Re-Evaluation of the *N*-Terminal Substitution and the D-Residues on Teixobactin

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Materials

All reagents and solvents were obtained from commercial suppliers and were used without further purification. Analytical HPLC was performed on an Agilent 1100 system, and Chemstation software was used for data processing. Buffer A: 0.1 % trifluoroacetic acid in H_2O; buffer B: 0.1 % trifluoroacetic acid in CH_3CN. LC–MS was performed on a Shimadzu 2020 UFLC-MS using an YMC-Triart C_{18} (5 μm, 4.6 × 150 mm) column and data processing was carried out by Lab Solution software. Buffer A: 0.1 % formic acid in H_2O; buffer B: 0.1 % formic acid in CH_3CN. Crude peptide was purified on Shimadzu LC-8A preparative HPLC using a PhenomenexLumaC_{18}(2) column (10 μm, 10 × 250 mm). High resolution mass spectrometry (HRMS) was performed using a Bruker ESI-QTOF mass spectrometer in positive mode.

Experimental section

Synthesis of Alloc amino acids[1]

1.68 mL of Alloc-Cl is dissolved in 5 ml of dioxane and 1.54g of sodium azide dissolved in 4 mL of water is added and stirred for 2 hours. Then, 2.5g of N-Ile-OH are dissolved in 50 mL water containing 4g of sodium carbonate is added to the mixture followed by 50mL dioxane. The reaction mixture stirred at rt 24 hours monitoring the pH which should be between 8 and 10. The pH is adjusted by adding sodium carbonate 10%.

Once the reaction is finished, the solvent mixture is evaporated using vaccum evaporator then wash the crude several times using hexane. 100 mL of water is added keeping the pH adjusted to between 9 and 10 and washed (3v X 50mL) with diethylether.

The aqueous phase is acidified with HCl to pH 2 and extracted with DCM, dried with anhydro magnesium sulfate, the ethyl acetate was evaporated and characterized HPLC, HPL-MS, NMR and they matched the spectra in literature. [2]

Synthesis of L-Arg analogue of teixobactin

a) Synthesis of tetrapeptide

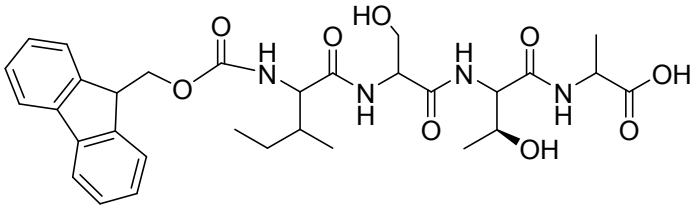
166 mg of 2-Cl-Trt resin (1.69 $mmolg^{-1}$) was placed in a 10 mL falcon tube and then was preactivated by 10 % $SOCl_2$ in DCM for 12 h. The resin was transferred into 10 mL polypropylene syringe fitted with a polyethylene filter disc and washed several times with DCM. Then, it washed with DCM (3 × 10 mL, 1 min) followed by adding the first amino acid Fmoc-L-Ala-OH (31 mg, 0.1 mmol) and DIEA (174 µL, 1 mmol, 10 equiv) in 0.5 mL DCM and shacked for 1 h. Then 100 µL of MeOH was added and shacked for 30 min to ensure full capping of the resin. Then the resin was washed with DMF (2 × 10 mL, 1 min), DCM (2 × 10 mL, 1 min), Methanol (2 × 10 mL, 1 min), DCM (2 × 10 mL, 1 min) and DMF (2 × 10 mL, 1 min). Then, Fmoc removal was achieved by 20 % piperidine in DMF (2 × 10 mL, 5 min). Then, the next amino acids were added by using the following coupling condition:

For coupling:Fmoc-AA-OH/HATU/DIEA (3:3:6) in 1 mL DMF for 30 min

For Fmoc removal: 20 % piperidine in DMF (2 × 10 mL, 5 min)

Washing: DMF (2 × 10 mL, 1 min), DCM (2 × 10 mL, 1 min) and DMF (2 × 10 mL, 1 min)

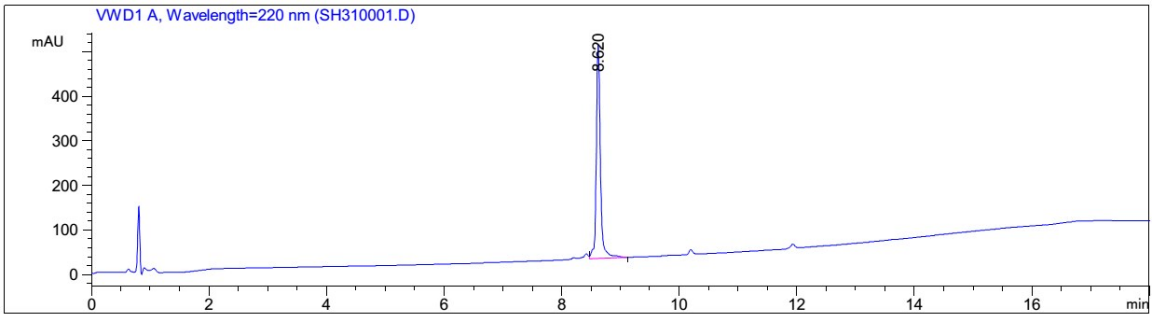
Until getting tetrapeptide**6** (Fmoc-L-Ile-L-Ser-D-Thr-L-Ala-OH), mini-cleavage was performed in order to monitor the esterification step

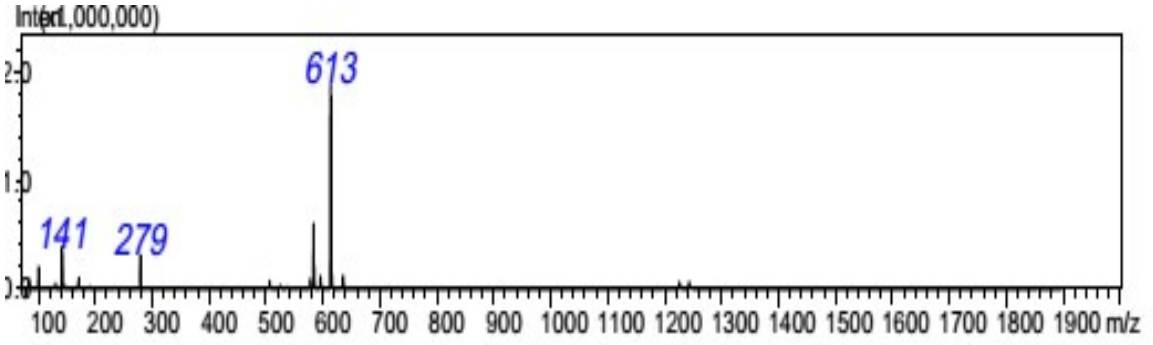


Molecular Weight: 612.67

HPLC chromatogram of Fmoc-L-Ile-L-Ser-D-Thr(OH)-L-Ala-OH:

A linear gradient of 5–95% CH_3CN/H_2O and 0.1% TFA over 15 min was applied, with a flow rate of 1.0 mL/min and detection at 220 nm using a Phenomenex C_{18} (3 µm, 4.6 × 50 mm) column.



