Design and syntheses of highly potent teixobactin analogues against *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant Enterococci (VRE) *in vitro* and *in vivo*

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I: Materials

All L amino acids, Fmoc-D-Arg(pbf)-OH, Fmoc-D-Gln(Trt)-OH, Boc-N-methyl-D-phenylalanine, 1 [Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium3-oxidhexafluorophosphate (HATU), Phenylsilane ($PhSiH_3$), Tetrakis(triphenylphosphine)palladium(0) [$Pd(PPh_3)$], Diisoproplycarbodiimide (DIC) and Triisopropylsilane (TIS) were purchased from Fluorochem, UK. Fmoc-D-*allo*-Ile-OH and oxyma pure were purchased from Merck Millipore. The side chain protecting groups for the amino acids are $_t$Bu for Ser, Pbf for Arg and Trt for Gln and Thr unless specified otherwise. Diisopropylethylamine (DIPEA), supplied as extra dry, redistilled, 99.5 % pure, Acetic anhydride, allyl chloroformate, $CDCl_3$ and polysorbate 80 and were purchased from Sigma Aldrich. Tritylchloride and 4-(Dimethylamino)pyridine were purchased from Alfa Aesar. Dimmethylformamide (DMF) peptide synthesis grade was purchased from Rathburn chemicals. Triethylamine, Diethyl ether (Et_2O), Dimethylsulfoxide (DMSO), Dichloromethane (DCM), Tetrahydrofuran (extra dry with molecular sieves), Formic acid 98-100% purity and Acetonitrile (HPLC grade) were purchased from Fisher Scientific. Water with the Milli-Q grade standard was obtained in-house from an ELGA Purelab Flex system. 2-Chlorotritylchloride resin (manufacturer's loading: 1.20 mmol/g) was purchased from Fluorochem. All chemicals were used without further purification.

II: Equipment used for the analysis and purification of compounds

All peptides were analysed on a Thermo Scientific Dionex Ultimate 3000 RP-HPLC equipped with a Phenomenex Gemini NX C18 110 Å (150 x 4.6 mm) column using the following buffer systems: A: 0.1% HCOOH in milliQ water. B: ACN using a flow rate of 1 ml/min. The column was flushed with 95% A for 5 min prior to an injection and was flushed for 5 min with 95% B and 5% A after the run was finished. Peptides were dissolved in (1:1) 0.1% HCOOH buffer in water and acetonitrile (ACN) and analysed using the following gradient: 95% A for 2 min. 5-95% B in 25 min. 95% B for 5 min. 5% A for 4 min. Peptides were dissolved in 0.1% HCOOH buffer in water and in ACN (10-30% ACN) and purified using the same gradient as mentioned above, on a Thermo Scientific Dionex Ultimate 3000 RP-HPLC with a flow rate of 5 mL/min using a Phenomenex Gemini NX C18 110 Å (150 x 10 mm) semi-prep column.
HRMS spectra were recorded on a Thermo Scientific Q Exactive Plus Orbitrap Mass Spectrometer in the positive ion mode.

III: HPLC/MS analysis

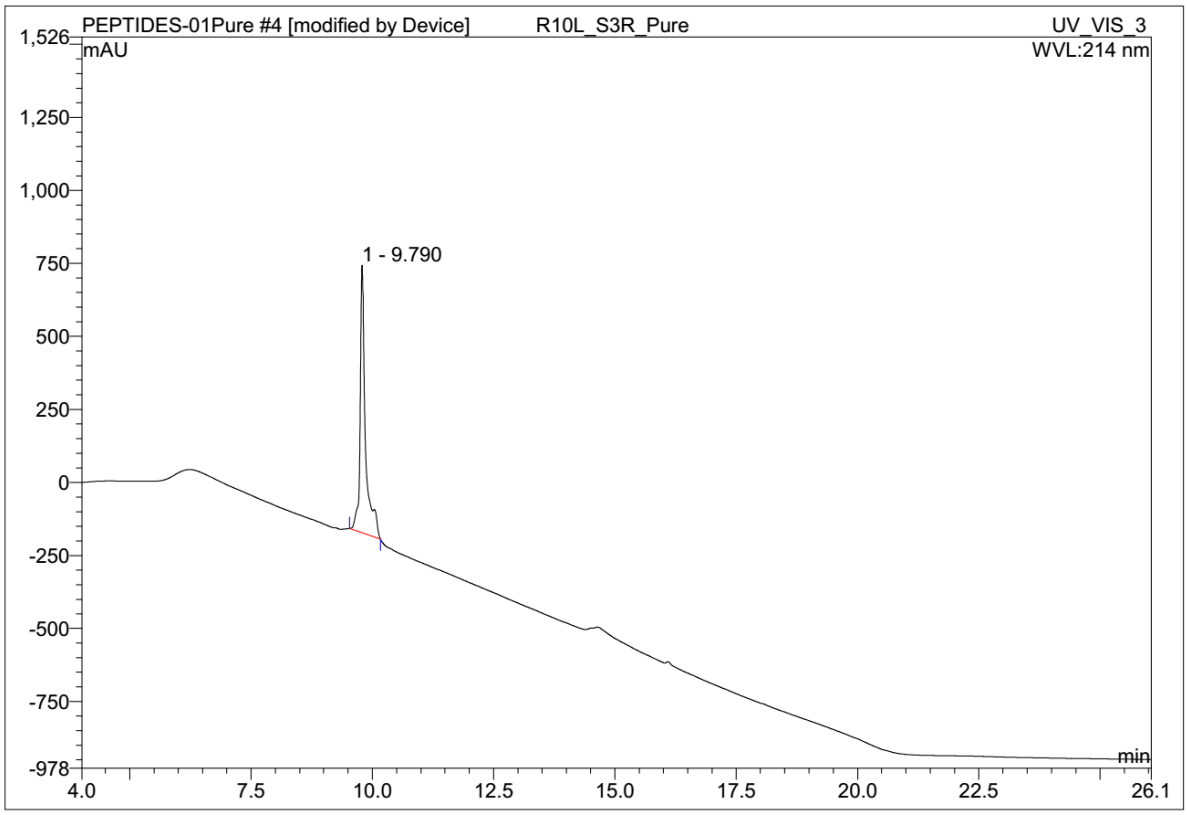


Fig. S1: HPLC trace of HPLC purified teixobactin analogue **1** (gradient: 5-95% ACN in 25 min using A: 0.1% HCOOH in water, B: ACN)

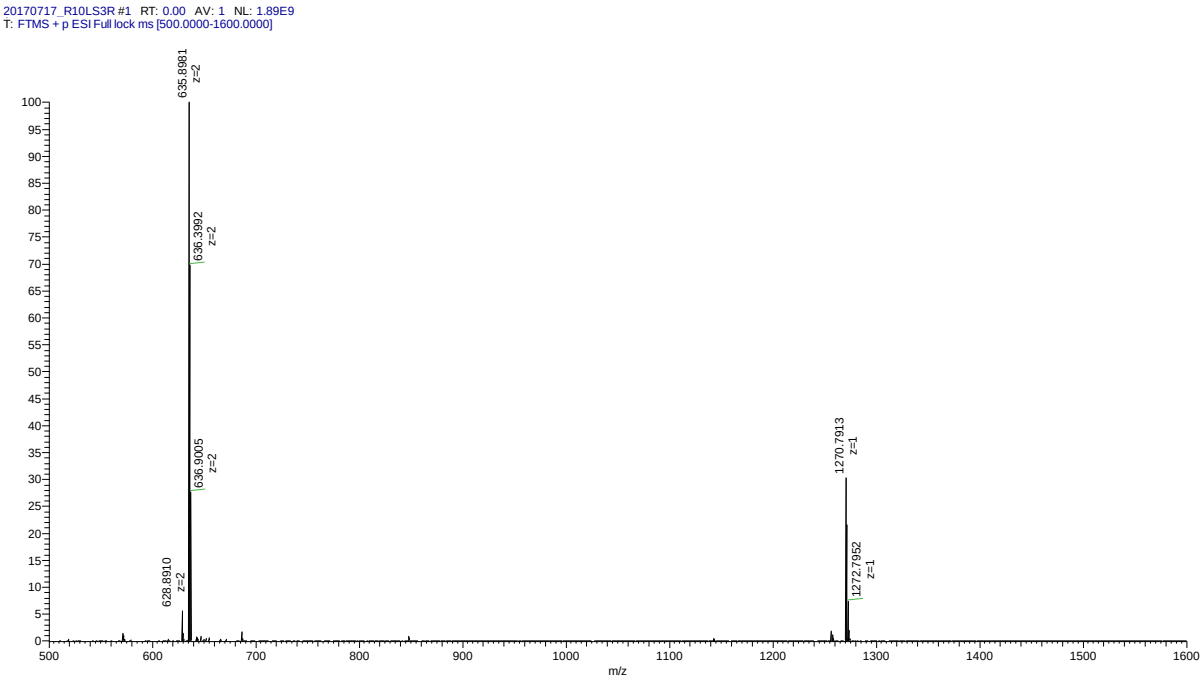


Fig. S2: HRMS of HPLC purified teixobactin analogue **1**. Mass calcd. for $C_{61}H_{104}N_{15}O_{14}$ = 1270.7887, found M + H^+ = 1270.7913, $[M+2H]^{2+}/2$ = 635.8981.

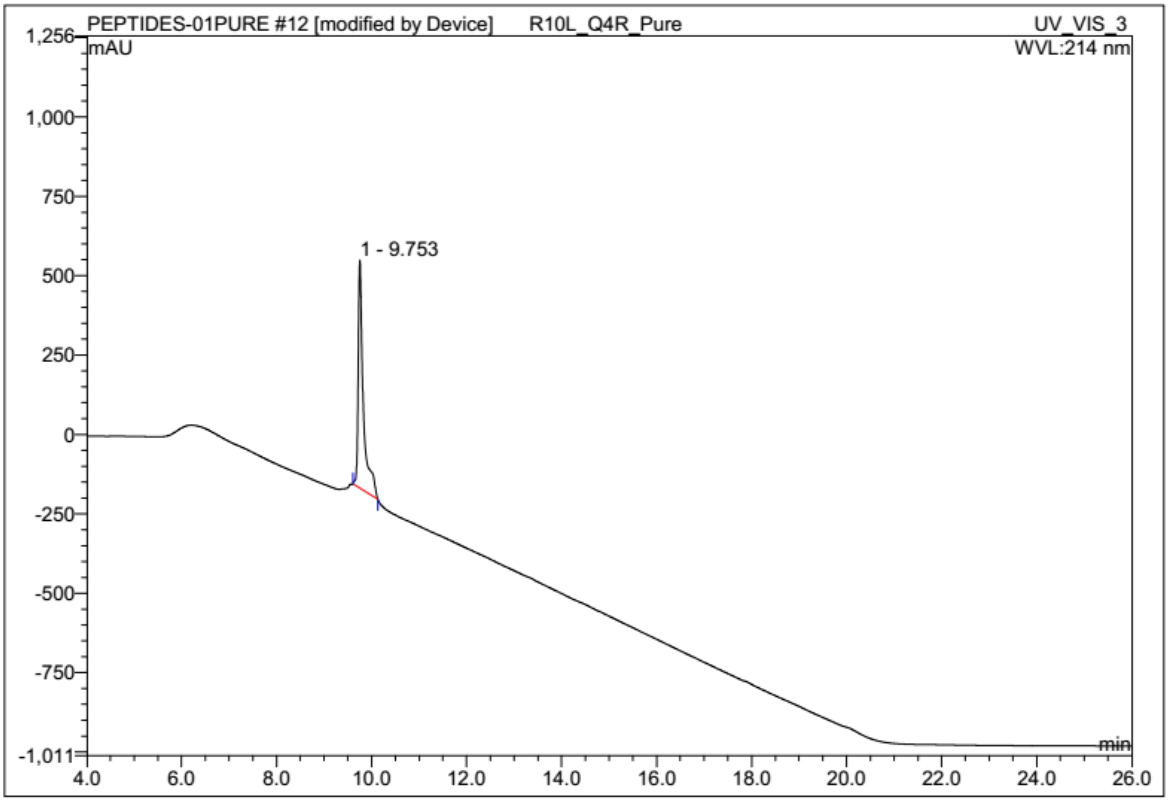


Fig. S3: HPLC trace of HPLC purified teixobactin analogue **2** (gradient: 5-95% ACN in 25 min using A: 0.1% HCOOH in water, B: ACN)

