Electronic Supplementary Information for

Discovery of reactive peptide inhibitors of human papillomavirus oncoprotein E6

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[§] Author Jason Tao was unable to be contacted following the changes at submission of the revised manuscript, therefore they do not take any responsibility for the changes in the revised manuscript.

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Contents

1. Supplementary figures and tables

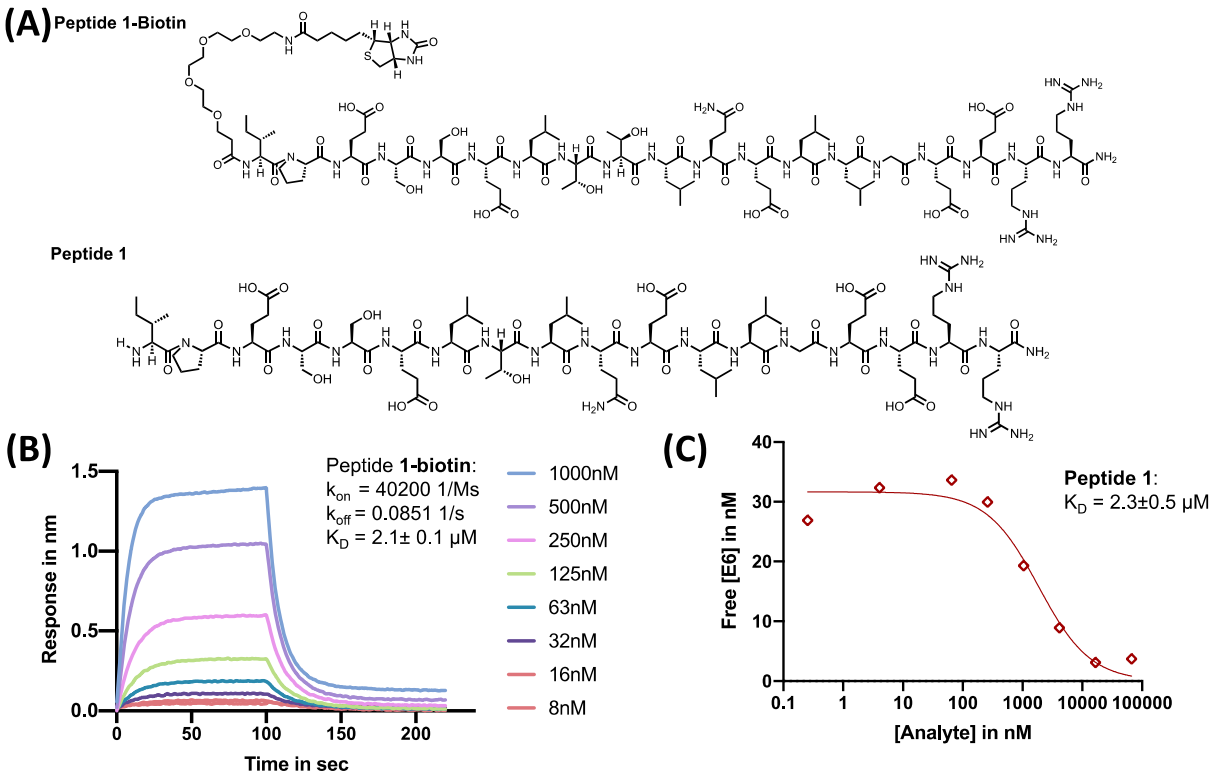


Figure S1. Determination of binding constant between peptide **1** and 16E6 by direct binding assay in BLI and competition BLI binding assay. (**A**) Structure of peptide **1** and peptide **1-biotin**. (**B**) BLI measurement of **1-biotin** and 16E6, with estimated parameters: k_{on} = 40200 $M^{-1}s^{-1}$, k_{off} = 0.0851 $s^{-1}$and K_D = 2.1± 0.1 µM (N=3). MBP-16E6 protein concentration used in each channel is indicated on the right. k_{on} is the on-rate constant, k_{off} is the off-rate constant, K_D is the kinetic apparent dissociation constant. **(C)** Peptide **1** binding was assessed by competition binding assay. Various concentrations of **1** were mixed with 30 nM MBP-16E6 protein. Peptide **1-biotin** was immobilized onto the streptavidin sensor tips to compete for MBP-16E6 protein with various concentrations of unlabeled peptide **1** in the solution (33,333 nM, 8,333 nM, 2,083 nM, 520 nM, 130 nM, 32 nM, 2.0 nM or 0.1 nM). Competition K_D was estimated as 2.3 ± 0.5 µM. Error is the quadratic curve fitting standard error of the mean (SEM) reported by the Prism 8 software (N=3).

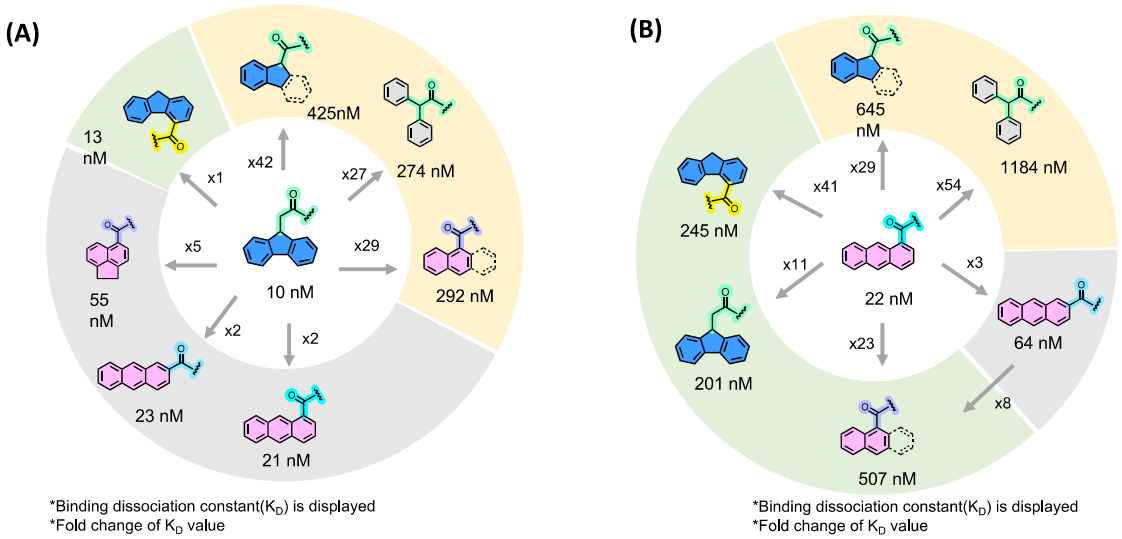


Figure S2. Structure-activity-relationship (SAR) study of small molecule modifications. (**A**) N-terminal modifications SAR. Green: modification is not sensitive to ring substitution position. Grey: tricyclic, planar, and aromatic molecules improve the affinity to the low nano-molar range. Yellow: Removing a phenyl ring from the structure or breaking the strain between two phenyl moieties will decrease the binding by 30-50-fold. (**B**) C-terminal modifications SAR. Green: binding improvement of anthracene modification is not sensitive to ring substitution position. Green: two phenyl rings are required for a mid-micromolar binding. Yellow: Removing a phenyl ring from the structure, breaking the strain between two phenyl moieties, will decrease the binding by 30-50-fold. K_D values are measured by BLI binding assay in a competition mode.

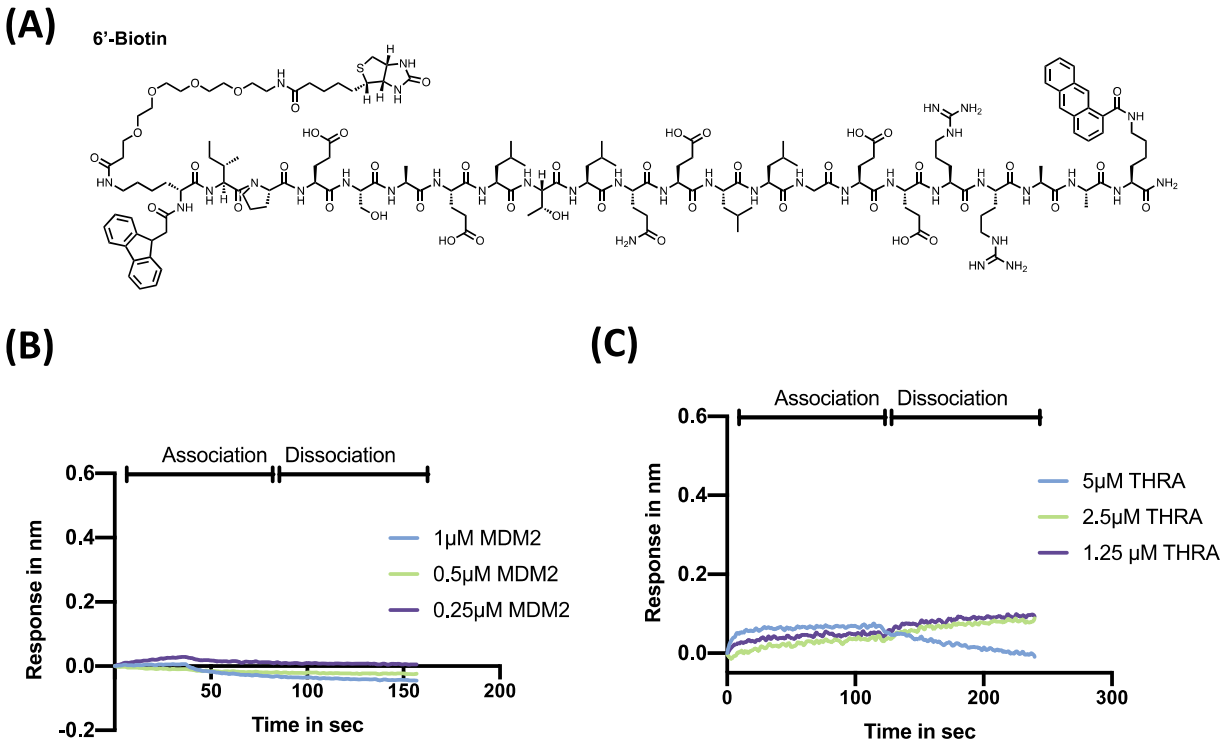


Figure S3. (**A**) Structure of **6'-biotin**. (**B**) Direct BLI binding assay of **6'-biotin** against SUMO-MDM2. No observable association and dissociation were observed (N=2). (**C**) Direct BLI binding assay of **6'-biotin** against THRA. No observable association and dissociation were observed (N = 2).

Entry	Name	N term	Spacer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Binding affinity in nM	Ratio to WT
1	IPES	-	bAla	I	P	E	S	S	E	L	T	L	Q	E	L	L	G	E	E	R	2589±1394	-
2	N-FITC	FITC	bAla	I	P	E	S	S	E	L	T	L	Q	E	L	L	G	E	E	R	186±32	1
3	A1	FITC	bAla	**A**	P	E	S	S	E	L	T	L	Q	E	L	L	G	E	E	R	944±182	5
4	A2	FITC	bAla	I	**A**	E	S	S	E	L	T	L	Q	E	L	L	G	E	E	R	691±157	4
5	A3	FITC	bAla	I	P	**A**	S	S	E	L	T	L	Q	E	L	L	G	E	E	R	638±54	3
6	A4	FITC	bAla	I	P	E	**A**	S	E	L	T	L	Q	E	L	L	G	E	E	R	739±113	4
7	A5	FITC	bAla	I	P	E	S	**A**	E	L	T	L	Q	E	L	L	G	E	E	R	125±17	0.6
8	A6	FITC	bAla	I	P	E	S	S	**A**	L	T	L	Q	E	L	L	G	E	E	R	1706±209	9
9	A7	FITC	bAla	I	P	E	S	S	E	**A**	T	L	Q	E	L	L	G	E	E	R	1114±152	6
10	A8	FITC	bAla	I	P	E	S	S	E	L	**A**	L	Q	E	L	L	G	E	E	R	3748±680	20
11	A9	FITC	bAla	I	P	E	S	S	E	L	T	**A**	Q	E	L	L	G	E	E	R	>10000	-
12	A10	FITC	bAla	I	P	E	S	S	E	L	T	L	**A**	E	L	L	G	E	E	R	242±31	1
13	A11	FITC	bAla	I	P	E	S	S	E	L	T	L	Q	**A**	L	L	G	E	E	R	3158±568	16
14	A12	FITC	bAla	I	P	E	S	S	E	L	T	L	Q	E	**A**	L	G	E	E	R	>10000	-
15	A13	FITC	bAla	I	P	E	S	S	E	L	T	L	Q	E	L	**A**	G	E	E	R	>10000	-
16	A14	FITC	bAla	I	P	E	S	S	E	L	T	L	Q	E	L	L	**A**	E	E	R	4342±1177	23
17	A15	FITC	bAla	I	P	E	S	S	E	L	T	L	Q	E	L	L	G	**A**	E	R	953±119	5
18	A16	FITC	bAla	I	P	E	S	S	E	L	T	L	Q	E	L	L	G	E	**A**	R	274±21	1
19	A17	FITC	bAla	I	P	E	S	S	E	L	T	L	Q	E	L	L	G	E	E	**A**	413±39	2

Table S1. Alanine scanning of the N-terminal modified E6AP peptide. Seventeen single alanine mutants of E6AP peptides were synthesized to investigate the critical residues for binding activity. Substituting residues Leu9, Leu12, and Leu13 to alanine or removing the N-terminal modification significantly decreases or disrupts binding. Four residues, Glu6, Thr8, Glu11, and Gly14, showed medium alanine tolerance. Binding affinity was measured by competition BLI binding assay (N = 3). *NB: no binding. *bAla= beta-Alanine.

Name	N-Term	Sequence	K_D in nM	Ratio to WT
WT	-	IPESSELTLQELLGEERRAAK	2441 ± 1334	1
N-FITC	Fluorescein-5-Isothiocyanate	IPESSELTLQELLGEERR	95±17	25.7
N1	9-fluorene acetamido	IPESSELTLQELLGEERR	10±6.8	244.6
N2	1-fluorene carboxamido	IPESSELTLQELLGEERR	13±6.3	188.1
N3	1-Indane acetamido	IPESSELTLQELLGEERR	425±78	5.7
N4	9-fluorenone-2-carboxamido	IPESSELTLQELLGEERR	79±14	30.9
N5	9-fluorenone-1-carboxamido	IPESSELTLQELLGEERR	110±21	22.2
N6	9-fluorenone-4-carboxamido	IPESSELTLQELLGEERR	102±18	23.9
N7	anthraquinone-2-carboxamido	IPESSELTLQELLGEERR	27±6.3	90.5
N8	xanthene-9-carboxamido	IPESSELTLQELLGEERR	75±19	32.6
N9	1-anthracene carboxamido	IPESSELTLQELLGEERR	22±7.2	111.1
N10	2-anthracene carboxamido	IPESSELTLQELLGEERR	23±9.0	106.3
N11	1-adamantane carboxamido	IPESSELTLQELLGEERR	595±152	4.1
N12	Triphenyl acetamido	IPESSELTLQELLGEERR	199±37	12.2
N13	Diphenyl acetamido	IPESSELTLQELLGEERR	274±51	8.9
N14	1-naphthyl carboxamido	IPESSELTLQELLGEERR	292±54	8.3
N15	1,6-dihydrophenyl carboxamido	IPESSELTLQELLGEERR	767±295	3.1
N16	Pentafluorophenyl carboxamido	IPESSELTLQELLGEERR	228±74	10.7
N17	6-hydroxy-2-naphthyl carboxamido	IPESSELTLQELLGEERR	159±47	15.3
N18	1-pyrenebutyl carboxamido	IPESSELTLQELLGEERR	106±27	23
N19	5-Acenaphthene carboxamide	IPESSELTLQELLGEERR	55±12	44.4
N20	7-Methoxycoumarin-4-acetamido	IPESSELTLQELLGEERR	213±47	11.4
N21	4-phenyl-phenylalanine	IPESSELTLQELLGEERR	1412±757	1.7
N22	Cyclohexyl-alanine	IPESSELTLQELLGEERR	1658±705	1.4

Table S2. N-terminal modified E6AP peptides. The binding affinity was measured by BLI competition assay. Error distribution is reported as the standard error of the mean (SEM) of curve fitting reported by Prism 8 software. The wild-type peptide was used as a reference. K_D values are measured by BLI binding assay in a competition mode (N = 2 or 3). The ratio is calculated by dividing competition K_D in this row to the competition K_D of wild-type (WT) showed in the first row.

Name	Sequence	C-term	K_D in nM	Ratio to wild-type
WT	IPESSELTLQELLGEERRAAK	-	2432 ± 1302	1
C-FITC	Ac-IPESSELTLQELLGEERRAA	Lys (Fluorescein-5-Isothiocyanate)	640±82	3.8
C1	Ac-IPESSELTLQELLGEERRAA	Lys(fluorene-9-acetamido)	165±35	9.9
C2	Ac-IPESSELTLQELLGEERRAA	Lys(fluorene-1-acetamido)	245±59	9.9
C3	Ac-IPESSELTLQELLGEERRAA	Lys(1-Indane acetamido)	645±92	3.7
C4	Ac-IPESSELTLQELLGEERRAA	Lys(9-fluorenone-2-carboxamido)	71±14	34.4
C5	Ac-IPESSELTLQELLGEERRAA	Lys(9-fluorenone-1-carboxamido)	81±13	30.1
C6	Ac-IPESSELTLQELLGEERRAA	Lys(9-fluorenone-4-carboxamido)	84±13	29.1
C7	Ac-IPESSELTLQELLGEERRAA	Lys(anthraquinone-2-carboxamido)	46±5.5	53.1
C8	Ac-IPESSELTLQELLGEERRAA	Lys(xanthene-9-carboxamido)	78±18	31.3
C9	Ac-IPESSELTLQELLGEERRAA	Lys(1-anthracene carboxamido)	22±5.8	111.1
C10	Ac-IPESSELTLQELLGEERRAA	Lys(2-anthracene carboxamido)	64±10	38.2
C11	Ac-IPESSELTLQELLGEERRAA	Lys(1-adamantane carboxamido)	1459±474	1.6
C12	Ac-IPESSELTLQELLGEERRAA	Lys(triphenylacetamido)	878±125	2.7
C13	Ac-IPESSELTLQELLGEERRAA	Lys(diphenylacetamido)	1184±264	2
C14	Ac-IPESSELTLQELLGEERRAA	Lys(1-naphthyl carboxamido)	507±65	4.8
C15	Ac-IPESSELTLQELLGEERRAA	Lys(Exo-norbornene-carboxamido)	756±128	3.2

Table S3. C-terminal modified E6AP peptides. The binding affinity was measured by BLI competition assay. Error distribution is reported as the standard error of the mean (SEM) of curve fitting reported by Prism 8 software. The wild-type peptide was used as a reference. K_D values are measured by BLI binding assay in a competition mode (N = 2 or 3). The ratio is calculated by dividing competition K_D in this row to the competition K_D of wild-type (WT) showed in the first row.

(A)

Name	Sequence	Cross-link yield (%)	Conditions[a]
E0	$AcE^{1}L^{2}T^{3}L^{4}Q^{5}E^{6}L^{7}L^{8}G^{9}E^{10}E^{11}R^{12}$	N/A	N/A
E1	AcELTLQELLC(Phacr)EER	0	protein 2μM; peptide 20 μM, x1 PBS, rt 24 h
E2	AcELTLQELLGC(Phacr)ER	0	protein 2μM; peptide 20 μM, x1 PBS, rt, 24 h
E3	AcELTLQELL(Dha)EER	74	protein 2 μM, peptide 4 μM, x1 PBS, rt, 2h
E4	AcELTLQELLG(Dha)ER	0	protein 2 μM, peptide 20 μM, x1 PBS, rt, 1h
E5	AcELTLQELLGE(Dha)R	0	protein 2 μM, peptide 20 μM, x1 PBS, rt, 2h
E6	AcELTLQELL(dap-acr)EER	0	protein 2μM; peptide 20 μM, x1 PBS, rt 2 h
E7	AcELTLQELLG(dap-acr)ER	0	protein 2μM; peptide 20 μM, x1 PBS, rt 2 h
E8	AcELTLQELLGE(dap-acr)R	0 (42%, 3 days)	protein 2μM; peptide 20 μM, x1 PBS, rt 3 d
E9	AcELTLQELLG(dab-acr)ER	0	protein 2μM; peptide 20 μM, x1 PBS, rt 2 h
E10	AcELTLQELLGE(dab-acr)R	0	protein 2μM; peptide 20 μM, x1 PBS, rt 2 h
E11	AcELTLQELLGE(dab-ppa)R	27	protein 2μM; pep 5 μM, x1 PBS, rt 2 h

(B)

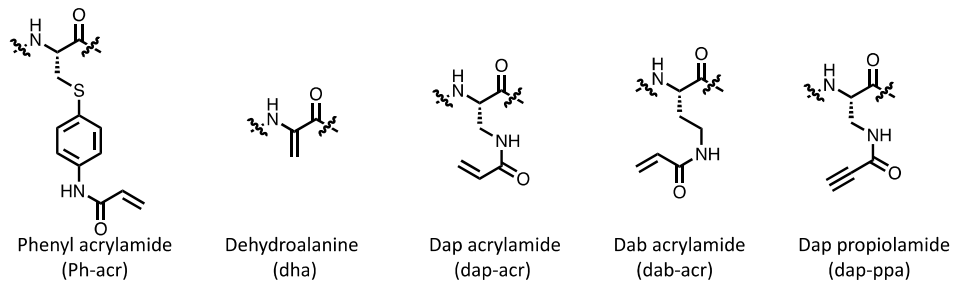


Figure S4. (**A**) Cross-linking experiments between MBP-16E6 and peptides E0 to E11 Reactions were performed in 1X PBS at pH = 7.4 under conditions listed for each experiment. Cross-link yield was measured by LC-MS protein deconvolution mass spectrum, and the percentage was calculated by dividing the peak area of cross-linked protein by the sum of uncross-linked and cross-linked protein peak area (N = 2). (**B**) Structures of electrophiles.

Name	N-term	Sequence	C-term	Charge	Apparent K_i in nM
7	9-fluorenyl acetamido	IPESAELTLQELL(Dha)EERRAA	K(1-anthracenyl acetamido)	-3	23±8.1
8	9-fluorenyl acetamido	IPESAELTLQELL(Dha)EERRNKK	K(1-anthracenyl acetamido)	-1	39±6.1
9	9-fluorenyl acetamido	IPQSAELTLQELL(Dha)EARRNKK	K(1-anthracenyl acetamido)	1	11±3.7
10	9-fluorenyl acetamido	IPQSAELTLQELL(Dha)QARRNKK	K(1-anthracenyl acetamido)	2	16±2.6
11	9-fluorenyl acetamido	IPQSAELTLQELL(Dha)QARRKK	K(1-anthracenyl acetamido)	2	11±4.9
12	9-fluorenyl acetamido	IPQSAELTLQELL(Dha)QRRKK	K(1-anthracenyl acetamido)	2	20±5.4
13	9-fluorenyl acetamido	IPQSAELTLQELL(Dha)RRKK	K(1-anthracenyl acetamido)	2	17±3.9
13-3L3A	9-fluorenyl acetamido	IPQSAELTAQEAA(Dha)RRKK	K(1-anthracenyl acetamido)	2	n.b.

Figure S5. Sequence table of E6AP-mimicking peptides designed to increase positive charge. Apparent K_i is determined by BLI. Peptides were in competition with immobilized **1-biotin** following a 30-min incubation with MBP-16E6. Dha: dehydroalanine.

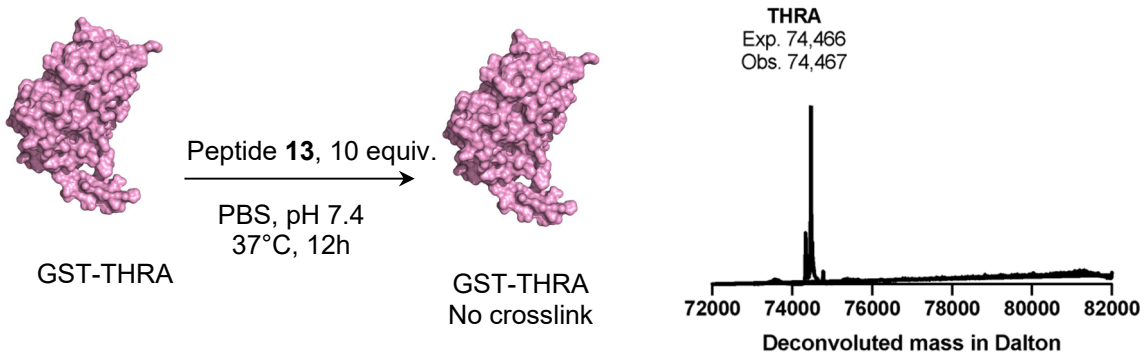


Figure S6. **13** showed no observable crosslink to THRA. The crosslinking reaction was performed with 1 μM THRA and 10 μM **13** at 37°C for 12 h. PDB code of THRA is 1nav.

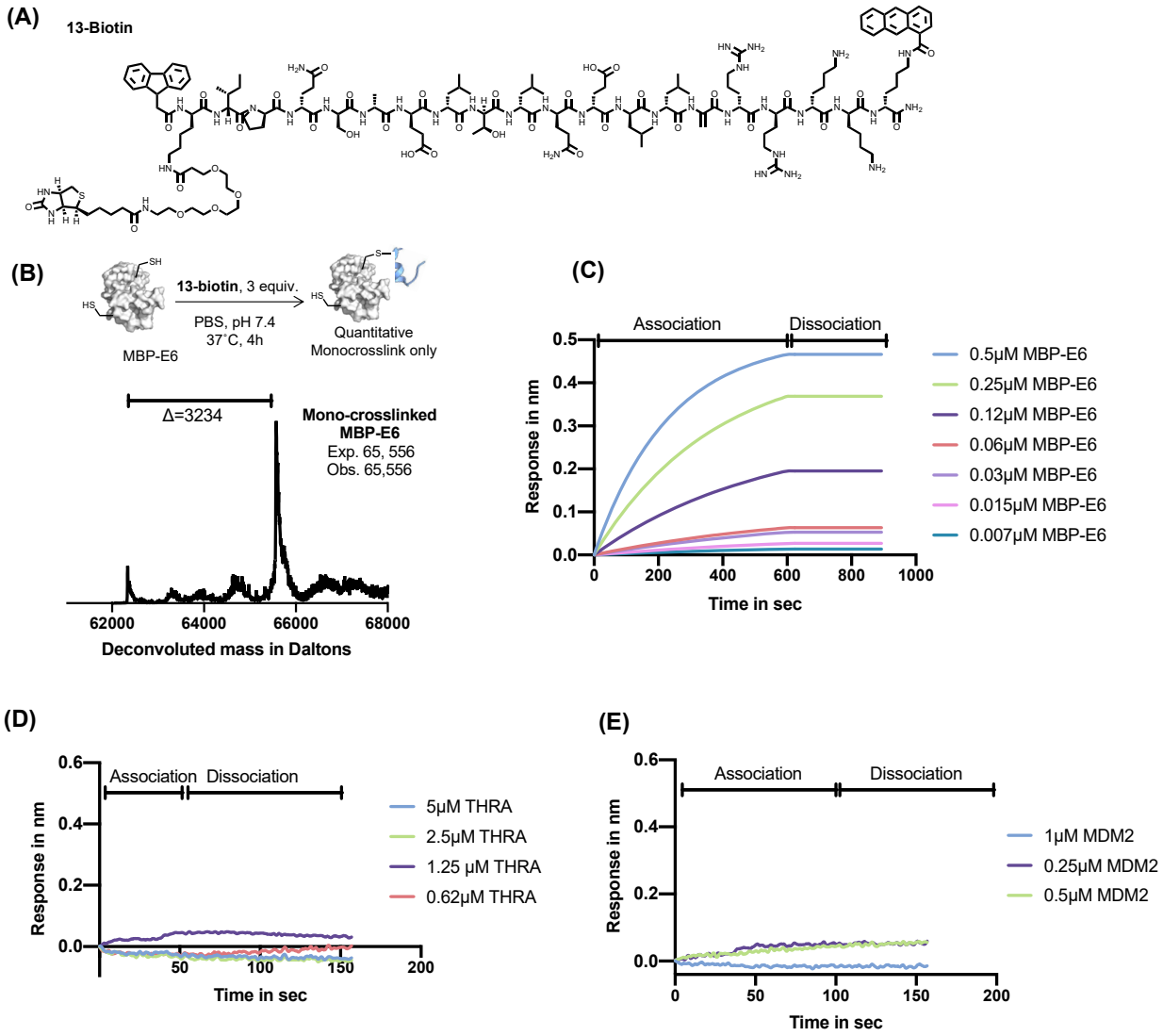


Figure S7. (A) Structure of **13-biotin** (B) Protein deconvolution mass spec of MBP-E6 crosslinking with peptide **13-biotin** (Molecular Weight: 3,231.90 Da). Three μM **13-biotin** and 1 μM MBP-E6 were incubated in 1 × PBS at 37°C for 4 h. Mass-spec was recorded using a 6550 Agilent Q-TOF coupled to Infinite II 1290 LC system, with an Aeries C4 column (Phenomenex) using 1 to 61 B% linear ramping gradients. (C) **13-biotin** direct K_i is determined by BLI assay. k_{off}= 6.79E-05 s^{-1}, k_{on}= 5.31E+04 $M^{-1}s^{-1}$, direct K_i= 1.3 ± 0.6 nM (N = 3). (D) **13-biotin** showed no observable association and dissociation to THRA as determined by direct BLI assay (N = 1). (E) **13-biotin** showed no observable association and dissociation to MDM2 as determined by direct BLI assay (N = 1).

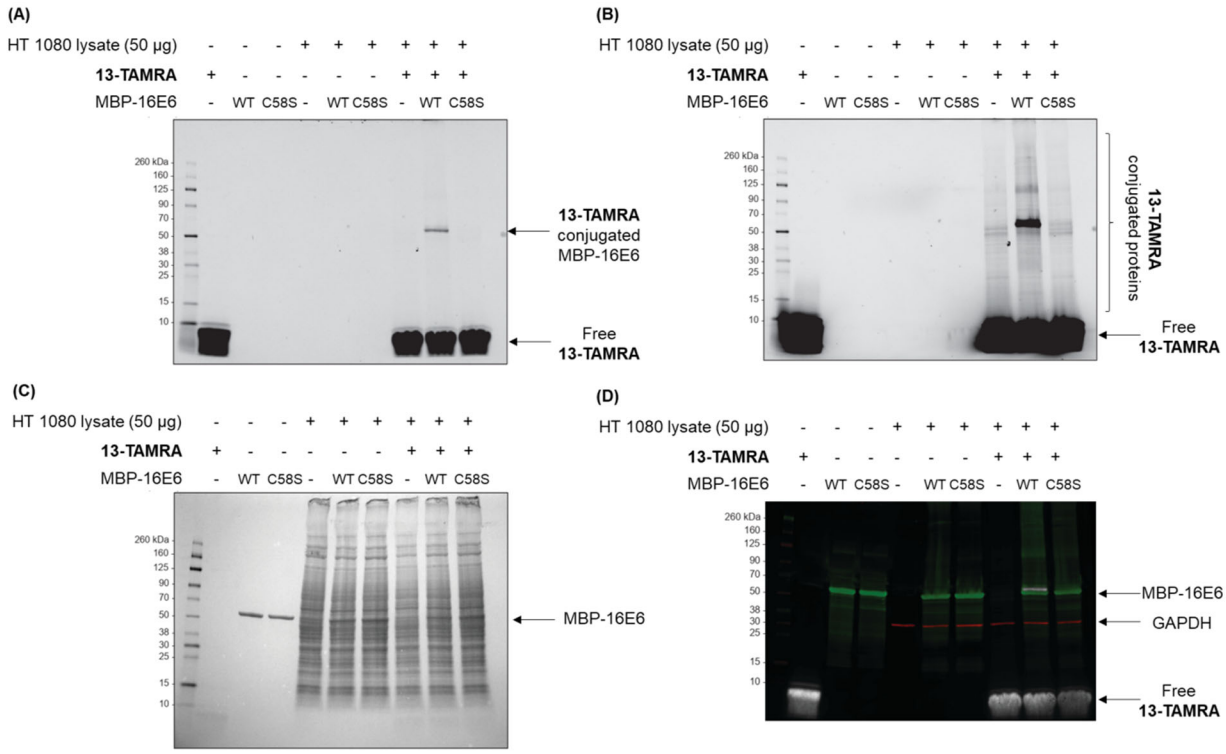


Figure S8. Crosslinking of **13-TAMRA** to MBP-16E6 (4C4S) and MBP-16E6 C58S (4C4S) in HPV-negative HT1080 lysate. HT1080 lysates (50 µg) were incubated overnight at 4°C with 2 µM each of MBP-16E6 WT or C58S (each 4C4S), and **13-TAMRA** (10 µM). The mixtures were then separated by SDS-PAGE and transferred onto nitrocellulose. Fluorescence scans of the protein gel provided a visualization of both the free **13-TAMRA** and **13-TAMRA** crosslinked to WT MBP-16E6 with (A) short and (B) long exposure times. Additional minor fluorescent signals from **13-TAMRA** crosslinked to unidentified proteins in the lysate at Figure S8B. (C) Ponceau S staining of the nitrocellulose membrane after transfer displayed the combination of MBP-16E6 (either WT or C58S), **13-TAMRA**, and HT1080 lysate. (D) The Western blot for MBP-16E6 and GAPDH, alongside a 488 nm fluorescence scan for **13-TAMRA**, showed selective binding of **13-TAMRA** to MBP-16E6 WT but not C58S.

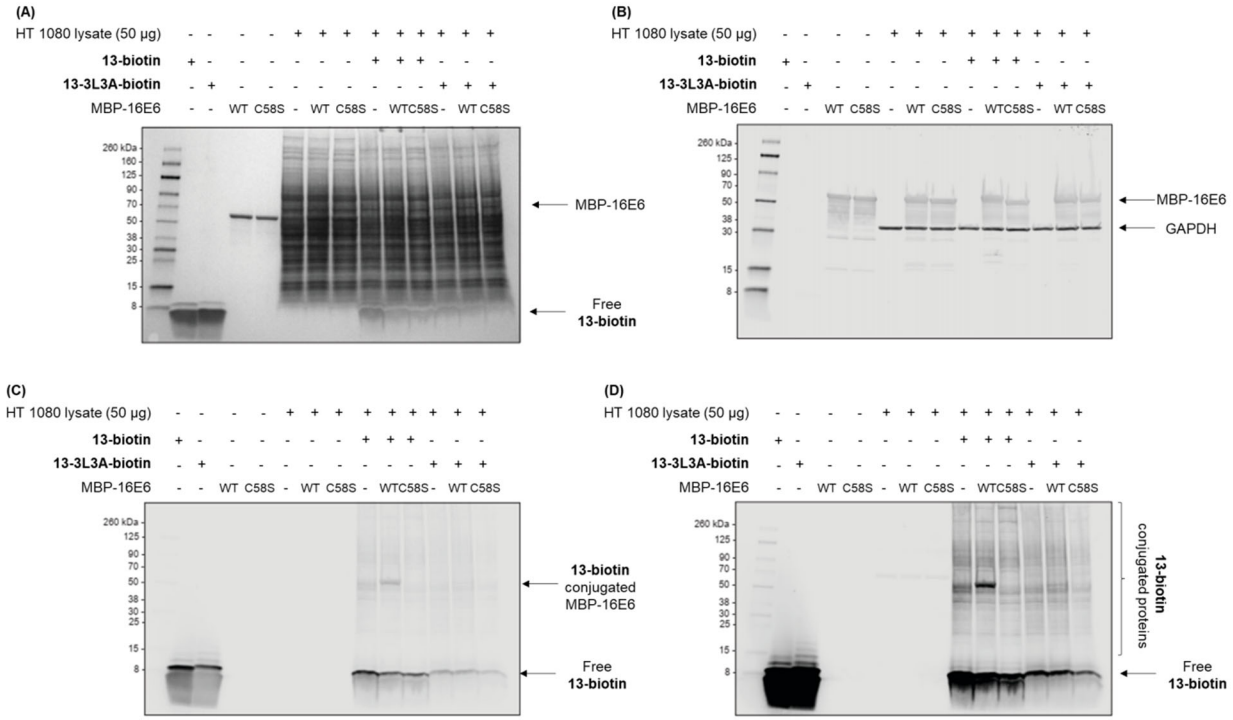


Figure S9. Crosslinking of **13-biotin** to MBP-16E6 (4C4S) and MBP-16E6 C58S (4C4S) in HPV-negative HT1080 lysate. HT1080 lysates (50 µg) were incubated overnight at 4°C with 2 µM each of MBP-16E6 WT or C58S (each 4C4S), and **13-biotin** (10 µM) or **13-3L3A-biotin** (10 µM). The mixtures were then separated by SDS-PAGE and transferred onto nitrocellulose. (A) Ponceau staining of the nitrocellulose membrane provides an overview of the distribution of MBP-16E6 (WT or C58S), **13-biotin** or **13-3L3A-biotin**, and HT1080 lysate. (B) Western blot analysis of MBP-16E6 and GAPDH, detailing antibody interactions. The same blot was subsequently incubated with streptavidin-680, enabling the visualization of **13-biotin** and **13-3L3A-biotin** under (C) short and (D) long exposures. The interaction between MBP-16E6 and **13-biotin** remains observable. Additional bands indicate at 10 µM, binding of **13-biotin** and **13-3L3A-biotin** to unidentified proteins present in the HT1080 lysate.

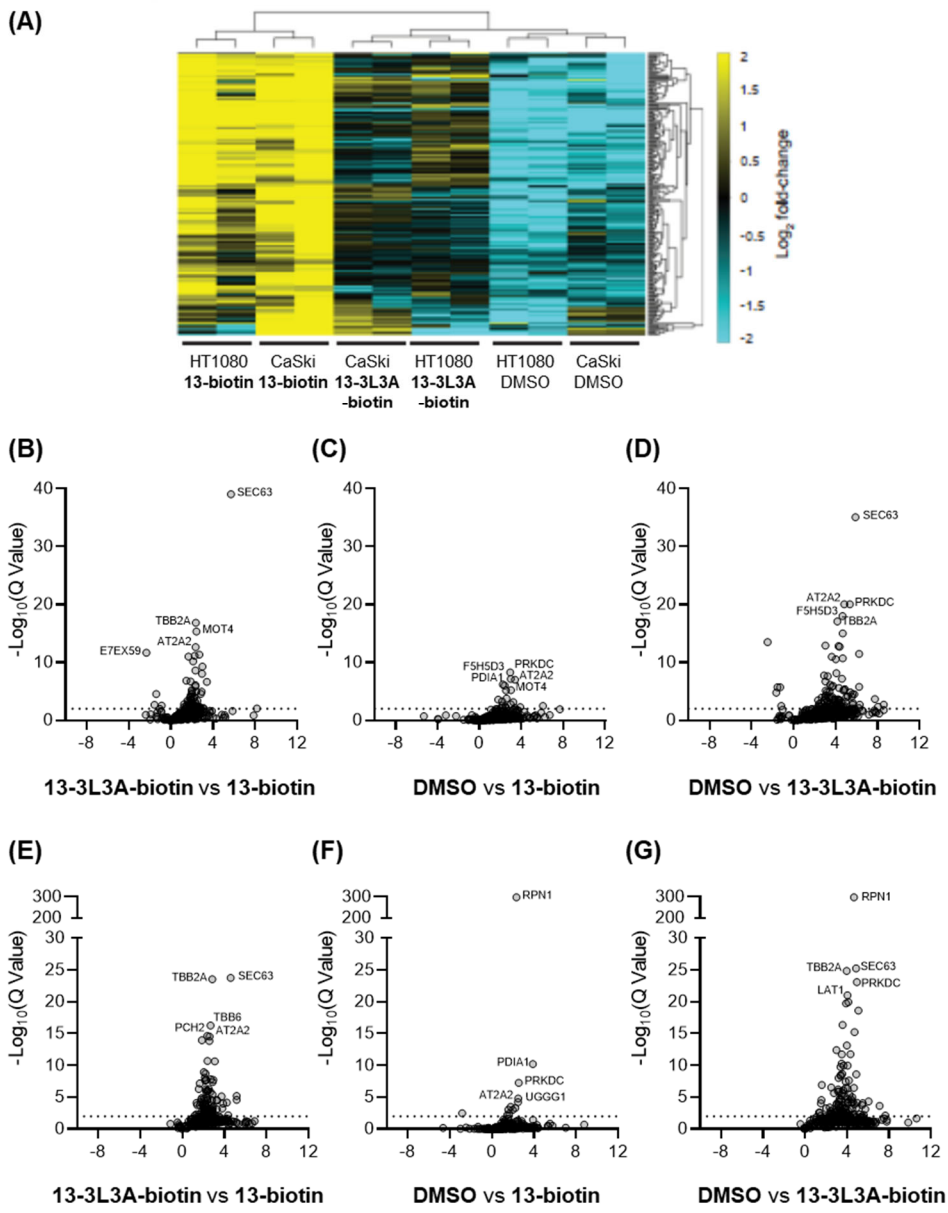


Figure S10: Streptavidin pull-down analysis of **13-biotin**, **13-3L3A-biotin**, or control (beads alone with DMSO) using quantitative multiplexed mass spectrometry. HPV- HT1080 or HPV16+ CaSki cell lysates (2 mg) were treated with **13-biotin** (5 μM), **13-3L3A-biotin** (5 μM), or DMSO (0.5%) overnight at 4 °C and subjected to pull-down using streptavidin beads. The samples were then TMT labeled, digested with trypsin, and analyzed by mass spectrometry. (A) Heat map showing the relative enrichment in CaSki cells treated with **13-biotin** relative to those treated with **13-3L3A-biotin**. Volcano plot comparisons for HT1080 cells are shown for (B) **13-3L3A-biotin** vs. **13-biotin**, (C) DMSO vs. **13-biotin**, and (D) DMSO vs. **13-3L3A-biotin**. Volcano plot comparisons for CaSki cells are presented for (E) **13-3L3A-biotin** vs. **13-biotin**, (F) DMSO vs. **13-biotin**, and (G) DMSO vs. **13-3L3A-biotin**. The data represent results from two independent experiments. The top five proteins with the highest -Log_{10}(q-value) are highlighted, and a comprehensive list of proteins along with their significance values can be found in Supplemental File S1.

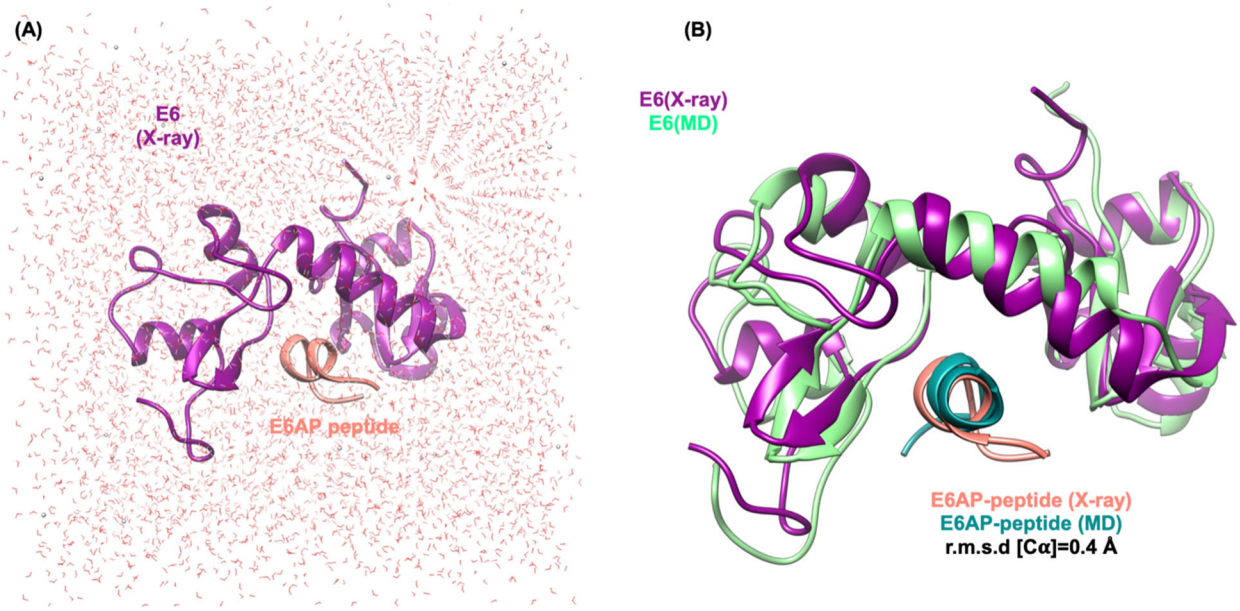


Figure S11. The native E6AP LXXLL peptide forms a stable alpha helix when in complex with 16E6. (A) E6AP peptide (salmon) bound to 16E6 protein (purple) with surrounding water molecules (red lines) and ions (white spheres). **(B)** Comparison of X-ray structure of 16E6 (green) bound to E6AP (salmon) (PDB ID: 4XR8) with relaxed 16E6(purple)-E6AP peptide (dark green) complex after 1.1 μs MD simulation. P53 and MBP were omitted for clarity. The RMSD was calculated for the Cα atoms.

