Peptide Tethering: Pocket-Directed Fragment Screening for Peptidomimetic Inhibitor Discovery

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A. Supporting Figures and Tables

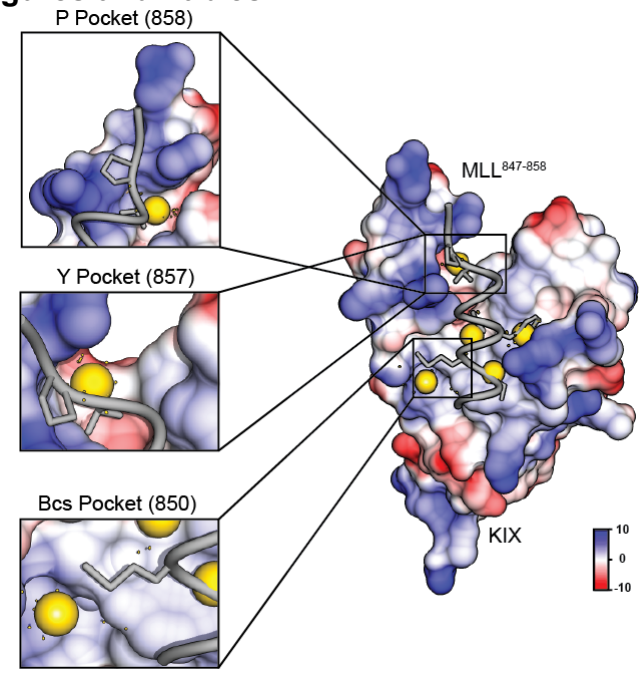


Figure S1: Electrostatic map of KIX highlighting the pockets screened. MLL$^{847-858}$ ribbon, docked to KIX, shown in electrostatic surface. Pockets screened are highlighted. The Bcs pocket is hydrophobic as well as the surrounding residues around the Y pocket, whereas the P pocket is surrounded by a positive patch on KIX. PDB 2AGH.

Table S1: Summary of binding constants for HBS derivatives. The MLL and unconstrained compounds (‡) are the same compounds tested and reported in [1]. Several mutations were made to identify **HBS I** as the best binder. Binding constants determined by a fluorescence polarization competition assay.

Compound	**Sequence**															**K_i (μM)**
MLL$^{847-858}$ ‡	Ac-	S	D	I	M	D	F	V	L	K	N	T	P	-NH_2		>1000‡
Peptide I‡	Ac-	S	D	I	Bcs	D	2meF	I	L	K	N	Y	P	-OH		22 ± 8‡
Peptide I-NH_2 ‡	Ac-	S	D	I	Bcs	D	2meF	I	L	K	N	Y	P	-NH_2		20 ± 26‡
HBS 0	X	S	D	G*	Bcs	D	2meF	I	L	K	N	Y	P	-NH_2		28 ± 10
HBS I	X	S	D	G*	Bcs	D	2meF	I	L	K	N	Y	P	W	-NH_2	13 ± 3

All single letter amino acid codes are used above in addition to the following abbreviations:
Ac = Acyl cap; X = Pentenoic Acid; Bcs = Benzylcysteine; 2meF = 2-methyl phenylalanine; G* = N-allyl glycine

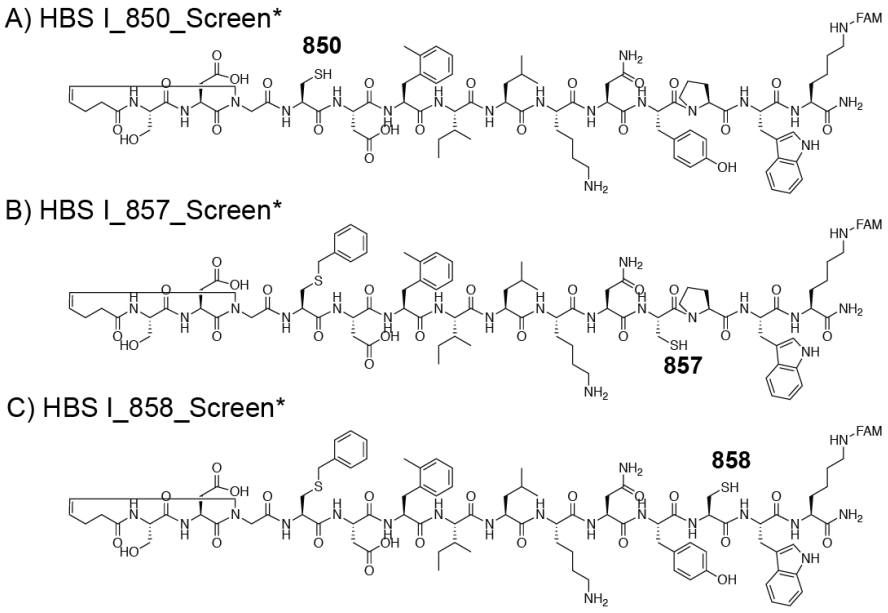


Figure S2: Peptides used for screening in this study: A) HBS I_850_Screen*to screen at the Bcs pocket, B) HBS I_857_Screen* to screen at the Y pocket, and C) HBS I_858_Screen* to screen at the P pocket.

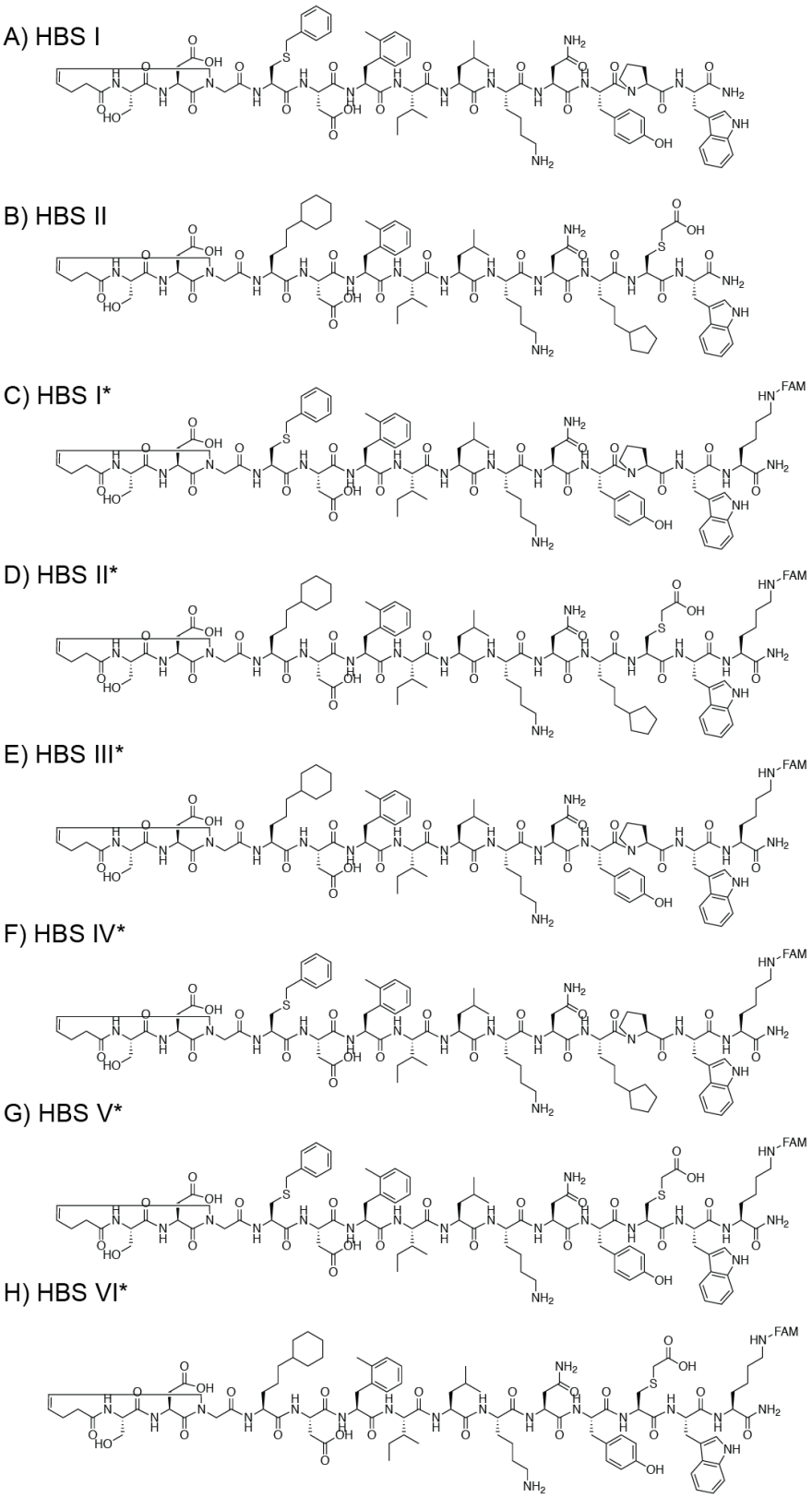


Figure S3: Structures of peptides used in this study. Baseline HBS (HBS I), optimized HBS (HBS II), and peptides containing fragment hits at one position (HBS III-V*), two positions (HBS VI*). * denotes fluorescently labeled.

Table S2: Summary of binding affinities with hits from the screen. Data is shown in Fig 5 in the manuscript. Binding Constants determined by FP.

Peptide	K_d (μM)
HBS I*	51 ± 18
HBS III*	17 ± 5
HBS IV *	13 ± 3
HBS V*	21 ± 5
HBS VI *	7.4 ± 1.4
HBS II*	2.0 ± 0.6

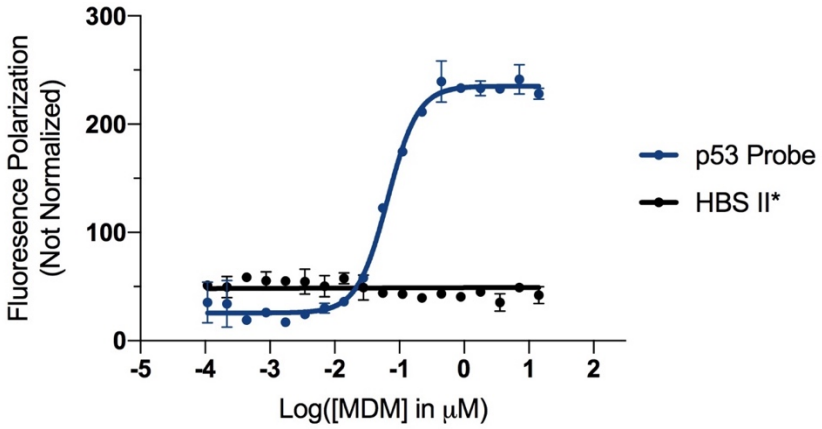


Figure S4: Investigating selectivity of the HBS II* peptide against MDM2. Fluorescence polarization direct binding studies with the p53 Probe from [2] (known binder to MDM2) and HBSII* fluorescently labeled peptide to MDM2. K_D p53 Probe for MDM2 is 47 ± 10 nM. K_D HBS II* for MDM2 is >> 15 μM.

A)

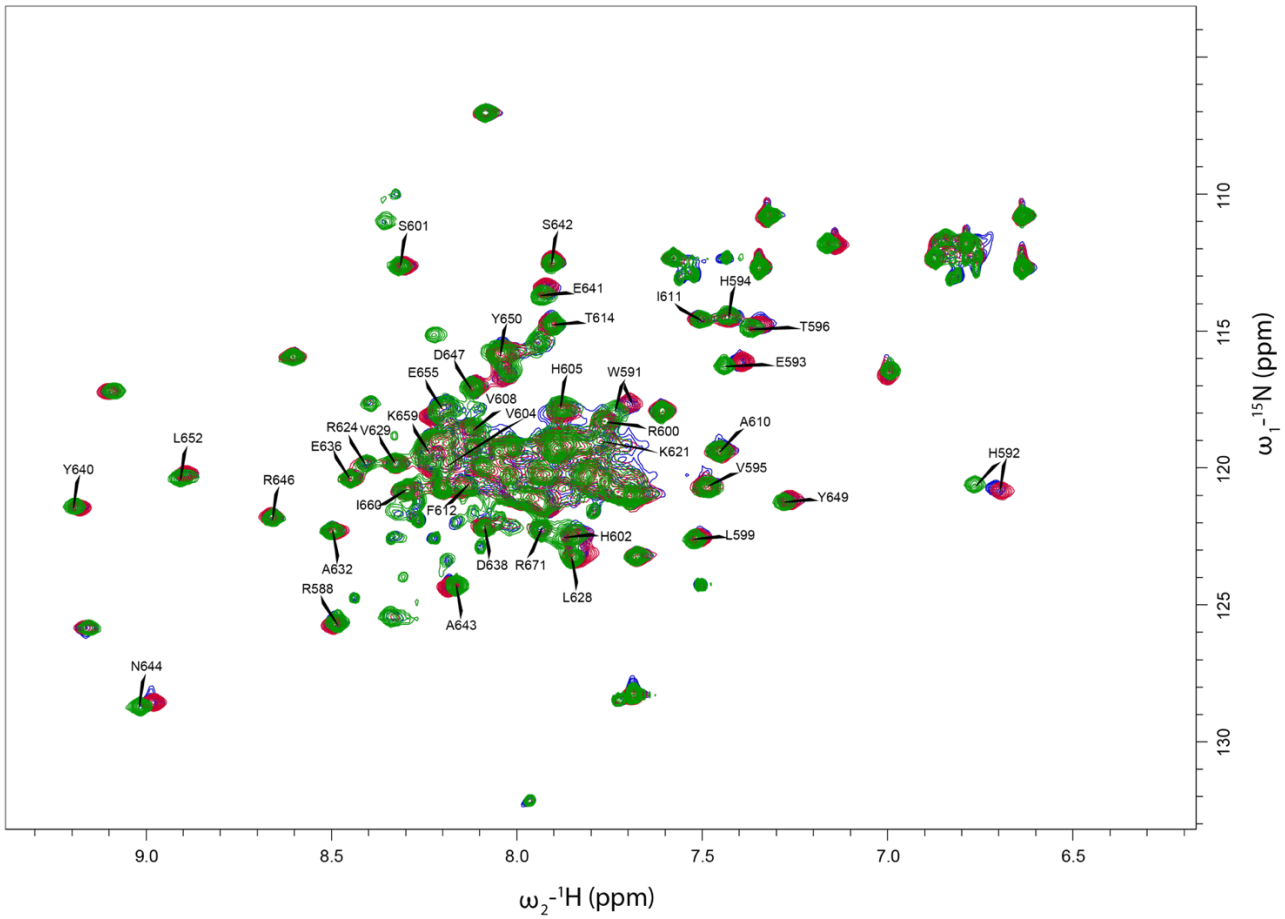
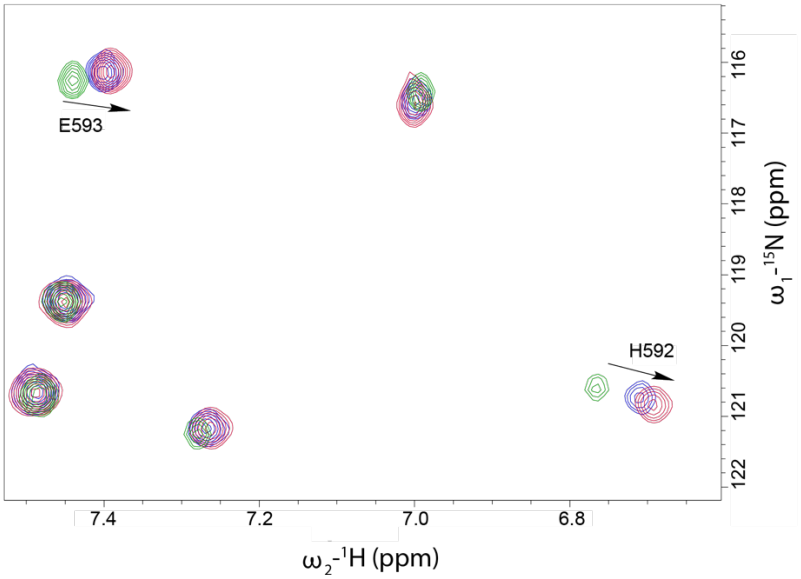


Figure S5: A) NMR shifts mapped on KIX in the presence of HBS II at 0, 0.5 and 1 molar equivalents.

B)



C)

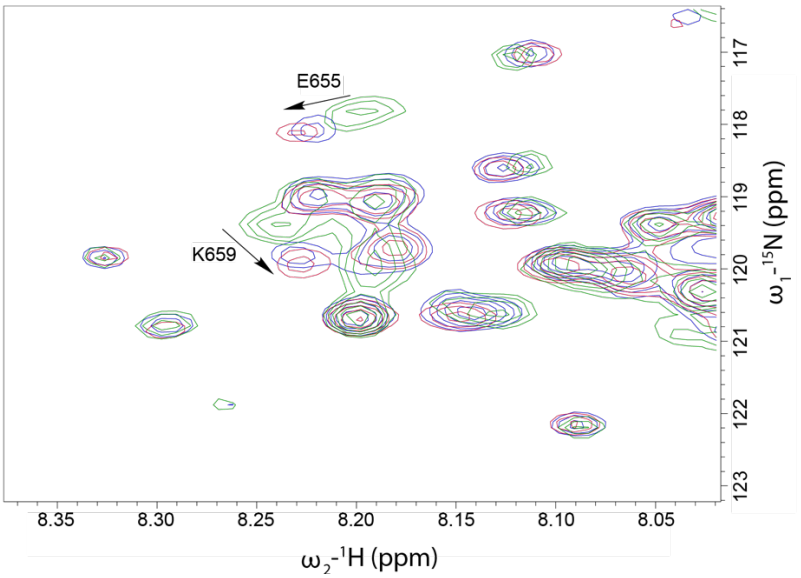


Figure S5. Cont. B) Unidirectional shifts of E593, H592, and C) E655 and K659 are shown. Color codes: Green = Apo, Blue = 0.5 molar eq., Red = 1 molar eq.

Table S3: Summary of NMR Shifts in the presence of HBS II.

Residue	Position	Shift
R	588	0.031
W	591	0.094
H	592	0.101
E	593	0.049
H	594	0.043
V	595	0.006
T	596	0.032
L	599	0.014
R	600	0.009
S	601	0.015
V	604	0.053
H	605	0.054
L	607	0.007
V	608	0.028
A	610	0.009
I	611	0.012
F	612	0.026
T	614	0.022
D	616	0.008
A	618	0.012
A	619	0.007
L	620	0.003
K	621	0.028
R	624	0.009
L	628	0.008
V	629	0.004
A	630	0.006
Y	631	0.009

Residue	Position	Shift
R	588	0.031
K	633	0.002
K	634	0.009
E	636	0.002
D	638	0.007
M	639	0.012
Y	640	0.026
E	641	0.076
S	642	0.005
A	643	0.029
N	644	0.047
R	646	0.009
D	647	0.008
Y	649	0.017
Y	650	0.012
H	651	0.019
L	652	0.038
L	653	0.005
A	654	0.018
E	655	0.071
I	657	0.013
K	659	0.155
I	660	0.027
Q	661	0.014
K	662	0.009
E	663	0.005
E	666	0.018
R	669	0.005

B. Materials

Commercial grade solvents and reagents were used without further purification. All reagents were purchased from ChemImpex, TCI, Sigma, Aldrich, Sigma-Aldrich, Alfa Aesar, VWR, Anaspec, ThermoScientific, Novabiochem, GyrosProtein Technologies, Combi-Blocks, Matrix Scientific, Enamine, and Acros. Bioexpress Media (10X) was purchased from Cambridge Isotopes.

C. Synthesis and Purification of Peptides

Solid-Phase Peptide Synthesis: High loading Rink Amide MBHA (0.80 mmol/g) was used for the HBS derivatives. Low loading Rink Amide MBHA (0.27 mmol/g) was used for peptides with C-terminal 5-carboxyfluoroscien additions. Standard Fmoc- synthesis was used and the resin was sufficiently washed in between steps with DMF, DCM, MeOH, DMF, DCM with 5 column volumes each. Synthesized amino acid monomers were coupled by hand using either 3 or 4 eq. of Amino Acid/HOAt/DIC in DMF overnight.

