Next generation bacitracin: reimagining a classic antibiotic

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Structural exploration of the bacitracin scaffold

Parts of this chapter have been published in:

N. Buijs, H. C. Vlaming, M. J. van Haren and N. I. Martin, *ChemBioChem*, 2022, **23**, e202200547.

Abstract

Bacitracin A is a macrocyclic peptide antibiotic commonly used in topical formulations to treat Gram-positive bacterial infections. It operates *via* a unique mechanism of action by selectively binding to undecaprenyl pyrophosphate (C_{55}PP), an essential lipid carrier involved in bacterial cell wall biosynthesis. However, the fragility of bacitracin's N-terminal aminothiazoline moiety, and its limited structural exploration, have limited its use as a systemic drug. In this chapter, we prepared novel analogues of bacitracin A, focusing on the replacement of the aminothiazoline motif with other known zinc-binding motifs. An alanine scan was also performed in order to reveal the contribution of individual amino acid residues to bacitracin's antibacterial activity. The antibacterial activities of these analogues were evaluated, providing insights into the structural requirements for potent activity while retaining bacitracin's unique mechanism of action. These findings may contribute to the future optimisation of bacitracin and its analogues as effective antibacterial agents.

Introduction

The bacitracins are a family of macrocyclic peptide antibiotics, the main component of which is bacitracin A (BacA **(1)**, **Fig. 1A**). Bacitracin was discovered in 1943 by Johnson, Anker and Meleney who reported its isolation from a strain of *Bacillus subtilis*.[1,2] Today, bacitracin is commonly used in topical formulations to treat skin infections caused by Gram-positive bacteria, including *Staphylococcus aureus* and *Streptococcus pyogenes*.

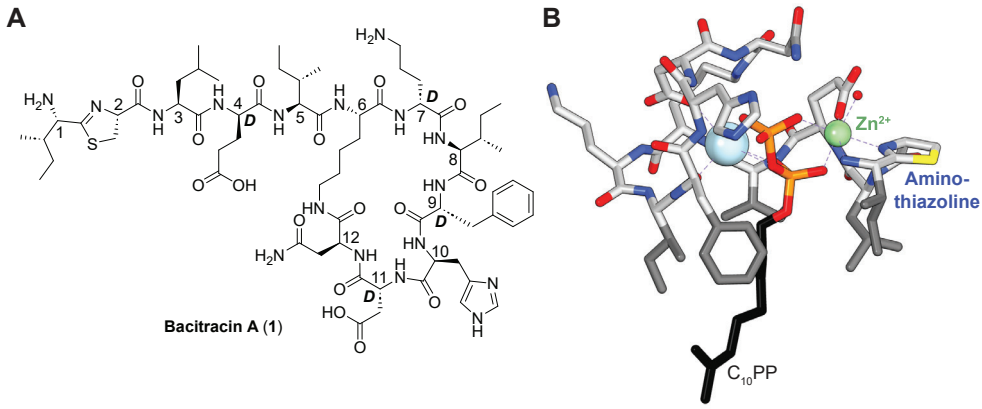


Figure 1. (A) The chemical structure of bacitracin A (**1**). **(B)** Crystal structure of bacitracin A bound to C_{10}PP mediated by Zn^{2+} (green) and Na^{+} (blue). The C_{10}PP pyrophosphate head group is coloured red and orange with the lipid tail in black. Produced using PyMol from PDB file 4K7T.[4]

Bacitracin operates *via* a unique mechanism of action not observed for any other clinically used antibiotics.[3] Bacitracin selectively binds to undecaprenyl pyrophosphate (C_{55}PP), an important lipid carrier involved in transporting the building blocks of the bacterial cell wall from the cytosol to the extracellular space. In binding to C_{55}PP bacitracin interrupts bacterial cell wall biosynthesis preventing the dephosphorylation of C_{55}PP and its subsequent recycling back into the cell wall biosynthetic cycle.[5,6]

Bacitracin's activity is enhanced in the presence of divalent metal ions, with the Zn^{2+} complex offering the highest potency.[7] In 2013 Loll and co-workers published the crystal structure of bacitracin bound to geranyl pyrophosphate in the presence of Zn^{2+}, revealing the role of this ion (**Fig. 1B**).[4] The Zn^{2+} ion bridges the peptide and pyrophosphate, with the N-terminal aminothiazoline playing an essential role in Zn^{2+} chelation. These structural insights also provide an explanation for the lack of activity observed for bacitracin F, the degradation product of bacitracin A formed by oxidative deamination of the aminothiazoline to the corresponding ketothiazole (**Scheme S1**).[8,9] Bacitracin F is also nephrotoxic and its

formation *in vivo* has limited the systemic use of bacitracin.[10] Moreover, it is well-known that the aminothiazoline moiety is sensitive to epimerisation at both low and high pHs, which generate lower potency isomeric forms of bacitracin.[11–13] Taken together, these factors encapsulate both the necessity and fragility of bacitracin's aminothiazoline. To date, attempts to replace this motif with alternate heterocycles have in all cases led to analogues with reduced antibacterial potency.[14]

Despite these challenges, bacitracin remains in widespread use.[3] One reason for this is that C_{55}PP is an excellent antibacterial target. It is essential to cell viability, ubiquitous in the bacterial world, and as a non-peptidic compound, less susceptible to mutation compared to other targets.[15] Considering that bacitracin is the only antibiotic that exploits this target, it stands to reason that structural exploration of this antibiotic may facilitate the optimisation of this compound. This, in turn, could further improve its efficacy while addressing the structural liabilities that have up to now limited its applications as a systemic drug. Indeed, it is already known that bacitracin can tolerate some level of structural variation while retaining antibacterial activity. Natural preparations of bacitracin contain a variety of related compounds, the most active of which are bacitracin A, B_1, B_2, and B_3.[16] Bacitracin B_1, B_2, and B_3, bear a valine in place of an isoleucine at positions 1, 8 and 5 respectively.[9]

In this chapter, we utilise our previously reported improved total synthesis of bacitracin A to further explore its peptidic scaffold.[12] In doing so, we aim to gain insights into the contribution of the individual amino acid residues within bacitracin towards its antibacterial activity. A number of analogues wherein the aminothiazoline moiety is replaced with alternate zinc-binding motifs were also prepared and their antibacterial activities evaluated. These findings will serve to inform the design of future bacitracin analogues that can more effectively exploit its unique mechanism of action.

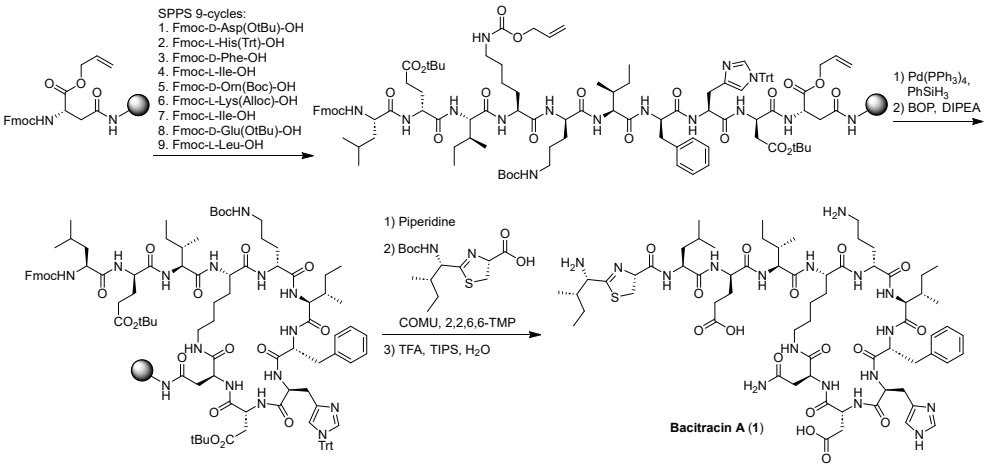
Results and Discussion

Exploring alternatives to the N-terminal aminothiazoline

In bacitracin, the N-terminal aminothiazoline moiety is formed biosynthetically through the enzymatic condensation of L-Ile and L-Cys.[3] The challenges associated with the synthesis and coupling of the aminothiazoline (discussed in **Chapter 2**), as well as its established role in the systemic toxicity of bacitracin F,[9,17] prompted us to explore alternative N-terminal moieties for bacitracin including known zinc-binding motifs. The crystal structure of bacitracin A

bound to geranyl pyrophosphate makes clear the role played by a Zn^{2+} ion in complexing both the antibiotic and phospholipid, a finding that also explains the zinc-dependency of bacitracin's antibacterial activity.[4] We therefore examined the potential for replacing the N-terminal aminothiazoline of bacitracin with alternative zinc-binding motifs such as bipyridyl and dipicolinic acid moieties. In addition, alternate dipeptides were incorporated that maintained the N-terminal Ile residue while replacing the Cys2 residue with L-alanine, D-alanine, or glycine. These analogues were prepared to probe the relative importance of the thiazoline heterocycle relative to the free N-terminal amino group. Finally, we were interested in preparing an alternate thiazoline dipeptide formed from alanine and cysteine. This would maintain the thiazoline ring and adjacent amino group, both involved in Zn^{2+} binding. At the same time, it would replace the naturally occurring isoleucine side chain with the simple methyl group present in alanine. This substitution aims to determine the relative impact of this hydrophobic moiety on the activity of bacitracin while preserving the aminothiazoline motif.

