Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supplementary Information

Thiol-ene enabled preparation of *S*-lipidated anti-HBV peptides

Oscar A. Shepperson[†,‡,§], Alan J. Cameron[*,†,‡,§], Carol J. Wang[‡], Paul W.R. Harris[†,‡,§], John A. Taylor[‡,§], Margaret A. Brimble[*,†,‡,§].

[*] Corresponding authors

[†]School of Chemical Sciences, The University of Auckland, 23 Symonds St., Auckland 1010, New Zealand.

[‡] School of Biological Sciences, The University of Auckland, 3A Symonds St., Auckland 1010, New Zealand.

[§] Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, 3A Symonds St., Auckland 1010, New Zealand.

Contents

S1. Abbreviations

3-MPA: 3-(tritylthio)propionic acid. 5-(6)-CF (CF): 5(6)-carboxyfluorescein. 6-Cl-HOBt: 6-chloro-1-hydroxybenzotriazole. CH_2Cl_2: dichloromethane. CLIA: chemiluminescence immunoassay. CLipPA: Cysteine Lipidation on a Peptide or Amino acid. Dde: 1-(4,4'-dimethyl-2,6-dioxocyclohexylidene)-3-ethyl. DIC: *N,N'*-diisopropylcarbodiimide. DIPEA: *N,N*-diisopropylethylamine. DMEM: Dublecco's modified eagle's medium. Et_2O: diethylether. EtOAc: ethyl acetate. FA: Formic acid. FACS: fluorescence-activated cell sorting. HATU: *O*-(7-azabenzo-triazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate. HBsAg: hepatitis B surface antigen. $Hg(OAc)_2$: mercury acetate. HPLC: high performance liquid chromatography. MeCN: acetonitrile. NEAA: non-essential amino acids. NH_2NH_2: hydrazine. NMM: *N*-methylmorpholine. NTCP: sodium (Na) taurocholate cotransporting polypeptide. P/S: penicillin/streptomycin. qPCR: quantitative polymerase chain reaction. RP: reverse-phase. SPPS: solid phase peptide synthesis. *t*BuSH: *tert*-butylthiol.. t_R: time of retention.

S2. General Information

All reagents were purchased as reagent grade and used without further purification unless otherwise noted. All Fmoc-amino acids, 3-MPA, mercury acetate ($Hg(OAc)_2$) and HATU were purchased from GL BioChem (Shanghai, China). The supplied amino-acids were side chain protected as follows: Boc-Gly-OH, Fmoc-Arg(Pdf)-OH (Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5- sulfonyl), Fmoc- Asp(OtBu)-OH, Fmoc-Asn(Trt)-OH, (Trt = trityl) Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu), Fmoc-His(Trt), Fmoc-Lys(Boc)-OH, Fmoc-Lys(Dde)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH and Fmoc-Trp(Boc)-OH. FA, piperidine, TIPS, *tert*-nonyl mercaptan (mixture of isomers), myrisitic acid, 5(6)-CF, vinyl acetate, hydrous sodium acetate (NaOAc.3H2O), EDT, NMM, DIC, DIPEA, hydrazine, DMPA, NMP, biological grade dimethyl sulfoxide (DMSO), tetracycline, foetal bovine serum (FBS), P/S and PEG 8000 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deutorochloroform ($CDCl_3$) and DMSO-d_6 were purchased from Cambridge Isotopes (Tewksbury, MA, USA). DMF (synthesis grade) and MeCN (HPLC grade) were purchased from Thermo Fisher Scientific (Hampshire, NH, USA). TFA was purchased from Oakwood Chemicals (Estill, SC, USA). 4-[(R,S)-α-[1-(9H-fluoren-9-yl)]methoxycarbonylamino]-2,4-dimethoxy]phenoxyacetic acid (Fmoc-Rink amide linker) and neodecanoic acid (mixture of isomers) were purchased from AK Scientific (Union City, CA, USA). 8-phenyloctanoic acid and 4-octylbenzoic acid were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Et_2O was purchased from Avantar Performance Chemicals (Center Valley, PA, USA). 6-Cl-HOBt was purchased from Aapptec (Louisville, KY, USA). Dichloromethane (CH_2Cl_2), sulphuric acid (H_2SO_4, 98%) was purchased from ECP Limited (Auckland, New Zealand). DMSO was purchased from Romil Limited (Cambridge, United Kingdom). Ethyl acetate and petroleum ether were purchased from Burdick & Jackson® (Muskegon, MI, USA). Aminomethyl ChemMatrix® resin was purchased from PCAS BioMatrix Inc. (Quebec, Canada). Polystyrene aminomethyl resin and TentaGel® resin were purchased from RAPP Polymere (Tubingen, Germany). Dulbecco's modified eagle media (#11330032) (DMEM/F12), trypsin-EDTA (0.25%), G418 and non-essential amino acids (NEAAs) from Life Technologies (Auckland, New Zealand). HBeAg 96 well ELISA chemiluminescence immunoassay plates from Autobio Diagnostics Co. (Zheng Zhou, China). Milli-Q high purity deionized water (MQ H_2O) was available from a Sartorius Arium® Pro Ultrapure Water System from Sartorius Stedim Biotech (Gottingen, Germany).

Analytical reverse phase high-performance liquid chromatography (RP-HPLC) was performed on either a Waters (Waltham, MA, USA) Alliance analytical HPLC or Dionex UltiMateR 3000 equipped with a Phenomenex (Torrance, CA, USA) Luna C18 column (100 A, 5 µm, 4.6 mm x 250 mm) operated at room temperature, with chromatograms recorded at 210/214 nm and 254 nm. Semi-preparative RP-HPLC was performed on a Waters 1525 Binary HPLC pump equipped with a Waters 2489 UV/visible detector using either a Waters XTerra Prep MS C18 OBD column (125 A, 10 µm, 300 x 19 mm) or Waters XTerra Prep MS C18 OBD column (125 A, 10 µm, 250 x 10 mm). For both

analytical and semi-preparative RP-HPLC, solvents used were as follows: solvent A = 0.1% TFA in water (MQ H_2O) and solvent B = 0.1% TFA in MeCN. For analytical RP-HPLC the gradient employed was 5-95% of solvent B over 30 minutes at flow rate 1 ml/min, unless specified otherwise. For semi-preparative RP-HPLC gradients were adjusted as indicated in the experimental procedures, according to elution times and peak profiles obtained during analytical analysis. Analytical thin-layer chromatography (TLC) was performed on Kieselgel F254 200 µm (Merck) silica plates. The compounds were then visualised by ultraviolet fluorescence or by staining with potassium permanganate or vanillin followed by heating of the plate. Column chromatography was performed using Grace Davison Discovery Sciences (Columbia, MD, USA), Davasil LC60A 40-63 Micron Chromatographic Silica Media with the indicated eluent. High-resolution mass spectra were obtained with a Bruker (Billerica, MA, USA) micrOTOFQ mass spectrometer by using electrospray ionisation (ESI) in the positive mode at a nominal accelerating voltage of 70 eV. Low-resolution mass spectrometry was performed on a Waters Quattro micro API Mass Spectrometer in ESI positive mode. Ultraviolet irradiation was performed using a handheld Spectroline UV lamp at a peak wavelength of 365 nm. Nuclear magnetic resonance (NMR) experiments were recorded as shown on a Bruker (Billerica, MA, USA) AVANCE 400 spectrometer operating at 400MHz for ^{1}H nuclei and 100MHz for ^{13}C nuclei. Chemical shifts were reported in parts per million (ppm) on the δ scale from tetramethylsilane (TMS), at 0.00 ppm. Shifts were referenced to residual solvent peaks of $CDCl_3$ at δ = 7.26 ppm for ^{1}H NMR, and $CDCl_3$ δ = 77.16 ppm for ^{13}C NMR or DMSO-d_6 at δ = 2.50 ppm for ^{1}H NMR and δ = 39.52 ppm for ^{13}C NMR. The ^{1}H NMR shift values are reported as chemical shift δ, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, td = triplet of doublets, qd = quartet of doublets), coupling constant (J in Hz), relative integral and assignments. ^{13}C NMR values are reported as the chemical shift δ, the degree of hybridisation and assignments.

The AD38 and HepG2 NTCP cell lines were gifted from the WHO Regional Reference Library (Melbourne, Australia). The HepG2 cell lines was purchased from the ATCC.

S3. General Methods

Linear peptides were synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS) at room temperature on a 0.1 mmol scale either manually using a fritted glass reaction vessel or using a PS3 automated peptide synthesizer (Gyros Protein Technologies, Sweden).

Method 1. Construction of peptides by automatic synthesis

TentaGel Resin (0.370 g, 0.1 mmol, 0.27 mmol/g loading) was swollen in CH_2Cl_2 (3 ml) for 5 mins followed by DMF (3 ml) for 5 mins. Fmoc-Rink amide linker (216 mg, 0.4 mmol, 4 equiv.) was used as a linker to the solid TentaGel support and was coupled to the resin using the same method as the subsequent amino acids. Using the PS3 Peptide Synthesizer, all amino acids were coupled in single, hour long cycles at room temperature. Protected amino-acids were coupled using a mixture of Fmoc-AA_x-OH (0.4 mmol, 4 equiv.), HATU (144 mg, 0.38 mmol, 3.8 equiv.) and NMM (1mmol, 10 equiv.) in DMF (3 ml). The Fmoc protecting group was removed with a solution of 20% piperidine in DMF (v/v) containing 5% formic acid (v/v) (3 ml, 2 x 5 min). Between couplings and deprotection the resin was washed with DMF (6 x 5 ml).

Method 2. General method for coupling of 3-(tritylthio)propionic acid

3-(Tritylthio)propionic acid was coupled as the terminal acid using a similar method to that described in **Method 1**. A mixture of 3-(tritylthio)propionic acid (0.139 mg, 0.4 mmol, 4 equiv.) HATU (144 mg, 0.38 mmol, 3.8 equiv.) and DIPEA (175 µL, 1 mmol, 10 equiv.) in DMF (3 ml) was coupled for 2 hours at room temperature to the deprotected *N*-terminus of the peptidyl resin.

Method 3. General method for coupling of Myristic Acid

A mixture of myristic acid (90 mg, 0.4 mmol, 4 equiv.) HATU (144 mg, 0.38 mmol, 3.8 equiv.) and DIPEA (175 µL, 1 mmol, 10 equiv.) in DMF (3 ml) was coupled for 1 hour at room temperature to the deprotected *N*-terminus of the peptidyl resin.

Method 4. General method for removal of Dde protecting group from amino acid side chain

The resin-bound peptide was treated with 2% hydrazine monohydrate in DMF (*v/v*, 3 x 3 min) at room temperature and vigorously agitated.

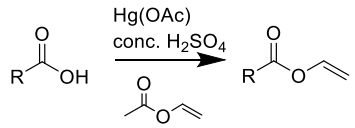
Method 5. General method for coupling of 5(6)-CF

After removal of the sidechain Dde group, the resin-bound peptide was treated with a mixture of 5(6)-CF (151 mg, 0.4 mmol, 3 equiv.) 6-Cl HOBT (50.8 mg, 0.400 mmol, 3 equiv.) and DIC (46.0 µL, 0.4 mmol, 3 equiv.) in DMF and vigorously agitated for 4 hours at room temperature. The resin was then washed with 20% piperidine in DMF (v/v) containing 5% formic acid (v/v) (3 ml, 3 x 3 min).

Method 6. General method for cleavage of peptide from resin

After washing the resin with CH_2Cl_2 (3 x 5 ml) and air drying the resin, a cleavage mixture of TFA/TIS/EDT/H_2O (10 ml, *v/v/v/v*; 94/2/2/2) was added to the on resin peptide and agitated for 2 hours at room temperature. The cleavage mixture was drained and chilled diethyl ether (45 ml) was used to precipitate the peptide. The peptide was isolated by centrifugation and the pellet washed with diethyl ether (45 ml), dissolved in MeCN:MQ H_2O (1:1) and lyophilised.

Method 7. Synthesis of vinyl esters



The desired lipid starting material (1 equiv.) was added to an excess of vinyl acetate (10 equiv.), mercury acetate (0.025 equiv.) and fuming sulphuric acid (H_2SO_4, 15.0 µL). The reaction proceeded under reflux (74 ^oC) under a nitrogen atmosphere until completion, as judged by TLC monitoring of the reaction mixture. Upon completion of the reaction the reaction mixture was cooled, the sulphuric acid quenched with a portion wise addition of $NaOAc.3H_2O$ (3 x 25.0 mg) and filtered through Celite. Excess vinyl acetate was evaporated and the crude reaction mixture purified by flash column chromatography on silica gel with an eluent of ethyl actetate:petroleum ether (1:99).

Method 8. Attachment of vinyl esters using CLipPA chemistry

The desired peptide (1 equiv.) containing a thiol functionality was added to a solution of NMP (volume adjusted to ensure peptide concentration of 2.50 µM) together with DMPA (0.5 equiv.), *tert*-nonyl mercaptan (80 equiv.), TIPS (80 equiv.) and TFA (5% *v/v* of final volume). To this solution the respective vinyl ester (35 equiv.) was added and the reaction mixture agitated at room temperature under UV light (365 nm) for 60 min. Upon completion of the reaction, as determined by RP-HPLC, the peptidyl products were precipitated out by addition of diethyl ether (45 ml), isolated by centrifugation, and washed with diethyl ether (2 x 45 ml). The final crude CLipPA peptide was dissolved in a 1:1 solution of MeCN:MQ H_2O and lyophilized. The lyophilized crude peptide was dissolved into a solution of MeCN:MQ H_2O and purified using semipreparative RP-HPLC using linear gradients determined from observation of the analytical RP-HPLC traces. For each peptide the linear gradient for purification can be found in the peptide experimental section (*vide infra*).

S4. ^{1}H NMR, ^{13}C NMR, ESI-HRMS and IR profiles of vinyl esters 12a-d

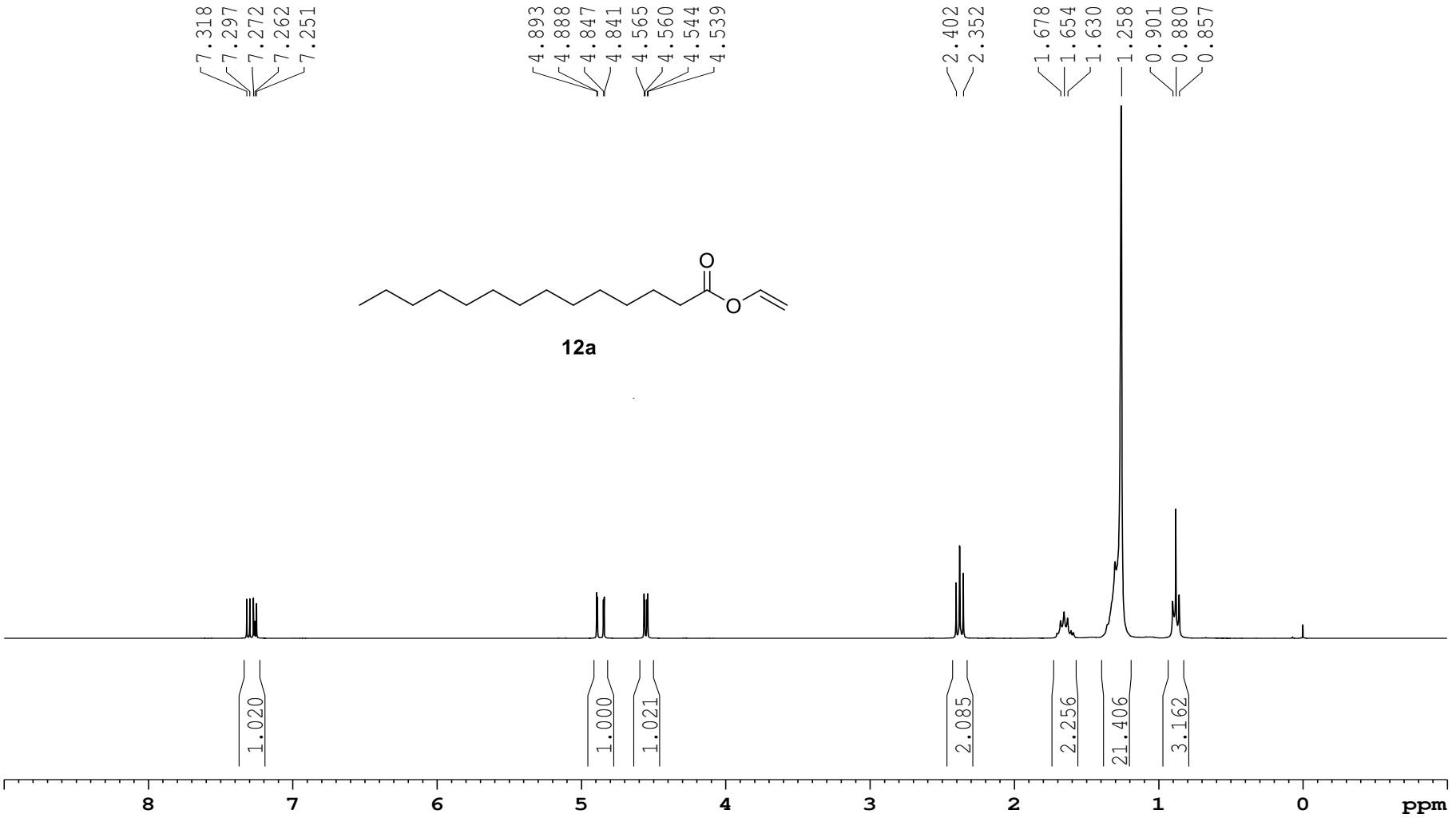


Figure S4.1: Compound **12a** ^{1}H NMR (400 MHz, $CDCl_3$)

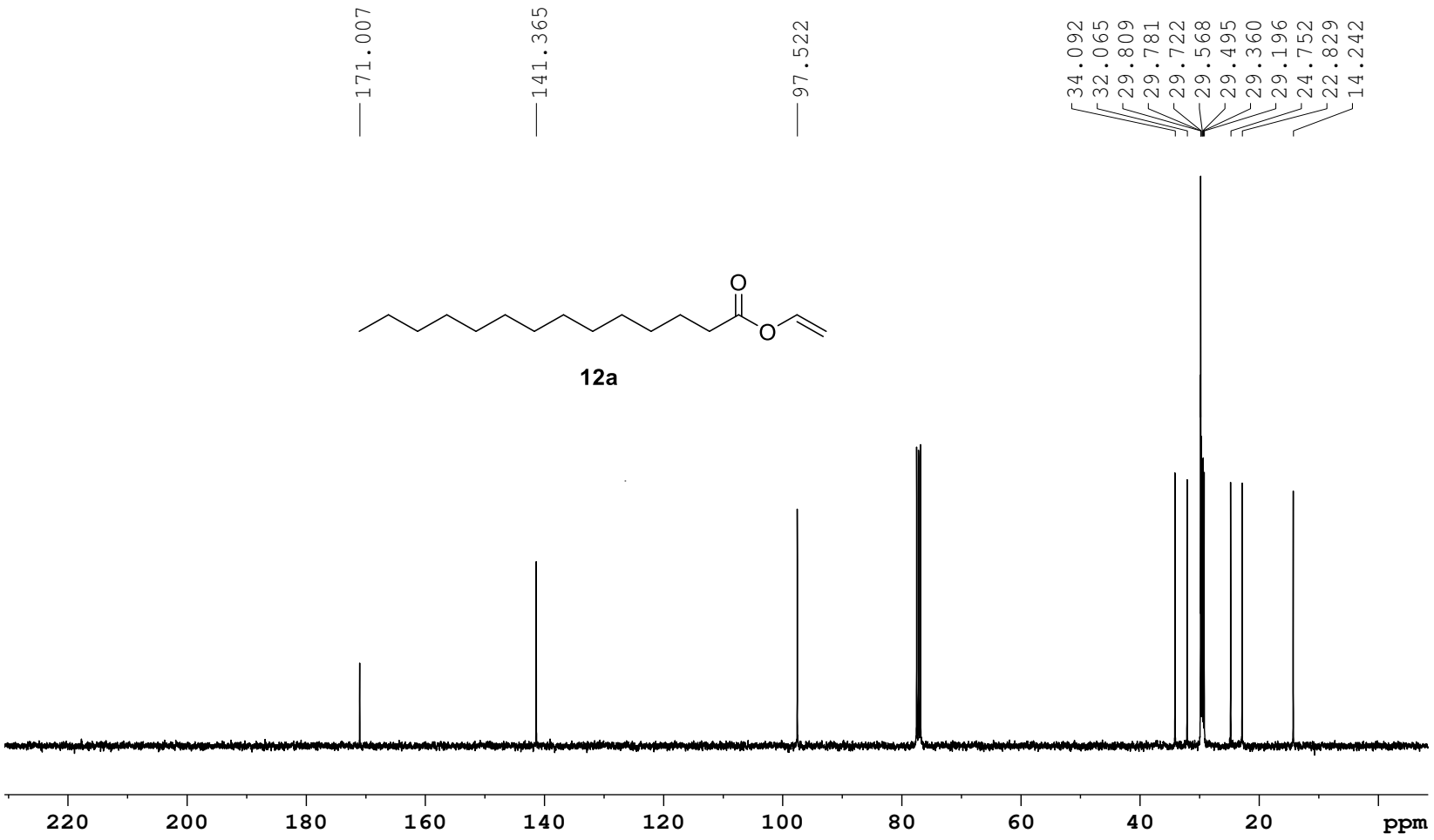


Figure S4.2: Compound **12a** ^{13}C NMR (100 MHz, $CDCl_3$)

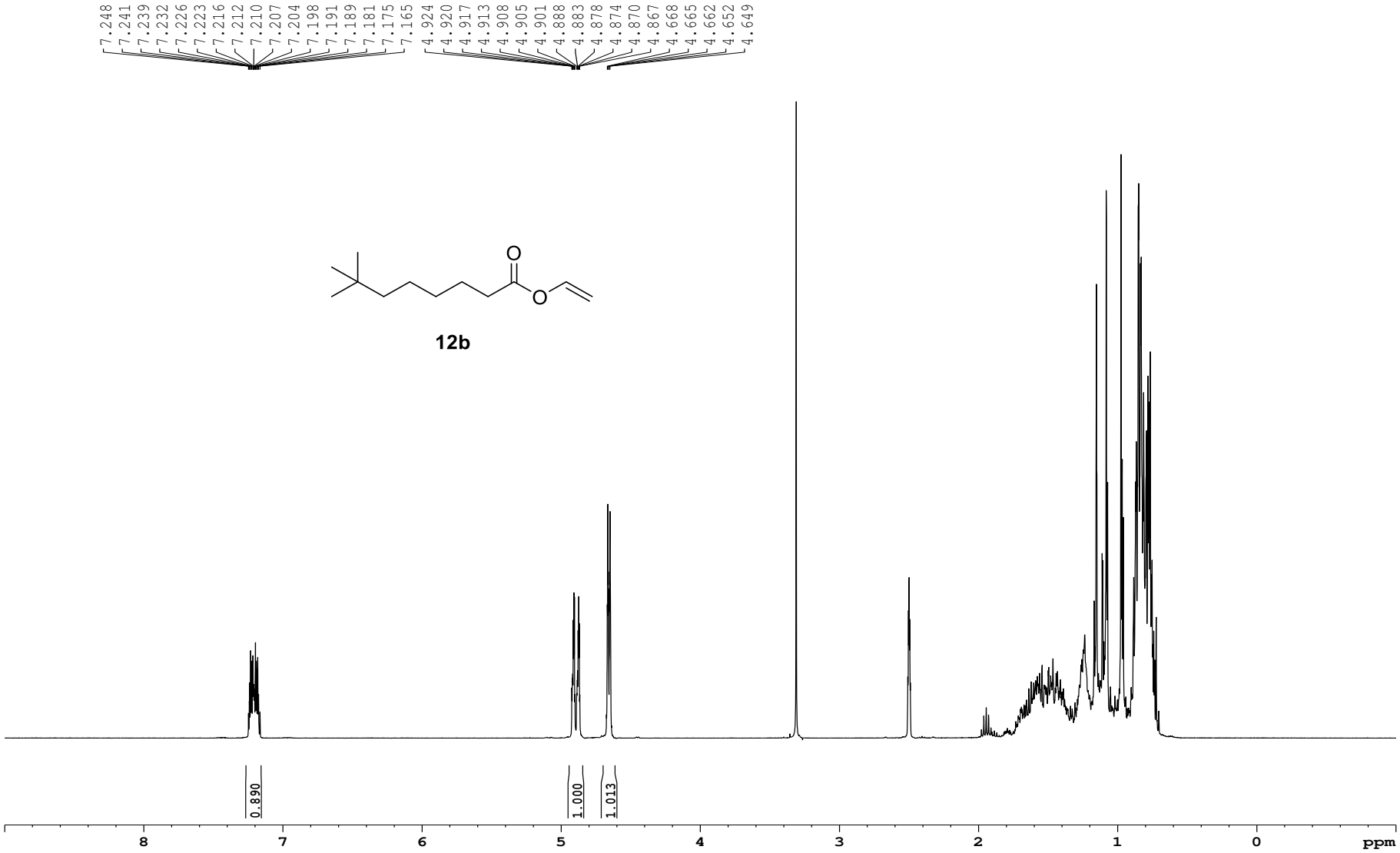


Figure S4.3: Compound **12b** ^{1}H NMR (400 MHz, DMSO-d_6) – structure indicative only (mixture of vinyl neodecanoate isomers)

