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Supporting Information for

Alanine Scan Reveals Modifiable Residues in Teixobactin

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Materials and Methods

General information

All natural and unnatural amino acids were purchased from LC Sciences, Chem-Impex, and Santa Cruz Biotechnology. 2-Chlorotrityl chloride resin was purchased from Chem-Impex. Trifluoroacetic acid (TFA), and HPLC grade acetonitrile (ACN) were purchased from Fischer Scientific. Water was purified with Barnstead NANOpure Diamond lab water purification system. All other solvents and chemicals were purchased from Alfa Aesar and Sigma Aldrich. All amino acids, resins, solvents, and chemicals were used as received, with the exception that dichloromethane (DCM) and *N*,*N*-dimethylformamide (DMF) was dried by passage through dry alumina under argon.[1] Preparative reverse-phase HPLC was performed on a Beckman Gold Series P instrument equipped with an Agilent Zorbax SB-C18 column. Analytical reverse-phase HPLC was performed on either an Agilent 1200 or an Agilent 1260 Infinity II instrument, both equipped with a Phenomenex Aeris PEPTIDE 2.6μ XB-C18 column. HPLC grade acetonitrile and purified water containing 0.1% trifluoroacetic acid were used as solvents for both preparative and analytical reverse-phase HPLC.

Synthesis of Lys_{10}-teixobactin alanine scan analogues

Lys_{10}-teixobactin and the alanine scan analogues were synthesized as the trifluoroacetic acid (TFA) salts following procedures we have previously reported.[2] Dry DMF was used instead of a mixture of ACN/THF/DCM for the cyclization step.

Table S1. Chemical yield of purified Lys_{10}-teixobactin alanine scan analogues

teixobactin analogues	yield (mg)	% yield	Calcd. MW as TFA salt
Lys_{10}-teixobactin	12.5 mg	8.7 %	1444.77 (·2 TFA)
N-Me-D-Ala_1,Lys_{10}-teixobactin	4.7 mg	3.4 %	1368.74 (·2 TFA)
Ala_2,Lys_{10}-teixobactin	7.2 mg	5.1 %	1402.72 (·2 TFA)
Ala_3,Lys_{10}-teixobactin	9.7 mg	6.8 %	1428.78 (·2 TFA)
D-Ala_4,Lys_{10}-teixobactin	4.1 mg	3.0 %	1387.75 (·2 TFA)
D-Ala_5,Lys_{10}-teixobactin	5.9 mg	4.2 %	1402.72 (·2 TFA)
Ala_6,Lys_{10}-teixobactin	15.1 mg	10.7 %	1428.78 (·2 TFA)
Ala_7,Lys_{10}-teixobactin	13.2 mg	9.4 %	1402.72 (·2 TFA)
Ala_{10}-teixobactin	5.6 mg	4.0 %	1387.71 (·1 TFA)
Lys_{10},Ala_{11}-teixobactin	10.5 mg	7.5 %	1402.72 (·2 TFA)

Sample synthesis of Lys10-teixobactin

Resin loading. 2-Chlorotrityl chloride resin (300 mg, 1.12 mmol/g) was added to a 10-mL Bio-Rad Poly-Prep chromatography column (8 mm x 40 mm). The resin was suspended in dry DCM (10 mL) and allowed to swell undisturbed for 30 min. The solution was drained from the resin using nitrogen and a solution of Fmoc-Lys(Boc)-OH (84 mg, 0.18 mmol, 0.50 equiv) in 20% (v/v) 2,4,6-collidine in dry DCM (5 mL) was added immediately. The suspension was gently agitated for 12 h. The solution was then drained using nitrogen and washed with dry DCM (2x). After washing, a mixture of DCM/MeOH/DIPEA (8.5:1:0.5, 10 mL) was added immediately. The suspension was gently agitated for 1 h to cap any unreacted sites on the resin. The resin was washed with dry DMF (2x) and dried by passing nitrogen through the chromatography column. The resin loading was determined to be 0.10 mmol (0.32 mmol/g, 53% loading) based on UV analysis (290 nm) of the Fmoc cleavage product.

Solid-phase amino acid couplings. The Fmoc-Lys(Boc)-OH loaded resin was transferred to a solid-phase peptide synthesizer reaction vessel designed for an automated peptide synthesizer (Protein Technologies). The resin was subjected to cycles of automated amino acid couplings using Fmoc-protected amino acid building blocks. The linear peptide was synthesized from the *C*-terminus to the *N*-terminus. Each coupling consisted of: (1) Fmoc deprotection with 20% (v/v) piperidine in dry DMF for 5 min; (2) resin washing with dry DMF (3x); (3) activation of the Fmoc-protected amino acid (0.40 mmol, 4 equiv) with 20% (v/v) 2,4,6-collidine in dry DMF (5 mL) in the presence of HCTU (0.40 mmol, 4 equiv); (4) coupling of the activated Fmoc-protected amino acid; (5) resin washing with dry DMF (3x). After completing the linear synthesis, the resin was transferred from the peptide synthesizer reaction vessel to a new 10-mL Bio-Rad Poly-Prep chromatography column. The resin was washed with dry DCM (3x), and dried by passing nitrogen through the chromatography column.

Esterification. In a test tube, Fmoc-Ile-OH (353 mg, 1.0 mmol, 10 equiv) and diisopropylcarbodiimide (155 µL, 1.0 mmol, 10 equiv) were dissolved in dry DCM (5 mL). The resulting solution was filtered through 0.20-µm nylon filter, and then 4-dimethylaminopyridine (12 mg, 0.1 mmol, 1 equiv) was added to the filtrate. The resulting solution was transferred to the resin and was gently agitated for 4 h. The solution was drained and the resin was washed with dry DCM (3x) and DMF (3x).

*Fmoc deprotection of Ile_{11}.*The Fmoc protecting group on Ile_{11} was removed by adding 20% (v/v) piperidine in DMF for 30 min. The solution was drained, and the resin was washed with dry DMF (3x) and DCM (3x).

Cleavage of the linear peptide from the resin. The linear peptide was cleaved from the resin by subjecting the resin to a cleavage solution of 20% (v/v) HFIP in dry DCM (5 mL). The resin and the suspension immediately turned red. The suspension was gently agitated for 1 h. The suspension was filtered, and the filtrate was collected in a 250-mL round-bottom flask. The resin was washed with additional cleavage solution (5 mL) and then with dry DCM (2x 5 mL) until the resin was no longer red. The combined filtrates were concentrated under reduced pressure to afford a colorless oil. The crude product was dried using a vacuum pump to remove any residual solvents.

Solution-phase cyclization. The crude oil was dissolved in dry DMF (125 mL) in the same 250-mL round-bottom flask as the previous step. HOBt (67 mg, 0.5 mmol, 5 equiv) and HBTU (189 mg, 0.5 mmol, 5 equiv) were added to the solution. The reaction mixture was then stirred under nitrogen at room temperature for 10 min. DIPEA (0.3 mL, 1.5 mmol, 15 equiv) was added to the solution and the mixture was stirred under nitrogen at room temperature for 12 h. The reaction mixture was concentrated under reduced pressure to give a brown oil. The crude product was dried using a vacuum pump to remove any residual solvents.

G*lobal deprotection.* The crude protected peptide was dissolved in a mixture of TFA/TIPS/H_2O (9.5:0.5:0.5, 10 mL) in the same 250-mL round-bottom flask as the previous step. The reaction mixture was then stirred under nitrogen at room temperature for 1 h. The reaction mixture was concentrated under reduced pressure to give a brown oil. The crude product was dried using a vacuum pump to remove any residual solvents.

Purification of Lys_{10}-teixobactin with preparative reverse-phase HPLC. The crude peptide was dissolved in 40% (v/v) ACN/H_2O (10 mL), and the solution was centrifuged at

14,000 rpm for 5 min to pellet any insoluble material. [The presence of a small amount of insoluble pellet is normal.] The supernatant was then filtered through a 0.2 μm syringe filter. The crude macrocyclic β-sheet **1** was purified by reverse-phase HPLC. The purification was performed on a Agilent Zorbax SB-C18 PrepHT column (21.2 mm x 250 mm, 7-μm particle size) on a Beckman HPLC with a flow of 15.0 mL/min. The UV detector was set to 214 nm. The gradient started with 20% to 40% ACN/H_2O for 100 min. The pure fractions were lyophilized to afford Lys_{10}-teixobactin as a white powder (12.5 mg, 8.7%).

MIC assays of Lys_{10}-teixobactin alanine scan analogues and teixobactin[3]

MIC assays of Lys_{10}-teixobactin alanine scan analogues and vancomycin were determined by using a broth microdilution method according to CLSI.[3] *Escherichia coli* (ATCC 10798), *Enterococcus durans* (ATCC 6056), *Streptococcus salivarius* (ATCC 13419), *Staphylococcus epidermidis* (ATCC 14990), *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 29213) were acquired as freeze-dried powders from ATCC.

Preparation of bacterial plate stocks. A portion of freeze-dried bacteria powder was removed with a sterile loop and suspended in 5 mL of Mueller-Hinton broth in a 14 mL polypropylene round-bottom culture tube. The mixture was incubated at 37 °C while shaking overnight. The mixture was streaked on Mueller-Hinton agar plates, and the plates were incubated at 37 °C overnight to allow colonies to grow. The plates were Parafilm wrapped and stored for subsequent experiment.