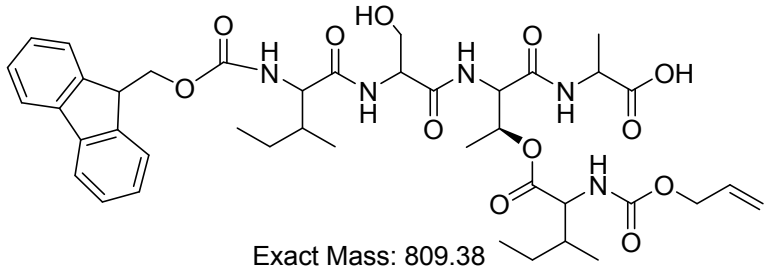


b) Ester-bond formation

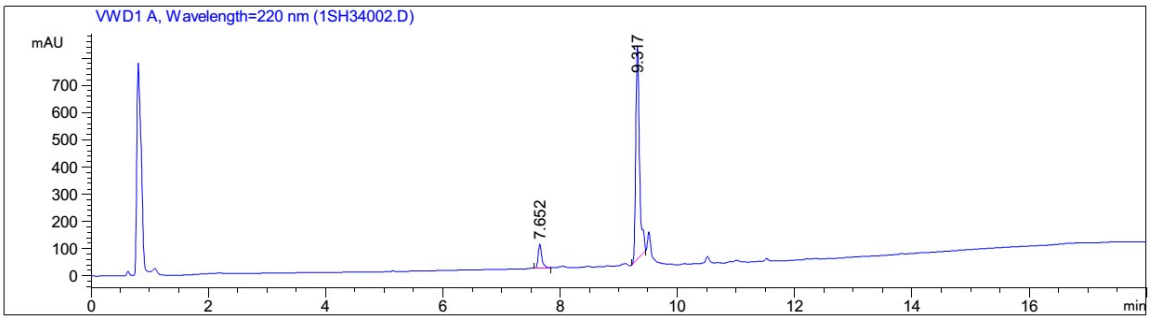
The ester bond formation was achieved by using the following condition: Alloc-L-Ile-OH (215 mg, 1 mmol, 10equiv), DIC (77 µL, 0.5 mmol, 5 equiv) and DMAP (12 mg, 0.1 mmol, 1 equiv) in DCM/DMF (8:2) (2 ×2 h).

HPLC chromatogram of Fmoc-L-Ile-L-Ser-D-Thr(Alloc-L-Ile)-L-Ala-OH:

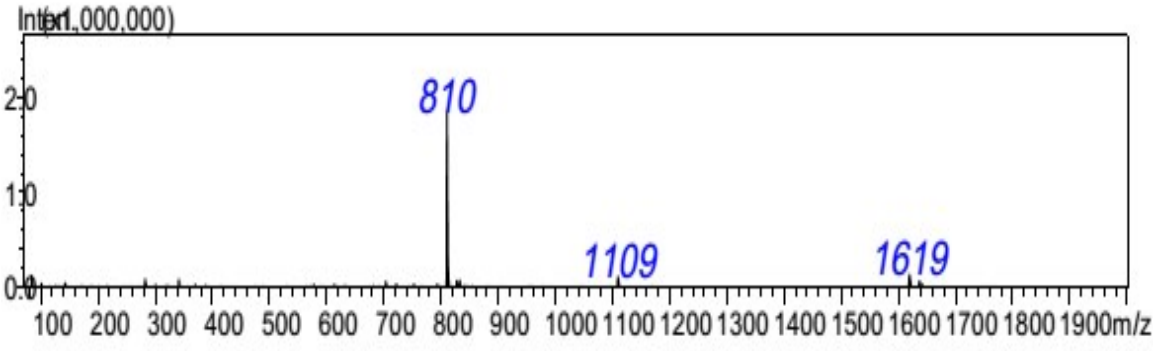
A linear gradient of 5–95% CH_3CN/H_2O and 0.1% TFA over 15 min was applied, with a flow rate of 1.0 mL/min and detection at 220 nm using a Phenomenex C_{18} (3 µm, 4.6 × 50 mm) column.



Exact Mass: 809.38



MS

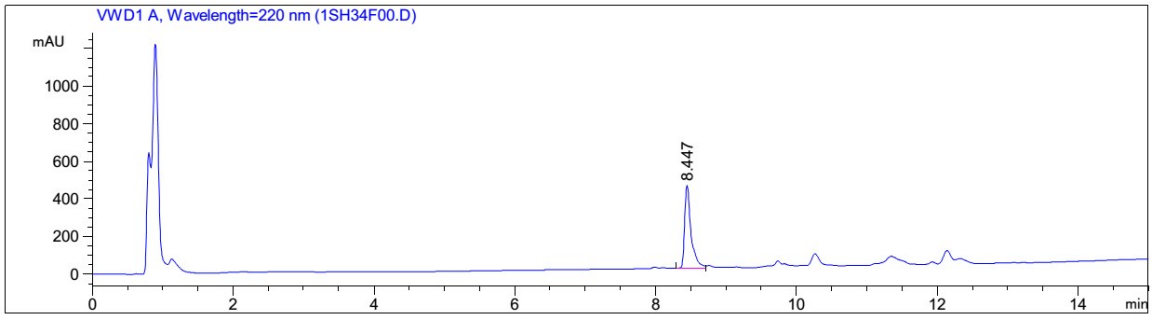


c) Synthesis of protected precursor peptide:

The resin was dried under vacuum and transferred onto microwave vial shielded from light by aluminum foil. Then, A solution of phenylsilane (124 µL, 1 mmol, 10 equiv) and a catalytic amount of tetrakistriphenylphosphine palladium (0) (11 mg,0.01mmol, 0.1equiv) in dry DCM (1 mL) was added. The reaction vessel was flushed with nitrogen and shacked for 15 min. Mini-cleavage was performed in order to control the reaction

HPLC chromatogram of 5-mer depsipeptide after Alloc deprotection:

A linear gradient of 5–95% CH_3CN/H_2O and 0.1% TFA over 15 min was applied, with a flow rate of 1.0 mL/min and detection at 220 nm using a Phenomenex C_{18} (3 µm, 4.6 × 50 mm) column



Then, Alloc-L-Arg(Pbf)-OH was added to the resin followed by removal of the Fmoc group and adding the following building blocks on the same order using the same protocol that mentioned above: Fmoc-L-Ile-OH, Fmoc-L-Gln(Trt)-OH, Fmoc-L-Ser(*t*Bu)-OH, Fmoc-L-Ile-OH and Boc-L-*N*-Me-Phe-OH in case **2b,** and Fmoc-D-*allo*-Ile-OH, Fmoc-D-Gln(Trt)-OH, Fmoc-L-Ser(*t*Bu)-OH, Fmoc-L-Ile-OH and Fmoc-D-Phe-OH in case **3a.**

In case of **3a:** the acetylation on D-Phe takes place after Fmoc deprotection using 20% piperidine in DMF, followed by acylation with acetic acid in presence of K-oxyma and DIC (3:3:6) for 2h.