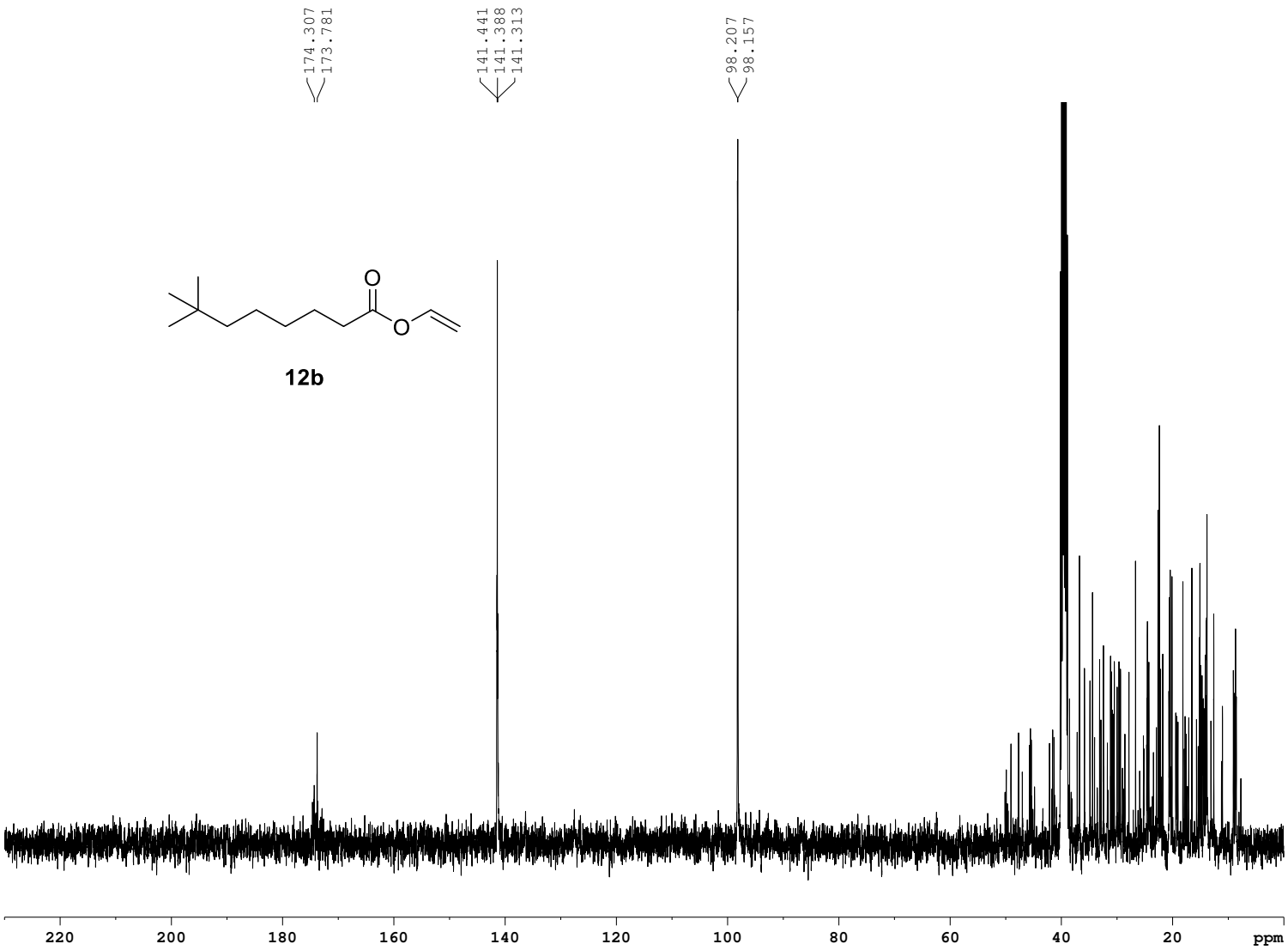


Figure S4.4: Compound **12b** ^{13}C NMR (100 MHz, DMSO-d_6) – structure indicative only (mixture of vinyl neodecanoate isomers)

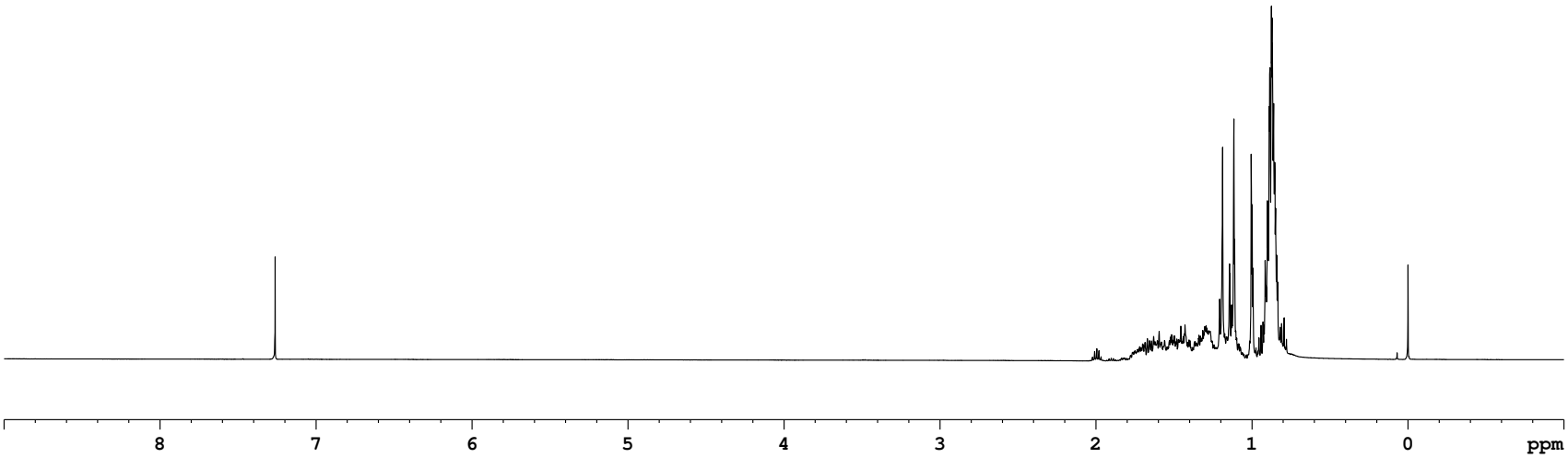


Figure S4.5: ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of the neodecanoic acid (mixture of isomers) starting material for the synthesis vinyl ester **12b**

Auckland Uni Mass Spectrum SmartFormula Report

Analysis Info

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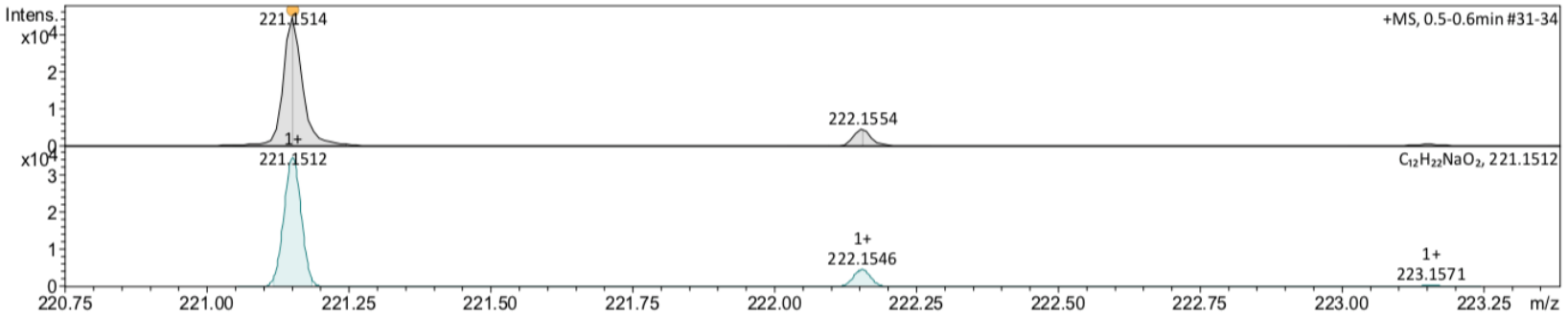
Method	may2014 - low - hplc.m	Operator	Admin
Sample Name	Vinyl Neodecanoate - VE2	Instrument / Ser#	micrOTOF-Q 228888.10191
Comment	Sample dissovled in 0.5ml DCM Sampe diluted 3ul in 1ml MeOH		

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e⁻ Conf	N-Rule
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	1	C12H19N3O	221.1523	3.8	10.1	1	100.00	5.0	odd	ok
	1	C12H22NaO2	221.1512	-1.0	7.3	1	100.00	1.5	even	ok

Figure S4.6: ESI-HRMS formula analysis of vinyl ester **12b**

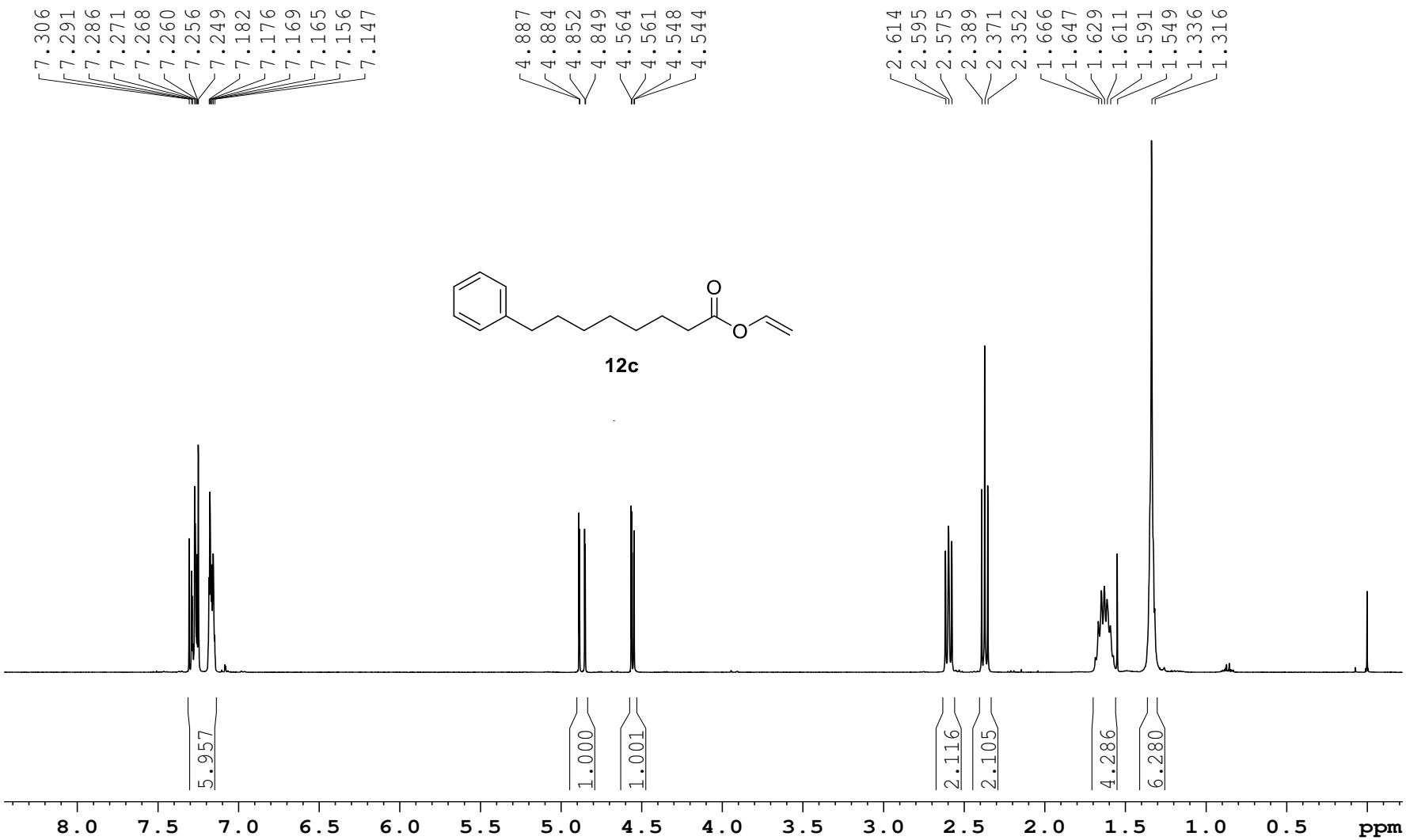

Figure S4.7: Compound **12c** ^{1}H NMR (400 MHz, $CDCl_3$)

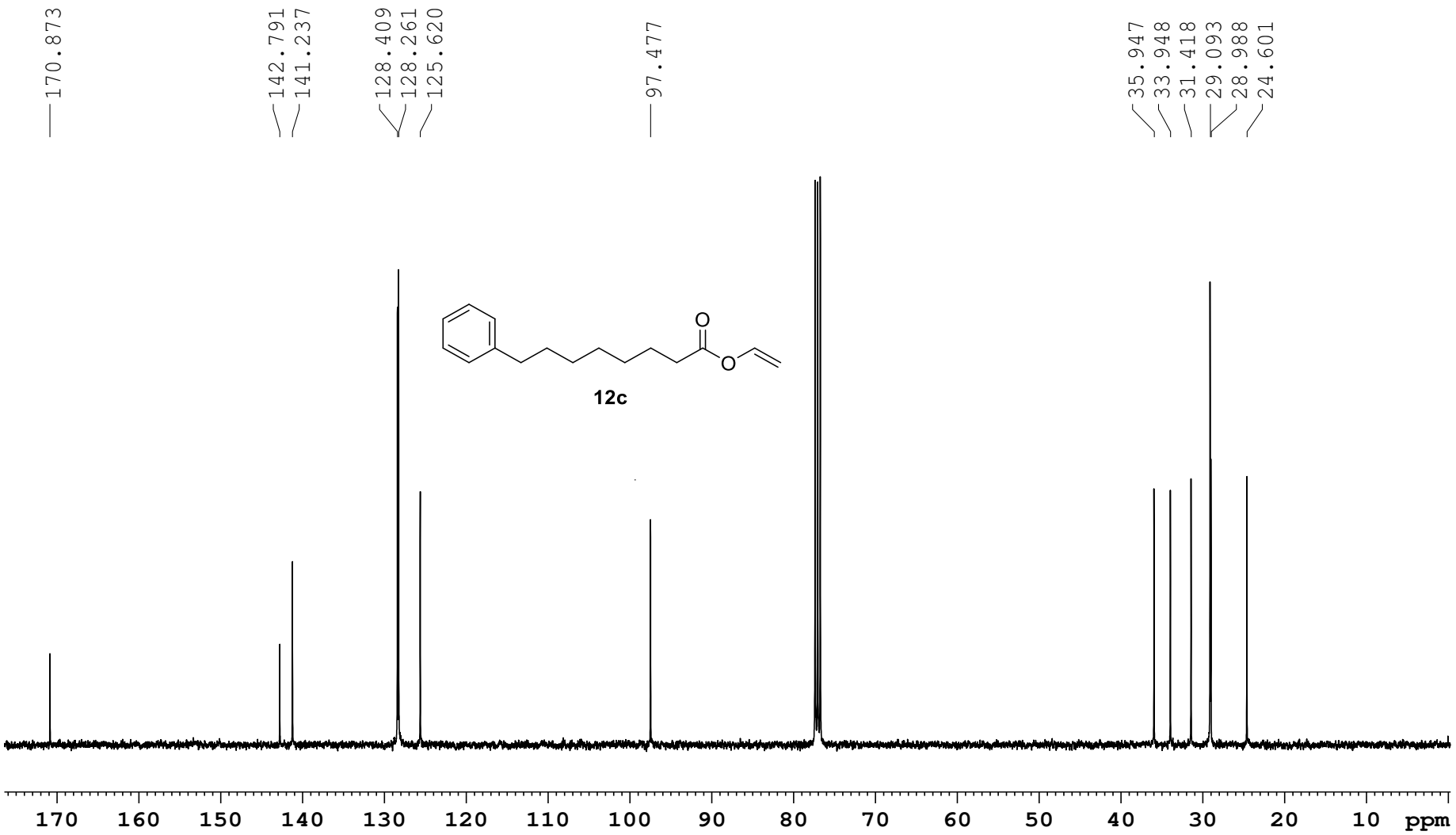

Figure S4.8: Compound **12c** ^{13}C NMR (100 MHz, $CDCl_3$)

Auckland Uni Mass Spectrum SmartFormula Report

Analysis Info

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Method	may2014 - low - hplc.m	Operator	Admin
Sample Name	Vinyl 8-Phenyloctanoate - VE3	Instrument / Ser#	micrOTOF-Q 228888.10191
Comment	Sample dissovled in 0.5ml DCM Sampe diluted 3ul in 1ml MeOH		

Acquisition Parameter

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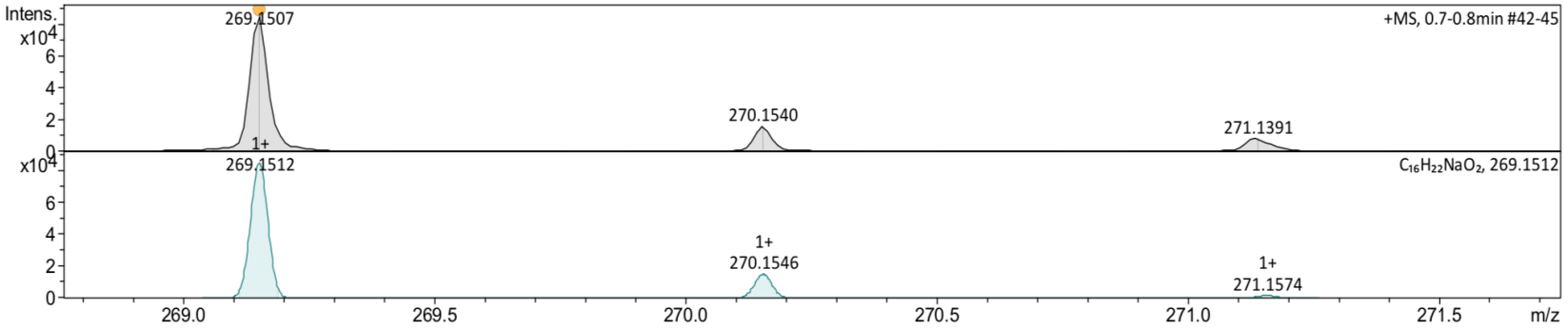
Scan End	1000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e⁻ Conf	N-Rule
269.1507	1	C16H22NaO2	269.1512	2.0	45.2	1	100.00	5.5	even	ok

Figure S4.9: ESI-HRMS formula analysis of vinyl ester **12c**

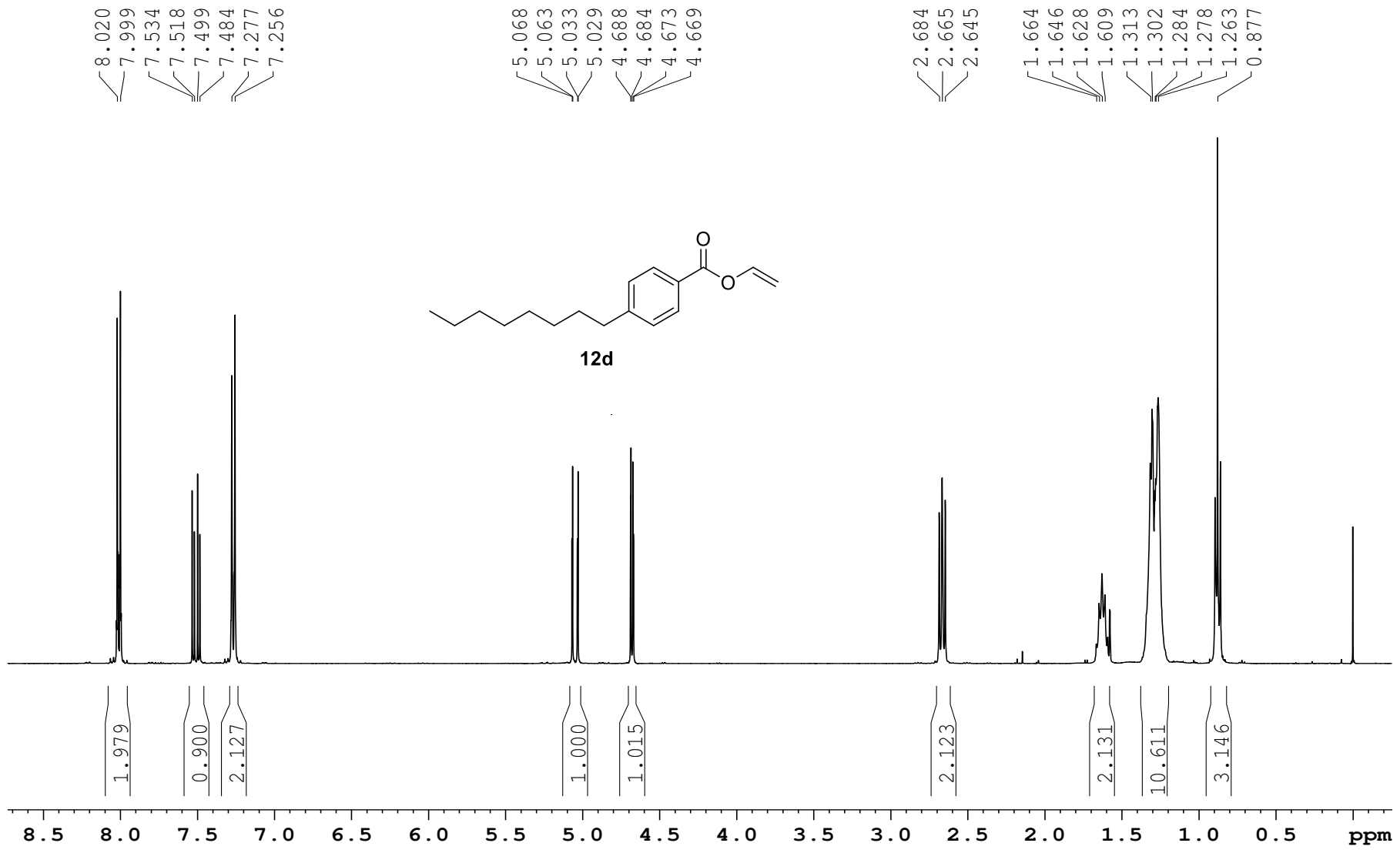


Figure S4.10: Compound **12d** ^{1}H NMR (400 MHz, $CDCl_3$)

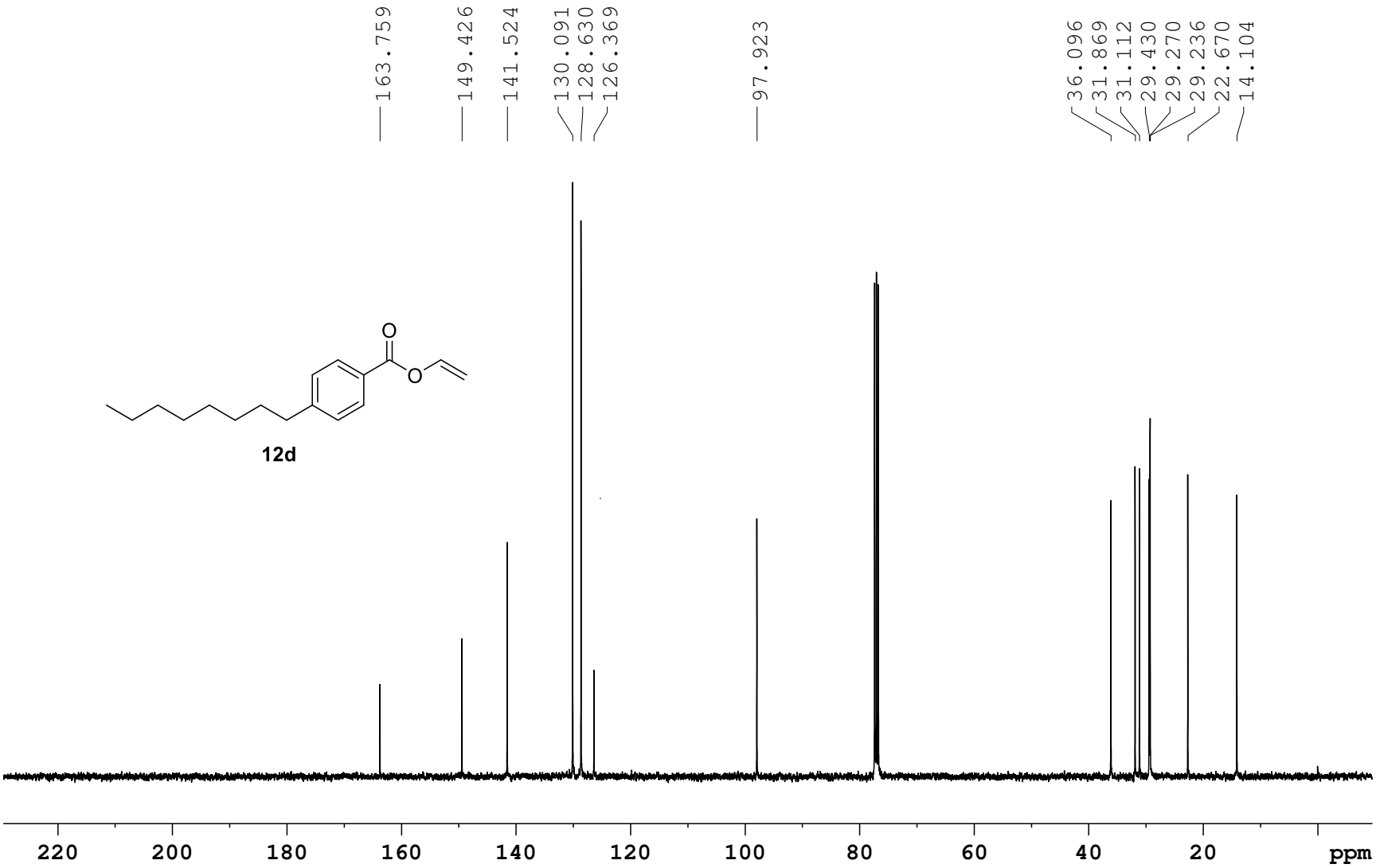


Figure S4.11: Compound **12d** ^{13}C NMR (100 MHz, $CDCl_3$)

Auckland Uni Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	W:\Tony19\06\2019-06-06\run1\Vinyl 4-Octylbenzoate - VE4_RB6_01_10745.d	Acquisition Date	6/06/2019 5:52:21 PM
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Sample Name	Vinyl 4-Octylbenzoate - VE4	Instrument / Ser#	micrOTOF-Q 228888.10191
Comment	Sample dissovled in 0.5ml DCM Sampe diluted 3ul in 1ml MeOH		

Acquisition Parameter

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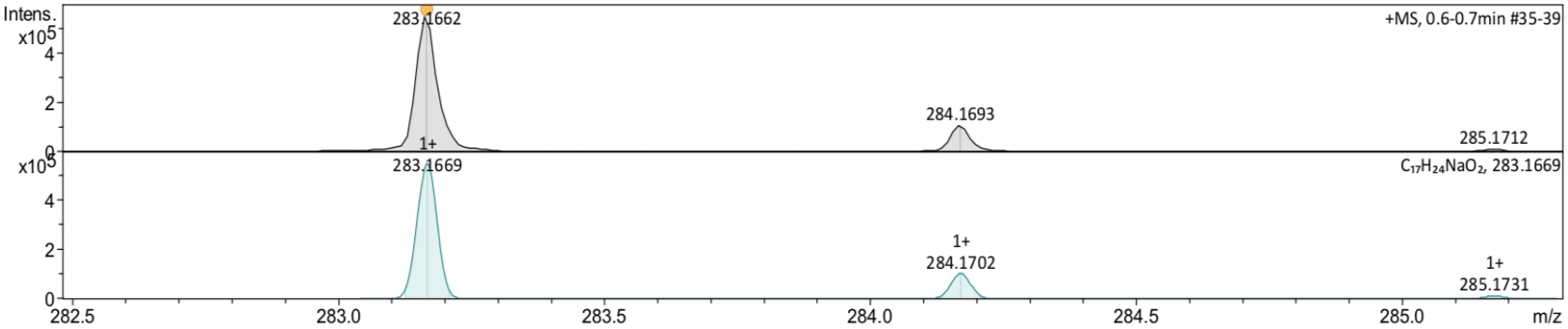
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste

