Enhancing the Cell-Permeability of Stapled Peptides with a Cyclic Cell-Penetrating Peptide

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Supporting Information

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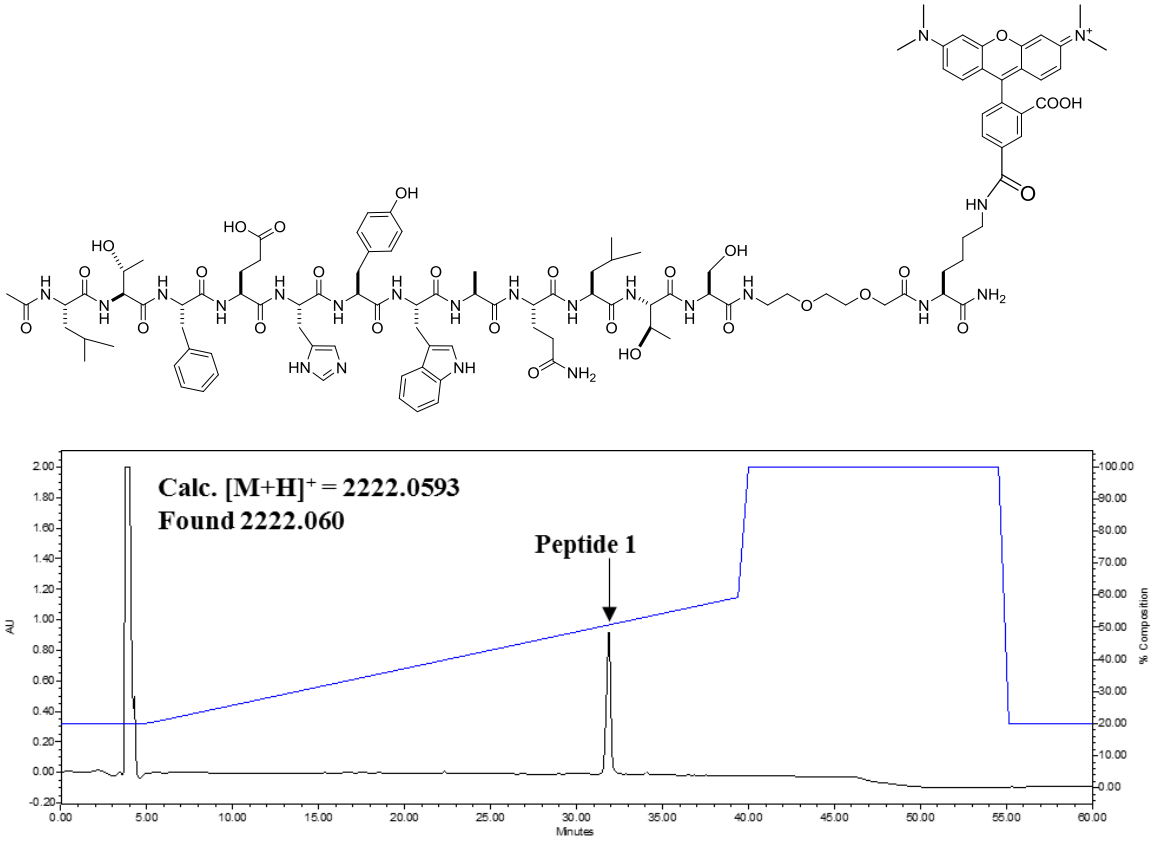
Table S1. Purity and MS data of compounds **1-25**

Compound	HPLC Purity (%)	$[M+H]^+$ calculated	HRMS $[M+H]^+$ observed
1	95.23	2222.0593	2222.060
2	95.71	1834.9486	1834.949
3	95.59	2744.4320	2744.436
4	97.71	2914.5375	2914.546
5	95.62	2838.5062	2838.509
6	97.27	3417.8991	3417.887
7	98.81	3281.8692	3281.860
8	97.50	2388.0827	2388.074
9	96.41	3444.8763	3444.855
10	97.95	3948.2379	3948.206
11	96.62	3812.2079	1906.607 $[M+2H]^{2+}$
12	95.08 (combined 2 peaks)	1702.7339	1702.733
13	97.75 (combined 2 peaks)	3098.4969	3098.505
14	95.22	2415.9871	2415.989
15	96.70	3811.7507	3811.728
16	95.12 (combined 2 peaks)	2286.9445	2286.943
17	95.76	3682.7081	1841.858 $[M+2H]^{2+}$
18	98.7	2355.9956	2355.990
19	98.56	3751.7592	1876.382 $[M+2H]^{2+}$
20	98.53 (combined 2 peaks)	2275.9503	2275.950
21	97.04	3671.7139	3671.692
22	96.15 (combined 2 peaks)	2291.9088	2291.909
23	94.13 (combined 2 peaks)	3687.6725	3687.656
24	92.30 (combined 2 peaks)	2420.9514	2420.952
25	98.73	3816.7151	3816.707

Figure S1. Structures, purity (by reversed-phase HPLC), and HR-MS (FT-ICR) of peptides **1-25** used in this work. Note: Some of the dye-labeled peptides (**12-25**) eluted as two separate peaks, because the commercially available NF and FAM are mixtures of 5- and 6-carboxy isomers.

Peptide **1**



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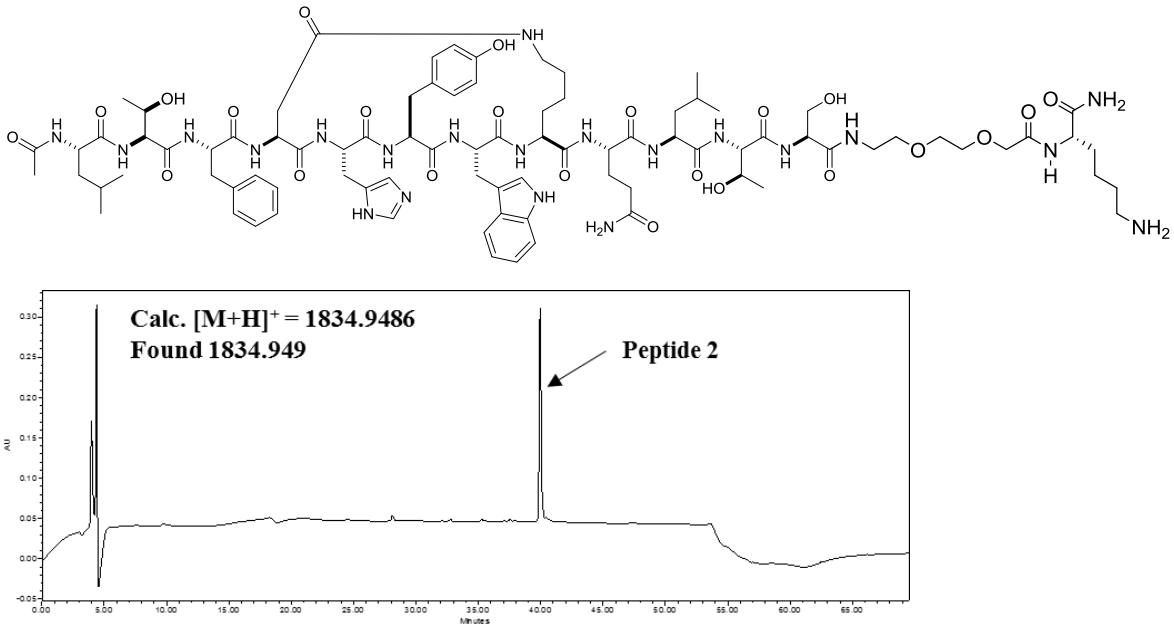

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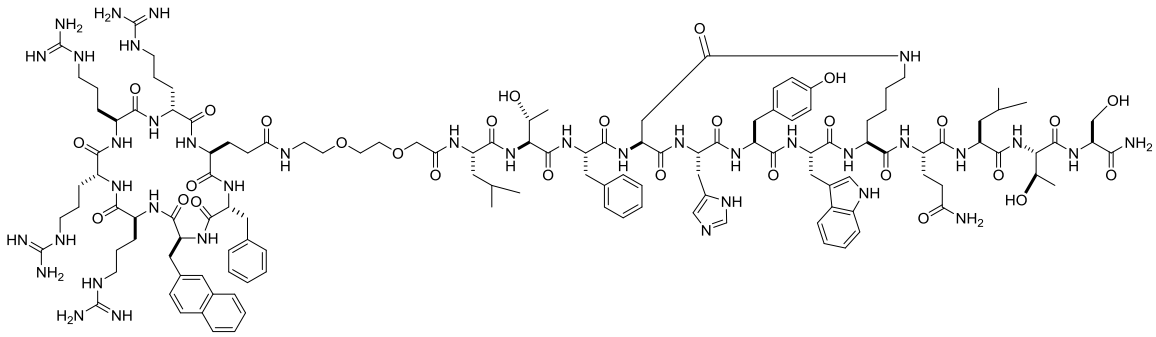

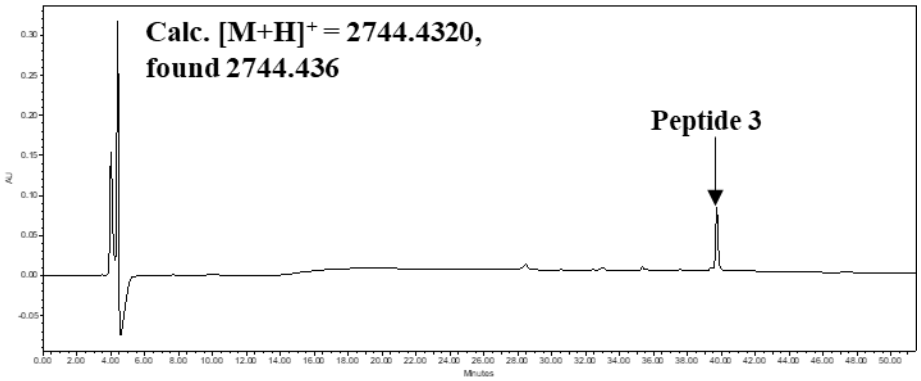

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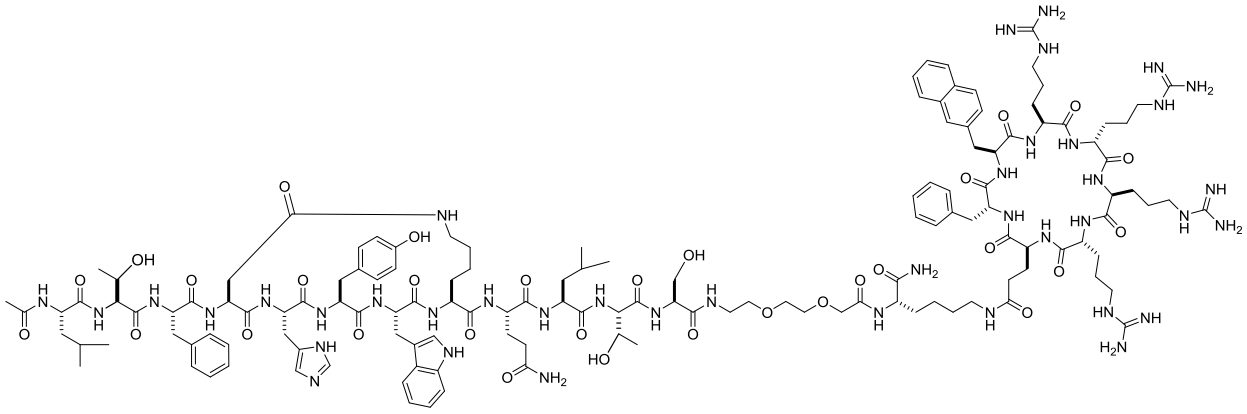

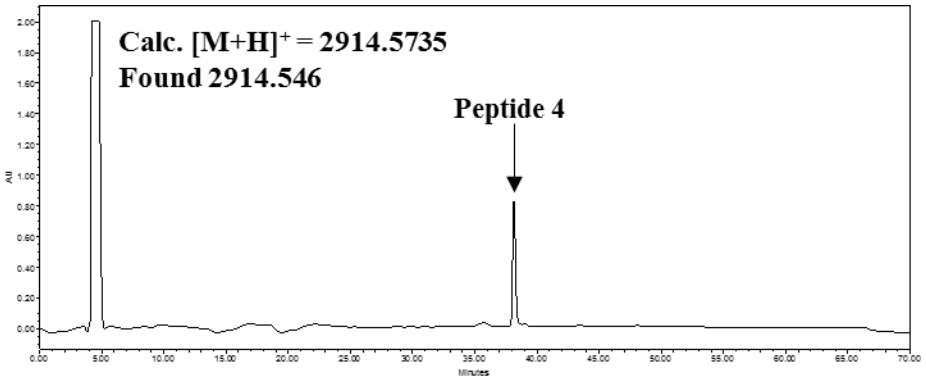

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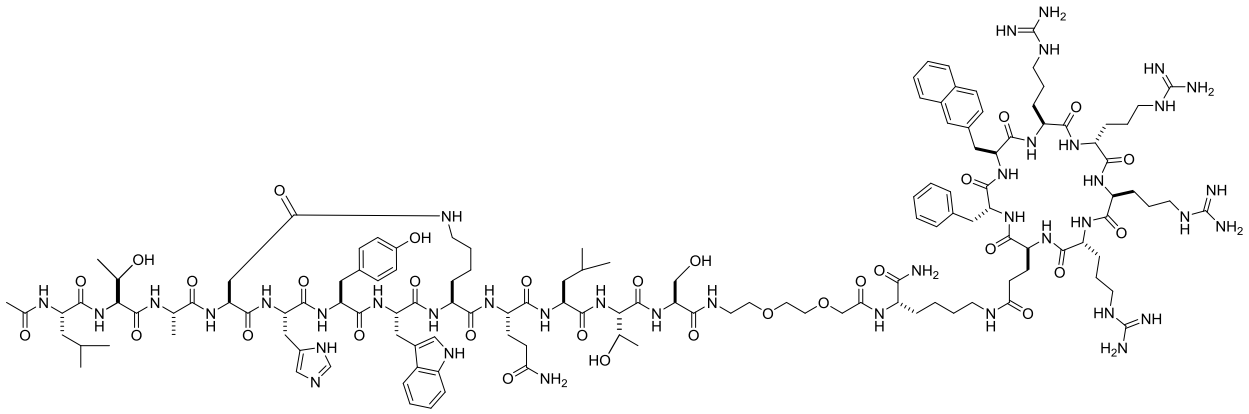