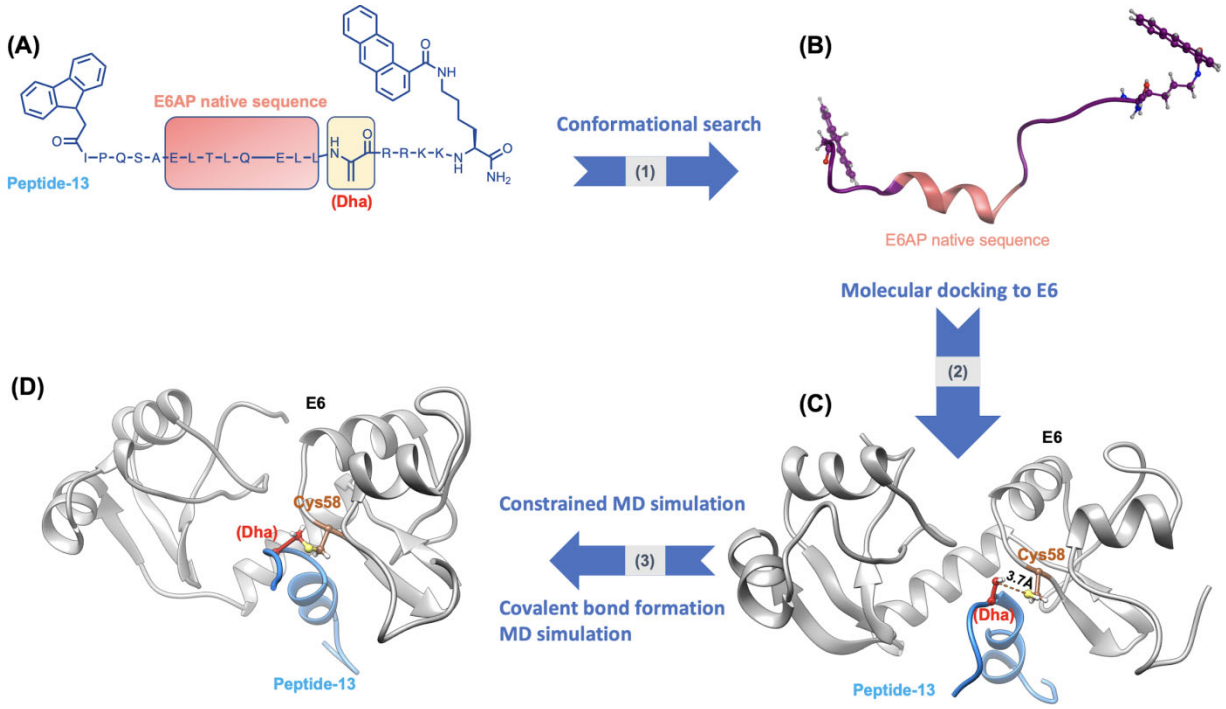


Figure S12. Structural modeling of 16E6 bound to Peptide-13. (**A**) Structure of Peptide **13**. (**B**) E6AP LXXLL peptide native core adopts an alpha helical conformation in the peptide 13 structural model obtained by computational conformational sampling. (**C**) Molecular docking of Peptide-13 (blue) to 16E6 protein (gray). (**D**) Molecular modeling of 16E6 (gray) and peptide 13 (blue) in complex with the covalent thioester bond between the peptide Dha warhead of peptide 13 and Cys58 of 16E6 highlighted.

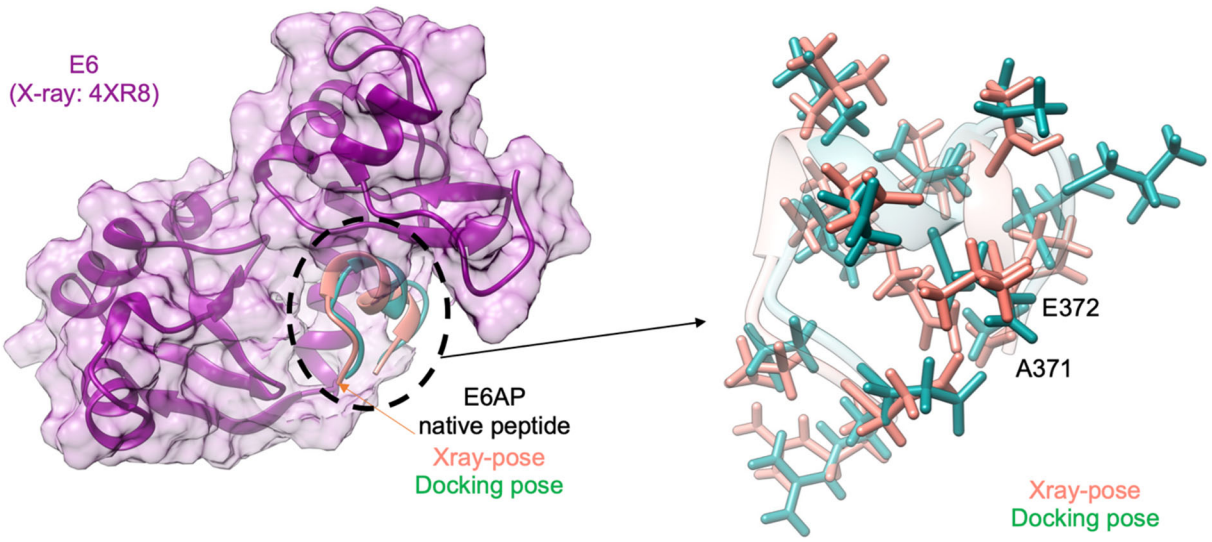


Figure S13. Molecular docking of the native E6AP peptide in the 16E6 (left). The MOE molecular docking procedure accurately describes the interactions between 16E6 and E6AP native peptide (right).

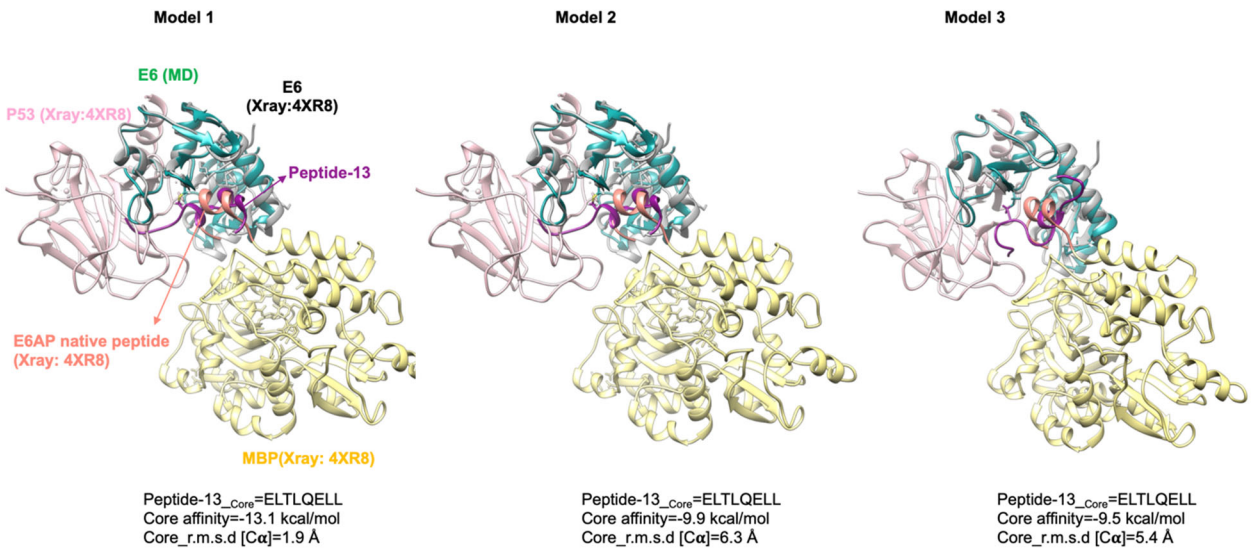


Figure S14. Molecular modeling of 16E6 and peptide 13 complexes. Complexes are ordered from highest binding affinity (mode 1) to lowest (mode 3) based on RMSD of peptide **13** core sequence compared to the native E6AP LXXLL peptide in PDB: 4XR8.

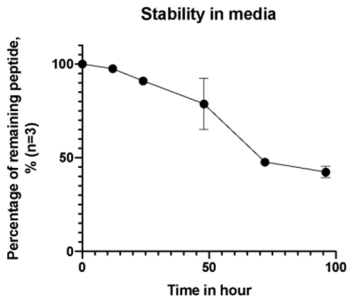


Figure S15. Reactide 13 stability in media. Ten μM peptide was incubated in RPMI with 10% FBS at 37°C for the indicated time. Proteins were removed from the mixture and analyzed by LC-MS. Percentage of the remaining peptide was estimated by dividing the area under extract ion chromatogram (EIC) to the area of time zero (N = 3).

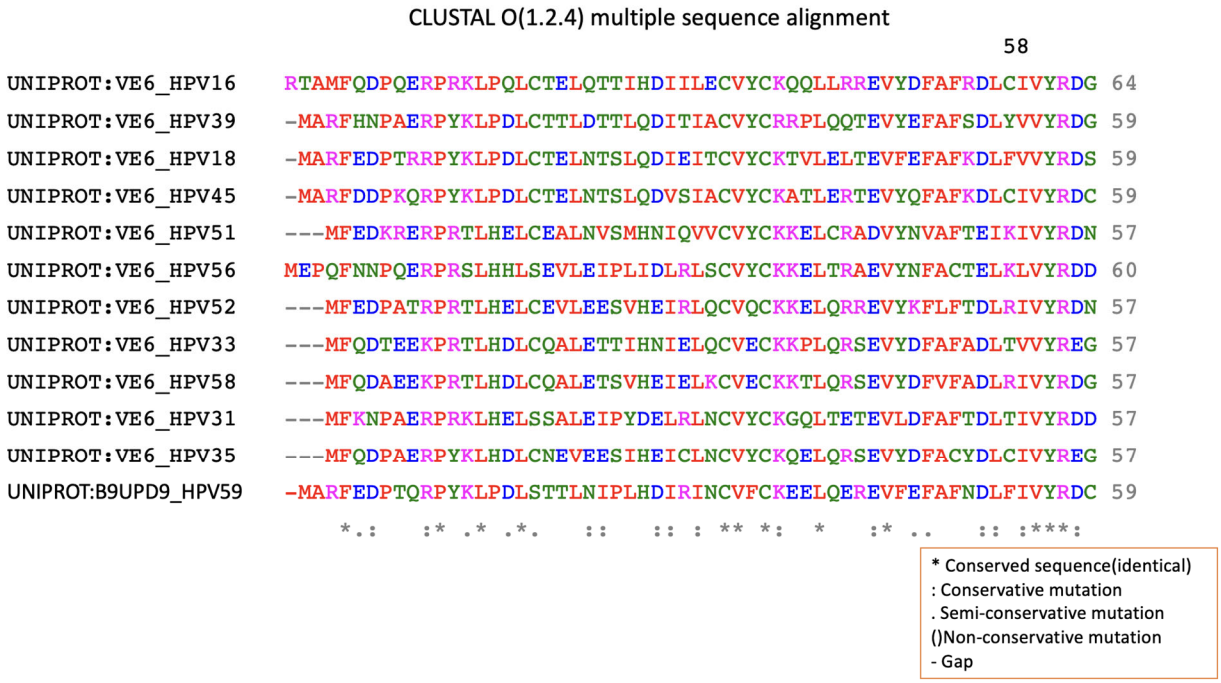


Figure S16. Protein sequence alignment of High-risk type HPV E6 using CLUSTAL O (1.2.4) sequence alignment tool. The alignment includes for the following high-risk subtypes: 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. For example, in HPV16E6 (48-64) and HPV18E6 (43-59), the Phe53 of HPV18 E6 aligns with the Cys58 of HPV16 E6. Occurrence of Phe53 in HPV18 cysteine bind-and-react inhibition strategy.

Methods and Materials

1.1 Materials

H-Rink Amide-ChemMatrix resin was purchased from PCAS BioMatrix Inc. Amino acids: Fmoc-Ala-OH, Fmoc-β-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH, and Fmoc-Val-OH were purchased from Novabiochem (Billerica, MA). Other amino acids: Fmoc-Lys(ivdde)-OH, Fmoc-Lys(Alloc)-OH, Fmoc-Lys(Fmoc)-OH were purchased from Novabiochem (Billerica, MA). Palladium tetrakistriphenylphosphine(0) ($Pd(PPh_3)_4$) was purchased from Sigma Aldrich. Reagents used in solid phase peptide synthesis: Piperidine (ReagentPlus; 99%), formic acid (≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Diisopropylethylamine (DIEA; biotech. grade; 99.5%) was purchased from Millipore Sigma and purified by a Seca Solvent Purification system from Pure Process Technology (Nashua, NH). Reagents (cleavage): Trifluoroacetic acid (TFA; for HPLC, ≥99%), triisopropylsilane (TIPS; 98%) were purchased from Sigma-Aldrich (St. Louis, MO). Reagents used in peptide post-synthesis modifications: Acetic anhydride (≥98%) was purchased from Sigma-Aldrich (St. Louis, MO), 5-TAMRA (5-carboxytetramethylrhodamine) and Biotin-PEG4-carboxylic acid from ChemPep. Bovine serum albumin (BSA) from VWR, PA. Recombinant Human Thyroid Hormone Receptor alpha 1 protein (THRA) from Abcam, UK.

Cell culture media (RPMI-1640 ATCC modified, EMEM and MEM), fetal bovine serum (FBS), penicillin-streptomycin, 10× PBS, and 0.25% trypsin-EDTA were obtained from Gibco. Culture flasks, plates, and serological pipettes were obtained from Fisher Scientific.

Tandem mass tag (TMTpro) isobaric reagents, BondBreaker TCEP, iodoacetamide, water and organic solvents were purchased from Thermo Fisher Scientific (Waltham, MA). HEPPS buffer was from Boston BioProducts (Milford, MA) and trypsin was from Promega Corporation (Madison, WI). Unless otherwise stated, all other chemicals were purchased from Sigma (St. Louis, MO).

1.2 Fast flow synthesis of peptides

H-Rink Amide-ChemMatrix resin (200 mg, 0.49 mmol/g, 0.10 mmol) was used to prepare peptide-α-carboxamides. Peptides containing noncanonical amino acids were prepared by manual SPPS. Peptides without noncanonical amino acids were prepared by fully automated SPPS[1]. Upon completion, resins were washed with dichloromethane (DCM) three times and dried under reduced pressure.

1.3 Automated flow peptide synthesis (AFPS) set-up

All peptides were synthesized on automated-flow systems built in the Pentelute lab (“Amidator” and “Peptidator”), which are similar to the published AFPS system[2]. The synthesis conditions were published previously[2]:

The following settings were used for protein synthesis: flowrate = 40 mL/min, temperature = 90°C (loop) and 85–90°C (reactor). The 50 mL/min pump head pumps 400 µL of liquid per pump stroke; the 5 mL/min pump head pumps 40 µL of liquid per pump stroke. The standard synthetic cycle involves a first step of prewashing the resin at elevated temperatures for 60 s at 40 mL/min. During the coupling step, three HPLC pumps are used: a 50 mL/min pump head pumps the activating agent, a second 50 mL/min pump head pumps the amino acid and a 5 mL/min pump head pumps DIEA. The first two pumps are activated for 8 pumping strokes to prime the coupling agent and amino acid before the DIEA pump is activated. The three pumps are then actuated together for a period of 7 pumping strokes, after which the activating agent pump and amino acid pump are switched using a rotary valve to select DMF. The three pumps are actuated together for a final 8

pumping strokes, after which the DIEA pump is shut off, and the other two pumps continue to wash the resin for another 40 pump strokes. During the deprotection step, two HPLC pumps are used. Using a rotary valve, one HPLC pump selects deprotection stock solution and DMF. The pumps are activated for 13 pump strokes. Both solutions are mixed in a 1:1 ratio. Next, the rotary valves select DMF for both HPLC pumps, and the resin is washed for an additional 40 pump strokes. The coupling–deprotection cycle is repeated for all additional monomers.

1.4 Method for peptide acetylation

A 100 mg portion of peptidyl resin from section 1.3 was placed into a 5 mL Torviq fritted syringe and subsequently swelled in DMF. After removing DMF, a solution of Ac_2O, DIEA, and DMF (2 mL, 85:315:1600, v/v) were added to the peptidyl resin. The resulting mixture was occasionally agitated for 45 min. After draining the solution, the remaining resin was washed with DMF three times, DCM three times, and dried under reduced pressure.

1.5 Method for Alloc deprotection

Peptidyl resin (~10 μmol theoretical loading) was washed with DCM (3 × 5 mL) and then treated with $Pd(PPh_3)_4$ (11.0 mg, 10 μmol, 1 equiv) in DCM/piperidine (8:2, 1 mL) for 30 minutes at room temperature under exclusion of light. The resin was then drained and washed with DCM (3 × 5 mL).

1.6 Method for biotin labeling

Peptidyl resin (~10 μmol theoretical loading) was loaded into a fritted syringe (6 mL), swollen in DMF (4 mL) for 5 minutes, and then drained. Biotin-PEG4-propionic acid (Biotin-PEG4-OH, 22 mg, 50 μmol, 5 equivalents) and HATU (17 mg, 45 μmol, 4.5 equivalents) were dissolved in DMF (500 μL), activated with DIEA (19 mg, 26 μL, 150 μmol), added to the peptidyl resin and incubated for 30 minutes under exclusion of light. After this time, the resin was drained, washed with DMF (3 × 5 mL), and stored until cleavage.

1.7 Method for TAMRA labeling

Peptidyl resin (~10 μmol theoretical loading) was loaded into a fritted syringe (6 mL), swollen in DMF (4 mL) for 5 minutes, and then drained. 5-Carboxytetramethylrhodamine (5-TAMRA, 22 mg, 50 μmol, 5 equivalents) and HATU (17 mg, 45 μmol, 4.5 equivalents) were dissolved in DMF (500 μL), activated with DIEA (19 mg, 26 μL, 150 μmol), added to the peptidyl resin and incubated for 30 minutes under exclusion of light. After this time, the resin was drained, washed with DMF (3 × 5 mL), and stored until cleavage.

1.8 Method for FITC labeling

Peptidyl resin (~10 μmol theoretical loading) was loaded into a fritted syringe (6 mL), swollen in DMF (4 mL) for 5 minutes, and then drained. 5-Carboxytetramethylrhodamine (Fluorescein isomer I, 22 mg, 50 μmol, 5 equivalents) was dissolved in DMF (500 μL), activated with DIEA (19 mg, 26 μL, 150 μmol), added to the peptidyl resin and incubated for 30 minutes under exclusion of light. After this time, the resin was drained, washed with DMF (3 × 5 mL) and stored until cleavage.

1.9 Cleavage of peptides

The synthesized peptide was cleaved from the resin and globally deprotected by treating the peptidyl resin with a cleavage cocktail containing 94% TFA, 2.5% water, and 2.5% TIPS (*v/v*), for 2 h at room temperature. TFA was removed under a gentle stream of nitrogen gas, and the crude peptide was precipitated by adding cold Et_2O (-80°C). After centrifugation at 3220 rcf for 3 min, the supernatant was removed, and the precipitated peptide was triturated three times with cold

Et_2O. The resulting material was dissolved in 50% MeCN in water with 0.1% TFA and lyophilized as crude.

1.10 Pd Mediated Conjugation

In a 50 mL falcon tube: Dissolve peptide (5.0 mg, 2.75 mmol, 1.0 equiv) in water (8.5 mL) and 500 mM HEPES (1.5 mL, pH = 8.0). In a 20 mL glass vial: Dissolve Pd OAC (5.0 mg, 6.19 mmol, 2.25 equiv) in MeCN and add to peptide solution over 30 seconds (final volume = 15 mL, final peptide concentration = 184 μM). Mix by vortexing and let stand for 20 minutes. Add 10 mL AcOH and 30 mL H_2O and mix and then purify by reversed phase flash chromatography using a Sfär Bio C18 D (300 Å 20 μm, 6 g) column (mobile phase 5% MeCN/H_2O to 55% MeCN in H_2O + 0.1% TFA).

1.11 Dehydroalanine formation

In a 1.5 mL microcentrifuge tube, cysteine-containing peptide (7 mg) was dissolved in DMF (0.5 mL), and to this, a 10 mg/mL potassium carbonate solution (3.1 mg, 22.3 μmol, 5 equiv) was added. To this mixture, a 10 mg/mL solution of Diethylmeso-2,5-dibromoadipate (151 μL, 1.1 equiv) was added. The mixture was mixed by vortexing for 5 seconds and allowed to react for 4 h. The reaction mixture was diluted with 5% MeCN in H_2O + 0.1% TFA and purified by reversed-phase HPLC (Zorbax 300SB-C3, 300Å, 5 μm, 9.4 mm x 250 mm) Mobile phase 5% to 55% MeCN in H_2O + 0.1% TFA.

1.12 General procedure for Cross-linking MBP-16E6 with Covalent Peptides

A solution of cross-linker (250 μM solution in 10% DMSO/H_2O, 2 to 10 equiv, 0.66 μL) was diluted with H_2O (30 μL) and 10× PBS (4.1 μL, pH = 7.4). A 30 μM solution of MBP-6 (2.4 μL, 1 equiv) and then incubated at room temperature for 2 h. An 8 μL aliquot of the reaction mixture was removed and quenched with 92 μL of 50:50 MeCN/H_2O + 0.1% TFA and analyzed by LC/MS. Yields were obtained by extracting all protein-containing species' total ion current (TIC) spectra in the chromatogram utilizing Agilent MassHunter Bioconfirm Software 10.0. The extracted chromatograms were deconvoluted utilizing a maximum entropy algorithm, and the abundance of each species was determined using total ion count. $\%yield = \frac{P_c}{P_c+P_0} \times 100$ where P_c is the peak area of the peptide-protein conjugate, and P_0 is the peak area of the unmodified protein.

1.13 Purification of the crude peptide.

Crude peptides were purified by a Biotage Selekt flash purification system. Water with 0.1% TFA (solvent A) and MeCN with 0.1% TFA (solvent B) was utilized as mobile phases for purifications. The crude peptide was dissolved in a minimal amount of 10% MeCN in water with 0.1% TFA and then loaded onto a 10 g Biotage SNAP Bio C4 20 μm column. The purification was performed using a gradient as follows: 10% B for 2 column volume (CV), the linear ramp from 30% B to 50% B for 20 CV, 25 mL/min flow rate.

1.14 Method for LC-MS characterization.

LC-MS characterizations were carried out using an Agilent 6550 quadrupole time-of-flight LC-MS. Total ion current (TIC) chromatograms were plotted. Mass spectra were integrated over the principal TIC peaks. High-performance liquid chromatography was done by the following methods: (solvent A: water with 0.1% formic acid; solvent B: MeCN with 0.1% formic acid).

Method A: Column: Phenomenex Jupiter C4 column (1.0 × 150 mm, 5 μm particle size, 300 Å pore size) Gradient: 1% B (0-2 min), linearly ramp from 1% B to 91% B (2-8 min). The flow rate is 100 μL/min. MS acquisition is from 2 to 8 min.

Method B: Column: Phenomenex Jupiter C4 column (1.0 × 150 mm, 5 μm particle size, 300 Å

pore size) Gradient: 1% B (0-2 min), linearly ramp from 1% B to 61% B (2-12 min), 61% B to 95% B (11-16 min). The flow rate is 100 µL/min. MS acquisition is from 4 to 12 min.
Method C: Column: Agilent Zorbax 300SB C3 column (2.1 × 150 mm, 5 µm particle size, 300 Å pore size) Gradient: 1% B (0-2 min), linearly ramp from 1% B to 91% B (2-12 min), 91% B to 91% B (12-13 min). The flow rate is 500 µL/min. MS acquisition is from 4 to 12 min.

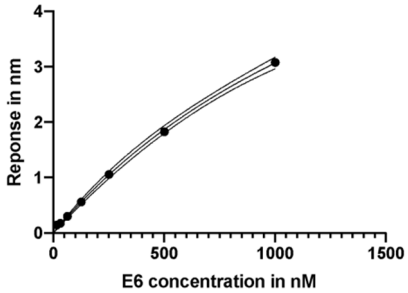
1.15 Direct binding measurement by BLI

Streptavidin sensors were soaked in blocking buffer (1× PBS supplemented with 0.05% Tween-20 and 1 mg/mL bovine serum albumin) for 5 min. After immobilizing the **1-biotin** peptide (200 nM onto streptavidin sensors, serial dilutions of MBP-16E6 in the blocking buffer were analyzed for binding. The response was recorded at equilibrium after 2 min. Association lasted for 100 seconds, and dissociation lasted for another 120 seconds. The curve was reported by GatorPlus software (version 2.7.3.1013) and replotted by Prism 8 software.

1.16 Competitive binding assay by BLI

A competition binding assay was performed as described below using GatorPlus bio-layer interferometry (GatorBio) to estimate the binding affinity of peptides.

Calibration curve: Streptavidin sensors were soaked in blocking buffer (PBS supplemented with 0.05% Tween-20 and 1 mg/mL bovine serum albumin) for 5 min. After immobilizing the PEG4-Biotinylated **1** peptide (200 nM of Biotin-PEG4-IPESSELTLQELLGEER) onto streptavidin sensors, 1:1 serial dilutions from 1000 nM of E6 in blocking buffer were analyzed for binding (All concentrations: 1,000nM, 500 nM, 250 nM, 125 nM, 62 nM, 31nM, 15nM, and 7.8nM). The response was recorded at equilibrium after 2 min. A curve of sensor response (nm) vs. MBP-16E6 concentration (nM) was generated to calibrate the free MBP-16E6 concentration in the solution observed in the competition assay. The curve was generated using Prism 8 software.



Competition assay. Increasing concentrations of peptides were incubated in wells with MBP-16E6 protein in the blocking buffer for 30 min. The PEG4-Biotinylated **1** peptide was immobilized onto streptavidin sensors and dipped into preincubated sample wells. The association events were measured at 30°C, 1,000 rpm. Response at equilibrium after 2 min was recorded. Based on the binding response (nm) values, the concentration of 'free' MBP-16E6 was interpolated for each sample using the calibration curve. The apparent dissociation constant, K_D, can be obtained from the non-linear regression analysis using the equation:
$[Y] = 0.5 \times [b - K_d - [X] + \sqrt{([X] + K_d - b)^2 + 4b \times K_d}]$, where [Y] is the free [MBP-16E6] in nM, [X] is the total [peptide] in nM, K_D is the binding dissociation constant to be fitted by the equation, and b is the maximal possible E6 concentration to be fitted by the equation. By fitting the free [MBP-16E6] and [peptide] to the equation, a binding constant with a fitting error was generated by Prism 8 software.

1.17 Protein expression and purification methods

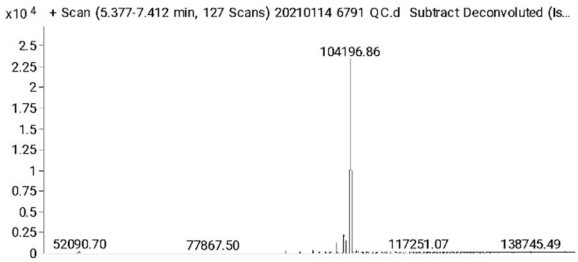
1.17.1 **E6AP**

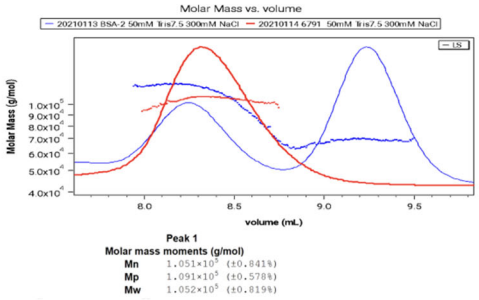
Expression and Purification Method:
E6AP(1-875) protein with a C-terminal TEV-6xHis-Avi sequence was subcloned into a pFastBac1 vector (Genscript). Bacmid and viruses of E6AP prepared as described by vendor's instructions (Invitrogen Version A, A10606) were amplified in *Sf9* cells (ThermoFisher, cat no. 11496-015). P2 viruses at 2 µL/mL virus to media were used to infect *Sf21* cells for protein expression. Cells were harvested 48 hours post-infection.

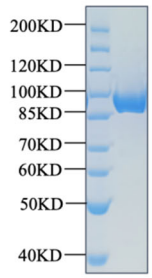
Sf21 cells were lysed by French Press in 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM Imidazole, 5% glycerol, 1 mM PMSF, and cOmplete Protease Inhibitor (Roche, cat no. 53002800). The supernatant was collected after centrifugation at 39,800 RCF for 30 minutes and loaded onto Ni Resin (Bestchrom, cat no. AA0053) and washed with 10 CVs of 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 1mM PMSF, and 20 mM imidazole before eluting with the same buffer supplemented with 500mM imidazole. The elution was diluted five-fold with 50 mM Tris-HCl pH 7.5 before loading onto a Mono Q 10/100 GL column (Cytiva, Cat no. 17516701) and eluted with a 20 CV linear gradient (Buffer A: 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5% glycerol, 1 mM PMSF; Buffer B: 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 5% glycerol, 1mM PMSF). Fractions containing E6AP as determined by SDS-PAGE and Coomassie staining were pooled and concentrated using Amicon Centrifugal Filters (cat no. R1SB42368) and loaded onto a HiLoad 16/600 Superdex 200pg column (Fisher Scientific, cat no. 28989335) equilibrated in 25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM TCEP.

Sequence:
MEKLHQCYWKSGEPQSDDIEASRMKRAAAKHLIERYYHQLTEGCGNEACTNEFCASCPTFLR
MDNNAAAIKALELYKINAKLCDPHPSKKGASSAYLENSKGAPNNSCSEIKMNKKGARIDFKDVT
YLTEEKVYEILELCREREDYSPLIRVIGRVFSSAEALVQSFRKVKQHTKEELKSLQAKDEDKDED
EKEKAACSAAAMEEDSEASSSRIGDSSQGDNNLQKLGPDDVSVDIDAIRRVYTRLLSNEKIETA
FLNALVYLSPNVECDLTYHNVYSRDPNYLNLFIIVMENRNLHSPEYLEMALPLFCKAMSKLPLAA
QGKLIRLWSKYNADQIRRMMETFQQLITYKVISNEFNSRNLVNDDDAIVAASKCLKMVYYANVV
GGEVDTNHNEEDDEEPIPESSELTLQELLGEERRNKKGPRVDPLETELGVKTLDCRKPLIPFEE
FINEPLNEVLEMDKDYTFFKVETENKFSFMTCPFILNAVTKNLGLYYDNRIRMYSERRITVLYSLV
QGQQLNPYLRLKVRRDHIIDDALVRLEMIAMENPADLKKQLYVEFEGEQGVDEGGVSKEFFQL
VVEEIFNPDIGMFTYDESTKLFWFNPSSFETEGQFTLIGIVLGLAIYNNCILDVHFPMVVYRKLMG
KKGTFRDLGDSHPVLYQSLKDLLEYEGNVEDDMMITFQISQTDLFGNPMMYDLKENGDKIPITN
ENRKEFVNLYSDYILNKSVEKQFKAFRRGFHMVTNESPLKYLFRPEEIELLICGSRNLDFQALEE
TTEYDGGYTRDSVLIREFWEIVHSFTDEQKRLFLQFTTGTDRAPVGGLGKLKMIIAKNGPDTER
LPTSHTCFNVLLLPEYSSKEKLKERLLKAITYAKGFGMLENLYFQGHHHHHHGLNDIFEAQKIEW
HE*

QC: LC-MS, MALS and Gel







1.17.2 **MBP-16E6**

Expression and Purification Method:

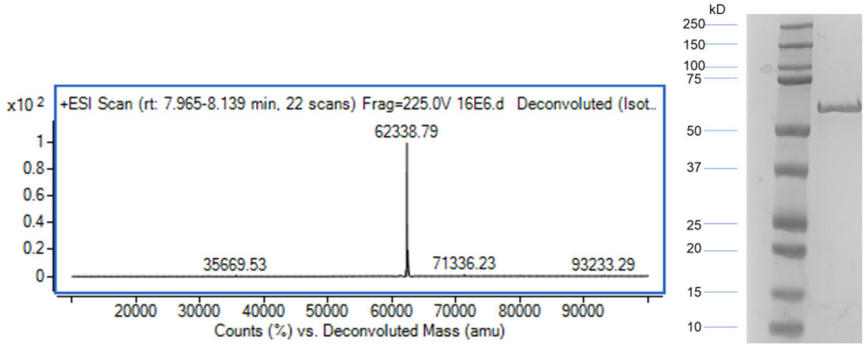
HPV16 E6(8-158) with a N-terminal HisMBP and four cysteine to serine point mutations to increase solubility was subcloned into a pET45b vector (Genscript). The plasmid was transformed into BL21(DE3) competent cells (NEB, Cat no. C2527H). The cells were grown in LB media supplemented with 50 µg/mL carbenicillin shaking at 190 RPM in a 37°C incubator to an optical density of 0.6 at 600 nm before induction with IPTG at a final concentration of 0.5 mM. Protein expression was allowed to proceed for 16 hours shaking at 190 RPM in an 18°C incubator before harvest.

BL21(DE3) cells were resuspended in 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 2 mM DTT, and cOmplete Protease Inhibitor before lysis by sonication . The supernatant was collected after centrifugation at 30,000 RCF for 45 minutes and loaded onto a MBPTrap HP column (Cytiva, cat no. 28918779) equilibrated in the same resuspension buffer and washed for 30 CV. The protein was eluted with 50 mM Tris-HCl pH 7.5, 500 mM NaCl, 2 mM DTT, and 15 mM maltose. The elution was further purified by size exclusion chromatography on a HiLoad 26/600 Superdex 200pg column (Cytiva, cat no. 28989336) equilibrated in PBS supplemented with 1 mM DTT.

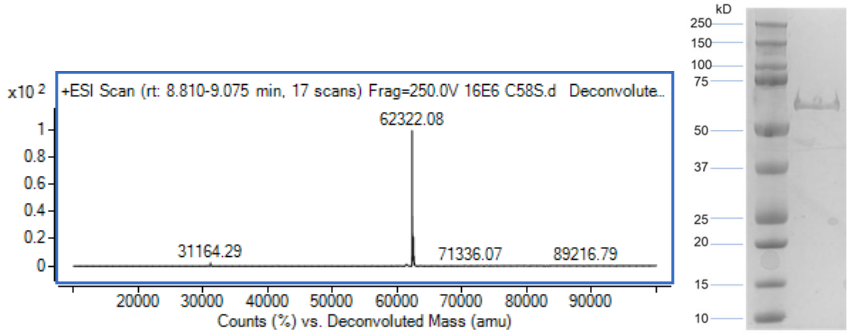
Sequence:

MAHHHHHHPMKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAAT
GDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYN
KDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVG
VDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTV
LPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEE
LAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTNSSSNNN
NNNNNNNPMSENLYFQGAMFQDPQERPRKLPQL**C**TELQTTIHDIILE**C**VY**C**KQQLLRREVYDF
AFRDL**C**IVYRDGNPYAV**C**DK**C**LKFYSKISEYRHYSYSLYGTTLEQQYNKPLSDLLIR**C**IN**C**QKPL
SPEEKQRHLDKKQRFHNIRGRWTGR**C**MS**C**SRSSRTRRETQL*

QC: LC-MS and Gel of MBP 16E6 4C4S



QC: LC-MS and Gel of MBP 16E6 4C4S C58S



1.17.3 **SUMO-MDM2**

Recombinant SUMO-MDM2 was expressed as previously reported[3].

In brief, the 25-109MDM2 gene was purchased from Addgene (pGEX-4T MDM2 wild type (WT), 16237). The SUMO tag was incorporated using the Champion™ pET SUMO Expression System (Invitrogen, CA). SUMO-25-109MDM2 was expressed in Rosetta (DE3) pLysS cells. The bacteria were inoculated to reach OD600 = 0.5 at 37°C, induced with 0.4 mM IPTG for 4 hours, and pelleted. Approximately 1 L broth produced 10 g cell pellet, which was resuspended in 30 mL of 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 buffer containing 40 mg lysozyme, 1 mg Roche DNAase I, and one tablet of Roche protease inhibitor cocktail, and then sonicated for three times for 20 s. The suspension was then centrifuged at 30,000 rcf for 30 min to clarify the lysate. The supernatant was loaded onto a 5 mL HisTrap FF crude Ni-NTA columns (GE Healthcare, UK), and washed sequentially with 30 mL of 20 mM Tris-HCl, 150 mM NaCl, pH 8.5 and then 30 mL 20 mM Tris-HCl 150 mM NaCl, 80 mM imidazole pH 8.5. The protein was eluted with 10 mL of 20 mM Tris-HCl, 500 mM imidazole, 500 mM NaCl, pH 8.5. The eluted protein was buffer exchanged into 20 mM Tris-HCl, 50 mM NaCl, pH 8.5 using a HiPrep 26/10 desalting column (GE Healthcare, UK). The resulting protein solution was purified the same day using a 5 mL HiTrap Q HP (GE Healthcare, UK) anion exchange columns with a linear NaCl gradient (B% graded from 5% to 25%, B = 20 mM Tris-HCl, 500 mM NaCl, pH 8.5). Fractions containing pure SUMO-25-109MDM2, as determined by SDS-PAGE, were collected, and concentrated to 0.5 mg/mL using a 10 kDa nominal molecular weight limit Amicon Ultra-15 Centrifuge Filter Unit (EMD Millipore), and immediately flash frozen and stored at −80 °C.

1.18 16E6/E6AP interaction disruption assay

E6AP protein was biotinylated by the commercial BirA kit (AVIDITY, CA). In brief, 40 µL of E6AP was added directly into a tube containing 20 µl of 1 mg/mL BirA biotin-protein ligase. Then the

solution was added with 5 µL of BiomixB (10× concentration: 100 mM Adenosine 5′-triphosphate, 100 mM MgOAc, 500 µM d-biotin), followed by the addition of 5 µL of BIO200 (d-biotin; 10× concentration: 500 µM). The whole mixture was incubated at 4°C for 16 hrs, followed by a spinning filter through 0.2 µm. Excess biotin was purified away on Superdex200 or AdvanceBio 150mm (Agilent, MA) equilibrated with PBS + 1mM DTT. Biotin-E6AP fractions were collected and frozen at -80°C before use. 50 µL of 12 µM MBP-16E6 in PBS was added with 0.5 µL of 10 mM reactive peptide of interest (final concentration 100 µM). The mixture was incubated at 4°C for 16 hrs. One µM of Biotin-E6AP protein was immobilized onto streptavidin sensors soaked in a blocking buffer. The sensors were then dipped into the mixture of MBP-16E6 and reactive peptide. The signal response was recorded.

1.19 Assessment of 13-biotin and 13-TAMRA specificity in HT1080 cells

HT1080 cells (CCL-121, ATCC) were cultured until they reached ~80% confluency, at which point they were harvested using trypsinization and subsequent centrifugation at 500 x g for 5 minutes. Resulting cell pellets were washed with 1x PBS and lysed via the introduction of cold Pierce IP lysis buffer (catalog no. 87787), supplemented with 1x Halt Protease Phosphatase inhibitor cocktail (ThermoFisher, catalog no. 1861284). This lysis process was conducted on ice for 10 minutes, with periodic pipetting to ensure adequate mixing. Post-lysis, the supernatant was cleared by centrifuging at maximum speed using a bench-top Eppendorf centrifuge (model #5425R) for 10 minutes at 4°C. After removing the supernatant, the resulting lysates were quantified using the Pierce 660 nm Protein Assay (catalog no. 22660), with BSA standards serving as a reference.

HT1080 lysate (50 µg) was supplemented with either MBP-16E6 (2 µM) or MBP-16E6 C58S (2 µM), followed by an overnight incubation at 4°C with the presence of **13-TAMRA** (10 µM), **13-biotin** (10 µM), or **13-3L3A-biotin** (10 µM). Each reaction was diluted in 1 x PBS supplemented with 1 mM DTT and 5% glycerol to a final volume of 20 µL while maintaining DMSO concentrations constant at 0.5%. Resulting reactions were subjected to 4-20% Criterion TGX polyacrylamide gel electrophoresis (BioRad). Samples containing **13-TAMRA** were visualized on an Amersham ImageQuant 800 using the Cy2 settings. Protein ladder (LI-COR; catalog no. 928-60000) was also imaged using IR short and IR long settings. Following this, all gels underwent transfer onto 0.2 µm nitrocellulose (catalog no. 1704159, BioRad) using the 'Mixed Mw Turbo' mode of the Trans-Blot Turbo Transfer System (catalog no. 1704150, BioRad). Post-transfer, membranes were rinsed with ultrapure water, stained with Ponceau S staining solution (Thermo Scientific; catalog no. A40000278) for 5 minutes, washed again, and imaged on a BioRad ChemiDoc MP Imaging System with visible light.

Membranes were then blocked with Intercept (TBS) blocking buffer (catalog no. 9276001, LI-COR), followed by overnight incubation at 4°C with shaking in Intercept (T20, TBS) Antibody Diluent buffer (catalog no. 92765001, LI-COR) incubated with α-HPV16E6 antibody (catalog no. GTX132686, Genetex, lot no. 44601) and α-GAPDH (catalog no. MA5-15738, lot no. WL332983), each diluted to 1:1,000. Blots were then washed four times for 10 minutes each with TBS-T, and incubated with goat-anti-mouse (680 nm conjugate; catalog no. 925-68070, lot no. D10901-11) and goat-anti-rabbit (800 nm conjugate; catalog no. 926-32211, lot no. D20322-15) secondary antibodies for 2 hours at room temperature with shaking. This was followed by an additional series of four 10-minute washes with TBS-T.

13-biotin and **13-3L3A-biotin** blots were visualized using the ODYSSEY CLx LI-COR instrument with Image Studio software (version no. 5.2). The scanning parameters included automated intensity, 169 µm pixel size, and 0.0 mm offset. After imaging for GAPDH and MBP-16E6, the **13-**

biotin and **13-3L3A-biotin** blots were incubated with IRDye 680 RD streptavidin (LI-COR, catalog no. 926-68079, lot no. D20803-07), at a 1:2,000 dilution in Intercept antibody diluent for 30 minutes. The blots were then washed four times for 10 minutes each with TBS-T and subsequently imaged. These initial conditions led to signal saturation, prompting the blots to be stripped using the NewBlot Nitrocellulose stripping buffer (LI-COR, catalog no. 928-40030, lot no. D30111-04) for 5 minutes, following the manufacturer's instructions. After stripping, the blots were re-imaged as previously described, resulting in an unsaturated signal.

Blots containing **13-TAMRA** were visualized using the ODYSSEY M LI-COR instrument paired with LI-COR acquisition 1.1 software. Scanning was carried out in the 700, 800, and 488 nm channels using the 'membrane' assay settings and 100 µm resolution. To ensure reproducibility, all experiments were conducted at least three times. The figures presented in the paper represent typical gels and blots from these repeated experiments.

1.20 Structural modeling of 16E6-bound Peptide 13

Our Peptide **13** consists of a flexible N-terminal region {*9-fluorenyl acetamido*-IPQSA}, the native E6AP core {ELTLQEELL}, and flexible C-terminal {Dha-RRKK-K *(1-anthracenyl acetamido)*} segments, as shown by its sequencing (**Figure 3B**). The E6AP peptide, which interacts with the 16E6 protein and functions as a ubiquitin ligase, has an alpha-helical conformation according to the x-ray 16E6-MBP(E6AP)-p53 ternary complex [PDB ID: 4XR8]. To investigate whether the native core sequence of E6AP in Peptide **13** also adopts an alpha helix, we used the x-ray complex as a starting point, removed the MBP and p53 proteins, and kept the E6-bound E6AP peptide. We then conducted a 1.1 µs MD simulation in a box (60.0Å * 60.0Å * 60.0Å) of water (~6K water molecules) and ions and found that the E6AP peptide remained stable (**Figure S11**), with an RMSD of 0.4 Å (measured using the Cα atoms) compared to the E6AP peptide in the ternary complex.

Using the Molecular Operating Environment (MOE) software (Molecular Operating Environment (MOE), Canada**)**, we expanded upon the alpha helix E6AP core by incorporating the flexible N-terminal and C-terminal sequences to construct our Peptide **13** molecule (**Figure S12B**). To optimize the Peptide **13** structure, we employed the LowModeMD algorithm[4], which is integrated into MOE. We limited the sampling by imposing the following parameters defined in the MOE package: {Rejection Limit=100; Iteration Limit=10000; RMS Gradient=0.005, MM Iteration Limit=500; RMSD limit=0.75 Å; Energy Window =10.0 kcal/mol; Conformational Limit=10,000}. Additionally, we treated the backbone atoms of the E6AP peptide as a rigid segment to maintain the alpha-helix conformation during the calculations.

To determine how Peptide **13** binds to the 16E6 protein (**Figure S12C** and **S12D**), we used the MOE docking module for molecular docking, which is known to be reliable for capturing peptide/receptor interactions[5,6]. To ensure the accuracy of our approach, we first performed molecular docking to determine the binding mode of the native E6AP peptide to the crystallographic 16E6 protein, using the X-ray E6-bound native E6AP peptide (PDB ID: 4XR8) as the starting point after eliminating the MBP and p53 from the ternary complex. We employed a docking protocol that started with the triangular matcher algorithm to quickly generate 1,000 poses of docked peptides based on the receptor shape. From the resulting docked poses, we used the London dG scoring function to screen among the resulting poses to keep the top 100 structures for further refinement. We then used the rigid receptor replacement method combined with the GBVI/WSA dG scoring function to refine the resulting poses, obtaining an E6-bound E6AP peptide that closely matched the crystallographic binding mode of E6AP (**Figure S13**). Similarly, we

followed the same docking procedure to obtain top 3 docking poses of 16E6-Peptide-13 complexes using our pre-generated conformations of peptide **13** (described above) to dock to the binding pocket of the native E6AP peptide in the 16E6.

Next, we performed ~60 ns of constrained MD simulations to further refine the E6-Peptide-13 complexes in the presence of water and ions. Our goal was to push the peptide-13 warhead (Dha) toward the 16E6 Cys58 hotspot and create a covalent link between the two. To achieve this, we placed a harmonic restraint at 3.0 Å between Cys58 (S atom) and Dha (C atom), with a force constant of 0.36 kcal mol^{-1} $Å^{-2}$. These simulations allowed us to prepare the 16E6-Peptide-13 constructs for the formation of the covalent link between Dha and Cys58.

Subsequently, we covalently linked Cys58 and Dha using MOE followed by energetically minimizing the covalent link and the 16E6-Pepitde-13 complexes. We then subjected each of three complexes to a ~120 ns classical MD simulation to characterize the binding mode of Peptide-13 covalently linked to the 16E6 protein. The resulting final complexes closely match the crystallographic E6AP binding peptide (**Figure S14**). For further analysis, we selected the structure featuring the largest binding affinity between the peptide-13 core sequence and E6 while exhibiting the lowest RMSD of the peptide-13 core sequence compared to the crystallographic native E6AP peptide. For the results presented in **Figure 5**, we used **GROMOS** algorithm[7] with RMSD cutoff=0.18Å to cluster the entire MD trajectory of the best 16E6-Pepitde-13 complex.