Determination of bacterial concentration (CFU/mL). Five colonies from the bacterial plate stocks were transferred to a single 14 mL polypropylene round-bottom tube that contained

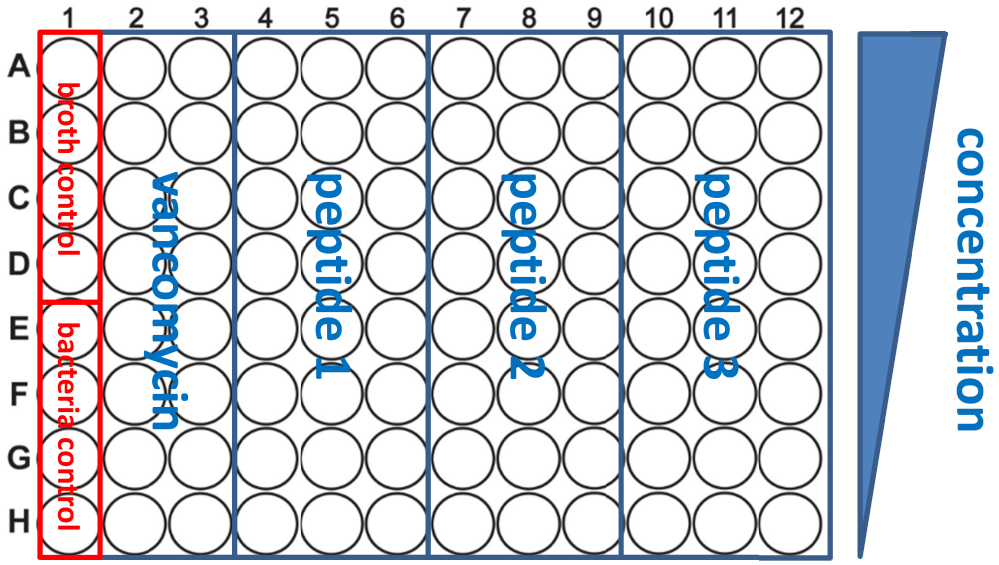
Mueller-Hinton broth (2 mL) and the mixture was incubated at 37 °C while shaking. As the turbidity of the cell suspension mixture visually increased, a 200 µL aliquot was transferred to a 96-well plate for OD_{600} measurement. The cell suspension mixture was diluted with Mueller-Hinton broth to an OD_{600} of 0.075 as measured in a 96-well plate (equivalent to a 0.5 McFarland standard). A 10 µL aliquot of the diluted cell suspension was diluted 1:1000 with Mueller-Hinton broth. A 10 µL aliquot of the 1:1000 diluted cell suspension mixture was further diluted 1:200 with Mueller-Hinton broth. A 100 µL aliquot of the resulting mixture was then streaked on a Mueller-Hinton agar plate (repeated four times). The agar plates were incubated at 37 °C overnight. The number of colonies on each agar plate were counted, and the average of four plates was used to back calculate the bacterial concentration (CFU/mL) at an OD_{600} of 0.075 as measured in a 96-well plate (equivalent to a 0.5 McFarland standard).

Bacteria	Average number of colonies	Concentration at a 0.5 McFarland standard
Staphylococcus epidermidis ATCC 14990	5	1×10^7 CFU/mL
Streptococcus salivarius ATCC 13419	25	5×10^7 CFU/mL
Enterococcus durans ATCC 6056	32	6.4×10^7 CFU/mL
Bacillus subtilis ATCC 6051	25	5×10^7 CFU/mL
Escherichia coli ATCC 10798	24	4.8×10^7 CFU/mL
Staphylococcus aureus ATCC 29213	213.5	4.3×10^8 CFU/mL

Preparation of peptide stocks. Solutions of Lys_{10}-teixobactin, the Lys_{10}-teixobactin alanine scan analogues and teixobactin were prepared gravimetrically by dissolving an appropriate amount of peptide in an appropriate volume of sterile DMSO to make 10 mg/mL

stock solutions. The concentration of the vancomycin stock solution was 20 mg/mL. The stock solutions were stored at -20 °C for subsequent experiments.

Preparation of the minimum inhibitory concentration (MIC) assays. An aliquot of the 10 or 20 mg/mL peptide stock solutions diluted to 64 µg/mL with Mueller-Hinton broth. A 200 µL aliquot of the 64 µg/mL solution was transferred to a 96-well plate. Two-fold serial dilutions were made with Mueller-Hinton broth vertically down a 96-well plate to achieve a final volume of 100 µL in each well. 100 µL serial diluted solutions had the following concentrations: 64, 32, 16, 8, 4, 2, 1, and 0.5 µg/mL. [This vertical 96-well plate setup allows for testing one additional peptide, at the expense of testing the lower range of peptide concentrations (0.125, 0.0625, and 0.03125 µg/mL) achieved by a horizontal setup].



Performing the minimum inhibitory concentration (MIC) assays. Five colonies from the bacterial plate stocks were selected and transferred to a single 14 mL polypropylene round-bottom tube that contained Mueller-Hinton broth (2 mL) and the mixture was incubated at 37 °C while shaking. As the turbidity of the cell suspension mixture visually increased, the mixture was

diluted with Mueller-Hinton broth to OD_{600} of 0.075 as measured in a 96-well plate (equivalent to a 0.5 McFarland standard). Based on the previously determined CFU/mL, the diluted mixture was further diluted to 1 x 10^6 CFU/mL with Mueller-Hinton broth. A 100 μL aliquot of the 1 x 10^6 CFU/mL bacterial solution was added to each well in 96-well plates, resulting final bacteria concentration of 5 x 10^5 CFU/mL in each well. As 100 μL of bacteria were added to each well, peptide homologue solution was also diluted to the following concentrations: 32, 16, 8, 4, 2, 1, 0.5, and 0.25 μg/mL. The plate was covered with a lid and incubated at 37 °C for 16 h. The OD_{600} was measured using a 96-well UV/Vis plate reader (MultiSkan GO, Thermo Scientific). The MIC values were taken as the lowest concentration that had no bacteria growth. Each MIC assay was run in triplicate in three independent runs to ensure reproducibility.

Solubility assay of Lys_{10}-teixobactin alanine scan analogues and teixobactin in PBS buffer

A 20 μL drop of PBS buffer at pH 7.4 containing a small amount of crystal violet was placed onto a glass depression well microscope slide. [The addition of crystal violet into the PBS buffer provides a good color contrast that aids in the identification of insoluble, gelatinous mass.] A 1 μL aliquot of the peptide stock solution in DMSO was placed directly into the center of the well. A low magnification stereoscopic microscope was used to visually observe the drop. Alanine scan analogues with poor solubility formed a gelatinous mass upon addition of the DMSO peptide stock solution into the PBS buffer. Alanine scan analogues with good solubility readily dissolved into the PBS buffer, forming a homogenous solution upon stirring with a pipet tip.

LDH release assay of Lys_{10}-teixobactin alanine scan analogues and teixobactin[4]

The cytotoxicity of Lys_{10}-teixobactin alanine scan analogues and teixobactin toward HepG2 cells was assessed by an LDH release assay. The LDH release assay was performed using the Pierce LDH Cytotoxicity Assay Kit from Thermo Scientific. The HepG2 cells were incubated with 100, 50, and 25 μg/mL of each Lys_{10}-teixobactin alanine analogue and teixobactin. Experiments were performed in quadruplicate (replicate) in flat-bottom 96-well plate. Triton X-100, included in the kit, was used as a positive control; vehicle (1% DMSO) was used as a negative control. Melittin (5 μg/mL) was used as an additional peptidic positive control. The plates were covered and incubated for 24 h. The cells were cultured in the inner 60 wells (rows B–G, columns 2–11) of the 96-well plate. Low-glucose DMEM media (100 μL) was added to the outer wells (rows A and H and columns 1 and 12), to ensure reproducibility of data generated from the inner wells.

Preparation of peptide stocks. The following procedure was conducted inside a certified biosafety cabinet on the day of the cell treatment. 10 mg/mL peptide stock solutions in sterilized DMSO were 2-fold serial diluted with sterilized DMSO. [Typically, 4.5 μL of 10 mg/mL peptide stock was needed to make 450 μL of compound-containing media.] The appropriate amount of serum-free low-glucose DMEM media (without fetal bovine serum, penicillin, and streptomycin) was added to make a 450 μL of peptide stock solutions of 100, 50, and 25 μg/mL. [450 μL is enough volume to conduct a quadruplicate experiment with 50 μL leftover.]. The Eppendorf tubes were then removed from the biosafety cabinet and were vortexed to mix the peptide and the media. The tubes were then centrifuged. The vortexing and centrifugation process was repeated once more to ensure a homogenous peptide stock in DMEM was prepared that is also

free of precipitate. The Eppendorf tubes were transferred back into the biosafety cabinet while observing the proper sterile techniques.

Preparation of HepG2 cells for LDH release assay. HepG2 cells were cultured in 37 ˚C incubator with 5% CO_2. 20,000 cells were plated into each of the 60 inner wells. Cells were incubated in 100 µL of low-glucose DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 ˚C in a 5% CO_2 atmosphere. The cells were allowed to adhere to the bottom of the plate for 24 h.

Treatment of HepG2 cells with peptide stocks. After 24 h, the culture media was removed four wells at a time with a multichannel micropipet fitted with four tips. The peptide samples were mixed multiple times with a micropipet to ensure homogenous solutions were being used. The empty wells were replaced with 100 µL of serum-free, low-glucose DMEM media containing the peptides. Experiments were performed in quadruplicate (replicate). Four wells were used for a negative control and received 100 µL of serum-free low-glucose DMEM containing 1% DMSO. Four wells were used for a positive control with lysis buffer (Triton X-100) provided in the LDH release assay kit. Four wells were used for a peptidic positive control consisting 5 µg/mL of melittin, the active cytotoxic compound in bee venom. The cells were incubated at 37 ˚C in a 5% CO_2 atmosphere for 24 h.

LDH release assay readout. After 24 h incubation, a 50-µL aliquot of the supernatant media from each well was transferred to a new flat-bottom 96-well plate and 50-µL aliquot of LDH substrate solution, prepared according to manufacturer's protocol, was added to each well. The LDH treated 96-well plate was stored in the dark for 30 min. The absorbance of each well was measured at 490 and 680 (A_{490} and A_{680}). Data were processed by calculating the differential

absorbance for each well (A_{490}-A_{680}) and comparing those value to those of the lysis buffer controls and the untreated controls:

$$\% \text{ cell death} = [(A_{490}\text{-}A_{680})_{compound} - (A_{490}\text{-}A_{680})_{vehicle}]/[(A_{490}\text{-}A_{680})_{lysis} - (A_{490}\text{-}A_{680})_{vehicle}]$$