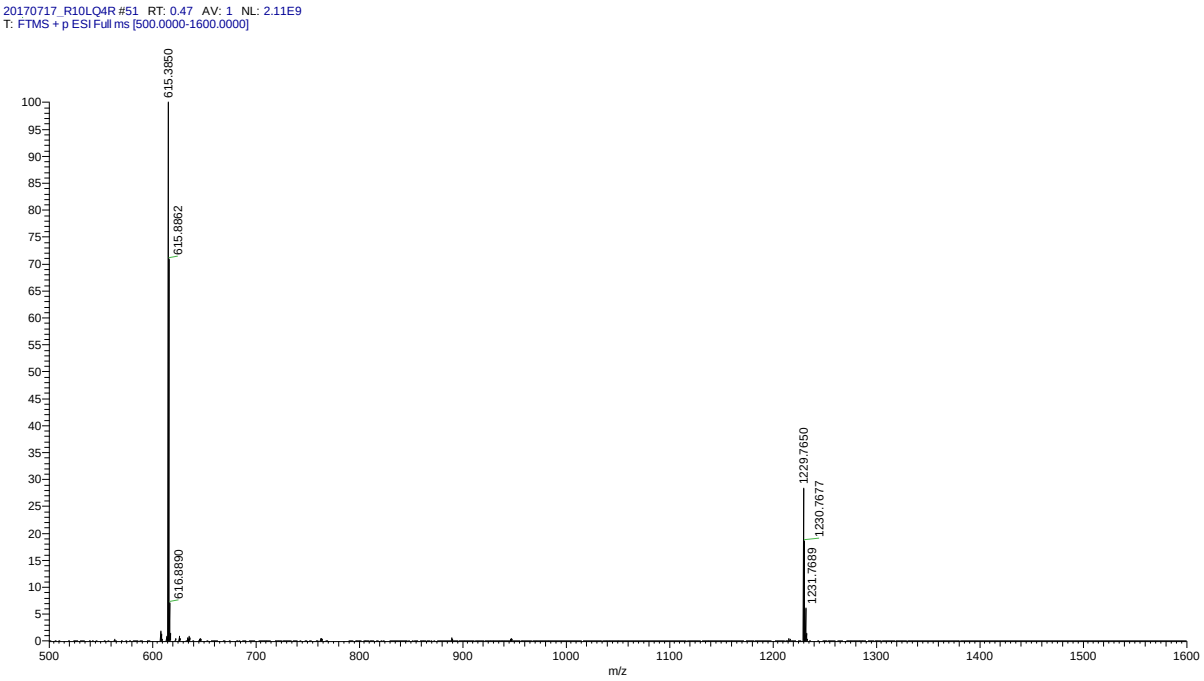


Fig. S4: HRMS of HPLC purified teixobactin analogue **2**. Mass calcd. for $C_{59}H_{101}N_{14}O_{14}$ = 1229.7622, found M + H^+ = 1229.7650, $[M+2H]^{2+}/2$ = 615.8850.

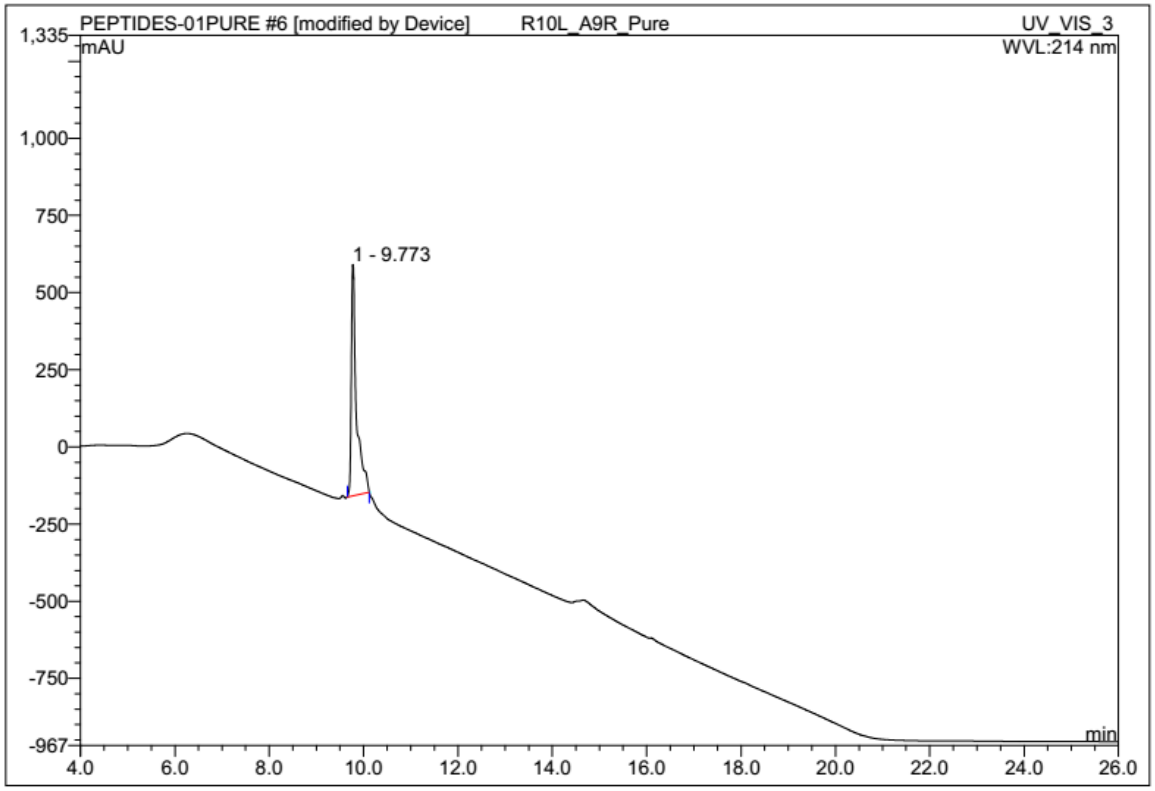


Fig. S5: HPLC trace of HPLC purified teixobactin analogue **3** (gradient: 5-95% ACN in 25 min using A: 0.1% HCOOH in water, B: ACN)

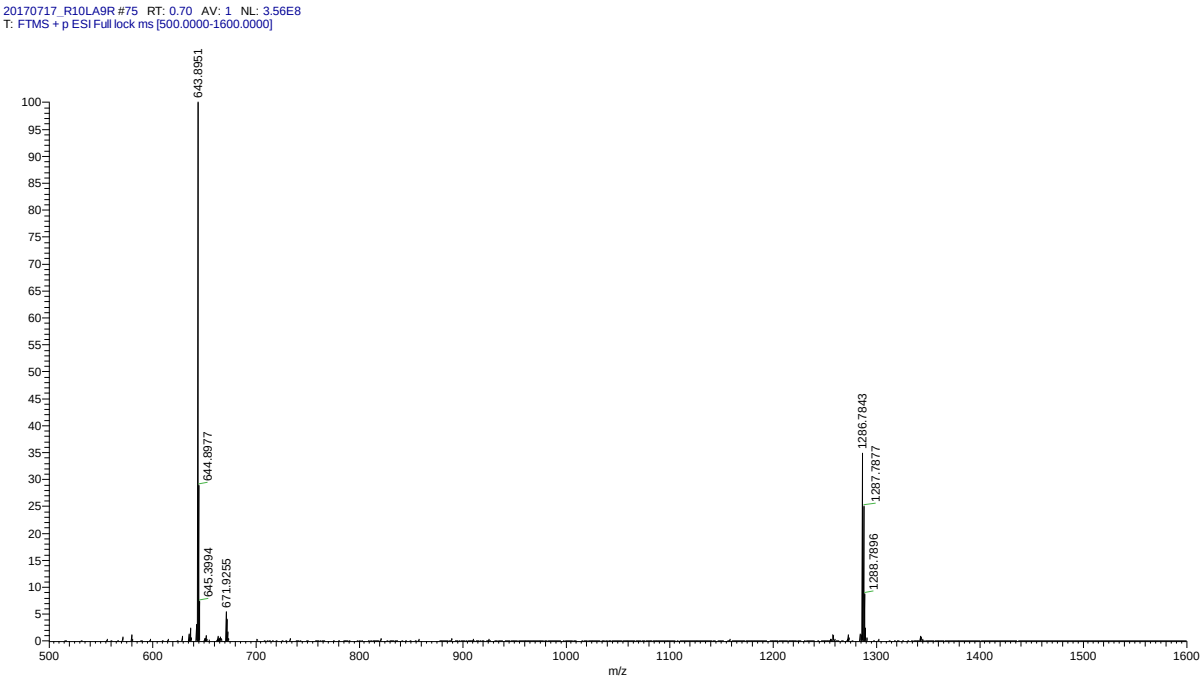


Fig. S6: HRMS of HPLC purified teixobactin analogue **3**. Mass calcd. for $C_{61}H_{104}N_{15}O_{15}$ = 1286.7836, found M + H^+ = 1286.7843, $[M+2H]^{2+}/2$ = 643.8951.

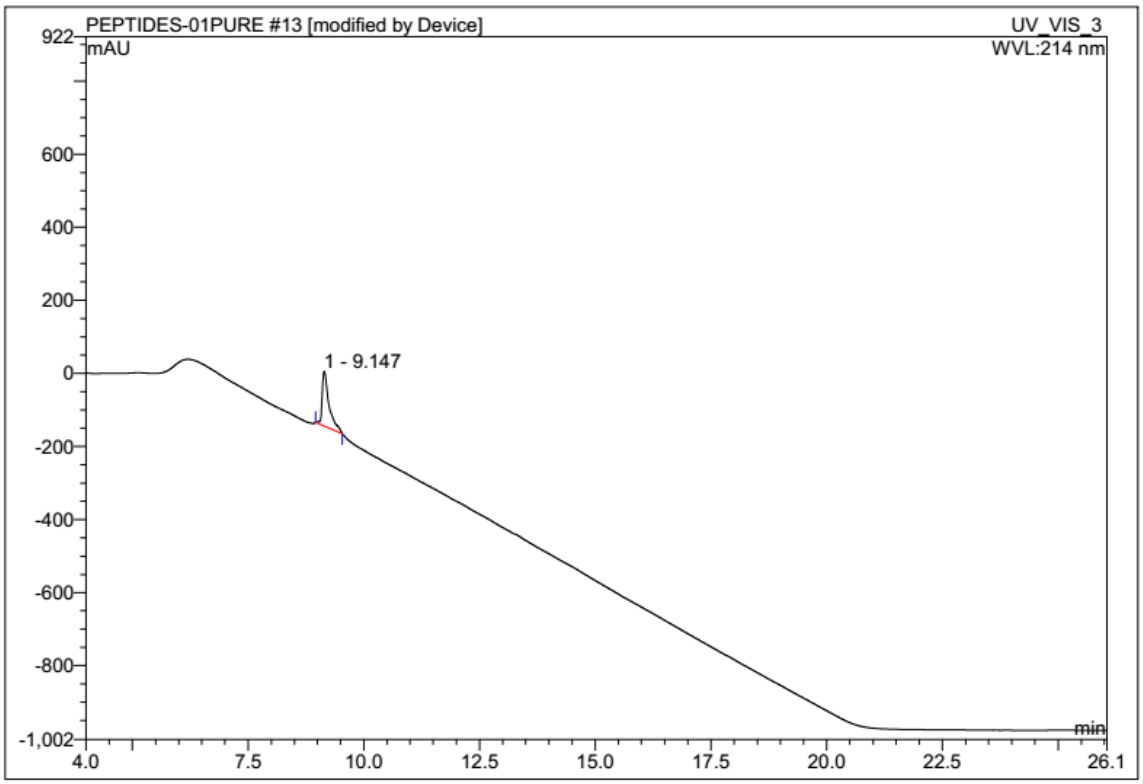


Fig. S7: HPLC trace of HPLC purified teixobactin analogue **4** (gradient: 5-95% ACN in 25 min using A: 0.1% HCOOH in water, B: ACN)

