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Supporting Information

A Tag-Free Platform for Synthesis and Screening of Cyclic Peptide Libraries

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Supplemental Information

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I. General All commercial materials without further purification. All solvents were reagent or HPLC (Fisher) grade. All reactions were performed under air in glass vials or round bottom flasks. HPLC and MS were used for the analysis of each reaction.

Materials Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Cys(Trt)-OH, (Fmoc-Gln(Trt)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH, and Fmoc-Val-OH, Fmoc-aminooxy acetic acid, Rink Amide resin 100-200 mesh (0.59 mmole/g loading), and Merrifield Resin were purchased from Advanced ChemTech (Louisville, KY). Fmoc-Thr-OH was purchased from AAPPtec (Louisville, KY). Fmoc-Ala-CHO was purchased from Combi-Blocks (San Diego, CA). 1-hydroxy-7-azabenzotriazole (HOAt), N,N′-diisopropylcarbodiimide (DIC), and N,N-diisopropylethylamine (DIPEA) were obtained from Chem-Impex (Wood Dale, IL). Ultralink Hydrazide Resin and Anti-HA-Biotin (12ca5) were obtained from Thermo Fisher Scientific. Glacial acetic acid (AcOH), formic acid (FA), N,N-dimethylformamide (DMF), dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), water (H_2O) were obtained from VWR International (Philadelphia, PA). Lenacapavir (LEN) was purchased from MedChemExpress (Monmouth Junction, NJ) and suspended in ≥99.9% DMSO (Sigma-Aldrich).

II. Instrumentation and Sample Analysis

Analytical HPLC. Analysis of peptides was performed on an Agilent 1100 series HPLC equipped with either an Agilent Eclipse Plus C18 column (4.6x100 mm, 5 µm particle size) or an Ascentis Express C18 column (2.1x50 mm, 2.7 µm particle size). All separations were performed utilizing a gradient of 0.1% formic acid in water (solvent A) vs. 0.1% formic acid in acetonitrile (solvent B).

LC/MS. High resolution LC/MS was performed on a Dionex 3000 Ultimate UPLC system connected to a ThermoFisherLTQ Orbitrap Velos mass spectrometer with a heated electrospray source. For direct infusion experiments, the solution was infused at a rate of 75 µl min^{-1} and the positive ion spray voltage was set to 3.0 kV. The instrument parameters were as follows: scan range = 200-2000 m/z; capillary temp = 320 °C, RF lens = 60%. For LC/MS experiments, samples were run on an Ascentis Express C18 column (2.1x50 mm, 2.7 µm particle size. The standard method for analysis of peptide mixtures was run with a flow rate of 300 µL min^{-1} and ramped from 2% to 60% of solvent B over 45 minutes at ambient room temperature.

nanoLC/MS. High resolution nanoLC/MS-MS analysis of affinity selection eluates was performed on a Waters Acquity nanoLC Orbitrap Ascend mass spectrometer. Custom packed fused silica C18 trapping and analytical columns were used for separation of peptide eluate.

III. Fmoc-Solid Phase Peptide Synthesis.[1] Model peptide H_2N-AVGPFEYA-CHO as well as all peptide libraries were manually synthesized on Rink Amide resin preloaded with Fmoc-Ala-CHO (preloading procedure described below) using standard protocols. Resin was swelled in 50:50 DMF:DCM cosolvent for 1 hour. To facilitate initial Fmoc deprotection, the swelling solution was replaced with 20% piperidine in DMF and the resin was placed on a wrist action shaker for 20 minutes at room temperature. After Fmoc deprotection, the resin was washed with twice with DMF, MeOH, and DCM. Subsequent amino acid couplings were performed using 5 equivalents of Fmoc-protected amino acid, HOAt, and DIC in DMF, shaking for 30

minutes at room temperature. Iterative deprotection and coupling steps were performed until the full sequence was achieved. Fmoc deprotection was reduced to 15 minutes for these subsequent steps.

Procedure for preloading of Rink Resin. Fmoc-Gly-OH and Fmoc-Thr-OH (unprotected side chain) were coupled with the general peptide synthesis procedure to swollen Rink resin. Following terminal Fmoc deprotection, the resin was added to a round bottom flask and stirred slowly (60 rpm) while refluxing in a solution of Fmoc-Ala-CHO (5 equivalents) in 1% DIEA v/v in MeOH for 5 h at 60 °C. The resin was transferred to a SPPS tube and washed with MeOH (5 x 3 mL), DMF (5 x 3 mL), DCM (5 x 3 mL), and THF (5 x 3 mL). The resin was then returned to a round bottom flask and stirred slowly for 5 h at 50°C in a solution of di-tert-butyl dicarbonate (Boc_2O, 5 equiv.), N-methyl morpholine (5 equiv.) in THF (final conc. 0.1 M). The resin was again transferred to an SPPS tubed and washed with THF (5 x 3 mL), DCM (5 x 3), and DMF (5 x 3 mL).

Procedure for split-and-pool synthesis. After the first deprotection step, the resin was washed as described above and dried on vacuum after the last DCM wash. The dried resin was weighed and split into equivalent portions for amino acid coupling. Coupling for each portion was performed as described above. After coupling and washing, the resin portions were recombined for the deprotection step. This strategy was employed for each variable position in the library design.

Procedure for FITC abeling of peptides. After synthesis and purification, CyClick cyclized peptides with a single Lys residue were incubated at a 5mM concentration with 1.5 equivalents of fluorescein isothiocyanate and 5 equivalents of diisopropylethylamine in DMF for 2 hours at room temperature.

IV. PEPTIC Workflow

Hydrazide Resin-based Peptide Aldehyde Purification. Crude model peptide aldehyde or peptide aldehyde libraries were dissolved in either ACN or ACN:DMF cosolvent containing 2% acetic acid. Hydrazide resin slurry was transferred to a solid phase synthesis tube and storage buffer was filtered off. The resin was then washed 2x with MeOH, 2x with DCM, and allowed to dry over vacuum. The resin was then transferred to either a glass scintillation vial (for heating in an incubator shaker) or a round-bottomed flask with a magnetic stir bar (for heating in an oil bath) and the peptide aldehyde solution was added. For both heating strategies the temperature was set to 60° C. If using the incubator shaker, shaking was set to 250 rpm. If using an oil bath on a heated stir-plate, stirring was set to the lowest setting of 60 rpm. Functional group loading of the Hydrazide Ultralink Resin is not provided by the manufacturer, but good yields were observed when utilizing 2 mL of hydrazide resin slurry for every 100 mg of Rink Resin used in peptide synthesis. The peptide/resin slurry was allowed to shake/stir for 4 hours before being transferred back to the solid phase synthesis tube. The eluate was collected into a 15 mL centrifuge tube and the resin was washed 2x with DMF, MeOH, and DCM. After washing, the resin was dried over vacuum and added to a new reaction vessel. The resin was then incubated with a solution of 50:50 ACN:H_2O with 2% AcOH for another 4 hours and 60°C. After incubation, the resin was again transferred to a solid phase synthesis tube and the eluate collected in a 15 mL centrifuge tube. The eluate was subjected to centrifugation and the supernatant was frozen and lyophilized. If excess peptide was detected in the scavenging eluate, the procedure could be repeated with the same hydrazide resin used in the initial purification.

CyClick Chemistry. CyClick reaction was performed by dissolving the purified linear peptide aldehyde or peptide aldehyde library in 1% DIEA in DMF at a 5mM concentration. The solution was added to a round-bottom flask with a stir bar. The reaction was allowed to proceed for 12-16 hours at 60°C with fast stirring. For peptide libraries, total mmoles were estimated by first dividing the total mass of enriched peptide (m) by the number of theoretical sequences in the library (s) to get an "individual" peptide mass (i). This incorrectly assumes each peptide contributes equally to the total mass but provides a straightforward estimation strategy. The molecular weight of each theoretical sequence is calculated (w_n) and the mmoles determined by dividing the "individual" peptide mass by the molecular weight. The calculated mmoles for all peptides are summed (t) and this is used to estimate concentration.

$$i = \frac{m}{s}$$

$$t = \sum_{n=1}^{s} \frac{i}{w_n}$$

Thr-Gly Resin-based Macrocycle Purification. To the CyClick cyclization mixture, 5 equivalents sodium sulfate and 5 equivalents of rink resin functionalized with a TG dipeptide (utilizing a Fmoc-Thr-OH residue) were added. The stirring was slowed to 60 rpm and the solution was incubated at a maintained 60 °C for 8 hours. Afterwards, the slurry was transferred to a solid phase synthesis tube and the eluate along with two washes in DMF were collected in a 15 mL centrifuge tube. The final solution was evaporated on speed vacuum concentrator and stored at -80 °C until further use.

One-pot Linearization and Derivatization. Peptide linearization and derivatization was performed with 2 equivalents (relative to peptide) of the H_2N-O-GGRG tetrapeptide probe in either 50:50 ACN:H_2O with 2% formic acid or PBS buffer at pH 3. The reaction was performed in an incubator shaker at 60 °C for 8-10 hours. In the context of the peptide libraries, mmoles were estimated using the same method described above in the CyClick cyclization section.

V. Affinity Selections

Magnetic Bead Strategy. 100 µL portions of MyOne Streptavidin T1 Dynabeads (10 mg/mL; 1 mg; 0.13 nmol IgG binding capacity) were transferred to 1.7 mL plastic centrifuge tubes, and placed in a magnetic separation rack (New England Biolabs, cat# S1506S). The beads were washed three times with blocking buffer (1 mg/mL BSA, 0.02% Tween 20, 1M PBS), and subsequently incubated with Anti-HA-Biotin. The tubes were transferred to a rocking shaker for 1 hour at 4 °C. Afterwards, the beads were returned to the separating rack and the supernatant was removed. The beads were washed again with blocking buffer and incubated with 1 mL of peptide library (200 pM per member concentration) or positive control antigen sequence (200 pM) in blocking buffer with 2.5 % DMF for 1 hour at 4 °C on a rocking shaker. After incubation the tubes were again transferred to a magnetic separation rack, the supernatant removed, and the beads washed 3x with 1M PBS. Beads were then washed 2x with 150 µL of 200 mM PBS with 6M guanidinium hydrocholoride. All 300 µL were kept and subjected to peptide linearization and derivatization conditions.

Size Exclusion Strategy. 24 mg of the peptide library was dissolved in 5 mL of 5% DMSO in PBS at pH 7.5 to achieve a ~400 nM per library member concentration. 0.13 nmol of 12ca5 was dissolved in 100 µL of PBS pH 7.4 and added to the peptide library and incubated at room temperature for 30 mins. Size Exclusion Chromatography was done with the incubate mixture using a flow rate of 1 mL/min with a 3 µm Agilent SEC Column with dimension 7.8 x 150mm. 100 µL of the peptide library with the target protein was injected into the HPLC with an isocratic mobile phase using PBS Buffer pH 7.5. The HPLC run was for 15 min. The target protein-binder complexes eluted at 4.155 min. The affinity selection experiment was monitored by UV (214 nm). After the run, the size exclusion column was cleaned with deionized water.

VI. Microscale Thermophoresis

All microscale thermophoresis experiments were performed on a Nanotemper Monolith NT.115 Pico. Each sample was prepared in Nanotemper Monolith Premium Capillaries and measurements of fluorescence intensity were performed using the BLUE filter set at a set temperature of 25°C. Peptides were dissolved in DMSO and diluted in analysis buffer (1M PBS with 0.1% pluronic F-127) to 80 nM concentrations. The anti-HA antibody was dissolved in the same analysis buffer to a 6 µm concentration. Peptide only traces were prepared by mixing 25 µL of diluted peptide solution with 25 µL of analysis buffer. Peptide/antibody traces were prepared by mixing 25µof diluted peptide solution with 25 µL of antibody solution. Laser power was set using the instrument's auto-detect feature.

VII. Peptide Stability Experiments

Peptide stability studies were conducted using the macrocyclized sequence AVGAFEYA. For each condition, 100 µg (0.000126 mmole) of peptide was incubated in affinity selection conditions, MST conditions, or affinity selection conditions in the presence of a nucleophilic small molecule. Nucleophiles studied included Lys, Cys, glutathione, and O-benzyl hydroxylamine. Ten equivalents of each nucleophile was used relative to the cyclic peptide and four equivalents of TCEP was used relative to the thiol in the case of Cys and glutathione. The mixtures were analyzed at the start of the incubation, 1 hour, and 3 hour time points.