These unnatural analogues of bacitracin were prepared using our combined solid- and solution-phase synthesis (**Scheme 1**).[12] This route was described in detail in **Chapter 2**. Briefly, the general synthesis of the linear peptide precursors commences by first loading Fmoc-L-Asp(OH)-OAllyl through its side chain onto Rink amide resin. Following final acid treatment, this generates the C-terminal L-Asn12. The peptides were then extended through nine cycles of SPPS until L-Leu3, generating linear peptides ten amino acids in length. Notably, the side chain amine of L-Lys6 was Alloc protected. These resin-bound 10mers were then treated with palladium tetrakis which led to simultaneous deprotection of the Allyl and Alloc



Scheme 1. The general synthetic route used for the total synthesis of all bacitracin analogues with bacitracin A (**1**) shown as an example. For an in depth discussion of the specifics of the synthetic route see **Chapter 2**.

protecting groups of Asn12 and Lys6 respectively. Next, on-resin cyclisation was achieved through treatment with BOP and DIPEA, cleanly generating the heptapeptide macrocyclic core. The cyclised peptides were then treated with piperidine, mediating N-terminal Fmoc deprotection. Subsequent coupling of various N-terminal dipeptides afforded the finished protected analogues. Finally, simultaneous global deprotection and resin-cleavage was achieved with a TFA cleavage cocktail, affording the crude peptides. These were subsequently purified using RP-HPLC.

The synthesis of bacitracin analogues **2–6** followed that of bacitracin itself as outlined in **Scheme 1** up to the resin-bound cyclic precursor terminating at Leu3. The alternative N-terminal groups were conveniently introduced using the corresponding carboxylic acid building blocks. In the case of bipyridyl and dipicolinic acid analogues **2** and **3** respectively these were directly coupled, while for analogues **4–6**, two additional rounds of SPPS were applied to introduce the L-Ile-L-Ala, L-Ile-D-Ala, and L-Ile-Gly dipeptides respectively (**Fig. 2**).

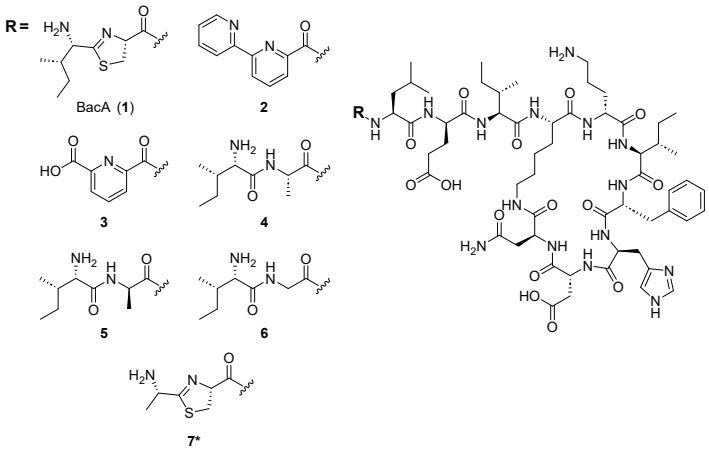
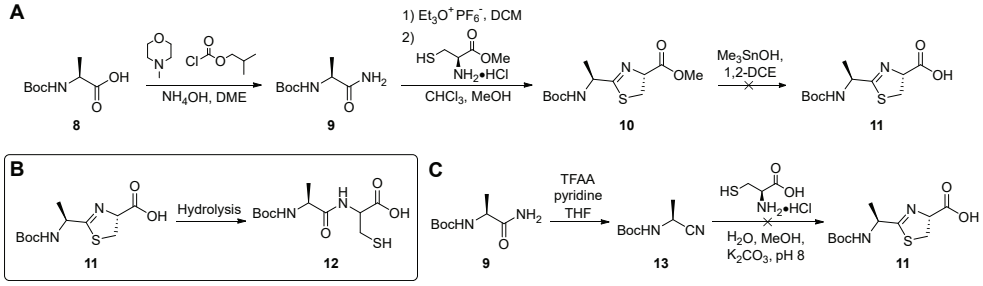


Figure 2. The structures of the bacitracin analogues bearing unnatural N-terminal dipeptides. *Analogue **7** was not successfully synthesised (see **Scheme 2B**).

Preparation of the alanine aminothiazoline analogue **7** first required the synthesis of a novel alanine thiazoline carboxylic acid **11** (**Scheme 2A**). This was attempted by following a similar route to that described in **Chapter 2** for the synthesis of the naturally occurring isoleucine derived aminothiazoline carboxylic acid. The synthesis commenced with commercially available Boc protected alanine **8**. This was smoothly converted to its corresponding amide **9** through the formation of a mixed anhydride, followed by treatment with a concentrated ammonia solution. Next, the Boc-Ala-amide intermediate **9** was reacted with the alkylating

agent Et_3OPF_6 leading to the formation of the corresponding imidate. Following a simple work up, this was then immediately combined with cysteine methyl ester in a solution of chloroform and methanol which led to the formation of the desired alanine based thiazoline methyl ester **10**. In order to couple this building block to the rest of the bacitracin peptide structure, the methyl ester needed first to be hydrolysed to its corresponding free carboxylic acid **11**. Our previous work with thiazolines had revealed the importance of using mild conditions for this step as strongly basic conditions cause scrambling of the adjacent α-stereocenter. As such, we employed trimethyltin hydroxide, a mild and selective reagent for the hydrolysis of methyl esters previously reported by Nicolaou and co-workers.[18] This reagent had previously proved effective in the preparation of the naturally occurring isoleucine-derived thiazoline carboxylic acid. It was therefore somewhat surprising that none of the expected product **11** was detected in the crude reaction mixture. LC-MS analysis revealed a primary by-product which possessed a mass of +18 amu relative to **11**, suggestive of a hydrolytic opening of the thiazoline (**Scheme 2B**). Subsequent NMR analysis confirmed this, with **12** identified as the by-product.

An alternative method widely used for the preparation of thiazolines is the reaction of unprotected cysteine with nitriles (**Scheme 2C**). We hypothesised that this route may prove more effective as it avoids the formation of the thiazoline methyl ester **10**, thereby negating the need for any kind of methyl ester hydrolysis. As such, amide **9** was dehydrated through treatment with trifluoroacetic anhydride in the presence of pyridine, which cleanly formed the nitrile intermediate **13**. Next, **13** was stirred with cysteine (HCl salt) in a solution of water and methanol. To our disappointment, this also lead to the formation of the hydrolysis by-product **12**. Notably, hydrolysis of thiazoline compounds via our proposed mechanism (**Scheme 2B**) has been reported previously.[19] We hypothesised that in both routes **2A** and **2C**, the desired **11** is successfully formed, but that this is then rapidly hydrolysed to **12** in the reaction mixture. This is indicative of inherent instability in the chemical structure of



Scheme 2. Synthetic scheme for **(A)** The unsuccessful synthesis of alanine aminothiazoline **11** *via* the imidate approach utilised in **Chapter 2**. **(B)** The proposed mechanism of degradation of **11** to the observed by-product **12**. **(C)** The second unsuccessful synthesis of alanine aminothiazoline **11** via alanine derived nitrile **13**.

compound **11**, which interestingly, is not observed for the isoleucine derived aminothiazoline carboxylic acid that we previously synthesised successfully. Due to the inherent instability of compound **11**, bacitracin analogue **7** was not prepared and was excluded from this study.

