Supporting Information

Synthesis and Structure-activity Relationship Studies of Teixobactin Analogues

Chunlei Wu[+], Zhengyin Pan[+], Guiyang Yao, Wei Wang, Lijing Fang,* and Wu Su*[a]

[a]*Guangdong Key Laboratory of Nanomedicine, Institute of Biomedicine and Biotechnology, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, Guangdong, 518055, P. R. China.*

lj.fang@siat.ac.cn and wu.su@siat.ac.cn

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General Information

Abbreviations: CH_3CN, acetonitrile; CH_3OH, methanol; DIEA, N,N'-diisopropylethylamine; DMF, N,N'-dimethylformamide; TFA, trifluoroacetic acid; DCM, dichloromethane; $NaBH_3CN$, sodium cyanoborohydride; HATU, 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate; DCC, N,N'-dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; $Cu(OAc)_2$, cupric acetate monohydrate.

Dry DMF were purchased from Sigma-Aldrich. Collidine (2,4,6-collidine) and the HPLC grade solvents (CH_3CN and CH_3OH), DMAP, $NaBH_3CN$ were purchased from J&K Scientific. DIEA, TFA, 2,2-Dihydroxyindane-1,3-dione, chloranil, acetaldehyde, $Cu(OAc)_2$ were purchased from Aladdin. Fmoc-hydrazinobenzoyl AM resin was purchased from Novabiochem. HATU and Amino acids such as Fmoc-Ala-OH, Fmoc-D-Thr(OH)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ile-OH, Fmoc-D-allo-Ile-OH, Fmoc-D-Gln(Trt)-OH, Boc-D-*N*-Me-Phe-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, Fmoc-His(Trt)-OH, Fmoc-D-Asn(Trt)-OH, Fmoc-D-Arg(Pbf)-OH et al, were purchased from GL Biochem. Acetic acid, piperidine, *n*-butanol, DMF, DCM were purchased from Shanghai Lingfeng. All commercial reagents were used as received.

Analytical RP-HPLC was performed at room temperature on the Shimadzu LC 20 with UV detector SPD-20A using Inertsil ODS-SP column (4.6 x 250 mm, 5 μm, 100 Å). The RP-HPLC gradient was started at 10% of B (CH_3CN), then increased to 100% of B over 30 min (A: 0.1% TFA in water). Semi-preparative RP-HPLC was performed on the ULTIMAT 3000 Instrument (DIONEX). UV absorbance was measured using a photodiode array detector at 220 and 254 nm. The RP-HPLC gradient was started at 10% of B (CH_3CN), then increased to 100% of B over 30 min (A: 0.1% TFA in water). ^1H NMR (^{13}C NMR) spectrums were recorded with a Bruker AV600 or Bruker AV400, at 600 (150) or 400 (100) MHz respectively. Chemical shifts are referenced to either tetramethylsilane as an internal standard or the signals resulting from the residual solvent. High resolution mass spectrums were measured with an ABI Q-star Elite.

Experimental Procedure

1. Solid-phase synthesis of peptidyl resin 2 on Fmoc-hydrazinobenzoyl AM resin

Resin loading: Commercially available Fmoc-hydrazinobenzoyl AM resin (800 mg, 0.61mmol/g) was pre-swelled for 20min in DCM in a manual solid phase peptide synthesis vessel (25 mL). Fmoc removal was achieved by 20 % piperidine in DMF (2 × 3 mL, 5 min). The solution was drained and the resin was washed with DMF (6 x 3 mL) and dry DMF (3 mL). Meanwhile, Fmoc-Ala-OH (75 mg, 0.24 mmol) and HATU (91 mg, 0.24 mmol) were dissolved in dry DMF (3 mL). DIEA (120 μL, 0.72 mmol) was added to the DMF solution. After activating for 5 min, the resulting solution was added to the deprotected resin. The mixture was shaken for 2 h. The solvent was drained and the resin was washed with DMF (6 x 3 mL) and dry DMF (3 mL). Then, a mixture of pivalic anhydride (186 μL, 0.96 mmol) and DIEA (500 μL, 2.88 mmol) in dry DMF (3 mL) was added to the resin and agitated for 15 min to cap any unreacted resin sites. The solution was drained, and the resin was washed with DMF (6 x 3 mL). The resin loading was determined to be 0.3 mmol/g in the case of complete reaction of Fmoc-Ala-OH (0.5 equiv) with resin.

The next synthetic steps can use the following general methods until getting peptidyl resin **2**.

Method A: Fmoc deprotection

A solution of 20% piperidine in DMF (3 mL) was added to the resin and the resulting suspension was shaken for 5 min. Then the solution was removed from the resin. Again, a solution of 20% piperidine in DMF (3 mL) was added to the resin and the resulting suspension was shaken for another 5 min. The solution was drained and the resin was washed with DMF (6 x 3 mL) and dry DMF (3 mL).

Method B: Coupling of amino acid

Fmoc-Xaa-OH (or Boc-D-*N*-Me-Phe-OH) (0.96 mmol, 4 eq.) and HATU (365 mg, 0.96 mmol, 4 eq.) were dissolved in dry DMF (3 mL). DIEA (500 μL, 2.88 mmol, 12 eq.) was added to the DMF solution. The mixture was stirred at room temperature for 2 min, before it was transferred to the deprotected resin (800 mg, 0.3 mmol/g, 0.24

mmol, 1 eq.). The mixture was shaken until a negative Kaiser test was observed (ca. 30 min). Then the solvent was drained and the resin was rinsed with DMF (4 x 3 mL).

Method C: Kaiser test

During the coupling reaction, a few resin beads were taken out and rinsed with DMF (2 x 1 mL). To the resin beads were added 2 drops of a solution of 2,2-Dihydroxyindane-1,3-dione (15 g) and acetic acid (3 ml) in *n*-butanol (100 ml). The resulting solution was heated for 3 min at 90 ℃. No change in color of the resin indicated complete reaction. Blue- to black-stained beads indicated the presence of primary amines.

Method D: Chloranil test

A few resin beads were taken out and rinsed with DMF (2 x 1 mL). To the resin were added 2 drops of a 2% solution of acetaldehyde and 2 drops of a 2% solution of chloranil in DMF. The resulting suspension was allowed to stand for 5 min at room temperature. Blue- to green-stained beads indicated the presence of secondary amines.

2. Synthesis of peptidyl resin 3

Esterification: To a solution of Fmoc-Ile-OH (1.70 g, 4.8 mmol, 20 eq.) in DCM (2 ml) and DMF (2 ml) was added DCC (494 mg, 2.4 mmol, 10 eq.) at 0 ^{o}C. The resulting mixture was stirred at 0 ^{o}C for 1h and then at room temperature for 1 h. The resulting dicyclohexylurea was separated by centrifuge, and the solvent was transferred to peptidyl resin **2** followed by the addition of DMAP (293mg, 2.4 mmol, 10 eq.). The reaction mixture was shaken at room temperature for 2 h. Then the resin was washed with DCM (2 x 3 mL) and DMF (4 x 3 mL). This process was repeated once.

After esterification, Fmoc-Arg(Pbf)-OH was coupled to the resin according to **Method B** to provide peptidyl resin **3**.

3. **Cyclization**

After removal of the Fmoc group from **3** according to **Method A,** the peptidyl resin was suspended in a solution of $Cu(OAc)_2$ (40 mg) and pyridine (400 μL) in dry DMF (4 mL). The resulting mixture was shaken vigorously at room temperature for 12 h. Then the resin was removed by filtration through a disposable propylene filter

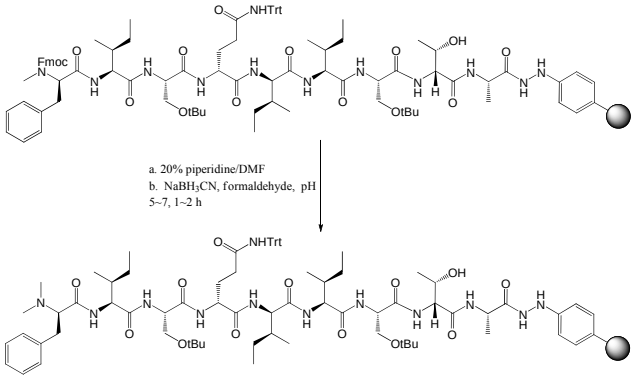
and washed with DCM (20 mL). The organic solution was concentrated under reduced pressure and the residue was purified by semi-preparative RP-HPLC. After lyophilization, peptide **4** was obtained as a white solid. HRMS (ESI) m/z: calcd for $C_{103}H_{151}N_{15}O_{20}S$ $[M+H]^+$ 1951.1059, found 1951.1050.