VIII. HIV Capsid Experimental Procedures

Expression and Purification of HIV-1 Capsid (CA). HIV-1 capsid protein monomers (CA) was cloned in a pET11a expression plasmid, provided by Dr. Chun Tang (Peking University). *E. coli* BL21(DE3)RIL was used for protein expression and CA was purified by ammonium sulfate precipitation followed by anion exchange chromatography as previously described.[2]

Cross-linked CA hexamers, containing A14C/E45C/W184A/M185A mutations for disulfide stabilization, (CA121 or CA_{HEX}) were cloned in a pET11a expression plasmid, provided by Dr. Owen Pornillos (University of Virginia).[3] Subsequently, a 6xHIS tag was added to the C-terminal end of CA_{HEX} to create the $CA_{HEX:6HIS}$ construct in pET11a vector (Genscript). *E. coli* BL21(DE3)RIL was used for protein expression, and both CA_{HEX} and $CA_{HEX:6HIS}$ were purified as previously described,[3] with additional size-exclusion chromatography step for added protein purity to remove non-crosslinked CA using HiLoad™ 26/600 Superdex 200 pg in storage buffer (20 mM Tris pH 8.2 and 40 mM NaCl).

CA Assembly Assay

The CA assembly assay was modified from a previously described method.[4] Briefly, a 2X solution was made of 100 μM of CA monomer, diluted from frozen aliquots in 50 mM Tris (pH 8.0), and treated with equimolar (100 μM) of LEN, cyFG-3, or a DMSO vehicle control (0.4 %) for approximately 30 minutes on ice. These 2X Solutions were dispensed into a 96-well plate and mixed 1:1 with 2 M NaCl in 50 mM Tris (pH 8.0) to initiate assembly. Absorbance at 350 nm (A_{350}) was measured every 25 seconds for 60 minutes at room temperature with a Synergy Neo 2 (BioTek) plate reader. Samples containing the 1X solution of CA, compound and 2 M NaCl were background subtracted from a blank well that lacked NaCl. In the LEN-treated and cyFG-3 background, we note CA assembly occurs in the absence of NaCl (time = 60 minutes), thus leading to an apparent lower A_{350} reading of the sample compared to the DMSO. The data are transformed by background subtraction, and the rate of A_{350} increase remains higher in LEN and cyFG-3 than the DMSO-treated control initially, however, soon after NaCl is added, CA polymerization rapidly completes, and the LEN-treated and cyFG-3 samples plateau.

Biolayer Interferometry (BLI)

The BLI protocol was modified from the default parameters in the Octet® BLI Discovery program (version 13.0.0.17, Sartorius). LEN conditions adapted from reported Surface Plasmon Resonance experiments for a biotinylated CA_{HEX}.[2] Frozen aliquots of $CA_{HEX:6HIS}$ were diluted to 100 μg/mL in BLI buffer [20 mM Tris (pH 8.2) with 40 mM NaCl, 20 mM imidazole, 1% Bovine Serum Albumin (BSA), 600 mM sucrose; modified from Dubrow et al. (2022)[5]. Anti-penta-HIS (HIS1K) Dip and Read™ Biosensors (FortéBio #18-5120) were first hydrated in 200 μL of buffer for 10-30 minutes before sample loading. All experiments were performed in 96-well microplates (Greiner, 655209), agitated at 1000 rpm, at 25 °C, and at a volume of 200 μL per well.

Experiments were initiated with a 120 second baseline step, followed by loading of CA_{HEX} at 100 μg/mL in BLI buffer for 600 seconds. The CA_{HEX}-loaded probe was washed twice in BLI buffer for 60 seconds. For the LEN experiments, the protein-loaded probes were dipped into a 200 μL solution of BLI buffer containing 7.5, 5.0, or 2.5 μM LEN for 200 seconds of association time, then 1,000 seconds of dissociation time in a BLI buffer. For the cyFG-3 experiments, the protein-loaded probes were prepared as described above; the concentrations of cyFG-3 tested were 100, 75, or 50 μM with an association time of 50 seconds and a dissociation time of 100 seconds. The Octet Analysis Studio (version 13.0.0.32, Sartorius) was used to perform double background subtraction for both LEN and cyFG-3. The first subtraction is the protein-loaded biosensor from the parallel, protein-absent biosensor. The second data transformation is done by subtracting an acquired measurement of protein and an equivalent volume of DMSO. Then, data were corrected using the “Average of Baseline Step” from time = 115.0–119.8 and “Baseline Inter-step Correct” at time = 0. Association and Dissociation were fit with a continuous 1:1 protein:ligand binding model. The resulting K_D, k_{on}, and k_{off} values were determined in triplicate for each concentration of drug (except 50 μM cyFG-3, which was performed in duplicate), and these were reported as an average and error of globally fit values.

Thermal Shift Assay (TSA)

Prior to TSA, 20 μM $CA121_{HEX}$ was incubated with 0.08 mg/mL of the library of cyclic peptides (≤1% DMSO) in 50 mM Tris (pH 8.0). Samples were then mixed with 1X SYPRO™ Orange dye in a qPCR plate and samples were heated from 25–95°C QuantStudio 3 Real-Time PCR Systems (Thermo Fisher Scientific) as previously described.[6] Thermal profiles were analyzed with Protein Thermal Shift Software v1.3 (Applied Biosystems) and visualized with TSAR.[7] Statistical significance was determined by comparing the treated condition to the DMSO vehicle with a two-sided unpaired t-test.

IX. Supplementary Tables and Figures

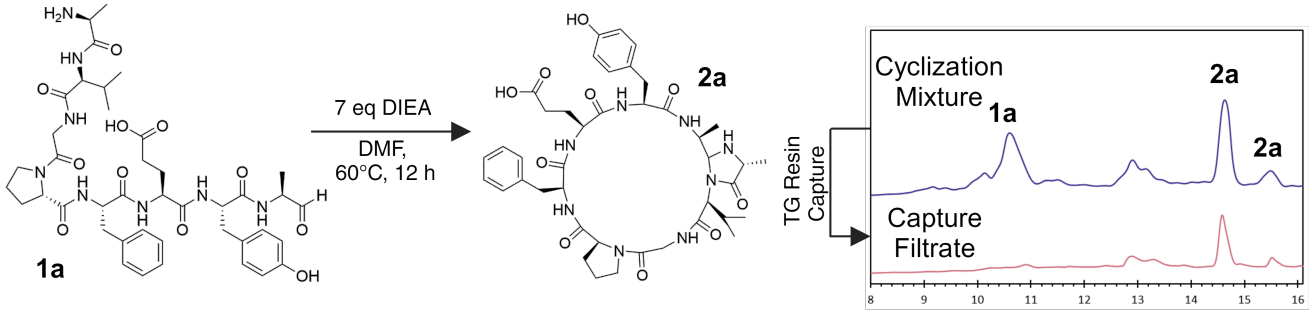
Supplementary Table 1. Optimization table for a model linear peptide H_2N-AVGPFEYA-CHO capture with aldehyde scavenging resins.

Functionalized Resin	Conditions	Temp. (°C)	Time (hours)	% Capture
Hydrazide	2% AcOH in DMF	RT	4	20%
Hydrazide	2% AcOH in DMF	60	4	83%
Hydrazide	2% AcOH in ACN	60	4	100%
Sulfonyl Hydrazine	2% FA in ACN	60	4	0%
Rink-GT	1% DIEA in DMF	60	4	55%
Rink-GC	1% DIEA in DMF	60	4	28%
Merrifield-GT	1% DIEA in MeOH	60	4	3%
Merrifield-GC	1% DIEA in MeOH	60	4	0%
Merrifield-GT	1% DIEA in DMF	60	16	13%
Merrifield-GC	1% DIEA in DMF	60	16	65%

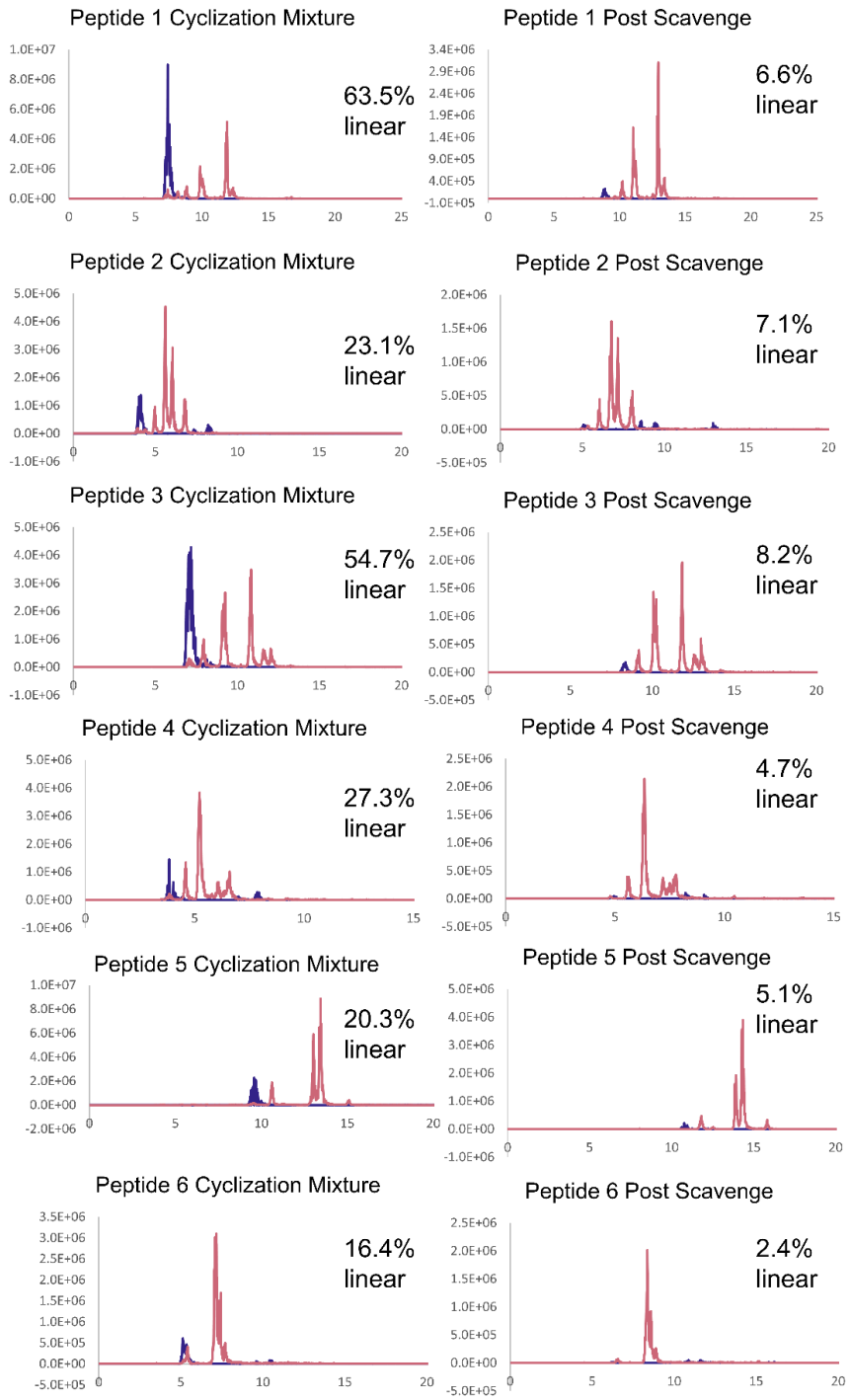
Supplementary Table 2. Optimization table for a model cyclic peptide H_2N-AVGPFEYA-CHO purification with aldehyde scavenging resins.

