final designation of the heterocycle, as they can further be modified by FMN containing oxidase domains (Ox), which catalyze the two-electron oxidation of the five-membered heterocycles into their corresponding thiazoles or oxazoles,[3] or reduced by NADPH-dependent reductases to the tetrahydro heterocycles.[4] Since heterocycles play an important role for the bioactivity of natural products, their alteration and variation are of major interest for the derivatization of these molecules. Bacitracin is a mixture of closely related metal-dependent peptide antibiotics that are produced nonribosomally by strains of *Bacillus licheniformis* and *Bacillus subtilis*.[5] It is recognized as a potent antibiotic directed primarily against Gram-positive pathogens, such as staphylococci, streptococci, corynebacteria, and anaerobe cocci such as clostridia, neisseria, and most gonococci.[6] The major compound of commercially available bacitracin is Bacitracin A (Figure 1b), mainly responsible for its antibiotic activity. Bacitracin A is a branched cyclic dodecapeptidolactam antibiotic that is synthesized via the thiotemplate mechanism of nonribosomal peptide synthesis (Figure 1a).[2a] It represents a primary structure of NH_2−L-Ile$_1$-L-thiazoline$_2$-L-Leu$_3$-D-Glu$_4$-L-Ile$_5$-L-Lys$_6$-D-Orn$_7$-L-Ile$_8$-D-Phe$_9$-L-His$_{10}$-D-Asp$_{11}$-L-Asn$_{12}$−COOH and consists of an N-terminal linear pentapeptide and a C-terminal heptapeptide lariat cycle which is formed between the ϵ-amino group of L-Lys$_6$ and the α-carboxyl group of L-Asn$_{12}$ (Figure 1b).[7] The most interesting structural feature of bacitracin is the thiazoline ring in position 2 of the molecule that is mainly responsible for the metal binding activity of this compound.[24] In the case of Bacitracin A, the

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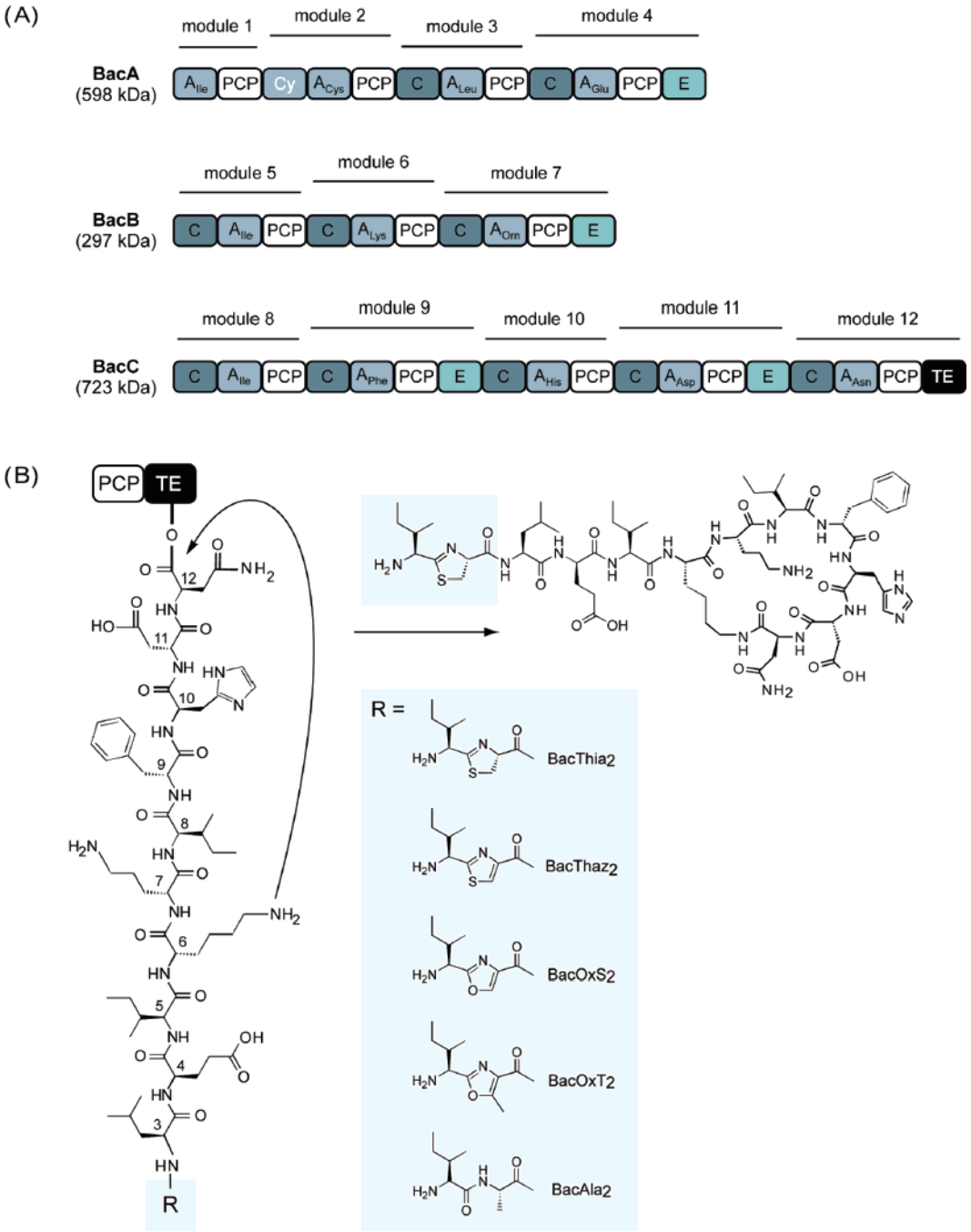

