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The Impact of Backbone *N*-Methylation on the Structure-activity Relationship of Leu_{10}-teixobactin

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Abstract

Antimicrobial resistance is a serious threat to global human health; therefore, new anti-infective therapeutics are required. The cyclic *depsi*-peptide natural product teixobactin exhibits potent antimicrobial activity against several Gram-positive pathogens. To study the natural product's mechanism of action and improve its pharmacological properties, efficient chemical methods for preparing teixobactin analogues are required to expedite structure-activity relationship studies. Described herein is a synthetic route that enables rapid access to analogues. Furthermore, our new *N*-methylated analogues highlight that hydrogen bonding along the *N*-terminal tail is likely to be important for antimicrobial activity.

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Introduction

According to the World Health Organisation, the spectre of a post-antibiotic era is a serious threat to global human health.[1] It has been estimated that global deaths could reach up to 10 million annually by 2050 and the cumulative financial burden is projected to be in excess of US $100 trillion.[2] The problem is caused by the rapid rise in antimicrobial resistance (AMR), which has been driven by the overuse of antibiotics (in farming and the clinic) and a lack of antimicrobial agents with novel mechanisms of action in the developmental pipeline.[3] Better stewardship of antibiotic use has been identified as one way of slowing the spread of AMR but there is still an urgent need for the development of new, more effective antimicrobial drugs.

Teixobactin is a novel cyclic *depsi*-peptide (**Figure 1**), recently isolated from an uncultivable soil bacteria species *Eleftheria terrae* via iChip technology.[4] It has potent antimicrobial activity against several Gram-positive pathogens, including the problematic methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE) and *Mycobacterium tuberculosis*.[4] However, the most remarkable property of teixobactin is that following serial passaging and time-kill studies, no detectable resistance was generated, which highlights its enormous therapeutic potential.[4] Teixobactin consists of 11 residues including the non-proteinogenic L-*allo*-enduracididine (L-*allo*-End) at position 10, four D-amino acids, a methylated *N*-terminus and a 13-membered macrocycle that is formed between the side chain of D-Thr_8 and the *C*-terminus. Its unique mode of action involves binding to and inhibition of lipid II (a precursor of the peptidoglycan structural component of the cell wall), which leads to cell death via autolysis.[4] There is strong evidence that the macrocycle binds to the pyrophosphate group of lipid II via hydrogen bonding[5] but the role of the hydrophobic *N*-terminal tail is less clear. Initially, it was purported to be involved in membrane insertion and/or binding directly to the isoprenyl tail of lipid II.[5] Based on crystallographic data, it has recently been put forward that the peptide forms an amyloid-like assembly and acts as a multivalent receptor for the pyrophosphate moiety in lipid II.[6]

Several groups have reported the chemical synthesis of both native teixobactin[7, 8] and various analogues[9, 10] by 9-fluorenylmethyloxycarbonyl solid-phase peptide synthesis (Fmoc SPPS). To date, three generic synthetic strategies have been established: (i) stepwise synthesis and solution phase cyclisation;[6, 8, 11-14] (ii) stepwise synthesis and ‘on-resin’ cyclisation;[15, 16] and, (iii) a convergent strategy.[7, 17] Effective structure-activity

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relationship (SAR) studies of teixobactin necessitates a synthetic route that is both rapid and efficient. To this end, we were attracted to a convergent strategy whereby modification is performed on shorter precursors.

There are two reported procedures that describe a convergent synthetic route to Teixobactin analogues. One method utilises a serine ligation to prepare the native peptide from unprotected precursors via a 6+5 strategy.[7] A second method also adopts this strategy[17] but with protected fragments, although epimerisation of L-Ile_6 poses a risk during activation. We preferred a 7+4 approach which would involve assembly of the protected *N*-terminal fragment and the macrocyclic core. This enables incorporation of an Ile-Ser *pseudo*-proline dipeptide at the *C*-terminus, to mitigate the potential for epimerisation at L-Ser_7 during activation.[18-20]

Previous studies have found that the activity of teixobactin is highly sensitive to modification, but substitutions are moderately tolerated at residues 3, 4, 9 and 10.[9] Replacement of L-*allo*-End_{10} with natural commercially available amino acids such as L-leucine[21, 22] is desirable for rapid SAR studies since the former is both synthetically challenging and difficult to incorporate into the peptide. We report a rapid synthetic approach that utilises standard Fmoc SPPS protocols and precursors for ready access to Leu_{10}-teixobactin analogues in good overall yields and in high purity.

Materials and Methods

Materials. Synthesis of peptide precursors was performed manually using fritted syringes on a vacuum manifold. All reagents were purchased from commercial sources and used without further purification. 2-chlorotrityl chloride resin (sub. = 0.81 mmol/g) was sourced from Chempep (USA). All standard Fmoc amino acids were purchased from CEM Corporation (USA). Boc-NMe-D-Phe-OH, Fmoc-D-Gln(Trt)-OH, Fmoc-D-*allo*-Ile-OH, Fmoc-Ile-Ser(ψ(Me,Me)pro)-OH and Boc-D-Thr-OH were sourced from Combi Blocks (USA). Fmoc-NMe-L-Ile-OH and Fmoc-NMe-L-Ser(tBu)-OH were obtained from GL Biochem (China). (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) was purchased from Iris Biotech. Diisopropylcarbodiimide (DIC), 4-dimethylaminopyridine (DMAP), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl), 1,4-dioxane, triisopropylsilane (TIPS), 2-nitrobenzenesulfonyl chloride, 7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene (MTBD), methyl 4-nitrobenzenesulfonate, 2,2′-(Ethylenedioxy)diethanethiol (DODT) and 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU)

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were sourced from Sigma-Aldrich. Piperidine, diisopropylethylamine (DIEA), dimethylformamide (DMF), dichloromethane (DCM), diethyl ether and acetonitrile were purchased from Merck Millipore (Australia). Trifluoroacetic acid (TFA) was purchased from Auspep Pty Ltd (Australia).

Analysis. All precursors and final products were analysed on an Agilent 6120 Quadrupole LC/MS with an Agilent 1260 infinity II high performance liquid chromatography (HPLC) system. The mobile phases were: 0.05% TFA in water (buffer A) and 0.05% TFA in acetonitrile (buffer B). Column: Agilent Poroshell 120, EC-C8, 3 x 50 mm, 2.7µm HPLC column. Gradient: 0-100% buffer B over 10 m. Flow rate: 0.5 mL/m. Detection: 215 nm. High resolution mass spectrometry was performed on an Agilent 6500 Series Q-TOF.