4. Global deprotection and purification of Arg_{10}-teixobactin (1a)

To peptide **4** was added a mixture of trifluoroacetic acid (TFA)/triisopropylsilane/H_2O (95:2.5:2.5, 2 mL) and the resulting solution was stirred at room temperature for 1 h. After the solvent was removed under reduced pressure, the residue was dissolved in 50% (v/v) CH_3CN in water (4 mL) and centrifuged at 14,000 rpm for 5 min. The solution was purified by reverse-phase HPLC (A: 0.1% TFA in H_2O, B: CH_3CN, gradient elution of 10-100% CH_3CN, 30 min). Pure fractions analyzed by analytical HPLC and electrospray ionization (ESI) mass spectrometry were combined and lyophilized. Arg_{10}-teixobactin (**1a**) was obtained in 18% yield with respect to the first loading of the resin. ^1H NMR (DMSO-d6, 400 MHz): δ 0.52-0.66 (m, 6H), 0.66-0.92 (m, 19H), 0.99-1.23 (m, 7H), 1.24-1.60 (m, 9H), 1.60-1.96 (m, 7H), 2.01-2.14 (m, 2H), 2.46 (s, 3H), 2.90-3.01 (m, 1H), 3.05-3.20 (m, 4H), 3.50-3.80 (m, 4H), 3.89-3.98 (m, 1H), 3.98-4.07 (m, 1H), 4.10-4.24 (m, 3H), 4.24-4.44 (m, 5H), 4.52-4.65 (s, 1H), 4.92-5.08 (m, 1H), 5.20-5.43 (m, 2H), 6.71-6.86 (s, 1H), 7.13-7.38 (m, 5H), 7.50-7.80 (m, 6H), 7.82-8.15 (m, 5H), 8.40-8.55 (m, 2H), 8.89-9.17 (m, 2H) ppm; HRMS (ESI) m/z: calcd for $C_{58}H_{97}N_{15}O_{15}$ $[M+H]^+$ 1244.7367, found 1244.7368.

5. Synthesis of analogues 1b–1i

Analogues 1b–**1g** and **1i** were prepared according to the similar procedures used to synthesize Arg_{10}-teixobactin (**1a**). Exceptionally, D-Me_2Phe-Arg_{10}-teixobactin (**1h**) was achieved according to the following method:



Fmoc-D-*N*-Me-Phe-OH was incorporated (**Method B**) to access the main peptidyl chain with *N*-terminal protected by Fmoc. After removal of the Fmoc group using **Method A**, the peptidyl resin was swollen with a mixture of CH_3CN/H_2O (3:1) for 15 min. The solvent was drained from the resin, 30% formaldehyde solution (1.5 ml, 12 mmol, 50 eq.) was added followed by a solution of $NaBH_3CN$ (302 mg, 4.8 mmol, 20 eq.) in CH_3CN/H_2O (3:1, 2 ml). Afterward, the pH of the reaction mixture was adjusted to between 5 and 7 by addition of acetic acid, and the resin was agitated for 1-2 h until a negative Chloranil test (**Method D**) was recorded. Other steps were similar to the synthesis of Arg_{10}-teixobactin (**1a**)

All these compounds were characterized by ^1H NMR and HRMS:

Lys_{10}-teixobactin (1b): ^1H NMR (DMSO-d6, 400 MHz): δ 0.52-0.64 (m, 6H), 0.68-0.97 (m, 19H), 0.97-1.20 (m, 7H), 1.20-1.47 (m, 8H), 1.47-1.95 (m, 10H), 1.95-2.19 (m, 2H), 2.46 (s, 3H), 2.62-2.89 (m, 2H), 2.89-3.01 (m, 1H), 3.02-3.18 (m, 1H), 3.49-3.78 (m, 4H), 3.87-4.08 (m, 2H), 4.08-4.50 (m, 9H), 4.50-4.70 (s, 1H), 4.87-5.13 (s, 1H), 5.20-5.45 (m, 2H), 6.70-6.88 (s, 1H), 7.11-7.38 (m, 5H), 7.50-7.85 (m, 6H), 7.88-8.20 (m, 5H), 8.30-8.59 (m, 2H), 8.92-9.30 (m, 2H) ppm; HRMS (ESI) m/z: calcd for $C_{58}H_{98}N_{13}O_{15}$ $[M+H]^+$ 1216.7305, found 1216.7280.

His_{10}-teixobactin (1c): ^1H NMR (DMSO-d6, 400 MHz): δ 0.50-0.69 (m, 6H), 0.69-0.91 (m, 16H), 0.95-1.35 (m, 11H), 1.35-1.59 (m, 3H), 1.61-2.00 (m, 5H), 1.95-2.20 (m, 2H), 2.46 (s, 3H), 2.90-3.20 (m, 4H), 3.49-3.77 (m, 5H), 3.82-3.92 (m, 1H), 3.95-4.03 (m, 1H), 4.09-4.28 (m, 3H), 4.28-4.43 (m, 3H), 4.51-4.78 (m, 2H), 4.90-5.10 (m, 1H), 5.15-5.40 (m, 2H), 6.71-6.85 (m, 1H), 7.15-7.37 (m, 5H), 7.70-7.90 (m, 2H), 7.90-8.19 (m, 5H), 8.32-8.57 (m, 2H), 8.87-8.79 (s, 1H) ppm;

HRMS (ESI) m/z: calcd for $C_{58}H_{92}N_{14}O_{15}$ $[M+H]^+$ 1225.6945, found 1225.6944.

Ala$_7$-Arg$_{10}$-teixobactin (1d): ^{1}H NMR (DMSO-d6, 400 MHz): δ 0.65-0.88 (m, 7H), 0.88-1.05 (m, 16H), 1.05-1.42 (m, 11H), 1.40-1.76 (m, 12H), 1.76-2.24 (m, 9H), 2.27-2.39 (m, 2H), 2.69 (s, 3H), 3.05-3.15 (m, 1H), 3.17-3.30 (m, 3H), 3.70-3.87 (m, 2H), 4.00-4.34 (m, 8H), 4.37-4.47 (m, 2H), 4.49-4.60 (m, 1H), 4.71-4.85 (m, 1H), 5.52-5.62 (m, 1H), 7.73-7.61 (m, 5H), 8.09-8.30 (m, 3H), 8.35-8.54 (m, 3H) ppm; HRMS (ESI) m/z: calcd for $C_{58}H_{98}N_{15}O_{114}$ $[M+H]^+$ 1228.7418, found 1228.7411.

D-Asn$_4$-Arg$_{10}$-teixobactin (1e): ^{1}H NMR (DMSO-d6, 400 MHz): δ 0.58-0.67 (m, 7H), 0.67-0.90 (m, 21H), 0.99-1.20 (m, 8H), 1.20-1.30 (m, 2H), 1.30-1.60 (m, 11H), 1.65-1.89 (m, 5H), 2.30-2.62 (m, 10H), 2.85-3.02 (m, 1H), 3.02-3.21 (m, 4H), 3.48-3.62 (m, 2H), 3.62-3.79 (m, 2H), 3.85-4.00 (m, 1H), 4.00-4.10 (m, 1H), 4.10-4.26 (m, 3H), 4.26-4.40 (m, 4H), 4.50-4.67 (m, 1H), 4.82-5.10 (s, 1H), 5.18-5.40 (m, 2H), 6.75-6.69 (s, 2H), 7.19-7.37(m, 5H), 7.40-7.59 (m, 2H), 7.60-7.69 (m, 2H), 7.69-7.80 (m, 1H), 7.95-8.30 (m, 4H), 8.35-8.60 (m, 2H), 8.90-9.25 (m, 2H) ppm; HRMS (ESI) m/z: calcd for $C_{57}H_{96}N_{15}O_{15}$ $[M+H]^+$ 1230.7210, found 1230.7219.