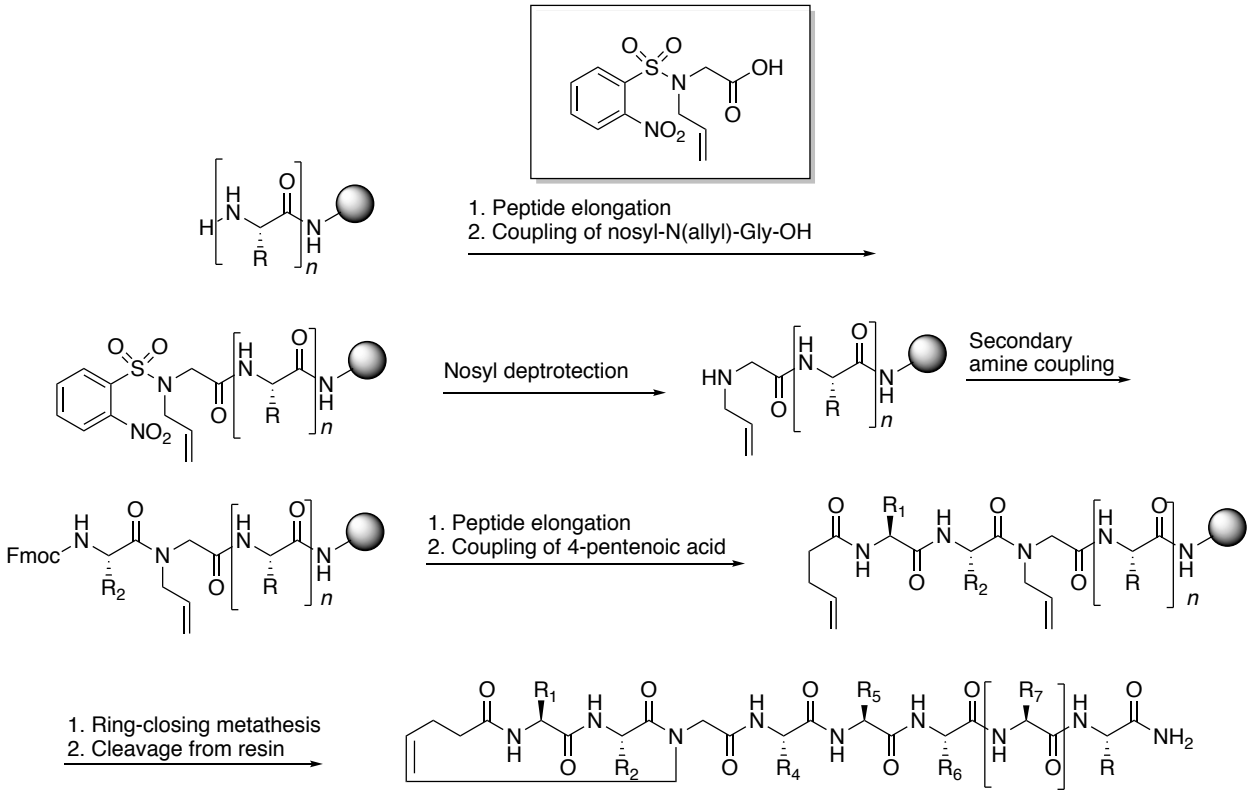
For HBS derivatives containing glycine at the bridge: peptides were synthesized until the bridge, followed by coupling of Nosyl-N(allyl)-glycine-OH which has been previously synthesized and described [3]. For HBS derivatives containing alternative residues at the bridge: peptides were synthesized until the bridgehead carbon, protected with nosyl chloride before performing a mitsunobu reaction with allyl alcohol as described [4]. The nosyl group was then deprotected using 10 equivalents of thiophenol and 10 equivalents of triethylamine in DMF for 2 hours. Secondary amine couplings were performed overnight with 10/10/10 equivalents of AA/HOAT/DIC in DMF. Standard Fmoc- coupling was used to complete the bis-olefin.

All other peptides were synthesized using standard Fmoc-solid phases synthesis using a Prelude X Protein Technologies synthesizer until the bis olefin. Nosyl deprotection of nosyl glycine was carried out on the synthesizer using 120 mM DBU and 100 mM β-mercaptoethanol in DMF. The Fmoc- deprotection solution (20% Piperidine/DMF) was supplemented with 0.1 M HOBt to prevent aspartimide formation [5].

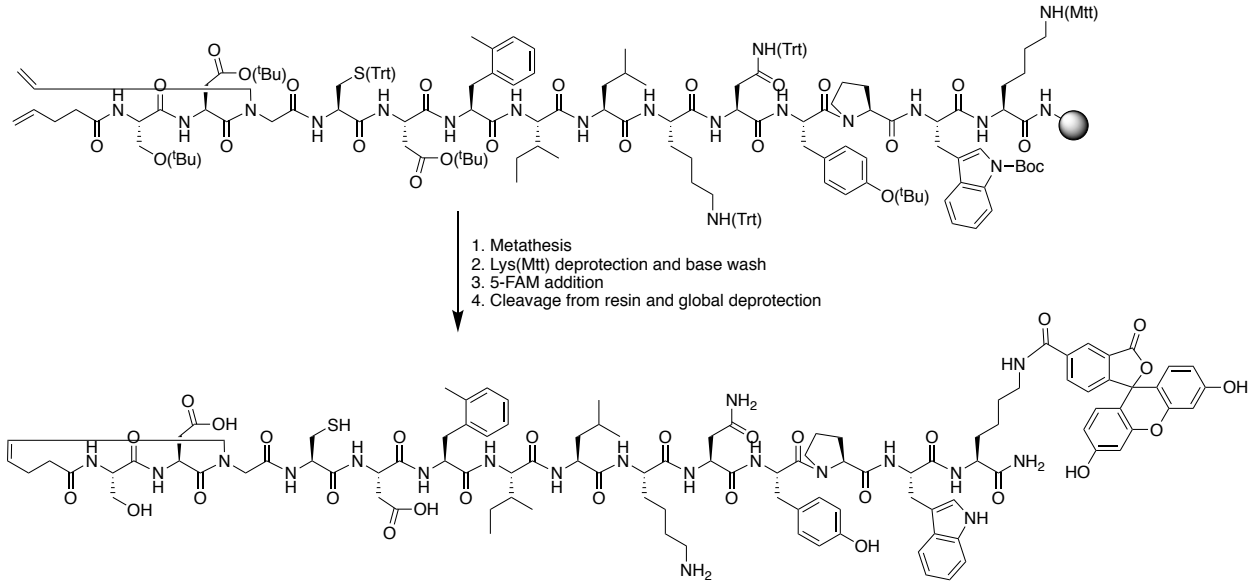
For all peptides, ring closing metathesis was performed in the microwave on 0.1 mmol scales with 3 mLs of dry DCE, 0.20% of Hoveyda-Grubbs II using 120°C for 12 minutes at 150 W power (**Scheme S1**).

After ring closing metathesis, Lys(Mtt)-containing peptides were deprotected using 1.5% TFA, 5% TIPS in DCM for 5 minutes and repeated for a total of 5 times. The resin was then treated with 5% DIEA in DMF for 5 minutes each for a total of 3 times. 4 equivalents of 5-carboxyfluoroscein (5-FAM) was activated with 4 equivalents of HBTU and 8 equivalents of DIEA in DMF and was then added to the resin. The 5-FAM was at a 0.1 M final concentration. The reaction proceeded overnight and was confirmed by MALDI-TOF (**Scheme S2**).

All peptides were cleaved from the resin and globally deprotected by applying a solution of trifluoroacetic acid/TIPS/water (95/2.5/2.5) for 2 hours at room temperature or trifluoroacetic acid/thioanisole/EDT/anisole (90/5/3/2) for peptides with free thiols. The resin was filtered and the peptide was concentrated in vacuo. The pellet was precipitated with cold diethyl ether and dried under nitrogen gas. Reversed-phase HPLC using a gradient of acetonitrile in water supplemented with 0.1% TFA was performed. Each peptide was lyophilized and yielded a white to off-white powder. Purity of the compounds were determined using a reversed-phase HPLC analytical column (C-18, 3.5 μm, 2.1 x 150 mm) run with 5-95% acetonitrile in water with 0.1% TFA over 12 minutes. Exact masses were determined using the Bruker UltrafleXtreme MALDI-TOF.



Scheme S1: Solid phase HBS synthesis.



Scheme S2: Solid phase synthesis of screen and hit compounds, example peptide.

D. Characterization of purified peptides

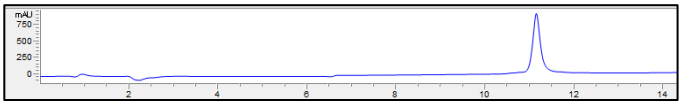
Table S4: Peptide Characterization summary of mass spectrometry results.

Compound	Sequence	$[M+H]^+$ Calc'd	$[M+H]^+$ Obs.
HBS Derivatives			
HBS I	XSDG*(Bcs)D(2meF)ILKNYPW-NH_2	1754.817	1755.203
Screening Peptides			
HBS I_850_Screen*	XSDG*CD(2meF)ILKNYPW(K-5-FAM)-NH_2	2150.913	2151.408
HBS I_857_Screen*	XSDG*(Bcs)D(2meF)ILKNCPW(K-5-FAM)-NH_2	2180.906	2180.981
HBS I_858_Screen*	XSDG*(Bcs)D(2meF)ILKNYCW(K-5-FAM)-NH_2	2246.917	2247.198
Baseline Peptide			
HBS I*	XSDG*(Bcs) D(2meF)ILKNYPW(K-5-FAM)-NH_2	2240.960	2241.082
Single Hits			
HBS III*	XSDG*(CyHex)D(2meF)ILKNYPW(K-5-FAM)-NH_2	2229.051	2228.700
HBS IV*	XSDG*(Bcs)D(2meF)ILKN(CyPent)PW(K-5-FAM)-NH_2	2245.028	2244.743
HBS V*	XSDG*(Bcs)D(2meF)ILKNY(C-IAA) W(K-5-FAM)-NH_2	2304.922	2304.770
Double Hit			
HBS VI*	XSDG*(CyHex)D(2meF)ILKNY(C-IAA)W(K-5-FAM)-NH_2	2293.013	2292.731
Triple Hit			
HBS II*	XSDG*(CyHex)D(2meF)ILKN(CyPent)(C-IAA)W(K-5-FAM)-NH_2	2297.080	2297.486
HBS II	XSDG*(CyHex)D(2meF)ILKN(CyPent)(C-IAA)W-NH_2	1810.938	1811.094

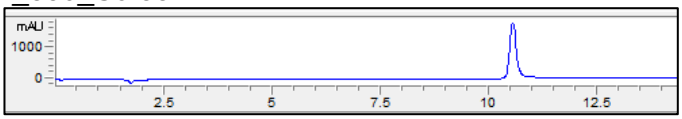
All single letter amino acid codes are used above in addition to the following abbreviations:
Ac = Acyl cap; X = Pentenoic Acid; Bcs = Benzylcysteine; 2meF = 2-methyl phenylalanine; CyHex = 5-cyclohexylpentanoic acid from Fmoc-5-cyclohexylpentanoic acid, synthesized; CyPent = 5-cyclopentylpentanoic acid from Fmoc-5-cyclohexylpentanoic acid, synthesized; C-IAA = Cys containing iodoacetic acid from Fmoc-Cys(OtBu), synthesized; 5-FAM = 5-carboxyfluoroscien
* in compound name denotes the compound has a fluorophore

Figure S6: Analytical traces for peptides in this study (A-K).

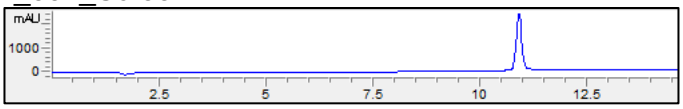
A) HBS I



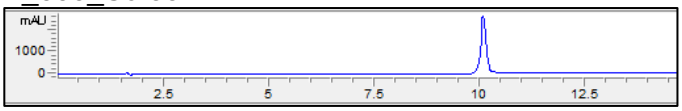
B) HBS I_850_Screen*



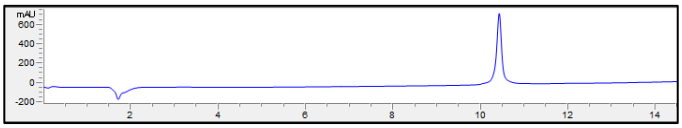
C) HBS I_857_Screen*



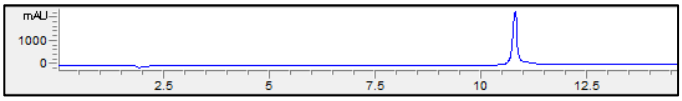
D) HBS I_858_Screen*



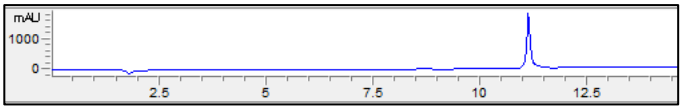
E) HBS I*



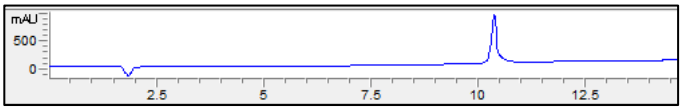
F) HBS III*



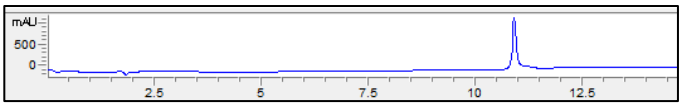
G) HBS IV *



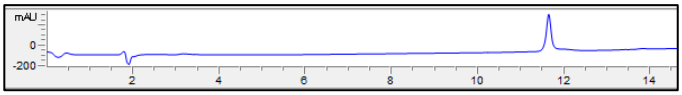
H) HBS V*



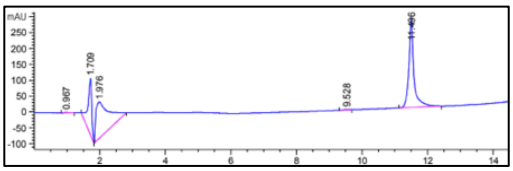
I) HBS VI*



J) HBS II*



K) HBS II



Synthesis Characterization of other peptides used in this study:

The following peptides can be found in [1]:

MLL$^{847-858}$: Ac-SDIMDFVLKNTP-NH_2

Peptide I: Ac-SDI(Bcs)D(2meF)ILKNYP-OH

Peptide I-NH_2: Ac-SDI(Bcs)D(2meF)ILKNYP-NH_2

The following peptide can be found in [2]:

p53 Probe: Ac-EAFSDLWKLLPENNVC^{Flu}-NH_2

Where all single letter amino acid codes are used above in addition to the following abbreviations:

Ac = Acyl cap

Bcs = Benzylcysteine

2meF = 2-methyl phenylalanine

^{Flu} = 5-acetamidofluorescein.

E. Circular Dichroism Studies

Spectra were recorded using AVIV 202SF CD spectrometer or JASCO CS spectrometer in 1 mm cells at 25.00 °C, 1.000 nm bandwidth slits, from 260.00 nm to 185.00 nm with increments of 0.500 nm, averaging time of 0.500 seconds, with 4 scans per sample. Baselines were subtracted from analogous conditions as that from samples. Each sample was prepared at 50 μM in 0.1X PBS (13.7 mM NaCl, 0.27 mM KCl, 1 mM Na_2HPO_4, 0.18 mM KH_2PO_4, pH 7.4) as performed in Joy, S. T., *et al*. and Rooklin, D., et al [1, 3].

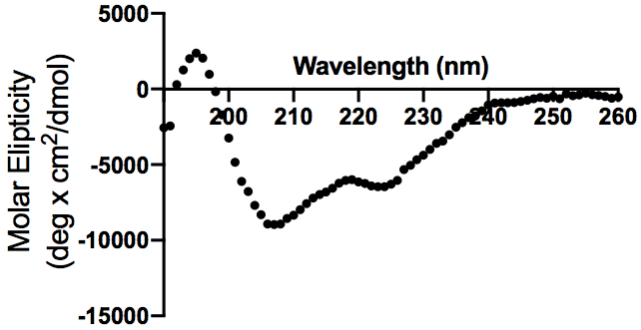


Figure S7: Representative CD data. CD for HBS II.

F. Protein Expression and Purification

His-KIX expression and purification: was carried out in the same manner as it was in [1].

MDM2 expression and purification: was carried out as performed in [6], with supplementing lysis buffers with DNAse and adjusting the NaCl concentration to 1M throughout.

^{15}N Labeled His-KIX expression and purification: All media was supplemented with Ampicillin (100 mg/mL in water) and Chlroamphenicol (25 mg/mL in ethanol). LB was inoculated for an overnight growth and grown at 37 °C. Cells were pelleted and the supernatant was discarded. Cells were then added to and grown in M9 media (M9 salts,^{15}N-NH_4Cl, $MgSO_4$, $CaCl_2$,Glucose, and 2.5 mL

of 10X Bioexpress Media from Cambridge Isotopes). At OD 600 = 0.6, the temperature was lowered to 25 °C, and the culture was induced with 0.1 mM IPTG. The cells were grown for 12 hours at 25 °C before harvesting and storing them at -80 °C with 10% glycerol. Purification was performed in the same manner for KIX in [1].

G. Fluorescence Polarization Methods

Sample Preparation:

Concentrations of protein and peptide samples were determined using Nanodrop 2000c (ThermoScientific).

Direct Binding Experiments:

Experiments were performed in triplicate in a 96-well plate format. His-KIX or MDM2 was concentrated in 10% glycerol in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) using Amicon Ultra Centrifugal filters according to the manufacturer's protocol. Pluronic F-127 (Sigma) was added to a final concentration of 0.1% in each experiment. All fluorescently-tagged peptides were dissolved in the same buffer as the protein and were at a final concentration of 15 nM. Each plate incubated for 60 minutes at room temperature and was read on a DTX 880 Multimode Detector (Beckman) at 25 °C with excitation of 485 nm and emission of 525 nm. Raw values were fit to a Sigmoidal, 4PL, model where X is log(concentration) in GraphPad Prism 8.0. to obtain the EC_{50} value. The binding affinities (K_d) for each peptide was determined using the following equation as performed in Lao, *et al*. [7] The following equation from [8] was used:

$$K_d = (R_T \times (1-F_{SB}) + L_{ST} \times F_{SB}^2)/F_{SB} - L_{ST} \qquad \textbf{(1)}$$

R_T = EC_{50} value determined by prism
F_{SB} = fraction bound = 0.5 (in the case of EC_{50})
L_{ST} = total concentration of Flu-peptide = 15 nM (for all experiments)

Competition Binding Experiments:

Inhibition studies were performed using WT-MLL^{Flu}, His-KIX and unlabeled peptides. The WT-MLL^{Flu} used in these studies is the same used in [1] with FITC-βAla-DCGNILPSDIMDFVLKNTP-NH_2. All studies were performed in triplicate in a 96 well plate format and contained final concentrations of 10% glycerol and 0.1% Pluronic F-127 (Sigma) in PBS, pH 7.4. The MLL analogues were dissolved in DMSO and added so that the final concentration of DMSO in the well was 5%. As performed in direct binding studies, the WT-MLL^{Flu} was at a final concentration of 15 nM. A blank direct binding study was performed with WT-MLL^{Flu} and DMSO with no compound present to determine the concentration of His-KIX at 70% saturation unless except for HBS-FT and HBS-W, which were tested at 65% saturation. In each well were varying amounts of MLL analogues in DMSO. Samples were incubated at room temperature for 60 minutes before reading the plate. The detection was read with the same parameters as done in the direct binding studies. The competition binding affinities (K_i) for each peptide was determined using the following equation as performed in Lao, *et al*. [7] For compounds that did not fully compete out the probe, the K_i was estimated by plugging in the Y = 50% saturation into the K_d calculator. 95% confidence intervals were determined by taking the difference between the lower confidence interval and value determined by Prism. The following equation from [8] was used:

$$K_i = K_d * F_{SB} * ((L_T/L_{ST} * F_{SB} - (K_d + L_{ST} + R_T) * F_{SB} + R_T)) - 1/(1- F_{SB})) \qquad \textbf{(2)}$$

K_d = K_d of WT-MLL^{Flu} to His-KIX from equation Direct Binding **(1)**

F_{SB} = Fraction of WT-MLL^{Flu} bound to His-KIX at EC_{50}
L_T = Total concentration of WT-MLL^{Flu}
L_{ST} = EC_{50} of peptide from competition curve using equation **(1)** as determined by Prism
R_T = Total concentration of His-KIX protein

H. Protein NMR Titration Studies

The ^{1}H-^{15}N-HSQC experiments were carried out using a 600 MHz Bruker 4-channel NMR spectrometer equipped with a 5 mm TCI cryogenic probe. Experiments were performed at 27 °C using a standard pulse sequence and analyzed by Topspin 4.0.6 (Bruker) and MestReNova 6.0.2. Uniformly ^{15}N-labelled KIX was buffer exchanged into NMR buffer (9:1 H_2O:D_2O, 10 mM phosphate buffer containing 100 mM NaCl at pH 7.2) using Amicon Ultra centrifugal filter (Millipore). ^{1}H-^{15}N-HSQC NMR of ^{15}N-labeled KIX (100 μM) was collected, and peaks were assigned based on published data [9, 10]. For peptide titration experiments, 0.5 and 1 molar equivalents of HBS II dissolved in NMR buffer were added to the protein solution. The change in chemical shift after the addition of peptide was plotted for each KIX residue. Chemical shift changes ^{1}H and ^{15}N were averaged by using the formula:

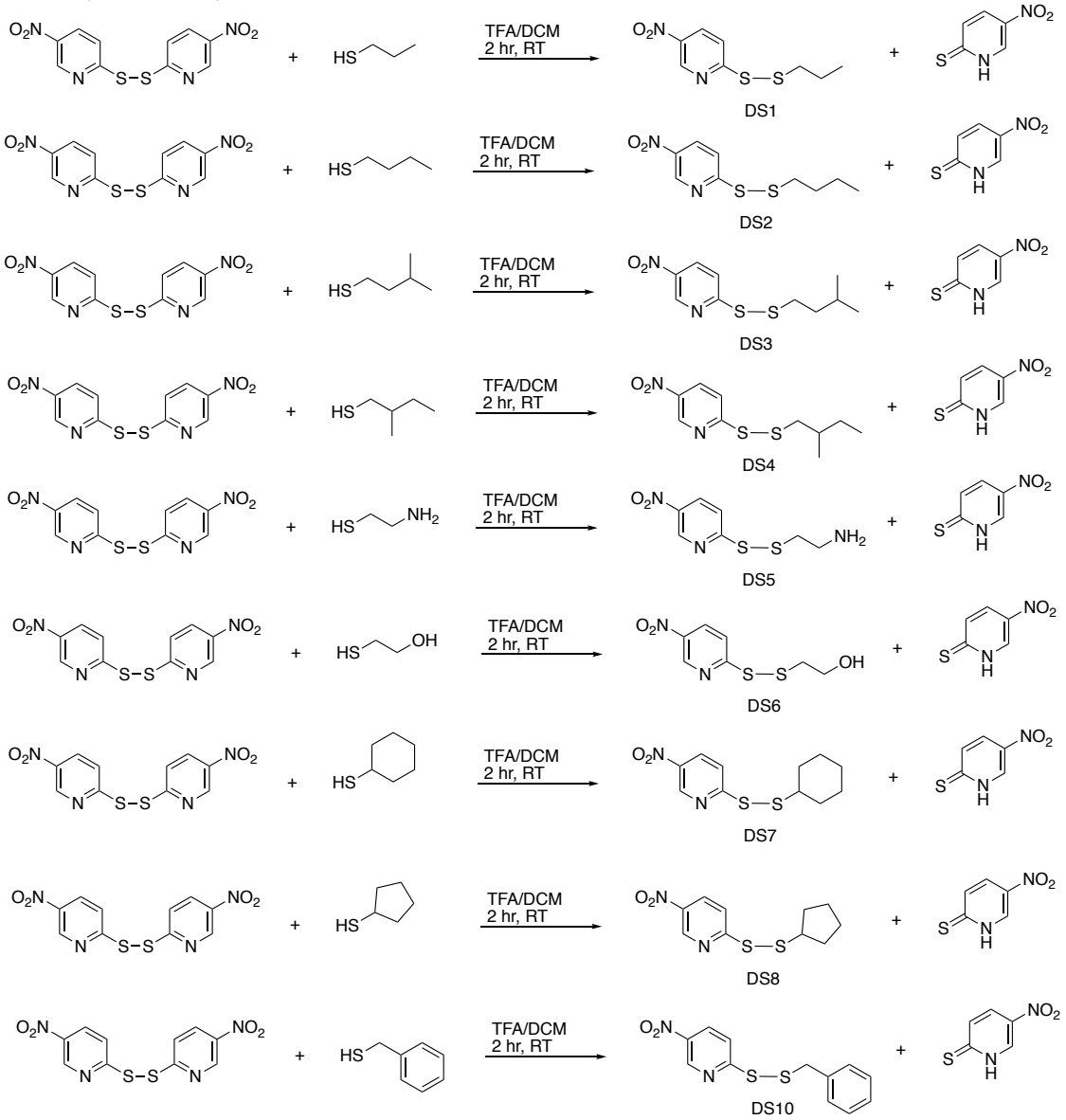
$$Shift = \sqrt{0.5 \times [\delta_{\mathrm{H}}^2 + (\alpha \times \delta_{\mathrm{N}}^2)]},$$

with the scale factor α taken to be 0.14 [11].

