Supporting Information

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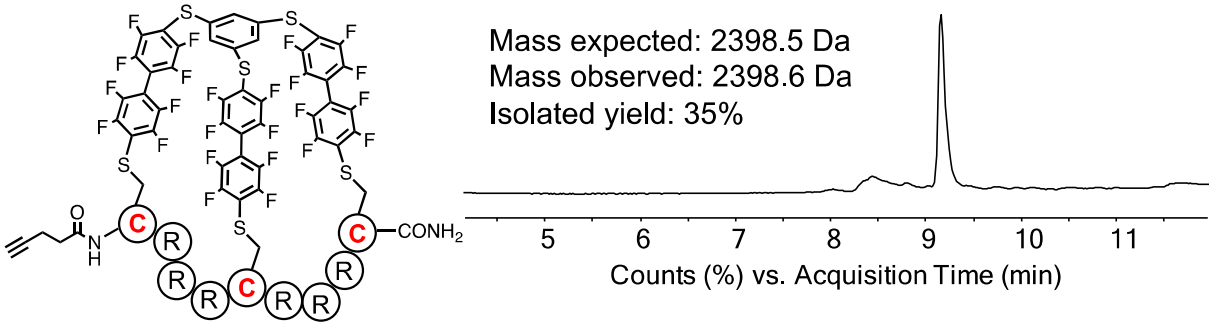
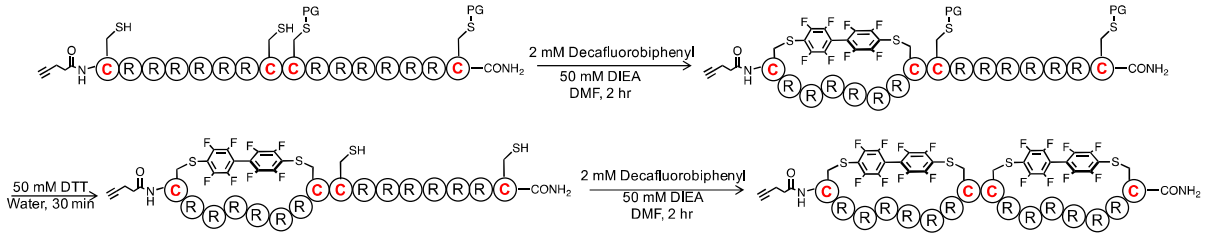
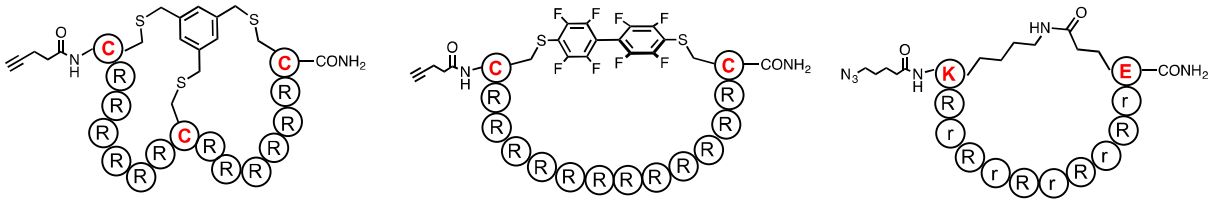
Supplemental Figures



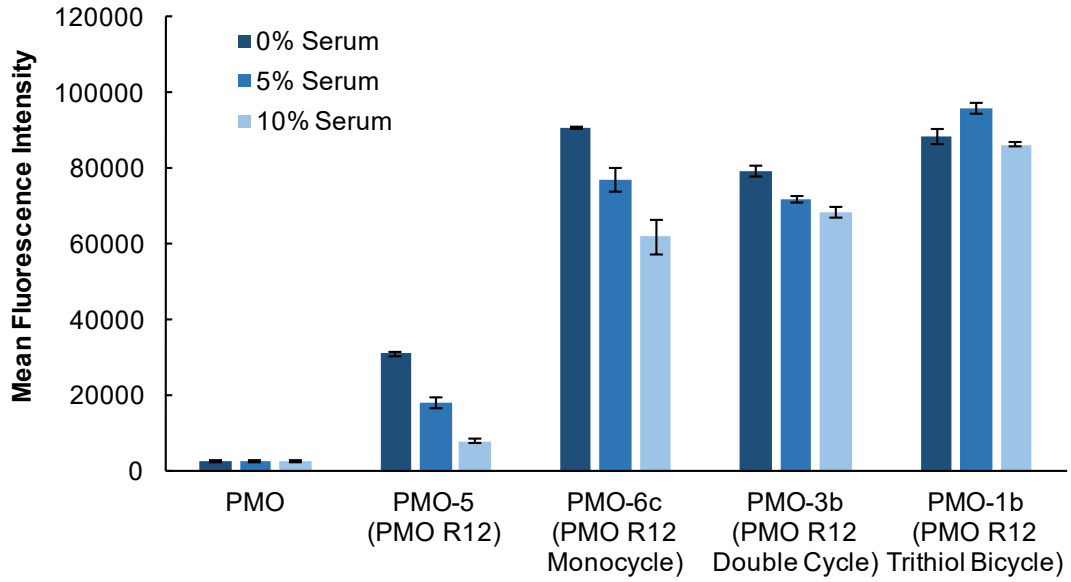
Supplementary Figure 1: Structure and LC-MS analysis of bicyclic peptide **2b**.



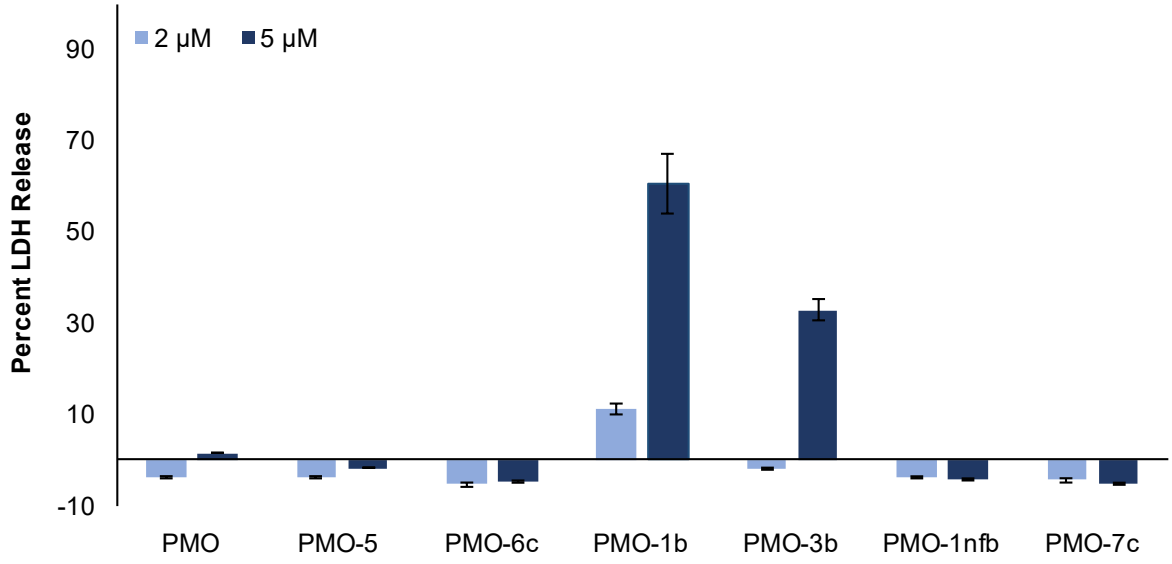
Supplementary Figure 2: Synthetic scheme of bicyclic peptide **3b** using orthogonal protection. PG refers to *tert*-butylthiol.



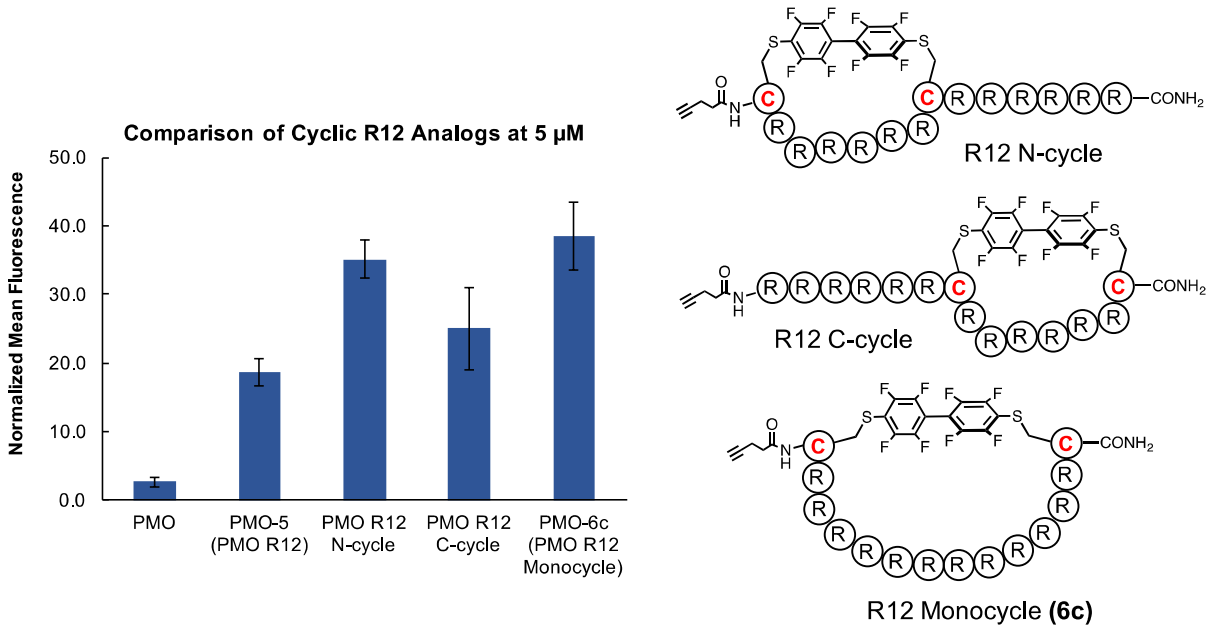
Supplementary Figure 3: Structures of peptide **1nfb**, **6c**, and **7c**. The non-fluorinated bicyclic peptide **1nfb** was synthesized through the reaction between peptide **1** and tris(bromomethyl)benzene. The cyclic peptide **6c** was synthesized through the reaction between peptide **6** and decafluorobiphenyl. The lactam-cyclized peptide **7c** was cyclized on-resin using standard active-ester chemistry.



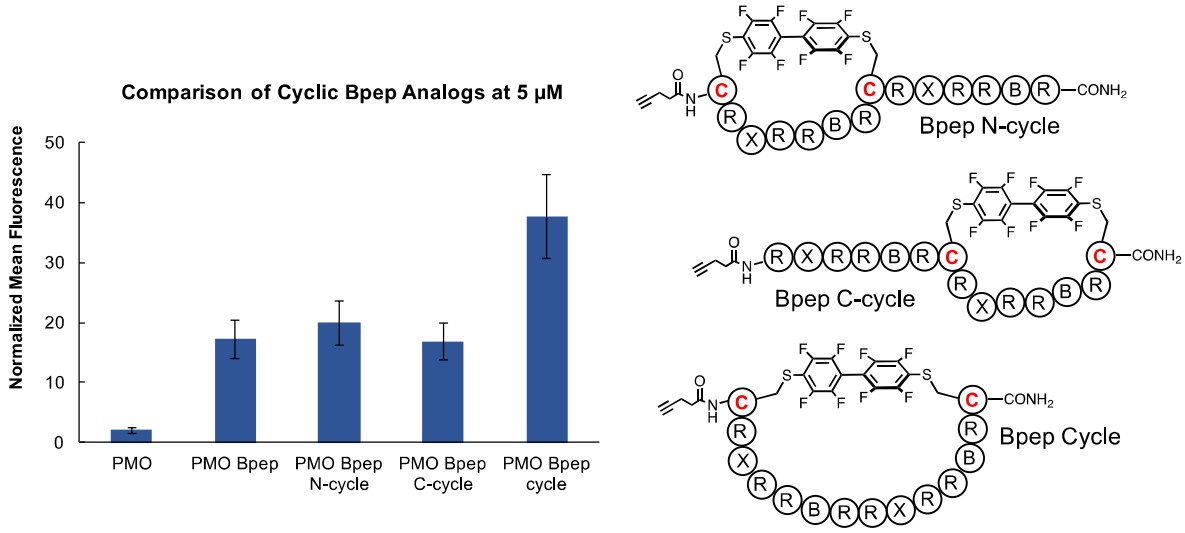
Supplementary Figure 4: Treatment of HeLa-654 cells using media with different amounts of serum. HeLa-654 cells were treated in a pulse-chase style experiment using 5 μM of each PMO conjugate in media containing 0, 5, or 10% fetal bovine serum. After three hours, the treatment media was removed and replaced with untreated media containing 10% FBS. The cells were allowed to grow for 22 hours, and then analyzed by flow cytometry. Errors bars are standard deviation (n=3 biological replicates). Both PMO-**5** and PMO-**6c** showed a reduction in activity with increasing amounts of serum, while PMO-**3b** and PMO-**1b** showed less reduction in activity.



Supplementary Figure 5: Lactate Dehydrogenase (LDH) release assay. Hela-654 cells were incubated with 2 or 5 µM of each PMO-peptide conjugate for 22 hours. The media was removed and analyzed for the amount of LDH present. The plot shows the percent of LDH released, compared to total cell lysis as 100% and untreated cells as 0%. PMO-**1b** showed LDH release at both concentrations, suggesting membrane disruption and early signs of cytotoxicity, while PMO-**3b** only showed LDH release at 5µM concentration.

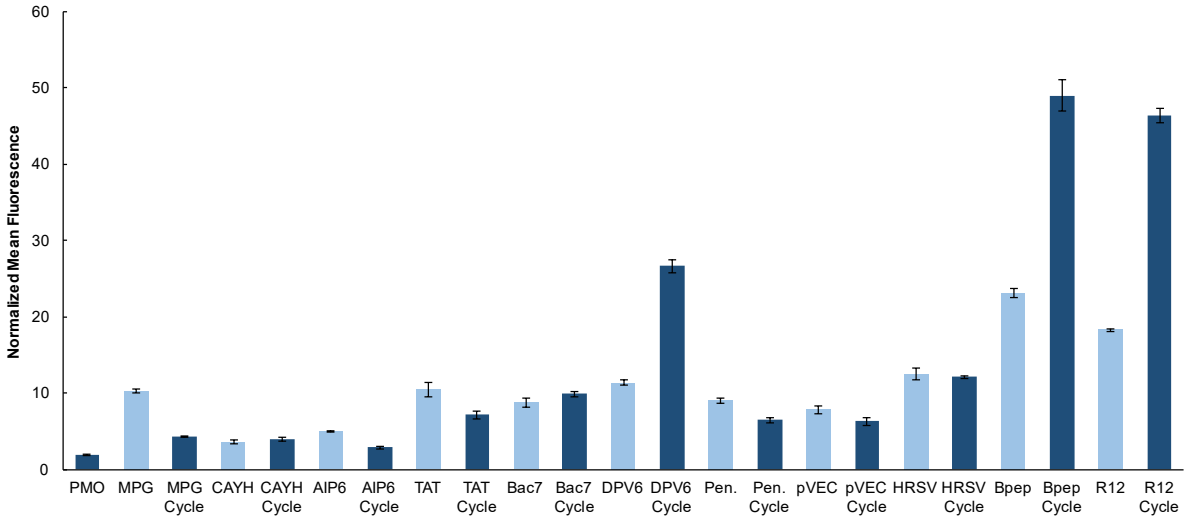


Supplementary Figure 6: In addition to R12 monocycle **6c**, R12 was cyclized across either the 6 N-terminal arginine residues or the 6 C-terminal residues. The peptides were conjugated to the IVS2-654 PMO and assayed for exon-skipping activity in HeLa-654 cells. Cells were treated with 5µM of each PMO conjugate for 22 hours and the cellular fluorescence was analyzed by flow cytometry. Each sample was normalized to the background fluorescence of untreated cells. Error bars are standard deviation (three independent experimental replicates, each with n=3 biological replicates). The PMO R12 C-cycle conjugate exhibited reduced cellular fluorescence, suggesting that the increase in activity for PMO-**6c** is not solely a result of hydrophobicity and cationic nature.



