SUPPORTING INFORMATION

Chromodomain Ligand Optimization via Target-Class Directed Combinatorial Repurposing

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TABLE OF CONTENTS

Materials and Methods

Expression constructs

The chromo domains of CBX5 (residues 18-75 of NP_036429), CBX8 (residues 8-61 of NP_065700) and MPP8 (residues 55-116 of NP_059990) were expressed with N-terminal His-tags in pET28 expression vectors. The chromo domains of CBX7 (residues 8-62 of NP_783640) and CDYL2 (residues 1-75 of NP_689555) were expressed with C-terminal His-tags in pET30 expression vectors. The chromo domains of CDY1 (residues 1-101 of NP_733841) and CDYL (residues 1-78 of NP_004815) and were expressed with N-terminal GST-tags in pGEX4T expression vectors.

Protein expression and purification

All expression constructs were transformed into Rosetta2 BL21(DE3)pLysS competent cells (Novagen, EMD Chemicals, San Diego, CA). Protein expression was induced by growing cells at 37°C with shaking until the OD_{600} reached ~0.6-0.8 at which time the temperature was lowered to 18°C and expression was induced by adding 0.5mM IPTG and continuing shaking overnight. Cells were harvested by centrifugation and pellets were stored at -80°C.

His-tagged proteins were purified by resuspending thawed cell pellets in 30ml of lysis buffer (50mM sodium phosphate pH 7.2, 50mM NaCl, 30mM imidazole, 1X EDTA free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)) per liter of culture. Cells were lysed on ice by sonication with a Branson Digital 450 Sonifier (Branson Ultrasonics, Danbury, CT) at 40% amplitude for 12 cycles with each cycle consisting of a 20 second pulse followed by a 40 second rest. The cell lysate was clarified by centrifugation and loaded onto a HisTrap FF column (GE Healthcare, Piscataway, NJ) that had been pre-equilibrated with 10 column volumes of binding buffer (50mM sodium phosphate pH 7.2, 500mM NaCl, 30mM imidazole) using an AKTA FPLC (GE Healthcare, Piscataway, NJ). The column was washed with 15 column volumes of binding buffer and protein was eluted in a linear gradient to 100% elution buffer (50mM sodium phosphate pH 7.2, 500mM NaCl, 500mM imidazole) over 20 column volumes. Peak fractions containing the desired protein were pooled and concentrated to 2ml in Amicon Ultra-15 concentrators 3,000 molecular weight cut-off (Merck Millipore, Carrigtwohill Co. Cork

IRL). Concentrated protein was loaded onto a HiLoad 26/60 Superdex 75 prep grade column (GE Healthcare, Piscataway, NJ) that had been pre-equilibrated with 1.2 column volumes of sizing buffer (25mM Tris pH 7.5, 250mM NaCl, 2mM DTT, 5% glycerol) using an ATKA Purifier (GE Healthcare, Piscataway, NJ). Protein was eluted isocratically in sizing buffer over 1.3 column volumes at a flow rate of 2ml/min collecting 3ml fractions. Peak fractions were analyzed for purity by SDS-PAGE and those containing pure protein were pooled and concentrated using Amicon Ultra-15 concentrators 3,000 molecular weight cut-off (Merck Millipore, Carrigtwohill Co. Cork IRL).

GST-tagged proteins were purified by resuspening thawed cell pellets in 30ml of lysis buffer (1xPBS, 5mM DTT, 1X EDTA free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)) per liter of culture. Cells were lysed on ice by sonication as described for His-tagged proteins. Clarified cell lysate was loaded onto a GSTrap FF column (GE Healthcare, Piscataway, NJ) that had been pre-equilibrated with 10 column volumes of binding buffer (1xPBS, 5mM DTT) using a AKTA FPLC (GE Healthcare, Piscataway, NJ). The column was washed with 10 column volumes of binding buffer and protein was eluted in 100% elution buffer (50mM Tris pH 7.5, 150mM NaCl, 10mM reduced glutathione) over 10 column volumes. Peak fractions containing the desired protein were pooled and concentrated to 2ml in Amicon Ultra-15 concentrators, 10,000 molecular weight cut-off (Merck Millipore, Carrigtwohill Co. Cork IRL). Concentrated protein was loaded onto a HiLoad 26/60 Superdex 200 prep grade column (GE Healthcare, Piscataway, NJ) that had been pre-equilibrated with 1.2 column volumes of sizing buffer (25mM Tris pH 7.5, 250mM NaCl, 2mM DTT, 5% glycerol) using an ATKA FPLC (GE Healthcare, Piscataway, NJ). Protein was eluted isocratically in sizing buffer over 1.3 column volumes at a flow rate of 2ml/min collecting 3ml fractions. Peak fractions were analyzed for purity by SDS-PAGE and those containing pure protein were pooled and concentrated using Amicon Ultra-15 concentrators 10,000 molecular weight cut-off (Merck Millipore, Carrigtwohill Co. Cork IRL).

Proteins were dialyzed into a buffer containing 25mM Tris pH 7.5, 150mM NaCl, 2mM β-mercaptoethanol prior to use for ITC.

Affinity tag removal

The N-terminal GST-tag was removed from CDYL by thrombin cleavage according to manufacturer's recommendations (Novagen, EMD Chemicals, San Diego, CA). Briefly, purified protein was incubated with biotinylated thrombin at a final concentration of 1 unit thrombin per milligram tagged protein for 16 hours at 4^0C. The cleavage reaction was then passed over a GSTrap FF column (GST-tagged proteins) to remove any protein that still retained the tag. The column flow through was collected and incubated with streptavidin agarose to remove thrombin from the sample. Streptavidin agarose was removed from the sample by centrifugation and the protein was concentrated using Amico Ultra-15 concentrators, 3,000 molecular weight cut-off (Merck Millipore, Carrigtwohill Co. Cork IRL). Protein was exchanged into a buffer containing 25mM Tris pH 7.5, 150mM NaCl, 2mM β-mercaptoethanol prior to use in ITC.

Library Synthesis

Fmoc-protected amino acids were purchased from Chem-Impex and Sigma-Aldrich with the exception of Fmoc lysine derivatives (synthesis described below). All other chemicals and solvents were purchased from TCI America and Sigma Aldrich, unless otherwise stated. Syringe reaction vessels were made in-house with 2 mL and 10 mL syringes (Norm-Ject) and frits (Teledyne). Approximately 200 mg of PEGA resin (140-300 μm; EMD Millipore) swollen in ethanol was removed by pipette to a 10 mL syringe reaction vessel. The resin was rinsed once with N,N-dimethylformamide (DMF), drained, and left to equilibrate in DMF for 2 hours. Standard Fmoc solid phase peptide synthesis was combined with the split-and-pool method to generate the library. Briefly, Fmoc-protected amino acids (15 eq) were mixed for 5 minutes with HBTU (15 eq), HOAt (15 eq), and DIPEA (30 eq) in 5 mL of DMF and 3 mL of dichloromethane (DCM). The solution was then added to the resin and left on a shaker at room temperature for 1 hr. The resin was filtered and washed twice with DCM, DMF, methanol, and DMF again. Fmoc protecting groups were removed in a solution of 2.5% 1,8-diazabicycloundec-7-ene and 2.5% pyrrolidine in DMF for 10 min. The resin was filtered and washed twice with DMF, methanol, DMF, and DCM before adding the next amino acid for coupling. After

completion of the linker synthesis, splitting of the library was conducted following Fmoc deprotection. Resin in DMF was split equally by pipette into 2 mL syringe reaction vessels. Following the coupling reactions, the resin in DMF was pooled back together into a 10 mL syringe reaction vessel and Fmoc was deprotected before splitting again. The carboxylic acid caps were coupled under the same conditions as the amino acids described above, but the couplings were left overnight. The resin was then pooled back into a new 10 mL syringe reaction vessel. Following several DCM rinses, the on-bead peptides were deprotected in 95% trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% water for 3 hours. The resin was rinsed again with DCM 10 times followed by several ethanol washes. The on-bead library was stored in ethanol until use.

Library Screening

Half of the synthesized library was removed to a 15 mL conical tube and equilibrated in 25 mM Tris, pH 7.8, 150 mM NaCl, and 0.1% Tween-20 (TBST) overnight. Individual magnetic screens follow a similar protocol to that originally described by Astle et al[1]. For 1 hour, the beads were blocked in 5% BSA in TBST (10 mL) at room temperature. The resin was washed once (10 mL TBST) and then incubated with 1 µM His-tagged MPP8 in 2.5% BSA in TBST (10 mL) for 1 hour. Simultaneously, mouse anti-His antibody (10 µL of a 1 µg/µL solution; Pierce MA1-21315) was incubated with Protein G Dynabeads (50 µL; Life Technologies) in 2.5% BSA in TBST (200 µL). After incubation, the Dynabeads were washed 3 times with TBST (10 mL), and the library was washed 3 times with TBST (10 mL). 2.5% BSA in TBST (10 mL) was added to the library followed by Dynabead addition. The library beads and magnetic beads were left to mix at room temperature for 1 hour. A magnet was used to remove MPP8 hits and non-specific binders. The remaining library was stripped with 1% SDS (10 mL) at 95°C for 3 minutes and rinsed repeatedly with TBST. The library was then left in TBST overnight at 4°C on the rotator. The next day the library was screened under the same conditions as above except 1 µM His-CDYL2 was used as the target protein. Magnetized hits from this screen were isolated to a 2 mL Eppendorf tube. Hit PEGA beads were left to settle at the bottom of the tube while excess magnetic beads were mostly removed by pulling off the solution by pipette. Fresh 2.5% BSA in TBST (1.8 mL) was added to the hit beads. 10 mM UNC3866 in DMSO was added to the

solution to a final concentration of 10 μM. The hits were left to rotate in this solution for 2 hours. At 120 minutes the beads were subjected to a magnet and unmagnetized beads were removed to a separate Eppendorf tube. The hit beads that remained magnetized throughout the soluble competitor treatment were stripped with 1% SDS at 95°C for 3 minutes then rinsed repeatedly with TBST. Negative selection screens with these CDYL2 hit beads were performed with 1 μM His-CBX8, His-CBX7, and His-CBX5 under the same conditions but with the following modifications: screening volumes of the PEGA hit beads were all adjusted to 1.5 mL, and 2 μL of anti-His antibody was incubated with 5 μL of Protein G Dynabead in 50 μL 2.5% BSA in TBST. These negative selections were done sequentially and after each screen the unmagnetized PEGA beads were stripped of protein by SDS treatment and stringently washed before proceeding to the next negative selection. After all three negative selections, the CDYL2-selective hit beads were rinsed repeatedly in ethanol and then equilibrated overnight in ethanol.

Hit Cleavage and MALDI-TOF/TOF Sequencing

The hit beads were isolated (1-2 beads per well) into a 96-well plate (Thermo Scientific 0.2 mL 96-well PCR plate). The ethanol solutions were left to evaporate. Cyanogen bromide cleavage followed previously described protocols[1,2]. In brief, a mixture of 0.25 M cyanogen bromide in 10% water, 40% acetic acid, and 50% acetonitrile was prepared. 20 μL of this mixture was added to each well. The plates were loosely covered and left to mix overnight on a shaker platform at room temperature in a fume hood. Cleaved compounds were redissolved in 10 μL of 1:1 water:acetonitrile. From this solution, 0.5 μL was spotted on an Opti-TOF 384-well MALDI plate and 0.5 μL of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 50% water with 0.1% TFA was spotted on top. The solutions were left to evaporate. Spectra were recorded on an ABSciex 5800 MALDI-TOF/TOF in positive, linear mode with a laser power between 2200 and 2600.

Hit Resynthesis

Resynthesis was conducted on Fmoc Rink amide MBHA resin (50 mg per peptide, Anaspec). The resin was initially swollen in DCM followed by DMF (10 minutes each). Fmoc deprotection

was conducted as described previously. For FP ligands, the first residue was an alkynyl peptoid that was installed by coupling on bromoacetic acid (1 M) with N,N-diisopropylcarbodiimide (1 M) for 30 minutes followed by 7 DMF washes. Next, the displacement reaction was conducted with 1 M of propargylamine in DMF for 2 hours. Following installation of the peptoid residue, standard Fmoc synthesis protocols (as described in the Library Synthesis section above) were used for the remaining residues and for the entirety of the 6-mer peptides, UNC4990 and UNC4991. Following installation of the capping residue, the resin was rinsed 10 times with DCM. Cleavage cocktail (95% trifluoroacetic acid, 2.5% triisopropylsilane, and 2.5% water) was added to the resin, the mixture was left on the shaker for 2 hours, and the filtrate was collected. Resin was rinsed twice with DCM and filtrates were pooled then concentrated under vacuum. The mixture was dissolved in water and ether was added. Two aqueous extractions followed. The aqueous layers were combined then concentrated under vacuum. Preparative HPLC was performed using an Agilent Prep 1200 series with the UV detector set to 220 nm. Samples were dissolved in a 1 mL 1:1 water: acetonitrile mixture and injected onto a Phenomenex Luna 75 x 30 mm (5 µm) C18 column at room temperature. Mobile phases of A (H_2O + 0.1% TFA) and B (MeCN) were used with a flow rate of 30 mL/min. Products peaks were identified via an Agilent 6110 Series LCMS with a UV detector set to 220 nm and a single quadrupole mass spectrometer. LCMS samples were run on an analytical Agilent Eclipse Plus 4.6 x 50mm, 1.8 µm, C18 column at room temperature with mobile phases A (H_2O + 0.1% acetic acid) and B (MeOH + 0.1% acetic acid). Product fractions were pooled and volatiles were removed under vacuum. Products were redissolved in water and lyophilized to white powders.

Click Labeling of Peptides

Generally, a 1:1 DMF:water (2 mL) mixture of azido-fluorescein (2 eq, Tenova Pharmaceuticals) or Biotin-PEG3-azide (3 eq, Click Chemistry Tools), copper sulfate (2 eq), and ascorbic acid (6 eq) was added to purified peptide. The mixture was heated to 60°C while stirring for 2 hr. The reaction was then left to cool down to room temperature and stirred overnight prior to concentration under vacuum. Crude mixtures were redissolved in 1:1 water:acetonitrile (1 mL), filtered, and purified via HPLC. Product fractions as determined by LCMS were pooled and

volatiles were removed under vacuum. Products were redissolved in water and lyophilized to yellow (FAM-labelled) or white (Biotin-labeled) powders.

FP Assays

Binding assays were carried out in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, and 1mM CHAPS in black 384-well microplates (Greiner) with total reaction volumes of 25 µL. To each well, 15 µL of buffer was added followed by the addition of 5 µL of a 500 nM compound stock (UNC4979, UNC4980, UNC4981, UNC4982, UNC3866-FAM, or FAM-H3K9me3 in FP buffer with 2% DMSO). Serial dilutions of the proteins (CDYL2, CBX7, CBX8, CBX5, MPP8, CDY1, and CDYL) were prepared and 5 µL was added to give final protein concentrations ranging from 0-100 µM, with the exception of CBX8 where concentrations ranged from 0-50 µM. Plates were incubated for 30 minutes at room temperature and then FP measurements were taken on an AcQuest (LJL BioSystems) plate reader, with an excitation wavelength of 485 nm and the emission collected at 530 nm. All assays were run in triplicate. Data was analyzed with GraphPad Prism, and curves were fit using nonlinear regression with a three parameter curve fit. The K_d was calculated as the average of the three K_d's as determined via three independent curve fits. Error was calculated as the standard deviation of the three K_d values.

ITC Experiments

ITC measurements were recorded at 25°C using an AutoITC200 microcalorimeter (MicroCal Inc., MA). Protein stocks were prepared in ITC buffer (25 mM Tris-HCl, pH 8, 150 mM NaCl, and 2 mM β-mercaptoethanol) and then diluted into ITC buffer to achieve a final concentration of 50 µM (325 µL). UNC4990 was dissolved in water to a stock concentration of 10 mM and then diluted to a final concentration of 500 µM. Due to limited solubility, UNC4991 was dissolved in water to a stock concentration of 1 mM and diluted into a high salt ITC buffer (25 mM Tris-HCl, pH 8, 250 mM NaCl, and 2 mM β-mercaptoethanol) to a final concentration of 500 µM. A typical experiment included a single 0.2 µl compound injection into a 200 µl cell filled with protein, followed by 26 subsequent 1.5 µl injections of compound. Injections were performed with a spacing of 180 s and a reference power of 8 µcal/s. The titration data was

analyzed using Origin Software (MicroCal Inc., USA) by nonlinear least-squares, fitting the heats of binding as a function of the compound:protein ratio to a one site binding model. The first data point was deleted from all analyses. All assays were run in duplicate. The data was fit separately for each experiment and the reported K_d is the average of the two runs. Error was calculated as the standard deviation of the two K_d values.

Molecular modeling

Homology model: The homology model of the CDYL2 chromodomain was built using Maestro 2015.2 (Schrödinger). The structure of the CBX7 chromodomain in complex with UNC3866 (PDB: 5EPJ) was used as a structural template. The binding mode of the peptide ligand UNC4991 was hypothesized to be the same as for UNC3866 in 5EPJ. The structure of the resulting complex was energy-minimized and equilibrated in 1 ns NPT- followed by a 5ns NVT MD simulation (see below).

