Supplementary information
for
Synthesis of CLipPA Analogues of the Antimicrobial Peptide, Paenipeptin C'

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S1. Abbreviations

˚C: degrees Celsius. 6-Cl-HOBt: 6-chloro-1-hydroxybenzotriazole. AA: amino acid. Ala: alanine. AMP: antimicrobial peptide. AMR: antimicrobial resistance. ATCC: American Type Culture Collection. BHI: brain heart infusion. Boc: *tert*-butoxycarbonyl. CH_2Cl_2: dichloromethane. CLipPA: Cysteine Lipidation on a Peptide or Amino acid. COMU: (1-[(1-(cyano-2-ethoxy-2-oxoethylidene. CM: ChemMatrix. Cys (C): cysteine. Dab: 2,4-diaminobutyric acid. DIC: *N*,*N*'-diisopropylcarbodiimide. DIPEA: *N*,*N*-diisopropylethylamine. DMF: *N*,*N*'-dimethylformamide. *E. coli*: *Escherichia coli*. EDT: ethanediothiol. ESI-MS: electronspray ionization-mass spectrometry. Et_2O: diethyl ether. Eq: equivalent(s). Fmoc: 9-fluorenylmethyloxycarbonyl. FDA: Food and Drug Administration. g: gram. Gly (G): glycine. h: hour(s). HATU: 1-((dimethylamino)(dimethyliminio)methyl)-1*H*-[1,2,3]-triazolo[4,5-*b*]pyridine-3-oxide hexafluorophosphate. HOAt: 1-hydroxy-7-azabenzotriazole. HPLC: high-performance liquid chromatography. Ile (I): isoleucine. LC-MS: liquid chromatography-mass spectrometry. Leu(L): leucine. log

D: distribution coefficient. LPS: lipopolysaccharide. LTA: lipoteichoic acid. M (moles per litre (mol L^{-1}). MBC: minimum bactericidal concentration. Me: methyl. MeCN: acetonitrile. mg: milligram. MH: Mueller Hinton. MIC: minimum inhibitory concentration. min: minute(s). mL (milliliter). mm: millimeter. mmol: millimole. mol: mole. MRSA: methicillin-resistant *Staphylococcus aureus*. N_2: nitrogen (g). nm: nanometer. NMP: N-methyl-2-pyrrolidone. NMM: *N*-methylmorpholine. PBS: phosphate-buffered saline. Phe (F): phenylalanine. PS: polystyrene. R&D: research and development. Rink amide: 4-[(*R*,*S*)-α-[α-(9*H*-fluoren-9-yl)]-methoxycarbonylamino]-2,4-dimethoxy]phenoxyacetic acid. RNA: ribonucleic acid. RP: reverse phase. rpm: revolutions per minute. r.t.: room temperature. *S. aureus*: *Staphylococcus aureus*. SAR: structure activity relationship. Ser (S): serine. SP-CLipPA: solid phase CLipPA. Spp.: species. SPPS: solid phase peptide synthesis. *t*Bu: *tert*-butyl. TFA: trifluoroacetic acid. TIPS: triisopropylsilane. *t*-nonyl-SH: *tert*-nonyl mercaptan. t_R: retention time. Trt: trityl. USA: United States of America. UV: ultraviolet. Val (V): valine. *v/v*: volume per volume. W: watts. WHO: World Health Organisation. μL: microliter(s). μm: micrometre(s). μM: micromolar. μmol: micromole(s).

S2. General Information

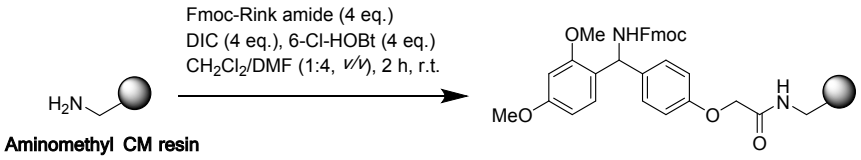
All reagents were used as supplied. Solvents for RP-HPLC were purchased as HPLC grade and used without further purification. 3-(Tritylthio)propionic acid, HATU, and Fmoc-L-amino acids were purchased from GL Biochem (Shanghai, China) with the following side-chain protection: Fmoc-Ser(Trt)-OH, and Fmoc-Dab(Boc)-OH. Fmoc-D-Val-OH was purchased from Auspep (Tullamarine, Australia). Fmoc-Dab(Boc)-OH and Fmoc-D-Phe-OH were purchased from ChemPep (Wellington, FL, USA). Fmoc-amino acids were supplied. Formic acid, DIPEA, piperidine, NMM, NMP, DIC, TIPS, DMPA, *tert*-nonyl mercaptan (mixture of isomers), EDT, decanoic acid, vinyl propionate, vinyl butyrate, vinyl benzoate, vinyl ^{t}Bu-benzoate, and vinyl cinnamate were purchased from Sigma Aldrich (St. Louis, MO, USA). Vinyl hexanoate and vinyl octanoate were purchased from Tokyo Chemical Industry Co., LTD (Tokyo, Japan). DMF (synthesis grade), and MeCN (HPLC grade and LC-MS grade) were purchased from Thermo Fischer Scientific (Hampshire, NH, USA). Octanoic acid was purchased from Honeywell Fluka, (Charlotte, NC, USA). TFA was purchased from Oakwood Chemicals (Estill, SC, USA). 6-Cl-HOBt was purchased from Aapptec (Louisville, KY, USA). Et_2O was purchased from Avantar Performance Chemicals (Center Valley, PA, USA). CH_2Cl_2 was purchased from ECP Limited (Auckland, New Zealand). Petroleum ether was purchased from Burdick & Jackson® (Muskegon, MI, USA). Fmoc-Rink amide linker was purchased from AK Scientific (Union City, CA, USA). Aminomethyl ChemMatrix® resin was purchased from PCAS BioMatrix Inc. (Quebec, Canada). Milli-Q high purity deionised water (MQ H_2O) was available from a Satorius Arium® Pro Ultrapure Water System from Sartorius Stedim Biotech (Göttingen, Germany).

Either a PS3 (Gyros Protein Technologies, Tuscon, AZ, USA) synthesiser or CEM (Mathews, NC, USA) Discover microwave synthesiser was used for peptide synthesis. Analytical liquid chromatography mass spectrometry (LC-MS) was performed on an Agilent Technologies (Santa Clara, CA, USA) 1260 infinity LC connected to an Agilent Technologies 6120 quadrupole MSD spectrophotometer. Data processing was performed on OpenLAB software. An Agilent Zorbax 300SB-C3; 3.0 × 150 mm; 3.5 μm (0.3 mL/min) column and a linear gradient was used as specified. For LC-MS analysis, solvent A was H_2O containing 0.1% formic acid and solvent B was MeCN containing 0.1% formic acid. Mass spectrometry (MS) was performed on a Waters (Milford, MA, USA) Quattro micro API Mass Spectrometer in ESI positive mode. Analytical reverse phase high-performance liquid chromatography (RP-HPLC) was performed on a Dionex (Sunnyvale, CA, USA) UltiMate® 3000 with either a Phenomenex (Torrance, CA, USA) Gemini C18 110 Å, 150 × 4.60 mm, 5 μm (1.0 mL/min), or Waters Xterra® MS C18 110 Å 5 μm; 4.6 × 150 mm (1.0 mL/min), as the column and using a linear gradient as specified, where solvent A was H_2O containing 0.1% TFA and B was MeCN containing 0.1% TFA. Semi-preparative RP-HPLC was performed using a Dionex Ultimate® 3000 equipped with a four-channel UV detector, using either a Phenomenex Gemini C18 110 Å, 10.0 × 250 mm; 5 μm (4 mL/min) or Agilent Zorbax C18, 9.4 × 250 mm; 5 μm (4 mL/min), as the column. Gradient systems used for semi-preparative RP-HPLC were altered according to the elution time and peak profiles obtained from the analytical RP-HPLC chromatograms.

Nuclear magnetic resonance (NMR) spectra were recorded as indicated on a Bruker AVANCE 400 spectrometer (^{1}H, 400 MHz; ^{13}C, 101 MHz). Chemical shifts are reported in parts per million (ppm) from tetramethylsilane (δ = 0) and were measured relative to the solvent in which the sample was analysed (CDCl3: δ 7.26 for ^{1}H NMR, δ 77.0 for ^{13}C NMR). The ^{1}H NMR shift values are reported as chemical shift (δ_H), the corresponding integral, multiplicity (m = multiplet, dd = doublet of doublets), coupling constant (J in Hz) and assignments. ^{13}C NMR values are reported as the chemical shift (δ_C) and assignment. High-resolution mass spectra were recorded using a Bruker micrOTOF-Q II mass spectrometer.

S3. General Methods

General Method A: Coupling of Fmoc-Rink amide to aminomethyl ChemMatrix® (CM) resin



Aminomethyl Chemmatrix® (CM) resin (1 eq., 0.62 mmol/g) on either a 0.1 or 0.2 mmol scale, was swollen in CH_2Cl_2 (2 mL) for 15 min. The CH_2Cl_2 was drained from the resin and Fmoc-Rink amide (4 eq.), 6-Cl-HOBt (4 eq.) and DIC (4 eq.) in CH_2Cl_2/DMF (1:4, *v/v*) were added to the resin. The reaction was agitated for 2 h at r.t., then the resin was drained. Completion of coupling was confirmed by the ninhydrin test (**General Method F**).

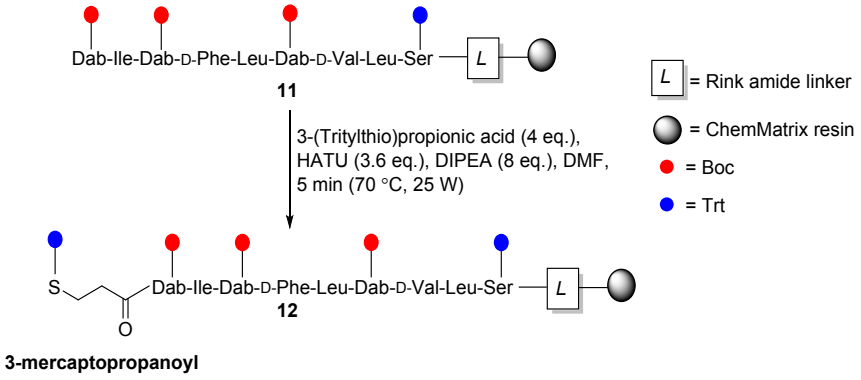
General Method B: Standard removal of *N*$^{\alpha}$-Fmoc protecting group

The peptidyl resin was treated with piperidine in DMF (1:4, *v/v*, 3 mL) for 2 × 5 min at r.t., then drained, followed by resin washing using DMF (5 × 3 mL).

General Method C: Global deprotection and full cleavage of peptide from resin

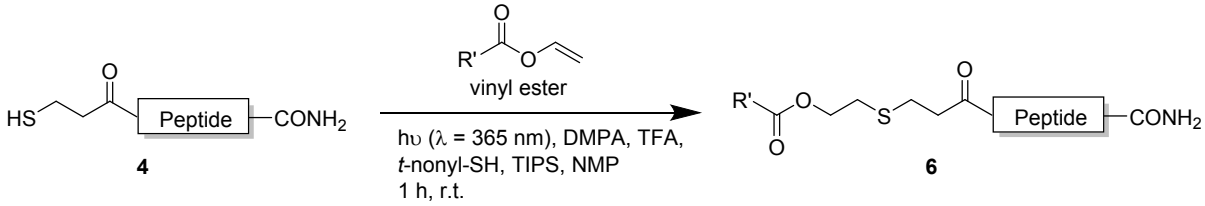
After washing the resin with CH_2Cl_2 (5 × 3 mL) and drying under vacuum, a cleavage cocktail of TFA/EDT/H_2O/TIPS (94:2.5:2.5:1, *v/v/v/v*, 10 mL) was added to the peptidyl resin and agitated for 2 h at r.t.. The cleavage mixture was drained from the resin, and the resin was washed with a further aliquot of TFA (5 mL). The collected TFA mixture was evaporated by a flow of N_2 and the peptide was precipitated from cold diethyl ether (2 × 40 mL), isolated (centrifugation, 4000 rpm, 10 min), concentrated under a light stream of N_2 then the peptide pellet was dissolved in 0.1% (*v/v*) TFA in H_2O/MeCN (1:1, *v/v*, 5 mL). The peptide was then lyophilised.

