Supporting Information

Discovery of a potent and selective targeted NSD2 degrader for reduction of H3K36me2

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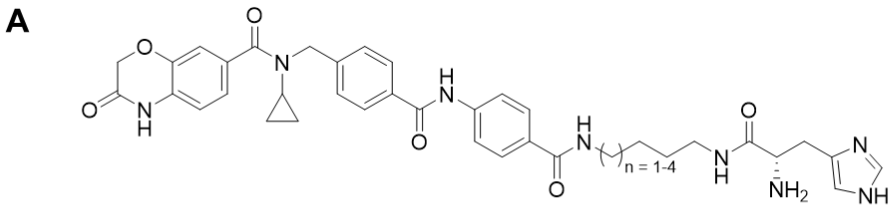


Compound	n =	SPR k_D (nM)	D_{max}	DC_{50} (μM)
UNC7985	1	25	13%	N.D.
UNC7895	2	30	19%	N.D.
UNC7749	3	23	58%	8.50 [5.81-13.37]
UNC8010	4	55	31%	N.D.

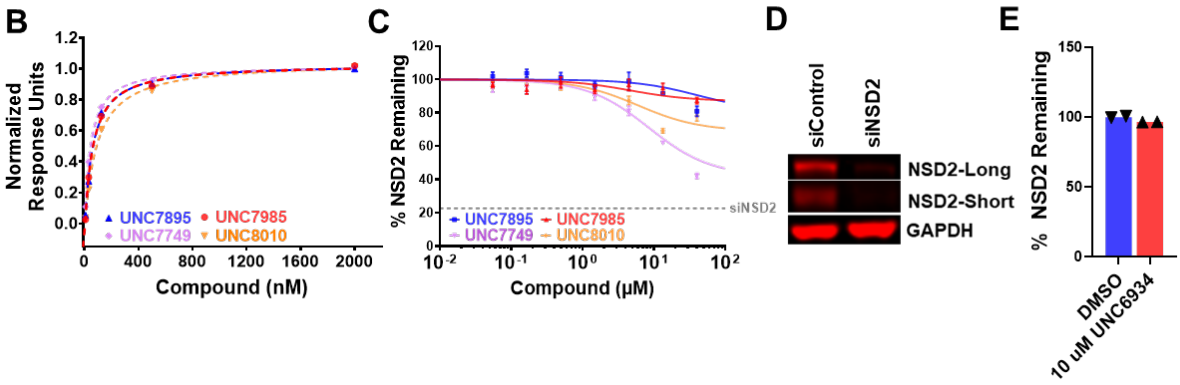


Figure S1, Relates to Figure 2.

(A) Chemical structures of histidine-containing degraders. Compound binding affinities for recombinant NSD2-PWWP1 were determined by SPR in a single experiment. NSD2 degradation was evaluated by an ICW assay in U2OS cells dosed with compound for 24h. D_{max} and DC_{50} [95% CI] values from one experiment with 4 technical replicates are shown. N.D: DC_{50} values not determined for compounds with D_{max} values < 50%.

(B) Representative NSD2-PWWP1 SPR traces of all compounds listed in (A). SPR response units were normalized with the highest response unit = 1.00 for each set of curves.

(C): ICW dose-response degradation curves for compounds listed in (A). 4 technical replicates were performed for each condition. Grey dashed line denotes the NSD2 levels measured by ICW upon siRNA knockdown of NSD2 in U2OS cells.

(D): Immunoblot validation of NSD2 siRNA knockdown.

(E): NSD2 levels measured by ICW upon treatment of U2OS cells with 10 μM UNC6934 relative to DMSO for 24h. Two technical replicates were performed for each condition.

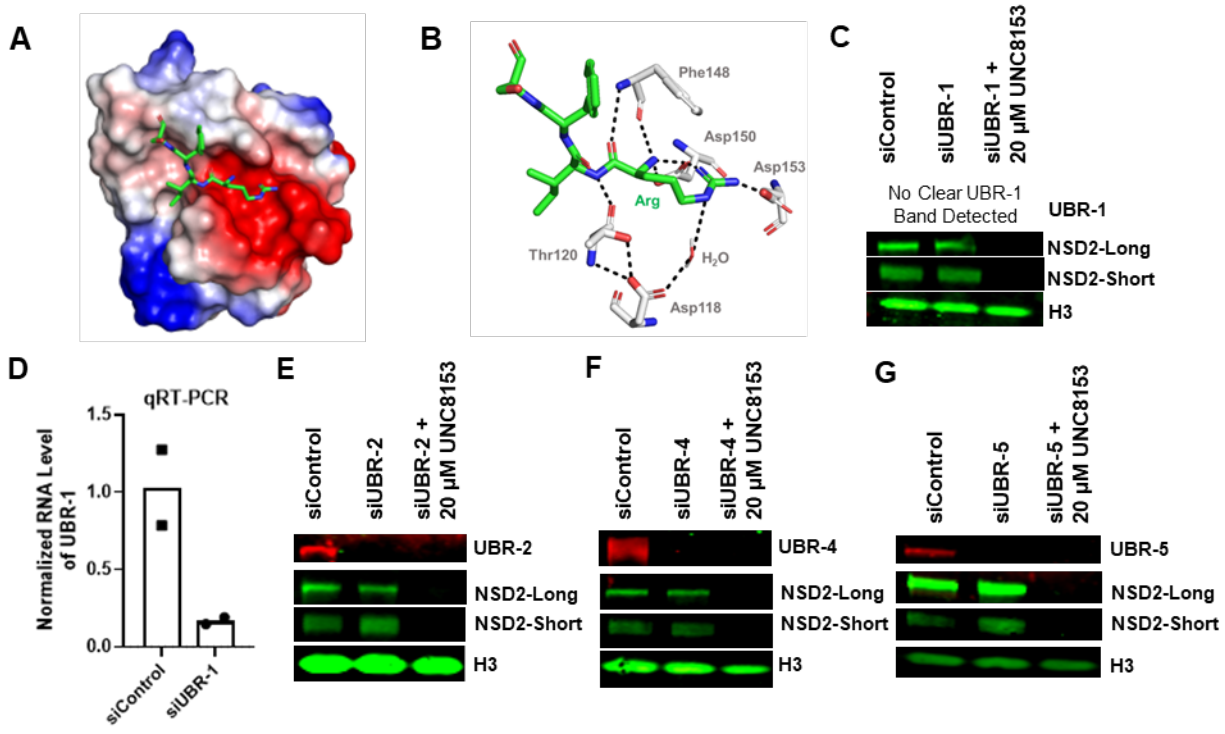


Figure S2, Relates to Figure 3.

(A): Structure of an Arg N-degron (green) interacting with the negatively charged binding pocket of the UBR-box of UBR2 (acidic residues shown in red) (PDB: 3NY3).

(B): Molecular interactions for the structure shown in (A) (PDB: 3NY3).

(C): Evaluation of the reduction of NSD2 levels upon UBR-1 knockdown and UBR-1 knockdown in combination with 20 μM UNC8153 in U2OS cells. Samples immunoblotted for NSD2, H3, and UBR-1. No clear band was observed for UBR-1.

(D): Validation of UBR-1 siRNA knockdown by qRT-PCR due to the absence of a clear UBR-1 band in immunoblot in (C).

(E): Evaluation of the reduction of NSD2 levels upon UBR-2 knockdown and UBR-2 knockdown in combination with 20 μM UNC8153 in U2OS cells. Samples immunoblotted for NSD2, H3 and UBR-2.

(F): Evaluation of the reduction of NSD2 levels upon UBR-4 knockdown and UBR-4 knockdown in combination with 20 μM UNC8153 in U2OS cells. Samples immunoblotted for NSD2, H3 and UBR-4.

(G): Evaluation of the reduction of NSD2 levels upon UBR-5 knockdown and UBR-5 knockdown in combination with 20 μM UNC8153 in U2OS cells. Samples immunoblotted for NSD2, H3 and UBR-5.

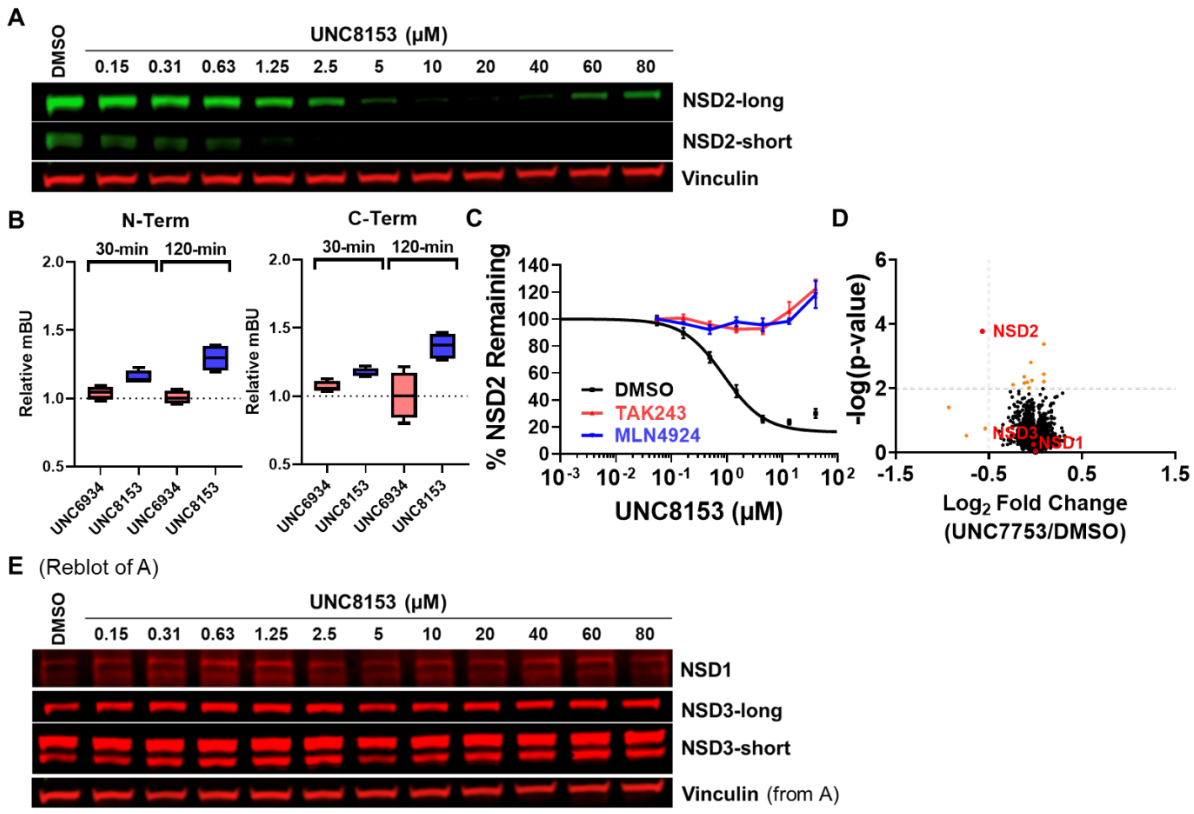


Figure S3, Relates to Figure 4.

(A): Immunoblot of NSD2 following dose-response treatment of UNC8153 in U2OS cells. Protein loading and membrane exposure intensity were increased to visualize weak bands.

(B): NanoBRET ubiquitination assay following treatment with 20 μM UNC6934 (control) or UNC8153 using N-terminal or C-terminal NanoLuc-tagged NSD2-PWWP1 and HaloTag-Ubiquitin. All BRET Units (mBU) were normalized to DMSO control treatment (=1.00) in the same experiment. Each condition was analyzed in 4 independent experiments with each represented as the average of 3 technical replicates.

(C): In-cell western dose-response curves upon UNC8153 treatment for 6h in the absence (black) or presence of 1 μM TAK243 ubiquitin E1 inhibitor (red) or 1 μM MLN4924 neddylation inhibitor (blue) in U2OS cells. Each condition was analyzed with 3 technical replicates.

(D): Unbiased global proteomics experiments using tandem mass tag quantification comparing DMSO vs. 5 μM UNC7753-treated U2OS cells for 6h.

(E): Immunoblot from Figure S3A reblotted for NSD1 and NSD3.

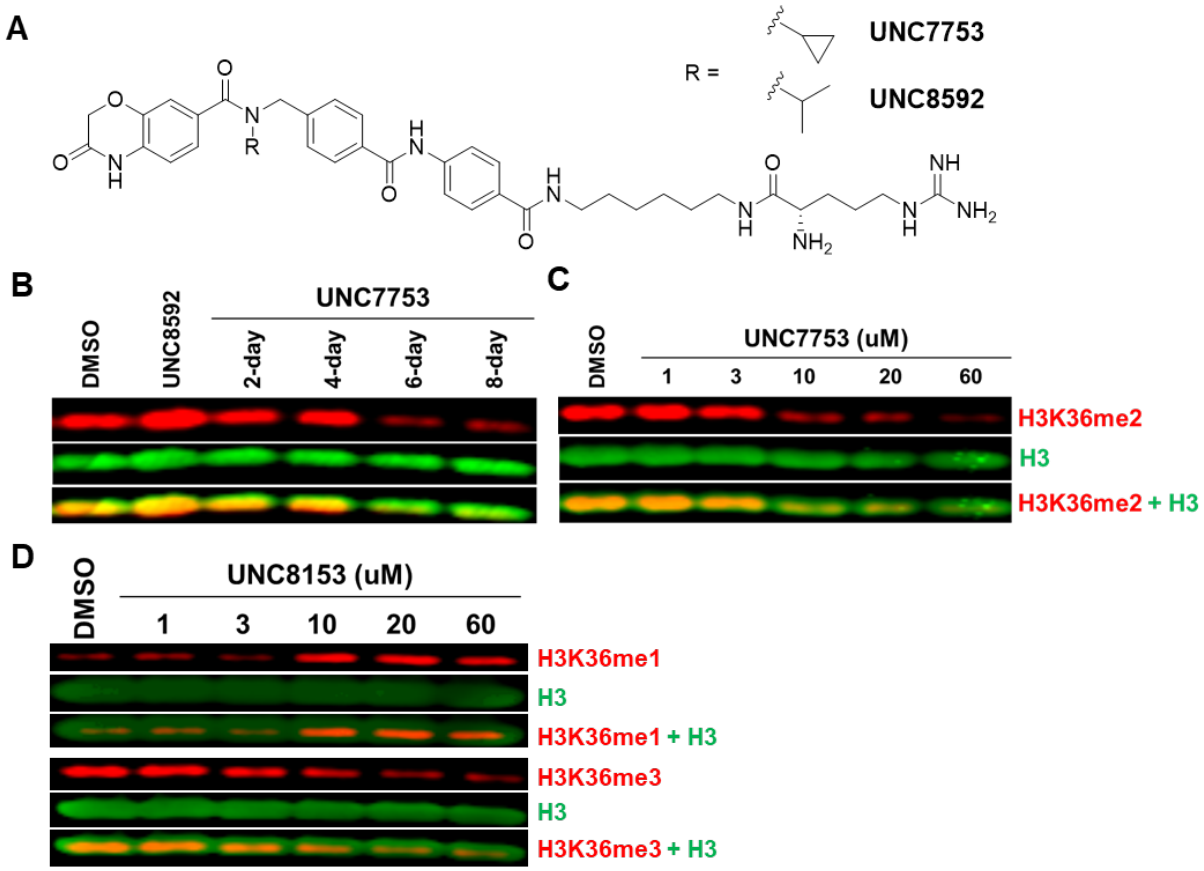


Figure S4, Relates to Figure 5.
(A): Structure of Arg-containing active degrader, UNC7753, and corresponding negative control, UNC8592.
(B): H3K36me2 immunoblot of KMS11 cell pellets from a 2 to 8 day time course treatment with 20 µM UNC7753 and an 8 day treatment with 20 µM UNC8592 and DMSO. H3 is a loading control.
(C): H3K36me2 immunoblot of a 6-day treatment of KMS11 cells with UNC7753 at 1 – 60 µM. H3 is a loading control.
(D): H3K36me1 and H3K36me3 immunoblot of a 6-day treatment of KMS11 cells with UNC8153 at 1 – 60 µM. H3 is a loading control.

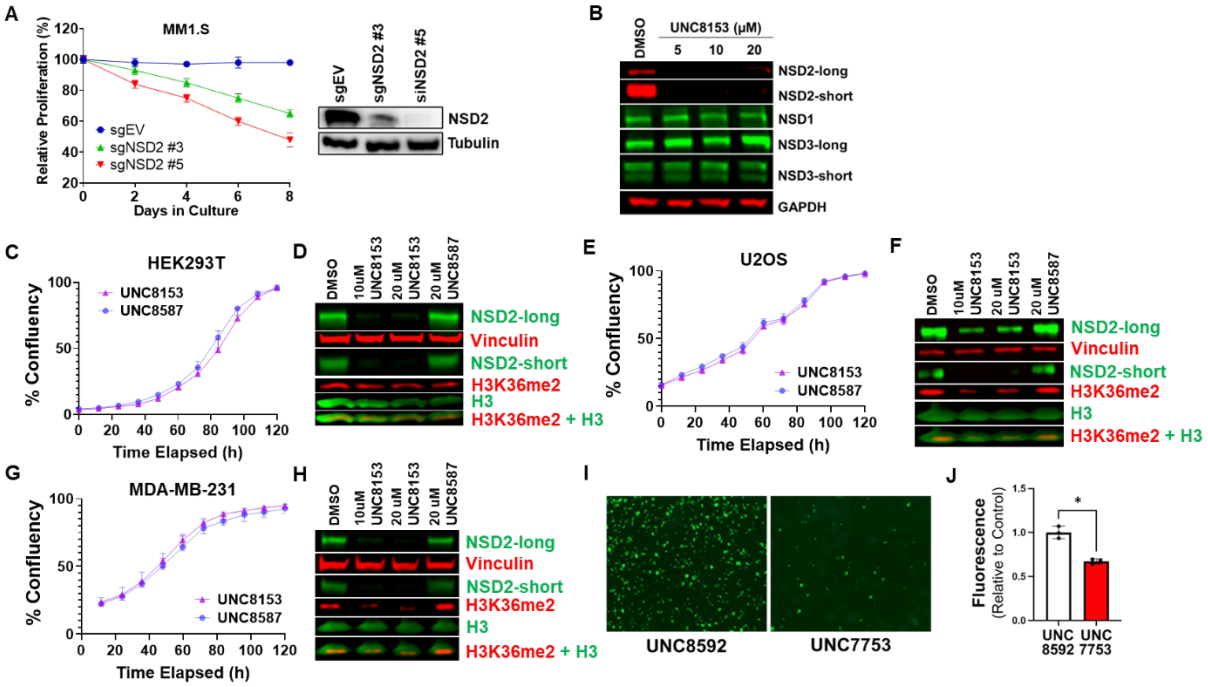


Figure S5, Relates to Figure 6.

(A): Relative proliferation of MM1.S cells after CRISPR/cas9-mediated gene depletion of NSD2 by two sgRNA sequences compared to empty vector control (left). Proliferation was measured by cell viability counts after trypan blue staining. Data are reported as the mean of three replicates ± SD. CRISPR/cas9 sgRNAs targeting NSD2 were validated by immunobloting (right).

(B): Immunoblot of NSD1-3 after 24h of UNC8153 treatment at specified concentrations in MM1S cells.

(C) Growth curve of HEK293T cells treated with 20 µM UNC8587 (control) or 20 µM UNC8153 up to 5 days (n = 3 cell culture wells, mean ± S.D.).

(D) Immunoblot of NSD2 and H3K36me2 from HEK293T cells treated with 10 µM UNC8153, 20 µM UNC8153, or 20 µM UNC8587 for 5 days as shown in (A). Vinculin and H3 are loading controls.

(E) Growth curve of U2OS cells treated with 20 µM UNC8587 (control) or 20 µM UNC8153 up to 5 days (n = 3 cell culture wells, mean ± S.D.).

(F) Immunoblot of NSD2 and H3K36me2 from U2OS cells treated with 10 µM UNC8153, 20 µM UNC8153, or 20 µM UNC8587 for 5 days as shown in (C). Vinculin and H3 are loading controls.

(G) Growth curve of MDA-MB-231 cells treated with 20 µM UNC8587 (control) or 20 µM UNC8153 up to 5 days (n = 3 cell culture wells, mean ± S.D.).

(H) Immunoblot of NSD2 and H3K36me2 from MDA-MB-231 cells treated with 10 µM UNC8153, 20 µM UNC8153, or 20 µM UNC8587 for 5 days as shown in (E). Vinculin and H3 are loading controls.

(I) Representative microscopic images from adhesion assays with green fluorescent KMS11 cells treated with 25 µM UNC8592 (control) or UNC7753 for 14 days. Cells were plated in Matrigel-coated plates for 12 h, and images were acquired after washing plates with PBS at 10X magnification.

(J) Fluorescence intensity of adherent cells treated with 25 μM UNC8587 or 25 μM UNC8153. Data are represented as mean fluorescence intensities (from 3 independent experiments) relative to control ± SD. * $P<0.05$ (one-tailed t-test).

Table S1, Relates to Methods: Reagents Used.

Gene	siRNA Product Name	Cat. ID
Control	MISSION® siRNA Universal Negative Control #1	Millipore Sigma SIC001
UBR1	siGENOME Human UBR1 siRNA, SMARTPool	Horizon Discovery M-010691-02-0005
UBR2	siGENOME Human UBR2 siRNA, SMARTPool	Horizon Discovery M-006954-01-0005
UBR4	siGENOME Human UBR4 siRNA, SMARTPool	Horizon Discovery M-014021-01-0005
UBR5	siGENOME Human UBR5 siRNA, SMARTPool	Horizon Discovery M-007189-02-0005
NSD2	siGENOME Human NSD2 siRNA, SMARTPool	Horizon Discovery M-006571-01-0005

qRT-PCR Primers		
Gene	**Forward (5'-3')**	**Reverse (5'-3')**
α-tubulin	TCTGTTAGTGGGAGATCCTT	TGGGTTCCAAGTCTACAAAC
UBR1	TTTGTGGGAGGGTTTTCAAAAGT	CAGTTTTCCATGCCTCTGTGT

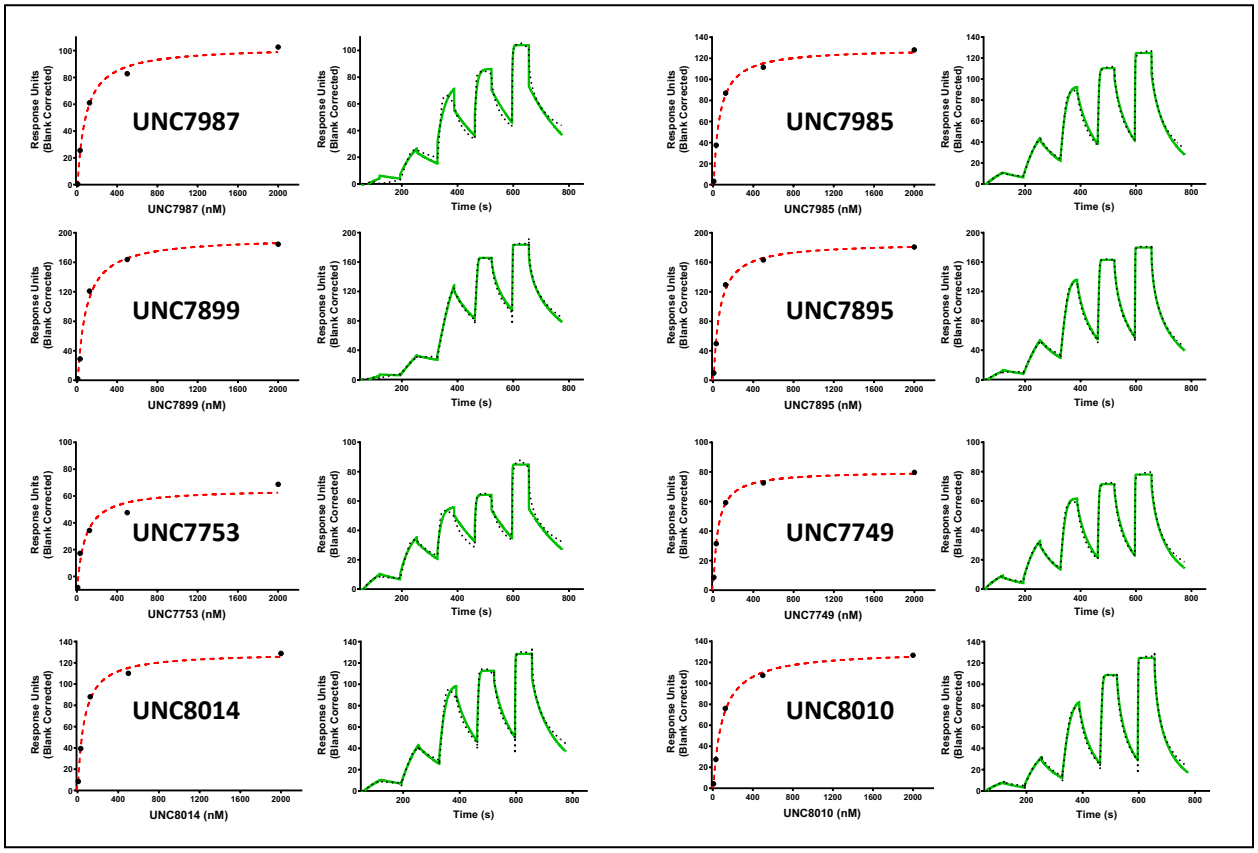
Antibodies			
Protein	**Product Name**	**Cat. ID**	**Dilution**
UBR1	Rabbit anti-UBR1 Antibody	Cedarlane A302-988A-T	1:1000
UBR2	Rabbit anti-UBR2 Antibody	Cedarlane A305-416A-T	1:1000
UBR4 (p600)	Rabbit anti-p600 Antibody	Cedarlane A302-278A-T	1:1000
UBR5	UBR5 (D6O8Z) Rabbit mAb #65344	Cell Signaling 65344S	1:1000
NSD2	Anti-WHSC1/NSD2 antibody [29D1] (ab75359)	Abcam ab75359	1:1000 for both IB and ICW
NSD1	Anti-NSD1 Antibody	Millipore ABE1009	1:500
NSD3	WHSC1L1 (D4N9N) Rabbit mAb #92056	Cell Signaling 92056	1:1000
H3K36me1	Anti-Histone H3 (mono methyl K36) antibody	Abcam ab9048	1:3000
H3K36me2	Recombinant Anti-Histone H3 (di methyl K36) antibody [EPR16994(2)]	Abcam ab176921	1:2000 to 1:4000
H3K36me3	Histone H3K36me3 antibody (pAb)	Active Motif 61101	1:3000
H3	Histone H3 antibody (mAb)	Active Motif 39763	1:2000 to 1:4000
Vinculin	Vinculin (E1E9V) XP® Rabbit mAb #13901	Cell Signaling 13901S	1:1000
GAPDH	Anti-GAPDH Antibody (6C5): sc-32233	Santa Cruz sc-32233	1:2000
Rabbit	IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody	LiCOR 926-32211	1:5000
Rabbit	IRDye® 680RD Donkey anti-Rabbit IgG Secondary Antibody	LiCOR 926-68073	1:5000
Mouse	IRDye® 800CW Donkey anti-Mouse IgG Secondary Antibody	LiCOR 926-32212	1:5000 for IB 1:1000 for ICW
Mouse	IRDye® 680RD Goat anti-Mouse IgG Secondary Antibody	LiCOR 926-68070	1:5000

Table S2. Raw data supporting Figure 3D: Concentration of UNC7753 and UNC8153 in cell pellet homogenate after treating with 10 µM of UNC7753 for the indicated amount of time.

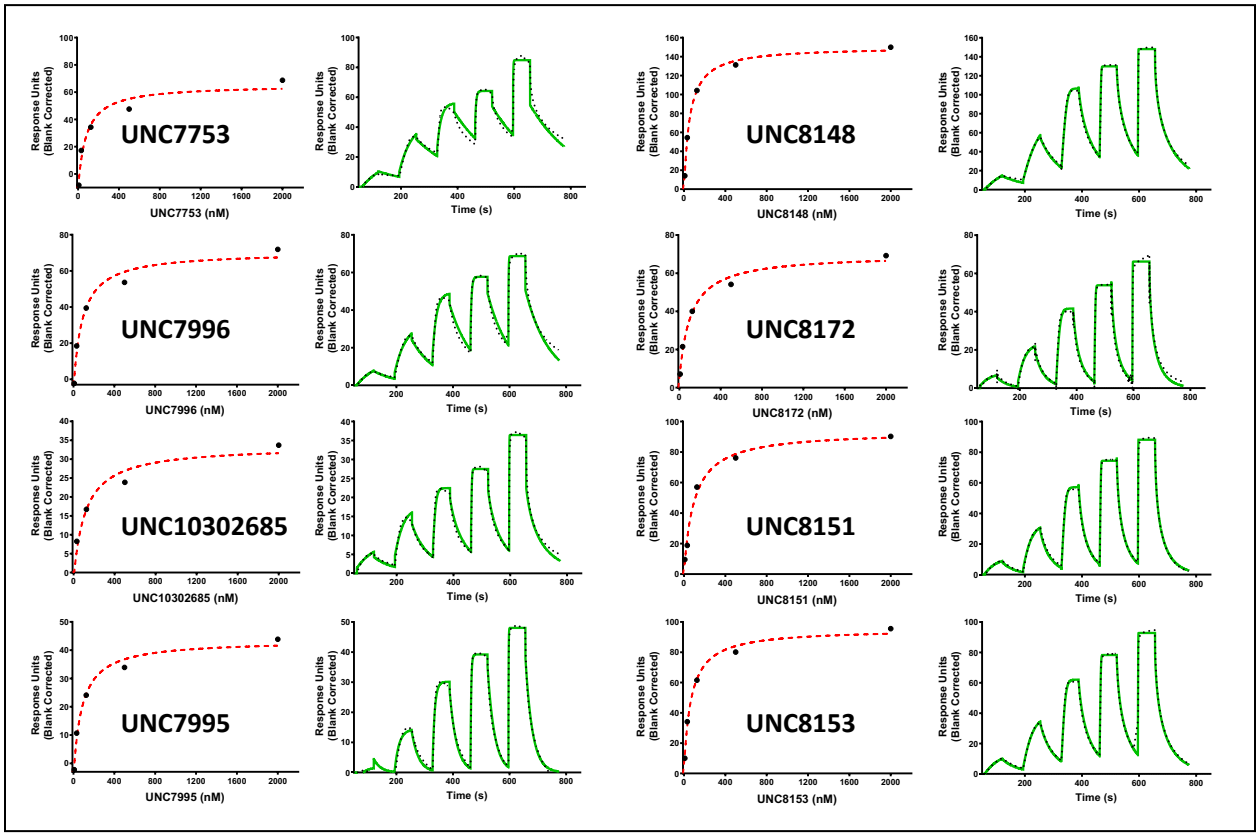
Ref. Std.	Sample ID	Conc. (ng/mL)	Average (ng/mL)	Standard Deviation (ng/mL)
UNC7753	001-UNC7753-10uM-1-hr-CellPellet-A	2.24	5.98	6.8
	002-UNC7753-10uM-1-hr-CellPellet-B	1.9		
	003-UNC7753-10uM-1-hr-CellPellet-C	13.8		
	004-UNC7753-10uM-2-hr-CellPellet-A	1.78	1.56	0.32
	005-UNC7753-10uM-2-hr-CellPellet-B	1.72		
	006-UNC7753-10uM-2-hr-CellPellet-C	1.19		
	007-UNC7753-10uM-4-hr-CellPellet-A	2.73	2.46	0.36
	008-UNC7753-10uM-4-hr-CellPellet-B	2.05		
	009-UNC7753-10uM-4-hr-CellPellet-C	2.6		
	010-UNC7753-10uM-8-hr-CellPellet-A	1.95	2.39	0.38
	011-UNC7753-10uM-8-hr-CellPellet-B	2.65		
	012-UNC7753-10uM-8-hr-CellPellet-C	2.57		
	013-UNC7753-10uM-24-hr-CellPellet-A	2.49	2.35	0.28
	014-UNC7753-10uM-24-hr-CellPellet-B	2.03		
	015-UNC7753-10uM-24-hr-CellPellet-C	2.53		
	016-UNC7753-10uM-48-hr-CellPellet-A	0.824	2.66	2
	017-UNC7753-10uM-48-hr-CellPellet-B	4.8		
	018-UNC7753-10uM-48-hr-CellPellet-C	2.36		
UNC8153	001-UNC7753-10uM-1-hr-CellPellet-A	1.02	3.7	2.9
	002-UNC7753-10uM-1-hr-CellPellet-B	3.29		
	003-UNC7753-10uM-1-hr-CellPellet-C	6.79		
	004-UNC7753-10uM-2-hr-CellPellet-A	3.77	4.77	1.4
	005-UNC7753-10uM-2-hr-CellPellet-B	6.31		
	006-UNC7753-10uM-2-hr-CellPellet-C	4.24		
	007-UNC7753-10uM-4-hr-CellPellet-A	6.79	6.96	0.94
	008-UNC7753-10uM-4-hr-CellPellet-B	6.12		
	009-UNC7753-10uM-4-hr-CellPellet-C	7.98		
	010-UNC7753-10uM-8-hr-CellPellet-A	9.01	12.6	3.1
	011-UNC7753-10uM-8-hr-CellPellet-B	14.4		
	012-UNC7753-10uM-8-hr-CellPellet-C	14.4		
	013-UNC7753-10uM-24-hr-CellPellet-A	15.4	16.7	3.3
	014-UNC7753-10uM-24-hr-CellPellet-B	14.3		
	015-UNC7753-10uM-24-hr-CellPellet-C	20.5		
	016-UNC7753-10uM-48-hr-CellPellet-A	8.43	23.6	14
	017-UNC7753-10uM-48-hr-CellPellet-B	36.1		
	018-UNC7753-10uM-48-hr-CellPellet-C	26.4		
Concentration in ng/mL is expressed in free form, not salt form, if applicable.				

Raw SPR Data and Curve Fitting for All Compounds

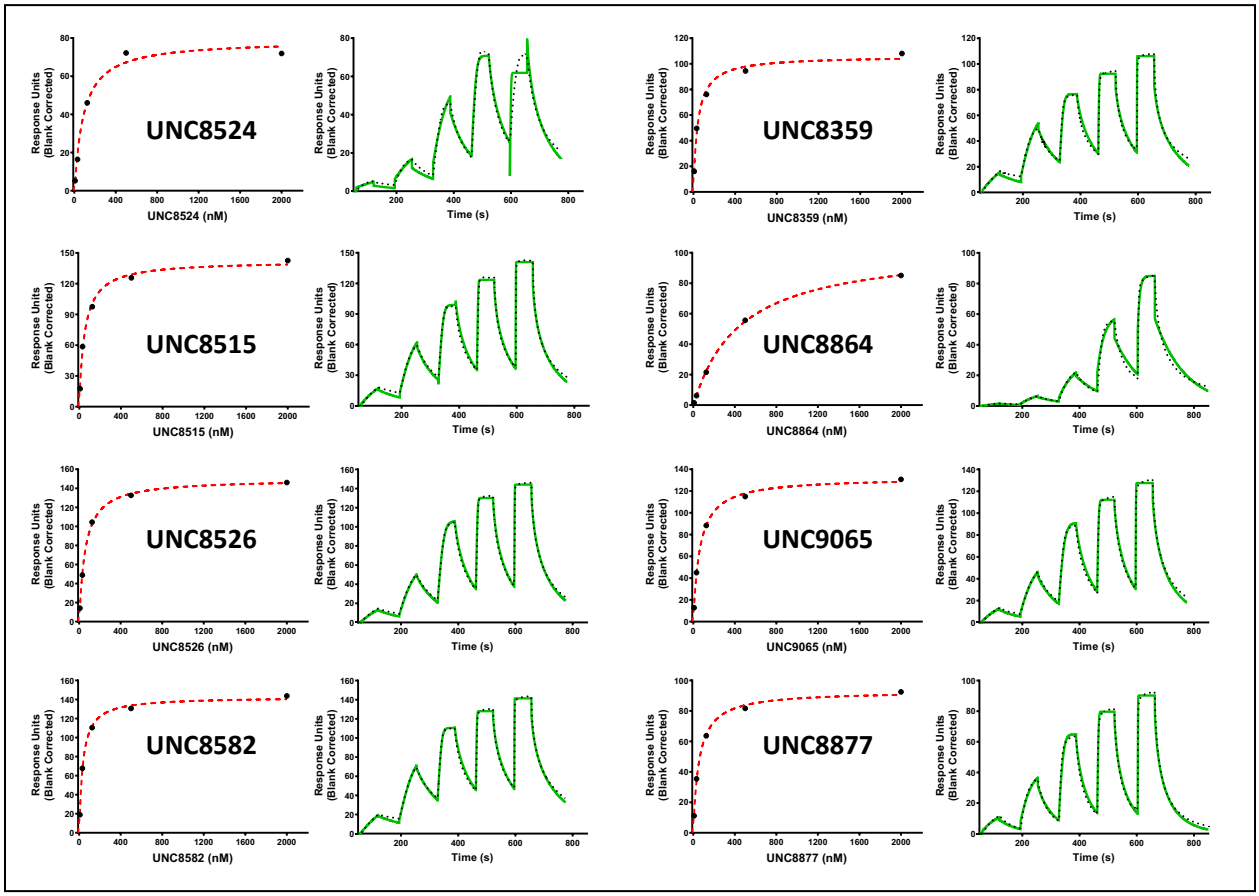
SPR curve fitting raw data for compounds in Figure 2 and Figure S1:



SPR curve fitting raw data for compounds in Figure 3A:

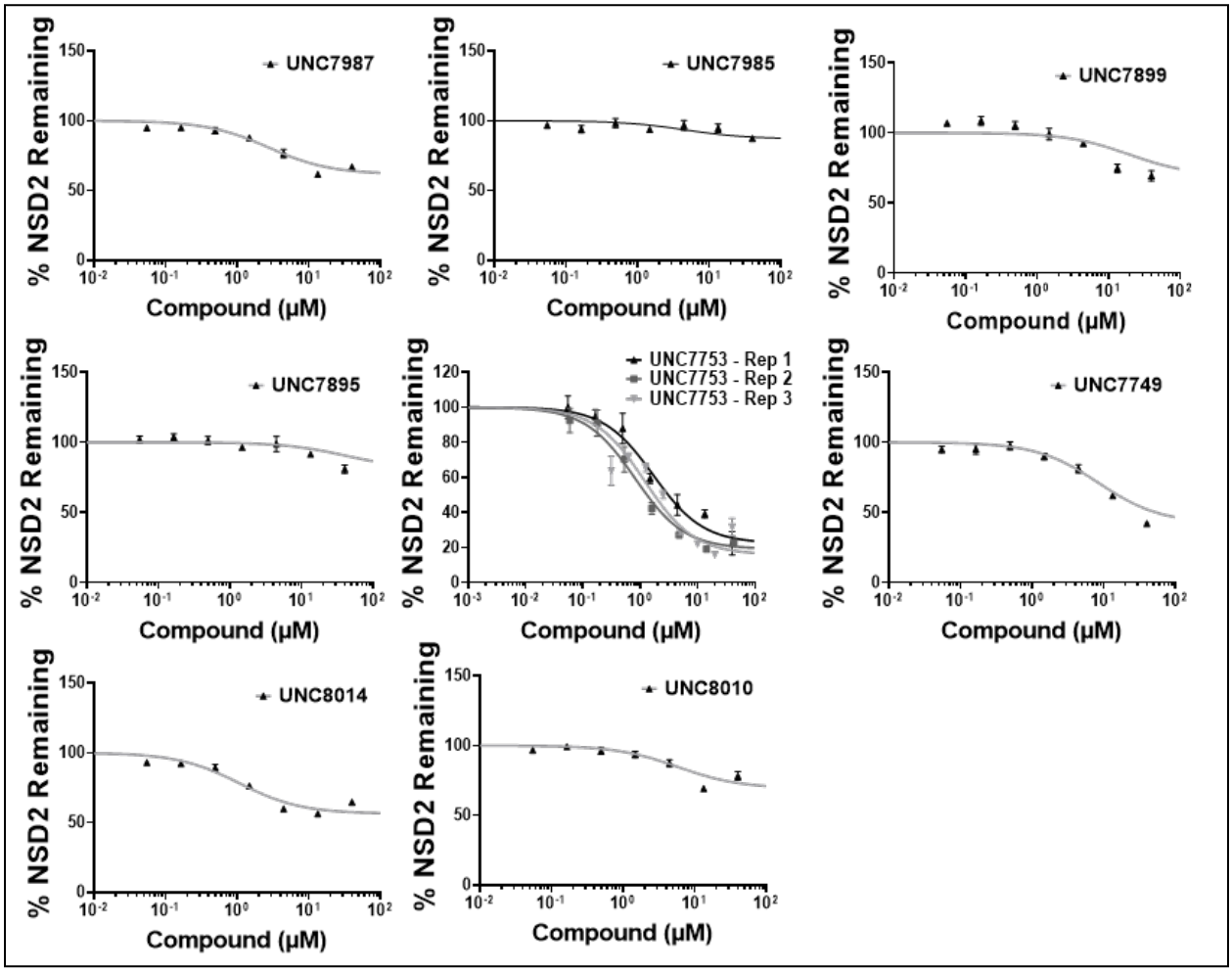


SPR curve fitting raw data for compounds in Figure 3E:

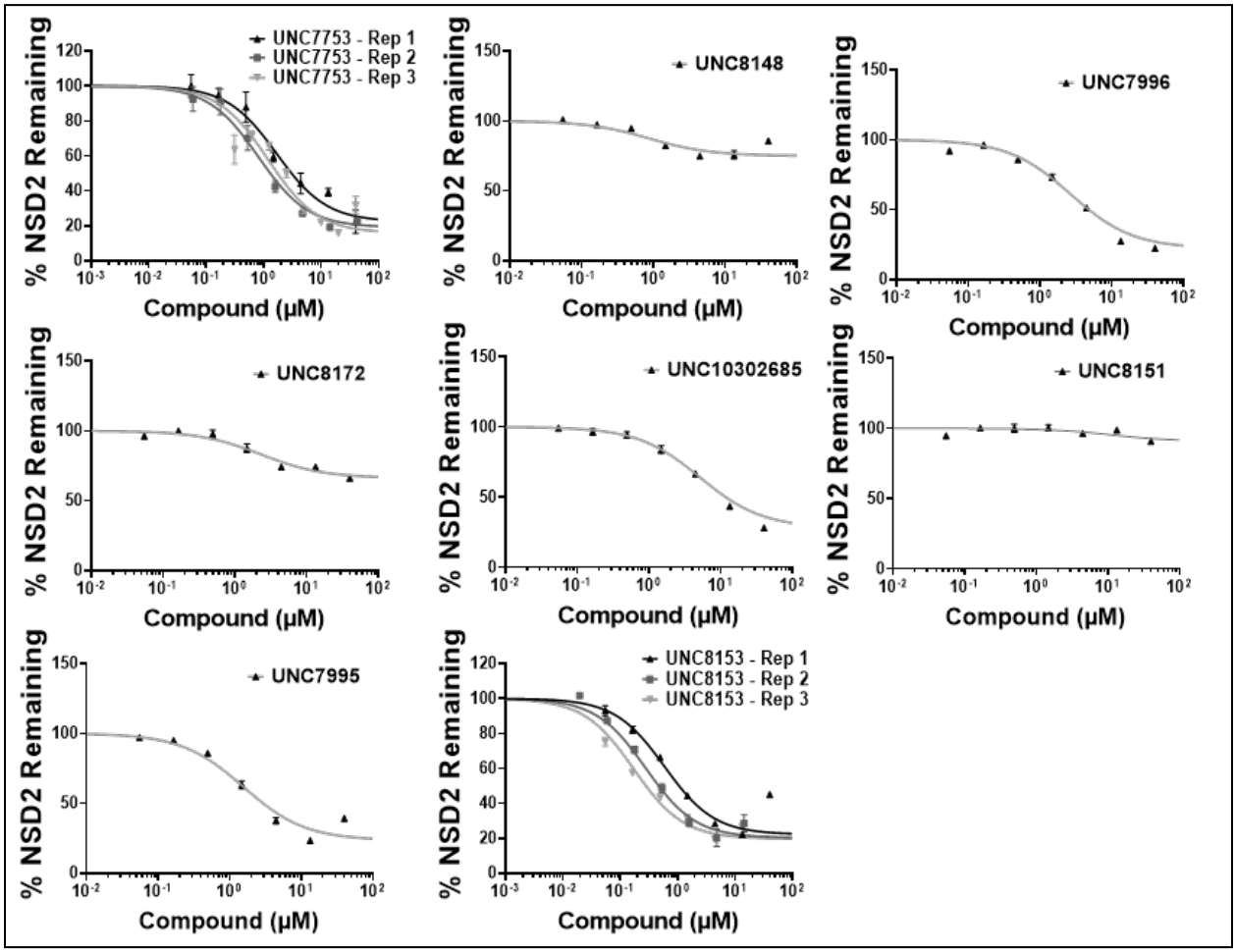


In-Cell Western Dose-Response Curves for All Compounds

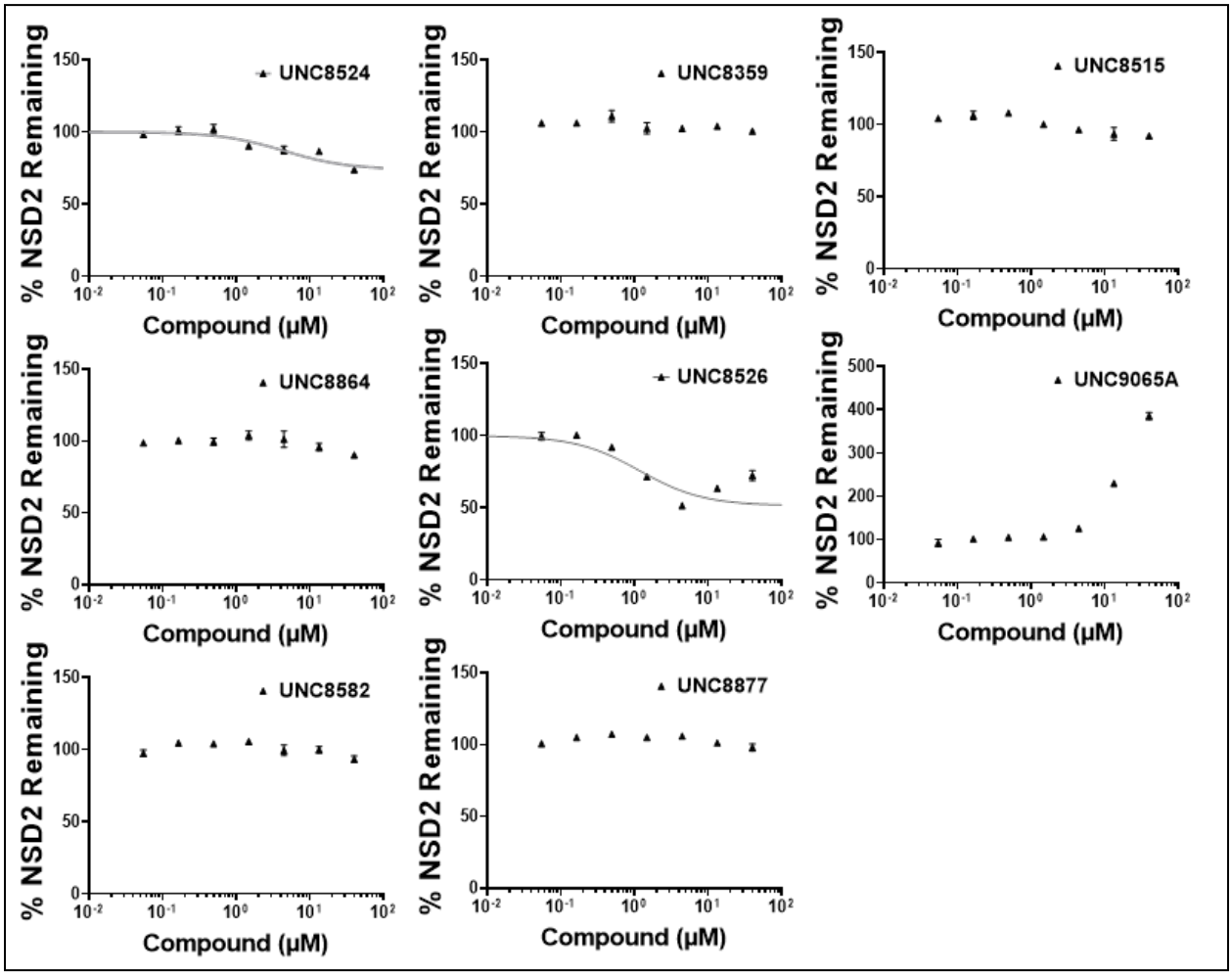
In-Cell Western Dose-Response Curves for compounds in Figure 2 and Figure S1:



In-Cell Western Dose-Response Curves for compounds in Figure 3A:

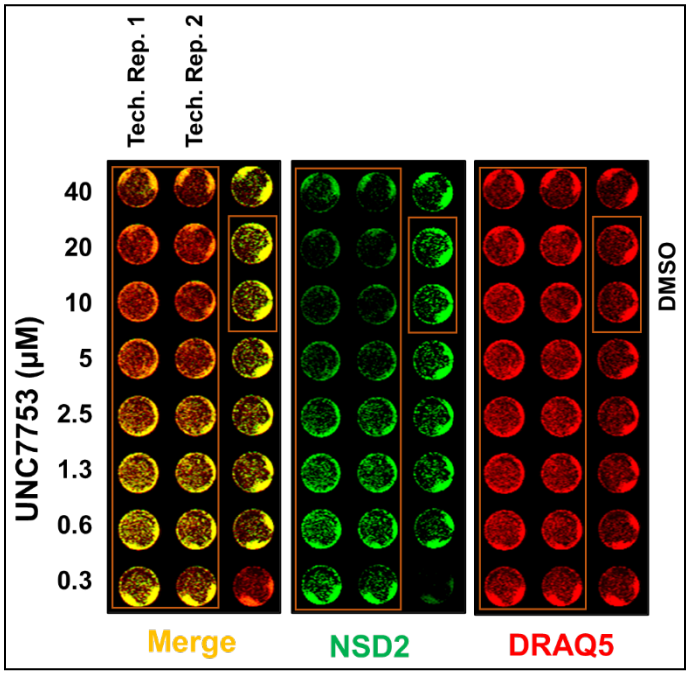


In-Cell Western Dose-Response Curves for compounds in Figure 3E:



Complete ICW Plate Scans

Figure 2D: ICW Plate Scan



Full Western Blot Images

Figure 2E: Raw Blot

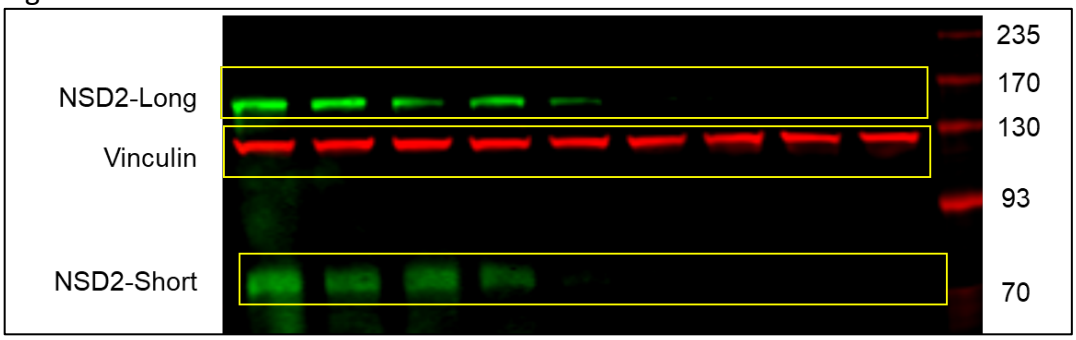


Figure 4D: Raw Blot

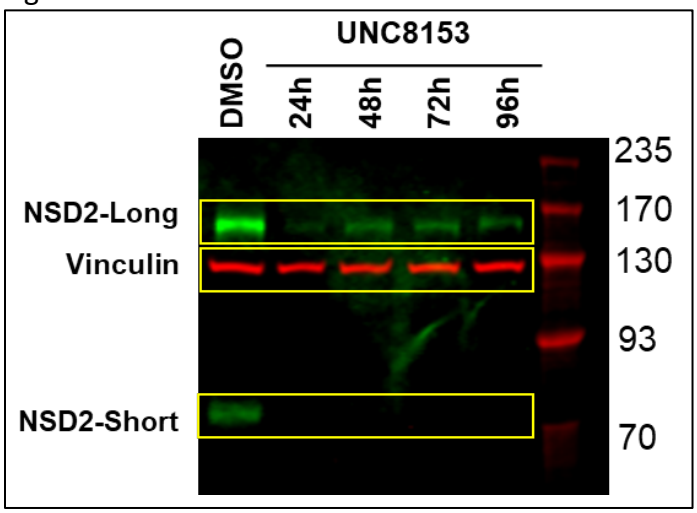


Figure 5B: Raw Blot

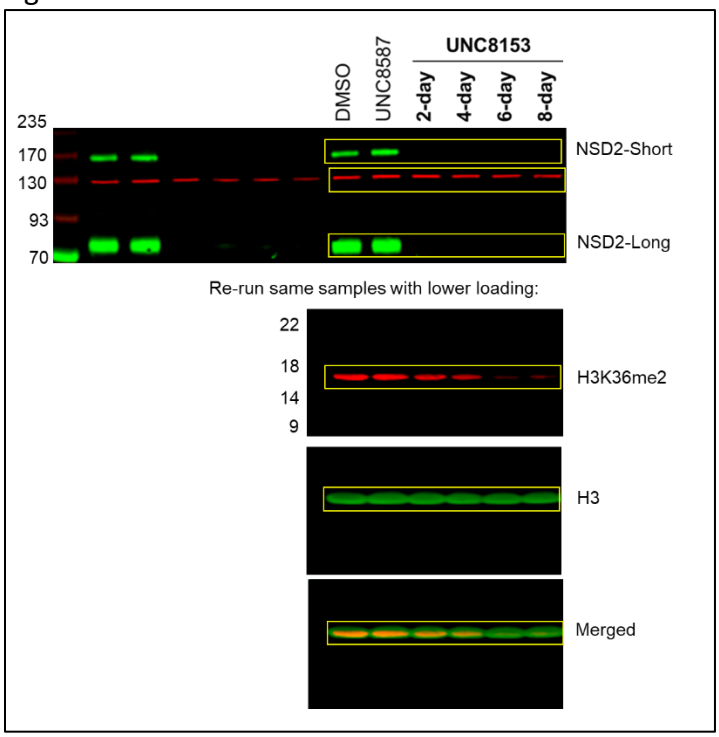


Figure 5C: Raw Blot

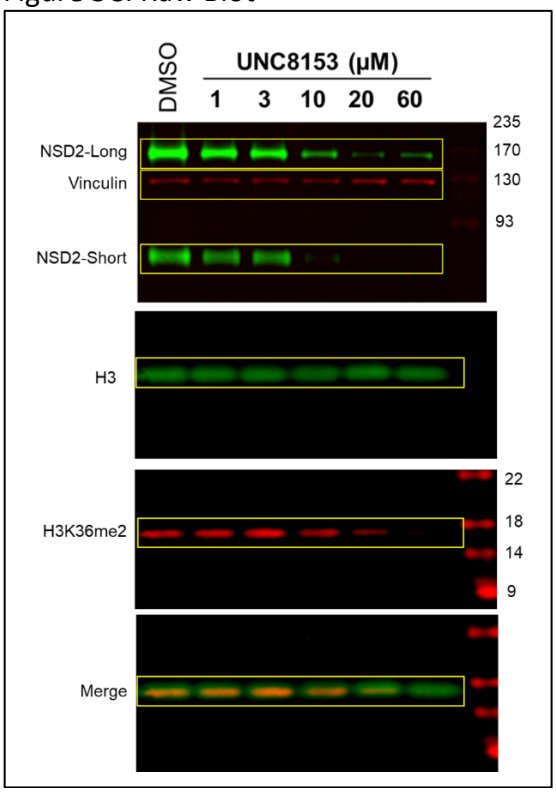


Figure 5E: Raw Blot

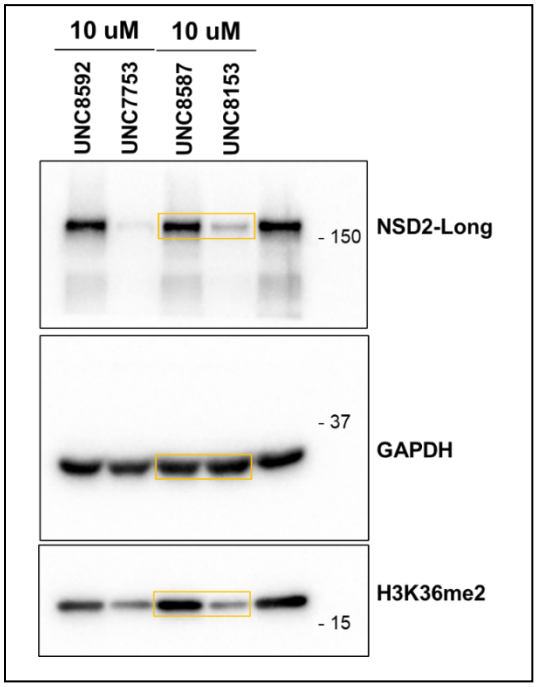


Figure 5F & 5G: Raw Blot

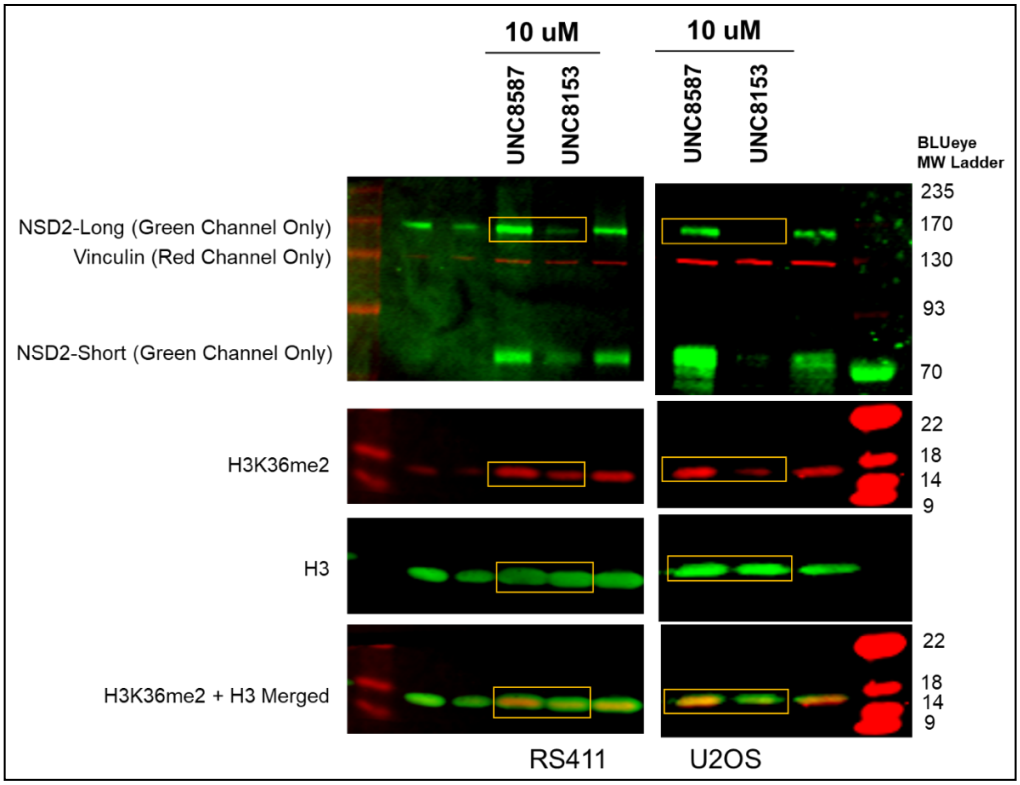


Figure S1D: Raw Blot

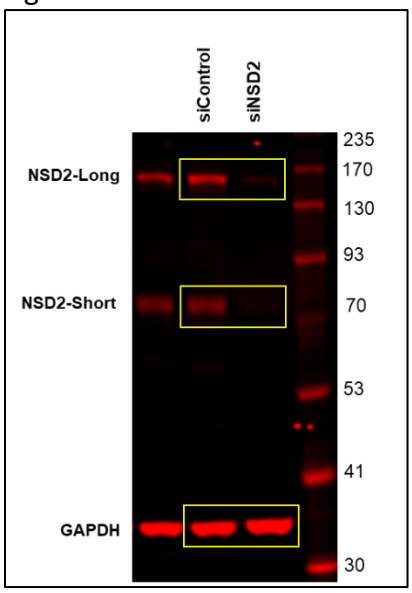


Figure S2C: Raw Blot

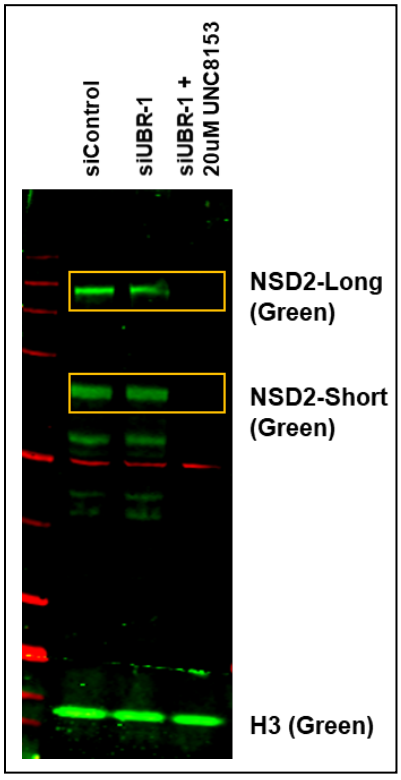


Figure S2E: Raw Blot

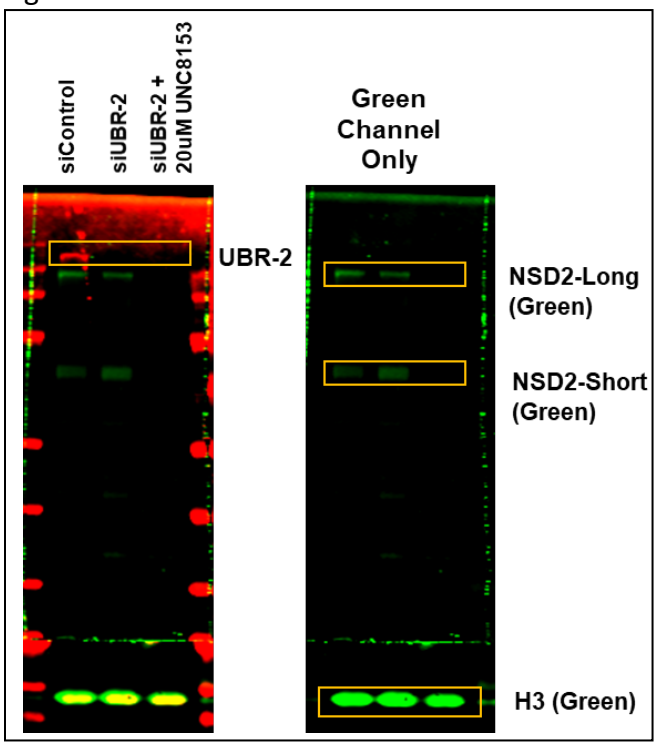


Figure S2F: Raw Blot

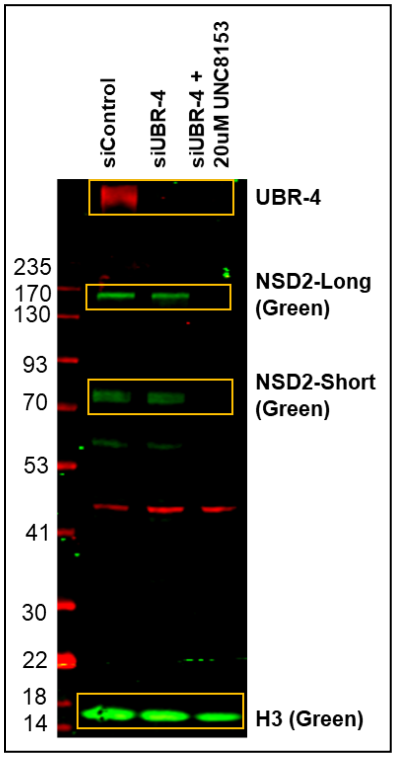


Figure S2G: Raw Blot

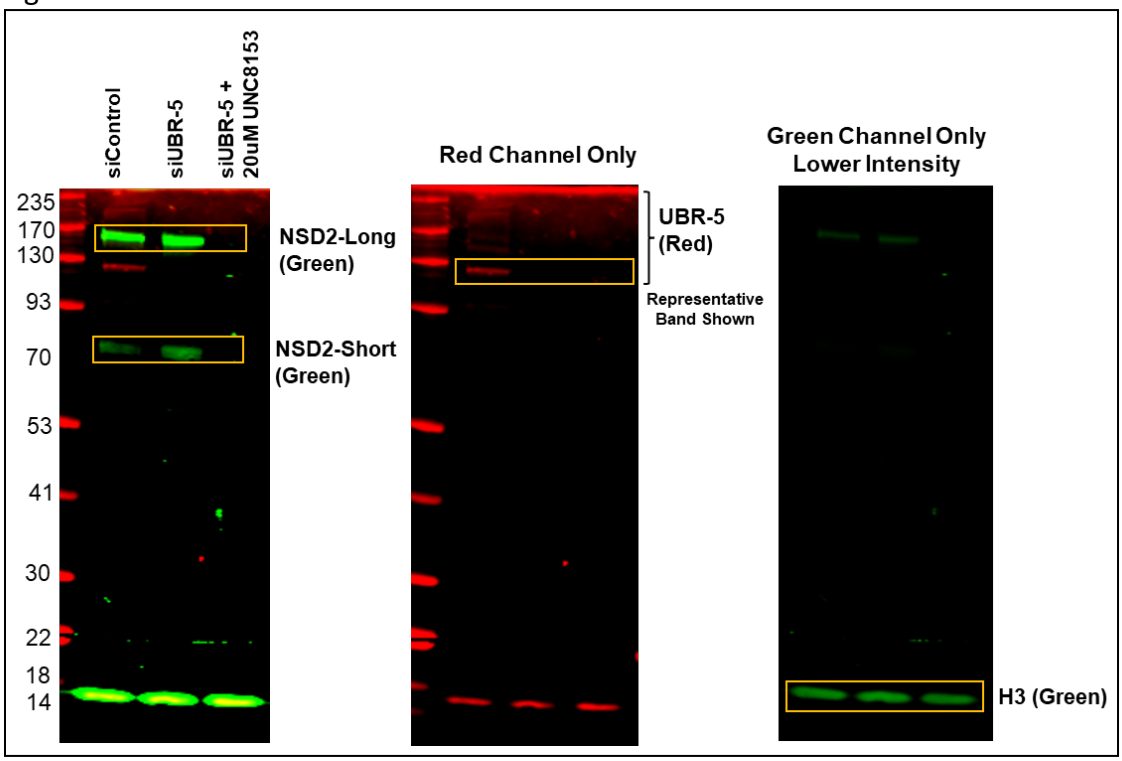


Figure S3A, S3E: Raw Blot

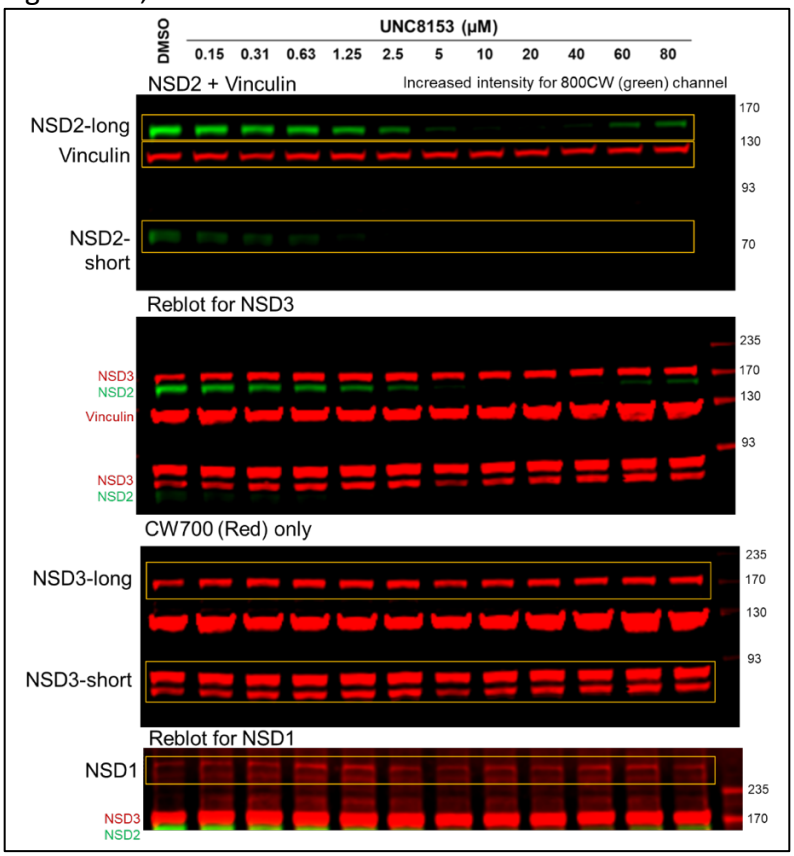


Figure S4B &S4C: Raw Blot

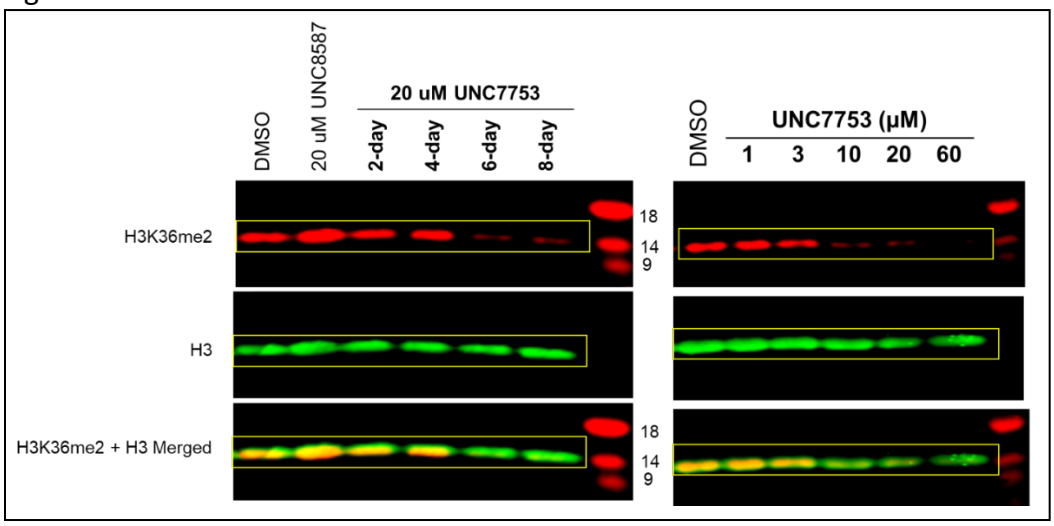


Figure S4D: Raw Blot

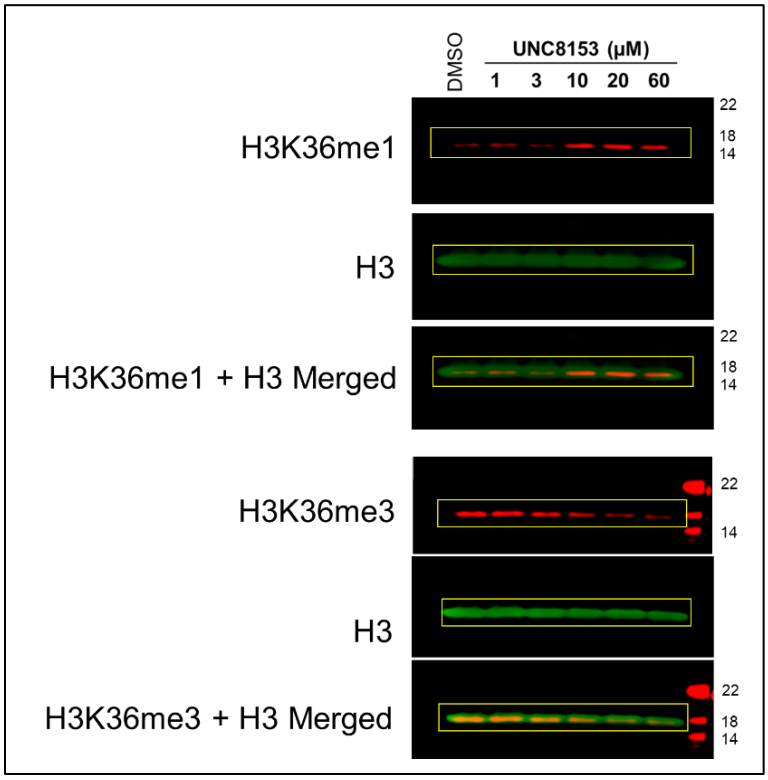


Figure S5A: Raw Blot

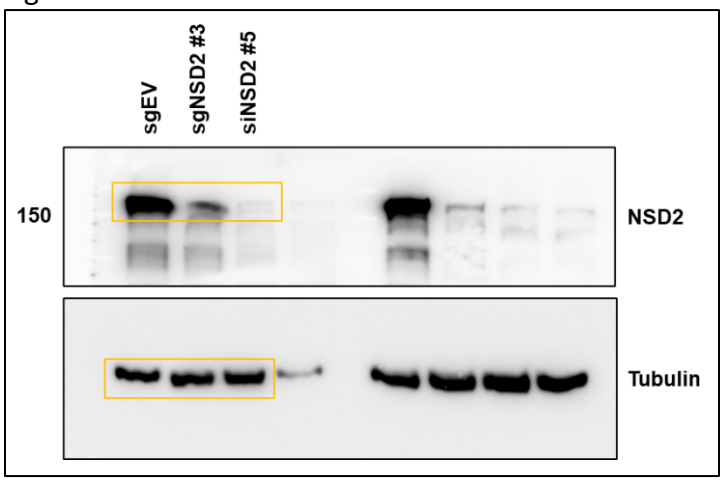


Figure S5B: Raw Blot

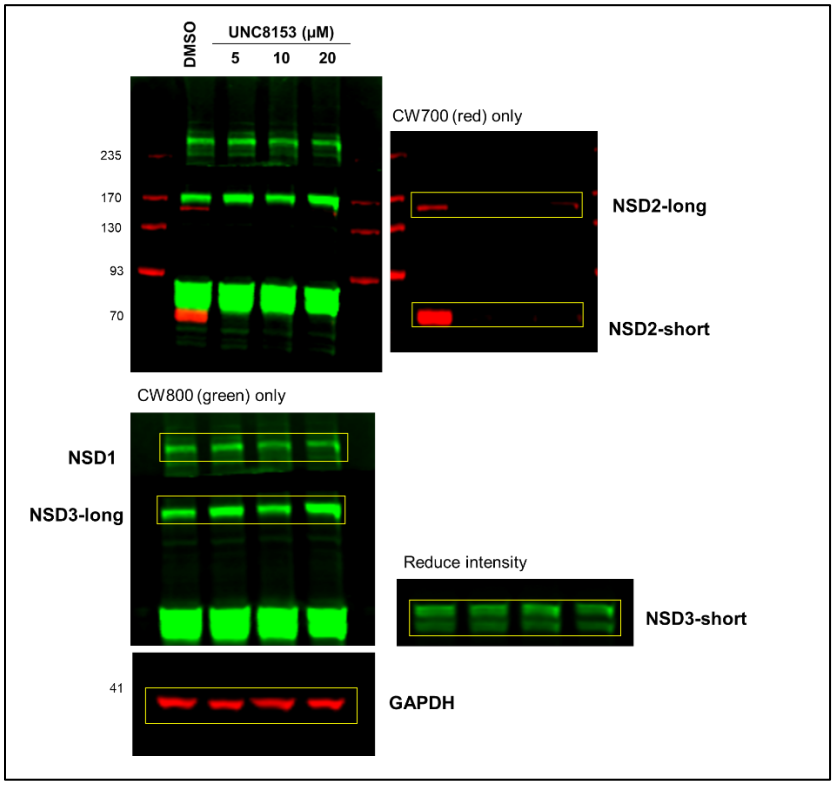


Figure S5D: Raw Blot

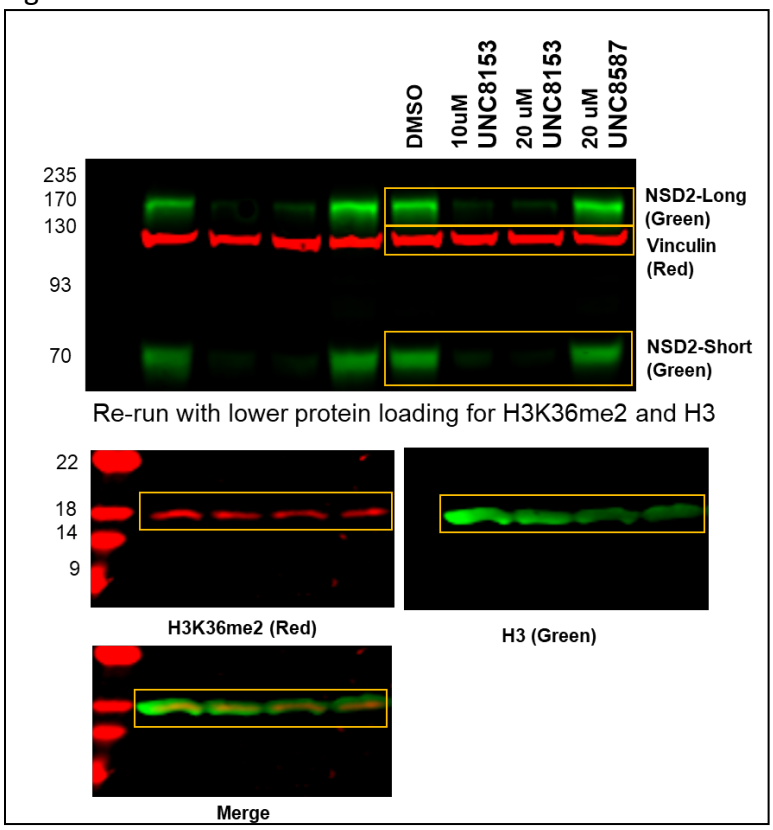


Figure S5F: Raw Blot

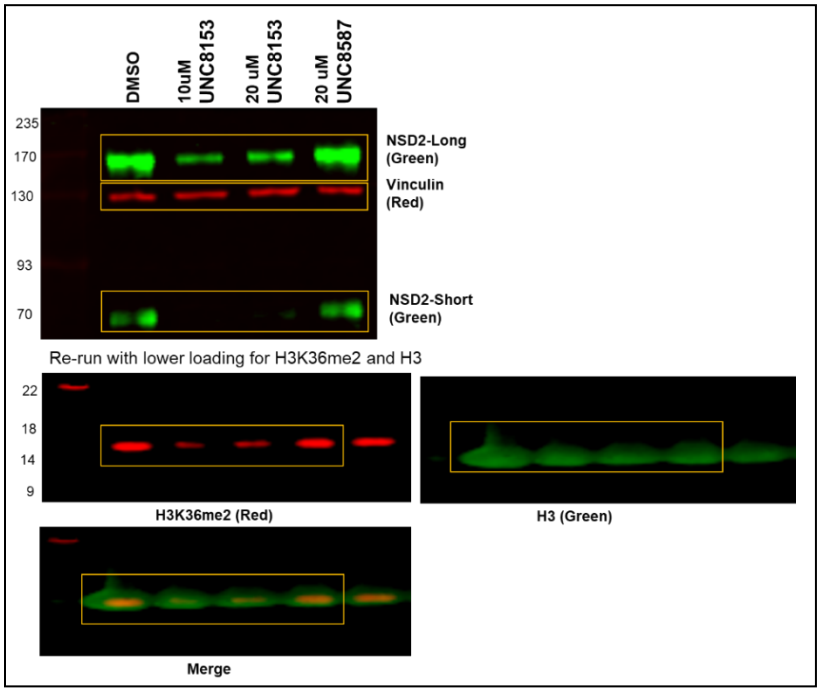
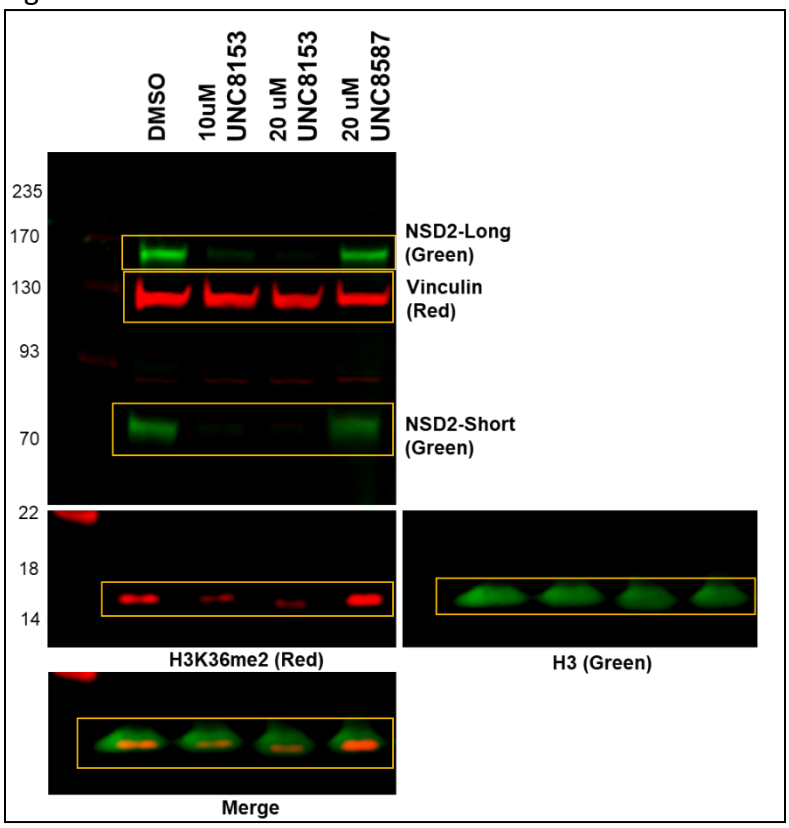


Figure S5H: Raw Blot



General Chemistry Procedures

Reactions were carried out using conventional glassware. All reagents and solvents were used as received unless otherwise stated. Reagents were of 95% purity or greater, and solvents were reagent grade unless otherwise stated. Any anhydrous solvents used were purchased as "anhydrous" grade and used without further drying. "Room" or ambient temperature varied between 20-25 °C. Analytical thin layer chromatography (TLC) was carried out using glass plates pre-coated with silica gel (Merck) impregnated with fluorescent indicator (254 nm). TLC plates were visualized by illumination with a 254 nm UV lamp. Analytical LCMS data for all compounds were acquired using an Agilent 1260 Infinity II system with the UV detector set to 254 nm. Samples were injected (<25 µL) onto an Agilent ZORBAX Eclipse Plus C18, 600 Bar, 4.6 x 50 mm, 1.8 µM column at 25 °C. Mobile phases A (H_2O + 0.1% acetic acid), B (MeOH + 0.1% acetic acid), and C (99% MeCN + 1% H_2O + 0.1% acetic acid) were used with a linear gradient from 10% to 100% B or C in 5 min, followed by a flush at 100% B or C for another 2 minutes with a flow rate of 1 mL/min. Low resolution mass spectra (MS) data were acquired in positive ion mode using an Agilent InfinityLab LC/MSD single quadrupole mass spectrometer with an electrospray ionization (ESI) source (see below for HRMS details). Normal phase column chromatography was performed with a Teledyne Isco CombiFlash®R_f 200 using RediSep®R_f SILICA columns with the UV detector set to 254 nm and 280 nm. Reverse phase column chromatography was performed with a Teledyne Isco CombiFlash®R_f 200 using C18 RediSep®R_f Gold columns with the UV detector set to 220 nm and 254 nm. Mobile phases A (H_2O + 0.1% TFA) and B (MeOH or MeCN) were used. Preparative HPLC was performed as follows unless otherwise noted: Preparative HPLC was performed using an Agilent Prep 1200 series with the UV detector set to 220 nm and 254 nm. Samples were injected onto either a Phenomenex Luna 250 x 30 mm (5 µm) C18 column, a Phenomenex Luna 250 x 50mm (10 µm) C18 column, a Phenomenex Luna 80 x 40mm (3 µm) C18 column, or a Phenomenex Luna 75 x 30 mm (5 µm) C18 column at rt. Mobile phases A (H_2O + 0.1% TFA) and B (MeOH or MeCN) were used with a flow rate of 40 mL/min for the larger column and 30 mL/min for the smaller column. Analytical LCMS (at 254 nm) was used to establish the purity of targeted compounds. All compounds that were evaluated in biochemical and biophysical assays had >95% purity as determined by LCMS or NMR (spectra provided in Supplementary Note).

Nuclear Magnetic Resonance Spectroscopy (NMR)

^1H and ^{13}C NMR spectra were obtained on a Varian 400MR at 400 MHz and 101 MHz respectively. Chemical shifts are reported in ppm and coupling constants are reported in Hz with $CDCl_3$ referenced at 7.26 (^1H) and 77.1 ppm (^{13}C), DMSO-d_6 referenced at 2.50 (^1H) and 39.5 ppm (^{13}C), acetone-d_6 referenced at 2.05 (^1H) and 29.8 ppm (^{13}C), and MeOH-d_4 referenced at 3.31 (^1H) and 49.0 ppm (^{13}C). All compounds that were evaluated in biochemical, biophysical and cellular assays had >95% purity as determined by ^1H NMR and LCMS (spectra provided in Supplementary Note).

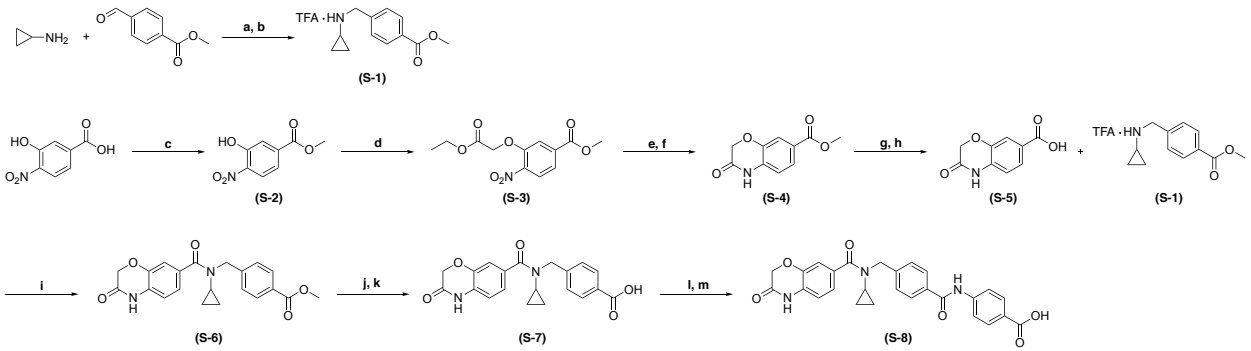
High-Resolution Mass Spectrometry

Samples were analyzed with a ThermoFisher Q Exactive HF-X (ThermoFisher, Bremen, Germany) mass spectrometer coupled with a Waters Acquity H-class liquid chromatograph system. Samples were introduced via a heated electrospray source (HESI) at a flow rate of 0.3 mL/min. Electrospray source conditions were set as: spray voltage 3.0 kV, sheath gas (nitrogen) 60 arb, auxiliary gas (nitrogen) 20 arb, sweep gas (nitrogen) 0 arb, nebulizer temperature 375 °C, capillary temperature 380 °C, RF funnel 45 V. The mass range was set to 150-2000 *m/z*. All measurements were recorded at a resolution setting of 120,000. Separations were conducted on a Waters Acquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm particle size). LC conditions were set at 95 % water with 0.1% formic acid (A) ramped linearly over 5.0 mins to 100% acetonitrile with 0.1% formic acid (B) and held until 6.0 mins. At 7.0 mins the gradient was switched back to 95% A and allowed to re-equilibrate until 9.0 mins. Injection volume for all samples was 3 µL. Xcalibur (ThermoFisher, Breman, Germany) was used to analyze the data. Solutions were analyzed at 0.1 mg/mL or less based on responsiveness to the ESI mechanism. Molecular formula assignments were determined with Molecular Formula Calculator (v 1.2.3). All observed species were singly charged, as verified by unit *m/z* separation between mass spectral peaks corresponding to the ^{12}C and $^{13}C^{12}C_{c-1}$ isotope for each elemental composition.

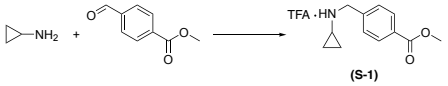
Abbreviations used

CDMT	2-Chloro-4,6-dimethoxy-1,3,5-triazine
DCM	Dichloromethane
DIBAL-H	Diisobutylaluminum hydride
DIPEA	N,N-Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulfoxide
EDC	N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
ESI	Electrospray Ionization
HOAt	1-Hydroxy-7-azabenzotriazole
HPLC	High-Perfomance Liquid Chromatography
HRMS	High-Resolution Mass Spectrometry
LAH	Lithium Aluminum Hydride
LCMS	Liquid Chromatography-Mass Spectrometry
NMM	N-methylmorpholine
NMR	Nuclear Magnetic Resonance
RBF	Round-bottom Flask
TBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate
TFA	2,2,2-Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography

Synthetic Schemes



Supplemental Scheme 1: Synthesis of intermediate (S-8). Reagents and conditions: a) MeOH, $NaBH_4$, 0 °C; b) TFA, Et_2O, 0 °C; c) H_2SO_4, MeOH, reflux; d) K_2CO_3, ethyl 2-bromoacetate, DMF, 70 °C; e) Pd/C, H_2, MeOH; f) AcOH, reflux; g) LiOH-H_2O, H_2O, THF; h) HCl; i) EDC, HOAt, Et_3N, MeCN; j) LiOH-H_2O, H_2O, THF; k) HCl; l) tert-butyl 4-aminobenzoate, DMAP, EDC, DMF, 50 ˚C; m) TFA/DCM

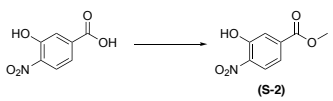


methyl 4-((cyclopropylamino)methyl)benzoate 2,2,2-trifluoroacetate (S-1)

To a 50 mL flask equipped with a stir bar was added methyl 4-formylbenzoate (1.0 g, 1 Eq, 6.1 mmol) and methanol (10 mL), followed by cyclopropylamine (0.35 g, 0.43 mL, 1 Eq, 6.1 mmol). The flask was capped and stirred at room temperature overnight. The next day, the flask was cooled in an ice water bath and sodium borohydride (0.46 g, 2 Eq, 12 mmol) was added portionwise. Borohydride addition was accompanied by effervescence and heating of the solution. After 4 hours, at which time the reaction had come to room temperature, the reaction was quenched by addition of saturated sodium bicarbonate and extracted three times with ethyl acetate. The combined organic layers were washed once more with saturated sodium bicarbonate, once with brine, then dried over sodium sulfate and concentrated to an oil. Normal phase chromatography over silica gel (0-100% ethyl acetate in hexanes) provided the free base as a colorless free-flowing oil. The oil was dissolved in 25 mL of diethyl ether and cooled in an ice water bath, and trifluoroacetic acid (1.2 g, 0.80 mL, 1.7 Eq, 10 mmol) was added dropwise. A voluminous white solid formed, which was collected by filtration and washed rigorously with diethyl ether to provide (**S-1)** (1.586 g, 4.976 mmol, 82%) as a fluffy white powder.

^{1}H NMR (400 MHz, MeOH-d_4) δ 8.09 (d, J = 8.5 Hz, 2H), 7.62 (d, J = 8.4 Hz, 2H), 4.39 (s, 2H), 3.92 (s, 3H), 2.83 – 2.75 (m, 1H), 0.96 – 0.85 (m, 4H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{12}H_{16}NO_2$, 206.1; found, 206.2.

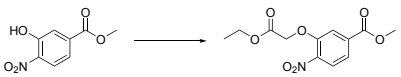


methyl 3-hydroxy-4-nitrobenzoate (S-2)

To a 250 mL RBF charged with a stir bar was added 3-hydroxy-4-nitrobenzoic acid (20 g, 1 Eq, 0.11 mol), methanol (100 mL) then sulfuric acid (22 g, 12 mL, 2 Eq, 0.22 mol). The reaction was equipped with a reflux condenser and heated to reflux overnight. The next day, the reaction was allowed to cool to room temperature. The solvent was evaporated under reduced pressure to give a residue. The residue was neutralized with saturated $NaHCO_3$ and extracted with ethyl acetate (200 mL). The organic phase was washed with brine (2 × 50 mL), dried over Na_2SO_4, and filtered. Volatiles were removed *in vacuo* to give **(S-2)** (20.1525 g, 102.22 mmol, 93 %) as a red solid. The crude solid was used in the next step without purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.50 (s, 1H, -OH), 8.17 (d, *J* = 8.9 Hz, 1H), 7.83 (d, *J* = 1.8 Hz, 1H), 7.61 (dd, *J* = 8.8, 1.8 Hz, 1H), 3.96 (s, 3H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_8H_8NO_5$, 198.03; found, 198.0.

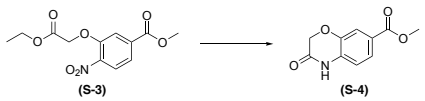


methyl 3-(2-ethoxy-2-oxoethoxy)-4-nitrobenzoate (S-3)

To a flask charged with a stirbar was added crude **(S-2)** (20.1 g, 1 Eq, 102 mmol), potassium carbonate (33.5 g, 2.38 Eq, 243 mmol), and DMF (150 mL). To the stirring blood-orange colored solution was added ethyl 2-bromoacetate (23.0 g, 15.3 mL, 1.35 Eq, 138 mmol) dropwise, and the mixture was capped and heated to 70 °C for 18 hours. The resulting blood-red solution was cooled and poured into 1L of water which was then stirred rapidly to produce a yellowish precipitate. The solid was collected by filtration and washed copiously with water to give **(S-3)** (27.968 g, 98.743 mmol, 96.8 %) as a beige powder.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, *J* = 8.3 Hz, 1H), 7.75 (dd, *J* = 8.3, 1.5 Hz, 1H), 7.65 (d, *J* = 1.6 Hz, 1H), 4.83 (s, 2H), 4.28 (q, *J* = 7.1 Hz, 2H), 3.95 (s, 3H), 1.29 (t, *J* = 7.1 Hz, 3H).

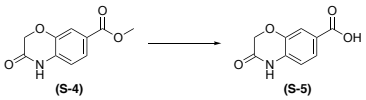
LCMS (ESI, +ve mode) (*m/z*): $[M+Na]^+$ calculated for $C_{12}H_{13}NO_7Na$, 306.1; found, 306.1.



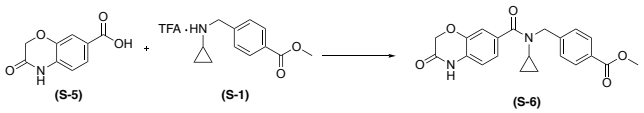
methyl 3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxylate (S-4)

To a round-bottom flask charged with a stirbar was added **(S-3)** (3.776 g, 1 Eq, 13.33 mmol) and methanol (80 mL). The flask was sealed with a septum then evacuated and backfilled with nitrogen 3 times before addition of palladium on carbon (383.0 mg, 10% Wt, 0.027 Eq, 359.9 µmol). The evacuation/backfill procedure was repeated another 3 times before attachment of a hydrogen balloon. After stirring for 6 hours, TLC analysis (3:1 hexanes/ethyl acetate) showed complete consumption of starting material. The solution was filtered through Celite, which was then flushed with additional methanol. Volatiles were reduced by about half *in vacuo*, then acetic acid (8.005 g, 7.62 mL, 10 Eq, 133.3 mmol) was added and the reaction heated to reflux for 30 minutes. A voluminous solid formed during this time and the crystals separated from the solution, which was now olive-colored and clear. The solids were collected

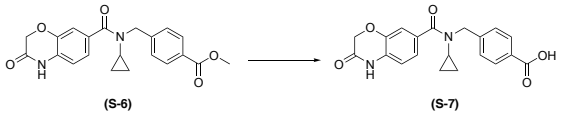
by filtration, rinsed with minimal ice-cold methanol then dried on high-vac overnight to provide **(S-4)** (2.0262 g, 9.7794 mmol, 73.36 %) as a fluffy, off-white powder.
^{1}H NMR (400 MHz, DMSO-d_6) δ 11.04 (s, 1H, -NH), 7.56 (dd, *J* = 8.2, 1.8 Hz, 1H), 7.41 (d, *J* = 1.5 Hz, 1H), 6.96 (d, *J* = 8.2 Hz, 1H), 4.62 (s, 2H), 3.79 (s, 3H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{10}H_{10}NO_4$, 208.1; found, 208.1.