Figure 1. (a) Nonribosomal peptide synthetases (BacA-C) are responsible for the biosynthesis of Bacitracin A in *B. licheniformis* ATTC 10716. BacA (598 kDa) consists of four modules of which module 2 catalyzes the heterocyclization of PCP bound L-Cys by the Cy-domain. BacB (297 kDa) comprises three modules, and BacC (723 kDa), five modules with the C-terminal PCP-TE-bidomain, which was utilized for chemoenzymatic synthesis of the bacitracin derivatives. A = Adenylation domain, PCP = Peptidyl Carrier Protein, C = Condensation domain, Cy = Cyclization domain, E = Epimerization domain, TE = Thioesterase (Cyclase) domain. (b) Structure of Bacitracin A and the bacitracin derivatives presented in this study, highlighting the heterocyclic metal binding subunit in gray. Chemoenzymatic synthesis is obtained by the transfer of the dodecapeptide to an active site serine of the excised PCP-TE-bidomain in vitro, forming the acyl-O-TE intermediate. Subsequent product release is carried out by the attack of an internal nucleophile (L-Lys_6) on the oxoester bond to give the cyclic branched macrolactam. R = Heterocyclic subunit.

thiazoline moiety can form a strong complex in conjugation with a bivalent transition metal ion such as Zn^{2+}or Mn^{2+}.[8] This complex binds very tightly to the long chain C_{55}-isoprenyl pyrophosphate (IPP) lipid carrier which is responsible for transporting bacterial cell wall intermediates through the membrane. The formation of this complex prevents the dephosphorylation of this carrier and therefore leads to an accumulation

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of cell wall synthesis precursors and the subsequent disruption of cell wall synthesis.[9]

The formation of the macrocyclic structure is a common characteristic of nonribosomally synthesized peptides. It ensures both stability against proteolytic digestion and bioactivity. Macrocyclization is catalyzed by so-called thioesterase domains (TE-domains, cyclases) that are located at the C-termini of the most downstream peptide synthetases.[10] In the terminal step of biosynthesis the TE-domain of the bacitracin synthetase BacC catalyses the formation of a branched cyclic peptidolactam, through the nucleophilic attack of the L-Lys_6 residue onto the C-terminal L-Asn_1 bound acyl-enzyme oxoester intermediate (Figure 1b). To investigate the importance of the heterocyclic moiety of bacitracin, we made use of a chemoenzymatic approach using the excised TE-domain of bacitracin in combination with solid-phase derived linear substrates. Chemoenzymatic cyclization using excised TE-domains from different nonribosomal peptide synthetases (NRPSs) has been reported to be a versatile synthetic tool for the easy and convenient production of cyclic natural products from linear peptidyl thioester substrates.[11] Due to their great tolerance for substrate variations in length, side chains, and nucleophiles of the recognized peptide, an enormous potential arises for the derivatization of the formed macrocyclic products.[12] To create macrocyclic derivatives of Bacitracin A, we took advantage of this well-established chemoenzymatic approach. The utilization of the bacitracin thioesterase as a tool for the macrocyclization of novel synthesized precursors allows easy access to a variety of derivatives and overcomes the limiting factor of macrocyclization via total synthesis of Bacitracin A.[15]

Since unfractionated bacitracin is not suitable for systemic use due to severe side effects of Bacitracin F, bacitracin is only accessible for topical antibacterial ointments and an additive in animal feeds. Bacitracin F is a degradation product that exerts a vasoconstrictor effect in the kidneys and causes nephrotoxicity.[13] The formation of Bacitracin F is believed to occur systemically by an N-terminal deamination of Bacitracin A and the subsequent oxidation of the thiazoline moiety, resulting in this very reactive ketone species.[14] We introduce a synthetic strategy suitable to vary the heterocyclic subunit of Bacitracin A followed by thioesterase mediated cyclization to generate novel bioactive Bacitracin A derivatives.

Experimental Procedures

Cloning, Expression, and Purification of the Bacitracin PCP-TE Didomain: The bacitracin gene fragment *bac pcp-te* was amplified from chromosomal DNA of *Bacillus licheniformis* ATCC 10716 by PCR using Pfu Turbo DNA Polymerase (Stratagene) and the following oligonucleotides: Bac PCP-TE, 5′-GCG CTC CAT GGA ATA CGA GCC TC-3′ and 5′-GGA TGC CGT TTG GAT CCT ATT TTG A-3′. The PCR product was digested using restriction endonucleases *Nco*I/*Bam*HI and subsequently ligated into the expression vector pQE60 (Qiagen), which appends a C-terminal His_6 tag to the expressed protein. *Escherichia coli* TOP10 cells were used for the preparation of the recombinant plasmid. The identity of the cloning product was confirmed by DNA sequencing (GATC Biotech). Expression plasmids were used to transform *Escherichia coli* BL21 cells (Amersham Bioscience). Cells were grown to an $OD_{600} = 0.6$, induced with 0.1% IPTG, and cultivation was continued for 3 h at 30 °C. The cells were harvested by centrifugation (15 min, 4 °C, 6000 rpm) and resuspended in HEPES buffer before disruption using a french pressure cell (Avestin). The recombinant protein was purified by Ni^{2+}-NTA affinity chromatography (Amersham Pharmacia Biotech) and dialyzed against assay buffer (25mM HEPES, 50mM NaCl, pH 8.0) using HiTrap desalting columns (Amersham Pharmacia Biotech). Gel filtration (HiLoad Superdex prep grade 26/60, Amersham) was applied as final step, and the purity of the enzyme was analyzed by SDS-PAGE (15%) (Supporting Information). The protein concentration was determined spectrophotometrically using the estimated extinction coefficient (1.13 mol^{-1} dm^3 cm^{-1}) at 280 nm, and the protein was immediately flash frozen in liquid nitrogen for storage at −80 °C.