General Method D: Coupling of 3-(tritylthio)propionic acid to 11



A mixture of 3-(tritylthio)propionic acid (4 eq., 0.8 mmol, 279 mg), HATU (3.6 eq., 0.72 mmol, 274 mg), and DIPEA (8 eq., 1.6 mmol, 280 μL) in DMF (2 mL) was added to **11** (preswollen in CH_2Cl_2) and irradiated at 70 °C, 25 W, for 5 min. The solution was drained and washed with DMF (5 × 3 mL). Completion of coupling was confirmed by a negative result from the ninhydrin test (**General Method F**).

General Method E: Attachment of vinyl esters using solution phase CLipPA methodology



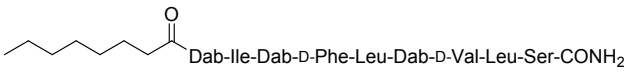
Purified peptide **4** (1 eq., 9 μmol, 10 mg) equipped with the *N*-terminal 3-mercaptopropanoyl handle was added to a solution of vinyl ester (70 eq.), DMPA (1 eq., 9 μmol, 2.4 mg), *t*-nonyl-SH (80 eq., 0.74 mmol, 139 μL), TIPS (80 eq., 0.74 mmol, 152 μL) with 5% TFA in NMP (*v/v*, 50 μL) to give a final concentration of the peptide of *ca*. 10 mg/mL). The NMP solvent was sparged with argon for 20 min at r.t. prior to addition of the peptide and other CLipPA reagents. The reaction mixture was irradiated under UV light (365 nm) for 60 min at r.t. with agitation. Upon completion of the reaction, as determined by analytical RP-HPLC, the peptide was precipitated from a mixture of diethyl ether/petroleum ether (1:1, *v/v*, 2 × 40 mL), isolated (centrifugation, 4000 rpm, 10 min), concentrated under a light stream of N_2 then dissolved in MeCN/H_2O (1:1, *v/v*) containing 0.1% TFA (*v/v*), then lyophilised.

General Method F: Ninhydrin test

To assess completion of linker or AA coupling, a small sample of the Fmoc-protected resin was extracted, washed with CH_2Cl_2 and allowed to dry. A single drop each of the solutions 5% (*w/v*) ninhydrin in ethanol 80% (*w/v*) phenol in EtOH, and 2% (*v/v*) potassium cyanide (KCN) in pyridine were successively added to the dried resin. The mixture was heated at 90 °C for 3 min. A yellow-coloured solution indicated that there were no free primary amines present and hence coupling was complete (negative test), whereas blue-coloured beads and solution indicated that free amino groups were present and therefore coupling was incomplete (positive test).

S4. Synthesis, structures, and chromatograms for the synthetic peptides

S4.1. Synthesis of paenipeptin C′ 3



Paenipeptin C′

3

Paenipeptin C′ was synthesised by Fmoc-SPPS on a 0.1 mmol scale and was used as a control for comparison to the synthesised paenipeptin C′ analogues.

Fmoc-Rink amide linker (4 eq., 0.4 mmol, 216 mg) was coupled to aminomethyl PS resin (1 eq., 0.1 mmol, 79 mg), according to **General Method A**. Automated Fmoc-SPPS using the PS3^{TM} synthesizer was used for synthesis of **3** on a 0.1 mmol scale. This first involved transferring the linker-resin to the required PS3 reaction vessel. All amino acid couplings were performed as single coupling cycles using Fmoc-AA-OH (5 eq., 0.5 mmol), HATU (4.5 eq., 0,45 mmol), and NMM (0.4 M, 1.0 mmol) for 60 min at r.t.. After completion of each amino acid coupling, the resin was drained and washed with DMF, and Fmoc removal was accomplished by treating the peptidyl resin with piperidine/DMF (1:4, *v/v*, 2 × 5 min).

Following this, a mixture of octanoic acid (4 eq., 0.4 mmol, 64 μL), COMU (4 eq., 0.4 mmol, 171 mg), Oxyma Pure (4 eq., 0.4 mmol, 72 mg), and DIPEA (8 eq., 0.8 mmol, 140 μL), in DMF (2 mL) were added to the peptidyl-resin. The reaction mixture was agitated for 3 h at r.t., drained and washed with DMF, then CH_2Cl_2. A small sample of resin was then cleaved using a cleavage cocktail solution of TFA/H_2O/TIPS (95:2.5:2.5, *v/v/v*, 500 μL) for 60 min at r.t.. Subsequent LC-MS and analytical RP-HPLC analysis (at λ = 210 nm and 254 nm) indicated the presence of both the desired paenipeptin C′ **3** and a side product with mass difference of +96 Da (*m/z* 1212.7) **Figure S1** and **S2**. This side product was attributed to the peptide with the final Dab residue still Fmoc-protected. Although subsequent treatment of the crude peptide mixture with piperidine in DMF (1:4, *v/v*) for 20 min at r.t. was seen to reduce the intensity of the undesired side product, the Fmoc-protected peptide was still present. To further increase the amount of Fmoc removal, microwave-assisted Fmoc group removal with 20% piperidine in DMF (*v/v*) at 70 ˚C, 25 W for 2 × 3 min was then employed. Cleavage of a small sample of the peptide from the resin followed by LC-MS analysis indicated that trace amounts of Fmoc-protected peptide were still present, however, it was decided to continue with lipidation as previously stated. The resin was then dried, and the peptide was cleaved from the resin and globally deprotected using the conditions outlined in **General Method C** to afford crude paenipeptin C´ **3** as a white solid (47.5 mg, 42% yield).

Note when paenipeptin C′ was synthesised previously by Huang and co-workers,[1] an extended Fmoc deprotection time of 1 h using 20% piperdine in DMF (v/v) was employed.

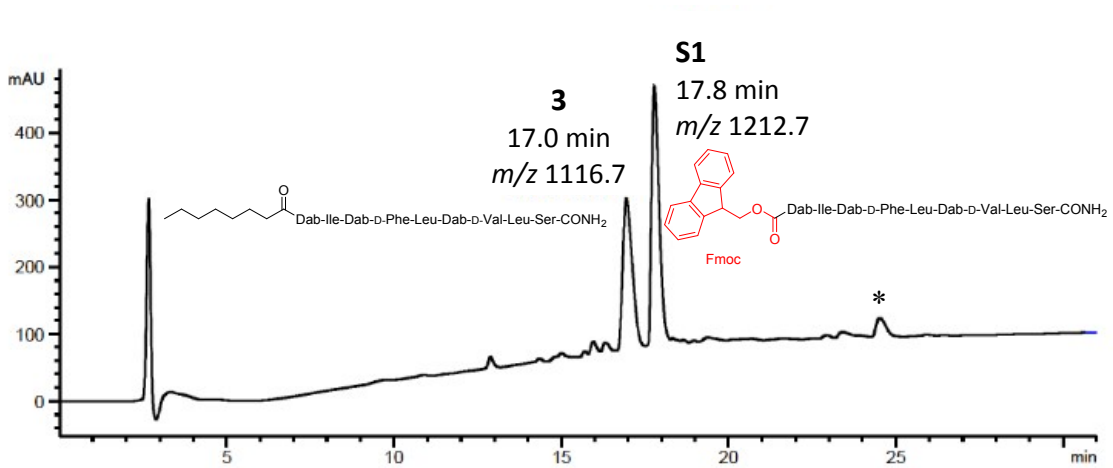


Figure S1. LC chromatogram of mini cleavage of paenipeptin C´ **3** *m/z* (ESI-MS) 1116.7 and **S1** *m/z* 1212.7 prior to the modified Fmoc-removal conditions. ([M+H]$^+$ requires *m/z* 1116.7). Agilent Zorbax 300SB-C3 (5 μm; 3.0 x 150 mm) 5-95% B over 31 min at 0.3 mL/min, r.t., where A: 0.1% formic acid in H_2O, and B: 0.1% formic acid in MeCN. * denotes non-peptidyl side product.

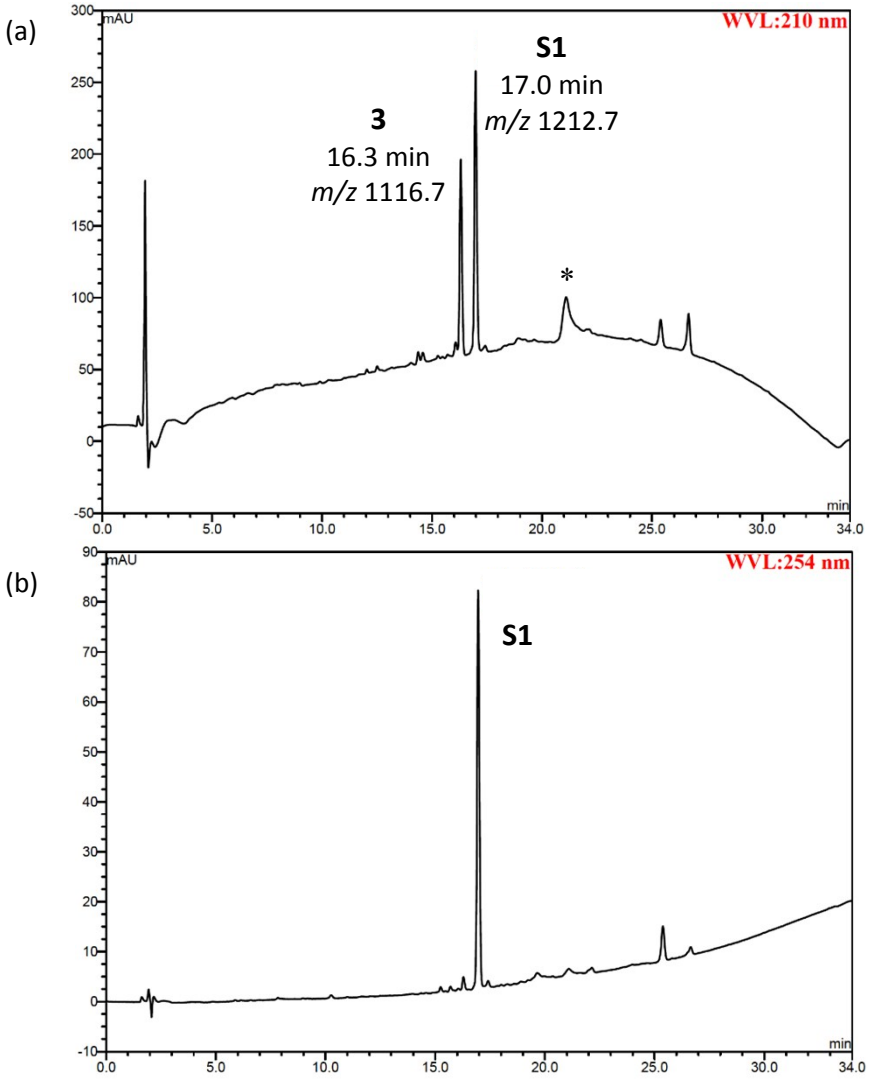


Figure S2. Analytical RP-HPLC chromatograms after cleavage of a small resin sample, (a) showing **3** (*m/z* 1116.7) and **S1** (*m/z* 1212.7) at 210 nm, (b) showing **S1** (*m/z* 1212.7) at 254 nm indicating the presence of the Fmoc group. Xterra MS 110 Å C18 (5 μm; 4.6 × 150 mm) 5-95% B over 34 min, 3% B/min at 1 mL/min, r.t., where A: 0.1% TFA in H_2O, and B: 0.1% TFA in MeCN. * denotes column impurity.

The crude paenipeptin C´ **3** (19.0 mg) was purified by semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å, 10.0 × 250 mm; 5 μm (4 mL/min) using a gradient of 5-95% B at 1% B/min. Fractions were collected and analysed by ESI-MS and analytical RP-HPLC. Fractions identified with the correct *m/z* were combined and lyophilised to afford **3** as a white solid (5.4 mg, 12% yield (based on 0.1 mmol scale), >98% purity); t_R 16.3 min; *m/z* (ESI-MS) 1116.7 ($[M+H]^+$ requires 1116.7), **Figure S3**.