3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxylic acid (S-5)
In a round-bottom flask, **(S-4)** (2.0 g, 1 Eq, 9.5 mmol) was suspended in THF (24 mL). A solution of lithium hydroxide hydrate (2.4 g, 6 Eq, 57 mmol) in water (24 mL) was added and the solution was stirred overnight and consumption of starting material was monitored by TLC. Upon completion, the still-basic aqueous layer was washed five times with ether. The aqueous layer was then acidified to a pH of 2-3 with 2 M HCl. Immediately upon addition of HCl, white solid began to crash out. The suspension was stirred for several minutes, then collected by filtration and washed with water. The solid was air dried overnight to give **(S-5)** (1.4123 g, 7.3116 mmol, 77 %) as an off-white solid.
^{1}H NMR (400 MHz, DMSO-d_6) δ 12.79 (s, 1H, -OH), 11.03 (s, 1H), 7.56 (dd, *J* = 8.2, 1.7 Hz, 1H), 7.41 (d, *J* = 1.3 Hz, 1H), 6.97 (d, *J* = 8.2 Hz, 1H), 4.63 (s, 2H).



methyl 4-((N-cyclopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamido) methyl)benzoate (S-6)
To a flask was added (**S-5)** (200 mg, 1 Eq, 1.04 mmol), HOAt (211 mg, 1.5 Eq, 1.55 mmol), EDC (298 mg, 1.5 Eq, 1.55 mmol), and acetonitrile (5 mL). The mixture was stirred for 15 minutes at room temperature to preactivate the acid. To the flask was then added (**S-1)** (397 mg, 1.2 Eq, 1.24 mmol) and triethylamine (314 mg, 0.43 mL, 3 Eq, 3.11 mmol), and the reaction stirred at room temperature overnight. The next day, the reaction was quenched with water and extracted 3 times with ethyl acetate. The combined organic fractions were washed once with 0.5 M citric acid, once with water, once with saturated sodium bicarbonate, and once with brine, then dried over sodium sulfate, filtered, and concentrated to a white solid. Normal phase chromatography over silica gel (0- 100% ethyl acetate in DCM) provided (**S-6)** (292.7 mg, 769.5 µmol, 74.3%) as a white solid.
^{1}H NMR (400 MHz, DMSO-d_6) δ 10.87 (s, 1H), 7.95 (d, *J* = 7.7 Hz, 2H), 7.44 (d, *J* = 7.4 Hz, 2H), 7.32 – 7.04 (m, 2H), 6.92 (d, *J* = 7.9 Hz, 1H), 4.71 (s, 2H), 4.61 (s, 2H), 3.85 (s, 3H), 2.79 (m, 1H), 0.52 (m, 2H), 0.45 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{21}H_{21}N_2O_5$, 381.1; found, 381.2.

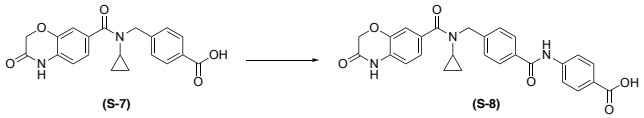


4-((N-cyclopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamido)methyl) benzoic acid (S-7)

To a round-bottom flask was added (**S-6)** (904 mg, 1 Eq, 2.38 mmol), THF (20 mL), and lithium hydroxide hydrate (10% in water, 5 mL, 5 Eq, 11.9 mmol). The reaction was stirred at room temperature for 24 hours, then extracted 5 times with 20 mL portions of diethyl ether. The aqueous layer was then acidified to pH 2 with 1 M HCl, and the precipitate produced was collected by filtration, washed with cold water, and dried to provide (**S-7)** (771.1 mg, 2.105 mmol, 88.6%) as a white solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.87 (s, 1H), 7.93 (d, *J* = 8.2 Hz, 2H), 7.41 (d, *J* = 8.0 Hz, 2H), 7.18 (dd, *J* = 8.1, 1.8 Hz, 1H), 7.14 (d, *J* = 1.7 Hz, 1H), 6.92 (d, *J* = 8.1 Hz, 1H), 4.70 (s, 2H), 4.61 (s, 2H), 2.84 – 2.73 (m, 1H), 0.59 – 0.49 (m, 2H), 0.49 – 0.38 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{20}H_{19}N_2O_5$, 367.1; found, 367.1.

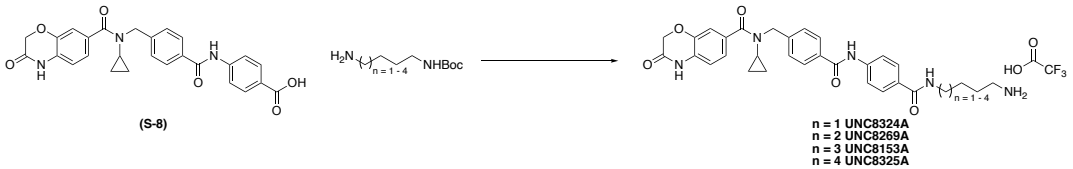


4-(4-((N-cyclopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamido)methyl) benzamido)benzoic acid (S-8)

To a scintillation vial was added (**S-7)** (1.04 g, 1 Eq, 2.84 mmol), tert-butyl 4-aminobenzoate (1.10 g, 2 Eq, 5.68 mmol), EDC (1.09 g, 2 Eq, 5.68 mmol), DMAP (694 mg, 2 Eq, 5.68 mmol), and DMF (10 mL). The reaction was heated to 50 ˚C and stirred overnight. The next day, the reaction was partitioned between water and ethyl acetate. The layers were separated, and the aqueous layer was extracted twice more with ethyl acetate. The combined organic layers were washed twice with water, once with saturated sodium bicarbonate, and once with brine, then dried over sodium sulfate and concentrated to an off-white residue. Purification by normal phase chromatography over silica gel (0-100% ethyl acetate in DCM) provided the intermediate ester as a white solid. To the ester was added TFA in DCM (10.9 mL, 20% Wt, 10 Eq, 28.4 mmol), and the solution was stirred at room temperature overnight. The next day, the volatiles were removed *in vacuo* to provide (**S-8)** (1.06 g, 2.18 mmol, 76.9%) as a tan solid.

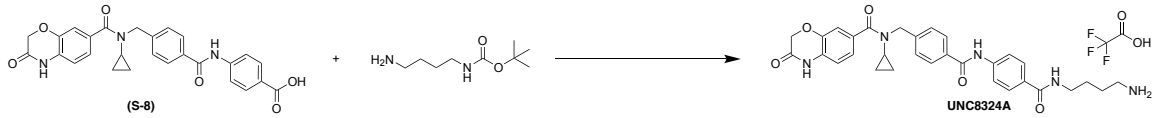
^{1}H NMR (400 MHz, DMSO-d_6) δ 10.86 (s, 1H), 10.50 (s, 1H), 8.14 – 7.70 (m, 6H), 7.45 (d, *J* = 7.9 Hz, 2H), 7.22 – 7.05 (m, 2H), 6.91 (d, *J* = 8.0 Hz, 1H), 4.70 (s, 2H), 4.60 (s, 2H), 2.78 (s, 1H), 0.69 – 0.49 (m, 2H), 0.49 – 0.31 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{27}H_{24}N_3O_6$, 486.2; found, 486.1.



General Procedure A: For the coupling of (S-8) with aliphatic amines

To a scintillation vial charged with a stirbar was added **(S-8)** (1 Eq), EDC (1.5 Eq), HOAt (1.5 Eq) and DMF (1 – 2 mL). The mixture was left to stir at room temperature for 30 minutes. To the vial was then added aliphatic amine (1.2 – 1.5 Eq) followed by triethylamine (2.1 – 4 Eq). The reaction was stirred at room temperature for 24 hours. The reaction was then diluted with distilled water and extracted 3 times with ethyl acetate. The combined organic layers were washed once with water, twice with saturated sodium bicarbonate, once with brine, then dried over sodium sulfate, filtered and concentrated *in vacuo*. To a scintillation vial containing a stirbar and crude reaction mixture was added TFA in DCM (10 Eq, 20% v/v). The mixture was stirred overnight. Volatiles were removed *in vacuo* and residual TFA was removed by co-evaporation with methanol. Following purification (see individual details below), all free aliphatic amine products were isolated as trifluoroacetate salts (unless otherwise noted). All final products were suspended in water, flash frozen and lyophilized to dryness.

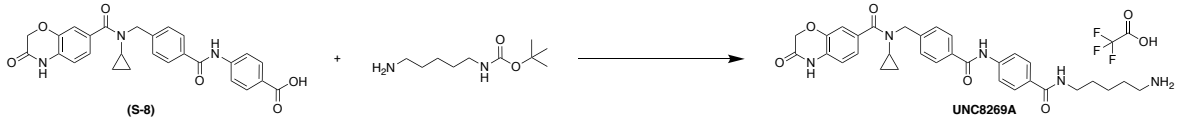


N-(4-((4-((4-aminobutyl)carbamoyl)phenyl)carbamoyl)benzyl)-N-cyclopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamide 2,2,2-trifluoroacetate (UNC8324A)

Prepared according to General Procedure A using **(S-8)** (50.0 mg, 1 Eq, 103 µmol), EDC (29.6 mg, 1.5 Eq, 155 µmol), HOAt (21.0 mg, 1.5 Eq, 155 µmol), DMF (1 mL), tert-butyl (4-aminobutyl)carbamate (29.1 mg, 1.5 Eq, 155 µmol) and triethylamine (41.7 mg, 57 µL, 4 Eq, 412 µmol). Following *N-Boc* deprotection, the crude material was purified by reverse phase chromatography (10-100% methanol in water + 0.1% TFA) to give **UNC8324A** (8.6 mg, 13 µmol, 12 % over two steps) as a white solid.

^{1}H NMR (400 MHz, MeOH-d_4) δ 7.96 (d, J = 8.3 Hz, 2H), 7.85 (s, 4H), 7.50 (d, J = 7.4 Hz, 2H), 7.30 – 7.10 (m, 2H), 6.96 (d, J = 8.1 Hz, 1H), 4.83 (s, 2H), 4.62 (s, 2H), 3.44 (t, J = 6.0 Hz, 2H), 3.06 – 2.92 (m, 2H), 2.83 (s, 1H), 1.83 – 1.62 (m, 4H), 0.78 – 0.61 (m, 2H), 0.60 – 0.41 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{31}H_{34}N_5O_5$, 556.3; found, 556.2.

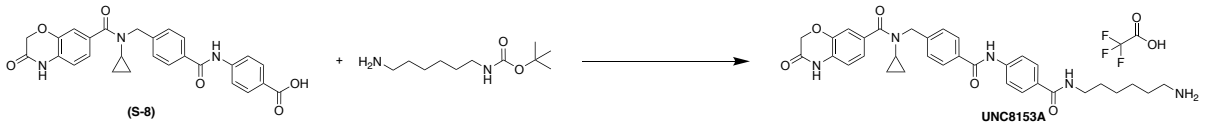


N-(4-((4-((5-aminopentyl)carbamoyl)phenyl)carbamoyl)benzyl)-N-cyclopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamide 2,2,2-trifluoroacetate (UNC8269A)

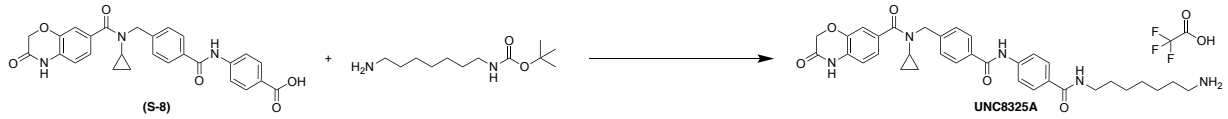
Prepared according to General Procedure A using **(S-8)** (20 mg, 1 Eq, 41 µmol), EDC (12 mg, 1.5 Eq, 62 µmol), HOAt (8.4 mg, 1.5 Eq, 62 µmol), DMF (1 mL), tert-butyl (5-aminopentyl)carbamate (10 mg, 1.2 Eq, 49 µmol) and triethylamine (8.8 mg, 12 µL, 2.1 Eq, 87 µmol). Following *N-Boc* deprotection, the crude material was purified by reverse phase chromatography (10-100% methanol in water + 0.1% TFA) to give **UNC8269A** (26.6 mg, 38.9 µmol, 94 % over two steps) as a white solid.

^{1}H NMR (400 MHz, MeOH-d_4) δ 7.95 (d, J = 8.4 Hz, 2H), 7.84 (s, 4H), 7.50 (d, J = 7.9 Hz, 2H), 7.20 (dd, J = 8.0, 1.7 Hz, 1H), 7.17 (d, J = 1.7 Hz, 1H), 6.96 (d, J = 8.0 Hz, 1H), 4.83 (s, 2H), 4.62 (s, 2H),

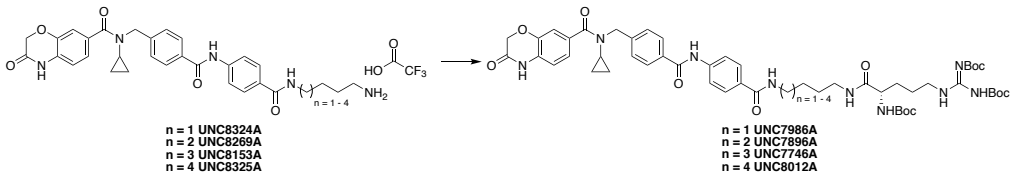
3.41 (t, *J* = 7.0 Hz, 2H), 2.94 (t, *J* = 7.6 Hz, 2H), 2.88 – 2.78 (m, 1H), 1.70 (tt, *J* = 13.0, 7.4 Hz, 4H), 1.49 (q, *J* = 8.3 Hz, 2H), 0.73 – 0.61 (m, 2H), 0.61 – 0.48 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{32}H_{36}N_5O_5$, 570.27; found, 570.25.



N-(4-((4-((6-aminohexyl)carbamoyl)phenyl)carbamoyl)benzyl)-N-cyclopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamide 2,2,2-trifluoroacetate (UNC8153A)
Prepared according to General Procedure A using **(S-8)** (200 mg, 1 Eq, 412 µmol), EDC (118 mg, 1.5 Eq, 618 µmol), HOAt (84.1 mg, 1.5 Eq, 618 µmol), DMF (2 mL), tert-butyl (6-aminohexyl)carbamate (134 mg, 1.5 Eq, 618 µmol) and triethylamine (167 mg, 0.23 mL, 4 Eq, 1.65 mmol), except that the crude reaction was diluted with water and the white precipitate formed was isolated by centrifugation. Following *N-Boc* deprotection, the crude material was purified by reverse phase chromatography (10-100% methanol in water + 0.1% TFA) to give **UNC8153A** (139.8 mg, 200.4 µmol, 48.6 % over two steps) as a white solid.
^{1}H NMR (400 MHz, MeOH-d_4) δ 7.94 (d, *J* = 8.3 Hz, 2H), 7.89 – 7.78 (m, 4H), 7.47 (d, *J* = 7.9 Hz, 2H), 7.18 (dd, *J* = 8.2, 1.7 Hz, 1H), 7.15 (d, *J* = 1.7 Hz, 1H), 6.95 (d, *J* = 8.1 Hz, 1H), 4.80 (s, 2H), 4.60 (s, 2H), 3.39 (t, *J* = 7.1 Hz, 2H), 2.92 (t, *J* = 7.6 Hz, 2H), 2.86 – 2.74 (m, 1H), 1.73 – 1.59 (m, 4H), 1.49 – 1.38 (m, 4H), 0.70 – 0.58 (m, 2H), 0.58 – 0.47 (m, 2H).
^{13}C NMR (101 MHz, MeOH-d_4) δ 169.52, 168.53, 167.30, 144.58, 143.57, 143.11, 135.04, 133.26, 131.09, 129.87, 129.16, 129.06, 128.84, 123.20, 121.38, 121.28, 116.88, 116.72, 68.11, 49.85, 40.68, 40.60, 30.29, 28.44, 27.39, 27.00, 10.54.
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{33}H_{38}N_5O_5$, 584.3; found, 584.2.
HRMS (*m/z*): $[M+H]^+$ calculated for $C_{33}H_{38}N_5O_5$, 584.28675; found, 584.28629.

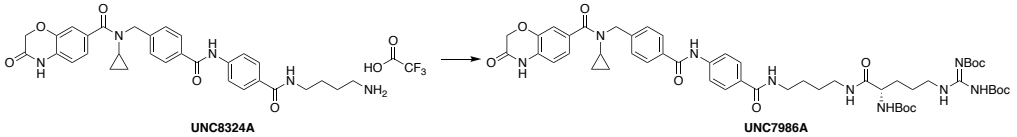


N-(4-((4-((7-aminoheptyl)carbamoyl)phenyl)carbamoyl)benzyl)-N-cyclopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamide 2,2,2-trifluoroacetate (UNC8325A)
Prepared according to General Procedure A using **(S-8)** (100 mg, 1 Eq, 206 µmol), EDC (59.2 mg, 1.5 Eq, 309 µmol), HOAt (42.1 mg, 1.5 Eq, 309 µmol), DMF (2 mL), tert-butyl (7-aminoheptyl)carbamate (71.2 mg, 1.5 Eq, 309 µmol) and triethylamine (83.4 mg, 0.11 mL, 4 Eq, 824 µmol). Following *N-Boc* deprotection, the crude material was purified by reverse phase chromatography (10-100% methanol in water + 0.1% TFA) to give **UNC8325A** (18.94 mg, 26.61 µmol, 12.9 %) as a white solid.
^{1}H NMR (400 MHz, MeOH-d_4) δ 7.95 (d, *J* = 8.2 Hz, 2H), 7.84 (s, 4H), 7.49 (d, *J* = 7.5 Hz, 2H), 7.24 – 7.09 (m, 2H), 6.96 (d, *J* = 8.0 Hz, 1H), 4.82 (s, 2H), 4.62 (s, 2H), 3.38 (t, *J* = 7.1 Hz, 2H), 2.92 (t, *J* = 7.6 Hz, 2H), 2.88 – 2.71 (m, 1H), 1.78 – 1.53 (m, 4H), 1.52 – 1.28 (m, 6H), 0.89 – 0.60 (m, 2H), 0.60 – 0.41 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{34}H_{40}N_5O_5$, 598.3; found, 598.3.



General Procedure B: For the arginine amidation of free amines

To a vial were charged Nα,Nω,Nω'-Tris-Boc-L-arginine (1.1 Eq), TBTU (1.3 Eq), and DMF (0.4M). The reaction was stirred for 10 minutes, then the amine (1 Eq) was added, followed by DIPEA (3.3 Eq). The reaction was stirred at room temperature overnight. The next day, the reaction was diluted with water and extracted 3 times with ethyl acetate. The combined organic extracts were washed twice with water, once with saturated sodium bicarbonate, and once with brine, then dried by passage through a phase separator and concentrated to a residue. If starting amine was detected by LCMS of the crude, normal phase chromatography over silica gel (0-10% methanol in DCM) was performed.

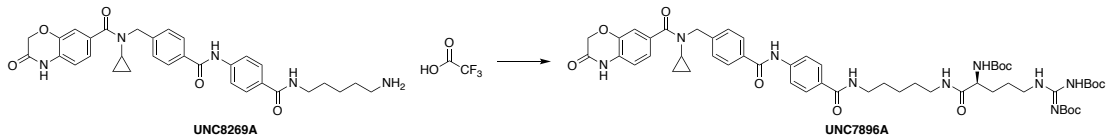


Tris-*N*-*Boc*-(*S*)-*N*-(4-((4-((4-(2-amino-5-guanidinopentanamido)butyl)carbamoyl)phenyl) carbamoyl) benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide (UNC7986A)

Prepared according to General Procedure B using Nα,Nω,Nω'-Tris-Boc-L-arginine (12 mg, 1.1 Eq, 25 µmol), HOAt (9.3 mg, 1.3 Eq, 29 µmol), DIPEA (9.6 mg, 13 µL, 3.3 Eq, 74 µmol), DMF (1 mL) and **UNC8324A** (15 mg, 1 Eq, 22 µmol). Normal phase chromatography over silica gel (0-10% MeOH in DCM) provided **UNC7986A** (17.2 mg, 17.0 µmol, 76 %) as a clear gum.

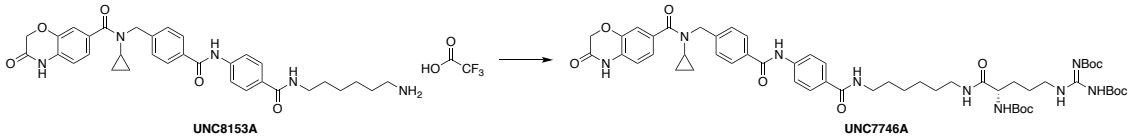
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.34 (s, 2H, -NH), 9.14 (s, 1H, -NH), 7.89 (d, *J* = 8.0 Hz, 2H), 7.74 (s, 4H), 7.32 (d, *J* = 7.9 Hz, 2H), 7.19 – 7.11 (m, 1H, -NH), 7.11 – 7.05 (m, 2H), 7.05 – 6.99 (m, 1H, -NH), 6.85 (d, *J* = 8.0 Hz, 1H), 5.99 (d, *J* = 8.7 Hz, 1H, -NH), 4.74 (s, 2H), 4.58 (s, 2H), 4.29 – 4.16 (m, 1H), 3.96 – 3.83 (m, 1H), 3.77 – 3.65 (m, 1H), 3.48 – 3.36 (m, 2H), 3.36 – 3.11 (m, 2H), 2.70 – 2.58 (m, 1H), 2.03 – 1.64 (m, 8H), 1.49 (s, 9H), 1.47 (s, 9H), 1.42 (s, 9H), 0.68 – 0.55 (m, 2H), 0.55 – 0.38 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{52}H_{70}N_9O_{12}$, 1012.5; found, 1012.4.

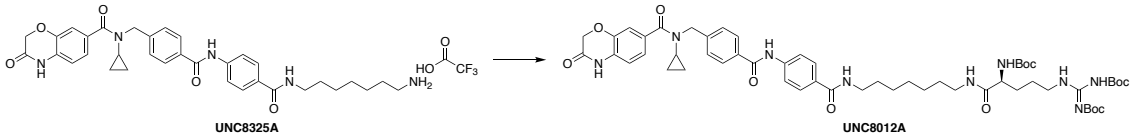


Tris-*N*-*Boc*-(*S*)-*N*-(4-((4-((5-(2-amino-5-guanidinopentanamido)pentyl)carbamoyl)phenyl) carbamoyl)benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide (UNC7896A)

Prepared according to General Procedure B using Nα,Nω,Nω'-Tris-Boc-L-arginine (3.8 mg, 1.1 Eq, 8.0 µmol), TBTU (3.1 mg, 1.3 Eq, 9.5 µmol), DIPEA (3.1 mg, 3.3 Eq, 24 µmol), DMF (0.5 mL) and **UNC8269A** (5.0 mg, 1 Eq, 7.3 µmol). Normal phase chromatography over silica gel (0-10% MeOH in DCM) provided **UNC7896A** (7.2 mg, 7.0 µmol, 96 %) as a white solid.
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.33 (s, 2H, -NH), 8.97 (s, 1H, -NH), 8.85 (s, 1H, -NH), 7.89 (d, *J* = 8.2 Hz, 2H), 7.79 (s, 4H), 7.36 (d, *J* = 7.9 Hz, 2H), 7.16 – 7.06 (m, 2H), 7.01 – 6.91 (m, 1H, -NH), 6.84 (d, *J* = 8.0 Hz, 1H), 6.74 (t, *J* = 5.6 Hz, 1H, -NH), 5.89 (d, *J* = 8.9 Hz, 1H, -NH), 4.76 (s, 2H), 4.61 (s, 2H), 4.23 – 4.14 (m, 1H), 3.97 – 3.78 (m, 1H), 3.75 – 3.55 (m, 1H), 3.53 – 3.33 (m, 2H), 3.33 – 3.08 (m, 2H), 2.64 (tt, *J* = 7.0, 3.9 Hz, 1H), 1.86 – 1.60 (m, 10H), 1.49 (s, 9H), 1.45 (s, 9H), 1.44 (s, 9H), 0.68 – 0.56 (m, 2H), 0.56 – 0.38 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{53}H_{72}N_9O_{12}$, 1026.52; found, 1026.40.

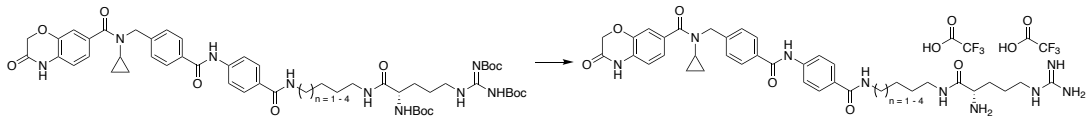


Tris-*N*-*Boc*-(*S*)-*N*-(4-((4-((6-(2-amino-5-guanidinopentanamido)hexyl)carbamoyl)phenyl) carbamoyl)benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide (UNC7746A)
Prepared according to General Procedure B using Nα,Nω,Nω'-Tris-Boc-L-arginine (4 mg, 1.1 Eq, 8 µmol), TBTU (3 mg, 1.3 Eq, 9 µmol), DIPEA (2 mg, 3 µL, 2.3 Eq, 0.02 mmol), DMF (0.5 mL) and **UNC8153A** (5 mg, 1 Eq, 7 µmol). Normal phase chromatography over silica gel (0-10% MeOH in DCM) provided **UNC7746A** (8.9 mg, 8.6 µmol, 100 %) as a clear gum.
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.31 (s, 2H, -NH), 8.89 (s, 1H, -NH), 8.83 (s, 1H, -NH), 7.90 (d, *J* = 8.2 Hz, 2H), 7.77 (s, 4H), 7.36 (d, *J* = 7.9 Hz, 2H), 7.16 – 7.07 (m, 2H), 6.98 – 6.90 (m, 1H, -NH), 6.84 (d, *J* = 8.0 Hz, 1H), 6.69 (t, *J* = 5.8 Hz, 1H, -NH), 5.91 (d, *J* = 8.7 Hz, 1H, -NH), 4.76 (s, 2H), 4.60 (s, 2H), 4.24 – 4.15 (m, 1H), 4.00 – 3.87 (m, 1H), 3.75 – 3.62 (m, 1H), 3.41 (hept, *J* = 6.6 Hz, 2H), 3.34 – 3.14 (m, 2H), 2.68 – 2.61 (m, 1H), 1.70 – 1.54 (m, 6H), 1.50 (s, 9H), 1.47 (s, 9H), 1.44 (s, 9H), 1.40 – 1.29 (m, 6H), 0.66 – 0.57 (m, 2H), 0.57 – 0.44 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{54}H_{74}N_9O_{12}$, 1040.55; found, 1040.40.

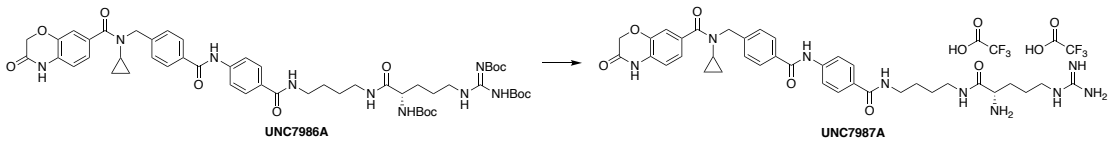


Tris-*N*-*Boc*-(*S*)-*N*-(4-((4-((7-(2-amino-5-guanidinopentanamido)heptyl)carbamoyl)phenyl) carbamoyl)benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide (UNC8012A)
Prepared according to General Procedure B using Nα,Nω,Nω'-Tris-Boc-L-arginine (8.0 mg, 1.2 Eq, 17 µmol), TBTU (5.9 mg, 1.3 Eq, 18 µmol), DIPEA (6.0 mg, 8.1 µL, 3.3 Eq, 46 µmol), DMF (1 mL) and **UNC8325A** (10 mg, 1 Eq, 14 µmol). Normal phase chromatography over silica gel (0-10% MeOH in DCM) provided **UNC8012A** (14.04 mg, 13.32 µmol, 95 %) as a clear gum.

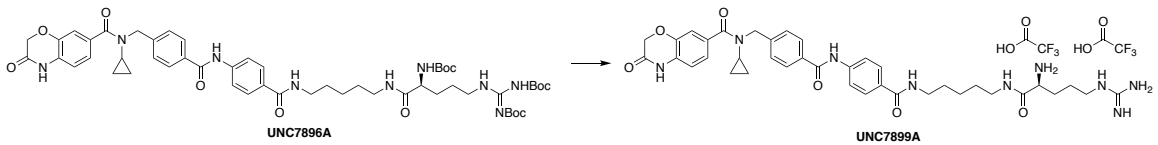
^{1}H NMR (400 MHz, $CDCl_3$) δ 9.29 (s, 3H, -NH), 9.06 (s, 1H, -NH), 7.88 (d, *J* = 8.0 Hz, 2H), 7.74 (s, 4H), 7.32 (d, *J* = 7.9 Hz, 2H), 7.14 – 7.05 (m, 2H), 7.03 – 6.90 (m, 1H, -NH), 6.85 (d, *J* = 8.0 Hz, 1H), 6.76 (t, *J* = 5.7 Hz, 1H, -NH), 5.98 (d, *J* = 8.7 Hz, 1H, -NH), 4.74 (s, 2H), 4.59 (s, 2H), 4.27 – 4.09 (m, 1H), 4.00 – 3.85 (m, 1H), 3.74 – 3.53 (m, 1H), 3.37 (q, *J* = 6.9 Hz, 2H), 3.21 (ddq, *J* = 49.7, 13.2, 6.7 Hz, 2H), 2.70 – 2.55 (m, 1H), 2.07 – 1.75 (m, 4H), 1.74 – 1.69 (m, 2H), 1.49 (s, 9H), 1.47 (s, 9H), 1.42 (s, 9H), 1.32 – 1.19 (m, 8H), 0.71 – 0.55 (m, 2H), 0.55 – 0.36 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{55}H_{76}N_9O_{12}$, 1054.56; found, 1054.40.



General Procedure C: N-Boc deprotection and purification of final products
To the *N-Boc*-protected product was added 50% TFA in DCM, and the solution was stirred at room temperature overnight. The next day the volatiles were removed under reduced pressure, the residue co-evaporated with methanol to remove residual TFA and purified by reverse phase chromatography (10-100% methanol in water + 0.1% TFA). The final product was suspended in water, flash frozen and lyophilized to dryness.



(*S*)-*N*-(4-((4-((4-(2-amino-5-guanidinopentanamido)butyl)carbamoyl)phenyl)carbamoyl) benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide bis-trifluoroacetate (UNC7987A)
Prepared according to General Procedure C using **UNC7986A** (12.58 mg, 1 Eq, 12.43 µmol) and 50% TFA in DCM to give **UNC7987A** (7.02 mg, 7.47 µmol, 60.1 %) as a white solid.
^{1}H NMR (400 MHz, MeOH-d_4) δ 7.96 (d, *J* = 8.3 Hz, 2H), 7.88 – 7.81 (m, 4H), 7.50 (d, *J* = 8.0 Hz, 2H), 7.20 (d, *J* = 8.5 Hz, 1H), 7.17 (s, 1H), 6.97 (d, *J* = 8.0 Hz, 1H), 4.83 (s, 2H), 4.62 (s, 2H), 3.83 (t, *J* = 6.5 Hz, 1H), 3.41 (t, *J* = 6.5 Hz, 2H), 3.38 – 3.34 (m, 1H), 3.30 – 3.26 (m, 1H), 3.23 (t, *J* = 7.1 Hz, 2H), 2.89 – 2.79 (m, 1H), 1.97 – 1.82 (m, 2H), 1.74 – 1.59 (m, 6H), 0.71 – 0.61 (m, 2H), 0.61 – 0.51 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{37}H_{46}N_9O_6$, 712.36; found, 712.30; $[M+2H]^{2+}$ calculated for $C_{37}H_{47}N_9O_6$, 356.68, found 356.70.

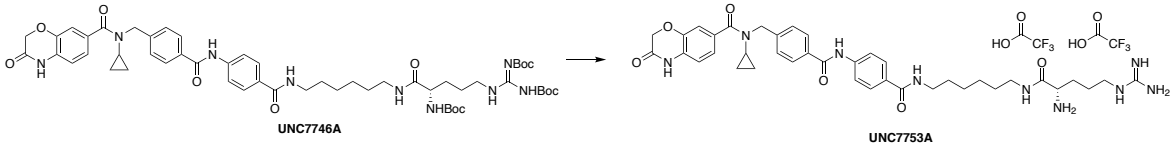


(*S*)-*N*-(4-((4-((5-(2-amino-5-guanidinopentanamido)pentyl)carbamoyl)phenyl)carbamoyl) benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide bis-trifluoroacetate (UNC7899A)

Prepared according to General Procedure C using **UNC7896A** (3.57 mg, 1 Eq, 3.48 µmol) and 50% TFA in DCM to give **UNC7899A** (3.23 mg, 3.39 µmol, 97.3 %) as a white solid.

^{1}H NMR (400 MHz, MeOH-*d*$_4$) δ 7.96 (d, *J* = 8.2 Hz, 2H), 7.87 – 7.82 (m, 4H), 7.51 (d, *J* = 7.9 Hz, 2H), 7.19 (d, *J* = 8.4 Hz, 1H), 7.18 (s, 1H), 6.97 (d, *J* = 8.0 Hz, 1H), 4.84 (s, 2H), 4.63 (s, 2H), 3.82 (t, *J* = 6.5 Hz, 1H), 3.40 (td, *J* = 7.0, 2.9 Hz, 2H), 3.37 – 3.33 (m, 1H), 3.28 – 3.24 (m, 1H), 3.24 – 3.19 (m, 2H), 2.88 – 2.79 (m, 1H), 1.94 – 1.82 (m, 2H), 1.71 – 1.58 (m, 6H), 1.49 – 1.39 (m, 2H), 0.71 – 0.61 (m, 2H), 0.61 – 0.51 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): [M+H]$^+$ calculated for $C_{38}H_{48}N_9O_7$, 726.37; found, 726.30; [M+2H]$^{2+}$ calculated for $C_{38}H_{49}N_9O_7$ 363.69, found 363.80.



(*S*)-*N*-(4-((4-((6-(2-amino-5-guanidinopentanamido)hexyl)carbamoyl)phenyl)carbamoyl) benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide bis-trifluoroacetate (UNC7753A)

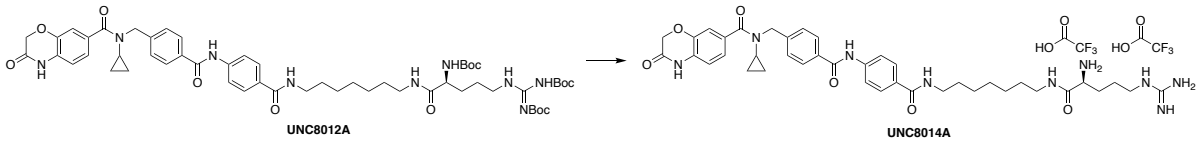
Prepared according to General Procedure C using **UNC7746A** (30 mg, 1 Eq, 43 µmol) and 50% TFA in DCM to give **UNC7753A** (24.63 mg, 25.45 µmol, 59 %) as a white fluffy powder.

^{1}H NMR (400 MHz, MeOH-*d*$_4$) δ 7.96 (d, *J* = 8.1 Hz, 2H), 7.84 (s, 4H), 7.51 (d, *J* = 7.9 Hz, 2H), 7.20 (d, *J* = 8.6 Hz, 1H), 7.18 (s, 1H), 6.97 (d, *J* = 8.0 Hz, 1H), 4.84 (s, 2H), 4.63 (s, 2H), 3.83 (t, *J* = 6.6 Hz, 1H), 3.39 (t, *J* = 7.0 Hz, 2H), 3.27 (m, 2H), 3.24 (t, *J* = 7.0 Hz, 2H), 2.89 – 2.79 (m, 1H), 1.88 (m, 2H), 1.75 – 1.52 (m, 6H), 1.43 (m, 4H), 0.71 – 0.61 (m, 2H), 0.57 (m, 2H).

^{13}C NMR (101 MHz, MeOH-*d*$_4$) δ 169.70, 169.63, 168.63, 167.37, 158.71, 144.64, 143.12, 135.08, 131.19, 129.92, 129.16, 129.06, 128.88, 123.22, 121.36, 116.90, 116.73, 68.13, 54.11, 49.64, 49.43, 49.21, 49.00, 48.79, 48.57, 48.36, 41.72, 40.76, 40.52, 30.36, 30.12, 29.77, 27.51, 27.48, 25.49, 10.47.

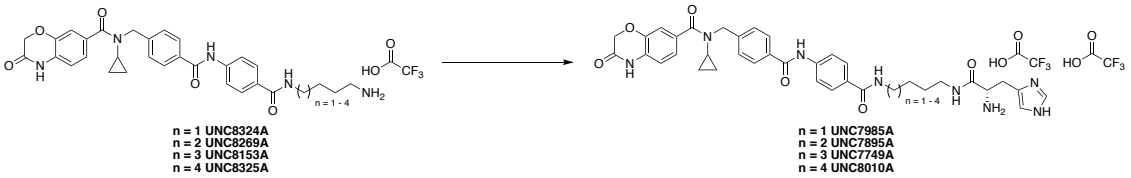
LCMS (ESI, +ve mode) (*m/z*): [M+H]$^+$ calculated for $C_{39}H_{50}N_9O_6$, 740.39; found, 740.30; [M+2H]$^{2+}$ calculated for $C_{39}H_{51}N_9O_6$, 370.70, found 370.80.

HRMS (*m/z*): [M+H]$^+$ calculated for $C_{39}H_{50}N_9O_6$, 740.38786; found, 740.38723.

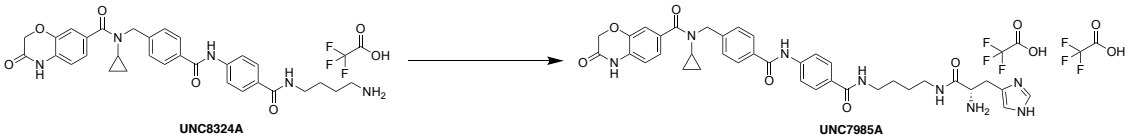


(*S*)-*N*-(4-((4-((7-(2-amino-5-guanidinopentanamido)heptyl)carbamoyl)phenyl)carbamoyl) benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide bis-trifluoroacetate (UNC8014A)

Prepared according to General Procedure C using **UNC8012A** (9.5 mg, 1 Eq, 9.0 µmol) and 50% TFA in DCM to give **UNC8014A** (5.4 mg, 5.5 µmol, 61 %) as a white solid.
^{1}H NMR (400 MHz, MeOH-d_4) δ 7.96 (d, *J* = 8.2 Hz, 2H), 7.87 – 7.81 (m, 4H), 7.50 (d, *J* = 7.9 Hz, 2H), 7.20 (d, *J* = 8.6 Hz, 1H), 7.18 (s, 1H), 6.97 (d, *J* = 8.1 Hz, 1H), 4.84 (s, 2H), 4.63 (s, 2H), 3.83 (t, *J* = 6.6 Hz, 1H), 3.38 (t, *J* = 7.1 Hz, 2H), 3.29 – 3.19 (m, 4H), 2.88 – 2.79 (m, 1H), 1.93 – 1.83 (m, 2H), 1.71 – 1.59 (m, 4H), 1.59 – 1.51 (m, 2H), 1.47 – 1.34 (m, 6H), 0.71 – 0.61 (m, 2H), 0.61 – 0.51 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{40}H_{52}N_9O_6$, 754.40; found, 754.30; $[M+2H]^{2+}$ calculated for $C_{40}H_{53}N_9O_6$ 377.70, found 377.85.

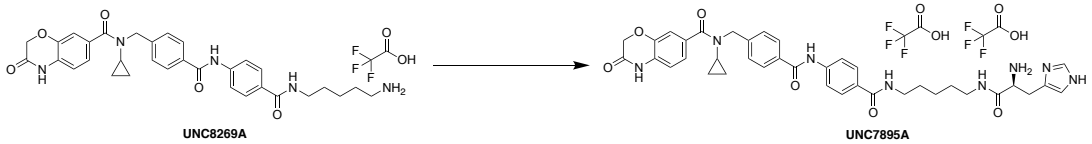


General Procedure D: Histidine Amidation
To a vial charged with a stirbar was added *N*-*Boc*-1-trityl-L-histidine (1.1 Eq), TBTU (1.3 Eq), and DMF (0.4M). The reaction was stirred for 10 minutes, then the amine (1 Eq) was added, followed by DIPEA (3.3 Eq). The reaction was stirred at room temperature overnight. The next day, the reaction was diluted with water and extracted 3 times with ethyl acetate. The combined organic extracts were washed twice with water, once with saturated sodium bicarbonate, and once with brine, then dried by passage through a phase separator and concentrated to a residue. To the protected product was added neat formic acid, and the reaction was stirred at room temperature overnight. The next day, a drop of methanol was added and the volatiles were removed *in vacuo*, and the residue co-evaporated with methanol to remove residual formic acid. The residue was taken up in methanol/water, filtered, and purified by reverse phase chromatography (10-100% methanol in water + 0.1% TFA). The final product was suspended in water, flash frozen and lyophilized to dryness.



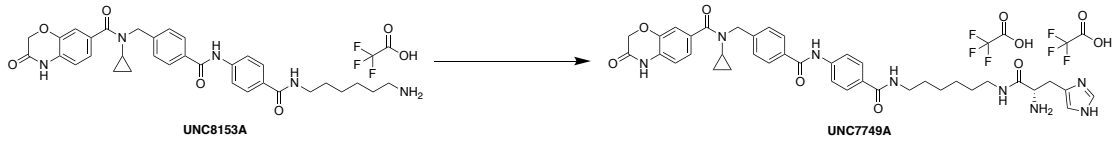
(*S*)-*N*-(4-((4-((4-(2-amino-3-(1*H*-imidazol-4-yl)propanamido)butyl)carbamoyl)phenyl) carbamoyl)benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide bis(2,2,2-trifluoroacetate) (UNC7985A)
Prepared and purified according to General Procedure D using *N*-*Boc*-1-trityl-L-histidine (8.2 mg, 1.1 Eq, 16 µmol), TBTU (6.2 mg, 1.3 Eq, 19 µmol), DIPEA (6.4 mg, 8.6 µL, 3.3 Eq, 49 µmol), DMF (1 mL) and **UNC8324A** (10 mg, 1 Eq, 15 µmol) to give **UNC7985A** (14 mg, 11.03 µmol, 74% over two steps) as a white solid.
^{1}H NMR (400 MHz, MeOH-d_4) δ 8.90 (d, *J* = 1.4 Hz, 1H), 7.96 (d, *J* = 8.3 Hz, 2H), 7.88 – 7.80 (m, 4H), 7.50 (d, *J* = 7.9 Hz, 2H), 7.46 (s, 1H), 7.20 (d, *J* = 8.1 Hz, 1H), 7.17 (d, *J* = 1.8 Hz, 1H), 6.96 (d, *J* = 8.1 Hz, 1H), 4.83 (s, 2H), 4.62 (s, 2H), 4.15 (t, *J* = 7.0 Hz, 1H), 3.42 – 3.36 (m, 2H), 3.36 –

3.32 (m, 2H), 3.30 – 3.25 (m, 2H), 2.88 – 2.77 (m, 1H), 1.66 – 1.51 (m, 4H), 0.71 – 0.61 (m, 2H), 0.61 – 0.52 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+2H]^{2+}$ calculated for $C_{37}H_{43}N_8O_6$, 694.3; found, 694.2.



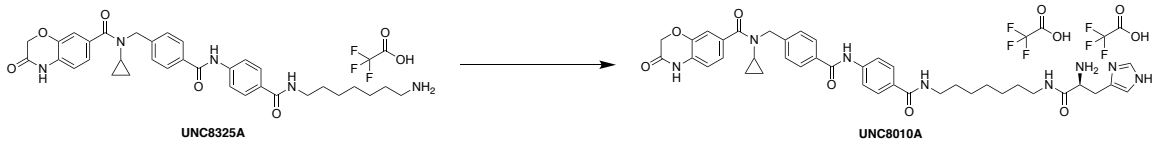
(*S*)-*N*-(4-((4-((5-(2-amino-3-(1*H*-imidazol-4-yl)propanamido)pentyl)carbamoyl)phenyl) carbamoyl)benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide bis(2,2,2-trifluoroacetate) (UNC7895A)

Prepared and purified according to General Procedure D using *N-Boc*-1-trityl-L-histidine (2.4 mg, 1.1 Eq, 4.8 µmol), TBTU (1.8 mg, 1.3 Eq, 5.7 µmol), DIPEA (1.9 mg, 2.5 µL, 3.3 Eq, 14 µmol), DMF (0.5 mL) and **UNC8269A** (3.0 mg, 1 Eq, 4.4 µmol) to give **UNC7895A** (3.28 mg, 3.51 µmol, 80 % over two steps) as a white solid.
^{1}H NMR (400 MHz, MeOH-d_4) δ 8.93 (d, *J* = 1.4 Hz, 1H), 7.96 (d, *J* = 8.3 Hz, 2H), 7.84 (s, 4H), 7.51 (d, *J* = 7.9 Hz, 2H), 7.45 (d, *J* = 1.3 Hz, 1H), 7.20 (d, *J* = 8.3 Hz, 1H), 7.18 (s, 1H), 6.97 (d, *J* = 8.0 Hz, 1H), 4.83 (s, 2H), 4.63 (s, 2H), 4.12 (d, *J* = 7.3 Hz, 1H), 3.38 (td, *J* = 6.7, 3.2 Hz, 2H), 3.30 – 3.16 (m, 4H), 2.88 – 2.80 (m, 1H), 1.63 (p, *J* = 7.3 Hz, 2H), 1.58 – 1.47 (m, 2H), 1.39 – 1.29 (m, 2H), 0.72 – 0.61 (m, 2H), 0.61 – 0.49 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+2H]^{2+}$ calculated for $C_{38}H_{43}N_8O_6$, 708.32; found, 708.2.



(*S*)-*N*-(4-((4-((6-(2-amino-3-(1*H*-imidazol-4-yl)propanamido)hexyl)carbamoyl)phenyl) carbamoyl)benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide bis(2,2,2-trifluoroacetate) (UNC7749A)

Prepared and purified according to General Procedure D using *N-Boc*-1-trityl-L-histidine (3.9 mg, 1.1 Eq, 7.9 µmol), TBTU (3.0 mg, 1.3 Eq, 9.3 µmol), DIPEA (2.1 mg, 2.9 µL, 2.3 Eq, 16 µmol), DMF (0.5 mL) and **UNC8153A** (5.0 mg, 1 Eq, 7.2 µmol) to give **UNC7749A** (5.89 mg, 6.21 µmol, 87 % over two steps) as a white solid.
^{1}H NMR (400 MHz, MeOH-d_4) δ 8.92 (d, *J* = 1.4 Hz, 1H), 7.96 (d, *J* = 8.3 Hz, 2H), 7.84 (s, 4H), 7.54 – 7.44 (m, 3H), 7.24 – 7.15 (m, 2H), 6.96 (d, *J* = 8.1 Hz, 1H), 4.83 (s, 2H), 4.62 (s, 2H), 4.14 (dd, *J* = 7.5, 6.6 Hz, 1H), 3.38 (td, *J* = 6.9, 2.6 Hz, 2H), 3.36 – 3.32 (m, 2H), 3.25 – 3.19 (m, 2H), 2.90 – 2.71 (m, 1H), 1.63 (p, *J* = 7.1 Hz, 2H), 1.50 (t, *J* = 7.5 Hz, 2H), 1.38 (dt, *J* = 21.7, 7.6 Hz, 4H), 0.65 (s, 2H), 0.57 (s, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{39}H_{45}N_8O_6$, 721.34; found, 721.30.

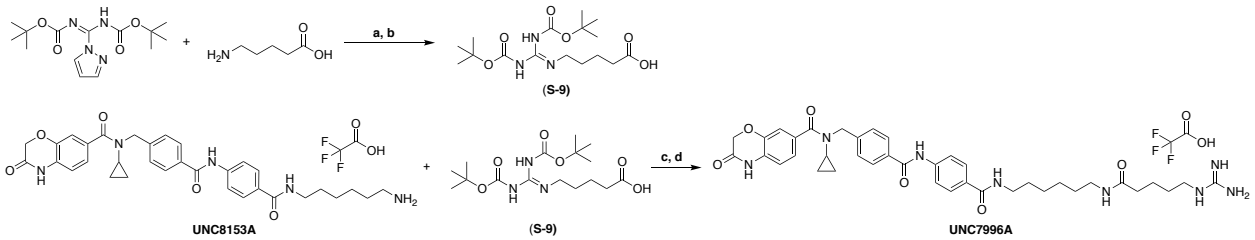


(*S*)-*N*-(4-((4-((7-(2-amino-3-(1*H*-imidazol-4-yl)propanamido)heptyl)carbamoyl)phenyl) carbamoyl)benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide bis(2,2,2-trifluoroacetate) (UNC8010A)

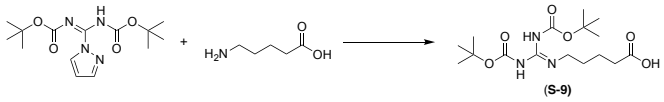
Prepared and purified according to General Procedure D using *N-Boc*-1-trityl-L-histidine (5.0 mg, 1.2 Eq, 10 µmol), TBTU (3.5 mg, 1.3 Eq, 11 µmol), DIPEA (3.6 mg, 3.3 Eq, 28 µmol), DMF (0.5 mL) and **UNC8325A** (6.0 mg, 1 Eq, 8.4 µmol) to give **UNC8010A** (8.1 mg, 6.5 µmol, 78% over two steps) as a white solid.