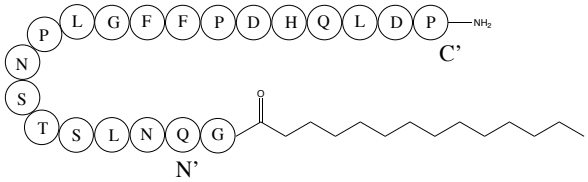


Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e⁻ Conf	N-Rule
283.1662	1	C17H24NaO2	283.1669	2.2	5.6	1	100.00	5.5	even	ok

Figure S4.12: ESI-HRMS formula analysis of vinyl ester **12d**

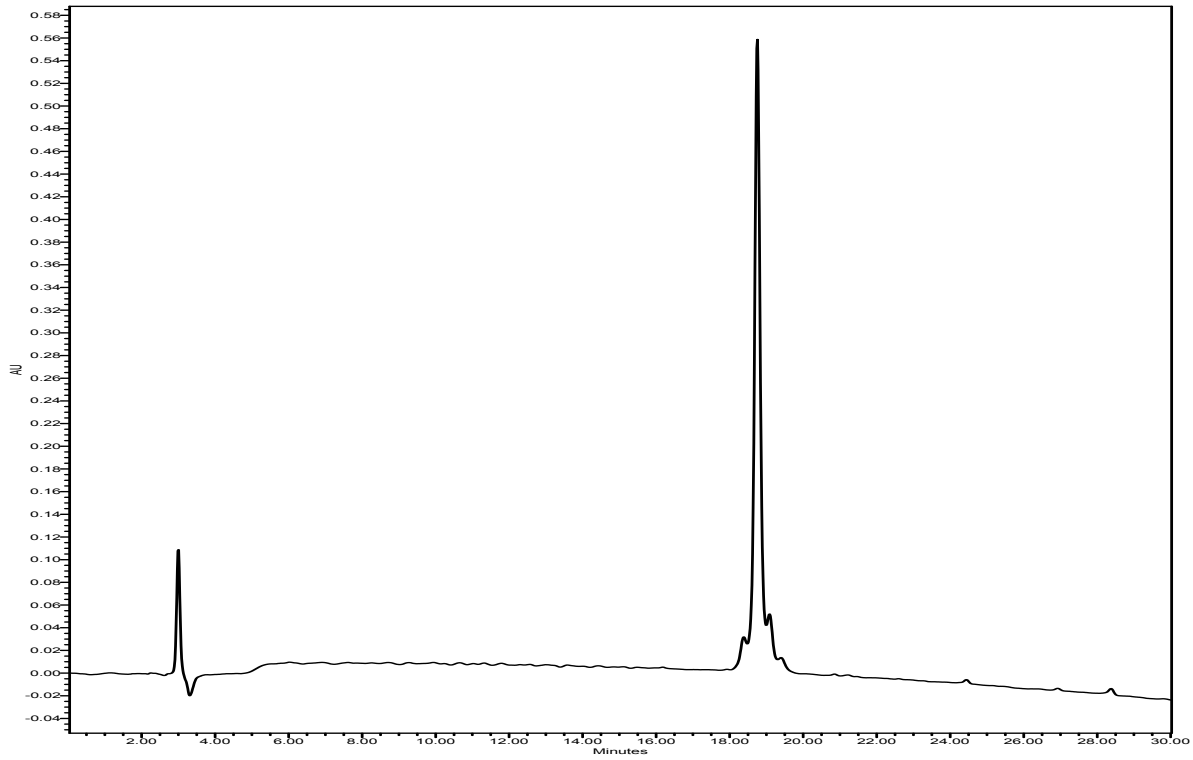
S5. Peptide experimental, structures, RP-HPLC and ESI-MS profiles

S5.1. Synthesis of truncated Myrcludex B analogue 1



The truncated parent peptide was assembled by automated Fmoc SPPS using the PS3 Peptide Synthesizer as described in **Method 1.** The final myristic acid was coupled using **Method 3**. The final cleavage of the peptide from the resin was performed using **Method 6 (**reduced to a scale of 0.04 mmol) to afford 66.8 mg of crude peptide at approximately 77% purity.

The crude peptide analogue **1** was dissolved in 1:1 MeCN/MQ H_2O at a concentration of 10 mg/ml and purified by RP-HPLC with a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 300 x 19 mm), employing a gradient of 45%B to 65%B over 28 min (ca. 0.7% B/min) at a flow rate of 10 ml/min. Fractions were collected based on deduction of main peak from analytical HPLC and confirmed by ESI-MS. Fractions from multiple runs were combined and lyophilised to afford *title compound* **1** as a white amorphous powder (24.7 mg, 21% yield, 95.7% purity).



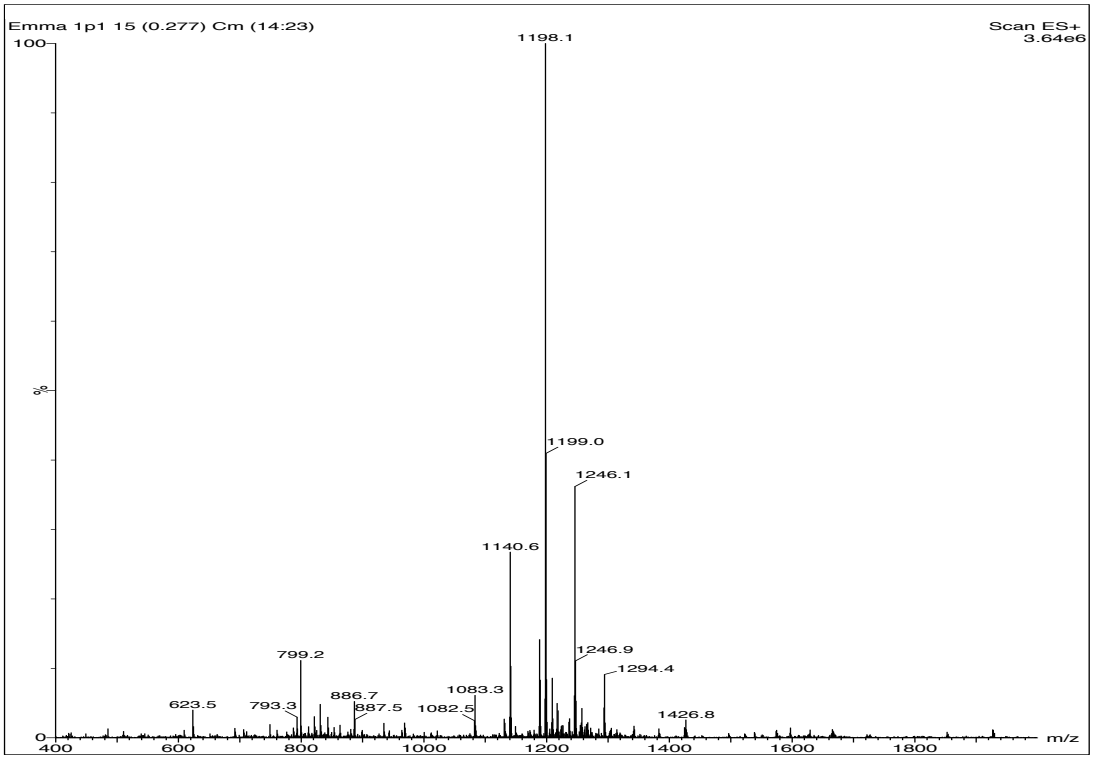
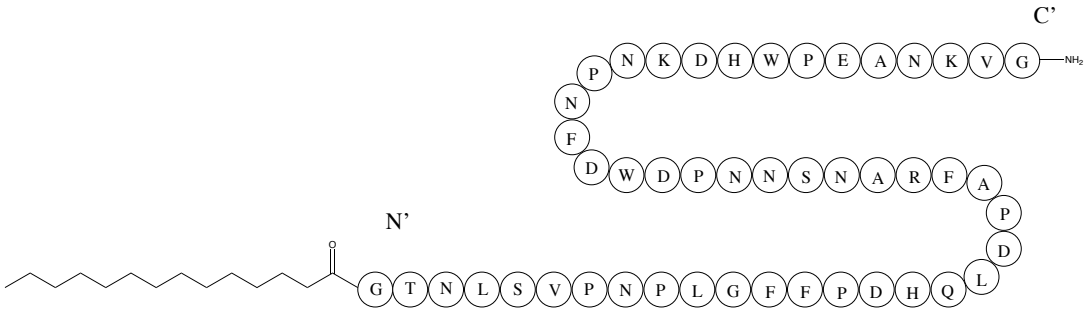


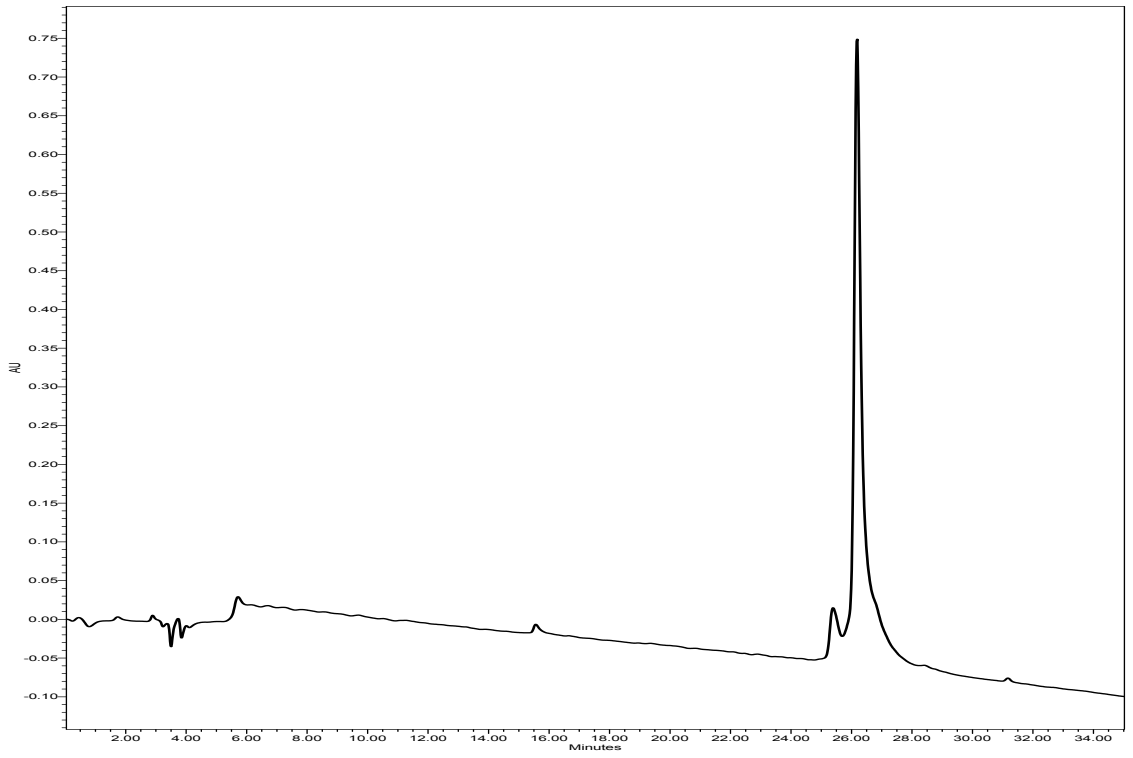
Figure S5.1: (upper) analytical **RP-HPLC** trace of pure truncated native peptide, **1** (ca. 95.7% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 75%B over 35 min (ca. 2%B/min) at 1 ml/min. t_R = 18.8 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 2394.10, observed: 2394.20.

S5.2. Synthesis of native Mycludex B 2



The parent peptide was assembled by automated Fmoc SPPS using the PS3 Peptide Synthesizer as described in **Method 1.** The final myristic acid was coupled using **Method 3**. The final cleavage of the peptide from the resin was performed using **Method 6 (**reduced to a scale of 0.04 mmol), to afford 167 mg of crude peptide at approximately 90% purity.

The crude peptide analogue **2** was dissolved in 1:4 MeCN/MQ H_2O at a concentration of 10 mg/ml and purified by RP-HPLC with a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 300 x 19 mm), employing a gradient of 22.5%B to 30%B over 5 min (ca. 1.5% B/min), then 30%B to 70%B over 20 min (ca. 2% B/min) at a flow rate of 10 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed by ESI-MS. Fractions from multiple runs were combined and lyophilised to afford *title compound* **2** as a white amorphous powder (65 mg, 30% yield, 95% purity).



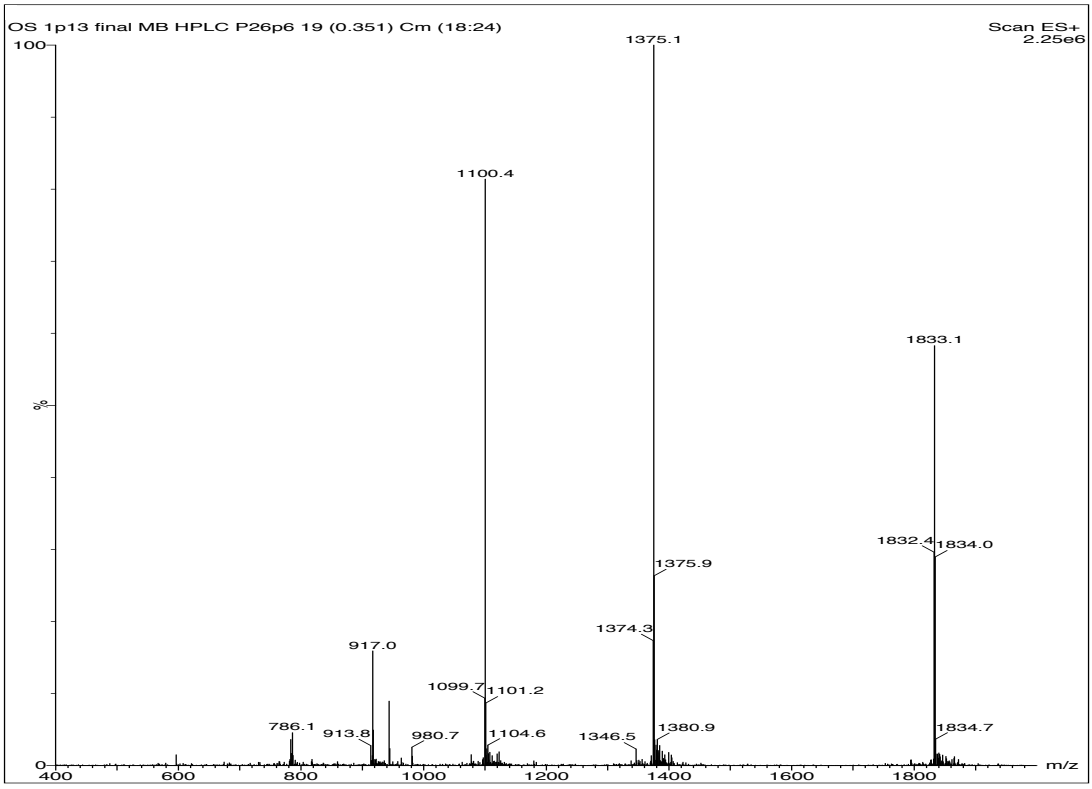
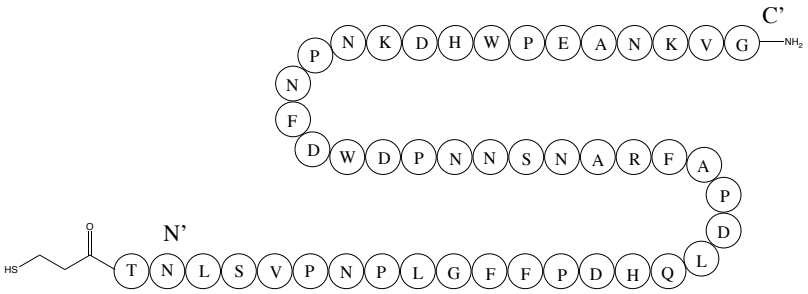


Figure 5.S2: (upper) **a**nalytical **RP-HPLC** trace of pure peptide Myrcludex B, **2** (ca. 95.0% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 75%B over 35 min (ca. 2%B/min) at 1 ml/min. t_R = 26.9 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5496.70, observed: 5496.57 ± 0.38.

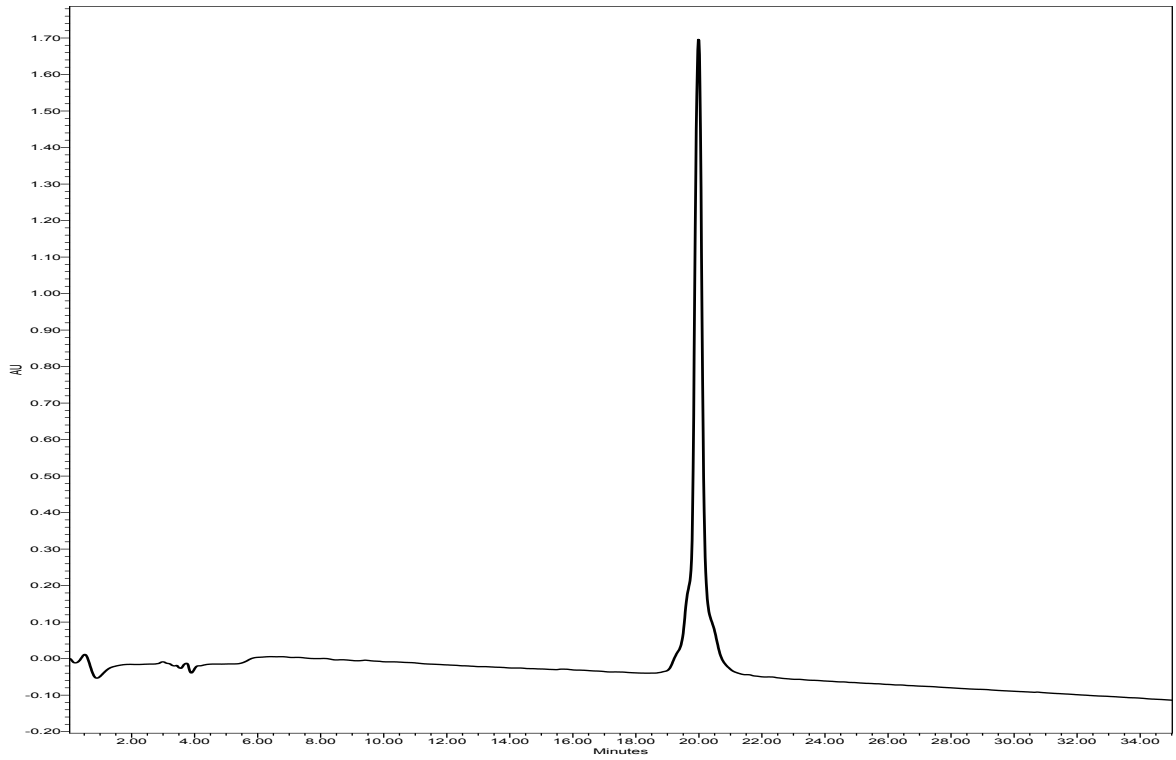
S5.3. Synthesis of Mycludex B intermediate 2s



The parent peptide intermediate **2s** was assembled by automated Fmoc SPPS using the PS3 Peptide Synthesizer as described in **Method 1.** The final mercaptopropionic acid was coupled as described by **Method 2**. The final cleavage of the peptide from the resin was performed as described by **Method 6** to afford 424 mg of crude intermediate peptide at approximately 90% purity.

The crude peptide intermediate **2s** was dissolved in 1:4 MeCN/MQ H_2O at a concentration of 10 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC with a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 300 x 19 mm). The gradient employed was as follows; 12.5%B to 30%B over 5 min (ca. 3.5%B/min), then 30%B to 50%B over 20 min (ca. 1%B/min) at a flow rate of 10 ml/min. Fractions were collected based on deduction of main peak from analytical HPLC and confirmed by ESI-MS. Fractions of multiple runs were lyophilised to afford the intermediate pure peptide of **2s** as a white amorphous powder (120 mg, 22.5% yield, 99.1% purity).

For the synthesis of analogues **2a-d,** peptide **2s** was split into portions as described below for CLipPA reactions.



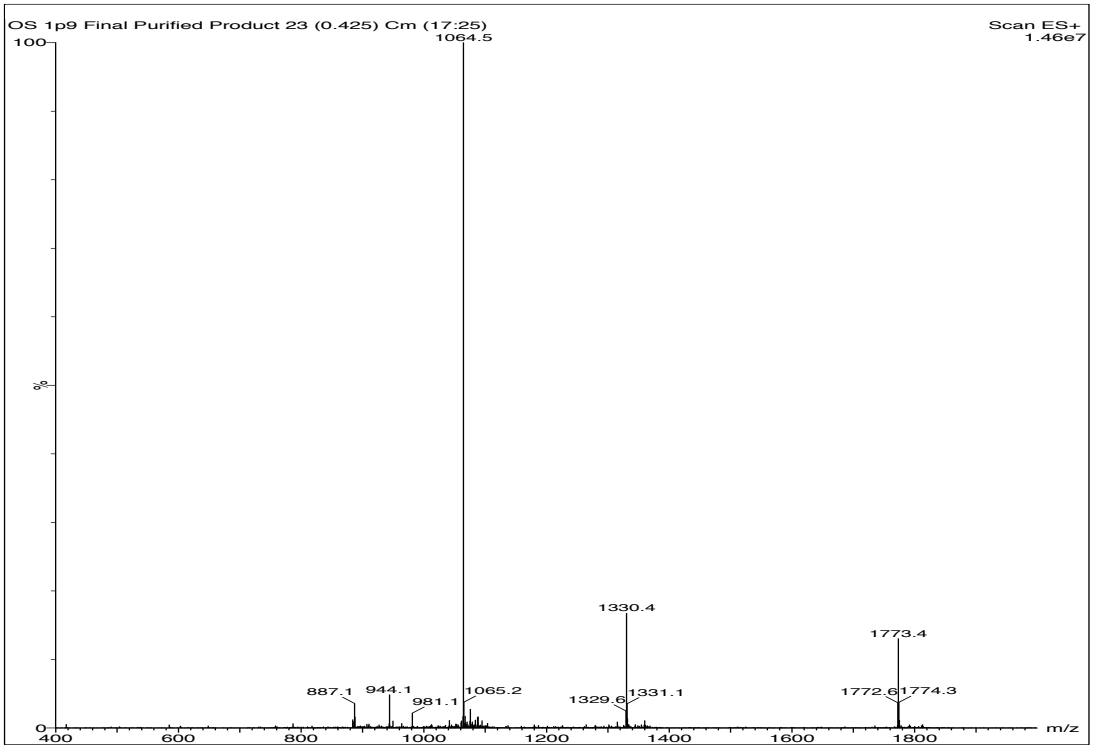
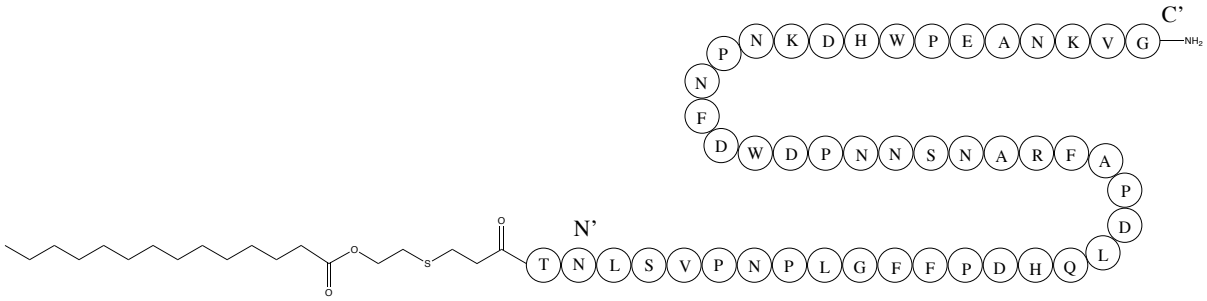


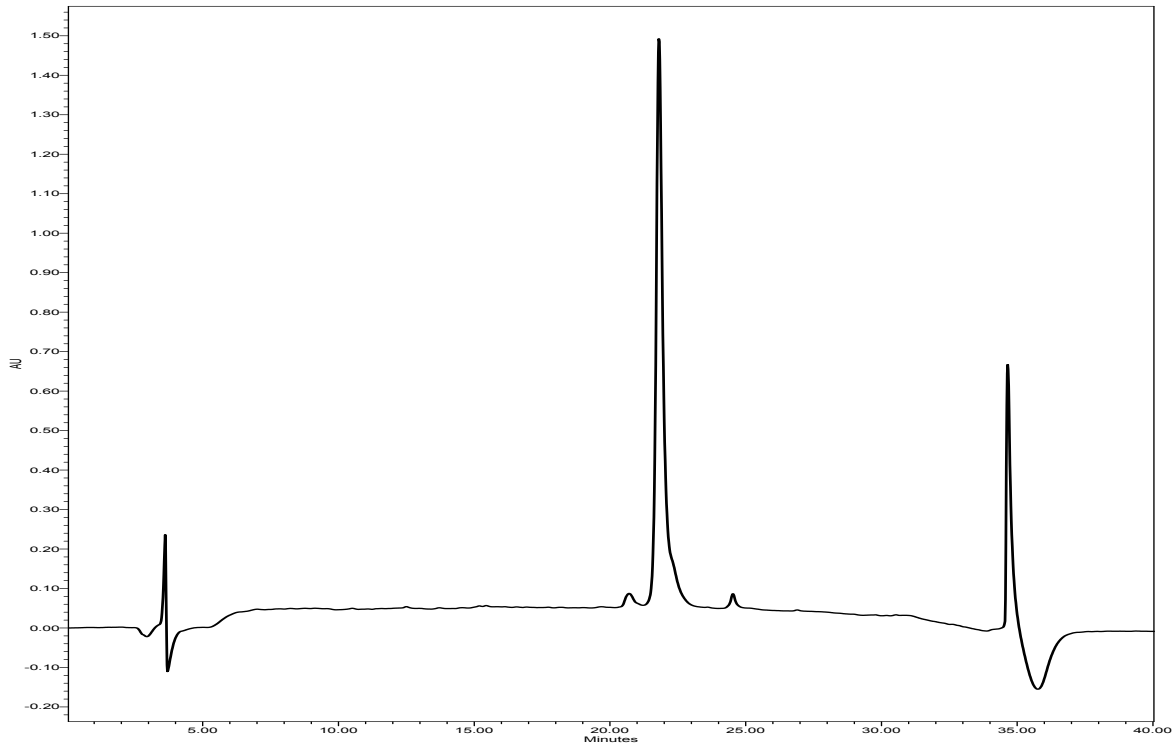
Figure S5.3: (upper) analytical **RP-HPLC** trace of pure peptide Myrcludex B, **2s** (ca. 99.1% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 75%B over 35 min (ca. 2%B/min) at 1 ml/min. t_R = 20.2 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5317.48, observed: 5317.40 ± 0.72.

