Cell-Permeable Bicyclic Peptidyl Inhibitors against NEMO-IkB kinase Interaction Directly from a Combinatorial Library

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Supporting Information

Materials. Peptide synthesis reagents were purchased from Chem-Impex (Wood Dale,IL), NovaBiochem (La Jolla, CA), or Anaspec (San Jose, CA). Rink amide resin LS (100-200 mesh, 0.27 mmol/g) was purchased from Advanced ChemTech (Louisville, KY). DMEM and RPMI cell culture media, fetal bovine serum, penicillin-streptomycin, 0.25% trypsin-EDTA, DPBS, and Dynabeads M-280 streptavidin were purchased from Invitrogen (Carlsbad, CA). N-(9-Fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) was from Advanced ChemTech. Biotin(peg)$_4$-NHS ester, isopropyl β-D-1-thiogalactopyranoside (IPTG), protease inhibitor cocktail tablets and ampicillin were purchased from Sigma-Aldrich (St. Louis, MO). Phenyl isothiocyanate was also purchased in 1-mL sealed ampules from Sigma-Aldrich, and a freshly opened ampule was used in each experiment. All solvents and other chemical reagents were obtained from Sigma-Aldrich, Fisher Scientific (Pittsburgh, PA), or VWR (West Chester, PA) and were used without further purification unless noted otherwise. Anti-GST-Tb and streptavidin-d2 were purchased from Cisbio (Bedford, MA). The NF-κB reporter (Luc)-HEK293 cell line and One-StepTM luciferase assay system were purchased from BPS Bioscience (San Diego, CA). The A375M cell line was obtained from ATCC (Manassas, VA). Ovarian Epitheial Cell Medium and trypsin neutralization solution (TNS) were purchased from ScienCell (Carlsbad, CA) for culture of the OSE cells. The cell proliferation kit (MTT) was purchased from Roche (Indianapolis, IN).

Library Synthesis. The peptide library was synthesized on 2 g of TentaGel S NH2 resin (130 μm) by modifying a previously reported protocol (**Figure S1a**).[1] The linker sequence (BBFM) was prepared using standard Fmoc/HATU chemistry (4 equiv fmoc-amino acid, 4 equiv HATU, 8 equiv diisopropyl ethylamine (DIPEA)). The coupling reactions were performed at room temperature for 2 h. The Fmoc protecting group was removed by treating the resin with 20% piperidine in DMF (2 x 10 min). Standard washing with DMF and DCM was performed between coupling and deprotection steps. To create a one bead-two compound library, the washed resin was soaked in DMF for 15 min followed by stepwise DMF/water mixtures (3:1, 1:1, 1:3). The dried resin was then soaked in degassed water overnight at room temperature. The water was drained and a solution of Fmoc-N-hydroxysuccinimide ester (Fmoc-OSu; 0.5 equiv) in 1:1 (v/v) DCM/diethyl ether was added to the resin and the suspension was incubated on a rotary shaker for 30 min. With the surface peptides N-terminally protected with Fmoc, the beads were washed with 1:1 DCM/diethyl ether and treated with a solution of di-*tert*-butyl dicarbonate (10 equiv) and 4-dimethylaminopyridine (DMAP, 0.05 equiv) in DMF for 45 min. After washing with DMF, the Fmoc protecting group was removed from the surface peptides with piperidine and 4-hydroxymethyl benzoic acid (HMBA) was coupled using standard HATU conditions. Next, Fmoc-β-Ala-OH was coupled to the HMBA moiety using DIC and DMAP to create an ester linkage for the surface peptides. Following Fmoc deprotection, Fmoc-L-propargylglycine-OH, two Fmoc-β-Ala-OH, and Fmoc-L-Dap(Alloc)-OH were coupled to the outer peptide using standard Fmoc/HATU conditions. Next, the Boc group on the inner peptides was removed by incubating

the resin in 95:5 (v/v) TFA/H_2O for 2 h and Fmoc-L-arginine(pbf)-OH was selectively coupled to the inner peptides to facilitate later MALDI analysis of hit peptides. The resin was treated with 20% piperidine to remove the Fmoc groups and split into twelve equal portions for the parallel synthesis of the 12 CPP sequences (with a different CPP sequence for each resin aliquot; **Table S1**). During the coupling of the N-terminal two residues of the CPP sequences, a small amount of capping agents (5% mol/mol CD_3CO_2D and/or $CH_3CD_2CO_2D$) were added to the coupling reactions to generate a few percent of chain termination products, which facilitates the determination of the CPP sequences of the library hits.[1] After the CPP sequence was completed, the beads were pooled and coupled with Fmoc-L-Dap(Mtt)-OH. Following removal of the Fmoc group, the resin was split into 24 equal portions, transferred into 24 different reaction vessels, and each was coupled with a different Fmoc-amino acid (**Figure S1b**). The resin was pooled, treated with 20% piperidine in DMF to remoce the Fmoc group, split again into 24 equal portions, and the split-and-pool synthesis was repeated. After coupling of the third, fourth, and fifth residue in the randomized sequence region, 25% of the resin was removed from the pool each time and set aside. For the last 25% of the resin, a sixth residue was added to the random region. Next, the four portions of resin were combined to generate a library containing 3-6 residues in the randomized region. Finally, the Mtt group on the internal Dap residue was removed with 2% TFA in DCM (v/v) and replaced with Fmoc (10 equiv Fmoc-OSu, 5 equiv DIPEA, 20 min.). The resin was incubated with 0.5 equiv $Pd(PPh_3)_4$ and 10 equiv. phenylsilane in DCM (3 x 20 min) to remove the Alloc protecting group on the C-terminal Dap residue. The resin was washed with 1% sodium dimethyldithiocarbamate (SDDC) in DMF (w/v) and a trimesic acid (TMA) was coupled to the Dap side chain amine. Next, the Fmoc protecting groups on the N-terminal and internal Dap resiudes were removed with piperidine and the resin was incubated in 1 M hydroxybenzotriazole (HOBt) in DMF for 15 min. After washing with DMF, the resin was treated with PyBOP (10 equiv), HOBt (10 equiv), and DIPEA (20 equiv) for 1 h to cyclize the surface peptides (twice). The resin was washed exhaustively with DMF and DCM and treated with a modified reagent K solution (5% phenol, 5% water, 5% thioanisole, 2.5% ethanedithiol, 2.5% TIPS, 1.25% anisole in TFA) for 3 h to deprotect the side chains. The resin was washed with DCM, dried in vacuum and stored at -20 °C until use.

Peptide Sequencing by PED-MS. Positive beads from screening were pooled into a glass reaction vessel and subjected to 9 rounds of partial Edman degradation. In each round, 160 μL of pyridine/water (v/v 2:1) containing 0.1% triethylamine was rapidly mixed with 160 μL of Fmoc-OSu/PITC (1:60 solution in pyridine) and immediately added to the vessel. After six minutes at RT the solution was drained, the beads were washed (pyridine 2x, DCM 3x, TFA 1x) and resuspended in 500 μL of TFA for 6 min (2x). After the incubation in TFA the beads were washed with DCM 2x and pyridine 2x and another PED cycle was initiated. After the final PED cycle was completed, the N-terminal Fmoc group was removed by treatment with 20% Piperidine in DMF. After washing, the beads are incubated in TFA containing ammonium iodide (5 mg) and dimethylsulfide (350 μL) for 30 minutes to reduce any oxidized methionine. Finally, the beads were washed with water and transferred to individual microcentrifuge tubes. The water was evaporated and 20 μL of CNBr (40 mg/mL) in 70% TFA/water was added to each tube. The tubes were left in the dark overnight at RT. Cleaved peptides were dissolved in 5 μL of 0.1% TFA/water and one μL of each peptide solution was mixed with one μL α-cyano-4-hydroxycinnamic acid solution (10 mg/mL in 1:1 acetonitrile/0.1% TFA in H_2O) in a separate tube. One μL of this mixture was spotted onto a MALDI sample plate for MS analysis with a Bruker Microflex

MALDI-TOF instrument. The spectra were analyzed on Bruker Baltonics FlexAnalysis 3.3 (Bruker Daltonic Gmb, Germany). Two representative PED-MS spectra are shown in **Figure S6**.

Individual Peptide Synthesis. Peptides were synthesized with standard Fmoc/HATU chemistry on Rink amide resin (0.27 mmol/g). Peptides were deprotected and cleaved from the resin by using 92.5:2.5:2.5:2.5 (v/v) TFA/water/1,3-dimethoxybenzene/triisoproplysilane (TIPS) for 3 h. The resultant solution was triturated with 5 mL of cold ethyl ether (3x). After centrifugation the ether was poured off and the solid peptide was dried overnight under vacuum. Dried peptide was purified to at least 90% homogeneity on a reversed-phase HPLC equipped with a C18 column. To label the peptides with FITC, 10 μL of FITC solution in DMF (100 mg/mL) was added to the purified peptide in 300 μL of 2:1 DMF/0.15 M sodium bicarbonate (pH 8.5). After 30-40 min at RT, the labeling mixture was re-purified with reversed-phase HPLC to obtain the labeled peptide. The purity and authenticity of each peptide was established by analytical HPLC and MALDI-TOF MS analysis (**Figure S7**).

A ^{1}H NMR spectrum of peptide **7** (**Figure S8**) was obtained by dissolving the peptide in 500 μL of 5:1 $CDCl_3$/DMSO-D_6 (final concentration 5 mM) and collecting data on a Bruker Ascend 700 MHz spectrometer at 298 K.

Fluorescence Anisotropy. FA titration experiments were performed by incubating 50 nM FITC-labeled Peptide 7 with varying concentrations of the respective protein in PBS + 0.01% Triton™ X-100 at RT. For the NEMO protein the concentration was kept low (<2 μM) to avoid problems with NEMO oligomerization at higher concentrations, which can lead to abberant FA increases and prevent curve saturation. After waiting one hour to establish an equilibrium, the FA values were measured on a Tecan Infinite m1000 Pro, with excitation and emission wavelengths at 470 and 535 nm, respectively. Equilibrium dissociation constants (K_D) were determined by plotting the fluorescence anisotropy values as a function of protein concentration. The titration curves were fitted to the following equation

$$Y = \frac{\left(A_{min} + \left(A_{max} \times \frac{Q_b}{Q_f} - A_{min}\right)\left(\frac{(L+x+K_d)-\sqrt{((L+x+K_d)^2-4Lx)}}{2L}\right)\right)}{\left(1 + \left(\frac{Q_b}{Q_f} - 1\right)\left(\frac{(L+x+K_d)-\sqrt{((L+x+K_d)^2-4Lx)}}{2L}\right)\right)}$$

where Y is the measured anisotropy at a given protein concentration x; L is the bicyclic peptide concentration; Qb/Qf is the correction factor for dye-protein interaction; A_{max} is the maximum anisotropy when all the peptides are bound, while A_{min} is the minimum anisotropy for peptide alone. Data presented are the mean ± SD of three independent experiments.

Cell Culture. HeLa, HEK293, and A375M cells were cultured in DMEM media containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin sulfate. The same media plus 50 μg/mL hygromycin B was used to maintain the HEK293(Luc) cells (NF-kB reporter strain). A2780 and CP70 cells were maintained in RPMI media containing 10% FBS and 1% penicillin/streptomycin sulfate. OSE cells were cultured in complete ovarian epithelial cell culture medium (ScienCell). All cells were cultured at 37 °C in the presence of 5% CO_2 in a humidified incubator.

NF-κB Luciferase Reporter Assay. The NF-κB luciferase assay was conducted similarly to previously published protocols.[2] Culture media was exchanged for DMEM containing 10% FBS and 1% penn/strep the day before seeding to remove hygromycin B. HEK293(Luc) cells were seeded in 50 μL assay medium (DMEM, 10% FBS, and 1% penicillin/streptomycin) in an opaque 96-well microplate (3000 cells per well) and incubated overnight. The peptide inhibitors were added to the cells the next day in 5 μL of assay medium and the plate was incubated at 37 °C for 2 h. After that, 5 μL of assay media containing recombinant TNFα (final concentration 5 ng/mL) was added to the wells. The plate was then returned to the incubator for 4 h at 37 °C. Finally, 50 μL of ONE-Step luciferase assay reagent was added to each well and after 10 min the luminescence was measured on a Tecan Infinite M1000 Pro microplate reader. The luciferase activity for the TNFα induced and un-induced control wells was reported as AU^- and AU^+. The luciferase activity of TNFα induced wells incubated with the peptide inhibitors is reported as AU^{pep}. The following equation was then used to calculate the percentage of luciferase activity in each treatment well. Data presented are the mean ± SD of three independent experiments.

$$\% \text{ Inhibition of TNF}\alpha \text{ Activation} = (AU^{pep}-AU^-)(AU^+-AU^-)$$

To assess the effects of this inhibitor on basal NF-kB activation the same assay was performed without the addition of TNFα. Data presented are the mean ± SD of six independent experiments.

Serum Stability Assay. This assay was performed according to previous literature protocols.[3] Diluted human serum (25%) was first centrifuged for 10 min at 15,000 rpm in a microcentrifuge. The supernatant was removed and used to dilute the peptide stock to ~10 μM. The peptide was incubated at this concentration in serum at 37 °C and 200-μL aliquots were withdrawn at various time points. This solution was mixed with 50 μL of 15% trichloroacetic acid and 200 μL of acetonitrile and this mixture was left at 4 °C overnight. Finally, the samples were centrifuged at 15,000 rpm for 10 min in a microcentrifuge and the supernatant was analyzed on a reversed-phase HPLC equipped with an analytical C18 column (Waters). The amount of peptide remaining at each time point was determined by integrating the area under the peptide peak in the resulting HPLC chromatogram (monitored at 214 nm) and comparing to the peptide amount at the time zero (**Figure S4**).

References

(1) Lian, W. L.; Upadhyaya, P.; Rhodes, C. A.; Liu, Y.; Pei, D. *J. Am. Chem. Soc.* **2013**, *135*, 11990-11995.

(2) Qian, Z.; Rhodes, C. A.; McCroskey, L.; Wen, J.; Appiah-Kubi, G.; Wang, D. J.; Guttridge, D. C.; Pei, D. *Angew. Chem. Int. Ed. Engl.* **2017**, 56, 1525-1529.

(3) Nguyen, L. T.; Chau, J. K.; Perry, N. A.; de Boer, L.; Zaat, S. A.; Vogel, H. J. *PLoS One* **2010**, 5, e12684.

Table S1. Sequences of CPP motifs incorporated into the bicyclic library

CPP ID	CPP Sequence[a]	Capping and Mass Pattern
1	F-Φ-R-R-R-R	No capping
2	R-R-R-R-Φ-F	No capping
3	F-Φ-R-R-R	Φ + 58
4	R-R-R-Φ-F	R + 45
5	F-Φ-R-F-R-R	N/A
6	R-R-F-R-Φ-R	R + 58
7	F-R-R-R-R-Φ	No capping
8	Φ-R-R-R-F	No capping
9	f-Φ-R-r-R-r	f + 45
10	f-Φ-R-r-R	f + 58
11	r-R-r-R-Φ-f	R + 45
12	F-ϕ-r-R-r-R	ϕ + 45

[a]Residues shown in red indicate where 5% capping agent was added during the coupling reaction of the indicated (red colored) amino acid. Φ = L-2-napthylalanine; ϕ, D-2- napthylalanine; lowercase letter = D-AA.