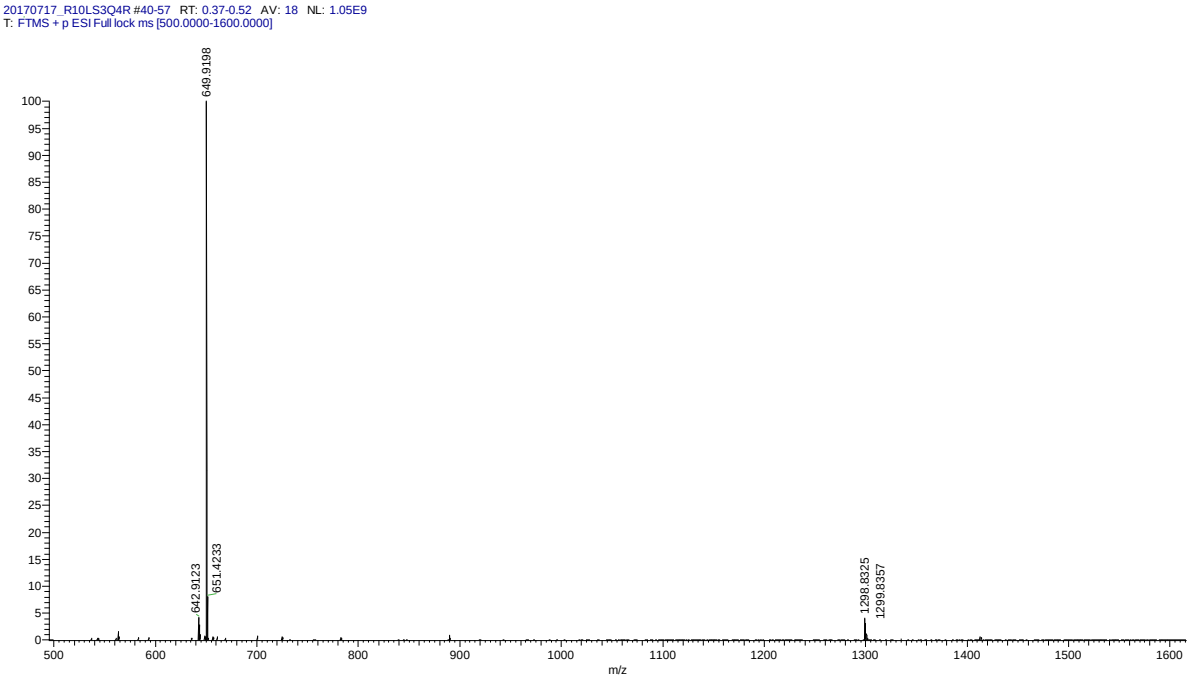


Fig. S8: HRMS of HPLC purified teixobactin analogue **4**. Mass calcd. for $C_{62}H_{108}N_{17}O_{13}$ = 1298.8313, found M + H^+ = 1298.8325, $[M+2H]^{2+}/2$ = 649.9198.

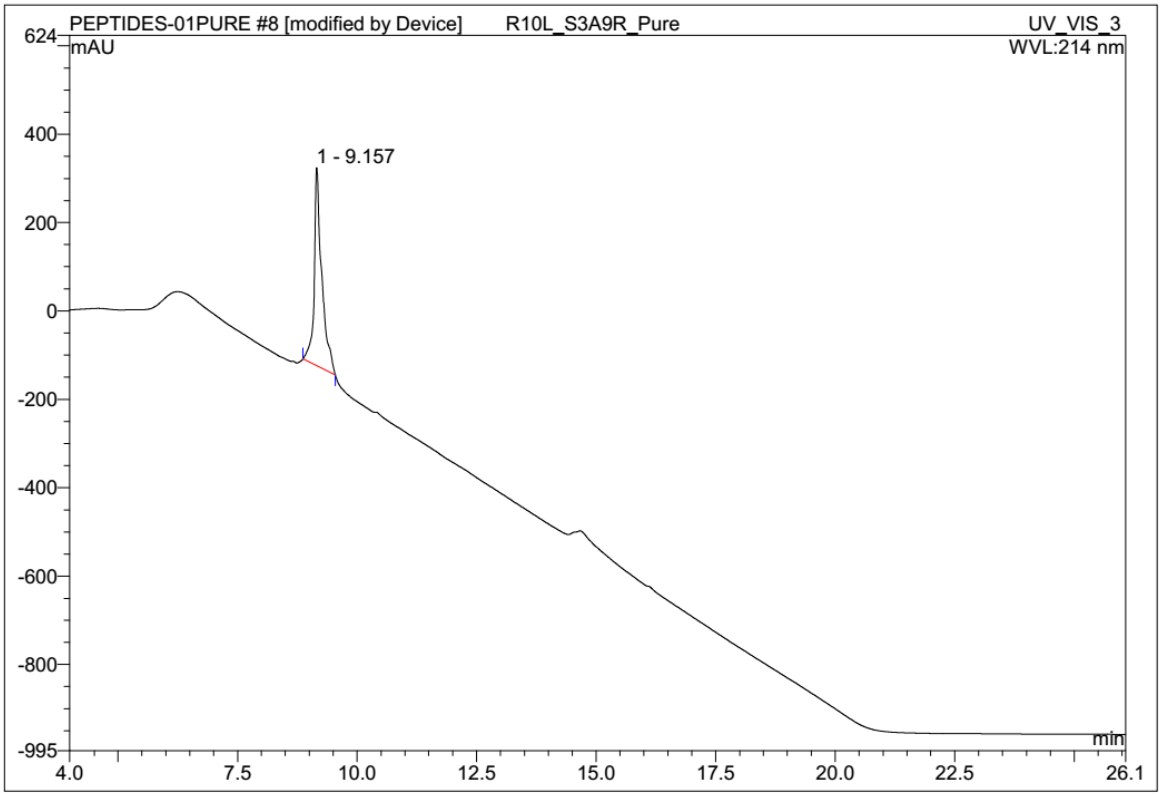


Fig. S9: HPLC trace of HPLC purified teixobactin analogue **5** (gradient: 5-95% ACN in 25 min using A: 0.1% HCOOH in water, B: ACN)

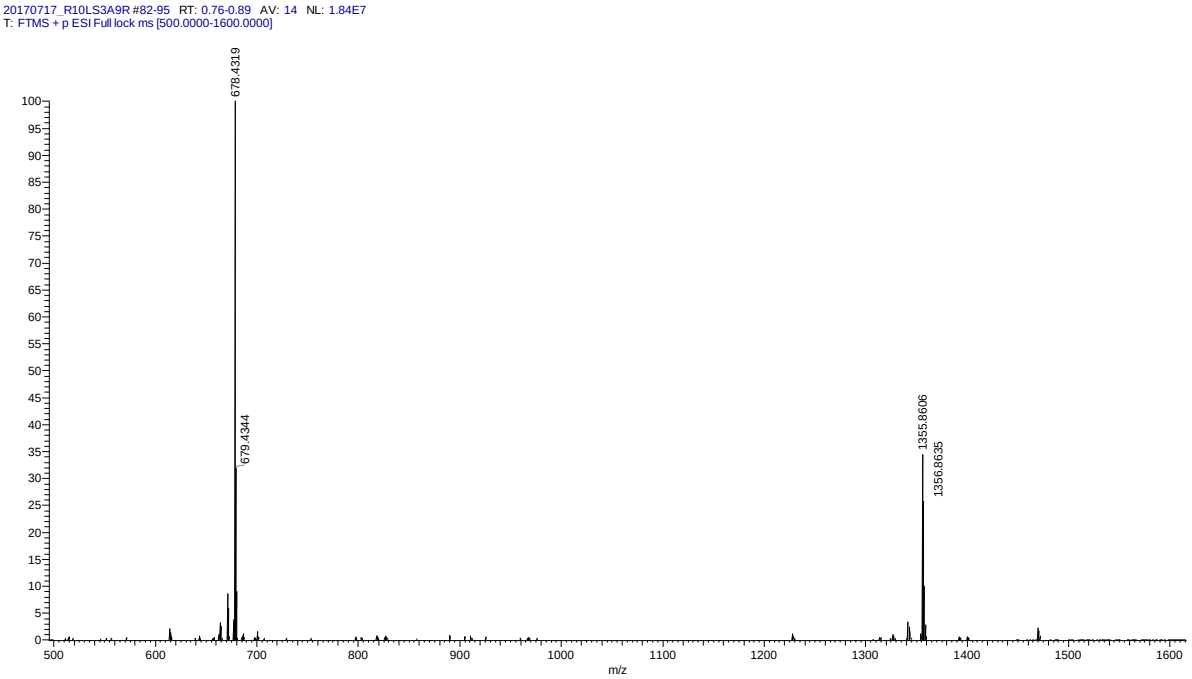


Fig. S10: HRMS of HPLC purified teixobactin analogue **5**. Mass calcd. for $C_{64}H_{111}N_{18}O_{14}$ = 1355.8527, found M + H^+ = 1355.8606, $[M+2H]^{2+}/2$ = 678.4319.