Alanine scanning of bacitracin A

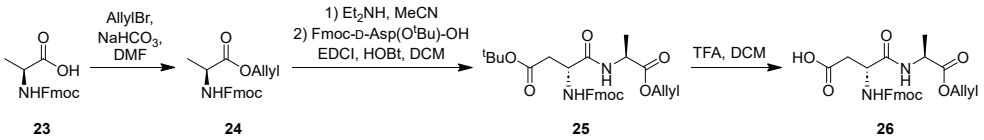
To assess the contribution to antibacterial activity of the amino acids in bacitracin A that are not involved in the thiazoline dipeptide motif, an alanine scan of the peptide scaffold was also performed. Bacitracin's heptapeptide macrocyclic core is formed by the reaction of the ε-amino group of Lys6 with the C-terminus of the peptide at Asn12. Considering that the substitution of Lys6 for an alanine would result in a linear peptide, fundamentally different from bacitracin, this alanine analogue was excluded from this study. The synthesis of the alanine scan analogues was achieved by simply modifying the synthesis of bacitracin A to replace the naturally occurring amino acid with an alanine during its respective SPPS step. Worth noting, was the decision to maintain the stereochemistry of these amino acid substitutions, *i.e.* L- amino acids were replaced with L-alanine while D- amino acids were replaced with D-alanine. Using this approach, bacitracin analogues **14**–**21** were readily prepared (**Table 1**).

Table 1. Structures of the synthetic bacitracin analogues.

	Amino Acid											
Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
BacA (**1**)	L-Ile-thiazoline		L-Leu	D-Glu	L-Ile	L-Lys	D-Orn	L-Ile	D-Phe	L-His	D-Asp	L-Asn
2	**bipyridyl**		L-Leu	D-Glu	L-Ile	L-Lys	D-Orn	L-Ile	D-Phe	L-His	D-Asp	L-Asn
3	**picolinic acid**		L-Leu	D-Glu	L-Ile	L-Lys	D-Orn	L-Ile	D-Phe	L-His	D-Asp	L-Asn
L-Ala2 (**4**)	L-Ile	**L-Ala**	L-Leu	D-Glu	L-Ile	L-Lys	D-Orn	L-Ile	D-Phe	L-His	D-Asp	L-Asn
D-Ala2 (**5**)	L-Ile	**D-Ala**	L-Leu	D-Glu	L-Ile	L-Lys	D-Orn	L-Ile	D-Phe	L-His	D-Asp	L-Asn
Gly2 (**6**)	L-Ile	**Gly**	L-Leu	D-Glu	L-Ile	L-Lys	D-Orn	L-Ile	D-Phe	L-His	D-Asp	L-Asn
Ala1 (**7**)*	**L-Ala**-thiazoline		L-Leu	D-Glu	L-Ile	L-Lys	D-Orn	L-Ile	D-Phe	L-His	D-Asp	L-Asn
Ala3 (**14**)	L-Ile-thiazoline		**L-Ala**	D-Glu	L-Ile	L-Lys	D-Orn	L-Ile	D-Phe	L-His	D-Asp	L-Asn
Ala4 (**15**)	L-Ile-thiazoline		L-Leu	**D-Ala**	L-Ile	L-Lys	D-Orn	L-Ile	D-Phe	L-His	D-Asp	L-Asn
Ala5 (**16**)	L-Ile-thiazoline		L-Leu	D-Glu	**L-Ala**	L-Lys	D-Orn	L-Ile	D-Phe	L-His	D-Asp	L-Asn
Ala7 (**17**)	L-Ile-thiazoline		L-Leu	D-Glu	L-Ile	L-Lys	**D-Ala**	L-Ile	D-Phe	L-His	D-Asp	L-Asn
Ala8 (**18**)	L-Ile-thiazoline		L-Leu	D-Glu	L-Ile	L-Lys	D-Orn	**L-Ala**	D-Phe	L-His	D-Asp	L-Asn
Ala9 (**19**)	L-Ile-thiazoline		L-Leu	D-Glu	L-Ile	L-Lys	D-Orn	L-Ile	**D-Ala**	L-His	D-Asp	L-Asn
Ala10 (**20**)	L-Ile-thiazoline		L-Leu	D-Glu	L-Ile	L-Lys	D-Orn	L-Ile	D-Phe	**L-Ala**	D-Asp	L-Asn
Ala11 (**21**)	L-Ile-thiazoline		L-Leu	D-Glu	L-Ile	L-Lys	D-Orn	L-Ile	D-Phe	L-His	**D-Ala**	L-Asn
Ala12 (**22**)	L-Ile-thiazoline		L-Leu	D-Glu	L-Ile	L-Lys	D-Orn	L-Ile	D-Phe	L-His	D-Asp	**L-Ala**

*Analogue **7** not synthesised

The bacitracin synthesis outlined in **Scheme 1** relies on anchoring the peptide to the resin through the side chain of L-Asn12. As a result, the final alanine analogue corresponding to a substitution of L-Asn12 for L-Ala (analogue **22**) required a different synthetic approach. Fortunately, immediately adjacent to L-Asn12 is D-Asp11, whose side chain carboxylic acid is a suitable alternate location for resin linkage. Since this residue must ultimately remain an aspartic acid, the acid-sensitive 2-Chlorotrityl Chloride (CTC) resin was employed. With this in mind, we first prepared the required L-Ala-D-Asp dipeptide (**Scheme 3**). In doing so, we started from the commercially available Fmoc-Ala **23** which was subsequently Allyl protected with Allyl bromide and sodium bicarbonate in DMF yielding **24**. Next, solution-phase Fmoc deprotection with diethylamine, followed by amide coupling with Fmoc-D-Asp(O^{t}Bu)-OH, generated the protected dipeptide **25**. Finally, treatment with TFA in DCM selectively deprotected the side chain carboxylic acid of the D-Asp moiety, yielding the partially protected dipeptide **26** ready to be loaded onto CTC resin for elaboration with SPPS. The linear peptide was subsequently assembled using standard Fmoc-SPPS techniques following the same approach utilised for the preparation of the other bacitracin variants, yielding analogue **22**.



Scheme 3. The synthetic route for the preparation of dipeptide **26** for use in the preparation of alanine analogue **22.**

Antibacterial assays

We next evaluated the antibacterial activities of bacitracin analogues **2**–**22** against the panel of bacteria indicated in **Table 2**. The haemolytic properties of the analogues were also evaluated and were all found to be, like bacitracin A, not haemolytic. Among the analogues bearing modified N-termini (**2**–**6**) no antibacterial activity was detected, even at the highest tested concentration of 64 μg mL^{-1}. This finding is in line with our previous assessment of the diastereomers of bacitracin A (**Chapter 2**), which revealed that the orientation of the N-terminal zinc chelating moiety is finely tuned, with small variations in its positioning leading to a precipitous loss of activity.[12] Notably, the zinc binding motifs in analogues **2** and **3** are planar and are therefore likely misaligned with bacitracin's zinc binding pocket. Furthermore, the lack of activity observed for analogues **4**–**6** makes clear the important role the thiazoline moiety plays in bacitracin's antibacterial activity. Our findings are also in agreement with the results of a previous study reported by Marahiel and co-workers who

Table 2. Minimum inhibitory concentration (MIC) values for bacitracin A analogues.