Molecular dynamics: Molecular dynamics simulations were used to probe the conformational spaces of CDYL2 and CBX7 in complexes with UNC4991 and UNC3866. The systems were fully atomistic and explicitly solvated with TIP3P [doi: 10.1063/1.445869], and it contains about 35,500 atoms. All production runs were performed in the canonical NVT ensemble, using a periodic box size obtained from an isothermal–isobaric NPT equilibration of the initial structure. The structure was energy minimized for 3000 steps and equilibrated for 200 ps in the NPT ensemble with no restrained prior to production runs. A temperature of 300 K was maintained using Langevin dynamics, with a damping constant of 5 ps^{-1}. A time step of 2 fs was used, with the lengths of all bonds involving hydrogens constrained. Short-range nonbonded interactions were cut off at 8.0 Å, with shifting beginning at 7.0 Å. Bonded and short-range nonbonded interactions were evaluated every time step, and full electrostatics evaluated using the particle mesh Ewald method [doi: 10.1063/1.470117] every second time step included in GROMACS software [10.1093/bioinformatics/btt055]. The production run was 200 ns using NVT ensemble after the equilibration.

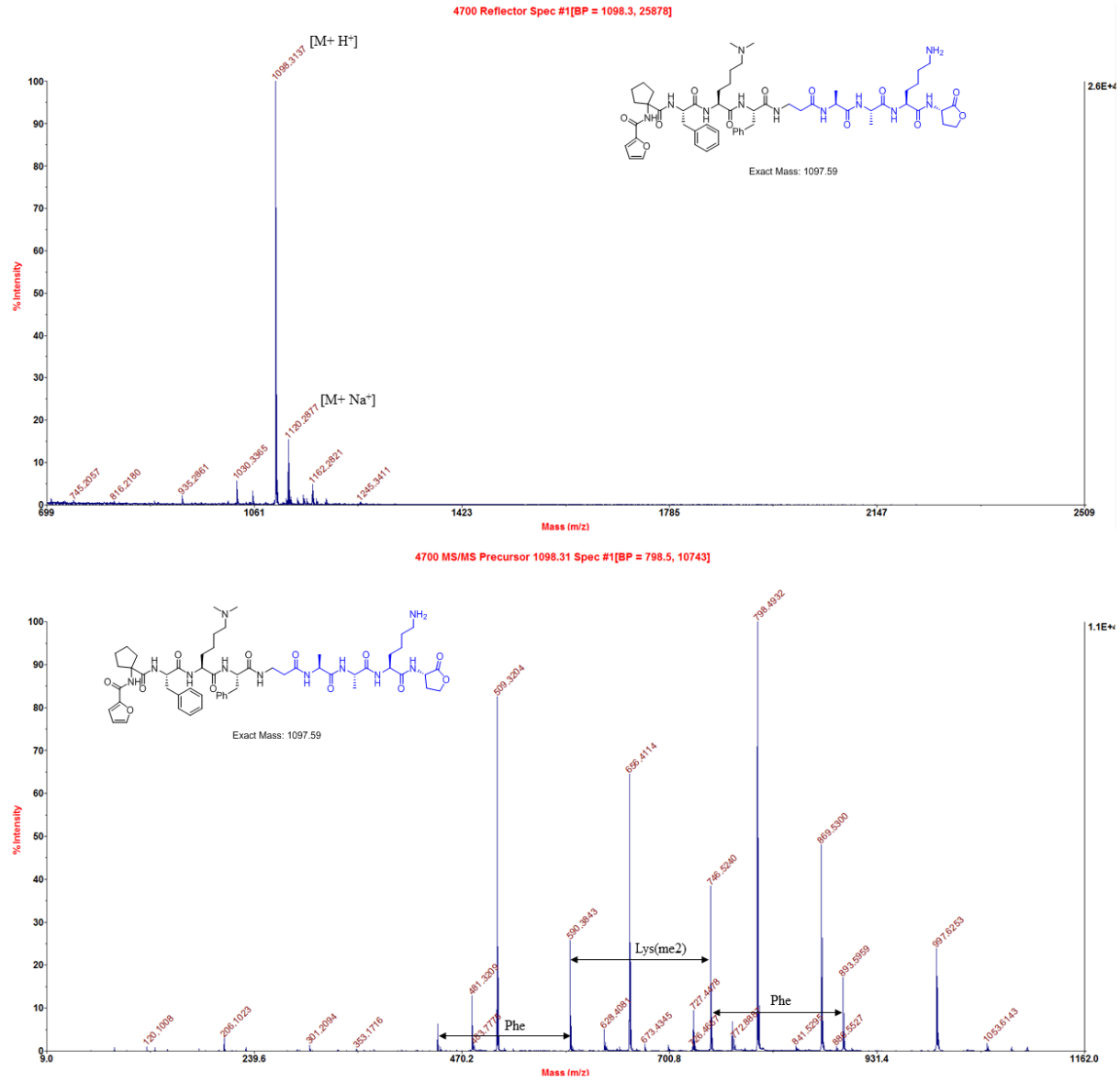
Pull-down Experiments

HeLa cell pellets was obtained from the UNC Lineberger Tissue Culture Facility. Nuclear extracts were prepared as previously described[3] with the substitution of two spins at 2500 x g for 10 min each in place of ultracentrifugation to clarify the nuclear lysate. Pulldown reagent

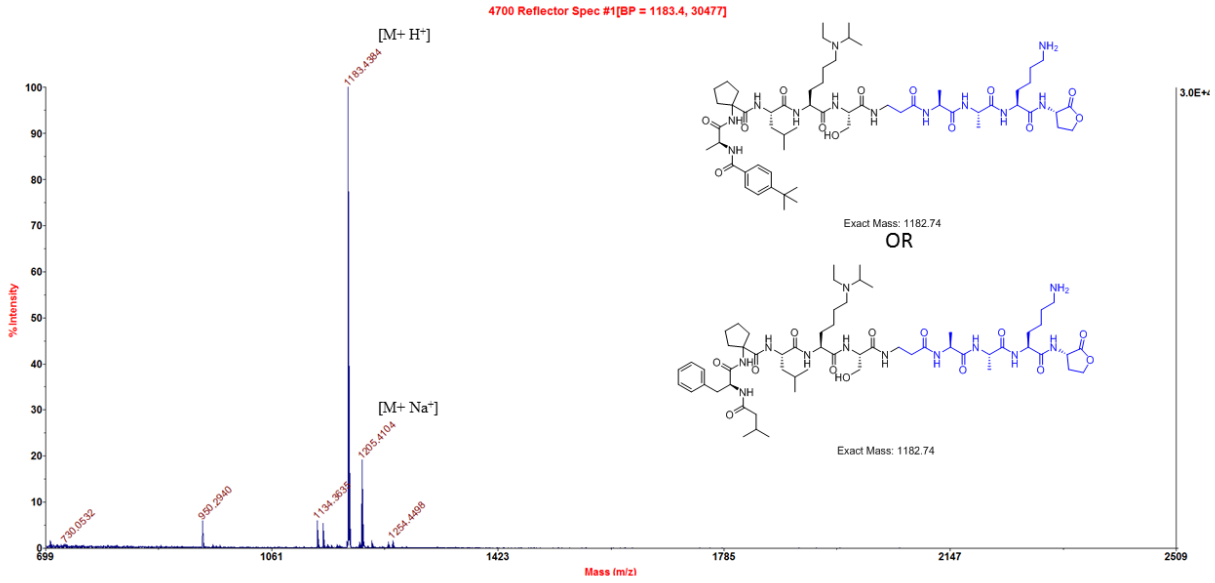
(UNC4991-Biotin) was bound to magnetic Streptavidin M-280 Dynabeads (30 µL beads per pulldown) at 20-fold excess (2 µL of 10 mM stock) in TBST. The beads were left to rotator for 30 min at room temperature. Concurrently, 500 µg of nuclear extract was diluted to 500 µL in TBST in the presence or absence of 100 µM UNC4991. Next, the Dynabeads were washed with TBST to remove excess UNC4991-biotin and then added to the lysate solution. The mixture was rotated at 4°C overnight, and then the beads were isolated by magnetization and washed with TBST. Beads were then re-suspended with 15 µL TBST and 15 µL 2x Laemmi sample buffer and heated at 95°C for 5 min. Half of each sample was used for analysis by SDS-PAGE and western blotting, excluding for CDYL where the full 500 µg was used. Input of 10 µg of nuclear extract was diluted to 7.5 µL and 2x Laemmi sample buffer was added and heated at 95°C for 5 min. Primary antibodies were purchased from Abcam for CDYL2 (ab183854), CDYL (ab137423), and CBX7 (ab21873). After the blot was incubated with primary antibody, IRDye 650 goat anti-mouse IgG (1:10,000 in PBST) was used to visualize the blot on a LI-COR Odyssey instrument.

Figure S1. Library validation. Examples of random beads that were picked and sequenced from the library to confirm that the library was synthesized with high integrity. MALDI-TOF and MALDI-TOF/TOF characterization of products from CNBr cleavage of picked beads.

a)



b)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OR
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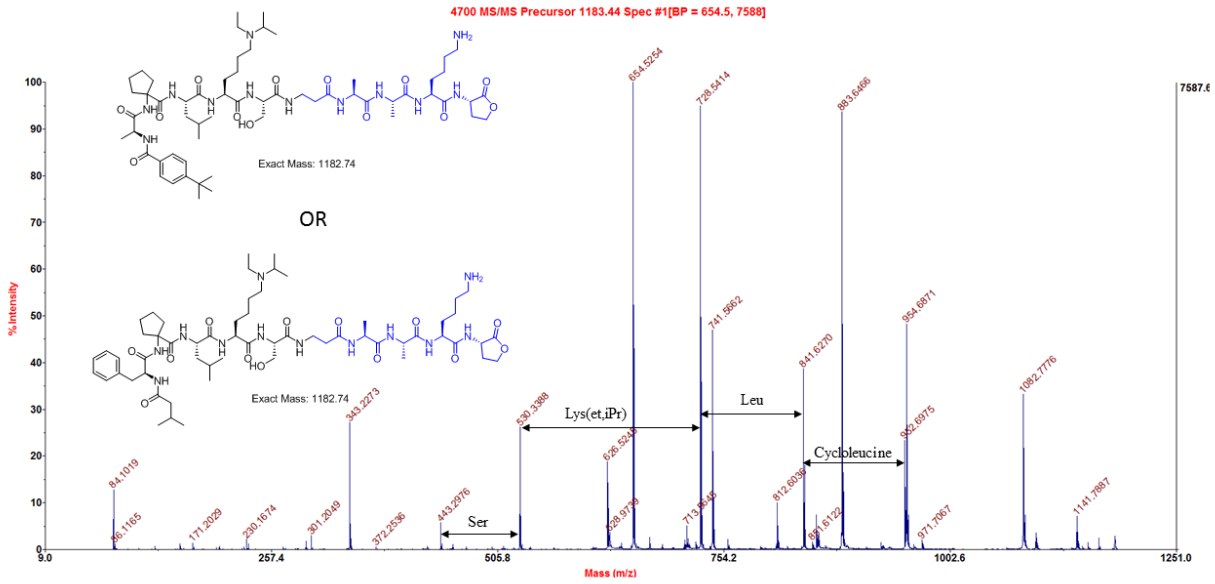
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Ser
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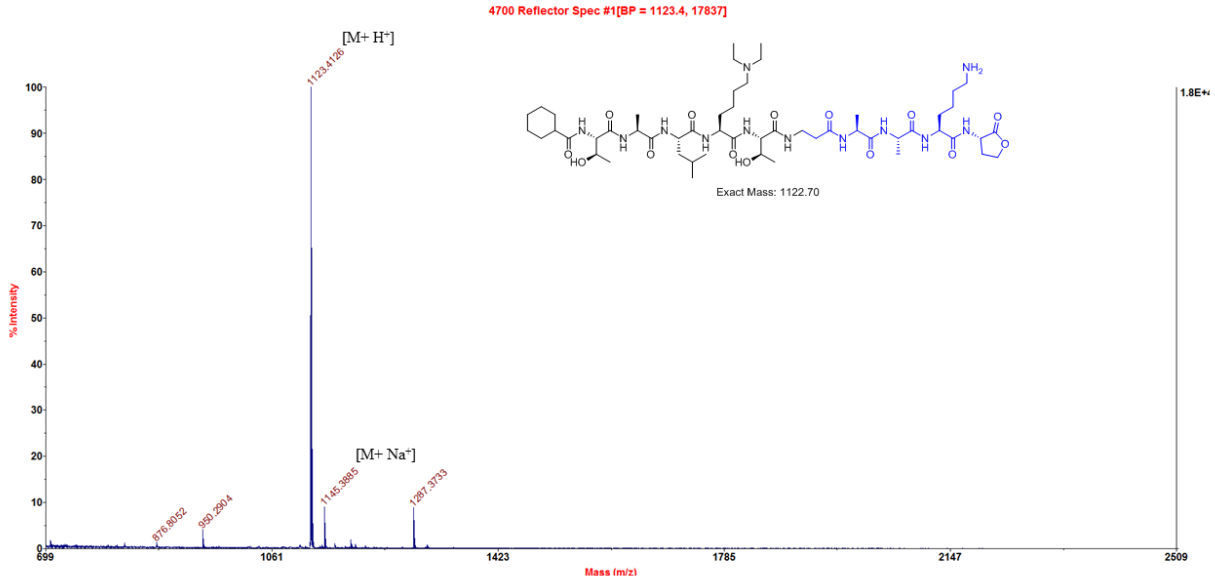
% Intensity
Mass (m/z)