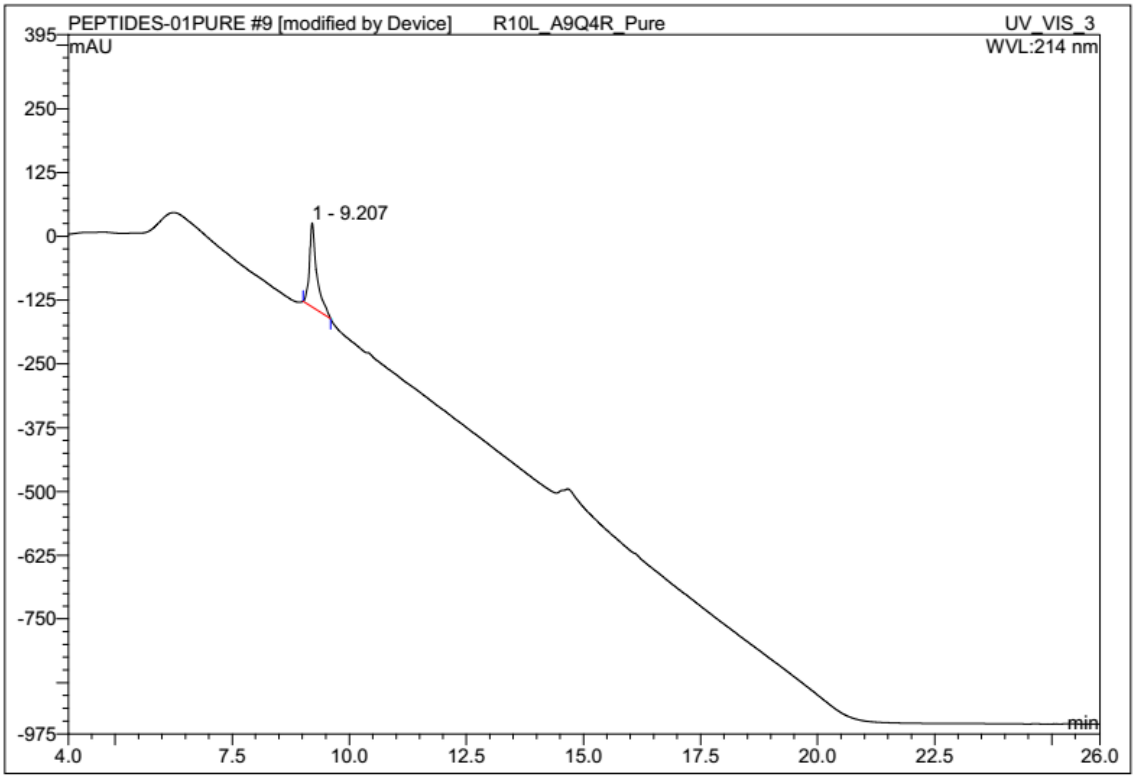


Fig. S11: HPLC trace of HPLC purified teixobactin analogue **6** (gradient: 5-95% ACN in 25 min using A: 0.1% HCOOH in water, B: ACN)

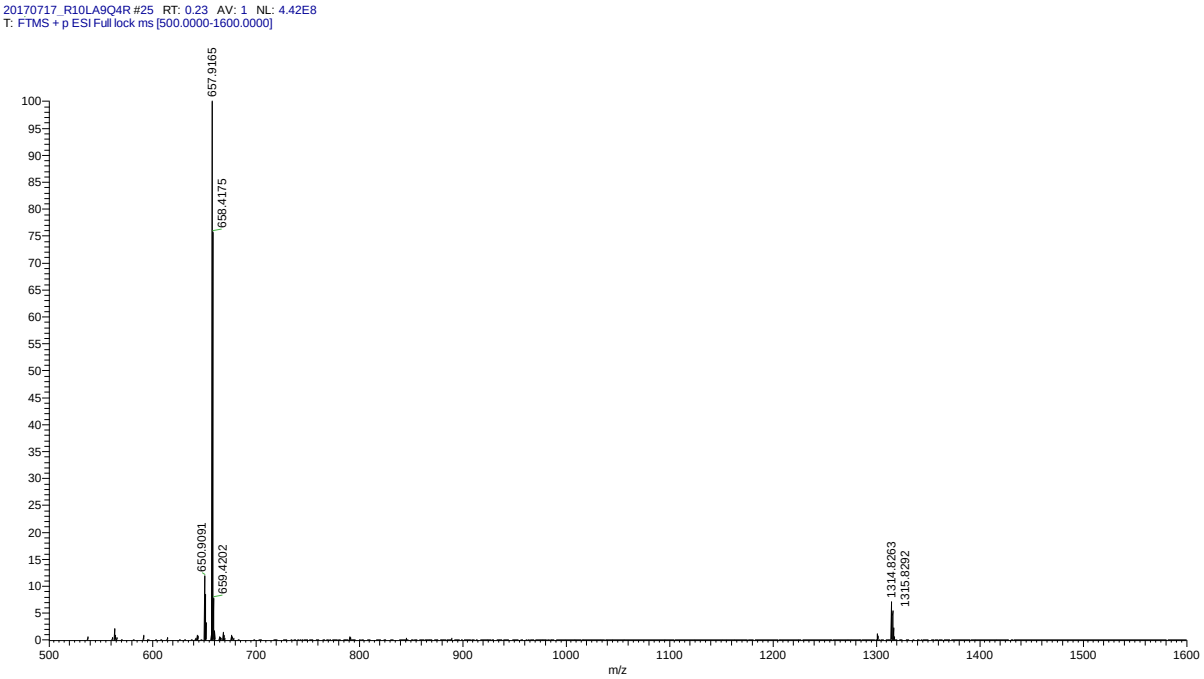


Fig. S12: HRMS of HPLC purified teixobactin analogue **6**. Mass calcd. for $C_{62}H_{108}N_{17}O_{14}$ = 1314.8262, found M + H^+ = 1314.8263, $[M+2H]^{2+}/2$ = 657.9165.

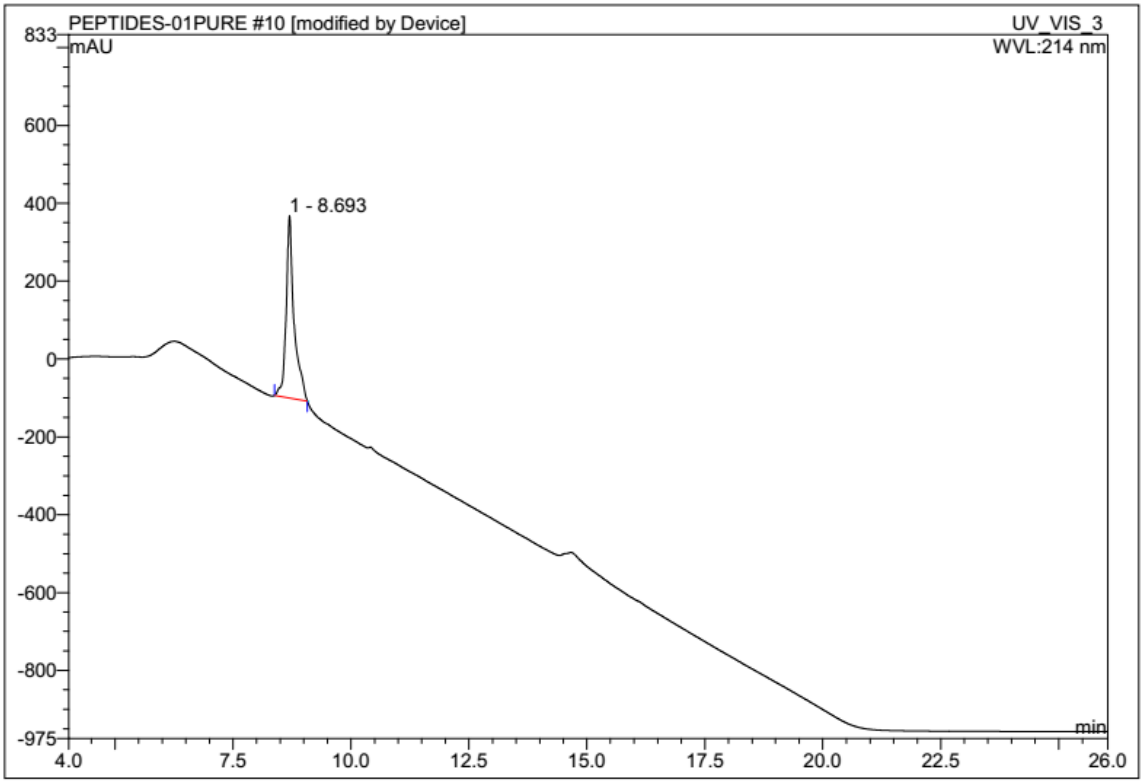


Fig. S13: HPLC trace of HPLC purified teixobactin analogue **7** (gradient: 5-95% ACN in 25 min using A: 0.1% HCOOH in water, B: ACN)

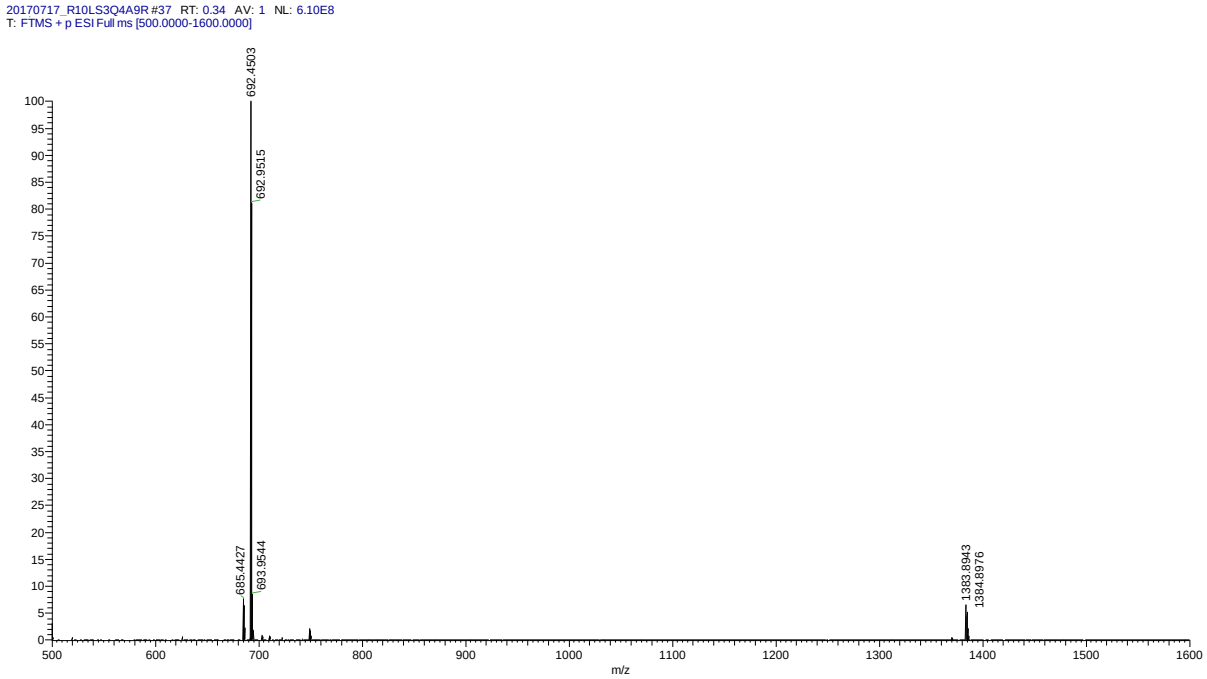


Fig. S14: HRMS of HPLC purified teixobactin analogue **7**. Mass calcd. for $C_{65}H_{115}N_{20}O_{13}$ = 1383.8952, found M + H^+ = 1383.8943, $[M+2H]^{2+}/2$ = 692.4503.