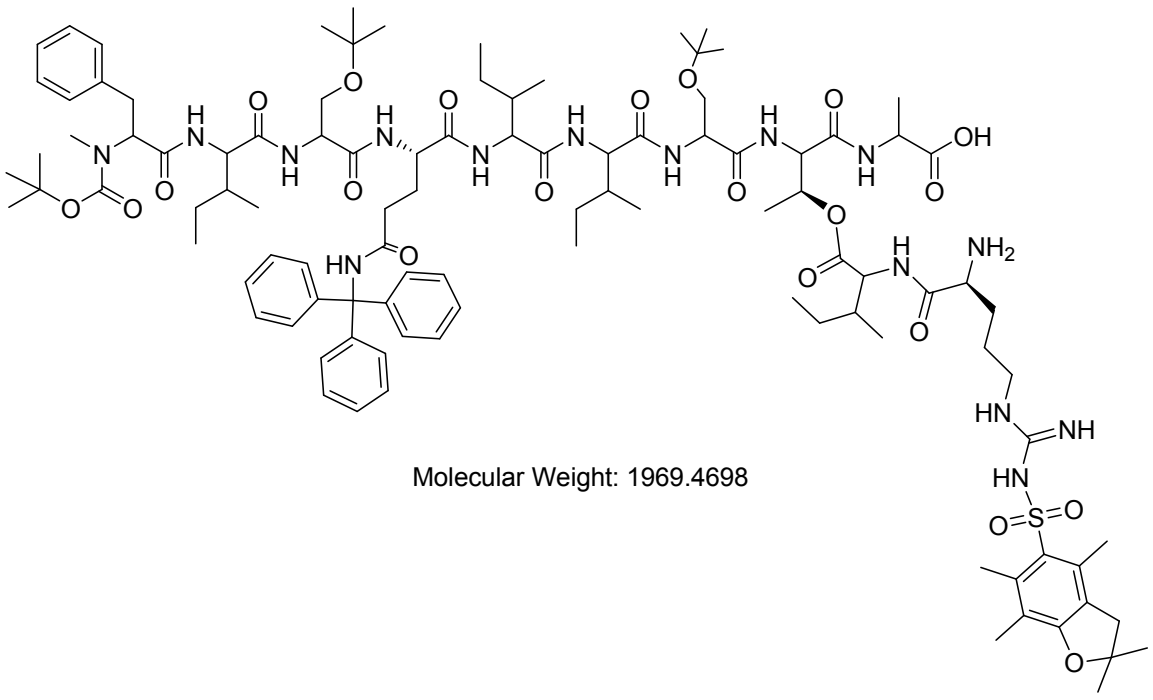
For both **2b** and **3a**: Alloc deprotection takes place to give the free L-Arg(Pbf)-OH before cyclization. The resin was dried under vacuum and treated with 1 % TFA in DCM (5 × 30 s) and collected over water. Then, TFA and DCM were evaporated, and then lyophilization takes place to get the protected powder before cyclization.

d) Cyclization and removing of all protecting groups:

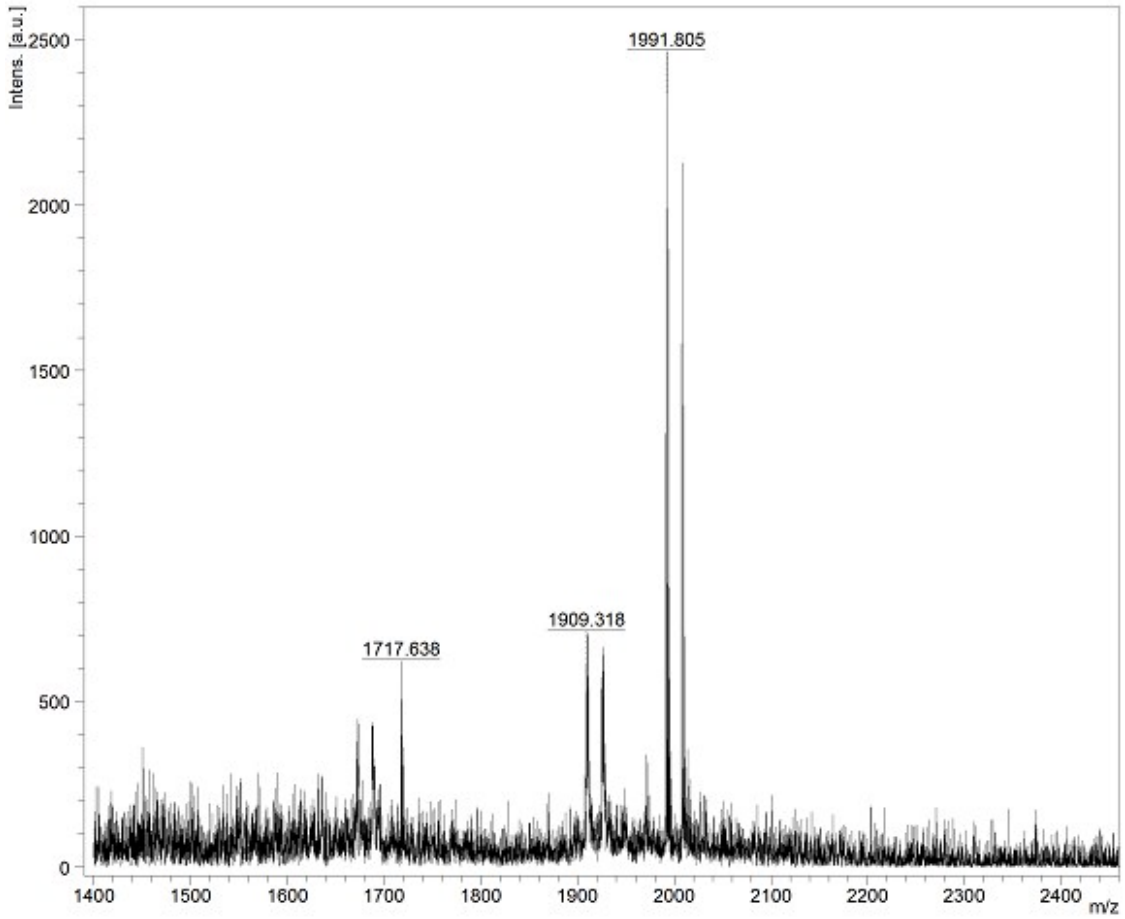
2b (L-teixobactin) analogue

In 1000 mL round flask, DIEA (52 µL, 0.05 mmol, 6 equiv), Oxyma Pure (21.3 mg, 0.05 mmol, 3 equiv) and 500 mL DCM were added to 11-mer depsipeptide (98.47 mg, 0.05mmol) in 1 mL DMF. Then, it cooled to 0 °C by ice bath followed by adding PyAOP (78.2 mg, 0.05 mmol, 3equiv) to the reaction mixture. The reaction was stirred 24h.