c)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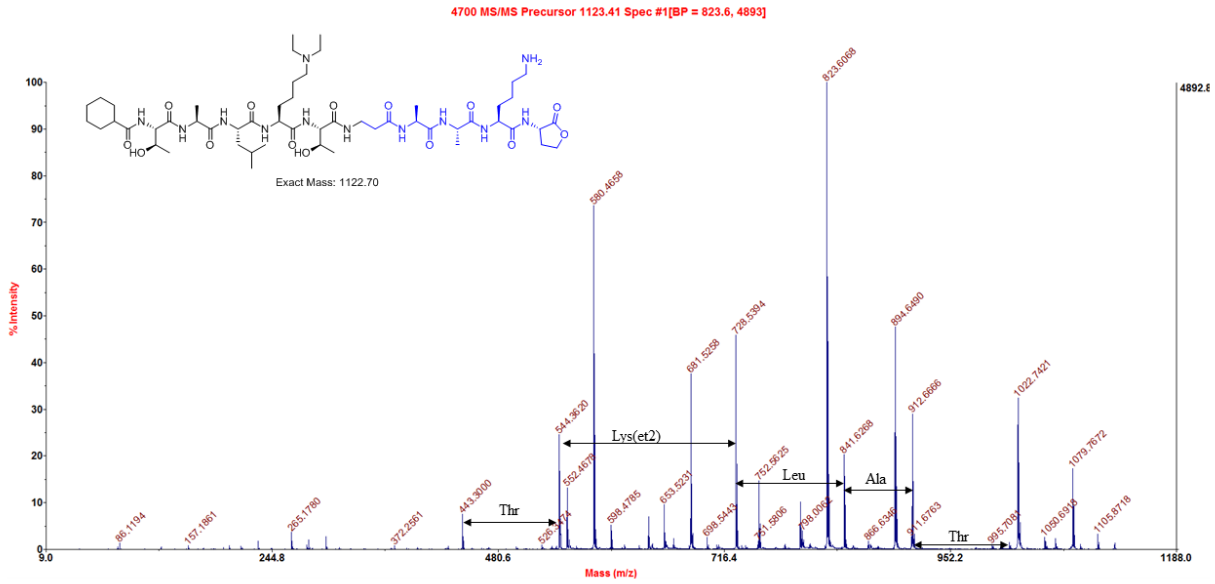
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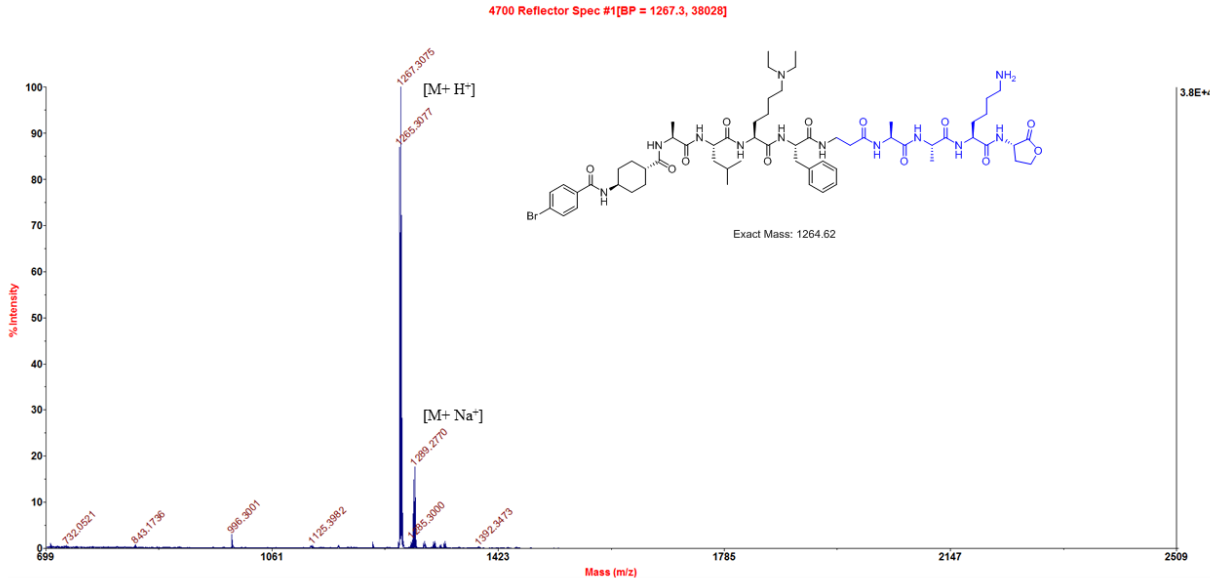
d)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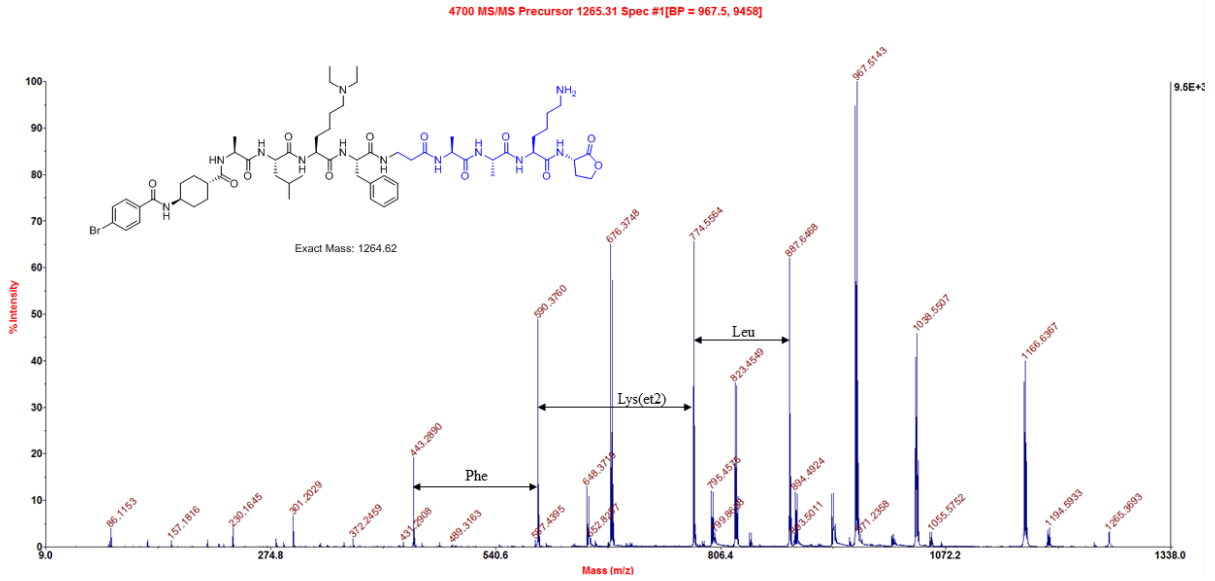
Mass (m/z)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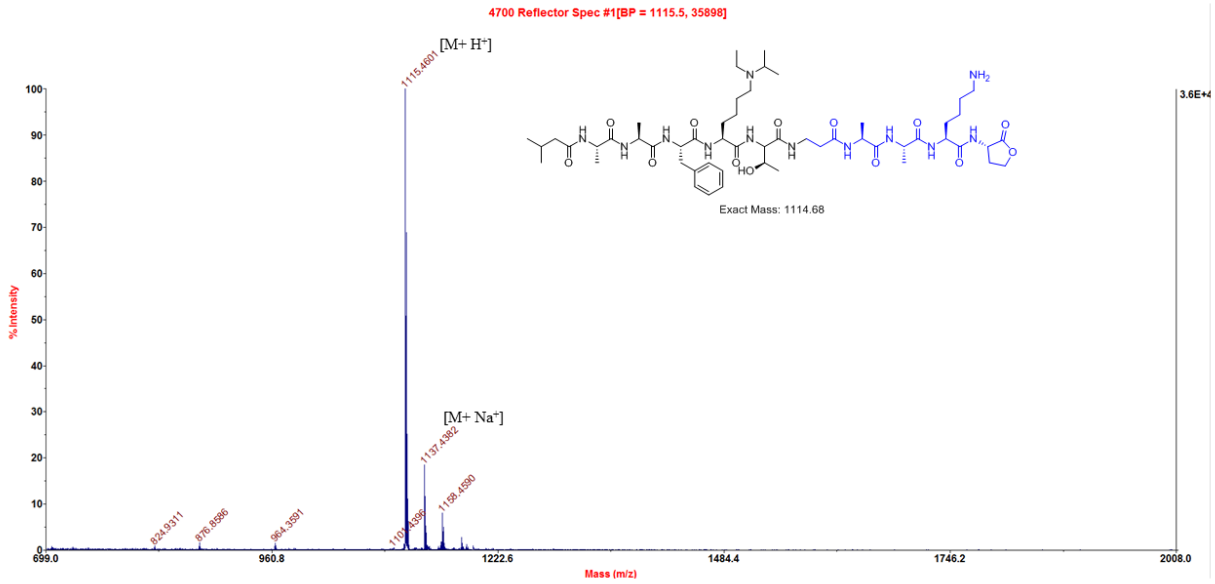
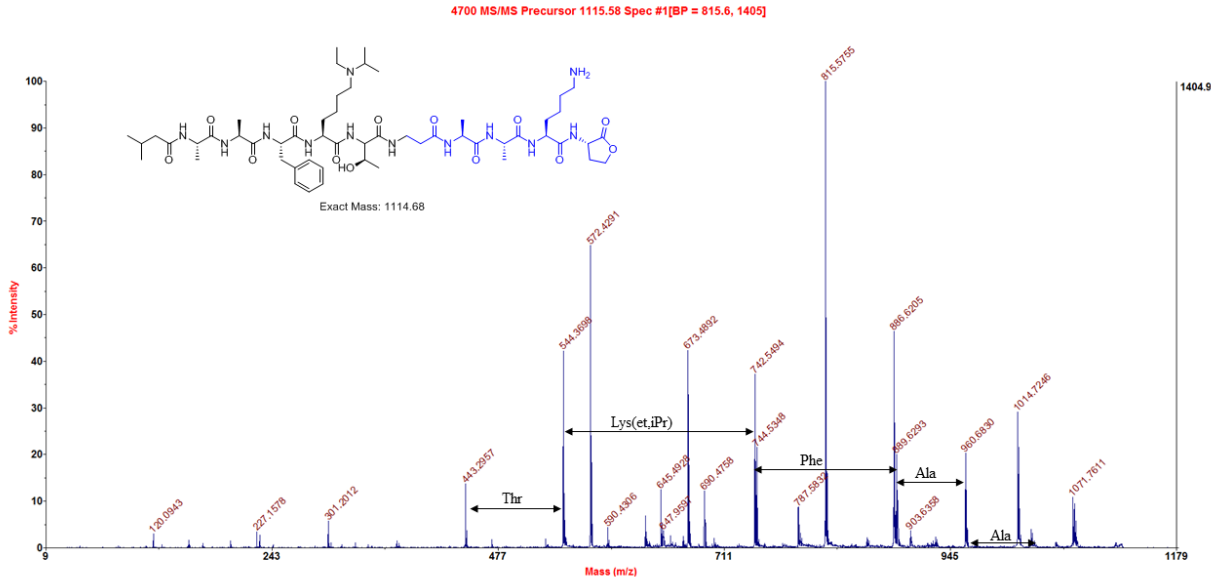
Phe
% Intensity
Mass (m/z)

Figure S2. Examples of CDYL2-selective, highest redundancy hits. MALDI-TOF and MALDI-TOF/TOF characterization of products from CNBr cleavage of hit beads.

a)





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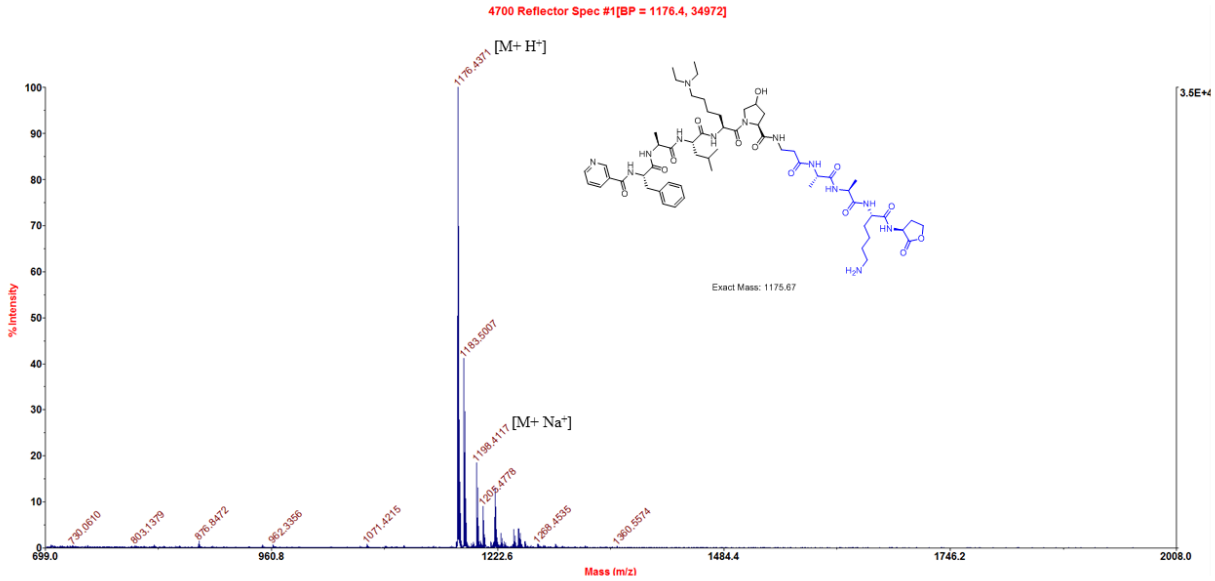
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Mass (m/z)
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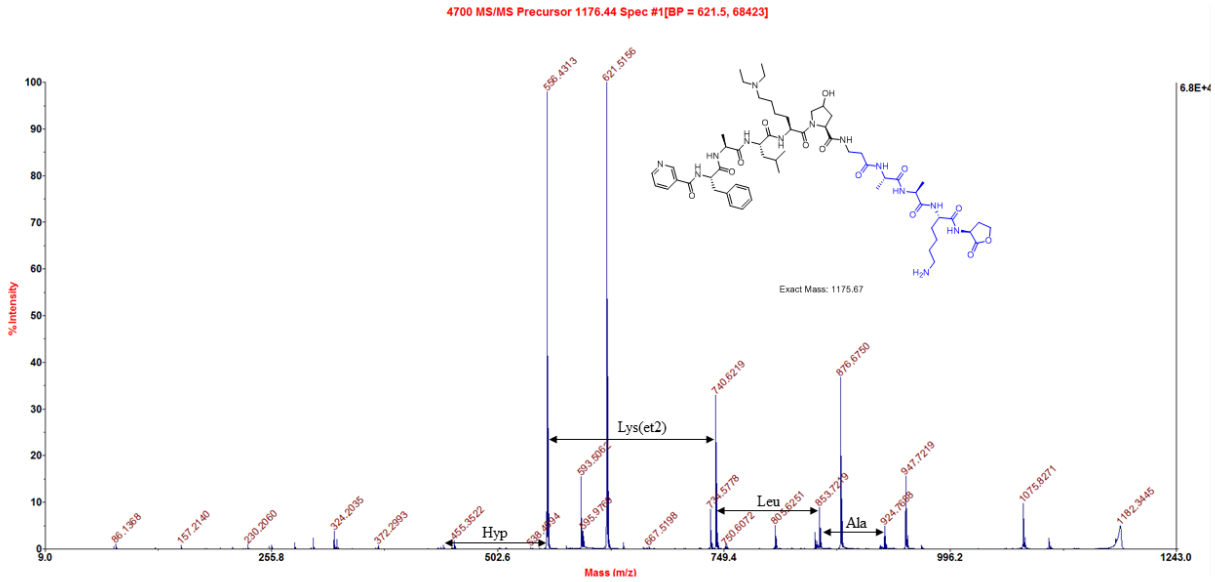
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% Intensity
Mass (m/z)
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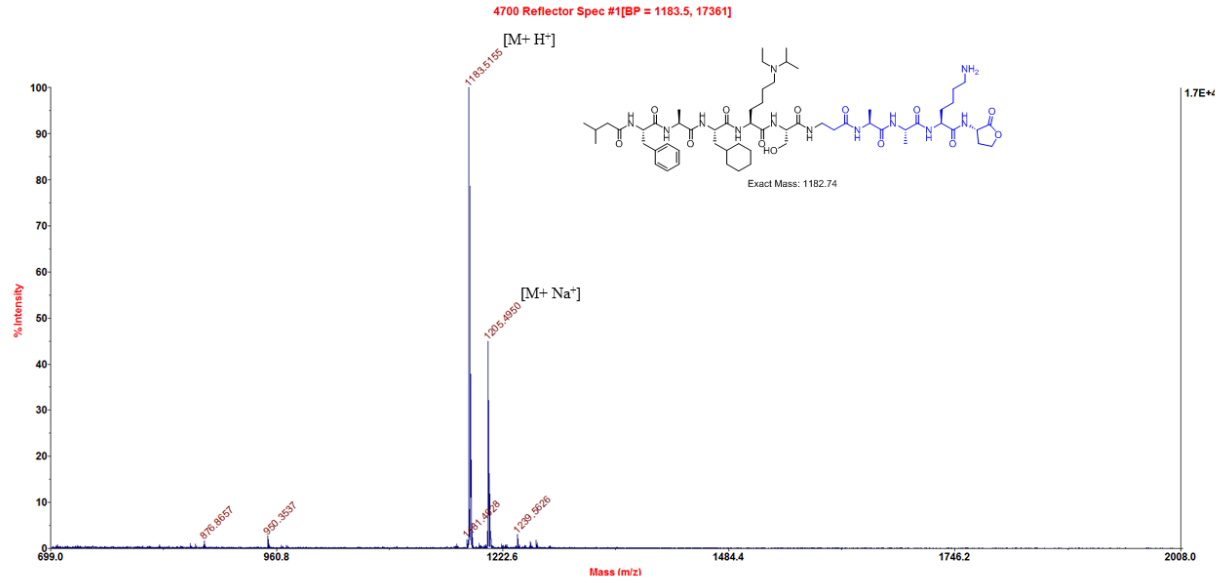
c)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% Intensity
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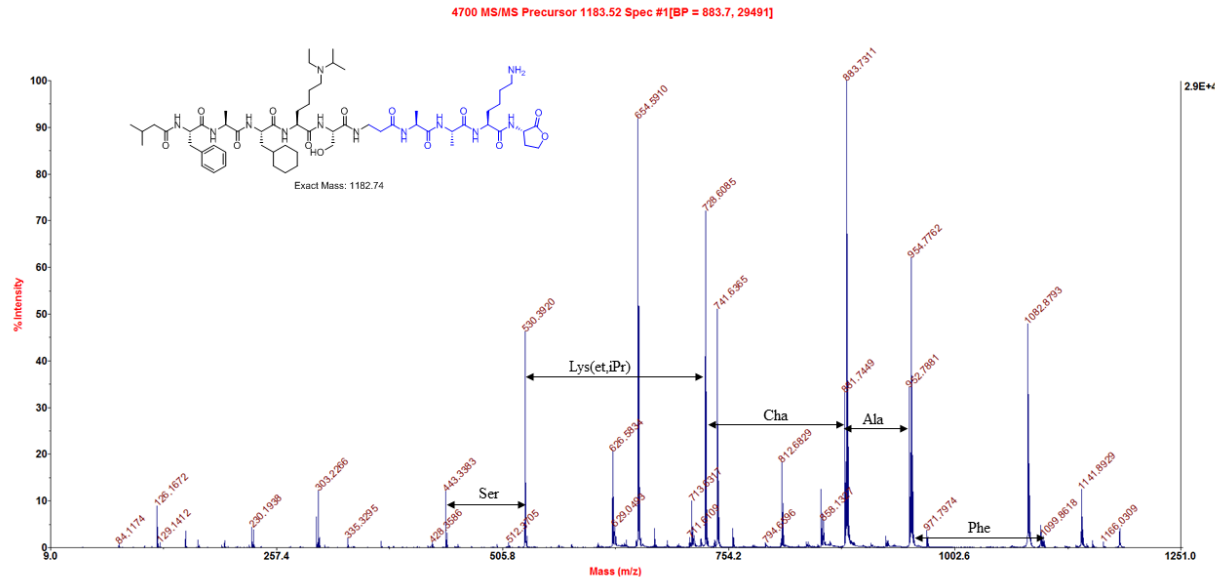
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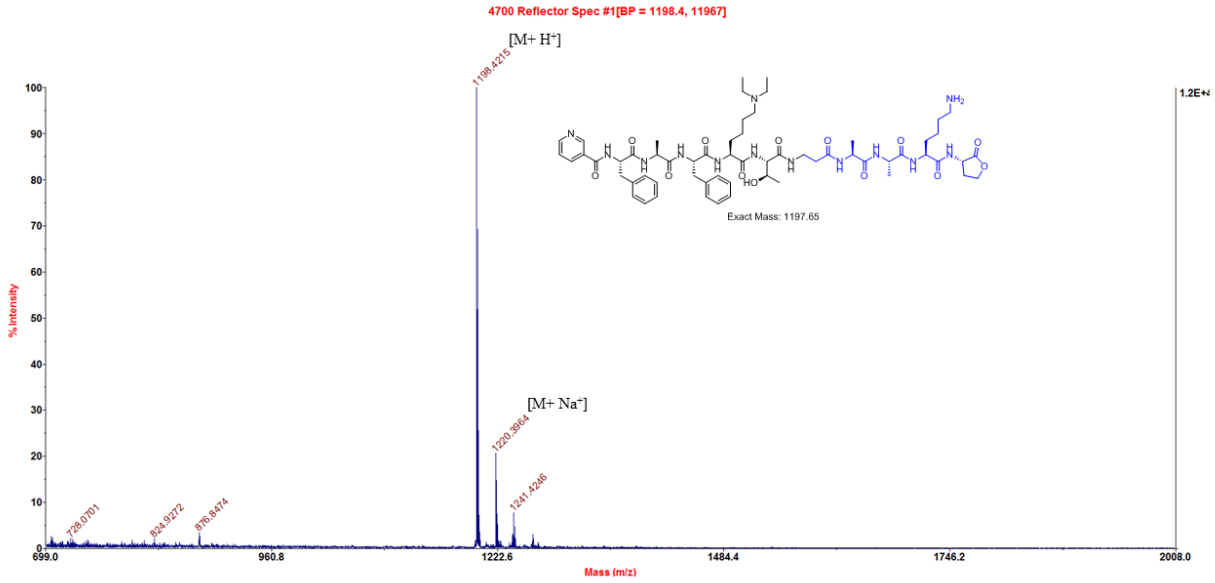
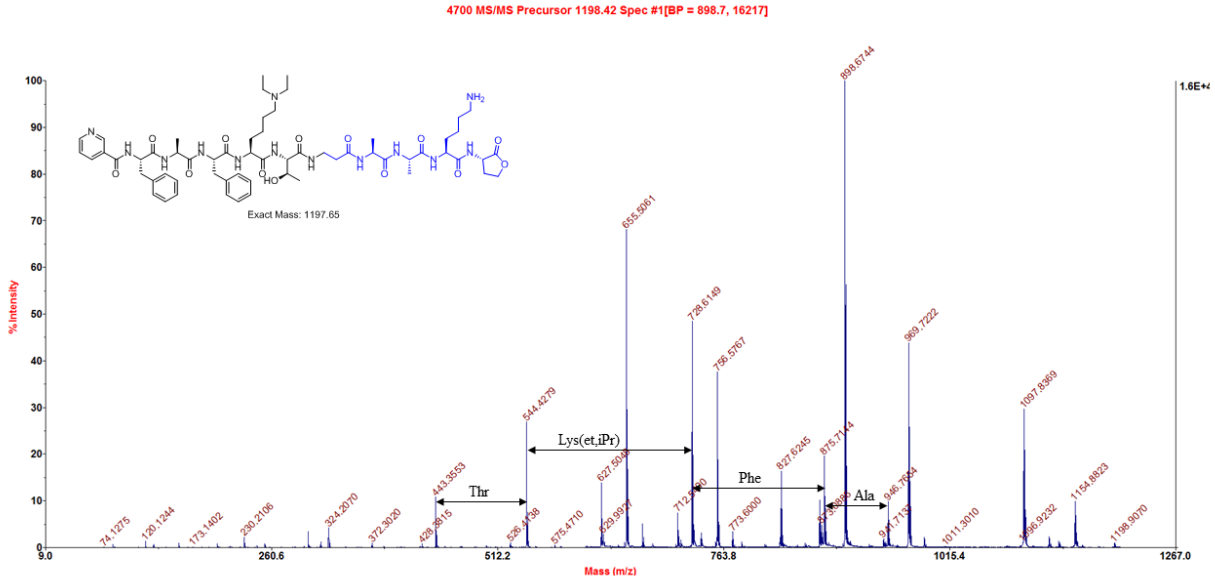
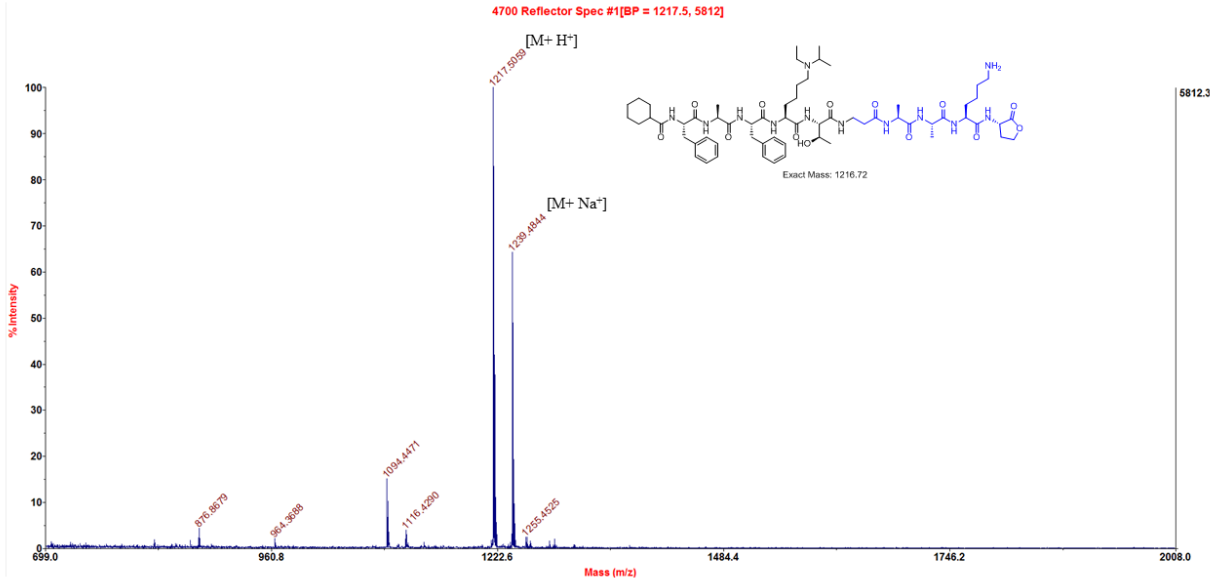
741.8365
530.3920
1082.8793
Lys(et,iPr)
861.7449
962.7861
Cha
Ala
626.4934
812.6829
Ser
Phe
% Intensity
Mass (m/z)
9.0
257.4
505.8
754.2
1002.6
1251.0