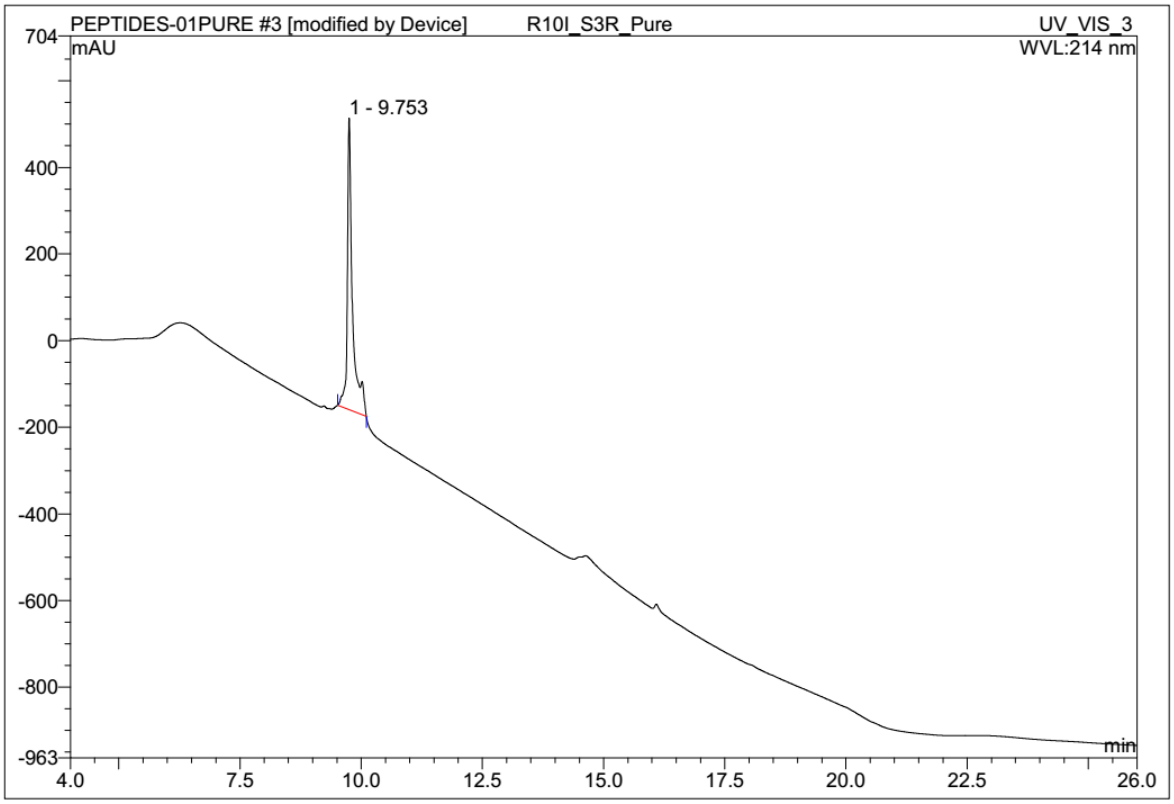


Fig. S15: HPLC trace of HPLC purified teixobactin analogue **8** (gradient: 5-95% ACN in 25 min using A: 0.1% HCOOH in water, B: ACN)

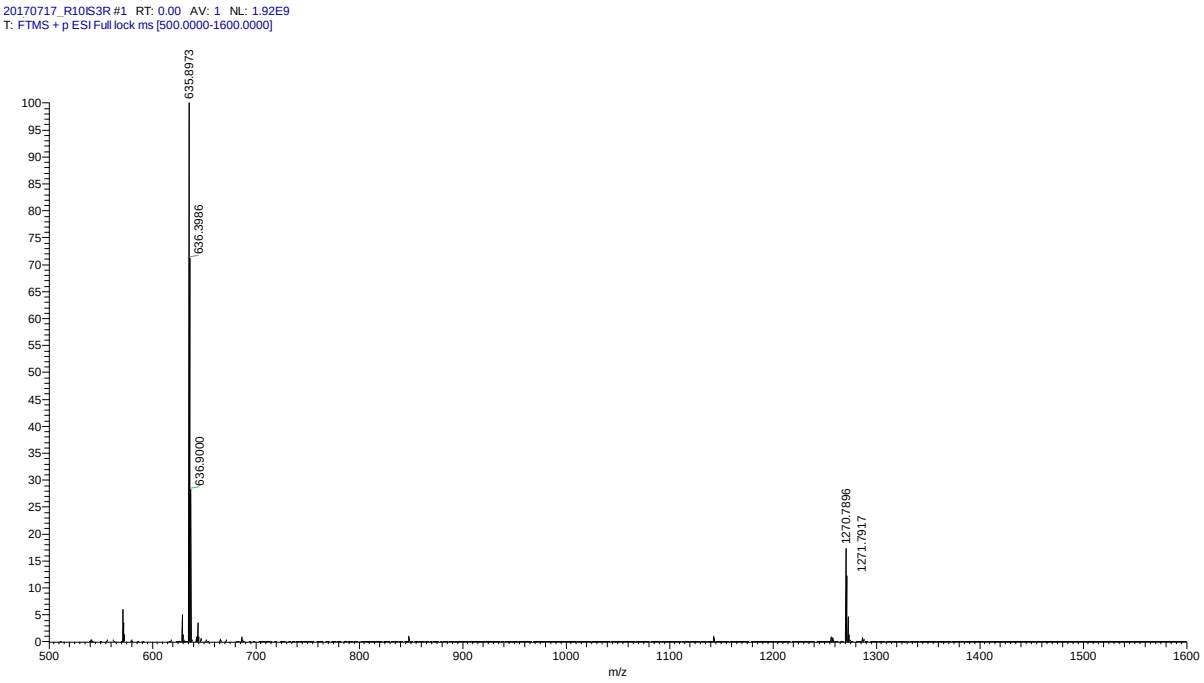


Fig. S16: HRMS of HPLC purified teixobactin analogue **8**. Mass calcd. for $C_{61}H_{104}N_{15}O_{14}$ = 1270.7887, found M + H^+ = 1270.7896, $[M+2H]^{2+}/2$ = 635.8973.

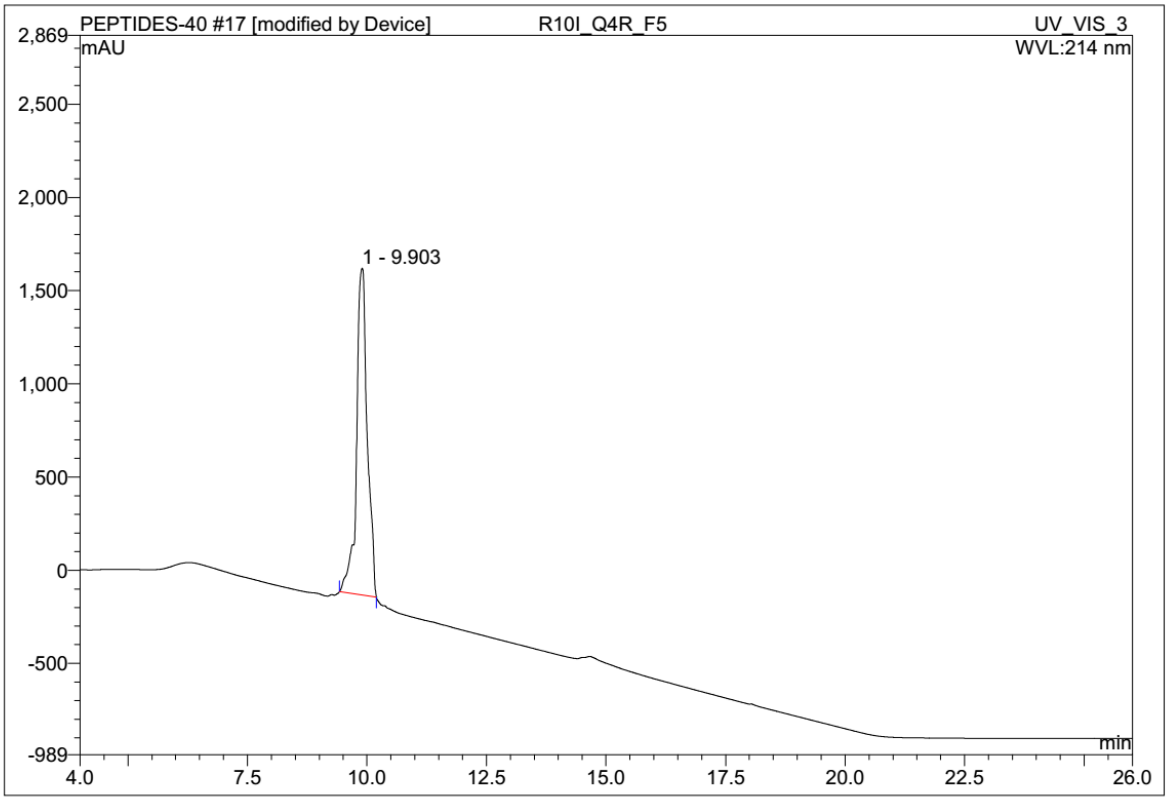


Fig. S17: HPLC trace of HPLC purified teixobactin analogue **9** (gradient: 5-95% ACN in 25 min using A: 0.1% HCOOH in water, B: ACN)

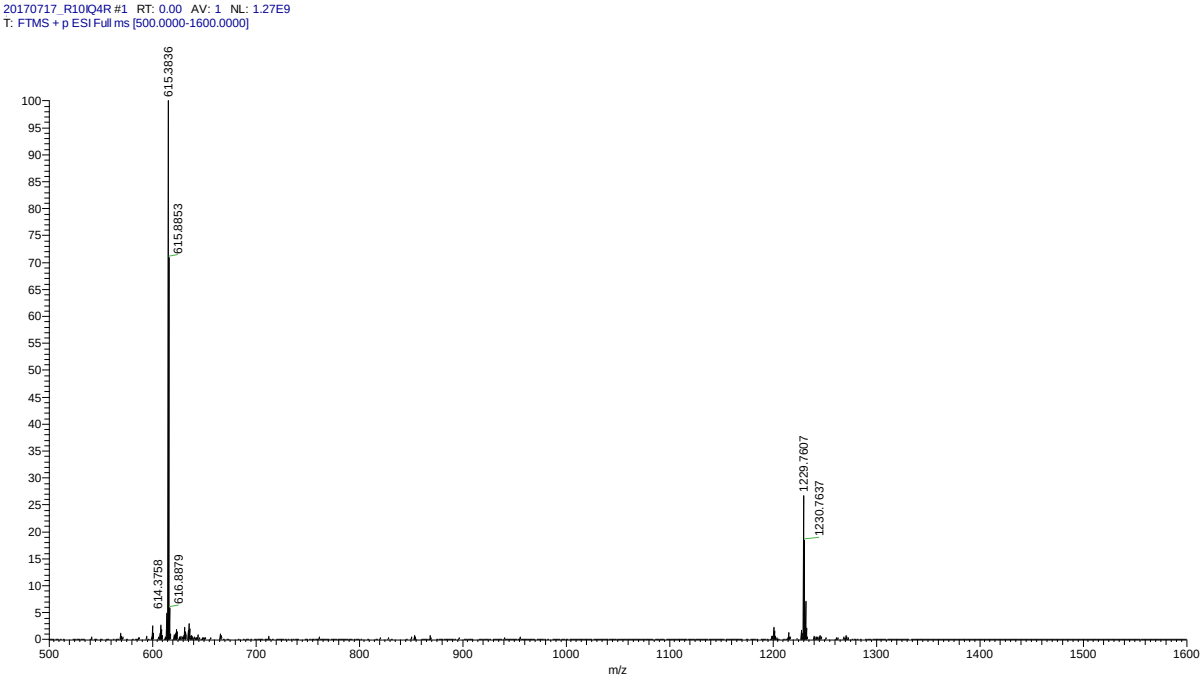


Fig. S18: HRMS of HPLC purified teixobactin analogue **9**. Mass calcd. for $C_{59}H_{101}N_{14}O_{14}$ = 1229.7622, found M + H^+ = 1229.7607, $[M+2H]^{2+}/2$ = 615.3836.