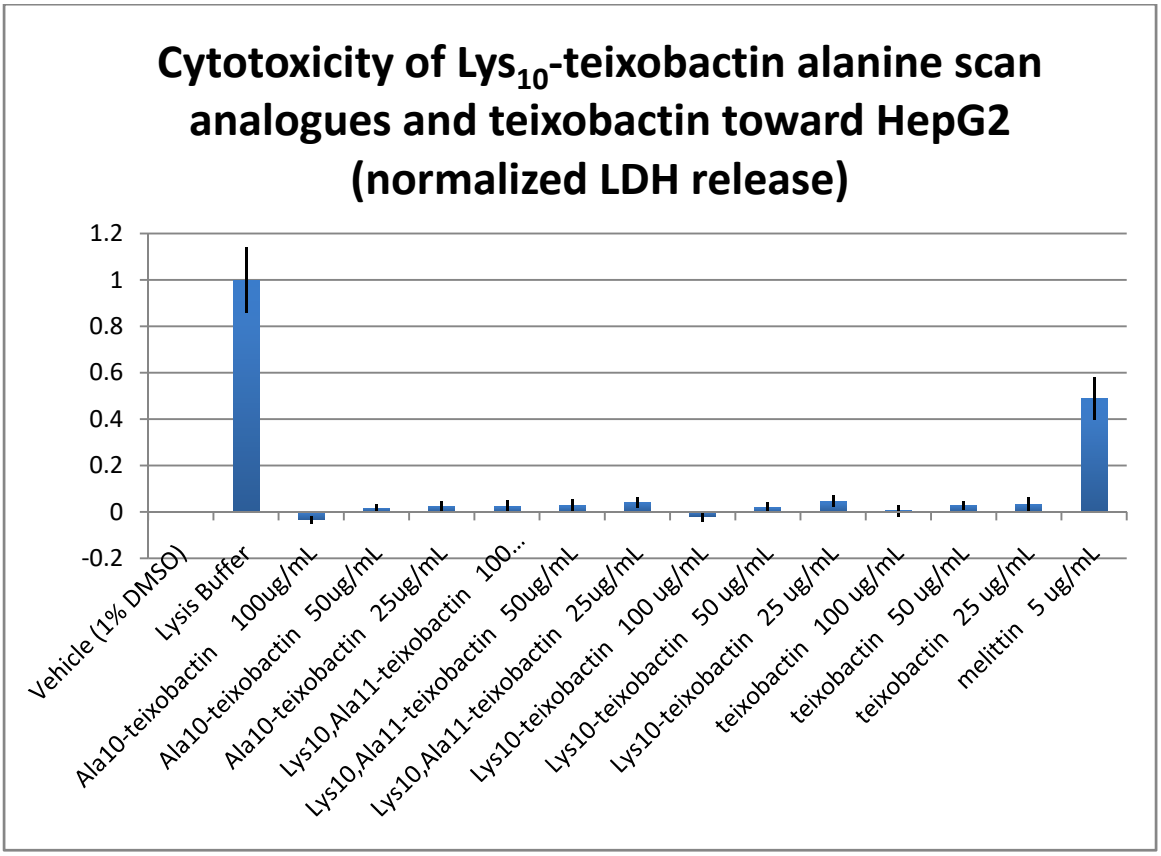


Figure S1. Representative result of a LDH release assay with HepG2 cells. Experiments were performed in quadruplicate. Four peptides at three different concentrations can be tested in one plate.

Hemolytic assay of Lys_{10}-teixobactin alanine scan analogues and teixobactin[5]

The hemolytic activity of Lys_{10}-teixobactin alanine scan analogues and teixobactin toward fresh human red blood cells (RBCs) was assessed in a hemolytic assay. The hemolytic assay was performed loosely following an established protocol [citation]. The RBCs were

purchased from Innovative Research Inc. and stored at 4 ˚C cold room. The RBCs were incubated with 100, 50, and 25 μg/mL of each Lys_{10}-teixobactin alanine analogue and teixobactin. Experiments were performed in quadruplicate (replicate) in V-bottom 96-well plate. Triton X-100, included in the LDH release assay kit, was used as a positive control; vehicle (1% DMSO) was used as a negative control. Melittin (5 μg/mL) was used as an additional peptidic positive control. The peptide solutions were added first followed by the RBCs in the inner 60 wells (rows B–G, columns 2–11) of the 96-well plate. The plates were covered and incubated for 1 h.

Preparation of peptide stocks. 10 mg/mL peptide stock solutions in DMSO were 2-fold serial diluted with sterilized DMSO in 2-mL Eppendorf tubes. [Typically, 5 μL of 10 mg/mL peptide stock was needed to make 250 μL of peptide in 1X PBS buffer.] The appropriate amount of 1X PBS buffer at pH of 7.3 was used to diluted the peptide to 200, 100, and 50 μg/mL. [250 μL is enough volume to conduct a quadruplicate experiment with 50 μL leftover.] The Eppendorf tubes were vortexed and centrifuged. The vortexing and centrifugation process was repeated once more to ensure a homogenous peptide stock in 1X PBS was prepared that is free of precipitate. The diluted peptide stock solutions for cell treatment were prepared on the day of the cell treatment.

Preparation of human red blood cells for hemolytic assay. Human red blood cells (RBCs) were stored in 4 ˚C cold room. Approximately 1 mL of RBC was needed for each plate. The RBCs were transferred to a 5-mL glass test-tube coated with EDTA to prevent blood coagulation. Approximately 2 mL of 1X PBS at pH of 7.3 was added to the red blood cells. The red blood cells in 1X PBS were centrifuged at 1000 g for 10 min at 4 ˚C. The supernatant was removed and the cells were washed multiple times (typically, 2–4 times) to ensure that the supernatant is

visibly transparent and free of any color from preexisting lysed RBCs. After PBS washes, the RBCs were resuspended in approximately 2 mL of 1X PBS buffer. After gently mixing by inversion, the number of RBCs were counted. [Typically, it will require a 10000-fold dilution in order to get a countable number.] The original RBCs stock is then diluted to a concentration that equates 20 million red blood cells per 50 µL.

Treatment of red blood cells with peptide stocks. The samples were mixed multiple times with a micropipet to ensure that they were homogenous. Experiments were performed in quadruplicate (replicate) in V-bottom 96-well plate. 50-µL aliquots of each peptide at 200, 100, and 50 µg/mL were added to the wells. Four wells were used for a negative control and received 50 µL of 1X PBS containing 2% DMSO. Four wells were used for a positive control with lysis buffer (Triton-X100) provided in the LDH release assay kit. Four wells were used for a peptidic positive control consisting 5 µg/mL of melittin, the active cytotoxic compound in bee venom. After the peptides had been plated, 50-µL aliquots of RBCs were added to each well using a multichannel micropipet fitted with ten tips. [The red blood cells should be pipetted very slowly to prevent shearing.] The final concentration in each peptide wells was 100, 50, and 25 µg/mL. The final number of RBCs in each well was 10 million. The cells were covered and incubated at 37 ˚C in a 5% CO_2 atmosphere for 1 h.

Hemolytic assay readout. A replica plate was made by aliquoting 50 uL of 1X PBS into each of the inner wells of a new flat-bottom 96-well plate. After 1 h incubation, the 96-well plate was centrifuged at 1000 g for 10 min at 4 ˚C. A 50-µL aliquot of the supernatant from each well was transferred to the new 96-well plate. [Extreme care was taken to avoid disturbing the red blood cell pellet. A good technique is to tilt the plate.] If any RBCs were disturbed, the V-bottom 96-well plate should be centrifuged again to re-pellet the RBC. The final volume of each well is

100 µL. The absorbance of each wells as measured at 540 (A_{540}). Data were processed by comparing those values to those of the lysis buffer controls and the untreated controls:

$$\% \text{ hemolytic activity} = [(A_{540})_{compound} - (A_{540})_{vehicle}]/[(A_{540})_{lysis} - (A_{540})_{vehicle}]$$

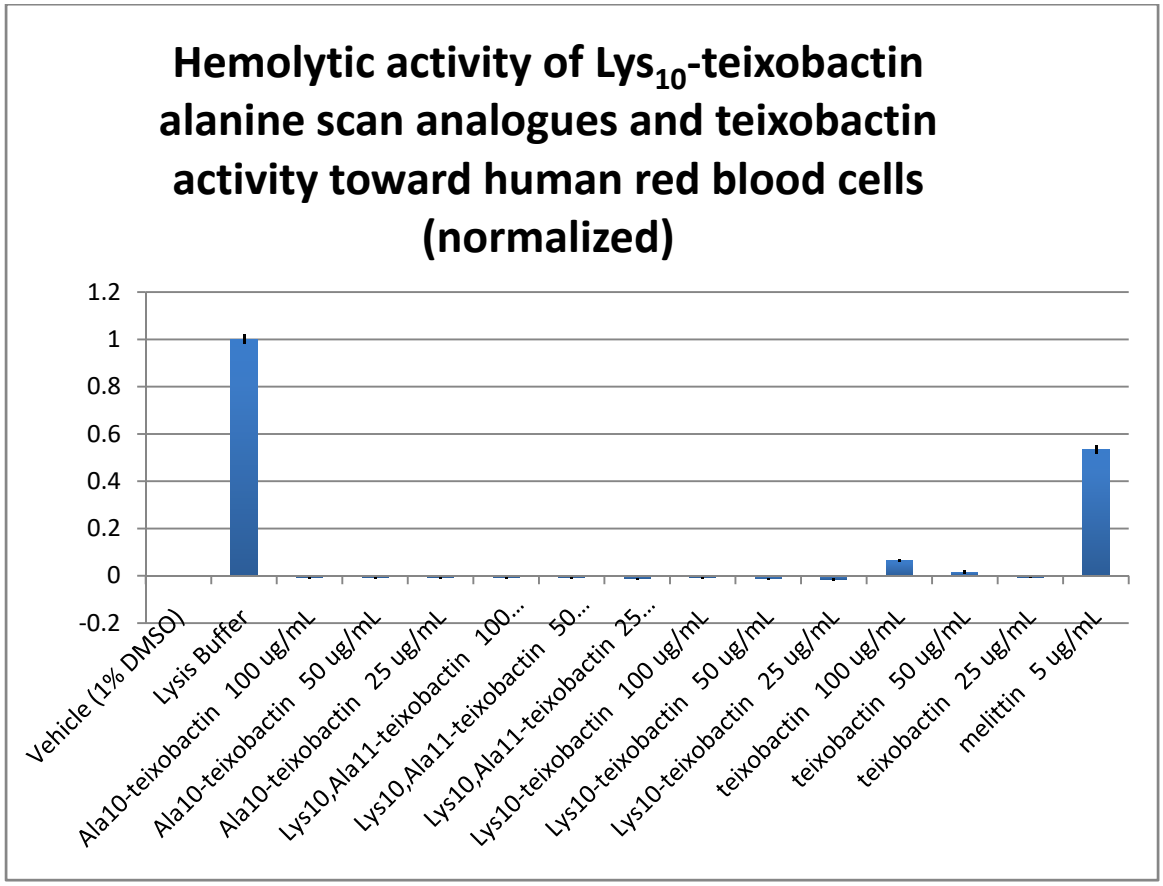
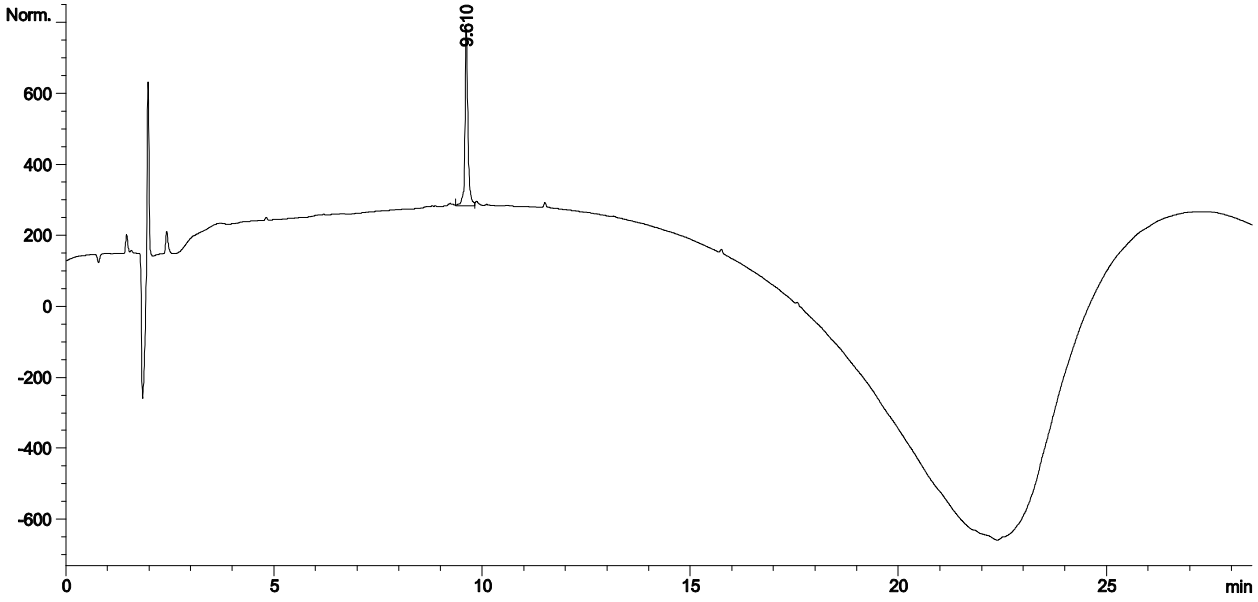