Synthesis of the Heterocyclic Building Blocks: A. 2-[1′(*S*)-(*tert*-Butyloxycarbonylamino)-2′(*S*)-methylbutyl]-4(*R*)-carboxy-thiazoline (1): The synthesis of the thiazoline unit **1** was performed as described by Lee et al.[15]

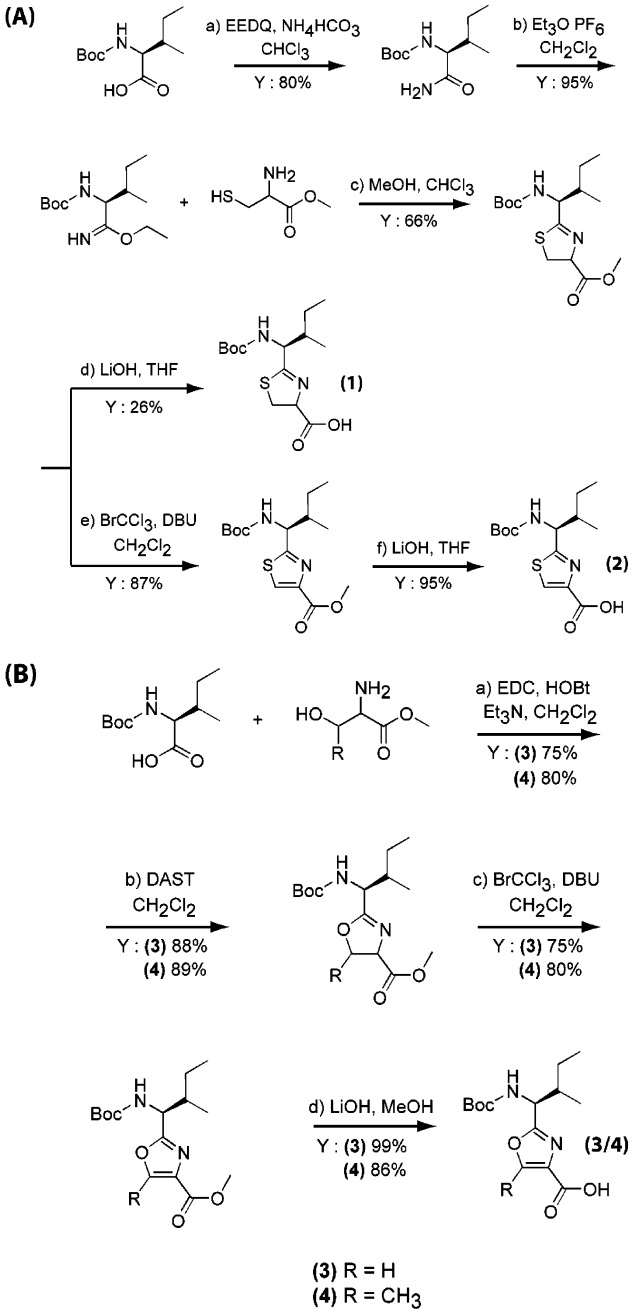
B. 2-[1′(*S*)-(*tert*-Butyloxycarbonylamino)-2′(*S*)-methylbutyl]-4-carboxy-thiazole (2): For compound **2** we used an alternative synthesis strategy as described by Hamada et al.[16] *N*-Boc-L-isoleucinyl-thiazoline methyl ester afforded in the synthesis of **1** was treated with DBU and $BrCCl_3$ to obtain the oxidized thiazole methylester (87%). Basic hydrolysis using LiOH resulted in the formation of the desired *N*-Boc-L-isoleucyl-thiazole-carboxylic acid **2** (95%)[17] (Scheme 1a); ^{1}H NMR (300 MHz, $CDCl_3$, 23 °C) $\delta = 0.80-1.00$ (m, 6H, Ile-γ,δCH_3), 1.16−1.73 (m, 11H, Ile-γCH_2, BOC−CH_3), 1.97−2.17 (m, 1H, Ile-βH), 4.82 (d, $J = 6.7$ Hz, 1H, Ile-αH), 8.28 (s, 1H, Thz-H); ^{13}C NMR (75 MHz, $CDCl_3$, 23 °C) $\delta = 11.6$ (Ile-δCH_3), 16.2 (Ile-γCH_3), 25.9 (Ile-γCH_2), 28.7 (BOC−CH_3), 40.7 (Ile-βCH), 59.0 (Ile-αCH), 80.8 (BOC−C_q), 128.7, 148.3, 157.9, 164.1, 176.2; HRMS (ESI-FT ICR): calcd for $C_{14}H_{22}N_2O_4S_1$ $[M + Na]^+$ 337.1192, found 337.1202.

C. 2-[1′(*S*)-(*tert*-Butyloxycarbonylamino)-2′(*S*)-methylbutyl]-4-carboxy-oxazole (3): The condensation of *N*-Boc-L-isoleucine and L-serine methyl ester hydrochloride provided a dipeptide (75%) that was subsequently cyclized with diethylaminosulfur trifluoride (DAST) to give the oxazoline (88%). This oxazoline ester was then oxidized using DBU and $BrCCl_3$ to the corresponding oxazole (75%). Basic hydrolysis using LiOH in aqueous methanol gave the desired *N*-Boc-L-isoleucyl-oxazole-carboxylic acid **3** (99%)[18] (Scheme 1b); ^{1}H NMR (300 MHz, $CDCl_3$, 23 °C) $\delta = 0.83-0.96$ (m, 6H, Ile-γ,δCH_3), 1.14−1.33 (m, 1H, Ile-γCH_2), 1.40 (s, 9H, BOC−CH_3), 1.44−1.64 (m, 1H, Ile-γCH_2), 1.86−2.08 (m, 1H, Ile-βH), 4.90 (m, 1H, Ile-αH), 6.33 (br s, 1H), 8.29 (s, 1H), 9.83 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, 23 °C) $\delta = 11.3$ (Ile-δCH_3), 15.3 (Ile-γCH_3), 25.2 (Ile-γCH_2), 28.3 (BOC−CH_3), 39.3 (Ile-βCH), 53.6 (Ile-αCH), 80.0 (BOC−C_q), 134.1, 144.5, 155.7, 164.0, 166.5; HRMS (ESI-FT ICR): calcd for $C_{14}H_{22}N_2O_5$ $[M + Na]^+$ 321.1421, found 321.1429.