MD Simulation protocol

Before running molecular docking and MD simulations, we used the MOE preparation module to refine the crystallographic E6 protein and added the missing sidechains/residues. The 16E6-pepitde complexes were then immersed in a water/ion (150 mM excess ions) box and underwent 500 steps of energy minimization using the steepest descents algorithm in GROMACS [8]. The refinement was followed by an MD simulation in a canonical ensemble, where the system was heated gradually from 0 K to 310 K in 20 ps, followed by MD simulations in an isobaric-isothermal ensemble for an aggregated 80 ps, during which the pressure was maintained at 1 bar to relax the simulation box. Throughout these pre-equilibration steps, the positional restraints were placed on all heavy atoms, gradually reduced to 0 kcal.$mol^{-1}Å^2$ for the final equilibration step. Finally, we optimized the 16E6-pepitde constructs by removing the positional restraints.

In all simulations, the AMBER-14 force field parameter set[9] was used to describe 16E6, Peptide-13, E6AP peptide, and ions. Non-canonical amino acid residues were parameterized with the Antechamber program[10] to ensure compatibility with the AMBER force field, while the force field parameters for two tagged molecules were borrowed from the Generalized AMBER force field (GAFF)[11] . The TIP3P model was utilized to describe water.

The temperature was maintained at 310 K using a velocity-rescale[12] thermostat with a damping constant of 1.0 ps for temperature coupling and the pressure was controlled at 1 bar using a Parrinello-Rahman barostat algorithm[13] with a 5.0 ps damping constant for the pressure coupling. Isotropic pressure coupling was used during this calculation. The Lennard-Jones cutoff radius was 12 Å, where the interaction was smoothly shifted to 0 after 10 Å. Periodic boundary conditions were applied to all three directions. The Particle Mesh Ewald algorithm [14] was used to calculate long-range coulombic interactions with a real cutoff radius of 10 Å and a grid spacing of 1.2 Å. A compressibility of 4.5×10^{-5} bar^{-1} was used to relax the box volume. In all the above simulations, water OH bonds were constrained by the SETTLE algorithm[15]. The remaining H-bonds were constrained using the P-LINCS algorithm[16]. All MD simulations were carried out using GROMACS, with constrained MD simulations aided by PULMED[17].

1.21 LC-MS/MS analysis of pull-down by biotinylated peptides from cells

HT1080 and CaSki (CRL-1550, ATCC) cells were grown to 70-80% confluency and harvested by trypsinization and centrifugation (500 x g, 5 min). Pellets were subsequently washed with 1 x PBS and lysed by the addition of cold Pierce IP lysis buffer (cat. no. 87787) supplemented with 1x Halt protease phosphatase inhibitor cocktail (ThermoFisher, cat. no. 1861284) for 10 minutes on ice, with intermittent pipetting to mix. The supernatant was cleared by centrifugation at max speed on a bench-top centrifuge (10 min, 4°C). Supernatant was removed and lysates quantified using a 660 nm protein assay (Pierce, cat. no. 22660). Each lysate replicate (2 mg) was incubated with either DMSO (final conc. 0.5%), 5 µM of **13-biotin** or 5 µM of **13-3L3A-biotin** in a final volume of 200 µL in IP lysis buffer (Pierce) overnight at 4°C. For each condition, 200 µL of treptavidin magnetic beads (Pierce, cat. no. 88816) were prepared according to the manufacturer's instructions and incubated with the biotin-peptide treated lysate for 2 h at RT with rotation. Beads were subsequently washed 3× 1 mL with RIPA buffer (ThermoScientific; cat. no. 8990) with the addition of 1× Halt Protease Phosphatase inhibitor cocktail, and 3× 1 mL with 50 mM HEPPS, pH 8.5 buffer. After the final wash, beads were resuspended in 50 mM HEPPS (pH 8.5) for on-bead reduction, alkylation and trypsin digestion. The samples were reduced with 5 mM BondBreaker TCEP (1h, RT, shaking), then alkylated with 10 mM iodoacetamide (30 min in the dark,RT, with shaking). Trypsin was added at a final concentration of 0.5 mg/ml for overnight digestion at 37°C with shaking. Peptides were collected in new tubes and placed on ice until ready for Tandem mass tag (TMT) labeling.

TMTpro reagents were resuspended in anhydrous acetonitrile to a concentration of 20 mg/mL, then 15 µl of each TMTpro reagent (126, 127n, 127c, 128n, 128c, 129n, 129c, 130n, 130c, 131n, 131c, 132n) was used to label the individual samples. Following incubation at 25°C for 1 hour, the reaction was quenched with 5% hydroxylamine to a final concentration of 0.3% hydroxylamine. The samples were acidified to a final concentration of 0.5% TFA and then all samples combined combined. The combined sample was desalted with StageTip as previously described and dried under vacuum[18].

Peptides were analyzed on an Orbitrap Fusion Lumos mass spectrometer coupled to an EASY-nLC 1200 (Thermo Fisher Scientific). Peptides were separated on an IonOpticks Aurora Ultimate C18 column (1.7 µm particle size, 120 Å pore size, 25 cm length × 75 µm internal diameter) with the system operating at a flow rate of 300 nL/min and the column heated to 60°C. Peptides were eluted into the mass spectrometer using a 180-minute method with acetonitrile increasing over a 165-minute linear gradient from 8 to 30% in 0.125% formic acid.

Data-dependent acquisition (DDA) mode was used for mass spectrometry data collection. A high resolution MS1 scan was collected in the Orbitrap (500-1200 m/z range, 60,000 resolution, AGC 5×105, 30% RF lens, 100 ms max. injection time), and the top 10 precursors were selected for MS2 followed by MS3 analysis. For MS2, ions were isolated using a 0.5 m/z window. The MS2 scan was performed in the quadrupole ion trap (CID, AGC 1x104, 34% fixed collision energy, 35 ms max. injection time). The MS3 scan was analyzed in the Orbitrap (HCD, 60k resolution, max. AGC 5×104, 45% normalized collision energy, 250 ms max. injection time). For TMT reporter ion quantification, up to 6 fragment ions from each MS2 spectra were selected for MS3 analysis using synchronous precursor selection (SPS).

An in-house software pipeline was used to process all proteomics data[19]. Raw files were converted to mzXML files and searched against a composite human UniProt database containing forward and reverse sequences with HPV16 and HPV18 E6 sequences added (P03126 and P06463) using the Sequest algorithm. Database searching matched MS/MS spectra with fully

tryptic peptides from this composite dataset with a precursor ion tolerance of 20 ppm and product ion tolerance of 0.6 Da. TMTpro modification of peptide N-termini and lysine residues (+304.207146 Da) and carbamidomethylation of cysteine residues (+57.021464 Da) were set as static modifications. Oxidation of methionine residues (+15.994914 Da) was set as a differential modification. Peptide spectral matches were filtered to a 1% false discovery rate (FDR) using linear discriminant analysis (LDA) as previously described[19]. Non-unique peptides that matched to multiple proteins were assigned to proteins that contained the largest number of matched redundant peptide sequences using the principle of Occam's razor[19]. TMT reporter ion intensities were quantified by extracting the most intense ion within a 0.003 m/z window at the predicted m/z value for each reporter ion. Peptide intensities and signal-to-noise ratios were exported and analyzed using the msTrawler software package. Default settings were used with the exception of turning off the column/TMT channel normalization since differences in abundance between samples was to be expected[20].

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD044352.

2. LC-MS characterization of peptides

2.1 LC-MS methods

LC-MS characterizations were carried out using an Agilent 6550 quadrupole time-of-flight LC-MS. Total ion current (TIC) chromatograms were plotted. Mass spectra were integrated over the principal TIC peaks. High-performance liquid chromatography was done by the following methods: (solvent A: water with 0.1% formic acid; solvent B: MeCN with 0.1% formic acid).

Method A: Column: Phenomenex Aeris C4 column (1.0 × 150 mm, 5 µm particle size, 300 Å pore size) Gradient: 1% B (0-2 min), linear ramp from 1% B to 91% B (2-10 min.). The flow rate is 100 µL/min. MS acquisition is from 2 to 10 min.

Method B: Column: Phenomenex Aeris C4 column (1.0 × 150 mm, 5 µm particle size, 300 Å pore size) Gradient: 1% B (0-2 min), linear ramp from 1% B to 91% B (2-8 min.), 61% B to 95% B (8-10 min.). The flow rate is 100 µL/min. MS acquisition is from 2 to 8 min.

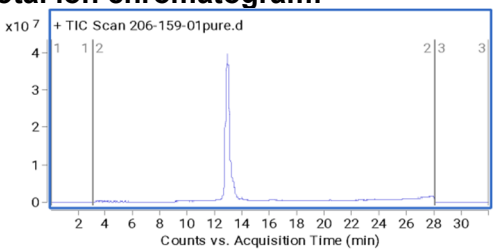
Method C: Column: Agilent Zorbax 300SB C3 column (2.1 × 150 mm, 5 µm particle size, 300 Å pore size) Gradient: 1% B (0-2 min), linear ramp from 1% B to 91% B (2-12 min), 91% B to 91% B (12-13 min.). The flow rate is 500 µL/min. MS acquisition is from 4 to 12 min.

Method D:
Column: Phenomenex Jupiter C4 column (1.0 × 150 mm, 5 µm particle size, 300 Å pore size)
Gradient: 1% B (0-2 min.), linear ramp from 1% B to 91% B (2-18 min.), 91% B to 91% B (18-21 min). The flow rate is 100 µL/min. MS acquisition is from 4 to 18 min.

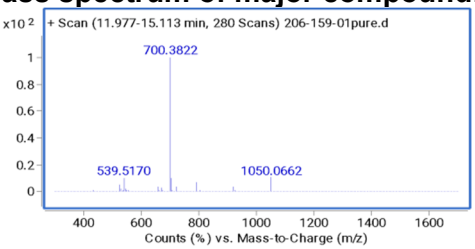
Method E:
Column: Agilent Zorbax 300SB C3 column (2.1 × 150 mm, 5 µm particle size, 300 Å pore size)
Gradient: 1% B (0-1 min.), linear ramp from 1% B to 91% B (1-11 min.), 91% B to 91% B (11-15 min.). The flow rate is 500 µL/min. MS acquisition is from 0 to 11 min.

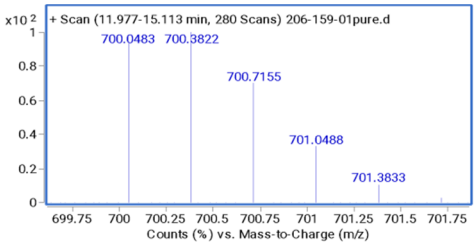
2.2 Peptides **1-13**

Name: Peptide **1**
Sequence**:** IPESSELTLQELLGEERR-NH_2
HPLC: method A
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 2097.10, found: 2097.15.
Total ion chromatogram:

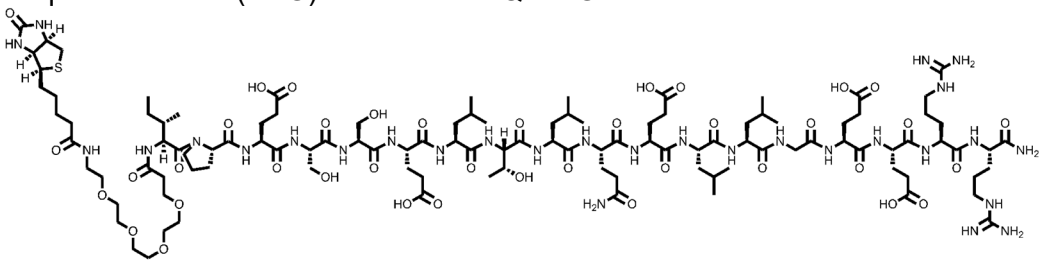


Mass spectrum of major compound:



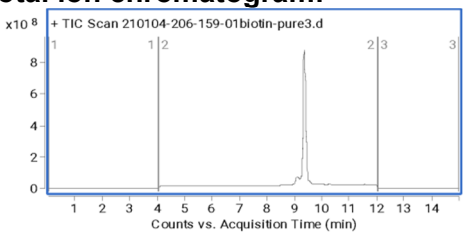


Name: Peptide **1-biotin**
Sequence**:** Biotin-(PEG)4-IPESSELTLQELLGEERR

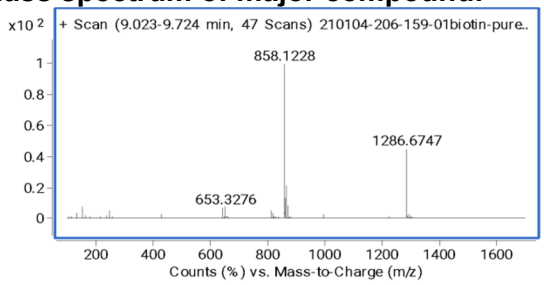


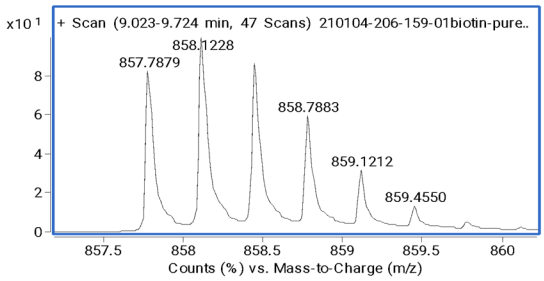
HPLC: method A
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 2570.65, found: 2570.64.

Total ion chromatogram:



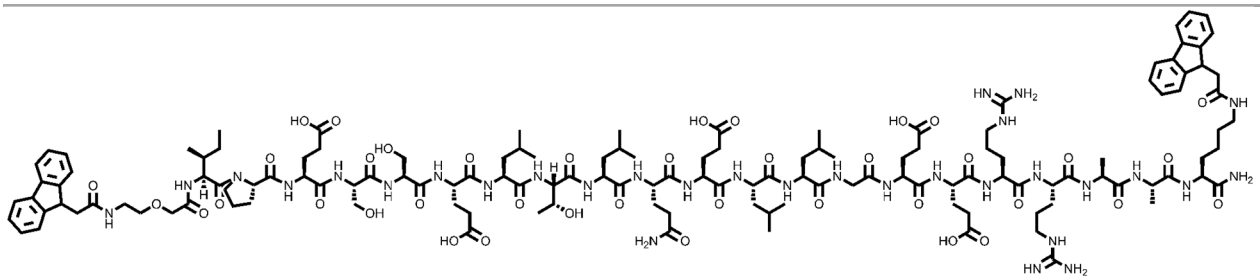
Mass spectrum of major compound:





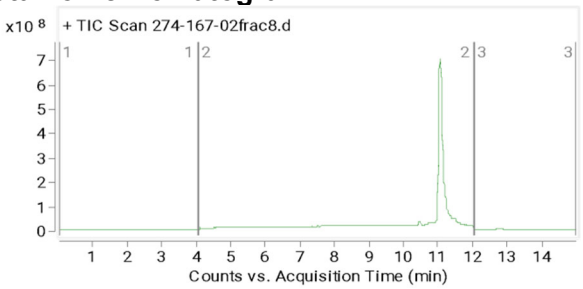
Name: Peptide **2**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido-PEG1	IPESSELTLQELLGEERRAA	Lys(9-fluorene acetamido)

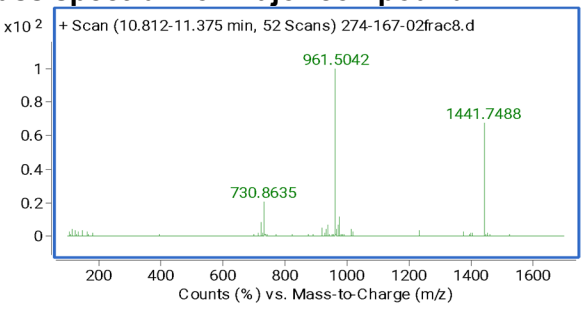


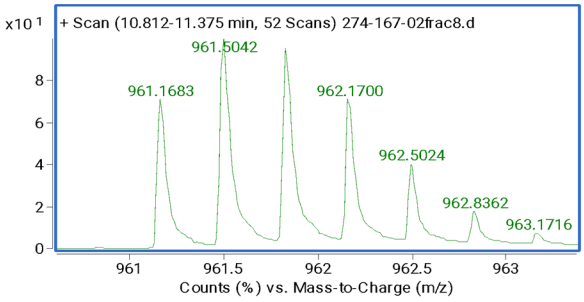
HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + H)^+$: 2880.50, found: 2880.50.

Total ion chromatogram:



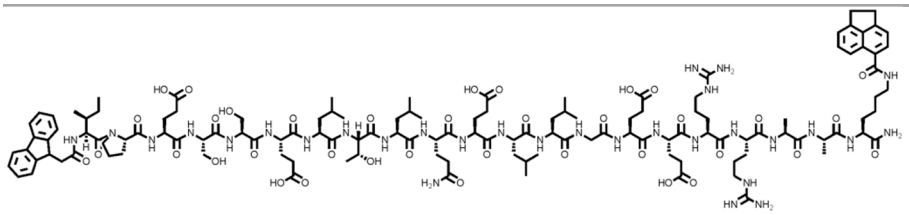
Mass spectrum of major compound:





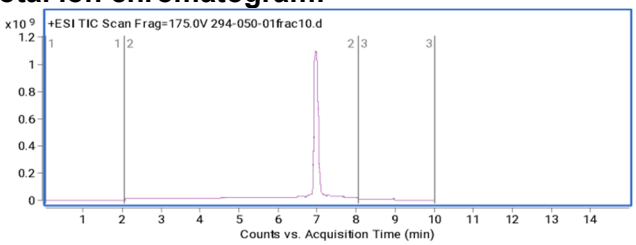
Name: Peptide **3**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido	IPESSELTLQELLGEERRAA	Lys(5-Acenaphthyl carboxamido)

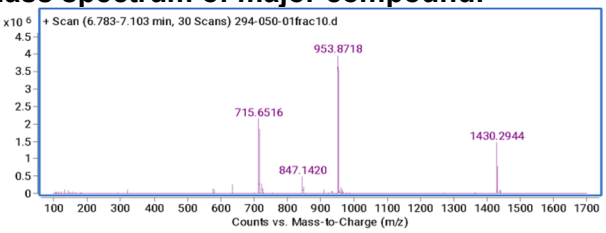


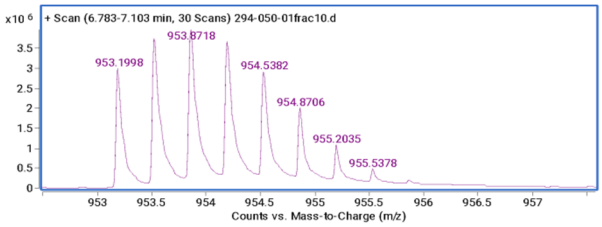
HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + H)^+$: 2760.46, found: 2760.46

Total ion chromatogram:



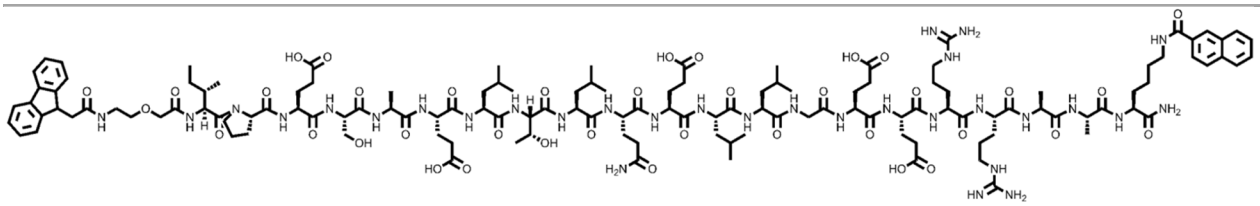
Mass spectrum of major compound:





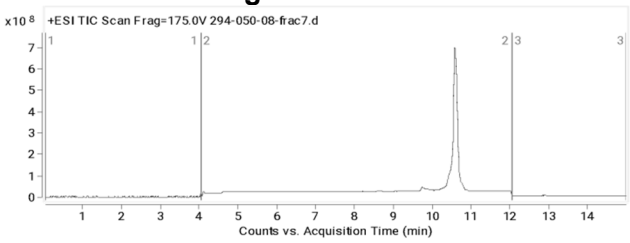
Name: Peptide **4**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido	IPESSELTLQELLGEERRAA	Lys(1-naphthylcarboxamido)

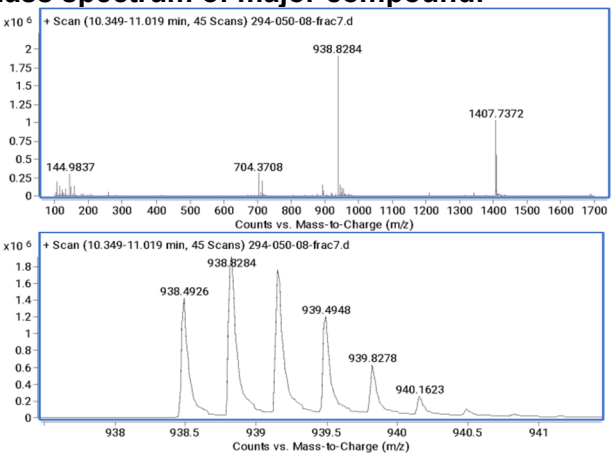


HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 3H)^{3+}$: 938.49, found:938.49.

Total ion chromatogram:

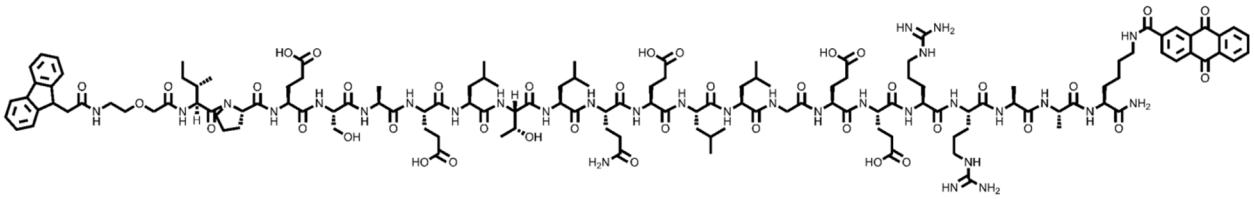


Mass spectrum of major compound:



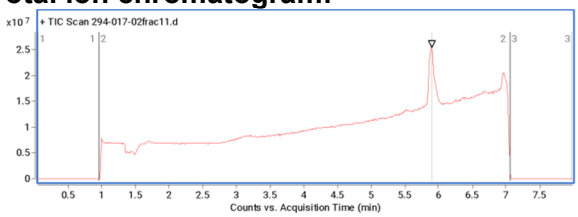
Name: Peptide **5**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido-PEG_1	IPESSELTLQELLGEERRAA	Lys(anthraquinone-2-carboxamido)

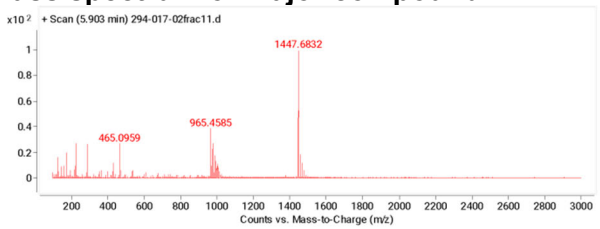


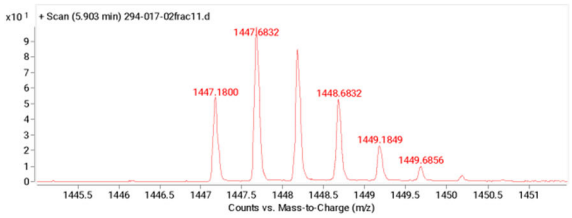
HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1447.18, found: 1447.18

Total ion chromatogram:

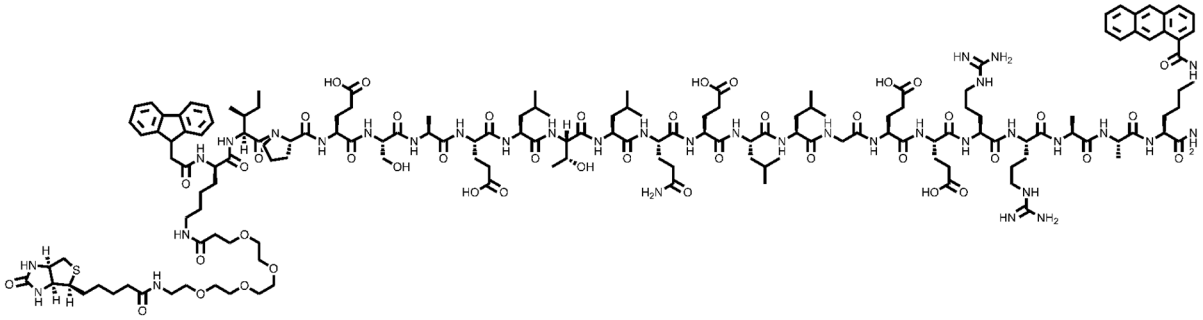


Mass spectrum of major compound:



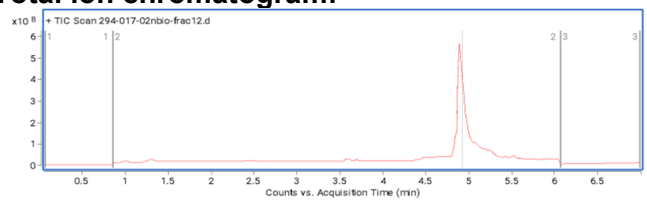


Name: Peptide **6'-biotin**
Structure**:**

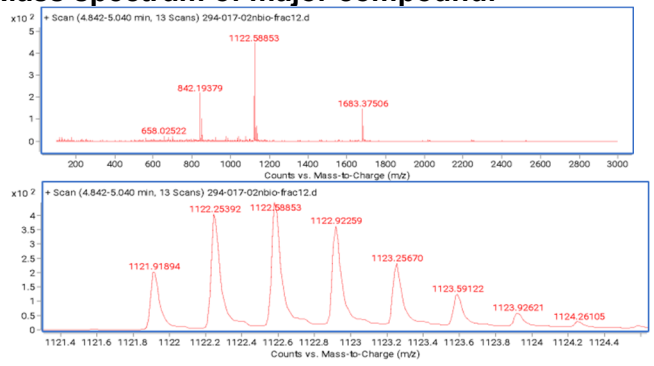


HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 3H)^{3+}$: 1121.92, found: 1121.92

Total ion chromatogram:

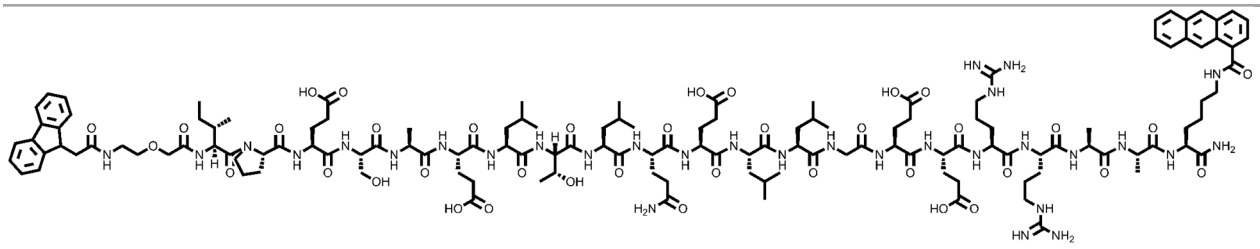


Mass spectrum of major compound:



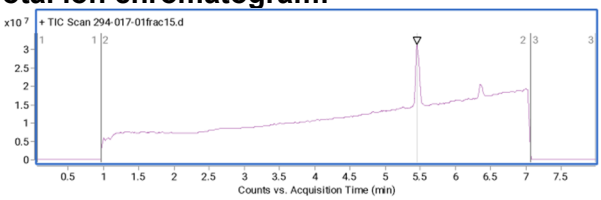
Name: Peptide **6'**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido-PEG_1	IPESAELTLQELLGEERRAA	Lys(1-anthracene carboxamido)

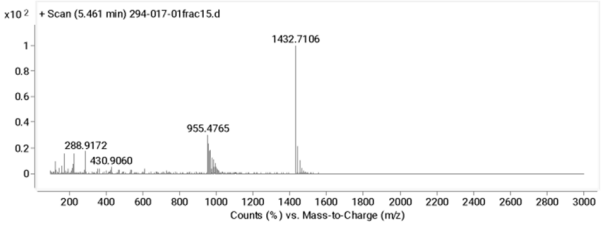


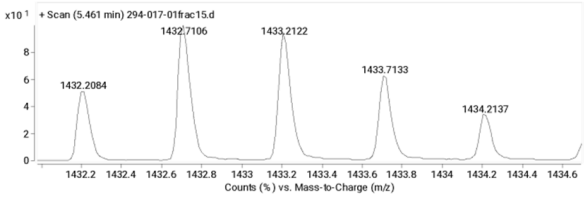
HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1432.20, found: 1432.21.

Total ion chromatogram:



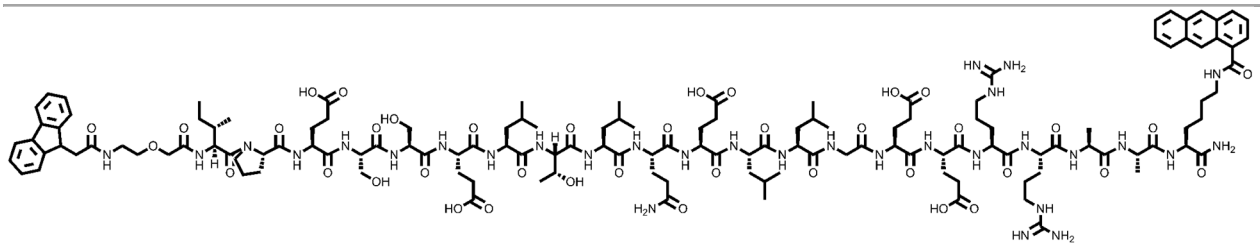
Mass spectrum of major compound:





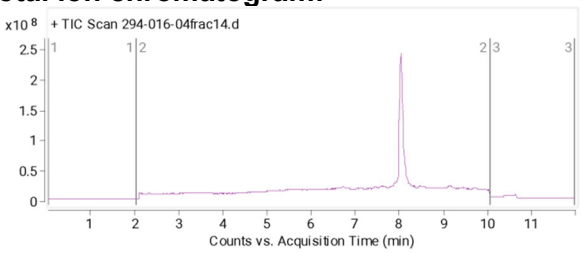
Name: Peptide **6**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido-PEG_1	IPES**S**ELTLQELLGEERRAA	Lys(1-anthracene carboxamido)

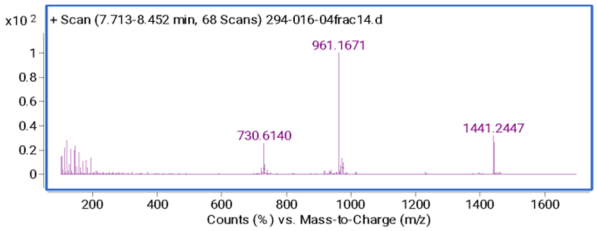


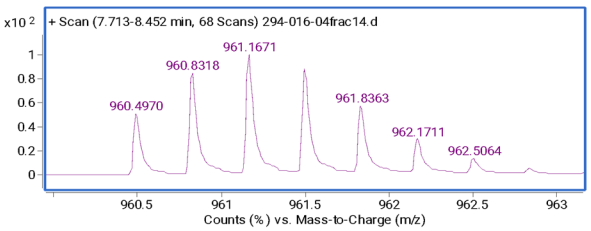
HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 3H)^{3+}$: 960.50, found: 960.497,

Total ion chromatogram:



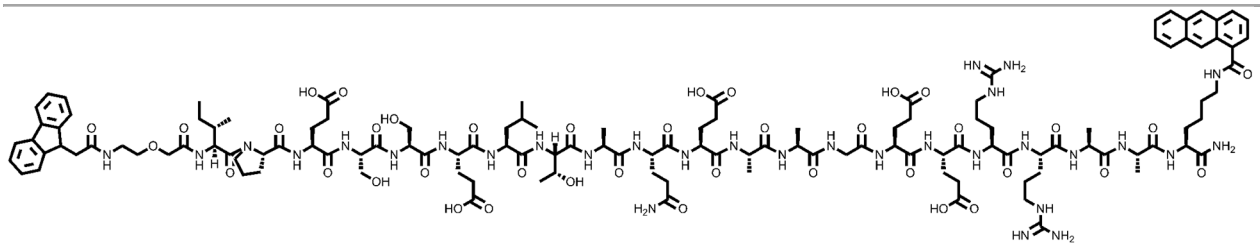
Mass spectrum of major compound:





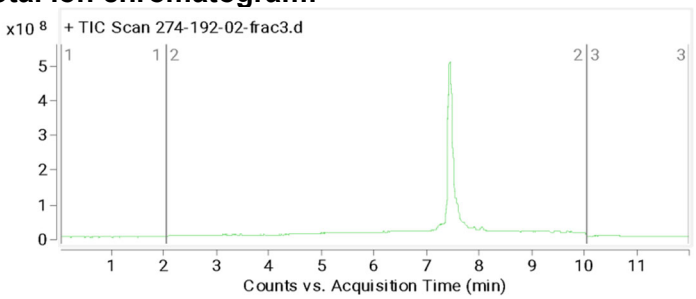
Name: Peptide **6'-3L3A**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido-PEG_1	IPESAELTAQEAAGEERRAA	Lys(1-anthracene carboxamido)

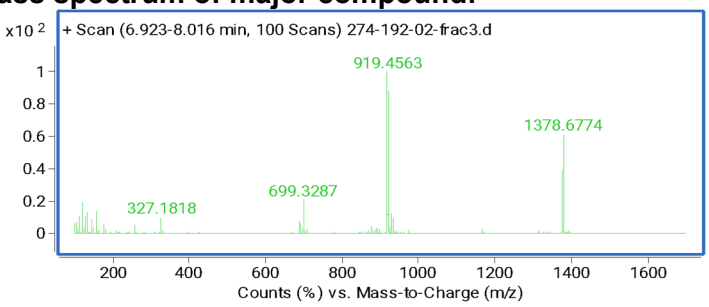


HRMS (ESI-QTOF): Calcd. for $(M + H)^+$: 2754.3548, found: 2754.3548.

Total ion chromatogram:

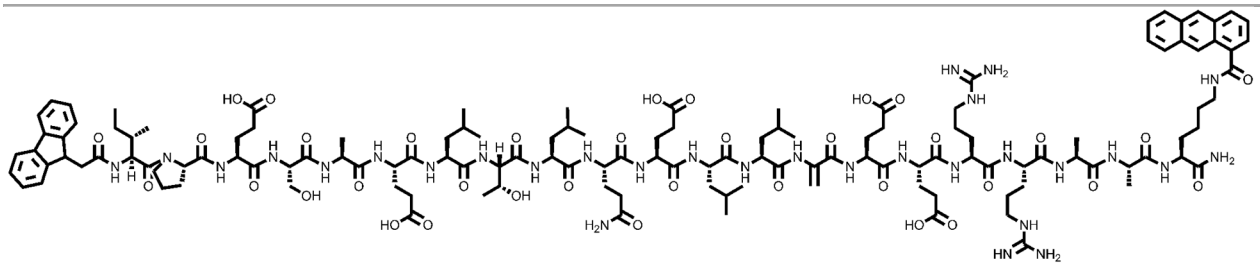


Mass spectrum of major compound:



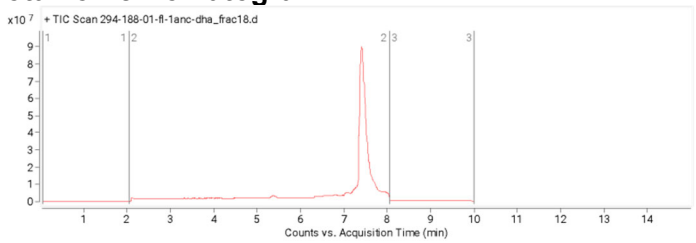
Name: Peptide **7**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido	IPESAELTLQELL(DHA)EERRAA	Lys(1-anthracene carboxamido)

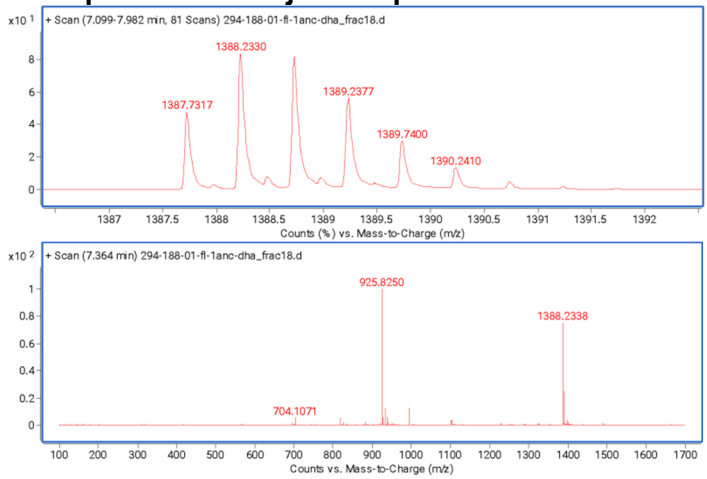


HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1387.73, found: 1387.73

Total ion chromatogram:

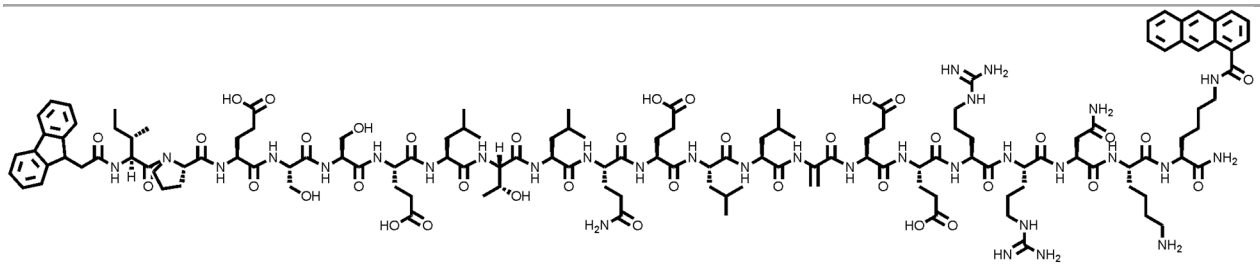


Mass spectrum of major compound:



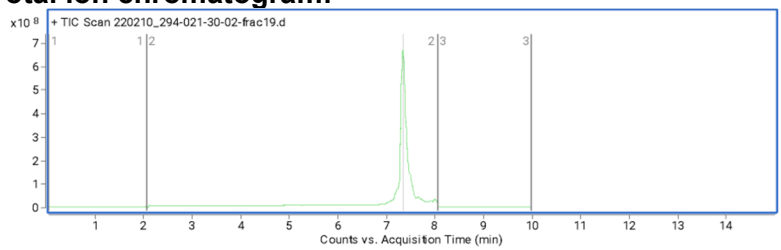
Name: Peptide **8**
Structure:

N-terminal	Sequence	C-terminal
9-fluorene acetamido	IPESSELTLQELL(DHA)EERRNK	Lys(1-anthracene carboxamido)

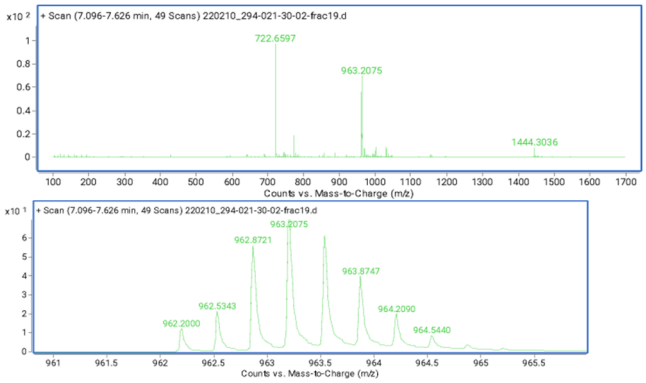


HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 3H)^{3+}$:962.20, found: 962.20.

Total ion chromatogram:

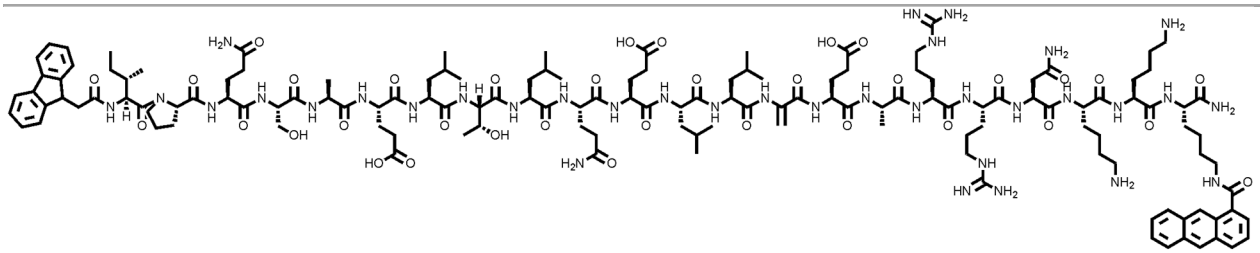


Mass spectrum of major compound:



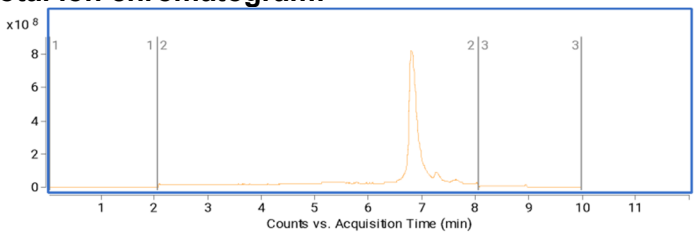
Name: Peptide **9**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido	IPQSAELTLQELL(DHA)EARRNKK	Lys(1-anthracene carboxamido)

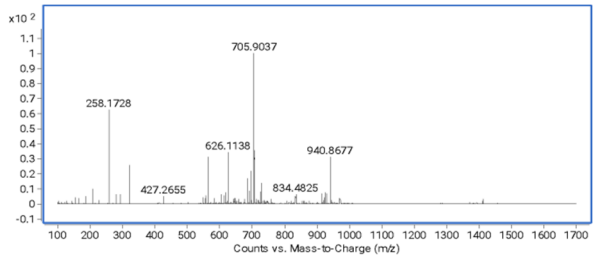


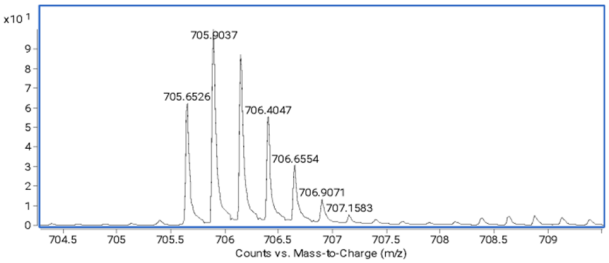
HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 4H)^{4+}$: 705.65, found: 705.65.

Total ion chromatogram:



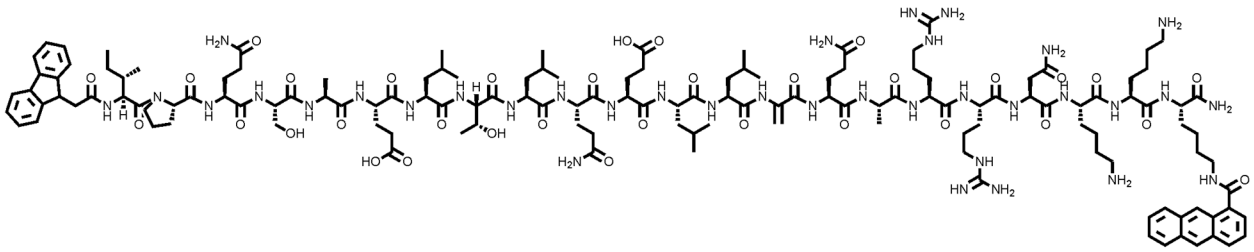
Mass spectrum of major compound:





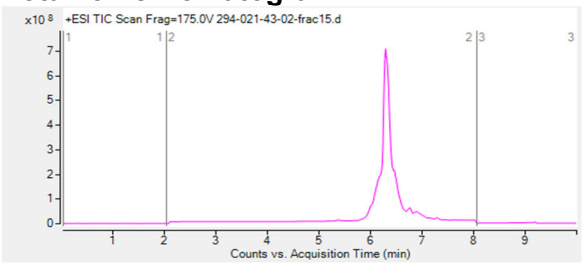
Name: Peptide **10**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido	IPQSAELTLQELL(DHA)QARRNKK	Lys(1-anthracene carboxamido)

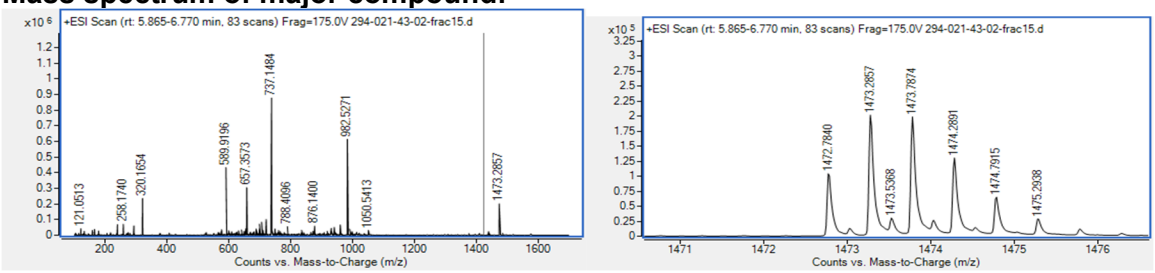


HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1472.89, found: 1472.78.