Figure S2. Representative results of a hemolytic assay experiment with human red blood cells. Experiments were performed in quadruplicate. Four peptides at three different concentrations can be tested in one plate.

HPLC Traces and Mass Spectra of Lys_{10}-teixobactin Alanine Scan Analogues

Lys_{10}-teixobactin: analytical RP-HPLC and mass spectrum

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Signal 1: VWD1 A, Wavelength=214 nm

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]  mAU   *s   [mAU   ]      %
----|-------|----|-------|----------|----------|--------|
   1   9.610 VV    0.0721 2433.12549  493.14777 100.0000
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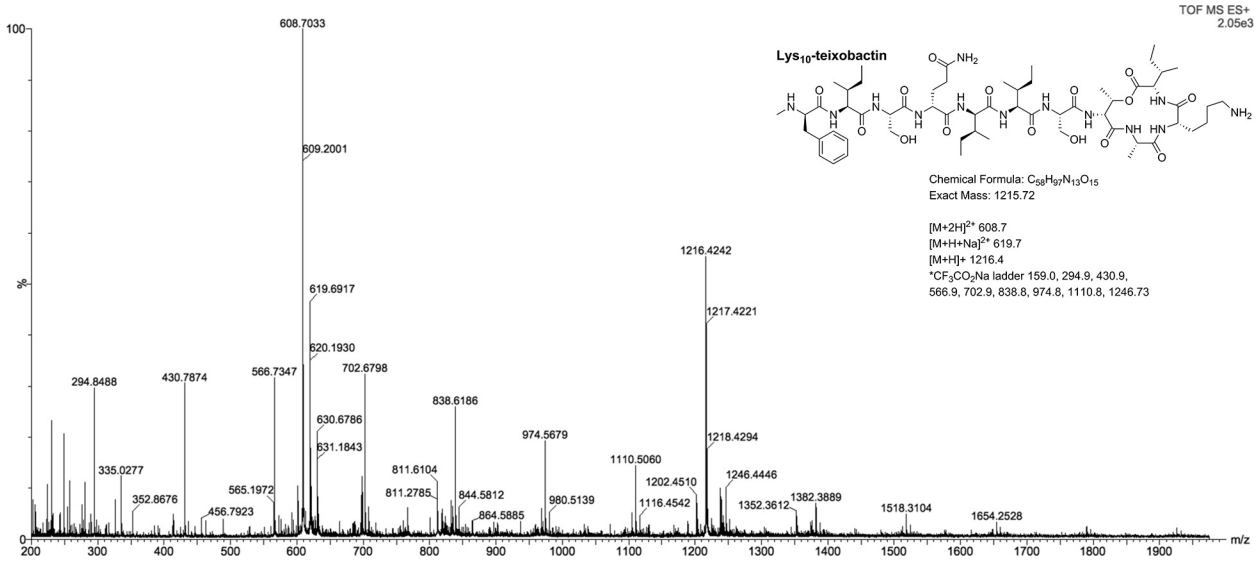
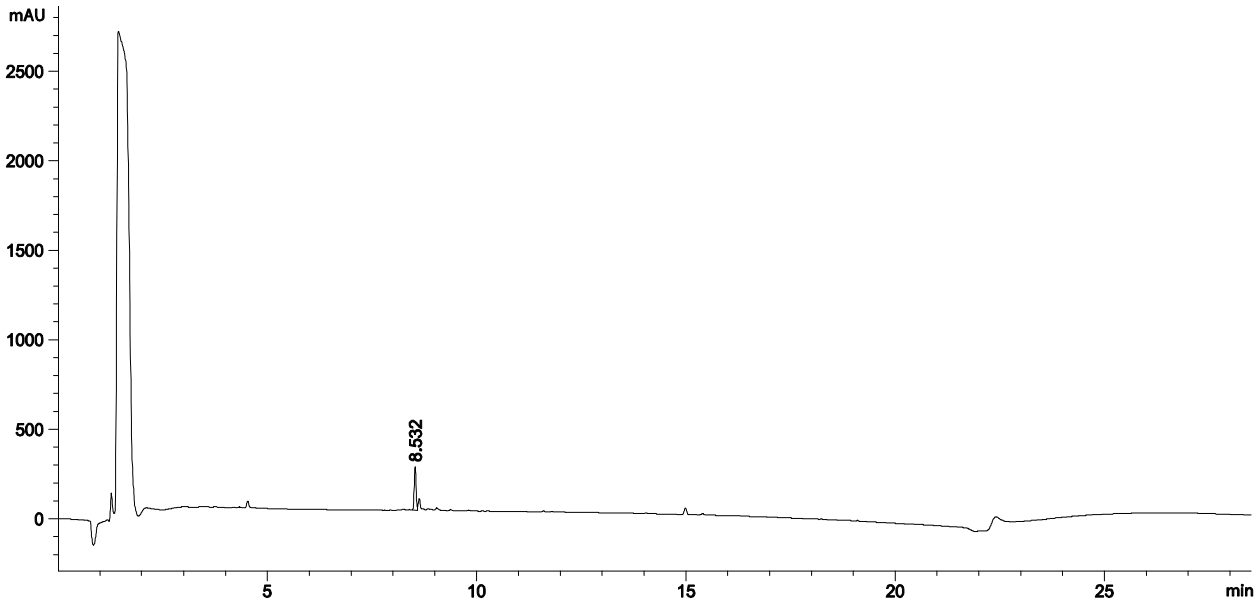
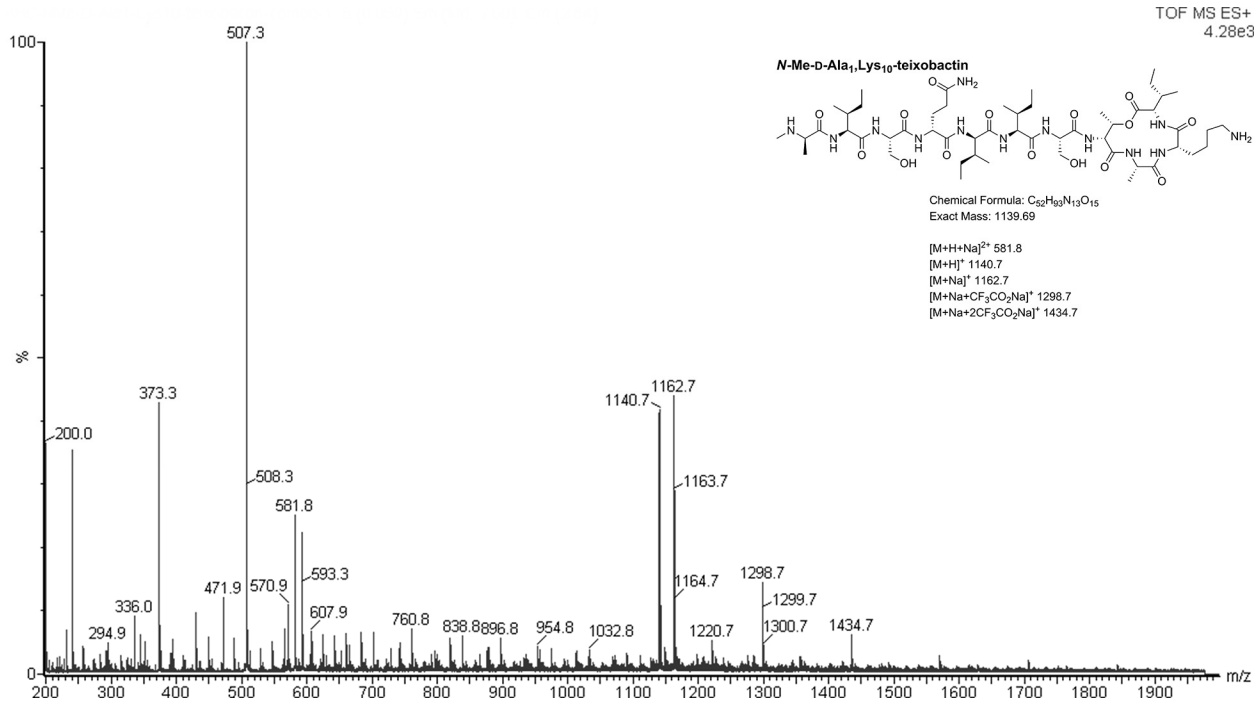