^{1}H NMR (400 MHz, MeOH-d_4) δ 8.91 (d, *J* = 1.4 Hz, 1H), 7.96 (d, *J* = 8.3 Hz, 2H), 7.84 (s, 4H), 7.50 (d, *J* = 7.8 Hz, 2H), 7.44 (d, *J* = 1.3 Hz, 1H), 7.20 (dd, *J* = 8.1, 1.8 Hz, 1H), 7.17 (s, 1H), 6.97 (d, *J* = 8.0 Hz, 1H), 4.83 (s, 2H), 4.62 (s, 2H), 4.14 (t, *J* = 7.0 Hz, 1H), 3.38 (t, *J* = 7.2 Hz, 2H), 3.36 – 3.33 (m, 1H), 3.30 – 3.24 (m, 1H), 3.21 (t, *J* = 7.0 Hz, 2H), 2.88 – 2.79 (m, 1H), 1.68 – 1.58 (m, 2H), 1.53 – 1.44 (m, 2H), 1.44 – 1.36 (m, 4H), 1.33 – 1.28 (m, 2H), 0.71 – 0.61 (m, 2H), 0.61 – 0.50 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): $[M+2H]^{2+}$ calculated for $C_{40}H_{47}N_8O_6$, 736.35; found, 736.30.



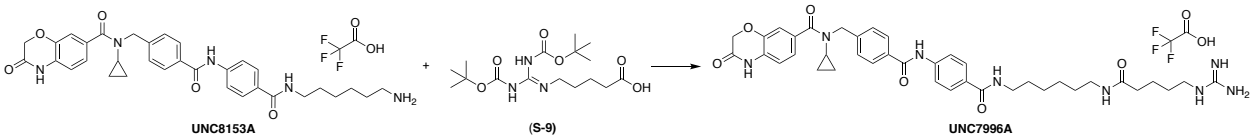
Supplemental Scheme 2: Synthesis of UNC7996A. Reagents and conditions: a) MeOH, Na_2CO_3 (aq); b) HCl; c) TBTU, DIPEA, DMF; d) TFA/DCM.



5-((2,2,10,10-tetramethyl-4,8-dioxo-3,9-dioxa-5,7-diazaundecan-6-ylidene)amino)pentanoic acid (S-9)

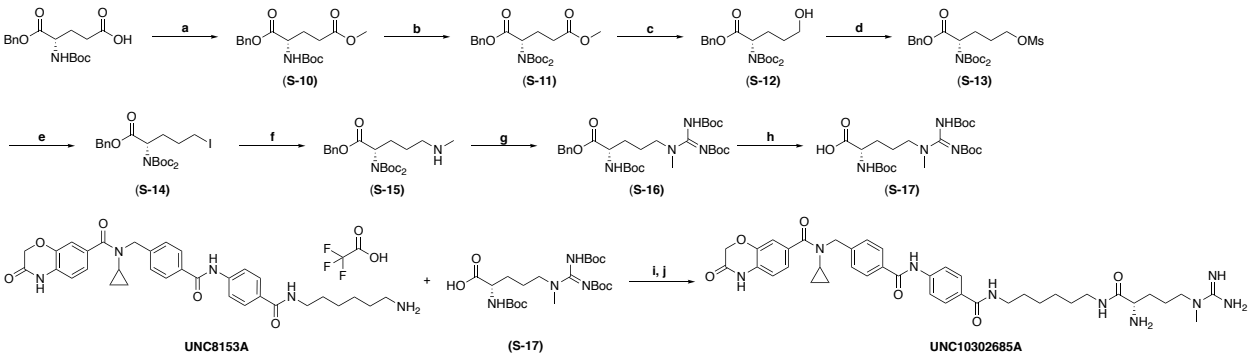
To a 2-dram vial charged with a stirbar was added 5-aminopentanoic acid (28 mg, 1.5 Eq, 0.24 mmol), methanol (1 mL), and an aqueous solution of sodium carbonate (0.16 mL, 1 M, 1 Eq, 0.6 mmol). Next, N,N'-Bis-Boc-1-guanylpyrazole (50 mg, 1 Eq, 0.16 mmol) was added, and the reaction stirred overnight. TLC of the reaction (3:1 hexanes/ethyl acetate) showed consumption of the N,N'-Bis-Boc-1-guanylpyrazole. Volatiles were removed *in vacuo*, and the aqueous layer washed 3 times with ethyl acetate (organics discarded). The reaction was diluted with ethyl acetate, and 1 mL of 1M HCl was added. The layers were separated, and the aqueous layer extracted twice more with ethyl acetate. The combined organic layers were washed once with water, once with brine, then dried by passage through a phase separator and concentrated to a

sticky residue. The residue was dissolved in minimal DCM and co-evaporated with hexanes to provide **(S-9)** (36 mg, 0.10 mmol, 62 %) as a white solid.
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 (s, 1H, -OH), 3.41 (q, *J* = 6.5 Hz, 2H), 2.38 (t, *J* = 7.0 Hz, 2H), 1.73 – 1.57 (m, 4H), 1.48 (s, 9H), 1.47 (s, 9H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{16}H_{30}N_3O_6$, 360.2; found, 360.2.



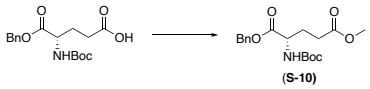
_N_-cyclopropyl-_N_-(4-((4-((6-(5-guanidinopentanamido)hexyl)carbamoyl) phenyl)carbamoyl)benzyl)-3-oxo-3,4-dihydro-2_H_-benzo[_b_][1,4]oxazine-7-carboxamide 2,2,2-trifluoroacetate (UNC7996A)

To a 2-dram vial was added **(S-9)** (5.7 mg, 1.1 Eq, 16 µmol), TBTU (6.0 mg, 1.3 Eq, 19 µmol), DIPEA (6.1 mg, 8.2 µL, 3.3 Eq, 47 µmol), and 200 µL DMF. The reaction was left to stir at room temperature for 30 minutes. To the vial was then added **UNC8153A** (10 mg, 1 Eq, 14 µmol) dissolved in 300 µL DMF, and the reaction was left to stir overnight. The reaction was quenched with 4 mL of distilled water and extracted 3 times with 4 mL portions of ethyl acetate. The combined organic layers were washed twice with water, once with saturated sodium bicarbonate, and once with brine, then dried by passage through a phase separator and concentrated to a clear residue. To the residue was added 1 mL of 50% TFA in DCM, and the reaction was stirred overnight. The volatiles were removed *in vacuo*, and the residue co-evaporated with methanol before purification by reverse phase chromatography (10-100% methanol in water + 0.1% TFA) and lyophilization to provide **UNC7996A** (1.83 mg, 14 µmol, 15% over two steps) as a white solid.
^{1}H NMR (400 MHz, MeOH-d_4) δ 7.96 (d, *J* = 8.1 Hz, 2H), 7.86 – 7.81 (m, 4H), 7.51 (d, *J* = 7.9 Hz, 2H), 7.20 (d, *J* = 8.6 Hz, 1H), 7.18 (s, 1H), 6.96 (d, *J* = 8.0 Hz, 1H), 4.84 (s, 2H), 4.63 (s, 2H), 3.38 (t, *J* = 7.1 Hz, 2H), 3.22 – 3.15 (m, 4H), 2.86 – 2.81 (m, 1H), 2.23 (t, *J* = 7.0 Hz, 2H), 1.71 – 1.50 (m, 8H), 1.47 – 1.36 (m, 4H), 0.70 – 0.62 (m, 2H), 0.62 – 0.53 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{39}H_{49}N_8O_6$, 725.4; found, 725.3.



Supplemental Scheme 3: Synthesis of UNC10302685A. Reagents and conditions: a) $ClCO_2Me$, Et_3N, DMAP, DCM; b) $(Boc)_2O$, DMAP, MeCN; c) DIBAL-H, THF, -78 °C; d) MsCl, Et_3N, DCM, 0

°C; e) NaI, acetone; f) NH_2Me-HCl, Na_2CO_3, THF, water, 80 °C; g) $(HNBoc)_2C=S$, DIPEA, EDC, DCM; h) LiOH-H_2O, MeOH, water; i) CDMT, NMM, DMF; j) HCl/EtOAc, DCM.



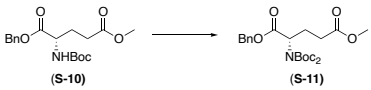
(**S-10**)

1-benzyl 5-methyl (*tert*-butoxycarbonyl)-*L*-glutamate (S-10)

To a round-bottom flask was added (4S)-5-benzyloxy-4-(tert-butoxycarbonylamino)-5-oxo-pentanoic acid (2 g, 1 Eq, 5.93 mmol), methyl chloroformate (672.2 mg, 7.11 mmol, 551.0 µL, 1.2 Eq), triethylamine (899.8 mg, 8.89 mmol, 1.24 mL, 1.5 Eq) and DCM (20 mL) at 25 °C. Then to the solution was added DMAP (72.4 mg, 592.82 µmol, 0.1 *Eq*). The solution was stirred for 12 h at 25 °C, and consumption of starting material was monitored by TLC. The reaction solution was quenched with $NaHCO_3$ (aq, 30 mL), extracted with DCM (3 x 10 mL), washed with brine (30 mL), dried over Na_2SO_4, concentrated to give the crude product. The crude product was purified by normal phase chromatography over silica gel (Petroleum ether/Ethyl acetate=1/0 to 1/1) to give **(S-10)** (1.6 g, 4.55 mmol, 76.81% yield) as colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.41 - 7.31 (m, 5H), 5.18 (d, *J*=2.4 Hz, 2H), 4.39 (d, *J*=4.8 Hz, 1H), 3.67 (s, 3H), 2.48 - 2.32 (m, 2H), 2.29 - 2.15 (m, 1H), 2.03 - 1.93 (m, 1H), 1.44 (s, 9H)

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{18}H_{26}NO_6$, 352.2; found, 352.2.

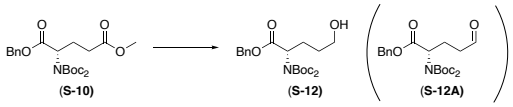


(**S-10**) (**S-11**)

1-benzyl 5-methyl *N*,*N*-bis(*tert*-butoxycarbonyl)-*L*-glutamate (S-11)

To a round-bottom flask was added **(S-10)** (5.8 g, 16.51 mmol, 1 Eq), Di-*tert*-butyl dicarbonate (14.41 g, 66.02 mmol, 15.17 mL, 4 Eq) and MeCN (60 mL), followed by DMAP (404.0 mg, 3.30 mmol, 0.2 Eq). The mixture was stirred at 25 °C for 12 h. TLC indicated that the starting material was consumed completely. The solution was concentrated *in vacuo* to give the crude product. The crude product was purified by normal phase chromatography over silica gel (Petroleum ether**/**Ethyl acetate = 1**/**0 to 3**/**1) to give **(S-11)** (6 g, 13.29 mmol, 80.51% yield) as a colorless oil.

^{1}H NMR (400MHz, $CDCl_3$) δ = 7.38 - 7.29 (m, 5H), 5.16 (s, 2H), 4.98 (dd, J=4.8, 9.6 Hz, 1H), 3.68 (s, 3H), 2.58 - 2.38 (m, 3H), 2.29 - 2.16 (m, 1H), 1.46 (s, 18H).



(**S-10**) (**S-12**) (**S-12A**)

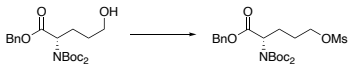
benzyl (*S*)-2-(bis(*tert*-butoxycarbonyl)amino)-5-hydroxypentanoate (S-12)
benzyl (*S*)-2-(bis(*tert*-butoxycarbonyl)amino)-5-oxopentanoate (S-12A)

To a three-necked round-bottom flask was added **(S-10)** (5 g, 11.07 mmol, 1 Eq) and THF (80 mL), followed by DIBAL-H (1 M, 24.36 mL, 2.2 Eq) at -78 °C dropwise. The mixture was allowed to warm from -78 °C to 0 °C over 5 hours with stirring. TLC indicated the reaction was completed. The reaction mixture was quenched with saturated NH_4Cl (150 mL), extracted with

EtOAc (3 x 50 mL), washed with brine (150 mL) then dried over Na_2SO_4. The mixture was filtered, and then the filtrate was concentrated to give the crude product. The crude product was purified by normal phase chromatography over silica gel (Petroleum ether/Ethyl acetate = 1/1) to give **(S-12)** (2.7 g, 6.38 mmol, 57.57% yield) and **(S-12A)** (1.4 g, 3.32 mmol, 29.99% yield), both as colorless oils.

(S-12): ^{1}H NMR (400MHz, $CDCl_3$) δ = 7.41 - 7.28 (m, 5H), 5.22 - 5.10 (m, 2H), 4.93 (dd, J=5.4, 9.2 Hz, 1H), 3.68 (t, J=6.4 Hz, 2H), 2.33 - 2.21 (m, 1H), 2.02 - 1.91 (m, 1H), 1.69 - 1.61 (m, 2H), 1.46 (s, 18H)

(S-12A): ^{1}H NMR (400MHz, $CDCl_3$) δ = 9.78 (s, 1H), 7.36 - 7.29 (m, 5H), 5.23 - 5.11 (m, 2H), 4.99 - 4.89 (m, 1H), 2.64 - 2.47 (m, 3H), 2.31 - 2.15 (m, 1H), 1.46 (s, 18H)

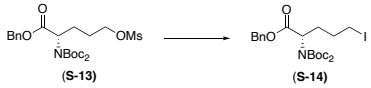


benzyl (*S*)-2-(bis(*tert*-butoxycarbonyl)amino)-5-((methylsulfonyl)oxy)pentanoate (S-13)

To a round-bottom flask was added **(S-12)** (2.7 g, 6.38 mmol, 413.22 µL, 1 Eq), DCM (30 mL) and triethylamine (1.29 g, 12.75 mmol, 1.77 mL, 2 Eq). Methanesulfonyl chloride (2.39 g, 20.86 mmol, 1.61 mL, 3.27 Eq) was added to the solution dropwise at 0 °C. The mixture was allowed to warm to 25 °C over 2 hours. TLC showed complete consumption of the starting material. The mixture was quenched with 50 mL of water and extracted with DCM (3 x 20 mL). The combined organic layers were washed with brine (40 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by normal phase chromatography over silica gel (Petroleum ether/Ethyl acetate=3:1) to give **(S-13)** (2.5 g, 4.98 mmol, 78.18% yield) as a colorless oil.

^{1}H NMR (400MHz, $CDCl_3$) δ = 7.38 - 7.30 (m, 5H), 5.21 - 5.10 (m, 2H), 4.91 (dd, J=5.2, 9.2 Hz, 1H), 4.26 (t, J=6.4 Hz, 2H), 3.01 (s, 3H), 2.35 - 2.23 (m, 1H), 2.05 - 1.95 (m, 1H), 1.91 - 1.80 (m, 2H), 1.46 (s, 18H).

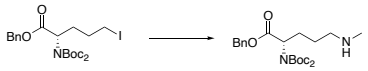
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{23}H_{36}NO_9S$, 502.2; found, 502.2.



benzyl (*S*)-2-(bis(*tert*-butoxycarbonyl)amino)-5-iodopentanoate (S-14)

To a round-bottom flask was added **(S-13)** (1.48 g, 2.95 mmol, 1 Eq) and acetone (10 mL). NaI (884.5 mg, 5.90 mmol, 2 Eq) was added and the solution was stirred at 25 °C for 12 h. TLC showed complete consumption of starting material. Volatiles were removed *in vacuo*. To the solid was added water (20 mL), and the mixture was extracted with EtOAc (3 x 10 mL). Combined organic layers were washed with brine (30 mL), dried over Na_2SO_4, filtered and concentrated to give crude **(S-14)** (1.25 g) as yellow oil, which was used in next step without further purification.

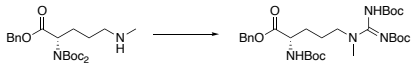
^{1}H NMR (400MHz, $CDCl_3$) δ = 7.40 - 7.30 (m, 5H), 5.23 - 5.11 (m, 2H), 4.91 (dd, J=5.1, 9.5 Hz, 1H), 3.31 - 3.13 (m, 2H), 2.31 - 2.20 (m, 1H), 2.12 - 1.83 (m, 3H), 1.46 (s, 18H).



(S-14) (S-15)

benzyl (*S*)-2-(bis(*tert*-butoxycarbonyl)amino)-5-(methylamino)pentanoate (S-15)

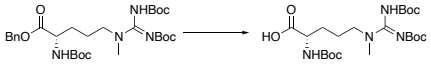
To a round-bottom flask was added **(S-14)** (1 g, 1.87 mmol, 1 Eq), THF (10 mL) and water (10 mL). Next were added Na_2CO_3 (1.99 g, 18.75 mmol, 10 Eq) and $MeNH_2$-HCl (1.30 g, 18.70 mmol, 9.97 Eq). The mixture was stirred at 80 °C for 12 h. LCMS showed complete consumption of starting material. Volatiles were removed *in vacuo*, and water (20 mL) was added to the solution. The mixture was extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with brine (2 x 30 mL), dried over Na_2SO_4, filtered and concentrated to give the crude product. The crude material was purified by prep-HPLC [mobile phase: (35-55% MeCN in water + 0.1%TFA,10min)] to give **(S-15)** (150 mg, 343.61 µmol, 18.33% yield) as a colorless oil.
^{1}H NMR (400MHz, $CDCl_3$) δ 7.39 - 7.28 (m, 8H), 5.14 (d, J=4.8 Hz, 2H), 4.87 - 4.82 (m, 1H), 3.49 (s, 2H), 2.99 (br s, 3H), 2.64 (br s, 2H), 2.24 (br d, J=7.7 Hz, 1H), 1.93 (br s, 1H), 1.43 (s, 18H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{23}H_{37}N_2O_6$, 437.3; found, 437.3.



(S-15) (S-16)

benzyl (*E*)-N^2,N^ω,$N^{\omega'}$-tris(*tert*-butoxycarbonyl)-N^δ-methyl-*L*-argininate (S-16)

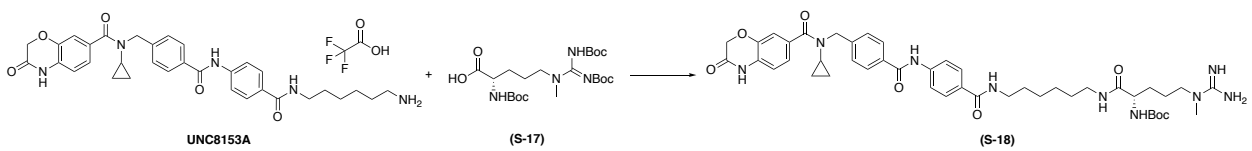
To a round-bottom flask was added **(S-15)** (120 mg, 274.89 µmol, 1 Eq), DCM (5 mL), *N,N*′−Di-(*tert*-butoxycarbonyl)thiourea (0.1 g, 361.86 µmol, 1.32 Eq) and DIPEA (74.2 mg, 574.13 µmol, 0.1 mL, 2.09 Eq). The mixture was cooled to 0 °C then EDC (120.0 mg, 625.98 µmol, 2.28 Eq) was added. The reaction was stirred at 25 °C for 3 h. LCMS showed the reaction was completed. Volatiles were removed *in vacuo*. The crude was purified by prep-TLC (Petroleum ether/Ethyl acetate = 3/1) to give **(S-16)** (70 mg, 103.12 µmol, 37.5% yield) as a colorless oil.
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{29}H_{47}N_4O_8$, 579.3; found, 579.3.



(S-16) (S-17)

(*E*)-N^2,N^ω,$N^{\omega'}$-tris(*tert*-butoxycarbonyl)-N^δ-methyl-*L*-arginine (S-17)

To a round-bottom flask was added **(S-16)** (55 mg, 81.02 µmol, 1 Eq), MeOH (2.5 mL), water (1 mL) and LiOH-H_2O (22.0 mg, 524.31 µmol, 6.47 Eq). The mixture was stirred at 25 °C for 12 h. LCMS showed the reaction was completed. Volatiles were removed *in vacuo*. The crude product was purified by prep-HPLC [mobile phase: (25-45% MeCN in water + 0.04 % HCl, 7min)] to give **(S-17)** (25 mg, 51.17 µmol, 63.1% yield) as a brown solid.
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{22}H_{41}N_4O_8$, 489.3; found, 489.3.



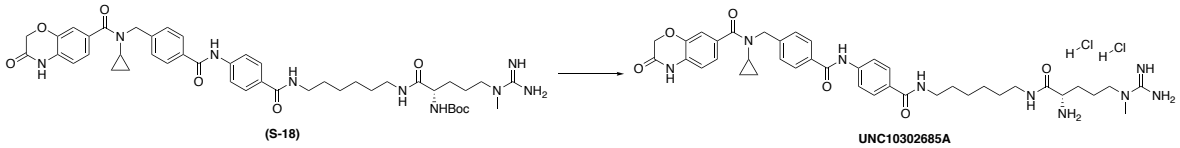
UNC8153A (S-17) (S-18)

***tert*-butyl (*S*)-(1-((6-(4-(4-((*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamido)methyl)benzamido)benzamido)hexyl)amino)-5-(1-methylguanidino)-1-oxopentan-2-yl)carbamate (S-18)**

To a round-bottom flask was added **(S-17)** (25 mg, 51.17 µmol, 1 Eq), **UNC8153A** (29.87 mg, 51.17 µmol, 1 Eq) and DMF (1 mL), followed by CDMT (12 mg, 61.40 µmol, 1.2 Eq) and NMM (11 mg, 102.34 µmol, 11.25 µL, 2 Eq). The mixture was stirred at 25 °C for 2 h. LCMS showed starting material was completely consumed and the desired mass was detected. The solution was concentrated to a residue. The residue was purified by prep-HPLC [mobile phase: (20-50% MeCN in water + 0.2 % formic acid, 8 min)] to give **(S-18)** (10 mg, 11.71 µmol, 22.88% yield) as a white solid.

^{1}H NMR (400MHz, MeOH-d_4) δ = 7.96 (d, J=8.4 Hz, 2H), 7.84 (s, 4H), 7.51 (d, J=7.6 Hz, 2H), 7.23 - 7.16 (m, 2H), 6.97 (d, J=8.0 Hz, 1H), 4.63 (s, 2H), 4.60 (s, 2H), 4.02 (s, 1H), 3.40 - 3.36 (m, 4H), 3.22 (dd, J=6.6, 12.4 Hz, 2H), 3.02 (s, 3H), 2.92 (s, 1H), 2.85 (s, 1H), 1.70 (s, 2H), 1.68 - 1.58 (m, 5H), 1.58 - 1.49 (m, 3H), 1.44 (s, 9H), 1.42 (s, 3H), 0.70 - 0.53 (m, 4H)

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{45}H_{60}N_9O_8$, 854.6; found, 854.3.



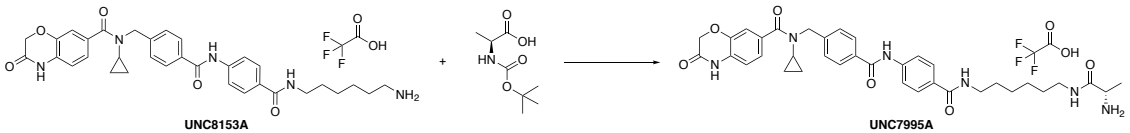
(*S*)-*N*-(4-((4-((6-(2-amino-5-(1-methylguanidino)pentanamido)hexyl)carbamoyl) phenyl)carbamoyl)benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide dihydrochloride (UNC10302685A)

To a 4 mL flask equipped with a stir bar was added **(S-18)** (10 mg, 11.71 µmol, 1 Eq) and DCM (1 mL), followed by HCl/EtOAc (1 mL, 4 M). The resulting mixture was stirred at 25 °C for 1 h. Volatiles were removed *in vacuo* to give **UNC10302685A** (10 mg, 11.72 µmol, 100%) as a white solid.

^{1}H NMR (400MHz, MeOH-d_4) 7.96 (d, J=8.4 Hz, 2H), 7.84 (s, 4H), 7.51 (d, J=7.2 Hz, 2H), 7.27 - 7.14 (m, 2H), 6.97 (d, J=8.0 Hz, 1H), 4.80 - 4.77 (m, 2H), 4.62 (s, 2H), 3.86 (t, J=6.4 Hz, 1H), 3.45 - 3.37 (m, 7H), 3.05 (s, 3H), 2.85 (s, 1H), 1.85 (d, J=8.0 Hz, 2H), 1.75 - 1.63 (m, 4H), 1.59 (s, 2H), 1.43 (s, 4H), 0.73 - 0.52 (m, 4H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{40}H_{52}N_9O_6$, 754.4; found, 754.4.

SFC: RT=11.130 min, 100% purity



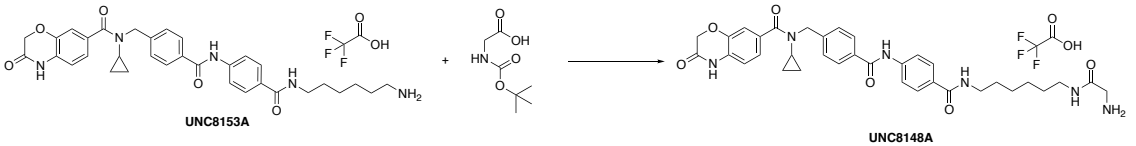
(*S*)-*N*-(4-((4-((6-(2-aminopropanamido)hexyl)carbamoyl)phenyl)carbamoyl)benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide 2,2,2-trifluoroacetate (UNC7995A)

To a 2-dram vial was added *N*-(*tert*-Butoxycarbonyl)-L-alanine (3.0 mg, 1.1 Eq, 16 µmol), TBTU (6.0 mg, 1.3 Eq, 19 µmol), DIPEA (6.1 mg, 3.3 Eq, 47 µmol), and 200 µL DMF. The reaction was

left to stir at room temperature for 30 minutes. To the vial was then added **UNC8153A** (10 mg, 1 Eq, 14 µmol) dissolved in 300 µL DMF, and the reaction was left to stir overnight. The reaction was quenched with 4 mL of distilled water and extracted 3 times with 4 mL portions of ethyl acetate. The combined organic layers were washed twice with water, once with saturated sodium bicarbonate, and once with brine, then dried by passage through a phase separator and concentrated to a clear residue. To the residue was added 1 mL of 50 % TFA in DCM, and the reaction was stirred overnight. The volatiles were removed *in vacuo*, and the residue co-evaporated with methanol before purification by reverse phase chromatography (10-100% methanol in water + 0.1% TFA) and lyophilization to give **UNC7995A** (11 mg, 14 µmol, 77%) as a white solid.

^{1}H NMR (400 MHz, MeOH-d_4) δ 7.96 (d, *J* = 8.3 Hz, 2H), 7.84 (s, 4H), 7.50 (d, *J* = 7.9 Hz, 2H), 7.20 (d, *J* = 8.6 Hz, 1H), 7.17 (d, *J* = 1.7 Hz, 1H), 6.96 (d, *J* = 8.0 Hz, 1H), 4.83 (s, 2H), 4.62 (s, 2H), 3.87 (q, *J* = 7.0 Hz, 1H), 3.39 (t, *J* = 7.1 Hz, 2H), 3.25 (t, *J* = 6.9 Hz, 2H), 2.88 – 2.78 (m, 1H), 1.69 – 1.61 (m, 2H), 1.59 – 1.52 (m, 2H), 1.49 (d, *J* = 7.0 Hz, 3H), 1.45 – 1.40 (m, 4H), 0.71 – 0.62 (m, 2H), 0.61 – 0.50 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{36}H_{43}N_6O_6$, 655.3; found, 655.2.

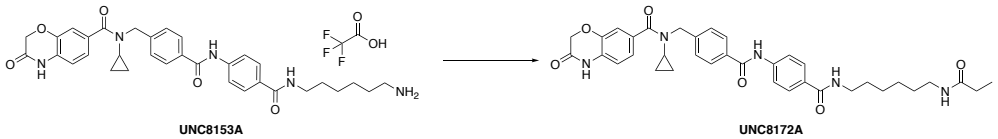


***N*-(4-((4-((6-(2-aminoacetamido)hexyl)carbamoyl)phenyl)carbamoyl)benzyl)-*N*-cyclopropyl -3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide 2,2,2-trifluoroacetate (UNC8148A)**

To a 2-dram vial was added *N*-(*tert*-butoxycarbonyl)glycine (2.5 mg, 1 Eq, 14 µmol), TBTU (6.0 mg, 1.3 Eq, 19 µmol), DIPEA (6.1 mg, 8.2 µL, 3.3 Eq, 47 µmol), and 200 µL DMF. The reaction was left to stir at room temperature for 30 minutes. To the vial was then added **UNC8153A** (10 mg, 1 Eq, 14 µmol) dissolved in 300 µL DMF, and the reaction was left to stir overnight. The reaction was quenched with 4 mL of distilled water and extracted 3 times with 4 mL portions of ethyl acetate. The combined organic layers were washed twice with water, once with saturated sodium bicarbonate, and once with brine, then dried by passage through a phase separator and concentrated to a clear residue. To the residue was added 1 mL of 50 % TFA in DCM, and the reaction was stirred overnight. The volatiles were removed *in vacuo*, and the residue co-evaporated with methanol before purification by reverse phase chromatography (10-100% methanol in water + 0.1% TFA) followed by lyophilization to give **UNC8148A** (8.33 mg, 11.0 µmol, 77 %) as a white solid.

^{1}H NMR (400 MHz, MeOH-d_4) δ 7.96 (d, *J* = 8.2 Hz, 2H), 7.85 – 7.82 (m, 4H), 7.50 (d, *J* = 7.9 Hz, 2H), 7.20 (d, *J* = 8.5 Hz, 1H), 7.17 (d, *J* = 1.7 Hz, 1H), 6.96 (d, *J* = 8.1 Hz, 1H), 4.83 (s, 2H), 4.62 (s, 2H), 3.65 (s, 2H), 3.39 (t, *J* = 7.1 Hz, 2H), 3.26 (t, *J* = 6.9 Hz, 2H), 2.87 – 2.79 (m, 1H), 1.69 – 1.60 (m, 2H), 1.60 – 1.51 (m, 2H), 1.48 – 1.37 (m, 4H), 0.70 – 0.61 (m, 2H), 0.61 – 0.51 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{35}H_{41}N_6O_6$, 641.3; found, 641.3.

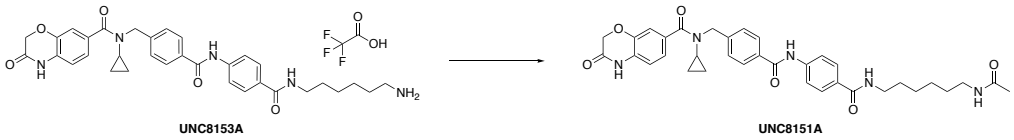


***N*-cyclopropyl-3-oxo-*N*-(4-((4-((6-propionamidohexyl)carbamoyl)phenyl)carbamoyl) benzyl)-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide (UNC8172A)**

To a 2-dram vial was added **UNC8153A** (10 mg, 1 Eq, 14 µmol), 4-methylmorpholine (5.8 mg, 6.3 µL, 4.0 Eq, 57 µmol) and 1 mL DCM. The vial was cooled to 0 °C and propionyl chloride (2 mg, 2 µL, 2 Eq, 0.02 mmol) was added. The vial was stirred overnight. Volatiles were removed *in vacuo* and the residue was purified by normal phase chromatography over silica gel (0-15% methanol in DCM) to give **UNC8172A** (5.08 mg, 7.94 µmol, 55 %) as a white solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.88 (s, 1H, -NH), 10.42 (s, 1H, -NH), 8.35 (t, *J* = 5.7 Hz, 1H, -NH), 7.95 (d, *J* = 8.3 Hz, 2H), 7.88 – 7.81 (m, 4H), 7.72 – 7.67 (m, 1H, -NH), 7.46 (d, *J* = 8.0 Hz, 2H), 7.19 (d, *J* = 8.1 Hz, 1H), 7.14 (d, *J* = 1.7 Hz, 1H), 6.93 (d, *J* = 8.0 Hz, 1H), 4.72 (s, 2H), 4.61 (s, 2H), 3.27 – 3.20 (m, 2H), 3.01 (q, *J* = 6.5 Hz, 2H), 2.83 – 2.77 (m, 1H), 2.04 (q, *J* = 7.6 Hz, 2H), 1.56 – 1.46 (m, 2H), 1.43 – 1.34 (m, 2H), 1.34 – 1.24 (m, 4H), 0.97 (t, *J* = 7.6 Hz, 3H), 0.59 – 0.51 (m, 2H), 0.51 – 0.43 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{36}H_{42}N_5O_6$, 640.3; found, 640.3.

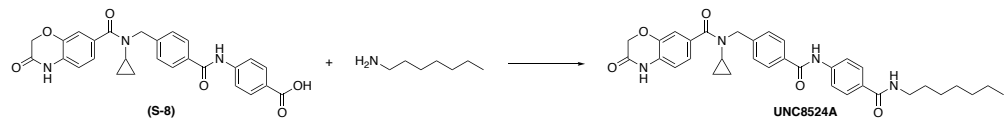


***N*-(4-((4-((6-acetamidohexyl)carbamoyl)phenyl)carbamoyl)benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide (UNC8151A)**

To a 2-dram vial was added **UNC8153A** (10 mg, 1 Eq, 14 µmol), triethylamine (4.8 mg, 6.6 µL, 3.3 Eq, 47 µmol) and 0.5 mL ethanol. The vial was cooled to 0 °C and acetic anhydride (1.9 mg, 1.8 µL, 1.3 Eq, 19 µmol) was added. The vial was stirred overnight. Volatiles were removed *in vacuo* and the residue was purified by normal phase chromatography over silica gel (0-15% methanol in DCM) to give **UNC8151A** (8.2 mg, 13 µmol, 91 %) as a white solid.

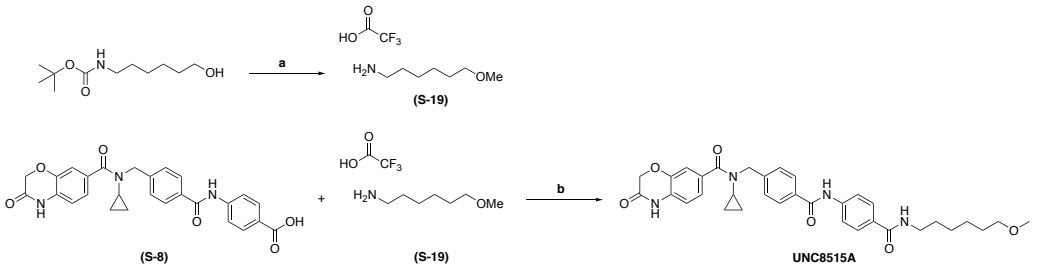
^{1}H NMR (400 MHz, DMSO-d_6) δ 10.88 (s, 1H, -NH), 10.42 (s, 1H, -NH), 8.35 (t, *J* = 5.7 Hz, 1H, -NH), 7.95 (d, *J* = 8.2 Hz, 2H), 7.84 (s, 4H), 7.81 – 7.71 (m, 1H, -NH), 7.46 (d, *J* = 7.9 Hz, 2H), 7.19 (d, *J* = 8.8 Hz, 1H), 7.14 (d, *J* = 1.7 Hz, 1H), 6.93 (d, *J* = 8.0 Hz, 1H), 4.72 (s, 2H), 4.61 (s, 2H), 3.24 (q, *J* = 6.7 Hz, 2H), 3.01 (q, *J* = 6.5 Hz, 2H), 2.87 – 2.71 (m, 1H), 1.78 (s, 3H), 1.58 – 1.45 (m, 2H), 1.45 – 1.35 (m, 2H), 1.35 – 1.26 (m, 4H), 0.70 – 0.51 (m, 2H), 0.51 – 0.30 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{35}H_{40}N_5O_6$, 626.3; found, 626.2.

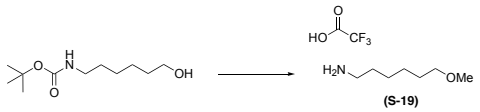


***N*-cyclopropyl-*N*-(4-((4-(heptylcarbamoyl)phenyl)carbamoyl)benzyl)-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide (UNC8524A)**

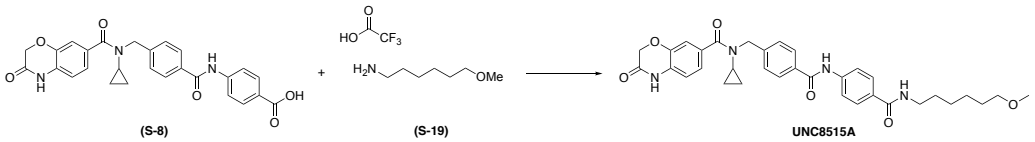
To a 2-dram vial were added **(S-8)** (50 mg, 1 Eq, 0.10 mmol), EDC (30 mg, 1.5 Eq, 0.15 mmol), HOAt (21 mg, 1.5 Eq, 0.15 mmol), and DMF (0.5 mL). The reaction was left to stir at room temperature for 30 minutes. To the vial was then added 1-aminoheptane (18 mg, 23 µL, 1.5 Eq, 0.15 mmol), followed by triethylamine (31 mg, 3 Eq, 0.31 mmol), and the reaction was left to stir overnight. The next day, the reaction was diluted with water and the white precipitate formed was isolated by filtration, then recrystallized from absolute ethanol to provide **UNC8524A** (10.73 mg, 18.41 µmol, 18 %) as a white solid.
^{1}H NMR (400 MHz, DMSO-d_6) δ 10.87 (s, 1H, -NH), 10.41 (s, 1H, -NH), 8.34 (t, *J* = 5.6 Hz, 1H, -NH), 7.96 (d, *J* = 8.5 Hz, 2H), 7.89 – 7.80 (m, 4H), 7.46 (d, *J* = 7.9 Hz, 2H), 7.19 (dd, *J* = 8.0, 1.7 Hz, 1H), 7.15 (d, *J* = 1.7 Hz, 1H), 6.93 (d, *J* = 8.1 Hz, 1H), 4.72 (s, 2H), 4.62 (s, 2H), 3.24 (q, *J* = 6.6 Hz, 2H), 2.80 (s, 1H), 1.57 – 1.46 (m, 2H), 1.34 – 1.22 (m, 8H), 0.86 (t, *J* = 7.0 Hz, 3H), 0.60 – 0.52 (m, 2H), 0.52 – 0.42 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{34}H_{39}N_4O_5$, 583.3; found, 583.2.



Supplemental Scheme 4: Synthesis of UNC8515A. Reagents and conditions: a) NaH, MeI, DMF, 0 °C to rt; b) EDC, HOAt, Et_3N, DMF.



6-methoxyhexan-1-amine 2,2,2-trifluoroacetate (S-19)
A solution of tert-butyl (6-hydroxyhexyl)carbamate (0.50 g, 1 Eq, 2.3 mmol) in DMF (5 mL) was cooled to 0 °C followed by the addition of sodium hydride (0.12 g, 60% Wt, 1.3 Eq, 3.0 mmol) and iodomethane (0.42 g, 0.19 mL, 1.3 Eq, 3.0 mmol). The reaction mixture was stirred at room temperature for 48 hours, after which the reaction mixture was quenched with H_2O (20 mL) and extracted 3x with EtOAc. The combined organic layers were washed with H_2O, brine (20 mL), and dried over $MgSO_4$. Volatiles were removed under reduced pressure and normal phase chromatography over silica gel (0-25% EtOAc in hexanes) provided the *N-Boc* protected product as a colorless oil. NMR confirmed formation of desired product. Following *N-Boc* deprotection, solvent was removed to give **(S-19)** (350.5 mg, 1.429 mmol, 62 %), which was used without further purification.
(*N-Boc*)-(S-18): ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.50 (br s, 1H, -NH), 3.35 (t, *J* = 6.5 Hz, 4H), 3.32 (s, 3H), 3.16 – 3.00 (m, 2H), 1.56 (p, *J* = 6.5 Hz, 2H), 1.51 – 1.46 (m, 2H), 1.44 (s, 9H), 1.41 – 1.29 (m, 2H).

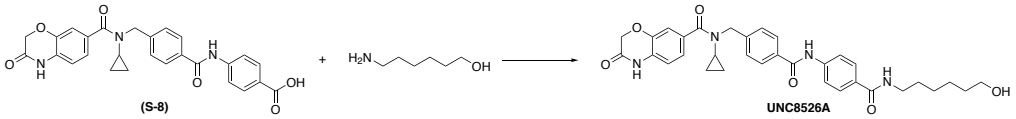


***N*-cyclopropyl-*N*-(4-((4-((6-methoxyhexyl)carbamoyl)phenyl)carbamoyl)benzyl)-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide (UNC8515A)**

Prepared and purified according to General Procedure A using **(S-8)** (50 mg, 1 Eq, 0.10 mmol), EDC (30 mg, 1.5 Eq, 0.15 mmol), HOAt (21 mg, 1.5 Eq, 0.15 mmol), DMF (1 mL), **(S-19)** (25 mg, 1 Eq, 0.10 mmol) and triethylamine (42 mg, 57 µL, 4 Eq, 0.41 mmol). The product was purified by normal phase chromatography over silica gel (0-10% MeOH in DCM) to give **UNC8515A** (2.64 mg, 4.41 µmol, 4.3 %) as a white solid.

^{1}H NMR (400 MHz, MeOH-d_4) δ 7.96 (d, *J* = 8.3 Hz, 2H), 7.84 (s, 4H), 7.50 (d, *J* = 7.8 Hz, 2H), 7.27 – 7.08 (m, 2H), 6.96 (d, *J* = 8.0 Hz, 1H), 4.83 (s, 2H), 4.63 (s, 2H), 3.49 – 3.33 (m, 4H), 3.32 (s, 3H), 2.94 – 2.74 (m, 1H), 1.74 – 1.51 (m, 4H), 1.51 – 1.34 (m, 4H), 0.77 – 0.62 (m, 2H), 0.62 – 0.38 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{34}H_{39}N_4O_6$, 599.3; found, 599.2.

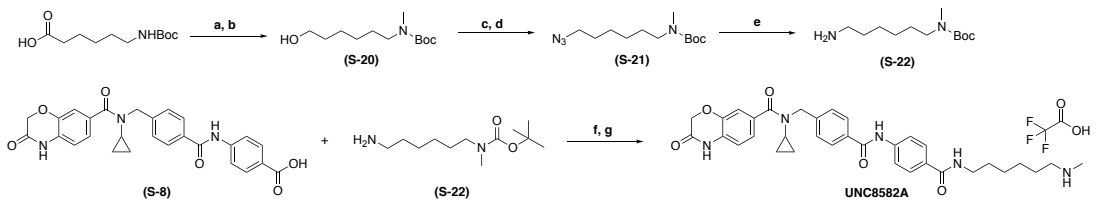


***N*-cyclopropyl-*N*-(4-((4-((6-hydroxyhexyl)carbamoyl)phenyl)carbamoyl)benzyl)-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide (UNC8526A)**

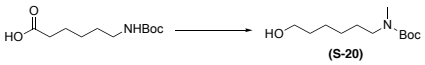
To a 2-dram vial were added **(S-8)** (50 mg, 1 Eq, 0.10 mmol), EDC (30 mg, 1.5 Eq, 0.15 mmol), HOAt (21 mg, 1.5 Eq, 0.15 mmol), and DMF (0.5 mL). The reaction was left to stir at room temperature for 30 minutes. To the vial was then added 6-aminohexan-1-ol (18 mg, 1.5 Eq, 0.15 mmol) dissolved in DMF (0.5 mL), followed by triethylamine (31 mg, 43 µL, 3 Eq, 0.31 mmol), and the reaction was left to stir overnight. The next day, the reaction was diluted with water and the white precipitate formed was collected by centrifugation (supernatant discarded), washed 5 times with distilled water, and dried to provide **UNC8526A** (11.20 mg, 19.16 µmol, 19 %) as a white solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.87 (s, 1H, -NH), 10.41 (s, 1H, -NH), 8.34 (t, *J* = 5.6 Hz, 1H, -NH), 7.96 (d, *J* = 8.3 Hz, 2H), 7.90 – 7.80 (m, 4H), 7.46 (d, *J* = 7.9 Hz, 2H), 7.19 (dd, *J* = 8.0, 1.8 Hz, 1H), 7.15 (d, *J* = 1.7 Hz, 1H), 6.93 (d, *J* = 8.1 Hz, 1H), 4.72 (s, 2H), 4.62 (s, 2H), 4.34 (t, *J* = 5.2 Hz, 1H, -OH), 3.38 (q, *J* = 6.4 Hz, 2H), 3.24 (q, *J* = 6.6 Hz, 2H), 2.80 (s, 1H), 1.56 – 1.47 (m, 2H), 1.47 – 1.38 (m, 2H), 1.36 – 1.27 (m, 4H), 0.60 – 0.51 (m, 2H), 0.51 – 0.43 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{33}H_{37}N_4O_6$, 585.3; found, 585.2.



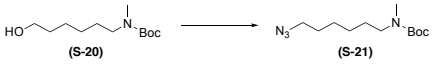
Supplemental Scheme 5: Synthesis of UNC8582A. Reagents and conditions: a) LAH, THF, 0 °C then reflux; b) Et_3N, Boc_2O, MeOH; c) MsCl, Et_3N, DCM, 0 °C; d) NaN_3, DMF, 70˚C; e) PtO_2, H_2, EtOH; f) EDC, HOAt, Et_3N, DMF; g) TFA, DCM.



***tert*-butyl (6-hydroxyhexyl)(methyl)carbamate (S-20)**

To a 50 mL flask charged with a stirbar was added 6-(Boc-amino)hexanoic acid (500 mg, 1 Eq, 2.16 mmol). The flask was sealed with a septum, and under positive pressure of nitrogen anhydrous THF (20 mL) was added. The flask was cooled in an ice bath, and lithium aluminum hydride (410 mg, 5 Eq, 10.8 mmol) was added cautiously. The reaction was allowed to stir for 1 hour while warming to room temperature, and was then heated to reflux overnight. The reaction was cooled to room temperature then immersed in an ice bath. The LAH was quenched first with 400 µL water, then 400 µL 4M KOH, then finally 1.2 mL water and left to stir while coming to room temperature. A pinch of $MgSO_4$ was added, and the solution filtered through Celite, washing with more THF, before being concentrated *in vacuo*. To the crude material were added MeOH (10 mL), triethylamine (284 mg, 1.3 Eq, 2.81 mmol), and di-tert-butyl dicarbonate (613 mg, 1.3 Eq, 2.81 mmol). The reaction was stirred for 18 hours, then the volatiles were removed *in vacuo*. The residue was purified by normal phase chromatography over silica gel (0-50% ethyl acetate in hexanes) to provide **(S-20)** (103 mg, 445 µmol, 20.6 % over two steps) as a clear oil.

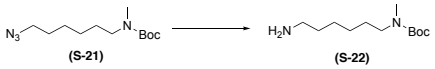
^{1}H NMR (400 MHz, $CDCl_3$) δ 3.56 (t, *J* = 6.6 Hz, 2H), 3.22 – 3.06 (m, 2H), 2.76 (s, 3H), 2.39 (s, 1H, -OH), 1.56 – 1.41 (m, 4H), 1.39 (s, 9H), 1.37 – 1.28 (m, 2H), 1.28 – 1.18 (m, 2H).



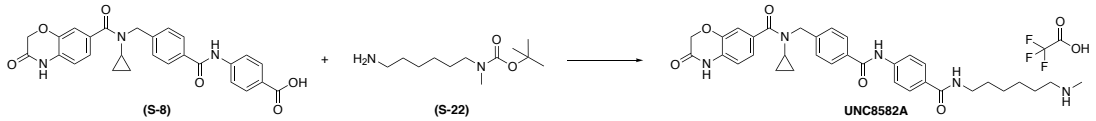
***tert*-butyl (6-azidohexyl)(methyl)carbamate (S-21)**

To a flask were added **(S-20)** (103 mg, 1 Eq, 445 µmol), DCM (2 mL), and triethylamine (81.3 mg, 112 µL, 1.80 Eq, 804 µmol). The flask was cooled in an ice bath, and mesyl chloride (74 mg, 50 µL, 1.4 Eq, 0.64 mmol) was added dropwise. The reaction vessel was capped and left to stir overnight while coming to room temperature. The next day, the reaction was quenched with 1M HCl and extracted 3 times with DCM. The combined organic extracts were washed once with water, once with saturated sodium bicarbonate, and once with brine, then dried over sodium sulfate and concentrated to a residue. NMR confirmed complete conversion to the mesylate. To the crude mesylate in a flask were added sodium azide (43.4 mg, 1.5 Eq, 668 µmol) and DMF (1 mL). The reaction was sealed and heated to 70˚C overnight. The next day, the reaction was quenched with water and extracted 3 times with ethyl acetate. The combined organic extracts were washed 3 times with water and once with brine, then dried over sodium

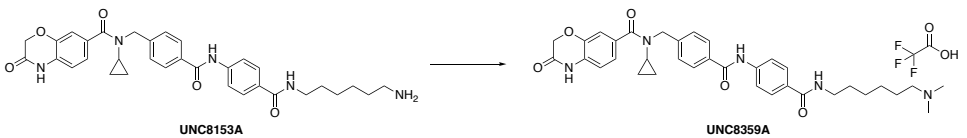
sulfate and concentrated to a residue. Normal phase chromatography over silica gel (0-50% ethyl acetate in hexanes) provided **(S-21)** (88.4 mg, 345 µmol, 77.5 % over two steps) as a clear oil.
^{1}H NMR (400 MHz, $CDCl_3$) δ 3.23 (t, *J* = 6.9 Hz, 2H), 3.16 (t, *J* = 7.2 Hz, 2H), 2.80 (s, 3H), 1.57 (p, *J* = 7.0 Hz, 2H), 1.48 (p, *J* = 7.4 Hz, 2H), 1.42 (s, 9H), 1.41 – 1.32 (m, 2H), 1.31 – 1.21 (m, 2H).



