Supporting Information

Introducing CLipPA Lipid Chemodiversity to Truncated Polymyxin B: A Soft Drug Strategy to Combat Gram-Negative Pathogens

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General Information

All solvents and reagents were used as supplied, unless otherwise noted. Solvents for RP-HPLC were purchased as RP-HPLC grade and used without further purification. Polymyxin B sulfate, methoxyamine hydrochloride ($CH_3ONH_2 \cdot HCl$), benzotriazole-1-yl-oxy-tris-pyrolidino-phosphonium hexafluorophosphate (PyBOP), triisopropylsilane (TIPS), 1,4-dithiothreitol (DTT), 2-methylbutyric acid, Boc-Cys(Trt)-OH (Boc = *tert*-butoxycarbonyl and Trt = trityl), Boc-Thz-OH (Thz = thiazolidine) and 2-(*tert*-butyloxycarbonyl-oxyimino)-2-phenylacetonitrile (Boc-ON) were purchased from AK scientific (Union city, CA, USA). Boc-D-Cys(Trt)-OH was purchased from Chempep (Florida, USA). 1-[*bis*(Dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinum *N*-oxide hexafluorophosphate (HATU) was purchased from AAPPTec (Louisville, KY, USA). Petroleum ether was purchased from Macron Fine Chemicals™ (Radnor, PA, USA). Papain from papaya latex (crude powder, 1.5–10 units/mg solid, unit definition:1 µmol of N^{α}-benzoyl-L-Arg-ethyl ester [BAEE] is hydrolysed at pH 6.2 and 25 °C per min by 1 unit of enzyme) was purchased from Sigma Aldrich (St Louis, MO, USA) and homogenised in a mortar and pestle. *N*,*N*'-Diisopropylcarbodiimide (DIC), *N*,*N*-diisopropylethylamine (DIPEA), 2,2-dimethoxy-2-phenylacetophenone (DMPA), hydroxylamine hydrochloride ($NH_2OH \cdot HCl$), *N*-methylpyrrolidone (NMP), triethylamine (Et_3N), formic acid (FA), palladium (II) acetate [$[Pd(OAc)_2]_3$], Val-Tyr-Val (VYV), vinyl acetate, vinyl propionate, vinyl butyrate, vinyl valerate, vinyl decanoate, vinyl pivalate, vinyl benzoate, vinyl 4-*t*Bu-benzoate, cyclopropanecarboxylic acid, cyclobutanecarboxylic acid, cyclopentanecarboxylic acid, cyclohexanecarboxylic acid, 3-methylbutyric acid, 2,2-dimethylbutyric acid, 2-methylvaleric acid, 3-methylvaleric acid, 4-methylvaleric acid, trichloroacetic acid (TCA), *tert*-nonyl mercaptan, Celite®, silica gel, ammonium hydroxide (NH_4OH) and human serum from human male AB plasma were purchased from Sigma Aldrich (St Louis, MO, USA). Vinyl hexanoate, vinyl octanoate and vinyl laurate were purchased from TCI America (Portland, OR, USA). Sodium phosphate dibasic (Na_2HPO_4) was purchased from VWR (Radnor, PA, USA). Chloroform-d ($CDCl_3$) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Dulbecco's phosphate-buffered saline (DPBS) was purchased from Thermo Fisher Scientific. 1-Hydroxy-7-azabenzotriazole (HOAt), dichloromethane (CH_2Cl_2), methanol (MeOH), ethanol (EtOH), ethyl acetate (EtOAc), magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), calcium chloride ($CaCl_2$),

hydrochloric acid (HCl), sodium hydroxide (NaOH) and potassium hydroxide (KOH) were purchased from ECP (Northcote, Auckland, NZ). Acetonitrile (MeCN, HPLC grade) and methanol (MeOH, Optima™ LC/MS grade) were purchased from Fisher Scientific (Waltham, MA, USA). *N*,*N*-dimethylformamide (DMF, AR grade) and diethyl ether (Et_2O) were purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). Trifluoroacetic acid (TFA) was purchased from Sigma Aldrich (St Louis, MO, USA) or Oakwood chemicals (Estill, SC, USA). Milli-Q water was dispensed from Sartorius (Göttingen, Germany) Arium® Pro, supplied with the resistivity of 18.2 MΩ·cm.

Yields calculated as a percentage (% yield) refer to chromatographically homogenous materials (≥95 %) unless otherwise stated. Analytical high performance liquid chromatography (HPLC) chromatograms were acquired on either a Dionex (Dionex Corporation, Sunnyvale, CA, USA) UltiMate 3000 HPLC system (spectra provided at 210 nm), or a Waters (Waters Corporation, Milford, MA, USA) Alliance analytical RP-HPLC equipped with a Waters 2998 PDA detector or a Waters 2489 UV/Vis detector (spectra provided at 214 nm). A linear gradient of 5–95 % B (unless otherwise stated) was used to elute peptides where Solvent A = MQ H_2O containing 0.1 % TFA and Solvent B = MeCN containing 0.1 % TFA (unless otherwise stated). Mass spectra (ESI-MS) were obtained with either a Waters Quattro micro API mass spectrometer or an Agilent (Agilent Technologies, Santa Clara, CA, USA) 6120 Quadrupole LC/MS Series mass spectrometer, both operated in ESI+ve mode. Semipreparative RP HPLC was performed on a Dionex UltiMate 3000 HPLC system using an appropriate linear gradient based on analytical HPLC profiles. Hydrolytic stability was monitored on an Agilent (Agilent Technologies, Santa Clara, CA, USA) 1260 Infinity II LC/MS system equipped with G7115A DAD and G6135B MSD XT. Phenex syringe filters (26 mm, 0.45 µm) used in the filtration of crude peptides prior to HPLC injection were purchased from Phenomenex (Torrance, CA, USA). The photochemical apparatus used for photoinitiation of CLipPA was a Spectroline EA-160 UV (365 nm) lamp purchased from Spectronics Corporation (Melville, NY, USA). ^{1}H and ^{13}C nuclear magnetic resonance experiments (^{1}H and ^{13}C NMR) were performed with a Bruker (Billerica, MA, USA) AVANCE 400 MHz (^{1}H 400 MHz, ^{13}C 100 MHz) spectrometer. Chemical shifts were recorded in parts per million (ppm) and referenced to the residual solvent peak for trimethylsilane (TMS) δ = 0.00 ppm for ^{1}H NMR and referenced to chloroform-d ($CDCl_3$) δ = 77.16 ppm for ^{13}C NMR. Infrared (IR) spectra

were obtained using a Perkin Elmer (Waltham, MA, USA) Spectrum 100 FT-IR spectrometer on a film ATR sampling accessory. Mueller-Hinton Broth (MHB) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Shimadzu (Kyoto, Japan) UV-1280 UV-Vis spectrophotometer was used to measure the optical density of bacterial culture in media at 600 nm. Polypropylene 96 well microplates (flat-bottom) were purchased from Greiner Bio-One (Kremsmünster, Austria). Kidney organoids were cultured in Stage II medium.[24] Nephrotoxicity assays were performed in 24 well ultra-low attachment plates (Corning® Costar®, Corning, NY, USA). Organoids were imaged on an EVOS® XL imaging system (Thermo Fisher Scientific, Waltham, MA, USA).

General Methods

General Method A: Peptide Purification and Final Yield Calculation

The resultant crude peptide dissolved and filtered in an appropriate mixture of H_2O/MeCN containing 0.1 % TFA (*v*/*v*) was eluted through a Phenomenex Gemini C18 column (110 Å, 250 mm × 10.0 mm; 5 µm) or Phenomenex Luna C18 column (100 Å, 250 mm × 10.0 mm; 5 µm) *via* a linear gradient (1 % B per min) with Solvent A and B consisting of MQ H_2O and MeCN, respectively, containing 0.1 % (*v*/*v*) TFA. Purity was confirmed by analytical HPLC (210 nm or 214 nm), in all cases final compounds were obtained in ≥95 % purity. Final % yields of purified semi-synthetic peptides were calculated over all enzymatic and chemical reaction steps (5–6 steps total) considering the original amount (mol) of starting material submitted at the start of synthesis and subsequent divisions.

General Method B: Column-Phase Enrichment

The resultant crude peptide was dissolved and filtered in an appropriate mixture of H_2O/MeCN containing 0.1 % TFA (*v*/*v*) and eluted through a Phenomenex Gemini C18 column (110 Å, 250 mm × 10.0 mm; 5 µm) or Phenomenex Luna C18 column (100 Å, 250 mm × 10.0 mm; 5 µm) *via* a linear gradient (1 % B per min) with Solvent A and B consisting of MQ H_2O and MeCN, respectively, containing 0.1 % (*v*/*v*) TFA. A crude enrichment of the peptide intermediate was undertaken by collecting a broad range of combined peaks containing the compound of interest, to achieve desalting or extraction of the compound from any impurity (e.g., PMB, **1**, carried from enzymatic

cleavage) that could interfere with the product isolation after the final CLipPA step, due to co-elution.

General Method C: Pd-Catalysed Vinyl Ester Formation

Performed according to the procedure of Mastihubová and Mastihuba[35].

Carboxylic acid (1 equiv.) was dissolved in vinyl acetate (80–150 mL), to which $[Pd(OAc)_2]_3$ (0.156 equiv.) and KOH (0.1 equiv.) were added. The resultant mixture was left to stir for 18 h at rt, filtered through a layer of Celite® and concentrated *in vacuo*. The crude product was purified by flash chromatography (Et_2O/pet. ether, 1:19, *v*/*v*) to give the isolated vinyl ester.

General Method D: Conjugation of Vinyl Esters to Thiol-Bearing Peptides (CLipPA)

Performed according to the procedure of Brimble and co-workers[28,41].

In NMP degassed by sparging with argon, were dissolved the crude or enriched Cys-coupled PMBN (1 equiv.; either **(a)** the number of moles (mol) of crude material was deduced based on that of starting material (PMB [**1**] submitted at the start of the synthesis) and subsequent divisions in the following steps, or **(b)** enriched material was assumed to be pure and thus, the number of moles (mol) was calculated based on the molecular weight (g/mol) and measured mass (g) of the resulting compound) and the reagents including vinyl ester (70 equiv.), *tert*-nonyl mercaptan (80 equiv.), TIPS (80 equiv.), DMPA (2 equiv.) and TFA (5 % of total solvent volume). The resultant mixture was irradiated with UV light (λ = 365 nm) for 40 min to 1 h 20 min at rt. Subsequently, the crude product was triturated in cold Et_2O (3 × 40 mL, 4 °C) and purified according to **General Method A** to afford the isolated final semi-synthetic peptides upon lyophilisation.

Evaluation of Antimicrobial Activity (MIC) towards Gram-Negative Bacteria

MIC assays were performed as previously described:[11]

Briefly, MIC assays and compound preparation were carried out according to CLSI guidelines for broth microdilution in cation-adjusted MHB with the median concentration of compound resulting in the complete inhibition of bacterial growth being determined as the MIC.[42]

Nephrotoxicity Assays towards Human Kidney Organoids

Nephrotoxicity assays were performed as previously described:[11]

Briefly, Kidney organoids were prepared according to the protocol by Sander *et al*.[24], from the human MANZ-2 and MANZ-4 induced pluripotent stem cell lines (Oh *et al*.[43]). The organoids were treated with peptide samples on day 12 of organoid development and nephrotoxicity was scored after 48 h, according to Harris *et al*.[18].

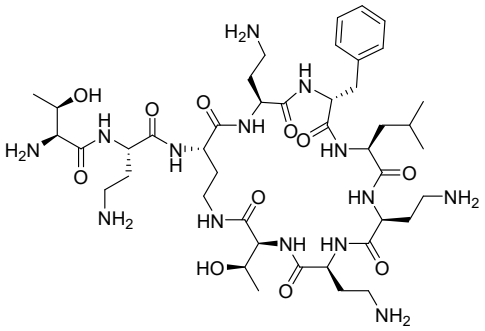
Stability Experiments in Human Serum and DPBS

Stability experiments were performed as previously described:[18]

Briefly, peptides were incubated over 24 h at 37 °C in each of DPBS and human serum diluted with DPBS. Aliquots were taken at three timepoints (0, 4, and 24 h) which were appropriately treated according to Harris *et al*.[18], lyophilised and hydrolysis monitored by LCMS.

S1. Preparation of Boc-protected polymyxin B nonapeptide intermediate

Enzymatic Cleavage of Polymyxin B Sulfate



2

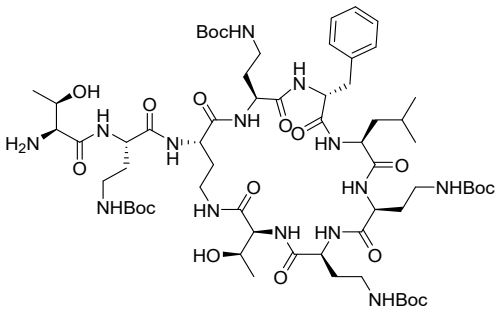
Enzymatic cleavage of commercially obtained polymyxin B sulfate (**1**) performed following the procedure in our previous report[11], modified from the procedure reported by Danner *et al*.[19].

Briefly, polymyxin B sulfate (**1**, 1 equiv.) was dissolved in 0.1 M phosphate buffer (pH 6.8, see **Table S1**). Crude papain extract (1.5–10 units/mg, enzyme:substrate, 1:18–25, *w*/*w*) and DTT (1:1 to papain, *w*/*w*) were added to above solution. The reaction was warmed to 37 °C and agitated for 18–48 h. The resultant mixture was centrifuged, filtered, dissolved in MQ H_2O and lyophilised to give crude peptide **2** which was directly used in the next step without further purification.

Table S1: Reaction conditions for enzymatic cleavage of **1**.

Repeat	Reagent and solvent				Crude yield (g)
	1 (mg, µmol)	**0.1 M Phosphate buffer (mL)**	**Papain (mg)**	**DTT (mg, µmol)**	
1	530, 383	37	21.2	21.2, 137	1.28
2	200, 144	14	8	8, 51.9	0.421
3	245, 177	17	13.6	13.6, 88.2	0.525
4	700, 505	49	34	34, 220	1.49

Synthesis of Tetrakis-*N*$^{\gamma}$-Boc-Polymyxin B Nonapeptide (3)



3

Selective tetra-*N*$^{\gamma}$-Boc-protection of polymyxin B nonapeptide (**2**) performed following the procedure in our previous report[11], modified from the procedures reported by O'Dowd *et al*.[26] and Hamill *et al*.[27].

Crude peptide **2** (1 equiv.) and Boc-ON (4 equiv.) were dissolved in MeOH/H_2O (2:1, *v*/*v*, see table below) to which Et_3N (15 equiv.) was added and the mixture agitated for 30 min at rt, Boc-ON (0.5 equiv.) was added portionwise every 30 min until complete conversion was observed upon monitoring by analytical RP-HPLC (5–95% B, 3% B/min, 1 mL/min), with peak identity confirmed by ESI-MS. Upon completion, the reaction was quenched with aq. NH_3 (30 %, *v*/*v*, see **Table S2**), split into smaller batches (10–15 mL) for work up, and concentrated under a stream of N_2. Each batch was then triturated in Et_2O/pet. ether (3 × 40 mL, 4 °C, 2:1, *v*/*v*). Subsequently, the crude material was dissolved in 0.1 % TFA (*v*/*v*) in H_2O/MeCN (1:1, *v*/*v*), combined and lyophilised to give product **3** which was used in the subsequent reaction without further purification.

Table S2: Reaction conditions for tetra-Boc protection of **2**.

Repeat		Reagent and solvent					Crude yield (mg)
		2 (mg, µmol)[a]	**Boc-ON (mg, mmol)[b]**	**2:1 MeOH/H_2O (mL)**	**Et_3N (µL, mmol)**	**30 % aq. NH_3 (mL)**	
1*	**a**	200, 59.8	58,9, 0.239	9	125, 0.897	0.2	214
	b	200, 59.8	58.9, 0.239	9	125, 0.897	0.2	192
	c	92.2, 27.6	27.1, 0.11	4	57.7, 0.414	0.1	85
	d	200, 59.8	58.9, 0.239	9	125, 0.897	0.2	116
2		421, 144	142, 0.576	18	301, 2.16	0.4	400

3	525, 177	174, 0.708	21	371, 2.66	0.5	691
4	1490, 505	497, 2.02	45	1.06, 7.58	1.5	1570

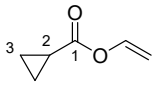
[a] The number of moles (μmol) of crude starting material **2** was deduced based on that of polymyxin B sulfate submitted in the previous step and subsequent divisions in the current step.

[b] Indicates the initial amount of Boc-ON added before the subsequent portionwise additions. Added more as needed, usually requires 0.5–1 more equiv. for satisfactory conversion.

***** The crude starting material was divided into portions **a**–**d**.

S2. Vinyl ester synthesis

Synthesis of Vinyl Cyclopropionate (9a)

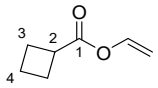


9a

Cyclopropanecarboxylic acid (1.19 mL, 15 mmol), $[Pd(OAc)_2]_3$ (525 mg, 2.34 mmol) and KOH (84.2 mg, 1.5 mmol) were dissolved in vinyl acetate (150 mL). Vinyl ester formation was performed according to **General Method C** to give the title compound **9a** (538 mg, 32.0 % yield) as a liquid; **IR** (film) ν_{max} 2924, 2865, 2845, 1749, 1648, 1158 cm^{-1}; **^{1}H NMR** (400 MHz; $CDCl_3$): δ 7.28 (dd, 1H, *J* = 14.2 Hz and 6.2 Hz, CO_2CHCH_2), 4.88 (dd, 1H, *J* = 14.1 Hz and 1.1 Hz, CO_2CHCH_2), 4.54 (dd, 1H, *J* = 6.0 Hz and 1.5 Hz, CO_2CHCH_2), 1.70–1.63 (m, 1H, H-2), 1.10-0.92 (m, 4H, H-3); **^{13}C NMR** (100 MHz; $CDCl_3$): δ 172.1 (C-1), 141.4 (CO_2CHCH_2), 97.2 (CO_2CHCH_2), 12.6 (C-2), 9.1 (C-3).

HRMS data could not be obtained due to the incompatibility caused by compound volatility.

Synthesis of Vinyl Cyclobutyrate (9b)

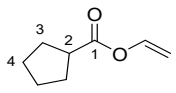


9b

Cyclobutanecarboxylic acid (1.43 mL, 15 mmol), $[Pd(OAc)_2]_3$ (525 mg, 2.34 mmol) and KOH (84.2 mg, 1.5 mmol) were dissolved in vinyl acetate (150 mL). Vinyl ester formation was performed according to **General Method C** to give the title compound **9b** (1433 mg, 75.7 % yield) as a liquid; **IR** (film) ν_{max} 2992, 2951, 2872, 1748, 1646, 1135 cm^{-1}; **^{1}H NMR** (400 MHz; $CDCl_3$): δ 7.29 (dd, 1H, *J* = 14.2 Hz and 6.2 Hz, CO_2CHCH_2) 4.88 (dd, 1H, *J* = 14.0 Hz and 1.5 Hz, CO_2CHCH_2), 4.56 (dd, 1H, *J* = 6.2 Hz and 1.7 Hz, CO_2CHCH_2), 3.25–3.16 (m, 1H, H-2), 2.39–1.88 (m, 6H, H-3, H-4); **^{13}C NMR** (100 MHz; $CDCl_3$): δ 172.7 (C-1), 141.5 (CO_2CHCH_2), 97.6 (CO_2CHCH_2), 37.9 (C-2), 25.2 (C-3), 18.6 (C-4).

HRMS data could not be obtained due to the incompatibility caused by compound volatility.

Synthesis of Vinyl Cyclovalerate (9c)

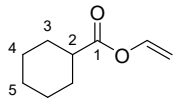


9c

Cyclopentanecarboxylic acid (1.63 mL, 15 mmol), $[Pd(OAc)_2]_3$ (525 mg, 2.34 mmol) and KOH (84.2 mg, 1.5 mmol) were dissolved in vinyl acetate (150 mL). Vinyl ester formation was performed according to **General Method C** to give the title compound **9c** (1134 mg, 53.9 % yield) as a liquid; **IR** (film) ν_{max} 2961, 2874, 1748, 1646, 1135 cm^{-1}; **^{1}H NMR** (400 MHz; $CDCl_3$): δ 7.29 (dd, 1H, *J* = 13.9 Hz and 6.5 Hz, CO_2CHCH_2) 4.88 (dd, 1H, *J* = 14.0 Hz and 1.5 Hz, CO_2CHCH_2), 4.56 (dd, 1H, *J* = 6.5 Hz and 1.5 Hz, CO_2CHCH_2), 2.80 (tt, 1H, *J* = 8.5 Hz and 7.4 Hz, H-2), 1.98–1.55 (m, 8H, H-3 and H-4); **^{13}C NMR** (100 MHz; $CDCl_3$): δ 174.0 (C-1), 141.6 (CO_2CHCH_2), 97.4 (CO_2CHCH_2), 43.7 (C-2), 30.0 (C-3), 26.0 (C-4).

HRMS data could not be obtained due to the incompatibility caused by compound volatility.

Synthesis of Vinyl Cyclohexanoate (9d)

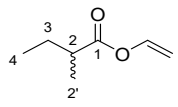


9d

Cyclohexanecarboxylic acid (1.24 mL, 10 mmol), $[Pd(OAc)_2]_3$ (350 mg, 1.56 mmol) and KOH (56.1 mg, 1 mmol) were dissolved in vinyl acetate (80 mL). Vinyl ester formation was performed according to **General Method C** to give the title compound **9d** (730 mg, 47.3 % yield) as a liquid; **IR** (film) ν_{max} 2934, 2858, 1748, 1646, 1156, 1138, 1121 cm^{-1}; **^{1}H NMR** (400 MHz; $CDCl_3$): δ 7.21 (dd, 1H, *J* = 14.0 Hz and 6.5 Hz, CO_2CHCH_2) 4.79 (dd, 1H, *J* = 14.0 Hz and 1.5 Hz, CO_2CHCH_2), 4.47 (dd, 1H, *J* = 6.4 Hz and 1.4 Hz, CO_2CHCH_2), 2.29 (tt, 1H, *J* = 11.2 Hz and 3.6 Hz, H-2), 1.88–1.11 (m, 10H, H-3, H-4 and H-5); **^{13}C NMR** (100 MHz; $CDCl_3$): δ 173.1 (C-1), 141.4 (CO_2CHCH_2), 97.3 (CO_2CHCH_2), 42.9 (C-2), 28.8 (C-3), 25.7 (C-5), 25.4 (C-4).

HRMS data could not be obtained due to the incompatibility caused by compound volatility.

Synthesis of Vinyl 2-Me-Butyrate (9e)

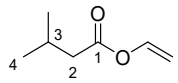


9e

2-Methylbutyric acid (1.15 mL, 10.5 mmol), $[Pd(OAc)_2]_3$ (368 mg, 1.64 mmol) and KOH (58.9 mg, 1.05 mmol) were dissolved in vinyl acetate (80 mL). Vinyl ester formation was performed according to **General Method C** to give the title compound **9e** (150 mg, 11.1 % yield) as a liquid; **IR** (film) ν_{max} 2970, 2938, 2881, 1751, 1647, 1140 cm^{-1}; **^{1}H NMR** (400 MHz; $CDCl_3$): δ 7.29 (dd, 1H, *J* = 13.7 Hz and 6.2 Hz, CO_2CHCH_2) 4.89 (dd, 1H, *J* = 14.0 Hz and 1.5 Hz, CO_2CHCH_2), 4.57 (dd, 1H, *J* = 6.5 Hz and 1.5 Hz, CO_2CHCH_2), 2.45 (qdd, 1H, *J* = 7.0, 7.0 and 7.0 Hz, H-2), 1.73 (qdd, 1H, *J* = 7.1 Hz, 7.1 Hz and 13.8 Hz, H-3a), 1.57–1.49 (m, 1H, H-3b), 1.19 (d, 3H, *J* = 7.0, H-2'), 0.93 (dd, 3H, *J* = 7.5 Hz and 7.5 Hz, H-4); **^{13}C NMR** (100 MHz; $CDCl_3$): δ 173.9 (C-1), 141.5 (CO_2CHCH_2), 97.6 (CO_2CHCH_2), 40.9 (C-2), 26.7 (C-3), 16.4 (C-2'), 11.6 (C-4).

HRMS data could not be obtained due to the incompatibility caused by compound volatility.

Synthesis of Vinyl 3-Me-Butyrate (9f)

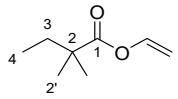


9f

3-Methylbutyric acid (1.10 mL, 10 mmol), $[Pd(OAc)_2]_3$ (350 mg, 1.56 mmol) and KOH (56.1 mg, 1 mmol) were dissolved in vinyl acetate (80 mL). Vinyl ester formation was performed according to **General Method C** to give the title compound **9f** (170 mg, 13.3 % yield) as a liquid; **IR** (film) ν_{max} 2962, 2929, 2875, 1755, 1647, 1199, 1136 cm^{-1}; **^{1}H NMR** (400 MHz; $CDCl_3$): δ 7.29 (dd, 1H, *J* = 14.0 Hz and 6.5 Hz, CO_2CHCH_2) 4.87 (dd, 1H, *J* = 14.0 Hz and 1.5 Hz, CO_2CHCH_2), 4.56 (dd, 1H, *J* = 6.5 Hz and 1.5 Hz, CO_2CHCH_2), 2.27 (d, 2H, *J* = 7.0 Hz, H-2), 2.19–2.09 (m, 1H, H-3), 0.98 (d, 6H, *J* = 6.5, H-4); **^{13}C NMR** (100 MHz; $CDCl_3$): δ 170.2 (C-1), 141.3 (CO_2CHCH_2), 97.6 (CO_2CHCH_2), 43.1 (C-2), 25.6 (C-3), 22.5 (C-4).

HRMS data could not be obtained due to the incompatibility caused by compound volatility.

Synthesis of Vinyl 2,2-Di-Me-Butyrate (9g)

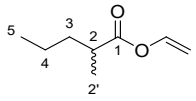


9g

2,2-Dimethylbutyric acid (1.88 mL, 15 mmol), $[Pd(OAc)_2]_3$ (525 mg, 2.34 mmol) and KOH (84.2 mg, 1.5 mmol) were dissolved in vinyl acetate (150 mL). Vinyl ester formation was performed according to **General Method C** to give the title compound **9g** (497 mg, 23.3 % yield) as a liquid; **IR** (film) ν_{max} 2971, 2883, 1747, 1646, 1138, 1122 cm^{-1}; **^{1}H NMR** (400 MHz; $CDCl_3$): δ 7.27 (dd, 1H, *J* = 13.9 Hz and 6.5 Hz, CO_2CHCH_2) 4.88 (dd, 1H, *J* = 14.0 Hz and 1.5 Hz, CO_2CHCH_2), 4.56 (dd, 1H, *J* = 6.5 Hz and 1.5 Hz, CO_2CHCH_2), 1.61 (q, 2H, *J* = 7.2 Hz, H-3), 1.20 (s, 6H, H-2'), 0.86 (t, 3H, *J* = 7.7 Hz, H-4); **^{13}C NMR** (100 MHz; $CDCl_3$): δ 175.2 (C-1), 141.7 (CO_2CHCH_2), 97.5 (CO_2CHCH_2), 42.8 (C-2), 33.3 (C-3), 24.5 (C-2'), 9.3 (C-4).

HRMS data could not be obtained due to the incompatibility caused by compound volatility.

Synthesis of Vinyl 2-Me-Valerate (9h)

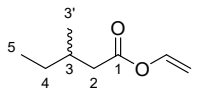


9h

2-Methylvaleric acid (1.3 mL, 10.4 mmol), $[Pd(OAc)_2]_3$ (364 mg, 1.62 mmol) and KOH (58.4 mg, 1.04 mmol) were dissolved in vinyl acetate (80 mL). Vinyl ester formation was performed according to **General Method C** to give the title compound **9h** (580 mg, 39.2 % yield) as a liquid; **IR** (film) ν_{max} 2963, 2938, 2876, 1751, 1646, 1139 cm^{-1}; **^{1}H NMR** (400 MHz; $CDCl_3$): δ 7.29 (dd, 1H, *J* = 14.0 Hz and 6.5 Hz, CO_2CHCH_2) 4.88 (dd, 1H, *J* = 14.0 Hz and 1.5 Hz, CO_2CHCH_2), 4.56 (dd, 1H, *J* = 6.5 Hz and 1.5 Hz, CO_2CHCH_2), 2.52 (qt, 1H, *J* = 7.0 Hz and 7.0 Hz, H-2), 1.74–1.65 (m, 1H, H-3a), 1.48–1.39 (m, 1H, H-3b overlaps partially with H-4), 1.38–1.29 (m, 2H, H-4 overlaps partially with H-3b), 1.19 (d, 3H, *J* = 7.0 Hz, H-2'), 0.92 (t, 3H, *J* = 7.2 Hz, H-5); **^{13}C NMR** (100 MHz; $CDCl_3$): δ 174.0 (C-1), 141.5 (CO_2CHCH_2), 97.5 (CO_2CHCH_2), 39.2 (C-2), 35.8 (C-3), 20.4 (C-4), 16.8 (C-2'), 14.0 (C-5).

HRMS data could not be obtained due to the incompatibility caused by compound volatility.

Synthesis of Vinyl 3-Me-Valerate (9i)

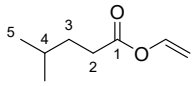


9i

3-Methylvaleric acid (1.25 mL, 10 mmol), $[Pd(OAc)_2]_3$ (350 mg, 1.56 mmol) and KOH (56.1 mg, 1 mmol) were dissolved in vinyl acetate (80 mL). Vinyl ester formation was performed according to **General Method C** to give the title compound **9i** (680 mg, 47.8 % yield) as a liquid; **IR** (film) ν_{max} 2964, 2931, 2879, 1754, 1646, 1135 cm^{-1}; **^{1}H NMR** (400 MHz; $CDCl_3$): δ 7.29 (dd, 1H, *J* = 14.1 Hz and 6.1 Hz, CO_2CHCH_2) 4.87 (dd, 1H, *J* = 14.0 Hz and 1.5 Hz, CO_2CHCH_2), 4.56 (dd, 1H, *J* = 6.0 Hz and 1.5 Hz, CO_2CHCH_2), 2.39 (dd, 1H, *J* = 14.9 Hz and 6.0 Hz, H-2a), 2.19 (dd, 1H, *J* = 14.9 Hz and 8.0 Hz, H-2b), 1.93 (qtdd, 1H, *J* = 6.8 Hz, 6.8 Hz, 6.8 Hz and 6.8 Hz, H-3), 1.44-1.20 (m, 2H, H-4), 0.96 (d, 3H, *J* = 6.5 Hz, H-3'), 0.91 (t, 3H, *J* = 7.3 Hz, H-5); **^{13}C NMR** (100 MHz; $CDCl_3$): δ 170.5 (C-1), 141.3 (CO_2CHCH_2), 97.5 (CO_2CHCH_2), 41.2 (C-2), 31.9 (C-3), 29.4 (C-4), 19.4 (C-3'), 11.4 (C-5).

HRMS data could not be obtained due to the incompatibility caused by compound volatility.

Synthesis of Vinyl 4-Me-Valerate (9j)



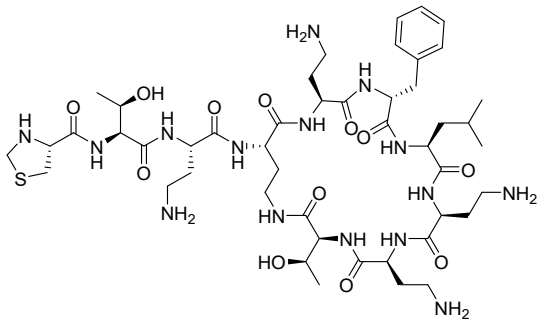
9j

4-Methylvaleric acid (1.25 mL, 9.93 mmol), $[Pd(OAc)_2]_3$ (348 mg, 1.55 mmol) and KOH (55.7 mg, 0.993 mmol) were dissolved in vinyl acetate (80 mL). Vinyl ester formation was performed according to **General Method C** to give the title compound **9j** (590 mg, 41.8 % yield) as a liquid; **IR** (film) ν_{max} 2960, 2873, 1755, 1647, 1138 cm^{-1}; **^{1}H NMR** (400 MHz; $CDCl_3$): δ 7.28 (dd, 1H, *J* = 14.0 Hz and 6.4 Hz, CO_2CHCH_2), 4.87 (dd, 1H, *J* = 14.0 Hz and 1.5 Hz, CO_2CHCH_2), 4.55 (dd, 1H, *J* = 6.5 Hz and 1.5 Hz, CO_2CHCH_2), 2.39 (t, 2H, *J* = 7.5 Hz, H-2), 1.65–1.53 (m, 3H, H-4 and H-3), 0.91 (d, 6H, *J* = 6.5 Hz, H-5); **^{13}C NMR** (100 MHz; $CDCl_3$): δ 171.1 (C-1), 141.4 (CO_2CHCH_2), 97.5 (CO_2CHCH_2), 33.5 (C-3), 32.1 (C-2), 27.7 (C-4), 22.3 (C-5).

HRMS data could not be obtained due to the incompatibility caused by compound volatility.

*S3. Preparation of L-Cys-coupled PMBN (**4**) and L-Cys-CLipPA analogues*

Synthesis of L-Thz-Polymyxin B Nonapeptide (S1)



S1

N^{α}-Boc-L-Thz-OH (4 equiv.) was dissolved in CH_2Cl_2 to which HATU (3.8 equiv.) and DIPEA (6 equiv.) were added. The resultant solution was added to crude peptide **3** (1 equiv.) prepared *via* **Repeats 1a**–**1d**, dissolved in CH_2Cl_2 and agitated for 2 h at rt. The completion of coupling was monitored using analytical RP-HPLC (5–95 % B, 3 % B/min, 1 mL/min). The N^{α}- and N^{γ}-Boc-protecting groups were removed by treatment with a mixture of TFA/CH_2Cl_2 (1:1, *v*/*v*) for 30 min at rt, after which the solvent was reduced with a stream of N_2, the crude oil triturated with Et_2O (3 × 40 mL, 4 °C) and dried to give crude product **S1** which was directly used in the next step without further purification.

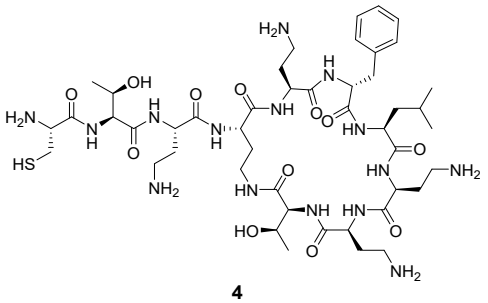
Table S3: Reaction conditions for Thz coupling of **3** and Boc-removal.

Repeat			Reagent and solvent					
			Boc-L-Thz-OH (mg, µmol)	CH_2Cl_2 (mL)	HATU (mg, µmol)	DIPEA (µL, µmol)	3 (mg, µmol)[a]	TFA
1	a*	i	13.1, 56.0	2.5	20.2, 53.2	14.6, 84.0	50, 14.0	2.5
		ii	20.0, 85.6	3.75	30.9, 81.3	22.3, 128	76.4, 21.4	3.75
	b		55.8, 239	9.5	86.3, 227	62.5, 359	192, 59.8	9.5
	c		25.7, 110	4.25	39.9, 105	28.9, 166	85, 27.6	4.25
	d		55.8, 239	9.5	86.3, 227	62.5, 359	116, 59.8	9.5

[a] The number of moles (µmol) of crude material **3** was deduced based on that of polymyxin B sulfate (**1**) submitted in the enzymatic cleavage step and subsequent divisions made in the following steps.

*The crude starting material was divided into portions **i** and **ii**.

Synthesis of L-Cys-Polymyxin B Nonapeptide (4) *via* Thz-ring opening



4

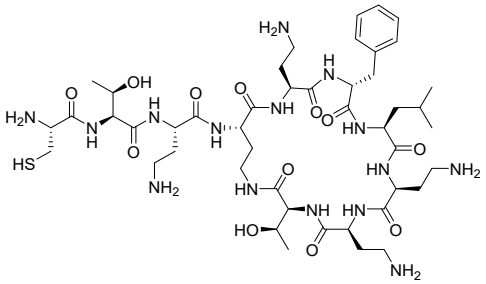
Crude peptide **S1** (1 equiv.) prepared *via* **Repeats 1a**–**1d** was dissolved in 0.2 M $CH_3ONH_2 \cdot HCl$ and the solution adjusted to pH 4 using 1 M NaOH. The resulting mixture was agitated for 18 h at 37 °C, after which it was centrifuged, filtered and enriched according to **General Method B**. Combined fractions were lyophilised to give enriched product **4** which was directly used in the next step without further purification.

Table S4: Reaction conditions for Thz ring opening of **S1**.

Repeat			Reagent and solvent		Enriched yield (mg)
			S1 (μmol)[a]	**0.2 M $CH_3ONH_2 \cdot HCl$ (mL)**	
1	a	i	14.0	5	12.4
		ii	21.0	7.5	9.5
	b		59.8	10	40.4
	c		27.6	5.5	16
	d		59.8	12	17.3

[a] The number of moles (μmol) of crude material **S1** was deduced based on that of polymyxin B sulfate (**1**) submitted in the enzymatic cleavage step and subsequent divisions made in the following steps.

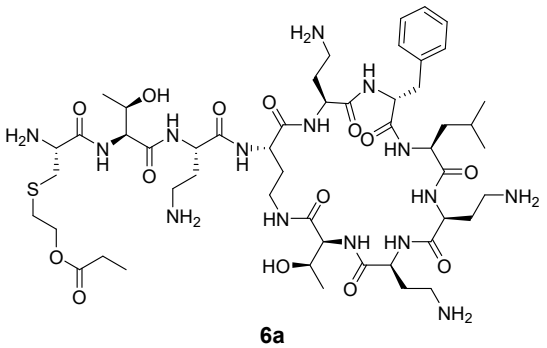
Synthesis of L-Cys-Polymyxin B Nonapeptide (4) *via* Cys coupling and deprotection



4

N$^{\alpha}$-Boc-L-Cys(Trt)-OH (16.7 mg, 36.0 µmol) was dissolved in CH_2Cl_2/MeCN (3 mL, 1:1, *v*/*v*) to which DIC (5.13 µL, 33.1 µmol) and HOAt (4.51 mg, 33.1 µmol) were added. The above solution was added to crude peptide **3** (40 mg, 14.4 µmol) prepared *via* **Repeat 2**, dissolved in CH_2Cl_2/MeCN (3 mL, 1:1, *v*/*v*). The reaction was agitated for 2 h at rt, and dried with a stream of N_2 or concentrated *in vacuo.* The resultant oil was treated with a mixture of TFA/TIPS (5 mL, 98:2, *v*/*v*) for 30 min at rt. Subsequently, the mixture was dried with a stream of N_2, triturated in Et_2O/pet. ether (3 × 40 mL, 4 °C, 2:1, *v*/*v*), dissolved in 0.1 % TFA (*v*/*v*) in H_2O/MeCN (5 mL, 1:1, *v*/*v*) and lyophilised to give crude product **4** (52.1 mg) which was directly used in the next step without further purification.

Synthesis of L-Cys-propionate (CLipPA) PMBN analogue 6a



6a

Enriched peptide **4** (11.8 mg, 11.1 µmol) prepared *via* **Repeat 1ai**, was dissolved in sparged NMP (1.13 mL) to which vinyl propionate (84.6 µL, 777 µmol), *tert*-nonyl mercaptan (166 µL, 888 µmol), TIPS (182 µL, 888 µmol), DMPA (5.69 mg, 22.2 µmol) and TFA (59.5 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **6a** as a white powder (3.33 mg, 21.5 % overall yield [6 steps], 99 % purity); **RP-HPLC**: t_R = 9.1 min; **ESI-MS**: $[M + H]^+$ found 1166.7, $[C_{51}H_{87}N_{15}O_{14}S + H]^+$ requires 1166.64.

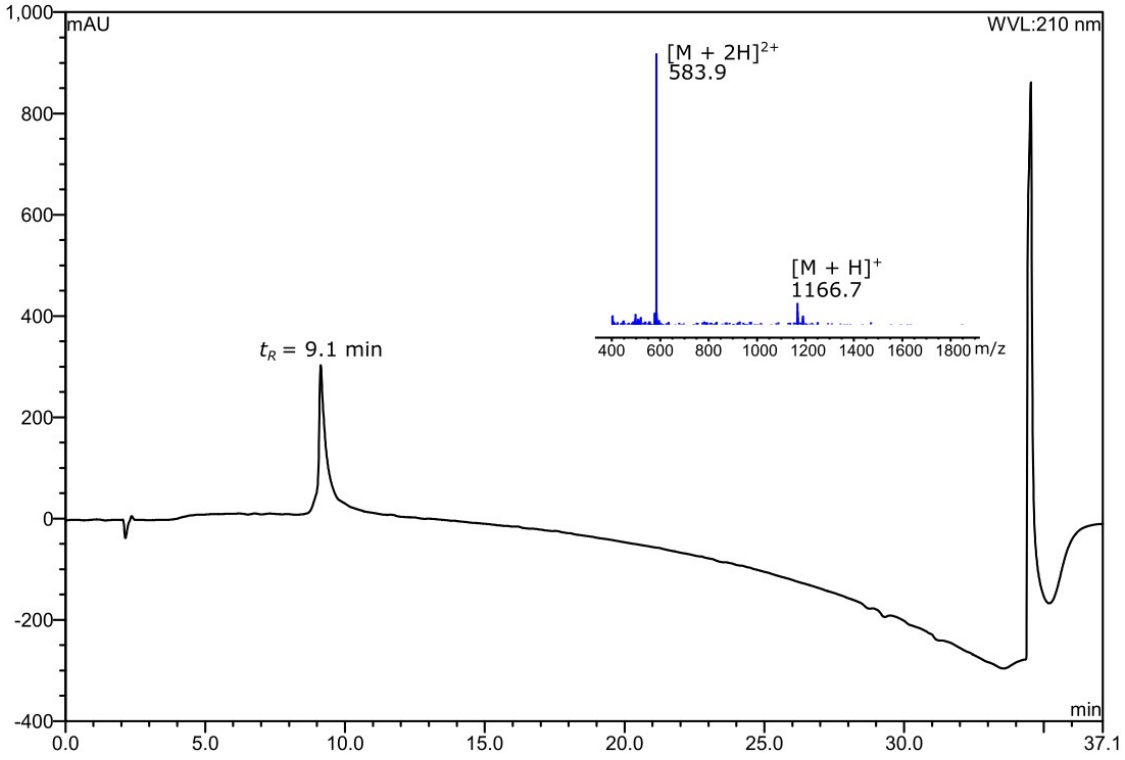
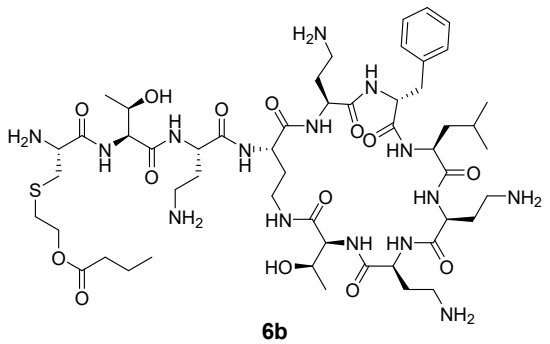


Figure S1: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **6a** (*ca.* 98 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of L-Cys-butyrate (CLipPA) PMBN analogue 6b



6b

Enriched peptide **4** (24.2 mg, 22.7 µmol) prepared *via* **Repeat 1b**, was dissolved in sparged NMP (2.3 mL) to which vinyl butyrate (202 µL, 1.59 mmol), *tert*-nonyl mercaptan (341 µL, 1.82 mmol), TIPS (373 µL, 1.82 mmol), DMPA (11.6 mg, 45.4 µmol) and TFA (121 µL) were added. CLipPA was performed for 1.5 h according to **General Method D** to afford purified product **6b** as a white powder (3.83 mg, 9.1 % overall yield [6 steps], 99 % purity); **RP-HPLC**: t_R = 9.3 min; **ESI-MS**: $[M + H]^+$ found 1180.5, $[C_{52}H_{89}N_{15}O_{14}S + H]^+$ requires 1180.65.

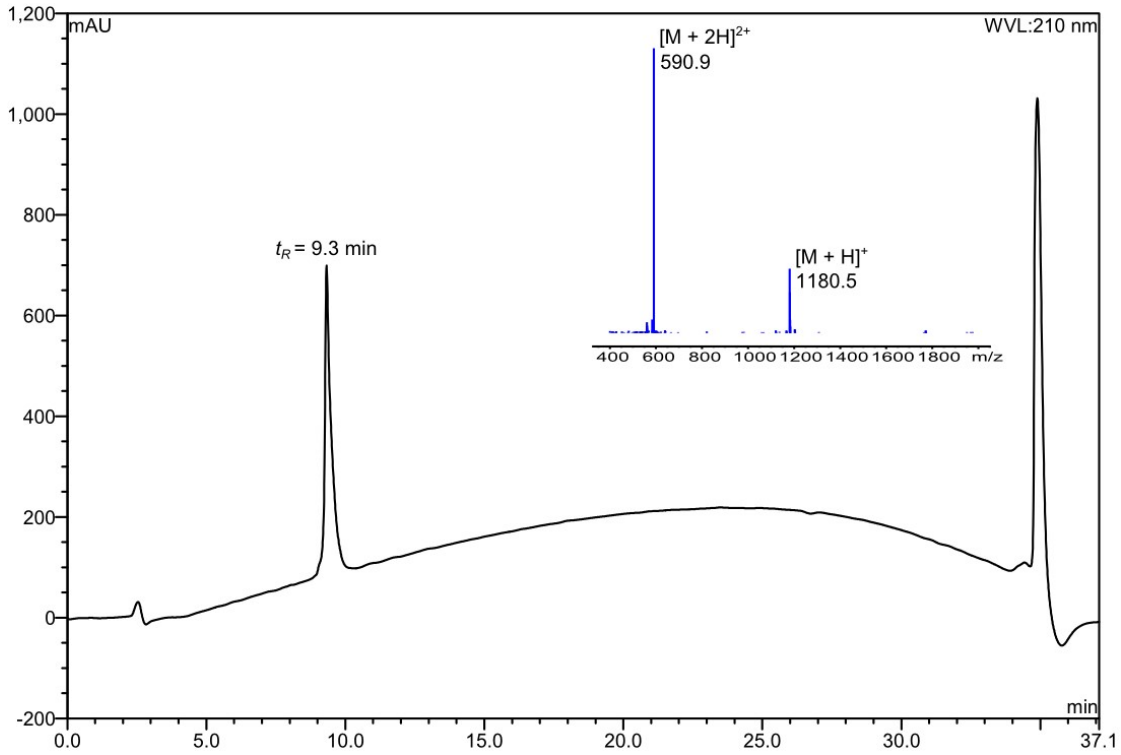
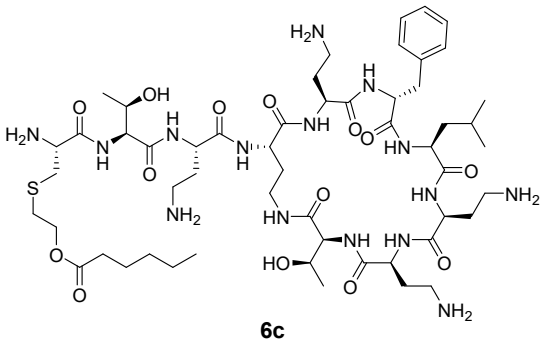


Figure S2: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **6b** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of L-Cys-hexanoate (CLipPA) PMBN analogue 6c



6c

Enriched product **4** (8.25 mg, 7.74 µmol) prepared *via* **Repeat 1c**, was dissolved in sparged NMP (790 µL) to which vinyl hexanoate (86.6 µL, 542 µmol), *tert*-nonyl mercaptan (116 µL, 619 µmol), TIPS (127 µL, 619 µmol), DMPA (3.97 mg, 15.5 µmol) and TFA (41.6 µL) were added. CLipPA was performed for 40 min according to **General Method D** to afford purified product **6c** as a white powder (2.60 mg, 15.1 % overall yield [6 steps], 99 % purity); RP-HPLC: t_R = 10.6 min; ESI-MS: $[M + H]^+$ found 1208.6, $[C_{54}H_{93}N_{15}O_{14}S + H]^+$ requires 1208.68.

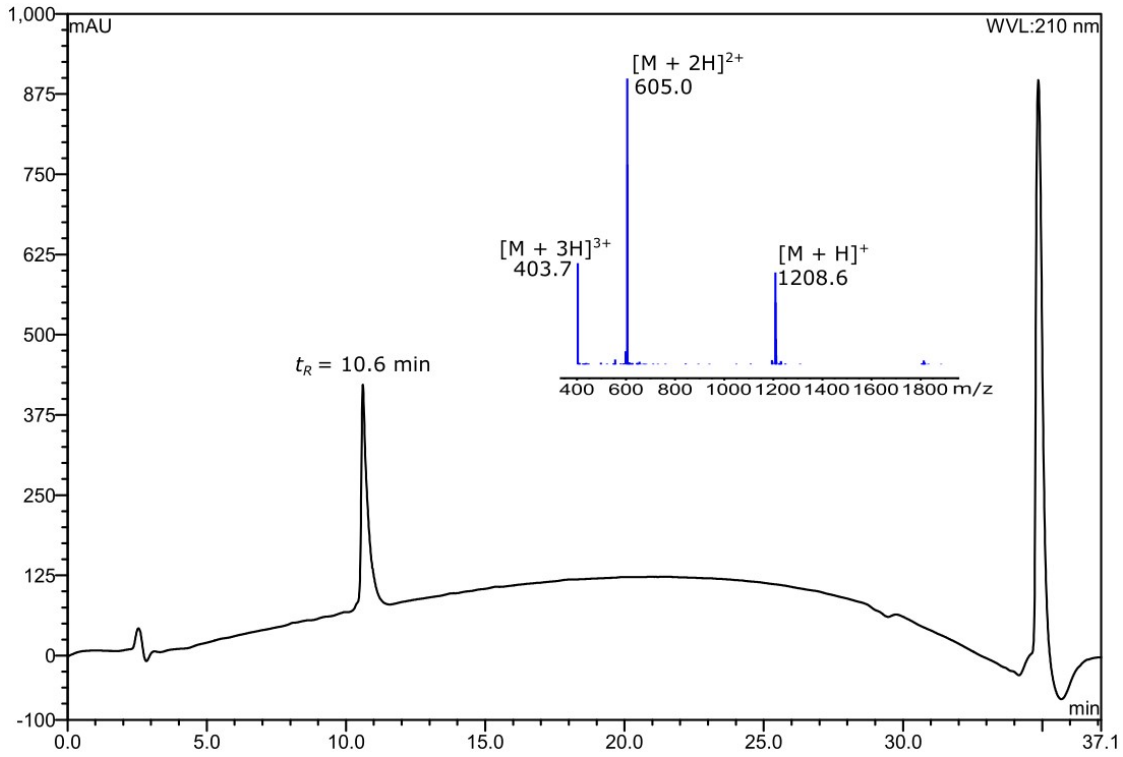
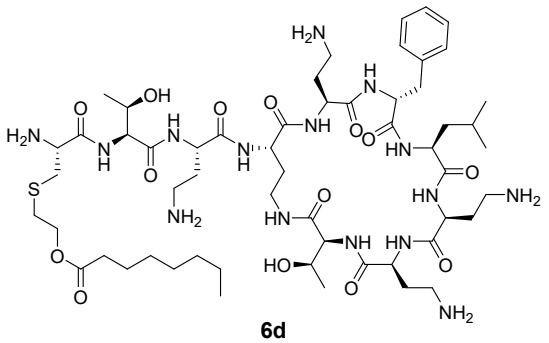


Figure S3: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **6c** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of L-Cys-octanoate (CLipPA) PMBN analogue 6d



6d

Enriched product **4** (7.70 mg, 7.22 µmol) prepared *via* **Repeat 1c**, was dissolved in sparged NMP (732 µL) to which vinyl octanoate (97.7 µL, 505 µmol), *tert*-nonyl mercaptan (108 µL, 578 µmol), TIPS (118 µL, 578 µmol), DMPA (3.69 mg, 14.4 µmol) and TFA (38.5 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **6d** as a white powder (2.15 mg, 13.1 % overall yield [6 steps], 96 % purity); **RP-HPLC**: t_R = 15.0 min; **ESI-MS**: $[M + H]^+$ found 1236.9, $[C_{56}H_{97}N_{15}O_{14}S + H]^+$ requires 1236.71.

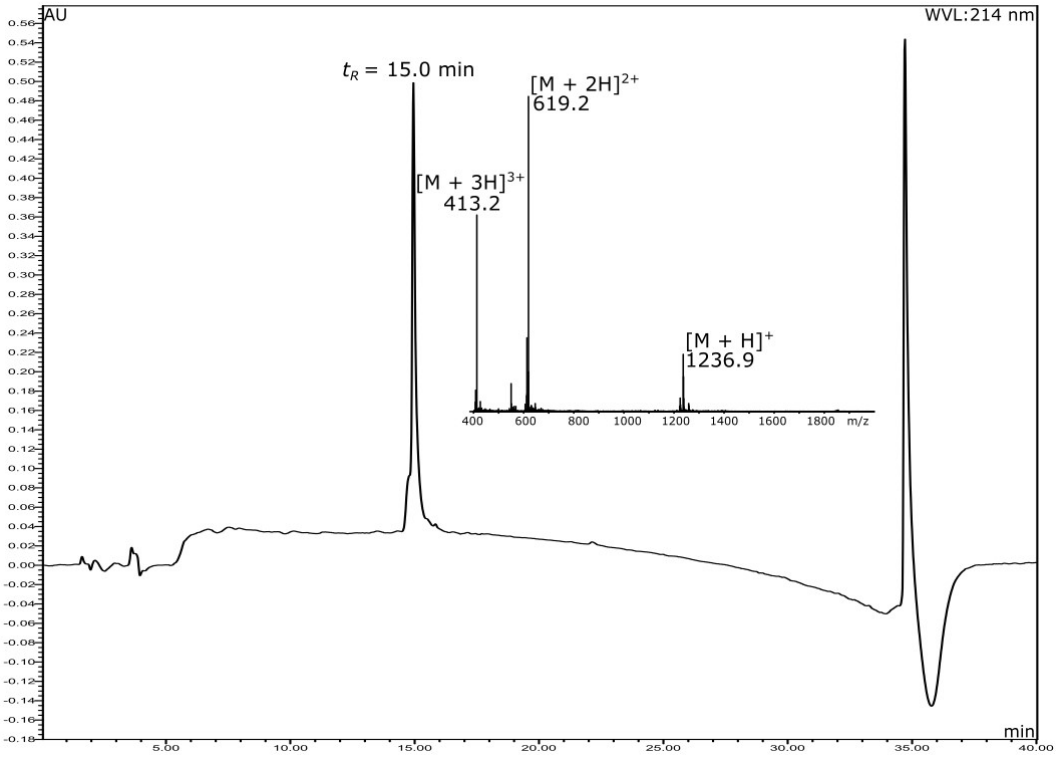
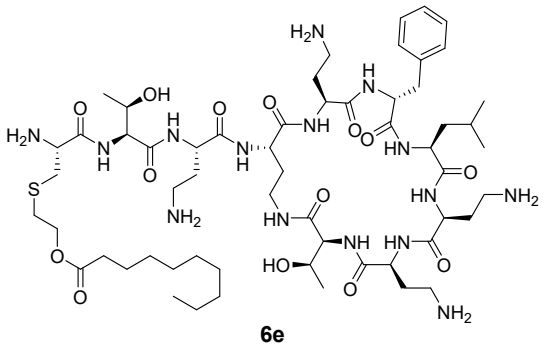


Figure S4: RP-HPLC (214 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **6d** (*ca.* 96 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm).

Synthesis of L-Cys-decanoate (CLipPA) PMBN analogue 6e



6e

Crude product **4** (26 mg, 7.19 µmol) prepared *via* **Repeat 2**, was dissolved in sparged NMP (1 mL) to which vinyl decanoate (113 µL, 503 µmol), *tert*-nonyl mercaptan (108 µL, 575 µmol), TIPS (118 µL, 575 µmol), DMPA (3.69 mg, 14.4 µmol) and TFA (52.6 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **6e** as a white powder (0.77 mg, 8.5 % overall yield [5 steps], 98 % purity); **RP-HPLC**: t_R = 14.6 min; **ESI-MS**: $[M + H]^+$ found 1265.0, $[C_{58}H_{101}N_{15}O_{14}S + H]^+$ requires 1264.75.

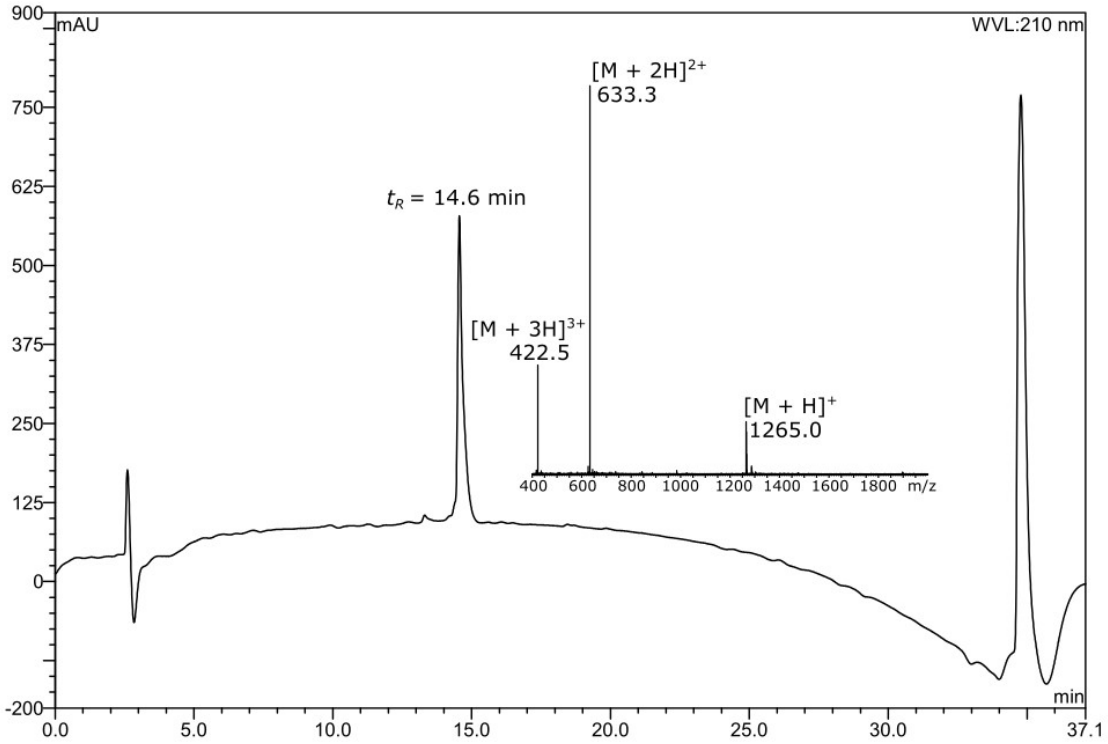
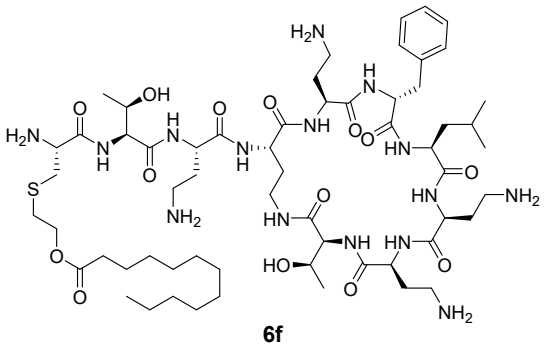


Figure S5: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **6e** (*ca.* 98 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of L-Cys-laurate (CLipPA) PMBN analogue 6f



6f

Crude product **4** (26 mg, 7.19 µmol) prepared *via* **Repeat 2**, was dissolved in sparged NMP (1 mL) to which vinyl laurate (131 µL, 503 µmol), *tert*-nonyl mercaptan (108 µL, 575 µmol), TIPS (118 µL, 575 µmol), DMPA (3.69 mg, 14.4 µmol) and TFA (52.6 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **6f** as a white powder (0.66 mg, 7.1 % overall yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 15.8 min; **ESI-MS**: $[M + H]^+$ found 1293.1, $[C_{60}H_{105}N_{15}O_{14}S + H]^+$ requires 1292.78.

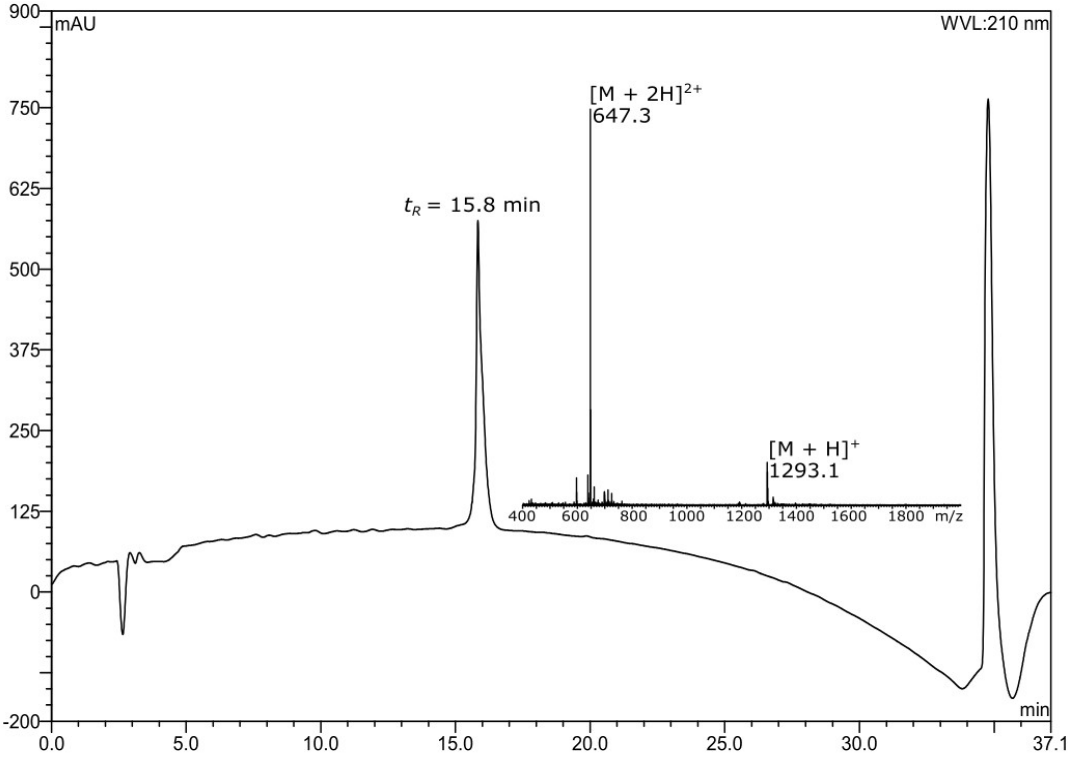
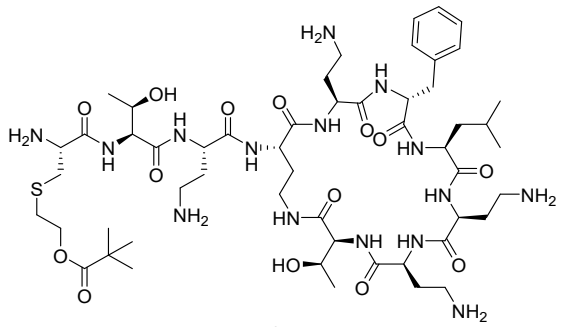


Figure S6: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **6f** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of L-Cys-pivalate (CLipPA) PMBN analogue 6g



6g

Enriched product **4** (9.5 mg, 8.91 µmol) prepared *via* **Repeat 1aii**, was dissolved in sparged NMP (914 µL) to which vinyl pivalate (92.4 µL, 624 µmol), *tert*-nonyl mercaptan (134 µL, 713 µmol), TIPS (146 µL, 713 µmol), DMPA (4.56 mg, 17.8 µmol) and TFA (48.1 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **6g** as a white powder (1.41 mg, 5.5 % overall yield [6 steps], 99 % purity); **RP-HPLC**: t_R = 10.1 min; **ESI-MS**: $[M + H]^+$ found 1194.6, $[C_{53}H_{91}N_{15}O_{14}S + H]^+$ requires 1194.67.

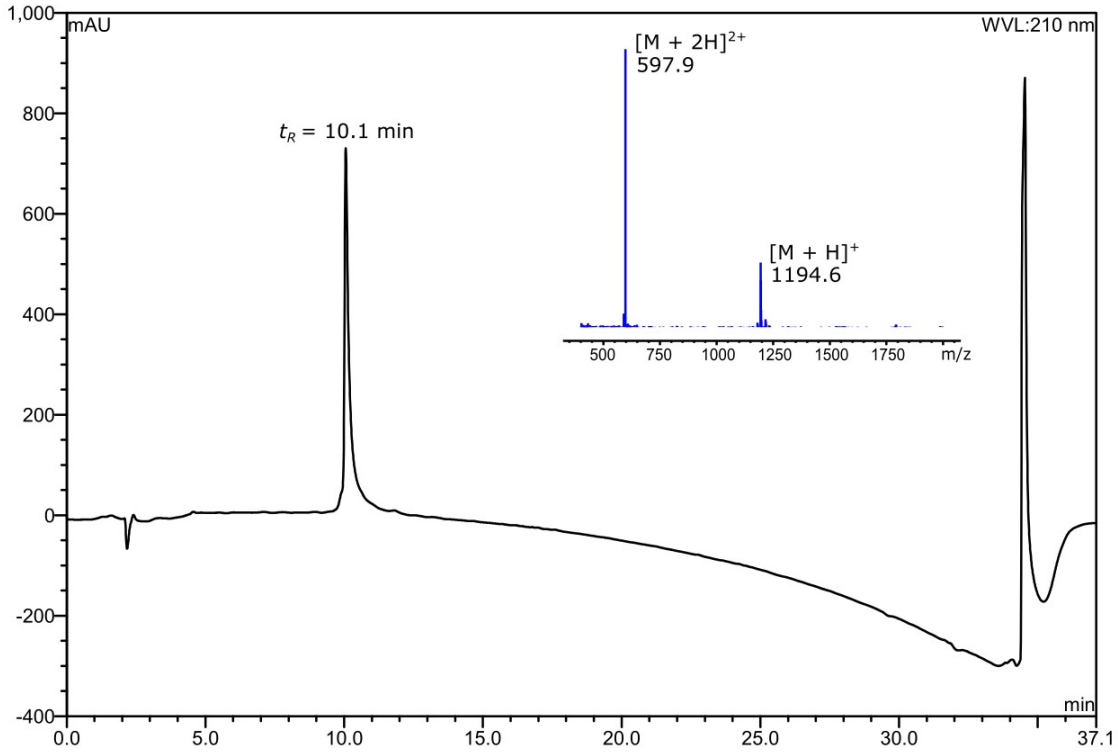
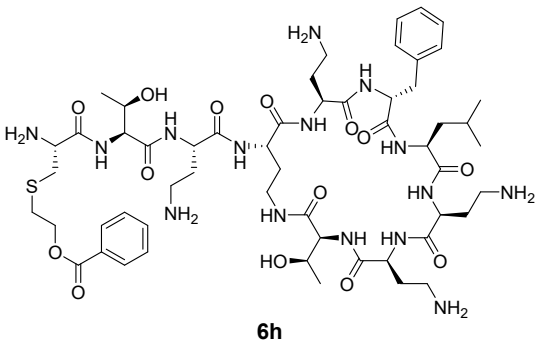


Figure S7: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **6g** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of L-Cys-benzoate (CLipPA) analogue 6h



6h

Enriched product **4** (7.75 mg, 7.27 µmol) prepared *via* **Repeat 1d**, was dissolved in sparged NMP (744 µL) to which vinyl benzoate (70.5 µL, 509 µmol), *tert*-nonyl mercaptan (109 µL, 582 µmol), TIPS (119 µL, 582 µmol), DMPA (3.72 mg, 14.5 µmol) and TFA (39.2 µL) were added. CLipPA was performed 1 h 20 min according to **General Method D** to afford purified product **6h** as a white powder (2.03 mg, 6.2 % overall yield [6 steps], 99 % purity); **RP-HPLC**: t_R = 10.2 min; **ESI-MS**: $[M + H]^+$ found 1214.6, $[C_{55}H_{87}N_{15}O_{14}S + H]^+$ requires 1214.64.