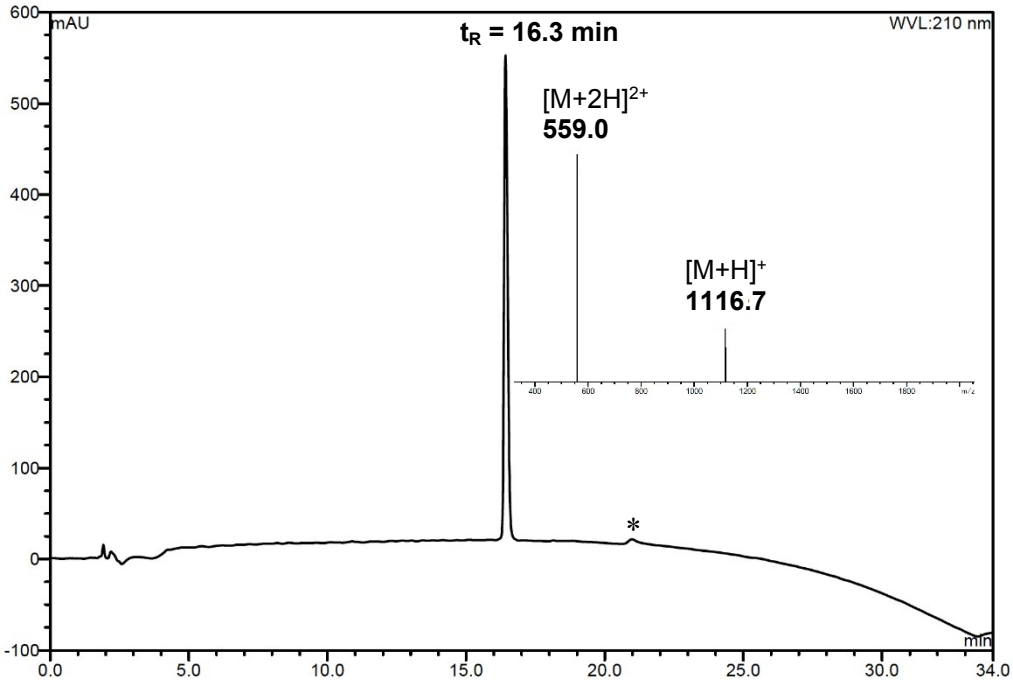
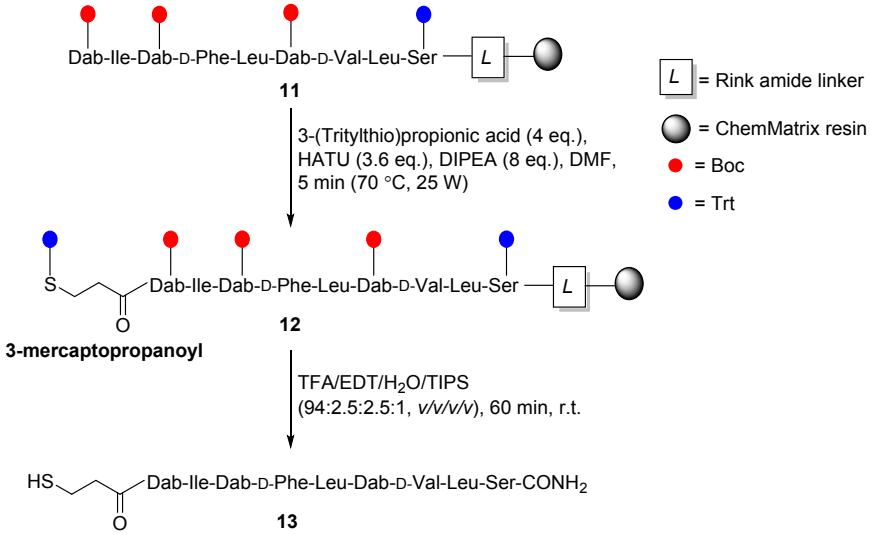


Figure S3. Analytical RP-HPLC (210 nm) and ESI-MS chromatograms of purified paenipeptin C´ **3** (*ca.* >98% as analysed by RP-HPLC peak area at 210 nm); Xterra® MS C18 110 Å 5 μm; 4.6 × 150 mm (1.0 mL/min), linear gradient of 5-95% B over 34 min at 1 mL/min, r.t.. t_R = 16.3 min; *m/z* (ESI-MS) 1116.7 ([M+H]$^+$ requires 1116.7). *denotes column impurity.

Synthesis of paenipeptin C′ analogues

For synthesis of the paenipeptin C′ analogues **15–22**, the native paenipeptin C´ peptide sequence **11** was synthesised under manual microwave assistance. Thiol-functionalisation was then achieved by coupling 3-(trityl)thiopropionic acid to the peptidyl resin, yielding **12**. Cleavage of the peptide from the resin with simultaneous side chain deprotection was achieved by treating the peptide with a cleavage cocktail solution of TFA/EDT/H_2O/TIPS (94:2.5:2.5:1, *v/v/v/v*). Subsequently, **13** was derivatised and the CLipPA reaction was performed in solution with the desired vinyl esters to yield crude analogues **15–22**. Purification of the peptide analogues was achieved by employing semi-preparative RP-HPLC. Fractions were collected and analysed by ESI-MS positive mode, and those identified as having the correct mass were combined and lyophilised to afford the purified lipidated peptide.

Detailed synthesis of common precursor 13



The native paenipeptin C´ peptide sequence **11** was synthesised using a manual microwave-assisted Fmoc/*t*Bu strategy for Fmoc-AA-OH coupling, and Fmoc-group removal at r.t.. **11** was prepared on a 0.2 mmol scale. Coupling of Fmoc-Rink amide linker to aminomethyl CM resin (pre-swollen in CH_2Cl_2) was achieved according to **General Method A**. Removal of the Fmoc-group from the linker was achieved using **General Method B** and subsequent Fmoc-AA-OH couplings, starting with Fmoc-Ser(Trt)-OH (4 eq., 0.8 mmol, 456 mg), were performed by manual microwave-assisted Fmoc-SPPS using a CEM® Discover SP Microwave Synthesiser. A mixture of Fmoc-AA-OH (4 eq., 0.8 mmol), HATU (3.6 eq., 0.72 mmol), and DIPEA (8 eq., 1.6 mmol) in DMF (2 mL) were added to the peptidyl resin. The reaction mixture was irradiated at 70 °C, 25 W for 5 min, drained and the resin washed with DMF (5 × 3 mL). Amino acid coupling was followed by a ninhydrin test (**General method F**) and if a negative result was obtained, standard Fmoc-group removal was performed according to **General method B**. Optimised conditions for complete Fmoc removal from the final Dab residue included treatment of the peptidyl-resin with 50% piperidine in DMF (*v/v*) for 2 × 20 min at r.t., thus affording crude **11**.

Thiol functionalisation of the *N*-terminus was achieved according to **General Method D**, and completion of coupling was confirmed by a negative result from the ninhydrin test (**General Method F**). **12** was then cleaved from the resin and globally deprotected according to **General Method C**. Subsequently, the peptide was lyophilised to afford the crude peptide **13** as a white solid (96.0 mg, *ca*. 44% yield, based on 0.2 mmol scale). LC-MS analysis indicated the presence of a +56 side product **S2** (*m/z* 1134.6) in addition to the desired peptide **13** (**Figure S4**).

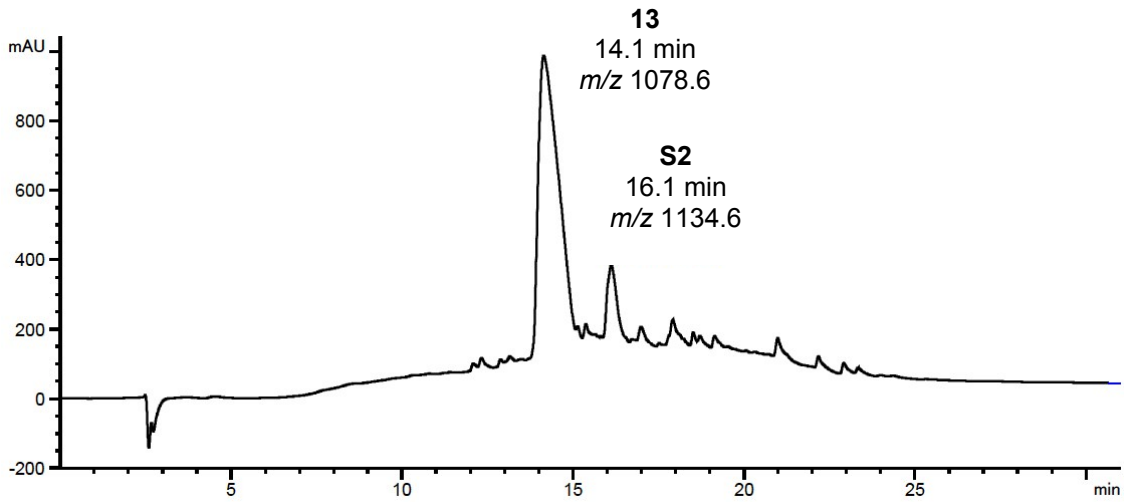
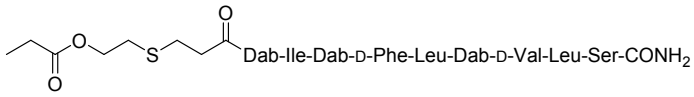


Figure S4. LC chromatogram showing crude **13** (*m/z* 1078.6; *ca*. 41% crude purity based on LC) and a +56 side product **S2** (*m/z* 1134.6). Agilent Zorbax 300SB-C3 (5 μm; 3.0 × 150 mm) 5-95% B over 31 min, 3% B/min at 0.3 mL/min, r.t., where A: 0.1% formic acid in H_2O, and B: 0.1% formic acid in MeCN.

The crude material (75.6 mg) was purified by semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å, 10.0 × 250 mm; 5 μm (4 mL/min) using a gradient of 5-65% B at 1% B/min. Fractions were collected based on UV absorbance at wavelengths of 210, 225, 254, and 280 nm, and analysed by ESI-MS (positive-mode) and analytical RP-HPLC. Fractions identified with the correct *m/z* were combined and lyophilised to afford purified **13** (49.1 mg, *ca*. 65% yield).

S4.2. Synthesis of paenipeptin CLipPA analogue 15



Purified **13** (1 eq., 9 μmol, 10 mg) was lipidated according to **General Method E**, using vinyl propionate (70 eq., 0.65 mmol, 71 μL) as the vinyl ester. Complete conversion was confirmed by analytical RP-HPLC analysis (**Figure S5**). The peptide was then precipitated and lyophilised to afford the crude lipidated analogue **15** as a white amorphous solid (*ca*. 10 mg).

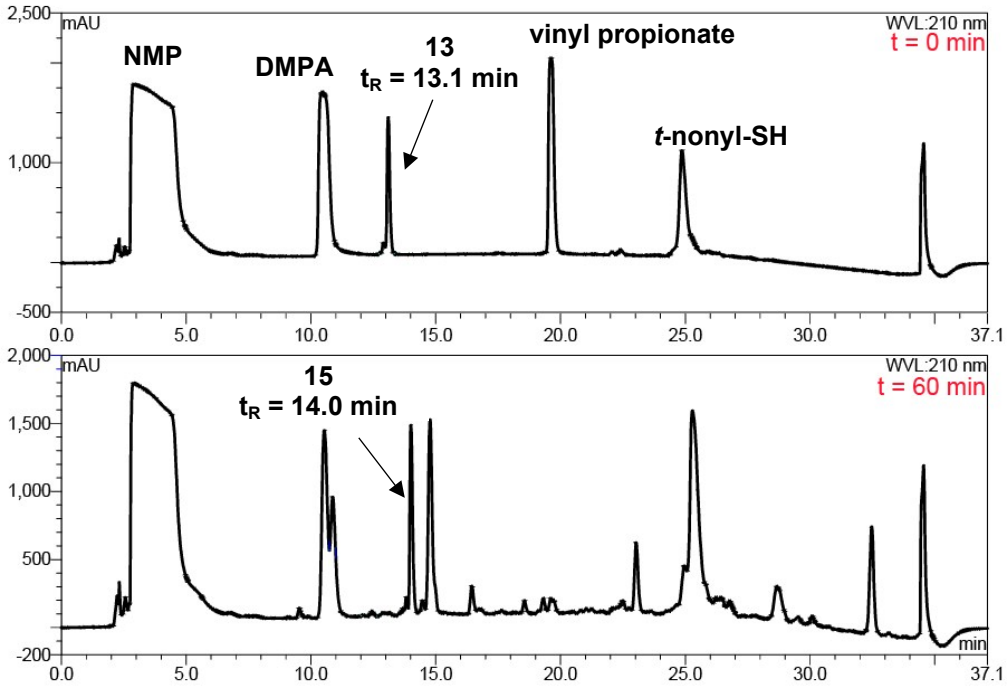


Figure S5. RP-HPLC chromatogram of thiol-ene conjugation of peptide **13** (*m/z* (ESI-MS) 1078.6) and vinyl propionate, using *t*-nonyl mercaptan scavenger at t = 0 and t = 60 min. Phenomenex Gemini C18 column (5 μm; 4.60 × 150 mm) and a linear gradient of 5-95% B over 31 min (*ca* 3% B/min) at a flow rate of 1.0 mL/min, r.t.. Where A: 0.1% TFA in H_2O (*v/v*), and B: 0.1% TFA in MeCN (*v/v*).