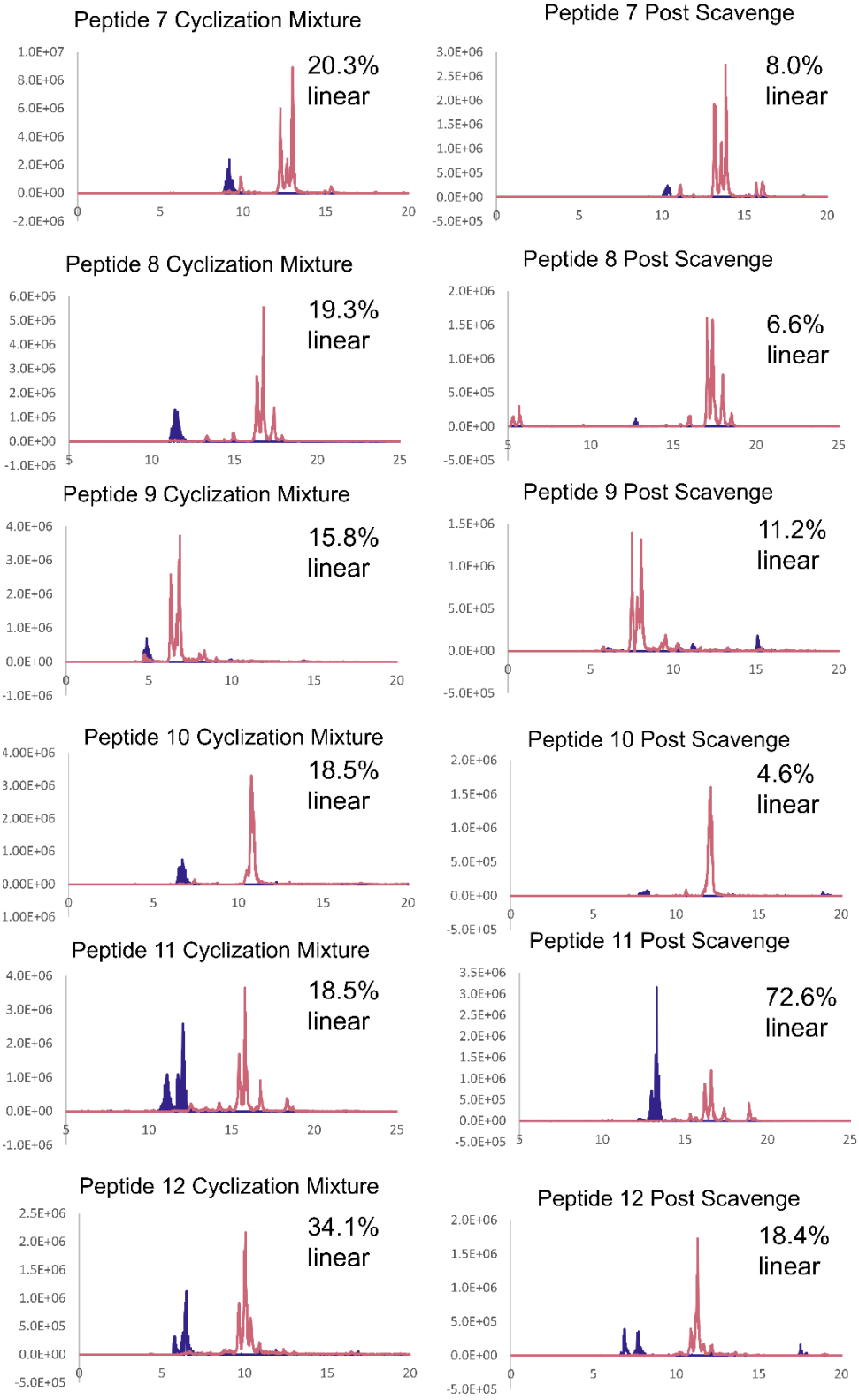
Functionalized Resin	Conditions	Temp. (°C)	Time (hours)	Purity of CyClick Peptide
Hydrazide	2% AcOH in ACN	60	4	Blank Chromatogram
Hydrazide	2% AcOH in ACN	RT	4	Mixed Results
Hydrazide	2% AcOH in ACN	0	4	65%
Rink-GT	1% DIEA in DMF, Na_2SO_4	60	Overnight	100%
Rink-GT	1% DIEA in DMF, Na_2SO_4	60	4	100%

Supplementary Figure 1. Model peptide N_2H-AVGPFEYA-CHO cyclization scheme and stacked chromatograms (DAD) of cyclization mixture and TG resin purification analysis.

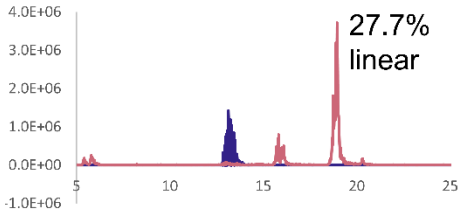


Supplementary Figure 2. Extracted ion chromatograms from LC/MS analysis of linear (blue) and CyClick (pink) peptide mini library mixtures after cyclization (left column) and after two round of TG resin scavenging (right column). The percent linear peptide is calculated as the percentage of total ion intensities of both the linear and CyClick species.

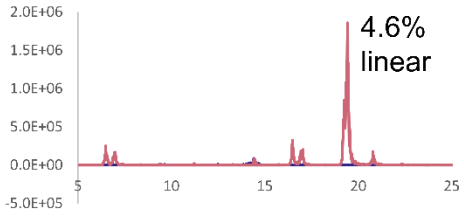



Peptide 7 Cyclization Mixture
20.3%
linear
Peptide 7 Post Scavenge
8.0%
linear
Peptide 8 Cyclization Mixture
19.3%
linear
Peptide 8 Post Scavenge
6.6%
linear
Peptide 9 Cyclization Mixture
15.8%
linear
Peptide 9 Post Scavenge
11.2%
linear
Peptide 10 Cyclization Mixture
18.5%
linear
Peptide 10 Post Scavenge
4.6%
linear
Peptide 11 Cyclization Mixture
18.5%
linear
Peptide 11 Post Scavenge
72.6%
linear
Peptide 12 Cyclization Mixture
34.1%
linear
Peptide 12 Post Scavenge
18.4%
linear

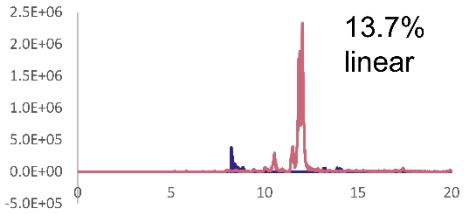
Peptide 13 Cyclization Mixture



Peptide 13 Post Scavenge



Peptide 14 Cyclization Mixture



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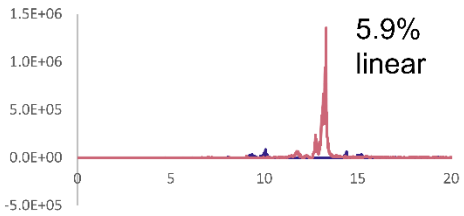

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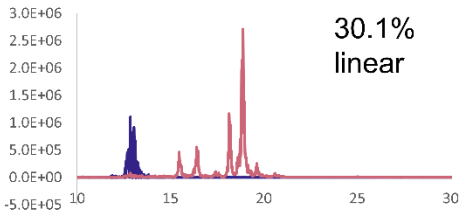
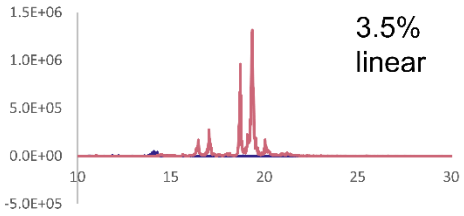
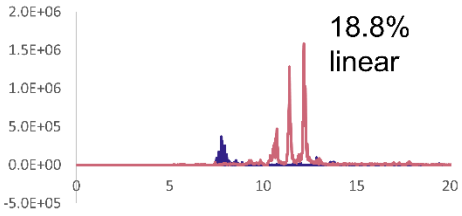


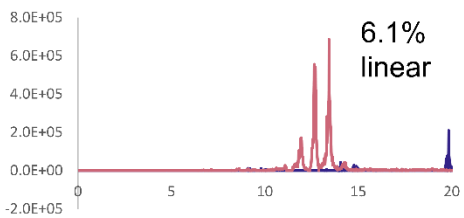
Peptide 15 Post Scavenge



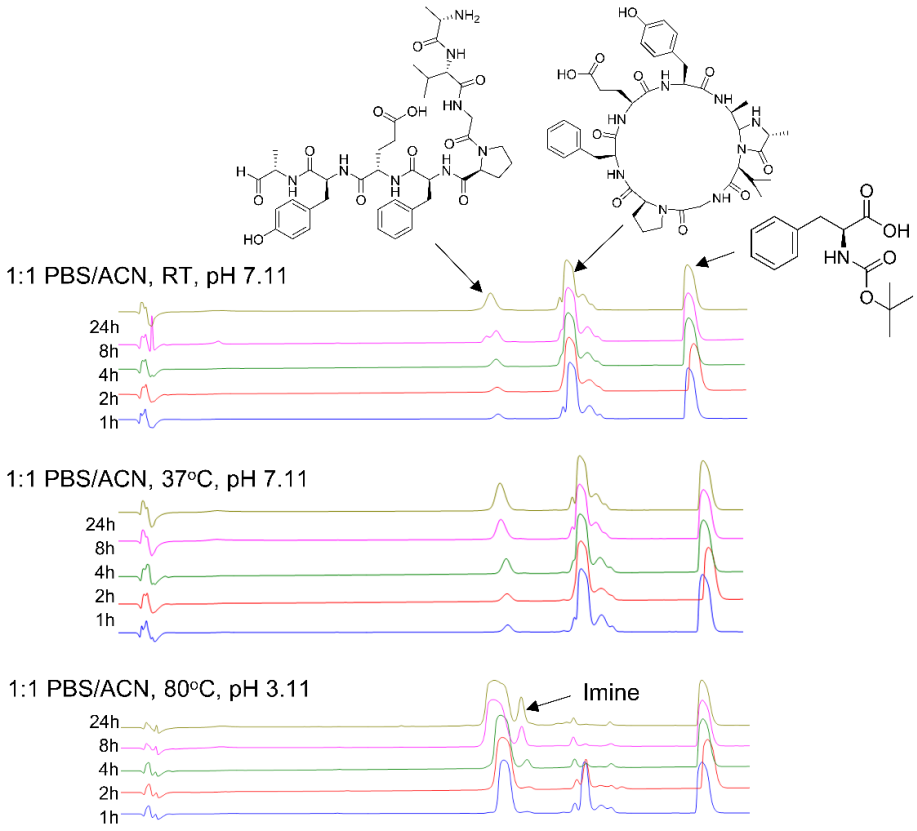
Peptide 16 Cyclization Mixture



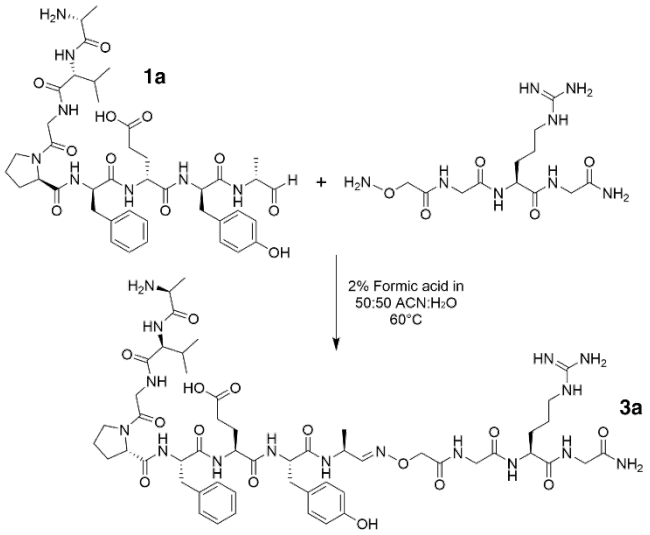
Peptide 16 Post Scavenge

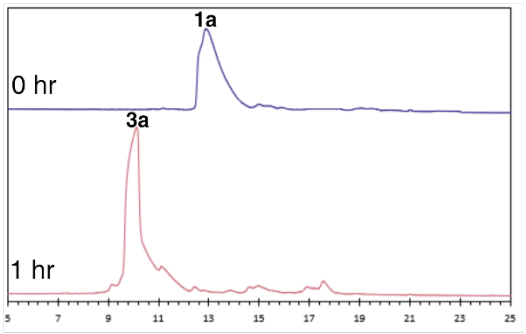


Supplementary Figure 3. Stacked chromatograms (DAD) for time point analysis of model CyClick peptide **2a** linearization.



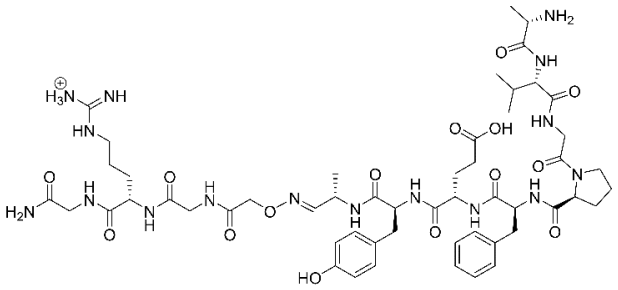
Supplementary Figure 4. Model peptide N_2H-AVGPFEYA-CHO derivatization scheme and stacked chromatograms (DAD) of reaction analysis.