Table S2. Sequences of library screening hits and their NEMO-binding affinity

Hit No.	Sequence[a]	IC_{50} (μM)
#1	(D-Val-Ala-Fpa-Phg-Gly)-Dap-(D-Arg-Arg-D-Arg-Arg-Nal-D-Phe-Dap)	5.8 ± 1.8
#2	(Trp-Ile-D-Glu)-Dap-(Phe-Nal-Arg-Arg-Arg-Dap)	17 ± 4
#3	(His-Gly-Ala-Trp-D-Phe)-Dap-(Phe-Nal-Arg-Arg-Arg-Dap)	25 ± 4
#4	(Gly-Trp-Ile-Tyr)-Dap-(Phe-Nal-Arg-Arg-Arg-Dap)	3.4 ± 0.6
#5	(Nle-Asp-D-Nal)-Dap-(D-Arg-Arg-D-Arg-Arg-Nal-D-Phe-Dap)	8.4 ± 1.4
#6	(D-Glu-Trp-D-Phe-D-Val)-Dap-(Nal-Arg-Arg-Arg-Phe-Dap)	13 ± 3
#7	(Trp-Phg-Gly-D-Phe)-Dap-(Arg-Arg-Arg-Nal-Phe-Dap)	6.6 ± 1.7
#8	(D-Val-Gly-Ile-Pro)-Dap-(Phe-Nal-Arg-Arg-Arg-Dap)	11 ± 4
#9	(Nle-Trp-Ser)-Dap-(Nal-Arg-Arg-Arg-Phe-Dap)	28 ± 4
#10	(His-Asp-Phg)-Dap-(D-Phe-Nal-Arg-D-Arg-Arg-Dap)	8.1 ± 2.0
#11	(Gln-Asp-D-Nal)-Dap-(D-Phe-Nal-Arg-D-Arg-Arg-D-Arg-Dap)	N/A
#12	(Ala-Ile-D-Phe-Asp)-Dap-(Arg-Arg-Arg-Nal-Phe-Dap)	N/A

[a]All peptides contained a β-Ala-β-Ala-Lys (BBK) motif at the α-carboxyl group of the C-terminal Dap as a handle for fluorescent labeling.

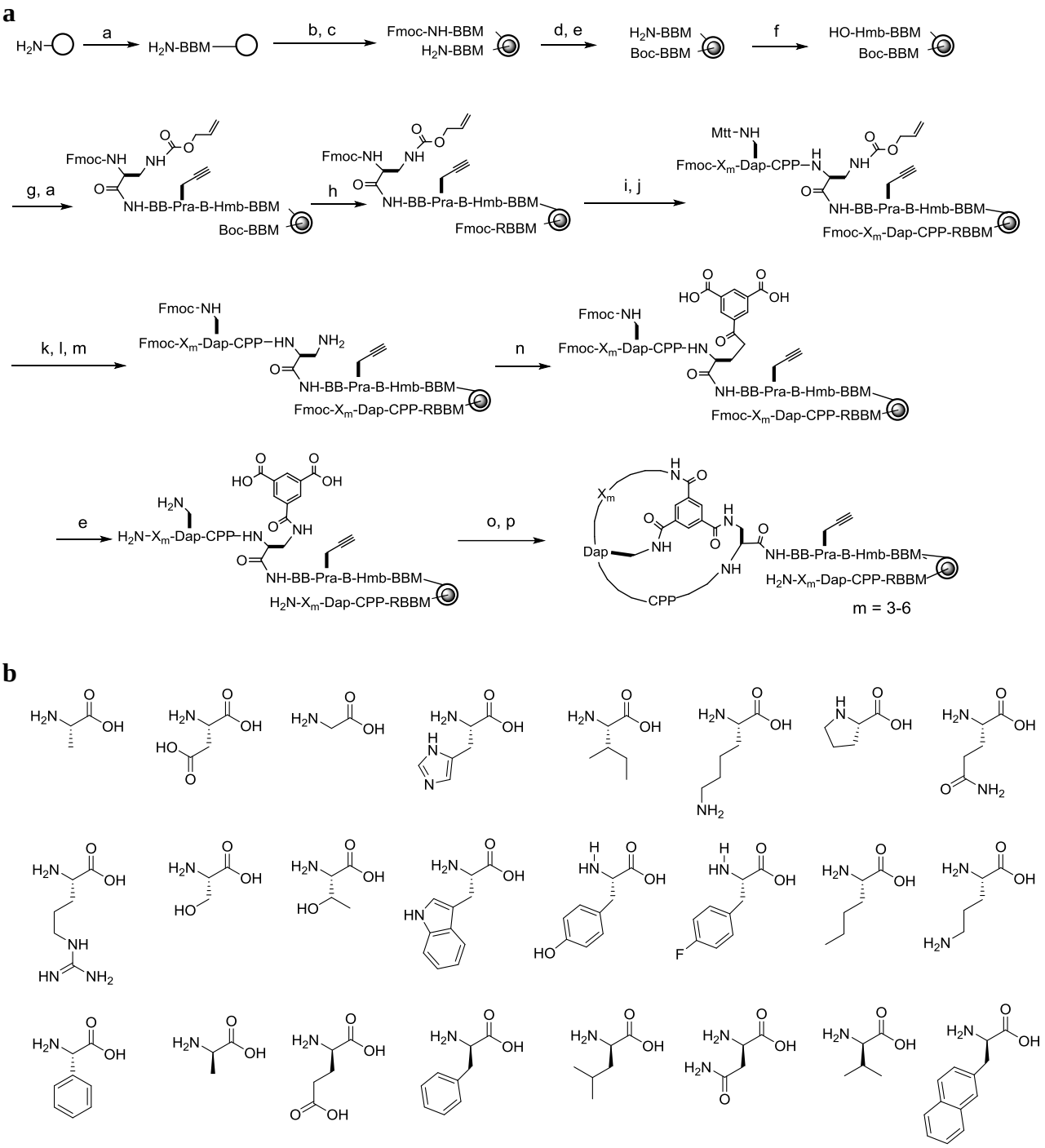


Figure S1. a) Synthesis of the bicyclic peptide library. Reagents and Conditions: (a) standard Fmoc/HATU chemistry; (b) soak in water; (c) 0.5 equiv Fmoc-OSu in Et_2O/CH_2Cl_2; (d) di-t-butyl dicarbonate; (e) piperidine; (f) 4-hydroxybenzoic acid/ HBTU/HOBT; (g) Fmoc-β-Ala-OH/DIC; (h) 50% TFA and then Fmoc-Arg(Pbf)-OH/HATU; (i) split resin and couple 12 CPP sequences by Fmoc/HATU chemistry; (j) split-and-pool synthesis by Fmoc/HATU chemistry; (k) 2% TFA in DCM; (l) Fmoc-OSu/DIPEA in DCM; (m) $Pd(PPh_3)_4$; (n) trimesic acid/HATU; (o) PyBOP/HOBt/DIPEA; and (p) reagent K. **b**) Structures of the 24 amino acid building blocks.

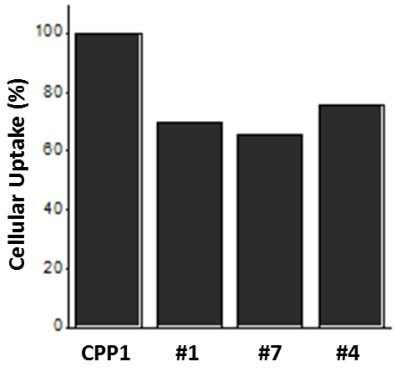


Figure S2. Cellular uptake efficiency of library hits No. #1, #4, and #7 (**Table S2**). HeLa cells were treated with 5 μM FITC-labeled peptides for 2 h and then analyzed by flow cytometry. All values are relative to that of CPP1 (100%).

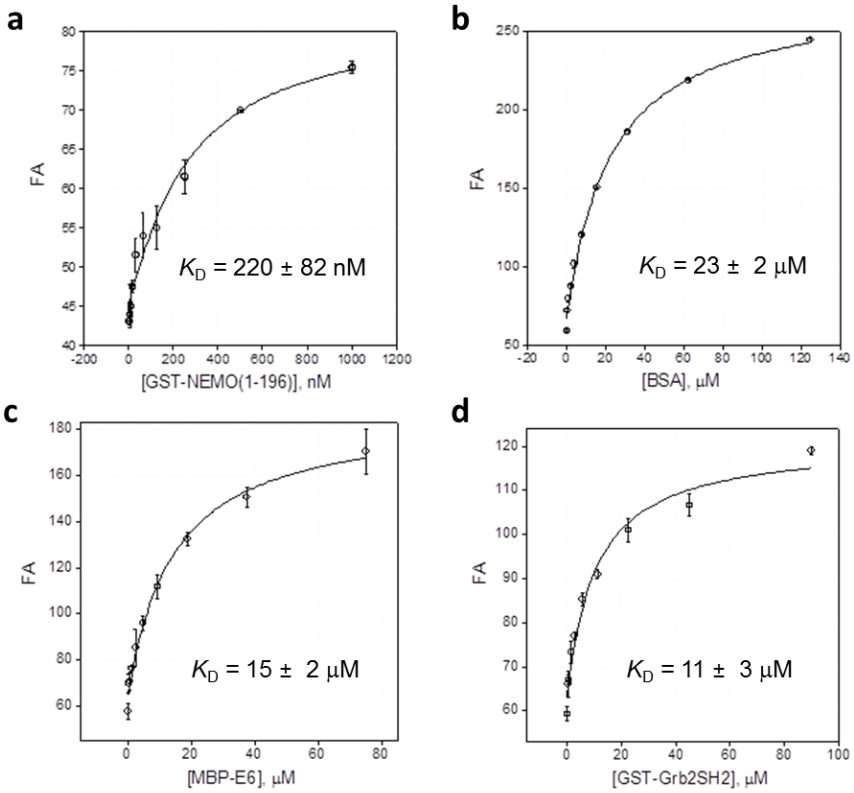


Figure S3. Binding of FITC-labeled peptide **7** (50 nM) to GST-NEMO (**a**) and three arbiturily selected control proteins: bovine serum albumin (**b**), maltose binding protein-E6 fusion (**c**), and GST-Grb2-SH2 (**d**) as monitored by fluorescence anisotropy (FA). The results demonstrate that peptide **7** is a selective ligand of NEMO. Note that GST-NEMO undergoes oligomerization at concentrations above 1 μM, complicating the FA-based binding assays. Therefore, the HTRF assay, which involved a constant NEMO concentration, was employed in this study to determine the IC_{50} values against the NEMO-IKK interaction.

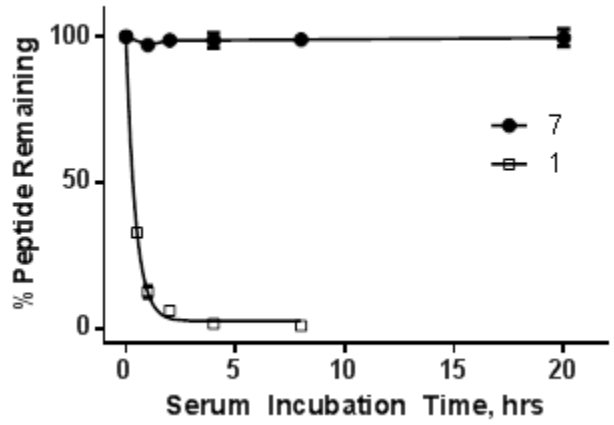


Figure S4. Comparison of the serum stability of peptides **1** (Antp-NBD) and **7**. Data presented are the mean ± SD of three independent experiments.

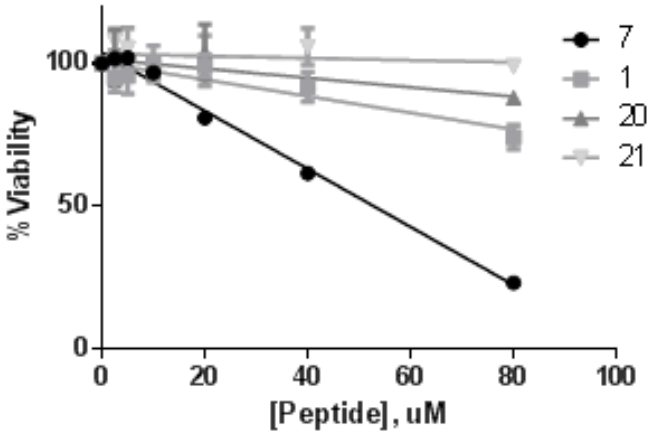
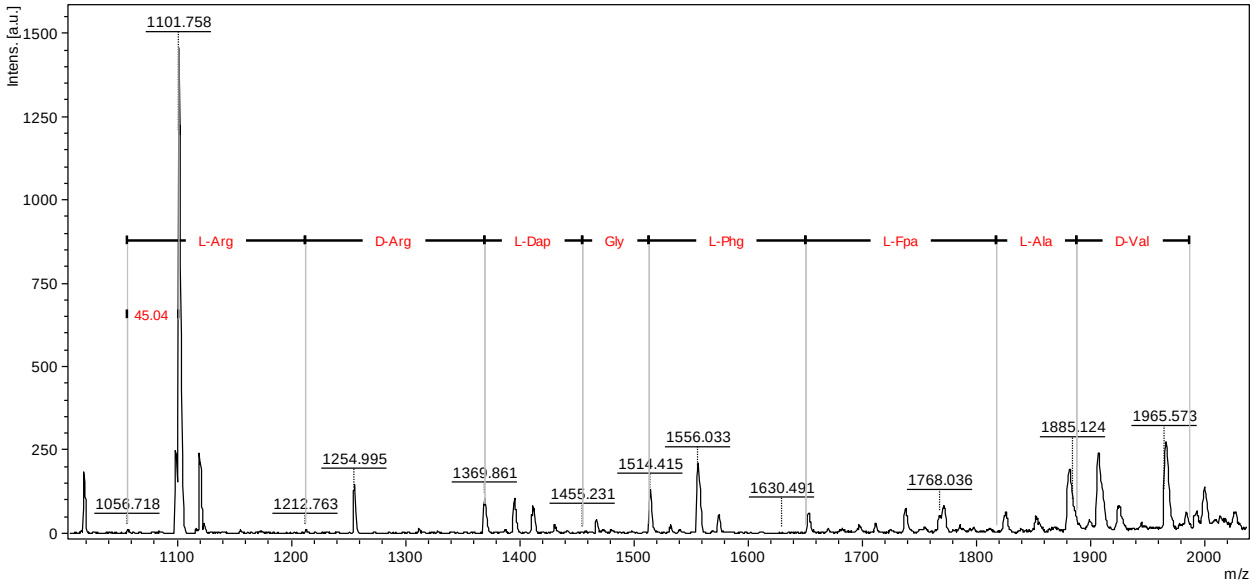


Figure S5. Effect of peptides **1** (Antp-NBD), **7**, **20**, and **21** on the viability of melanoma A375 cells, as monitored by the MTT assay.

