Materials and Methods

General analytical procedures

Analytical RT-HPLC were performed on a Shimadzu reverse-phase HPLC system, equipped with Shimadzu LC-20AT pumps, a Shimadzu SIL-20A autosampler and a Shimadzu SPD-20A UV-Vis detector monitoring at 214 nm and 280 nm. The system was equipped with a Phenomenex, Aeris, 5 µm, peptide XB-C18, 150 x 4.6 mm column and run at a flow rate of 1 mL/min. Gradients were run using a binary solvent system consisting of solvent A (H_2O +0.1% TFA) and solvent B (MeCN + 0.1 TFA). Analytical HPLC data are reported as column retention time (RT) in minutes (min) as well as with the gradient.

LC-MS analysis was performed on an Agilent Infinity Lab liquid chromatography mass spectrometer, using positive mode electrospray ionization (ESI+), fitted with either a Shim-pack XR-ODS, C18, 2.2 µm, 2.0 x 50 mm column or a Gemini 5µm C18 110 Å, 150x2 mm LC column. The gradients were run at 0.3 mL/min flow rate using a binary solvent system of buffer A (H_2O + 0.1% formic acid), and buffer B (MeCN + 0.1% formic acid) over 20 mins.

In accordance with best practice for large molecules accurate mass spectra were not determined and masses are reported to one decimal place.[1,2]

Peptides were purified on a reverse-phase Dionex HPLC system equipped with Dionex P680 pumps and a Dionex UVD170U UV-vis detector (monitoring at 214 nm and 280 nm), using a Phenomenex, Gemini, C18, 5 µm, 250 x 21.2 mm column at a flow rate of 8 ml/min. Gradients were run using a solvent system consisting of A (H_2O + 0.1 % TFA) and B (MeCN + 0.1 % TFA). Collected fractions were lyophilized on a Christ Alpha 2-4 LO plus freeze dryer.

General procedure for peptide synthesis

Peptides were synthesized using a CEM Liberty Blue peptide synthesizer, using DIC/Oxyma activation and 20% morpholine in DMF for Fmoc deprotection. Peptides were synthesized on a 0.1 mmol scale using Rink amide polystyrene resins. Peptides were cleaved from the solid support using a cocktail of TFA/TIPS/water in 95:2.5:2.5 proportions. Peptides were precipitated by dilution in ice cold diethyl ether and recovered by centrifugation, then redissolved in 1:1 water/acetonitrile and freeze dried.

Computational

Models were run using colabfold local (https://github.com/YoshitakaMo/localcolabfold) on a workstation equipped with an RTX4080 GPU.

FP Assays

Fluorescence polarization assays were performed using a BMG Labtech CLARIOstar plate reader with monochrome filters measuring excitation wavelengths of 482-16 nm and emission wavelength of 530-40 nm. The assays were run using the top optics, a settling time of 0.5 s and 500 flashes per well. Assays were run in Corning 384 well, black, low volume, round bottom polystyrene non-binding surface well plates.

FP assays were performed based on the basic protocol by Moerke in 2009.[3] The assays were run in MES buffer consisting of 0.2 g MES w/v, 0.09 g w/v and 0.05% Tween 20 in H_2O. A stock solution of the protein of interest was prepared by adding the appropriate

amount of protein to an Eppendorf tube with 5 µL of 10x MES buffer. Distilled water was added to the Eppendorf tub to a total volume of 50 µL. A 10 point 1:2 serial dilution of the protein was performed by sequentially mixing 25 µL of 1xMES buffer with 25 µL of the previous protein solution in a new Eppendorf tube. 25 µL of a 100 nM stock of the fluorescently labelled peptide in 1xMES buffer was added to each of the protein dilution tubes for a final concentration of 50 nM labelled peptide. 10 µL of protein dilution/labelled peptide solution was transferred to a black opaque 384-well plate in triplicate. 3x10 µL of 1xMES buffer was also transferred for background measurements as well as 3x10 µL of a 50 nM labelled peptide solution for gain adjustment. The plate was added to the plate reader and the gain was adjusted to 30 mP using the well with the 50 nM labelled peptide solution. The plate was measured, and the results were exported to a .csv file. The data was corrected for background contributions from the buffer by subtracting the values from the blank wells. The data was analyzed using a Jupyter notebook, and the average measured polarization for each datapoint was plotted as a function of protein concentration. The data were fitted to a 1:1 binding model using the 'PyBindingCurve' Python package (https://stevenshave.github.io/pybindingcurve/)'

FP titrations from DMSO were performed similarly to the standard FP assay. An additional 23 µL of 1xMES buffer was added to the tubes containing the serially diluted protein. 2 µL of a 1.25 µM stock of the fluorescently labelled peptide in DMSO were added to each of the protein dilution tubes for a final concentration of 50 nM labelled peptide, and 4% v/v DMSO. The peptide was transferred to the 384-well plate and measured using as described in the general procedure.

CD

Circular dichroism measurements were performed on an Applied Photophysics Chirascan VX circular dichroism spectropolarimeter, equipped with a Quantum Northwest TC Temperature controller and a CW-3000 industrial chiller, or on a JASCO J-810 circular dichroism spectropolarimeter equipped with a Peltier sample stage. CD spectra were recorded in MOPS buffer (20mM MOPS, 100mM NaCl) at pH 7, concentration of 50 µM and 20 °C unless otherwise stated. The HT signal stayed below 500 V to at least 180 nM unless otherwise stated.

Peptide synthesis
Peptides were synthesized on a 0.10 mmol scale using a Fmoc-Rink Amide resin (FluoroChem, loading 0.33 mmol/g) following the general procedure for SPPS. Alloc protection groups were selectively removed using $Pd(PPh_3)_4$ (0.25 equiv.) and morpholine (48 equiv.) in dry CH_2Cl_2 for 2 h. The resin was then washed with 5 mL of CH_2Cl_2 three times.

Capping of peptide
Acetic anhydride (50 equiv.) and pyridine (80 equiv.) were added to the peptide in DMF, and reaction was carried out over 1h. The resin was then washed with 5 mL DMF followed by 3 x 5mL washes with CH_2Cl_2.

Alloc deprotection
Alloc protection groups were removed using $Pd(PPh_3)_4$ (0.25 equiv.) and morpholine (48 equiv.) in dry DCM for 2 h. The resin was then washed with 5 mL of DCM three times.

Coupling of Fluorescein
Fluorescein isothiocyanate isomer 1 (3 equiv.) and DIPEA (3 equiv.) in DMF was added to the resin and reacted for 18 h. The resin was washed with 5 mL DMF and three times with 5 mL DCM.

Alkyne coupling
Peptides were synthesized following the general procedure for microwave assisted SPPS. The resins were split and 0.025 mmol were used for further reaction. 4-pentynoic acid was coupled using the standard procedure and the peptides were cleaved using the standard cleaving procedure.

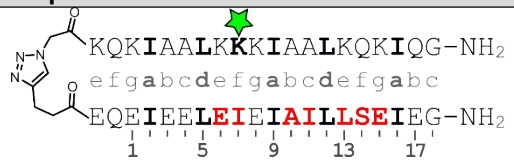
Azide coupling
Peptides were synthesized following the general procedure for microwave assisted SPPS. Bromoacetic acid (4 equiv.) was coupled using the strand procedure. The resin was washed with DMF three times. NaN_3 (2 equiv.) and 18-crown-6 (0.8 equiv.) was dissolved in DMF and reacted overnight. The resin was washed three times with CH_2Cl_2. The peptides were cleaved using the non-reductive cleaving procedure.

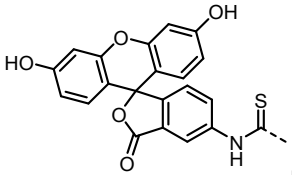
Triazole formation
Procedure A:
The alkyne containing peptide (1 equiv.) and the azide containing peptide (1 equiv.) were dissolved in H_2O to a concentration of 2 mM and added together (final concentration 1 mM). Sodium ascorbate (200 mM, 3 equiv.) and $CuSO_4 \cdot H_2O$ (100 mM, 1 equiv.) was added, and pH was adjusted to 7 using 1 M NaOH. The reaction was monitored using HPLC.

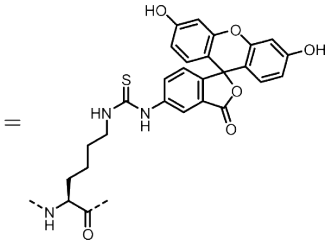
Procedure B:
The alkyne containing peptide (1 equiv.) were dissolved in 2-propanol and the azide containing peptide (1 equiv.) were dissolved in H_2O and the concentration was adjusted to 2 mM using 2-propanol (final concentration 1 mM). Sodium ascorbate (200 mM, 3 equiv.) and $CuSO_4 \cdot H_2O$ (100 mM, 1 equiv.) was added, and pH was adjusted to 7 using 1 M NaOH. The reaction was monitored using HPLC.

Table S1: Structural details and characterization for all peptides. Note that in accordance with best practice, and as described above, HRMS are not quoted for these peptides.

Peptide	Sequence	Purification gradient	Purity HPLC	Mass spectroscopy (ESI+)
1		NA	26.30 min 93.2 % (50 min gradient, 30-60% B).	Calculated for $C_{92}H_{141}N_{21}O_{34}$ $[M+H]^+$ 2085.0 found 2085.0
2		NA	14.64 min 84.9 % (50 min gradient, 20-80% B).	Calculated for $C_{95}H_{168}N_{30}O_{22}$ $[M+Na]^+$ 2104.3 found 2104.3
4		20-60% B	25.13 min 94.2 % (50 min gradient, 10-60% B).	Calculated for $C_{89}H_{139}N_{21}O_{34}$ $[M+2H]^{2+}$ 2048.0 found 2048.6
3		20-60%B Gemini Col	16.14 min 97.1 % (50 min gradient, 20-80% B).	Calculated for $C_{187}H_{309}N_{51}O_{56}$ $[M+H]^+$ 4169.3 found 4169.3
5		20-60% B	26.75 min 93.9 % (50 min gradient, 20-80% B).	Calculated for $C_{95}H_{169}N_{27}O_{22}$ $[M+2H]^{2+}$ 2042.3 found 2042.6
6		40-90% B	27.94 min 91.8% (20 min gradient, 40-80% B).	Calculated for $C_{106}H_{172}N_{22}O_{39}$ $[M+H2]^{2+}$ 2380.2 found 2380.2
7		25-45% B	17.87 min 100 % (50 min gradient, 20-80% B).	Calculated for $C_{106}H_{187}N_{33}O_{25}$ $[M+Na]^+$ 2346.9 found 2346.4
8		25-45% B	15.58 min 97.08% (20 min gradient, 20-60% B).	Calculated for $C_{124}H_{202}N_{36}O_{30}$ $[M+H3]^{3+}$ 2678.6 found 2678.5
9		20-30% B	25.66 min 100% (50 min gradient, 20-60% B).	Calculated for $C_{212}H_{359}N_{55}O_{64}$ $[M+H4]^{4+}$ 4703.7 found 4704.1

Peptide	Sequence	Purification gradient	Purity HPLC	Mass spectroscopy (ESI+)
10		25-55% B	13.43 min 97.1% (50 min gradient, 30-60% B).	Calculated for $C_{230}H_{374}N_{58}O_{69}$ $[M+H4]^{4+}$ 5057.8 found 5057.8
11		25-70% B	13.53 min 95.7% (50 min gradient, 30-60% B).	Calculated for $C_{97}H_{164}N_{26}O_{37}$ $[M+H2]^{2+}$ 2287.2 found 2287.2
12		35-60% B	31.46 min 94.0% (50 min gradient, 20-60% B)	Calculated for $C_{124}H_{187}N_{29}O_{43}S$ $[M+H2]^{2+}$ 2804.3 found 2803.3
S1	Fluor-βA-GEDEEELIRKAIELSLKESG-NH_2	24-70% B	35.96 min 100% (50 min gradient, 20-50% B).	Calculated for $C_{119}H_{178}N_{28}O_{42}S$ $[M+H4]^{4+}$ 2705.3 found 2705.1
S2	Fluor-βA-GEDEEEEIRKAIELSLKESG-NH_2	25-55% B	32.39 min 100% (50 min gradient, 20-50% B).	Calculated for $C_{118}H_{174}N_{28}O_{44}S$ $[M+H2]^{2+}$ 2721.2 found 2721.1
S3	Fluor-βA-GEDEEELIRKAIELSEKESG-NH_2	30-55% B	32.62 min 96.3% (50 min gradient, 20-50% B).	Calculated for $C_{118}H_{174}N_{28}O_{44}S$ $[M+H3]^{3+}$ 2722.2 found 2722.2
S4	Fluor-βA-GEDEEEEIRKAIELSEKESG-NH_2	25-60% B	27.98 min 91.6 % (50 min gradient, 20-50% B).	Calculated for $C_{117}H_{170}N_{28}O_{46}S$ $[M+H2]^{2+}$ 2737.2 found 2737.1
S5	Ac-βA-GEDEEEEIRKAIELSLKESG-NH_2	25-50% B	26.36 min 95.0% (50 min gradient, 20-50% B).	Calculated for $C_{99}H_{165}N_{27}O_{40}$ $[M+H2]^{2+}$ 2376.2 found 2377.5
S6	Ac-βA-GEDEEELIRKAIELSEKESG-NH_2	25-50% B	26.24 min 92.2% (50 min gradient, 20-50% B)	Calculated for $C_{99}H_{165}N_{27}O_{40}$ $[M+H2]^{2+}$ 2374.2 found 2374.1
S7	Ac-βA-GEDEEEEIRKAIELSEKESG-NH_2	20-60% B	21.03 min 92.2 % (50 min gradient, 20-50% B).	Calculated for $C_{98}H_{161}N_{27}O_{42}$ $[M+H2]^{2+}$ 2390.2 found 2390.1

Fluor = 

, **K** = 

Expression and purification of Ub

Ub was expressed and purified as described previously using pRSF_Duet vector containing a hexahistidine tag followed by a TEV cleavage site and a Gly-Gly-Ser linker at the N-terminus of Ub.[4] Ub was expressed in *E. coli* Rosetta (DE3) cells. Cells were grown in LB broth at 37° C and isopropyl β-D-1 thiogalactopyranoside (0.2 mM) was added at OD600nm of 0.6-0.8. The cells were then grown overnight at 23° C. Cells were harvested in 25 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 20 mM imidazole, 2.5 mM phenylmethanesulfonyl fluoride and 5 mM β-mercaptoethanol and lysed by a microfluidizer. The clarified lysates were applied onto Ni^{2+}-affinity column, washed with 25 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 20 mM imidazole and 5 mM β-mercaptoethanol, and eluted with 25 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 200 mM imidazole and 5 mM β-mercaptoethanol. Subsequently, TEV protease was added and dialyzed against buffer containing 25 mM Tris-HCl, pH 7.6, 0.15 M NaCl and 5 mM β-mercaptoethanol at room temperature overnight. The cleaved Ub was purified by Ni^{2+}-affinity column pass-back and gel filtration chromatography with SD75 1660 column. The purified Ub was stored in buffer containing 25 mM Tris-HCl, pH 7.6, 0.15 M NaCl and 1 mM DTT. Ub concentration was determined by using absorbance at 280 nm and the calculated molar extinction coefficient.