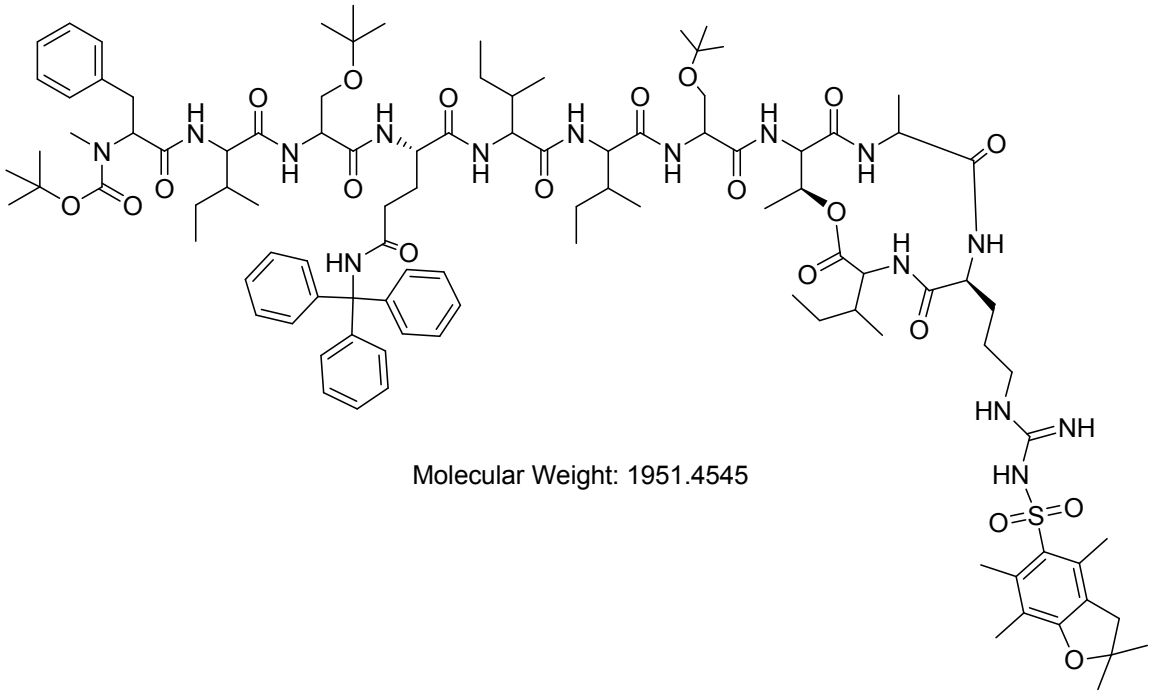
MALDI before cyclization with all protecting groups:[M+Na]



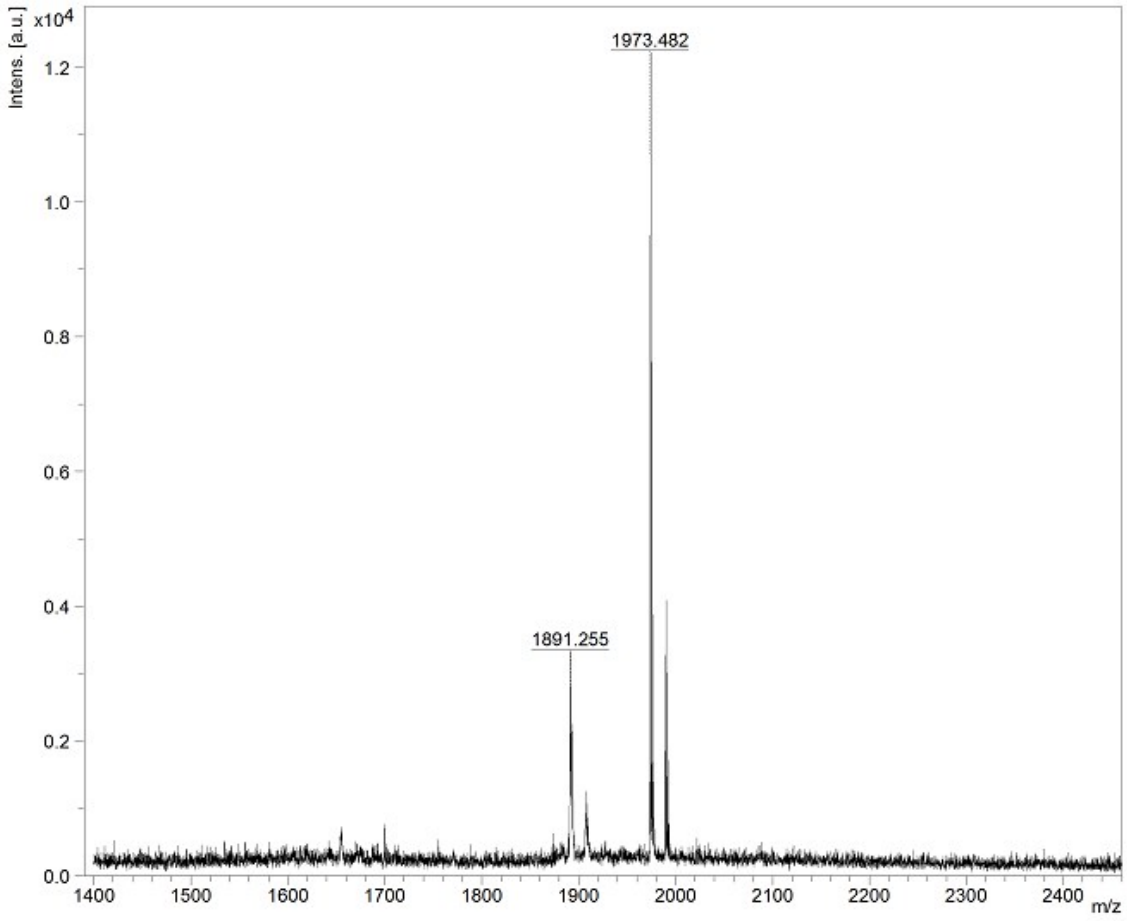
Molecular Weight: 1969.4698



MALDI after cyclization with all protecting groups: [M+Na]



