Supplementary Information

"CLipP"ing on lipids to generate antibacterial lipopeptides

Victor Yim,[a,c] Iman Kavianinia,[a,b,c] Melanie K. Knottenbelt,[d] Scott A. Ferguson,[d] Gregory M. Cook,[d] Simon Swift,[e] Aparajita Chakraborty[a,b] Jane R. Allison[a,b] ,Paul W. R. Harris*,[a,b,c] and Margaret A. Brimble*[a,b,c]

[a] *School of Biological Sciences, The University of Auckland, 3A Symonds Street, Auckland 1010, New Zealand*

[b] *Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, 3A Symonds Street, Auckland 1010, New Zealand*

[c] *School of Chemical Sciences, The University of Auckland, 23 Symonds Street, Auckland 1010, New Zealand*

[d] *Department of Microbiology and Immunology, School of Biomedical Sciences, University of Otago, 720 Cumberland Street, Dunedin 9054, New Zealand*

[e] *Department of Molecular Medicine and Pathology, School of Medical Sciences, University of Auckland, 85 Park Road, Grafton, Auckland 1023, New Zealand*

**Email: m.brimble@auckland.ac.nz, paul.harris@auckland.ac.nz*

Table of Contents

S1. Abbreviations

°C: degrees celcius. 6-Cl-HOBt: 6-chloro-1-hydroxybenzotriazole. *A. calcoaceticus*: *Acinetobacter calcoaceticus.* ATCC: American Type Culture Collection. BHI: brain heart infusion. Boc: *tert*-butyloxycaronyl. CFU: colony-forming unit. CH_2Cl_2: dichloromethane. CLipPA: Cysteine Lipidation on a Peptide or Amino acid. Dab: 2,4-diaminobutyric acid. DIC: *N,N'*-diisopropylcarbodiimide. DIPEA: *N,N*-diisopropylethylamine. DMF: dimethylformamide. DMPA: 2,2-dimethoxy-2-phenylacetophenone. DODT: 2,2'-(ethylenedioxy)-diethanethiol. DPG: disphosphatidylglycerol. *E. coli*: *Escherichia coli*. eq.: equivalent. ESI-MS: electrospray ionisation mass spectrometry. Fmoc: (9*H*-fluoren-9-yl)methoxycarbonyl. fs: femtoseconds. h: hour(s). HATU: *O*-(7-azabenzo-triazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate. K: kelvin. kJ: kilojoules. LC-MS: liquid chromatography-mass spectrometry. MBC: minimum bactericidal concentration. MeCN: acetonitrile. MH: Mueller Hinton. MHA: (±)-4-methylhexanoic acid. MIC: minimum inhibitory concentration. min: minute (s). mL: millilitre(s). mm: millimetre(s). mmol: millimoles. MW: Microwave. nm: nanometres. ns: nanoseconds. NMP: *N*-methyl-2-pyrrolidone. NZRM: New Zealand Reference Culture Collection. *P. aeruginosa*: *Pseudomonas aeruginosa.* PBS: phosphate-buffered saline. PE: phosphatidylethanolamine. PG: Phosphatidylglycerol. ps: picoseconds. PV: 1-palmitoyl, 2-*cis*-vaccenyl. r.t.: room temperature. Rink acid: 4-((2,4-dimethoxyphenyl)aminomethyl)phenoxyacetic acid. RP-HPLC: reverse-phase high performance-liquid chromatography. rpm: revolutions per minute. *S. aureus*: *Staphylococcus aureus*. *t*BuSH: *tert*-butylthiol. TFA: trifluoroacetic acid. TIPS: triisopropylsilane. UV: ultraviolet. V: volts. *v/v:* volume per volume. W: Watts. µL: microliters. µm: micrometres. µM: micromolar. µmol: micromoles.

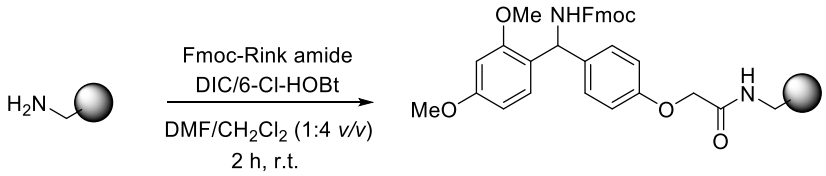
S2. General Information

All reagents were purchased as reagent grade and used without further purification unless otherwise noted. Vinyl propionate, vinyl butyrate, vinyl decanoate, vinyl pivalate, vinyl benzoate, vinyl 4-*tert*-butylbenzoate, DMPA, *t*BuSH, DODT, TIPS, DIPEA, piperidine, DIC and NMP were purchased from Sigma-Aldrich (St. Louis, Missouri). HATU and Fmoc-Leu-OH were purchased from GL Biochem (Shanghai, China). 6-Cl-HOBt was purchased from AAPPTec (Louisville, Kentucky). Fmoc-Dab(Boc)-OH was purchased from AK Scientific (Union City, California). Fmoc-D-Dab(Boc) and Fmoc-D-Phe-OH) were purchased from ChemPep (Wellingon, Florida). DMF was purchased from Scharlau (Barcelona, Spain), MeCN was purchased from Fisher Scientific (Fair Lawn, New Jersey) and TFA was obtained from Oakwood Chemicals (Estill, NC). H_2O was purified using a Sartorius arium® pro ultrapure water system.

Microwave assisted reactions were performed in a CEM (Matthews, NC) Discover SP, Model 908010 reactor. Analytical RP-HPLC was used to analyse final compounds and performed on a Dionex (Sunnyvale, CA) UltiMate 3000 system using a Waters (Milford, MA) Xterra MS C18 (5 µm 4.6 × 150 mm) column, and Chromeleon software was used for data processing. Buffer A: 0.1% (*v/v*) TFA in H_2O; buffer B: 0.1% (*v/v*) TFA in MeCN. LC-MS was performed on an Agilent (Santa Clara, CA) 1260 Infinity with UV absorbance at λ = 214 nm equipped with an Agilent 6120 Quadrupole LC-MS using an Agilent Zorbax 300SB-C3 column (3.5 µm, 3.0 × 150 mm). Data processing was carried out by Agilent OpenLAB software. Buffer A: 0.1% (*v/v*) formic acid in H_2O; buffer B: 0.1% (*v/v*) formic acid in MeCN. Crude peptides was purified on Dionex UltiMate 3000 preparative HPLC using Agilent Zorbax 300SB-C18 column (5 µm, 9.4 × 250 mm) and Chromeleon software was used for data processing. Ultraviolet irradiation was carried out using Spectroline (Westbury, NY) hand-held lamp EA-160/FA, 6 W integrally filtered tube at 50 Hz, 0.17 A and λ = 365 nm. Equipment used for biological testing carried out in separate labs and details are given within the methods. Details on softwares and parameters used for computational methods are given within the methods.

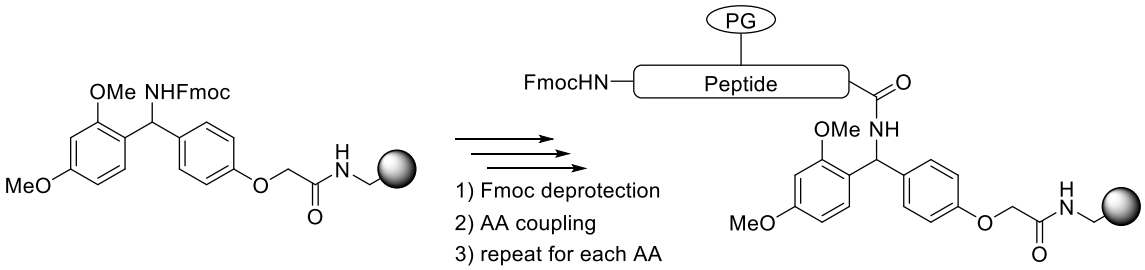
S3. General Methods

General Method A: attachment of Rink-amide linker to aminomethyl-polystyrene resin



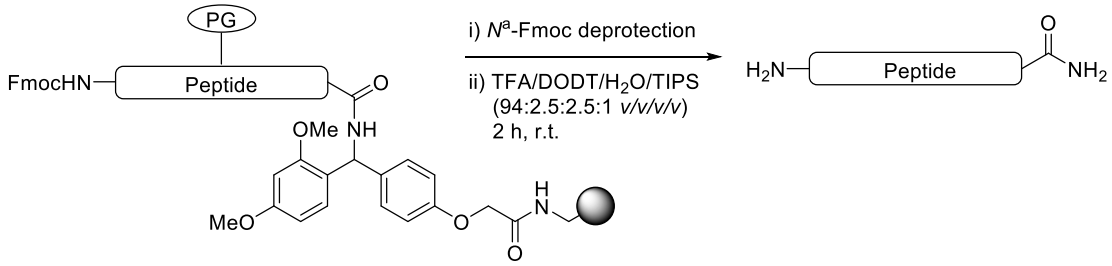
Aminomethyl-polystyrene resin (880 mg, 0.8 mmol, 0.91 mmol/g) was swollen in DMF/CH_2Cl_2 (8 mL, 1:1 *v/v*) for 15 min and the solvent was drained. Fmoc-Rink amide (1.2 g, 3.2 mmol, 4 eq.) was dissolved in DMF/CH_2Cl_2 (8 mL, 1:4 *v/v*), then DIC (501 µL, 3.2 mmol, 4 eq.) and 6-Cl-HOBt (542 mg, 4.0 mmol, 4 eq.) were added. The reaction mixture was added to the resin and agitated for 3 h. The solution was drained and the resin was washed with DMF (3 × 8 mL) and dried by washing with MeOH (3 × 8 mL). A negative ninhydrin test indicated the completion of the reaction. The N^{α}-protecting group was removed by treatment with piperidine/DMF (1:4 *v/v*, 8 mL, 2 × 5 min). The solution was drained and the resin was washed with DMF (3 × 8 mL).

General Method B: microwave coupling of amino acid



Fmoc protected amino acid (2 mmol, 2.5 eq.), HATU (700 mg, 1.8 mmol, 2.3 eq.) was dissolved in DMF (8 mL). DIPEA (700 µL, 4.0 mmol, 5 eq.) was added to the amino acid mixture and allowed to activate for 30 s before adding the activated amino acid to the linker-resin. The reaction mixture was irradiated under microwave (25 W, 50 °C, 5 min), the solution drained and the resin was washed with DMF (3 × 8 mL) and dried by washing with MeOH (3 × 8 mL). The completion of the coupling was monitored using the ninhydrin test.[1] The N^{α}-protecting group was removed by treatment with piperidine/DMF (1:4 *v/v*, 2 × 8 mL) under microwave irradiation (50 W, 75 °C, 2 × 3 min).

General Method C: acid cleavage of the peptide off the resin



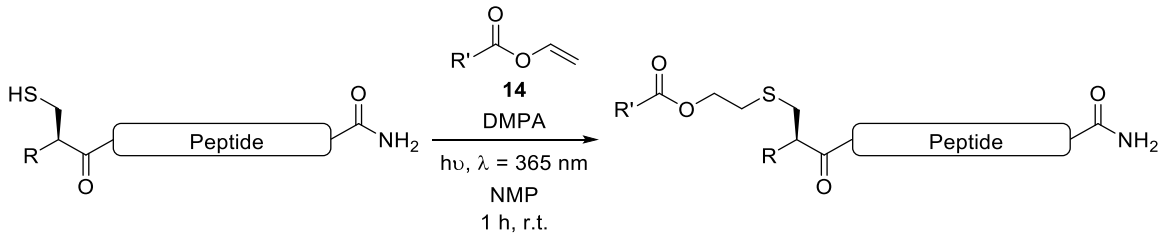
Elongated peptidyl resin was cleaved from the resin by treatment with TFA:DODT:H_2O:TIPS (94:2.5:2.5:1 *v/v/v/v*, 20 mL) for 2 h. The TFA was evaporated by sparging under a stream of nitrogen and the peptide was triturated

with diethyl ether. Centrifuging gave a peptide pellet which was dissolved in 0.1% (*v/v*) TFA in H_2O:MeCN (1:1 *v/v*).The identity of the peptide product was confirmed using LC-MS (5 – 95% B, 3% B/min, 0.3 mL/min, Agilent® C3 column (Zorbax, 3.0 × 150 mm; 3.5 µm), wherein solvent A = H_2O (+ 0.1% TFA, *v/v*), and solvent B = MeCN (+ 0.1% TFA, *v/v*). The peptide was then lyophilised or directly purified according to **General Method D**.

General Method D: purification

Crude peptide was dissolved in 0.1% (*v/v*) TFA in H_2O:MeCN (4:1 *v/v*), centrifuged and filtered using Phenomenex (Torrance, CA) Phenex syringe filters (26 mm, 0.45 µm). The filtrate was injected in 2500 µL aliquots and purified using a slow gradient on RP-HPLC (1 – 95%B, 1% B/min, 4 mL/min).[2] The fractions were collected based on UV absorbance at wavelengths of 210 nm, 230 nm, 254 nm and 280 nm, followed mass-spectrometry analysis (ESI^+ 100 V; H_2O:MeCN; 1:1 *v/v*, 0.2 mL/min). The fractions containing the purified peptide were combined and lyophilised.