Supplementary Figure 7: The effect of cyclization for Bpep, another arginine-rich peptide. Bpep was either cyclized across the entire sequence or cyclized on the 6 N-terminal or C-terminal residues. Next, the peptides were conjugated to the IVS2-654 PMO and assayed for exon-skipping activity in HeLa-654 cells. Cells were treated with 5µM of each PMO conjugate for 22 hours and the cellular fluorescence was analyzed by flow cytometry. Each sample was normalized to the background fluorescence of untreated cells. Error bars are standard deviation (n=3 biological replicates). The PMO Bpep cycle conjugate exhibited a two-fold increase in cellular fluorescence relative to Bpep, Bpep N-cycle, and Bpep C-cycle.

*X = aminohexanoic acid; B = beta-alanine



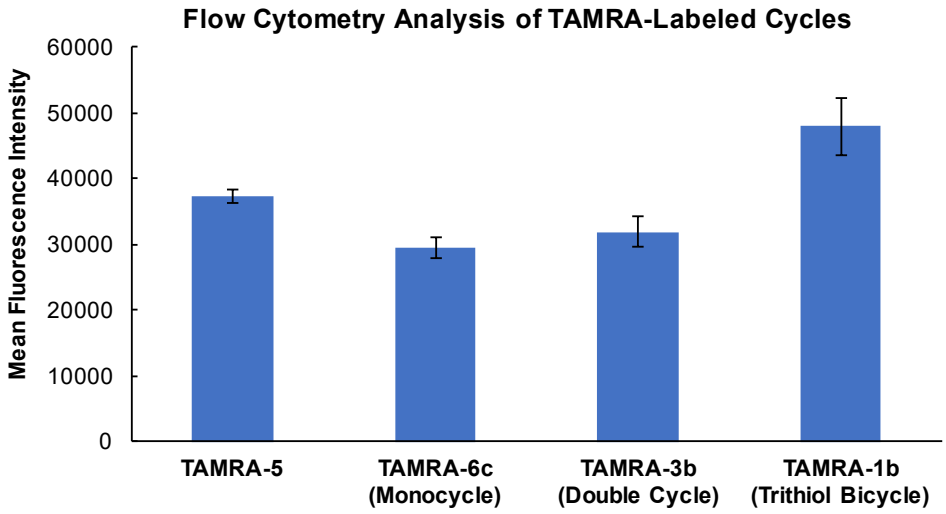
Supplementary Figure 8: Linear and perfluoroaryl-cyclic cell-penetrating peptides (CPPs) were compared for PMO delivery. For 11 known CPPs, each peptide was synthesized both as a linear peptide and as a cyclic variant with cysteine residues on the N and C-termini of the peptide and linked across the sequence using decafluorobiphenyl. The peptides were conjugated to PMO and assayed for activity in HeLa-654 cells. Cells were treated with 5µM of each PMO conjugate for 22 hours and the cellular fluorescence was analyzed by flow cytometry. Each sample was normalized to the background fluorescence of untreated cells. Error bars are standard deviation (n=3 biological replicates). Many decafluorobiphenyl-cyclized CPPs did not improve PMO activity relative to their linear counterparts. However, cyclic arginine-rich sequences (DPV6, Bpep, and R12) all demonstrated roughly a 2-fold increase in activity relative to the linear sequences.

Amino acid sequences and LC-MS analysis can be found starting on page 37.

Note: R12 is identical to PMO-5 and R12 cycle is identical to PMO-6c.

Note: Penetratin is abbreviated as Pen.

Note: the linear PMO-CPP conjugates were assayed in the context of a library screen (to be published), and these data are partial results from the larger library.



Supplementary Figure 9: Peptides **1b**, **3b**, **5**, and **6c** were conjugated to the fluorescent dye 5-TAMRA-azide by copper-catalyzed click chemistry and analyzed for cellular uptake by flow cytometry. Briefly, HeLa-654 cells were treated with 5 μM of each conjugate for 2 hours in serum-containing media and the average cellular fluorescence was measured by flow cytometry. Error bars are standard deviation (n=3 biological replicates). In contrast to the PMO conjugates, the TAMRA-conjugated bicyclic and cyclic compounds **3b** and **6c** did not exhibit improved uptake relative to linear peptide **5**.

1. Materials

H-Rink Amide-ChemMatrix resin was obtained from PCAS BioMatrix Inc. (St-Jean-sur-Richelieu, Quebec, Canada). 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium-3-oxid-hexafluorophosphate (HATU), Fmoc-L-Cys(StBu)-OH, 4-pentynoic acid, and 5-azidopentanoic acid were purchased from Chem-Impex International (Wood Dale, IL). All other Fmoc-protected amino acids were purchased from Advanced ChemTech (Louisville, KY). Peptide synthesis-grade N,N-dimethylformamide (DMF), dichloromethane (DCM), diethyl ether, and HPLC-grade acetonitrile were obtained from VWR International (Philadelphia, PA). Decafluorobiphenyl was purchased from Oakwood Products, Inc. (Estill, SC). IVS-654 PMO was provided by Sarepta Therapeutics. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), water was deionized before use, and reactions were conducted in open-air on bench. For experiments performed at MIT, HeLa-654 cells were obtained from the University of North Carolina Tissue Culture Core facility.

2. Methods for LC-MS Analysis

For all experiments except for the proteolysis assays, LC-MS chromatograms and associated mass spectra were acquired using an Agilent 6520 ESI-Q-TOF mass spectrometer equipped with a C_3 Zorbax column. Mobile phases were: 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Column: Zorbax SB C3 column: 2.1 x 150 mm, 5 μm.

General Method: 1% B from 0 to 2 min, linear ramp from 1% B to 61% B from 2 to 11 min, 61% B to 99% B from 11 to 12 min and 3 min of post-time at 1% B for equilibration, flow rate: 0.8 mL/min.

PMO library method: 5% B from 0 to 2 min, linear ramp from 5% B to 65% B from 2 to 11 min, 65% B from 11 to 12 min and 3 min of post-time at 5% B for equilibration, flow rate: 0.8 mL/min

All data were processed using Agilent MassHunter software package. Y-axis in all chromatograms shown represents total ion current (TIC) unless noted. For the proteolysis assays and bicyclization comparison assay, LC-MS chromatograms and associated mass spectra were acquired using an Agilent 6550 iFunnel Q-TOF mass spectrometer equipped with a Jupiter C_4 Phenomenex column. The following LC-MS method was used: Phenomenex Jupiter C4: 150 x 1.0 mm ID, 5 μm, linear gradient from 1% B to 61% B over 10 minutes, pre- and post-time: 2 minutes. Flow rate: 0.1 mL/min.

3. General Method for Peptide Preparation and Purification

Fast-flow Peptide Synthesis

Peptides were synthesized on a 0.1-mmol scale using an automated flow peptide synthesizer as previously described.[1] ChemMatrix Rink Amide HYR resin (200 mg) was loaded into a reactor maintained at 90 ºC. All reagents were flowed at 80 mL/min with HPLC pumps through a stainless-steel loop maintained at 90 ºC before introduction into the reactor. For each coupling, 10 mL of a solution containing 0.2 M amino acid and 0.17 M HATU in DMF were mixed with 200 µL diisopropylethylamine and delivered to the reactor. Fmoc removal was accomplished using 10.4 mL of 20% (v/v) piperidine. Between each step, DMF (15 mL) was used to wash out the reactor. Special coupling conditions were used for arginine, in which the flow rate was reduced to 40 mL/min and 10 mL of a solution containing 0.2 M Fmoc-L-Arg(Pbf)-OH and 0.17 M PyAOP in DMF were mixed with 200 µL diisopropylethylamine and delivered to the reactor. The final coupling was with 4-pentynoic acid, rather than an amino acid, but using the same conditions for activation. After completion of the synthesis, the resins were washed 3 times with DCM and dried under vacuum.

Peptide Cleavage and Deprotection

Each peptide was subjected to simultaneous global side-chain deprotection and cleavage from resin by treatment with 6 mL of Reagent K (82.5% trifluoroacetic acid, 5% phenol, 5% water, 5% thioanisole, and 2.5% 1,2-ethanedithiol (EDT)). For arginine-rich peptides, cleavages were left at room temperature for 16 hours to ensure complete removal of the Pbf protecting group. The cleavage cocktail was filtered to remove the resin and the TFA was evaporated by bubbling N_2 through the mixture. Then ~35 mL of cold ether was added and the crude product was pelleted through centrifugation for three minutes. This ether trituration and centrifugation was repeated two more times. After the third wash, the pellet was redissolved in 50% water and 50% acetonitrile and lyophilized.

Peptide Purification

Solvent A: water containing 0.1% TFA and Solvent B: acetonitrile containing 0.1% TFA

Lyophilized peptide was dissolved into a minimum volume of mobile phase (95% A, 5% B). The solution was loaded onto a reversed-phase HPLC column (Agilent Zorbax SB C18 column: 9.4 x 250 mm, 5 µm or Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 µm) attached to a mass-based purification system. A linear gradient was run at 0.5% B / min from 5% B to 55% B. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

4. Macrocyclization, Arylation, and Bicyclization

General conditions for peptide macrocyclization

To the purified peptide dissolved in DMF, decafluorobiphenyl and diisopropylethylamine (DIEA) were added such that the final concentration in the reaction vessel was 1 mM peptide, 2 mM decafluorobiphenyl, and 50 mM DIEA. After two hours, DMF was removed by rotary evaporation to a final volume of 1 mL and the reaction was quenched by adding 39 mL of water containing 2% TFA. The crude reaction was purified by mass-directed semi-preparative reversed-phase HPLC.

*Procedure for synthesis of **1a, 2a***

Peptides **1** (28 mg, 10 µmol) or **2** (20 mg, 12 µmol) were dissolved in DMF, and stock solutions of decafluorobiphenyl in DMF and diisopropylethylamine (DIEA) were added for a final concentration in the reaction vessel of 1 mM peptide, 100 mM decafluorobiphenyl, and 50 mM DIEA. After two hours, DMF was removed by rotary evaporation to a final volume of 1 mL and the reaction was quenched by adding 39 mL of 85:15 water:acetonitrile containing 2% TFA. The crude reaction was centrifuged, filtered, and purified by mass-directed semi-preparative reversed-phase HPLC (C3 column) to provide **1a** (13 mg, 35%) or **2a** (11 mg, 35%).

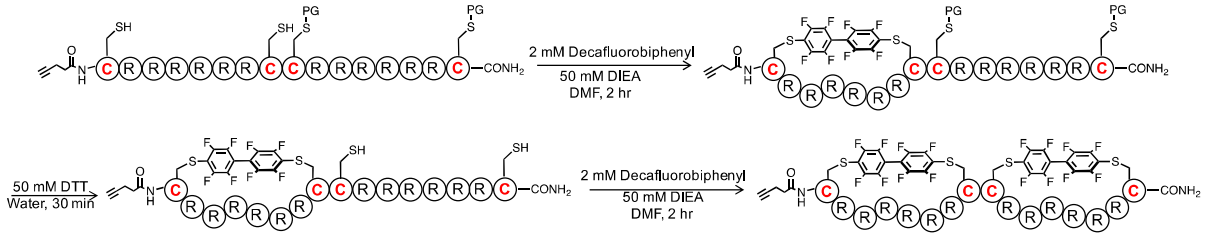
*Procedure for synthesis of bicycles **1b, 2b***

Purified peptides **1a** (10 mg, 2.56 µmol) or **2a** (5 mg, 1.9 µmol) were dissolved in DMF, and stock solutions of 1,3,5-benzenetrithiol in DMF and DIEA were added such that the final concentration in the reaction vessel was 1 mM peptide, 1 mM 1,3,5-benzenetrithiol, and 50 mM DIEA. After two hours, DMF was removed by rotary evaporation to a final volume of 1 mL and the reaction was quenched by adding 39 mL of 85:15 water:acetonitrile containing 2% TFA. The crude reaction was purified by mass-directed semi-preparative reversed-phase HPLC (C3 column) to provide **1b** (6.4 mg, 62%) or **2b** (1.8 mg, 35%).

*Procedure for synthesis of non-fluorinated bicyclic peptide **1nfb***

Purified peptide **1** (5 mg, 2.2 µmol) was dissolved in 2.2 mL of a degassed solution containing 50 mM Tris buffer pH=8 in 2:1 water:acetonitrile. Then 23 µL of a 100 mM solution of tris(bromomethyl)benzene in acetonitrile was added (2.3 µmol). After 1 hour, the reaction was diluted to 20 mL with water containing 0.1% TFA and purified by mass-directed semi-preparative reversed-phase HPLC (C18 column) to provide **1nfb** (4.7 mg, 89% yield)

*Procedure for synthesis of bicycle **3b** using orthogonal deprotection*



Synthetic scheme for **3b** using orthogonal deprotection

Peptide **3** was synthesized using standard automated flow chemistry. *Tert*-butyl thiol protected cysteine was incorporated for cysteine residues 1 and 2 and trityl protected cysteine was incorporated for cysteine residues 3 and 4. After cleavage and deprotection, the peptide was purified by reversed-phase HPLC. The peptide was macrocyclized using the above procedure for macrocyclization with a decafluorobiphenyl using 29 mg of peptide (9.5 mg, 30.3% yield). Next, *tert*-butylthiol protected cysteine residues 1 and 2 were deprotected by incubating the peptide (1 mM) with DTT (50 mM) and Tris (25 mM pH 8) in water for 30 minutes, followed by solid-phase extraction (SPE) to remove residual DTT (6.5 mg, 73% yield). The remaining two free cysteine residues were macrocyclized using the general procedure and purified by reversed-phase HPLC (C3 column) (1.5 mg, 21% yield). Overall yield 4.5%.

*Procedure for synthesis of **3a***

Peptide **3** was synthesized using standard automated flow chemistry using 200 mg of resin. *Tert*-butyl thiol protected cysteine was incorporated for cysteine residues 1 and 3 and trityl protected cysteine was incorporated for cysteine residues 2 and 4. The *tert*-butyl thiol groups were deprotected on resin using 3.8 mmol DTT in 2.5 mL DMF with 0.25 mL DIEA. The reaction proceeded at 60 ºC for 20 minutes. The resin was washed 3 times with DMF, followed by the addition of decafluorobiphenyl (1 mmol) with 2.5 mL DMF and 0.25 mL DIEA. The reaction proceeded at room temperature for 2 hours, and then the resin was washed with DMF (3x) and DCM (3x). The peptide was then cleaved from the resin and purified by reversed-phase HPLC (C3 column) to afford **3a**.

*Procedure for synthesis of bicycle **3b** controlled bicyclization*

Peptide **3a** (15 mg, 4 µmol, assuming 6 coordinated TFA salts) was suspended in 4 mL DMF. Neat DIEA (35.4 µL) was added to achieve a concentration of 50 mM. The reaction was allowed to proceed for 5 minutes, after which complete conversion is observed. DMF was removed by rotary evaporation to a final volume of 1 mL and the reaction was quenched by adding 39 mL of water containing 2% TFA. The

reaction was purified by mass-directed semi-preparative reversed-phase HPLC (C3 column) to provide peptide **3b** (12.0 mg, 81% yield).

*Procedure for synthesis of arylated control peptide **4a***

Peptide **4** was synthesized using standard automated flow chemistry using 200 mg of resin except *tert*-butyl thiol protected cysteine was incorporated for cysteine residue 1 and trityl protected cysteine was incorporated for cysteine residue 2. The *tert*-butyl groups were deprotected on resin using 3.8 mmol DTT in 2.5 mL DMF with 0.25 mL DIEA. The reaction proceeded at 60 ºC for 20 minutes. The resin was washed 3x with DMF, followed by the addition of decafluorobiphenyl (1 mmol) with 2.5 mL DMF and 0.25 mL DIEA. The reaction proceeded at room temperature for 2 hours, and then the resin was washed with DMF (3x) and DCM (3x). The peptide was then cleaved from the resin and purified using mass-directed semi-preparative reversed-phase HPLC (C3 column) to afford **4a**.

*Procedure for synthesis of cyclic peptide **6c***

Peptide **6** was synthesized using standard automated flow chemistry using 200 mg of resin. *Tert*-butyl thiol protected cysteine was incorporated for cysteine residue 1 and trityl protected cysteine was incorporated for cysteine residue 2. The *tert*-butyl groups were deprotected on resin using 3.8 mmol DTT in 2.5 mL DMF with 0.25 mL DIEA. The reaction proceeded at 60 ºC for 20 minutes. The resin was washed with DMF (3x), followed by the addition of decafluorobiphenyl (1 mmol) with 2.5 mL DMF and 0.25 mL DIEA. The reaction proceeded at room temperature for 2 hours, and then the resin was washed with DMF (3x) and DCM (3x). The peptide was then cleaved from the resin and purified using mass-directed semi-preparative reversed-phase HPLC (C3 column) to afford the monoaryl **6a**.

