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Rational design and synthesis of modified teixobactin analogues: in vitro antibacterial activity against *Staphylococcus aureus*, *Propionibacterium acnes* and *Pseudomonas aeruginosa*

Vivian Ng,[a] Sarah A. Kuehne[b] and Weng C. Chan*[a]

Abstract: Teixobactin, a recently discovered depsipeptide that binds to bacterial lipid II and lipid III, provides a promising molecular scaffold for the design of new antimicrobials. Herein, we describe the synthesis and antimicrobial evaluation of systematically modified teixobactin analogues. The replacement of Ile^{11} residue with aliphatic isosteres, the modification of the guanidino group at residue 10 and the introduction of a rigidifying residue, dehydroamino acid into the macrocyclic ring generated useful structure-activity information. Extensive antimicrobial susceptibility assessment against a panel of clinically relevant *Staphylococcus aureus* and *Propionibacterium acnes* led to the identification of a new lead compound, [Arg(Me)^{10},Nle^{11}]teixobactin **63**, with excellent bactericidal activity (MIC 2–4 μg/mL). Significantly, the antimicrobial activity of several of the teixobactin analogues against the pathogenic Gram-negative *Pseudomonas aeruginosa* was 'restored' when combined with sub-MIC concentration of the outer membrane-disruptive antibiotic, colistin. The antimicrobial effectiveness of [Tfn^{10},Nle^{11}]teixobactin **66** (32 μg/mL)-colistin (2 μg/mL; 0.5x MIC) combination against *P. aeruginosa* PAO1 reveals, for the first time, an alternative therapeutic option in the treatment of Gram-negative infections.

Introduction

Life-saving antibiotics are rapidly losing the race against the development of bacterial resistance to most, if not all, antibiotics. The resultant health and financial implications have spurred the deployment of antimicrobial stewardship programmes across the globe to ensure evidence-based prescribing of antibiotics that are still effective.[1–3] Meanwhile, scientists are working hand-in-hand to tackle the resistance crisis through drug discovery and development initiatives.

Natural antimicrobial peptides serve as invaluable molecular scaffolds for the development of the next generation of antimicrobial therapeutics. The recently discovered depsipeptide, teixobactin **1** (Figure 1), has great potential as a lead compound due to its favourable potency against many Gram-positive pathogens.[4] Among them, teixobactin has demonstrated excellent bactericidal activity against methicillin-resistant *Staphylococcus aureus* (MRSA) which is associated with a wide range of infections in both the community (e.g. cellulitis, abscesses) and the hospital settings (e.g. bacteraemia, pneumonia).[4–6] Vancomycin is currently the last line of defence against MRSA infections but strains with reduced susceptibility to this antibiotic have surfaced.[7–10] Teixobactin offers a potential solution to this predicament since it remains effective against MRSA, as well as vancomycin-intermediate *S. aureus* (VISA) due to its unique mode of action.[4] It has been shown to synergistically block the biosyntheses of peptidoglycan and teichoic acid, thereby resulting in a weakened cell wall and autolysin-mediated cell lysis.[4,11]

Teixobactin **1** is also a potent antimicrobial against another human skin commensal, *Propionibacterium acnes*.[4] This Gram-positive anaerobe is commonly associated with acnes vulgaris.[12] In recent years, however, it is increasingly recognised as an opportunistic pathogen that can cause invasive infections, especially those associated with medical implants.[13,14] There have been several reports on the isolation of *P. acnes* from prosthetic joints, cardiovascular devices and ophthalmic implants.[14–18] To aggravate matters, the widespread use of antibiotics to treat acne vulgaris has led to the emergence of *P. acnes* strains that are resistant to numerous antibiotics, including the macrolides, tetracycline and metronidazole.[13,19–22] The need for novel antimicrobials is therefore more pressing than ever. It is hoped that teixobactin and its analogues may serve as a timely solution to this clinically important pathogen.

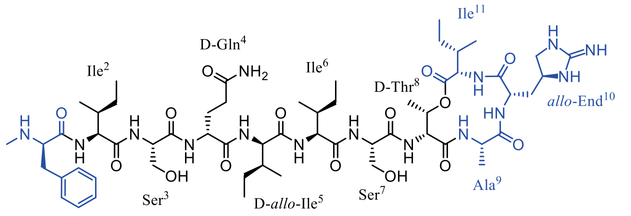

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Figure 1. Structure of teixobactin **1** and the four sites (blue) of modification presented in this work.

Teixobactin **1**, comprised of a 13-membered depsipeptide core and a tethered linear heptapeptide, offers multiple sites for synthetic modifications to improve its potency and efficacy. In less than three years since its discovery, more than a hundred analogues have been synthesized by various research groups in the hope of elucidating its structure-activity relationships (SARs).[23–37] The biological activities of these analogues and the different synthetic strategies reported have been comprehensively reviewed.[38,39] X-ray crystallographic, molecular dynamic and NMR structural studies have also been conducted to construct possible binding models of the native

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peptide and its analogues.[26,27,40] Additionally, in a recent mini-review, we provided an insight into the structural similarities of teixobactin with other lipid II inhibitors.[41] Together, these resources provide tremendous information that could aid the design of optimised analogues.

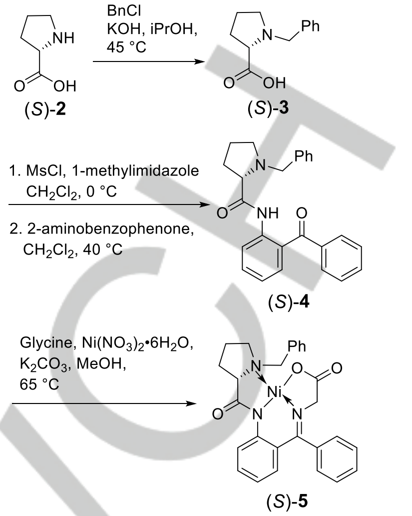
Early synthetic endeavours focused primarily on the exocyclic tail and the backbone stereochemistry of the native peptide. The replacement of any D-amino acid residues with its L-counterparts abolished activity, suggesting a significant contribution of these residues for the optimal conformation of teixobactin.[25,26,30] Yang *et al.* further demonstrated the importance of the *N*-terminal tail as the removal of the first five residues detrimentally affected antimicrobial potency.[24] Teixobactin appears to bind to the pyrophosphate and *N*-acetylmuramic acid amino sugar of lipid II. As such, its cyclic ring is believed to act as the main site of recognition.[4] With these considerations in mind, we have developed a series of analogues with modifications mainly on the macrocyclic core to examine the significance of hydrophobicity at position 11, the cationic feature of the guanidino group at position 10, and the effect of introducing conformational rigidity at position 9. The *N*-Me-D-Phe^1 was also replaced with D-Trp in an attempt to investigate the contribution of the phenyl group.

Apart from replacing the Ile residue at position 11 with readily available aliphatic isosteres, we sought to investigate the effect of introducing fluorine atoms and unsaturated side-chain at this position. Thus, Fmoc-(*S*)-6,6,6-trifluoronorleucine-OH (Fmoc-Tfn-OH) and Fmoc-(*S*)-homoallylglycine-OH (Fmoc-Hag-OH) were synthesized and their preparation will be discussed prior to the synthesis of the teixobactin analogues. All analogues were extensively evaluated for their antimicrobial activity against both *S. aureus* and *P. acnes*. Thus far, most biological assessments of reported teixobactin analogues are focused on *S. aureus* and only the activity of the native peptide is known against *P. acnes*. Herein, we report detailed antimicrobial activity of teixobactin analogues against several *P. acnes* strains. Although teixobactin and analogues thereof are considered inactive against Gram-negative bacteria (MIC >256 µg/mL), the effect of using teixobactin analogues in combination with colistin was also investigated against the Gram-negative pathogen *Pseudomonas aeruginosa*.