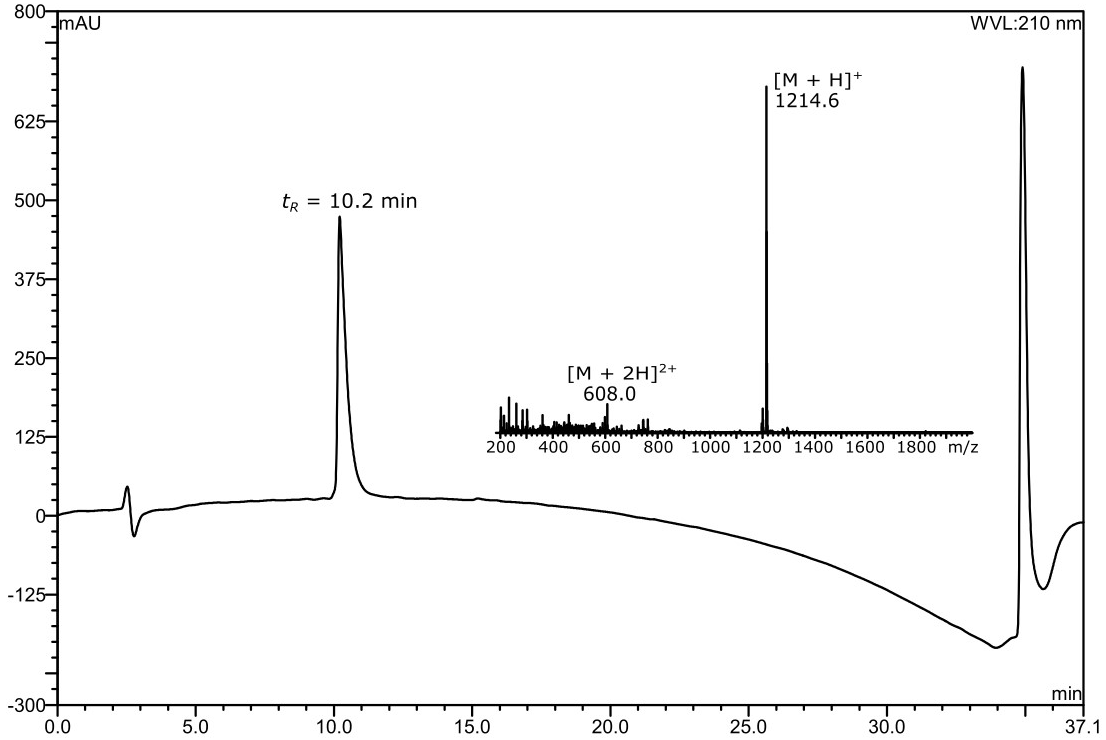
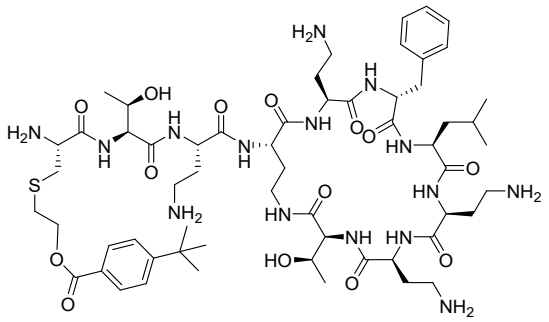


Figure S8: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **6h** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of L-Cys-4-*t*Bu-benzoate (CLipPA) analogue 6i



6i

Enriched product **4** (9 mg, 8.44 µmol) prepared *via* **Repeat 1d**, was dissolved in sparged NMP (865 µL) to which vinyl 4-*t*Bu-benzoate (121 µL, 591 µmol), *tert*-nonyl mercaptan (126 µL, 675 µmol), TIPS (138 µL, 675 µmol), DMPA (4.33 mg, 16.9 µmol) and TFA (45.5 µL) were added. CLipPA was performed for 1 h 20 min according to **General Method D** to afford purified product **6i** as a white powder (2.21 mg, 5.6 % overall yield [6 steps], 99 % purity); **RP-HPLC**: t_R = 12.3 min; **ESI-MS**: $[M + H]^+$ found 1270.6, $[C_{59}H_{95}N_{15}O_{14}S + H]^+$ requires 1270.70.

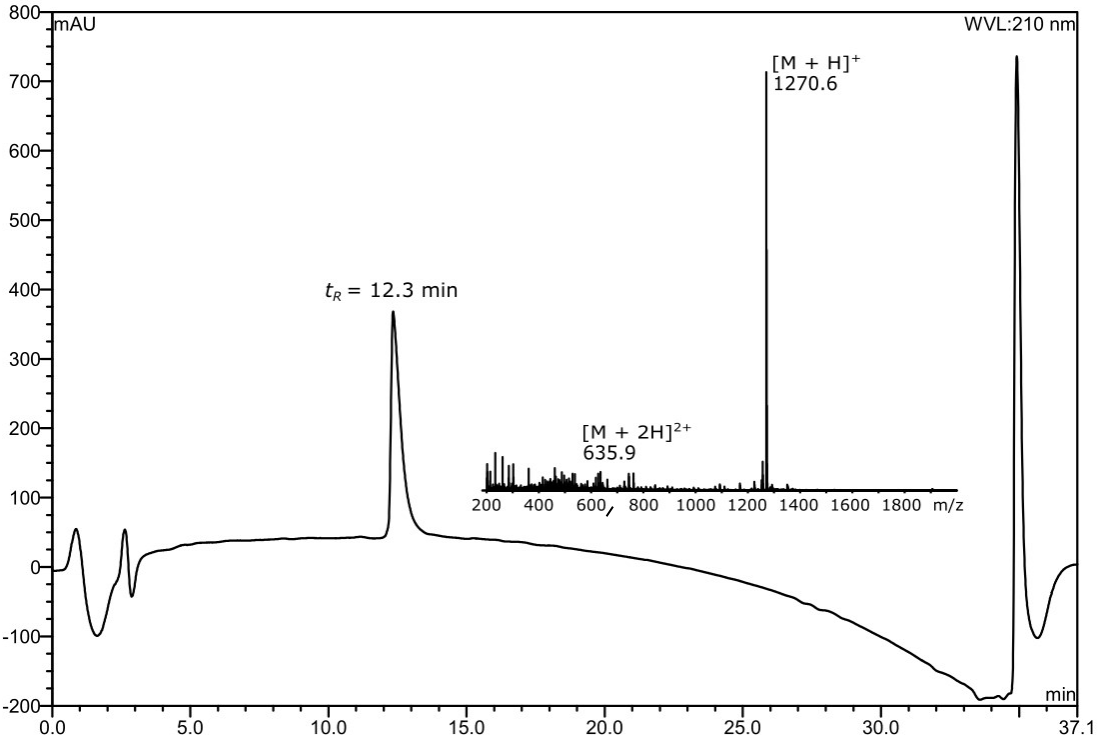
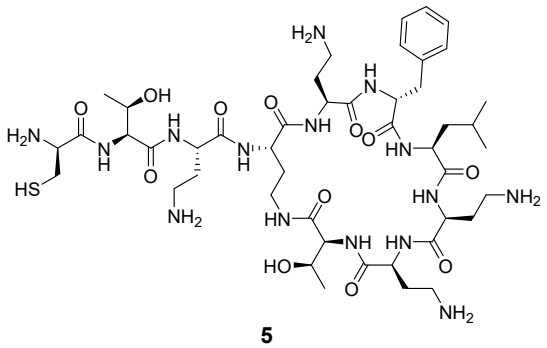


Figure S9: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **6i** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm).

*S4. Preparation of D-Cys-coupled PMBN (**5**) and D-Cys-CLipPA analogues*

Synthesis of D-Cys-Polymyxin B Nonapeptide (5)



5

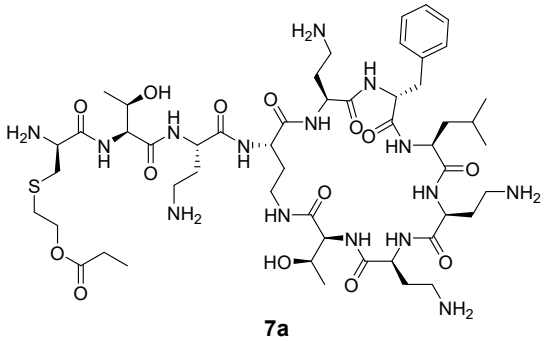
N^{α}-Boc-D-Cys(Trt)-OH (2.5 equiv.) was dissolved in CH_2Cl_2/MeCN (1:1, *v*/*v*) to which DIC (2.3 equiv.) and HOAt (2.3 equiv.) were added. The above solution was added to crude peptide **3** (1 equiv.) prepared *via* **Repeat 2**–**4**, dissolved in CH_2Cl_2/MeCN (1:1, *v*/*v*). The reaction was agitated for 2 h at rt, and concentrated *in vacuo.* The resultant oil was treated with a mixture of TFA/TIPS (5 mL, 98:2, *v*/*v*) for 30 min at rt. Subsequently, the mixture was dried with a stream of N_2, triturated in Et_2O/pet. ether (3 × 40 mL, 4 °C, 2:1, *v*/*v*), dissolved in 0.1 % TFA (*v*/*v*) in H_2O/MeCN (10 mL, 1:1, *v*/*v*) and lyophilised to give crude product **5** which was either used directly in the next step without further purification or was enriched according to **General Method B** to give enriched product **5**.

Table S5: Reaction conditions for D-Cys coupling of **3**.

Repeat		Reagent and solvent					Yield (mg)	
		Boc-D-Cys(Trt)-OH (mg, µmol)	**CH_2Cl_2 /MeCN (mL)**	**DIC (µL, µmol)**	**HOAt (mg, µmol)**	**3 (mg, µmol)**	**Crude**	**Enriched**
2*	**a**	45.8, 98.8	11	14.1, 90.9	12.4, 90.9	109.6, 39.5	149	-
	b	37.7, 81.3	9	11.6, 74.8	10.2, 74.8	90.4, 32.5	-	15
3		113, 243	20	34.7, 224	30.5, 224	380, 97.3	-	76.2
4		584, 1260	30	180, 1160	158, 1160	1570, 505	-	354

*The crude starting material was divided into portions **a** and **b**

Synthesis of D-Cys-propionate (CLipPA) analogue 7a



7a

Crude product **5** (21 mg, 5.56 µmol) prepared *via* **Repeat 2a**, was dissolved in sparged NMP (1 mL) to which vinyl propionate (42.4 µL, 389 µmol), *tert*-nonyl mercaptan (83.3 µL, 445 µmol), TIPS (91.2 µL, 445 µmol), DMPA (2.84 mg, 11.1 µmol) and TFA (52.6 µL) were added. CLipPA was performed for 1 h 20 min according to **General Method D** to afford purified product **7a** as a white powder (1.52 mg, 23.4 % overall yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 12.3 min; **ESI-MS**: $[M + H]^+$ found 1166.8, $[C_{51}H_{87}N_{15}O_{14}S + H]^+$ requires 1166.64.

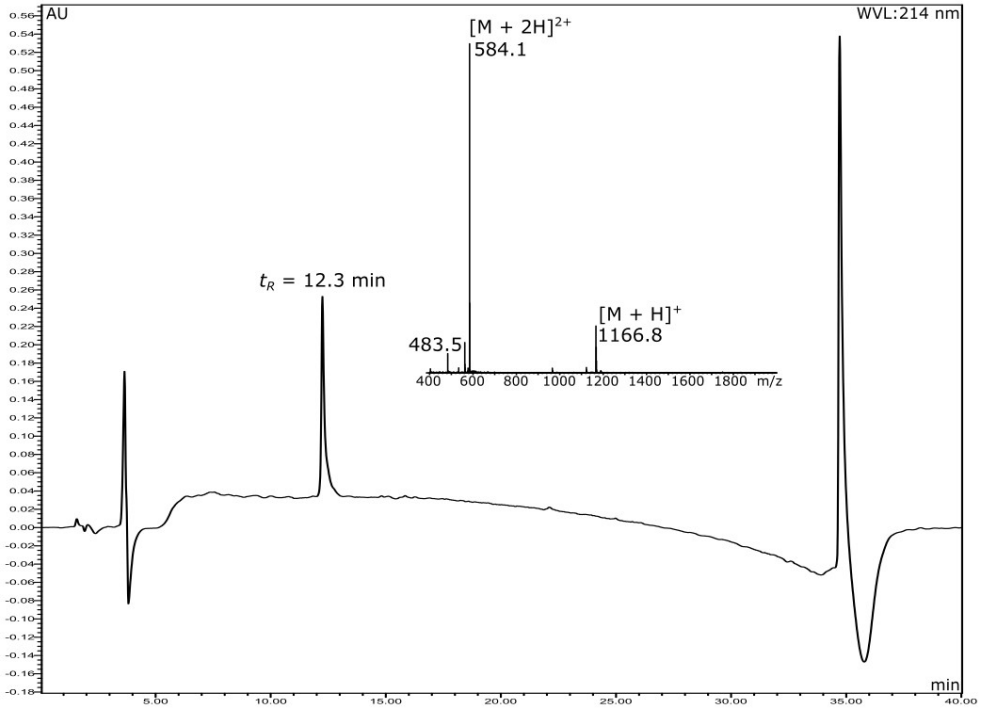
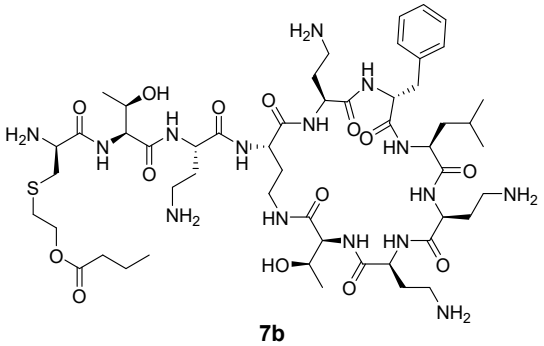


Figure S10: RP-HPLC (214 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7a** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm). Compound **2** (polymyxin B nonapeptide) was observed in ESI-MS as a result of ion fragmentation.

Synthesis of D-Cys-butyrate (CLipPA) analogue 7b



7b

Crude product **5** (21 mg, 5.56 µmol) prepared *via* **Repeat 2a**, was dissolved in sparged NMP (1 mL) to which vinyl butyrate (49.4 µL, 389 µmol), *tert*-nonyl mercaptan (83.3 µL, 445 µmol), TIPS (91.2 µL, 445 µmol), DMPA (2.84 mg, 11.1 µmol) and TFA (52.6 µL) were added. CLipPA was performed for 1 h 20 min according to **General Method D** to afford purified product **7b** as a white powder (0.80 mg, 12.2 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 12.8 min; **ESI-MS**: $[M + H]^+$ found 1180.8, $[C_{52}H_{89}N_{15}O_{14}S + H]^+$ requires 1180.65.

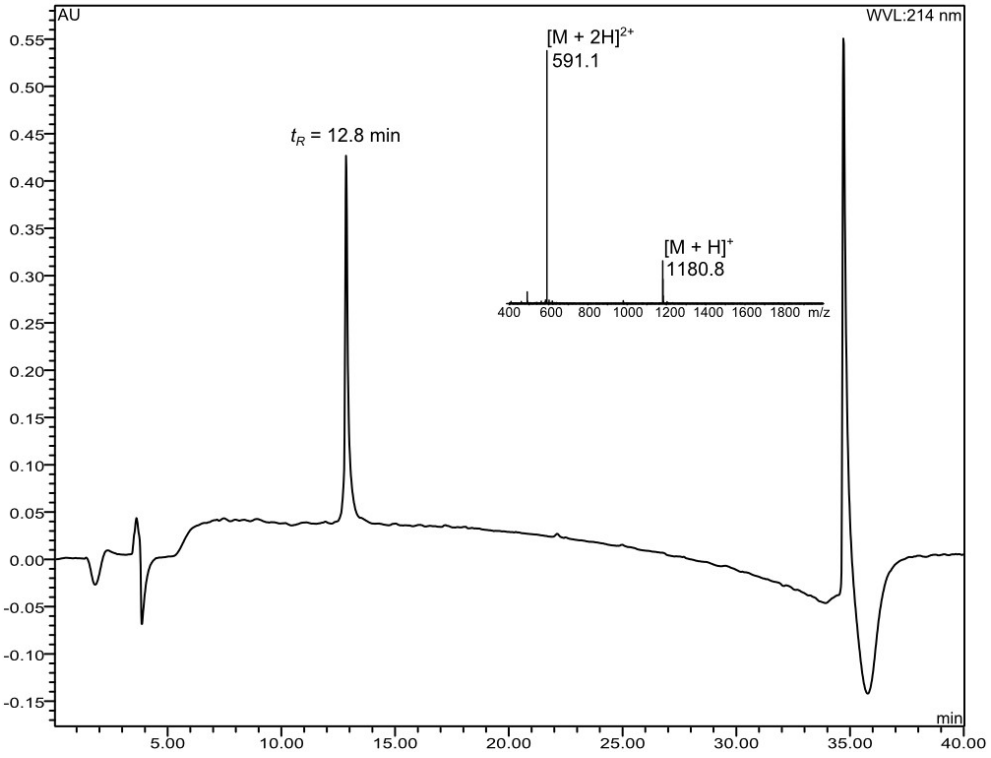
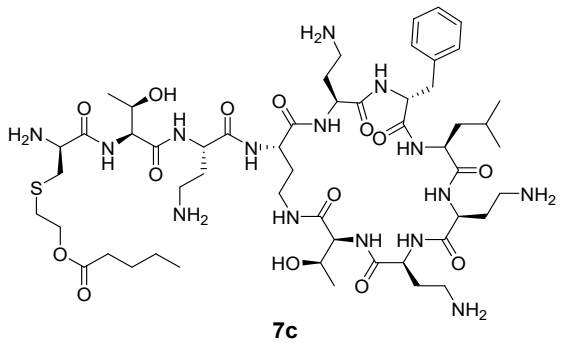


Figure S11: RP-HPLC (214 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7b** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-valerate (CLipPA) analogue 7c



7c

Enriched product **5** (34.3 mg, 32.2 µmol) prepared *via* **Repeat 3**, was dissolved in sparged NMP (2 mL) to which vinyl valerate (321 µL, 2.25 mmol), *tert*-nonyl mercaptan (483 µL, 2.58 mmol), TIPS (529 µL, 2.58 mmol), DMPA (16.5 mg, 64.4 µmol) and TFA (105 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7c** as a white powder (6.42 mg, 12.3 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 13.8 min; **ESI-MS**: $[M + H]^+$ found 1194.2, $[C_{53}H_{91}N_{15}O_{14}S + H]^+$ requires 1194.67.

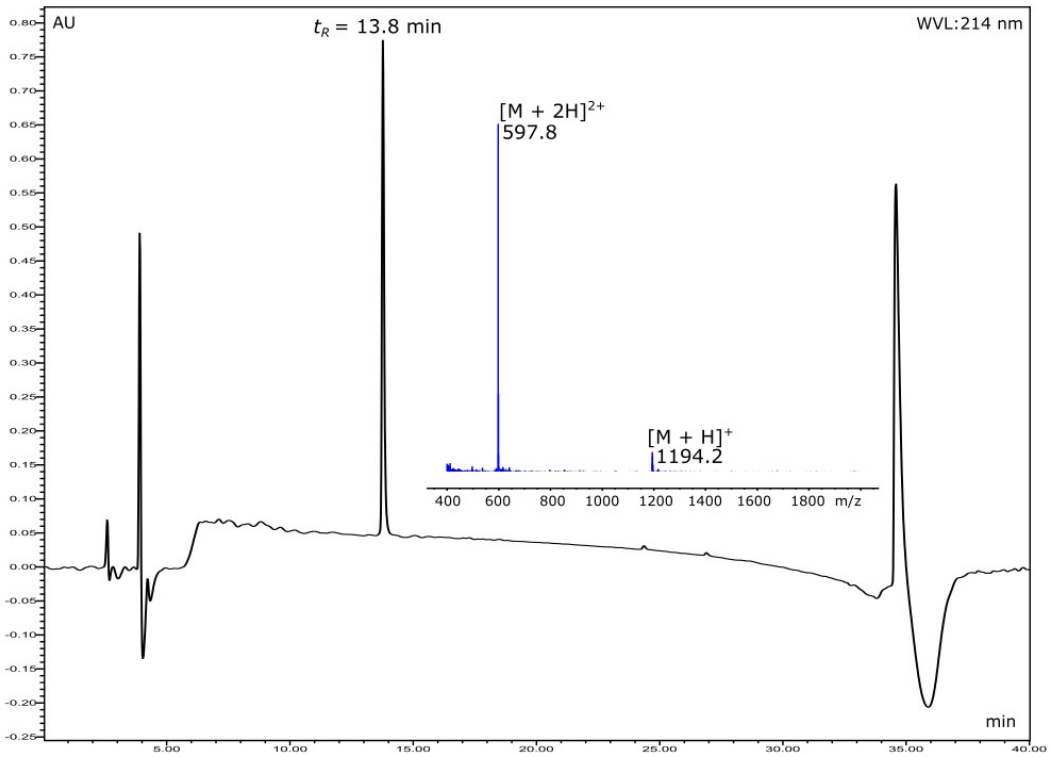
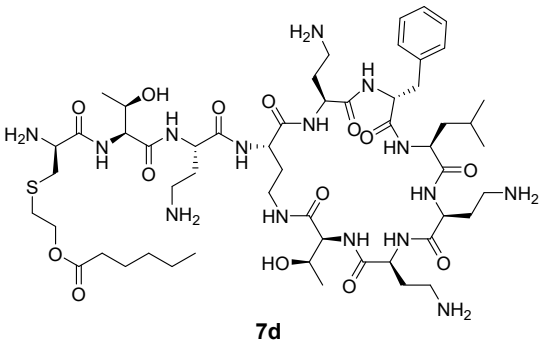


Figure S12: RP-HPLC (214 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7c** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-hexanoate (CLipPA) analogue 7d



7d

Enriched product **5** (7.5 mg, 7.03 µmol) prepared *via* **Repeat 2b**, was dissolved in sparged NMP (790 µL) to which vinyl hexanoate (78.6 µL, 492 µmol), *tert*-nonyl mercaptan (105 µL, 562 µmol), TIPS (115 µL, 562 µmol), DMPA (3.61 mg, 14.1 µmol) and TFA (41.6 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7d** as a white powder (3.14 mg, 16.0 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 14.2 min; **ESI-MS**: $[M + H]^+$ found 1208.1, $[C_{54}H_{93}N_{15}O_{14}S + H]^+$ requires 1208.68.

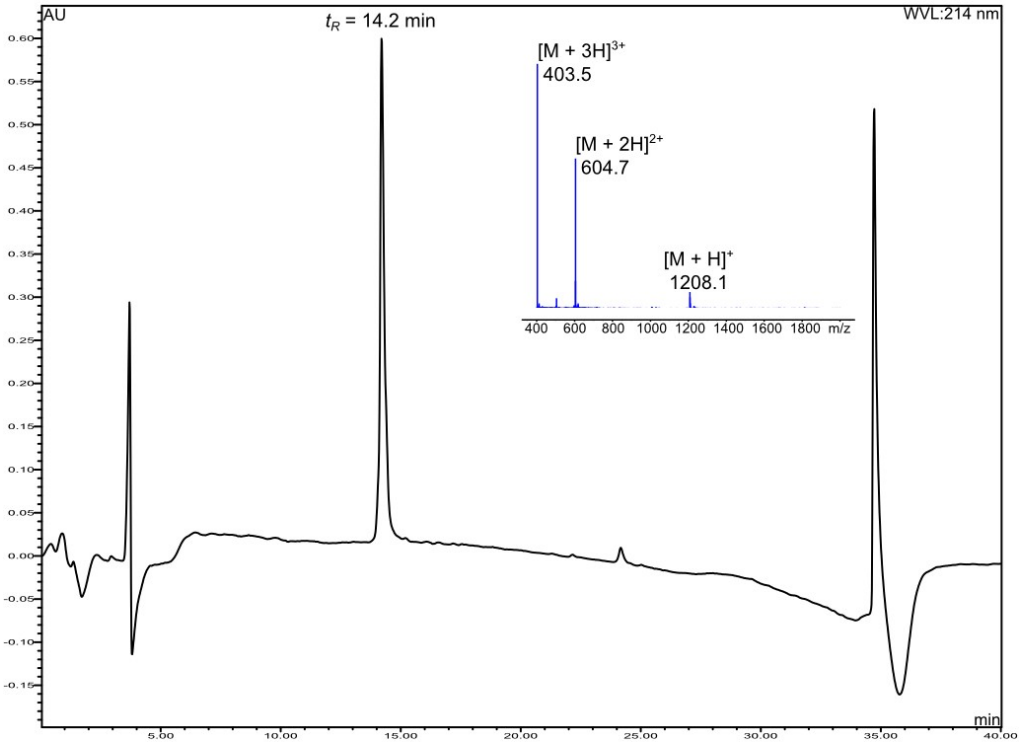
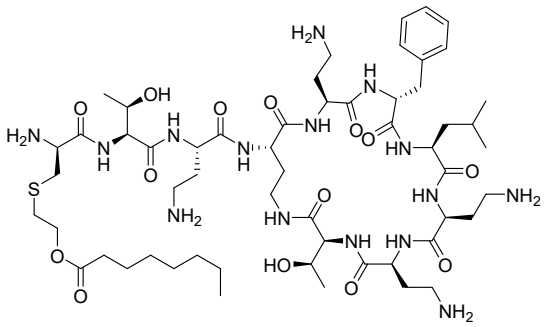


Figure S13: RP-HPLC (214 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7d** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-octanoate (CLipPA) analogue 7e



7e

Crude product **5** (21 mg, 5.56 µmol) prepared *via* **Repeat 2a**, was dissolved in sparged NMP (1 mL) to which vinyl octanoate (75.3 µL, 389 µmol), *tert*-nonyl mercaptan (83.3 µL, 445 µmol), TIPS (91.2 µL, 445 µmol), DMPA (2.84 mg, 11.1 µmol) and TFA (52.6 µL) were added. CLipPA was performed for 1 h 20 min according to **General Method D** to afford purified product **7e** as a white powder (1.20 mg, 17.5 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 15.7 min; **ESI-MS**: $[M + H]^+$ found 1236.8, $[C_{56}H_{97}N_{15}O_{14}S + H]^+$ requires 1236.71.

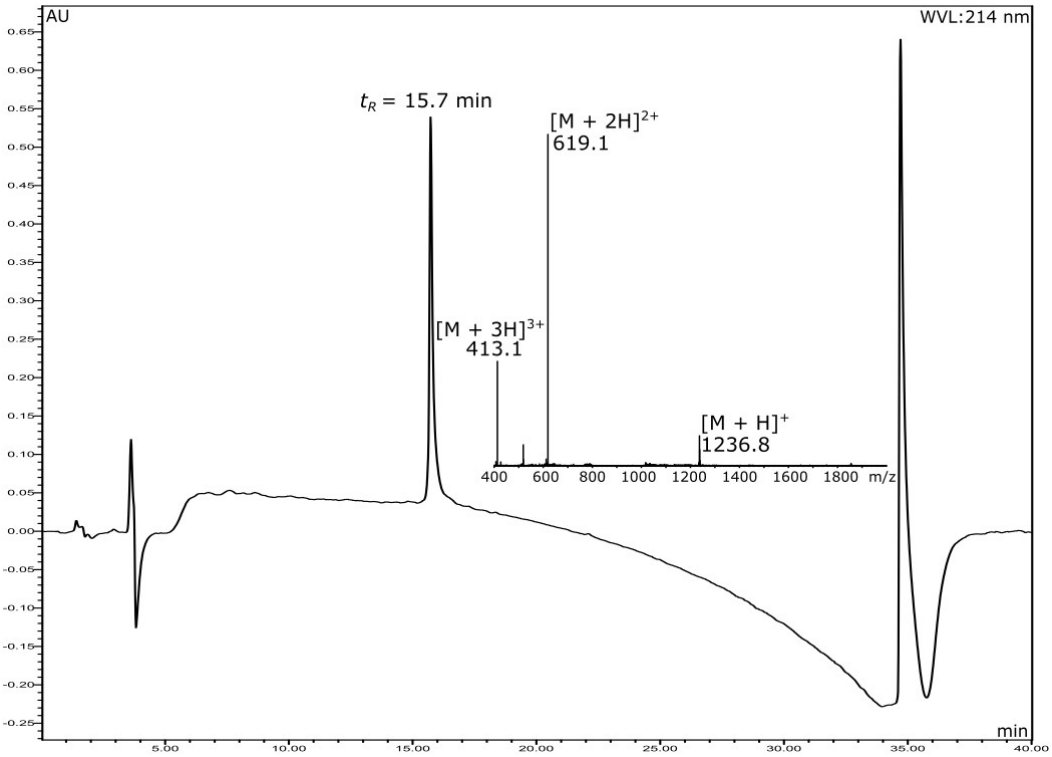
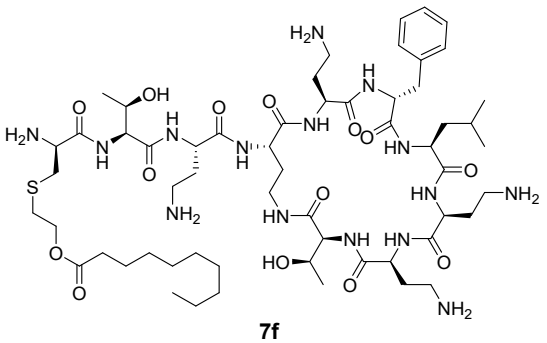


Figure S14: RP-HPLC (214 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7e** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-decanoate (CLipPA) analogue 7f



7f

Crude product **5** (21 mg, 5.56 µmol) prepared *via* **Repeat 2a**, was dissolved in sparged NMP (1 mL) to which vinyl decanoate (87.1 µL, 389 µmol), *tert*-nonyl mercaptan (83.3 µL, 445 µmol), TIPS (91.2 µL, 445 µmol), DMPA (2.84 mg, 11.1 µmol) and TFA (52.6 µL) were added. CLipPA was performed for 1 h 20 min according to **General Method D** to afford purified product **7f** as a white powder (1.19 mg, 16.9 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 17.2 min; **ESI-MS**: $[M + H]^+$ found 1265.0, $[C_{58}H_{101}N_{15}O_{14}S + H]^+$ requires 1264.75.

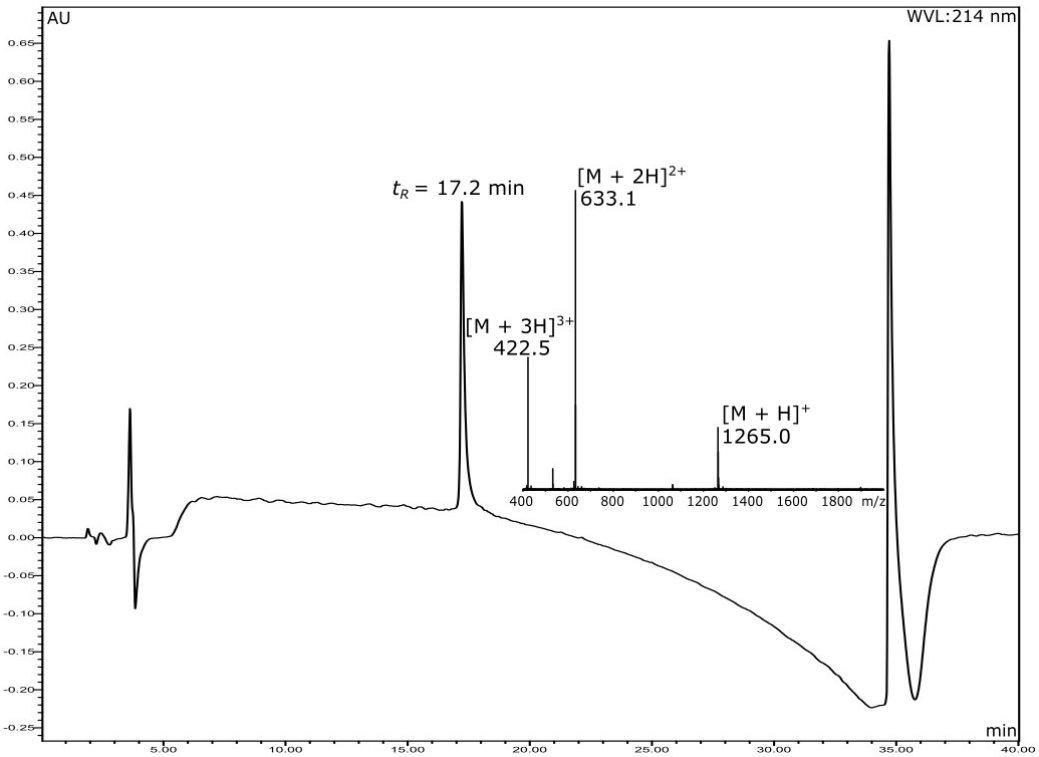
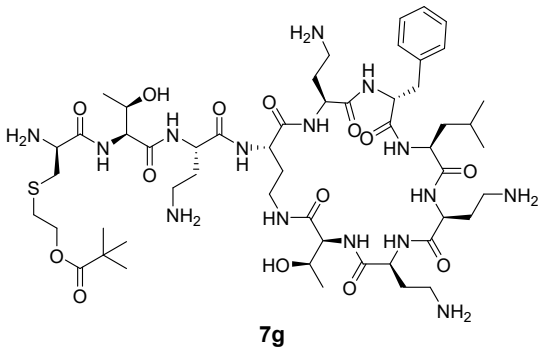


Figure S15: RP-HPLC (214 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7f** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-pivalate (CLipPA) analogue 7g



7g

Enriched product **5** (7.5 mg, 7.03 µmol) prepared *via* **Repeat 2b**, was dissolved in sparged NMP (790 µL) to which vinyl pivalate (72.8 µL, 492 µmol), *tert*-nonyl mercaptan (105 µL, 562 µmol), TIPS (115 µL, 562 µmol), DMPA (3.61 mg, 14.1 µmol) and TFA (41.6 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7g** as a white powder (1.86 mg, 9.6 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 13.4 min; **ESI-MS**: $[M + H]^+$ found 1194.1, $[C_{53}H_{91}N_{15}O_{14}S + H]^+$ requires 1194.67.

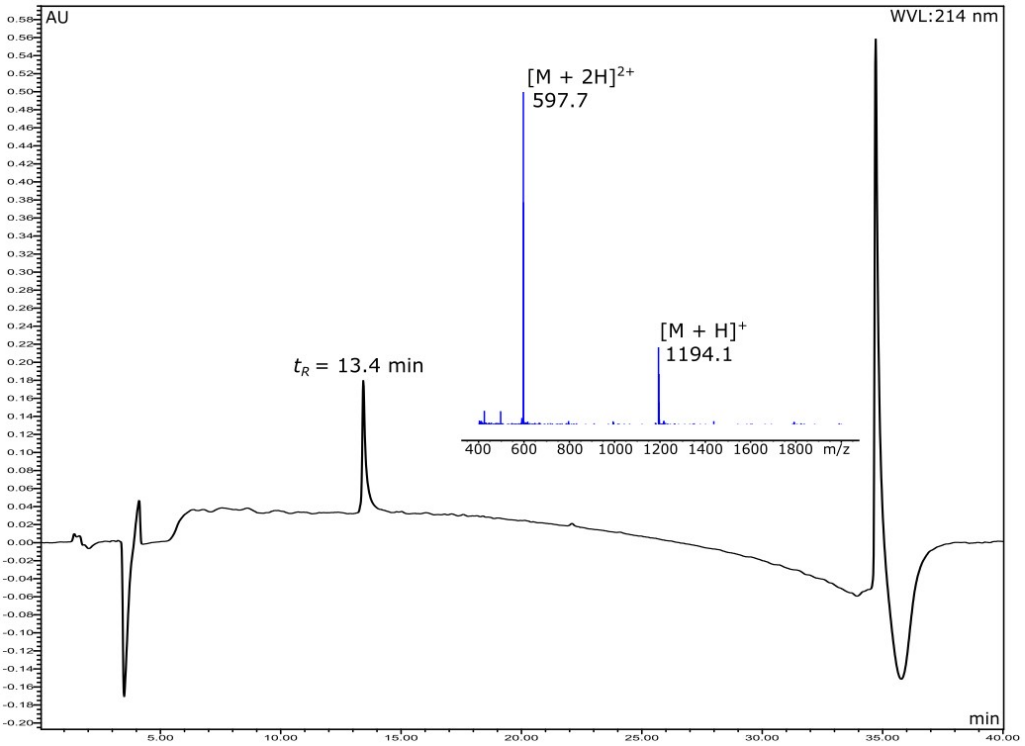
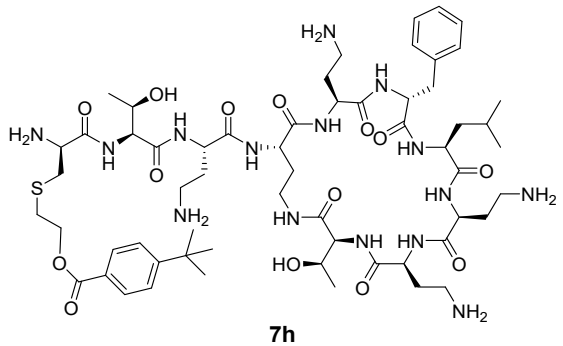


Figure S16: RP-HPLC (214 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7g** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-4-*t*Bu-benzoate (CLipPA) analogue 7h



7h

Crude product **5** (21 mg, 5.56 µmol) prepared *via* **Repeat 2a**, was dissolved in sparged NMP (1 mL) to which vinyl 4-*t*Bu-benzoate (79.5 µL, 389 µmol), *tert*-nonyl mercaptan (83.3 µL, 445 µmol), TIPS (91.2 µL, 445 µmol), DMPA (2.84 mg, 11.1 µmol) and TFA (52.6 µL) were added. CLipPA was performed for 1 h 20 min according to **General Method D** to afford purified product **7h** as a white powder (1.00 mg, 14.2 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 15.8 min; **ESI-MS**: $[M + H]^+$ found 1270.8, $[C_{59}H_{95}N_{15}O_{14}S + H]^+$ requires 1270.70.

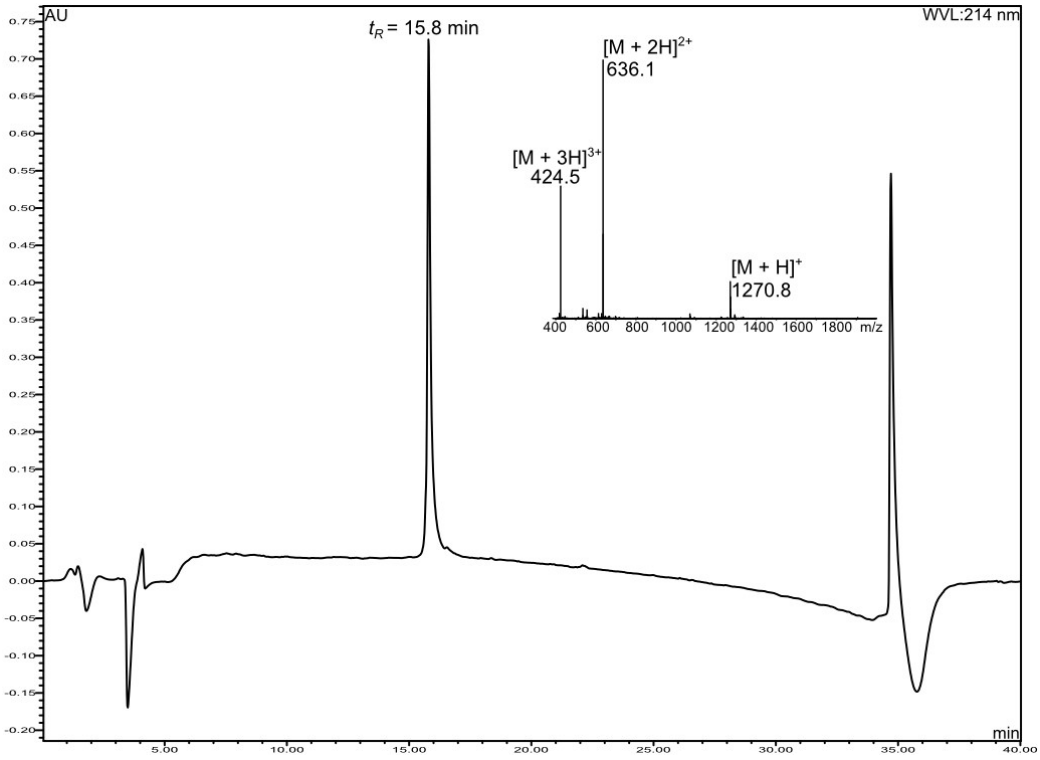
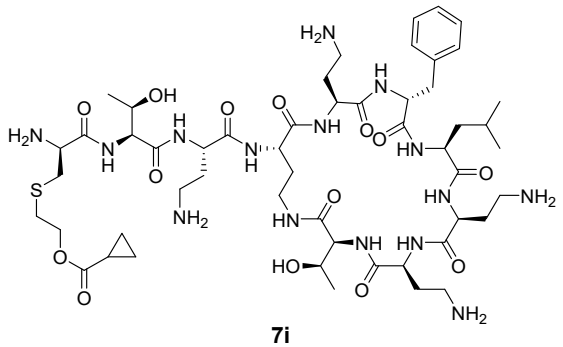


Figure S17: RP-HPLC (214 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7h** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-cyclopropionate (CLipPA) analogue 7i



7i

Enriched product **5** (3.72 mg, 3.49 µmol) prepared *via* **Repeat 3**, was dissolved in sparged NMP (350 µL) to which vinyl cyclopropionate (**9a**, 27.4 mg, 244 µmol), *tert*-nonyl mercaptan (52.3 µL, 279 µmol), TIPS (57.2 µL, 279 µmol), DMPA (1.79 mg, 6.98 µmol) and TFA (18.4 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7i** as a white powder (0.65 mg, 11.6 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 13.0 min; **ESI-MS**: $[M + H]^+$ found 1178.1, $[C_{52}H_{87}N_{15}O_{14}S + H]^+$ requires 1178.64.

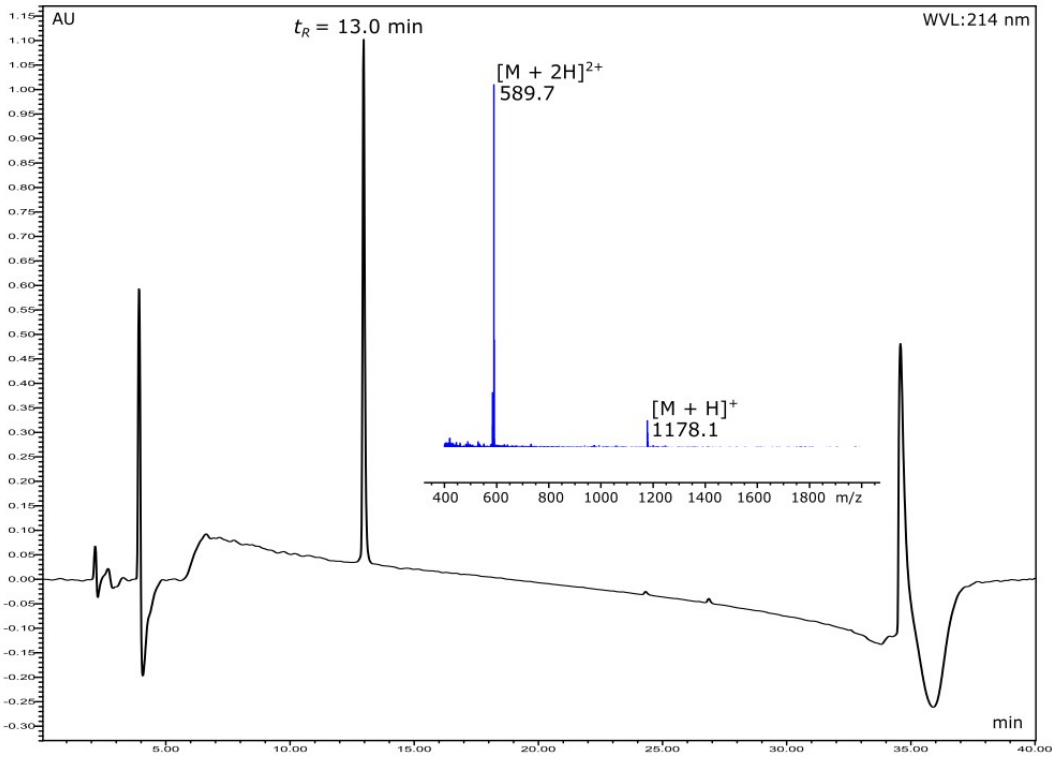
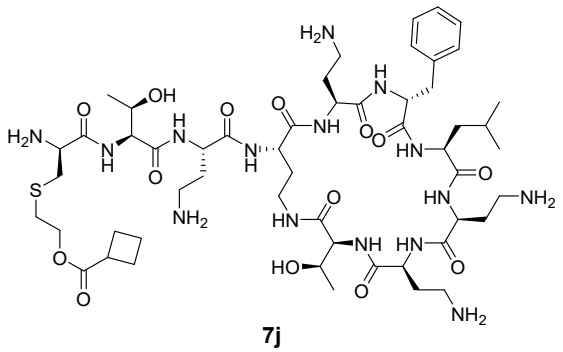


Figure S18: RP-HPLC (214 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7i** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-cyclobutyrate (CLipPA) analogue 7j



7j

Enriched product **5** (30 mg, 28.1 µmol) prepared *via* **Repeat 4**, was dissolved in sparged NMP (2 mL) to which vinyl cyclobutyrate (**9b**, 249 mg**,** 1.97 mmol), *tert*-nonyl mercaptan (421 µL, 2.25 mmol), TIPS (461 µL, 2.25 mmol), DMPA (14.4 mg, 56.2 µmol) and TFA (105 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7j** as a white powder (2.05 mg, 4.0 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 11.7 min; **ESI-MS**: $[M + H]^+$ found 1192.8, $[C_{53}H_{89}N_{15}O_{14}S + H]^+$ requires 1192.65.

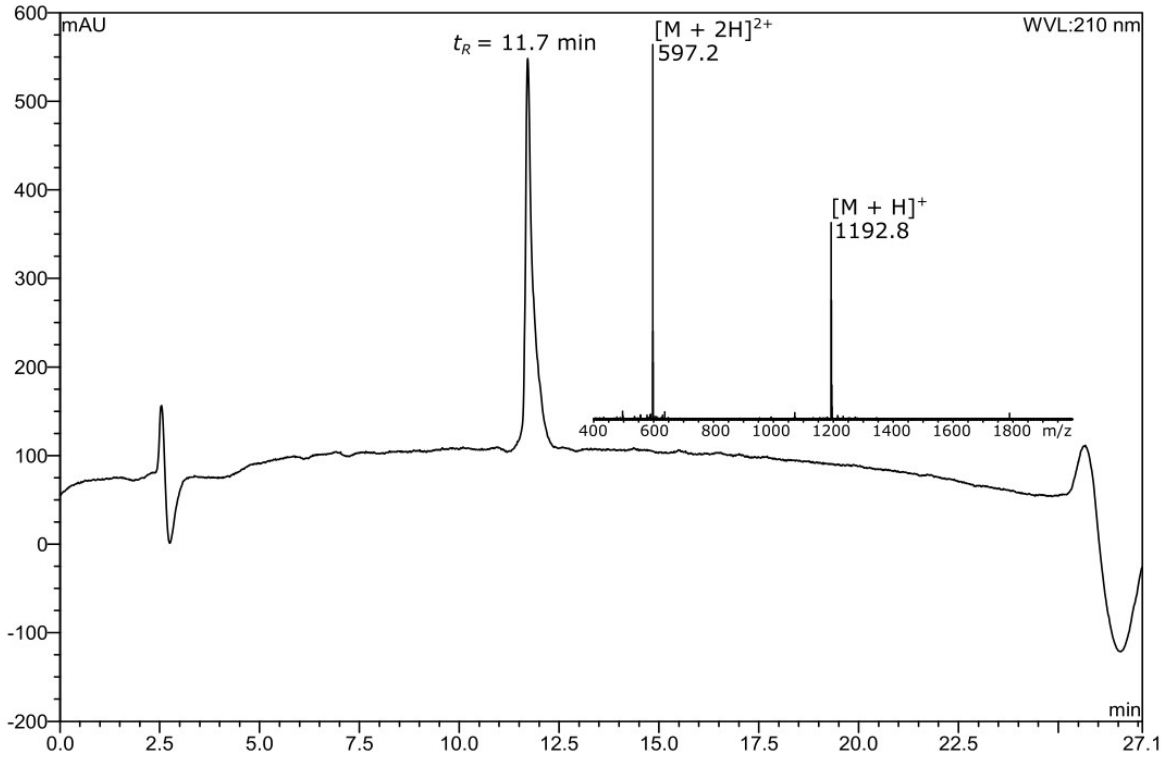
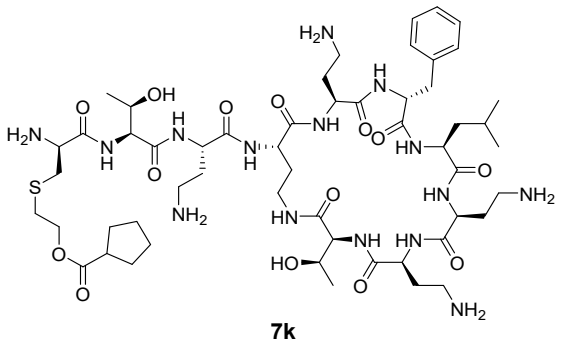


Figure S19: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7j** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 65 % B over 20 min), 1 mL/min flow rate at rt, through a Waters Xterra C18 column (125 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-cyclovalerate (CLipPA) analogue 7k



7k

Enriched product **5** (30 mg, 28.1 µmol) prepared *via* **Repeat 4**, was dissolved in sparged NMP (2 mL) to which vinyl cyclovalerate (**9c**, 276 mg**,** 1.97 mmol), *tert*-nonyl mercaptan (421 µL, 2.25 mmol), TIPS (461 µL, 2.25 mmol), DMPA (14.4 mg, 56.2 µmol) and TFA (105 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7k** as a white powder (2.28 mg, 4.4 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 12.3 min; **ESI-MS**: $[M + H]^+$ found 1206.8, $[C_{54}H_{91}N_{15}O_{14}S + H]^+$ requires 1206.67.

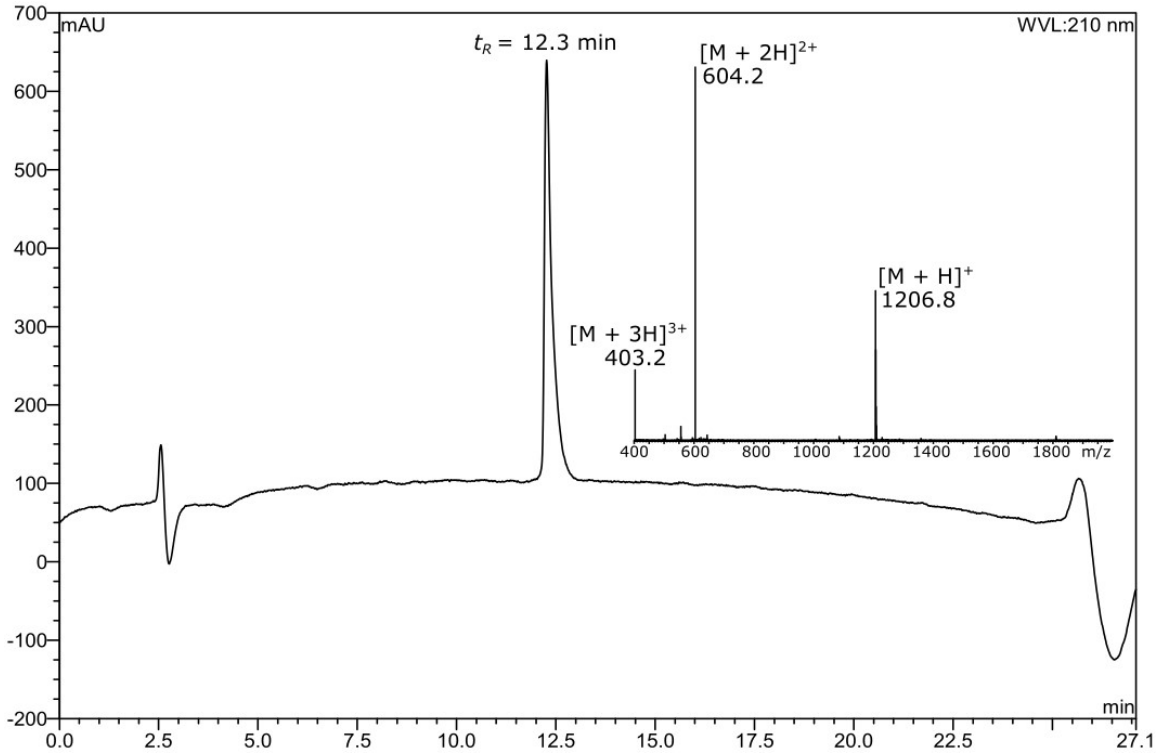
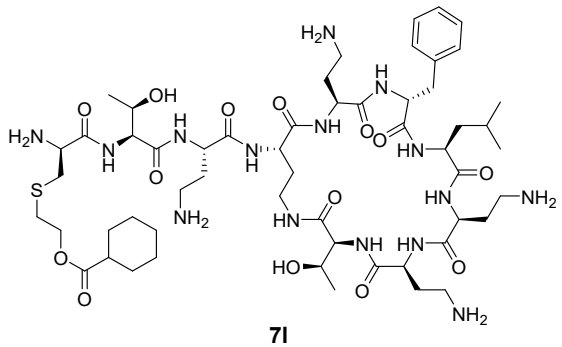


Figure S20: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7k** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 65 % B over 20 min), 1 mL/min flow rate at rt, through a Waters Xterra C18 column (125 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-cyclohexanoate (CLipPA) analogue 7l



7l

Enriched product **5** (3.72 mg, 3.49 µmol) prepared *via* **Repeat 3**, was dissolved in sparged NMP (350 µL) to which vinyl cyclohexanoate (**9d**, 37.6 mg**,** 244 µmol), *tert*-nonyl mercaptan (52.3 µL, 279 µmol), TIPS (57.2 µL, 279 µmol), DMPA (1.79 mg, 6.98 µmol) and TFA (18.4 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7l** as a white powder (0.79 mg, 13.6 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 14.5 min; **ESI-MS**: $[M + H]^+$ found 1220.9, $[C_{55}H_{93}N_{15}O_{14}S + H]^+$ requires 1220.68.

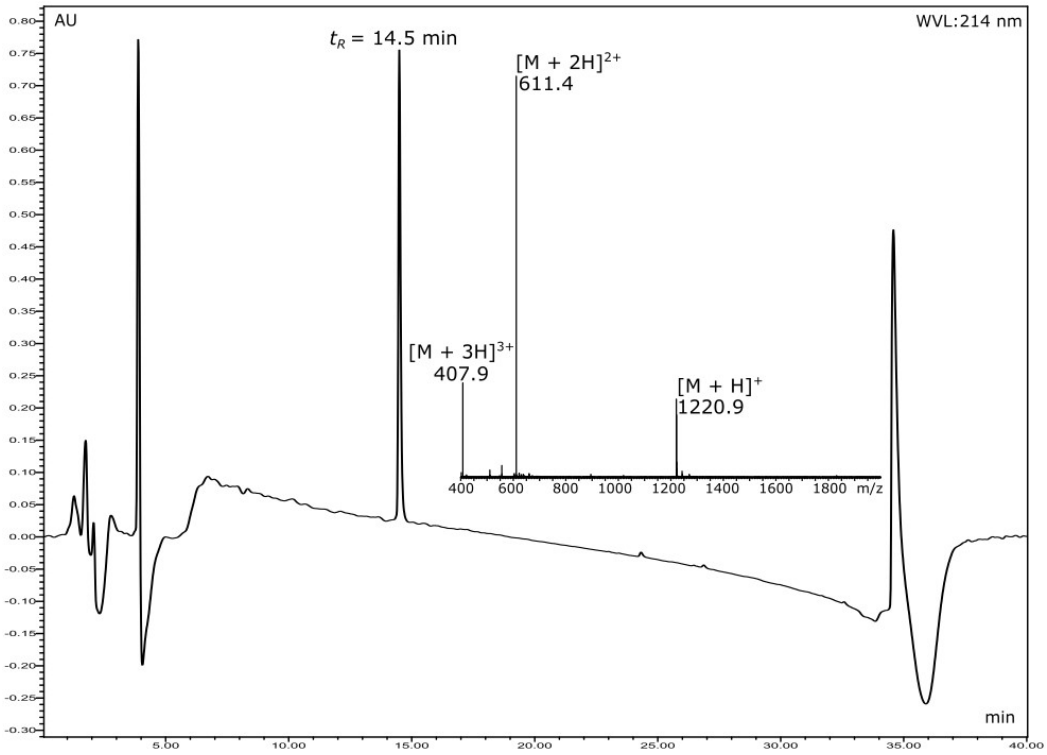
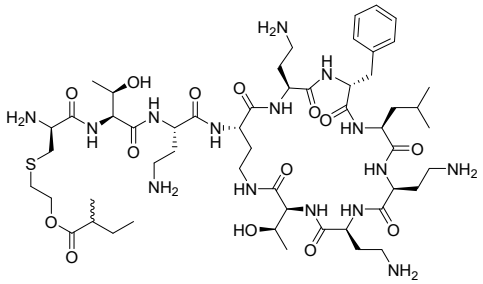


Figure S21: RP-HPLC (214 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7l** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-2-Me-butyrate (CLipPA) analogue 7m



7m

Enriched product **5** (10 mg, 9.38 µmol) prepared *via* **Repeat 4**, was dissolved in sparged NMP (1 mL) to which vinyl 2-Me-butyrate (**9e**, 84.2 mg**,** 657 µmol), *tert*-nonyl mercaptan (140 µL, 750 µmol), TIPS (154 µL, 750 µmol), DMPA (4.82 mg, 18.8 µmol) and TFA (52.6 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7m** as a white powder (0.76 mg, 4.5 % yield [5 steps], 98 % purity); **RP-HPLC**: t_R = 12.0 min; **ESI-MS**: $[M + H]^+$ found 1194.9, $[C_{53}H_{91}N_{15}O_{14}S + H]^+$ requires 1194.67.

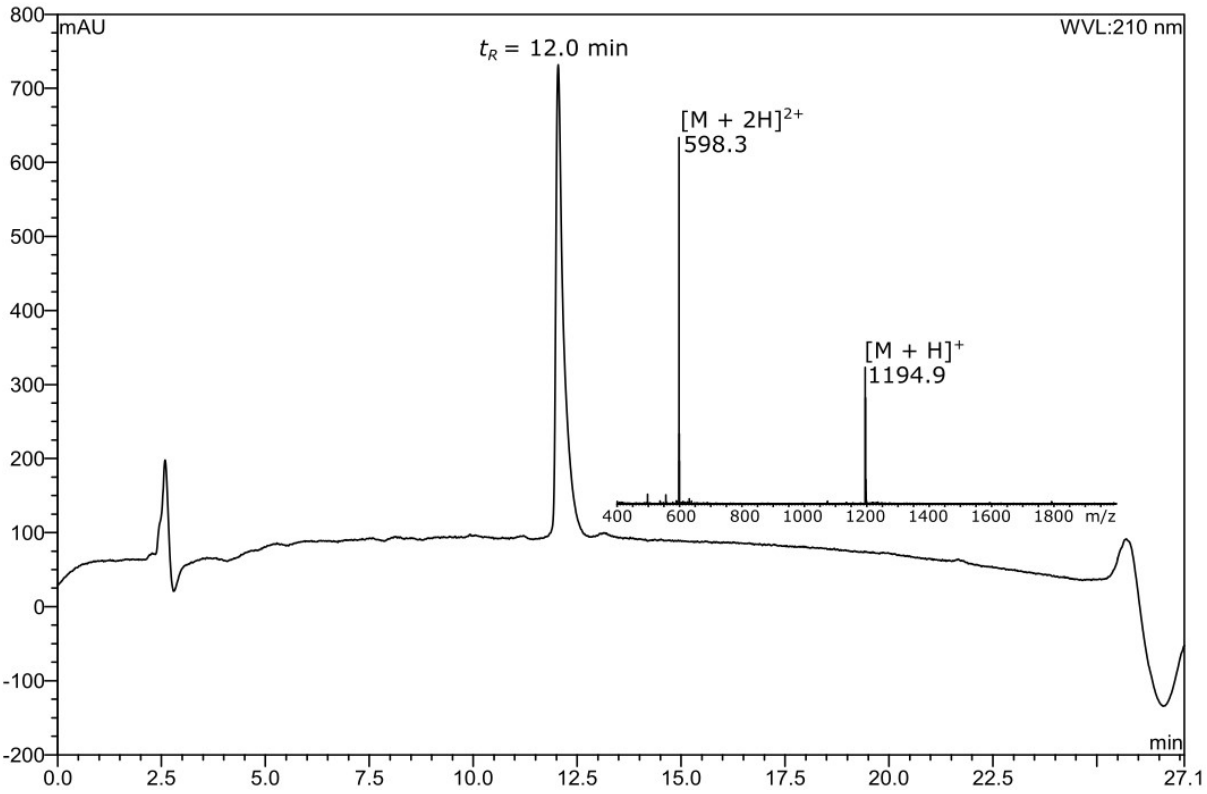
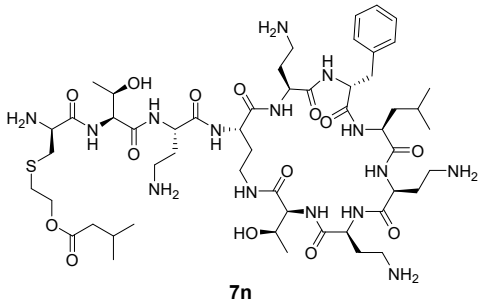


Figure S22: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7m** (*ca.* 98 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 65 % B over 20 min), 1 mL/min flow rate at rt, through a Waters Xterra C18 column (125 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-3-Me-butyrate (CLipPA) analogue 7n



7n

Enriched product **5** (30 mg, 28.1 µmol) prepared *via* **Repeat 4**, was dissolved in sparged NMP (2 mL) to which vinyl 3-Me-butyrate (**9f**, 252 mg, 1.97 mmol), *tert*-nonyl mercaptan (421 µL, 2.25 mmol), TIPS (461 µL, 2.25 mmol), DMPA (14.4 mg, 56.2 µmol) and TFA (105 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7n** as a white powder (2.10 mg, 4.1 % yield [5 steps], 98 % purity); **RP-HPLC**: t_R = 12.1 min; **ESI-MS**: $[M + H]^+$ found 1194.9, $[C_{53}H_{91}N_{15}O_{14}S + H]^+$ requires 1194.67.

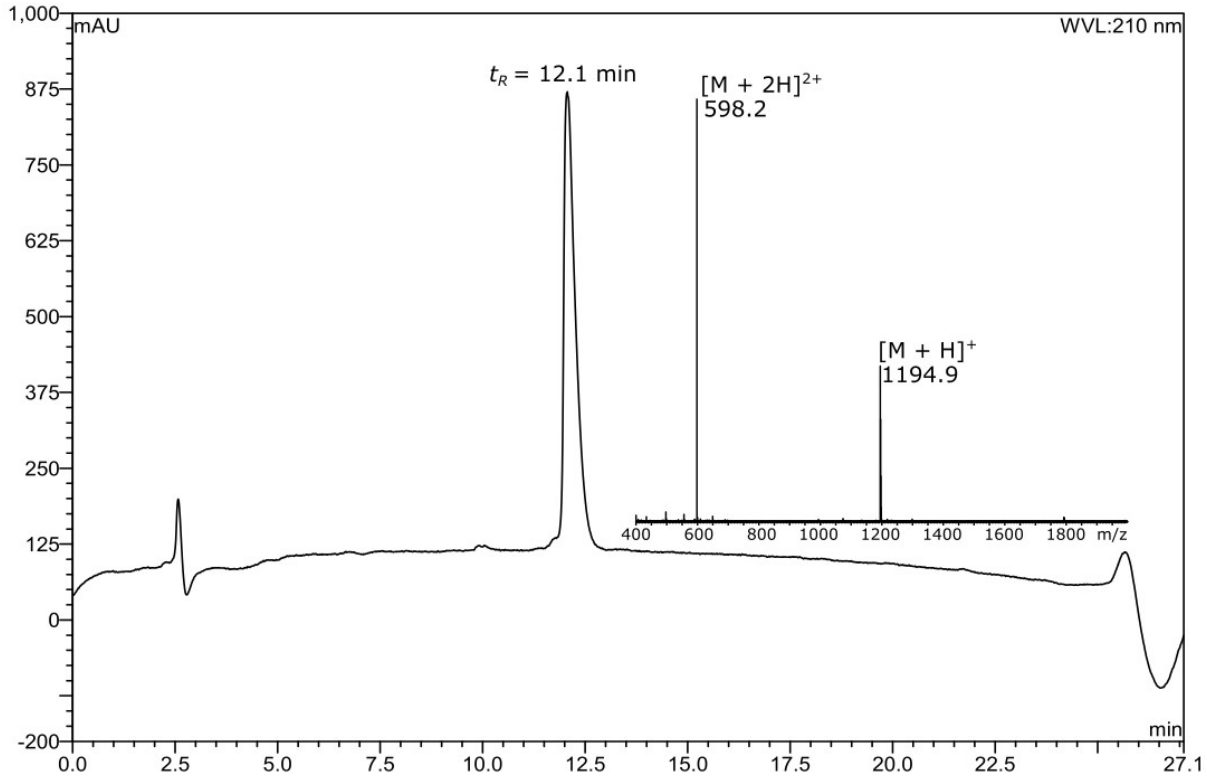
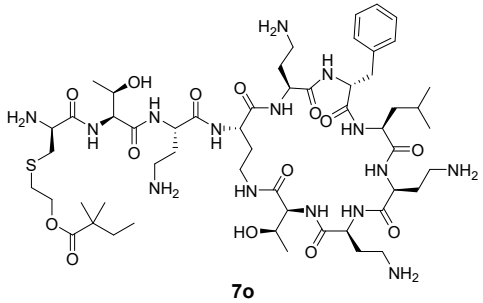


Figure S23: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7n** (*ca.* 98 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 65 % B over 20 min), 1 mL/min flow rate at rt, through a Waters Xterra C18 column (125 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-2,2-di-Me-butyrate (CLipPA) analogue 7o



7o

Enriched product **5** (30 mg, 28.1 µmol) prepared *via* **Repeat 4**, was dissolved in sparged NMP (2 mL) to which vinyl 2,2-di-Me-butyrate (**9g**, 280 mg, 1.97 mmol), *tert*-nonyl mercaptan (421 µL, 2.25 mmol), TIPS (461 µL, 2.25 mmol), DMPA (14.4 mg, 56.2 µmol) and TFA (105 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7o** as a white powder (1.69 mg, 3.3 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 12.6 min; **ESI-MS**: $[M + H]^+$ found 1208.9, $[C_{54}H_{93}N_{15}O_{14}S + H]^+$ requires 1208.68.