S5.4. Synthesis of Mycludex B CLipPA analogue 2a



In two separate batches, peptide intermediate **2s** (13 mg, 2.44 µmol, 1 equiv.) was treated with vinyl myristate, **12a** (22 µL, 85.4 µmol, 35 equiv.), using **Method 8** to afford a total of 22 mg of crude peptide **2b** at approximately 70% purity.

The crude peptide **2b** was dissolved in 1:3 MeCN/MQ H_2O at a concentration of 10 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC on a Waters 1525 HPLC using a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 250 x 10 mm). The gradient employed was as follows; 25%B to 65%B over 35 min (ca. 1.1%B/min) at a flow rate of 5 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed with ESI-MS. Fractions of multiple runs were lyophilised to afford *title compound* **2a** as a white amorphous powder (10.2 mg, 37% yield, 97.5% purity).



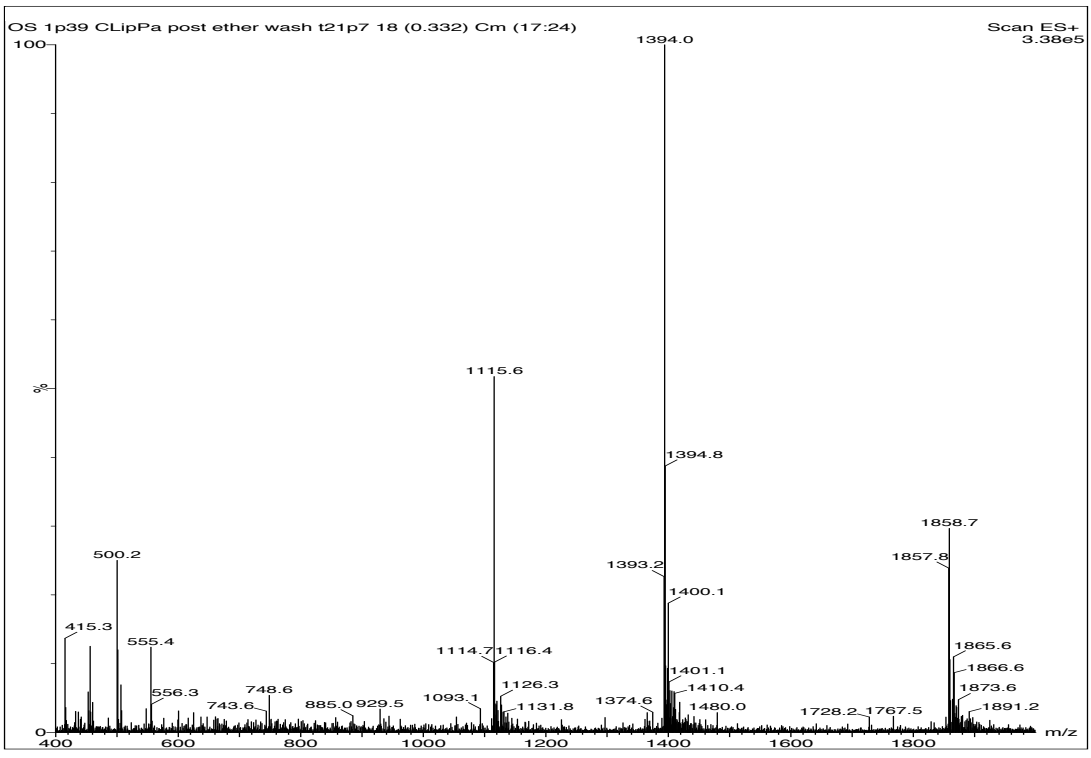
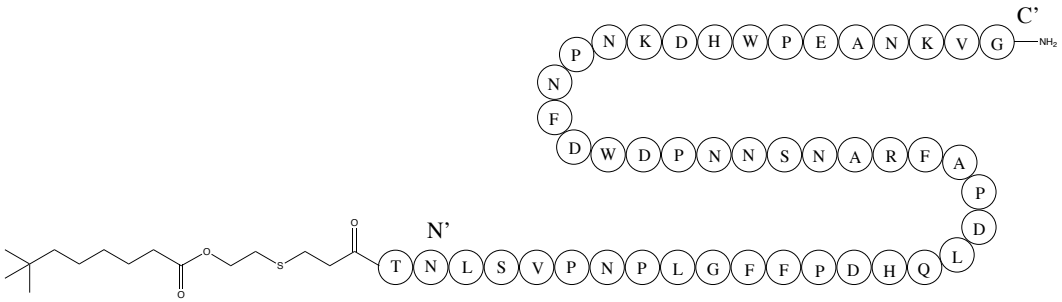


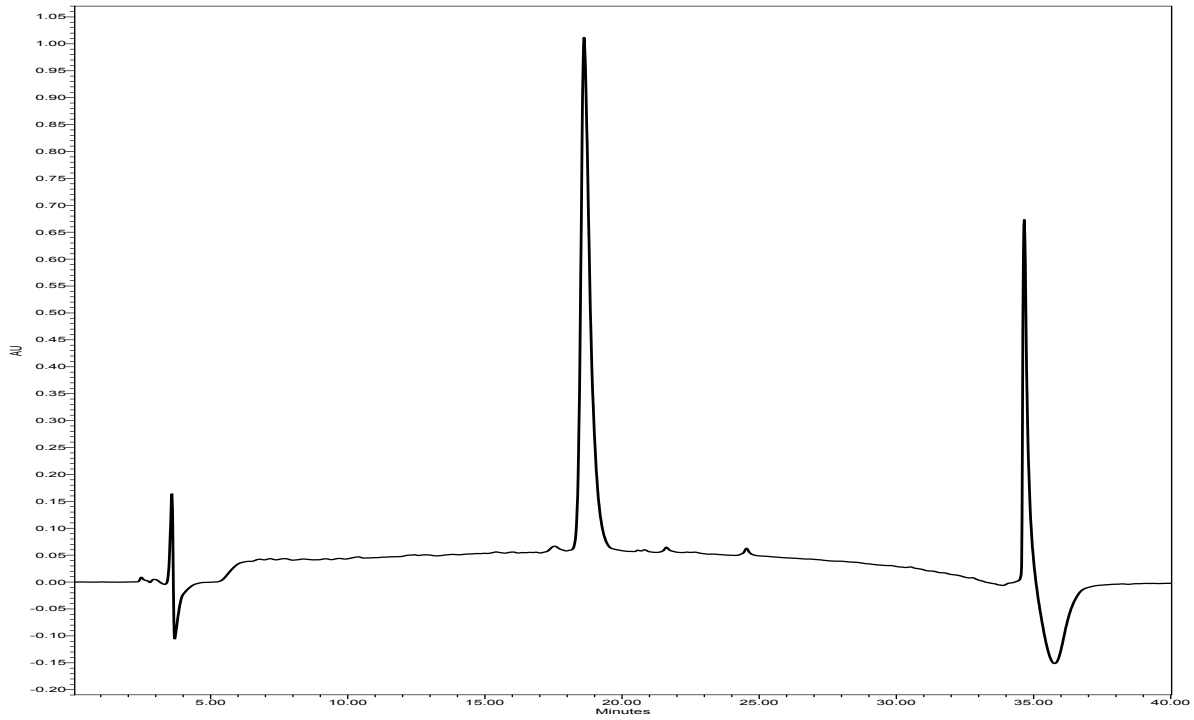
Figure S5.4: (upper) analytical **RP-HPLC** trace of pure CLipPA peptide, **2a** (ca. 97.5% as analysed by peak area at 214 nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 30 min (ca. 3%B/min) at 1 ml/min. t_R = 21.8 min (Note: peak at 24.4 min is column impurity as was also present in blank run previous to sample); (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5571.70, observed: 5572.70 ± 0.61.

S5.5. Synthesis of Mycludex B CLipPA analogue 2b



In two separate batches, peptide intermediate **2s** (13 mg, 2.44 µmol, 1 equiv.) was treated with vinyl neodecanoate, **12b** (17 µL, 85.4 µmol, 35 equiv.), using **Method 8** to afford a total of 12 mg of crude peptide **2b** at approximately 75% purity.

The crude peptide **2b** was dissolved in 1:3 MeCN/MQ H_2O at a concentration of 10 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC on a Waters 1525 HPLC using a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 250 x 10 mm). The gradient employed was as follows; 25%B to 65%B over 35 min (ca. 1.1%B/min) at a flow rate of 5 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed with ESI-MS. Fractions of multiple runs were lyophilised to afford *title compound* **2b** as a white amorphous powder (5 mg, 19% yield, 99% purity).



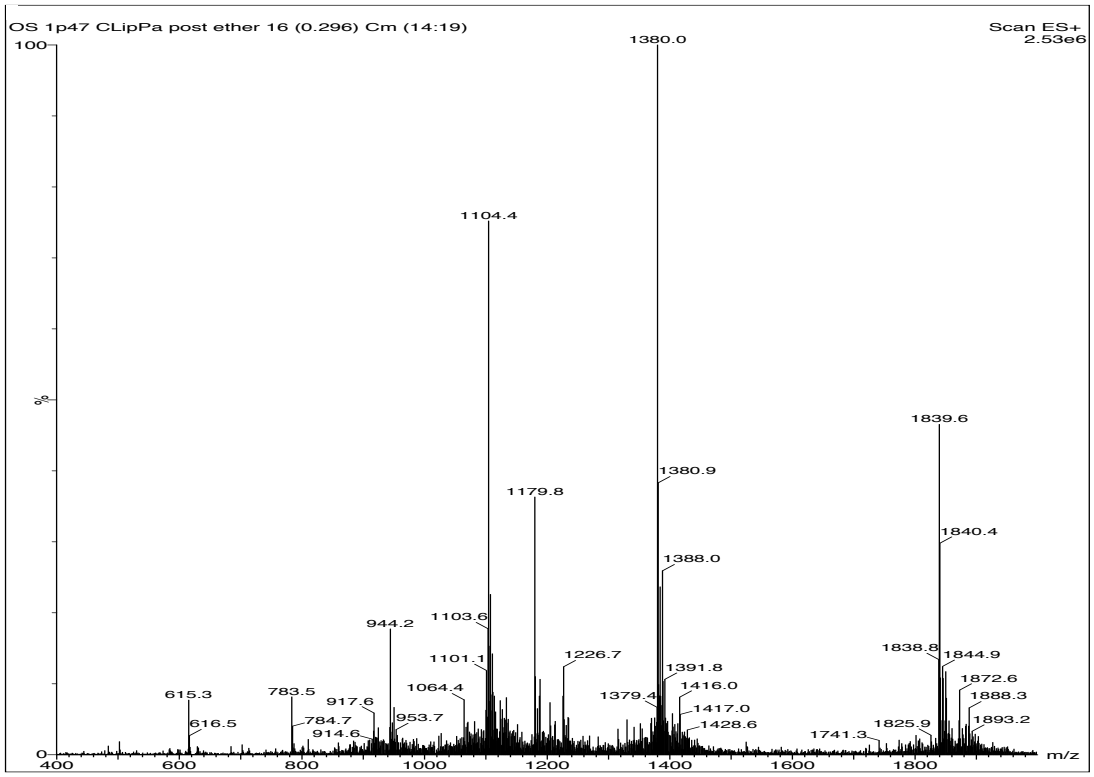
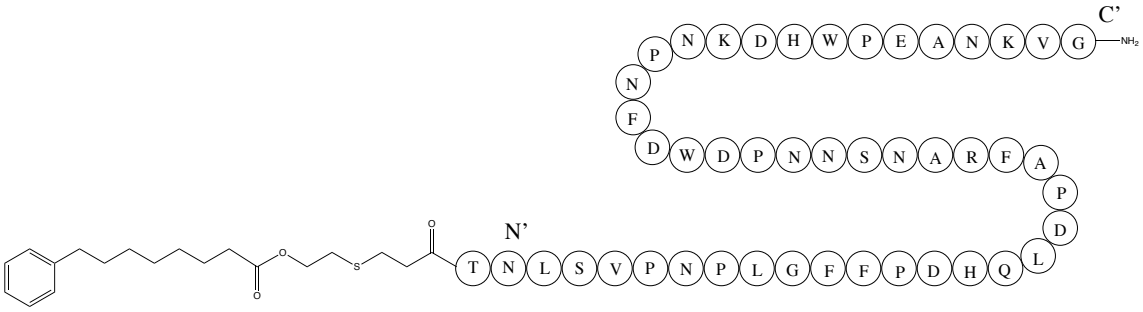


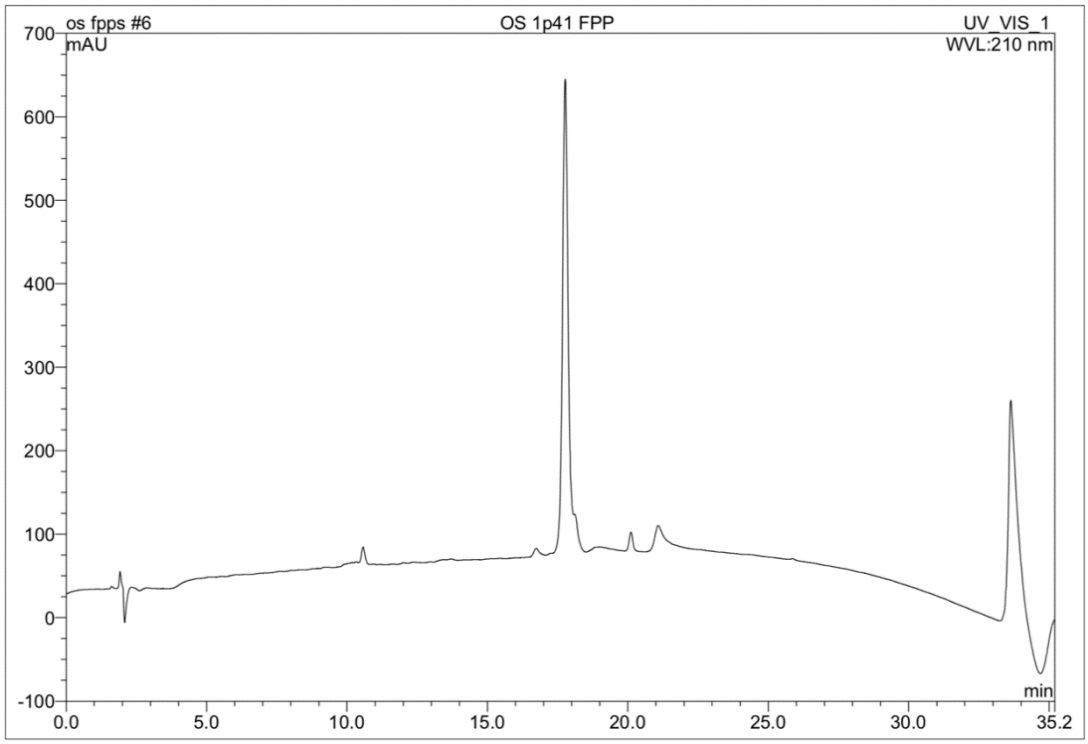
Figure S5.5; (upper) analytical **RP-HPLC** trace of pure CLipPA peptide, **2b** (ca. 99.0% as analysed by peak area at 214 nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 30 min (ca. 3%B/min) at 1 ml/min. t_R = 18.6 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5515.64, observed: 5516.27 ± 0.64.

S5.6. Synthesis of Mycludex B CLipPA analogue 2c



In two separate batches, peptide intermediate **2s** (13 mg, 2.44 µmol, 1 equiv.) was treated with vinyl 8-phenyloctanoate, **12c** (22.1 µL, 85.4 µmol, 35 equiv.), using **Method 8** to afford a total of 16 mg of crude lipopeptide **2c** at approximately 74% purity.

The crude lipopeptide **2c** was dissolved in 1:3 MeCN/MQ H_2O at a concentration of 10 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC on a Waters 1525 HPLC using a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 250 x 10 mm). The gradient employed was as follows; 25%B to 65%B over 35 min (ca. 1.1%B/min) at a flow rate of 5 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed with ESI-MS. Fractions of multiple runs were lyophilised to afford *title compound* **2c** as a white amorphous powder (6 mg, 22%, 95% purity).



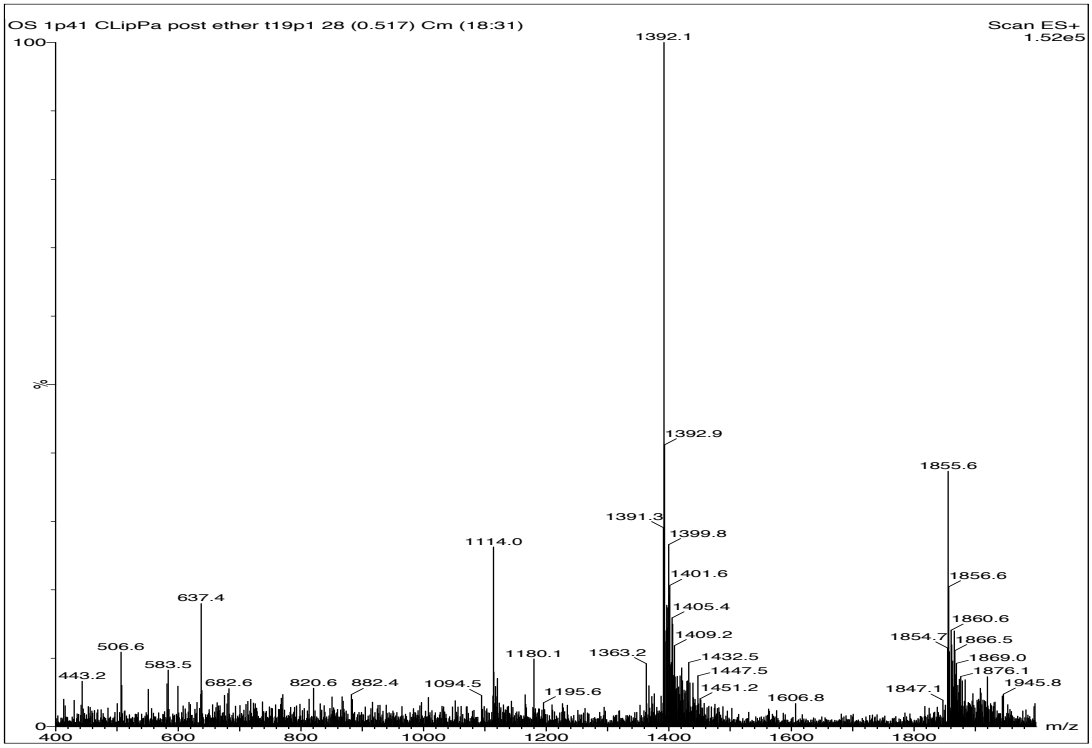
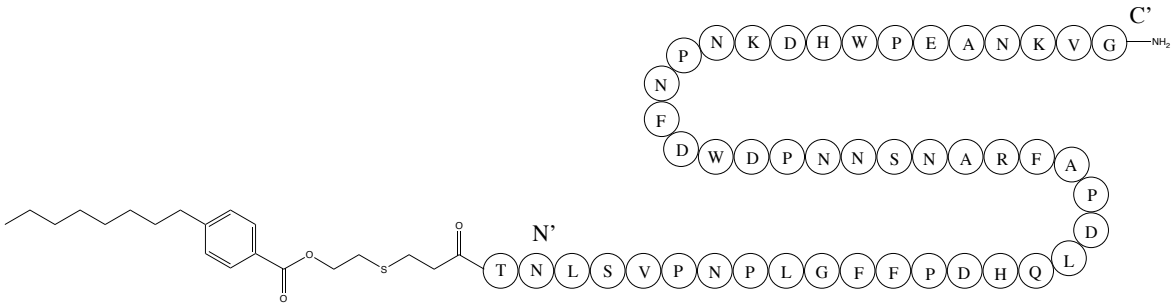


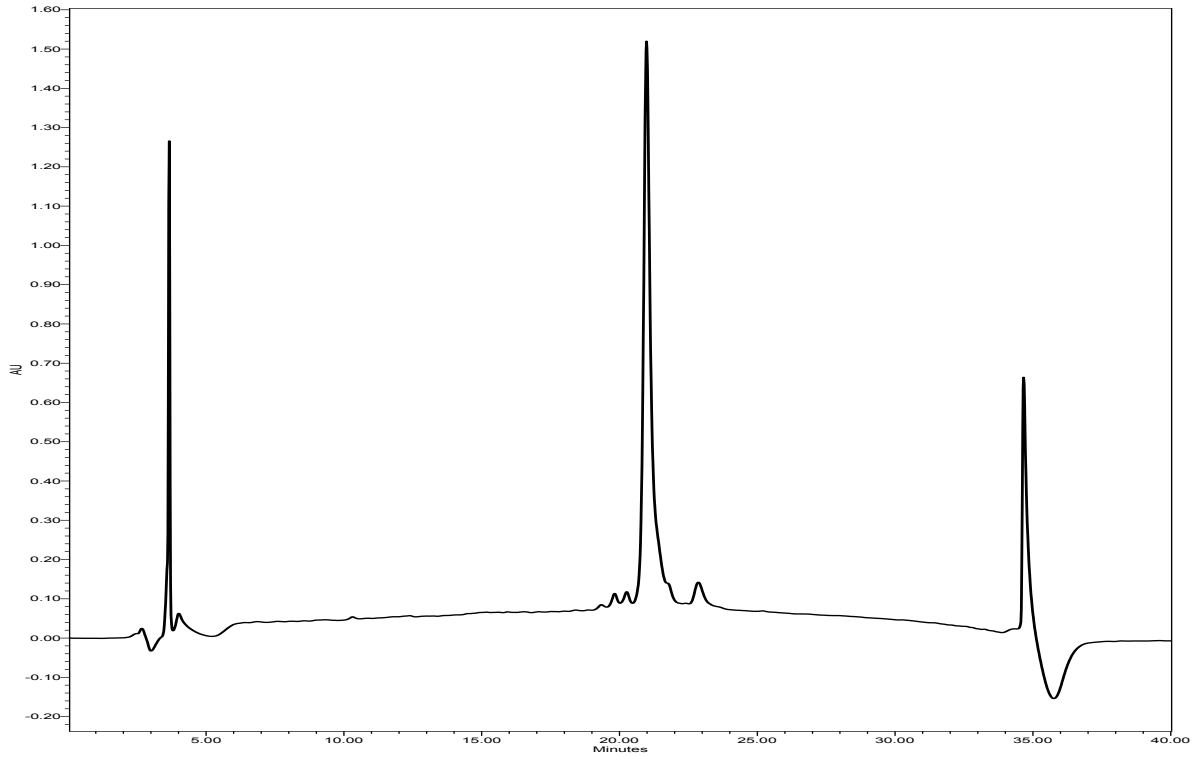
Figure S5.6: (upper) analytical **RP-HPLC** trace of pure CLipPA peptide, **2c** (ca. 95% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 30 min (ca. 3%B/min) at 1 ml/min. t_R = 17.9. (Note: peak at 21.1 min is column impurity as was also seen in blank run previous to sample); (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5563.64, observed: 5564.40 ± 0.60.

S5.7. Synthesis of Mycludex B CLipPA analogue 2d



In two separate batches, peptide intermediate **2s** (13 mg, 2.44 µmol, 1 equiv.) was treated with vinyl 4-octylbenzoate, **12d** (23.1 µL, 85.4 µmol, 35 equiv.), using **Method 8** to afford a total of 14 mg of crude peptide **2d** at approximately 66% purity.

The crude peptide **2d** was dissolved in 1:3 MeCN/MQ H_2O at a concentration of 10 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC on a Waters 1525 HPLC using a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 250 x 10 mm). The gradient employed was as follows; 25%B to 65%B over 35 min (ca. 1.1%B/min) at a flow rate of 5 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed with ESI-MS. Fractions of multiple runs were lyophilised to afford *title compound* **2d** as a white amorphous powder (4.96 mg, 18%, 95.8% purity).