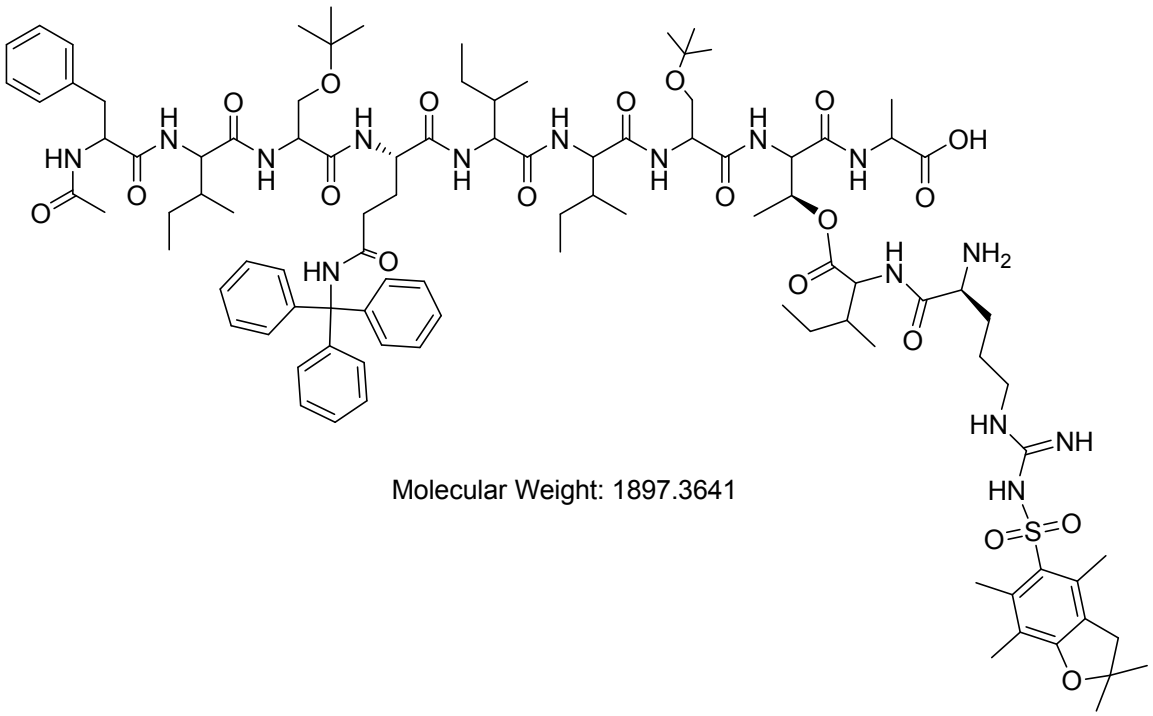
Molecular Weight: 1951.4545



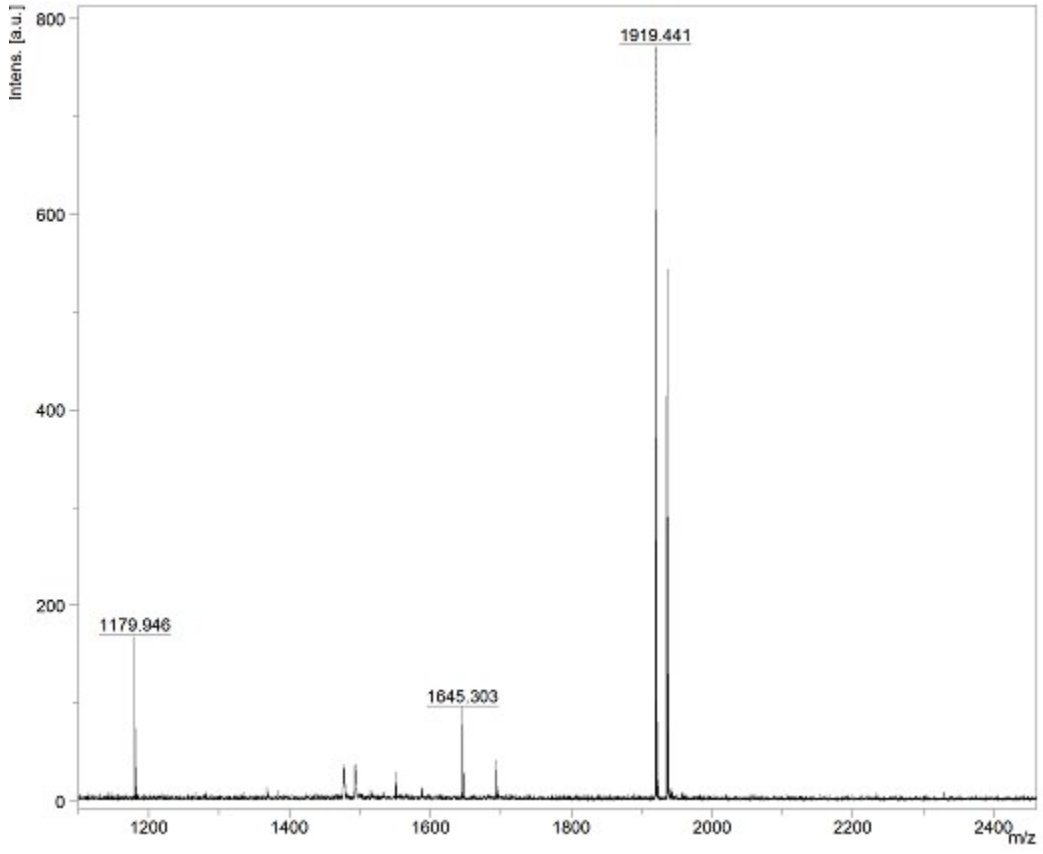
3a (Ac-D-Phe-teixobactin) analogue

In 1000 mL round flask, DIEA (58 µL, 0.05 mmol, 6 equiv), Oxyma Pure (23.3 mg, 0.05 mmol, 3 equiv) and 556 mL DCM were added to Ac- 11-mer depsipeptide (105.5 mg, 0.05mmol) in 1 mL DMF. Then, it cooled to 0 °C by ice bath followed by adding PyAOP (86.2 mg, 0.05 mmol, 3equiv) to the reaction mixture. The reaction was stirred 24h.