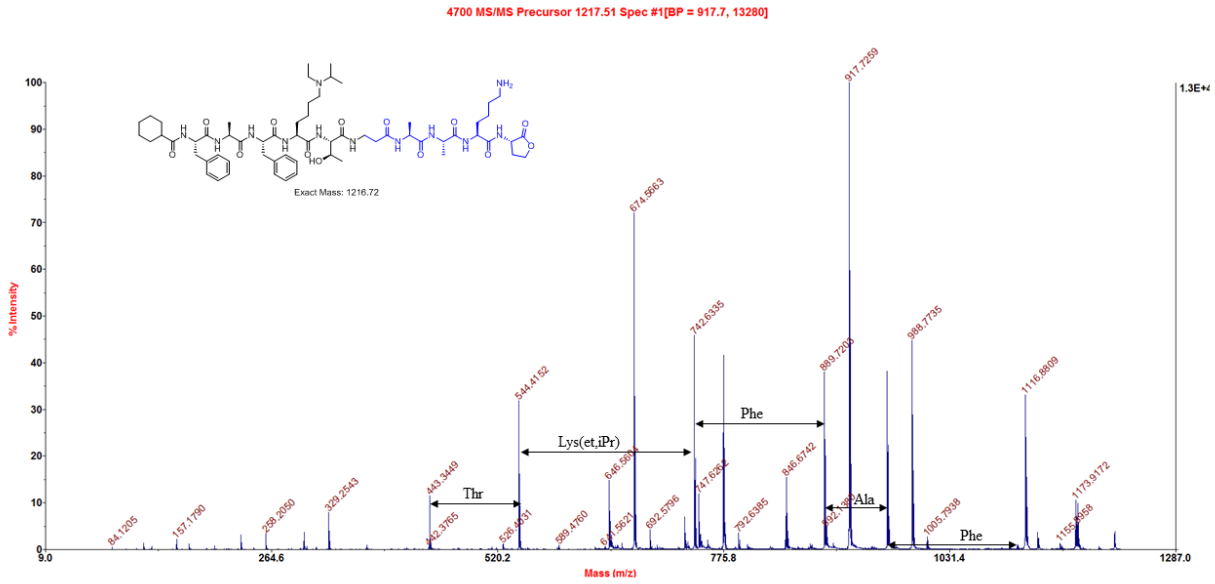
d)


4700 Reflector Spec #1[BP = 1198.4, 11967]
[M+ H+]
1198.4215
[M+ Na+]
1220.3964
1241.4246
728.0701
824.9272
876.8474
Exact Mass: 1197.65
% Intensity
Mass (m/z)
699.0
960.8
1222.6
1484.4
1746.2
2008.0

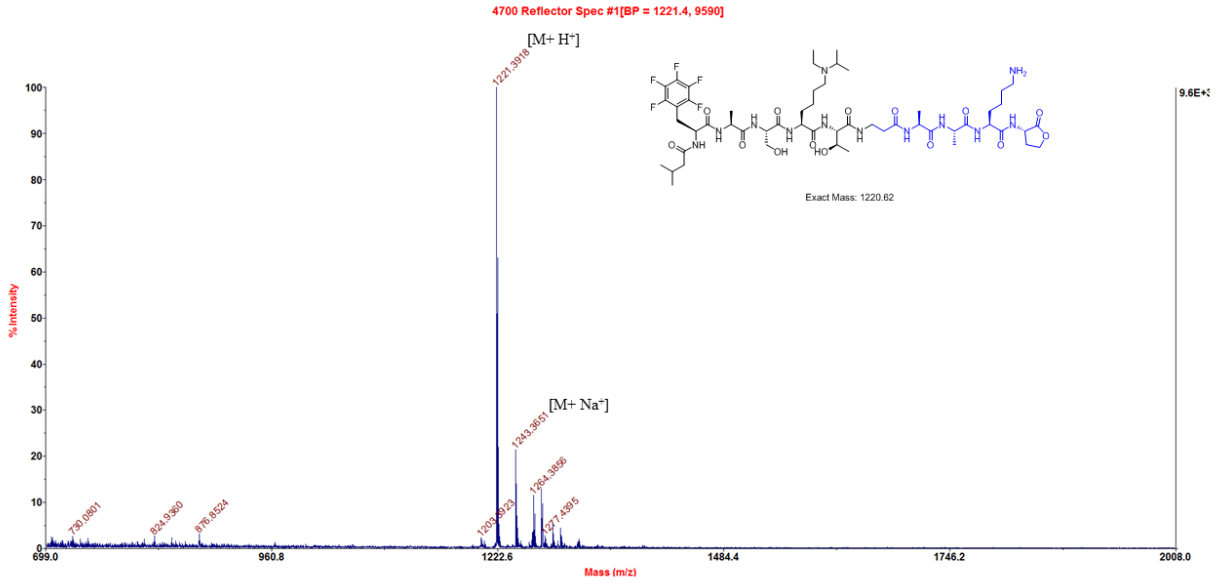

4700 MS/MS Precursor 1198.42 Spec #1[BP = 898.7, 16217]
Exact Mass: 1197.65
898.6744
655.5061
728.6149
795.5767
969.7222
544.4279
Lys(et,iPr)
627.5038
827.6245
875.7144
1097.8369
Phe
Thr
Ala
443.3553
946.7684
1154.8823
74.1275
120.1244
173.1402
230.2106
324.2070
372.3020
526.4138
575.4710
773.6000
1011.3010
1196.9070
% Intensity
Mass (m/z)
9.0
260.6
512.2
763.8
1015.4
1267.0

e)


4700 Reflector Spec #1[BP = 1217.5, 5812]
[M+ H+]
[M+ Na+]
1217.5059
1239.4844
1094.4471
876.8679
964.3698
1116.4290
1255.4525
Exact Mass: 1216.72
% Intensity
Mass (m/z)
699.0
960.8
1222.6
1484.4
1746.2
2008.0


4700 MS/MS Precursor 1217.51 Spec #1[BP = 917.7, 13280]
Exact Mass: 1216.72
917.7259
674.5663
742.6335
544.4152
Phe
Lys(et,iPr)
Thr
Ala
Phe
% Intensity
Mass (m/z)
9.0
264.6
520.2
775.8
1031.4
1287.0

f)


4700 Reflector Spec #1[BP = 1221.4, 9590]
[M+ H+]
[M+ Na+]
1221.3918
1243.3651
1264.3856
1277.4395
1203.3923
730.0801
824.9360
876.8524
Exact Mass: 1220.62
% Intensity
Mass (m/z)
699.0
960.8
1222.6
1484.4
1746.2
2008.0

