

Supporting Information

Triple-Targeting Gram-Negative Selective antimicrobial peptides capable of Disrupting the Cell Membrane and Lipid A Biosynthesis

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General Procedures

Fmoc-amino acids and *N,N,N',N'*-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were obtained from Activotec (Cambridge, United Kingdom). Oxyma Pure and Tentagel S RAM resin were obtained from IRIS Biotech (Marktredwitz, Germany). Methyl *tert*-butyl ether (MTBE), Hexane (HPLC grade) and TFA were obtained from Aldrich (Milwaukee, USA). DMF (Peptide grade) was obtained from VWR (Lutterworth, United Kingdom). Piperidine and DIPEA were obtained from AGTC Bioproducts (Hessle, United Kingdom). HPLC grade CH₂Cl₂ and acetonitrile were obtained from Fischer Scientific (Loughborough, United Kingdom). All reagents and solvents were used as received.

Solid-phase syntheses were carried out on an automated CS BIO 336X peptide synthesizer at room temperature.

Analytical high pressure liquid chromatography (HPLC) was carried out on a Shimadzu instrument comprising a communication module (CBM-20A), autosampler (SIL-20HT), pump modules (LC-20AT), UV/Vis detector (SPD-20A) and system controller (Labsolutions V5.54 SP), with an Dr Maisch Reprosil Gold 200 C18 reversed-phase analytical column (6.4 mm × 250 mm, 5 μm). UV measurements were recorded at 214 and 254 nm, and linear gradients of buffer B (acetonitrile/H₂O 95:5 with 0.1% TFA) into buffer A (acetonitrile/H₂O 5:95 with 0.1% TFA) over 30 min were used at a flow rate of 1.0 mL·min⁻¹ and a run time of 40 min.

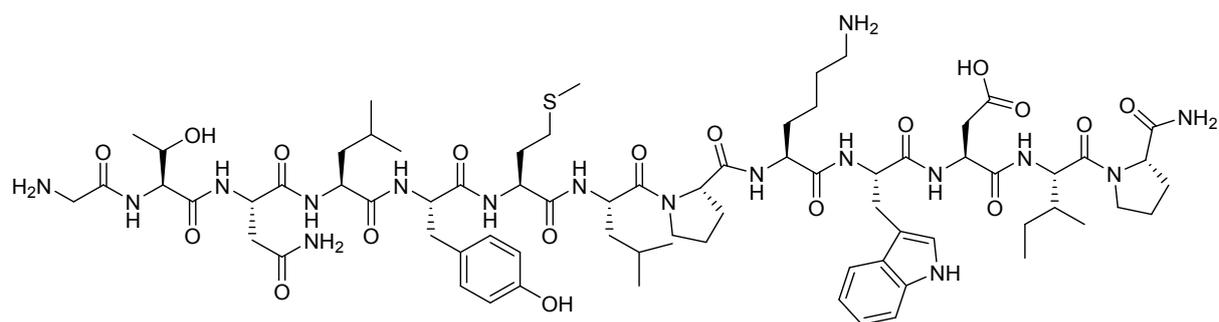
Semi-preparative high pressure liquid chromatography (prep-HPLC) was carried out on an Agilent Technologies 1260 infinity preparative system using both UV and ELSD detectors with a Phenomenex Gemini AXIA PA C18 reversed-phase semi-preparative column (21 mm × 250 mm, 10 μm). Auto-collection of fractions was used based on the UV measurements at 214 nm, and linear gradients of buffer B (acetonitrile/H₂O 95:5 with 0.1% TFA) into buffer A (acetonitrile/H₂O 5:95 with 0.1% TFA) over 65 min were used at a flow rate of 1.0 mL·min⁻¹ and a run time of 80 min.

Liquid chromatography mass spectrometry (LCMS) was carried out on a Thermo Scientific LCQ Fleet quadrupole mass spectrometer with a Dionex Ultimate 3000 LC using a Dr. Maisch Reprosil Gold 120 C18 reversed-phase HPLC column (4.0 mm × 150 mm, 3 μm). Linear gradients of buffer B into buffer A over 30 min were used at a flow rate of 1.0 mL·min⁻¹ and a run time of 40 min.