D. 2-[1′(*S*)-(*tert*-Butyloxycarbonylamino)-2′(*S*)-methylbutyl]-4-carboxy-5-methyl-oxazole (4): The preparation of the oxazole unit **4** derived from threonine was achieved in accordance. Condensation of *N*-Boc-L-isoleucine and L-threonine methyl ester hydrochloride led to the dipeptide (80%). Cyclodehydration was achieved by treatment with DAST in CH_2Cl_2 to give 5-methyl-oxazoline (89%).This oxazoline ester was then oxidized using DBU and $BrCCl_3$ to the corresponding 5-methyl-oxazole (80%). Basic hydrolysis using LiOH in aqueous methanol gave *N*-Boc-L-isoleucyl-5-methyl-oxazole-carboxylic acid **4** (86%)[19] (Scheme 1b); ^{1}H NMR (300 MHz, $CDCl_3$, 23 °C) $\delta = 0.81-0.96$ (m, 6H, Ile-γ,δCH_3), 1.14−1.35 (m, 1H, Ile-γCH_2), 1.39 (s, 9H, BOC−CH_3), 1.45−1.63 (m, 1H, Ile-γCH_2), 1.90−2.05 (m, 1H, Ile-βH), 2.66 (s, 3H, Oxa-CH_3), 4.77−4.89 (m, 1H, Ile-αH), 6.77 (br s, 1H), 11.18 (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$, 23 °C) $\delta = 11.2$ (Ile-δCH_3), 12.0 (Oxa-CH_3), 15.3 (Ile-γCH_3), 25.1 (Ile-γCH_2), 28.3 (BOC−CH_3), 39.2 (Ile-βCH), 53.4 (Ile-αCH), 79.6 (BOC−C_q), 127.1, 155.9, 156.9, 163.8, 164.6; HRMS (ESI-FT ICR): calcd for $C_{15}H_{24}N_2O_5$ $[M + Na]^+$ 335.1577, found 335.1577.

Synthesis of Linear Thiophenol Bacitracin Substrates: All linear peptides were synthesized by solid-phase peptide synthesis (SPPS) on an Advanced Chem Tech APEX 396 synthesizer (0.1 mmol scale). The N-terminal heterocyclic building blocks were coupled to the resin bound decapeptide in the final synthesis step according to standard

Scheme 1. (A) Synthesis of Thiazoline **1** and Thiazole **2** Building Block; (B) Synthesis of Oxazole (Ser) **3** and Oxazole (Thr) **4** Building Block[a]



[a] Reagents and conditions: (A) (a) *N*-Boc-L-Ile-OH (1.0 equiv), EEDQ (1.1 equiv), NH_4HCO_3 (3.0 equiv), $CHCl_3$, rt, 12 h; (b) Et_3OPF_6 (1.1 equiv), CH_2Cl_2, 25 °C, 18 h; (c) L-Cys-methyl ester (1.5 equiv), $CHCl_3$, 0 °C, 2 h, then rt, 36 h; (d) LiOH (2.0 equiv), THF, 0 °C, 1 h; (e) BrCCl3 (1.1 equiv), DBU (1.1 equiv), CH_2Cl_2, 0°, 3 h; (f) LiOH (2.0 equiv), THF, 0 °C, 15 min, then rt, 1 h. (B) (a) *N*-Boc-L-Ile-OH (1.0 equiv), L-Ser(Thr)-methyl ester (1.0 equiv), EDC (1.1 equiv), HOBt (1.1 equiv), CH_2Cl_2, rt, 5 min; then Et_3N (2.0 equiv), rt, 15 h (12 h); (b) DAST (1.5 equiv), CH_2Cl_2, −78 °C, 1 h; (c) $BrCCl_3$ (1.1 equiv), DBU (1.1 equiv), CH_2Cl_2, 0 °C, 6 h; (d) LiOH (2.0 equiv), THF, 0 °C, 15 min, then rt, 1 h.

procedure. Preparation and purification of the peptidyl-thiophenol substrates were performed as described elsewhere.[20] All products were verified by MALDI-TOF and ESI-FT-ICR mass spectrometry (Table 2). **Ala**$_2$ was generally synthesized on a solid phase support using Fmoc-L-alanine for position 2 of the molecule.

Assays of the Peptidyl-thiophenol Substrates: Initial enzymatic reactions were carried out in assay buffer (25 mM Hepes, 50 mM NaCl, pH 8.0) in a total volume of 50 μL. The substrate concentration was 150 μM for standard reactions and varied during kinetic investigations. The reaction was initiated by the addition of Bac PCP-TE to a final concentration of 1 μM (Figure 2). Reactions were quenched by the addition of 50 μL of 4% TFA/H_2O after various time points, and cyclization reactions were monitored by analytic HPLC-MS (Macherey and Nagel, Nucleodur CC 125−2 C-ec$_{18}$ RP column) under the following conditions: flow rate, 0.7 mL min^{-1}; column temperature, 40 °C; applied gradient, 20%−50% MeCN (0.1% TFA) in H_2O (0.1% TFA) for 20 min. The identity of the products was analyzed by both accurate mass (ESI−FT-ICR) and MS−MS sequencing, to verify the connection regiospecificity of the cyclic products (Supporting Information). Kinetic characterization of the cyclization reaction was performed by determining initial rates of eight substrate concentrations using two time points at each concentration within the linear region of the enzyme verified by time courses.

Preparation of Cyclic Bacitracin A and Bacitracin A Derivatives for Bioactivity Assays: For bioactivity studies semipreparative scale generation of the cyclic products was carried out in a total volume of 2 mL utilizing 5 μM purified Bac PCP-TE and 300 μM peptidyl thiophenol substrate for 1 h at ambient temperature. After quenching with 4% TFA/H_2O the mixture was directly injected on a 250/21 Nucleodur 100-5 C_{18} RP column (Macherey and Nagel) by applying a gradient from 20%−50% MeCN (0.1% TFA) in H_2O (0.1% TFA) over 30 min at a flow rate of 12 mL min^{-1}. The purity and identity of the obtained products were analyzed by ESI-FT-ICR measurements (Supporting Information). After flash freezing in liquid nitrogen, the products were lyophilized and the concentrations were calculated based on an internal standard (**Bac**$_{Ref}$) by HPLC analysis at 220 nm. The extinctions coefficients were assumed to be identical to all synthesized cyclic products. The yield of the obtained macrocyclic products was ∼0.7 mg as calculated for **BacThia**$_2$.

Determination of Bioactivity: For MIC determination of the synthesized Bacitracin A and its derivatives 2-fold serial dilutions of the cyclic products and authentic Bacitracin A (for purification, see Supporting Information) were prepared in 96-well microtiter trays as described previously,[12a] using LB media containing 67.6 mg of Zn^{2+}/L. 80 μL of a 1/10 000 diluted overnight culture of *Bacillus subtilis* PY79 were added to each well and incubated at 37 °C for 20 h prior to optical determination of turbidity and calculation of corresponding MICs.

