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,c 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........................................................................................................................................... 2
S2. General Information.................................................................................................................................... 2
S3. General Methods......................................................................................................................................... 3
S4. Synthesis, structures and LC-MS profiles of synthetic peptides ............................................................. 5
  S4.1. Synthesis of linear battacin analogue 2........................................................................................................ 5
  S4.2. Synthesis of linear battacin sequence 9......................................................................................................... 6
  S4.3. Synthesis of linear battacin analogue 12..................................................................................................... 7
  S4.4. Synthesis of linear battacin CLipPA analogue 12a..................................................................................... 8
  S4.5. Synthesis of linear battacin CLipPA analogue 12b..................................................................................... 9
  S4.6. Synthesis of linear battacin CLipPA analogue 12c.................................................................................... 10
  S4.7. Synthesis of linear battacin CLipPA analogue 12d.................................................................................... 11
  S4.8. Synthesis of linear battacin CLipPA analogue 12e.................................................................................... 12
  S4.9. Synthesis of linear battacin CLipPA analogue 12f.................................................................................... 13
  S4.10. Synthesis of linear battacin analogue 13.................................................................................................. 14
  S4.11. Synthesis of linear battacin CLipPA analogue 13a.................................................................................. 15
  S4.12. Synthesis of linear battacin CLipPA analogue 13b.................................................................................. 16
  S4.13. Synthesis of linear battacin CLipPA analogue 13c................................................................................ 17
  S4.15. Synthesis of linear battacin CLipPA analogue 13e............................................................................... 19
  S4.16. Synthesis of linear battacin CLipPA analogue 13f............................................................................... 20
S5. Antibacterial susceptibility testing............................................................................................................... 21
S6. Computational methods............................................................................................................................... 22
  S6.1. Coordinates.............................................................................................................................................. 22
  S6.2. Parameters............................................................................................................................................... 22
  S6.3. Molecular dynamics simulations............................................................................................................ 22
  S6.4. Analysis.................................................................................................................................................. 23
S7. References.................................................................................................................................................... 25
S1. Abbreviations


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, tBuSH, 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 (Wellington, Florida). DMF was purchased from Fischer Scientific (Fair Lawn, New Jersey) and TFA was obtained from Oakwood Chemicals (Estill, NC). H₂O was purified using a Sartorius arium® pro ultrapure water system.

Microwave assisted reactions were carried out 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 Chromlelon software was used for data processing. Buffer A: 0.1% (v/v) TFA in H₂O; 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 H₂O; buffer B: 0.1% (v/v) formic acid in MeCN. Crude peptides were purified on Dionex UltiMate 3000 preparative HPLC using Agilent Zorbax 300SB-C18 column (5 µm, 9.4 × 250 mm) and Chromlelon 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$_2$Cl$_2$ (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$_2$Cl$_2$ (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$^\alpha$-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. The N$^\alpha$-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$_2$O: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 H₂O: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 = H₂O (+ 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 H₂O: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). 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; H₂O: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 tBuSH (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 H₂O: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

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 4R/4S-methylhexanoic acid (MHA) (57 μL, 0.4 mmol, 4 eq.) onto the N-terminus of 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](image-url)
S4.2. Synthesis of linear battacin sequence 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.** 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

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$_9$S + H]$^+$ requires 994.6, Figure S3.

![Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence 12](image)

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

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 tBuSH (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 battecin CLipPA analogue 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 tBuSH (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 battecin 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 battecin 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

![Diagram of the linear battacin CLipPA analogue 12c](image)

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 tBuSH (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.

![Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence 12c](image)

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

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 tBuSH (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.

![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 320 nm); Agilent Zorbax 300SB-C3, (3.0 mm x 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.](image)

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 x 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

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 tBuSH (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, \([C_{48}H_{87}N_{15}O_{11}S + H]^+\) 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

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 fBuSH (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.

![Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence 12f](image)

**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

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)mercapto propionic 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**

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 tBuSH (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**.

![Analytical RP-HPLC trace with inset ESI-MS spectrum of linear battacin sequence 13a](image)

**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

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 (1.46 mL, 10 mg/mL final concentration w.r.t. 13) and degassed with argon for 15 min. Volatile liquids tBuSH (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

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 fBuSH (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: \( t_R = 18.5 \text{ min} \), 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.

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 tBuSH (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: $t_R = 14.7 \text{ min}$, 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

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 tBuSH (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

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 fBuSH (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, [C₄₉H₈₆N₁₄O₁₁S + 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>
<thead>
<tr>
<th>Peptide</th>
<th>E. coli ATCC 10546 MIC</th>
<th>P. aeruginosa PA01 MIC</th>
<th>A. calcoaceticus NZRM 150 MIC</th>
<th>S. aureus ATCC 6538 MIC</th>
<th>MBC</th>
<th>MBC</th>
<th>MBC</th>
<th>MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>8</td>
<td>&gt;128</td>
<td>128</td>
<td>128</td>
<td>64</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>32</td>
<td>32</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>128</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12a</td>
<td>32</td>
<td>32</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>12b</td>
<td>16</td>
<td>16</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>12c</td>
<td>16</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>12d</td>
<td>16</td>
<td>128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>12e</td>
<td>8</td>
<td>8</td>
<td>64</td>
<td>128</td>
<td>&gt;128</td>
<td>64</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>12f</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>32</td>
<td>8</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>&gt;128</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>13a</td>
<td>16</td>
<td>32</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td>13b</td>
<td>16</td>
<td>16</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>128</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>13c</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>32</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>13d</td>
<td>16</td>
<td>16</td>
<td>64</td>
<td>&gt;128</td>
<td>128</td>
<td>64</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>13e</td>
<td>8</td>
<td>16</td>
<td>64</td>
<td>128</td>
<td>64</td>
<td>64</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>13f</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>16</td>
<td>32</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Tetracycline (µg/ml)</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>128</td>
<td>2</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ampicillin (µg/ml)</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>0.125</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 \textit{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.

\textbf{Table 1.} Lipid composition of the \textit{E. coli} inner membrane model bilayer. The two leaflets of the bilayer had identical lipid composition.

<table>
<thead>
<tr>
<th>Lipid headgroup</th>
<th>Lipid tail</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylethanolamine (PE)</td>
<td>1-palmitoyl, 2-cis-vaccenyl (PV)</td>
<td>70%</td>
</tr>
<tr>
<td>Phosphatidylglycerol (PG)</td>
<td>1-palmitoyl, 2-cis-vaccenyl (PV)</td>
<td>15%</td>
</tr>
<tr>
<td>Diphosphatidylglycerol (DPG/cardiolipin)</td>
<td>1-palmitoyl, 2-cis-vaccenyl (PV)</td>
<td>5%</td>
</tr>
</tbody>
</table>

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\(_\alpha\) 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⋅mol\(^{-1}\)⋅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⋅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⋅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.
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) 13c 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