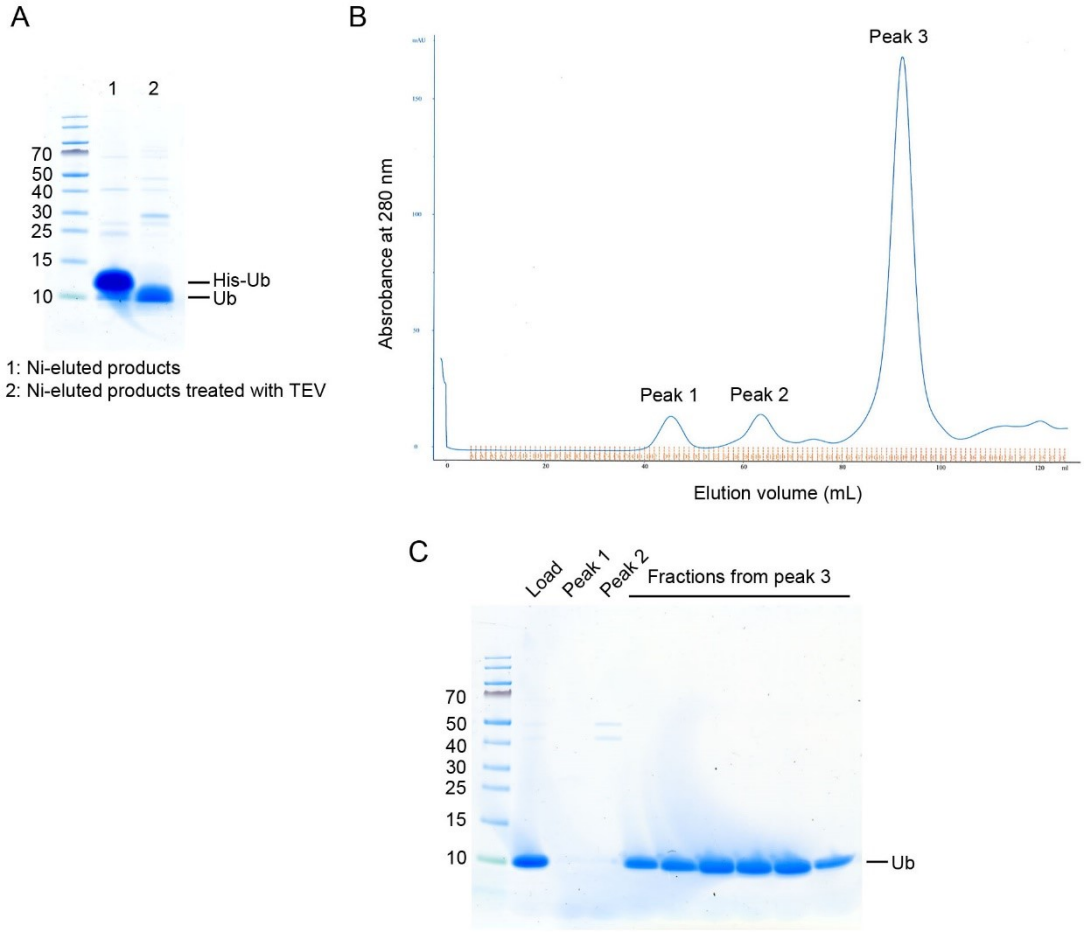


Figure S1: Purification of Ub. (A) SDS-PAGE showing Ni-eluted products before and after TEV treatment. Overexpressed His-Ub was subjected to Ni2+-affinity purification. Eluted products were treated with TEV to remove the His-tag. (B) Superdex 75 1660 gel filtration elution profile showing the purification of Ub. Peaks 1-3 are indicated. (C) SDS-PAGE showing the fractions from peaks 1-3 in B. Peak 3 contained pure Ub. Fractions in peak 3 were pooled and concentrated.

Protein crystallization

CC-UIM peptide was dissolved in MQ water to yield 11.7 mM and mixed with Ub (10 mg/mL) at 1.2:1 molar ratio. The complex was screened by sitting drop vapor diffusion method in a range of commercially available screens at 19 °C. Crystals were obtained in Morpheus screen condition H1 (Molecular Dimensions) containing 0.1 M amino acids, 0.1 M buffer system 1, pH 6.5 and 30 %(v/v) precipitant mix 1. Crystal optimization was performed using buffer system 1 and precipitant mix 1 (Molecular Dimension). The best diffracting crystals were obtained in 0.1 M amino acids, 0.1 M buffer system 1, pH 6.6-6.7 and 36-38 %(v/v) precipitant mix 1 and flash-frozen in the same condition.

Data Collection and Structure Determination

Data were collected at Diamond Light Source (DLS) beamlines I04, and processed using the automated xia2 pipeline[5] and CCP4 program suite.[6] Initial phase was obtained by molecular replacement with PHASER[7] using the structure of Ub (PDB: 1UBQ) as the search model. Subsequently, CC-UIM and triazole linker were built manually into the electron density. Refinement was performed using COOT[8] *and* PHENIX.[9] All data processing and refinement statistics are presented in **Table S2**.

Table S2. Data collection and refinement statistics

	CC-UIM:Ub (Form 1)	CC-UIM:Ub (Form 2)
PDB code	9FJ4	9FJ3
Data collection		
Space group	C 1 2 1	P 1 21 1
Cell dimensions		
a, *b*, *c* (Å)	61.36, 49.71, 42.29	42.45, 50.09, 52.20
α, β, γ (°)	90.0, 123.78, 90.0	90, 101.26, 90
Resolution (Å)	35.60 – 1.54 (1.59 – 1.54)*	41.63-1.40 (1.44 – 1.40)*
R_{merge}	0.026 (0.724)	0.031 (0.757)
$I / \sigma I$	12.9 (1.0)	10.5 (1.4)
CC1/2	0.999 (0.618)	0.999 (0.781)
Completeness (%)	99.9 (100.0)	97.0 (94.4)
Redundancy	3.4 (3.3)	3.4 (3.6)
Refinement		
Resolution (Å)	35.60 – 1.54 (1.59 – 1.54)*	41.63-1.40 (1.44 – 1.40)*
No. reflections	15709	40902
R_{work} / R_{free}	0.2251 / 0.2664	0.2169 / 0.2377
No. atoms		
Protein	892	1825
Ligand	12	24
Water	45	202
B-factors		
Protein	32.58	29.99
Ligand	33.36	34.51
Water	38.87	37.72
R.m.s. deviations		
Bond lengths (Å)	0.006	0.004
Bond angles (°)	0.777	0.721

*Values in parentheses are for highest-resolution shell.

Triazole formation HPLC monitoring

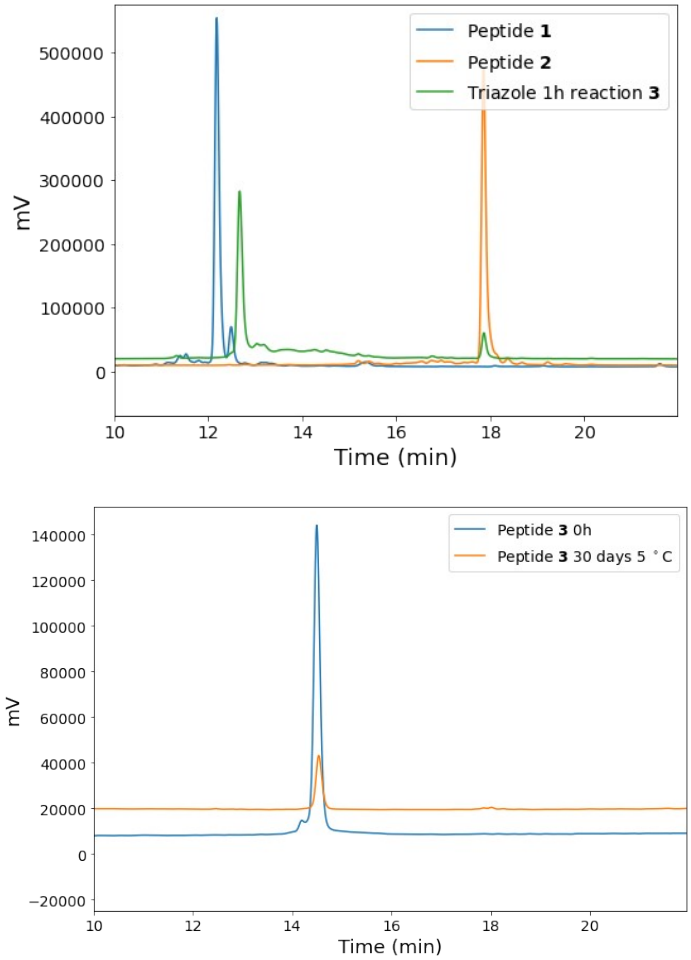


Figure S2: HPLC monitoring of triazole stability.

Peptide 3 CD Data

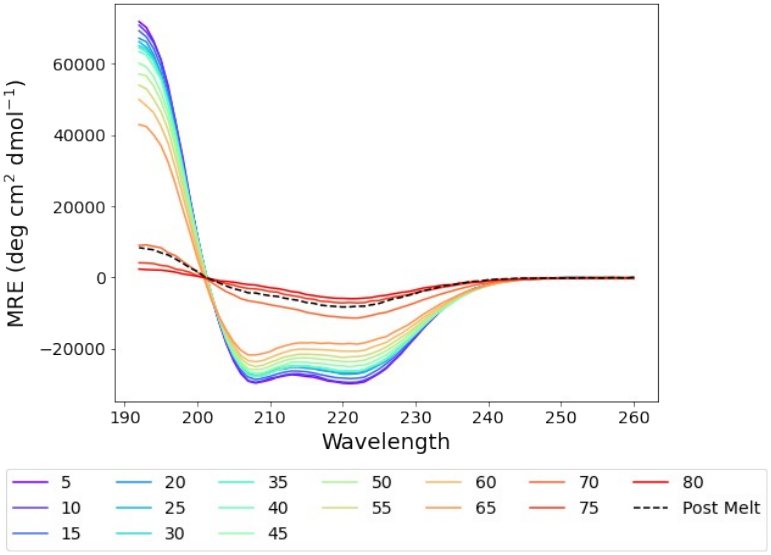


*Figure S3: Thermal melt CD spectra of peptide **3**.*

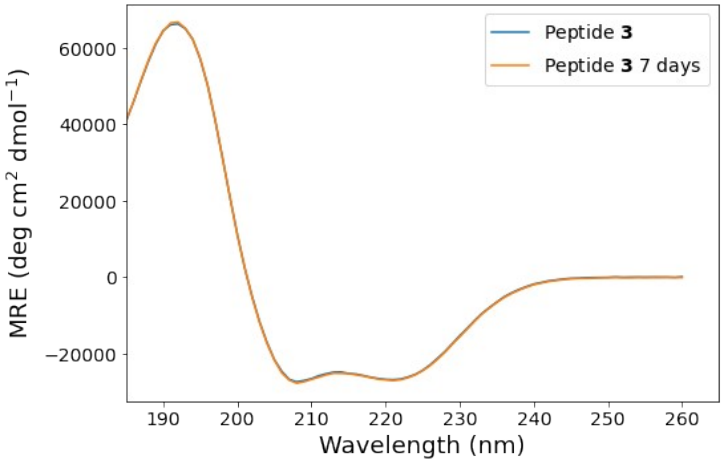


*Figure S4: CD spectra of peptide **3** and peptide **3** after 7 days at room temperature.*

Peptides S1-S4 FP and CD

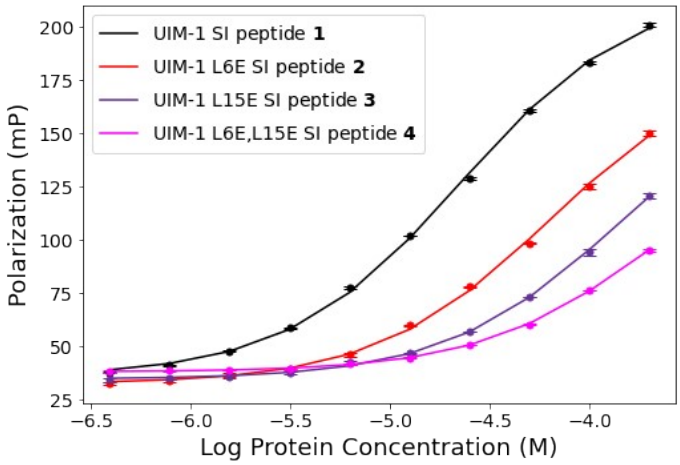


Figure S5: FP binding assays for peptides S1-S4. K_d values from fitting:

*Peptide **S1**, 21.5 ± 1.4 μM*

*Peptide **S2**, 67.2 ± 2.4 μM;*

*Peptide **S3**, 135.8 ± 4.9 μM;*

*Peptide **S4,** 195.7 ± 6.0 μM;*

*Note that peptide **S1** binds more strongly than the equivalent side chain labelled **12**, probably due to nonspecific interactions between the C-terminal tail of Ub and the fluorophore. We have made use of this enhanced affinity to increase the dynamic range of the titration experiments, on the assumption that the structurally similar series of peptides **S1**-**S4** are likely to be affected to the same degree.*

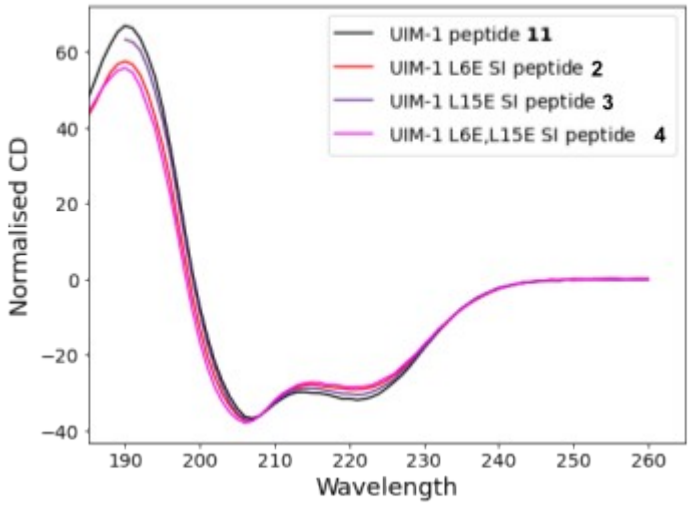


*Figure S6: CD comparison of peptides **11** and **S3-S4.***

Data are normalized to CD signal at 222 nm

Peptide 9 CD Data

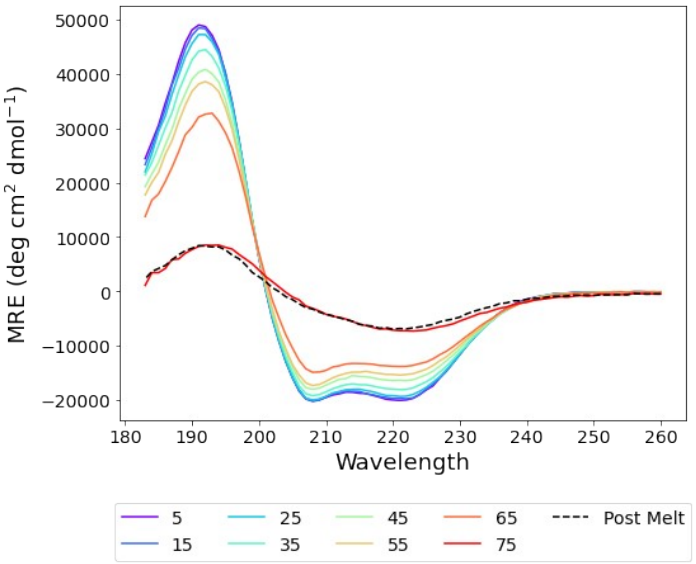


*Figure S7: Thermal melt CD spectra of peptide **9**.*

Peptide 10 FP data

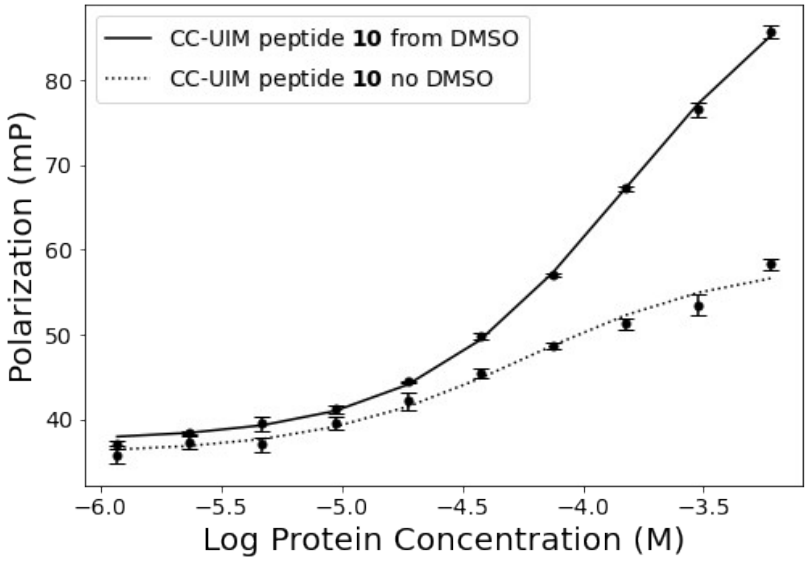


*Figure S8: Titration of peptide **10** against Ub, with peptide stocks in DMSO vs water*

Crystal Structure Views of Ub:CC-UIM Complex

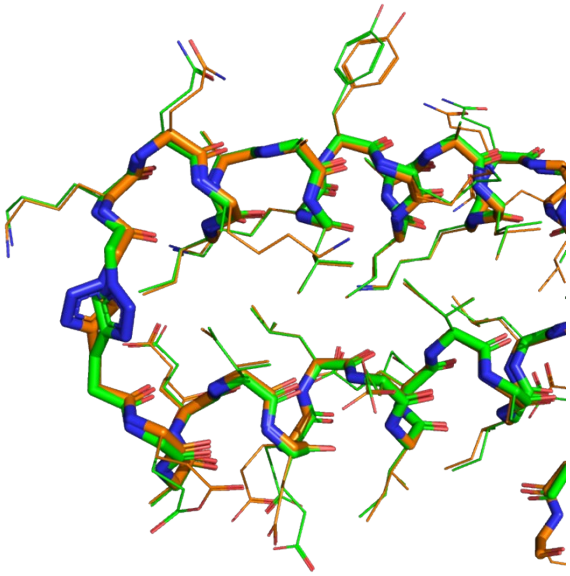


Figure S9: Overlay of two conformations of the CC-UIM triazole linker. The C121 crystal is shown in green and the P121 is shown in orange.

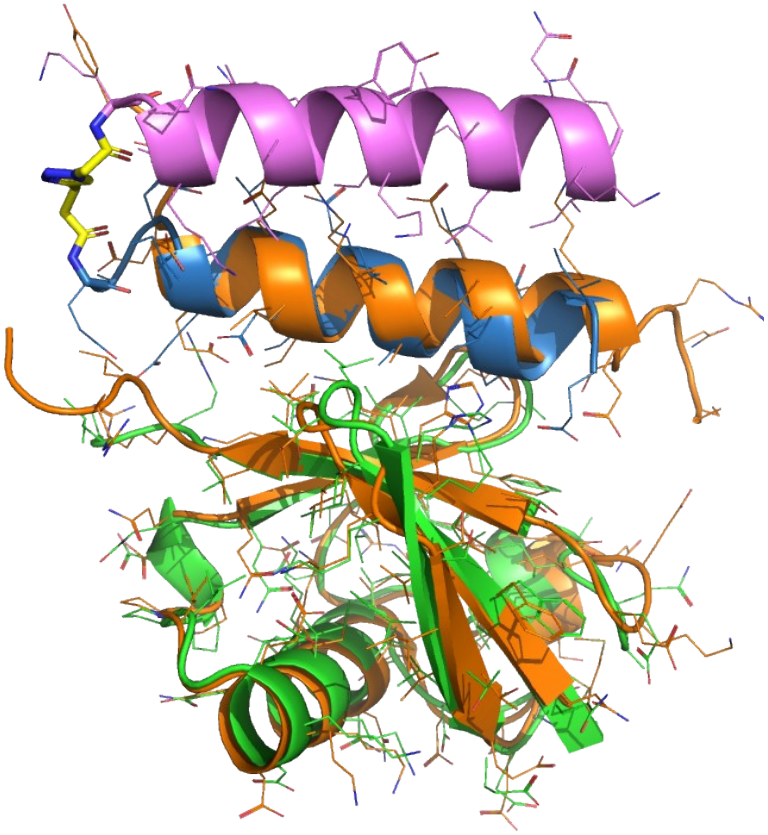


Figure S10: Overlay of Ub:CC-UIM crystal structure P121 form with NMR structure of the Ub:Vps27 UIM-1 complex (orange).

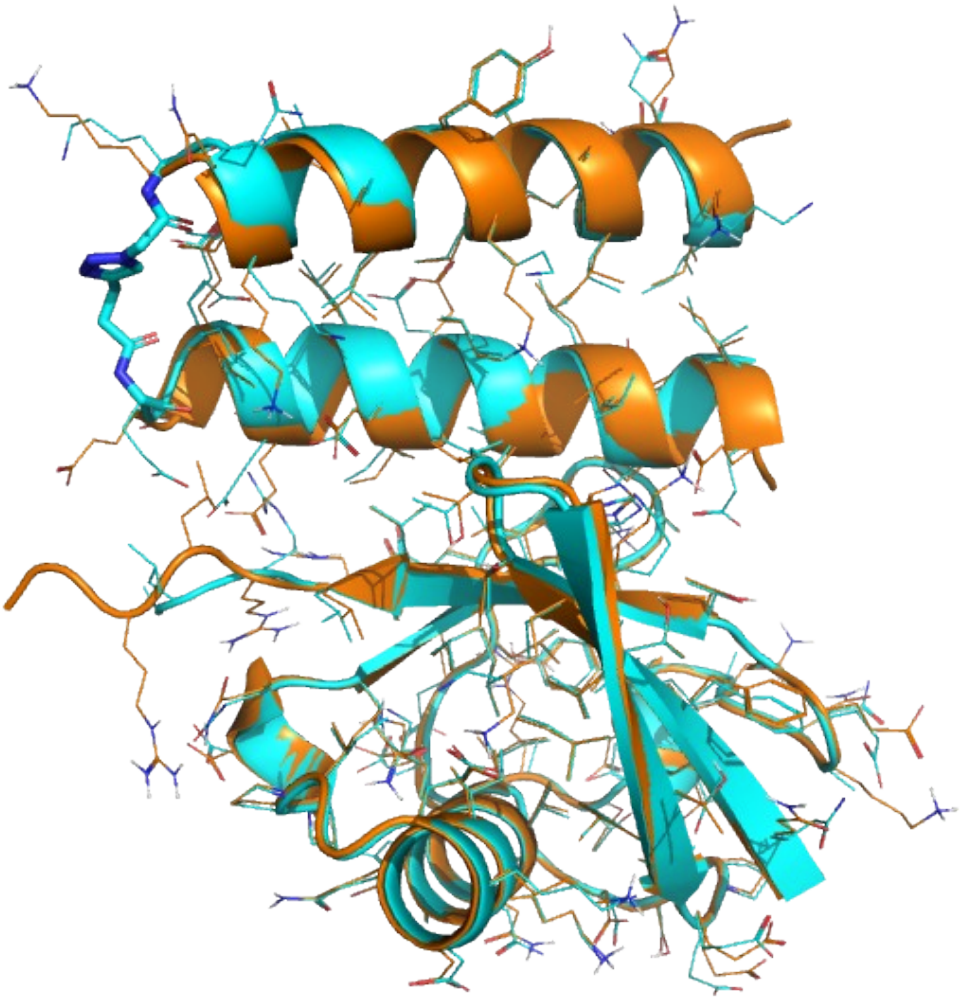


Figure S11: Overlay of CC-UIM crystal structure P121 form (cyan) with AlphaFold Multimer model of the same peptide sequence (orange).

HPLC Characterization for all peptides

Peptide 1

14.64 min 84.86% (50 min gradient, 20-80% B).

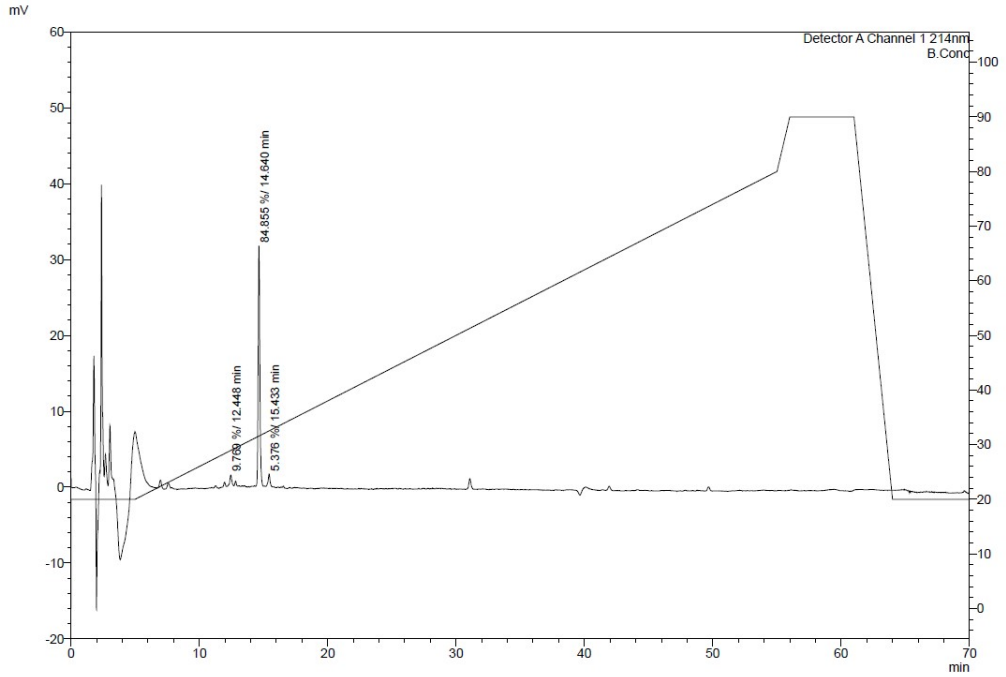


*Figure S12: 50 min gradient 20-80% B HPLC for peptide **1***

Peptide 2

26.30 min 93.24% (50 min gradient, 30-60% B).

