Development of Teixobactin Analogues Containing Hydrophobic, Non-Proteogenic Amino Acids that are Highly Potent Against Multidrug-Resistant Bacteria and Biofilms

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Abbreviations:

AA, Amino acid; Ac_2O, Acetic anhydride; Alloc, Allyloxycarbonyl; AMR, Antimicrobial resistance; ATCC, American Type Cell Culture; Boc, tert-butyloxycarbonyl; CFU, Colony-forming unit, DCM, Dichloromethane; DIC, Diisopropylcarbodiimide; DIPEA/DIEA, Diisopropylethylamine; DMAP, 4-Dimethylaminopyridine; DMF, *N*,*N*-Dimethylformamide; Et_3N, Triethylamine; ESI, Electrospray Ionization; HATU, N-[(dimethylamino-1H-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide; HRMS, High-resolution mass spectroscopy, HPLC , High-performance liquid chromatography; MeOH, Methanol; MIC, Minimum inhibitory concentration; MRSA, Methicillin-resistant *Staphylococcus aureus*; NMR, Nuclear magnetic resonance; PBS, Phosphate-buffered saline; $[Pd(PPh_3)_4]^0$, Tetrakis(triphenylphosphine)palladium(0); PG, Protecting group; $PhSiH_3$, Phenylsilane; *S. aureus* (SA), *Staphylococcus aureus*; TFA, Trifluoroacetic acid; TIS, Triisopropylsilane; Trt, Trityl; VRE, Vancomycin-resistant Enterococci

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Abstract: Teixobactin is a cyclic undecadepsipeptide that has shown excellent potency against multidrug-resistant pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE). In this article, we present the design, synthesis, and antibacterial evaluations of 16 different teixobactin analogues. These simplified analogues contain commercially available hydrophobic, non-proteogenic amino acid residues instead of synthetically challenging expensive L-*allo*-enduracididine amino acid residue at position 10 together with different combinations of arginines at positions 3, 4 and 9. The new teixobactin analogues showed potent antibacterial activity against a broad panel of Gram-positive bacteria, including MRSA and VRE strains. Our work also presents the first demonstration of the potent antibiofilm activity of teixobactin analogoues against *Staphylococcus* species associated with serious chronic infections. Our results suggest that the use of hydrophobic, non-proteogenic amino acids at position 10 in combination with arginine at positions 3, 4 and 9 holds the key to synthesising a new

generation of highly potent teixobactin analogues to tackle resistant bacterial infections and biofilms.

Keywords

Teixobactin, Antimicrobial Resistance, MRSA, VRE, Biofilms, Antibiofilm

Introduction

The recently discovered antibiotic teixobactin[1] has shown excellent activity against a broad range of Gram-positive bacteria, including multidrug-resistant pathogens, such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci, (VRE). Importantly, teixobactin kills Gram-positive bacteria without detectable resistance[1] and is known to operate via several modes of action, thereby making it difficult for bacteria to evolve resistance against the antibiotic.[2] The above reasons make the teixobactin scaffold an attractive starting point to develop a new class of antibiotics.[3]

Since the first publication by Ling *et al*. ,[1] several notable contributions have been made to teixobactin research, describing the total synthesis of teixobactin[4 5] and the syntheses and biological activities of teixobactin analogues.[6 7 8] Our group has established the importance of the D configuration of the amino acids of teixobactin in terms of antibacterial activity.[9] A lysine scan of Arg_{10}-teixobactin reported by Albericio *et al*. [10] showed that replacement of any one of the four isoleucine residues with lysine leads to a complete loss of antimicrobial activity. Furthermore,

Lys_{10}/Arg_{10}, Ser_7 and the NH- group of the *N*-terminal phenylalanine are important for the biological activity of teixobactin analogues.[11] A series of teixobactin analogues using convergent Ser/Thr ligation was reported, and the study suggested that substituting L-*allo*-enduracididine (End_{10}) with norarginine resulted in 16- to 32-fold increases in the MIC values.[12]

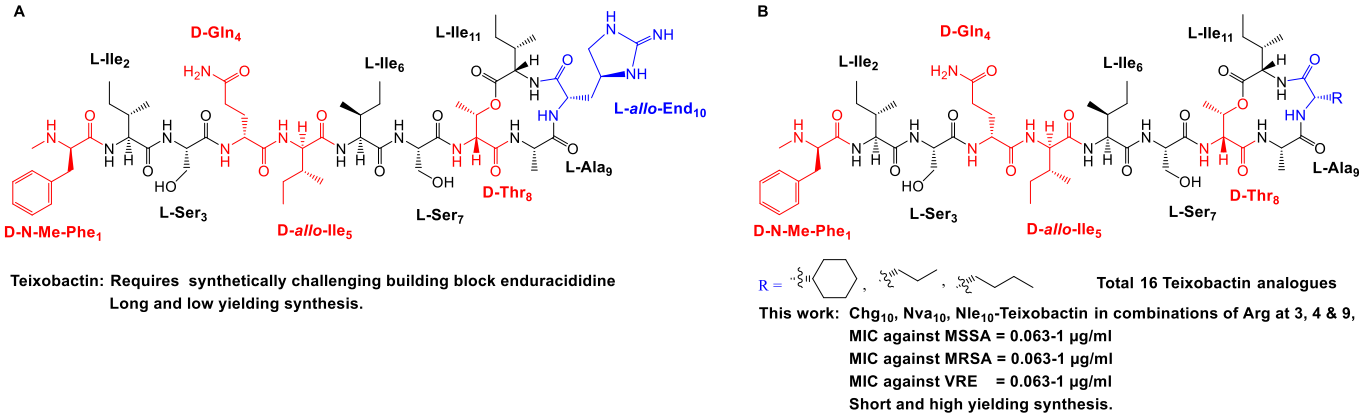


Fig. 1 A: Teixobactin and **1B:** its analogues containing hydrophobic non-proteogenic amino acids at position 10 and combination of arginine at positions 3, 4, 9.

We have also reported a series of potent teixobactin analogues by replacing L-*allo*-enduracididine with its isosteres.[13] In our out-of-box discovery, we showed that replacing End_{10} with Leu or Ileu retained the antimicrobial potency of native teixobactin, suggesting that a cationic amino acid residue at position 10 is not essential for high antibacterial activity.[14] Other important SAR publications include the synthesis and antibacterial activities of teixobactin analogues (containing End_{10} isosteres)[15] and teixobactin analogues containing lipid tails, such as farnesyl and geranyl.[16] An acyclic analogue of teixobactin or cyclisation through Cys_8 and Cys_{11}

resulted in reduced antimicrobial activity.[17] We have reported the highly potent teixobactin analogues against MRSA and VRE by using proteogenic amino acids instead of the challenging End_{10}.[18] Moreover, Li *et al*. independently confirmed our findings that a cationic amino acid residue at position 10 is not essential for high antibacterial activity and reported teixobactin analogues with substitution of End_{10} with proteogenic and non-proteogenic amino acids.[19] Despite these studies, the roles of overall net charge and the substitution of hydrophobic non-proteogenic residues on antibacterial and antibiofilm activity remain elusive. We hypothesise that increasing the overall net charge with a balance of hydrophobicity of the peptide may improve the solubility of teixobactin analogues and could also enhance the membrane interactions. Therefore, in this work, we used the design and synthesis of teixobactin analogues containing non-proteogenic amino acids and investigated the contributions of these residues and the increase in overall net charge to the antibiotic's antimicrobial, membrane permeability and antibiofilm properties against different bacterial strains such as *Staphylococcus* species.

