Supporting Information

Design, Synthesis and Biological Activity of Potential Retrometabolic Polymyxins via Thiol-ene Chemistry

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S1. General Information

All reagents and solvents for peptide synthesis and reversed-phase high-performance liquid chromatography (RP-HPLC) were purchased as synthesis and HPLC grade, respectively, and used without further purification. Ethyl acetate (EtOAc) and petroleum ether (Pet.ether) were purchased from Burdick & Jackson® (Muskegon, MI, USA). All standard Fmoc amino acids were purchased from CS Bio (Shanghai, China) and were side-chain protected as follows: Fmoc-Cys(Trt)-OH (Trt= trityl), Fmoc-Thr(^{t}Bu)-OH (^{t}Bu=*tert*-butyl). 2-Chlorotrityl chloride (2-CTC) polystyrene resin with a reported loading of 0.89mmol/g, Fmoc-homocysteine(Trt)-OH (Fmoc-Hcy(Trt)-OH), Fmoc-D-Phe-OH were purchased from Chempep Inc. (Wellington, FL, USA). Fmoc-D-Cys(Trt)-OH, Boc-Thr(^{t}Bu)-OH, (Boc=*tert*-butyloxycarbonyl), Fmoc-Dab(Boc)-OH and tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) were purchased from AK Scientific (Union City, CA, USA). Dichloromethane (CH_2Cl_2), sodium bicarbonate ($NaHCO_3$), and anhydrous sodium sulfate (Na_2SO_4) were purchased from ECP Limited (Auckland, New Zealand). *H*-1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5- b]pyridinium 3-oxid hexafluorophosphate (HATU), *N,N*-diisopropylethylamine (DIPEA), triisopropylsilane (TIPS), sodium carbonate (Na_2CO_3), *N*-methylpyrrolidone (NMP), thionyl chloride ($SOCl_2$), *tert*-nonyl mercaptan (mixture of isomers), vinyl acetate, 1,2-ethanedithiol (EDT), palladium acetate $(Pd(OAc)_2)_n$, piperidine, sodium diethyldithiocarbamate trihydrate, 2,2-dimethoxy-2-phenylacetophenone (DMPA), 2,4,6-trimethylpyridine, Aliquat 336®, phenylsilane ($PhSiH_3$) and allyl bromide, were purchased from SigmaAldrich (St. Louis, MO, USA). 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate. (HCTU) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC.HCl) were purchased from Aapptec (Louisville, KY, USA). Trifluoroacetic acid (TFA) was purchased from Oakwood Chemicals (Estill, SC, USA). Octanoic acid was purchased from Honeywell Fluka (Charlotte, NC, USA). Diethyl ether (Et_2O) was purchased from Macron Fine Chemicals (Radnor, PA, USA). Deuterochloroform ($CDCl_3$) and deuteromethanol (CD_3OD) were purchased from Cambridge Isotopes (Tewksbury, MA, USA). Milli-Q high purity deionized water (MQ H_2O) was available from a Sartorius Arium® Pro Ultrapure Water System from Sartorius Stedim Biotech (Gottingen, Germany). Potassium hydroxide (KOH), *N,N'*-dimethylformamide (DMF) (synthesis grade) and acetonitrile (MeCN) (HPLC grade) were purchased from Thermo Fisher Scientific (Hampshire, NH, USA). 1-Hydroxy-7-azabenzotriazole (HOAt) was purchased from Manchester Organics Ltd (Runcorn, UK).

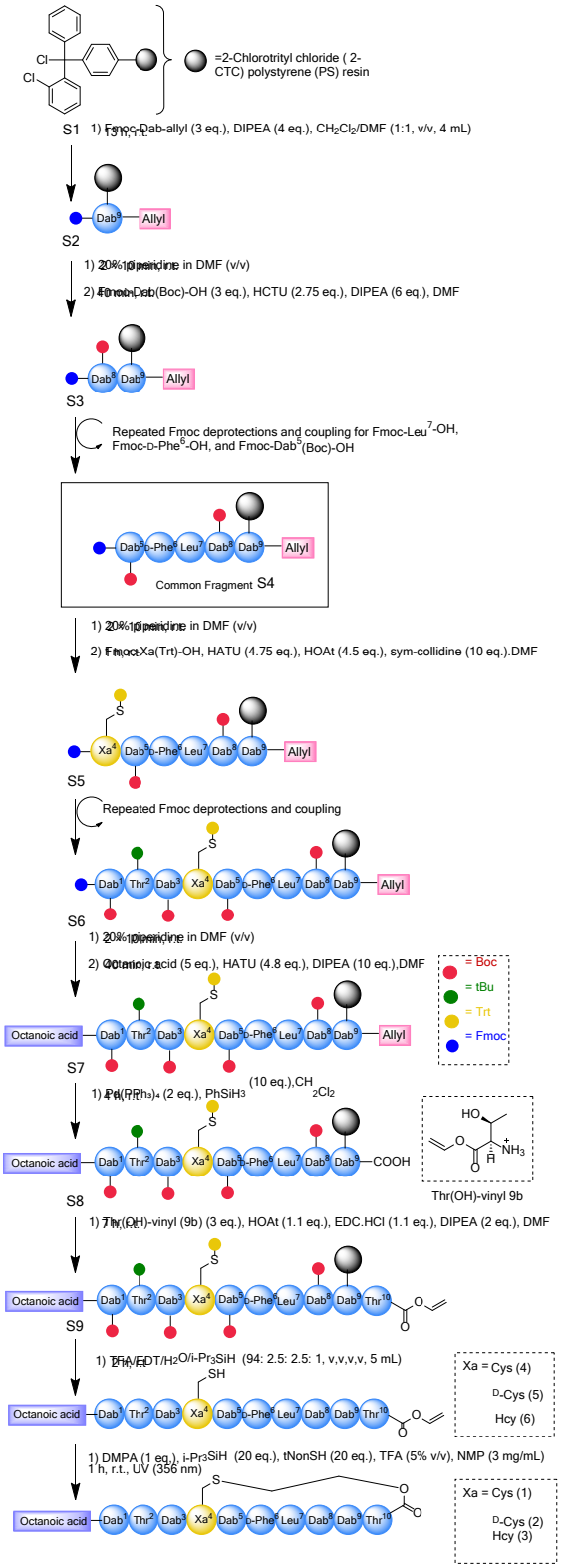
Analytical reverse-phase high-performance liquid chromatography (RP-HPLC) was performed on a Waters (Waltham, MA, USA) Alliance analytical HPLC equipped with a Phenomenex (Torrance, CA, USA) Luna C18 column (100 Å, 5 μm, 4.6 mm × 250 mm) operated at room temperature, with chromatograms recorded at 210/214 nm and 254 nm. Semi-preparative RP-HPLC was performed on a Waters 1525 Binary HPLC pump equipped with a Waters 2489 UV/visible detector using a Phenomenex Gemini-NX C18 (110 Å, 5 μm, 10 mm × 250 mm) at a flow rate of 5 mL/min. For both analytical and

semi-preparative RP-HPLC, solvents used were as follows: solvent A = 0.1% TFA in water (MQ H_2O) and solvent B = 0.1% TFA in MeCN. For analytical RP-HPLC, the gradient employed was 1-91% of solvent B over 40 minutes at a flow rate of 1 mL/min, or 1-91% of solvent B over 100 minutes at a flow rate of 1 mL/min, unless specified otherwise. Gradient systems used for semi-preparative RP-HPLC were adjusted according to the elution and peak profiles obtained from the analytical RP-HPLC chromatograms and are specified in the experimental procedures section.

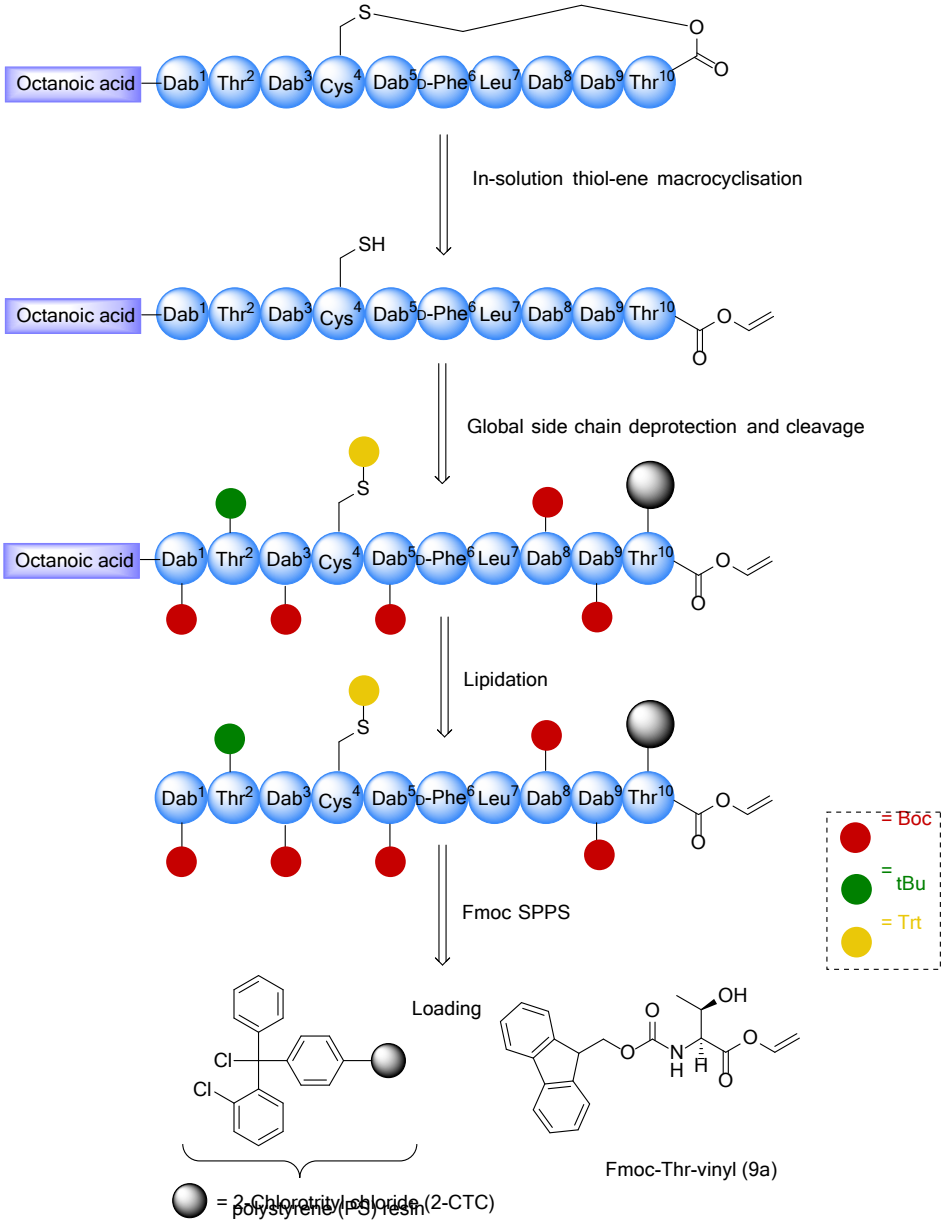
Analytical thin-layer chromatography (TLC) was performed on Kieselgel F254 200 μm (Merck) silica plates using UV light as the visualising agent with either potassium permanganate solution or ninhydrin, followed by heat development of the plate. Manual column chromatography was performed using ECP LabChem Silica Gel (230-400 mesh) with the indicated eluent. Low-resolution mass spectrometry was performed on a Waters Quattro micro API mass spectrometer in ESI positive mode. Ultraviolet irradiation was performed using a handheld Spectroline UV lamp at a peak wavelength of 365 nm.

Infrared (IR) spectra were recorded as a thin film on a composite of zinc selenide and diamond crystal on either a Perkin Elmer Spectrum 100 FT-IR spectrometer (Shelton, CT, USA) or a Bruker (Billerica, MA, USA) VERTEX 70 FT-IR spectrometer as neat samples, with absorption expressed in wavenumbers (cm^{-1}). Nuclear magnetic resonance (NMR) spectra were recorded as indicated on a Bruker AVANCE 400 spectrometer operating at 400 MHz for $CDCl_3$ or CD_3OD solutions on either a spectrometer operating at 400 MHz for ^1H nuclei and 100 MHz for ^{13}C nuclei or a spectrometer operating at 500 MHz for ^1H nuclei and 125 MHz for ^{13}C nuclei. Chemical shifts are reported in parts per million (ppm) calibrated relative to the solvent in which the sample was analysed: TMS (δ_H 0.00 ppm), $CDCl_3$ (δ_H 7.26 ppm, δ_C 77.2 ppm) or CD_3OD (δ_H 3.31 and 4.78, δ_C 49.1 ppm). Multiplicities are reported as “s” (singlet), “d” (doublet), “dd” (doublet of doublets), “dt” (doublet of triplets), “ddt” (doublet of doublet of triplets), “dtd” (doublet of triplet of doublets), “t” (triplet), “q” (quartet), and “m” (multiplet). Optical rotations were measured with an Autopol® IV automatic polarimeter (Hackettstown, NJ, USA) using the sodium-D line (589 nm), with the concentration measured in grams per 100 mL. High resolution mass spectra (HRMS) were obtained using the Bruker micrOTOF-Q spectrometer operating at a nominal accelerating voltage of 70 eV. LC-MS spectra were acquired on either an Agilent Technologies (Santa Clara, CA) 1120 Compact LC equipped with a Hewlett-Packard (Palo Alto, CA) 1100 MSD mass spectrometer or an Agilent Technologies (Santa Clara, CA) 1260 Infinity LC equipped with an Agilent Technologies 6120 Quadrupole mass spectrometer. An analytical Agilent column, Agilent C3 (3.5 μm; 3.0 × 150 mm) was used at a flow rate of 0.3 mL min^{-1} using a linear gradient of 5% B to 95% B over 30 min, where solvent A was 0.1% formic acid in H_2O and B was 0.1% formic acid in acetonitrile.

S2. Main Text Supporting Schemes and Images



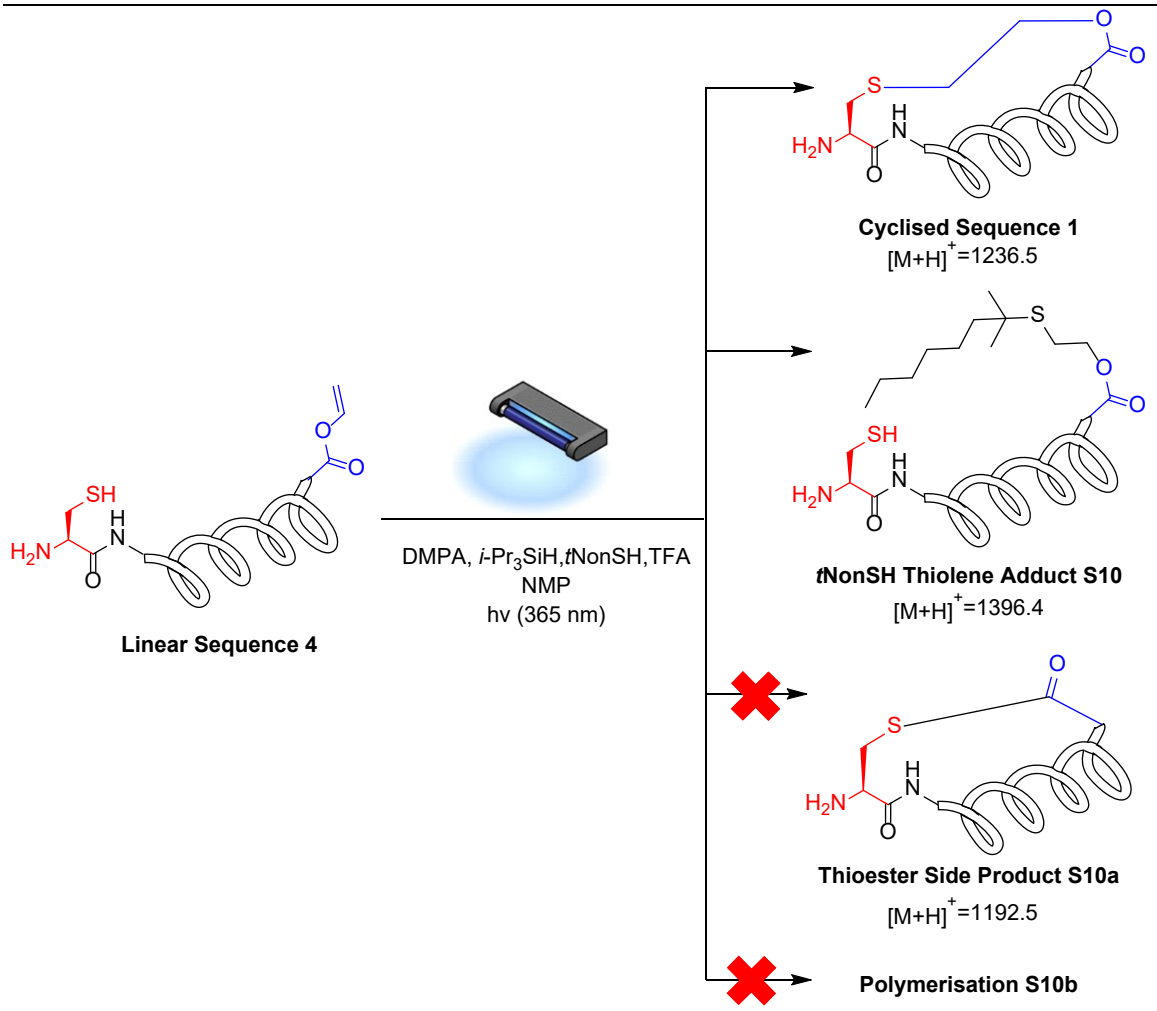
Scheme S1. Proposed scheme to synthesise the polymyxin analogues utilising a late-stage incorporation of the vinyl ester using **9b**.



Scheme S2. Proposed retrosynthetic scheme to synthesise the polymyxin analogue, utilising an early-stage incorporation of the vinyl ester using **9a**.

Table S1. Summary of attempted Fmoc removal conditions for **8a**.

Entry Number	Reaction Conditions	Temperature	Reaction Time	Results
1	Triethylamine:Methanol (1:5 *v/v*)	r.t.	Overnight	Both Fmoc and vinyl were removed, difficult to purify via column chromatography.
2	Diethylamine:Methanol (1:5 *v/v*)	r.t.	Overnight	Both Fmoc and vinyl were removed, difficult to purify via column chromatography.
3	1 M NaOH:Methanol(1:3 *v/v*)	r.t.	20 min	Both Fmoc and vinyl removed
4	1 M NaOH:Methanol (1:3 *v/v*)	r.t.	5 min	Both Fmoc and vinyl removed.
5	1 M NaOH:Methanol (1:3 *v/v*)	r.t.	3 min	Both Fmoc and vinyl removed.
6	1 M NaOH:Methanol (1:5 *v/v*)	r.t.	5 min	Both Fmoc and vinyl removed.
7	1 M NaOH:Methanol (1:20 *v/v*)	4 °C	20 sec	Both Fmoc and vinyl retained.
8	Piperidine:Methanol (1:5 *v/v*)	r.t.	10 min	Both Fmoc and vinyl removed.
9	Piperidine:Methanol (1:5 *v/v*)	r.t.	2 min	Both Fmoc and vinyl removed.