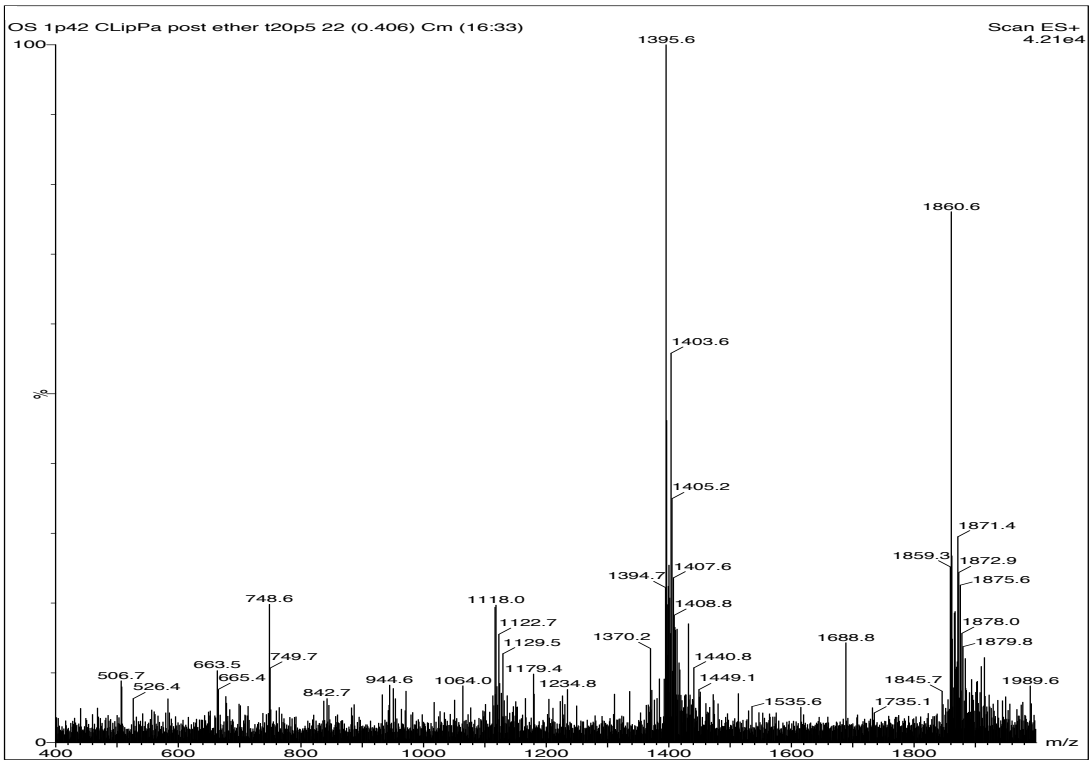
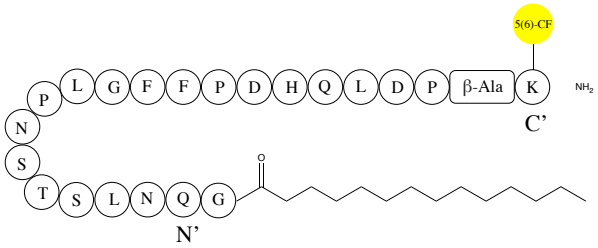


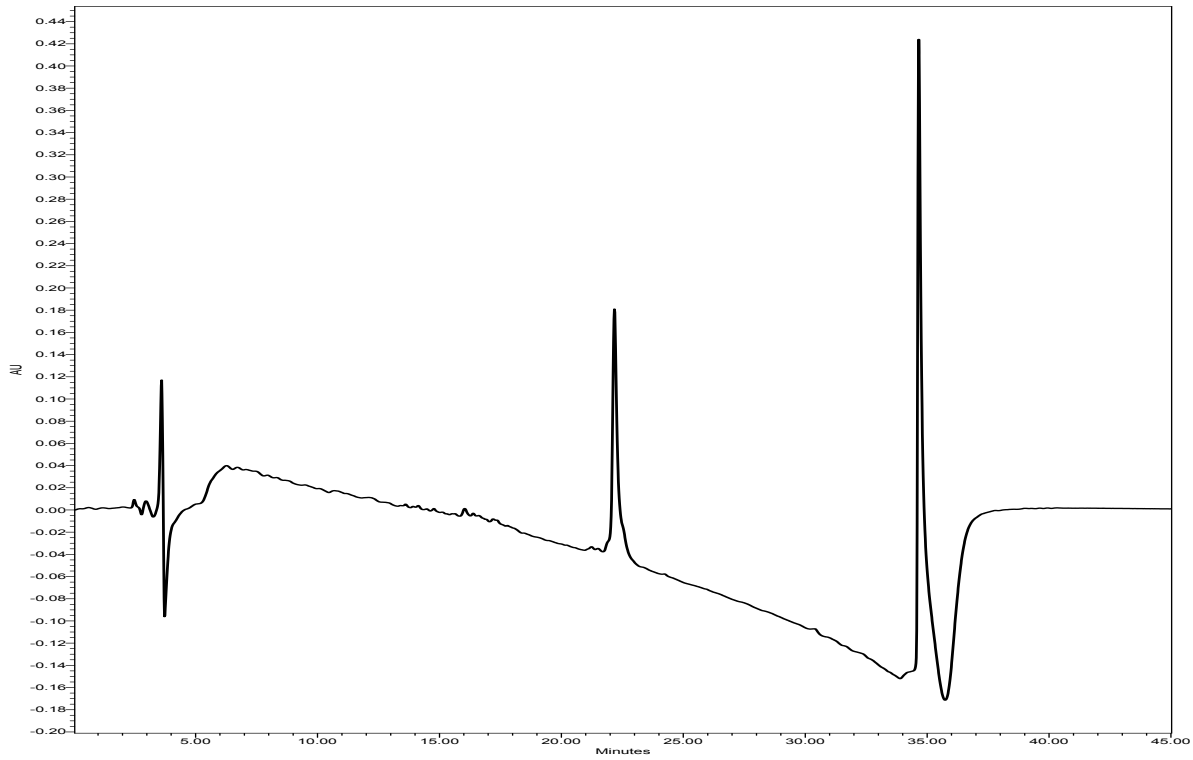
Figure S5.7: (upper) analytical **RP-HPLC** trace of pure CLipPA peptide, **2d** (ca. 95.8% as analysed by peak area at 214 nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 30 min (ca. 3%B/min) at 1 ml/min. t_R = 20.9 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5577.65, observed: 5580.73 ± 3.70.

S5.8. Synthesis of fluorescently tagged truncated Myrcludex B analogue 3



The truncated parent peptide was assembled by automated Fmoc SPPS using the PS3 Peptide Synthesizer as described in **Method 1.** The final myristic acid was coupled using **Method 3**. The Dde protecting group was removed as described in **Method 4** and 5(6)-CF attached as described in **Method 5.** The final cleavage of the peptide from the resin was performed using **Method 6**, decreased to a scale of 0.05 mmol to afford 94.2 mg of crude peptide at approximately 90% purity.

The crude truncated peptide analogue 3 was dissolved in 1:1 MeCN/MQ H_2O at a concentration of 10 mg/ml and purified by RP-HPLC with a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 300 x 19 mm), employing a gradient of 45.0%B to 65.0%B over 28 min (ca. 0.7% B/min) at a flow rate of 10 ml/min. Fractions were collected based on deduction of main peak from analytical HPLC and confirmed by ESI-MS. Fractions from multiple runs were combined and lyophilised to afford *title compound* **3** as a yellow amorphous powder (52.3 mg, 35% yield, 96.1% purity).



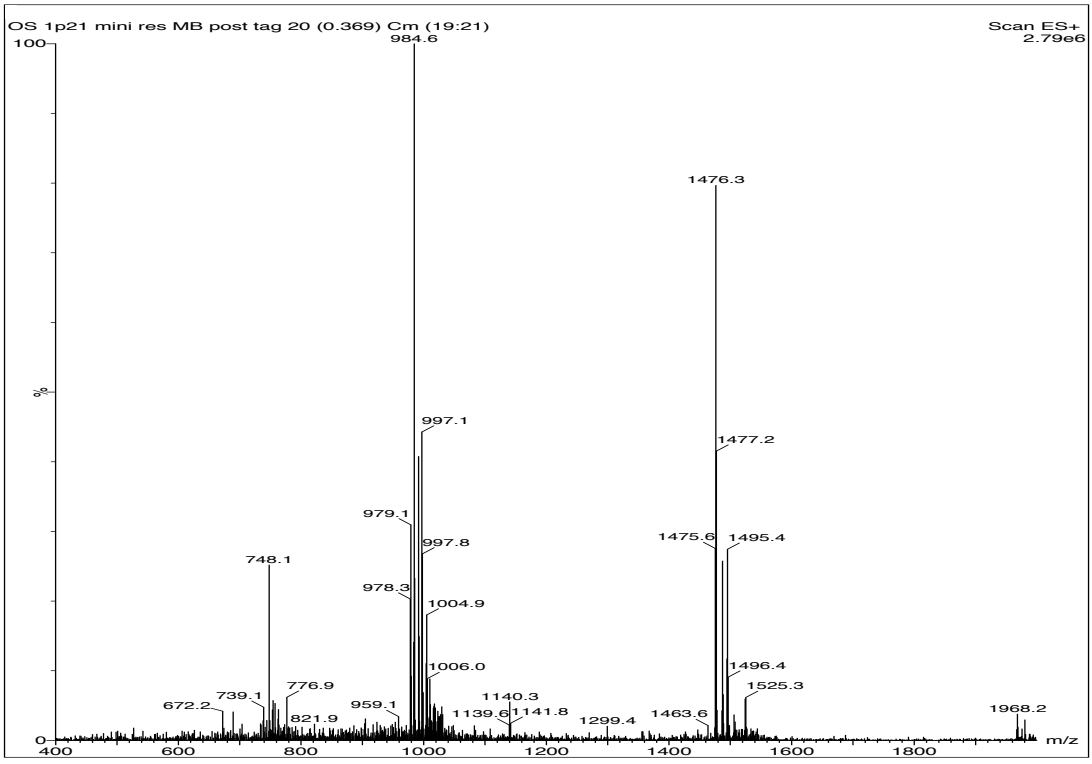
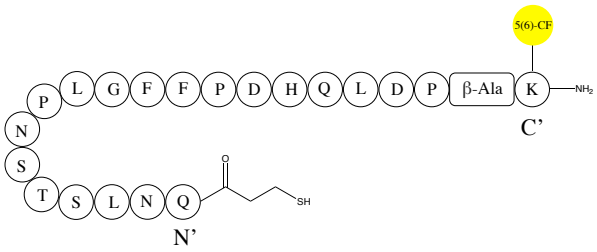


Figure S5.8: (upper) analytical **RP-HPLC** trace of pure fluorescently labelled native peptide peptide, **3** (ca. 96.1% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 35 min (ca. 3%B/min) at 1 ml/min. t_R = 22.2 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 2950.43, observed: 2950.80 $\pm$ 0.14.

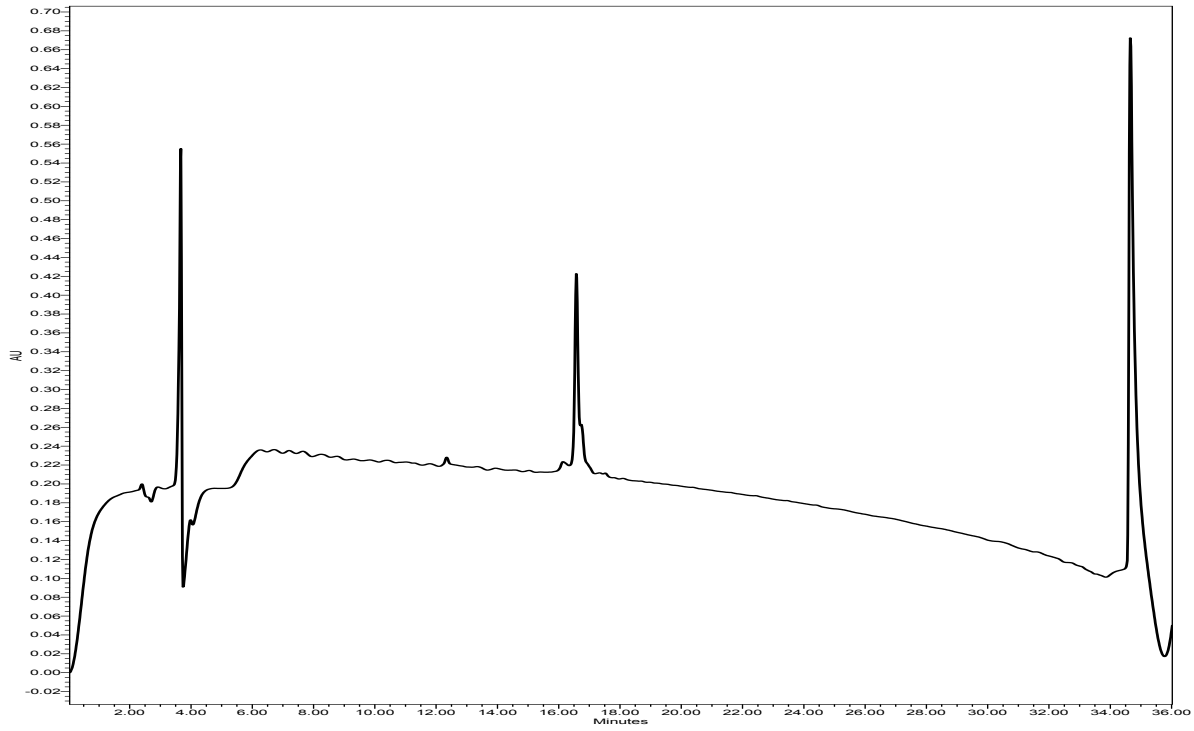
S5.9. Synthesis of fluorescently tagged truncated Mycludex B CLipPA intermediate 3s



The truncated peptide was assembled by automated Fmoc SPPS using the PS3 Peptide Synthesizer as described in **Method 1.** The final mercaptopropionic acid was coupled as described by **Method 2**. The Dde protecting group was removed as described in **Method 4** and 5(6)-carboxyfluoroscein attached as described in **Method 5.** The final cleavage of the peptide from the resin was performed as described by **Method 6** to afford 270 mg of crude peptide at approximately 76% purity.

The crude peptide intermediate **3s** was dissolved in 1:2 MeCN/MQ H_2O at a concentration of 10 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC with a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 300 x 19 mm). The gradient employed was as follows; 25.0%B to 60%B over 30 min (ca. 1.2%B/min) at a flow rate of 10 ml/min. Fractions were collected based on deduction of main peak from analytical HPLC and confirmed by ESI-MS. Fractions of multiple runs were lyophilised to afford the intermediate pure peptide of **3s** as a yellow amorphous powder (102 mg, 12% yield, 96.5% purity).

For the synthesis of analogues **3a-d,** peptide **3s** was split into portions as described below for CLipPA reactions.



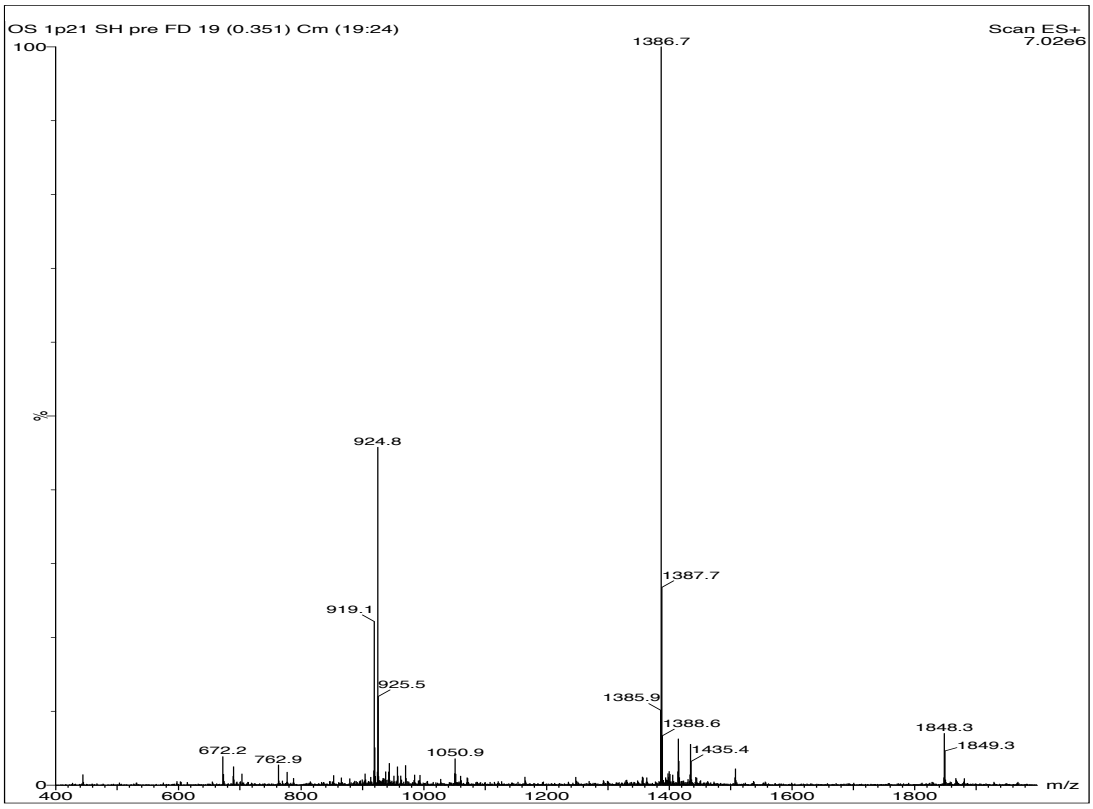
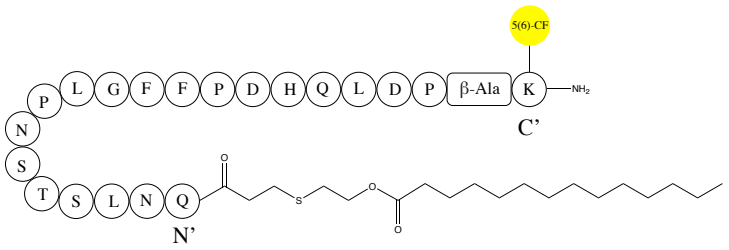


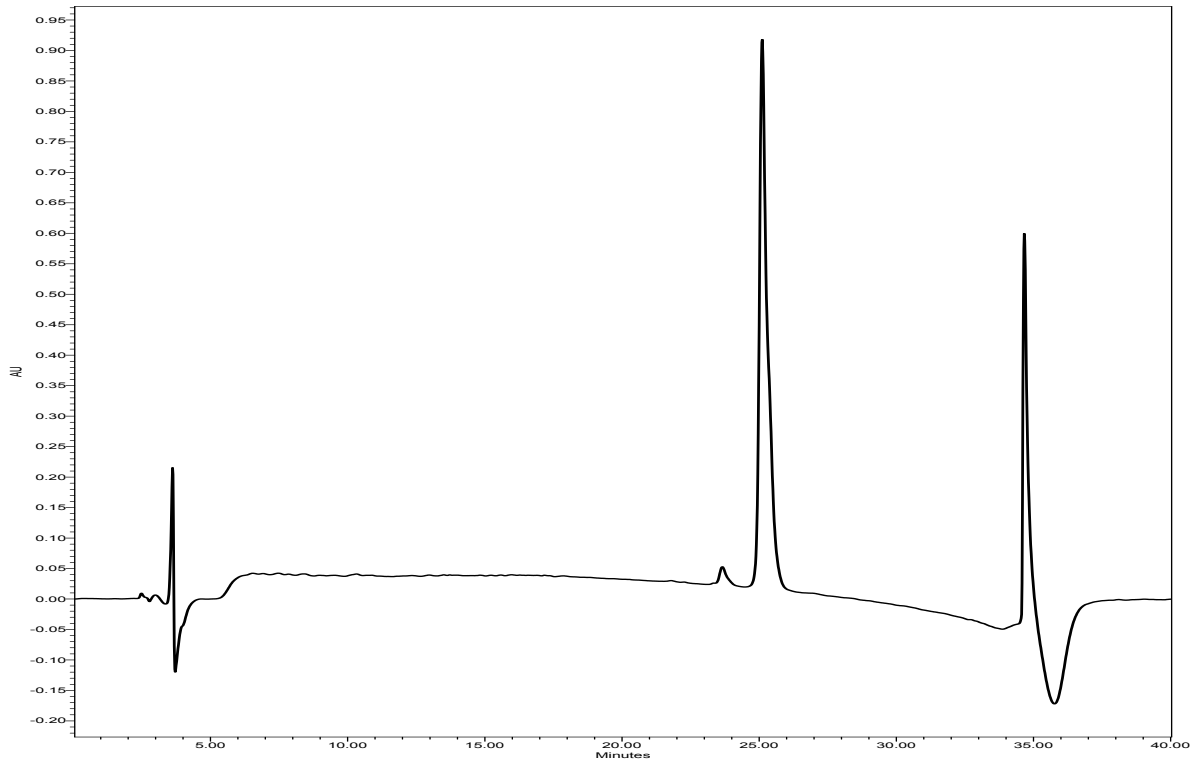
Figure S5.9: (upper) analytical **RP-HPLC** trace of pure peptide Myrcludex B, **3s** (ca. 96.5% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 75%B over 35 min (ca. 2%B/min) at 1 ml/min. t_R = 16.5 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 2773.2, observed: 2771.4 ± 0.0.

S5.10. Synthesis of fluorescently tagged truncated Mycludex B CLipPA analogue 3a



In two separate batches, peptide intermediate, **3s** (5.55 mg, 4 µmol, 1 equiv.), was treated with vinyl myristate, **12a** (35.6 µL, 140 µmol, 35 equiv.), using **Method 8** to afford a total of 9.5 mg of crude peptide **3a** at approximately 62% purity.

The crude peptide **3a** was dissolved in 1:1 MeCN/MQ H_2O at a concentration of 5 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC on a Waters 1525 HPLC using a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 250 x 10 mm). The gradient employed was as follows; 45%B to 75%B over 25 min (ca. 1.2%B/min) at a flow rate of 5 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed with ESI-MS. Fractions of multiple runs were lyophilised to afford *title compound* **3a** as a yellow amorphous powder (0.75 mg, 6% yield, 97.7% purity).



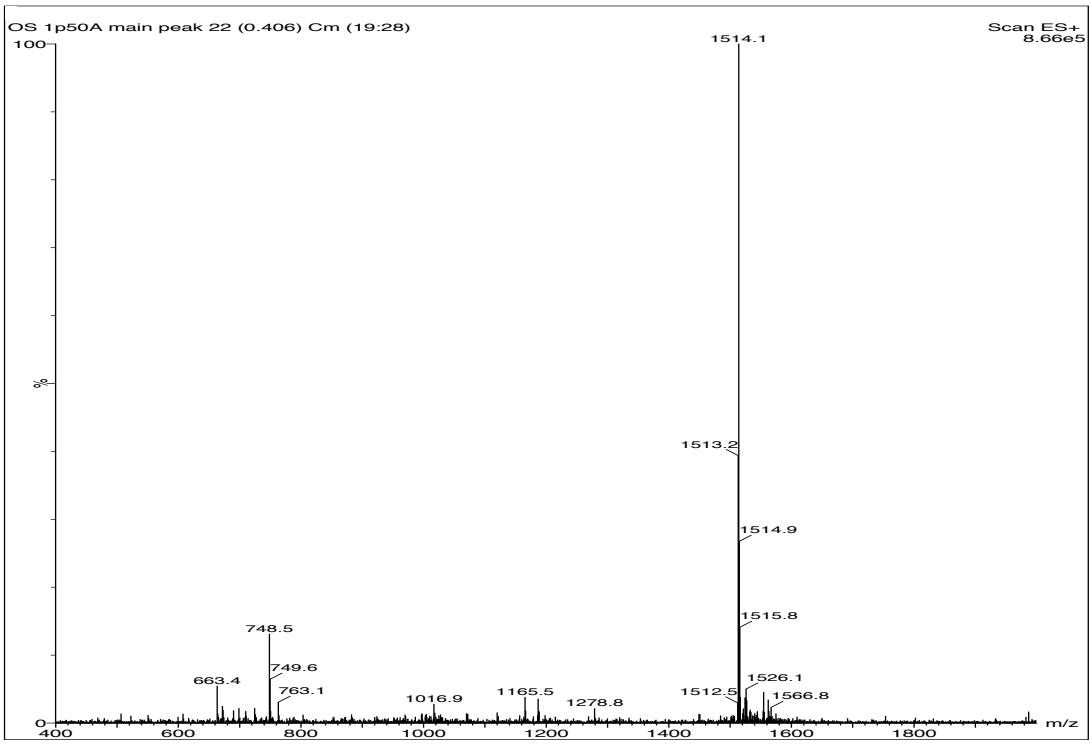
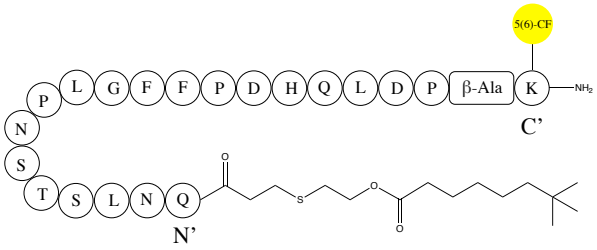


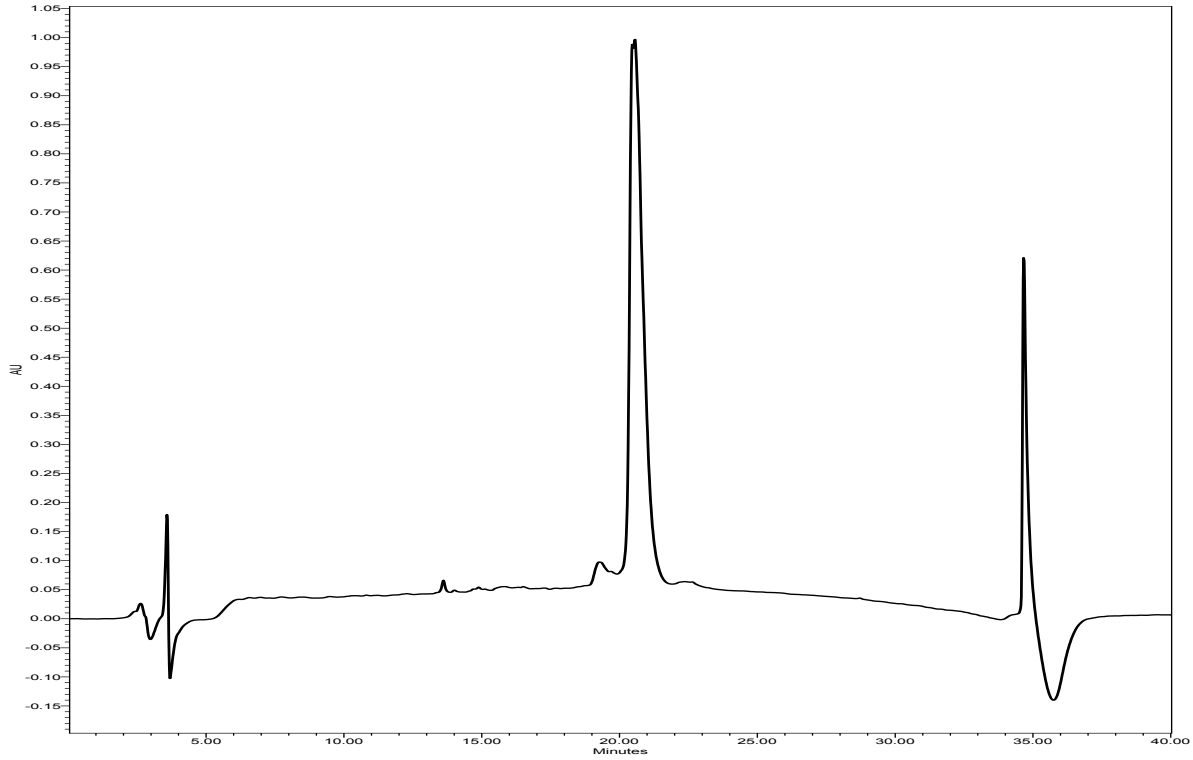
Figure S5.10: (upper) analytical **RP-HPLC** trace of fluorescently labelled pure truncated CLipPA peptide, **3a** (ca. 97.7% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 35 min (ca. 3%B/min) at 1 ml/min. t_R = 25.1 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 3027.44, observed: 3026.40 ± 0.14.

S5.11. Synthesis of fluorescently tagged truncated Mycludex B CLipPA analogue 3b



In two separate batches, peptide intermediate **3s** (5.55 mg, 4 µmol, 1 equiv.) was treated with vinyl neodecanoate, **12b** (27.8 µL, 140 µmol, 35 equiv.), using **Method 8** to afford a total of 9.3 mg of crude peptide **3b** at approximately 70% purity.

The crude peptide **3b** was dissolved in 1:2 MeCN/MQ H_2O at a concentration of 10 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC on a Waters 1525 HPLC using a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 250 x 10 mm). The gradient employed was as follows; 25%B to 65%B over 35 min (ca. 1.1%B/min) at a flow rate of 5 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed with ESI-MS. Fractions of multiple runs were lyophilised to afford *title compound* **3b** as a yellow amorphous powder (1.91 mg, 17% yield, 97.5% purity).