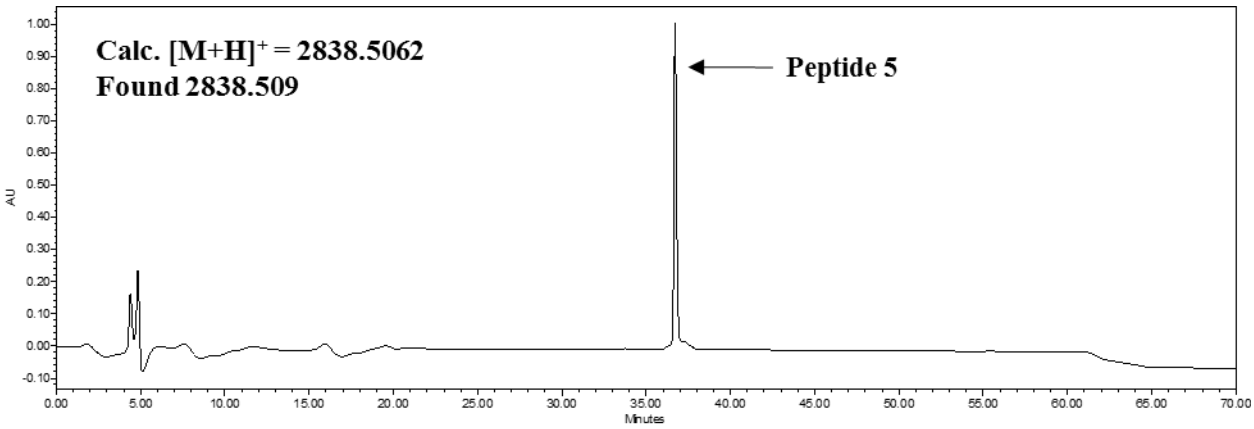

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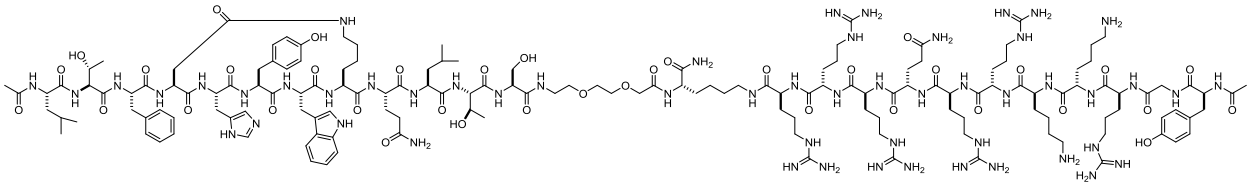

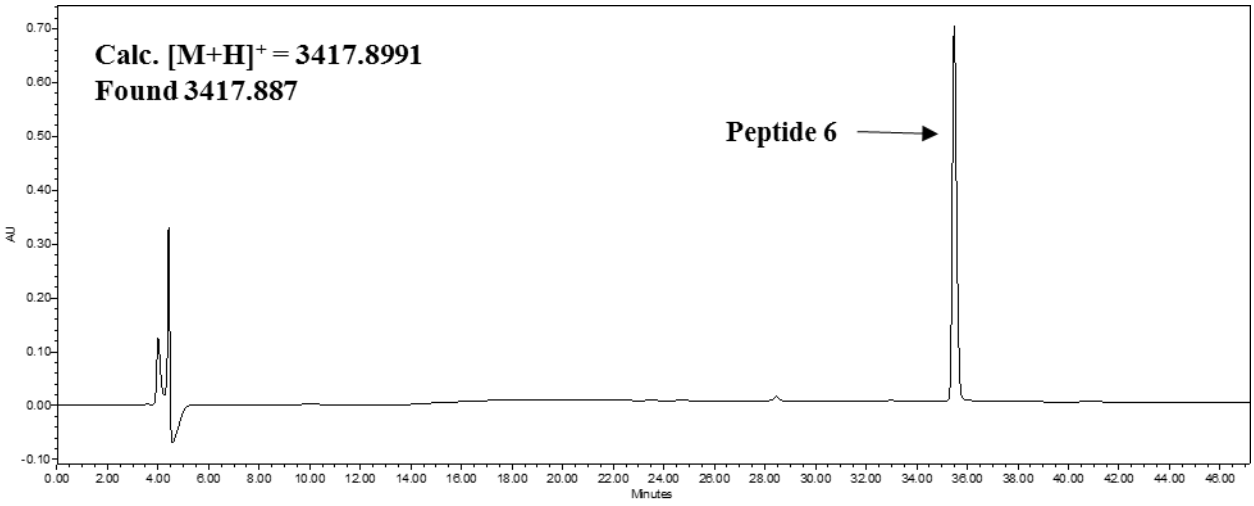

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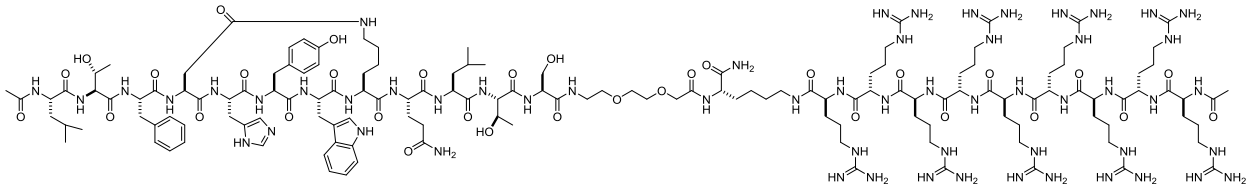

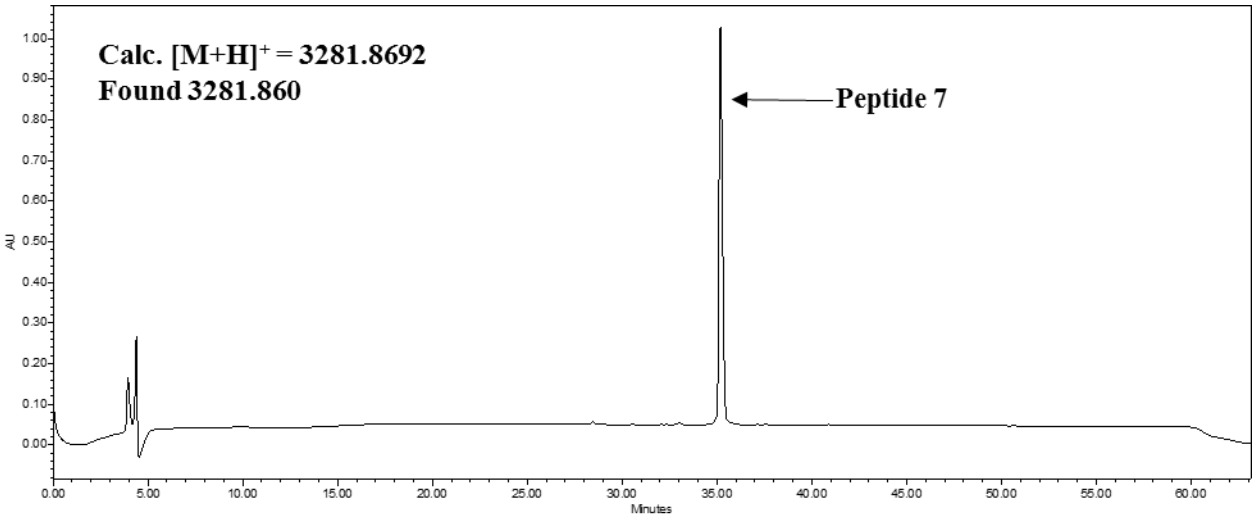

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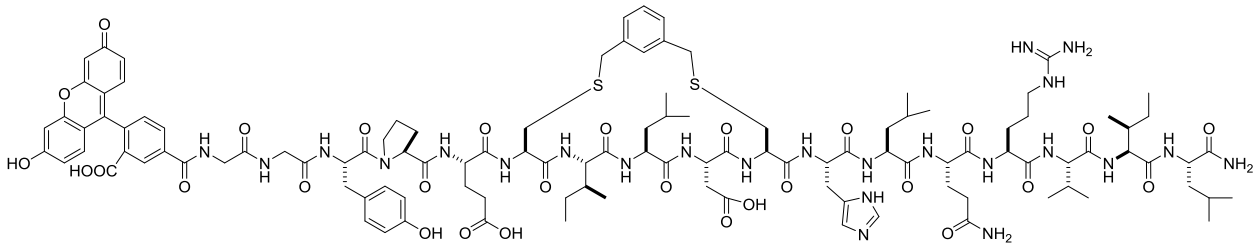

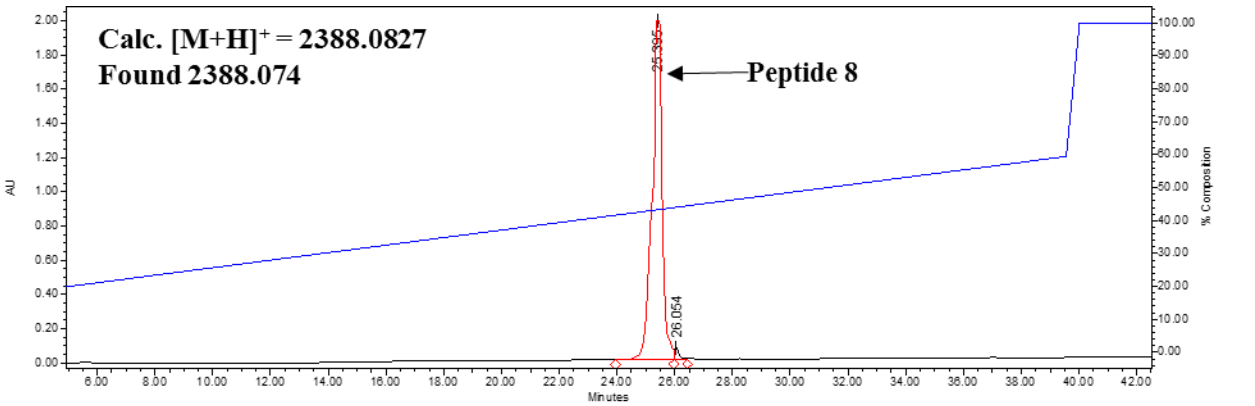

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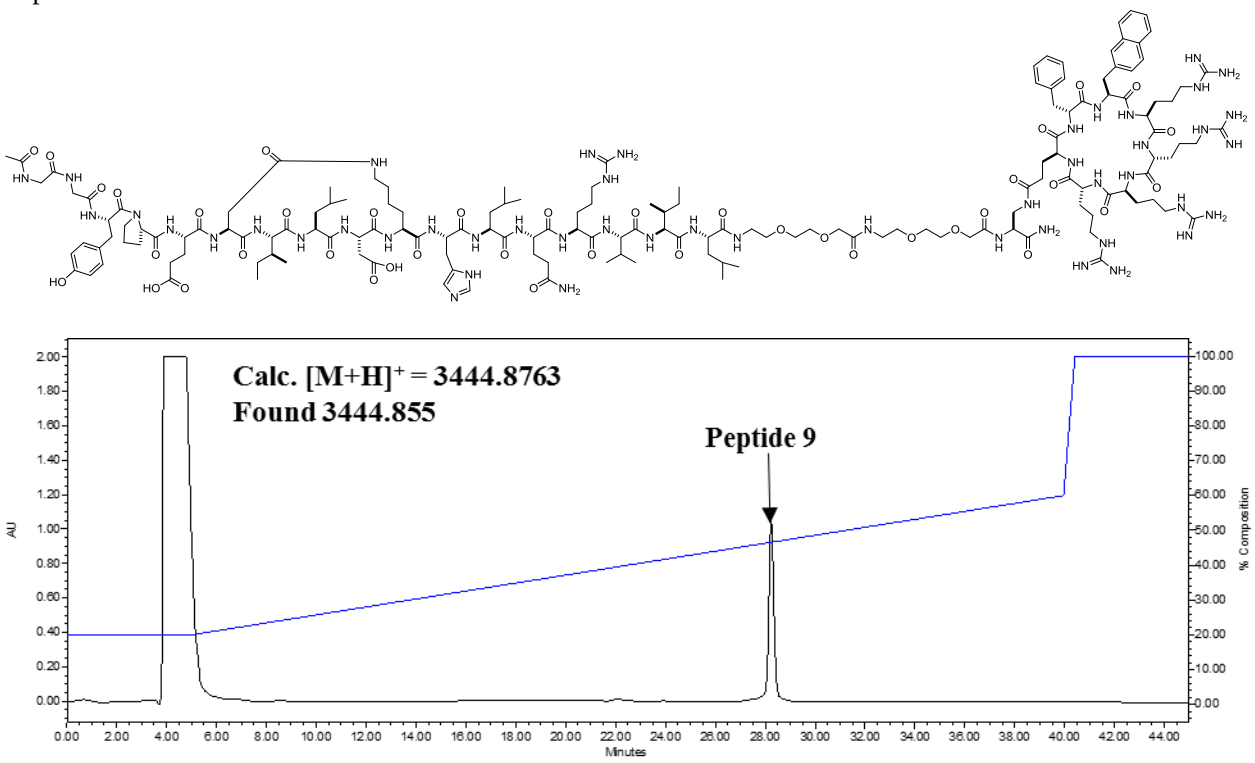