The crude lipidated analogue **15** (7.1 mg) was purified by semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å, 10.0 × 250 mm; 5 μm (4 mL/min) with a gradient of 5-95% B at 1% B/min. Fractions were collected and analysed by ESI-MS and analytical RP-HPLC. Fractions identified with the correct *m/z* were combined and lyophilised to afford **15** as a white solid (3.1 mg, *ca*. 43% yield) in high purity, >98%; t_R 29.9 min; *m/z* (ESI-MS) 1178.6, ($[M+H]^+$ requires 1178.7), **Figure S6**.

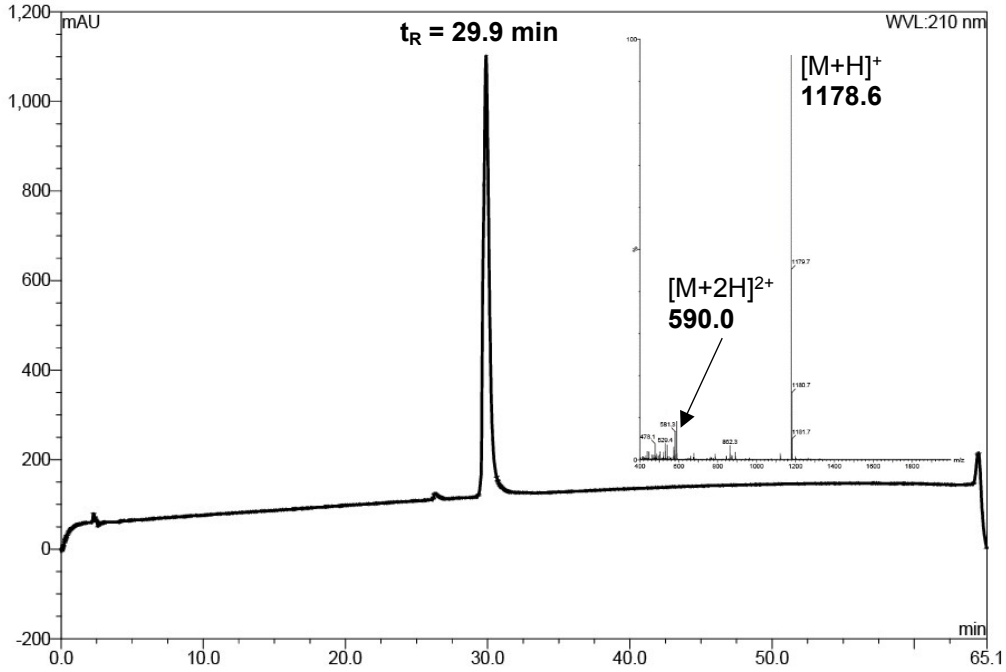
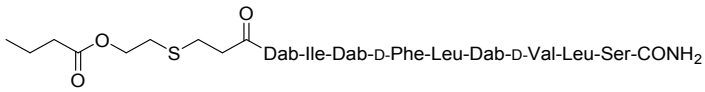


Figure S6. Analytical RP-HPLC (210 nm) and ESI-MS chromatograms of purified vinyl propionate lipidated analogue **15** (*ca.* >98% as analysed by RP-HPLC peak area at 210 nm); Phenomenex Gemini C18 110 Å, 150 × 4.60 mm, 5 μm (1.0 mL/min), linear gradient of 5-65% B at 1% B/min, r.t.. t_R = 29.9 min; *m/z* (ESI-MS) 1178.6 ([M+H]$^+$ requires 1178.7).

S4.3. Synthesis of paenipeptin CLipPA analogue 16



Purified peptide **13** (1 eq., 9 μmol, 10 mg) was lipidated according to **General Method E**, using vinyl butyrate (70 eq., 0.65 mmol, 82 μL) as the vinyl ester. Complete conversion was confirmed by analytical RP-HPLC analysis (**Figure S7**). The peptide was then precipitated and lyophilised to afford the crude lipidated analogue **16** as a white amorphous solid (*ca*. 10 mg).

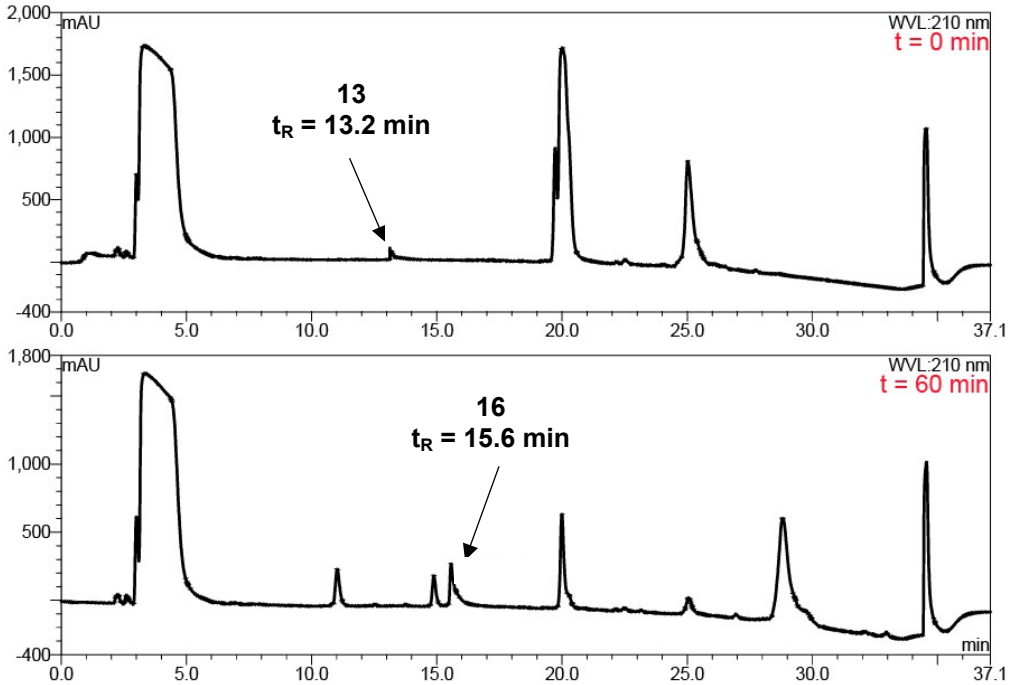


Figure S7. RP-HPLC chromatogram of thiol-ene conjugation of peptide **13** (*m/z* (ESI-MS) 1078.6) and vinyl butyrate, using *t*-nonyl mercaptan scavenger at t = 0 and t = 60 min. Phenomenex Gemini C18 column (5 μm; 4.60 × 150 mm) and a linear gradient of 5-95% B over 31 min (*ca* 3% B/min) at a flow rate of 1.0 mL/min, r.t.. Where A: 0.1% TFA in H_2O (*v/v*), and B: 0.1% TFA in MeCN (*v/v*).

The crude lipidated analogue **16** (5.3 mg) was purified by semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å, 10.0 × 250 mm; 5 μm (4 mL/min) with a gradient of 5-65% B at 1% B/min. Fractions were collected and analysed by ESI-MS and analytical RP-HPLC. Fractions identified with the correct *m/z* were combined and lyophilised to afford **16** as a white solid (3.3 mg, *ca*. 63% yield) in high purity, >98%; t_R 31.4 min; *m/z* (ESI-MS) 1192.6, ($[M+H]^+$ requires 1192.5), **Figure S8**.

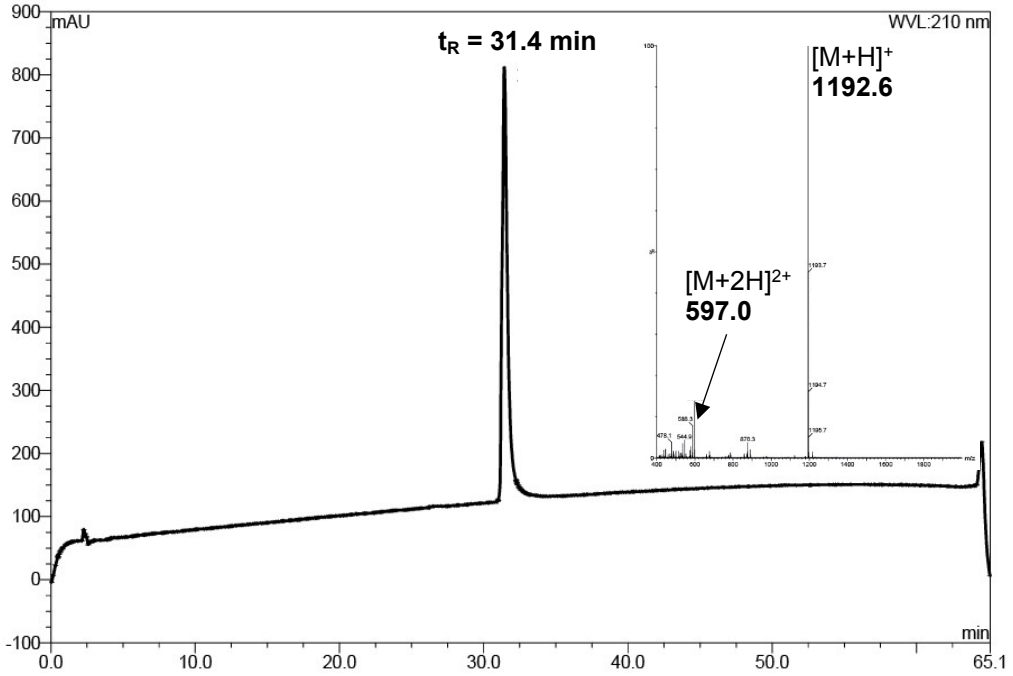
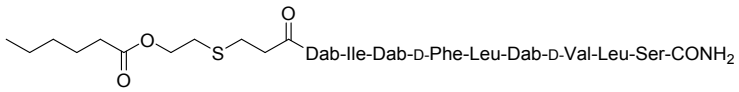


Figure S8. Analytical RP-HPLC (210 nm) and ESI-MS chromatograms of purified vinyl butyrate lipidated analogue **16** (*ca.* >98% as analysed by RP-HPLC peak area at 210 nm); Phenomenex Gemini C18 110 Å, 150 × 4.60 mm, 5 μm (1.0 mL/min), linear gradient of 5-65% B at 1% B/min, r.t.. t_R = 31.4 min; *m/z* (ESI-MS) 1192.6 ($[M+H]^+$ requires 1192.5).

S4.4. Synthesis of paenipeptin CLipPA analogue 17



Purified **13** (1 eq., 9 μmol, 10 mg) was lipidated according to **General Method E**, using vinyl hexanoate (70 eq., 0.65 mmol, 104 μL) as the vinyl ester. Complete conversion was confirmed by analytical RP-HPLC analysis (**Figure S9**). The peptide was then precipitated and lyophilised to afford the crude lipidated analogue **17** as a white amorphous solid (*ca*. 10 mg).

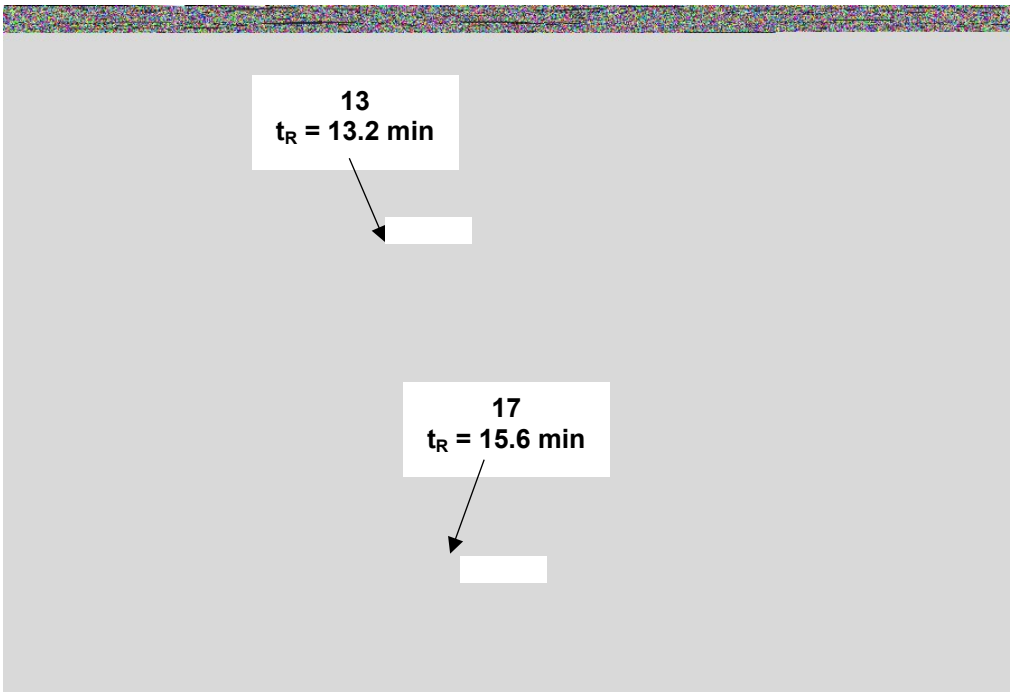


Figure S9. RP-HPLC chromatogram of thiol-ene conjugation of peptide **13** (*m/z* (ESI-MS) 1078.6) and vinyl hexanoate, using *t*-nonyl mercaptan scavenger at t = 0 and t = 60 min. Phenomenex Gemini C18 column (5 μm; 4.60 × 150 mm) and a linear gradient of 5-95% B over 31 min (*ca* 3% B/min) at a flow rate of 1.0 mL/min, r.t.. Where A: 0.1% TFA in H_2O (*v/v*), and B: 0.1% TFA in MeCN (*v/v*).