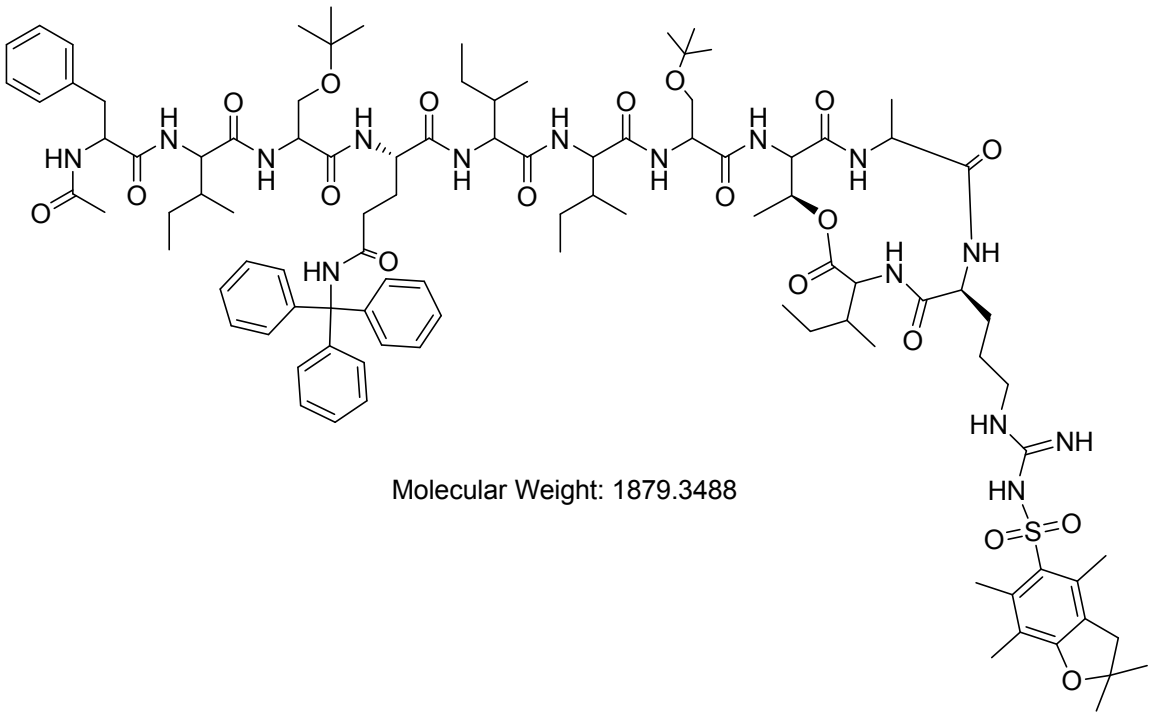
MALDI before cyclization with all protecting groups: [M+Na]



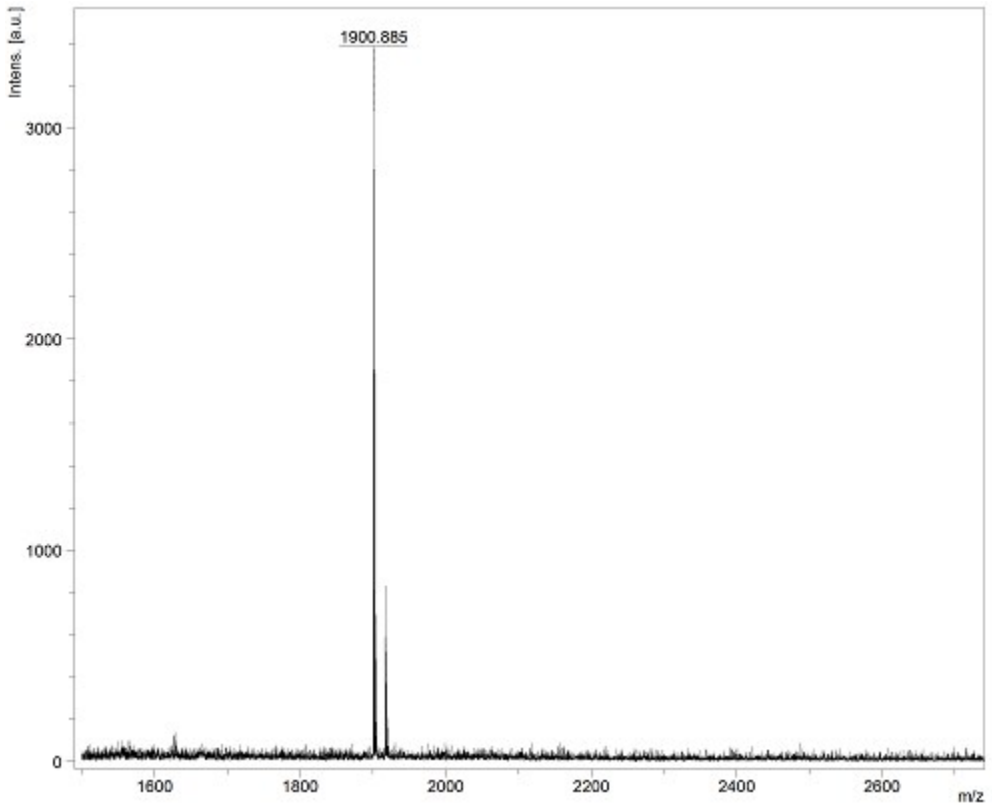
Molecular Weight: 1897.3641



MALDI after cyclization with all protecting groups: [M+Na]



Molecular Weight: 1879.3488



Then, DCM was removed by rotary evaporator while DMF was removed using phase drying. 5 mL of the TFA/TIS/H_2O (95:2.5:2.5) was added and stirred for 4 h. The solvent and residues from the cleavage cocktail were concentrated under nitrogen. The crude

peptide was precipitated with cold Et_2O (3 × 10 mL). The crude peptide was confirmed by HPLC and MALDI-TOF.

The crude peptide was purified by prep-HPLC

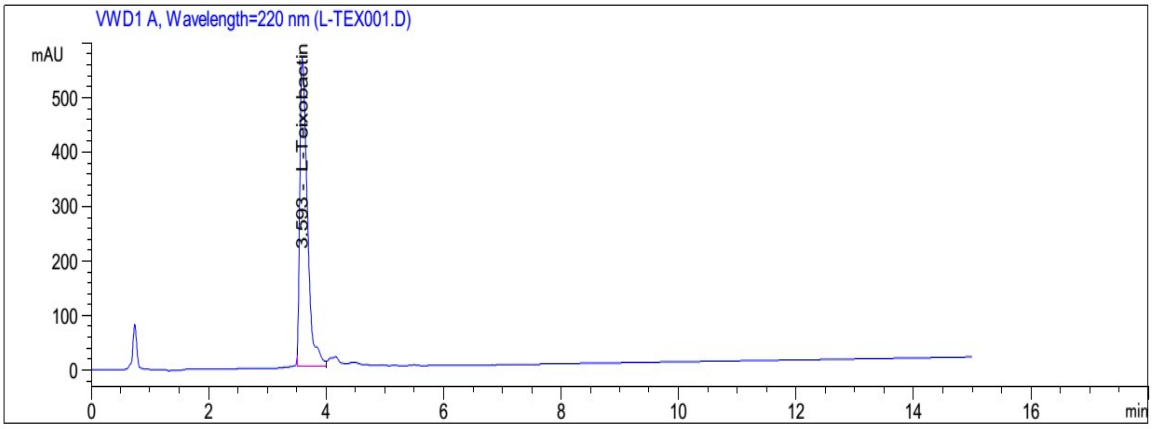
Condition:

A linear gradient of 25–50% for **2b** and 30-50% for **3a** CH_3CN/H_2O and 0.1% TFA over 30 min was applied, with a flow rate of 15.0 mL/min and detection at 220 nm using a PhenomenexLumaC_{18}(2) column (10 µm, 21.1 × 250 mm).