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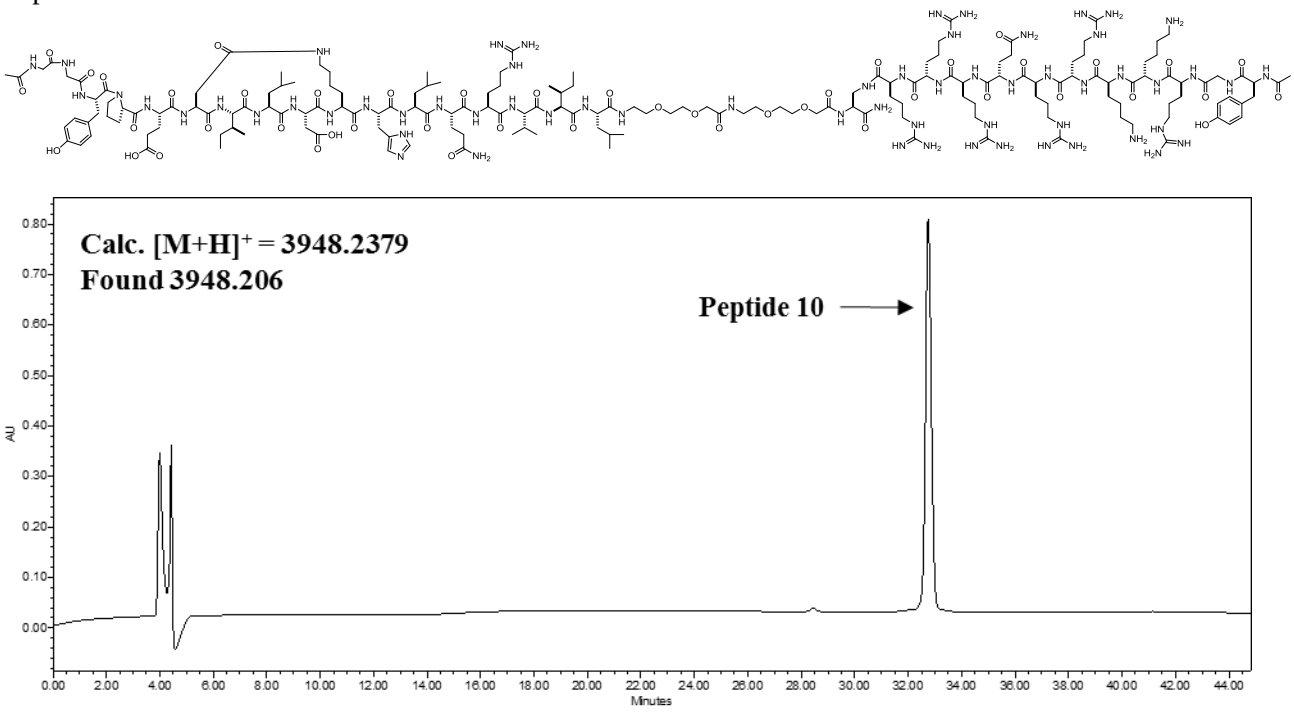

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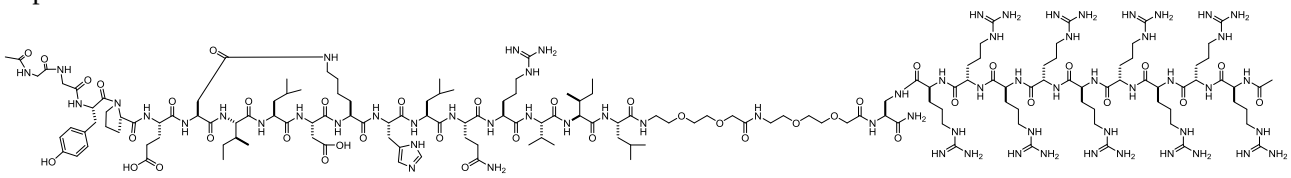

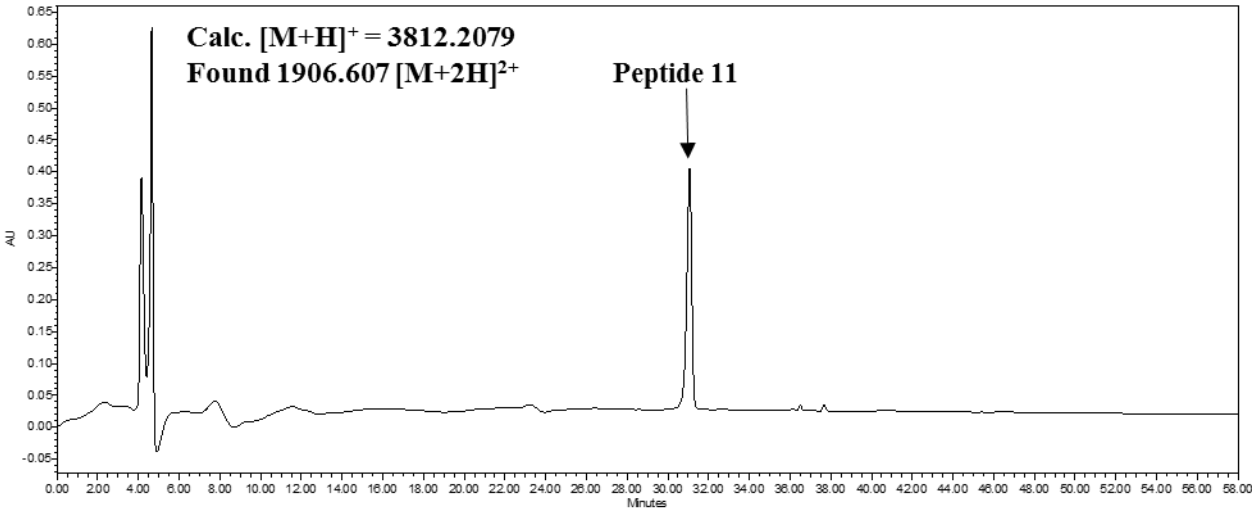

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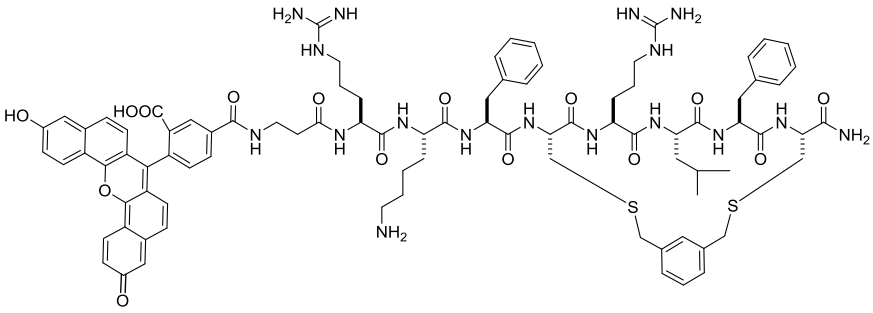

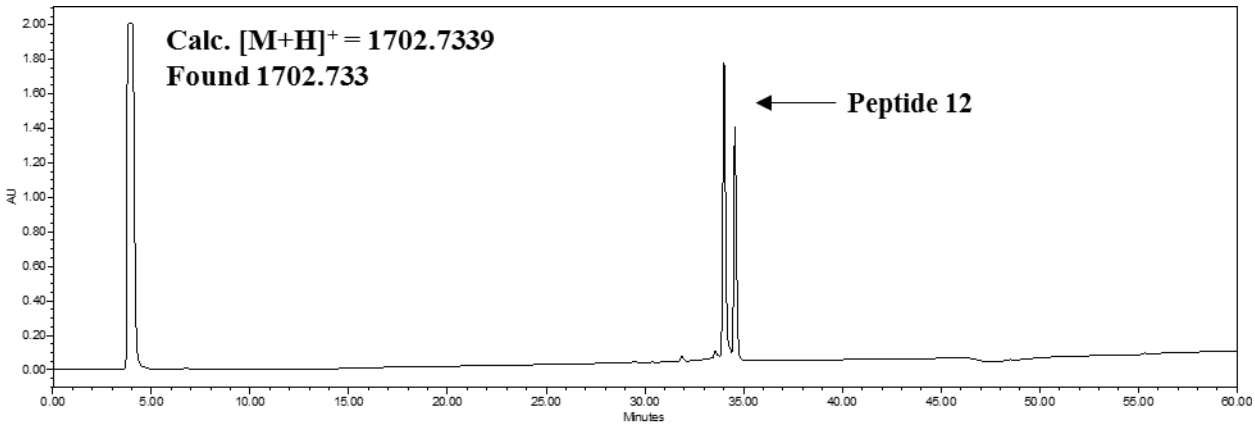

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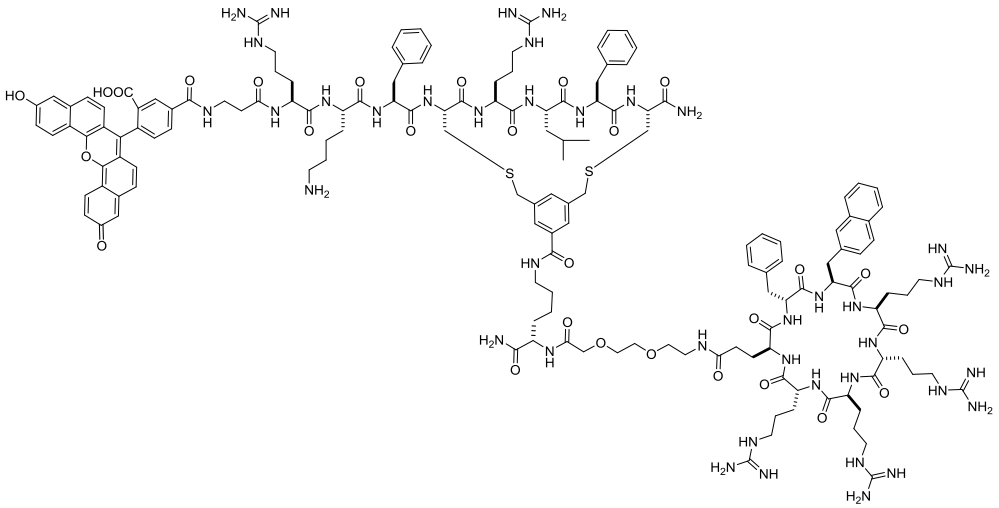

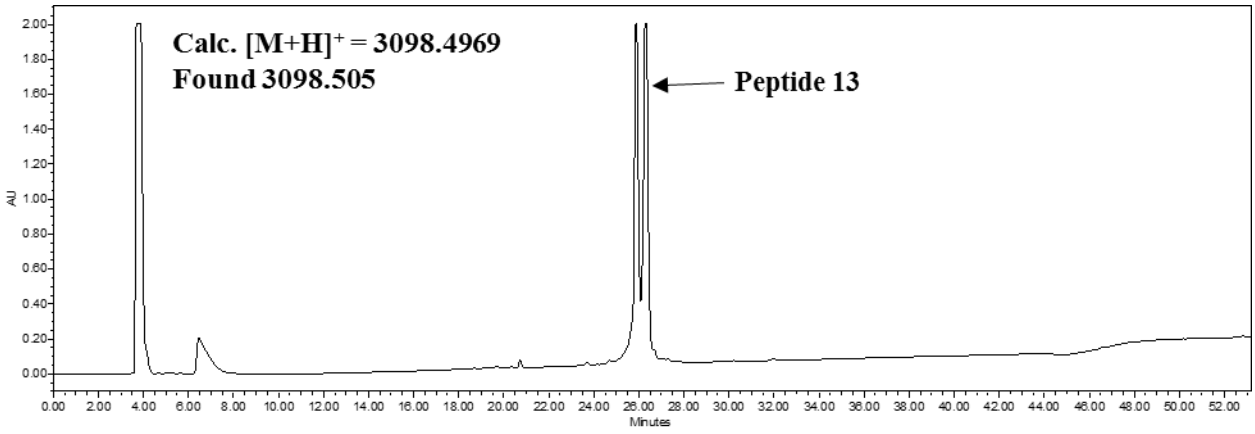

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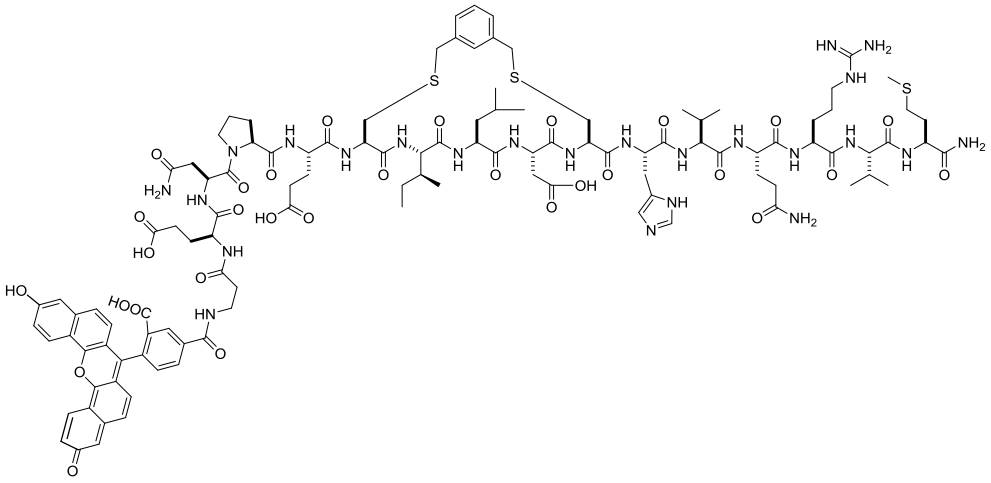
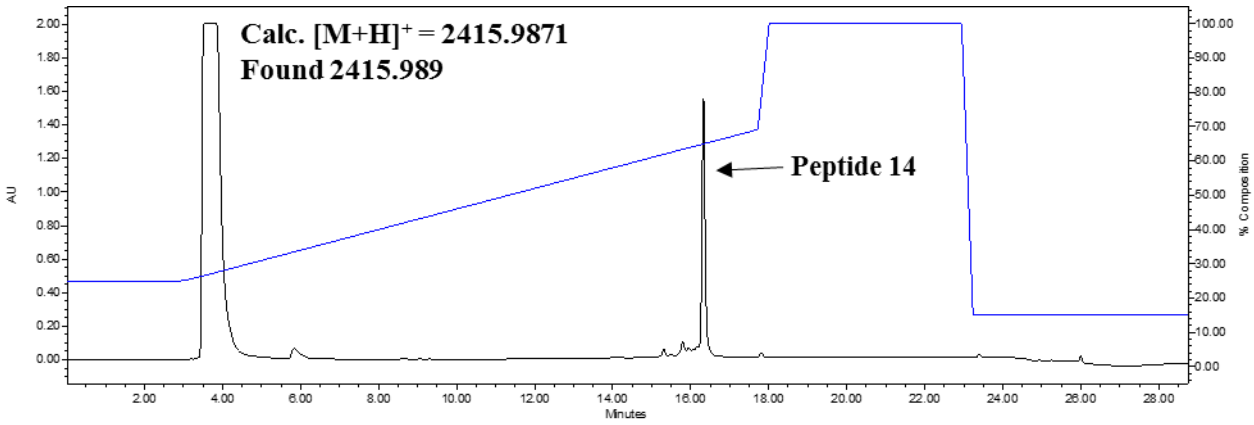
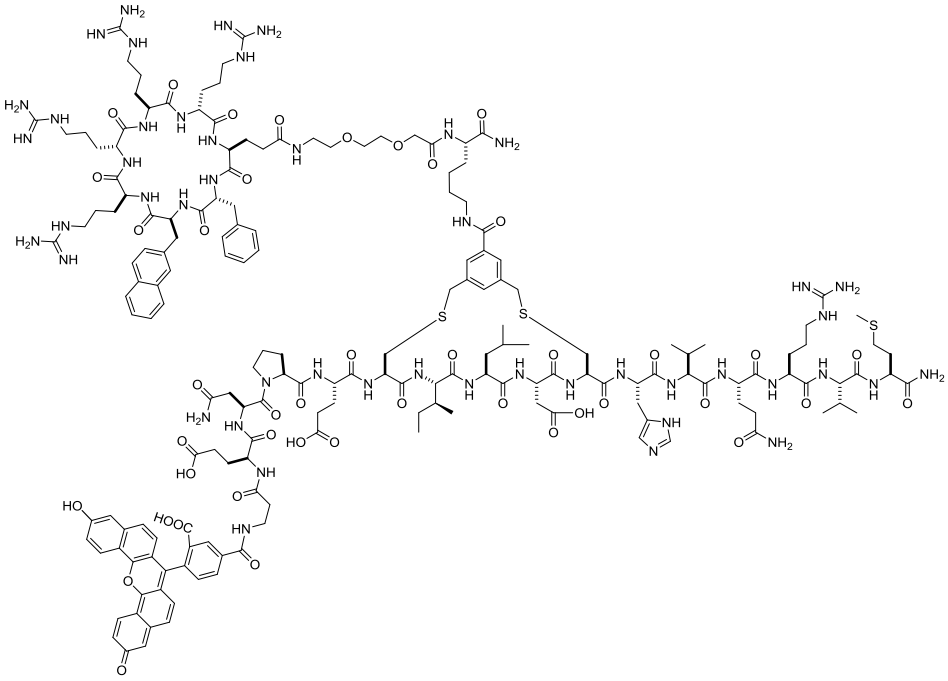
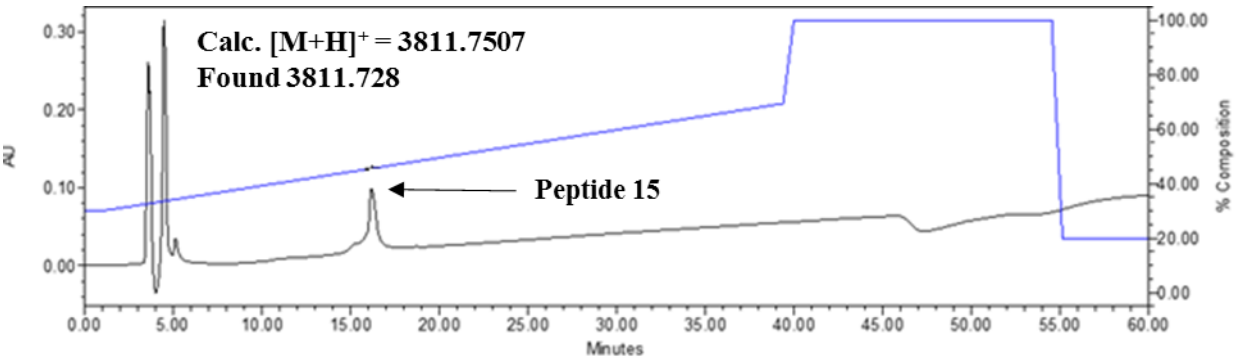