N-Me-D-Ala$_1$,Lys$_{10}$-teixobactin: RP-HPLC and mass spectrum

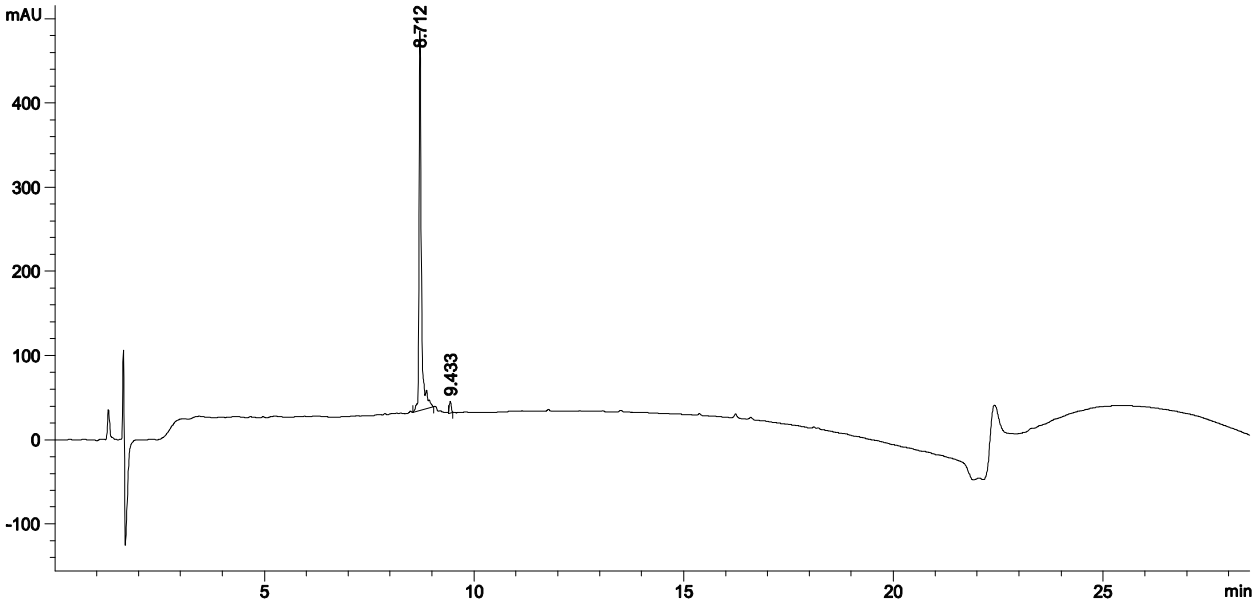


Signal 1: VWD1 A, Wavelength=214 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	8.532	MM	0.0489	717.85504	244.54773	100.0000

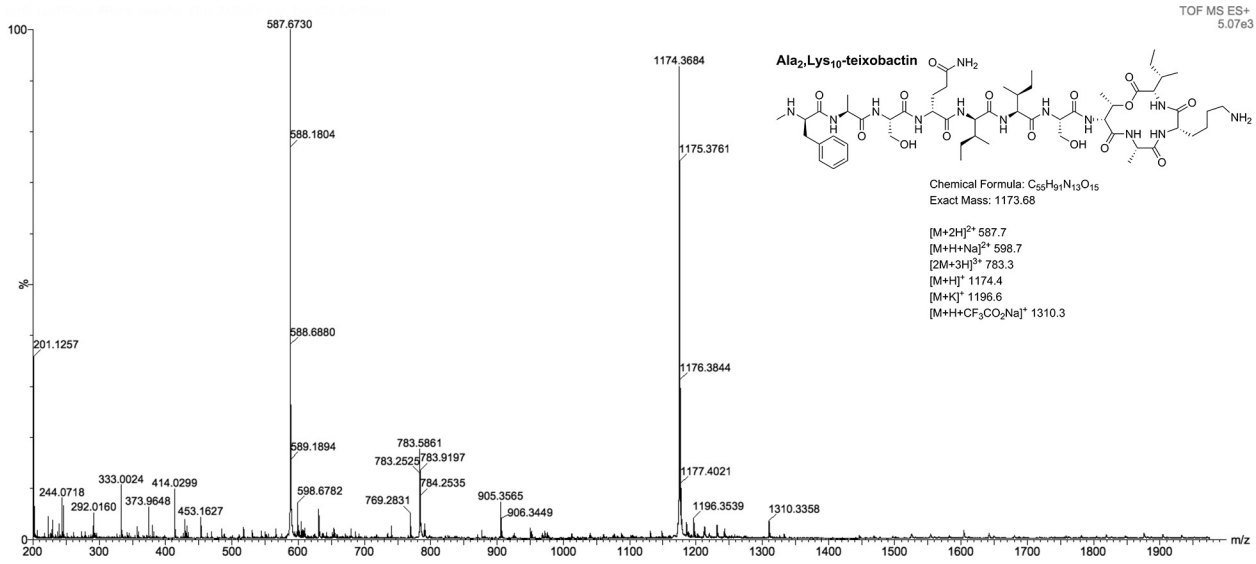


Ala_2,Lys_{10}-teixobactin: analytical RP-HPLC and mass spectrum

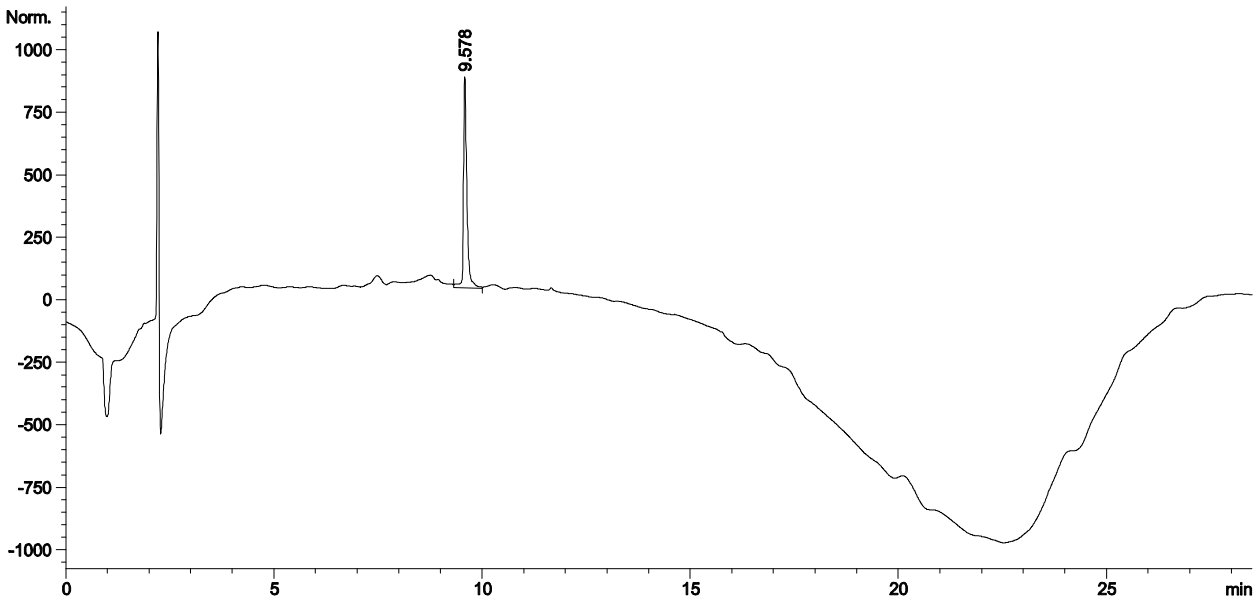


Signal 1: VWD1 A, Wavelength=214 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	8.712	MM	0.0654	1783.49731	454.38245	97.6010
2	9.433	MM	0.0520	43.83836	14.04287	2.3990

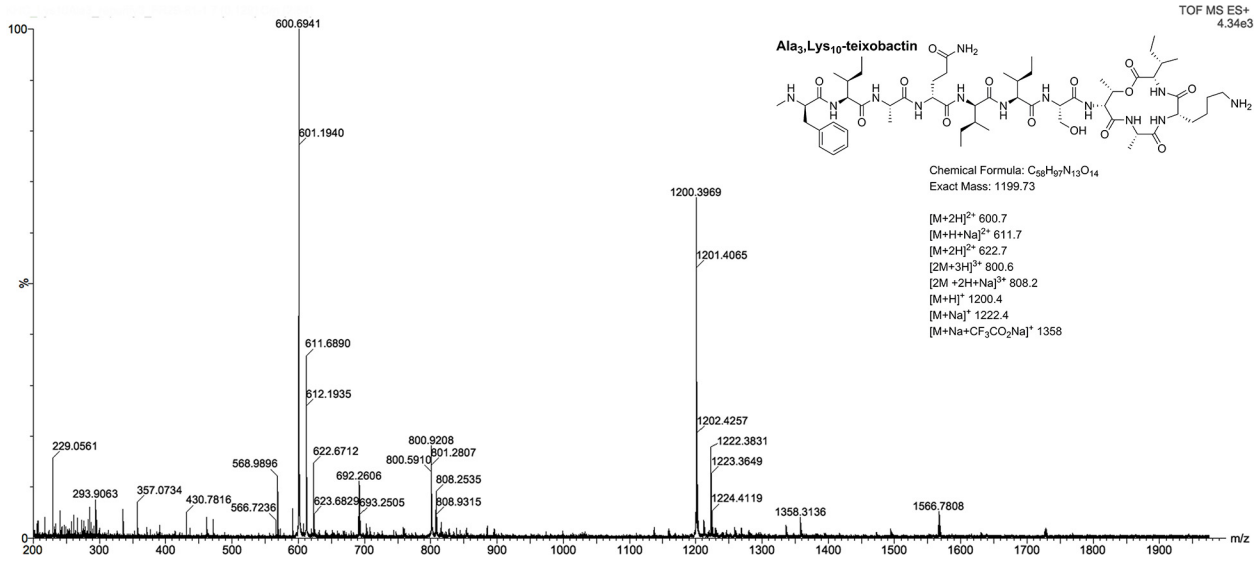


Ala_3,Lys_{10}-teixobactin: analytical RP-HPLC and mass spectrum

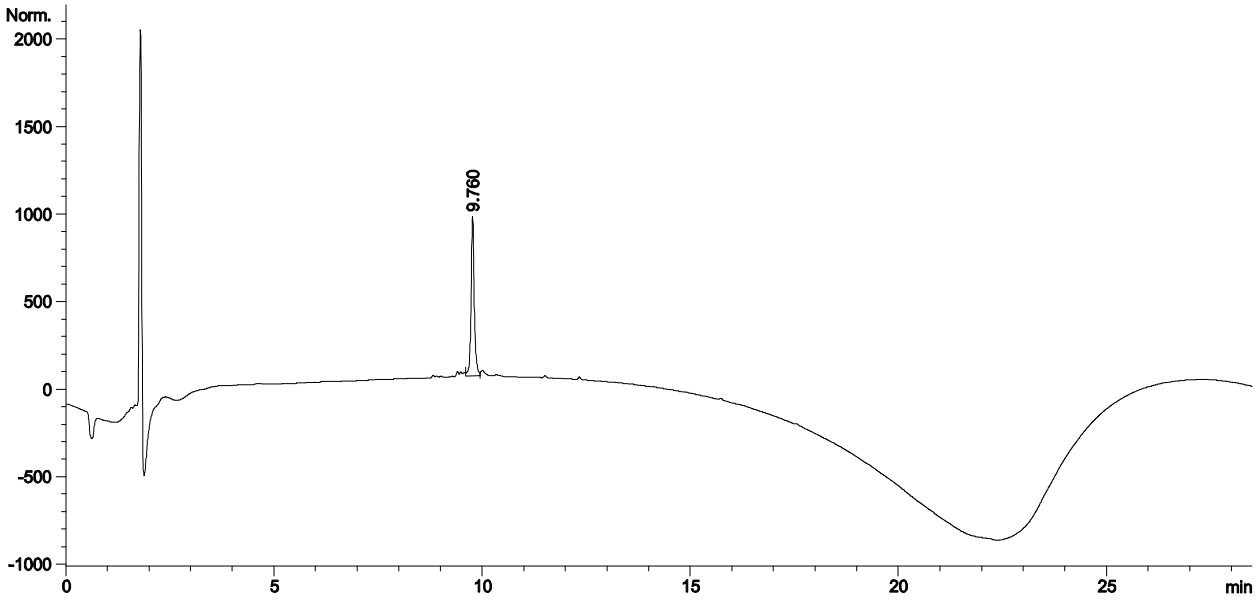


Signal 1: VWD1 A, Wavelength=214 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	9.578	VV	0.0868	4808.52539	845.21588	100.0000