D-Arg$_4$-Arg$_{10}$-teixobactin (1f): ^{1}H NMR (DMSO-d6, 400 MHz): δ 0.52-0.69 (m, 6H), 0.74-0.91 (m, 19H), 0.95-1.20 (m, 7H), 1.25-1.59 (m, 12H), 1.59-1.73 (m, 3H), 1.73-1.90 (m, 3H), 2.46 (s, 3H), 2.90-3.00 (m, 1H), 3.00-3.20 (m, 5H), 3.50-3.62 (m, 2H), 3.62-3.80 (m, 2H), 3.83-3.99 (m, 1H), 3.99-4.10 (m, 1H), 4.10-4.42 (m, 8H), 4.55-4.65 (m, 1H), 4.95-5.07 (br s, 1H), 5.20-5.45 (m, 2H), 6.73-7.11 (m, 4H), 7.11-7.38 (m, 5H), 7.50-7.66 (m, 2H), 7.70-7.82 (m, 2H), 7.90-8.05 (m, 2H), 8.05-8.55 (m, 3H), 8.92-9.25 (m, 1H) ppm; HRMS (ESI) m/z: calcd for $C_{59}H_{102}N_{17}O_{14}$ $[M+H]^+$ 1272.7792, found 1272.7782.

D-Phe$_1$-Arg$_{10}$-teixobactin (1g): ^{1}H NMR (DMSO-d6, 400 MHz): δ 0.60-0.80 (m, 23H), 1.00-1.55 (m, 18H), 1.55-1.91 (m, 9H), 1.95-2.16 (m, 3H), 2.87-3.20 (m, 6H), 3.50-3.83 (m, 8H), 3.90-4.07 (m, 2H), 4.11-4.40 (m, 8H), 4.50-4.68 (s, 1H), 4.97-5.08 (m, 1H), 5.19-5.40 (m, 2H), 6.74-6.90 (s, 2H), 7.20-7.40 (m, 6H), 7.54-7.85 (m, 4H), 7.88-8.30 (m, 8H), 8.38-8.48 (s, 1H), 8.50-8.60 (d, 1H) pm; HRMS (ESI) m/z: calcd for $C_{57}H_{96}N_{15}O_{15}$ $[M+H]^+$ 1230.7210, found 1230.7190.

D-Me_2Phe_1-Arg_{10}-teixobactin (1h): ^{1}H NMR (DMSO-d6, 400 MHz): δ 0.42-0.70 (m, 7H), 0.70-0.98 (m, 20H), 1.00-1.20 (m, 8H), 1.20-1.55 (m, 2H), 1.55-1.90 (m, 3H), 1.95-2.17 (m, 3H), 2.67-3.04 (m, 2H), 3.04-3.20 (m, 4H), 3.47-3.80 (m, 10H), 3.88-4.09 (m, 1H), 4.09-4.40 (m, 4H), 4.50-4.66 (s, 2H), 4.90-5.04 (s, 2H), 5.15-5.41 (m, 1H), 6.70-6.95 (s, 1H), 7.10-7.37 (m, 3H), 7.50-7.81 (m, 4H), 7.84-8.18 (m, 1H), 8.32-8.58 (s, 1H), 10.03-10.32 (d, 2H) ppm; HRMS (ESI) m/z: calcd for $C_{59}H_{100}N_{15}O_{15}$ $[M+H]^+$ 1258.7523, found 1258.7512.

D-Tyr_1-Arg_{10}-teixobactin (1i): ^{1}H NMR (DMSO-d6, 400 MHz): δ 0.70-0.95 (m, 24H), 1.02-1.20 (m, 6H), 1.20-1.56 (m, 11H), 1.56-1.95 (m, 9H), 1.96-2.17 (m, 3H), 2.73-2.89 (m, 1.5H), 2.89-3.00 (m, 1.5H), 3.06-3.20 (m, 3H), 3.50-3.80 (m, 5H), 3.90-4.15 (m, 3H), 4.15-4.23 (m, 1H), 4.23-4.42 (m, 6H), 4.50-4.65 (s, 1H), 4.92-5.08 (s, 1H), 5.15-5.40 (m, 2H), 6.63-6.78 (d, 2H), 6.78-6.84 (s, 1H), 7.03-7.12 (d, 3H), 7.20-7.30 (s, 2H), 7.55-7.83 (m, 5H), 7.90-8.15 (m, 7H), 8.15-8.29 (d, 1H), 8.38-8.49 (s, 1H), 8.49-8.57 (d, 1H), 9.31-9.40 (s, 1H) ppm; HRMS (ESI) m/z: calcd for $C_{57}H_{96}N_{15}O_{16}$ $[M+H]^+$ 1246.7159, found 1246.7161.

6. Minimum inhibitory concentration (MIC) assay

MIC assays of teixobactin analogues **1a-1i** were determined by using a broth microdilution method according to CLSI[1]. *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633) and *Escherichia coli* (ATCC 25922) were acquired as freeze-dried powders from ATCC. The MIC was defined as the lowest concentration of antibiotic which resulted in no visible growth. Meropenem was also tested as drug control. The assay was done in duplicate in three independent runs to confirm results.

Copies of HRMS spectrums and ^{1}H NMR spectrums of teixobactin analogues

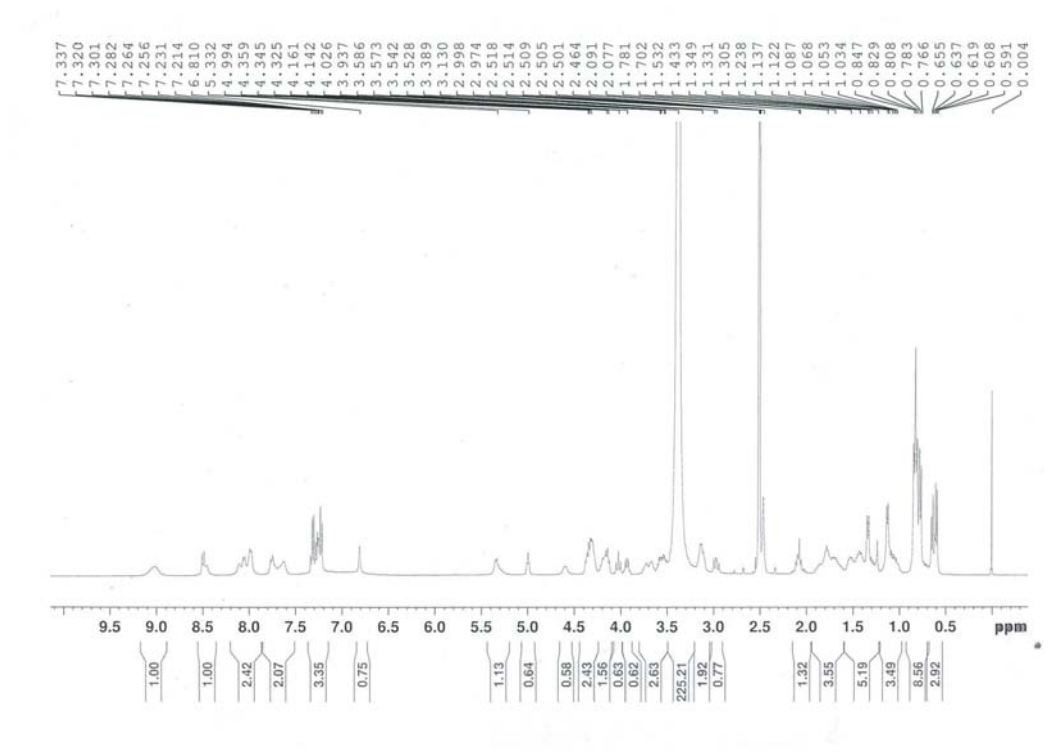


Figure S1. ^{1}H NMR spectra of Arg_{10}-teixobactin (**1a**)