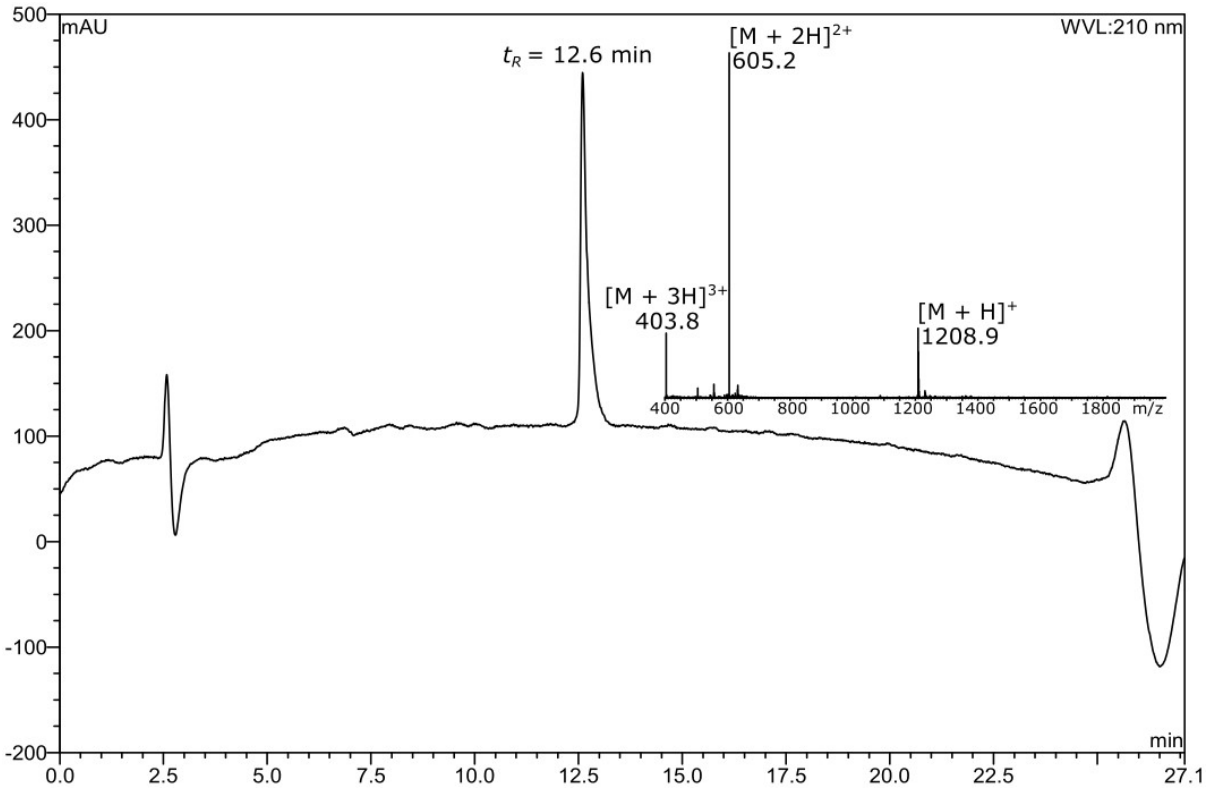
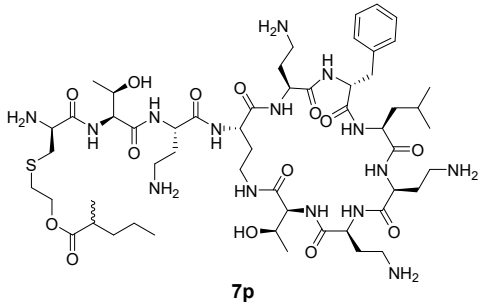


Figure S24: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7o** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 65 % B over 20 min), 1 mL/min flow rate at rt, through a Waters Xterra C18 column (125 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-2-Me-valerate (CLipPA) analogue 7p



7p

Enriched product **5** (30 mg, 28.1 µmol) prepared *via* **Repeat 4**, was dissolved in sparged NMP (2 mL) to which vinyl 2-Me-valerate (**9h**, 280 mg, 1.97 mmol), *tert*-nonyl mercaptan (421 µL, 2.25 mmol), TIPS (461 µL, 2.25 mmol), DMPA (14.4 mg, 56.2 µmol) and TFA (105 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7p** as a white powder (4.36 mg, 8.4 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 14.7 min; **ESI-MS**: $[M + H]^+$ found 1208.8, $[C_{54}H_{93}N_{15}O_{14}S + H]^+$ requires 1208.68.

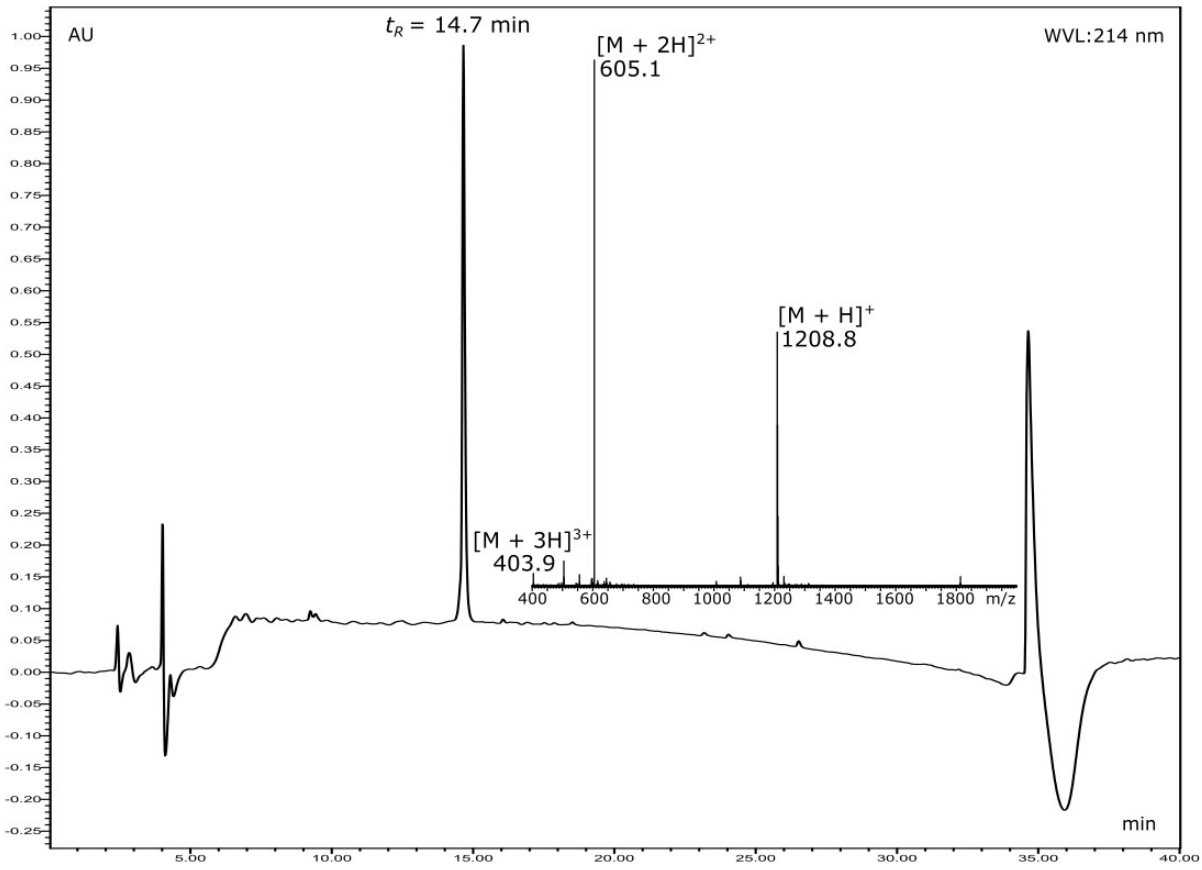
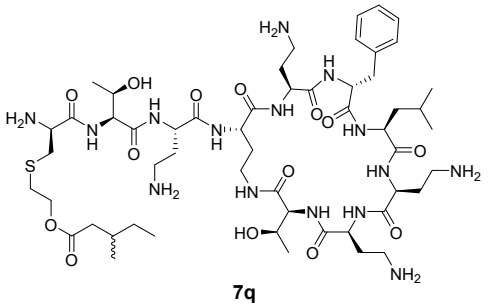


Figure S25: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7p** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow rate at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-3-Me-valerate (CLipPA) analogue 7q



7q

Enriched product **5** (30 mg, 28.1 µmol) prepared *via* **Repeat 4**, was dissolved in sparged NMP (2 mL) to which vinyl 3-Me-valerate (**9i**, 280 mg, 1.97 mmol), *tert*-nonyl mercaptan (421 µL, 2.25 mmol), TIPS (461 µL, 2.25 mmol), DMPA (14.4 mg, 56.2 µmol) and TFA (105 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7q** as a white powder (1.23 mg, 2.4 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 12.7 min; **ESI-MS**: $[M + H]^+$ found 1208.8, $[C_{54}H_{93}N_{15}O_{14}S + H]^+$ requires 1208.68.

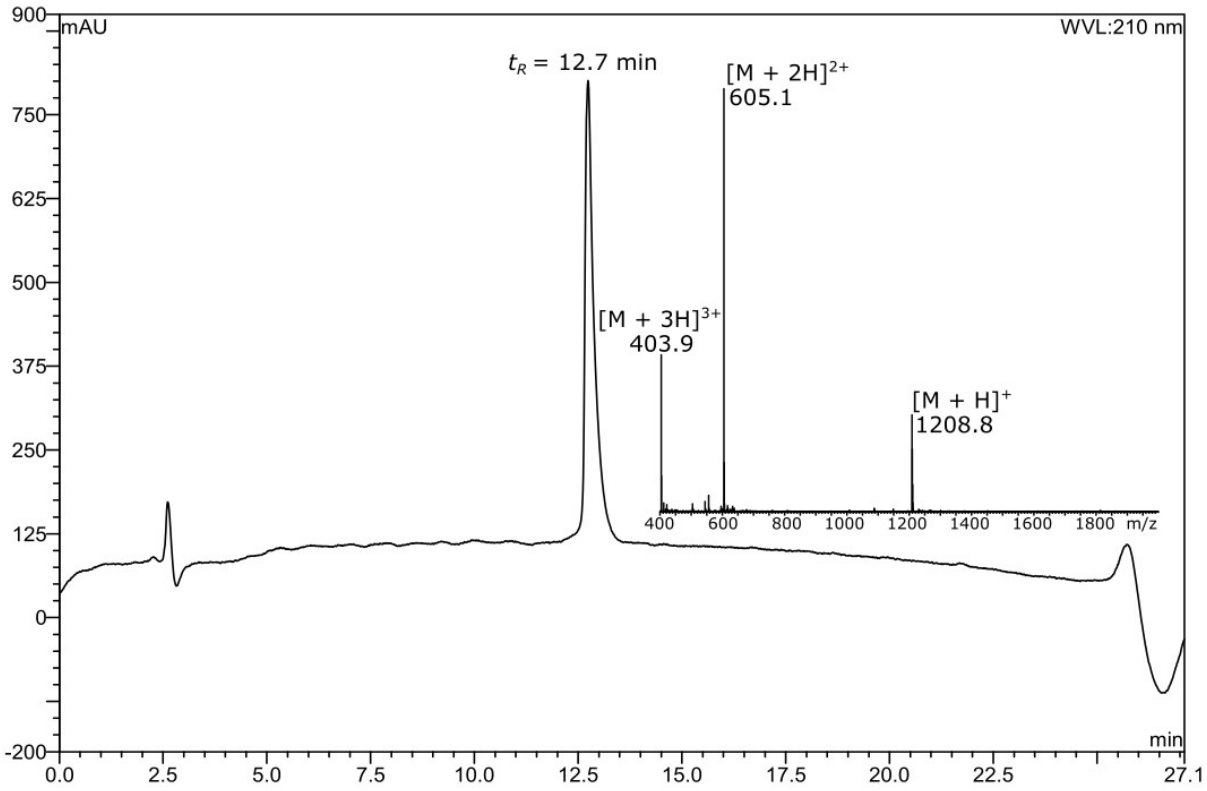
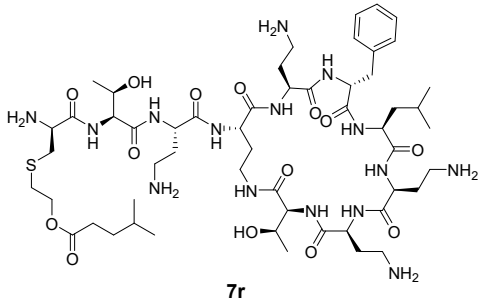


Figure S26: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7q** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 65 % B over 20 min), 1 mL/min flow rate at rt, through a Waters Xterra C18 column (125 Å, 150 mm × 4.60 mm; 5 µm).

Synthesis of D-Cys-4-Me-valerate (CLipPA) analogue 7r



7r

Enriched product **5** (30 mg, 28.1 µmol) prepared *via* **Repeat 4**, was dissolved in sparged NMP (2 mL) to which vinyl 4-Me-valerate (**9j**, 280 mg, 1.97 mmol), *tert*-nonyl mercaptan (421 µL, 2.25 mmol), TIPS (461 µL, 2.25 mmol), DMPA (14.4 mg, 56.2 µmol) and TFA (105 µL) were added. CLipPA was performed for 1 h according to **General Method D** to afford purified product **7r** as a white powder (3.26 mg, 6.3 % yield [5 steps], 99 % purity); **RP-HPLC**: t_R = 12.8 min; **ESI-MS**: $[M + H]^+$ found 1208.8, $[C_{54}H_{93}N_{15}O_{14}S + H]^+$ requires 1208.68.

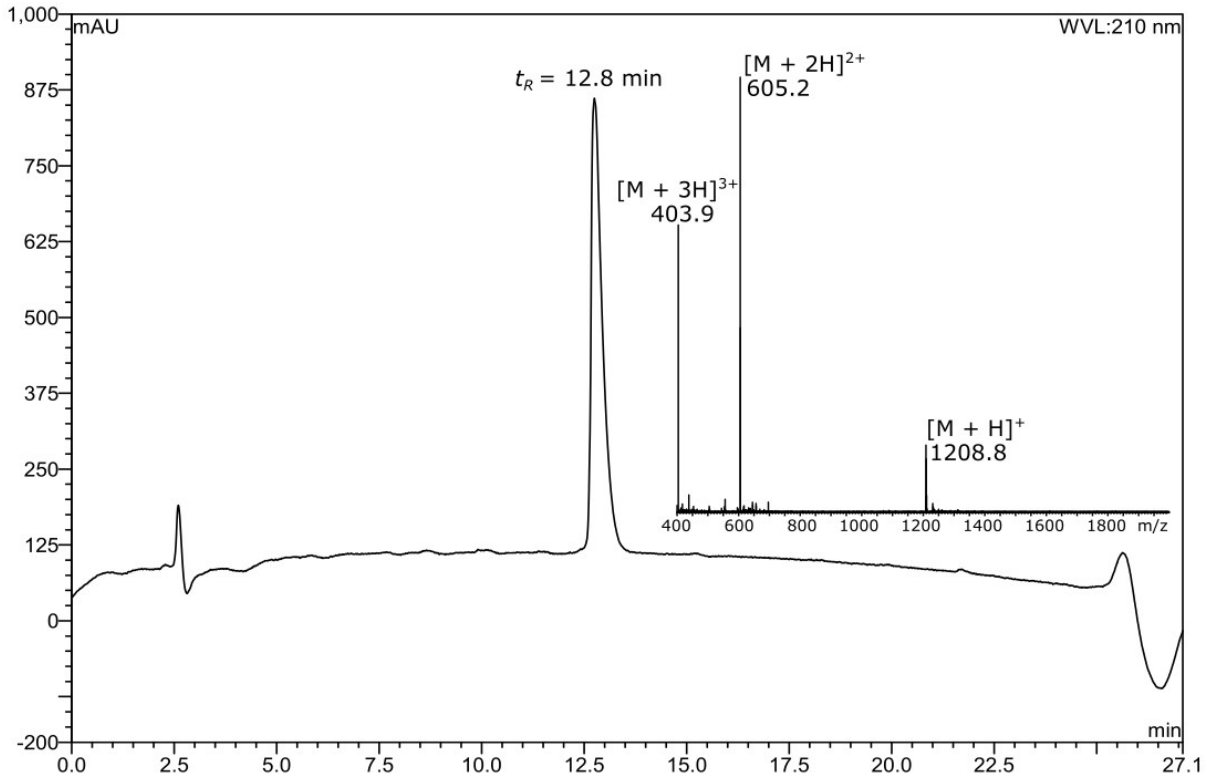


Figure S27: RP-HPLC (210 nm) and ESI-MS (see appendix for original copy) spectra of CLipPA analogue **7r** (*ca.* 99 % estimated purity by peak integration); RP-HPLC setup: linear, 3 % B per min gradient (5 % B to 65 % B over 20 min), 1 mL/min flow rate at rt, through a Waters Xterra C18 column (125 Å, 150 mm × 4.60 mm; 5 µm).

S5. HPLC, ESI-MS and NMR analyses

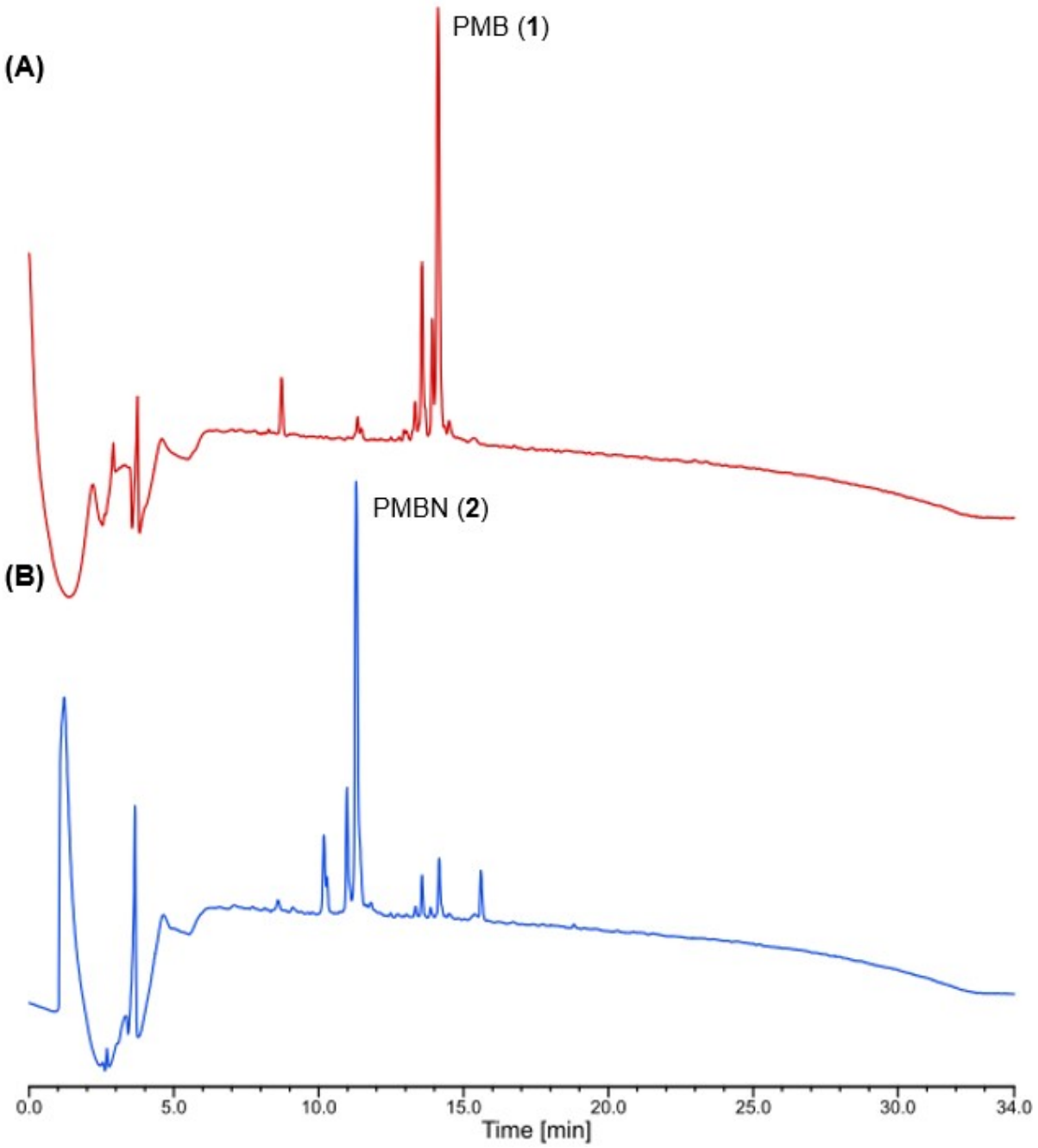


Figure S28: RP-HPLC traces [210 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm x 4.60 mm; 5 µm)] for the enzymatic cleavage of commercial mixture of PMB sulfate (**1**); **(A)** PMB mixture (**1**), **(B)** 20 h reaction.

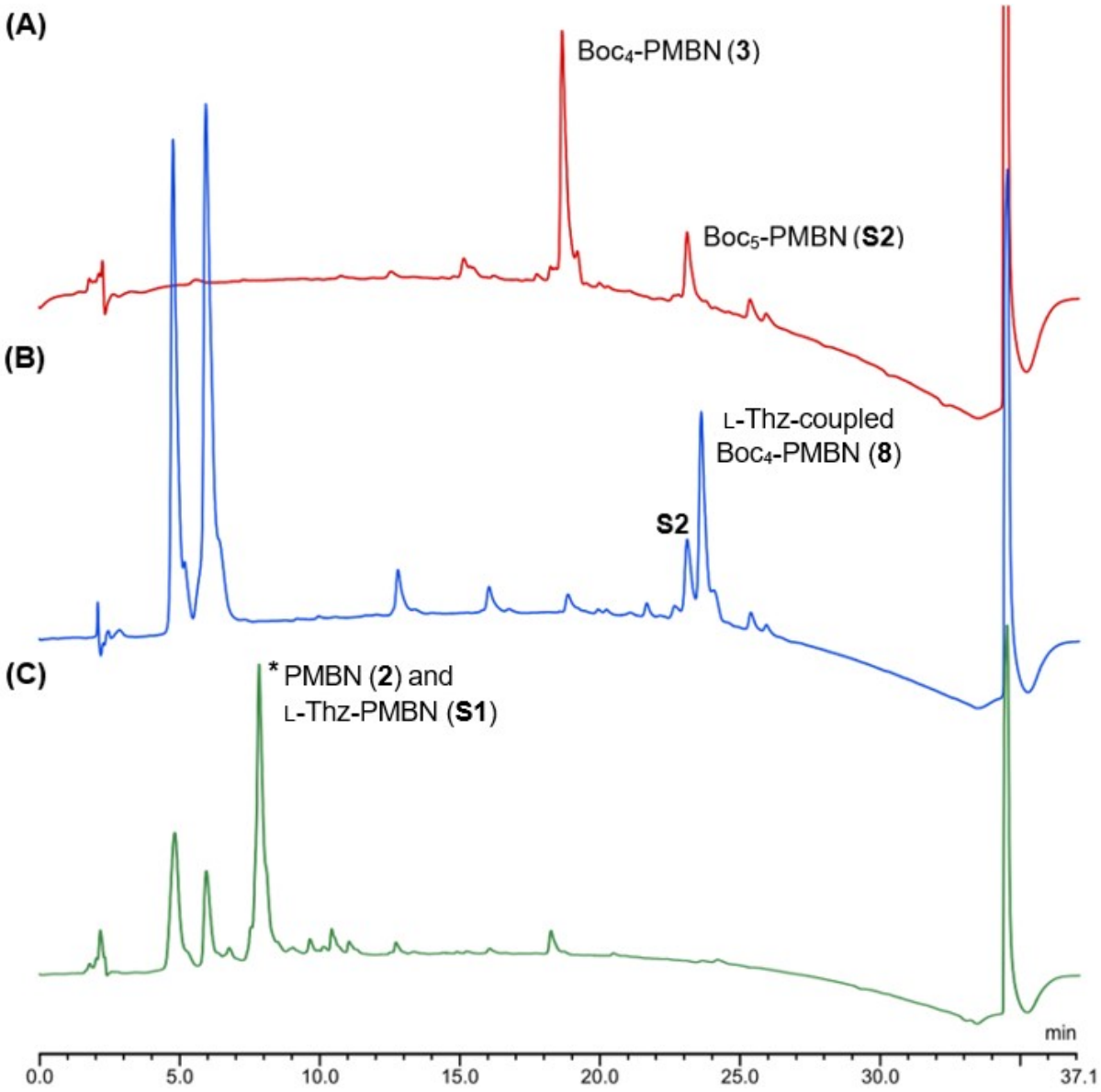


Figure S29: RP-HPLC traces [210 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm)] for the formation of L-Thz-PMBN (**S1**) from the intermediate **3**; **(A)** Tetra-Boc-protected crude intermediate **3**, afforded as a result of side chain Boc-protection of PMBN (**2**) **(B)** Thz-coupling (90 min), and **(C)** after Boc-removal and trituration in Et_2O.

* PMBN (**2**) generated from Boc_5-PMBN (**S2**) during Boc-removal, co-eluted with L-Thz-PMBN (**S1**).

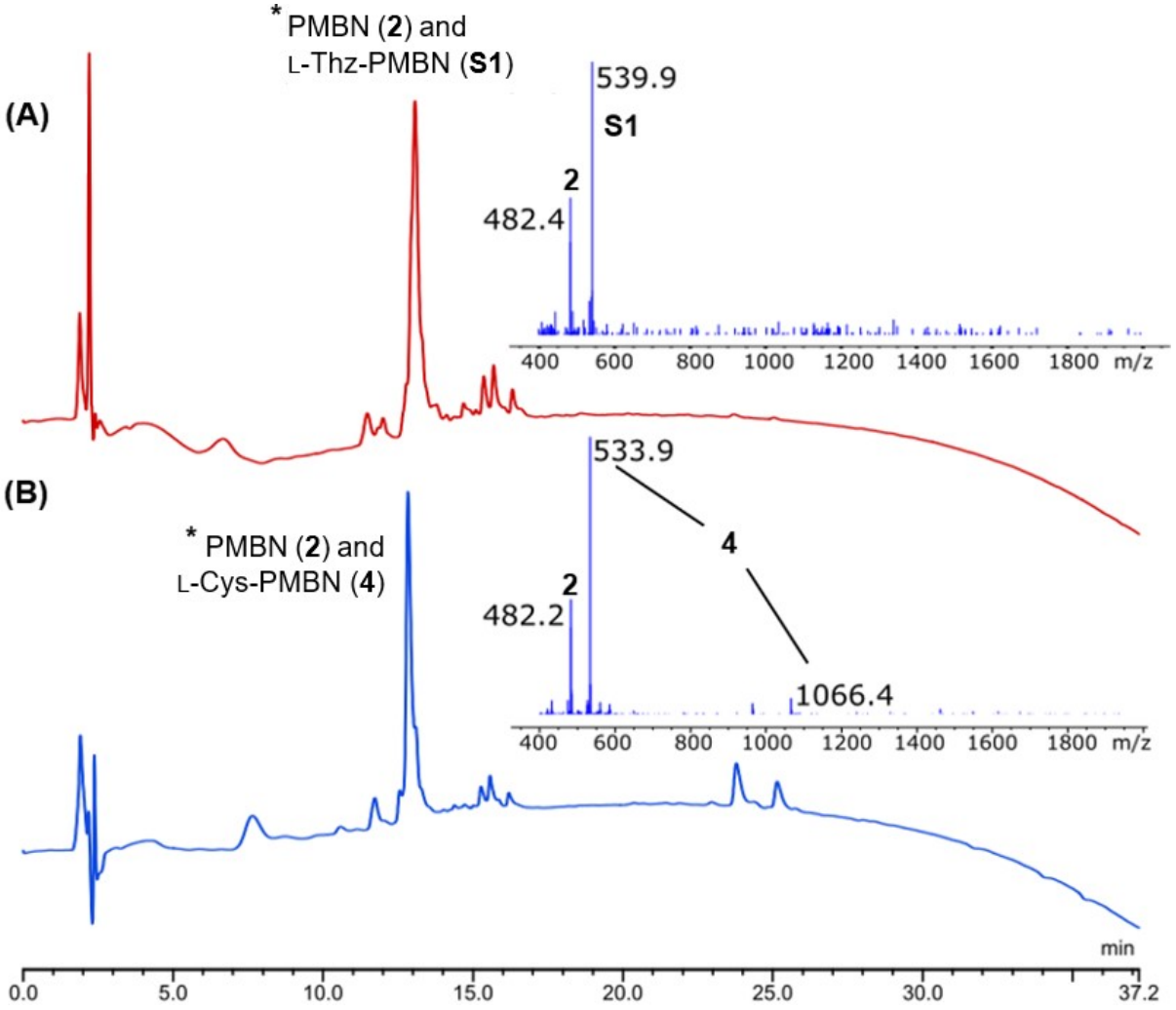


Figure S30: RP-HPLC [210 nm, linear, 3 % B per min gradient (1 % B to 91 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm), 5 min initial divert] and ESI-MS monitoring of Thz ring opening to form L-Cys intermediate **4**; **(A)** t = 0 h and **(B)** t = overnight.

* PMBN (**2**) generated from Boc_5-PMBN (**S2**) during Boc-removal, co-eluted with L-Thz-PMBN (**S1**) and L-Cys-PMBN (**4**).

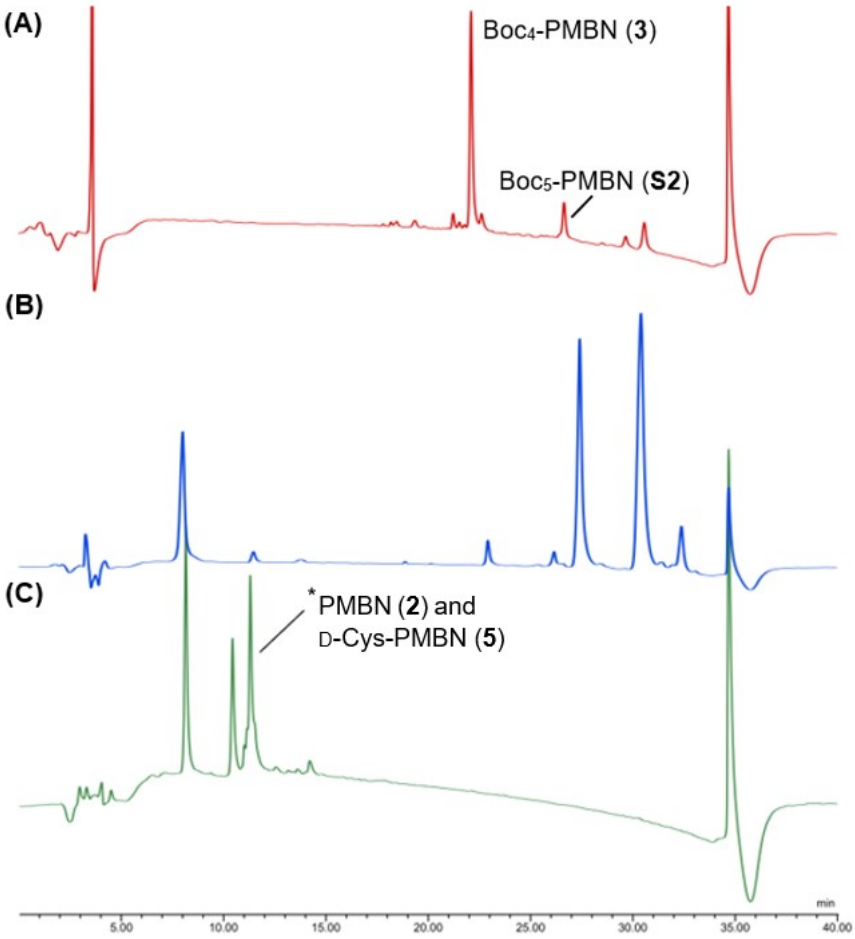


Figure S31: RP-HPLC traces [214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] of synthesis of D-Cys-PMBN (**5**) from intermediate **3**; **(A)** intermediate **3**, **(B)** t = 1 h, and **(C)** after TFA treatment and trituration in Et_2O.

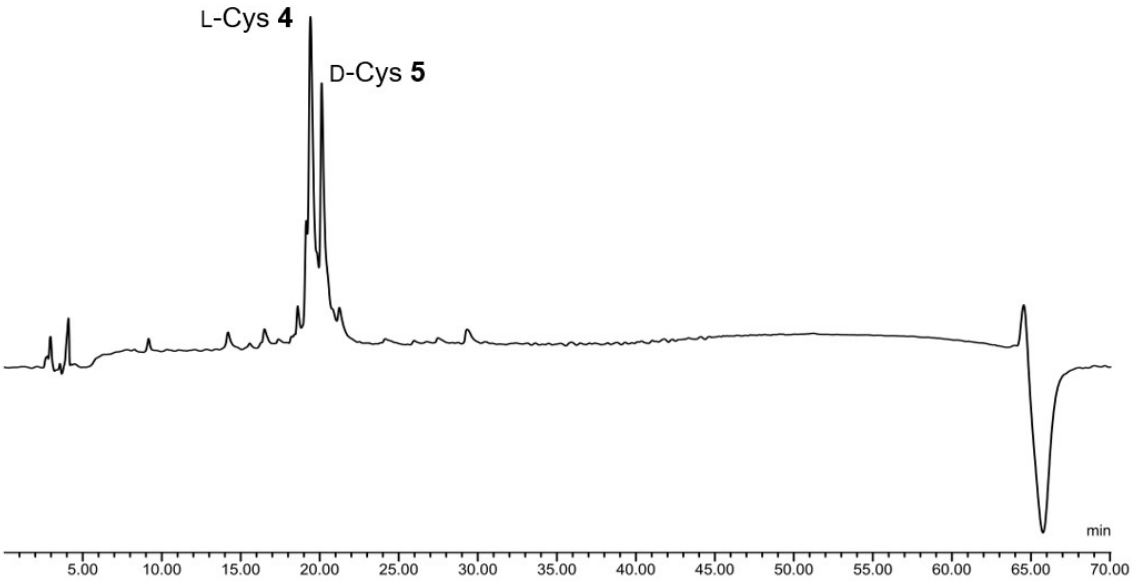


Figure S32: RP-HPLC trace (214 nm) of the co-injection of L-Cys (**4**) and D-Cys (**5**) intermediates showing their distinct retention profiles on a 1 % B per min gradient.

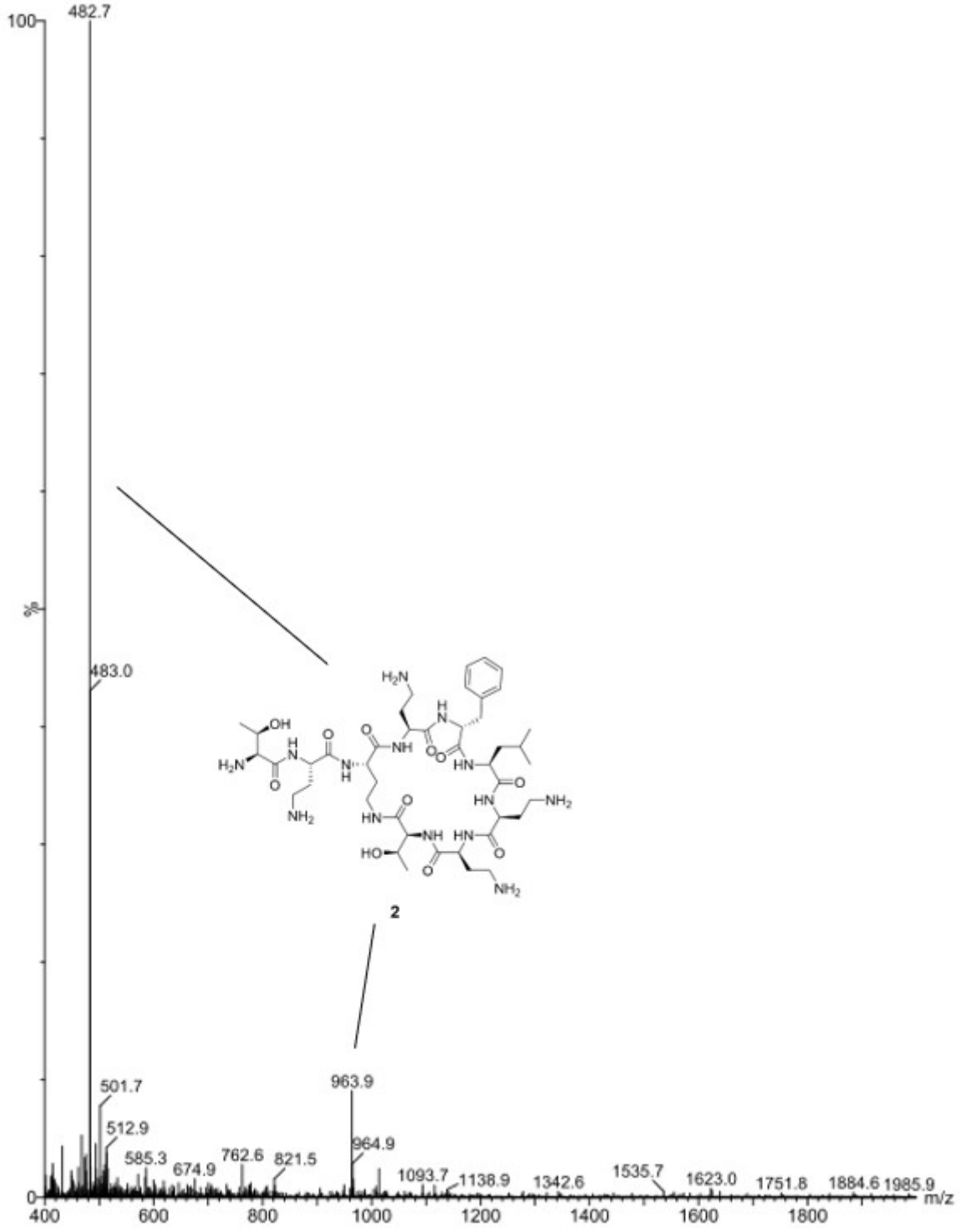


Figure S33: ESI-MS (+ve) spectrum of PMBN (**2**).

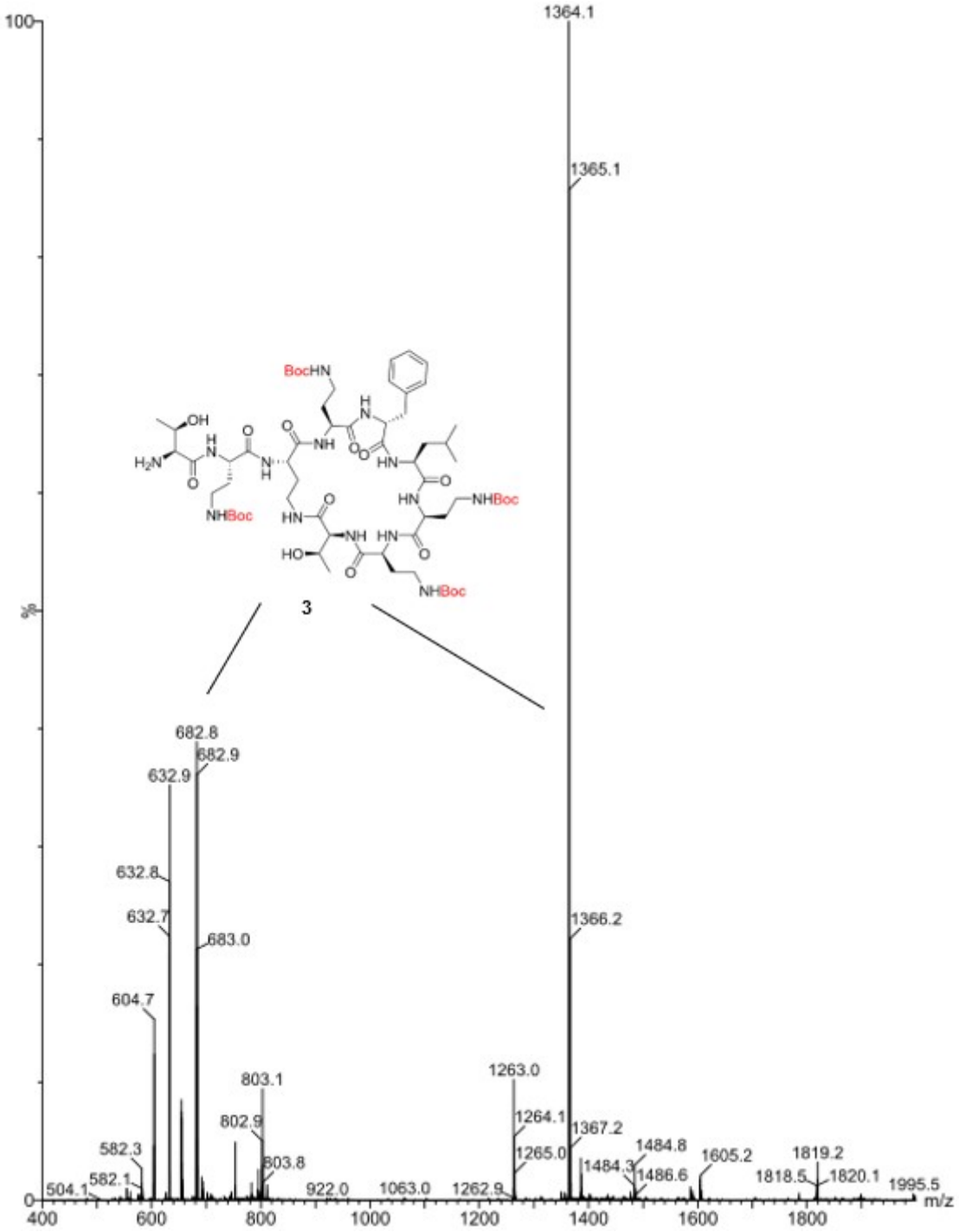


Figure S34: ESI-MS (+ve) spectrum of Boc_4-PMBN (**3**).

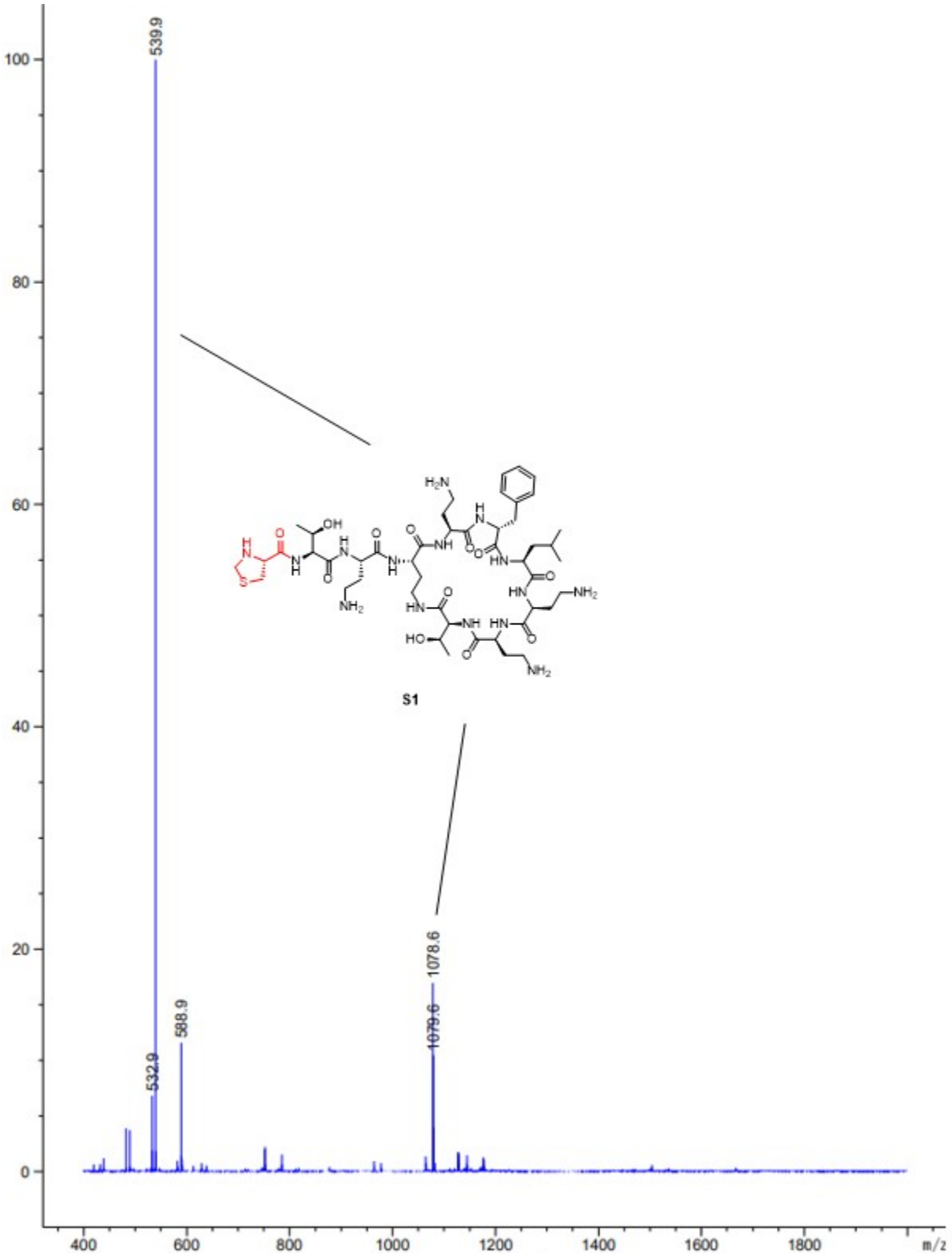


Figure S35: ESI-MS (+ve) spectrum of L-Thz-PMBN (**S1**).

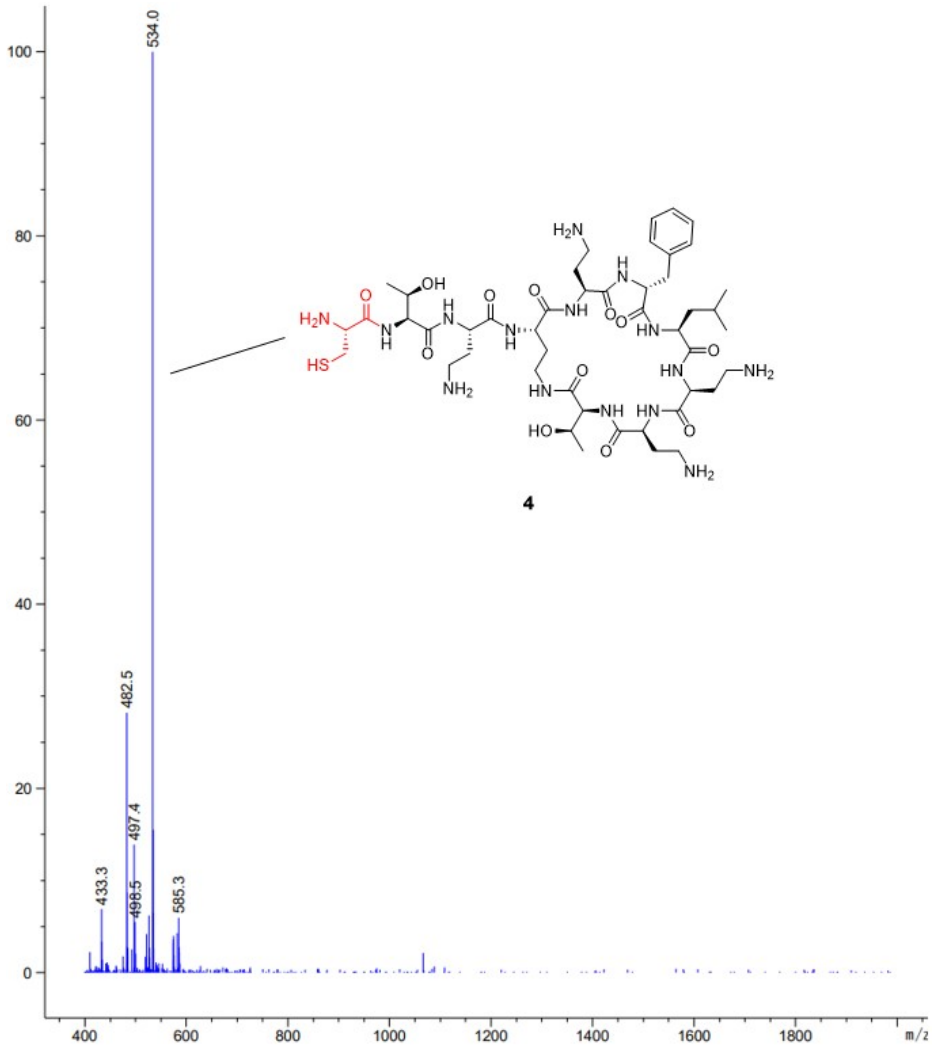


Figure S36: ESI-MS (+ve) spectrum of L-Cys-PMBN (**4**).

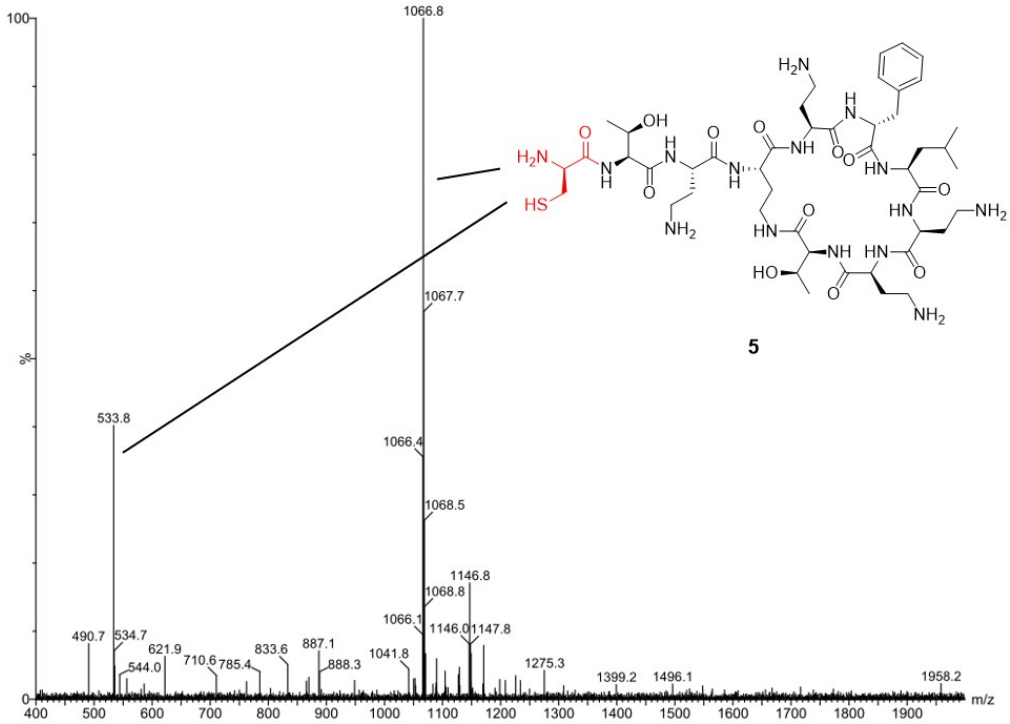


Figure S37: ESI-MS (+ve) spectrum of D-Cys-PMBN (**5**).

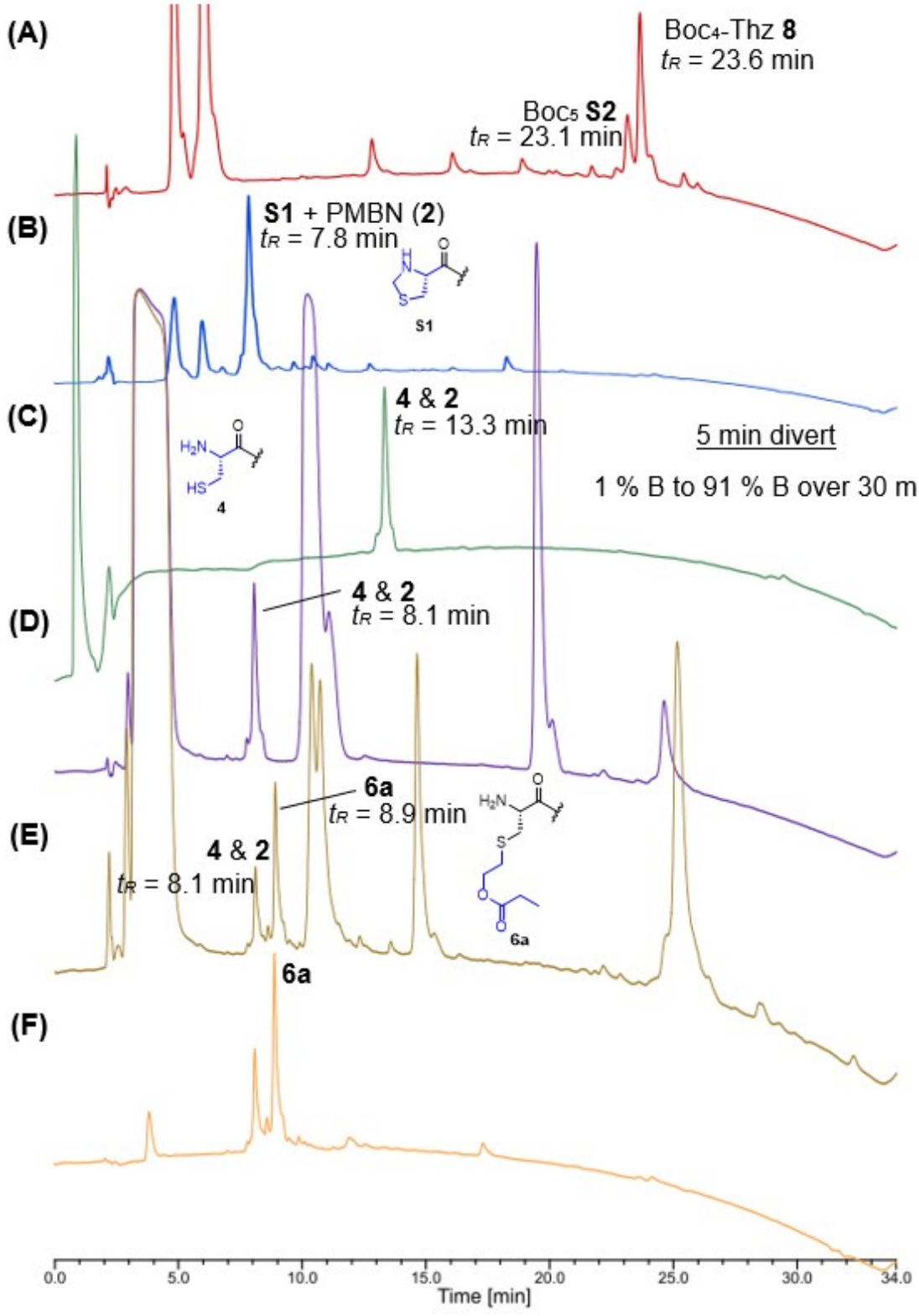


Figure S38: RP-HPLC [210 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm), for **(C)**, 1 % B to 91 % B over 30 min with 5 min initial divert] monitoring of the synthesis of L-Cys propionate CLipPA analogue **6a**, presented in the order of events; **(A)** 1.5 h Thz coupling, **(B)** after Boc-removal and trituration in Et_2O, **(C)** Thz-decryption (18 h, 5 m) and column-phase enrichment, **(D)** CLipPA t = 0 h, **(E)** CLipPA t = 1 h, and **(F)** after trituration in Et_2O.

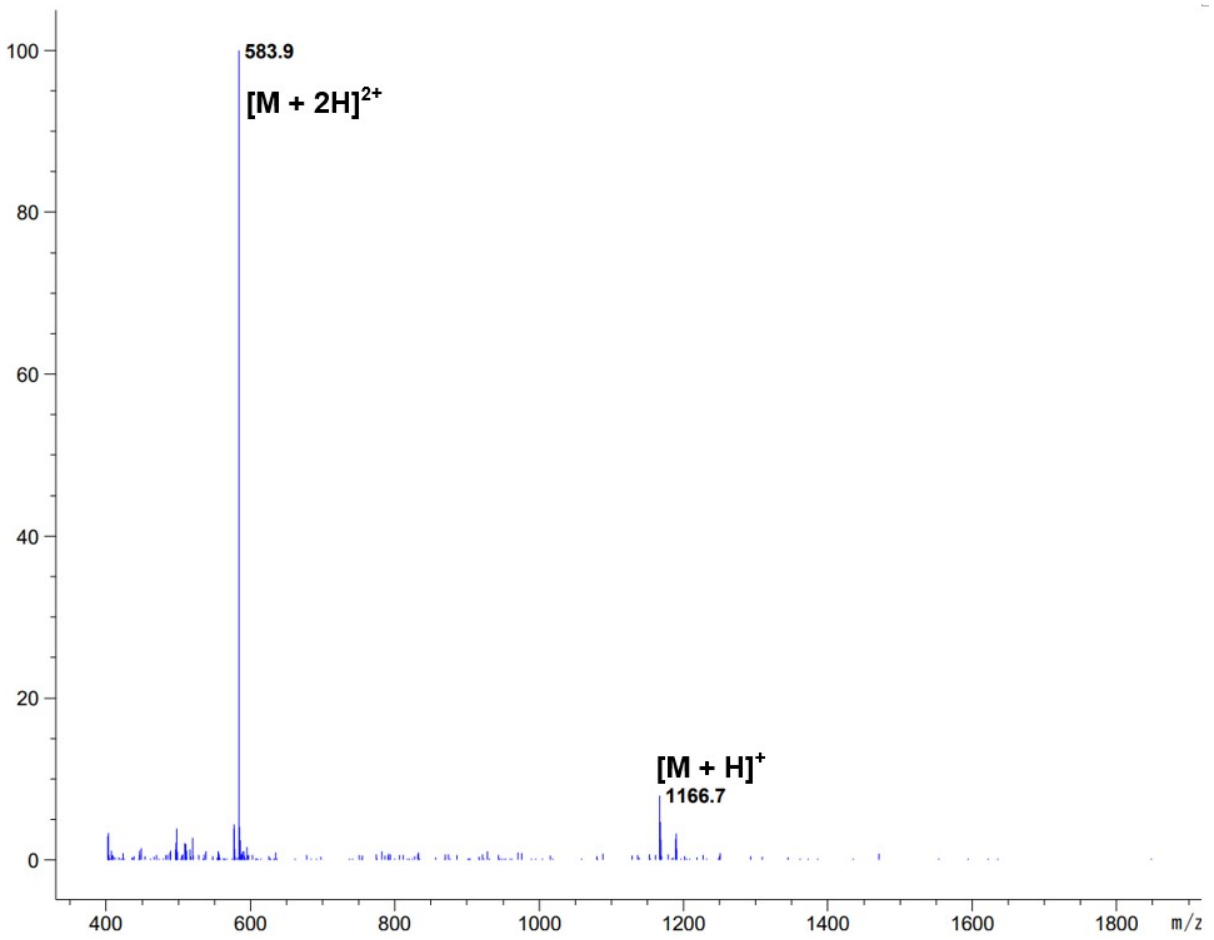


Figure S39: ESI-MS (+ve) spectrum of isolated L-Cys propionate CLipPA analogue **6a**.

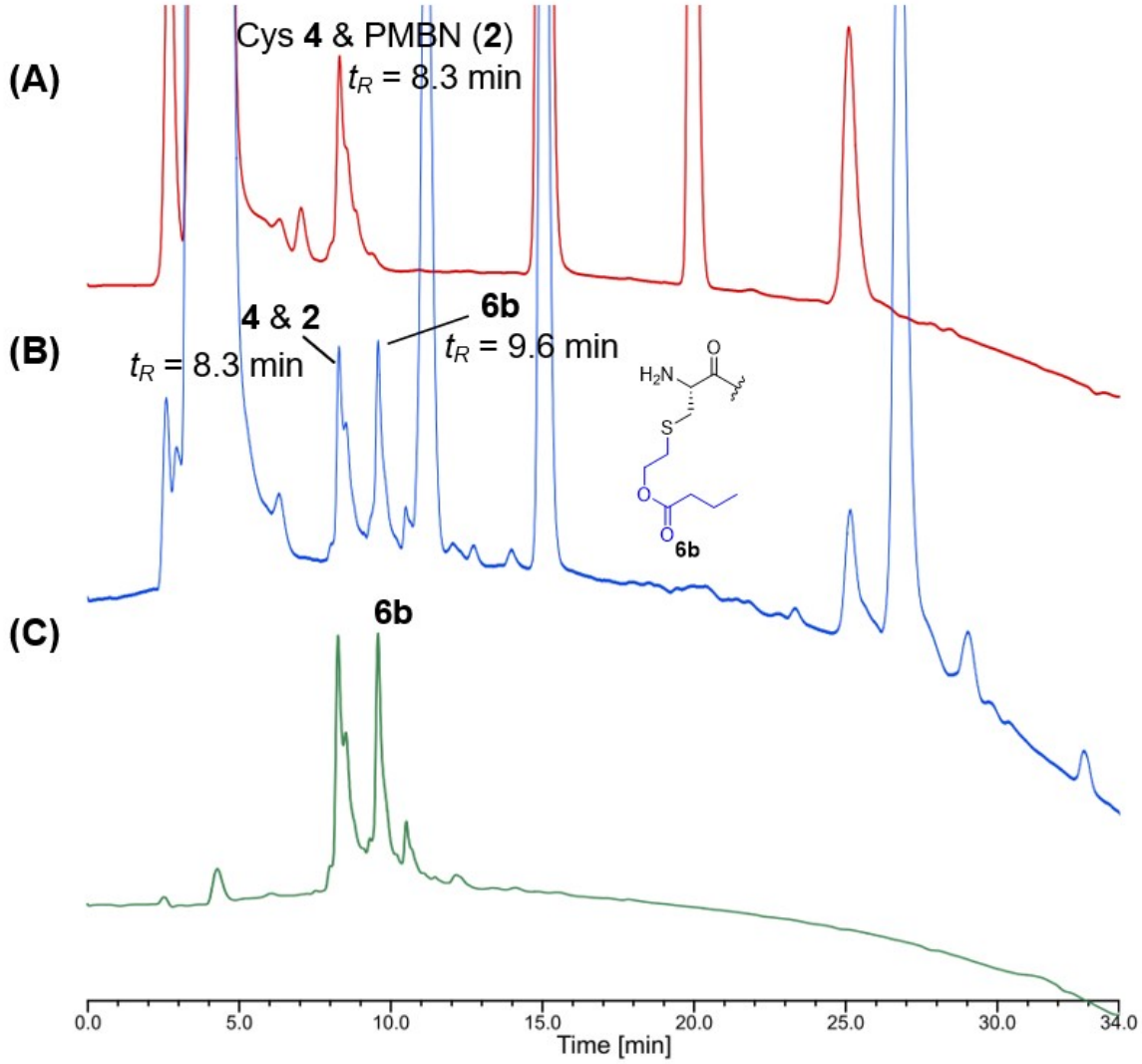


Figure S40: RP-HPLC [210 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of L-Cys butyrate CLipPA analogue **6b**, presented in the order of events; **(A)** CLipPA t = 0 h, **(B)** CLipPA t = 1.5 h, and **(C)** after trituration in Et_2O.

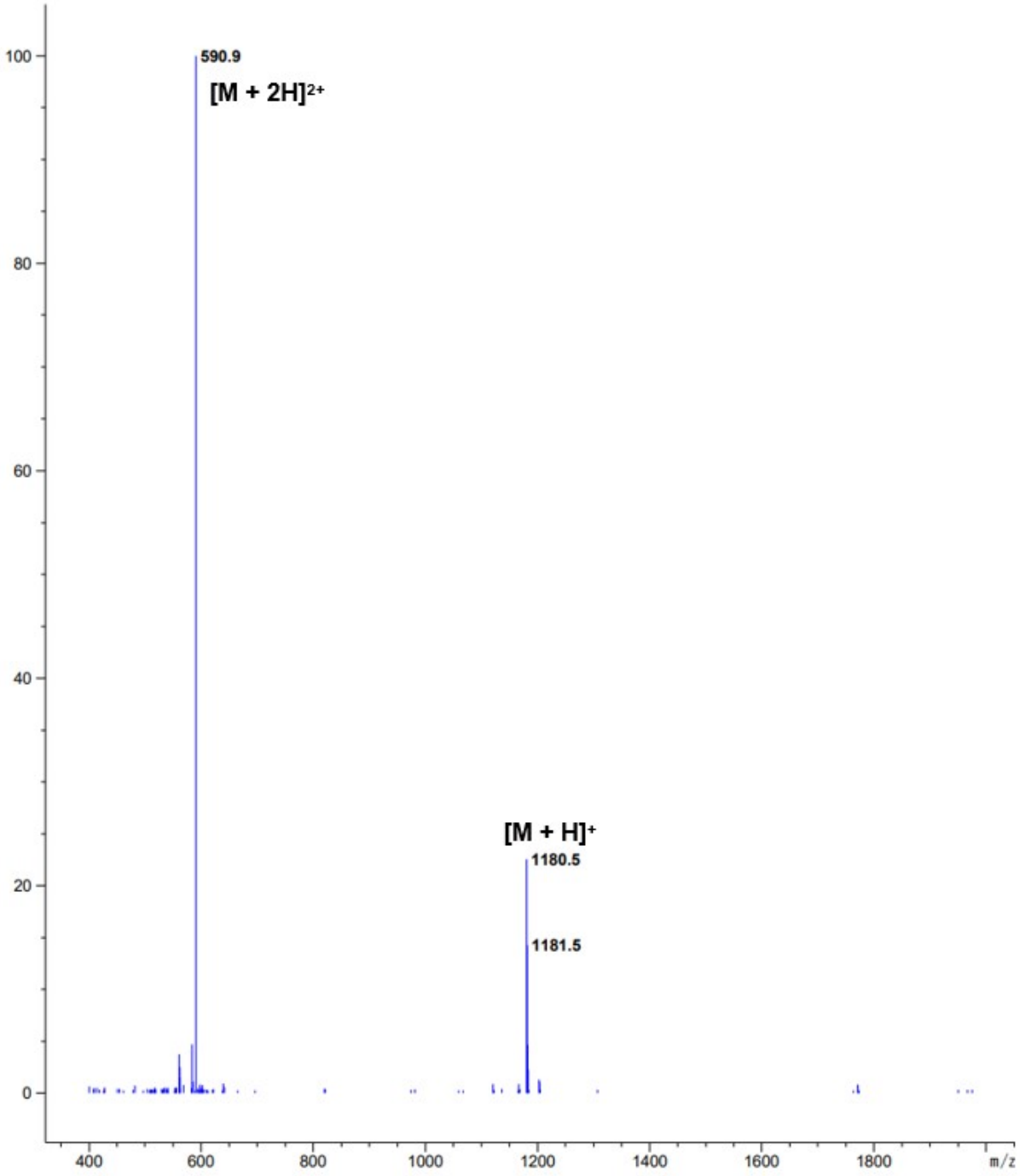


Figure S41: ESI-MS (+ve) spectrum of isolated L-Cys butyrate CLipPA analogue **6b**.