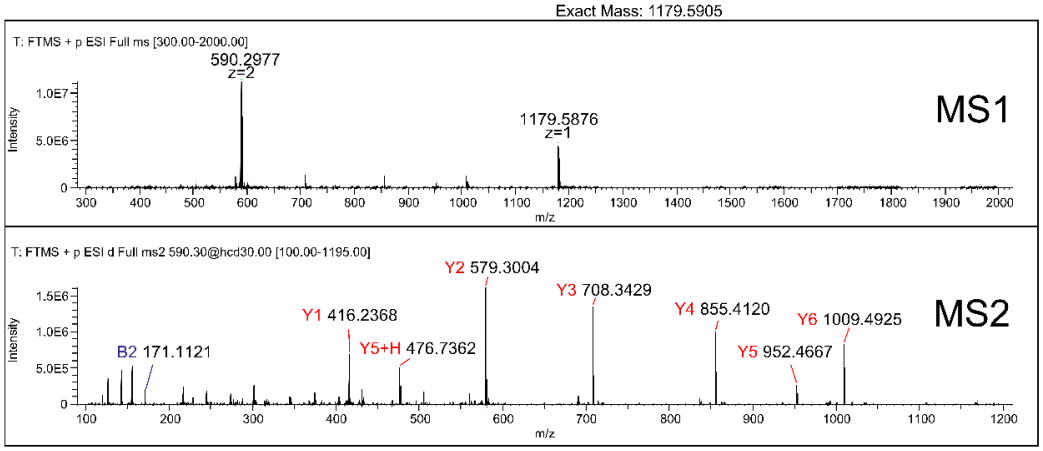


Supplementary Figure 5. MS1 and MS2 spectra for the derivatized (top) and underivatized (bottom) model peptide N_2H-AVGPFEYA-CHO **3a**.

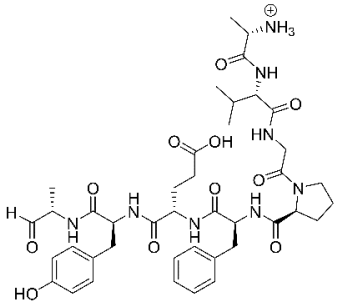
B ions	Y ions
72.04444	1179.59
171.1129	1108.553
228.1343	1009.485
325.1871	952.4635
472.2555	855.4107
601.2981	708.3423
764.3614	579.2997
1161.58	416.2364



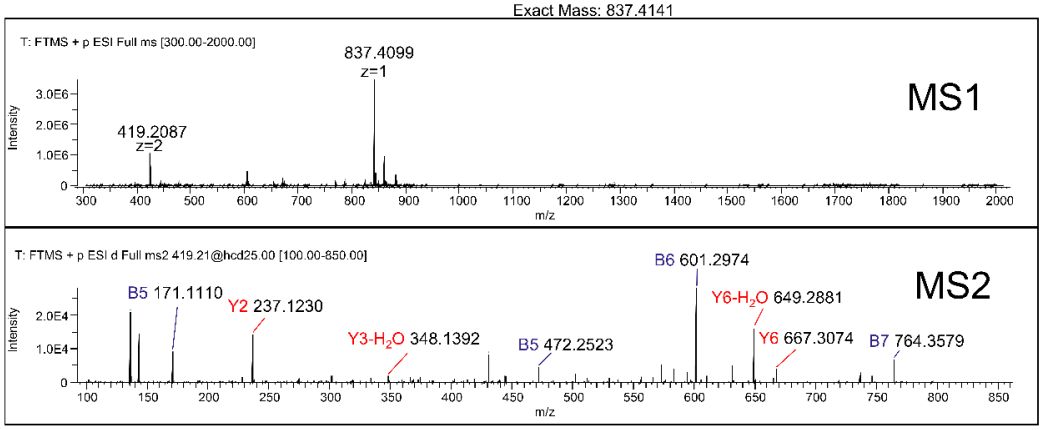
Exact Mass: 1179.5905



B ions	Y ions
72.04444	837.4142
171.1129	766.3771
228.1343	667.3087
325.1871	610.2872
472.2555	513.2344
601.2981	366.166
764.3614	237.1234
835.3985	74.0601

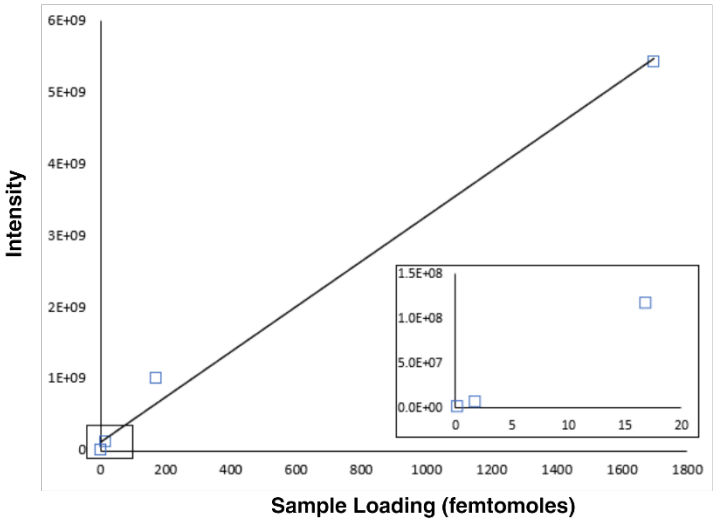


Exact Mass: 837.4141



Supplementary Figure 6. Limit of detection experiments with derivatized model peptide **3a**. a) Sample loading (femtomoles) vs. intensity counts of derivatized model peptide **3a**. b) MS2 spectra of highest and lowest sample loading for model peptide.

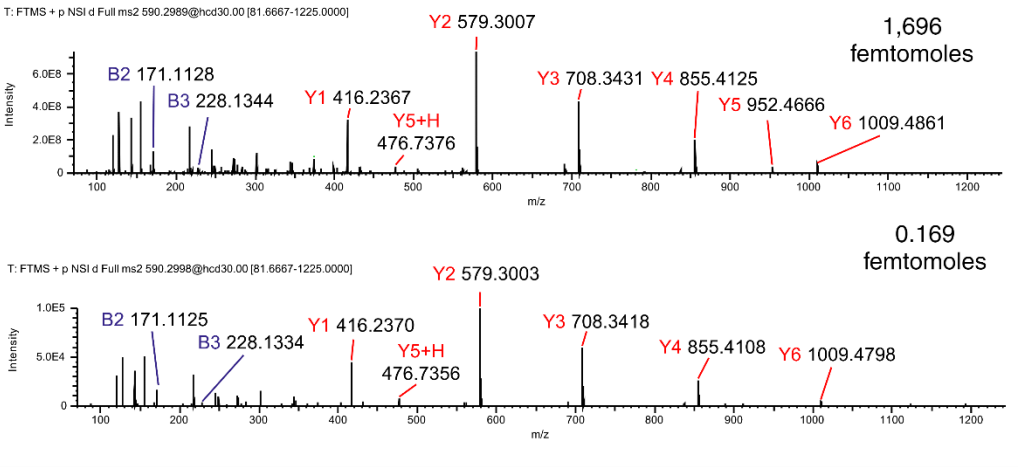
a



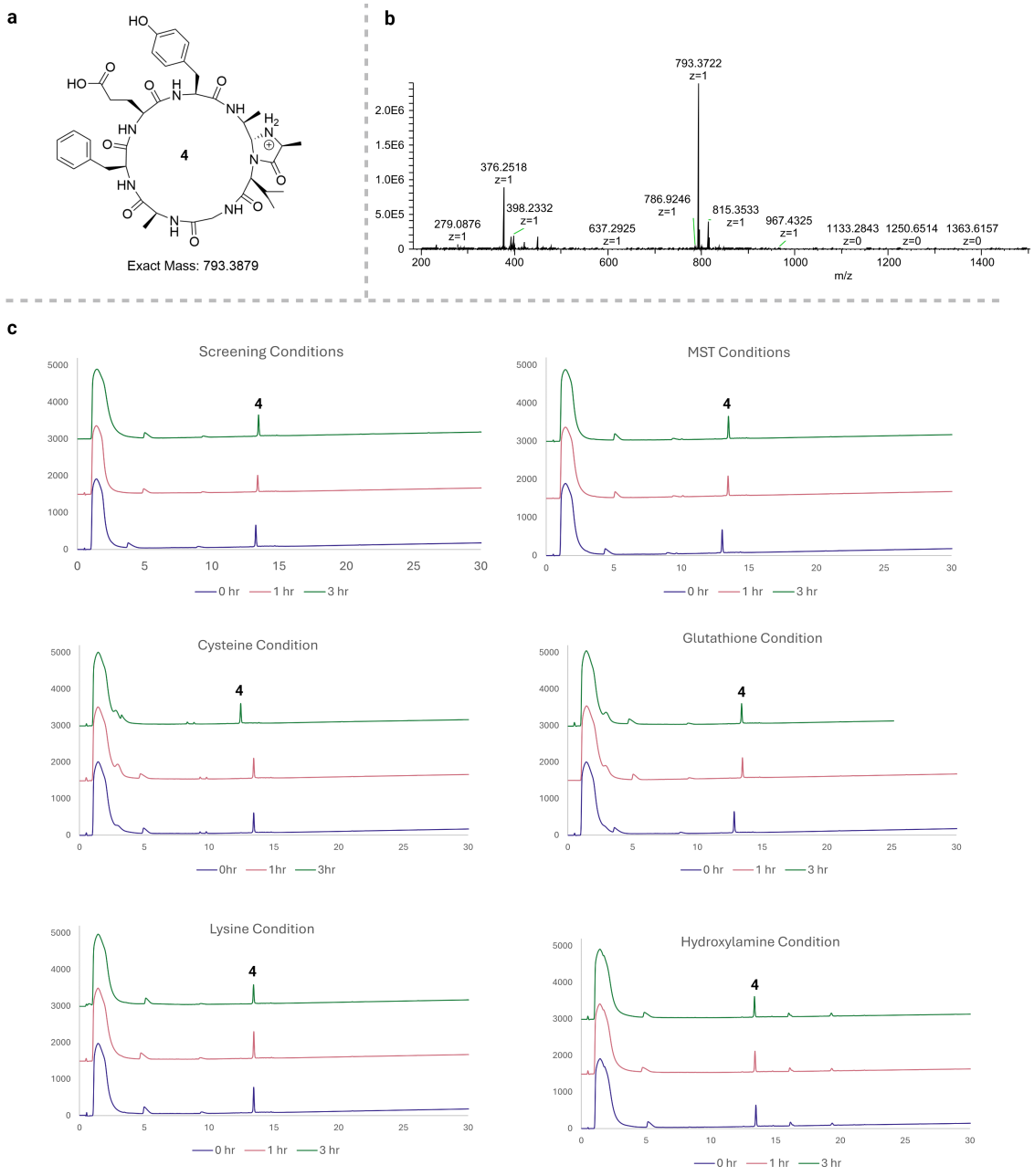
Femtomoles	Intensity
1,696	5.44E9
169.6	1.01E9
16.96	1.17E8
1.69	7.69E6
0.169	8.94E5

b

B ions	Y ions
72.04444	1179.59
171.1129	1108.553
228.1343	1009.485
325.1871	952.4635
472.2555	855.4107
601.2981	708.3423
764.3614	579.2997
1161.58	416.2364



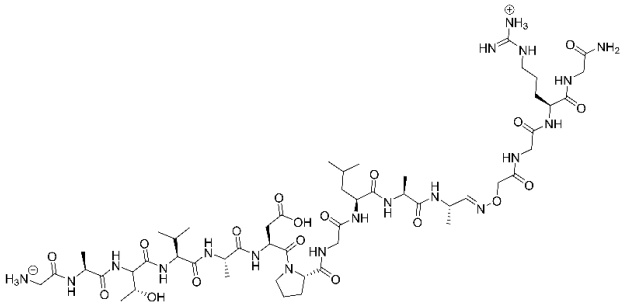
Supplementary Figure 7. Additional stability studies of CyClick macrocycle. (a) Macrocycle **4** structure (b) HRMS of macrocycle **4** (c) Stacked HPLC traces of macrocycle **4** at different time points in affinity selection coniditons, microscale thermophoresis conditions, and in the presence of relevant nucleophiles. Nucleophiles used include Lys, Cys, glutathione, and O-benzyl hydroxylamine at 10 equivalents relative to the peptide.


a
HO
HO
O
O
H
N
O
NH
H2
N
HN
O
4
N
O
HN
O
N
H
NH
O
O
Exact Mass: 793.3879
b
793.3722
z=1
2.0E6
1.5E6
1.0E6
5.0E5
0
376.2518
z=1
398.2332
z=1
279.0876
z=1
637.2925
z=1
786.9246
z=1
815.3533
z=1
967.4325
z=1
1133.2843
z=0
1250.6514
z=0
1363.6157
z=0
200
400
600
800
1000
1200
1400
m/z
c
Screening Conditions
MST Conditions
Cysteine Condition
Glutathione Condition
Lysine Condition
Hydroxylamine Condition
4
0 hr
1 hr
3 hr
0hr
1hr
3hr

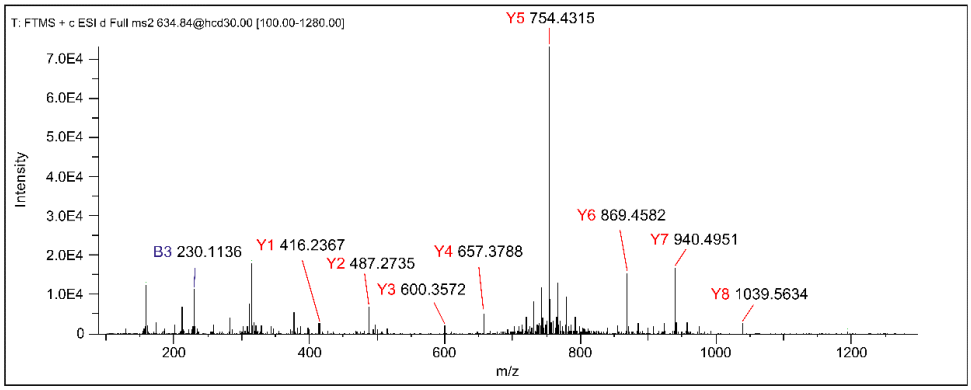
Supplementary Figure 8. MS2 spectra of the peptide mini library after linearization and derivatization with mass ionization tag. Structure and HRMS of FITC labeled cyclic peptide binders utilized for MST binding studies.