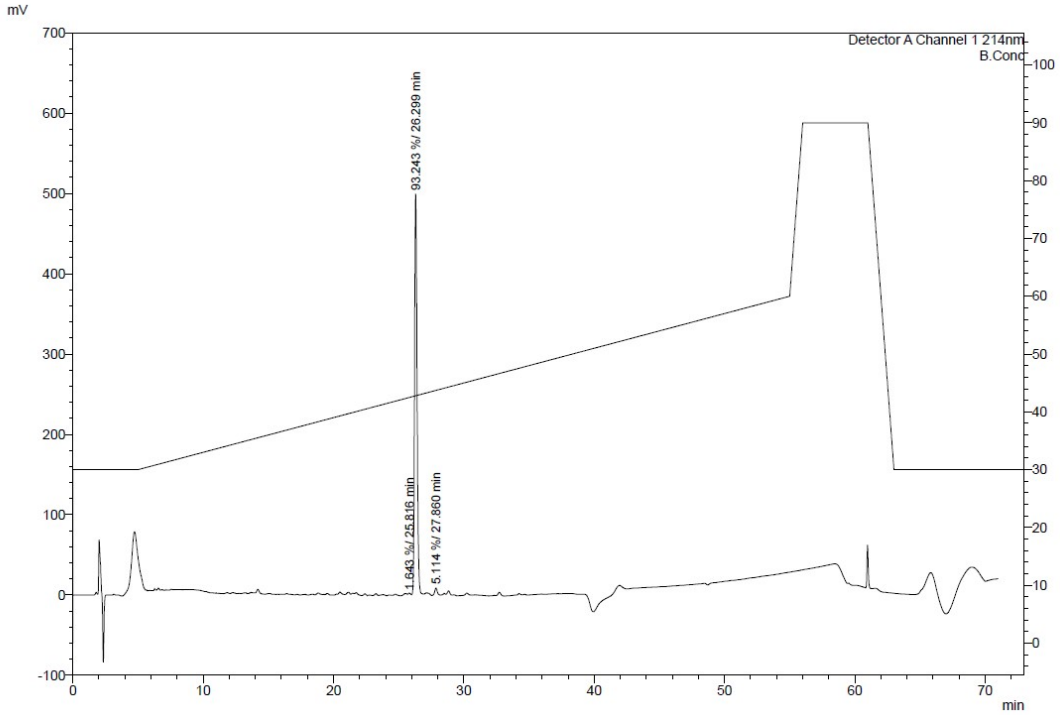


*Figure S13: 50 min gradient 20-80% B HPLC for peptide **2.***

Peptide 3
16.14 min 97.07% (50 min gradient, 20-80% B).

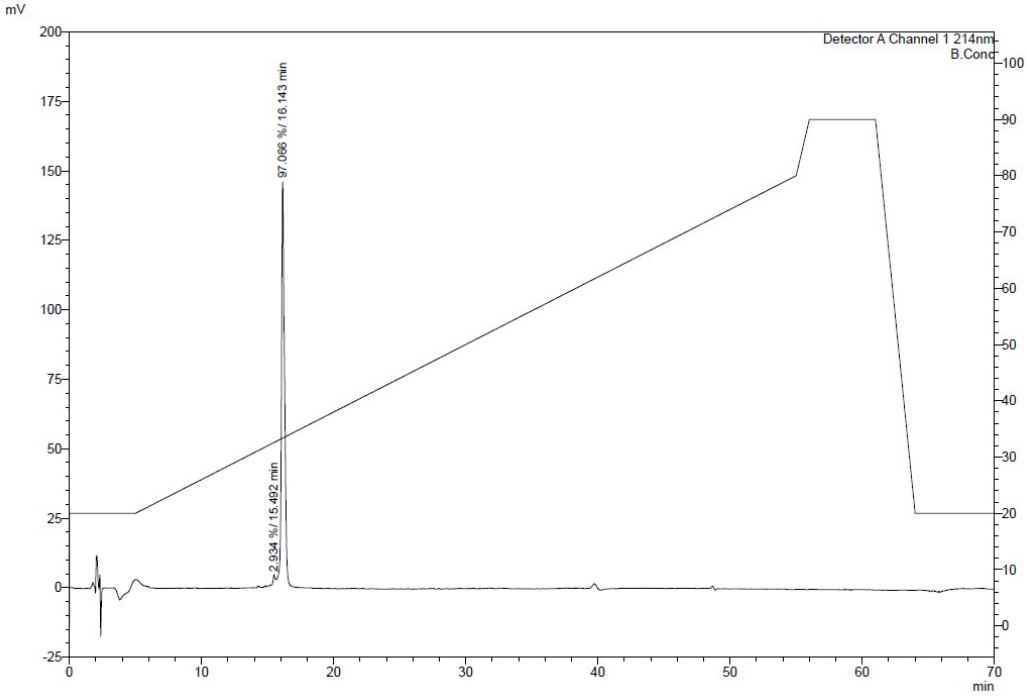


*Figure S14: 50 min gradient 20-80% B HPLC for peptide **3***

Peptide 4
25.13 min 94.16% (50 min gradient, 10-60% B).

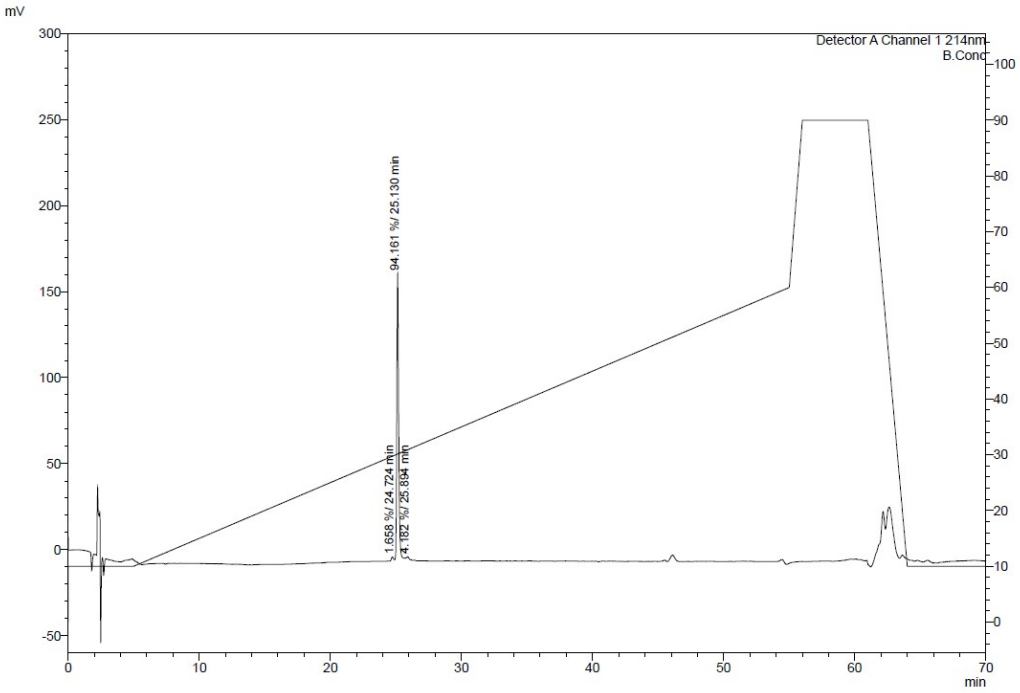


*Figure S15: 50 min gradient 10-60% B HPLC for peptide **4**.*

Peptide 5
26.75 min 93.90% (50 min gradient, 20-80% B).

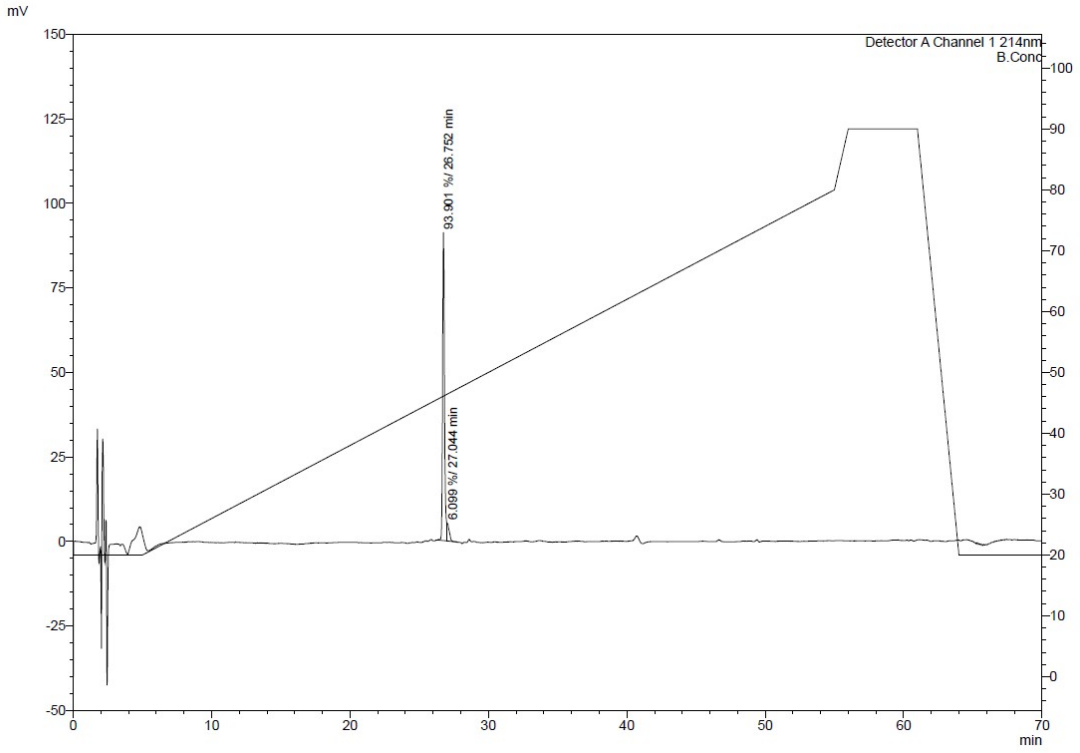


*Figure S16: 50 min gradient 20-80% B HPLC for peptide **5**.*

Peptide 6
27.94 min 91.75% (20 min gradient, 40-80% B).

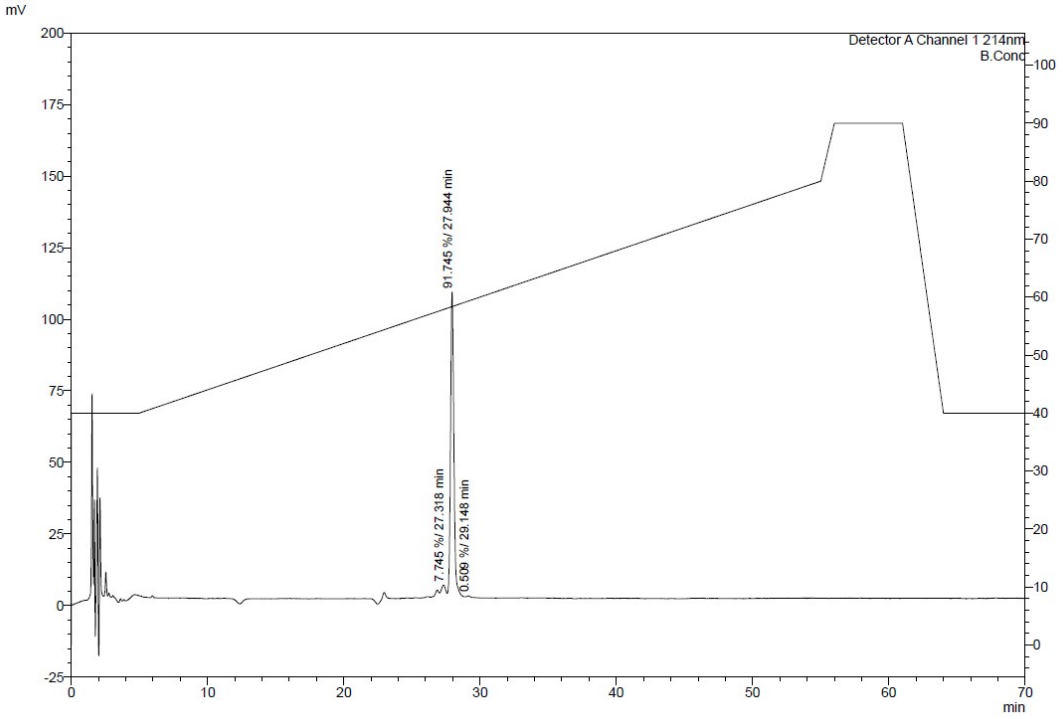


*Figure S17: 50 min gradient 40-80% B HPLC for peptide **6**.*

Peptide 7
17.87 min 100% (50 min gradient, 20-80% B).