Materials and Methods

Synthesis of Chg_{10}-teixobactin (1)

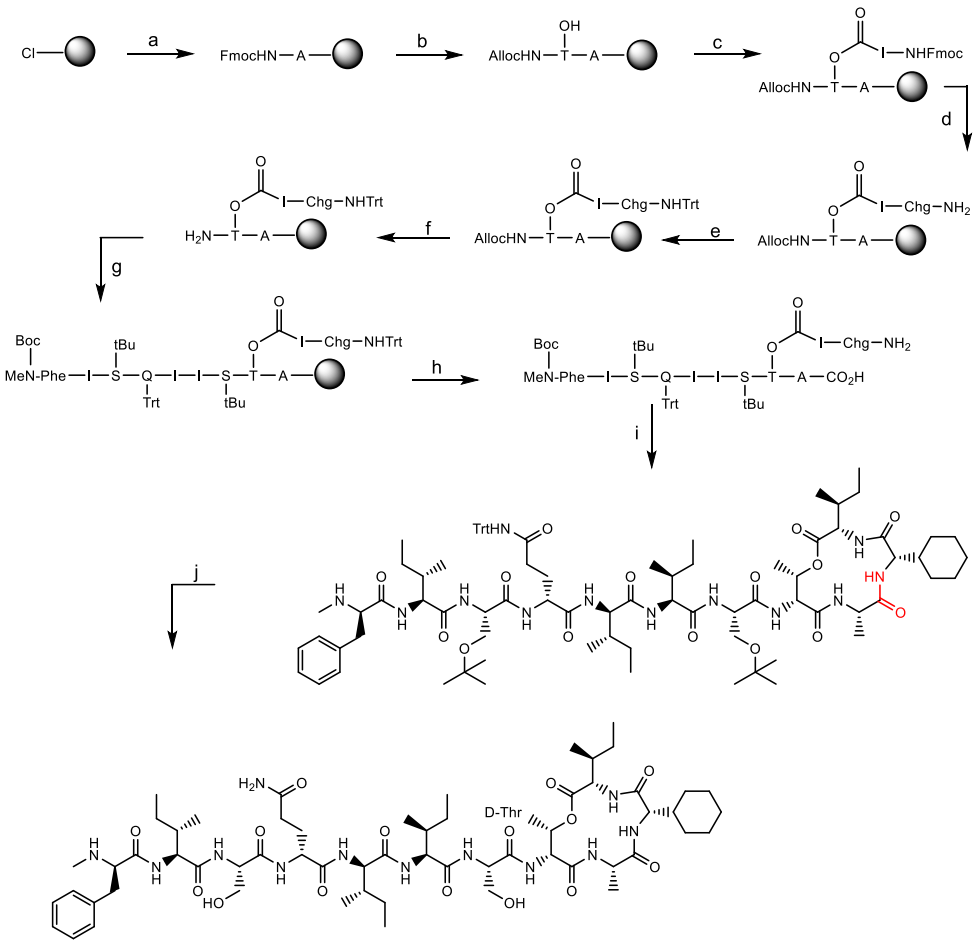
Chg_{10}-teixobactin (**1**) was synthesised as described in **Scheme 1** using methods adapted from our previously reported procedure.[14] (step a) Commercially available 2-chlorotrityl chloride resin (manufacturer's loading = 1.2 mmol/g, 160 mg resin) was swelled in DCM in a reactor. To this resin was added 4 eq. Fmoc-Ala-OH/8 eq. DIPEA in DCM, and the reactor

was shaken for 3 h. The loading, determined by UV absorption of the piperidine-dibenzofulvene adduct, was calculated to be 0.6 mmol/g, (160 mg resin, 0.096 mmol). Any unreacted resin was capped with MeOH:DIPEA: DCM = 1:2:7 by shaking for 1 h. (step b) The Fmoc protecting group was deprotected using 20% piperidine in DMF by shaking for 3 min, followed by draining and shaking again with 20% piperidine in DMF for 10 min. AllocHN-D-Thr-OH was then coupled to the resin by adding 3 eq. of the AA, 3 eq. HATU and 6 eq. DIPEA in DMF and shaking for 1.5 h at room temperature. (step c) Esterification was performed using 10 eq. of Fmoc-Ile-OH, 10 eq. DIC and 5 mol% DMAP in DCM and shaking the reaction for 1 h. This was followed by capping the unreacted alcohol using 10% Ac_2O/DIPEA in DMF shaking for 30 min, and Fmoc was removed using the protocol described earlier in step (b). (step d) Fmoc-Chg-OH was coupled using 4 eq. of AA, 4 eq. HATU and 8 eq. DIPEA in DMF and shaking for 1 h followed by Fmoc deprotection using 20% piperidine in DMF as described earlier. (step e) The N terminus of Leu was protected using 10 eq. Trt-Cl and 15% Et_3N in DCM and shaking for 1 h. The protection was verified by the Ninhydrin color test. (step f) The Alloc protecting group of D-Thr was removed using 0.2 eq. $[Pd(PPh^3)]^0$ and 24 eq. $PhSiH_3$ in dry DCM under argon for 20 min. This procedure was repeated again, increasing the time to 45 min, and the resin was washed thoroughly with DCM and DMF to remove any Pd stuck to the resin. (step g) All amino acids were coupled using 4 eq. amino Acid and 4 eq. DIC/Oxyma using a microwave peptide synthesiser. The coupling time was 10 min. Deprotection cycles were performed as described earlier. (step h) The peptide was cleaved from the resin without cleaving off the protecting groups of the amino acid side chains using TFA:TIS:DCM = 2:5:93 and shaking for 1 h. (step i) The

solvent was evaporated, and the peptide was redissolved in DMF, to which 1 eq. HATU and 10 eq. DIPEA were added, and the reaction was stirred for 30 min to perform the cyclisation. (step j) The sidechain protecting groups were then cleaved off using TFA:TIS: H_2O = 95:2.5:2.5 by stirring for 1 h. The peptide was precipitated using cold Et_2O (-20°C) and centrifuging at 7000 rpm to obtain a white solid. This solid was further purified by RP-HPLC using the protocols described in supporting information SII.

All teixobactin analogues were synthesised using the method described above. The overall yields after HPLC purifications were typically in the range of 12-22%. Teixobactin analogues **1**-**16** were characterised by HRMS (ESI) in positive mode (see Table S1 and Figs. S1-S32).

Chg_{10}-teixobactin **1** was also characterised by NMR (SV, Table S2, Figs. S33-34.). The homogeneity of HPLC-purified fractions was analysed by mass spectrometry. All of the teixobactin analogues used were purified to >95% purity, as indicated by HPLC.



Scheme 1. Synthesis of D-Arg$_4$-Leu$_{10}$-teixobactin starting from 2-chlorotritylchloride resin: a. 4 eq. Fmoc-Ala-OH/8 eq. DIPEA in DCM, 3 h. b. 20% piperidine in DMF followed by 3 eq. AllocHN-D-Thr-OH, 3 eq. HATU/6 eq. DIPEA, 1.5 h. c. 10 eq. Fmoc-Ile-OH, 10 eq. DIC, 5 mol% DMAP in DCM, 1 h followed by capping with Ac_2O/DIPEA 10% in DMF, 20% piperidine in DMF. d. 4 eq. Fmoc-Chg-OH, 4 eq. HATU/8 eq. DIPEA in DMF, 1 h followed by 20% piperidine in DMF. e. 10 eq. Trt-Cl, 15% Et_3N in DCM, 1 h. f. 0.2 eq. $[Pd(PPh_3)_4]^0$ + 24 eq. $PhSiH_3$ in dry DCM, 1 x 20 min, 1 x 45 min. g. 4 eq. Fmoc/Boc-AA(PG)-OH (AA = amino acid, PG = protecting group), 4 eq. DIC/Oxyma (µwave, 10 min) followed by 20% piperidine in DMF (3 min, 10 min). h. TFA:TIS:DCM = 2:5:93, 1 h. i. 1 eq. HATU/10 eq. DIPEA in DMF, 30 min. j. TFA:TIS: H_2O = 95:2.5:2.5, 1 h.