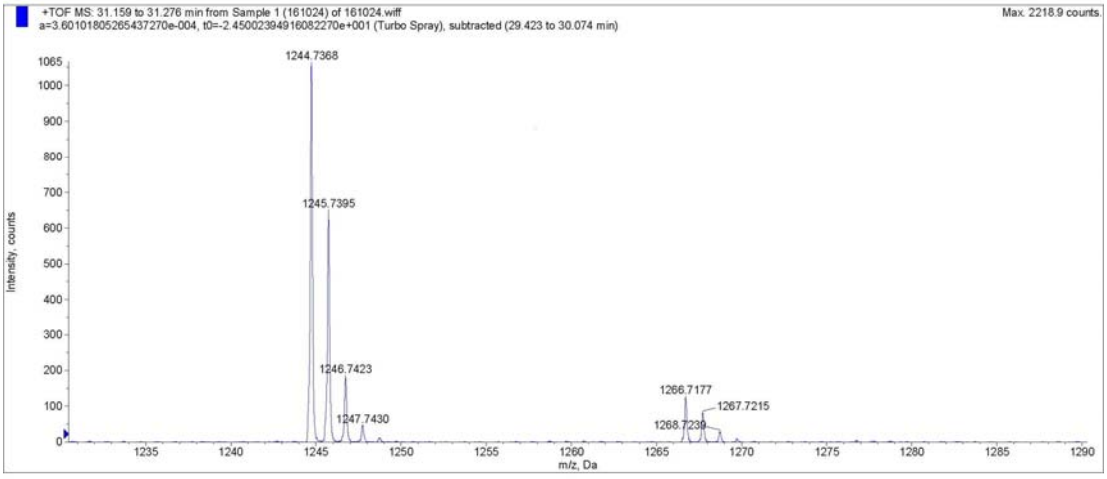


Figure S2. HRMS spectra of Arg_{10}-teixobactin (**1a**)

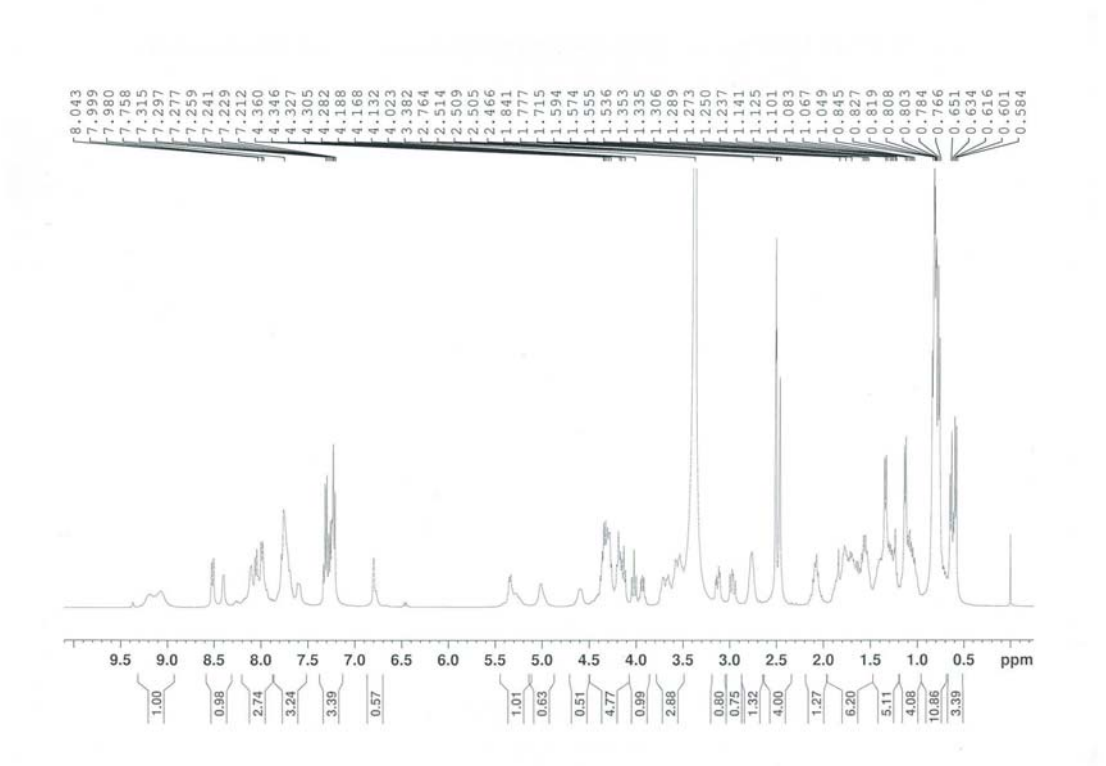


Figure S3. ^{1}H NMR spectra of Lys_{10}-teixobactin (**1b**)

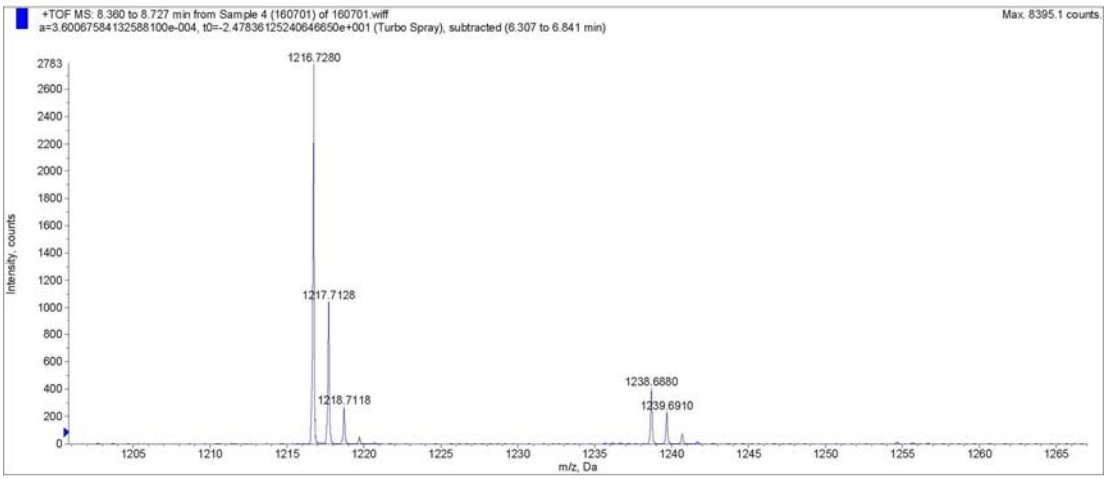


Figure S4. HRMS spectra of Lys_{10}-teixobactin (**1b**)