Preparative HPLC. All purification was carried out on an Agilent 1200 Series HPLC. For the *C*-terminal macrocyclic precursor, the mobile phases were: 5 mM HCl in water (buffer A) and 5 mM HCl in 80% aqueous acetonitrile (buffer B). Column: Agilent Pursuit XRs 100Å C18, 21.2 x 150 mm, 5 µm. Gradient: 20-80% buffer B over 60 m. Flow rate: 5 mL/m. Detection: 230 nm. For the *N*-terminal protected precursors that weren't of sufficient purity (NMe-D-*allo*-Ile_5 and NMe-Ser_7), the mobile phases were: 0.1% TFA in water (buffer A) and 0.1% TFA in acetonitrile (buffer B). Column: Agilent Pursuit XRs 100Å C8, 21.2 x 100 mm, 5 µm. Gradient: 50-100% buffer B over 50 m. Flow rate: 5 mL/m. Detection: 230 nm. For the Leu_{10}-teixobactin analogues, the mobile phases were 0.1% TFA in water (buffer A) and 0.1% TFA in acetonitrile (buffer B). Column: Agilent Pursuit XRs 100Å C18, 21.2 x 150 mm, 5 µm. Gradient: 20-80% buffer B over 60 m. Flow rate: 5 mL/m. Detection: 230 nm.

*Synthesis of teixobactin cyclo-(8-11) (**3**).* 2-chlorotrityl resin (2.47 g, 2 mmol) was solvated with DCM in a 50 mL tube, followed by the addition of a solution of Fmoc-Ala-OH (686 mg, 2.2 mmol) in DCM (30 mL). DIEA (2.088 mL, 12 mmol) was then added and the slurry was agitated. After 2 h, methanol (5 mL) was poured in and the mixture was agitated for a further 30 m. The resin was then filtered in a 25 mL fritted syringe, treated with 20% piperidine in DMF, followed by the addition of Boc-D-Thr-OH (482 mg, 2.2 mmol), activated with Oxyma Pure (312 mg, 2.2 mmol) and DIC (346 µL, 2.2 mmol) in DMF. After 1 h, the resin was filtered and Fmoc-Ile-OH (1.416 g, 4 mmol), activated with DIC (628 µL, 4 mmol) in DCM was added. DMAP (48 mg, 0.4 mmol) was then added to catalyse formation of the ester bond. After 2 h, the resin was filtered and the

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Fmoc group was cleaved with 20% piperidine in DMF, followed by the addition of Fmoc-L-Leu-OH (778 mg, 2.2 mmol) with COMU (942 mg, 2.2 mmol) and DIEA (766 μL, 4.4 mmol) in DMF. After coupling, the resin was again treated with the piperidine solution to cleave the final Fmoc group. After cleavage with 1% TFA in DCM, the solvent was removed *in vacuo*, followed by resolubilisaiton in acetonitrile/water and lyophilisation. 855 mg of crude product was obtained. 200 mg was isolated via HPLC to generate 102 mg of purified product. For the cyclisation reaction, EDC.HCl (93 mg, 0.484 mmol) and DIEA (141 μL, 0.810 mmol) were added to neat DCM (81 mL). The linear peptide precursor (102 mg, 0.162 mmol) was slowly added over 15 m and the reaction was stirred for 4 h and monitored via LC-MS. Note that the cyclisation can also be performed with the crude linear precursor. The solvent was washed with brine then removed in vacuo. TFA (5 mL) was then added and the reaction stirred for 30 m. After aspiration with N_2, the product was solubilised and purified via RP-HPLC purification using HCl buffers (20-80% B over 60 m, C18). The clean fractions were pooled and lyophilised. 49.0 mg was recovered (70%).

*Synthesis of protected teixobactin (1-7) (**4**).* 2-chlorotrityl resin (1.235 g, 1 mmol) was solvated with DCM in a 50 mL tube, followed by the addition of a solution of Fmoc-L-Ile-L-Ser(ψ(Me,Me)pro)-OH (529 mg, 1.1 mmol) in DCM (20 mL). DIEA (1.044 mL, 6 mmol) was then added and the slurry was agitated for 2 h. Methanol (5 mL) was then added and the mixture was agitated for a further 30 m. The resin was then filtered in a 25 mL fritted syringe, followed by Fmoc SPPS using 1.1 equivalents of reagent. After peptide assembly, the resin-bound peptide was cleaved with 1% TFA in DCM (100 mL), followed by two brine washes. The solvent was then removed in vacuo and the oily residue was resolubilised with an acetonitrile/water mixture and lyophilised. 960 mg of crude peptide was recovered (76% yield).

*Synthesis of Leu_{10}-teixobactin (**6**).* **4** (108 mg, 0.086 mmol) was dissolved in 1,4-dioxane (~4 mL) in a 25 mL round-bottom flask (RBF). COMU (74 mg, 0.173 mmol) was added, followed by DIEA (40 μL, 0.230 mmol). **3** (25 mg, 0.057 mmol) was dissolved in 1,4-dioxane (1 mL) separately and DIEA (10 μL, 0.057 mmol) was added. This solution was then added to the RBF and the reaction mixture was heated to 60°C and stirred for 3 h to generate **5**. The reaction was monitored via LC-MS. The solvent was then removed *in vacuo* and the oily residue was redissolved in EtOAc (10 mL), followed by two brine washes (2 x 10 mL). The solvent was again removed *in vacuo*, followed by addition of TFA/TIPS/H_2O (96/2/2). The reaction mixture was transferred to a 50 mL tube and

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agitated for 2 h, followed by aspiration with N_2 to reduce the volume to ~1 mL. Diethyl ether (40 mL) was then added and the mixture was cooled with ice. After centrifugation, the supernatant was removed and the precipitate air dried, followed by solubilisation in a mixture of acetonitrile/water, LC-MS analysis and HPLC purification. The clean fractions were pooled and lyophilised. 13.1 mg was recovered (17% yield). Analysis via high resolution mass spectrometry was as follows: monoisotopic $[M+H^+]^+_{exp.}$ = 1201.724 ($[M+H^+]^+_{th.}$ = 1201.719).

*Synthesis of NMe-Ile$_2$, Leu$_{10}$-teixobactin (**7**).* Protected teixobactin (1-7) with NMe-L-Ile$_2$ was prepared in the same manner as **4** on a 0.25 mmol scale. A portion of the crude material (88 mg, 0.069 mmol) was dissolved in 1,4-dioxane (~4 mL) in a 25 mL RBF. COMU (79 mg, 0.185 mmol) was added, followed by DIEA (32 µL, 0.184 mmol). **3** (20 mg, 0.046 mmol) was dissolved in 1,4-dioxane (1 mL) separately and DIEA (8 µL, 0.046 mmol) was added. The solution was then added to the RBF and the reaction mixture was heated to 60°C, stirred for 3 h and worked up in a similar fashion to **6**. 6.7 mg was recovered (11 % yield).