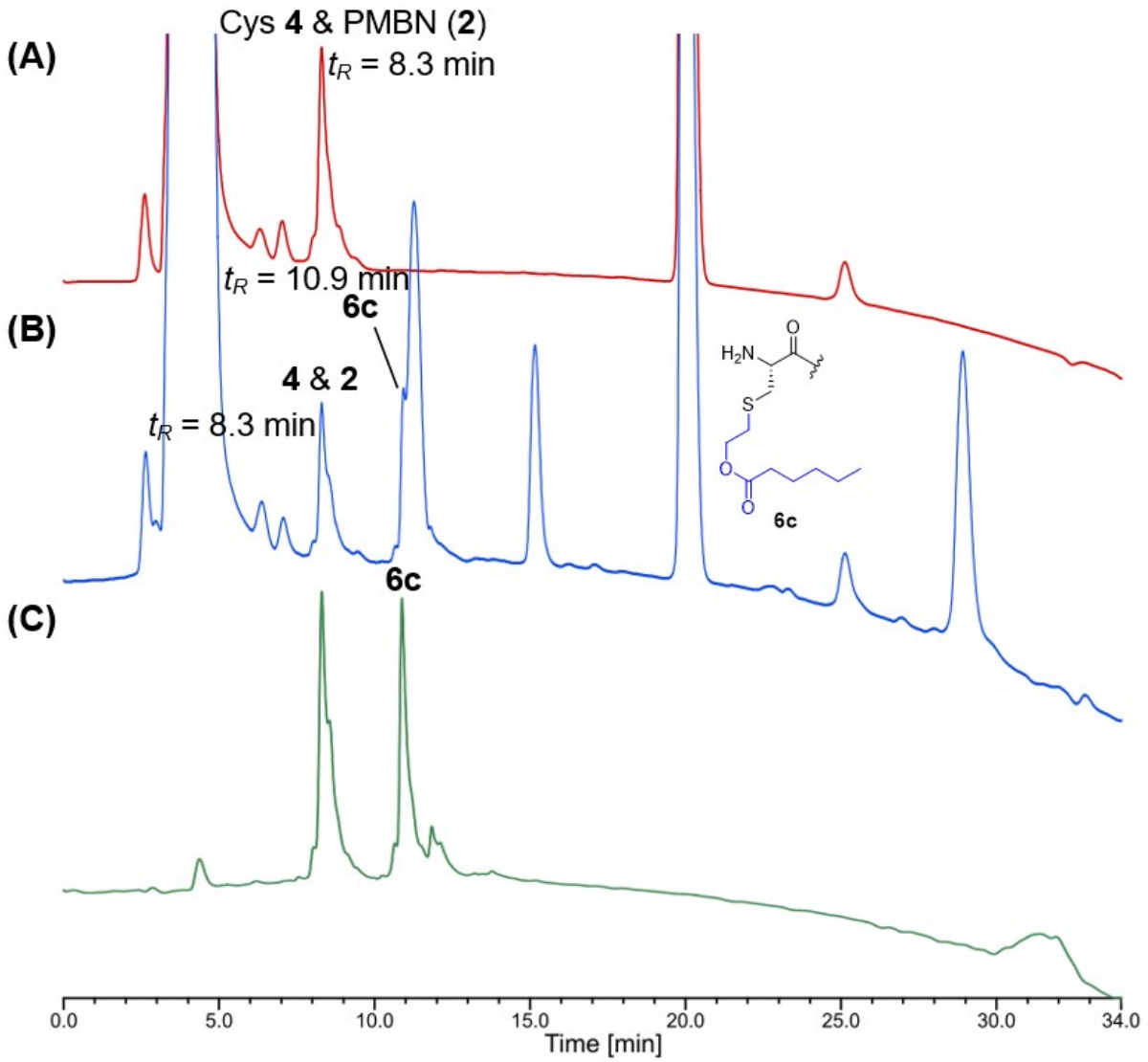


Figure S42: RP-HPLC [210 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of L-Cys hexanoate CLipPA analogue **6c**, presented in the order of events; **(A)** CLipPA t = 0 h, **(B)** CLipPA t = 40 min, and **(C)** after trituration in Et_2O.

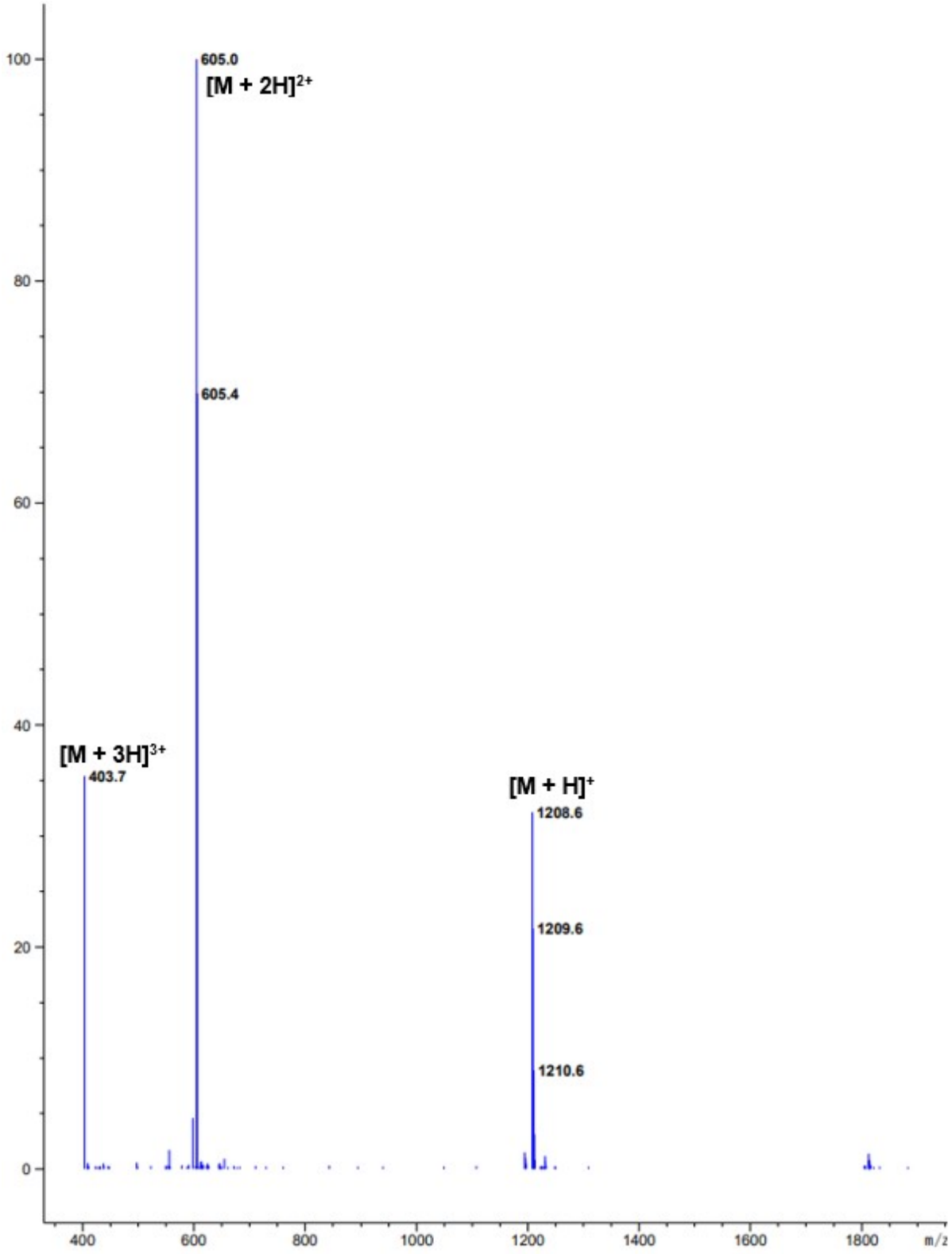


Figure S43: ESI-MS (+ve) spectrum of isolated L-Cys hexanoate CLipPA analogue **6c**.

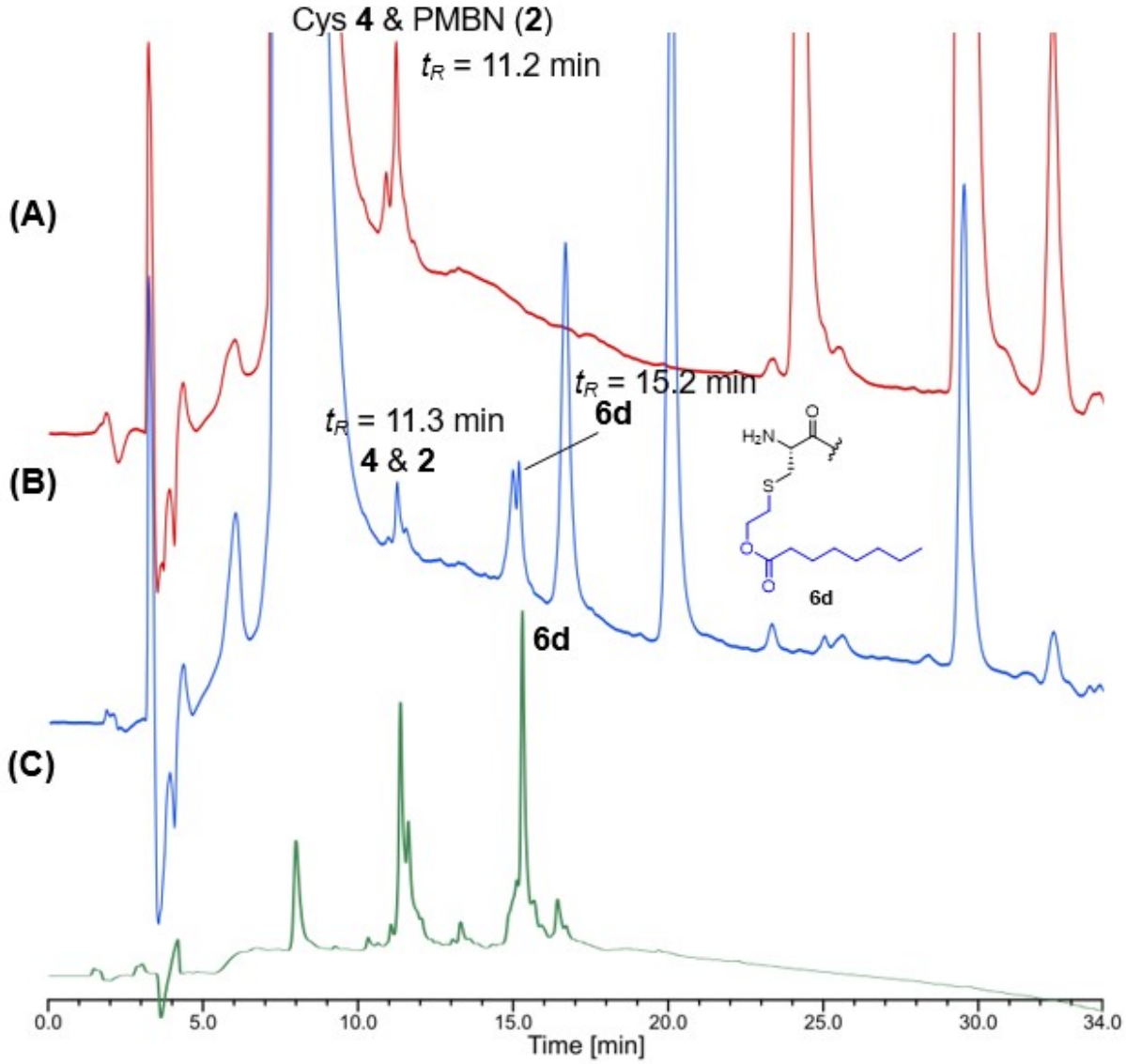


Figure S44: RP-HPLC [214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of L-Cys octanoate CLipPA analogue **6d**, presented in the order of events; **(A)** CLipPA t = 0 h, **(B)** CLipPA t = 1 h, and **(C)** after trituration in Et_2O.

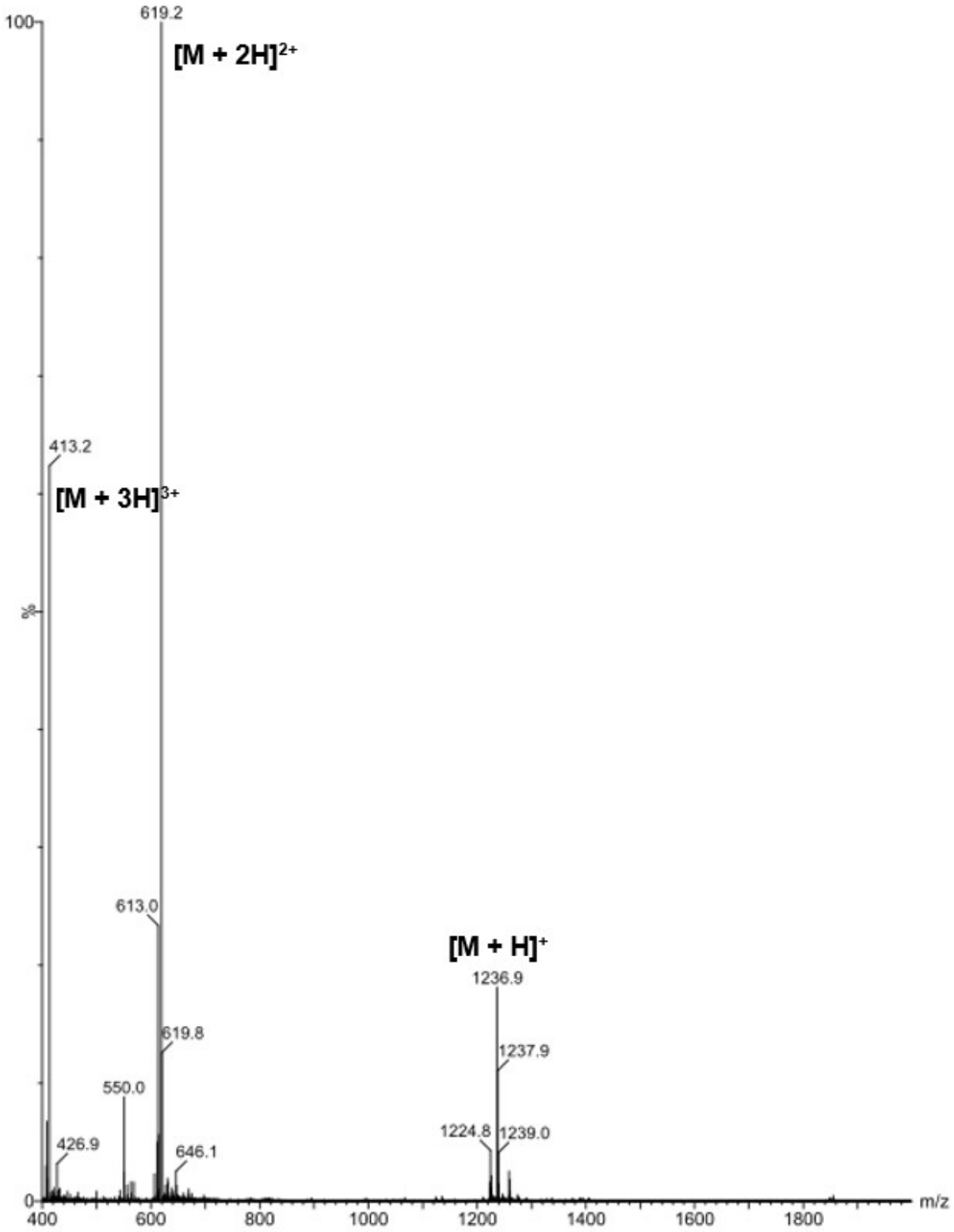


Figure S45: ESI-MS (+ve) spectrum of isolated L-Cys octanoate CLipPA analogue **6d**.

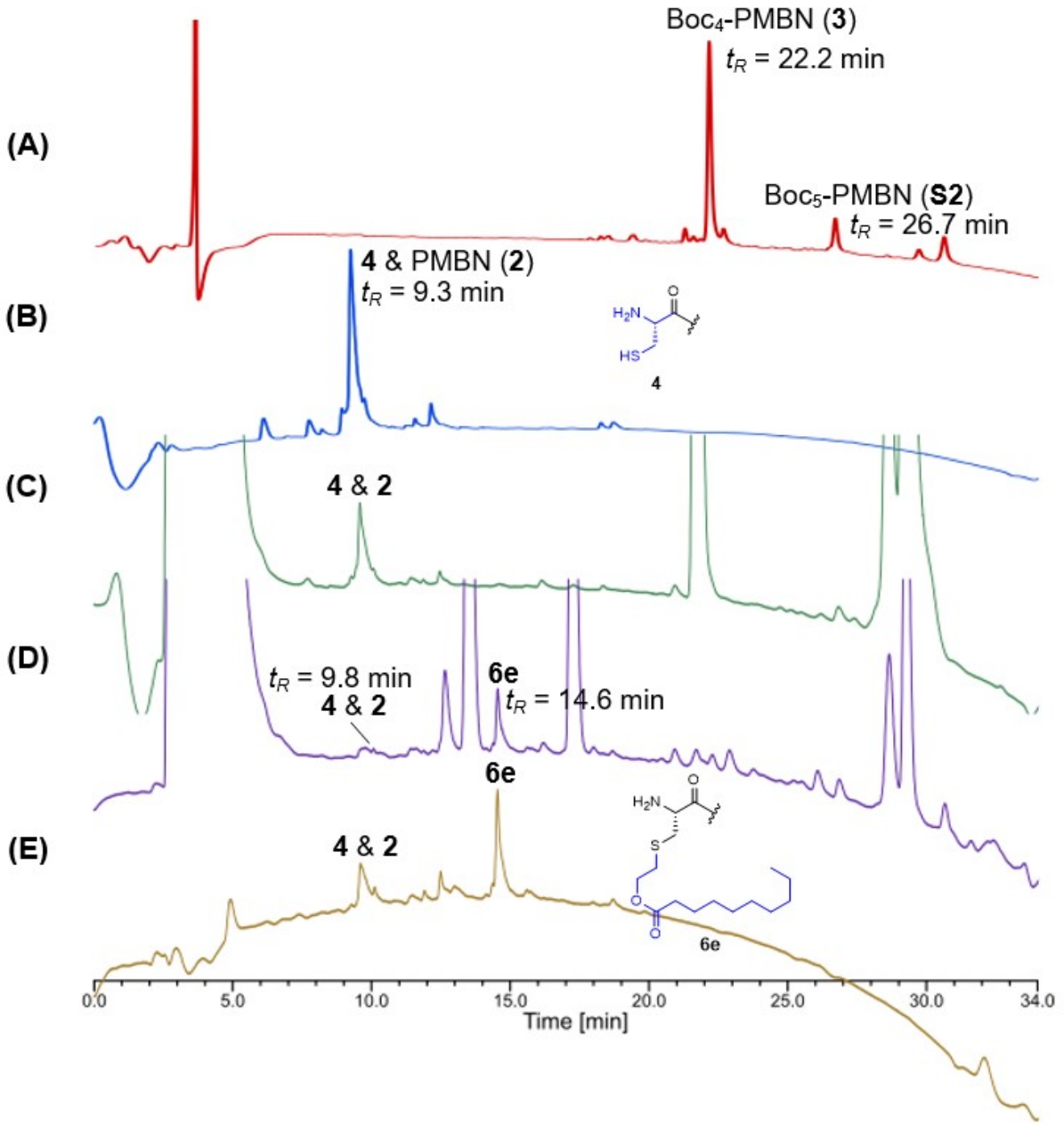


Figure S46: RP-HPLC [210/214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm) or a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of L-Cys decanoate CLipPA analogue **6e**, presented in the order of events; **(A)** Boc_4-PMBN (**3**), **(B)** L-Cys-coupling (2 h) and Boc-removal, **(C)** CLipPA t = 0 h, **(D)** CLipPA t = 1 h, and **(E)** after trituration in Et_2O.

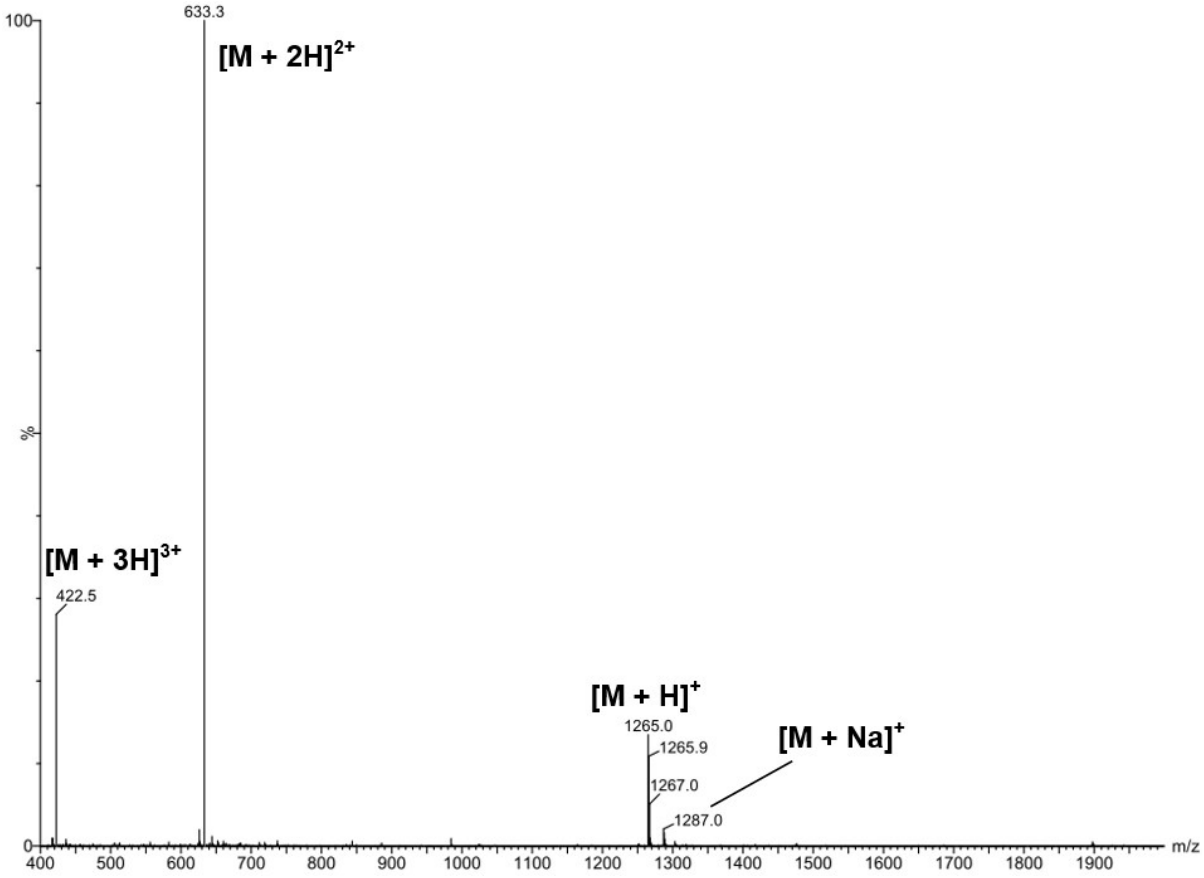


Figure S47: ESI-MS (+ve) spectrum of isolated L-Cys decanoate CLipPA analogue **6e**.

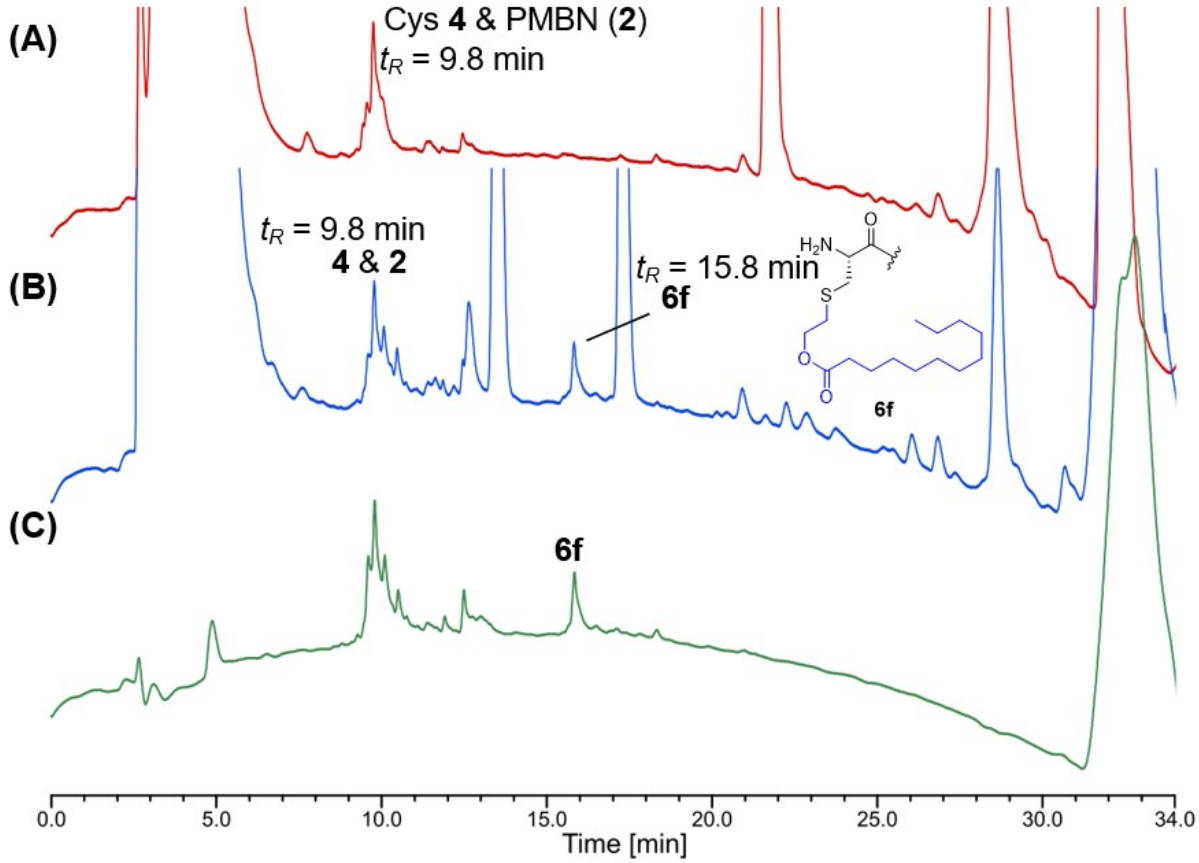


Figure S48: RP-HPLC [210 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of L-Cys laurate CLipPA analogue **6f**, presented in the order of events; **(A)** CLipPA t = 0 h, **(B)** CLipPA t = 1 h, and **(C)** after trituration in Et_2O.

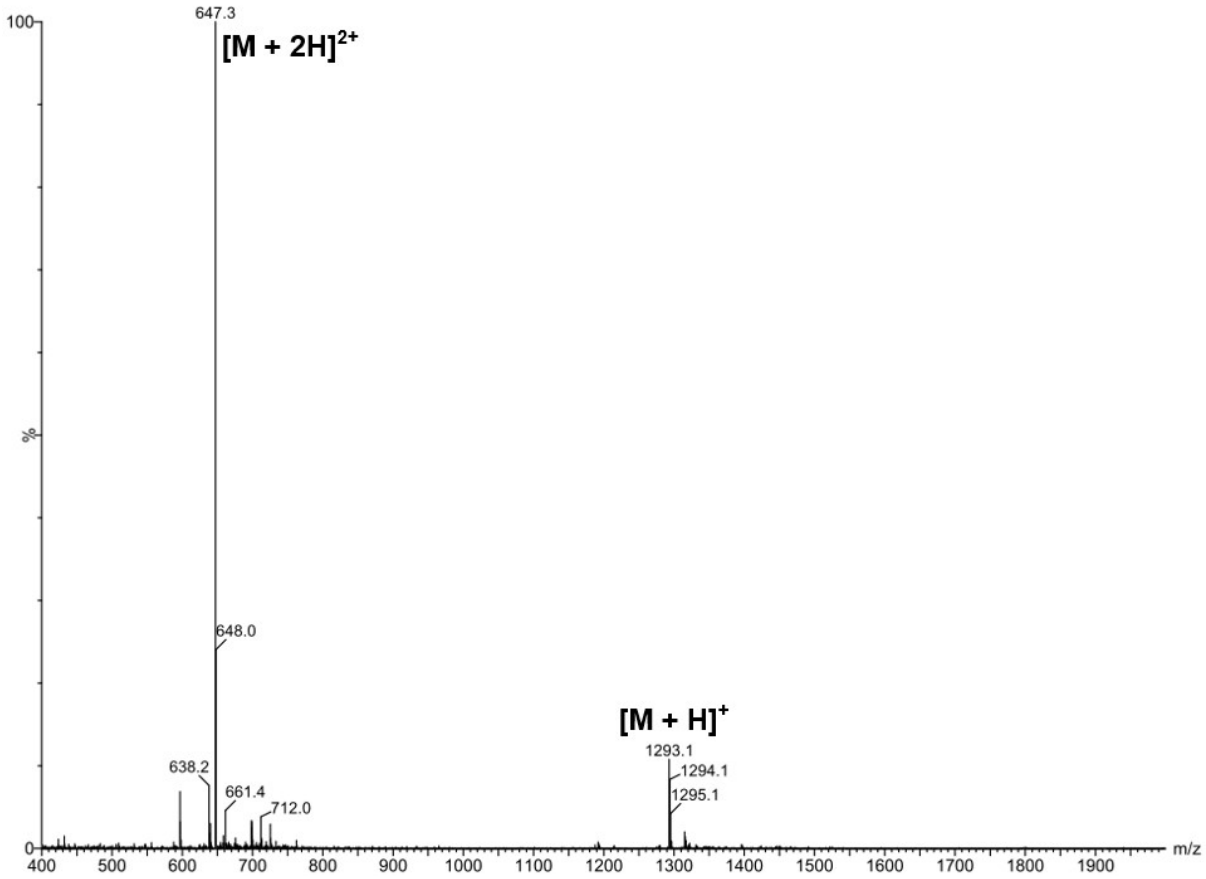


Figure S49: ESI-MS (+ve) spectrum of isolated L-Cys laurate CLipPA analogue **6f**.

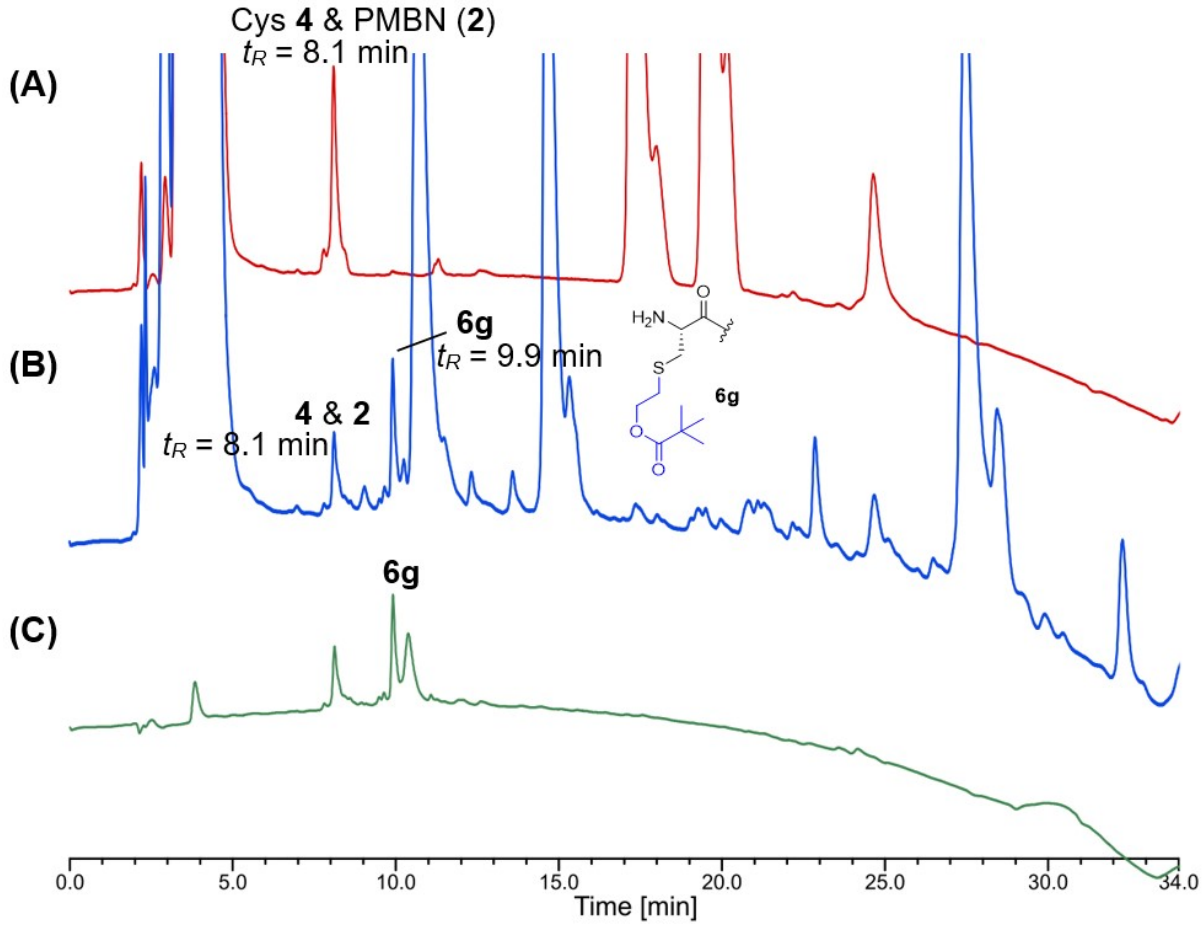


Figure S50: RP-HPLC [210 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of L-Cys pivalate CLipPA analogue **6g**, presented in the order of events; **(A)** CLipPA t = 0 h, **(B)** CLipPA t = 1 h, and **(C)** after trituration in Et_2O.

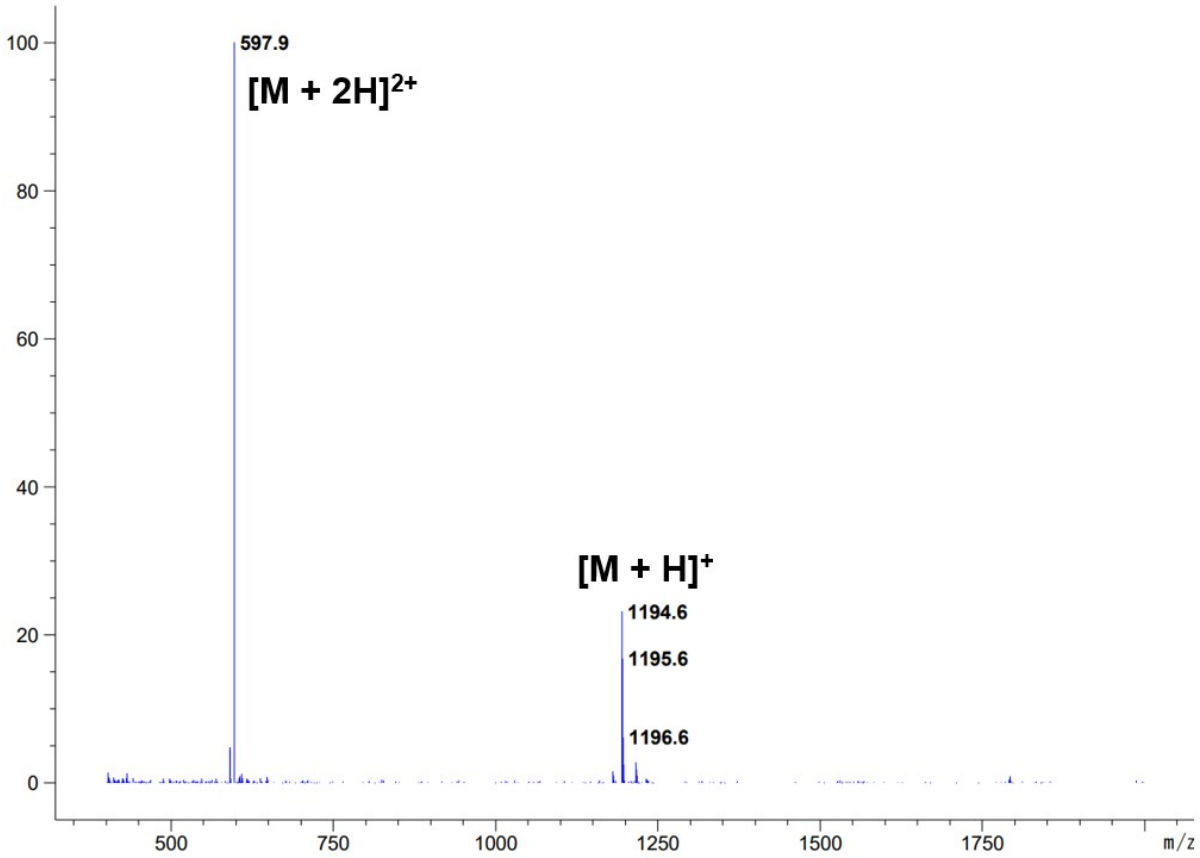


Figure S51: ESI-MS (+ve) spectrum of isolated L-Cys pivalate CLipPA analogue **6g**.

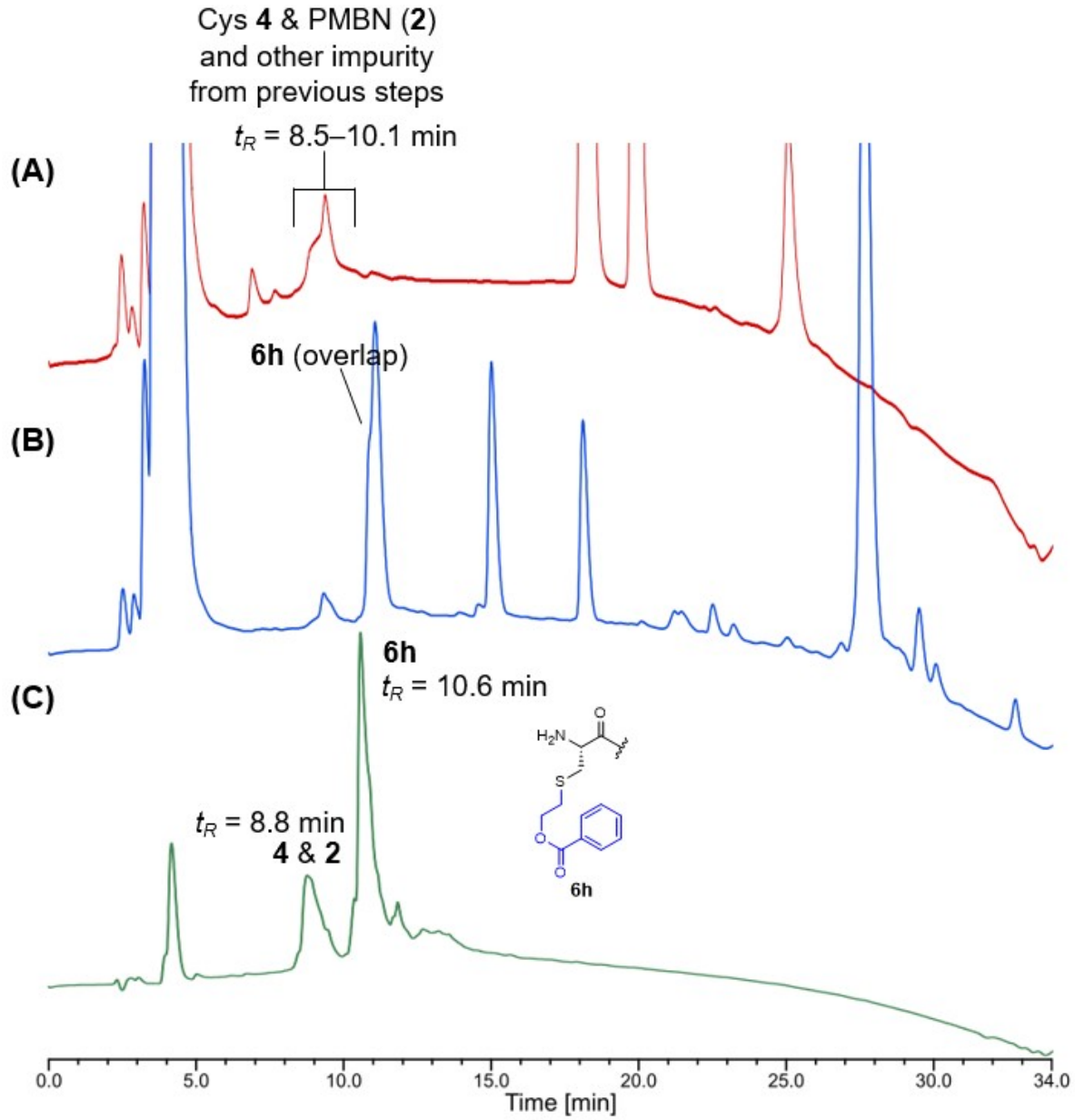


Figure S52: RP-HPLC [210 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of L-Cys benzoate CLipPA analogue **6h**, presented in the order of events; **(A)** CLipPA t = 0 h, **(B)** CLipPA t = 1 h 20 min, and **(C)** after trituration in Et_2O.

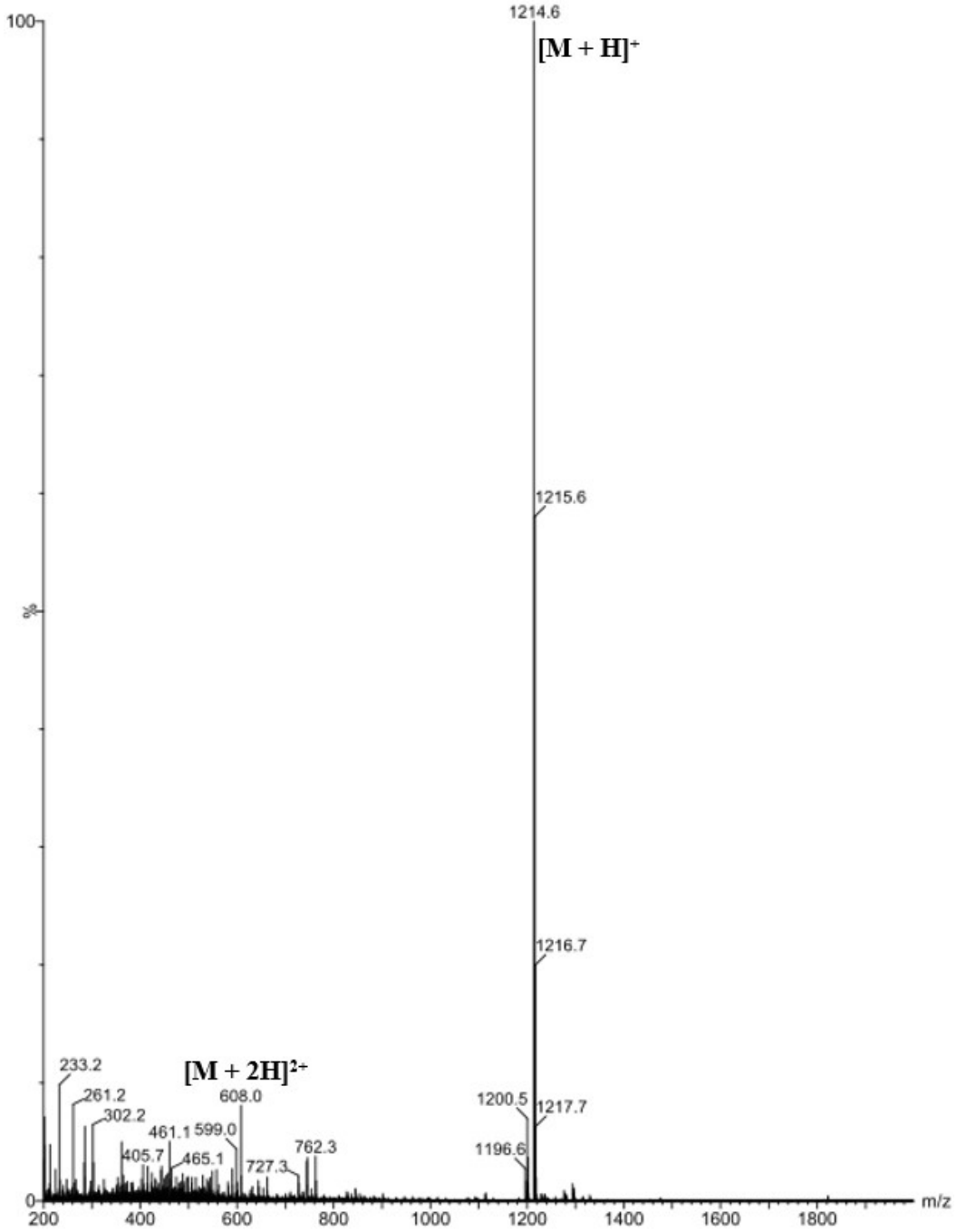


Figure S53: ESI-MS (+ve) spectrum of isolated L-Cys benzoate CLipPA analogue **6h**.

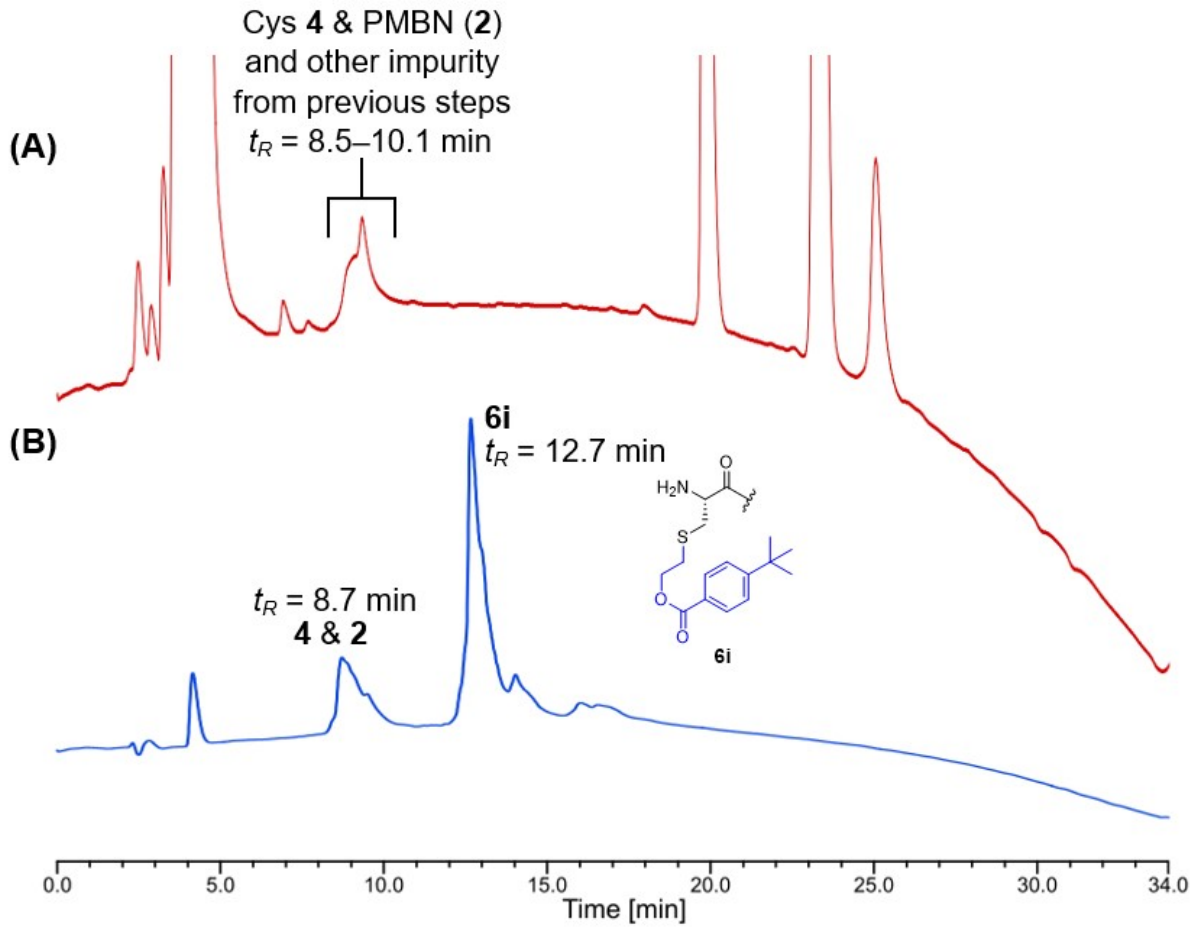


Figure S54: RP-HPLC [210 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of L-Cys 4-*t*Bu-benzoate CLipPA analogue **6i**, presented in the order of events; **(A)** CLipPA t = 0 h, **(B)** CLipPA t = 1 h 20 min, and **(C)** after trituration in Et_2O.

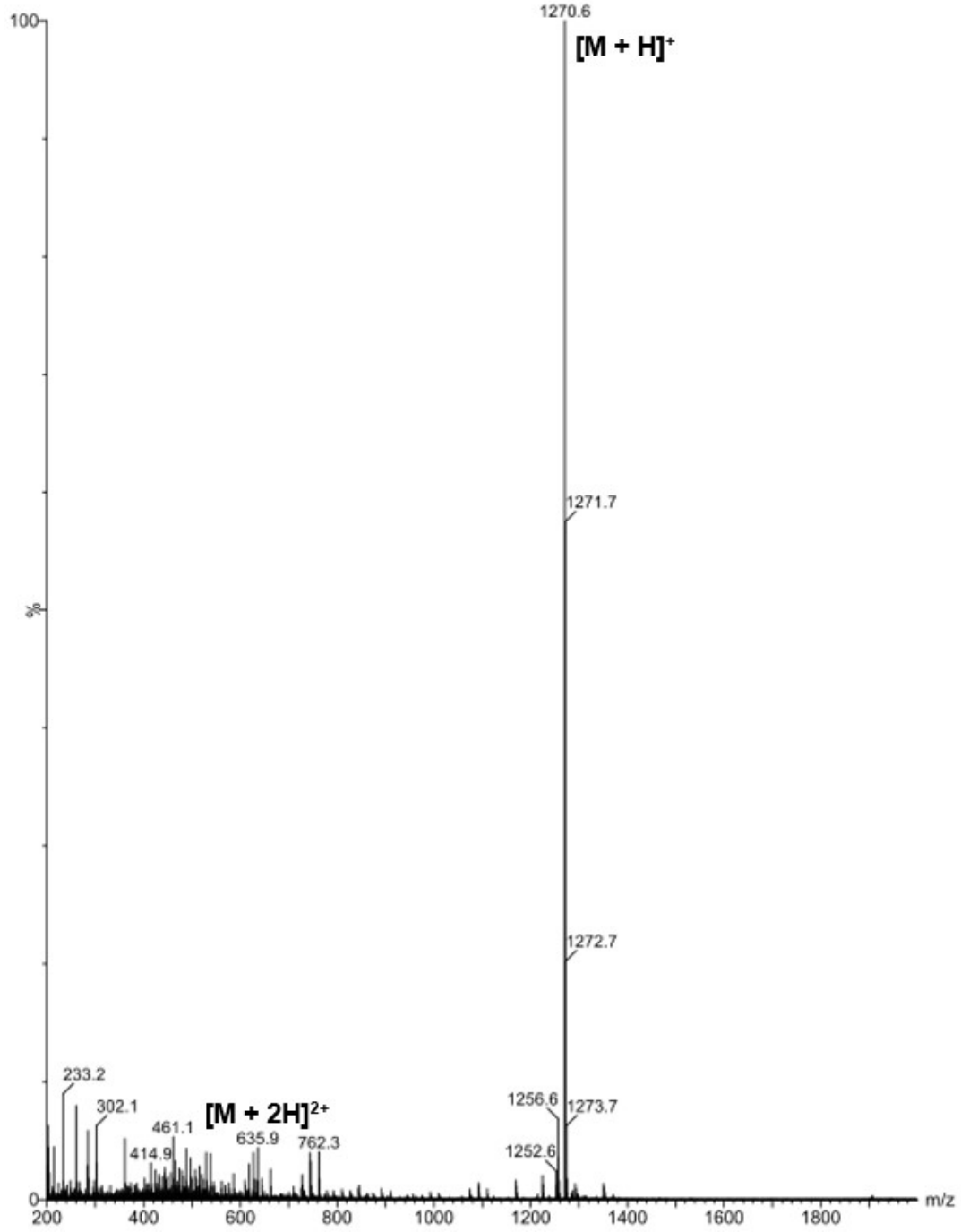


Figure S55: ESI-MS (+ve) spectrum of isolated L-Cys 4-*t*Bu-benzoate CLipPA analogue **6i**.

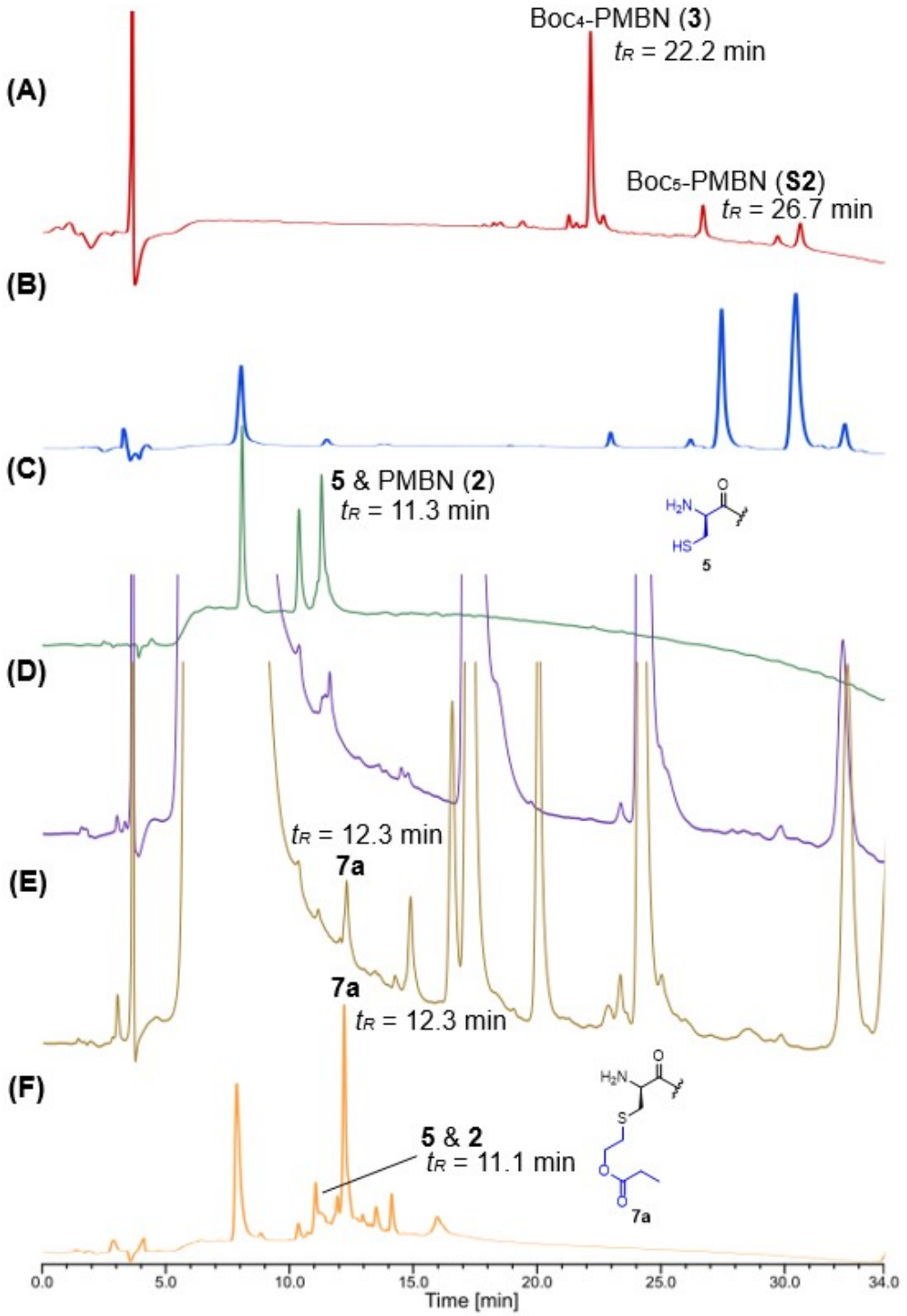


Figure S56: RP-HPLC [214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys propionate CLipPA analogue **7a**, presented in the order of events; **(A)** Boc_4-PMBN (**3**), **(B)** D-Cys-coupling (1 h), **(C)** after Boc-removal and column-phase enrichment, **(D)** CLipPA t = 0 h, **(E)** CLipPA t = 1 h 20 min, and **(F)** after trituration in Et_2O.

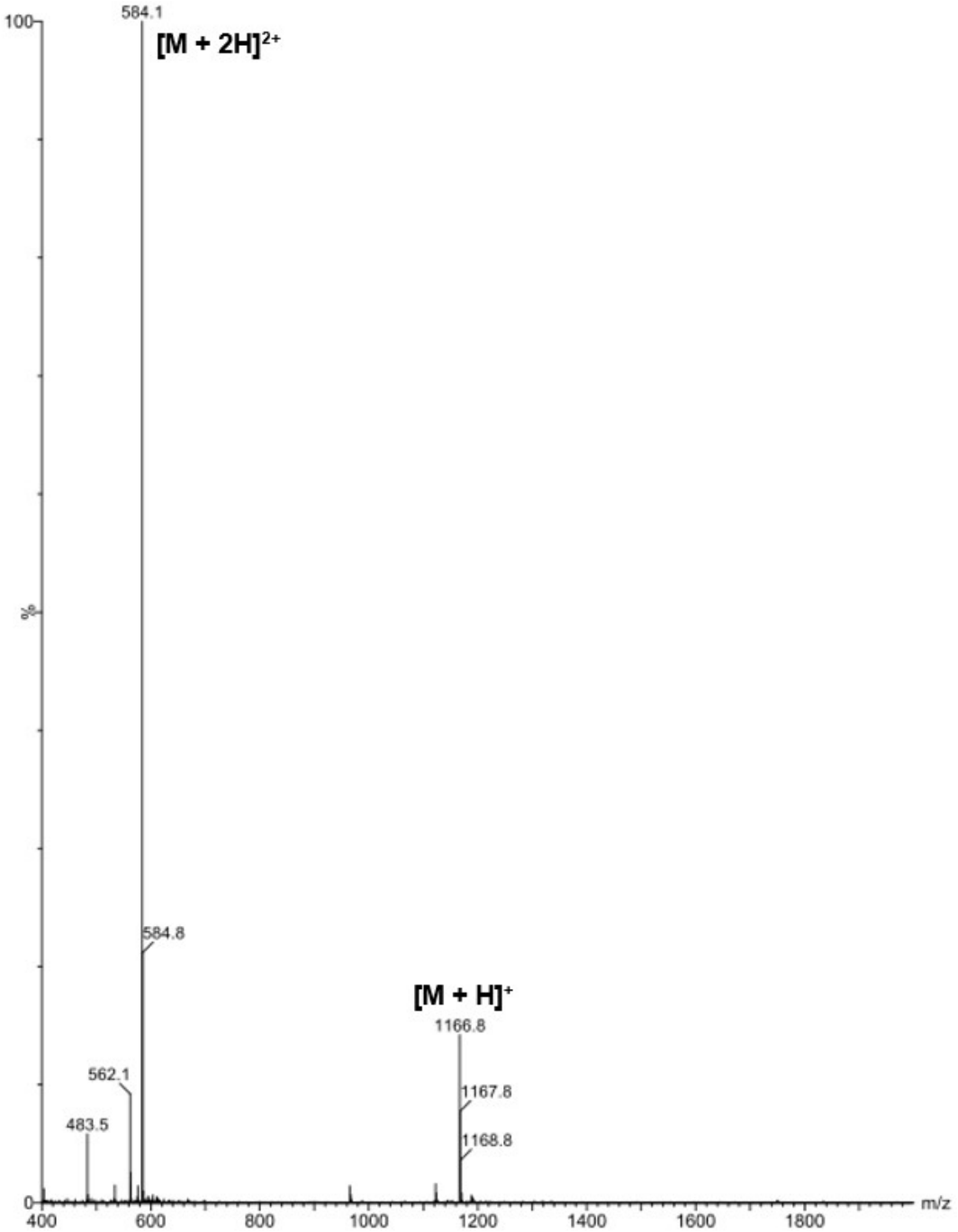


Figure S57: ESI-MS (+ve) spectrum of isolated D-Cys propionate CLipPA analogue **7a**.

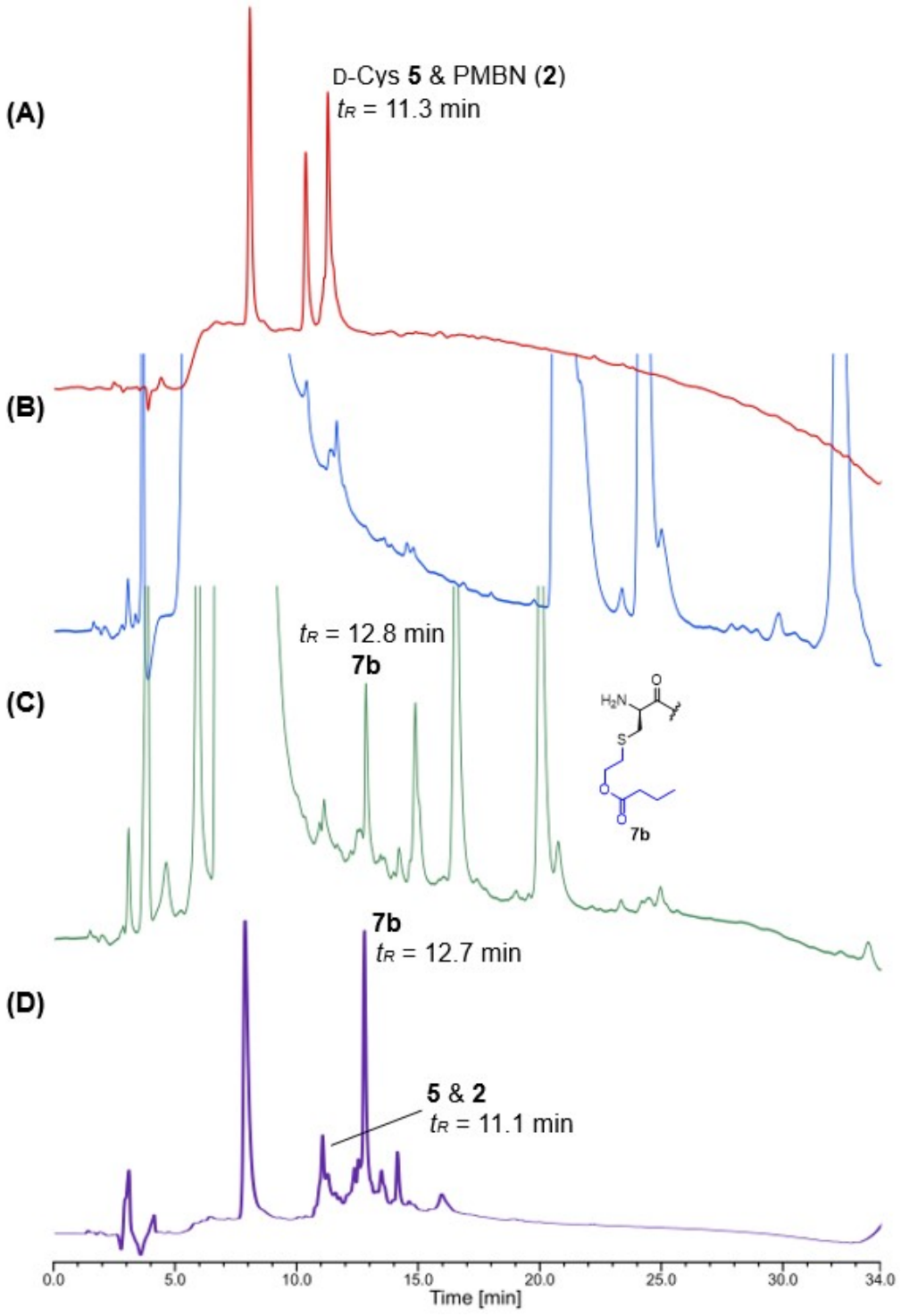


Figure S58: RP-HPLC [214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys butyrate CLipPA analogue **7b**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h 20 min, and **(D)** after trituration in Et_2O.

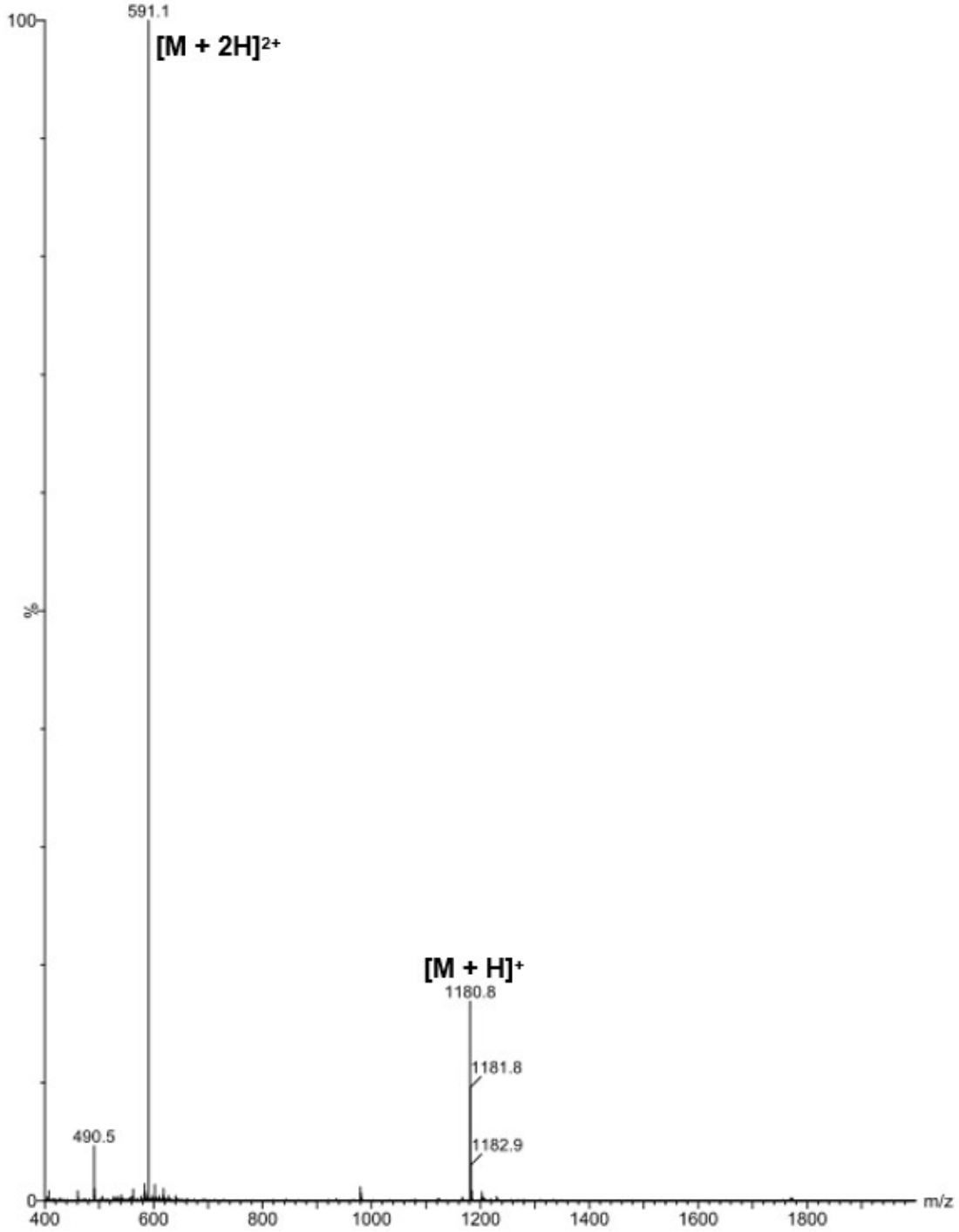


Figure S59: ESI-MS (+ve) spectrum of isolated D-Cys butyrate CLipPA analogue **7b**.