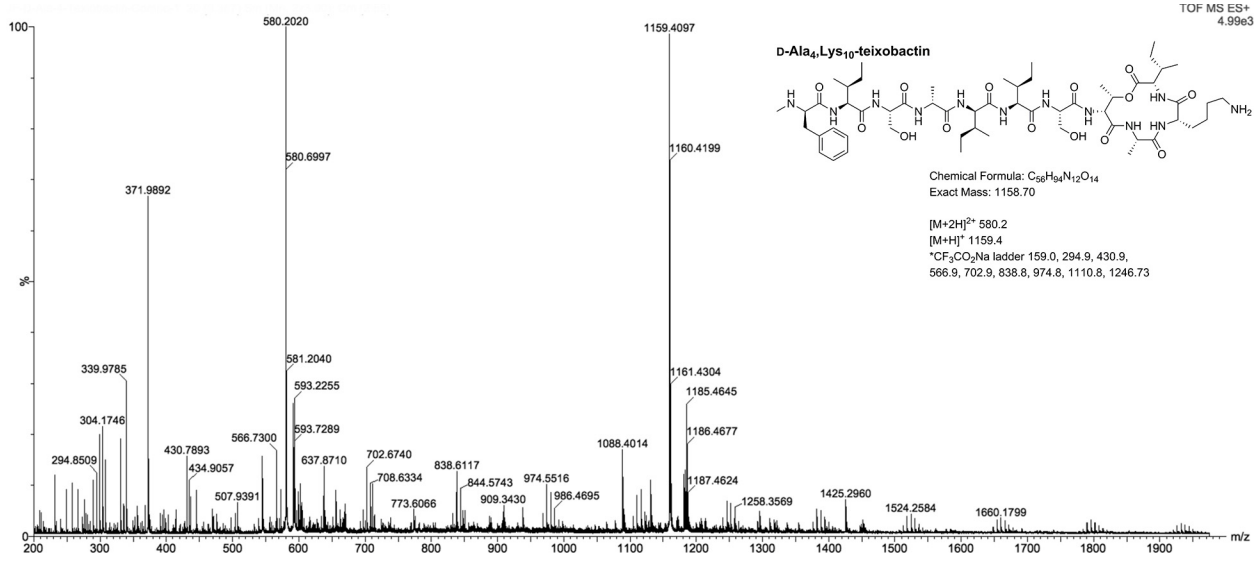


D-Ala$_4$,Lys$_{10}$-teixobactin: analytical RP-HPLC and mass spectrum

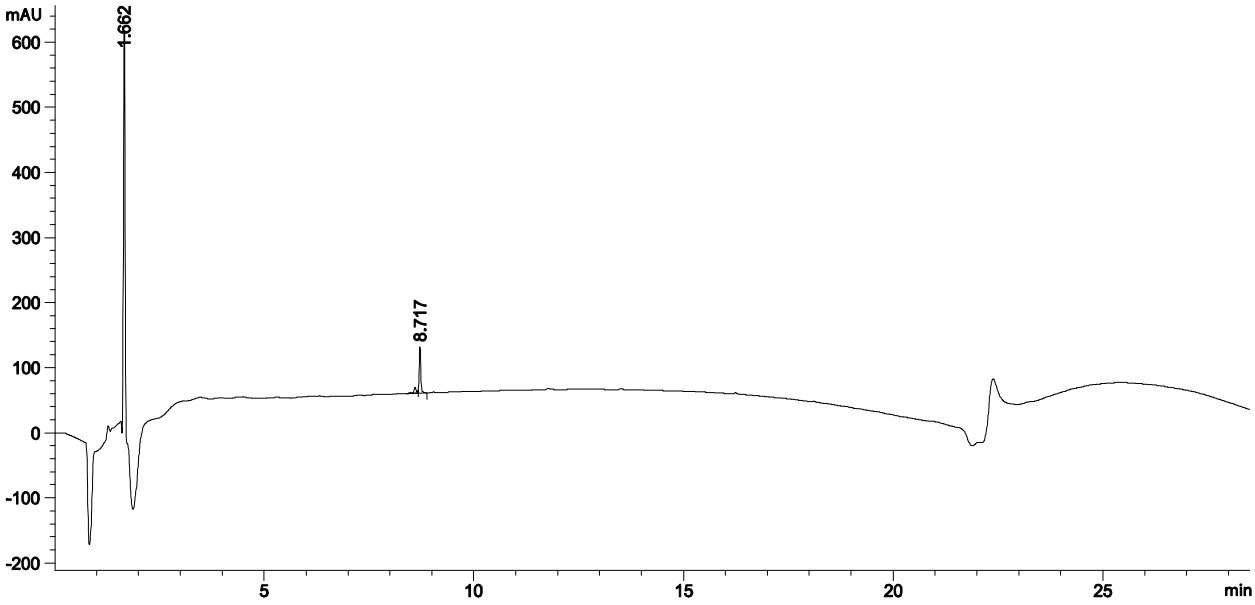


Signal 1: VWD1 A, Wavelength=214 nm

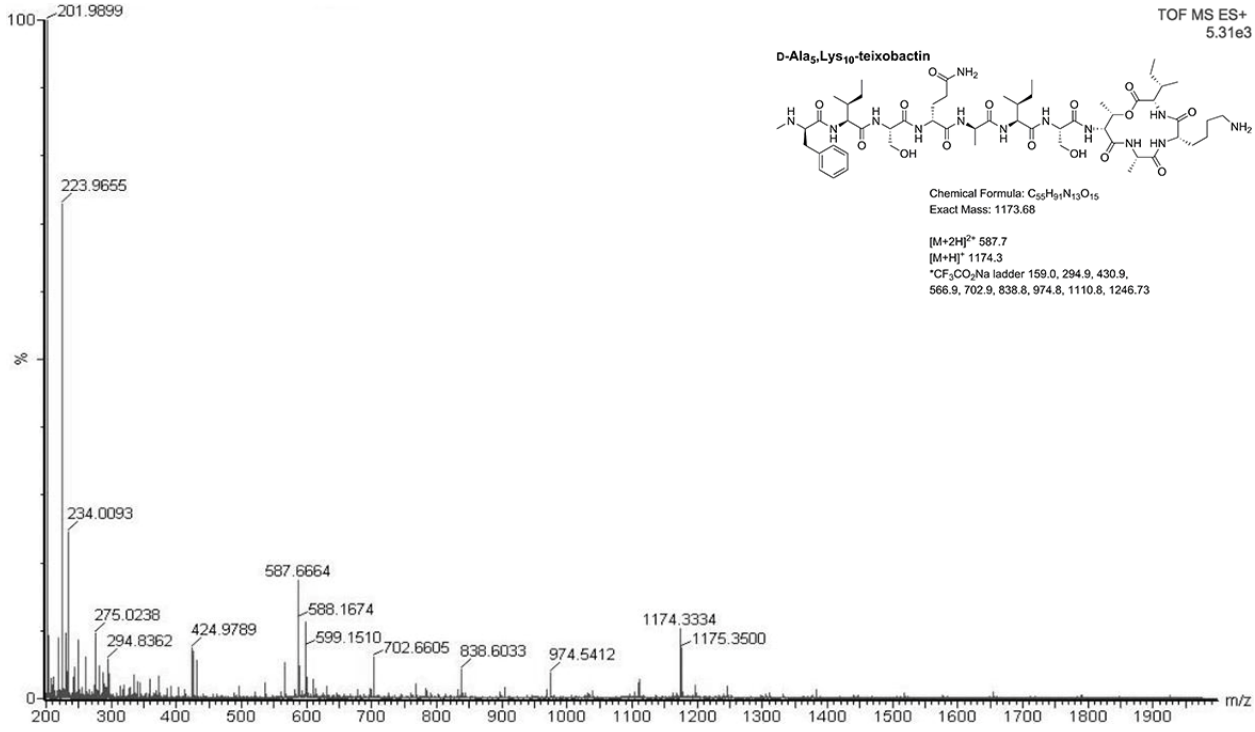
Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	9.760	VV	0.0804	4900.87695	909.23907	100.0000



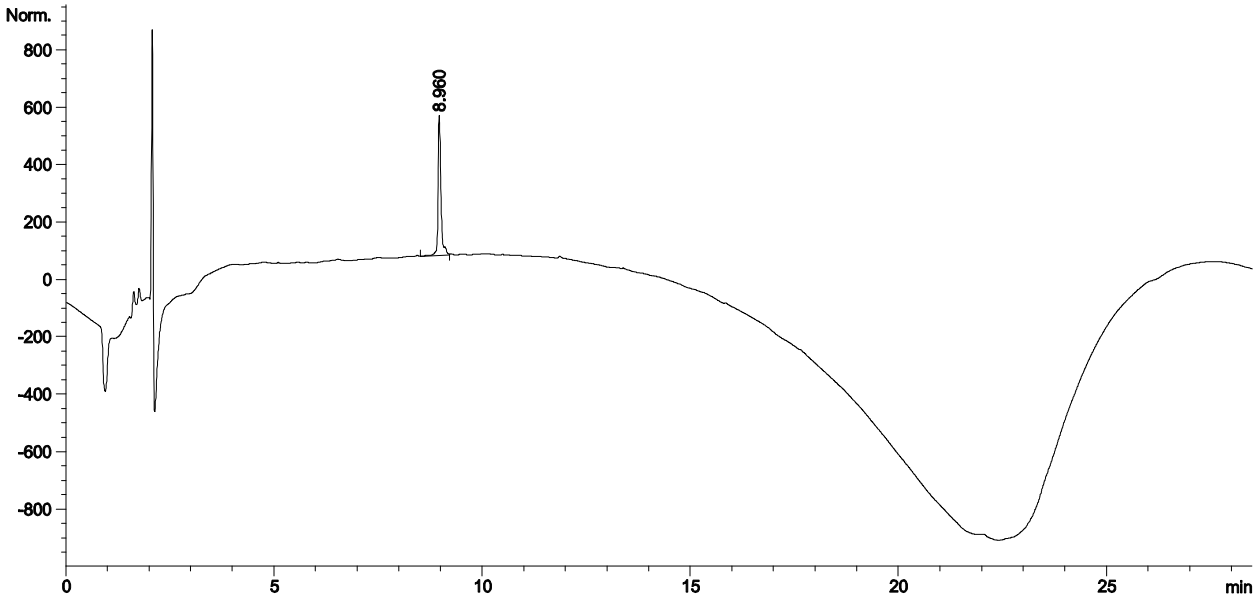
D-Ala_5,Lys_{10}-teixobactin: analytical RP-HPLC and mass spectrum