*Synthesis of NMe-Ser$_3$, Leu$_{10}$-teixobactin (**8**).* Protected teixobactin (1-7) with NMe-L-Ser$_3$ was prepared in the same manner as **4** on a 0.25 mmol scale. A portion of the crude material (88 mg, 0.069 mmol) was dissolved in 1,4-dioxane (~4 mL) in a 25 mL RBF. COMU (79 mg, 0.185 mmol) was added, followed by DIEA (32 µL, 0.184 mmol). **3** (20 mg, 0.046 mmol) was dissolved in 1,4-dioxane (1 mL) separately and DIEA (8 µL, 0.046 mmol) was added. The solution was then added to the RBF and the reaction mixture was heated to 60°C, stirred for 3 h and worked up in a similar fashion to **6**. 5.4 mg was recovered (9% yield).

*Synthesis of NMe-D-Gln$_4$, Leu$_{10}$-teixobactin (**9**).* Protected teixobactin (1-7) with NMe-D-Gln$_4$ incorporated in situ was prepared on a 0.25 mmol scale. After Fmoc deprotection at D-Gln$_4$, 2-nitrobenzenesulfonyl chloride (111 mg, 0.5 mmol) was dissolved in DCM and added to the resin bound peptide. DIEA (174 µL, 1 mmol) was also added and the resin was agitated for 1 h. After rinsing, methyl 4-nitrobenzenesulfonate (107 mg, 0.5 mmol) was dissolved in DMF and added to the resin. MTBD (72 µL, 0.5 mmol) was also added and the slurry was agitated for 1 h. After further rinsing, DMF was added, followed by DODT (407 µL, 2.5 mmol) and DBU (187 µL, 1.25 mmol). The reaction mixture was agitated for 30 m and the procedure repeated twice more. The protected precursor was then prepared in the same manner as **4**. A portion of the crude material

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(88 mg, 0.069 mmol) was dissolved in 1,4-dioxane (~4 mL) in a 25 mL RBF. COMU (79 mg, 0.185 mmol) was added, followed by DIEA (32 μL, 0.184 mmol). **3** (20 mg, 0.046 mmol) was dissolved in 1,4-dioxane (1 mL) separately and DIEA (8 μL, 0.046 mmol) was added. The solution was then added to the RBF and the reaction mixture was heated to 60°C, stirred for 3 h and worked up in a similar fashion to **6**. 6.2 mg was recovered (10% yield).

*Synthesis of NMe-D-allo-Ile$_5$, Leu$_{10}$-teixobactin (**10**).* Protected teixobactin (1-7) with NMe-D-*allo*-Ile$_5$ incorporated in situ was prepared in a similar manner to the protected NMe-Gln$_4$ precursor on a 0.25 mmol scale using the exact same quantity of reagents. The crude product was then purified as described above to remove excessive deletion sequences. A portion of the purified material (32 mg, 0.025 mmol) was dissolved in 1,4-dioxane (~4 mL) in a 25 mL RBF. COMU (39 mg, 0.091 mmol) was added, followed by DIEA (16 μL, 0.092 mmol). **3** (10 mg, 0.023 mmol) was dissolved in 1,4-dioxane (1 mL) separately and DIEA (4 μL, 0.023 mmol) was added. The solution was then added to the RBF and the reaction mixture was heated to 60°C, stirred for 3 h and worked up in a similar fashion to **6**. 5.0 mg was recovered (16% yield).

*Synthesis of NMe-Ile$_6$, Leu$_{10}$-teixobactin (**11**).* Protected teixobactin (1-7) with NMe-L-Ile$_6$ and L-Ser(tBu)$_7$ was prepared in the same manner as **4** on a 0.25 mmol scale. A portion of the material (89 mg, 0.069 mmol) was dissolved in 1,4-dioxane (~4 mL) in a 25 mL RBF. COMU (98 mg, 0.229 mmol) was added, followed by DIEA (40 μL, 0.230 mmol). **3** (25 mg, 0.057 mmol) was dissolved in 1,4-dioxane (1 mL) separately and DIEA (10 μL, 0.057 mmol) was added. The solution was then added to the RBF and the reaction mixture was heated to 60°C, stirred for 3 h and worked up in a similar fashion to **6**. 1.0 mg was recovered (1.3% yield).

*NMe-Ser$_7$, Leu$_{10}$-teixobactin (**12**).* Protected teixobactin with NMe-L-Ser$_7$ was prepared in the same manner as **4** on a 0.25 mmol scale. The crude product was purified as described above to remove excessive deletion sequences and a portion of the material (39 mg, 0.031 mmol) was dissolved in 1,4-dioxane (~4 mL) in a 25 mL RBF. COMU (47 mg, 0.110 mmol) was added, followed by DIEA (19 μL, 0.109 mmol). **3** (12 mg, 0.028 mmol) was dissolved in 1,4-dioxane (1 mL) separately and DIEA (10 μL, 0.057 mmol) was added. The solution was then added to the RBF and the reaction mixture was heated to 60°C, stirred for 3 h and worked up in a similar fashion to **6**. 4.4 mg was recovered (12% yield).

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MIC experiments. A broth microdilution checkerboard method was performed according to the Clinical and Laboratory Standards Institute guidelines.[23] Mueller–Hinton broth (MHB) supplemented with 0.1 % Tween 80 was used to avoid the adsorption of the compounds onto the plastic of the 96-well microtiter plates. The bacterial cell density was adjusted to ~0.5 McFarland turbidity standard, and cells were incubated for 20 h at 37°C. MICs were determined for all isolates in three replicates on separate days. MICs were determined as the lowest concentrations that inhibited the visible growth of the bacteria.

Results and Discussion

The macrocycle of teixobactin contains 13 backbone atoms, therefore the point of cyclisation is critical because an efficient reaction is dependent on the conformation that the peptide adopts. A solution-phase synthesis of the macrocyclic core was previously achieved with yields in the order of 50-55%.[24] For our strategy (**Scheme 1a**), we assembled the linear precursor such that the more flexible side-chain of D-Thr_8 was located in the middle of the linear peptide. It was thought that this might enable a favourable peptide conformation for cyclisation.