General Method: Automated peptide synthesis

The peptides were synthesized on a CS BIO 336X peptide synthesizer. Tentagel S RAM resin (1g, 0.25mmol, 1 equiv.) was washed with CH_2Cl_2 (1 × 5 min) and DMF (3 × 0.5 min). Deprotection of the Fmoc group was achieved by treatment of the resin with 20% piperidine/DMF (1 × 5 min and 1 × 20 min) followed by washing with DMF (3 × 0.5 min). The protected Fmoc-amino acids (4 equiv.) were coupled using HBTU (4 equiv.), Oxyma Pure (4 equiv.) and DIPEA (8 equiv.) in DMF, as a coupling system, with 2 min pre-activation for 45 min at rt. Washes between couplings and Fmoc deprotection steps were performed with DMF (3 × 0.5 min). Following elongation the resin was dried in vacuo and acidolytic cleavage was performed. Cleavage and deprotection was effected by treatment of the dry resin with TFA/TIS/ H_2O (95:2.5:2.5, 15 mL) for 1 h at rt. The peptide was precipitated in MTBE/hexane (1:1, 170 mL), centrifuged, the supernatant decanted and the pellet washed 3 times with MTBE/hexane (1:1, 50 mL). The resulting pellet was redissolved in $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (1:1) and lyophilized.

Synthesis of Peptide 1 – H-GTNLVMLPKWDIP-NH₂



Peptide **1** was synthesized according to the General Method. The peptide was purified with prep-HPLC using a linear gradient from 20% to 45% acetonitrile over 65 min and analytical HPLC analysis found that peptide **1** was obtained in >99% purity (linear gradient from 5% to 95% acetonitrile over 30 min, t_R peptide **1**: 18.2 min). LCMS peptide **1** observed $[M+H]^+$ 1546.9, required $[M+H]^+$ 1546.8.

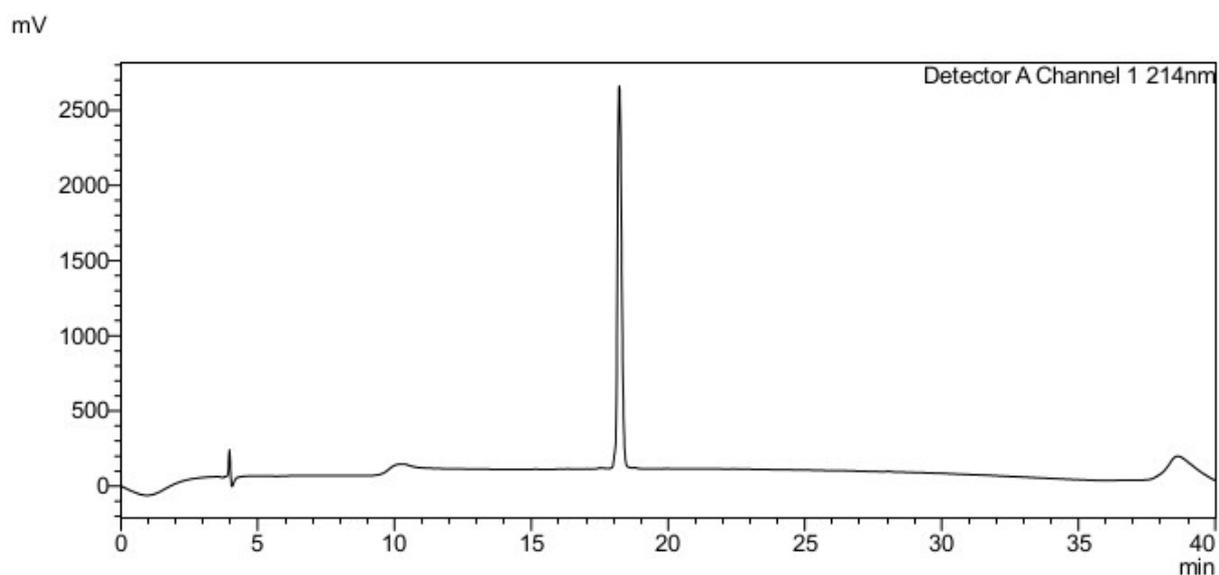
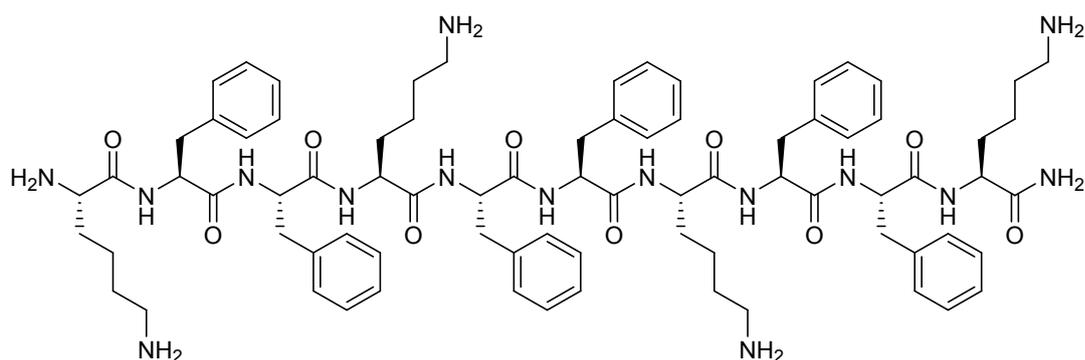