Calc. [M+H]+ = 2415.9871
Found 2415.989
Peptide 14
AU
% Composition
Minutes

Peptide **15**

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Peptide 15
AU
% Composition
Minutes

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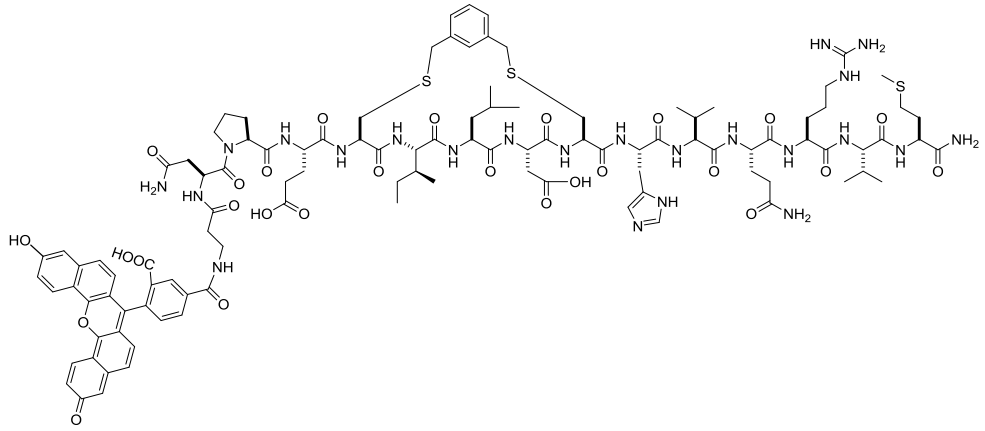

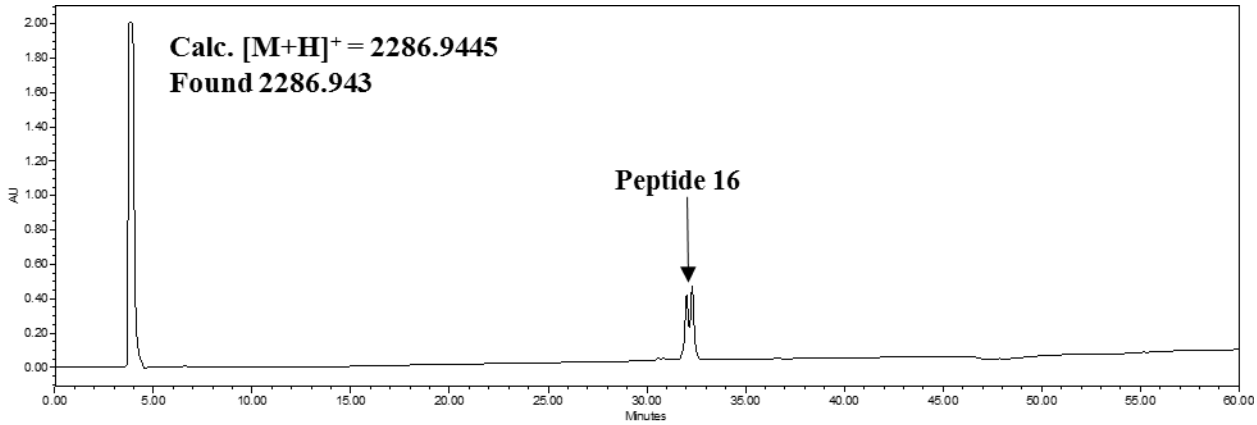

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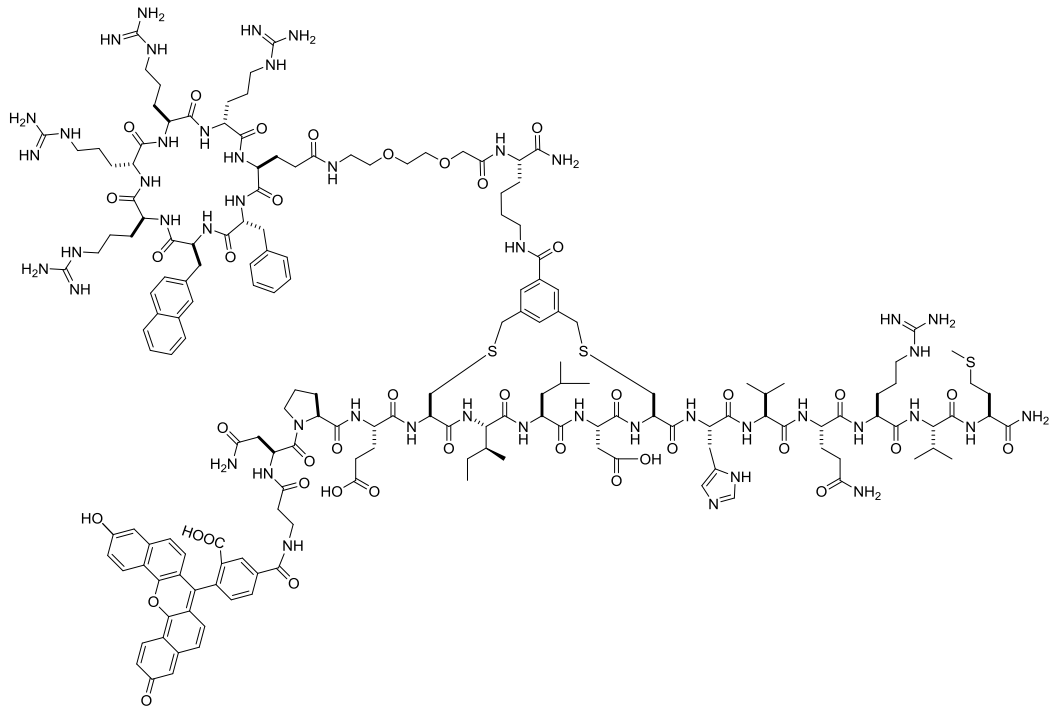

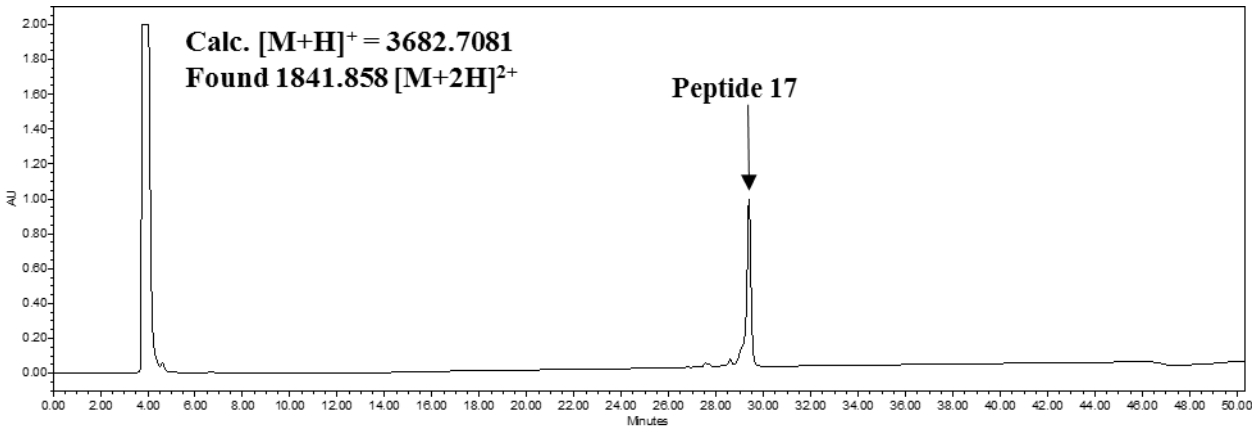
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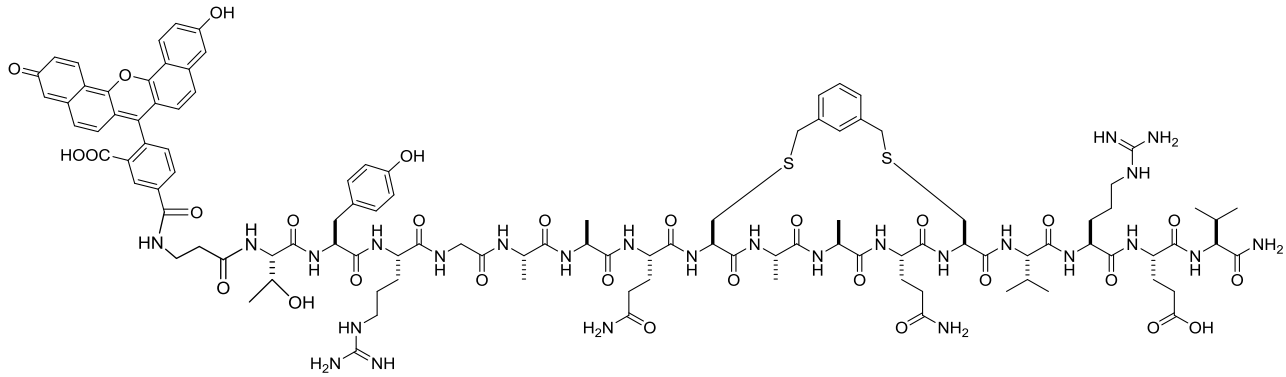
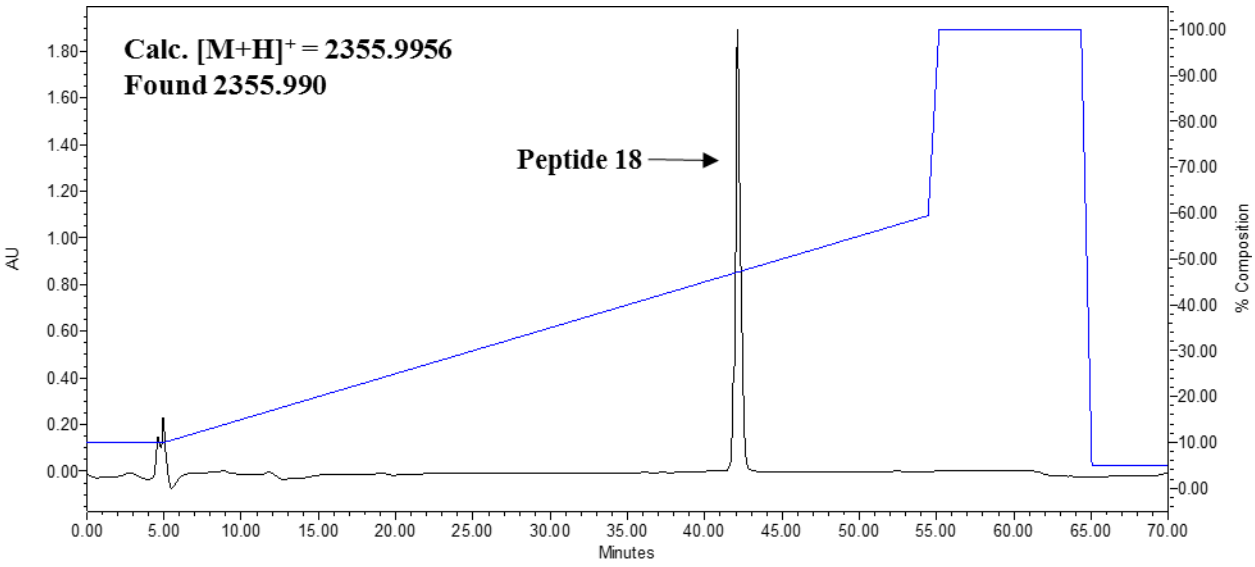
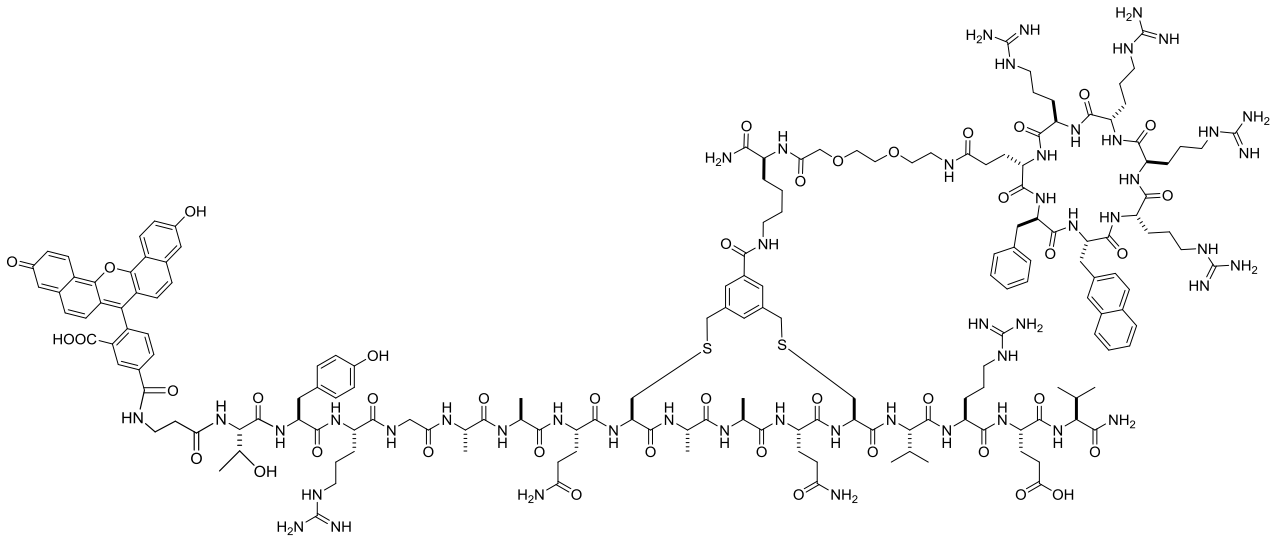
Peptide 17
AU
Minutes