	MIC[a] (μg mL^{-1})							
	Strain							
Compound	*S. aureus* ATCC 29213	*S. aureus* USA300 (MRSA)	S. *aureus* VRS3b (VRSA)	*E. faecium* E980	*E. faecium* E155 (*vanA*)	*E. faecium* E7314 (*vanB*)	*E. coli* ATCC 25922	Haemolysis[b]
BacA (**1**)	1	2	>64	4	16	2	>64	0%
2	>64	>64	>64	>64	>64	>64	>64	0%
3	>64	>64	>64	>64	>64	>64	>64	0%
L-Ala2 (**4**)	>64	>64	>64	>64	>64	>64	>64	0%
D-Ala2 (**5**)	>64	>64	>64	>64	>64	>64	>64	0%
Gly2 (**6**)	>64	>64	>64	>64	>64	>64	>64	0%
Ala3 (**14**)	16	16	>64	>64	64	64	>64	1%
Ala4 (**15**)	4	4	>64	32	32	16	>64	0%
Ala5 (**16**)	32	32	>64	>64	64	32	>64	0%
Ala7 (**17**)	8	8	>64	16	16	4	>64	1%
Ala8 (**18**)	8	16	>64	32	64	32	>64	0%
Ala9 (**19**)	32	32	>64	>64	>64	64	>64	1%
Ala10 (**20**)	8	4	>64	8	16	32	>64	1%
Ala11 (**21**)	>64	>64	>64	>64	>64	64	>64	2%
Ala12 (**22**)	16	8	>64	>64	>64	32	>64	2%
Vancomycin	1	1	4	0.5	>64	64	-	-
Polymyxin B	-	-	-	-	-	-	0.5	-

[a]MIC values displayed in μg ml^{-1} and measured in Lysogeny broth (LB) supplemented with 0.3 mM $ZnSO_4$; [b]Hemolysis determined after 1 h incubation of the compounds (128 μg mL^{-1}) in defibrinated sheep blood.

applied a chemoenzymatic approach to generate unnatural analogues of bacitracin A.[14] They found similarly diminished activities for analogues bearing alternative heterocycles at the bacitracin N-terminus.

The antibacterial activities of alanine scan analogues **14**–**22** were also investigated and are presented in **Table 2**. All of these analogues displayed attenuated antibacterial activity relative to bacitracin A. The degree of impairment varied significantly between analogues, providing new insight into the relative importance of each residue. The substitution of Asp11 with alanine in analogue **21** was the least tolerated substitution. This lead to a near complete loss of activity against the test strains with MICs of 64 μg mL^{-1} or greater. This is presumably a result of Asp11's role in recognising and binding the pyrophosphate head group of C_{55}PP. In the published crystal structure of bacitracin A bound to C_{10}PP, Asp11 is found to be primarily coordinating a sodium ion which itself bridges the binding to the pyrophosphate headgroup. As seen in the crystal structure of bacitracin A bound to C_{10}PP, a network of six hydrogen bonds serves to stabilise the complex.[4] Five of these are formed by the peptide backbone

and the sixth by the amide side chain of Asn12. A recent study suggested that the enzymatic hydrolysis of the Asn12 side chain amide to a negatively charged carboxylic acid represents a mechanism of bacitracin resistance that is exploited by certain organisms.[20] This amide-to-carboxylate conversion is proposed to generate a repulsive interaction between the antibiotic and the negatively charged pyrophosphate target. Considering this, it was surprising that substitution of Asn12 for alanine as in compound **22** led to only a moderate loss of antibacterial activity (4–16-fold). This is presumably a result of the loss of the favourable hydrogen-bond interaction, but without the introduction of a simultaneous repulsive interaction that the presence of a carboxylate would produce.

Analogues **16** and **19**, corresponding to the replacement of Ile5, or Phe9, with alanine respectively, were the other analogues with the most diminished antibacterial activity. Bacitracin B_3, bearing a valine in position 5, is known to be less potent than bacitracin A.[21] It is thought that the reduction in hydrophobicity in this position is poorly tolerated and may explain the loss of activity for analogue **16**. A similar rationale may apply for the severely reduced activity of analogue **19**. The other analogues with alanine substitutions at hydrophobic residues are analogues **14** and **18**, replacing the natural Leu3 and Ile8 respectively. The antibacterial properties of these two peptides were also reduced, but to a lesser degree than analogues **16** and **19**. These findings further emphasise the essential role of the hydrophobic residues within the structure of bacitracin A, and makes clear that substitutions for less hydrophobic side chains leads to a loss of antibacterial potency.

Interestingly, analogue **15** wherein Glu4 is replaced by alanine, retained a significant amount of bacitracin's potency. As seen in the bacitracin–C_{10}PP complex, this glutamic acid residue is implicated in direct coordination of the zinc ion, so the finding that its substitution for alanine had little impact on antibacterial activity was rather unexpected.[4] In keeping with this finding, we found that in general, the substitution of bacitracin's polar residues has less of an impact on antibacterial potency compared to the substitution of its nonpolar residues. Notably, the replacement of Orn7 and His10 with alanine in analogues **17** and **20** respectively, resulted in only a moderate 2–4 fold increase in MIC. These findings suggest that Glu4, Orn7, and His10 may provide sites amenable to further structural elaboration, for example in conjugating to fluorophores to generate tools for visualising bacterial cells or for linking to other active compounds in the preparation of novel hybrids.

Conclusions

In summary, we here describe the preparation and evaluation of a series of novel bacitracin analogues that provide new structural insights into the contribution towards antibacterial activity of the structural motifs within bacitracin. Utilising our combined solution- and solid-phase synthesis analogues were first prepared to investigate the possibility of replacing the N-terminal aminothiazoline moiety with other zinc-binding motifs and acyclic dipeptides. These new analogues were found to be inactive as antibacterials, demonstrating the key role played by the aminothiazoline moiety.

An alanine scan of the bacitracin framework revealed the important role that bacitracin's hydrophobic residues have on bacitracin's antibacterial activity. Substitution with the less hydrophobic alanine generally led to a precipitous loss in activity, particularly for positions 5 and 9. Conversely, the majority of the polar residues in bacitracin were more amenable to replacement, resulting in only small losses in antibacterial activity. The key exception in this regard relates to previously unappreciated role of D-Asp11, where a near total loss of activity was observed following its substitution.

Although attempts to replace the aminothiazoline moiety with more chemically stable motifs led to a marked loss in the compound's antibacterial activity, the hope remains that in future, other commensurate structural alterations could potentially recover this lost activity, resulting in equipotent analogues with an enhanced stability profile. This could be the key to unlock the true potential of this unique antibiotic. Future work will be aimed at applying these structural insights with a targeted strategy to improve efficacy.

Experimental Procedures

All reagents employed were of American Chemical Society (ACS) grade or higher and were used without further purification unless otherwise stated. LC-MS- and HPLC-grade acetonitrile, peptide grade *N,N*-dimethylformamide (DMF) and dichloromethane (DCM) for peptide synthesis were purchased from Biosolve Chimie SARL and VWR, respectively. Buffers and salts were purchased from Carl Roth GmbH (Karlsruhe, Germany) and VWR International (Leuven, Belgium). Unless otherwise stated, ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV-400 spectrometer. The chemical shifts are noted as δ-values in parts per million (ppm) relative to the signal of $CDCl_3$ for ^{1}H NMR (δ = 7.26 ppm) and ^{13}C NMR (δ = 77.16 ppm). Coupling constants (J) are given in Hz.

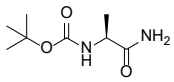
HPLC analyses were performed on a Shimadzu Prominence-i LC-2030 system with a Dr. Maisch ReproSil Gold 120 C18 column (4.6 × 250 mm, 10 µm) at 30 °C and equipped with a UV detector monitoring at 214 and 254 nm. The following solvent system, at a flow rate of 1 mL/min, was used: solvent A (0.1 % TFA in water/acetonitrile 95/5); solvent B (0.1 % TFA in water/acetonitrile 5/95). Gradient elution was as follows: 100:0 (A/B) for 2 min, 100:0 to 0:100 (A/B) over 23 min, 0:100 (A/B) for 1 min, then reversion back to 100:0 (A/B) over 1 min, 100:5 (A/B) for 4 min.

LC-MS analysis were performed on a Shimadzu LC-20AD system with a Shimadzu Shim-Pack GISS-HP C18 column (3.0 x 150 mm, 3 µm) at 30°C and equipped with a UV detector. As solvent system, at a flow rate of 0.5 mL/min, solvent A (0.1% formic acid in water) and solvent B (acetonitrile) was used. Gradient elution was as follows: 95:5 A/B for 2 min, 95:5 to 0:100 A/B over 13 min, 0:100 A/B for 2 min, then reversion back to 95:5 A/B over 1 min, 95:5 A/B for 3 min. This system was connected to a Shimadzu 8040 triple quadrupole mass spectrometer (ESI ionisation).