Results and Discussion

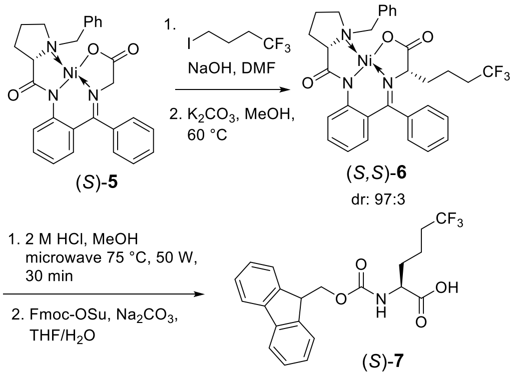
Synthesis of Fmoc-(*S*)-6,6,6-trifluoronorleucine-OH and Fmoc-(*S*)-homoallylglycine-OH

An operationally simple and cost-effective approach for the asymmetric synthesis of Fmoc-Tfn-OH (*S*)-**7** and Fmoc-Hag-OH (*S*)-**9** is by alkylation of an achiral auxiliary reagent Ni(II)-glycine Schiff base (*S*)-**5**.[42,43] The Ni(II)-complex (*S*)-**5** was synthesized in large scale in three straightforward steps (Scheme 1). The coordination of Ni(II) ion to the glycine greatly increased the acidity of the *α*-proton, enabling subsequent rapid alkylation with an alkyl halide.[42]



Scheme 1. An optimized synthesis of Ni(II)-Gly-(*S*)-2-[*N*-(*N*-benzylprolyl)amino]-benzophenone (BPB).

Thus, using the protocol developed by Belokon *et al.*, the first step in the synthesis progressed smoothly to give *N*-benzylated L-proline (*S*)-(**3**) in high yield.[43] Although the condensation between (*S*)-(**3**) and 2-aminobenzophenone did not proceed to completion, a reasonable yield of 45–60 % was obtained. To our dismay, the use of KOH in the final step, i.e. the transformation of (*S*)-**4** to (*S*)-**5**, gave a disappointing 50 % recovery of (*S*)-**5** after three recrystallizations. A review of the literature indicated that K_2CO_3 was previously employed by Soloshonok and co-workers to prepare a closely related Schiff base,[44] thereby suggesting that this alternative base could be more effective for synthesizing (*S*)-**5**. Gratifyingly, K_2CO_3 (20 equiv.) drove the final reaction step to completion within an hour and (*S*)-**5** was recrystallized from MeOH/H_2O in >85 % yield.



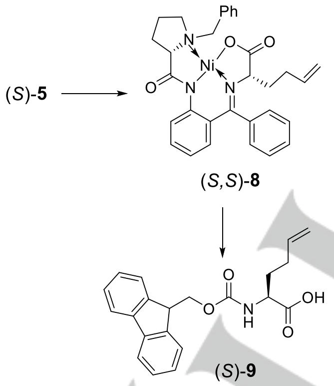
Scheme 2. An optimised synthesis of Fmoc-6,6,6-trifluoronorleucine-OH.

Having successfully prepared the Ni(II)-Schiff base (*S*)-**5**, we then sought to optimise the alkylation of the complex with 1,1,1-trifluoro-4-iodobutane (Scheme 2). Wang *et al.* have previously reported a high diastereoselectivity (97%) was achieved with only 1.1 equiv. of NaOH.[45] We have, however, obtained contradictory results. Although the rate of reaction increased with an increased amount of NaOH, the diastereoselectivity between (*S*,*S*)-**6** and (*S*,*R*)-**6** was disappointingly low (Table 1). It has been shown in other studies that when a monoalkylated complex is subjected to epimerisation under basic condition, thermodynamic control would dominate and eventually drives the equilibrium towards the favoured (*S*,*S*)-epimer.[46–48] Thus, a mixture of the (*S*,*S*)- and (*S*,*R*)-alkylated complex **6** was partially purified in a reaction work-up, and subsequently treated with K_2CO_3 in MeOH at 60 °C (Table 1, entry 3). A satisfactory diastereomeric ratio of 97:3 was obtained. In a series of pilot experiments, the addition of K_2CO_3 directly into the DMF reaction mixture at 60 °C did not afford the desirable level of diastereoselectivity whereas increasing the amount of NaOH led to the formation of other by-products. As such, the rate of epimerization and the shift of equilibrium towards the (*S*,*S*)-epimer seemed to be highly dependent on the use of a polar protic solvent.

Table 1. Conditions investigated for the alkylation of (*S*)-**5** with 1,1,1-trifluoro-4-iodobutane.[a]

Entry	Condition	Total reaction time (h)	(*S*,*S*):(*S*,*R*)[b]	Yield (%)
1	NaOH (2 equiv.), DMF, r.t.	1.5	77/23	75
2	NaOH (5 equiv.), DMF, r.t.	0.5	77/23	61
3	NaOH (5 equiv.), DMF, r.t. followed by K_2CO_3 (5 equiv.), MeOH, 60 °C	2.5	97/3	73

[a] Reactions were performed using 0.40 mmol (*S*)-**5** and 0.44 mmol alkyl halide. [b] Diastereomeric ratio determined using analytical RP-HPLC.



Scheme 3. The synthetic route to Fmoc-homoallylglycine-OH.

Finally, the de-assembly of (*S*,*S*)-**6** to yield the desired amino acid was achieved by microwave-assisted acid-mediated hydrolysis. Without further purification, the released amino acid was *N*-protected with a Fmoc group. Following a simple work-up, trituration with hexane afforded sufficiently pure Fmoc-Tfn-OH (*S*)-**7** for incorporation into the macrocycle of teixobactin. Fmoc-Hag-OH (*S*)-**9** was prepared under similar conditions by alkylating Schiff base (*S*)-**5** with 4-bromo-but-1-ene (Scheme 3). These Fmoc-protected amino acids were used in the synthesis of our teixobactin analogues.

Synthesis of teixobactin analogues

The substitution of the L-*allo*-enduracididine^{10} in teixobactin **1** with arginine has been shown to retain appreciable activity.[23–25] Hence, (Arg^{10})teixobactin **55** was initially prepared (Scheme 4) to serve as the positive control in our microbiological evaluation. The robust synthetic method reported by Yang *et al.* was adapted with several adjustments.[24] This synthetic strategy enabled the use of the resin-anchored linear decapeptide **16** as a common intermediate for subsequent on-resin esterification with different protected amino acid building blocks, thus providing an expedient access to teixobactin analogues **57–62**.

The 2-chlorotrityl chloride resin was used as the polymer support as it allowed the liberation of the branched peptide by mild acidolysis while retaining the side-chain protecting groups. In the synthesis of (Arg^{10})teixobactin **55**, the resin was first loaded with Fmoc-Arg(Pbf)-OH and the peptide chain was elongated stepwise by acylation with Fmoc-amino acids that were pre-activated by HATU (1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate) in the presence of iPr$_2$EtN. In order to minimise steric hindrance effects, Fmoc-D-Thr-OH and Fmoc-D-Gln-OH were used without side-chain protecting groups and no significant problems were observed during their couplings.