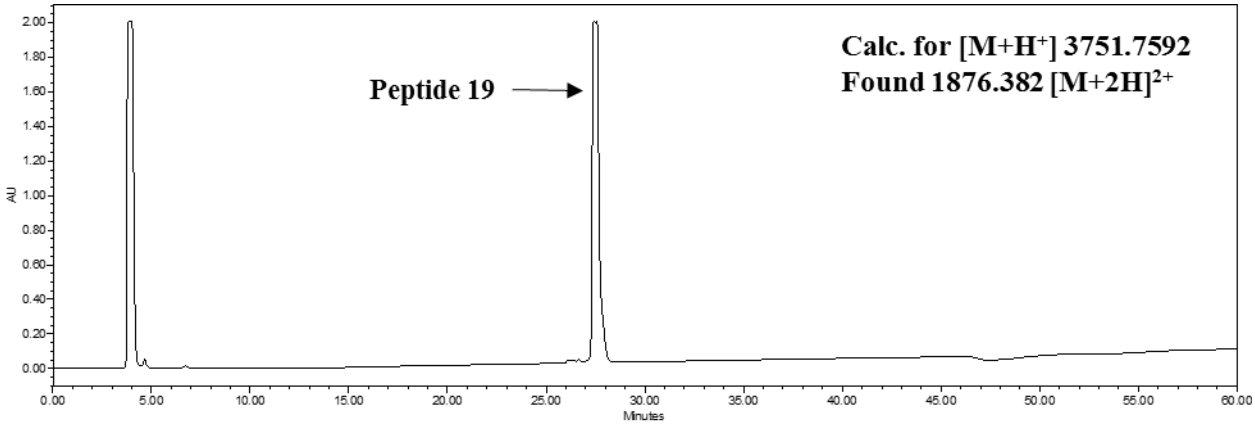
Peptide **18**




Calc. [M+H]+ = 2355.9956
Found 2355.990
Peptide 18
AU
% Composition
Minutes

Peptide **19**





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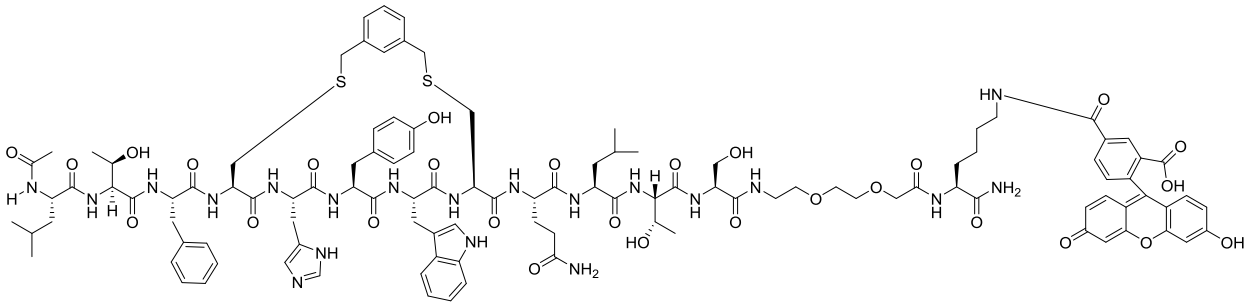

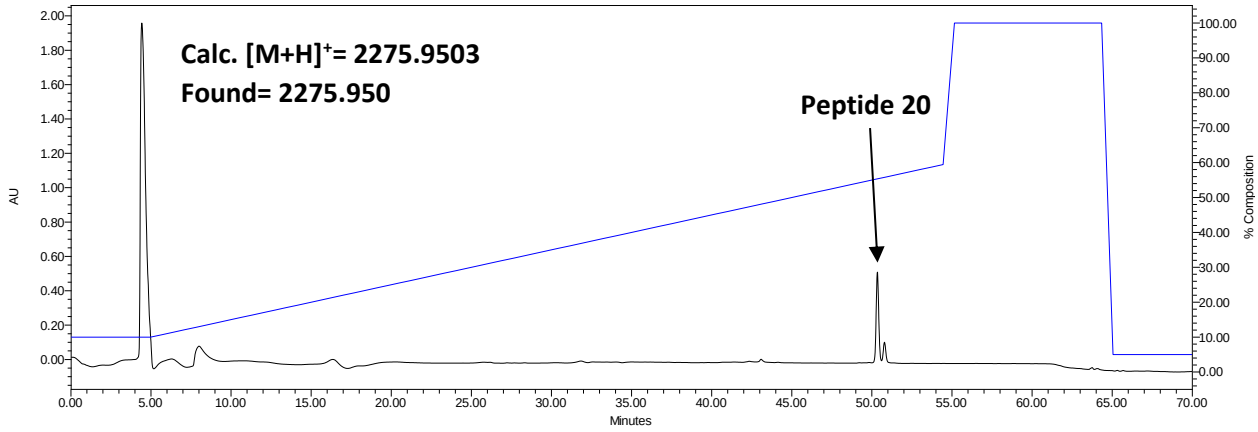
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Found= 2275.950
Peptide 20
AU
% Composition
Minutes

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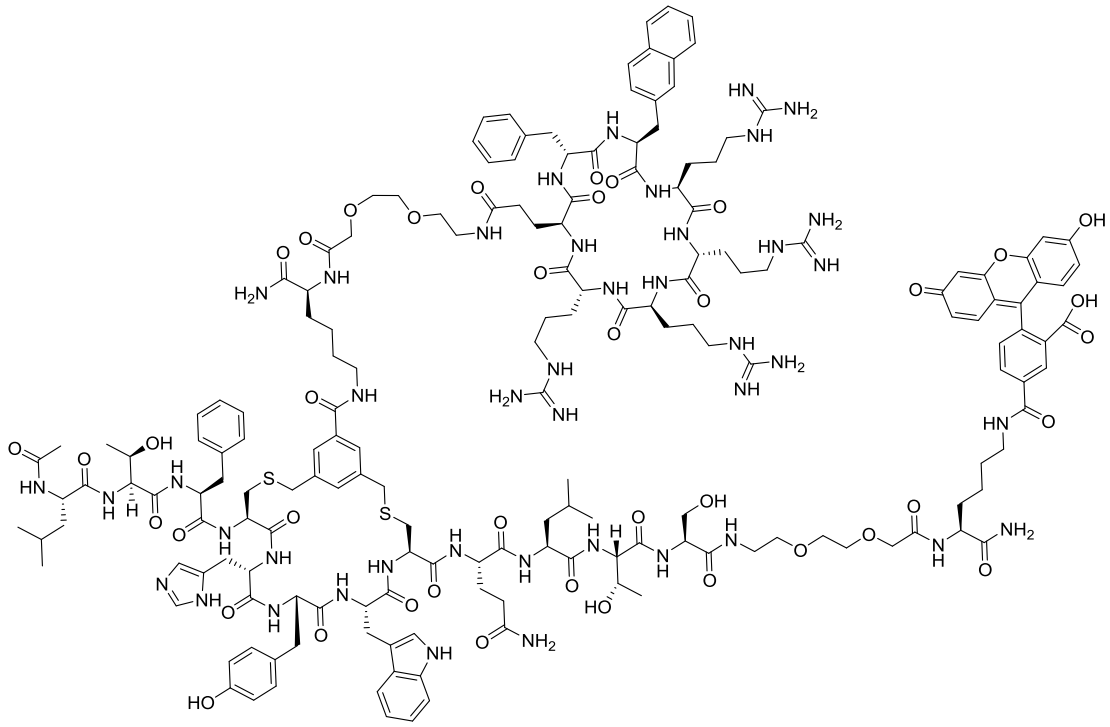

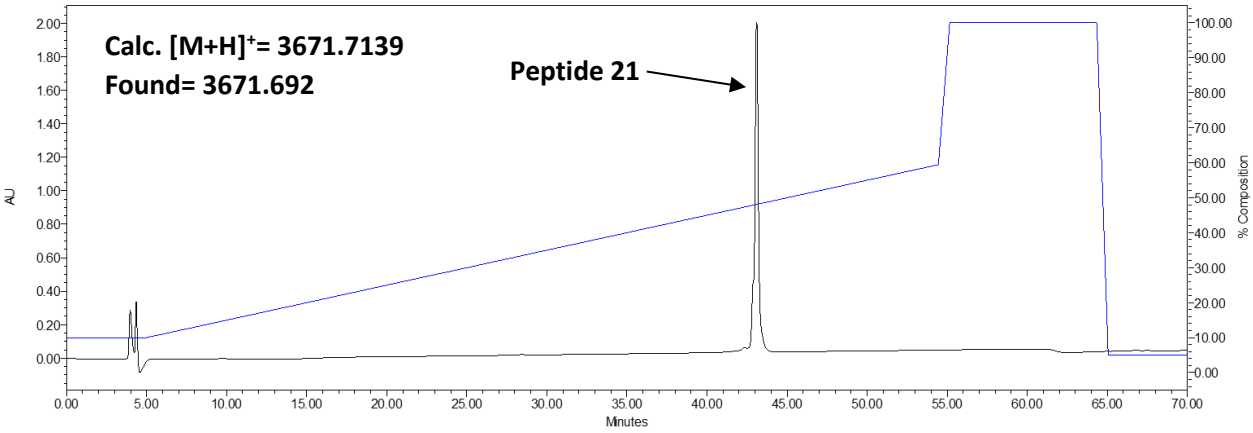
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Found= 3671.692
Peptide 21
AU
% Composition
Minutes

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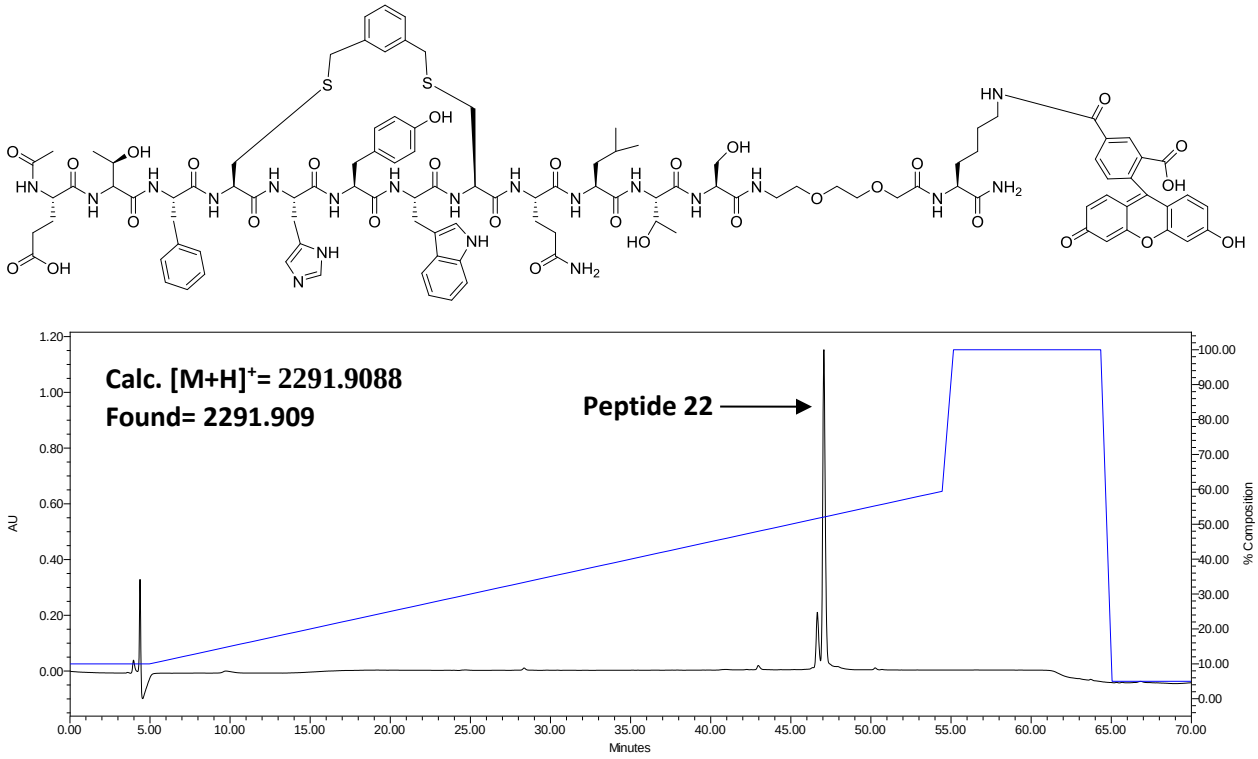