Total ion chromatogram:

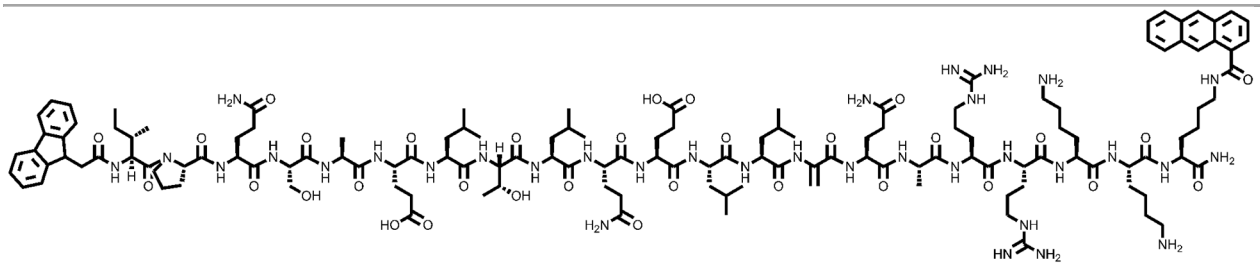


Mass spectrum of major compound:



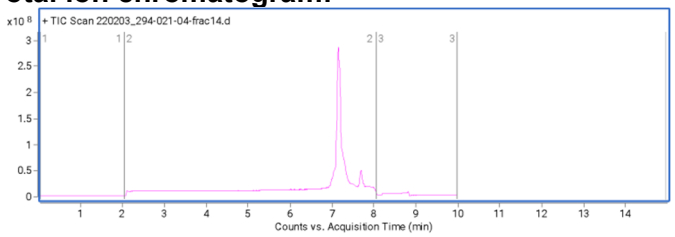
Name: Peptide **11**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido	IPQSAELTLQELL(DHA)QARRKK	Lys(1-anthracene carboxamido)

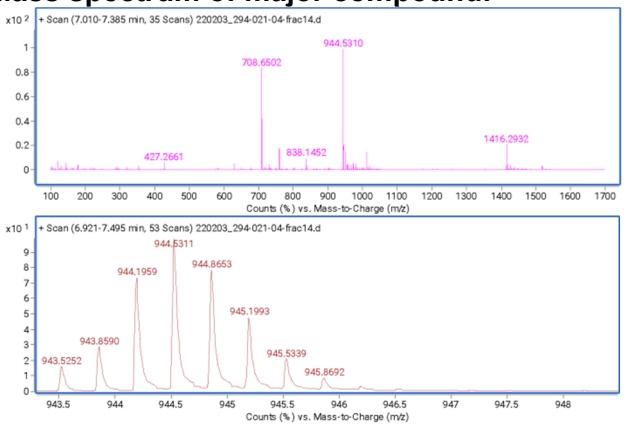


HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 3H)^{3+}$: 943.52, found: 943.52.

Total ion chromatogram:

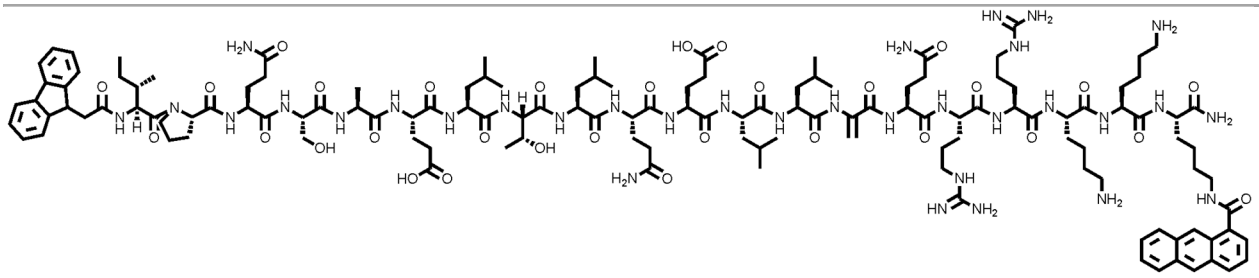


Mass spectrum of major compound:



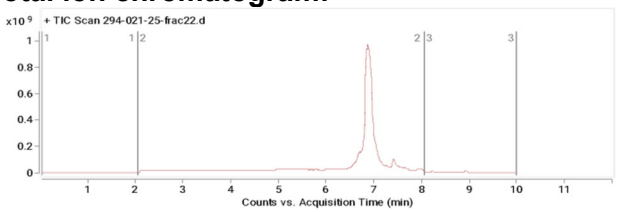
Name: Peptide **12**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido	IPQSAELTLQELL(DHA)QRRKK	Lys(1-anthracene carboxamido)

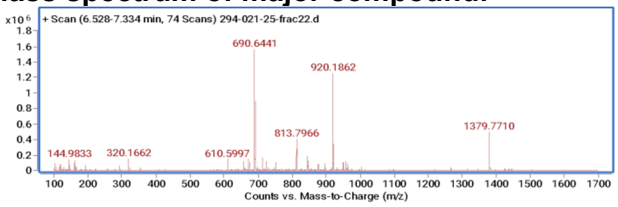


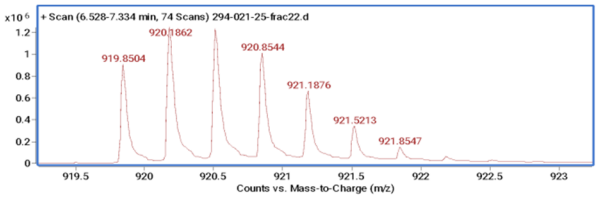
HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 3H)^{3+}$: 919.87, found: 919.85.

Total ion chromatogram:



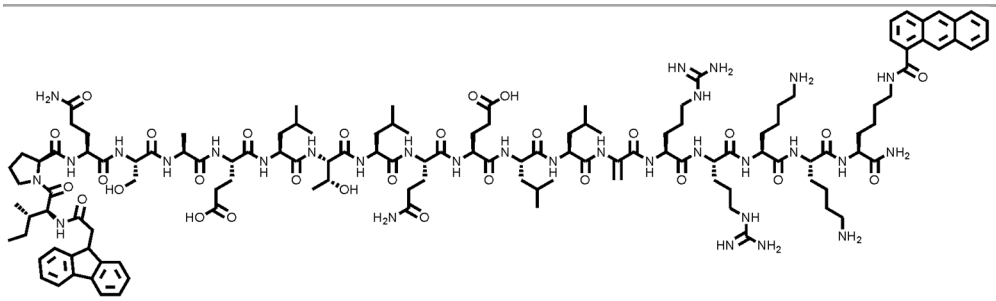
Mass spectrum of major compound:





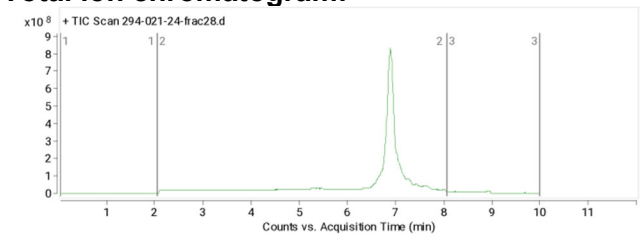
Name: Peptide **13**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido	IPQSAELTLQELL(DHA)RRKK	Lys(1-anthracene carboxamido)

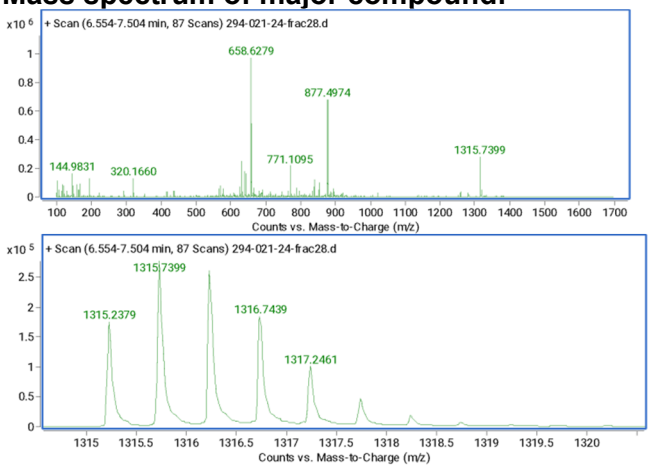


HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M+2H)^{2+}$: 1315.23, found: 1315.24.

Total ion chromatogram:

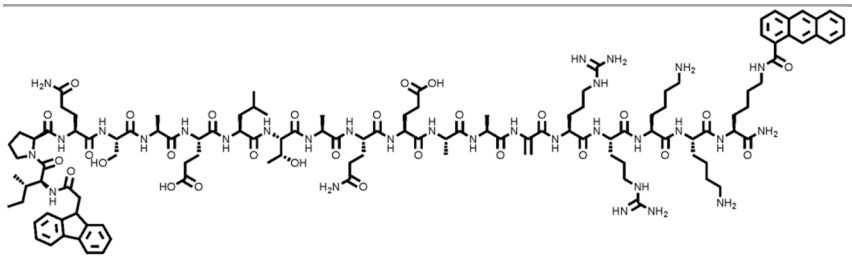


Mass spectrum of major compound:



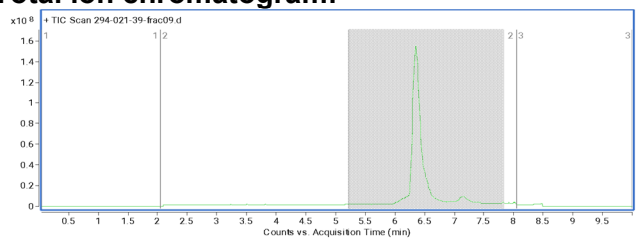
Name: Peptide **13-3L3A**
Sequence**:**

N-terminal	Sequence	C-terminal
9-fluorene acetamido	IPQSAELTAQEAA(DHA)RRKK	Lys(1-anthracene carboxamido)

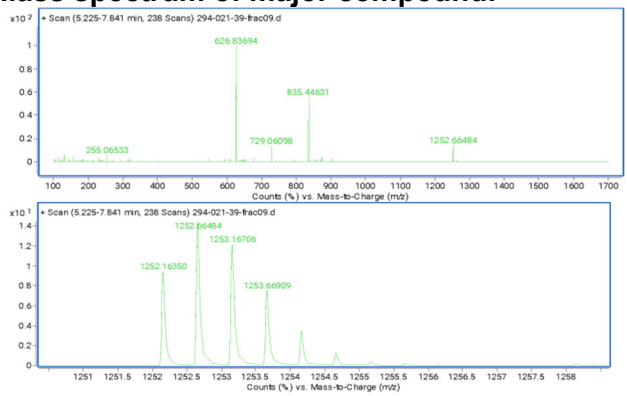


HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + H)^+$: 2502.32, found:2502.32.

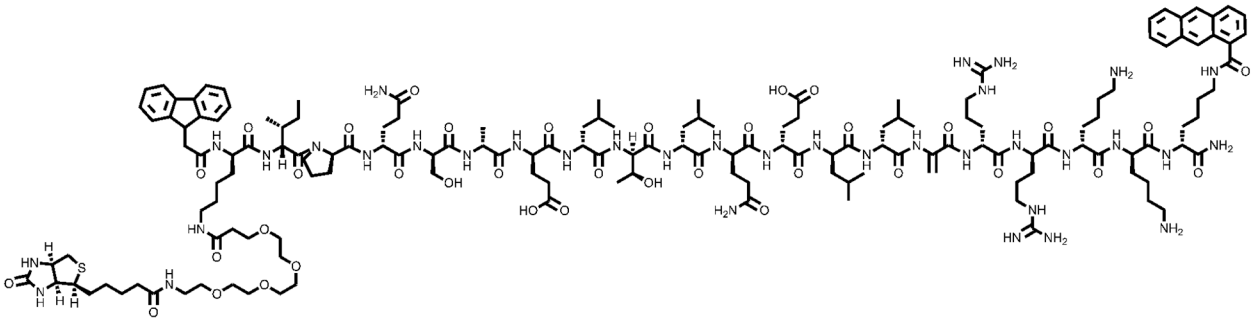
Total ion chromatogram:



Mass spectrum of major compound:

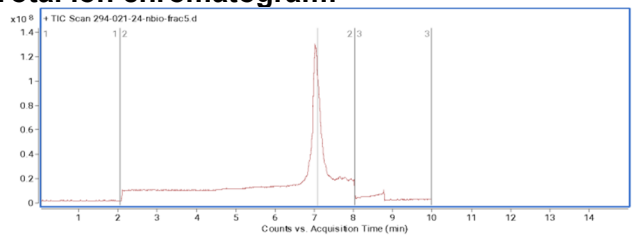


Name: Peptide **13-biotin**
Structure**:**

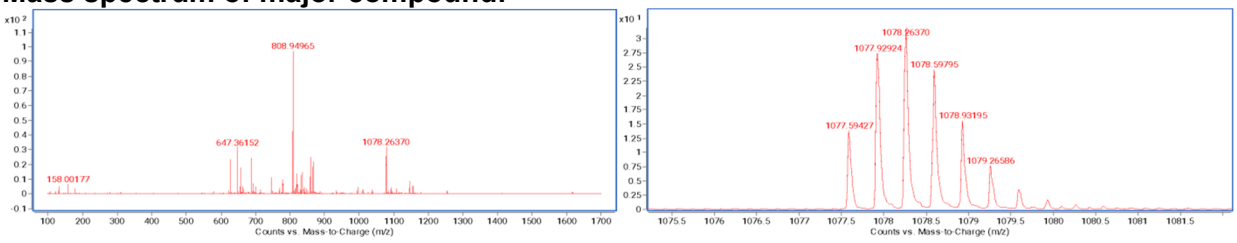


HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + H)^+$: 3229.77, found: 3229.78

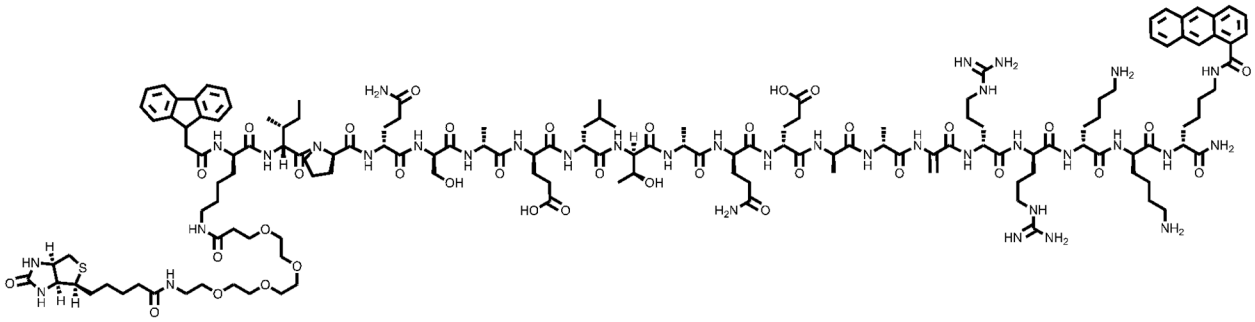
Total ion chromatogram:



Mass spectrum of major compound:

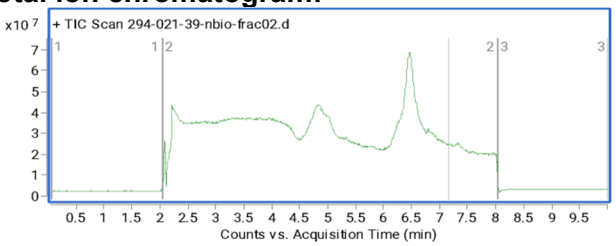


Name: Peptide **13-3L3A-biotin**
Structure**:**

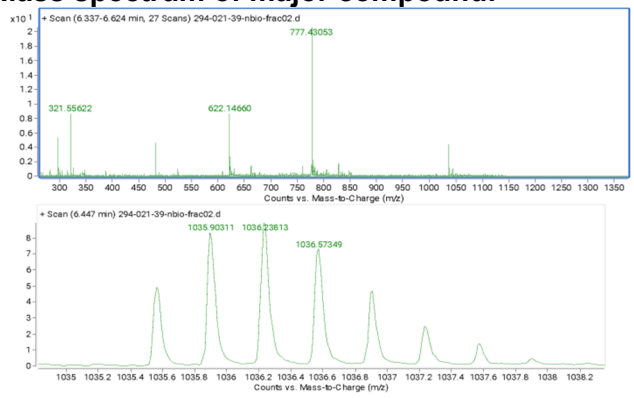


HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + H)^+$: 3103.63, found: 3103.69.

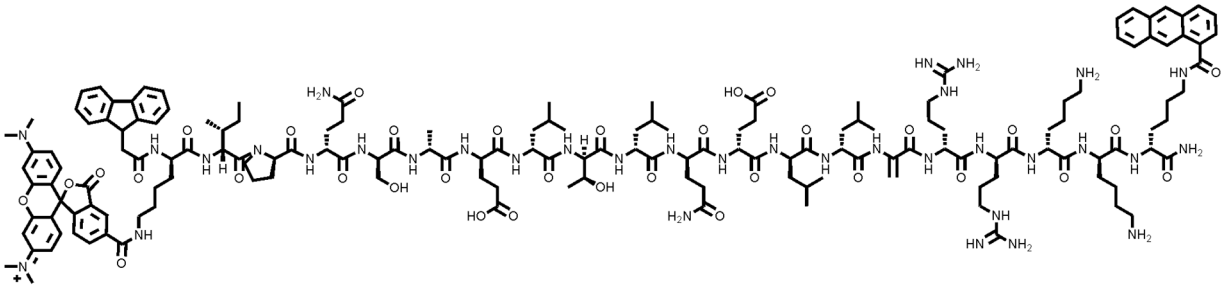
Total ion chromatogram:



Mass spectrum of major compound:

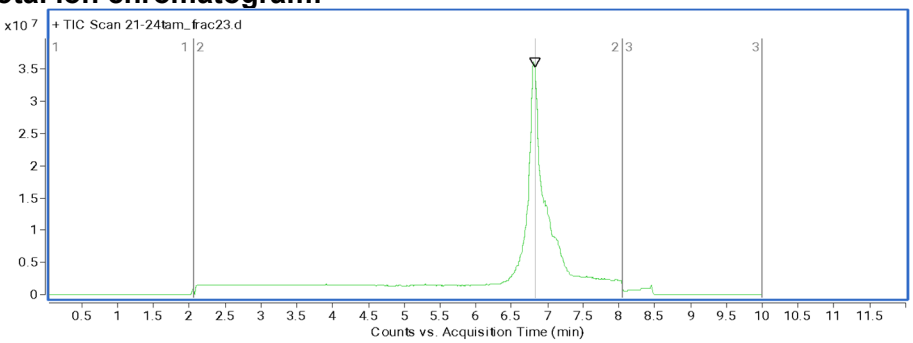


Name: Peptide **13-TAMRA**
Structure**:**

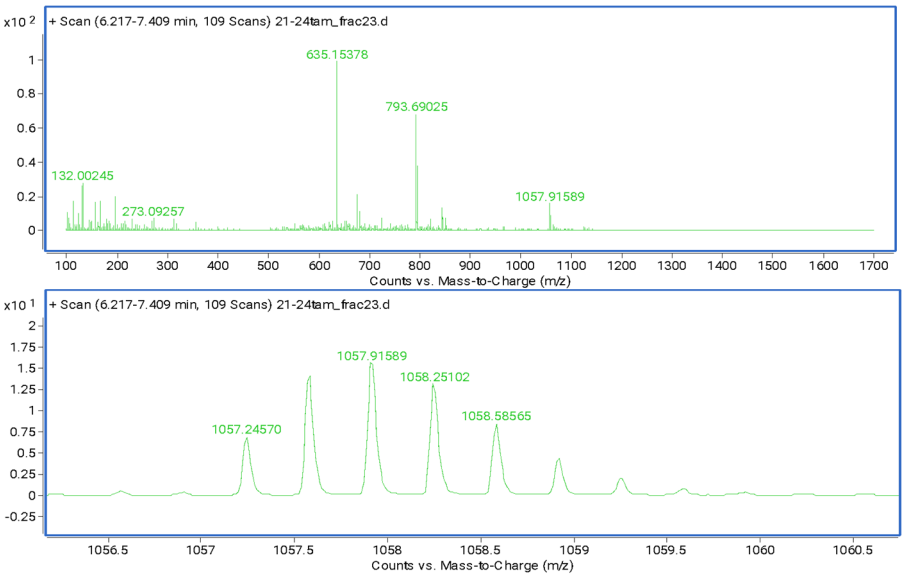


HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + H)^+$: 3168.70, found: 3168.73

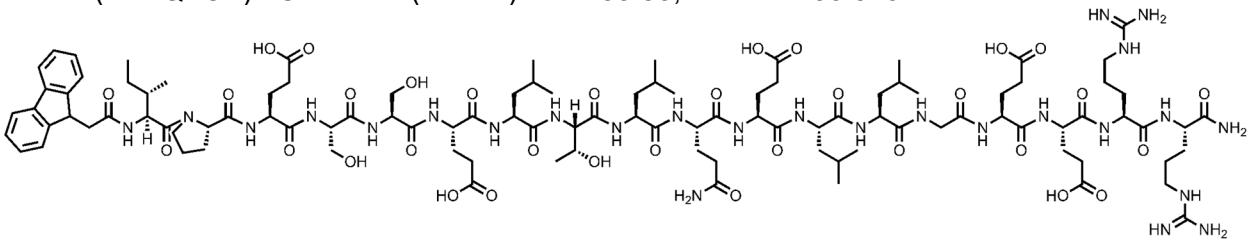
Total ion chromatogram:



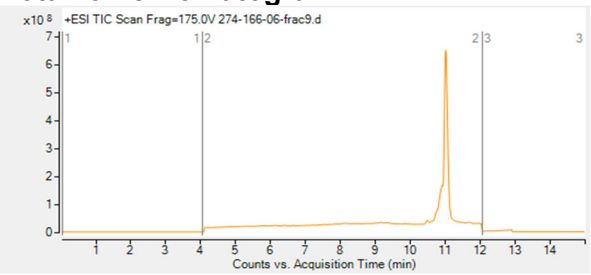
Mass spectrum of major compound:



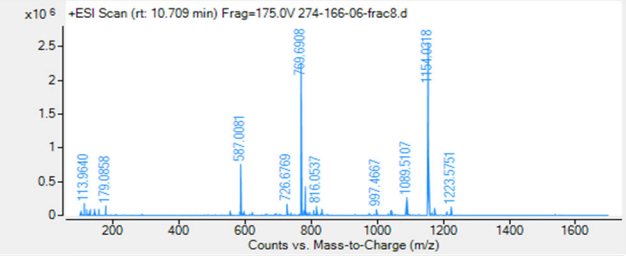
2.3 Peptides **N1-N22** and **N-FITC**
Name: Peptide **N1**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1153.53, found: 1153.5297.

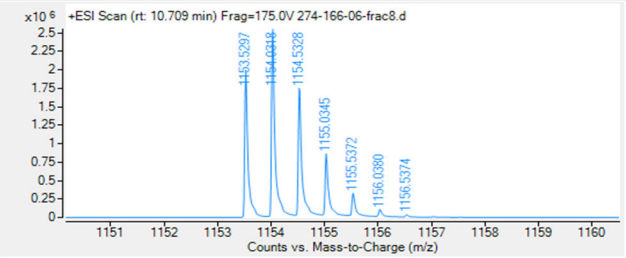


Total ion chromatogram:

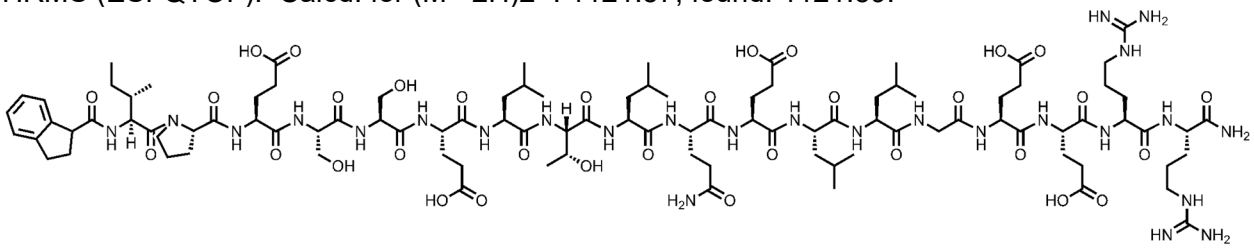


Mass spectrum of major compound:

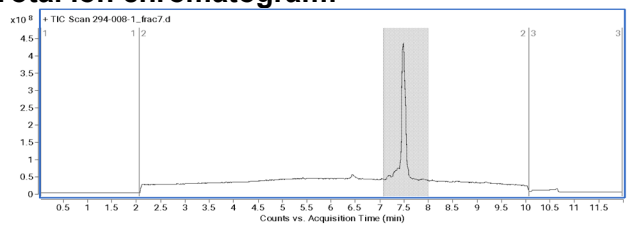




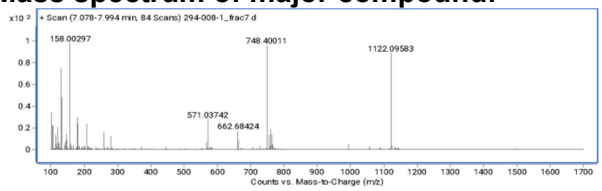
Name: Peptide **N2**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1121.57, found: 1121.59.

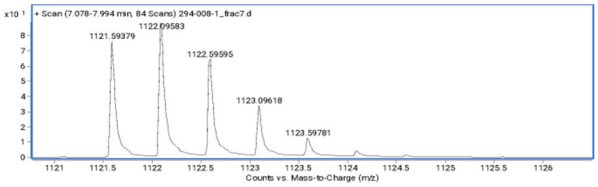


Total ion chromatogram:

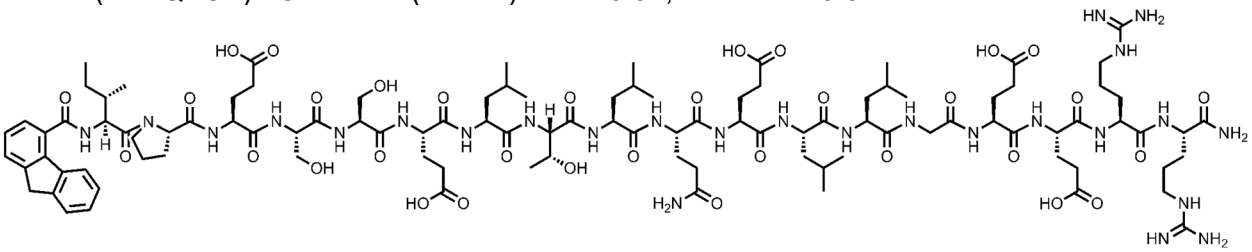


Mass spectrum of major compound:

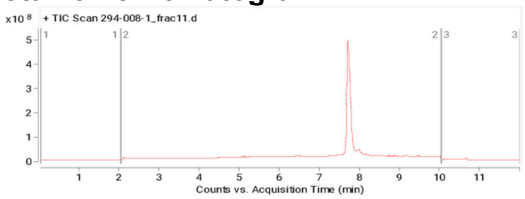




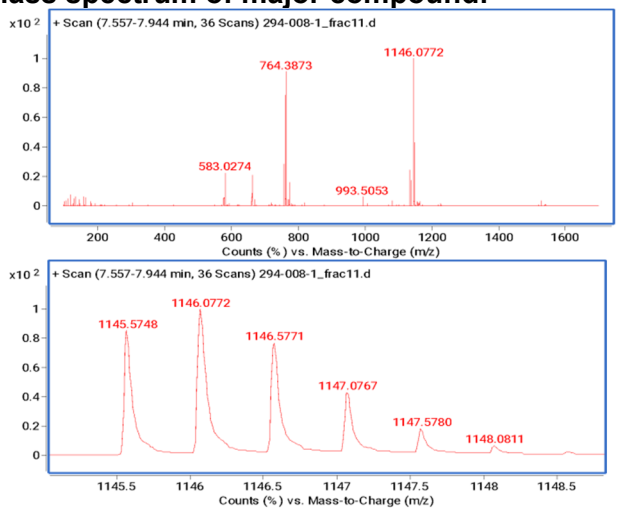
Name: Peptide **N3**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for (M +2H)2+:1145.57, found: 1145.57.



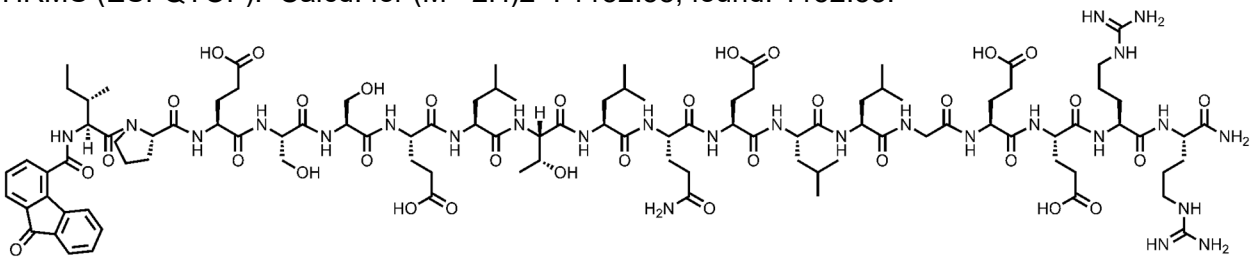
Total ion chromatogram:



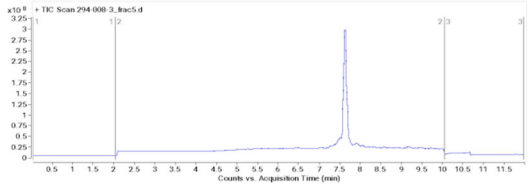
Mass spectrum of major compound:



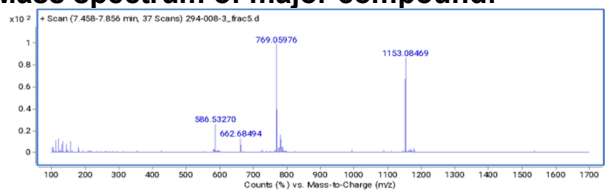
Name: Peptide **N4**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1152.58, found: 1152.58.

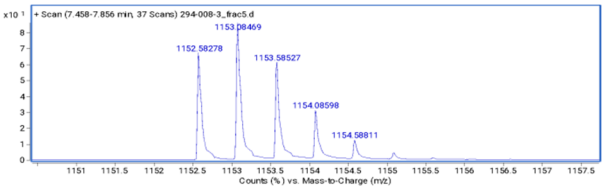


Total ion chromatogram:

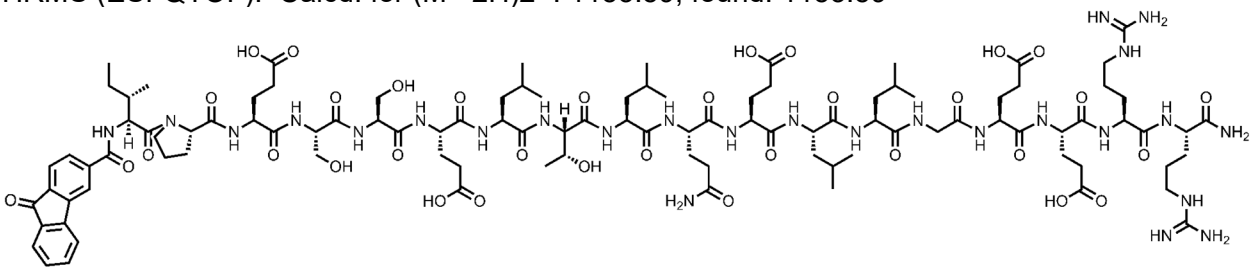


Mass spectrum of major compound:

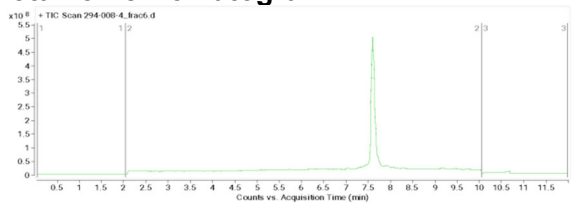




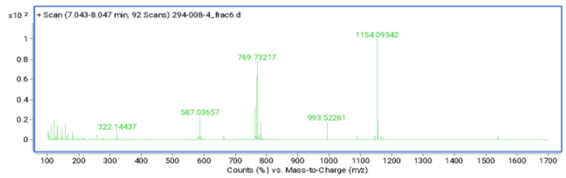
Name: Peptide **N5**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1153.59, found: 1153.59

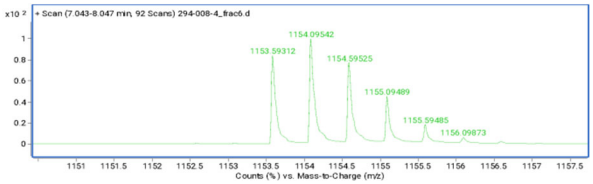


Total ion chromatogram:

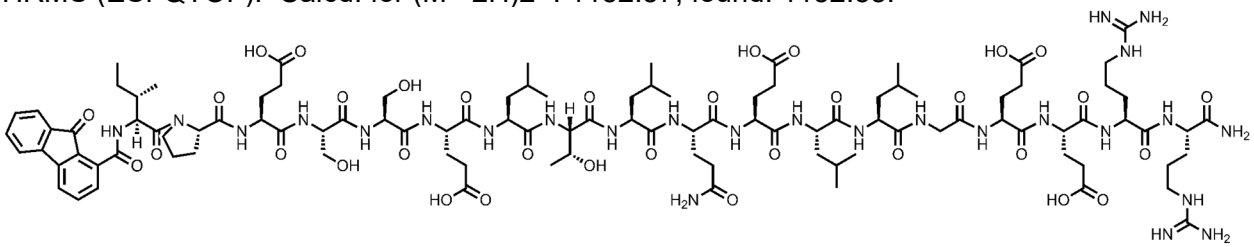


Mass spectrum of major compound:

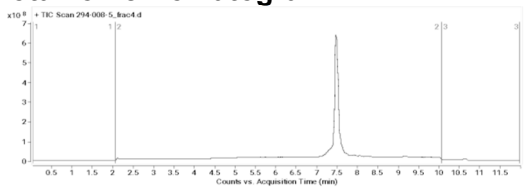




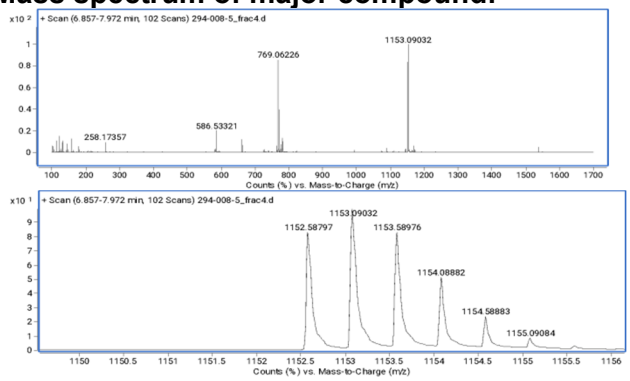
Name: Peptide **N6**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1152.57, found: 1152.58.



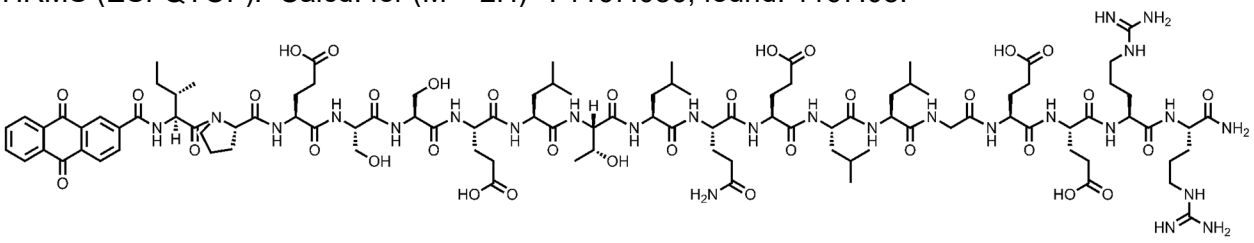
Total ion chromatogram:



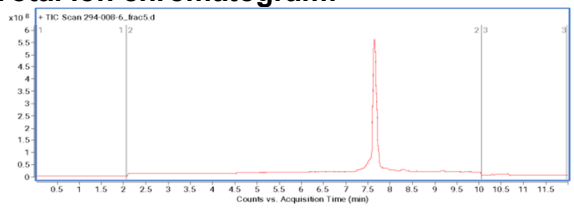
Mass spectrum of major compound:



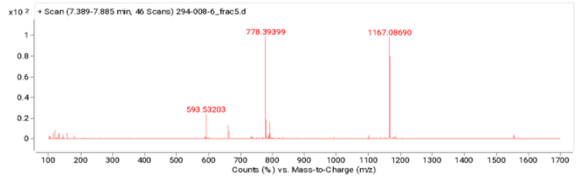
Name: Peptide **N7**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1167.086, found: 1167.08.

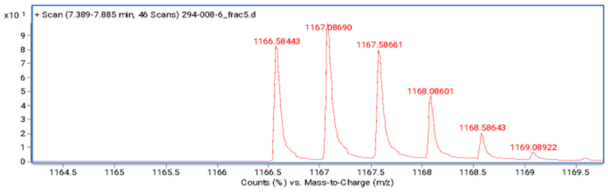


Total ion chromatogram:

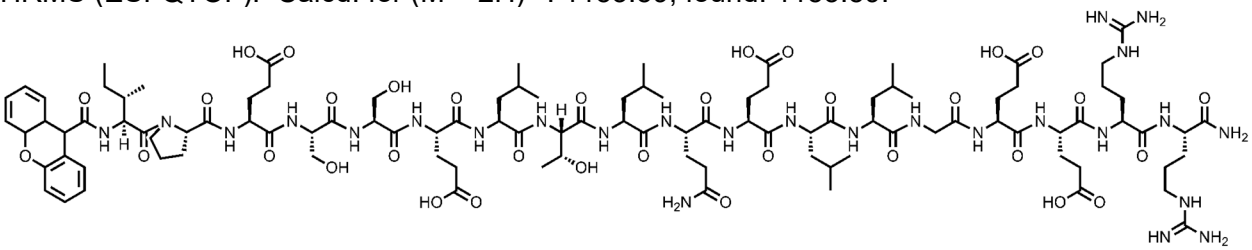


Mass spectrum of major compound:

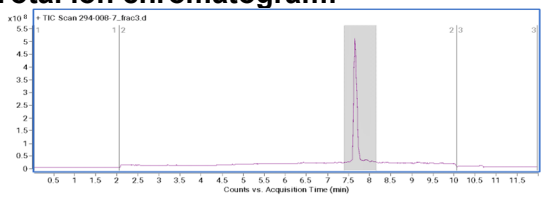




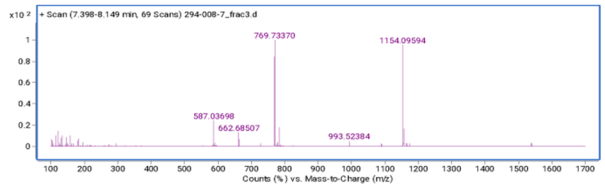
Name: Peptide **N8**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1153.59, found: 1153.59.

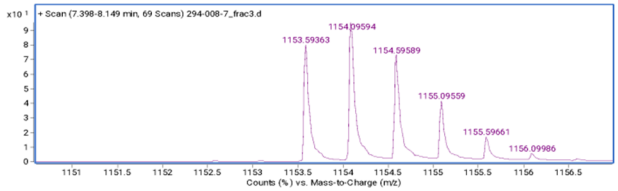


Total ion chromatogram:

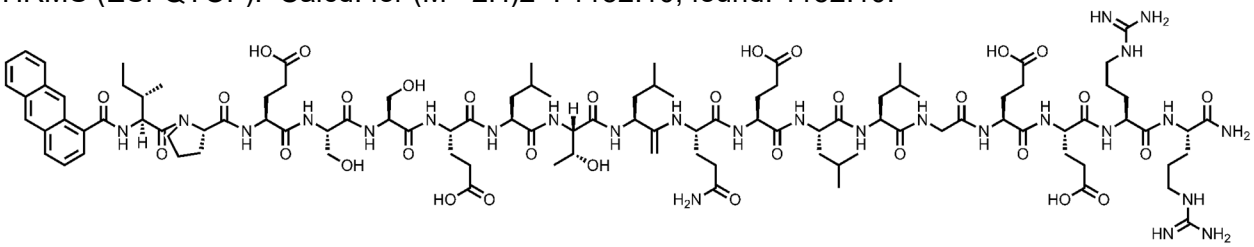


Mass spectrum of major compound:

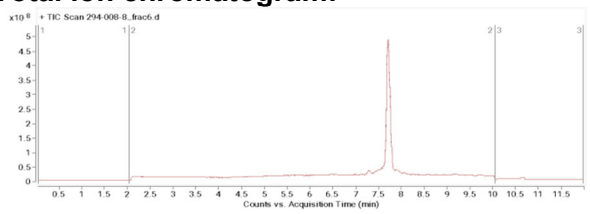




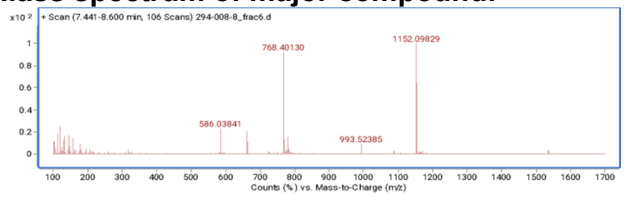
Name: Peptide **N9**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1152.10, found: 1152.10.

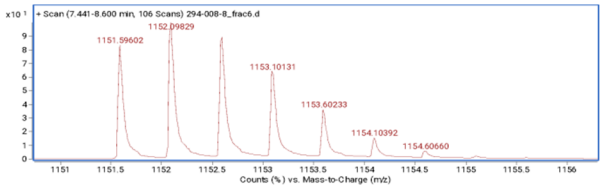


Total ion chromatogram:

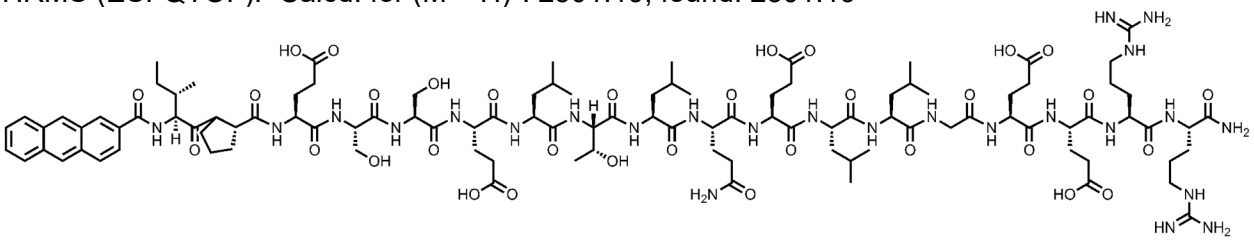


Mass spectrum of major compound:

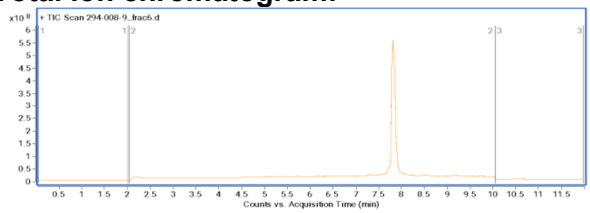




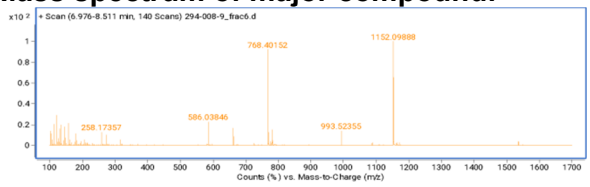
Name: Peptide **N10**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + H)^+$: 2301.19, found: 2301.19

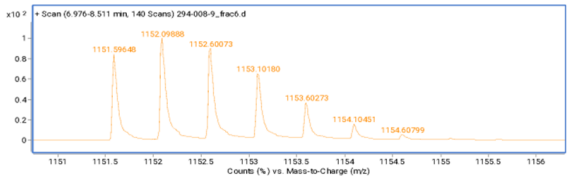


Total ion chromatogram:

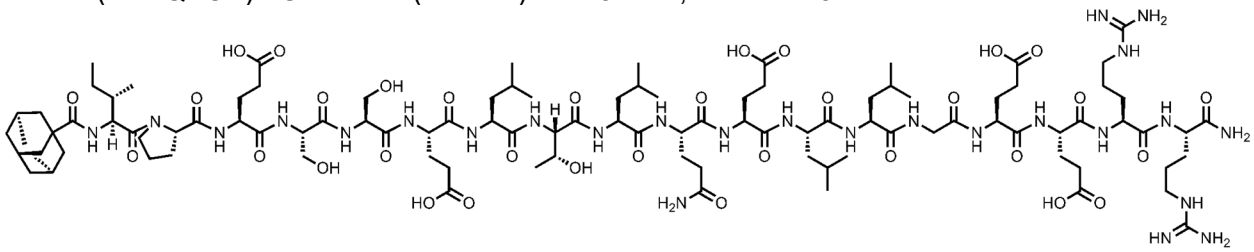


Mass spectrum of major compound:

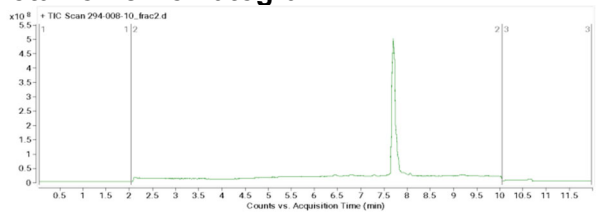




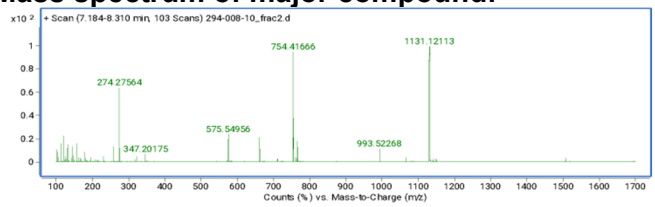
Name: Peptide **N11**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1131.12 , found: 1131.12.