Peptide **6a** was macrocyclized by suspending the peptide (12 mg, 3.9 μmol, assuming 6 coordinated TFA salts) in 3.9 mL DMF. Neat DIEA (33.8 μL) was added to achieve a concentration of 50 mM. The reaction was allowed to proceed for 5 minutes, after which complete conversion was observed. DMF was removed by rotary evaporation to a final volume of 1 mL and the reaction was quenched by adding 39 mL of water containing 2% TFA. The reaction was purified by mass-directed semi-preparative reversed-phase HPLC (C3 column) to provide peptide **6c** (8.4 mg, 88% yield).

*Procedure for synthesis of cyclic peptide **7c***

Peptide **7**, XK(Alloc)RrRrRrRrRrE(OAll) was synthesized using a combination of standard manual flow solid-phase peptide synthesis and published protocols synthesis of cR10 as detailed below.[2,3] "R" refers to Fmoc-L-Arg(Pbf) while "r' refers to Fmoc-D-Arg(Pbf). Lysine was Alloc-protected and Glu was OAll-protected to enable selective side-chain deprotection on resin for lactam cyclization. "X" is 5-azidopentanoic acid, which was incorporated instead of 4-pentynoic acid to avoid side-reactions between palladium and alkynes.

Synthesis proceeded as follows: 140 mg of H-Rink-Amide PEG resin was downloaded to 0.1667 mmol/g using a 1:2 mixture of Fmoc-Glu(OAll)-OH and Boc-Glu(OBnzl)-OH. The remaining amino-acids were coupled using manual flow peptide synthesis conditions, as previously described.[2] To remove Alloc- and OAll- protecting groups, the peptidyl resin was mixed with 0.01 mmol tetrakis(triphenylphosphine) palladium and 2.5 mmol phenylsilane in dry DCM. After 30 minutes, the resin was washed with DCM (3x), with 20% DIEA in DMF (3x), and with DMF (3x). To cyclize, 1 eq. of HBTU (0.023 mmol, based upon resin loading) was diluted into 6 mL of DMF with 8 μL DIEA and added to the resin. After 2 hours, the resin was washed with DMF (3x) and with DCM (3x). The peptide was then cleaved from the resin and purified using mass-directed semi-preparative reversed-phase HPLC (C18 column) to afford the cyclic peptide **7c**.

5. PMO-Peptide Conjugation

Procedure for coupling 5-azidopentanoic to PMO

PMO IVS2-654 (200 mg, 32 μmol) was dissolved in 600 μL DMSO. To the DMSO solution was added a solution containing 4 equivalents of 5-azidopentanoic acid (13.6 μL, 128 μmol) activated with HBTU (320 μL of 0.4 M HBTU in DMF, 128 μmol) and DIEA (22.3 μL, 128 μmol) in 244 μL DMF (Final reaction volume = 1.2 mL). The reaction proceeded for 25 minutes before being quenched with 1 mL of water and 2 mL of ammonium hydroxide. The ammonium hydroxide will hydrolyze any ester formed during the course of the reaction. After 1 hour, the solution was diluted to 40 mL and purified using reversed-phase HPLC (Agilent Zorbax SB C3 column: 21.2 x 100 mm, 5 μm) and a linear gradient from 2 to 60% B (solvent A: water; solvent B: acetonitrile) over 58 minutes (1 %B / min). Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS. Lyophilization afforded 171 mg of dry powder (84% yield).

Procedure for coupling 4-pentynoic acid to PMO

PMO IVS2-654 (200 mg, 32 μmol) was dissolved in 600 μL DMSO. To the DMSO solution was added a solution containing 4 equivalents of 4-pentynoic acid (128 μmol) activated with HBTU (320 μL of 0.4 M HBTU in DMF, 128 μmol) and DIEA (22.3 μL, 128 μmol) in 244 μL DMF (Final reaction volume = 1.2 mL). The reaction proceeded for 25 minutes before being quenched with 1 mL of water and 2 mL of ammonium hydroxide. The ammonium hydroxide will hydrolyze any ester formed during the course of the reaction. After 1 hour, the solution was diluted to 40 mL and purified using reversed-phase HPLC (Agilent Zorbax SB C3 column: 21.2 x 100 mm, 5 μm) and a linear gradient from 2 to 60% B (solvent A: water; solvent B: acetonitrile) over 58 minutes (1 %B / min). Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

General Procedure for PMO-peptide conjugation by azide/alkyne Huisgen Cycloaddition

A 20 mL scintillation vial with a septum cap was charged with peptide alkyne (1.1 μmol), ISV2-654 azide (0.95 μmol), and copper bromide (0.05 mmol). The vial was purged with nitrogen for 5 minutes to ensure the removal of oxygen before the addition of ~ 1 mL of degassed DMF through the septum. The reaction mixture was vortexed for 1 minute. After 2 hours, the reaction mixture was diluted with 10 mL of 50 mM Tris (pH 8), and loaded onto a reversed-phase HPLC column (Agilent Zorbax SB C3 9.4 x 50 mm, 5 μm). Chromatography was performed using a linear gradient from 5-45 %B over 20 minutes. Solvent A: 100 mM ammonium acetate, pH = 8 in water; solvent B: acetonitrile. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

Because peptide **7c** contained an azide group, for the synthesis of PMO-**7c** an identical protocol was followed using ISV2-654-alkyne.

PMO-**3b** and PMO-**6c** were purified a second time to remove co-eluting PMO, except now using solvent A: water containing 0.1% formic acid and solvent B: acetonitrile containing 0.1% formic acid. After lyophilization, the conjugates were then resuspended in water containing 100 mM ammonium acetate and lyophilized again.

6. Proteolysis assay

Proteolysis Assay of bicyclic peptides

For each peptide, 19.6 µL of PBS, 0.2 µL of Trypsin (0.005 mg/mL stock solution in 1 mM HCl), and 0.2 µL of peptide (1 mM DMSO stock solution) were combined in a PCR tube. The resulting reaction mixture was capped and incubated at 37 °C. At each time point, 1.0 µL of the crude reaction was transferred to a LC-MS vial and quenched by addition of 99 µL of 50:50 water:acetonitrile containing 0.1% TFA. 1.0 µL of the quenched reaction was injected onto the Agilent 6550 iFunnel Q-TOF MS. Time points were taken at t = 0 min, 20 min, 40 min, and 60 min. An extracted ion current (EIC) for the +5 charge state m/z was analyzed using the MassHunter software. The EIC peak was integrated and percent peptide intact was determined by ($EICt_1/EICt_0$) * 100 in which $EICt_1$ is the peak integration at a given time point and $EICt_0$ is the peak integration at time t = 0.

7. Comparison of *i,i+1* and *i,i+7* cyclization

*Procedure for comparing cyclization of **3a** and **4a***

A 0.5 mL microcentrifuge tube was charged with 1 mM **3a** in DMF. The cyclization reaction began with the addition of DIEA (50 mM) and the reaction vessel was vortexed for 30 seconds. After 0, 30, 60, 120, and 300 seconds, a 1 µL aliquot was quenched with 19 µL of 50:50 water:acetonitrile containing 0.1% TFA and subjected to LC-MS analysis on an Agilent 6550 iFunnel Q-TOF MS. An extracted ion current (EIC) for the +5 charge state m/z was analyzed using the MassHunter software. The EIC peak was integrated and percent peptide intact was determined by ($EICt_1/EICt_0$) * 100 in which $EICt_1$ is the peak integration at a given time point and $EICt_0$ is the peak integration at time t = 0. The process was repeated exactly for **4a**.

8. eGFP Exon-Skipping Assay

HeLa-654 cells were maintained in MEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin at 37 °C and 5% CO_2. Each PMO-peptide conjugate stock solution was freshly prepared on the same day as the experiment using phosphate-buffered serum (PBS). The concentration of the stocks was determined by measuring the absorbance at λ=260 nm (PMO extinction coefficient of ε=168,700 L mol^{-1} cm^{-1}). One day before treatment, cells were plated in a 96-well plate at a density of 5,000 cells/well. For treatment, cells were incubated with 100 µL of each respective conjugate at a concentration of either 2 or 5 µM in MEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C and 5% CO_2 for 22 hours. Next, the treatment media was aspirated and each well was incubated with 20 µL Trypsin-EDTA 0.25% for 15 min at 37 °C and 5% CO_2. To quench the trypsin, 80 µL of media was added to each well. The dissociated cells were transferred to a 96-well plate and spun at 500 rcf for 3 min. The supernatant was removed and the pellets were each washed with 200 µL of phosphate-buffered saline (PBS), and the 96-well plate was centrifuged again. The supernatant was again removed and the pellets were resuspended in 300 µL PBS with 2% FBS (v/v) and 2 µg/mL propidium iodide in water.

Flow cytometry analysis was carried out on a BD LSRII flow cytometer. Gates were applied to the data to ensure that cells that were highly positive for propidium iodide or exhibited forward/side scatter readings that were sufficiently different from the main cell population were excluded. The gated cell population for each sample well contains at least 2,000 events, with the exception of PMO-**1b** which contains 100-2,600 events for 5 µM treatment and 1,300-5,000 events for 2 µM treatment.

The mean fluorescence intensity was calculated for each sample and normalized to the background cellular fluorescence of untreated cells. For each PMO-peptide conjugate, the mean fluorescence intensity was averaged across the biological replicates (n=3). The experiment was then repeated on 3 different days. The average normalized fluorescence data for each experiment are shown in the table.

Construct at 5µM	Experiment 1 Average Normalized Mean Fluorescence	Experiment 2 Average Normalized Mean Fluorescence	Experiment 3 Average Normalized Mean Fluorescence
PMO	2.6	3.4	2.0
PMO-5	18.4	16.9	20.7
PMO-6c	33.2	39.2	43.1
PMO-1b	38.8	36.7	33.6
PMO-3b	39.5	37.3	39.3
PMO-1nfb	17.4	25.2	30.9
PMO-7c	13.3	17.8	17.3
PMO-R12 N-cycle	36.6	31.9	36.8
PMO-R12 C-cycle	18.7	25.9	30.6

Construct at 2 µM	Experiment 1 Average Normalized Mean Fluorescence	Experiment 2 Average Normalized Mean Fluorescence	Experiment 3 Average Normalized Mean Fluorescence
PMO	2.0	1.3	1.7
PMO-5	3.5	3.3	8.3
PMO-6c	11.6	17.1	24.9
PMO-1b	9.2	8.8	16.8
PMO-3b	22.2	28.7	27.5
PMO-1nfb	6.9	7.6	6.6
PMO-7c	5.7	5.0	6.6

All HeLa-654 experiments were performed in the Pentelute lab, with the exception of experiments testing the additional linear and cyclic peptides shown in supplemental figures 7 and 8. These experiments were performed at Sarepta Therapeutics as part of a library screen. In these cases, the mean fluorescence intensity was calculated for each sample and normalized to the background cellular fluorescence of untreated cells. Then, for each PMO-peptide conjugate, the mean fluorescence intensity was averaged across the biological replicates (n=3). The experiment was not repeated.

9. Effect of Serum

To test the role of serum in the treatment media, a pulse-chase experiment was performed. Briefly, HeLa 654 cells were plated at a density of 5,000 cells per well in a 96-well plate in MEM supplemented with 10% FBS and 1% penicillin-streptomycin. The next day, PMO-peptide conjugate stocks were prepared in PBS and added to media supplemented with 0%, 5%, or 10% (v/v) FBS to a final concentration of 5 μM. After preparation, the culture media was aspirated and the treatment media was added to each well. After incubation at 37 °C and 5% CO_2 for 3 hours, the treatment media was replaced with fresh untreated media (containing 10% serum and no PMO-peptide conjugate) and the cells were allowed to grow for another 22 hours at 37 °C and 5% CO_2. Sample preparation and flow cytometry was then performed as described above. The gated cell population for each sample well contains at least 2,000 events, with the exception of PMO-**1b** which contained between 500 and 2500 events per sample.

10. LDH Assay

This experiment was performed using the treatment media from the eGFP exon-skipping assay experiment. After the 22-hour treatment, the supernatant treatment media was transferred to another clear-bottom 96-well plate for the assay. The assay was performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to the included technical bulletin. After completion of the assay, the final solution in each well was diluted 5x with PBS such that the measured absorbance was in the linear range of the instrument. The absorbance was measured on a BioTek Epoch Microplate Spectrophotometer. Two sets of cell only wells were used as controls - one set was the negative control and the other was a positive control in which the cells were lysed using the assay lysis buffer to provide data for total LDH release. The results are expressed as a ratio of (Absorbance of sample – absorbance of negative control)/(Absorbance of total lysis - absorbance of negative control).

11. TAMRA-Labeled Peptide Synthesis and Flow Cytometry

5-TAMRA Conjugation

Purified peptide constructs (0.5 μmol) were each dissolved in 200 μL 50:50 t-butanol:water in a 1.7 mL microcentrifuge tube. The following solutions were added to the microcentrifuge tube:
530 μL 50:50 t-butanol:water, 10 μL of 50 mM 5-tetramethylrhodamine azide (5-TAMRA azide) in DMSO, 100 μL of 500 mM Tris pH 8 in water, 50 μL of 100 mM copper(II) sulfate in water, 10 μL of 10 mM Tris(benzyltriazolylmethyl)amine (TBTA) in DMSO, and 100 μL of 1 M ascorbic acid in water. After one hour, the reaction was diluted with 9 mL of 85:15 water:acetonitrile containing 0.1% TFA, filtered and purified by semi-preparative RP-HPLC (Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5 μm). Solvent A was water with 0.1% TFA additive and Solvent B was acetonitrile with 0.1% TFA additive. A linear gradient that changed at a rate of 0.5%/min was run from 20% B to 60% B. Using mass data abouteach fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS.

HeLa-654 Treatment and Flow Cytometry

HeLa-654 cells were maintained in MEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) Pen Strep at 37 °C and 5% CO_2. Twelve hours before treatment, HeLa cells were plated at a density of 5,000 cells per well in a 96-well plate. 1 mM stocks of each of the peptides were prepared in DMSO. Concentration of the stocks was quantified by absorbance using a UV/Vis spectrophotometer after dilution into PBS. A 5-TAMRA extinction coefficient of ε=54,124 $M^{-1}cm^{-1}$ at λ=552 nm was used (determined experimentally by creating a standard curve of 5-TAMRA azide dissolved in PBS. Then, each peptide was diluted to a final concentration of 5 μM in MEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) Pen Strep. To treat the cells, the overnight growth media was aspirated from each well and 100 μL of a given 5 μM peptide stock was applied to each well. Cells were incubated for 2 hours at 37 °C and 5% CO_2 with the peptide treatment, and then the treatment media was aspirated. Trypsin-EDTA 0.25 % (20 μL) was added to the cells and incubated for 10 min at 37 °C and 5% CO_2. To quench the trypsin, 80 μL of MEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) Pen Strep was added to each well. The dissociated cells in media were transferred to a 96-well plate and spun at 500 rcf for 3 min. The supernatant was removed and the pellets were each washed with 200 μL of phosphate-buffered saline (PBS), and the tubes were spun again. The supernatant was again removed and the pellets were resuspended in 300 μL PBS with 2% FBS (v/v) (no propidium iodide). Flow cytometry analysis was carried out on a BD LSRII flow cytometer. Gates were applied to the data to ensure that only data from healthy, living cells were taken into account. Cells that had forward/side scatter readings that were sufficiently different from the main cell population were excluded. The gated cell population for each sample well contains between 1,300 and 3,300 events.

13. References

[1] A. J. Mijalis, D. A. Thomas III, M. D. Simon, A. Adamo, R. Beaumont, K. F. Jensen, B. L. Pentelute, *Nat. Chem. Biol.* **2017**, *13*, 464–466.