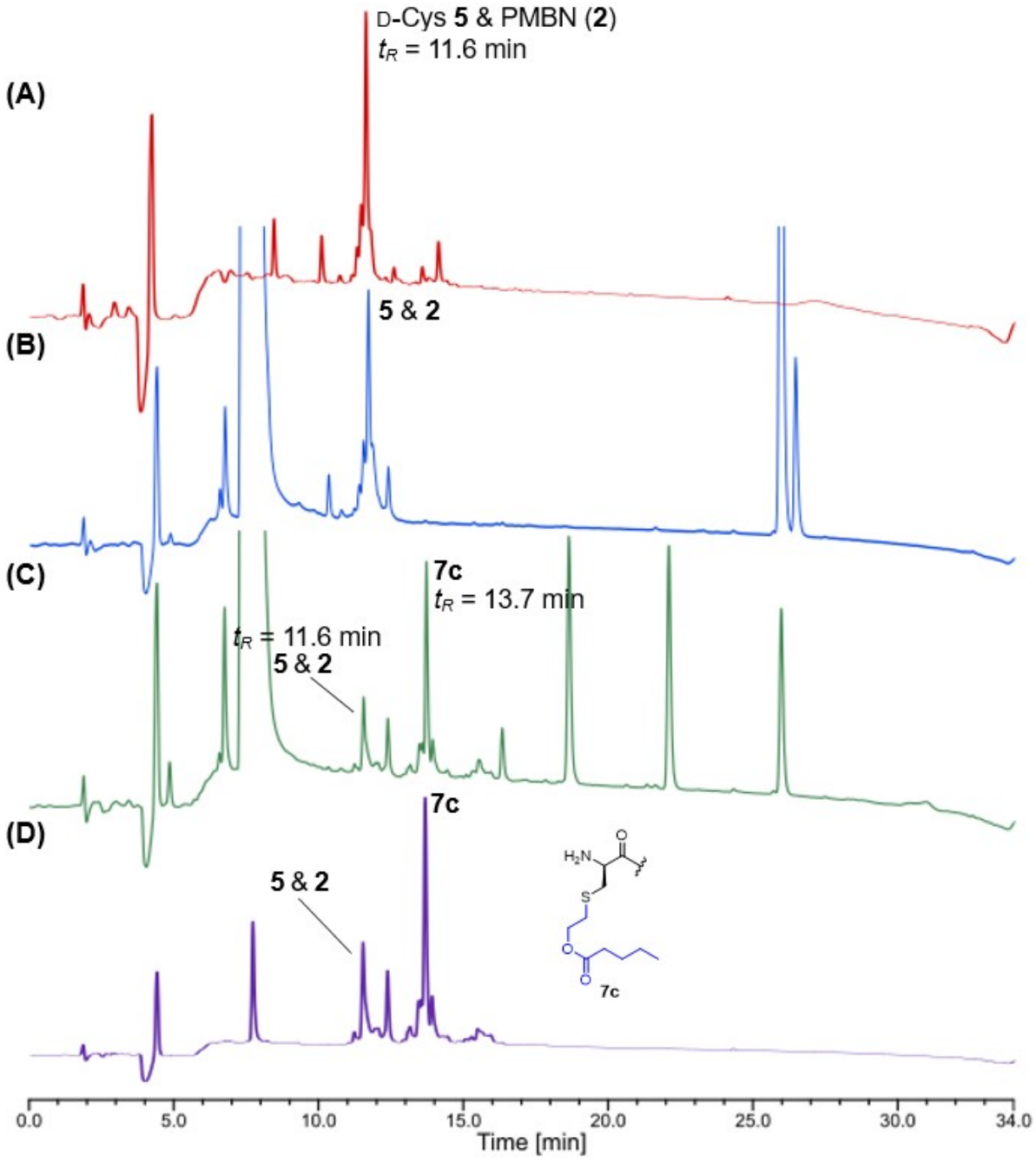


Figure S60: RP-HPLC [214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys valerate CLipPA analogue **7c**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h, and **(D)** after trituration in Et_2O.

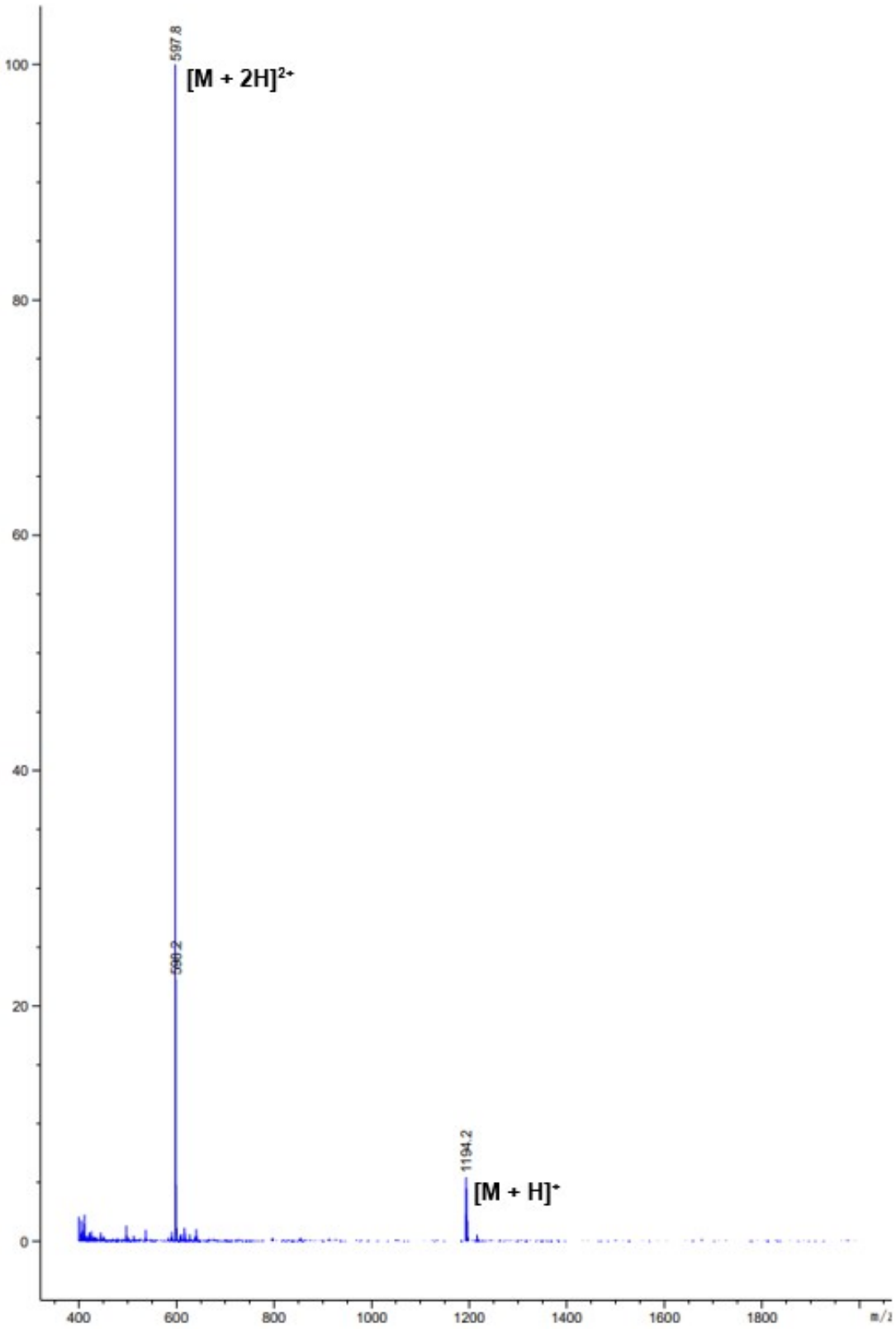


Figure S61: ESI-MS (+ve) spectrum of isolated D-Cys valerate CLipPA analogue **7c**.

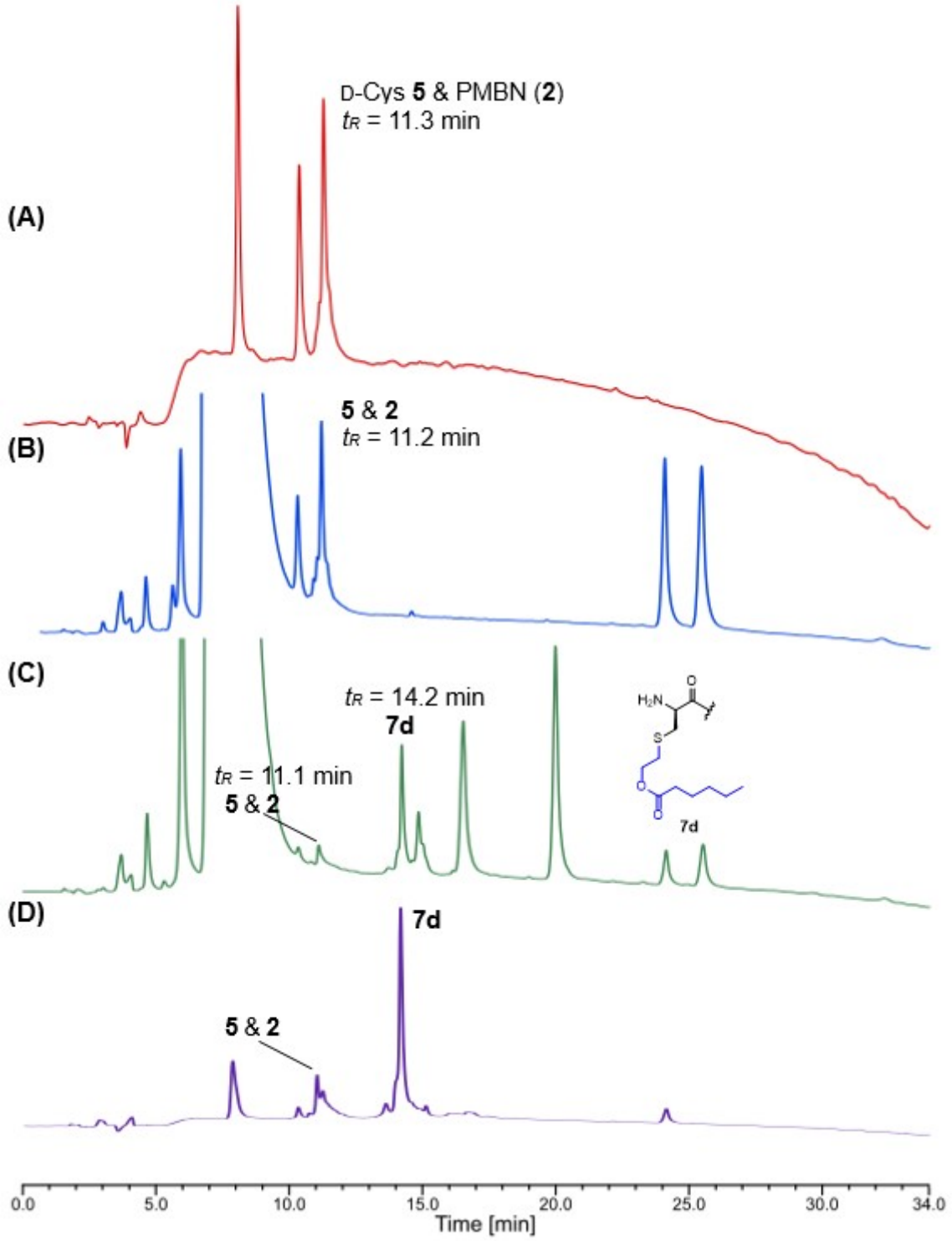


Figure S62: RP-HPLC [214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys hexanoate CLipPA analogue **7d**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h, and **(D)** after trituration in Et_2O.

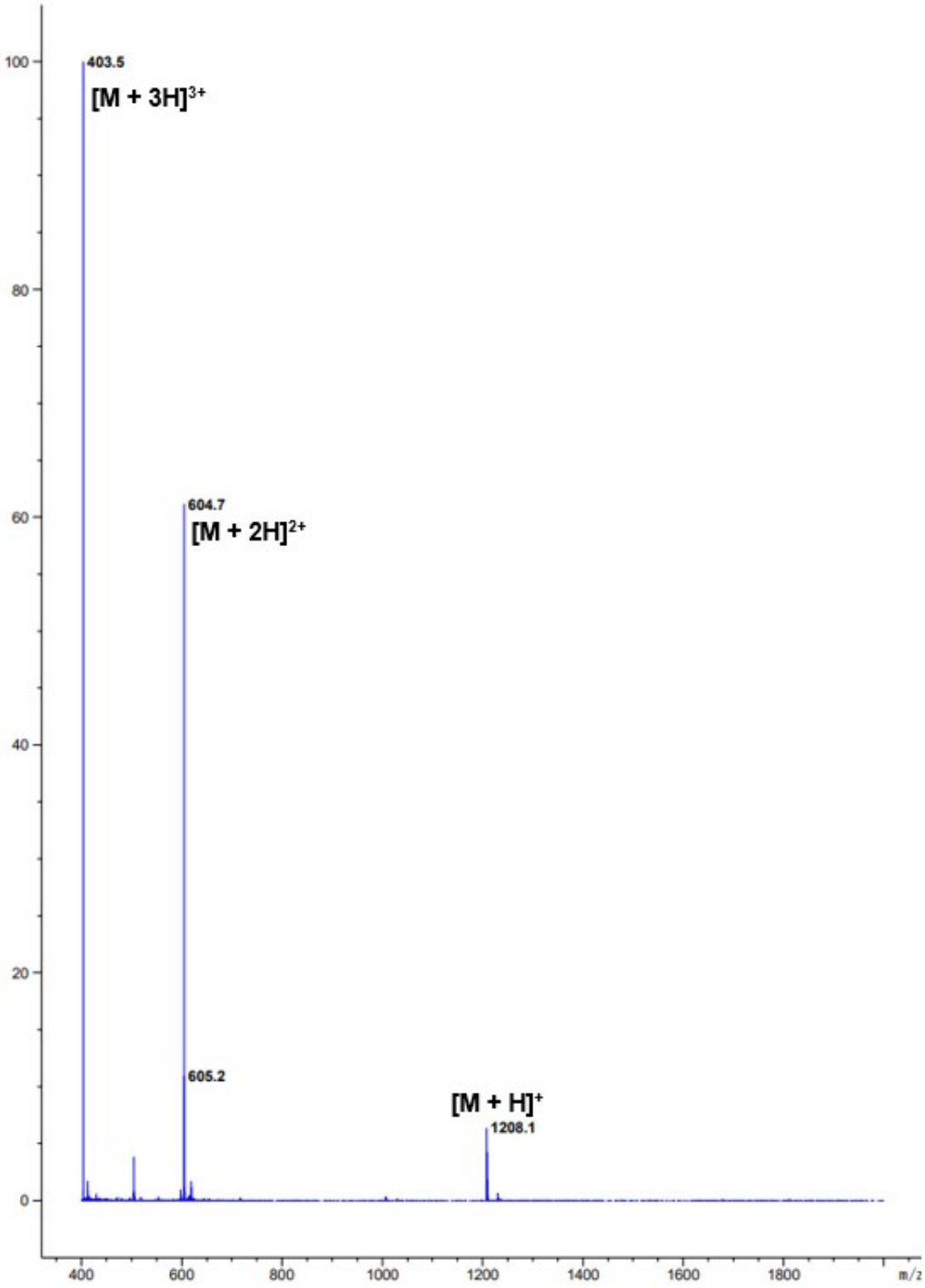


Figure S63: ESI-MS (+ve) spectrum of isolated D-Cys hexanoate CLipPA analogue **7d**.

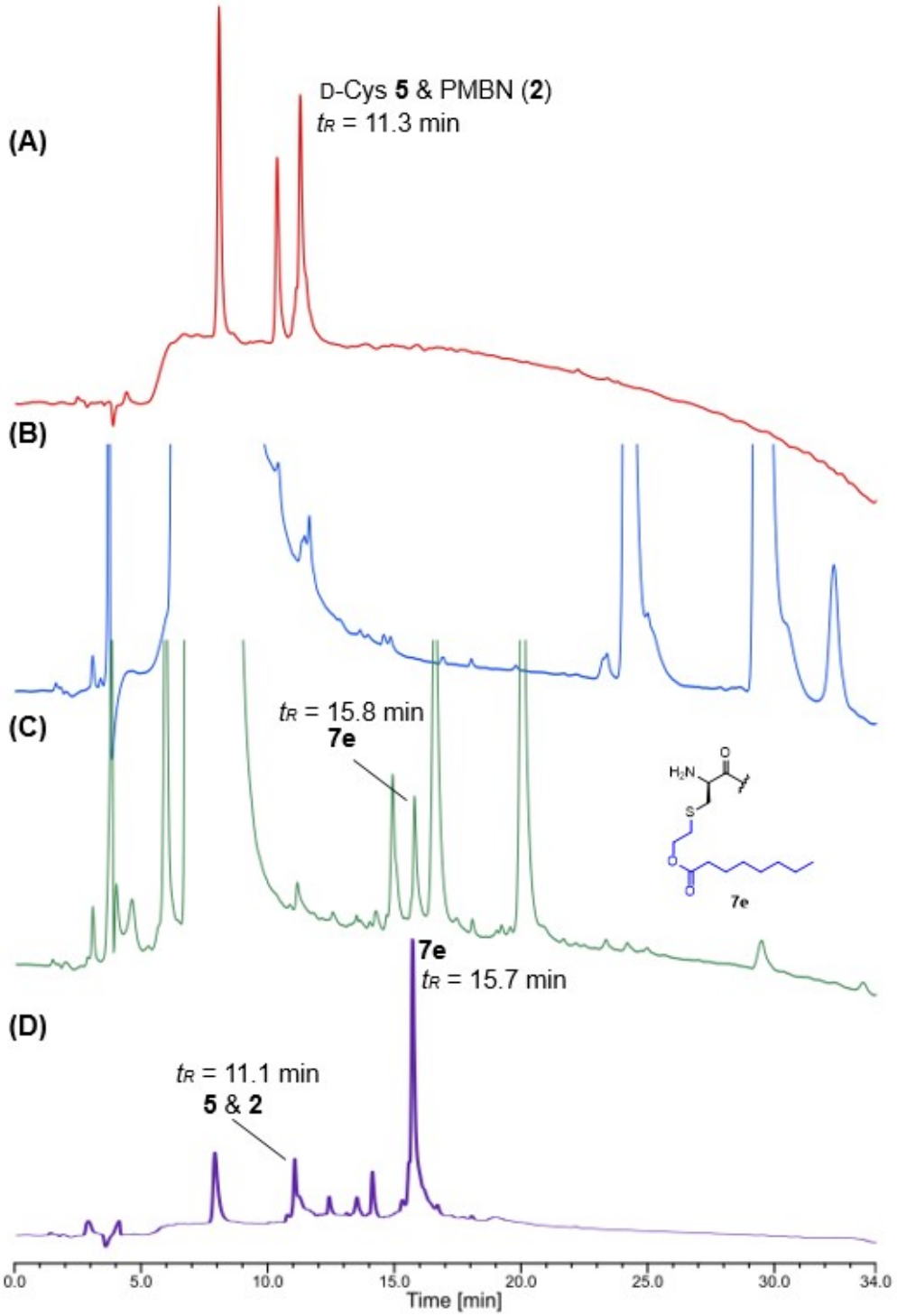


Figure S64: RP-HPLC [214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys octanoate CLipPA analogue **7e**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h 20 min, and **(D)** after trituration in Et_2O.

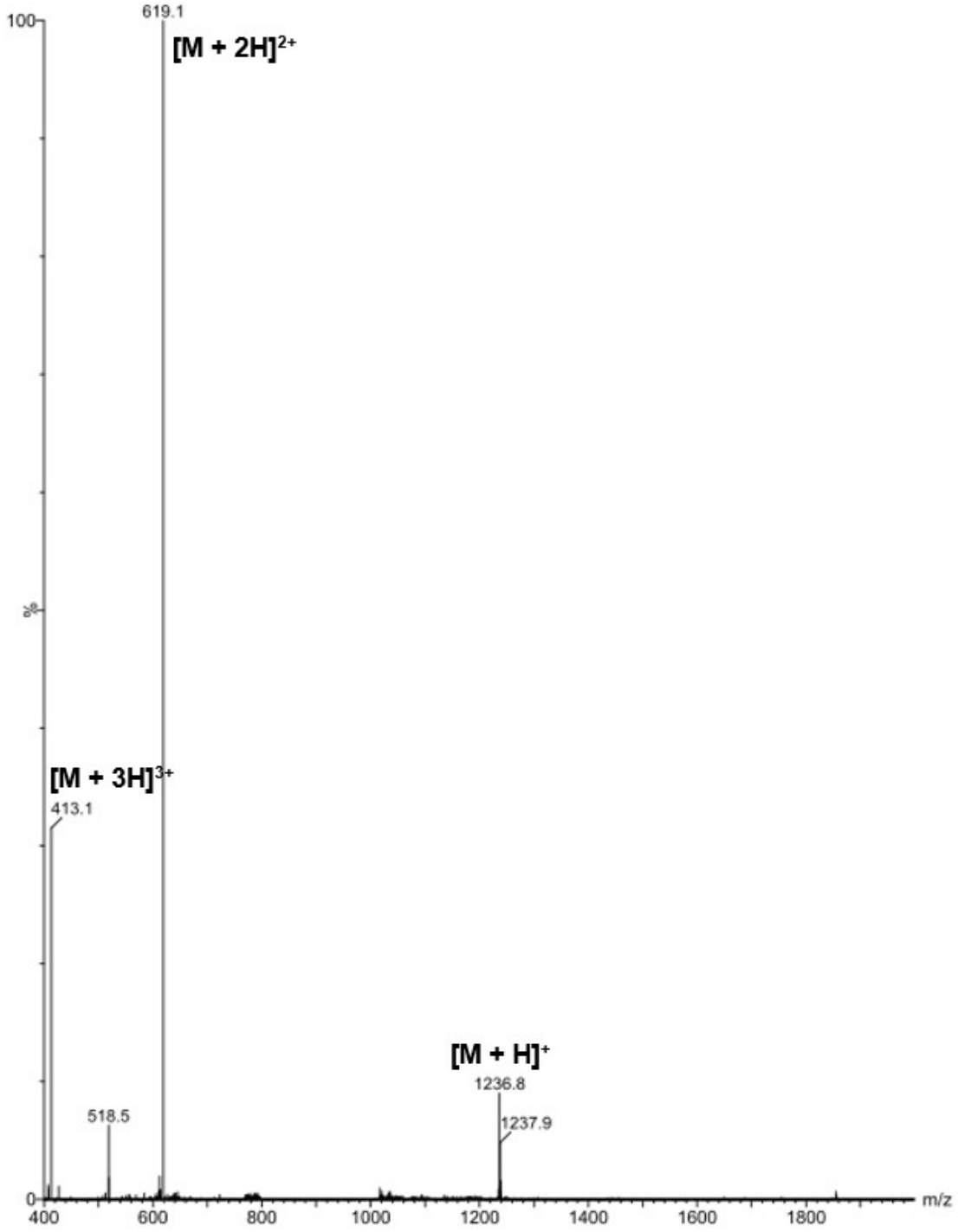


Figure S65: ESI-MS (+ve) spectrum of isolated D-Cys octanoate CLipPA analogue **7e**.

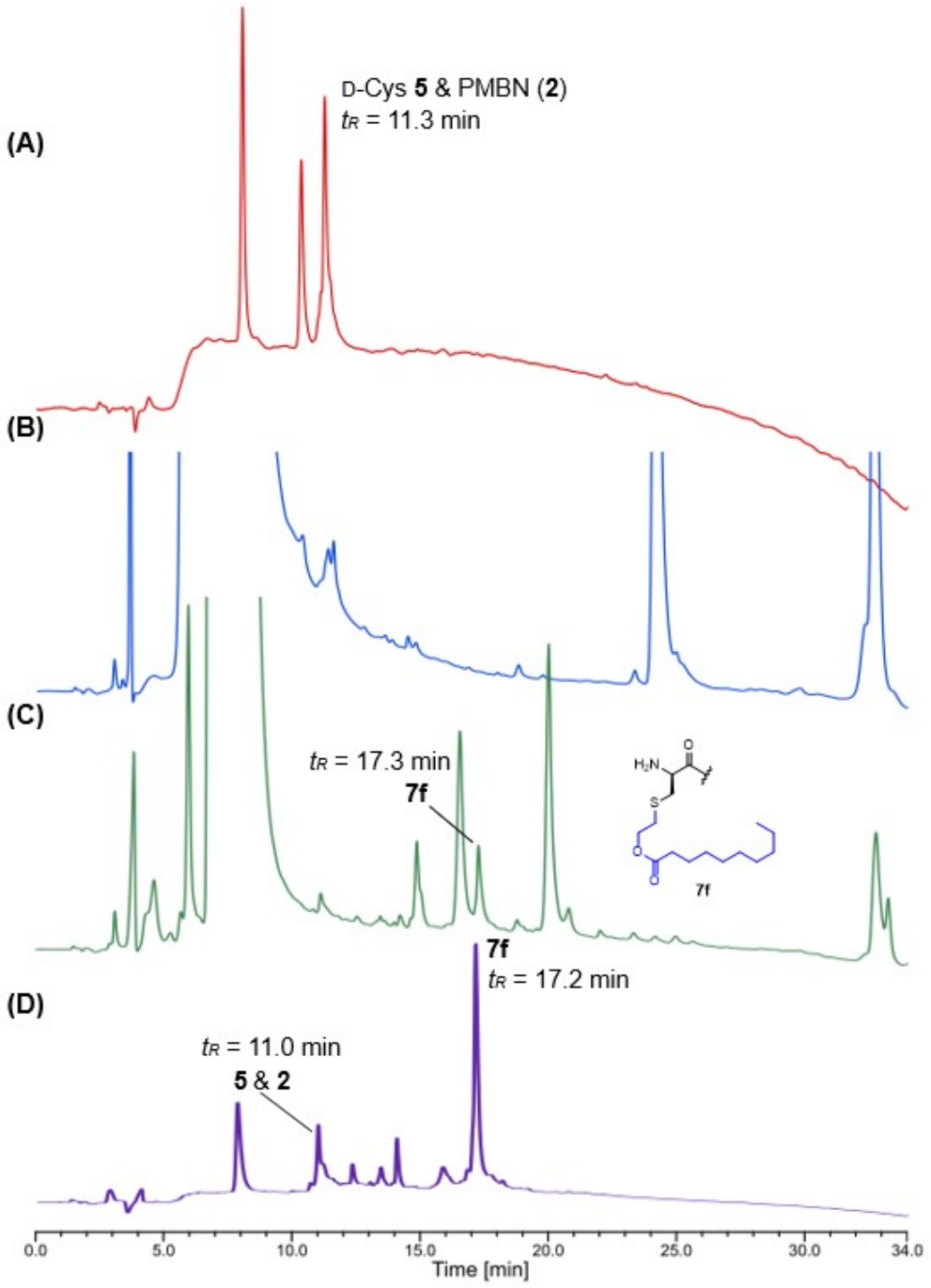


Figure S66: RP-HPLC [214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys decanoate CLipPA analogue **7f**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h 20 min, and **(D)** after trituration in Et_2O.

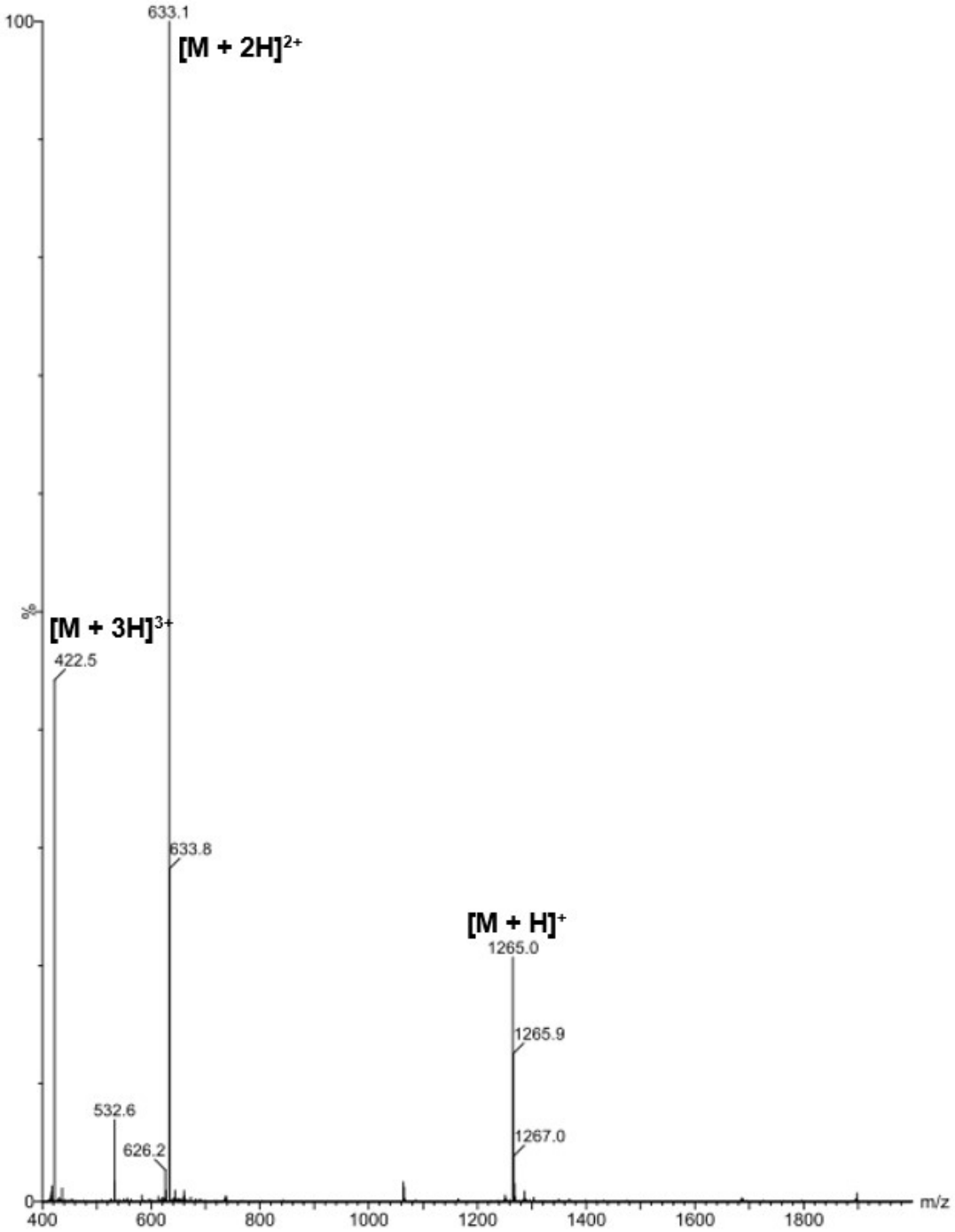


Figure S67: ESI-MS (+ve) spectrum of isolated D-Cys decanoate CLipPA analogue **7f**.

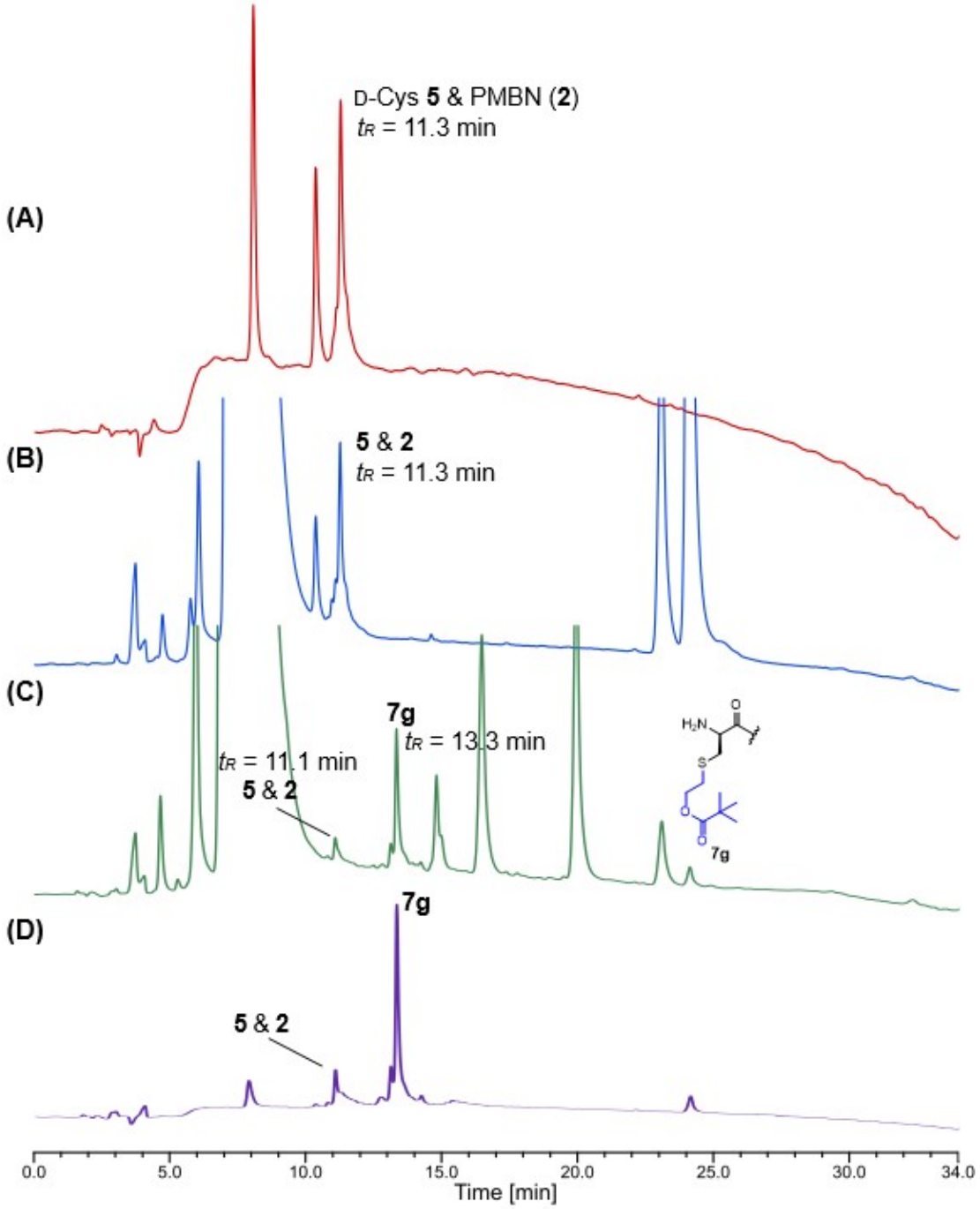


Figure S68: RP-HPLC [214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys pivalate CLipPA analogue **7g**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h, and **(D)** after trituration in Et_2O.

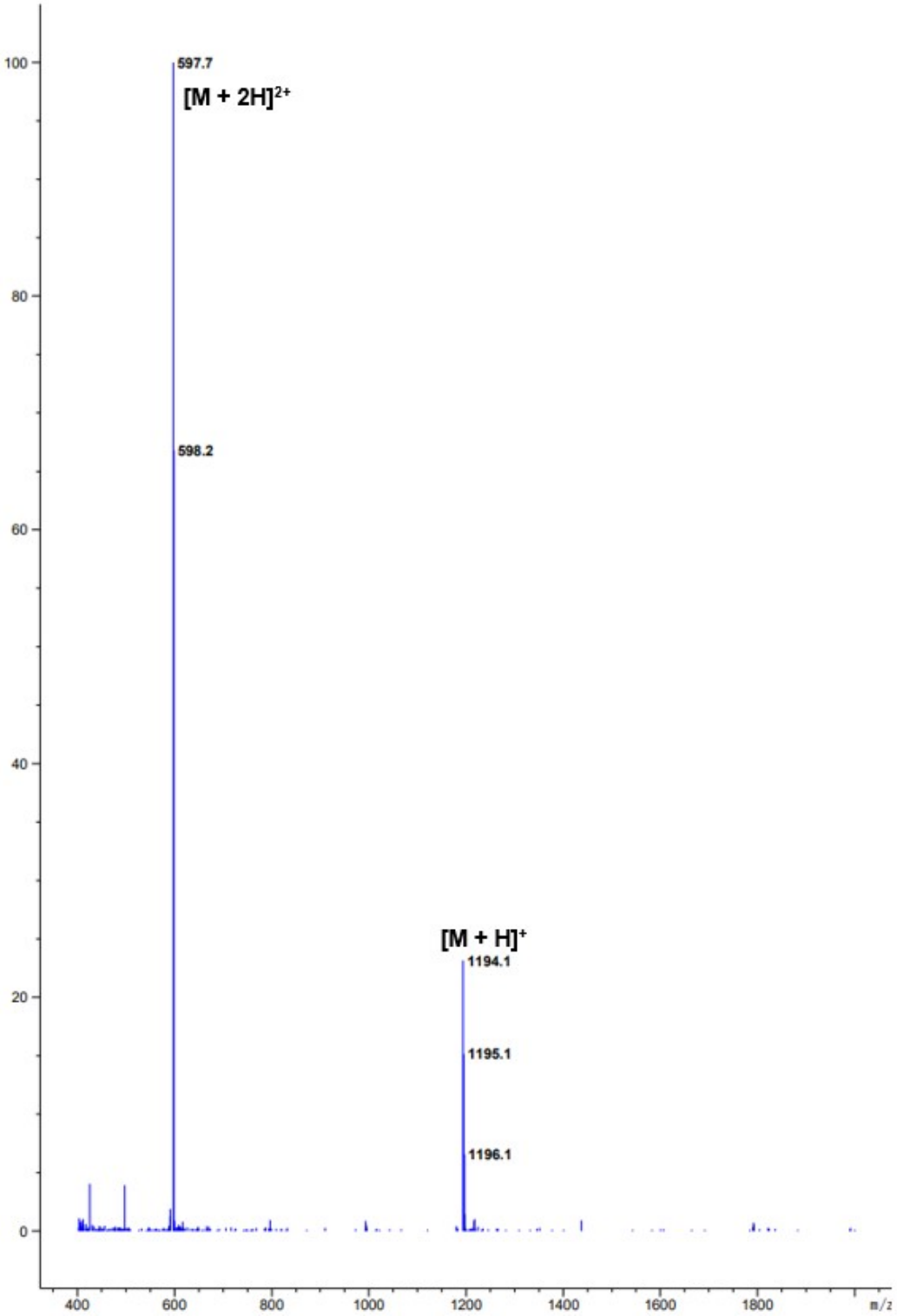


Figure S69: ESI-MS (+ve) spectrum of isolated D-Cys pivalate CLipPA analogue **7g**.

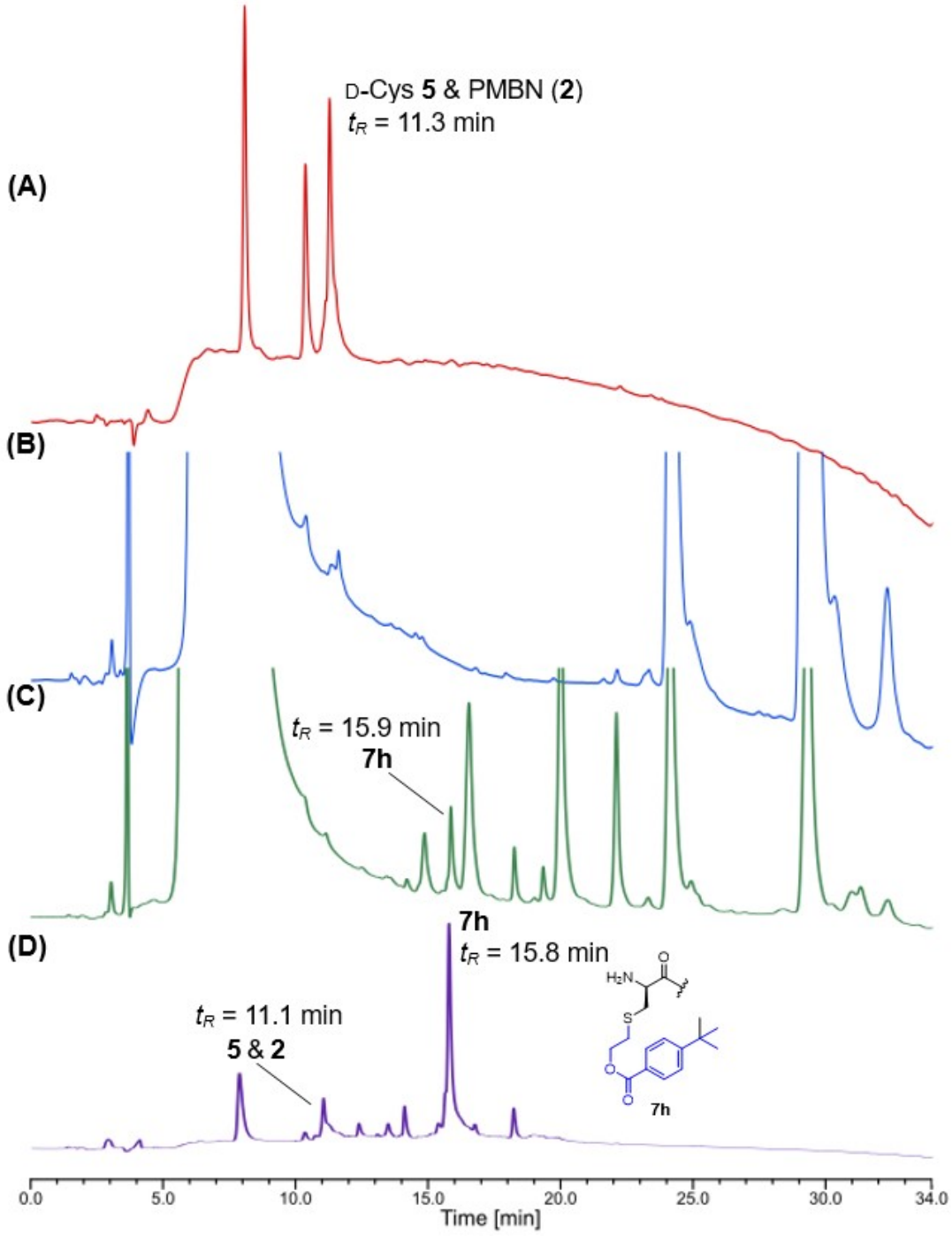


Figure S70: RP-HPLC [214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys 4-*t*Bu-benzoate CLipPA analogue **7h**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h 20 min, and **(D)** after trituration in Et_2O.

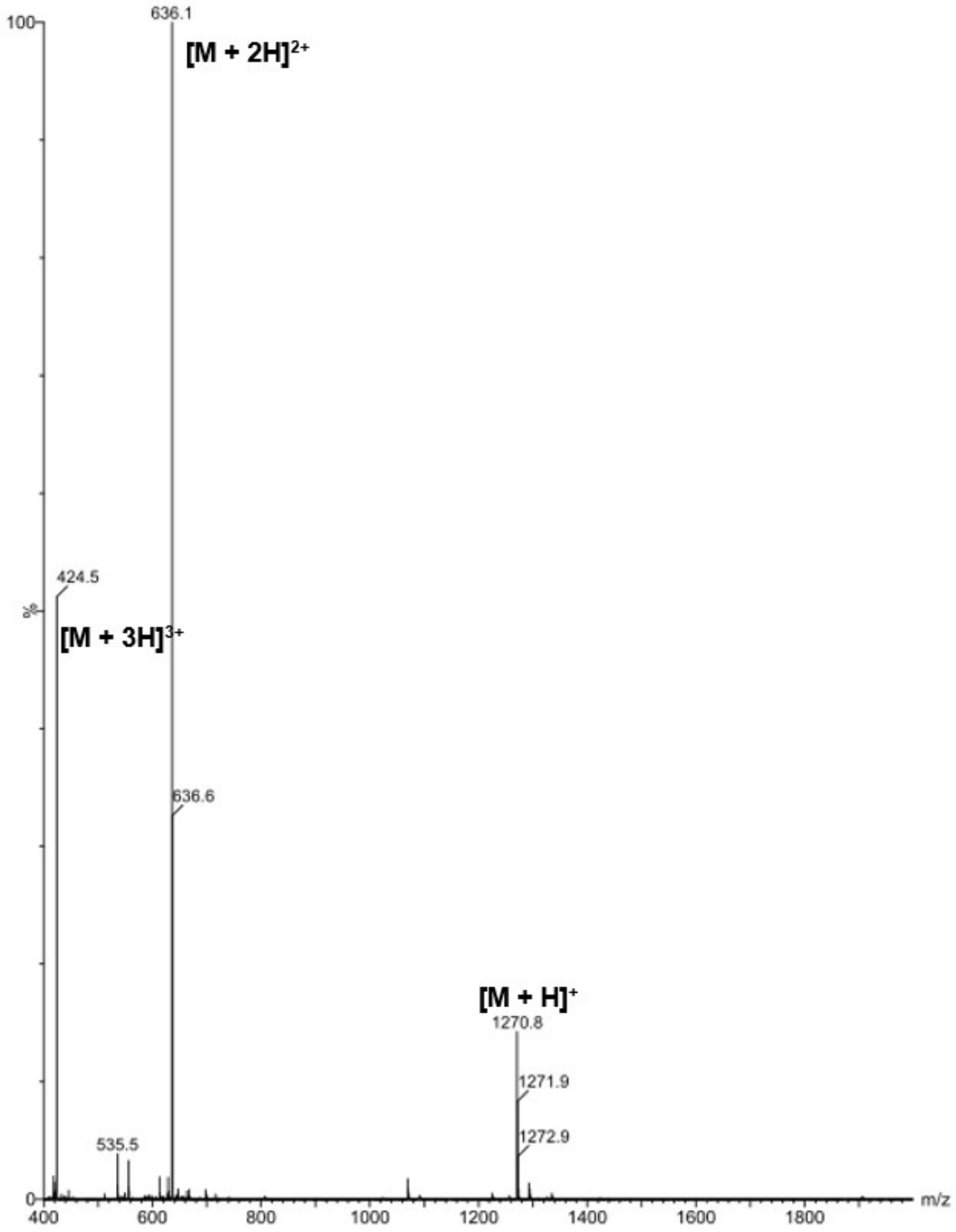


Figure S71: ESI-MS (+ve) spectrum of isolated D-Cys 4-*t*Bu-benzoate CLipPA analogue **7h**.

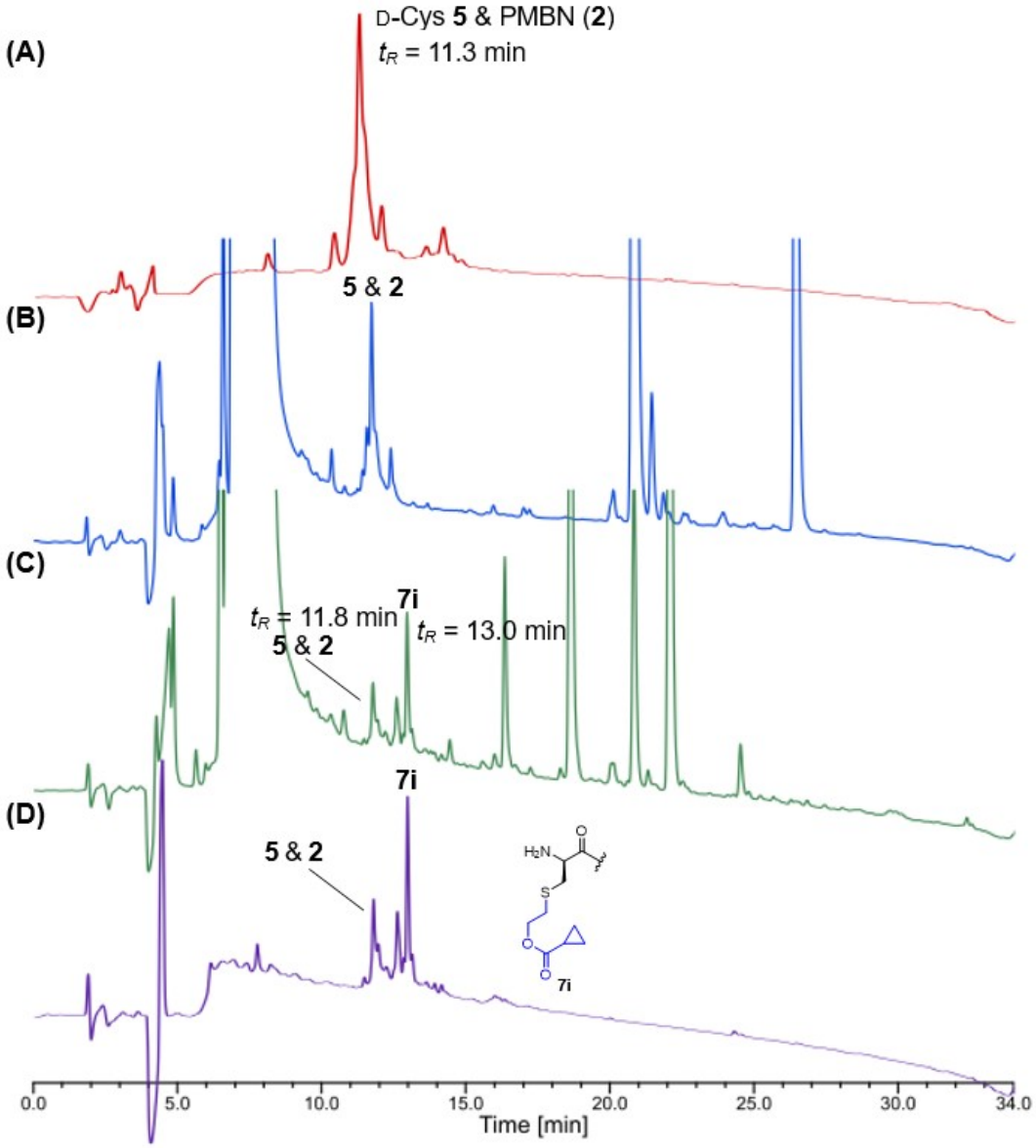


Figure S72: RP-HPLC [214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys cyclopropionate CLipPA analogue **7i**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h, and **(D)** after trituration in Et_2O.

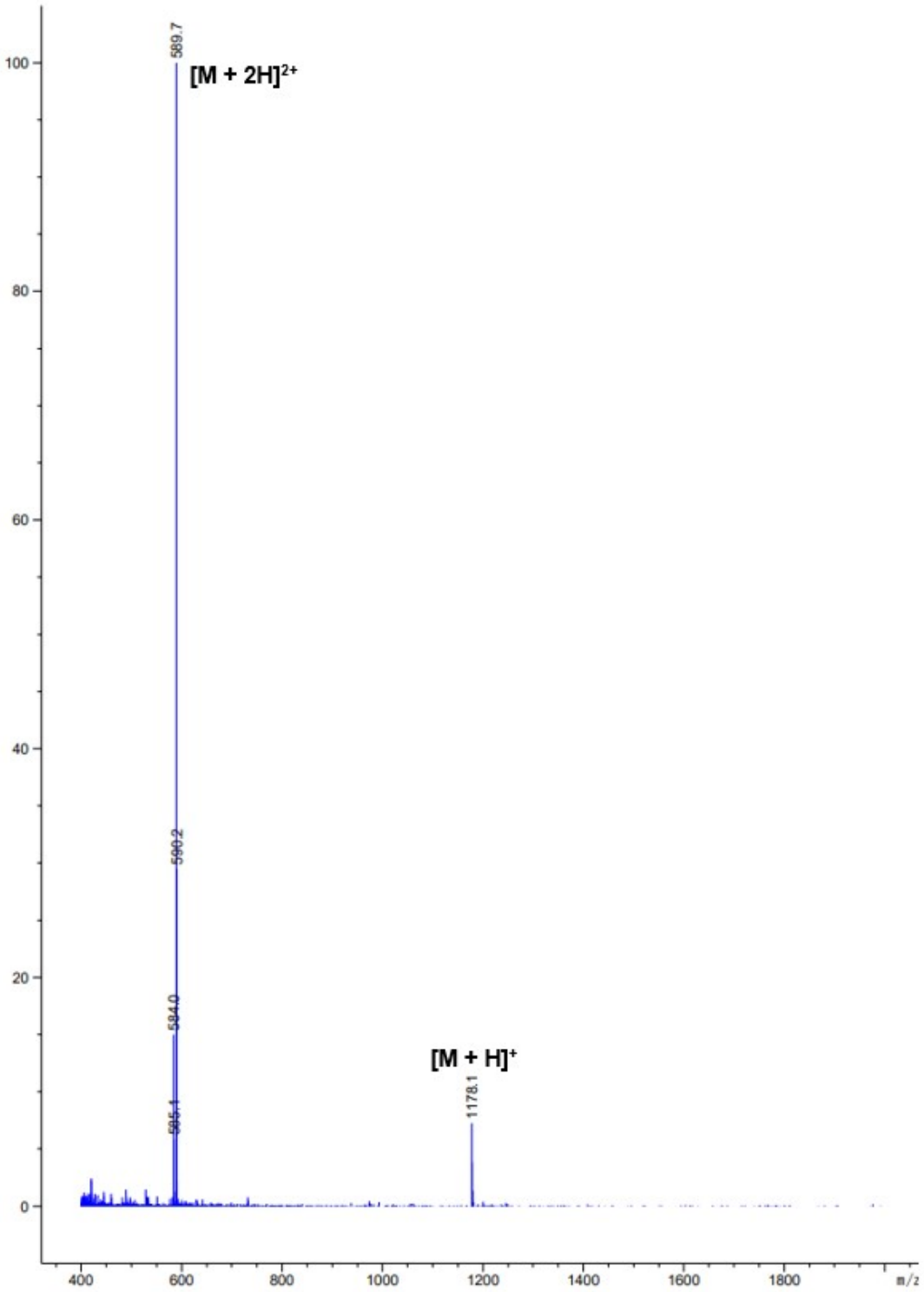


Figure S73: ESI-MS (+ve) spectrum of isolated D-Cys cyclopropionate CLipPA analogue **7i**.

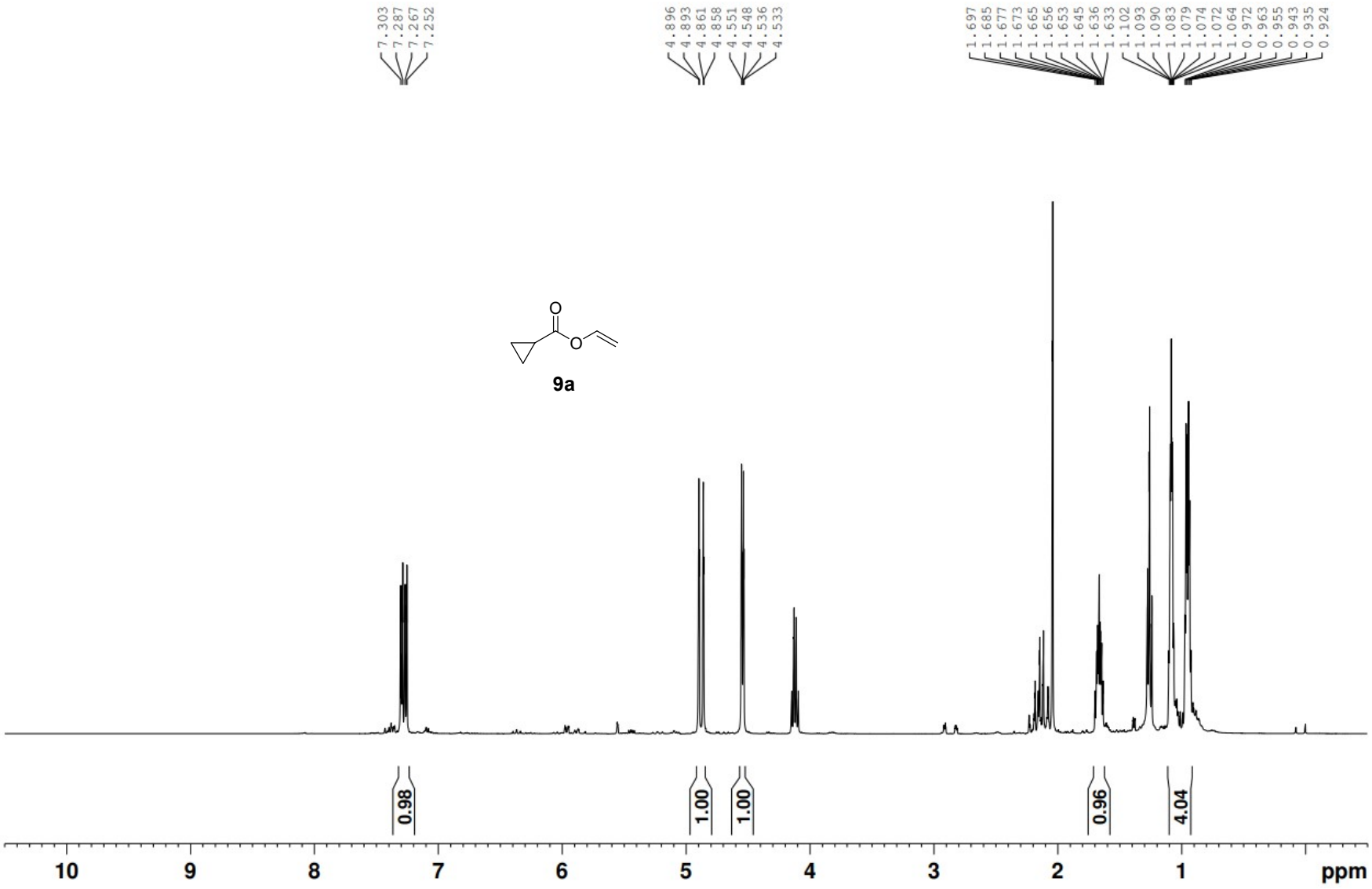


Figure S74: ^{1}H NMR spectrum ($CDCl_3$, 298 K, 400 MHz) of vinyl cyclopropionate (**9a**).

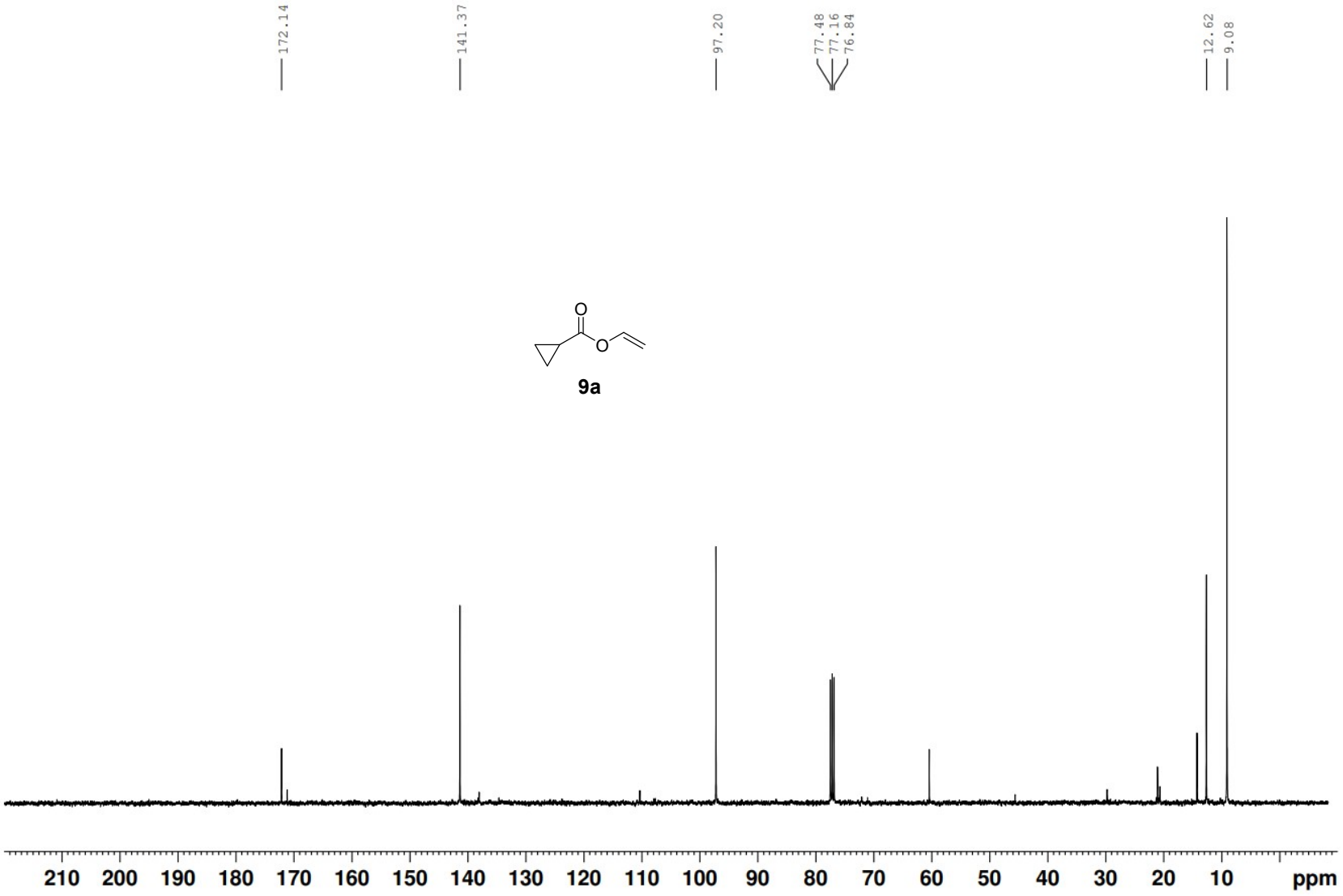


Figure S75: ^{13}C NMR spectrum ($CDCl_3$, 298 K, 100 MHz) of vinyl cyclopropionate (**9a**).

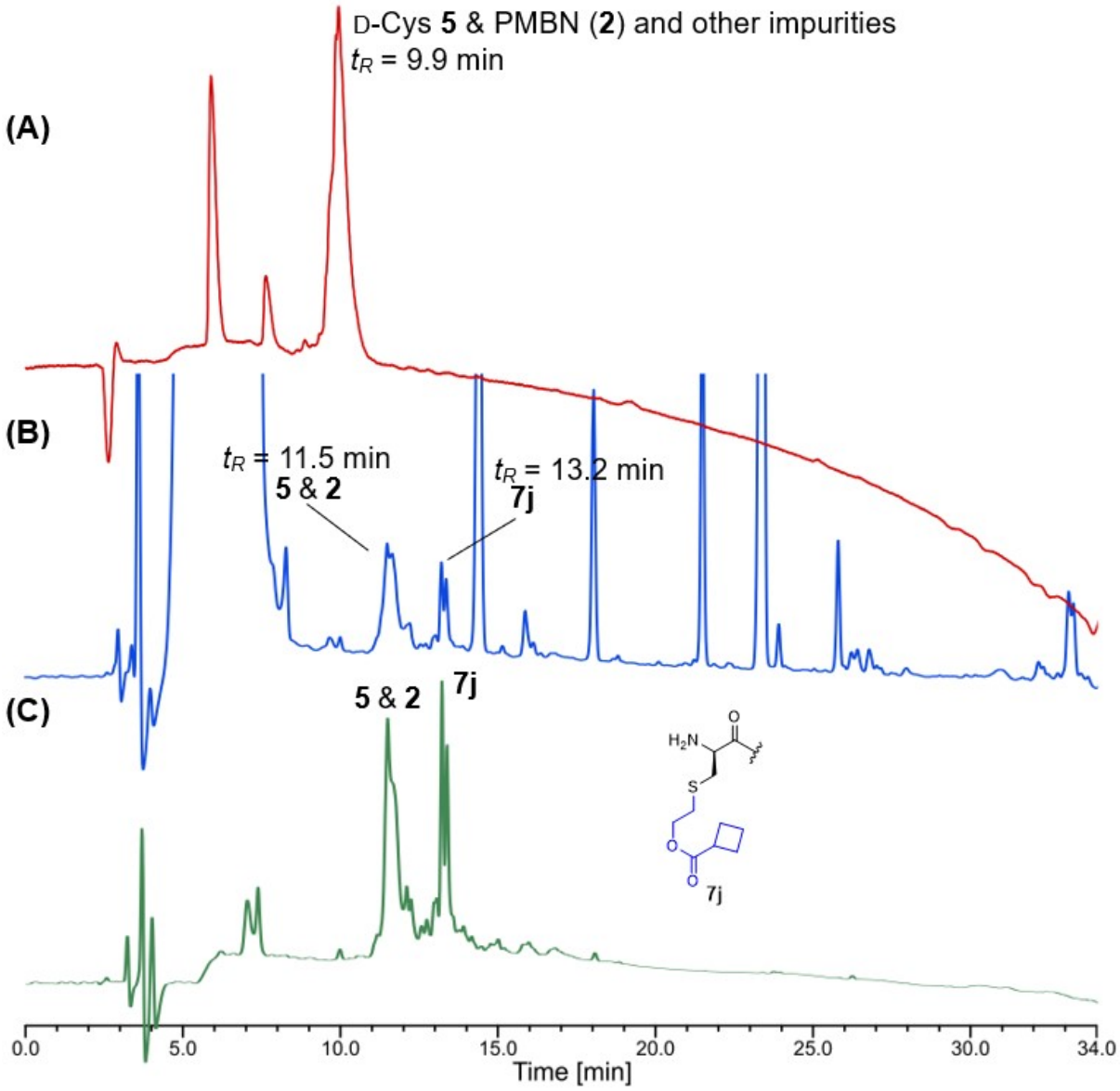


Figure S76: RP-HPLC [210/214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm) or a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys cyclobutyrate CLipPA analogue **7j**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 1 h, and **(C)** after trituration in Et_2O.