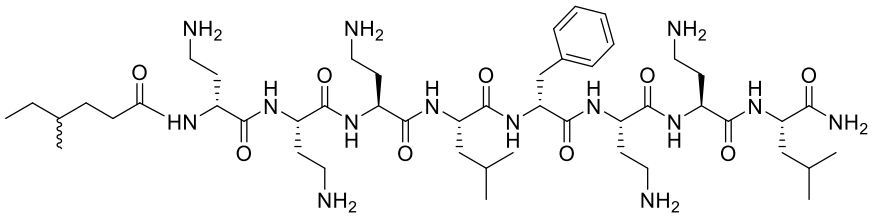
General Method E: direct conjugation of vinyl esters to free thiol-containing peptides using CLipPA



NMP was sparged with argon for 15 min, to which purified linear peptide was added (10 mg/mL), along with the desired vinyl ester (20 eq.), DMPA (1 eq.) and TIPS (80 eq.). TFA (5% *v/v*) and *t*BuSH (80 eq.) were then added to the mixture under argon and the mixture irradiated under at 365 nm, r.t., *ca.* 1 h. The reaction was monitored using LC-MS (5 – 95% B, 3% B/min, 0.3 mL/min). Upon completion of the reaction, the mixture was triturated with diethyl ether (40 mL, 4 °C). Centrifuging gave a peptide pellet which was dissolved in 0.1% (*v/v*) TFA in H_2O:MeCN (1:1 *v/v*). Purification according to **General Method D** gave purified peptides **12**, **12a** – **12f**, **13**, **13a** – **13f**.

S4. Synthesis, structures and LC-MS profiles of synthetic peptides

S4.1. Synthesis of linear battacin analogue 2



2

Peptidyl resin **8** was synthesised using microwave-enhanced Fmoc SPPS according to **General Method A and B**. A portion of **8** (0.1 mmol) was used for coupling 4*R*/4*S*-methylhexanoic acid (MHA) (57 µL, 0.4 mmol, 4 eq.) onto the *N*-terminus of D-Dab using HATU (87 mg, 0.2 mmol, 2.3 eq.) and DIPEA (84 µL, 0.5 mmol, 5 eq.) according to **General Method B**. The peptide was cleaved from the resin according to **General Method C** and then purified according to **General Method D**. Lyophilisation yielded purified linear battacin analogue **2** (58 mg, 58% yield, 99% purity) as a white solid; RP-HPLC: t_R = 14.7 min, ESI-MS: $[M + H]^+$ found 1004.6, $[C_{48}H_{86}N_{14}O_9 + H]^+$ requires 1003.7, **Figure S1**.

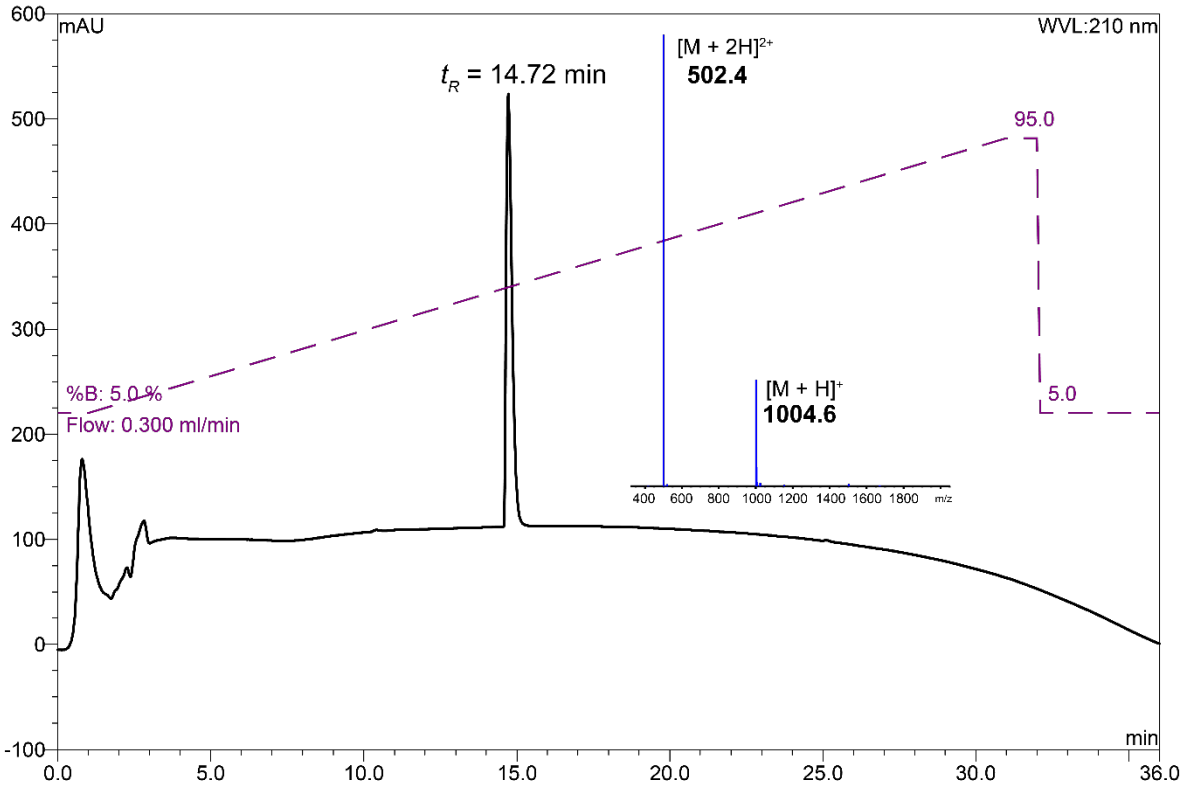
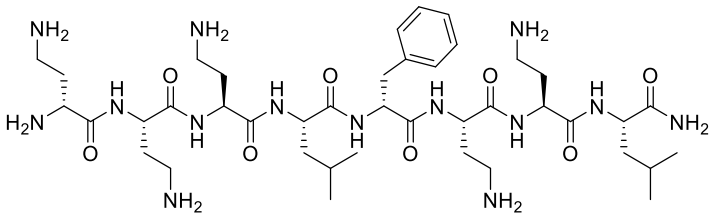


Figure S1 Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin analogue **2** (*ca.* 99% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

S4.2. Synthesis of linear battacin sequence 9



9

Peptidyl resin **8** (0.1 mmol) was synthesised using microwave-enhanced Fmoc SPPS according to **General Method A and B**. The peptide was cleaved from the resin according to **General Method C** and then purified according to **General Method D**. Lyophilisation yielded purified linear battacin sequence **9** (69 mg, 77% yield, 96% purity) as a white solid; RP-HPLC: t_R = 11.3 min, ESI-MS: $[M + H]^+$ found 891.5, $[C_{41}H_{74}N_{14}O_{18} + H]^+$ requires 891.6, **Figure S2**.

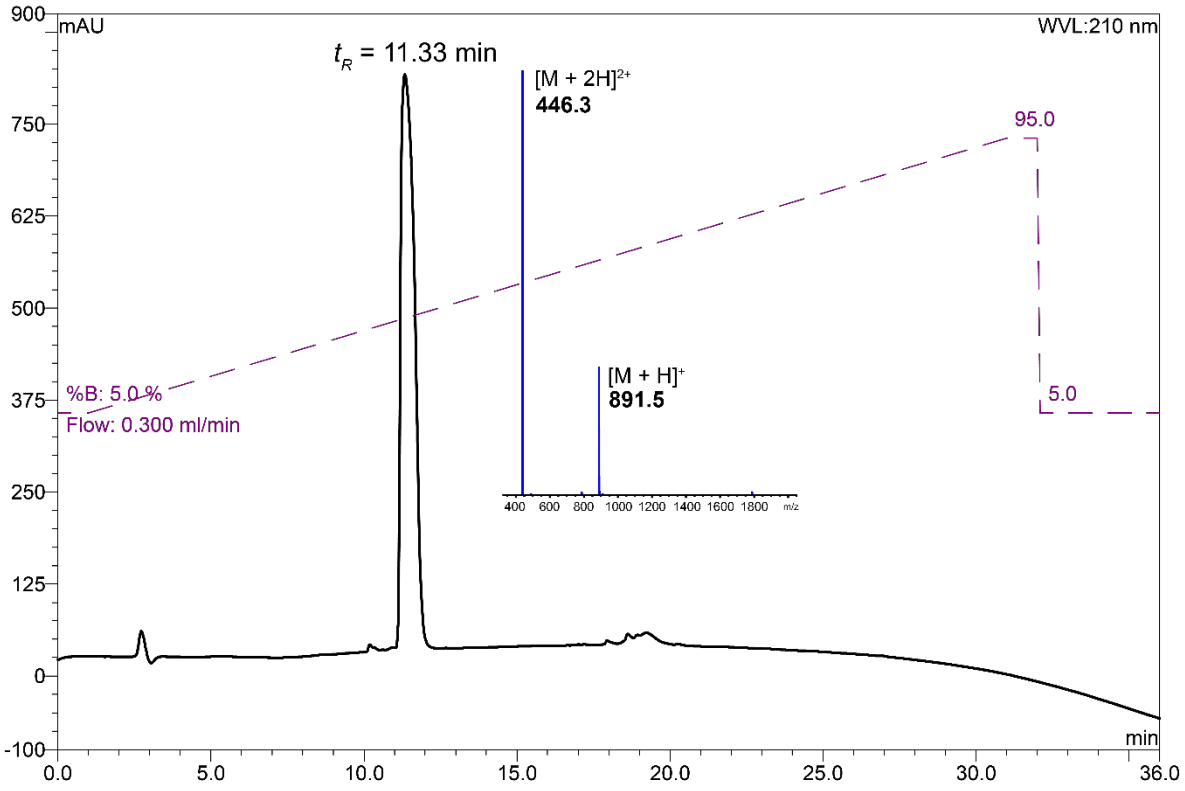
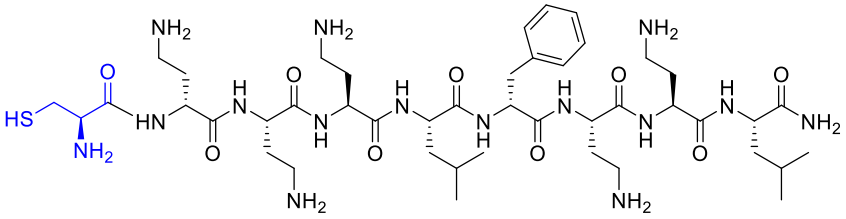


Figure S2. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **9** (*ca.* 96% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

S4.3. Synthesis of linear battacin analogue 12



12

Peptidyl resin **8** was synthesised using microwave-enhanced Fmoc SPPS according to **General Method A and B**. A portion of **8** (0.3 mmol) was used for coupling Fmoc-Cys(Trt)-OH (703 mg, 1.2 mmol, 4 eq.) onto the *N*-terminus of D-Dab using HATU (433 mg, 1.1 mmol, 3.8 eq.) and DIPEA (402 µL, 2.4 mmol, 8 eq.) according to **General Method B**. The peptide was cleaved from the resin according to **General Method C** and then purified according to **General Method D**. Lyophilisation yielded purified linear battacin analogue **12** (88.8 mg, 30% yield, 98% purity) as a white solid; RP-HPLC: t_R = 12.5 min, ESI-MS: $[M + H]^+$ found 994.5, $[C_{44}H_{79}N_{15}O_9S + H]^+$ requires 994.6, **Figure S3**.