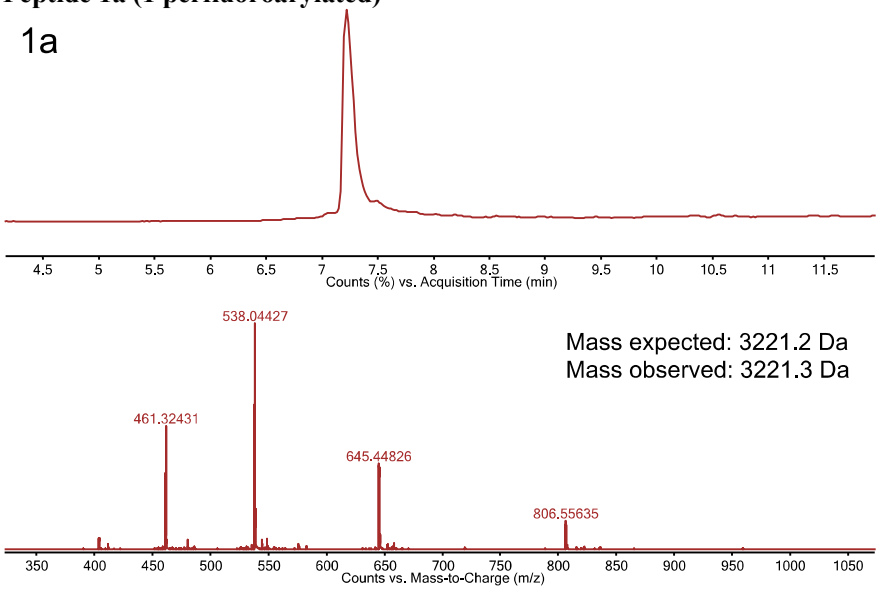
[2] M. D. Simon, P. L. Heider, A. Adamo, A. A. Vinogradov, S. K. Mong, X. Li, T. Berger, R. L. Policarpo, C. Zhang, Y. Zou, et al., *ChemBioChem* **2014**, *15*, 713–720.

[3] H. D. Herce, D. Schumacher, A. F. L. Schneider, A. K. Ludwig, F. A. Mann, M. Fillies, M.-A. Kasper, S. Reinke, E. Krause, H. Leonhardt, et al., *Nat. Chem.* **2017**, *9*, 762.

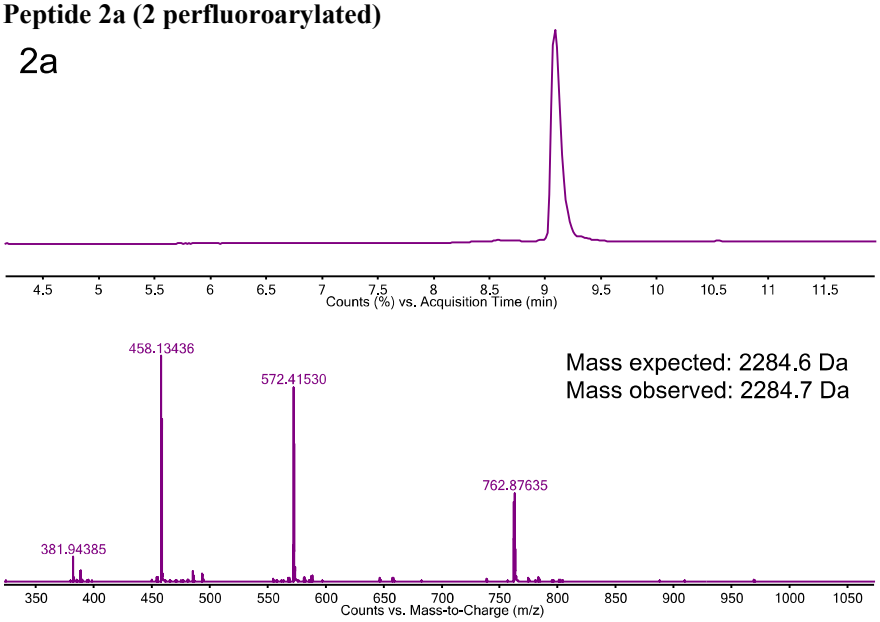
15. Chromatograms

Peptide chromatograms

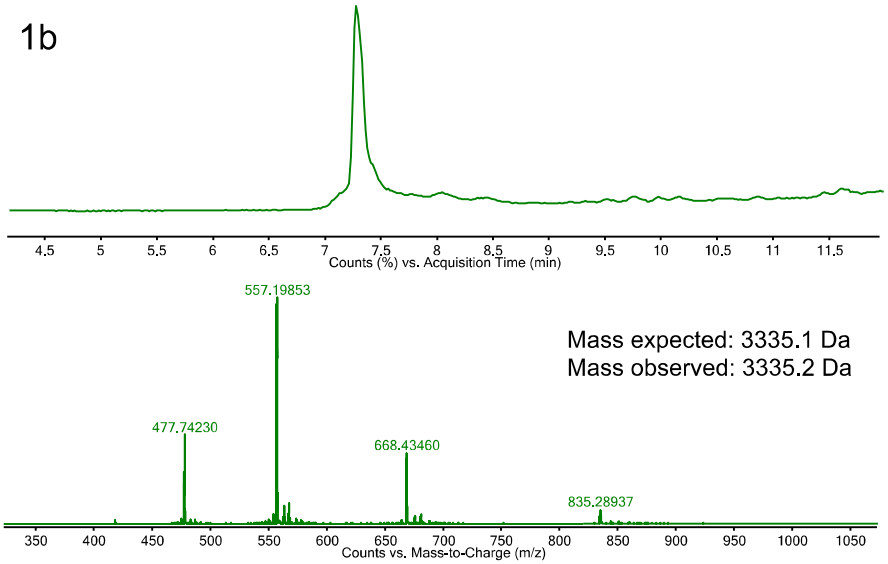
Peptide 1a (1 perfluoroarylated)



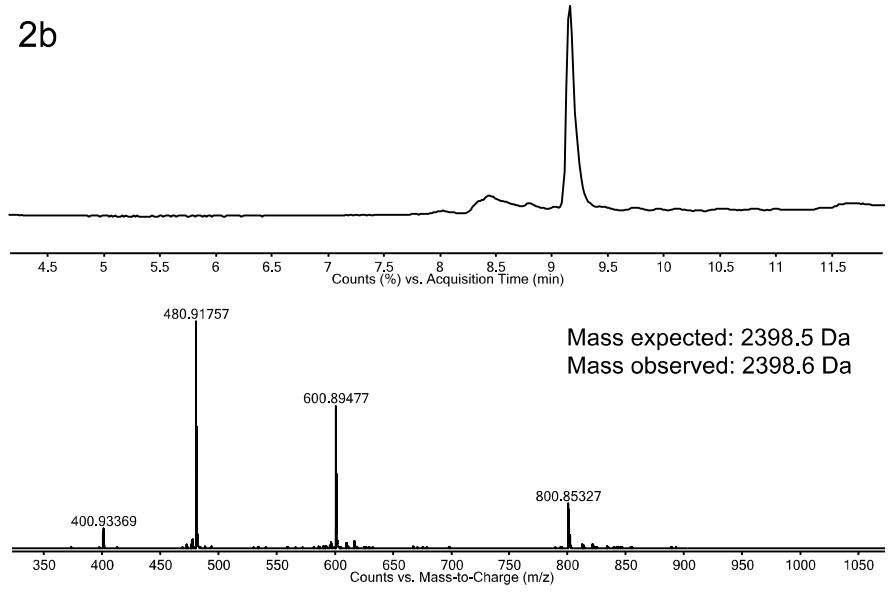
Peptide 2a (2 perfluoroarylated)



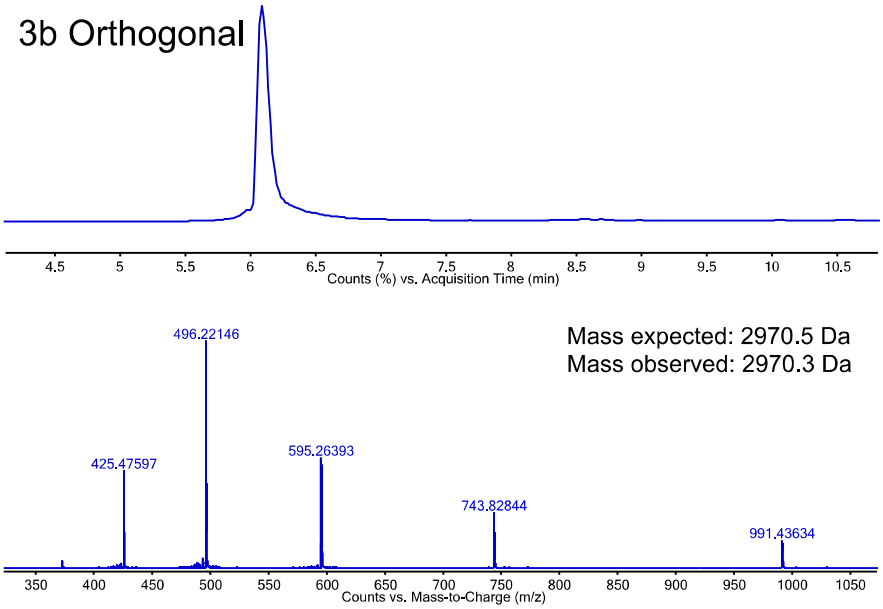
Peptide 1b (1 bicycle)



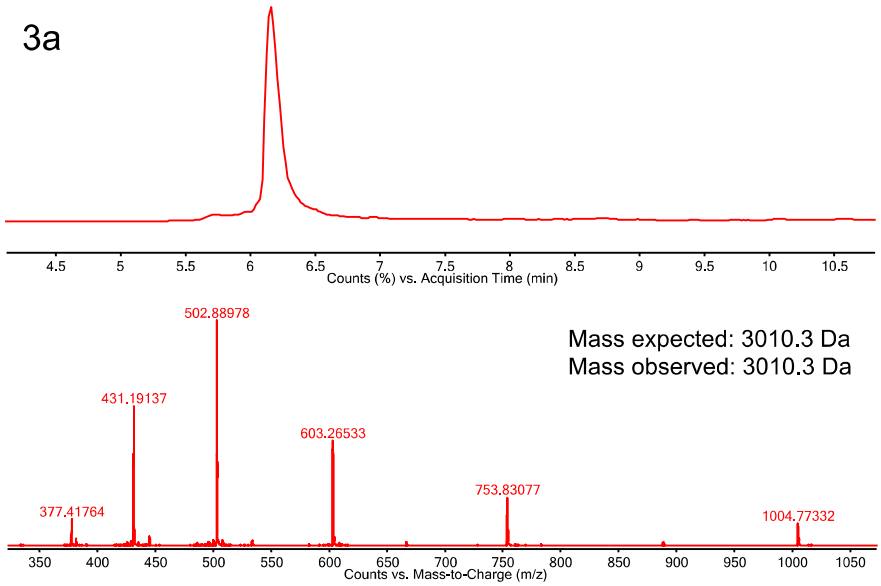
Peptide 2b (2 bicycle)



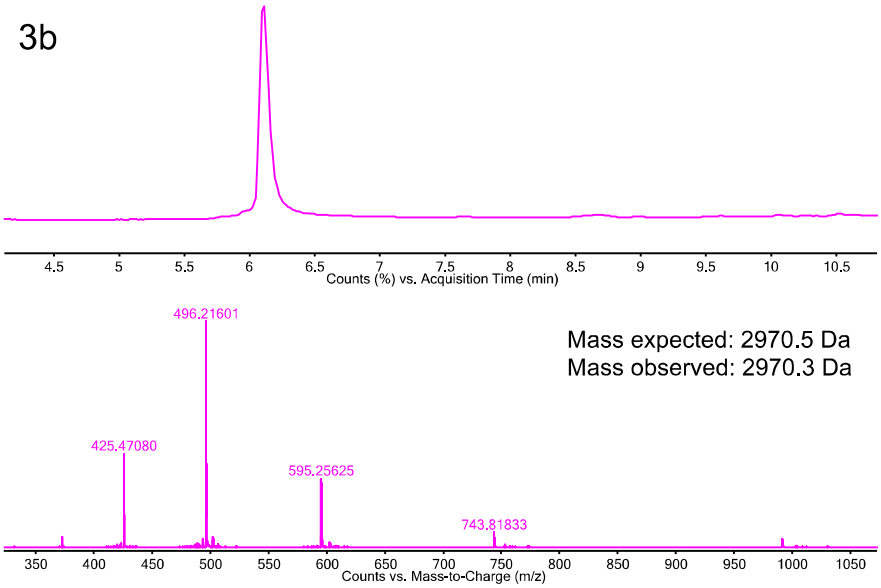
Peptide 3b (3 double cycle, synthesized through orthogonal deprotection)



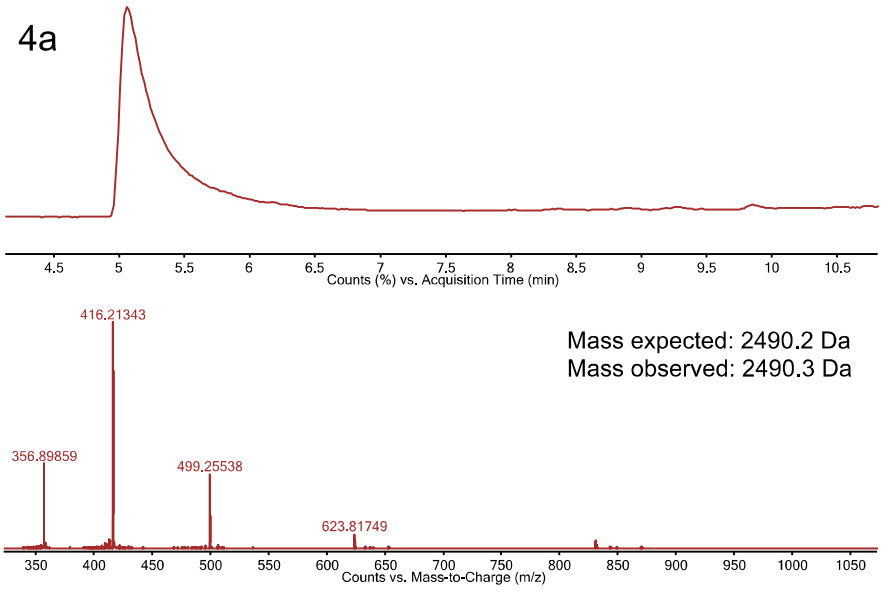
Peptide 3a (3 perfluoroarylated at position 1 and 9)



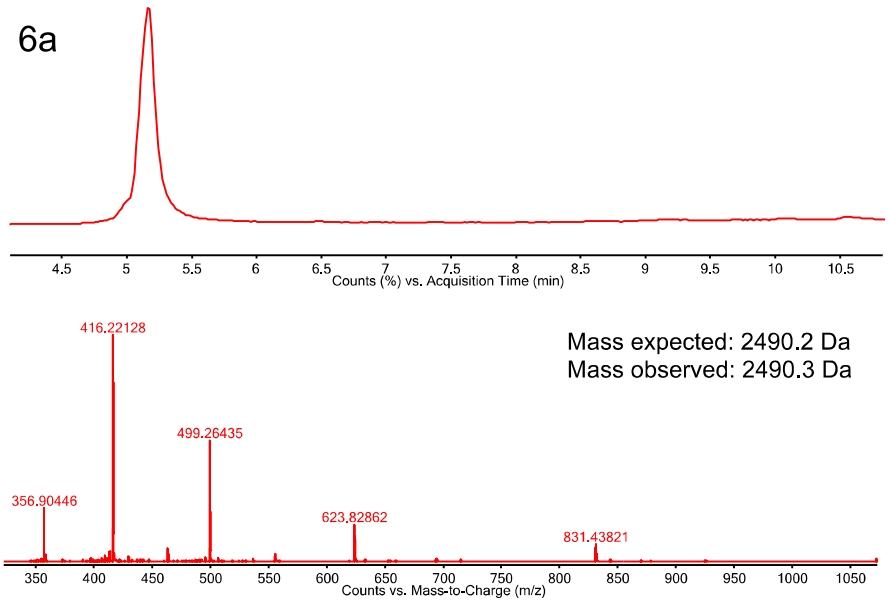
Peptide 3b (3 double cycle, synthesized through kinetic control)