The crude lipidated analogue **17** (5.2 mg) was purified by semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å, 10.0 × 250 mm; 5 μm (4 mL/min) with a gradient of 5-65% B at 1% B/min. Fractions were collected and analysed by ESI-MS and analytical RP-HPLC. Fractions identified with the correct *m/z* were combined and lyophilised to afford **17** as a white solid (3.3 mg, *ca*. 63% yield) in high purity, >98%; t_R 34.2 min; *m/z* (ESI-MS) 1220.6, ($[M+H]^+$ requires 1220.7), **Figure S10**.

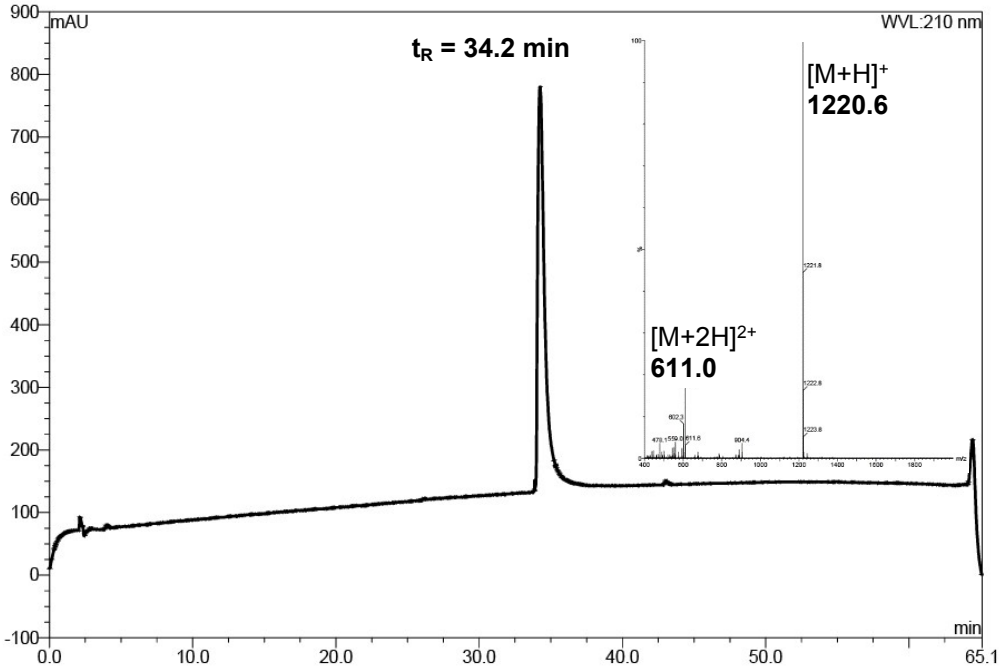
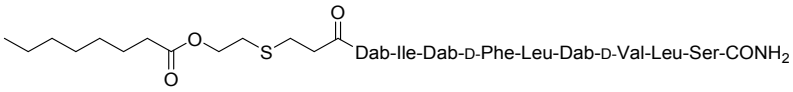


Figure S10. Analytical RP-HPLC (210 nm) and ESI-MS chromatograms of purified vinyl hexanoate lipidated analogue **17** (*ca.* >98% as analysed by RP-HPLC peak area at 210 nm); Phenomenex Gemini C18 110 Å, 150 × 4.60 mm, 5 μm (1.0 mL/min), linear gradient of 5-65% B at 1% B/min, r.t.. t_R = 34.2 min; *m/z* (ESI-MS) 1220.6 ([M+H]$^+$ requires 1220.7).

S4.5. Synthesis of paenipeptin CLipPA analogue 18



Purified **13** (1 eq., 9 μmol, 10 mg) was lipidated according to **General Method E**, employing vinyl octanoate (70 eq., 0.65 mmol, 126 μL) as the vinyl ester. Complete conversion was confirmed by analytical RP-HPLC analysis (**Figure S11**). The peptide was then precipitated and lyophilised to afford the crude lipidated analogue **18** as a white amorphous solid (*ca*. 10 mg).

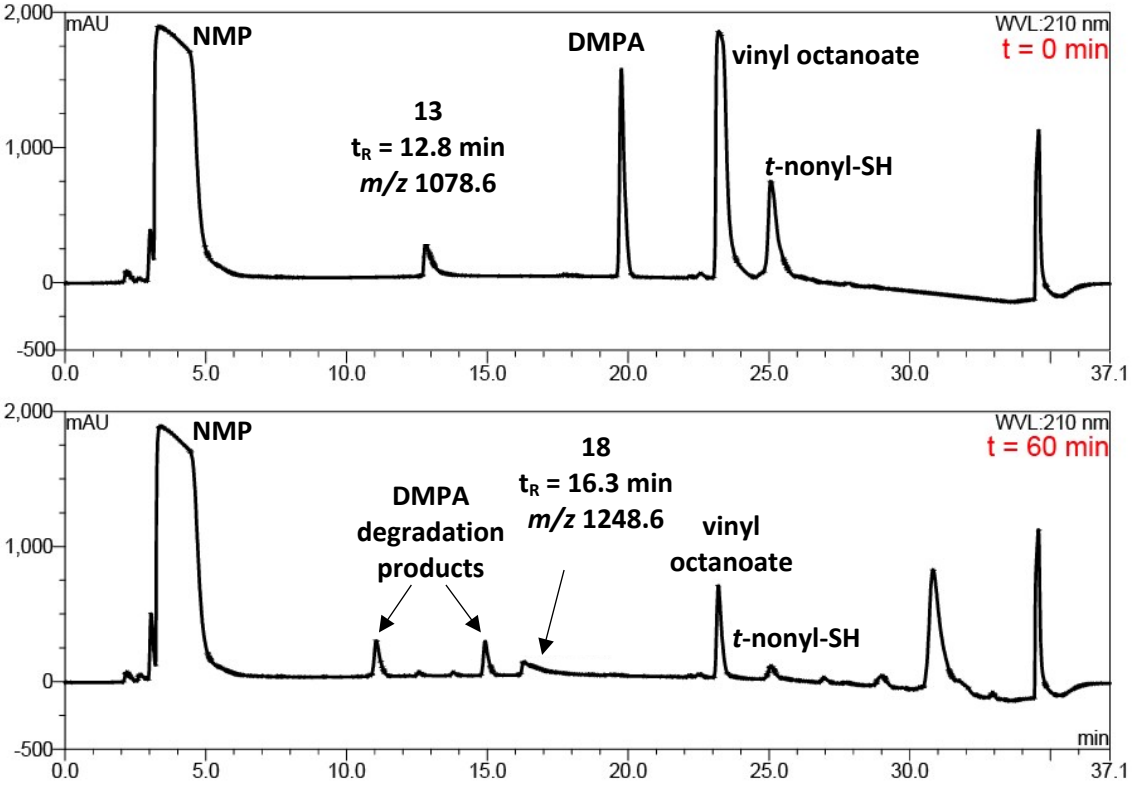


Figure S11. RP-HPLC chromatogram of thiol-ene conjugation of peptide **13** (m/z (ESI-MS) 1078.6) and vinyl octanoate, using *t*-nonyl mercaptan scavenger at t = 0 and t = 60 min. Phenomenex Gemini C18 column (5 μm; 4.60 × 150 mm) and a linear gradient of 5-95% B over 31 min (ca 3% B/min) at a flow rate of 1.0 mL/min, r.t.. Where A: 0.1% TFA in H_2O (v/v), and B: 0.1% TFA in MeCN (*v/v*).

The crude lipidated analogue **18** (5.6 mg) was purified by semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å, 10.0 × 250 mm; 5 μm (4 mL/min) using a gradient of 5-65% B at 1% B/min. Fractions were collected and analysed by ESI-MS and analytical RP-HPLC. Fractions identified with the correct *m/z* were combined and lyophilised to afford **18** as a white amorphous solid (2.2 mg, *ca*. 39% yield), in high purity, >98%; t_R 38.8 min; *m/z* (ESI-MS) 1248.6, ($[M+H]^+$ requires 1248.6), **Figure S12**.

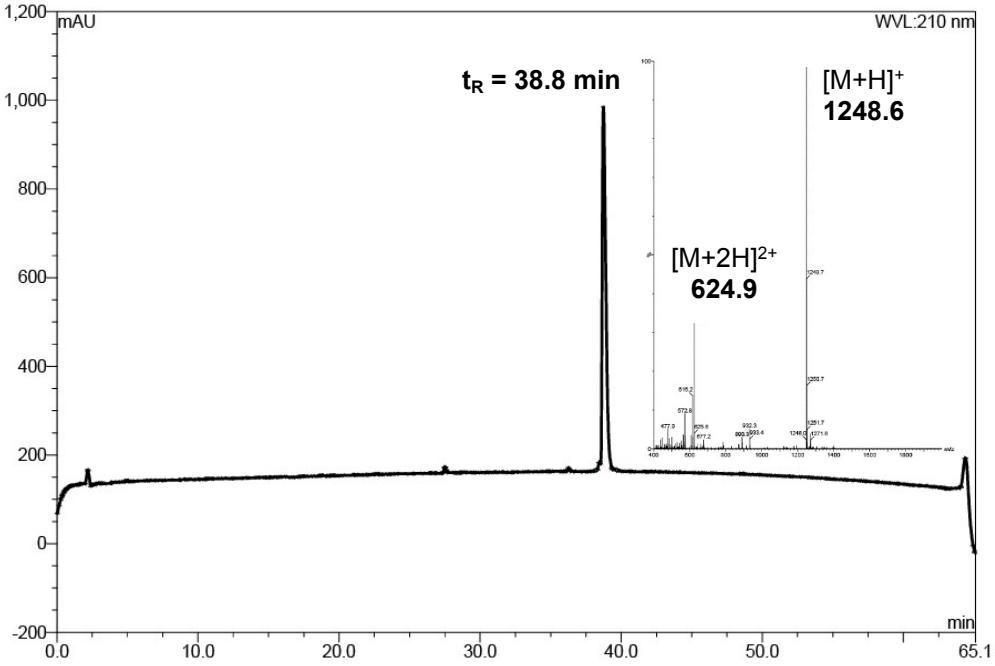
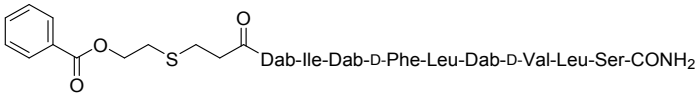


Figure S12. Analytical RP-HPLC and ESI-MS chromatograms of purified vinyl octanoate lipidated analogue **18** (*m/z* (ESI-MS) 1248.6). Phenomenex Gemini C18 column (5 μm; 4.60 × 150 mm) and a linear gradient of 5-65% B at 1% B/min at a flow rate of 1.0 mL/min, r.t.. Where A: 0.1% TFA in H_2O (*v/v*), and B: 0.1% TFA in MeCN (*v/v*).

S4.6. Synthesis of paenipeptin CLipPA analogue 19



Purified **13** (1 eq., 9 μmol, 10 mg) was lipidated according to **General Method E**, employing vinyl benzoate (70 eq., 0.65 mmol, 90 μL) as the vinyl ester. Complete conversion was confirmed by analytical RP-HPLC analysis (**Figure S13**). Following lyophilisation, the crude lipidated analogue **19** was afforded as a white amorphous solid (*ca*. 10 mg).