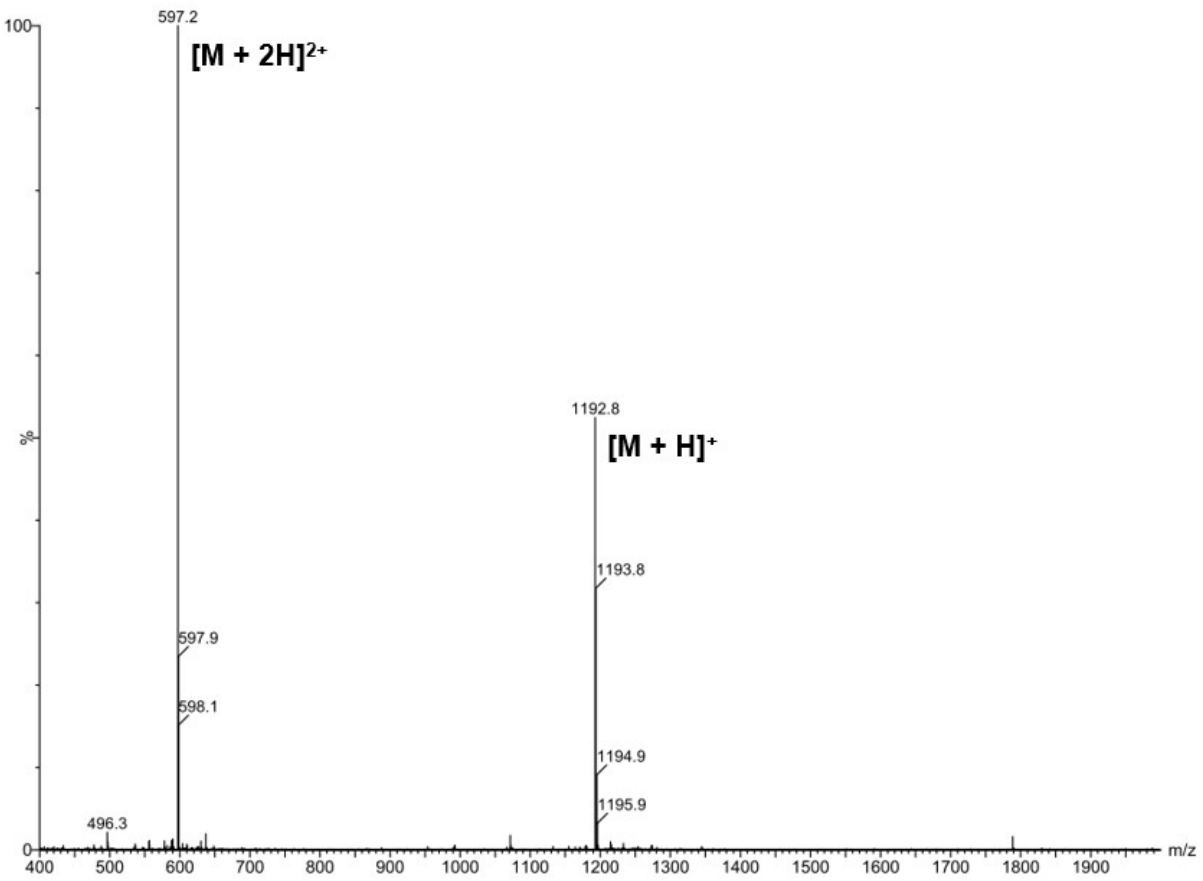


Figure S77: ESI-MS (+ve) spectrum of isolated D-Cys cyclobutyrate CLipPA analogue **7j**.

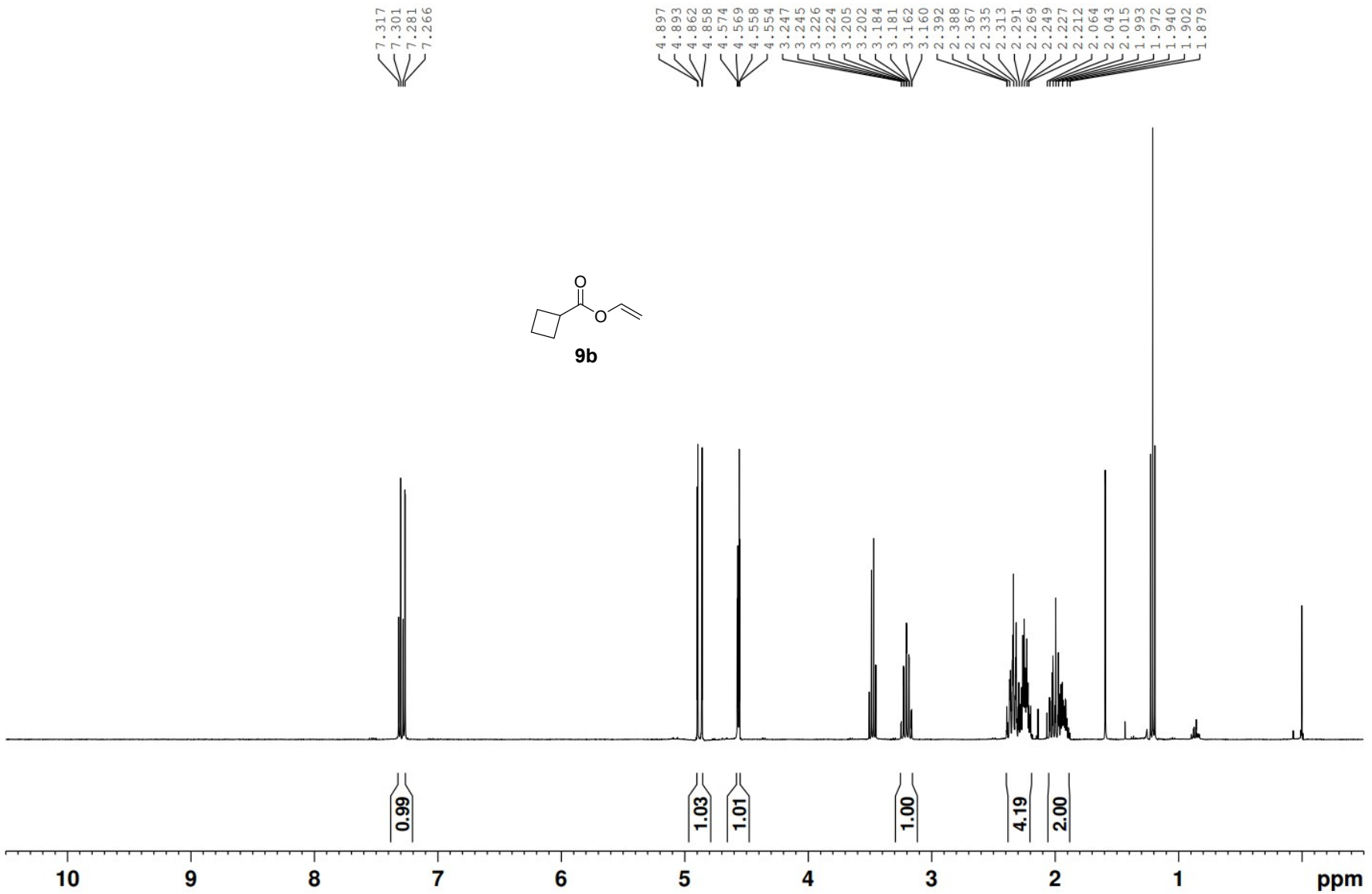


Figure S78: ^{1}H NMR spectrum ($CDCl_3$, 298 K, 400 MHz) of vinyl cyclobutyrate (**9b**).

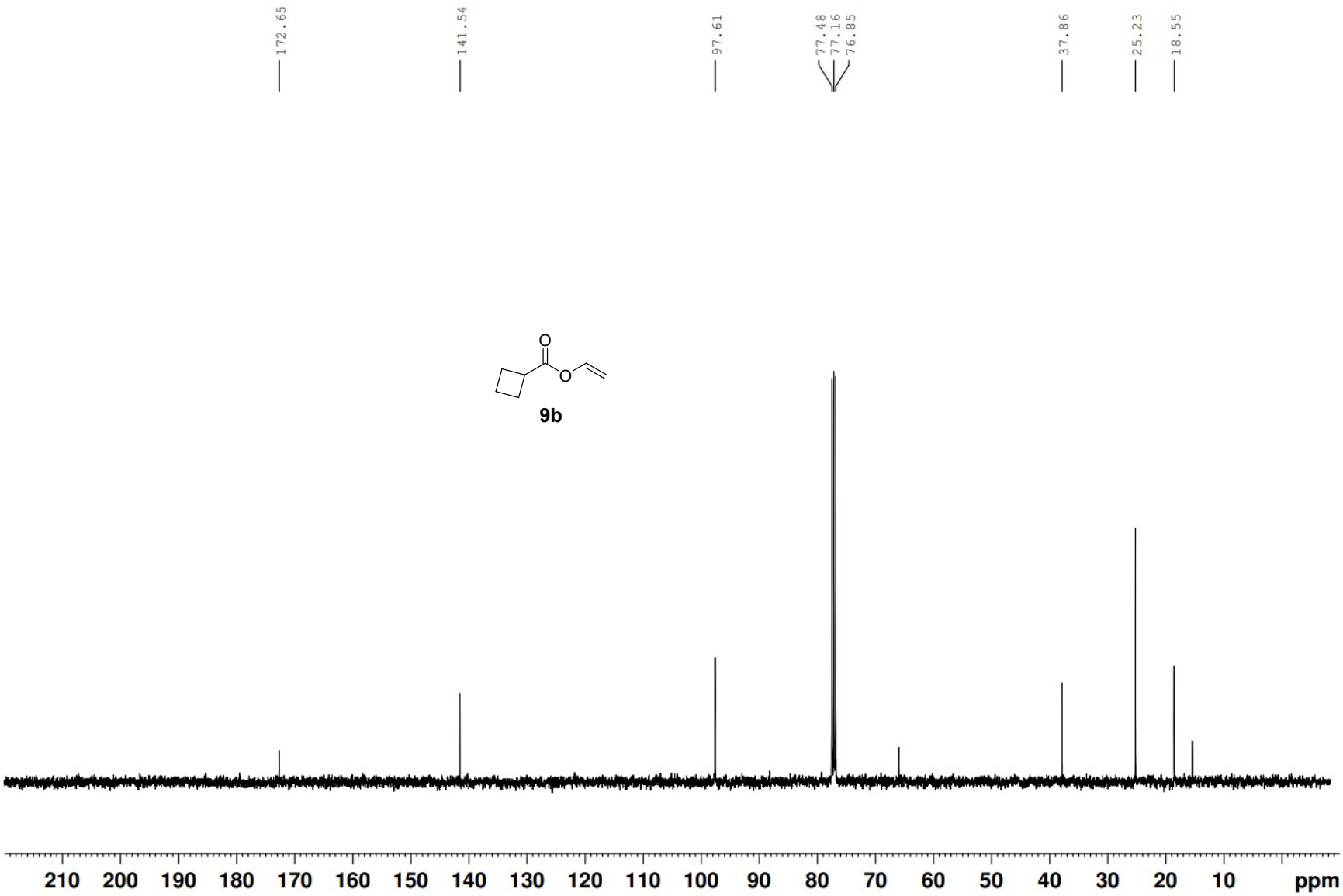


Figure S79: ^{13}C NMR spectrum ($CDCl_3$, 298 K, 100 MHz) of vinyl cyclobutyrate (**9b**).

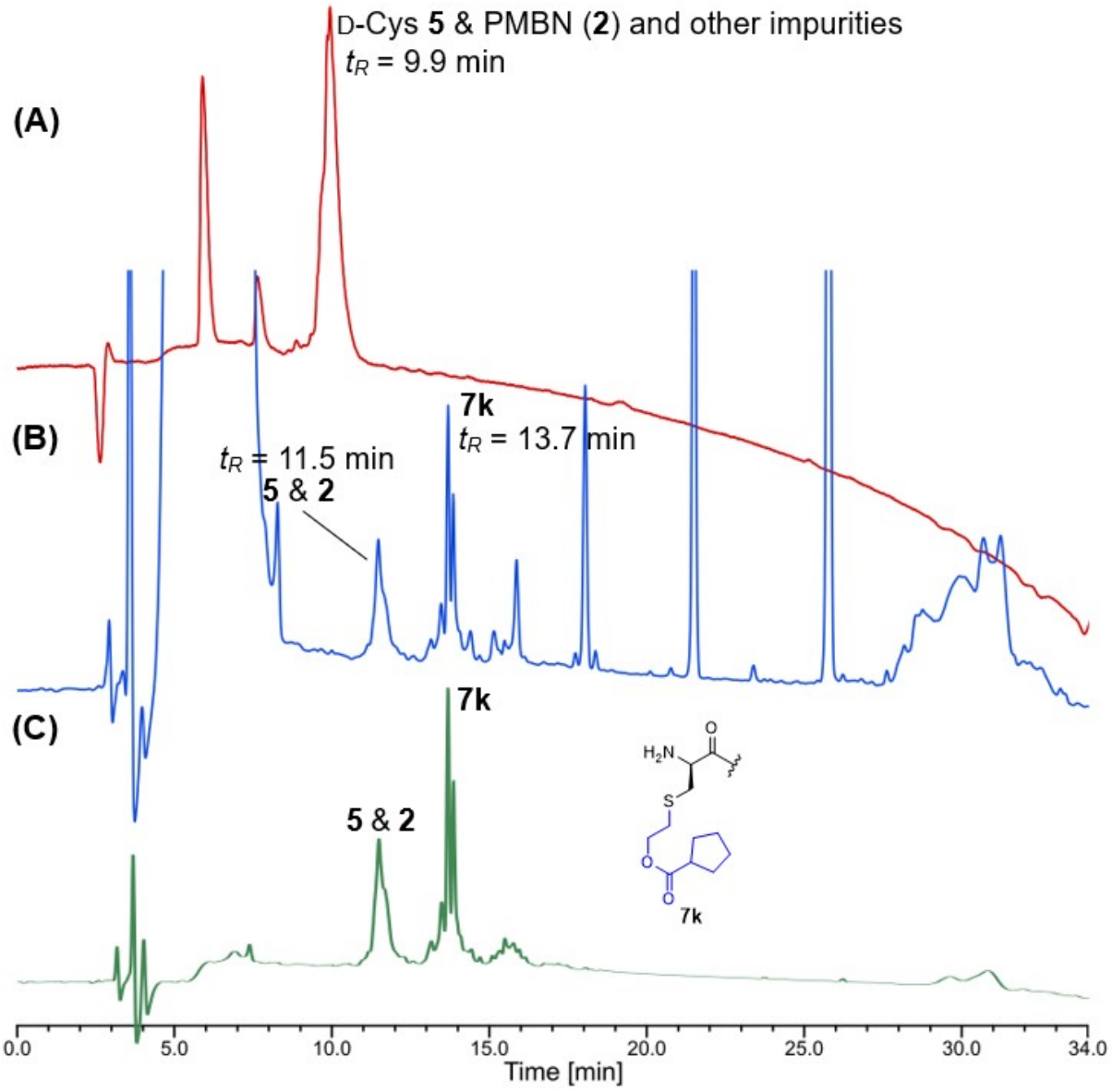


Figure S80: RP-HPLC [210/214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm) or a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys cyclovalerate CLipPA analogue **7k**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 1 h, and **(C)** after trituration in Et_2O.

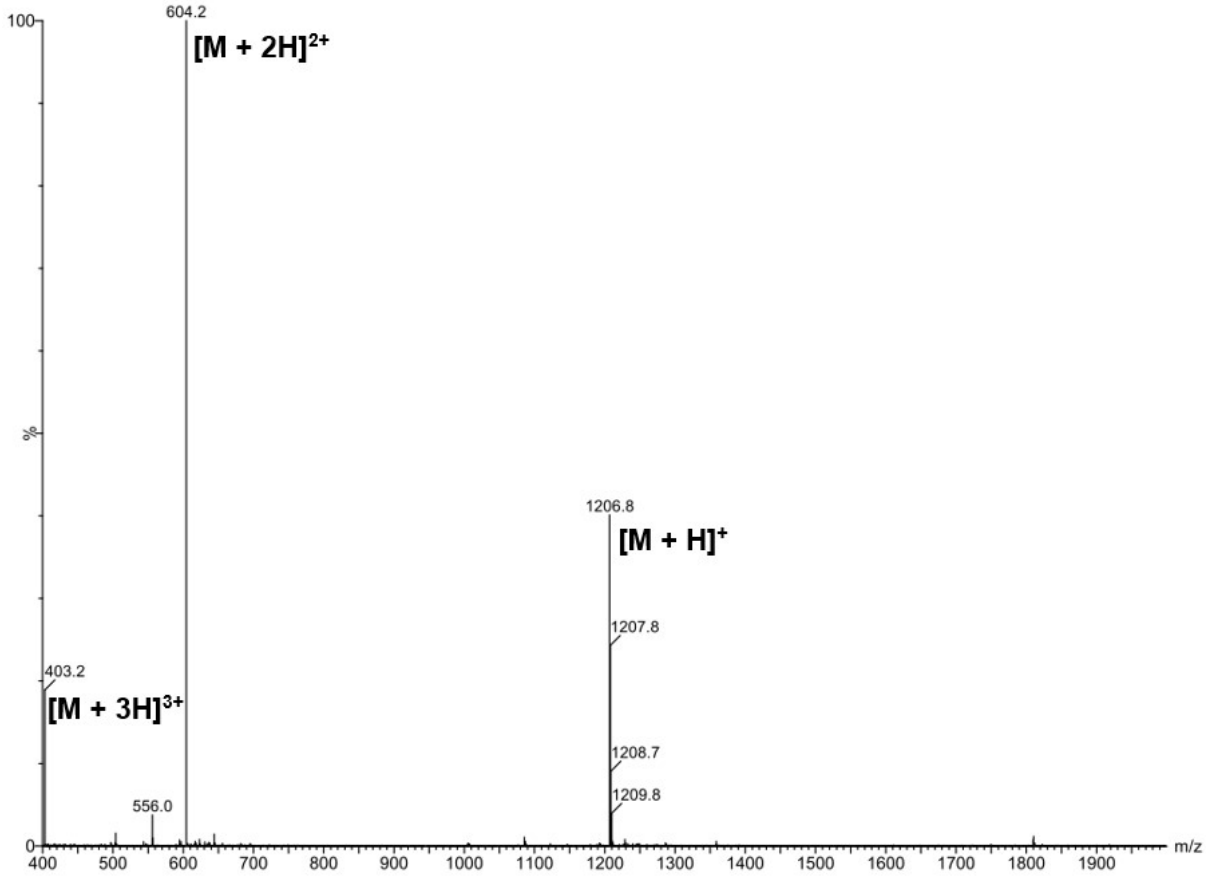


Figure S81: ESI-MS (+ve) spectrum of isolated D-Cys cyclovalerate CLipPA analogue **7k**.

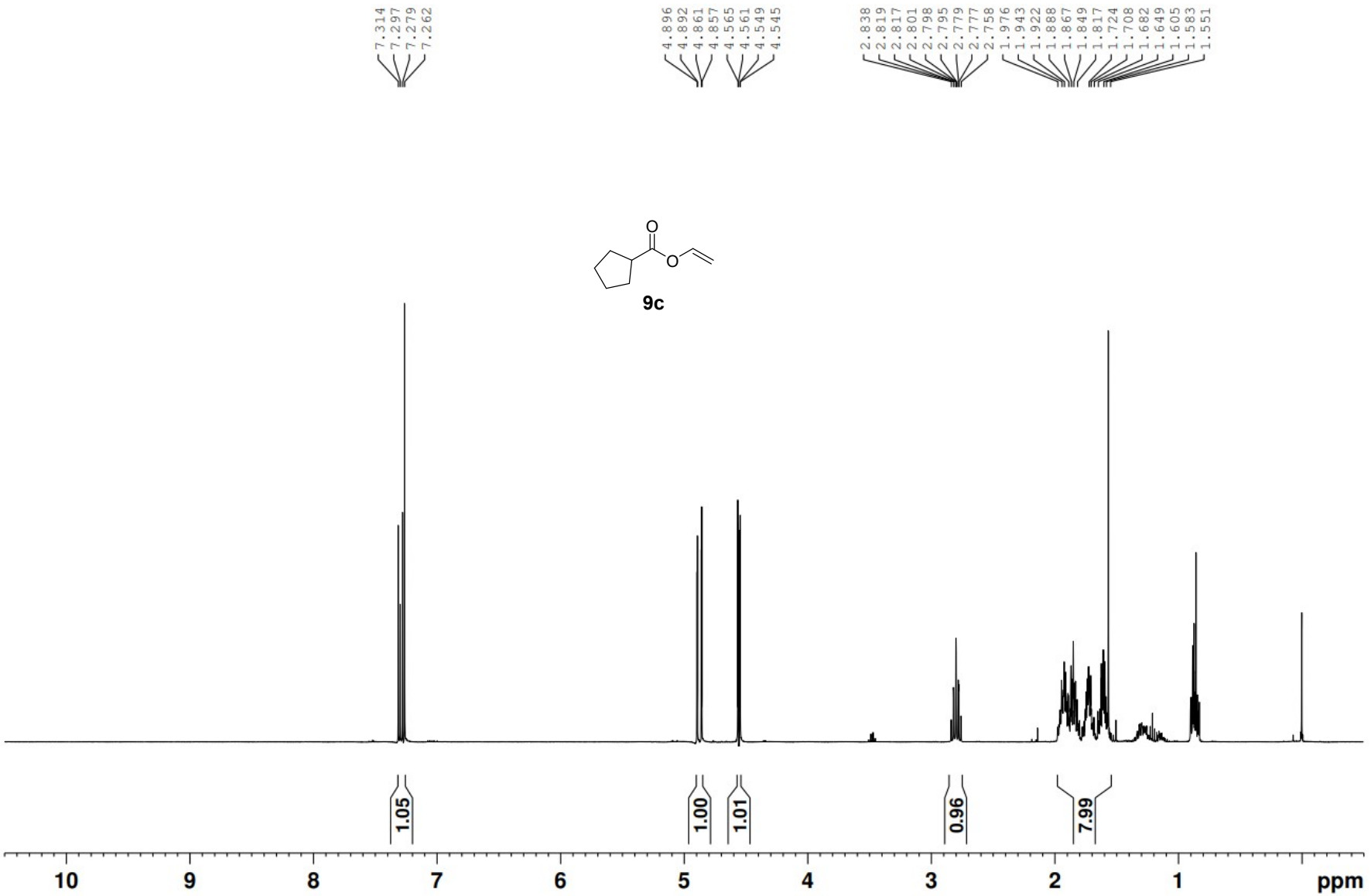


Figure S82: ^{1}H NMR spectrum ($CDCl_3$, 298 K, 400 MHz) of vinyl cyclovalerate (**9c**).

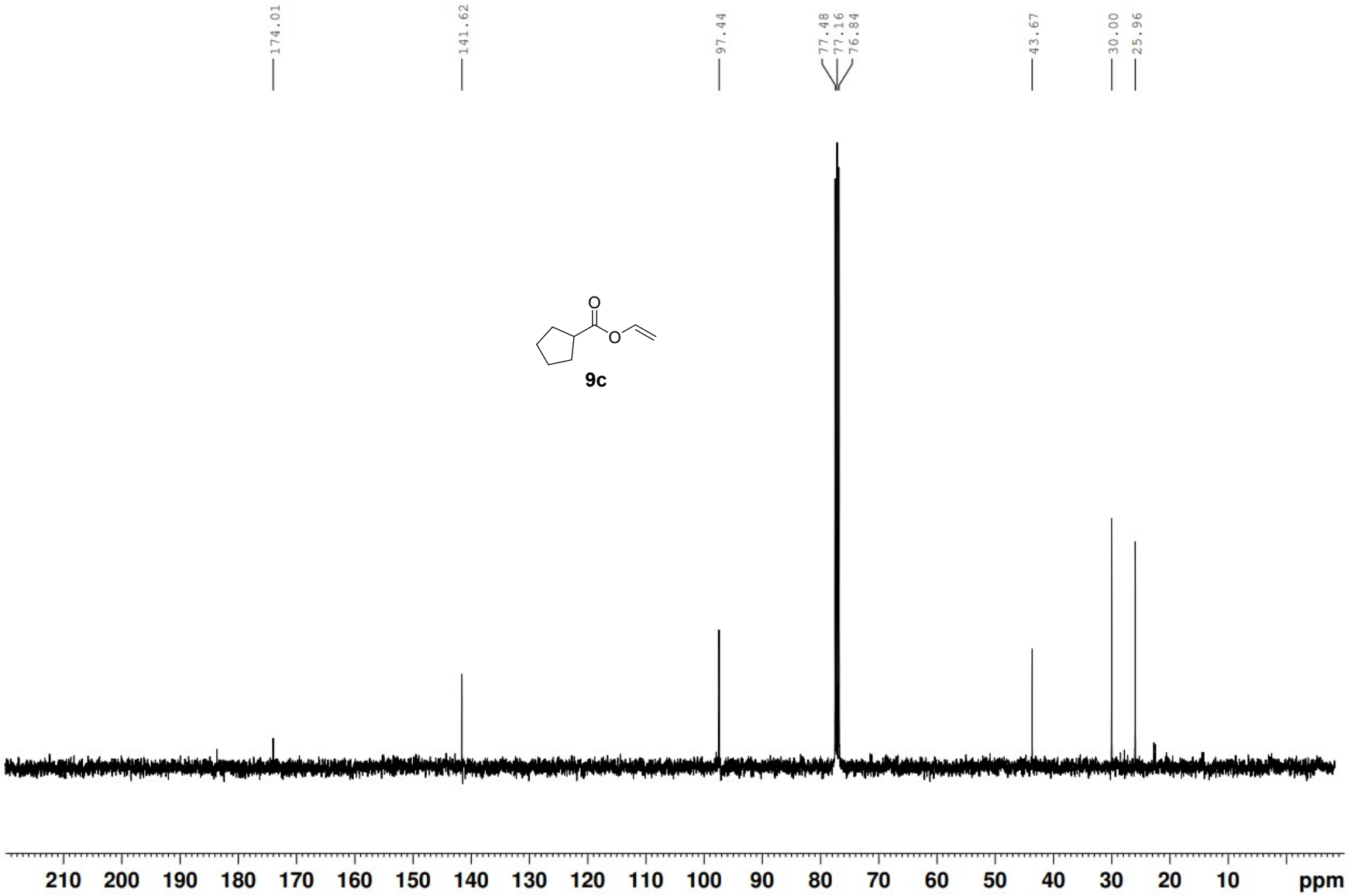


Figure S83: ^{13}C NMR spectrum ($CDCl_3$, 298 K, 100 MHz) of vinyl cyclovalerate (**9c**).

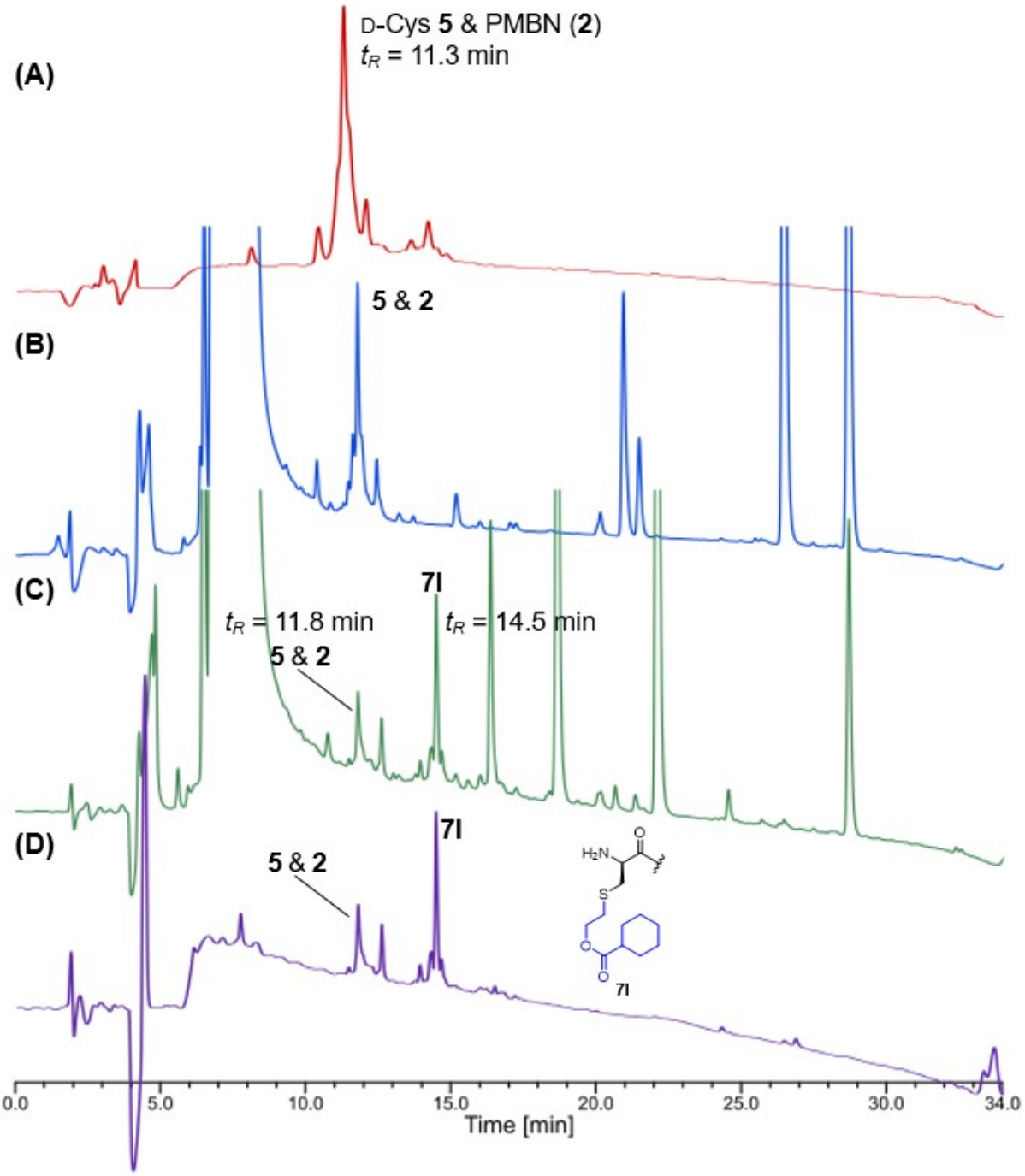


Figure S84: RP-HPLC [214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys cyclohexanoate CLipPA analogue **7I**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h, and **(D)** after trituration in Et_2O.

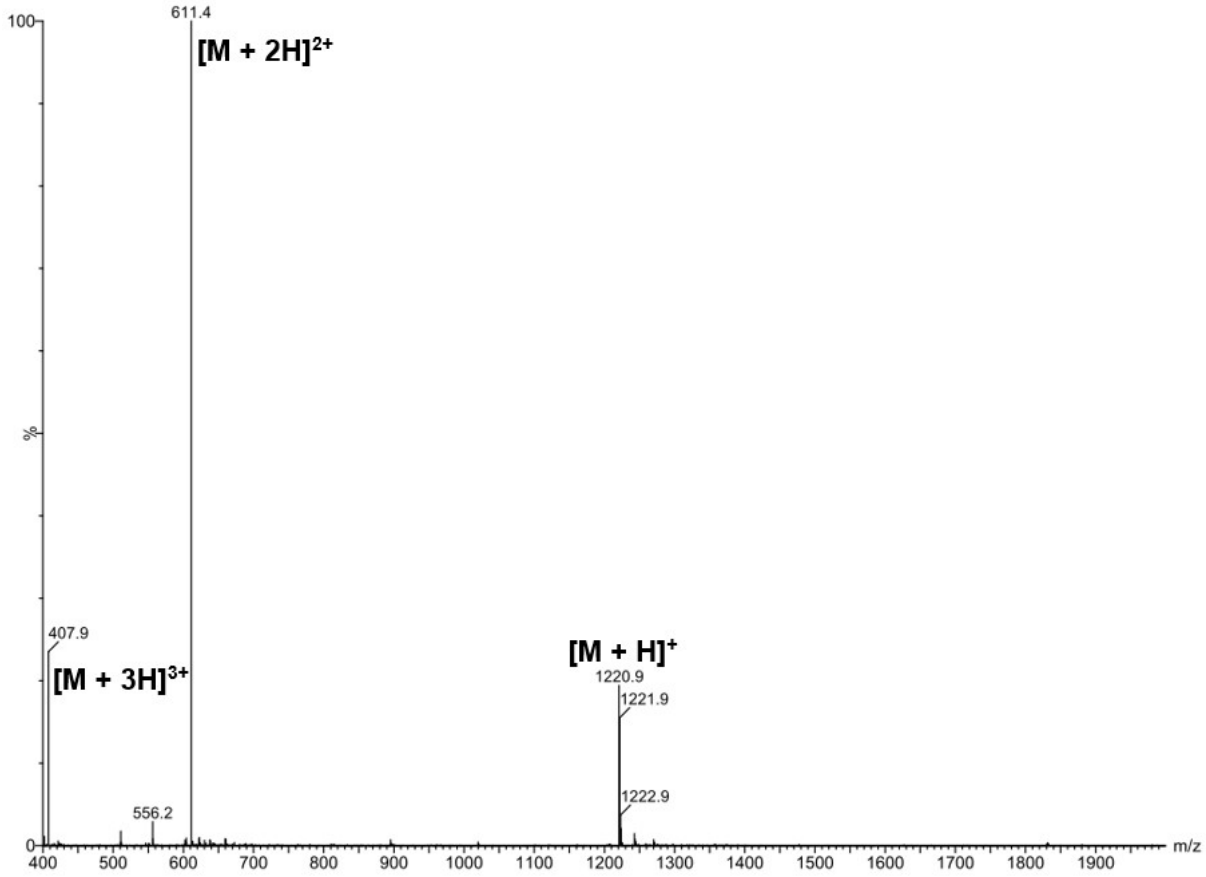


Figure S85: ESI-MS (+ve) spectrum of isolated D-Cys cyclohexanoate CLipPA analogue **7l**.

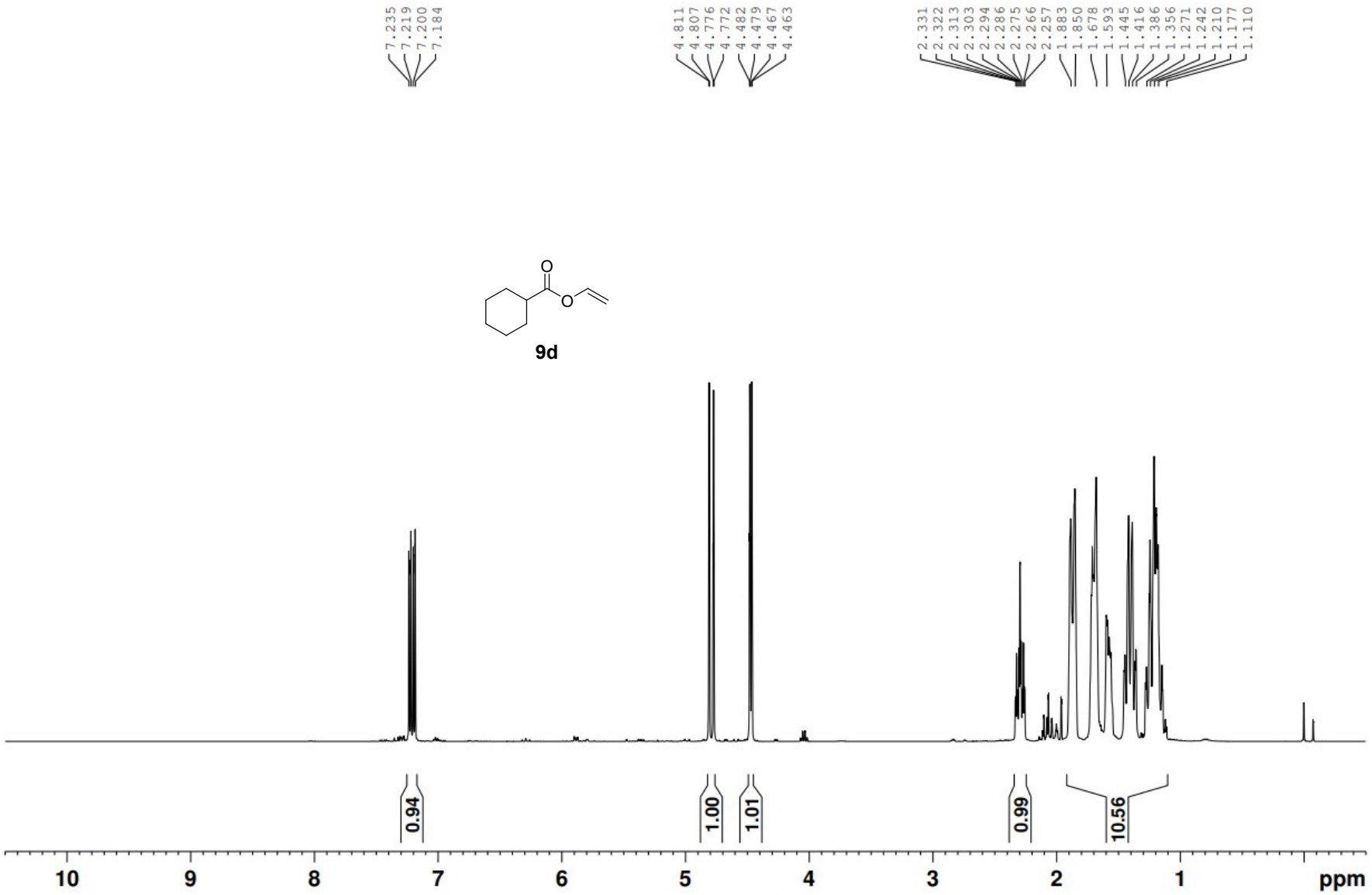


Figure S86: ^{1}H NMR spectrum ($CDCl_3$, 298 K, 400 MHz) of vinyl cyclohexanoate (**9d**).

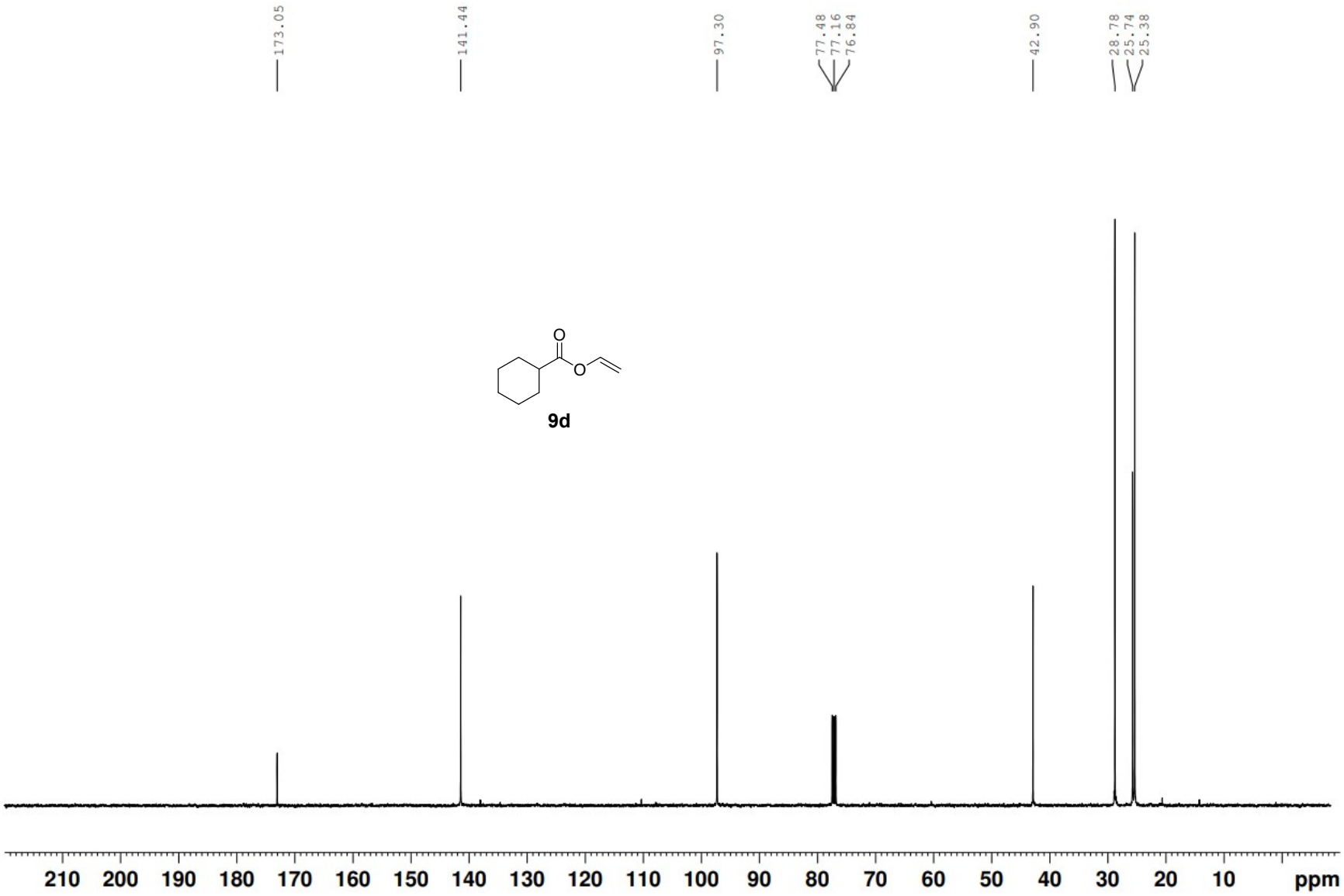


Figure S87: ^{13}C NMR spectrum ($CDCl_3$, 298 K, 100 MHz) of vinyl cyclohexanoate (**9d**).

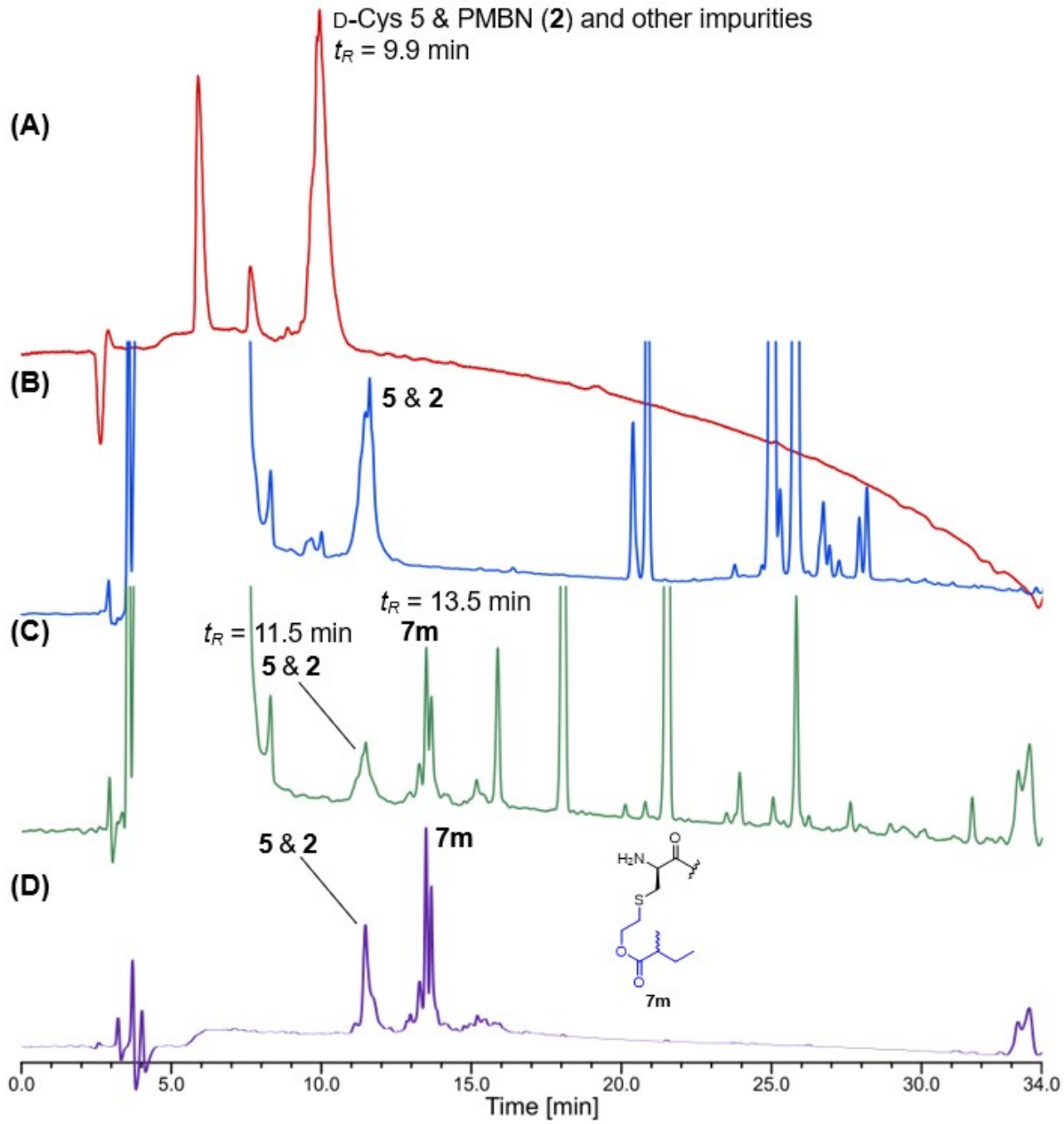


Figure S88: RP-HPLC [210/214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm) or a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys 2-Me-butyrate CLipPA analogue **7m**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h, and **(D)** after trituration in Et_2O.

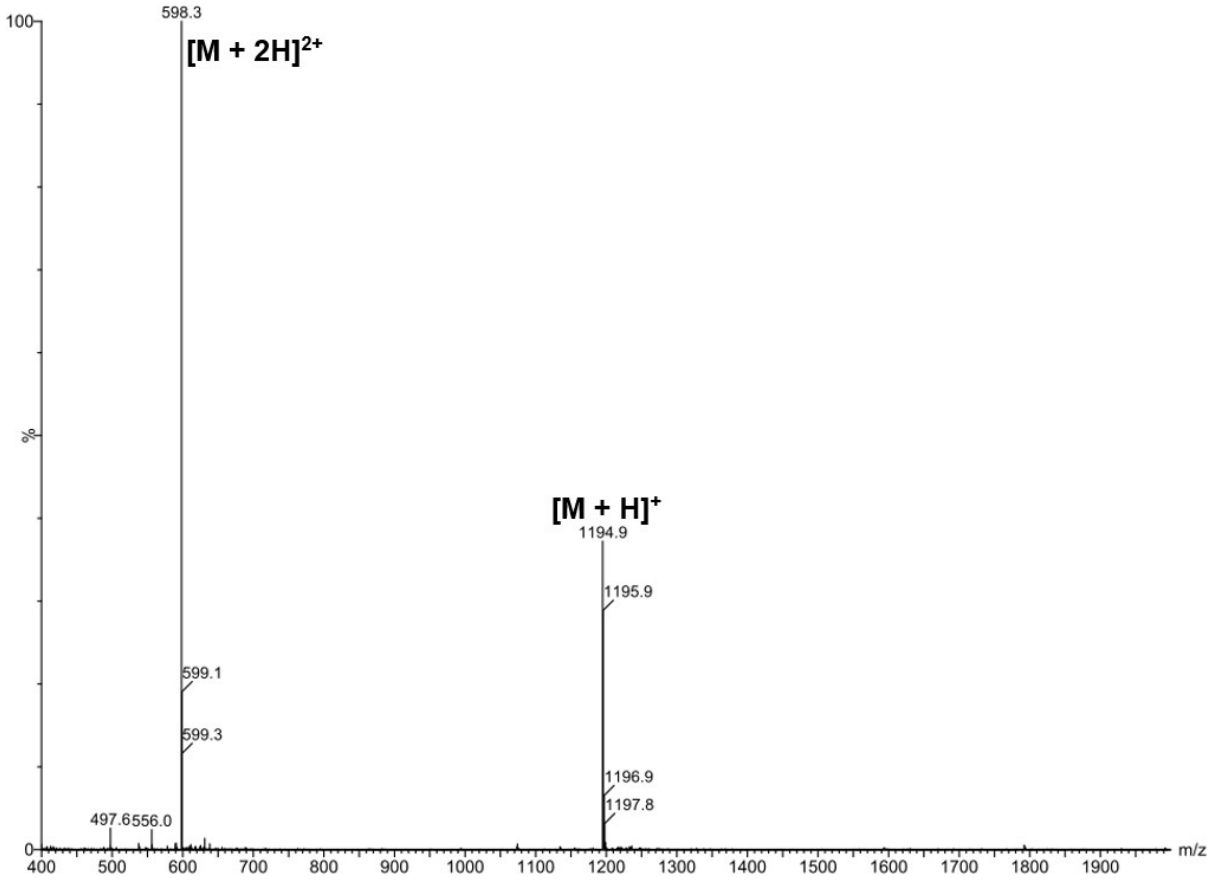


Figure S89: ESI-MS (+ve) spectrum of isolated D-Cys 2-Me-butyrate CLipPA analogue **7m**.

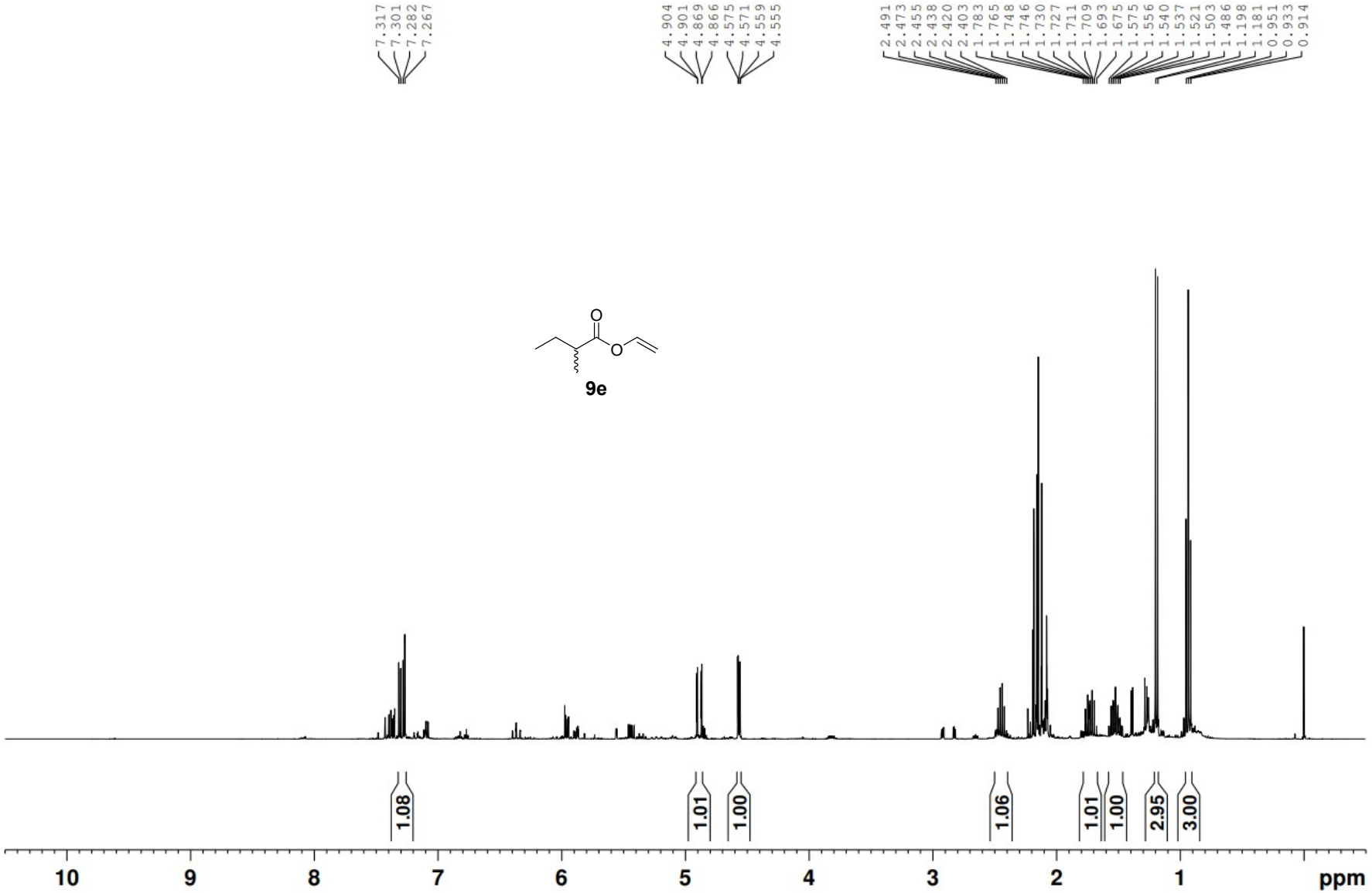


Figure S90: ^{1}H NMR spectrum ($CDCl_3$, 298 K, 400 MHz) of vinyl 2-Me-butyrate (**9e**).

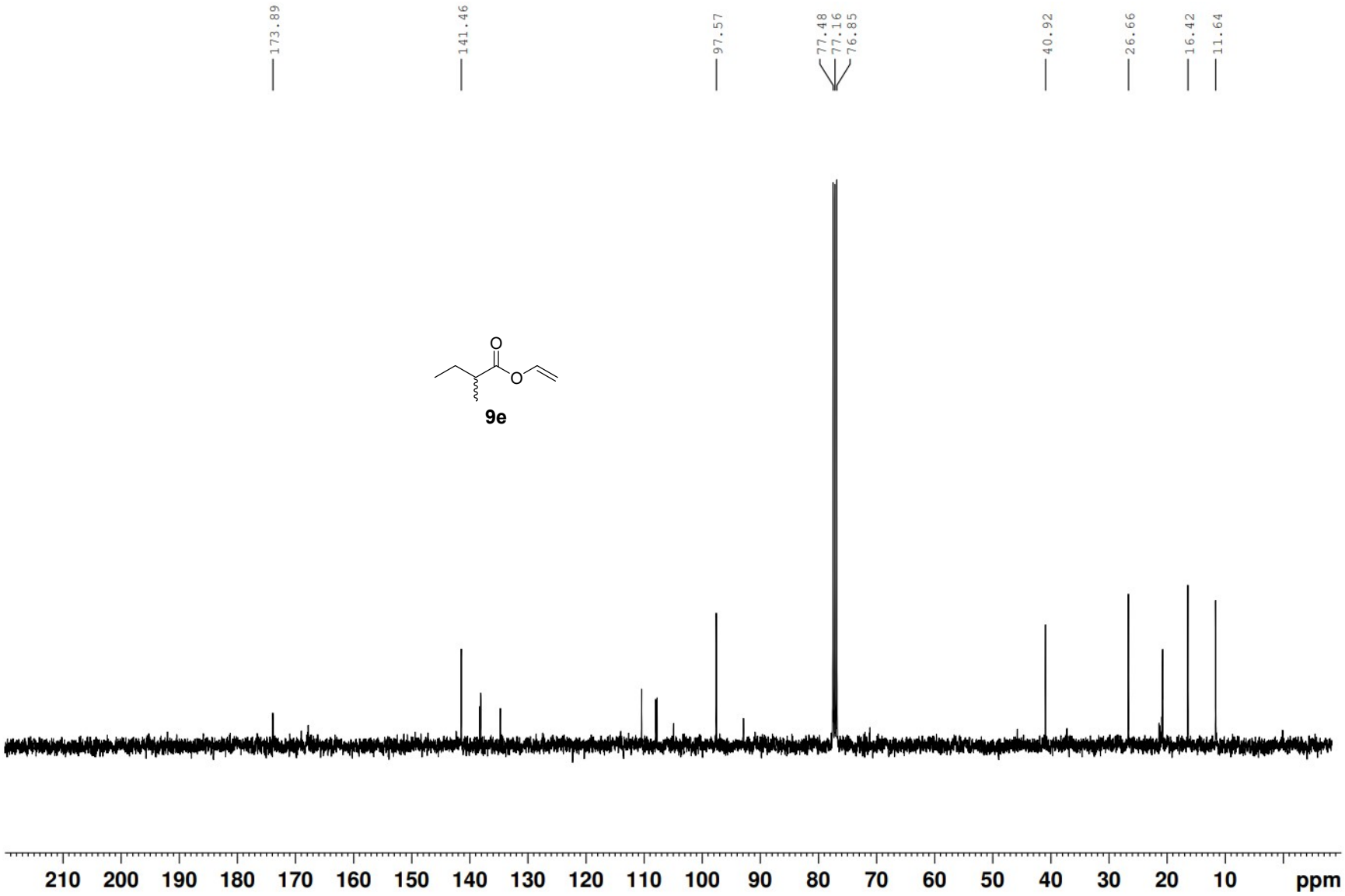


Figure S91: ^{13}C NMR spectrum ($CDCl_3$, 298 K, 100 MHz) of vinyl 2-Me-butyrate (**9e**).

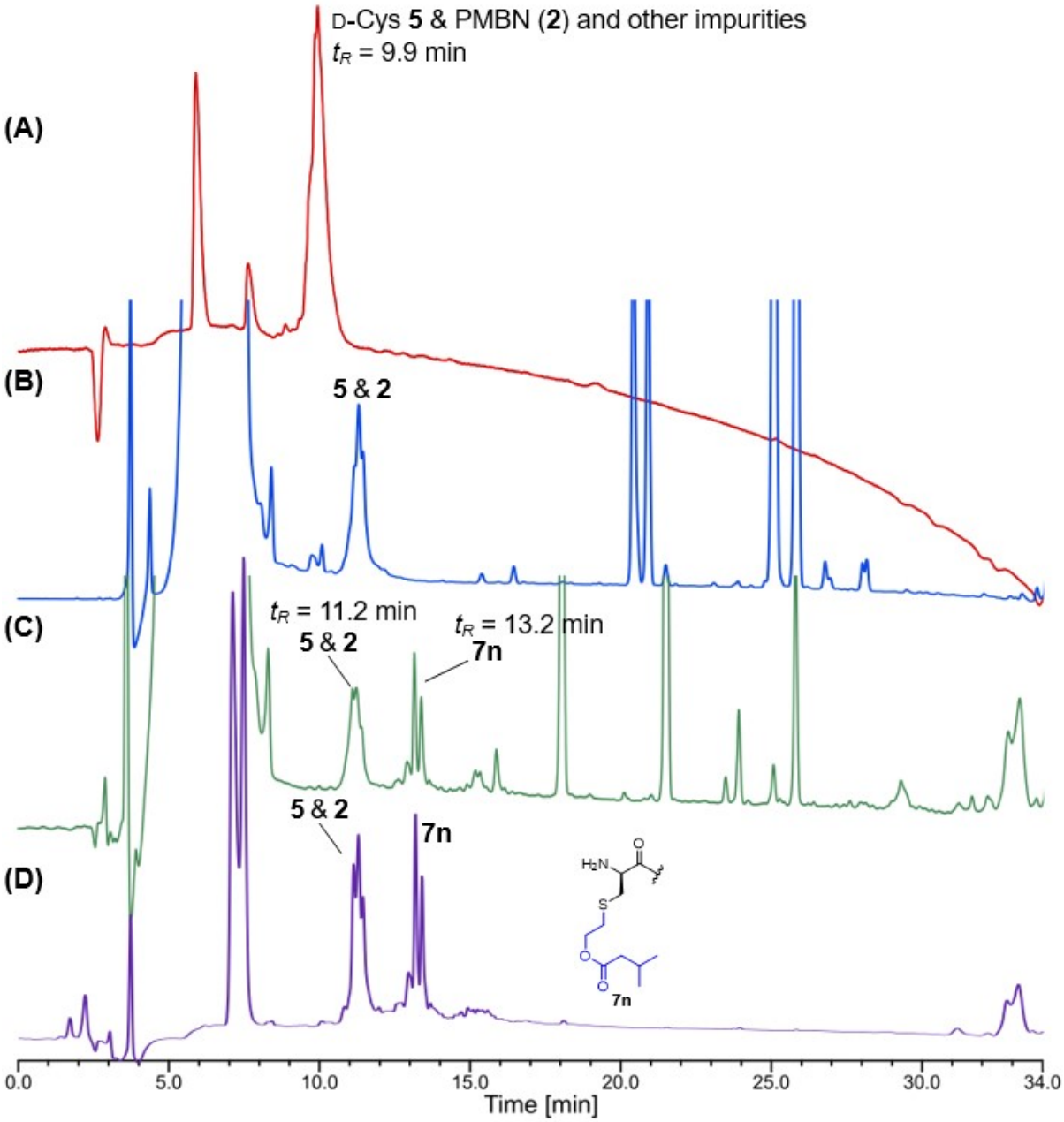


Figure S92: RP-HPLC [210/214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm) or a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys 3-Me-butyrate CLipPA analogue **7n**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h, and **(D)** after trituration in Et_2O.

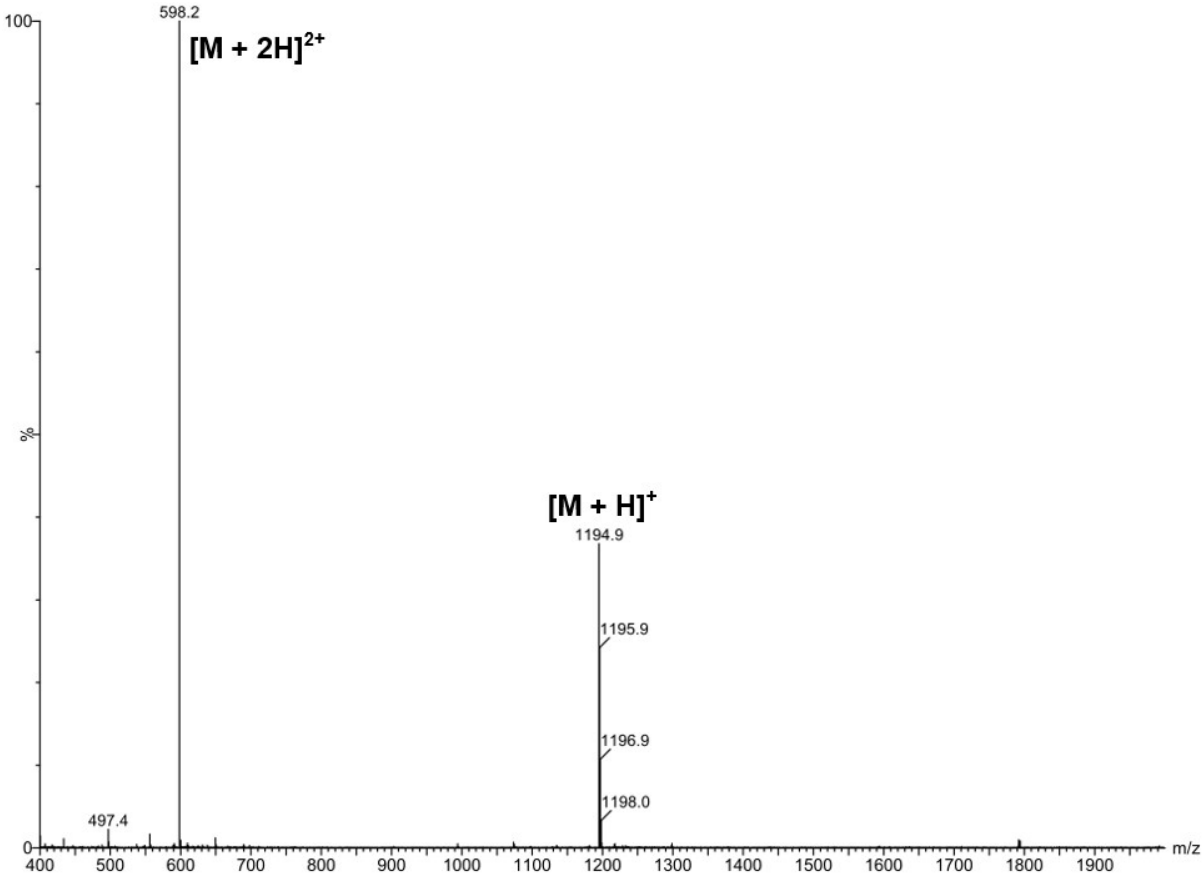


Figure S93: ESI-MS (+ve) spectrum of isolated D-Cys 3-Me-butyrate CLipPA analogue **7n**.

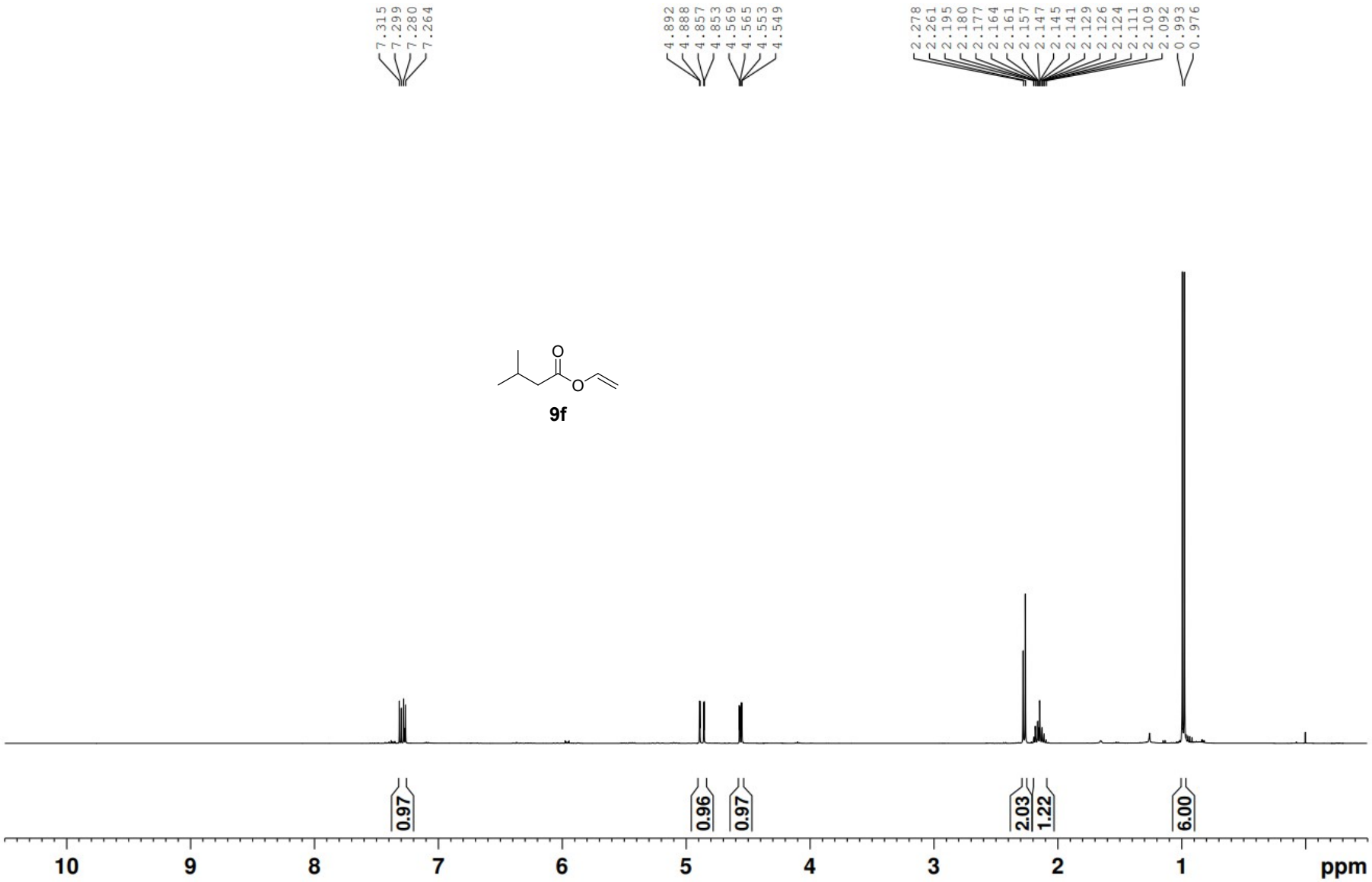


Figure S94: ^{1}H NMR spectrum ($CDCl_3$, 298 K, 400 MHz) of vinyl 3-Me-butyrate (**9f**).

