Supporting Information

Materials and General Methods. Reagents for peptide synthesis and Rink amide resin (100-200 mesh, 0.54 mmol/g) were purchased from Chem-Impex (Wood Dale, IL). Unless noted otherwise, the purity of peptides was assessed by reversed-phase analytical HPLC equipped with a C18 column and the identity was confirmed by MALDI-TOF mass spectrometric analyses on a Bruker UltrafleXtreme MALTI-TOF-TOF instrument (Campus Chemical Instrument Center, The Ohio State University). The purity and MS data are presented in Table S1 and Figure S3.

Synthesis of Cyclic Peptides 1, 6, CPP1, CPP9, and CPP12. Peptides were manually synthesized on 100 mg of Rink amide resin (0.54 mmol/g) by standard Fmoc chemistry. For each cycle, the resin was swollen in DMF for 20 min and Fmoc group was then removed by treatment with 20% piperidine in DMF for 5 min (twice). The resin was washed with DMF/DCM/DMF (3 times) and the next amino acid was coupled in standard Fmoc/HATU chemistry by using Fmoc-amino acid/HATU/DIPAE (4, 4, and 8 equiv) in DMF (2 h at RT). The coupling reactions were monitored by ninhydrin test after each residue. After completion of the linear sequence, the allyl group on the α-carboxyl group of the C-terminal L-Glu was removed by treatment with tetrakis(triphenylphosphine)palladium/phenylsilane (0.3 and 10 equiv, respectively) in DCM for 15 min (3 times). The resin was washed twice with sodium dimethyldithiocarbamate dihydrate (SDDNa, 0.5 M in DMF) and the N-terminal Fmoc group was removed by treatment with 20% piperidine. The resin was washed with DMF, DCM, and DMF (3 times each) and incubated with 1 M HOBt for 5 min. The peptide was cyclized on the resin by treatment with PyBOP/HOBt/DIPEA (5, 5, and 10 equiv, respectively) for 2 h (twice). The resin was then washed with DMF and DCM (3 times each). The cyclic peptide was released from the resin and deprotected by treating the resin with 92.5% TFA, 2.5% triisopropylsilane (TIPS), 2.5% H_2O and 2.5% dimethoxybenzene (DMB) for 3 h. After evaporation of the solvents, the crude peptides were triturated with cold diethylether 3 times and purified by reversed-phase HPLC on a semi-preparative C18 column. For labeling with naphthofluorescein, the purified peptide (~1 mg) was dissolved in 25 μL of DMF and the pH was adjusted to ~8 with the addition of 1 M $NaHCO_3$, and 1 mg of naphthofluorescein O-succinimidyl ester (NF-OSu) in 25 μL of DMF was added. The mixture was incubated for 2 h at RT. The labeled peptide was purified again by reversed-phase HPLC on a C18 column.

Synthesis of Peptides 2-5, 8, 9-16. Synthesis was carried out as described above, but with the following modifications. After the coupling of Fmoc-L-Dap(Mtt)-OH [or Fmoc-L-Lys(Mtt)-OH, Fmoc-L-Orn(Mtt)-OH], the Mtt group was removed by treatment with 2% TFA and 2% triisopropylsilane in DCM (6 x 5 min). The exposed side-chain amino group was amidated by treatment with decanoic acid/HATU/DIPEA (4, 4, 8 equiv) in DMF (2 h at RT). The resin was washed with DCM and DMF (3 times each).

Synthesis of Peptide 7. Synthesis was carried out as described above, but with the following modifications. After the coupling of Fmoc-L-Dap(Mtt)-OH, the resin was washed with DCM and DMF (3 times each). The allyl group on the α-carboxyl group of the C-terminal L-Glu was removed by treatment with tetrakis(triphenylphosphine)palladium/phenylsilane (0.3 and 10 equiv, respectively) in DCM for 15 min (3 times). The resin was washed twice with sodium dimethyldithiocarbamate dihydrate (SDDNa, 0.5 M in DMF) and the N-terminal Fmoc group was

removed by treatment with 20% piperidine. The resin was washed with DMF, DCM, and DMF (3 times each) and incubated with 1 M HOBt for 5 min. The peptide was cyclized on the resin by treatment with PyBOP/HOBt/DIPEA (5, 5, and 10 equiv, respectively) for 2 h (twice). The resin was washed with DMF and DCM (3 times each). The Mtt group (on Dap) was removed by treatment with 2% TFA and 2% triisopropylsilane in DCM (6 x 5 min). The exposed side-chain amino group was then reacted with Fmoc-OSu (4 equiv) in DMF (2 h at RT).

Synthesis of Peptide 17. Synthesis was carried out as described above, but with the following modifications. After coupling of Fmoc-L-Asp(2-PhiPr)-OH (where 2-PhiPr is 2-phenylisopropyl), the 2-PhiPr group was removed by treatment with 2% TFA and 2% triisopropylsilane in DCM (6 x 5 min). The exposed side-chain carboxyl group was then amidated by treatment with decylamine/PyBOP/HOBt/DIPEA (1, 5, 5, and 10 equiv) twice in DMF solution. The resin was washed with DCM and DMF (3 times each).

Synthesis of FITC-Labeled Peptide 17. The HPLC purified peptide (~1 mg) was dissolved in 25 μL of DMF and the pH was adjusted to ~8 with the addition of 1 M $NaHCO_3$, and 1 mg of FITC in 25 μL of DMF was added. The mixture was incubated for 2 h at RT. The labeled peptide was purified again by reversed-phase HPLC on a C18 column.

Flow Cytometry. HeLa cells were seeded in 12-well plates at a density 1.5 x 10^5 cells per well overnight. Next day, 5 μM NF-labeled peptide was added in DMEM media supplemented with 1% or 10% FBS and 1% penicillin/streptomycin sulfate for 2 h. After 2 h, the media was removed, and the cells were washed with cold DPBS and harvested by incubating with 0.25% trypsin for 5 min. The detached cells were washed with DPBS, suspended in DPBS, and analyzed by flow cytometry (BD FACS Aria III), with excitation at 633 nm.

Confocal Microscopy. One mL of HeLa cell suspension (~5 x 10^4 cells) was seeded in a 35-mm glass-bottomed microwell dish (MatTek) and cultured overnight. For end-stage imaging, cells were gently washed with DPBS twice and treated for 2 h with FITC-labelled peptides (5 μM) in phenol-red free, HEPES supplemented DMEM containing 1% FBS. After removal of the medium, the cells were gently washed with DPBS twice and imaged on a Nikon A1R live-cell confocal equipped with 100X oil objective or a Visitech Infinity 3 Hawk 2D-array live cell confocal microscope equipped with 60X oil objective. Data were analyzed using NIS-Elemenets AR or MetaMorph Premier.

MTT Cell Viability Assay. HeLa cells were seeded in a 96-well plate at a density of 5000 cells/well (100 μL in each well) in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin sulfate overnight. Next day, peptide was added to the cells and incubated in 5% CO_2 incubator at 37 ^oC for 72 h. Ten μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was added to each well and incubated for 2 h. One hundred μL of the SDS-HCl solubilizing buffer was added to each well and the contents were carefully mixed by pipetting up and down. The plate was incubated for 4 h and the absorbance of the solubilized formazan was measured on a Molecular Devices Spectramax M5 plate reader at 570 nm.

Table S1. Sequences and Cytosolic Entry Efficiencies of Cyclic CPPs

Peptide	Sequence[a]	Molecular Mass (M + H^+)		Cellular Entry Efficiency[b]	
		Calcd	Obsd	In 10% FBS	In 1% FBS
CPP12	cyclo(FfΦRrRrQ)	1975.932	1975.839	100	100
1-NF	cyclo(Dap^{Deca} RRRRQ)	1687.842	1687.868	6.72 ± 0.40	
2-NF	cyclo(Dap^{Hexan}RRRRQ)	1668.832	1668.816	2.86 ± 0.22	
3-NF	cyclo(Dap^{Octan}RRRRQ)	1696.863	1696.889	5.67 ± 0.63	
4-NF	cyclo(Dap^{Deca}RRRRQ)	1746.895 ($M + Na^+$)	1746.906	57.5 ± 18.8	44.1 ± 22.2
5-NF	cyclo($Dap^{1\text{-}Pyren}$RRRRQ)	1798.816	1798.836	10.5 ± 1.5	
6-NF	cyclo($Dap^{3,3\text{-}dipheny}$RRRRQ)	1707.811	1707.834	3.89 ± 0.37	
7-NF	cyclo(Dap^{Fmoc}RRRRQ)	1792.827	1792.861	17.0 ± 0.2	
8-NF	cyclo($Dap^{1\text{-}Pyreneb}$RRRRQ)	1840.863	1840.907	23.7 ± 0.9	
9-NF	cyclo(Dap^{Deca}RrRrQ)	1724.895	1724.923	20.7 ± 9.0	
10-NF	cyclo(Dap^{Deca}rRrRQ)	1724.895	1724.895	19.5 ± 5.8	
11-NF	cyclo(Dap^{Deca}RRRAQ)	1639.831	1639.824	25.9 ± 3.9	
12-NF	cyclo(Dap^{Deca}ARRRQ)	1639.831	1639.834	32.4 ± 1.9	
13-NF	cyclo(Dap^{Deca}RRRQ)	1568.794	1568.692	3.03 ± 0.10	
14-NF	cyclo(Dap^{Deca}RRRRRQ)	1880.996	1880.979	11.6 ± 0.9	
15-NF	cyclo(Orn^{Deca}RRRRQ)	1752.926	1752.832	115 ± 32	58.3 ± 22.0
16-NF	cyclo(Lys^{Deca}RRRRQ)	1766.942	1767.638	76.3 ± 21.0	43.5 ± 10.9
17-NF	cyclo(Asp^{Decy}RRRRQ)	1738.910	1738.830	281 ± 59	94.3 ± 41.3
17-FITC	cyclo(Asp^{Decy}RRRRQ)	1668.867	1668.644		

[a]Single-letter codes for amino acids. Φ, 2-naphthylalanine.

[b]All values are relative to that of CPP12 (100%).

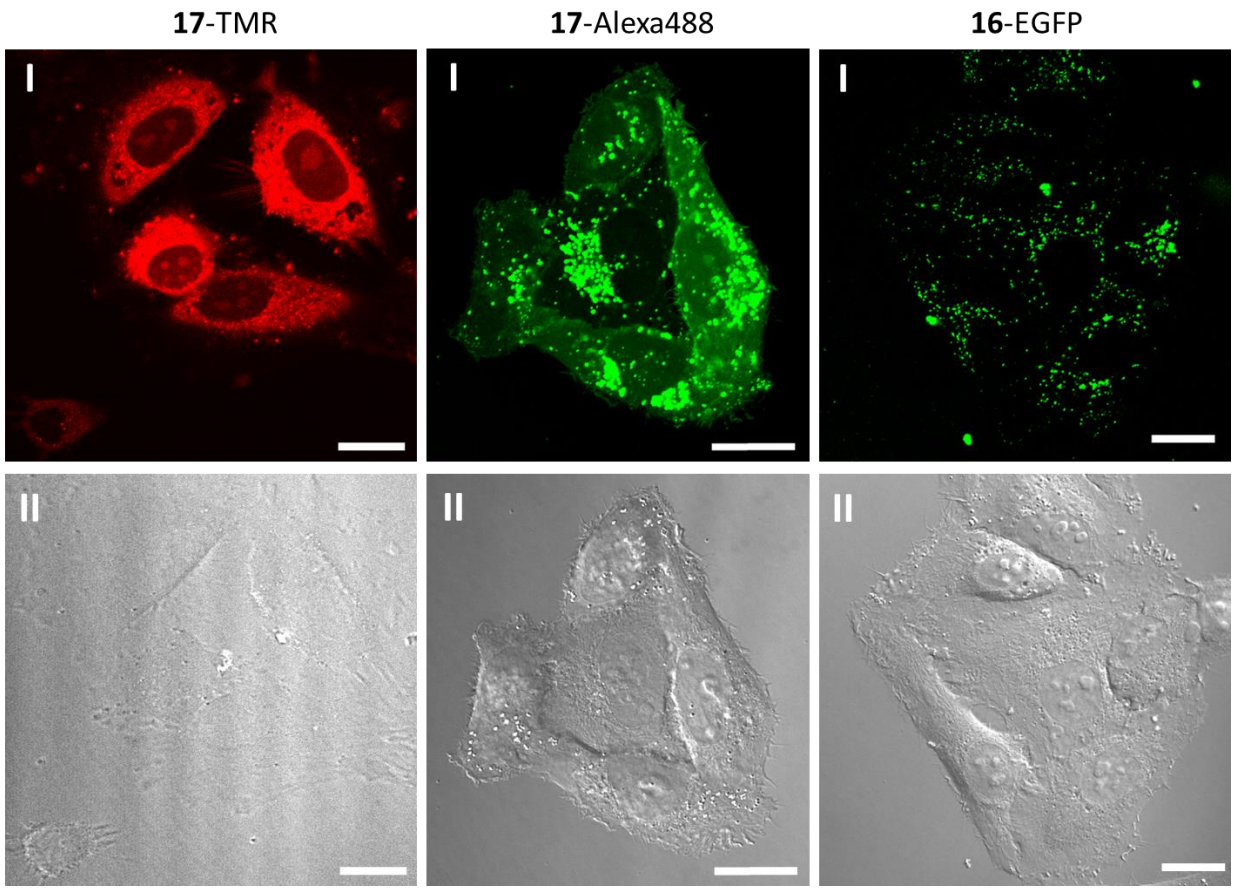


Figure S1. Live-cell confocal microscopic images of HeLa cells after 2-h treatment with 5 μM TMR-labeled peptide **17** (**17**-TMR), Alexa488-labeled peptide **17** (**17**-Alexa488), or a peptide **16**-enhanced green fluorescent protein conjugate (**16**-EGFP) in the presence of 1% FBS. **16**-EGFP was prepared by attaching peptide **16** to the N-terminus of EGFP through a ybbR tag (*Biochemistry* **2014**, *53*, 4034-4046). I, fluorescence channel; II, DIC. Bars, 20 μm.

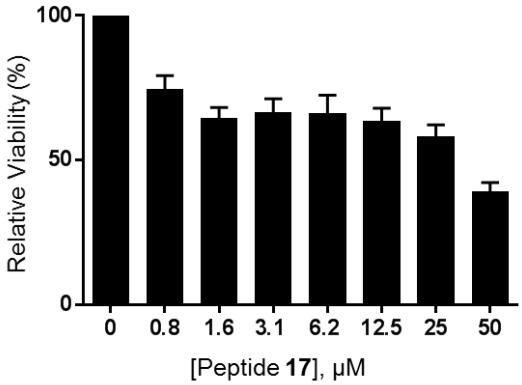
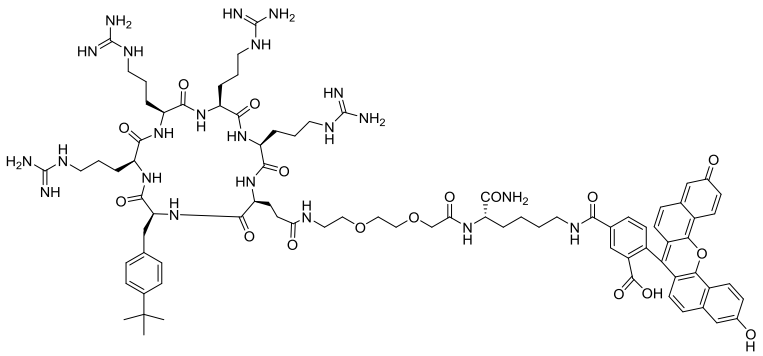


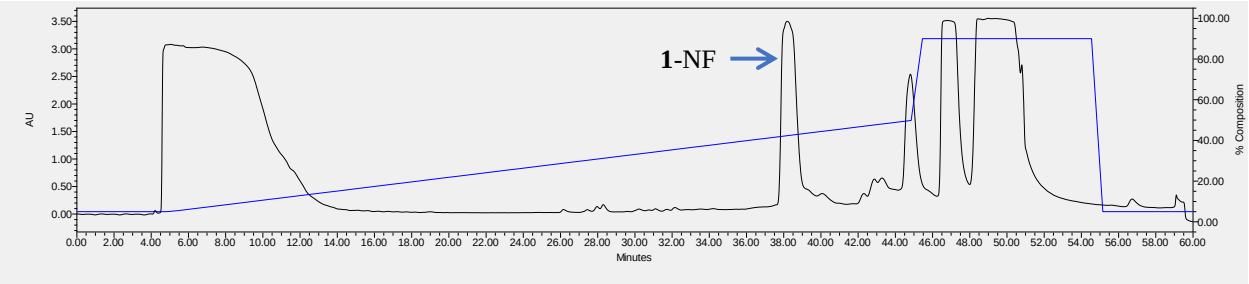
Figure S2. Effect of peptides **17** on the viability of HeLa cells as monitored by the MTT assay. Cells were incubated with the peptide for 72 h in the presence of 10% FBS (n = 6). The origin for the slight reduction of HeLa cell viability at ≤25 μM peptide **17** is yet unclear.