Scheme S3. Depiction of the desired cyclised sequence **1**, undesired *t*NonSH thiol-ene adduct **S10,** and the unobserved thioester (**S10a)** and polymerisation (**S10b**) side products**.** The thioester **10a** has a mass distinct (1192.5 m/z) to the thiolene product **10** (1236.5) m/z which we did observe

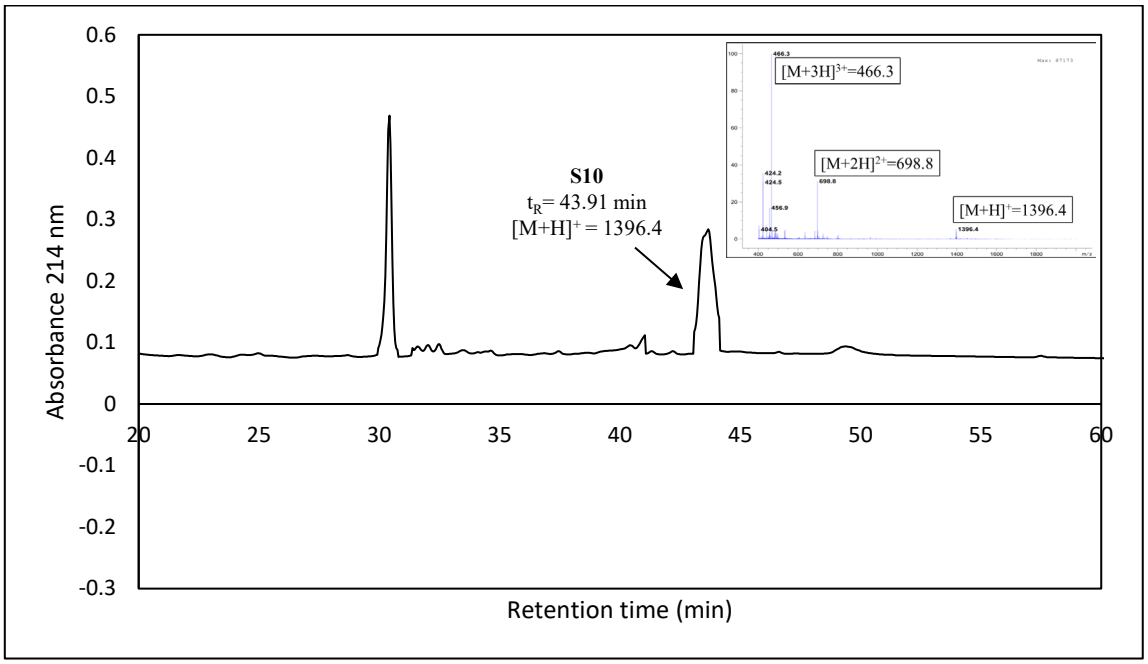


Figure S1. Analytical RP-HPLC and MS traces of an early attempt at cyclised Cys polymyxin analogue (**1**) demonstrating prominent *t*NonSH adduct (**S10**). Alliance HPLC by Waters™ Luna® (5µm, C18 100 Å, LC column 250 × 4.6 mm), 1 to 91%B over 90 minutes (*ca.* 1% B/min), 1 mL/min at room temperature, where A: 0.1% TFA in H_2O, and B: 0.1% TFA in acetonitrile (MeCN).

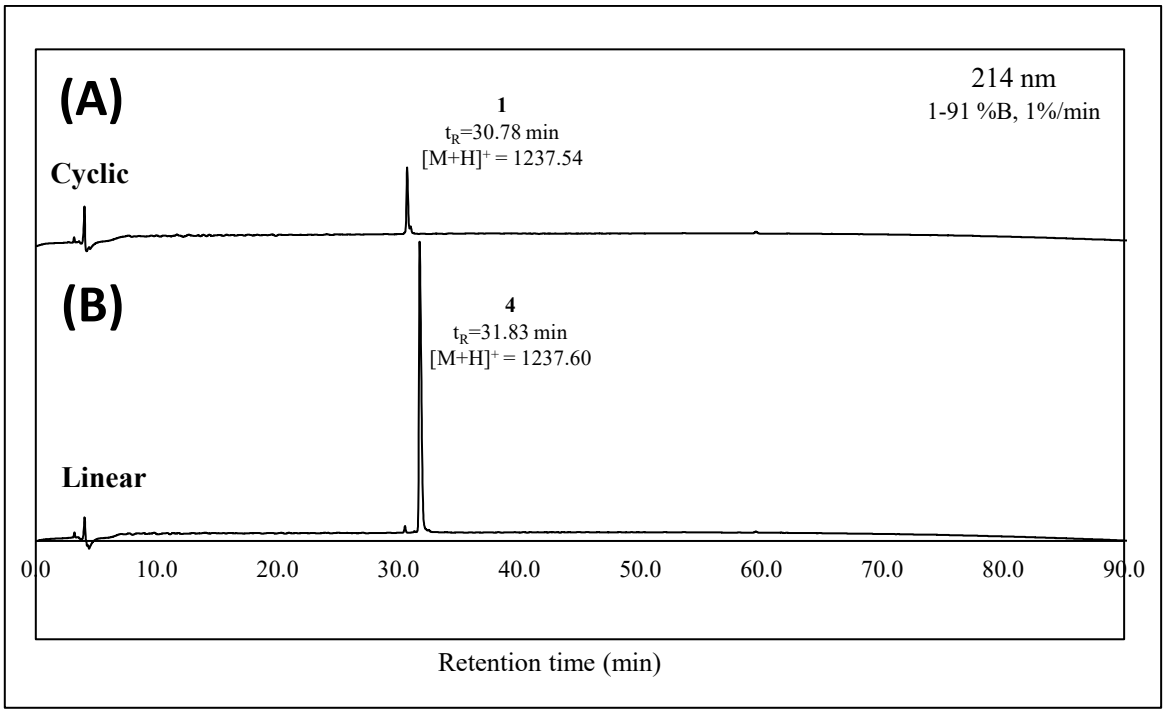


Figure S2. (A) Analytical RP-HPLC (214 nm) chromatogram performed on purified cyclised Cys polymyxin analogue (**1**) (*ca.* >99% as judged by peak area of RP-HPLC), t_R 30.78 min; *m/z* 413.0 $[M+3H]^{3+}$ requires 412.8, *m/z* 619.0 $[M + 2H]^{2+}$ requires 618.8, *m/z* 1237.5 $[M + H]^+$ requires 1236.6. Mass deconvolution calculated at 1236.17 Da with a standard deviation of 0.29; theoretical mass calculated at 1236.5 Da. **(B)** Analytical RP-HPLC (214 nm) chromatogram performed on purified linear Cys polymyxin analogue (**4**) (*ca.* >99% as judged by peak area of RP-HPLC). t_R 31.83 min; *m/z* 413.0 $[M+3H]^{3+}$ requires 412.8, *m/z* 619.0 $[M + 2H]^{2+}$ requires 618.8, m/z 1237.6 $[M + H]^+$ requires 1236.6 . Mass deconvolution calculated at 1236.20 Da with a standard deviation of 0.35; theoretical mass calculated at 1236.5.

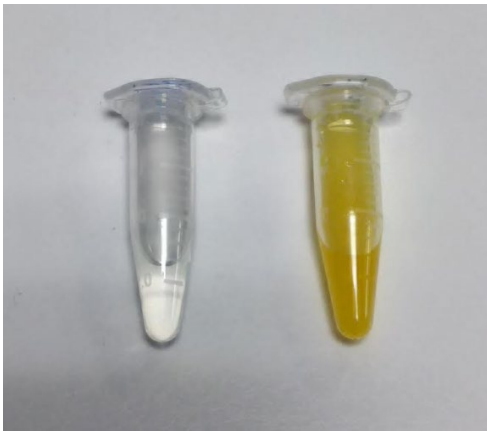
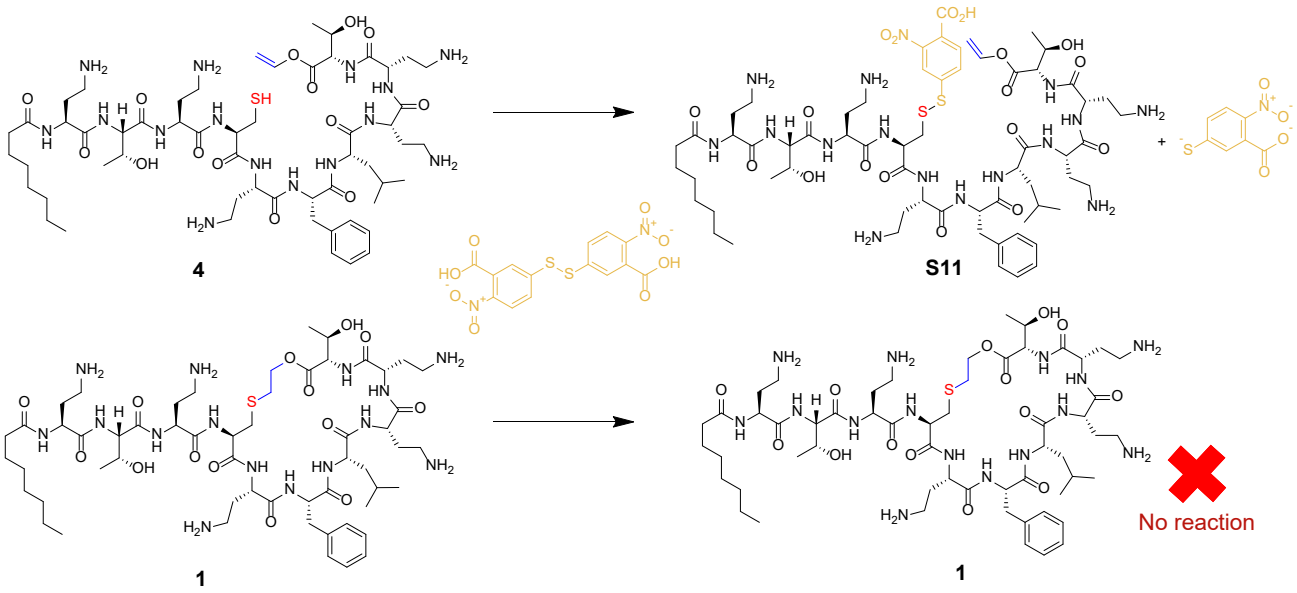


Figure S3. Linear Cys polymyxin analogue (**4**) (left) containing the free thiol at acidic pH, and Fmoc-cysteine (right, neutral pH), both in the presence of Ellman's reagent.



Scheme S4. Depiction of Ellman's reagent in the detection of successful polymyxin cyclisation.[1]

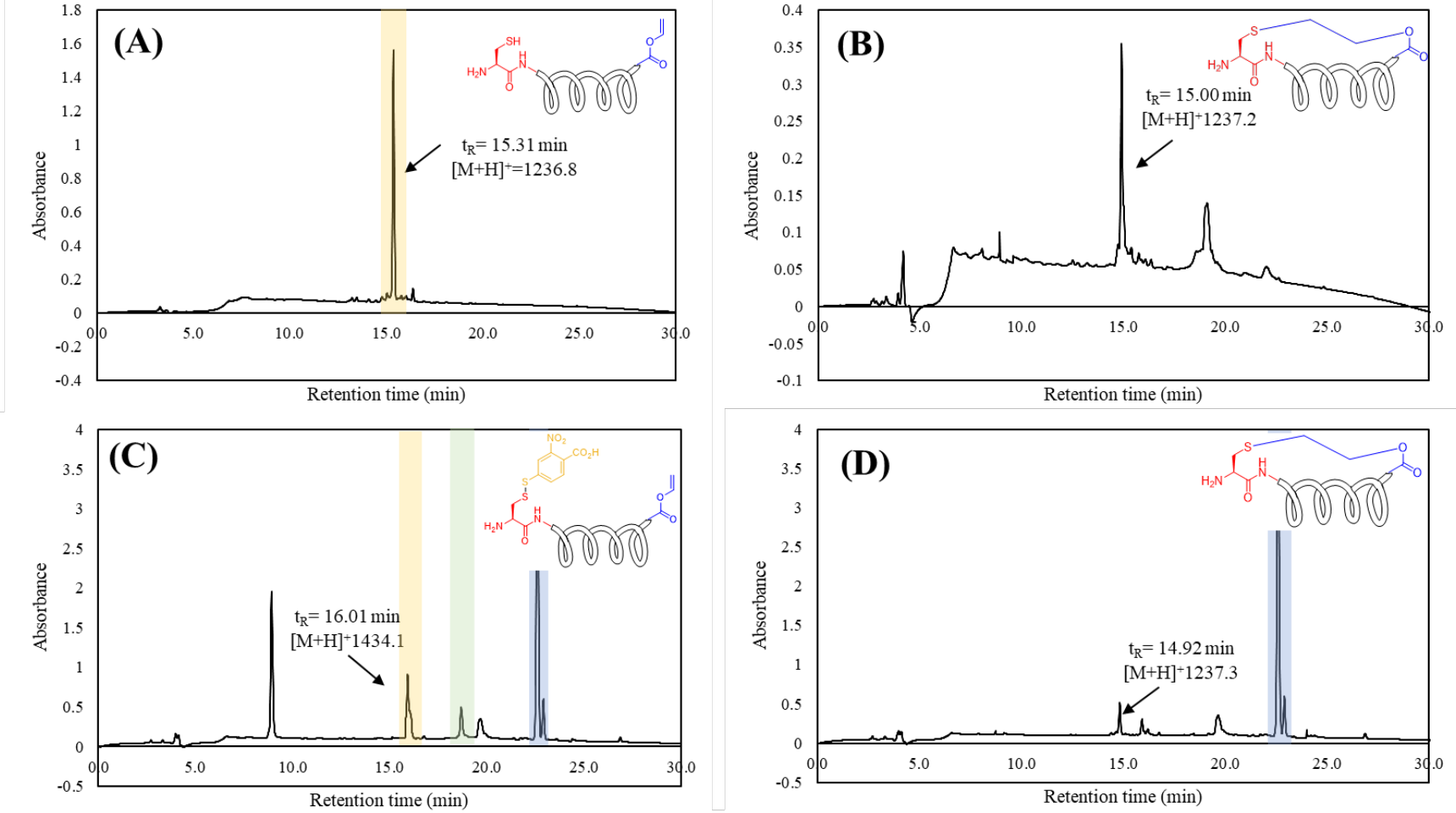


Figure S4. (A) Analytical RP-HPLC (214 nm) chromatograms performed on crude linear Cys polymyxin analogue (**4**) (yellow), t_R 15.31 min; *m/z* 619.2 $[M + 2H]^{2+}$ requires 618.8, *m/z* 1236.8 $[M + H]^+$ requires 1236.6 . Mass deconvolution calculated at 1236.10 Da with standard deviation of 0.42; theoretical mass calculated at 1236.5 Da. **(B)** Analytical RP-HPLC (214 nm) chromatograms performed on crude cyclised Cys polymyxin analogue (**1**), t_R 15.00 min; *m/z* 413.0 $[M + 3H]^{3+}$ requires 412.8, *m/z* 618.8 $[M + 2H]^{2+}$ requires 618.8, *m/z* 1237.2 $[M + H]^+$ requires 1236.6. Mass deconvolution calculated at 1235.93 Da with standard deviation of 0.31; theoretical mass calculated at 1236.5 Da. **(C)** Analytical RP-HPLC (214 nm) chromatograms performed on crude linear Cys polymyxin analogue (**4**) in the presence of Ellman's reagent. t_R18.8min TNB anion (green), t_R 22.7 min Ellman's reagent (blue), t_R 16.01 min TNB adduct on linear polymyxin (yellow); *m/z* 478.5 $[M + 3H]^{3+}$ requires 478.5, *m/z* 717.2 $[M + 2H]^{2+}$ requires 717.55, *m/z* 1434.1 $[M + H]^+$ requires 1434.2. Mass deconvolution calculated at 1432.7 Da with standard deviation of 0.34; theoretical mass calculated at 1433.7 **(D)** Analytical RP-HPLC (214 nm) chromatograms performed on crude cyclised Cys polymyxin analogue (**1**) in the presence of Ellman's reagent. t_R 22.51 min Ellman's reagent (blue), t_R 14.92 min cyclised polymyxin w/o TNB adduct; *m/z* 412.9 $[M + 3H]^{3+}$ requires 412.8, *m/z* 618.8 $[M + 2H]^{2+}$ requires 618.8, *m/z* 1237.3 $[M + H]^+$ requires 1236.6. Mass deconvolution calculated at 1235.87 Da with standard deviation of 0.38; theoretical mass calculated at 1236.5