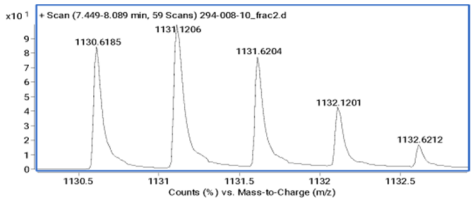


Total ion chromatogram:

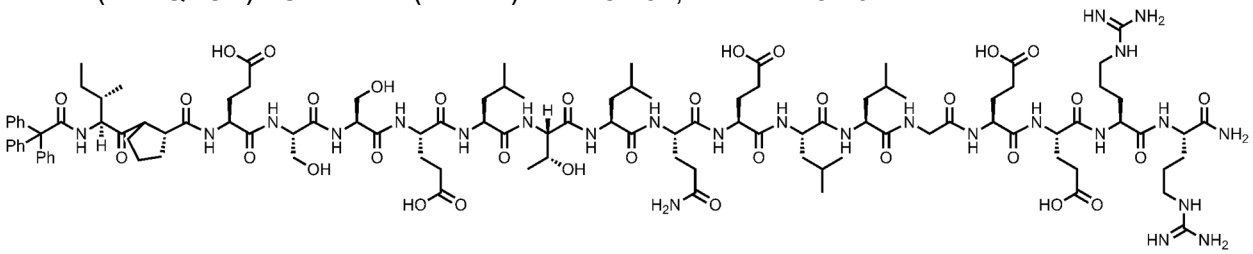


Mass spectrum of major compound:

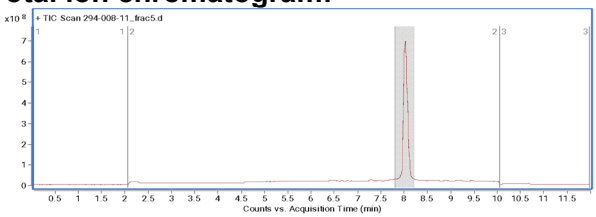




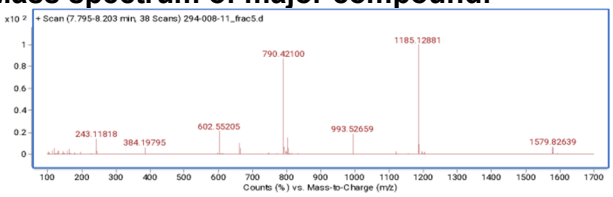
Name: Peptide **N12**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1184.62, found: 1184.62.

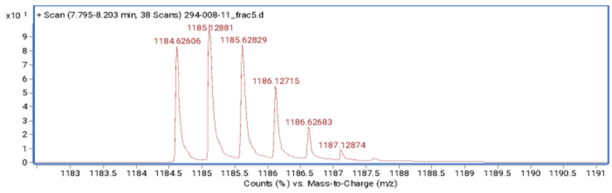


Total ion chromatogram:

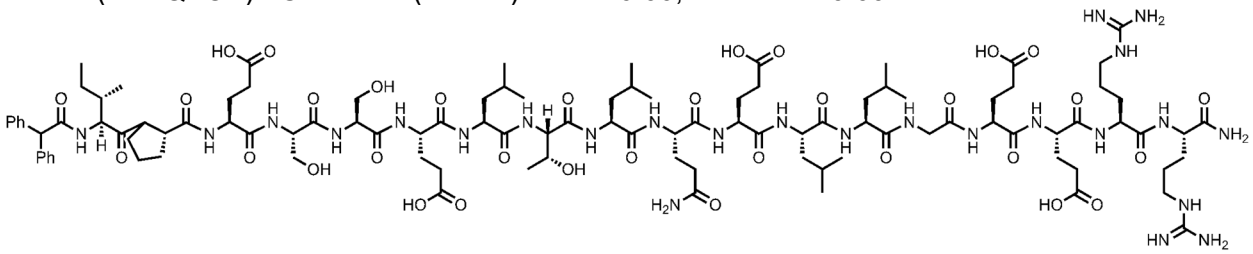


Mass spectrum of major compound:

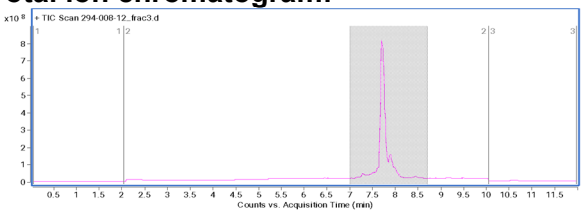




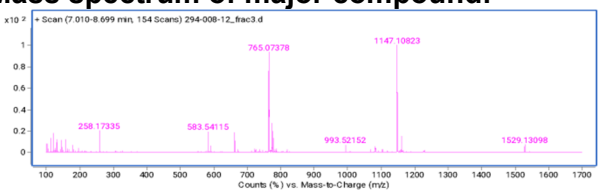
Name: Peptide **N13**
HPLC: method A
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1146.60, found: 1146.60.

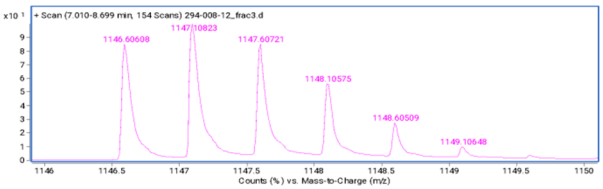


Total ion chromatogram:

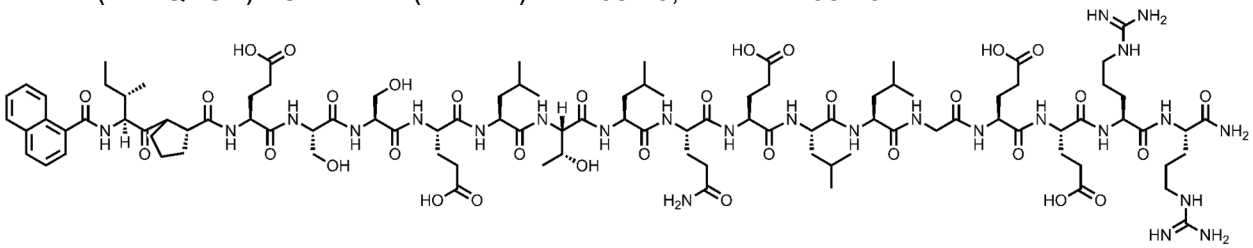


Mass spectrum of major compound:

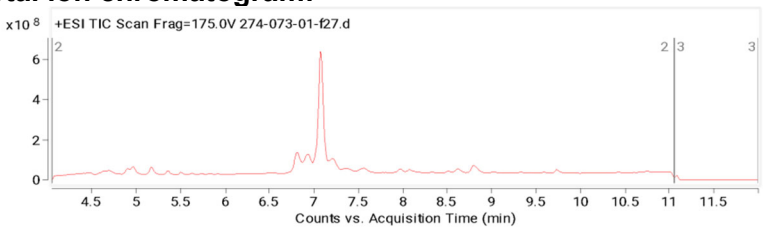




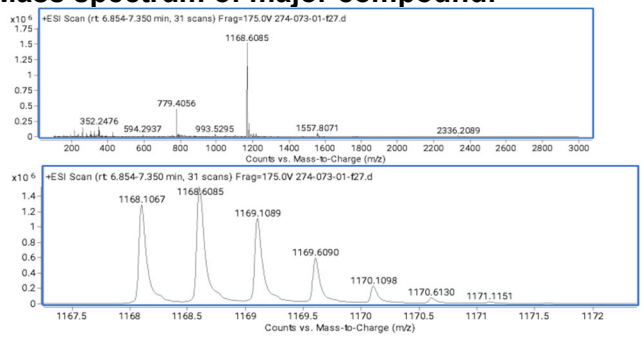
Name: Peptide **N14**
HPLC: method E
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1168.10, found: 1168.10.



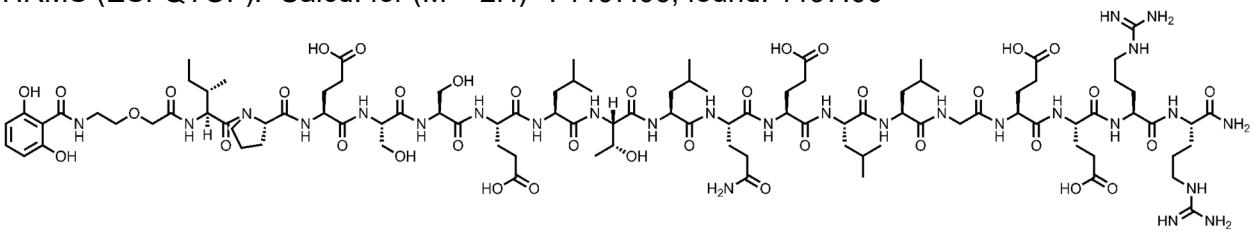
Total ion chromatogram:



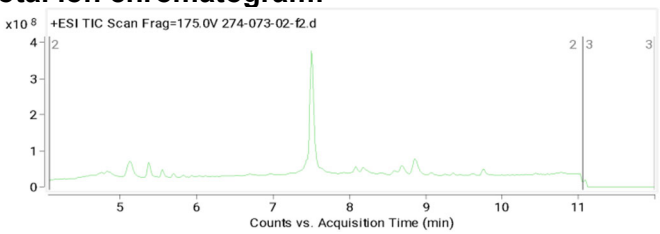
Mass spectrum of major compound:



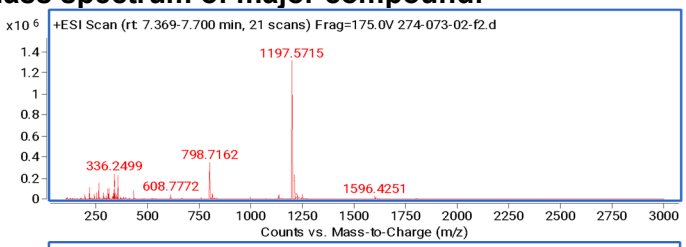
Name: Peptide **N15**
HPLC: method E
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1197.06, found: 1197.06

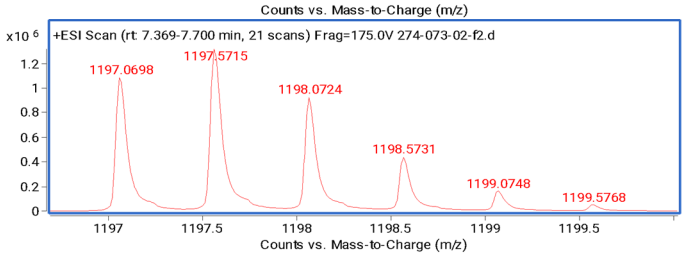


Total ion chromatogram:

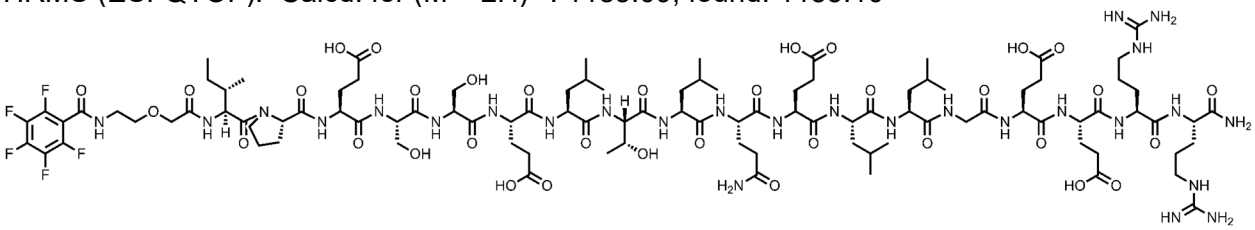


Mass spectrum of major compound:

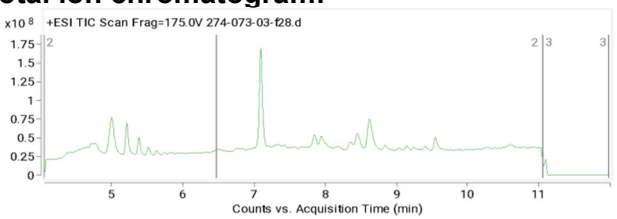




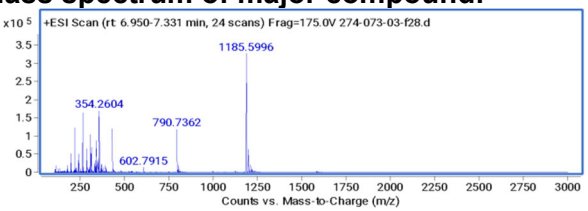
Name: Peptide **N16**
HPLC: method E
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1185.09, found: 1185.10

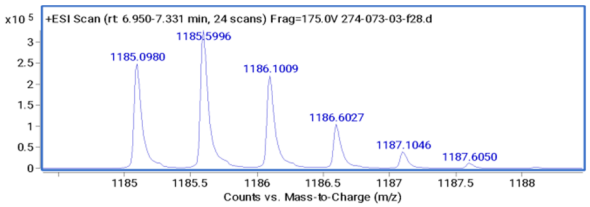


Total ion chromatogram:

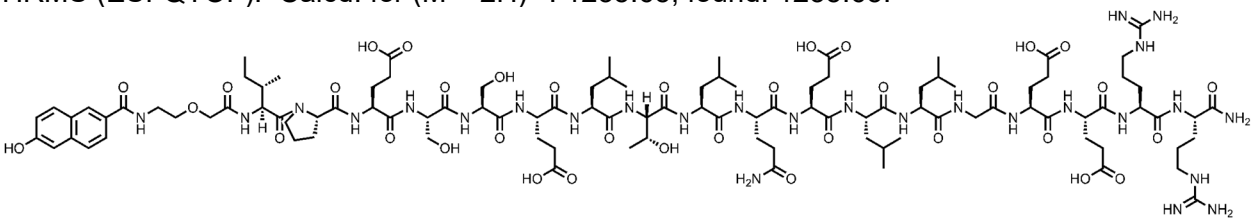


Mass spectrum of major compound:

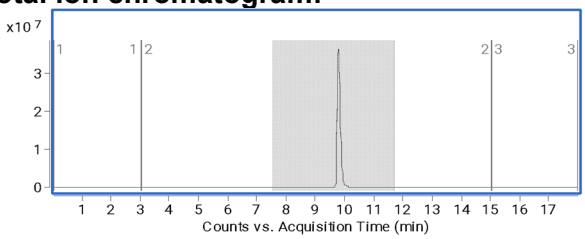




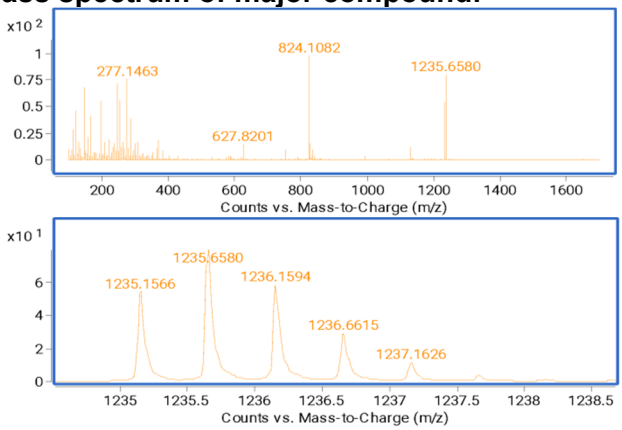
Name: Peptide **17**
HPLC: method D
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1235.65, found: 1235.65.



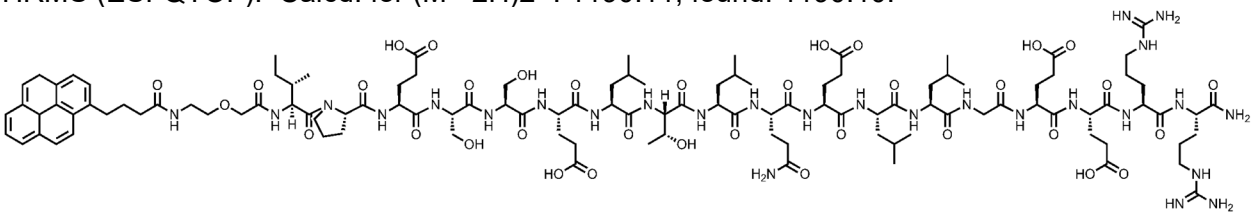
Total ion chromatogram:



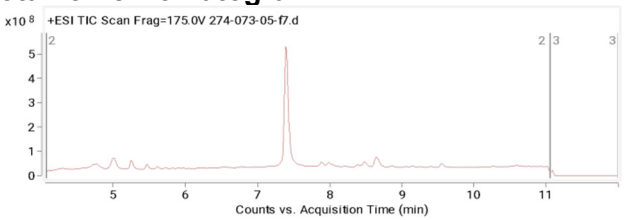
Mass spectrum of major compound:



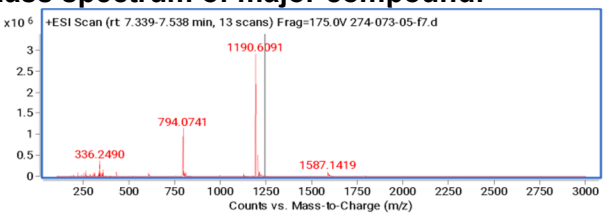
Name: Peptide **N18**
HPLC: method E
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1190.11, found: 1190.10.

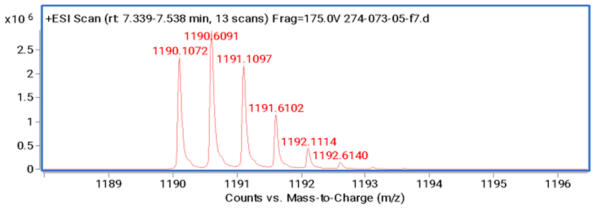


Total ion chromatogram:

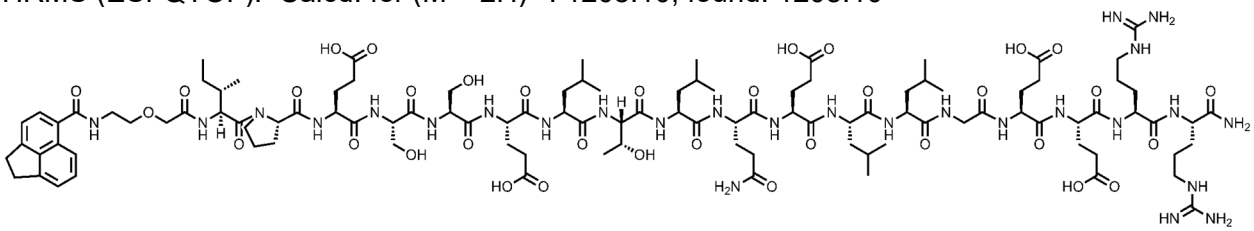


Mass spectrum of major compound:

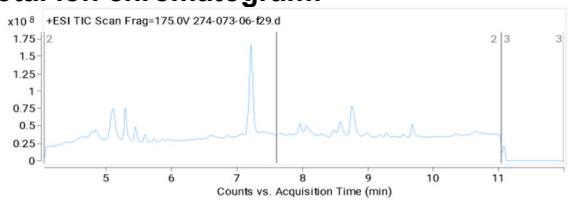




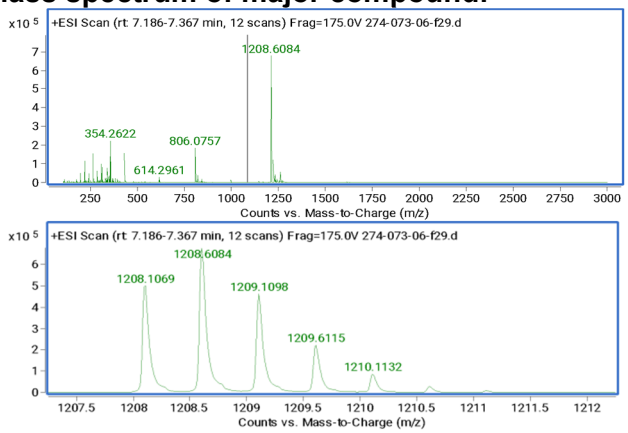
Name: Peptide **N19**
HPLC: method E
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1208.10, found: 1208.10



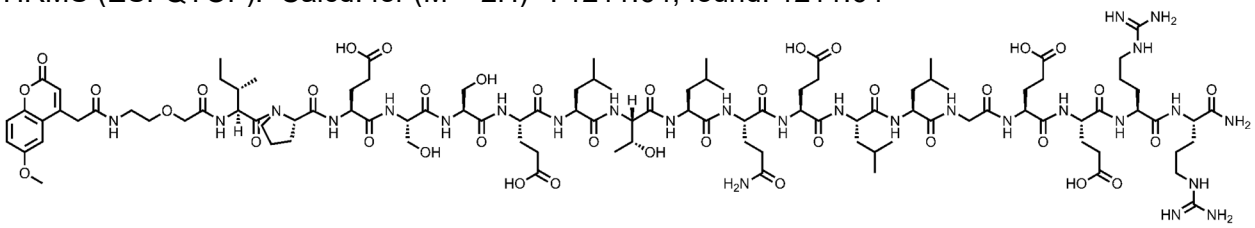
Total ion chromatogram:



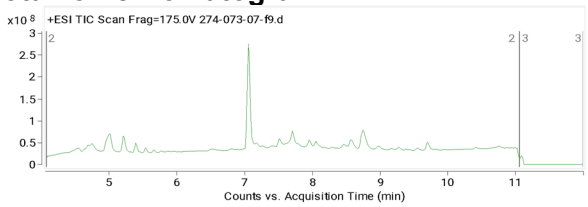
Mass spectrum of major compound:



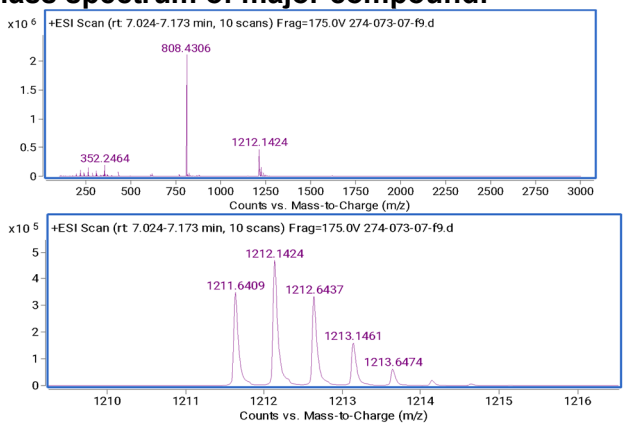
Name: Peptide **N20**
HPLC: method E
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1211.64, found: 1211.64



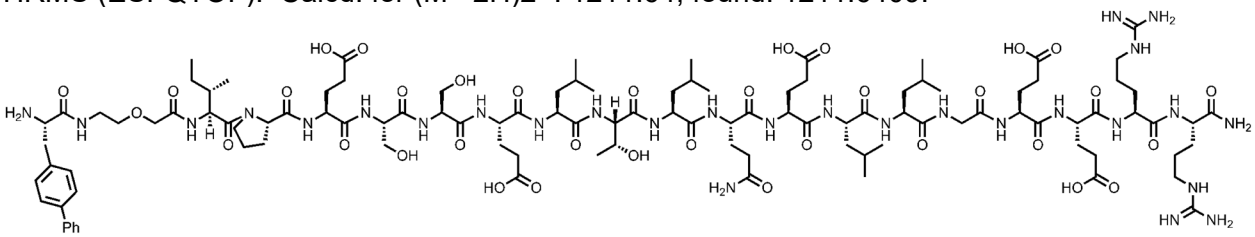
Total ion chromatogram:



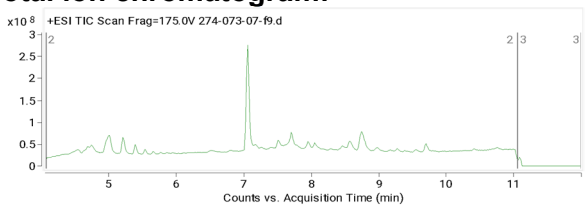
Mass spectrum of major compound:



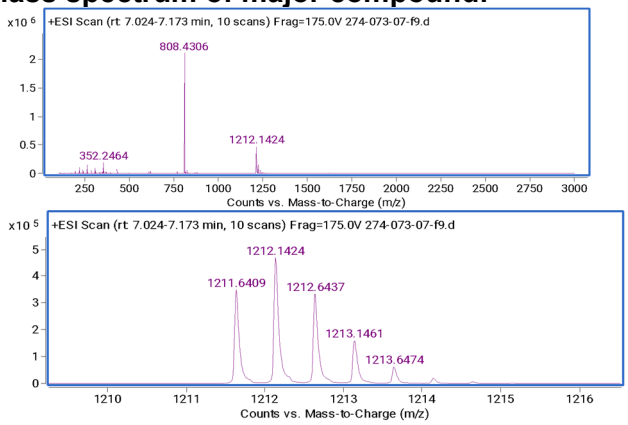
Name: Peptide **N21**
HPLC: method E
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1211.64, found: 1211.6409.



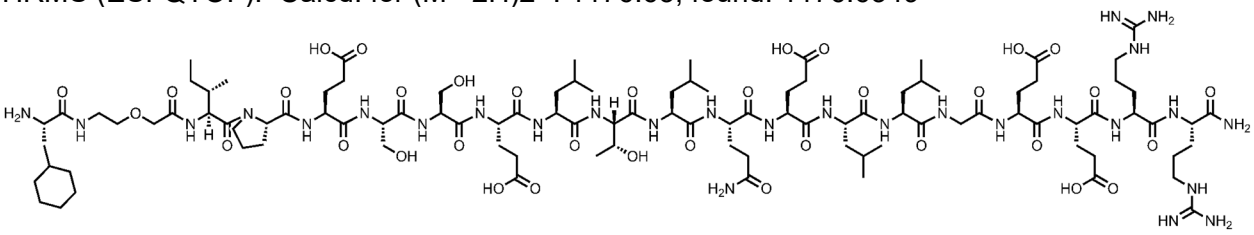
Total ion chromatogram:



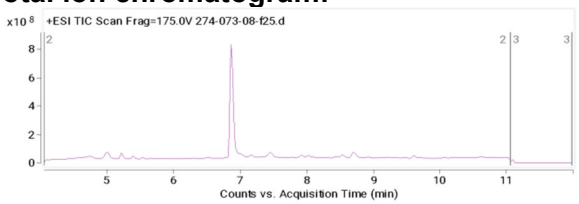
Mass spectrum of major compound:



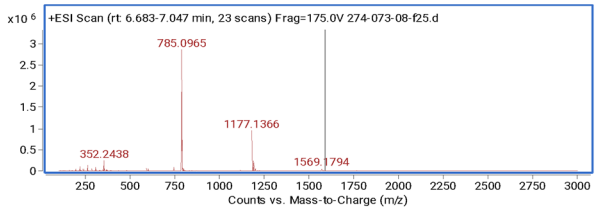
Name: Peptide **N22**
HPLC: method E
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1176.63, found: 1176.6349

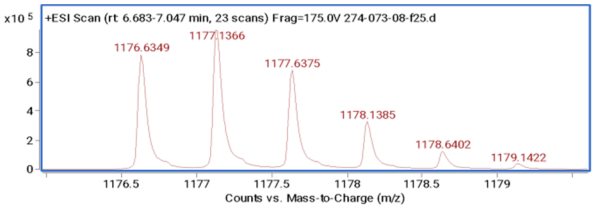


Total ion chromatogram:

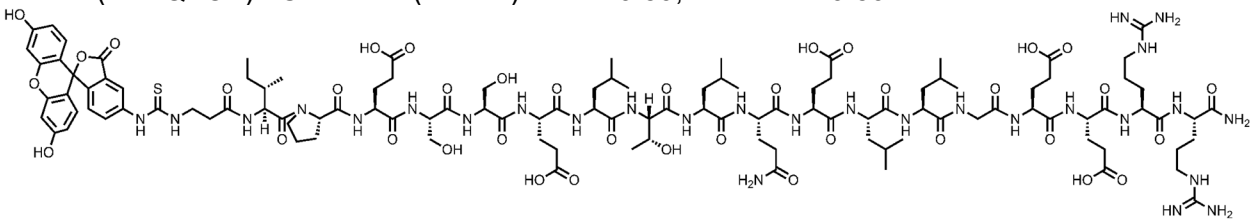


Mass spectrum of major compound:

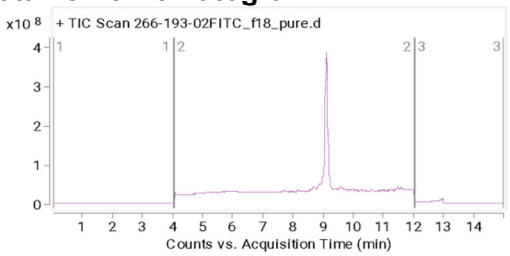




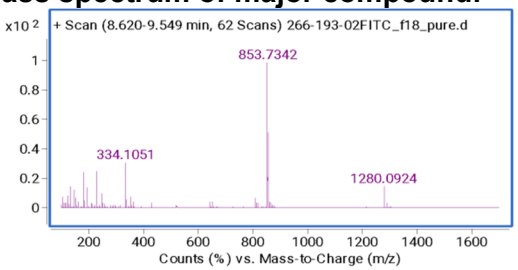
Name: Peptide **N-FITC**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1279.59, found: 1279.59

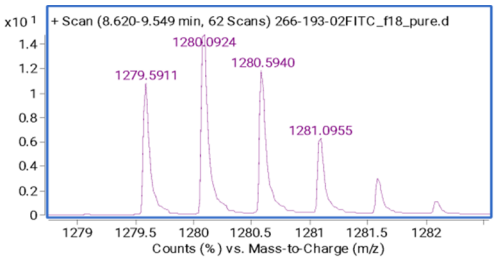


Total ion chromatogram:



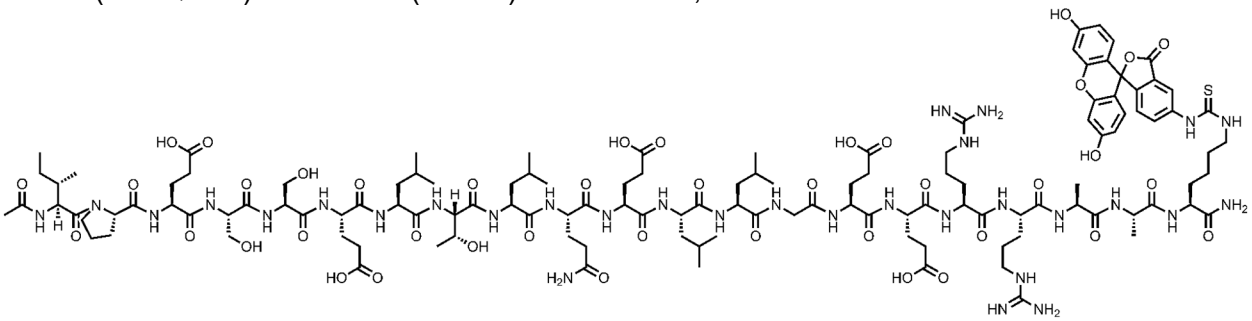
Mass spectrum of major compound:



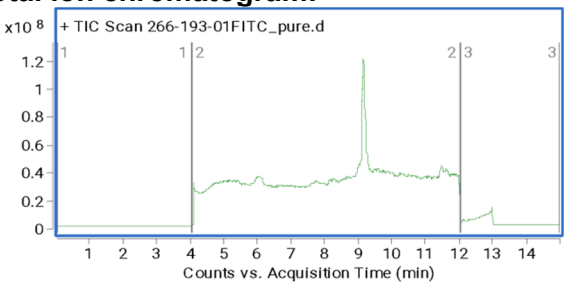


2.4 Peptides **C1-C13** and **C-FITC**

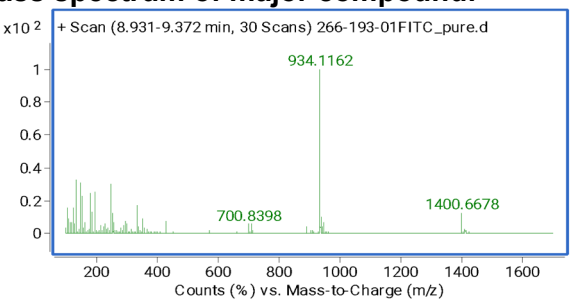
Name: Peptide **C-FITC**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M+2H)^{2+}$: 1400.1660, found: 1400.1660

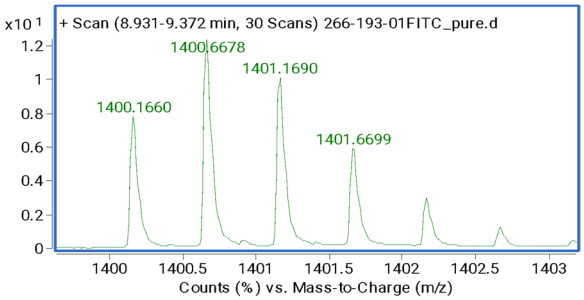


Total ion chromatogram:

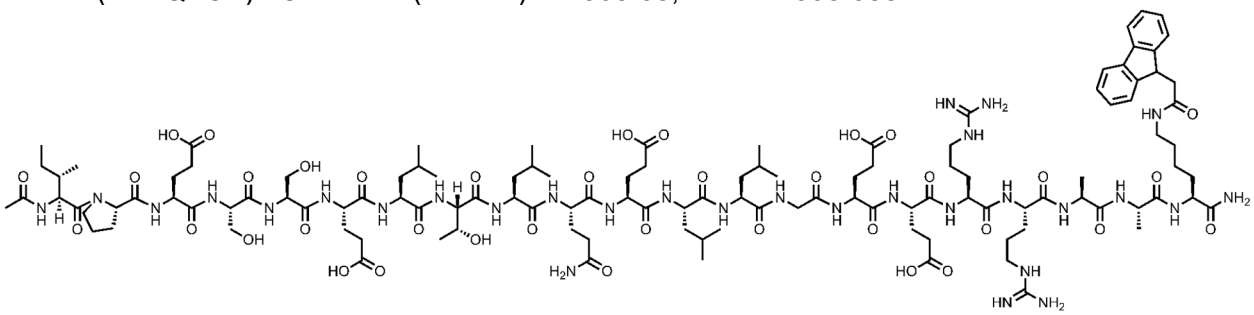


Mass spectrum of major compound:

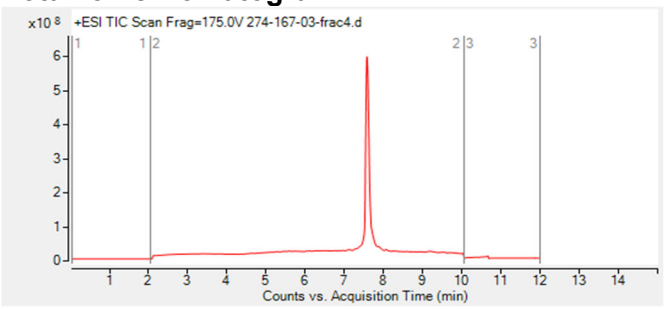




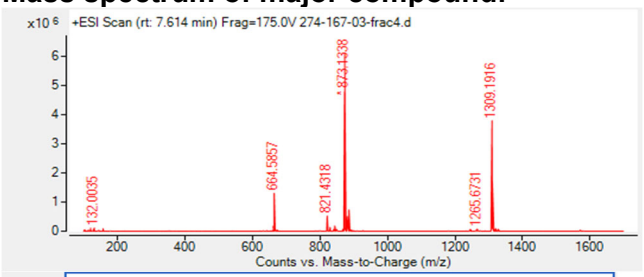
Name: Peptide **C1**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1308.68, found: 1308.6887

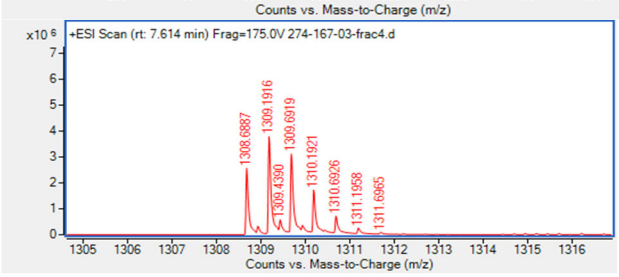


Total ion chromatogram:

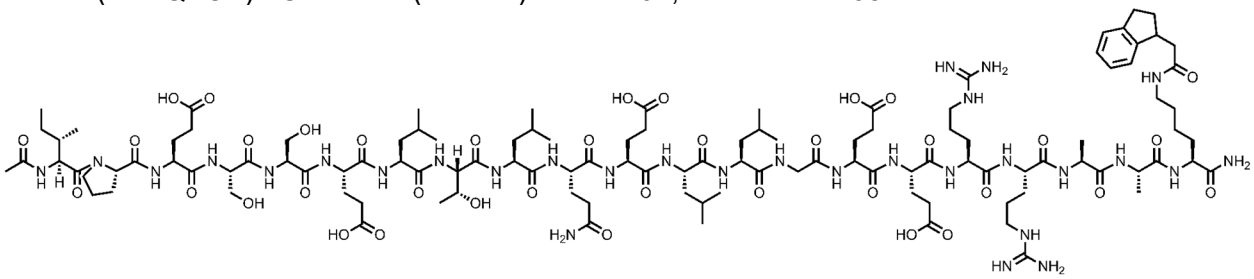


Mass spectrum of major compound:

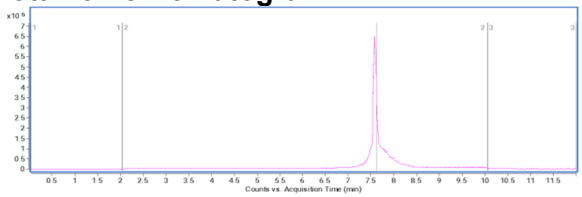




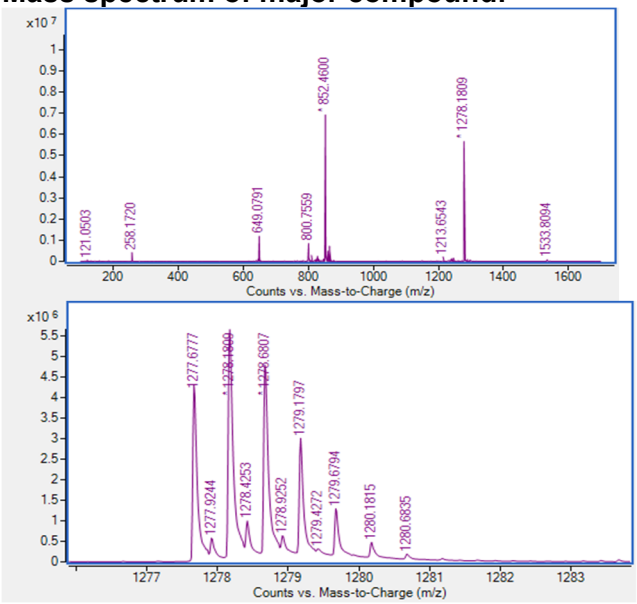
Name: Peptide **C2**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1277.67, found: 1277.68



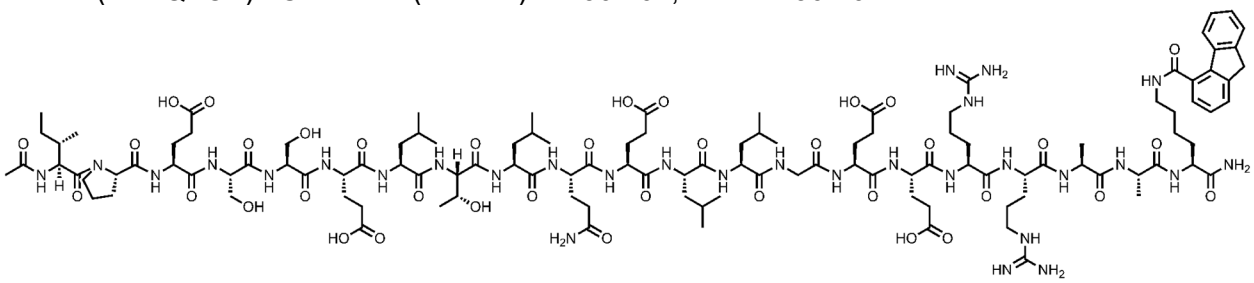
Total ion chromatogram:



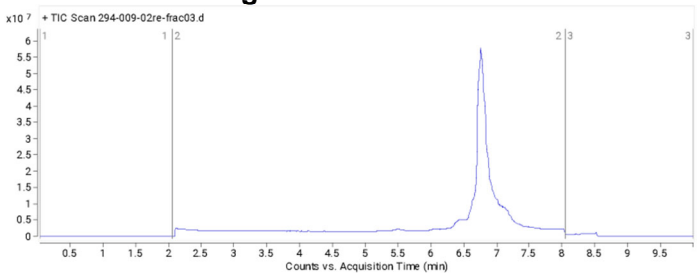
Mass spectrum of major compound:



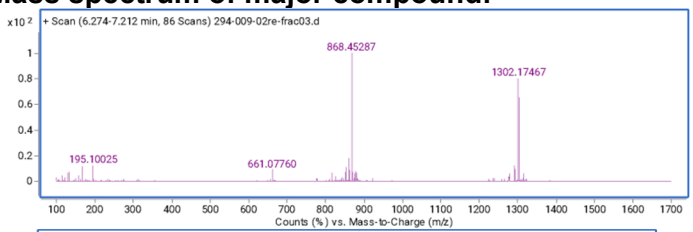
Name: Peptide **C3**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1301.67, found: 1301.67.

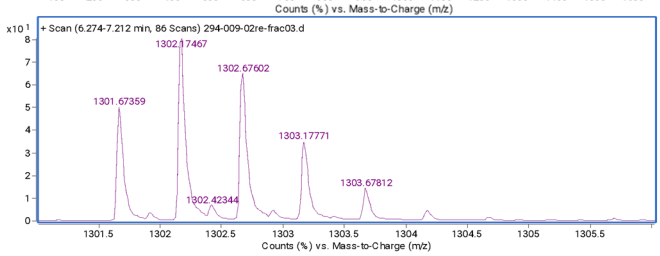


Total ion chromatogram:

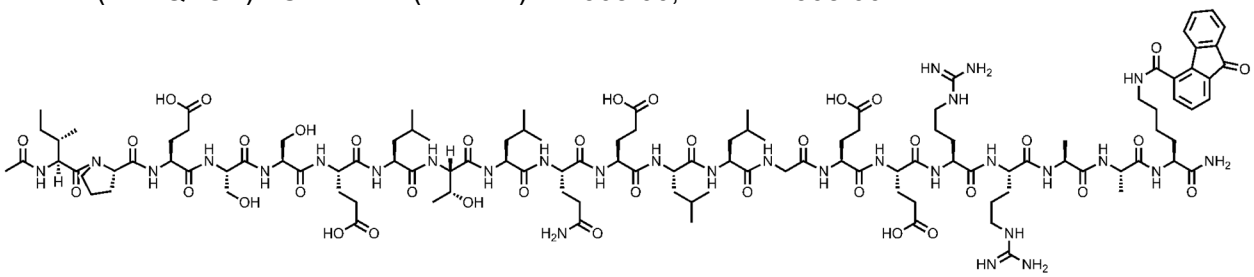


Mass spectrum of major compound:

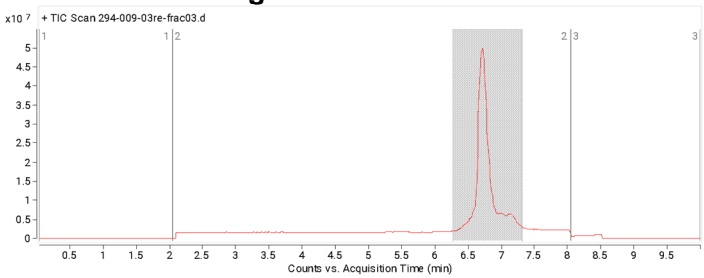




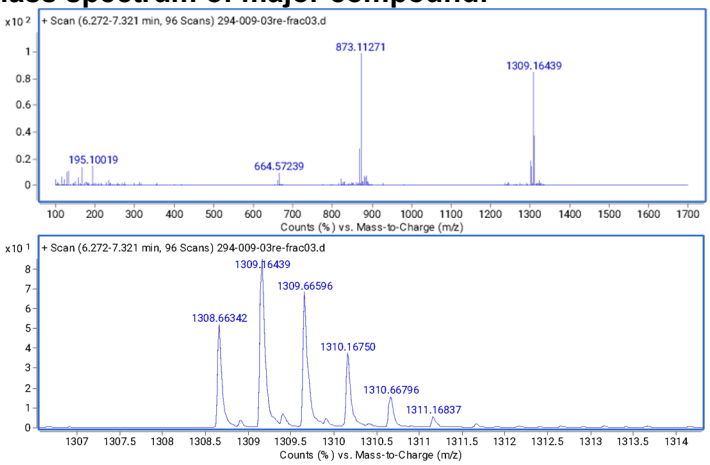
Name: Peptide **C4**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1308.66, found: 1308.66



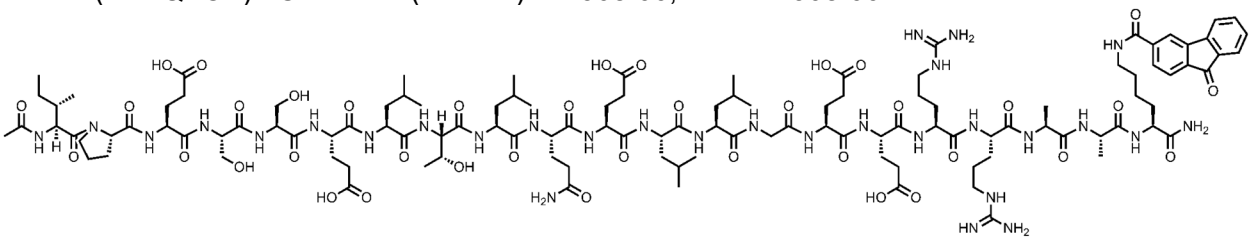
Total ion chromatogram:



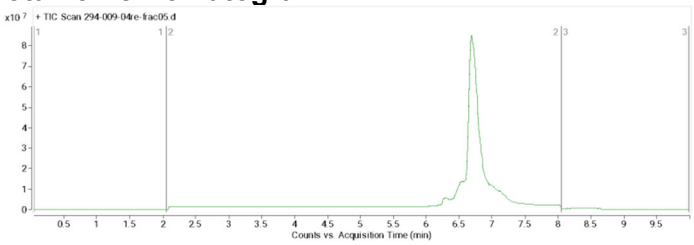
Mass spectrum of major compound:



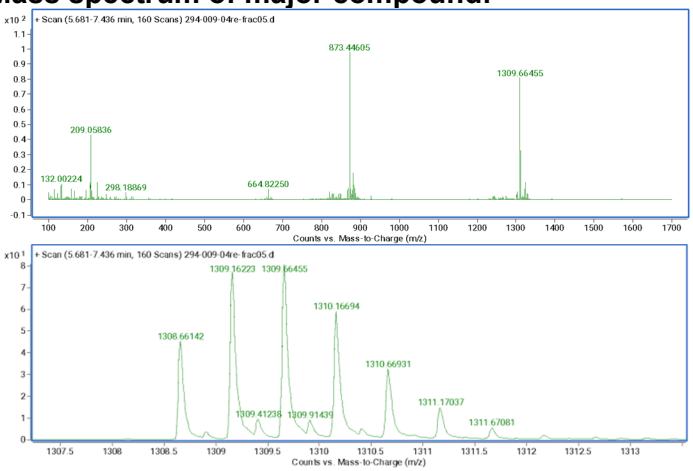
Name: Peptide **C5**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1308.66, found: 1308.66.