I. Synthesis, Purification, and Characterization of Small Molecules

General synthesis for disulfides:

Disulfide precursors were synthesized similar to those activated disulfides in [12]. 465 mgs (1.5 mmoles, 1 eq.) of 2,2'-Dithiobis(5-nitropyridine) aka DTNP were dissolved in 15 mLs of TFA/DCM 50/50. The respective thiols (1.8 mmoles, 1.2 eq.) were added directly to the solution and stirred for two hours at room temperature. TLC confirmed disappearance of the DTNP starting material and the product was concentrated in vacuo. The products were used as crude alkylators in the screening studies. DS-2 was also synthesized and purified and used in a crude versus purified disulfide alkylator study.



Scheme S3: Asymmetric disulfide synthesis.

DS1: DS1 was synthesized according to the general procedure for disulfides using 465 mgs (1.5 mmoles, 1 eq.) of 2,2'-Dithiobis(5-nitropyridine) (1.8 mmoles, 1.2 eq.) and 163 μLs (1.8 mmoles, 1.2 eq.) of propylmercaptan. The product was not isolated.

DS2 Crude: DS2 Crude was synthesized according to the general procedure for disulfides using 465 mgs (1.5 mmoles, 1 eq.) of 2,2'-Dithiobis(5-nitropyridine) (1.8 mmoles, 1.2 eq.) and 193 μLs (1.8 mmoles, 1.2 eq.) of n-butylmeraptan. The product was not isolated.

DS3: DS3 was synthesized according to the general procedure for disulfides using 465 mgs (1.5 mmoles, 1 eq.) of 2,2'-Dithiobis(5-nitropyridine) (1.8 mmoles, 1.2 eq.) and 225 μLs (1.8 mmoles, 1.2 eq.) of 3-methyl-1-butanethiol. The product was not isolated.

DS4: DS4 was synthesized according to the general procedure for disulfides using 465 mgs (1.5 mmoles, 1 eq.) of 2,2'-Dithiobis(5-nitropyridine) (1.8 mmoles, 1.2 eq.) and 221 μLs (1.8 mmoles, 1.2 eq.) of 2-methylbutane-1-thiol. The product was not isolated.

DS5: DS5 was synthesized according to the general procedure for disulfides using 465 mgs (1.5 mmoles, 1 eq.) of 2,2'-Dithiobis(5-nitropyridine) (1.8 mmoles, 1.2 eq.) and 203 mgs (1.8 mmoles, 1.2 eq.) of cysteamine-HCl. The product was not isolated.

DS6: DS6 was synthesized according to the general procedure for disulfides using 465 mgs (1.5 mmoles, 1 eq.) of 2,2'-Dithiobis(5-nitropyridine) (1.8 mmoles, 1.2 eq.) and 127 μLs (1.8 mmoles, 1.2 eq.) of mercaptoethanol. The product was not isolated.

DS7: DS7 was synthesized according to the general procedure for disulfides using 465 mgs (1.5 mmoles, 1 eq.) of 2,2'-Dithiobis(5-nitropyridine) (1.8 mmoles, 1.2 eq.) and 220 μLs (1.8 mmoles, 1.2 eq.) of cyclohexanethiol. The product was not isolated.

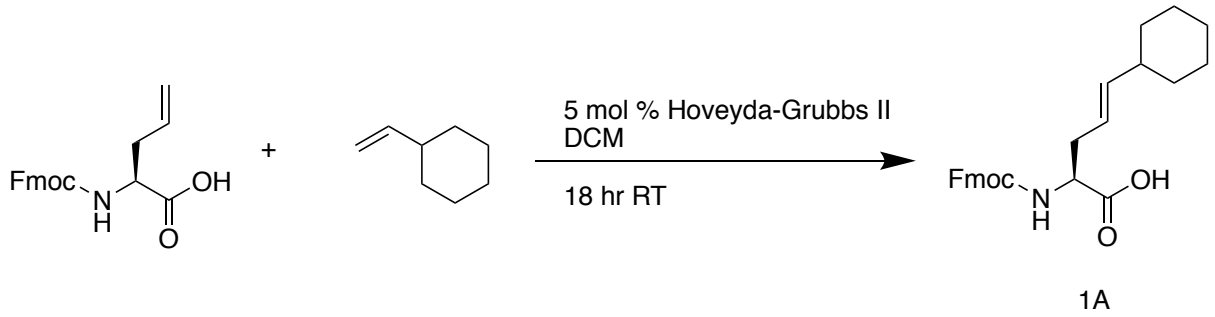
DS8: DS8 was synthesized according to the general procedure for disulfides using 465 mgs (1.5 mmoles, 1 eq.) of 2,2'-Dithiobis(5-nitropyridine) (1.8 mmoles, 1.2 eq.) and 193 μLs (1.8 mmoles, 1.2 eq.) of cyclopentanethiol. The product was not isolated.

DS10: DS10 was synthesized according to the general procedure for disulfides using 465 mgs (1.5 mmoles, 1 eq.) of 2,2'-Dithiobis(5-nitropyridine) (1.8 mmoles, 1.2 eq.) and 184 μLs (1.8 mmoles, 1.2 eq.) of benzylmercaptan. The product was not isolated.

DS2 Pure: See **Scheme S3** for scheme.
In addition to the crude variant, DS2 Pure was synthesized according to the general procedure for disulfides using 465 mgs (1.5 mmoles, 1 eq.) of 2,2'-Dithiobis(5-nitropyridine) (1.8 mmoles, 1.2 eq.) and 193 μLs (1.8 mmoles, 1.2 eq.) of n-butylmercaptan The product was purified on a Biotage Isolera One silica column using a 1-7% gradient of ethyl acetate in hexanes. The product was obtained as an off white solid (205 mgs, 56% yield).

R_f = 0.41 in 5% EtOAc/hexane
HRMS (APCI): Calculated $[M+H]^+$ 245.0340, Observed $[M+H]^+$ 245.0615.
^{1}H NMR (500 MHz, $CDCl_3$) δ 9.21 (d, *J* = 2.5 Hz, 1H), 8.38 (dd, *J* = 8.9, 2.6 Hz, 1H), 7.90 (d, *J* = 8.9 Hz, 1H), 2.83 – 2.78 (m, 2H), 1.69 – 1.61 (m, 2H), 1.45 – 1.35 (m, 2H), 0.88 (t, *J* = 7.4 Hz, 3H)
^{13}C NMR (126 MHz, $CDCl_3$) δ 169.39, 145.06, 141.92, 131.60, 119.09, 77.42, 77.16, 76.91, 38.74, 31.04, 21.64, 13.62.

Preparation of (1A) Fmoc-5-cyclohexylpent-4-enoic acid:



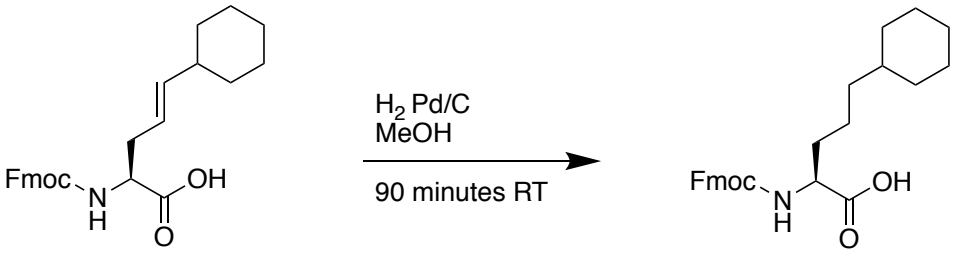
1A

Scheme S4: Preparation of 1A.

750 mgs (2.22 mmol, 1 eq.) of Fmoc-allyl-L-glycine-OH and 70 mgs (0.11 mmol, 0.05 eq.) of Hoveyda-Grubbs second generation catalyst were dissolved in 10 mLs of anhydrous, degassed dichloromethane. 1.52 mLs (11.11 mmol, 5 eq.) of vinylcyclohexne were added and the reaction was stirred overnight at room temperature. The product was purified by column chromatography using a Biotage Isolera One using a 5-45% ethyl acetate in hexanes gradient supplemented with 0.2% formic acid. 1A was obtained as an off-white solid (376 mgs, 40% yield).

R_f =0.58 in 35% EtOAc/hexane + 0.2% formic acid
HRMS (APCI): Calculated $[M+H]^+$ 420.2097, Observed $[M+H]^+$ 420.2178
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.59 (m, *J* = 7.0 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.31 (m, *J* = 7.4, 0.8 Hz, 2H), 5.58 – 5.44 (m, 1H), 5.33 – 5.19 (m, 2H), 4.49 – 4.335 (m, 3H), 4.24 (t, *J* = 6.6 Hz, 1H), 2.52 (m, *J* = 27.4, 13.7, 6.9 Hz, 2H), 1.94 (m, *J* = 3.8 Hz, 1H), 1.75 – 1.52 (overlapping, 5H), 1.32 – 0.98 (overlapping, 5H)
^{13}C NMR (126 MHz, $CDCl_3$) δ 176.40, 156.02, 143.83, 142.56, 141.46, 127.89, 127.21, 125.25, 120.37, 120.15, 77.16, 67.32, 53.43, 47.27, 40.85, 35.33, 33.05, 26.23, 26.08.

Preparation of (1B) Fmoc-5-cyclohexylpentanoic acid:



1A 1B

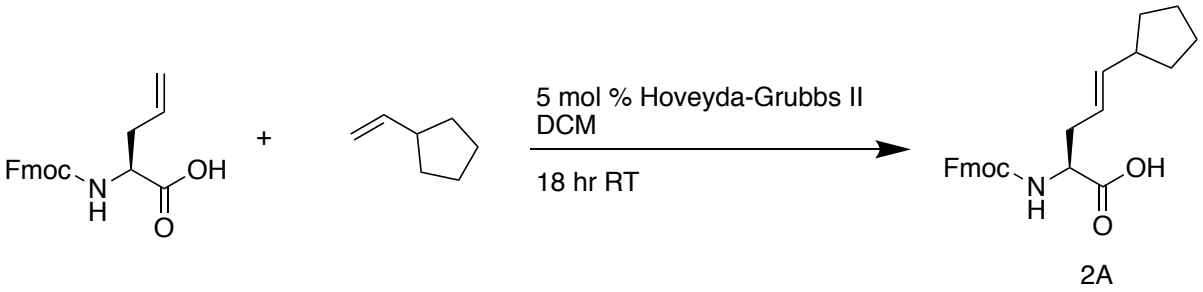
Scheme S5: Preparation of 1B.

376 mgs (0.90 mmoles, 1 eq.) of 1A was dissolved in 90 mLs of MeOH. The reaction vessel was flushed with nitrogen and 9.5 mgs of Pd/C (0.09 mmoles, 0.10 mol%, 0.1 eq.) was added to the solution. The reaction vessel was then flushed with hydrogen (g) and the reaction was allowed to proceed for 90 minutes. Upon completion, the reaction was filtered over Celite® and dried in vacuo to yield an off white solid (421 mgs, 85% yield).

R_f = 0.62 in 35 % EtOAc/hexane + 0.2% formic acid
HRMS (APCI): Calculated $[M+H]^+$ 422.2253, Observed $[M+H]^+$ 422.2369

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.2 Hz, 2H), 7.59 (m, 2H), 7.39 (m, *J* = 7.2 Hz, 2H), 7.34 – 7.28 (m, 2H), 5.24 (d, *J* = 6.9 Hz, 1H), 4.42 (d, *J* = 6.4 Hz, 2H), 4.23 (s, 1H), 1.88 (s, 1H), 1.64 (m, *J* = 33.8, 12.9 Hz, 5H), 1.53 (dd, *J* = 7.5, 2.0 Hz, 1H), 1.48 (s, 1H), 1.39 (s, 2H), 1.27 – 1.08 (m, 5H), 0.86 (d, *J* = 9.6 Hz, 2H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 141.47, 127.89, 127.23, 125.20, 47.29, 37.55, 33.48, 26.78, 26.48.

Preparation of fmoc-5-cyclopentylpent-4-enoic acid (2A):



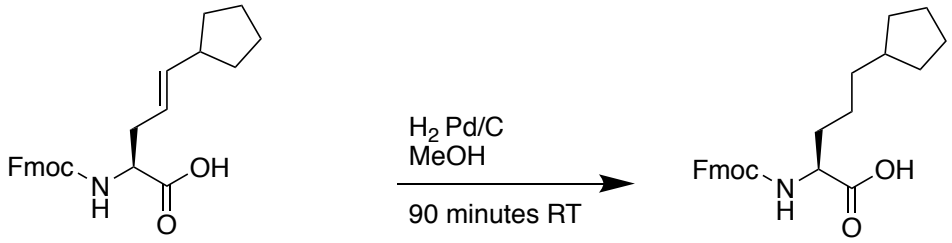
2A

Scheme S6: Preparation of 2A.

750 mgs (2.22 mmol, 1 eq.) of Fmoc-allyl-L-glycine-OH and 70 mgs (0.11 mmol, 0.05 eq.) of Hoveyda-Grubbs second generation catalyst were dissolved in 10 mLs of anhydrous, degassed dichloromethane. 1.52 mLs (11.11 mmol, 5 eq.) of vinylcyclopentane were added and the reaction was stirred overnight at room temperature. The product was purified by column chromatography using a 5-45% ethyl acetate in hexanes gradient supplemented with 0.2% formic acid. 2A was obtained as an off-white solid (254 mgs, 28% yield).

R_f =0.57 in 35% EtOAc/hexane + 0.2% formic acid
HRMS (APCI): Calculated $[M+H]^+$ 406.1940, Observed $[M+H]^+$ 406.2040
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H), 7.59 (m, *J* = 7.1 Hz, 2H), 7.40 (t, *J* = 7.4 Hz, 2H), 7.31 (t, *J* = 7.4 Hz, 2H), 5.56 (dt, *J* = 17.5, 8.8 Hz, 1H), 5.29 (m, *J* = 28.7, 14.3, 7.5 Hz, 2H), 4.53 – 4.33 (m, 2H), 4.23 (m, *J* = 8.9, 4.9 Hz, 1H), 2.77 – 2.46 (m, 2H), 2.42 (dd, *J* = 16.0, 8.0 Hz, 1H), 1.82 – 1.63 (m, 2H), 1.55 (dd, *J* = 7.2, 4.1 Hz, 4H), 1.31 – 1.18 (m, 2H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 176.66, 156.03, 143.83, 141.46, 141.24, 127.89, 127.21, 125.21, 120.91, 120.15, 77.16, 67.33, 53.47, 47.27, 43.32, 38.24, 35.27, 33.22, 25.58, 25.24.

Preparation of fmoc-5-cyclopentylpentanoic acid (2B):



2A 2B

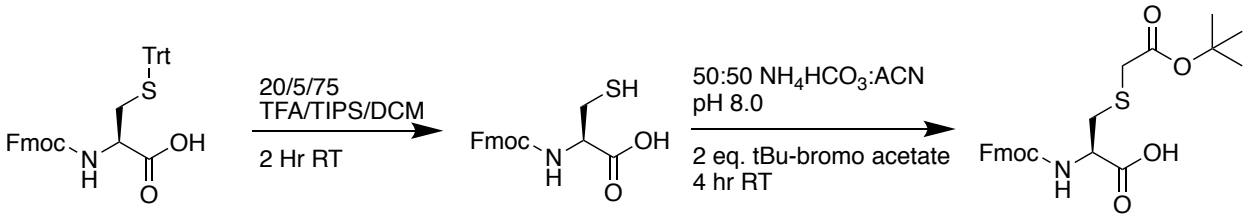
Scheme S7: Preparation of 2B.

254 mgs (0.63 mmol, 1 eq.) of 2A was dissolved in 66 mLs of MeOH. The reaction vessel was flushed with nitrogen and 6.6 mgs of Pd/C (10 mol %) were added to the solution. The reaction vessel was then flushed with hydrogen (g) and the reaction was allowed to proceed for 90

minutes. Upon completion, the reaction was filtered over Celite® and dried in vacuo to yield an off white solid (195 mgs, 76% yield).

R_f = 0.62 in 35% EtOAc/hexane + 0.2% formic acid
HRMS (APCI): Calculated $[M+H]^+$ 408.2098, Observed $[M+H]^+$ 408.2207
^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.2 Hz, 3H), 7.58 (m, *J* = 16.7, 10.8 Hz, 2H), 7.38 (m, *J* = 14.7, 7.1 Hz, 2H), 7.32 (m, *J* = 12.3, 3.7 Hz, 2H), 5.35 – 5.20 (m, 1H), 4.56 – 4.35 (m, 2H), 4.23 (t, *J* = 6.7 Hz, 1H), 1.88 (s, 1H), 1.73 (s, 4H), 1.59 (s, 2H), 1.55 – 1.45 (m, 2H), 1.43 – 1.22 (m, 4H), 1.06 (s, 2H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 149.13, 141.46, 127.88, 127.22, 127.07, 127.05, 124.15, 120.15, 119.98, 77.16, 67.27, 47.29, 42.57, 39.99, 35.72, 32.81, 32.75, 25.28, 24.56, 18.32.

Preparation of (3) Fmoc-Cys(OtBu)-OH:



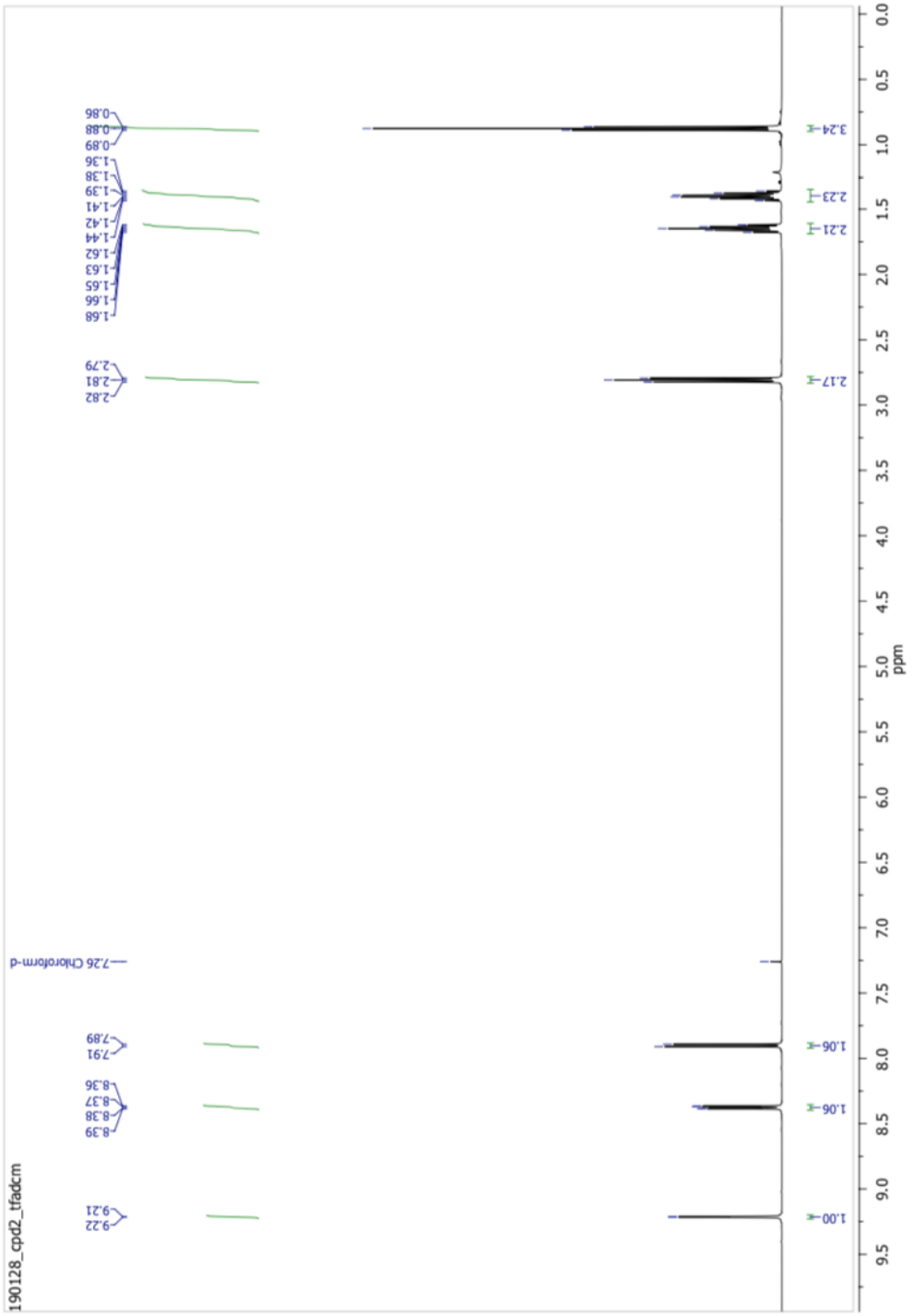
3

Scheme S8: Preparation of 3.