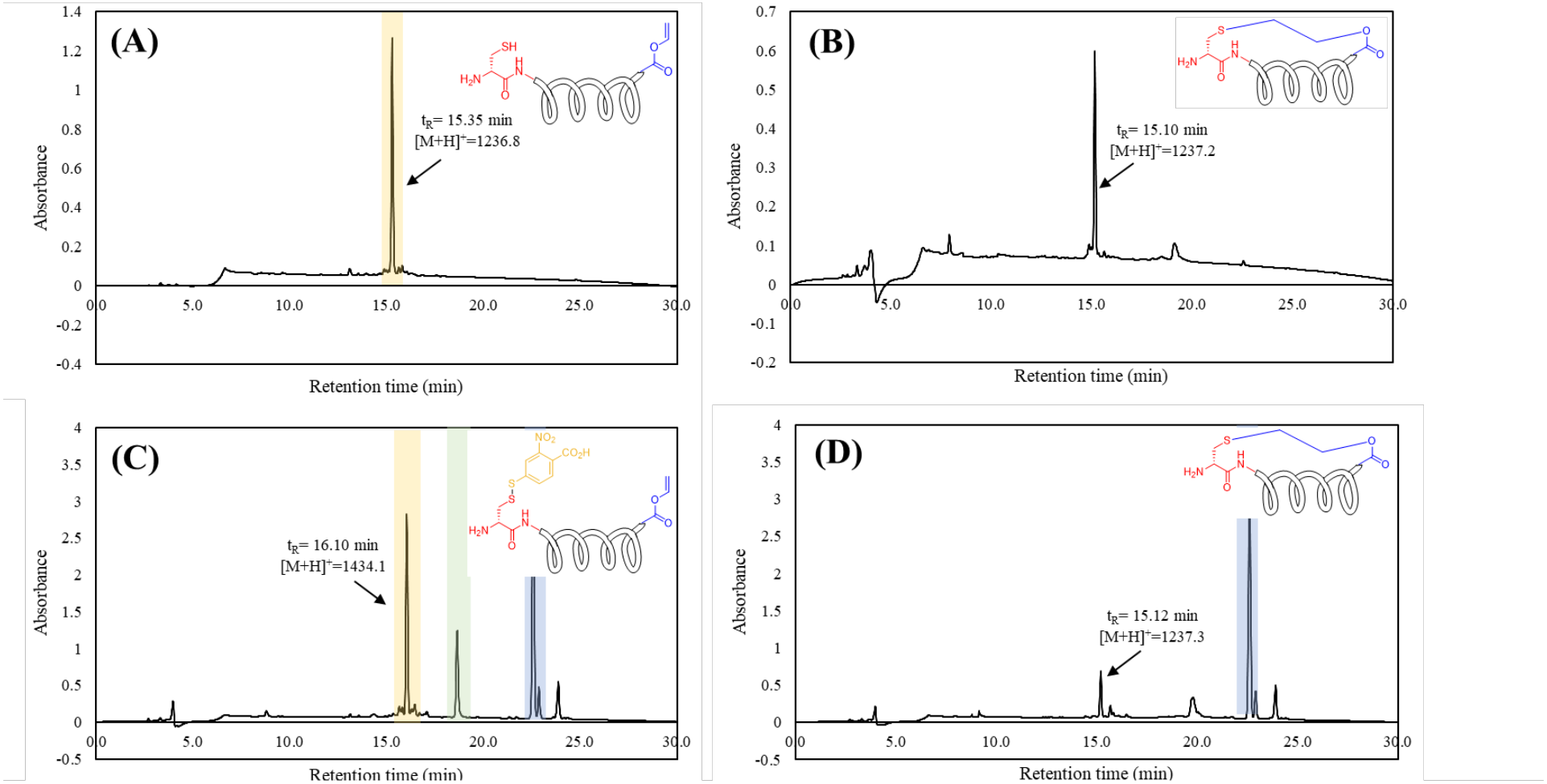


Figure S5. (A) Analytical RP-HPLC (214 nm) chromatograms performed on crude linear D-Cys polymyxin analogue (**5**) (yellow), t_R 15.35 min; *m/z* 619.2 [M + 2H]2+ requires 618.8, *m/z* 1236.8 [M + H]$^+$ requires 1236.6. Mass deconvolution calculated at 1236.10 Da with standard deviation of 0.42; theoretical mass calculated at 1236.5 Da. **(B)** Analytical RP-HPLC (214 nm) chromatograms performed on crude cyclised D-Cys polymyxin analogue (**2**), tR 15.10 min; *m/z* 413.0 [M + 3H]$^{3+}$ requires 412.8, *m/z* 618.8 [M + 2H]$^{2+}$ requires 618.8, *m/z* 1237.2 [M + H]$^+$ requires 1236.6. Mass deconvolution calculated at 1235.93 Da with standard deviation of 0.31; theoretical mass calculated at 1236.5 Da. **(C)** Analytical RP-HPLC (214 nm) chromatograms performed on crude linear D-Cys polymyxin analogue (**5**) in the presence of Ellman's reagent. t_R 18.8min TNB anion (green), tR 22.7 min Ellman's reagent (blue), t_R 16.10 min TNB adduct on linear polymyxin (yellow); *m/z* 478.5 [M + 3H]$^{3+}$ requires 478.5, *m/z* 717.2 [M + 2H]$^{2+}$ requires 717.55, *m/z* 1434.1 [M + H]$^+$ requires 1434.2. Mass deconvolution calculated at 1432.7 Da with standard deviation of 0.34; theoretical mass calculated at 1433.7 **(D)** Analytical RP-HPLC (214 nm) chromatograms performed on crude cyclised D-Cys polymyxin analogue (**2**) in the presence of Ellman's reagent. t_R 22.51 min Ellman's reagent (blue), t_R 14.93 min cyclised polymyxin w/o TNB adduct ; *m/z* 412.9 [M + 3H]$^{3+}$ requires 412.8, *m/z* 618.8 [M + 2H]$^{2+}$ requires 618.8, *m/z* 1237.3 [M + H]+ requires 1236.6. Mass deconvolution calculated at 1235.87 Da with standard deviation of 0.38; theoretical mass calculated at 1236.5.

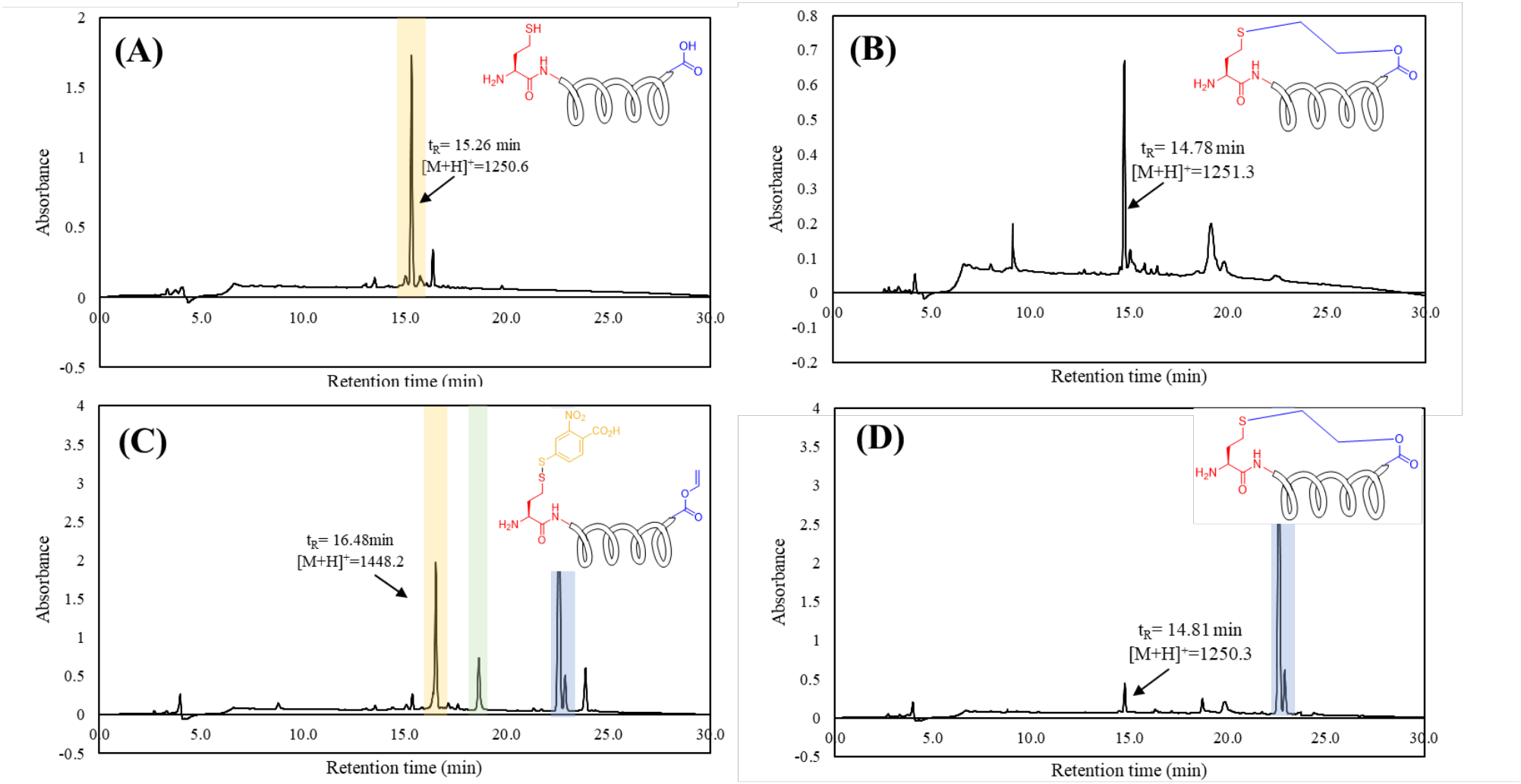
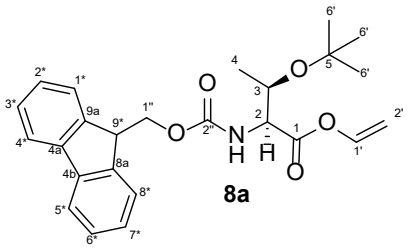


Figure S6. (A) Analytical RP-HPLC (214 nm) chromatograms performed on crude linear Hcy polymyxin analogue (**6**) (yellow), t_R 15.26 min; *m/z* 626.2 $[M + 2H]^{2+}$ requires 625.7, *m/z* 1250.6 $[M + H]^+$ requires 1250.4. Mass deconvolution calculated at 1250.4 Da with standard deviation of 1.13; theoretical mass calculated at 1250.0 Da. **(B)** Analytical RP-HPLC (214 nm) chromatograms performed on crude cyclised Hcy polymyxin analogue (**3**), t_R 14.78 min; *m/z* 417.6 $[M + 3H]^{3+}$ requires 417.5, *m/z* 625.8 $[M + 2H]^{2+}$ requires 625.7, *m/z* 1251.3 $[M + H]^+$ requires 1250.4 . Mass deconvolution calculated at 1249.90 Da with standard deviation of 0.36; theoretical mass calculated at 1250.0 Da. **(C)** Analytical RP-HPLC (214 nm) chromatograms performed on crude linear Hcy polymyxin analogue (**6**) in the presence of Ellman's reagent. t_R 18.68min TNB anion (green), t_R 22.5 min Ellman's reagent (blue), t_R 16.48 min TNB adduct on linear polymyxin (yellow); *m/z* 483.2 $[M + 3H]^+$ requires 483.5, *m/z* 724.3 $[M + 2H]^{2+}$ requires 724.7, *m/z* 1448.2 $[M + H]^+$ requires 1448.4 . Mass deconvolution calculated at 1446.80 Da with standard deviation of 0.35; theoretical mass calculated at 1447.73 . **(D)** Analytical RP-HPLC (214 nm) chromatograms performed on crude cyclised Hcy polymyxin analogue (**3**) in the presence of Ellman's reagent. t_R 22.56 min Ellman's reagent (blue), t_R 14.81 min cyclised polymyxin w/o TNB adduct ; *m/z* 417.6 $[M + 3H]^{3+}$ requires 417.5, *m/z* 625.8 $[M + 2H]^{2+}$ requires 625.7, *m/z* 1250.3 $[M + H]^+$ requires 1250.4 Mass deconvolution calculated at 1249.57 Da with standard deviation of 0.25; theoretical mass calculated at 1250.0.

S3. Synthesis and Characterisation of Small Molecules

vinyl *N*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*O*-(*tert*-butyl)-*L*-threoninate intermediate (**8a**)

(Fmoc-Thr(*t*Bu)-OVinyl)



This was prepared according to the method described by Lobell and Schnider.[2] Commercially available Fmoc-Thr(*t*Bu)-OH (**7a**) (1 g, 0.25 mmol), $Pd(OAc)_2$ (127 mg, 3.9 mmol), and KOH (14 mg, 0.25 mmol) were dissolved in vinyl acetate (25 mL, 2.7 mmol). This solution was then stirred overnight (14 h) at r.t. The crude mixture was filtered through celite, with the celite washed with vinyl acetate. Following evaporation of the vinyl acetate *in vacuo*, the crude product underwent purification using column chromatography on silica gel (EtOAc:Pet.ether, 1:8-1:6, *v/v*) to produce the title product (**8a**) as a pale translucent yellow oil (0.78 g, 74% yield).

R_f 0.6 (EtOAc-Pet ether 1:6)

$[\alpha]_D^{22.2}$ -4.4 (*c*1.10, DMF);

IR ν_{max} (neat): 3950, 1750, 1710, 1695, 1500, 1300, 1200, 1175, 1125, 1100, 1075, 1025 cm^{-1};

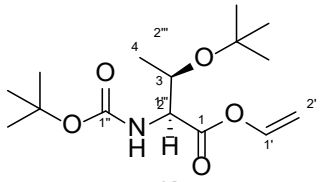
^{1}H NMR (400 MHz, $CDCl_3$): δ 7.7 (d, *J* = 7.7 Hz, 2H, 2 × Ar-*H*), 7.65 (t, *J* = 7.7 Hz, 2H, 2 × Ar-*H*), 7.41 (t, *J* = 7.5 Hz, 2H, 2 × Ar-*H*), 7.43-7.25 (m, 3H, 2 × Ar-*H, H*-1'), 5.64 (d, *J* = 9.8 Hz, 1H, N-*H*), 4.95 (dd, *J* = 13.8, 1.8 Hz, 1H, *H*-2'), 4.65 (dd, *J* = 6.3, 1.8 Hz, 1H, *H*-2'), 4.47-4.26 (m, 5H, *H*-1'', *H*-2, *H*-3, *H*-9*), 1.27 (d, *J* = 6.4 Hz, 3H, *H*-4), 1.14 (s, 9H, *H*-6');

^{13}C NMR (100 MHz, $CDCl_3$): δ 168.5 (C=O, *C*-1) 156.9 (C=O, *C*-2''), 144.1 (C, Ar-C), 143.9 (C, Ar-C), 141.4 (C-H, Ar-C), 141.1 (C-H, C-1'), 127.8 (C-H, 2 × Ar-C), 127.23 (C-H, Ar-C), 127.21 (C-H, 2 × Ar-C), 125.4 (C-H, Ar-C), 125.3 (C-H, Ar-C), 120.1 (C-H, 2 × Ar-C), 98.7 ($C-H_2$, C-2'), 74.5 (C-H, C-5), 67.4 (C-H, C-2), 67.3 (C-H2, C-1''), 59.9 (C-H, C-3), 47.3 (C-H, C-9*), 28.5 (3 × CH_3, C-6'), 21.0 (CH_3, C-4);

HRMS (ESI+) $[M + Na]^+$ calcd: for $C_{25}H_{29}NNaO_5$: 446.1938; found 446.1944.

See S8 for NMR and HRMS data.

vinyl *N*-(*tert*-butoxycarbonyl)-*O*-(*tert*-butyl)-*L*-threoninate intermediate (**8b**)
(Boc-Thr(*t*Bu)-OVinyl)



8b

This was prepared according to the method described by Lobell and Schnider.[2] Boc-Thr(*t*Bu)-OH (**7b**) (1 equiv.) (1 g, 0.36 mmol), $Pd(OAc)_2$ (127 mg, 0.56 mmol), and KOH (20 mg, 0.36 mmol) were dissolved in vinyl acetate (33 mL, 0.39 mol). This solution was then stirred overnight (14 h) at r.t. The crude mixture was filtered through celite, with the celite washed with vinyl acetate. Following evaporation of the vinyl acetate *in vacuo*, the crude product underwent purification using column chromatography on silica gel (EtOAc-Pet. ether, 1:8, *v/v*) to produce the *title product* (**8b**) as a clear oil (0.87 g, 80% yield).

R_f 0.5 (EtOAc-Pet ether 1:6);

$[\alpha]_D^{22.3}$ -9.7(*c* 1.09 DMF);

IR ν_{max}(neat): 3461, 2979, 1770, 1718, 1647, 1496, 1151, 1087 cm^{-1};

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.19 (m, 1H, *H*-1'), 5.36* (d, *J* = 7.28 Hz, 0.24H, N-H), 5.28 (d, *J* = 9.6 Hz, 1H, N-*H*), 4.89-4.85 (m, 1H, *H*-2'), 4.57-4.55 (m, 1H, *H*-2'), 4.22-4.18 (m, 2H, *H*-2, *H*-3), 1.41 (s, 9H, 3 × C*H*$_3$, *H*-1'''), 1.40* (s, 9H, 3 × C*H*$_3$, *H*-1'''), 1.18 (d, *J* = 6.3 Hz, 3H, *H*-4), 1.05 (s, 9H, 3 × C*H*$_3$, *H*-2''');

^{13}C NMR (100 MHz, $CDCl_3$): δ 168.7 (C=O, *C*-1), 156.3 (C=O, *C*-1''), 141.1 (C-H, *C*-1'), 98.4 (C-H_2, *C*-2'), 79.9 (C, *C*-1'''), 74.2 (C, C-2'''), 67.3 (C-H), 59.4 (C-H); 28.4 (3 × CH_3), 20.9 (CH_3, C-4)

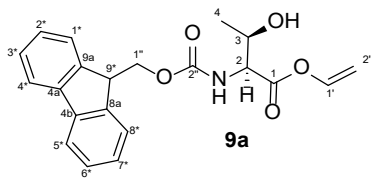
Peaks labeled with an asterisk (*) in ^{1}H-NMR and ^{13}C-NMR spectrum likely due to the presence of *N*-Boc rotamers

HRMS (ESI+) $[M + Na]^+$ calcd: for $C_{15}H_{27}NNaO_5$: 324.3728; found 324.1781.

See S8 for NMR and HRMS data.

vinyl (((9*H*-fluoren-9-yl)methoxy)carbonyl)-*L*-threoninate (**9a**)

(Fmoc-Thr-OVinyl)



The Fmoc-Thr(*t*Bu)-OVinyl (**8a**) (0.78 g) was stirred in a 70% solution of TFA in CH_2Cl_2 (1:1, *v/v*, 10 mL) and water (500 µl) for 45 min, with the solution concentrated via a stream of N_2, to yield the *title product* (**9a**) as a yellow oil (0.63 g, 92% crude yield). The product did not undergo further purification and was used as is.

R_f 0.3 **(**EtOAc-Pet ether 1:6);

$[\alpha]_D^{25.9}$ -6.3(*c* 1.11, DMF);

IR ν_{max}(neat): 3400, 2800, 2275, 2225, 1600, 1400, 1300, 1000, 990 cm^{-1};

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (d, *J* = 7.7 Hz, 2H, 2 × Ar-*H*), 7.62 (t, *J* = 6.2 Hz, 2H, 2 × Ar-*H*), 7.40 (t, *J* = 7.3 Hz, 2H, 2 × Ar-*H*), 7.33-7.25 (m, 3H), 5.66 (d, *J* = 9.0 Hz, 1H, N-*H*), 4.97 (dd, *J* = 13.9, 1.9 Hz, 1H, *H*-1'), 4.67 (dd, *J* = 6.2, 1.8 Hz, 1H, *H*-2'), 4.49-4.41 (m, 4H, *H*-1'', *H*-2, *H*-3), 4.25 (t, *J* = 7 Hz, 1H, *H*-9*), 1.28 (d, *J* = 6.1 Hz, 3H, *H*-4);

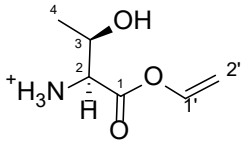
^{13}C NMR (100 MHz, $CDCl_3$): δ 168.5 (C=O, *C*-1) 156.9 (C=O, *C*-2''), 143.9 (C, Ar-C), 143.8 (C, Ar-C), 141.4 (C-H, Ar-C), 141.0 (C-H, C-1'), 127.9 (C-H, 2 × Ar-C), 127.2 (C-H, 2 × Ar-C), 125.2 (C-H, Ar-C), 120.1 (C-H, 2 × Ar-C), 99.3 ($C-H_2$, C-2'), 67.9 (C-H, C-2), 67.4 ($C-H_2$, C-1''), 59.1 (C-H, C-3), 47.3 (C-H, C-9*), 20.1 (CH_3, C-4);

HRMS (ESI+) $[M + Na]^+$ calcd: for $C_{21}H_{21}NNaO_5$: 390.1312; found 390.1309.