HPLC of the pure final product:

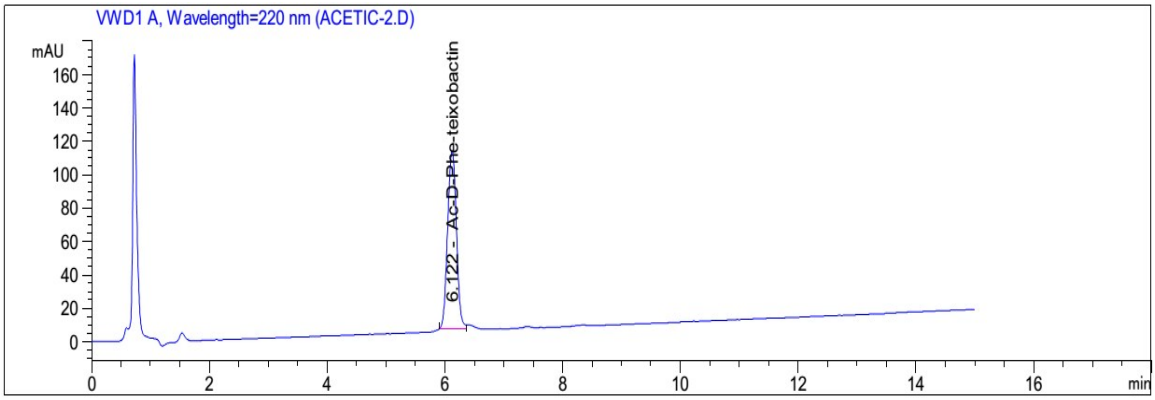
2b (L-teixobactin) analogue

A linear gradient of 25–50% CH_3CN/H_2O and 0.1% TFA over 15 min was applied, with a flow rate of 1.0 mL/min and detection at 220 nm using a Phenomenex C_{18} (3 µm, 4.6 × 50 mm) column,t_R= 3.59 min



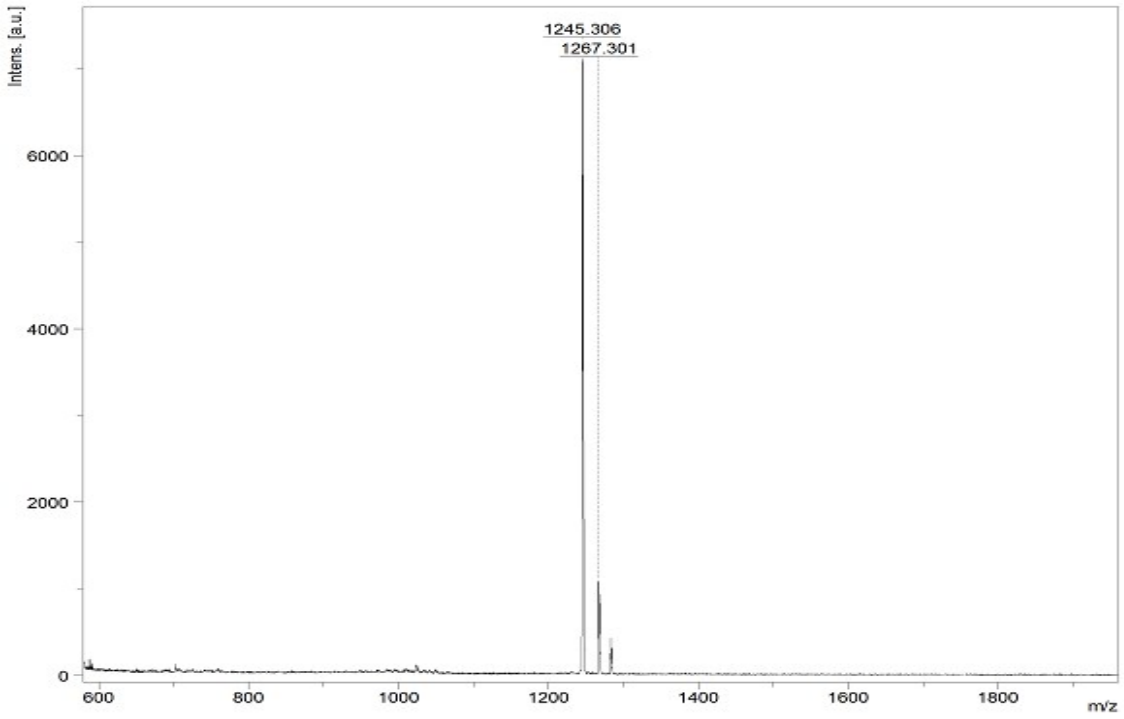
3a (Ac-D-Phe-teixobactin) analogue

A linear gradient of 30–50% CH_3CN/H_2O and 0.1% TFA over 15 min was applied, with a flow rate of 1.0 mL/min and detection at 220 nm using a Phenomenex C_{18} (3 µm, 4.6 × 50 mm) column,t_R= 6.12 min

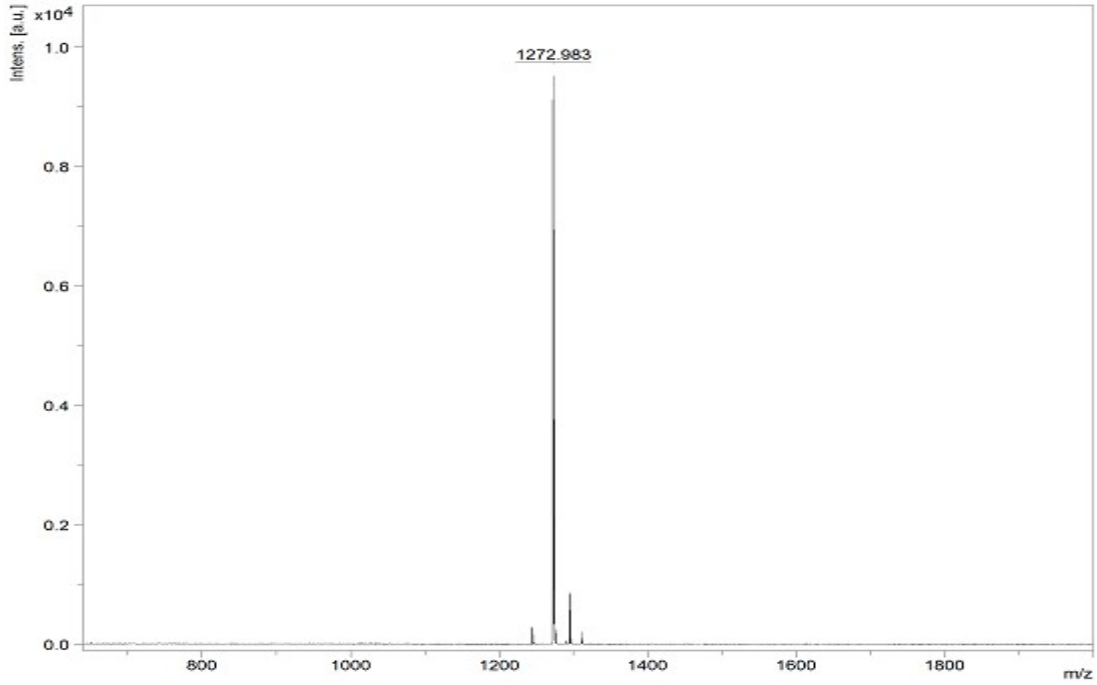

VWD1 A, Wavelength=220 nm (ACETIC-2.D)
mAU
160
140
120
100
80
60
40
20
0
6.122 - Ac-D-Phe-teixobactin
0
2
4
6
8
10
12
14
16
min