The esterification of the D-Thr residue in intermediate **16** was then carried out with a pre-formed symmetrical anhydride of Fmoc-Ile-OH. It was found that the esterification step required up to four repeated couplings, each requiring overnight (15–18 h) exposure, in order to achieve approximately 50 % transformation as monitored by RP-HPLC. The long reaction time could be attributed to the steric bulk of the Ile side-chain and the entrenched hydroxyl group of the D-Thr residue. In contrast, greater than 70 % *O*-acylation of the D-Thr residue was achieved with Nle, Nva, Abu and Ala as the acylating amino acids (analogues **57–60**), further corroborating the significance of steric effects. Following Fmoc-deprotection, the branched peptide **25** was cleaved from the resin in preparation for solution-phase intramolecular cyclisation between the Arg^{10} and Ile^{11} residues.

The use of (1-cyano-2-ethoxy-2-oxoethylidenamino-oxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) as the carboxy-activating reagent enabled the cyclisation to be visually monitored through colour changes.[49,50] Upon addition of base, the solution changed from yellowish-orange to colourless in under an hour and the reaction was completed within 2 h. Global side-chain deprotection by acidolysis of the macrocyclic intermediate, followed by RP-HPLC purification and lyophilisation afforded **55** as a white solid.

All the other analogues were similarly prepared by replacing Ile^{11} with various aliphatic residues, including the use of the synthesized Fmoc-Tfn-OH and Fmoc-Hag-OH (**57**–**62**), Arg^{10} with several arginine derivatives and Tfn (**63–66**), Ala^{9} with Abu and Z-dehydrobutyrine (Dhb) (**67**–**68**), and finally, *N*-Me-D-Phe^{1} with D-Trp (**69**) (Figure 2).

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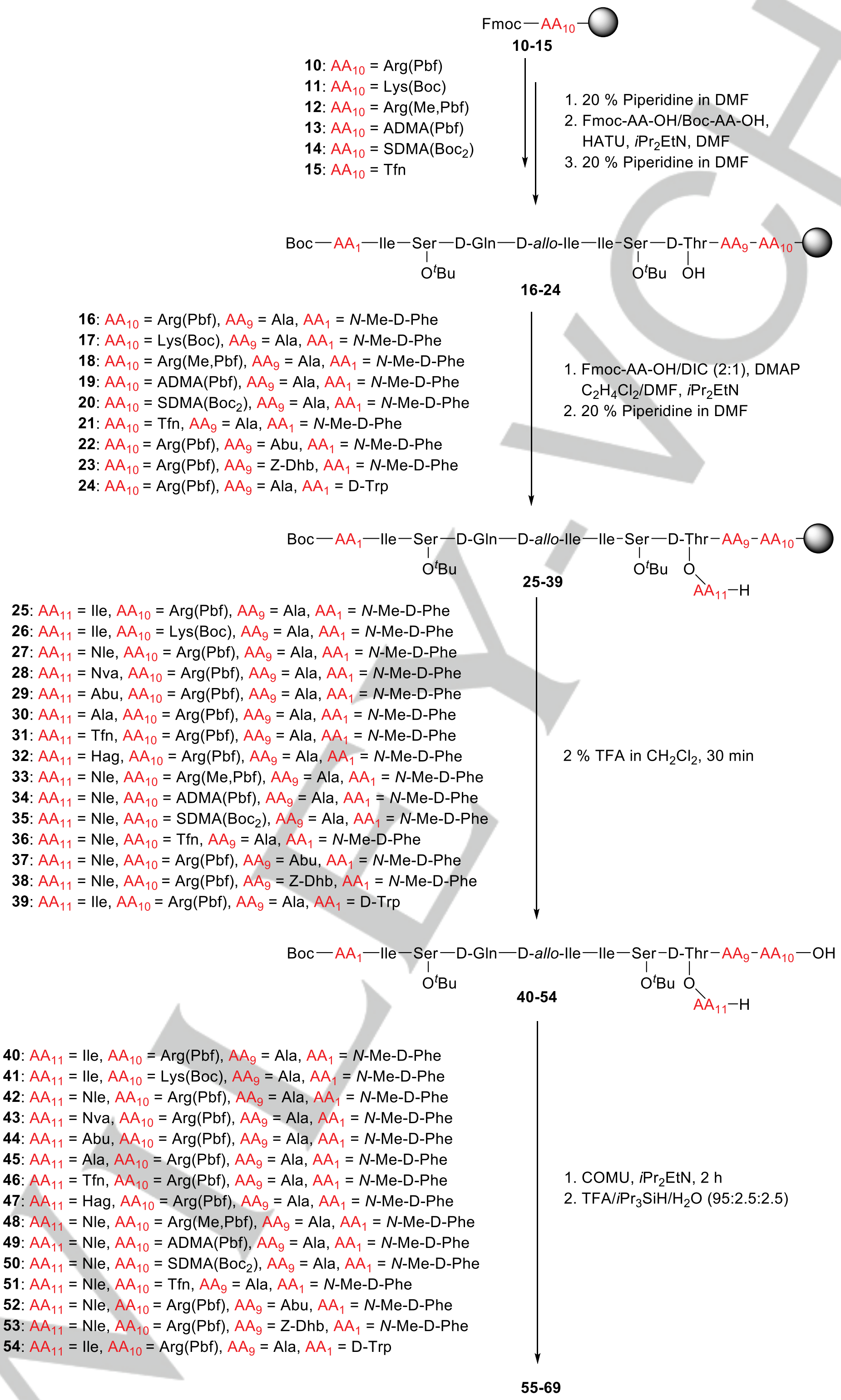

Scheme 4. Synthetic route for the preparation of teixobactin analogues. The synthesis of branched peptides **25**–**39** were performed solely on a polymer support (2-chlorotrityl chloride resin) using a 9-fluorenylmethyloxycarbonyl (Fmoc/*t*Bu) strategy. The cleaved peptides **40**–**54** were then subjected to solution-phase macrolactamisation.

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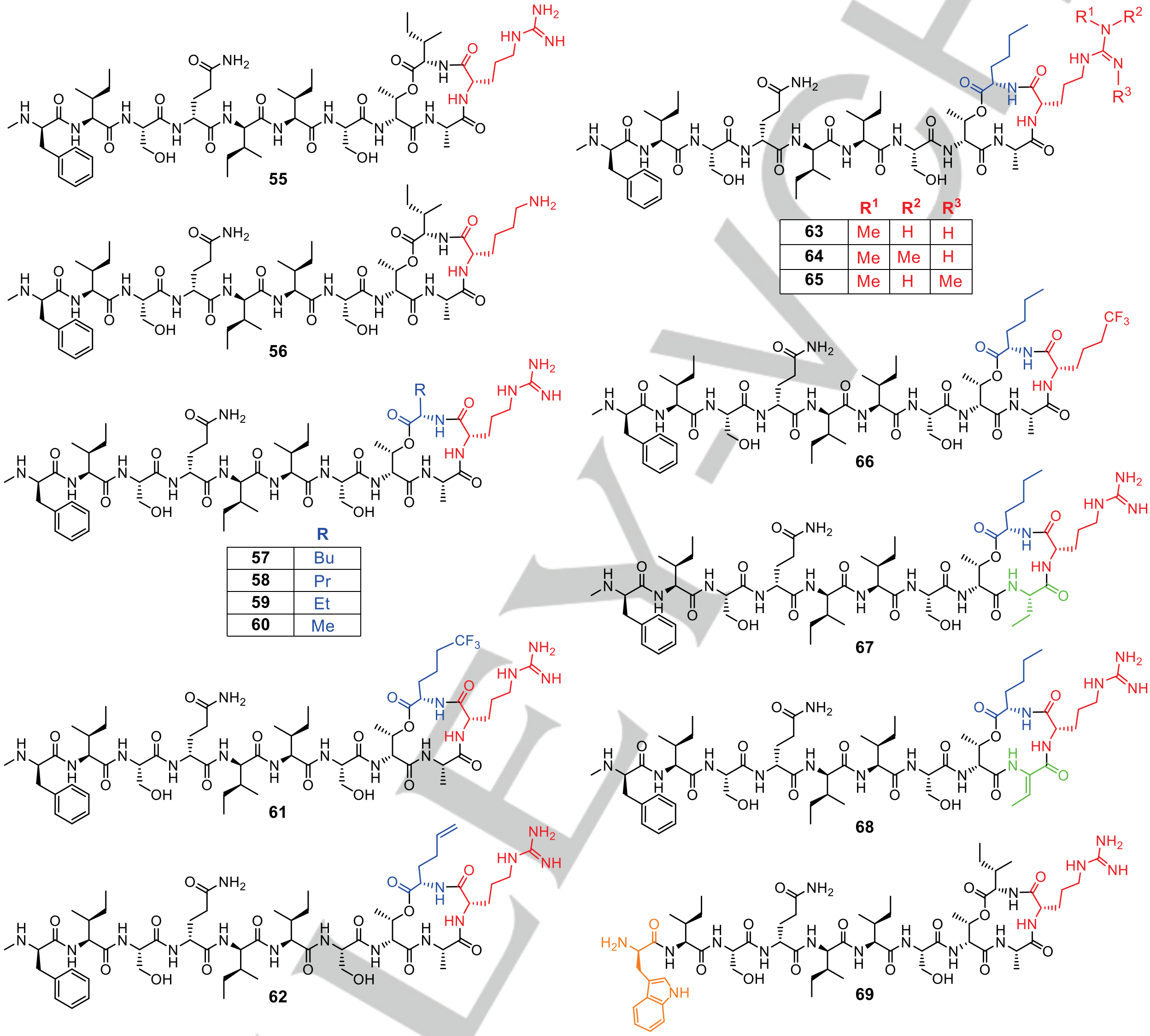