B ions	Y ions
58.0287	1268.6705
129.0659	1211.6491
230.1135	1140.612
329.1819	1039.5643
400.219	940.4958
515.2460	869.4587
612.2988	754.4318
669.3202	657.3790
782.4043	600.3575
853.4414	487.2735
1250.6599	416.2364

L1

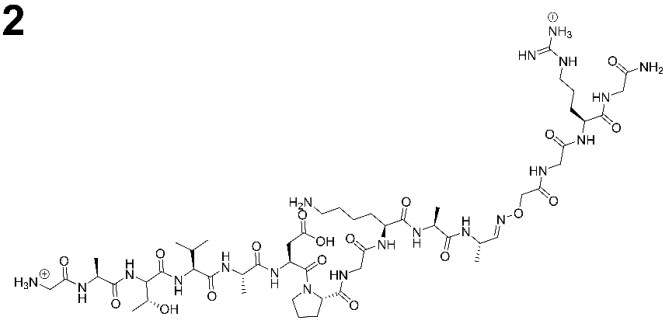


Exact Mass: 1269.6779
m/z: 634.8389

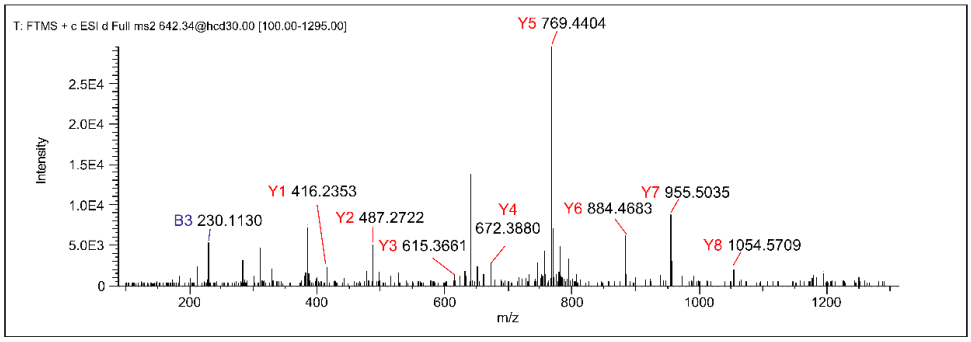


B ions	Y ions
58.0287	1283.6814
129.0659	1226.6599
230.1135	1155.6228
329.1819	1054.5751
400.2191	955.5067
515.2460	884.4696
612.2988	769.4427
669.3202	672.3899
797.4152	615.3684
868.4523	487.2735
1265.6708	416.2364

L2

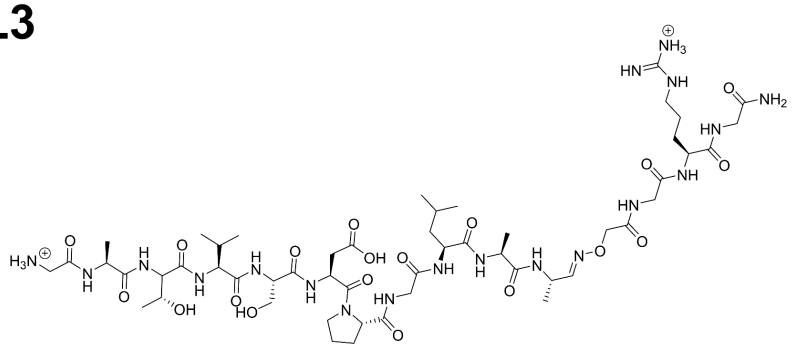


Exact Mass: 1284.6887
m/z: 642.3444

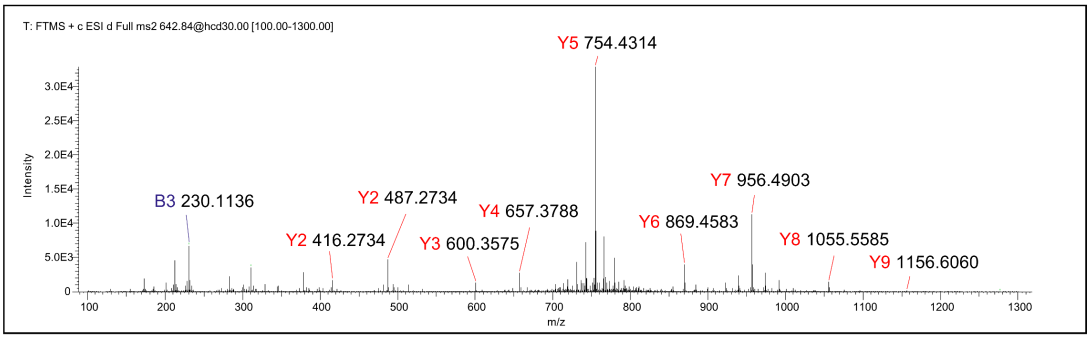


B ions	Y ions
58.0287	1284.6654
129.0659	1227.6439
230.1135	1156.6068
329.1819	1055.5591
416.2140	956.4907
531.2409	869.4587
628.2937	754.4318
685.3151	657.3790
798.3992	600.3575
869.4363	487.27351
1266.6548	416.2364

L3

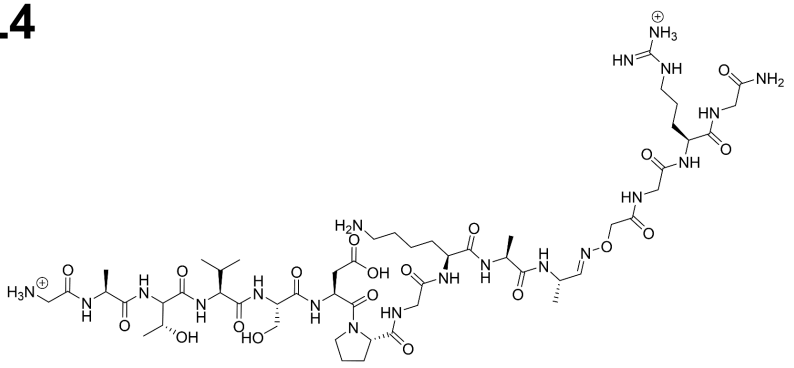


Exact Mass: 1285.6728
m/z: 642.8364

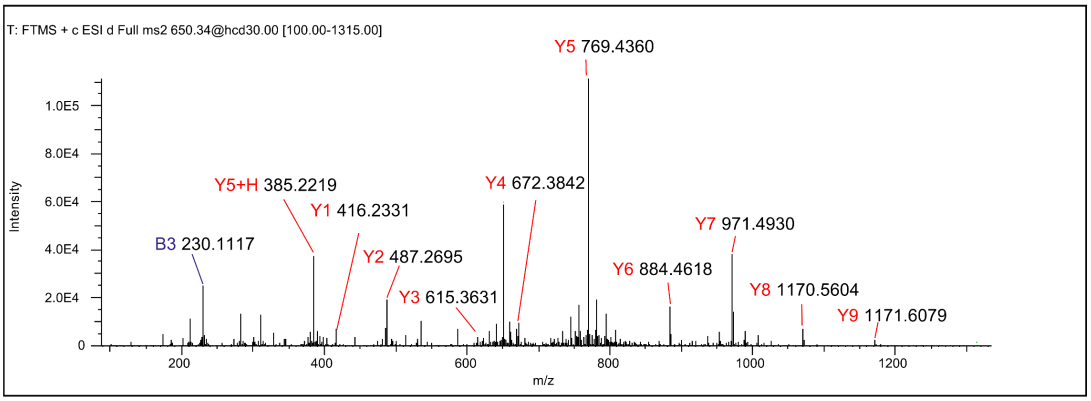


B ions	Y ions
58.02879	1299.676
129.0659	1242.655
230.1136	1171.618
329.182	1070.57
416.214	971.5017
531.241	884.4697
628.2937	769.4427
685.3152	672.3899
813.4102	615.3685
884.4473	487.2735
1281.666	416.2364

L4

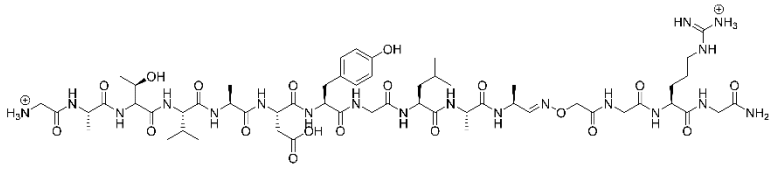


Exact Mass: 1300.6837
m/z: 650.3419

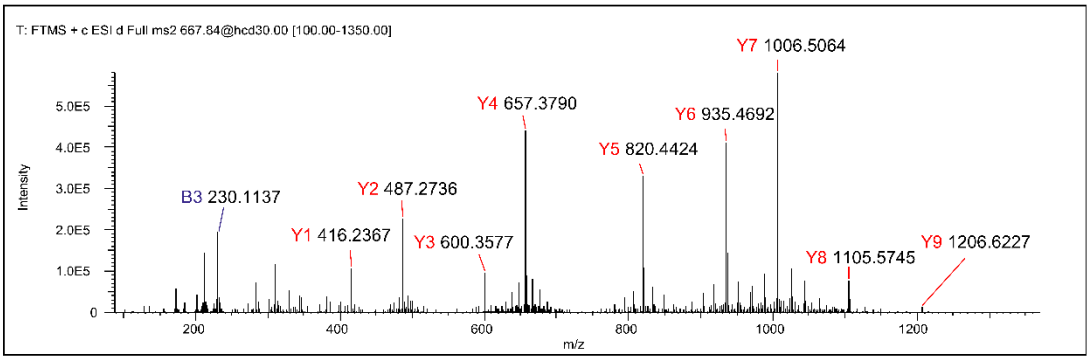


B ions	Y ions
58.02879	1334.681
129.0659	1277.66
230.1136	1206.623
329.182	1105.575
400.2191	1006.506
515.2461	935.4693
678.3094	820.4424
735.3308	657.379
848.4149	600.3576
919.452	487.2735
1316.671	416.2364