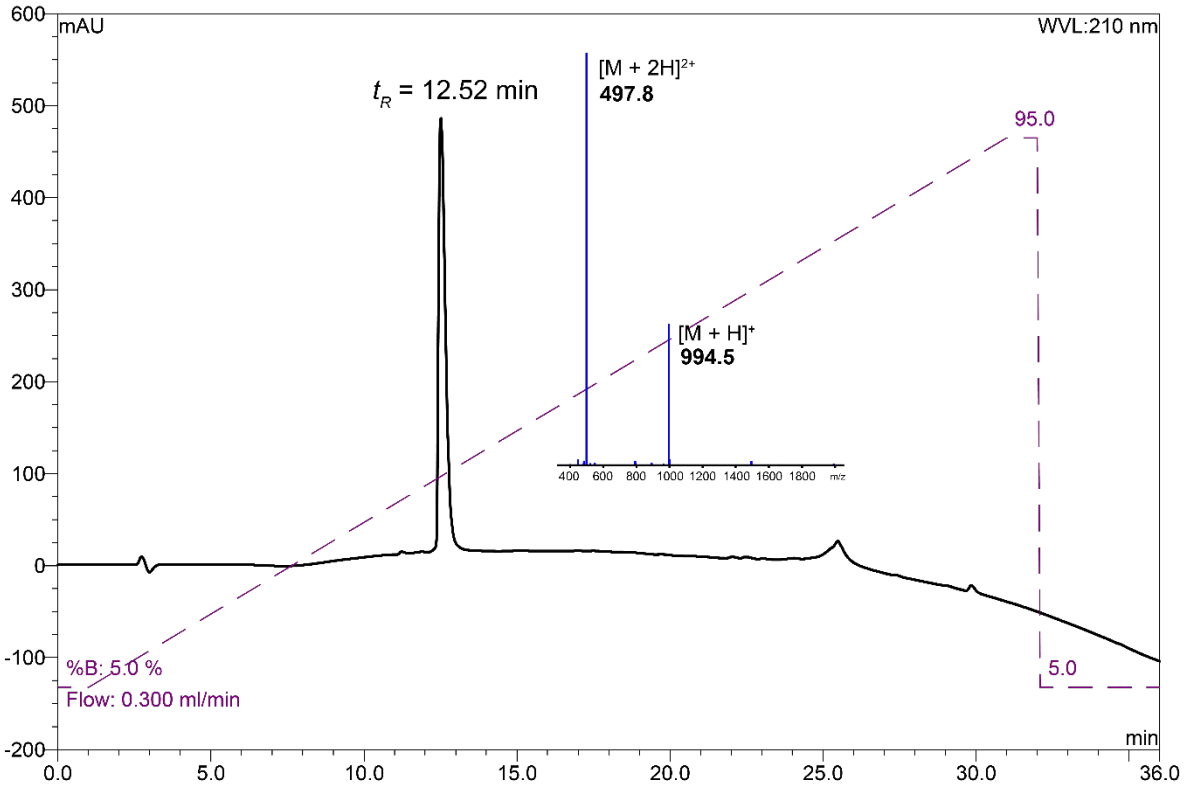
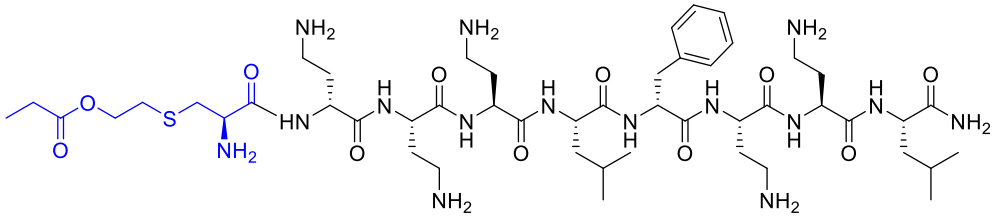


Figure S3. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **12** (*ca.* 98% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

S4.4. Synthesis of linear battacin CLipPA analogue 12a



12a

Purified peptide **12** (14.1 mg, 14.2 µmol), DMPA (3.6 mg, 14.2 µmol, 1 eq.), vinyl propionate (31 µL, 0.3 mmol, 20 eq.) and TIPS (233 µL, 1.1 mmol, 80 eq.) were dissolved in NMP (1.41 mL, 10 mg/mL final concentration w.r.t. **12**) and degassed with argon for 15 min. Volatile liquids *t*BuSH (128 µL, 1.1 mmol, 80 eq.) and TFA (71 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method E**. Purification according to **General Method D** yielded purified linear battacin CLipPA analogue **12a** (5.4 mg, 10% yield, 97% purity) as a white solid; RP-HPLC: t_R = 13.4 min, ESI-MS: $[M + H]^+$ found 1094.6, $[C_{49}H_{87}N_{15}O_{11}S + H]^+$ requires 1094.6, **Figure S4**.

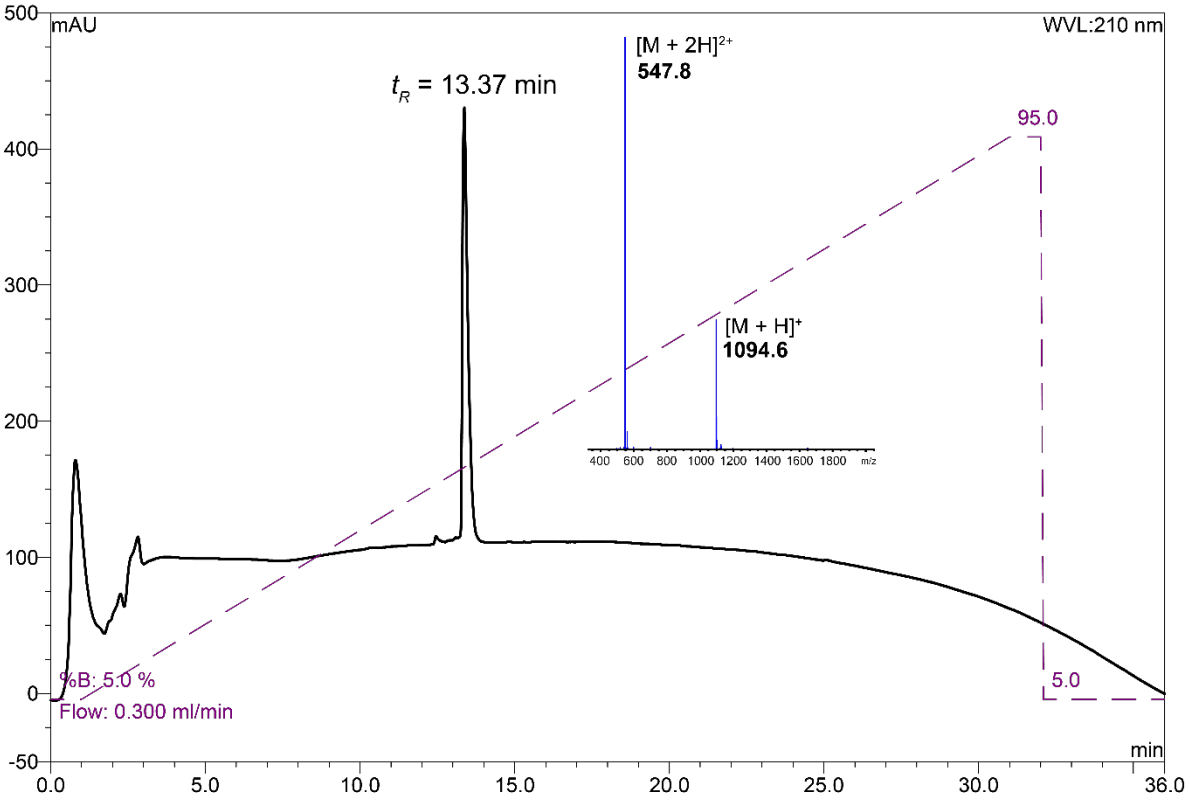
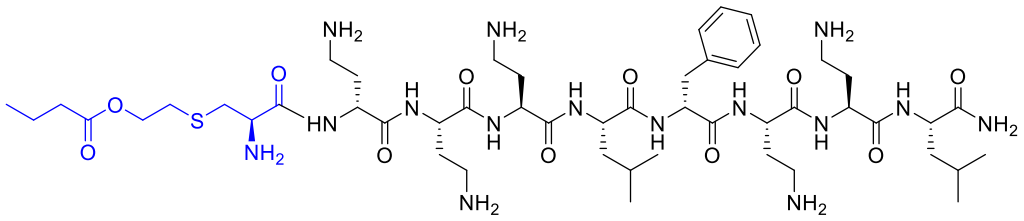


Figure S4. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **12a** (*ca.* 97% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

S4.5. Synthesis of linear battacin CLipPA analogue 12b



12b

Purified peptide **12** (14.3 mg, 14.4 µmol), DMPA (3.7 mg, 14.4 µmol, 1 eq.), vinyl butyrate (37 µL, 0.3 mmol, 20 eq.) and TIPS (236 µL, 1.2 mmol, 80 eq.) were dissolved in NMP (1.43 mL, 10 mg/mL final concentration w.r.t. **12**) and degassed with argon for 15 min. Volatile liquids *t*BuSH (130 µL, 1.2 mmol, 80 eq.) and TFA (72 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method E**. The peptide was purified according to **General Method D.** Lyophilisation yielded purified linear battacin CLipPA analogue **12b** (2.7 mg, 5% yield, 99% purity) as a white solid; RP-HPLC: t_R = 13.9 min, ESI-MS: $[M + H]^+$ found 1108.6, $[C_{50}H_{89}N_{15}O_{11}S + H]^+$ requires 1108.7, **Figure S5**.

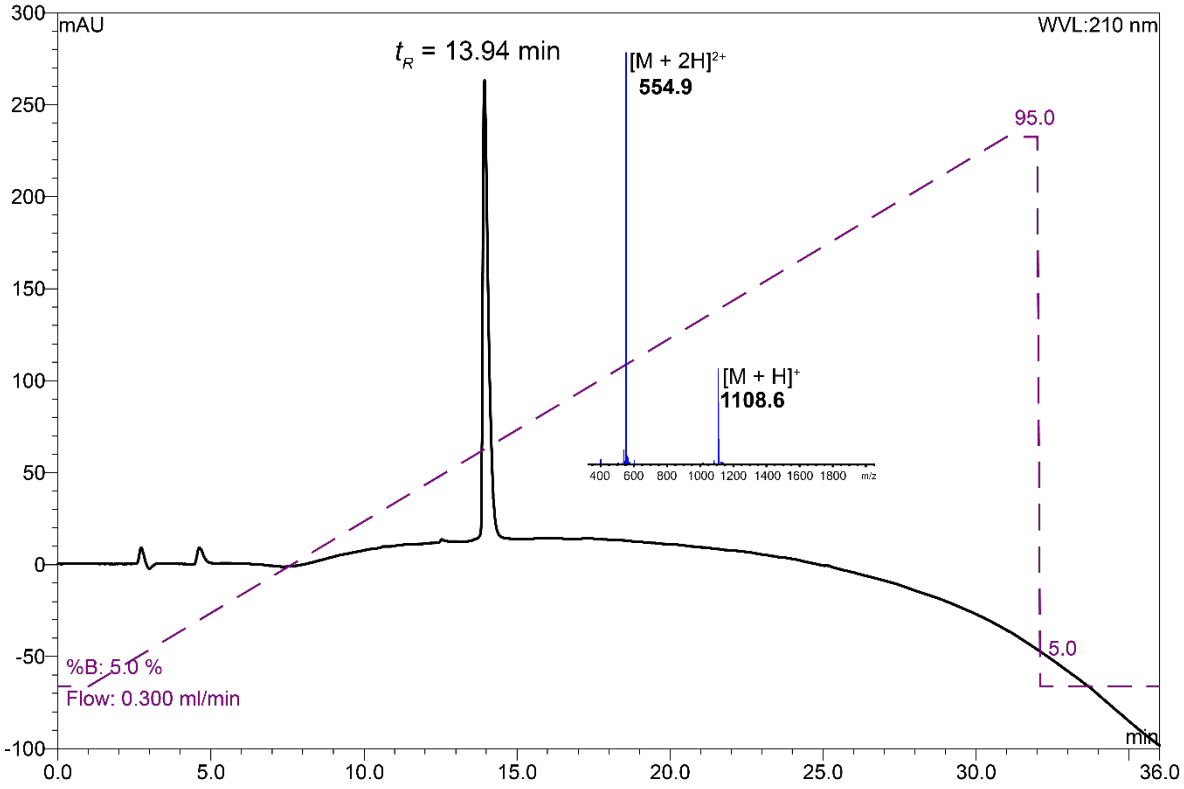
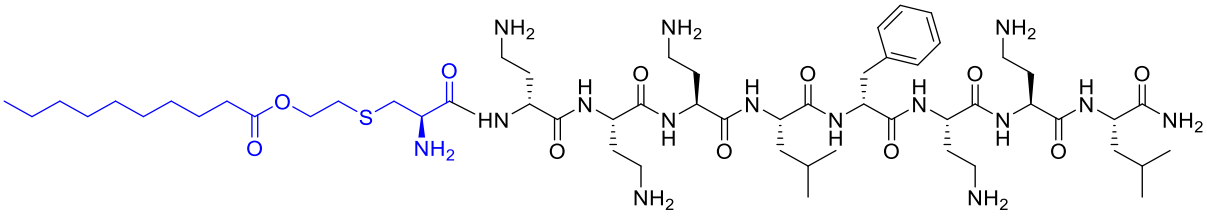


Figure S5. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **12b** (*ca.* 99% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

S4.6. Synthesis of linear battacin CLipPA analogue 12c



12c

Purified peptide **12** (14.6 mg, 14.7 μmol), DMPA (3.8 mg, 14.7 μmol, 1 eq.), vinyl decanoate (66 μL, 0.3 mmol, 20 eq.) and TIPS (241 μL, 1.2 mmol, 80 eq.) were dissolved in NMP (1.46 mL, 10 mg/mL final concentration w.r.t. **12**) and degassed with argon for 15 min. Volatile liquids *t*BuSH (133 μL, 1.2 mmol, 80 eq.) and TFA (73 μL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method E**. The peptide was purified according to **General Method D.** Lyophilisation yielded purified linear battacin CLipPA analogue **12c** (0.7 mg, 1% yield, 97% purity) as a white solid; RP-HPLC: t_R = 17.7 min, ESI-MS: $[M + H]^+$ found 1192.6, $[C_{56}H_{101}N_{15}O_{11}S + H]^+$ requires 1192.8, **Figure S6**.