Results

Characterization of the Chemoenzymatic Cyclization Reaction Utilizing Bacitracin PCP-TE: Various reports described the utility of excised thioesterases for the formation of macrocyclic products and their derivatives. To explore the cyclization activity of the recombinantly produced bacitracin thioesterase (Bac PCP-TE) with artificial linear bacitracin thioester substrates, we generated a bimodular system of the bacitracin TE-domain together with its native *peptidyl-carrier protein* (PCP). This ensures correct folding of the N-terminal part of the cyclase, and therefore optimal cyclization activities as PCPs seem to possess an impact on their neighboring domains.[20,21] The *bac pcp-te* fragment of the bacitracin biosynthetic cluster was amplified from chromosomal DNA of *Bacillus licheniformis* ATCC10716, expressed heterologously in *E. coli* at 30 °C, purified to homogeneity as soluble protein, and analyzed by SDS-PAGE (Supporting Information). The recombinant protein (36.4 kDa) was obtained with a total yield of 8.7 mg/L LB-medium. To investigate the ability of Bac PCP-TE to catalyze macrocyclization of peptidyl thioesters, we synthesized in an initial approach a peptide thioester analogue of the natural Bacitracin A replacing the thiazoline residue in position

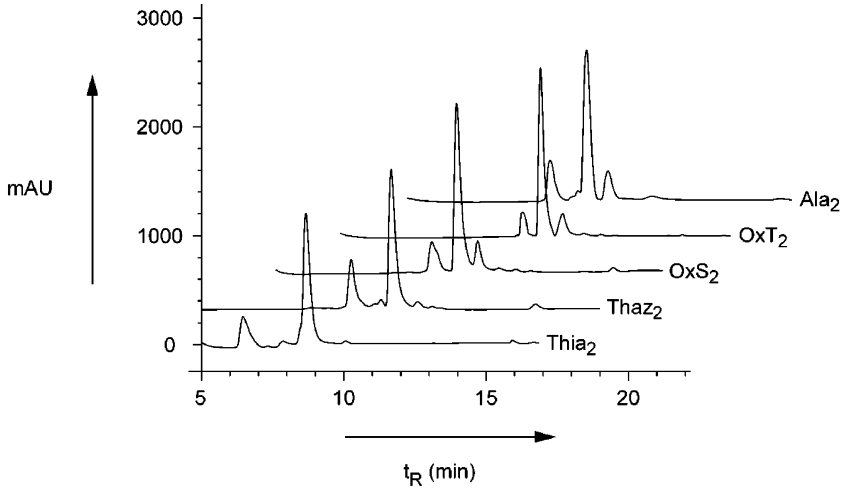


Figure 2. Three-dimensional overlay of HPLC traces of Bac PCP-TE cyclase (1 μM) incubated with presented linear bacitracin thiophenol substrates (150 μM) at ambient temperature. Reactions were stopped after 1 h with 4% TFA. Reaction volume was 50 μL. Leading peak = hydrolysis; dominant peaks represent collected seven-membered lariat cycle as controlled by MS−MS sequencing, minor peak following product in **OxS**$_2$, **OxT**$_2$, and **Ala**$_2$ = six-membered cycle.

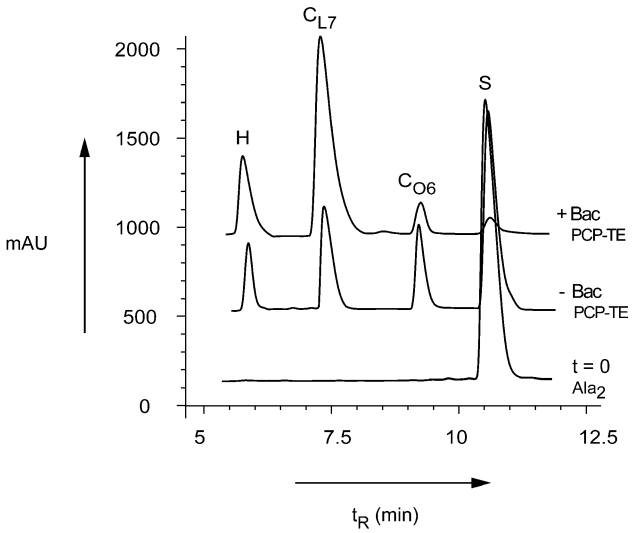


Figure 3. Trace 1 (t = 0; Ala$_2$): linear thiophenol substrate **Ala**$_2$ stopped with 4% TFA after 0 min of incubation with the cyclase. Trace 2 (− Bac PCP-TE): **Ala**$_2$ without incubation with the cyclase for 45 min. Trace 3 (+ Bac PCP-TE): Bac PCP-TE cyclase (1 μM) incubated with **Ala**$_2$ for 45 min. H = Hydrolysis, C_{L7} = seven-membered cycle (Lys$_6$), C_{O6} = six-membered cycle (Orn$_7$), S = substrate (**Ala**$_2$).

2 by alanine. The C-terminus of the peptide was activated with the SNAC leaving group that mimics the thiol-containing part of the ppant arm of the natural cofactor. This peptidyl SNAC substrate was subsequently incubated with the excised cyclase and analyzed by reversed-phase HPLC/MS. No formation of the desired bacitracin derivative could be detected which might be due to the insufficient reactivity of the SNAC leaving group for enzyme acylation. To address this problem we decided to use peptidyl thiophenol substrates which indeed allowed biochemical characterization of the Bac PCP-TE cyclase.[22] The incubation of this thiophenol substrate **Ala**$_2$ with Bac PCP-TE led to the formation of the expected cyclic product (Figure 3). Interestingly, the reaction profile of **Ala**$_2$ revealed two peaks with the expected mass (m/z = 1407.8) for the cyclization product. Taking into account that the linear bacitracin substrate possesses two neighboring nucleophiles, L-Lys$_6$ and D-Orn$_7$, it was assumed that both nucleophiles are involved in ring