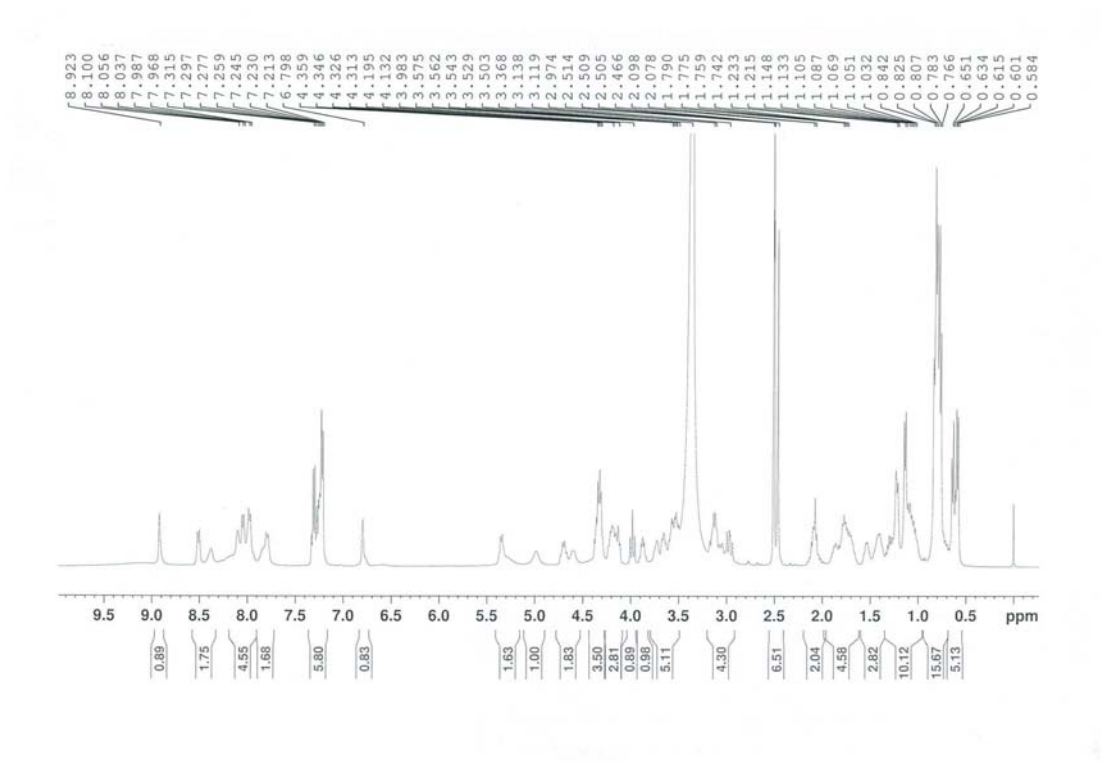


Figure S5. ^{1}H NMR spectra of His_{10}-teixobactin (**1c**)

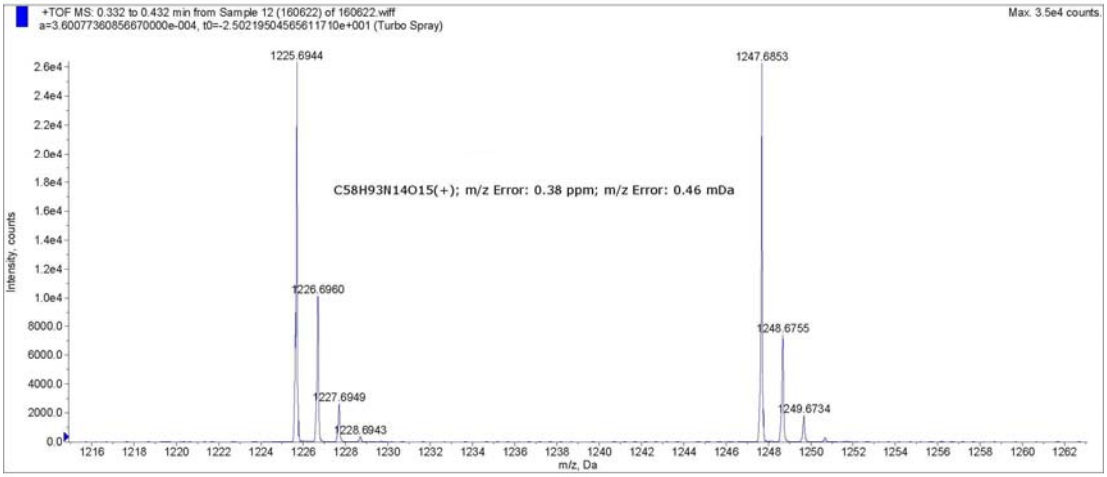


Figure S6. HRMS spectra of His_{10}-teixobactin (**1c**)

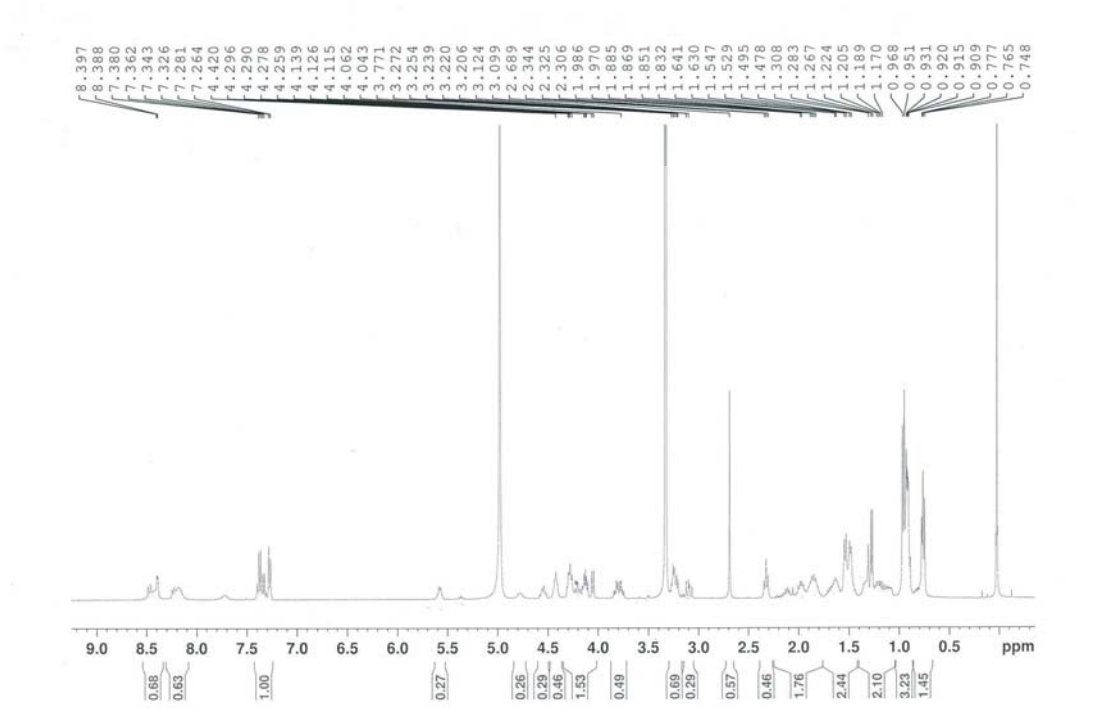


Figure S7. ^{1}H NMR spectra of Ala_7 -Arg_{10}-teixobactin (**1d**)

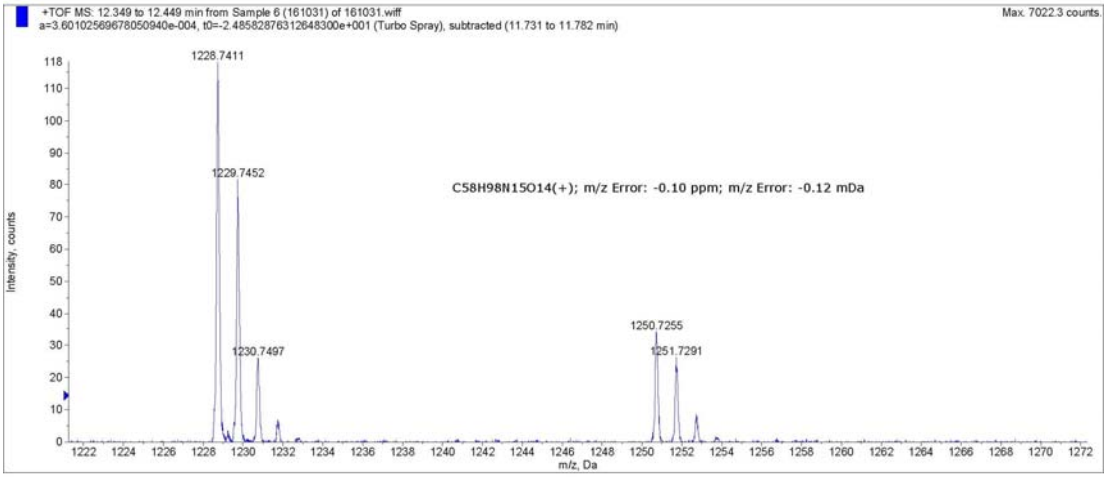


Figure S8. HRMS spectra of Ala_7- Arg_{10}-teixobactin (**1d**)