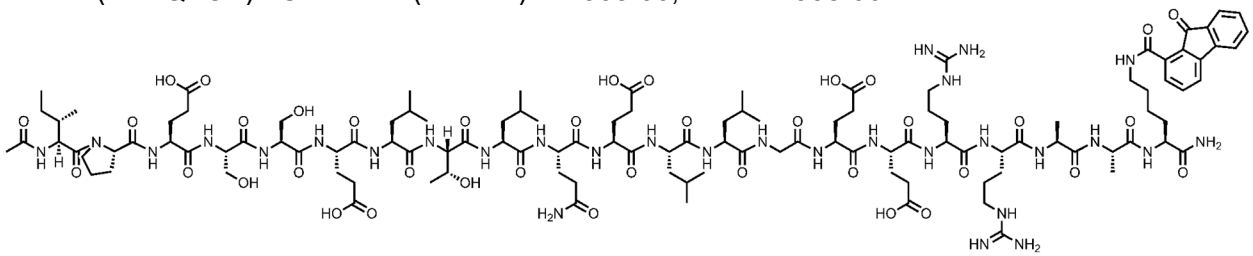
Total ion chromatogram:



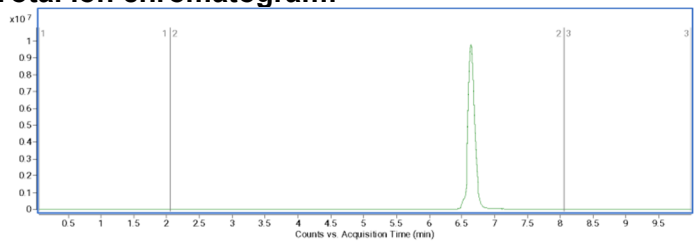
Mass spectrum of major compound:



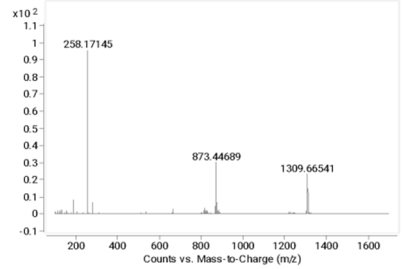
Name: Peptide **C6**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1308.66, found: 1308.66.

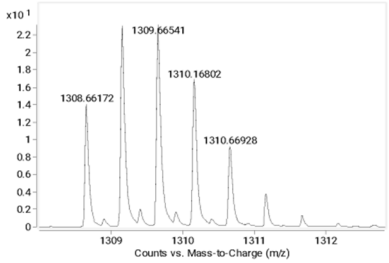


Total ion chromatogram:

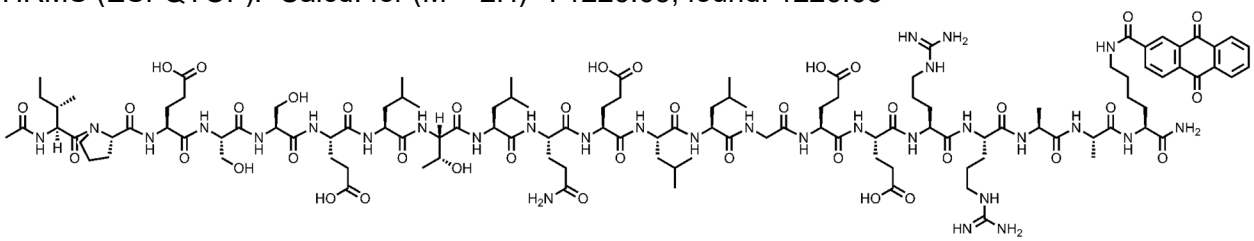


Mass spectrum of major compound:

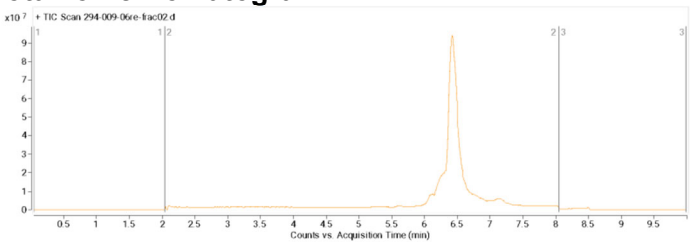




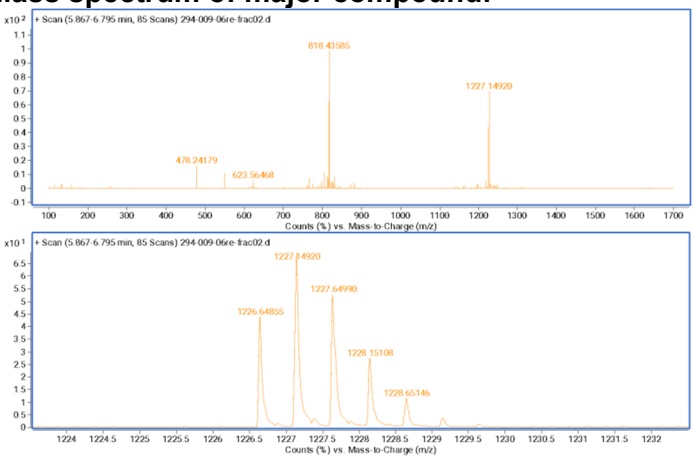
Name: Peptide **C7**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1226.65, found: 1226.65



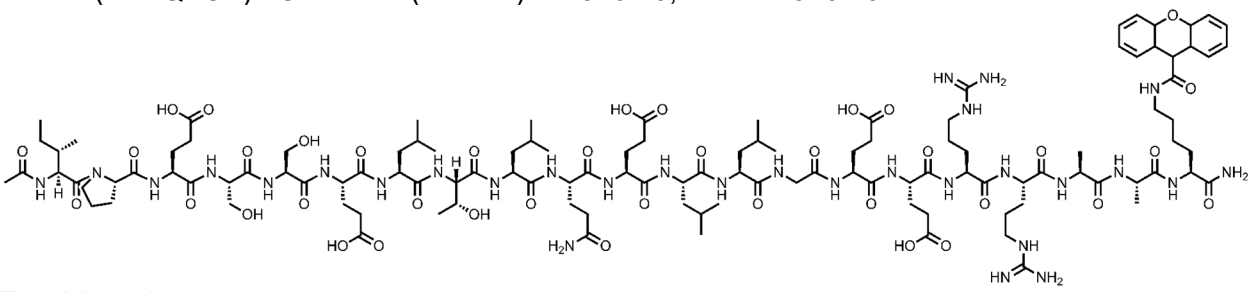
Total ion chromatogram:



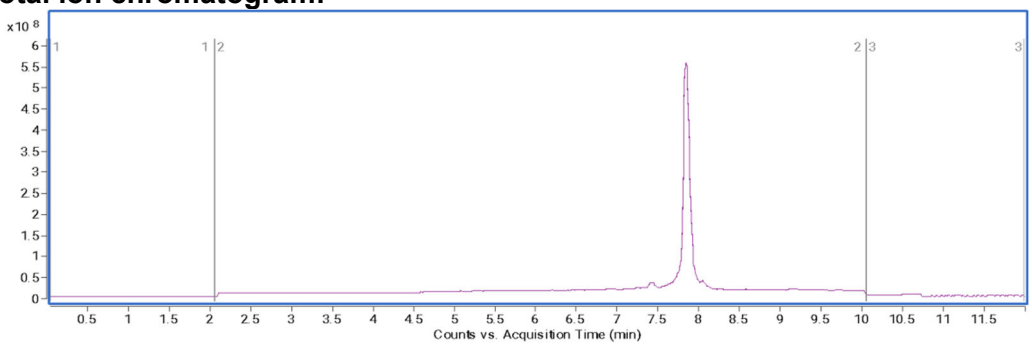
Mass spectrum of major compound:



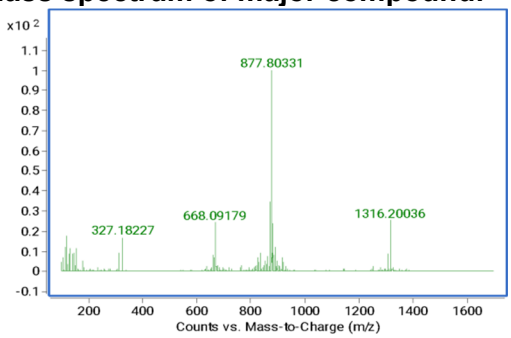
Name: Peptide **C8**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1316.70, found: 1316.70.

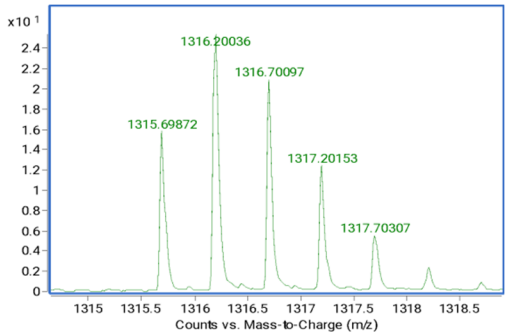


Total ion chromatogram:

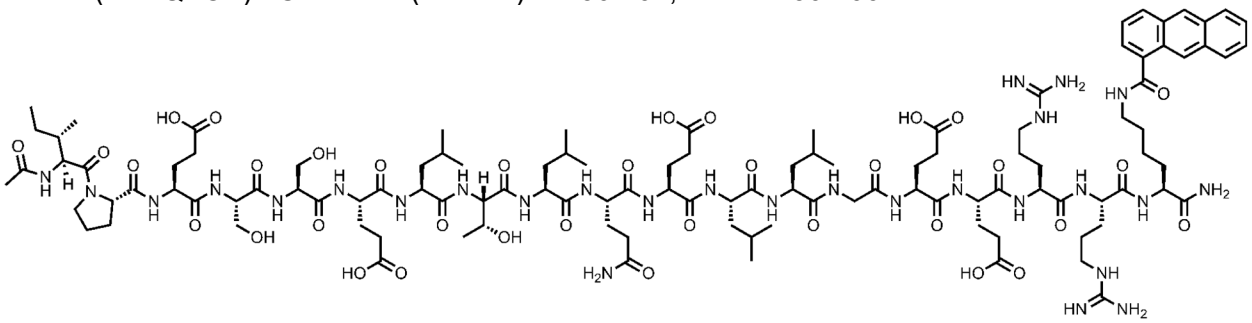


Mass spectrum of major compound:

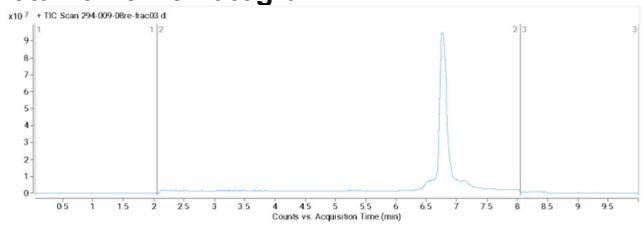




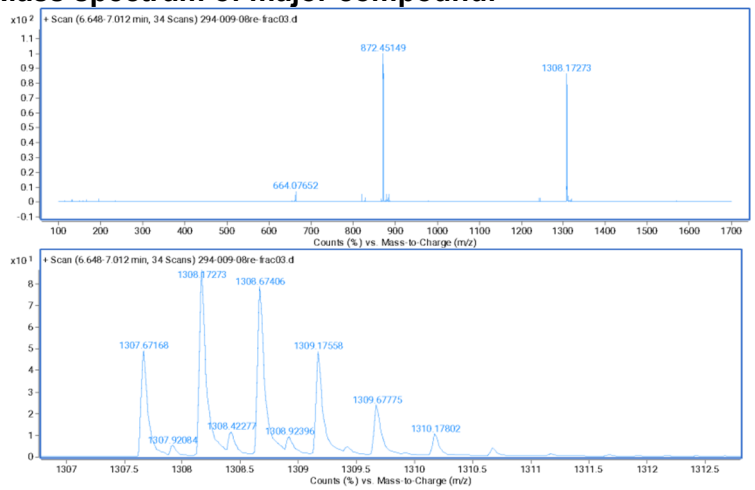
Name: Peptide **C9**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1307.67, found: 1307.66



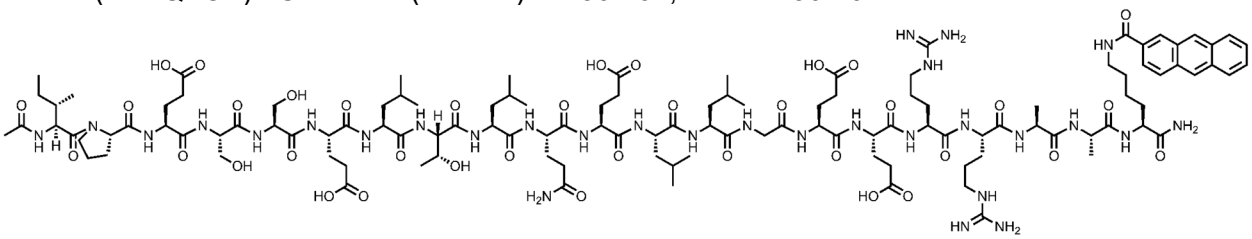
Total ion chromatogram:



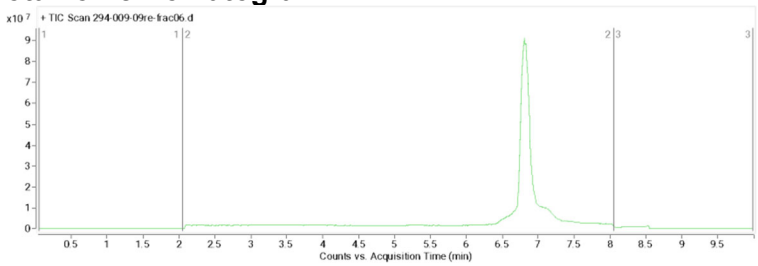
Mass spectrum of major compound:



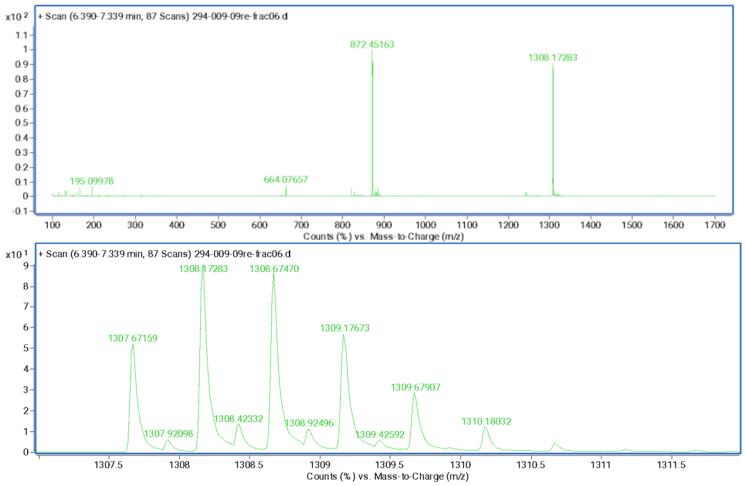
Name: Peptide **C10**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1307.67, found: 1307.67.



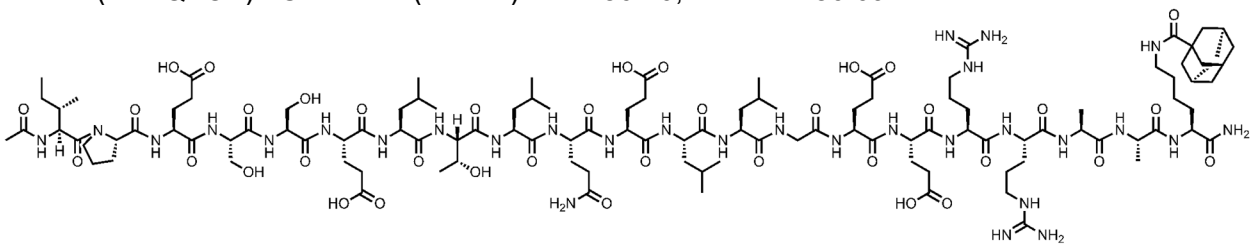
Total ion chromatogram:



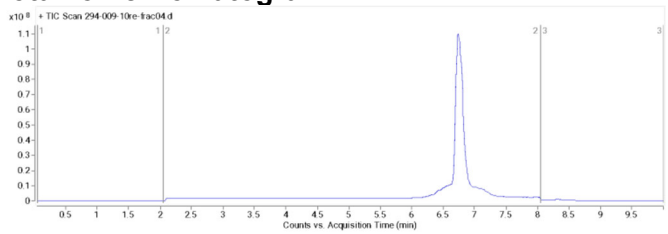
Mass spectrum of major compound:



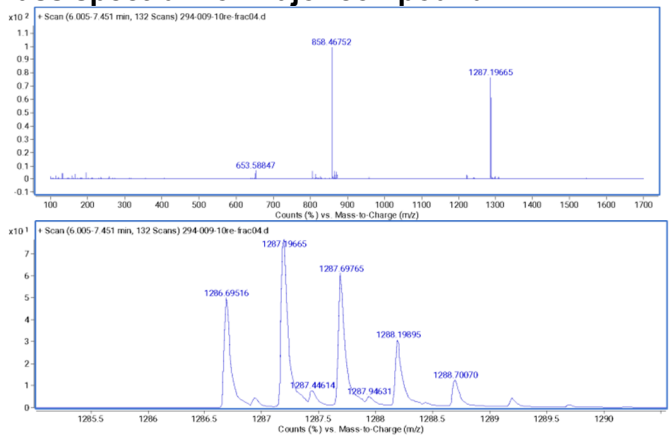
Name: Peptide **C11**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1286.70, found: 1286.69.



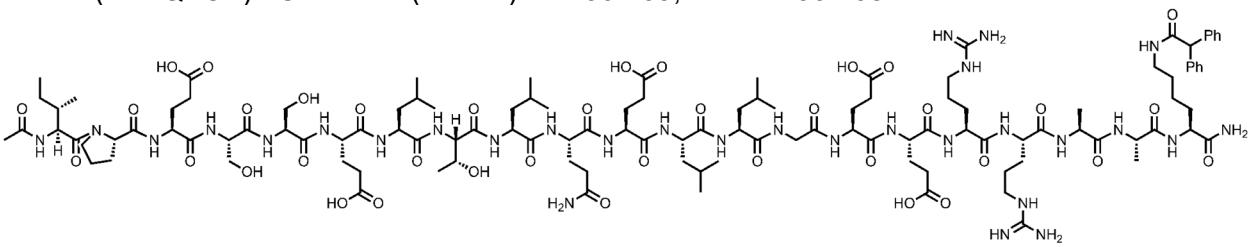
Total ion chromatogram:



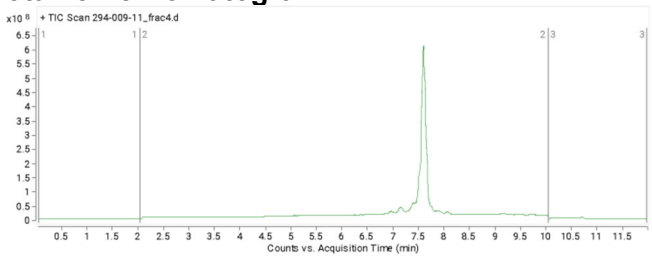
Mass spectrum of major compound:



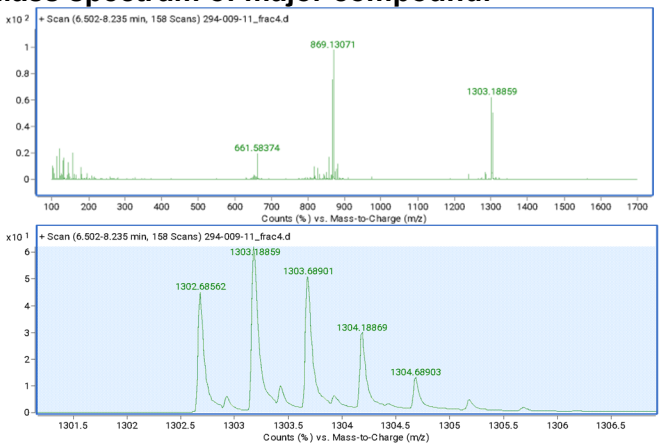
Name: Peptide **C12**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1302.69, found: 1302.68



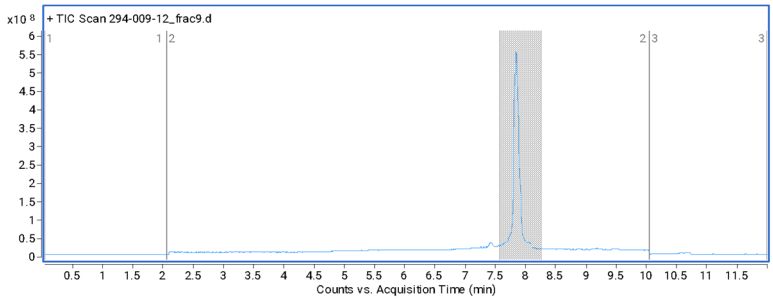
Total ion chromatogram:



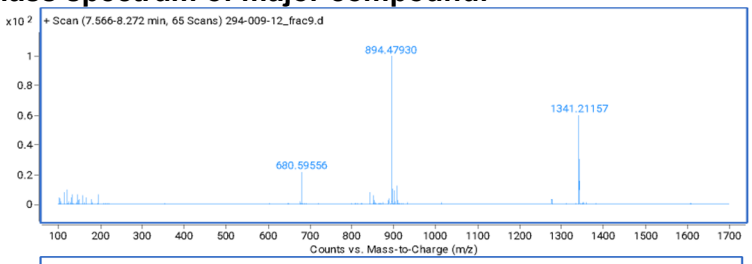
Mass spectrum of major compound:

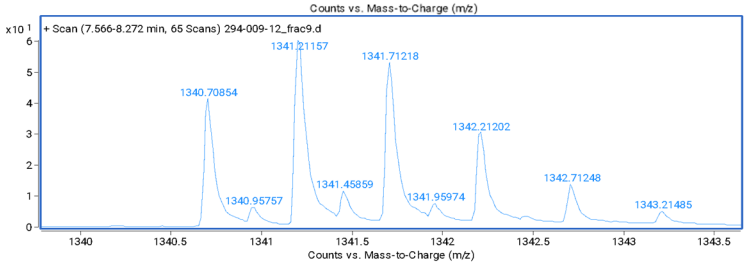


Name: Peptide **C13**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1340.69, found: 1340.70.
Total ion chromatogram:

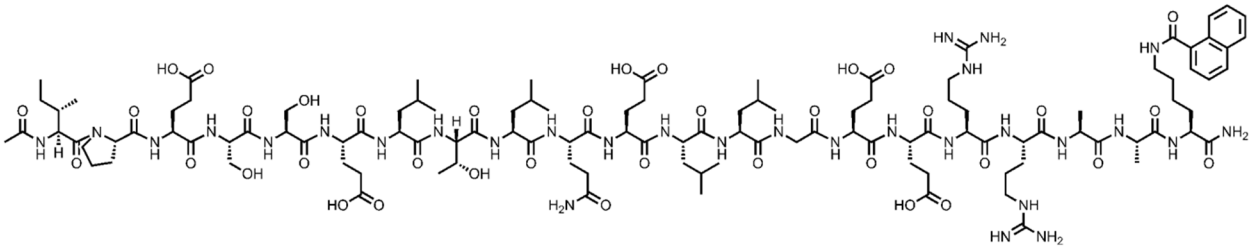


Mass spectrum of major compound:

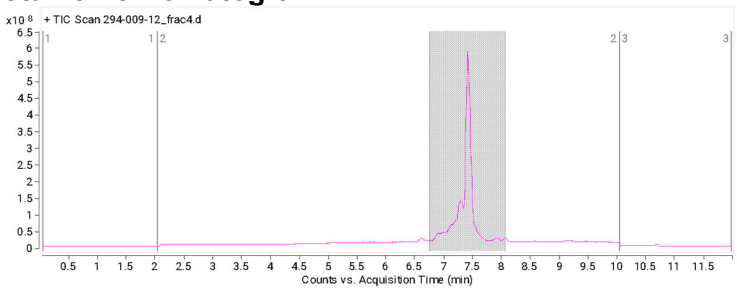




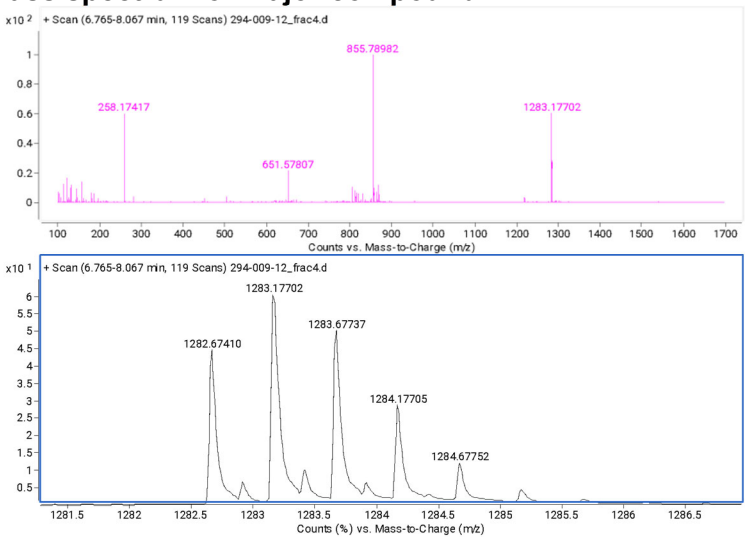
Name: Peptide **C14**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1282.66, found: 1282.67



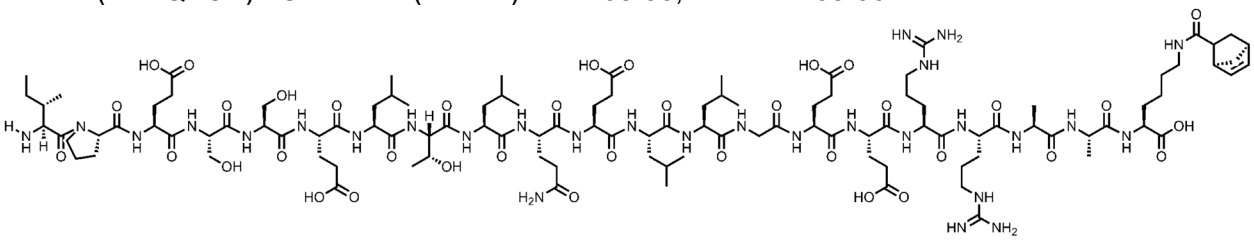
Total ion chromatogram:



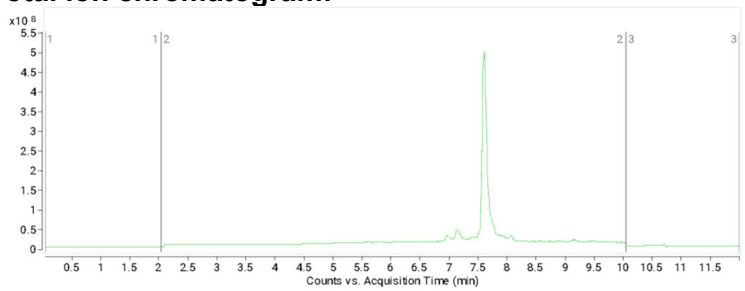
Mass spectrum of major compound:



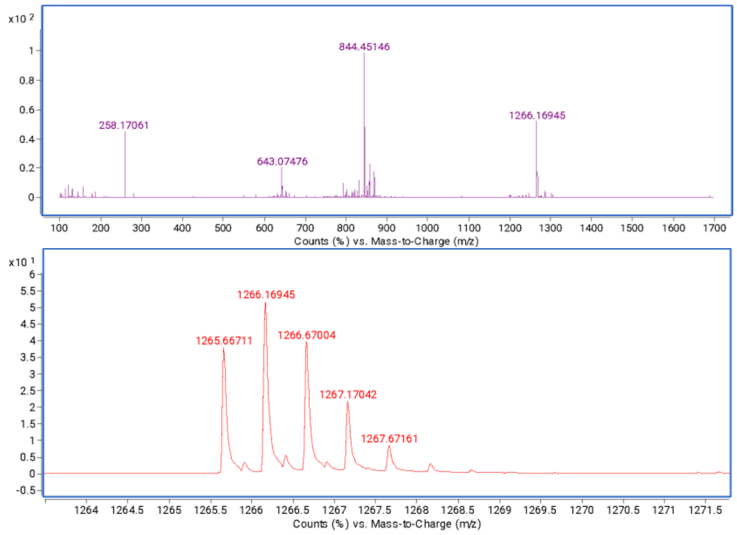
Name: Peptide **C15**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1265.66, found: 1265.66



Total ion chromatogram:

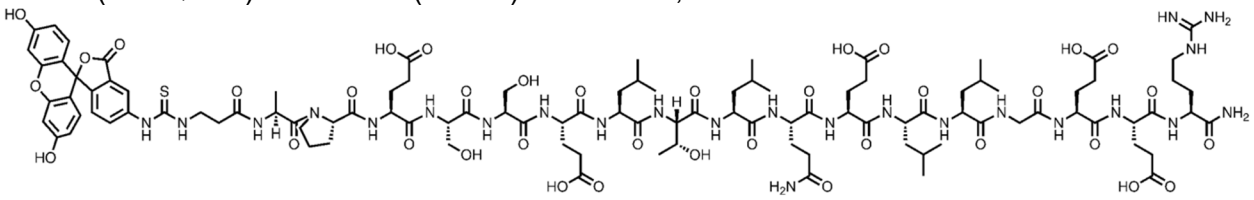


Mass spectrum of major compound:

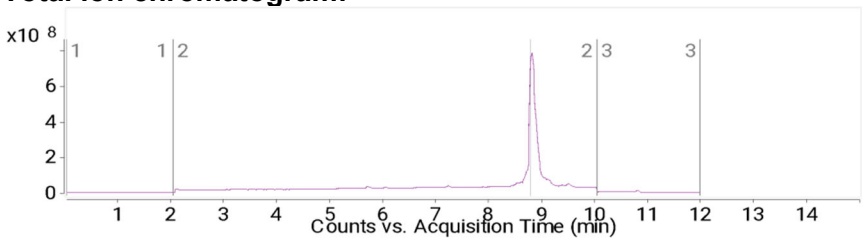


2.5 Peptides **A1-A17**

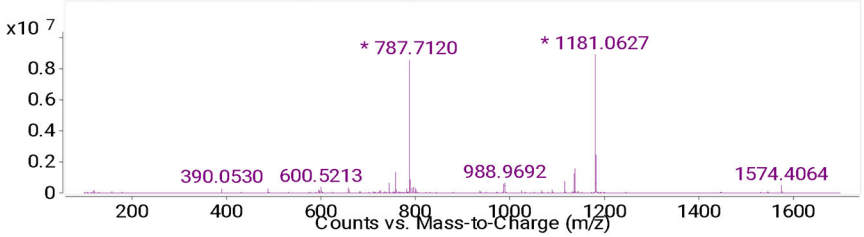
Name: Peptide **A1**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1181.06, found: 1181.06.



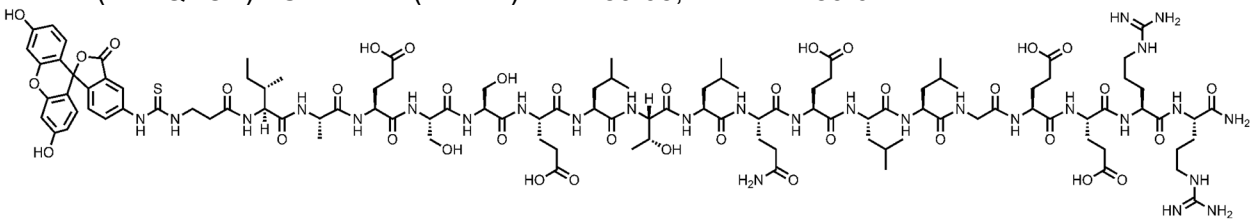
Total ion chromatogram:



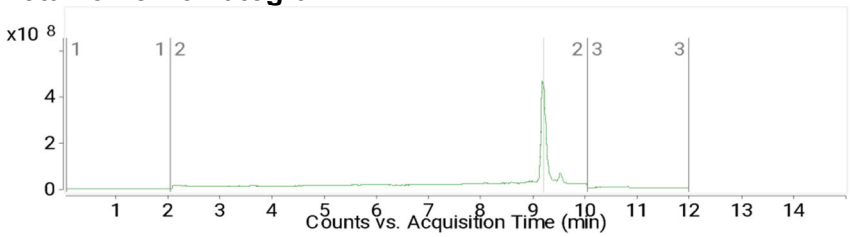
Mass spectrum of major compound:



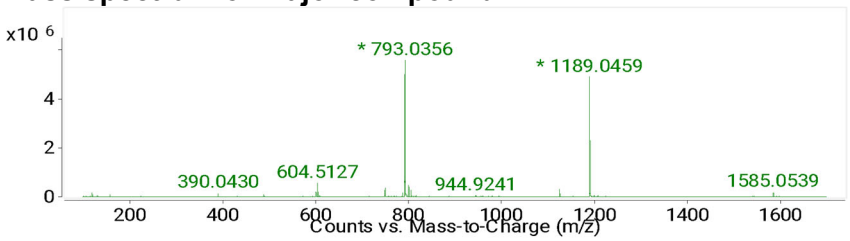
Name: Peptide **A2**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1189.05, found: 1189.04.



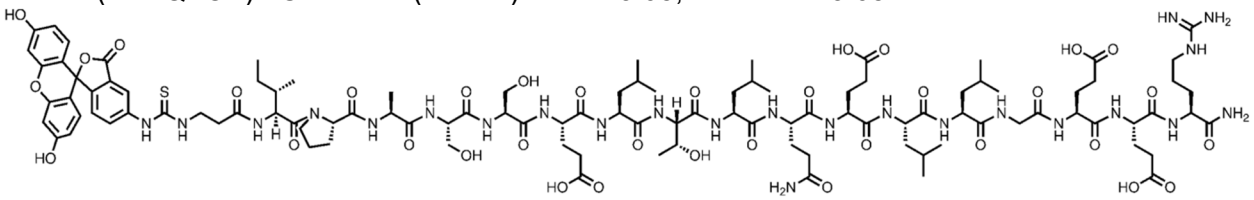
Total ion chromatogram:



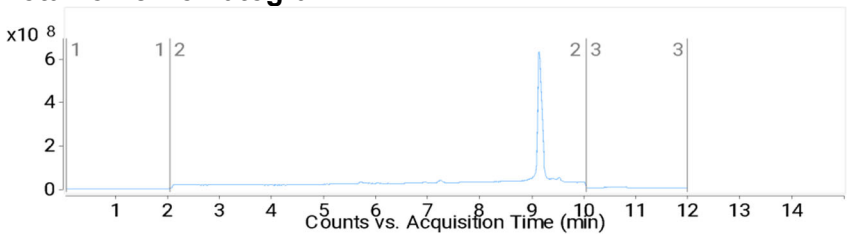
Mass spectrum of major compound:



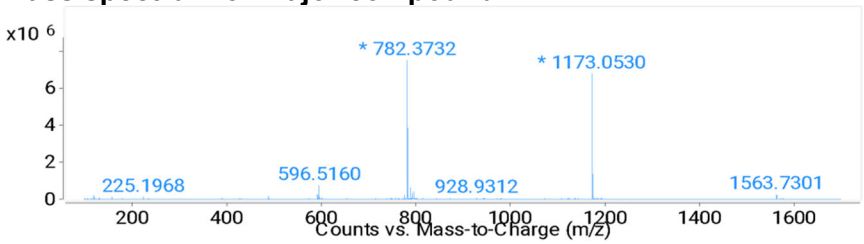
Name: Peptide **A3**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1173.05, found: 1173.05.



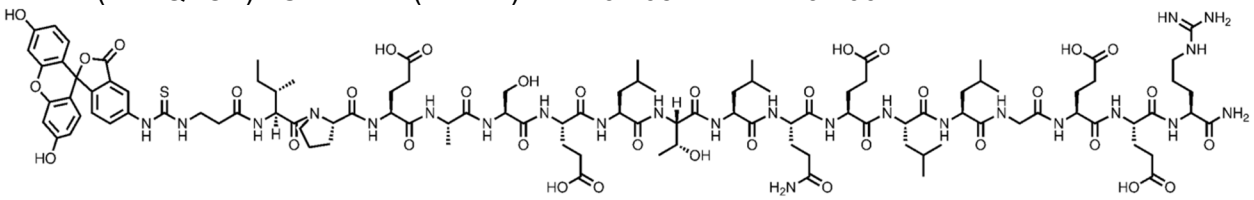
Total ion chromatogram:



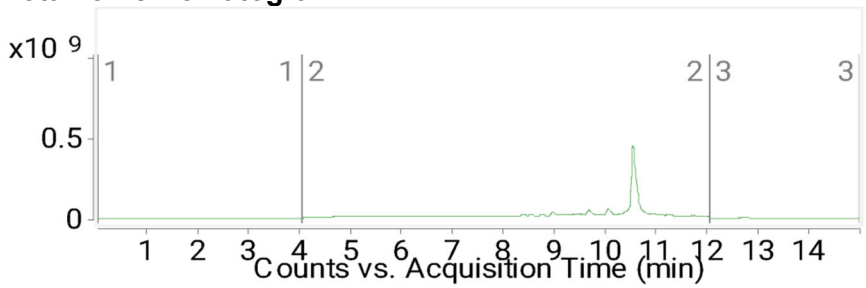
Mass spectrum of major compound:



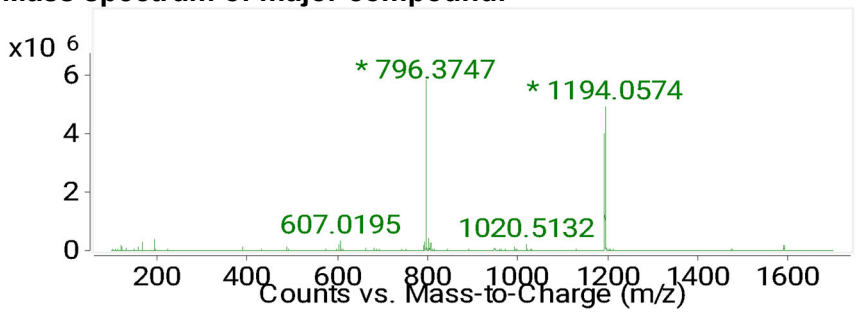
Name: Peptide **A4**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1194.05 found: 1194.06.



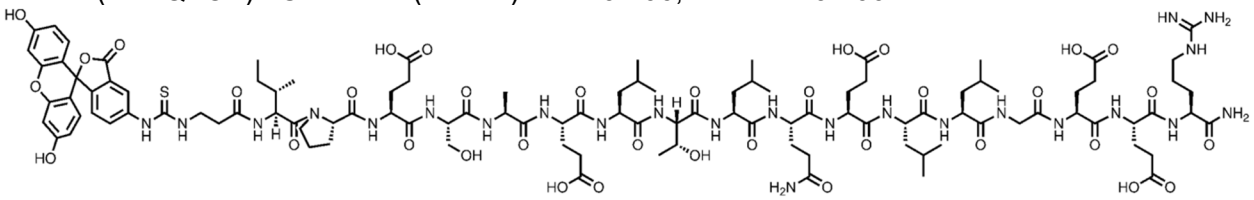
Total ion chromatogram:



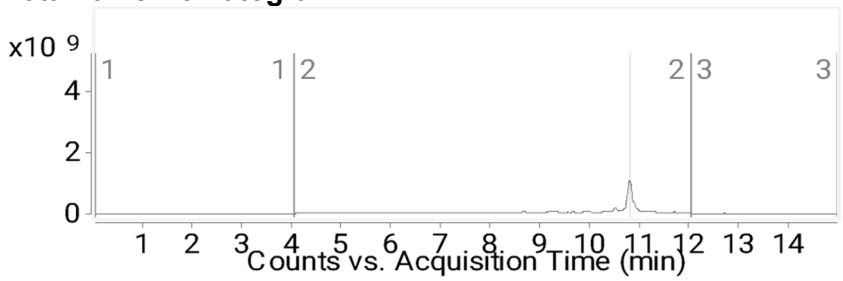
Mass spectrum of major compound:



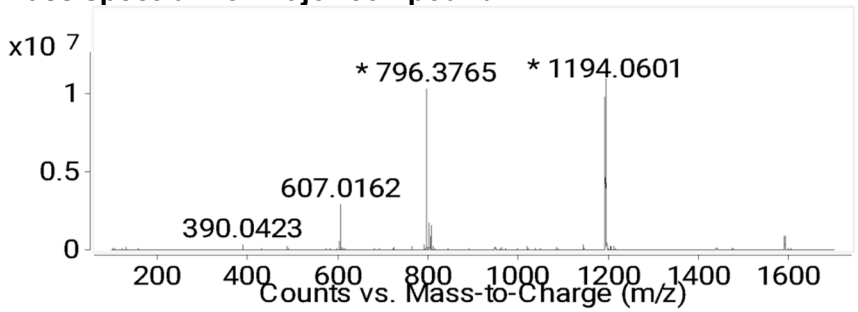
Name: Peptide **A5**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1194.06, found: 1194.06.



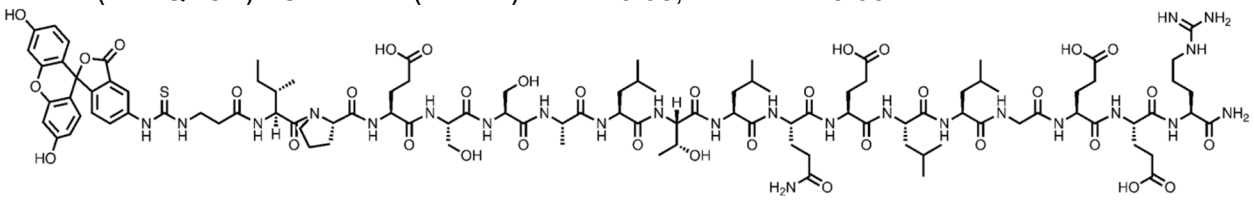
Total ion chromatogram:



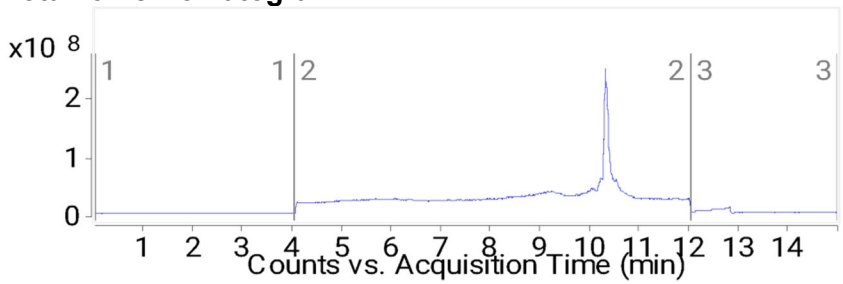
Mass spectrum of major compound:



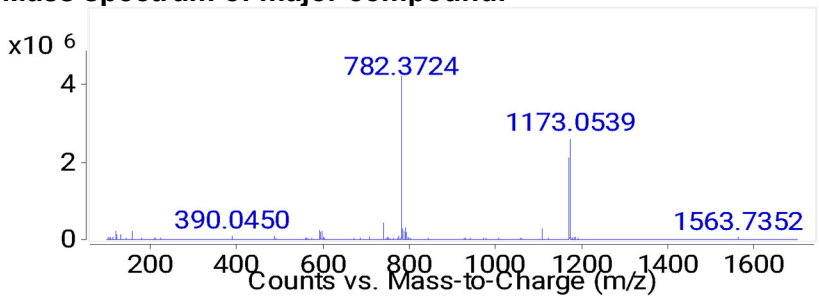
Name: Peptide **A6**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1173.05, found: 1173.05



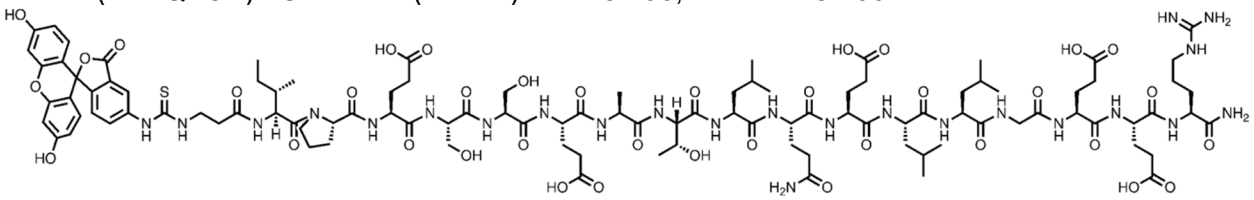
Total ion chromatogram:



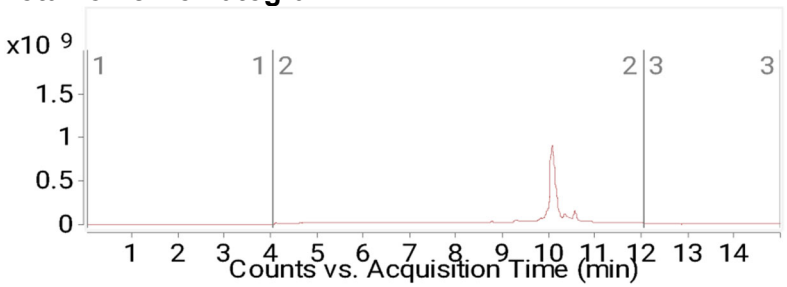
Mass spectrum of major compound:



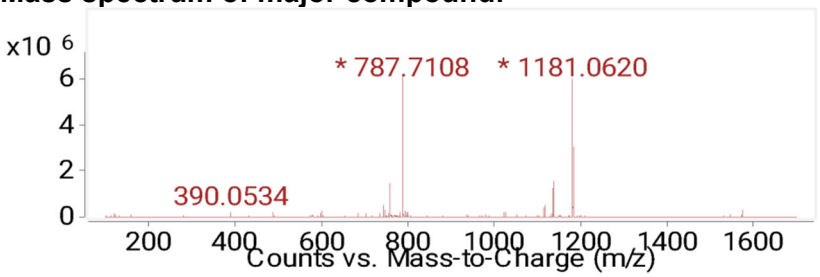
Name: Peptide **A7**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1181.06, found: 1181.06.