Table 1. Kinetic Values for the Cyclization Reactions Catalyzed by Bac PCP-TE[a]

compound	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}mM^{-1}$)	cyclization/ hydrolysis	$cycle_{L7}$/ $cycle_{O6}$
$BacThia_2$	0.197	0.79	4.01	3.8:1	91.0:1
$BacThaz_2$	0.226	0.77	3.41	2.9:1	72.2:1
$BacOxS_2$	0.248	0.70	2.94	4.6:1	8.1:1
$BacOxT_2$	0.262	0.66	2.52	5.2:1	8.8:1
$BacAla_2$	0.202	0.74	3.66	2.3:1	7.1:1

[a] All values were determined in triplicate. Results shown are the average of three independent assays and were corrected concerning spontaneous hydrolysis and cyclization. Standard deviation is <8% in all cases.

formation. To prove that no other nucleophiles except the mentioned L-Lys$_6$ and D-Orn$_7$ contributed to cyclization, we synthesized a thiophenol substrate, replacing both nucleophiles by alanine, respectively. HPLC analysis confirmed our expectation as no cyclization and only flux toward hydrolysis could be detected after incubation with Bac PCP-TE. To confirm the identity of both observed peaks, MS−MS sequencing was performed, assigning that the minor cyclization peak (t_R = 9.25 min) was constituted by the internal nucleophile D-Orn$_7$ and the major product (t_R = 7.14 min) obtained through the internal attack of the desired side chain amino group of L-Lys$_6$ (Supporting Information). The ratio between these regioisomeric macrolactams was determined as 7:1 in favor of the heptapeptide cycle (L-Lys$_6$). Kinetic studies revealed that cyclization occurred with a k_{cat}/K_M value of 3.66 min^{-1} mM^{-1} determined for the formation of the heptapeptide cycle (**BacAla**$_2$). Hydrolysis, occurring in almost all chemoenzymatic cyclization reactions, constitutes 34% of the obtained products (Table 1). To inhibit the formation of the hydrolytic side product, assays were carried out with a lyophilized enzyme in a variety of organic solvents but did not lead to significant product formation.[23] The reaction profile of the control reaction, the substrate without Bac PCP-TE for 1 h at ambient temperature, revealed the spontaneous formation of the two cyclic products and hydrolysis (Figure 3). The ratio between these regioisomeric macrolactams was determined as 1.3:1 in favor of the heptapeptide cycle (L-Lys$_6$). This spontaneous cyclization and hydrolysis pattern were observed for all bacitracin thiophenol substrates presented in

Table 2. ESI-FT-ICR Mass Spectrometric Characterization of Linear Thiophenol Substrates and Cyclic Products

	linear substrate			cyclic product		
compound	species (*m*/*z*)	calculated	observed	species (*m*/*z*)	calculated	observed
$BacA_{Ref}$	n.a.[a]	n.a.	n.a.	$([M + Na]^+)$	1444.7382	1444.7385
$BacThia_2$	$([M + H]^+)$	1532.7680	1532.7698	$([M + Na]^+)$	1444.7382	1444.7406
$BacThaz_2$	$([M + H]^+)$	1530.7596	1530.7654	$([M + 2H]^{2+})$	710.8739	710.8746
$BacOxS_2$	$([M + H]^+)$	1514.7824	1514.7903	$([M + 2Na]^{2+})$	724.8673	724.8689
$BacOxT_2$	$([M + H]^+)$	1528.7981	1528.7986	$([M + 2Na]^{2+})$	731.8751	731.8780
$BacAla_2$	$([M + H]^+)$	1518.8162	1518.8166	$([M + 2Na]^{2+})$	726.8829	726.8842

[a] n.a. = not applicable.

this study. To further characterize the cyclization potential of Bac PCP-TE, we synthesized two bacitracin thiophenol substrates, carrying either D-Asn_1 or D-Lys_6, to gain insight into the stereoselectivity of the cyclase. For the selective examination of the cyclic products the second nucleophile (D-Orn_7) was replaced by alanine. Incubation of these peptidyl substrates resulted only in the formation of hydrolysis product, indicating the importance of the L-configured nucleophile Lys_6 and the C-terminal Asn_1 for the enzyme-mediated cyclization (data not shown).

Chemoenzymatic Synthesis of Bacitracin A: Our results with the thiophenol substrate Ala_2 clearly showed that the recombinant Bacitracin PCP-TE catalyzes the macrocyclization of bacitracin thiophenol substrates in sufficient yields. In this context we focused on the synthesis of native Bacitracin A using the recombinant cyclase. Synthesis of the authentic linear bacitracin A thiophenol (**Thia**$_2$) precursor required the production of an N-terminal thiazoline unit **1** of the molecule since commercial precursors are not available. The synthesis of this precursor was performed from *N*-Boc-L-isoleucine and L-cysteine methyl ester according to Lee et al.[15] (Scheme A). The resulting building block was subsequently coupled to the resin-bound decapeptide, synthesized by traditional Fmoc chemistry. The activation with thiophenol led to the formation of the desired linear bacitracin substrate **Thia**$_2$. After incubation of this **Thia**$_2$ substrate with Bac PCP-TE for 1 h, the chromatographic analysis showed the formation of the expected cyclic product (t_R = 10.81 min) Bacitracin A (**BacThia**$_2$) in sufficient yield (79%) (Figure 4, Table 2). Remarkably, the conversion of the linear substrate into the macrocyclic **BacThia**$_2$ yielded mainly the desired heptapeptide product. With a ratio of 91:1 in favor of the cyclization by L-Lys_6, the formation of the undesired macrolactam derived from cyclization via L-Orn_7 was nearly abolished. The cyclization reaction of **BacThia**$_2$ follows Michaelis−Menten kinetics with a K_M of 0.197 mM and a k_{cat} value of 0.79 min^{-1} (Table 1). Probably due to the better alignment in the hydrophobic core of the enzyme's active site compared to the **Ala**$_2$ substrate, the cyclization reaction of **Thia**$_2$ shows a decrease in hydrolysis with a cyclization-to-hydrolysis ratio of 3.8:1. The identity of the obtained synthetic product was analyzed by FT- ICR measurement (Table 2) and comparison of the retention time with that of the commercially available purified Bacitracin A (**BacA**$_{Ref,}$ t_R = 10.81 min) (Figure 4).