L5

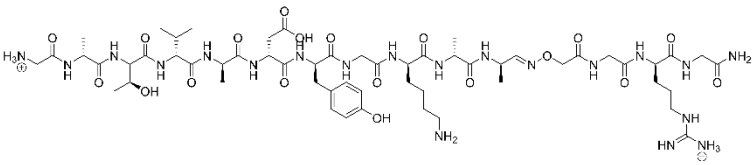


Exact Mass: 1335.6884
m/z: 667.8442

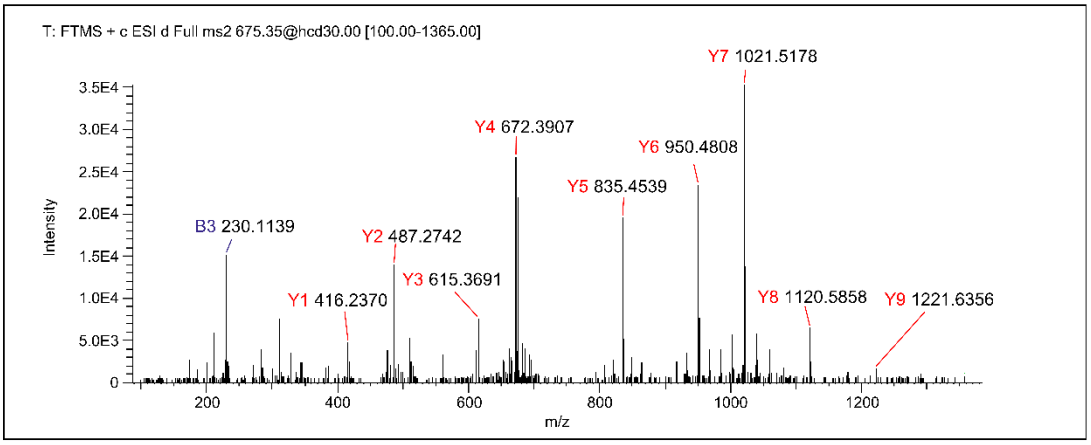


B ions	Y ions
58.0287	1349.6919
129.0659	1292.6705
230.1135	1221.6334
329.1819	1120.5857
400.2191	1021.5173
515.2460	950.4802
678.3093	835.4532
735.3308	672.3899
863.4258	615.3684
934.4629	487.2735
1331.6814	416.2364

L6

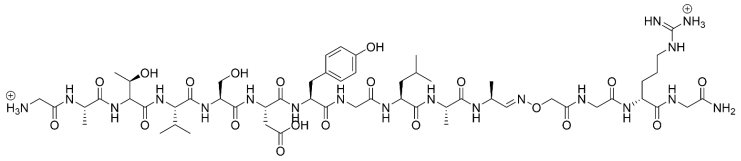


Exact Mass: 1350.6993
m/z: 675.3497

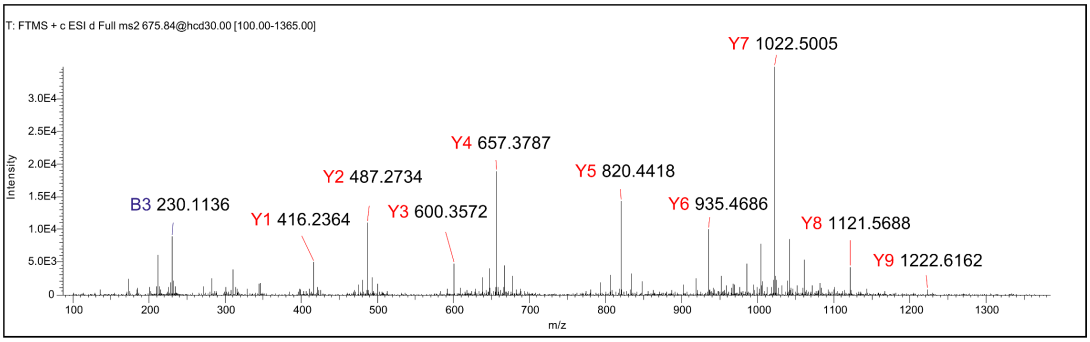


B ions	Y ions
58.02879	1350.67601
129.0659	1293.65455
230.11358	1222.61743
329.18199	1121.56975
416.21402	1022.50134
531.24096	935.46931
694.30429	820.44237
751.32575	657.37904
864.40982	600.35758
935.44693	487.27351
1332.66544	416.2364

L7

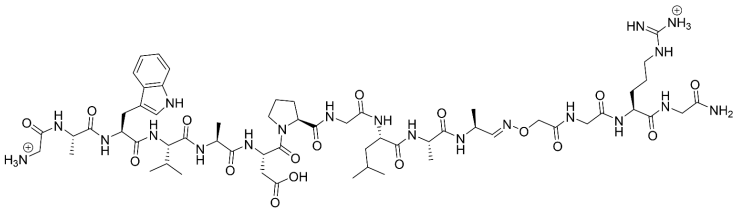


Exact Mass: 1351.6833
m/z: 675.8417

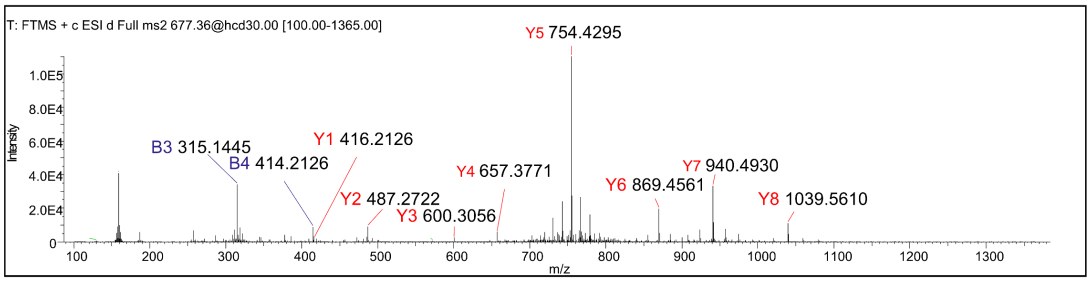


B ions	Y ions
58.0287	1353.7021
129.0659	1296.6807
315.1452	1225.6435
414.2136	1039.5642
485.2507	940.49586
600.2776	869.4587
697.3304	754.431
754.3519	657.3790
867.4359	600.3575
938.4730	487.2735
1335.6916	416.2364

L8

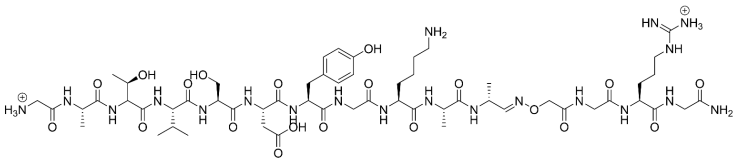


Exact Mass: 1354.7095
m/z: 677.3548

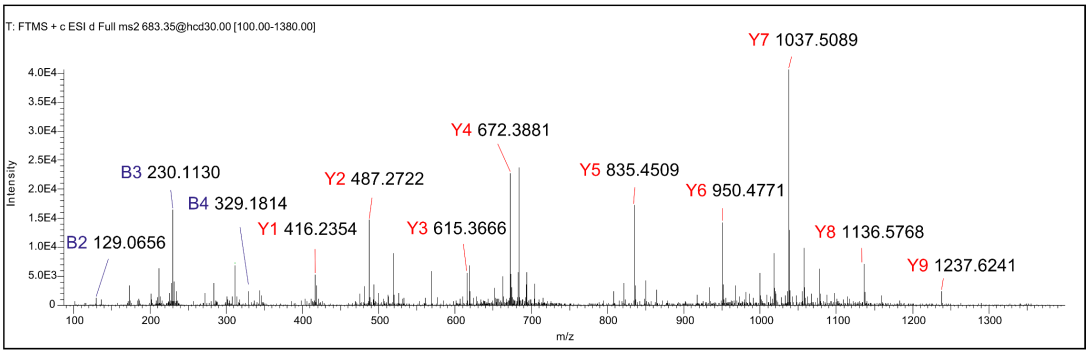


B ions	Y ions
58.0287	1365.6869
129.0659	1308.6654
230.1135	1237.6283
329.1819	1136.5806
416.2140	1037.5122
531.2409	950.4802
694.3042	835.4532
751.3257	672.3899
879.4207	615.3684
950.4578	487.2735
1347.6763	416.2364

L9

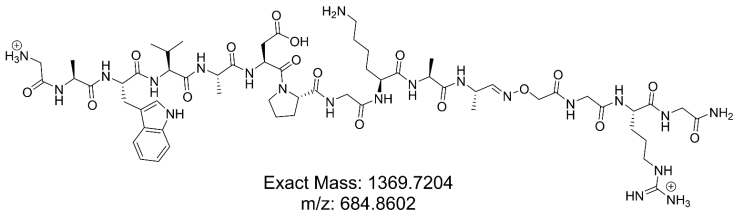


Exact Mass: 1366.6942
m/z: 683.3471

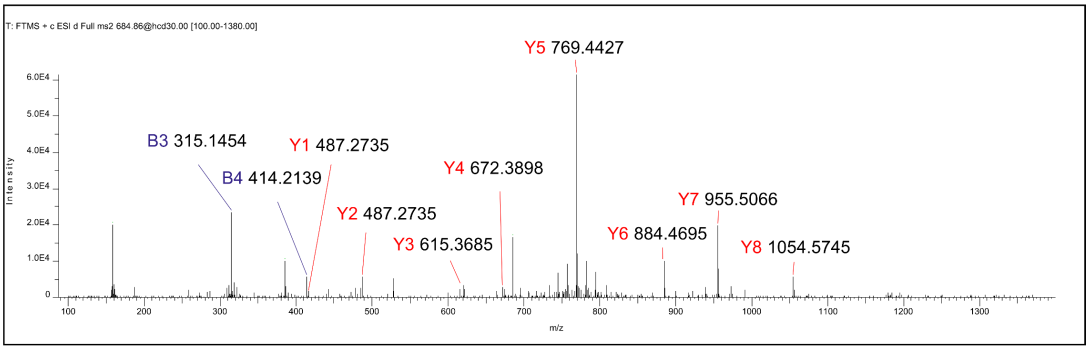


B ions	Y ions
58.0287	1368.7130
129.0659	1311.6916
315.1452	1240.6544
414.2136	1054.5751
485.2507	955.5067
600.2776	884.4696
697.3304	769.4427
754.3519	672.3899
882.4468	615.3684
953.4839	487.2735
1350.7025	416.2364

L10

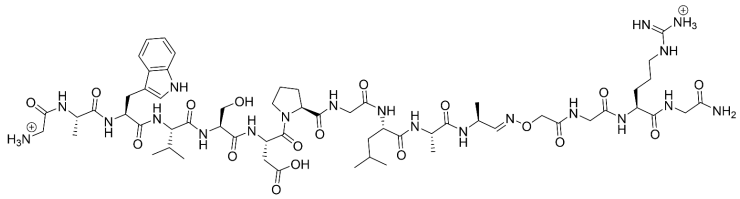


Exact Mass: 1369.7204
m/z: 684.8602

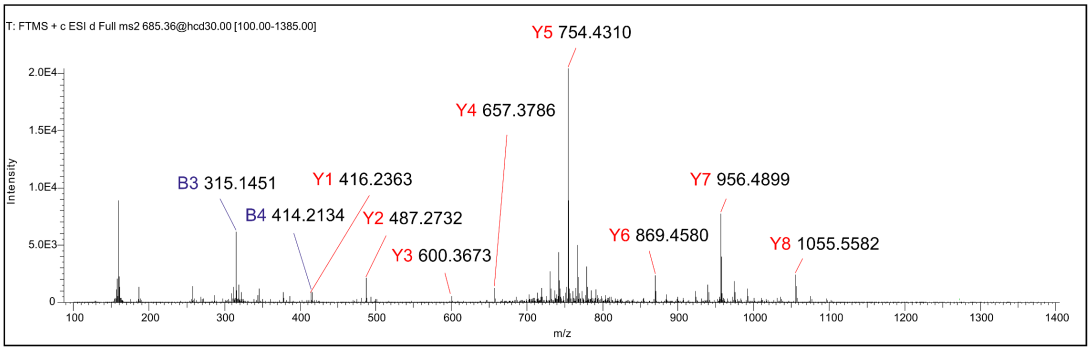


B ions	Y ions
58.0287	1369.6970
129.0659	1312.6756
315.1452	1241.6385
414.2136	1055.5591
501.2456	956.4907
616.2726	869.4587
713.3253	754.4318
770.3468	657.3790
883.4308	600.3575
954.468	487.2735
1351.6865	416.2364