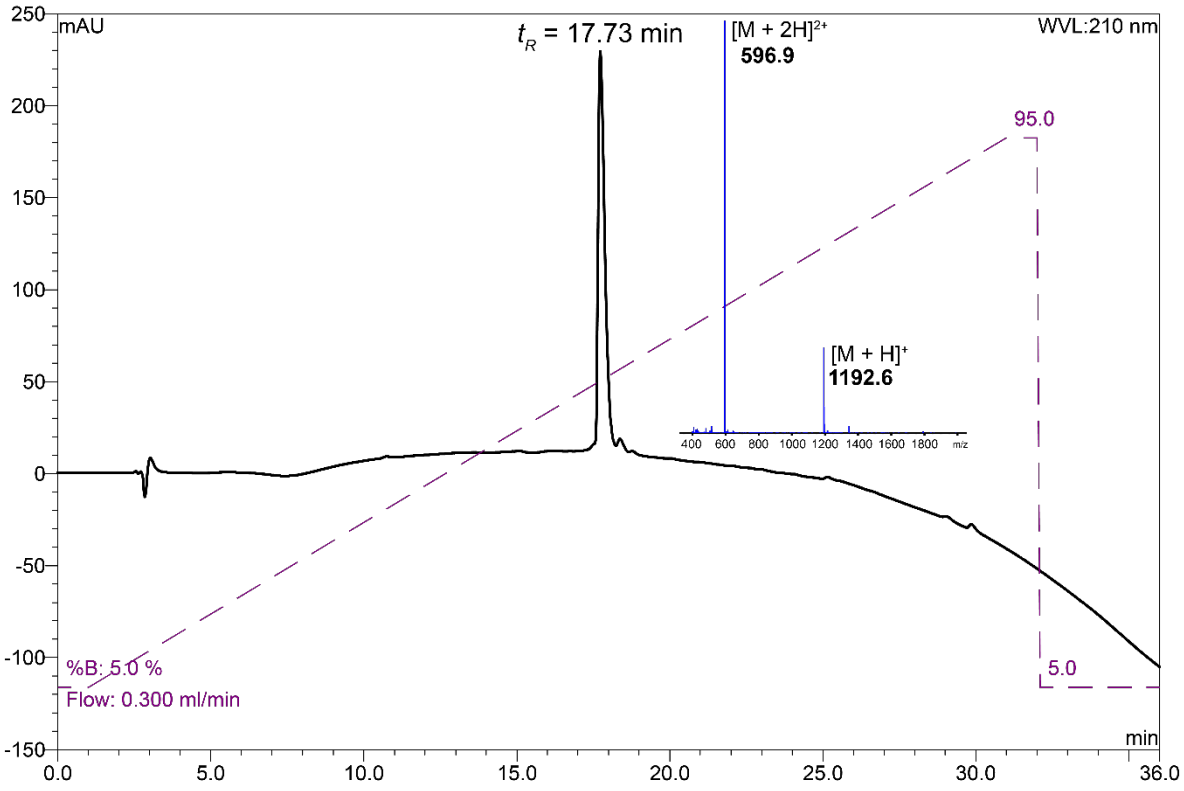
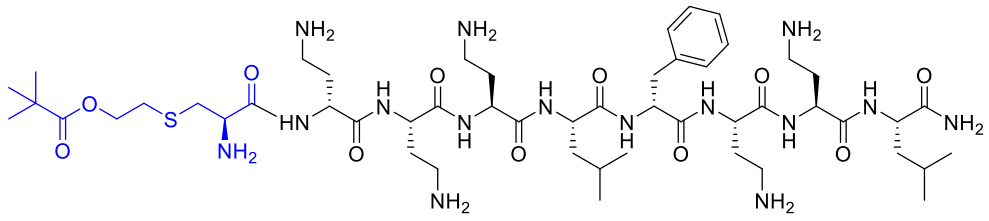


Figure S6. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **12c** (*ca*. 97% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 μm), linear gradient of 5% B to 95% B over 30 min, *ca*. 3% B per minute at r.t., 0.3 mL/min.

S4.7. Synthesis of linear battacin CLipPA analogue 12d



12d

Purified peptide **12** (14.6 mg, 14.7 µmol), DMPA (3.8 mg, 14.7 µmol, 1 eq.), vinyl pivalate (43 µL, 0.3 mmol, 20 eq.) and TIPS (241 µL, 1.2 mmol, 80 eq.) were dissolved in NMP (1.46 mL, 10 mg/mL final concentration w.r.t. **12**) and degassed with argon for 15 min. Volatile liquids *t*BuSH (133 µL, 1.2 mmol, 80 eq.) and TFA (73 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method E**. The peptide was purified according to **General Method D.** Lyophilisation yielded purified linear battacin CLipPA analogue **12d** (1.9 mg, 3% yield, 99% purity) as a white solid; RP-HPLC: t_R = 14.4 min, ESI-MS: $[M + H]^+$ found 1122.6, $[C_{49}H_{87}N_{15}O_{11}S + H]^+$ requires 1122.7, **Figure S7**.

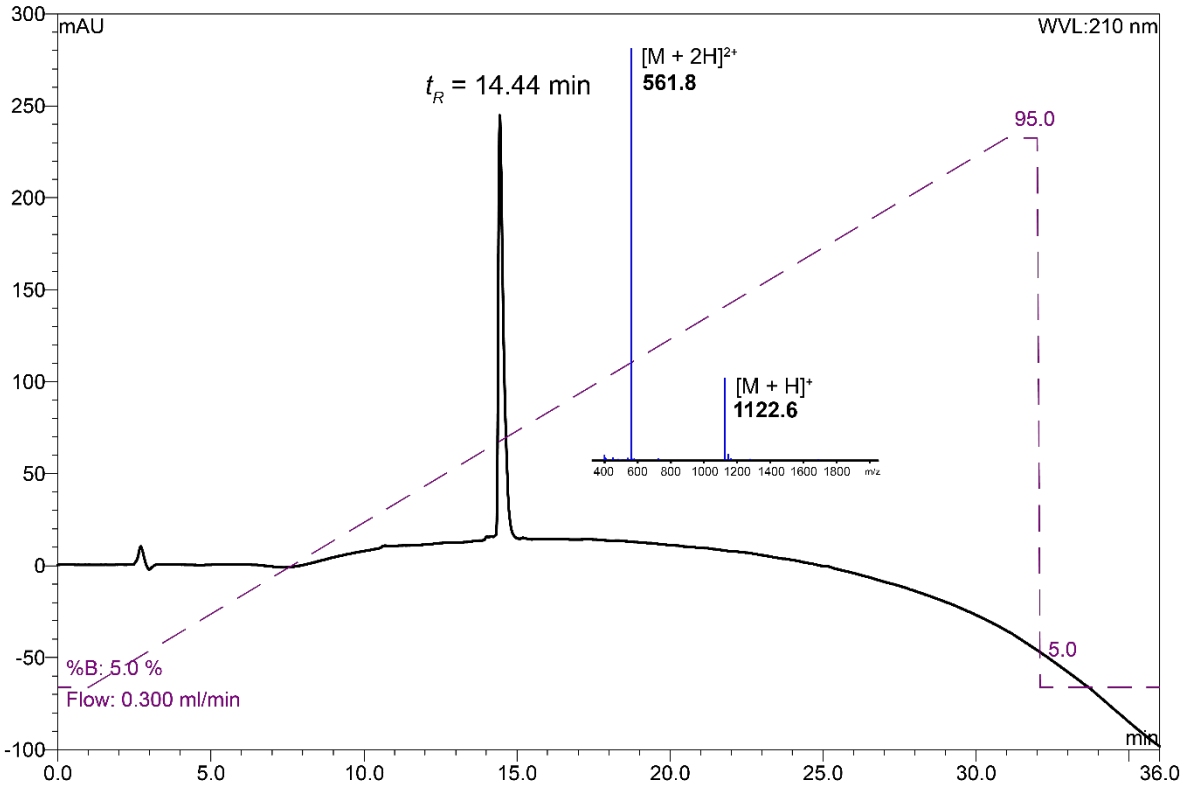
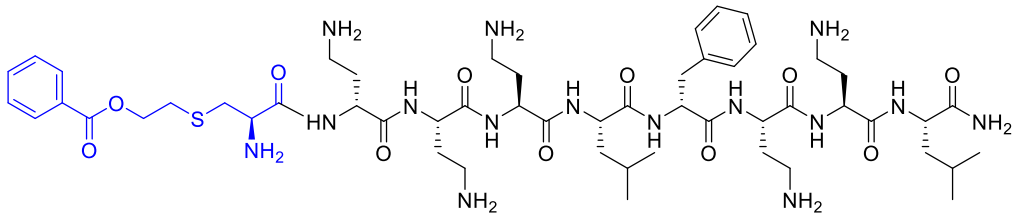


Figure S7. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **12d** (*ca.* 99% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

S4.8. Synthesis of linear battacin CLipPA analogue 12e



12e

Purified peptide **12** (15.1 mg, 15.2 µmol), DMPA (3.9 mg, 15.2 µmol, 1 eq.), vinyl benzoate (42 µL, 0.3 mmol, 20 eq.) and TIPS (249 µL, 1.2 mmol, 80 eq.) were dissolved in NMP (1.51 mL, 10 mg/mL final concentration w.r.t. **12**) and degassed with argon for 15 min. Volatile liquids *t*BuSH (137 µL, 1.2 mmol, 80 eq.) and TFA (76 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method E**. The peptide was purified according to **General Method D.** Lyophilisation yielded purified linear battacin CLipPA analogue **12e** (0.8 mg, 1% yield, 99% purity) as a white solid; RP-HPLC: t_R = 17.8 min, ESI-MS: $[M + H]^+$ found 1142.6, $[C_{49}H_{87}N_{15}O_{11}S + H]^+$ requires 1142.6, **Figure S8**.

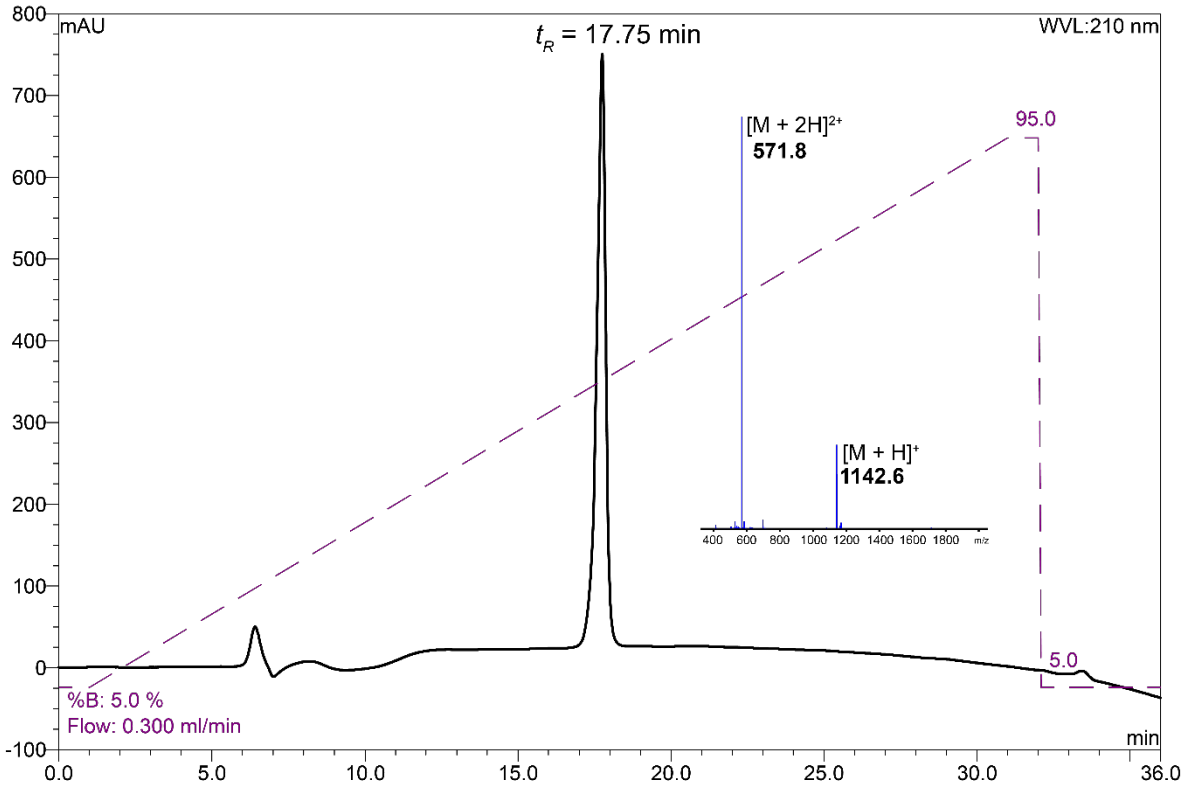
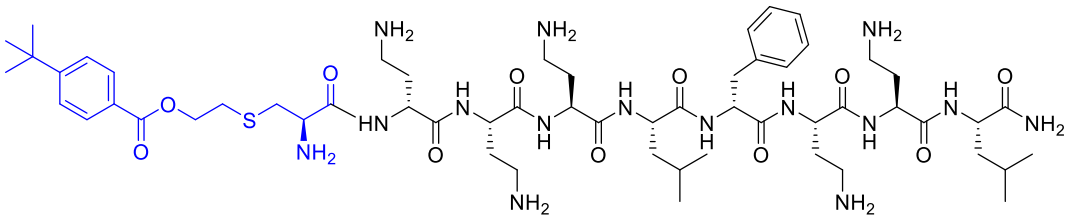


Figure S8. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **12e** (*ca*. 99% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca*. 3% B per minute at r.t., 0.3 mL/min.