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	8.717	VB R	0.0460	208.06128	71.32160	100.0000

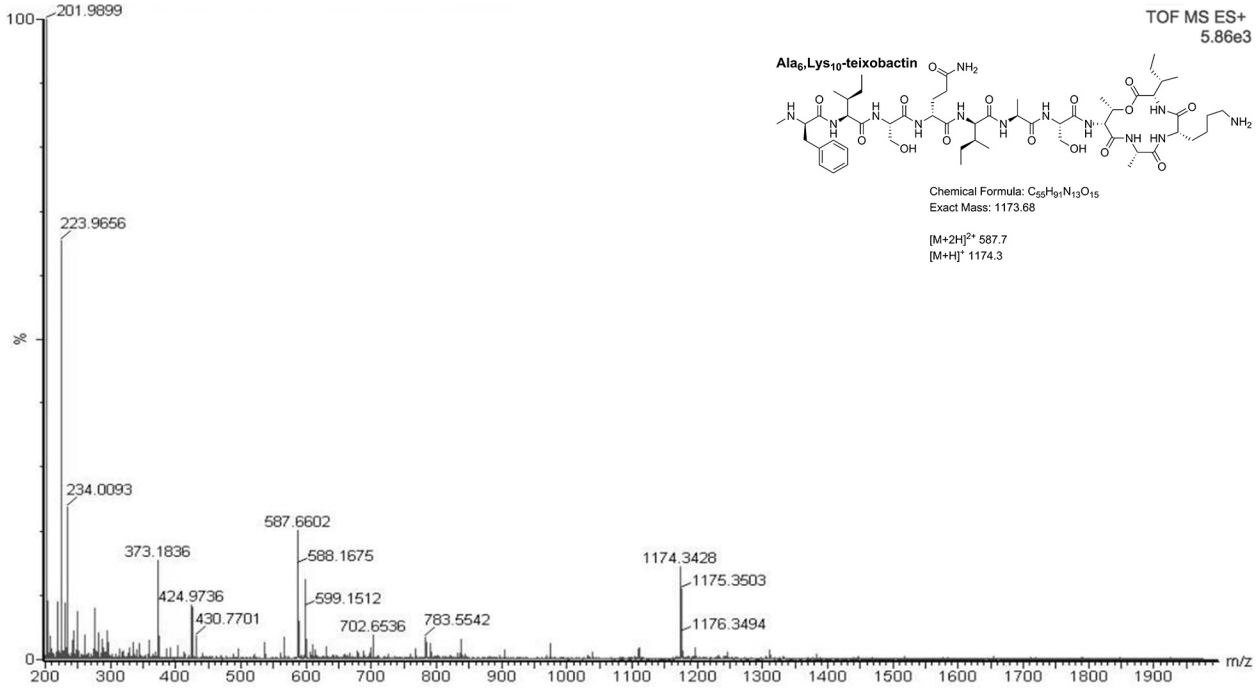


Ala_6,Lys_{10}-teixobactin: analytical RP-HPLC and mass spectrum

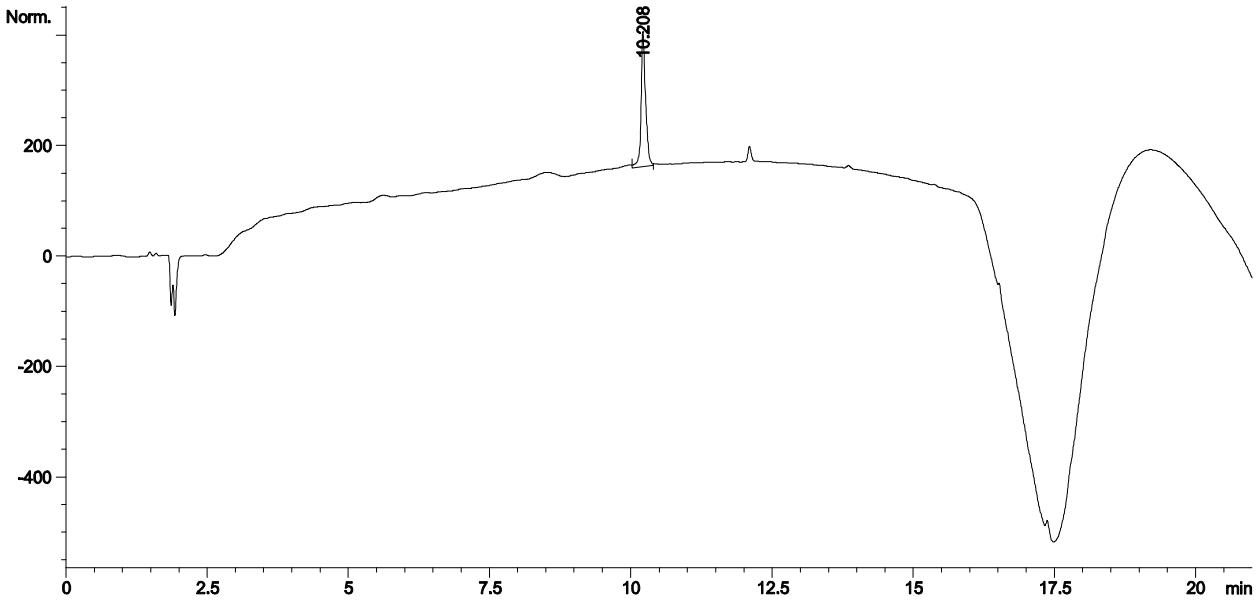


Signal 1: VWD1 A, Wavelength=214 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	8.960	VV	0.0757	2419.54810	484.55621	100.0000