a) Hit #1 (Table S2): D-Val-Ala-Fpa-Phg-Gly-Dap-D-Arg-Arg-D-Arg-Arg-Nal-Phe-RBBM*



b) Hit #5 (Table S2): Nle-Asp-D-Nal-Dap-D-Arg-Arg-D-Arg-Nal-D-Phe-Dap-RBBM*

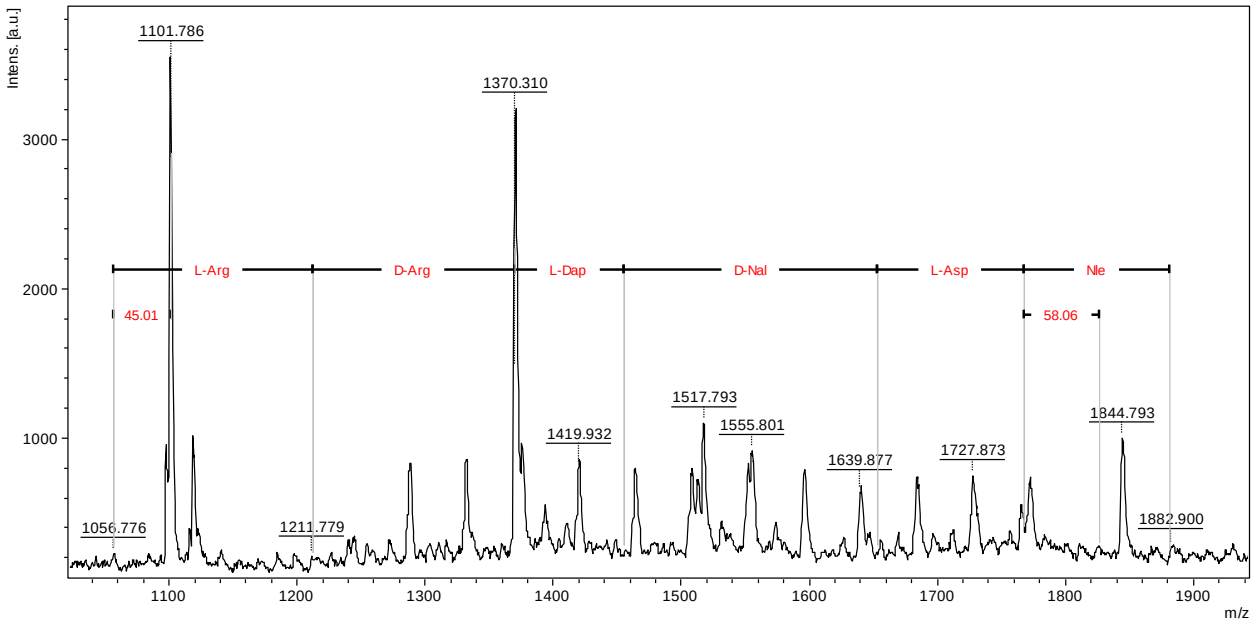
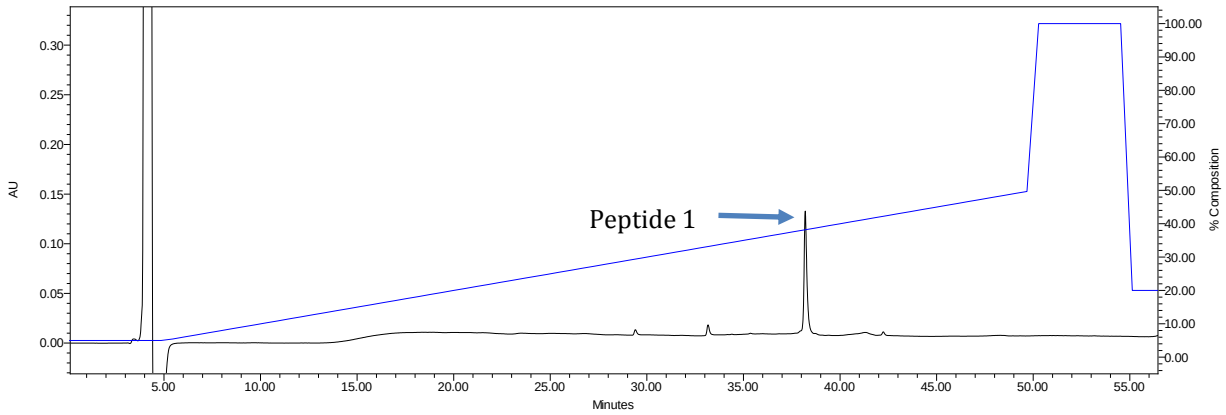


Figure S6. Representative PED-MS spectra of a) hit #1 and b) hit #5 (Table S2). M* = homoserine lactone.

Figure S7. HPLC and MS analysis of peptides used in this work. The purity of each peptide was assessed by reversed-phase HPLC equipped with an analytical C_{18} column. The authenticity of the peptide was confirmed by high-resolution MALDI-TOF MS analysis. Lowercase letter denotes D-amino acid. Φ = L-2-napthylalanine.

Peptide 1: RQIKIWFQNRRMKWKKGGTALDWSWLQTE



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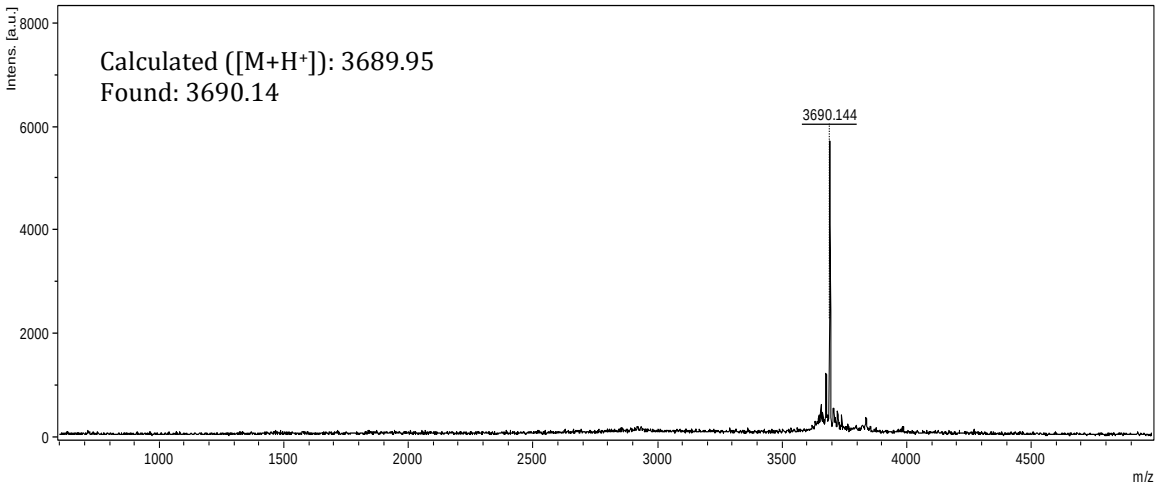
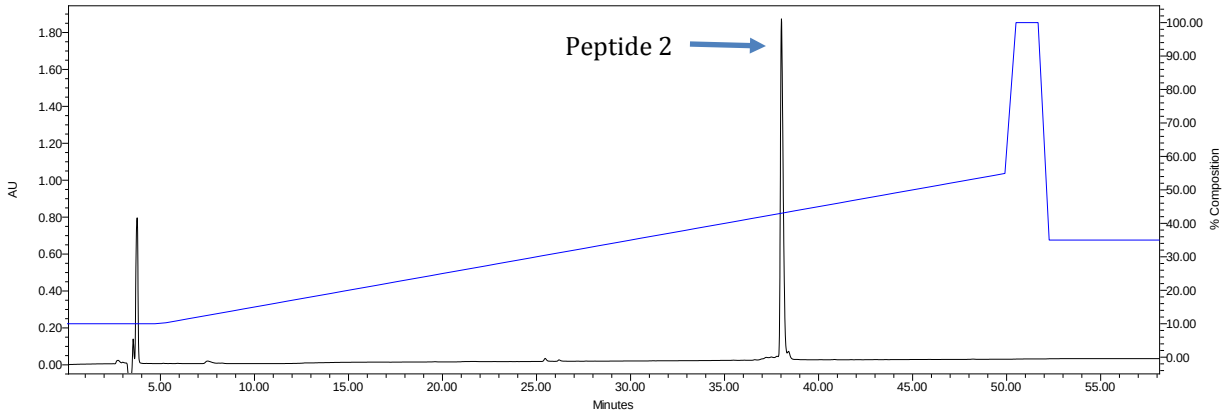


Peptide 2: bicyclo(GWIY)-Dap-(FΦRRR-Dap)-BBK



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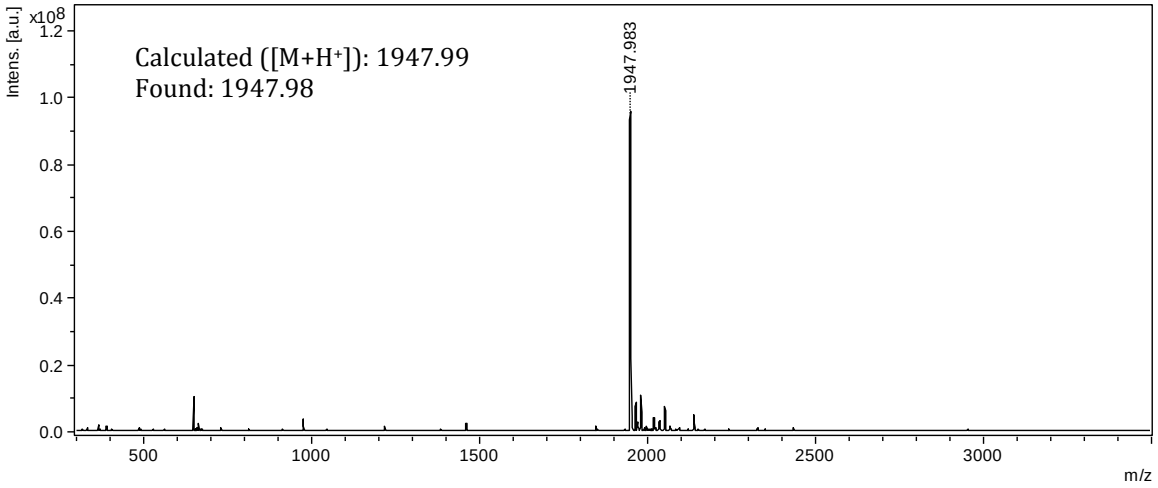
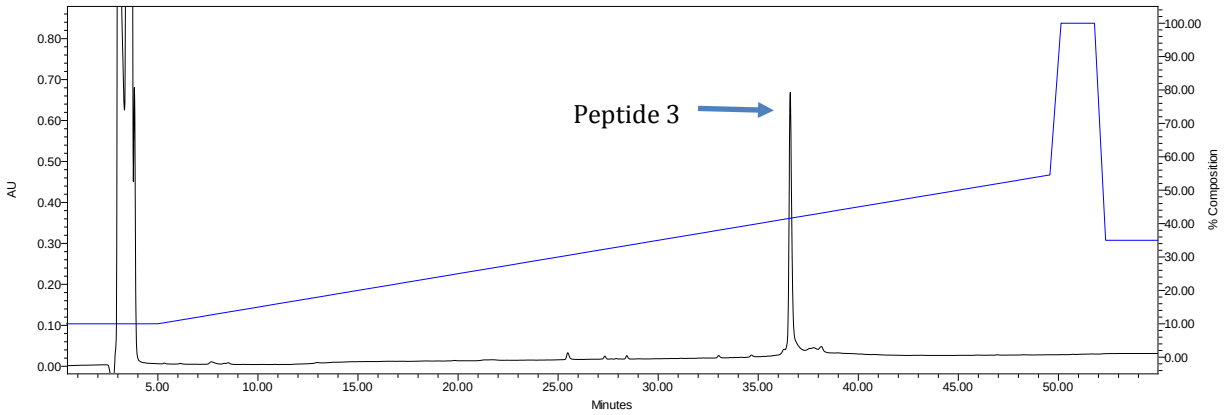


Peptide 3: bicyclo(GWIY)-Dap-(FΦRRRR-Dap)-BBK



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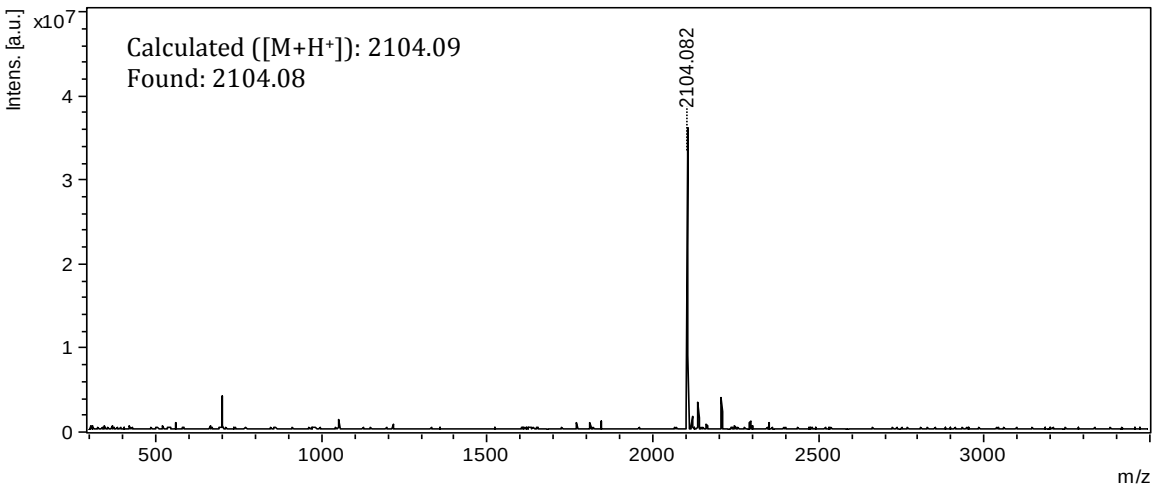
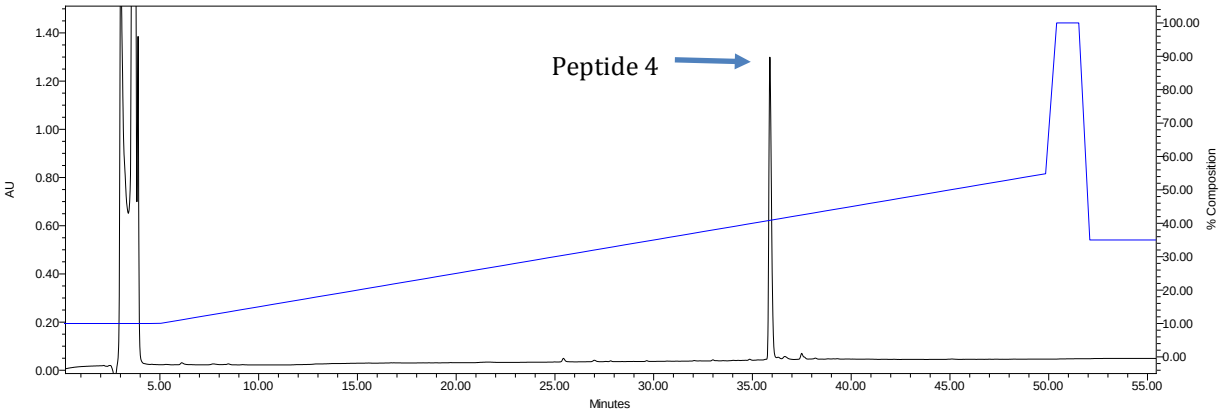