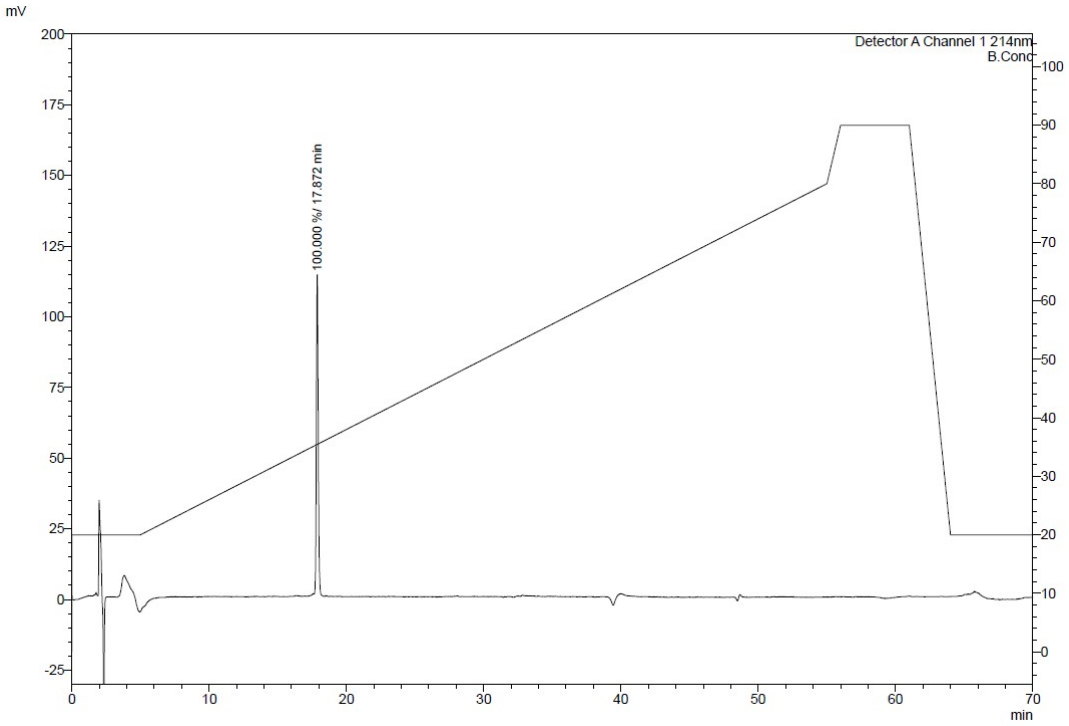


*Figure S18: 50 min gradient 20-80% B HPLC for peptide **7**.*

Peptide 8
15.58 min 97.08% (20 min gradient, 20-60% B).

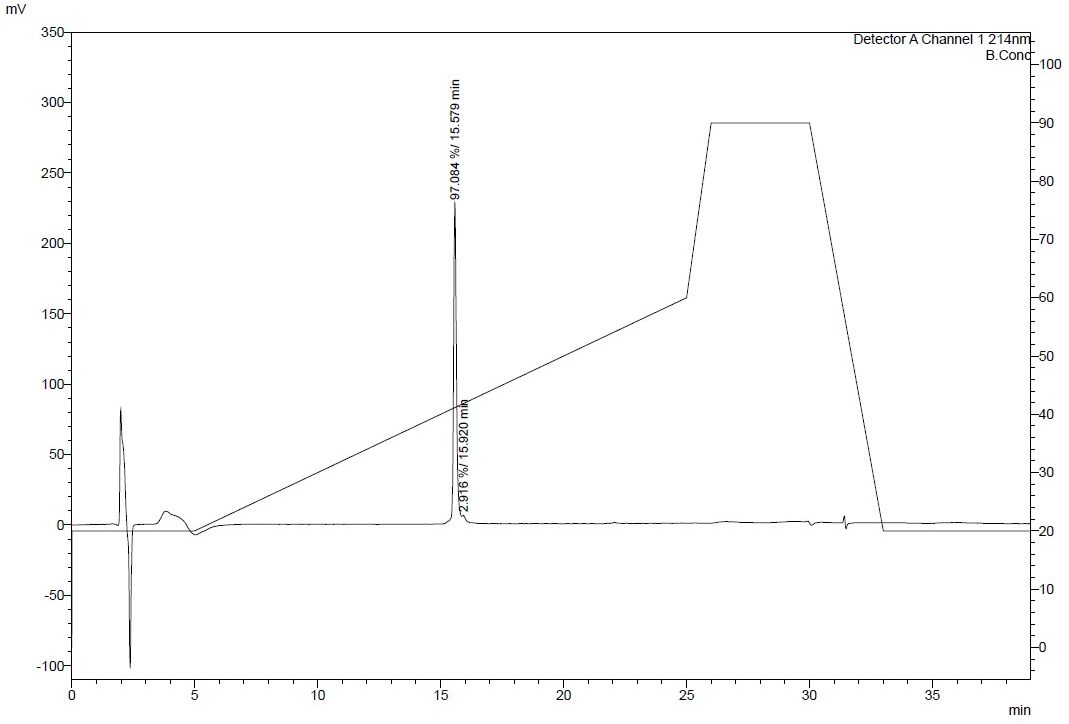


*Figure S19: 50 min gradient 20-60% B HPLC for peptide **8**.*

Peptide 9
25.66 min 100% (50 min gradient, 20-60% B).

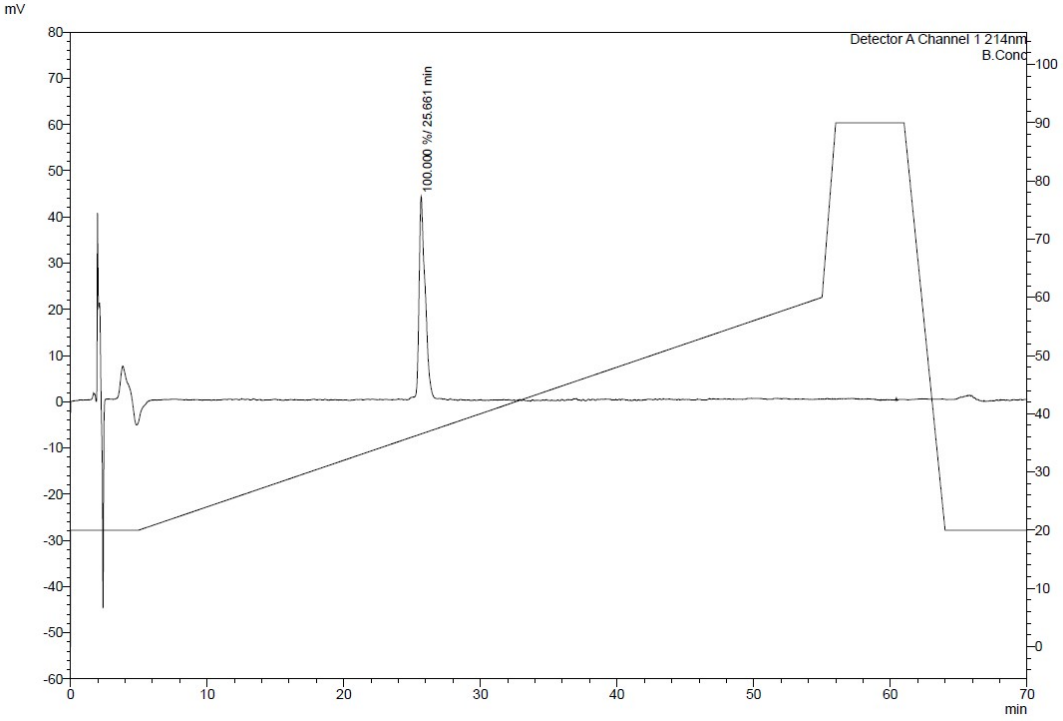


*Figure S20: 50 min gradient 20-60% B HPLC for peptide **9**.*

Peptide 10
13.43 min 97.06% (50 min gradient, 30-60% B).

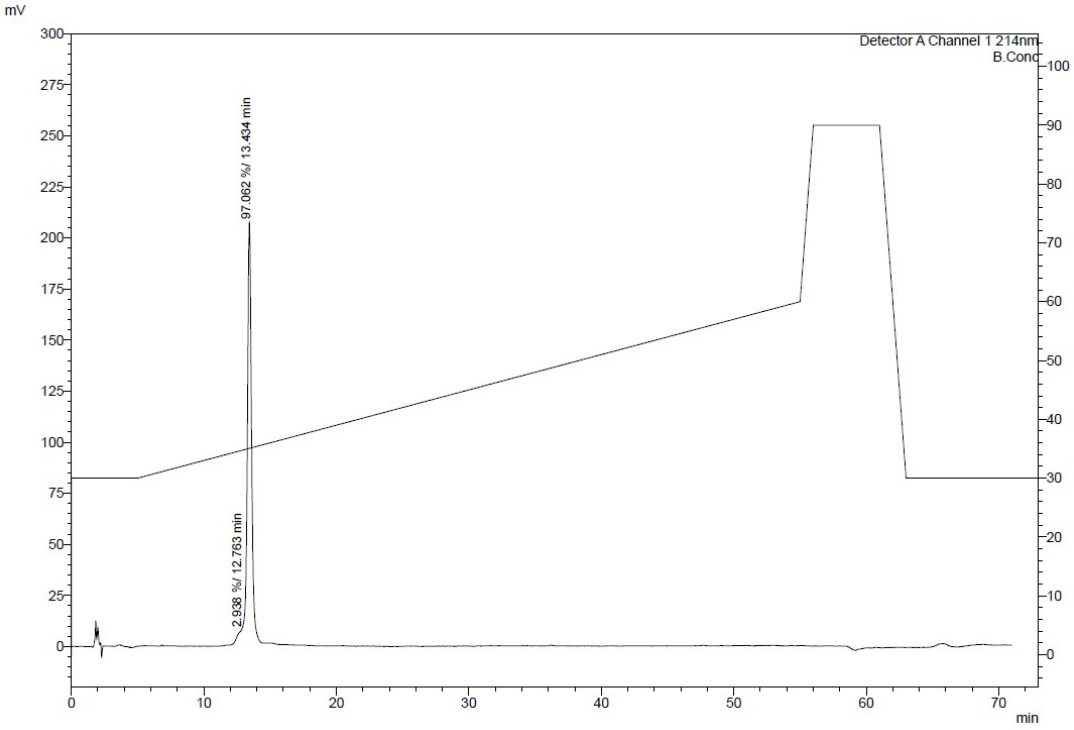


*Figure S21: 50 min gradient 30-60% B HPLC for peptide **10**.*

Peptide 11
31.46 min 93.99% (50 min gradient, 20-60% B).

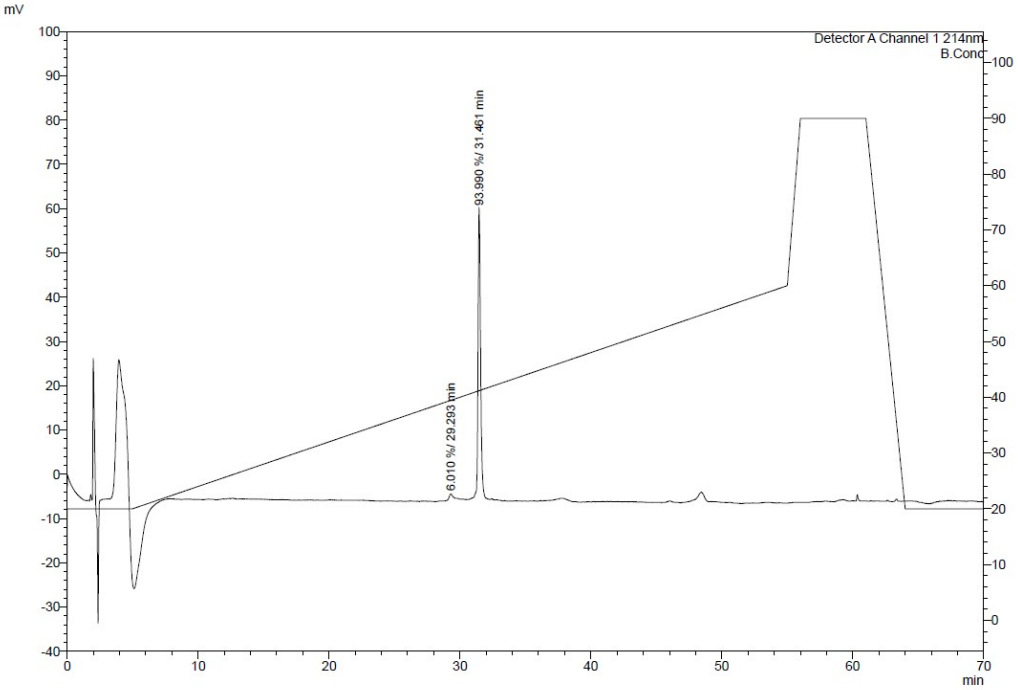


*Figure S22: 50 min gradient 20-60% B HPLC for Peptide **11**.*

Peptide 12
31.46 min 93.99% (50 min gradient, 20-60% B).