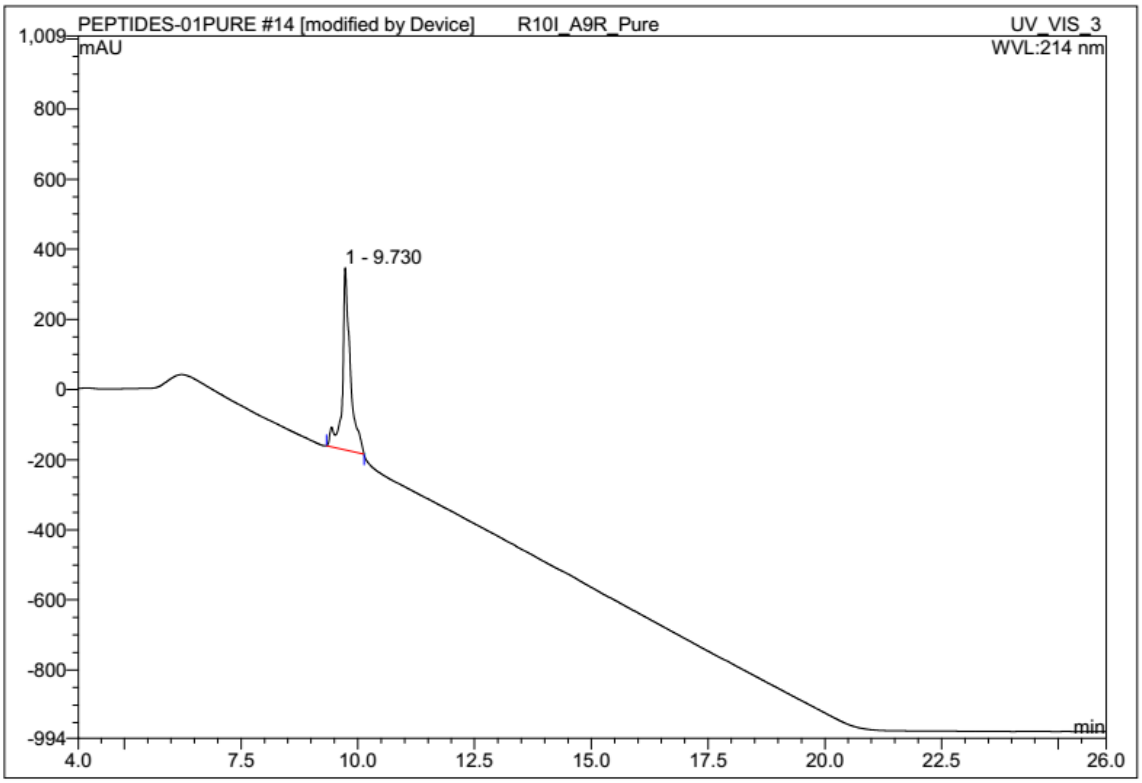


Fig. S19: HPLC trace of HPLC purified teixobactin analogue **10** (gradient: 5-95% ACN in 25 min using A: 0.1% HCOOH in water, B: ACN)

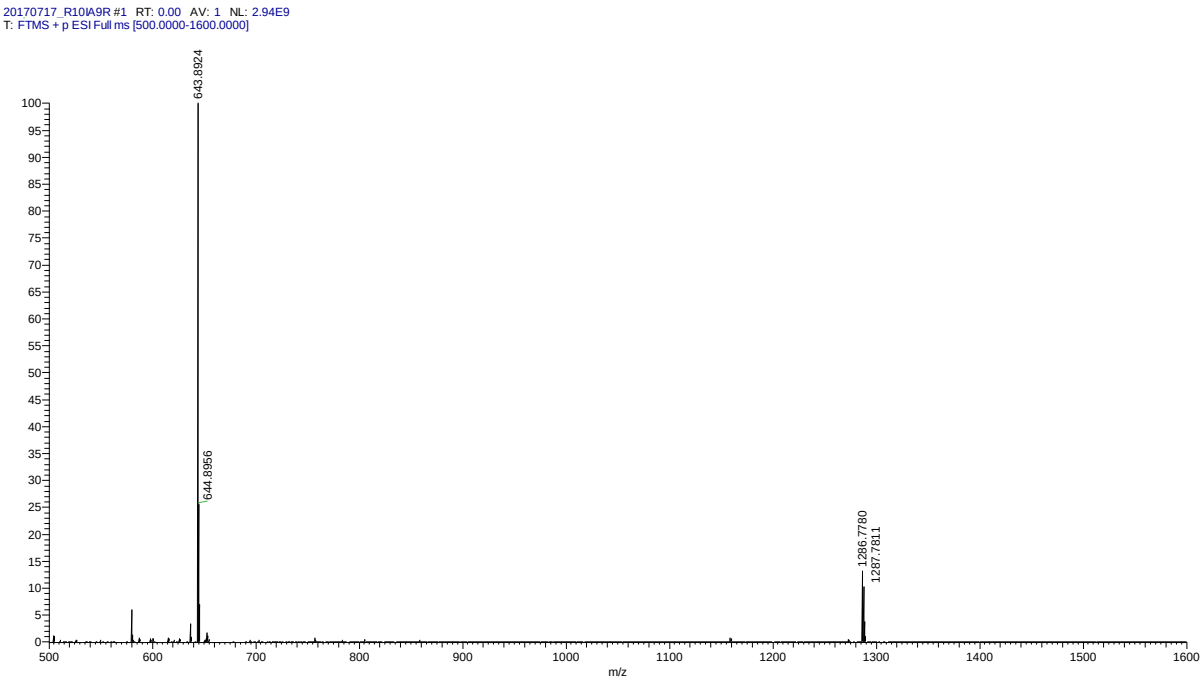
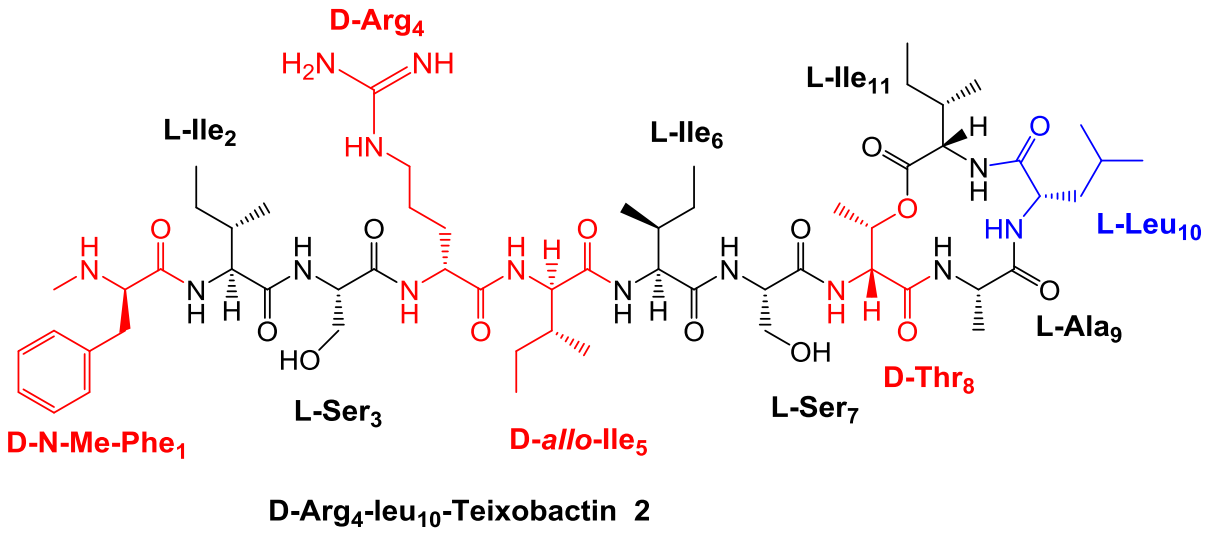


Fig. S20: HRMS of HPLC purified teixobactin analogue **10**. Mass calcd. for $C_{61}H_{104}N_{15}O_{15}$ = 1286.7836, found M + H^+ = 1286.7780, $[M+2H]^{2+}/2$ = 643.8924.

IV: NMR Analysis

All NMR was carried out in DMSO-d_6 at 27°C on a Bruker Avance III HD 500 MHz spectrometer equipped with a room-temperature broadband probe. The following spectra were utilised in the assignment of 1 mM solution of the teixobactin analogue **2**: ^{1}H (128k points, 16 scans); $^{13}C\{1H\}$ (64k points, 1024 scans); ^{1}H- ^{13}C HSQC (2k and 256 points in the direct and indirect dimensions, 4 scans.



D-Arg$_4$-leu$_{10}$-Teixobactin 2

Fig. S21 D-Arg$_4$-Leu$_{10}$-Teixobactin **2**

	H	H_α	C_α	
1 Nm-Phe		3.272	68.12	H_3: 2.170 H_b: 2.795,2.694 H_{d*}: 7.186 H_{e*}: 7.239 H_z: 7.171 C_3: 37.43 C_b: 42.27 C_{d*}: 132.27 C_{e*}: 131.13 C_z: 129.19
2 Ile	7.945	4.193	59.70	H_b: 1.676 H_{g1}: 0.919,1.262 H_{g2*}: 0.746 H_{d1*}: 0.742 C_b: 39.43 C_{g1}: 27.31 C_{g2}: 18.39 C_{d1}: 14.01
3 Ser	7.962	4.323	58.23	H_b: 3.609,3.543 H_g: 4.991 C_b: 64.90
4 Arg	7.923	4.355	32.15	H_b: 1.710,1.494 H_g: 1.476,1.476 H_d: 3.075 H_e: 7.537 C_b: 27.90 C_d: 43.59
5 Ile	7.707	4.391	58.96	H_b: 1.785 H_{g1}: 1.076,1.302 H_{g2*}: 0.781 H_{d1*}: 0.779 C_b: 40.06 C_{g1}: 28.89 C_{g2}: 17.41 C_{d1}: 13.79
6 Ile	7.956	4.264	59.57	H_b: 1.782 H_{g1}: 1.109,1.413 H_{g2*}: 0.831 H_{d1*}: 0.831 C_b: 39.36 C_{g1}: 27.46 C_{g2}: 18.47
7 Ser	9.040	4.376	58.96	H_b: 3.687,3.770 C_b: 64.99
8 Thr	8.546	4.634		H_b: 5.367 H_{g2*}: 1.104 C_b: 73.43 C_{g2}: 18.77
9 Ala	8.151	3.922	54.89	H_{b*}: 1.287 C_b: 20.12
10 Leu	7.908	4.364	55.27	H_b: 1.534,1.534 H_g: 1.535 H_{d*}: 0.881,0.906 C_b: 44.18 C_g: 27.67 C_d: 24.80,25.69
11 Ile	8.111	4.028	60.09	H_b: 1.678 H_{g1}: 1.419,1.098 H_{g2*}: 0.809 H_{d1*}: 0.807 C_b: 39.43 C_{g1}: 27.46 C_{g2}: 18.36