Figure S3. Reversed-phase HPLC chromatograms and HR MALDI-TOF MS of peptides used in this work. Doublet peaks for some peptides are due to the fact that commercial NF is a mixture of 5- and 6-carboxyl isomers. In some cases, epimerization at Gln residue during peptide cyclization may also contribute to multiple peaks.

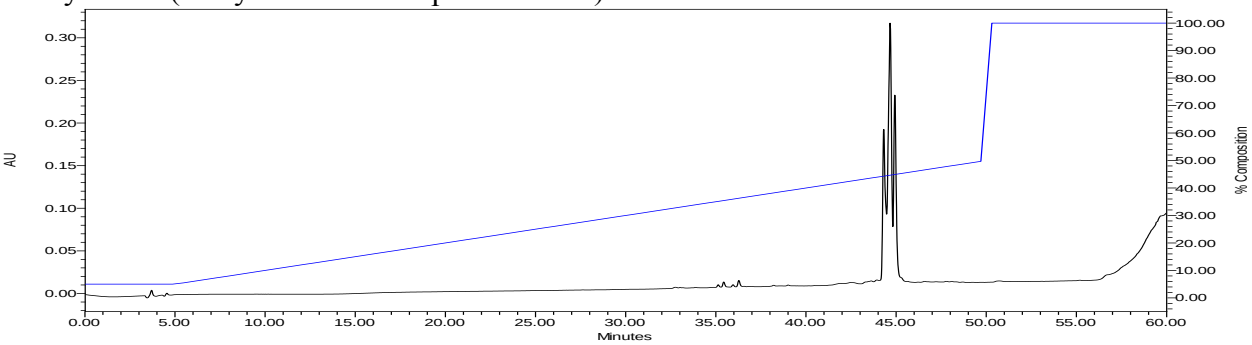
Peptide **1**-NF



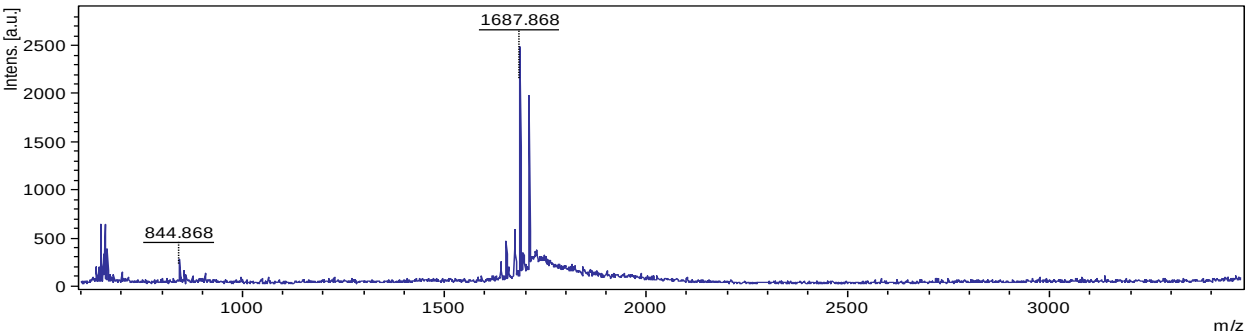
Crude peptide on semipreparative HPLC:



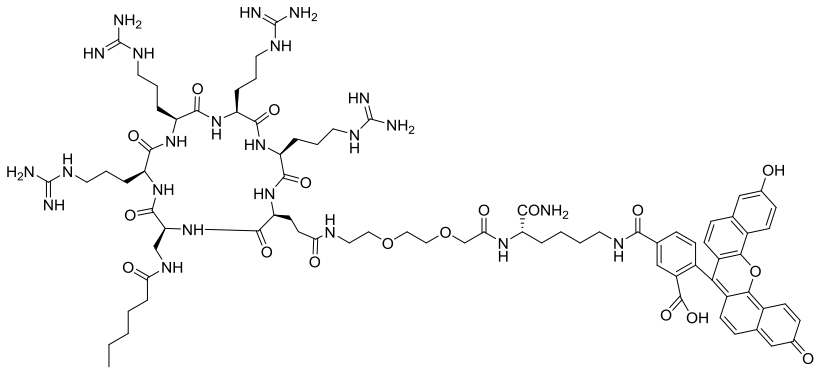
Purity check (analytical reversed-phase HPLC):



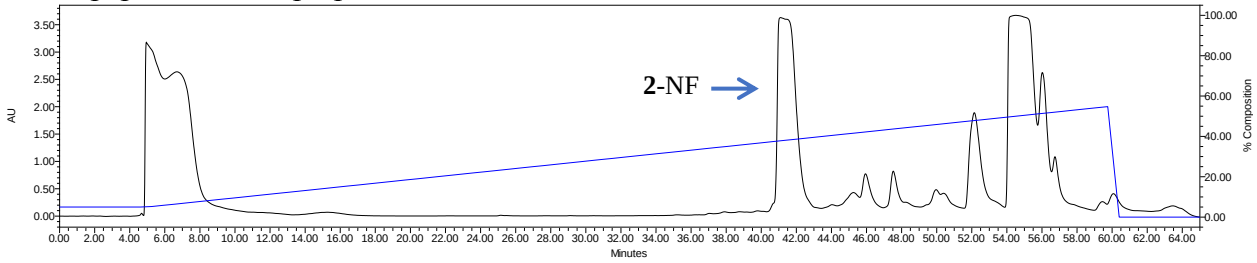
MALDI-TOF MS Expected for $M+H^+$: 1687.842; Found 1687.868



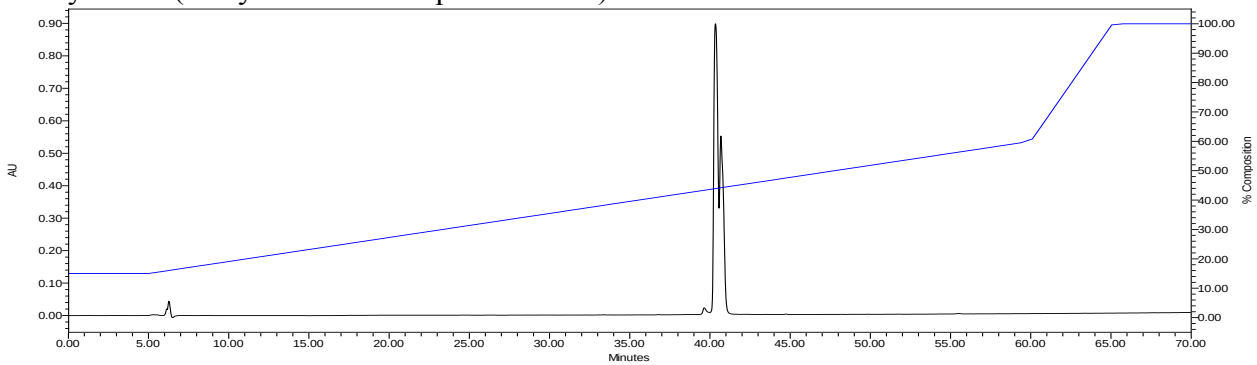
Peptide **2**-NF



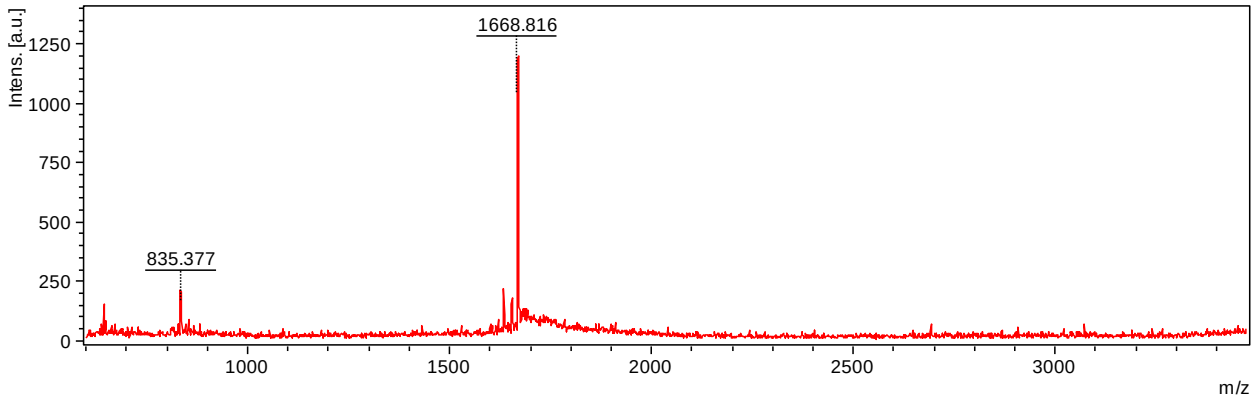
Crude peptide on semipreparative HPLC:



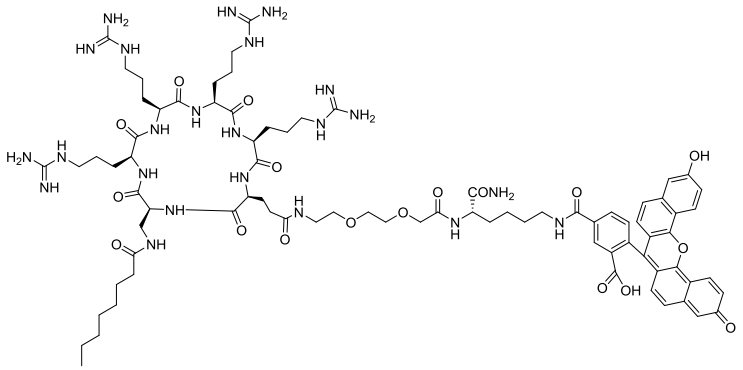
Purity check (analytical reversed-phase HPLC):



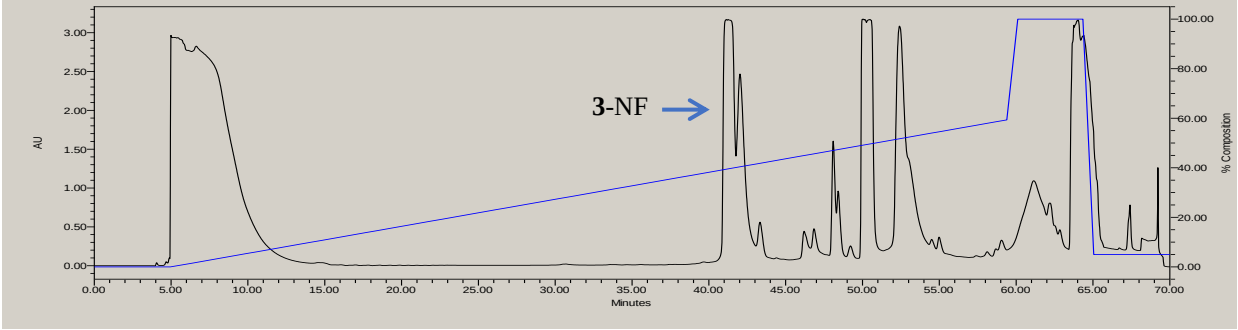
MALDI-TOF MS Expected for $M+H^+$: 1668.832; Found 1668.816



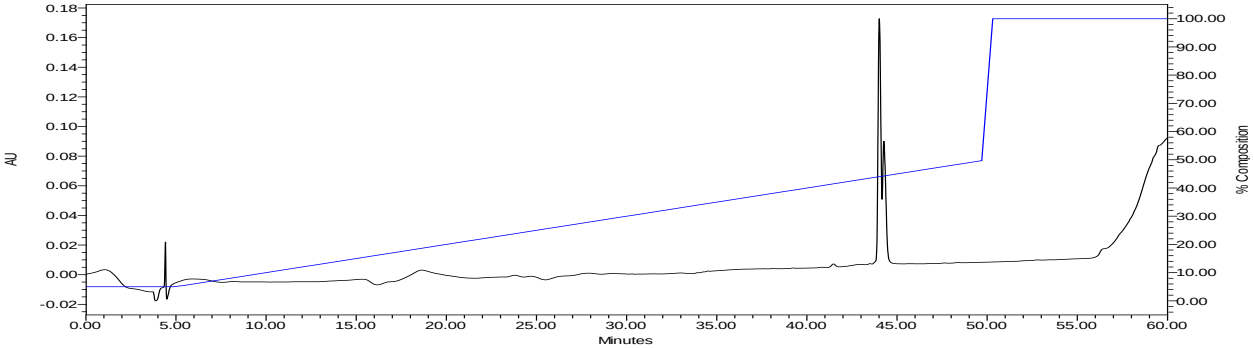
Peptide **3**-NF



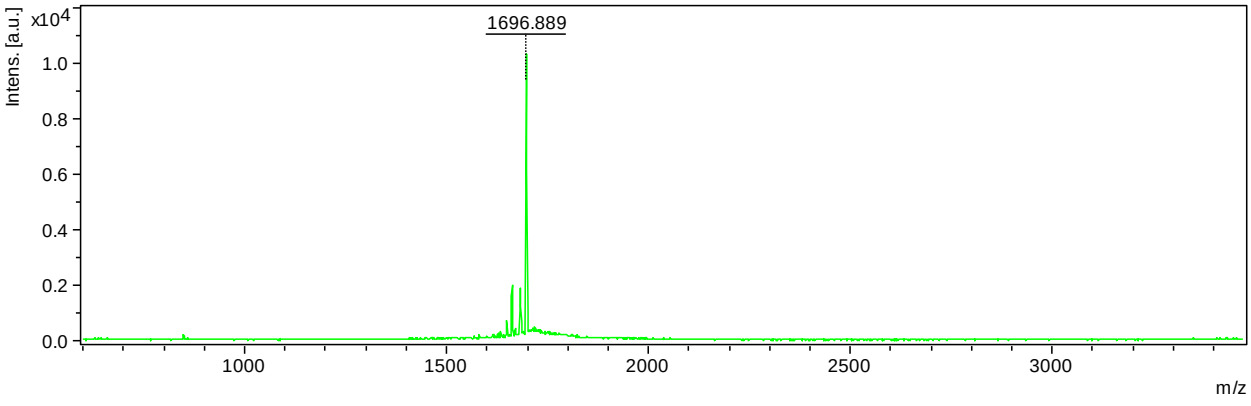
Crude peptide on semipreparative HPLC:



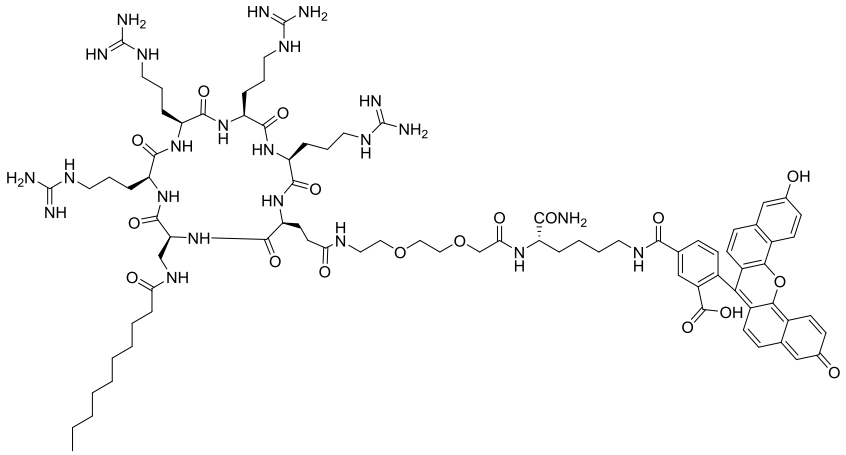
Purity check (analytical reversed-phase HPLC):