See S8 for NMR and HRMS data.

vinyl *L*-threoninate (**9b**)

(H_2N-Thr-OVinyl)



9b

Boc-Thr(*t*Bu)-OVinyl (**8b**) (0.87 g) was stirred in a 50% solution of TFA in CH_2Cl_2 (*v/v,* 10 mL) for 45 minutes, with the solution concentrated via a stream of N_2 to yield the *title product* (**9b**) as a dark brown oil (0.394 g, 94% yield). The product did not undergo further purification and was used as is.

R_f 0.6 (DCM-MeOH 1:3);

$[\alpha]_D^{22.3}$ -4.1(*c* 1.09 DMF);

IR ν_{max}(neat): 3421, 2975, 1762, 1670, 1369, 1188, 1135, 723 cm^{-1};

^{1}H NMR (400 MHz, CD_3OD): δ 7.34-7.29 (m, 1H, *H*-1'), 5.09-5.05 (m, 1H, *H*-2'), 4.80-4.78 (m, 1H, *H*-2'), 4.38-4.32 (m, 1H, *H*-3), 4.05 (d, *J* = 3.99 Hz, *H*-2), 1.35 (d, *J* = 6.48 Hz, *H*-4);

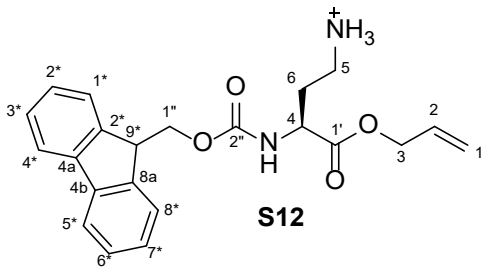
^{13}C NMR (100 MHz, CD_3OD): 166.8 (C=O, *C*-1), 141.9 (C-H, *C*-1'), 100.3 (C-H_2, *C*-2'), 66.2 (C-H, *C*-3), 59.6 (C-H, *C*-2), 20.4 (CH_3, *C*-4);

HRMS (ESI+) $[M + H]^+$ calcd: for $C_6H_{12}NO_3$:146.0739; found 146.0812.

See S8 for NMR and HRMS data.

(S)-3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-(allyloxy)-4-oxobutan-1-aminium (**S12**).

(Fmoc-Dab-OAll)



This was prepared according to the method described by Xu *et al.*[3] Fmoc-Dab(Boc)-OH (4 g, 9.08 mmol) was dissolved in MQ water (18 mL) containing sodium bicarbonate ($NaHCO_3$) (0.7 g, 8.3 mmol). Separately, Aliquat 336® (3 g, 6.7 mmol) and allyl bromide (4 mL, 46.6 mmol) were dissolved in CH_2Cl_2 (16 mL), and this mixture was slowly added to the Fmoc-Dab(Boc)-OH solution over 1 minute. The resulting solution was stirred overnight (14 h) at r.t. Water (90 mL) was then added to the solution, with the product extracted using CH_2Cl_2 (3 × 100 mL). The combined CH_2Cl_2 organic layer was then washed in brine (2 × 50 mL). The CH_2Cl_2 layer was then dried using Na_2SO_4, filtered, and concentrated *in vacuo*. The crude mixture then underwent column chromatography on silica gel (EtOAc-PET ether, 1:4, *v/v*) to produce the Fmoc-Dab(Boc)-OAll (3.56 g, 81% yield).

Fmoc-Dab(Boc)-OAll (3.56 g, 7.41 mmol) was stirred in a 20% solution of TFA in CH_2Cl_2 (*v/v*, 5 mL), for 2 h at r.t. The solution was then concentrated via an N_2 stream. Water (40 mL) was then added, inducing the formation of white solid clumps. The water was then removed *in vacuo* and the resulting solid washed with Et_2O (2 × 40 mL). The product was then dried *in vacuo* to render the *title product* (**S12**) as a white solid (2.71 g, 96% yield).

Rf: 0.8 (CH_2Cl_2:MeOH 1% Et_2NH);

***m/z* (ESI-MS)** 381.2([M+H]+ requires 381.2);

$[\alpha]_D^{22.3}$ -4.1(*c* 1.09 in DMF);

^{1}H NMR (400 MHz, $CDCl_3$) 8.12 (s, 3H, N-*H*), 7.71 (d, *J* = 6.8 Hz, 2H, 2 × Ar-*H*), 7.53 (d, *J* = 7.6 Hz, 2H, 2 × Ar-*H*), 7.35 (t, *J* = 7.44 Hz, 2H, 2 × Ar-*H*), 7.26 (q, *J* = 7.4 Hz, 3H, 2 × Ar-*H*, N-*H*), 5.81-5.76 (m, 1H, *H*-1'), 5.23 (q, *J* = 17.16 Hz, 2H, *H*-2'), 4.57 (d, *J* = 2.52 Hz, 2H, *H*-3), 4.35 (q, *J* = 7.76 Hz, 3H, *H*-9, 1''), 4.13 (t, *J* = 6.64, 1H, *H*-4), 3.08 (s, 2H, *H*-5), 2.95 (s, 2H, *H*-6);

^{13}C NMR (100 MHz, $CDCl_3$) δ 171.5 (C=O, *C*-1'), 156.6 (C=O,*C*-2''), 143.5 (C, 2 × Ar-C), 141.3 (C, 2 × Ar-C), 130.9 (C-H, *C*-2), 127.8 (C-H, 2 × Ar-C), 127.1 (C-H, 2 × Ar-C), 125.0 (C-H, 2 × Ar-C), 120.0 (C-H, 2 × Ar-C), 119.5 (C-H_2, *C*-1), 67.5 (C-H_2, *C*-1''), 66.7 (C-H_2, *C*-3), 52.4 (C-H, *C*-4), 47.0 (C, *C*-9*), 36.4 (C, *C*-5), 29.0 (C, *C*-6).

Spectroscopic data were in good agreement with those previously reported.[3]

S4. General Methods

General procedure for peptide synthesis.

Peptides were synthesised manually using 9-fluorenylmethoxycarbonyl solid phase peptide synthesis (Fmoc-SPPS) at room temperature using a fritted glass reaction vessel.

General Method 1. Removal of *N*α - Fmoc protecting group.

A solution of 20% piperidine in DMF (*v/v*, 7 mL) was added to the peptidyl resin. The resin was then shaken at r.t for 10 min. The solution was then drained, and the resin was washed with DMF (3 × 7 mL). Fresh 20% piperidine in DMF solution (*v/v*, 7 mL) was added to the resin and shaken at r.t for 10 min. Resin was drained and washed with DMF (5 × 7 mL). A positive Kaiser test[4] was used to indicate the presence of a free *N*α-amino group.

General Method 2. Coupling of Fmoc-Leu-OH and Fmoc-Thr(*t*Bu)-OH.

To the resin, a solution of Fmoc amino acid (5 equiv.), HCTU (4.75 equiv.), and DIPEA (10 equiv.) in DMF (3 mL), was added. The reaction mixture was shaken for 40 min at r.t., drained, then washed with DMF (3 × 7 mL). A negative Kaiser test[4] was used to indicate the lack of free *N*α-amino group.

General Method 3. Coupling of Fmoc-Dab(Boc)-OH and Fmoc-D-Phe-OH.

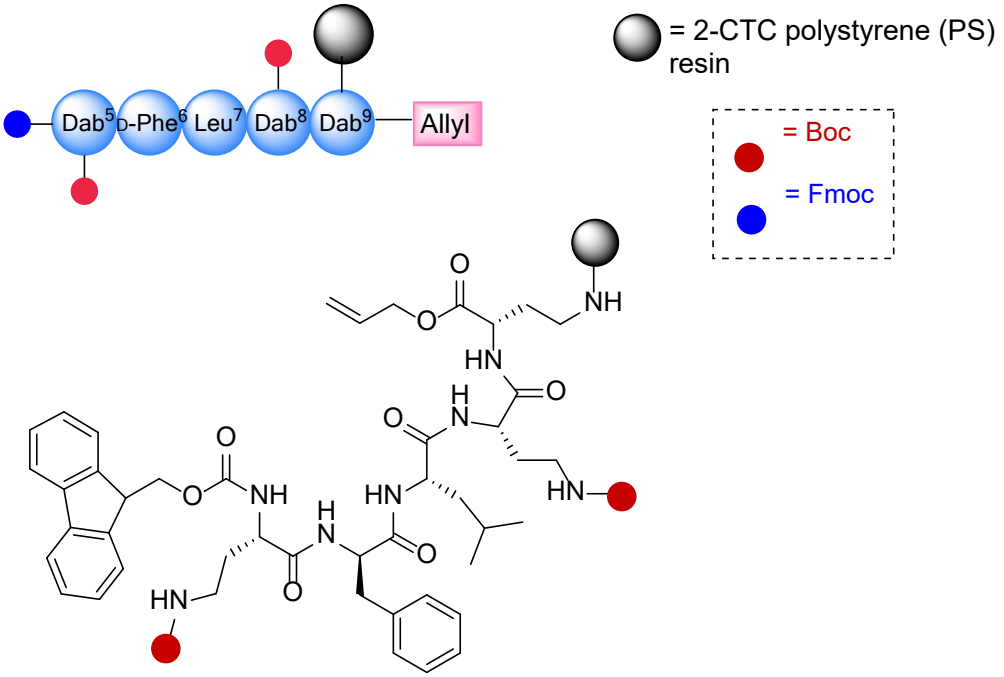
To the peptidyl resin, a solution of Fmoc amino acid (3 equiv.), HCTU (2.75 equiv.), and DIPEA (6 equiv.) in DMF (3 mL) was added. The reaction mixture was then shaken for 1 h at r.t., drained, then washed with DMF (3 × 7 mL). A negative Kaiser test[4] was used to indicate the lack of free *N*α-amino group.

General Method 4. Coupling of Fmoc-Cys(Trt)-OH, Fmoc-D-Cys(Trt)-OH, and Fmoc-Hcy(Trt)-OH.

To the peptidyl resin, a solution of Fmoc amino acid (5 equiv.), HATU (4.75 equiv.), HOAt (4.5 equiv.), *sym*-collidine (10 equiv.)[5] in DMF (3 mL) was added. The reaction mixture was then shaken for 1 h at r.t., drained, then washed with DMF (3 × 7 mL). A negative Kaiser test[4] was used to indicate the lack of free *N*α-amino group.

S5. Synthesis, Structure, and LC-MS Traces of Synthetic Peptides

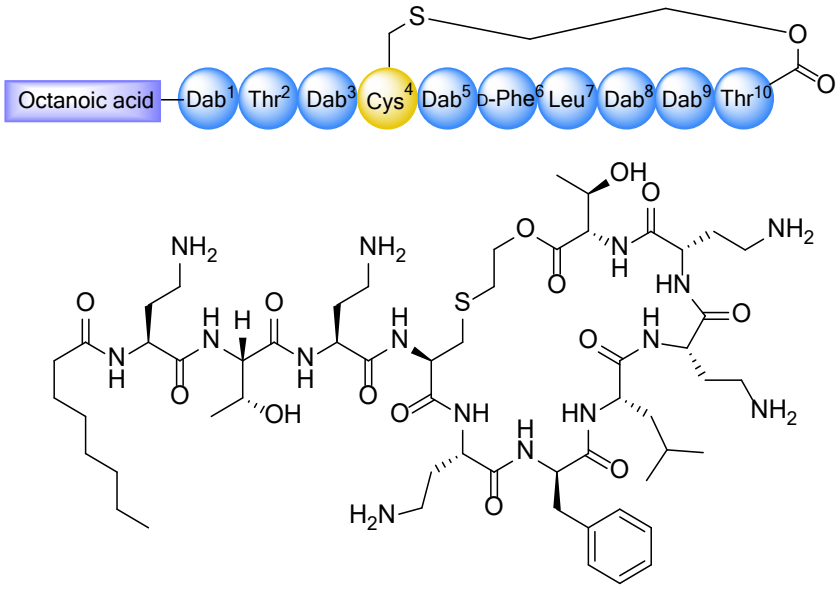
Synthesis of the Common Fragment (**S4**)



To the 2-CTC PS resin (0.7 mmol, 0.89 mmol/g) $SOCl_2$ (7 mL) was added, and mixture was shaken for 2 h. The $SOCl_2$ was then drained, and the resin washed with CH_2Cl_2 (6 × 5 mL) then with DMF (6 × 5 mL).[6] The DMF was drained, and fresh DMF added (7 mL) and mixture was shaken for 30 min. The DMF was removed from the resin, and a solution of Fmoc-Dab-OAll (**S12**) (2.1 mmol, 3 equiv) and DIPEA (4 equiv.) in a mixture of CH_2Cl_2/DMF (1:1, *v/v,* 4 mL) was added to the resin, and then shaken for 24 h at r.t. The solution was then drained and the resin washed with DMF (3 × 5 mL). A sample of the resin was washed in CH_2Cl_2 and dried and then subjected to a loading test (10 mg)[7] affording the loading of 0.57 mmol/g.

A solution of MeOH and CH_2Cl_2 (1:1, *v/v*, 4 mL), was added to the peptidyl resin, and shaken for 10 min at r.t. The resin was then washed with DMF (5 × 7 mL). The *N*α protecting group was then deprotected in accordance with general method 1, and peptide chain elongation was carried out using **General Method 1-3** to afford the common fragment Fmoc-Dab(Boc)-DPhe-Leu-Dab(Boc)-Dab(2-CTC PS)-OAll (**S4**). The common fragment (**S4**) was then washed with CH_2Cl_2 and dried *in vacuo* and split into three portions to synthesise the Cys (**1**), D-Cys (**2**), and Hcy (**3**) polymyxin analogues.

Synthesis of the Cys Polymyxin Analogue (**1**)



The common fragment Fmoc-Dab(Boc)-DPhe-Leu-Dab(Boc)-Dab(2-CTC PS)-OAll (**S4**) (0.23 mmol, 0.57 mmol/g) underwent *N*α deprotection in accordance with **General Method 1**, and peptide chain elongation was carried out using **General Method 1-4**, to afford H_2N-Dab(Boc)-Thr(*t*Bu)-Dab(Boc)-Cys(Trt)-Dab(Boc)-DPhe-Leu-Dab(Boc)-Dab(2-CTC PS)-OAll. To the peptidyl resin, a solution of octanoic acid (5 equiv.), HATU (4.75 equiv.), and DIPEA (10 equiv.) in DMF (3 mL) was added. The reaction mixture was then shaken for 40 min at r.t, drained then washed with DMF (3 × 5 mL). A negative Kaiser test[4] was used to indicate the lack of free *N*α-amino group.

To the peptidyl resin, a solution of $Pd(PPh_3)_4$ (2 equiv.), and $PhSiH_3$ (20 equiv.) in CH_2Cl_2 (4 mL) was added. The reaction mixture was then shaken for 4 h at r.t. The resin was then drained and washed with DMF (5 × 7 mL). A 0.5 M solution of sodium diethyldithiocarbamate trihydrate in DMF (10 mL) was added to the peptidyl resin, and mixture shaken for 10 min. The solution was drained, and fresh 0.5 M sodium diethyldithiocarbamate trihydrate (10 mL) was added to the resin and shaken at r.t. for 10 min. Resin was drained and washed with DMF (5 × 5 mL) affording octanoic acid-Dab(Boc)-Thr(*t*Bu)-Dab(Boc)-Cys(Trt)-Dab(Boc)-DPhe-Leu-Dab(Boc)-Dab(2-CTC PS)-COOH.

A solution of H_2N-Thr-OVinyl building block (**9b**) (3 equiv.), EDC.HCl (1.1 equiv.), HOAt (1.1 equiv.), and DIPEA (2 equiv.) in DMF (3 ml) was added to the allyl deprotected peptidyl resin and mixture shaken for 7 h at r.t. The resin was drained then washed with DMF (3 × 5 mL) and then with CH_2Cl_2 (3 × 5 mL). A solution of TFA/H_2O/EDT/*i*-Pr_3SiH (94, 2.5, 2.5, 1; *v/v/v/v,* 5 mL) was added and mixture shaken for 2 h at r.t. The solution was then concentrated via an N_2 stream. The peptide was then precipitated using cold Et_2O (45 mL) and was isolated (centrifugation, 4000 rpm, 5 min), with the supernatant carefully removed via decanting. The crude peptide was then dissolved in MeCN:MQ H_2O containing 0.1% TFA

(1:1, *v/v,* 1 mL), which was then filtered and lyophilised to afford the crude linear peptide octanoic acid-Dab-Thr-Dab-Cys-Dab-DPhe-Leu-Dab-Dab-Thr-OVinyl (**4**) (**Figure S4 A**).

The thiol-ene induced cyclisation of linear polymyxin analogue. The crude linear peptide (**4**) (1 equiv.) was added to a degassed solution of NMP (volume adjusted to ensure peptide concentration of 3 mg/mL) with DMPA (1 equiv.), *t*NonSH (20 equiv.), *i*-Pr_3SiH (20 equiv.), and TFA (5% *v/v* of final volume). The reaction mixture was then stirred at room temperature under UV light (365 nm) for 1 h. The crude cyclic peptide was then precipitated using cold Et_2O, isolated via centrifugation, and washed with cold Et_2O (2 × 45 mL) (**Figure S7**). Crude peptide was then dissolved in a solution of MeCN: MQ H_2O containing 0.1% TFA (1:1, *v/v*, 10 mL) and lyophilised. The crude cyclic peptide (**1**) was purified using a semi preparative Waters 1525 Binary HPLC pump equipped with a Waters 2489 UV/visible detector using a Phenomenex Gemini-NX C18 (110 Å, 5 μm, 10 mm × 250 mm) at a flow rate of 5 mL/min at a gradient of 1-91% B at 1% B/min over 100 min. Fractions were collected and analysed by ESI-MS and analytical RP-HPLC. Fractions which were identified as containing the correct *m/z* at a sufficient purity were combined and lyophilised to afford **1** as a white solid (12 mg, 6.5% yield (based on a 0.149 mmol scale), >99% purity); t_R 31.95 min; *m/z* (ESI-MS) 1236.6 ($[M+H]^+$ requires 1235.6) (**Figure S8**).