Peptide 4: bicyclo(GWIY)-Dap-(FΦRRR-Dap)-RBK



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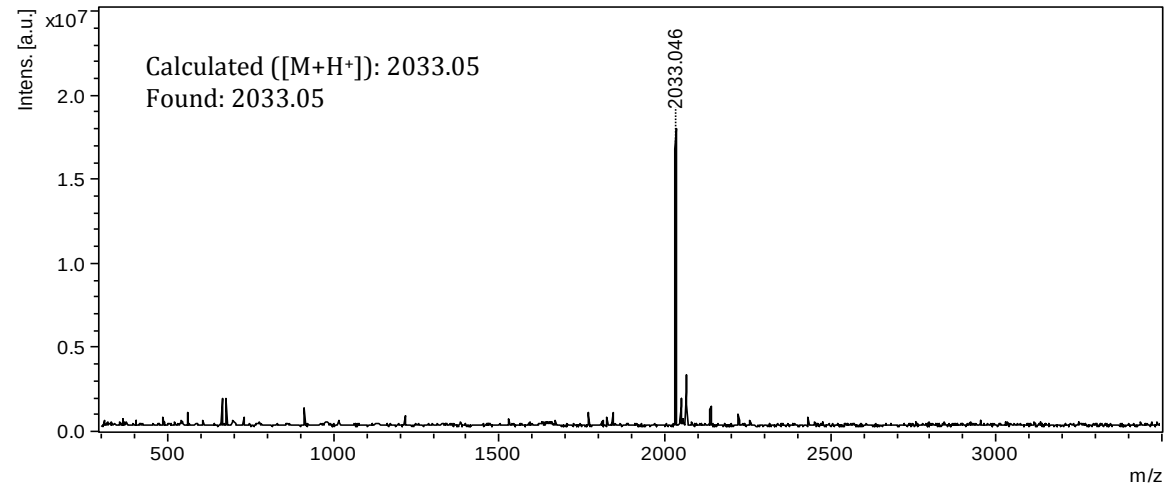
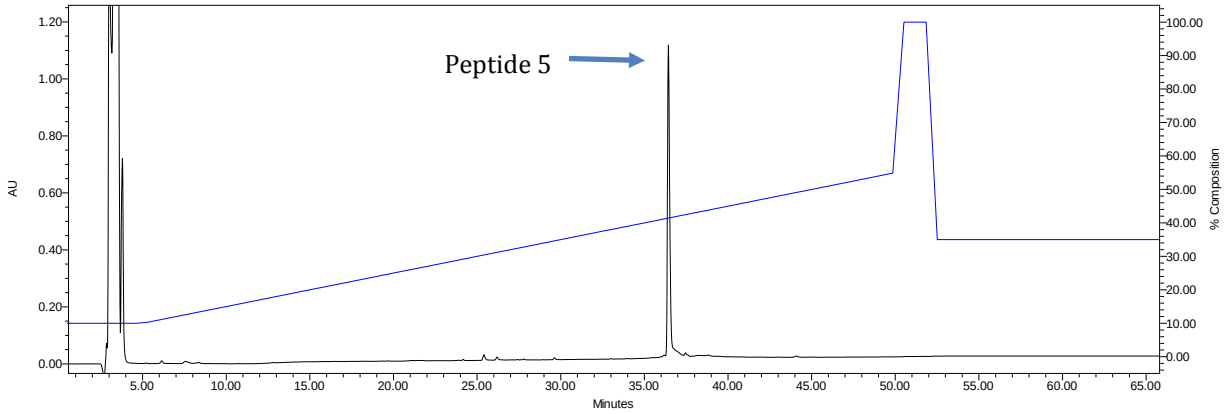


Peptide 5: bicyclo(GWIY)-Dap-(FΦRRR-Dap)-rBK



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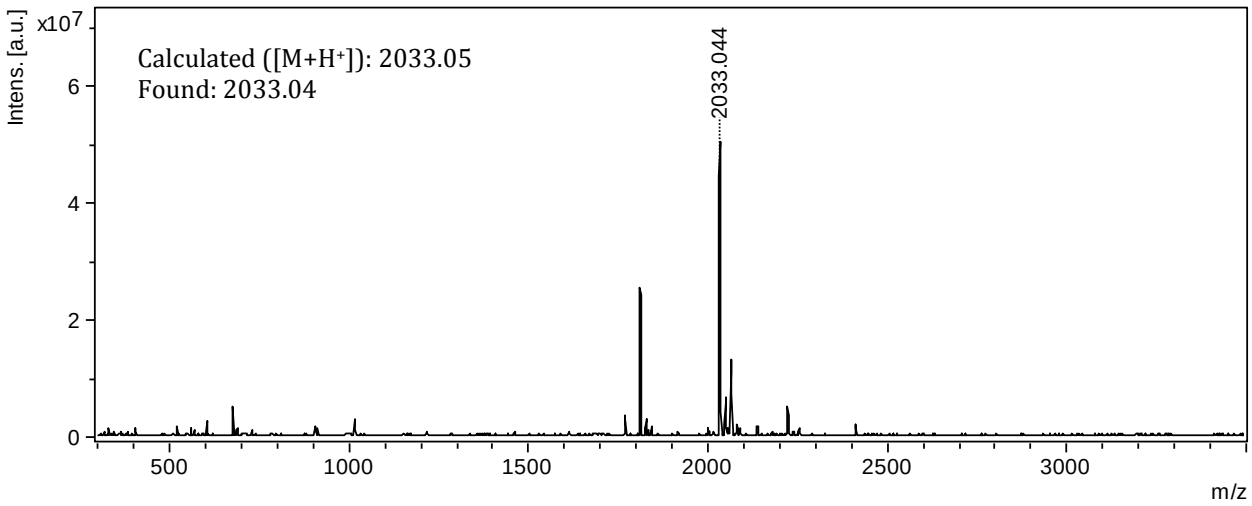
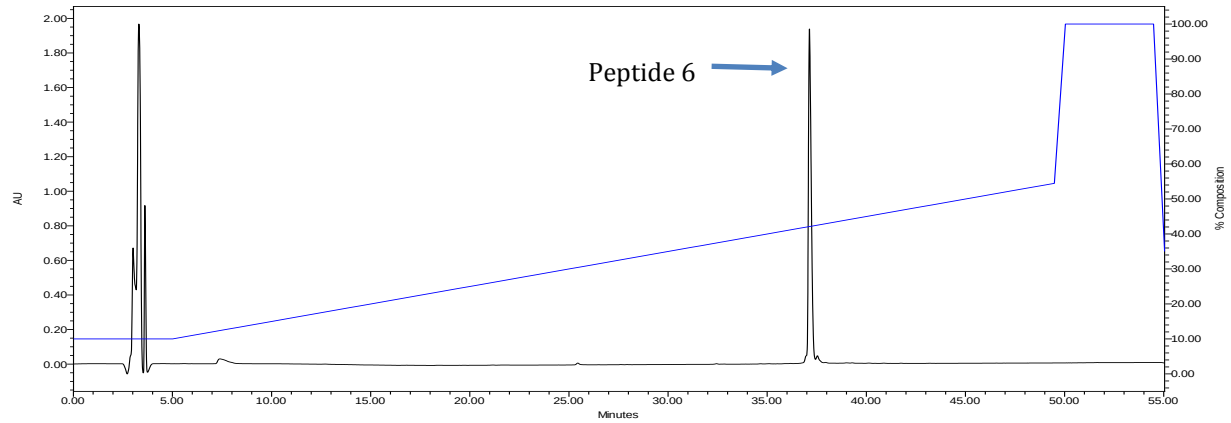


Peptide 6: bicyclo(GWIY)-Dap-(FΦRrR-Dap)-BBK



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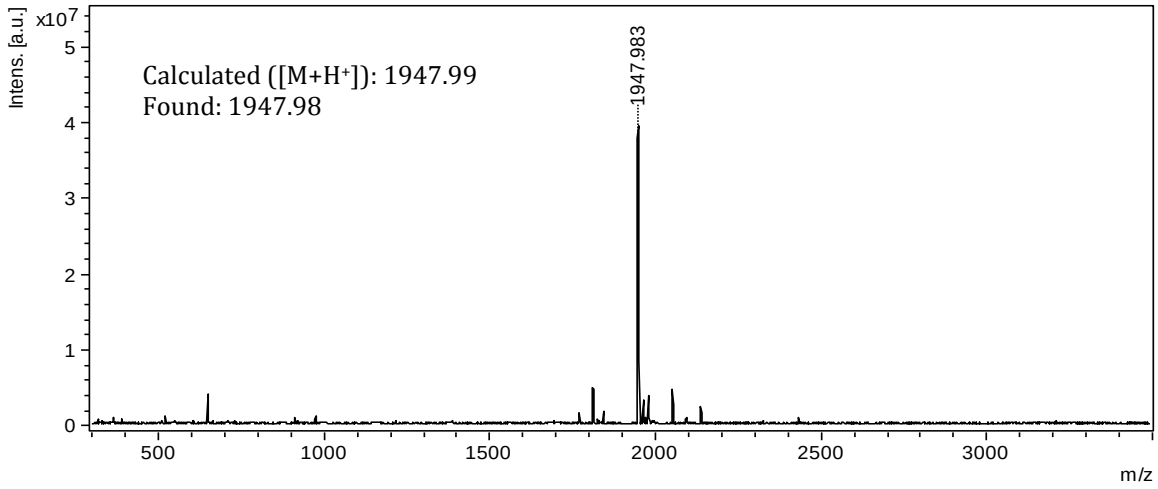
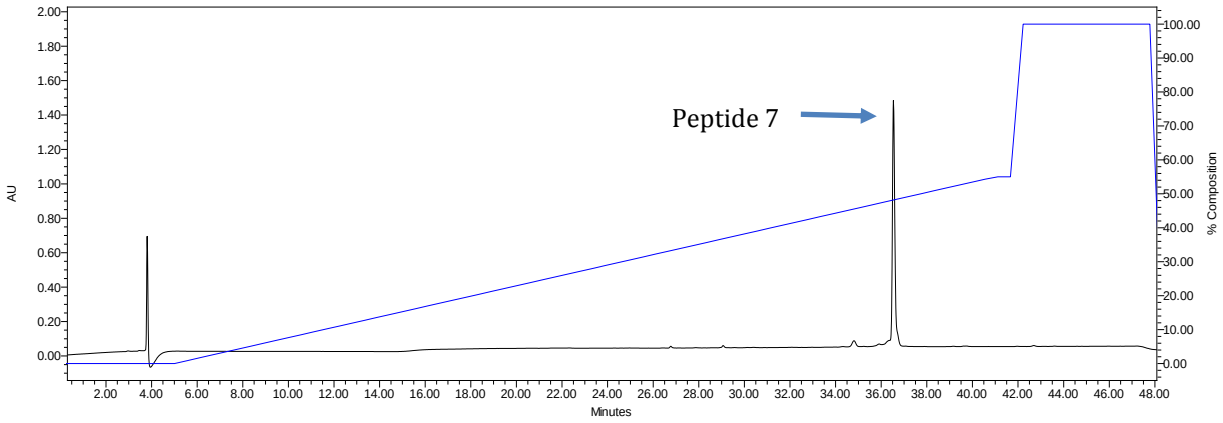


Peptide 7: bicyclo(GWIYA)-Dap-(FΦRrR-Dap)-BBK



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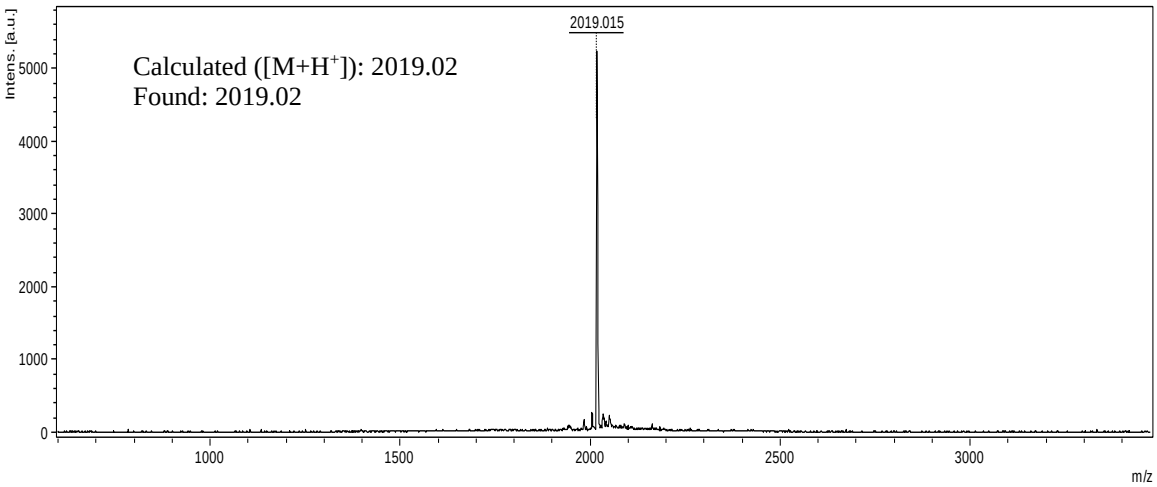
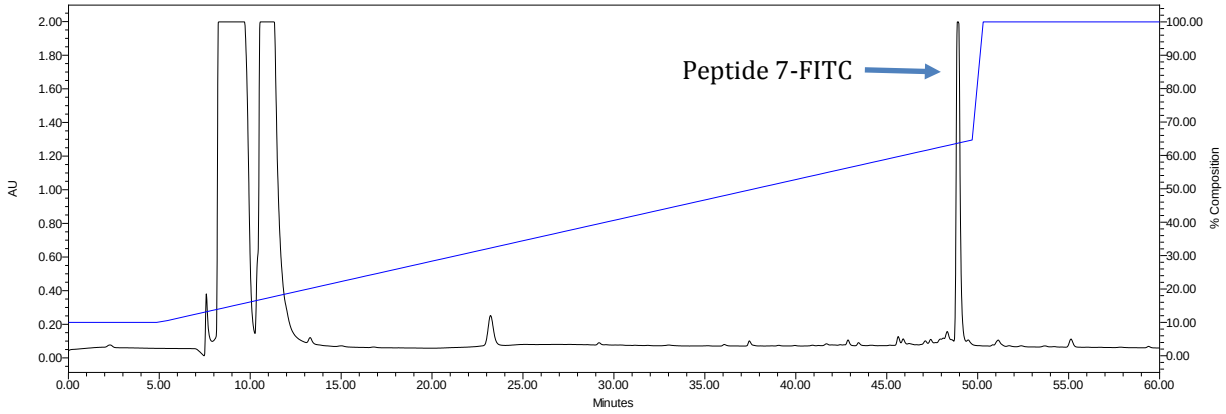


Peptide 7-FITC: bicyclo(GWIYA)-Dap-(FΦRrR-Dap)-BBK(FITC)