Figure S-1: HPLC chromatogram of peptide **1**

Synthesis of Peptide 2 – H-KFFKFFKFFK-NH₂



Peptide **2** was synthesized according to the General Method. The peptide was purified with prep-HPLC using a linear gradient from 5% to 60% acetonitrile over 65 min and analytical HPLC analysis found that peptide **2** was obtained in 98% purity (linear gradient from 5% to 95% acetonitrile over 30 min, t_R peptide **2**: 17.9 min). LCMS peptide **2** observed $[M+H]^+$ 1412.8, required $[M+H]^+$ 1412.8.

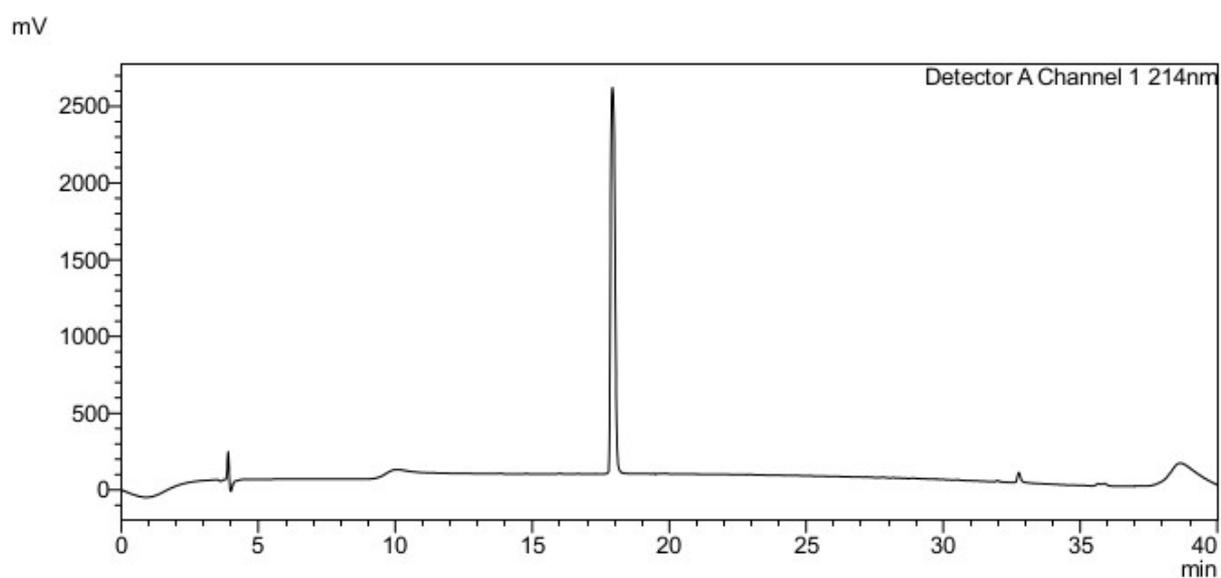
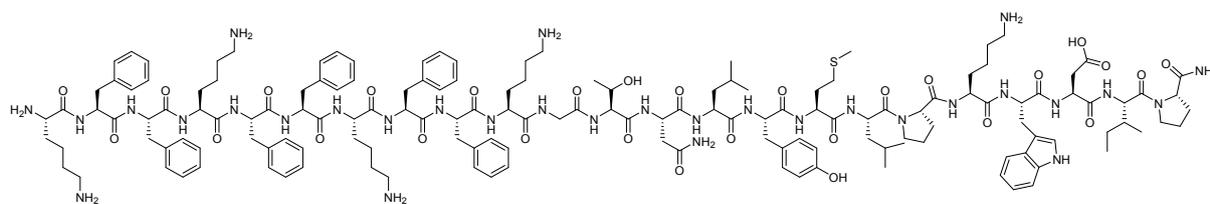