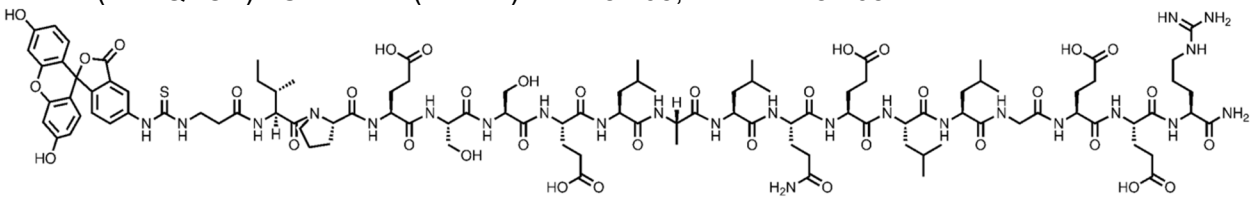
Total ion chromatogram:



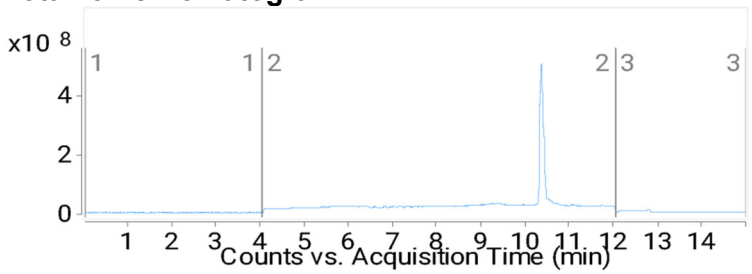
Mass spectrum of major compound:



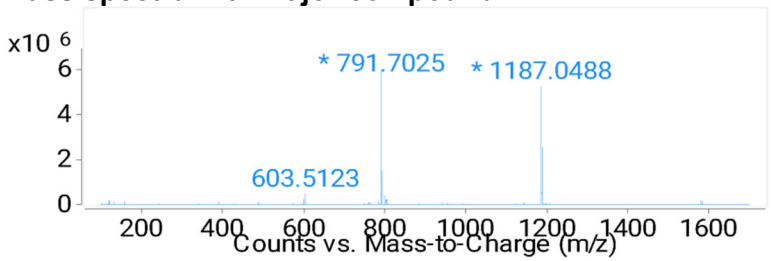
Name: Peptide **A8**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1187.05, found: 1187.05.



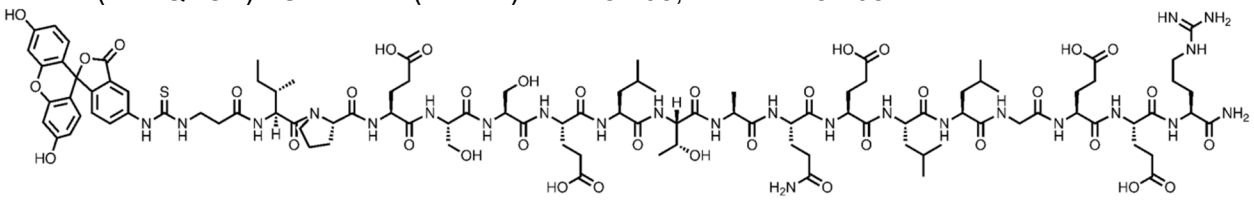
Total ion chromatogram:



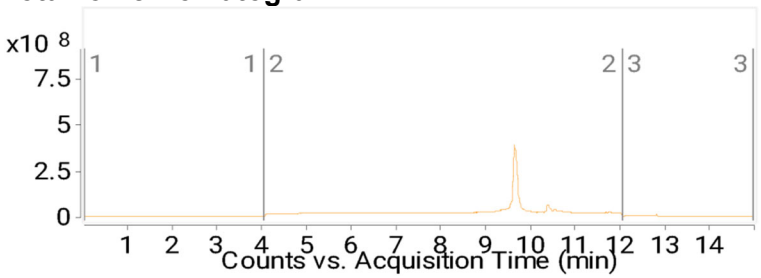
Mass spectrum of major compound:



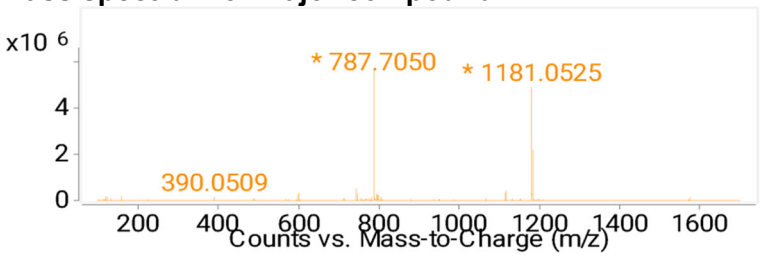
Name: Peptide **A9**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1181.05, found: 1181.05.



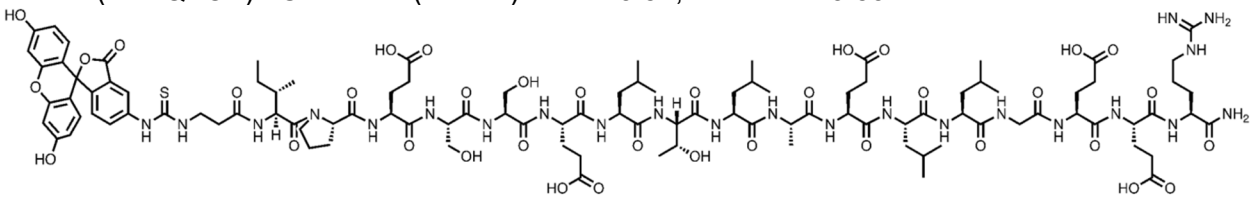
Total ion chromatogram:



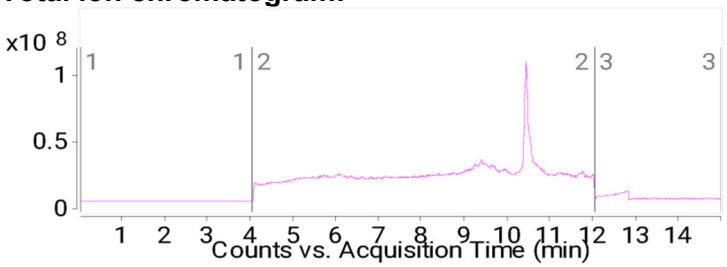
Mass spectrum of major compound:



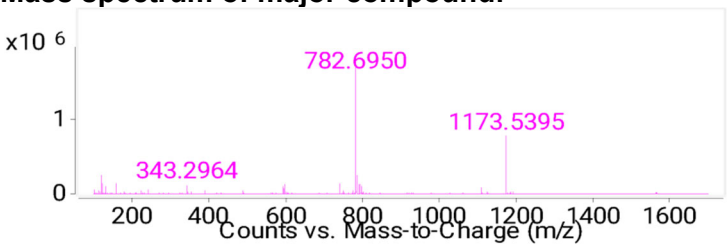
Name: Peptide **A10**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for (M +2H)2+: 1173.54, found: 1173.53.



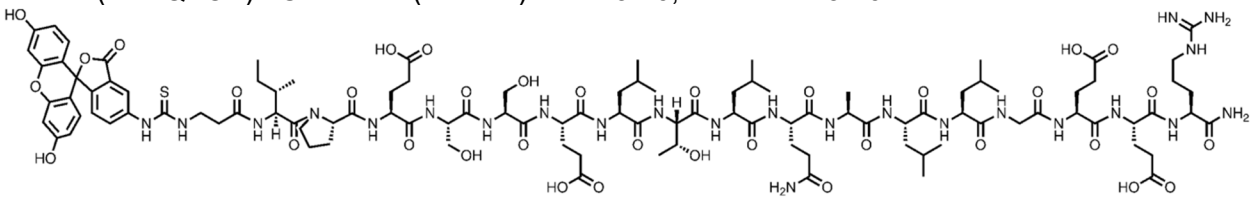
Total ion chromatogram:



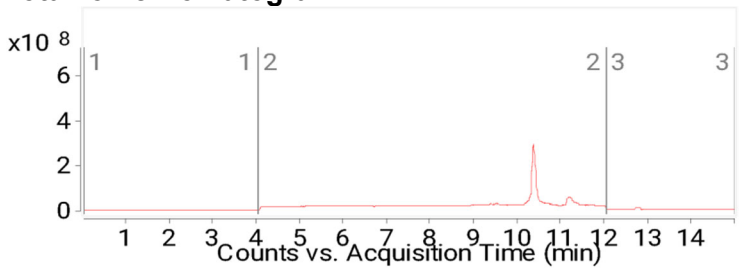
Mass spectrum of major compound:



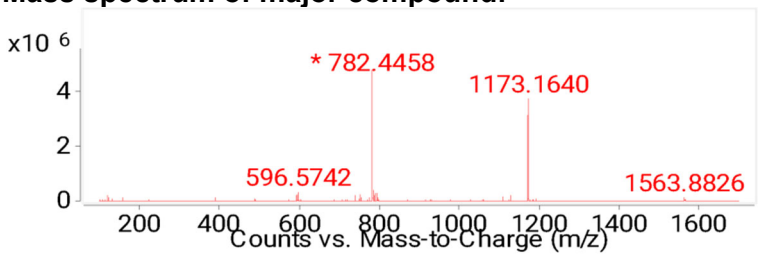
Name: Peptide **A11**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1173.16, found: 1173.16.



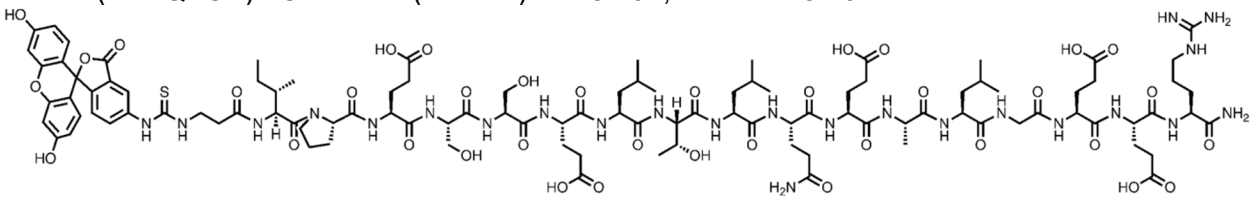
Total ion chromatogram:



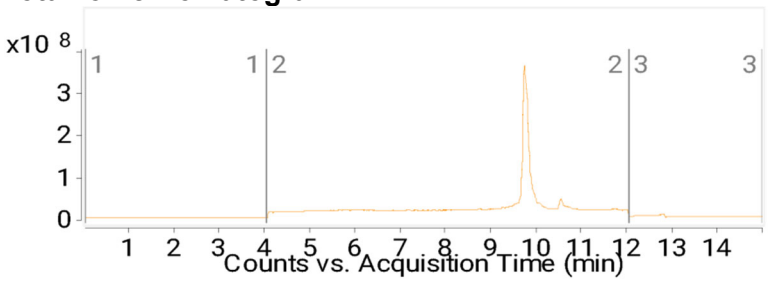
Mass spectrum of major compound:



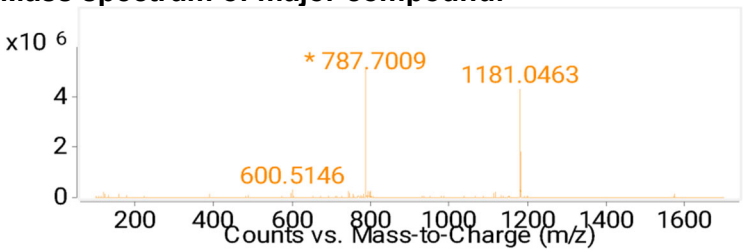
Name: Peptide **A12**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$:1181.04, found: 1181.04.



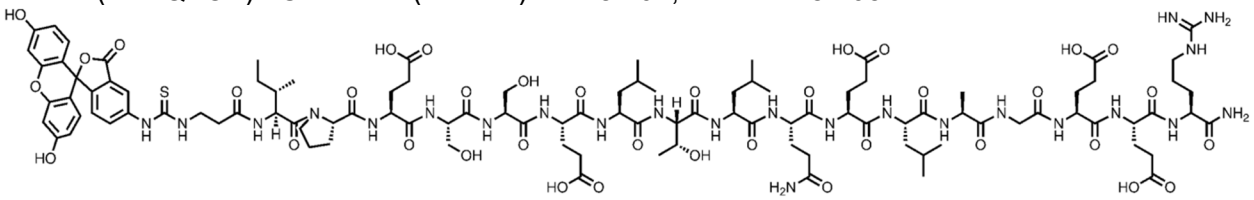
Total ion chromatogram:



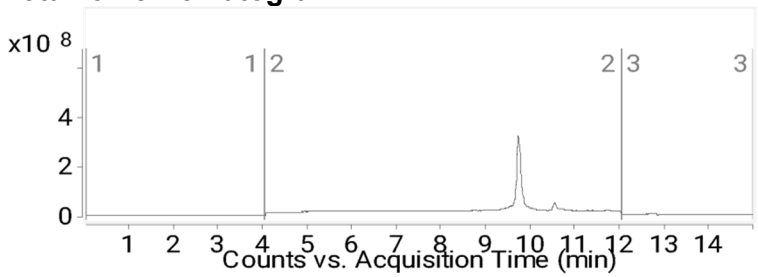
Mass spectrum of major compound:



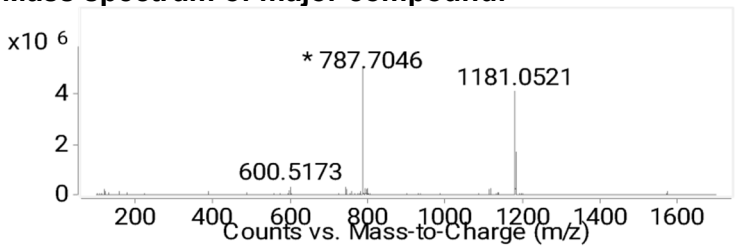
Name: Peptide **A13**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1181.04, found: 1181.05



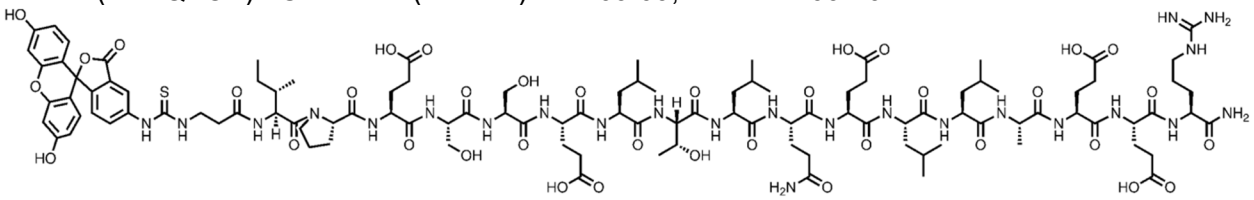
Total ion chromatogram:



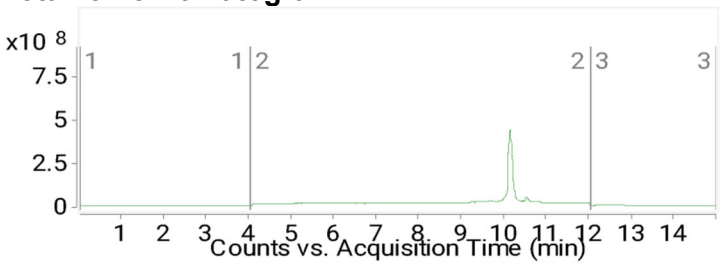
Mass spectrum of major compound:



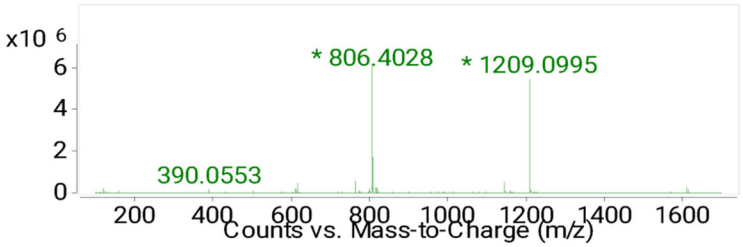
Name: Peptide **A14**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1209.09, found: 1209.10.



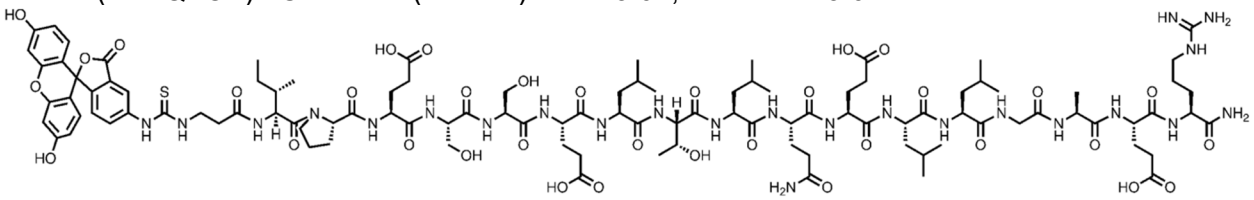
Total ion chromatogram:



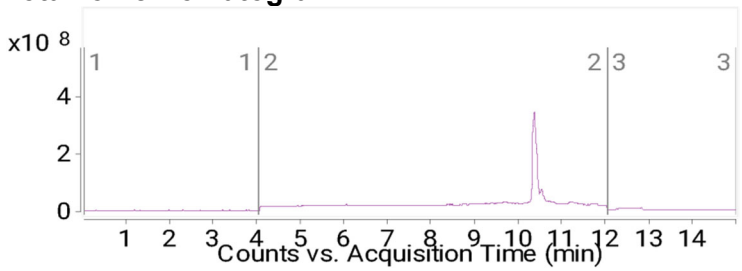
Mass spectrum of major compound:



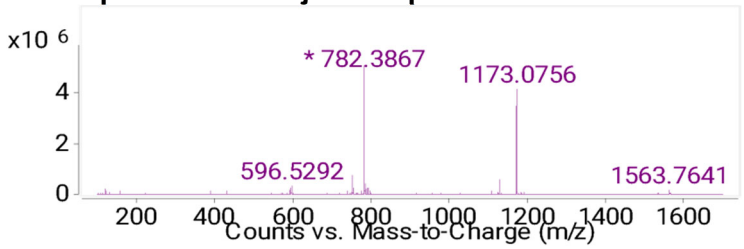
Name: Peptide **A15**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1173.07, found: 1173.07



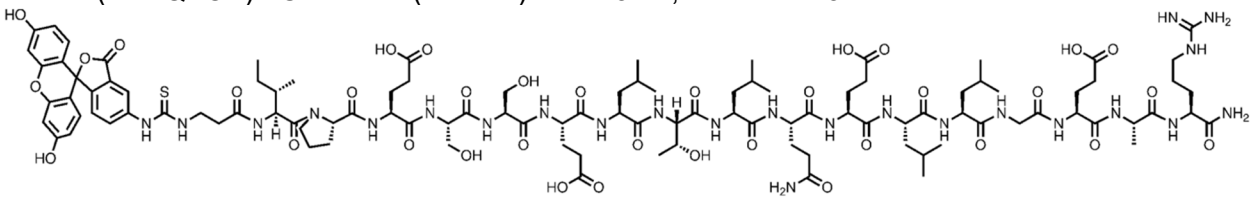
Total ion chromatogram:



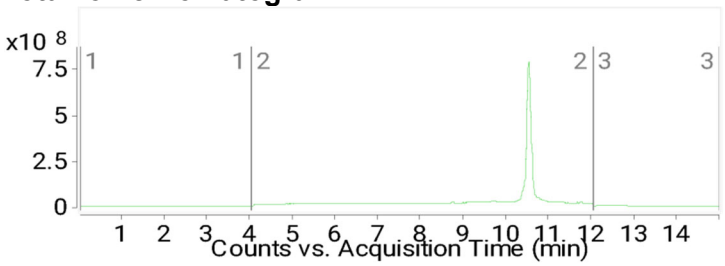
Mass spectrum of major compound:



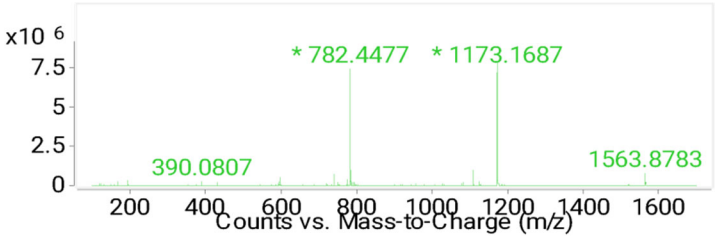
Name: Peptide **A16**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1173.17, found: 1173.17.



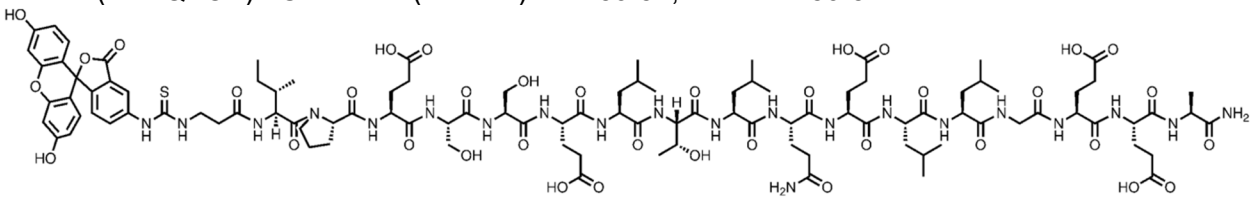
Total ion chromatogram:



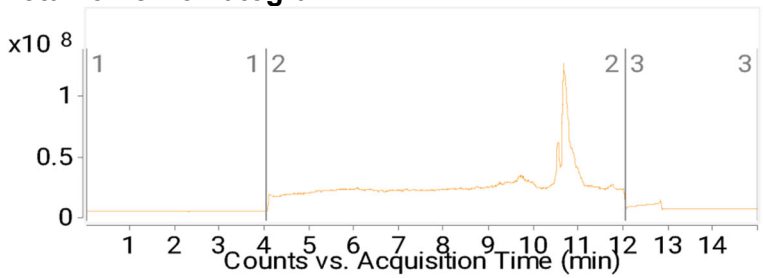
Mass spectrum of major compound:



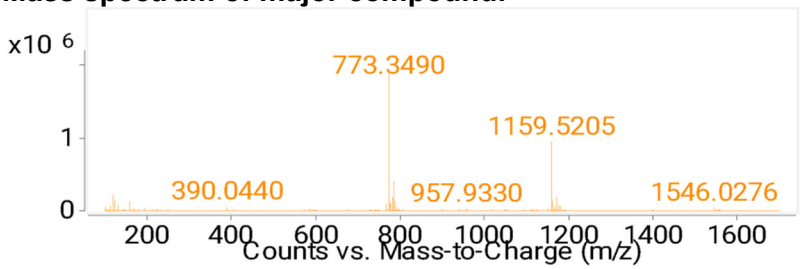
Name: Peptide **A17**
HPLC: method B
HRMS (ESI-QTOF): Calcd. for $(M + 2H)^{2+}$: 1159.52, found: 1159.52



Total ion chromatogram:

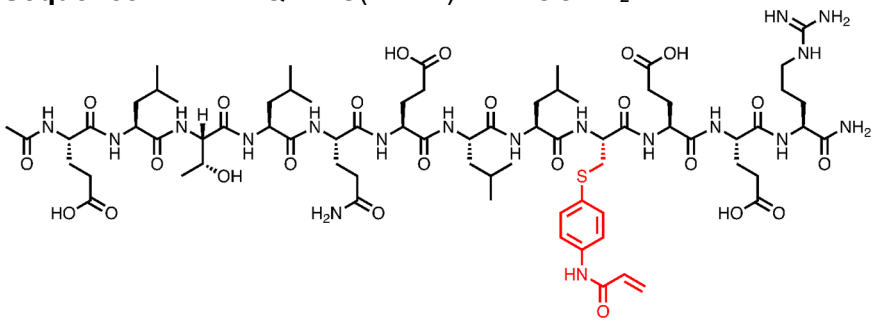


Mass spectrum of major compound:

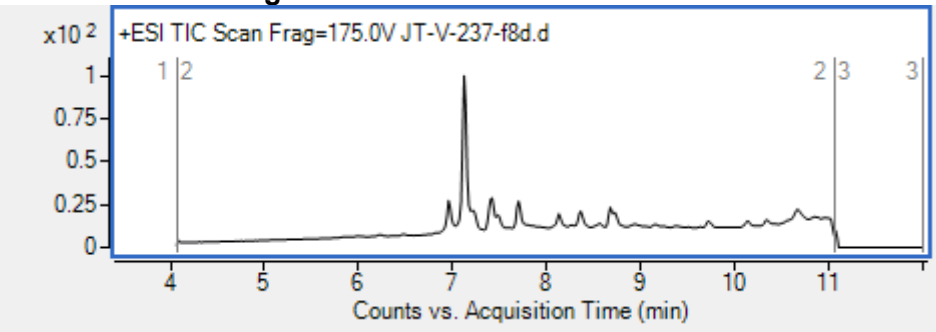


2.6 Peptides **E1-E11**

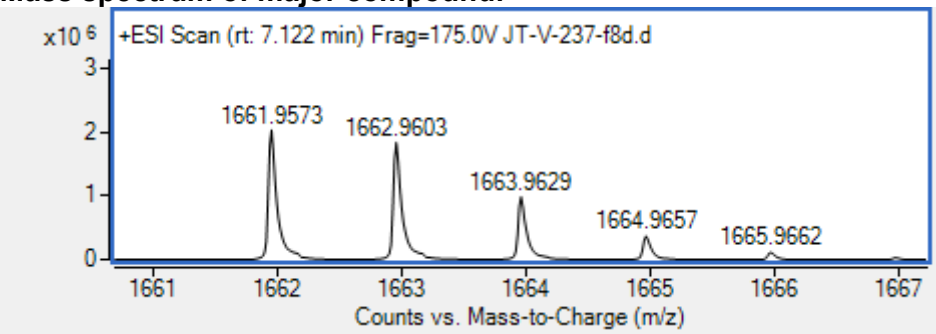
Peptide **E1**
Sequence: AcELTLQELLC(Phacr)EER -*CONH*$_2$



HPLC: Method C
Total ion chromatogram:



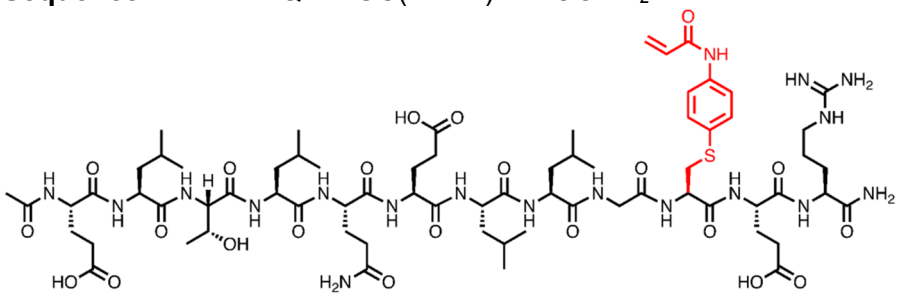
Mass spectrum of major compound:



Exact Mass:	Calculated for $C_{73}H_{117}N_{18}O_{24}S$ $[M+H]^+$	1661.82 Da
	found	1661.96 Da

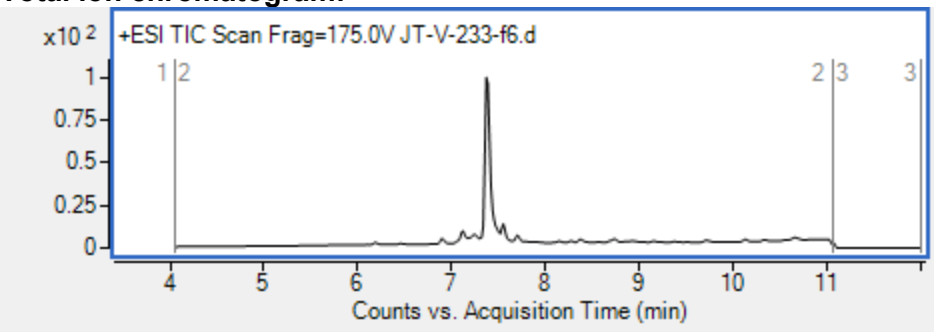
Peptide **E2**

Sequence: AcELTLQELLGC(Phacr)ER -*CONH*$_2$

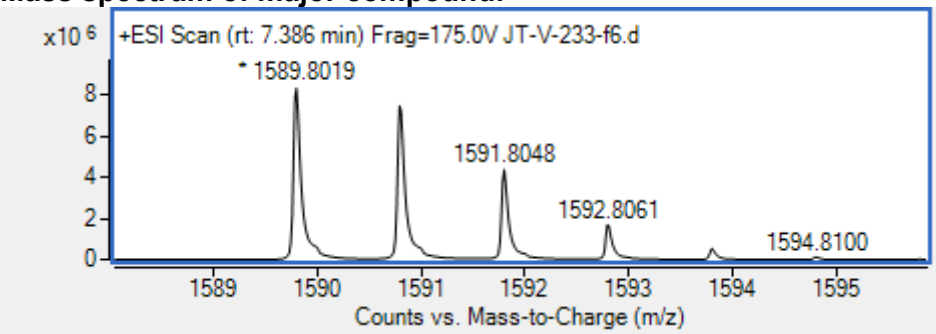


HPLC: Method C

Total ion chromatogram:



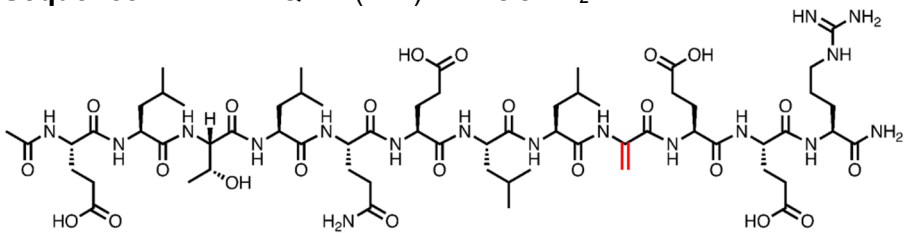
Mass spectrum of major compound:



Exact Mass:	Calculated $[M+H]^+$	for	$C_{70}H_{113}N_{18}O_{22}S$	1589.80 Da
	found			1589.80 Da

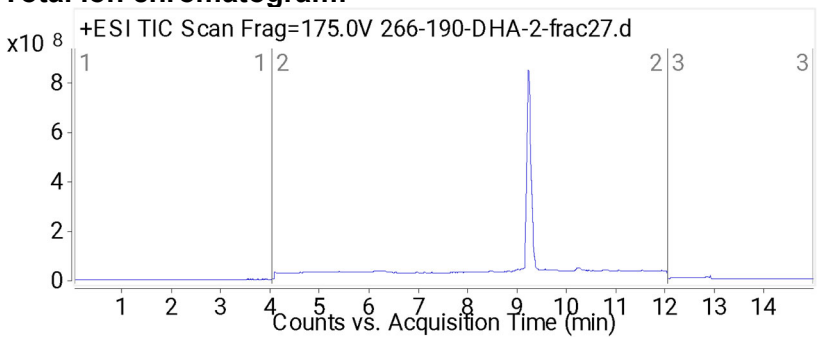
Peptide **E3**

Sequence: AcELTLQELL(dha)EER-*CONH*$_2$

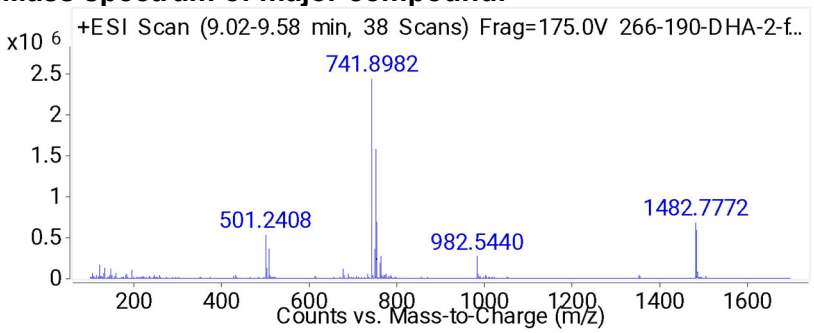


HPLC: Method C

Total ion chromatogram:



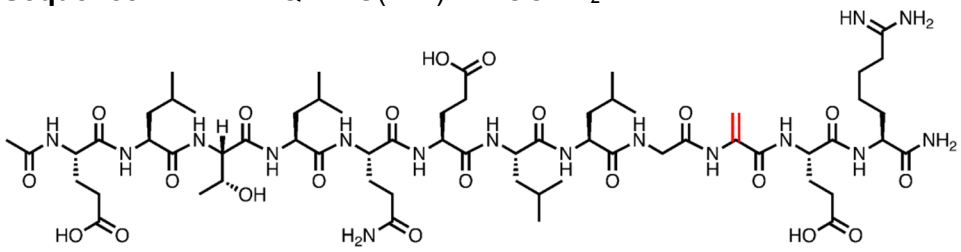
Mass spectrum of major compound:



HRMS (ESI-QTOF): Calcd. $C_{64}H_{107}N_{17}O_{23}$ for $(M + H)^+$: 1482.77, found: 1482.77.

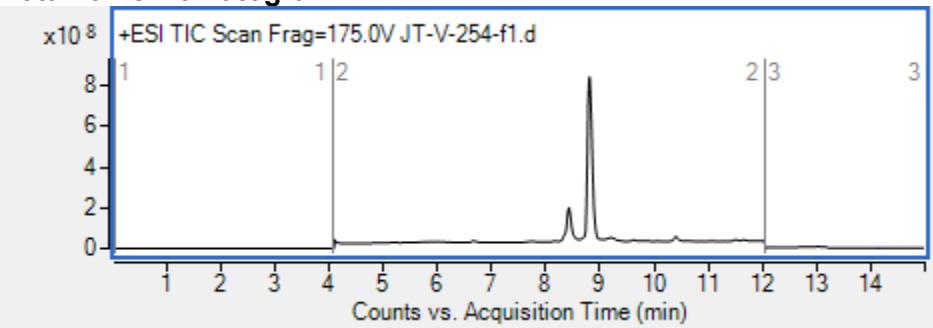
Peptide **E4**

Sequence: AcELTLQELLG(dha)ER -*CONH*$_2$

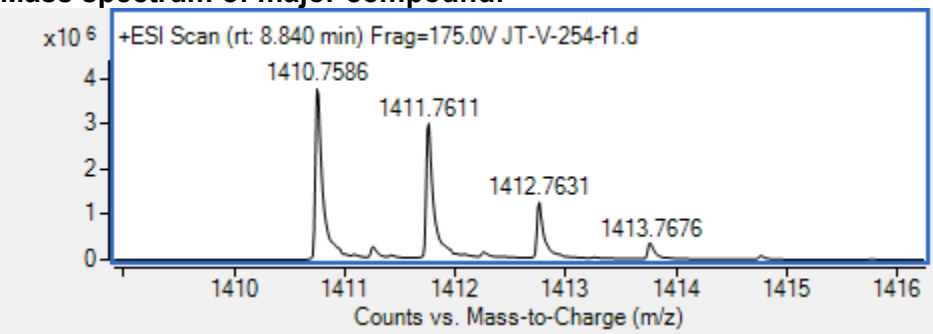


HPLC: Method C

Total ion chromatogram:



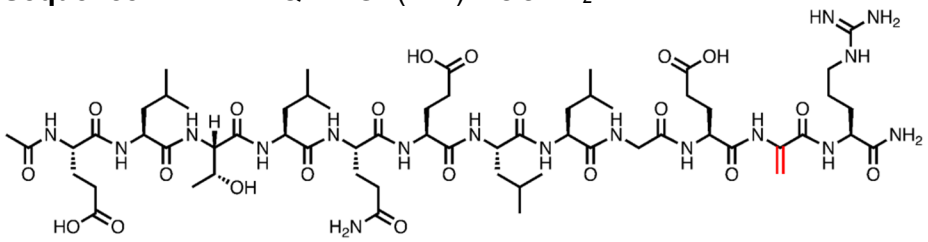
Mass spectrum of major compound:



Exact Mass:	Calculated for $C_{61}H_{104}N_{17}O_{21}$ $[M+H]^+$	1410.76 Da
	found	1410.76 Da

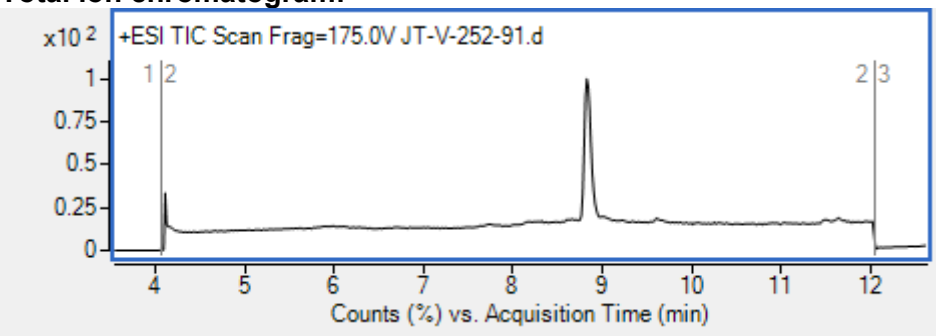
Peptide **E5**

Sequence: AcELTLQELLGE(dha)R-*CONH*$_2$

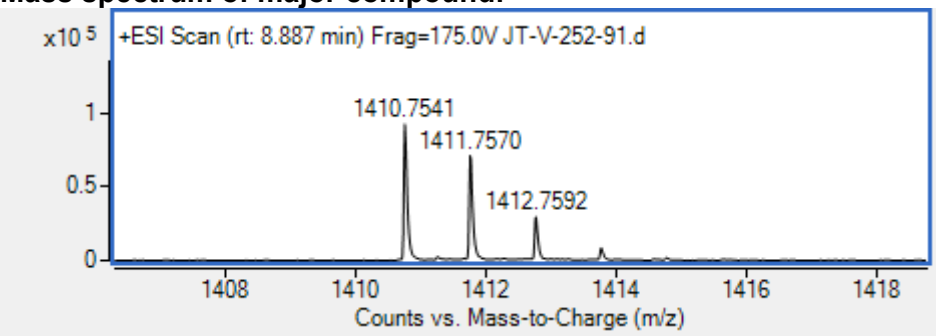


HPLC: Method C

Total ion chromatogram:



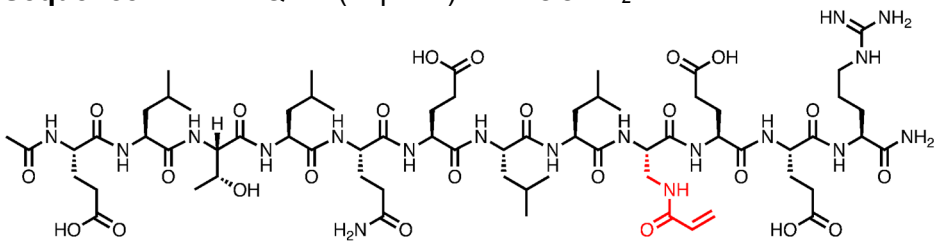
Mass spectrum of major compound:



Exact Mass:	Calculated for $C_{62}H_{105}N_{16}O_{21}$ $[M+H]^+$	1410.75 Da
	found	1410.75 Da

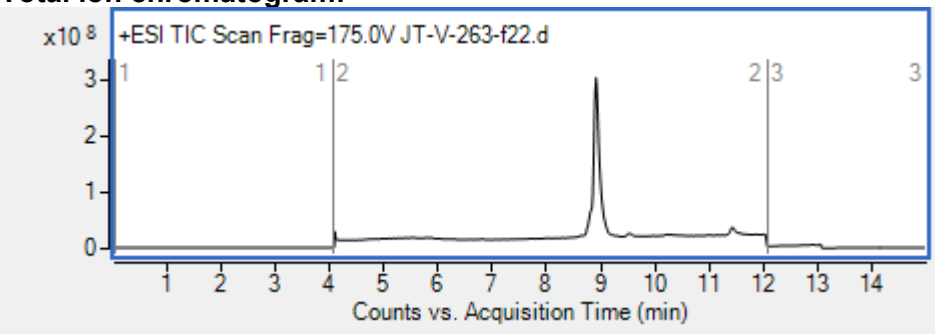
Peptide **E6**

Sequence: AcELTLQELL(dap-acr)EER -*CONH*$_2$

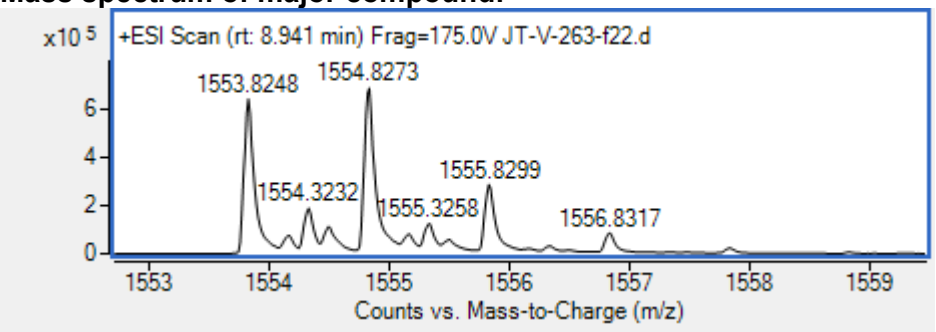


HPLC: Method C

Total ion chromatogram:



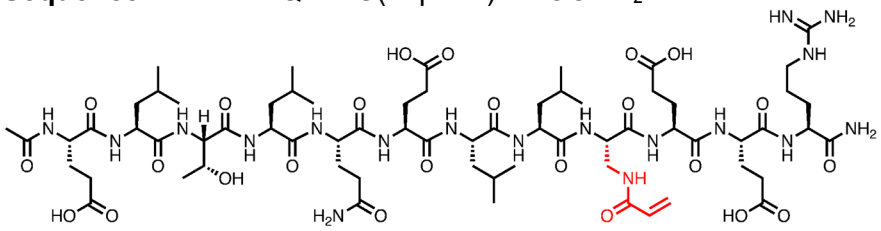
Mass spectrum of major compound:



Exact Mass:	Calculated for $C_{67}H_{113}N_{18}O_{24}[M+H]^+$	1553.82 Da
	found	1553.82 Da

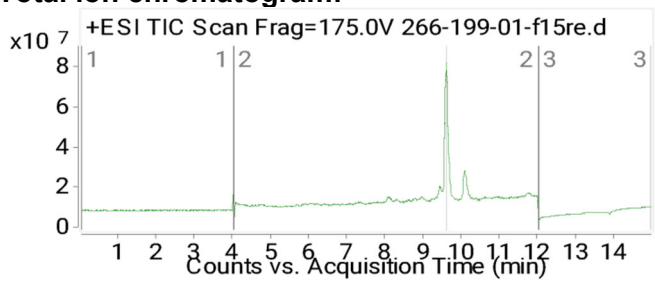
Peptide **E7**

Sequence: AcELTLQELLG(dap-acr)ER-*CONH*$_2$

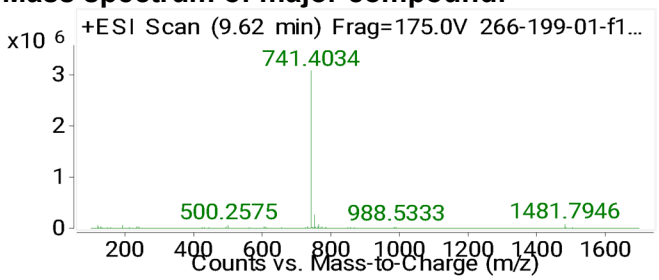


HPLC: Method D

Total ion chromatogram:



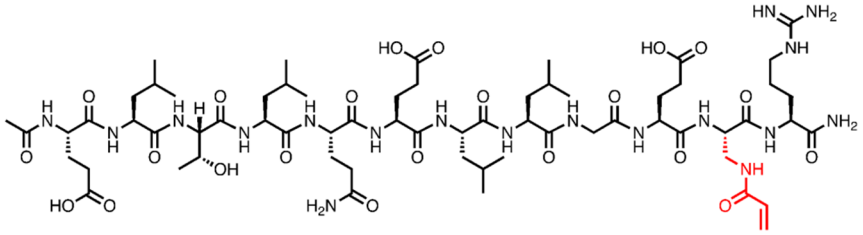
Mass spectrum of major compound:



HRMS (ESI-QTOF): Calcd. $C_{64}H_{108}N_{18}O_{22}$ for $(M + H)^+$: 1481.79, found: 1481.79.