Table S1. NMR assignment of teixobactin analogue **2.**

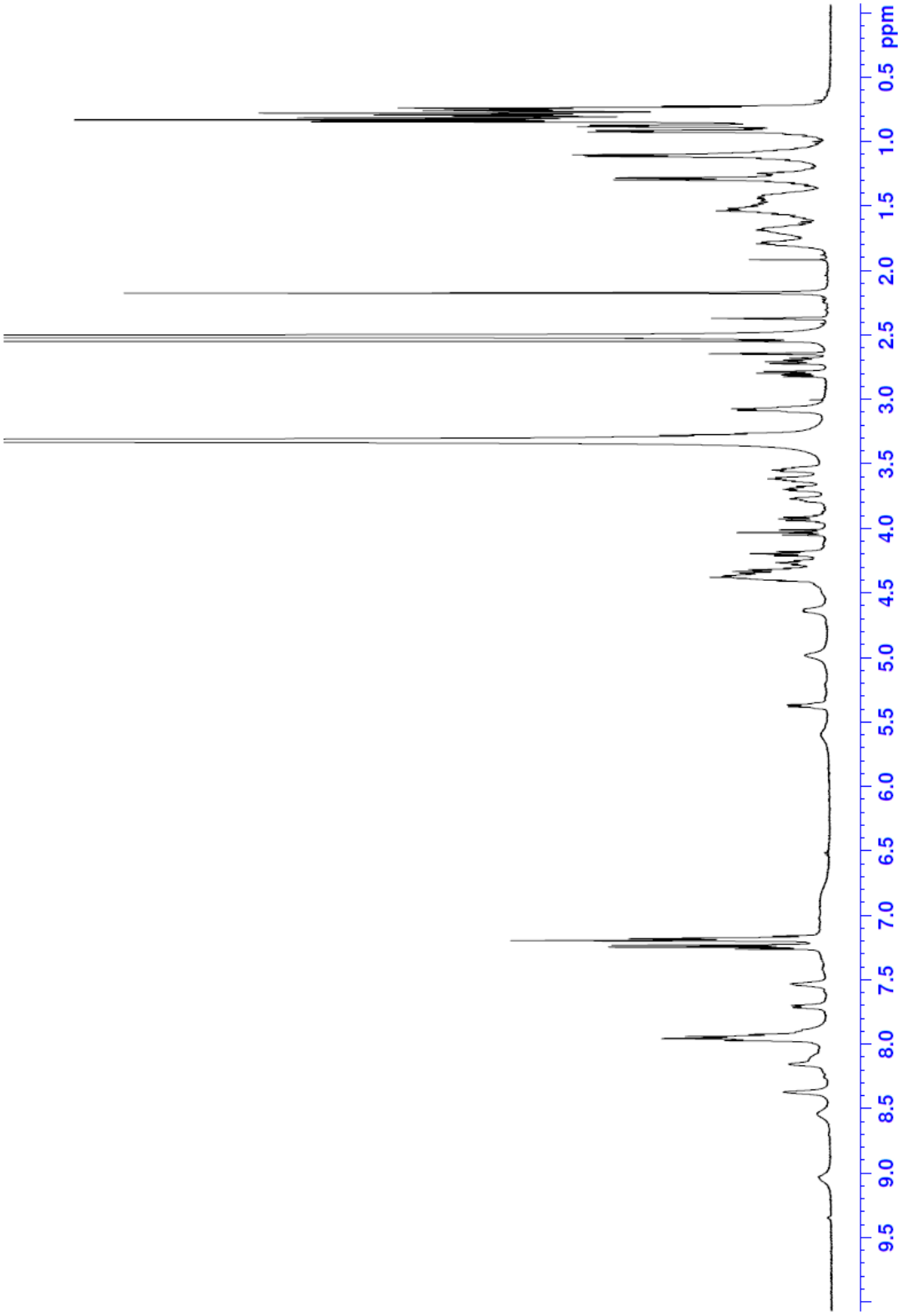


Fig. S22: ^{1}H NMR spectrum of teixobactin analogues **2**

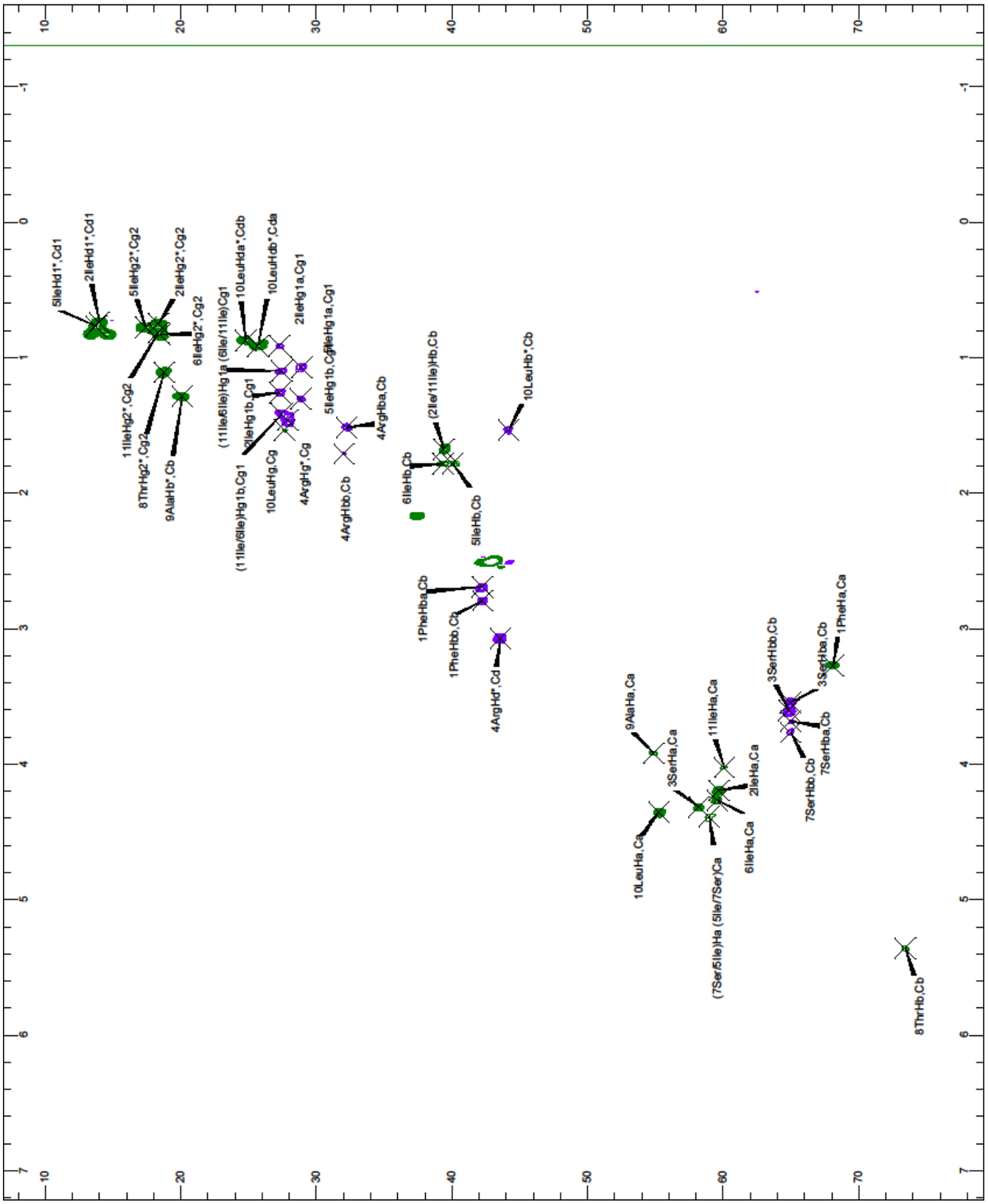


Fig. S23 ^{1}H-^{13}C HSQC spectrum of teixobactin analogues **2**, showing assignment.

V: MIC & MBC testing

For MRSA ATCC 33591: For MIC assays all peptides were dissolved in DMSO containing 0.002% polysorbate 80. MRSA ATCC 33591 was grown in Mueller Hinton broth (Oxoid) in triplicate. All incubations were at 37°C. Dilutions were carried out in triplicate. 100 µl of autoclaved Mueller Hinton broth was added to wells 2-12 in a 96-well plate. 200 µl of the peptide was added to well one at a concentration of 256 µg/mL. 100µl of peptide in well one was taken up and pipetted into well two. The mixture was then mixed via pipetting before 100µl was taken up and pipetted into well three. This process was repeated up to well 11. Once peptide was added to well 11 100 µl was taken up and then discarded ensuring the well 12 had no peptide present. Thus, the concentrations (in µg/mL) were: 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and no peptide present. Each well was then inoculated with 100µl of bacteria that had been diluted to an OD600nm of 0.1. This was repeated three times. The 96-well plates were then incubated at 37°C for 24 hours. The MIC was determined to be the lowest concentration at which there was no growth visible.

For all the compounds in which the MIC lower than 1 µg/ml for the initial test, the above procedure was repeated at an altered initial concentration of 64 µg/ml. Therefore, the new concentrations for MIC were: 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and no peptide present.

(Extended panel)

Bacterial cultures were grown overnight in Mueller-Hinton Agar (MHA) plates and adjusted to a final concentration of 105 – 106 CFU/ml. 100 µl of inoculum in Meuller-Hinton broth (MHB) was mixed with equal volume of peptides (dissolved in MHB) at 2x their concentration in a 96 well plate. In parallel experiments, MIC values were determined in the media containing polysorbate 80 (0.002%, v/v) to prevent non-specific adsorption of the peptides to plastic surfaces. The final peptides concentrations ranged from 0.0625 – 32 µg/ml (for lower range 0.031 – 16 µg/ml was used) . Positive and negative controls contained 200 µl of inoculum without any peptide dissolved in broth, respectively. The 96 well plates were then incubated at 37 °C for 24 h. All the experiments were performed in two independent duplicates and the MIC was determined as the lowest concentration in which no visible growth was observed. Minimum bactericidal concentration (MBC) was determined by plating out the dilution representing the MIC and concentrations up to 16x MIC on MHA plates kept at 37 °C for 24 h. The lowest concentration in which no visible colonies could be detected was taken as the MBC.

Resistance studies: For single step resistance, 100µl *S. aureus* ATCC 29213 or MRSA ATCC 33591 at 10^{10} c.f.u./ml were plated onto MHB containing 5 x , 10 x , 20 x MIC of teixobactin analogues **2**. Agarose was used as a solidifying agent. After 24 h of incubation at 37**°** C, no resistant colonies were detected, giving the calculated frequency of resistance to teixobactin analogues **2** of $<10^{-10}$.

VI: Time-dependent killing of bacteria by teixobactin analogue 2:
Time-kill kinetics against *S. aureus* ATCC 29213 was carried out in MHB. Cultures were grown overnight in MHA plates and adjusted to a final inoculum of 10^5 – 10^6 CFU/ml in MHB (containing 0.002% v/v, polysorbate 80) with teixobactin analogue **2** maintained at a final concentration of 0.5 and 1 µg/ml. The tubes were then incubated at 37 °C. 100 µl of cell suspension was withdrawn at various time points (0, 2, 4, 8h), serially diluted (10^1-10^5 fold dilutions) and plated onto a MHA plates and incubated for 24 h at 37 °C. Colonies were then enumerated using a haemocytometer. Colony counting too numerous to count (>300 colonies) was taken as 10^{10} CFU. Average values from two independent experiments are reported.

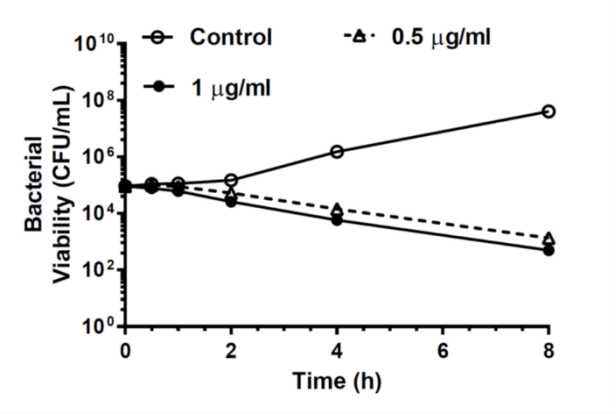


Fig. S24: Time-kill kinetics of teixobactin analogue **2** against *S. aureus* ATCC 29213. The concentration of teixobactin analogue **2** was maintained at 0.5 and 1 μg/ml.