***tert*-butyl (6-aminohexyl)(methyl)carbamate (S-22)**
To a round bottomed flask were added **(S-21)** (44 mg, 1 Eq, 0.17 mmol) and EtOH (4 mL). The flask was evacuated and backfilled with nitrogen three times, then platinum(IV) oxide (4.4 mg, 0.11 Eq, 19 µmol) was added. The flask was again evacuated and backfilled with nitrogen three times, and the nitrogen inlet was replaced with a balloon of hydrogen gas. The reaction was stirred at room temperature under hydrogen atmosphere for 18 hours. The mixture was filtered through Celite and the filtrate concentrated to provide **(S-22)** (38.5 mg, 167 µmol, 97 %) a clear oil that was used in the next step without further purification.
^{1}H NMR (400 MHz, MeOH-d_6) δ 3.22 (t, *J* = 7.2 Hz, 2H), 2.83 (s, 3H), 2.67 (t, *J* = 7.2 Hz, 2H), 1.67 – 1.48 (m, 4H), 1.45 (s, 9H), 1.41 – 1.28 (m, 4H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{12}H_{27}N_2O_2$, 231.2; found, 231.3.



***N*-cyclopropyl-*N*-(4-((4-((6-(methylamino)hexyl)carbamoyl)phenyl)carbamoyl)benzyl)-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide 2,2,2-trifluoroacetate (UNC8582A)**
To a 2-dram vial were added **(S-8)** (80 mg, 1 Eq, 0.16 mmol), EDC (47 mg, 1.5 Eq, 0.25 mmol), HOAt (34 mg, 1.5 Eq, 0.25 mmol), and DMF (1 mL). The reaction was left to stir at room temperature for 30 minutes. To the vial was then added **(S-22)** (38 mg, 1 Eq, 0.16 mmol), followed by triethylamine (50 mg, 69 µL, 3 Eq, 0.49 mmol), and the reaction was left to stir overnight. The next day, the reaction was diluted with water and the white precipitate formed was isolated by centrifugation (supernatant discarded), washed once with water, taken up in absolute ethanol and concentrated to a clear residue. To the residue was added 3 mL of 20% TFA in DCM, which was stirred until disappearance of starting material as monitored by LCMS. The reaction was concentrated, co-evaporated with methanol, and purified by reverse phase chromatography (10-100% methanol in water + 0.1% TFA) to provide **UNC8582A** (52.06 mg, 73.14 µmol, 44 % over two steps) as a white solid.
^{1}H NMR (400 MHz, MeOH-d_4) δ 7.94 (d, *J* = 8.3 Hz, 2H), 7.84 (s, 4H), 7.47 (d, *J* = 8.2 Hz, 2H), 7.22 – 7.12 (m, 2H), 6.95 (d, *J* = 8.1 Hz, 1H), 4.81 (s, 2H), 4.61 (s, 2H), 3.39 (t, *J* = 7.1 Hz, 2H), 3.02 – 2.94 (m, 2H), 2.81 (s, 1H), 2.69 (s, 3H), 1.75 – 1.59 (m, 4H), 1.50 – 1.39 (m, 4H), 0.63 (m, 2H), 0.54 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{34}H_{40}N_5O_5$, 598.3; found, 598.2.

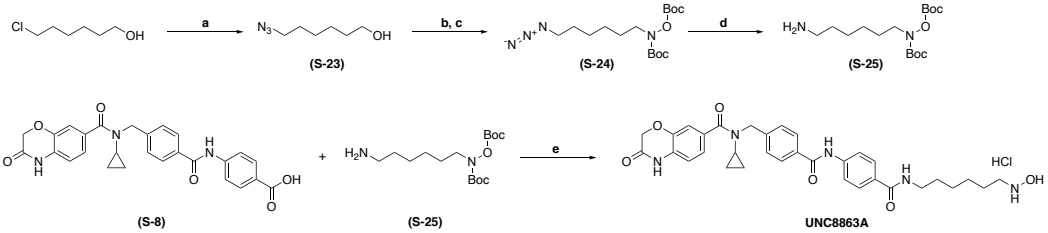


N-cyclopropyl-N-(4-((4-((6-(dimethylamino)hexyl)carbamoyl)phenyl)carbamoyl)benzyl)-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamide 2,2,2-trifluoroacetate (UNC8359A)

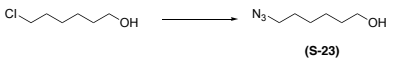
To a vial was added a solution of **UNC8153A** (10 mg, 1 Eq, 14 µmol) in methanol (1 mL). Next, formaldehyde (12 mg, 11 µL, 37% Wt, 10 Eq, 0.14 mmol) was added, followed by sodium cyanoborohydride (4.5 mg, 5 Eq, 72 µmol). The reaction was stirred at room temperature overnight. The next day, LCMS indicated consumption of starting material. The reaction was quenched with a few drops of saturated ammonium chloride, volatiles were removed *in vacuo*, and the residue redissolved in 50% aqueous methanol and purified by reverse phase chromatography (10-100% methanol in water + 0.1% TFA) to provide **UNC8359A** (4.22 mg, 5.81 µmol, 41 %).

^{1}H NMR (400 MHz, MeOH-d_4) δ 7.96 (d, *J* = 8.3 Hz, 2H), 7.86 – 7.81 (m, 4H), 7.50 (d, *J* = 7.9 Hz, 2H), 7.20 (d, *J* = 8.6 Hz, 1H), 7.18 (s, 1H), 6.96 (d, *J* = 8.1 Hz, 1H), 4.83 (s, 2H), 4.63 (s, 2H), 3.41 (t, *J* = 7.1 Hz, 2H), 3.16 – 3.09 (m, 2H), 2.88 (s, 6H), 2.86 – 2.80 (m, 1H), 1.79 – 1.70 (m, 2H), 1.70 – 1.63 (m, 2H), 1.51 – 1.41 (m, 4H), 0.70 – 0.61 (m, 2H), 0.61 – 0.52 (m, 2H).

LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{35}H_{42}N_5O_5$, 612.3; found, 612.3.



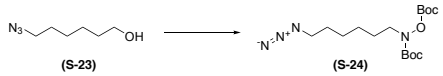
Supplemental Scheme 6: Synthesis of UNC8863A. Reagents and conditions: a) NaN_3, H_2O, reflux; b) MsCl, Et_3N, DCM, 0 °C; c) N,O-Bis(tert-butoxycarbonyl)hydroxylamine, DMF, 70 °C; d) Pt_2O, H_2, EtOH; e) EDC, HOAt, Et_3N, DMF.



6-azidohexan-1-ol (S-23)

To a flask charged with a stirbar were added sodium azide (0.42 g, 1.3 Eq, 6.5 mmol) and water (10 mL). To the reaction was then added 6-chlorohexan-1-ol (0.68 g, 1 Eq, 5.0 mmol), and the reaction was heated under reflux for 16 hours. The reaction was cooled and extracted 3 times with ethyl acetate, and the combined organic layers washed once with brine, dried over anhydrous sodium sulfate, filtered, and concentrated to yield **(S-23)** (635 mg, 4.43 mmol, 89 %) as a clear oil that was used without further purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.51 (s, 1H), 3.42 (t, *J* = 6.7 Hz, 2H), 3.11 (t, *J* = 6.9 Hz, 2H), 1.52 – 1.33 (m, 4H), 1.31 – 1.16 (m, 4H).

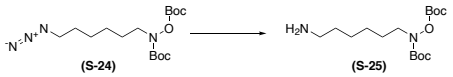


(S-23) (S-24)

***tert*-butyl (6-azidohexyl)((*tert*-butoxycarbonyl)oxy)carbamate (S-24)**

To a flask were added **(S-23)** (243 mg, 1 Eq, 1.70 mmol), DCM (10 mL), and triethylamine (309 mg, 426 µL, 1.8 Eq, 3.05 mmol). The flask was cooled in an ice bath, and mesyl chloride (272 mg, 185 µL, 1.4 Eq, 2.38 mmol) was added dropwise. The reaction vessel was capped and left to stir overnight while coming to room temperature. The next day, the reaction was quenched with 1M HCl and extracted 3 times with DCM. The combined organic extracts were washed once with water, once with saturated sodium bicarbonate, and once with brine, then dried over sodium sulfate and concentrated to a residue. NMR confirmed complete conversion to the mesylate. To the crude mesylate in a flask were added N,O-Bis(tert-butoxycarbonyl)hydroxylamine (475 mg, 1.2 Eq, 2.04 mmol), potassium carbonate (352 mg, 1.5 Eq, 2.55 mmol), and DMF (4 mL). The reaction was sealed and heated to 70˚C overnight. The next day, the reaction was quenched with water and extracted 3 times with ethyl acetate. The combined organic extracts were washed 3 times with water and once with brine, then dried over sodium sulfate and concentrated to a residue. Normal phase chromatography over silica gel (0-100% EtOAc in hexanes) provided **(S-24)** (420.5 mg, 1.173 mmol, 69.1 % over two steps) as a clear oil.

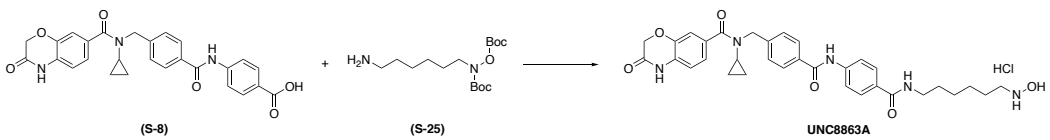
^{1}H NMR (400 MHz, $CDCl_3$) δ 3.57 (m, 2H), 3.25 (t, *J* = 6.9 Hz, 2H), 1.63 – 1.57 (m, 4H), 1.53 (s, 9H), 1.48 (s, 9H), 1.38 (m, 4H).



(S-24) (S-25)

***tert*-butyl (6-aminohexyl)((*tert*-butoxycarbonyl)oxy)carbamate (S-25)**

To a round bottomed flask were added **(S-24)** (0.18 g, 1 Eq, 0.50 mmol) and ethanol (5 mL). The flask was evacuated and backfilled with nitrogen three times, then platinum(IV) oxide (5.7 mg, 0.05 Eq, 25 µmol) was added. The flask was again evacuated and backfilled with nitrogen three times, and the nitrogen inlet was replaced with a balloon of hydrogen gas. The reaction was stirred at room temperature under hydrogen atmosphere for 18 hours. The mixture was filtered through Celite and the filtrate concentrated to provide **(S-25)** (159 mg, 478 µmol, 96 %) a clear oil that was used in the next step without further purification.

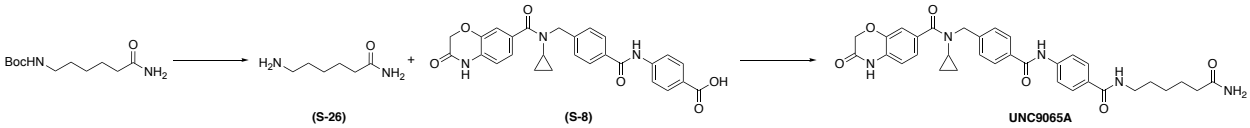


(S-8) (S-25) UNC8863A

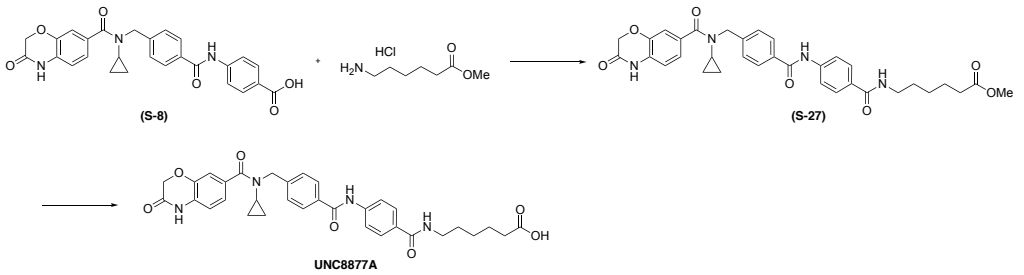
***N*-cyclopropyl-*N*-(4-((4-((6-(hydroxyamino)hexyl)carbamoyl)phenyl)carbamoyl)benzyl)-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide hydrochloride (UNC8863A)**

To a 2-dram vial were added **(S-8)** (50 mg, 1 Eq, 0.10 mmol), EDC (30 mg, 1.5 Eq, 0.15 mmol), HOAt (21 mg, 1.5 Eq, 0.15 mmol), and DMF (0.5 mL). The reaction was left to stir at room temperature for 30 minutes. To the vial was then added **(S-25)** (159 mg, 4.6 Eq, 0.477 mmol) dissolved in DMF (0.5 mL), followed by triethylamine (31 mg, 43 µL, 3 Eq, 0.31 mmol),

and the reaction was left to stir overnight. The reaction was quenched with water and extracted 3 times with ethyl acetate. The combined organic fractions were washed twice with water, once with saturated sodium bicarbonate, and once with brine, then dried by passage through a phase separator and concentrated to a residue. Normal phase chromatography over silica gel (0-10% methanol in DCM) provided partially pure material, to which was added neat formic acid. After 18 hours, volatiles were removed *in vacuo*, residual formic acid was co-evaporated with methanol, and the residue was purified by reverse phase chromatography (10-100% methanol in water + 0.1% HCl) to provide **UNC8863A** (28 mg, 44 µmol, 43 % over two steps) as a white solid after lyophilization.
^{1}H NMR (400 MHz, MeOH-d_4) δ 7.96 (d, *J* = 8.3 Hz, 2H), 7.89 – 7.79 (m, 4H), 7.50 (d, *J* = 7.9 Hz, 2H), 7.20 (dd, *J* = 8.2, 1.6 Hz, 1H), 7.17 (d, *J* = 1.7 Hz, 1H), 6.97 (d, *J* = 8.0 Hz, 1H), 4.83 (s, 2H), 4.62 (s, 2H), 3.40 (t, *J* = 7.1 Hz, 2H), 3.26 – 3.18 (m, 2H), 2.86 – 2.79 (m, 1H), 1.80 – 1.62 (m, 4H), 1.54 – 1.40 (m, 4H), 0.71 – 0.60 (m, 2H), 0.60 – 0.50 (m, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{33}H_{38}N_5O_6$, 600.3; found, 600.3.



***N*-(4-((4-((6-amino-6-oxohexyl)carbamoyl)phenyl)carbamoyl)benzyl)-*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamide (UNC9065A)**
To a scintillation vial was added tert-butyl (6-amino-6-oxohexyl)carbamate (50 mg, 1 Eq, 0.22 mmol) and 3 mL of 20% TFA in DCM. The mixture was allowed to stir overnight. Volatiles were removed *in vacuo* and residual volatiles were co-evaporated with DCM 3 times to give **(S-26)**.
To a separate scintillation vial was added **(S-8)** (0.11 g, 1 Eq, 0.22 mmol), EDC (62 mg, 1.5 Eq, 0.33 mmol), HOAt (44 mg, 1.5 Eq, 0.33 mmol), and DMF (1 mL). The reaction was left to stir at room temperature for 30 minutes. To the vial was then added **(S-26)** in 0.5 mL of DMF, followed by triethylamine (88 mg, 0.12 mL, 4 Eq, 0.87 mmol), and the reaction was left to stir overnight. The next day, the reaction was quenched with 10 mL of distilled water and extracted 3 times with ethyl acetate. The combined organic layers were washed once with water, twice with saturated sodium bicarbonate, and once with brine, then dried sodium sulfate and concentrated to a white solid. The solid was dissolved in MeOH/DCM and purified by normal phase chromatography over silica gel (0-10% MeOH in DCM) to give **UNC9065A** (32.93 mg, 55.10 µmol, 25 %) as a white solid.
^{1}H NMR (400 MHz, DMSO-d_6) δ 10.86 (s, 1H, -NH), 10.40 (s, 1H, -NH), 8.33 (t, *J* = 5.6 Hz, 1H), 7.94 (d, *J* = 8.2 Hz, 2H), 7.89 – 7.72 (m, 4H), 7.44 (d, *J* = 8.0 Hz, 2H), 7.28 – 7.04 (m, 2H), 6.91 (d, *J* = 8.1 Hz, 1H), 4.70 (s, 2H), 4.60 (s, 2H), 3.24 – 3.18 (m, 2H), 2.88 – 2.67 (m, 1H), 2.02 (t, *J* = 7.4 Hz, 2H), 1.56 – 1.42 (m, 4H), 1.32 – 1.23 (m, 2H), 0.70 – 0.50 (m, 2H), 0.50 – 0.29 (m, 2H).
^{1}H NMR (400 MHz, cd_3od) δ 8.92 (d, *J* = 1.4 Hz, 1H), 7.96 (d, *J* = 8.3 Hz, 2H), 7.84 (s, 4H), 7.54 – 7.44 (m, 3H), 7.24 – 7.15 (m, 2H), 6.96 (d, *J* = 8.1 Hz, 1H), 4.83 (s, 2H), 4.62 (s, 2H), 4.14 (dd, *J* = 7.5, 6.6 Hz, 1H), 3.38 (td, *J* = 6.9, 2.6 Hz, 2H), 3.36 – 3.32 (m, 2H), 3.25 – 3.19 (m, 2H), 2.90 – 2.71 (m, 1H), 1.63 (p, *J* = 7.1 Hz, 2H), 1.50 (t, *J* = 7.5 Hz, 2H), 1.38 (dt, *J* = 21.7, 7.6 Hz, 4H), 0.65 (s, 2H), 0.57 (s, 2H).
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{33}H_{36}N_5O_6$, 598.3; found, 598.2.



methyl 6-(4-(4-((*N*-cyclopropyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxamido) methyl)benzamido)benzamido)hexanoate (UNC8877A)

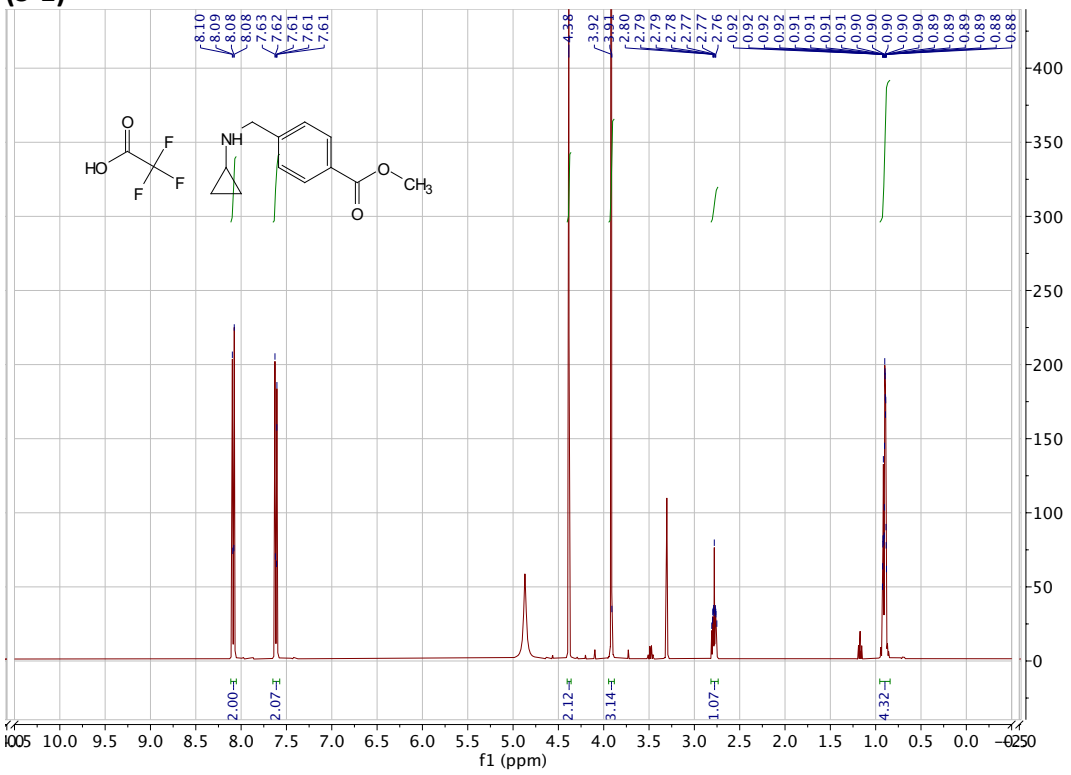
To a 2-dram vial were added **(S-8)** (50 mg, 1 Eq, 0.10 mmol), EDC (30 mg, 1.5 Eq, 0.15 mmol), HOAt (21 mg, 1.5 Eq, 0.15 mmol) and DMF (1 mL). The vial was allowed to stir for 30 minutes, then methyl 6-aminohexanoate hydrochloride (22 mg, 1.2 Eq, 0.12 mmol) and triethylamine (42 mg, 57 µL, 4 Eq, 0.41 mmol) were added, and the reaction was left to stir overnight. The next day, the reaction was quenched with 10 mL of distilled water and extracted 3 times with ethyl acetate. The combined organic layers were washed once with water, twice with saturated sodium bicarbonate, and once with brine, then dried sodium sulfate and concentrated to a white solid. The material was purified by normal phase chromatography over silica gel (0-10% MeOH in DCM) to give ester intermediate **(S-27)**. To the ester was added 1,4-dioxane (16 mL) and lithium hydroxide hydrate (22 mg, 5 Eq, 0.51 mmol) in water (4 mL). The mixture was stirred overnight. The still-basic aqueous layer was washed with ether 5 times. The solution was then acidifed to a pH of 2-3 with 2 M HCl. The acidic layer was extracted 3x with ethyl acetate. Combined ethyl acetate layers were washed with brine, dried over magnesium sulfate, filtered and concentrated *in vacuo*. The product was taken up in a minimal amount of MeOH/DCM and triturated into a large volume of vigorously stirring ether. The precipitate was collected by filtration and washed with additional ether. The solid was recrystallized from a minimal amount of boiling ethanol to give **UNC8877A** (4.14 mg, 6.92 µmol, 6.7 %) as a white solid.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.40 (s, 1H, -NH), 8.33 (t, J = 5.6 Hz, 1H, NH), 7.94 (d, J = 8.3 Hz, 2H), 7.89 – 7.73 (m, 4H), 7.44 (d, J = 8.0 Hz, 2H), 7.23 – 7.06 (m, 2H), 6.91 (d, J = 8.0 Hz, 1H), 4.70 (s, 2H), 4.60 (s, 2H), 3.22 (q, J = 6.5 Hz, 2H), 2.78 (s, 1H), 2.19 (t, J = 7.3 Hz, 2H), 1.59 – 1.42 (m, 4H), 1.39 – 1.24 (m, 2H), 0.60 – 0.50 (m, 2H), 0.50 – 0.32 (m, 2H).

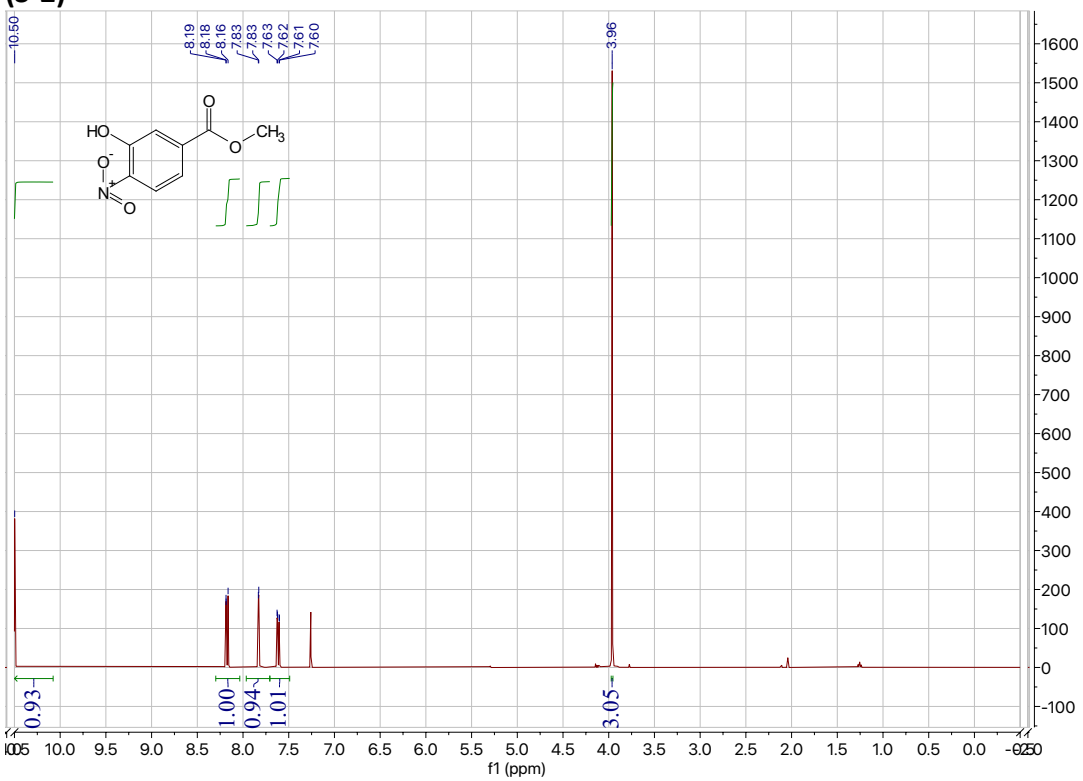
LCMS (ESI, +ve mode) (*m/z*): $[M+H]^+$ calculated for $C_{33}H_{35}N_4O_7$, 599.2; found, 599.2.

NMR Spectra

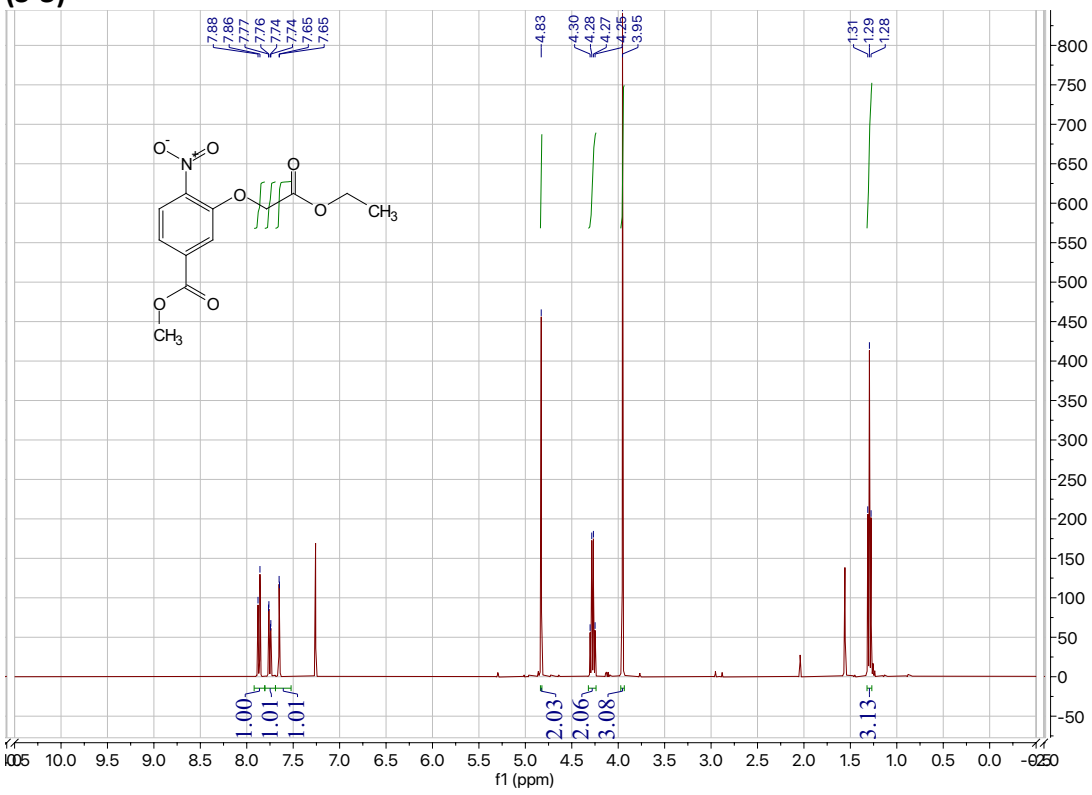
(S-1)



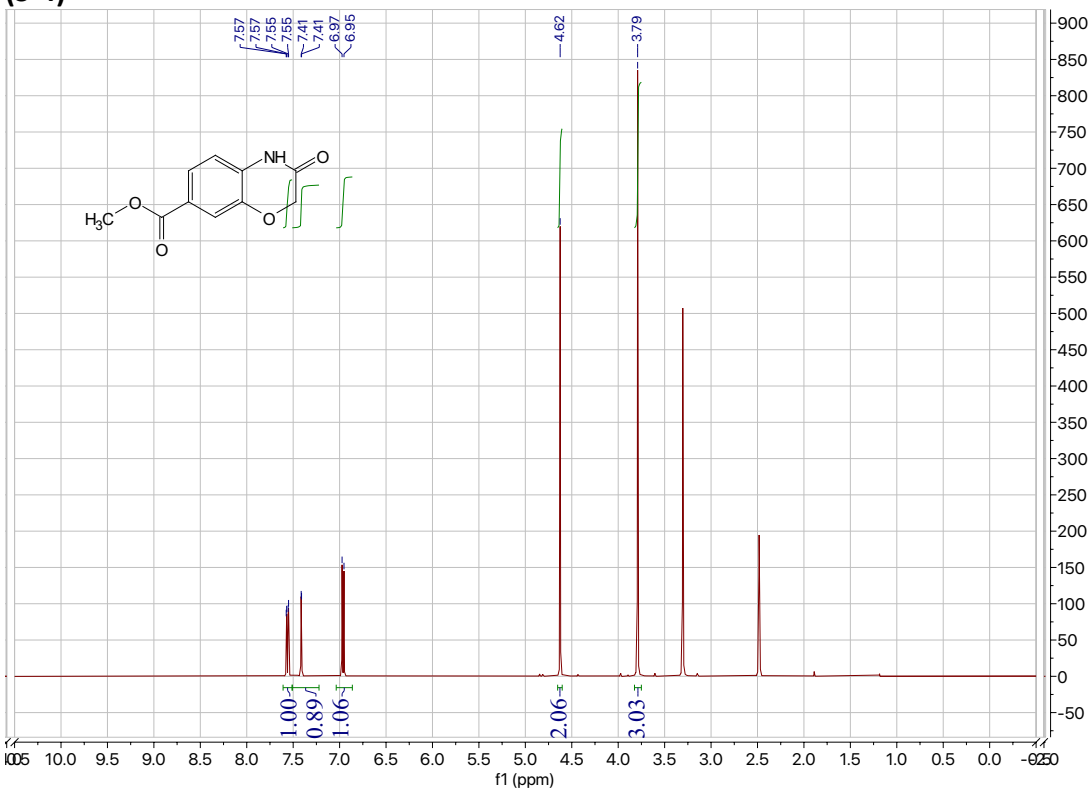
(S-2)



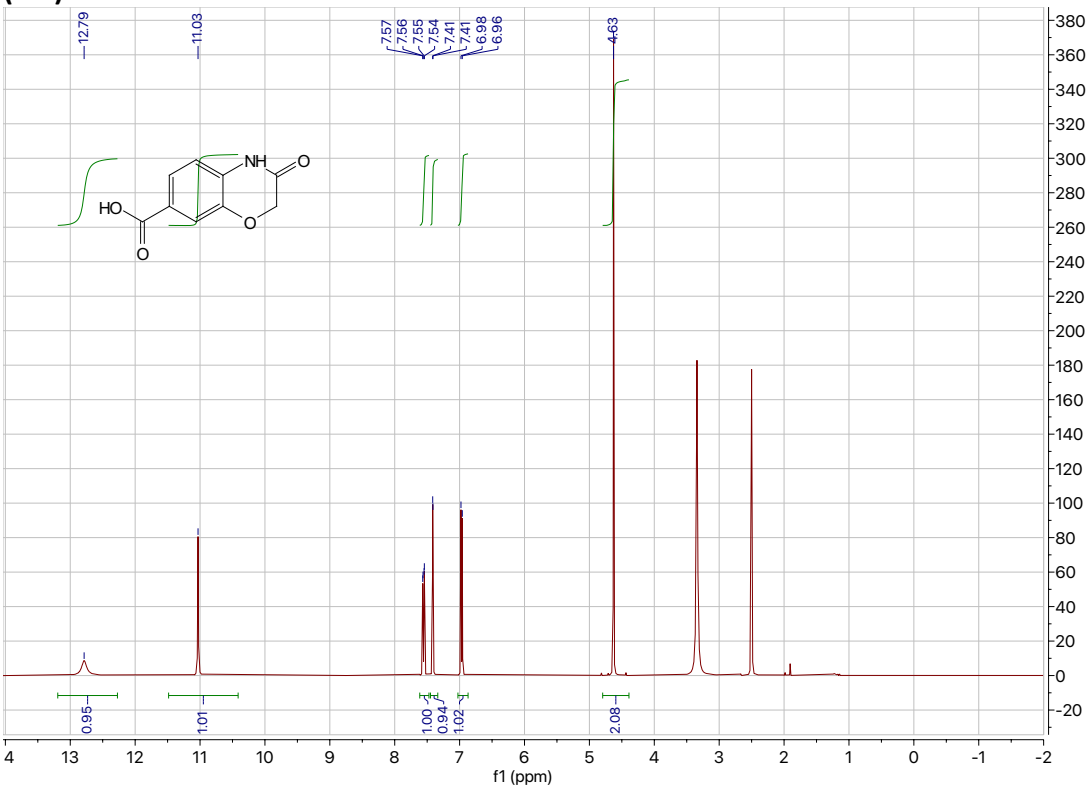
(S-3)


7.88
7.86
7.77
7.76
7.74
7.74
7.65
7.65
4.83
4.30
4.28
4.27
4.25
3.95
1.31
1.29
1.28
1.00
1.01
1.01
2.03
2.06
3.08
3.13
f1 (ppm)

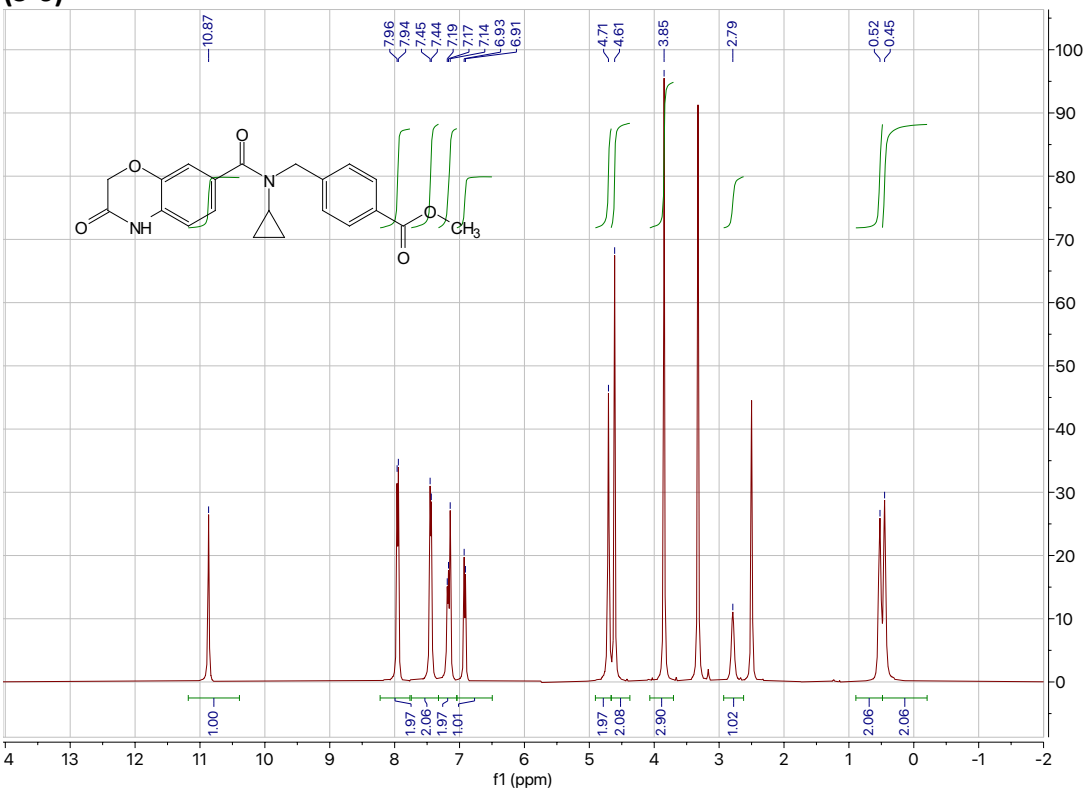
(S-4)


7.57
7.57
7.55
7.55
7.41
7.41
6.97
6.95
4.62
3.79
1.00
0.89
1.06
2.06
3.03
f1 (ppm)

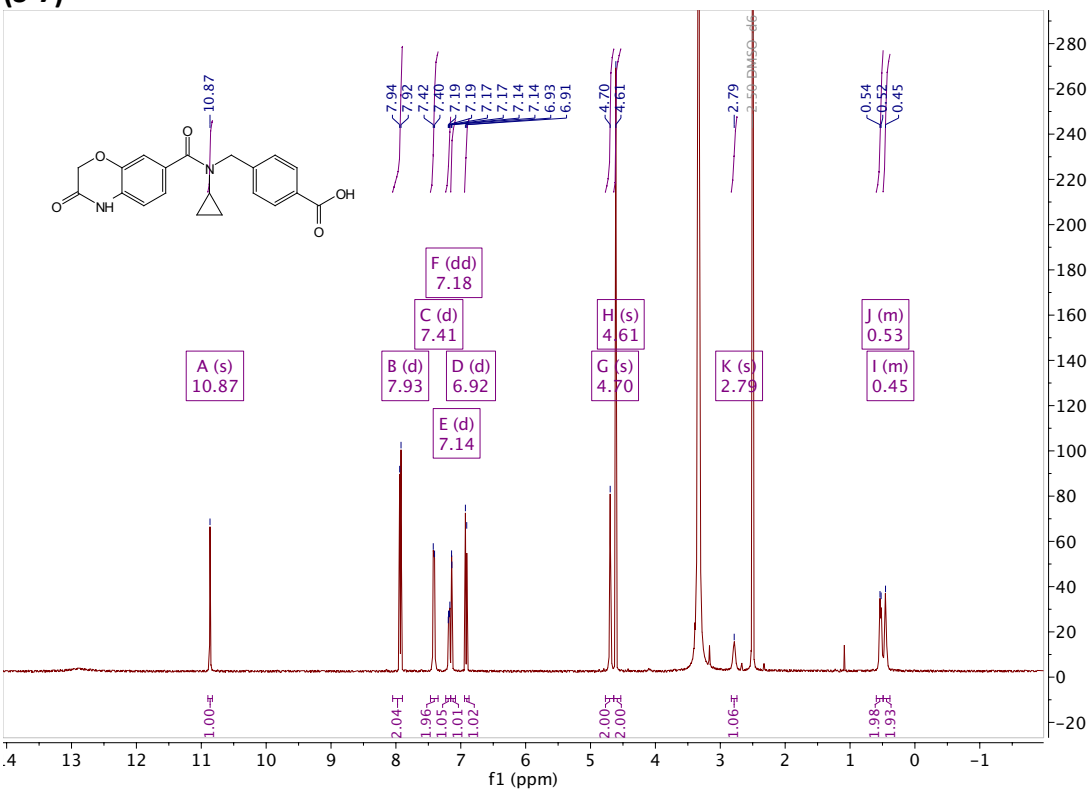
(S-5)


12.79
11.03
7.57
7.56
7.55
7.54
7.41
7.41
6.98
6.96
4.63
NH
O
HO
O
O
0.95
1.01
1.00
0.94
1.02
2.08
f1 (ppm)

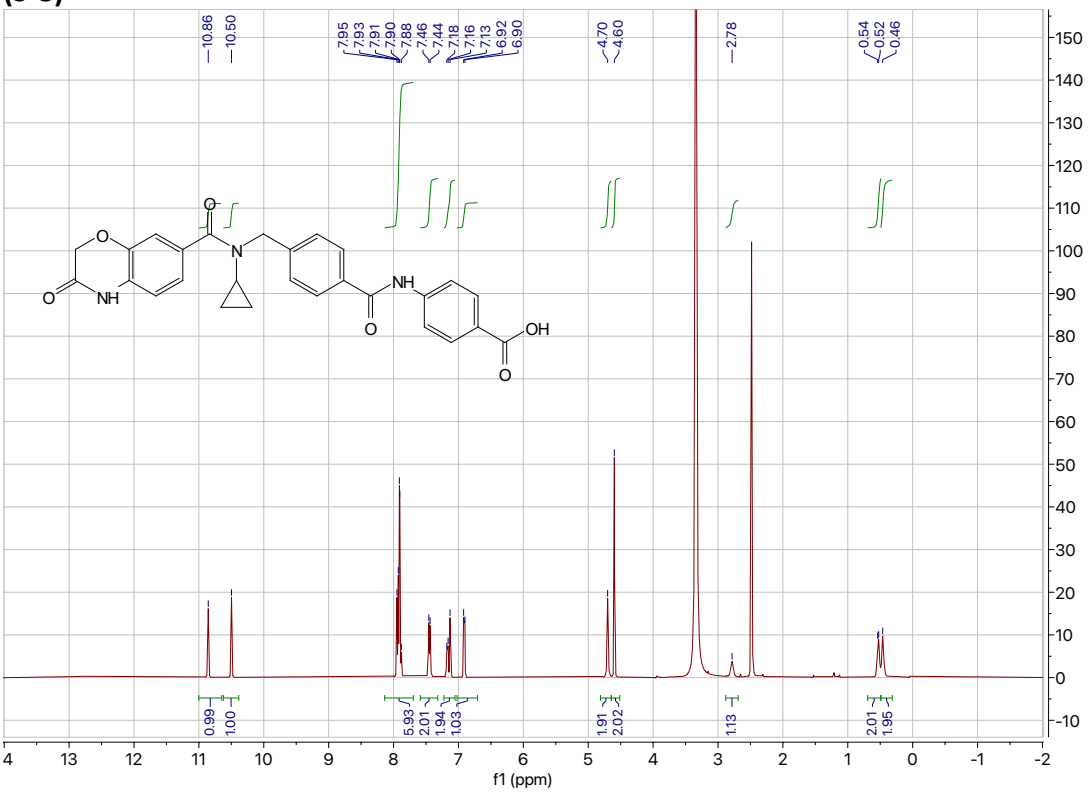
(S-6)


10.87
7.96
7.94
7.45
7.44
7.19
7.17
7.14
6.93
6.91
4.71
4.61
3.85
2.79
0.52
0.45
O
O
NH
N
O
O
CH3
1.00
1.97
2.06
1.97
1.01
1.97
2.08
2.90
1.02
2.06
2.06
f1 (ppm)

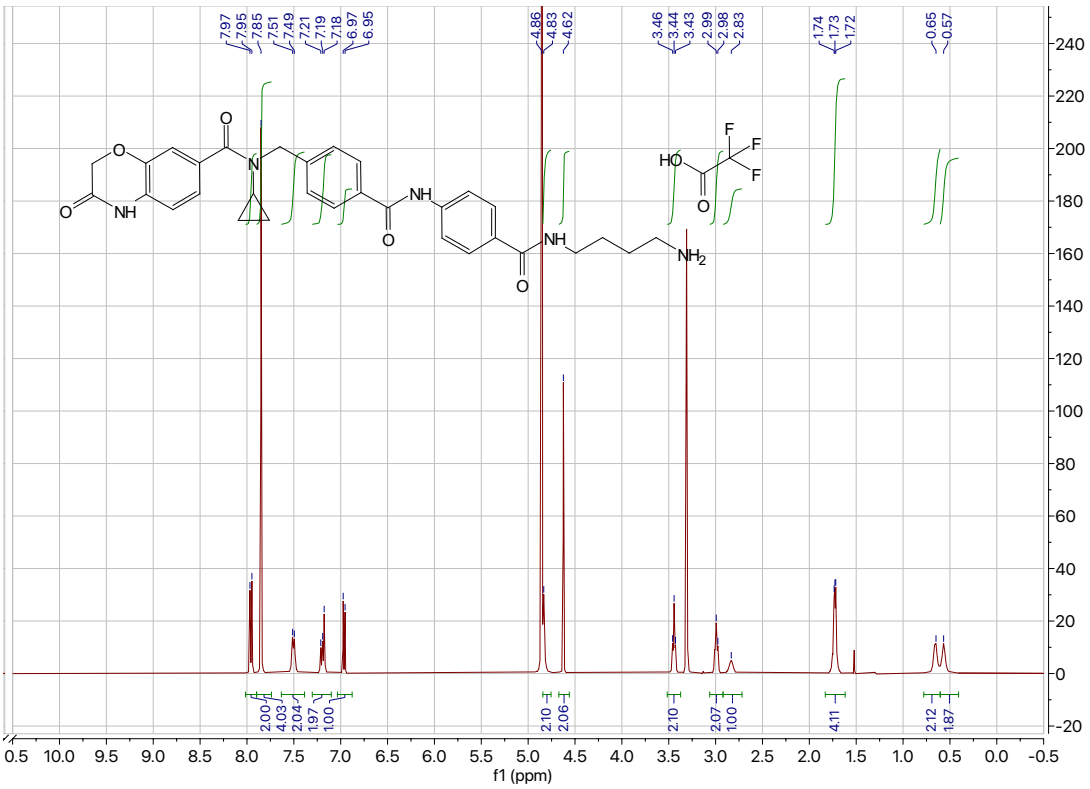
(S-7)


A (s)
10.87
B (d)
7.93
C (d)
7.41
D (d)
6.92
E (d)
7.14
F (dd)
7.18
G (s)
4.70
H (s)
4.61
I (m)
0.45
J (m)
0.53
K (s)
2.79
f1 (ppm)

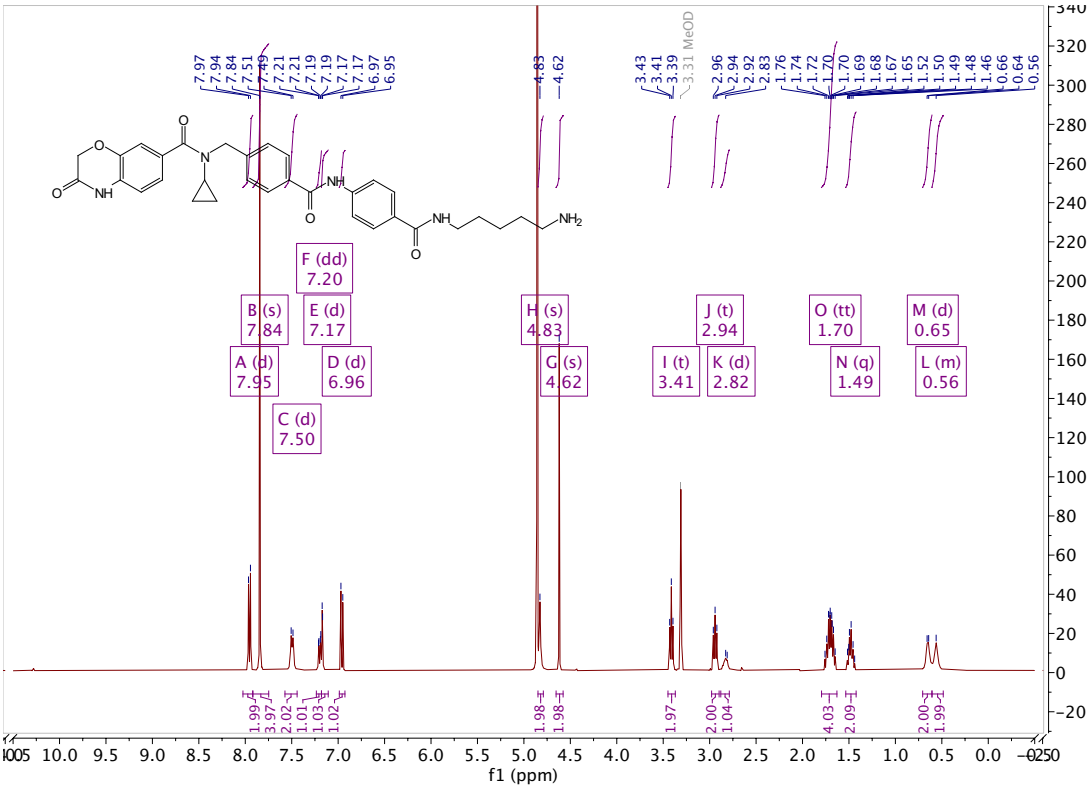
(S-8)


f1 (ppm)