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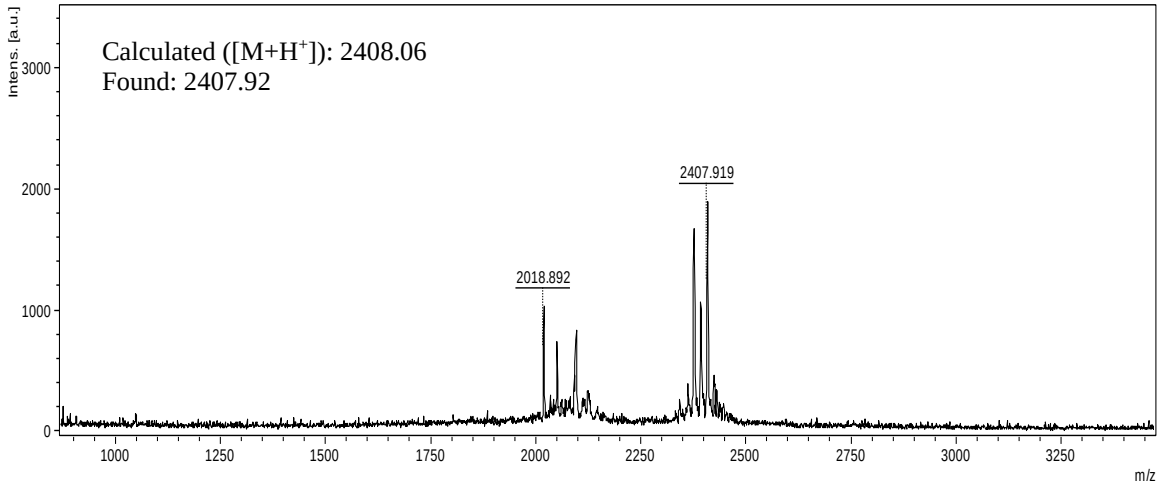
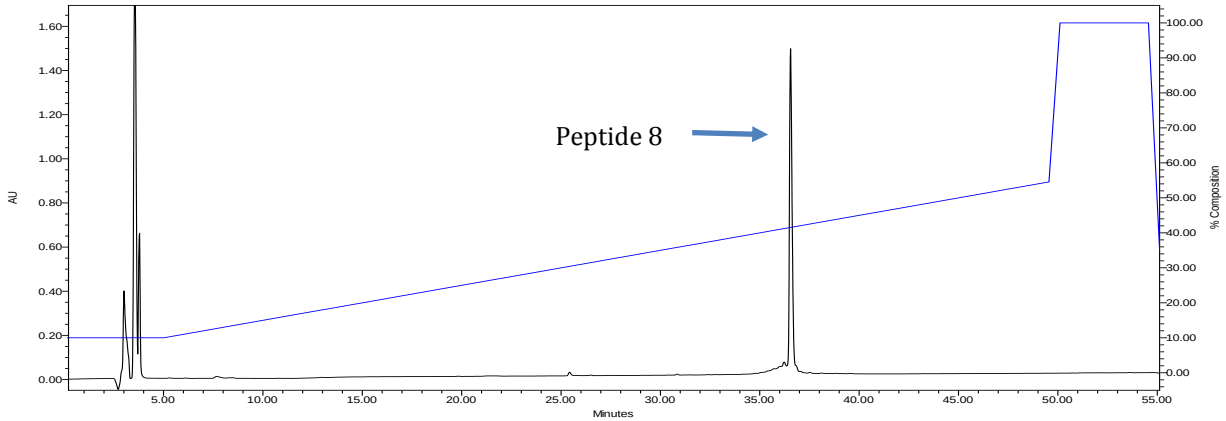


Peptide 8: bicyclo(GWIYa)-Dap-(FΦRrR-Dap)-BBK



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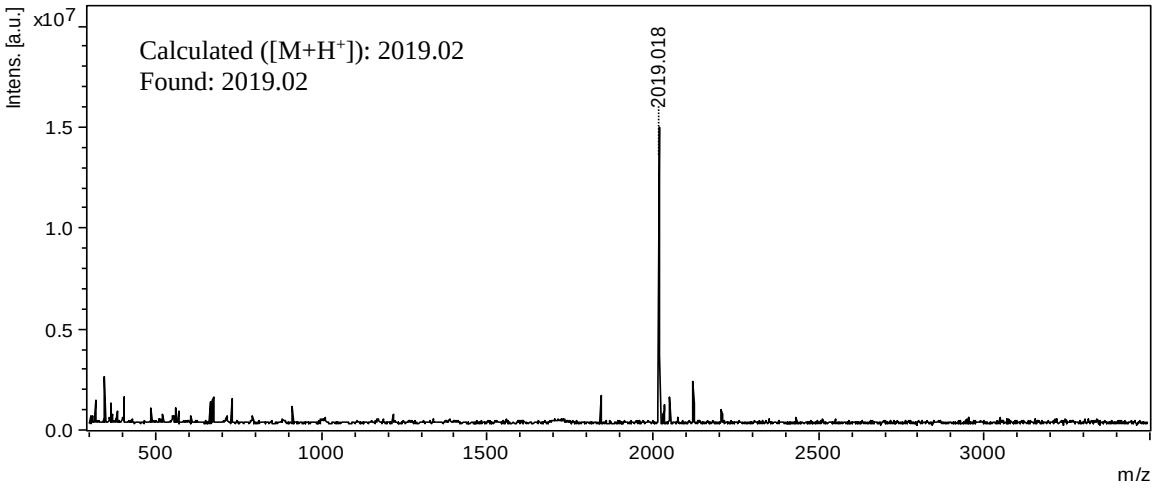
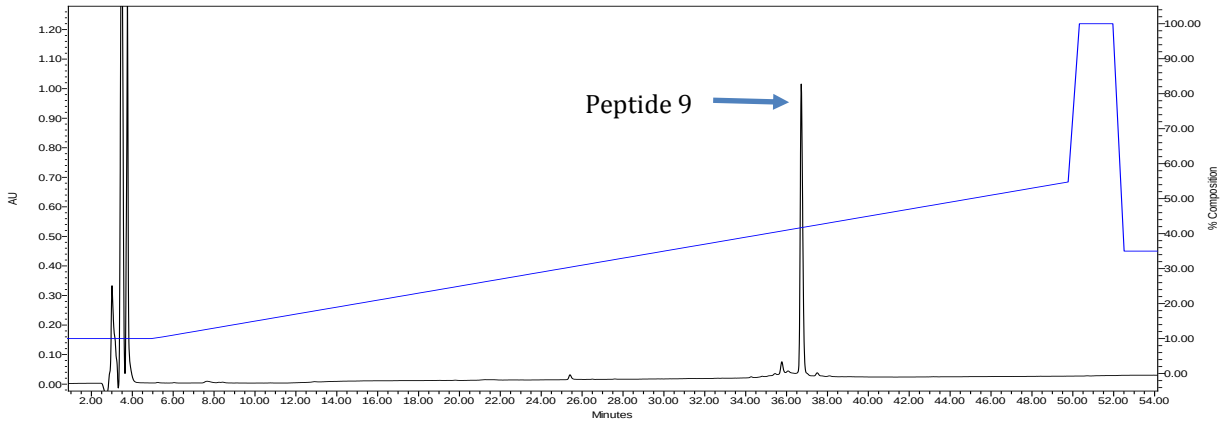


Peptide 9: bicyclo(AGWIY)-Dap-(FΦRrR-Dap)-BBK



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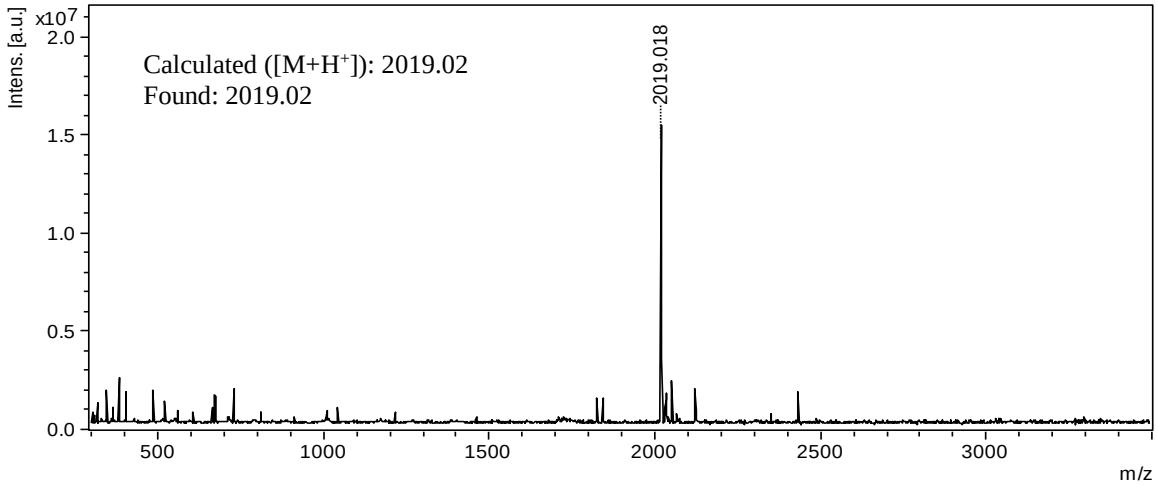
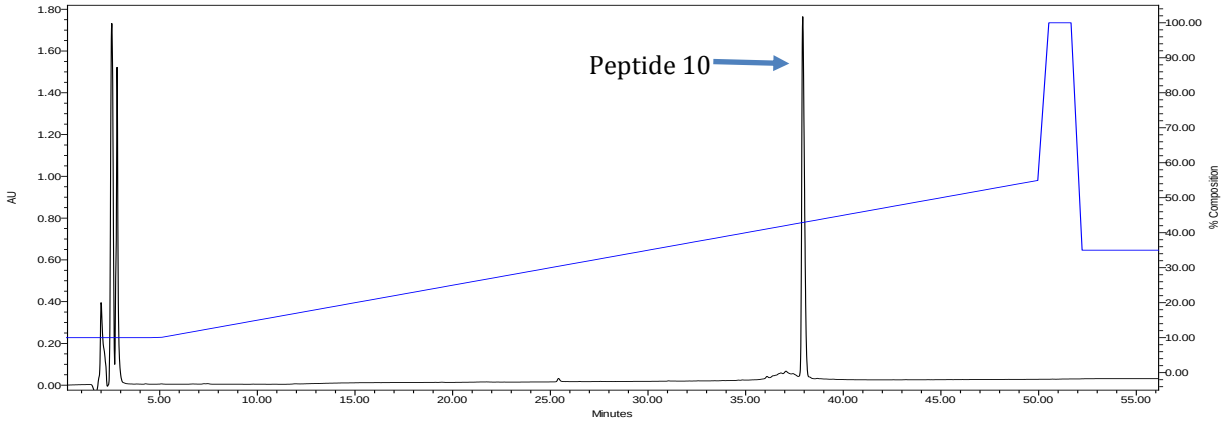


Peptide 10: bicyclo(aGWIY)-Dap-(FΦRrR-Dap)-BBK



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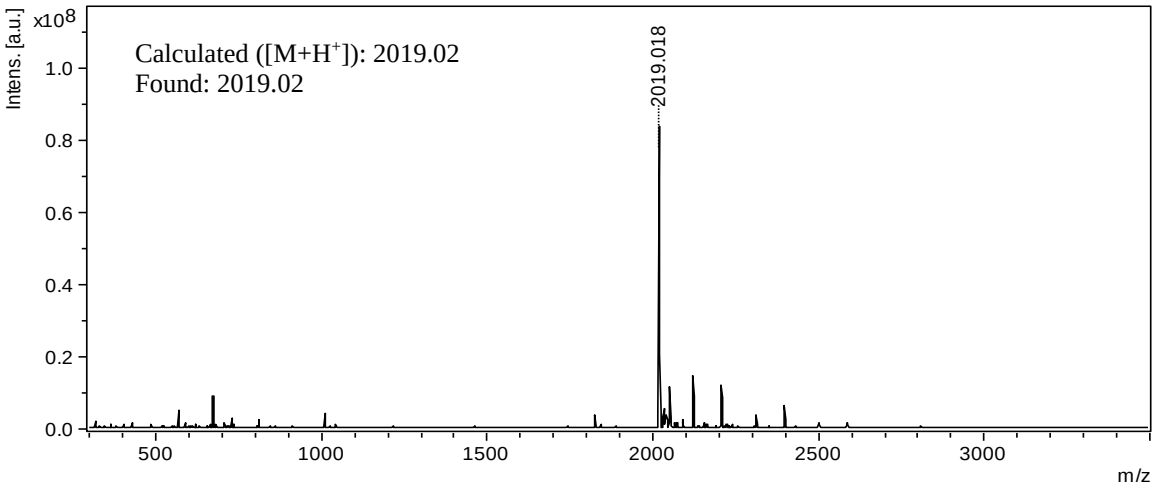
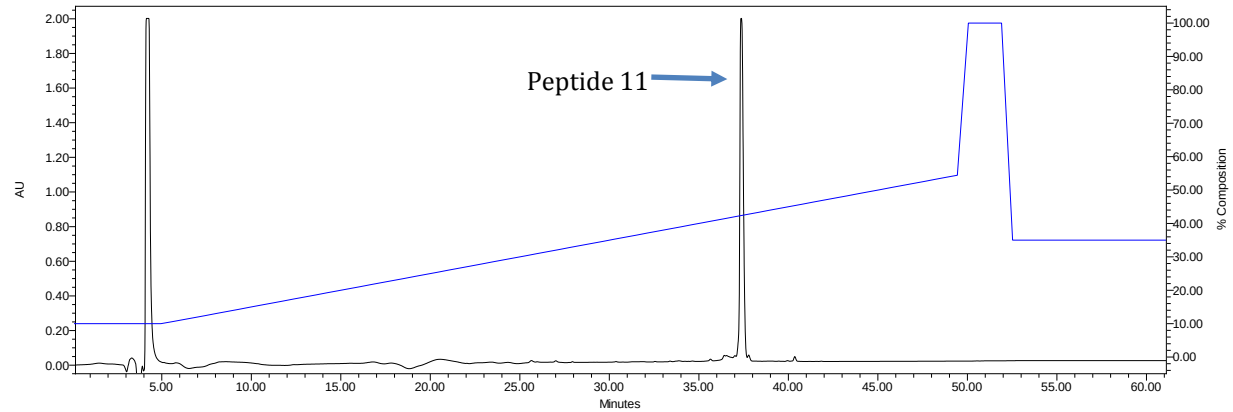