After derivatisation of 2-chlorotrityl resin with L-Ala_9 and Fmoc deprotection, D-Thr_8 was incorporated with N^{α} *tert.*-butyloxycarbonyl (Boc) protection and an unprotected side-chain. L-Ile_{11} was then installed via a Steglich esterification with minimal epimerisation, followed by further Fmoc SPPS to couple L-Leu_{10}. The Boc-protected linear precursor was then cleaved from the solid support with 1% trifluoroacetic acid (TFA) in dichloromethane (DCM) and isolated to generate **1**. Macro-lactamisation was achieved with the activator EDC.HCl and *N,N*-diisopropylethylamine (DIEA) in DCM to form **2**. EDC.HCl was used because it is inexpensive and can be readily extracted from the reaction mixture with aqueous washes prior to Boc deprotection. Each synthetic step was characterised via LC-MS; refer to **Figure 2a** for details.

The unprotected pure cyclic precursor (**3**) is readily accessible from the crude material with yields of ~35-40%. When the linear peptide is purified, the cyclisation yield is 70%, which to our knowledge is the highest reported for this pharmacophore and highlights the efficiency of our synthetic strategy. The precursor was purified in hydrochloric acid (HCl) buffers to remove all traces of TFA and prevent trifluoroacetylation, which was detected previously (data not shown). The *N*-terminal fragment (**4**) bearing the *C*-terminal *pseudo*-proline assembles readily via Fmoc SPPS on 2-chlorotrityl resin. To

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minimise unnecessary waste of the building blocks, 1.1 equivalents of Fmoc amino acid were used for each coupling. Cleavage was performed in mild acidic conditions and the protected peptide was washed with brine prior to isolation. Linear peptide **4** was isolated with a yield of 67% and without purification.

For the fragment condensation (**Scheme 1b**), several different reaction conditions were evaluated. Initially, the ligation was unsuccessful at ambient temperature, even by preparing an acyl fluoride (possibly due to steric hindrance by the β-methyl group of D-Thr_8). Therefore, the reaction was repeated at 60°C which led to successful coupling. The optimised conditions that were applied henceforth utilised COMU as the coupling reagent and DIEA as the base in 1,4-dioxane. 1.2-1.5 equivalents of the *N*-terminal precursor was used to ensure that the labour-intensive macrocyclic precursor was consumed in the reaction. After coupling (to form **5**) and global deprotection, crude **6** was immediately purified *via* RP-HPLC. The yield for the reaction was 17% and Leu_{10}-teixobactin was isolated at high purity. All relevant analytical data appear in **Figure 2b**. To determine whether our chemically synthesised Leu_{10}-teixobactin possessed antimicrobial activity, we evaluated its potency using checkerboard assays against a panel of *Staphylococcus aureus (S. aureus)* isolates. It was found that the minimum inhibitory concentrations (MICs) were comparable to those from previous reports[21] (**Table 1**) including matching MIC values for *S. aureus* ATCC29213 (0.5 μg/mL).[22]

Several hundred teixobactin analogues have already been evaluated, with most modifications only involving amino acid substitutions.[9, 10] *N*-methylation is observed in non-ribosomal *depsi*-peptides and can greatly impact their pharmacological activity.[25] Moreover, it has been shown to improve the *in vivo* stability[26] and efficacy[27] of peptide-based drugs such as cyclosporin A and somatostatin analogues.[28] Blocking backbone amides with a methyl group can also probe *intra*- or *inter*-molecular hydrogen bonding interactions. For example, adding an additional methyl group to the already *N*-methylated *N*-terminus reduced potency by eight-fold in the Arg_{10}-teixobactin analogue.[29] Given that *N*-methylation of one or more of the macrocyclic amides would likely introduce conformational or steric constraints and directly interfere with pyrophosphate binding, we focussed our current investigation to the effect of *N*-methylating[30] residues 2-7 of Leu_{10}-teixobactin (**Figure 3**). *N*-methylated D-Gln and D-*allo*-Ile building blocks are not commercially available, in which case the alkylation was performed *in situ*.[31] To avoid dimethylation, the *N*-terminus of the resin-bound peptide was first transformed to an *ortho*-nitrobenzenesulfonamide, followed by *N*-

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alkylation and then deprotection (see **Figure 4**). Initial attempts to synthesise **11** via *N*-methylation of the *pseudo*-proline dipeptide failed, which was likely due to diketopiperazine formation (despite use of the hindered 2-chlorotrityl linker). Analogue **11** was eventually synthesised with *tert.*-butyl (*t*Bu) protection at L-Ser_7; the level of epimerisation occurred during the fragment condensation was ~30% but was removed during purification. The remaining *N*-methylated analogues were prepared in good yield, although there were significant deletion sequences in the crude linear precursors for analogues **10** and **12**, most likely due to inefficient coupling onto the N-methylated residue; these required purification prior to the fragment coupling. Refer to **Figure 5** for respective analytical data of the purified analogues.

The antimicrobial activity of the *N*-methyl analogues against four *S. aureus* bacterial strains was determined using broth microdilution based on EUCAST guidelines (**Table 2**). Resistance was defined as an MIC >4 μg/mL. In general, *N*-methylation was detrimental to antimicrobial activity, although **11** still retained low potency (MIC = 16 μg/mL). This loss of activity indicates that conformational changes to the *N*-methylated analogues might have occurred and/or hydrogen bonding is critical for the monomeric or multimeric active structure; the latter as proposed by Nowick and co-workers,[6] which describes the formation of amyloid-like assemblies of teixobactin as intrinsic to its mechanism of action.

Conclusion

In summary, we have developed a convergent synthetic route that enables rapid production of highly pure teixobactin analogues. Cyclisation of the *C*-terminal macrocycle was achieved efficiently (70% yield) and epimerisation during the fragment condensation was suppressed by utilising a *pseudo*-proline dipeptide. Our synthetic strategy enabled the assembly of several *N*-methylated analogues. Backbone modification of teixobactin diminished antimicrobial activity in some cases and abolished it in others, which suggests that hydrogen bonding along the *N*-terminal tail is critical to its activity. These results will underpin future work in elucidating mechanism of action and lead to the design of new analogues with improved potency and a broader spectrum of activity, to help address the looming threat of AMR.

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Table 1. Comparison of the antimicrobial activity of our Leu$_{10}$-teixobactin analogue with previous reports of the same analogue (μg/mL).