	R
57	Bu
58	Pr
59	Et
60	Me

	R^1	R^2	R^3
63	Me	H	H
64	Me	Me	H
65	Me	H	Me

Figure 2. The chemical structures of teixobactin analogues synthesized for antimicrobial evaluation.

Broth microdilution and growth inhibition assays

Minimal inhibitory concentration (MIC) is the most commonly used parameter to define in vitro antimicrobial susceptibility. It is defined as the lowest concentration of a compound that inhibits visible growth. Using the broth microdilution method outlined in the Clinical and Laboratory Standards Institute (CLSI) guidelines[51], the MIC values of our teixobactin analogues against five prominent isolates of *S. aureus* from different clinical settings and with varying antibiotic sensitivity were obtained. Among them, *S. aureus* USA300 JE2 is particularly virulent due to the expression of Panton-Valentine Leukocidin (PVL). In fact, the incidence of skin and soft tissue infections caused by *S. aureus* has increased substantially since the late 1990s, which is largely driven by the emergence of the USA300 clone.[52,53] The MIC was also determined for three *P. acnes* strains and the Gram-negative *Pseudomonas aeruginosa* PAO1. A summary description of the bacteria strains used in this study is provided in Table 2.

Since MIC is determined at a fixed incubation time-point (typically 16 h or 24 h), detailed information on how bacterial growth rate is affected by antimicrobials at different concentrations is unavailable.[65] Hence, a sensitive assay that involved the generation of growth curves was concurrently used to evaluate the synthesized teixobactin analogues.

S. aureus SH1000, a fully sequenced strain that is representative of the species, was used as the test microorganism in our growth inhibition assay. The starting bacterial concentration was adjusted to give approximately 10^6 colony forming units/mL (CFU/mL) in each well of a 96-well microtitre plate. Following treatment with different concentrations of the compounds, bacterial growth was monitored for 20 h by measuring the optical density at 600 nm. The normalized percentage growth at 13 h was then used to construct a dose-response curve in order to determine the concentration at which 50 % of the bacterial growth was inhibited (IC_{50}). As illustration, the dose-response curves of two analogues, (Arg^{10},Nle^{11})teixobactin **57** (IC_{50} = 7.38 ± 0.09 μM) and (Arg^{10},Ala^{11})teixobactin **60** (IC_{50} >100 μM), are shown in Figure 3; detailed growth curves and their corresponding dose-response curves are provided in the Supporting Information (p. S37–S40).

The sigmoidal dose-response curves of all active analogues showed a sharp drop in percentage growth as the concentration of the test compounds were increased. This was also observed with vancomycin, a well-established lipid II inhibitor which was used as the positive control. This phenomenon where a small change in concentration causes substantial growth inhibition appeared to be a common attribute of lipid II and/or lipid III binders. In addition to a measurement of potency, the IC_{50} values (Table 3) provided useful and invaluable SAR information on the effect of subtle changes in the chemical composition of our teixobactin analogues.

Table 2. Bacterial strains used for antimicrobial susceptibility testing of teixobactin analogues.

Organism	Description	Reference
S. aureus		
SH1000	*S. aureus* 8325-4 strain with repaired *rsbU* gene	[54]
Newman	Clinical MSSA isolate with fully sequenced genome and lacks in antibiotic resistant genes	[55,56]
PM64	Healthcare-associated MRSA, isolate of the epidemic MRSA type 16 clonal group (EMRSA-16)	[57]
USA300 JE2	Community-acquired MRSA USA300 LAC cured of three plasmids	[58]
Mu50	Vancomycin-intermediate resistant strain (VISA) with thickened cell walls	[8,9,59]
P. acnes		
ATCC 11828	Representative strain of the type II phylotype	[60,61]
ATCC 6919	Representative strain of the type IA_1 phylotype	[62]
Asn12	Representative strain of type III phylotype isolated from intervertebral disc material	[63]
P. aeruginosa		
PAO1	Gram-negative, wild-type laboratory strain, Nottingham sub-line	[64]

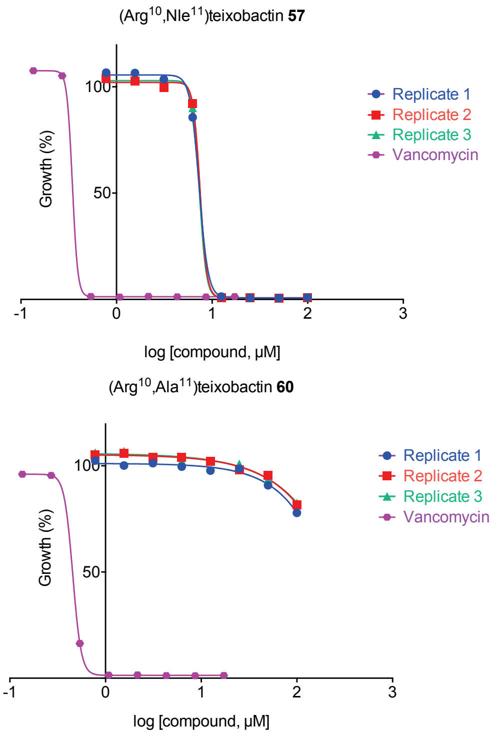

Figure 3. Dose-response curves of analogues **57** and **60** against *S. aureus* SH1000. Three independent experiments were conducted, with vancomycin (IC_{50} = 0.45 ± 0.13 μM) as the positive control.

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The (Arg^{10},Nle^{11})teixobactin (**57**) was also tested using the same methodology against *P. acnes* ATCC 11828 (Figure 4). Unlike *S. aureus*, the anaerobe *P. acnes* showed a more gradual decrease in growth with increasing concentrations of the test compound. This distinct dose-response pattern may be due to the slow-growing nature of the bacteria and/or the difference in its cell wall composition compared to other Gram-positives.[13] In future, further work will be performed to establish the lipid contents of the cell wall of *P. acnes* in order to shed light on the binding efficacy of teixobactin analogues.