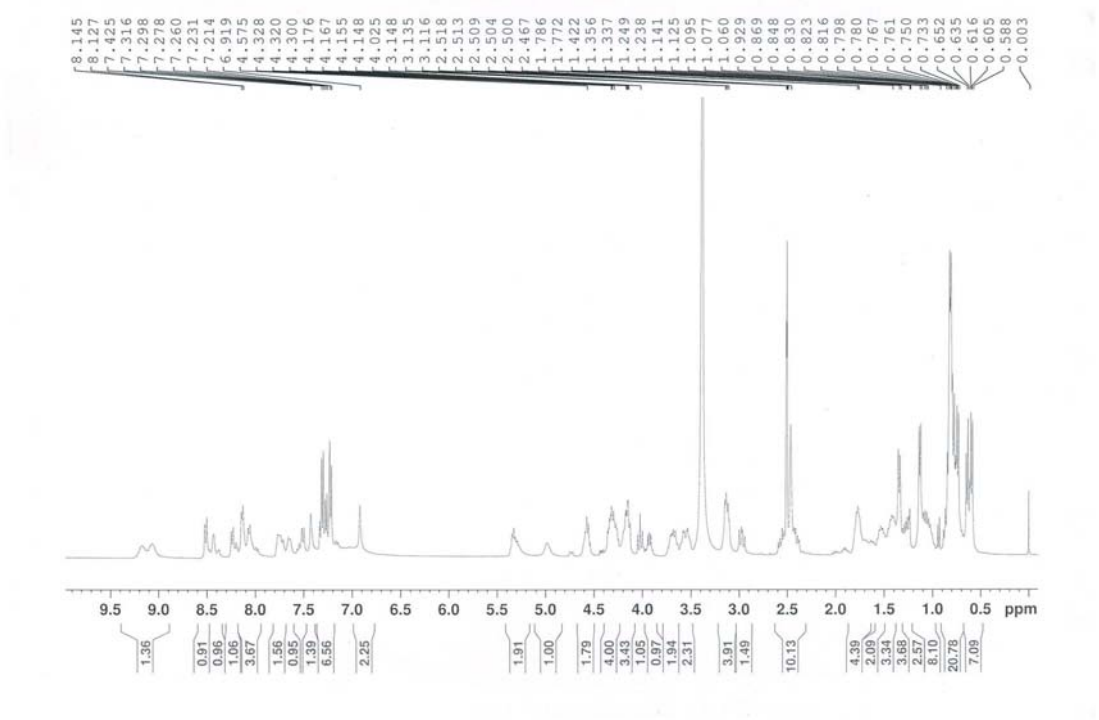


Figure S9. ^{1}H NMR spectra of D-Asn$_4$- Arg$_{10}$-teixobactin (**1e**)

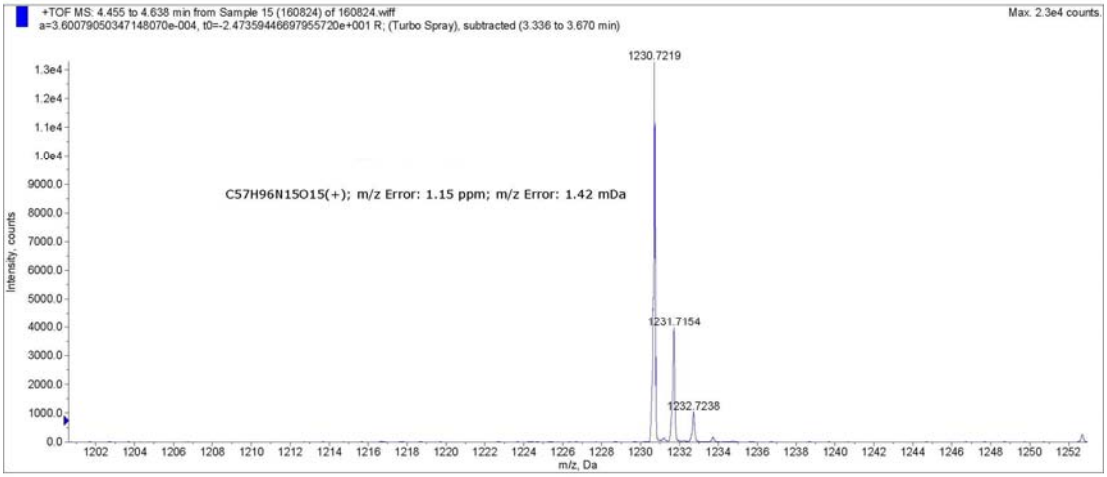


Figure S10. HRMS spectra of D-Asn$_4$- Arg$_{10}$-teixobactin (**1e**)

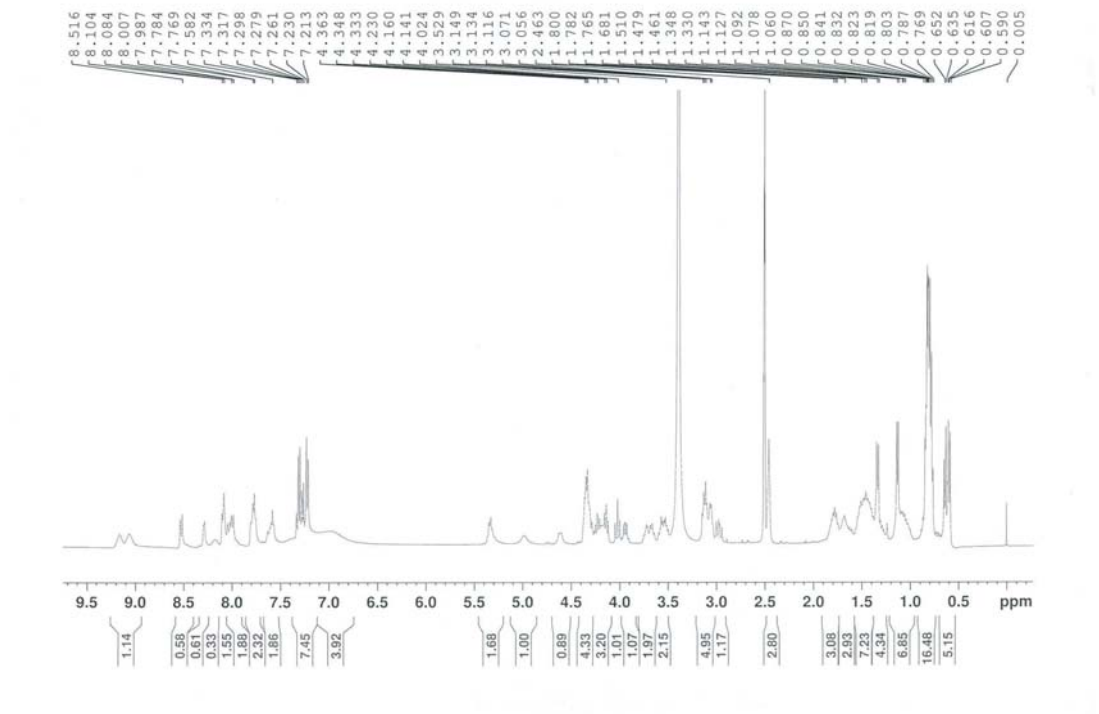


Figure S11. ^{1}H NMR spectra of D-Arg$_4$-Arg$_{10}$-teixobactin (**1f**)