This work	SA ATCC29213	SA ATCC700699 / Mu50	SA JKD6008	SA ATCC700698 / Mu30	SA JKD6009
	0.5	0.5	0.5	0.5	0.5
Li et al.[22]	SA ATCC29213	MRSA SA1114			
	0.5	2			
Singh et al.[21]	VRE ATCC700802	VRE ATCC29212	MRSA ATCC700699	MRSA 42412	MRSA 21455
	0.25	0.25	0.25	< 0.0625	< 0.0625

Table 2. Antimicrobial activity of *N*-methylated Leu$_{10}$-teixobactin analogues against several isolates of *S. Aureus* (μg/mL). *Note that **11** is >78% pure hence its MIC values may be lower.

***N*-Methylated Leu$_{10}$-teixobactins**	**SA ATCC 29213**	**SA ATCC 700698**	**SA ATCC 700698**	**SA ATCC 43300**
None (**6**)	0.5	0.5	0.5	0.5
NMe-L-Ile$_2$ (**7**)	>128	>128	32	>128
NMe-L-Ser$_3$ (**8**)	>128	>128	>128	>128
NMe-D-Gln$_4$ (**9**)	64	32	32	64
NMe-D-*allo*-Ile$_5$ (**10**)	>128	>128	32	>128
NMe-L-Ile$_6$ (**11**)*	16	16	16	16
NMe-L-Ser$_7$ (**12**)	>128	>128	32	>128

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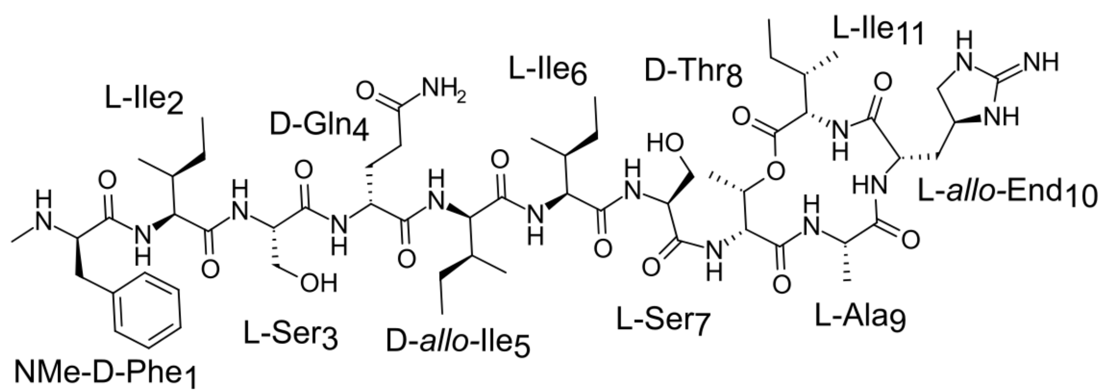

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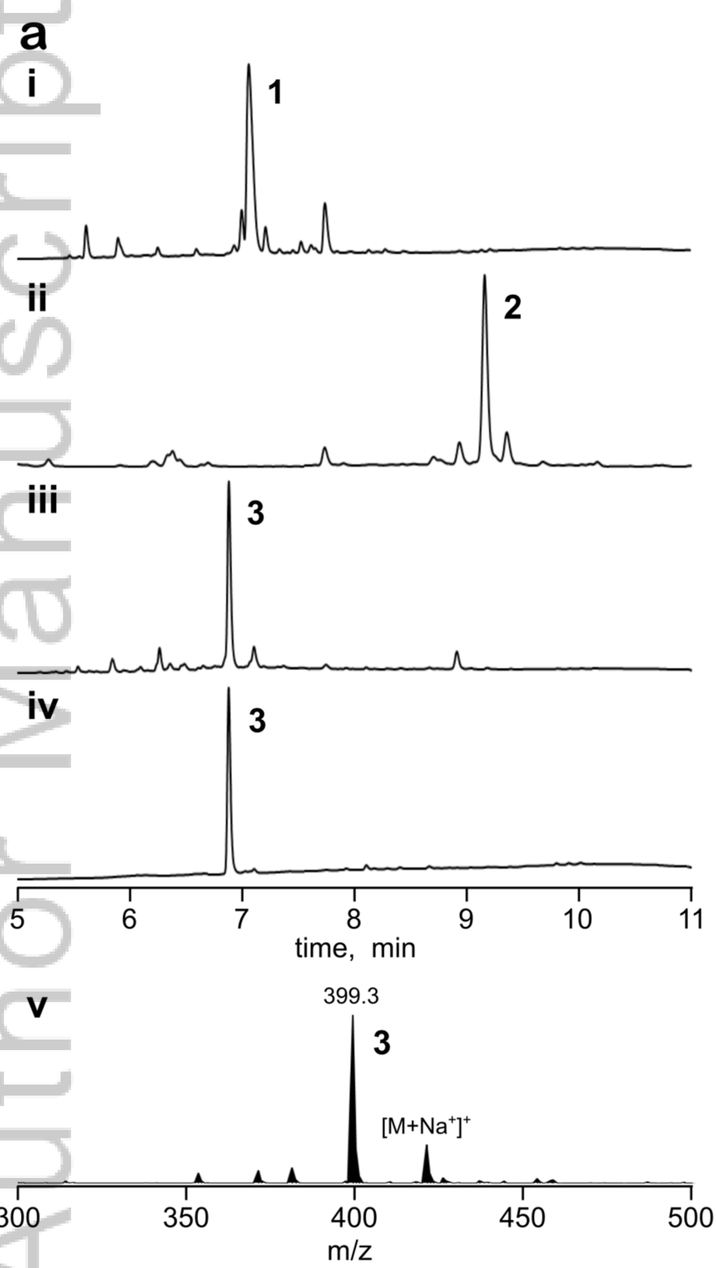