Results and discussion

Design and synthesis:

To further develop simplified, highly potent teixobactin analogues, we were interested in understanding the role of hydrophobic, non-proteogenic amino acids at position 10 of teixobactin and their impact on antibacterial activity. The replacement of the synthetically challenging L-*allo*-enduracididine amino acid with proteogenic leucine and isoleucine provided two highly potent teixobactin analogues, Leu_{10}-teixobactin and Ile_{10}-teixobactin.[14] However, the structure–activity relationships of teixobactin analogues containing hydrophobic non-proteogenic amino acids at position 10 and combinations of arginine with a polar side chain at positions 3, 4 and 9 have not yet been explored. Moreover, the use of non-proteogenic amino acids may offer improved proteolytic stability. For the purpose of this study, we have selected the hydrophobic non-proteogenic amino acids norvaline, norleucine and cyclohexylglycine, containing the hydrophobic side chains propyl, butyl and cyclohexyl, respectively, to replace L-*allo*-enduracididine. We have also combined these modifications with arginine at positions 3, 4 and 9.

Maintaining an amphipathic character is important for developing highly potent teixobactin analogues with drug like properties.[18 20] To maintain this characteristic in our analogues, we have substituted arginines sequentially at positions 3, 4 and 9. Subsequently, we synthesised compounds **1**-**16** (Fig. 2) using our previously reported procedure,[14] (Scheme 1), with short synthesis times and higher yields (Scheme 1, Table S1). Highlights of the synthesis include a shorter esterification time in step c,

rapid synthesis using μwave-assisted SPPS (10-minute coupling time) and a 30-minute cyclisation time despite using sterically hindered, hydrophobic amino acids such as Chg. These are significant reductions compared to the previously reported cyclisation time (24 h) in the case of nonproteinogenic amino acid residues.[19] Furthermore, we are able to achieve lower total synthesis times and higher yields due to our convergent approach compared to the fragment approach reported previously.[19] We further tested all compounds against a broad panel of resistant and antibiotic-susceptible Gram-positive pathogens, including multiple MRSA and VRE strains, clinical isolates and using daptomycin and moxifloxacin as a comparator antibiotics (Table 1A-B). The effects of charge/s on bactericidal and hemolytic activity were evaluated, along with the antibiofilm activities of teixobactin analogues. To the best of our knowledge, antibiofilm activities of any analogues of teixobactin have never been previously evaluated against *Staphylococcus* species.

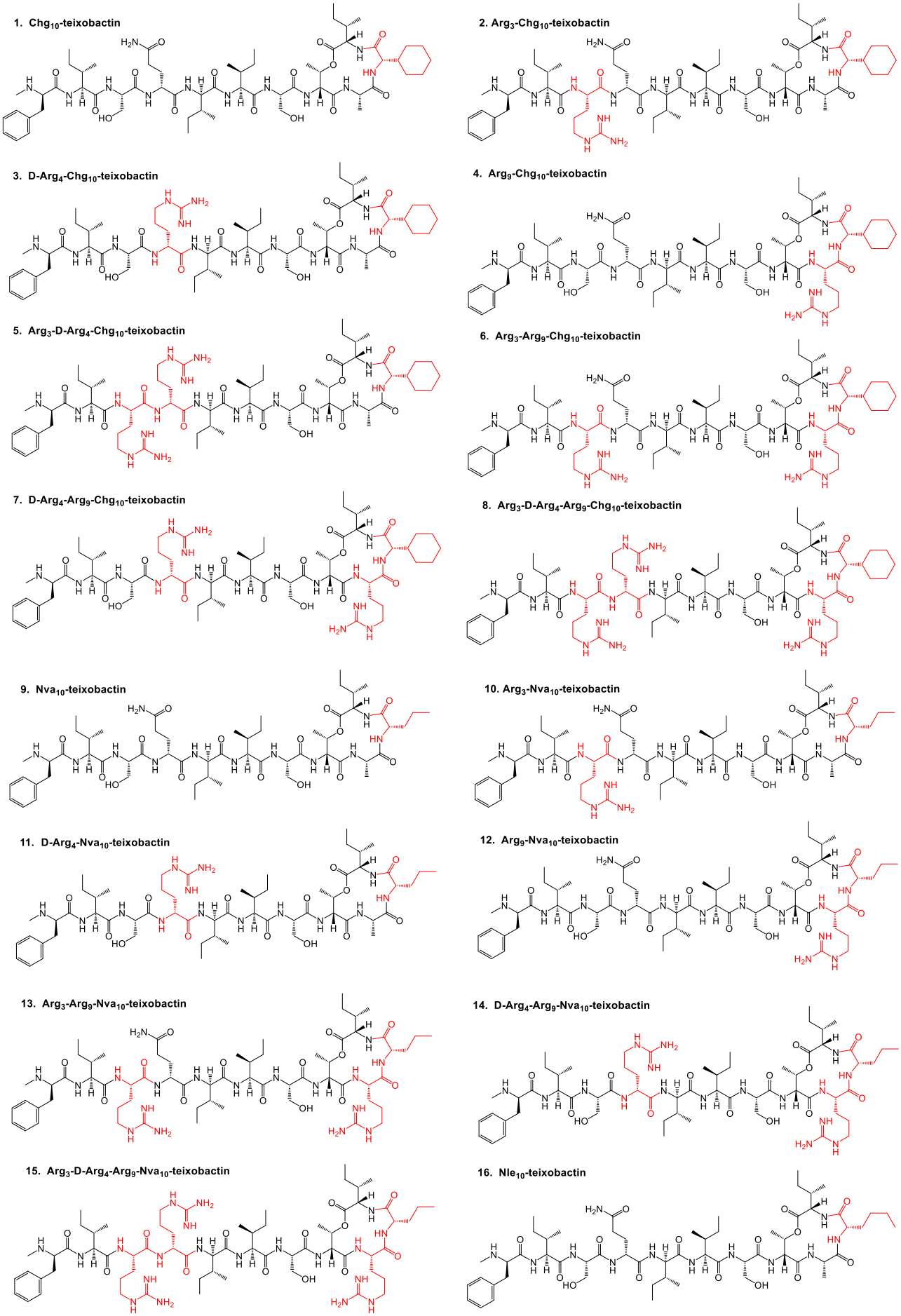


Fig. 2: Structures of teixobactin analogues **1-16,** with the modified amino acids highlighted in red

Table 1A-B: MICs (in µg/ml) of compounds **1-16** tested against a panel of Gram-positive bacteria. Table 1A compares compounds **1-8,** which contain a Chg_{10} substitutions Table 1B compares compounds **9-15,** which have Nva_{10} substitutions, while compound **16** has Nle_{10} substitutions. Both tables compare the synthesised teixobactin analogues to daptomycin as the control in our experiment. Colour codes: Lowest tested concentration = 0.0625- 0.25 µg/ml (green), 0.5-4 µg/ml (yellow), > 4 µg/ml (red). MRSA 1003, MRSA 21455, *Enterococcus faecium* (VRE 1014) are clinical isolates, ND = not determined.