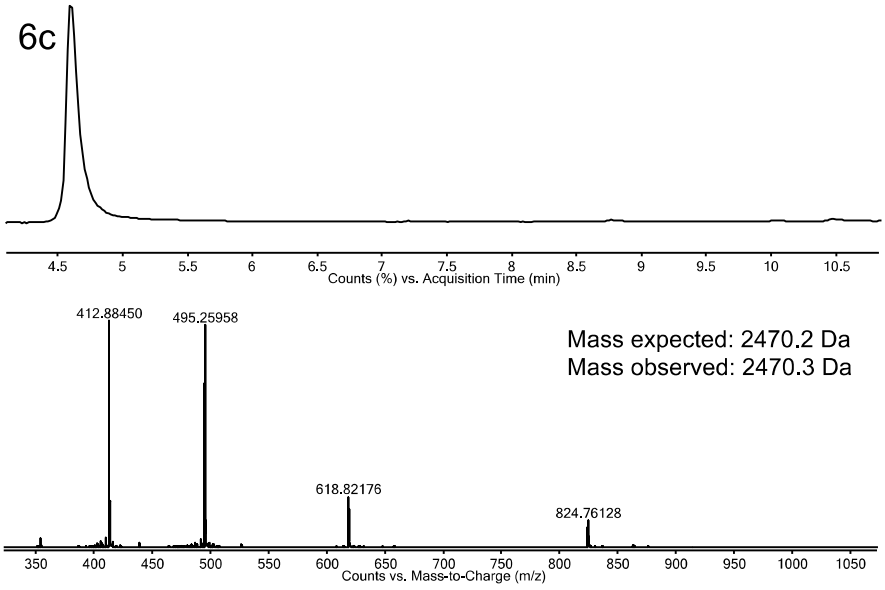
Peptide 4a (4 perfluoroarylated at position 9)



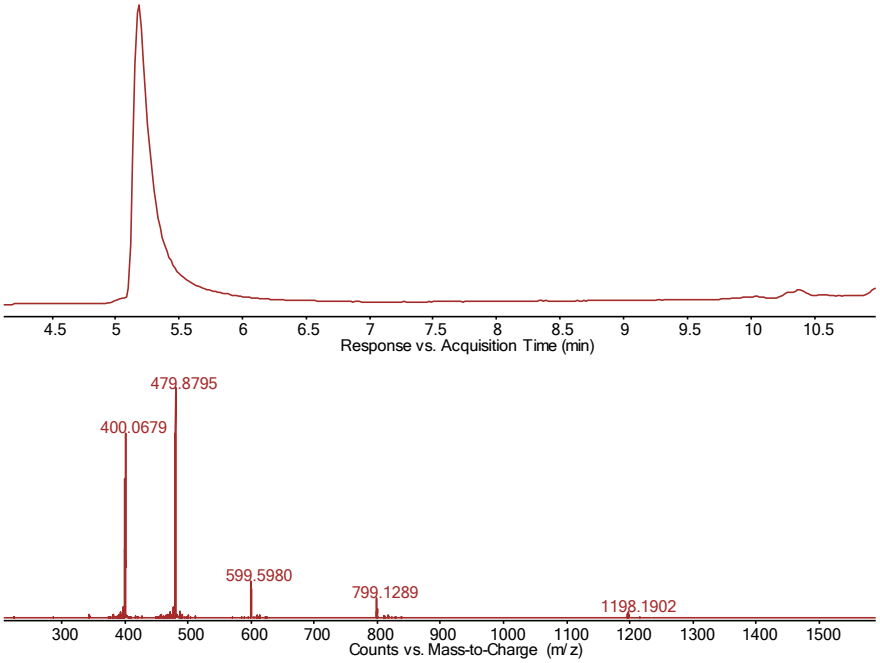
Peptide 6a



Peptide 6c (R12 monocycle)

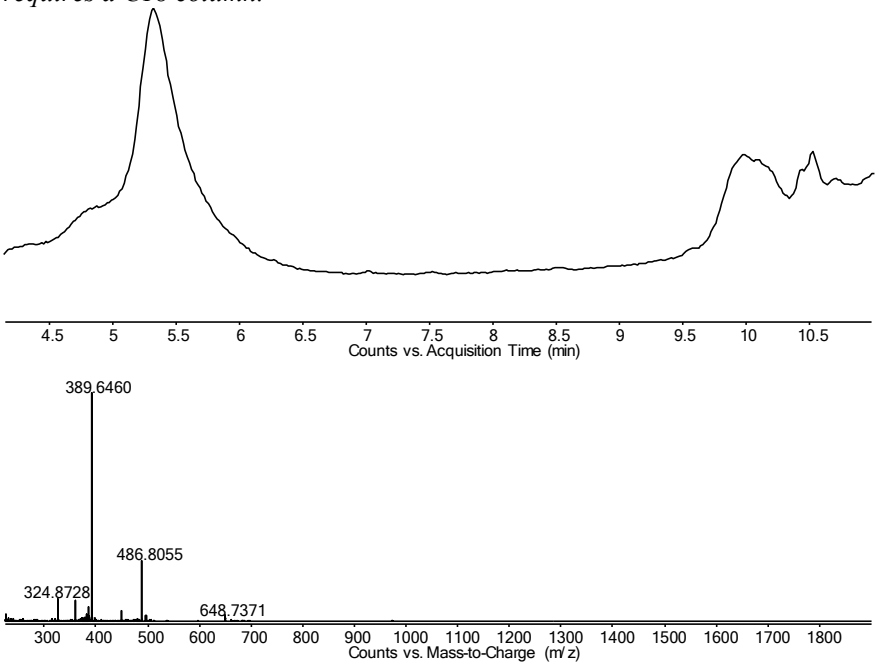


Peptide 1nfb (R12 non-fluorinated bicycle)
Mass expected: 2393.3 Da
Mass observed: 2393.4 Da

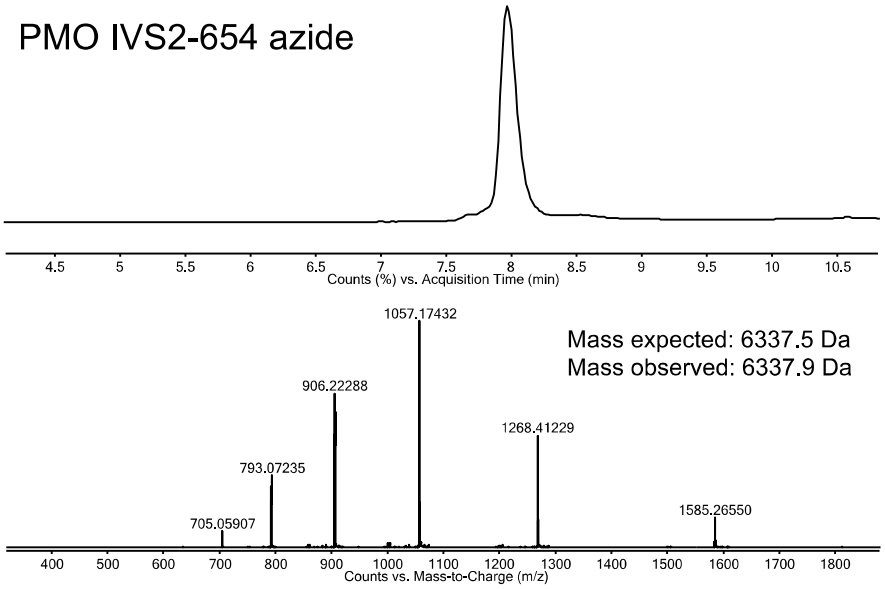


Peptide 7c (cR10)
Mass expected: 1942.2 Da
Mass observed: 1942.3 Da

**The peaks after 9 minutes result from column background. The hydrophilic nature of peptide 7c requires a C18 column.*



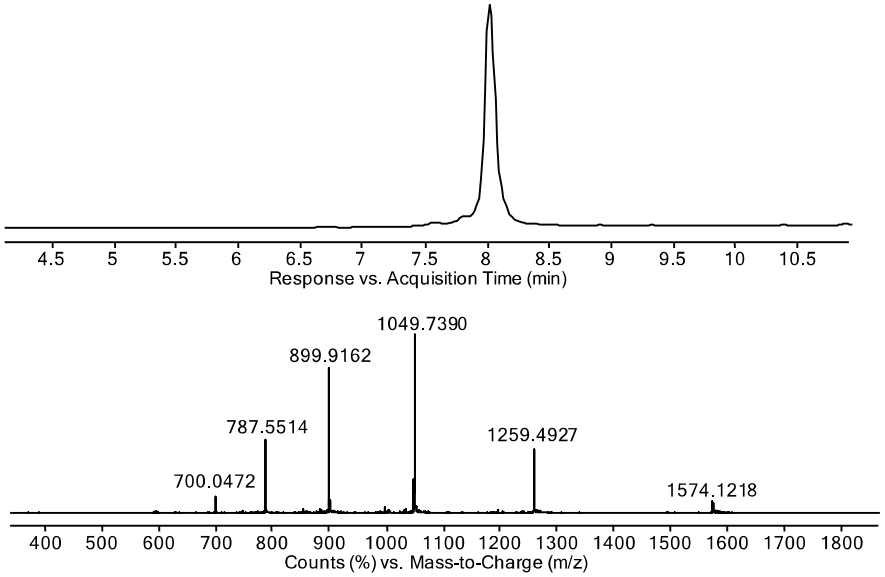
PMO IVS2-654 azide



PMO IVS2-654 alkyne

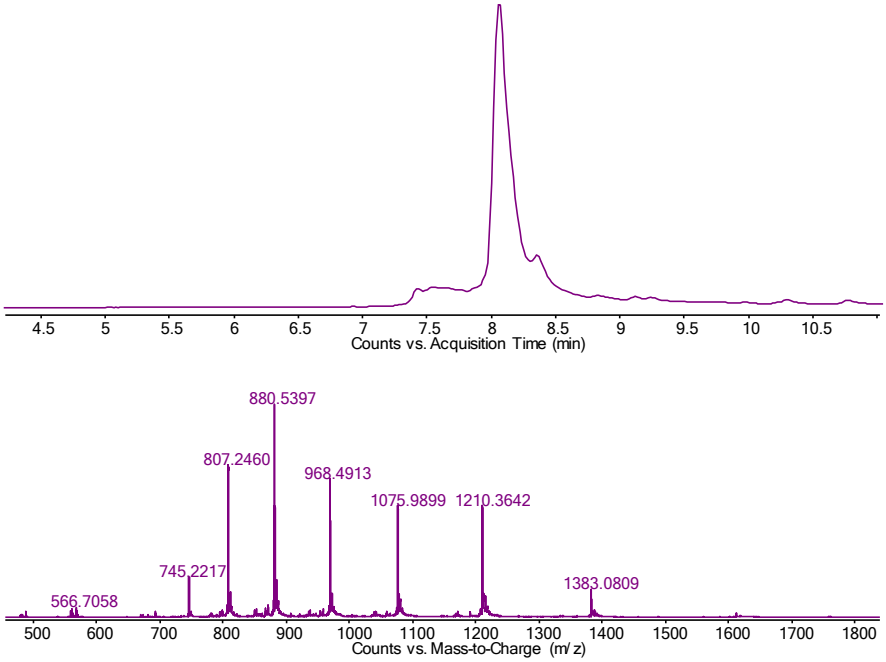
Mass expected: 6292.5 Da

Mass observed: 6293.6 Da

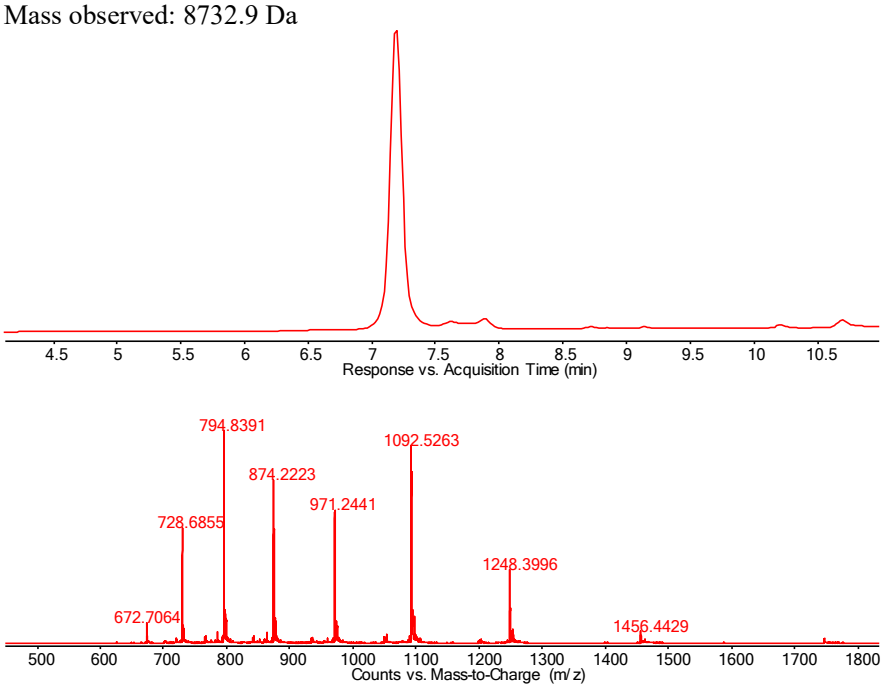


PMO-peptide conjugate chromatograms

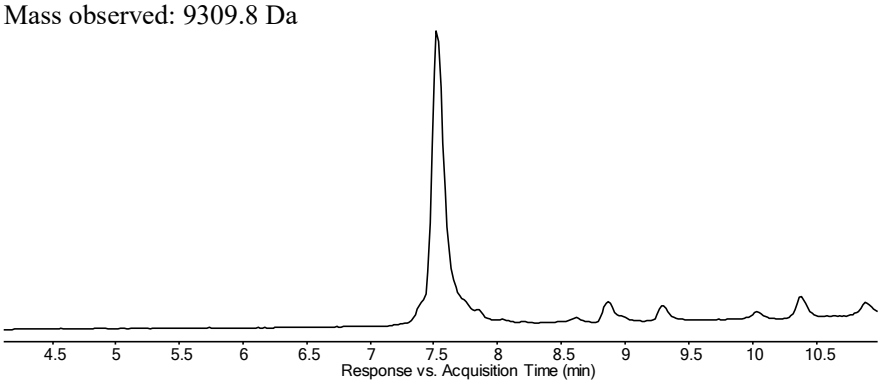
PMO-1b (PMO-R12 trithiol bicycle)
Mass expected: 9674.9 Da
Mass observed: 9675.3 Da

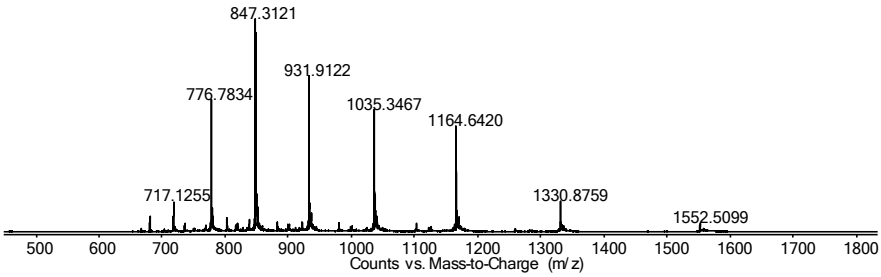


PMO-1nfb (PMO-R12 non-fluorinated bicycle)
Mass expected: 8732.5 Da
Mass observed: 8732.9 Da

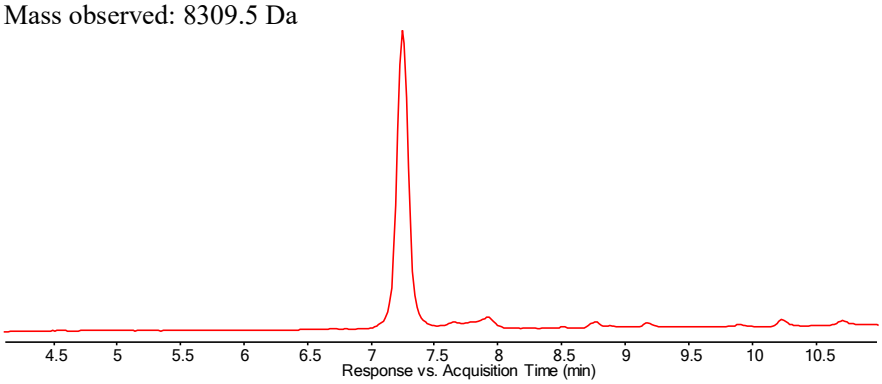


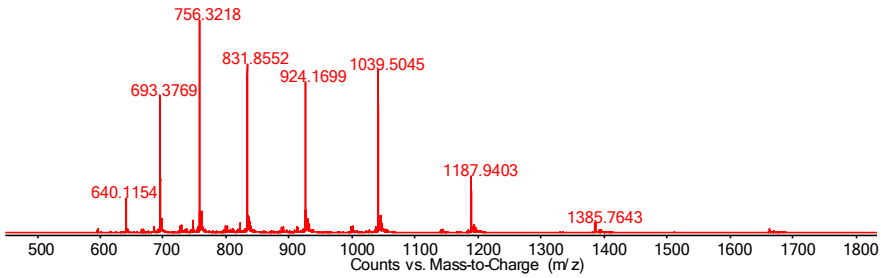
PMO-3b (PMO-R12 double cycle)
Mass expected: 9309.6 Da
Mass observed: 9309.8 Da