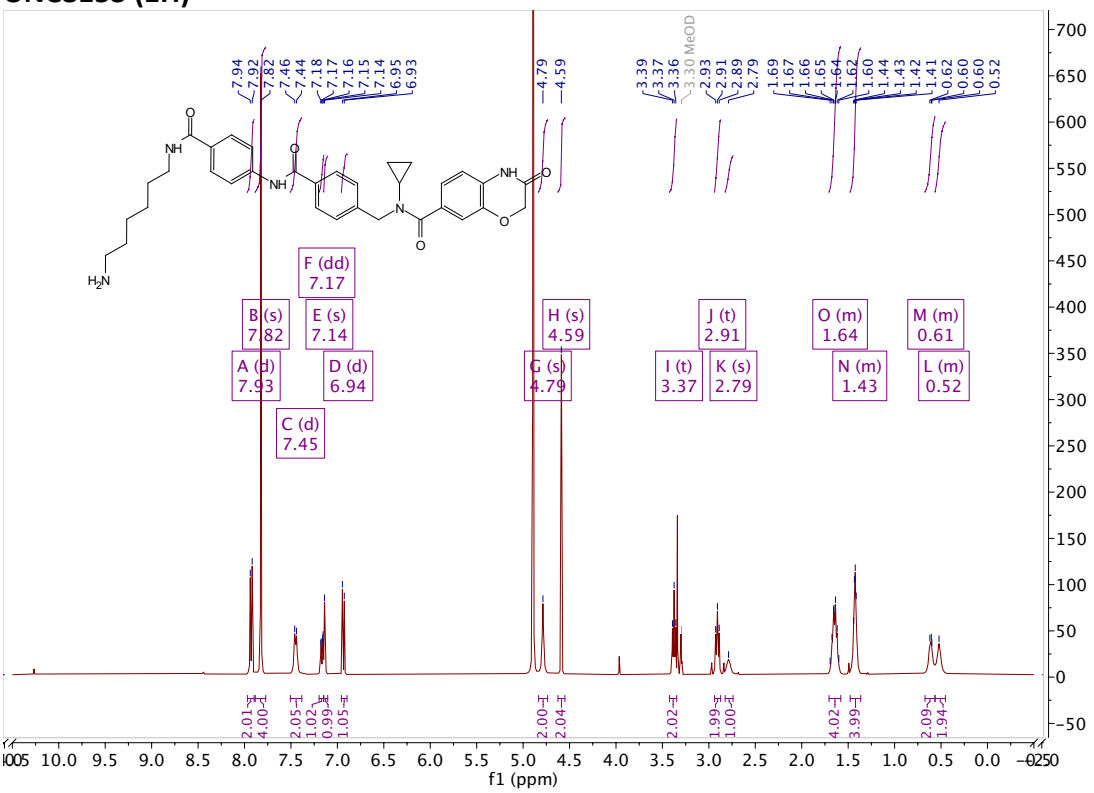
UNC8324



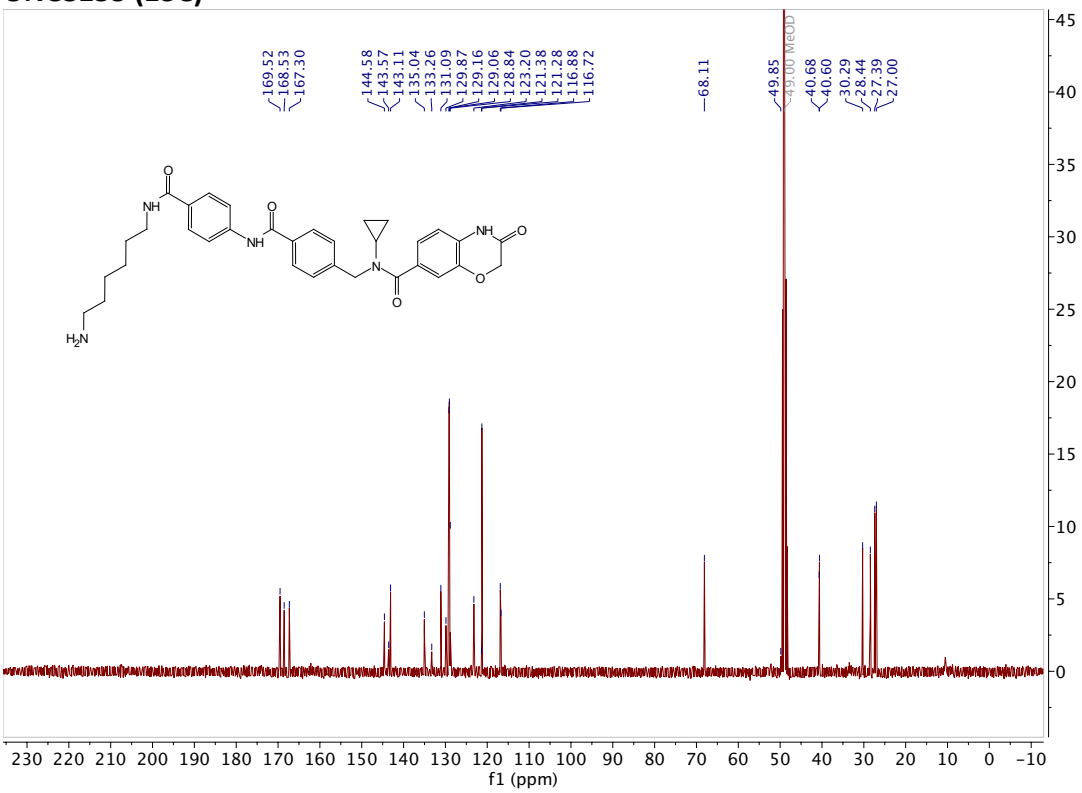
UNC8269



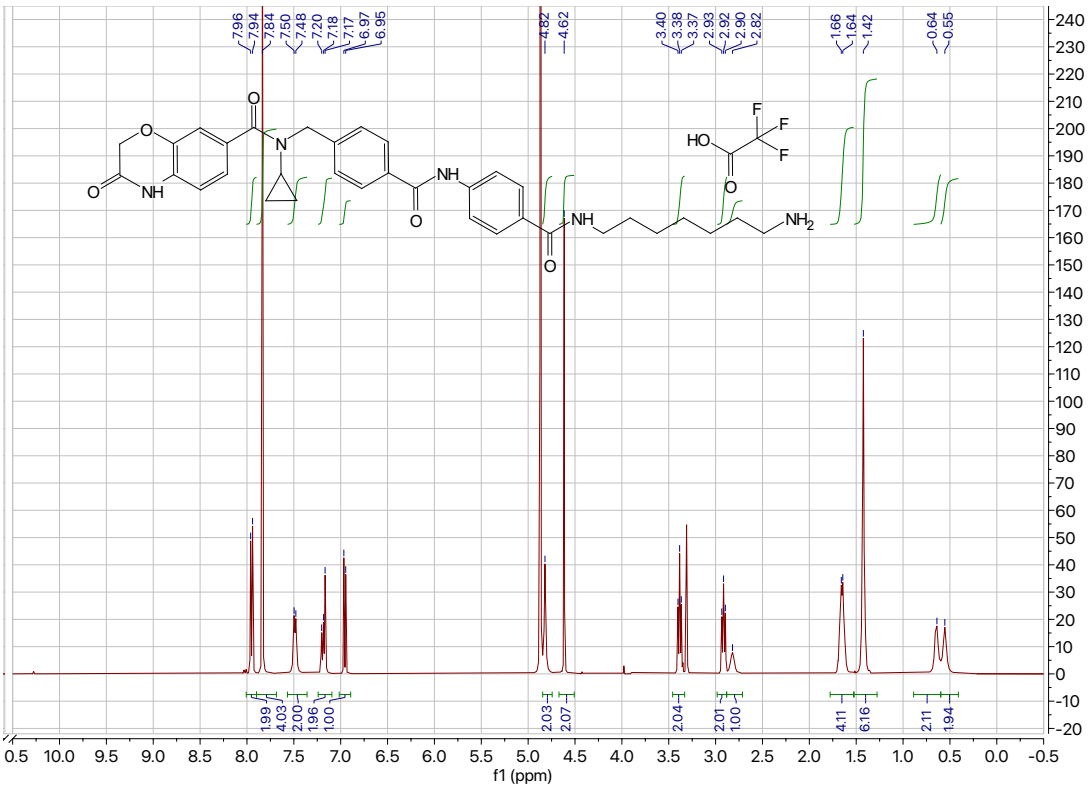
UNC8153 (1H)



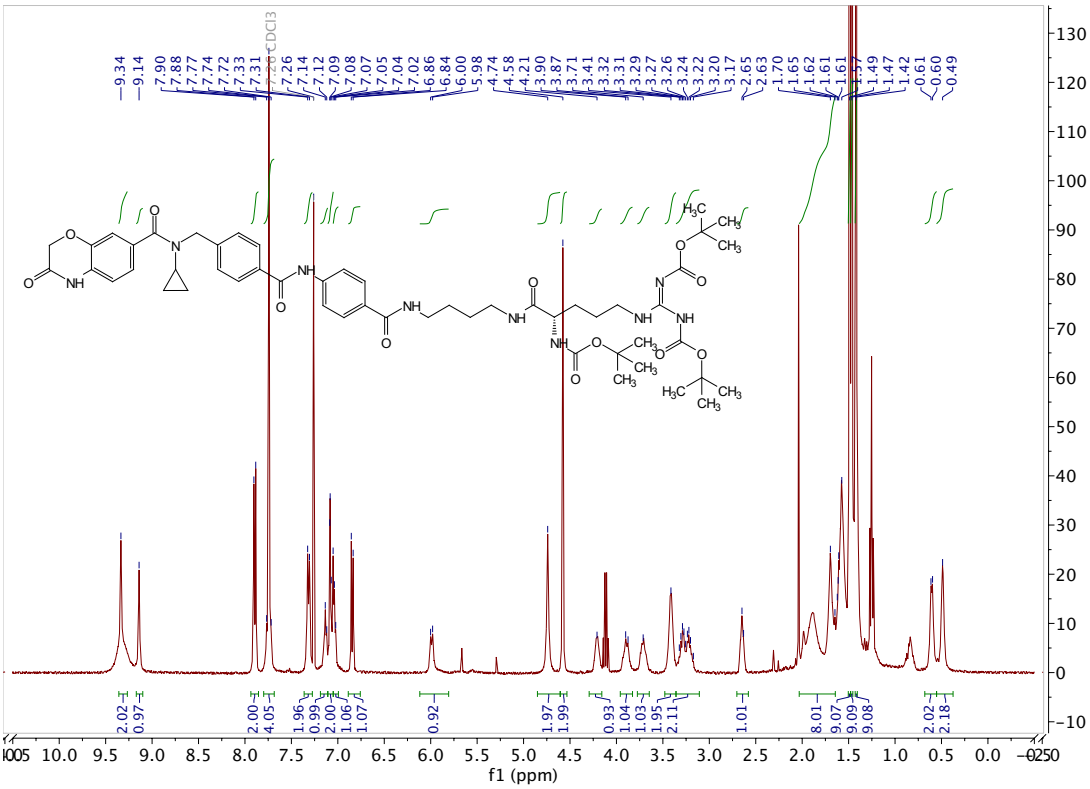
UNC8153 (13C)



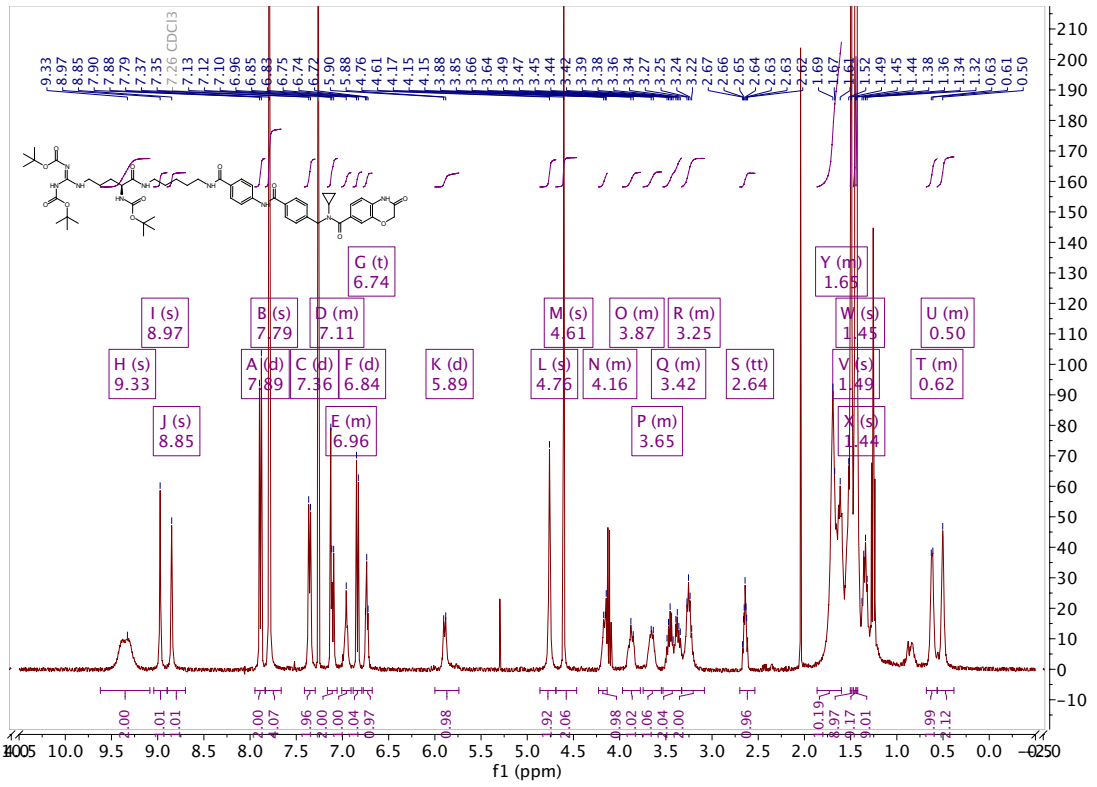
UNC8325



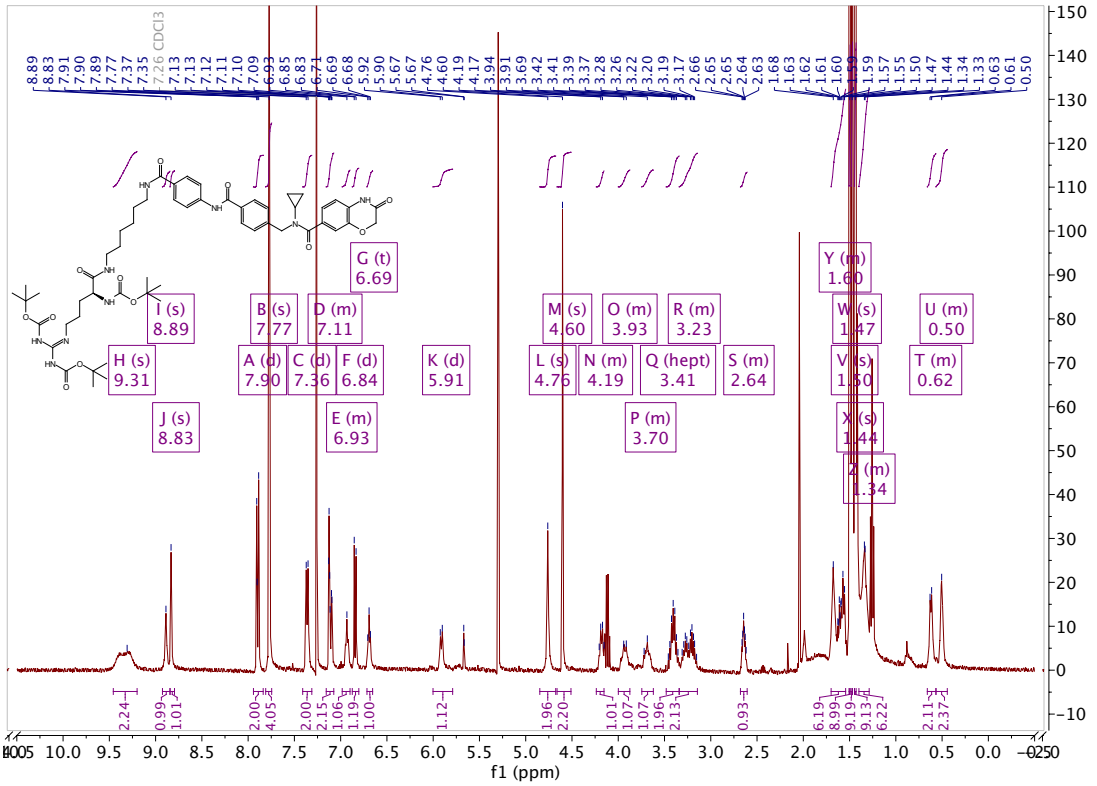
UNC7986



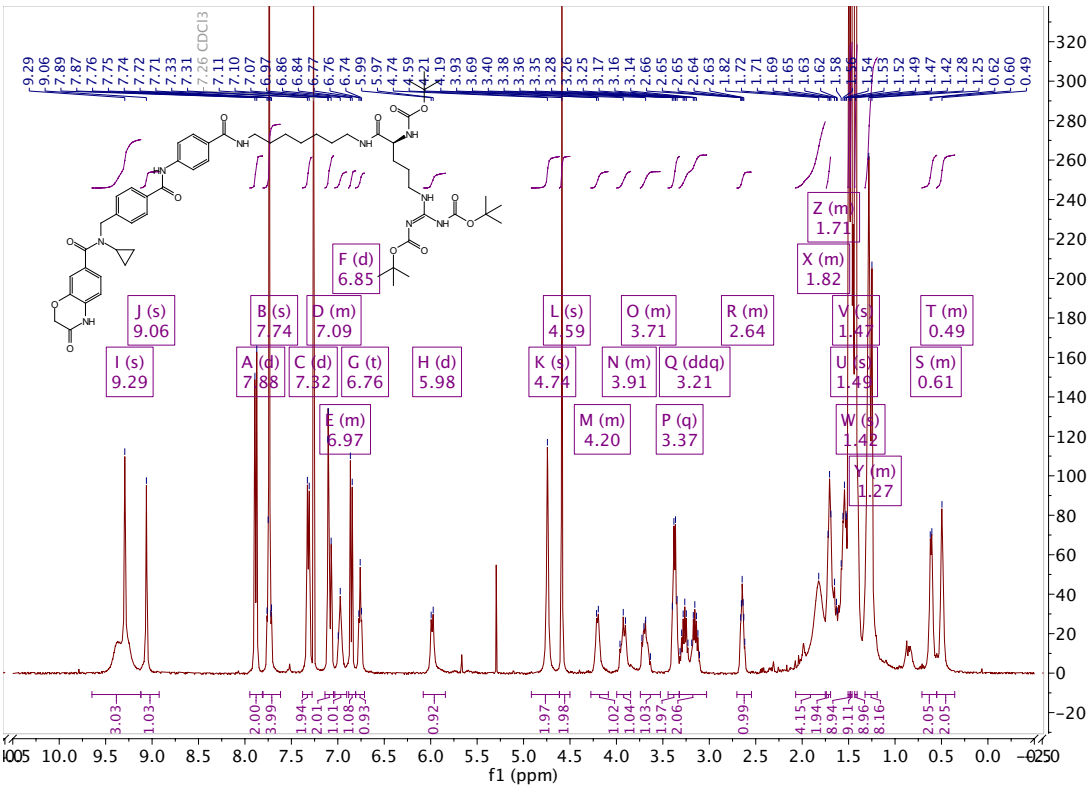
UNC7896



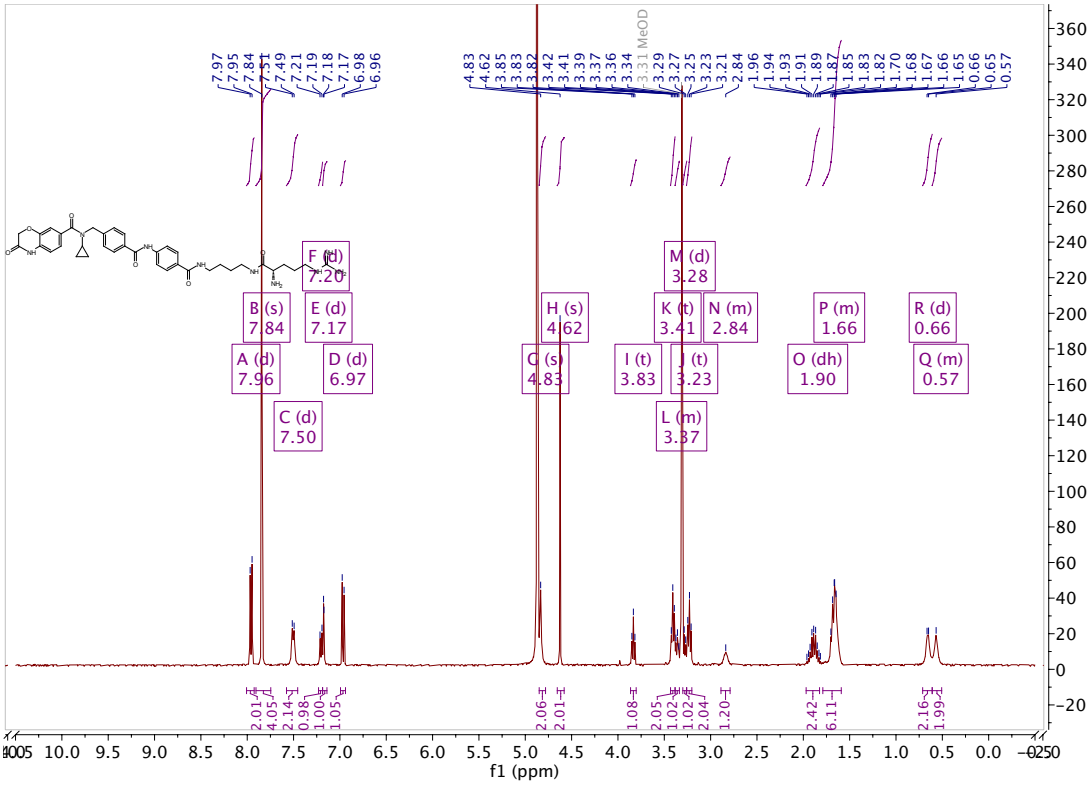
UNC7746



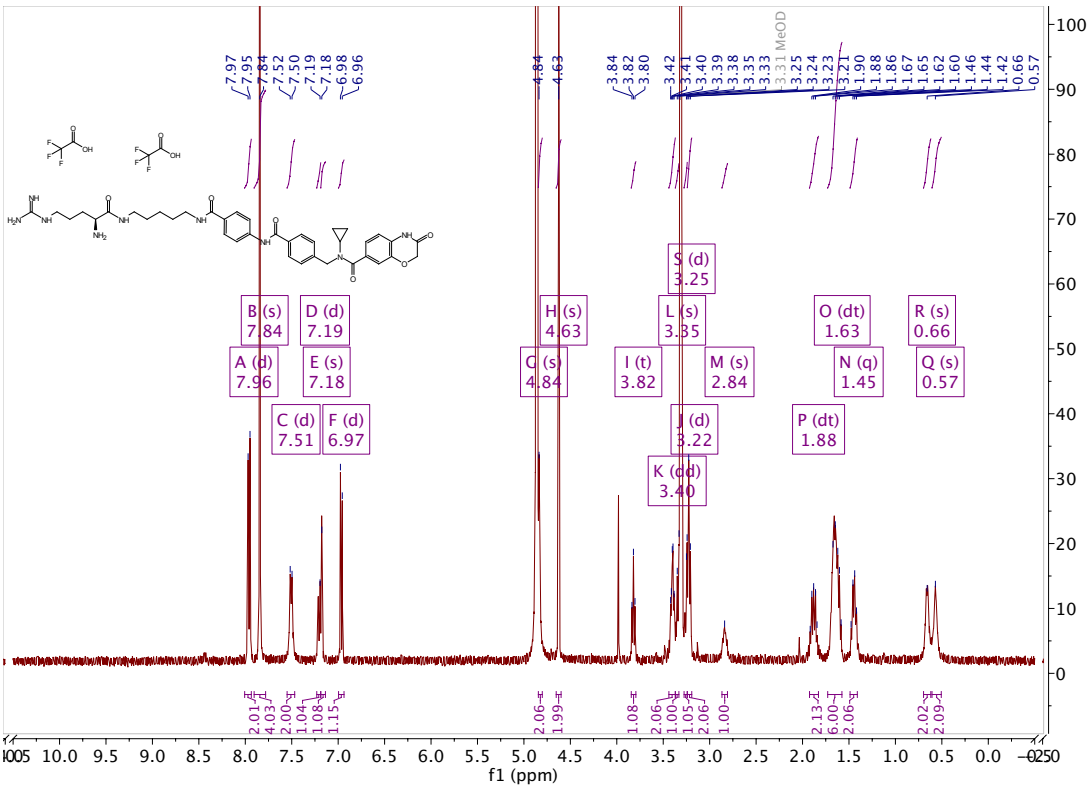
UNC8012



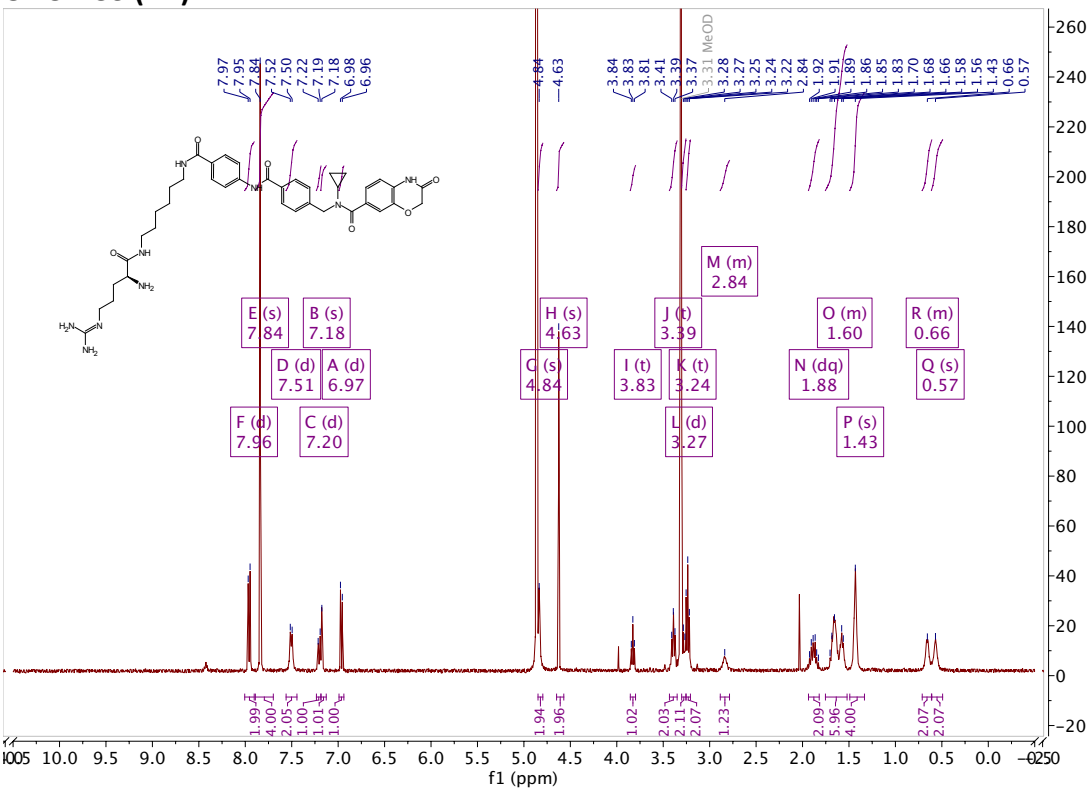
UNC7987



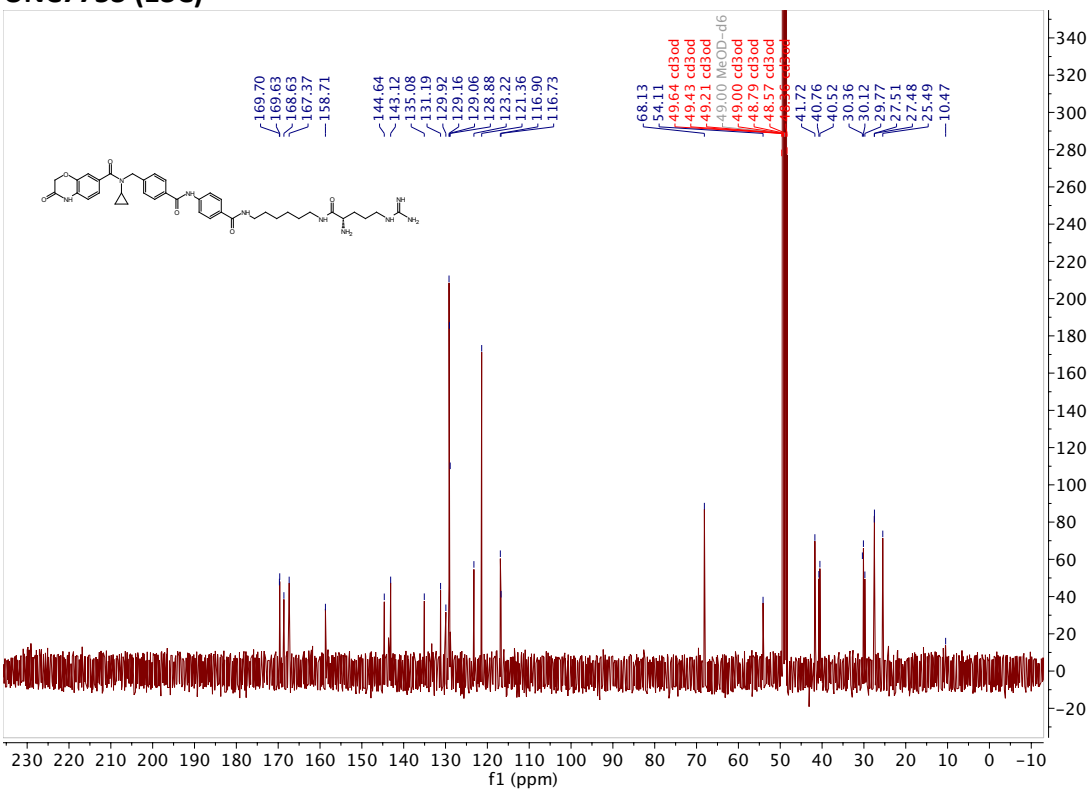
UNC7899



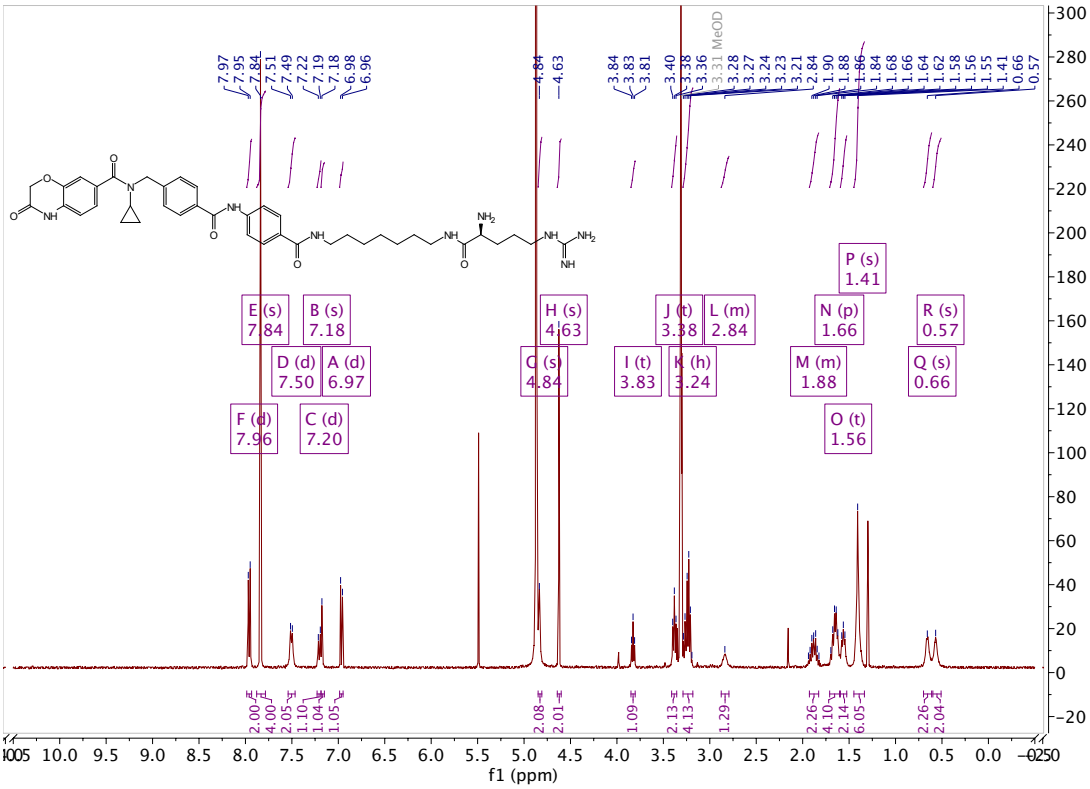
UNC7753 (1H)



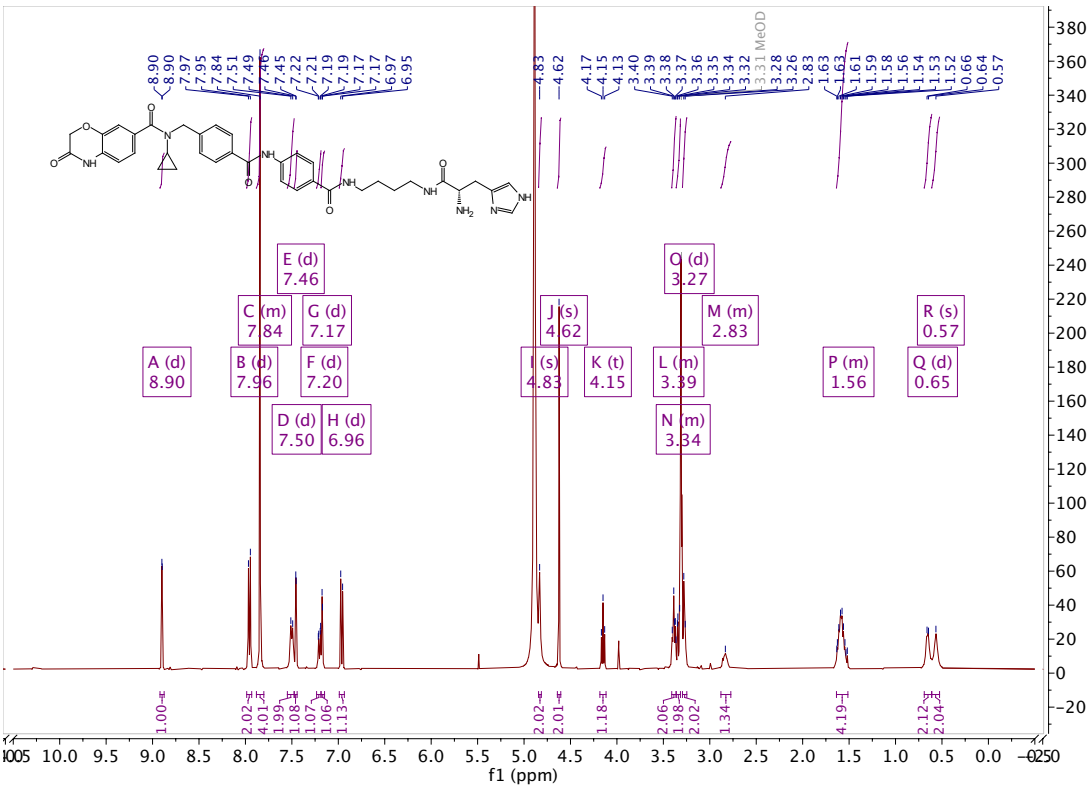
UNC7753 (13C)



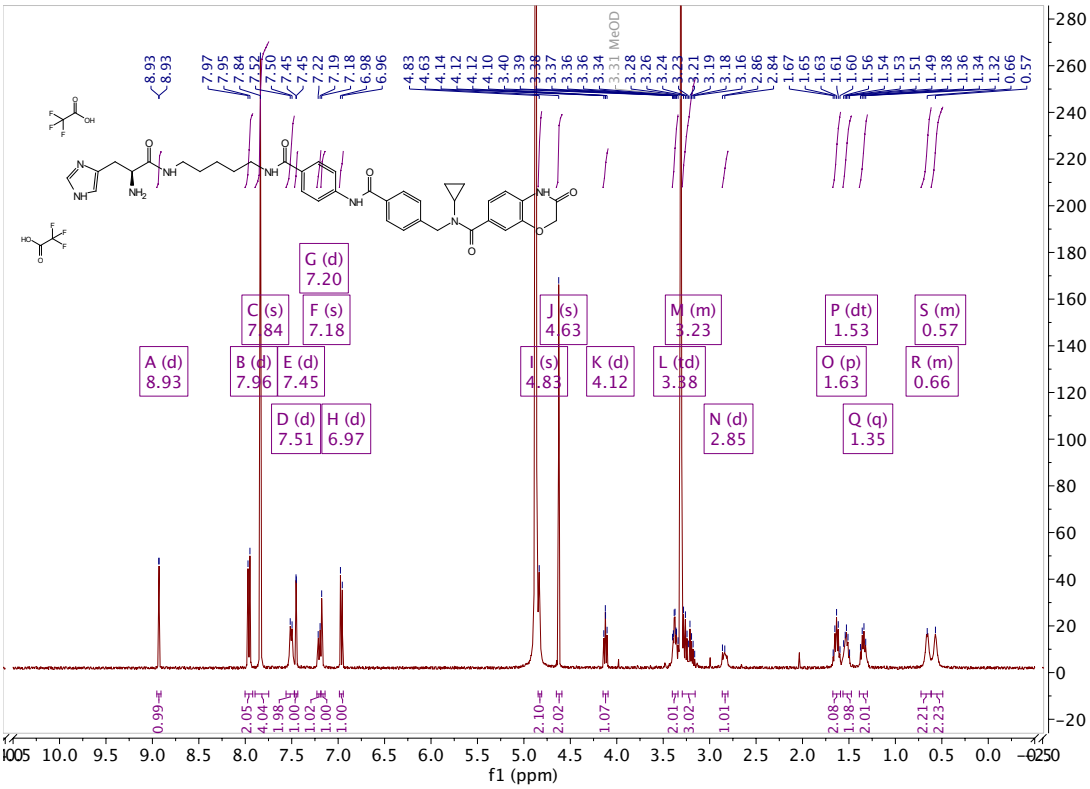
UNC8014



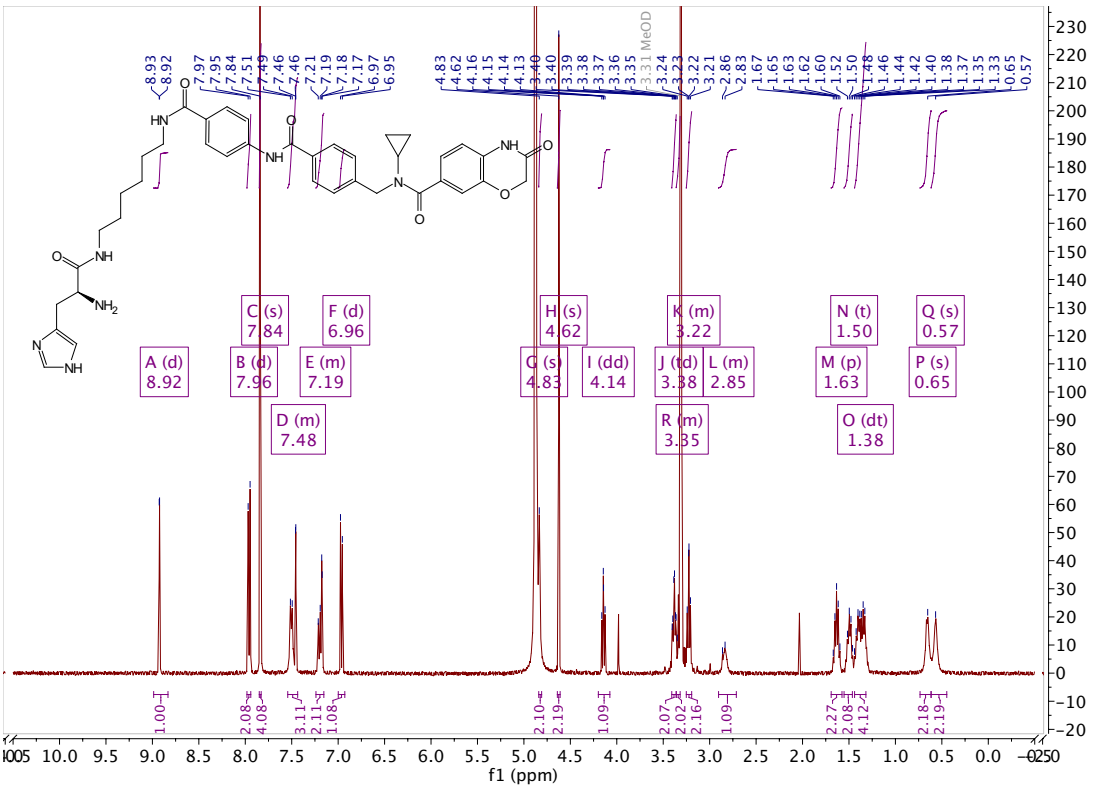
UNC7985



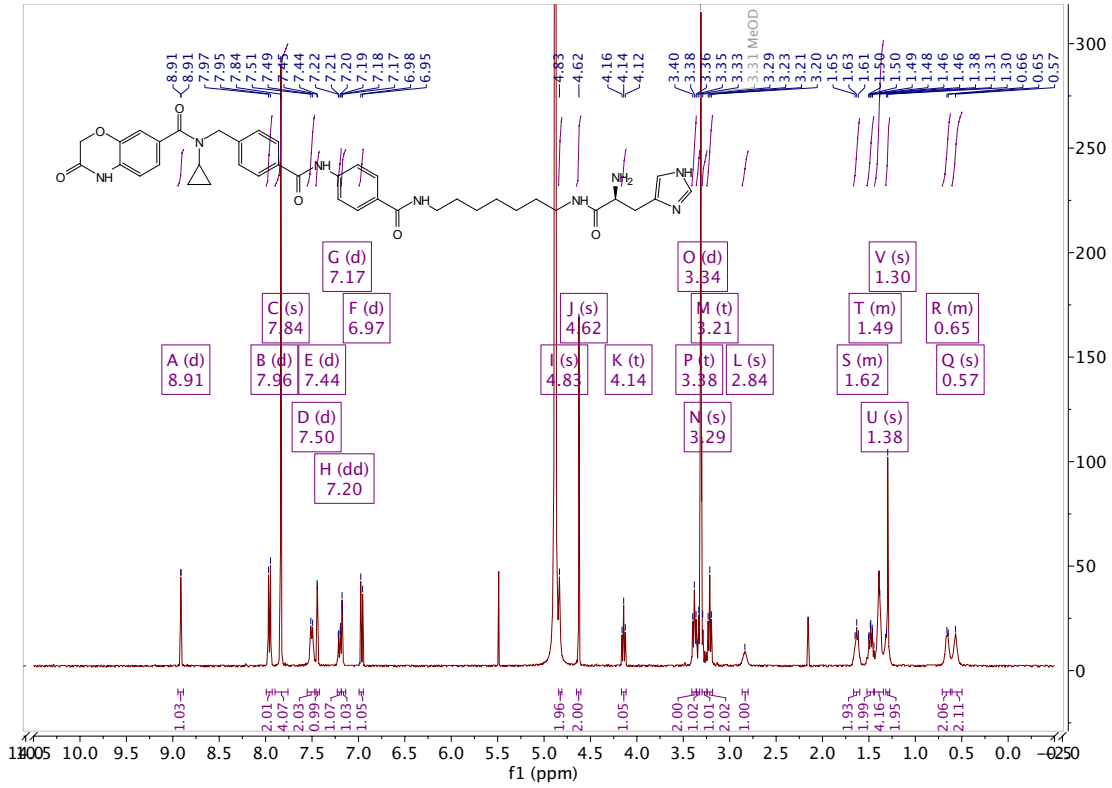
UNC7895



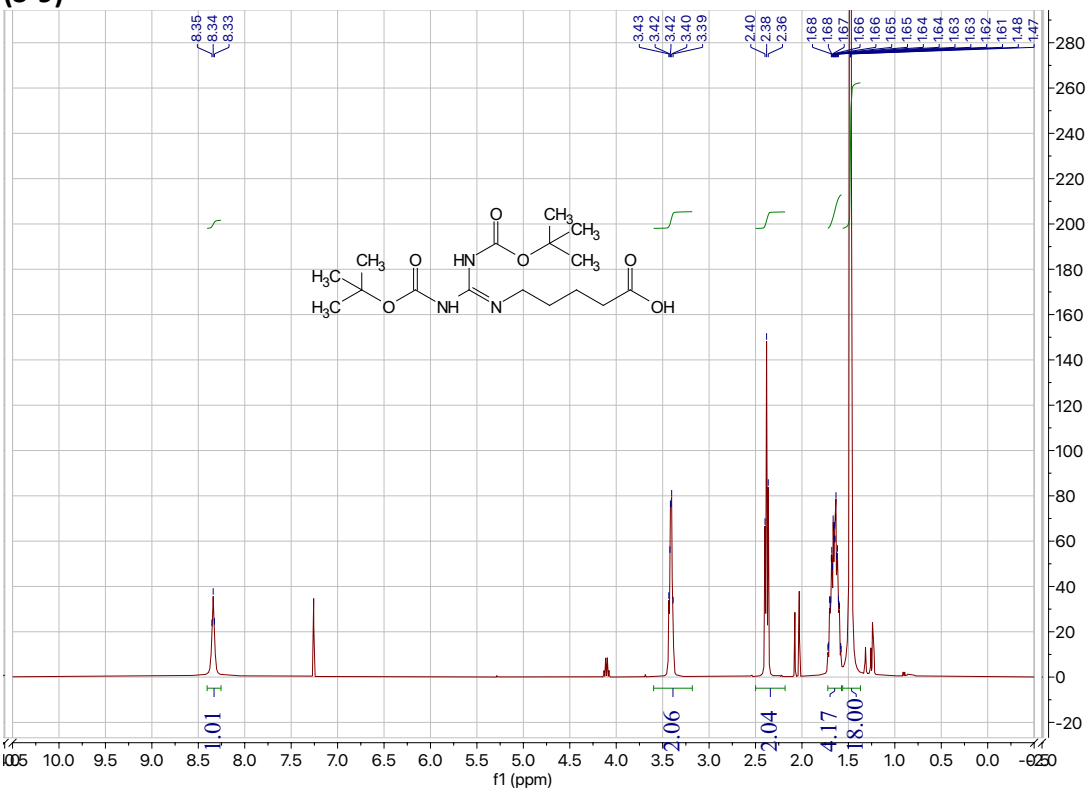
UNC7749



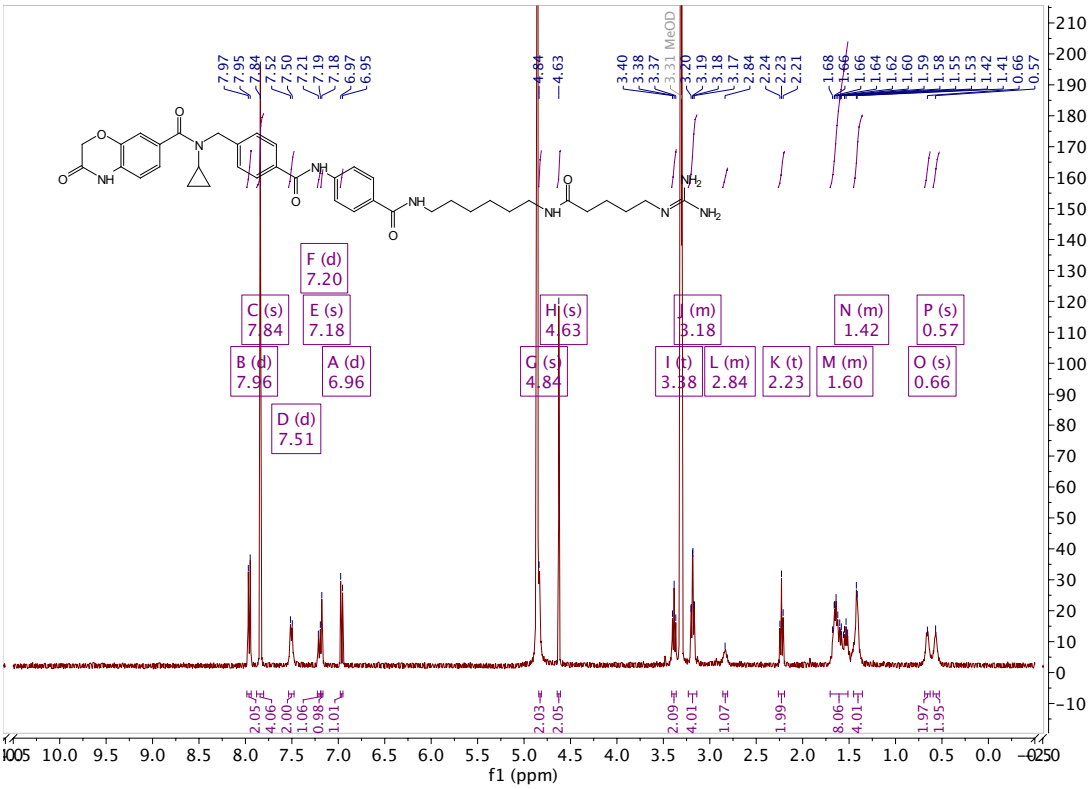
UNC8010



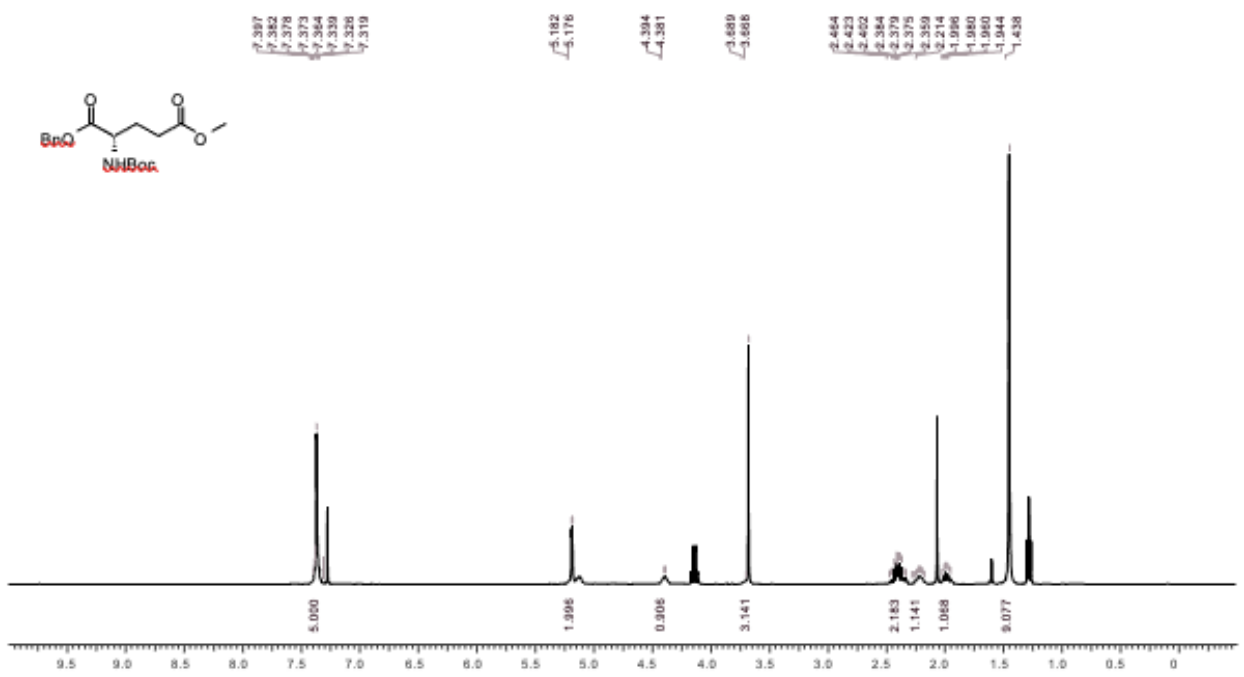
(S-9)


f1 (ppm)