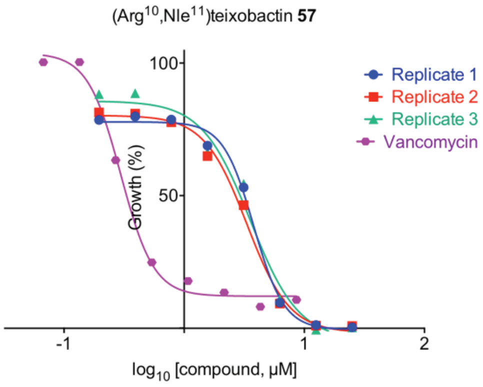

Figure 4. Dose-response curves of analogue **57** against *P. acnes* ATCC 11828 (IC_{50} = 3.56 ± 0.23 µM). Three independent trials were conducted with vancomycin (IC_{50} = 0.29 ± 0.03 µM) as the positive control.

Both arginine and lysine have been used as a substitute for L-*allo*-enduracididine at position 10. Yang *et al.* reported that (Lys^{10})teixobactin showed a 2- to 4-fold lower MIC values than (Arg^{10})teixobactin against specific strains of *Staphylococcus epidermidis*, *Streptococcus salivarus*, *Enterococcus durans* and *Bacillus subtilis*.[24] A similar result against MRSA ATCC 33591 has recently been observed by Singh and co-workers.[37] However, based on the MIC values, we observed that the (Lys^{10})teixobactin was in fact consistently 2-fold less potent compared to (Arg^{10})teixobactin across the *S. aureus* strains tested; the IC_{50} data similarly reported a 2.2-fold loss of activity. Since (Arg^{10})teixobactin showed a better antibacterial profile here, Arg was maintained at this position in analogues **57** to **62** in our initial SAR study.

The Ile^{11} was initially replaced with its aliphatic isostere, Nle. The resultant (Arg^{10},Nle^{11})teixobactin **57** (IC_{50} = 7.38 ± 0.09 µM) was found to have comparable potency, if not slightly better than (Arg^{10})teixobactin **55** (IC_{50} = 7.96 ± 0.36 µM) in inhibiting the growth of *S. aureus* SH1000. Additionally, both analogues showed the same MIC of 8 µg/mL against three different strains of *S. aureus.* It was surprising to observe that (Arg^{10},Nle^{11})teixobactin was 2-fold more potent against MRSA PM64 but 2-fold less active against VISA Mu50. Nevertheless, the overall results suggest that the flexible linear aliphatic chain of Nle seemed to confer similar hydrophobic interactions as the branched chain of Ile. Decreasing the alkyl chain length, however, has a negative impact. There was an increasing reduction in antimicrobial potency with the replacement of Nle with Nva and Abu (compounds **58** and **59**) followed by a complete loss of activity when Ala was installed (compound **60**). This indicates that a minimum of four carbons are necessary for optimal hydrophobic interactions. The importance of a non-polar group at position 11 was also evident in previous studies which showed that the substitution of Ile with a polar residue, such as Lys, abolished antibacterial activity.[27,31]

Having identified Nle as a beneficial substitute of Ile, we next investigated the effects of introducing fluorine atoms and an unsaturated functional group in the hydrocarbon side-chain. It is hypothesised that the replacement of the terminal methyl in Nle with a trifluoromethyl moiety could provide the extra hydrophobicity needed for effective binding. However, the antimicrobial potency against *S. aureus* SH1000 dropped by almost 4.0-fold when Nle was replaced with Tfn in compound **61**. The MIC values of (Arg^{10},Tfn^{11})teixobactin **61** against the other *S. aureus* strains were also observed to be 2- to 4-fold higher compared to (Arg^{10},Nle^{11})teixobactin **57**. The electronegativity of the fluorine atoms appeared to detrimentally affect the interaction of the (Arg^{10},Tfn^{11})-analogue **61** with its molecular targets. Alternatively, the steric effect from the bulkier CF_3 moiety might have caused the decrease in activity. Further work could be carried out by synthesizing a shorter trifluoromethylene analogue since the -CF_3 is considered an isosteric replacement of an ethyl moiety.[66–68] A similar increase in both IC_{50} and MIC was observed when a terminal alkenic bond was introduced (compound **62**). Nle was therefore chosen as the optimal residue at this position for the subsequent six analogues **63–68**, in which the effect of replacing the guanidine moiety in Arg^{10} was investigated. The less sterically demanding Nle additionally provided a synthetic advantage in the esterification step compared to Ile.

The cationic nature of L-*allo*-enduracididine^{10} in the native peptide is thought to be crucial in the electrostatic interaction with the negatively-charged pyrophosphate moiety in lipid II.[4] In this unusual amino acid, the terminal NH group of its guanidine moiety is connected to the γ-carbon to afford a heterocyclic structure. As such, it was envisaged that additional methyl group(s) on the guanidine in the side-chain would closely mimic the natural interaction of teixobactin with lipid II. Gratifyingly, compared to (Arg^{10},Nle^{11})-analogue **57**, the [Arg(Me)^{10},Nle^{11}]teixobactin **63** displayed a 2-fold increase in antimicrobial potency against several *S. aureus* strains (4-fold in *S. aureus* Mu50) when evaluated using the IC_{50} and MIC values. Evich *et al.* has shown that the pK_a of the guanidino group of Arg is not significantly altered by methylation.[69] Hence, the increase in hydrophobicity rather than basicity resulting from the N^G-methylation has contributed to a greater binding affinity. On the other hand, the asymmetric and symmetric N^G-dimethylated arginine analogues, **64** and **65**, respectively showed 3.4- and 2.4-fold reduction in potency (IC_{50}) compared to the corresponding Arg analogue **57**. The introduction of the second methyl group appeared to be detrimental, possibly due to disruption of potential hydrogen bond(s) or significant steric hindrance.[69] These results are consistent with other studies that showed a lack of activity when the guanidino group was tetra-alkylated.[35]

Table 3. The antibacterial activity of teixobactin analogues and several antibiotics.

Compound	IC_{50} (μM) against *S. aureus* SH1000[a]	Minimum inhibitory concentration (μg/mL)[b]								
		S. aureus					*P. acnes*			*P. aeruginosa*
		SH1000	Newman	USA300 JE2	PM64	Mu50	ATCC 11828	ATCC 6919	Asn12	PAO1
(Arg^{10})teixobactin **55**	7.96 ± 0.36	8	8	8	8	8	4	4	4	>256
(Lys^{10})teixobactin **56**	17.43 ± 2.31	16	16	16	16	16	8	8	8	>256
(Arg^{10},Nle^{11})teixobactin **57**	7.38 ± 0.09	8	8	8	4	16	4	4	4	>256
(Arg^{10},Nva^{11})teixobactin **58**	8.53 ± 0.37	8	8	16	8	16	8	8	4	>256
(Arg^{10},Abu^{11})teixobactin **59**	14.74 ± 0.25	16	16	16	16	32	8	8	8	>256
(Arg^{10},Ala^{11})teixobactin **60**	> 100	>32	>32	>32	>32	>32	32	>32	32	>256
(Arg^{10},Tfn^{11})teixobactin **61**	29.16 ± 1.51	32	32	32	16	32	16	32	32	>256
(Arg^{10},Hag^{11})teixobactin **62**	28.55 ± 0.24	32	32	32	32	32	16	32	32	>256
($Arg(Me)^{10}$,Nle^{11})teixobactin **63**	3.84 ± 0.26	4	4	4	2	4	2	4	2	>256
($ADMA^{10}$,Nle^{11})teixobactin **64**	24.83 ± 4.47	16	32	32	16	32	8	8	8	>256
($SDMA^{10}$,Nle^{11})teixobactin **65**	17.82 ± 3.42	16	16	16	16	32	8	8	8	>256
(Tfn^{10},Nle^{11})teixobactin **66**	8.02 ± 0.26	8	8	8	8	8	8	4	4	>256
(Abu^{9},Arg^{10},Nle^{11})teixobactin **67**	7.52 ± 0.24	8	8	8	8	8	4	4	4	>256
(Dhb^{9},Arg^{10},Nle^{11})teixobactin **68**	25.69 ± 4.57	32	32	32	16	32	16	16	16	>256
(D-Trp^{1},Arg^{10})teixobactin **69**	> 100	>32	>32	>32	>32	>32	>32	>32	>32	>256
Teixobactin[c]	-	-	-	0.5[11]	-	-	-	0.08[4]	-	>100[4]
Meropenem	n.d.	<0.0625	<0.0625	<0.0625	16	n.d.	n.d.	n.d.	n.d.	8
Vancomycin	0.45 ± 0.13	1	0.5	0.5	0.25	4	1	2	1	>256
Colistin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4
Tunicamycin	n.d.	n.d.	n.d.	8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] IC_{50} values were expressed as the mean ± SD of three independent growth inhibition assay; n.d. = not determined. [b] MIC determined by broth microdilution performed in triplicates according to CLSI guidelines. [c] MIC values of teixobactin were obtained from literature; Nle = norleucine; Nva = norvaline; Abu = aminobutyric acid; Tfn = trifluoronorleucine; Hag = homoallylglycine; Arg(Me) = monomethylarginine; ADMA = asymmetric dimethylarginine; SDMA = symmetric dimethylarginine; Dhb = dehydrobutyrine.