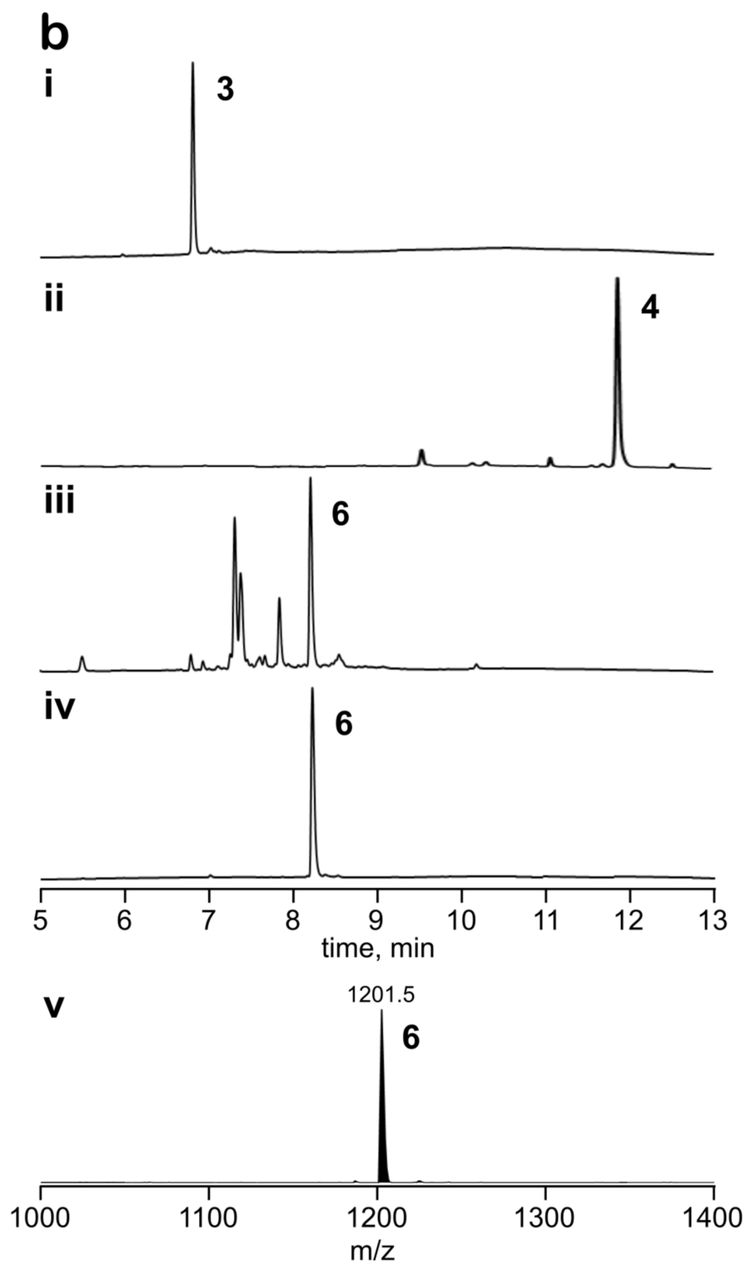

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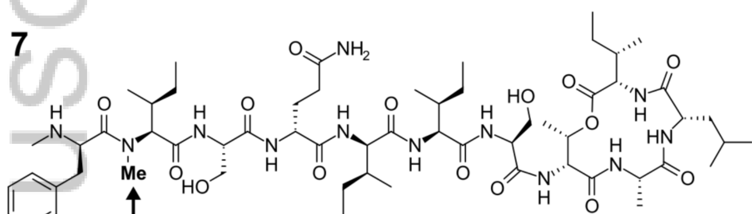

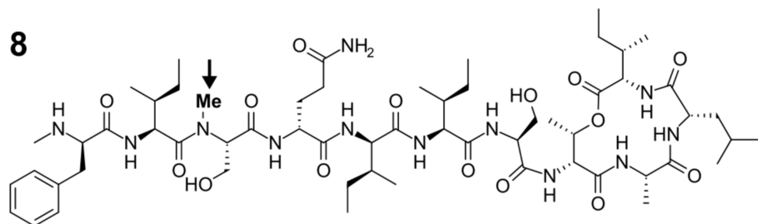

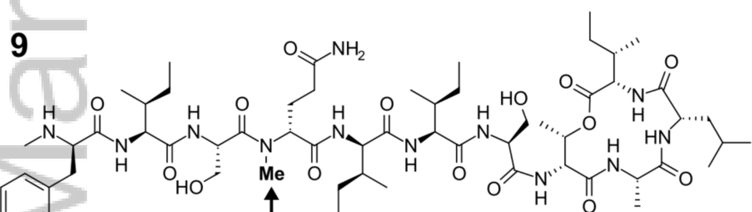

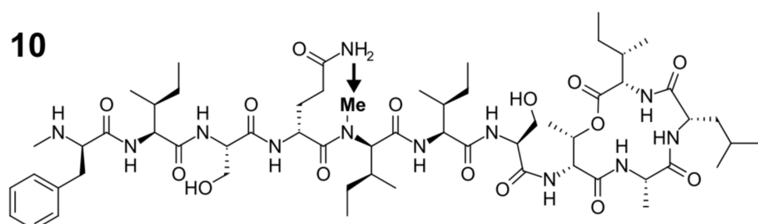

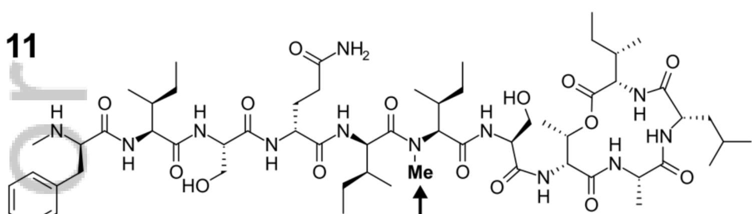

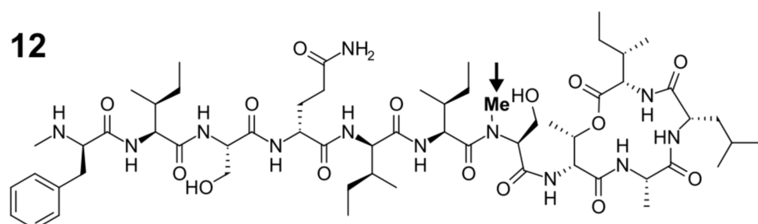

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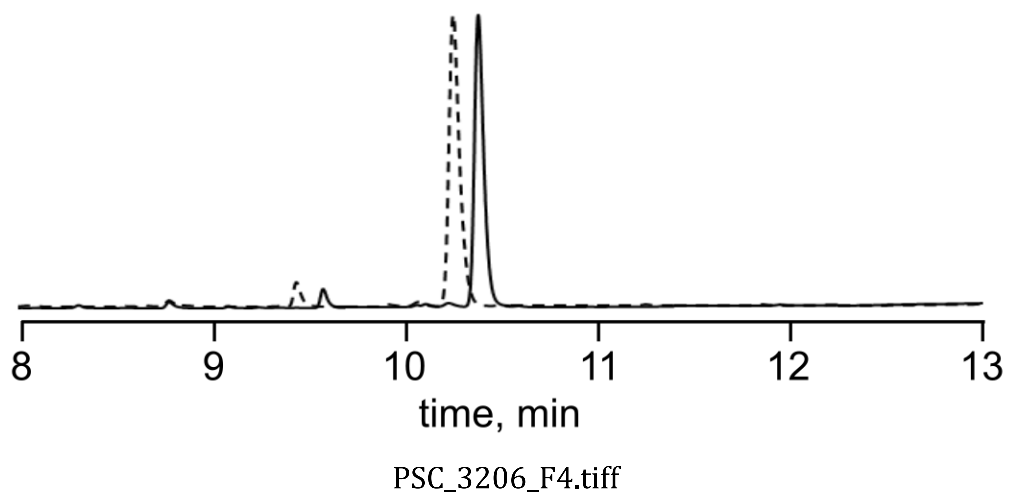