L11

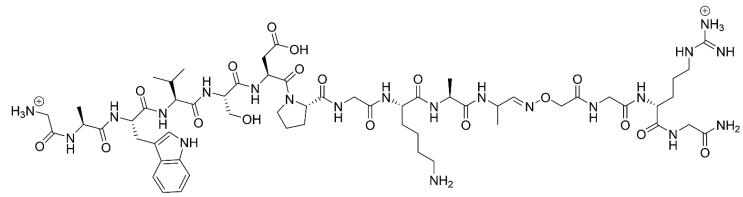


Exact Mass: 1370.7044
m/z: 685.3522

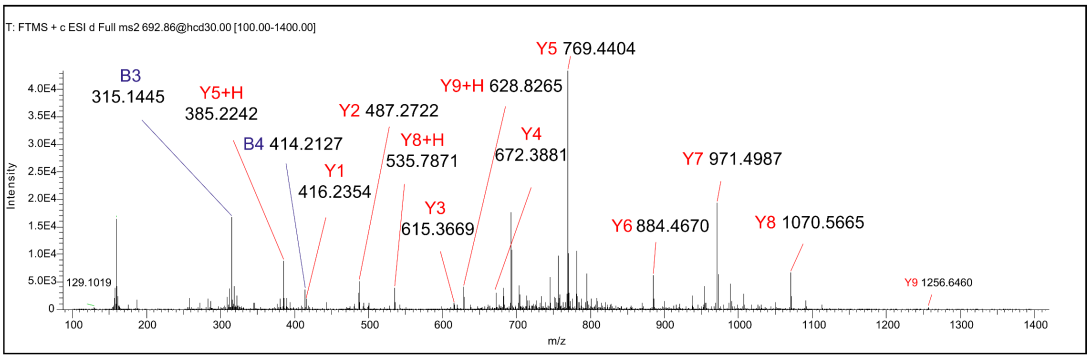


B ions	Y ions
58.0287	1384.7079
129.0659	1327.6865
315.1452	1256.6494
414.2136	1070.5700
501.2456	971.5016
616.2726	884.4696
713.3253	769.4427
770.3468	672.3899
898.4417	615.3684
969.4789	487.2735
1366.6974	416.2364

L12

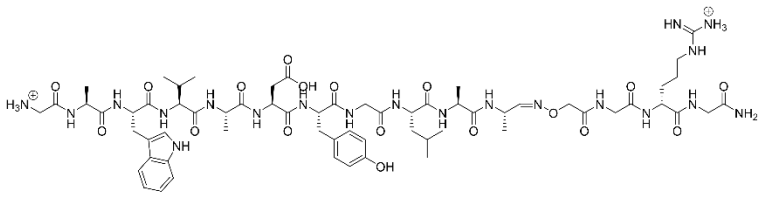


Exact Mass: 1385.7153
m/z: 692.8577

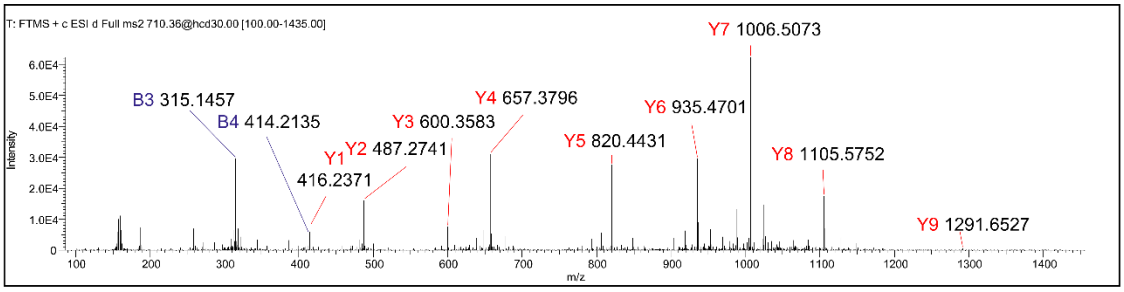


B ions	Y ions
58.0287	1419.7127
129.0659	1362.6912
315.1452	1291.6541
414.2136	1105.5748
485.2507	1006.5064
600.2776	935.4693
763.3410	820.4423
820.3624	657.3790
933.4465	600.3575
1004.4836	487.2735
1401.7021	416.2364

L13

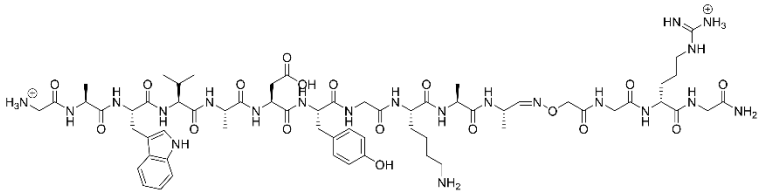


Exact Mass: 1420.7200
m/z: 710.3600

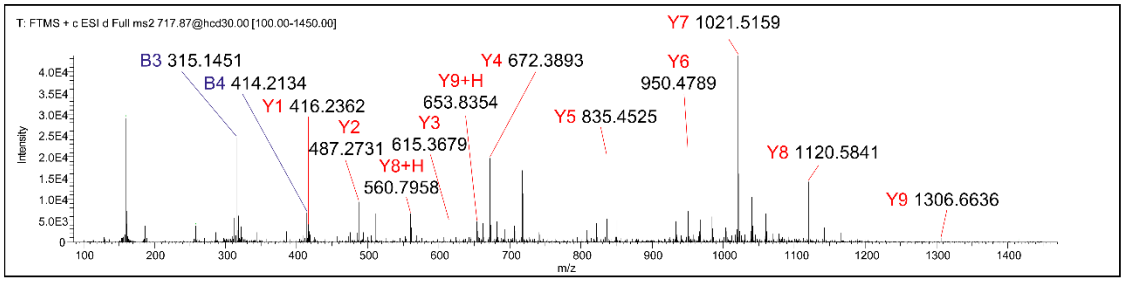


B ions	Y ions
58.02879	1434.7236
129.0659	1377.7021
315.1452	1306.6650
414.2136	1120.5857
485.2507	1021.5173
600.2776	950.4802
763.3410	835.4532
820.3624	672.3899
948.4574	615.3684
1019.4945	487.2735
1416.7130	416.2364

L14

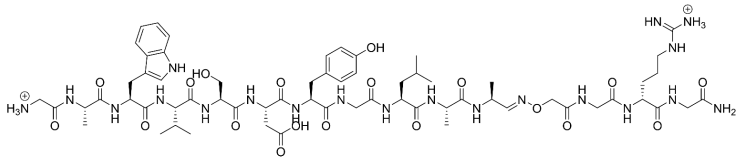


Exact Mass: 1435.7309
m/z: 717.8655

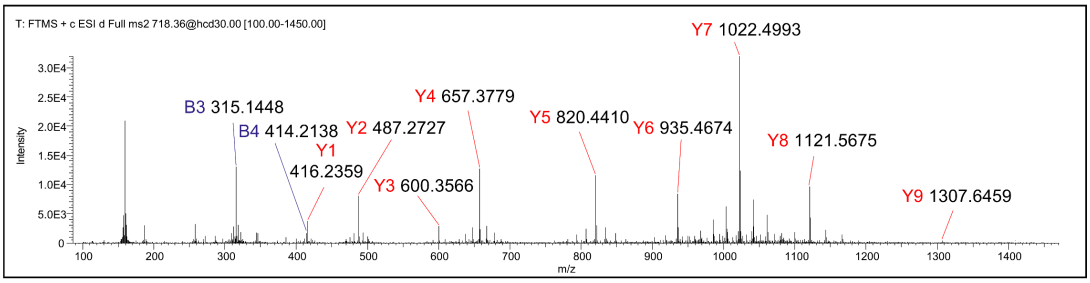


B ions	Y ions
58.0287	1435.7076
129.0659	1378.6861
315.1452	1307.6490
414.2136	1121.5697
501.2456	1022.5013
616.2726	935.4693
779.3359	820.4423
836.3573	657.3790
949.4414	600.3575
1020.4785	487.2735
1417.6970	416.2364

L15

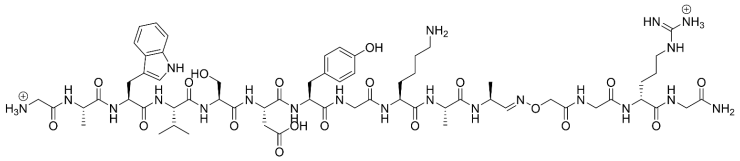


Exact Mass: 1436.7150
m/z: 718.3575

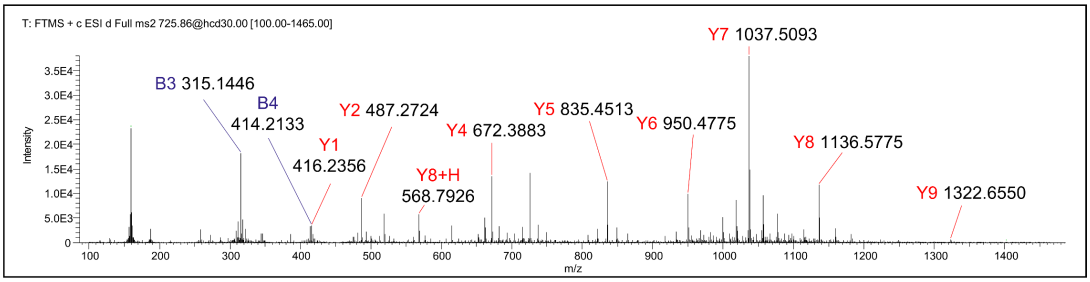


B ions	Y ions
58.0287	1450.7185
129.0659	1393.6970
315.1452	1322.6599
414.2136	1136.5806
501.2456	1037.5122
616.2726	950.4802
779.3359	835.4532
836.3573	672.3899
964.4523	615.3684
1035.4894	487.2735
1432.7079	416.2364

L16



Exact Mass: 1451.7259
m/z: 725.8630

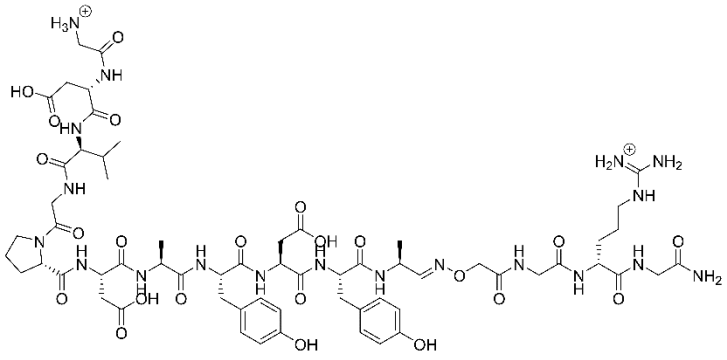


Supplementary Table 3. Database matched sequences from Comet search of size exclusion chromatography-based affinity selection.

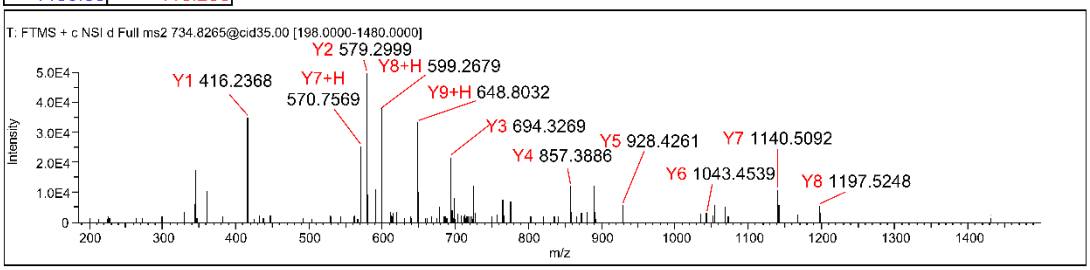
Expect	Number of ions	Sequences	Mass Error (ppm)
0.0389	14/20	GDAGPYDADYA	-1.9714
0.000771	16/20	GDVVVLDADYA	-1.6994
4.01E-06	15/20	GDVGPDYYDYA	-1.676
0.00326	12/20	GDDPPYDASYA	-1.0611
0.0103	14/20	GDAGPLDASYA	-0.6074
0.000244	16/20	GDVVVLDYDYA	-0.3212
0.00443	13/20	GDVGALAYDLA	-0.2857
3.30E-05	13/20	GDVGADDASYA	-0.093
0.0225	13/20	GDAPPYDYSLA	-0.0763
0.00041	12/20	GDAGPYDASYA	0.0085
0.00098	15/20	GDVVALDADLA	0.2826
6.95E-05	13/20	GDAPDDAYSYA	0.3491
2.62E-05	12/20	GDAPDDAYSYA	0.4321
2.94E-05	15/20	GDVGPDAYDYA	0.4443
0.000242	14/20	GDAGVLAADYA	0.6671
6.12E-05	15/20	GDAPPYDASLA	0.8547
0.000113	16/20	GDVGPLDYDYA	0.8677
0.000786	14/20	GDDGPYDASYA	0.8739
5.80E-06	15/20	GDAGVLAADYA	1.0292
1.04E-07	16/20	GDVGPDAASYA	1.0352
0.000156	13/20	GDAGALDASYA	1.2279
0.0225	11/20	GDVGADDADYA	1.8061
0.000232	16/20	GDVPADDASYA	1.9579
0.000328	14/20	GDVGPDAYDYA	2.1081
0.015	14/20	GDVGPLDADYA	2.2805
0.00901	14/20	GDVGPDAYDLA	3.1411

Supplementary Figure 9. MS2 spectra of two cyclic peptide binders after affinity selection, linearization and derivatization with mass ionization tag.