S4.9. Synthesis of linear battacin CLipPA analogue 12f



12f

Purified peptide **12** (15.4 mg, 15.5 µmol), DMPA (4.0 mg, 15.5 µmol, 1 eq.), vinyl 4-*tert*-butylbenzoate (63 µL, 0.3 mmol, 20 eq.) and TIPS (254 µL, 1.2 mmol, 80 eq.) were dissolved in NMP (1.54 mL, 10 mg/mL final concentration w.r.t. **12**) and degassed with argon for 15 min. Volatile liquids *t*BuSH (140 µL, 1.2 mmol, 80 eq.) and TFA (77 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method E**. The peptide was purified according to **General Method D.** Lyophilisation yielded purified linear battacin CLipPA analogue **12f** (3.9 mg, 6% yield, 99% purity) as a white solid; RP-HPLC: t_R = 16.6 min, ESI-MS: $[M + H]^+$ found 1198.6, $[C_{49}H_{87}N_{15}O_{11}S + H]^+$ requires 1198.7, **Figure S9**.

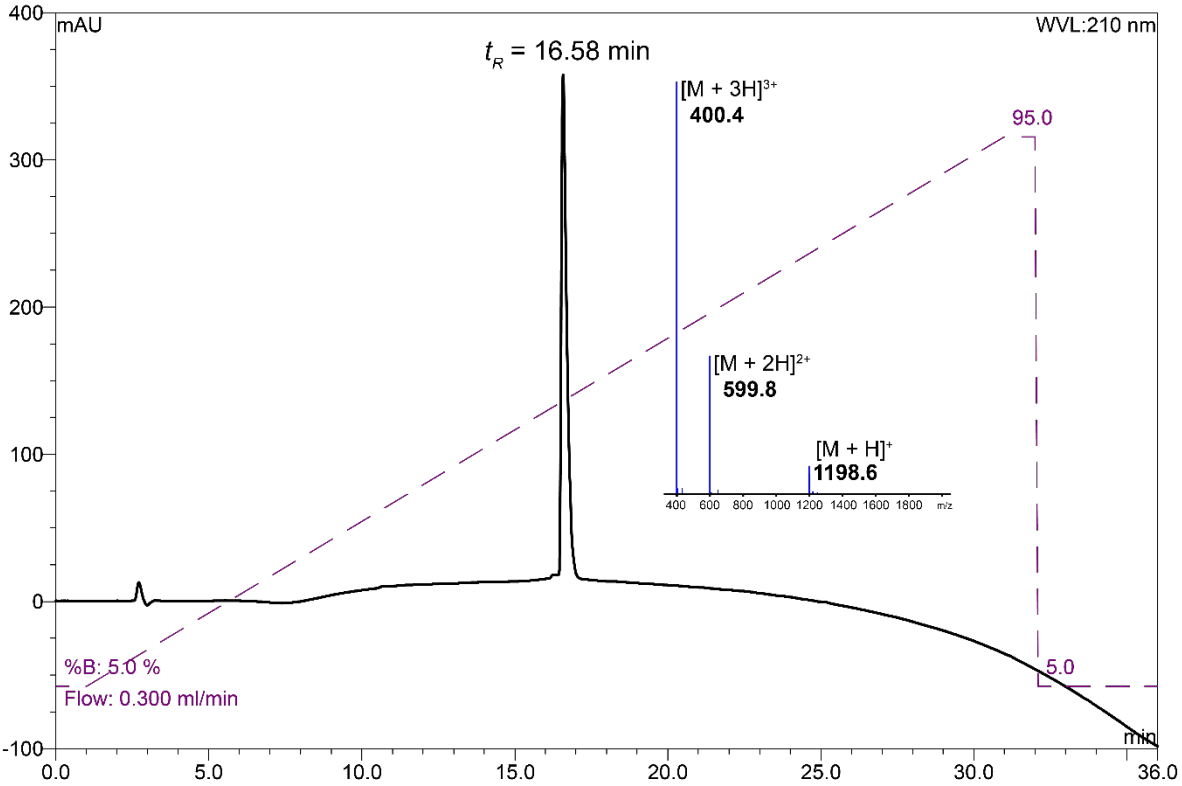
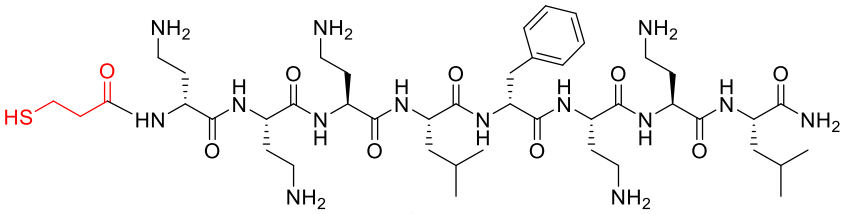


Figure S9. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **12f** (*ca.* 99% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

S4.10. Synthesis of linear battacin analogue 13



13

Peptidyl resin **8** was synthesised using microwave-enhanced Fmoc SPPS according to **General Method A and B**. A portion of **8** (0.3 mmol) was used for coupling (3-trityl)mercaptopropionic acid (418 mg, 1.2 mmol, 4 eq.) onto the *N*-terminus of D-Dab using HATU (433 mg, 1.1 mmol, 3.8 eq.) and DIPEA (402 µL, 2.4 mmol, 8 eq.) according to **General Method B**. The peptide was cleaved from the resin according to **General Method C** and then purified according to **General Method D**. Lyophilisation yielded purified linear battacin analogue **13** (93.8 mg, 32% yield, 95% purity) as a white solid; RP-HPLC: t_R = 16.8 min, ESI-MS: $[M + H]^+$ found 979.5, $[C_{44}H_{78}N_{14}O_9S + H]^+$ requires 979.6, **Figure S10**.

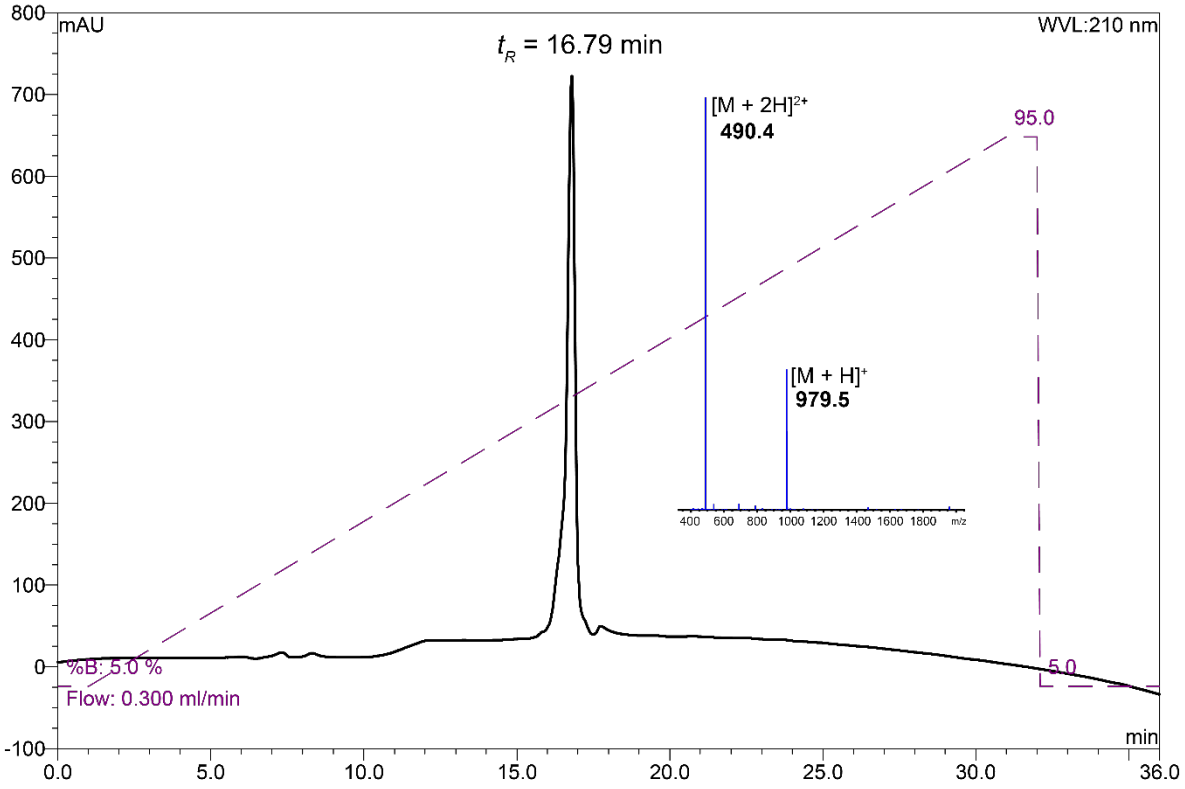
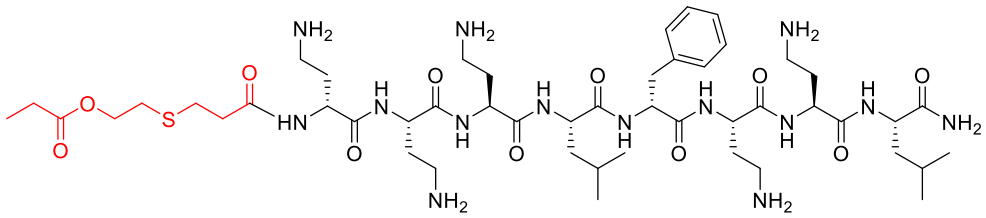


Figure S10. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **13** (*ca.* 95% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

S4.11. Synthesis of linear battacin CLipPA analogue 13a



13a

Purified peptide **13** (14.5 mg, 14.8 µmol), DMPA (3.8 mg, 14.8 µmol, 1 eq.), vinyl propionate (32 µL, 0.3 mmol, 20 eq.) and TIPS (243 µL, 1.2 mmol, 80 eq.) were dissolved in NMP (1.45 mL, 10 mg/mL final concentration w.r.t. **13**) and degassed with argon for 15 min. Volatile liquids *t*BuSH (134 µL, 1.2 mmol, 80 eq.) and TFA (73 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method E**. The peptide was purified according to **General Method D.** Lyophilisation yielded purified linear battacin CLipPA analogue **13a** (7.8 mg, 16% yield, 98% purity) as a white solid; RP-HPLC: t_R = 14.3 min, ESI-MS: $[M + H]^+$ found 1079.6, $[C_{49}H_{86}N_{14}O_{11}S + H]^+$ requires 1079.6, **Figure S11**.

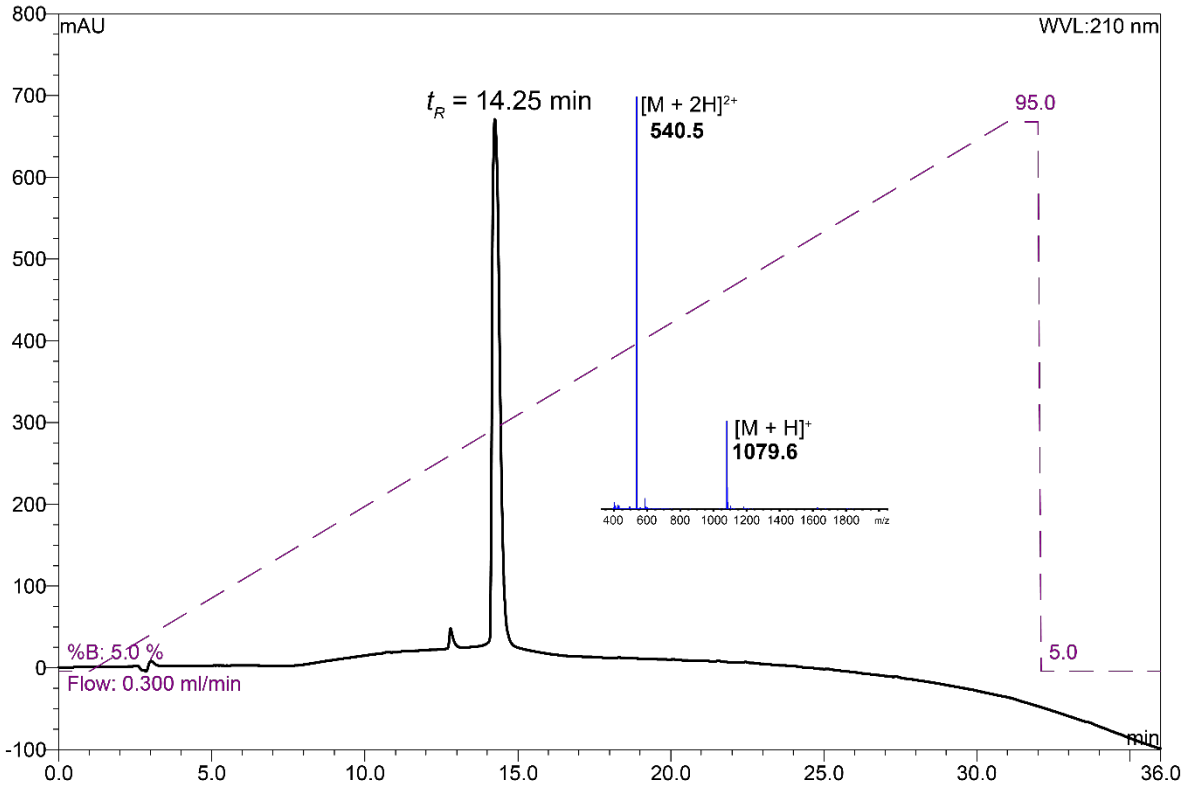
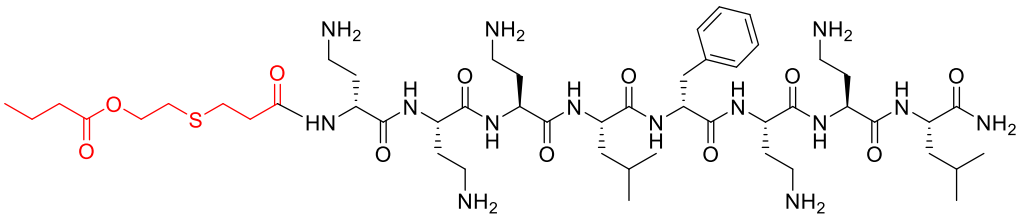


Figure S11. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **13a** (*ca.* 98% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca*. 3% B per minute at r.t., 0.3 mL/min.