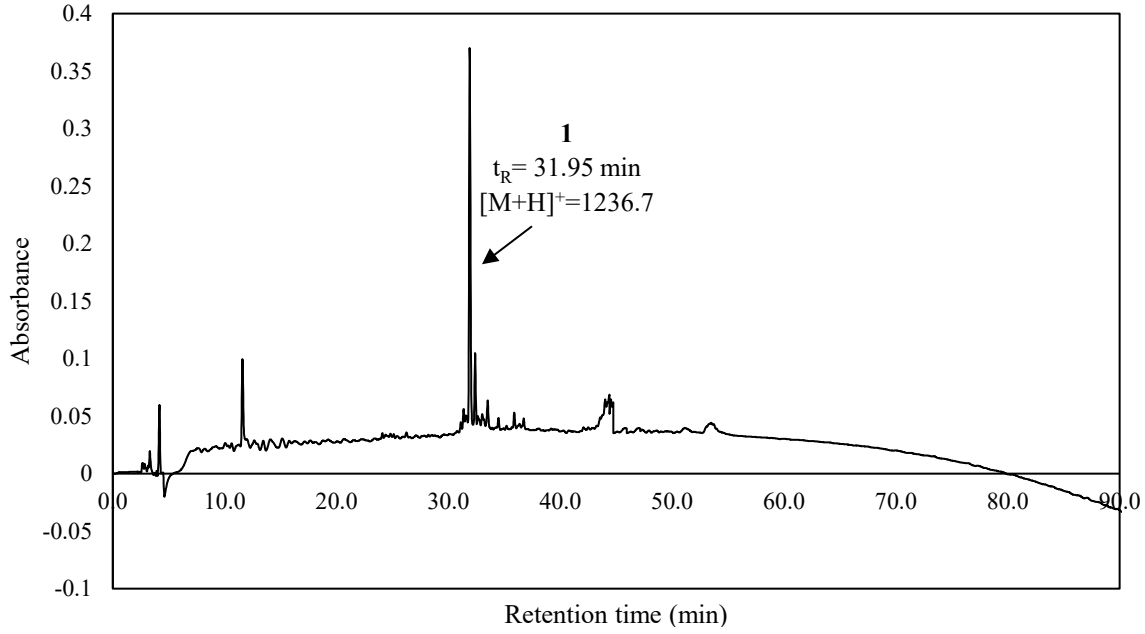


Figure S7. Analytical RP-HPLC of crude cyclised Cys polymyxin analogue (**1**) (*ca.* 34% as judged by peak area of RP-HPLC at 214 nm). t_R 31.95 min cyclised polymyxin; *m/z* 413.2 $[M + 3H]^{3+}$ requires 412.8, *m/z* 619.1 $[M + 2H]^{2+}$ requires 618.8, *m/z* 1236.7 $[M + H]^+$ requires 1235.6. Mass deconvolution calculated at 1236.17 Da with standard deviation 0.45; theoretical mass calculated at 1236.5 Da. Alliance HPLC by Waters™ Luna® (5µm, C18 100 Å, LC column 250 × 4.6 mm), 1 to 91%B over 90 minutes (*ca.* 1% B/min), 1 mL/min at room temperature, where A: 0.1% TFA in H_2O, and B: 0.1% TFA in acetonitrile (MeCN).

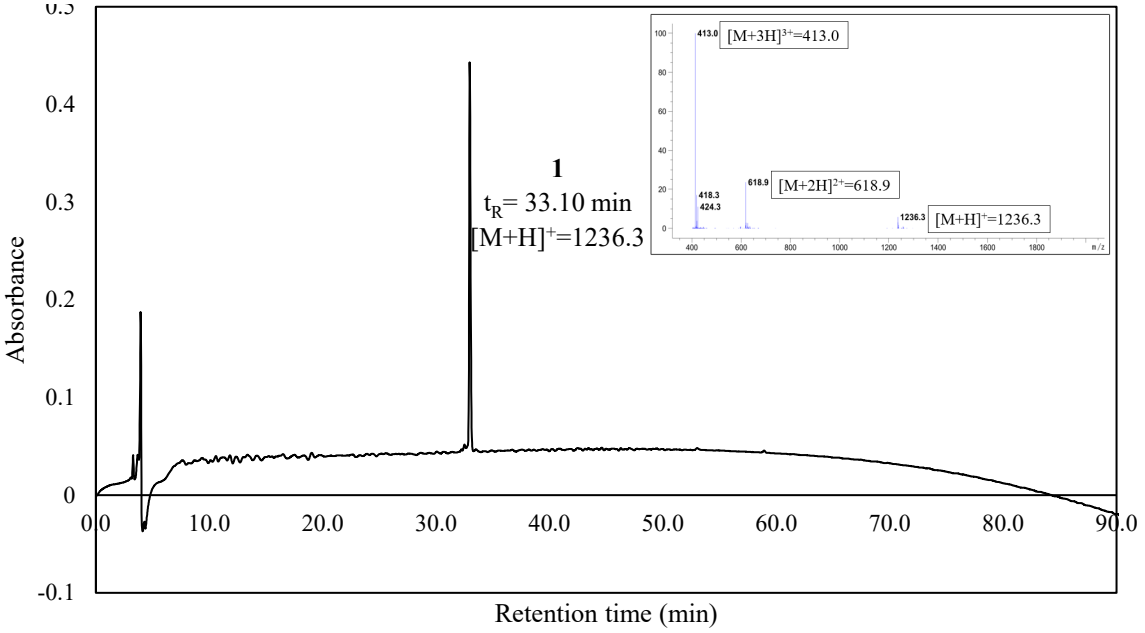
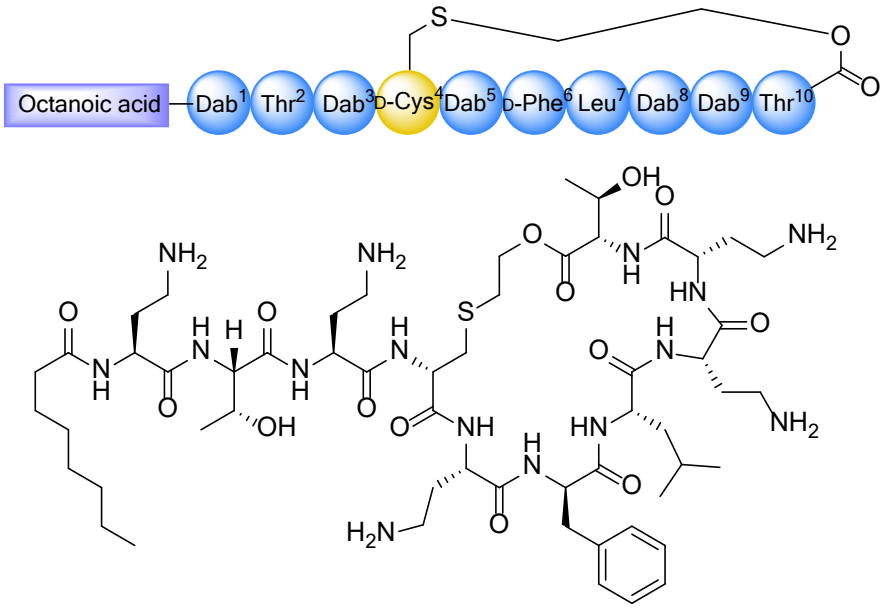


Figure S8. Analytical RP-HPLC and MS traces of purified cyclised Cys polymyxin analogue (**1**) (*ca.* 99% as judged by peak area of RP-HPLC at 214 nm). t_R 31.95 min cyclised polymyxin; *m/z* 413.0 $[M +3H]^{3+}$requires 412.8, *m/z* 618.9 $[M + 2H]^{2+}$ requires 618.8, *m/z* 1236.3 $[M + H]^+$ requires 1235.6. Mass deconvolution calculated at 1235.70 Da with standard deviation 0.36; theoretical mass calculated at 1236.5 Da. Alliance HPLC by Waters™ Luna® (5µm, C18 100 Å, LC column 250 × 4.6 mm), 1 to 91%B over 90 minutes (*ca.* 1% B/min), 1 mL/min at room temperature, where A: 0.1% TFA in H_2O, and B: 0.1% TFA in acetonitrile (MeCN).

Synthesis of the D-Cys Polymyxin Analogue (**2**)



The common fragment Fmoc-Dab(Boc)-DPhe-Leu-Dab(Boc)-Dab(2-CTC PS)-OAll (**S4**) (0.23 mmol, 0.57 mmol/g) underwent *N*α deprotection in accordance with **General Method 1**. Fmoc-D-Cys-OH was then coupled using **General Method 4**. The subsequent synthetic steps of peptide chain elongation, octanoic acid coupling, allyl deprotection, H_2N-Thr-OVinyl, followed the same procedure as those used for the Cys polymyxin analogue (**1**) affording the crude linear peptide octanoic acid-Dab-Thr-Dab-DCys-Dab-DPhe-Leu-Dab-Dab-Thr-OVinyl (**5**) (**Figure S5 A**). The thiol-ene induced macrocyclysation of the linear polymyxin analogue **5 f**ollowed the same procedure as those used for the Cys polymyxin analogue (**1**) affording the crude cyclic peptide (**2**) **(Figure S9).**

The crude cyclic peptide (**2**) was purified using a semi preparative Waters 1525 Binary HPLC pump equipped with a Waters 2489 UV/visible detector using a Phenomenex Gemini-NX C18 (110 Å, 5 µm, 10 mm × 250 mm) at a flow rate of 5 mL/min at a gradient of 1-91% B at 1% B/min over 100 min. Fractions were collected and analysed by ESI-MS and analytical RP-HPLC. Fractions which were identified as containing the correct *m/z* at a sufficient purity were combined and lyophilised to afford **2** as a white solid (16 mg, 8.7% yield (based on a 0.149 mmol scale), 99% purity); t_R 33.01 min; *m/z* (ESI-MS) 1236.3 ($[M+H]^+$ requires 1235.6) (**Figure S10**).

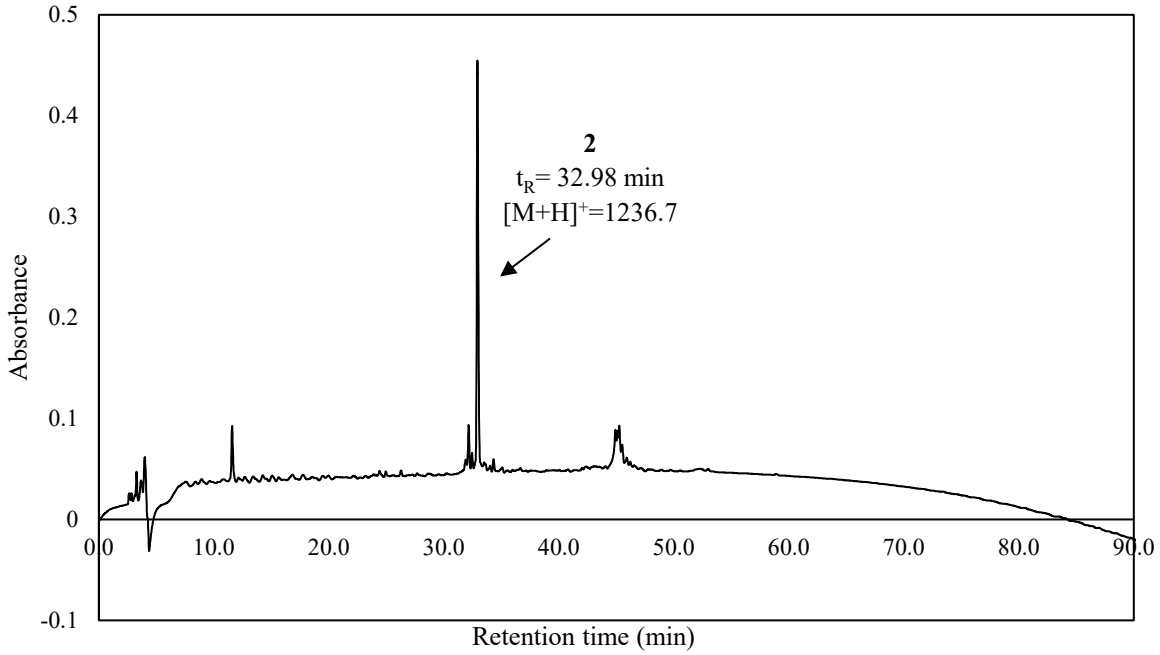


Figure S9. Analytical RP-HPLC of crude cyclised D-Cys polymyxin analogue (**2**) (*ca*. 39% as judged by peak area of RP-HPLC at 214 nm). t_R 32.98 min cyclised polymyxin; *m/z* 619.1 $[M + 2H]^{2+}$ requires 618.8, *m/z* 1236.7 $[M + H]^+$ requires 1235.6 . Mass deconvolution calculated at 1235.95 Da with standard deviation 0.35; theoretical mass calculated at 1236.5 Da. Alliance HPLC by Waters™ Luna® (5µm, C18 100 Å, LC column 250 × 4.6 mm), 1 to 91%B over 90 minutes (*ca.* 1% B/min), 1 mL/min at room temperature, where A: 0.1% TFA in H_2O, and B: 0.1% TFA in acetonitrile (MeCN).

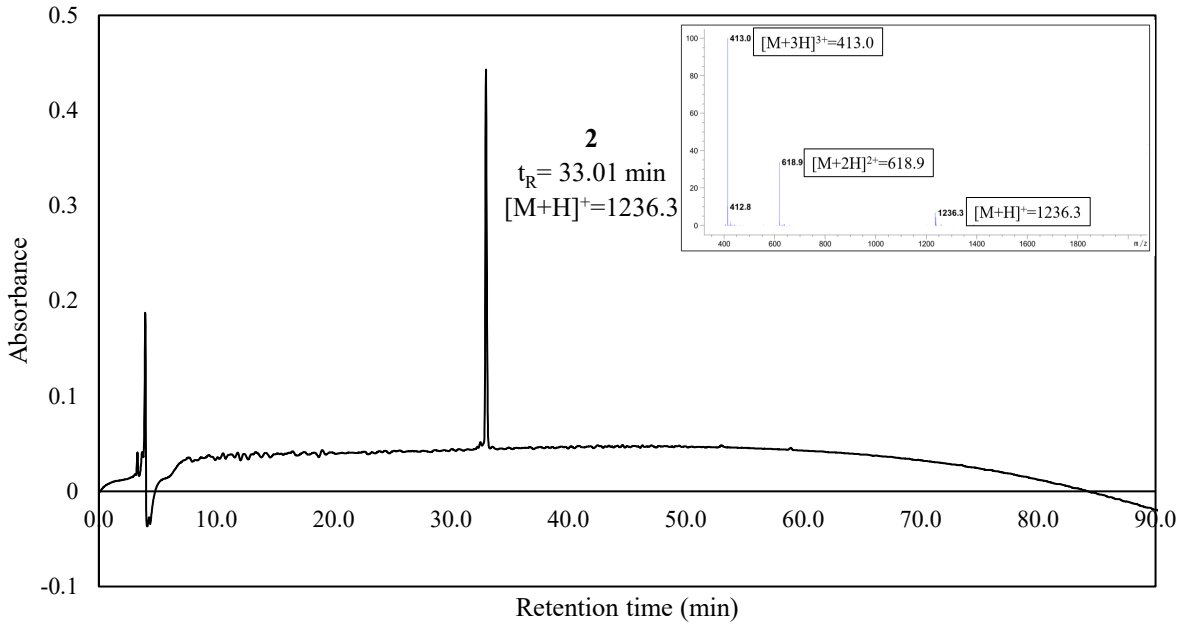
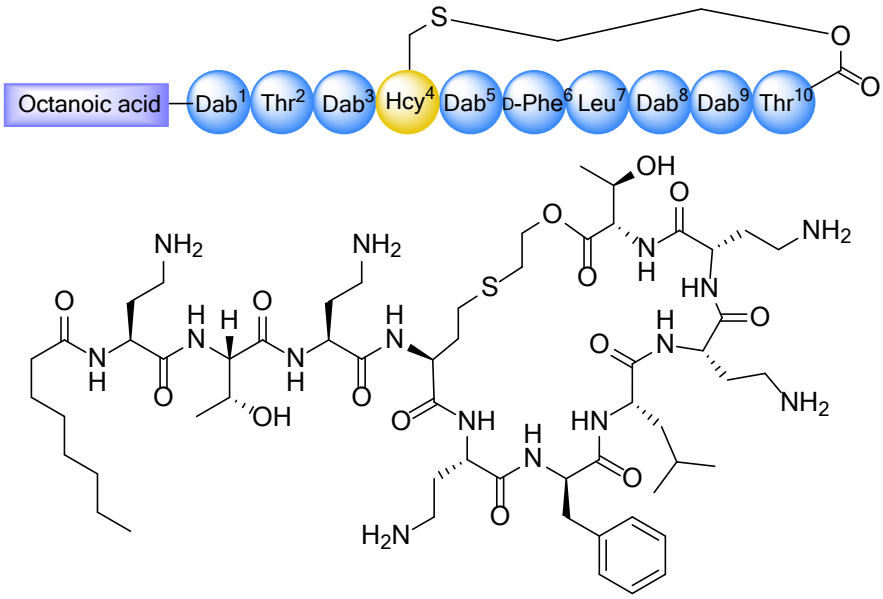


Figure S10. Analytical RP-HPLC and MS traces of purified cyclised D-Cys polymyxin analogue (**2**) (*ca*.99% as judged by peak area of RP-HPLC at 214 nm). t_R 33.01 min cyclised polymyxin; *m/z* 413.0 $[M + 3H]^{3+}$ requires 412.8, *m/z* 618.9 $[M + 2H]^{2+}$ requires 618.8, *m/z* 1236.3 $[M + H]^+$ requires 1235.6 .Mass deconvolution calculated at 1235.70 Da with standard deviation 0.36; theoretical mass calculated at 1236.5 Da. Alliance HPLC by Waters™ Luna® (5µm, C18 100 Å, LC column 250 × 4.6 mm), 1 to 91%B over 90 minutes (*ca.* 1% B/min), 1 mL/min at room temperature, where A: 0.1% TFA in H_2O, and B: 0.1% TFA in acetonitrile (MeCN).

Synthesis of the Hcy Analogue (**3**)



The common fragment Fmoc-Dab(Boc)-DPhe-Leu-Dab(Boc)-Dab(2-CTC PS)-OAll (**S4**) (0.23 mmol, 0.57 mmol/g) underwent *N*α deprotection in accordance with **General Method 1**. Fmoc-Hcy (Trt)-OH was then coupled using **General Method 4**. The subsequent synthetic steps of peptide chain elongation, octanoic acid coupling, allyl deprotection, H_2N-Thr-OVinyl, followed the same procedure as those used for the Cys polymyxin analogue (**1**) affording the crude linear peptide octanoic acid-Dab-Thr-Dab-Hcy-Dab-DPhe-Leu-Dab-Dab-Thr-OVinyl (**6**) (**Figure S6 A**). The thiol-ene induced macrocyclysation of the linear polymyxin analogue (**6**) **f**ollowed the same procedure as those used for the Cys polymyxin analogue (**1**) affording the crude cyclic peptide (**3**) (**Figure S11**).