High resolution mass spectra (HRMS) were recorded on a Shimadzu Nexera X2 UHPLC system with a Waters Acquity HSS C18 column (2.1 × 100 mm, 1.8 µm) at 30 °C and equipped with a diode array detector. The following solvent system, at a flow rate of 0.5 mL/min, was used: solvent A, 0.1 % formic acid in water; solvent B, 0.1 % formic acid in acetonitrile. Gradient elution was as follows: 95:5 (A/B) for 1 min, 95:5 to 15:85 (A/B) over 6 min, 15:85 to 0:100 (A/B) over 1 min, 0:100 (A/B) for 3 min, then reversion back to 95:5 (A/B) for 3 min. This system was connected to a Shimadzu 9030 QTOF mass spectrometer (ESI ionisation) calibrated internally with Agilent's API-TOF reference mass solution kit (5.0 mM purine, 100.0 mM ammonium trifluoroacetate and 2.5 mM hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine) diluted to achieve a mass count of 10,000.

Synthetic Chemistry

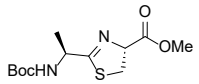
N-Boc-L-alanyl amide (9)



To a solution of *N*-Boc-L-alanine **8** (5.00 g, 26.4 mmol, 1 eq.) and *N*-methylmorpholine (3.21 g, 3.49 mL, 31.7 mmol, 1.2 eq.) in DME (100 mL) at 0 °C was added isobutyl chloroformate (4.33 g, 4.13 mL, 31.7 mmol, 1.2 eq.). After stirring for 2 minutes, an aqueous solution of ammonia (25 wt%, 12 mL) was added. The resulting slurry was stirred vigorously at room temperature and the reaction was followed by TLC. Once the reaction had reacted completion (1 hour), the reaction was quenched with water (8 mL). Next, the reaction mixture was concentrated under reduced vacuum in order to remove the majority of the DME. The residue was then diluted with 150 mL of EtOAc. The organic layer was then washed successively with 1 M HCl (50 mL), 1 M $NaHCO_3$ (50 mL), 1 M HCl (50 mL), and brine (50 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure to afford *N*-Boc-L-alanyl amide **9** (2.89 g, 58%) as a white crystalline solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.21 (s, 1H), 6.92 (s, 1H), 6.77 (d, *J* = 7.8 Hz, 1H), 3.88 (p, *J* = 7.3 Hz, 1H), 1.37 (s, 9H), 1.15 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.8 (C), 155.0 (C), 77.9 (C), 49.5 (CH), 28.2 ($(CH_3)_3$), 18.3 (CH_3).

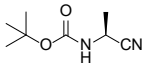
HRMS (ESI) *m/z*: $[M+H]^+$ calculated $C_8H_{16}N_2O_3$: 189.1234, found: 189.1230.

Methyl (*R*)-2-((*S*)-1-((*tert*-butoxycarbonyl)amino)ethyl)- Δ^2-thiazoline-4-carboxylate (10)



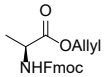
Triethyloxonium hexafluorophosphate (1.52 g, 6.11 mmol, 1.15 eq.) was dissolved in anhydrous DCM (5 mL), cooled to 0 °C and added to a solution of *N*-Boc-L-alanyl amide **9** (1.00 g, 5.31 mmol, 1 eq.) in anhydrous DCM (5 mL) at 0 °C. The resulting slurry was then allowed to reach room temperature and stirred 18 hours under an inert atmosphere. After full conversion by TLC, the now clear solution was diluted with DCM (20 mL) and washed using $NaHCO_3$ (3 × 20 mL) followed by brine (1 × 20 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to afford the crude imine ether product as an oil (0.69 g). This was used directly without further purification.
To a solution of the crude imine ether (0.69 g, 3.18 mmol, 1 eq.) in 8 mL of chloroform was added methyl L-cysteinate (0.82 g, 4.77 mmol, 1.5 eq.) in 6 mL of methanol at 0 °C. The reaction mixture was stirred under an inert atmosphere at 0 °C for 2 hours after which it was allowed to warm to room temperature. After 18 hours the white suspension was concentrated under reduced pressure and purified immediately by silica gel column chromatography eluting 30–60% Et_2O in petroleum ether to afford **10** (0.92 g, 41% over 2 steps) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.24 (br s, 1H), 5.09 (td, *J* = 9.3, 1.7 Hz, 1H), 4.57 (br s, 1H), 3.79 (s, 3H), 3.71–3.42 (m, 2H), 1.43 – 1.40 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.5 (C), 165.5 (C), 155.2 (C), 79.8 (C), 78.0 (CH), 52.9 (CH_3), 49.4 (CH), 35.3 (CH_2), 28.4 (CH_3)$_3$, 20.4 (CH_3). HRMS (ESI) *m/z*: $[M+H]^+$ calculated $C_{12}H_{20}N_2O_4S$: 289.1217, found: 289.1220.

Tert-butyl (*S*)-(1-cyanoethyl)carbamate (13)



To a stirred solution of *N*-Boc-L-alanyl amide **9** (1.00 g, 5.31 mmol, 1 eq.) in THF (25 mL) was added pyridine (1.68g, 1.71 mL, 21.3 mmol, 4 eq.). Trifluoroacetic anhydride (2.23 g, 1.48 mL, 10.6 mmol, 2 eq.) was then added dropwise at 0 °C. The reaction was then stirred room temperature for 2 hours. After completion of the reaction by TLC, the THF was removed under reduced pressure. The obtained residue was dissolved in EtOAc (20 mL), washed with 0.5 M HCl (5 × 10 mL), half saturated aqueous $NaHCO_3$ (10 mL), brine (10 mL), and dried over Na_2SO_4. The solvent was evaporated under reduced pressure to afford the crude product (0.856 g). The crude was then recrystalised in EtOAc/n-Hexane (1:4) to give the pure product **13** (0.563 g, 62%) as white crystals. ^{1}H NMR (500 MHz $CDCl_3$) δ 4.98 (br s, 1H), 4.61 (br s, 1H), 1.53 (d, *J* = 7.3 Hz, 3H), 1.45 (s, 9H). ^{13}C NMR (126 MHz $CDCl_3$) δ 154.2 (C), 119.6 (C), 81.3 (C), 37.6 (CH), 28.2 ((CH_3)$_3$), 19.6 (CH_3). HRMS (ESI) *m/z*: $[M+H]^+$ calculated $C_8H_{14}N_2O_2$: 171.1128, found: 171.1128.

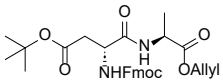
N-Fmoc-L-alanine-OAllyl (24)



To a magnetically stirred mixture of Fmoc-L-alanine-OH **23** (3.00 g, 9.64 mmol, 1.0 eq.) and $NaHCO_3$ (2.03 g, 24.1 mmol, 2.5 eq.) in DMF (50 mL), Allyl bromide (3.50 g, 2.5 mL, 28.9 mmol, 3.0 eq.) was added slowly. The reaction mixture was stirred for 48 hours at room temperature and monitored by TLC. After the reaction had reached completion, the solvents were removed under reduced pressure and the crude residue was dissolved in EtOAc (150 mL), washed with water (25 mL) and brine (25 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude

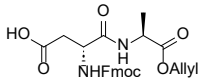
product was then purified by silica gel column chromatography eluting 10–20% EtOAc in petroleum ether to afford **24** (2.89 g, 85%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.61 (dd, *J* = 7.6, 4.2 Hz, 2H), 7.40 (t, *J* = 7.4, 2H), 7.32 (td, *J* = 7.4, 1.2 Hz, 2H), 5.92 (ddt, *J* = 16.4, 10.9, 5.7 Hz, 1H), 5.38 (d, *J* = 7.1 Hz, 1H), 5.34 (dq, *J* = 17.2, 1.7 Hz, 1H), 5.27 (dq, *J* = 10.4, 1.3 Hz, 1H), 4.66 (d, *J* = 5.4 Hz, 2H), 4.48 – 4.35 (m, 3H), 4.23 (t, *J* = 7.0 Hz, 1H), 1.46 (d, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.9 (C), 155.8 (C), 143.9 (C), 141.4 (C), 131.6 (CH), 127.8 (CH), 127.2 (CH), 125.2 (CH), 120.1 (CH), 118.9 (CH_2), 67.1(CH_2), 66.1 (CH_2), 49.8 (CH), 47.3 (CH), 18.9 (CH_3). HRMS (ESI) *m/z*: $[M+H]^+$ calculated $C_{21}H_{21}NO_4$: 352.1544, found: 352.1546.