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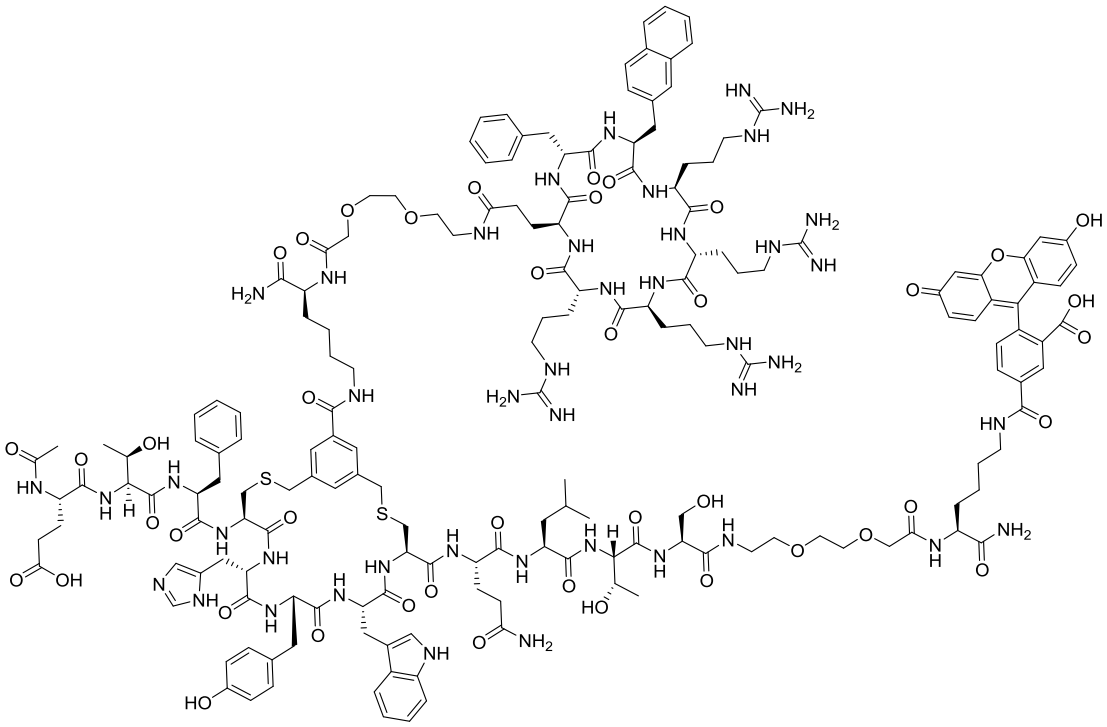

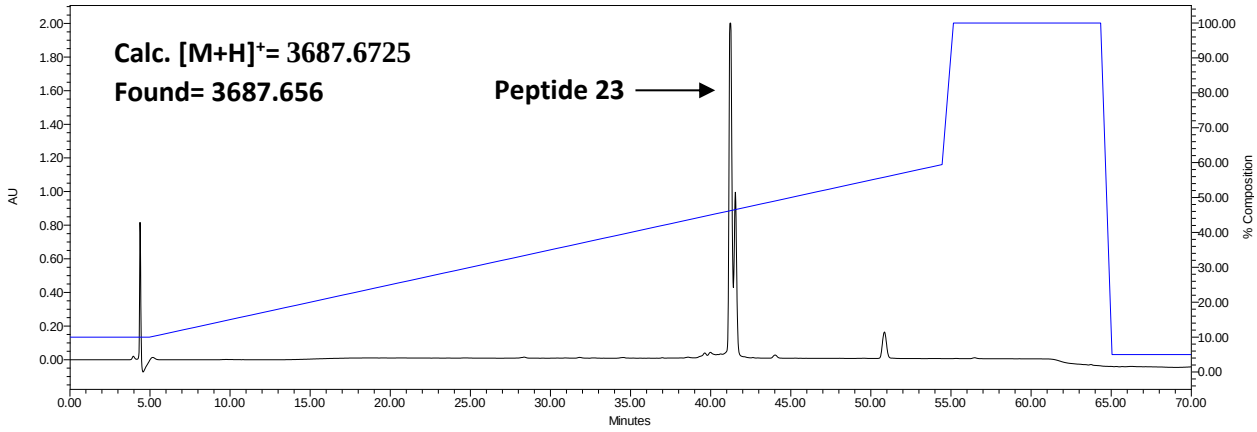
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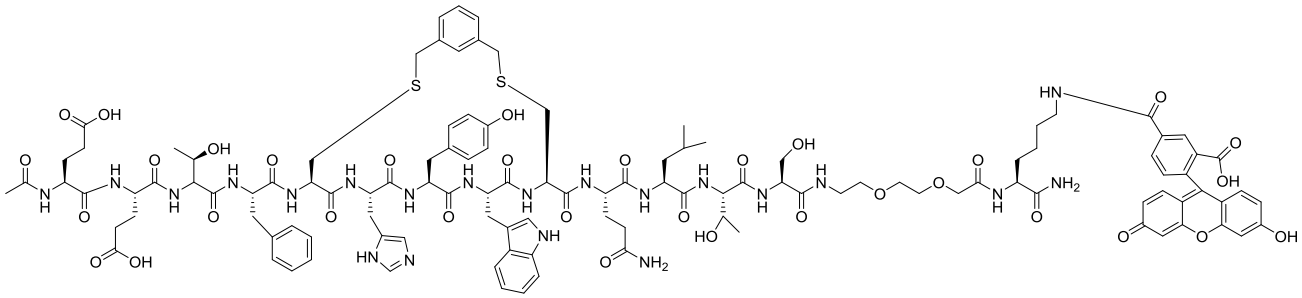
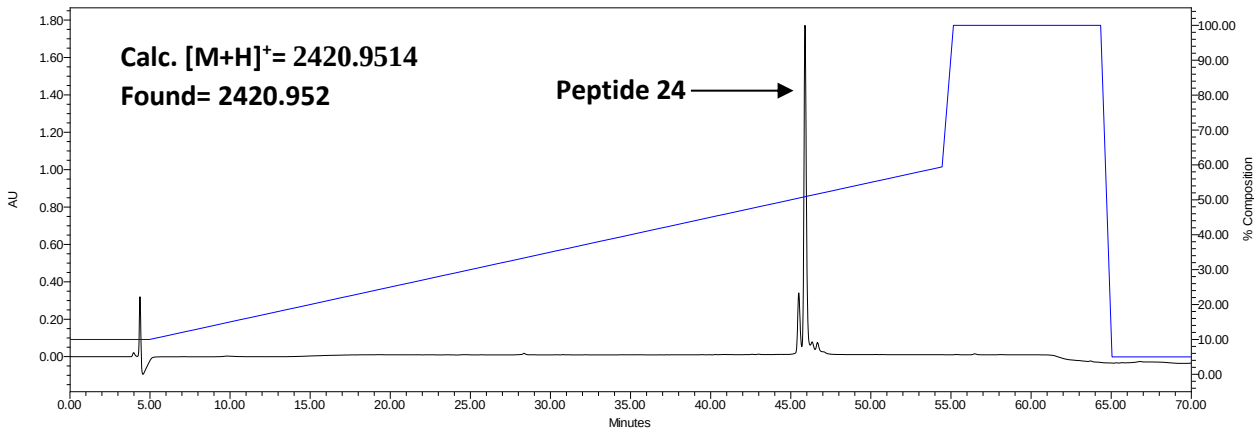
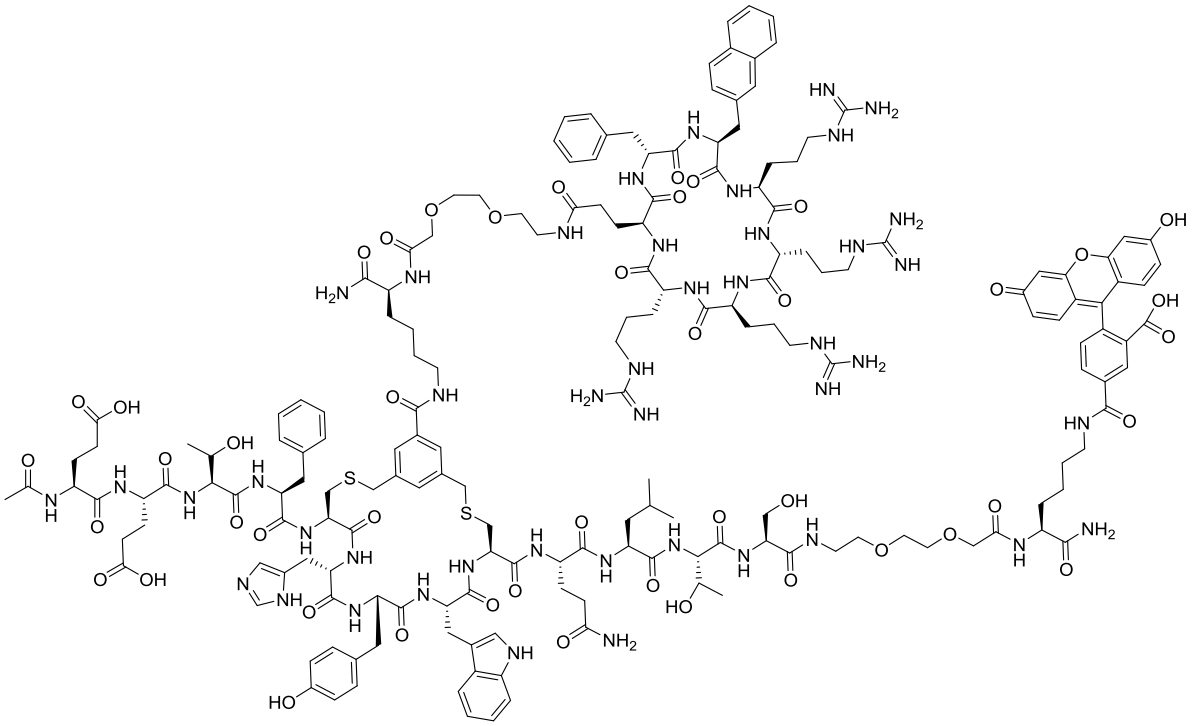
Found= 3687.656
Peptide 23
AU
% Composition
Minutes

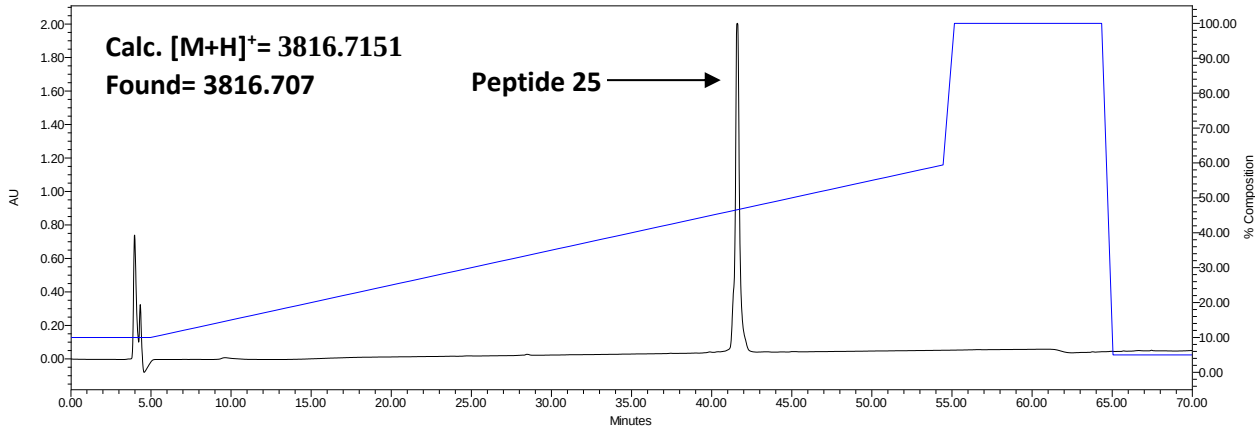
Peptide **24**




Calc. [M+H]+= 2420.9514
Found= 2420.952
Peptide 24
AU
% Composition
Minutes

Peptide **25**

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Found= 3816.707
Peptide 25
AU
% Composition
Minutes

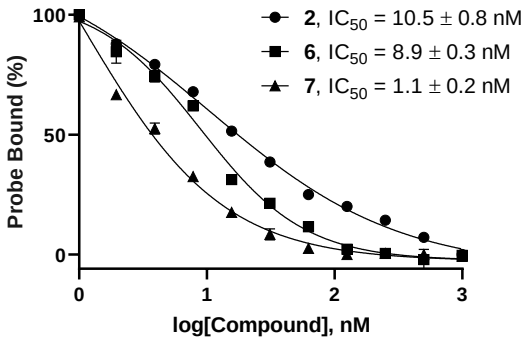

Figure S2. MDM2-binding affinity (IC_{50}) of stapled PDI conjugated to Tat or R_9 (peptides **6** and **7**) as measured by FP-base competition assay. Reaction contained 15 nM peptide **1** (probe), 15 nM GST-MDM2, and indicated concentration of peptide **2**, **6**, or **7** in PBS (pH 7.4) containing 5 mM DTT and 0.01% Triton-X100. FP values are relative to those at 0 (100%) and saturating concentration of competing peptide (0%). Data reported represent the mean ± SD of three sets of independent experiments.