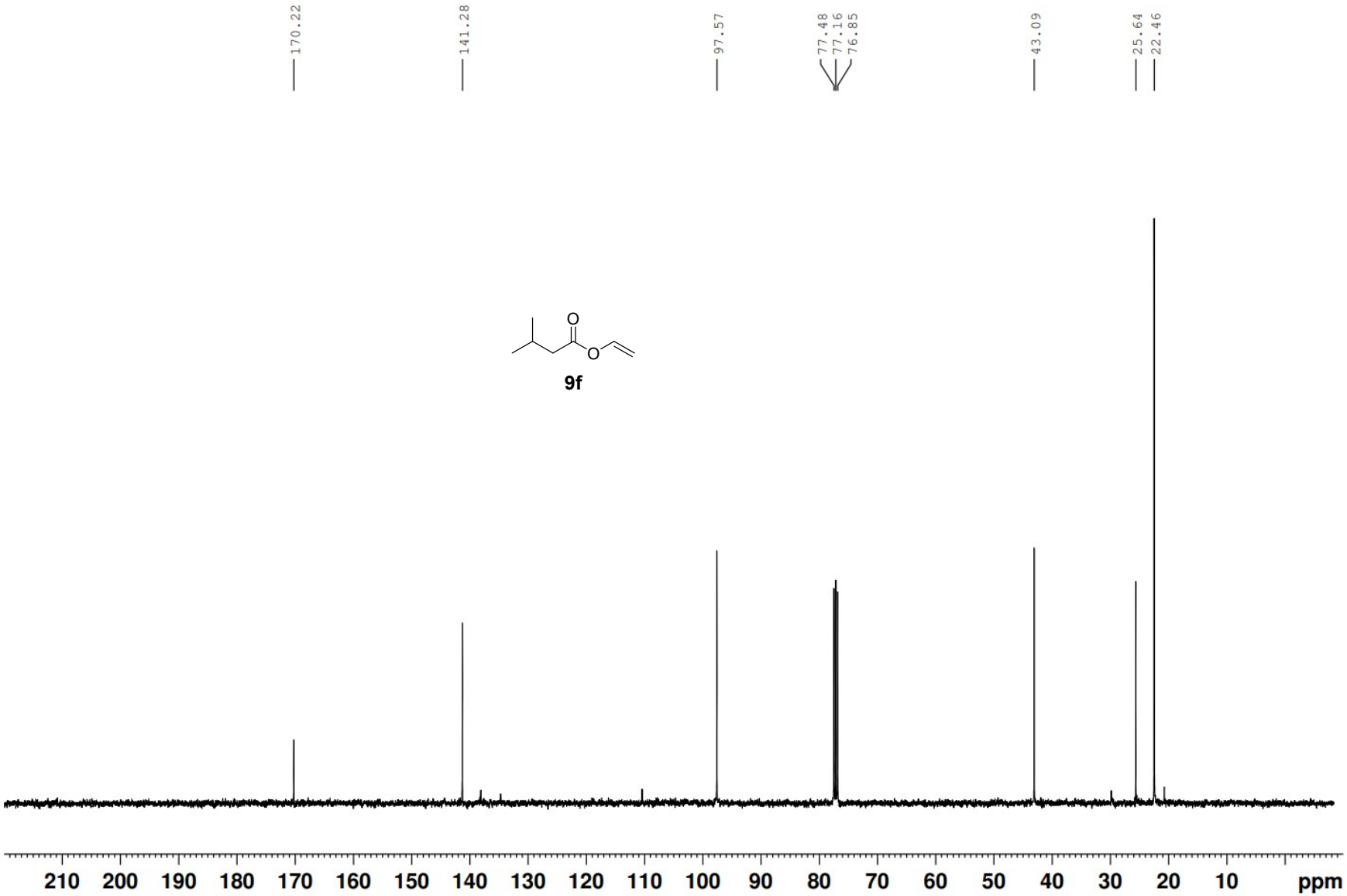


Figure S95: ^{13}C NMR spectrum ($CDCl_3$, 298 K, 100 MHz) of vinyl 3-Me-butyrate (**9f**).

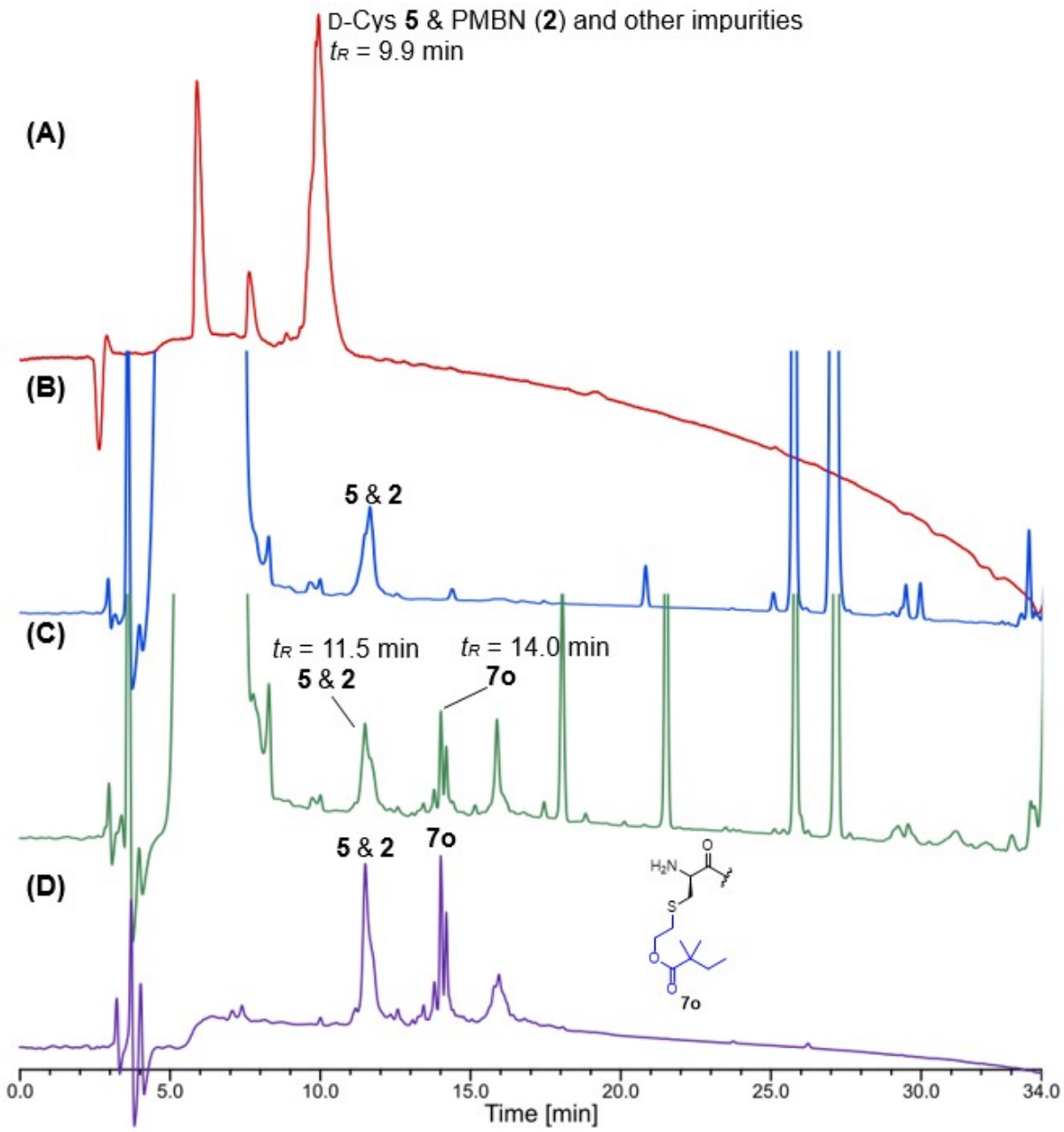


Figure S96: RP-HPLC [210/214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm) or a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys 2,2-di-Me-butyrate CLipPA analogue **7o**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h, and **(D)** after trituration in Et_2O.

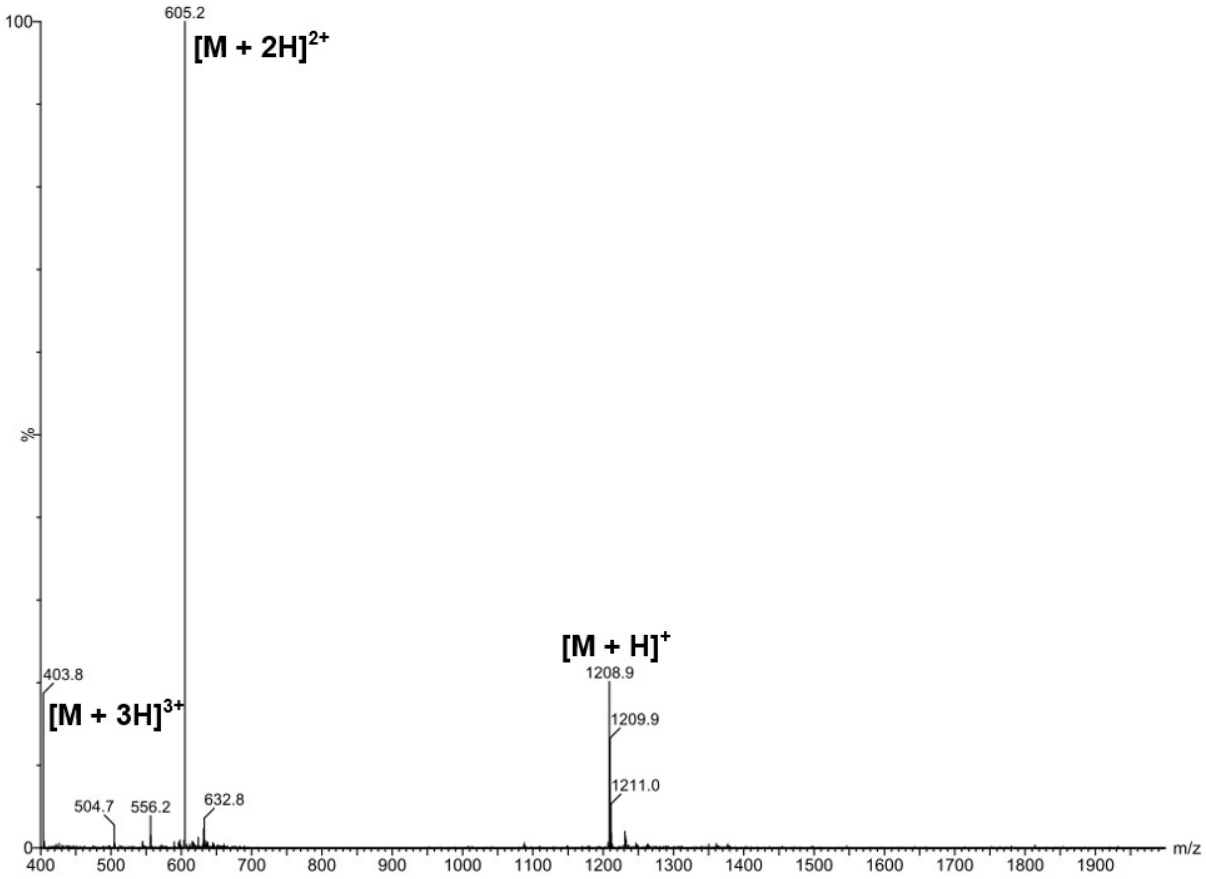


Figure S97: ESI-MS (+ve) spectrum of isolated D-Cys 2,2-di-Me-butyrate CLipPA analogue **7o**.

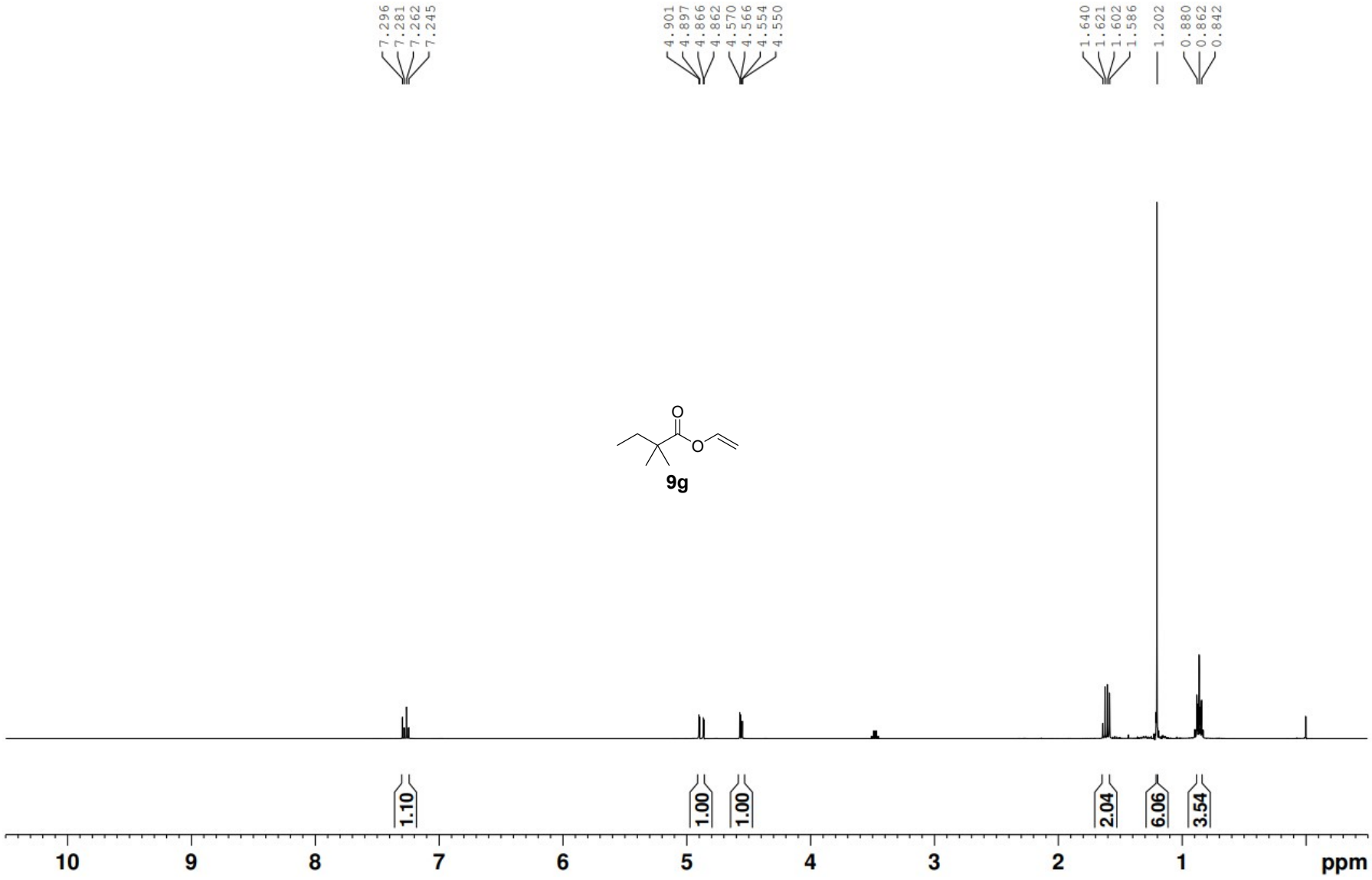


Figure S98: ^{1}H NMR spectrum ($CDCl_3$, 298 K, 400 MHz) of vinyl 2,2-di-Me-butyrate (**9g**).

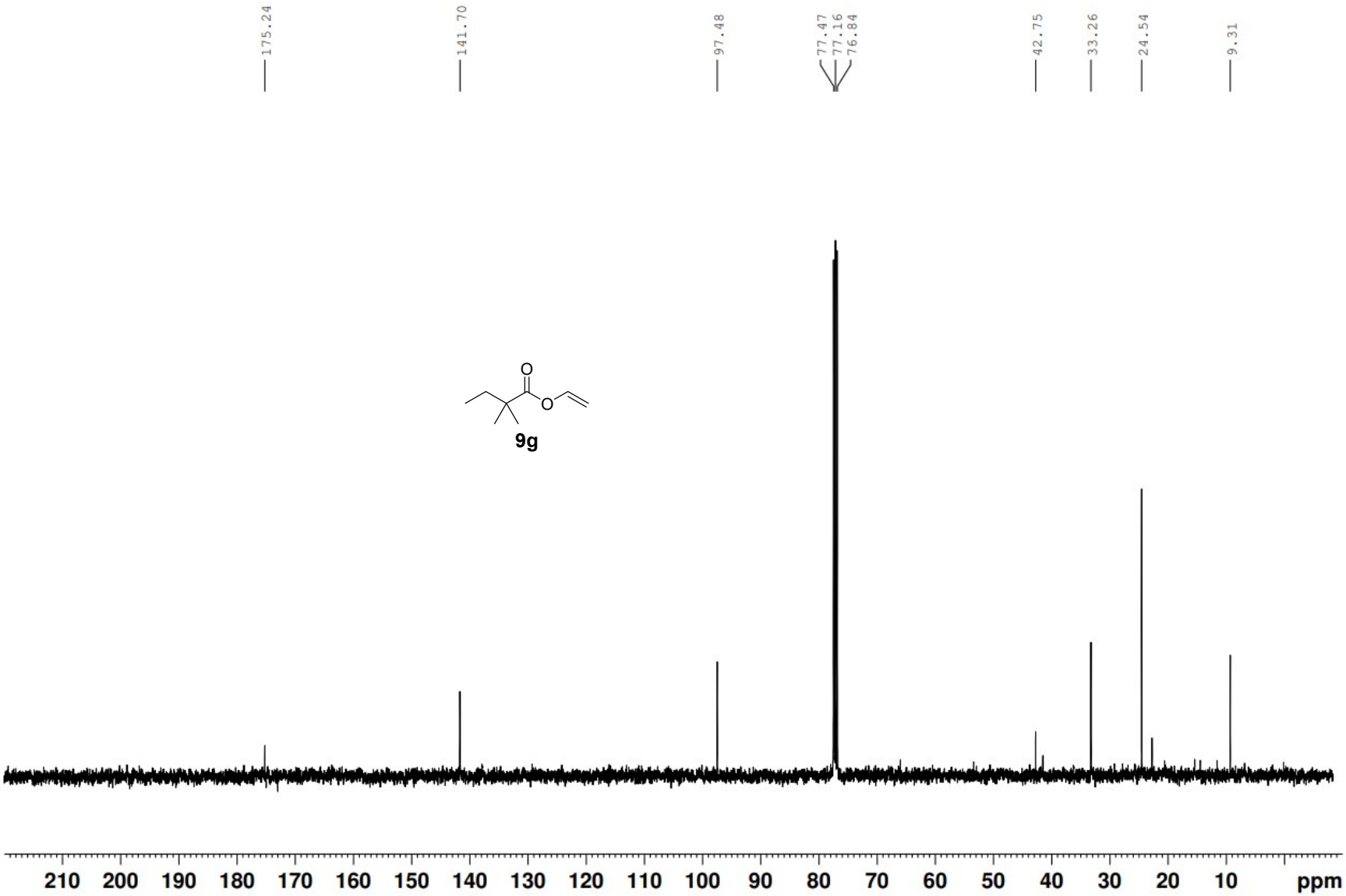


Figure S99: ^{13}C NMR spectrum ($CDCl_3$, 298 K, 100 MHz) of vinyl 2,2-di-Me-butyrate (**9g**).

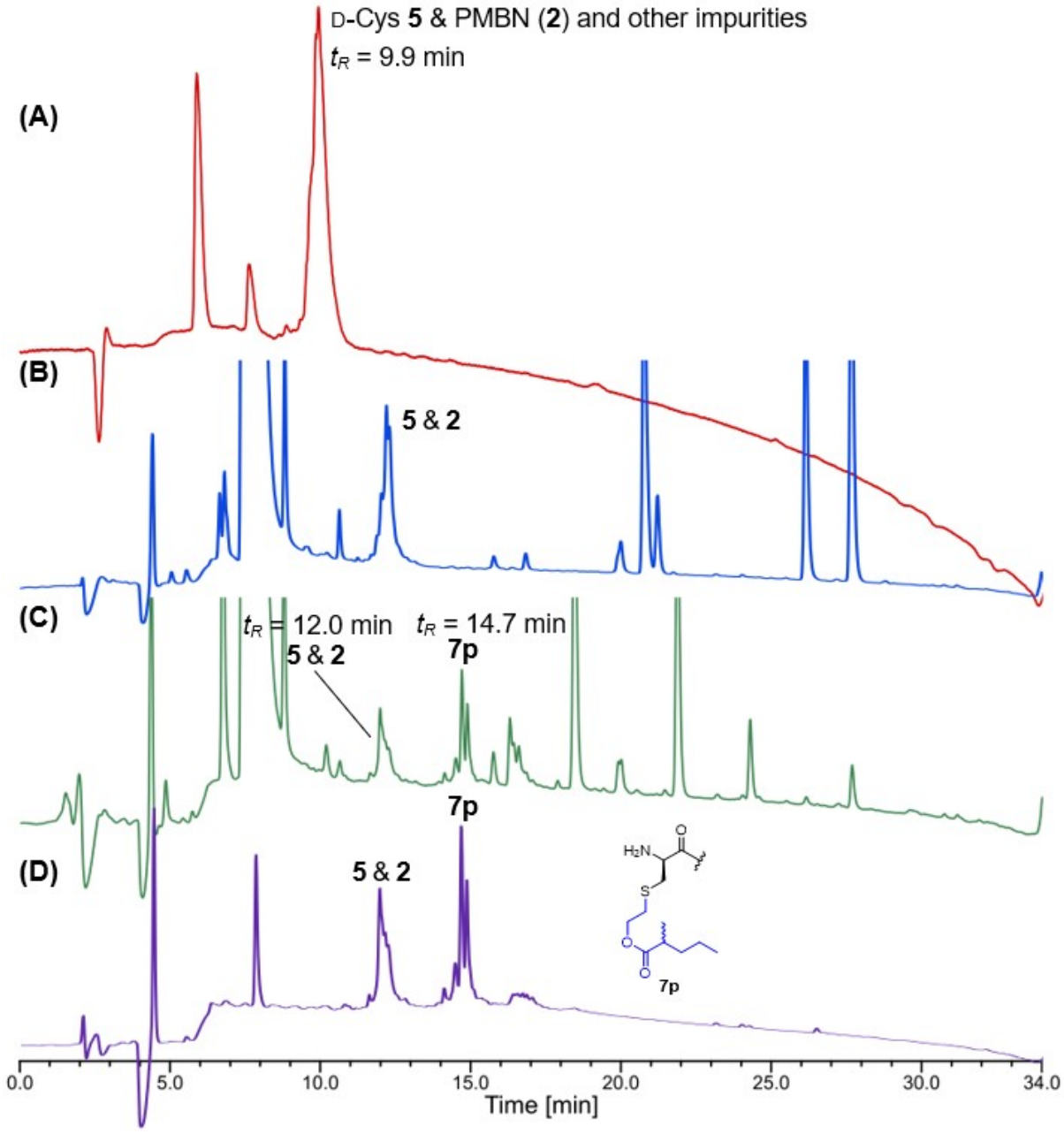


Figure S100: RP-HPLC [210/214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm) or a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys 2-Me-valerate CLipPA analogue **7p**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h, and **(D)** after trituration in Et_2O.

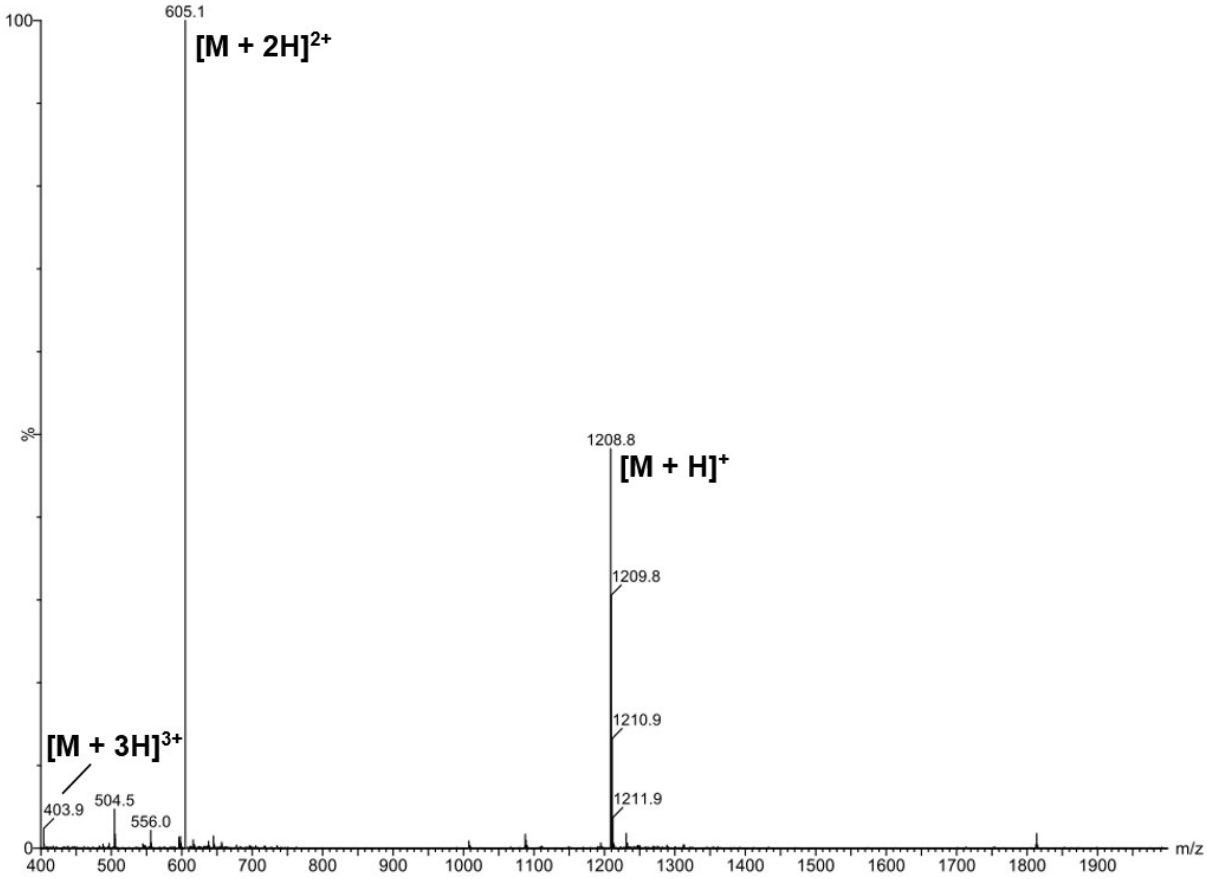


Figure S101: ESI-MS (+ve) spectrum of isolated D-Cys 2-Me-valerate CLipPA analogue **7p**.

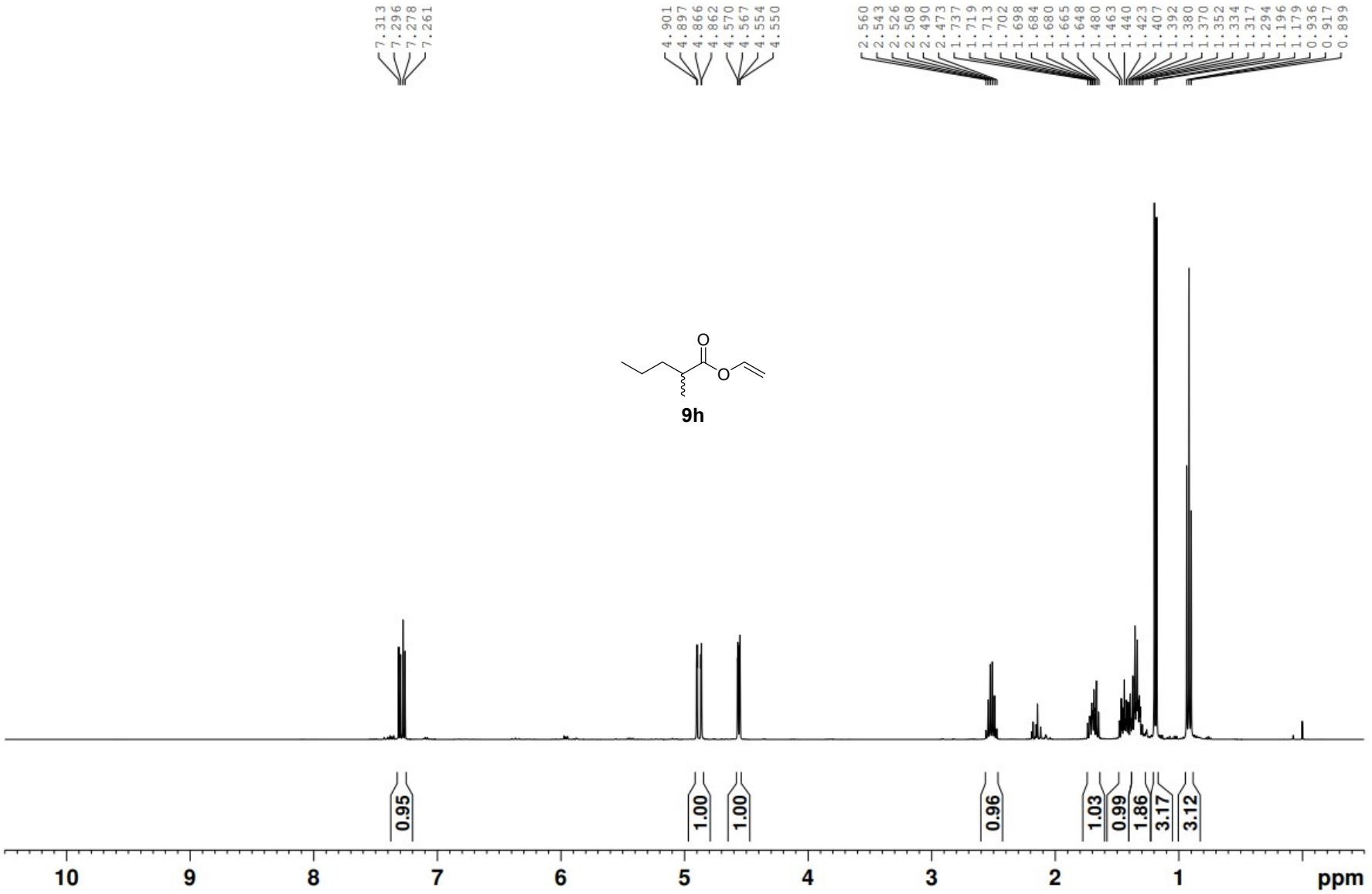


Figure S102: ^{1}H NMR spectrum ($CDCl_3$, 298 K, 400 MHz) of vinyl 2-Me-valerate (**9h**).

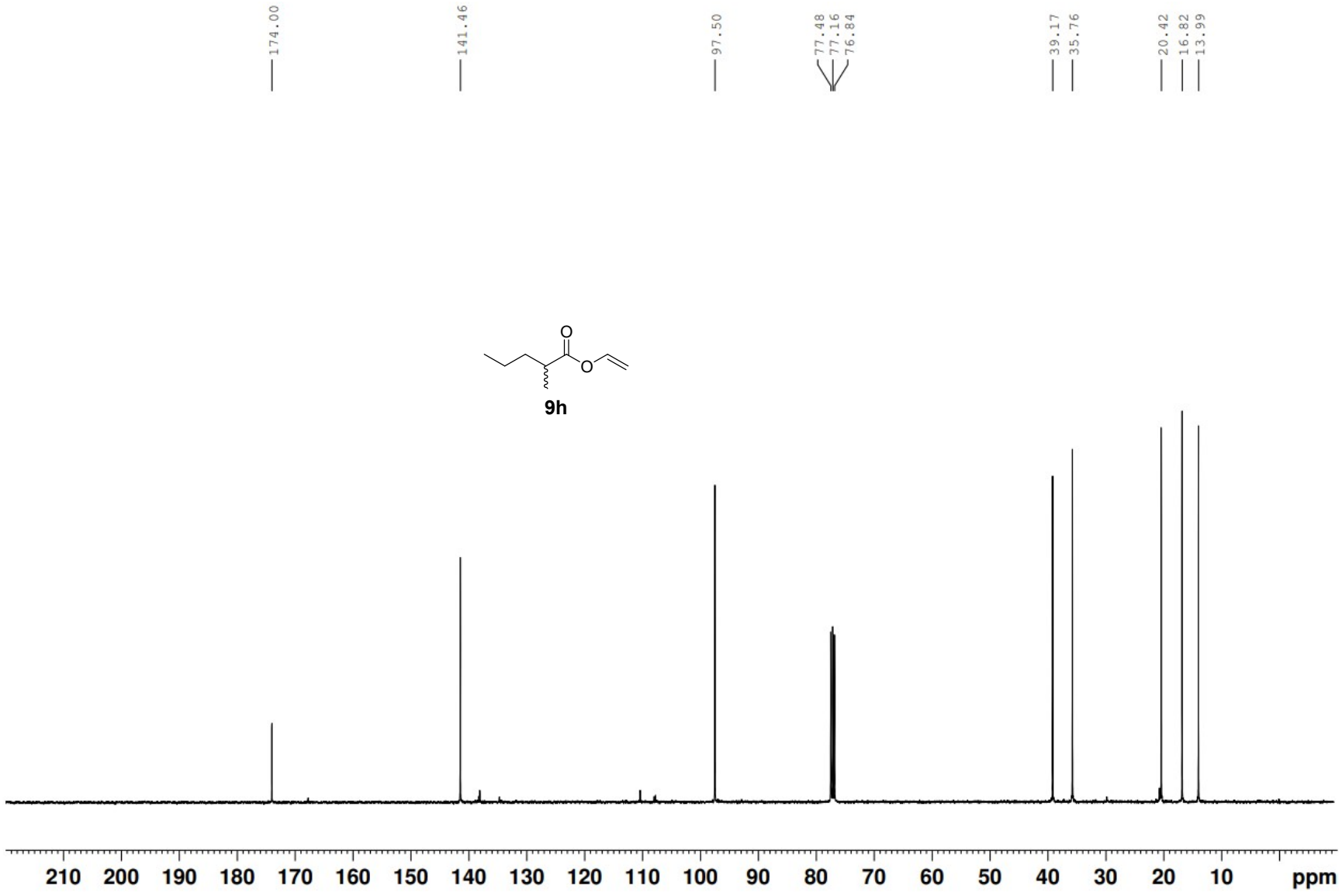


Figure S103: ^{13}C NMR spectrum ($CDCl_3$, 298 K, 100 MHz) of vinyl 2-Me-valerate (**9h**).

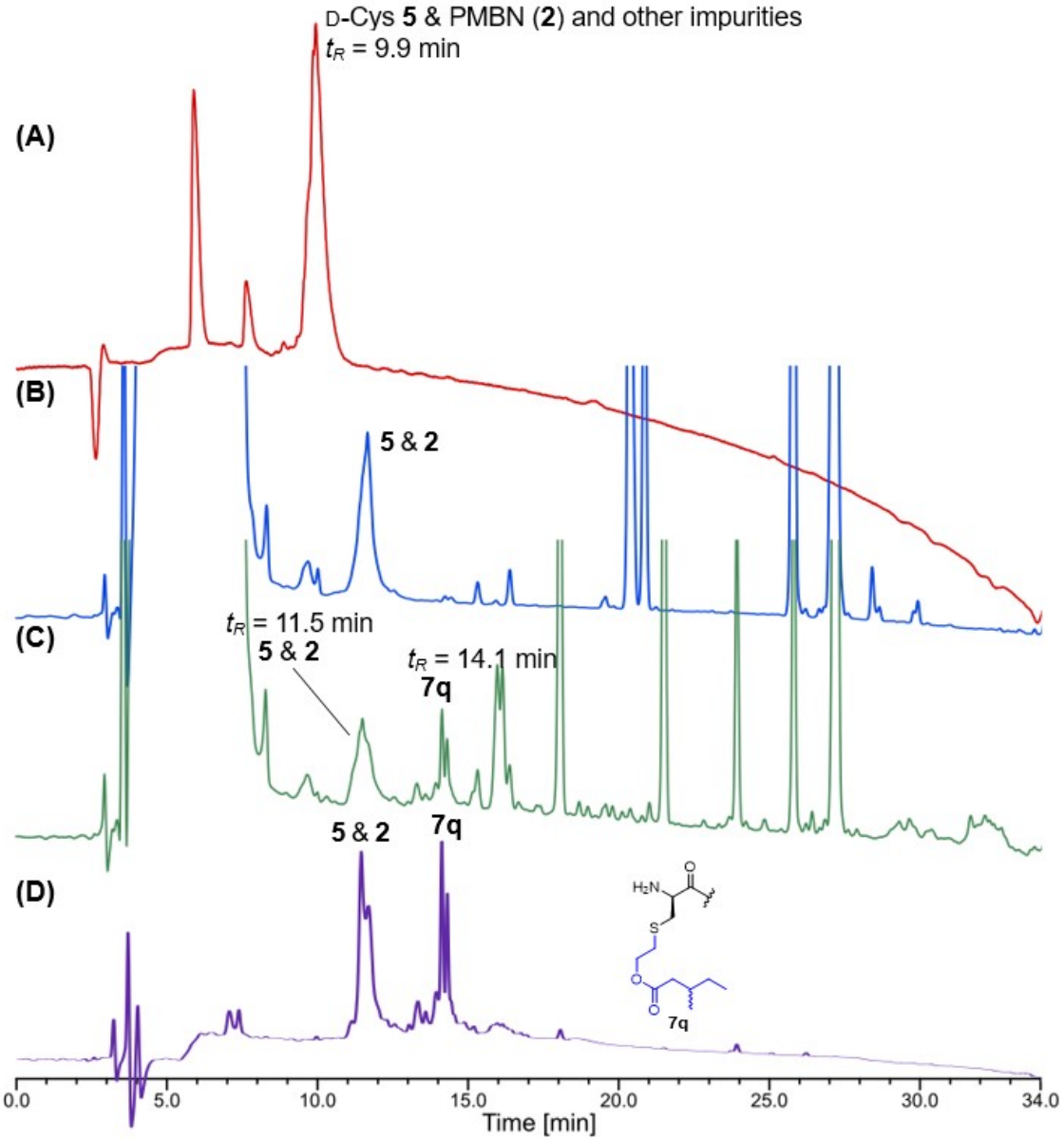


Figure S104: RP-HPLC [210/214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm) or a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys 3-Me-valerate CLipPA analogue **7q**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h, and **(D)** after trituration in Et_2O.

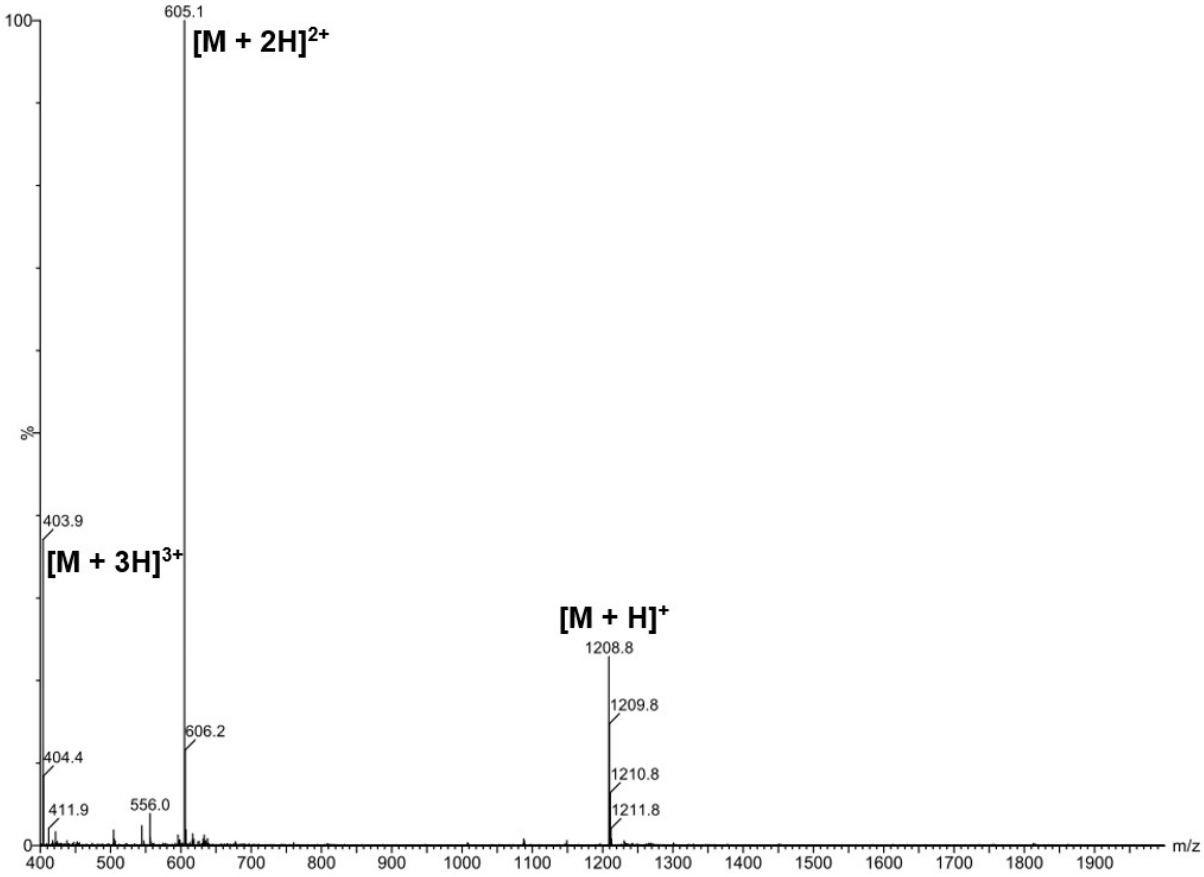


Figure S105: ESI-MS (+ve) spectrum of isolated D-Cys 3-Me-valerate CLipPA analogue **7q**.

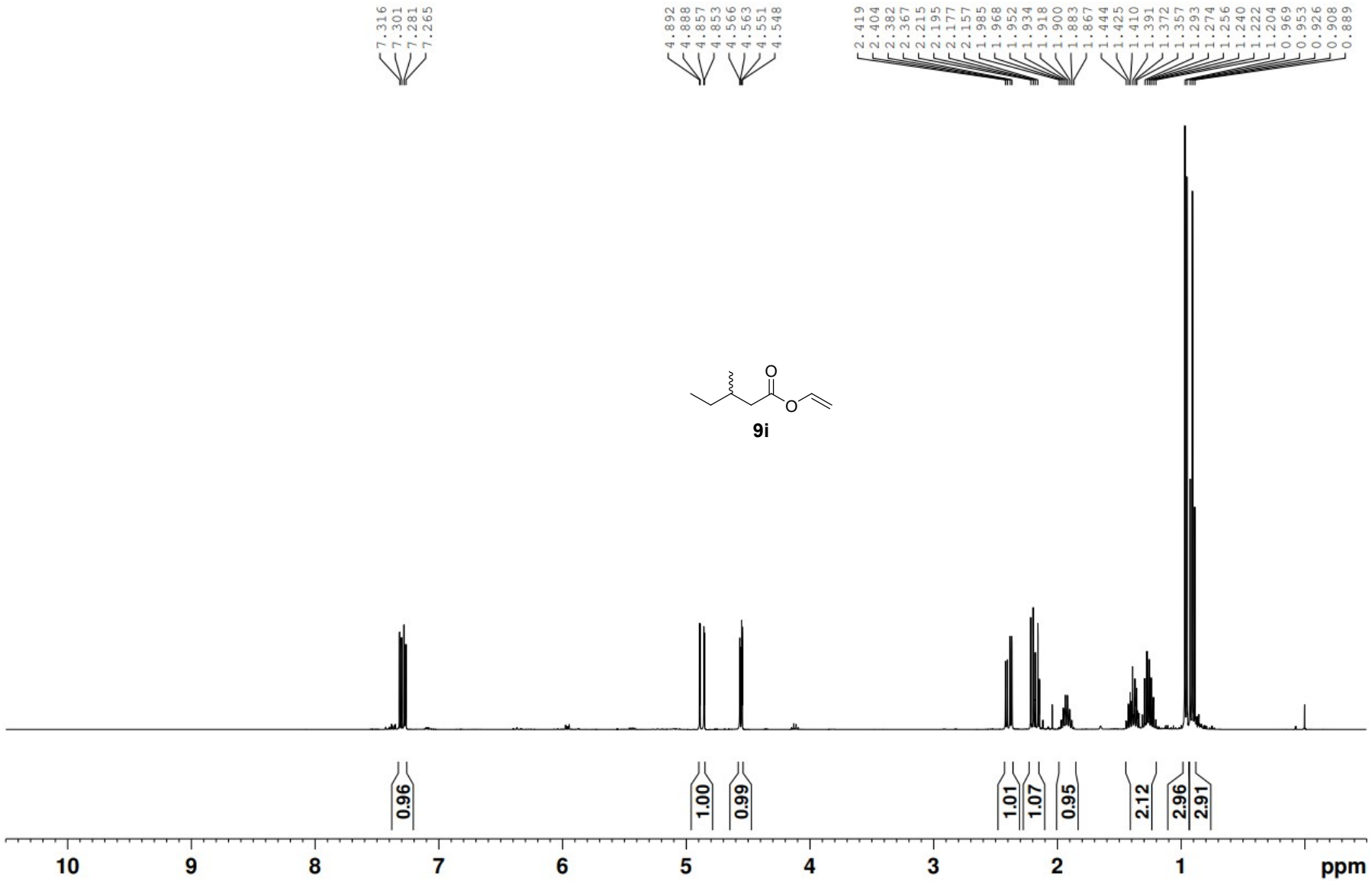


Figure S106: ^{1}H NMR spectrum ($CDCl_3$, 298 K, 400 MHz) of vinyl 3-Me-valerate (**9i**).

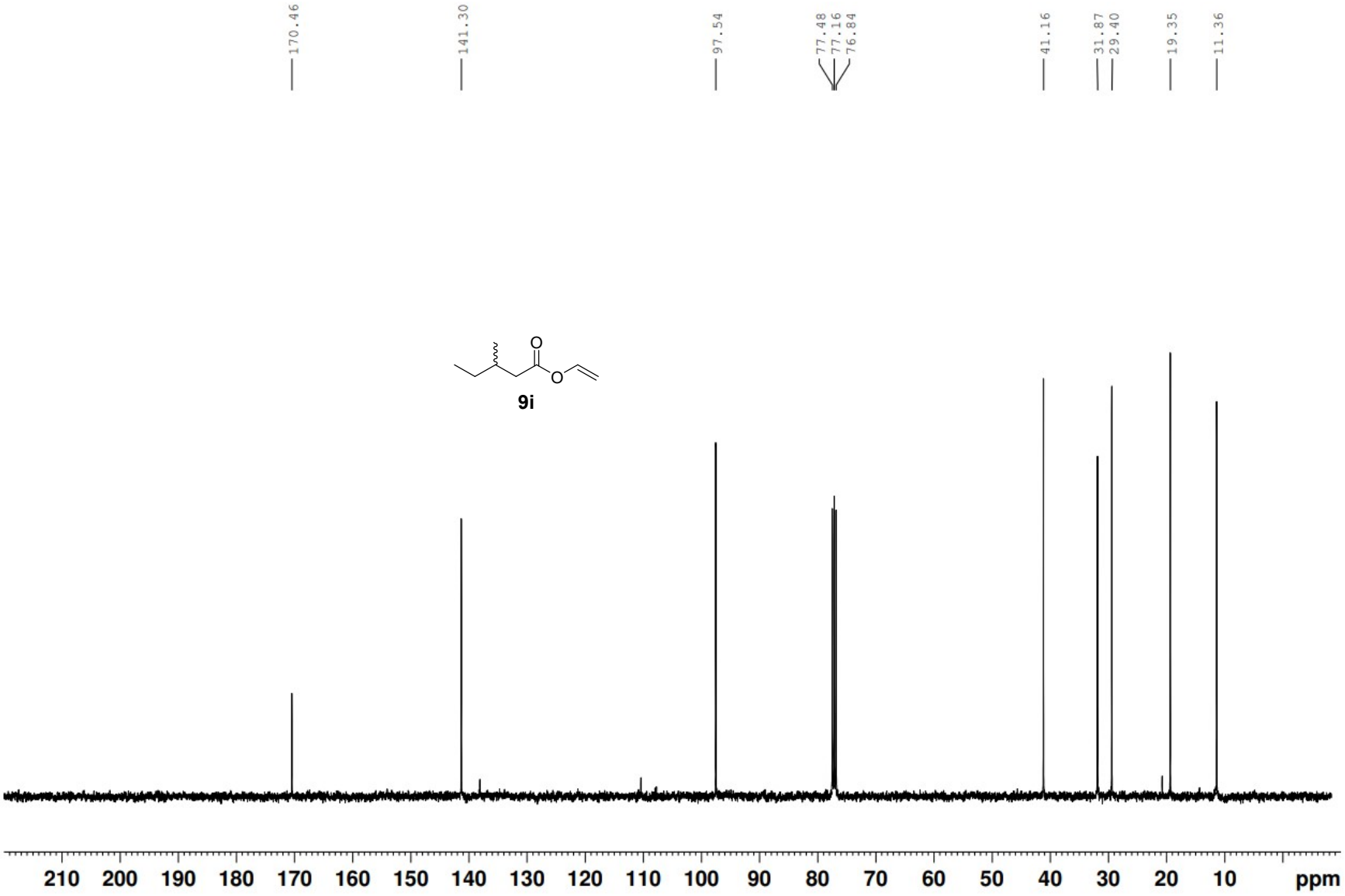


Figure S107: ^{13}C NMR spectrum ($CDCl_3$, 298 K, 100 MHz) of vinyl 3-Me-valerate (**9i**).

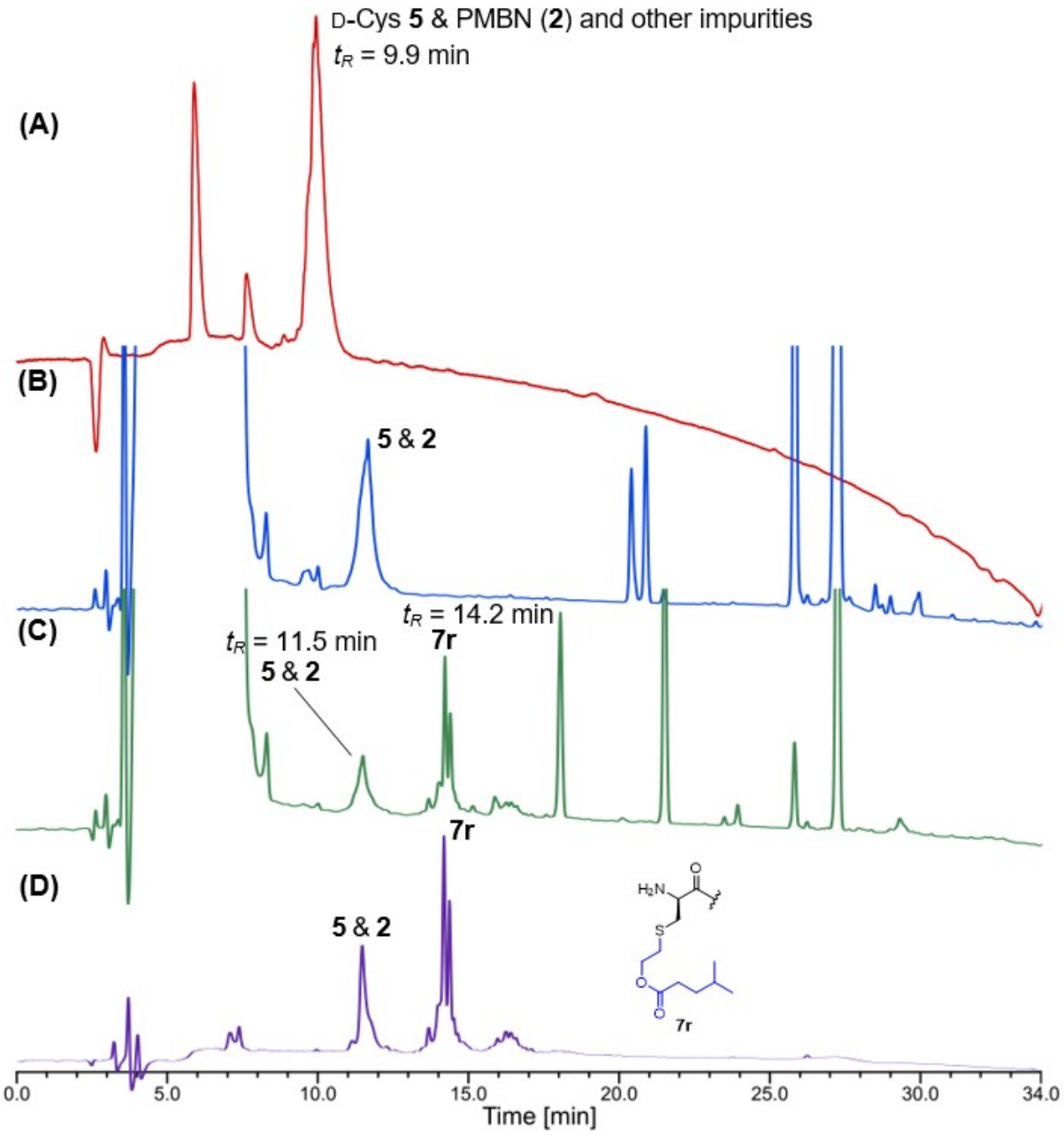


Figure S108: RP-HPLC [210/214 nm, linear, 3 % B per min gradient (5 % B to 95 % B over 30 min), 1 mL/min flow at rt, through a Phenomenex Gemini C18 column (110 Å, 150 mm × 4.60 mm; 5 µm) or a Phenomenex Luna C18 column (100 Å, 250 mm × 4.60 mm; 5 µm)] monitoring of the synthesis of D-Cys 4-Me-valerate CLipPA analogue **7r**, presented in the order of events; **(A)** D-Cys-PMBN (**5**), **(B)** CLipPA t = 0 h, **(C)** CLipPA t = 1 h, and **(D)** after trituration in Et_2O.

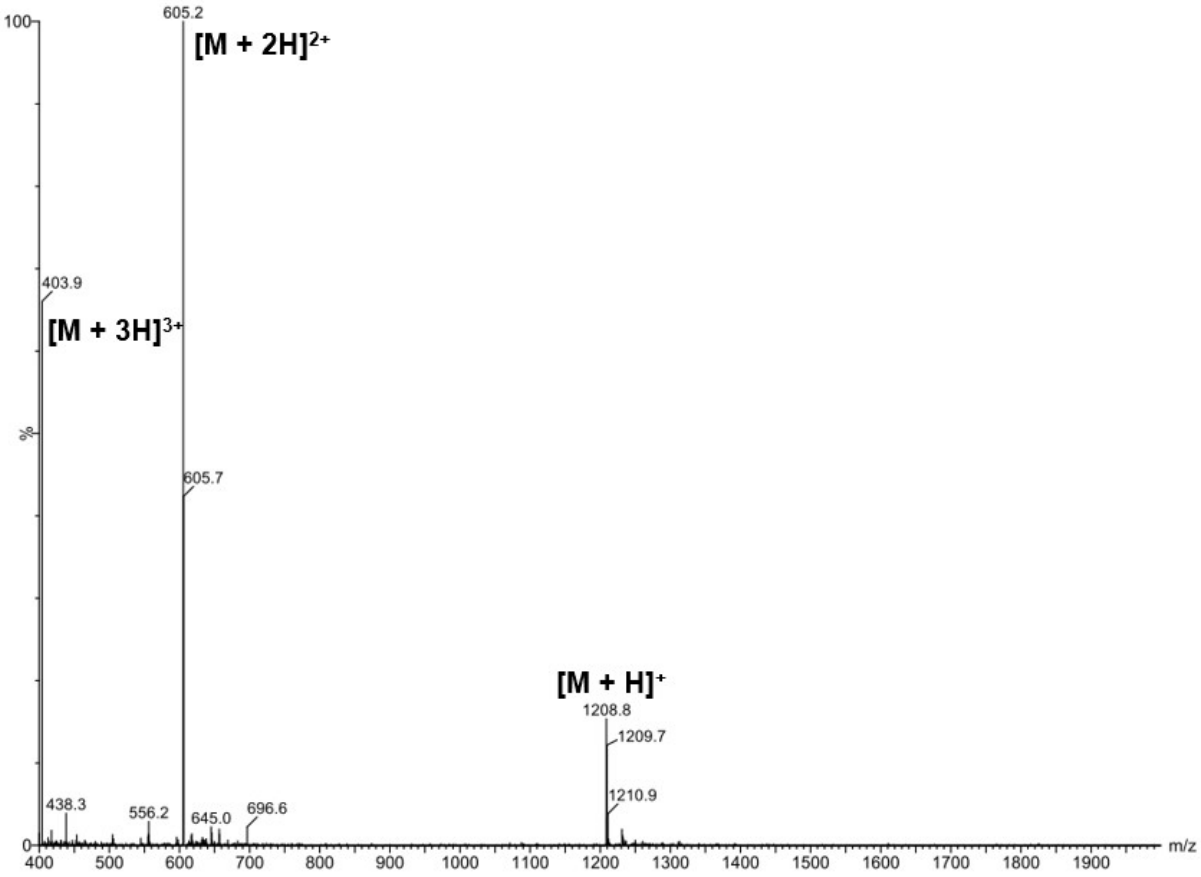


Figure S109: ESI-MS (+ve) spectrum of isolated D-Cys 4-Me-valerate CLipPA analogue **7r**.

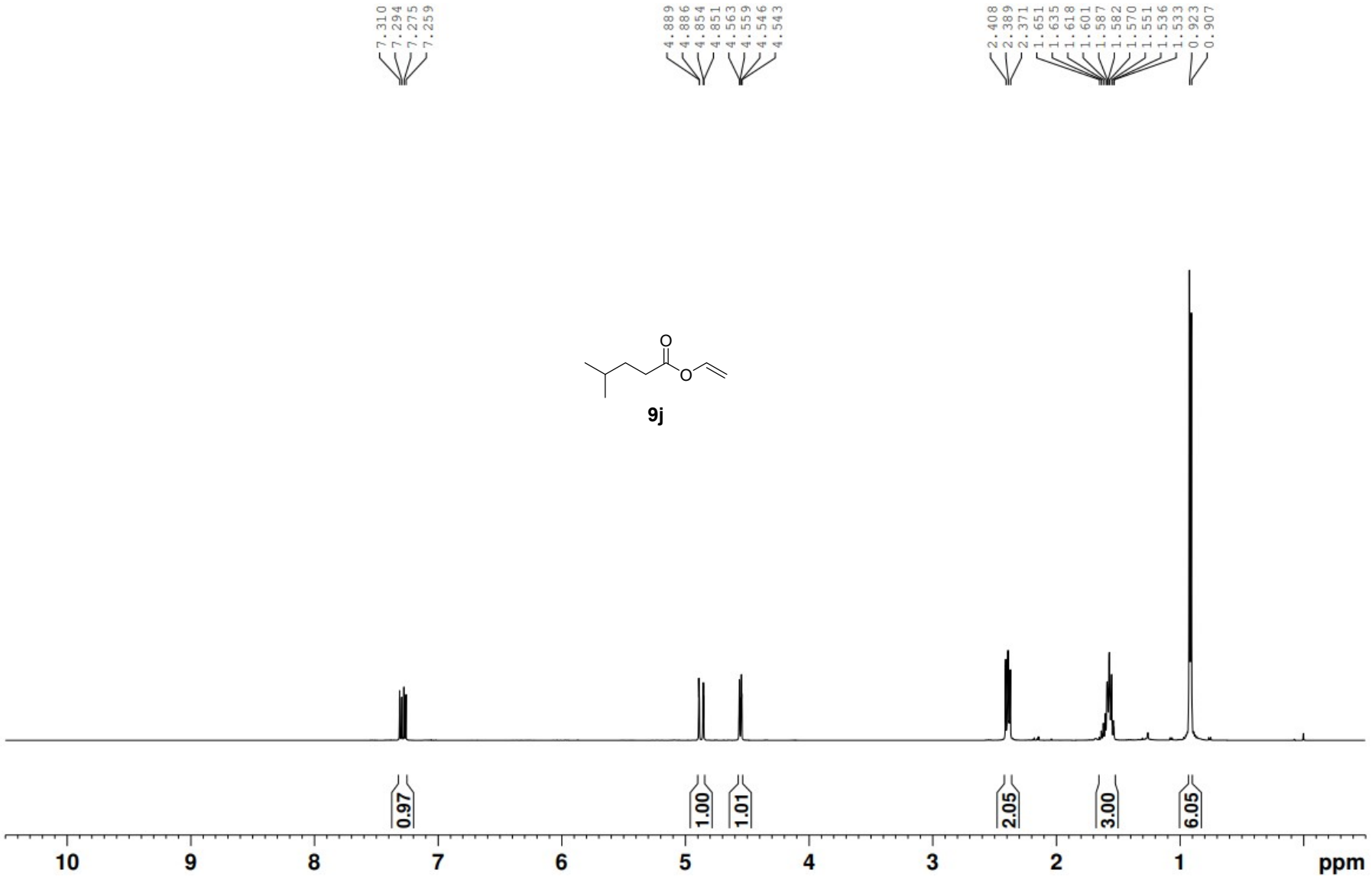


Figure S110: ^{1}H NMR spectrum ($CDCl_3$, 298 K, 400 MHz) of vinyl 4-Me-valerate (**9j**).

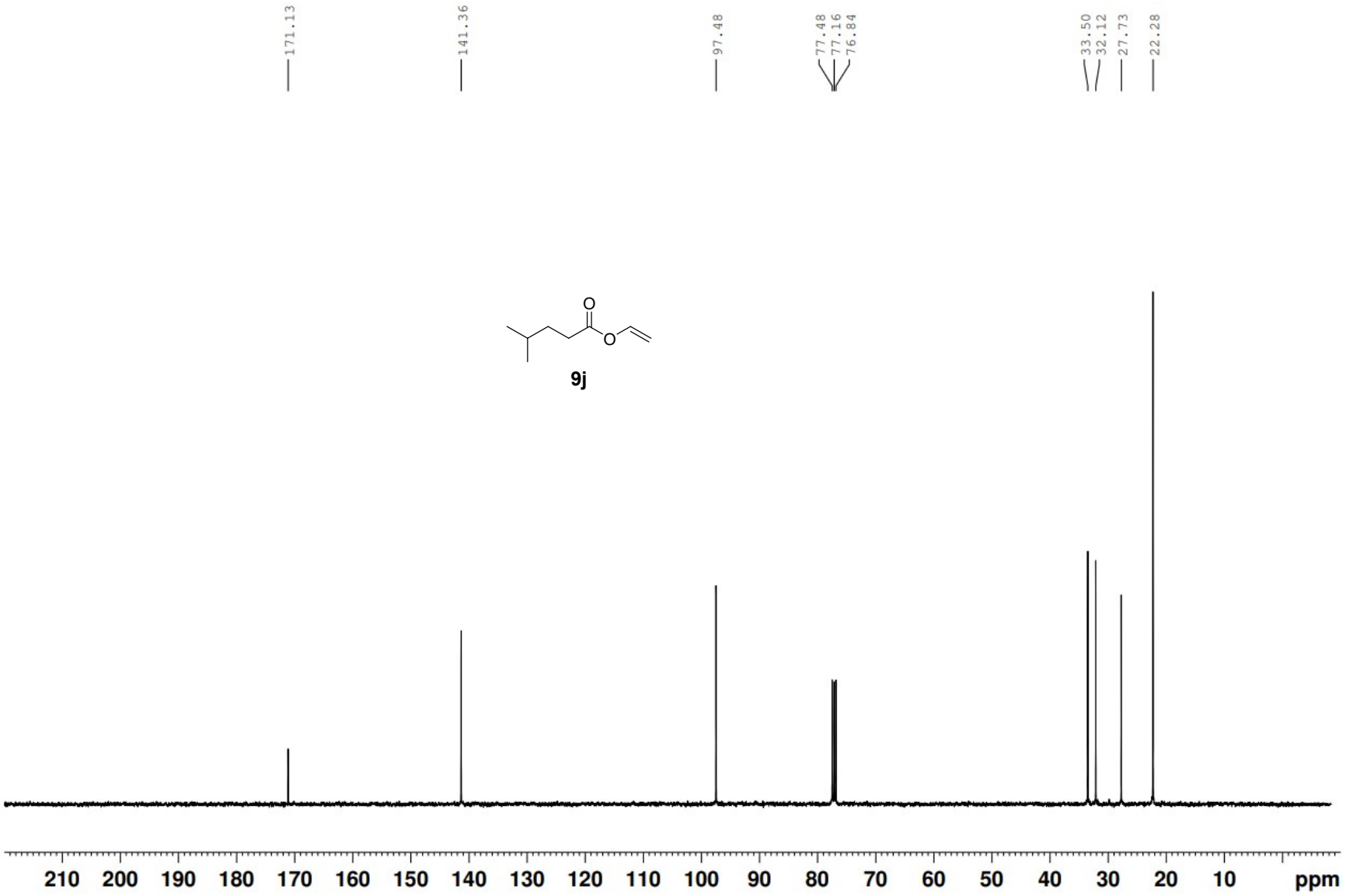


Figure S111: ^{13}C NMR spectrum ($CDCl_3$, 298 K, 100 MHz) of vinyl 4-Me-valerate (**9j**).

S6. Molecular dynamics simulations

Supplementary Methods

GROMACS 2021.3[44] was used to prepare, run and analyse the MD simulations except for the calculation of hydrogen bonds, which was carried using VMD and in-house python scripts. Graphs (aside from the hydrogen bonds) were made using QtGrace and the simulation was visualised and snapshots made using Visual Molecular Dynamics[45].

All MD simulations were run under periodic boundary conditions with bonds constrained using LINCS[46] to allow for a 2 fs integration step. Coulombic and van der Waals interactions were cut off at 1.4 nm, with long-range electrostatic interactions treated with the particle mesh Ewald method[47]. Translational centre of mass motion was removed very 100 integration steps.

The two polymyxin variants were modelled with the GROMOS 54A7 force field[48] with new building blocks for the Cys-linked lipid tails added manually with reference to standard Cys and lipid building blocks. Initial coordinates for both peptides were created from polymyxin B3 coordinates[18] using Avogadro[49]. Each peptide was placed in a cubic box such that the minimum distance from the peptide to the edge of the box was 1.4 nm, and energy minimised using the steepest descent algorithm for 10,000 steps or until the maximum force was less than 2 $kJ \cdot mol^{-1}$. Each peptide was solvated using SPC water[50] and 5 Cl^- ions added to neutralize the system before a second round of energy minimisation. Each system was heated from 50 to 298 K over 250 ps in the NVT ensemble, with temperature controlled using the velocity-rescale thermostat[51] with τ_T = 0.1 ps. Next, a 1 ns equilibration was run in the NpT ensemble, with the same settings as in the heating phase and pressure controlled using the Berendsen barostat[52] with τ_p = 1.0 ps and an isothermal compressibility of 4.5×10^{-5} UNITS. Finally, a 500 ns production simulation was run for each system using the same settings as in the equilibration phase except that temperature was controlled using the Nosé-Hoover thermostat[53] with τ_T = 1.0 ps and pressure was controlled with the Parinello-Rahman barostat[54] with τ_p = 5.0 ps.

Supplementary Tables

Table S6: Total number of conformational clusters¶ and population of the top five conformational clusters for each polymyxin variant.

Variant	Number of clusters	Cluster populations†				
		1	2	3	4	5
6b (L-Cys)	26	5962	1255	1084	600	372
7b (D-Cys)	39	2608	2431	1373	916	793

¶ Conformations were clustered by all-atom RMSD with a cutoff of 0.3 nm.