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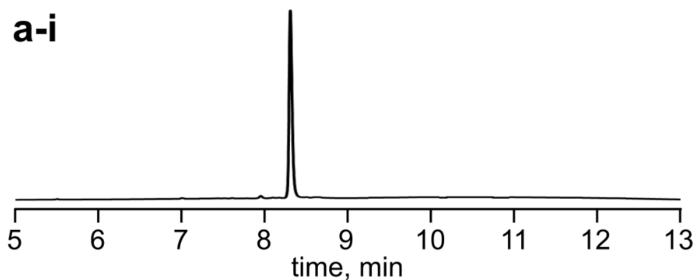

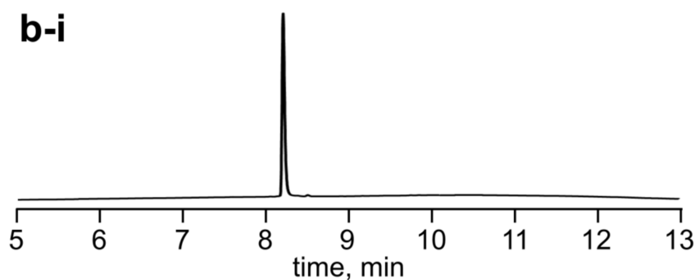

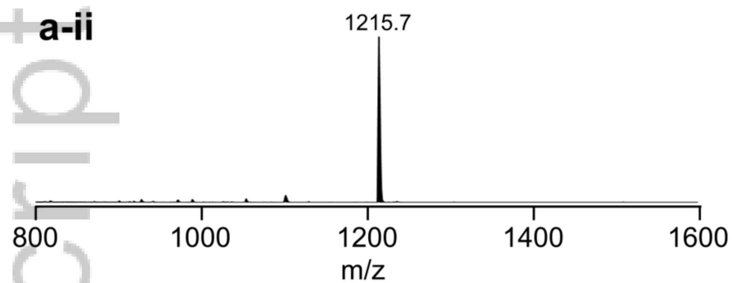

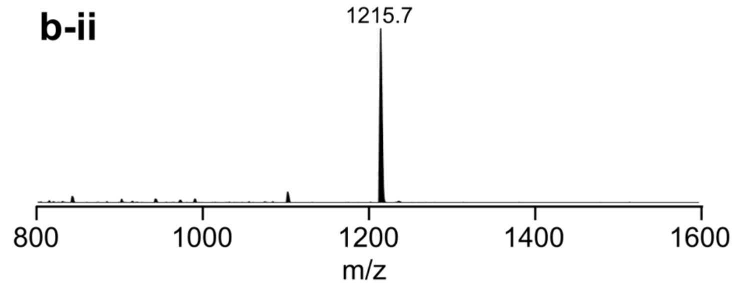

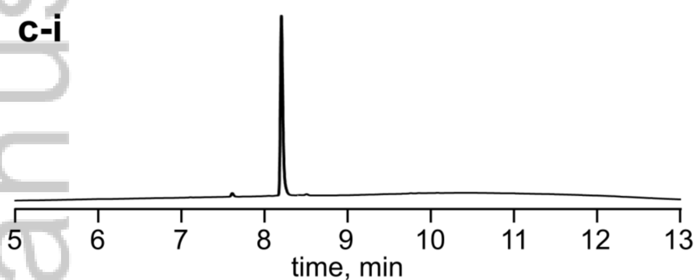

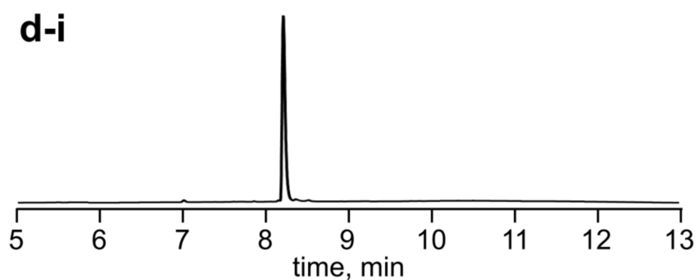

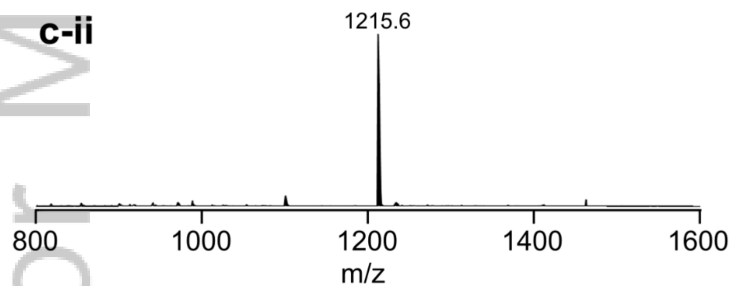

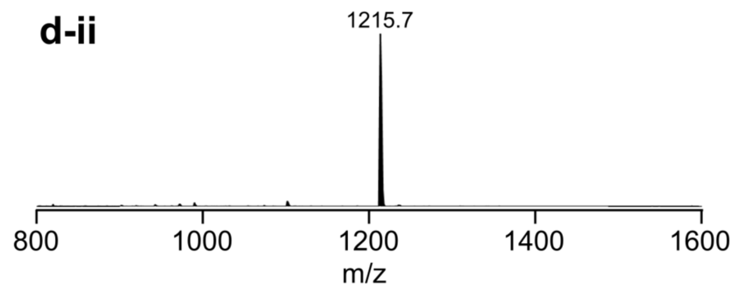