The crude cyclic peptide (**3**) was purified using a semi preparative Waters 1525 Binary HPLC pump equipped with a Waters 2489 UV/visible detector using a Phenomenex Gemini-NX C18 (110 Å, 5 µm, 10 mm × 250 mm) at a flow rate of 5 mL/min at a gradient of 1-91% B at 1% B/min over 100 min. Fractions were collected and analysed by ESI-MS and analytical RP-HPLC. Fractions which were identified as containing the correct *m/z* at a sufficient purity were combined and lyophilised to afford **3** as a white solid (9 mg, 4.76% yield (based on a 0.149 mmol scale), 99% purity); t_R 31.61 min; *m/z* (ESI-MS) 1250.3 ($[M+H]^+$ requires 1250.4) (**Figure S12**).

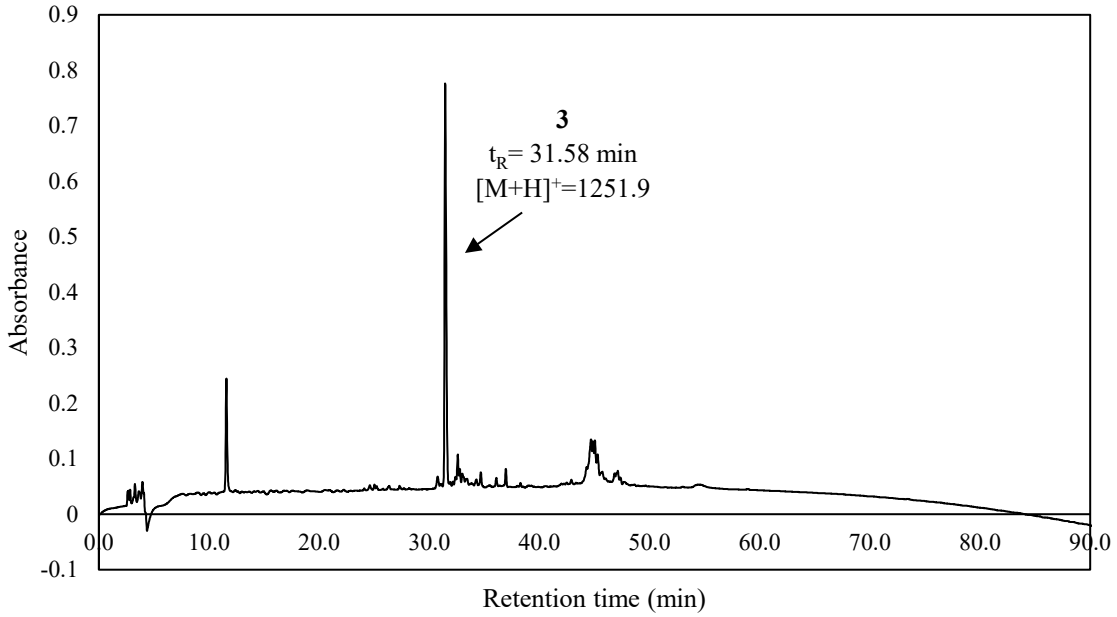


Figure S11. Analytical RP-HPLC of crude cyclised Hcy polymyxin analogue (**3**) (*ca.* 51% as judged by peak area of RP-HPLC at 214 nm). t_R 31.58 min cyclised polymyxin; *m/z* 626.1 $[M + 2H]^{2+}$ requires 625.7, *m/z* 1251.9 $[M + H]^+$ requires 1250.4. Mass deconvolution calculated at 1250.55 Da with standard deviation 0.49; theoretical mass calculated at 1250.57 Da. Alliance HPLC by Waters™ Luna® (5µm, C18 100 Å, LC column 250 × 4.6 mm), 1 to 91%B over 90 minutes (*ca.* 1% B/min), 1 mL/min at room temperature, where A: 0.1% TFA in H_2O, and B: 0.1% TFA in acetonitrile (MeCN).

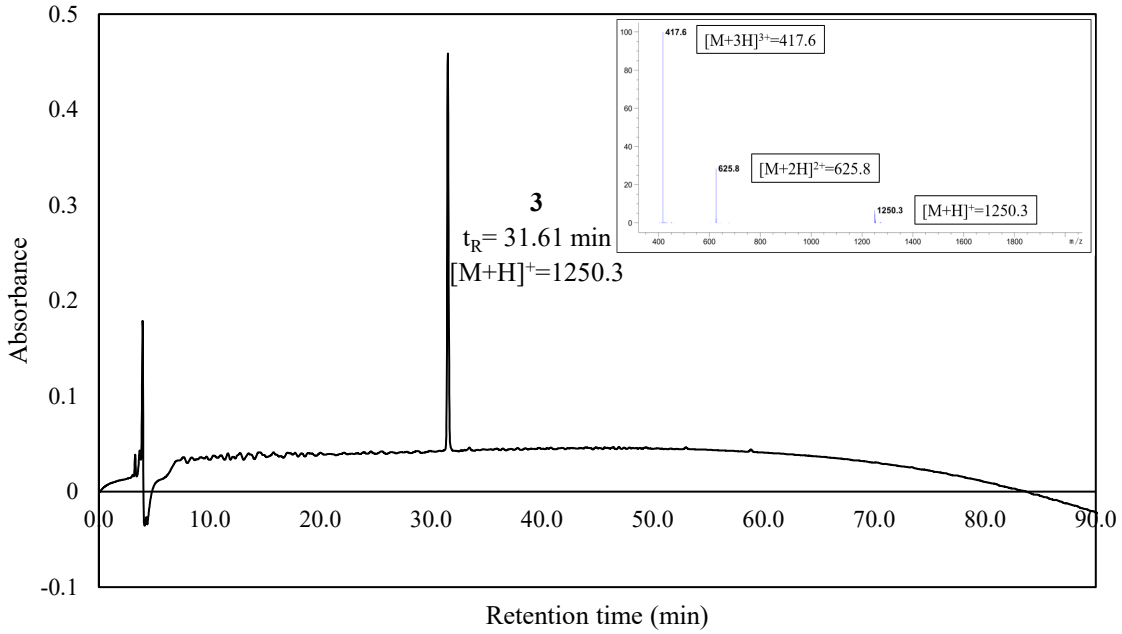


Figure S12. Analytical RP-HPLC and MS traces of purified cyclised Hcy polymyxin analogue (**3**) (*ca.*99% as judged by peak area of RP-HPLC at 214 nm). t_R 31.61 min cyclised polymyxin; *m/z* 417.6 $[M + 3H]^{3+}$ requires 417.5, *m/z* 625.8 $[M + 2H]^{2+}$ requires 625.7, *m/z* 1250.3 $[M + H]^+$ requires 1250.4 . Mass deconvolution calculated at 1249.57 Da with standard deviation 0.25; theoretical mass calculated at 1250.57 Da. Alliance HPLC by Waters™ Luna® (5µm, C18 100 Å, LC column 250 × 4.6 mm), 1 to 91%B over 90 minutes (*ca.* 1% B/min), 1 mL/min at room temperature, where A: 0.1% TFA in H_2O, and B: 0.1% TFA in acetonitrile (MeCN).

S6. Antibacterial Susceptibility Testing.

The MIC of all polymyxin analogues (**1–3**) in this study were determined by broth microdilution according to established methods.[8] Bacteria (listed in **Table S2**) were routinely cultured in cation-adjusted Mueller-Hinton (MH) broth at 37°C. Briefly, two-fold serial dilutions in 50 µL (final concentrations ranging from 32 µg/mL to 0.03125 µg/mL) of polymyxin analogues (**1–3**) or control antibiotics were prepared in polypropylene 96-well plates using MH broth. A bacterial inoculum, equivalent to a 0.5 McFarland turbidity standard (1.5×10^8 CFU/mL), was prepared by suspending fresh bacterial colonies in phosphate-buffered saline. This inoculum was then diluted 1:100 in MH broth, and 50 µL of the diluted suspension was added to each well, resulting in a final volume of 100 µL per well and achieving an initial bacterial inoculum of approximately 5×10^5 CFU/mL. Plates were incubated at 37°C for 16-20 h. The MIC was defined as the lowest concentration at which no visible bacterial growth occurred. Each compound was tested in biological triplicate, and the highest concordant concentration at which no growth was observed across all replicates was reported as the MIC.

Table S2. Minimum Inhibitory Concentrations (MIC) of Cys (**1**), D-Cys (**2**), and Hcy (**3**) polymyxin analogues against Gram-negative Strains of Bacteria.

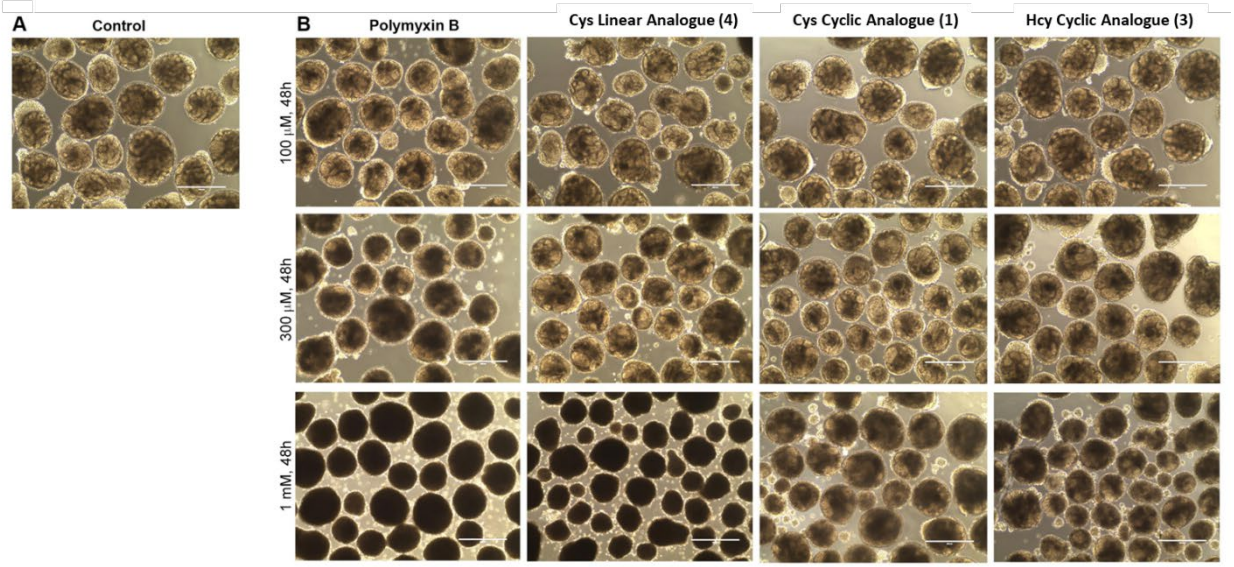
Compound	***E. coli* MS8345**	***A. baumannii* AB5075**	***K. pneumoniae* MS6671**	***E. coli* ATCC 25922**	***A. baumannii* ATCC 19606**	***P. aeruginosa* ATCC 27853**	***K. pneumoniae* ATCC 33495**	***K. pneumoniae* ATCC 700603**
Colistin	4	1	>32	0.5,0.5,1	1	1,2,2	0.5	0.5
Polymyxin B	4	1	>32	0.5	1	2	0.5	1
Meropenem	0.125	>32	>32	0.0312	1	1	0.0625	0.0625
Cys (**1**)	2	>32	>32	0.5	>32	2	1	1
D-Cys (**2**)	>32	>32	>32	>32	>32	>32	32	>32
Hcy (**3**)	2	>32	>32	1	>32	4,8,8	2	4

MICs are reported in µg/mL

S7. Nephrotoxicity Testing

Nephrotoxic effects of control compound polymyxin B and new polymyxin analogues on kidney organoids. (**A**) Bright field images of untreated organoids. (**B**) Organoids treated with 100 µM – 1 mM of polymyxin B and synthetic polymyxin analogues **1**, **3**, **4**. Hallmarks of tissue deterioration (loss of tubular structures, loss of defined edges, floating debris, and dark colouring of the whole organoid indicative of cell death) are observed upon exposure to polymyxin B, compounds **1**, **3**, **4** in a dose-dependent manner. Scale bar, 400 µm.

Figure S13. Bright-field imaging of (**A**) untreated control kidney organoids, and organoids following 48 hr treatment with (**B**) standard polymyxin B, Cys Linear (**4**), Cys Cyclic (**1**), and Hcy Cyclic (**3**), at 100 µM, 300 µM, and 1 mM concentration.

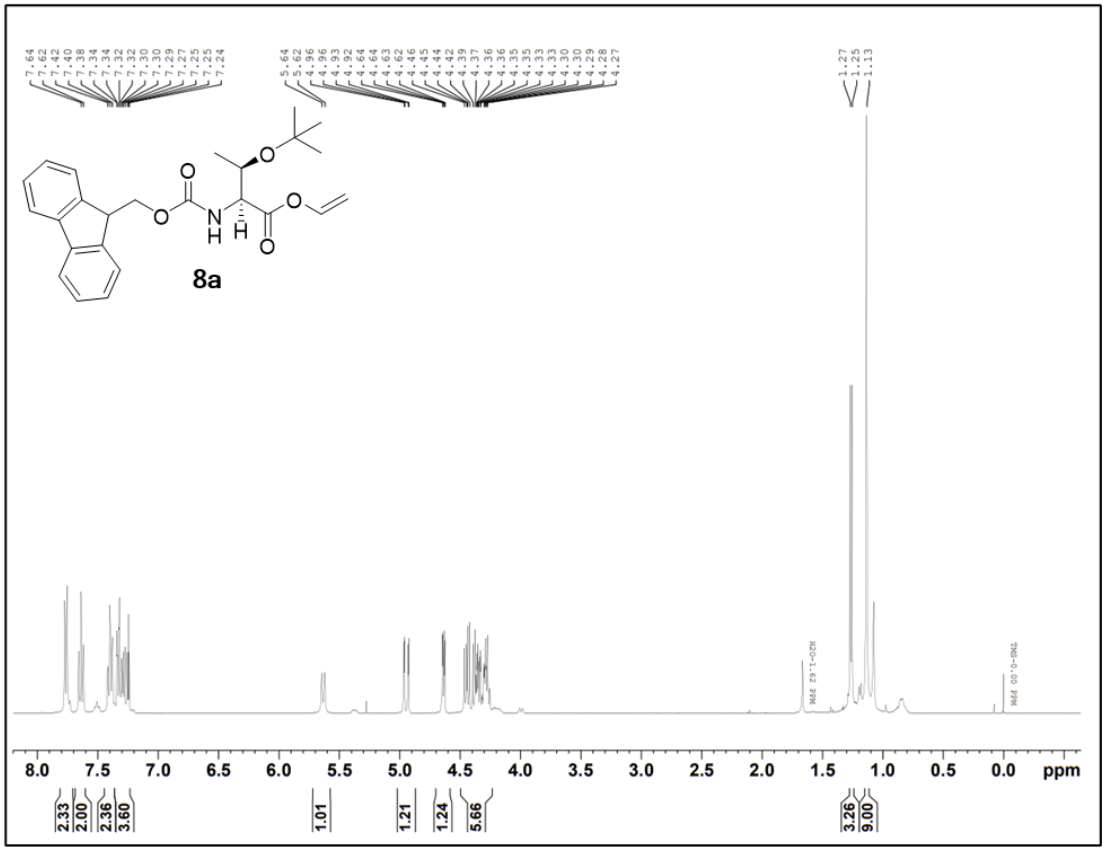


	Polymyxin B	Cys Linear (4)	Cys Cyclic (1)	Hcy Cyclic (3)
-ve control (H_2O)	100 µM	100 µM	100 µM	100 µM
	300 µM	300 µM	300 µM	300 µM
	1 mM	1 mM	1 mM	1 mM

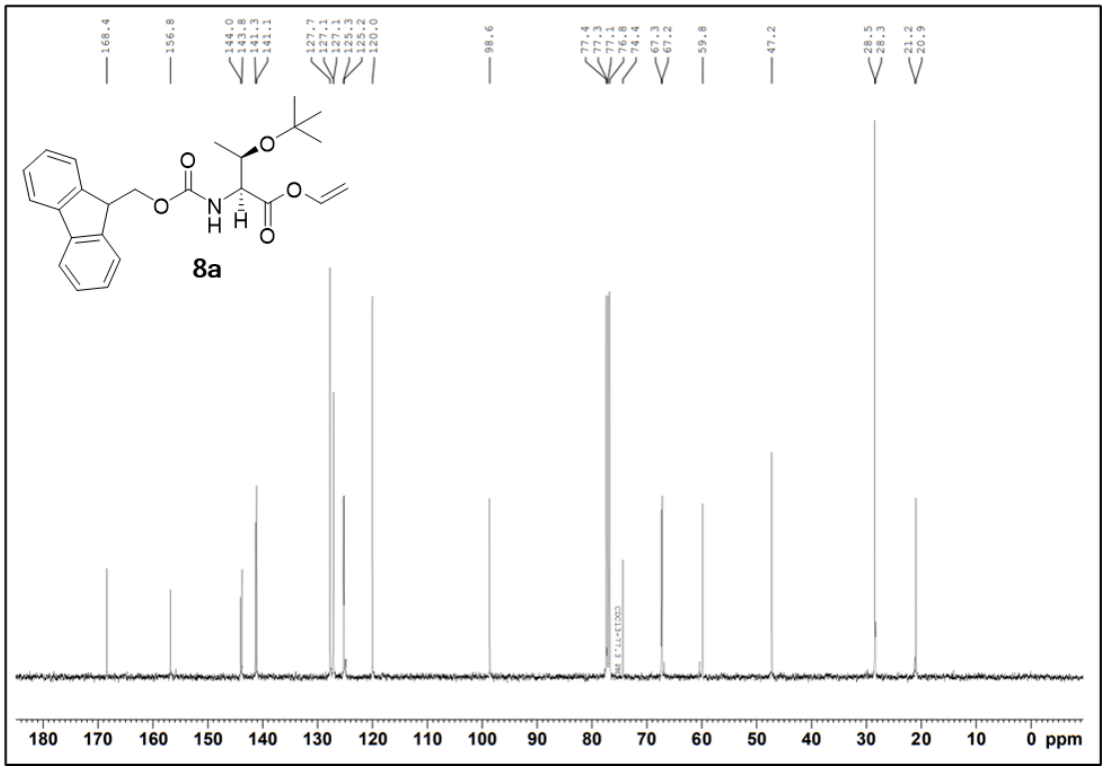
	Unaffected
	Mild Deterioration
	Severe Deterioration
	Complete Deterioration