Generation of Bacitracin A Derivatives through Alteration of the Heterocyclic Subunit: Successful incorporation of the thiazoline subunit into the bacitracin molecule encouraged us to synthesize a variety of different five-membered heterocyclic building blocks (Figure 1b). To obtain the oxidized thiazole unit **2** the thiazoline-methyl ester was oxidized using 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) and $BrCCl_3$ (Scheme 1a). After

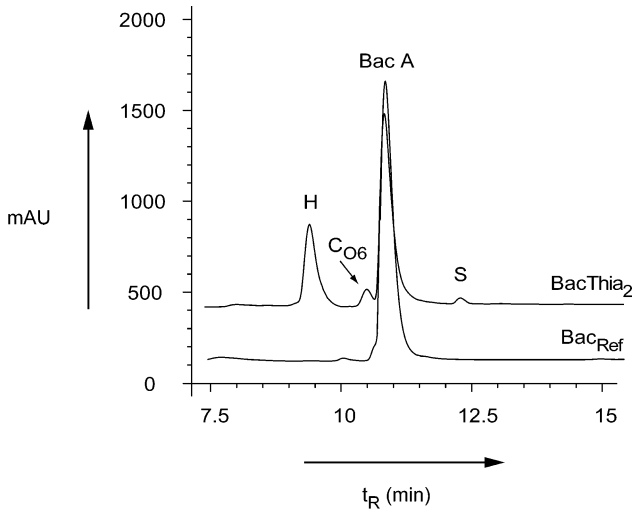


Figure 4. Overlay of HPLC traces of chemoenzymatic generated Bacitracin A (Trace **BacThia**$_2$) and HPLC-purified commercial Bacitracin A (Trace **Bac**$_{Ref}$). H = Hydrolysis, C_{O6} = six-membered cycle (Orn_7), Bac A = Bacitracin A, S = substrate (**Thia**$_2$).

coupling of this precursor to the decapeptide, the linear thiophenol substrate **Thaz**$_2$ was obtained. **Thaz**$_2$ was incubated with Bac PCP-TE, and as expected cyclic product formation of $BacThaz_2$ was detected by HPLC analysis. The kinetic parameters for the Bac PCP-TE catalyzed cyclization were determined as a K_M of 0.226 mM and k_{cat} of 0.77 min^{-1} (Table 1). The cyclization-to-hydrolysis ratio of 2.9:1 was higher than that for the corresponding thiophenol substrate carrying the thiazoline (**Thia**$_2$). In fact, this result and the regioisomeric ratio of 72:1, for the cyclization via the desired heptapeptide, provide evidence for the contribution of the oxidation level of the heterocyclic moiety of the molecule for the recognition of the substrate by Bac PCP-TE.

As oxazolines and oxazoles are a common feature of naturally occurring peptides for the formation of complexes with divalent ions, our goal was to incorporate oxazoline or oxazole moieties into the molecule to alter its pharmacological profile but retain the complex formation potential. For the synthesis of the oxazoline and the corresponding oxazole units **3** (serine) and **4** (threonine), we altered the synthesis approach of the building blocks accordingly (Scheme 1b). The conversion of dipeptides containing β-hydroxy amino acids into five-membered oxazolines using diethylaminosulfur trifluoride (DAST) and their further oxidation with DBU and $BCCl_3$ to the corresponding oxazoles was performed as described by Philipps et al.[18b] Both oxazole units could be synthesized in good yields (Supporting Information) and were coupled to the described decapeptide. The linear thiophenol substrates **OxS**$_2$ and **OxT**$_2$ were incubated with Bac PCP-TE and successfully converted into the desired

Table 3. Determination of the Minimum Inhibitory Concentration (MIC_{90}) of Cyclic Bacitracin Derivatives against *Bacillus subtilis* PY79[a]

compound	MIC_{90} (μg/mL) at 67.6 mg/L $ZnSO_4$
$BacA_{Ref}$	4
$BacThia_2$	4
$BacThaz_2$	12
$BacOxS_2$	40
$BacOxT_2$	12
$BacAla_2$	>800

[a] Cyclic Bacitracin derivatives including authentic Bacitracin A as reference were prepared in microtiter plates as described earlier.[12a]

cyclic products **BacOxS**$_2$ and **BacOxT**$_2$. The regioselectivity of the cyclization reaction was dramatically decreased to ratios of 8.1:1 for **BacOxS**$_2$ and 8.8:1 for **BacOxT**$_2$, indicating a weaker recognition of the substrates by the cyclase due to their exchanged heterocyclic subunits. Accordingly, the K_M value increased to 0.248 mM for **OxS**$_2$ and 0.262 mM for **OxT**$_2$ (Table 1). The determination of k_{cat} revealed a turnover of 0.70 min^{-1} for **OxS**$_2$ and 0.66 min^{-1} for **OxT**$_2$. The formation of the linear product due to hydrolysis of the acyl-enzyme intermediate was significantly lower with a ratio of 4.6:1 for **OxS**$_2$ and 5.2:1 for **OxT**$_2$ than that for the native linear bacitracin substrate **Thia**$_2$.

Bioactivity of the Generated Bacitracin A and Bacitracin Derivatives: To elucidate the structural importance of the heterocyclic moiety for the antibacterial activity of the bacitracin derivatives, we found Bac PCP-TE to be a suitable catalyst for the preparative generation of cyclic Bacitracin A and derivatives **BacThaz**$_2$, **BacOxS**$_2$, **BacOxT**$_2$, and **BacAla**$_2$ (Figure 2). Cyclization of the macrocyclic products was performed on a semipreparative scale, and the cyclic products were HPLC purified. To ensure the comparability of the obtained products the concentration was determined using an internal standard **BacA**$_{Ref}$. Antibiotic activity was determined against *Bacillus subtilis* PY79 using the dilution method.[12a] In accordance with previous studies, the bioactivity of bacitracin strongly depended on the concentration of Zn^{2+} ions.[24] A control reaction with $ZnSO_4$ (80 mg/L) revealed no effect on bacterial growth. Several concentrations were tested leading to the result that the addition of 67.6 mg/L $ZnSO_4$ was needed to obtain the lowest MIC values. Further increase in $ZnSO_4$ concentration had no influence on antibiotic activity. In contrast, decreasing the $ZnSO_4$ concentration lower than 50 mg/L resulted in dramatically higher MIC values, revealing the importance of sufficient Zn^{2+} concentration for complex formation and inhibition of cell wall biosynthesis.