Given the likely favourable electronic and hydrophobic properties of our synthesized trifluoromethyl-containing amino acid, the (Tfn^{10},Nle^{11})teixobactin **66** was synthesized and found to be only marginally less active than (Arg^{10},Nle^{11})teixobactin **57** (IC$_{50}$ 8.02 ± 0.26 *cf* 7.38 ± 0.09 μM). This result reveals that position 10 is a good location for the introduction of other fluorinated hydrocarbon amino acids. In fact, Chen *et al.* reported that (Ala^{10})teixobactin unexpectedly retained considerable activity against *S. aureus*; it was proposed that a positively-charged residue is not essential at position 10.[34]

The role of the Ala^{9} residue is not fully known except that it could be replaced with polar amino acids, such as Lys or Orn.[27,29,31,35] We observed that replacement of the methyl group with an ethyl moiety did not significantly change the antimicrobial activity since (Abu^{9},Arg^{10},Nle^{11})teixobactin **67** and (Arg^{10},Nle^{11})teixobactin **57** are essentially equipotent against the tested *S. aureus* strains. Subsequently, the possibility of improving the conformational characteristics of the peptide through the installation of an *α*,*β*-dehydroamino acid was investigated. The alkenic amino acid residue is known to rigidify the backbone of macrocyclic rings and could lead to a desirable peptide conformation that enhances binding to its target(s).[70] *Z*-Dhb was chosen as the test residue due to its natural abundance in antimicrobial peptides, including the lipid II-binder nisin.[71,72] However, compared to the corresponding Abu-containing analogue **67**, the potency of (Dhb^{9},Arg^{10},Nle^{11})teixobactin **68** was decreased by 3.4-fold; using the MIC values, a reduction of 2- to 4-fold in activity was observed against all the tested *S. aureus* strains. A similar result was found by Albericio and co-workers when 4-aminoproline was introduced at position 10 to constrict the macrocyclic ring.[35] Collectively, these results showed that conformational restriction has an unfavourable effect on target binding.

Meanwhile, hydrophobicity was previously found to play a significant role at position 1. Although the removal of the *N*-methyl group from *N*-Me-D-Phe^{1} has a minimal effect on potency, activity was lost when Wu *et al.* replaced D-Phe with D-Tyr.[28] The same observation was obtained with analogue **69**, in which the phenyl was substituted with an indole moiety; our (D-Trp^{1},Arg^{10})-analogue **69** is essentially inactive.

The treatment of MRSA infection is increasingly difficult due to the co-emergence of VISA strains. It is therefore not surprising that the MIC of vancomycin is increased by 4- to 16-fold when tested against VISA Mu50 (Table 3). This is attributed to thicker peptidoglycan layers and a lower degree of cross-linking that exposes more of the D-alanyl-D-alanine binding site of vancomycin.[8,9] A large proportion of the vancomycin molecules are consequently trapped or titrated out by the mature peptidoglycan, preventing them from reaching their vital targets near the cytoplasmic membrane.[9] In contrast, most of the teixobactin analogues tested showed the same MIC across all the *S. aureus* strains, including Mu50. Hence, these teixobactin analogues, similar to the parent teixobactin, are unaffected by the thicker peptidoglycan layers in the VISA strains.[11] On a side note, studies have suggested the addition of a surfactant in the broth microdilution assay to prevent possible adsorption of the peptide compounds to the plastic wall of the 96-well plate.[4,11,73,74] However, no difference in MIC was observed for [Arg(Me)^{10},Nle^{11}]teixobactin **63** in the presence of 0.002 % or 0.1 % Tween 80.

We next evaluated the antimicrobial utility of our teixobactin analogues against the opportunistic pathogen *P. acnes*. The majority of the analogues showed a 2-fold higher potency against the three *P. acnes* strains tested compared to *S. aureus* (Table 3). In the broth microdilution assay, the bacteria inoculum for *P. acnes* was approximately 100-fold higher than *S. aureus*. As such, the MIC values of the teixobactin analogues are likely to be considerably lower if a similar inoculum size was used. Overall, the results are in agreement with teixobactin which showed a 3-fold higher potency against *P. acnes* compared to *S. aureus*.[4] It is worth highlighting that the MIC of our most potent analogue, [Arg(Me)^{10},Nle^{11}]teixobactin (**63**), is comparable to that of vancomycin. These results demonstrate the promising application of our teixobactin analogue against the emerging pathogenic bacterium *P. acnes*.

Time-kill assay to establish bactericidal activity against *S. aureus* and *P. acnes*

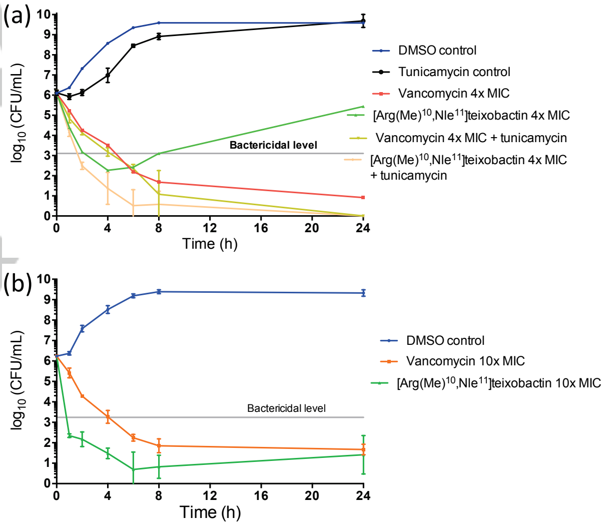

Figure 5. Time-kill curves of *S. aureus* USA300 JE2 treated with (a) 4x MIC [Arg(Me)^{10},Nle^{11}]teixobactin **63** or 4x MIC vancomycin in the presence and absence of tunicamycin (0.4 μg/mL) and (b) 10x MIC **63** or 10x MIC vancomycin. Data are presented as the mean log$_{10}$ viable counts (CFU/mL) ± SD from two independent experiments.