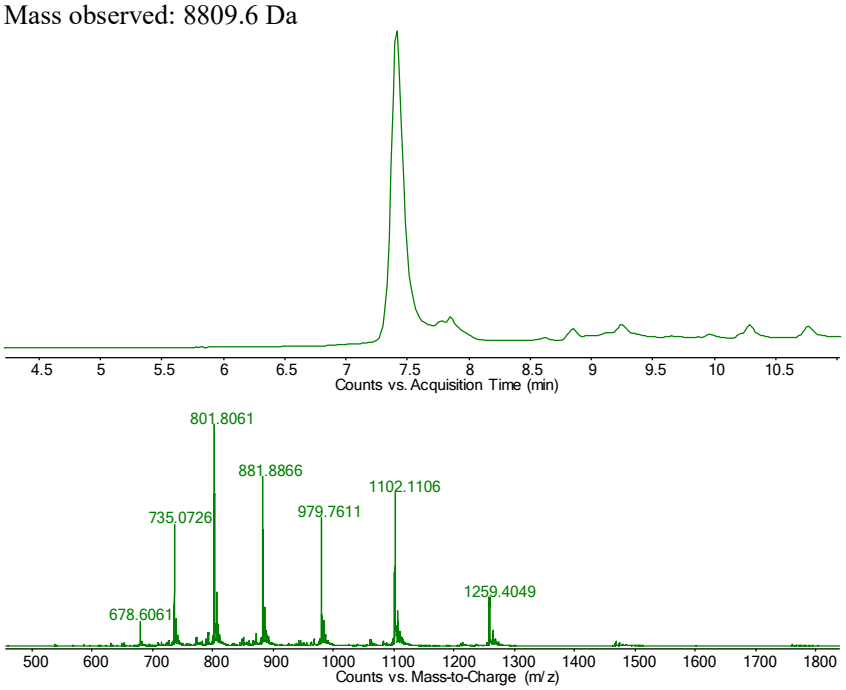


PMO-5 (PMO-R12)
Mass expected: 8309.0 Da
Mass observed: 8309.5 Da

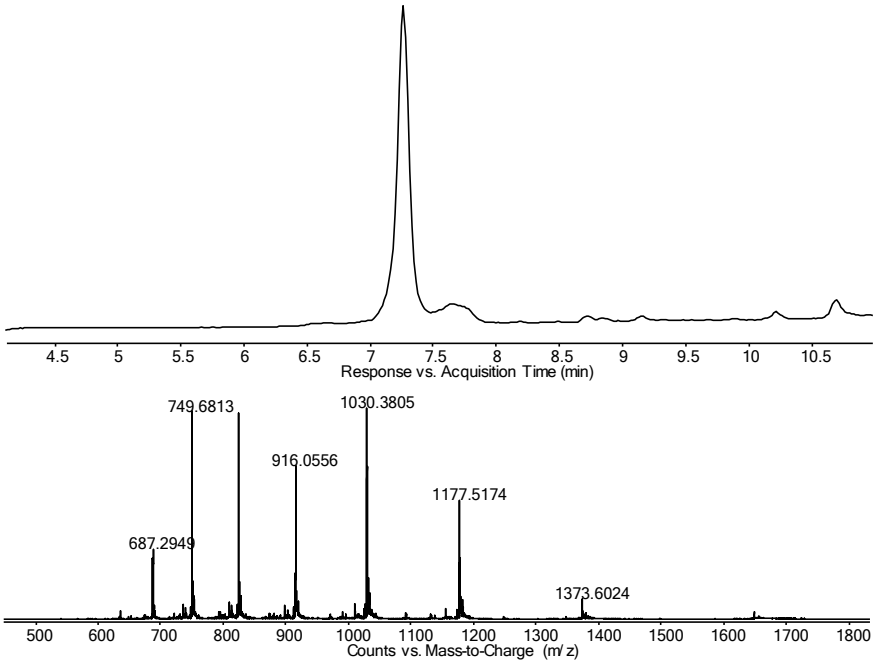




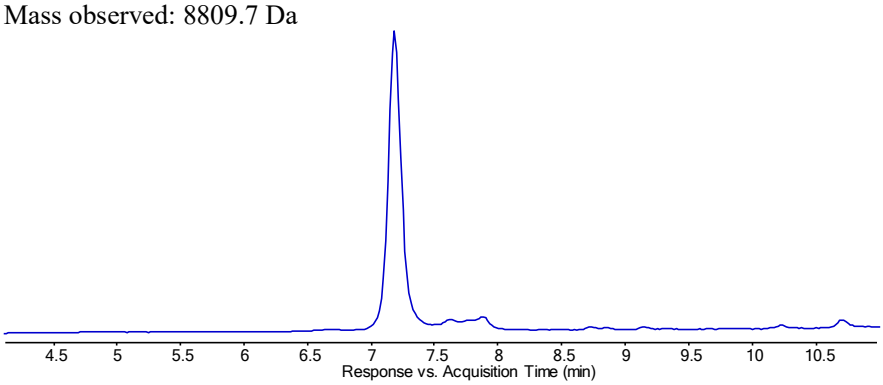
PMO-6c (PMO-R12 cycle)
Mass expected: 8809.2 Da
Mass observed: 8809.6 Da

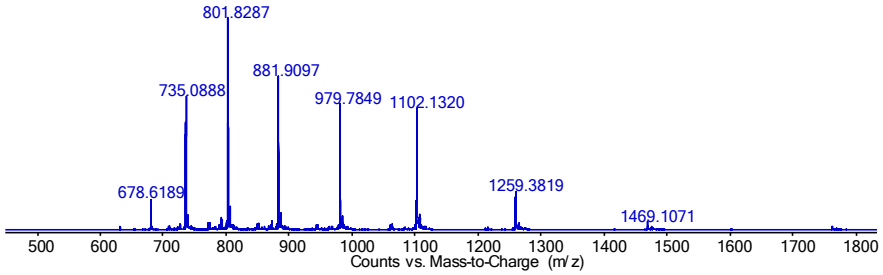


PMO-7c (PMO-cR10)
Mass expected: 8235.8 Da
Mass observed: 8236.3 Da

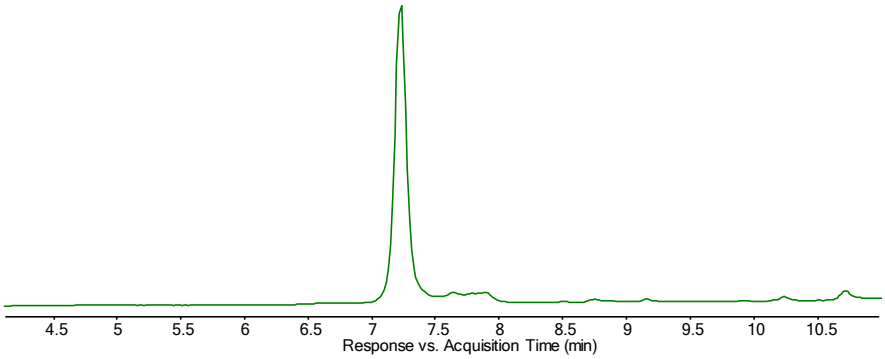


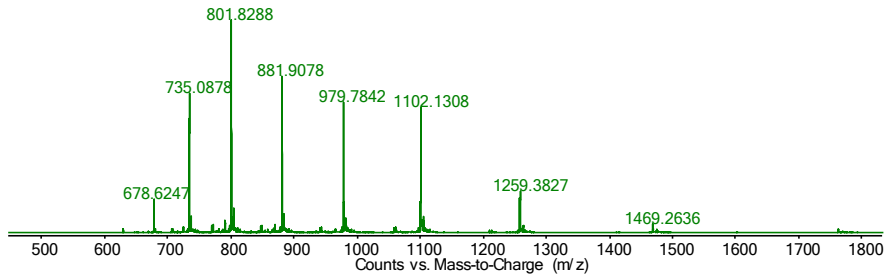
PMO-R12 cyclized on N-term
Mass expected: 8809.2 Da
Mass observed: 8809.7 Da





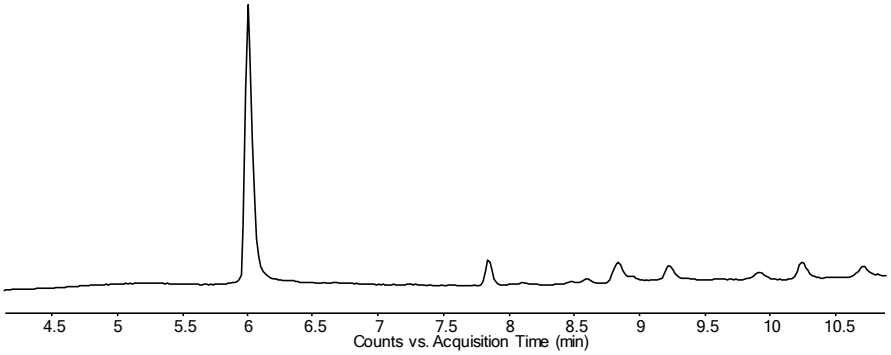
PMO-R12 cyclized on C-term
Mass expected: 8809.2 Da
Mass observed: 8809.7 Da

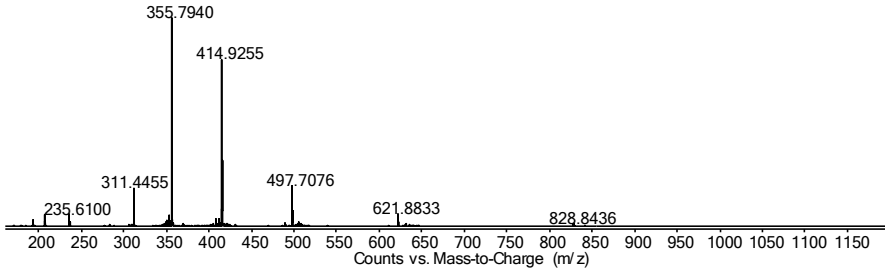




TAMRA-peptide Conjugate Chromatograms

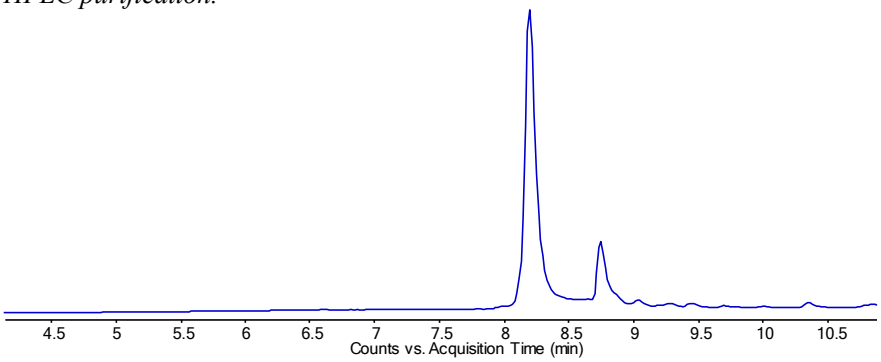
TAMRA-5 (TAMRA-R12)
Mass expected: 2482.4 Da
Mass observed: 2482.5 Da

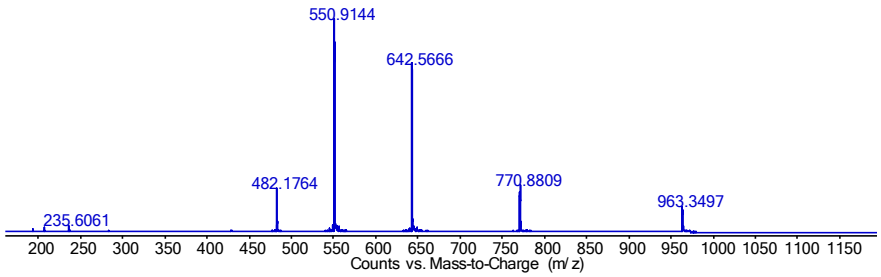




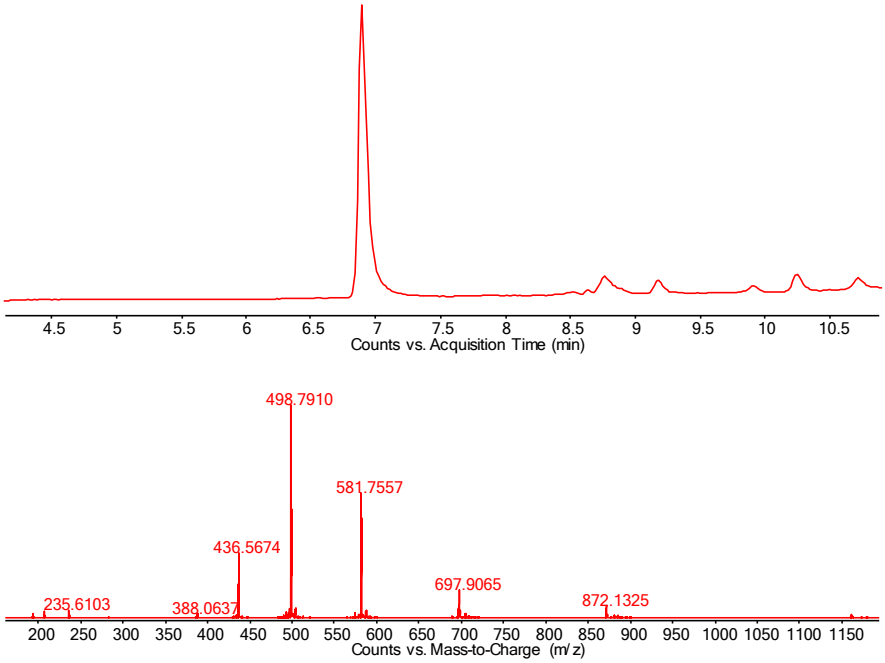
TAMRA-1b (TAMRA-R12 trithiol bicycle)
Mass expected: 3847.3 Da
Mass observed: 3847.4 Da

**The additional peak before 9 minutes is excess TAMRA-azide that could not be removed during HPLC purification.*

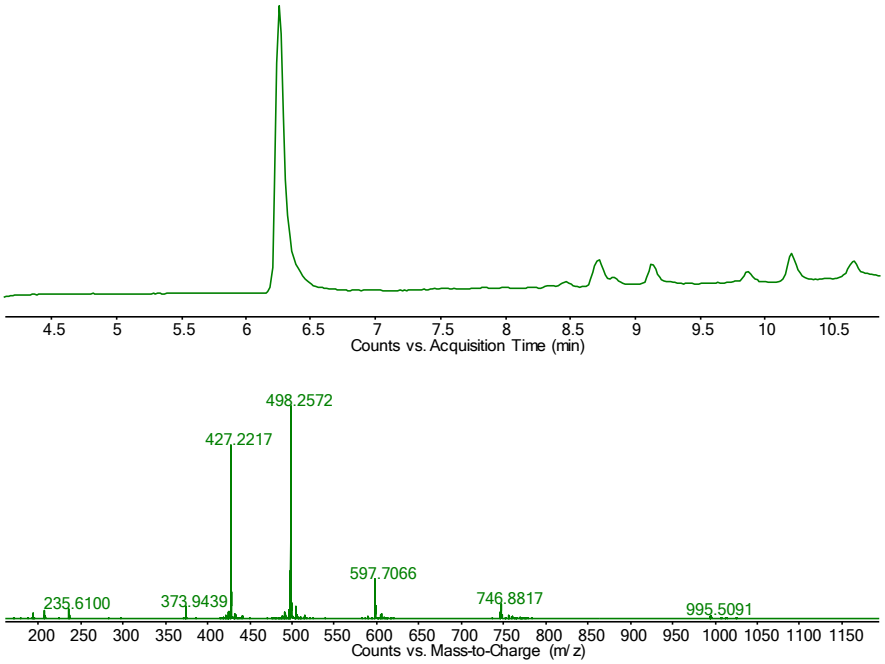




TAMRA-3b (TAMRA-R12 double cycle)
Mass expected: 3482.7 Da
Mass observed: 3482.6 Da