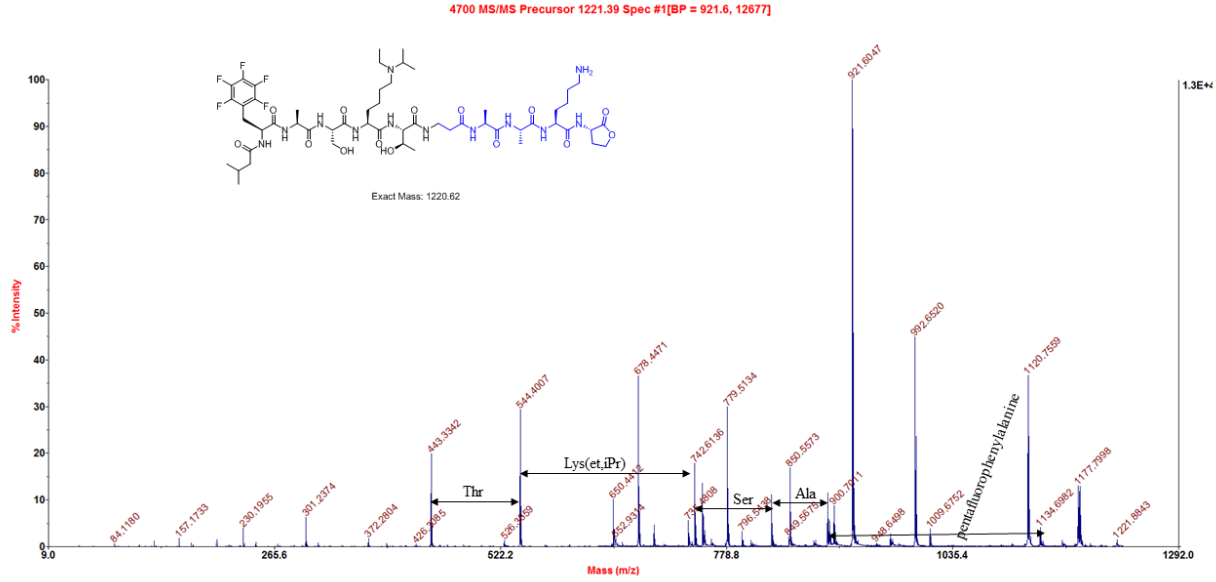
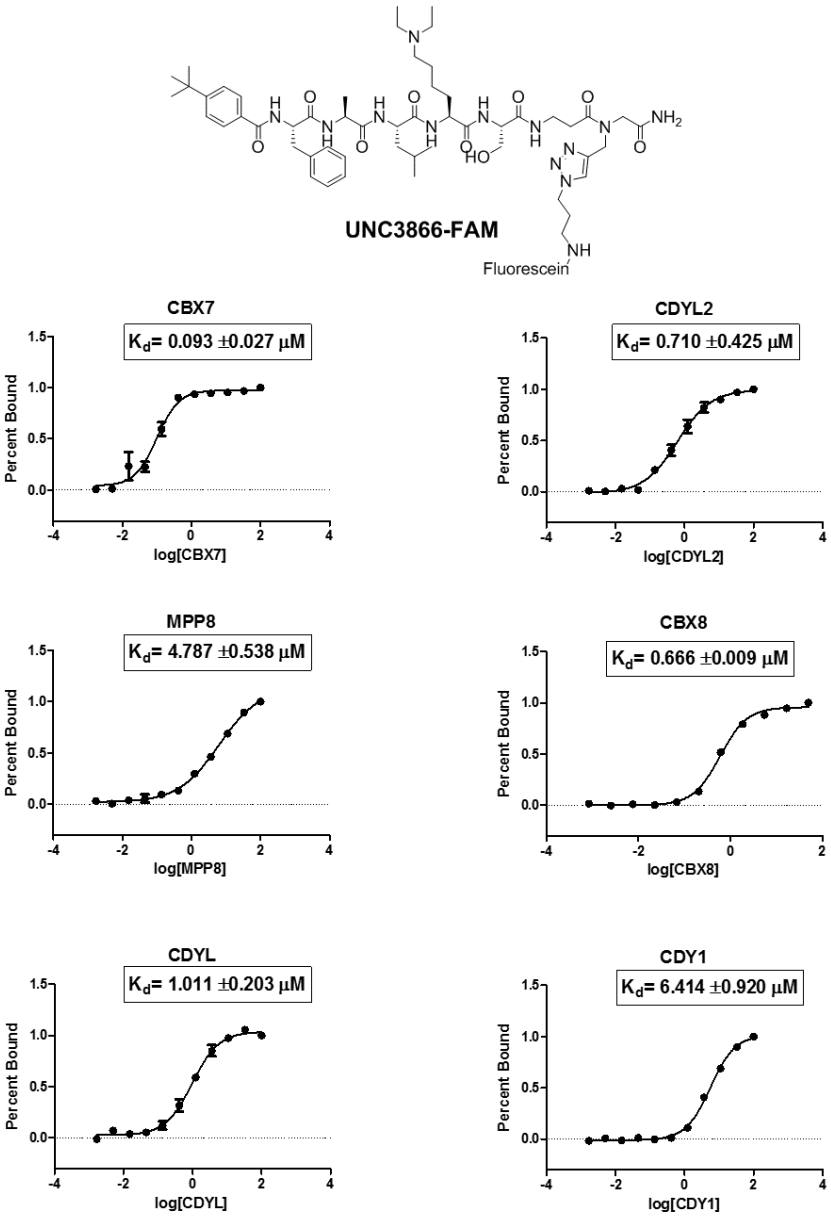
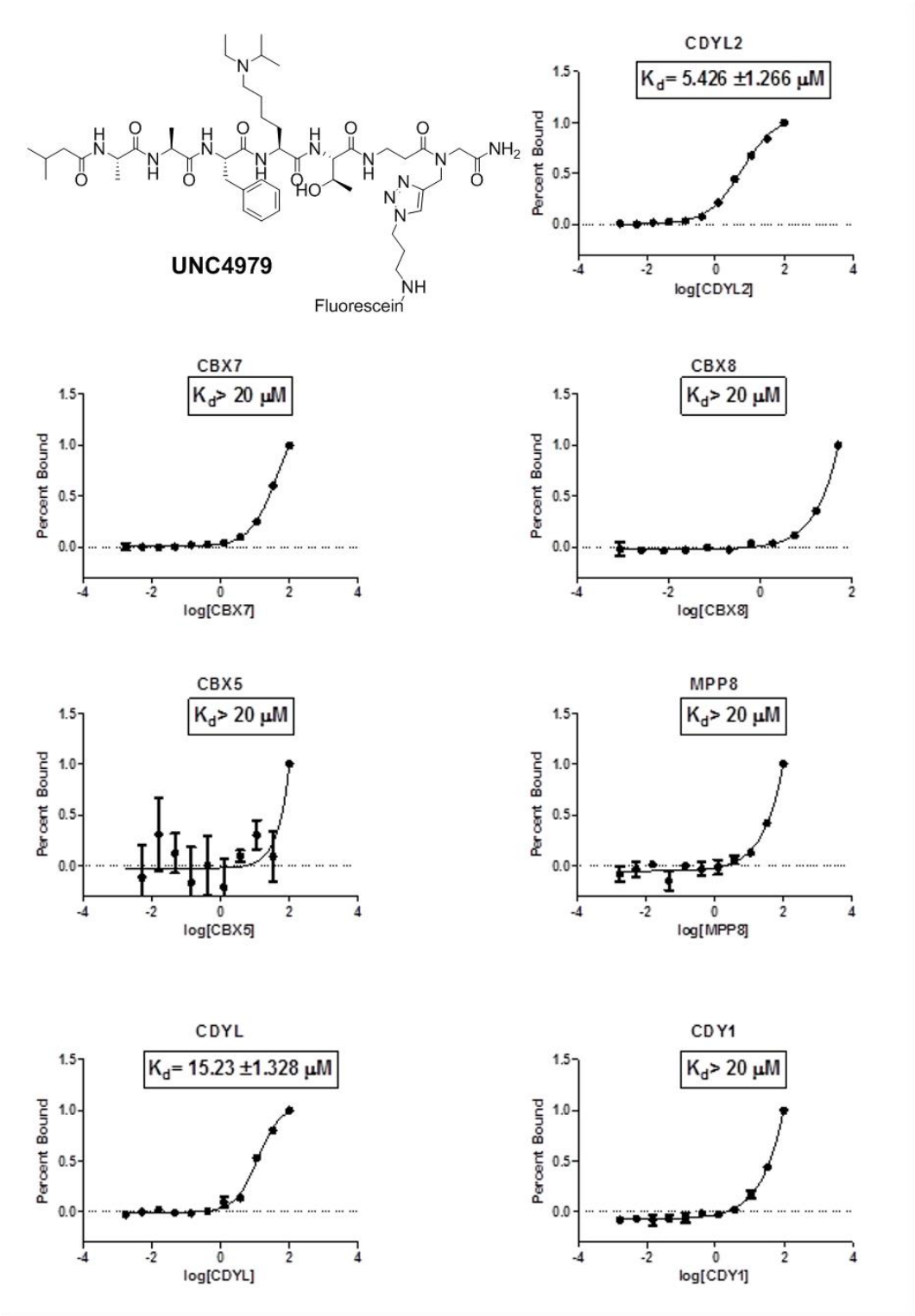

4700 MS/MS Precursor 1221.39 Spec #1[BP = 921.6, 12677]
Exact Mass: 1220.62
921.6047
992.6520
1120.7559
678.4471
544.4007
779.5134
443.3342
742.6136
850.5573
Thr
Lys(et,iPr)
Ser
Ala
pentafluorophenylalanine
84.1180
157.1733
230.1955
301.2374
372.2804
1009.6752
1134.6982
1177.7998
1221.8843
% Intensity
Mass (m/z)
9.0
265.6
522.2
778.8
1035.4
1292.0

Figure S3. Fluorescence Polarization Experiments. a) UNC3866-FAM was used as a positive control for the chromodomains of CBX7, CDYL2, MPP8, CBX8, CDYL, and CDY1. b-e) FP curves for UNC4979, UNC4980, UNC4981, and UNC4982 (see Figure 4b for additional results). All experiments were conducted in triplicate and the K_d is reported as the average.

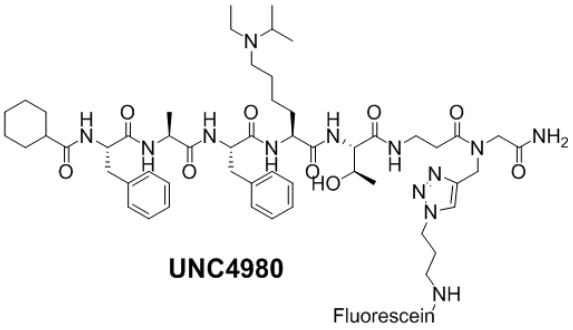
a)

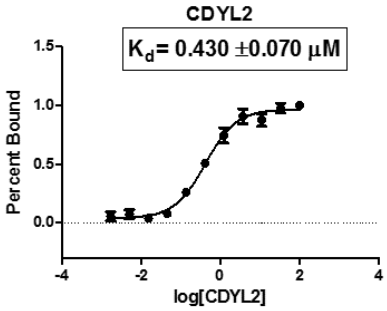


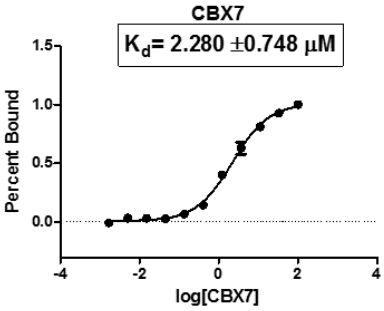
b)

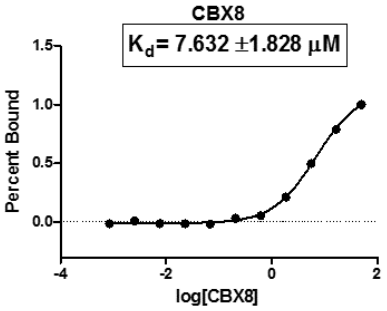

UNC4979
Fluorescein
CDYL2
K_d= 5.426 ±1.266 µM
log[CDYL2]
CBX7
K_d> 20 µM
log[CBX7]
CBX8
K_d> 20 µM
log[CBX8]
CBX5
K_d> 20 µM
log[CBX5]
MPP8
K_d> 20 µM
log[MPP8]
CDYL
K_d= 15.23 ±1.328 µM
log[CDYL]
CDY1
K_d> 20 µM
log[CDY1]
Percent Bound

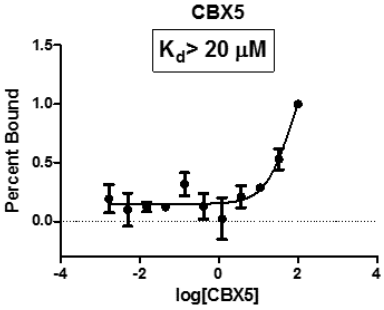
c)

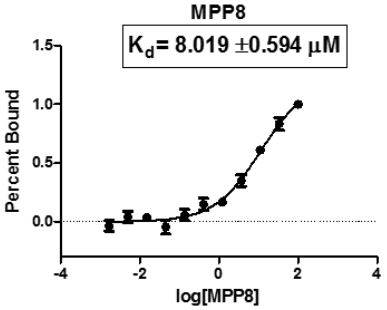

UNC4980
Fluorescein

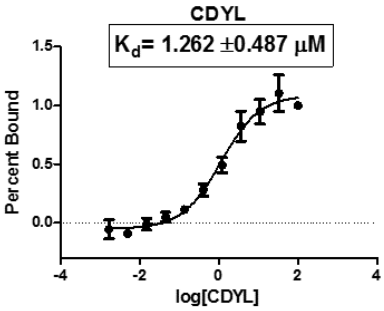

CDYL2
K_d= 0.430 ±0.070 μM
Percent Bound
log[CDYL2]

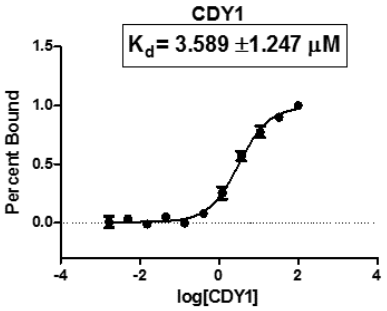

CBX7
K_d= 2.280 ±0.748 μM
Percent Bound
log[CBX7]


CBX8
K_d= 7.632 ±1.828 μM
Percent Bound
log[CBX8]

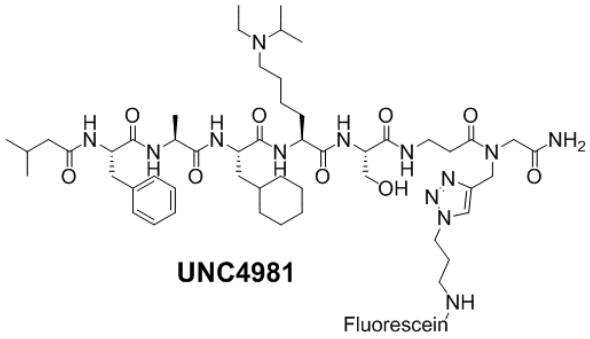

CBX5
K_d> 20 μM
Percent Bound
log[CBX5]

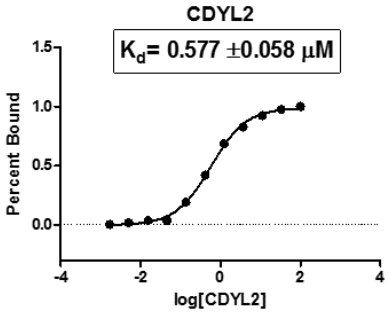

MPP8
K_d= 8.019 ±0.594 μM
Percent Bound
log[MPP8]

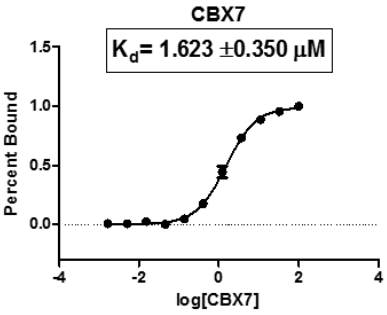

CDYL
K_d= 1.262 ±0.487 μM
Percent Bound
log[CDYL]

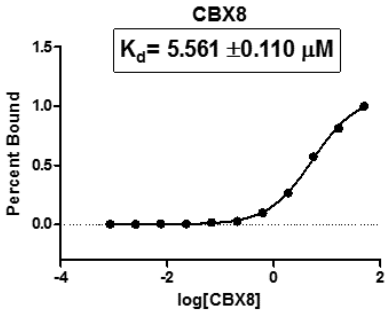

CDY1
K_d= 3.589 ±1.247 μM
Percent Bound
log[CDY1]

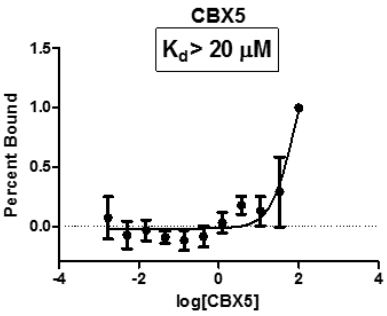
d)

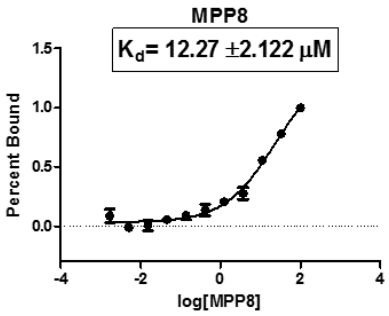

UNC4981
Fluorescein

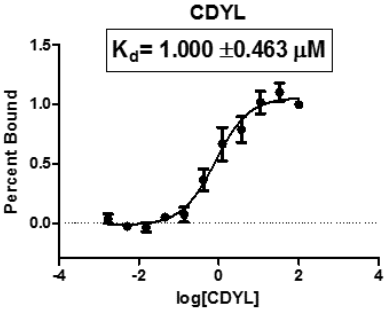

CDYL2
K_d= 0.577 ±0.058 μM
Percent Bound
log[CDYL2]

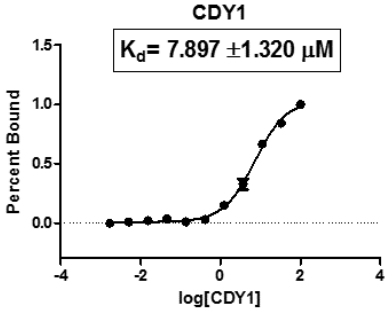

CBX7
K_d= 1.623 ±0.350 μM
Percent Bound
log[CBX7]


CBX8
K_d= 5.561 ±0.110 μM
Percent Bound
log[CBX8]

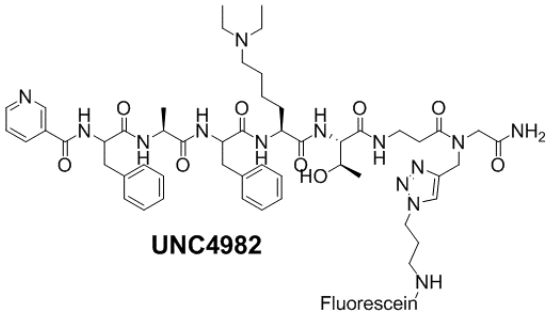

CBX5
K_d> 20 μM
Percent Bound
log[CBX5]

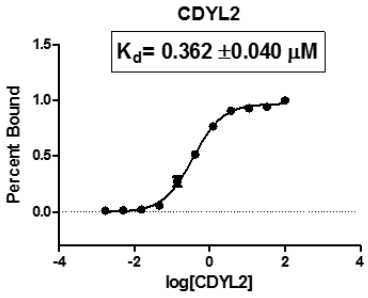

MPP8
K_d= 12.27 ±2.122 μM
Percent Bound
log[MPP8]

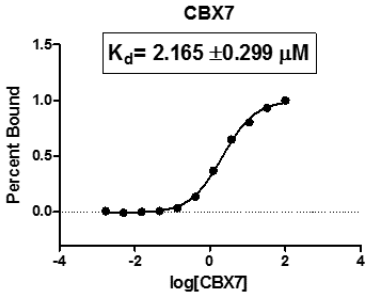

CDYL
K_d= 1.000 ±0.463 μM
Percent Bound
log[CDYL]

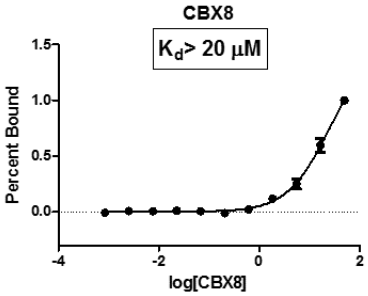

CDY1
K_d= 7.897 ±1.320 μM
Percent Bound
log[CDY1]

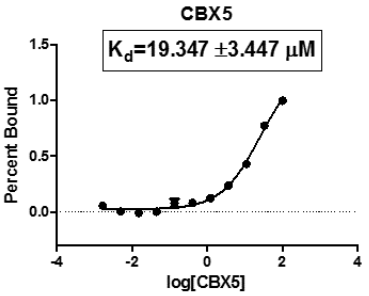
e)