Teixobactin has been shown to possess excellent bactericidal activity against *S. aureus*.[4,11] Although both IC$_{50}$ and MIC values are good indicator of potency, it remains unknown if our teixobactin analogues are bacteriostatic or bactericidal.[65] Hence, the killing kinetics of our most potent analogue, [Arg(Me)^{10},Nle^{11}]teixobactin **63**, were assessed against the highly virulent pathogen *S. aureus* USA300 JE2 using a validated time-kill method. Thus, *S. aureus* USA300 JE2 grown to mid-exponential phase was challenged with suprainhibitory

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concentrations (4x or 10x MIC) of **63**. Vancomycin, an established antibiotic for treating challenging *S. aureus* infections, was used as a comparator. Viable counts (CFU/mL) of the bacteria were then determined at specific times over 24 h. A compound that reduces bacterial viability by ≥ 3 $\log_{10}$ CFU/mL is defined as bactericidal.[75]

At 4x MIC, the [Arg(Me)^{10},Nle^{11}]teixobactin **63** showed a greater killing rate than vancomycin in the first six hours (Figure 5 (a)). There was, however, a regrowth of bacteria from 8 h onwards for compound **63**. To our delight, a decrease of >3 $\log_{10}$ CFU/mL was rapidly obtained with 10x MIC of [Arg(Me)^{10},Nle^{11}]teixobactin **63**, within an hour (Figure 5(b)). In contrast, vancomycin required up to 4 h to achieve bactericidal activity at 10x MIC. Thus, our lead compound **63** at 10x MIC showed highly efficient and sustained bactericidal activity against the pathogenic *S. aureus* USA300 JE2.

Vancomycin acts by binding solely to lipid II, an important intermediate for the synthesis of bacterial peptidoglycan.[10,76] On the other hand, teixobactin is reported to bind to both lipid II and lipid III, which may account for its remarkable bactericidal activity over vancomycin.[4,11] Lipid III is the precursor for the synthesis of wall teichoic acid (WTA) which is covalently attached to peptidoglycan.[77,78] It is formed by the TarO-mediated attachment of *N*-acetylglucosamine to undecaprenyl pyrophosphate. Subsequently, *N*-acetylmannosamine is transferred to lipid III by TarA. The resultant lipid-linked disaccharide intermediate is then modified by a series of other Tar enzymes before being transported through the cell membrane to be linked to the peptidoglycan network.[77,79,80] Paradoxically, the deletion of genes encoding TarO and/or TarA has no effects on the in vitro viability of bacteria. These early-stage enzymes have been deemed non-essential and are useful for studying potential WTA inhibitors.[80–83]

Under normal circumstances, WTA plays a pivotal role in anchoring the major autolysin Atl to prevent unregulated self-digestion. The absence of WTA results in the delocalisation of this enzyme throughout the cell surface that eventually leads to bacterial lysis.[84,85] It is anticipated that the rapid bactericidal activity of [Arg(Me)^{10},Nle^{11}]teixobactin **63** might also be due to the synergistic inhibition of both peptidoglycan and WTA synthesis.

To demonstrate the significance of WTA for bacterial survival, we determined the killing kinetics of both [Arg(Me)^{10},Nle^{11}]teixobactin and vancomycin (4x MIC) in the presence of sub-lethal tunicamycin (0.05x MIC), a highly selective inhibitor of TarO[86]. At the sub-MIC concentration, tunicamycin showed no effects on cell viability and growth of *S. aureus* USA300 JE2 (Figure 5(a)), though it is predicted that WTA production is substantially suppressed. In the initial eight hours, the bactericidal profile was very similar for vancomycin regardless of the presence of tunicamycin. However, the viable count was reduced to a greater extent (<0.1 $\log_{10}$ CFU/mL) at 24 h when vancomycin was used with tunicamycin compared to vancomycin alone (*ca.* 1 $\log_{10}$ CFU/mL remaining). Tunicamycin also enhanced the killing activity of 4x MIC [Arg(Me)^{10},Nle^{11}]teixobactin **63** since there was no regrowth of bacteria throughout the 24 h (Figure 5(a)). This suggests that compound **63** and tunicamycin are likely to perturb different stages of the WTA biosynthesis. The rapid killing activity of **63** could therefore be due to a more lethal target within the WTA biosynthetic pathway and further work is required to establish its target. Despite being viable in vitro, bacterial cells without WTA tend to show an altered morphology and defects in cellular division.[83,86] This may explain why the killing effects of both vancomycin and **63** were noticeably potentiated when used in combination with tunicamycin.

The time-kill assay was similarly conducted on *P. acnes* ATCC 11828. As expected, [Arg(Me)^{10},Nle^{11}]teixobactin **63** was bactericidal and exhibited a faster killing rate than vancomycin (Figure 6). Unlike in *S. aureus*, however, no regrowth of bacteria was observed up to 48 h with 4x MIC of compound **63**. Once again, this confirms our analogue **63** holds great promise for the treatment of *P. acnes* infection.

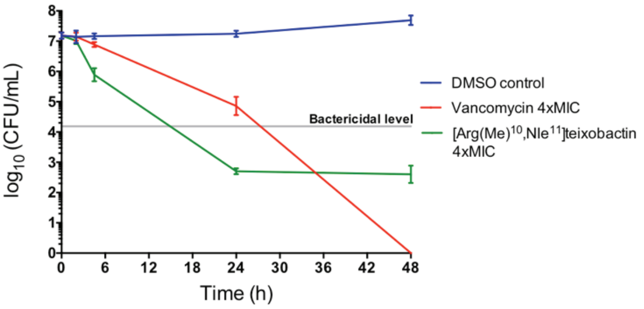

Figure 6. Time-kill curves of *P. acnes* ATCC 11828 treated with 4x MIC [Arg(Me)^{10},Nle^{11}]teixobactin **63** or 4x MIC vancomycin. Data are presented as the mean $\log_{10}$ viable count (CFU/mL) ± SD of two independent experiments.

Checkerboard assay to determine antimicrobial effect of teixobactin analogues-colistin combination against *P. aeruginosa*

Given the size (M.W. 1200–1300) and hydrophobic nature of the teixobactin analogues reported herein, it is not surprising that these compounds failed to display effective antimicrobial activity against the Gram-negative pathogen, *Pseudomonas aeruginosa* (MIC >256 µg/mL, Table 3). The bacterial outer membrane presents a significant permeability barrier[87] and hence, the teixobactin analogues are unable to reach their molecular target, i.e. lipid II, that is located in the periplasm; lipid III and teichoic acid are not found in Gram-negatives. The Gram-negative outer membrane is an asymmetric bilayer comprised of lipopolysaccharides (lipid A and O-antigen moieties) and glycerophospholipids. Colistin, a cationic polymyxin antibiotic, is known to disrupt Gram-negative outer membrane via its interactions with anionic lipopolysaccharides and phospholipids.[88] Thus, we hypothesized that the membrane disruptive capability of colistin would enable permeation of teixobactin analogues through the pseudomonal outer membrane and hence ‘restore’ antimicrobial susceptibility.

Indeed, growth inhibition was observed when several of the analogues were tested in combination with 0.5x MIC colistin against *P. aeruginosa* PAO1 (Figure 7). In fact, the MIC values of (Arg^{10},Nle^{11})teixobactin **57** and (Tfn^{10},Nle^{11})teixobactin **66** were markedly reduced from >256 µg/mL to 64 µg/mL and 32 µg/mL, respectively.