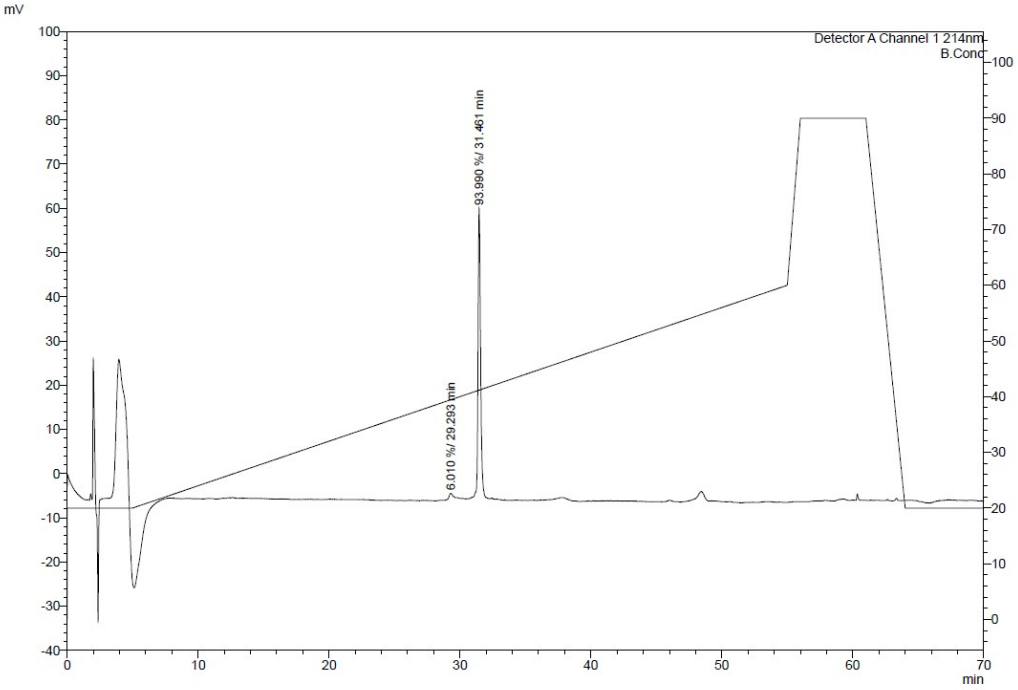


*Figure S23: Figure: 50 min gradient 20-60% B HPLC for peptide **12**.*

Peptide S1
35.96 min 100% (50 min gradient, 20-50% B).

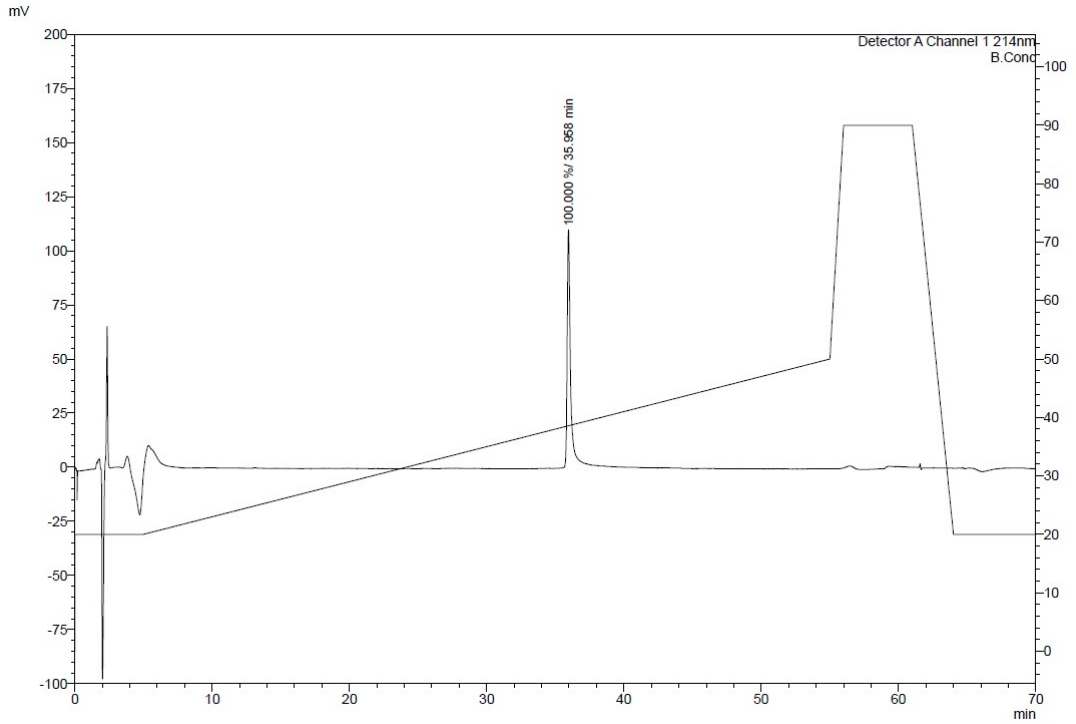


Figure S24: 50 min gradient 20-50% B HPLC for S1.

Peptide S2
32.39 min 100% (50 min gradient, 20-50% B).

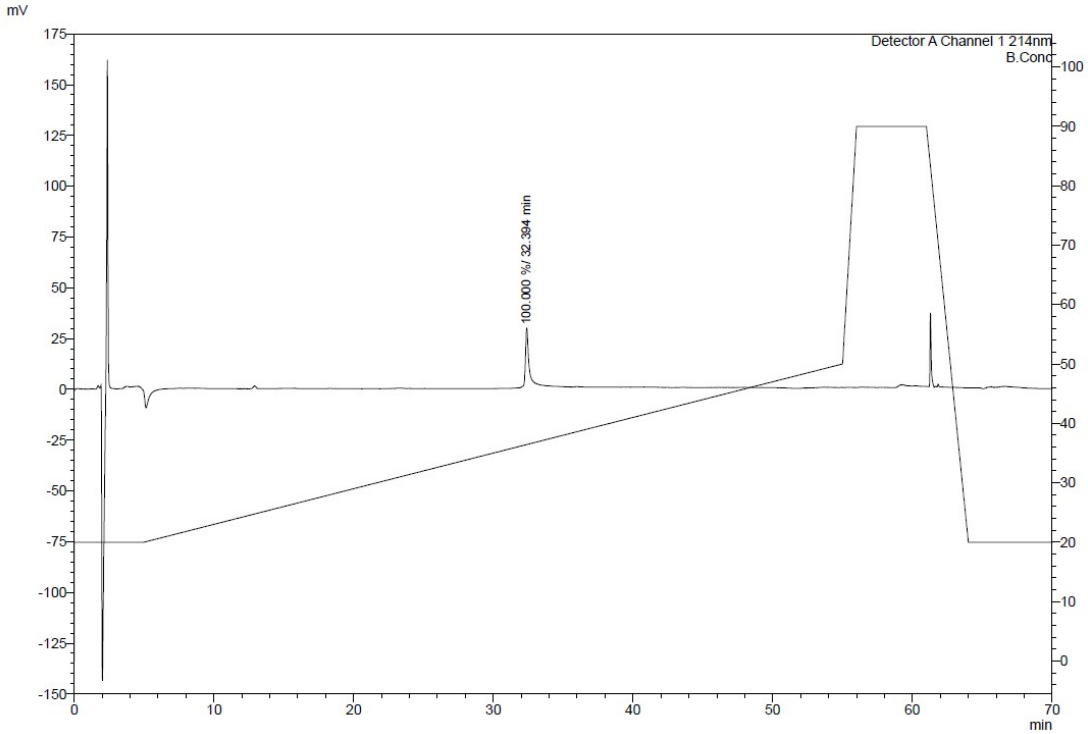


Figure S25: 50 min gradient 20-50% B HPLC for peptide S2.

Peptide S3
32.62 min 96.25% (50 min gradient, 20-50% B).

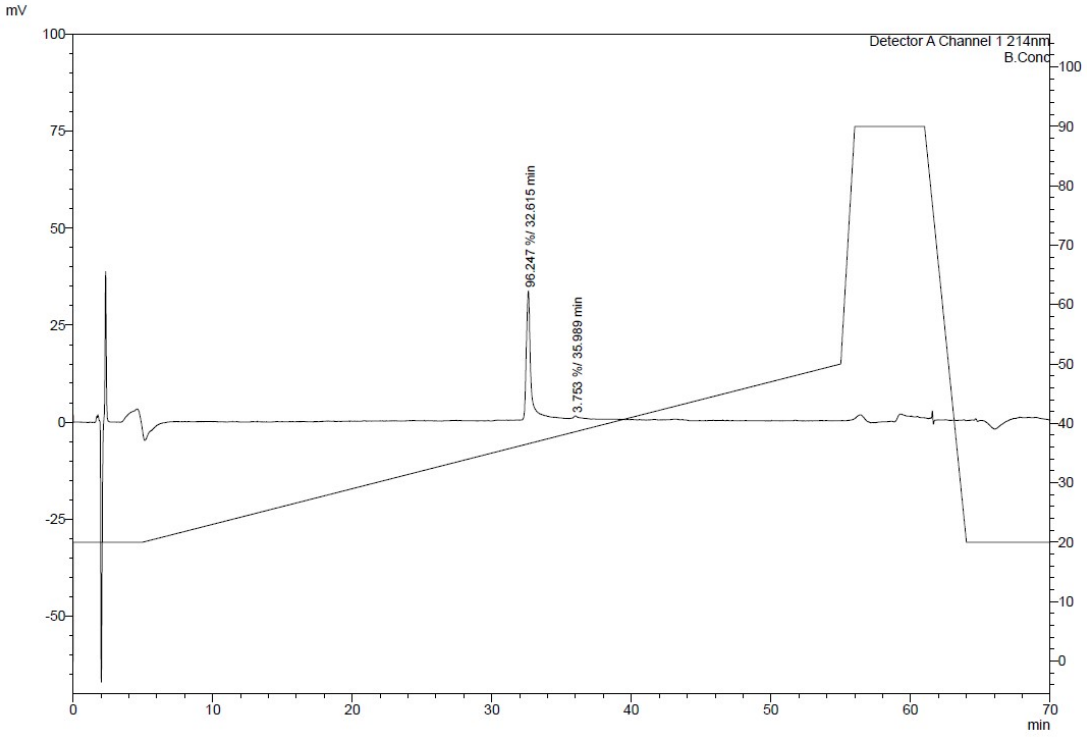


Figure S26: 50 min gradient 20-50% B HPLC for peptide S3.

Peptide S4
27.98 min 91.60% (50 min gradient, 20-50% B).

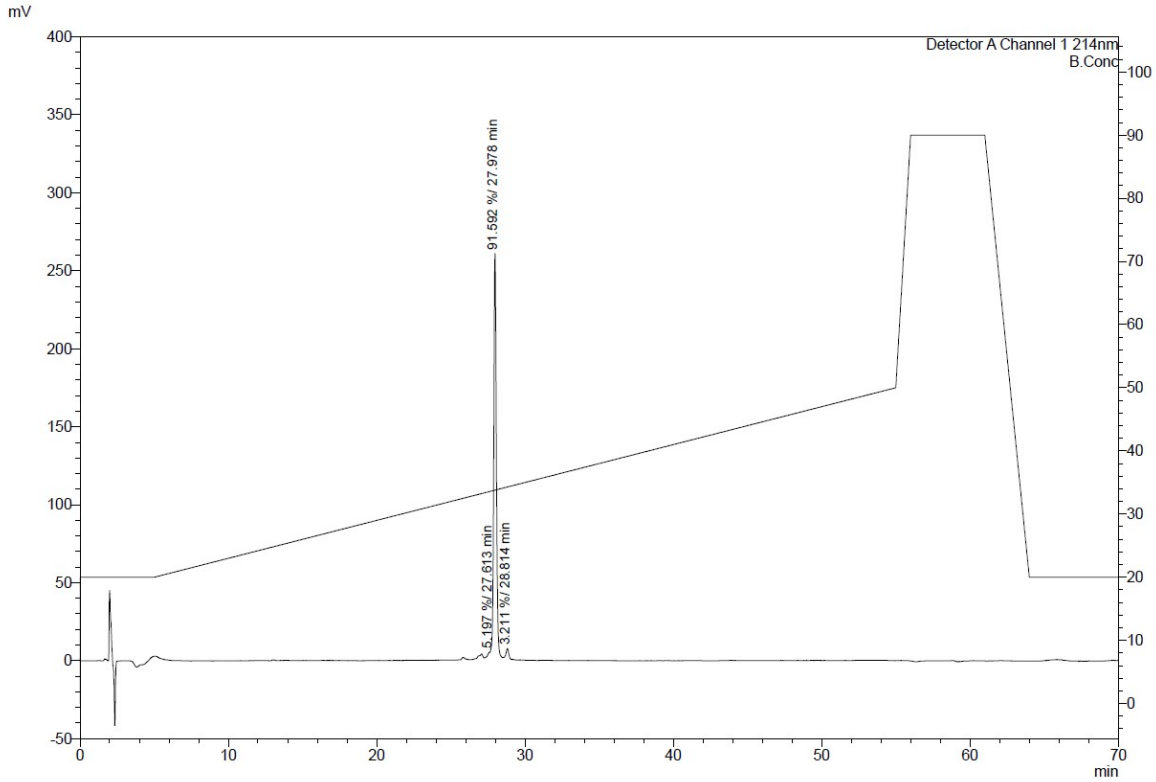


Figure S27: 50 min gradient 20-50% B HPLC for peptide S4.

Peptide S5
26.36 min 95.04% (50 min gradient, 20-50% B).