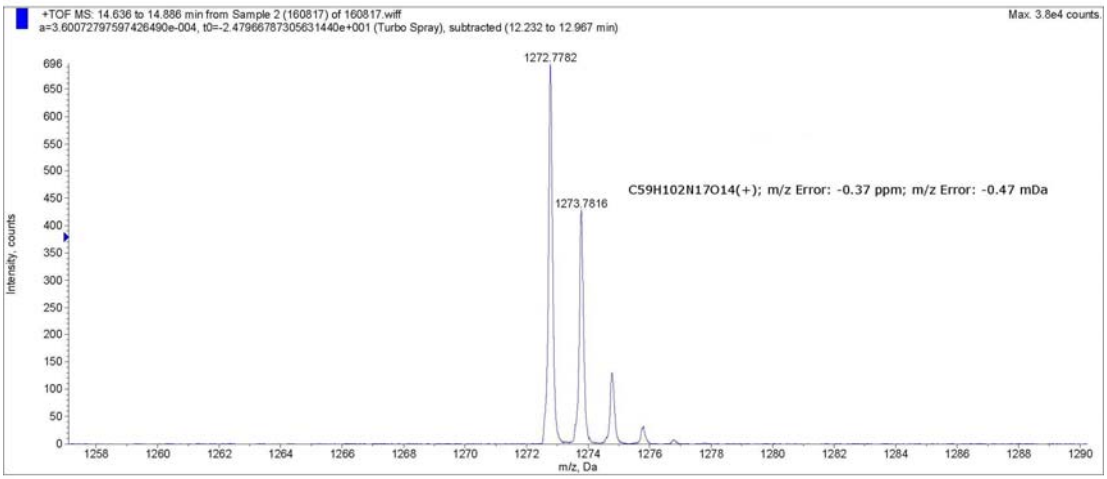


Figure S12. HRMS spectra of D-Arg$_4$- Arg$_{10}$-teixobactin (**1f**)

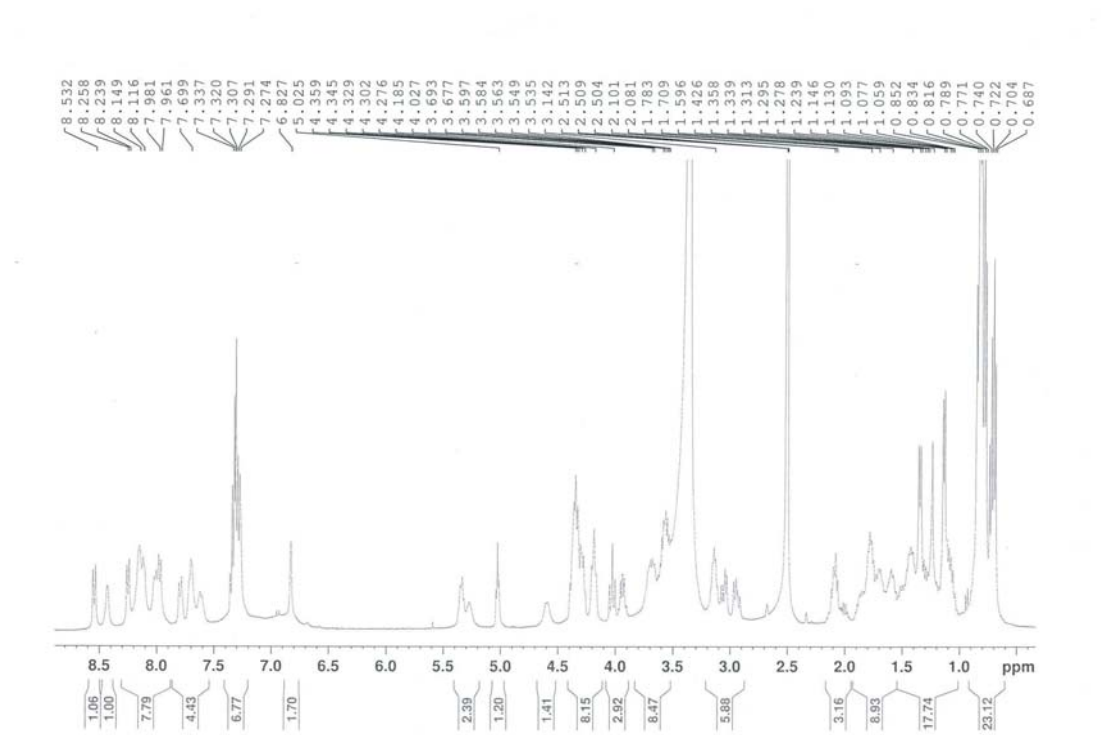


Figure S13. ^{1}H NMR spectra of D-Phe$_1$- Arg$_{10}$-teixobactin (**1g**)

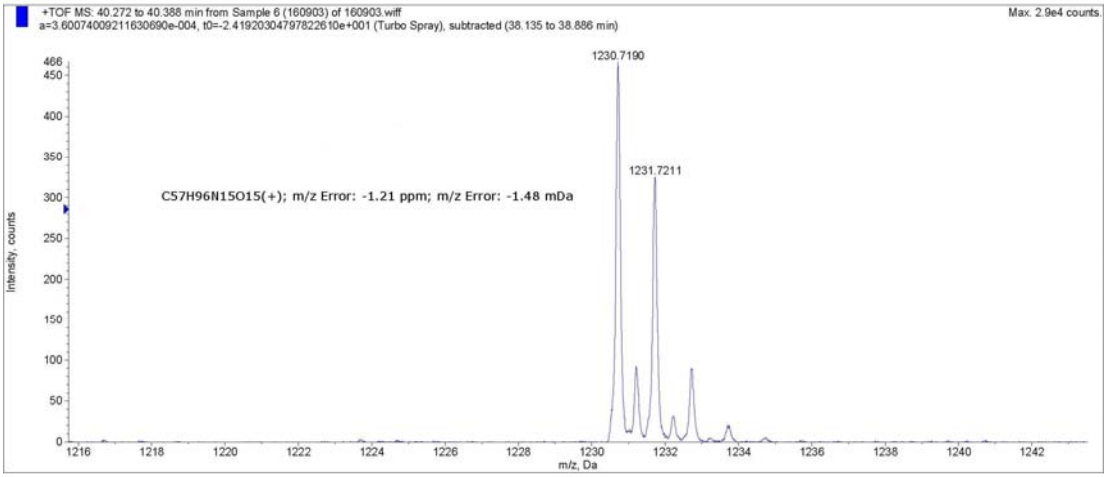


Figure S14. HRMS spectra of D-Phe$_1$- Arg$_{10}$-teixobactin (**1g**)

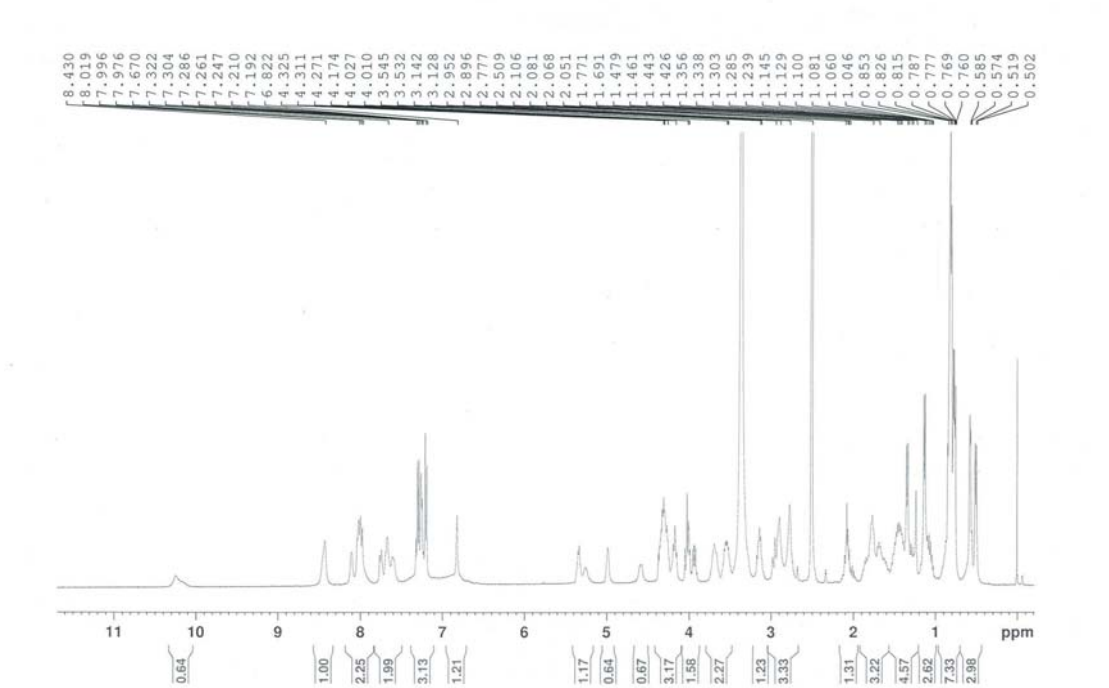


Figure S15. ^{1}H NMR spectra of D-Me$_2$Phe$_1$- Arg$_{10}$-teixobactin (**1h**)