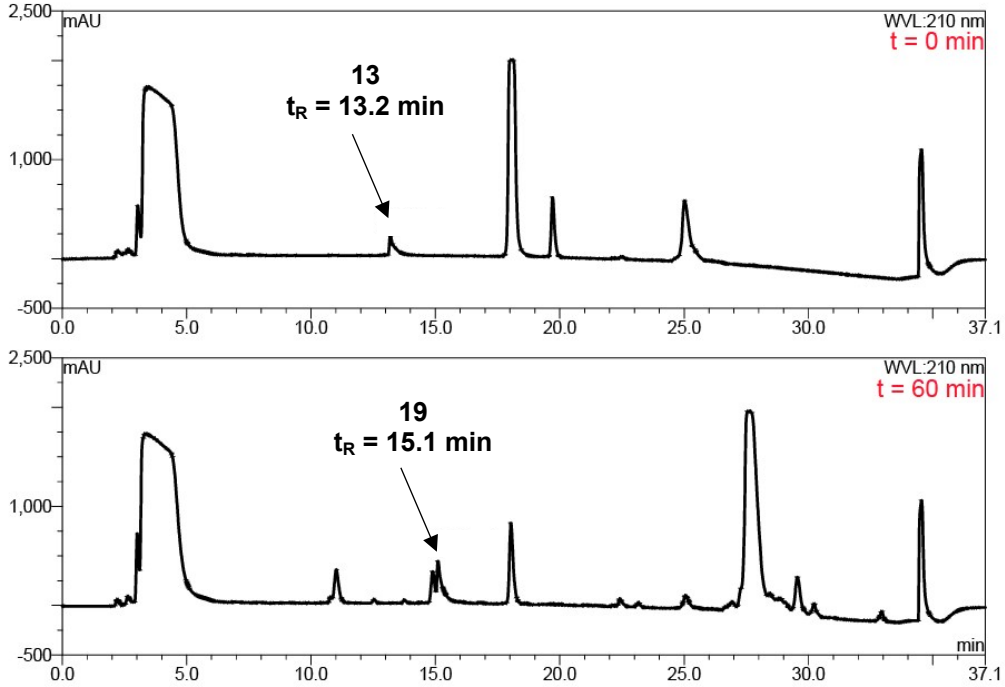


Figure S13. RP-HPLC chromatogram of thiol-ene conjugation of peptide **13** (*m/z* (ESI-MS) 1078.6) and vinyl benzoate, using *t*-nonyl mercaptan scavenger at t = 0 and t = 60 min. Phenomenex Gemini C18 column (5 μm; 4.60 × 150 mm) and a linear gradient of 5-95% B over 31 min (*ca* 3% B/min) at a flow rate of 1.0 mL/min, r.t.. Where A: 0.1% TFA in H_2O (*v/v*), and B: 0.1% TFA in MeCN (*v/v*).

The crude lipidated analogue **19** (4.0 mg) was purified by semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å, 10.0 × 250 mm; 5 μm (4 mL/min) using a gradient of 5-65% B at 1% B/min. Fractions were collected and analysed by ESI-MS and analytical RP-HPLC. Fractions identified with the correct *m/z* were combined and lyophilised to afford **19** as a white amorphous solid (1.3 mg, *ca*. 32% yield), in high purity, >98%; t_R 33.4 min; *m/z* (ESI-MS) 1226.5, ($[M+H]^+$ requires 1226.7), **Figure S14**.

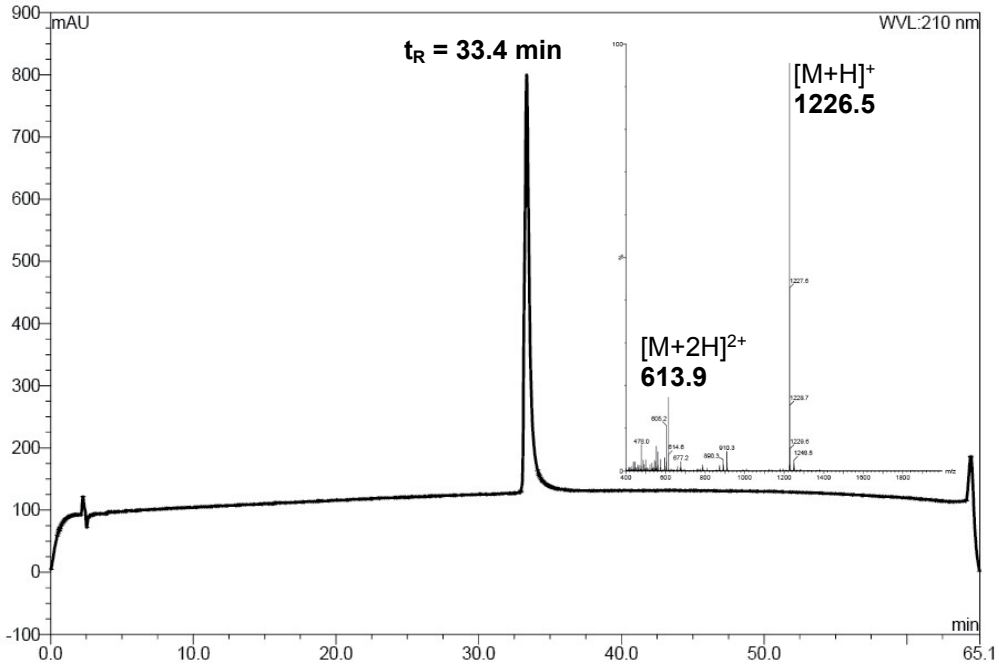
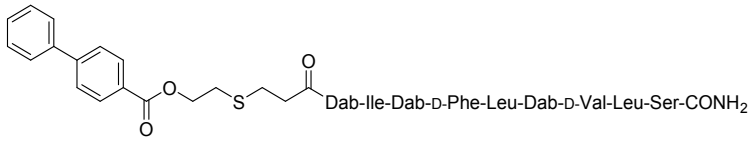


Figure S14. Analytical RP-HPLC (210 nm) and ESI-MS chromatograms of purified vinyl benzoate lipidated analogue **19** (*ca.* >98% as analysed by RP-HPLC peak area at 210 nm); Phenomenex Gemini C18 110 Å, 150 × 4.60 mm, 5 µm (1.0 mL/min), linear gradient of 5-65% B at 1% B/min, r.t.. t_R = 33.4 min; *m/z* (ESI-MS) 1226.5 ($[M+H]^+$ requires 1226.7).

S4.7. Synthesis of paenipeptin CLipPA analogue 20



Purified **13** (1 eq., 9 μmol, 10 mg) was lipidated according to **General Method E**, employing [1,1'-biphenyl]-4-carboxylate (70 eq., 0.65 mmol, 146 mg) as the vinyl ester. Complete conversion was confirmed by analytical RP-HPLC analysis (**Figure S15**). Following lyophilisation, the crude lipidated analogue **20** was afforded as a white amorphous solid (*ca*. 10 mg).

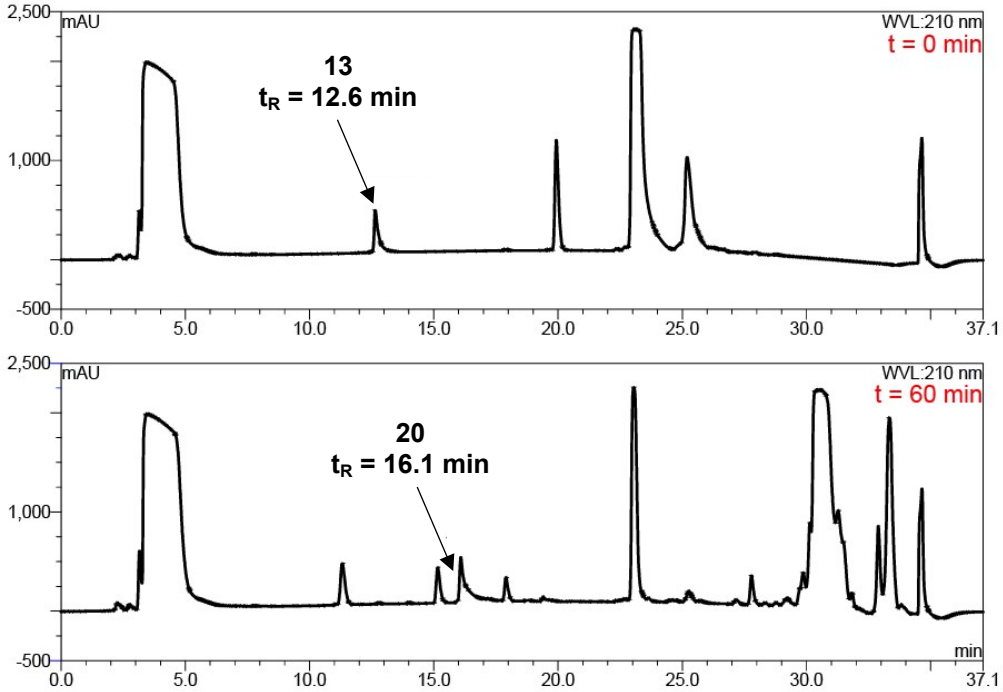


Figure S15. RP-HPLC chromatogram of thiol-ene conjugation of peptide **13** (*m/z* (ESI-MS) 1078.6) and [1.1'-biphenyl]-4-carboxylate, using *t*-nonyl mercaptan scavenger at t = 0 and t = 60 min. Phenomenex Gemini C18 column (5 μm; 4.60 × 150 mm) and a linear gradient of 5-95% B over 31 min (*ca* 3% B/min) at a flow rate of 1.0 mL/min, r.t.. Where A: 0.1% TFA in H_2O (*v/v*), and B: 0.1% TFA in MeCN (*v/v*).

The crude lipidated analogue **20** (7.1 mg) was purified by semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å, 10.0 × 250 mm; 5 μm (4 mL/min) using a gradient of 5-65% B at 1% B/min. Fractions were collected and analysed by ESI-MS and analytical RP-HPLC. Fractions identified with the correct *m/z* were combined and lyophilised to afford **20** as a white amorphous solid (2.6 mg, *ca*. 36% yield), in high purity, >98%; t_R 36.2 min; *m/z* (ESI-MS) 1302.6, ($[M+H]^+$ requires 1302.7), **Figure S16**.

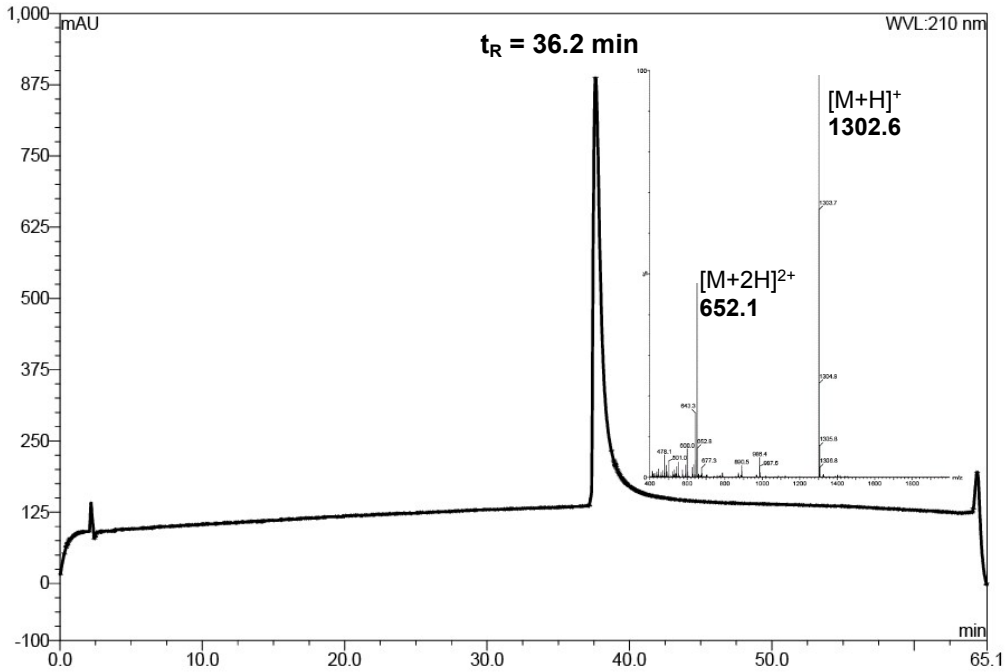
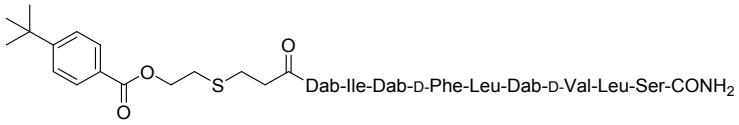


Figure S16. Analytical RP-HPLC (210 nm) and ESI-MS chromatograms of purified biphenyl vinyl ester lipidated analogue **20** (*ca.* >98% as analysed by RP-HPLC peak area at 210 nm); Phenomenex Gemini C18 110 Å, 150 × 4.60 mm, 5 μm (1.0 mL/min), linear gradient of 5-65% B at 1% B/min, r.t.. t_R = 36.2 min; *m/z* (ESI-MS) 1302.6 ($[M+H]^+$ requires 1302.7).