S8. Appendix NMR and HRMS Data

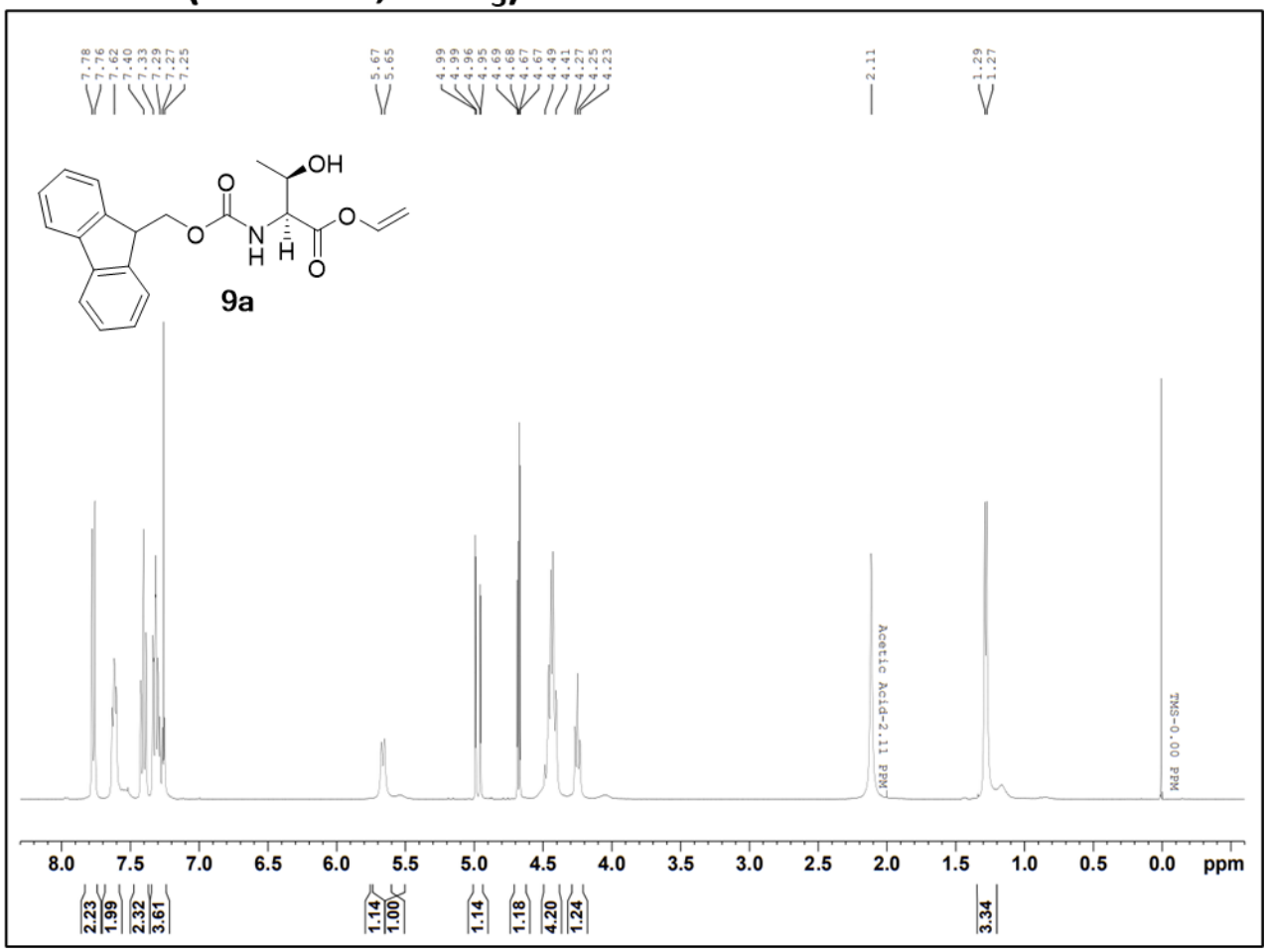
^{1}H NMR (400 MHz, $CDCl_3$)



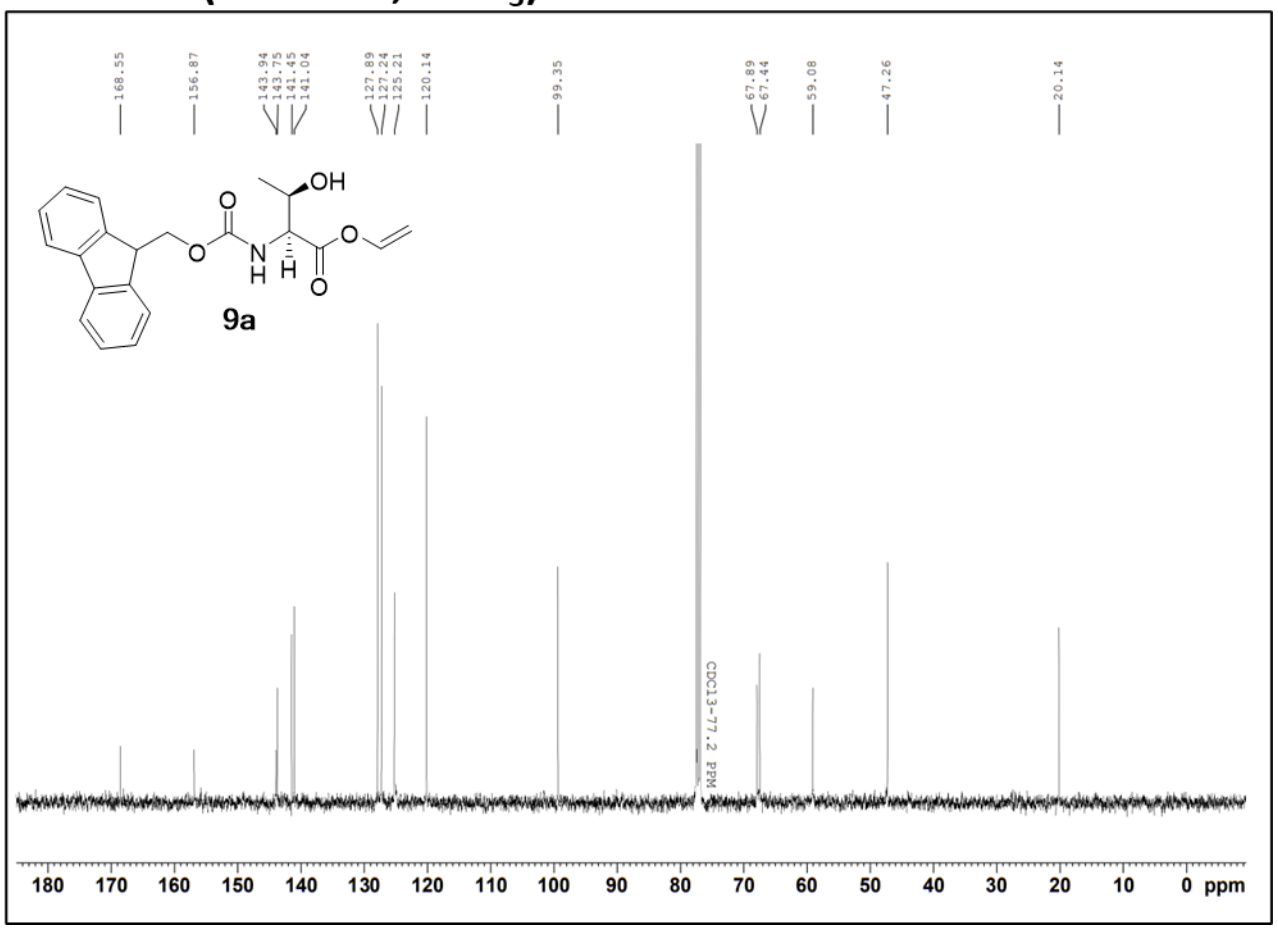
^{13}C NMR (100 MHz, $CDCl_3$)



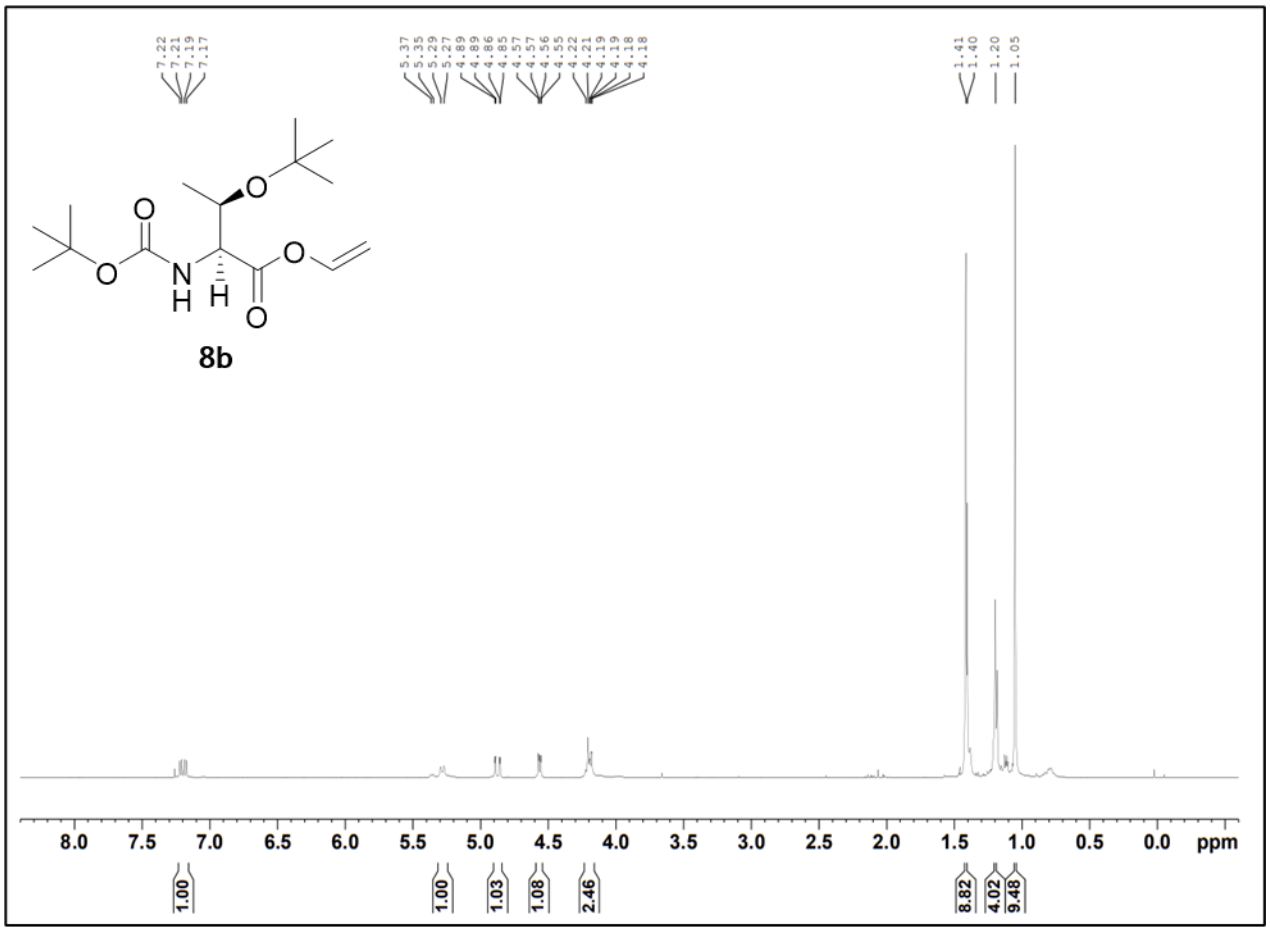
^{1}H NMR (400 MHz, $CDCl_3$)



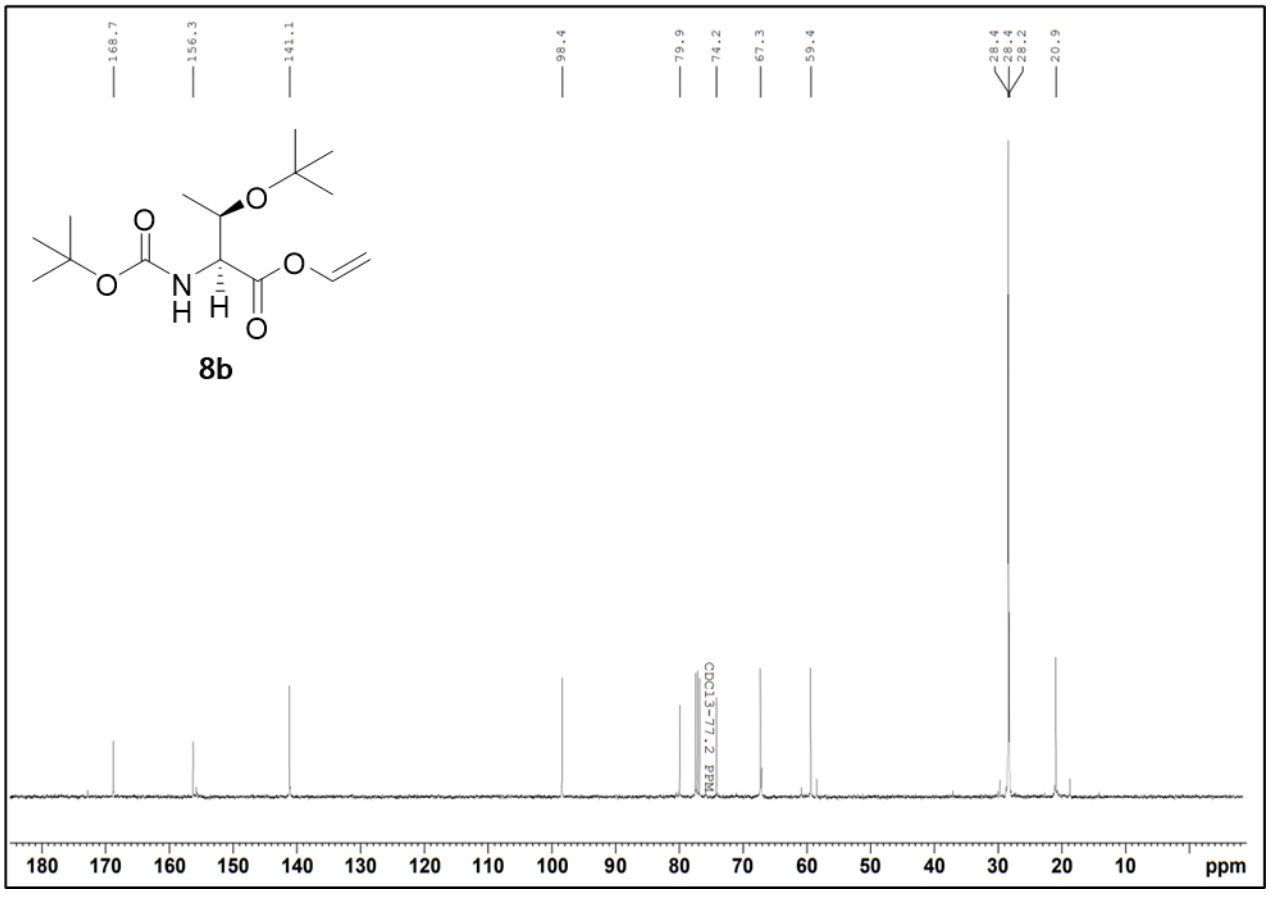
^{13}C NMR (100 MHz, $CDCl_3$)



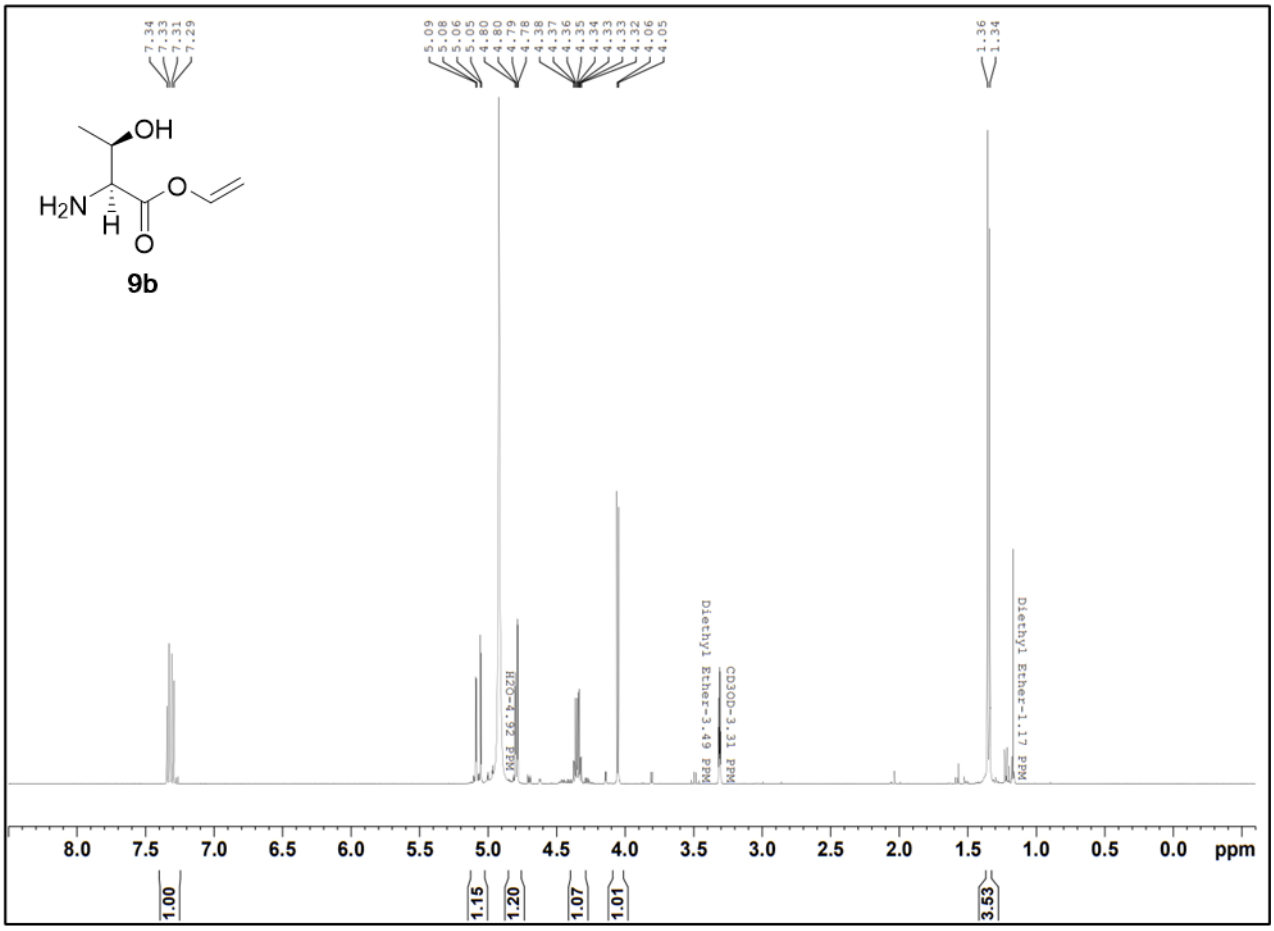
^{1}H NMR (400 MHz, $CDCl_3$)