S4.12. Synthesis of linear battacin CLipPA analogue 13b



13b

Purified peptide **13** (14.6 mg, 14.9 µmol), DMPA (3.8 mg, 14.9 µmol, 1 eq.), vinyl butyrate (38 µL, 0.3 mmol, 20 eq.) and TIPS (245 µL, 1.2 mmol, 80 eq.) were dissolved in NMP (1.46mL, 10 mg/mL final concentration w.r.t. **13**) and degassed with argon for 15 min. Volatile liquids *t*BuSH (135 µL, 1.2 mmol, 80 eq.) and TFA (73 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method E**. The peptide was purified according to **General Method D.** Lyophilisation yielded purified linear battacin CLipPA analogue **13b** (8.6 mg, 17% yield, 99% purity) as a white solid; RP-HPLC: t_R = 14.8 min, ESI-MS: $[M + H]^+$ found 1093.6, $[C_{50}H_{88}N_{14}O_{11}S + H]^+$ requires 1093.7, **Figure S12**.

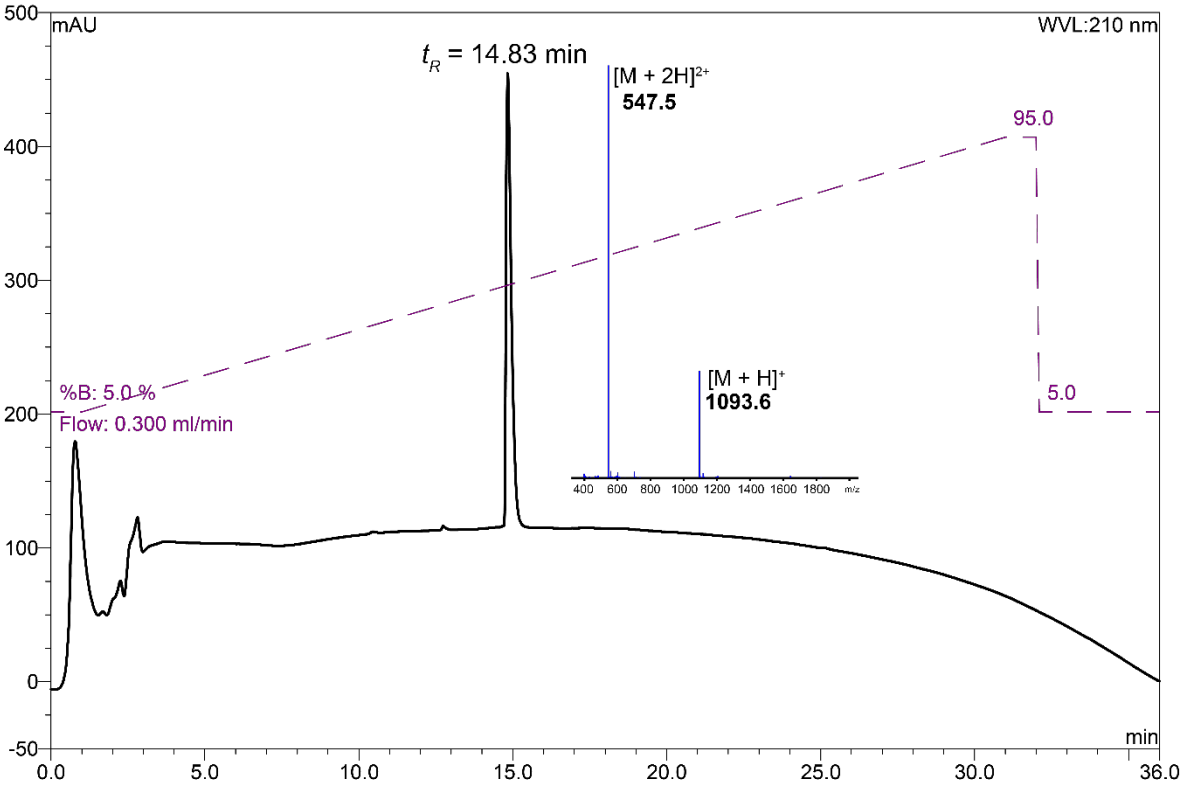
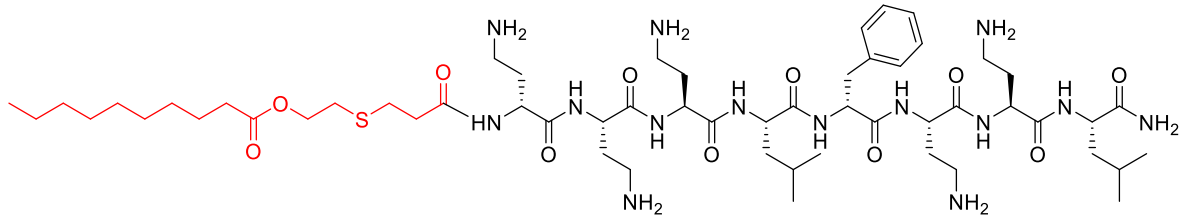


Figure S12. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **13b** (*ca.* 99% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

S4.13. Synthesis of linear battacin CLipPA analogue 13c



13c

Purified peptide **13** (11.6 mg, 11.9 μmol), DMPA (3.0 mg, 11.9 μmol, 1 eq.), vinyl decanoate (53 μL, 0.2 mmol, 20 eq.) and TIPS (194 μL, 0.9 mmol, 80 eq.) were dissolved in NMP (1.16 mL, 10 mg/mL final concentration w.r.t. **13**) and degassed with argon for 15 min. Volatile liquids *t*BuSH (107 μL, 0.9 mmol, 80 eq.) and TFA (58 μL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method E**. The peptide was purified according to **General Method D.** Lyophilisation yielded purified linear battacin CLipPA analogue **13c** (2.8 mg, 6% yield, 94% purity) as a white solid; RP-HPLC: t_R = 18.5 min, ESI-MS: $[M + H]^+$ found 1177.7, $[C_{56}H_{100}N_{14}O_{11}S + H]^+$ requires 1177.7, **Figure S13**.

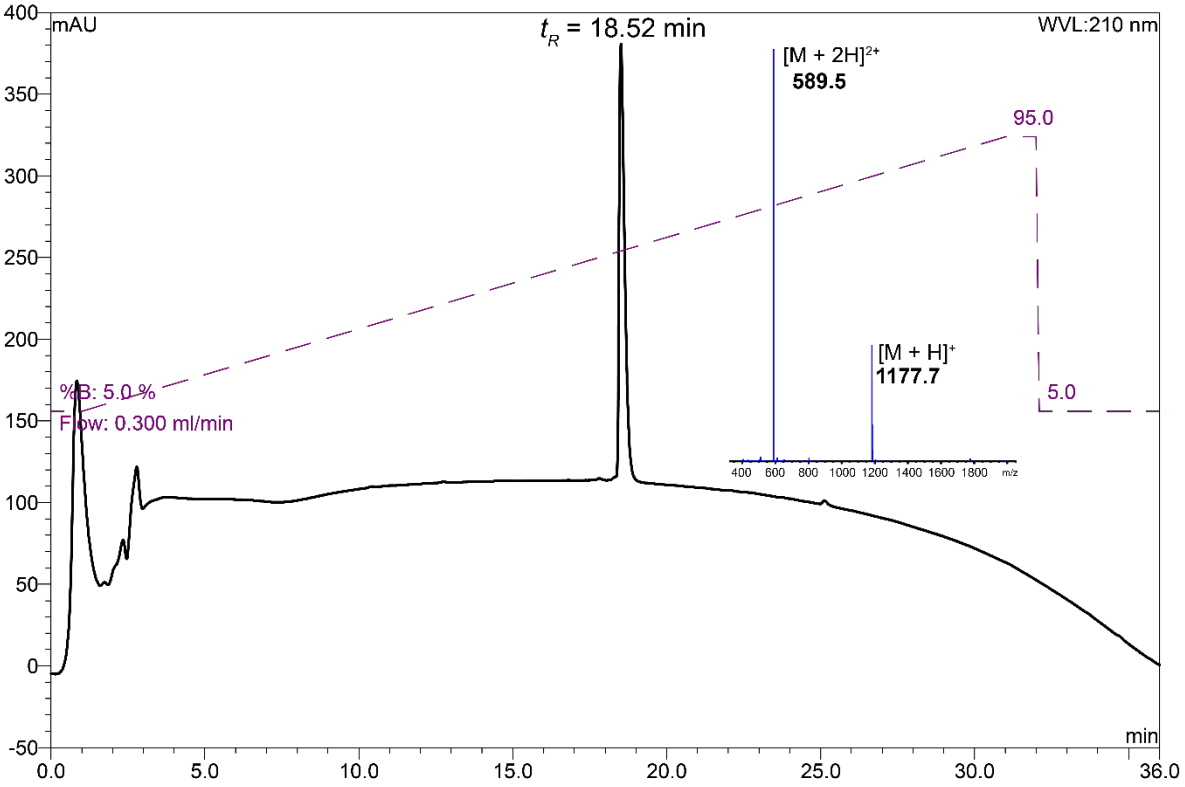
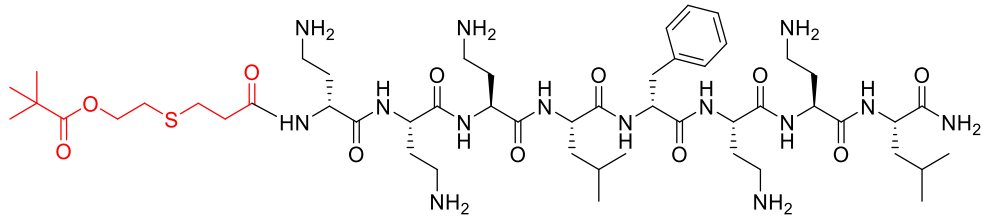


Figure S13. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **13c** (*ca.* 94% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 μm), linear gradient of 5% B to 95% B over 30 min, *ca*. 3% B per minute at r.t., 0.3 mL/min.

S4.14. Synthesis of linear battacin CLipPA analogue 13d



13d

Purified peptide **13** (15.3 mg, 15.6 µmol), DMPA (4.0 mg, 15.6 µmol, 1 eq.), vinyl pivalate (46 µL, 0.3 mmol, 20 eq.) and TIPS (256 µL, 1.3 mmol, 80 eq.) were dissolved in NMP (1.53 mL, 10 mg/mL final concentration w.r.t. **13**) and degassed with argon for 15 min. Volatile liquids *t*BuSH (141 µL, 1.3 mmol, 80 eq.) and TFA (77 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method E**. The peptide was purified according to **General Method D.** Lyophilisation yielded purified linear battacin CLipPA analogue **13d** (7.9 mg, 15% yield, 95% purity) as a white solid; RP-HPLC: t_R = 14.7 min, ESI-MS: $[M + H]^+$ found 1107.6, $[C_{51}H_{90}N_{14}O_{11}S + H]^+$ requires 1107.7, **Figure S14**.