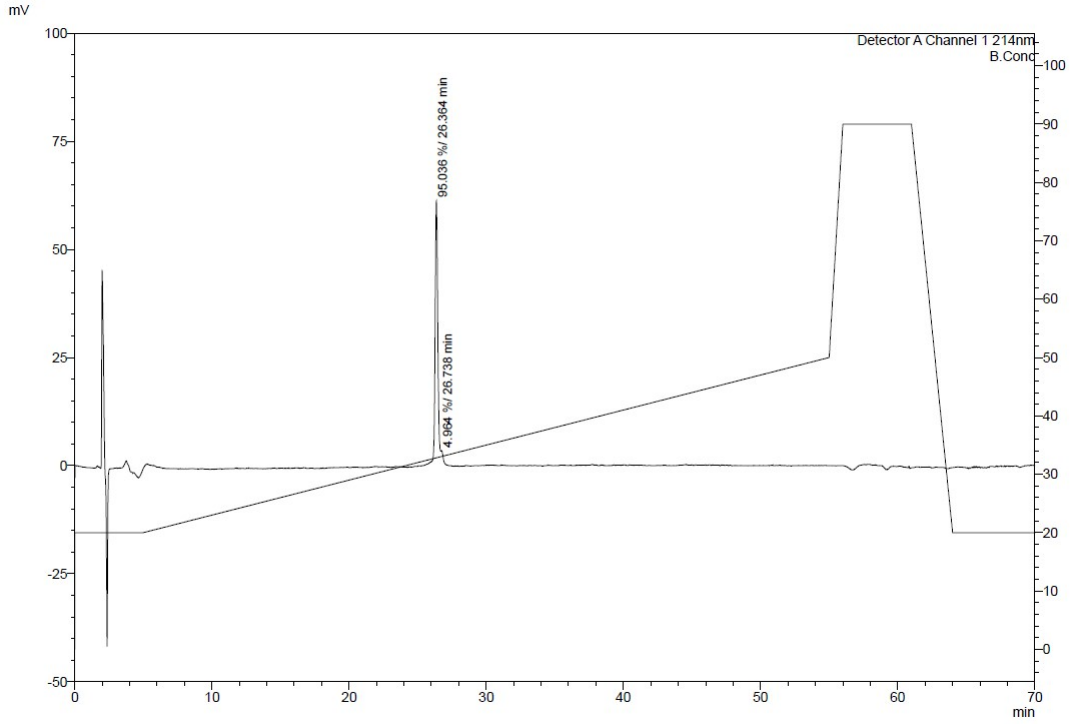


*Figure S28: 50 min gradient 20-50% B HPLC for peptide S**5**.*

Peptide S6
26.24 min 92.93% (50 min gradient, 20-50% B).

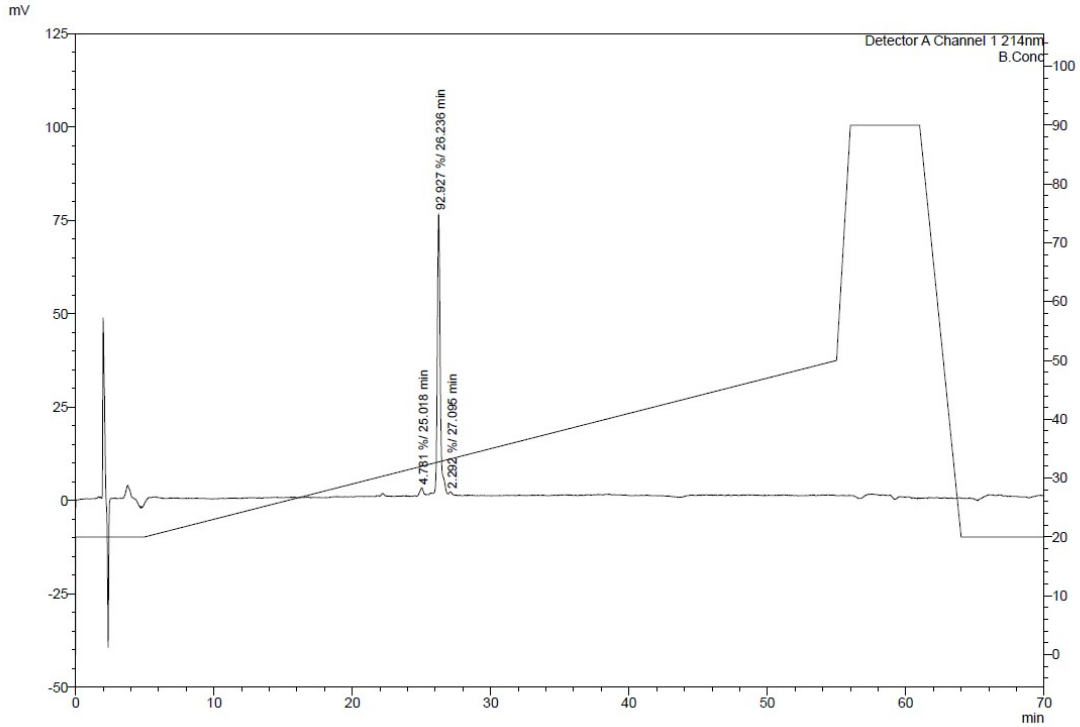


*Figure S29: 50 min gradient 20-50% B HPLC for peptide S**6**.*

Peptide S7
21.03 min 92.17% (50 min gradient, 20-50% B).

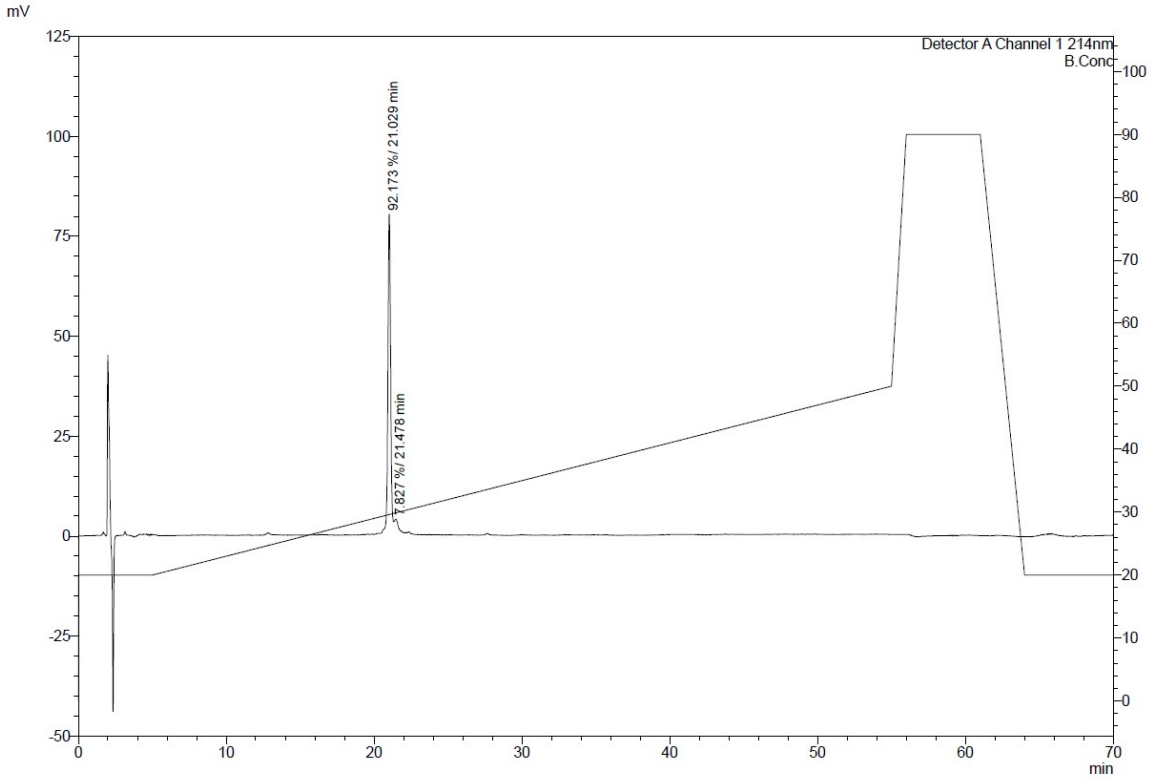


Figure S30: 50 min gradient 20-50% B HPLC for peptide S7.

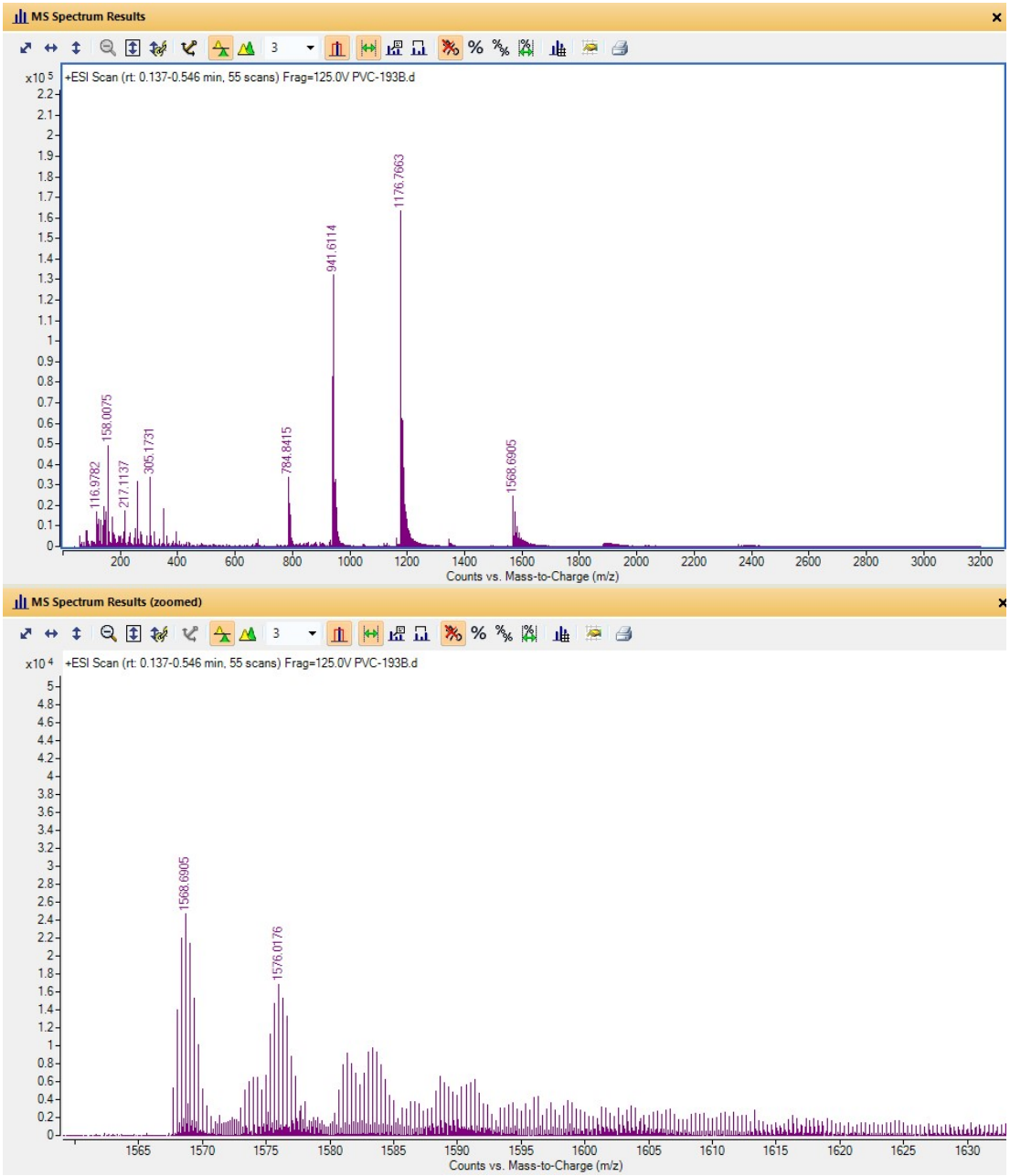


*Fig S31: Detailed view of Mass spectrometry data for peptide **9** showing $[M+3H]^{3+}$ peak.*

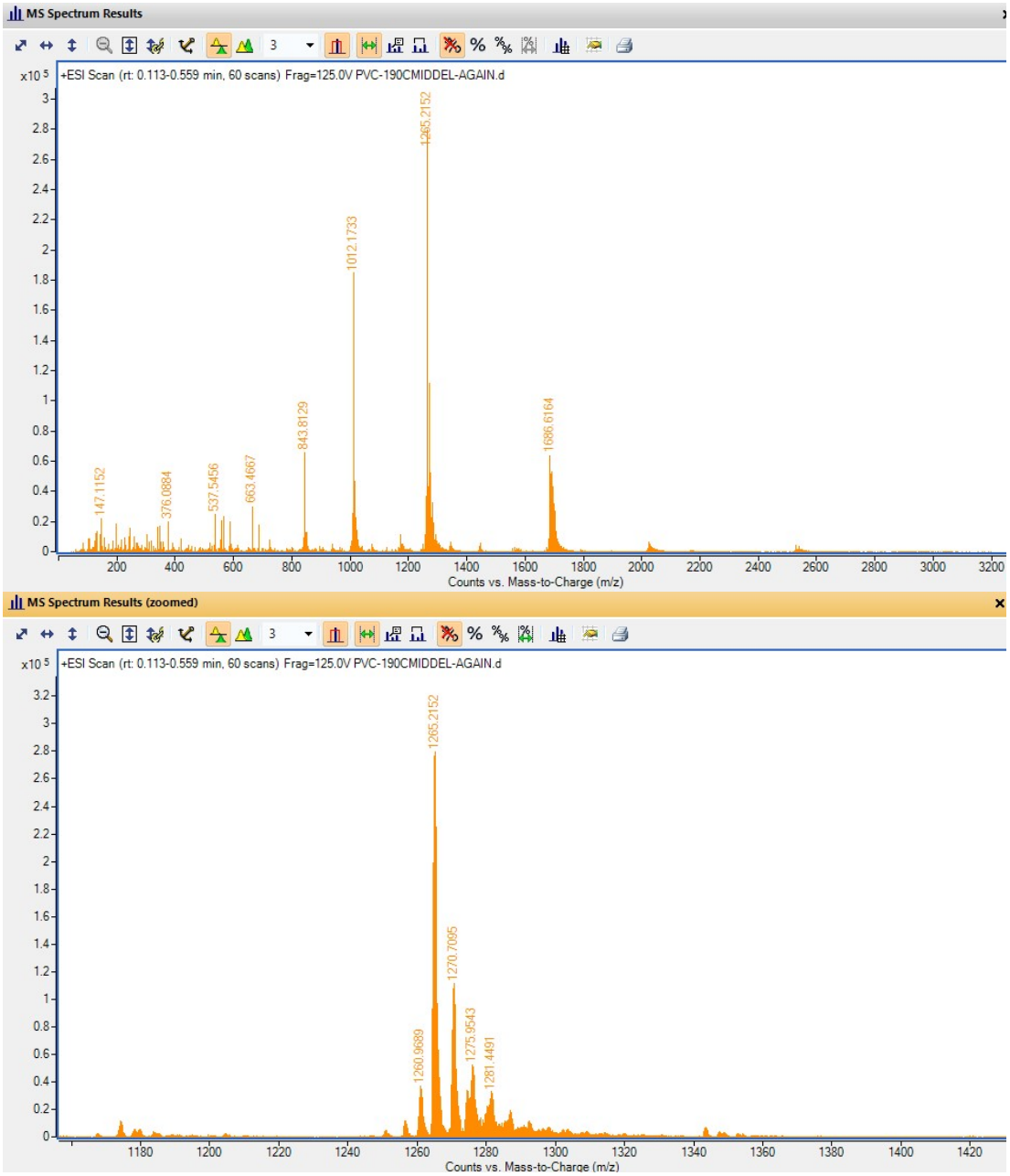


Figure S32: Detailed view of mass spectrometry data for peptide **10** *showing* $[M+4H]^{4+}$ *peak*.