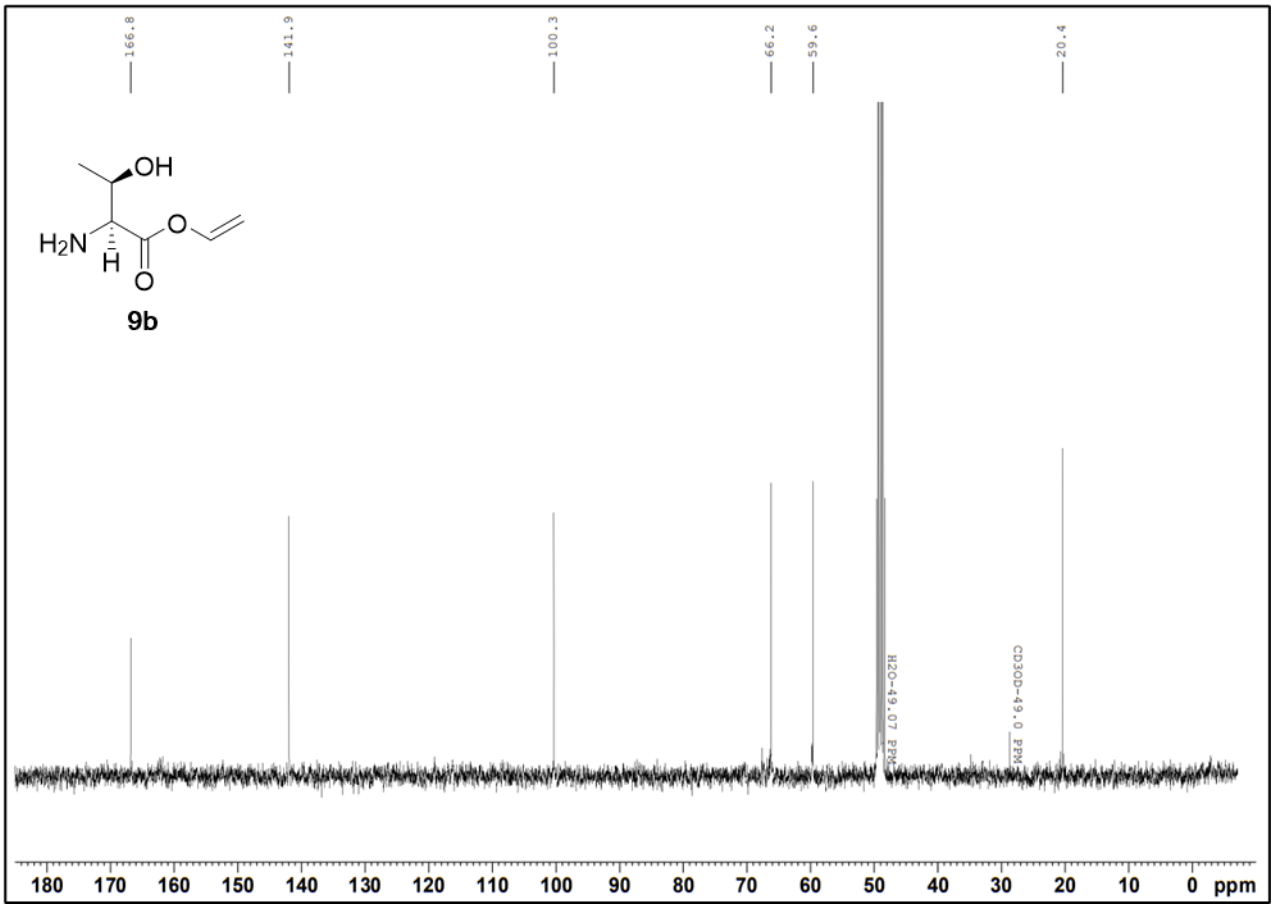
^{13}C NMR (100 MHz, $CDCl_3$)

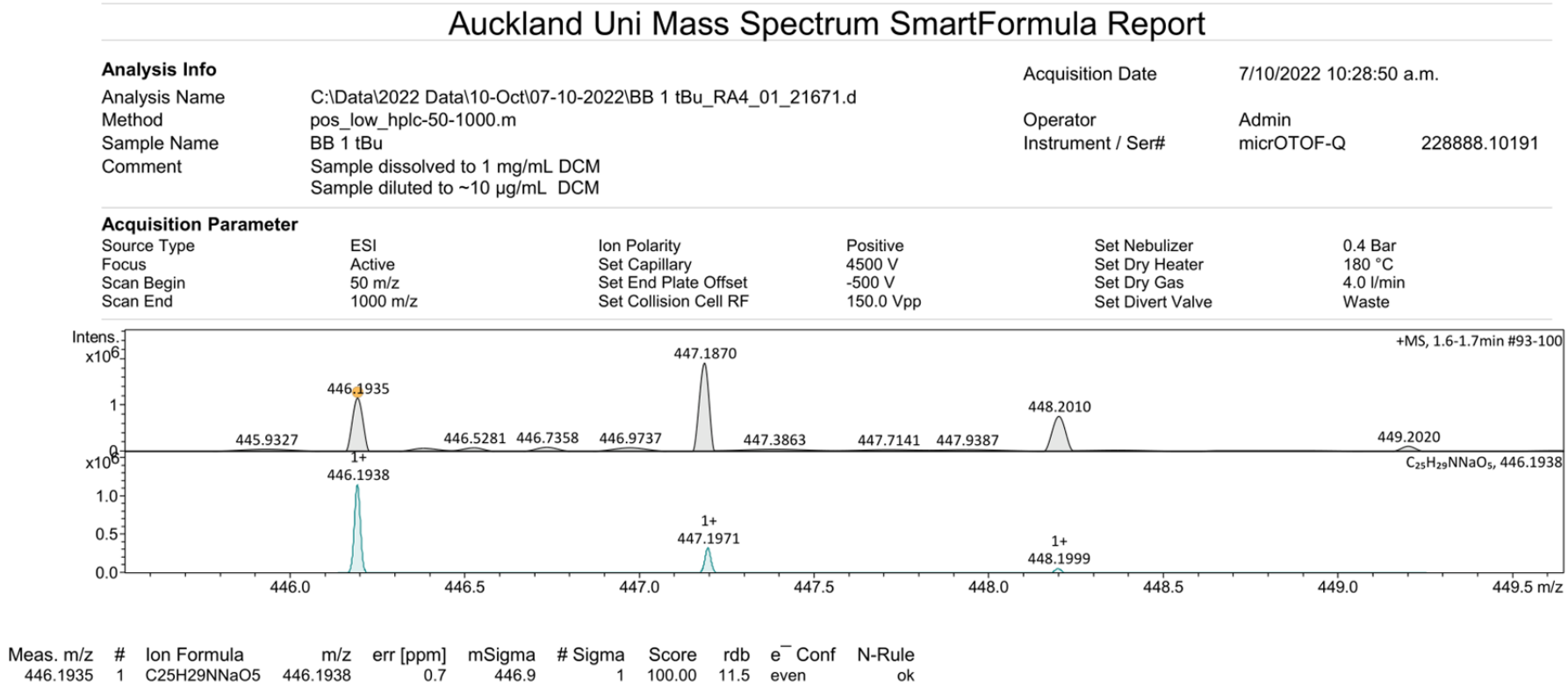


^{1}H NMR (400 MHz, $CDCl_3$)



^{13}C NMR (100 MHz, CD3OD)





Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e⁻ Conf	N-Rule
446.1935	1	C25H29NNaO5	446.1938	0.7	446.9	1	100.00	11.5	even	ok

Figure S14. High resolution mass spectrometry (HRMS, ESI/Q-TOF) positive-ion spectra of Fmoc-Thr(^{t}Bu)-vinyl (**8a**). *m/z*: $[M + Na]^+$ calcd: for $C_{25}H_{29}NNaO_5$: 446.1938; found 446.1944

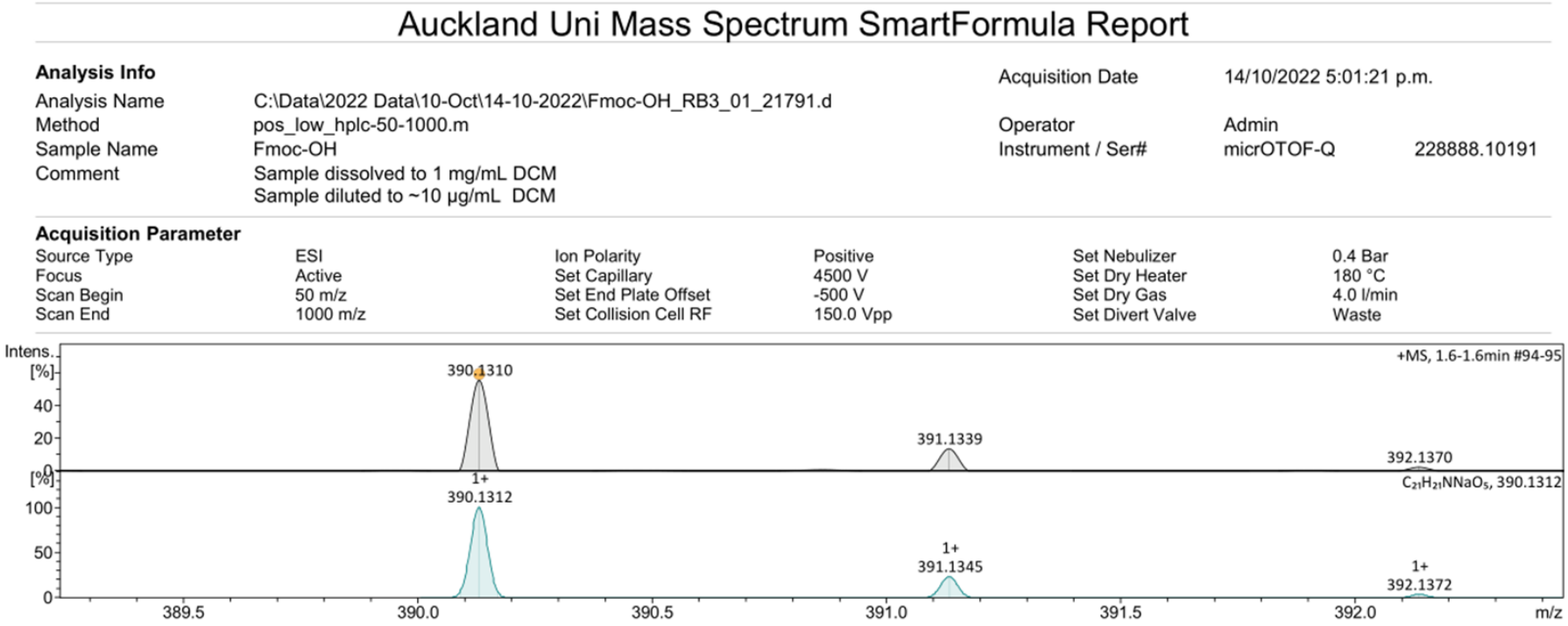


Figure S15. High resolution mass spectrometry (HRMS, ESI/Q-TOF) positive-ion spectra of Fmoc-Thr(OH)-vinyl (**9a**) *m/z*: $[M + Na]^+$ calcd: for $C_{21}H_{21}NNaO_5$: 390.1312; found 390.1309.

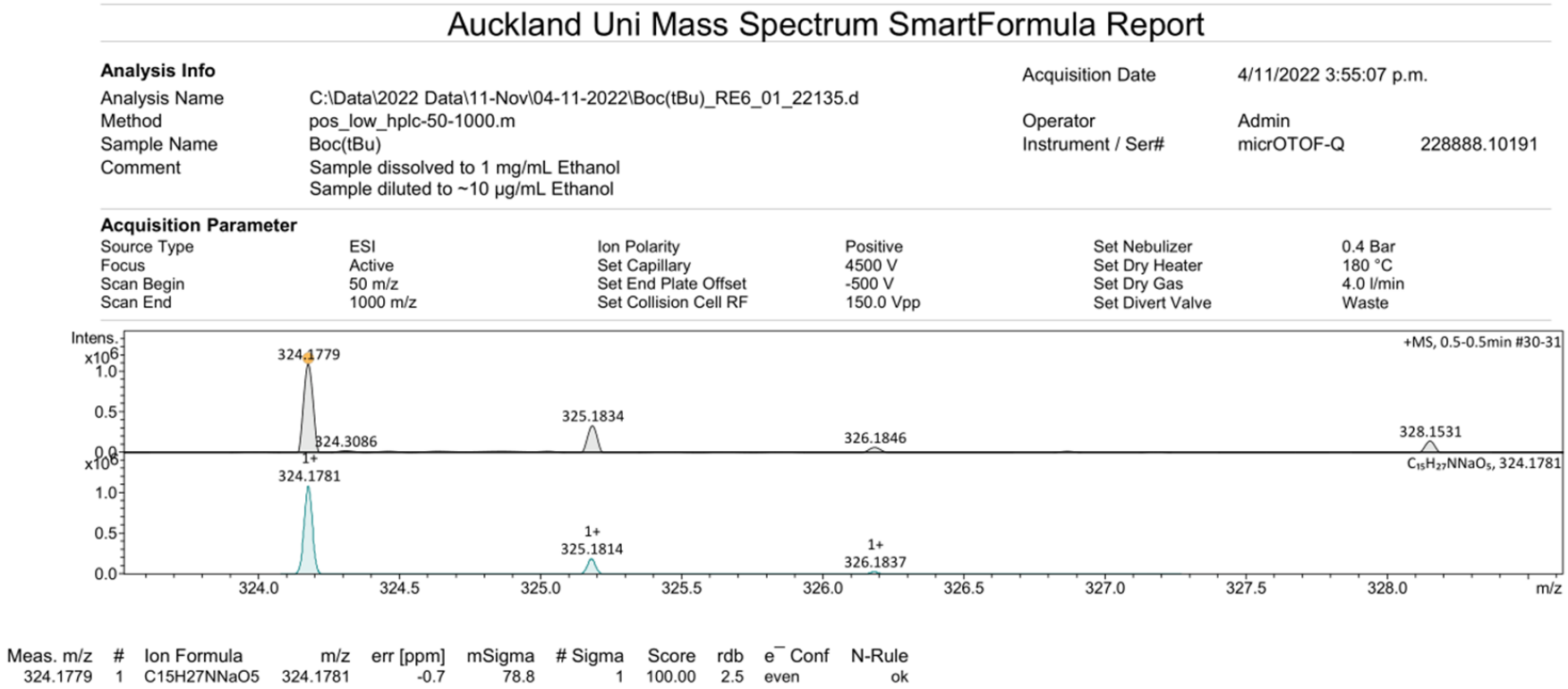
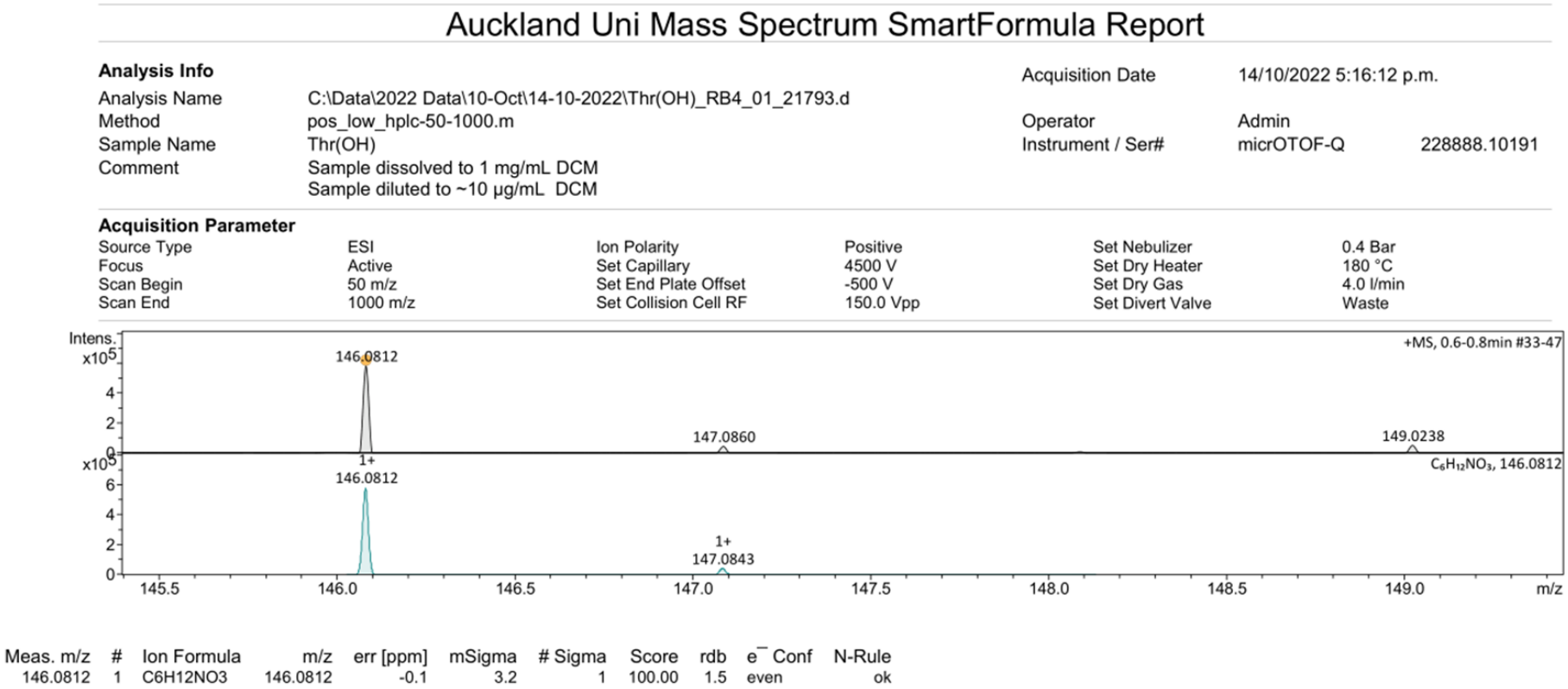


Figure S16. High resolution mass spectrometry (HRMS, ESI/Q-TOF) positive-ion spectra of Boc-Thr(tBu)-vinyl (**8b**). *m/z*: $[M + Na]^+$ calcd: for $C_{15}H_{27}NNaO_5$: 324.3728; found 324.1781.



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e⁻ Conf	N-Rule
146.0812	1	C6H12NO3	146.0812	-0.1	3.2	1	100.00	1.5	even	ok

Figure S17. High resolution mass spectrometry (HRMS, ESI/Q-TOF) positive-ion spectra of Thr(OH)-vinyl (**9b**). *m/z*: $[M + Na]^+$ calcd: for $C_6H_{12}NO_3$:146.0739; found 146.0812.

S9. Additional References

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