Peptide 11: bicyclo(AWIYA)-Dap-(FΦRrR-Dap)-BBK



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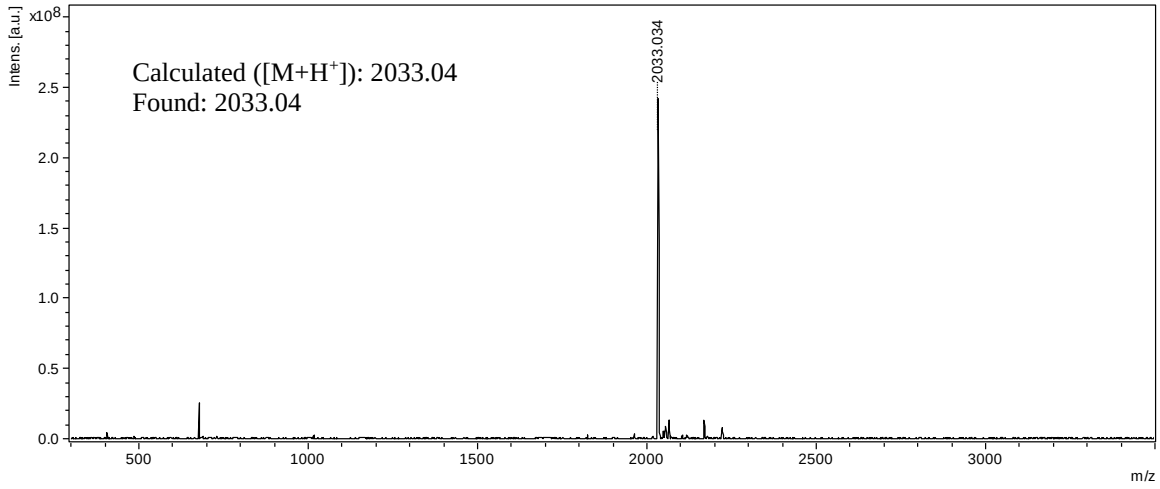
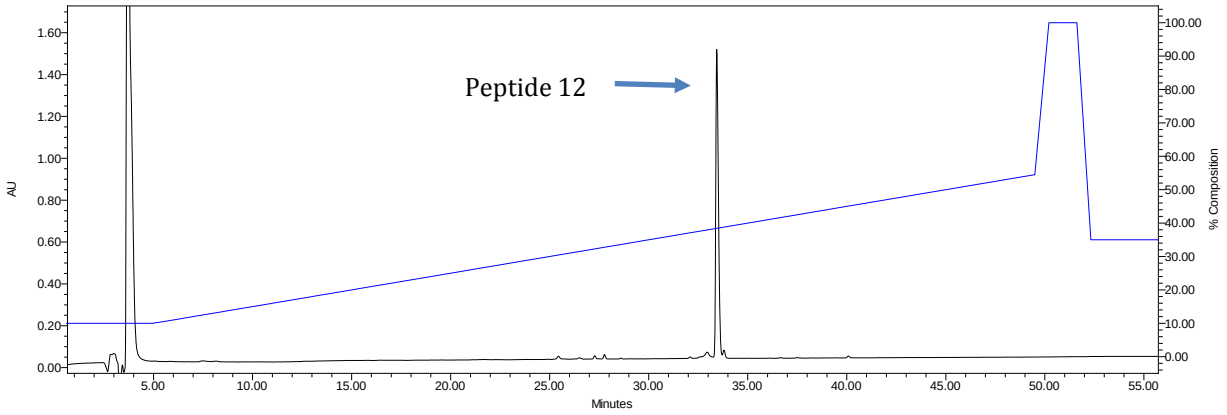


Peptide 12: bicyclo(GAIYA)-Dap-(FΦRrR-Dap)-BBK



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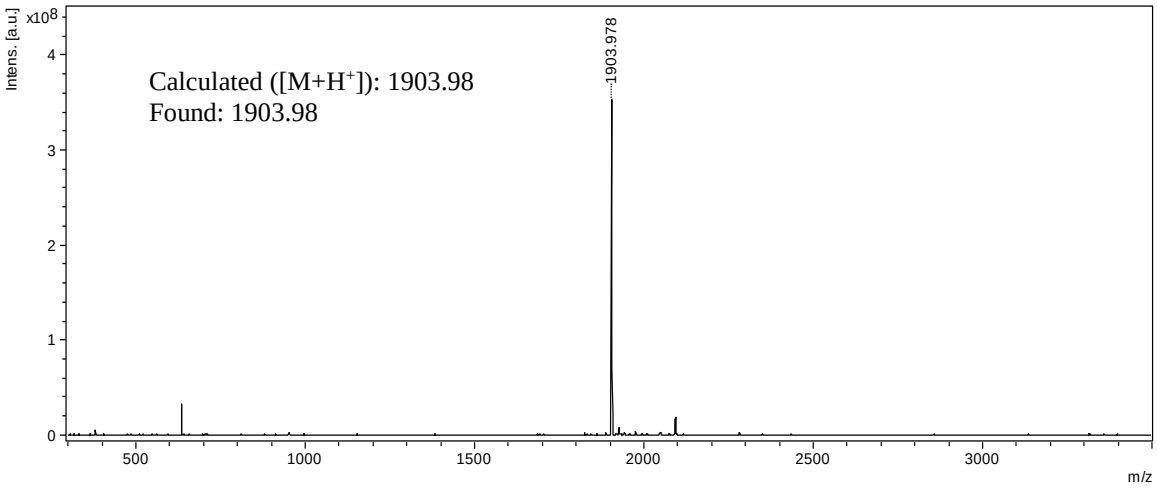
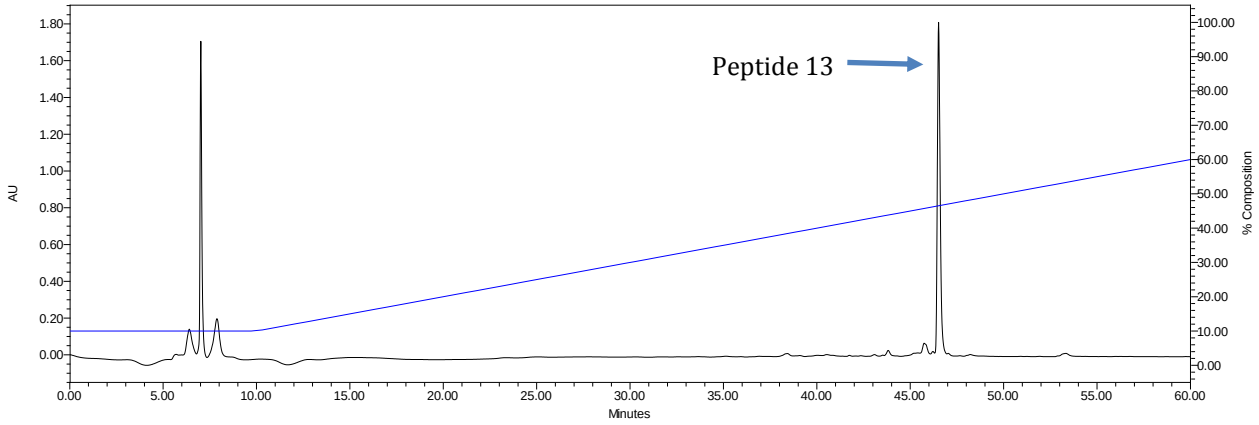


Peptide 13: bicyclo(GWAYA)-Dap-(FΦRrR-Dap)-BBK



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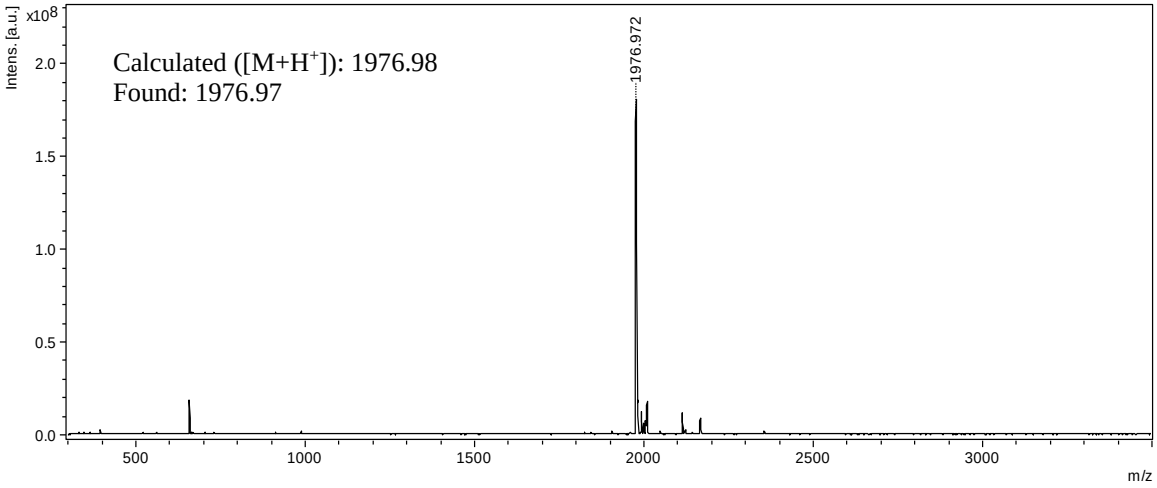
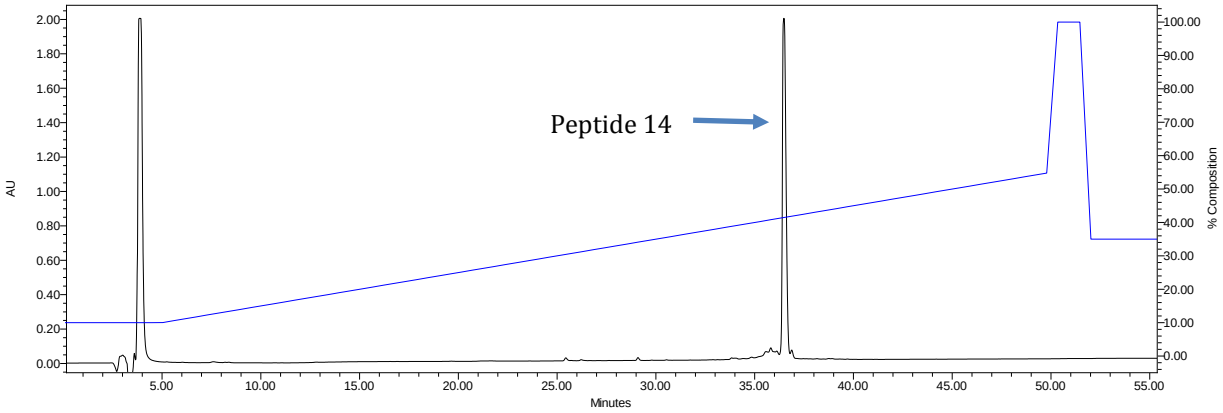


Peptide 14: bicyclo(GWIAA)-Dap-(FΦRrR-Dap)-BBK



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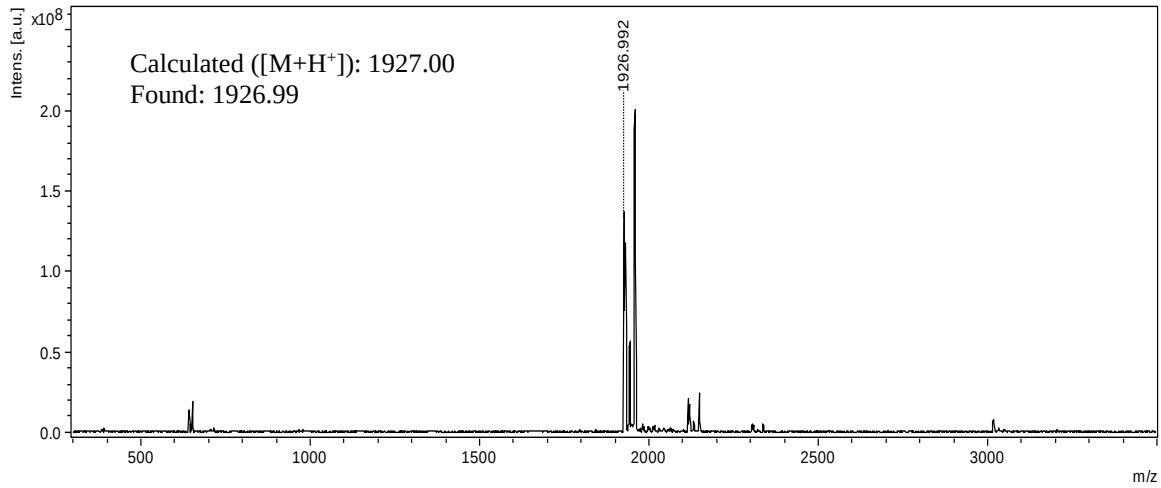
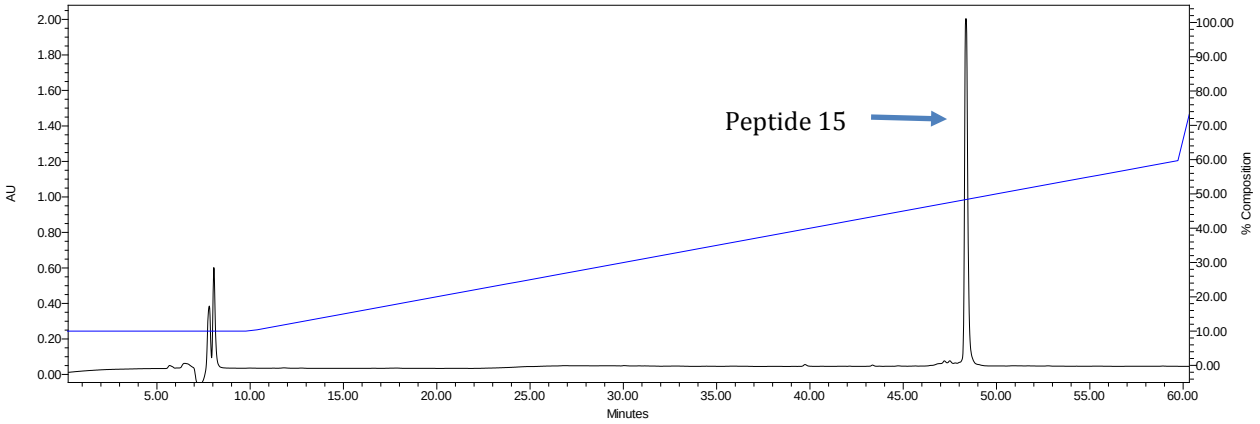


Peptide 15: bicyclo(GWIYA)-Dap-(AΦRrR-Dap)-BBK



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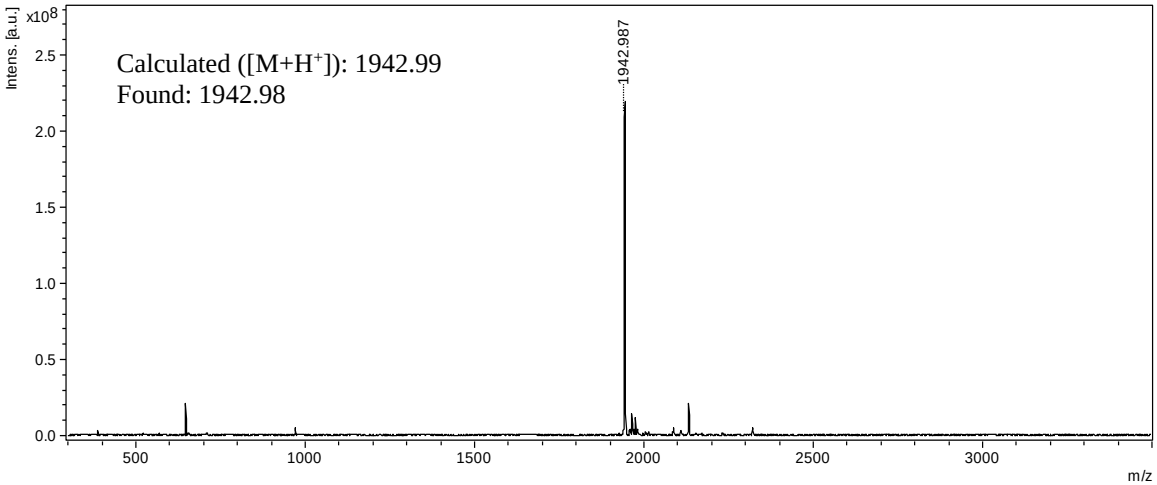
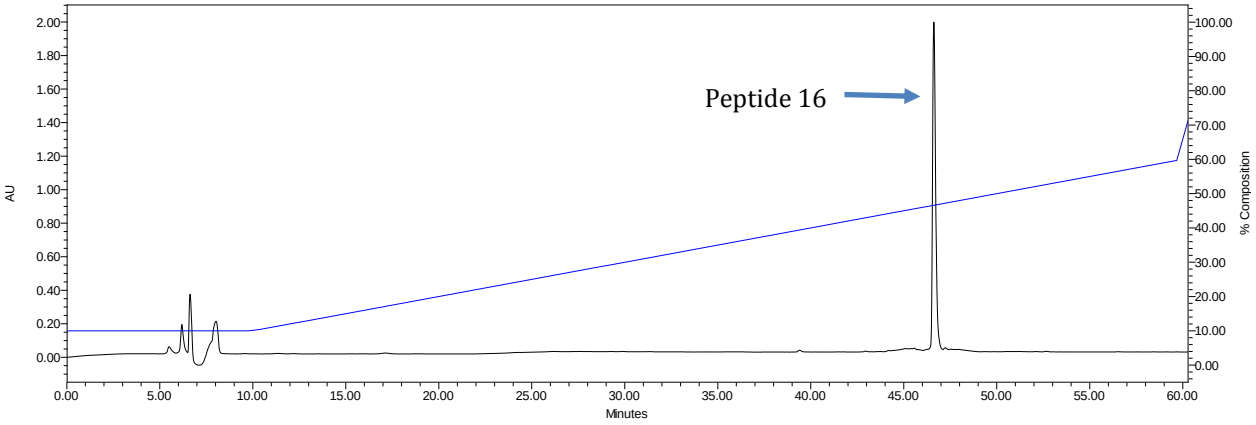