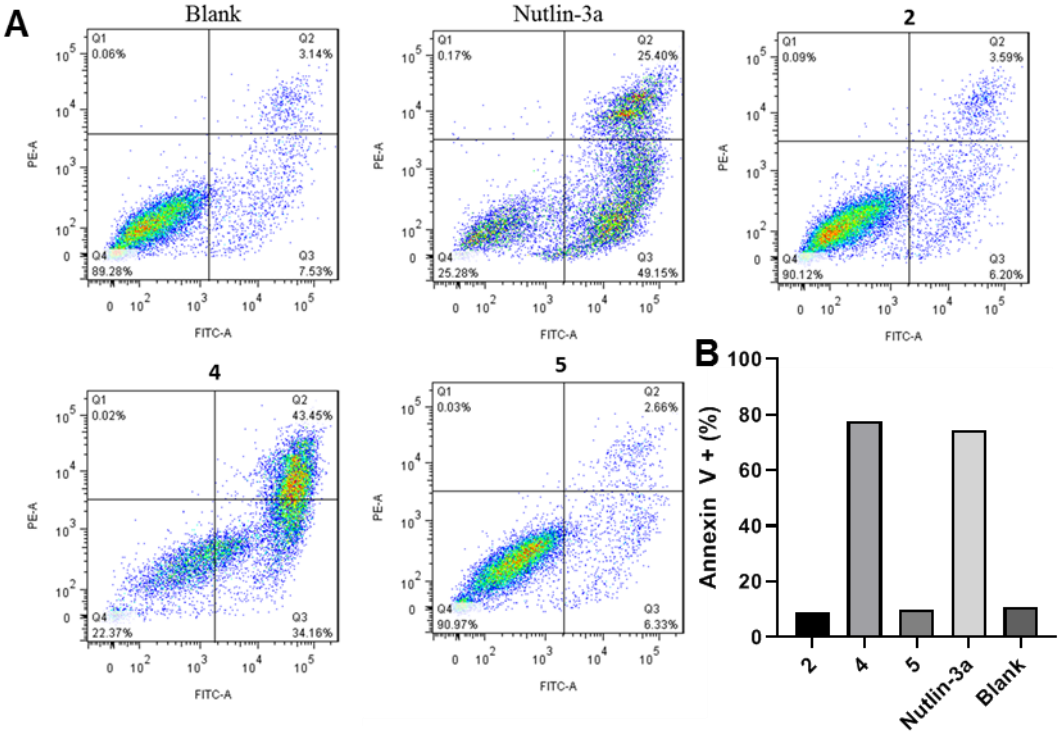

Figure S3. Apoptosis of SJSA-1 cells induced by MDM2 inhibitors. Cells were seeded in 12-well plates at a density of 1.0 x 10^5 cells/well in serum-free RPMI-1640 supplemented with 1% penicillin/streptomycin and incubated for 24 h at 37 °C in the presence of 5% CO_2. Compound was added to each well (final concentration = 10 µM for peptide **4** and Nutlin-3a or 25 µM for peptides **2** and **5**) in fresh RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were incubated for 48 h, stained with Annexin V and PI, and analyzed by flow cytometry. (A) Flow cytometry data for untreated cells (blank) and cells after treatment with nutlin-3a or peptides **2**, **4**, or **5**. (B) Percentage of apoptotic cells (populations in Q2 and Q3) after compound treatment, from (A).

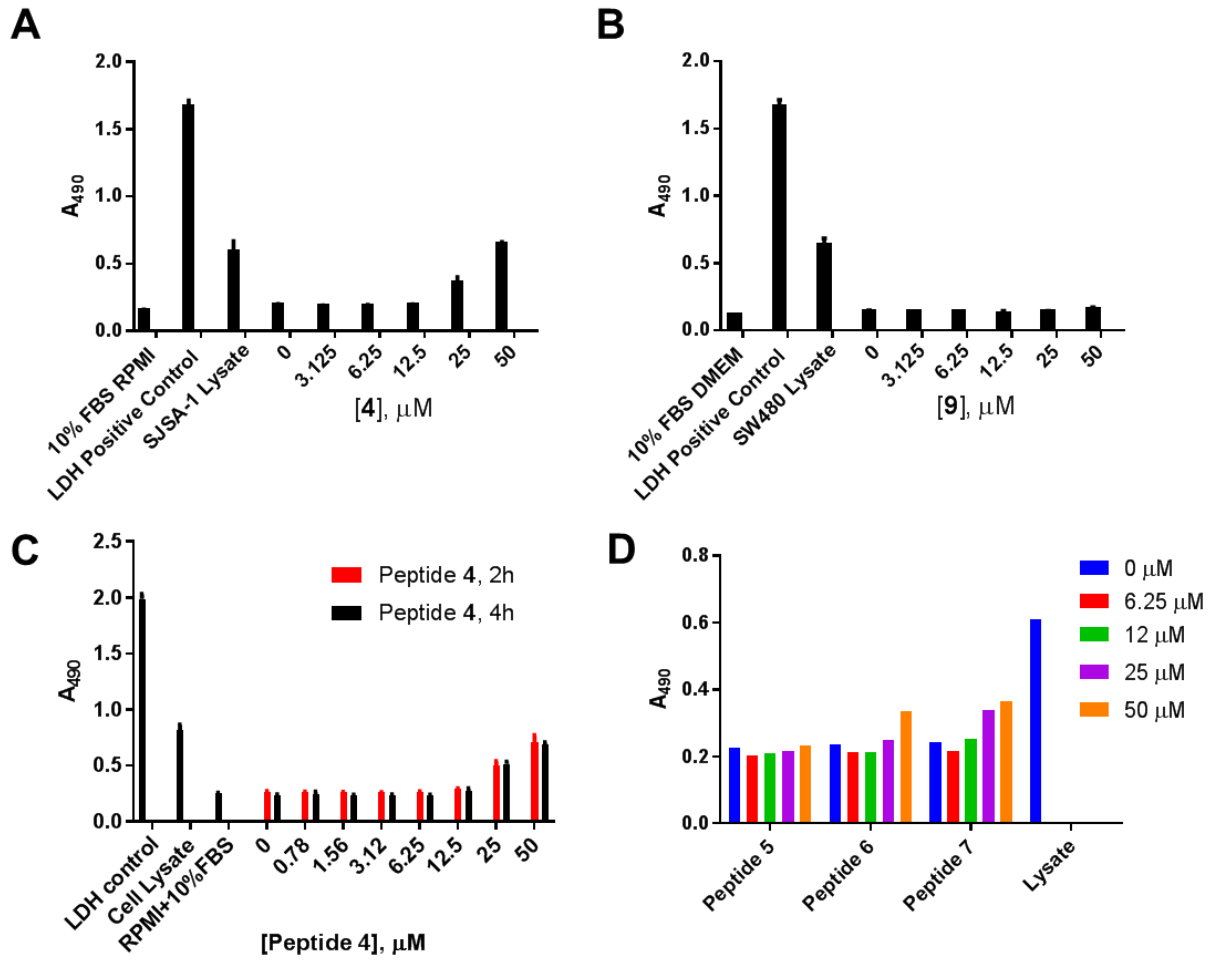

Figure S4. Lactate dehydrogenase (LDH) release caused by membrane disruption. (**A**) SJSA-1 cells treated for 45 min with 0-50 μM peptide **4.** Data shown represent the mean ± SD of three replicates from two independent experiments (n = 6). (**B**) SW480 cells treated for 45 min with 0-50 μM peptide **9**. Data shown represent the mean ± SD of three replicates from two independent experiments (n = 6). (**C**) SJSA-1 cells treated with peptide **4** for 2 or 4 h. Data shown represent the mean ± SD of three independent experiments (n = 3). (**D**) SJSA-1 cells treated for 45 min with 0-50 μM indicated peptides**.** Data shown were from a single set of experiments.

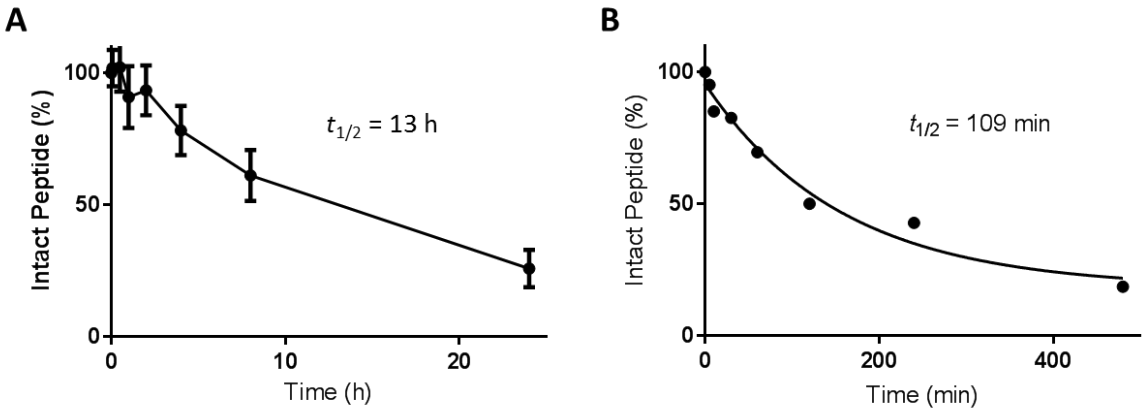

Figure S5. Stability of peptides **4** (**a**) and **9** (**b**) in human serum. Serum was diluted 1:4 in sterile DPBS (v/v) and equilibrated at 37 °C for 15 min. Peptide was added to the diluted serum to a final concentration of 100 µM and incubated at 37 °C. At the indicated time points, 100-µL aliquots were withdrawn, mixed with 100 µL of 15% trichloroacetic acid (TCA) in MeOH (w/v) and 100 µL of MeCN, and stored overnight at 4 °C. The samples were analyzed by RP-HPLC and the percentage of remaining peptide at a given time point was determined by integrating the peak area and compared it to that of the untreated control (100%). Data in (**a**) are the mean ± SD of three independent experiments, where data in (**b**) were from a single set of experiment.

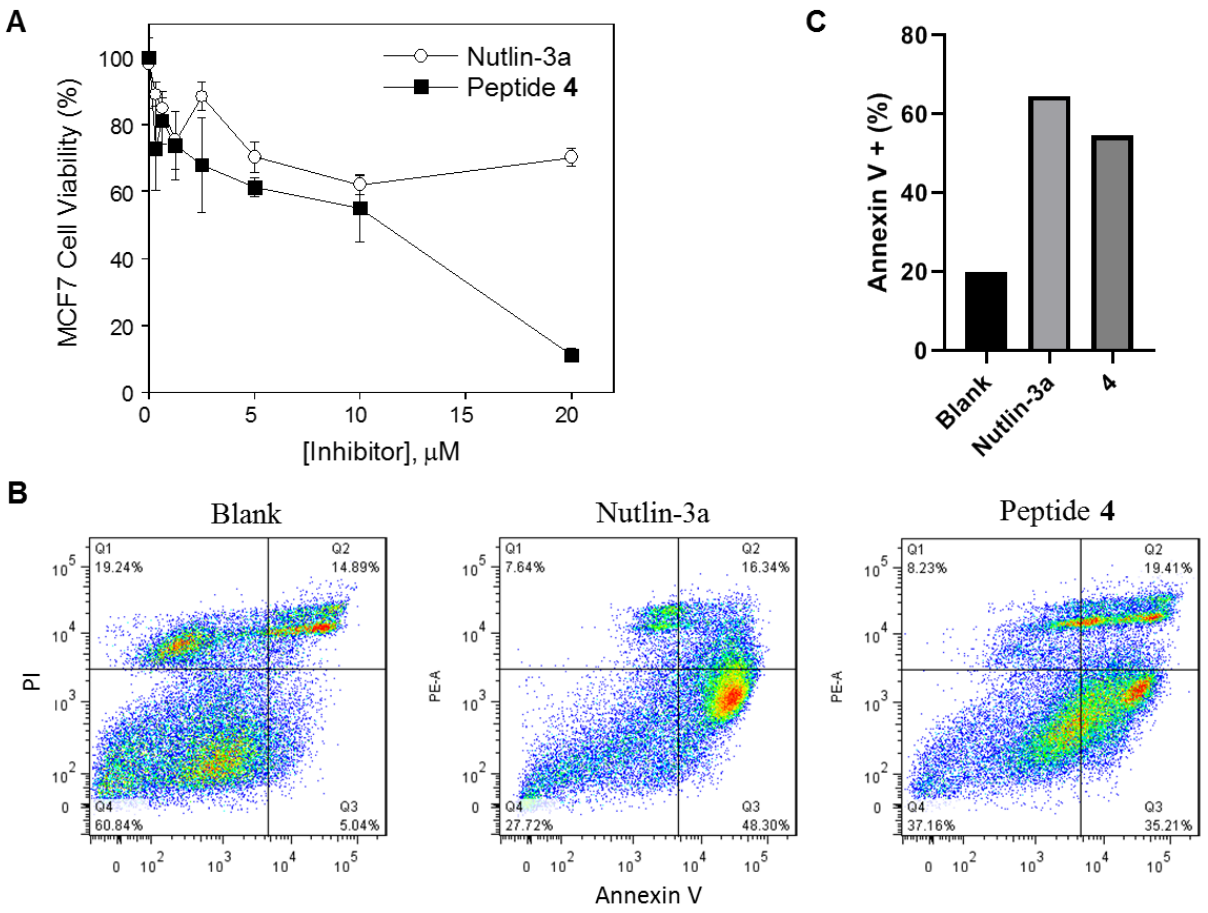

Figure S6. Induction of apoptotic death of MCF7 cells (which express WT p53 and display MDM2 and MDMX amplification) by peptide **4**. (**A**) Viability of MCF7 cells as a function of peptide **4** or nutlin-3a concentration (72 h treatment and in the presence of 10% FBS) as monitored by the MTT assay ($n \geq 3$). (**B**) Annexin V/PI staining of MCF7 cells after treatment with MDM2 inhibitors. Cells were seeded in 12-well plates at a density of 1.0 x 10^5 cells/well in MEM supplemented with 10% FBS, 1% penicillin/streptomycin and incubated for 24 h at 37 °C in the presence of 5% CO_2. Each well was washed with warm DPBS and compound (5 µM peptide **4** or Nutlin-3a) was added to each well in fresh MEM supplemented with 10% FBS, 1% penicillin/streptomycin. Cells were incubated for 48 h, stained with Annexin V and PI, and analyzed by flow cytometry. (**C**) Percentage of apoptotic cells (populations in Q2 and Q3) with and without compound treatment from (**B**).