Table 1A

	Compound No.									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Daptomycin**	**Moxifloxacin**
***Staphylococcus saprophyticus* ATCC BAA 750**	0.0625	0.0625	0.0625	0.0625	<0.0625	<0.0625	0.125	<0.0625	0.125	0.25
***Staphylococcus saprophyticus* ATCC 15305**	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	0.125	0.25
***Staphylococcus saprophyticus* ATCC 49453**	<0.0625	<0.0625	<0.0625	0.125	0.125	0.0625	0.0625	0.0625	0.125	0.25
***Staphylococcus saprophyticus* ATCC 49907**	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.125	0.125
VRE 1014	0.25	0.0625	0.0625	0.25	1	1	1	2	2	16
VRE ATCC 700802	0.0625	0.0625	0.0625	0.5	2	1	1	4	1	0.25
VRE ATCC 29212	0.125	0.125	0.0625	1	2	1	1	2	1	0.25
MRSA ATCC 700699	0.125	0.0625	0.0625	0.5	0.25	0.5	0.5	1	2	4
MRSA 42412	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.125	1	1	4
MRSA 21455	0.125	0.125	0.25	0.5	0.25	0.5	0.25	2	0.5	4
MRSA 1003	0.0625	0.0625	0.0625	0.125	0.5	0.5	0.5	1	2	4
***Staphylococcus aureus* 29213**	0.0625	0.0625	0.0625	0.0625	0.0625	0.25	0.5	0.5	0.25	0.0625
***Staphylococcus aureus* 4299**	0.0625	0.0625	0.0625	0.0625	0.125	0.0625	0.5	1	0.25	0.125
***Staphylococcus epidermidis* 12228**	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.125	0.0625
***Bacillus Cereus* ATCC 11788**	0.0625	0.0625	0.0625	0.25	2	1	1	0.5	0.25	0.125
***Bacillus Subtilis* ATCC 6633**	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.125	0.0312

Table 1B

	Compound No.									
	9	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**Daptomycin**	**Moxifloxacin**
***Staphylococcus saprophyticus* ATCC BAA 750**	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.5	0.0625	0.125	0.25
***Staphylococcus saprophyticus* ATCC 15305**	0.0625	0.0625	0.0625	0.0625	0.125	0.0625	0.25	0.0625	0.125	0.25
***Staphylococcus saprophyticus* ATCC 49453**	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.25	0.0625	0.125	0.25
***Staphylococcus saprophyticus* ATCC 49907**	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.5	0.0625	0.125	0.125
VRE 1014	1	1	1	2	2	2	8	1	2	16
VRE ATCC 700802	1	1	0.5	1	2	1	32	0.5	1	0.25
VRE ATCC 29212	1	2	1	2	2	2	32	1	1	0.25
MRSA ATCC 700699	0.5	0.25	0.0625	0.5	0.25	0.5	32	0.5	2	4
MRSA 42412	0.0625	0.0625	0.0625	0.0625	0.125	0.125	32	0.125	1	4
MRSA 21455	0.125	0.0625	0.125	0.25	0.5	0.5	16	0.25	0.5	4
MRSA 1003	0.25	0.125	0.125	1	1	1	>32	0.5	2	4
***Staphylococcus aureus* 29213**	0.125	0.125	0.125	0.5	0.5	0.25	16	0.0625	0.25	0.0625
***Staphylococcus aureus* 4299**	0.0625	0.0625	0.0625	0.25	0.25	0.0625	16	0.25	0.25	0.125
***Staphylococcus epidermidis* 12228**	0.125	0.0625	0.0625	0.0625	0.0625	0.0625	2	0.0625	0.125	0.0625
***Bacillus Cereus* ATCC 11788**	0.0625	0.0625	0.5	0.5	1	1	2	0.0625	0.25	0.125
***Bacillus Subtilis* ATCC 6633**	0.0625	0.0625	0.0625	0.0625	0.0625	0.5	16	0.0625	0.125	0.0312

Antibacterial studies:

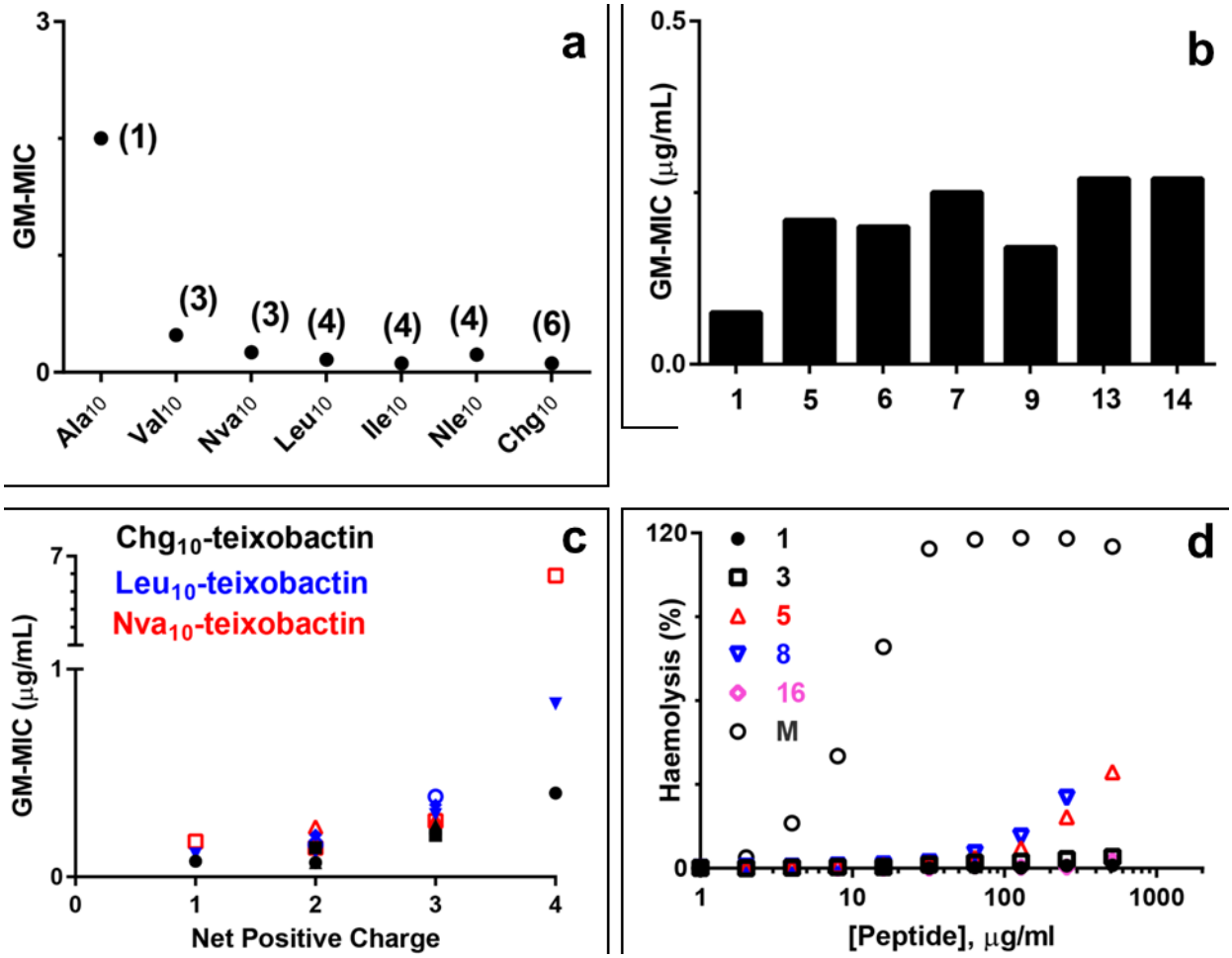


Fig. 3 a) Structure-activity correlation in teixobactin analogues showing the effect of the side chain alkyl group on GM-MIC values, as determined from the MIC values against 16 different Gram-positive strains. Note the decrease in the values with the increasing number of carbons in the side chain. b) Effect of two arginine substitutions on GM-MIC values for Chg_{10}- and Nva_{10}-teixobactins. c) Effect of overall cationic charge on antimicrobial properties of teixobactins. Note that for Chg_{10}-teixobactin, a linear increase in GM-MIC was observed, whereas for Nva_{10} and Leu_{10}-teixobactins, a nonlinear relationship was observed. GM-MIC values for Leu_{10}-teixobactin analogues were obtained from our previous report.[18] d) Concentration-dependent hemolytic

activity of teixobactins for rabbit erythrocytes. The prolific pore-forming peptide, melittin (M), was used as a negative control.