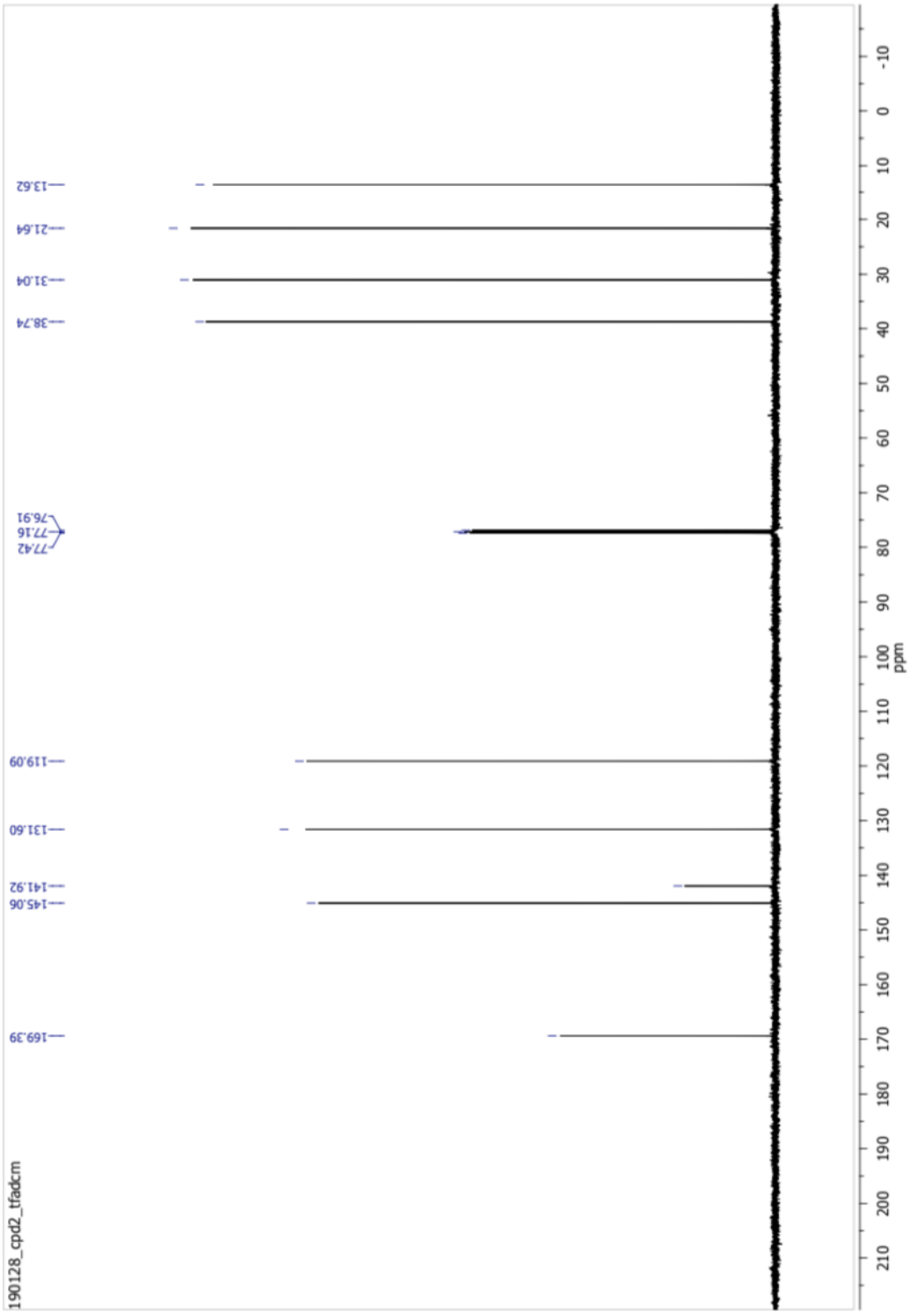
3 g (5.12 mmol, 1 eq.) of Fmoc-Cys(Trt)-OH was dissolved in 75 mLs of DCM. 20 mLs of TFA and 5 mLs of triisopropylsilane (TIPS) were added and stirred at room temperature for 2 hours. The solution was concentrated in vacuo and rinsed with DCM to remove excess TFA. Fmoc-Cys-OH was then dissolved in 250 mLs of 100 mM ammonium bicarbonate and 250 mL acetonitrile. The pH was adjusted to pH 8.0 using solid ammonium bicarbonate. 1.35 mLs (10.24 mmol, 2 eq.) of tert-butyl bromoacetate were added directly to the solution and the solution was stirred for 4 hours at room temperature. Upon completion, the pH was adjusted to 7 using 5% citric acid aqueous solution. The acetonitrile was removed in vacuo and compound 3 was extracted using ethyl acetate (2X). The organic layer was subsequently washed with water (3X) and brine (1X) before drying over magnesium sulfate and concentrating in vacuo. Compound 3 was purified using a 10-50% gradient of ethyl acetate in hexane supplemented with 0.2% formic acid (543 mgs, 23% yield over two steps). The proton NMR is in agreement with established literature results of compound 3 [13].

R_f = 0.43 in 35% EtOAc/hexane + 0.2% formic acid
HRMS (APCI): Calculated $[M+H]^+$ 458.1559, Observed $[M+H]^+$ 458.1663
^{1}H NMR (500 MHz, $CDCl_3$) δ 9.21 (s, 1H), 7.76 (d, *J* = 7.5 Hz, 2H), 7.61 (m, *J* = 29.8, 14.9 Hz, 2H), 7.44 – 7.35 (m, 2H), 7.32 (m, *J* = 7.5, 1.0 Hz, 2H), 6.17 (d, *J* = 7.9 Hz, 1H), 4.67 (m, *J* = 13.0, 5.7 Hz, 1H), 4.41 (d, *J* = 7.2 Hz, 2H), 4.24 (t, *J* = 7.1 Hz, 1H), 3.31 – 3.08 (m, 2H), 1.48 (s, 9H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 174.48, 170.12, 164.71, 156.34, 143.84, 143.72, 141.35, 127.81, 127.17, 125.25, 120.05, 82.67, 77.16, 67.48, 60.71, 53.69, 47.11, 35.49, 34.62, 28.01.