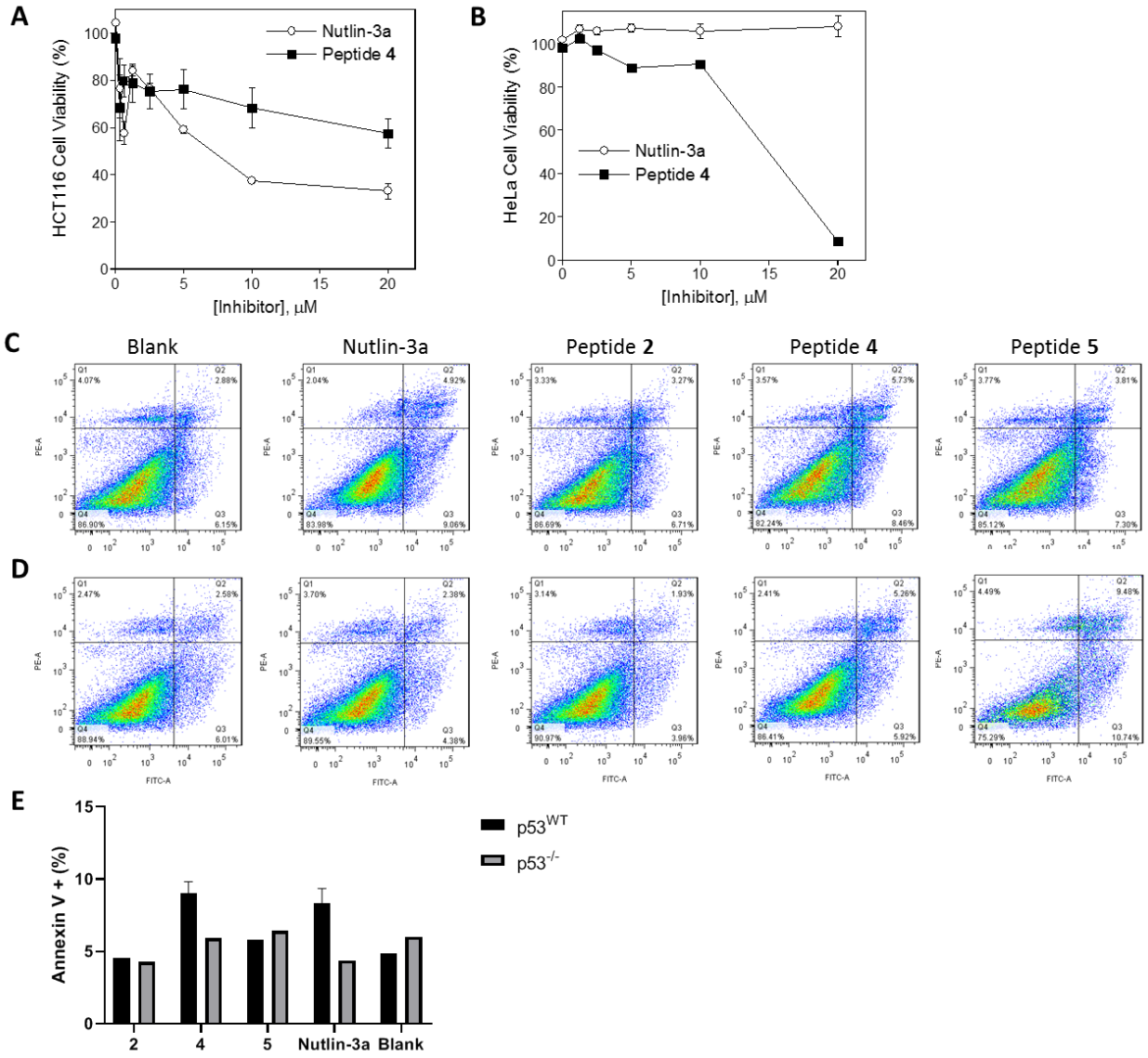

Figure S7. Effect of peptide **4** on the viability of HCT116 and HeLa cells. (**A**) Viability of HCT116 $p53^{+/+}$ cells as a function of peptide **4** or nutlin-3a concentration (72 h treatment and in the presence of 10% FBS) as monitored by the MTT assay (n = 3). (**B**) Viability of HeLa cells (which express WT p53) under the same condition as in (**A**) (n = 3). (**C, D**) Annexin V/PI staining in HCT116 $p53^{WT}$ (**C**) and $p53^{-/-}$ cells (**D**). HCT116 cells were seeded into 12-well plates at a final density of 1.0 x 10^5 cells/well in RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin and incubated for 24 h at 37 °C, 5% CO_2. Each well was washed with warm DPBS and compound (5 μM) was added to each well in fresh RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin and incubated for 48h. Cells were stained with Annexin V and PI, and analyzed by flow cytometry. (**E**) Percentages of HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells that are positive for annexin V (Q2 + Q3 populations) after treatment with 5 μM peptide **2**, **4**, **5**, or nutlin-3a in fresh RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin for 48 h. **Result**: Peptide **4** and nutlin-3a (but not peptide **2** or **5**) resulted in p53-dependent apoptosis in a smal fraction of $p53^{WT}$ cells (~5%), although HCT116 cells are less sensitive to MDM2-p53 inhibition than SJSA-1 and MCF7 cells.

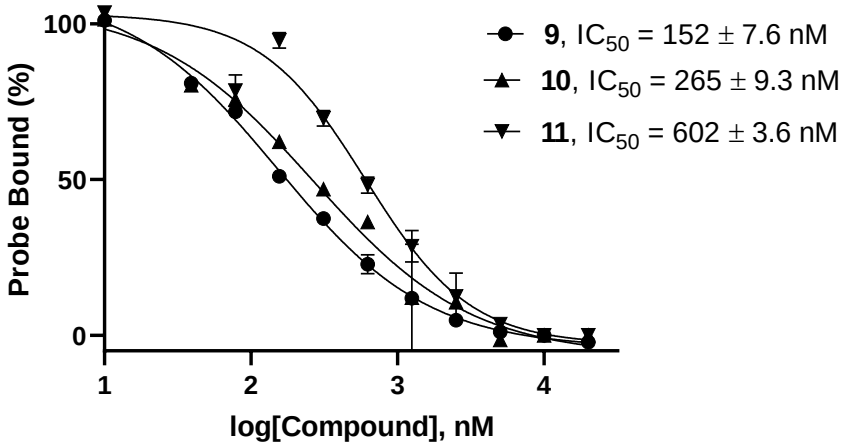

Figure S8. Competition for binding to β-catenin by peptides **9**-**11**. Reaction contained 10 nM FAM-labeled peptide **8** as probe and 50 nM GST-β-catenin in 20 mM Tris, pH 8.8, 300 mM NaCl, 0.01% Triton-X100 was pre-incubated for 1 h at RT. Serial dilutions of peptide **9**-**11** were prepared in the same buffer and mixed with the above equilibrated complex for 1 h at RT. Values are normalized to fully bound/unbound FP values for peptide **9**-FITC. Data shown represent the mean ± SD of three independent experiments.

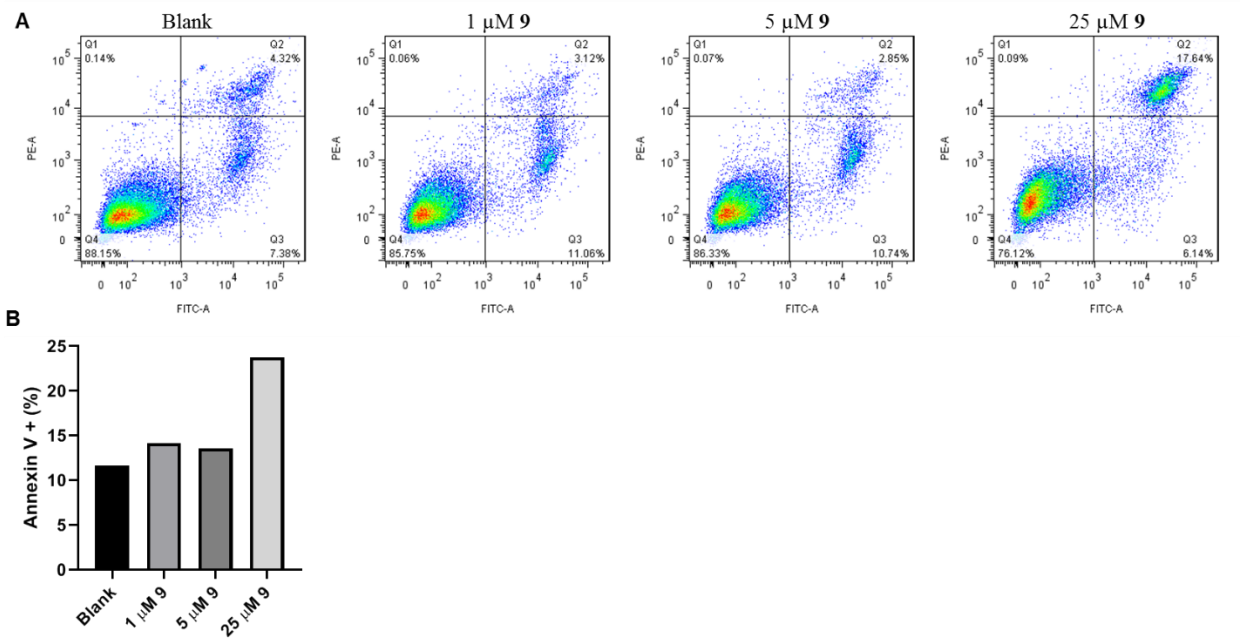

Figure S9. Annexin V/PI staining of SW480 cells after treatment with increasing concentrations of peptide **9**. Cells were seeded into 12-well plates at a final density of 1.0 x 10^5 cells/well in RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin and incubated for 24 h at 37 °C in the presence of 5% CO_2. The cells were washed with warm DPBS and treated with 0-25 µM peptide in fresh RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin for 48 h. Annexin V/PI staining was performed as described above. (**A**) Flow cytometry data for untreated cells (blank) and cells after treatment with 1, 5, or 25 µM peptide **9**. (**B**) Percentage of apoptotic cells (populations in Q2 and Q3) with and without compound treatment from (**A**).

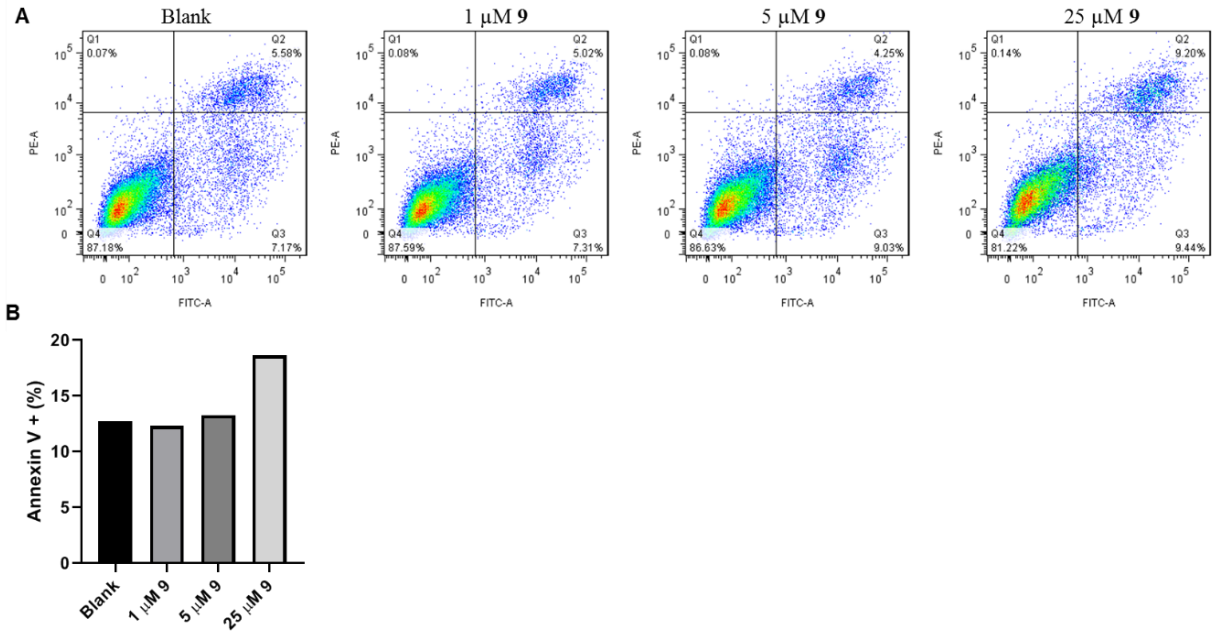

Figure S10. Annexin V/PI staining of DLD-1 cells after treatment with increasing concentrations of peptide **9**. Cells were seeded into 12-well plates at a final density of 1.0 x 10^5 cells/well in RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin and incubated for 24 h at 37 °C in the presence of 5% CO_2. The cells were washed with warm DPBS and treated with 0-25 µM peptide in fresh RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin for 48 h. Annexin V/PI staining was performed as described above. (**A**) Flow cytometry data for untreated cells (blank) and cells after treatment with 1, 5, or 25 µM peptide **9**. (**B**) Percentage of apoptotic cells (populations in Q2 and Q3) with and without compound treatment from (**A**).

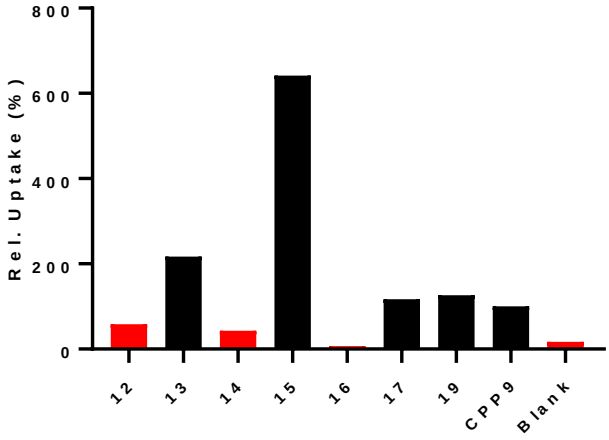

Figure S11. Comparison of the cytosolic entry efficiencies of unconjugated (peptides **12**, **14**, and **16**) and CPP9-conjugated peptides (peptides **13**, **15**, **17**, and **19**) as analyzed by flow cytometry at pH 6.5. HeLa cells (1.5 x 10^5 cells/well) were incubated with 0 (blank) or 5 µM NF-labeled peptide for 2 h in the presence of 10% FBS. Cells were harvested and washed. Immediately before flow cytometry analysis, the pH of the cell suspension was lowered to 6.5 by the addition of 0.2 M glycine-HCl (pH 2.0) to quench the fluorescence of any cell surface-associated peptide. All values are relative to that of CPP9 (100%).