The antibacterial properties of the synthesised teixobactin analogues were investigated against a panel of 16 Gram-positive strains, such as MRSA, VRE (Table 1). To gain better insight into the structure–activity relationship, we determined the geometric mean MIC (GM-MIC) values for all teixobactin analogues and compared the results. A clear correlation between the number of carbons in the sidechain at position 10 and GM-MIC was observed (**Fig. 3a**). The best overall results were obtained for compounds containing a Chg_{10} substitution (compounds **1**-**8**), which showed highly potent antibacterial activity against all bacteria tested with MIC values in the range of 0.0625-0.125 μg/mL and a GM-MIC of 0.07 μg/mL. Thus, natural or unnatural residues carrying bulky side chains of ≥4 carbons (Chg or Ile/Leu) at position 10 resulted in the lowest GM-MIC values (**Fig. 3a**). This was further supported by the fact that substitution of residues containing linear alkyl groups (Nle) (**16**) resulted in 2-fold higher GM-MIC values than Ile_{10}-teixobactin (**18**). Among the amino acids containing isopropyl or n-propyl (Val or Nva) side chains at position 10; Val_{10}-teixobactin[14] had a two-fold higher GM-MIC than Nva_{10}-teixobactin (**9**). Next, we investigated the importance of the overall net charge of the teixobactin analogues on GM-MIC values. In particular, we focused our attention on the substitution of L-Arg residues at positions 3 and 9 and D-

Arg at position 4 in Chg_{10}-texiobactin and Nva_{10}-teixobactin. The results indicated that substitution with cationic residues at all three positions did not affect the GM-MIC values for Chg_{10} and Nva_{10} teixobactin analogues. These results are consistent with the results obtained for Leu_{10}-teixobactin (**Fig. 3b**). However, the substitution of L-Arg at position 3 or 9 increased the GM-MIC values for Ile_{10}-teixobactin, suggesting that Chg_{10}/Nva_{10}-teixobactin analogues have broader scopes for the substitution of cationic residues.

We have also determined the antibacterial activity of teixobactin analogue **3** against a Gram-negative *Pseudomonas aeruginosa* (*P aeruginosa* ATCC 9027, *P aeruginosa* DSMZ 1117). Teixobactin analogue **3** did not display antibacterial activity (MIC = >32 μg/mL) against *P aeruginosa* strains.

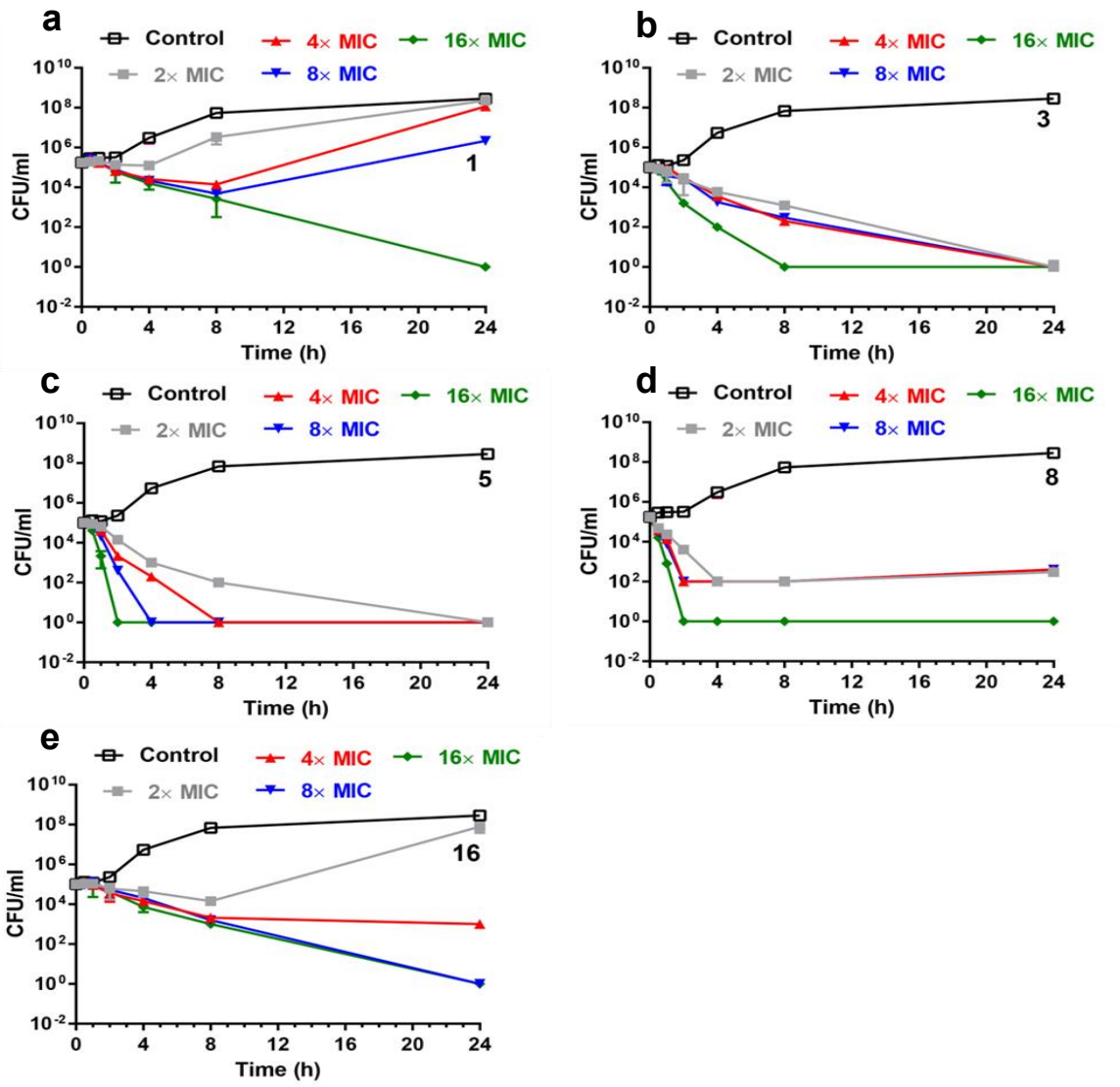


Fig. 4 Time-kill kinetics studies showing the concentration-dependent bactericidal properties of teixobactin analogues with increased cationicity (**1**, **3**, **5** and **8**) against MRSA 21455 strain. a) **1**, b) **3**, c) **5,** d) **8** and e) **16**. Note that the time required to yield a 3 $\log_{10}$-reduction in bacterial viability decreased with increasing cationicity of teixobactin analogues. For comparison, the effects of Chg_{10}- and Nle_{10}-teixobactins (**1** and **16**) are also shown.

Increasing the net cationicity by substituting two and three arginine residues resulted in a linear increase in the GM-MIC values for all of the teixobactin analogues (**Fig. 3c**). However, a further increase in the cationicity (4 cationic charges) resulted in a significant decrease in the GM-MIC. Arginine substitutions in the case of Chg_{10}-teixobactin had the lowest effect on the GM-MIC values compared to Leu_{10}- and Nva_{10}-teixobactin analogues, making it easier to balance the amphipathic nature of Chg_{10}-teixobactin.

Most of the analogues showed potent antibacterial activity (MIC) against MRSA/*S. aureus* strains (0.0625 - 2 µg/mL Table 1A-B, S3). Teixobactin analogues also showed potent antibacterial activity against *E faecalis* and *E faecium* (VRE strains) (MIC 0.0625 - 2 µg/mL, except compounds **8** and **15**, which showed higher MIC 1 - >32 µg/mL Table 1A-B, S3). Our teixobactin analogues containing non-proteogenic hydrophobic amino acids at position 10 showed comparable antibacterial potency with previously reported teixobactin analogues (with substitution of End_{10} with hydrophobic amino acids at position 10) by us and others.[14 19] Most of our teixobactins analogues showed superior antibacterial potency against bacterial pathogens including multidrug-resistant bacterial strains such as MRSA, VRE in comparison to different classes of clinical antibiotics daptomycin and moxifloxacin (Table 1A-B).