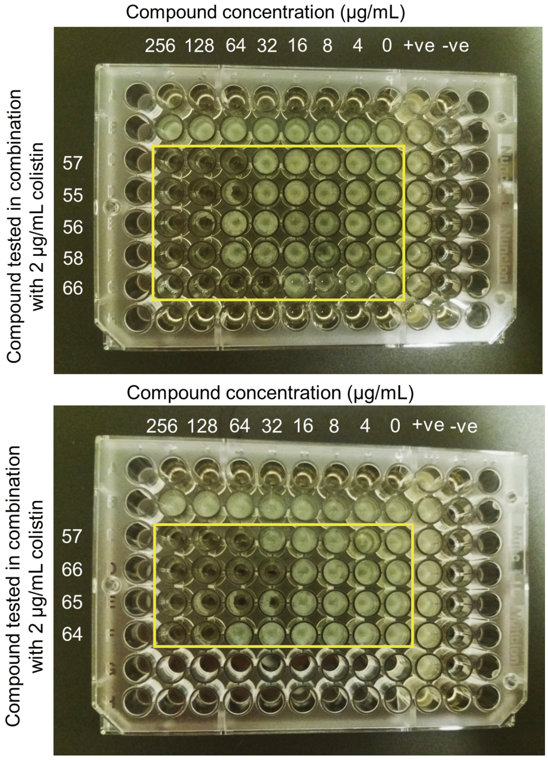

Figure 7. Antimicrobial susceptibility testing of analogues (Arg^{10})teixobactin **55**, (Lys^{10})teixobactin **56**, (Arg^{10},Nle^{11})teixobactin **57**, (Arg^{10},Nva^{11})teixobactin **58**, (ADMA^{10},Nle^{11})teixobactin **64**, (SDMA^{10},Nle^{11})teixobactin **65** and (Tfn^{10},Nle^{11})-teixobactin **66** in combination with colistin (2 µg/mL, 0.5x MIC) against *P. aeruginosa* PAO1. Wells A2–A8 and wells B2–B8 were set up as control wells containing colistin at 4 µg/mL and 2 µg/mL respectively. +ve = media and bacteria only, -ve = media only.

In order to comprehensively establish a synergistic or additive effect of the test compounds-colistin combination, an antimicrobial checkerboard assay against *P. aeruginosa* PAO1 was performed on analogue **66** and our lead compound [Arg(Me)^{10},Nle^{11}]teixobactin **63** (Figure 8); the results for analogue **57** and the comparator antibiotic vancomycin can be found in Supporting Information (Figure S1, p. S36). Surprisingly, despite its high potency against the tested Gram-positive pathogens, compound **63** remained inactive at 256 µg/mL even when combined with colistin (Figure 8(b)). The vancomycin (256 µg/mL)-colistin (0.125–0.5x MIC) combinations were similarly found to be ineffective. In sharp contrast, when (Tfn^{10},Nle^{11})teixobactin **66** was used in combination with colistin, an additive effect (FICI = 0.63) was observed in the checkerboard assay against *P. aeruginosa* PAO1 (Figure 8(a)).

Multidrug resistant (MDR) *P. aeruginosa* is increasingly responsible for infection in the critically ill patients. In many of these cases, colistin is used as the last resort treatment option. However, the use of high doses of colistin is limited by its nephrotoxicity.[88] The pronounced restoration of antimicrobial susceptibility of (Tfn^{10},Nle^{11})teixobactin **66** by sub-MIC level of colistin offers, for the first time, an unique therapeutic option for the treatment of infections caused by MDR *P. aeruginosa* and possibly other Gram-negative pathogens.

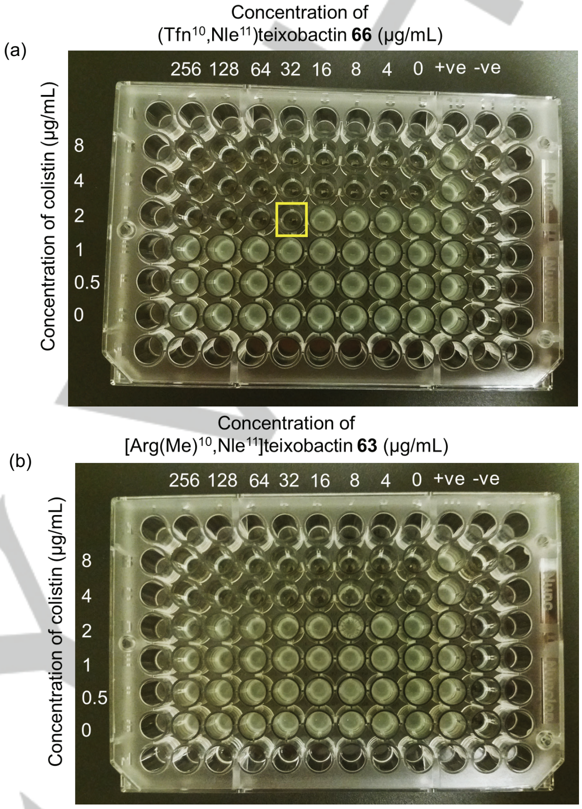

Figure 8. Checkerboard assay of (a) (Tfn^{10},Nle^{11})teixobactin **66** and (b) [Arg(Me)^{10},Nle^{11}]teixobactin **63** with varying concentration of colistin. +ve = media and bacteria only, -ve = media only.

Conclusions

In summary, a series of 14 unique teixobactin analogues have been designed and synthesized to critically examine the roles of the residues at positions 9, 10 and 11 within the macrocyclic structure. The antimicrobial activity of the analogues was determined against a panel of clinically important *S. aureus* isolates, including the highly virulent USA300 JE2 and Mu50 (a vancomycin intermediate-resistant *S. aureus*, VISA) strains. The teixobactin analogues were also tested against a panel of *P. acnes* strains and they were found to be more potent than against *S. aureus*. The in vitro antimicrobial effect of these analogues provided valuable SARs information and [Arg(Me)^{10},Nle^{11}]teixobactin **63** (IC_{50} = 3.84 ± 0.26 µM against *S. aureus* SH1000; MIC 2–4 µg/mL against 5 different *S. aureus* strains and 3 different *P. acnes* strains) has been identified as a lead candidate. The impressive bactericidal activity (characterised by the rate of kill) of compound **63** at 10x MIC, exceeding that of vancomycin, further reinforces its therapeutic potential. Importantly, when used in combination with an outer membrane-disruptive antibiotic, colistin at 0.5x MIC, the antimicrobial activity of a subset of teixobactin analogues against *P. aeruginosa* was ‘restored’, thereby providing a potential option for the treatment of infections caused by MDR *P. aeruginosa* and possibly other Gram-negative pathogens. In light of the pronounced antimicrobial effectiveness of the [Tfn^{10},Nle^{11}]teixobactin **66** (32 µg/mL)-colistin (2 µg/mL; 0.5x MIC) combination, its potency should be determined against a wider spectrum of clinical isolates of not only *P. aeruginosa* but also MDR *Acinetobacter baumannii* and *Klebsiella pneumoniae*. Furthermore, the antibiotic combination should be evaluated against colistin-resistant Gram-negatives. These future studies will be reported in due course.

Experimental section

For full experimental procedures, spectroscopic and analytical data for all new compounds, including copies of NMR spectra, see the Supporting Information.

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Keywords: macrocyclic peptides • lipid II inhibitors • teixobactin • antimicrobial • colistin

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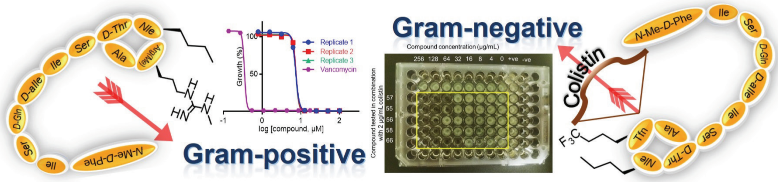

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