S4.8. Synthesis of paenipeptin CLipPA analogue 21



Purified **13** (1 eq., 9 μmol, 10 mg) was lipidated according to **General Method E**, employing [1,1'-biphenyl]-4-carboxylate (70 eq., 0.65 mmol, as the vinyl ester. Complete conversion was confirmed by analytical RP-HPLC analysis (**Figure S17**). Following lyophilisation, the crude lipidated analogue **21** was afforded as a white amorphous solid (*ca*. 10 mg).

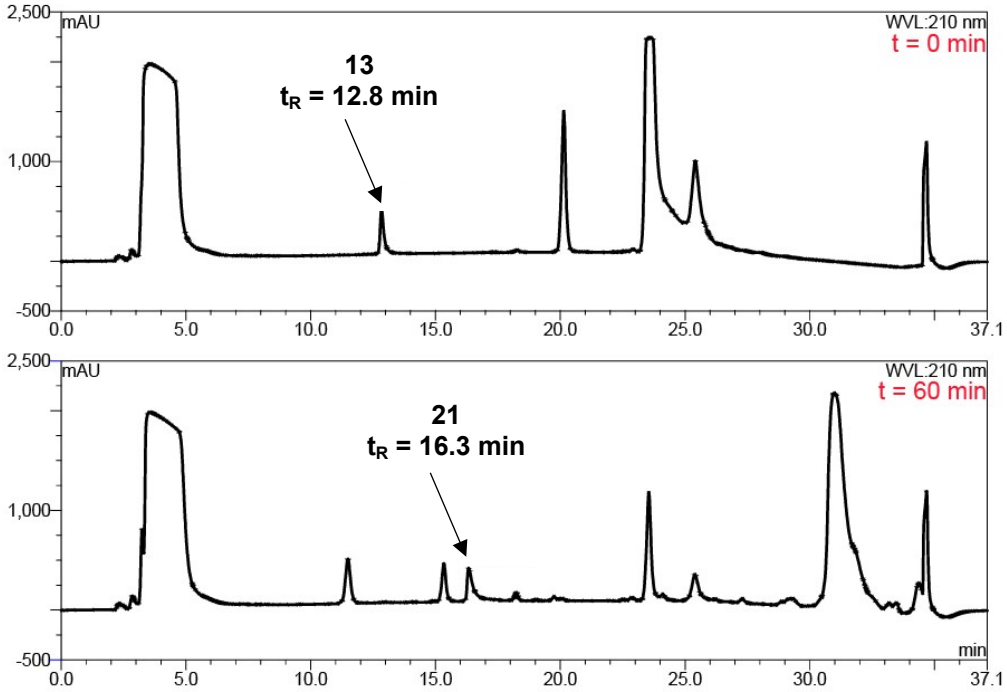


Figure S17. RP-HPLC chromatogram of thiol-ene conjugation of peptide **13** (*m/z* (ESI-MS) 1078.6) and vinyl 4-*t*Bu benzoate, using *t*-nonyl mercaptan scavenger at t = 0 and t = 60 min. Phenomenex Gemini C18 column (5 μm; 4.60 × 150 mm) and a linear gradient of 5-95% B over 31 min (*ca* 3% B/min) at a flow rate of 1.0 mL/min, r.t.. Where A: 0.1% TFA in H_2O (*v/v*), and B: 0.1% TFA in MeCN (*v/v*).

The crude lipidated analogue **21** (6.4 mg) was purified by semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å, 10.0 × 250 mm; 5 μm (4 mL/min) using a gradient of 5-65% B at 1% B/min. Fractions were collected and analysed by ESI-MS and analytical RP-HPLC. Fractions identified with the correct *m/z* were combined and lyophilised to afford **21** as a white amorphous solid (1.3 mg, *ca*. 20% yield), in high purity, >98%; t_R 37.7 min; *m/z* (ESI-MS) 1282.7, ($[M+H]^+$ requires 1282.8), **Figure S18**.

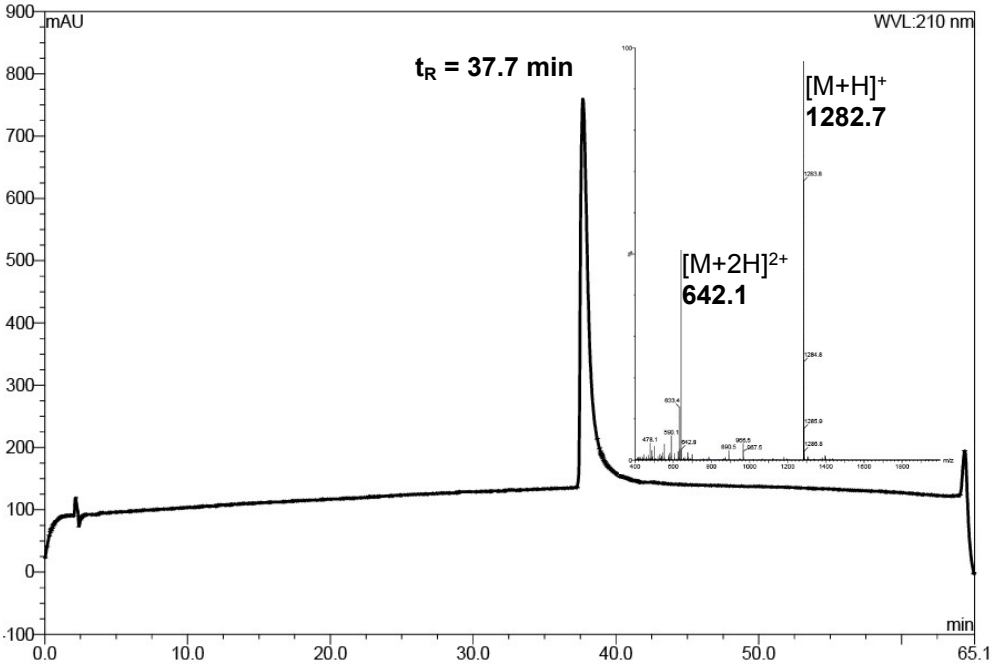
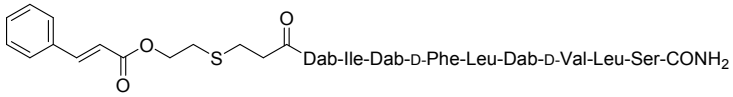


Figure S18. Analytical RP-HPLC (210 nm) and ESI-MS chromatograms of purified vinyl 4-*t*Bu benzoate lipidated analogue **21** (*ca.* >98% as analysed by RP-HPLC peak area at 210 nm); Phenomenex Gemini C18 110 Å, 150 × 4.60 mm, 5 μm (1.0 mL/min), linear gradient of 5-65% B at 1% B/min, r.t.. t_R = 37.7 min; *m/z* (ESI-MS) 1282.7 ($[M+H]^+$ requires 1282.8).

S4.9. Synthesis of paenipeptin CLipPA analogue 22



Purified **13** (1 eq., 9 μmol, 10 mg) was lipidated according to **General Method E**, employing vinyl cinnamate (70 eq., 0.65 mmol, 106 μL) as the vinyl ester. Complete conversion was confirmed by analytical RP-HPLC analysis (**Figure S19**). Following lyophilisation, the crude lipidated analogue **22** was afforded as a white amorphous solid (*ca*. 10 mg).

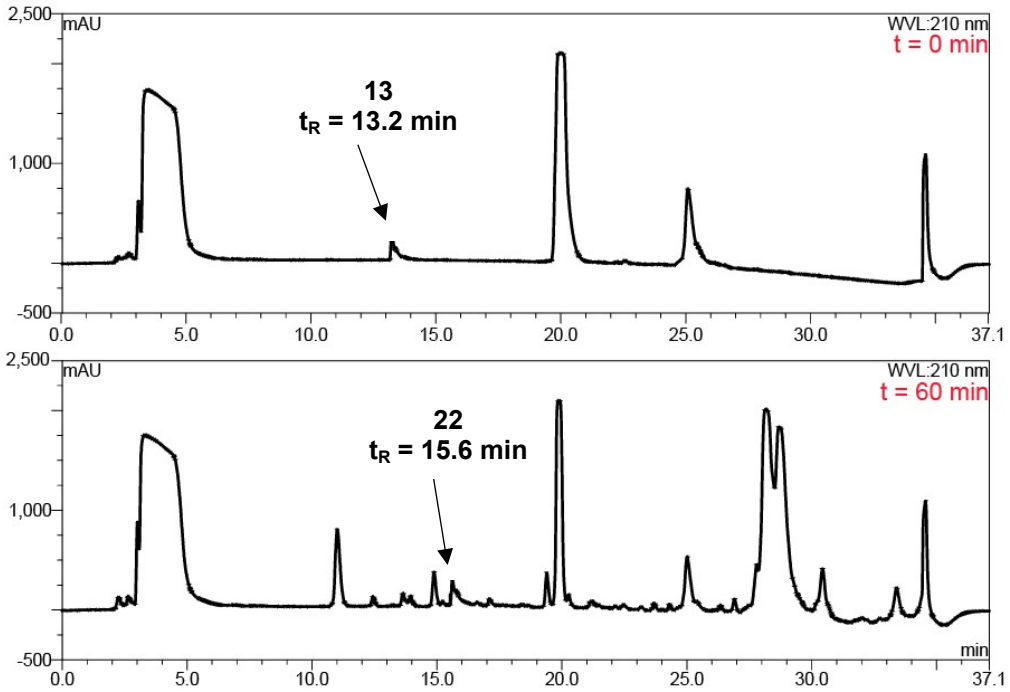


Figure S19. RP-HPLC chromatogram of thiol-ene conjugation of peptide **13** (*m/z* (ESI-MS) 1078.6) and vinyl cinnamate, using *t*-nonyl mercaptan scavenger at t = 0 and t = 60 min. Phenomenex Gemini C18 column (5 μm; 4.60 × 150 mm) and a linear gradient of 5-95% B over 31 min (*ca* 3% B/min) at a flow rate of 1.0 mL/min, r.t.. Where A: 0.1% TFA in H_2O (*v/v*), and B: 0.1% TFA in MeCN (*v/v*).

The crude lipidated analogue **22** (5.2 mg) was purified by semi-preparative RP-HPLC using a Dionex Ultimate® 3000 on a Phenomenex Gemini C18 110 Å, 10.0 × 250 mm; 5 μm (4 mL/min) using a gradient of 5-65% B at 1% B/min. Fractions were collected and analysed by ESI-MS and analytical RP-HPLC. Fractions identified with the correct *m/z* were combined and lyophilised to afford compound **22** as a white amorphous solid (2.2 mg, *ca*. 41% yield), in high purity, >98%; t_R 34.9 min; *m/z* (ESI-MS) 1252.5, ($[M+H]^+$ requires 1252.7), **Figure S20**.