To determine if substituting charged residues altered the cytotoxicity, we determined the hemolytic activity levels of teixobactin analogues **1**, **3**, **5**, **8** and **16** for rabbit erythrocytes. The results suggested that teixobactin analogues **1**, **3** and **16** did not show appreciable hemolytic activity (≤2%), even at 512 μg/mL, suggesting their excellent microbial cell selectivity (**Fig. 3d**). Teixobactin analogues **5** and **8** displayed good microbial cell selectivity (3.7%, 5.4% hemolytic activity) at 64 μg/mL (128 times higher than the MIC). However, teixobactin analogues **5** and **8** displayed 18% and 25% hemolytic activity at 256 μg/mL, respectively. These results suggest that increasing the cationicity of Chg_{10}-teixobactin above +2 resulted in heightened hemolytic activity. Nevertheless, the teixobactin analogues still required >200× GM-MIC to achieve a cytotoxic effect for rabbit erythrocytes. It is likely that the increase of cationicity may induce variable interactions with the zwitterionic phospholipids present in the cytoplasmic membranes of mammalian cells, thus causing membrane perturbation and eventual hemolytic activity.

An important hallmark of membrane-active antimicrobial peptides is their capacity to elicit rapid bactericidal properties.[21] To further study the effect of charged residues, we investigated the time-kill kinetics for teixobactin

analogues **1**, **3**, **5,** and **8,** which possess increased cationicity. To understand the effects of hydrophobic Chg_{10} and Nle_{10} substitutions, we investigated the kill-kinetics of teixobactin analogues **1** and **16**. All of the teixobactin analogues displayed concentration-dependent bactericidal activity against the MRSA (**Fig. 4a – e**) at the highest concentration. However, teixobactin analogue **1** required a higher concentration (≥8× MIC) to achieve complete lethality when compared to other teixobactin analogues. This may also be correlated with its hydrophobic characteristic as singly charged and, therefore, higher potential for adsorption to plastics. The effect of charged residues in altering the rate of killing was clearly evident, as the charged teixobactin analogues caused substantial lethality in a shorter time. At 16× MIC, teixobactin analogues **5** and **8** elicited rapid bactericidal properties, as ≥3 log_{10}-reductions in bacterial viability were achieved within 2 h for the two teixobactin analogues, whereas teixobactin analogues **1** and **3** required 8 h and 24 h, respectively to achieve similar endpoints. Between teixobactin analogues **5** and **8**, the latter teixobactin analogue elicited greater bactericidal properties, as the endpoint (≥99.9% reduction in bacterial viability) could be achieved in 4 h at 2× MIC. These results may indicate possible interactions between cationic residues and the cytoplasmic membrane at elevated concentrations, a mechanism akin to host defense peptides.[21] Analogue **16** also required a higher concentration to show a bactericidal effect similar to analogue **1**. This is also a

hydrophobic analogue and is likely to have increased adsorption to plastic. Among the two singly charged teixobactin analogues (**1** and **16**), Nle_{10}-teixobactin **16** displayed a better kill-kinetics profile than Chg_{10}-teixobactin, indicating that the linear alkyl side chain was effective in conferring potent bactericidal properties. To understand the kill kinetics trends of **1** and **16,** we determined these analogues' minimum bactericidal concentration (MBC) against MRSA 21455. The MBC of **1** was 2μg/ml, which is 16 x of MIC. For **16**, MBC was 1μg/ml, which is 4 x MIC. The MBCs values of **1** and **16** correlate well with bactericidal activity observed in kill-kinetics studies.

To evaluate teixobactin analogues containing non-proteogenic amino acids binding to the target lipid II, we have performed the lipid II TLC binding assay on Chg_{10}-teixobactin **1**. Chg_{10}-teixobactin binds to lipid II in a 2: 1 ratio, resulting in the complete disappearance of the lipid II spot on TLC (Fig. S36). Our previous study examined the binding modes of Arg_4-Leu_{10}-teixobactin analogue with target Lipid II in cellular membranes. In that report, the teixobactin macrocycle (ring motif) interacted with MurNAc and pyrophosphate (PPi) of Lipid II.[22] We anticipate comparable interactions of teixobactin analogues included in this work with Lipid II as they share the common feature of hydrophobic groups at position 10 (Fig. 5).

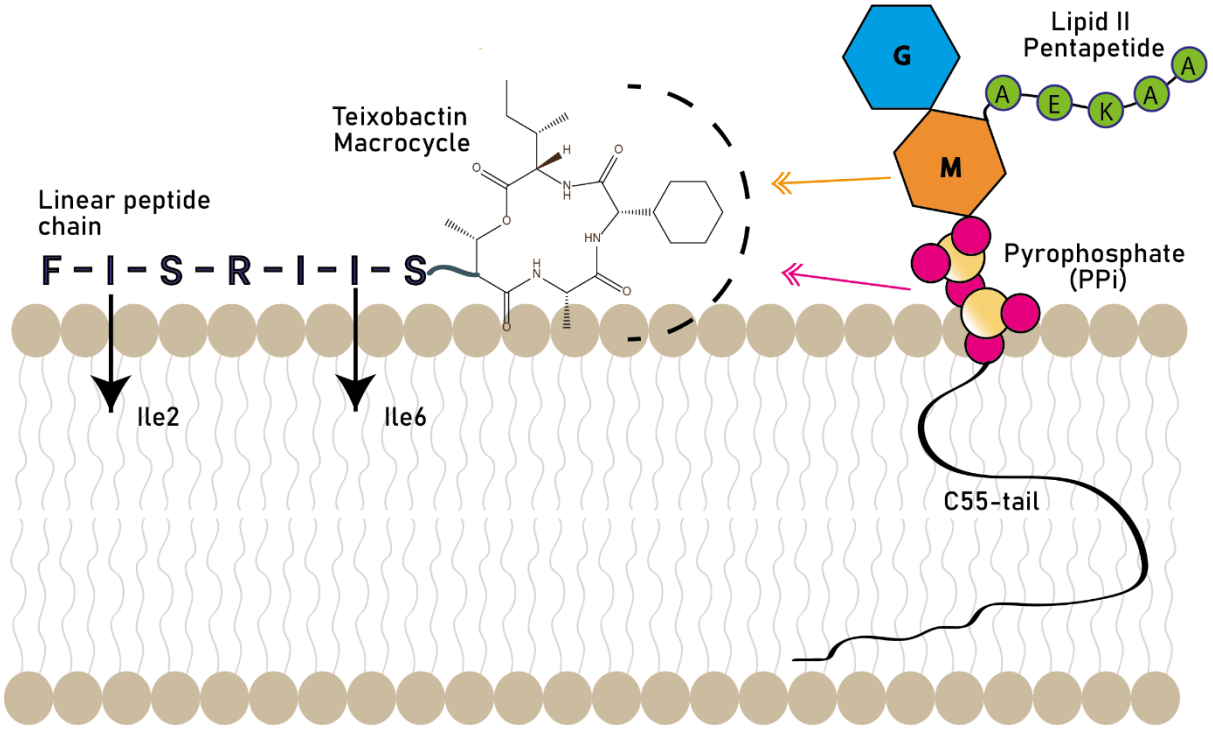


Fig. 5 Illustration depicting Teixobactin analogues binding to Lipid II. Teixobactin analogues reside at the water–lipid interface with Ile_2 and Ile_6 inserting into the membrane. The macrocycle coordinates with PPi and MurNAc (M, orange) predominantly, and to a lesser extent GlcNAc (G, blue).