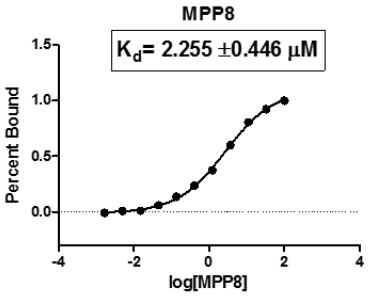

UNC4982
Fluorescein

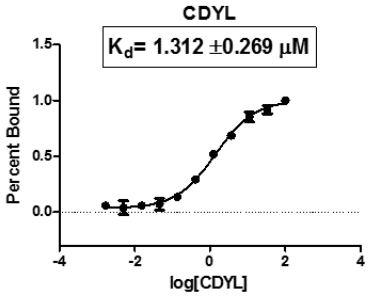

CDYL2
K_d= 0.362 ±0.040 μM
Percent Bound
log[CDYL2]


CBX7
K_d= 2.165 ±0.299 μM
Percent Bound
log[CBX7]


CBX8
K_d> 20 μM
Percent Bound
log[CBX8]


CBX5
K_d=19.347 ±3.447 μM
Percent Bound
log[CBX5]


MPP8
K_d= 2.255 ±0.446 μM
Percent Bound
log[MPP8]


CDYL
K_d= 1.312 ±0.269 μM
Percent Bound
log[CDYL]

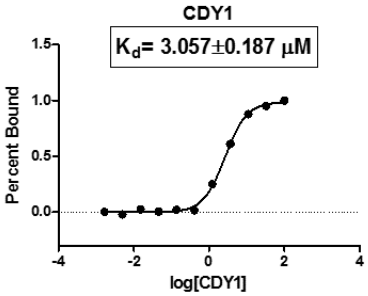
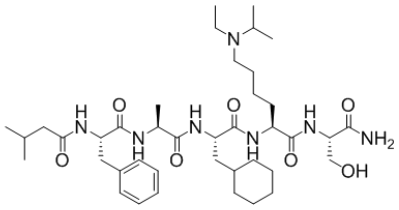
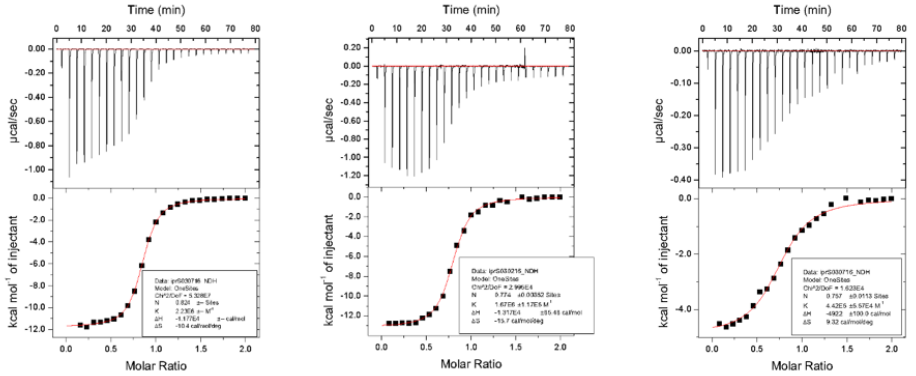

CDY1
K_d= 3.057±0.187 μM
Percent Bound
log[CDY1]

Figure S4. Isothermal Titration Calorimetry Experiments. a) UNC4990 and b) UNC4991 were run against CDYL2, CDYL, and CBX7 in duplicate and the K_d is reported as the average.

a)



UNC4990

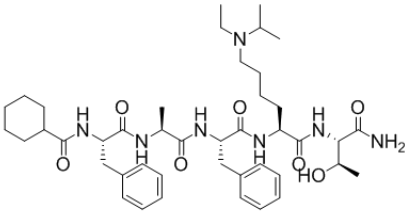


CDYL2 K_d= 0.574± 0.126 µM

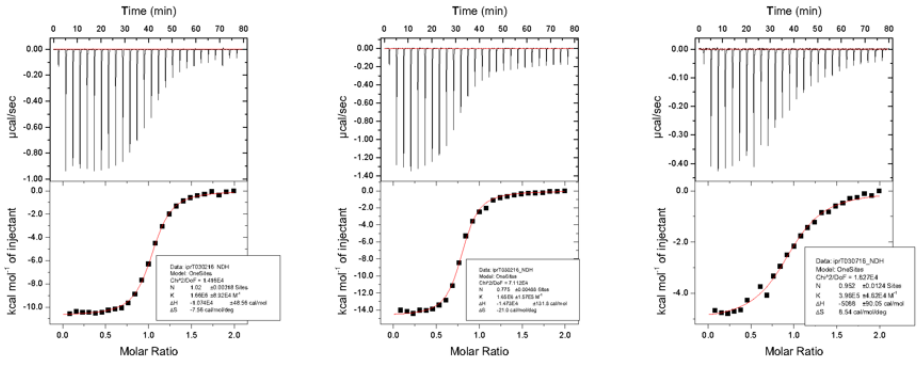
CDYL K_d= 0.659± 0.060 µM

CBX7 K_d= 2.380± 0.120 µM

b)



UNC4991



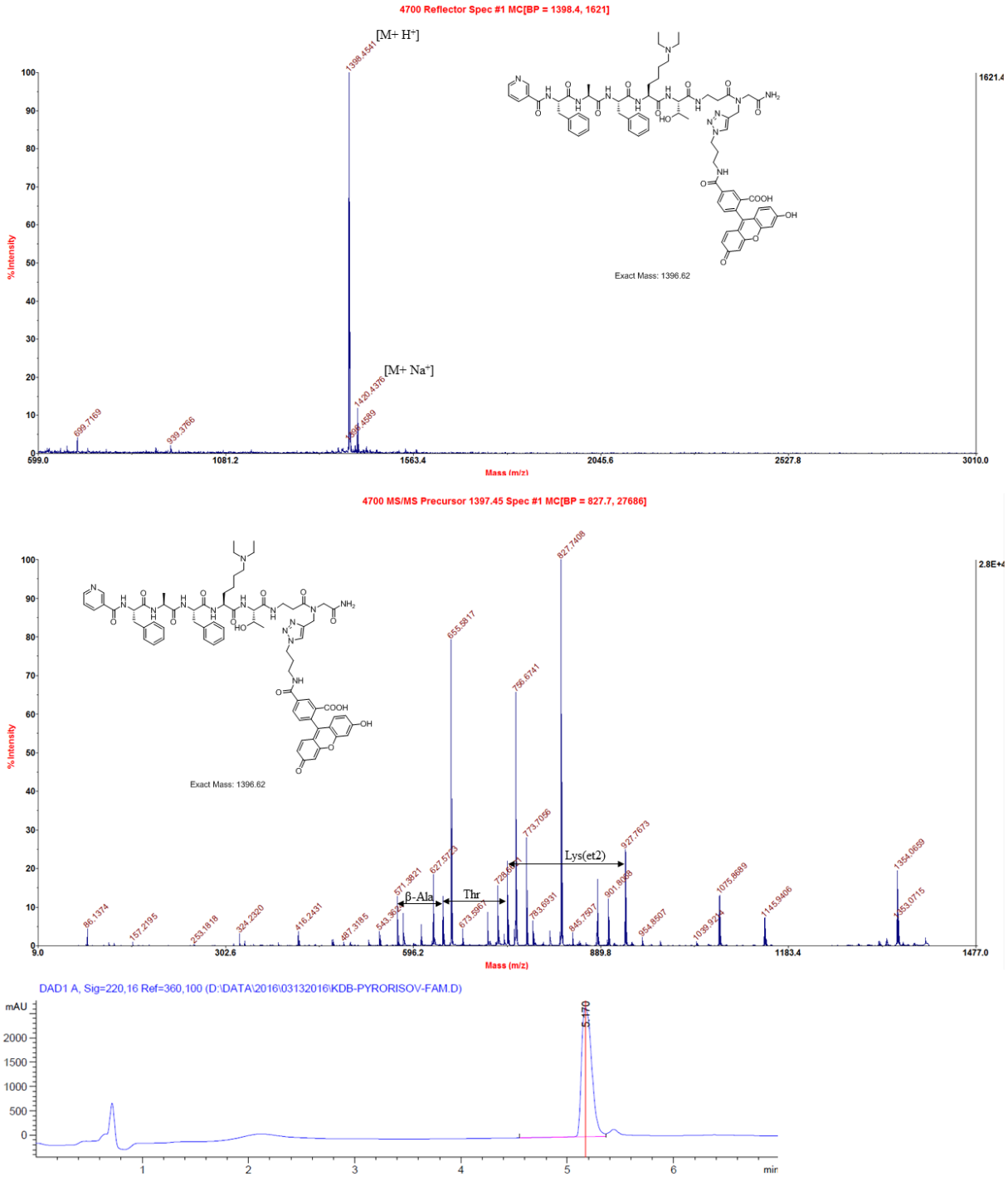
CDYL2 K_d= 0.637± 0.035 µM

CDYL K_d= 0.490± 0.117 µM

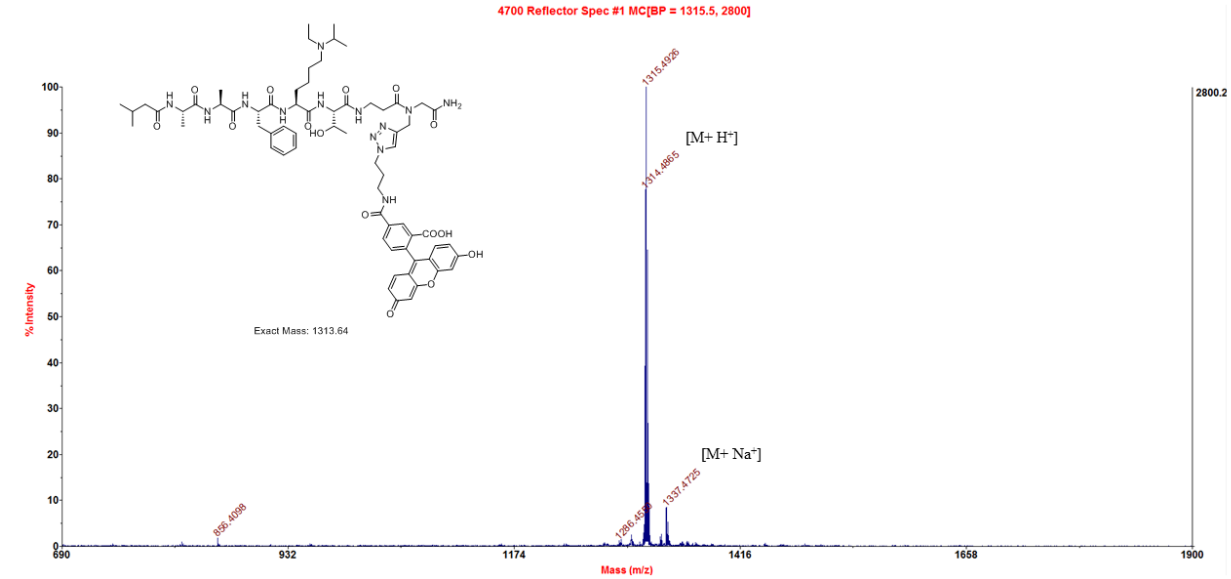
CBX7 K_d= 3.165± 0.635 µM

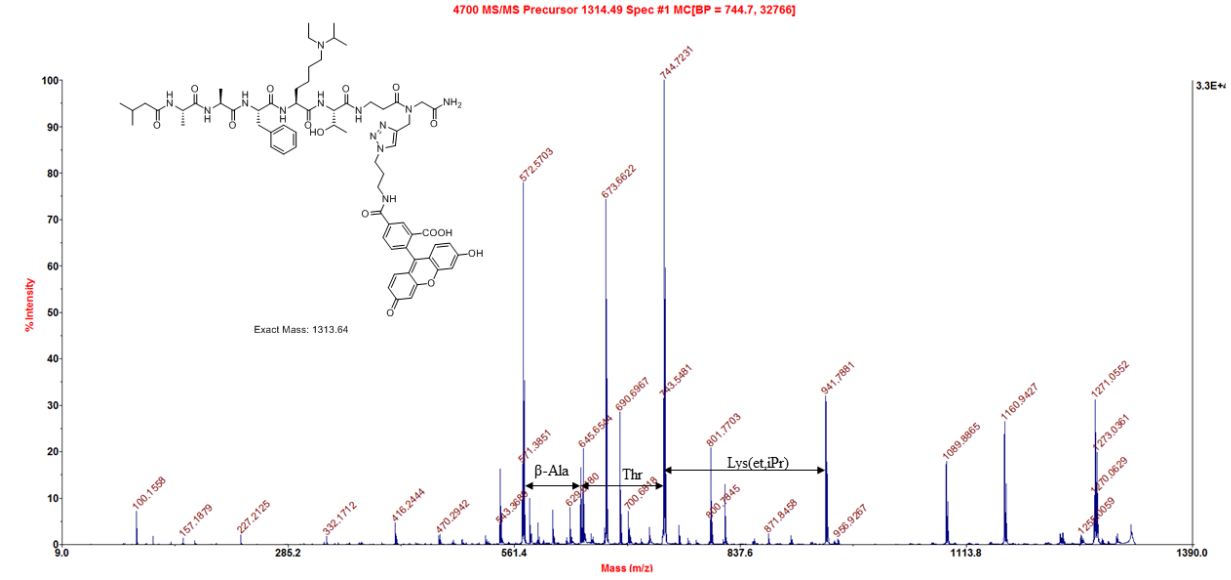
Figure S5. Compound characterization of C-terminally labeled peptides. Purity was determined to be > 95% by MALDI-TOF, MALDI-TOF/TOF, and LC analysis. a-d) FAM-labeled peptides and e) biotin-labeled UNC4991.

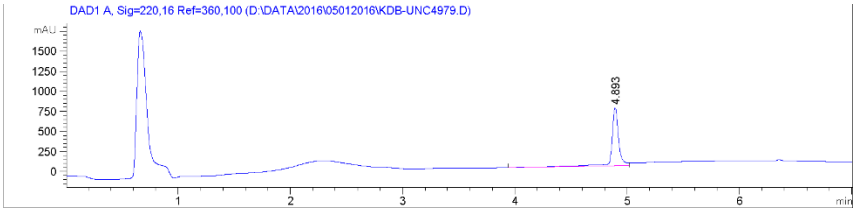
a)



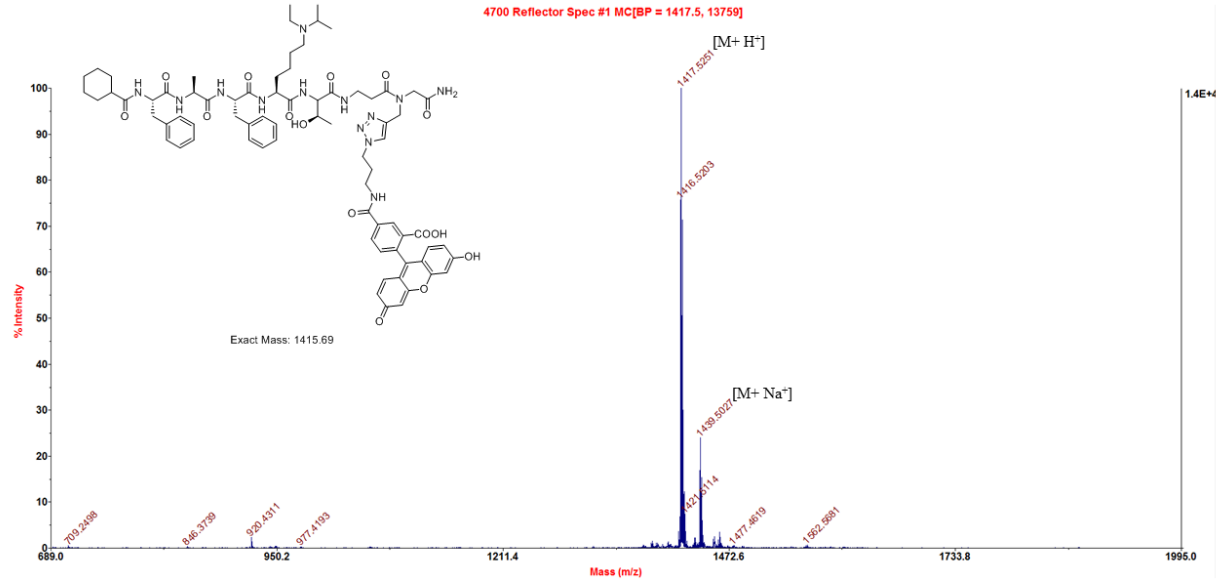
b)