Tert-butyl (*R*)-3-((Fmoc)amino)-4-(((*S*)-1-(allyloxy)-1-oxopropan-2-yl)amino)-4-oxobutanoate (25)



N-Fmoc-L-alanine-OAllyl **24** (1.50 g, 4.27 mmol, 1.0 eq.) was dissolved in MeCN (21 mL). Diethylamine (12.5 g, 17.6 mL, 170 mmol, 40 eq.) was added and the mixture was stirred for 3 hours at room temperature. Once the reaction was complete by TLC, the reaction mixture was concentrated under vacuum. to afford the crude amine product as a white solid. This was used directly without further purification. In a separate flask, HOBt (0.11 g, 0.85 mmol, 0.2 eq.) and EDCI (0.90 g, 4.70 mmol, 1.1 eq.) were added to a solution of Fmoc-D-Asp(OtBu)-OH (1.76 g, 4.27 mmol, 1 eq.) in DCM (17 mL) at 0 °C under N_2. The mixture was stirred for 5 minutes at 0 °C after which the crude NH_2-L-Ala-OAllyl was added. The reaction mixture was allowed to warm to room temperature and stirred for 15 hours, whereupon the mixture was successively washed with water (2 × 15 mL), saturated $NaHCO_3$(aq.) (2 × 15 mL), 0.1 M hydrochloric acid (2 × 15 mL) and brine (2 × 15 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude product was then purified by silica gel column chromatography eluting 10–25% EtOAc in petroleum ether to afford **25** (1.13 g, 51%) as white crystals. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (dt, *J* = 7.6, 0.9 Hz, 2H), 7.60 (d, *J* = 7.4 Hz, 2H), 7.41 (td, *J* = 7.5, 1.2 Hz, 2H), 7.32 (tt, *J* = 7.4, 1.4 Hz, 2H), 7.03 (d, *J* = 7.4 Hz, 1H), 5.98 (d, *J* = 8.5 Hz, 1H), 5.89 (ddt, *J* = 17.3, 10.5, 5.7 Hz, 1H), 5.32 (dq, *J* = 17.2, 1.5 Hz, 1H), 5.24 (dq, *J* = 10.4, 1.3 Hz, 1H), 4.63 (dq, *J* = 5.7, 1.5 Hz, 2H), 4.61 – 4.53 (m, 2H), 4.42 (dd, *J* = 7.3, 3.5 Hz, 2H), 4.24 (t, *J* = 7.1 Hz, 1H), 2.92 (dd, *J* = 16.8, 4.5 Hz, 1H), 2.62 (dd, *J* = 16.9, 6.7 Hz, 1H), 1.45 (s, 9H), 1.42 (d, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.1 (C), 170.9 (C), 167.0 (C), 157.2 (C), 143.7 (C), 141.3 (C), 131.5 (CH), 127.8 (CH), 127.1 (CH), 125.1 (CH), 120.1 (CH), 118.8 (CH_2), 81.9 (C), 67.4 (CH_2), 64.9 (CH_2), 51.1 (CH), 48.4 (CH), 47.1 (CH), 37.4 (CH_2), 28.1 (CH_3)$_3$), 18.2 (CH_3). HRMS (ESI) *m/z*: $[M+H]^+$ calculated $C_{29}H_{34}N_2O_7$: 523.2439, found: 523.2441.

(*R*)-3-((Fmoc)amino)-4-(((*S*)-1-(allyloxy)-1-oxopropan-2-yl)amino)-4-oxobutanoic acid (26)



tert-butyl (*R*)-3-((Fmoc)amino)-4-(((*S*)-1-(allyloxy)-1-oxopropan-2-yl)amino)-4-oxobutanoate **25** (0.54 g, 1.03 mmol 1eq.) was dissolved in TFA/DCM (10 mL, 1:1, *v*/*v*) and the resulting solution was stirred at room temperature for 3 hours. The solvent was subsequently evaporated under reduced pressure and the residue redissolved in ^{t}BuOH/H_2O (10 mL, 1:1, *v*/*v*). The solution was then frozen in liquid N_2 and lyophilised overnight affording **26** (0.43 g, 90%) as a white powder. ^{1}H NMR (400 MHz, MeOD) δ 7.79 (dt, *J* = 7.6, 1.0 Hz, 2H), 7.66 (d, *J* = 7.5 Hz, 2H), 7.39 (td, *J* = 7.3, 1.0 Hz, 2H), 7.31 (td, *J* = 7.4, 1.2 Hz, 2H), 5.90 (ddt, *J* = 16.1, 10.8, 5.6 Hz, 1H), 5.29 (dq, *J* = 17.3, 1.6 Hz, 1H), 5.17 (dq, *J* = 10.5, 1.4 Hz, 1H), 4.63 – 4.52 (m, 3H), 4.43 (q, *J* = 7.3 Hz, 1H), 4.36 (dt, *J* = 28.5, 7.6 Hz, 2H), 4.24 (t, *J* =

7.0 Hz, 1H), 2.84 (dd, *J* = 16.6, 6.0 Hz, 1H), 2.67 (dd, *J* = 16.6, 7.7 Hz, 1H), 1.40 (d, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, MeOD) δ 173.9 (C), 173.5 (C), 173.2 (C), 158.3 (C), 145.2 (C), 142.6 (C), 133.3 (CH), 128.8 (CH), 128.2 (CH), 126.3 (CH), 120.9 (CH), 118.6 (CH_2), 68.2 (CH_2), 66.8 (CH_2), 52.8 (CH), 49.7 (CH), 48.3 (CH), 37.1 (CH_2), 17.5 (CH_3). HRMS (ESI) *m/z*: $[M+H]^+$ calculated $C_{25}H_{26}N_2O_7$: 467.1813, found: 467.1817.

Solid-Phase Peptide Synthesis

General procedure A: Peptide backbone synthesis (automated): The resin bound linear decapeptide was assembled on a CEM HT12 Liberty Blue peptide synthesiser. This commenced with Fmoc-L-Asp-OAllyl being loaded onto MBHA Rink amide resin through its side chain. The linear peptide was then extended with 9 further SPPS cycles sequentially adding: Fmoc-D-Asp(tBu)-OH, Fmoc-L-His(Trt)-OH, Fmoc-D-Phe-OH, Fmoc-L-Ile-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-L-Lys(Alloc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Glu(tBu)-OH, and Fmoc-L-Leu-OH. This was achieved by first swelling MBHA Rink amide resin (163 mg, 0.1 mmol) in 10 mL of DMF for 5 minutes. Fmoc deprotections were carried out in a solution of piperidine in DMF (1:4 *v/v*, 3 mL) heated to 76 °C for 15 seconds followed by 50 seconds at 90 °C. The resin was then washed with DMF (3 × 2 mL). Single amino acid coupling cycles were performed in DMF (4 mL) and mediated by DIC (0.125 mol/L, 5 eq.), Oxyma (0.125 mol/L, 5 eq.) and the respective Fmoc amino acid (0.125 mol/L, 5 eq.) at 75 °C for 15 seconds followed by 110 seconds at 90°C. The only exception was Fmoc-L-His(Trt)-OH, where the coupling was performed at 25 °C for 2 minutes followed by 8 minutes at 50 °C. After the 10th amino acid was coupled, a final washing step was performed (3 × 2 mL DMF). At this stage the resin bound decapeptide was removed from the peptide synthesiser, manually washed with DCM, and dried under a continuous flow of nitrogen.

General procedure B: Cyclisation (manual): Next, the peptide was swelled in DCM (*ca.* 7 mL) for 10 minutes under a nitrogen atmosphere. To this was added $Pd(PPh_3)_4$ (30 mg, 0.03 mmol), phenylsilane (0.30 mL, 3.0 mmol) with a flow of nitrogen providing agitation. After 1 hour, the reaction mixture was drained thoroughly washed. Firstly with DCM (5 × 10 mL), then a solution of diethyldithiocarbamic acid trihydrate sodium salt (5 mg mL^{-1} in DMF, 5 × 10 mL), and finally DMF (5 × 10 mL). After confirmation of Alloc/Allyl deprotection by LC-MS, cyclisation was effected by treatment of the peptide with 4 equivalents of BOP and 8 equivalents of DIPEA in 4 mL of DMF for 1 hour. The resin was then washed with DMF (3 × 4 mL).