To ascertain the observations that the rapid killing of bacteria by teixobactin analogue **8** was linked to bacterial membrane perturbations, we performed two additional experiments to correlate the time-kill kinetics studies. The singly charged teixobactin analogue **1** was used for a comparison. We used a $diSC_3(5)$ assay, which employs a membrane-potential sensitive dye to determine the cytoplasmic membrane depolarisation of MRSA cells upon

the addition of teixobactin analogues. The lipophilic dye diffuses into the cytoplasmic membranes of intact bacterial cells with a concomitant decrease in fluorescence intensity. Any alterations in membrane potential result in efflux of the dye with a simultaneous increase in fluorescence intensity. No apparent change in membrane potential was observed upon the addition of teixobactin analogues **1** and **3,** whereas a weak perturbation was observed when teixobactin analogue **5** was added (**Fig. 6a-c**). However, rapid dissipation of membrane potential and the associated increase in the fluorescence intensity of $diSC_3(5)$ was observed upon the addition of teixobactin analogue **8** (**Fig. 6d**). Next, we determined the fluorescence increase of SYTOX Green (SG) upon the addition of teixobactin analogue to intact microbial cells. SG is a membrane-impermeant DNA-binding dye; therefore, any microbial membrane perturbation allows entry of the dye and a concomitant increase in fluorescence upon binding to intracellular DNAs.[23] The percentage of SG uptake was determined relative to the membranolytic prolific pore-forming antimicrobial peptide melittin. At 8× and 16× MIC values, the addition of teixobactin analogue **1** to intact bacteria resulted in a moderate increase in the fluorescence intensity (**Fig. 7a**) and, thus, a weak perturbation of the cell membrane (8.8% and 10.6% SG uptake at 8× and 16×, respectively). Teixobactin analogues **3** and **5** caused substantial increases in SG uptake when compared to teixobactin analogue **1**, indicating membrane

permeabilisation upon increasing the overall net charge of the teixobactin analogues (**Fig. 7b**). However, addition of teixobactin analogue **8** at 8× and 16× MIC values resulted in significant uptake of the dye and marked increases in the fluorescence intensity values (**Fig. 7d**). Teixobactin analogue **8** caused 65.5% and 70.2% SG uptake at 8× and 16× MIC, respectively, confirming the enhanced membrane permeability of the teixobactin analogues. These results confirm that the interaction of teixobactin analogue **8** with microbial cytoplasmic membranes causes rapid dissipation of membrane potential followed by more significant membrane perturbation, leading to enhanced SG uptake. Taken together, these results support the time kill-kinetics studies showing that increasing the overall net charge of the teixobactin analogue resulted in an increase in cytoplasmic membrane perturbations, thus potentiating rapid bactericidal properties.

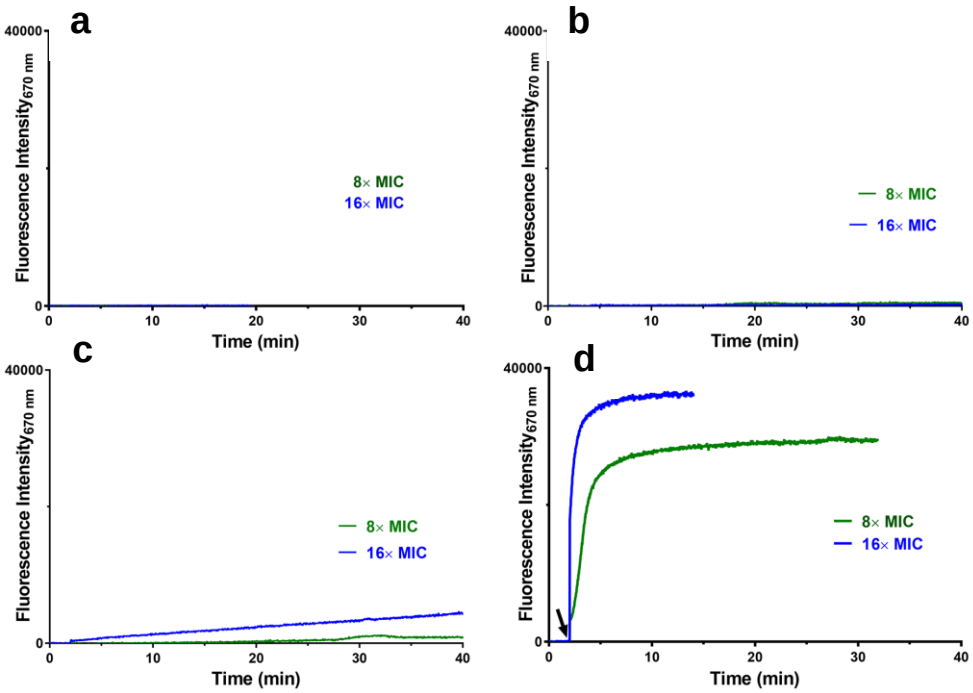


Fig. 6. Cytoplasmic membrane depolarisation of MRSA 21455 upon addition of teixobactin analogues: a) **1**, b) **3**, c) **5** and d) **8**. The black arrows in figure indicates time of addition of teixobactin analogues. Note that a rapid loss of membrane potential with concomitant increase in fluorescence intensity was evident upon the addition of teixobactin analogue **8**.

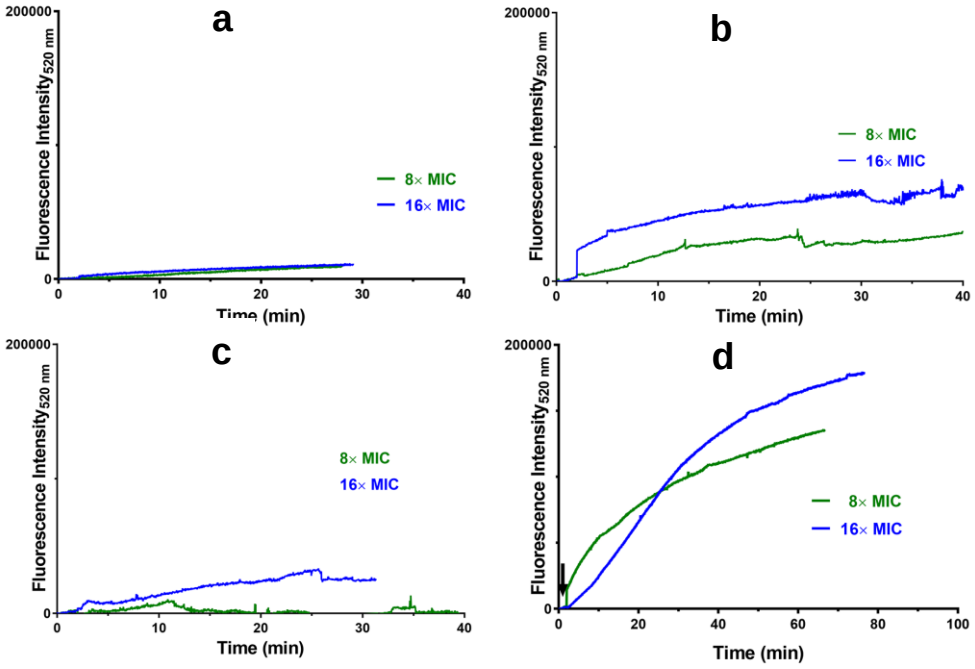


Fig. 7. SYTOX Green uptake of MRSA 21455 bacterial cells upon addition of teixobactin analogues: a) **1**, b) **3**, c) **5** and d) **8**. The black arrow in 'd' indicates time of addition of teixobactin analogues. Note the gradual uptake of the DNA-binding dye upon the addition of teixobactin analogue **8**.

Based on our results, we can infer that rapid bactericidal properties and excellent cell selectivity among the teixobactin analogues containing hydrophobic, non-proteogenic residues at position 10 are observed. It is likely that increasing the overall net charge beyond +3 in teixobactin analogues resulted in variable interactions with lipid II and the cytoplasmic membranes of the bacteria, leading to higher GM-MIC values and low cell selectivity.