	Compound ➔ No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Daptomycin**
	Strain ⬇											
1.	*Staphylococcus saprophyticus* ATCC BAA 750	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	0.125
2.	*Staphylococcus saprophyticus* ATCC 15305	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	0.25	<0.0625	<0.0625	<0.0625	0.125
3.	*Staphylococcus saprophyticus* ATCC 49453	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	0.125
4.	*Staphylococcus saprophyticus* ATCC 49907	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	0.125
5.	VRE 1001	0.25	0.5	0.5	1	0.5	1	2	1	0.5	1	2
6.	VRE 1002	0.5	1	1	1	1	1	8	1	1	1	4
7.	VRE 1004	<0.0625	0.25	0.25	0.5	0.5	1	4	1	0.5	1	1
8.	VRE 1008	0.125	0.5	0.25	0.5	0.5	1	8	1	0.5	1	4
9.	VRE ATCC 700802	0.5	0.5	0.5	2	1	1	4	1	0.25	1	0.25
10.	VRE ATCC 29212	0.5	0.5	1	1	1	1	4	1	0.25	1	0.25
11.	MRSA ATCC 700699	0.5	0.25	0.5	0.5	1	1	2	1	0.25	1	1
12.	MRSA 42412	<0.0625	0.0313	<0.0625	0.25	0.25	1	2	0.125	<0.0625	0.125	0.5
13.	MRSA 21455	0.03125	0.0313	0.25	0.5	1	1	2	0.25	0.03125	0.5	0.5
14.	MRSA 1003	<0.0625	0.5	0.25	1	2	0.5	2	0.125	<0.0625	0.5	0.5
15.	SA29213	0.25	<0.0625	0.5	0.25	1	1	1	0.5	0.0625	1	0.5
16.	SA4299	0.125	-	0.25	0.25	0.5	0.5	1	0.125	<0.0625	1	0.5
17.	SE12228	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	0.125
18.	*Bacillus Cereus* ATCC 11788	<0.0625	0.5	0.25	1	1	1	1	0.125	<0.0625	0.5	0.25
19.	*Bacillus Subtilis* ATCC 6633	<0.0625	0.125	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	<0.0625	0.125	0.125
20.	*P aeruginosa* ATCC 27853	-	>64	-	-	-	-	-	-	-	-	-

Table S2: MIC (in µg/mL) of the teixobactin analogues **1-10** and Daptomycin control against an extended panel of Gram positive bacteria in the presence of polysorbate 80.

Peptides	Minimum Bactericidal Concentration (in μg/ml) against			
	***S. aureus* 29213**	***S. aureus* 4299**	**MRSA 700699**	**MRSA 21455**
1	>2 (8×)	1 (>8×)	2 (4×)	0.0625 (2×)
2	0.125 (1×)	≤0.0625 (1×)	2 (4×)	≤0.0625 (1×)
3	>4 (8×)	2 (8)	>4 (>8×)	>2 (>8×)
4	>2 (>8×)	>2 (>8×)	>4 (>8×)	1 (2×)
5	2 (2×)	4 (8×)	2 (2×)	1 (2×)
6	4 (4×)	2 (4×)	8 (8×)	2 (2×)
7	2 (2×)	1 (1×)	>8 (>4×)	2 (1×)
8	4 (8×)	1 (8×)	2 (2×)	0.5 (2×)
9	>0.5 (>8×)	>0.5 (>8×)	0.5 (2×)	0.25 (8×)
10	8 (8×)	8 (8×)	1 (1×)	2 (4×)

Table S3: Minimum bactericidal concentrations of teixobactin peptides against *S. aureus* and MRSA strains MBC (in μg/mL) of the teixobactin analogues **1-10**

VII: Cytocompatibility of 2 for mammalian cells

Cytocompatibility assessment of **2** for A549 lung adenocarcinoma cell line and primary human dermal fibroblasts (hDFs) were determined by MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)) assay and high content analysis (HCA). Both A549 and hDF cells (2 X 10^3 cells/well) seeded on 96-well plates were treated with various concentrations of peptide (15.625 – 250 µg/ml) and incubated for 24 h at 37 °C and 5% CO_2. The stock solution of 2 (500 µg/ml) was prepared fresh by directly dissolving **2** in cell culture medium (Dulbecco's Modified Eagle Medium, Gibco®) and used. The metabolic activity was determined using CellTier 96® Aqueous One solution cell proliferation assay kit according to the manufacturer's instruction (Promega Corporation, Madison, WI). The relative cell viability was determined from UV readings of untreated control cells. The antineoplastic agent, nocodazole (5 µg/ml dissolved in DMSO) served as the negative control. Data represents mean ± standard error of the mean of three independent triplicate experiments. For HCA, cells treated with peptide **2** were washed with PBS and fixed in 3% paraformaldehyde. A549/hDF cells were fluorescently stained with Alex Fluor 488 anti-α-tubulin (green), Hoechst 33342 (blue) and Rhodamine-Phalloidin (red) to visualize cellular morphologies and imaged by IN Cell Analyzer 2200 automated microscope.

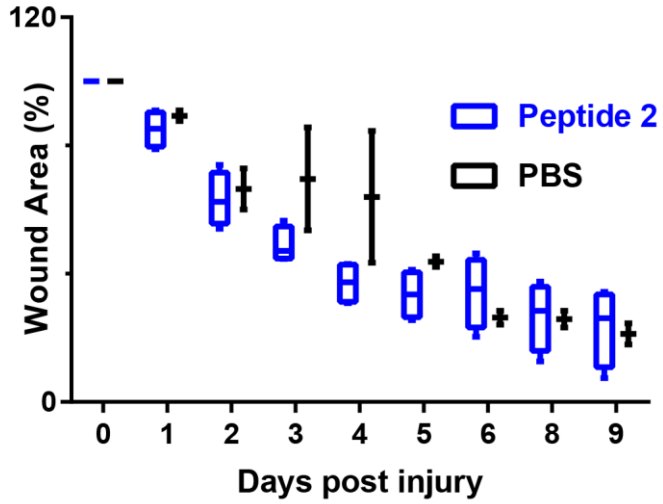


Fig. S25: Quantitative determination of corneal wound healing after topical instillation of PBS or peptide **2** after corneal injury in rabbits. The re-establishment of corneal epithelium after injury confirm that peptide **2** does not interfere with regular wound healing process, thus establishing its safety for topical applications.

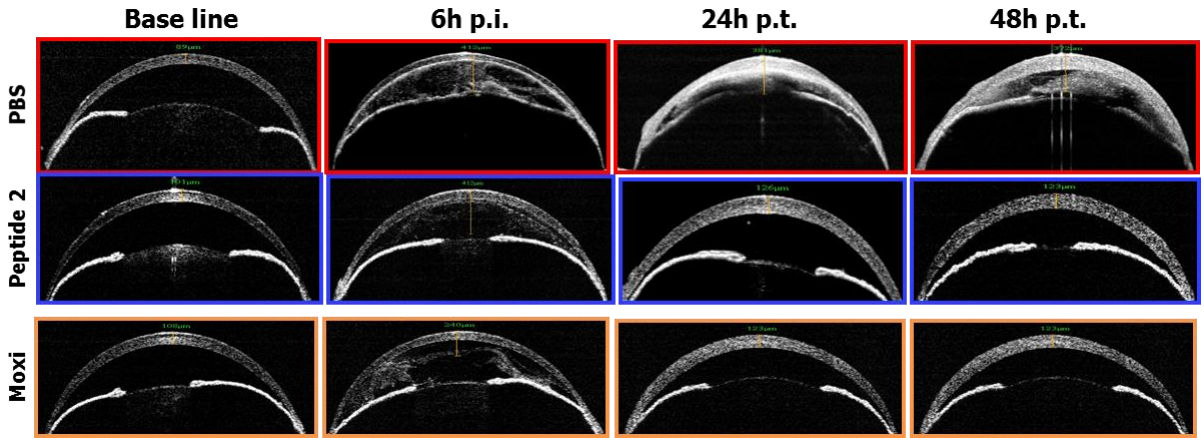


***Fig*. S26**: Representative AS-OCT images showing the changes in corneal thickness before and after infections or Treatment with various groups. Note the significant presence of corneal edema and hyper reflective materials throughout the cornea in the case of PBS treated groups but were minimized in peptide **2** or moxifloxacin treated groups.

VIII: The *in vivo* toxicity in a rabbit model of corneal epithelium-injured

All the animals used in this study were treated in accordance to the tenets of the Association for Research in Vision and Ophthalmology (ARVO) statement, and the protocol was approved by SingHealth Institutional Animal Care and Use Committee (IACUC) (AALAC accredited; protocol number 2012/SHS/775 for wound healing). Six New Zealand White rabbits, aged 8 months old and body weight 3-3.5 kg were used for the study. Prior to the creation of corneal wound, all the rabbit eyes were examined by slit-lamp photography to ensure absence of any ocular defects. The rabbits were anesthetized and a 7.5-mm-diameter region of the corneal surface was de-epithelialized with a sterile mini blade (BD Beaver, MA, USA) and divided into two groups. Rabbits received a 50 µl topical instillation of peptide **2** (0.3% w/v in PBS) (4 eyes) or PBS (2 eyes) 4 times/day for ten days. The corneal epithelial wound healing was visualized using 2% w/v fluorescein sodium (Bausch & Lomb) staining. The progression of wound healing was examined by illumination with cobalt blue light with a digital camera. The area of corneal abrassion was quantified using Image J software.

IX*: In vivo* efficacy of peptide in a mice model of infectious keratitis

We have used eighteen pathogen free 6-8 weeks old Female mice (wild type C57BL/6). As per the Sing-Health Institutional Animal Care and Use Committee (IACUC) guidelines, all the animals were handled, and for the animal experimentation , the guidelines of Association for Research in Vision and Ophthalmology (ARVO) were followed. The designated groups, with six mice each were categorized as *group I* treated with PBS, *group II* treated with 0.3% of moxifloxacin Hydrochloride, *group III* treated with 0.3% of peptide **2**. *Staphylococcus aureus* ATCC 29213 strains were grown overnight in Tryptic Soy Agar (TSA) plates at 35°C. Isolated single bacterial colonies were identified and suspended in sterile PBS at a final inoculum concentration of 3 x 10^6 CFU/mL. Slit-lamp biomicroscopy (FS-3V Zoom Photo Slit Lamp, Nikon, Tokyo, Japan) and AS-OCT (RTvue, Optovue, Fremont, CA) were carried out on the days before bacterial inoculation (Baseline), and 6 h post infection (p.i.), 24 h and 48 post treatment (p.t.).

Prior to infection all the mice eyes were examined by slit-lamp photography and AS-OCT to make sure that there was no corneal aberration, such as vascularization or any other ocular defects. Mice were anesthetized by an intraperitoneal injection of xylazine (10 mg/kg, Troy Laboratories, Smithfield, Australia) and ketamine (80 mg/kg, Ketamine, Parnell Laboratories, Australia) under the dissecting microscope (Zeiss, Stemi-2000C). The mice corneal epithelium were then scratched and removed using a sterile Beaver 6400 Mini-Blade to create a superficial wound without damaging the stroma and one drop of 1-5% lignocaine hydrochloride were used as topical anesthesia instilled before corneal wounding and then the cornea was irrigated with sterile saline to wash away any debris and residual topical anesthetic agent. Immediately following this procedure, 15µL of bacterial suspension containing 3 x 10^6 CFU/mL of *Staphylococcus aureus* ATCC 29213 was applied topically on the corneal surface. After 6 h post infection, mice were treated with peptide **2**, Moxifloxacin and PBS topically (15µL).

The dosage regimen are two times on Day 1 (2:30PM; 5:30PM), four times (8AM; 11AM; 2PM; 5PM) on day 2 and two times on day 3. The eyes were examined daily by slit lamp and OCT, sacrificed at 48 hr post-treatment (day 3) for evaluation of bacteria quantification analysis.
After treatment with various groups, the mice corneas were dissected and homogenized in sterile PBS by using Pellet pestles cordless motor (Z359971, Sigma) with sterile plastic pestles followed by fine homogenization with bead beating using sterile glass beads (2 mm). The homogenates were vortexed and 10-fold serial dilutions were prepared using sterile PBS to give 10^2 to 10^4 dilutions. A 0.1 mL of each suspension was inoculated onto Tryptic Soy Agar (TSA) plates in duplicate and incubated at 35°C for 48 h. The numbers of colonies were enumerated and the results were expressed as the $\log_{10}$ number of CFU/cornea.