Starting from this point we compared the antibiotic activity of synthetic **BacThia**$_2$ with commercially available purified **BacA**$_{Ref}$. Both products revealed an MIC value of 4 μg/mL (Table 3). Considering the structural coincidence of both products, the comparable MIC values proved the accuracy of our synthesis approach. To explore whether the exchange of the heterocyclic subunit would lead to a loss of antibacterial activity, we determined the MIC values of the generated derivatives. Remarkably, **BacThaz**$_2$ containing the thiazole moiety compared to authentic **BacA**$_{Ref}$ revealed only a 3-fold higher MIC (12 μg/mL). Taking into account that thiazoles as well as thiazolines can complex bivalent ions, this increase was unexpected since both products only vary in the oxidation state of the heterocycle. To elucidate if the heterocyclic variants, containing oxygen instead of sulfur, were able to form a complex with Zn^{2+} as efficiently as authentic Bacitracin A, we tested **BacOxS**$_2$ and **BacOxT**$_2$ for bioactivity against *B. subtilis* PY79. The MIC values of **BacOxS**$_2$ (40 μg/mL) and **BacOxT**$_2$ (12 μg/mL) were therefore 10-fold and 3-fold higher than those of authentic Bacitracin A. In contrast, the screen for bioactivity of the **BacAla**$_2$ derivative did not show any bactericidal activity (MIC > 800 μg/mL). In summary, these results emphasize the importance of the heterocycle for the bioactivity of bacitracin.

Discussion

Modification in the peptide backbone of natural compounds is accompanied with an increase of bioactivity and stability.[2b] The overall aim of our study was to elucidate the impact of the substitution of the heterocyclic thiazoline subunit of Bacitracin A with respect to the macrocyclization using a well established chemoenzymatic approach and the production of novel bioactive bacitracin derivatives. To obtain bacitracin derivatives, we generated a library of naturally derived heterocyclic building blocks that could successfully substitute the native heterocyclic subunit. These novel hybrid agents were cyclized utilizing the recombinantly produced Bacitracin PCP-TE and its substrate tolerance in vitro. Starting from a linear bacitracin thiophenol precursor carrying alanine at position 2, the formation of two regioisomeric macrolactams could be detected. These regioisomers might arise from the simultaneous nucleophilic attack of the two adjacent nucleophiles L-Lys_6 and D-Orn_7 onto the C-terminus of the acyl enzyme intermediate. The preference for the seven-membered lariat cycle might be explained by a referenced positioning of L-Lys_6 in the active site of the enzyme. Interestingly, both cyclic products were likewise detected spontaneously in the absence of the cyclase. As previously reported for the macrolactam tyrocidine, this observation is due to a preorganization of the linear precursor and the high reactivity of the thiophenol leaving group.[25] In contrast, the incubation of the cyclase with this bacitracin analogue favors the desired generation of the seven-membered lactam. Moreover, quantitative regioselective cyclization occurs if a substrate representing the authentic primary bacitracin sequence is incubated with the cyclase. These results are in accordance with previous studies in which the importance of the authentic fatty acid length for regiospecific cyclization of the lipopeptide antibiotic CDA cyclase was demonstrated.[26] In the case of the Bacitracin PCP-TE, the recognition of the heterocycle in the active site might be the explanation for regioselective enzymatic cyclization. Moreover, the efficient transformation of the linear bacitracin thiophenol substrate into the authentic Bacitracin A (yield 79%) utilizing the recombinant cyclase demonstrated the efficiency of this chemoenzymatic approach. It also raised the question if this approach could be used for the generation of bacitracin derivatives carrying heterocyclic variants in position 2 of the molecule. In fact, the macrocyclization of linear bacitracin derivatives revealed that the cyclase is tolerant toward substitutions of this position. Macrolactamization of all derivatives compared to authentic **Thia**$_2$ substrate occurred with comparable k_{cat}/K_M values, indicating that the substitution of

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the heterocyclic subunit did not significantly impair the recognition of the peptide by the cyclase. In conclusion, the identity of the heterocyclic subunit did not reduce the catalytic efficiency but was mainly important for the regiospecific macrocyclization of the cyclase. Bacitracin PCP-TE therefore provides a versatile tool for the cyclization of bacitracin derivatives with altered metal binding subunits. Alteration of the heterocyclic subunit enabled us to unveil the antibiotic potential of the selected cyclic variants **BacThia**$_2$, **BacThaz**$_2$, **BacOxS**$_2$, **BacOxT**$_2$, and **BacAla**$_2$ (Table 3). All chemoenzymatically produced bacitracin derivatives tested in this study displayed bioactivity against *B. subtilis* PY79. Interestingly, the serine derived oxazole, which is closely related to the authentic bacitracin heterocycle, did not show higher bioactivity, whereas the threonine derived oxazole carrying an extra methyl group revealed an MIC comparable to that of the authentic product (Table 3). BacAla$_2$, lacking the metal binding heterocycle, did not show any bactericidal activity against *B. subtilis* PY79. This underscores the importance of the ability to form a stable complex with a bivalent ion for bioactivity of this antibiotic class.

In summary, we demonstrated that the heterocyclic subunit of bacitracin is the key factor for the bioactive potential of this antibiotic against Gram positive bacterial infections. Moreover, we could demonstrate that through alteration of this heterocyclic moiety, neither bioactivity for the tested strain *B. subtilis* PY79 nor cyclization efficiency of Bac PCP-TE dramatically altered. In this context, the successful utilization of the excised Bac PCP-TE as a molecular tool in a chemoenzymatic approach was shown for the first time and opens the gate to a rapid screening of novel cyclic bioactive bacitracin derivatives. Our findings clearly point out that variation of the heterocyclic subunit in naturally derived compounds might lead to higher diversification of bacitracin antibiotics to gain more suitable derivatives to cope with acute medicinal infectious and microbial resistance problems.

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Supporting Information Available: Experimental Data for the Synthesized *N*-Boc heterocycles, SDS-gel of Bac PCP-TE, MS−MS sequencing data of the cyclic products, and purification procedures of Bacitracin A. This material is available free of charge via the Internet at http://pubs.acs.org.

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