Supporting Information for

Lysine Scanning of Arg₁₀-Teixobactin. Deciphering the Role of Hydrophobic and Hydrophilic Residues.

Shimaa A. H. Abdel Monaim, Yahya E. Jad, Estelle J. Ramchuran, Ayman El-Faham, Thavendran Govender, Hendrik G. Kruger, Beatriz G. de la Torre and Fernando Albericio

a Catalysis and Peptide Research Unit, School of Health Sciences, University of KwaZulu-Natal, University Road, Durban 4001, South Africa
b Department of Chemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh11451, Saudi Arabia
c Department of Chemistry, Faculty of Science, Alexandria University, P.O. Box 426, Ibrahimia, Alexandria 21321, Egypt
d School of Chemistry and Physics, University of KwaZulu-Natal, University Road, Durban 4001, South Africa
e Department of Organic Chemistry, University of Barcelona, Martí i Franquès 1-11, 08028-Barcelona, Spain
f CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028-Barcelona, Spain

Experimental section

Synthesis of Alloc amino acids
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 vacuum evaporator then wash the crude several times using hexane. 100mL 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, DCM was evaporated and characterized HPLC, HPL-MS, NMR and they matched the spectra in literature.¹

Synthesis of modified analogues of L-Arg teixobactin
a) Synthesis of tetrapeptide (in all cases except Lys₁₁-Teixobactin)
166 mg of 2-Cl-Trt resin (1.69 mmolg⁻¹) was placed in a 10mL falcon tube and then was activated by 10 % SOCl₂ in DCM for overnight. 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-A.A.-OH (0.1 mmol) and DIEA (174 µL, 1 mmol, 10 equiv) in 0.5mL DCM and shacked for 1 h. Then 100 µL MeOHwas 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-A.A.-OH/HATU/DIEA (3:3:6) in 0.5 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, mini-cleavage was performed in order to monitor the pre-esterification step using analytical HPLC and LC-MS using the same condition as our previous work.²

b) Ester-bond formation

In this report the esterification step was facilitated by the use of Alloc-L-Ile-OH/DIC/DMAP (10:10:1equiv.) in DCM/DMF (8:2) (1 ×2 h).

Figure S1: HPLC of esterification step (starting material appears at R.t=8.489min and the product at R.t= 10.7min in 94.3% yield).

Figure S2: LC-MS of esterification step:

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) (11mg, 0.01mmol, 0.1equiv) in dry DCM (1mL) was added. The reaction vessel was flushed with nitrogen and shaken for 15 min. Mini-cleavage was performed in order to control the reaction.

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.

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 × 30s) 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:

In 1000 mL round flask, DIEA (6 equiv), Oxyma Pure (3 equiv) dissolved in DCM were added to 11-mer depsipeptide(1 equiv) in 0.5 mL DMF. Then, it cooled to 0 °C by ice bath followed by adding PyAOP (3 equiv) to the reaction mixture. The reaction was stirred 24h. (The peptide concentration must not be more than 0.0001M).

Then, DCM was removed by rotary evaporator while DMF was removed using phase drying. 5 mL of the TFA/TIS/H$_2$O (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 and washed with cold Et$_2$O (3 × 10 mL). The crude peptide was confirmed by HPLC and MALDI-TOF. The crude peptide was purified by prep-HPLC using the following condition: A linear gradient of 30–50% CH$_3$CN/H$_2$O and 0.1% TFA over 30 min was applied, with a flow rate of 7.0 mL/min and detection at 220 nm using a PhenomenexLunaC18(2) 100Å column (10 µm × 250 mm).

Figure S3: Illustration of Lys scanning on the Arg$_{10}$-teixobactin analogue

Table S1: HPLC/ MALDI/ HR-MS for analogues after purification:

| Analogue number | Retention time (HPLC) | Theoretical Mass | HR-MS       |
|-----------------|-----------------------|-----------------|-------------|
| Lys$_2$-teixobactin | 6.634                 | 1259.7470       | 1259.7423   |
| Lys$_3$-teixobactin | 9.205                 | 1285.7991       | 1285.7945   |
| Peptide          | R (%) | MW (Da)          | M+H (Da)    |
|------------------|-------|------------------|-------------|
| Lys4-teixobactin | 9.358 | 1244.7725        | 1244.7688   |
| Lys5-teixobactin | 6.758 | 1259.7470        | 1259.7346   |
| Lys6-teixobactin | 5.205 | 1259.7470        | 1259.7423   |
| Lys7-teixobactin | 6.419 | 1285.7991        | 1285.7973   |
| Lys9-teixobactin | 7.249 | 651.4006(M+2)    | 651.3968(M+2) |
| Lys11-teixobactin | 6.388 | 1259.7470        | 1259.7489   |

Figure S4: HPLC of **Lys2-teixobactin** after purification

![HPLC graph of Lys2-teixobactin](image)

Figure S5: MALDI of **Lys2-teixobactin** after purification

![MALDI graph of Lys2-teixobactin](image)

Figure S6: HR-MS of **Lys2-teixobactin** after purification

![HR-MS graph of Lys2-teixobactin](image)
Figure S7: HPLC of Lys$_3$-teixobactin after purification

Figure S8: MALDI of Lys$_3$-teixobactin after purification
Figure S9: HR-MS of Lys$_3$-teixobactin after purification

Figure S10: HPLC of Lys$_4$-teixobactin after purification

Figure S11: MALDI of Lys$_4$-teixobactin after purification
Figure S12: HR-MS of **Lys$_4$-teixobactin** after purification

![HR-MS Graph]

Figure S13: HPLC of **Lys$_5$-teixobactin** after purification

![HPLC Graph]

Figure S14: MALDI of **Lys$_5$-teixobactin** after purification

![MALDI Graph]
Figure S15: HR-MS of Lys$_5$-teixobactin after purification

Figure S16: HPLC of Lys$_6$-teixobactin after purification

Figure S17: MALDI of Lys$_6$-teixobactin after purification
Figure S18: HR-MS of **Lys₆-teixobactin** after purification

Figure S19: HPLC of **Lys₇-teixobactin** after purification

Figure S20: MALDI of **Lys₇-teixobactin** after purification
Figure S21: HR-MS of \textbf{Lys}_7-teixobactin after purification

Figure S22: HPLC of \textbf{Lys}_9-teixobactin after purification

Figure S23: MALDI of \textbf{Lys}_9-teixobactin after purification
Figure S24: HR-MS of Lys$_9$-teixobactin after purification

Figure S25: HPLC of Lys$_{11}$-teixobactin after purification

Figure S26: MALDI of Lys$_{11}$-teixobactin after purification
Figure S27: HR-MS of Lys$_{11}$-teixobactin after purification
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
The MIC was determined using the broth microdilution 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 °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.

References:

(1) Jad, Y. E.; Acosta, G. A.; Naicker, T.; Ramtahal, M.; El-Faham, A.; Govender, T.; Kruger, H. G.; Torre, B. G. d. l.; Albericio, F., (2015) Synthesis and biological evaluation of a teixobactin analogue. Organic letters,17, 6182-6185.
(2) Abdel Monaim, S. A.; Jad, Y. E.; Acosta, G. A.; Naicker, T.; Ramtahal, M.; El-Faham, A.; Govender, T.; Kruger, H. G.; Torre, B. G. d. l.; Albericio, F., (2016) Re-evaluation of the N-terminal substitution and the D-residues of teixobactin. RSC Advances,6, 73827-73829.