Figure S-2: HPLC chromatogram of peptide **2**

Synthesis of Peptide 3 – H-KFFKFFKFFKGTNLYMLPKWDIP-NH₂



Peptide **3** was synthesized according to the General Method. The peptide was purified with prep-HPLC using a linear gradient from 20% to 50% acetonitrile over 65 min and analytical HPLC analysis found that peptide **3** was obtained in >99% purity (linear gradient from 5% to 95% acetonitrile over 30 min, t_R peptide **3**: 19.2 min). LCMS peptide **3** observed $[M+2H]^{2+}$ 1471.9, required $[M+2H]^{2+}$ 1471.3 and observed $[M+3H]^{3+}$ 982.0, required $[M+3H]^{3+}$ 981.5.

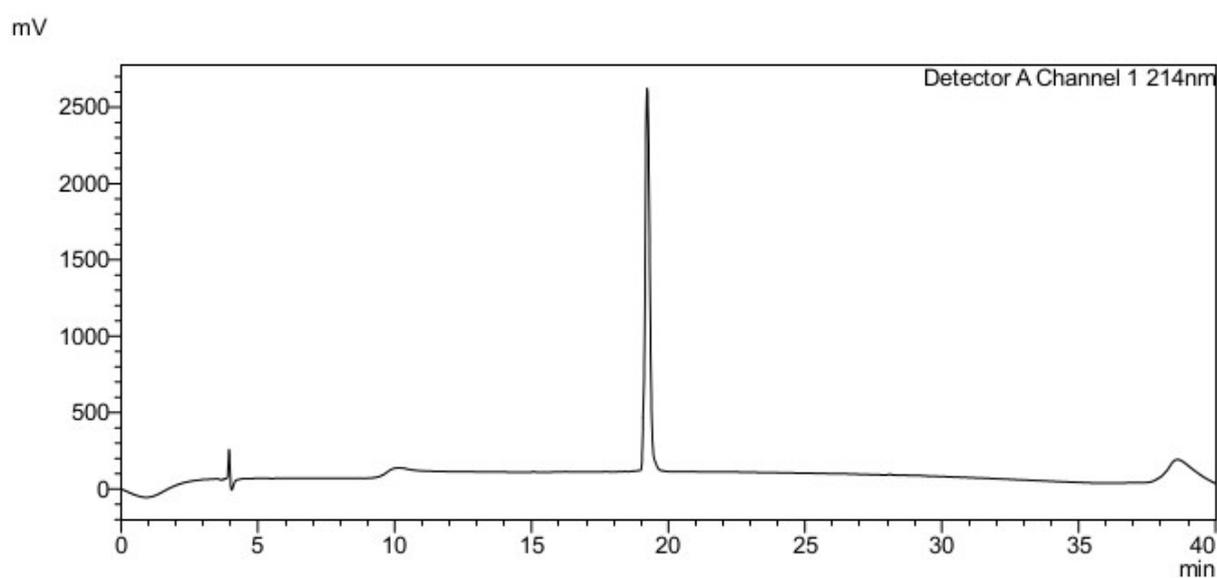


Figure S-3: HPLC chromatogram of peptide **3**

Biological Evaluation

MIC Determination

The antimicrobial activity of purified peptides against *Escherichia coli* DSM 1103, *Klebsiella pneumoniae* DSM 789, *Citrobacter freundii* DSM 30029, *Shigella sonnei* DSM 5570, *Salmonella enterica* DSM 17058, *Pseudomonas aeruginosa* DSM 1117, *Acinetobacter baumannii* DSM 30007 (ATCC 19606), *Acinetobacter baumannii* (AL1851 - Δ LpxA), *Acinetobacter baumannii* (AL1852 - Δ LpxD), *Staphylococcus aureus* DSM 11729 and *Staphylococcus epidermidis* DSM 28319 was quantified in a microbroth dilution assay. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of a compound that visibly inhibits bacterial growth after 24h at 37 °C. An established microbroth dilution assay was used in our group to determine the MIC values.¹ Overnight cultures in Mueller Hinton broth (MHB) were diluted to 1×10^8 colony forming units (cfu)/mL, using a McFarland 0.5 standard, and subsequently diluted 100 fold in fresh MHB to 1×10^6 cfu/mL.² Peptide stock solutions were prepared at a concentration of 1 mg/mL peptide in 0.2% bovine serum albumin (BSA) and 0.01% acetic acid. Aliquots (50 μ L) of twofold diluted peptide solutions in 0.2% BSA and 0.01% acetic acid (1000 – 0.48 μ g/mL) were placed in sterile 96-well low binding polypropylene microtiter plates (Corning 3879). The bacterial suspension (50 μ L) was added to the wells and the plates were incubated for 24h at 37 °C on a shaking incubator (200 rpm). The MIC values were determined by visual inspection of the plate and the well with the lowest concentration that inhibited growth was chosen. The plates included a positive control with ampicillin. All measurements were performed in triplicate and validated using two separate experiments.