TAMRA-6c (TAMRA-R12 monocycle)
Mass expected: 2982.4 Da
Mass observed: 2982.5 Da

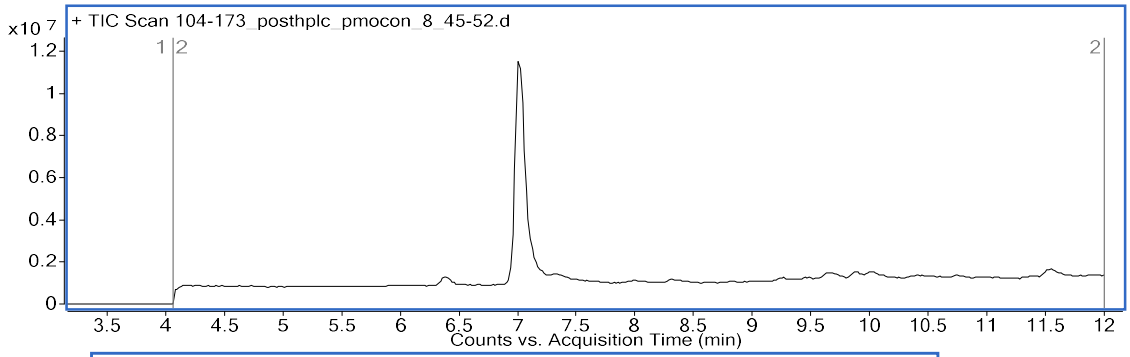


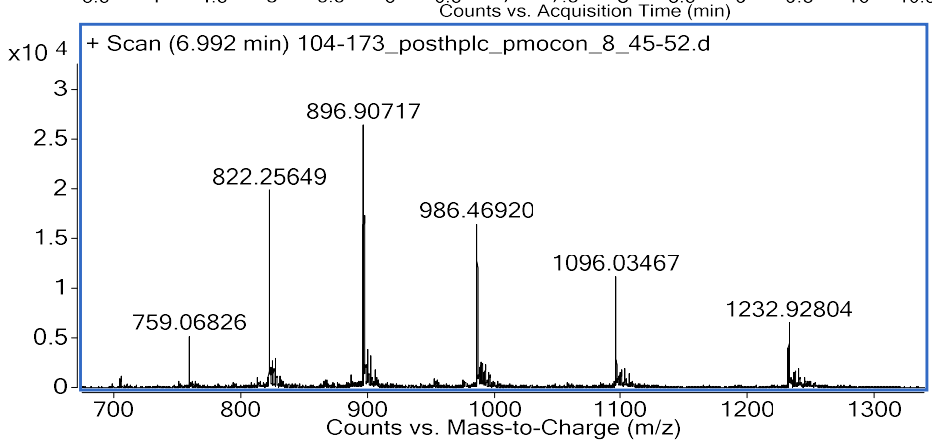
PMO-cyclic CPP conjugates

Bolded Cysteines are linked with decafluorophenyl

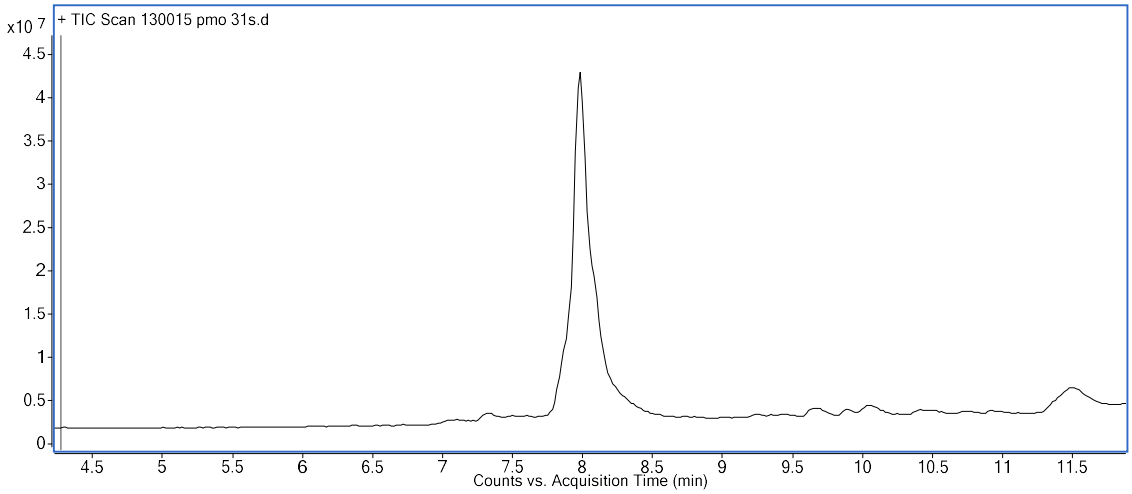
**Note: the linear CPP conjugates were assayed in the context of a larger library screen, and characterization data for the entire library will be provided elsewhere. The sequences of the linear peptide do not contain the cysteine residues.*

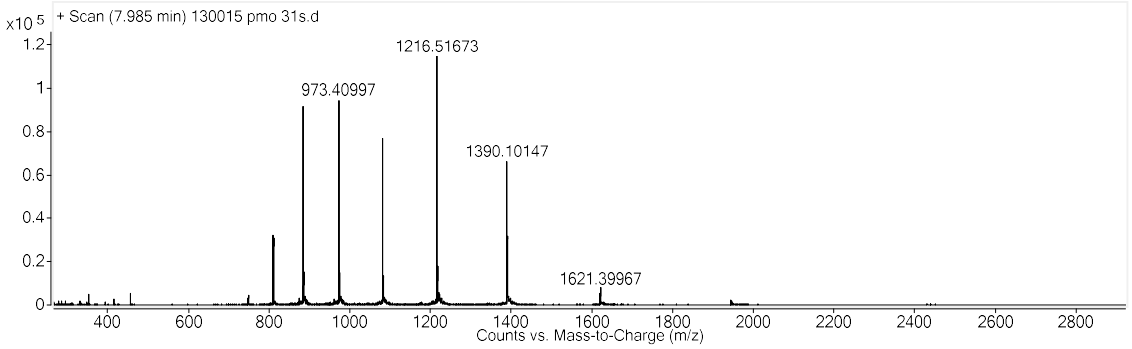
PMO-Bac7 monocycle
Mass expected: 9855.6 Da
Mass observed: 9856.0 Da
Peptide Sequence: **C**RRIRPRPPRLPRPRPRPLPFPRPG**C**



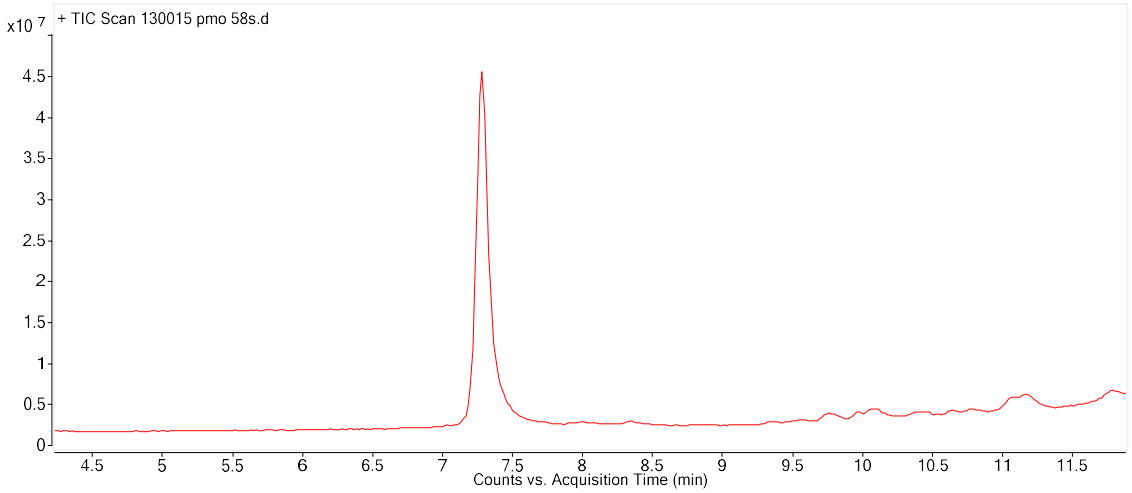


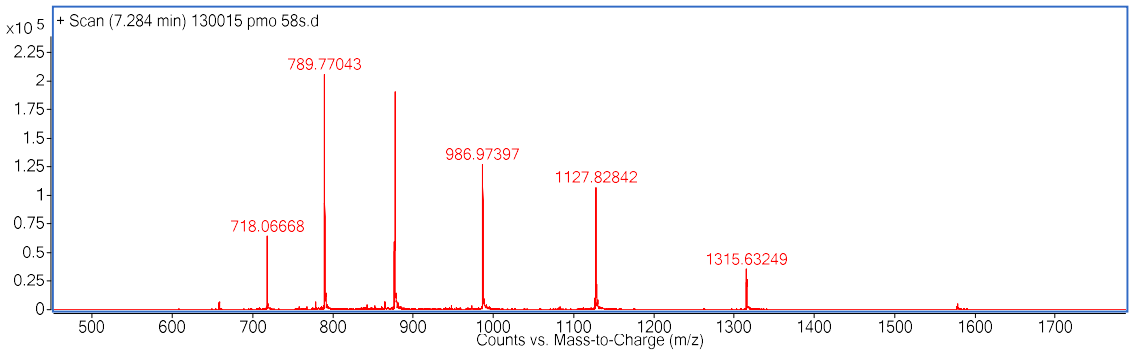
PMO-MPG monocycle
Mass expected: 9724.3 Da
Mass observed: 9724.7 Da
Peptide Sequence: **C**GLAFLGFLGAAGSTMGAWSQPKKKRKV**C**



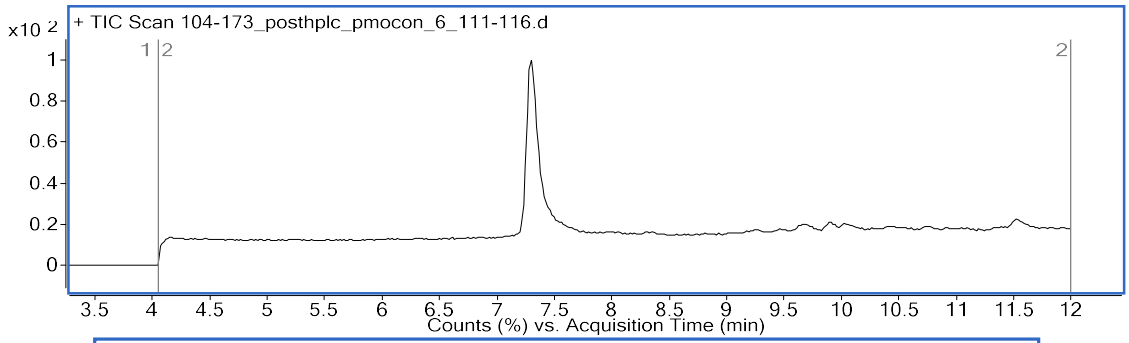


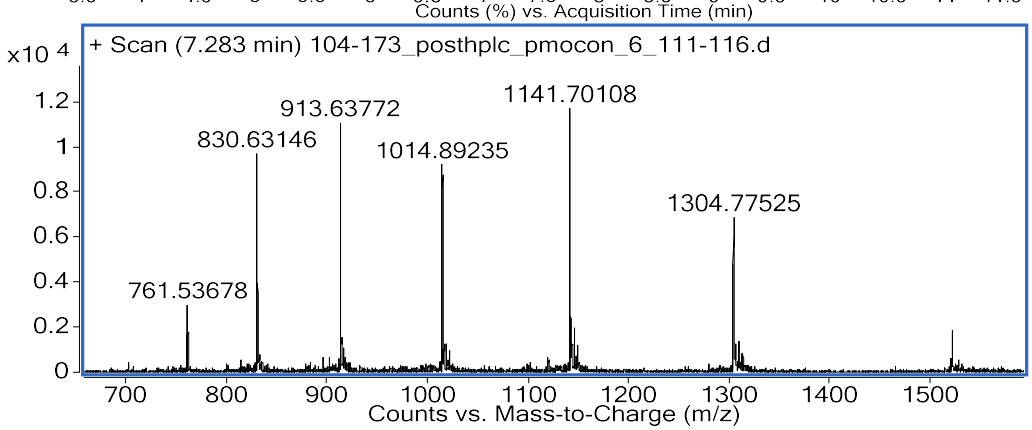
PMO-CAYH monocycle
Mass expected: 7888.0 Da
Mass observed: 7888.6 Da
Peptide Sequence: **C**AYHRLRR**C**



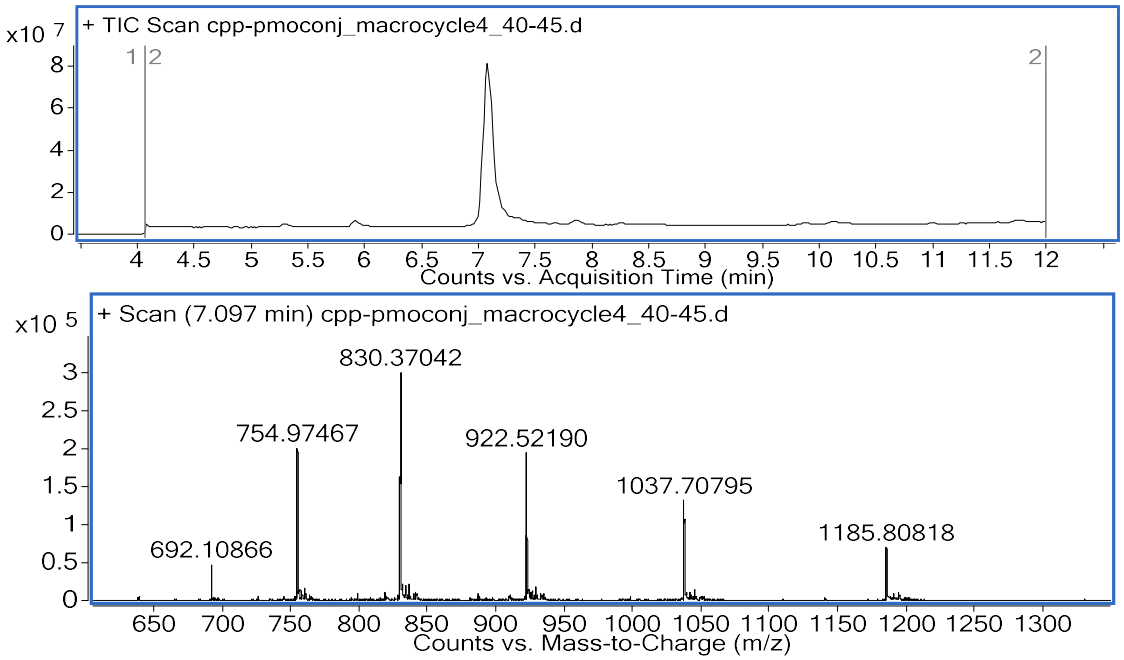


PMO-pVEC monocycle
Mass expected: 9126.7 Da
Mass observed: 9127.1 Da
Peptide Sequence: **C**LLIILRRRIRKQAHAHSK**C**