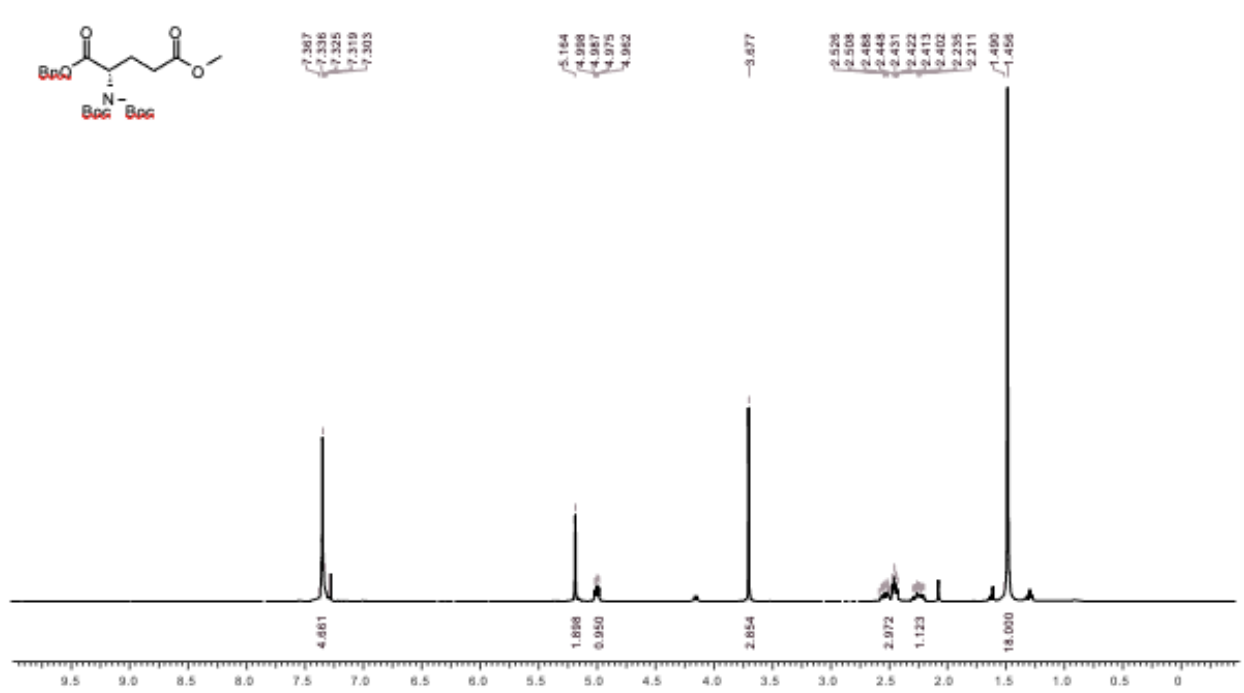
UNC7996


F (d) 7.20
C (s) 7.84
E (s) 7.18
H (s) 4.63
J (m) 3.18
N (m) 1.42
P (s) 0.57
B (d) 7.96
A (d) 6.96
G (s) 4.84
I (t) 3.38
L (m) 2.84
K (t) 2.23
M (m) 1.60
O (s) 0.66
D (d) 7.51
f1 (ppm)

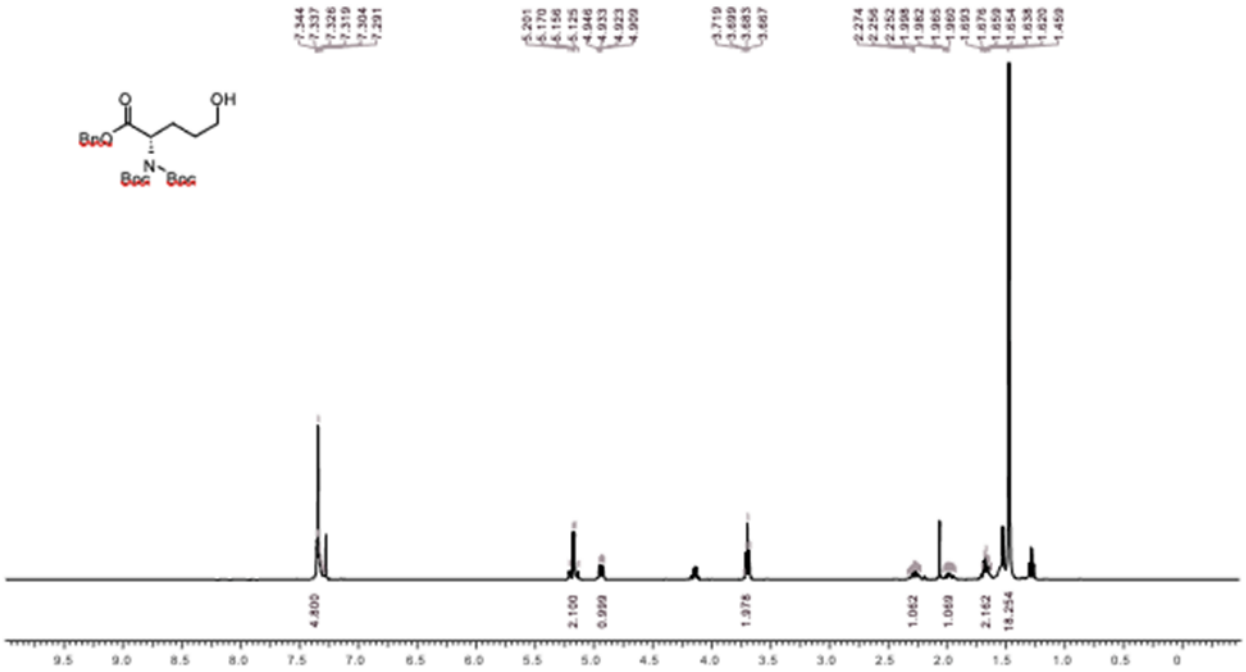
(S-10)



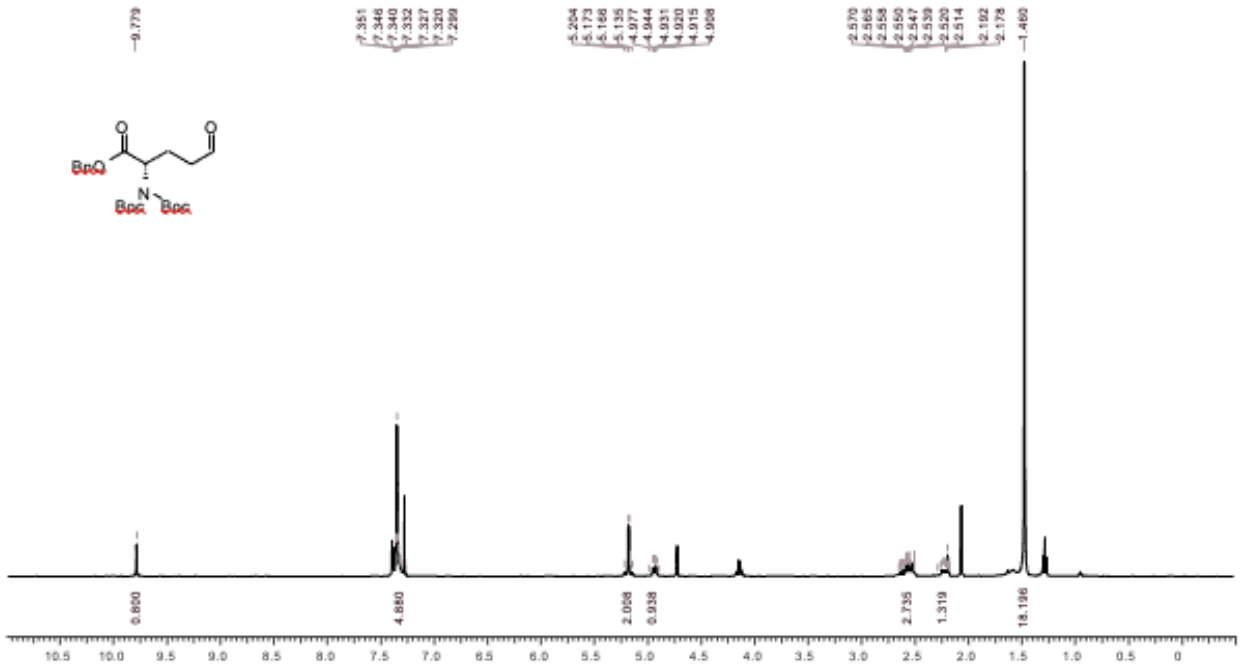
(S-11)



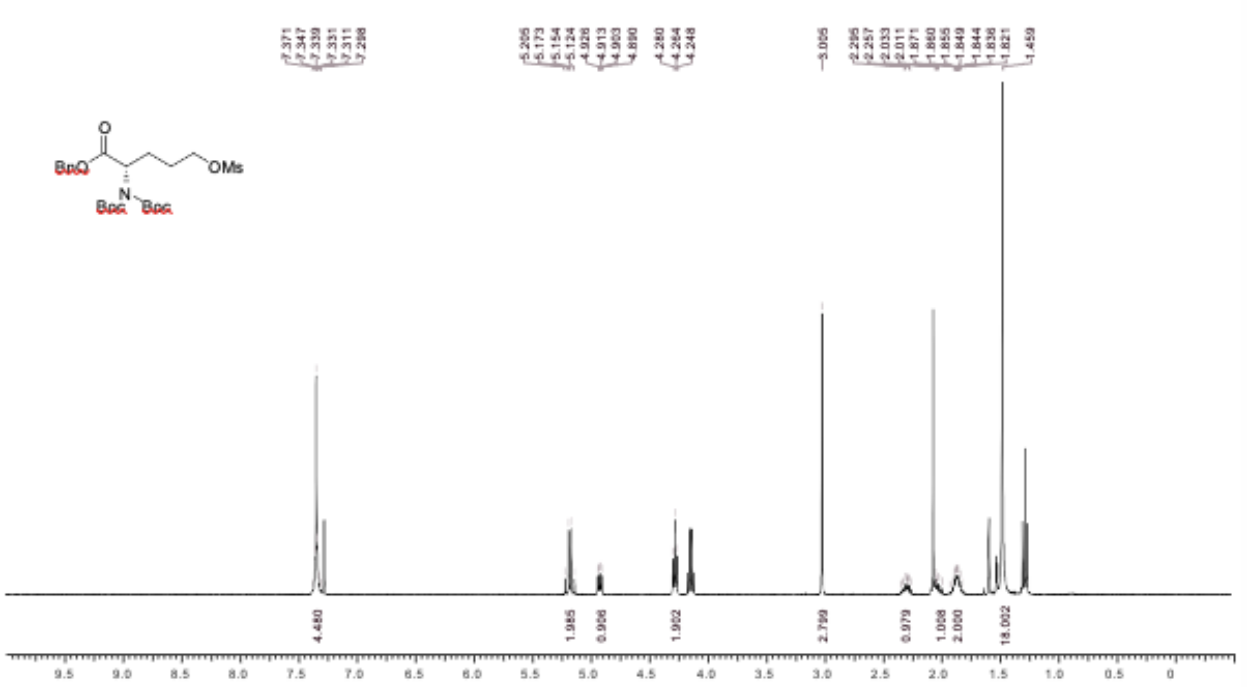
(S-12)



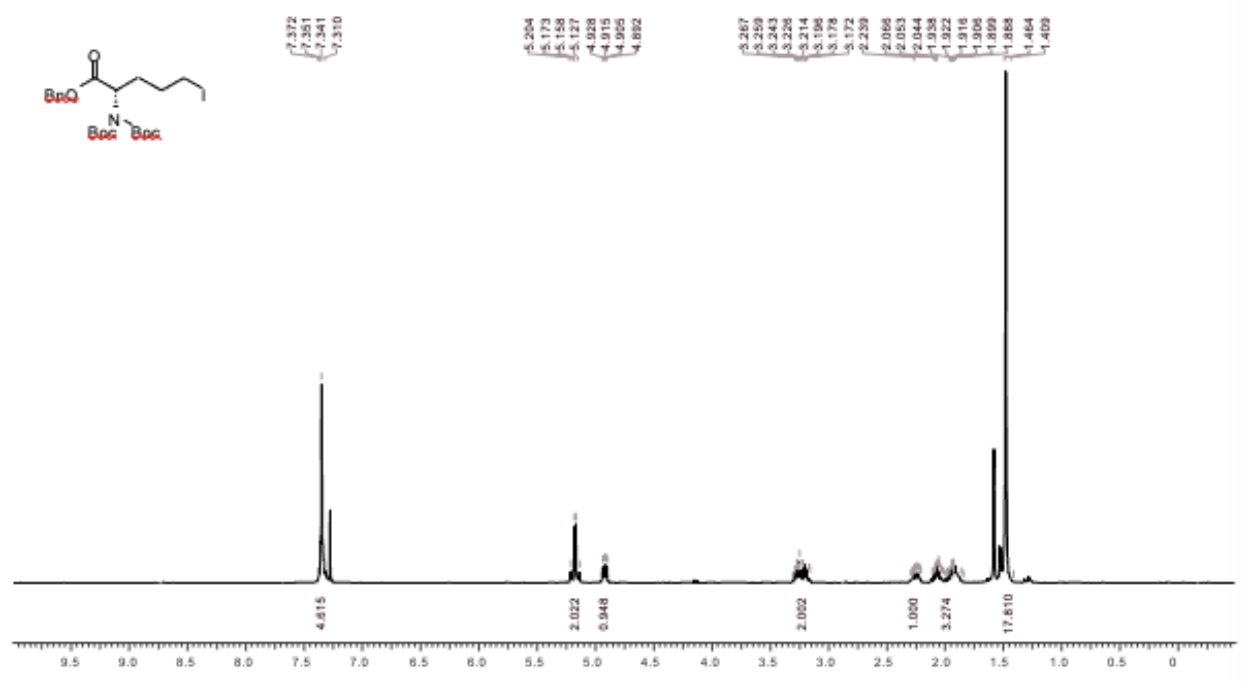
(S-12A)



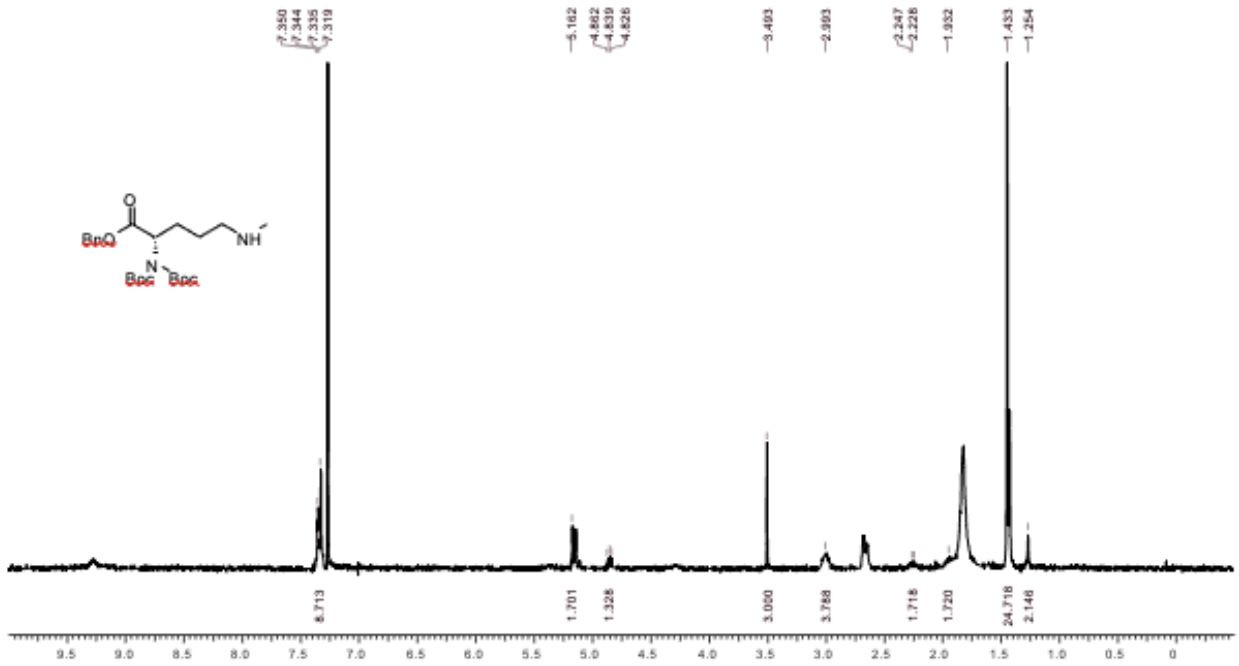
(S-13)


BnO
OMs
Bpc
Bpc
7.371
7.347
7.339
7.331
7.311
7.288
5.205
5.173
5.154
5.124
4.926
4.913
4.903
4.890
4.280
4.264
4.248
3.005
2.295
2.257
2.033
2.011
1.871
1.860
1.855
1.849
1.844
1.836
1.821
1.459
4.480
1.985
0.926
1.902
2.799
0.979
1.058
2.000
18.002

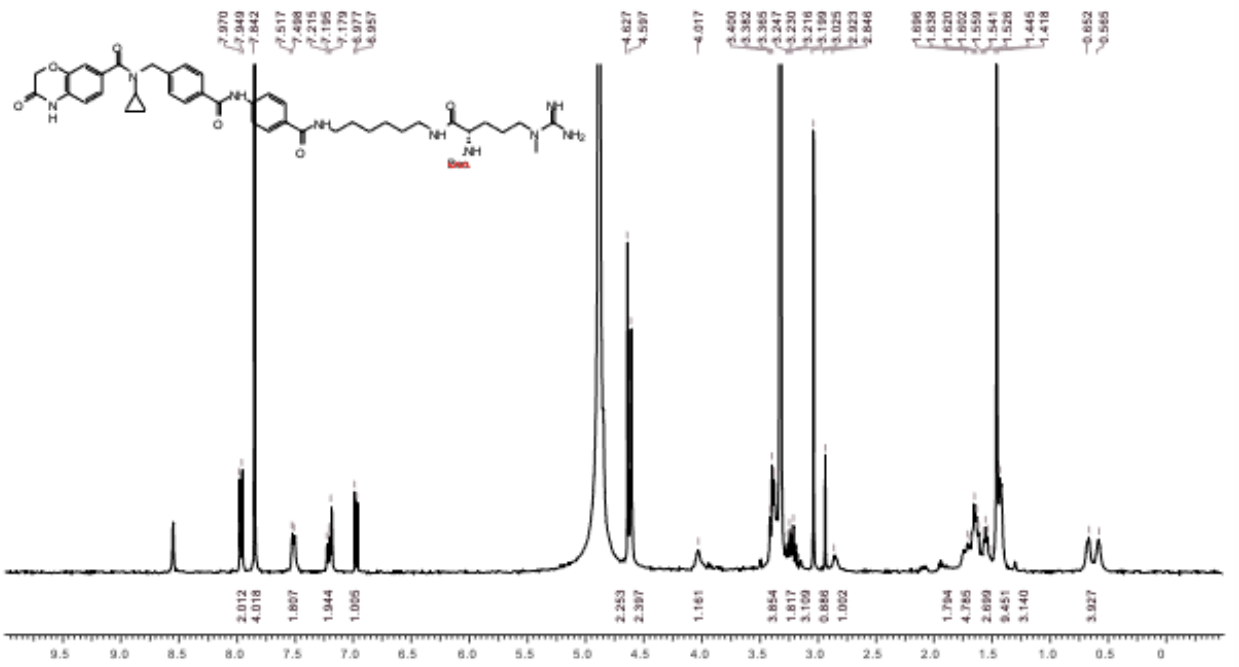
(S-14)


BnO
Bpc
Bpc
7.372
7.351
7.341
7.310
5.264
5.173
5.158
5.127
4.938
4.915
4.905
4.892
3.267
3.259
3.243
3.229
3.214
3.196
3.178
3.172
2.239
2.086
2.053
2.044
1.938
1.922
1.916
1.906
1.899
1.888
1.464
1.409
4.615
2.022
0.948
2.002
1.050
3.274
17.810

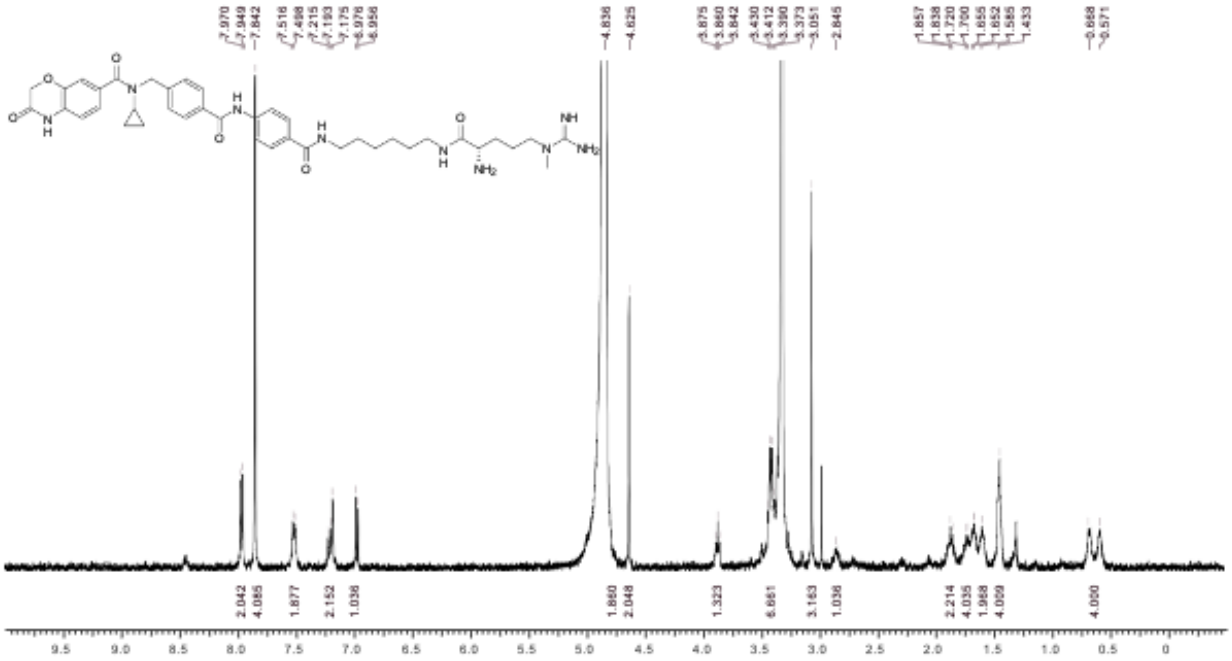
(S-15)



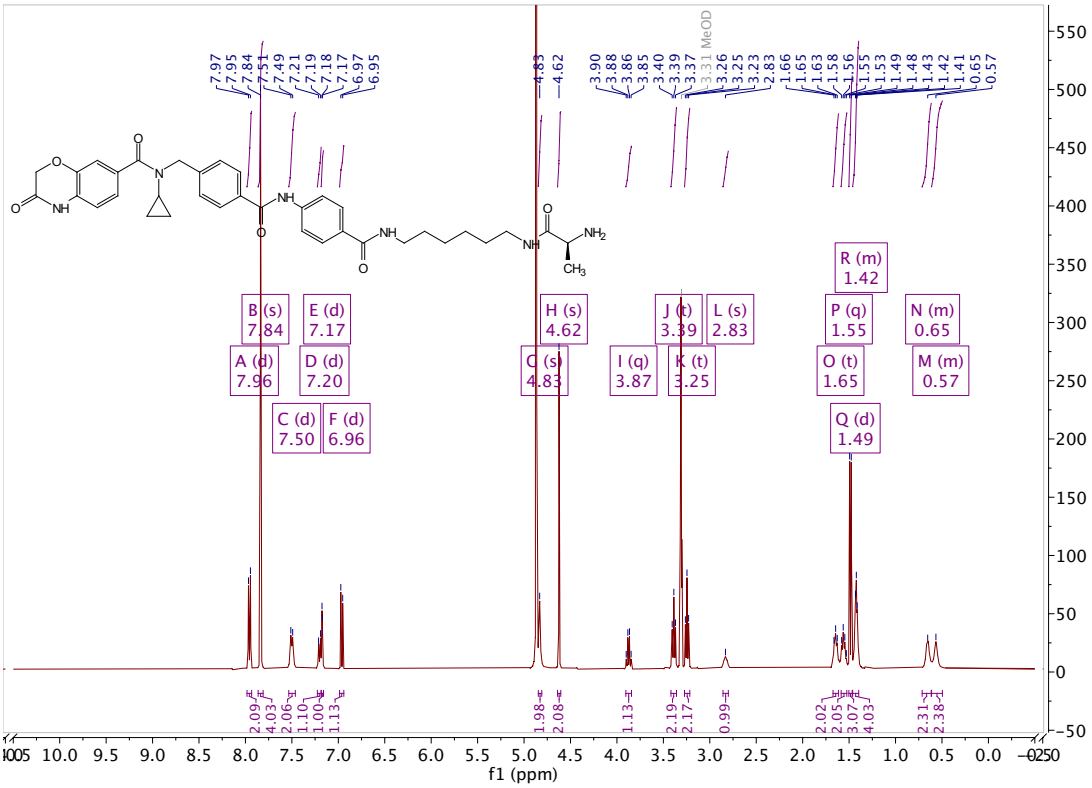
(S-18)



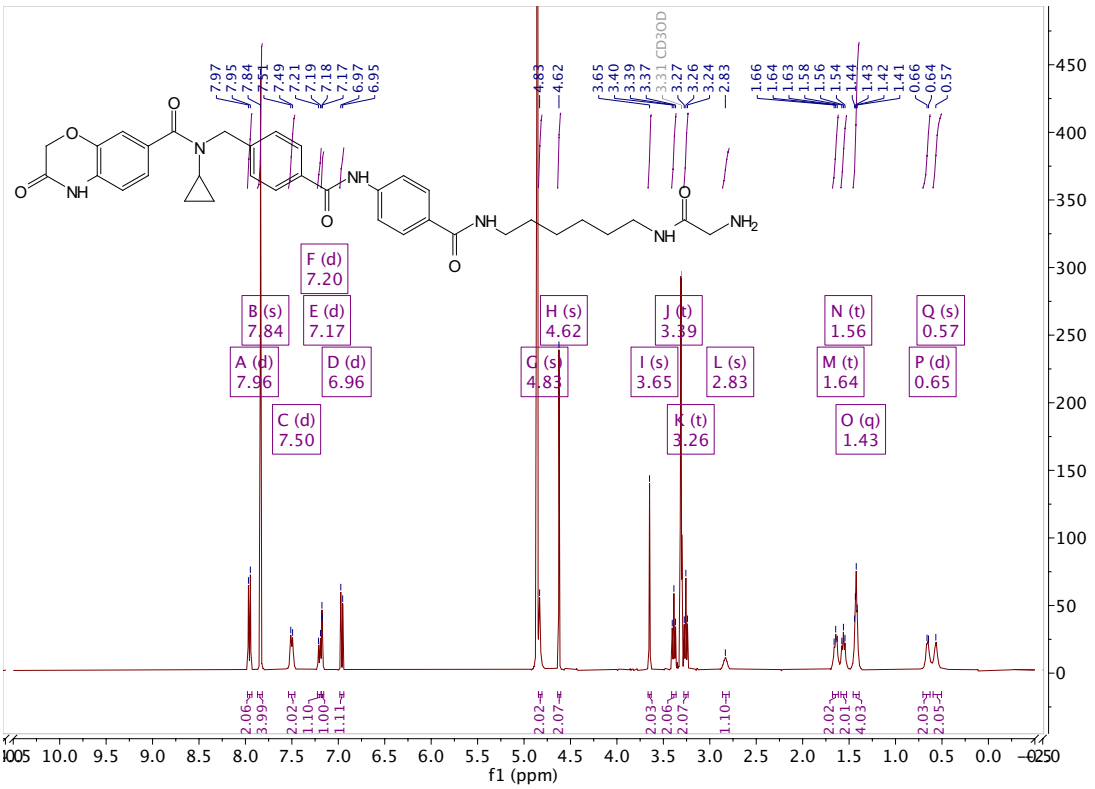
UNC10302685



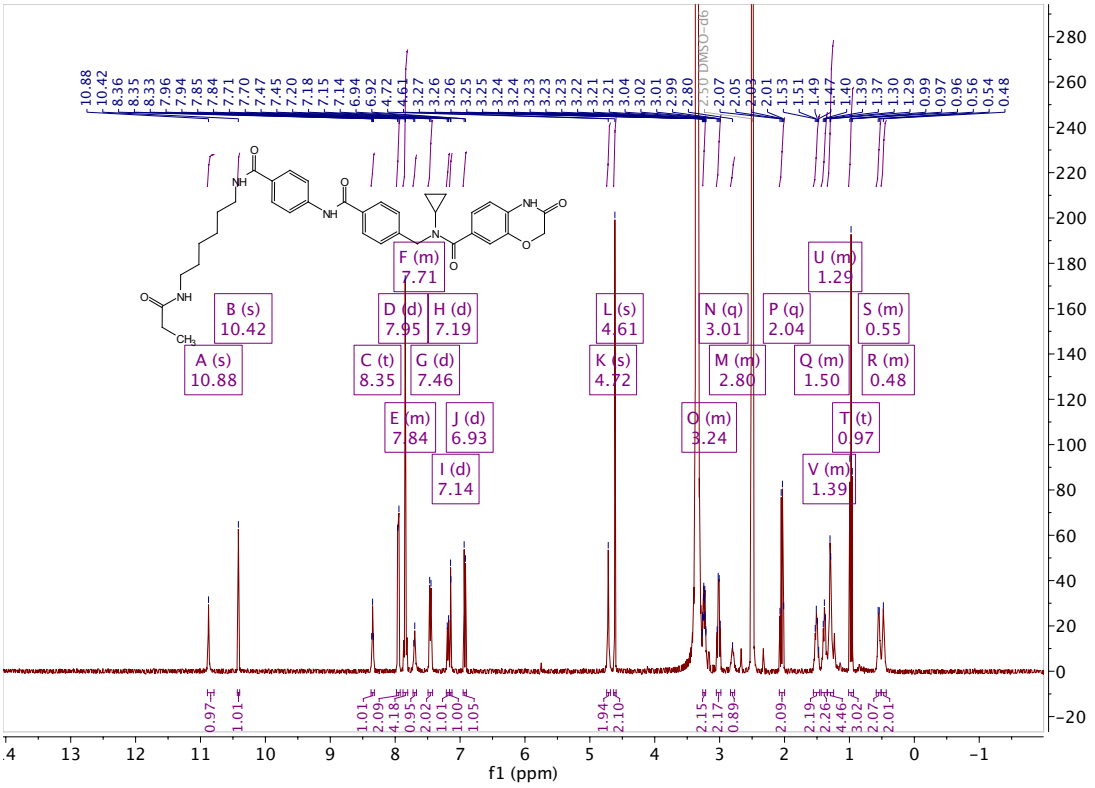
UNC7995



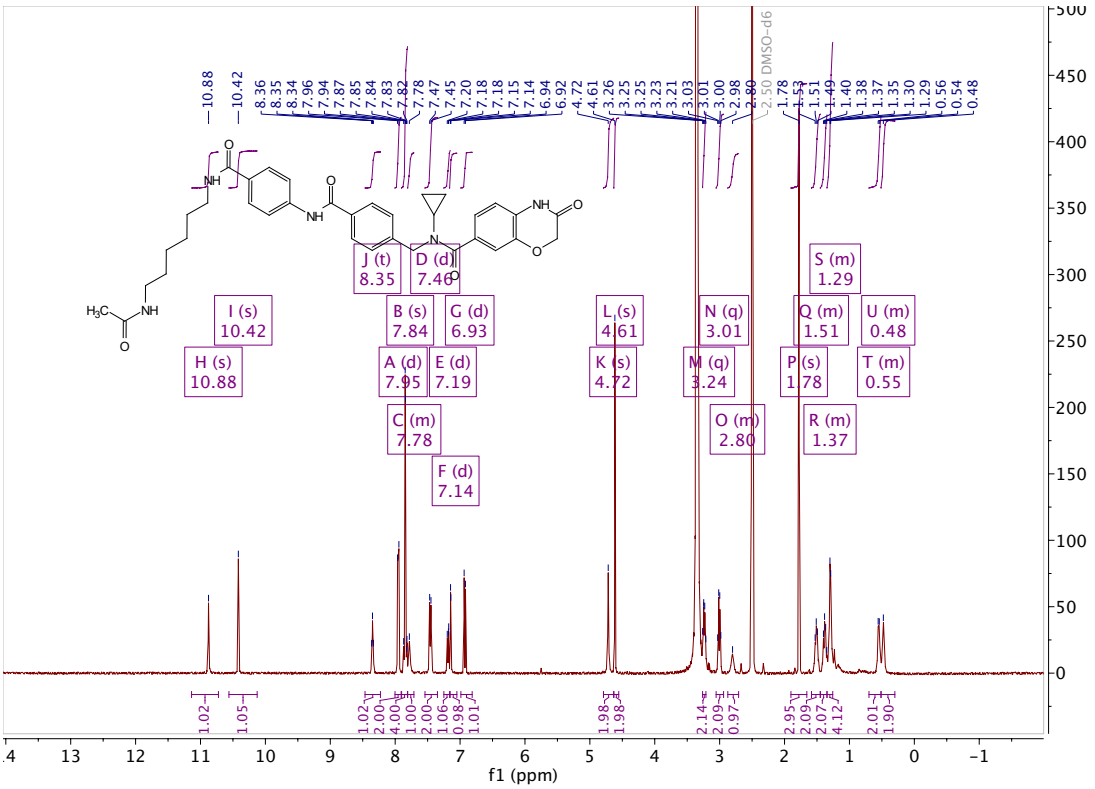
UNC8148



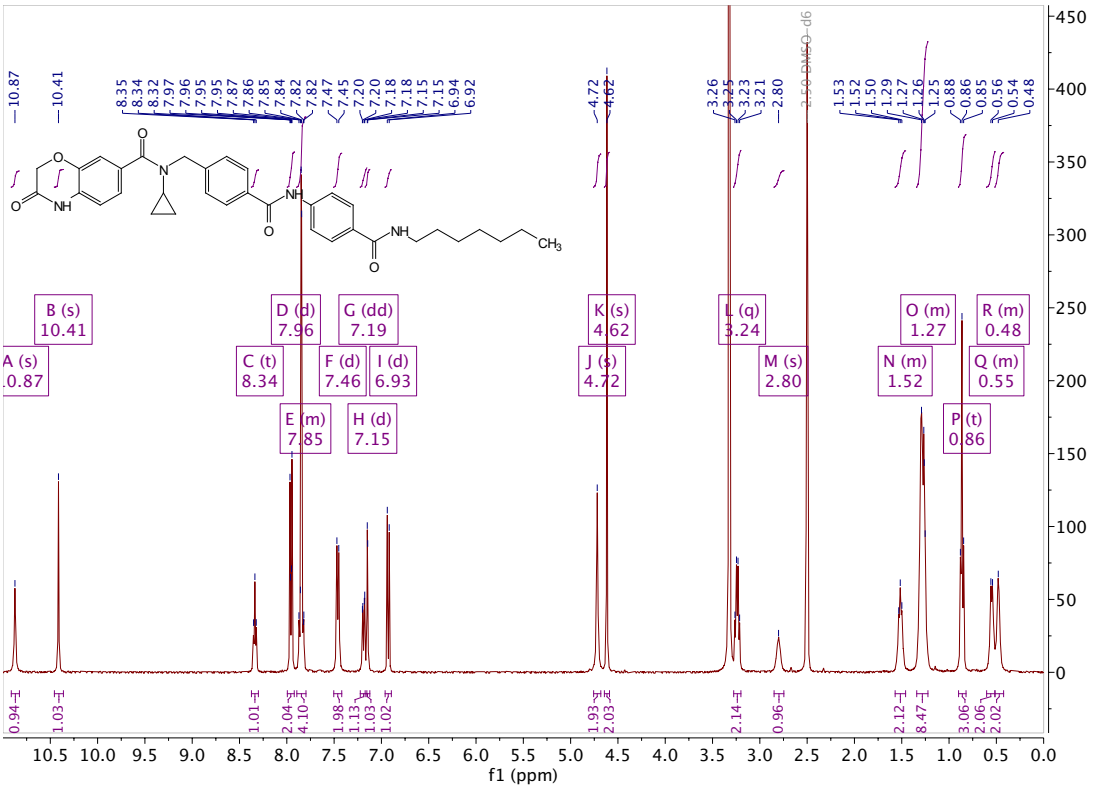
UNC8172



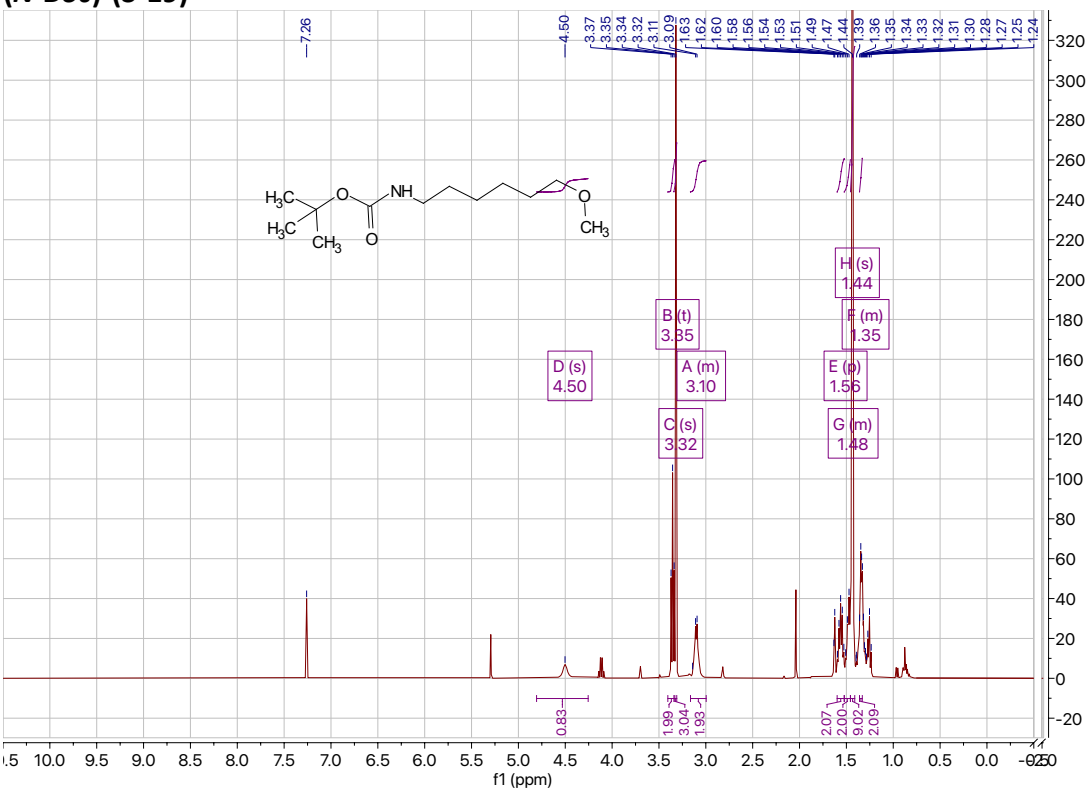
UNC8151



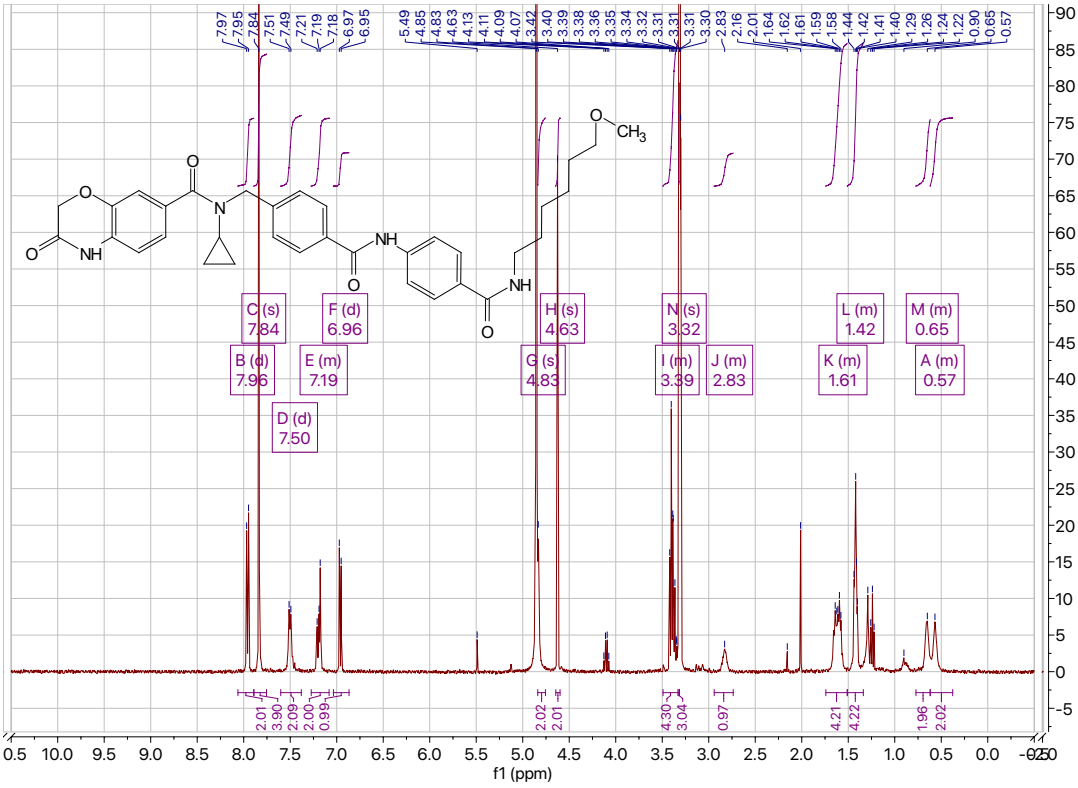
UNC8524



(*N-Boc*)-(S-19)