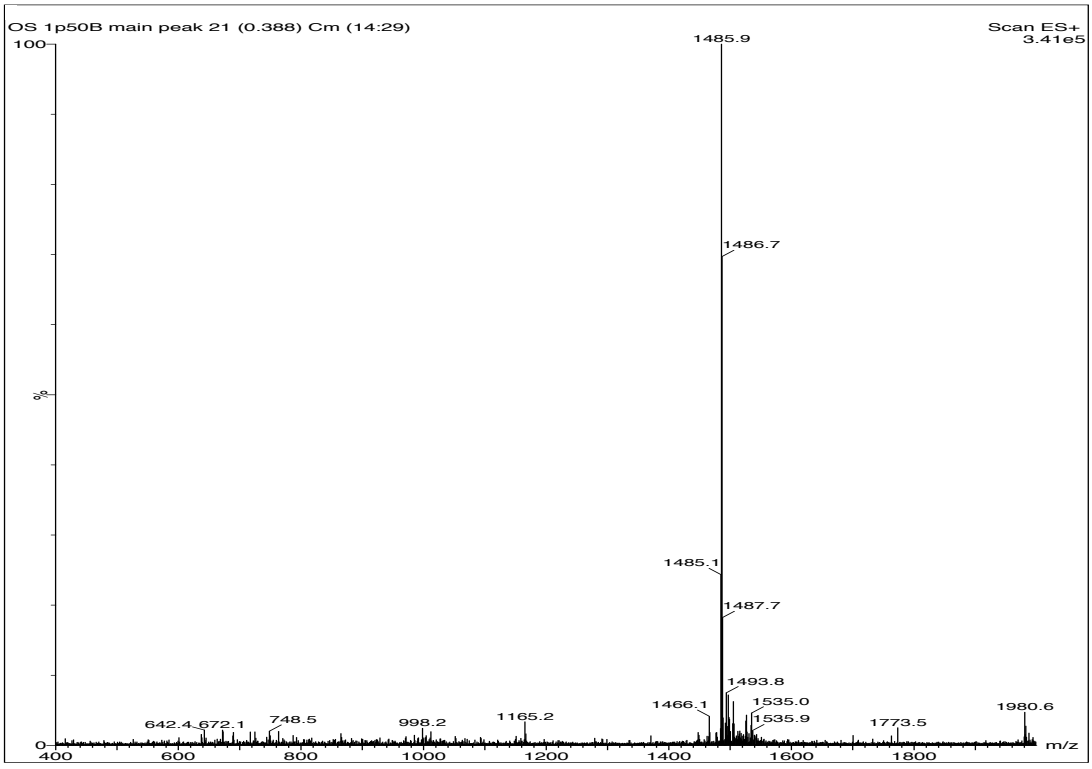
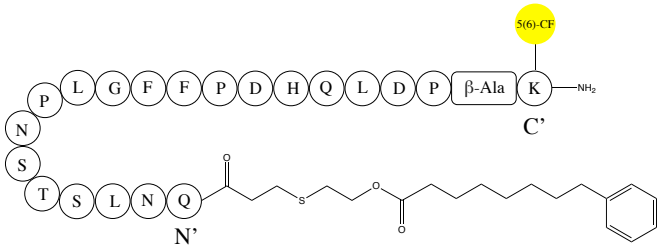


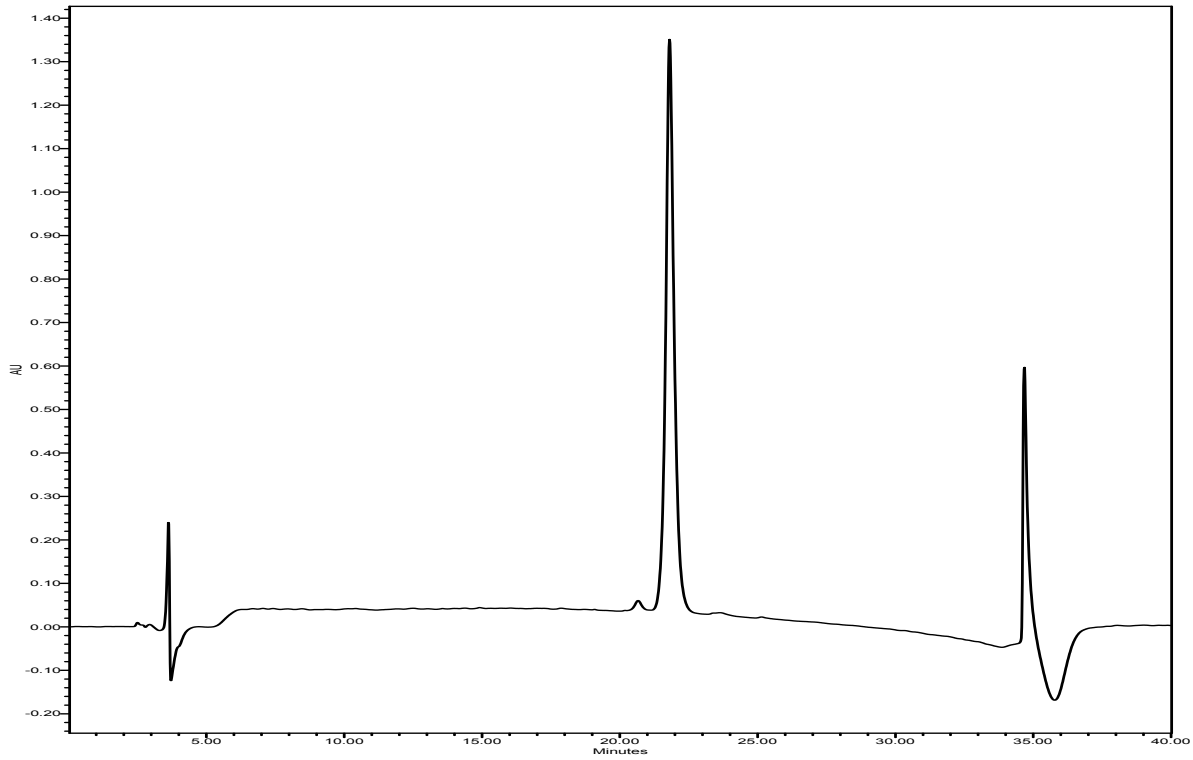
Figure S5.11: (upper) analytical **RP-HPLC** trace of fluorescently labelled pure truncated CLipPA peptide, **3b** (ca. 97.5% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 35 min (ca. 3%B/min) at 1 ml/min. t_R = 20.6 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 2971.38, observed: 2970.20.

S5.12. Synthesis of fluorescently tagged truncated Mycludex B CLipPA analogue 3c



In two separate batches, peptide intermediate **3s** (5.55 mg, 4 μmol, 1 equiv.) was treated with vinyl 8-phenyoctanoate, **12c** (34.5 μL, 140 μmol, 35 equiv.), using **Method 8** to afford a total of 7.4 mg of crude peptide **3c** at approximately 45% purity.

The crude peptide **3c** was dissolved in 1:2 MeCN/MQ H_2O at a concentration of 10 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC on a Waters 1525 HPLC using a Waters XTerra Prep MS C18 OBD column (125 Å, 10 μm, 250 x 10 mm). The gradient employed was as follows; 25%B to 65%B over 35 min (ca. 1.1%B/min) at a flow rate of 5 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed with ESI-MS. Fractions of multiple runs were lyophilised to afford *title compound* **3c** as a yellow amorphous powder (1.25 mg, 10% yield, 97.7% purity).



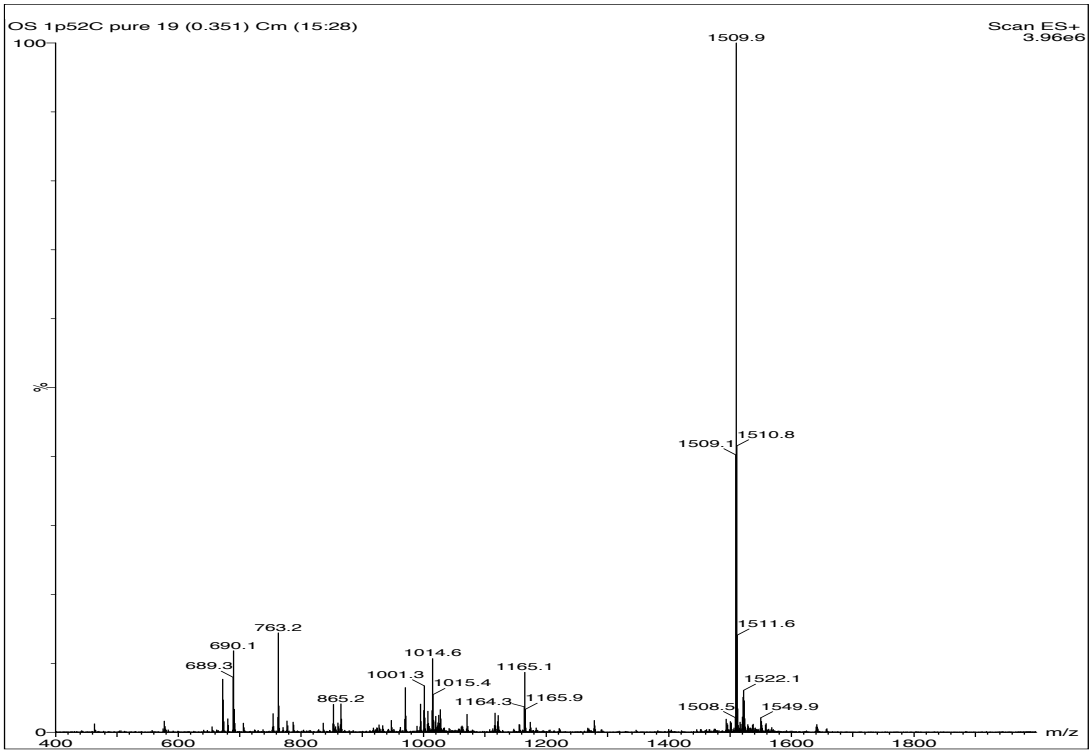
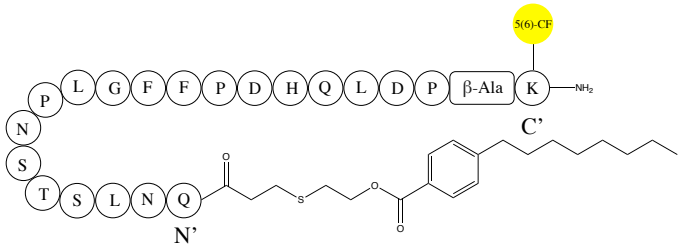


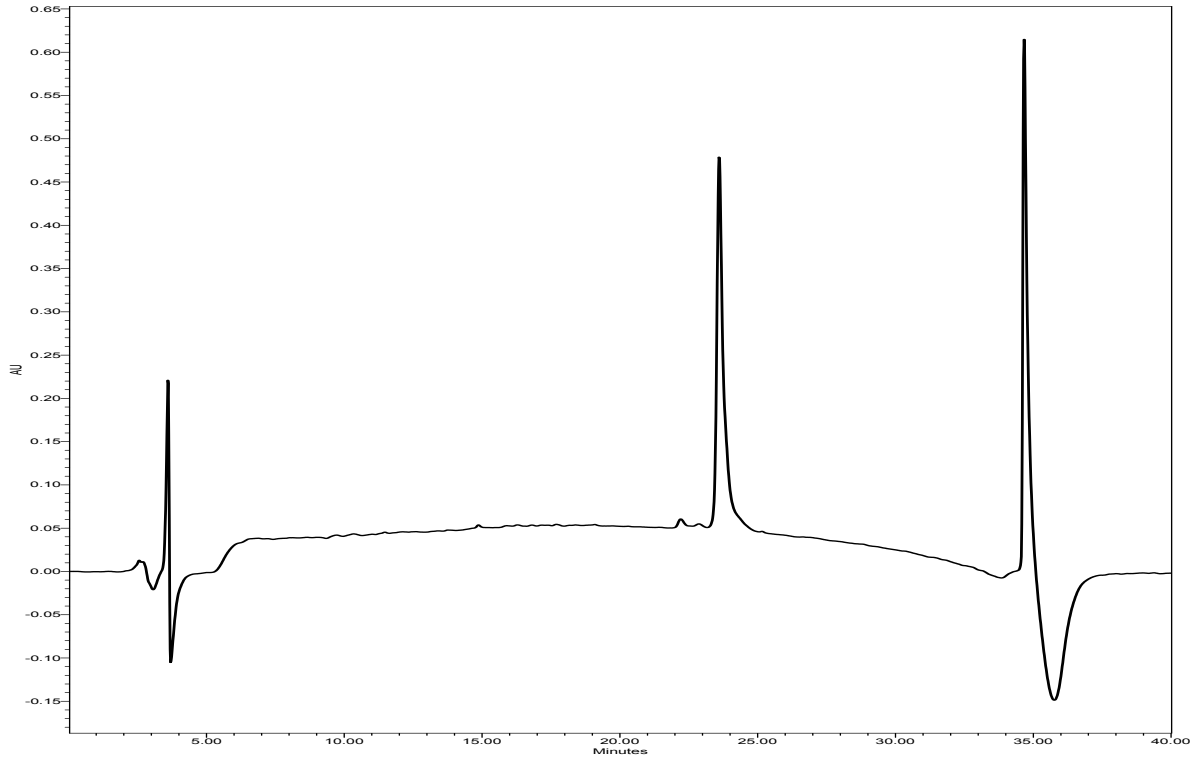
Figure S5.12: (upper) analytical **RP-HPLC** trace of fluorescently labelled pure truncated CLipPA peptide, **3c** (ca. 97.7% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 35 min (ca. 3%B/min) at 1 ml/min. t_R = 21.4 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 3019.38, observed: 3017.80.

S5.13. Synthesis of fluorescently tagged truncated Mycludex B CLipPA analogue 3d



In two separate batches, peptide intermediate **3d** (5.55 mg, 4 µmol, 1 equiv.) was treated with vinyl 4-octylbenzoate, **12d** (36.4 µL, 140 µmol, 35 equiv.), using **Method 8** to afford a total of 6.9 mg of crude peptide **3d** at approximately 77% purity.

The crude peptide **3d** was dissolved in 1:1 MeCN/MQ H_2O at a concentration of 5 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC on a Waters 1525 HPLC using a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 250 x 10 mm). The gradient employed was as follows; 35%B to 75%B over 35 min (ca. 0.9%B/min) at a flow rate of 5 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed with ESI-MS. Fractions of multiple runs were lyophilised to afford *title compound* **3d** as a yellow amorphous powder (1.25 mg, 10% yield, 98.5% purity).



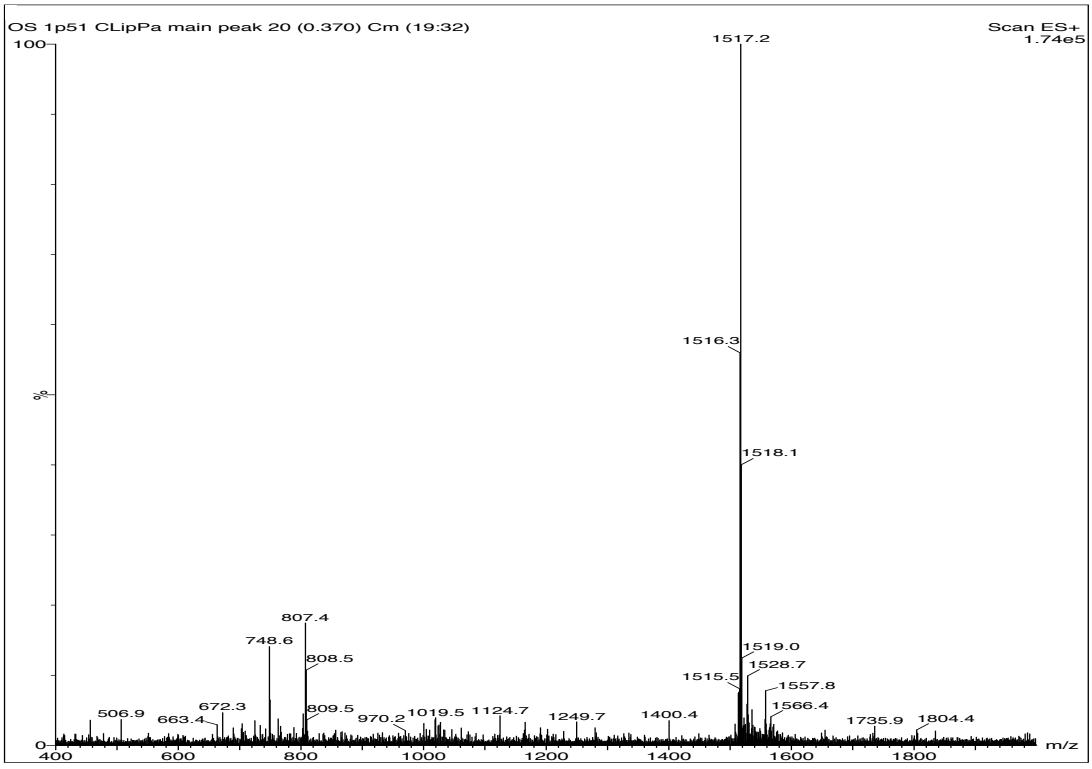
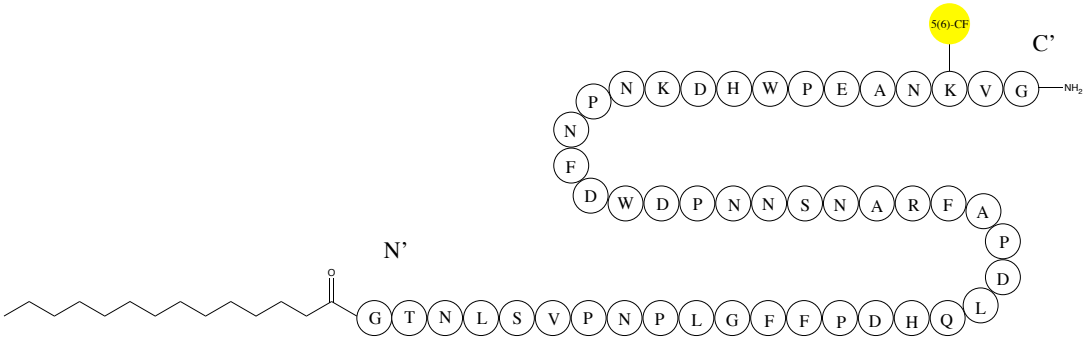


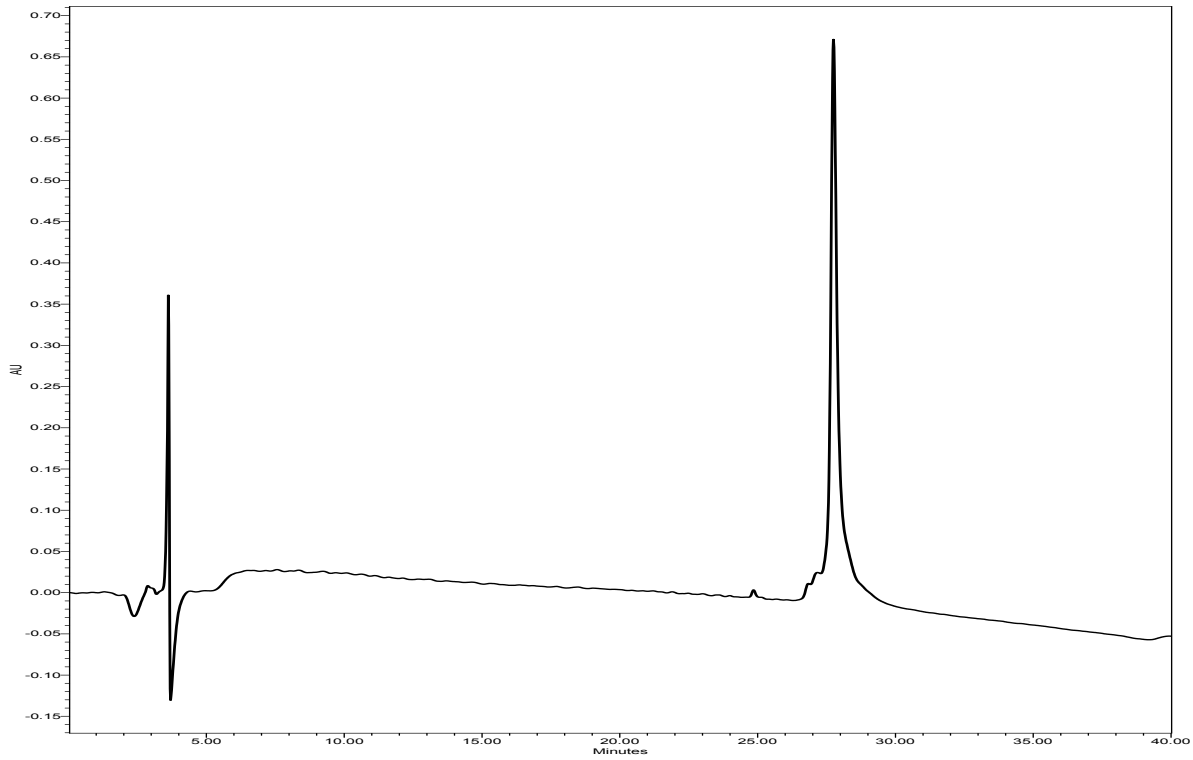
Figure S5.13: (upper) analytical **RP-HPLC** trace of fluorescently labelled pure truncated CLipPA peptide, **3d** (ca. 98.5% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 35 min (ca. 3%B/min) at 1 ml/min. t_R = 23.6 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 3033.40, observed: 3032.40.

S5.14. Synthesis of fluorescently tagged native Myrcludex B analogue 4



The parent peptide was assembled by automated Fmoc SPPS using the PS3 Peptide Synthesizer as described in **Method 1.** The final myristic acid was coupled using **Method 3**. The Dde protecting group was removed as described in **Method 4** and 5(6)-CF attached as described in **Method 5.** The final cleavage of the peptide from the resin was performed using **Method 6** (reduced to a 0.04 mmol scale), to afford 100 mg of crude peptide at approximately 70% purity.

The crude peptide analogue **4** was dissolved in 1:4 MeCN/MQ H_2O at a concentration of 10 mg/ml and purified by RP-HPLC with a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 300 x 19 mm), employing a gradient of 22.5%B to 30%B over 5 min (ca. 1.5% B/min), then 30%B to 70%B over 20 min (ca. 2% B/min) at a flow rate of 10 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed by ESI-MS. Fractions from multiple runs were combined and lyophilised to afford *title compound* **4** as a yellow amorphous powder (34.2 mg, 14% yield, 98.3% purity).



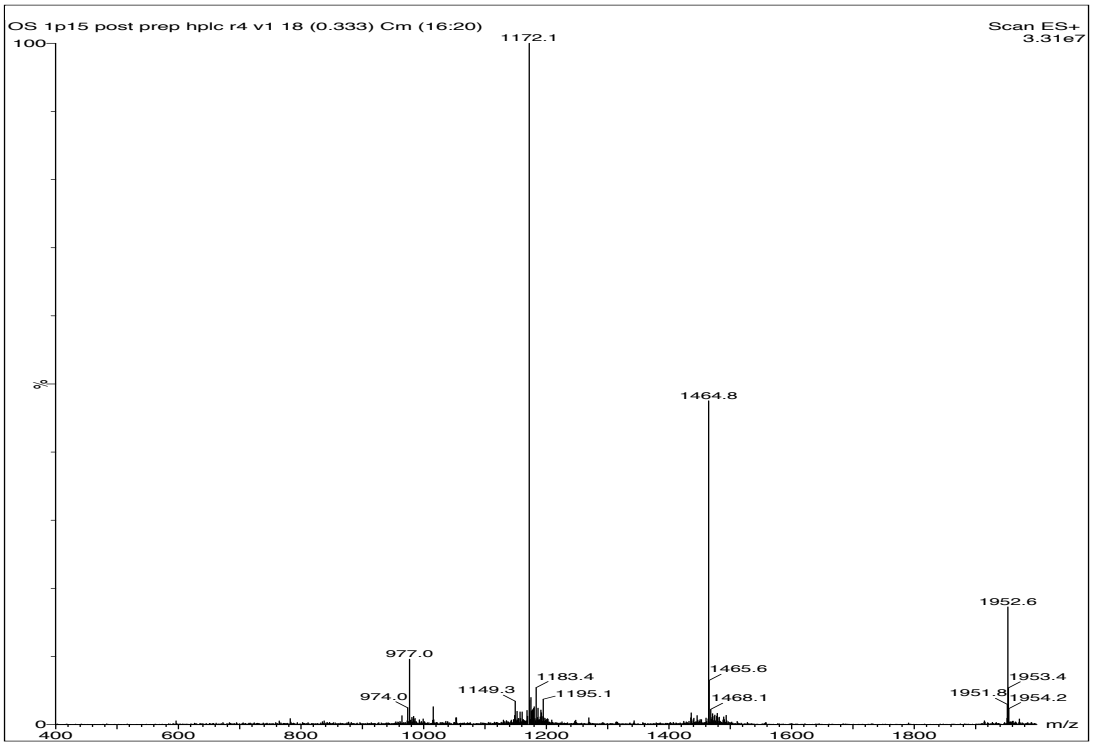
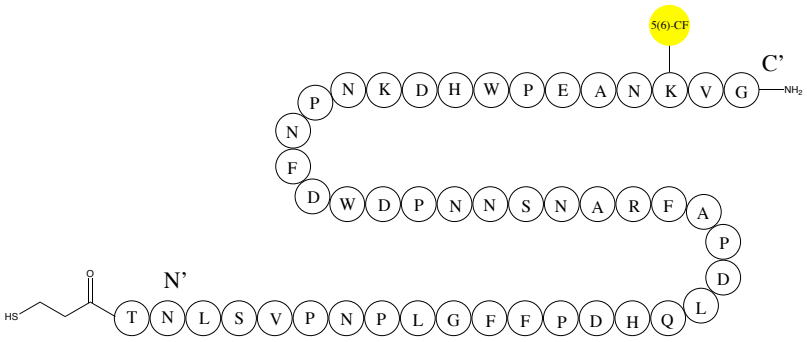


Figure S5.14: (upper) analytical **RP-HPLC** trace of fluorescently tagged pure native peptide Myrcludex B, **4** (ca. 98.3% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 75%B over 35 min (ca. 2%B/min) at 1 ml/min. t_R = 27.7 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5854.75, observed: 5855.38 ± 0.51.