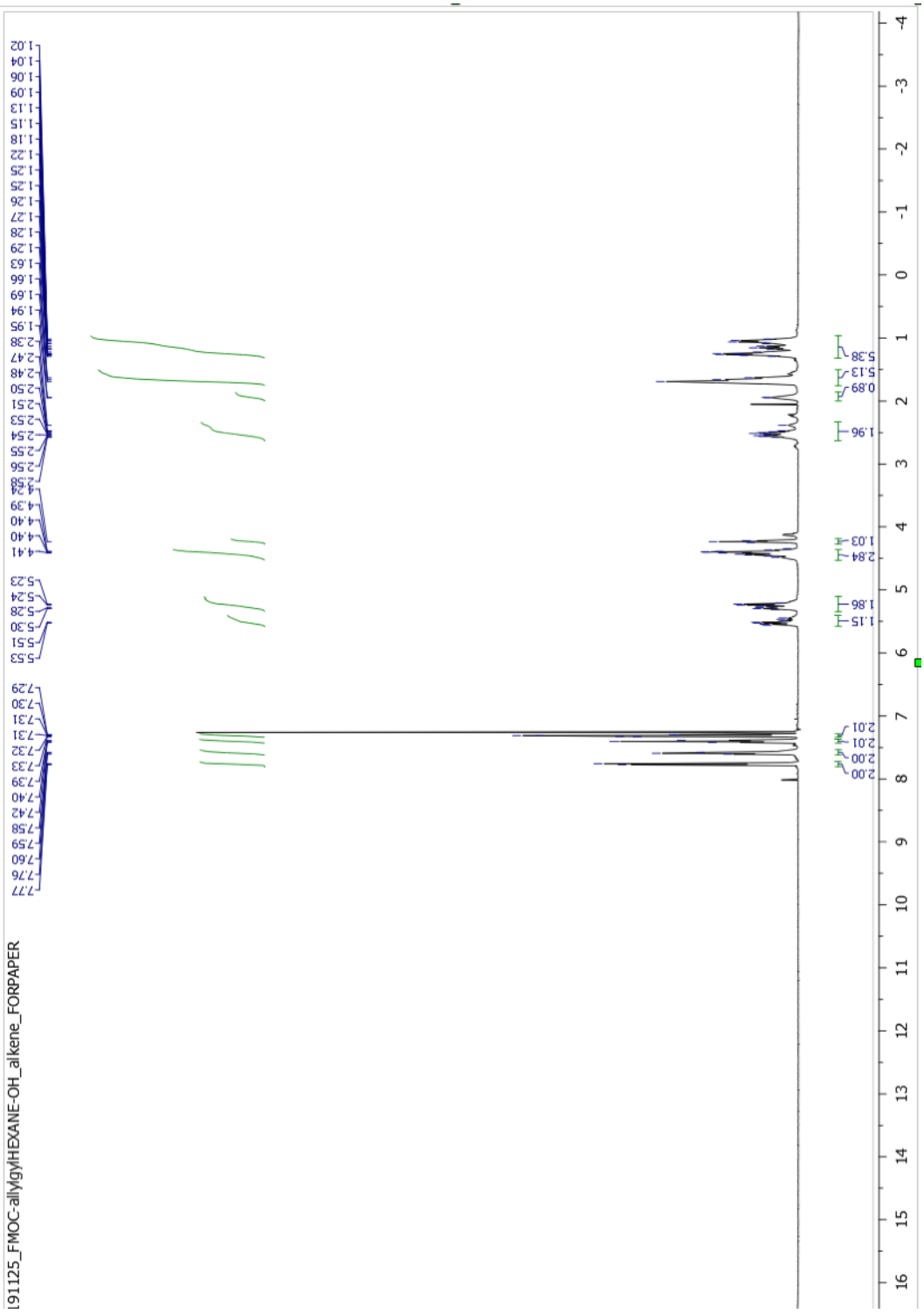
NMR spectra:
Compound DS-2 ^{1}H NMR



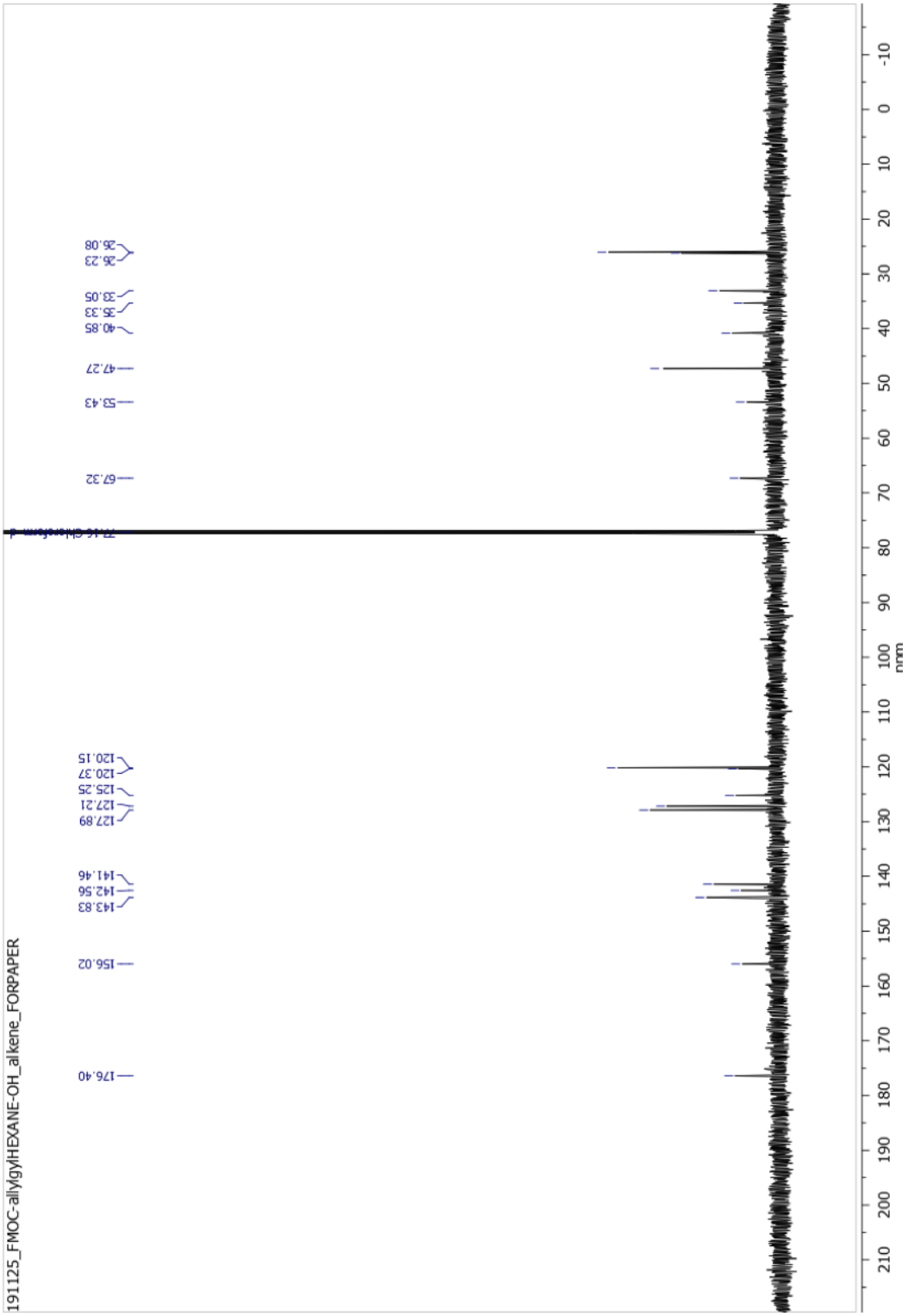
Compound DS-2 ^{13}C NMR



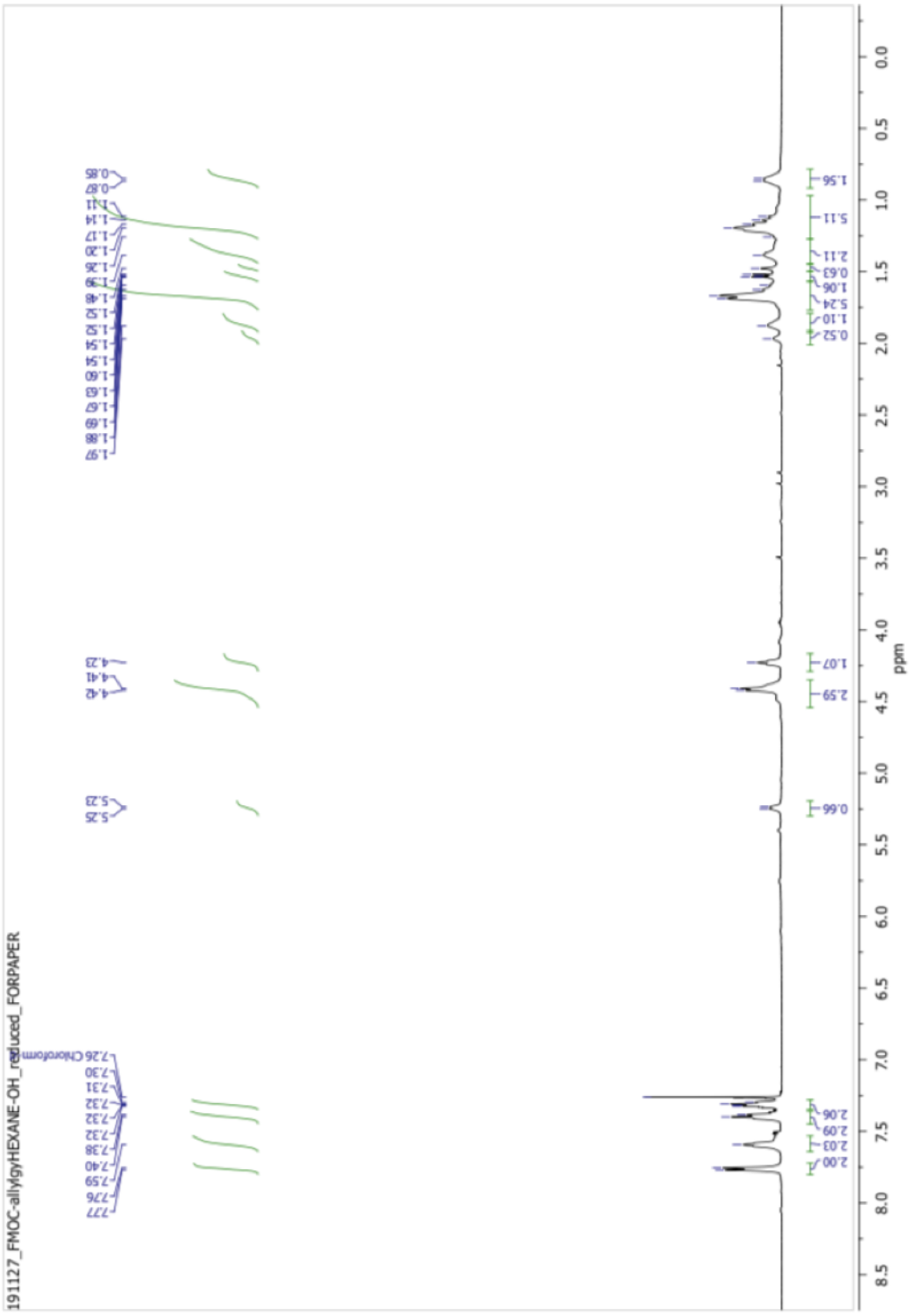
Compound 1A ^{1}H NMR



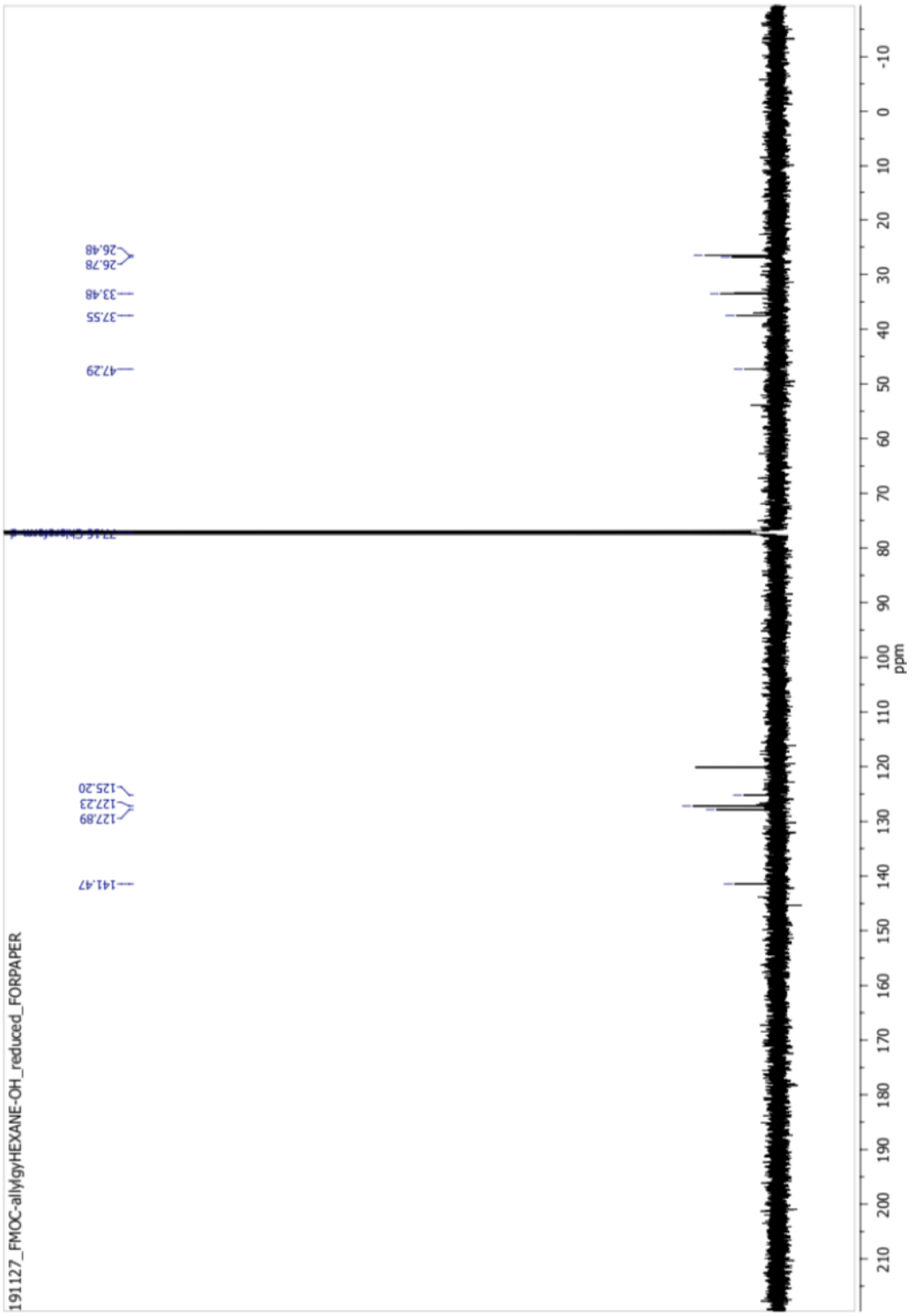
Compound 1A ^{13}C NMR



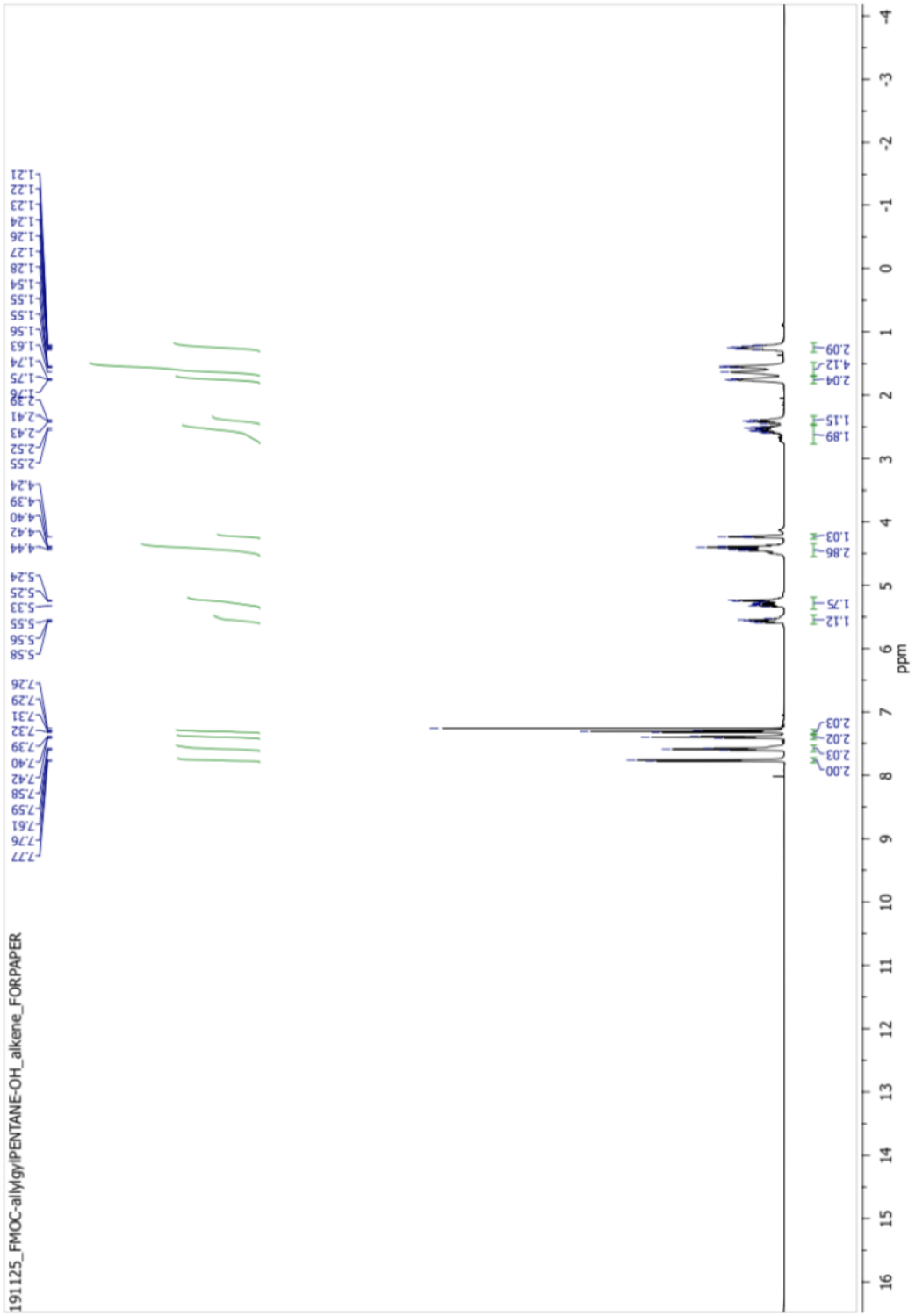
Compound 1B ^{1}H NMR



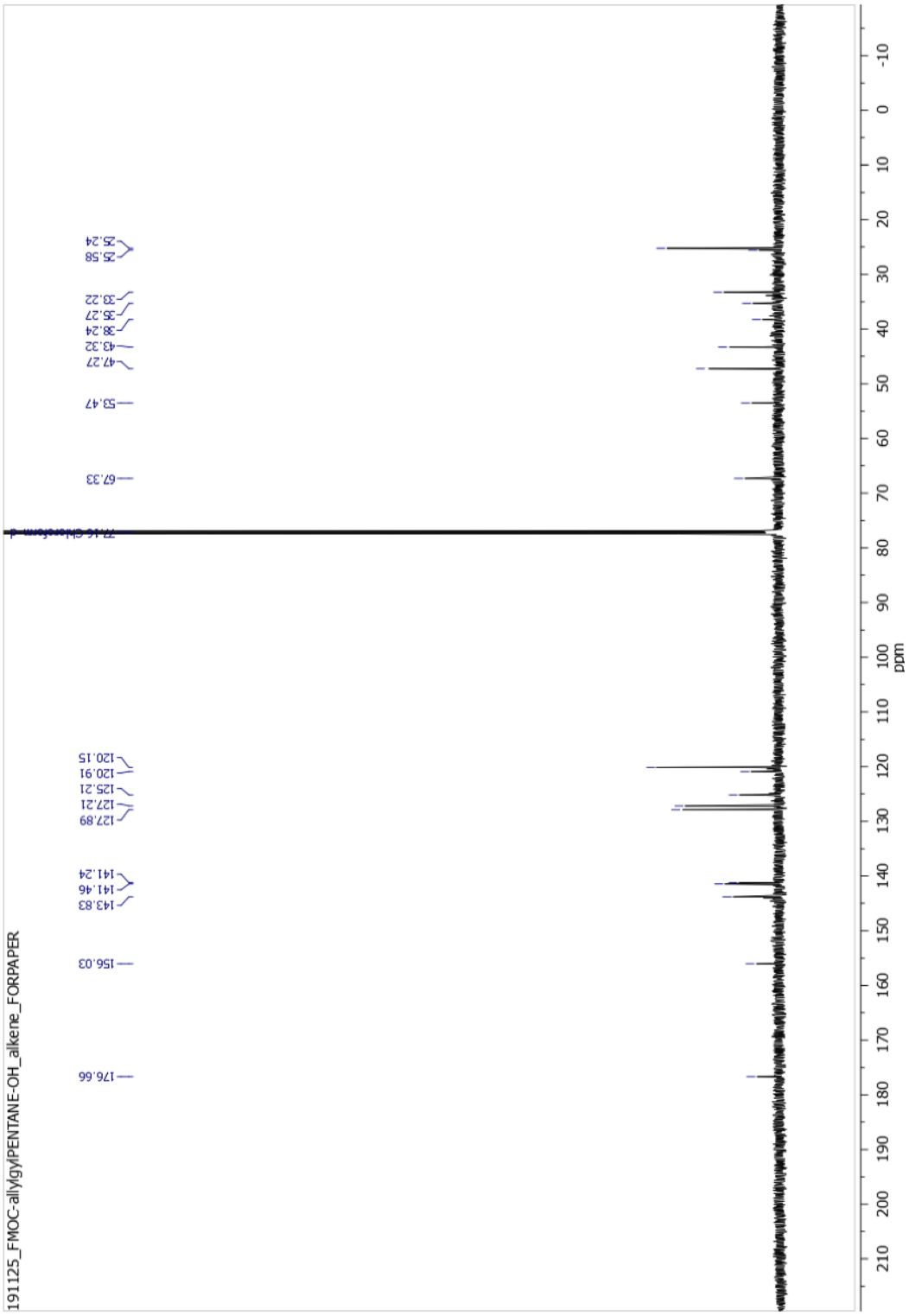
Compound 1B ^{13}C NMR



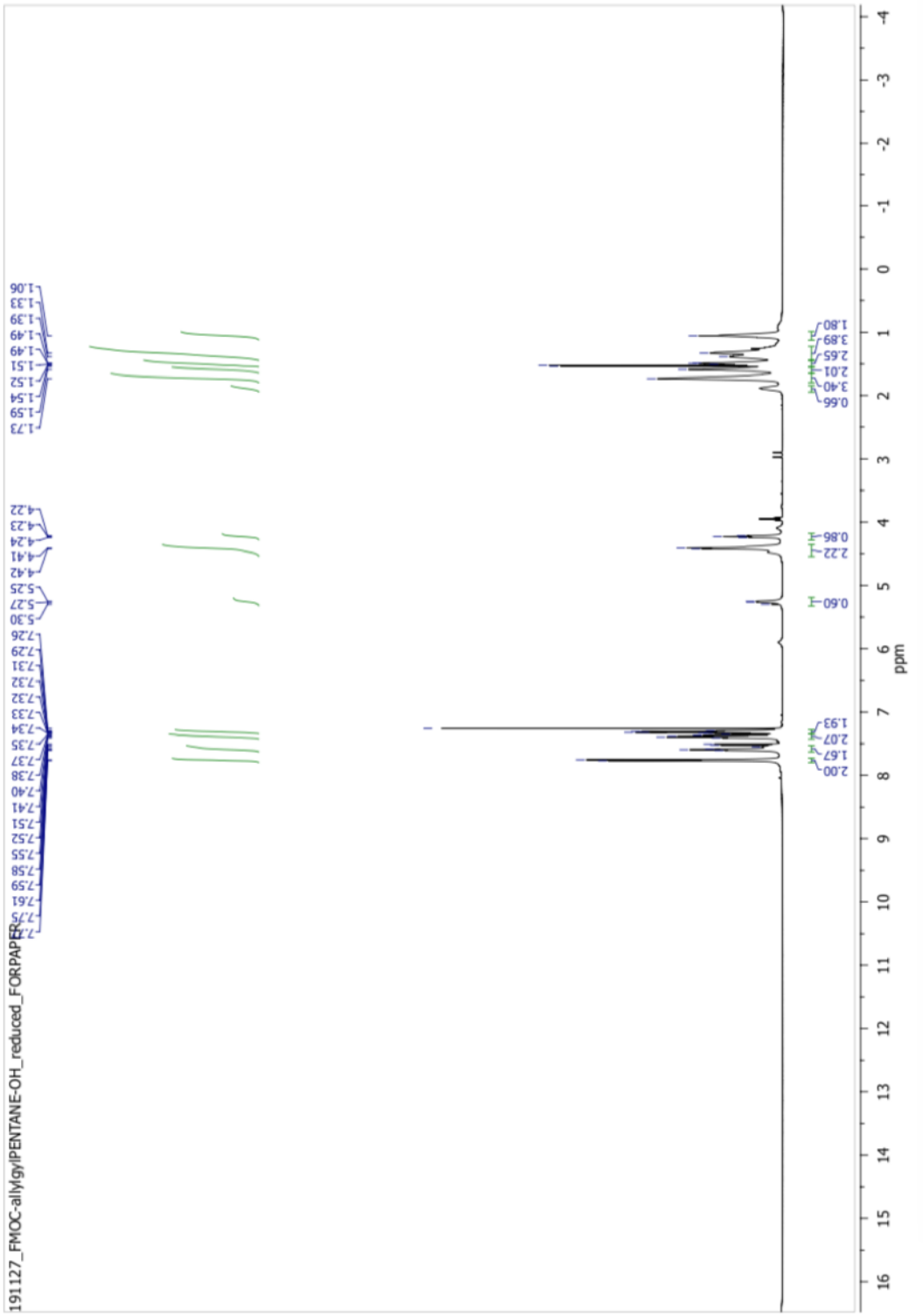
Compound 2A ^{1}H NMR



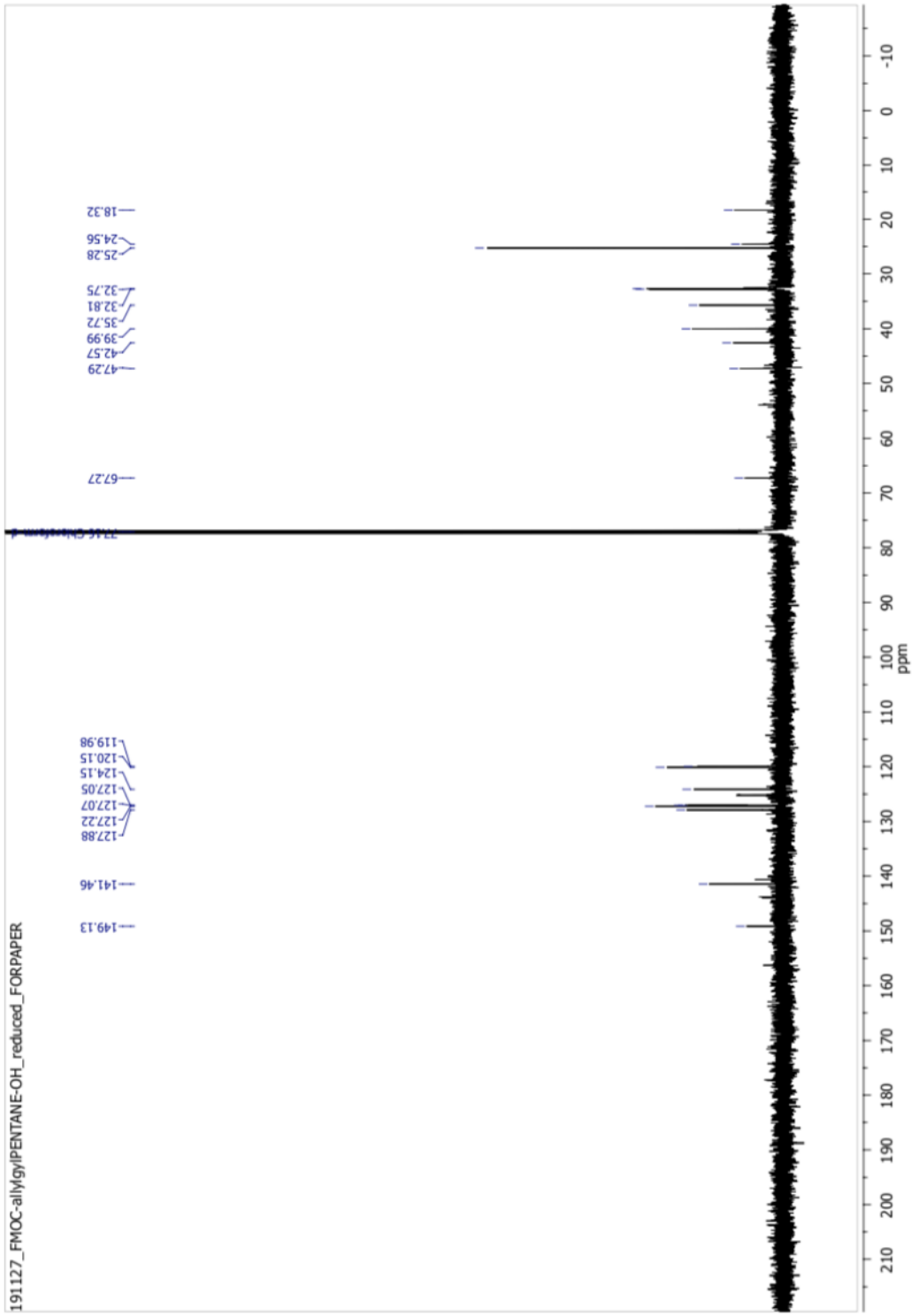
Compound 2A ^{13}C NMR



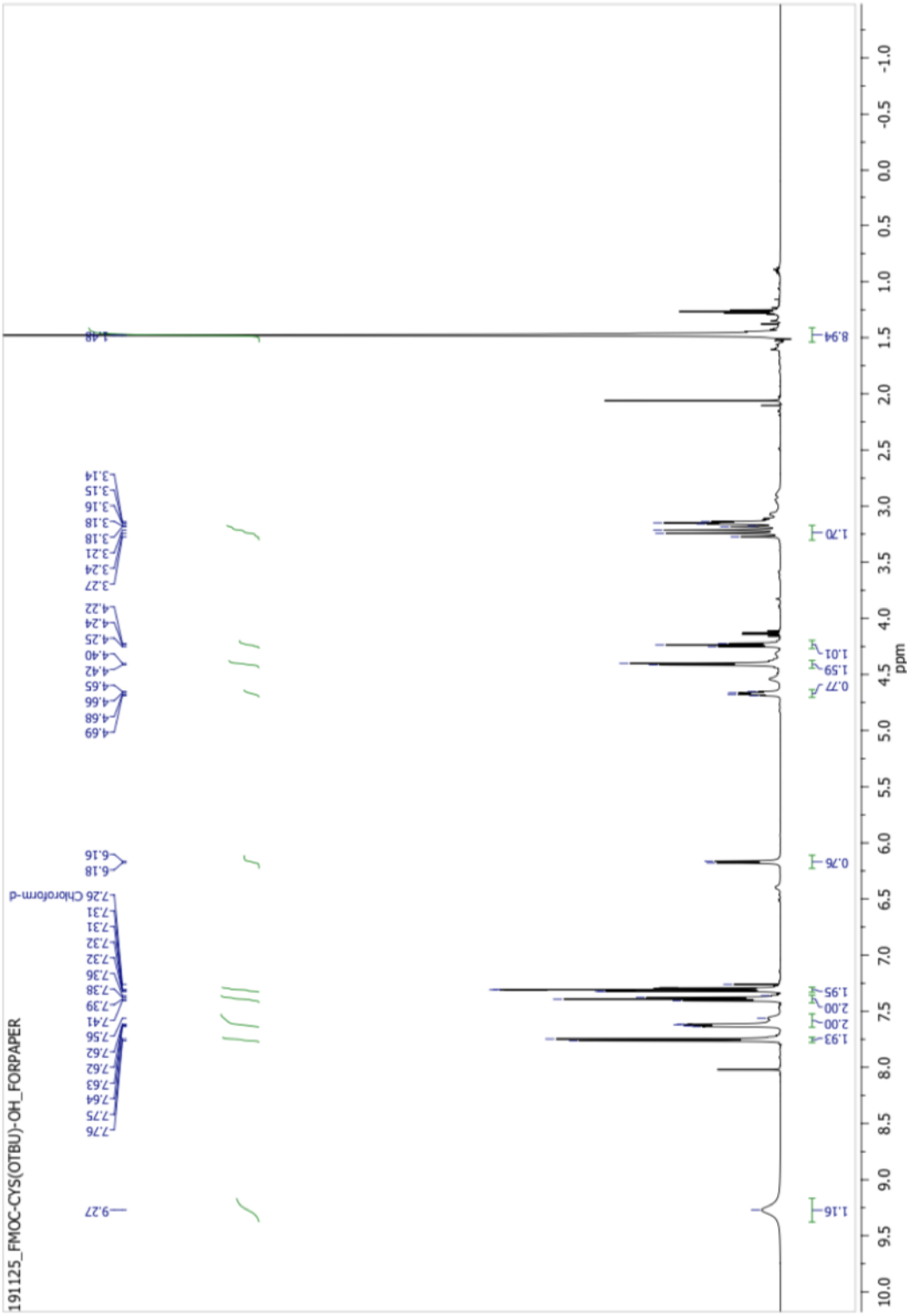
Compound 2B ^{1}H NMR



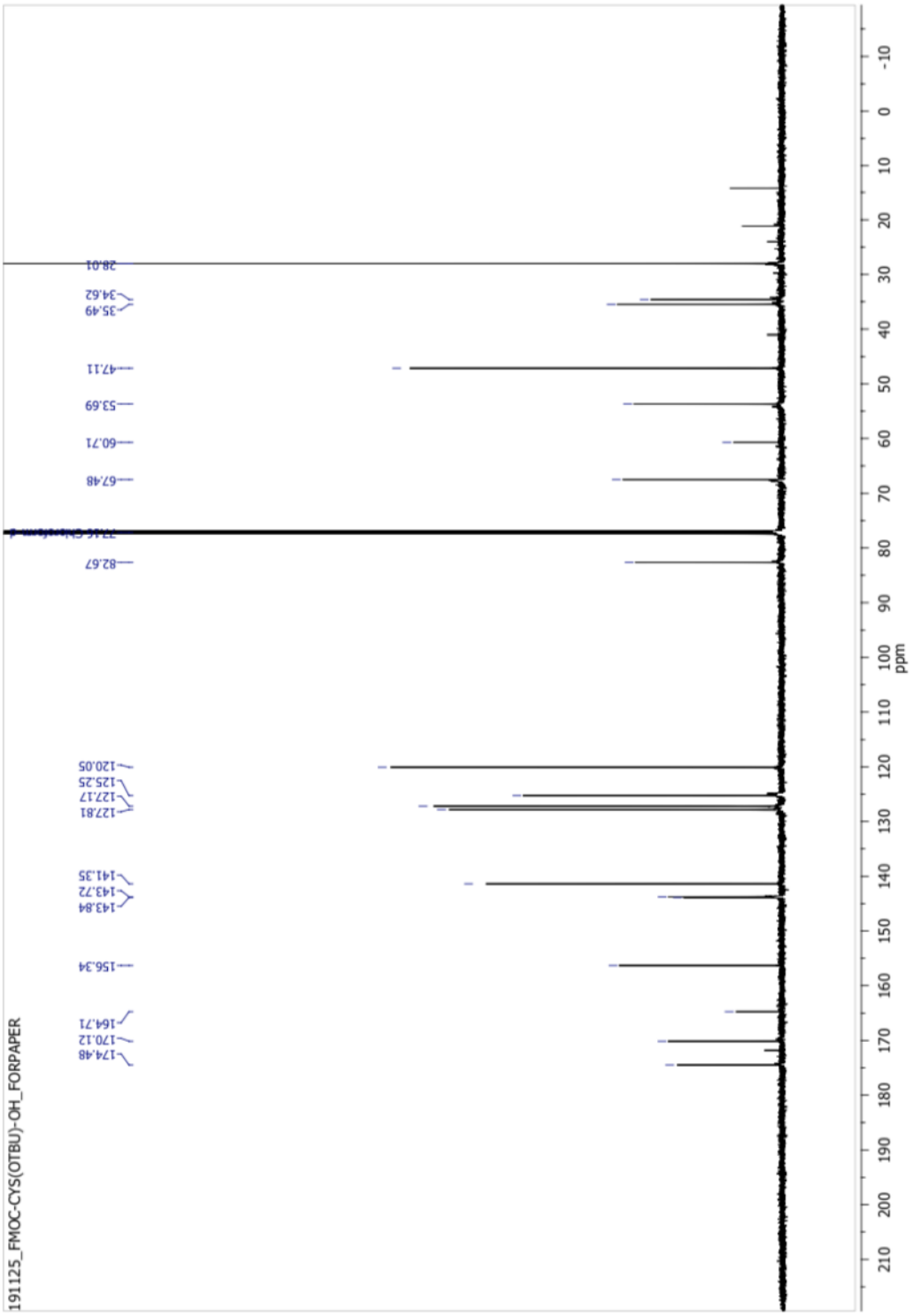
Compound 2B ^{13}C NMR



Compound 3 ^{1}H NMR



Compound 3 ^{13}C NMR



J. Alkylation Test Reactions Methods

General Alkylation Reaction Procedure:

Each alkylator was prepared and tested on the peptide individually. The HBS I_850_screen peptide (XSDG*CD(2meF)ILKNYPW(K-5-FAM)-NH_2) was used in all test reactions. For each test reaction, the fluorescent peptide was brought up in 60% 50 mM ammonium bicarbonate, pH 8.0 in 40% acetonitrile. The peptide's concentration was adjusted to 180 μM using E_{494} = 77,000 $L \cdot mol^{-1} \cdot cm^{-1}$. Concentrations of protein and peptide samples were determined using Nanodrop 2000c (ThermoScientific). Each alkylator was prepared individually to have a stock concentration of 4.5 mM in 100% acetonitrile. 2 μL of alkylator and 10 μL of 5-FAM-peptide were mixed in an Eppendorf so that the final concentration of the peptide in the well was 150 μM, and the alkylators were present at 5X the concentration at 750 μM. The final mixture yielded 50/50 acetonitrile/50 mM ammonium bicarbonate, pH 8.0. The reaction was agitated in the dark at room temperature. Alkylators containing benzyl bromide moieties or iodoacetamide (IAM) or iodoacetic acid (IAA) were purchased. Asymmetric disulfide compounds were synthesized and used crude with the exception of the purified compound DS2 to confirm that the crude alkylator produces the same results as the purified alkylator.