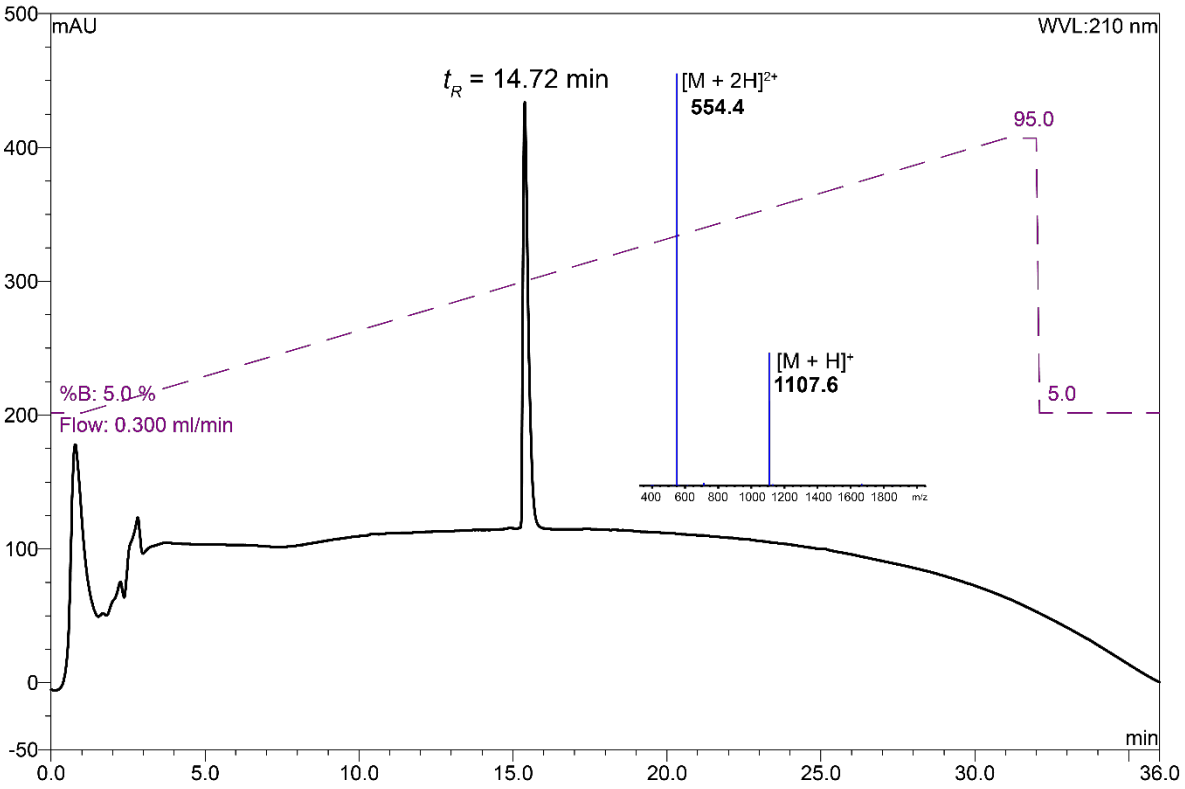
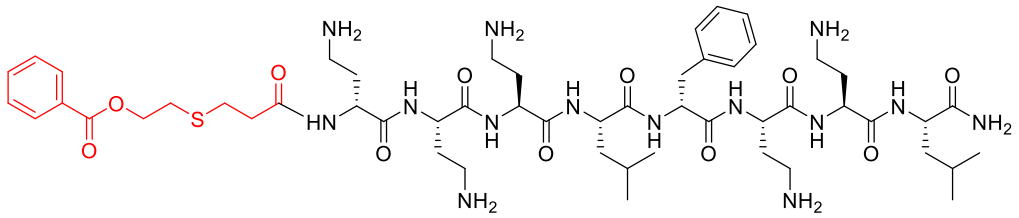


Figure S14. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **13d** (*ca.* 95% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

S4.15. Synthesis of linear battacin CLipPA analogue 13e



13e

Purified peptide **13** (15.7 mg, 16.0 µmol), DMPA (4.1 mg, 16.0 µmol, 1 eq.), vinyl benzoate (44 µL, 0.3 mmol, 20 eq.) and TIPS (263 µL, 1.3 mmol, 80 eq.) were dissolved in NMP (1.57 mL, 10 mg/mL final concentration w.r.t. **13**) and degassed with argon for 15 min. Volatile liquids *t*BuSH (145 µL, 1.3 mmol, 80 eq.) and TFA (79 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method E**. The peptide was purified according to **General Method D.** Lyophilisation yielded purified linear battacin CLipPA analogue **13e** (4.5 mg, 8% yield, 96% purity) as a white solid; RP-HPLC: t_R = 15.6 min, ESI-MS: $[M + H]^+$ found 1127.7, $[C_{53}H_{86}N_{14}O_{11}S + H]^+$ requires 1127.7, **Figure S15**.

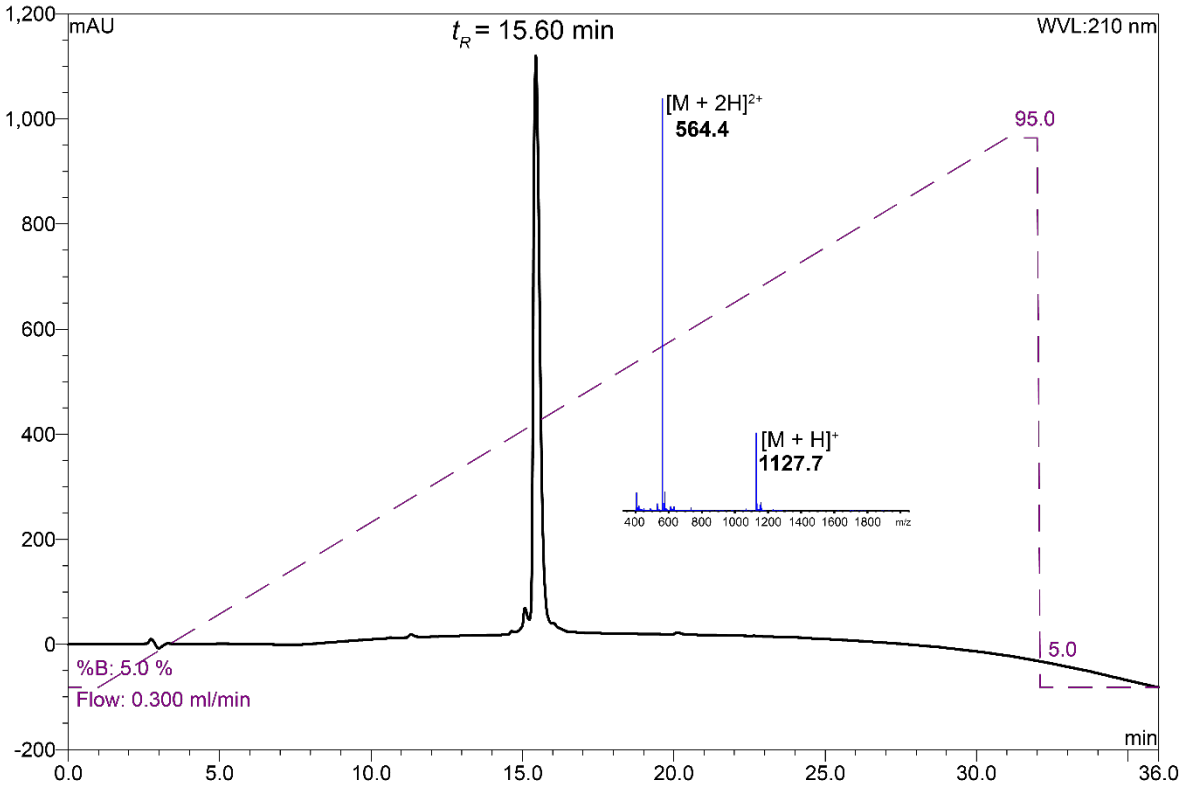
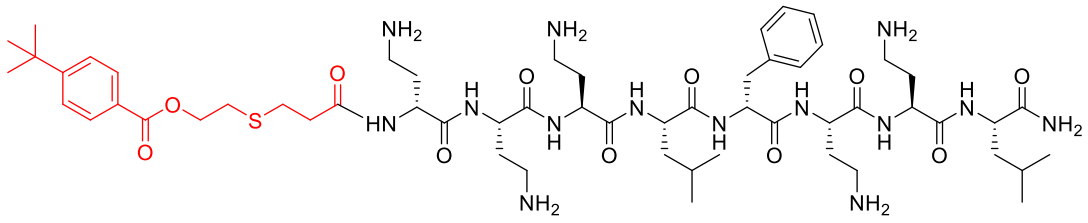


Figure S15. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **13e** (*ca.* 96% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca*. 3% B per minute at r.t., 0.3 mL/min.

S4.16. Synthesis of linear battacin CLipPA analogue 13f



13f

Purified peptide **13** (15.5 mg, 15.8 µmol), DMPA (4.1 mg, 15.8 µmol, 1 eq.), vinyl 4-*tert*-butylbenzoate (65 µL, 0.3 mmol, 20 eq.) and TIPS (260 µL, 1.3 mmol, 80 eq.) were dissolved in NMP (1.55 mL, 10 mg/mL final concentration w.r.t. **13**) and degassed with argon for 15 min. Volatile liquids *t*BuSH (143 µL, 1.3 mmol, 80 eq.) and TFA (78 µL, 5% *v/v*) were added under argon. The mixture underwent the CLipPA reaction according to **General Method E**. The peptide was purified according to **General Method D.** Lyophilisation yielded purified linear battacin CLipPA analogue **13f** (3.0 mg, 5% yield, 96% purity) as a white solid; RP-HPLC: t_R = 17.5 min, ESI-MS: $[M + H]^+$ found 1183.6, $[C_{49}H_{86}N_{14}O_{11}S + H]^+$ requires 1183.7, **Figure S16**.

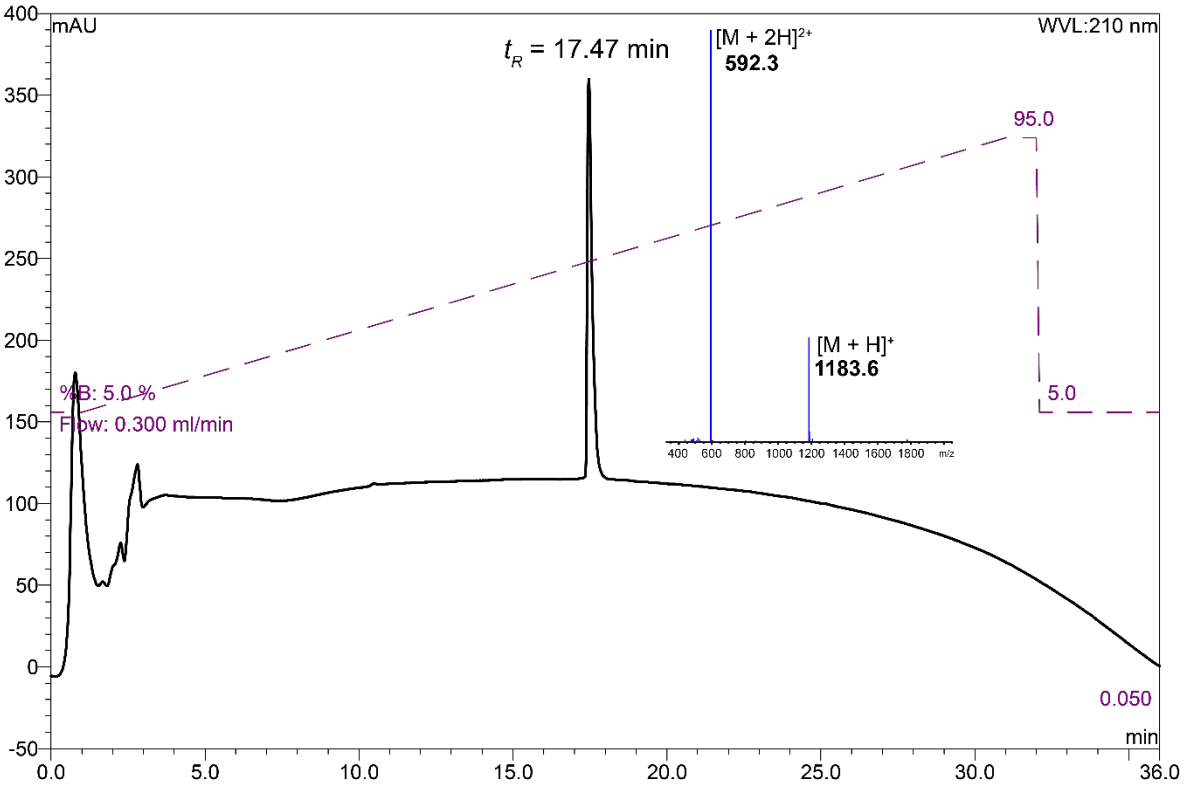


Figure S16. Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence **13f** (*ca.* 96% as judged by peak area of RP-HPLC at 210 nm); Agilent Zorbax 300SB-C3, (3.0 mm × 150 mm; 5 µm), linear gradient of 5% B to 95% B over 30 min, *ca.* 3% B per minute at r.t., 0.3 mL/min.

S5. Antibacterial susceptibility testing

Bacteria were routinely grown in non-cation adjusted Mueller Hinton (MH) broth at 37 °C with shaking (200 rpm). All MIC testing was performed as previously reported.[3] Briefly, a two-fold dilution series (from 128 μM to 0.0625 μM, final) was prepared in polypropylene 96-well plates using MH broth. Overnight cultures of bacteria were diluted accordingly in fresh MH before adding 50 μL of inoculum to each well of the MIC plate, to achieve a final volume of 100 μL with a uniform CFU/mL of ~5 × 10^5 in each well. Plates were incubated at 37 °C with shaking for 24 h before determining the MIC. MIC's were determined as the lowest concentration at which growth did not occur. MBC's were determined by diluting 10 μL of culture from the MIC plate in sterile PBS to 10^{-3}, before spot plating 10 μL onto Brain Heart Infusion (BHI) agar plates. Spots were left to dry before incubating at 37 °C for 24 h. MBC's were determined as the lowest concentration at which growth did not occur (**Table S1**). Compounds were assayed in technical duplicate and the assays were repeated independently on three occasions. The highest concordant result at which growth did not occur for all replicates was used to determine the MIC and MBC.