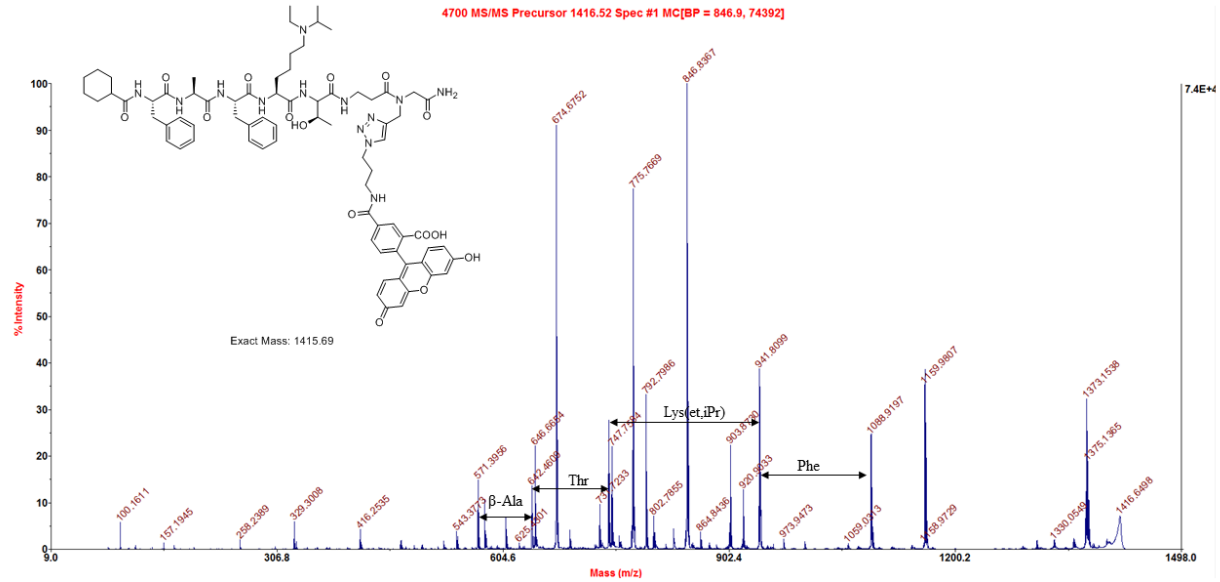

4700 Reflector Spec #1 MC[BP = 1315.5, 2800]
% Intensity
Mass (m/z)
Exact Mass: 1313.64
[M+ H+]
[M+ Na+]
1315.4926
1314.4865
1337.4725
856.4098
690
932
1174
1416
1658
1900
2800.2


4700 MS/MS Precursor 1314.49 Spec #1 MC[BP = 744.7, 32766]
% Intensity
Mass (m/z)
Exact Mass: 1313.64
β-Ala
Thr
Lys(et,iPr)
744.7231
572.5703
673.6622
9.0
285.2
561.4
837.6
1113.8
1390.0


DAD1 A, Sig=220,16 Ref=360,100 (D:\DATA\2016\05012016\KDB-UNC4979.D)
mAU
4.893
min

c)


4700 Reflector Spec #1 MC[BP = 1417.5, 13759]
[M+ H+]
[M+ Na+]
Exact Mass: 1415.69
% Intensity
Mass (m/z)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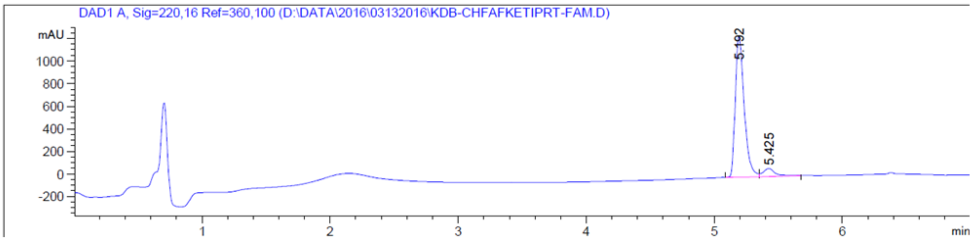
β-Ala
Thr
Lys(et,iPr)
Phe
% Intensity
Mass (m/z)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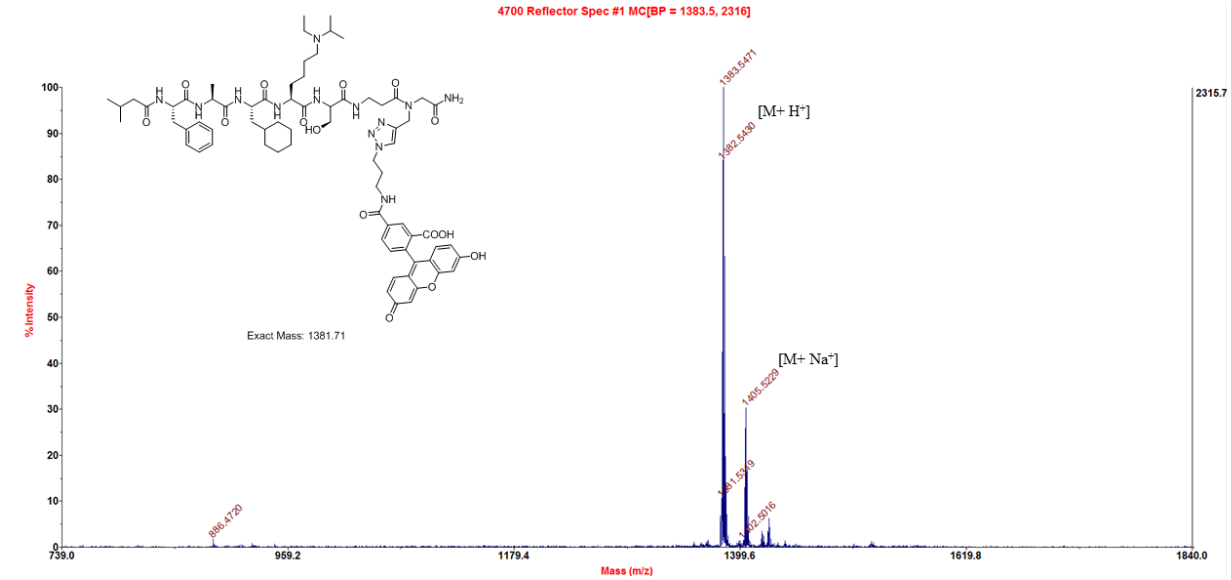
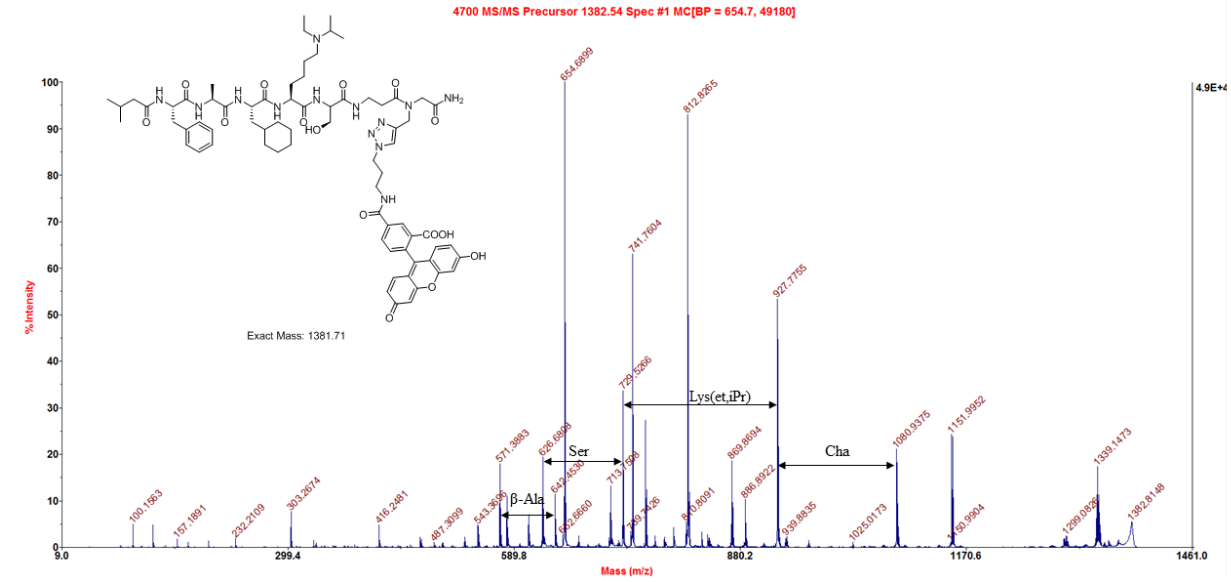
mAU
min
5.192
5.425

d)


4700 Reflector Spec #1 MC[BP = 1383.5, 2316]
[M+ H+]
[M+ Na+]
Exact Mass: 1381.71
% Intensity
Mass (m/z)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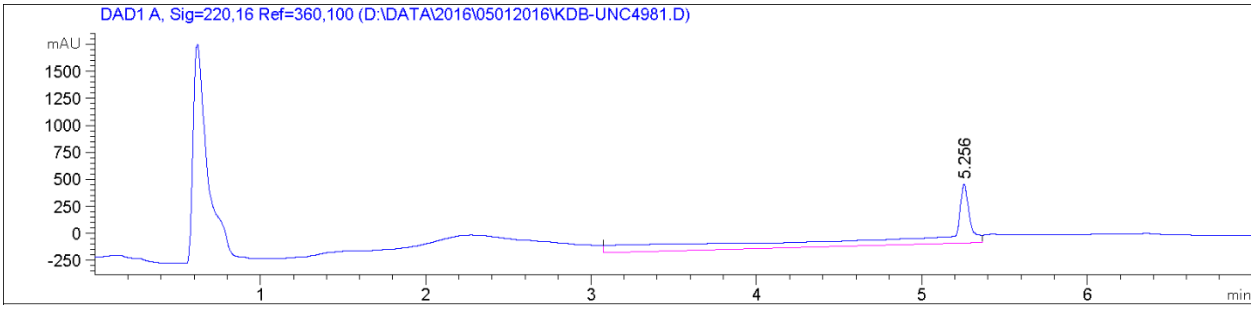
Lys(et,iPr)
Ser
β-Ala
Cha
% Intensity
Mass (m/z)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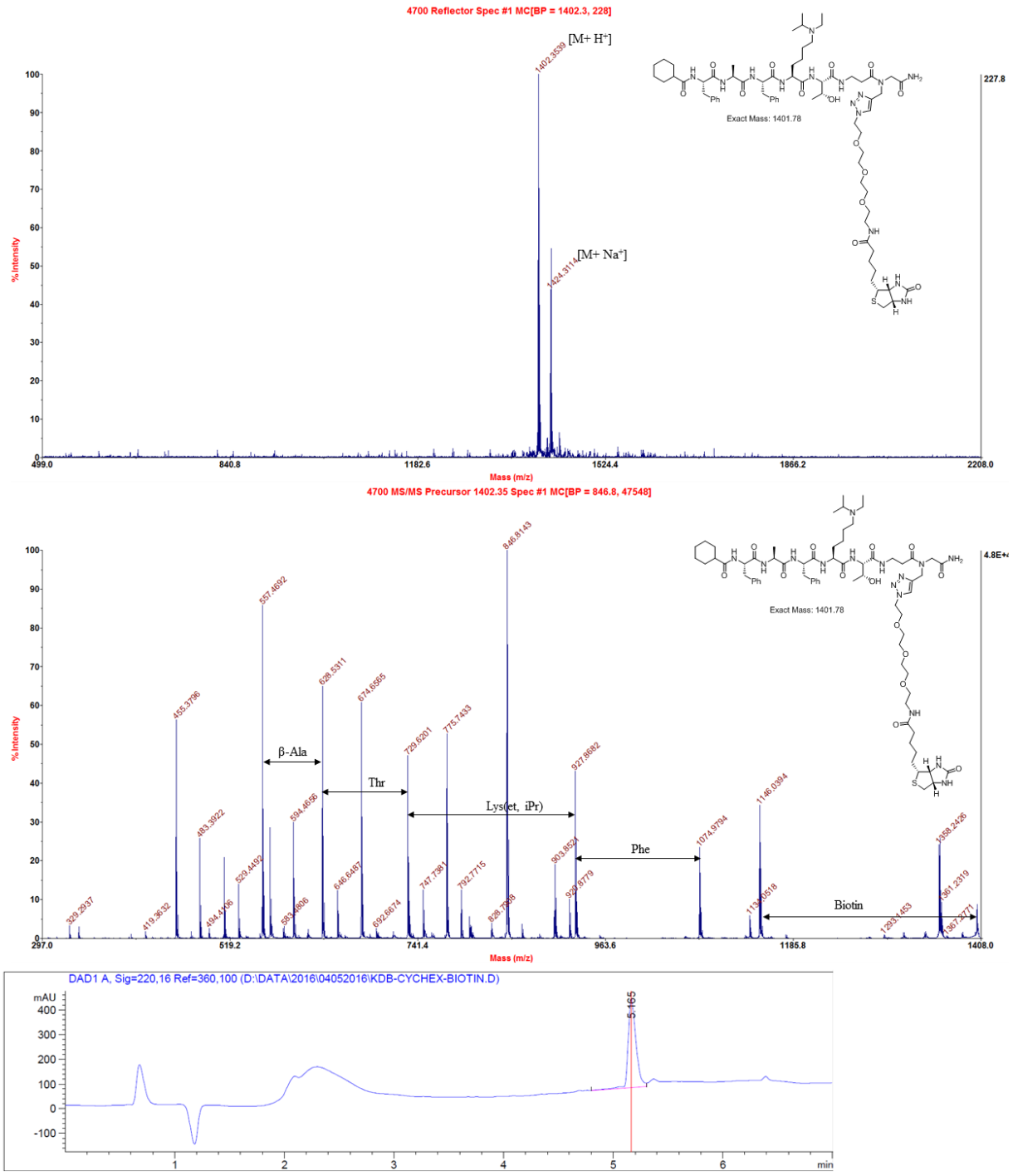
min

e)



Synthesis of Fmoc Lysine Derivatives

N2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N6,N6-dimethyl-L-lysine

Fmoc-Lys-OH (0.5 g, 1 eq, 1.36 mmol) was dissolved in ethanol and stirred at room temperature. Formaldehyde (30% w/v solution in water; 0.24 mL, 10 eq, 2.43 mmol) was added to the cloudy solution and the mixture was left to stir for 5 minutes followed by addition of acetic acid (0.23 mL, 3 eq, 4.08 mmol). Sodium cyanoborohydride (0.34 g, 4 eq, 5.44 mmol) was then added to the reaction. The mixture was stirred at room temperature for 2 hours. Next, the reaction was concentrated *in vacuo*. Purification via reverse phase flash chromatography (H_2O + 0.1 trifluoroacetic acid and acetonitrile) yielded the product. The product was lyophilized to a hygroscopic, white powder and yielded 0.46 g (66%) of product. ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.77 (d, J = 7.5 Hz, 2H), 7.65 (t, J = 7.5 Hz, 2H), 7.37 (t, J = 7.8 Hz, 2H), 7.29 (t, J = 7.4 Hz, 2H), 4.43 – 4.27 (m, 2H), 4.23 – 4.13 (m, 2H), 3.09 – 3.01 (m, 2H), 2.82 (s, 6H), 2.00 – 1.82 (m, 1H), 1.80 – 1.64 (m, 3H), 1.51 – 1.37 (m, 2H).

N2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N6,N6-diethyl-L-lysine

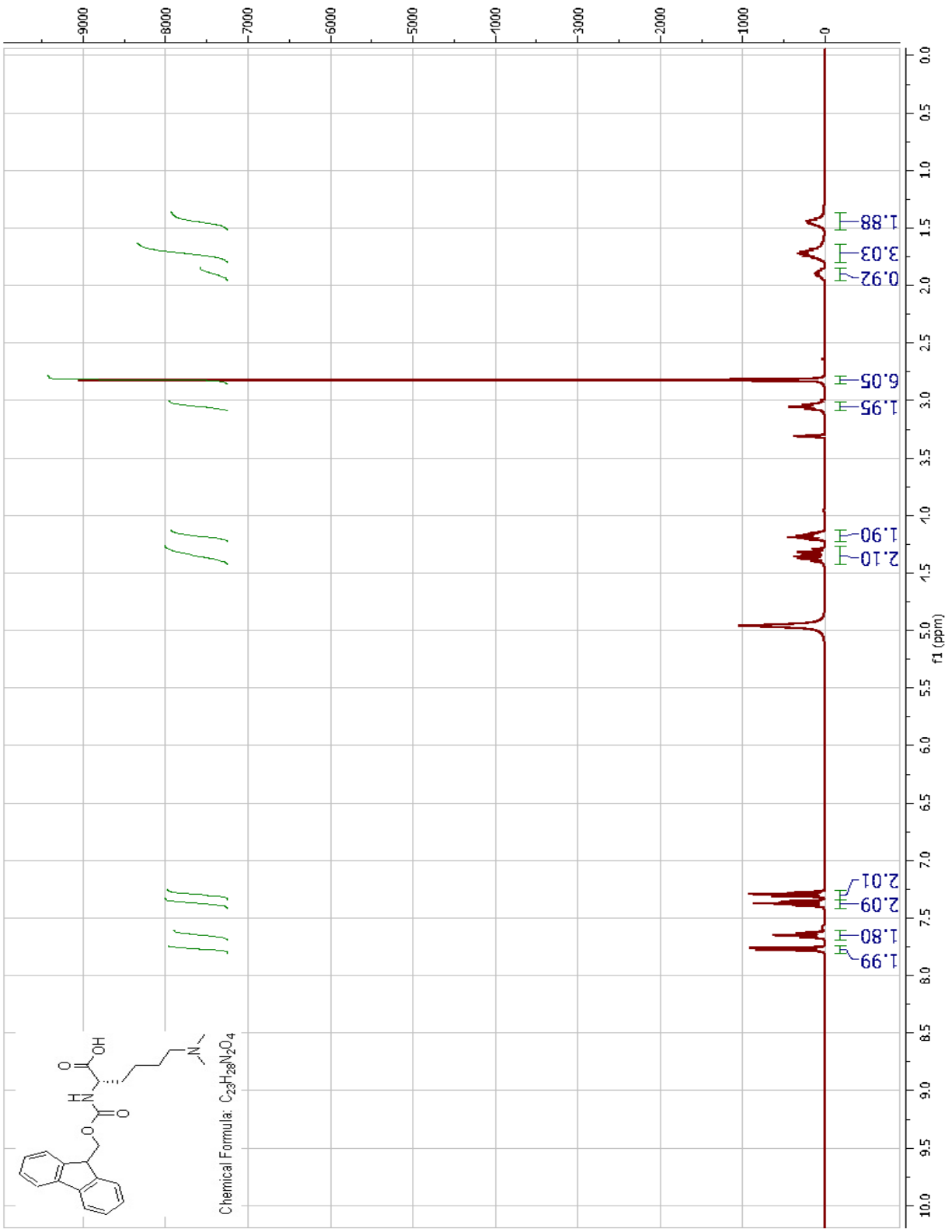
Fmoc-Lys-OH (0.5 g, 1 eq, 1.36 mmol) was dissolved in ethanol and stirred at room temperature. Acetaldehyde (0.61 mL, 8 eq, 10.88 mmol) was added to the cloudy solution and the mixture was left to stir for 5 minutes followed by addition of acetic acid (0.23 mL, 3 eq, 4.08 mmol). Sodium cyanoborohydride (0.34 g, 4 eq, 5.44 mmol) was then added to the reaction. The mixture was stirred at room temperature for 2 hours. Next, the reaction was concentrated *in vacuo*. Purification via reverse phase flash chromatography (H_2O + 0.1 trifluoroacetic acid and acetonitrile) yielded the product. The product was lyophilized to a hygroscopic, white powder and yielded 0.53 g (72%) of product. ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.80 (d, J = 7.5 Hz, 2H), 7.67 (t, J = 9.1 Hz, 2H), 7.39 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.4 Hz, 2H), 4.43 – 4.29 (m, 2H), 4.25 – 4.19 (m, 1H), 4.17 – 4.06 (m, 1H), 3.24 – 3.15 (m, 4H), 3.12 – 3.01 (m, 2H), 1.95 – 1.84 (m, 1H), 1.81 – 1.64 (m, 3H), 1.46 (p, J = 7.6 Hz, 2H), 1.28 (t, J = 7.4 Hz, 6H).