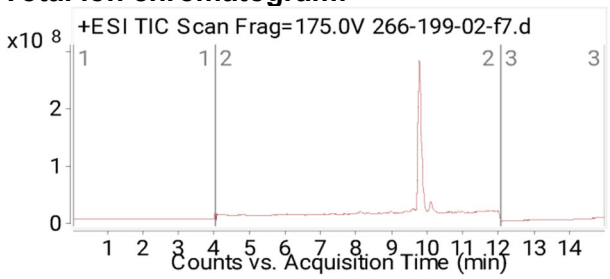
Peptide **E8**

Sequence: AcELTLQELLGE(dap-acr)R -*CONH*$_2$

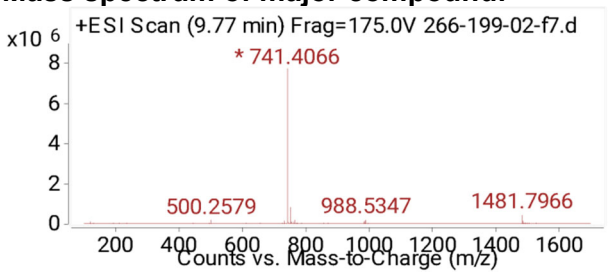


HPLC: Method D

Total ion chromatogram:



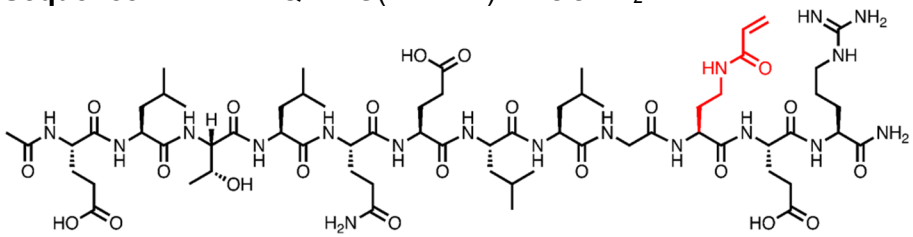
Mass spectrum of major compound:



HRMS (ESI-QTOF): Calcd. $C_{64}H_{108}N_{18}O_{22}$ for $(M + H)^+$: 1481.79, found: 1481.79.

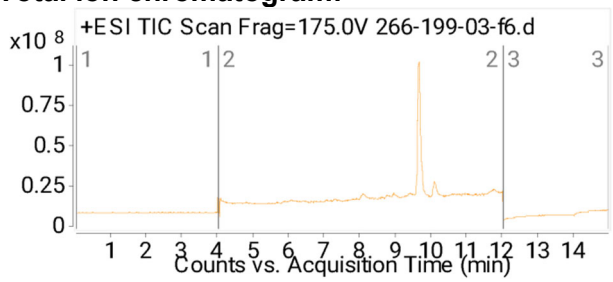
Peptide **E9**

Sequence: AcELTLQELLG(dab-acr)ER-*CONH*$_2$

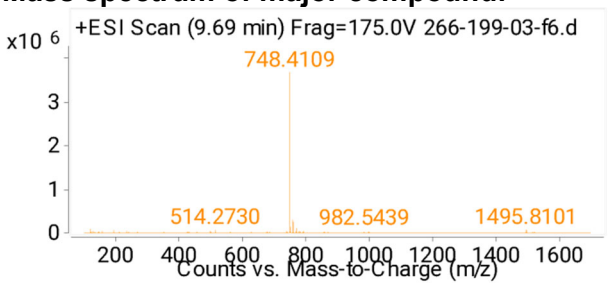


HPLC: Method D

Total ion chromatogram:



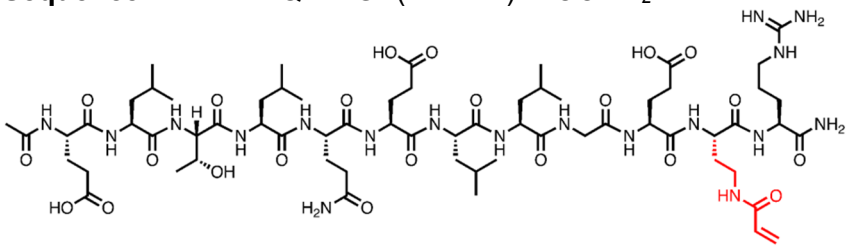
Mass spectrum of major compound:



HRMS (ESI-QTOF): Calcd. $C_{65}H_{110}N_{18}O_{22}$ for $(M + H)^+$: 1495.81, found: 1495.81.

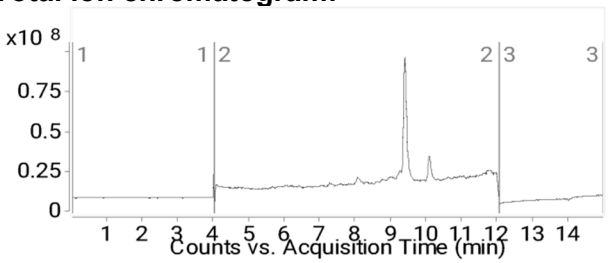
Peptide **E10**

Sequence: AcELTLQELLGE(dab-acr)R -*CONH*$_2$

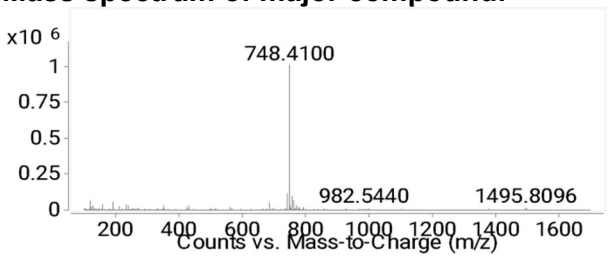


HPLC: Method D

Total ion chromatogram:



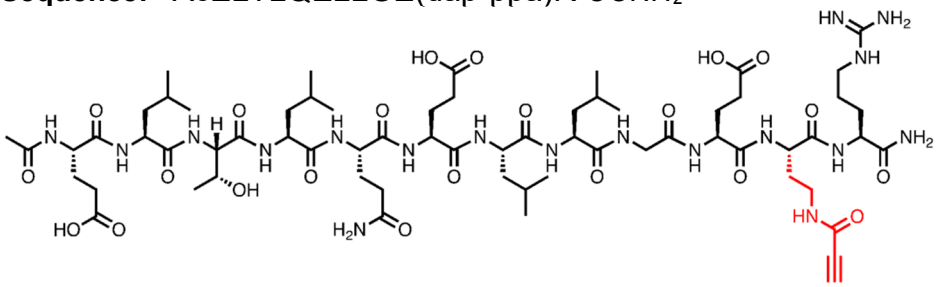
Mass spectrum of major compound:



HRMS (ESI-QTOF): Calcd. $C_{65}H_{110}N_{18}O_{22}$ for $(M + H)^+$: 1495.81, found: 1495.81.

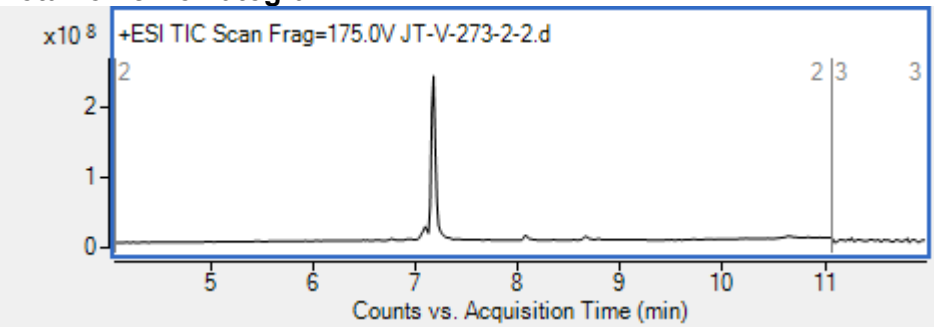
Peptide **E11**

Sequence: AcELTLQELLGE(dap-ppa)R-*CONH*$_2$

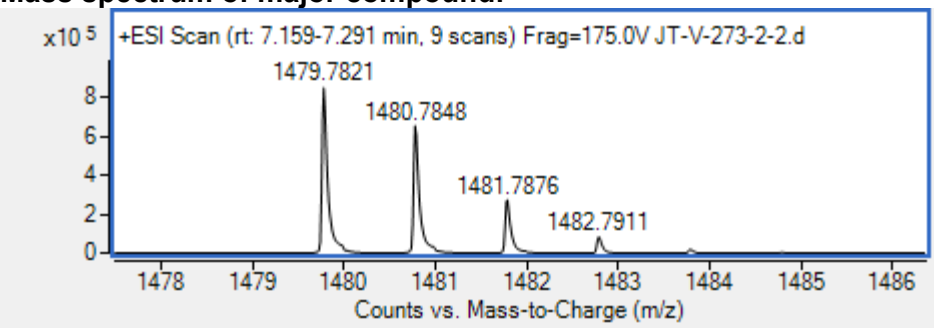


HPLC: Method C

Total ion chromatogram:



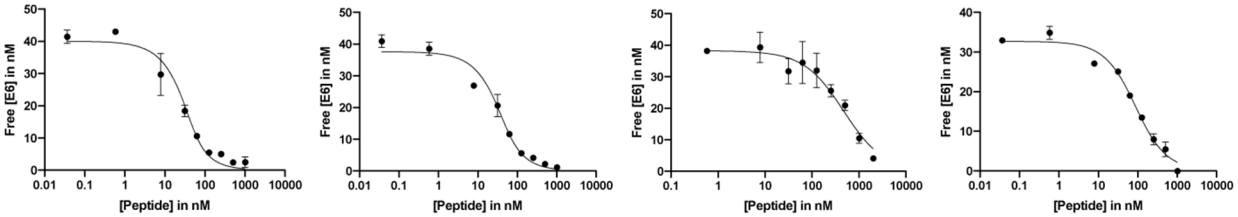
Mass spectrum of major compound:



Exact Mass:	Calculated $C_{64}H_{107}N_{18}O_{22}$ $[M+H]^+$	1479.78 Da
	found	1479.78 Da

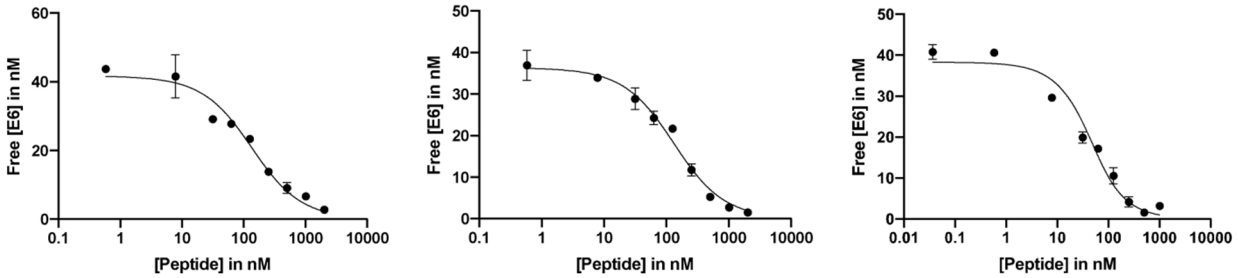
3. Binding characterization

Peptide **N1** binding validation. Approximately 100 nM MBP-16E6 and peptide were mixed following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.



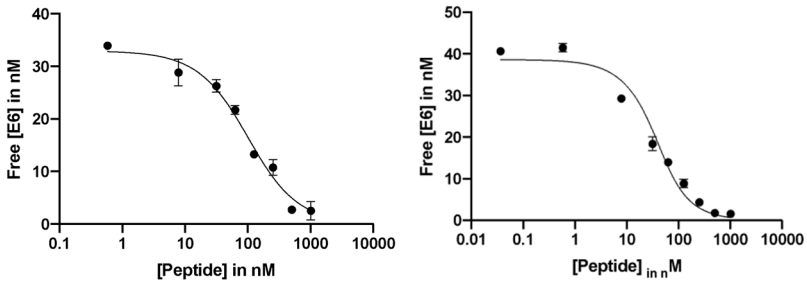
N1	N2	N3	N4
K_D= 10±6.8 nM	K_D= 13±6.3 nM	K_D= 425±78 nM	K_D= 79±14 nM

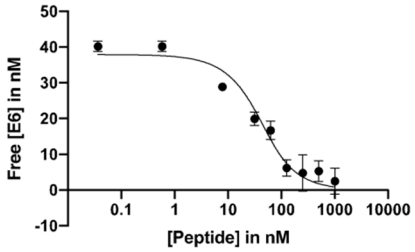
Peptide **N1, N2, N3 and N4** binding validation. Approximately 100 nM MBP-16E6 and peptide (1000 nM, 500 nM, 250 nM, 125nM, 63 nM, 32 nM, 7.8 nM, 0.6 nM, and 0.03 nM) were mixed following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.



N5	N6	N7
K_D= 110±21 nM	K_D= 102±18 nM	K_D= 27±6.3 nM

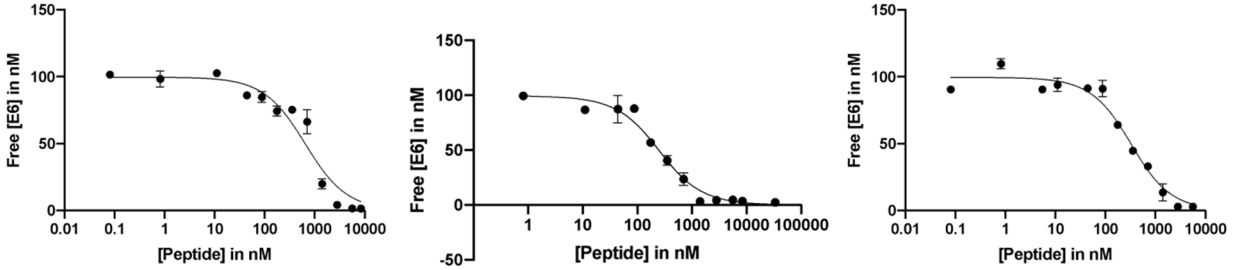
Peptide **N5, N6, and N7** binding validation. Approximately 40 nM MBP-16E6 and peptide (1000 nM, 500 nM, 250 nM, 125nM, 63 nM, 32 nM, 7.8 nM, 0.6 nM, and 0.03 nM) were mixed following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.





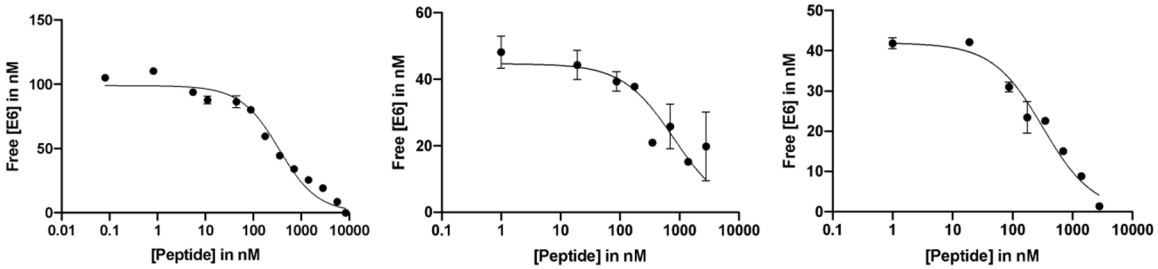
N8	N9	N10
K_D= 75±19 nM	K_D= 22±7.2 nM	K_D= 23±9.0 nM

Peptide **N8, N9, and N1** binding validation. Approximately 40 nM MBP-16E6 and peptide (1000 nM, 500 nM, 250 nM, 125nM, 63 nM, 32 nM, 7.8 nM, 0.6 nM, and 0.03 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.



N11	N12	N13
K_D= 595±152 nM	K_D= 199±37nM	K_D= 274±51nM

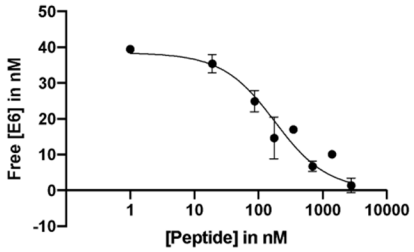
Peptide **N10, N11, and N12** binding validation. Approximately 100 nM MBP-16E6 and peptide (33333 nM, 16667 nM, 8333 nM, 4166 nM, 2083 nM, 1041 nM, 520 nM, 260 nM, 65 nM, 32nM, 4.8 nM 0.5 nM were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.

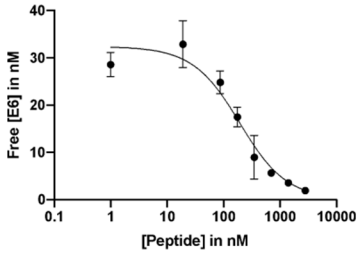


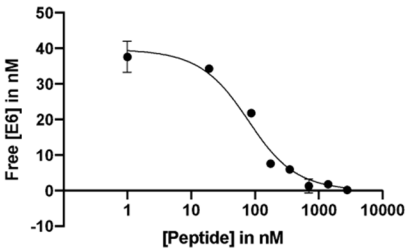
N14	N15	N16
K_D= 292±54 nM	K_D= 767±295 nM	K_D= 228±74 nM

Peptide **N14, N15, and N16** binding validation. Approximately 100 nM or 40nM MBP-16E6 and peptide **N14** (33333 nM, 16667 nM, 8333 nM, 4166 nM, 2083 nM, 1041 nM,

520 nM, 260 nM, 65 nM, 32nM, 4.8 nM, 0.5 nM) or **N15, N16** (2777 nM, 1388 nM, 694 nM, 347 nM, 173 nM, 86 nM, 18 nM, 1nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.

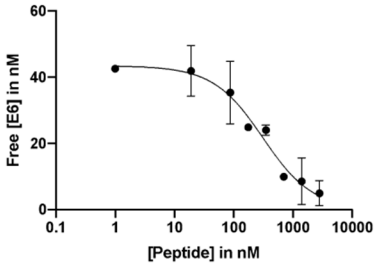


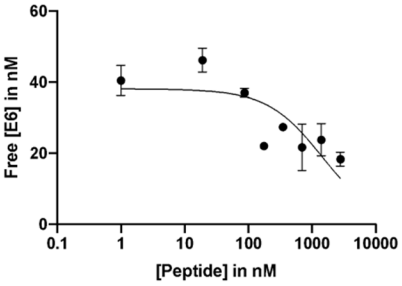


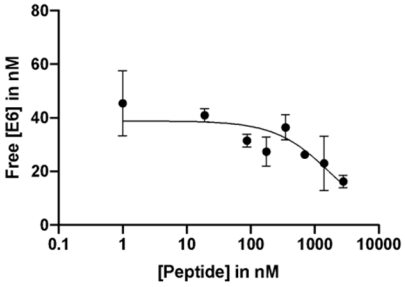


N17	N18	N19
K_D= 159±47 nM	K_D= 106±27 nM	K_D= 55±12 nM

Peptide **N16, N17, and N18** binding validation. Approximately 40 nM MBP-16E6 and peptide (2777 nM, 1388 nM, 694 nM, 347 nM, 173 nM, 86 nM, 18 nM, 1nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.

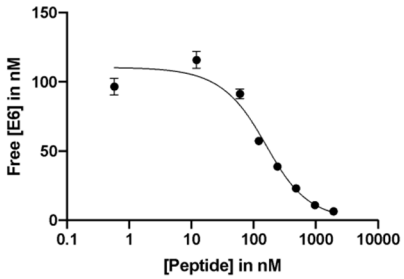


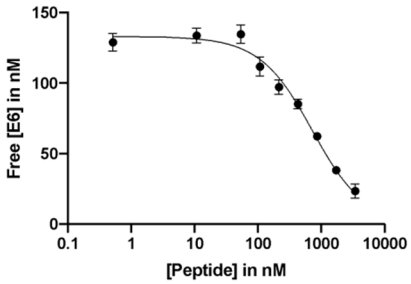




N20	N21	N22
K_D= 213±49 nM	K_D= 1412±822 nM	K_D= 1658±705 nM

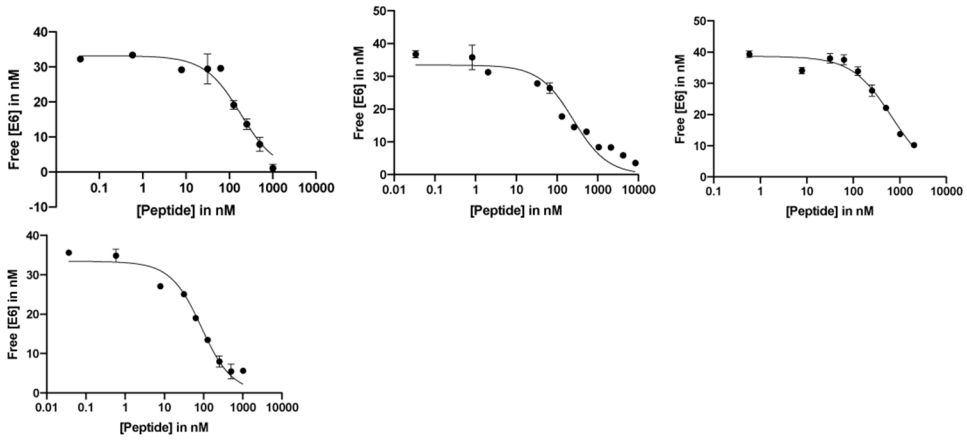
Peptide **N19, N20, and N21** binding validation. Approximately 40 nM MBP-16E6 and peptide (2777 nM, 1388 nM, 694 nM, 347 nM, 173 nM, 86 nM, 18 nM, 1nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.





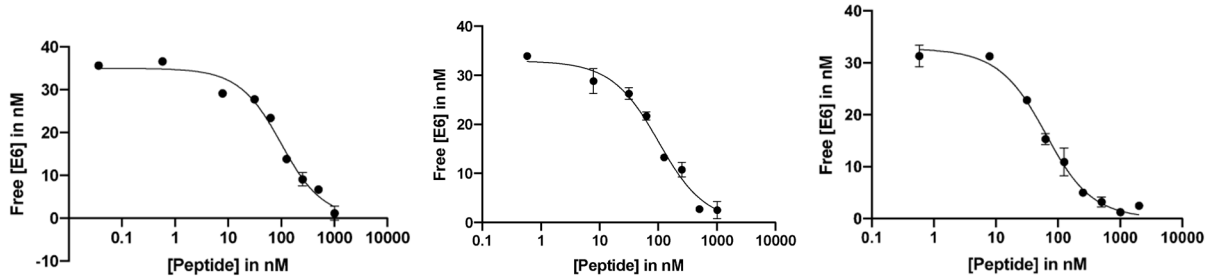
N-FITC	C-FITC
K_D= 95±17 nM	K_D= 640±82 nM

Peptide **N-FITC** and **C-FITC** binding validation. Approximately 100 nM or 120nM MBP-16E6 and peptide (1915 nM, 957nM, 478nM, 239nM, 119nM, 59nM, 12nM, and 0.5nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.



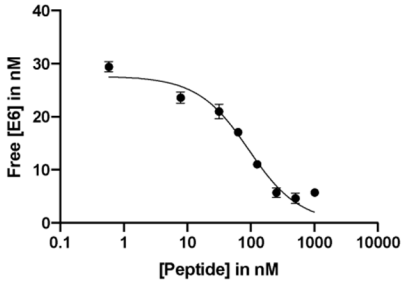
C1	C2	C3	C4
K_D=165±35 nM	K_D=245±59 nM	K_D=645±92 nM	K_D= 71±14 nM

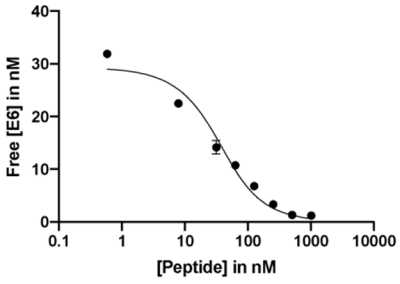
Peptide **C1, C2, C3, and C4** binding validation. Approximately 40 nM MBP-16E6 and peptide (1000 nM, 500 nM, 250 nM, 125nM, 63 nM, 32 nM, 7.8 nM, 0.6 nM, and 0.06 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.

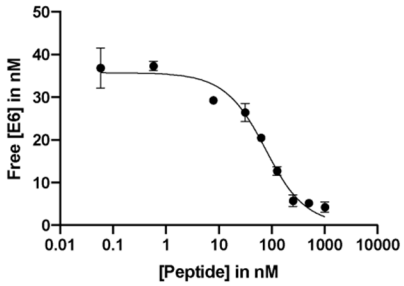


C5	C6	C7
K_D= 81±13 nM	K_D=84±13 nM	K_D= 46±5.5 nM

Peptide **C5, C6, and C7** binding validation. Approximately 30 nM MBP-16E6 and peptide (1000 nM, 500 nM, 250 nM, 125nM, 63 nM, 32 nM, 7.8 nM, 0.6 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.

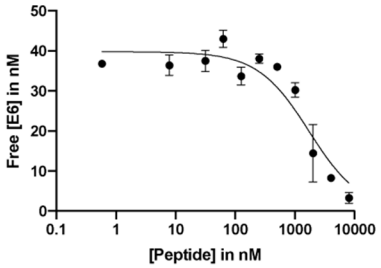


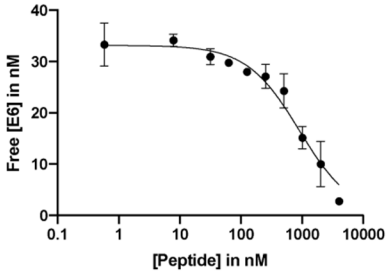


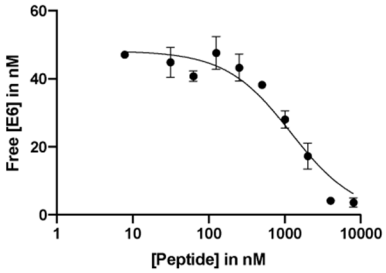


C8	C9	C10
K_D= 78±18 nM	K_D= 22±5.8 nM	K_D= 64±10 nM

Peptide **C8, C9, and C10** binding validation. Approximately 30 or 15 nM MBP-16E6 and peptide (1000 nM, 500 nM, 250 nM, 125nM, 63 nM, 32 nM, 7.8 nM, 0.6 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.

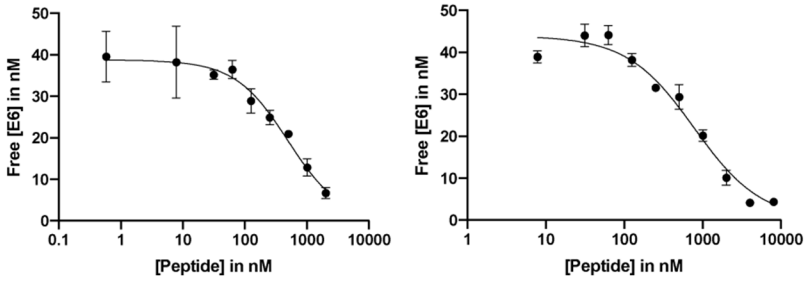






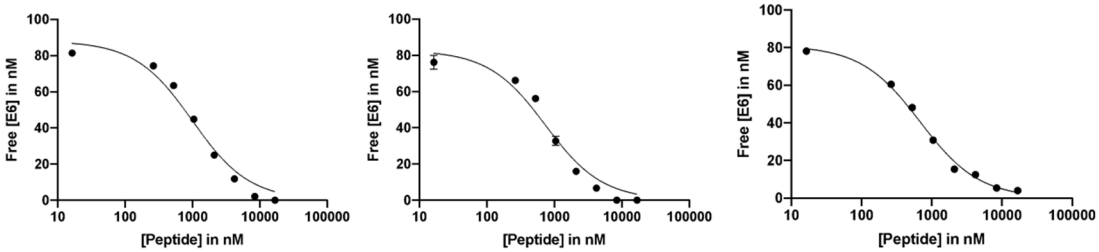
C11	C12	C13
K_D= 1459±474 nM	K_D= 878±125 nM	K_D= 1184±264 nM

Peptide **C11, C12, and C13** binding validation. Approximately 40 nM MBP-16E6 and peptide (8000 nM, 4000 nM, 2000 nM, 1000 nM, 500 nM, 250 nM, 125nM, 63 nM, 32 nM, 7.8 nM, 0.6 nM, and 0.03 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve is shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the titration curve was fitted.



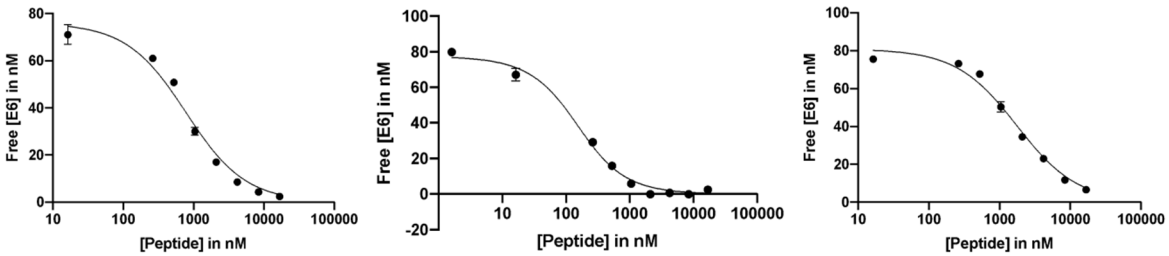
C14	C15
K_D= 507±65nM	K_D= 756±128 nM

Peptide **C14** and **C15** binding validation. Approximately 40 nM MBP-16E6 and peptide (4000 nM, 2000 nM, 1000 nM, 500 nM, 250 nM, 125nM, 63 nM, 32 nM, 7.8 nM, 0.6 nM, and 0.03 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve is shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.



A1	A2	A3
FITC-APESSELTLQELLGEER	FITC-IAESSELTLQELLGEER	FITC-IPASSELTLQELLGEER
K_D= 944±182 nM	K_D= 691±157 nM	K_D= 638±54 nM

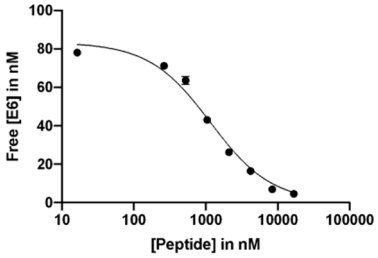
Peptide **A1, A2, and A3** binding validation. Approximately 80 nM MBP-16E6 and peptide (16667 nM, 8333 nM, 4166 nM, 2083 nM, 1041 nM, 520 nM, 260 nM, 16 nM, and 1.6 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.

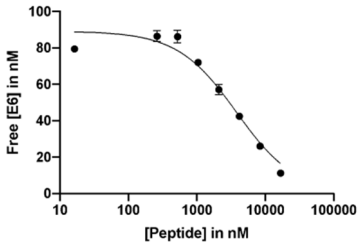


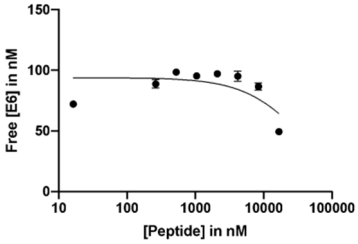
A4	A5	A6
FITC-IPEASELTLQELLGEER	FITC-IPESAELTLQELLGEER	FITC-IPESSALTLQELLGEER
K_D= 739±113 nM	K_D=125±17 nM	K_D= 1706±209 nM

Peptide **A4, A5, and A6** binding validation. Approximately 80 nM MBP-16E6 and peptide (16667 nM, 8333 nM, 4166 nM, 2083 nM, 1041 nM, 520 nM, 260 nM, 16 nM, and 1.6 nM)

were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.

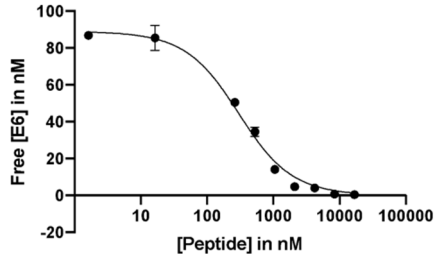


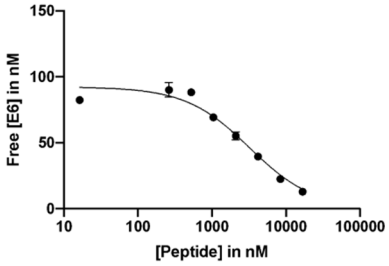


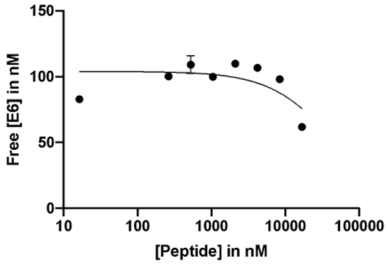


A7	A8	A9
FITC-IPESSEATLQELLGEER	FITC-IPESSELALQELLGEER	FITC-IPESSELTAQELLGEER
K_D= 1114±152 nM	K_D=3748±680 nM	K_D>10000 nM

Peptide **A7, A8, and A9** binding validation. Approximately 80 nM MBP-16E6 and peptide (16667 nM, 8333 nM, 4166 nM, 2083 nM, 1041 nM, 520 nM, 260 nM, 16 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.

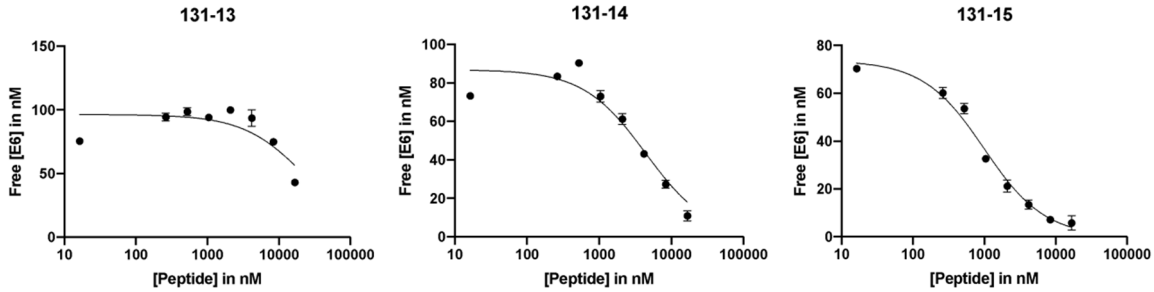






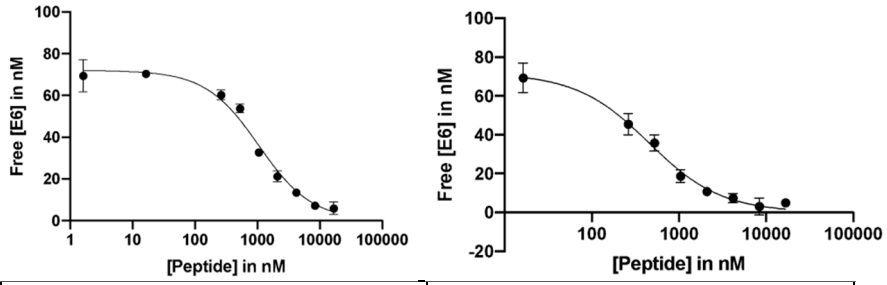
A10	A11	A12
FITC-IPESSELTLAELLGEER	FITC-IPESSELTLQALLGEER	FITC-IPESSELTLQEALGEER
K_D= 242±31 nM	K_D=3158±568 nM	K_D>10000 nM

Peptide **A10, A11, and A12** binding validation. Approximately 80 nM MBP-16E6 and peptide (16667 nM, 8333 nM, 4166 nM, 2083 nM, 1041 nM, 520 nM, 260 nM, 16 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.



A13	A14	A15
FITC-IPESSELTLQELAGEER	FITC-IPESSELTLQELLAEER	FITC-IPESSELTLQELLGAER
K_D>10000 nM	K_D= 4342±1177 nM	K_D=953±119 nM

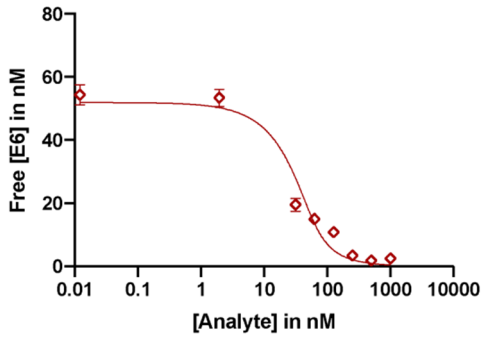
Peptide **A13, A14, and A15** binding validation. Approximately 80 nM MBP-16E6 and peptide (16667 nM, 8333 nM, 4166 nM, 2083 nM, 1041 nM, 520 nM, 260 nM, 16 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.



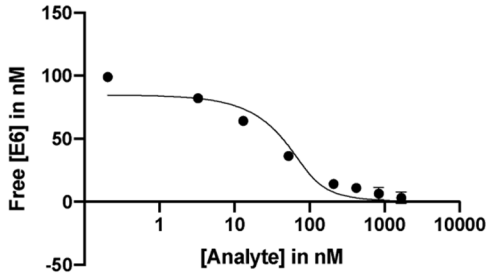
A16	A17
FITC-IPESSELTLQELLGEAR	FITC-IPESSELTLQELLGEEA
K_D= 274±21 nM	K_D=413±39 nM

Peptide **A16 and A17** binding validation. Approximately 70 nM MBP-16E6 and peptide (16667 nM, 8333 nM, 4166 nM, 2083 nM, 1041 nM, 520 nM, 260 nM, 16 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.

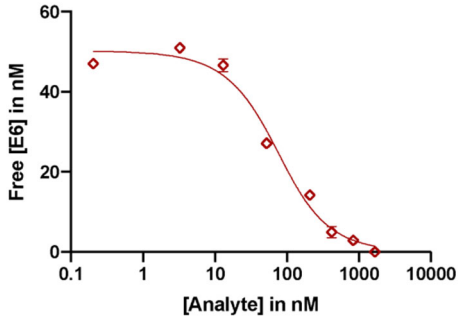
Peptide 1 binding see **Figure S1**.



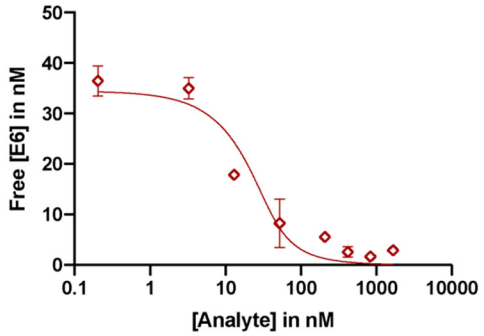
Peptide 2 binding validation. Approximately 50 nM MBP-16E6 and peptide (1000 nM, 500 nM, 250 nM, 125 nM, 62 nM, 31 nM, 1.9 nM, 0.012 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=3). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.



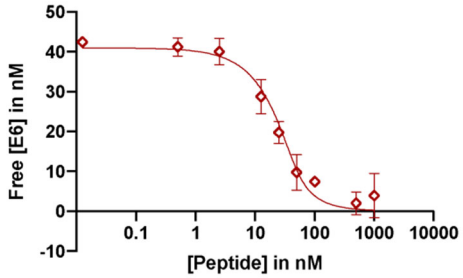
Peptide 3 binding validation. Approximately 100 nM MBP-16E6 and peptide (1667 nM, 833 nM, 416 nM, 208 nM, 52 nM, 13 nM, 3.0 nM, and 0.2 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=3). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.



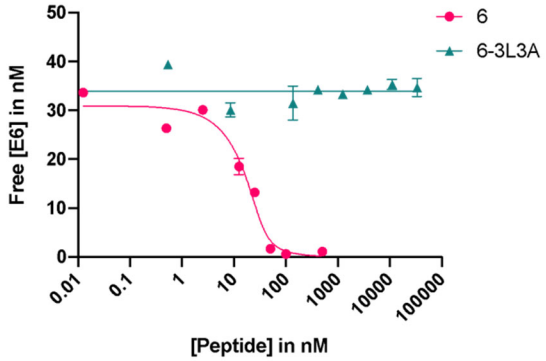
Peptide 4 binding validation. Approximately 50 nM MBP-16E6 and peptide (1667 nM, 833 nM, 416 nM, 208 nM, 52 nM, 13 nM, 3.0 nM, and 0.2 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=3). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted.



Peptide 5 binding validation. Approximately 40 nM MBP-16E6 and peptide (1667 nM, 833 nM, 416 nM, 208 nM, 52 nM, 13 nM, 3.0 nM, and 0.2 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=3). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted. Peptide **5** K_D= 6.7±3.9 nM,

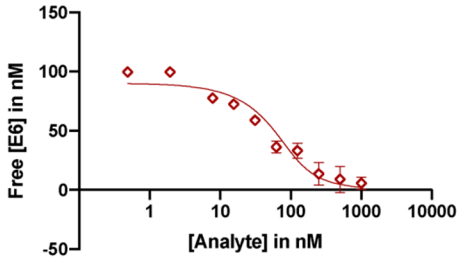


Peptide 6 binding validation. Approximately 40 nM MBP-16E6 and peptide (1000 nM, 500 nM, 100 nM, 50 nM, 25 nM, 12 nM, 2.5 nM, 0.5 nM, and 0.0125 nM)were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted. Peptide **6** K_D= 3.7±1.9 nM,

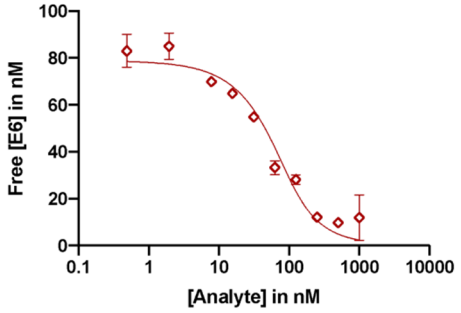


Peptide 6' and **6'-3L3A**. Binding validation. Approximately 40 nM MBP-16E6 and peptide **6** (500 nM, 100 nM, 50 nM, 25 nM, 12 nM, 2.5 nM, 0.5 nM, and 0.0125 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=3). With

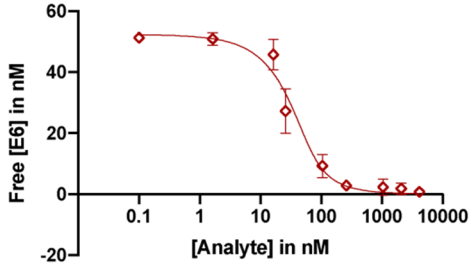
peptide **6-3L3A** 33333 nM, 11111 nM, 3703 nM, 1234 nM, 411 nM, 137 nM, 8.5 nM, and 0.5 nM (N=2). Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted. Peptide **6** K_D= 2.7±1.2 nM, Peptide **6-3L3A** K_D > 33,333 nM



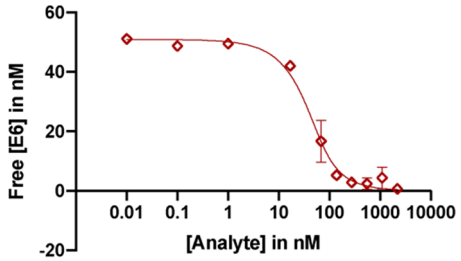
Peptide 7 binding validation. Approximately 100 nM MBP-16E6 and peptide (1000 nM, 500 nM, 250 nM, 125 nM, 62 nM, 31 nM, 15 nM, 7.8 nM, 3.9 nM, 1.9 nM, and 0.5 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=2). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted. Estimated apparent K_i = 23±8.1nM.



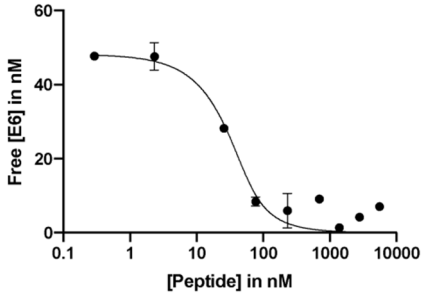
Peptide 8 binding validation. Approximately 80 nM MBP-16E6 and peptide (1000 nM, 500 nM, 250 nM, 125 nM, 62 nM, 31 nM, 15 nM, 7.8 nM, 3.9 nM, 1.9 nM, and 0.5 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=3). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted. Estimated apparent K_i = 39±6.1nM.



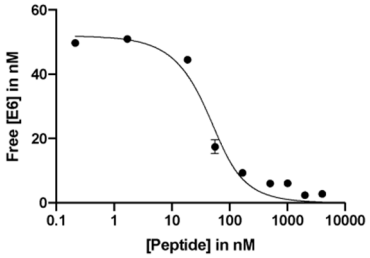
Peptide 9 binding validation. Approximately 80 nM MBP-16E6 and peptide (4167 nM, 2084 nM, 1042 nM, 260 nM, 104 nM, 26 nM, 16 nM, 1.6 nM, 0.1 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=3). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted. Estimated apparent K_i = 11±3.7 nM.



Peptide 10 binding validation. Approximately 80 nM MBP-16E6 and peptide (4167 nM, 2084 nM, 1042 nM, 260 nM, 104 nM, 26 nM, 16 nM, 1.6 nM, 0.1 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=3). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted. Estimated apparent K_i = 16±2.6 nM.

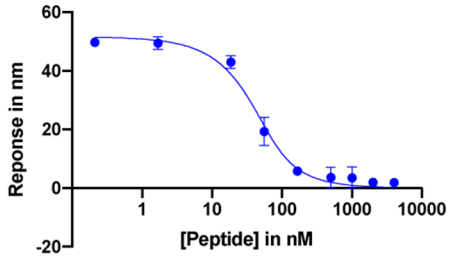


Peptide 11 binding validation. Approximately 50 nM MBP-16E6 and peptide (5500 nM, 2750 nM, 1375 nM, 687 nM, 229 nM, 76 nM, 25 nM, 2.3 nM, and 0.2 nM) were mixed together following the protocol for **Competitive binding assay by BLI** (N=3). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted. Estimated apparent K_i 11±4.9nM.



Peptide 12 binding validation. Approximately 80 nM MBP-16E6 and peptide (4000 nM, 2000 nM, 1000 nM, 500 nM, 166 nM, 55 nM, 18 nM, 1.7 nM, and 0.2 nM) were mixed

together following the protocol for in-solution competition assay (N=3). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted. Estimated apparent K_i = 20±5.4nM.



Peptide 13 binding validation. Approximately 80 nM MBP-16E6 and peptide (4000 nM, 2000 nM, 1000 nM, 500 nM, 166 nM, 55 nM, 18 nM, 1.7 nM, and 0.2 nM) were mixed together following the protocol for in-solution competition assay (N=3). A representative binding curve was shown. Free [MBP-16E6] was estimated using the biotin-IPESS calibration curve, and the obtained titration curve was fitted. Estimated apparent K_i = 17±3.9nM.

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