General procedure C: *N*-terminal coupling (manual): The terminal Fmoc group was removed with piperidine in DMF (1:4 *v/v*, 2 × 5 mL, 1 × 5 min., 1 × 15 min.). The resin was then washed with DMF (3 × 4 mL). Next, a solution of thiazoline dipeptide ((*R*)-2-[1'(*S*)-(*tert*-butoxycarbonylamino)-2'(*S*)-methylbutyl]-Δ^2-thiazoline-4-carboxylic acid[12] (2 eq.), COMU (2 eq.), and 2,2,6,6-tetramethylpiperidine (1 eq.) in 4 mL of DMF was allowed to react for 1 minute before being added to the resin bound cyclised decapeptide. Once the reaction was complete, visible by colour change, (*ca.* 30 min.) the resin was drained and washed with DMF (3 × 5 mL).

General procedure D: Global deprotection and cleavage from resin: The completed peptide was simultaneously deprotected and cleaved from the resin by treatment with a mixture of TFA:TIPS:H_2O (95:2.5:2.5 *v/v/v*, 5 mL) for 30 minutes. The reaction mixture was filtered and added to a cooled mixture of MTBE/hexanes (1:1 *v/v*, 45 mL). This lead to the precipitation of the peptide which was collected by centrifugation and washed twice more with MTBE/hexanes (2 × 50 mL), yielding crude bacitracin A analogue.

General Procedure E: Preparative HPLC purification: Bacitracin and derivatives were purified using a BESTA-Technik system with a Dr. Maisch Reprosil Gold 120 C18 column (25 × 250 mm, 10 μm) and equipped with a ECOM Flash UV detector monitoring at 214 and 254 nm. As solvent system, at a flow rate of 12 mL/min., was used: solvent A (0.1 % TFA in water/acetonitrile [95:5 v/v]); solvent B (0.1 % TFA in water/acetonitrile [95:5 v/v]). Gradient elution was as follows: 100:0 to 40:60 A/B over 80 min., 40:60 to 0:100 A/B over 1 min., 0:100 A/B for 5 min., then reversion back to 100:0 A/B over 1

min., 100:0 A/B for 3 min, total method = 90 min. After collection, fractions were frozen and lyophilised yielding pure peptide as a white powder.

Peptide 2 (2-Bipy-Bac)

Rink amide AM resin (250 mg, 0.125 mmol) was used for solid-phase peptide synthesis according to general procedure A. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. The terminal Fmoc group was then removed with piperidine in DMF (1:4 *v/v*, 2 × 5 mL, 1 × 5 min, 1 × 15 min). Next the resin bound peptide was treated a solution of [2,2'-bipyridine]-6-carboxylic acid (4 eq.), BOP (4 eq.), and DIPEA (8 eq.) in 4 mL of DMF. After 1 hour the resin was drained and washed with DMF (3 × 5 mL). Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 44 mg, 25%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{68}H_{95}N_{17}O_{16}$: 703.8645, found: 703.8649. HPLC R_t = 13.22 min.

Peptide 3 (2-Pic-Bac)

Rink amide AM resin (250 mg, 0.125 mmol) was used for solid-phase peptide synthesis according to general procedure A. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. The terminal Fmoc group was then removed with piperidine in DMF (1:4 *v/v*, 2 × 5 mL, 1 × 5 min, 1 × 15 min). Next the resin bound peptide was treated a solution of 6-((((4-methoxybenzyl)oxy)carbonyl)picolinic acid[22] (4 eq.), BOP (4 eq.), and DiPEA (8 eq.) in 4 mL of DMF. After 1 hour the resin was drained and washed with DMF (3 × 5 mL). Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 35 mg, 20%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{64}H_{92}N_{16}O_{18}$: 687.3461, found: 687.3466. HPLC R_t = 13.72 min.

Peptide 4 (2-L-Ala-Bac)

Rink amide AM resin (200 mg, 0.1 mmol) was used for solid-phase peptide synthesis according to general procedure A. The linear peptide was extended by an additional 2 SPPS cycles that sequentially incorporated Fmoc-L-Ala-OH, and Fmoc-L-Ile-OH generating a resin-bound linear dodecapeptide. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. The terminal Fmoc group was then deprotection with piperidine in DMF (1:4 *v/v*, 2 × 5 mL, 1 × 5 min, 1 × 15 min). Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 19 mg, 13%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{66}H_{105}N_{17}O_{17}$: 704.9010, found: 704.9012. HPLC R_t = 12.60 min.

Peptide 5 (2-D-Ala-Bac)

Rink amide AM resin (200 mg, 0.1 mmol) was used for solid-phase peptide synthesis according to general procedure A. The linear peptide was extended by an additional 2 SPPS cycles that sequentially incorporated Fmoc-D-Ala-OH, and Fmoc-L-Ile-OH generating a resin-bound linear dodecapeptide. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. The terminal Fmoc group was then deprotection with piperidine in DMF (1:4 *v/v*, 2 × 5 mL, 1 × 5 min, 1 × 15 min). Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 41 mg, 29%). HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{66}H_{105}N_{17}O_{17}$: 704.9010, found: 704.9012. HPLC R_t = 13.13 min.

Peptide 6 (2-Gly-Bac)

Rink amide AM resin (200 mg, 0.1 mmol) was used for solid-phase peptide synthesis according to general procedure A. The linear peptide was extended by an additional 2 SPPS cycles that sequentially

incorporated Fmoc-Gly-OH, and Fmoc-L-Ile-OH generating a resin-bound linear dodecapeptide. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. The terminal Fmoc group was then deprotection with piperidine in DMF (1:4 *v/v*, 2 × 5 mL, 1 × 5 min, 1 × 15 min). Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 34 mg, 24%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated for $C_{65}H_{103}N_{17}O_{17}$: 697.8932, found: 697.8936. HPLC R_t = 12.70 min.

Peptide 14 (Ala3)

Rink amide MBHA resin (82 mg, 0.05 mmol) was used for solid-phase peptide synthesis according to general procedure A. The sequence of amino acids coupled was Fmoc-L-Asp-OAllyl, Fmoc-D-Asp(tBu)-OH, Fmoc-L-His(Trt)-OH, Fmoc-D-Phe-OH, Fmoc-L-Ile-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-L-Lys(Alloc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Glu(tBu)-OH, Fmoc-L-Ala-OH. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. Next, the N-terminal thiazoline dipeptide[12] was coupled according to general procedure C. Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 2 mg, 3%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{63}H_{97}N_{17}O_{16}S$: 690.8583, found: 690.8592.

Peptide 15 (Ala4)

Rink amide MBHA resin (82 mg, 0.05 mmol) was used for solid-phase peptide synthesis according to general procedure A. The sequence of amino acids coupled was Fmoc-L-Asp-OAllyl, Fmoc-D-Asp(tBu)-OH, Fmoc-L-His(Trt)-OH, Fmoc-D-Phe-OH, Fmoc-L-Ile-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-L-Lys(Alloc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Ala-OH, Fmoc-L-Leu-OH. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. Next, the N-terminal thiazoline dipeptide[12] was coupled according to general procedure C. Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 4 mg, 6%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{64}H_{101}N_{17}O_{14}S$: 682.8791, found: 682.8802.

Peptide 16 (Ala5)

Rink amide MBHA resin (82 mg, 0.05 mmol) was used for solid-phase peptide synthesis according to general procedure A. The sequence of amino acids coupled was Fmoc-L-Asp-OAllyl, Fmoc-D-Asp(tBu)-OH, Fmoc-L-His(Trt)-OH, Fmoc-D-Phe-OH, Fmoc-L-Ile-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-L-Lys(Alloc)-OH, Fmoc-L-Ala-OH, Fmoc-D-Glu(tBu)-OH, Fmoc-L-Leu-OH. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. Next, the N-terminal thiazoline dipeptide[12] was coupled according to general procedure C. Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 4 mg, 6%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{63}H_{97}N_{17}O_{16}S$: 690.8583, found: 690.8591.

Peptide 17 (Ala7)

Rink amide MBHA resin (82 mg, 0.05 mmol) was used for solid-phase peptide synthesis according to general procedure A. The sequence of amino acids coupled was Fmoc-L-Asp-OAllyl, Fmoc-D-Asp(tBu)-OH, Fmoc-L-His(Trt)-OH, Fmoc-D-Phe-OH, Fmoc-L-Ile-OH, Fmoc-D-Ala-OH, Fmoc-L-Lys(Alloc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Glu(tBu)-OH, Fmoc-L-Leu-OH. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. Next, the N-terminal thiazoline dipeptide[12] was coupled according to general procedure C. Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E).