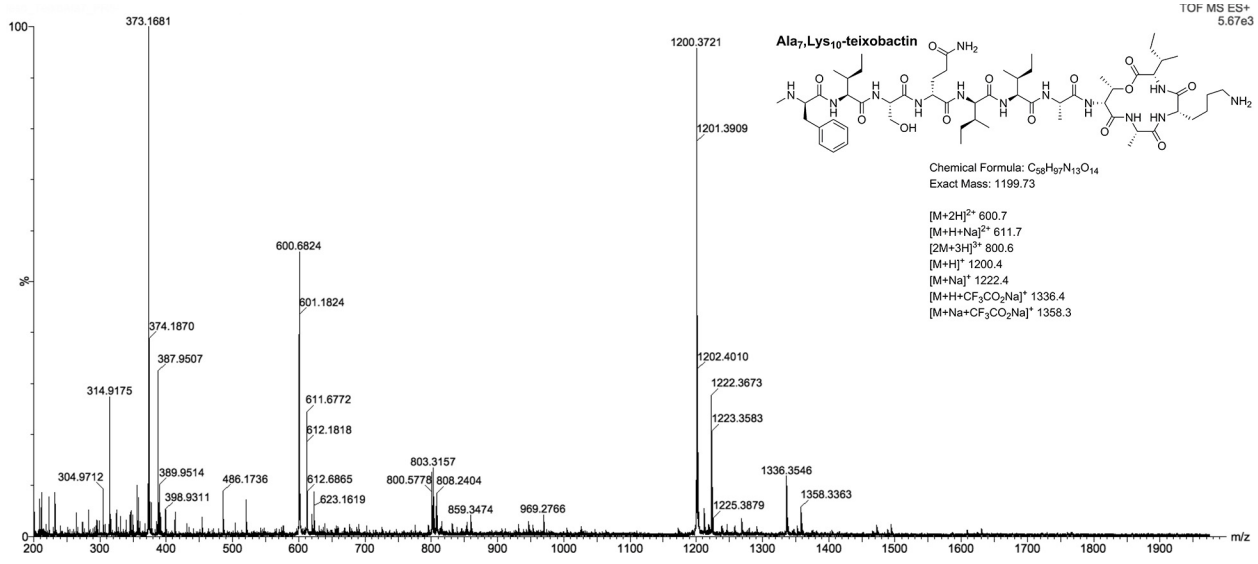


Ala_7,Lys_{10}-teixobactin: analytical RP-HPLC and mass spectrum

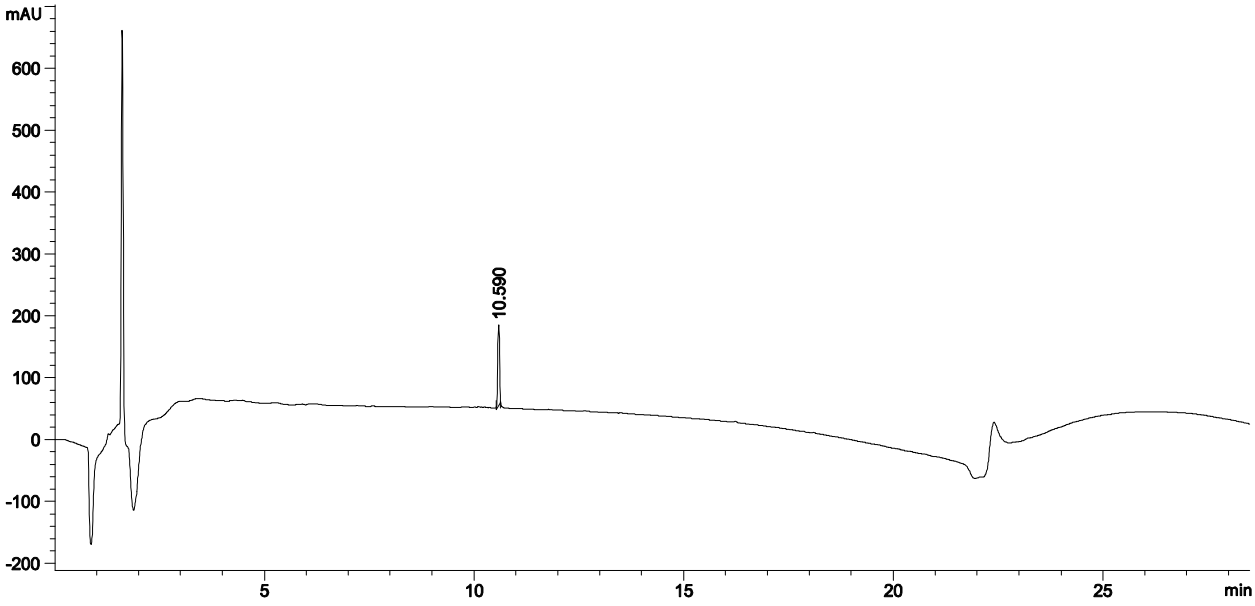


Signal 1: VWD1 A, Wavelength=214 nm

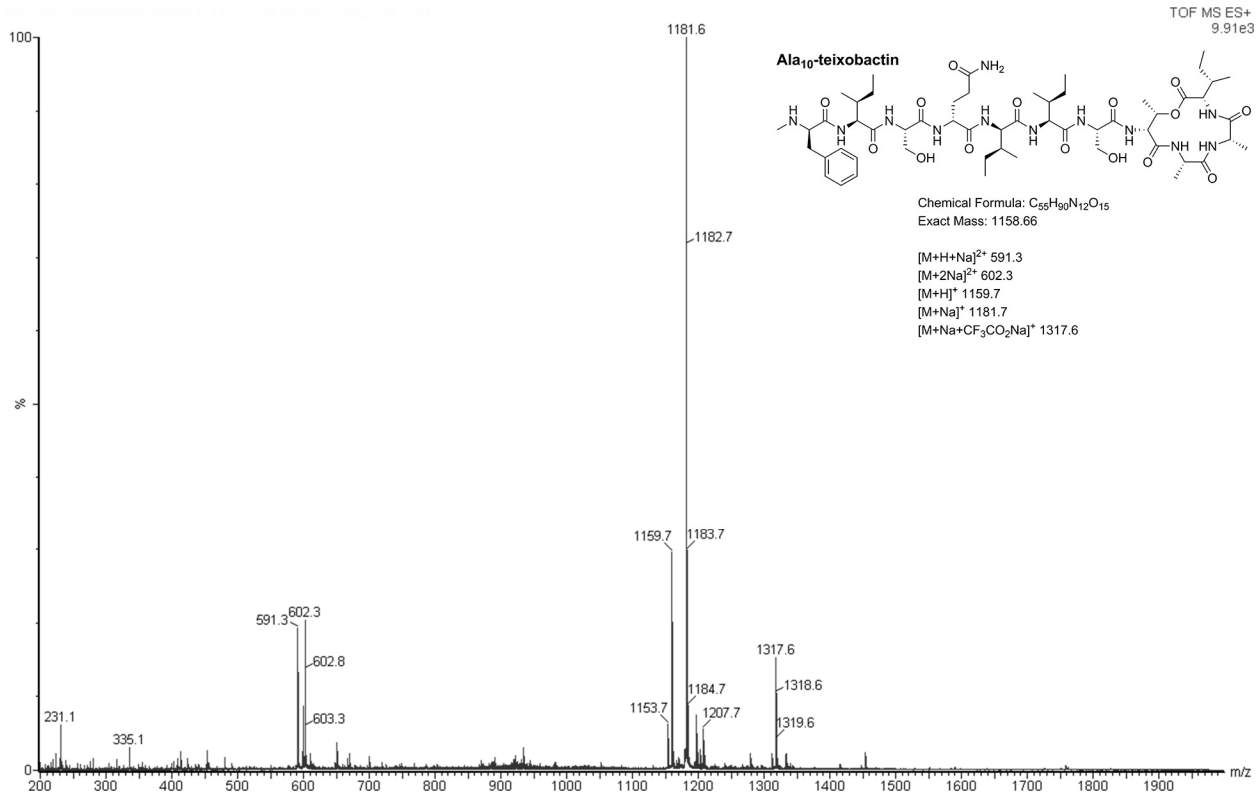
Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	10.208	VV	0.0831	1374.00317	244.24222	100.0000



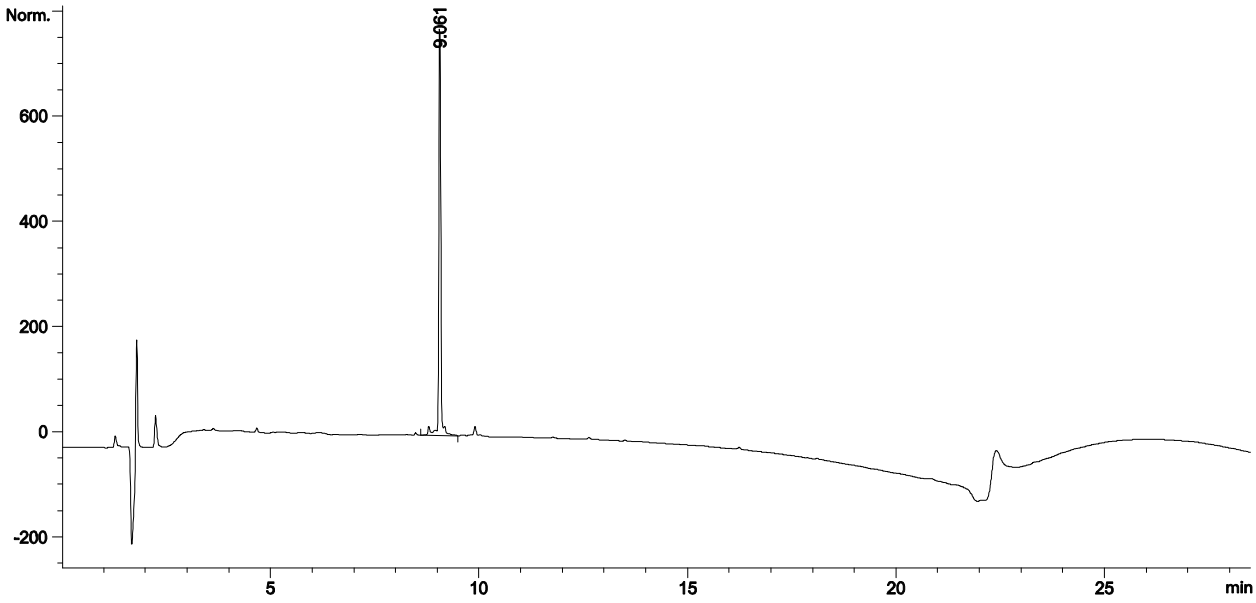
Ala_{10}-teixobactin: analytical RP-HPLC and mass spectrum



Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	10.590	MM	0.0469	371.83914	132.08942	100.0000

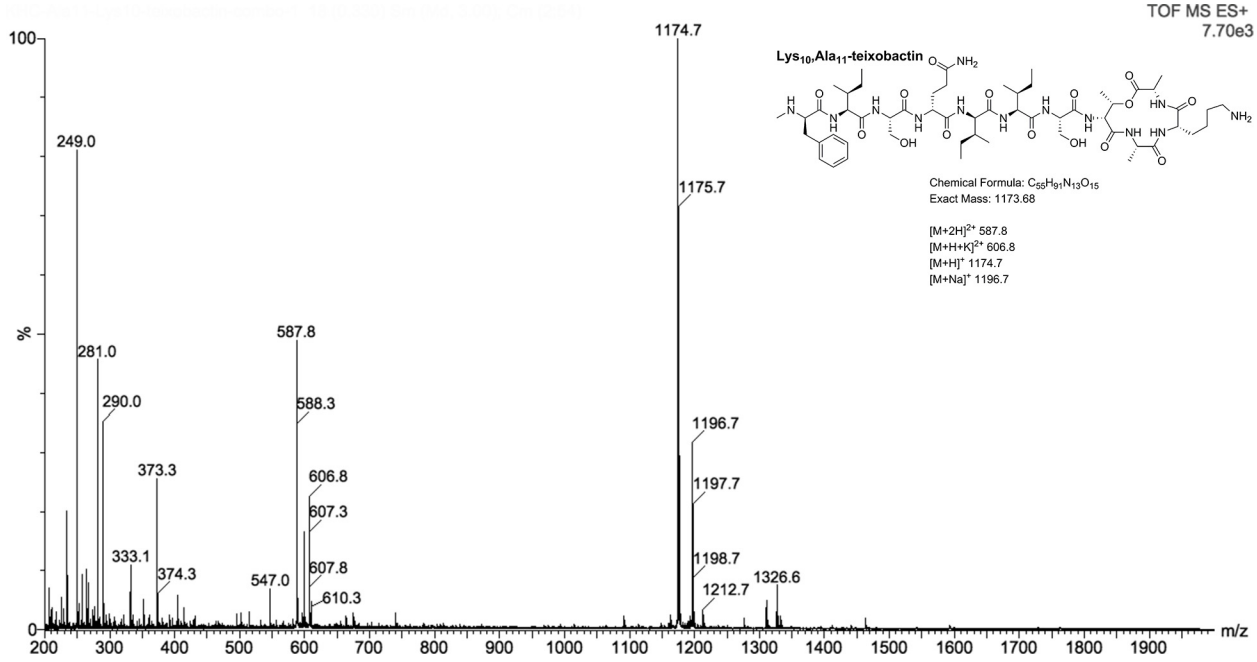


Lys_{10},Ala_{11}-teixobactin: analytical RP-HPLC and mass spectrum



Signal 1: VWD1 A, Wavelength=214 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	9.061	VB R	0.0494	2273.71558	692.86609	100.0000



Notes and References

1. A. B. Pangborn, M. A. Giardello, R. H. Grubbs, R. K. Rosen, F. J. Timmers, *Organometallics* 1996, **15**, 1518.
2. Lys_{10}-teixobactin and the alanine scan analogues were synthesized following a protocol similar to those published previously The procedures were either adapted from or taken verbatim from K. H. Chen, K. A. Corro, S. P. Le, and J. S. Nowick, *J. Am. Chem. Soc*., 2017, **139**, 8102, and H. Yang, K. H. Chen, and J. S. Nowick, *ACS Chem. Biol*., 2016, **11**, 1823.
3. Minimum inhibitory concentration assays were performed following a protocol similar to those published previously. The procedures were either adapted from or taken verbatim from H. Yang, K. H. Chen, and J. S. Nowick, *ACS Chem. Biol*., 2016, **11**, 1823.
4. LDH release assays were performed following a protocol similar to those published previously. The procedures either adapted from or taken verbatim from A. G. Kreutzer, S. Yoo, R. K. Spencer, J. S. Nowick, *J. Am. Chem. Soc*., 2017, **139**, 966.
5. Hemolytic assays were performed following a protocol adapted from B. C. Evans, C. E. Nelson, S. S. Yu, K. R. Beavers, A. J. Kim, H. Li, H. M. Nelson, T. D. Giorgio, C. L. Duvall, *J. Vis. Exp.,* 2013, **73**, e50166, doi:10.3791/50166.