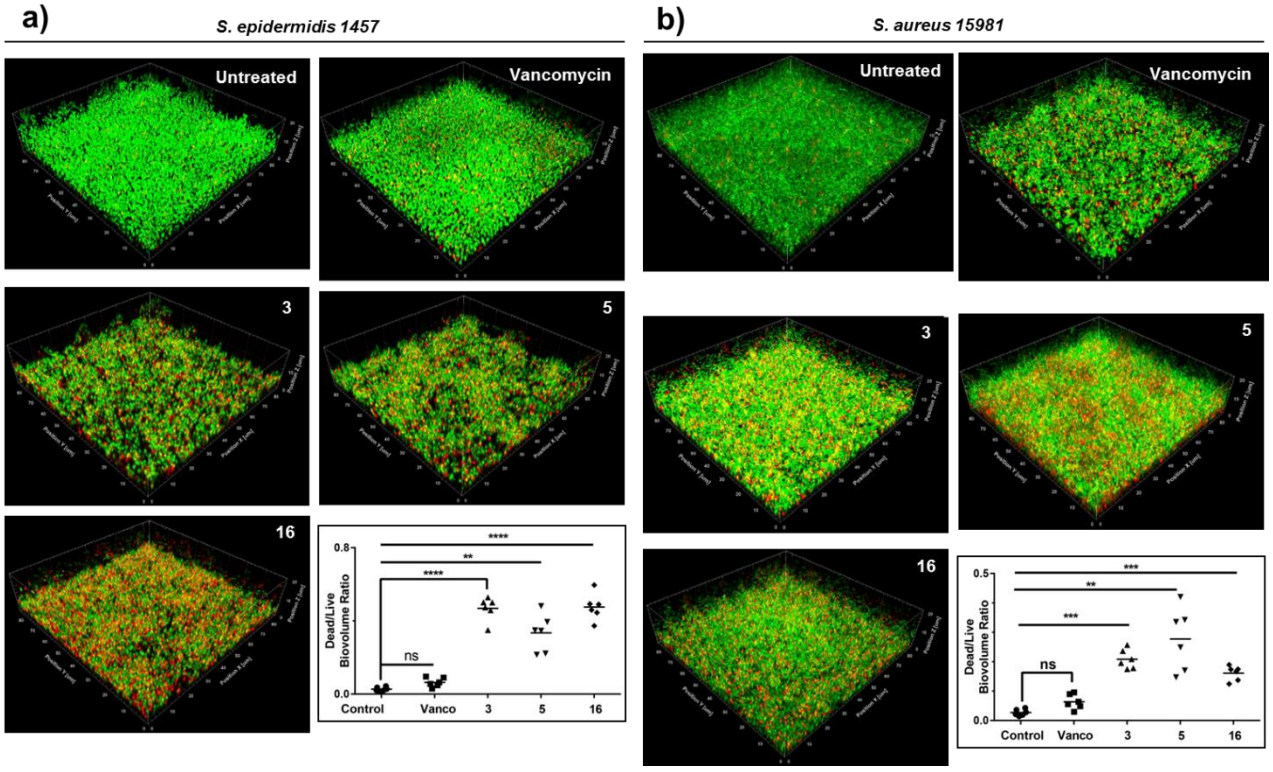


Fig. 8 Antibiofilm properties of teixobactin analogues against a) *S. epidermidis* 1457 and b) *S. aureus* 15981 biofilms. The biofilms were treated with either the teixobactin analogue (10 μg/ml) or vancomycin (10 μg/ml) for 5 h. Representative confocal images of the bacterial cells stained with live/dead fluorescent probes are shown. The scatter plot displays the dead/live biovolume ratio quantitatively and was estimated from 6 representative images. The results were analysed by two-way ANOVA, Turkey's multiple comparison test. ns, $p>0.05$; *, $p\leq0.05$; **, $p\leq0.01$; ***, $p\leq0.001$ and ****, $p\leq0.0001$.

Biofilm-forming bacterial strains such as *S. aureus* and *S. epidermidis* are known to colonise medical impacts such as insertion sites and catheters, which pose severe challenges in designing treatment strategies against bacterial biofilms. This is due to their inherent resistance to antimicrobials and the host immune responses.[24–26] To evaluate the potency of teixobactin analogues, we determined their antibiofilm properties against prolific biofilm strains *S. aureus* 15981 and *S. epidermidis* 1457 using a static biofilm model.[27,28] The preformed biofilms were treated with teixobactin analogues (**3**, **5** and **16**, selected based on antibacterial properties) for 5 h, and the teixobactin analogues displayed potent antibiofilm properties against both *S. aureus* and *S. epidermidis* biofilms when compared to comparator antibiotic (vancomycin at 10 μg/mL). In the case of *S. epidermidis* biofilm, the dead/live biovolume ratio remained similar for

untreated and vancomycin ($p>0.05$)-treated groups (**Fig. 8a**). However, the values increased by 2- to 18-fold upon treatment with teixobactin analogues, suggesting susceptibility and the teixobactin analogues triggered considerable cell death (as shown from the yellow- and red-stained cells). Interestingly, the ratios for analogues **3** and **5** are greater than vancomycin**,** of which both analogues, suggesting increased sensitivity of *S. epidermidis* biofilms. The results further indicated that teixobactin analogue **16** displayed superior antibiofilm properties compared to vancomycin, suggesting the importance of the free alkyl side chain in disrupting the preformed biofilms.

However, a contrasting trend was observed when we investigated the activity of teixobactin analogues against *S. aureus* biofilms (**Fig. 8b**). At similar concentrations, all of the teixobactin analogues displayed higher dead/live cell ratios than the comparator vancomycin ($p<0.0001$ for **1**, $p<0.001$ for **3**, $p<0.01$ and $p\leq0.05$ for teixobactin analogue **16**). These results establish the potent antibiofilm properties of teixobactin analogues against commensal pathogens, further suggesting that the two pathogenic bacteria had differential susceptibility to membrane-lytic peptides. To the best of our knowledge, this is the first report demonstrating the antibiofilm properties of synthetic teixobactin analogues against *Staphylococcus* species.

Teixobactin analogue **3** displayed the optimum antimicrobial/ antibiofilm properties and showed no hemolytic activity for rabbit erythrocytes.

Conclusion

In conclusion, we have designed and synthesised highly potent teixobactin analogues through the replacement of L-*allo*-enduracididine with the hydrophobic, non-proteogenic amino acids cyclohexylglycine, norvaline and norleucine. We have developed a better understanding of the structure–activity relationships of teixobactin analogues containing non-proteogenic amino acids at position 10. Most of the compounds were highly potent against multiple MRSA and VRE strains, including clinical isolates and showed superior antibacterial potency than clinical antibiotics daptomycin and moxifloxacin. For the first time, we have demonstrated the antibiofilm activity of teixobactin analogues against *Staphylococcus* species. We believe the work presented here will enable the development of new drugs, such as simplified highly potent teixobactin analogues, and their applications to address the challenges posed by antimicrobial resistance, including biofilm-related infections.

Supporting information

Supplementary Information (ESI) available: Teixobactin analogues HPLC, LC-MS analysis, NMR analysis, antibacterial assay (MIC, MBC, time kill kinetics,

antibiofilm assays), SYTOX Green uptake assay, cytoplasmic membrane potential disC_3(5) assay, static biofilm studies and hemolysis.

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Conflict of Interest

The authors declare no conflicts of interest.

Author contributions

† These authors have contributed equally to this work. The project was designed and directed by Ishwar Singh. The manuscript was written by Abhishek Iyer, Anish Parmar, Tsz Ying To, Enas Newire, Rajamani Lakshminarayanan and Ishwar Singh through contributions from all authors. Anish Parmar carried out the teixobactin analogues syntheses. Joey Kuok Hoong Yam and Yang Liang carried out the antibiofilm studies. Eefjan Breukink was responsible for lipid II.

Annemieke Madder was responsible for the LC-MS analyses. Eunice Tze Leng Goh, Enas Newire and Rajamani Lakshminarayanan were responsible for the antibacterial studies and hemolysis assays. Tsz Ying To carried out the data analysis. All authors have given approval to the final version of the manuscript.

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