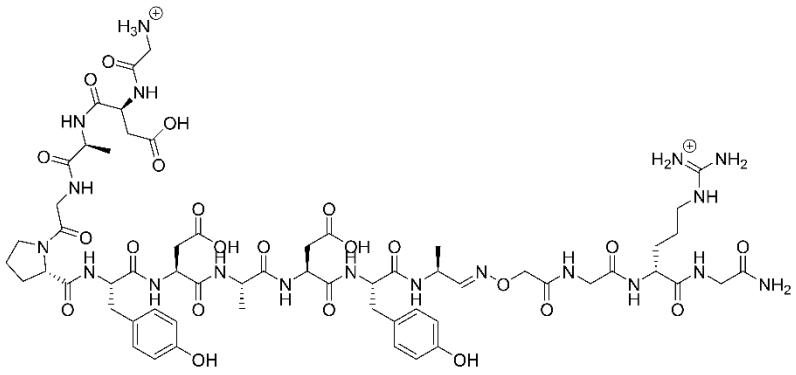
B ions	Y ions
58.0288	1468.65
173.056	1411.62
272.124	1296.6
329.146	1197.53
426.198	1140.51
541.225	1043.45
612.262	928.427
775.326	857.39
890.353	694.327
1053.42	579.2997
1450.63	416.236



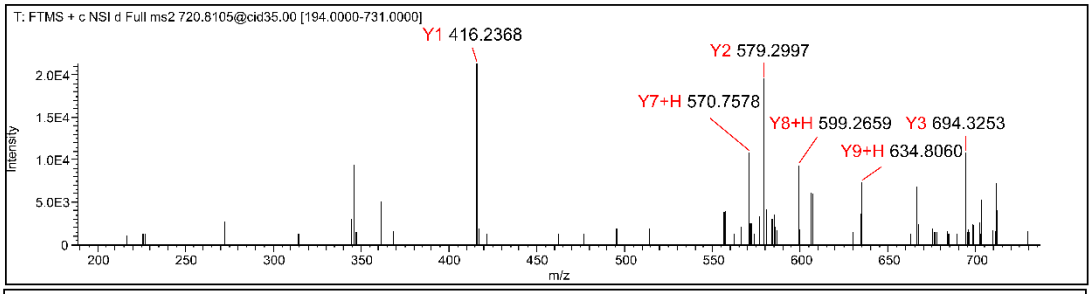
Exact Mass: 1469.6524
m/z: 734.8262

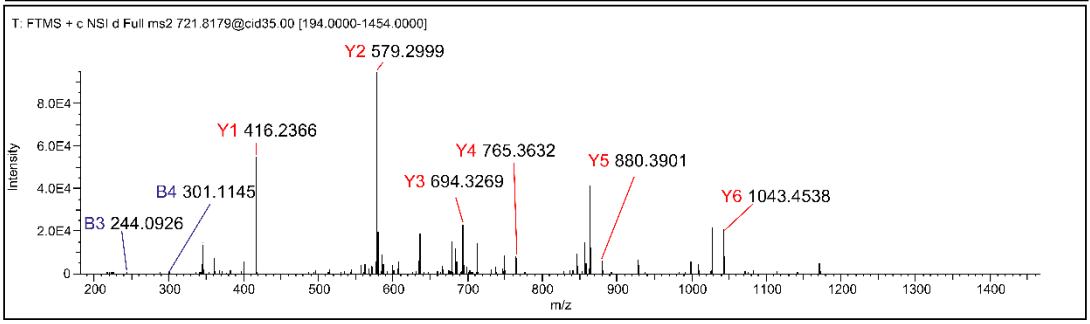


B ions	Y ions
58.0287	1440.614
173.0557	1383.592
244.0928	1268.565
301.1143	1197.528
398.1671	1140.507
561.2304	1043.454
676.2573	880.3907
747.2945	765.3638
862.3214	694.3267
1025.385	579.2997
1422.603	416.2364

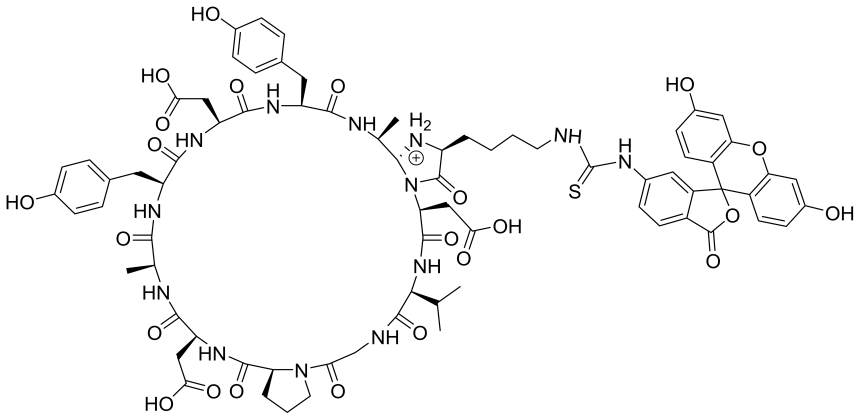


Exact Mass: 1441.6211
m/z: 720.8106

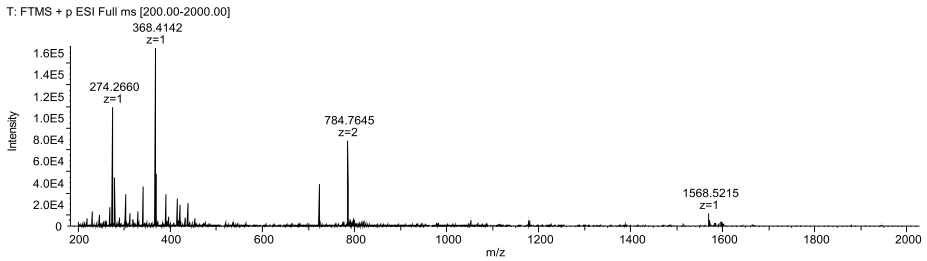


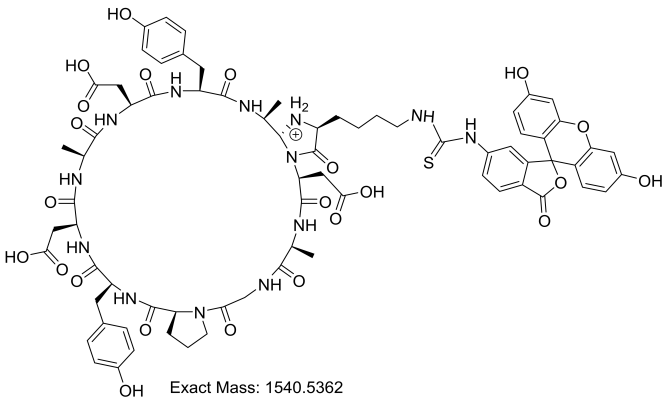


Supplementary Figure 10. Structure and MS data of the cyclic peptide binders with FITC.

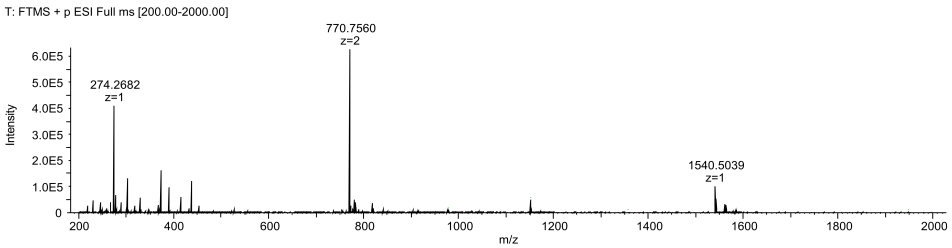


Exact Mass: 1568.5675

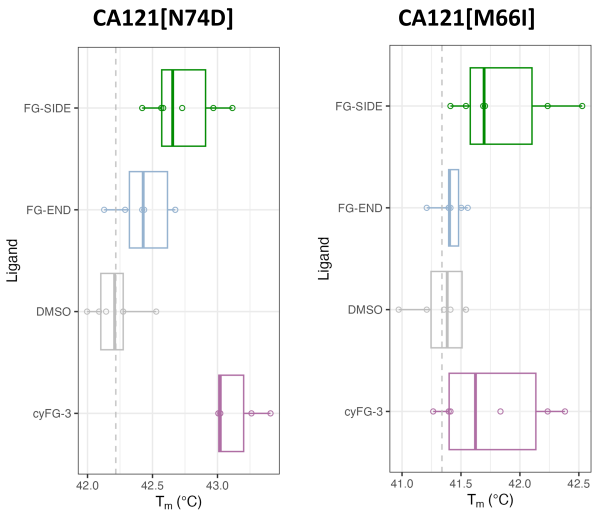




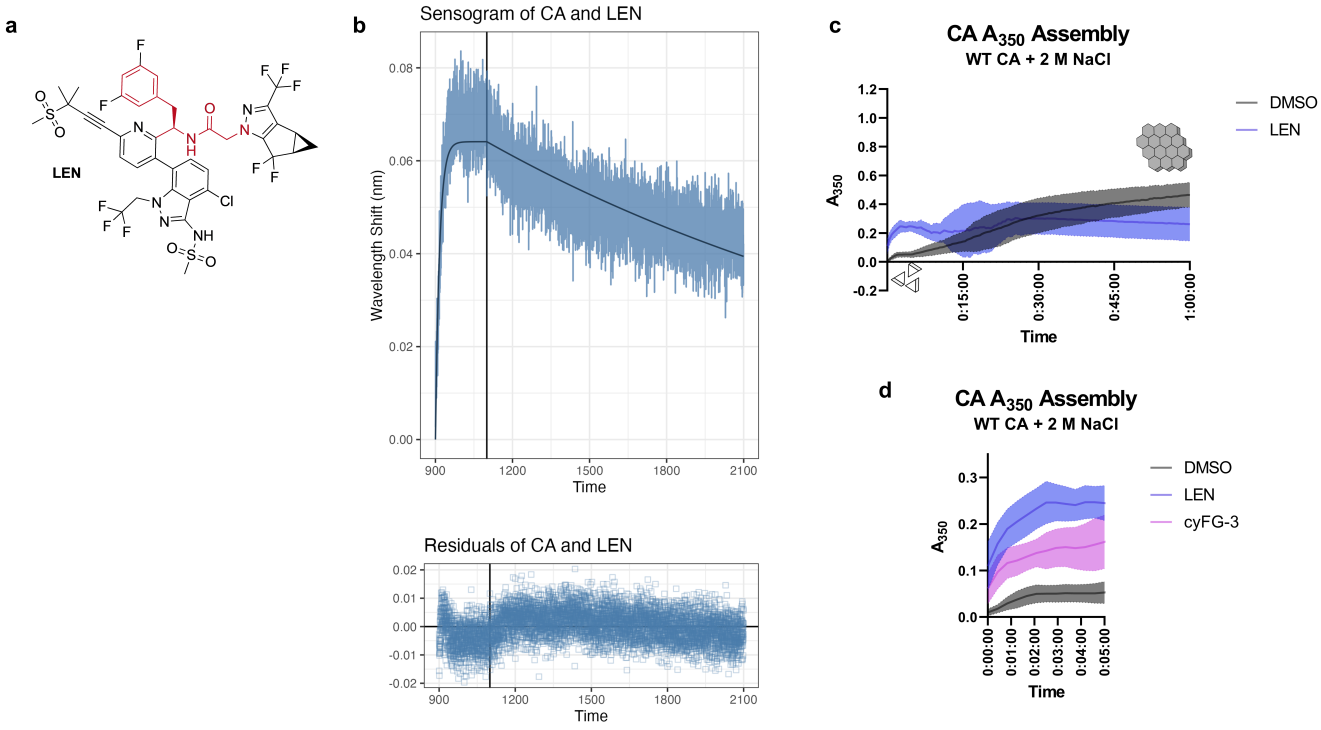
Exact Mass: 1540.5362



Supplementary Figure 11. Thermal Shift Assay data for FG CyClick peptides with point mutated HIV capsid protein (CAN74D and CAM66I).



Supplementary Figure 12. Lenacapavir (LEN) small molecule inhibitor (a) Structure (b) Representative Biolayer Interferometry (BLI) sensograms of double background subtracted data and modeled fit for CA Hexamers with LEN at 5 μM (c) Kinetics of CA assembly in the presence of DMSO or 5 μM LEN (d) Kinetics of CA assembly in the presence of DMSO, 5 μM LEN, or 75 μM cyFG-3



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