Peptide 16: bicyclo(GWIYA)-Dap-(FARrR-Dap)-BBK



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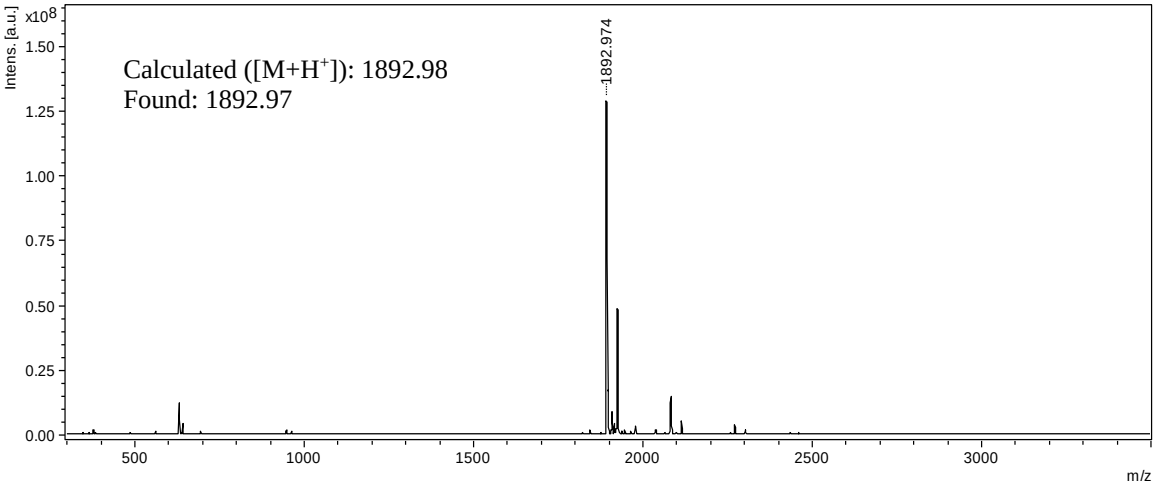
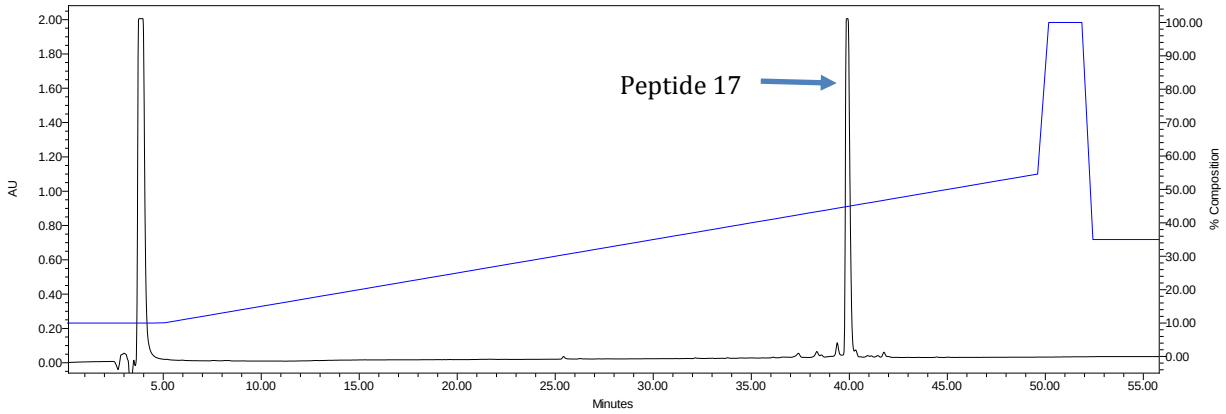


Peptide 17: bicyclo(GWIYA)-Dap-(FΦArR-Dap)-BBK



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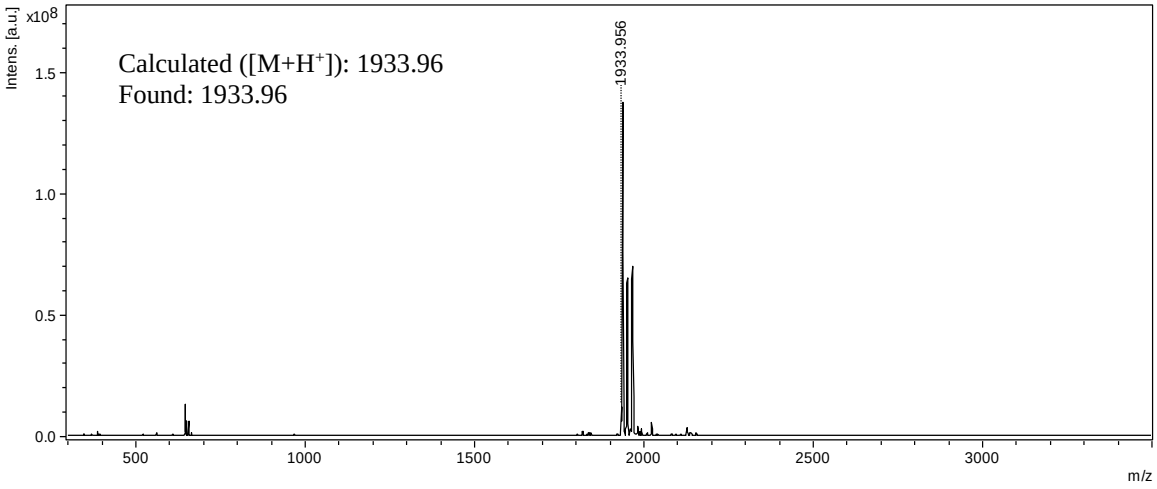
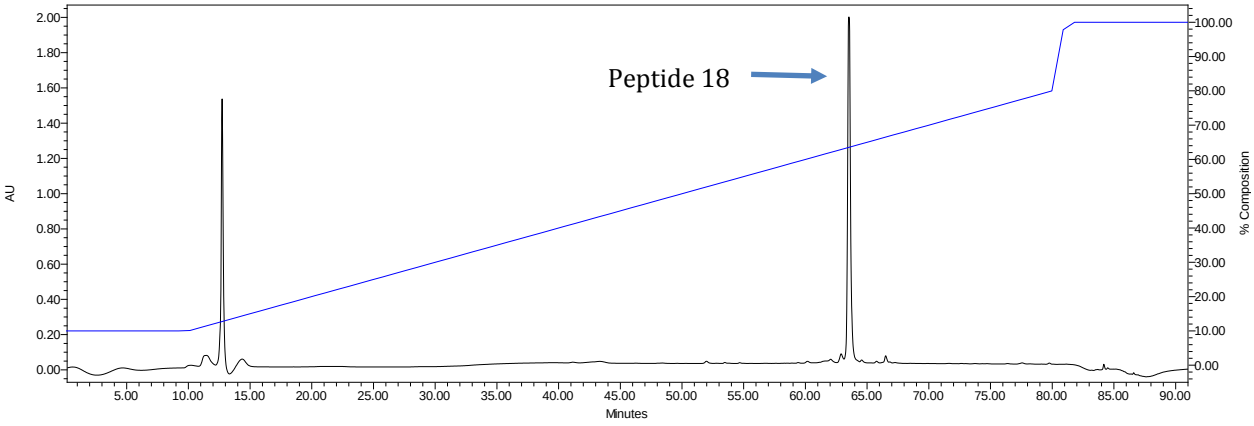


Peptide 18: bicyclo(GWIYA)-Dap-(FΦRaR-Dap)-BBK



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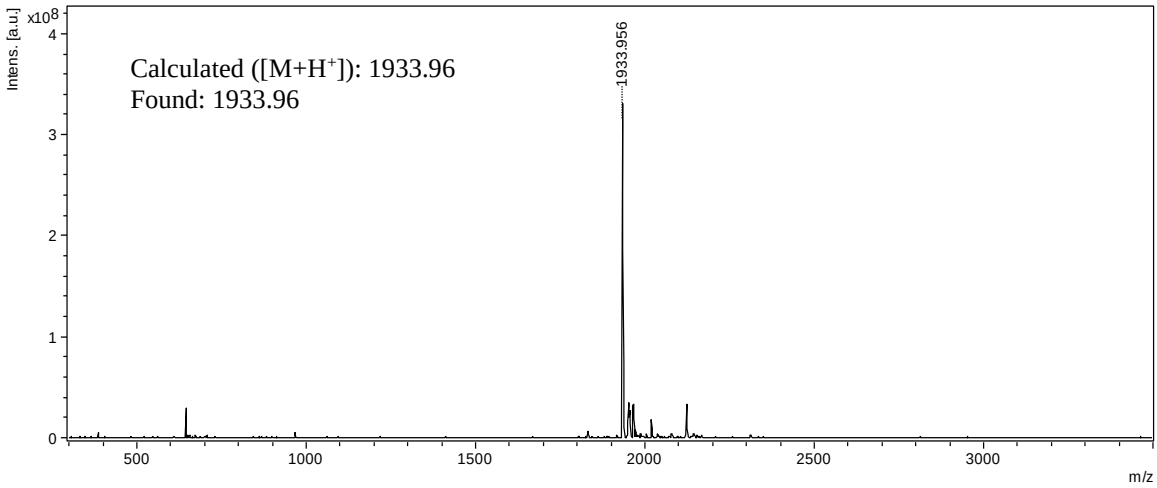
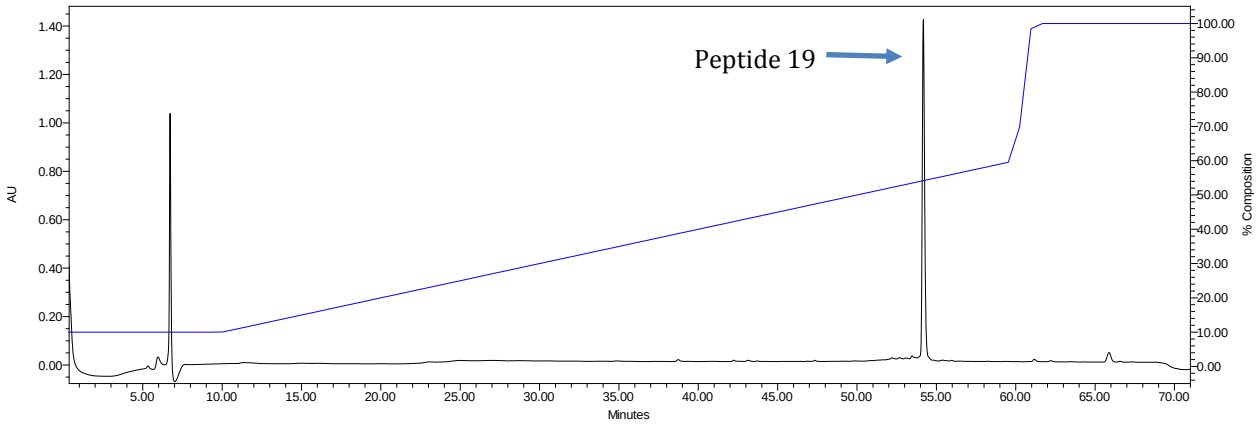


Peptide 19: bicyclo(GWIYA)-Dap-(FΦRrA-Dap)-BBK



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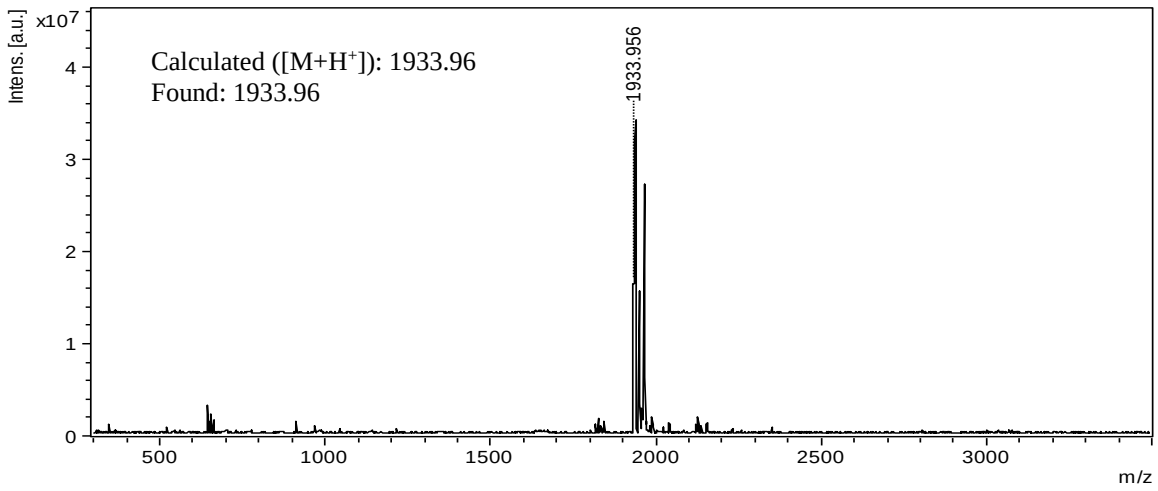
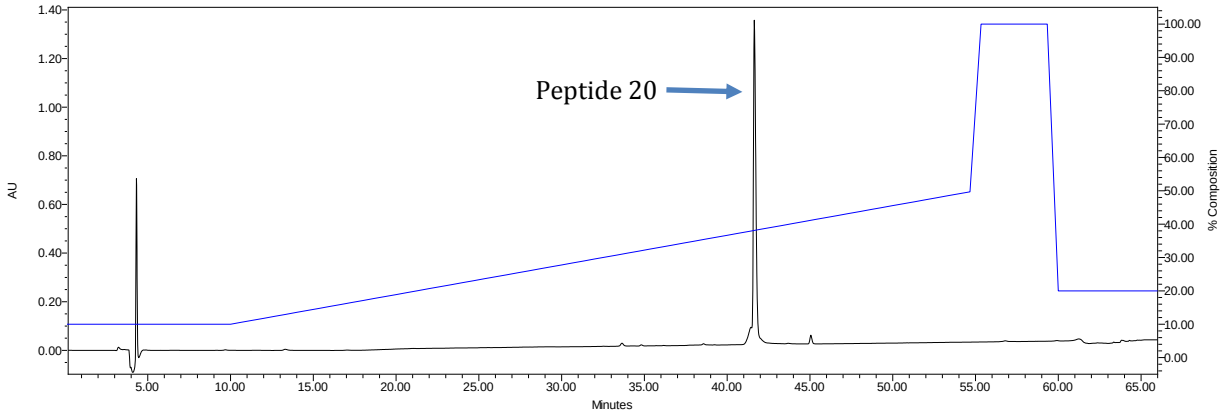