Table S1. MIC and MBC of linear battacin CLipPA analogues (μM)

Peptide	*E. coli* ATCC 10546		*P. aeruginosa* PA01		*A. calcoaceticus* NZRM 150		*S. aureus* ATCC 6538	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
9	128	128	>128	>128	>128	>128	>128	>128
2	8	8	>128	>128	128	128	64	128
12	32	32	64	128	64	64	128	128
12a	32	32	>128	>128	>128	>128	>128	>128
12b	16	16	>128	>128	>128	>128	128	>128
12c	16	32	32	32	32	32	8	8
12d	16	16	128	>128	>128	>128	>128	>128
12e	8	8	64	128	128	>128	64	64
12f	8	8	8	16	32	32	8	16
13	32	32	32	>128	64	64	64	64
13a	16	32	>128	>128	>128	>128	>128	>128
13b	16	16	>128	>128	>128	>128	128	128
13c	8	8	8	16	32	32	8	8
13d	16	16	64	>128	128	128	64	128
13e	8	16	64	128	64	64	32	32
13f	8	8	16	16	32	32	8	8
Tetracycline (μg/ml)	-	-	8	128	2	8	-	-
Ampicillin (μg/ml)	2	2	-	-	-	-	0.125	0.25

S6. Computational methods

S6.1. Coordinates

Initial coordinates for both peptides were obtained using the Avogadro software[7] and refined by energy minimization and a 500 ps equilibration as outlined below. Pre-equilibrated coordinates for a lipid bilayer representative of the *E. coli* inner membrane (Table 1) was obtained from Dr Tom Piggot[8]. Peptide molecules were placed at least 1.4 nm (the cutoff distance for calculation of inter-atomic interactions) from the membrane, and peptide and membrane coordinates were combined by simply concatenating the coordinate files.

Table 1. Lipid composition of the *E. coli* inner membrane model bilayer. The two leaflets of the bilayer had identical lipid composition.

Lipid headgroup	Lipid tail	%
Phosphatidylethanolamine (PE)	1-palmitoyl, 2-*cis*-vaccenyl (PV)	70%
Phosphatidylglycerol (PG)	1-palmitoyl, 2-*cis*-vaccenyl (PV)	15%
Diphosphatidylglycerol (DPG/cardiolipin)	1-palmitoyl, 2-*cis*-vaccenyl (PV)	5%

S6.2. Parameters

The natural amino acids in battacin were modelled using standard GROMOS 54A7[9] parameters. Parameters for L-Dab were obtained by removing one CH_2 group from the side chain of Lys, and D-Phe and D-Dab were obtained by inverting the stereochemistry of the C_α atom of the L-Phe and L-Dab parameters. The terminal amine of D-Dab was modelled as NH_3^+, representative of its state at pH 7, unless otherwise specified. Partial charges for the NH_2 state were obtained by analogy to the deprotonated state of lysine. GROMOS-CKP [8, 10-12] parameters, which are compatible with the GROMOS 54A7 force field, were used for the phospholipids and for the lipid portions of the battacin analogues.

S6.3. Molecular dynamics simulations

All simulations were performed using the GROMACS molecular dynamics software[13] version 2016.3. All bond lengths were constrained using the LINCS algorithm[14] allowing for a 2 fs time step, and periodic boundary conditions were applied. The energy of the complete peptide-membrane system was minimized using the steepest descent algorithm until the maximum force changed by less than 1000 $kJ{\cdot}mol^{-1}{\cdot}nm^{-1}$, and then solvated using the SPC water model[15] and minimised again. Each system was then neutralised by addition of Na^+ ions and again energy minimised. Each system was equilibrated for 500 ps under standard conditions with the temperature maintained at physiological temperature, 310 K, using the Berendsen thermostat [10] with a time constant of 1 ps, and the pressure maintained at 1 bar using the Berendsen barostat[16] with semi-isotropic pressure coupling, a time constant of 1 ps and an isothermal compressibility of 4.575×10^{-4} $(kJ{\cdot}mol^{-1}nm^{-3})^{-1}$. For the production runs, the temperature was maintained at 310 K using the Nosé-Hoover thermostat [17,18] with a time constant of 1 ps, and the pressure of 1 bar was maintained using semi-isotropic pressure coupling using the Parrinello-Rahman barostat[19] with a time constant of 5 ps and an isothermal compressibility of 4.575×10^{-4} $(kJ{\cdot}mol^{-1}nm^{-3})^{-1}$. For both the equilibration and production runs, long-range electrostatic interactions outside a cut-off of 1.4 nm were treated using the reaction field[20] algorithm and van der Waals interactions were truncated at 1.4 nm.

Each peptide was first simulated alone in solution for 500 ns. Each peptide-membrane system, comprising one copy of a given peptide with one of the two types of membrane, was first simulated in quintuplicate for 50 ns, and three of these were extended to 500 ns.

S6.4. Analysis

All analysis was carried out using GROMACS tools unless otherwise specified. Partial electron densities along the Z axis (perpendicular to the plane of the membrane) were calculated with the system vertically centred to the middle of the lipid bilayer.

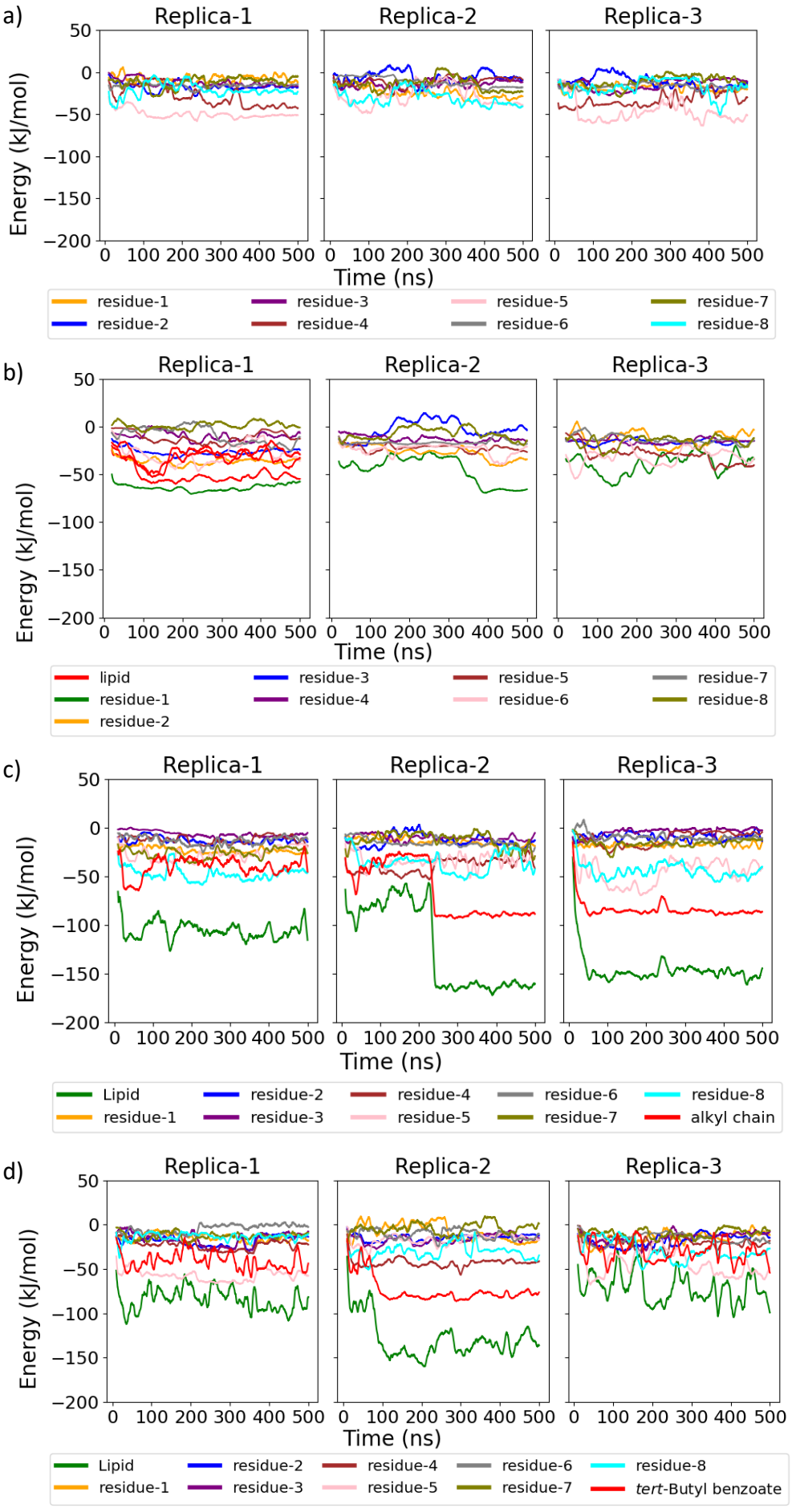

a)
Replica-1
Replica-2
Replica-3
Energy (kJ/mol)
50
0
−50
−100
−150
−200
0 100 200 300 400 500
Time (ns)
residue-1
residue-2
residue-3
residue-4
residue-5
residue-6
residue-7
residue-8
b)
Replica-1
Replica-2
Replica-3
Energy (kJ/mol)
Time (ns)
lipid
residue-1
residue-2
residue-3
residue-4
residue-5
residue-6
residue-7
residue-8
c)
Replica-1
Replica-2
Replica-3
Energy (kJ/mol)
Time (ns)
Lipid
residue-1
residue-2
residue-3
residue-4
residue-5
residue-6
residue-7
residue-8
alkyl chain
d)
Replica-1
Replica-2
Replica-3
Energy (kJ/mol)
Time (ns)
Lipid
residue-1
residue-2
residue-3
residue-4
residue-5
residue-6
residue-7
residue-8
tert-Butyl benzoate

Figure S17. The alkyl chain contributes substantially to the favourable interactions between inserted lipid tails and membrane lipids. The interaction energy between the membrane lipids and for each residue of battacin, as indicated, for analogues a) **9**, b) **2**, c) 1**3c** and d) **13f**. Residue 1 refers to the first amino acid (D-Dab) for analogue **9**, and to the lipid tail for analogues **2**, **12c**, **13c**, **12f** and **13f**. 'Lipid' refers to the entire lipid tail, including the linker region, and the contribution of only the alkyl chain or *tert*-butyl benzoate is indicated separately. The energies are plotted against simulation time for each of the three replicate simulations. The battacin analogues approach the membrane during the initial 100-200 ns of each simulation and thereafter, are bound to and, in some cases, penetrate, the membrane surface.

S7. References

1 V. K. Sarin, S. B. H. Kent, J. P. Tam and R. B. Merrifield, *Anal. Biochem.*, 1981, **117**, 147–157.
2 P. W. R. Harris, D. J. Lee and M. A. Brimble, *J. Pept. Sci.*, 2012, **18**, 549–555.
3 I. Wiegand, K. Hilpert and R. E. W. Hancock, *Nat. Protoc.*, 2008, **3**, 163.
4 N. Hattori, M.-O. Nakajima, K. O'Hara and T. Sawai, *Antimicrob. Agents Chemother.*, 1998, **42**, 1406–1411.
5 Y. Cai, C. L. Seah, H. Leck, T.-P. Lim, J. Q. Teo, W. Lee, T.-T. Tan, T.-H. Koh, P. L. R. Ee and A. L. Kwa, *Antimicrob. Agents Chemother.*, 2018, **62**, e00183-18.
6 A. Adan, G. Alizada, Y. Kiraz, Y. Baran and A. Nalbant, *Crit. Rev. Biotechnol.*, 2017, **37**, 163–176.
7 M. D. Hanwell, D. E. Curtis, D. C. Lonie, T. Vandermeersch, E. Zurek and G. R. Hutchison, *J. Cheminform.*, 2012, **4**, 17.
8 T. J. Piggot, D. A. Holdbrook and S. Khalid, *Biochim. Biophys. Acta - Biomembr.*, 2013, **1828**, 284–293.
9 N. Schmid, A. P. Eichenberger, A. Choutko, S. Riniker, M. Winger, A. E. Mark and W. F. Van Gunsteren, *Eur. Biophys. J.*, 2011, **40**, 843–856.
10 T. J. Piggot, D. A. Holdbrook and S. Khalid, *J. Phys. Chem. B*, 2011, **115**, 13381–13388.
11 A. Kukol, *J. Chem. Theory Comput.*, 2009, **5**, 615–626.
12 I. Chandrasekhar, M. Kastenholz, R. D. Lins, C. Oostenbrink, L. D. Schuler, D. P. Tieleman and W. F. Van Gunsteren, *Eur. Biophys. J.*, 2003, **32**, 67–77.
13 M. J. Abraham, T. Murtola, R. Schulz, S. Páll, J. C. Smith, B. Hess and E. Lindah, *SoftwareX*, 2015, **1**, 19–25.
14 B. Hess, H. Bekker, H. J. C. Berendsen and J. G. E. M. Fraaije, *J. Comput. Chem.*, 1997, **18**, 1463–1472.
15 H. J. C. Berendsen, J. P. M. Postma, W. F. van Gunsteren and J. Hermans, in *Intermolecular Interactions*, 1981, pp. 331–342.
16 H. J. C. Berendsen, J. P. M. Postma, W. F. Van Gunsteren, A. Dinola and J. R. Haak, *J. Chem. Phys.*, 1984, **81**, 3684–3690.
17 S. Nosé, *Mol. Phys.*, 1984, **52**, 255–268.
18 W. G. Hoover, *Phys. Rev. A*, 1985, **31**, 1695–1697.
19 M. Parrinello and A. Rahman, *J. Appl. Phys.*, 1981, **52**, 7182–7190.
20 W. F. Van Gunsteren, H. J. C. Berendsen and J. A. C. Rullmann, *Faraday Discuss. Chem. Soc.*, 1978, **66**, 58–70.