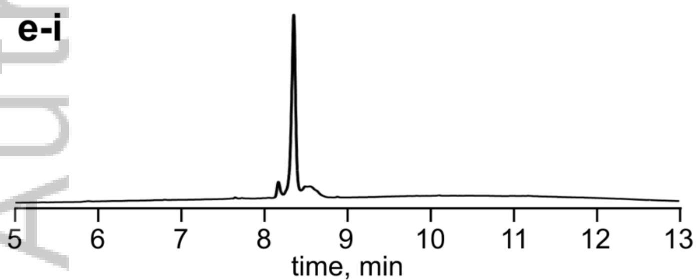

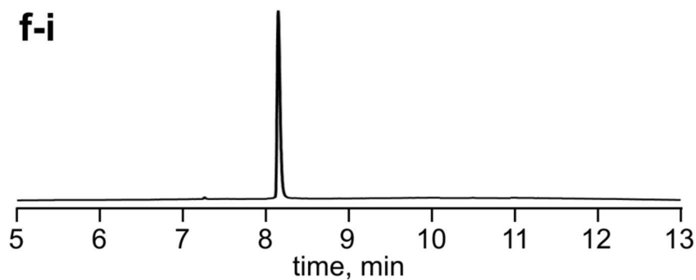

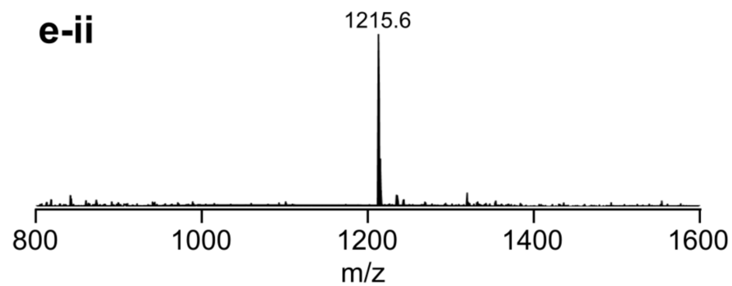

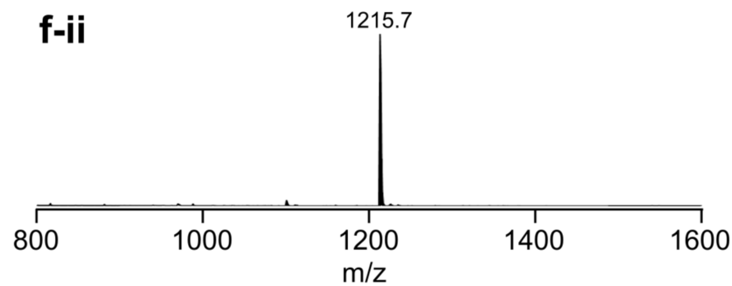

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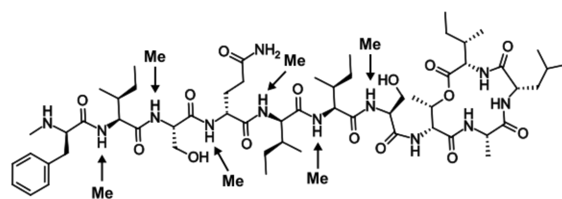

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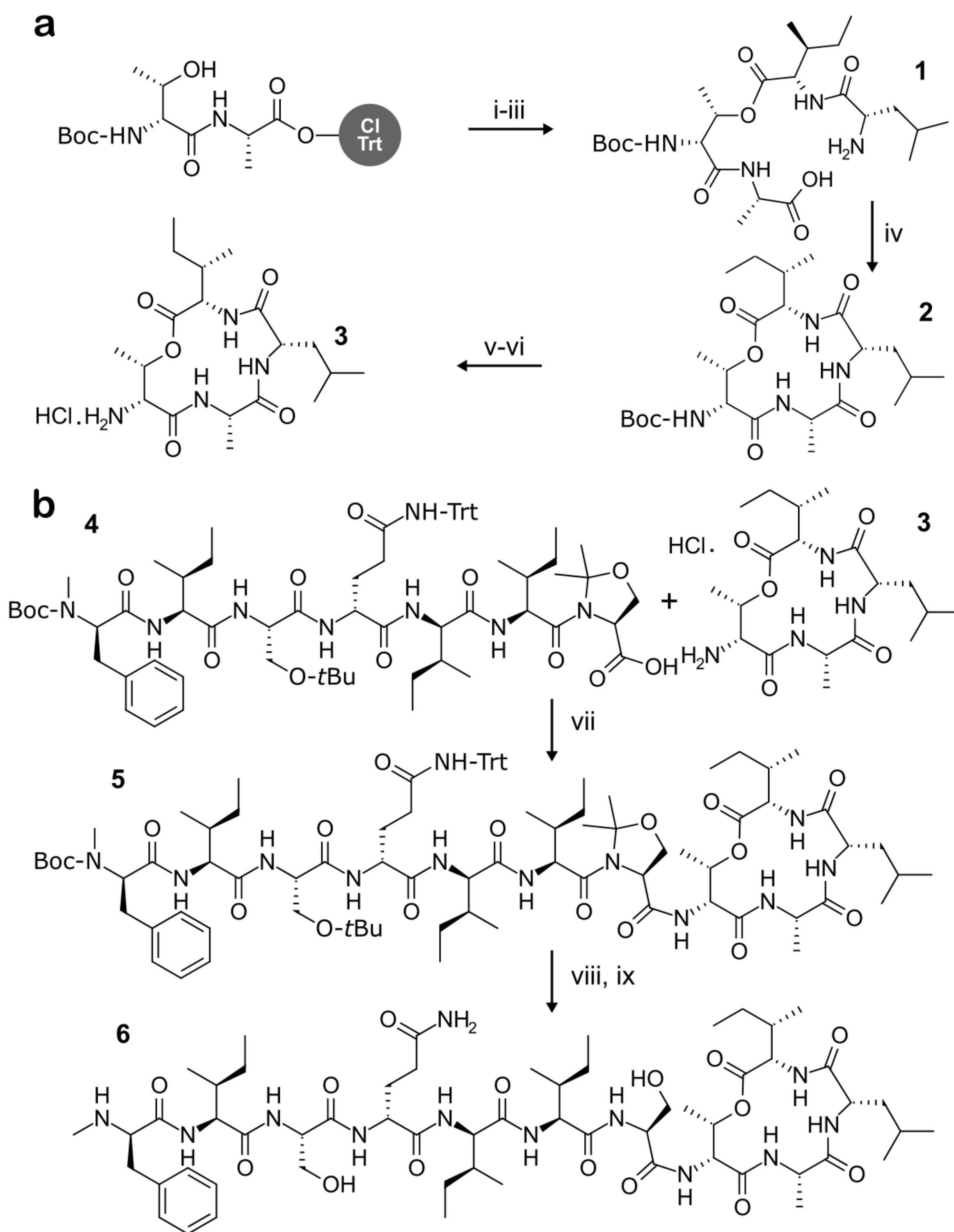

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