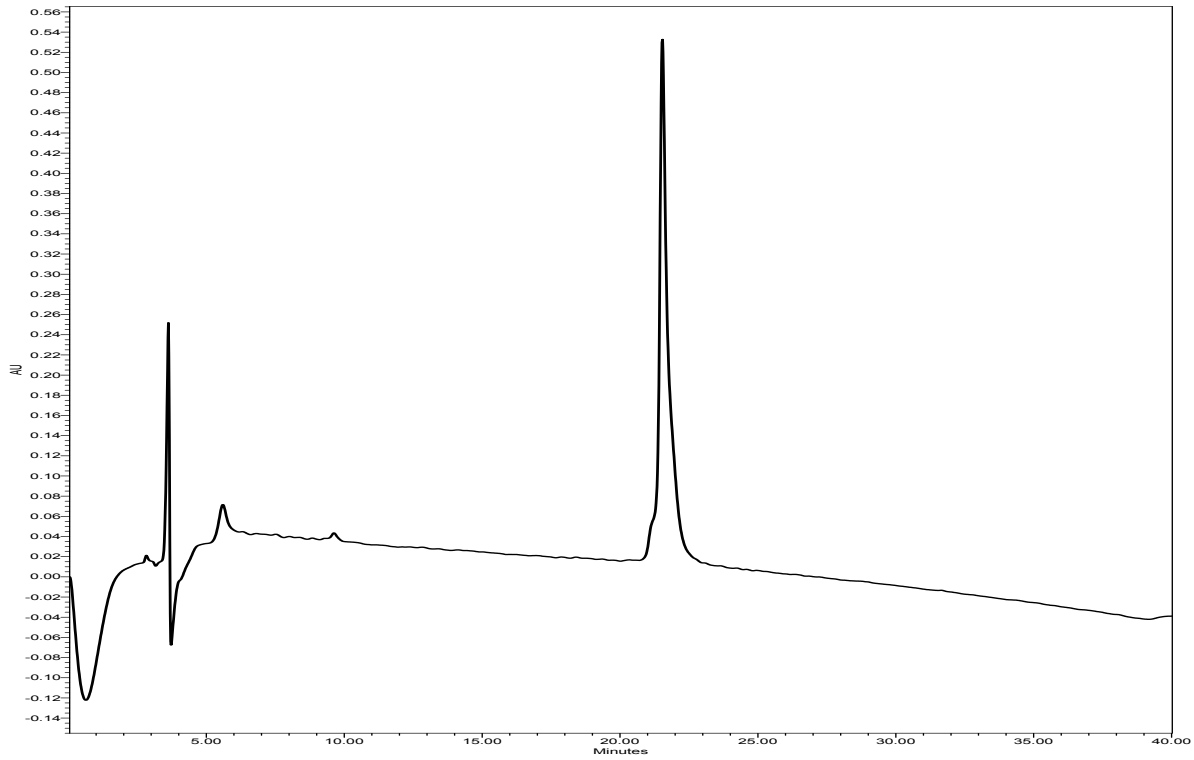
S5.15. Synthesis of fluorescently tagged Myrcludex B CLipPA intermediate 4s



The peptide was assembled by automated Fmoc SPPS using the PS3 Peptide Synthesizer as described in **Method 1.** The final mercaptopropionic acid was coupled as described by **Method 2**. The Dde protecting group was removed as described in **Method 4** and 5(6)-carboxyfluoroscein attached as described in **Method 5.** The final cleavage of the peptide from the resin was performed as described by **Method 6** to afford 494 mg of crude peptide at approximately 70% purity.

The crude peptide intermediate **4s** was dissolved in 1:4 MeCN/MQ H_2O at a concentration of 10 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC with a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 300 x 19 mm). The gradient employed was as follows; 12.5%B to 30%B over 5 min (ca. 3.5%B/min), then 30%B to 50%B over 20 min (ca. 1%B/min) at a flow rate of 10 ml/min. Fractions were collected based on deduction of main peak from analytical HPLC and confirmed by ESI-MS. Fractions of multiple runs were lyophilised to afford the intermediate pure peptide of **4s** as a yellow amorphous powder (115 mg, 22% yield, 97.5% purity).

For the synthesis of analogues **4a-d** peptide **4s** was split into portions as described below for CLipPA reactions.



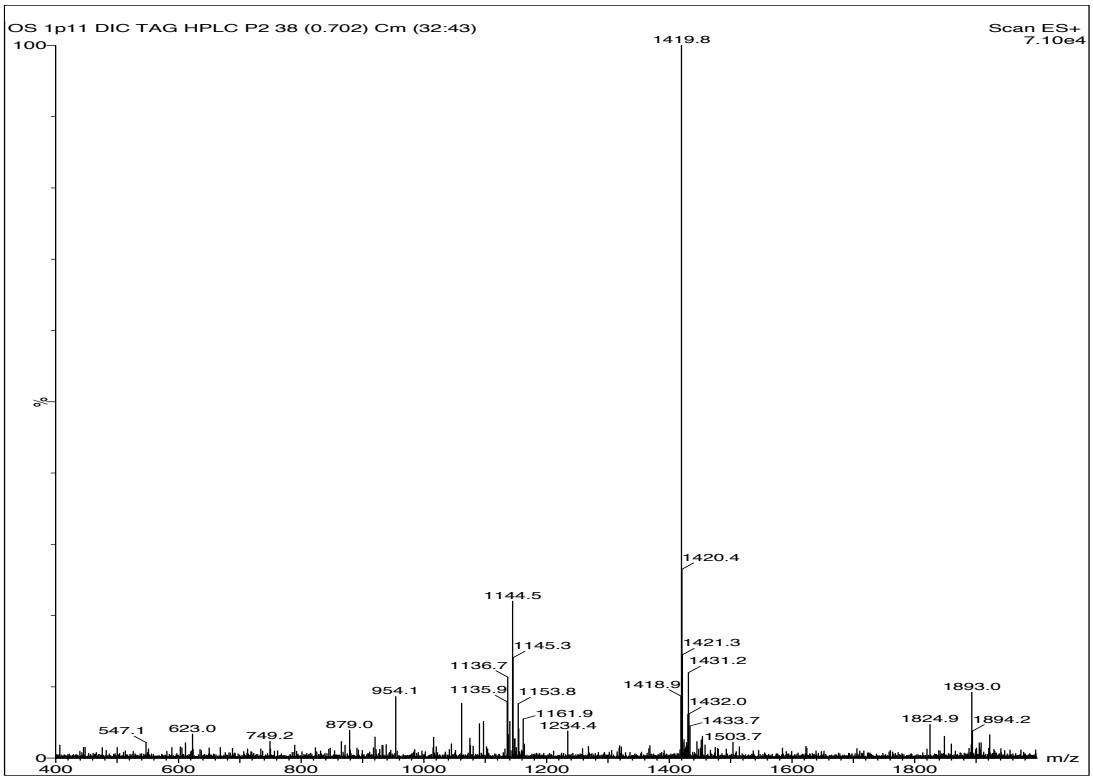
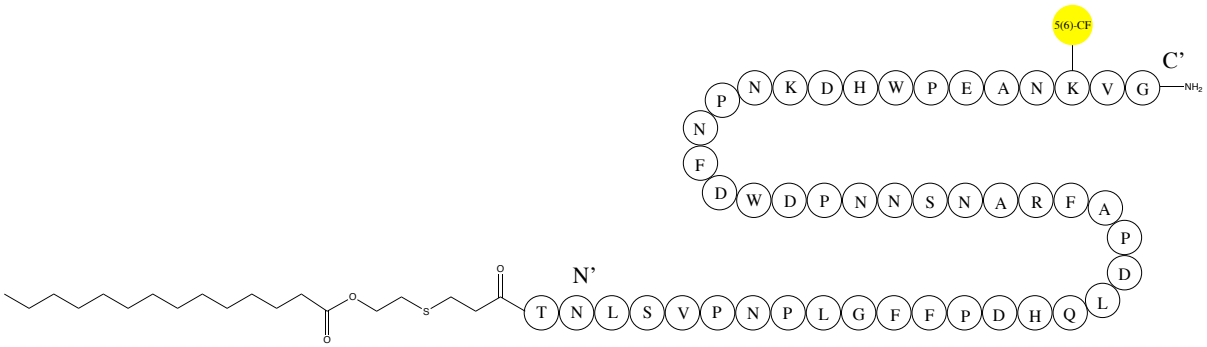


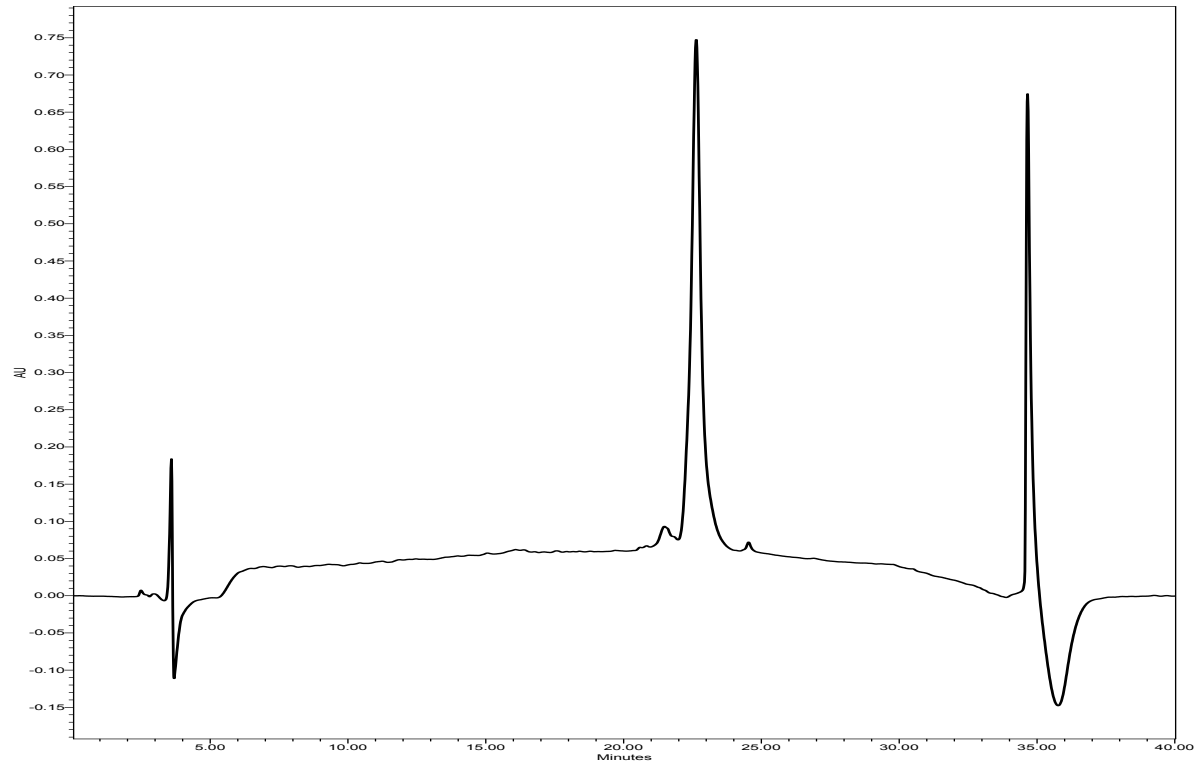
Figure S5.15: (upper) analytical **RP-HPLC** trace of pure peptide Myrcludex B, **4s** (ca. 97.5% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 75%B over 35 min (ca. 2%B/min) at 1 ml/min. t_R = 21.5 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5676.50, observed: 5676.20 ± 0.51.

S5.16. Synthesis of fluorescently tagged Myrcludex B CLipPA analogue 4a



In two separate batches, peptide intermediate **4s** (12.5 mg, 2.20 µmol, 1 equiv.) was treated with vinyl myristate, **12a** (19.6 µL, 77 µmol, 35 equiv.), using **Method 8** to afford a total of 19 mg of crude peptide **4a** at approximately 61% purity.

The crude peptide **4a** was dissolved in 1:3 MeCN/MQ H_2O at a concentration of 10mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC on a Waters 1525 HPLC using a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 250 x 10 mm). The gradient employed was as follows; 25%B to 65%B over 35 min (ca. 1.1%B/min) at a flow rate of 5 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed with ESI-MS. Fractions of multiple runs were lyophilised to afford *title compound* **4a** as a yellow amorphous powder (3 mg, 12% yield, 99.2% purity).



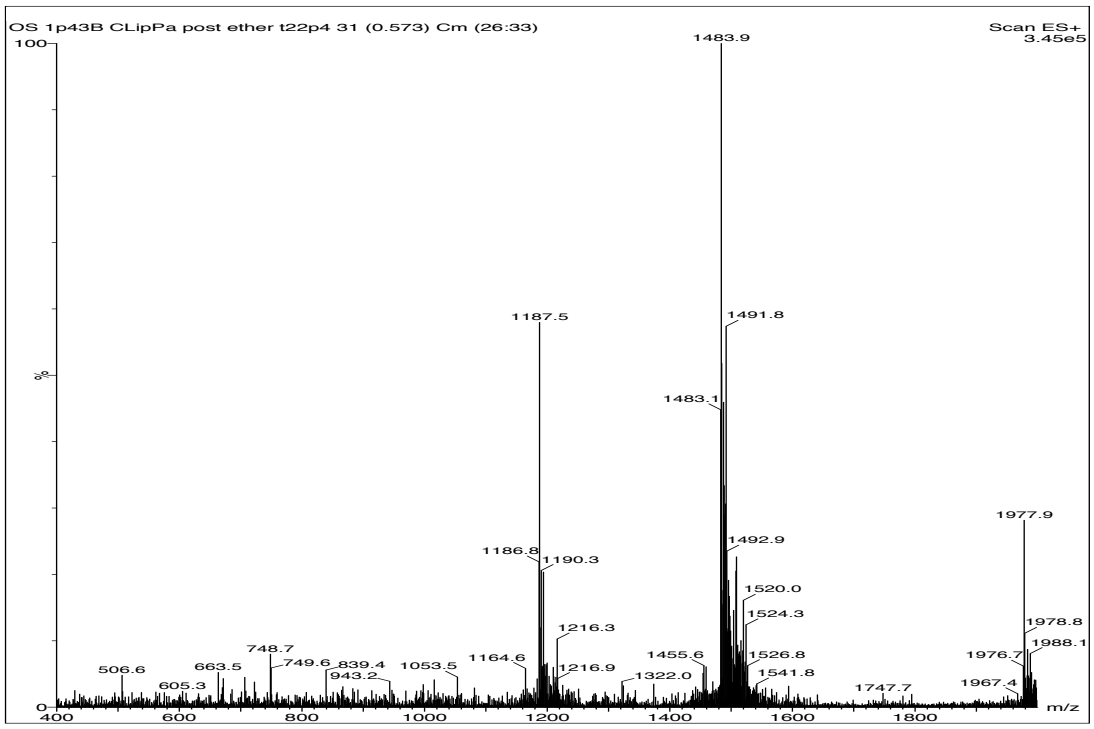
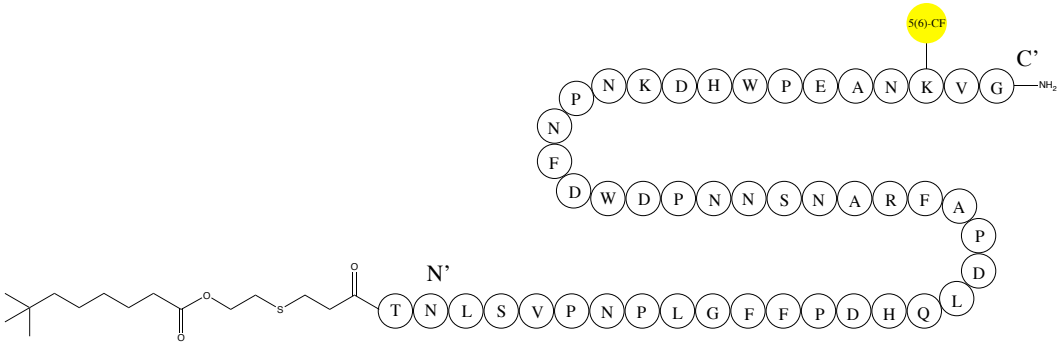


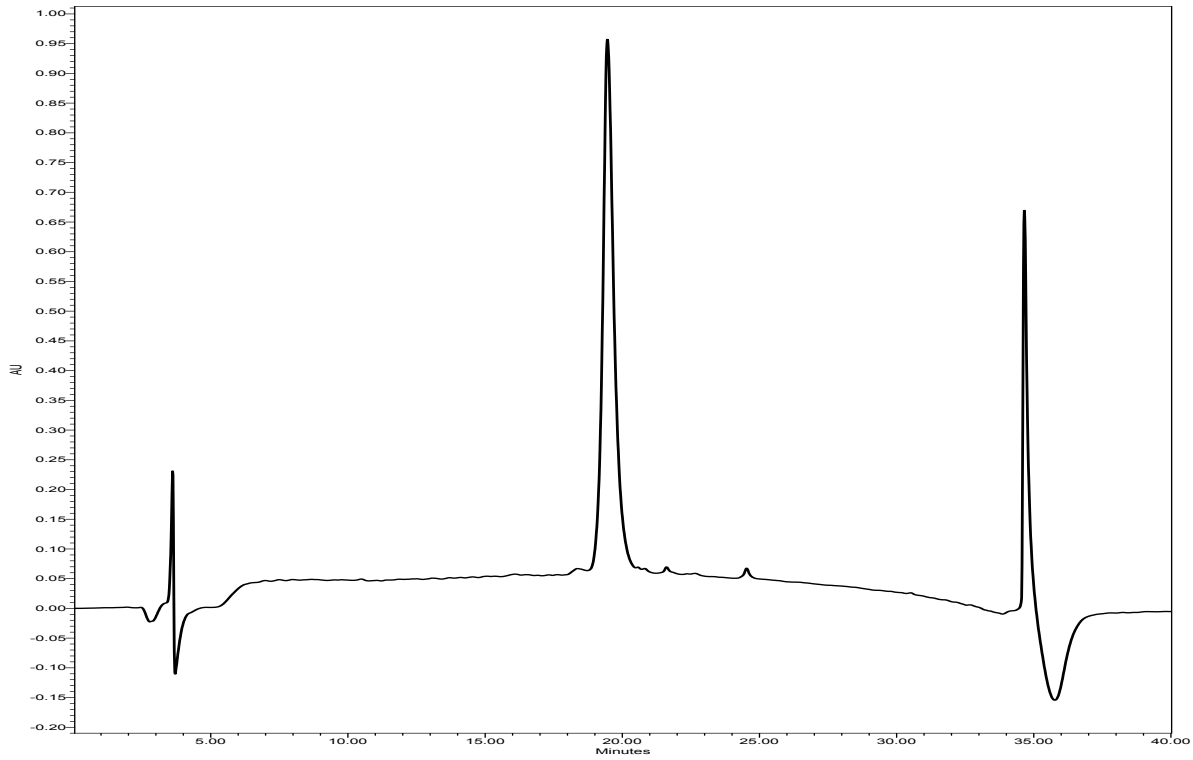
Figure S5.16: (upper) analytical **RP-HPLC** trace of fluorescently labelled pure CLipPA peptide, **4a** (ca. 99.2% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 35 min (ca. 3%B/min) at 1 ml/min. t_R = 22.6 min (Note: peak at 24.4 min is column impurity as was also present in blank run previous to sample); (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5929.75, observed: 5931.60 ± 0.90.

S5.17. Synthesis of fluorescently tagged Myrcludex B CLipPA analogue 4b



In two separate batches, peptide intermediate **4s** (12.5 mg, 2.20 µmol, 1 equiv.) was treated with vinyl neodecanoate, **12b** (15.3 µL, 77.0 µmol, 35 equiv.), using **Method 8** to afford a total of 14 mg of crude peptide **4b** at approximately 67% purity.

The crude peptide **4b** was dissolved in 1:3 MeCN/MQ H_2O at a concentration of 10 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC on a Waters 1525 HPLC using a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 250 x 10 mm). The gradient employed was as follows; 25%B to 65%B over 35 min (ca. 1.1%B/min) at a flow rate of 5 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed with ESI-MS. Fractions of multiple runs were lyophilised to afford *title compound* **4b** as a yellow amorphous powder (3.01 mg, 12% yield, 99.4% purity).



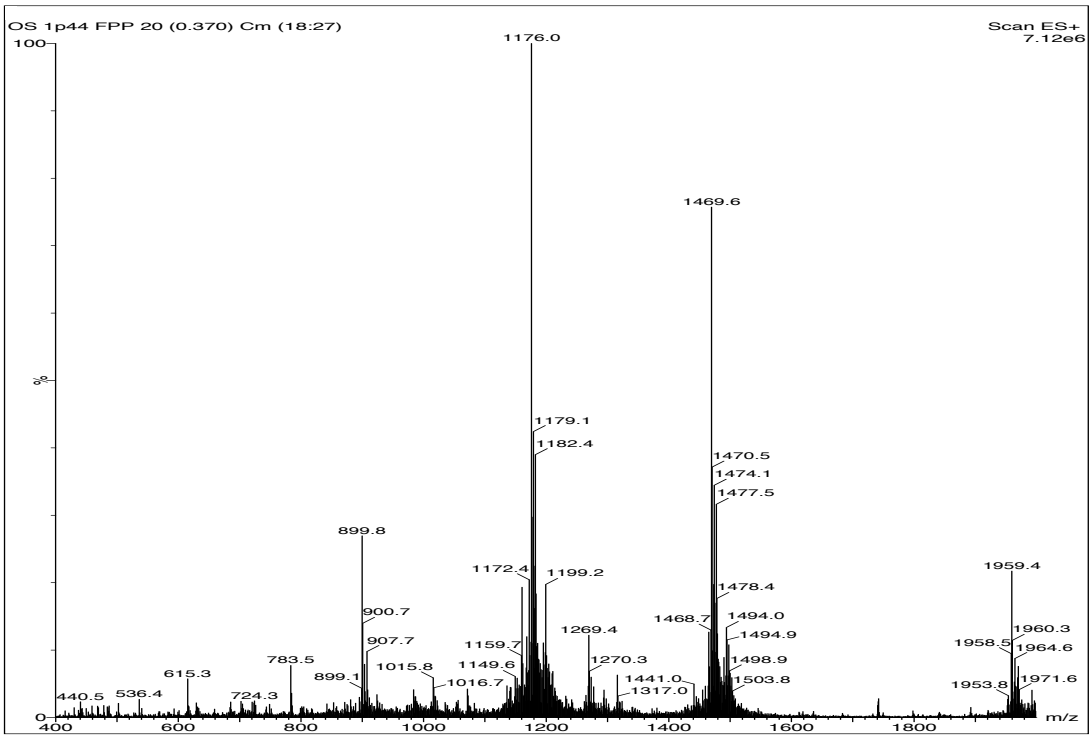
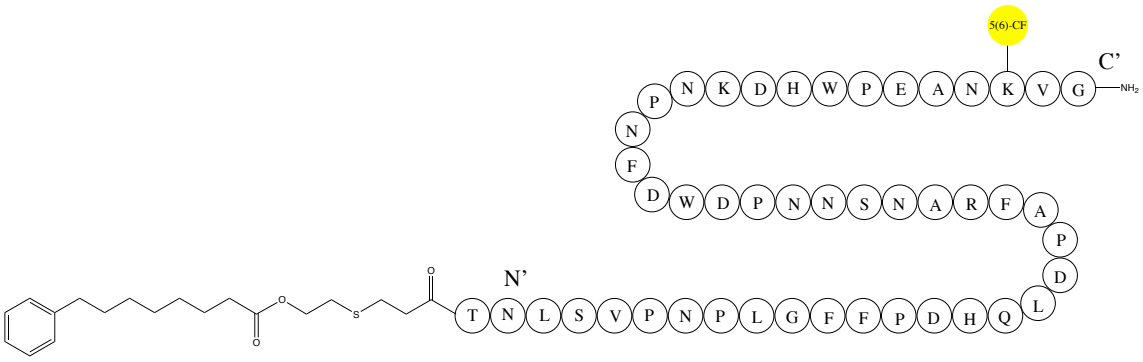


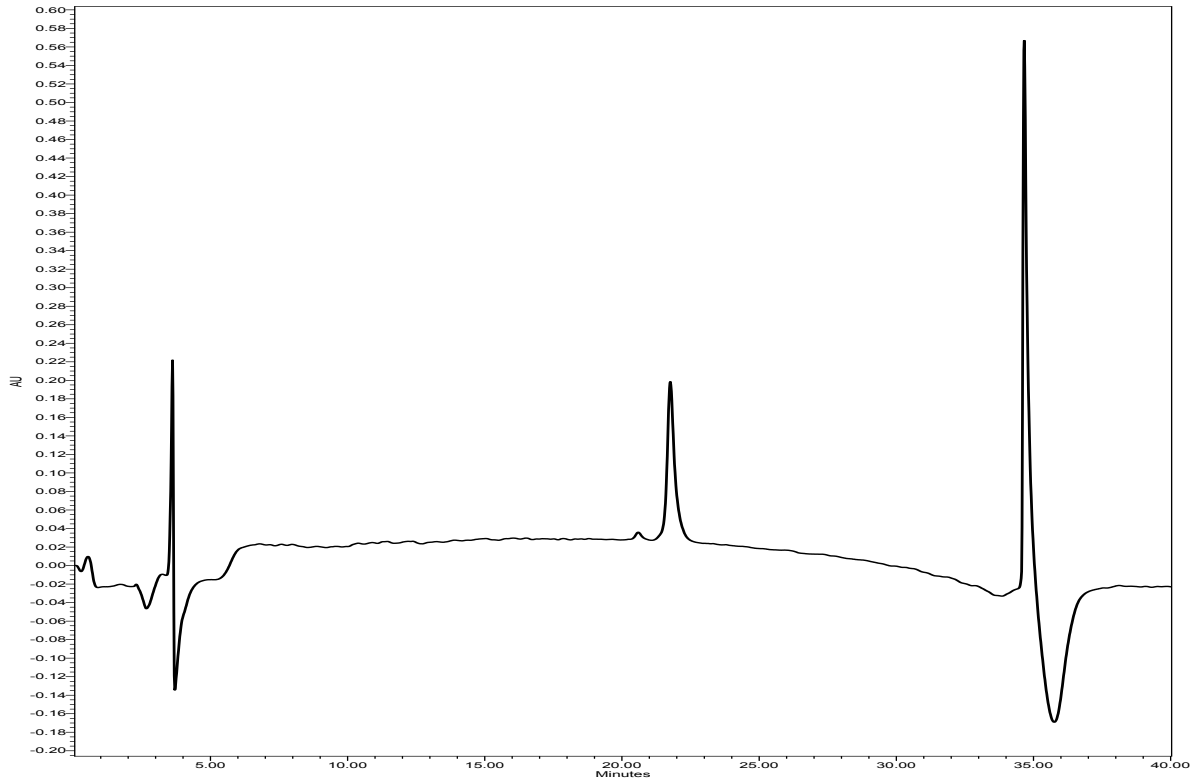
Figure S5.17: (upper) analytical **RP-HPLC** trace of fluorescently labelled pure CLipPA peptide, **4b** (ca. 99.4% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 35 min (ca. 3%B/min) at 1 ml/min. t_R = 19.5 min (Note: peak at 24.4 min is column impurity as was also present in blank run previous to sample); (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5873.69, observed: 5874.87 ± 0.42.