General Analysis Procedure:

Time points were removed from the test reaction at 0, 30, 60 and 90 minutes for Bruker UltrafleXtreme MALDI-TOF analysis. The sample was prepared by mixing the reaction with the α-cyano-4-hydroxycinnamic acid matrix, which was dissolved in 50/50 acetonitrile/water with 0.1% TFA. The peaks next to the labeled peaks are the Na^+ adduct and not labeled for clarity. Additionally, at the 90-minute time point, the sample was diluted with water and analyzed by analytical HPLC (C-18, 3.5 μm, 2.1 x 150 mm) using a 5-95% acetonitrile in water with 0.1% TFA gradient over 15 minutes. Both analytical techniques subject the sample to 0.1% TFA, which lowers the pH and thus quenches the reaction. A representative analytical HPLC trace of the sample peptide at t = 0 (with no alkylator) is shown below.

K. Alkylation Test Reactions Results

For all test reactions, HBS I_850_Screen was used.

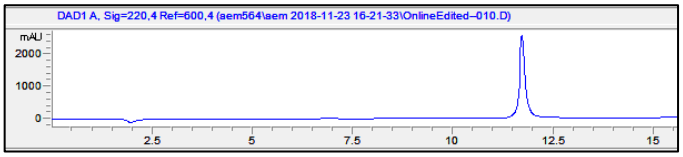
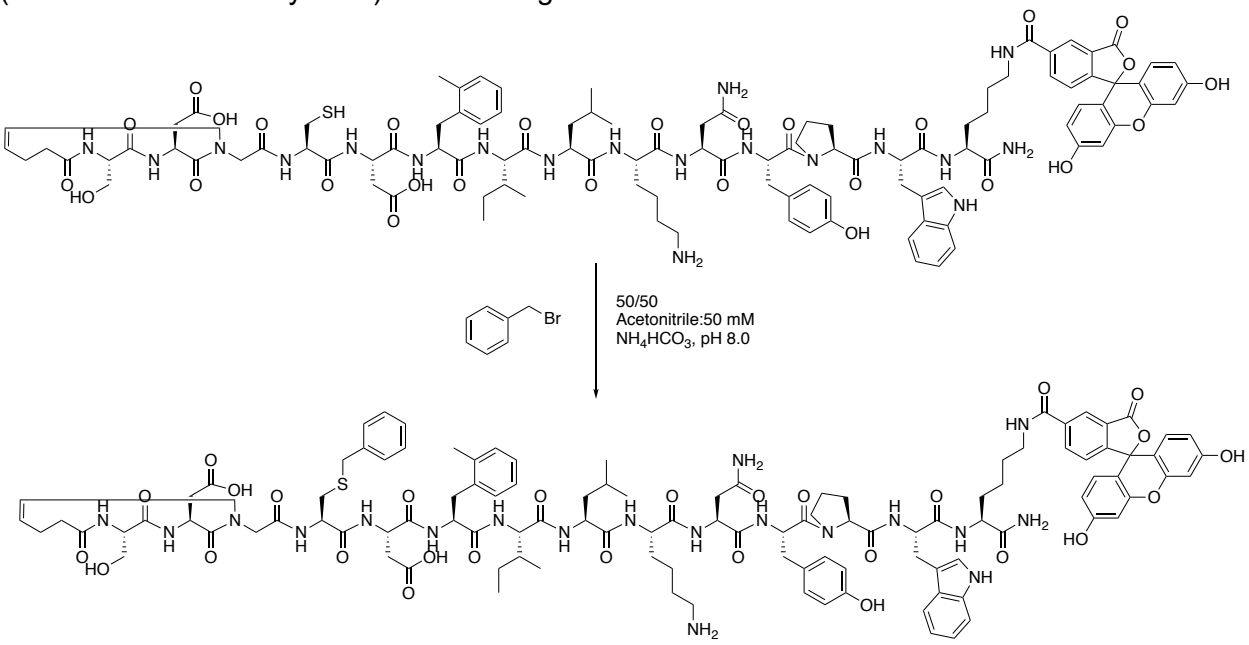


Figure S8: HPLC trace of peptide with no alkylator.

Bromide Alkylation

The benzyl bromide alkylation took place according to the general alkylation procedure. The MALDI-TOF analysis confirms that no disulfides were formed and alkylation only occurred once (there is no double alkylation). The starting material is consumed in 60 minutes.



Scheme S9: Benzyl bromide test reaction scheme.

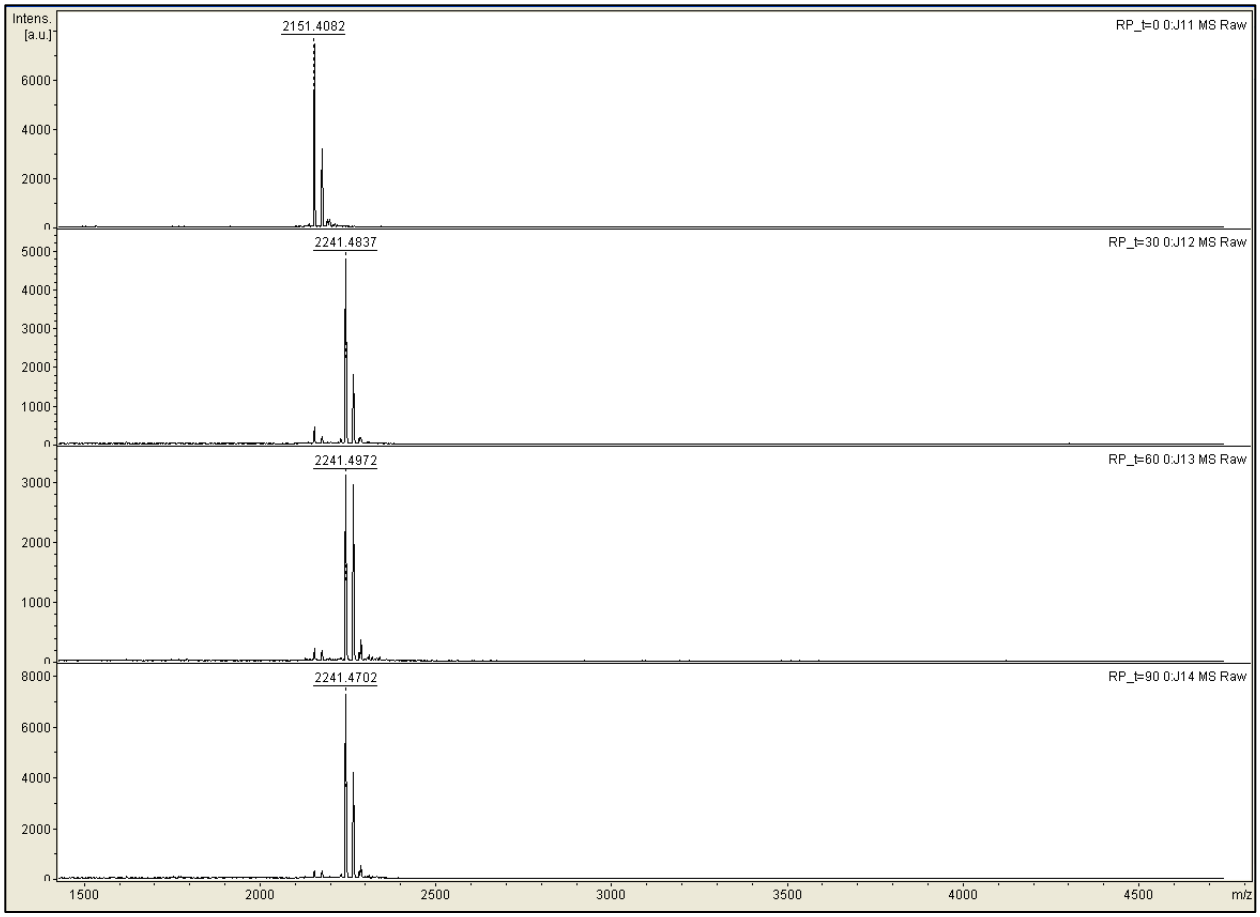


Figure S9: MALDI-TOF data of benzyl bromide test reaction. Masses showing time = 0 minutes (top), followed by 30 minutes, 60 minutes and 90 minutes.

Table S5: Summary of benzyl bromide (BnBr) alkylation test reaction masses.

Compound	Expected Mass $[M+H]^+$	Observed Mass $[M+H]^+$
Starting peptide (Free Thiol)	2150.913	2151.408
Peptide + BnBr Product	2240.960	2241.470
Change in mass	90.047	90.062

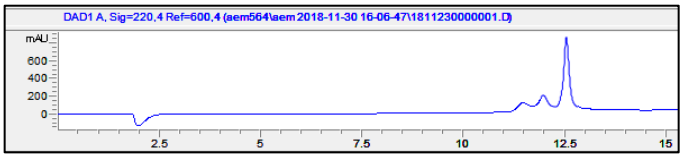
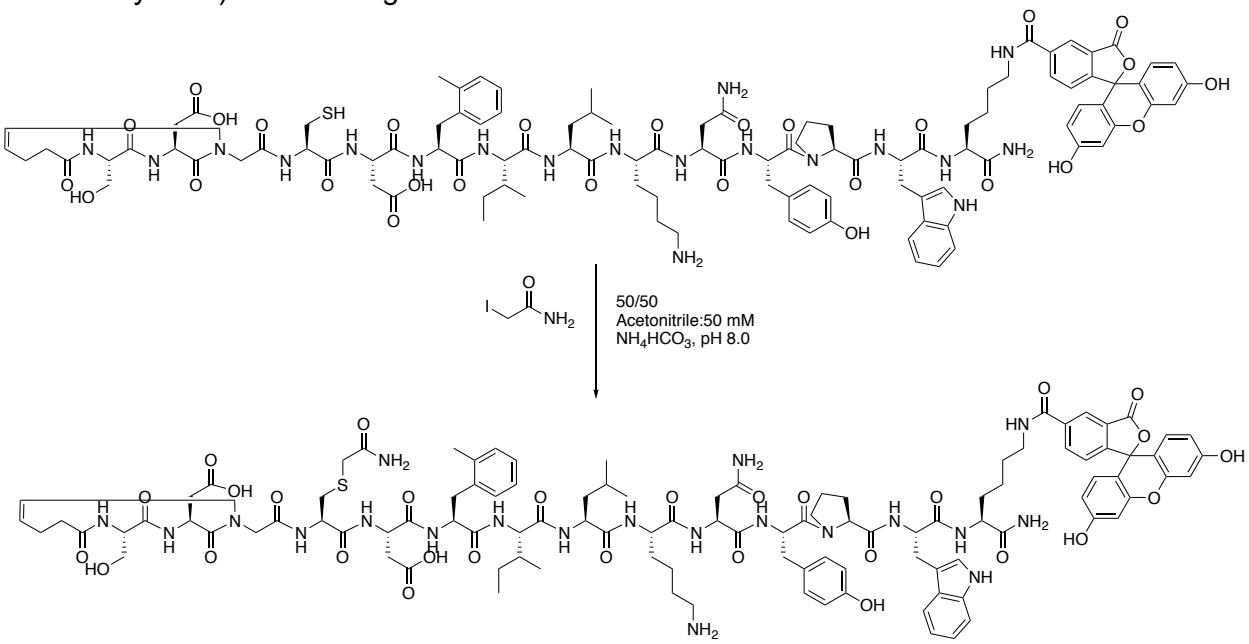


Figure S10: HPLC trace of alkylated peptide of benzyl bromide.

Iodoacetamide Alkylation

The iodoacetamide alkylation took place according to the general alkylation procedure. The MALDI confirms that no disulfides were formed and alkylation only happed once (there is no double alkylation). The starting material is consumed in 30 minutes.



Scheme S10: Iodoacetamide test reaction scheme.

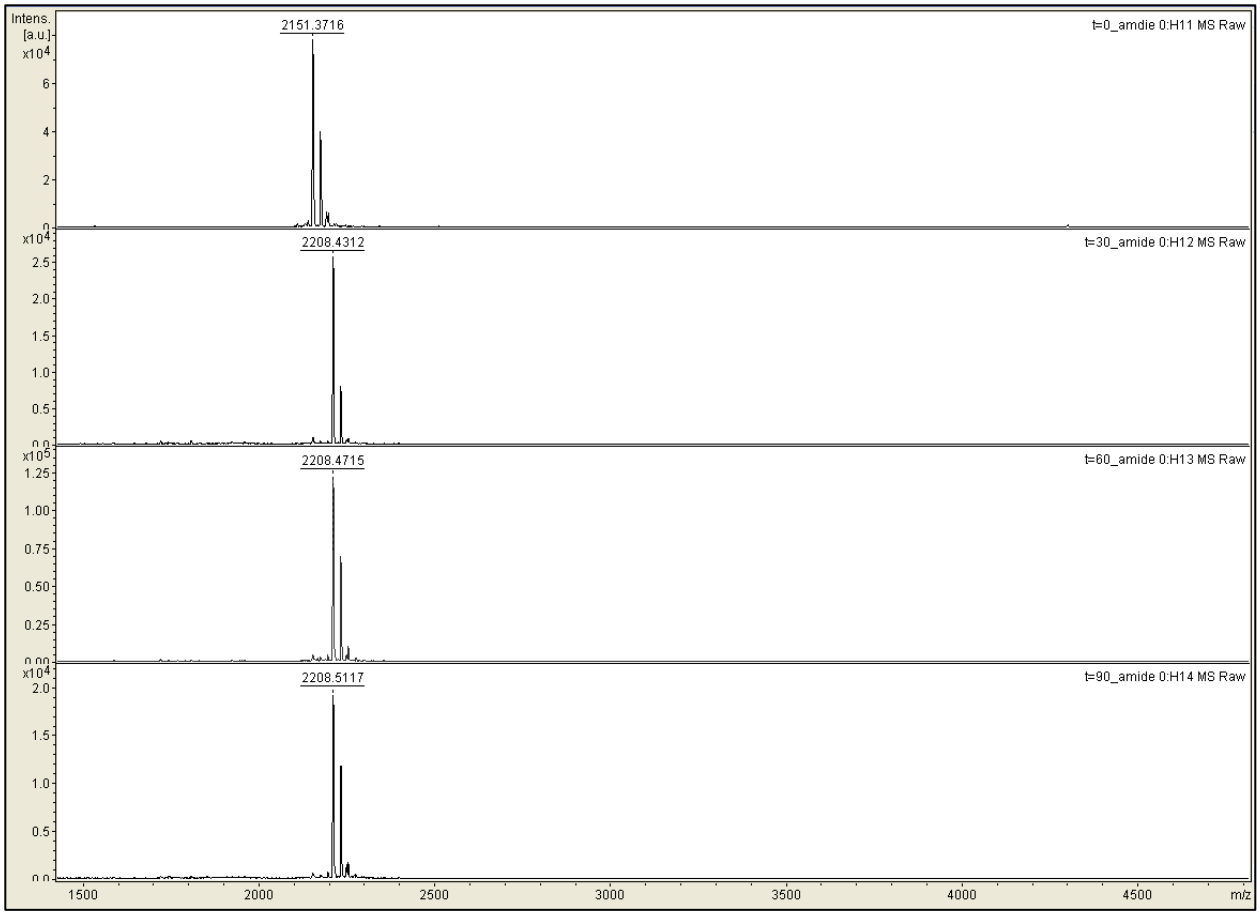


Figure S11: MALDI-TOF data of iodoacetamide test reaction. Masses showing time = 0 minutes (top), followed by 30 minutes, 60 minutes and 90 minutes.

Table S6: Summary of iodoacetamide (IAM) alkylation test reaction masses.

Compound	Expected Mass $[M+H]^+$	Observed Mass $[M+H]^+$
Starting peptide (Free Thiol)	2150.913	2151.372
Peptide + IAM Product	2207.935	2208.512
Change in mass	57.021	57.140

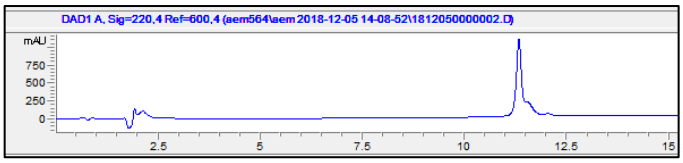
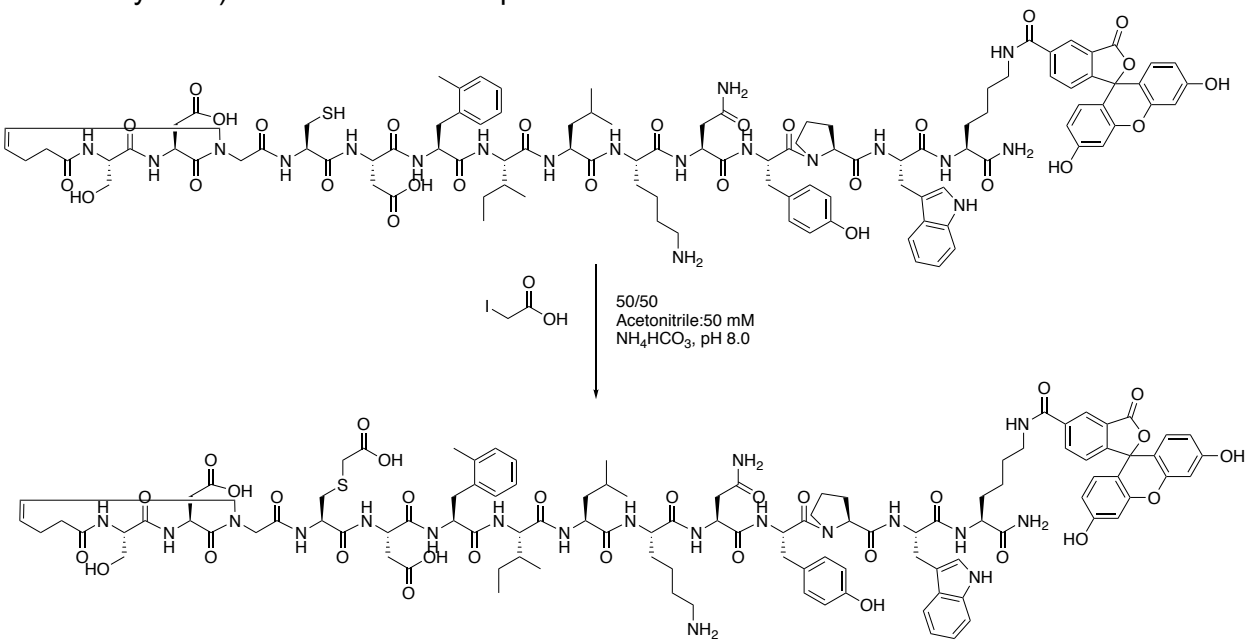


Figure S12: HPLC trace of alkylated peptide of iodoacetamide.

Iodoacetic Acid Alkylation

The iodoacetamide alkylation took place according to the general alkylation procedure. The MALDI confirms that no disulfides were formed and alkylation only happed once (there is no double alkylation). The reaction is completed in 90 minutes.



Scheme S11: Iodoacetic acid test reaction scheme.