Haemolytic activity Assay

The haemolytic activity of the purified peptides was determined using a method that was previously developed in our group with minor modifications.³ Erythrocytes were isolated from defibrinated sheep blood (E&O Laboratories LTD) using centrifugation (1000 \times G, 10 min) and subsequently the erythrocytes were washed three times with saline solution (0.9% NaCl) prior to use. The erythrocyte pellet was diluted to 5% (v:v) in phosphate buffered saline (PBS). Peptide solutions were prepared at a concentration of 256 μ M in PBS and aliquots (200 μ L / well) were transferred in triplicate to the first column of a sterile 96-well low binding polypropylene microtiter plate (Corning 3879). An aliquot (100 μ L / well) was taken from the first column and 2-fold diluted in PBS (100 μ L / well). A positive control was prepared to determine 100% hemolysis using Triton X-100 (1% in PBS) and PBS was used as a blank. Aliquots (100 μ L / well) of the 5% erythrocyte suspension were added to the microtiter plate and incubated for 1h at 37 °C. Following incubation, the microtiter plate was centrifuged (1000 \times G, 10 min) and 50 μ L of supernatant from each well was transferred to a 96-well flat bottom polystyrene microtiter plate (Nunc 269620). The OD at 414 nm was measured in triplicate and used to determine the percentage of haemolysis in comparison to 100% haemolysis (1% Triton X-100).

References

- 1 J. C. Slootweg, T. B. van Schaik, H. L. C. Quarles van Ufford, E. Breukink, R. M. J. Liskamp and D. T. S. Rijkers, *Bioorg. Med. Chem. Lett.*, 2013, **23**, 3749–52.
- 2 I. Wiegand, K. Hilpert and R. E. W. Hancock, *Nat. Protoc.*, 2008, **3**, 163–75.
- 3 R. T. C. Cleophas, M. Riool, H. (Linda) C. Quarles van Ufford, S. A. J. Zaat, J. A. W. Kruijtzter and R. M. J. Liskamp, *ACS Macro Lett.*, 2014, **3**, 477–480.