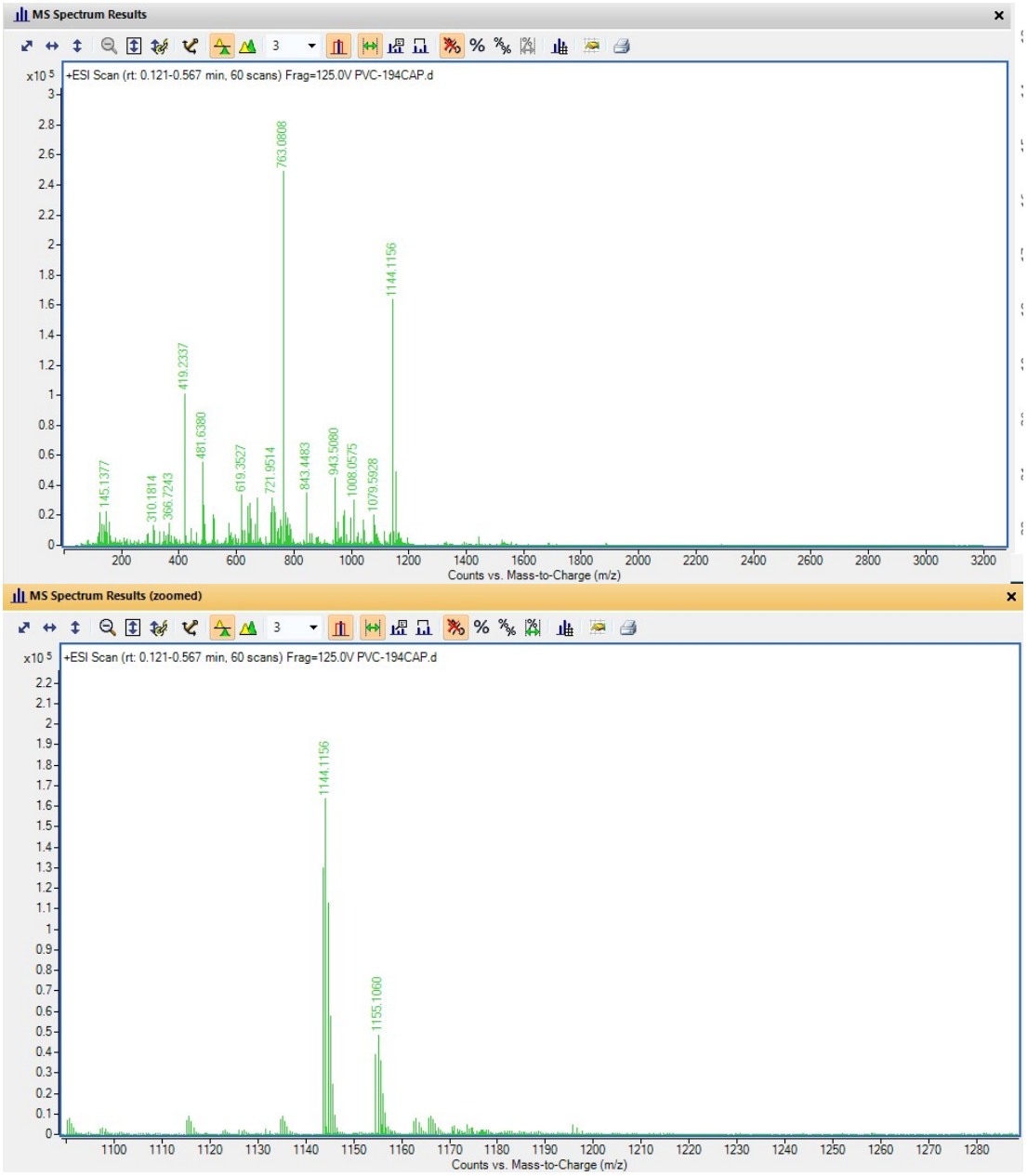


Figure S33: Detailed view of mass spectrometry data for peptide **11** *showing* $[M+2H]^{2+}$ *peak*.

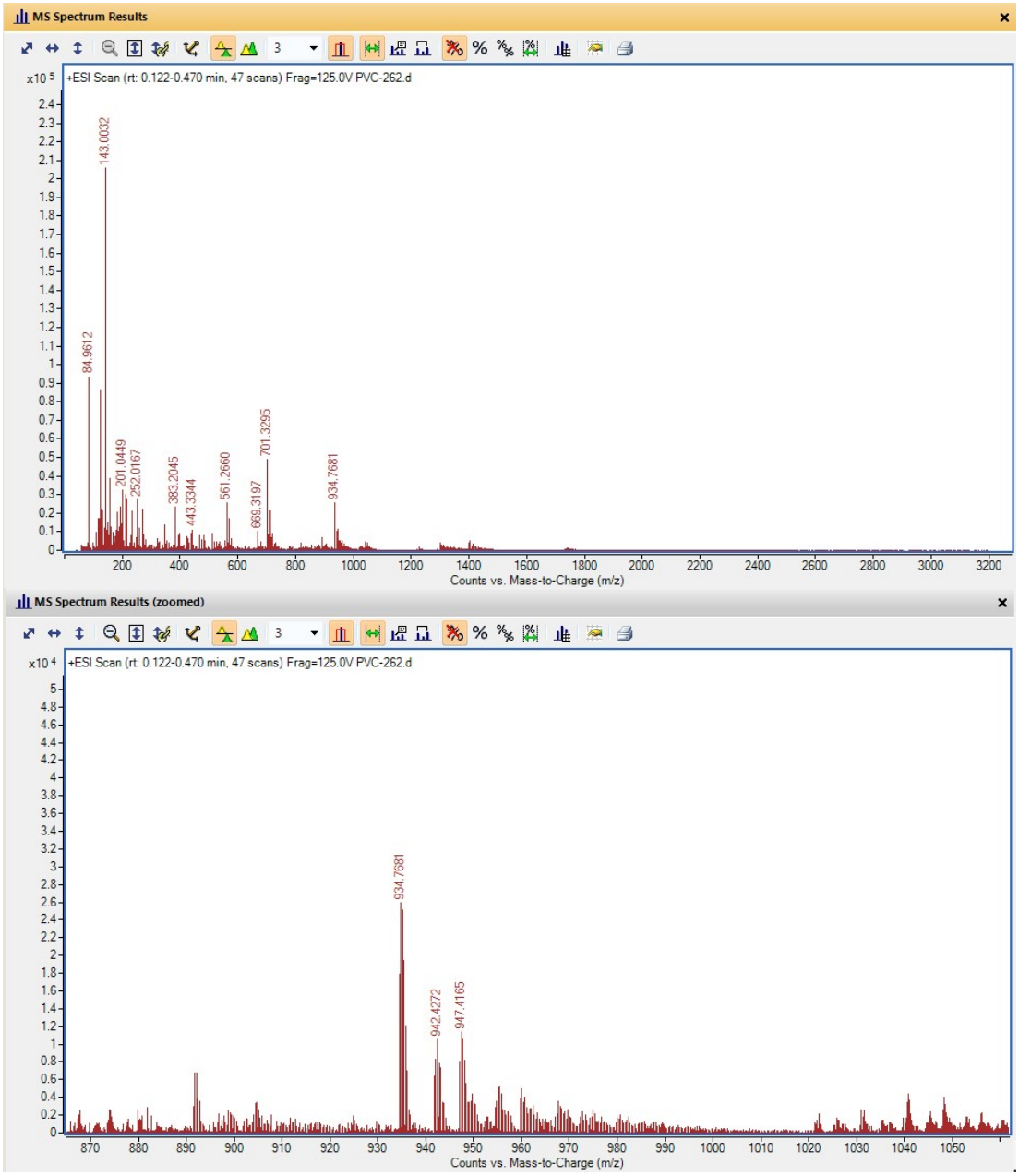


*Figure S34: Detailed view of mass spectrometry data for peptide **12** showing $[M+3H]^{3+}$ peak.*

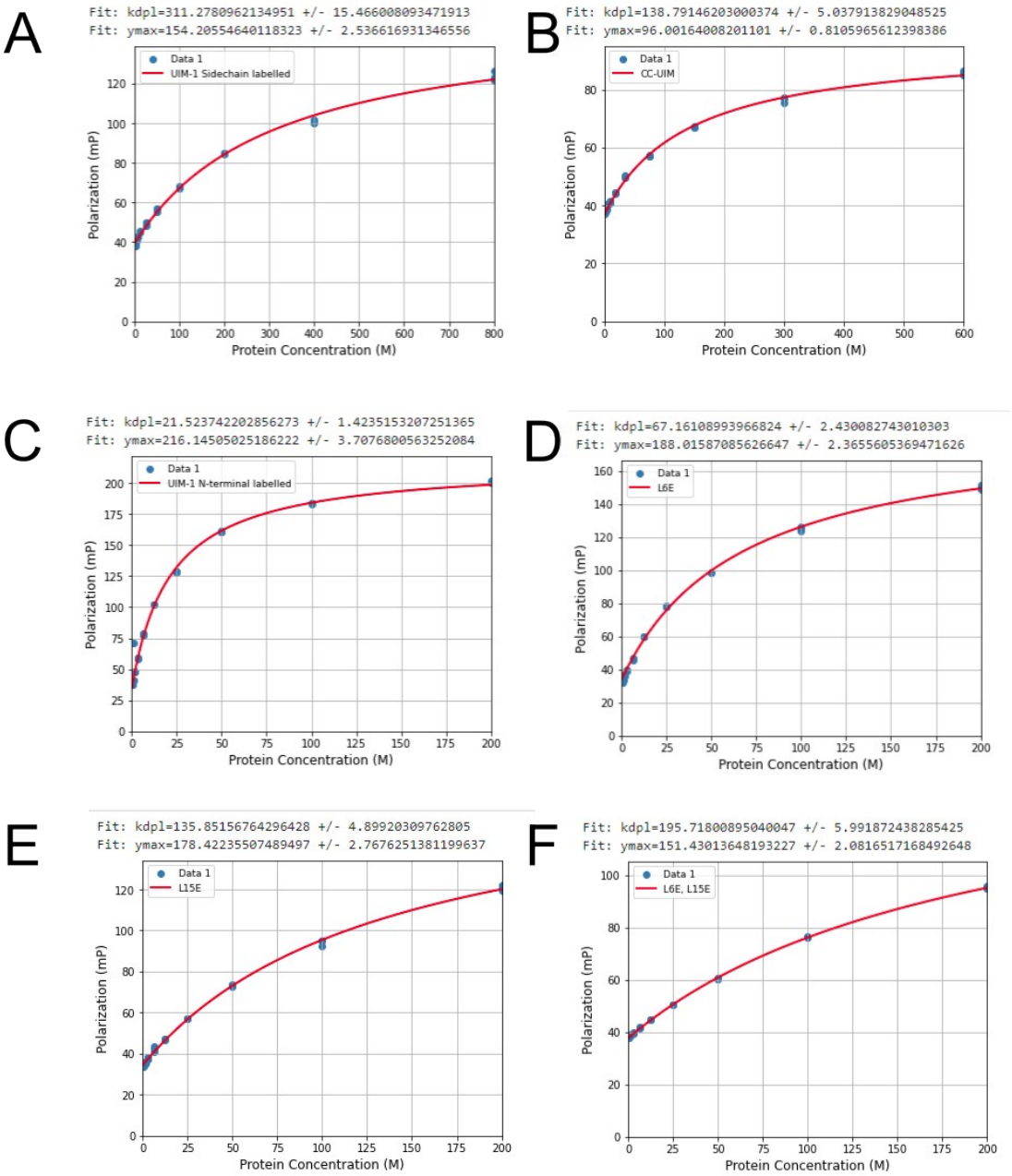


Figure S35: FP titration fits for A: peptide 12, B: peptide 10, C: peptide S1, D: peptide S2, E: peptide S3 and F: peptide S4

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