(S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-(diethylamino)pentanoic acid

Fmoc-Orn-OH (0.15 g, 1 eq, 0.423 mmol) was dissolved in ethanol and stirred at room temperature. Acetaldehyde (0.11 mL, 8 eq, 1.94 mmol) was added to the cloudy solution and the mixture was left to stir for 5 minutes followed by addition of acetic acid (0.04 mL, 3 eq, 0.73 mmol). Sodium cyanoborohydride (0.61 g, 4 eq, 0.97 mmol) was then added to the reaction. The mixture was stirred at room temperature for 2 hours. Next, the reaction was concentrated *in vacuo*. Purification via reverse phase flash chromatography (H_2O + 0.1 trifluoroacetic acid and acetonitrile) yielded the product. The product was lyophilized to a hygroscopic, white powder and yielded 0.11 g (90%) of product. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.81 (d, J = 7.5 Hz, 2H), 7.67 (t, J = 8.7 Hz, 2H), 7.40 (t, J = 7.3 Hz, 2H), 7.31 (t, J = 7.5, 1.2 Hz, 2H), 4.47 – 4.40 (m, 1H), 4.38 – 4.31 (m, 1H), 4.26 – 4.20 (m, 2H), 3.23 – 3.12 (m, 6H), 2.02 – 1.91 (m, 1H), 1.85 – 1.72 (m, 3H), 1.29 (t, J = 7.3 Hz, 6H).

N2-(((9H-fluoren-9-yl)methoxy)carbonyl)-N6-ethyl-N6-isopropyl-L-lysine

Fmoc-Lys-OH (1.0 g, 1 eq, 2.71 mmol) was dissolved in ethanol (35 mL) and stirred at room temperature. Acetone (15 mL) was added to the cloudy solution and the mixture was left to stir for 15 minutes followed by addition of acetic acid (0.46 mL, 3 eq, 8.13 mmol). Sodium cyanoborohydride (0.68 g, 4 eq, 10.84 mmol) was then added to the reaction. The mixture was stirred at room temperature overnight. Acetaldehyde (0.61 mL, 4 eq, 10.88 mmol) was added and the reaction was left to stir for 1 hour. Next, the reaction was concentrated *in vacuo*. Purification via reverse phase flash chromatography (H_2O + 0.1 trifluoroacetic acid and acetonitrile) yielded the product. The product was lyophilized to a hygroscopic, white powder and yielded 1.18 g (79%) of product. ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.80 (d, J = 7.5 Hz, 2H), 7.67 (t, J = 8.6 Hz, 2H), 7.39 (t, J = 7.6 Hz, 2H), 7.31 (td, J = 7.5, 1.2 Hz, 2H), 4.44 – 4.38 (m, 1H), 4.36 – 4.29 (m, 1H), 4.26 – 4.16 (m, 2H), 3.67 (p, J = 6.9 Hz, 1H), 3.27 – 2.94 (m, 4H), 1.98 – 1.88 (m, 1H), 1.82 – 1.63 (m, 3H), 1.55 – 1.44 (m, 2H), 1.35 – 1.29 (m, 9H).


1.88
3.03
0.92
6.05
1.95
1.90
2.10
2.01
2.09
1.80
1.99
f1 (ppm)
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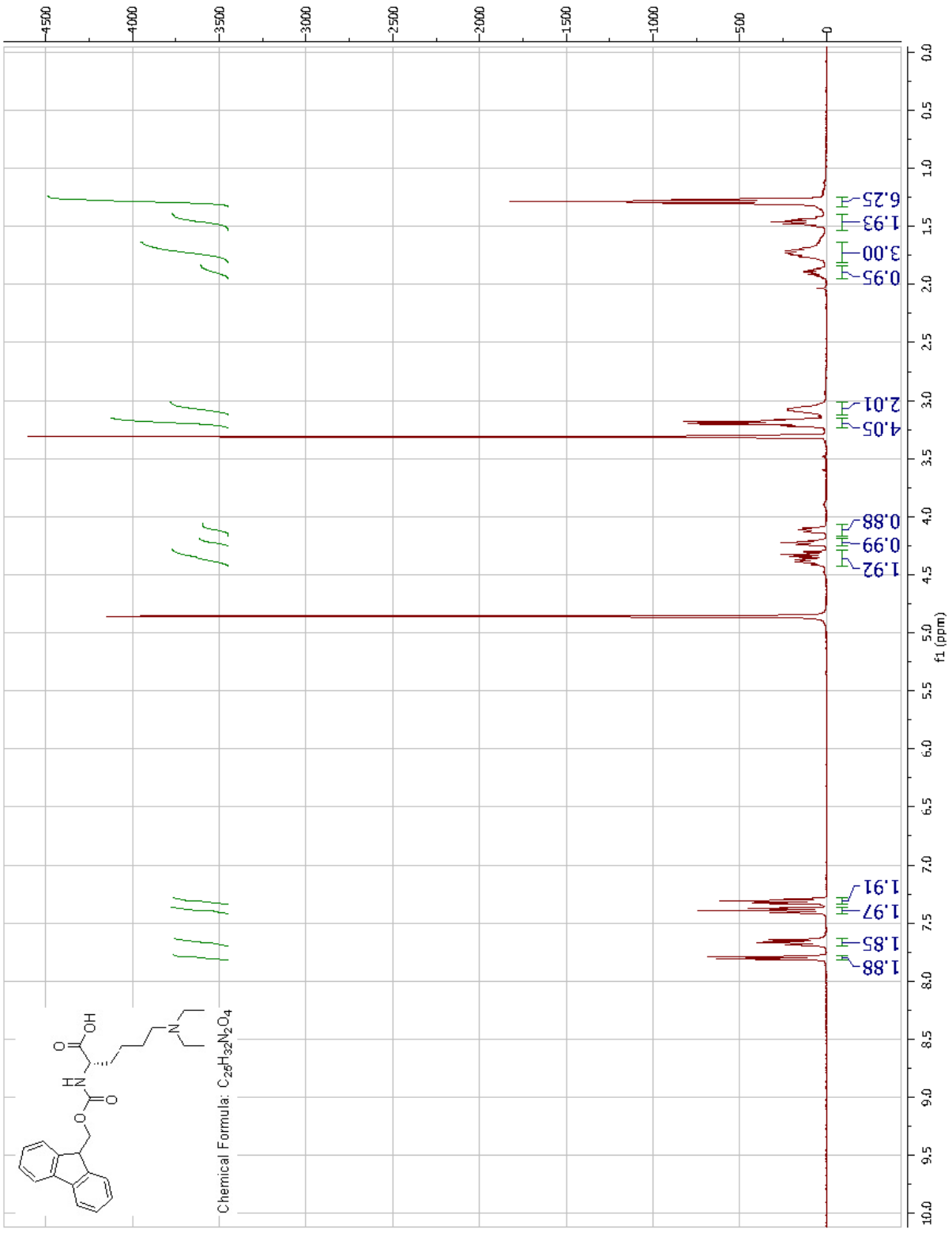
6.25
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2.01
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1.91
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1.88
f1 (ppm)
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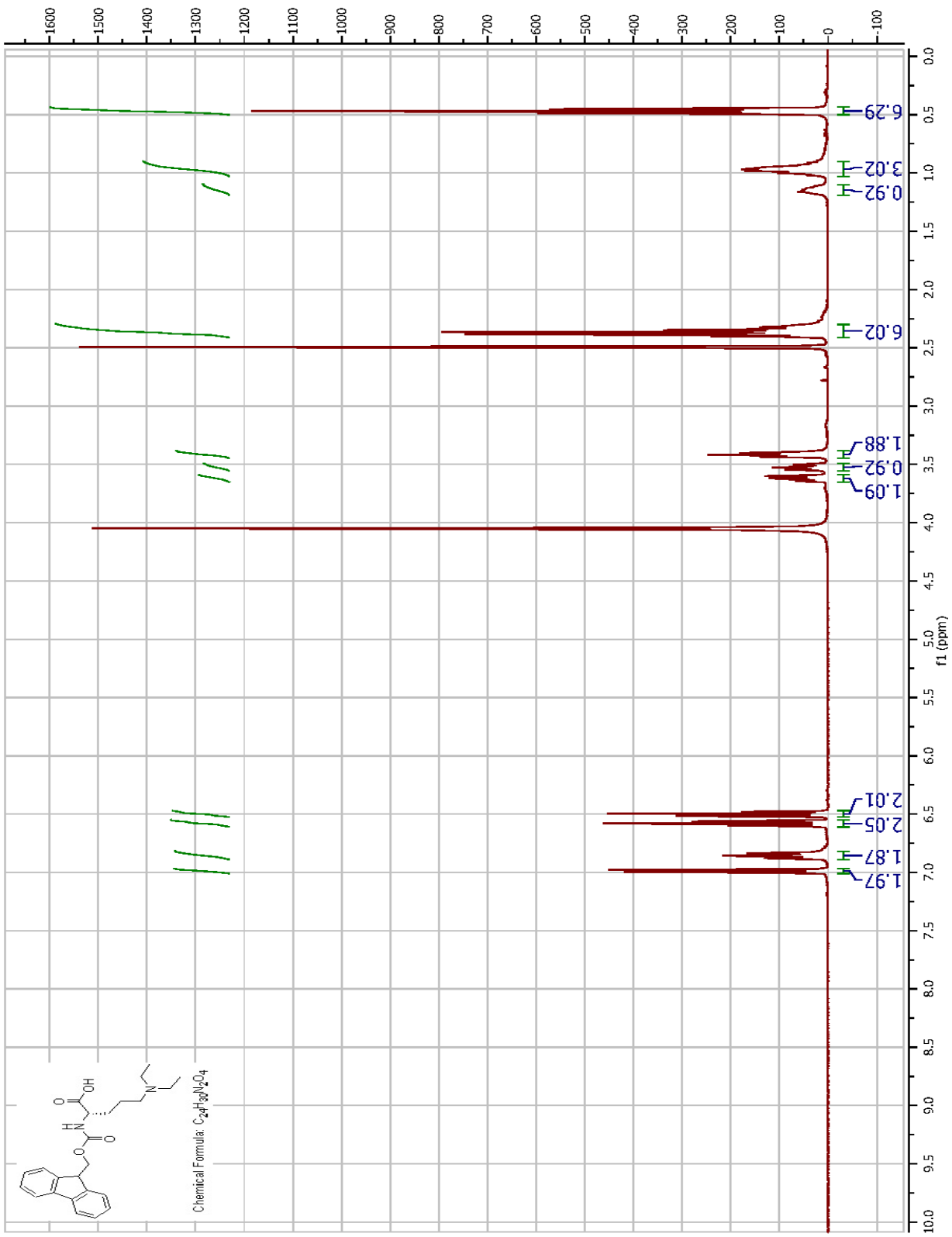
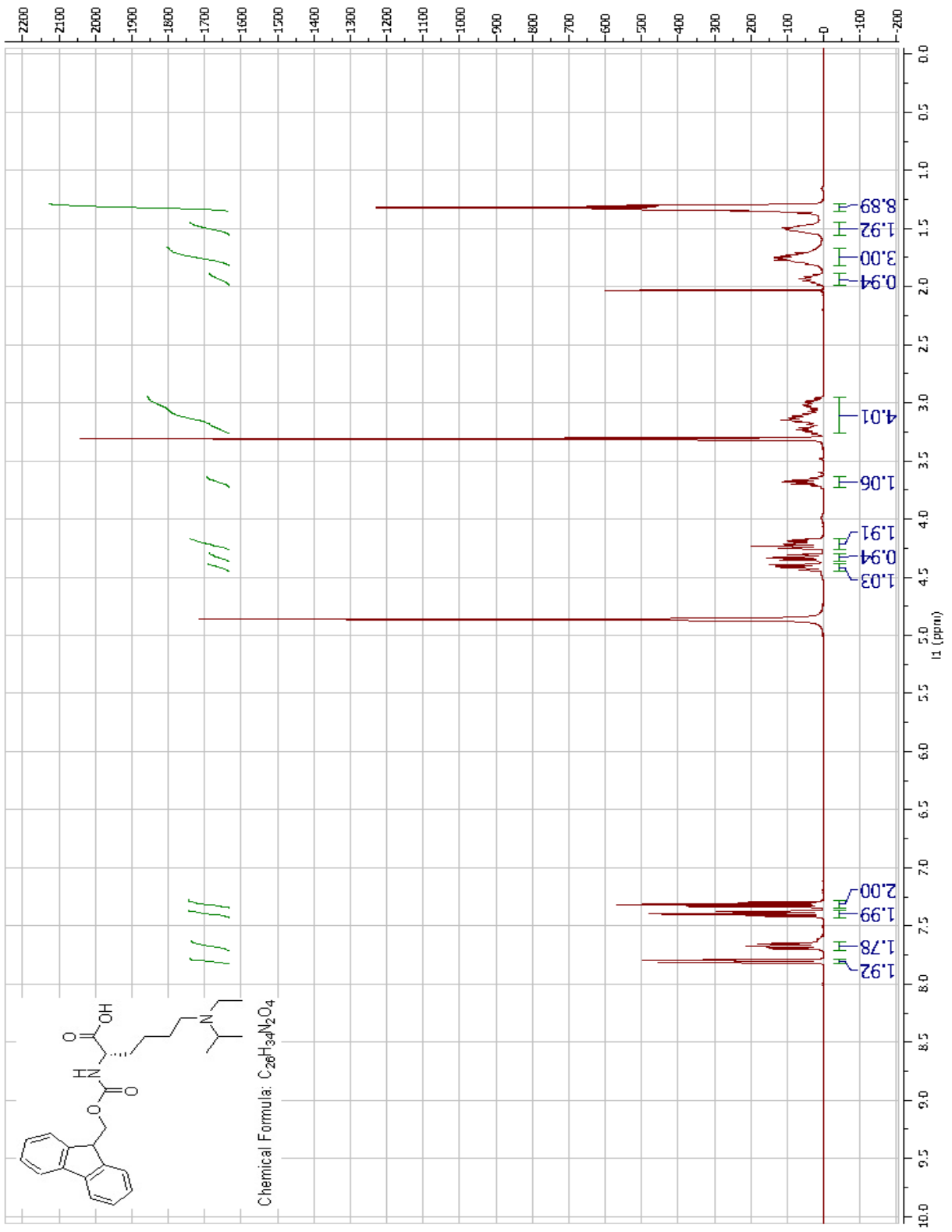

6.29
3.02
0.92
6.02
1.88
0.92
1.09
2.01
2.05
1.87
1.97
f1 (ppm)
Chemical Formula: C24H30N2O4


Chemical Formula: $C_{26}H_{34}N_2O_4$
8.89
1.92
3.00
0.94
4.01
1.06
1.03
0.94
1.91
2.00
1.99
1.78
1.92
f1 (ppm)

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2. Gao, Y.; Kodadek, T., Synthesis and Screening of Stereochemically Diverse Combinatorial Libraries of Peptide Tertiary Amides. *Chemistry & Biology* **2013,** *20* (3), 360-369.
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