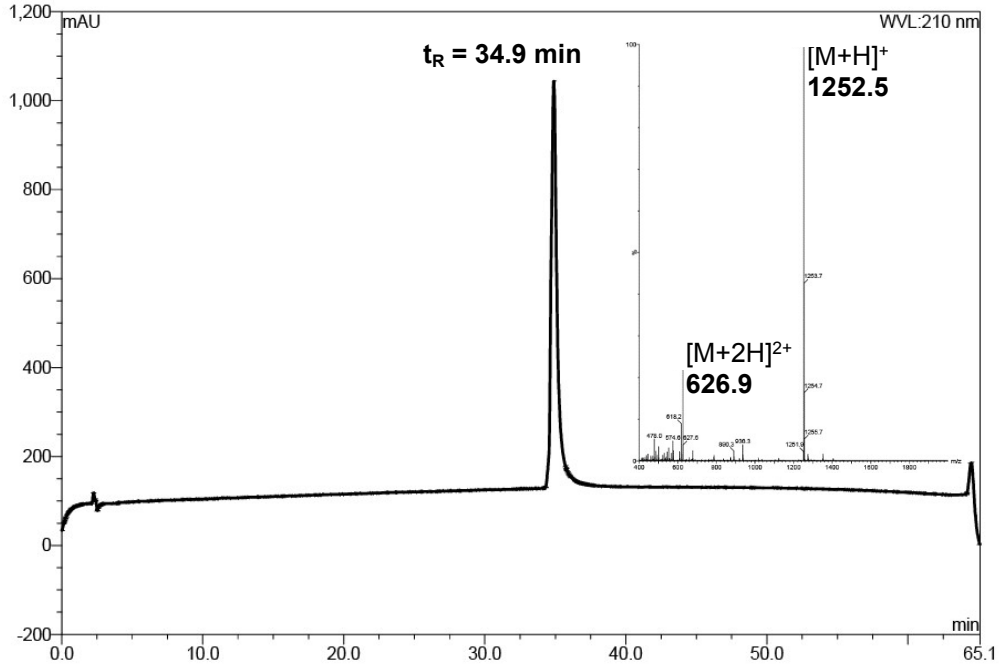
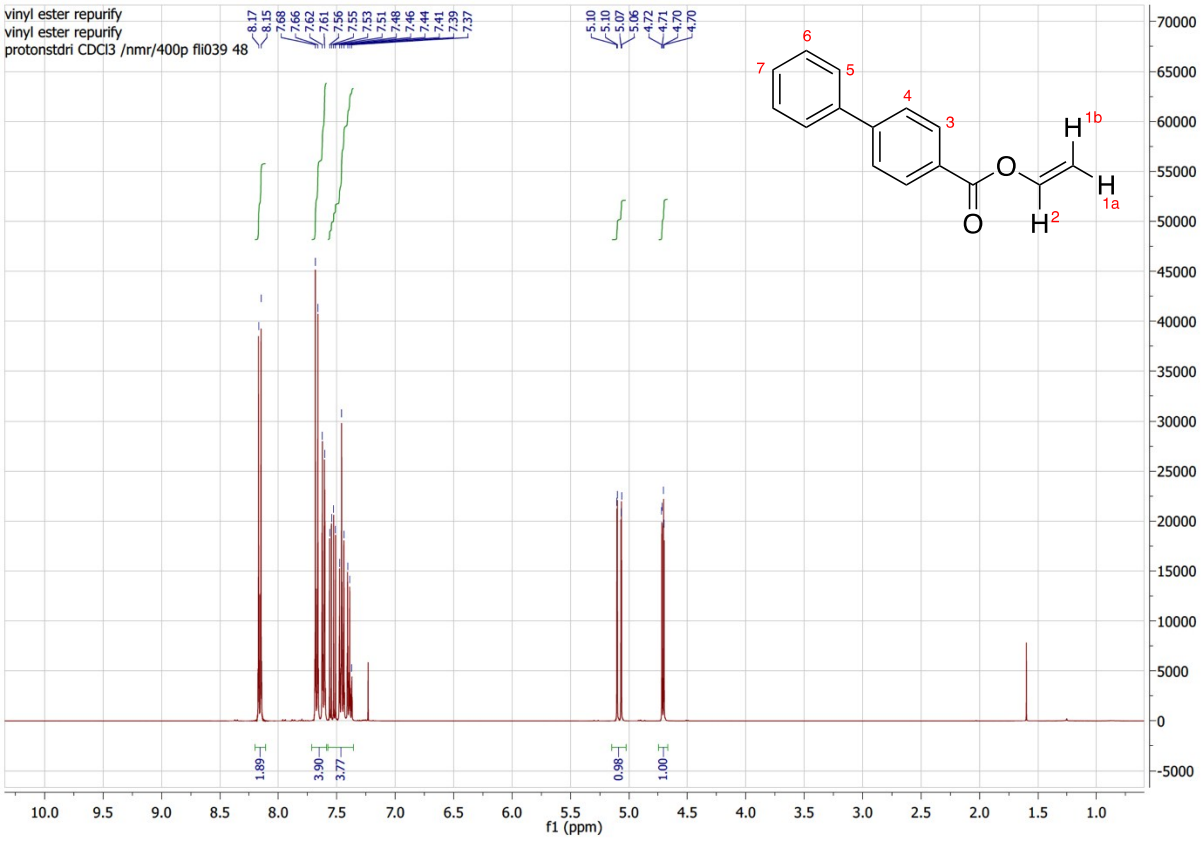


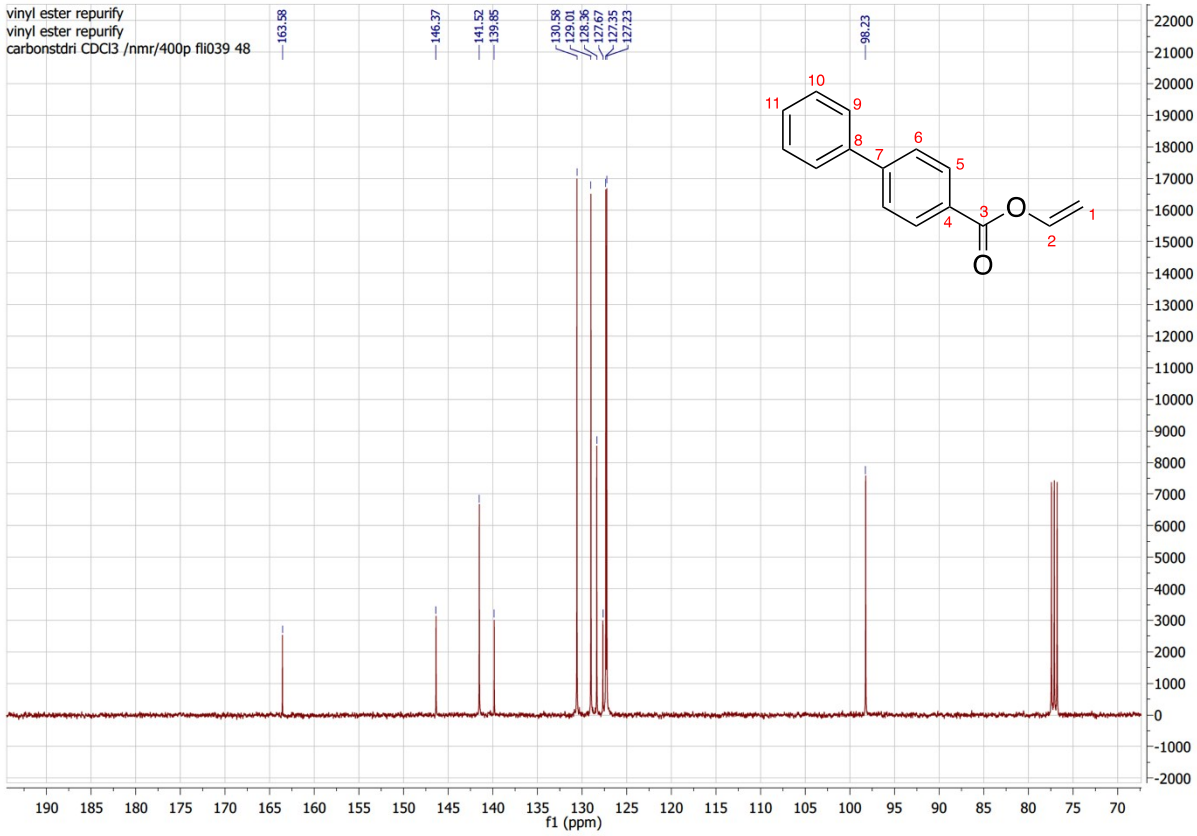
Figure S20. Analytical RP-HPLC (210 nm) and ESI-MS chromatograms of purified vinyl cinnamate lipidated analogue **22** (*ca.* >98% as analysed by RP-HPLC peak area at 210 nm); Phenomenex Gemini C18 110 Å, 150 × 4.60 mm, 5 μm (1.0 mL/min), linear gradient of 5-65% B at 1% B/min, r.t.. t_R = 34.9 min; *m/z* (ESI-MS) 1252.5 ($[M+H]^+$ requires 1252.7).

S5. Synthesis of biphenyl vinyl ester ([1,1'-biphenyl]-4-carboxylate)

To mercury(II) acetate (217 mg, 0.025 eq.) was added a solution of biphenyl-4-carboxylic acid (5.40 g, 1 eq.) in vinyl acetate (23.5 mL, 10 eq.). The reaction mixture was stirred for 10 min at r.t. before addition of a drop of sulfuric acid. The reaction was stirred at reflux for 24 h. After cooling at r.t., sodium acetate was added portion-wise (3 × 25 mg) and filtered on celite. After concentration *in vacuo*, the crude product was purified by column chromatography on silica gel (petroleum ether/ethyl acetate, 98:2, *v/v*). The pure compound was afforded as a white solid (3.87 g, 63%). HRMS Calcd for $C_{15}H_{12}NaO_2$ 247.0730; found, 247.0729



^{1}H NMR (400MHz, $CDCl_3$): δ_H = 8.14 – 8.17 (m, 2H, H-4), 7.59 – 7.69 (m, 4H, H-3 and H-5), 7.54 (dd, 1H, *J* = 14, 6 Hz, H-2), 7.43 – 7.48 (m, 2H, H-6), 7.37 – 7.42 (m, 1H, H-7), 5.08 (dd, 1H, *J* = 14, 2 Hz, H-1b), 4.71 ppm (dd, 1H, *J* = 6, 2 Hz, H-1a)



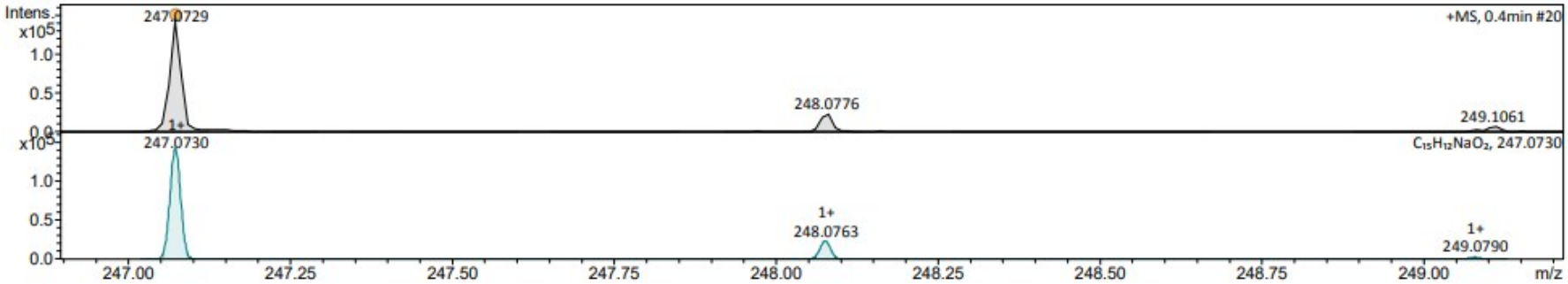
^{13}C NMR (400MHz, $CDCl_3$): δ_C = 163.6 (C=O, C-3), 146.4 (Ar-C, C-7), 141.5 (CH, C-2), 139.8 (Ar-C, C-8), 130.6 (2 × Ar-CH, C-5), 129.0 (2 × Ar-CH, C-10), 128.4 (Ar-CH, C-11), 127.7 (Ar-C, C-4), 127.4 (2 × Ar-CH, C-9), 127.2 (2 × Ar-CH, C-6), 98.2 ppm (CH_2, C-1)

Analysis Info

Analysis Name	C:\Data\Mansa 2020\samples run\May\20200520\JT-08044- pos_RA8_01_13648.d	Acquisition Date	20/05/2020 3:56:02 p.m.
Method	low_hplc.m	Operator	Admin
Sample Name	JT-08044- pos	Instrument / Ser#	micrOTOF-Q 228888.10191
Comment	pre-dissolved Sample diluted 3 µL in 1 mL MeOH		

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e¯ Conf	N-Rule
247.0729	1	C15H12NaO2	247.0730	0.1	10.7	1	100.00	9.5	even	ok

HRMS Calcd for $C_{15}H_{12}NaO_2$ 247.0730; found, 247.0729

S6. Antibacterial Susceptibility Testing

S. aureus ATCC 6538 and *E. coli* ATCC 10536 were grown in non-cation adjusted Mueller Hinton (MH) broth at 37 °C with shaking (200 rpm). The MIC assay was performed whereby a polypropylene 96-well plate was set up such that 100 μL of MH media was added to column 1 (A-H) and 50 μL of media was added to the remaining wells. Compound was added to column 1, achieving a final concentration of 128 μg/mL, and it was then serially diluted 2-fold (50 μL transfer) into the neighbouring wells, resulting in a serial dilution of each compound from 128 μg/mL to 0.0625 μg/mL. Overnight cultures of bacteria were diluted in fresh MH before adding 50 μL of culture to each well of the plate, thereby achieving a uniform CFU/mL of ~5×10^5 in the MIC plate. The plates were incubated at 37 °C with shaking for 24 h before determining the MIC. MICs were determined as the lowest concentration at which growth did not occur. In contrast, MBCs were identified by diluting 10 μL of culture from the MIC plate in sterile PBS down 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. MBCs were determined as the lowest concentration at which growth did not occur. For analogues **15**–**22**, polymyxin B, was used as the control.