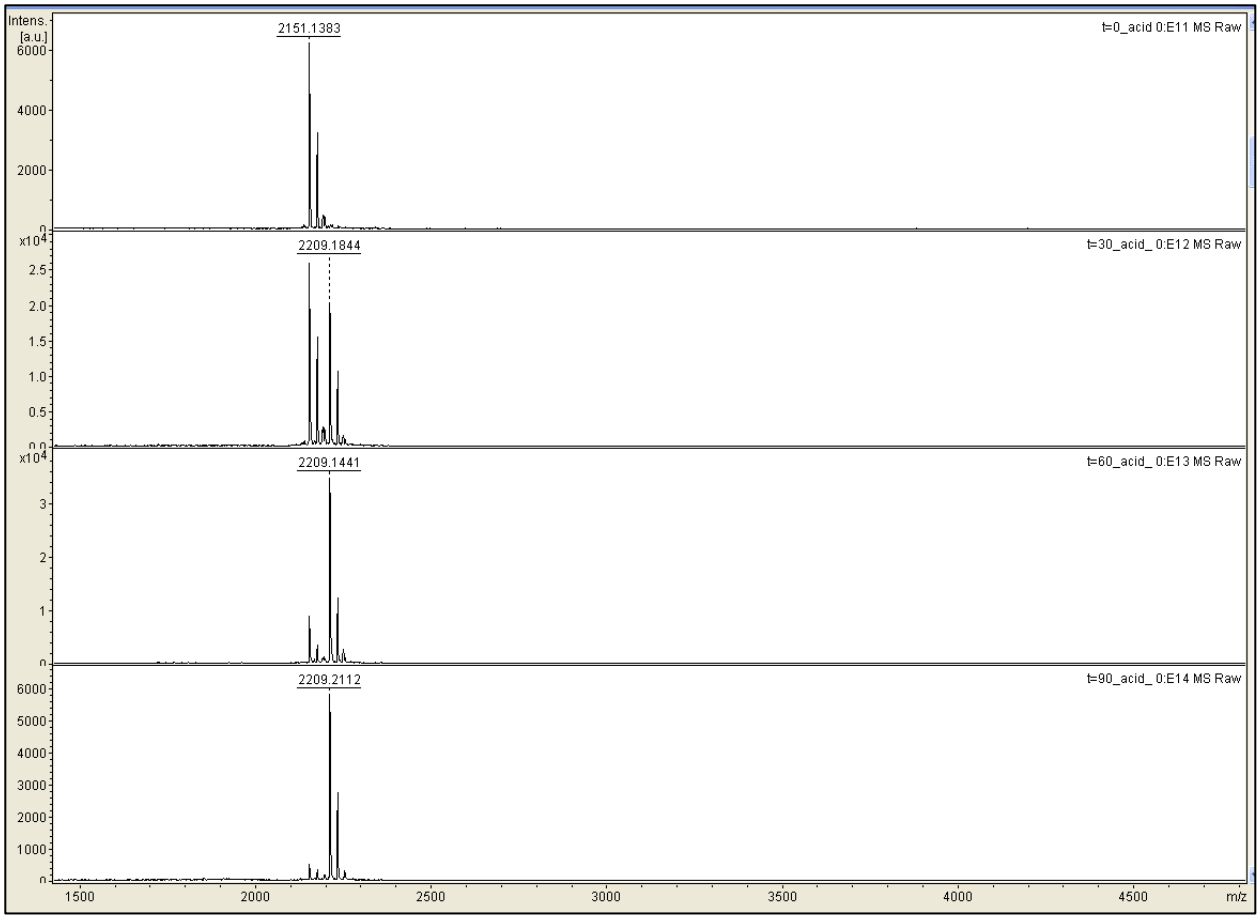


Figure S13: MALDI-TOF data of iodoacetic acid test reaction. Masses showing time = 0 minutes (top), followed by 30 minutes, 60 minutes and 90 minutes.

Table S7: Summary of iodoacetic acid (IAA) alkylation test reaction masses.

Compound	Expected Mass $[M+H]^+$	Observed Mass $[M+H]^+$
Starting Peptide (Free Thiol)	2150.913	2151.138
Peptide + IAA Product	2208.919	2209.211
Change in mass	58.005	58.073

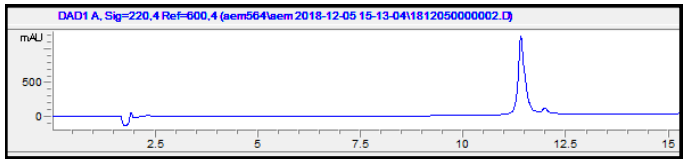
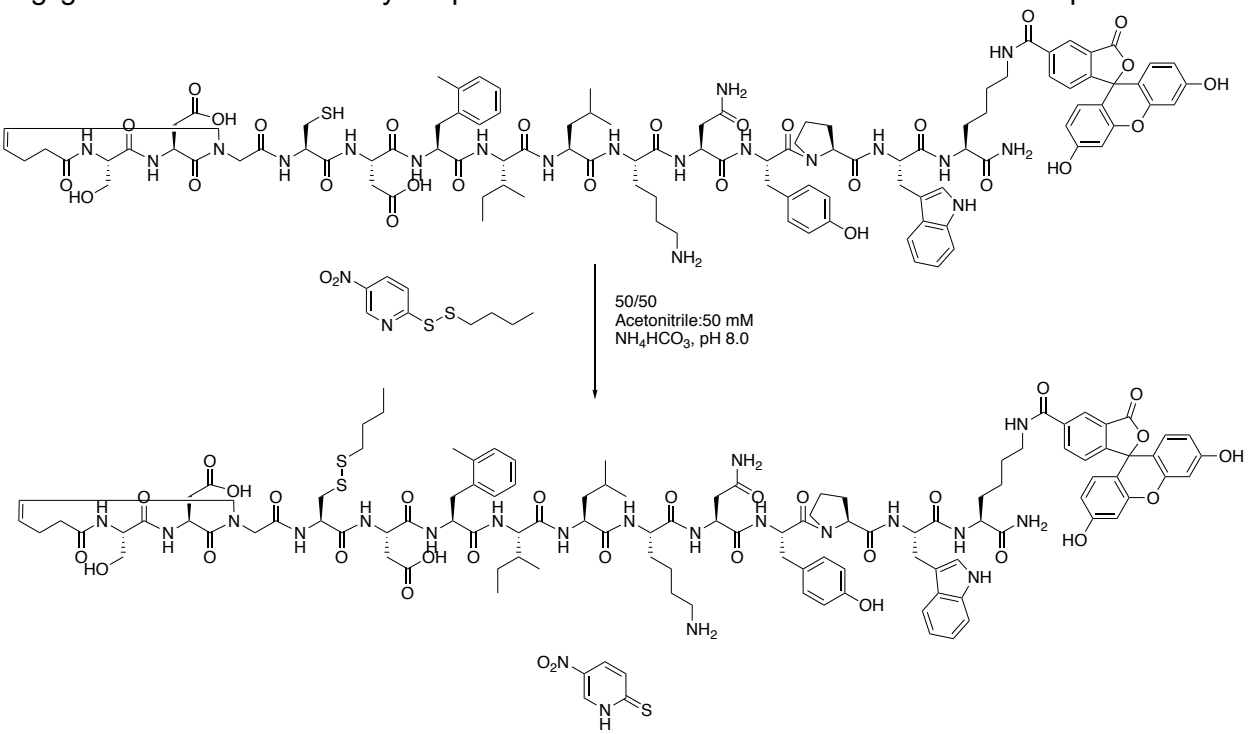


Figure S14: HPLC trace of alkylated peptide of iodoacetic acid.

Activated Disulfide Alkylation

The disulfide alkylation took place according to the general alkylation procedure. The MALDI confirms that no disulfides were formed and alkylation only happed once (there is no double alkylation). The reaction is completed in 90 minutes. The crude disulfide mass was calculated as 4.5 mM of the total weight of the crude product (i.e.,DS-2: 244.0340 + 155.9993 g/mol) to account for the side product produced at 1 equivalent. The 0.2 eq. of thiol leftover is accounted as negligible since it is most likely evaporated off after concentration of the crude compound.



Scheme S12: Activated disulfide test reaction scheme.

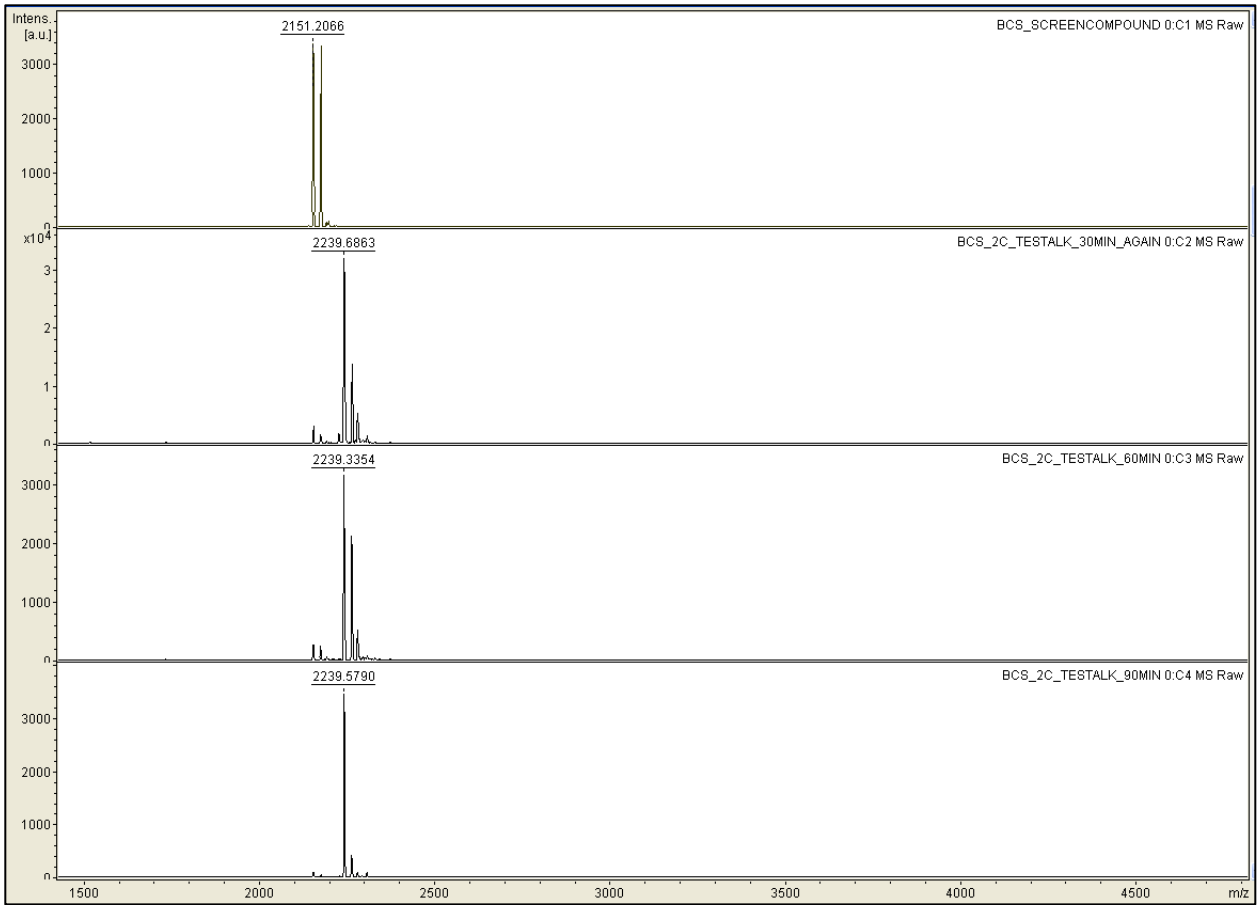


Figure S15: MALDI-TOF data of disulfide test reaction. Masses showing time = 0 minutes (top), followed by 30 minutes, 60 minutes and 90 minutes.

Table S8: Summary of disulfide alkylation test reaction masses.

Compound	Expected Mass $[M+H]^+$	Observed Mass $[M+H]^+$
Starting Peptide (Free Thiol)	2150.913	2151.207
Peptide + DS Product	2238.948	2239.57
Change in mass	88.035	88.366

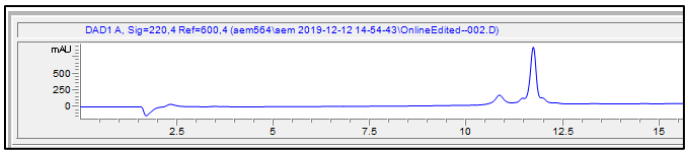


Figure S16: HPLC trace of alkylated peptide with disulfide using pure DS2.

L. Screening Assay Methods

General Screening Protocol:

Alkylators were prepared as performed in the test reactions with a final concentration of peptide at 150 μM and alkylator at 750 μM. All reactions proceeded for 90 minutes (the time it takes for all test reactions to be completed). Upon completion of the reaction (confirmed by MALDI-TOF consumption of starting material), each alkylated peptide was diluted in PBS pH 7.4 with 10% glycerol and 0.1% Pluronic acid F-127 (Sigma) to a concentration of 30 nM. Equal amounts of alkylated peptide and KIX protein were mixed to produce 15 nM of alkylated peptide in the well with 40 μM of KIX in triplicate in a 384 well plate format. The alkylators were also mixed with buffer (PBS pH 7.4 with 10% glycerol and 0.1% Pluronic acid) for a blank reading in triplicate. Fluorescence polarization was read after 60 minutes. The blank reading for each alkylated peptide was averaged and subtracted from the experiment wells. Screens were performed in triplicate and repeated at least once.

Initial disulfide crude versus pure disulfide test:

The pure disulfide mass was calculated as the purified product (244.0340 g/mol) in the test reaction and the screen protocol was used to compare the crude disulfide alkylator with the purified one. Results are within error and confirm that the crude alkylator produces similar results to the purified alkylator.

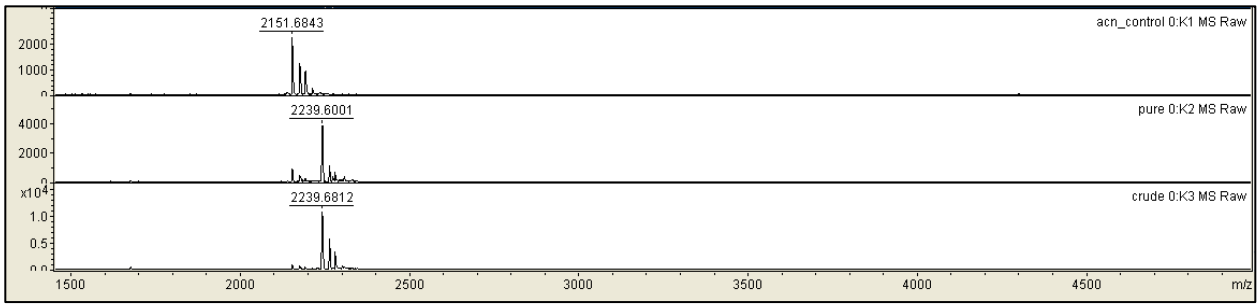


Figure S17: Alkylated peptide with pure and crude disulfide. Acetonitrile control is at the top. Alkylated mass with pure DS2 is in the middle, and alkylated mass with crude DS2 is shown at the bottom.

Table S9: Disulfide (DS) alkylation test reaction masses - Pure DS2.

Compound	Expected Mass $[M+H]^+$	Observed Mass $[M+H]^+$
Starting Peptide (Free Thiol)	2150.913	2151.684
Peptide + DS Product (Pure)	2238.948	2239.681
Change in mass	88.035	87.997

Table S10: Disulfide (DS) alkylation test reaction masses - Crude DS2.

Compound	Expected Mass $[M+H]^+$	Observed Mass $[M+H]^+$
Starting Peptide (Free Thiol)	2150.913	2151.684
Peptide + DS Product (Crude)	2238.948	2239.948
Change in mass	88.035	88.264

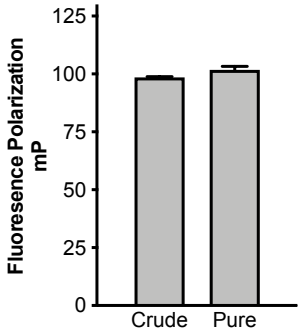


Figure S18: Crude versus pure disulfide results. Alkylation of the Bcs to C peptide with crude DS2 versus pure DS2.

M. Screening Assay Results

Bcs to C Screen using peptide HBS I_850_Screen

A)

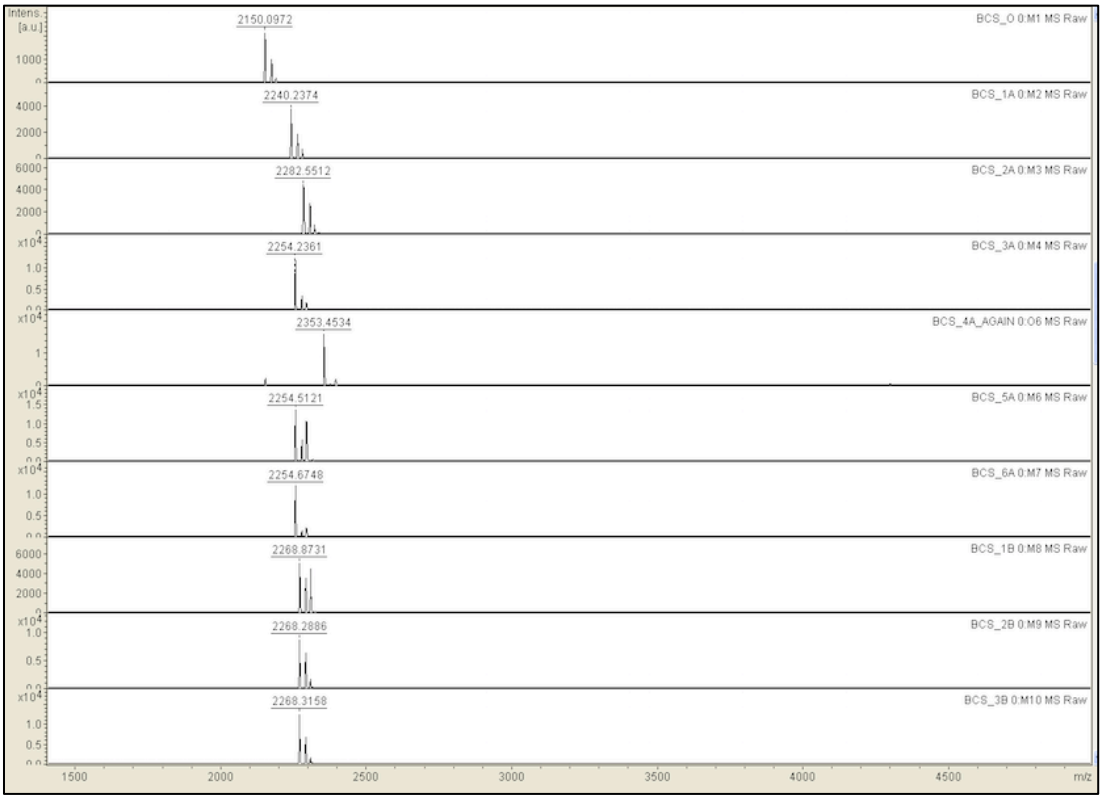
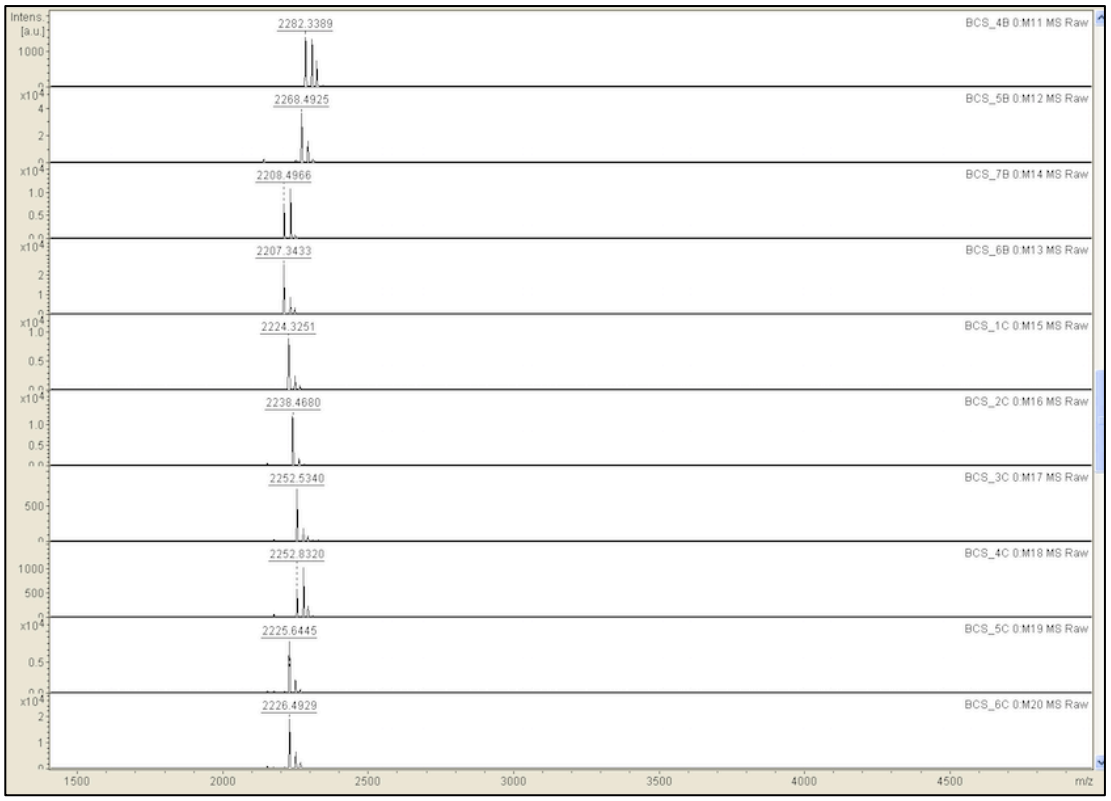


Figure S19: A) MALDI data showing alkylation of HBS I_850_Screen with fragments.