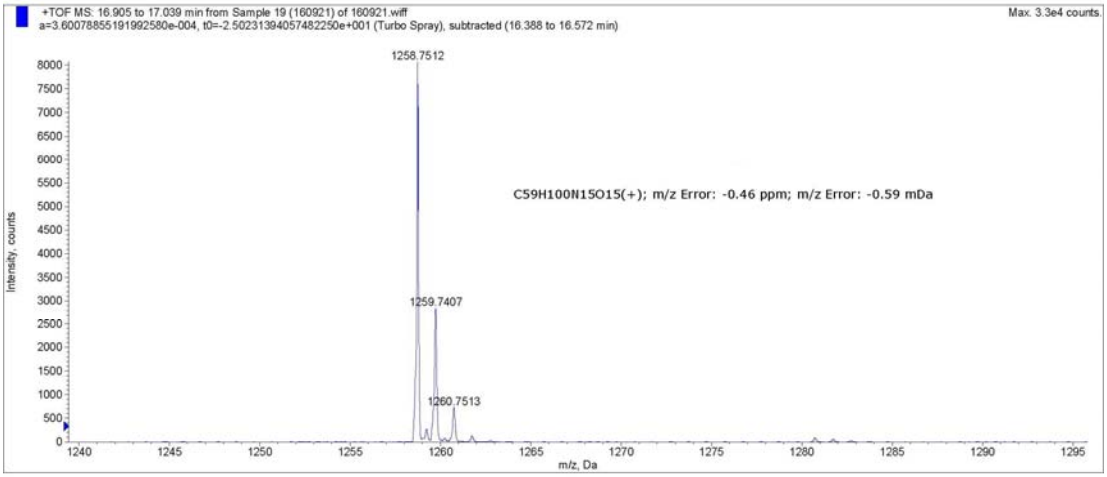


Figure S16. HRMS spectra of D-Me$_2$Phe$_1$- Arg$_{10}$-teixobactin (**1h**)

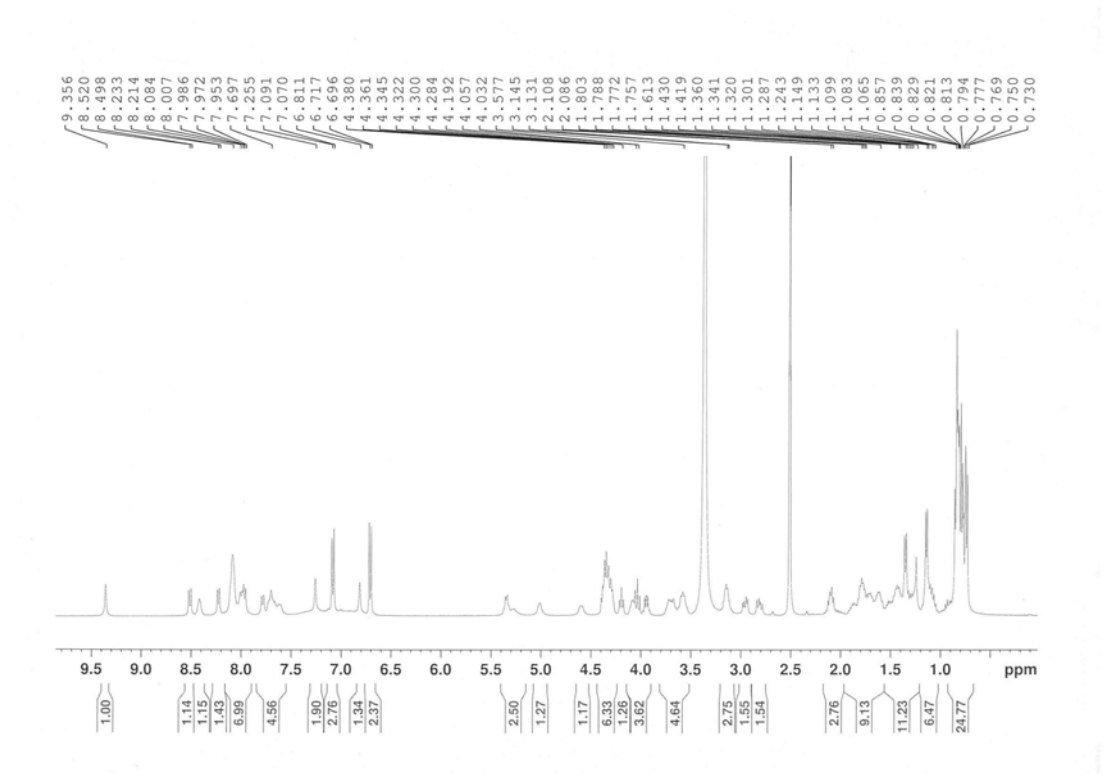


Figure S17. ^{1}H NMR spectra of D-Tyr$_1$- Arg$_{10}$-teixobactin (**1i**)

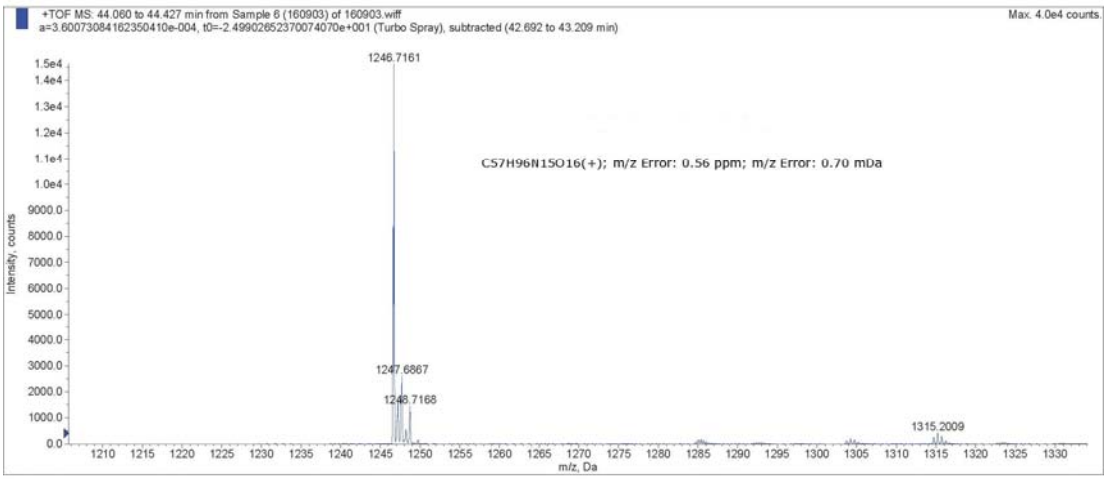


Figure S18. HRMS spectra of D-Tyr$_1$- Arg$_{10}$-teixobactin (**1i**)

1. CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. Approved Standard—Ninth Edition. CLSI document M07-A9. Wayne, PA: Clinical and Laboratory Standards Institute; 2012.