Yield: 3 mg, 4%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{64}H_{98}N_{16}O_{16}S$: 690.3607, found: 690.3619.

Peptide 18 (Ala8)

Rink amide MBHA resin (82 mg, 0.05 mmol) was used for solid-phase peptide synthesis according to general procedure A. The sequence of amino acids coupled was Fmoc-L-Asp-OAllyl, Fmoc-D-Asp(tBu)-OH, Fmoc-L-His(Trt)-OH, Fmoc-D-Phe-OH, Fmoc-L-Ala-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-L-Lys(Alloc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Glu(tBu)-OH, Fmoc-L-Leu-OH. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. Next, the N-terminal thiazoline dipeptide[12] was coupled according to general procedure C. Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 2 mg, 3%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{63}H_{97}N_{17}O_{16}S$: 690.8583, found: 690.8591.

Peptide 19 (Ala9)

Rink amide MBHA resin (82 mg, 0.05 mmol) was used for solid-phase peptide synthesis according to general procedure A. The sequence of amino acids coupled was Fmoc-L-Asp-OAllyl, Fmoc-D-Asp(tBu)-OH, Fmoc-L-His(Trt)-OH, Fmoc-D-Ala-OH, Fmoc-L-Ile-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-L-Lys(Alloc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Glu(tBu)-OH, Fmoc-L-Leu-OH. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. Next, the N-terminal thiazoline dipeptide[12] was coupled according to general procedure C. Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 2 mg, 3%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{60}H_{99}N_{17}O_{16}S$: 673.8661, found: 673.8670.

Peptide 20 (Ala10)

Rink amide MBHA resin (82 mg, 0.05 mmol) was used for solid-phase peptide synthesis according to general procedure A. The sequence of amino acids coupled was Fmoc-L-Asp-OAllyl, Fmoc-D-Asp(tBu)-OH, Fmoc-L-Ala-OH, Fmoc-D-Phe-OH, Fmoc-L-Ile-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-L-Lys(Alloc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Glu(tBu)-OH, Fmoc-L-Leu-OH. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. Next, the N-terminal thiazoline dipeptide[12] was coupled according to general procedure C. Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 3 mg, 4%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{63}H_{101}N_{15}O_{16}S$: 678.8709, found: 678.8722.

Peptide 21 (Ala11)

Rink amide MBHA resin (82 mg, 0.05 mmol) was used for solid-phase peptide synthesis according to general procedure A. The sequence of amino acids coupled was Fmoc-L-Asp-OAllyl, Fmoc-D-Ala-OH, Fmoc-L-His(Trt)-OH, Fmoc-D-Phe-OH, Fmoc-L-Ile-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-L-Lys(Alloc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Glu(tBu)-OH, Fmoc-L-Leu-OH. After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. Next, the N-terminal thiazoline dipeptide[12] was coupled according to general procedure C. Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 4 mg, 6%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{65}H_{103}N_{17}O_{14}S$: 689.8869, found: 689.8877.

Peptide 22 (Ala12)

(*R*)-3-((Fmoc)amino)-4-(((*S*)-1-(allyloxy)-1-oxopropan-2-yl)amino)-4-oxobutanoic acid **26** was manually loaded onto CTC resin, and the resin loading was determined to be 0.535 mmol g^{-1}. This preloaded CTC resin (187 mg, 0.1 mmol) was then used for solid phase peptide synthesis, with eight

cycles of manual SPPS sequentially adding: Fmoc-L-His(Trt)-OH, Fmoc-D-Phe-OH, Fmoc-L-Ile-OH, Fmoc-D-Orn(Boc)-OH, Fmoc-L-Lys(Alloc)-OH, Fmoc-L-Ile-OH, Fmoc-D-Glu(tBu)-OH, Fmoc-L-Leu-OH. This was achieved by first swelling CTC resin in 10 mL of DMF for 5 minutes. Couplings were mediated with BOP and DIPEA (resin bound AA:Fmoc-AA:BOP:DIPEA, 1:4:4:8 molar eq.) in DMF. Fmoc deprotections were carried out in a solution of piperidine in DMF (1 : 4 v/v, 2 × 5 mL, 1 × 5 min, 1 × 15 min). After checking the peptide by LC-MS, the peptide was cyclised according to general procedure B. Next, the N-terminal thiazoline dipeptide[12] was coupled according to general procedure C. Subsequently, the peptide was simultaneously deprotected and cleaved from resin (general procedure D) and purified by preparative HPLC (general procedure E). Yield: 2 mg, 3%. HRMS (ESI) *m/z*: $[M+2H]^{2+}$ calculated $C_{65}H_{102}N_{16}O_{15}S$: 690.3789, found: 690.3798.

MIC Determinations

From glycerol stocks, bacterial strains were cultured on blood agar plates and incubated overnight at 37 °C. Following incubation, 3 mL of tryptic soy broth (TSB) was inoculated with an individual colony. The cultures were grown to exponential phase (OD_{600} = 0.5) at 37 °C. The bacterial suspensions were then diluted 100-fold in lysogeny broth supplemented with 0.3 M $ZnSO_4$ (ZnLB) to reach a bacterial cell density of 10^6 CFU mL^{-1}. In polypropylene 96-well microtiter plates, test compounds in ZnLB were added in triplicate and 2-fold serially diluted to achieve a final volume of 50 µL per well. An equal volume of bacterial suspension (10^6 CFU mL^{-1}) was added to the wells. The plates were sealed with breathable membranes and incubated at 37 °C for 18 hours with constant shaking (600 rpm). MICs were determined by visual inspection as the median of a minimum of triplicates.

Haemolysis Assay

The haemolytic activity of each analogue was assessed in triplicate. Red blood cells from defibrillated sheep blood obtained from Thermo Fisher were centrifuged (400 g for 15 minutes at 4 °C) and washed with Phosphate-Buffered Saline (PBS) containing 0.002% Tween20 (buffer) for five times. Then, the red blood cells were normalised to obtain a positive control read-out of 2.5 at 415 nm to stay within the linear range with the maximum sensitivity. A serial dilution of the compounds (128 – 4 µg/mL, 75 µL) was prepared in a 96-well plate. The outer border of the plate was filled with 75 µL buffer. Each plate contained a positive control (0.1% Triton-X final concentration, 75 µL) and a negative control (buffer, 75 µL) in triplicate. The normalised blood cells (75 µL) were added and the plates were incubated at 37 °C for 1 hour while shaking at 500 rpm. A flat-bottom plate of polystyrene with 100 µL buffer in each well was prepared. After incubation, the plates were centrifuged (800 g for 5 minutes at room temperature) and 25 µL of the supernatant was transferred to their respective wells in the flat-bottom plate. The values obtained from a read-out at 415 nm were corrected for background (negative control) and transformed to a percentage relative to the positive control (0.1% Triton-X).

Acknowledgements

We thank Halana Vlaming and Yunhao Duan for their contributions relating to the chemical synthesis of some analogues, and Paolo Innocenti for acquiring the HRMS data. Thanks also to Matthijs van Haren for providing the 6-(((4-methoxybenzyl)oxy)carbonyl)picolinic acid building block.

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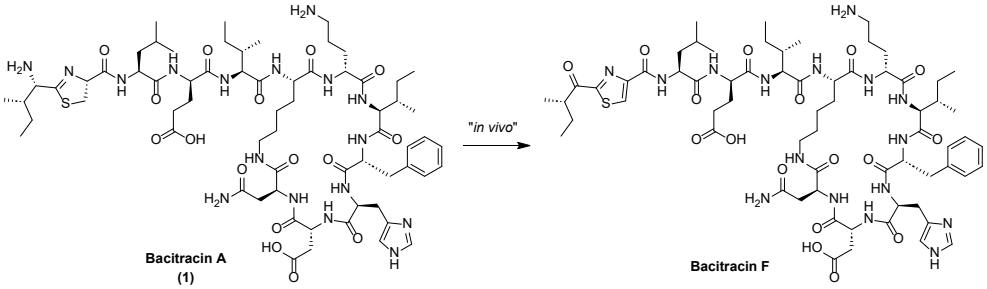
Supplementary Figures



Scheme S1. Bacitracin F, bearing an N-terminal ketothiazole moiety in place of bacitracin A's aminothiazoline.