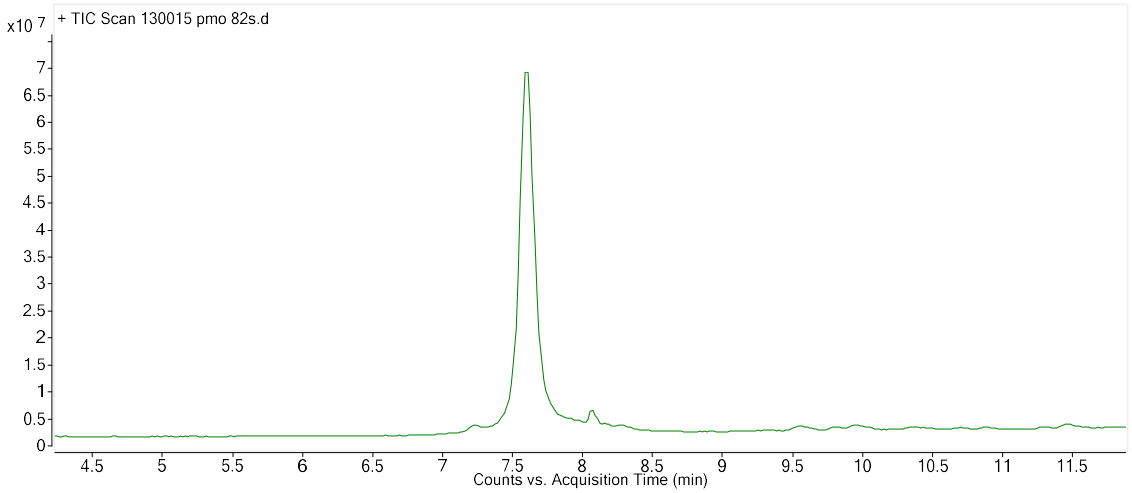


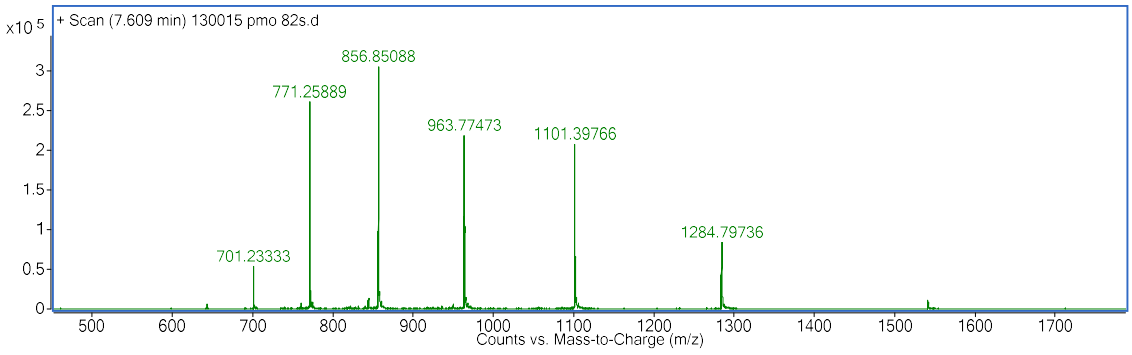


PMO-HRSV monocycle
Mass expected: 8293.7 Da
Mass observed: 8293.7 Da
Peptide Sequence: **C**RRIPNRRPRR**C**

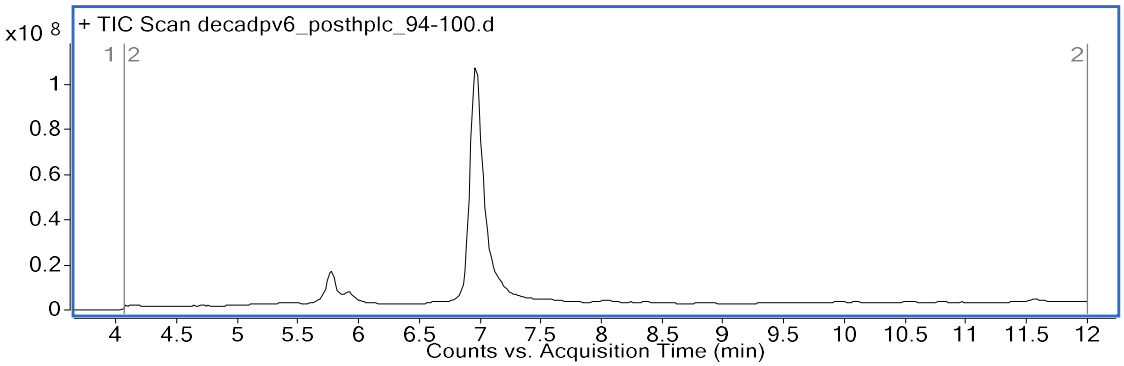


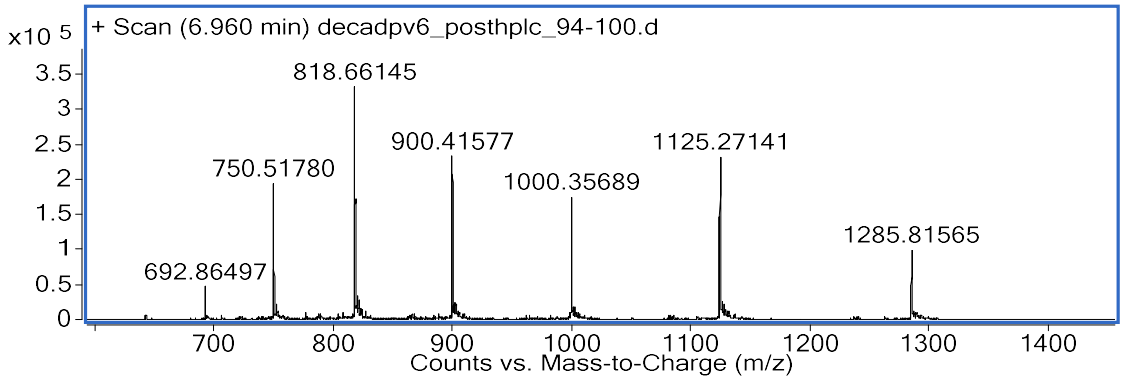
PMO-AIP6 monocycle
Mass expected: 7702.8 Da
Mass observed: 7704.0 Da
Peptide Sequence: **C**RLRWR**C**



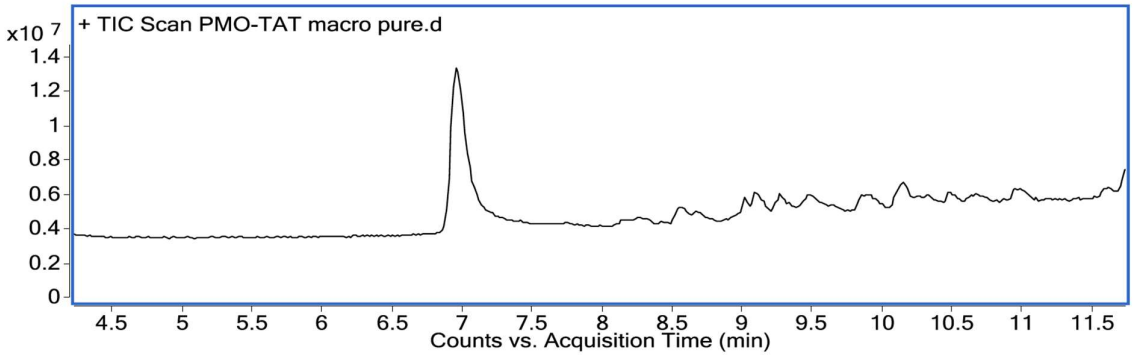


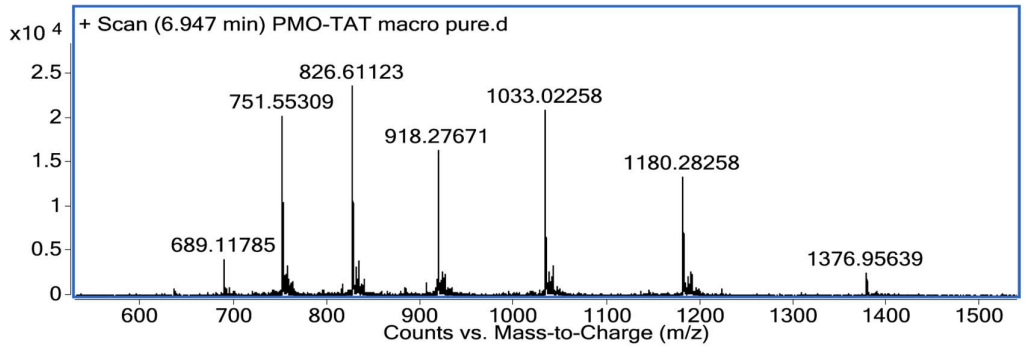
PMO-DPV6 monocycle
Mass expected: 8994.6 Da
Mass observed: 8995.1 Da
Peptide Sequence: **C**GRPRESGKKRKRKRLKP**C**





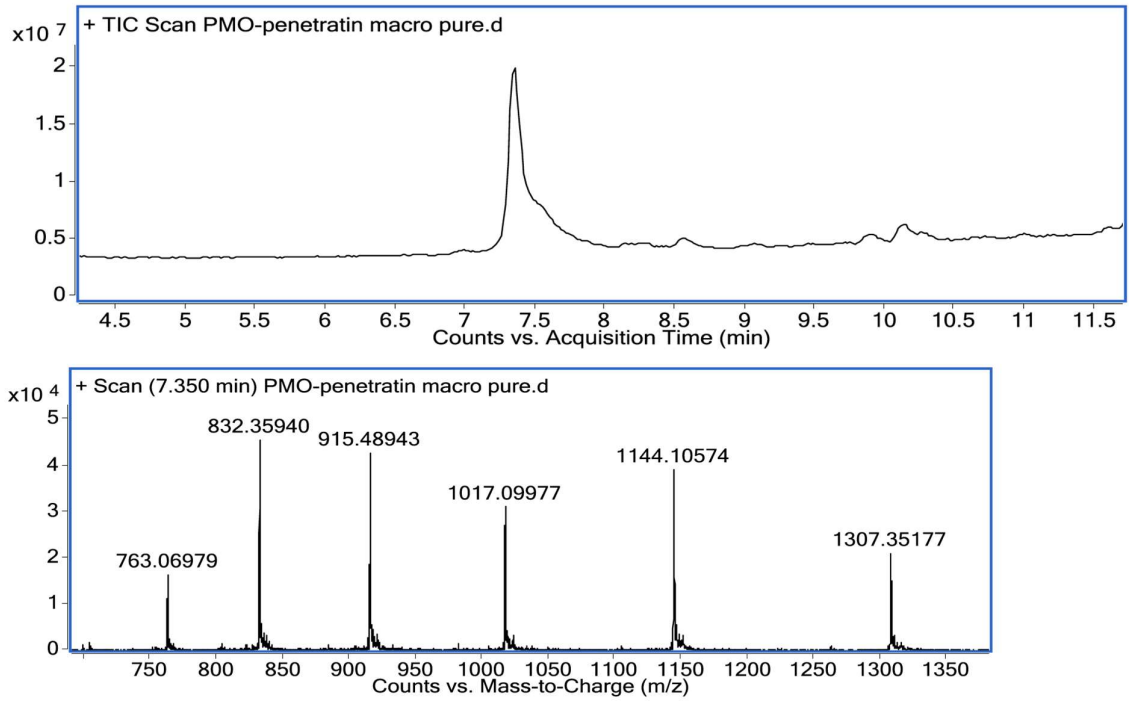
PMO-TAT monocycle
Mass expected: 8255.4 Da
Mass observed: 8257.0 Da
Peptide Sequence: **C**RKKRRQRRR**C**



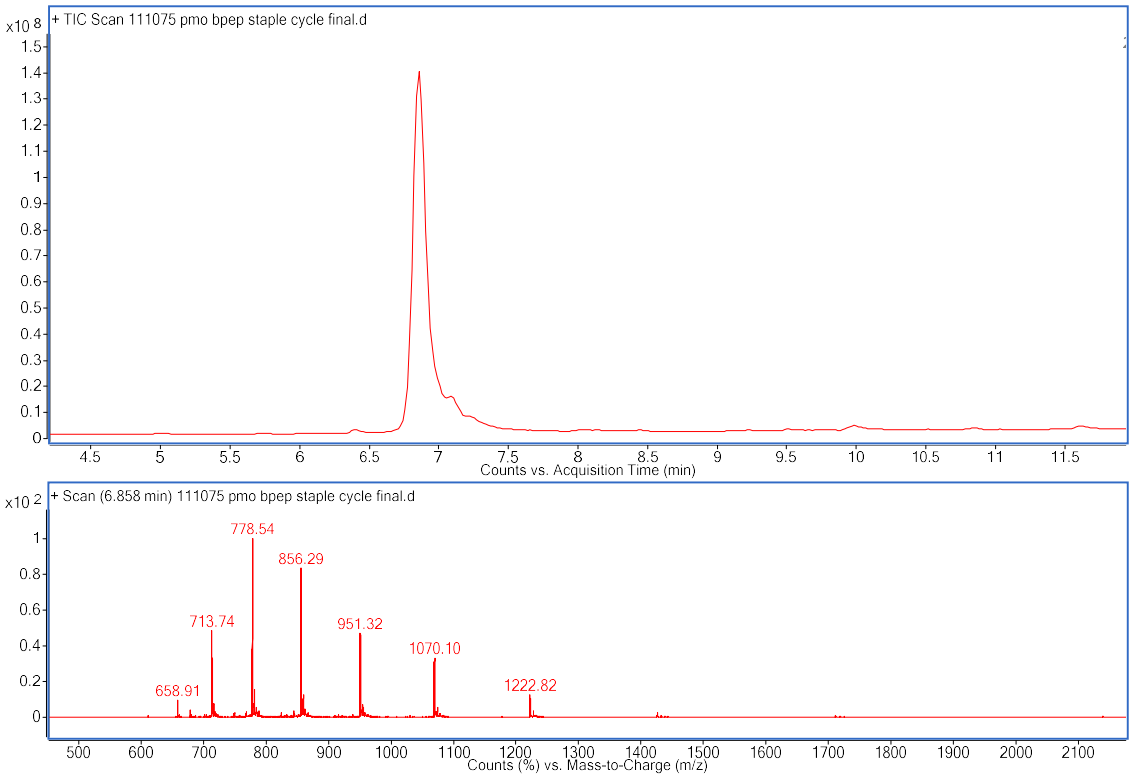


PMO-penetratin monocycle
Mass expected: 9143.9 Da
Mass observed: 9145.7 Da
Peptide Sequence: **C**RQIKIWFQNRRMKWKK**C**

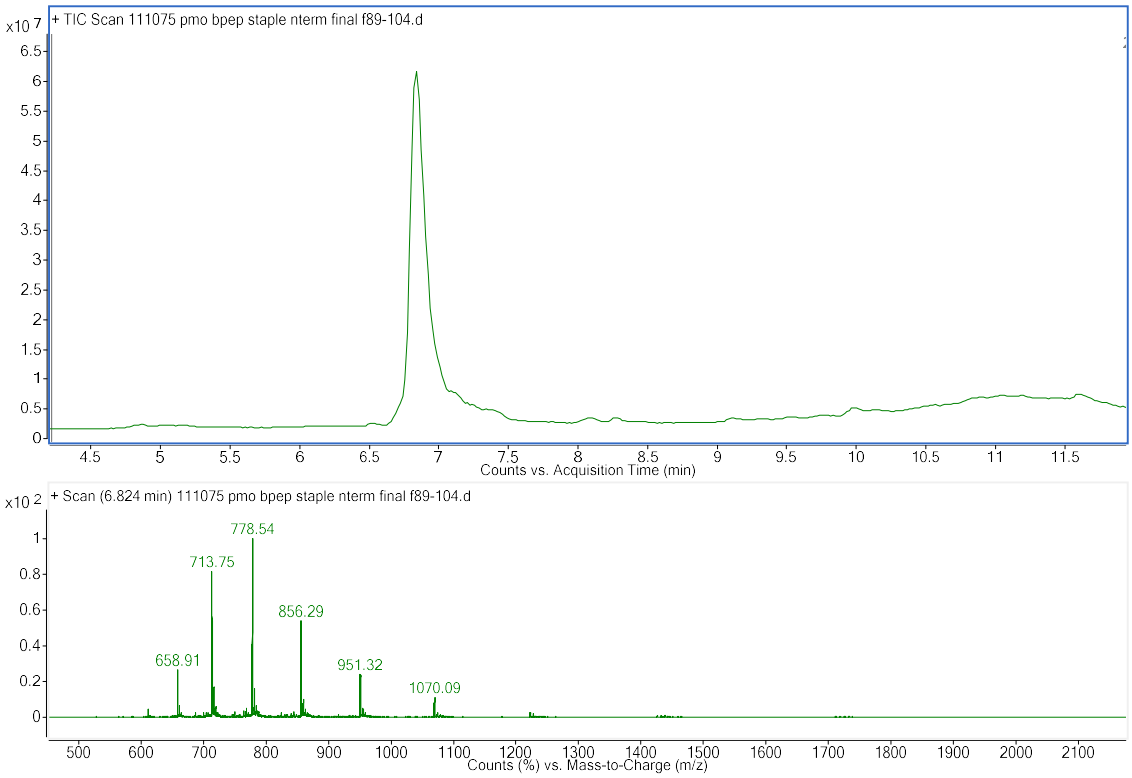
*Note: Shoulder in LC-MS trace corresponds to minor spontaneous oxidation products



PMO-Bpep monocycle
Mass expected: 8553.0 Da
Mass observed: 8552.4 Da
Peptide Sequence: **C** RXR RBR RXR RBR **C**



PMO-Bpep N-term cycle
Mass expected: 8553.0 Da
Mass observed: 8553.0 Da
Peptide Sequence: **C** RXR RBR **C** RXR RBR



PMO-Bpep C-term cycle
Mass expected: 8553.0 Da
Mass observed: 8552.1 Da
Peptide Sequence: RXR RBR **C** RXR RBR **C**