S5.18. Synthesis of fluorescently tagged Myrcludex B CLipPA analogue 4c



In two separate batches, peptide intermediate **4s** (12.5 mg, 2.2 µmol, 1 equiv.) was treated with vinyl 8-phenyloctanoate, **12c** (19.7 µL, 77 µmol, 35 equiv.), using **Method 8** to afford a total of 12.4 mg of crude peptide **4c** at approximately 59% purity.

The crude peptide **4c** was dissolved in 1:3 MeCN/MQ H_2O at a concentration of 10 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC on a Waters 1525 HPLC using a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 250 x 10 mm). The gradient employed was as follows; 25%B to 65%B over 35 min (ca. 1.1%B/min) at a flow rate of 5 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed with ESI-MS. Fractions of multiple runs were lyophilised to afford *title compound* **4c** as a yellow amorphous powder (3.7 mg, 15% yield, 96.5% purity).



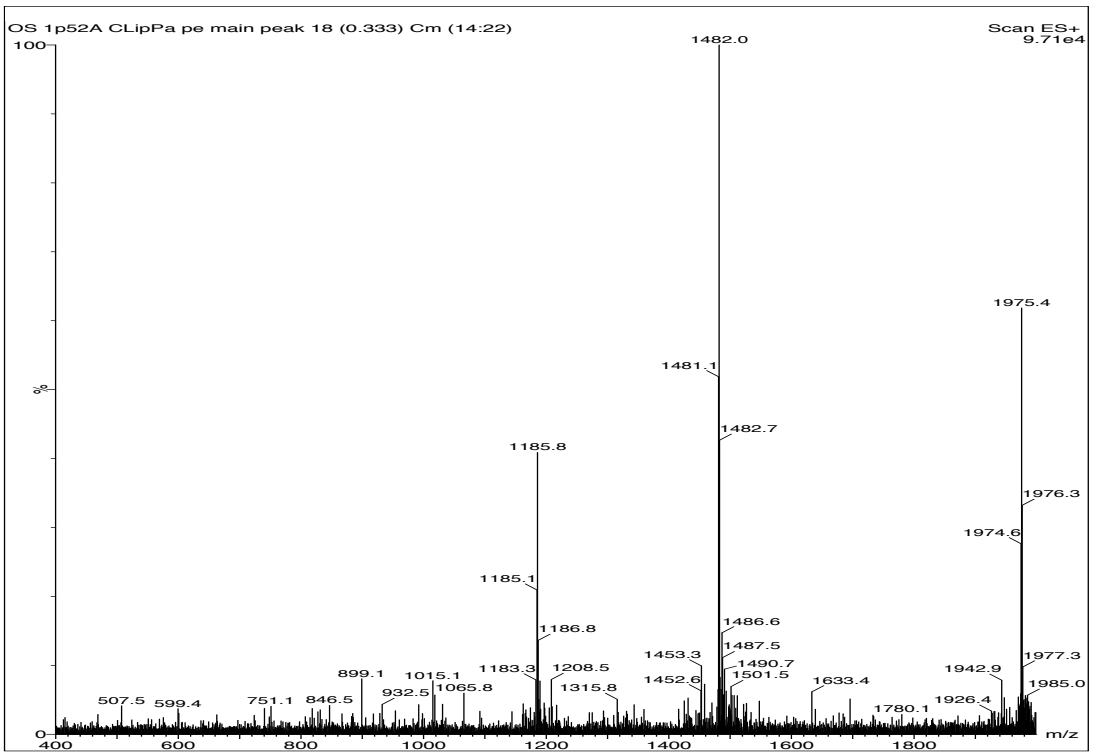
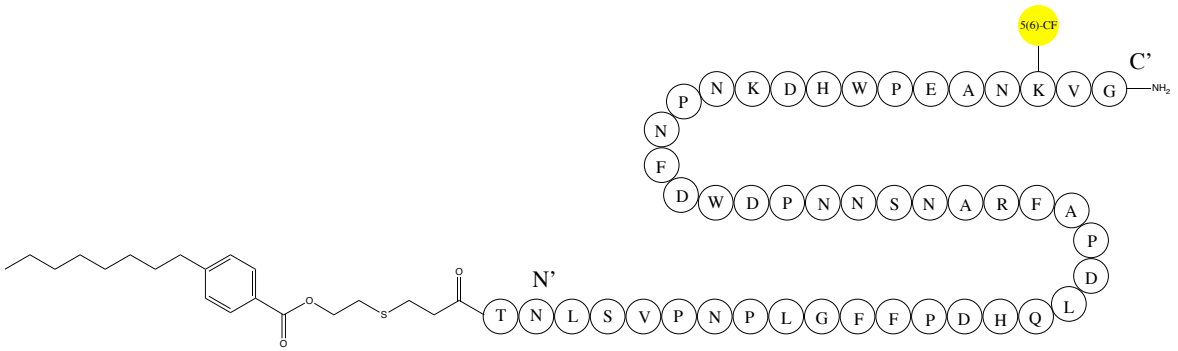


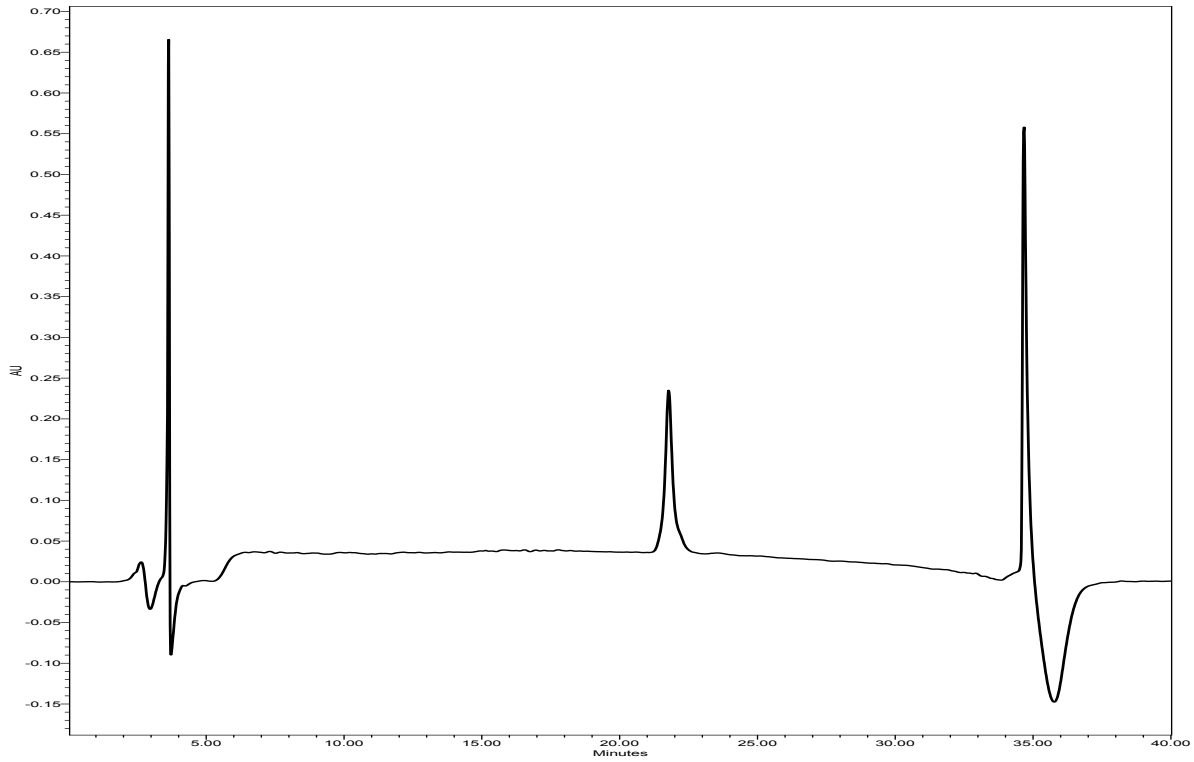
Figure S5.18: (upper) analytical **RP-HPLC** trace of fluorescently labelled pure CLipPA peptide, **4c** (ca. 96.5% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 35 min (ca. 3%B/min) at 1 ml/min. t_R = 21.8 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5921.69, observed: 5923.73 ± 0.46.

S5.19. Synthesis of fluorescently tagged Myrcludex B CLipPA analogue 4d



In two separate batches, peptide intermediate **4s** (12.5 mg, 2.2 µmol, 1 equiv.) was treated with vinyl 4-octylbenzoate, **12d** (20.8 µL, 77 µmol, 35 equiv.), using **Method 8** to afford a total of 11.6 mg of crude peptide **4d** at approximately 63% purity.

The crude peptide **4d** was dissolved in 1:3 MeCN/MQ H_2O at a concentration of 10 mg/ml. It was purified over a series of semi-preparative runs using RP-HPLC on a Waters 1525 HPLC using a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 250 x 10 mm). The gradient employed was as follows; 25%B to 65%B over 35 min (ca. 1.1%B/min) at a flow rate of 5 ml/min. Fractions were collected based on deduction of main peak from analytical RP-HPLC and confirmed with ESI-MS. Fractions of multiple runs were lyophilised to afford *title compound* **4d** as a yellow amorphous powder (2.3 mg, 9% yield, 98.2% purity).



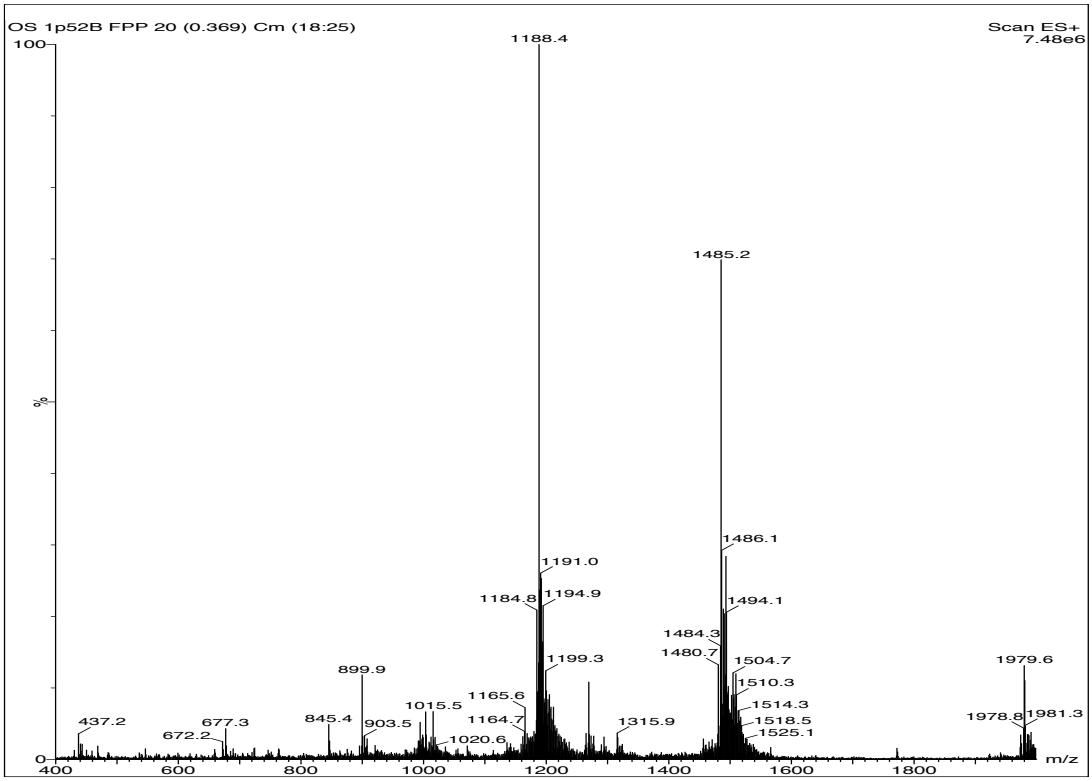
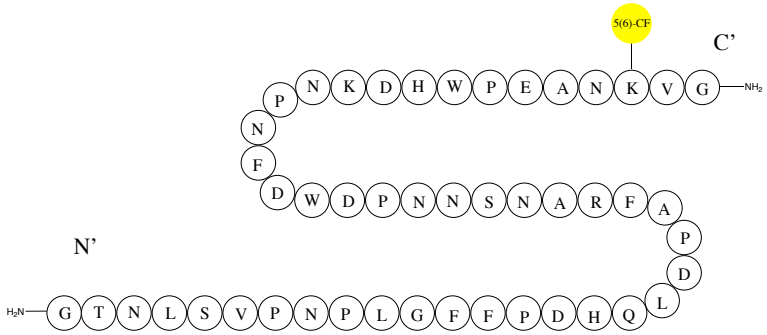


Figure S5.19: (upper) analytical **RP-HPLC** trace of fluorescently labelled pure CLipPA peptide, **4d** (ca. 98.2% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 35 min (ca. 3%B/min) at 1 ml/min. t_R = 21.8 min; (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5935.70, observed: 5936.52 ± 0.64.

S5.20. Synthesis of fluorescently tagged non lipidated analogue 5



The non-lipidated peptide was assembled by automated Fmoc SPPS using the PS3 Peptide Synthesizer as described in **Method 1**, with the final residue being coupled, Boc-Gly-OH. The Dde protecting group was removed as described in **Method 4** and 5(6)-CF attached as described in **Method 5.** The final cleavage of the peptide from the resin was performed using **Method 6,** to afford 112 mg of crude peptide at approximately 89% purity.

The crude peptide analogue **5** was dissolved in 1:4 MeCN/MQ H_2O at a concentration of 10 mg/ml and purified by RP-HPLC with a Waters XTerra Prep MS C18 OBD column (125 Å, 10 µm, 300 x 19 mm), employing a gradient of 22.5%B to 30%B over 5 min (ca. 1.5% B/min), then 30%B to 70%B over 20 min (ca. 2% B/min) at a flow rate of 10 ml/min. Fractions were collected based on deduction of main peak from analytical HPLC and confirmed by ESI-MS. Fractions from multiple runs were combined and lyophilised to afford *title compound* **5** as a yellow amorphous powder (66.1 mg, 29% yield, 99.5% purity).

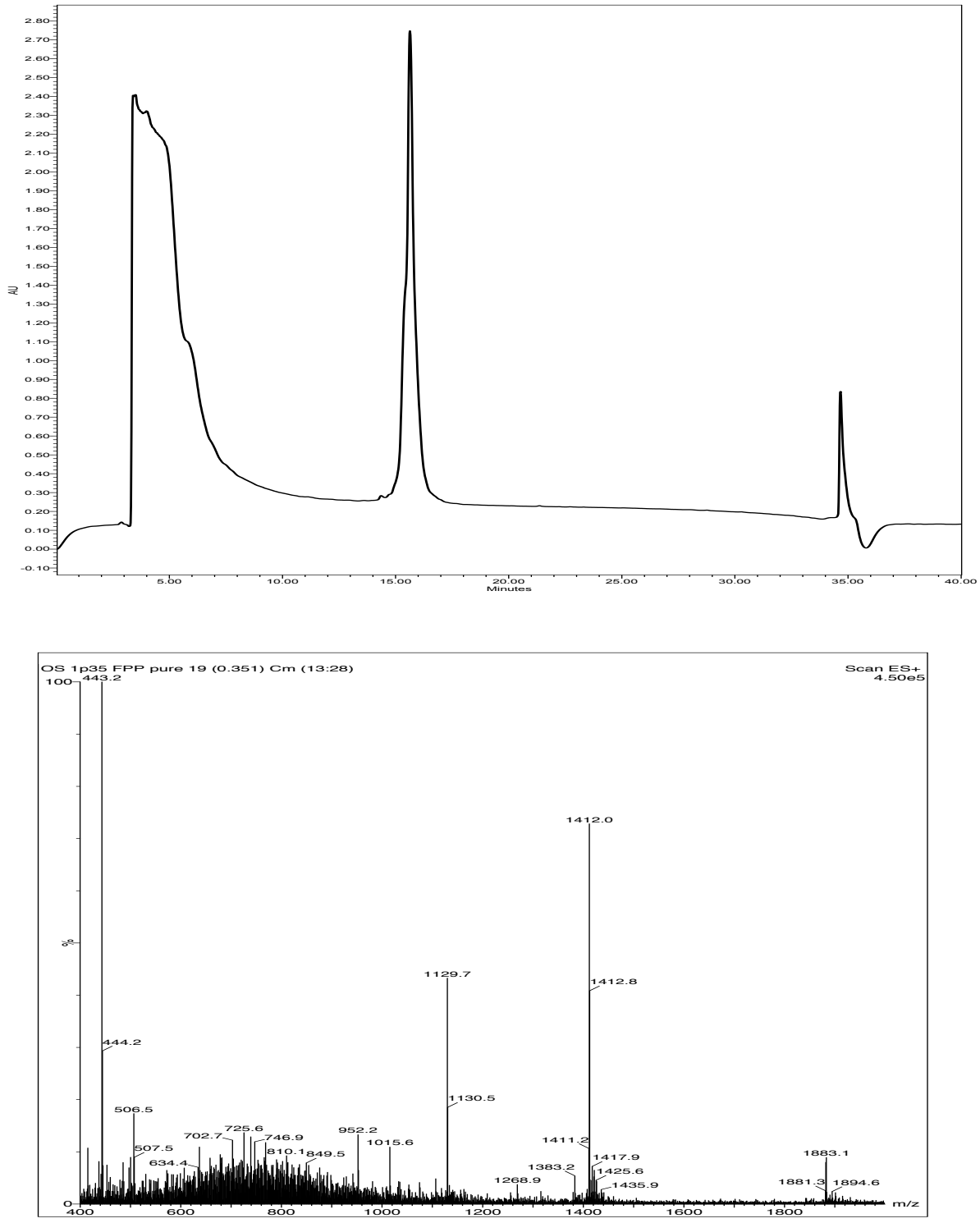


Figure S5.20: (upper) analytical **RP-HPLC** trace of fluorescently labelled pure non-lipidated peptide, **5** (ca. 99.5% as analysed by peak area at 214nm); Phenomenex Luna C18 column (100 Å, 5 µm, 4.6 mm x 250 mm), linear gradient of 5%B to 95%B over 35 min (ca. 3%B/min) at 1 ml/min. t_R = 15.6 min. (Broad peak at ~5 min. is DMSO as was used in stock solution for bioactivity assays); (lower) **ESI-MS** deconvoluted mass, m/z; calculated: 5644.55, observed: 5644.60 ± 1.49.

S6.1. IC_{50} Plot for Full Length Peptides

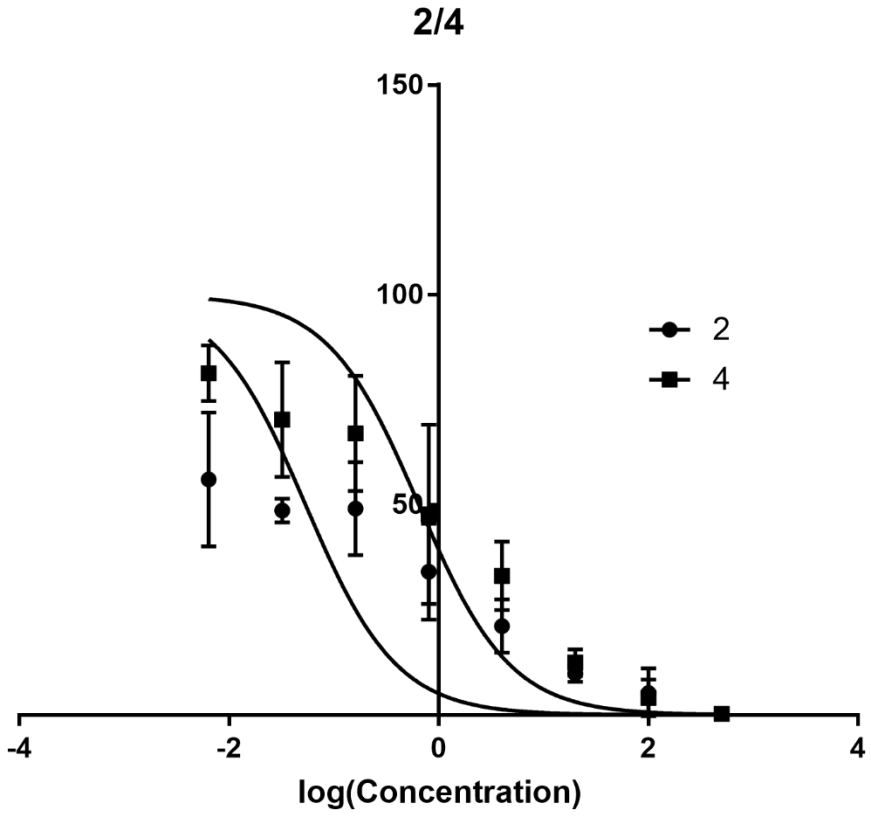


Figure S6.1: IC_{50} plots for 47-mer native lipopeptide (**2**) and 5(6)-CF labelled lipopeptide (**4**). Error bars correspond to ± 1 SE. Viral replication presented as % of uncompeted HBV infection in HepG2 NTCP cells as determined by HBeAg chemiluminescence immunoassay (CLIA) of secreted HBeAg present in the supernatant at day 9.

S6.2. IC_{50} Plot for Truncated Peptides

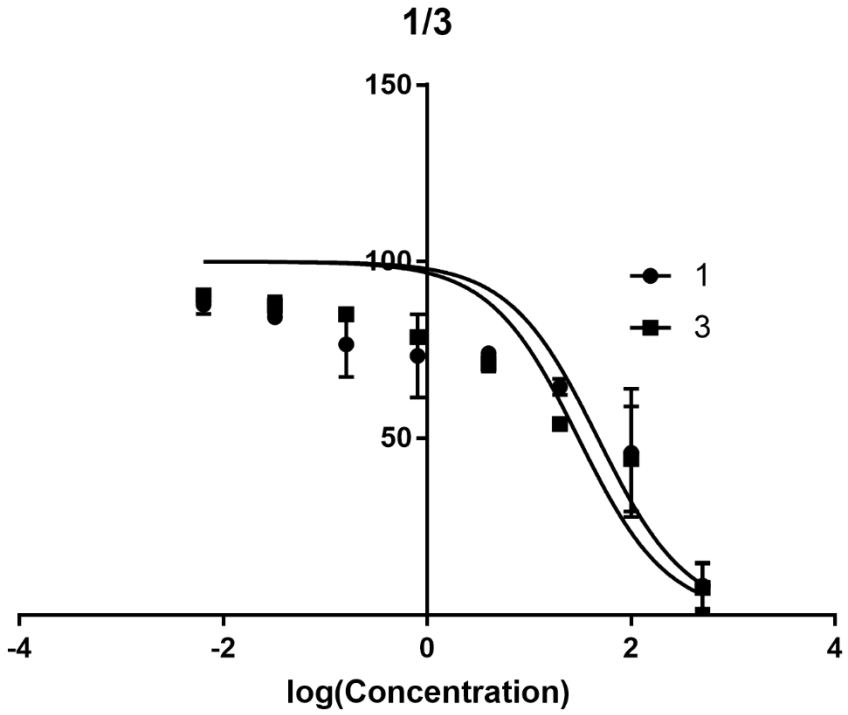


Figure S6.2: IC_{50} plots for truncated native lipopeptide (**1**) and truncated 5(6)-CF labelled lipopeptide (**3**). Error bars correspond to ± 1 SE. Viral replication presented as % of uncompeted HBV infection in HepG2 NTCP cells as determined by HBeAg chemiluminescence immunoassay (CLIA) of secreted HBeAg present in the supernatant at day 9.

S6.3. IC_{50} Plot for Full Length CLipPA Peptides

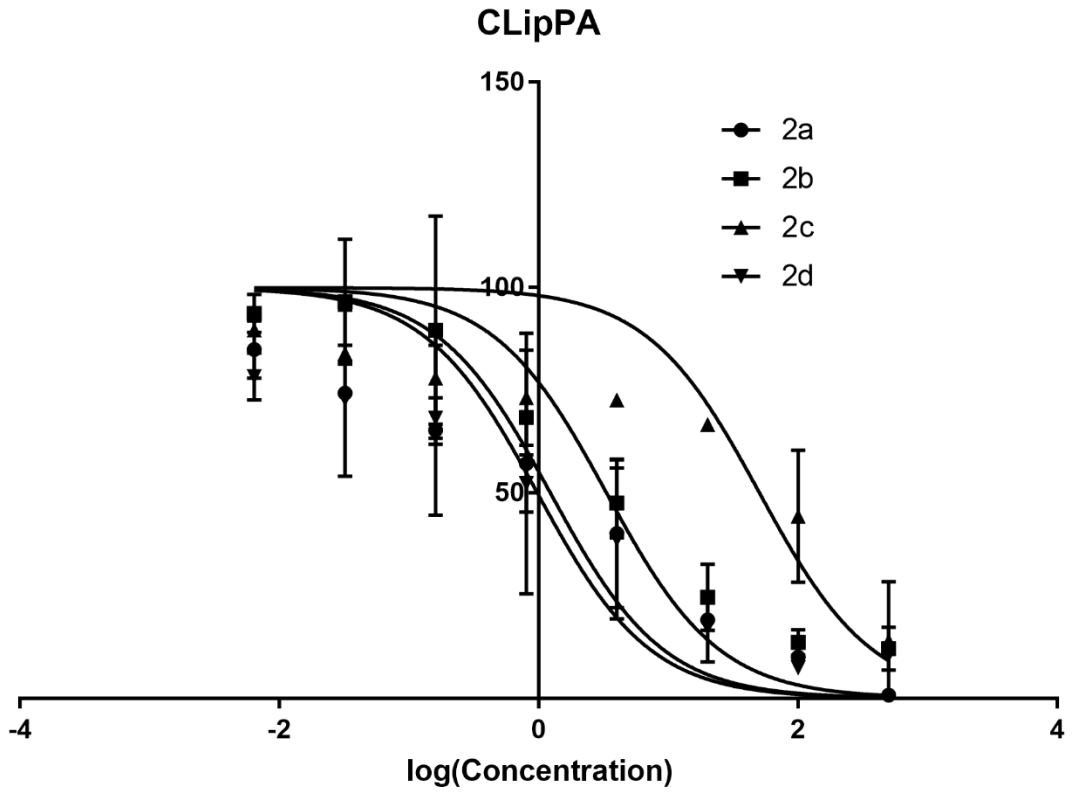


Figure S6.3: IC_{50} plots for 47-mer ClipPA lipidated lipopeptides (**2a-d**). Error bars correspond to ± 1 SE. Viral replication presented as % of uncompeted HBV infection in HepG2 NTCP cells as determined by HBeAg chemiluminescence immunoassay (CLIA) of secreted HBeAg present in the supernatant at day 9.