MALDI-TOF

2b (L-teixobactin) analogue

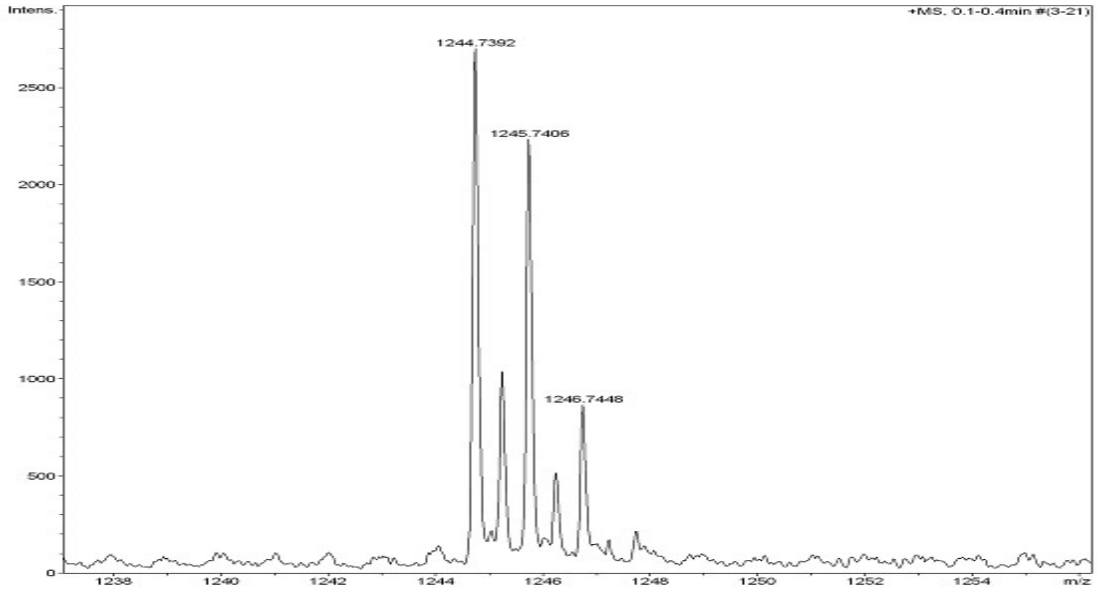


3a (Ac-D-Phe-teixobactin) analogue



HRMS

2b (L-teixobactin) analogue

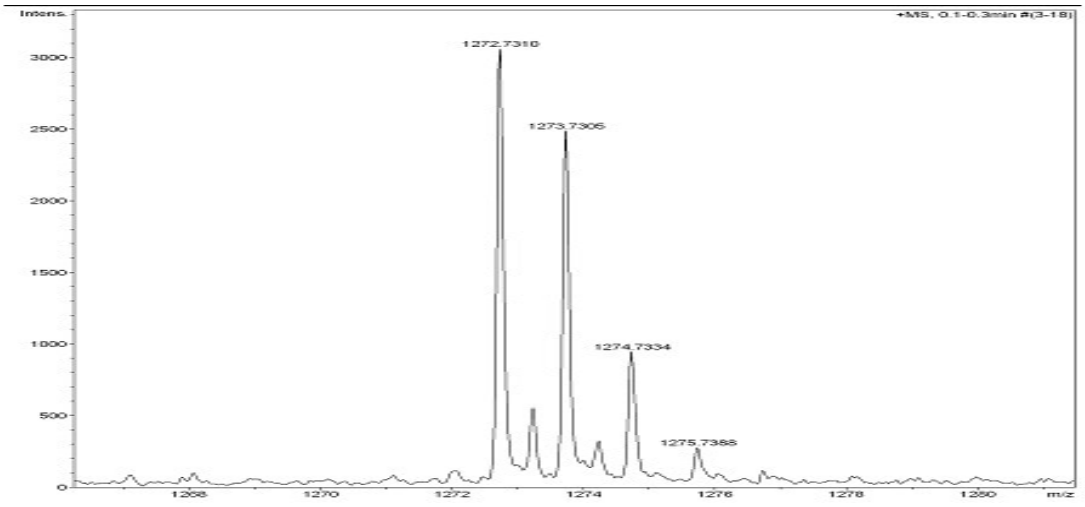


Expected exact mass of **2b** ($C_{58}H_{97}N_{15}O_{15}$) $[M+H]^+$: 1244.7361 Da

Observed exact mass of **1**$[M+H]^+$: 1244.7392 Da

Error = 1.8 ppm

3a (Ac-D-Phe-teixobactin) analogue



Expected exact mass of **3a** ($C_{59}H_{97}N_{15}O_{16}$) $[M+H]^+$: 1272.7310 Da

Observed exact mass of **1**$[M+H]^+$: 1272.7310 Da

Error = 0.0 ppm

Biological activity

Compounds and Reference bacterial strains

The compound was dissolved in sterile distilled water. The ATCC bacterial strains (2 Gram positive and 2 Gram negative) were subcultured onto Mueller Hinton agar and incubated at 37°C for 24 hours prior to use in the experiments.

Minimum Inhibitory Concentration (MIC) determination[3]

The MIC was determined using the broth micro dilution method as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines. Briefly, two-fold serial dilutions of each drug/compound were done in cation adjusted Mueller Hinton broth (CAMHB) in a 96 well microtitre plate. The bacterial inoculum was prepared in distilled water and matched to a 0.5 McFarland standard and added to make a final volume of 200µl in each microtitre well. The plates were incubated for 24 hours at 37 ^{o}C under aerobic conditions. The MIC was then recorded, as the lowest concentration at which there was no visible growth. A drug free and media control wells containing bacteria and CAMHB respectively were included in each plate. Meropenem was also tested as drug control. The assay was done in duplicate to confirm results

1 P. Gomez-Martinez, M. Dessolin, F. Guibé, F. Albericio, *Journal of the Chemical Society, Perkin Transactions 1* 1999, 2871-2874.

2 Y. E. Jad, G. A. Acosta, T. Naicker, M. Ramtahal, A. El-Faham, T. Govender, H. G. Kruger, B. G. d. l. Torre, F. Albericio, *Organic letters* 2015.

3 Clinical and Laboratory Standards Institute, 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard 8th ed. CLSI publication M07-A9. Clinical Laboratory Standard Institute, Wayne, PA