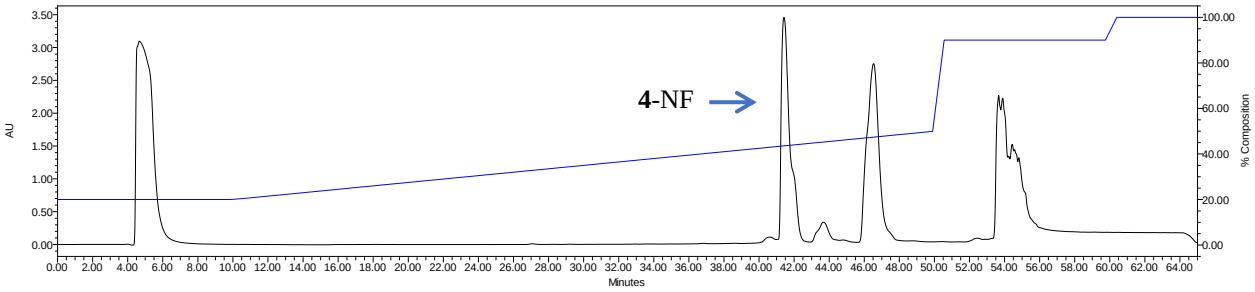
MALDI-TOF MS Expected for $M+H^+$: 1696.863; Found 1696.889



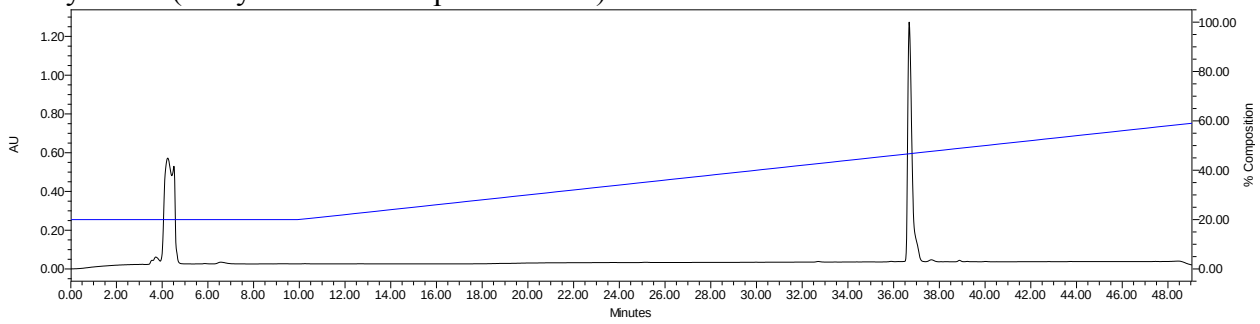
Peptide **4**-NF



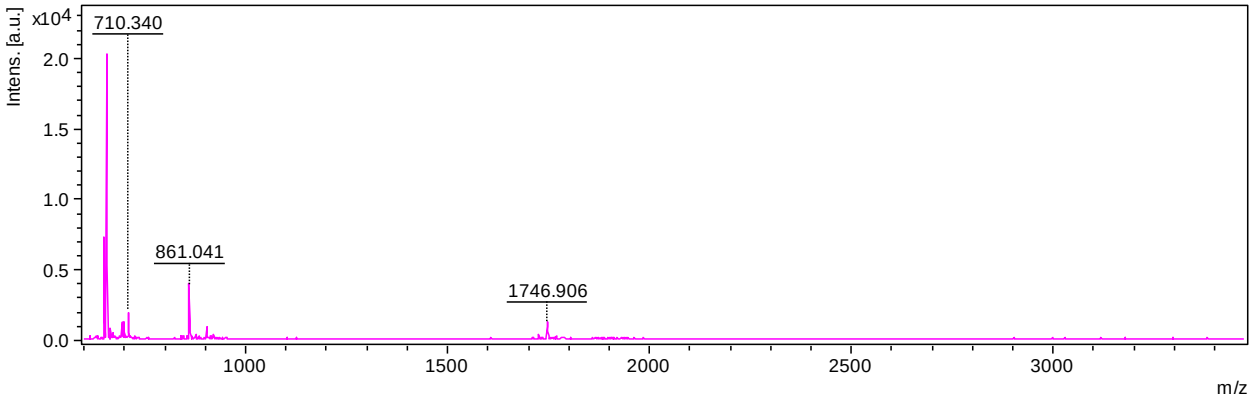
Crude peptide on semipreparative HPLC:



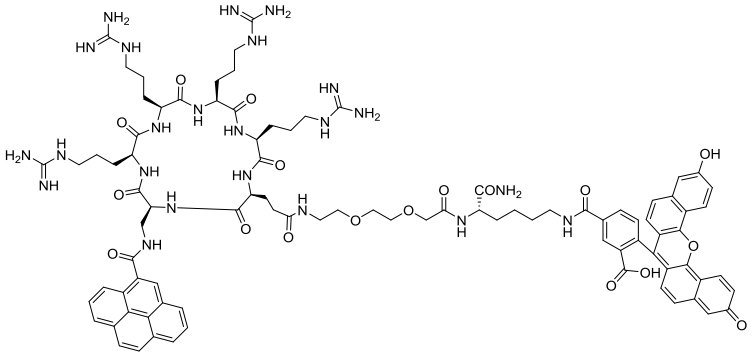
Purity check (analytical reversed-phase HPLC):



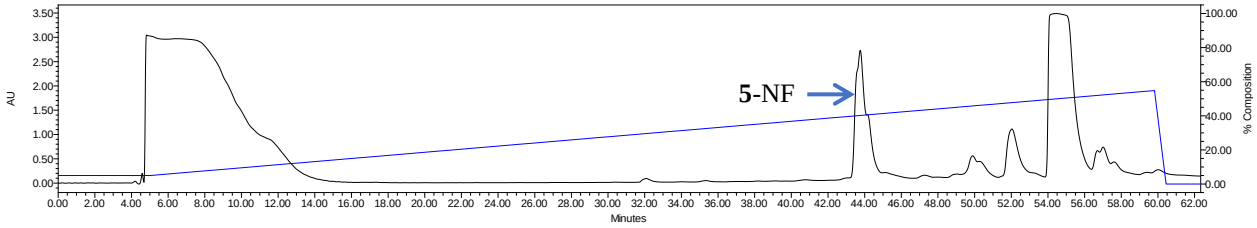
MALDI-TOF MS: Expected for $M+Na^+$: 1746.895; Found 1746.906



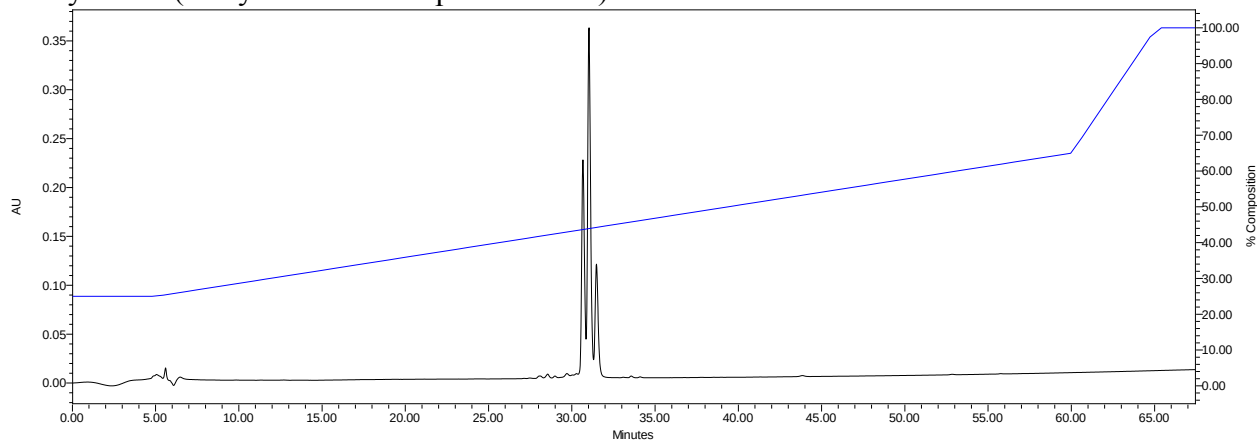
Peptide **5**-NF



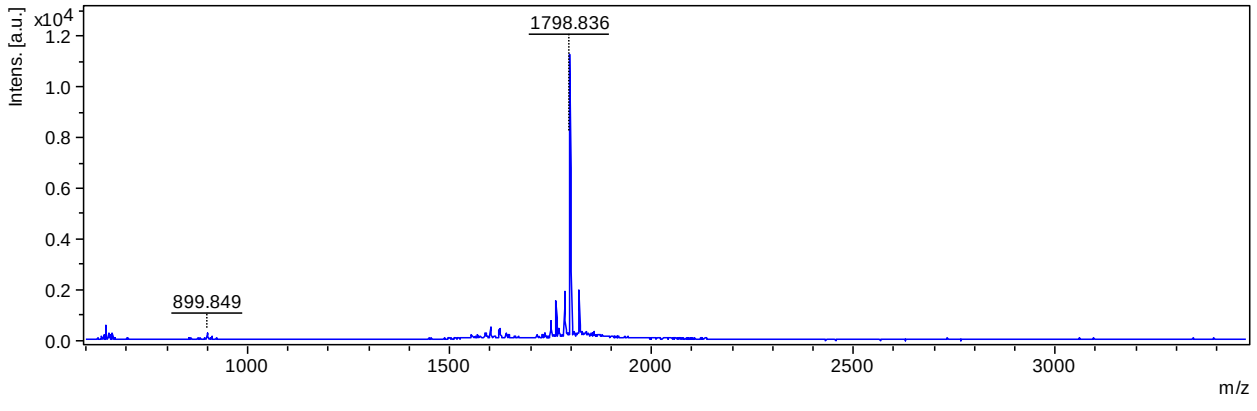
Crude peptide on semipreparative HPLC:



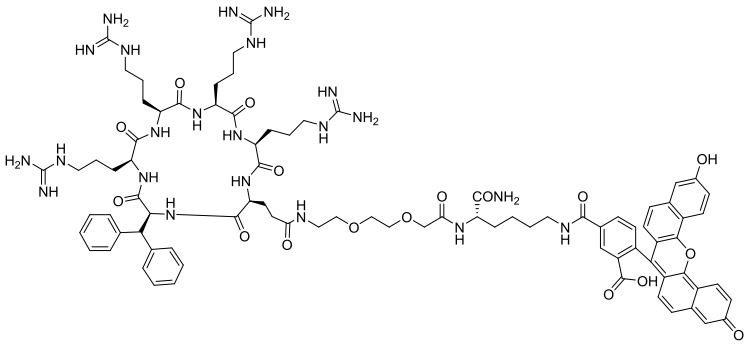
Purity check (analytical reversed-phase HPLC):



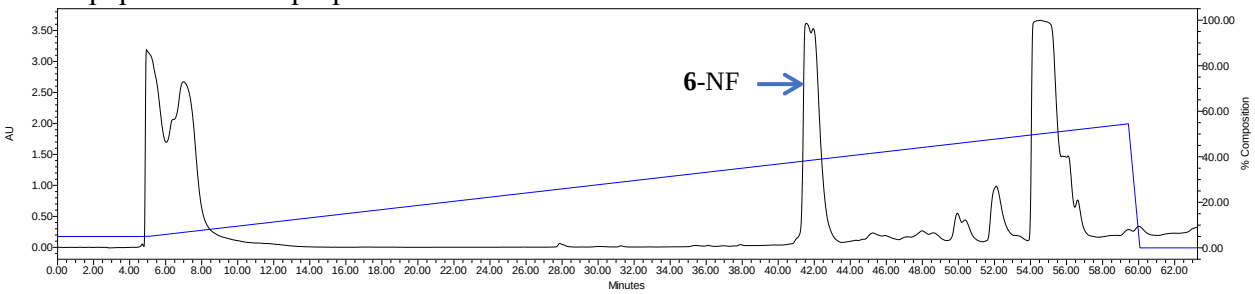
MALDI-TOF MS: Expected for $M+H^+$: 1798.816; Found 1798.836



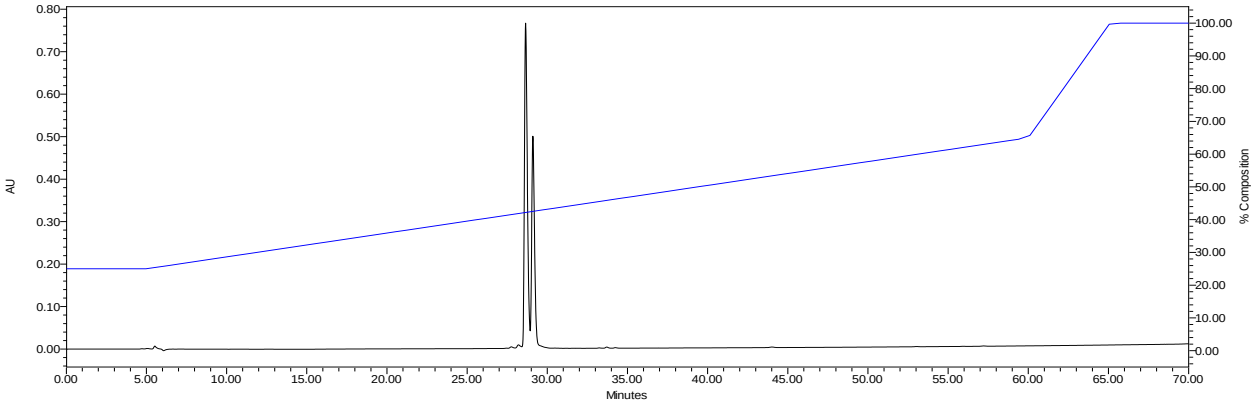
Peptide **6**-NF



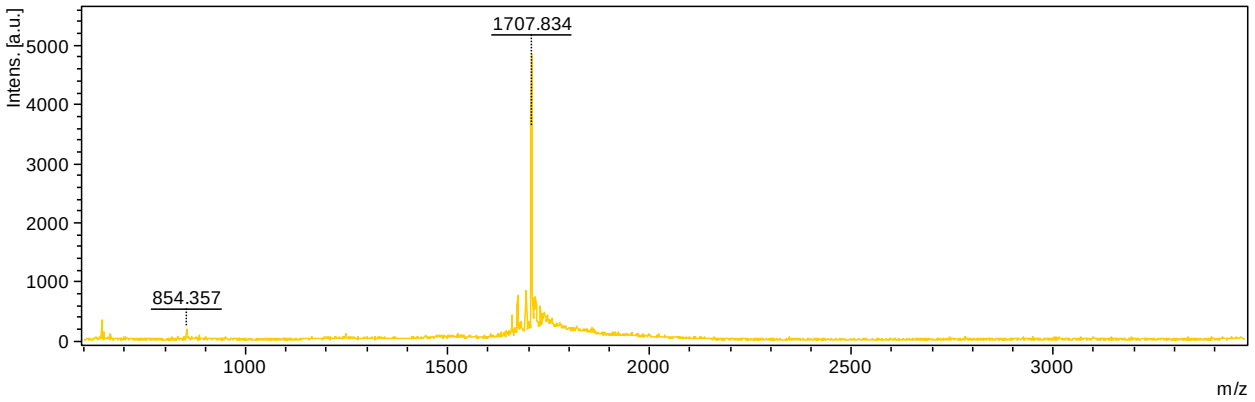
Crude peptide on semipreparative HPLC:



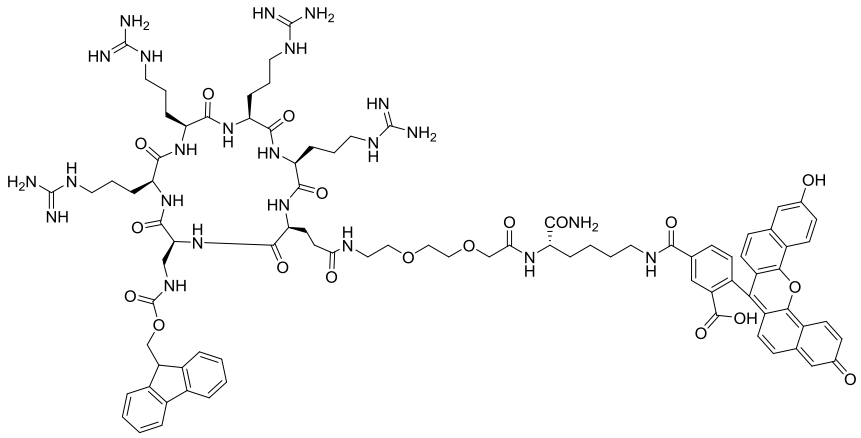
Purity check (analytical reversed-phase HPLC):



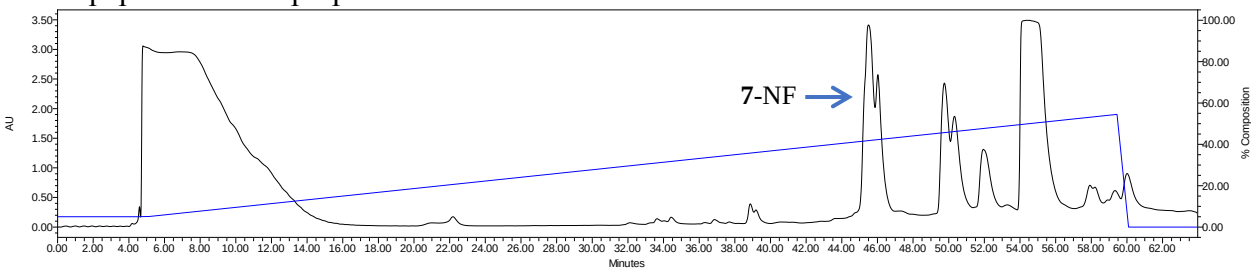
MALDI-TOF MS: Expected for $M+H^+$: 1707.811; Found 1707.834



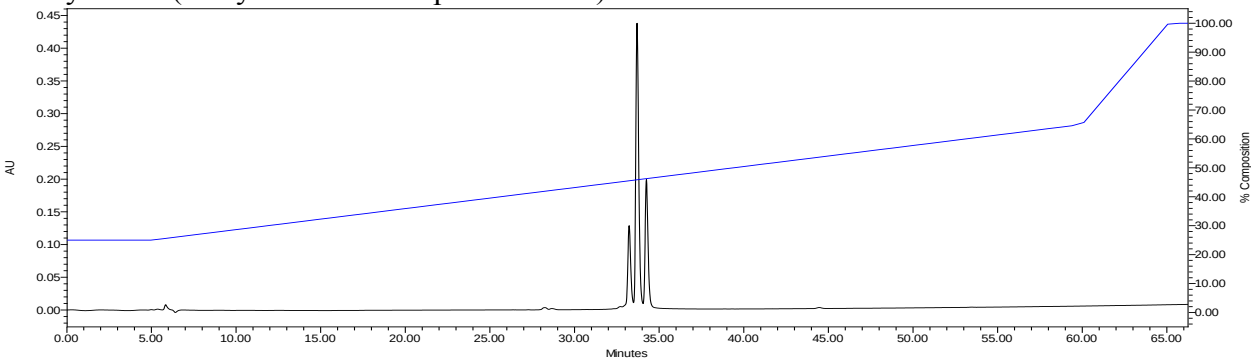
Peptide **7**-NF



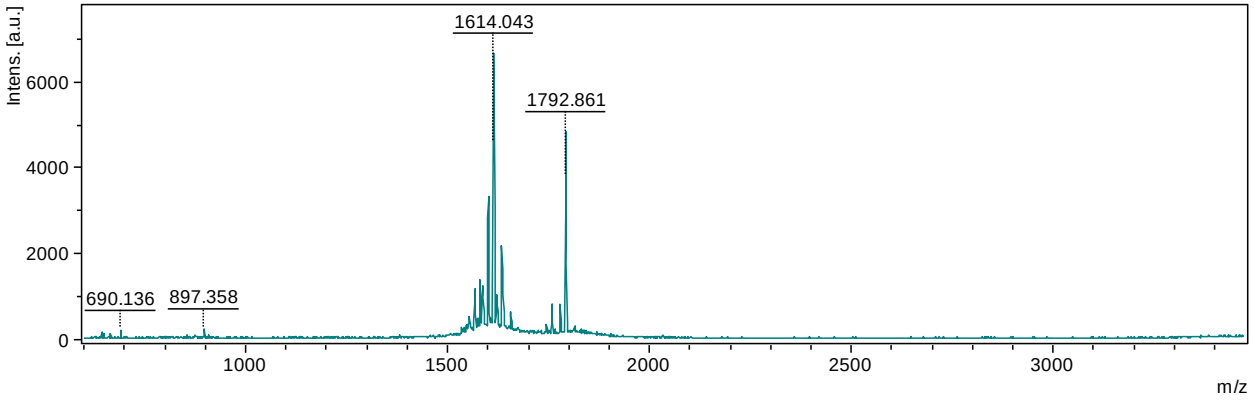
Crude peptide on semipreparative HPLC:



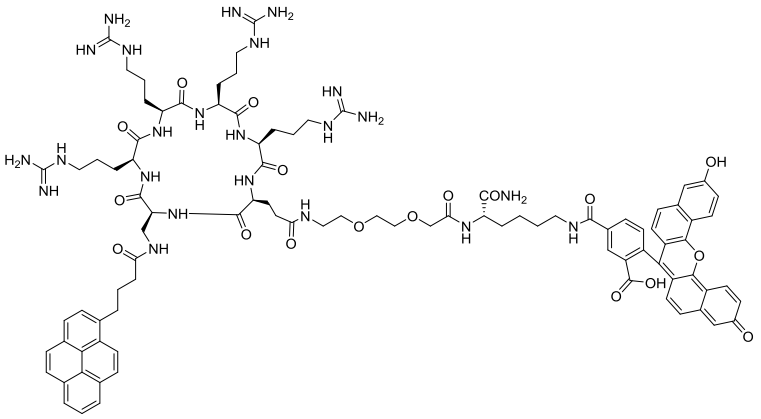
Purity check (analytical reversed-phase HPLC):



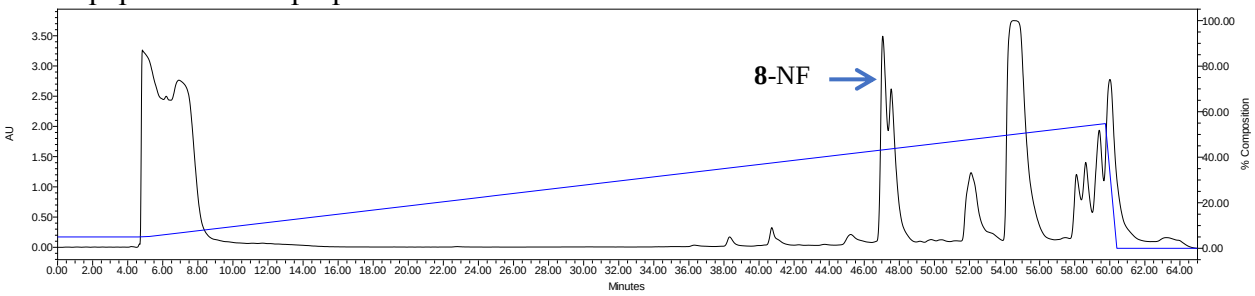
MALDI-TOF MS: Expected for $M+H^+$: 1792.827; Found 1792.861



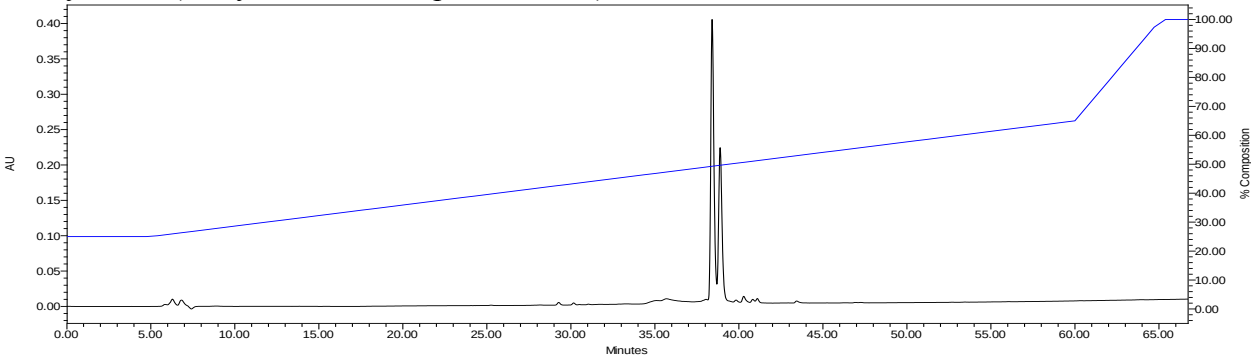
Peptide **8**-NF



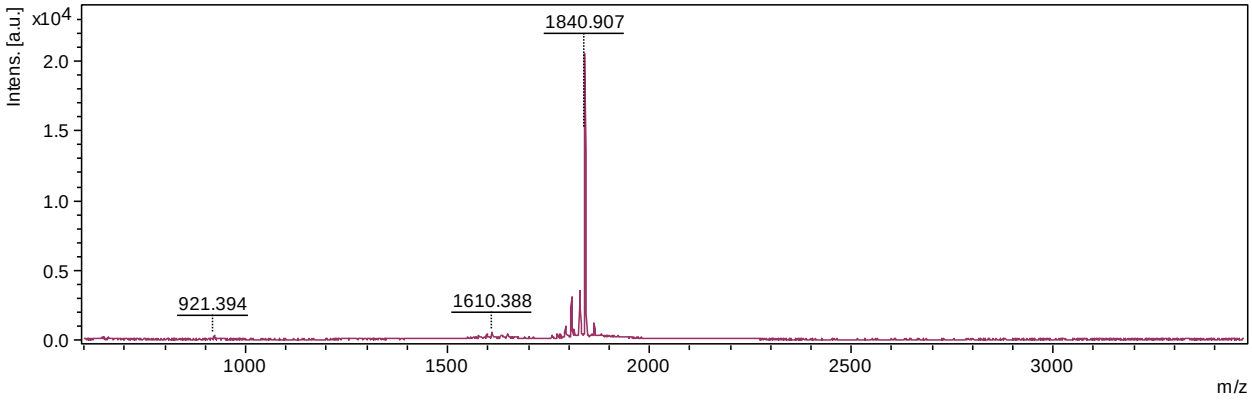
Crude peptide on semipreparative HPLC:



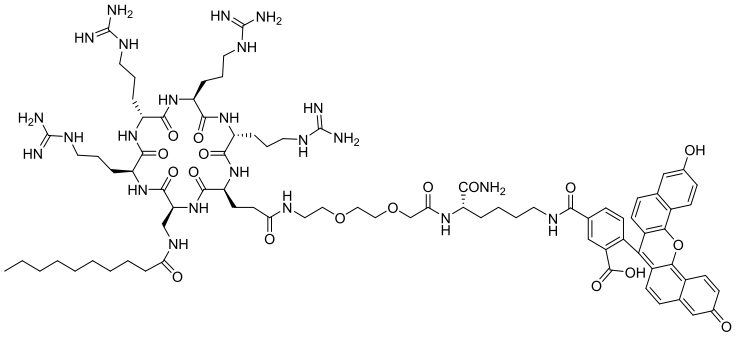
Purity check (analytical reversed-phase HPLC):



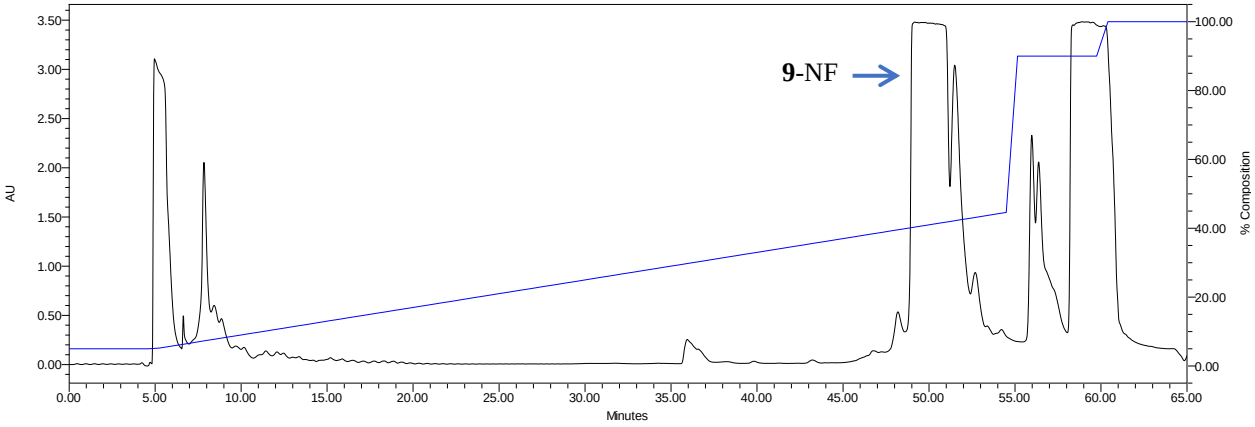
MALDI-TOF MS: Expected for $M+H^+$: 1840.863; Found 1840.907



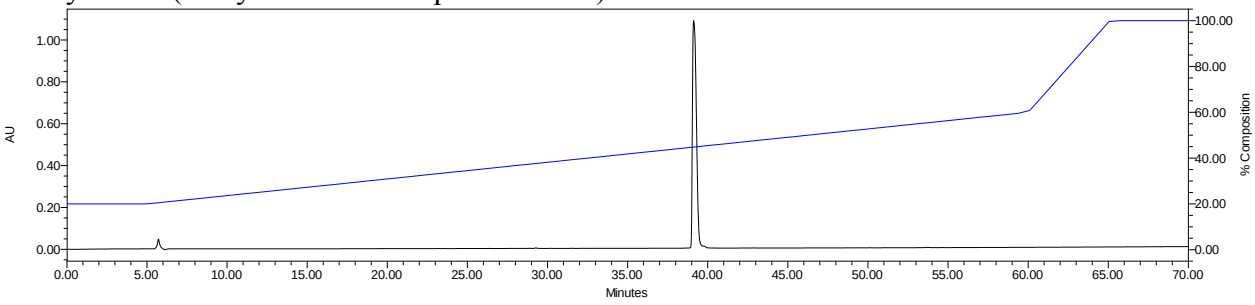
Peptide **9**-NF



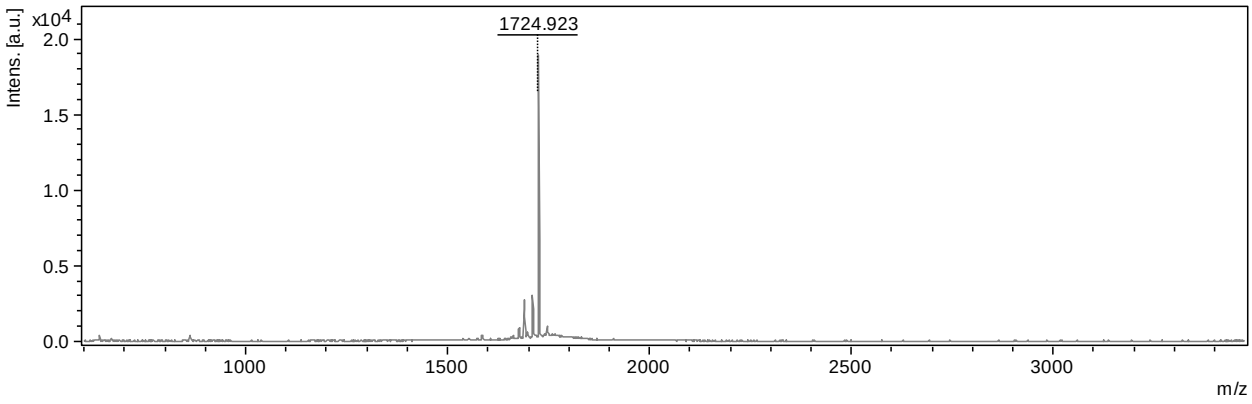
Crude peptide on semipreparative HPLC:



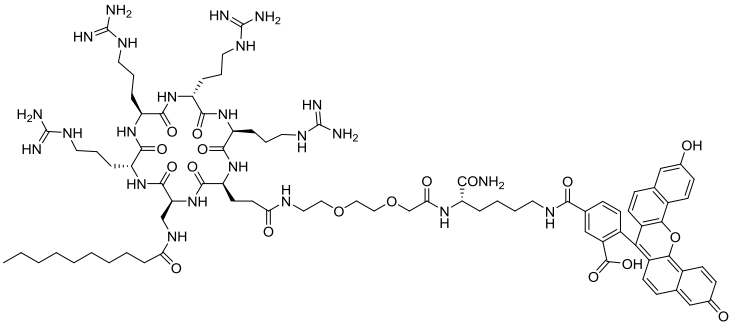
Purity check (analytical reversed-phase HPLC):



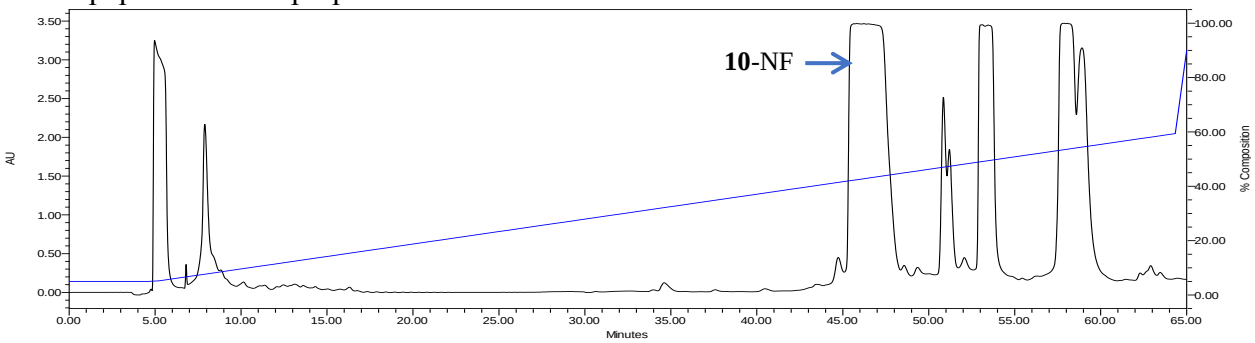
MALDI-TOF MS: Expected for $M+H^+$: 1724.895; Found 1724.923



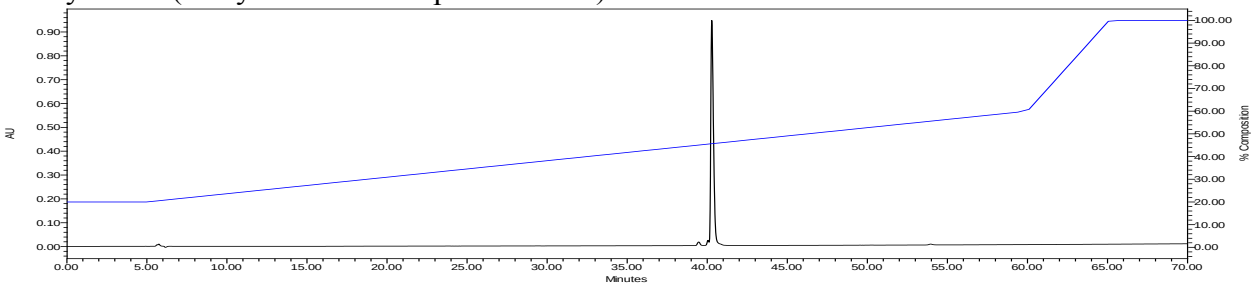
Peptide **10**-NF



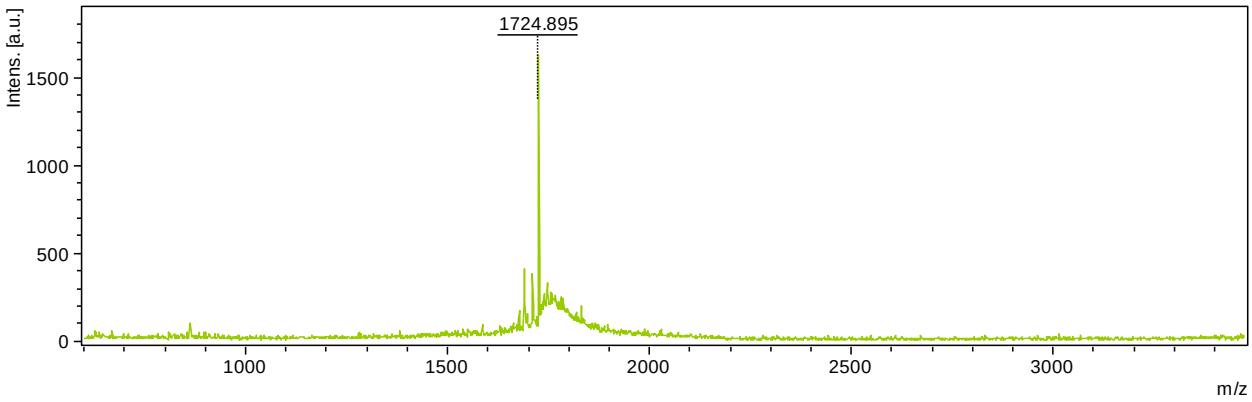
Crude peptide on semipreparative HPLC:



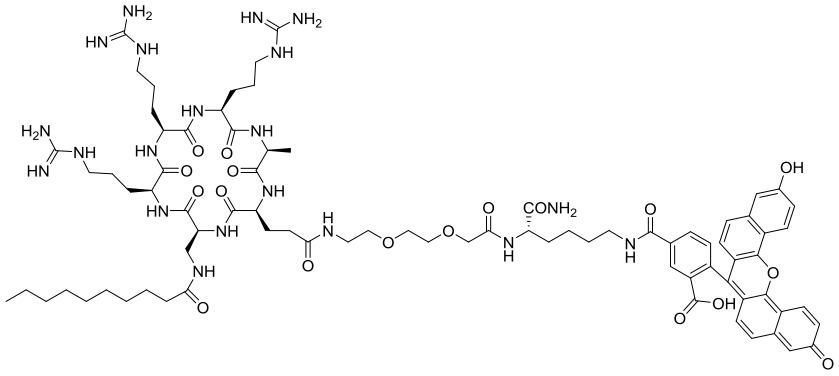
Purity check (analytical reversed-phase HPLC):



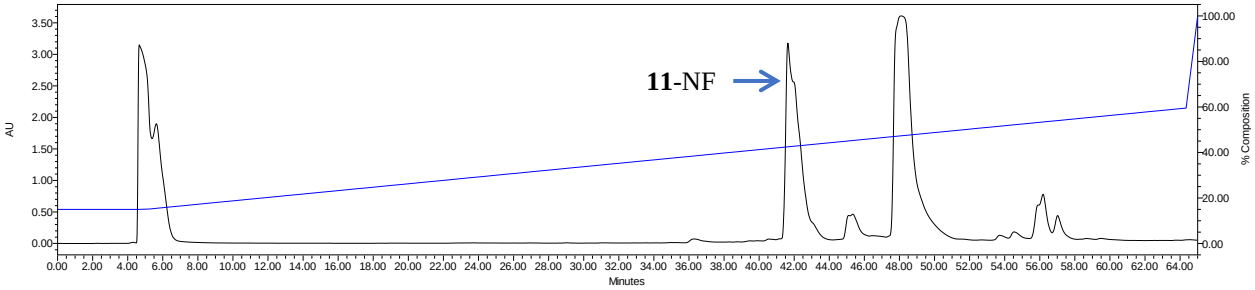
MALDI-TOF MS: Expected for $M+H^+$: 1724.895; Found 1724.895



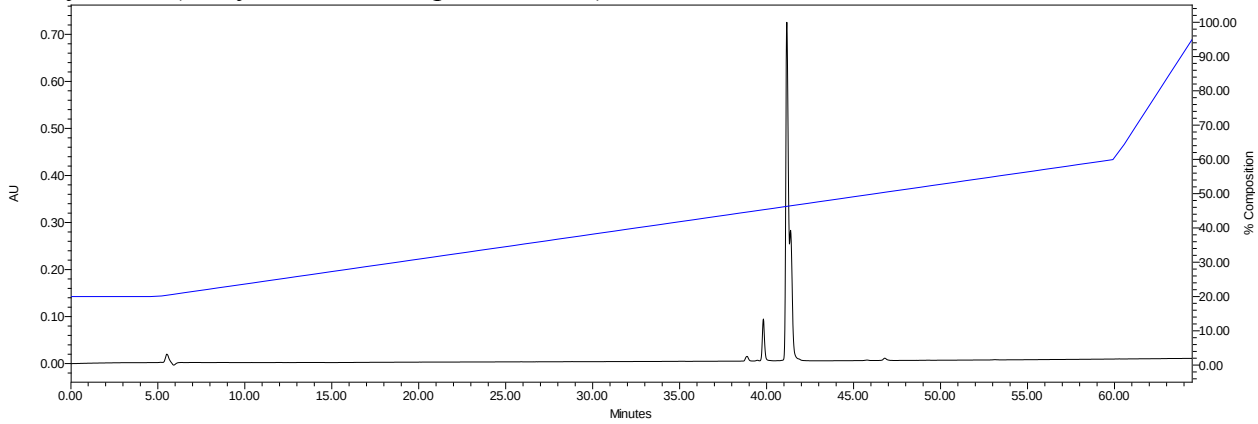
Peptide **11**-NF



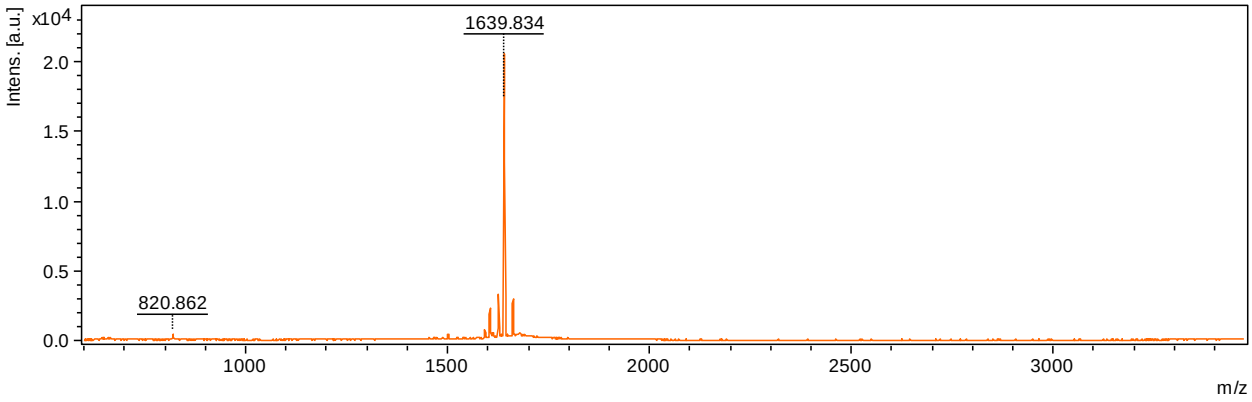
Crude peptide on semipreparative HPLC:



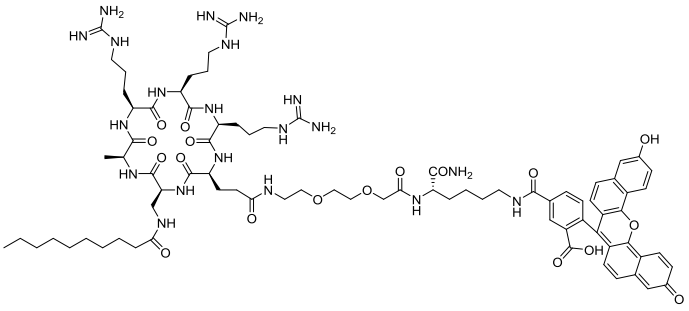
Purity check (analytical reversed-phase HPLC):



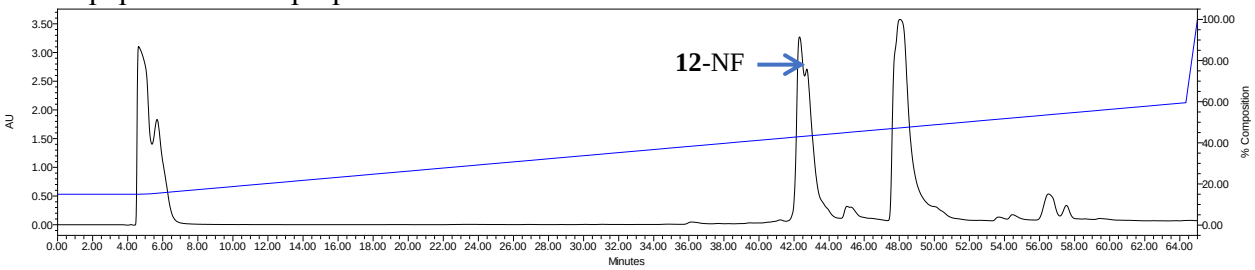
MALDI-TOF MS: Expected for $M+H^+$: 1639.831; Found 1639.834



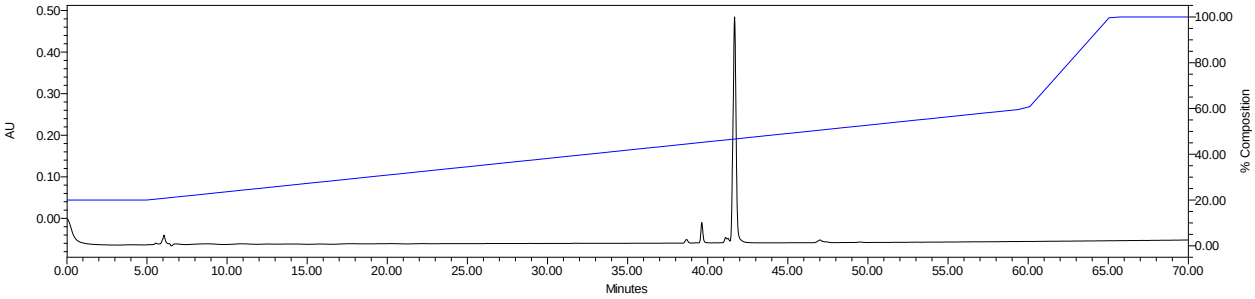
Peptide **12**-NF



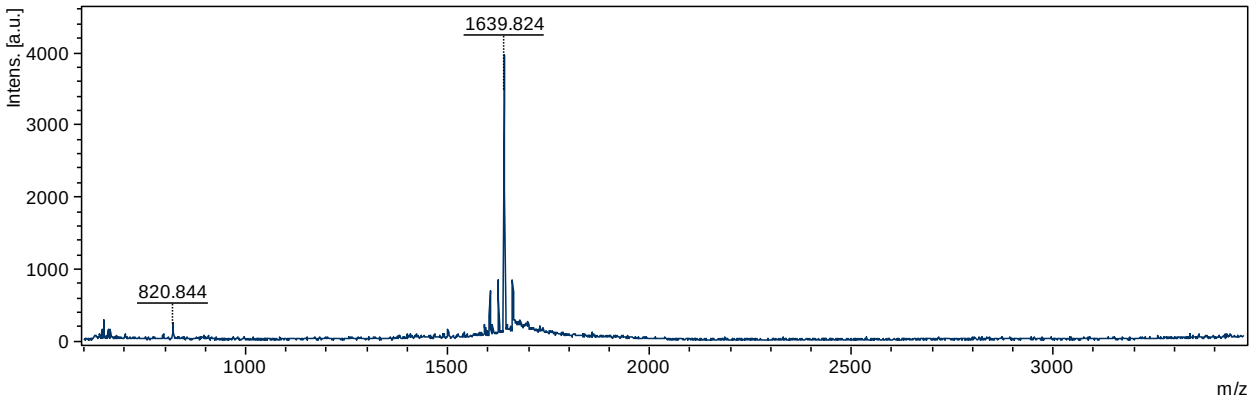
Crude peptide on semipreparative HPLC:



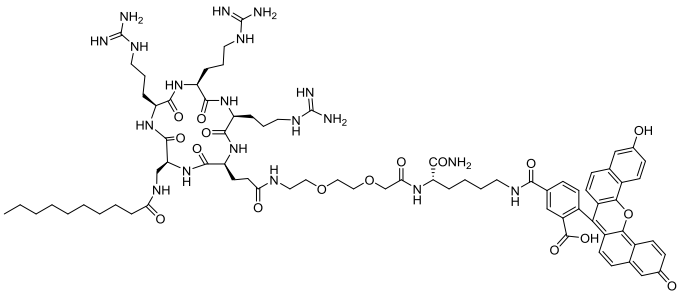
Purity check (analytical reversed-phase HPLC):



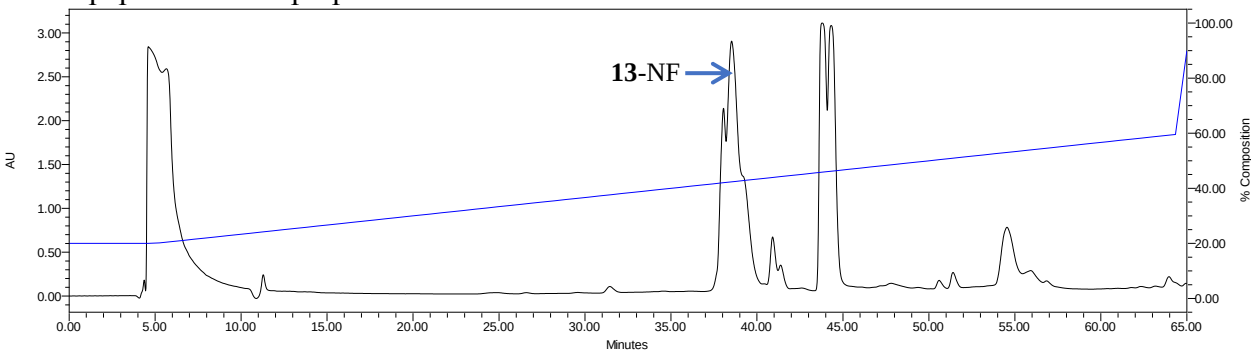
MALDI-TOF MS: Expected for $M+H^+$: 1639.831; Found 1639.824



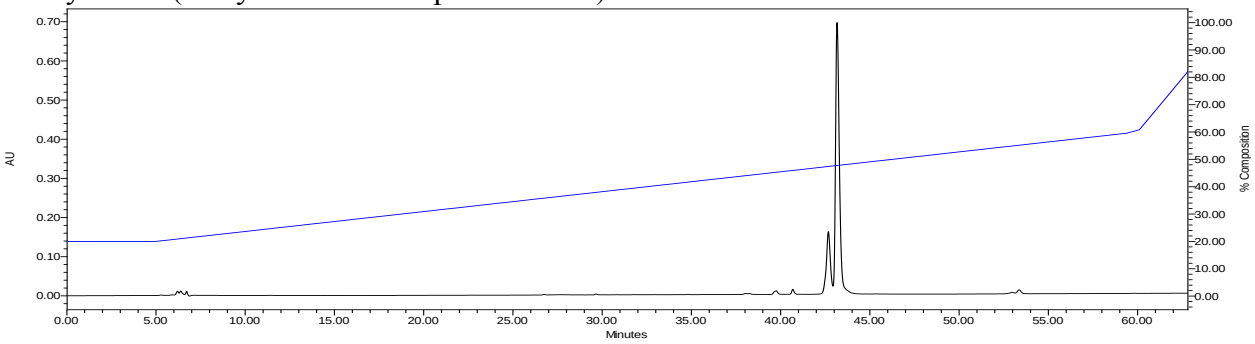
Peptide **13**-NF



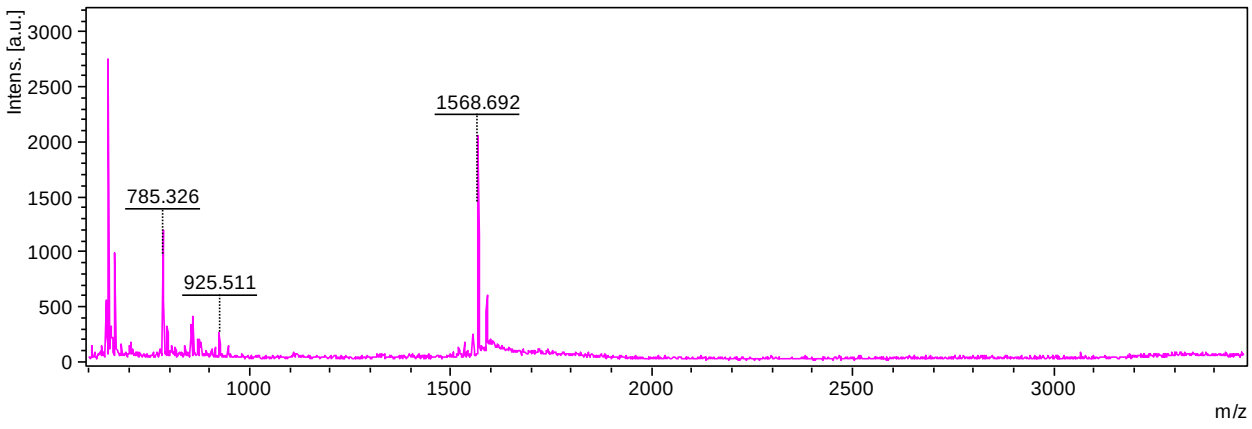
Crude peptide on semipreparative HPLC:



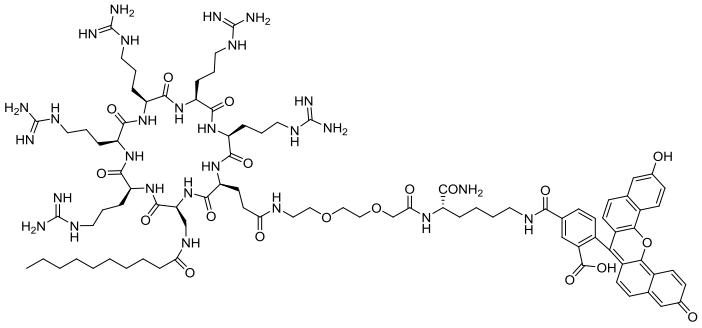
Purity check (analytical reversed-phase HPLC):



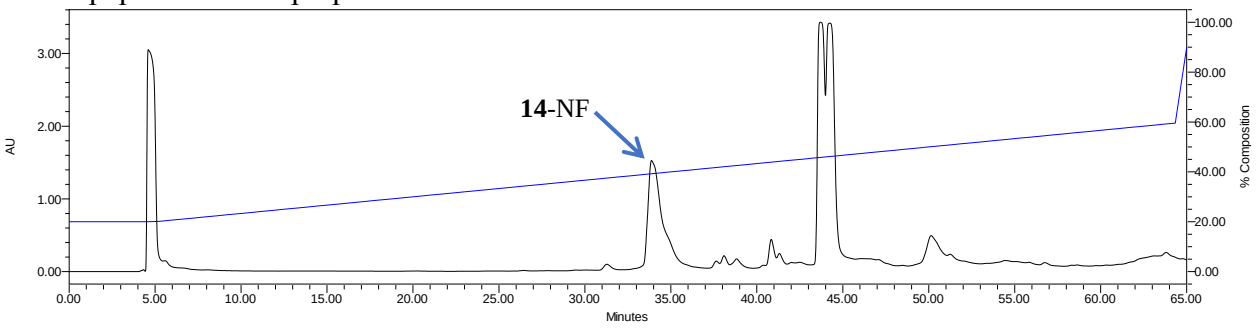
MALDI-TOF MS: Expected for $M+H^+$: 1568.794; Found 1568.692



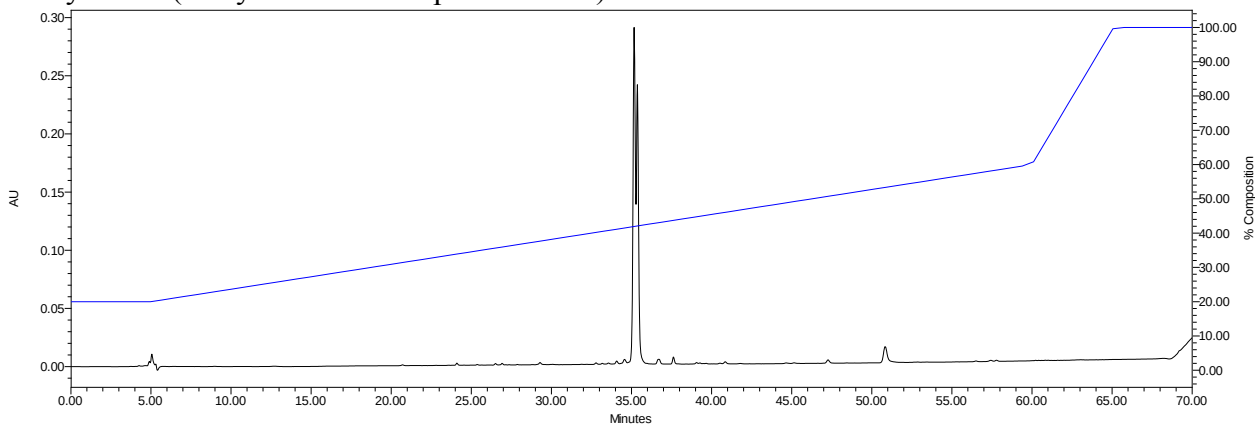
Peptide **14**-NF



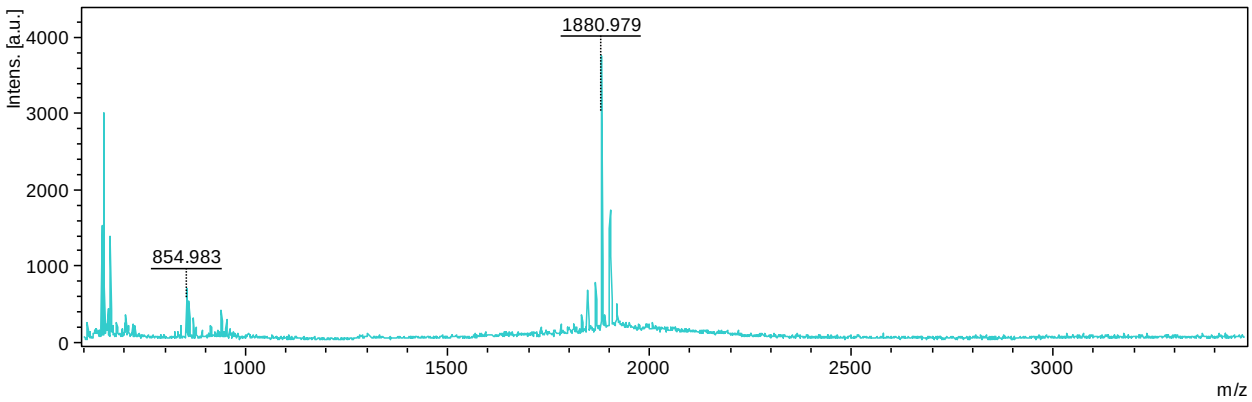
Crude peptide on semipreparative HPLC:



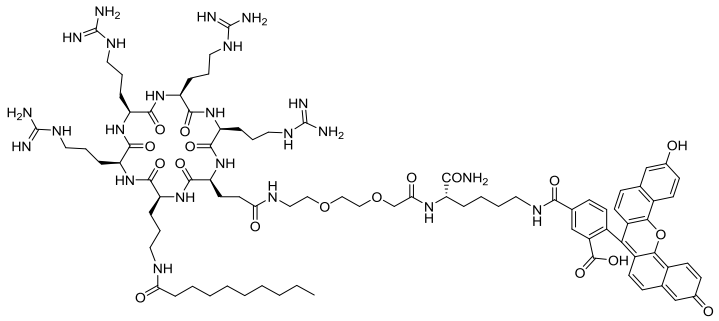
Purity check (analytical reversed-phase HPLC):



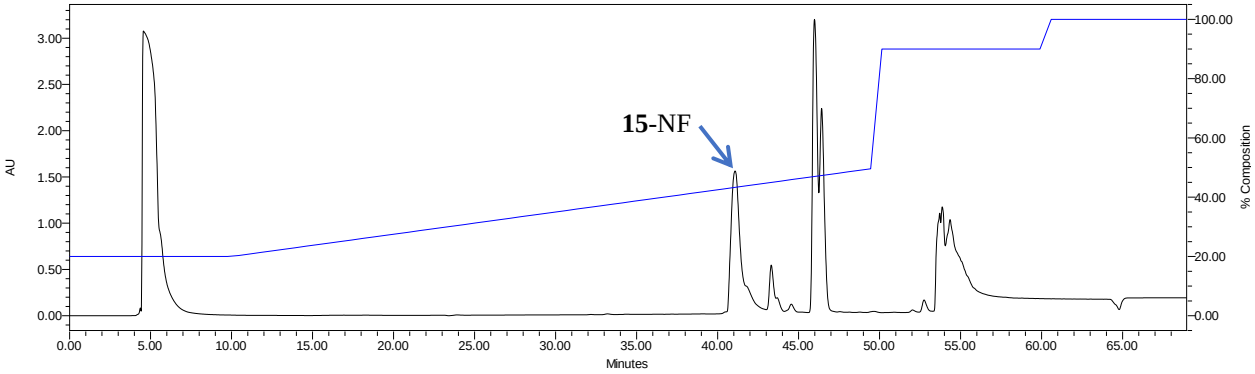
MALDI-TOF MS: Expected for $M+H^+$: 1880.996; Found 1880.979



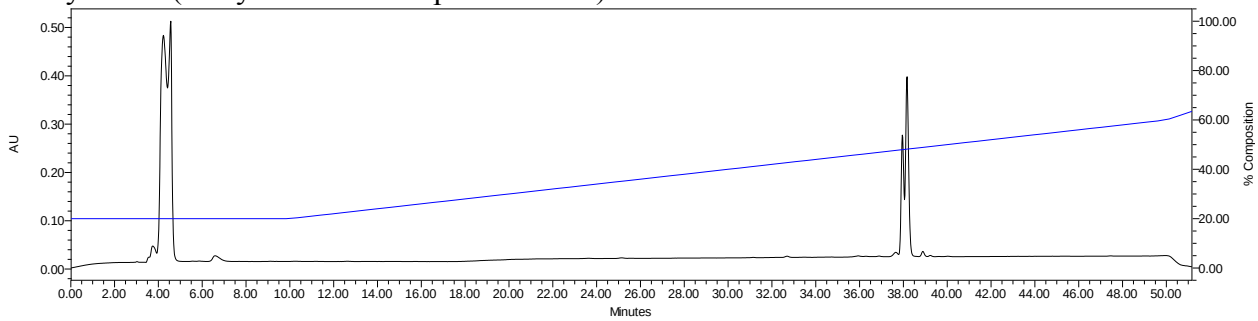
Peptide **15**-NF



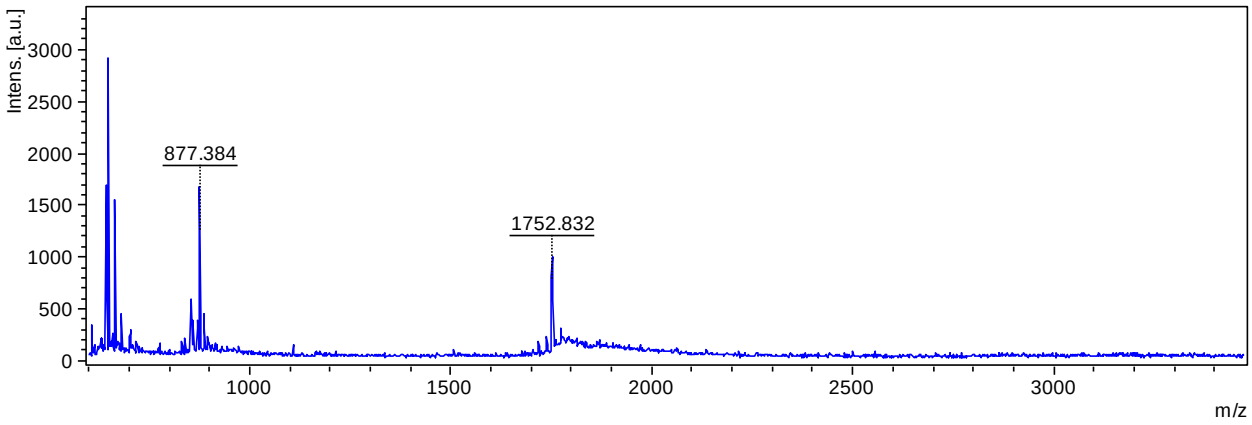
Crude peptide on semipreparative HPLC:



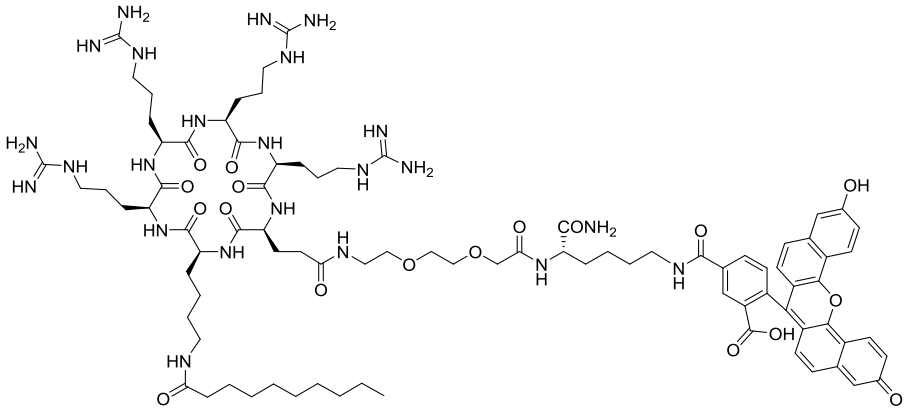
Purity check (analytical reversed-phase HPLC):



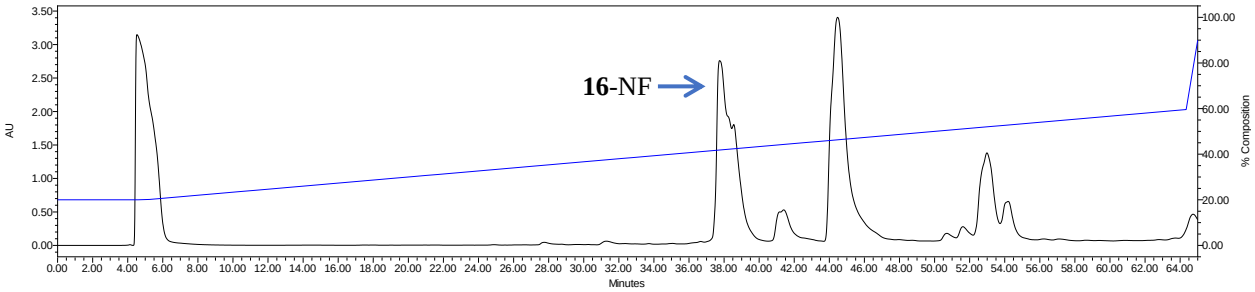
MALDI-TOF MS: Expected for $M+H^+$: 1752.926; Found 1752.832



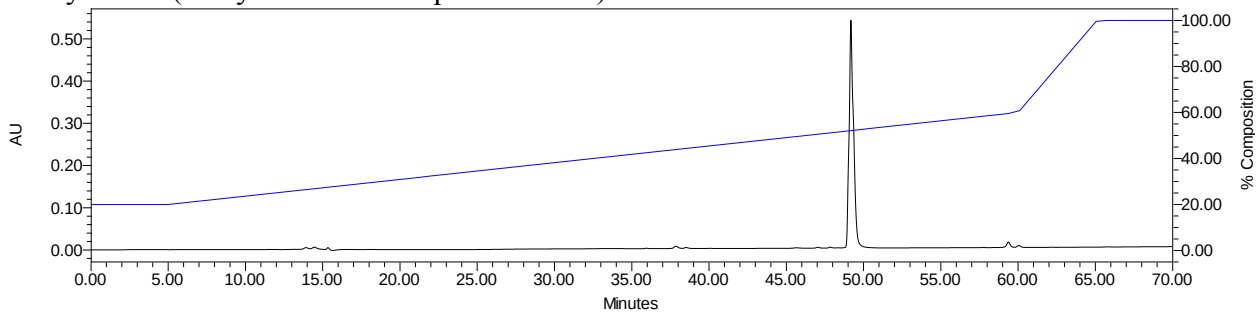
Peptide **16**-NF



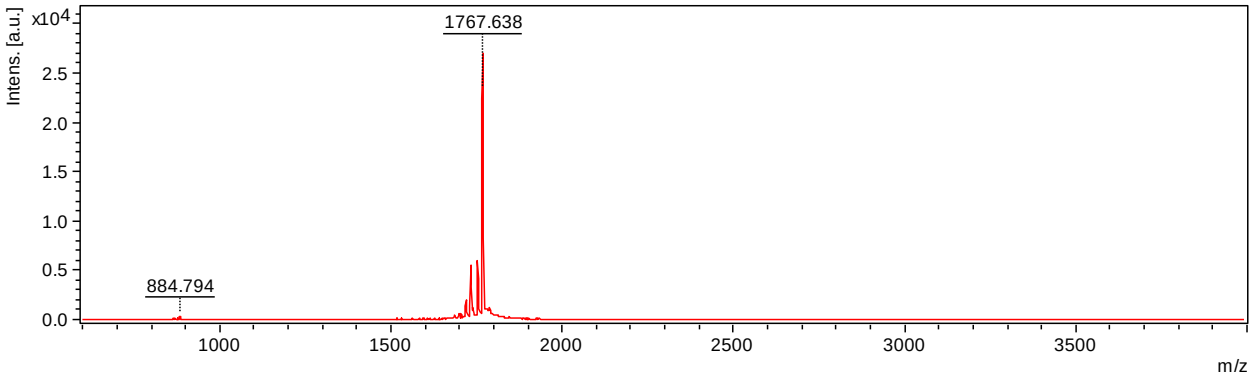
Crude peptide on semipreparative HPLC:



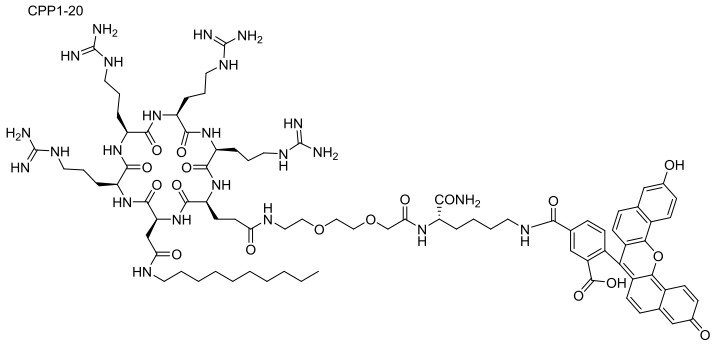
Purity check (analytical reversed-phase HPLC):



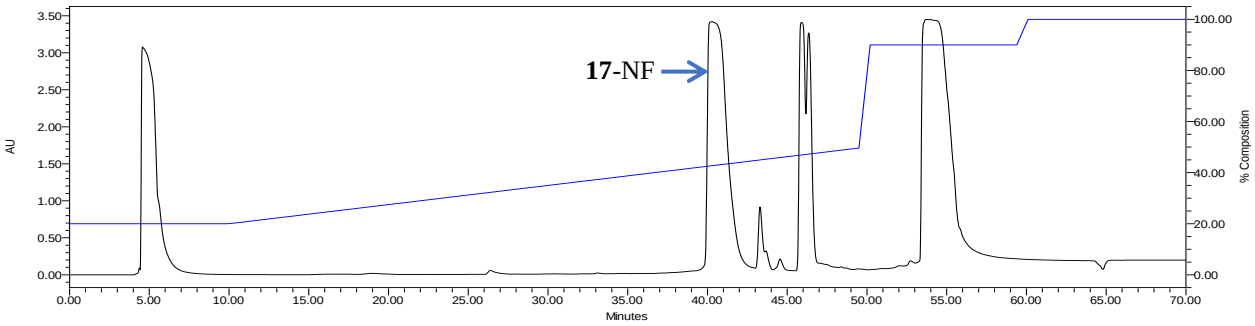
MALDI-TOF MS: Expected for $M+H^+$: 1766.942; Found 1767.638



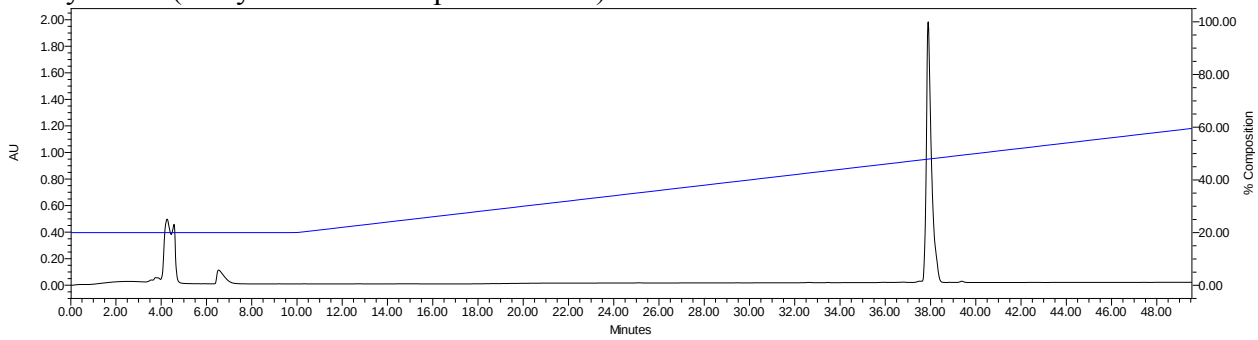
Peptide **17**-NF



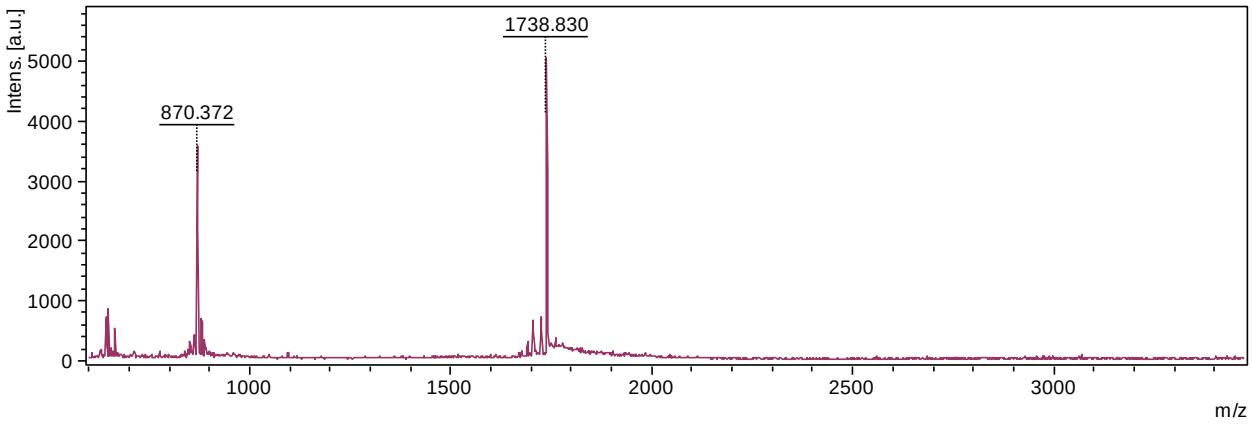
Crude peptide on semipreparative HPLC:



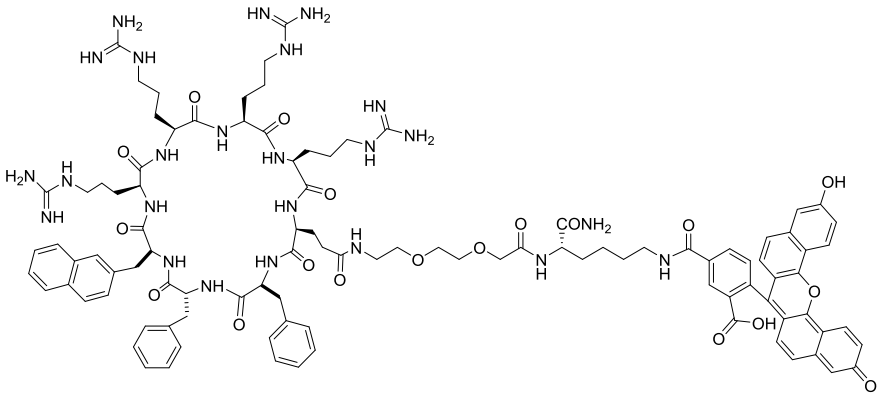
Purity check (analytical reversed-phase HPLC):



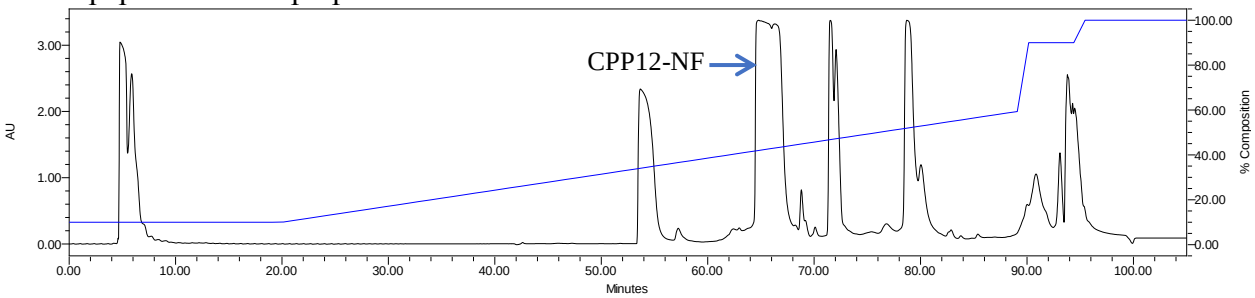
MALDI-TOF MS: Expected for $M+H^+$: 1738.910; Found 1738.830