† The total number of conformations for each simulation is 10,000.

Table S7: Total number of hydrogen bonds¶, and occupancy of hydrogen bonds occupied for more than 5 % of the simulation for each polymyxin variant.

Variant	Number of hydrogen bonds	Occupancy of most common hydrogen bonds†				Total lipid tail hydrogen bond occupancy
		Dab^5 Nɣ – Dab^9 O	Dab^5 N – Lipid OB	Dab^9 N – Dab^6 O	Dab^5 N – Thr^3 O	
6b (L-Cys)	62	21.53 %	11.69 %	7.22 %	6.09 %	19.16 %
7b (D-Cys)	95	13.08 %	<5 %	6.05 %	<5 %	12.33 %

¶ Hydrogen bonds were deemed to form if the D-H-A distance was <0.3 nm and the D-H-A angle deviated by no more than 30° from 180°.

† N is the backbone amide nitrogen; O is the backbone carbonyl oxygen; Nɣ is the side chain ɣ nitrogen; OB is the lipid carbonyl oxygen atom.

Supplementary Figures

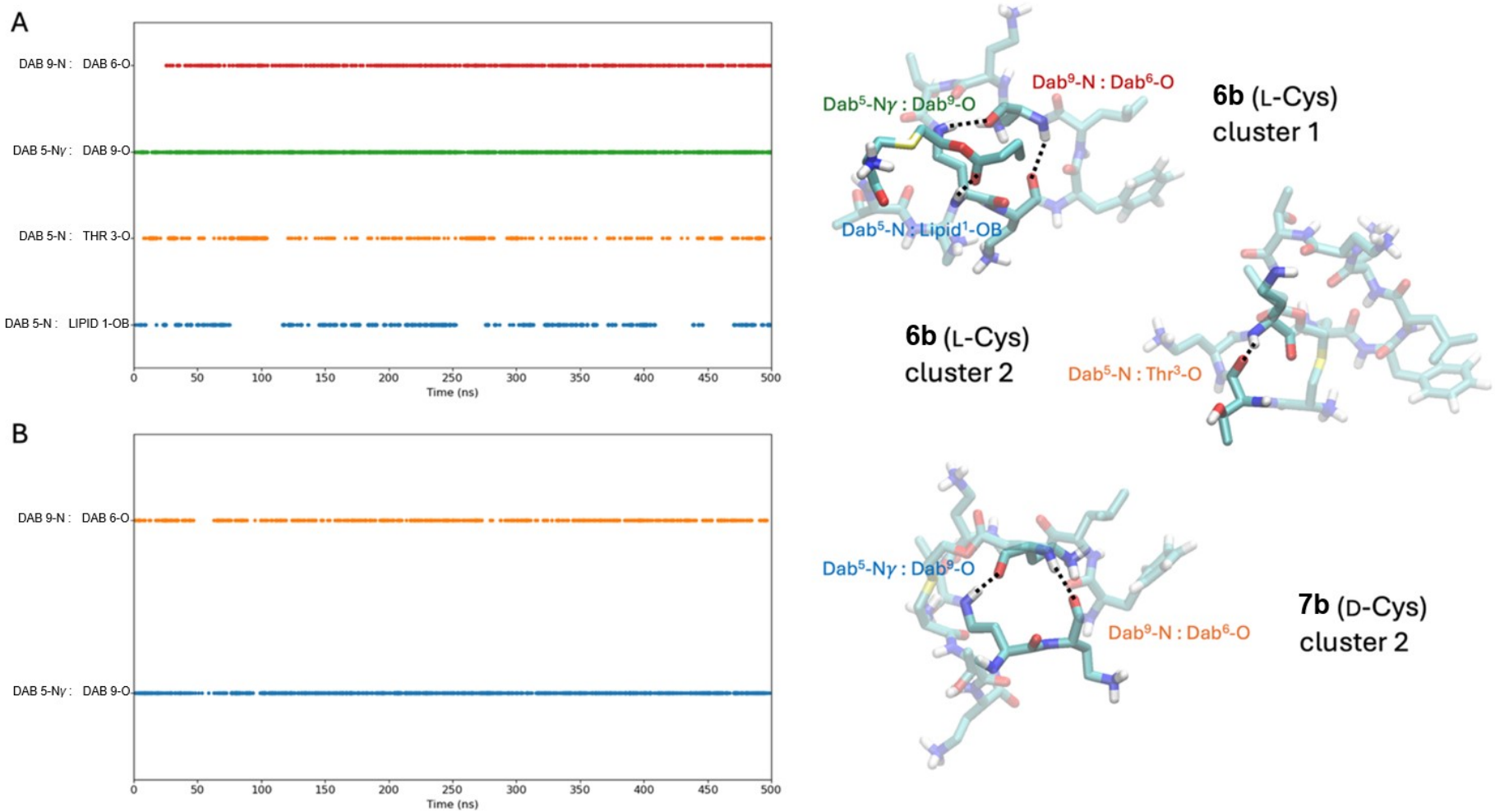


Figure S112: Time-series of hydrogen bond formation with representative snapshots. **A**. **6b** (L-Cys); **B**. **7b** (D-Cys). Hydrogen bonds were deemed to form if the D-H-A distance was <0.3 nm and the D-H-A angle deviated by no more than 30° from 180°, and plotted if occupied for more than 5 % of the simulation.

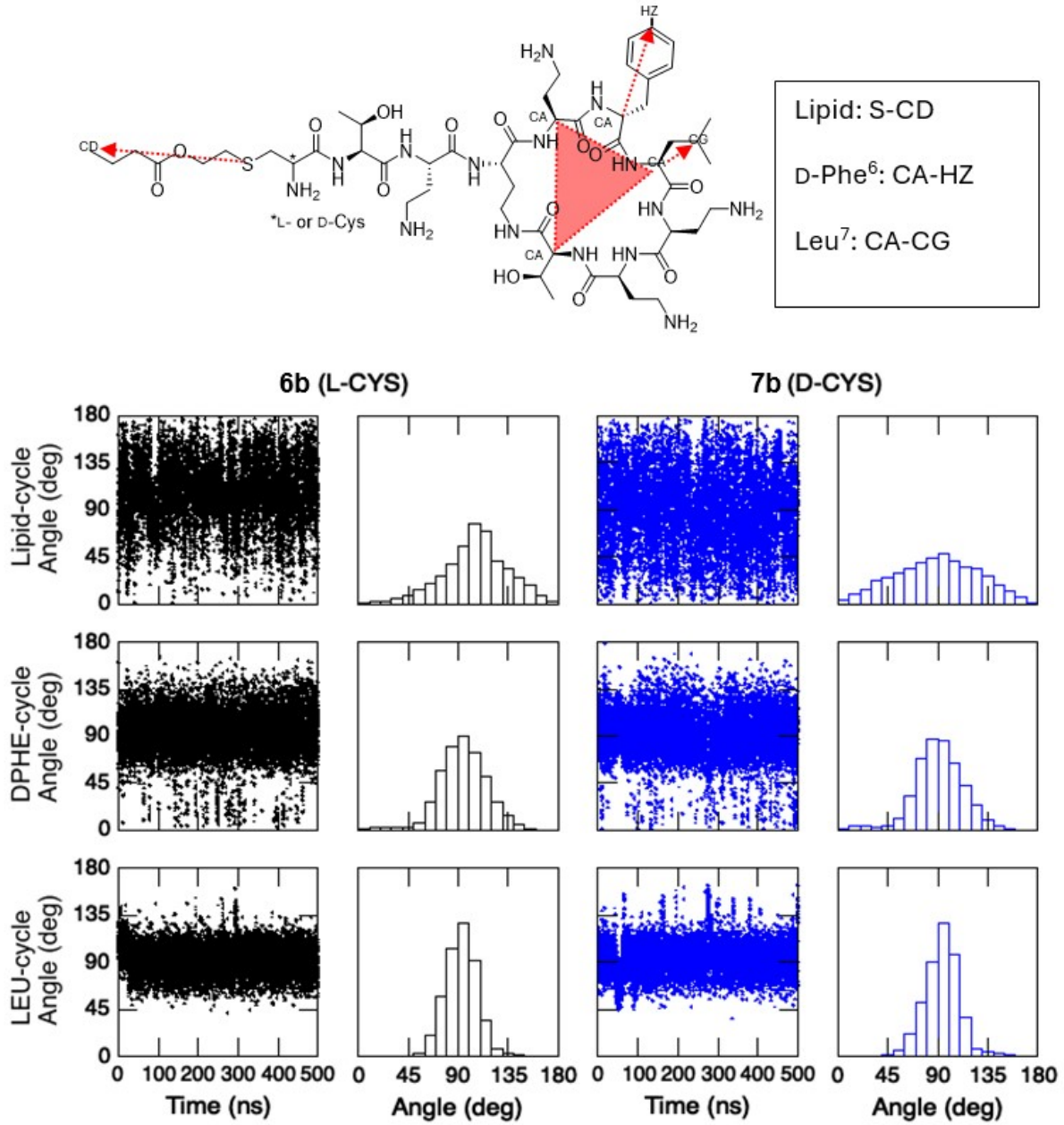


Figure S113: Angle between the cycle normal and the lipid tail, D-Phe or Leu residues of **6b** (L-Cys) or **7b** (D-Cys). At top is a schematic showing how the vectors for each residue (dotted vectors) and the plane of the cycle (shaded triangle) are defined. The angles are measured between the lipid tail, D-Phe^6 or Leu^7 vector and the normal of the plane of the cycle: angles around 90° therefore correspond to the vector lying in the plane of the cycle. The bin width for the histograms is 10°.

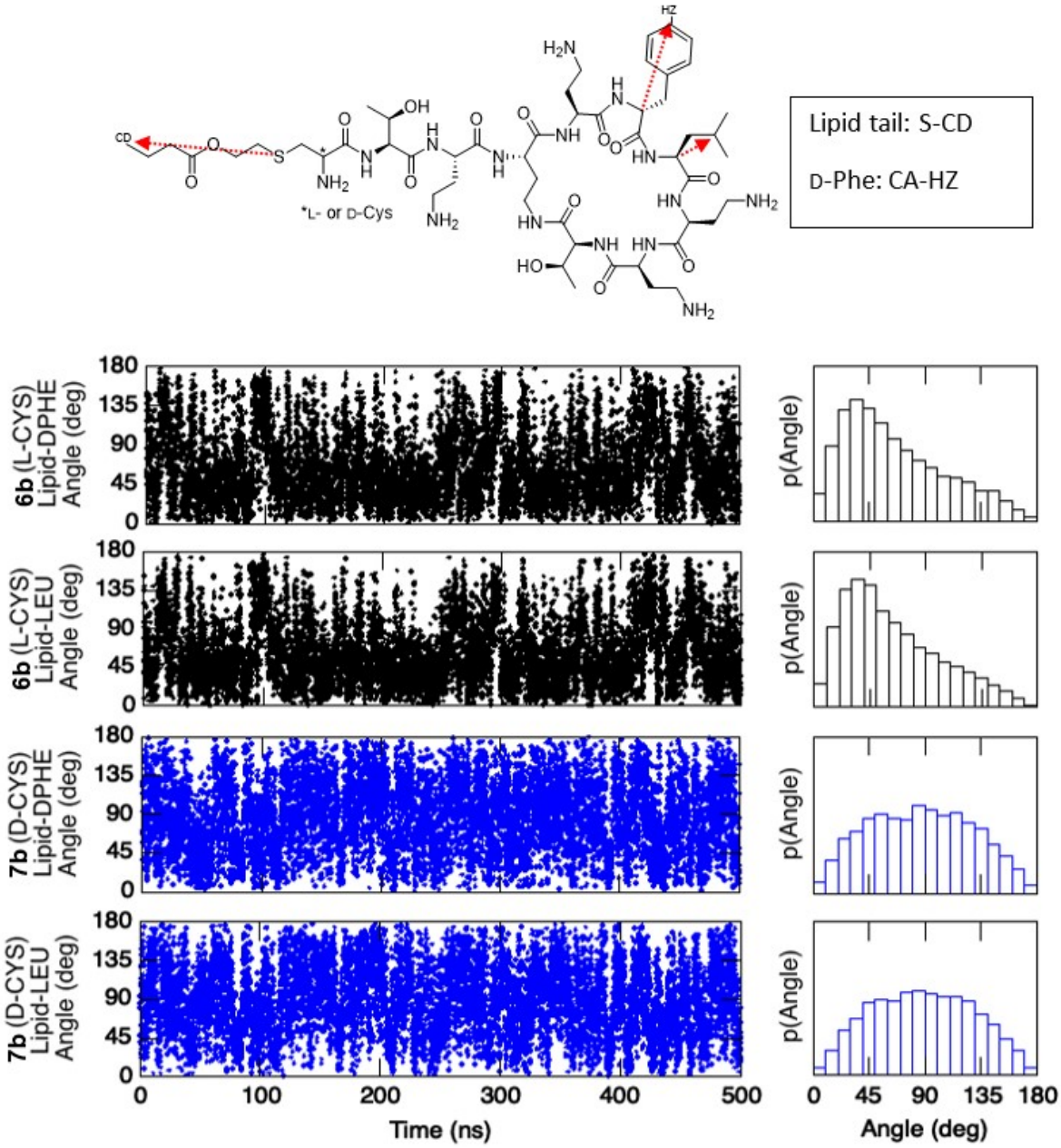


Figure S114: Angle between the lipid tail and the D-Phe or Leu residues of **6b** (L-Cys) or **7b** (D-Cys). At top is a schematic showing how the vectors for each residue are defined (red dotted arrows). The angles are measured between the lipid vector and either the D-Phe^{6} or Leu^{7} vector. The bin width for the histograms is 10°.

S7. Hydrolytic stability experiments

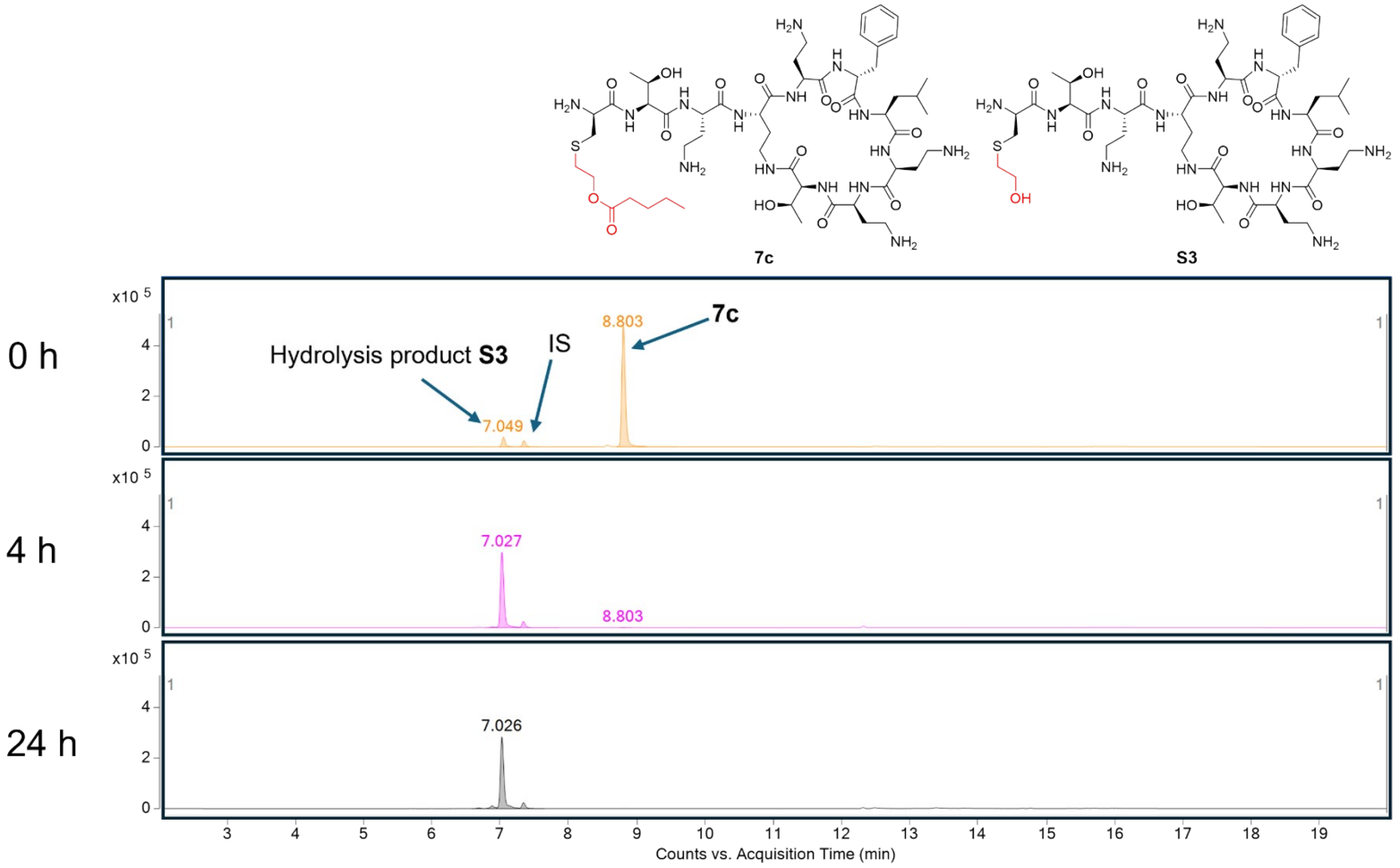


Figure S115: Stability monitoring of D-Cys valerate **7c** in human serum over 24 h; RP-HPLC [214 nm, linear gradient, 5 % B to 100 % B over 12 min with an initial 1 min divert and a 2 min hold at 100 % B, 0.6 mL/min flow at rt, through a Phenomenex Kinetex C18 column (100 Å, 100 mm × 3 mm; 2.6 µm)]; Solvent A = H_2O, containing 0.02 % TFA (*v*/*v*) and solvent B = MeOH. IS = internal standard, Val-Tyr-Val (VYV).

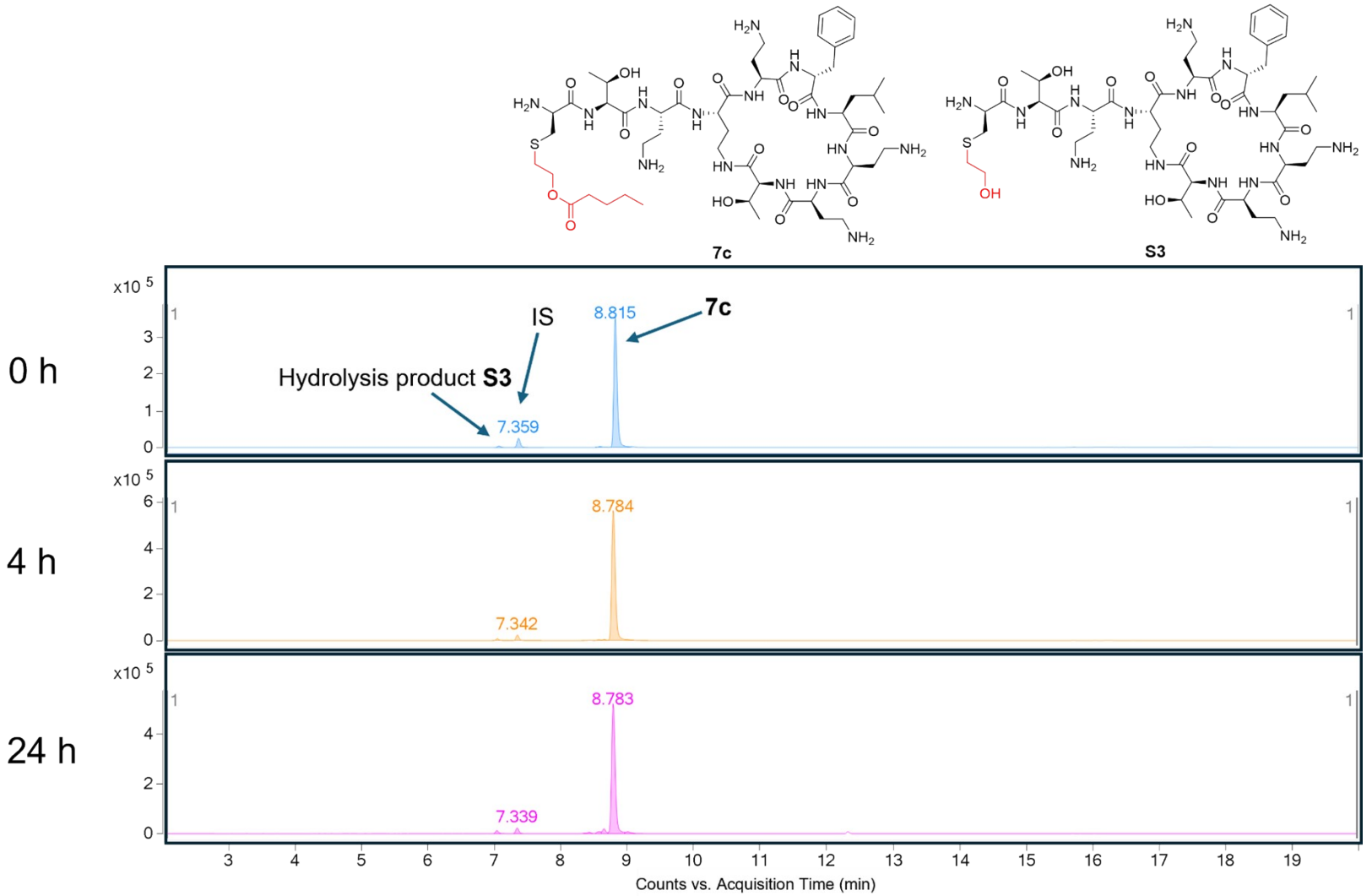


Figure S116: Stability monitoring of D-Cys valerate **7c** in DPBS over 24 h; RP-HPLC [214 nm, linear gradient, 5 % B to 100 % B over 12 min with an initial 1 min divert and a 2 min hold at 100 % B, 0.6 mL/min flow at rt, through a Phenomenex Kinetex C18 column (100 Å, 100 mm × 3 mm; 2.6 µm)]; Solvent A = H_2O, containing 0.02 % TFA (*v*/*v*) and solvent B = MeOH. IS = internal standard, Val-Tyr-Val (VYV).

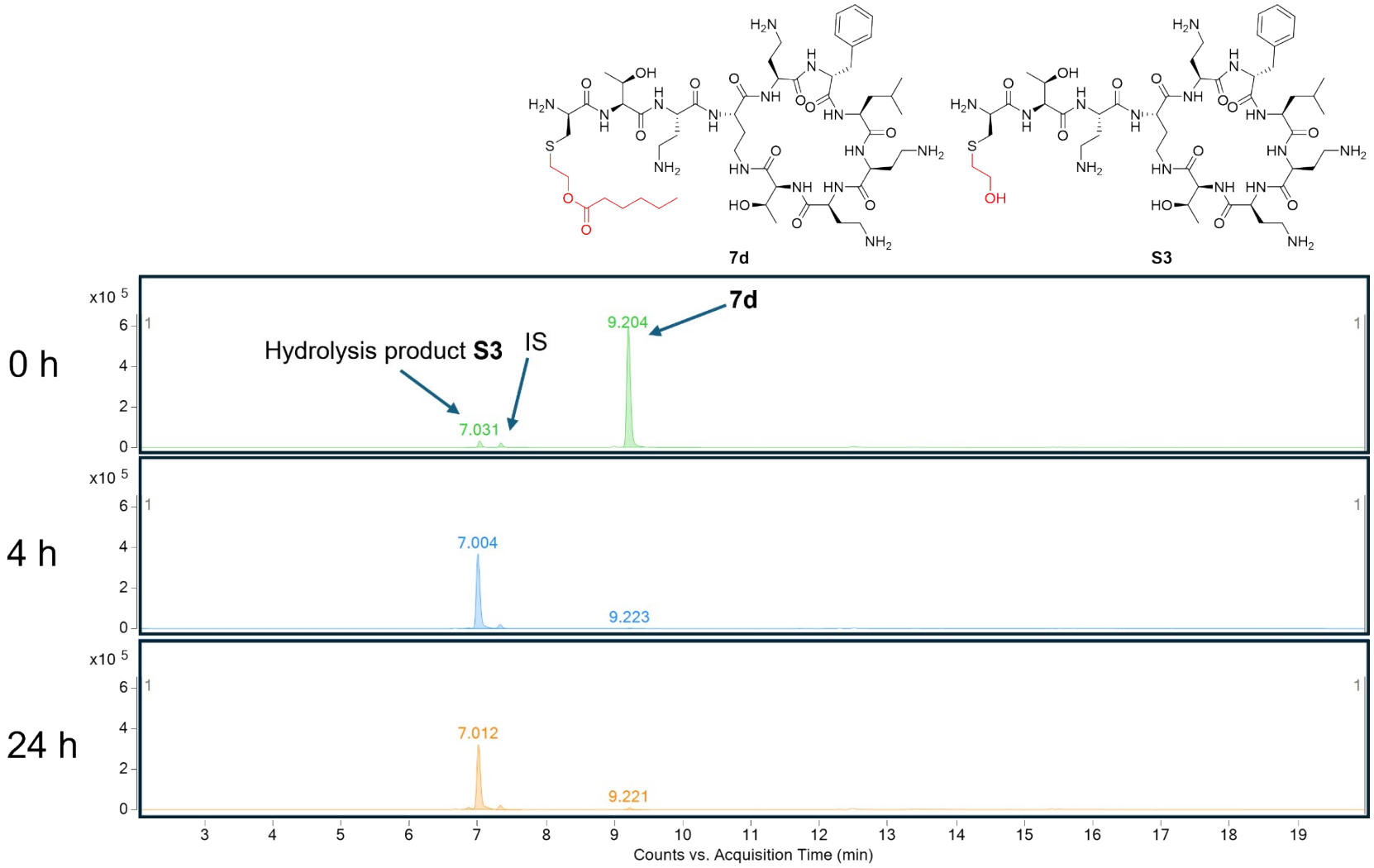


Figure S117: Stability monitoring of D-Cys hexanoate **7d** in human serum over 24 h; RP-HPLC [214 nm, linear gradient, 5 % B to 100 % B over 12 min with an initial 1 min divert and a 2 min hold at 100 % B, 0.6 mL/min flow at rt, through a Phenomenex Kinetex C18 column (100 Å, 100 mm × 3 mm; 2.6 µm)]; Solvent A = H_2O, containing 0.02 % TFA (*v*/*v*) and solvent B = MeOH. IS = internal standard, Val-Tyr-Val (VYV).

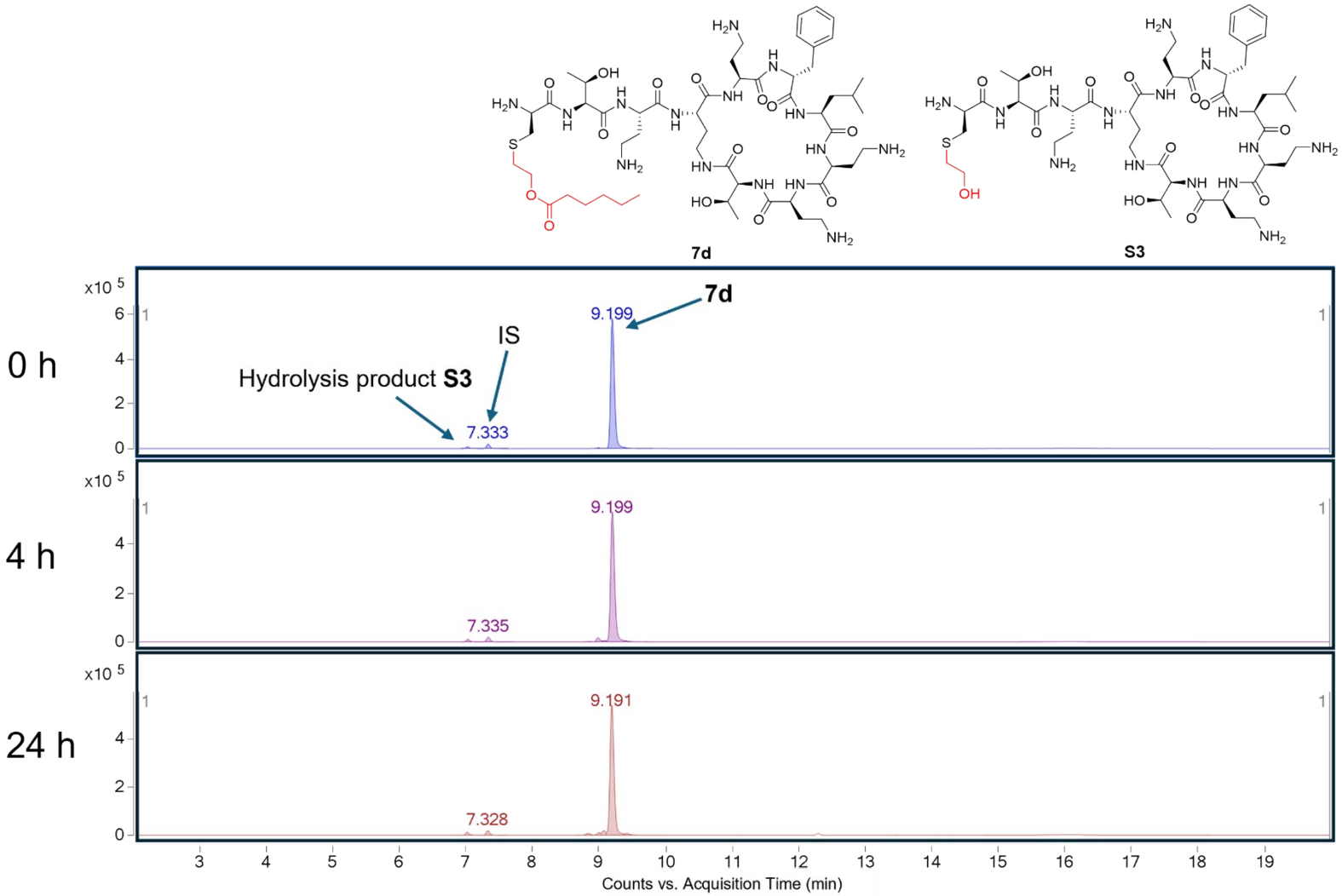


Figure S118: Stability monitoring of D-Cys hexanoate **7d** in DPBS over 24 h; RP-HPLC [214 nm, linear gradient, 5 % B to 100 % B over 12 min with an initial 1 min divert and a 2 min hold at 100 % B, 0.6 mL/min flow at rt, through a Phenomenex Kinetex C18 column (100 Å, 100 mm × 3 mm; 2.6 µm)]; Solvent A = H_2O, containing 0.02 % TFA (*v*/*v*) and solvent B = MeOH. IS = internal standard, Val-Tyr-Val (VYV).

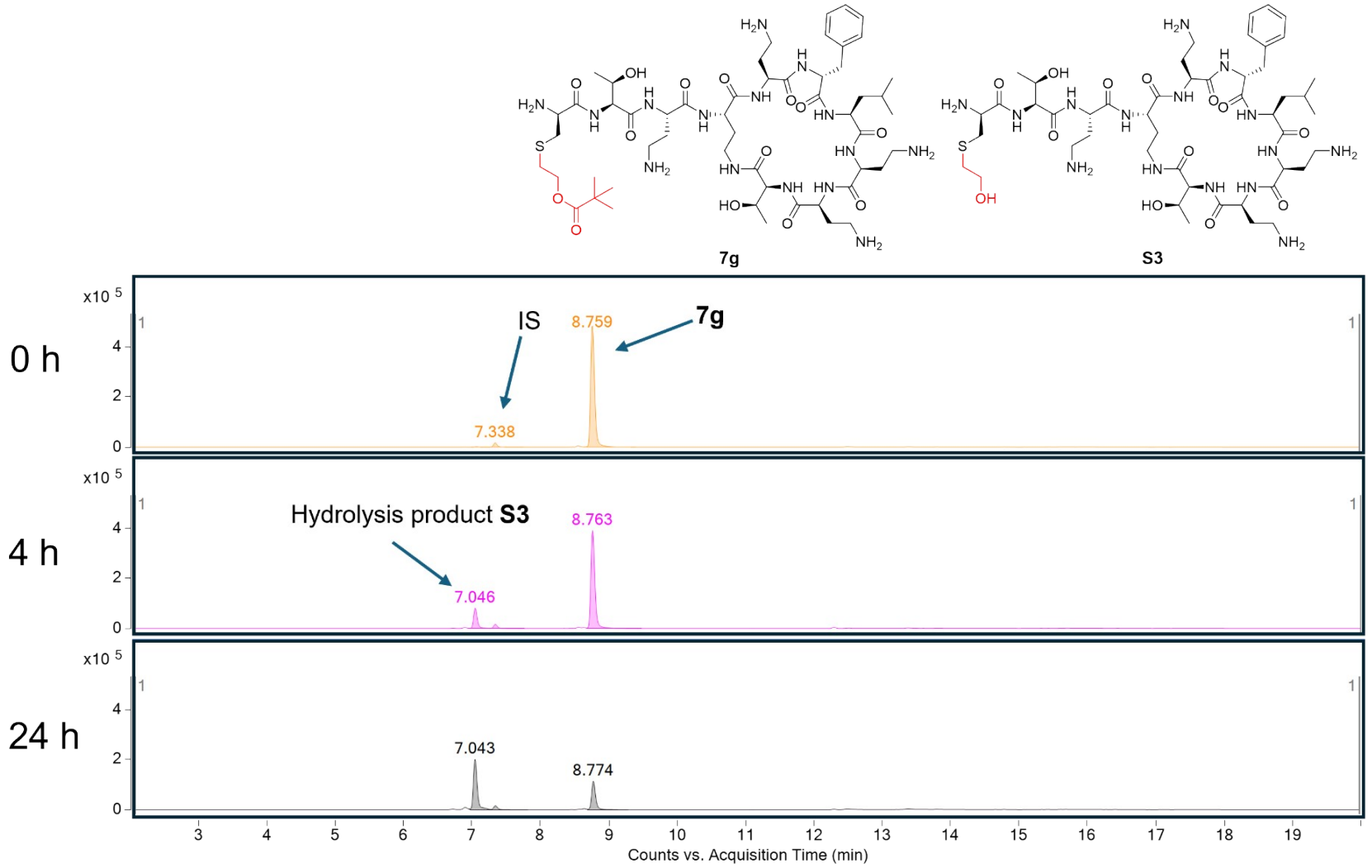


Figure S119: Stability monitoring of D-Cys pivalate **7g** in human serum over 24 h; RP-HPLC [214 nm, linear gradient, 5 % B to 100 % B over 12 min with an initial 1 min divert and a 2 min hold at 100 % B, 0.6 mL/min flow at rt, through a Phenomenex Kinetex C18 column (100 Å, 100 mm × 3 mm; 2.6 µm)]; Solvent A = H_2O, containing 0.02 % TFA (*v*/*v*) and solvent B = MeOH. IS = internal standard, Val-Tyr-Val (VYV).

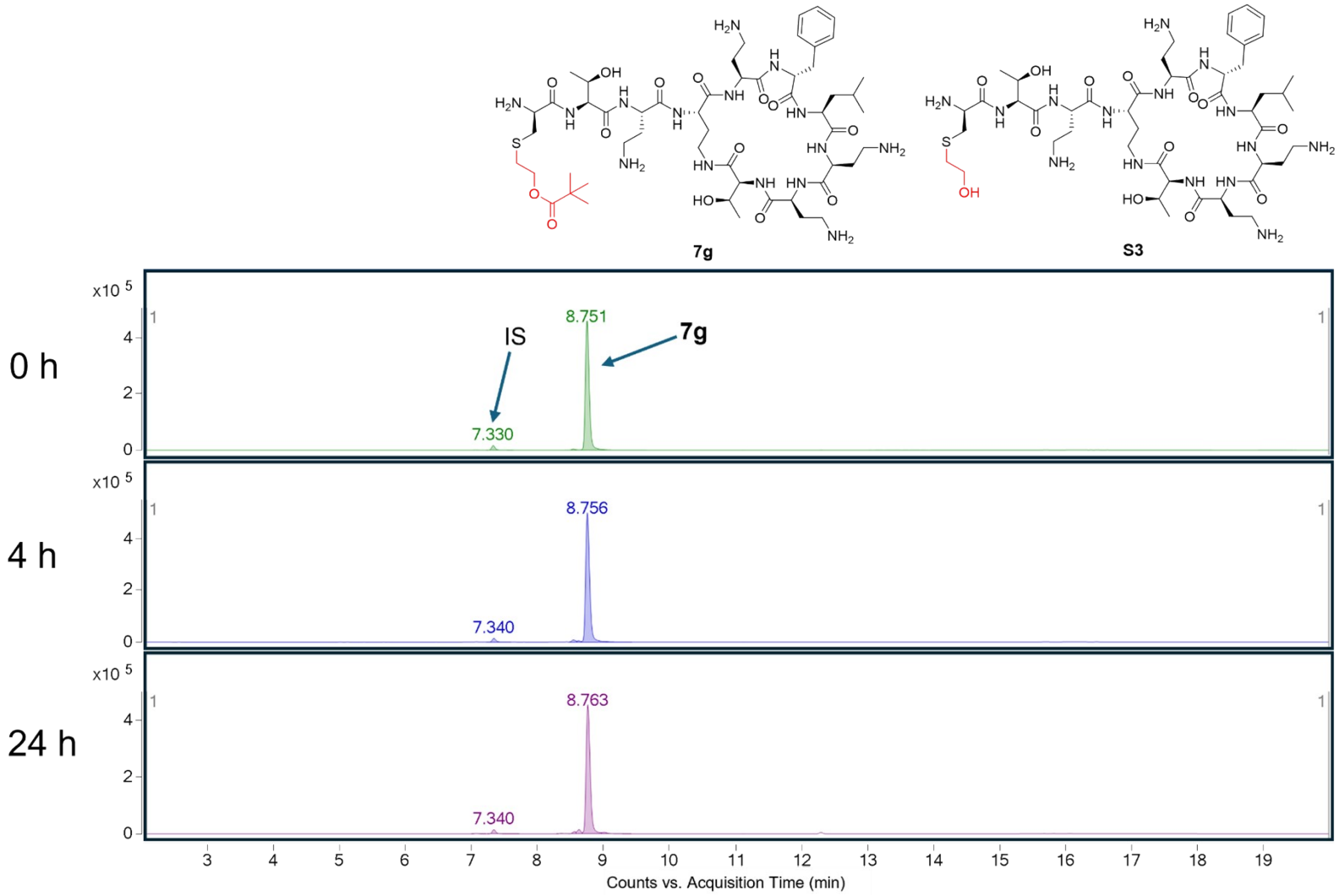


Figure S120: Stability monitoring of D-Cys pivalate **7g** in DPBS over 24 h; RP-HPLC [214 nm, linear gradient, 5 % B to 100 % B over 12 min with an initial 1 min divert and a 2 min hold at 100 % B, 0.6 mL/min flow at rt, through a Phenomenex Kinetex C18 column (100 Å, 100 mm × 3 mm; 2.6 µm)]; Solvent A = H_2O, containing 0.02 % TFA (*v*/*v*) and solvent B = MeOH. IS = internal standard, Val-Tyr-Val (VYV).

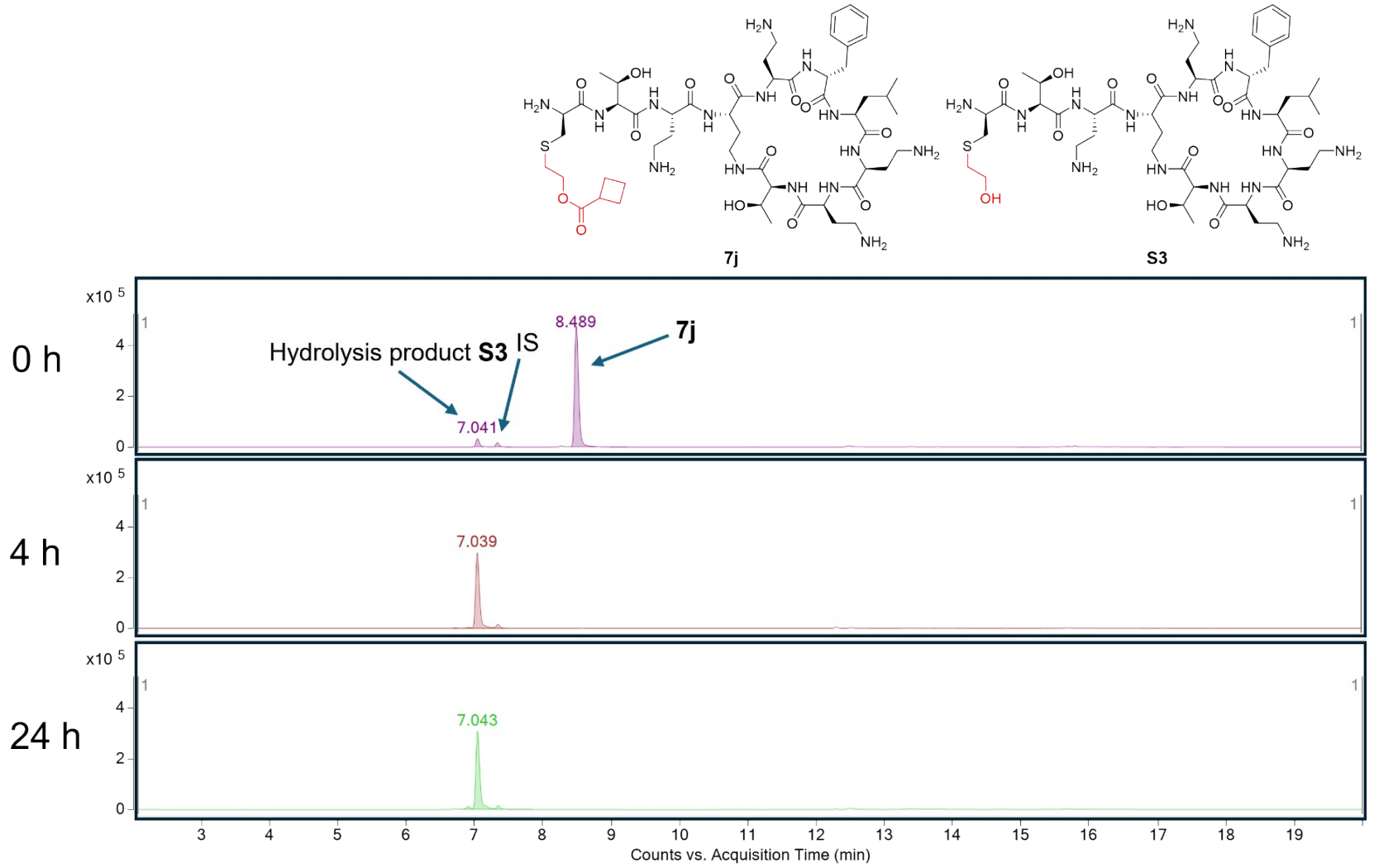


Figure S121: Stability monitoring of D-Cys cyclobutyrate **7j** in human serum over 24 h; RP-HPLC [214 nm, linear gradient, 5 % B to 100 % B over 12 min with an initial 1 min divert and a 2 min hold at 100 % B, 0.6 mL/min flow at rt, through a Phenomenex Kinetex C18 column (100 Å, 100 mm × 3 mm; 2.6 µm)]; Solvent A = H_2O, containing 0.02 % TFA (*v*/*v*) and solvent B = MeOH. IS = internal standard, Val-Tyr-Val (VYV).

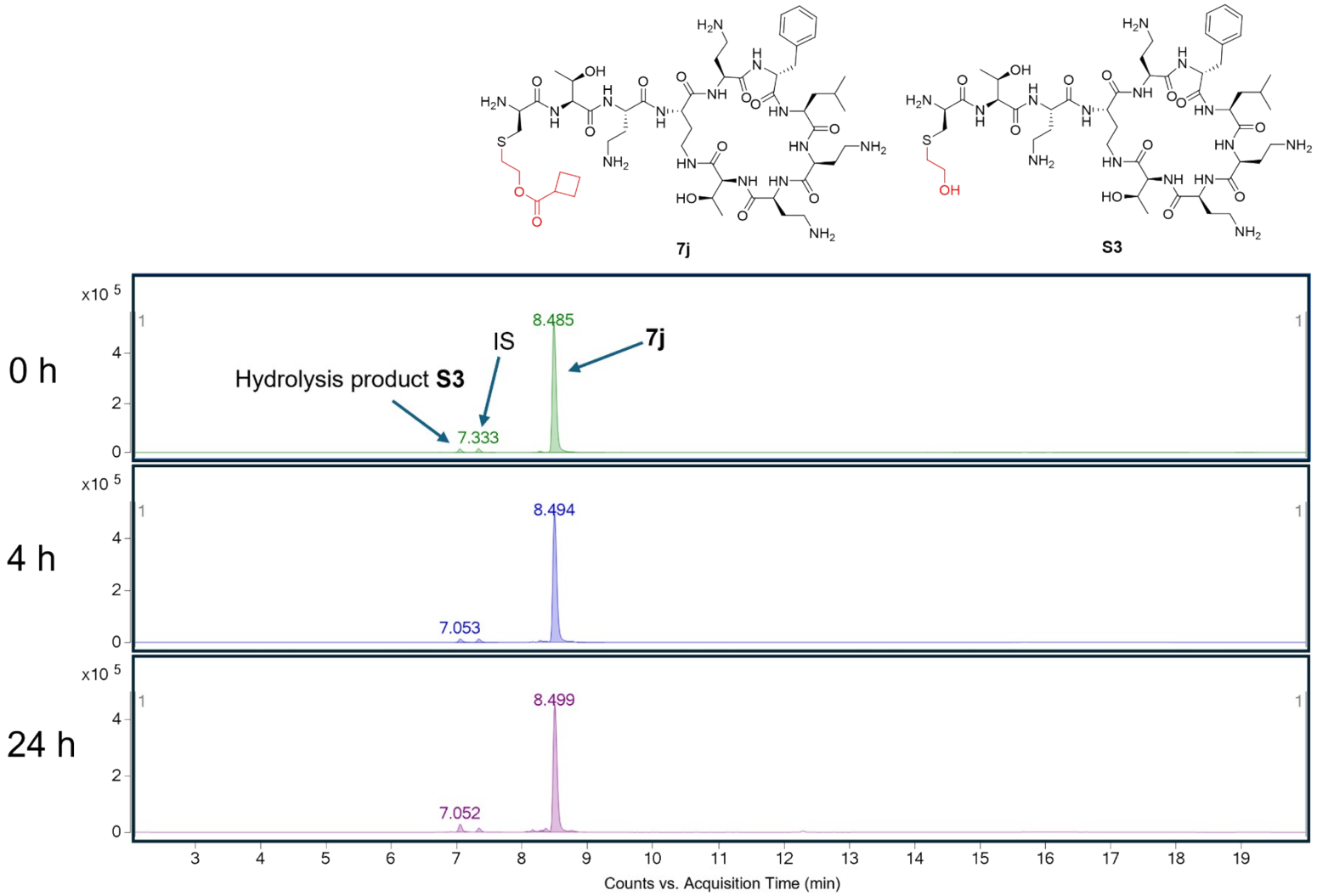


Figure S122: Stability monitoring of D-Cys cyclobutyrate **7j** in DPBS over 24 h; RP-HPLC [214 nm, linear gradient, 5 % B to 100 % B over 12 min with an initial 1 min divert and a 2 min hold at 100 % B, 0.6 mL/min flow at rt, through a Phenomenex Kinetex C18 column (100 Å, 100 mm × 3 mm; 2.6 µm)]; Solvent A = H_2O, containing 0.02 % TFA (*v*/*v*) and solvent B = MeOH. IS = internal standard, Val-Tyr-Val (VYV).

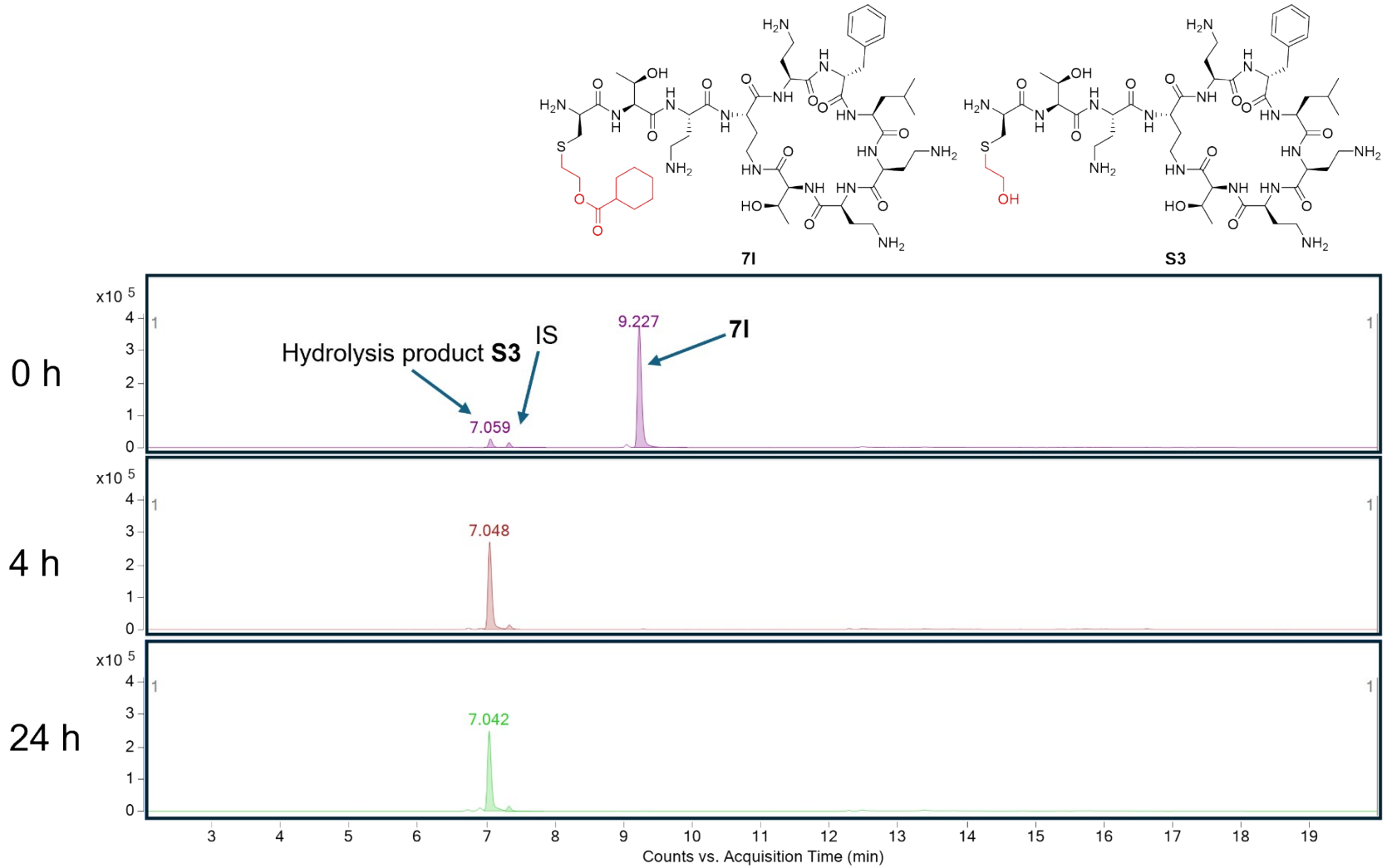


Figure S123: Stability monitoring of D-Cys cyclohexanoate **7I** in human serum over 24 h; RP-HPLC [214 nm, linear gradient, 5 % B to 100 % B over 12 min with an initial 1 min divert and a 2 min hold at 100 % B, 0.6 mL/min flow at rt, through a Phenomenex Kinetex C18 column (100 Å, 100 mm × 3 mm; 2.6 µm)]; Solvent A = H_2O, containing 0.02 % TFA (*v*/*v*) and solvent B = MeOH. IS = internal standard, Val-Tyr-Val (VYV).

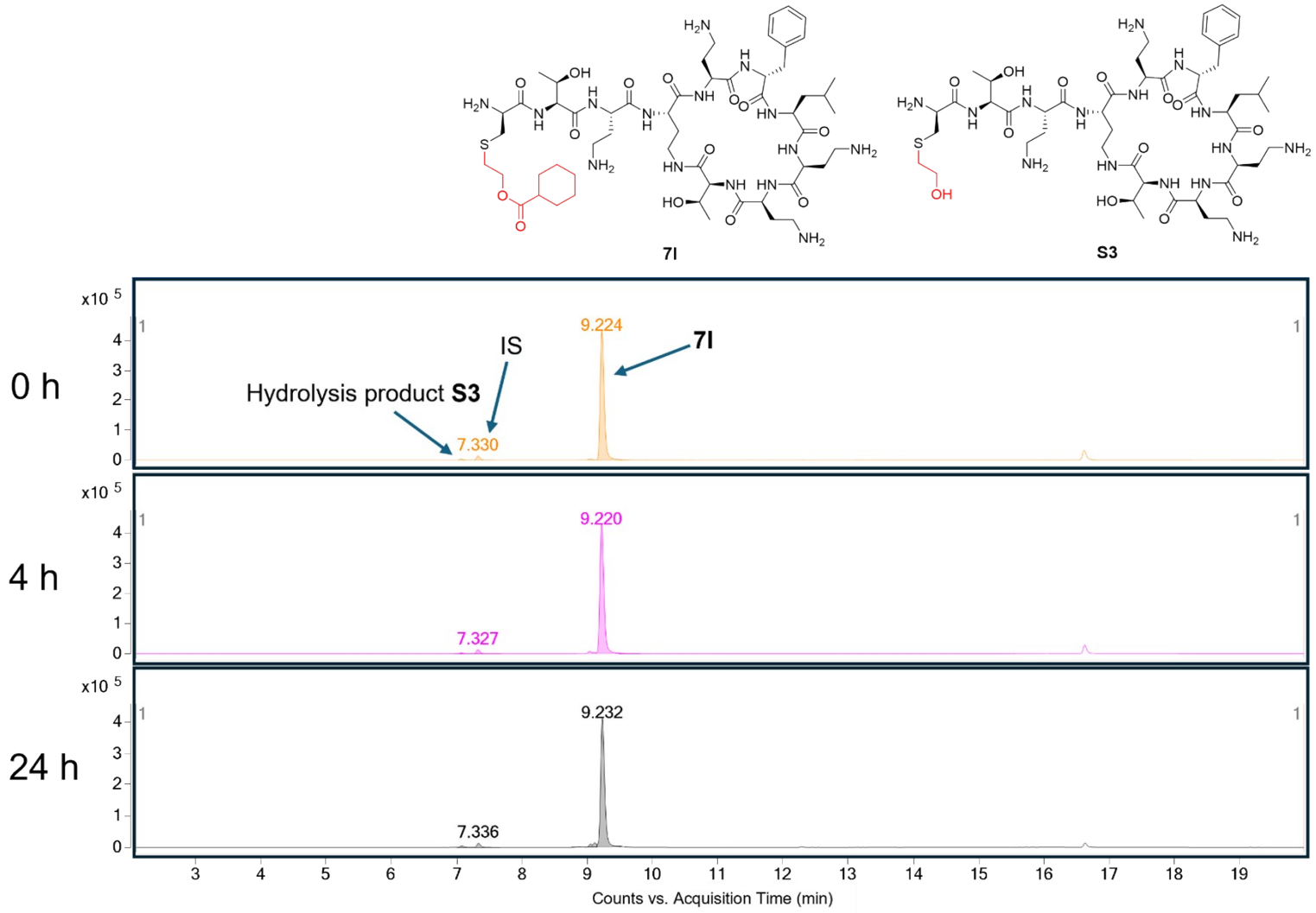


Figure S124: Stability monitoring of D-Cys cyclohexanoate **7l** in DPBS over 24 h; RP-HPLC [214 nm, linear gradient, 5 % B to 100 % B over 12 min with an initial 1 min divert and a 2 min hold at 100 % B, 0.6 mL/min flow at rt, through a Phenomenex Kinetex C18 column (100 Å, 100 mm × 3 mm; 2.6 µm)]; Solvent A = H_2O, containing 0.02 % TFA (*v*/*v*) and solvent B = MeOH. IS = internal standard, Val-Tyr-Val (VYV).

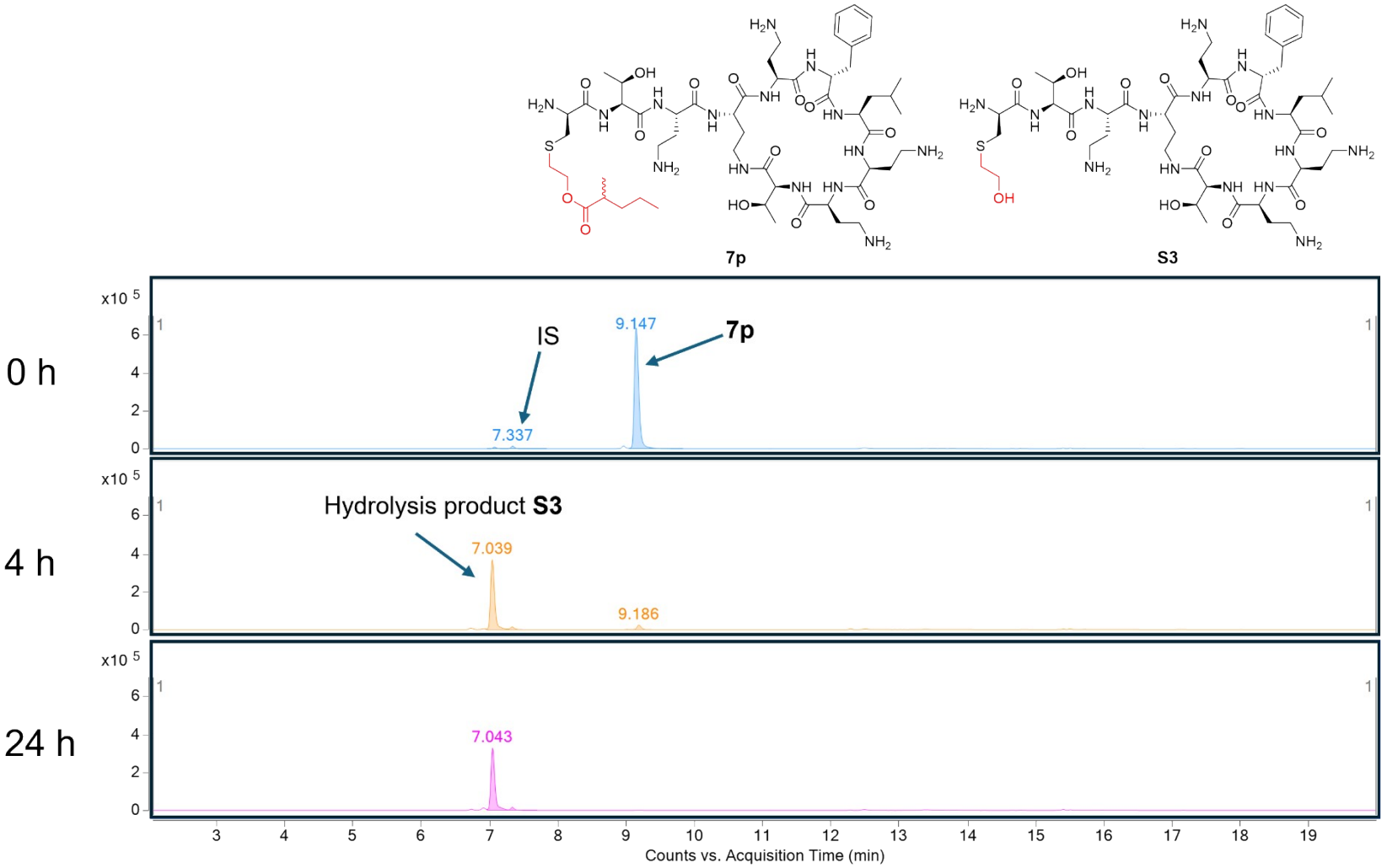


Figure S125: Stability monitoring of D-Cys 2-Me-valerate **7p** in human serum over 24 h; RP-HPLC [214 nm, linear gradient, 5 % B to 100 % B over 12 min with an initial 1 min divert and a 2 min hold at 100 % B, 0.6 mL/min flow at rt, through a Phenomenex Kinetex C18 column (100 Å, 100 mm × 3 mm; 2.6 µm)]; Solvent A = H_2O, containing 0.02 % TFA (*v*/*v*) and solvent B = MeOH IS = internal standard, Val-Tyr-Val (VYV).

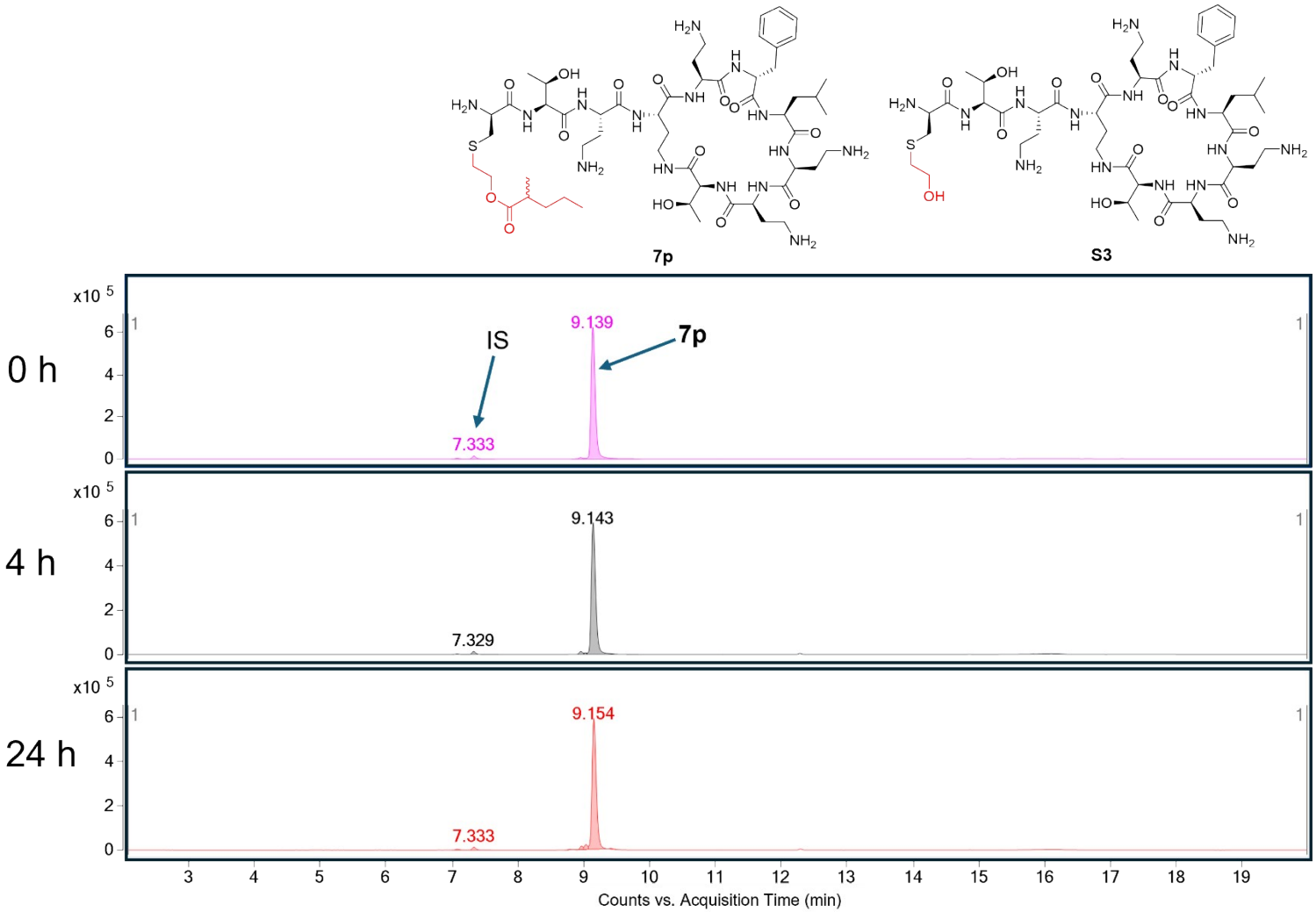
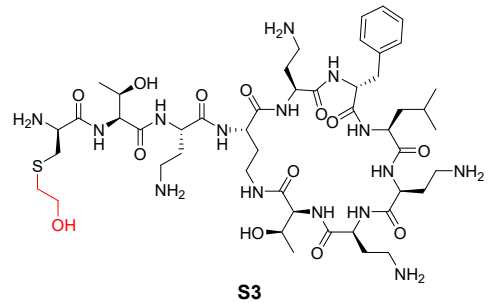


Figure S126: Stability monitoring of D-Cys 2-Me-valerate **7p** in DPBS over 24 h; RP-HPLC [214 nm, linear gradient, 5 % B to 100 % B over 12 min with an initial 1 min divert and a 2 min hold at 100 % B, 0.6 mL/min flow at rt, through a Phenomenex Kinetex C18 column (100 Å, 100 mm × 3 mm; 2.6 µm)]; Solvent A = H_2O, containing 0.02 % TFA (*v*/*v*) and solvent B = MeOH. IS = internal standard, Val-Tyr-Val (VYV).



S3

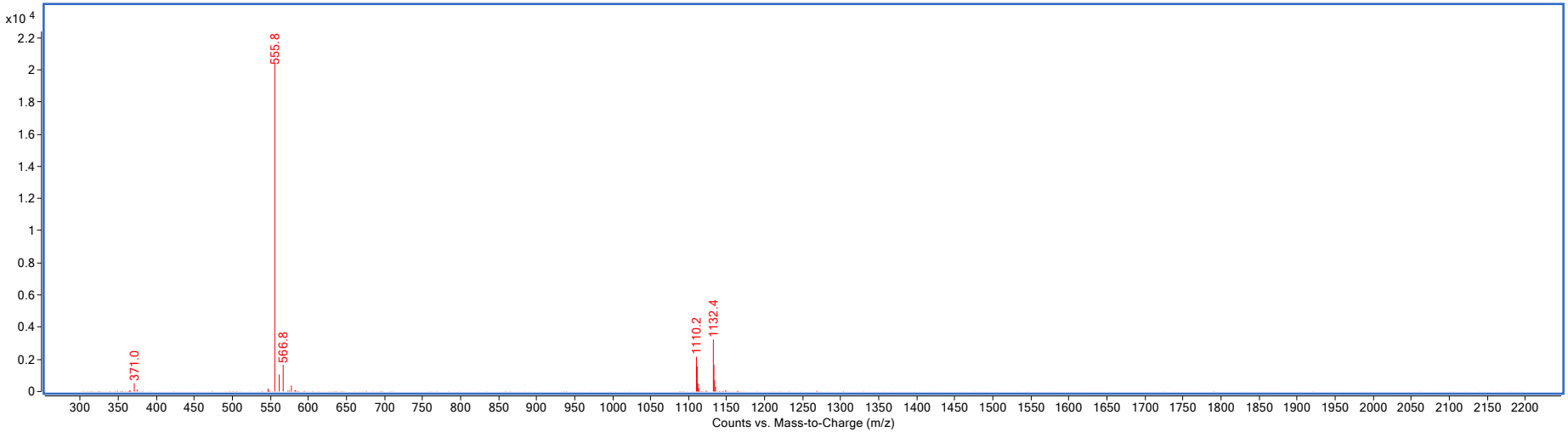


Figure S127: ESI-MS (+ve) analysis of hydrolysis product **S3**; $[M + H]^+$ found 1110.2, $[C_{48}H_{83}N_{15}O_{13}S + H]^+$ requires 1110.61.