B)



C)

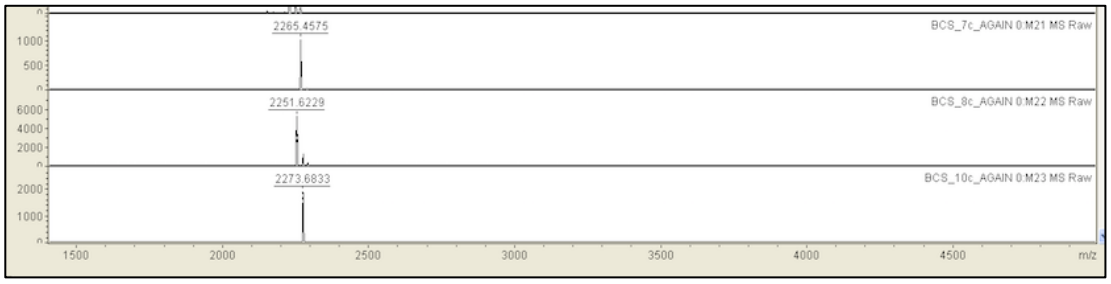


Figure S19: B-C) MALDI data showing alkylation of HBS I_850_Screen with fragments cont.

Table S11: MALDI summary of Bcs to C. Screen at 850.

Fragment	Expected Mass $[M+H]^+$	Observed Mass $[M+H]^+$
0 - No Alkylator	2150.913	2150.097
1	2240.960	2240.237
2	2283.007	2282.551
3	2254.976	2254.236
4	2353.085	2353.453
5	2254.976	2254.512
6	2254.976	2254.675
7	2268.991	2268.873
8	2268.991	2268.289
9	2268.991	2268.316
10	2283.007	2282.339
11	2268.991	2268.493
12	2208.919	2208.497
13	2207.935	2207.343
14	2224.932	2224.364
15	2238.948	2238.468
16	2252.964	2252.534
17	2252.964	2252.832
18	2225.927	2225.645
19	2226.912	2226.493
20	2264.964	2265.458
21	2250.948	2251.623
22	2272.932	2273.683

Y to C Screen using peptide HBS I_857_Screen

A)

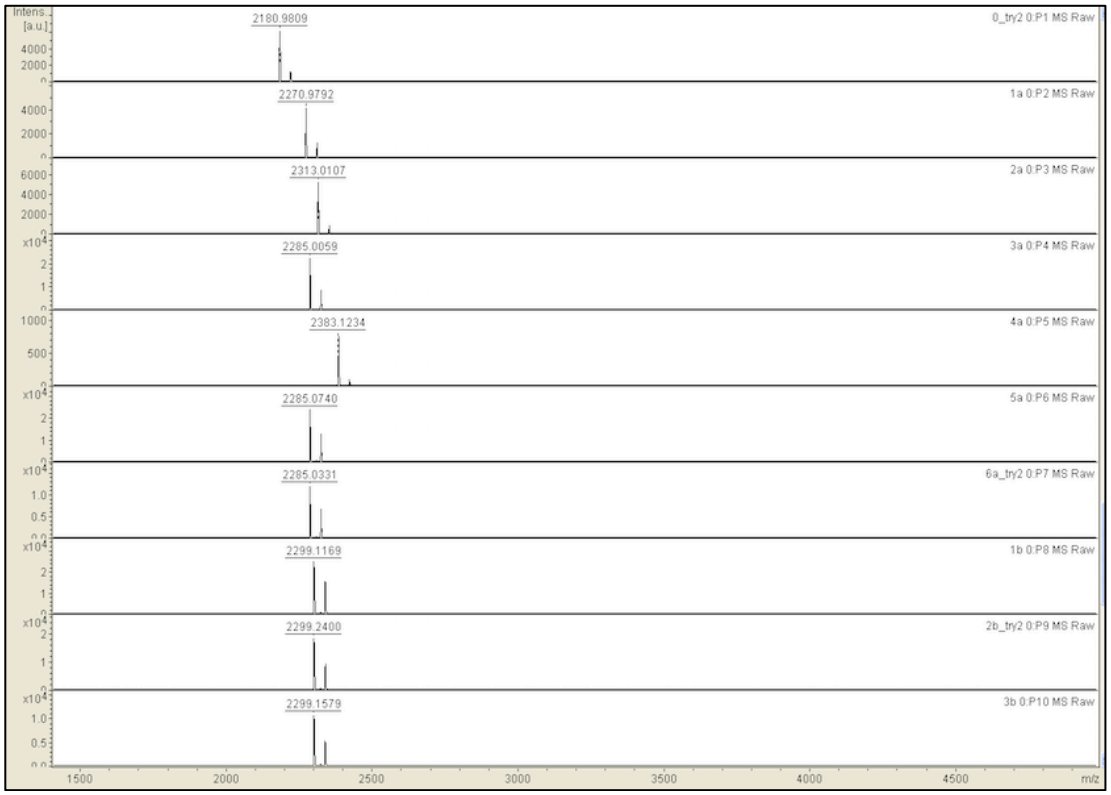
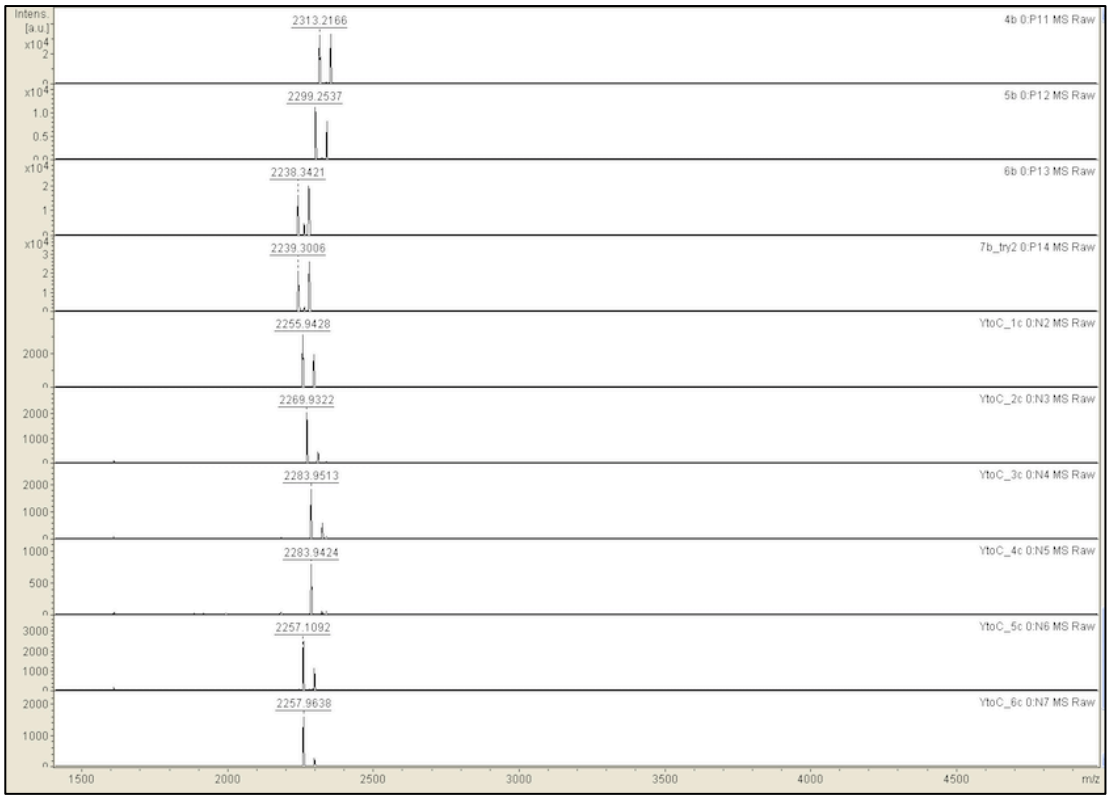


Figure S20: A) MALDI data showing alkylation of HBS I_857_Screen with fragments.

B)



C)

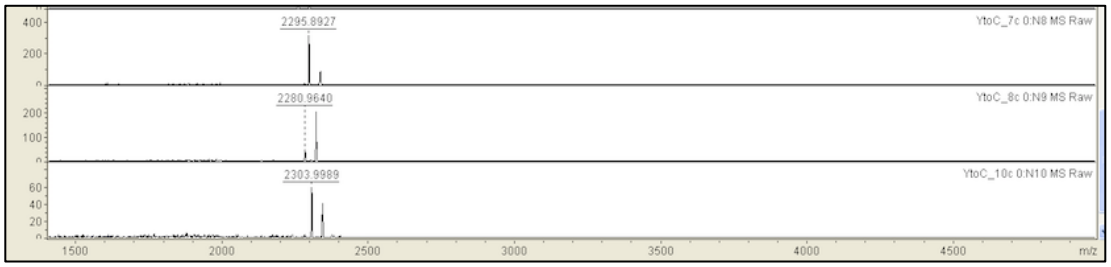


Figure S20: B-C) MALDI data showing alkylation of HBS I_857_Screen with fragments cont.

Table S12: MALDI summary of Y to C. Screen at 857.

Fragment	Expected Mass $[M+H]^+$	Observed Mass $[M+H]^+$
0 - No Alkylator	2180.906	2180.981
1	2270.953	2270.979
2	2313.000	2313.011
3	2284.969	2285.006
4	2383.078	2383.123
5	2284.969	2285.074
6	2284.969	2285.033
7	2298.984	2299.117
8	2298.984	2299.240
9	2298.984	2299.158
10	2313.000	2313.217
11	2298.984	2299.254
12	2238.911	2238.342
13	2237.927	2239.301
14	2254.925	2255.943
15	2268.941	2269.932
16	2282.956	2283.951
17	2282.956	2283.942
18	2255.920	2257.109
19	2256.904	2257.964
20	2294.956	2295.893
21	2280.941	2280.964
22	2302.925	2303.999

P to C Screen using peptide HBS I_858_Screen

A)

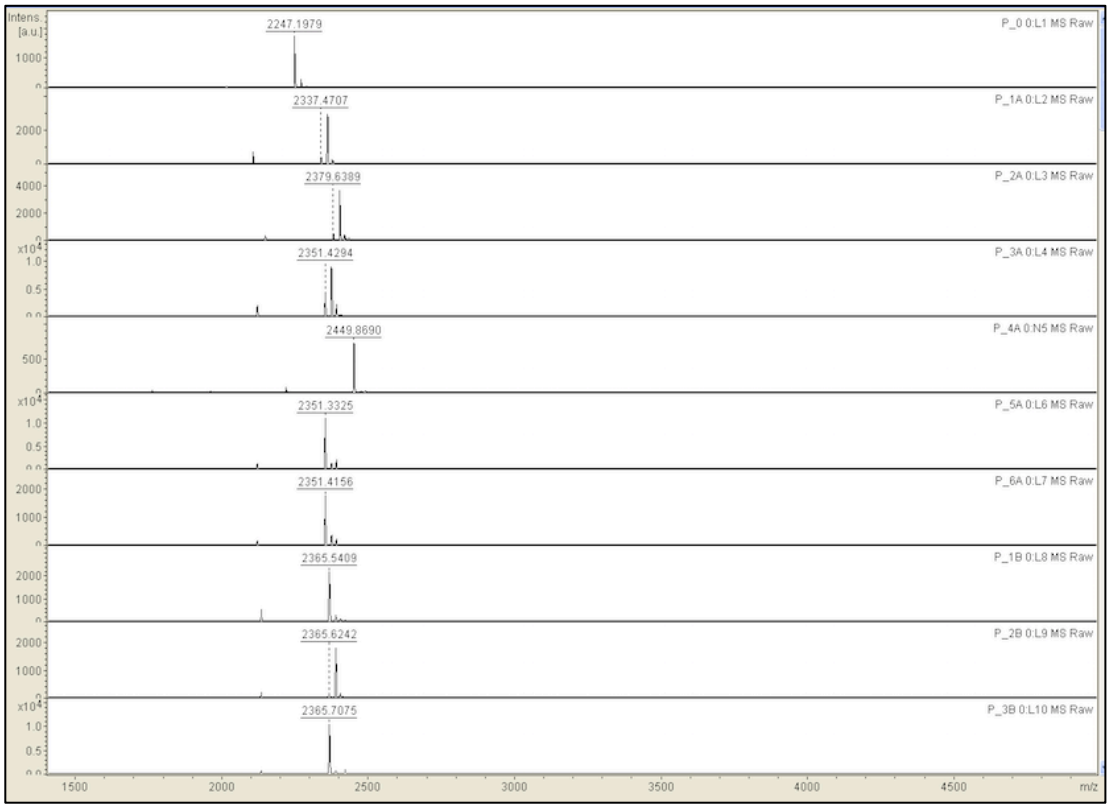
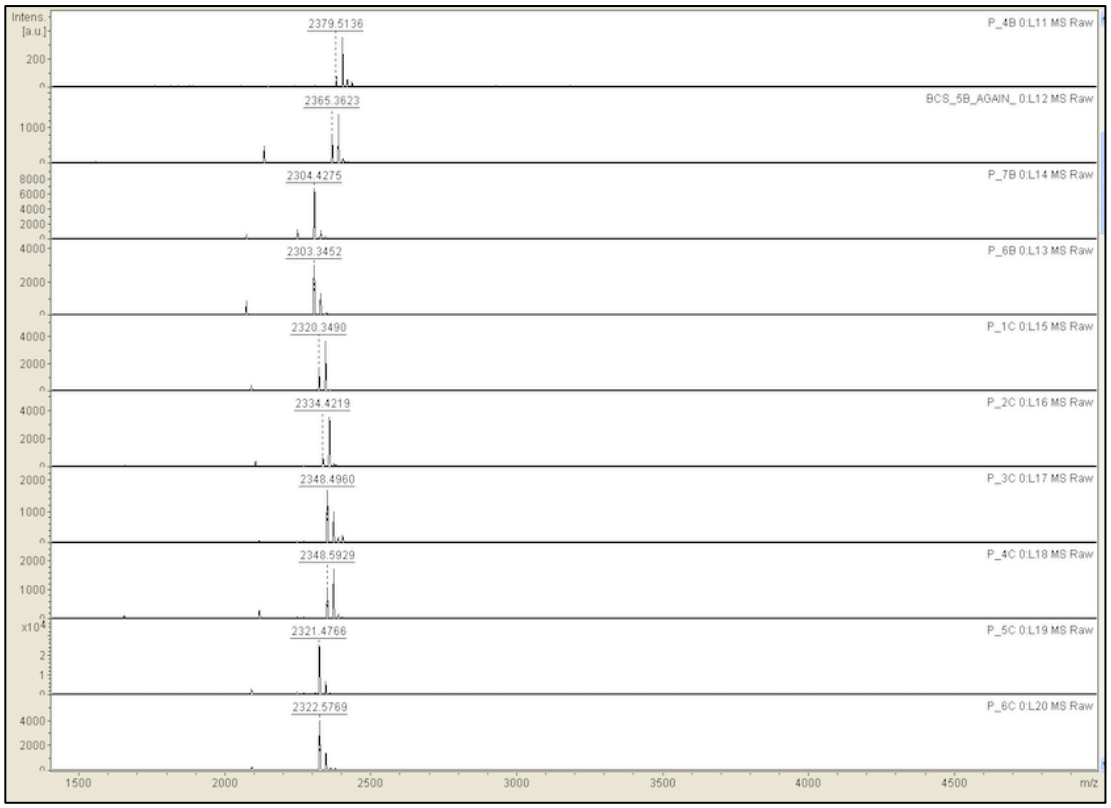


Figure S21: A) MALDI data showing alkylation of HBS I_858_Screen with fragments.

B)



C)

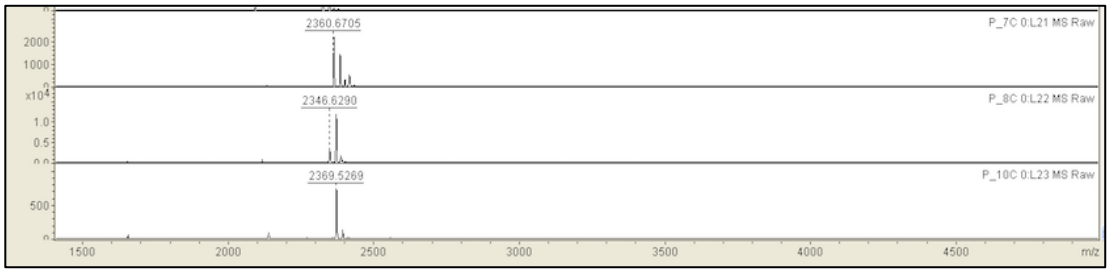


Figure S21: B-C) MALDI data showing alkylation of HBS I_858_Screen with fragments cont.

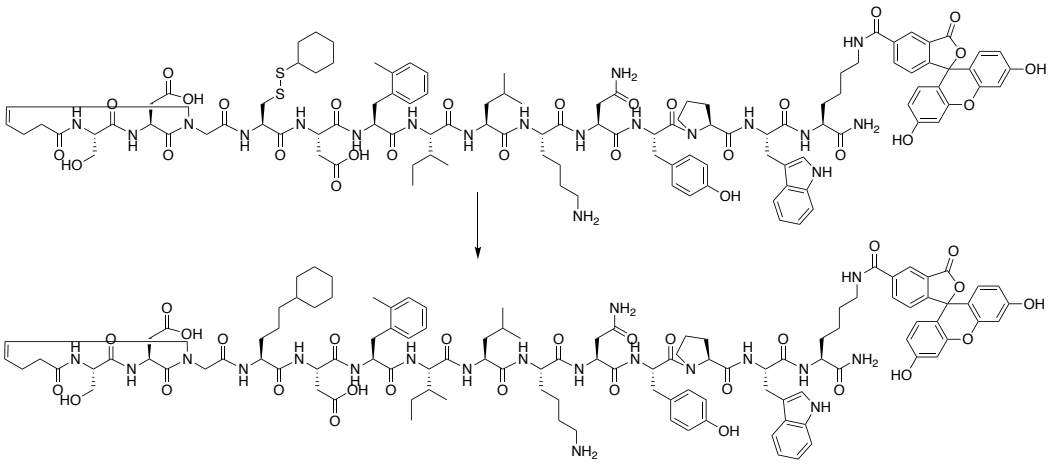
Table S13: MALDI summary of P to C. Screen at 850.

Fragment	Expected Mass $[M+H]^+$	Observed Mass $[M+H]^+$
0 - No Alkylator	2246.917	2247.198
1	2336.963	2337.471
2	2379.010	2379.639
3	2350.979	2351.429
4	2449.089	2449.869
5	2350.979	2351.333
6	2350.979	2351.416
7	2364.995	2365.541
8	2364.995	2365.624
9	2364.995	2365.708
10	2379.010	2379.514
11	2364.995	2365.362
12	2304.922	2304.428
13	2303.938	2303.345
14	2320.936	2320.349
15	2334.951	2334.422
16	2348.967	2348.496
17	2348.967	2348.593
18	2321.931	2321.477
19	2322.915	2322.577
20	2360.967	2360.671
21	2346.951	2346.629
22	2368.936	2369.527

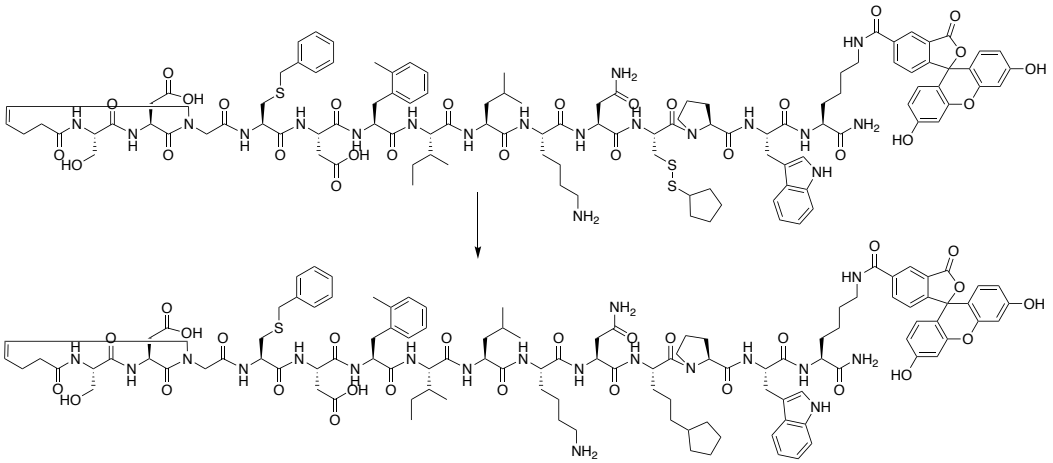
N. Designs of Cell-Stable Analogues from Screening Hits

Disulfide bonds were replaced with aliphatic linkages for cyclohexane and cyclopentane hits (Figure S22A and B), and the iodoacetic acid hit had no modifications (Figure S22C)

A)



B)



C)

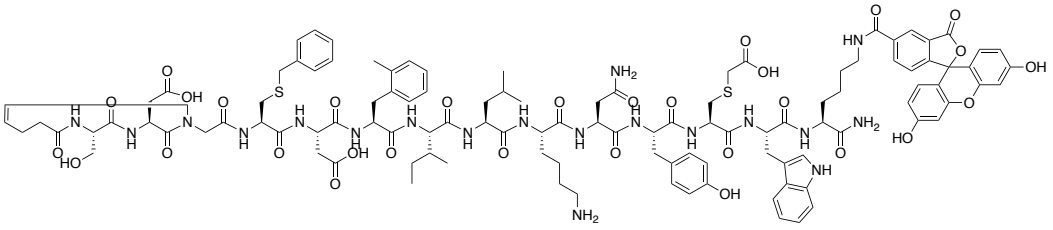


Figure S22: Design of stable analogues. (A) Top: hit from screen at the 850 Bcs position. Bottom: cell-stable analogue HBS III*. (B) Top: hit from screen at the 857 Y position. Bottom: cell-stable analogue HBS IV*. (C) Hit from screen at the 858 P position. Bottom: cell-stable analogue HBS V*.

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