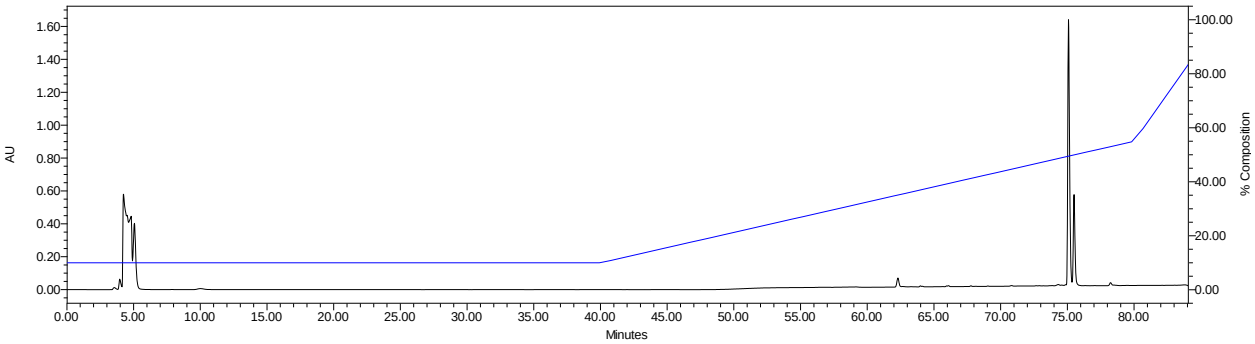
CPP12-NF



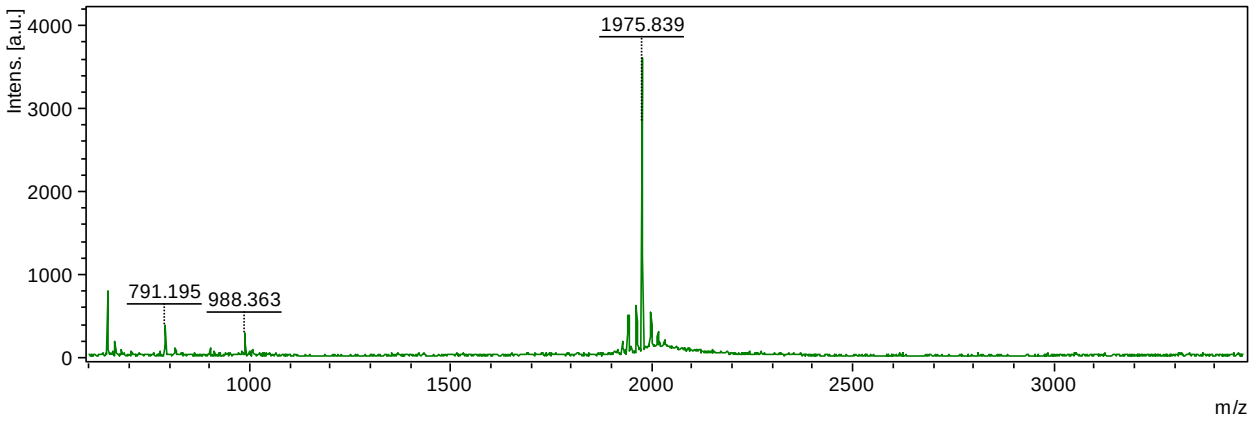
Crude peptide on semipreparative HPLC:



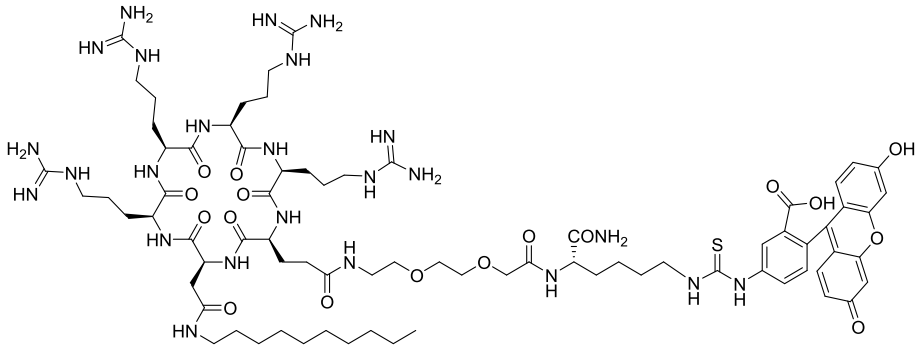
Purity check (analytical reversed-phase HPLC):



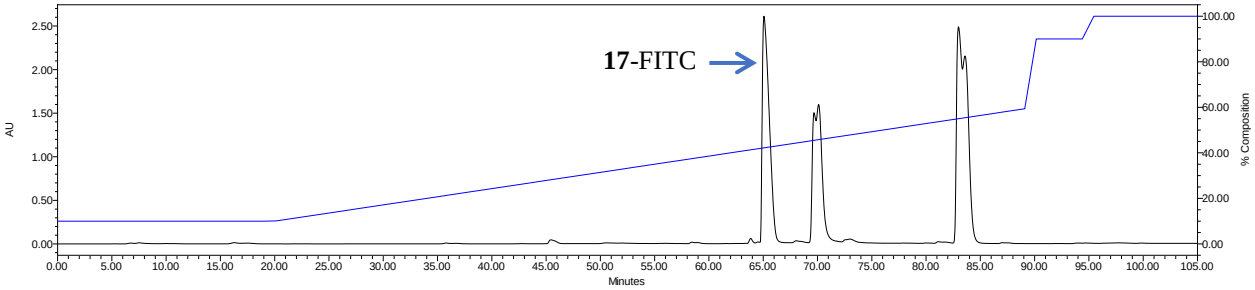
MALDI-TOF MS: Expected for $M+H^+$: 1975.932; Found 1975.839



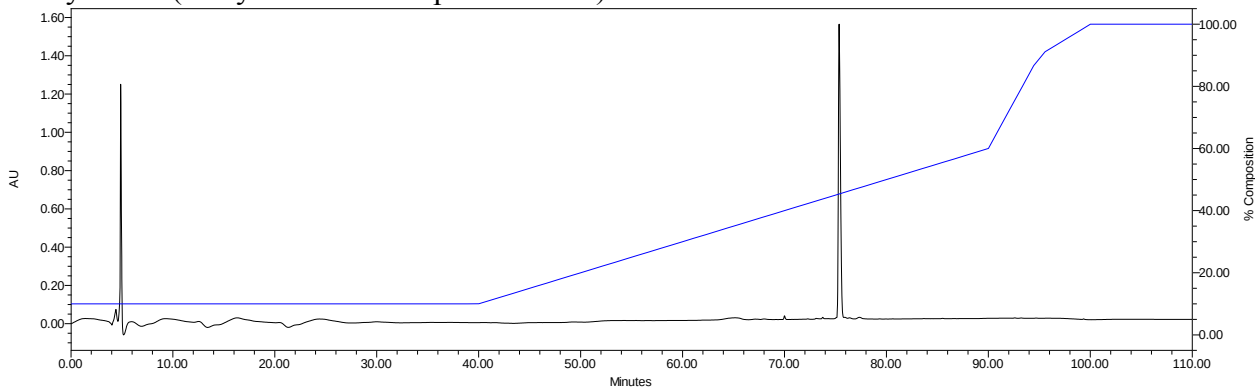
Peptide **17**-FITC



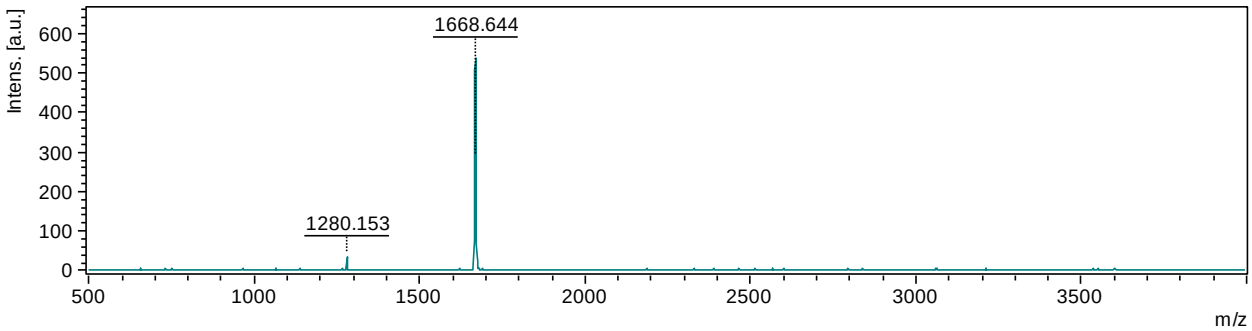
Crude peptide on semipreparative HPLC:



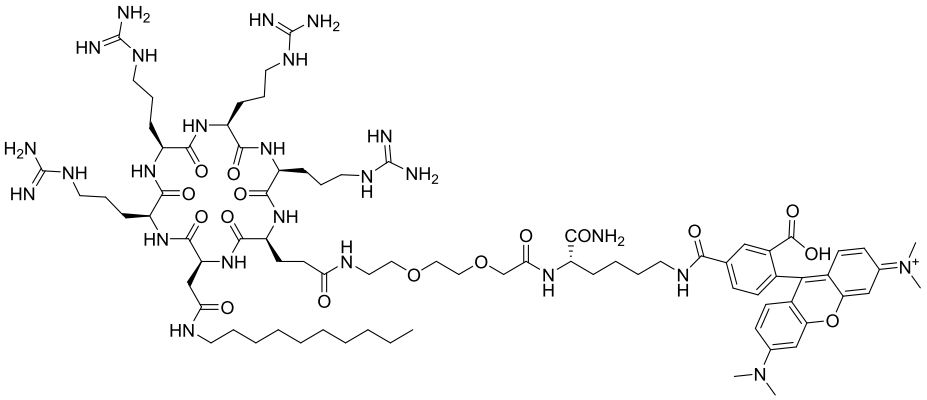
Purity check (analytical reversed-phase HPLC):



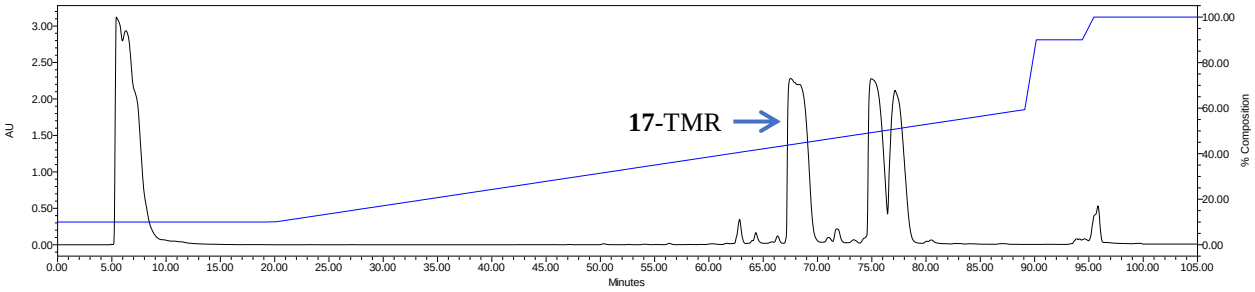
MALDI-TOF MS: Expected for $M+H^+$: 1668.867; Found 1668.644



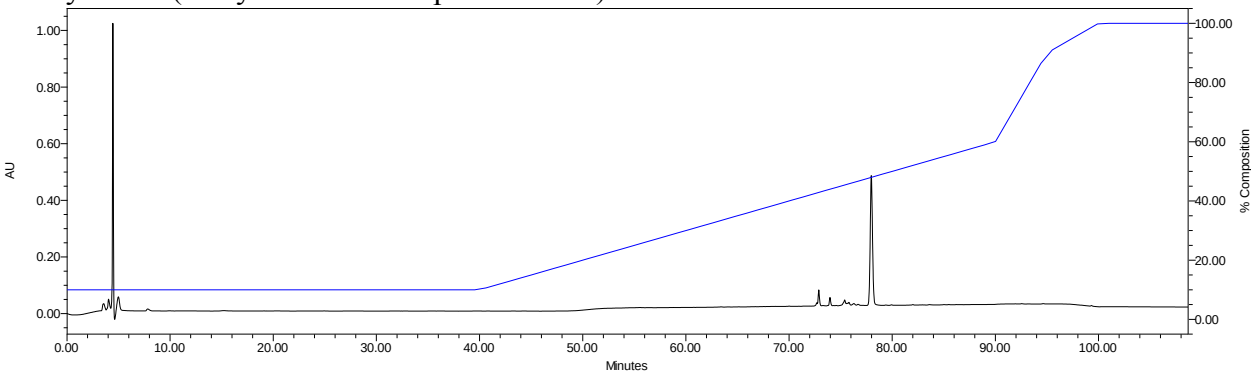
Peptide **17**-TMR



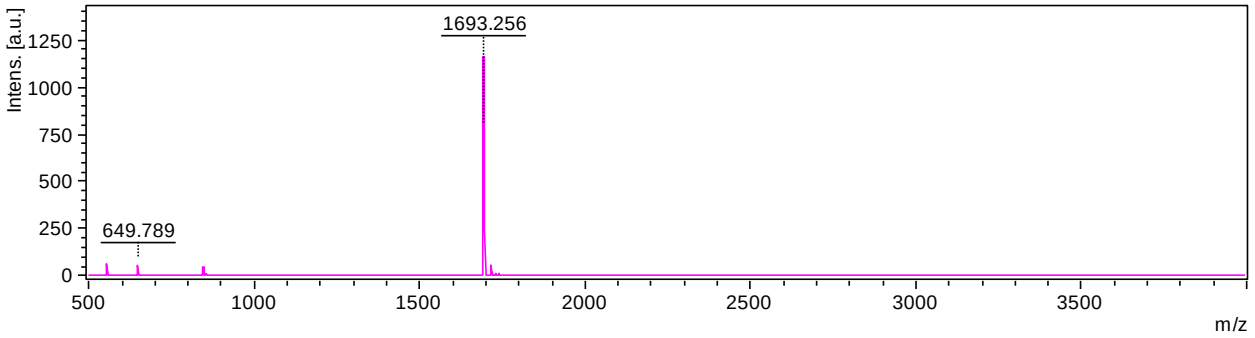
Crude peptide on semipreparative HPLC:



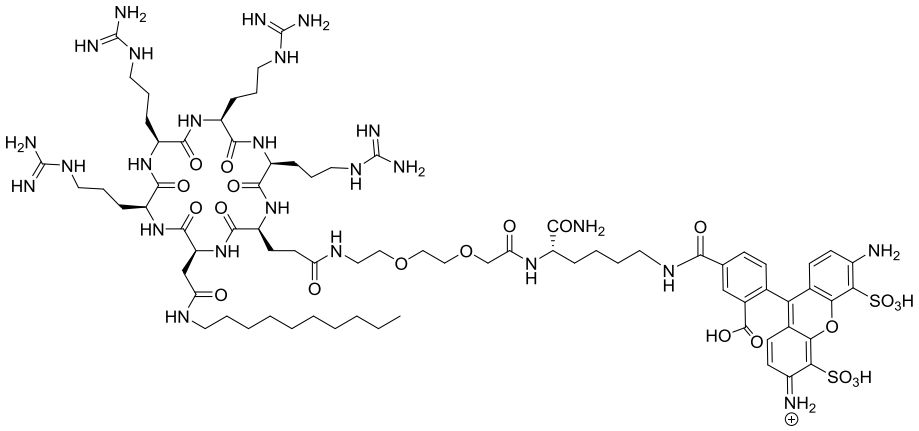
Purity check (analytical reversed-phase HPLC):

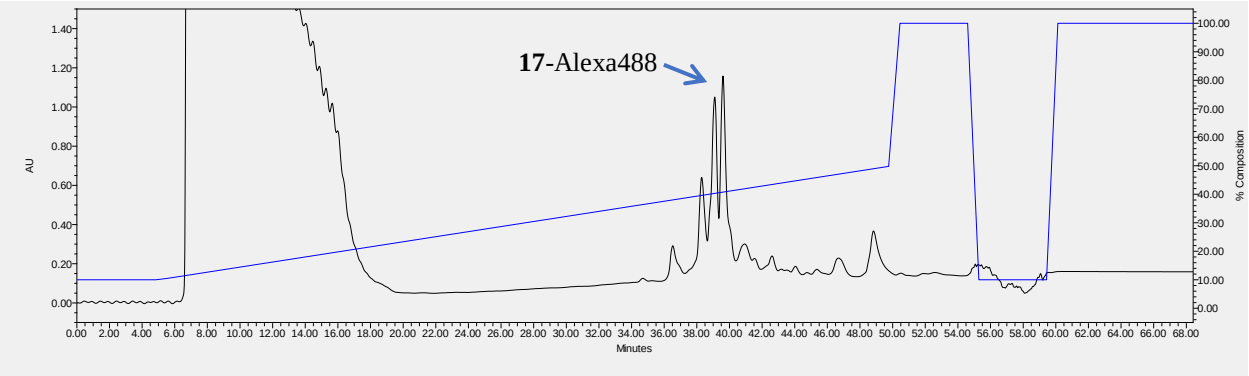


MALDI-TOF MS: Expected for $M+H^+$: 1692.98; Found 1693.256



Peptide **17**-Alexa488





MALDI-TOF MS: Expected for $M+H^+$: 1796.83; Found 1796.940