Peptide 20: bicyclo(GAIAA)-Dap-(FΦRrR-Dap)-BBK



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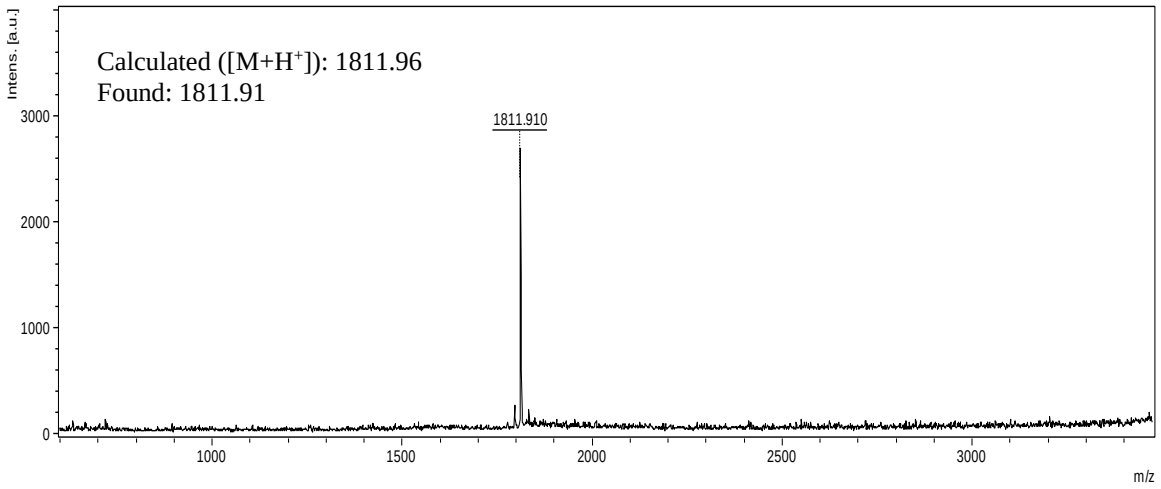
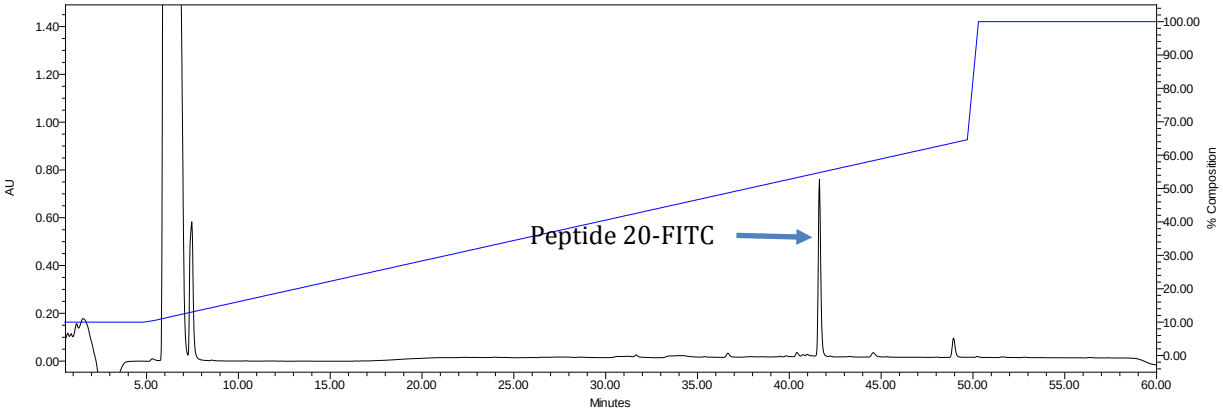


Peptide 20-FITC: bicyclo(GAIAA)-Dap-(FΦRrR-Dap)-BBK(FITC)



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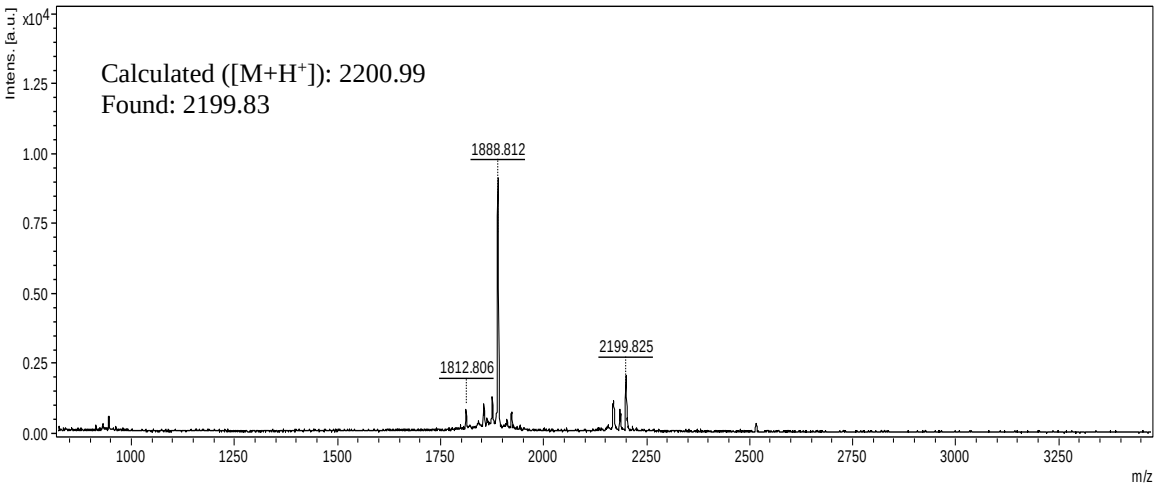
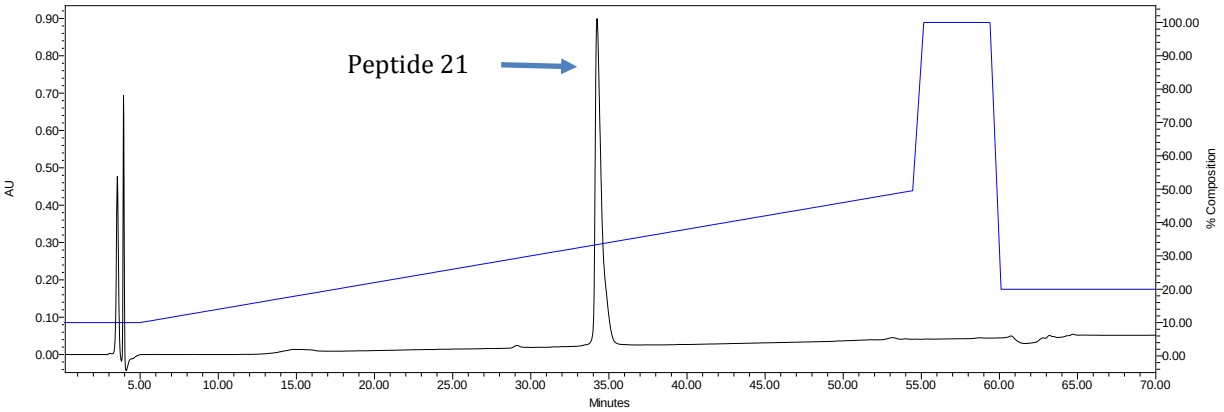


Peptide 21: bicyclo(GAAAA)-Dap-(FΦRrR-Dap)-BBK



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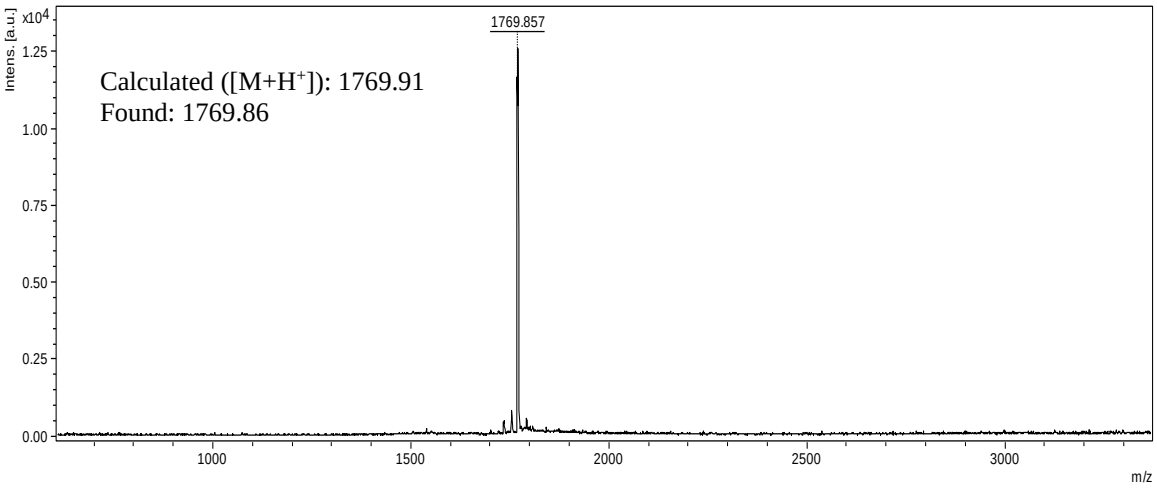
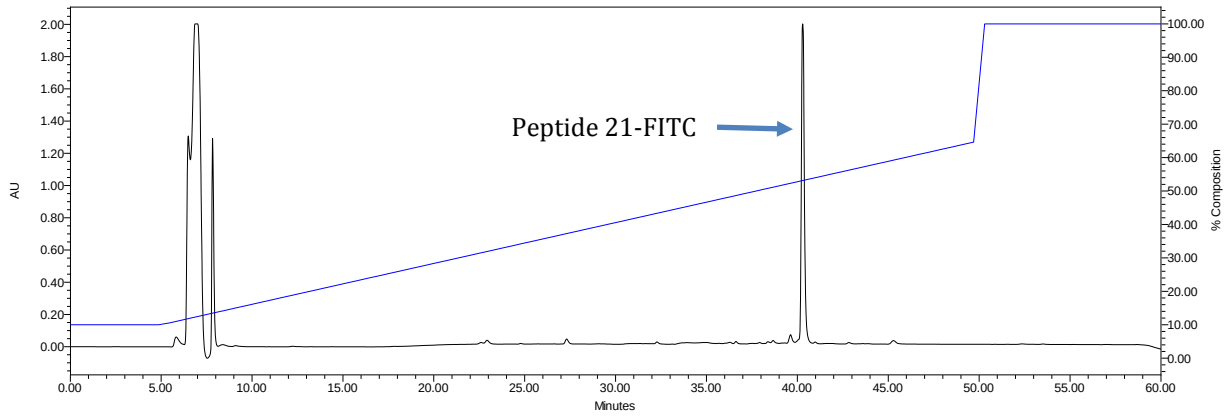


Peptide 21-FITC: bicyclo(GAAAA)-Dap-(FΦRrR-Dap)-BBK(FITC)



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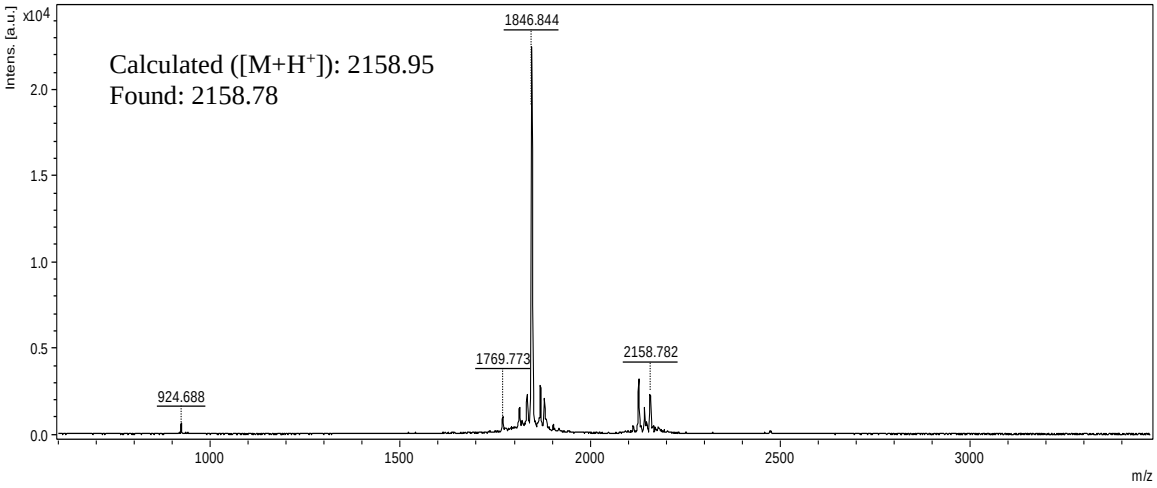

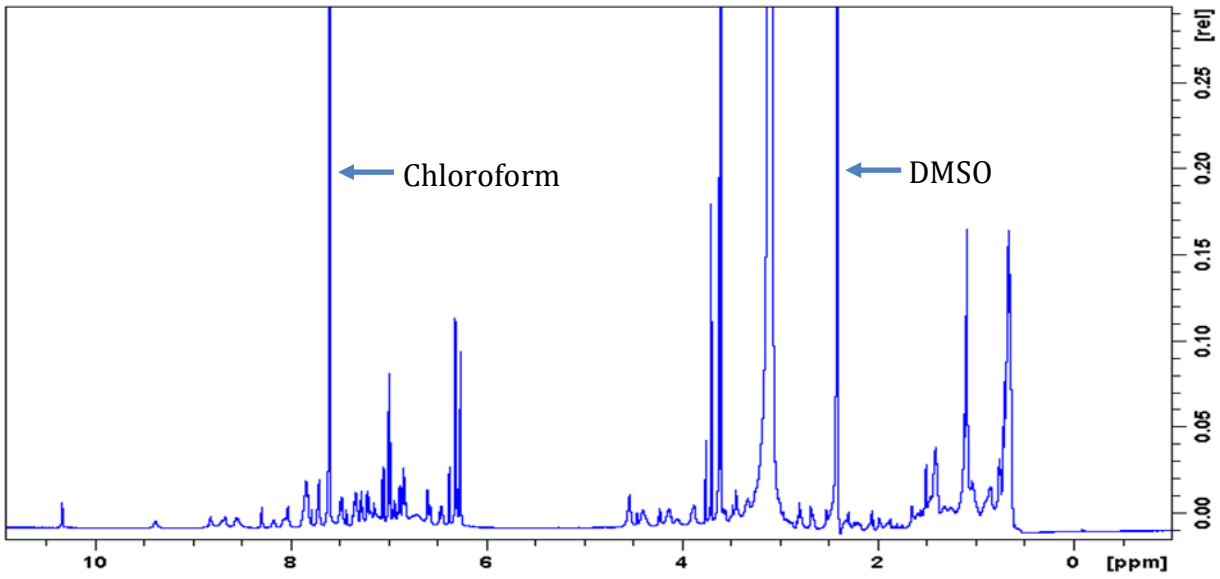

Figure S8. ^{1}H NMR spectrum for bicyclic peptide **7**.