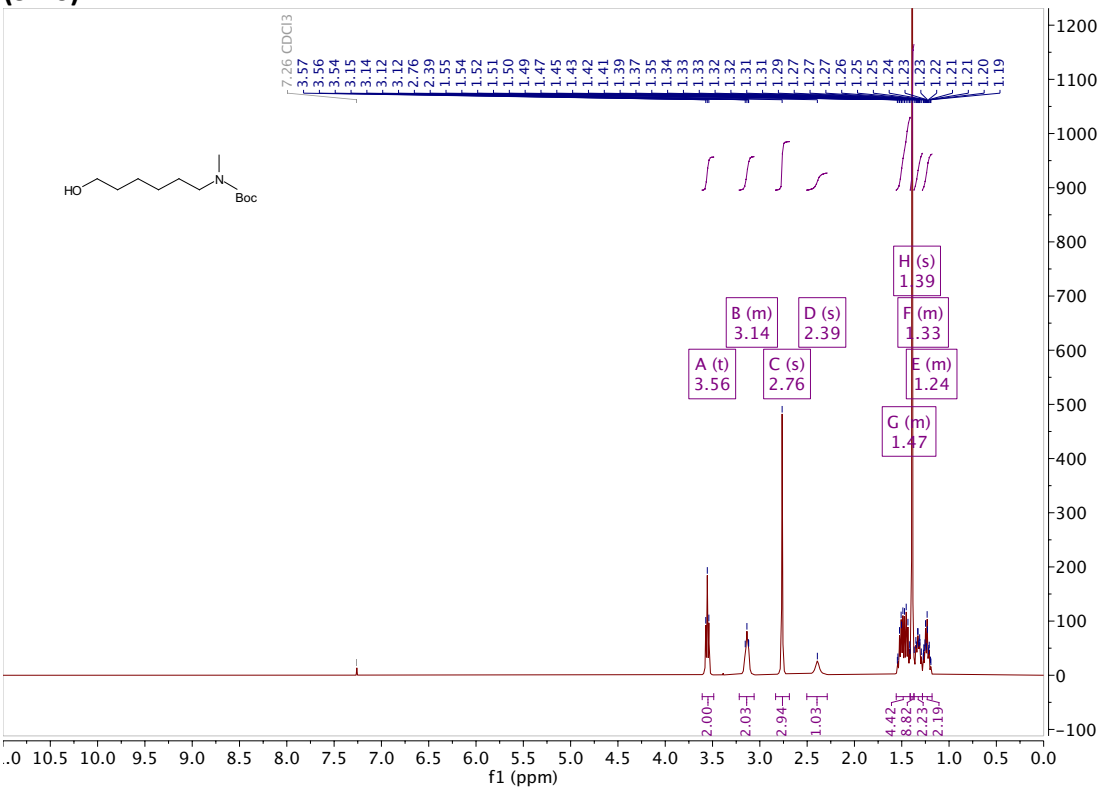
UNC8515



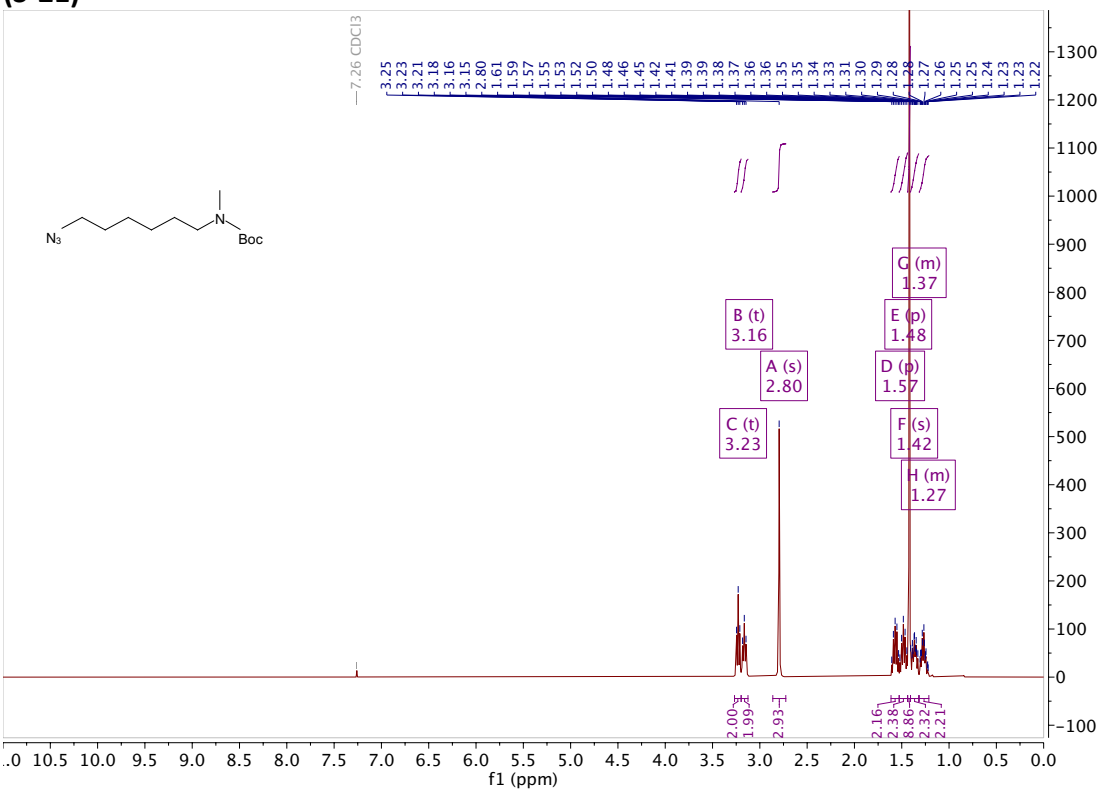
UNC8526


B (s) 10.41
D (d) 7.96
H (d) 7.15
K (s) 4.62
M (q) 3.38
O (s) 2.80
Q (m) 1.41
T (m) 0.48
A (s) 0.87
C (t) 8.34
F (d) 7.46
G (d) 6.93
J (s) 4.72
N (q) 3.24
P (m) 1.52
S (m) 0.55
E (m) 7.84
I (dd) 7.19
L (t) 4.34
R (m) 1.31
f1 (ppm)

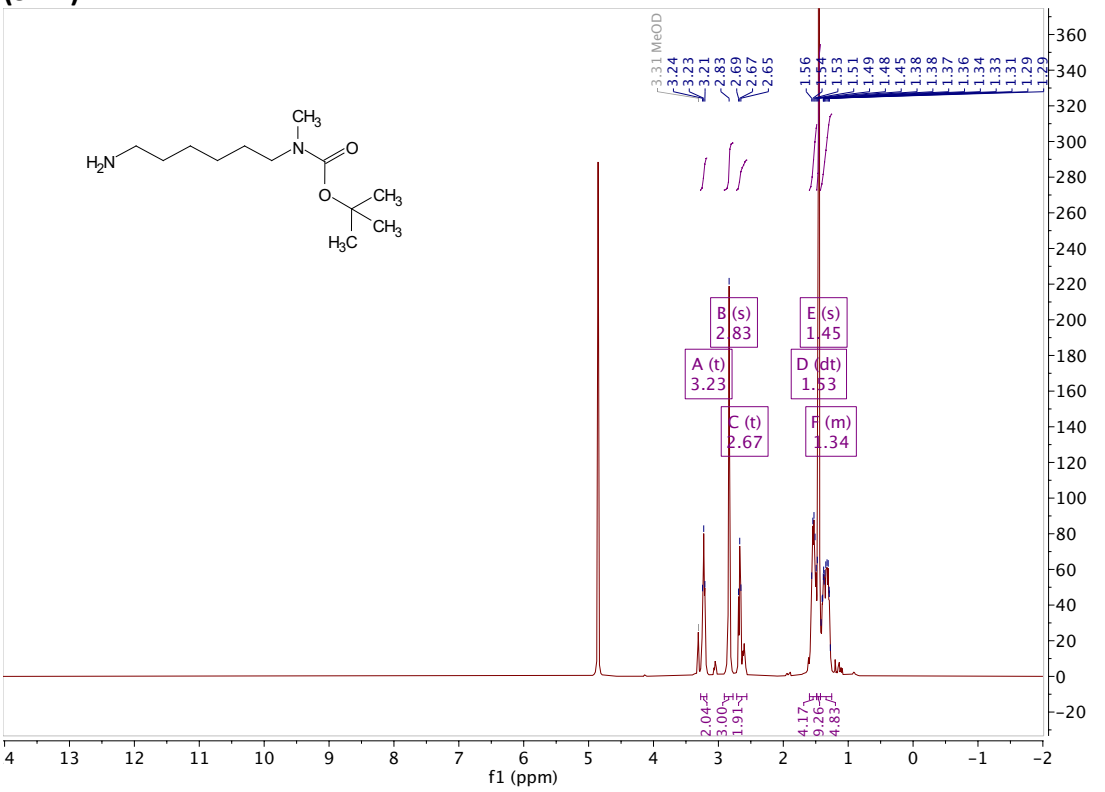
(S-20)


H (s) 1.39
B (m) 3.14
D (s) 2.39
F (m) 1.33
A (t) 3.56
C (s) 2.76
E (m) 1.24
G (m) 1.47
f1 (ppm)

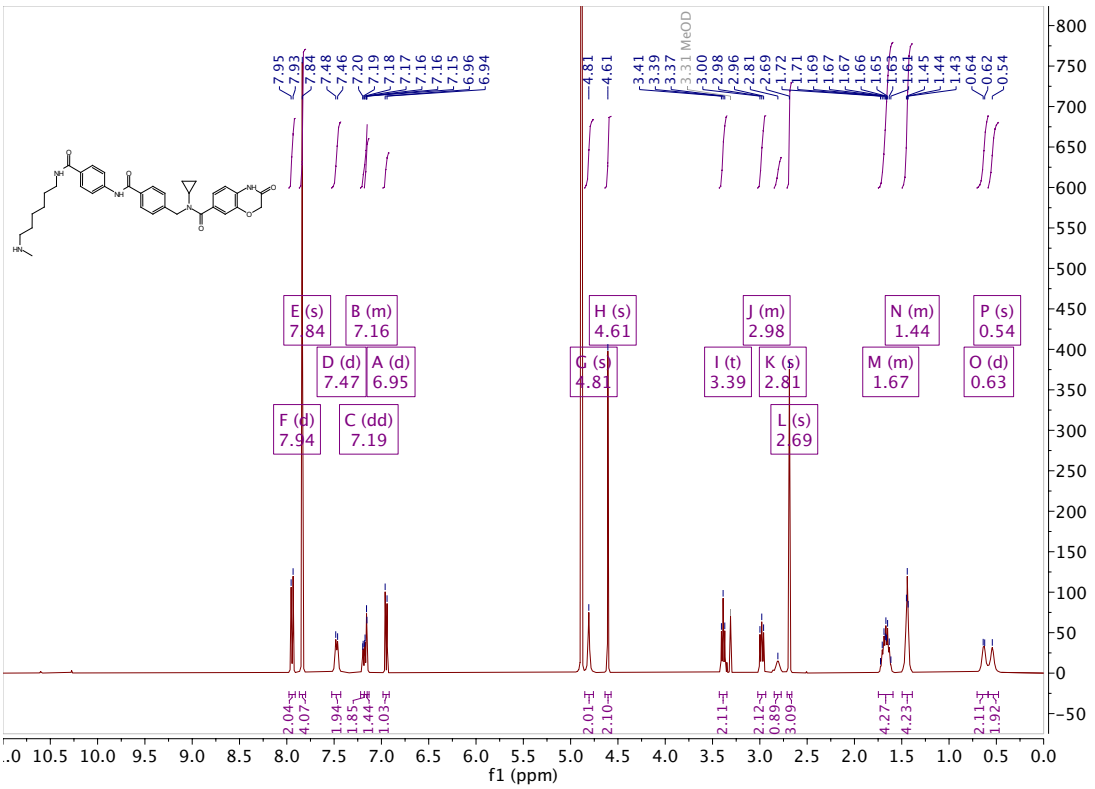
(S-21)


7.26 CDCl3
N3
Boc
B (t) 3.16
A (s) 2.80
C (t) 3.23
G (m) 1.37
E (p) 1.48
D (p) 1.57
F (s) 1.42
H (m) 1.27
f1 (ppm)

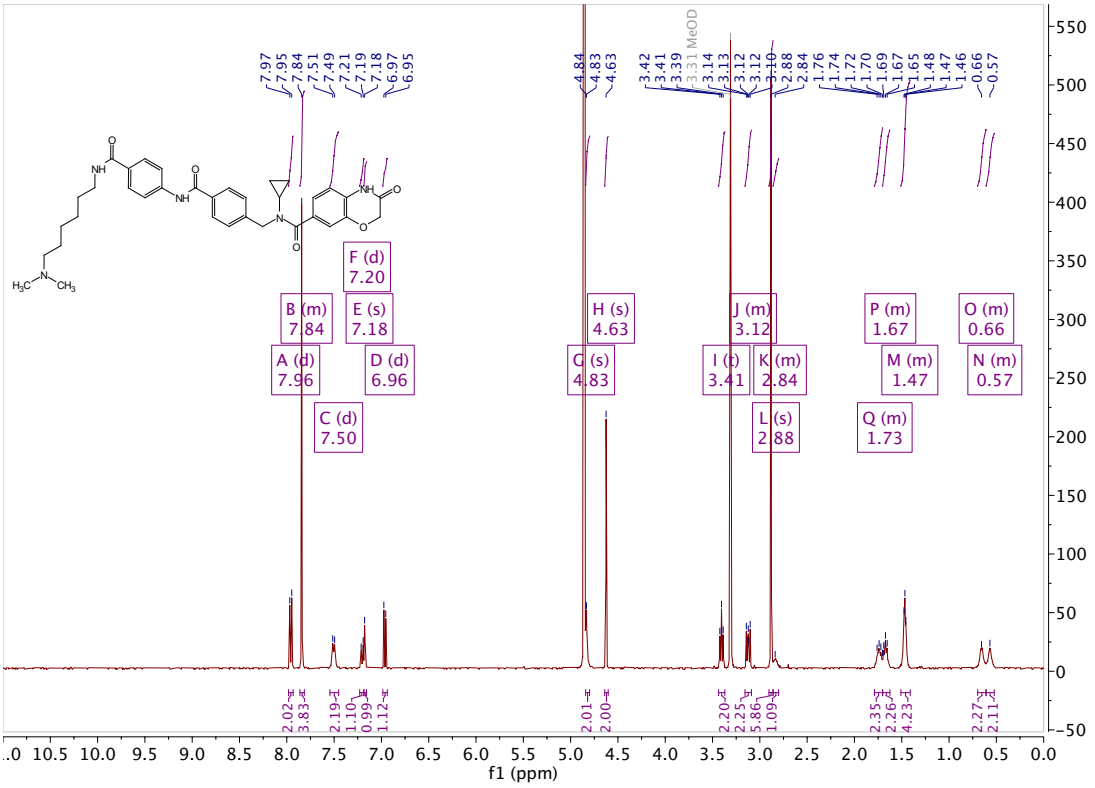
(S-22)


3.31 MeOD
H2N
CH3
N
O
O
CH3
CH3
H3C
B (s) 2.83
A (t) 3.23
C (t) 2.67
E (s) 1.45
D (dt) 1.53
F (m) 1.34
f1 (ppm)

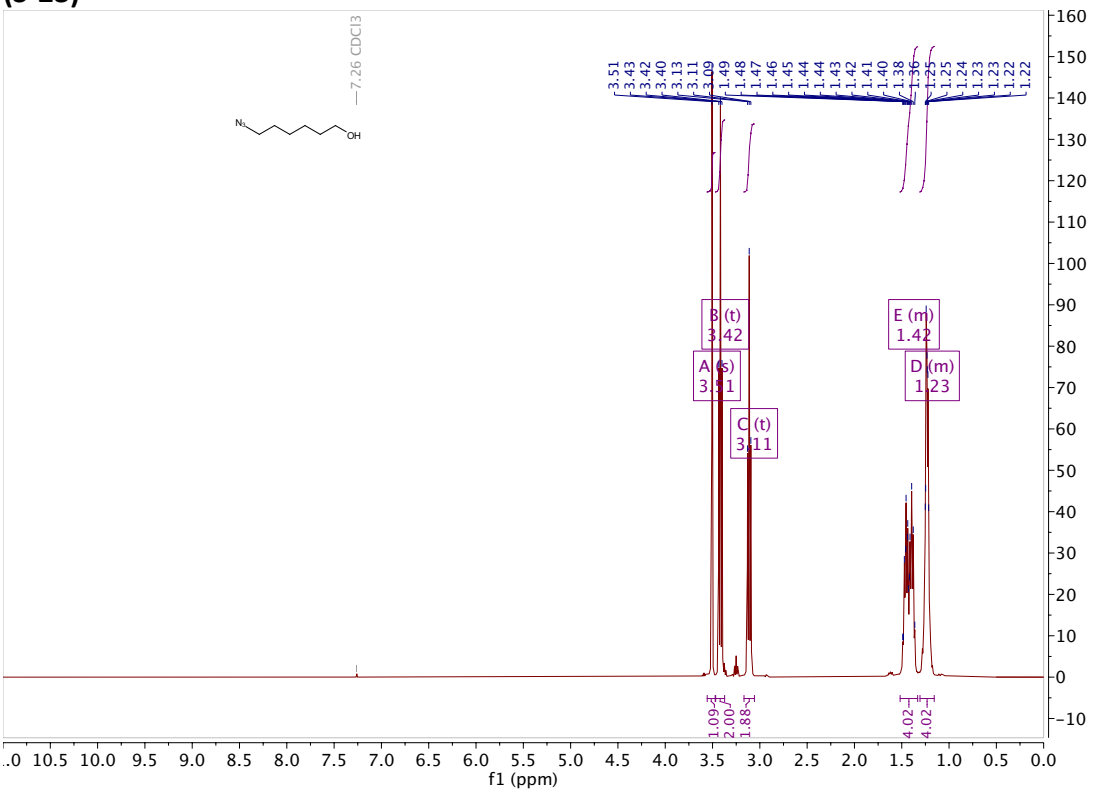
UNC8582



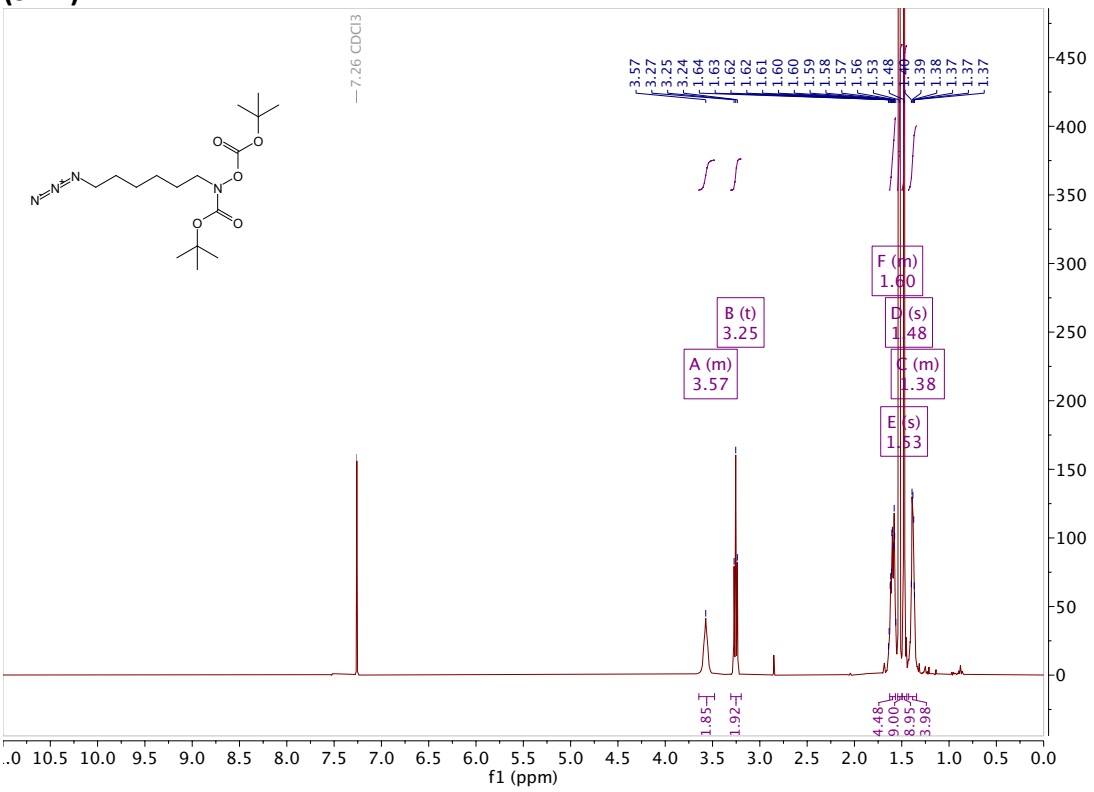
UNC8359



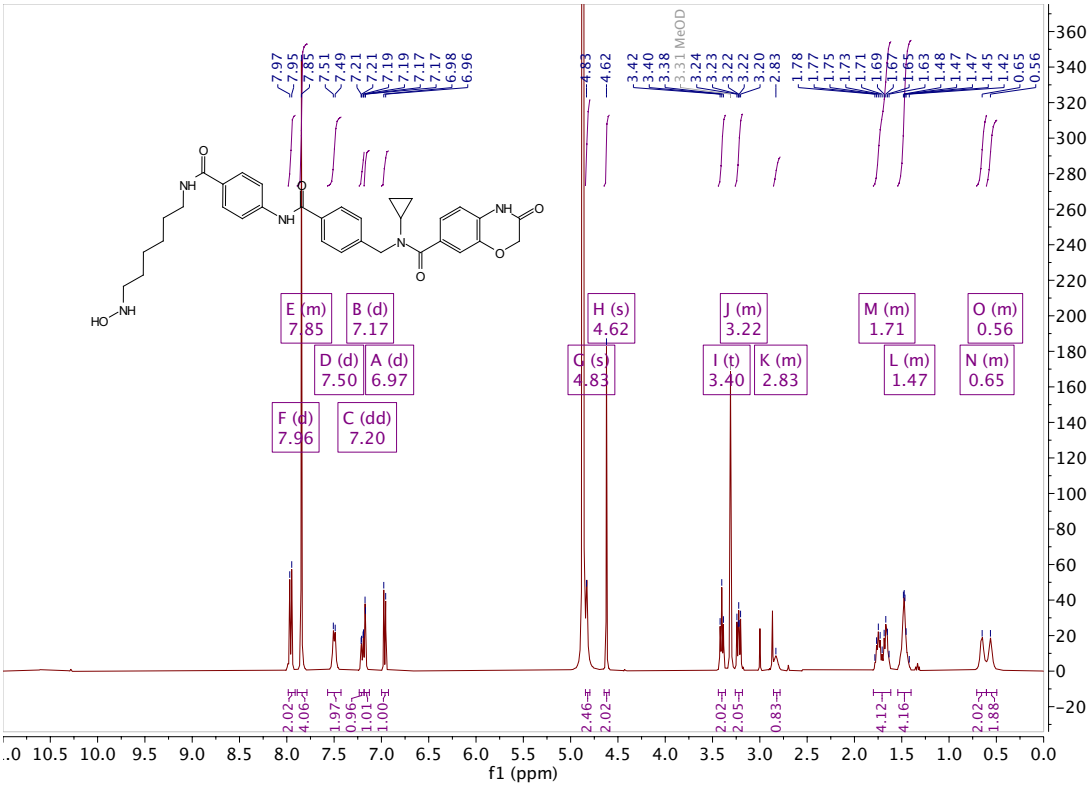
(S-23)


7.26 CDCl3
B (t) 3.42
A (s) 3.51
C (t) 3.11
E (m) 1.42
D (m) 1.23
f1 (ppm)

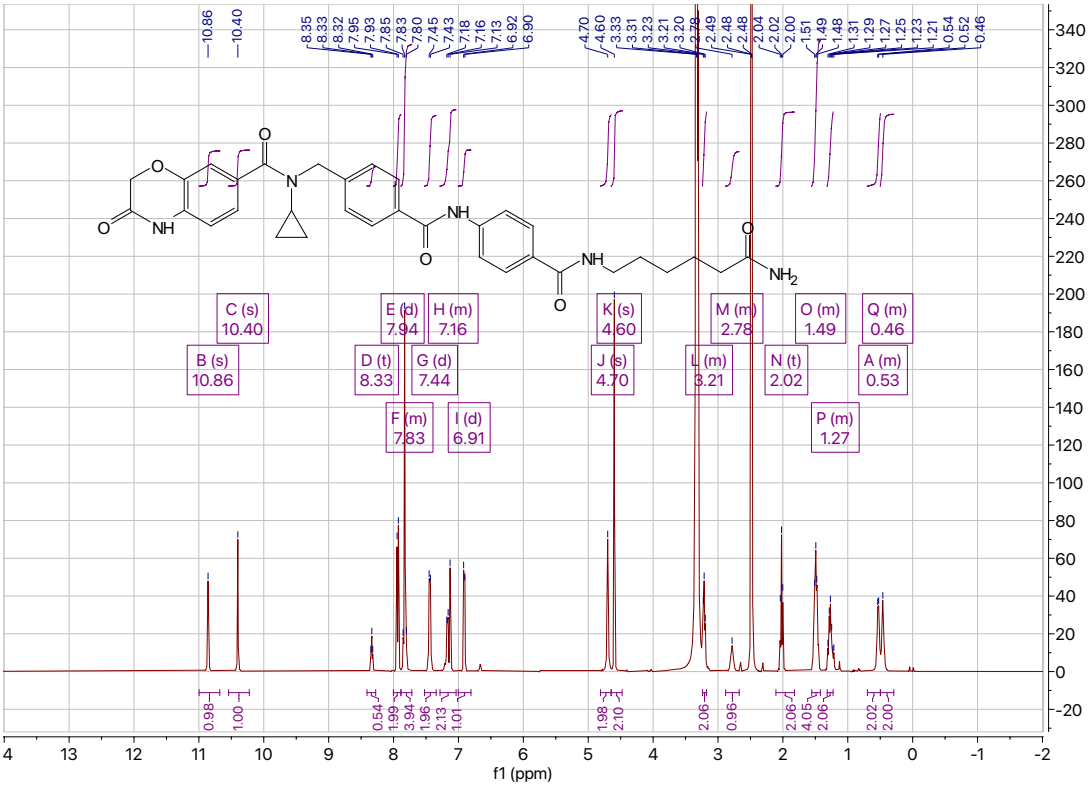
(S-24)


7.26 CDCl3
F (m) 1.60
B (t) 3.25
D (s) 1.48
A (m) 3.57
C (m) 1.38
E (s) 1.53
f1 (ppm)

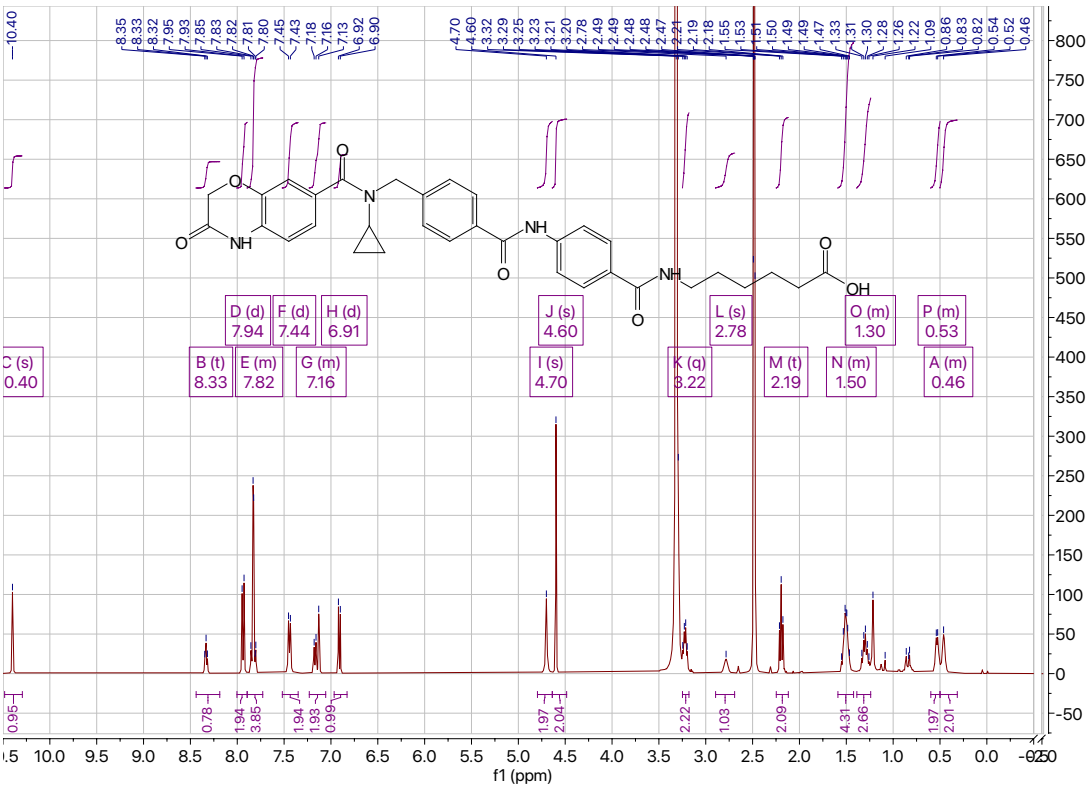
UNC8863



UNC9065

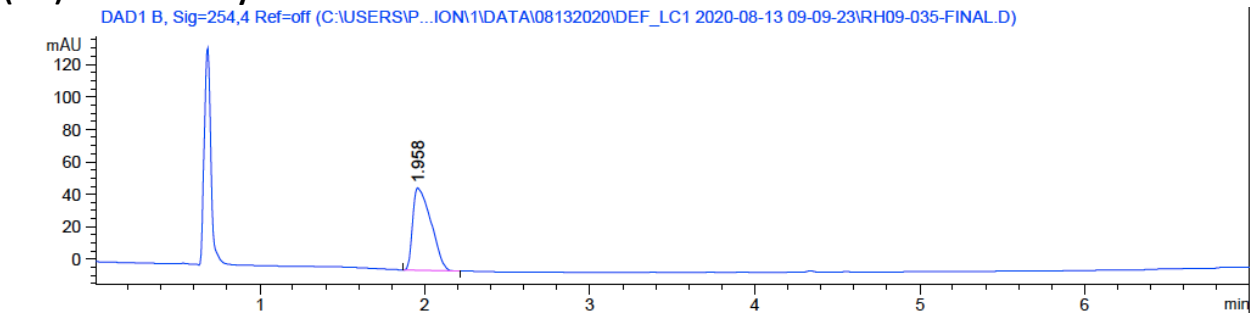


UNC8877

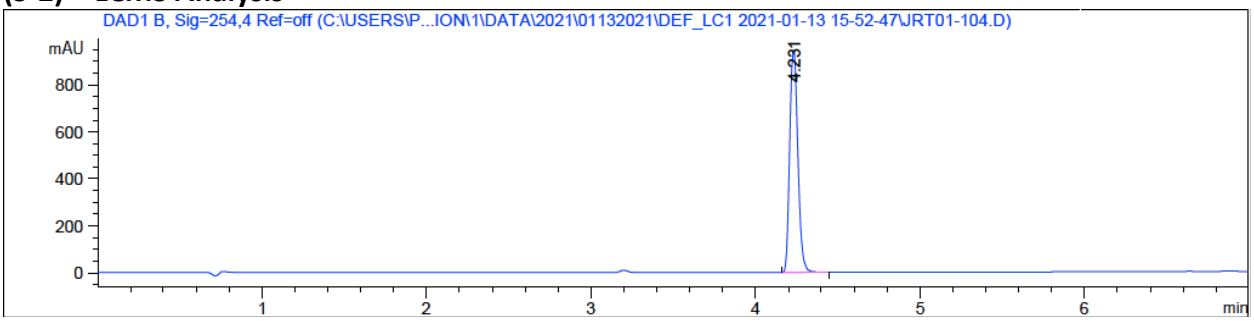

D (d) 7.94
F (d) 7.44
H (d) 6.91
J (s) 4.60
L (s) 2.78
O (m) 1.30
P (m) 0.53
B (t) 8.33
E (m) 7.82
G (m) 7.16
I (s) 4.70
K (q) 3.22
M (t) 2.19
N (m) 1.50
A (m) 0.46
f1 (ppm)

LC-MS Spectra

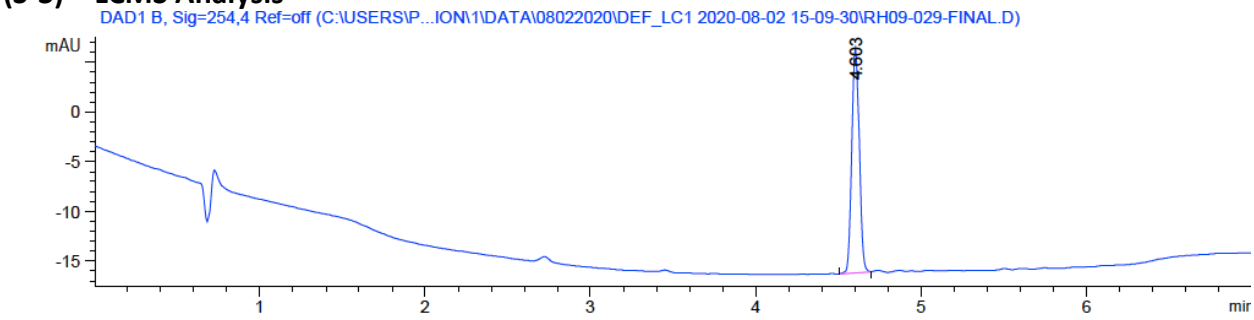
(S-1) – LCMS Analysis



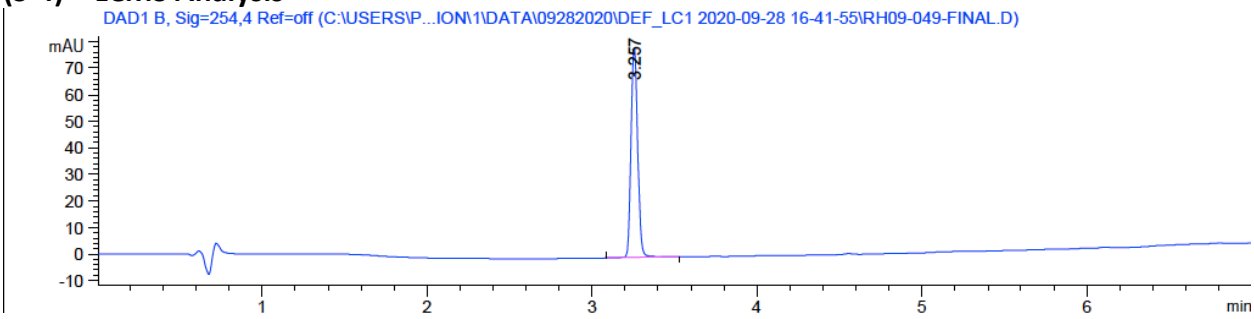
(S-2) – LCMS Analysis



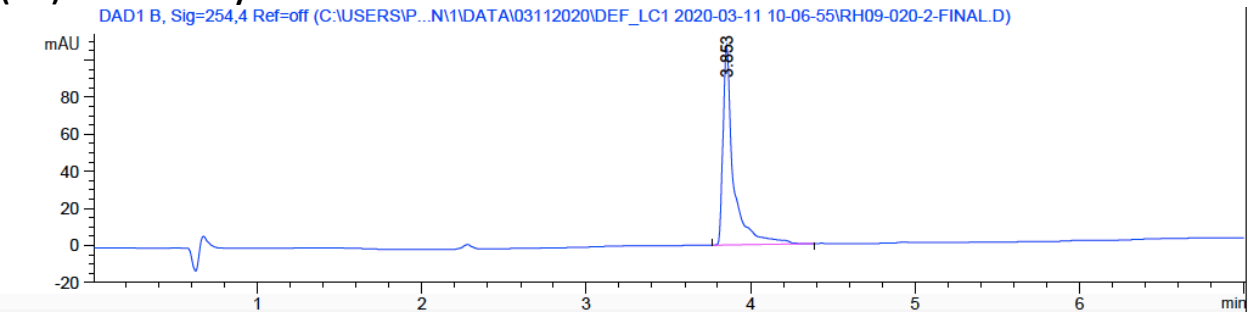
(S-3) – LCMS Analysis



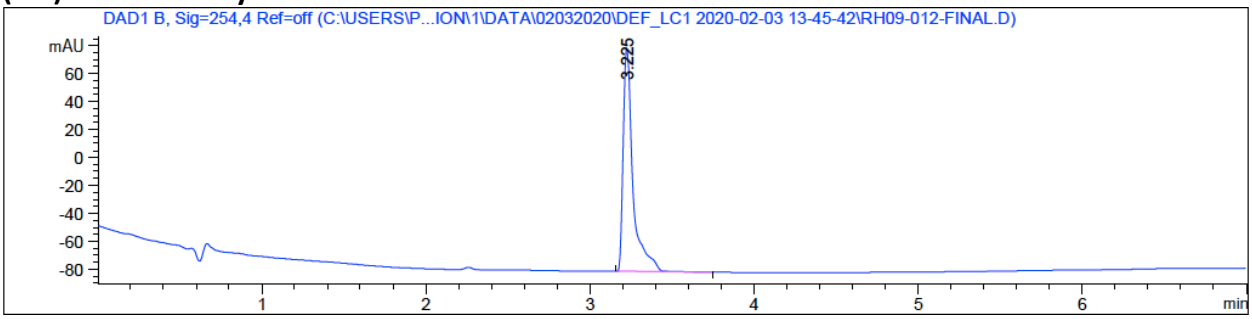
(S-4) – LCMS Analysis



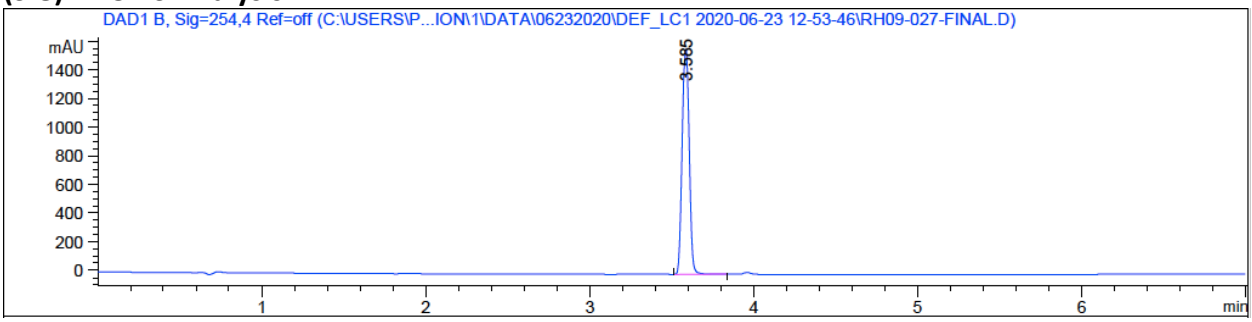
(S-6) – LCMS Analysis



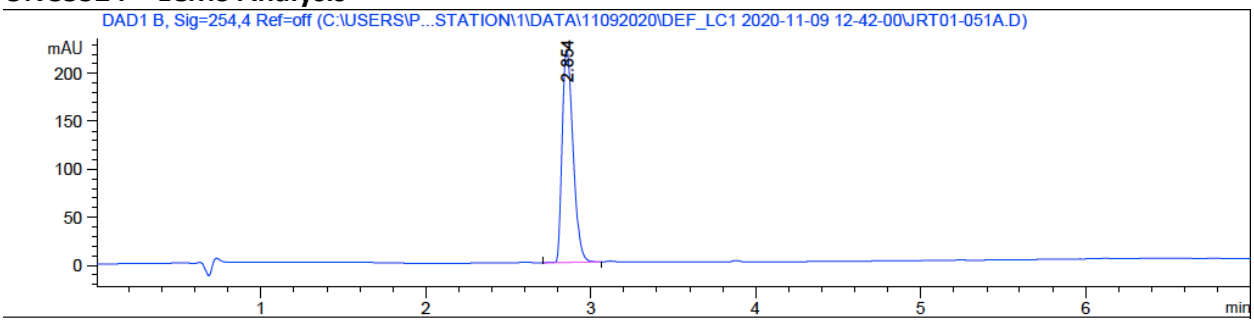
(S-7) – LCMS Analysis



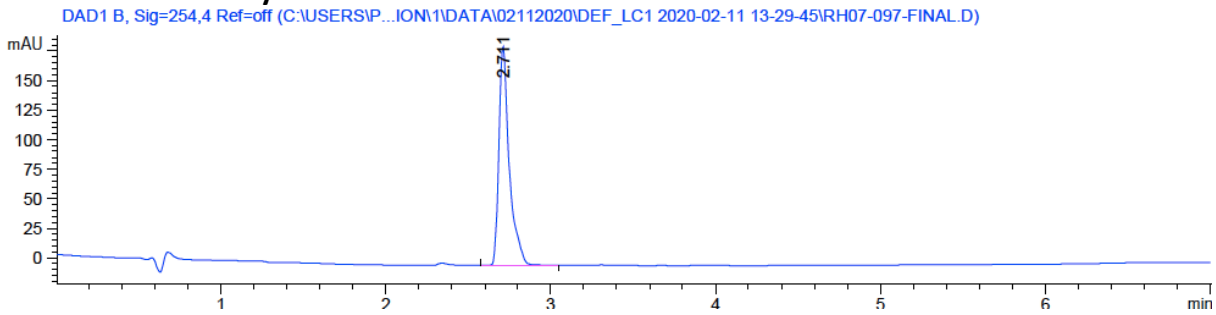
(S-8) – LCMS Analysis



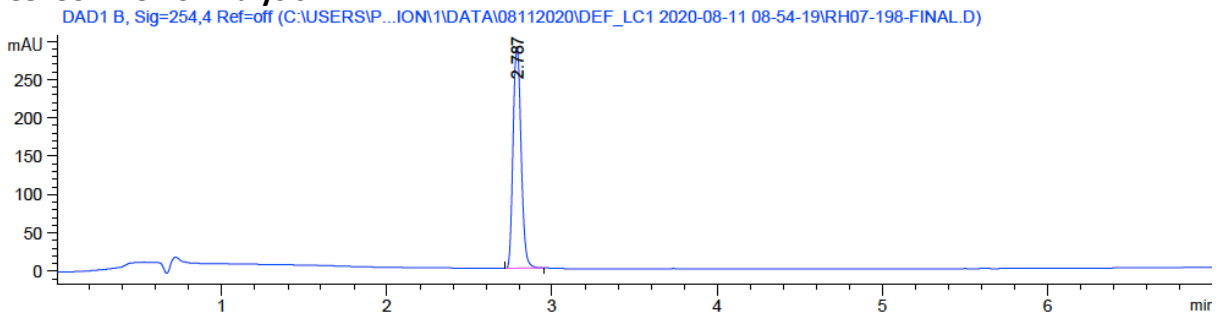
UNC8324 – LCMS Analysis



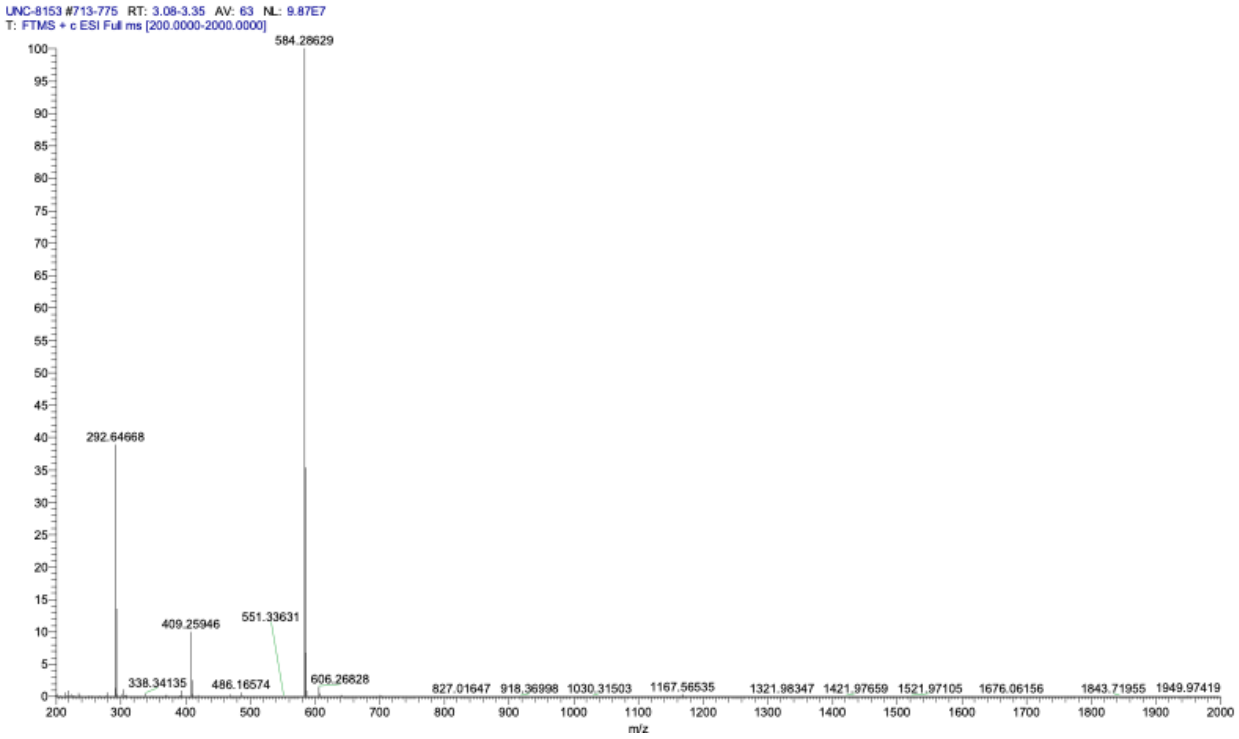
UNC8269 – LCMS Analysis



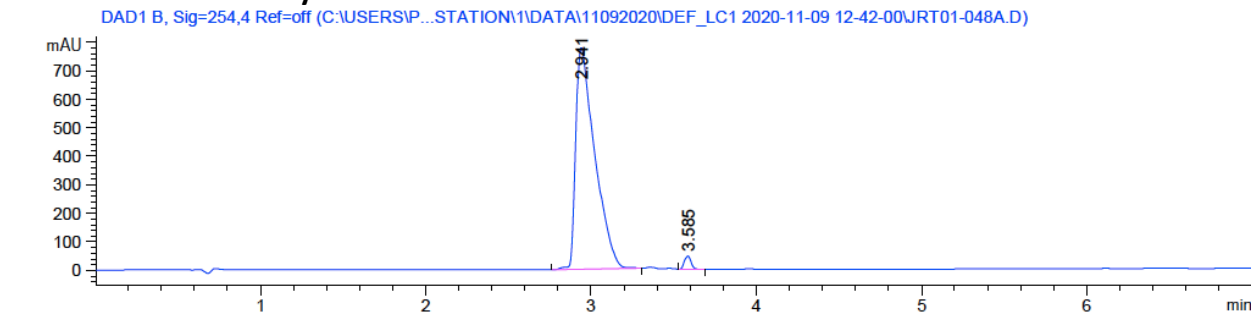
UNC8153 – LCMS Analysis



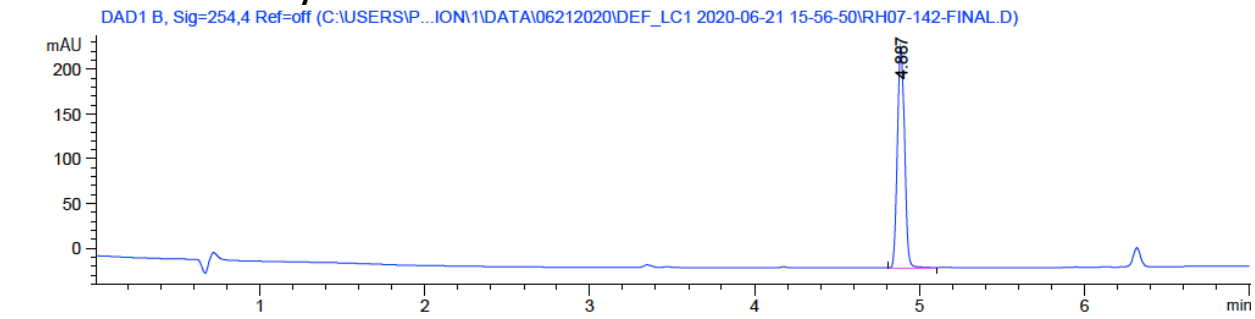
UNC8153 – HRMS Spectrum



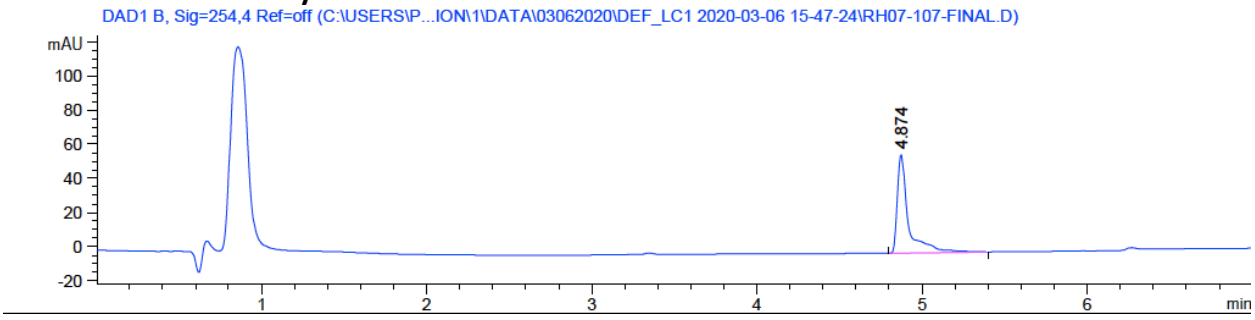
UNC8325 – LCMS Analysis



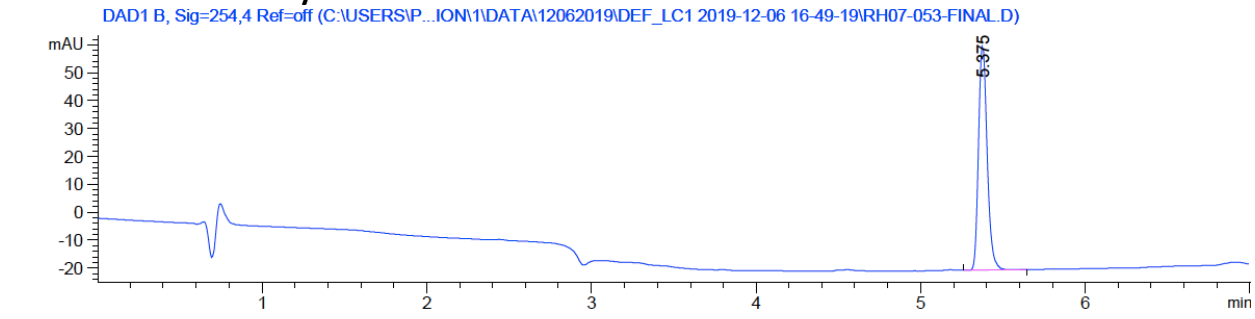
UNC7986 – LCMS Analysis



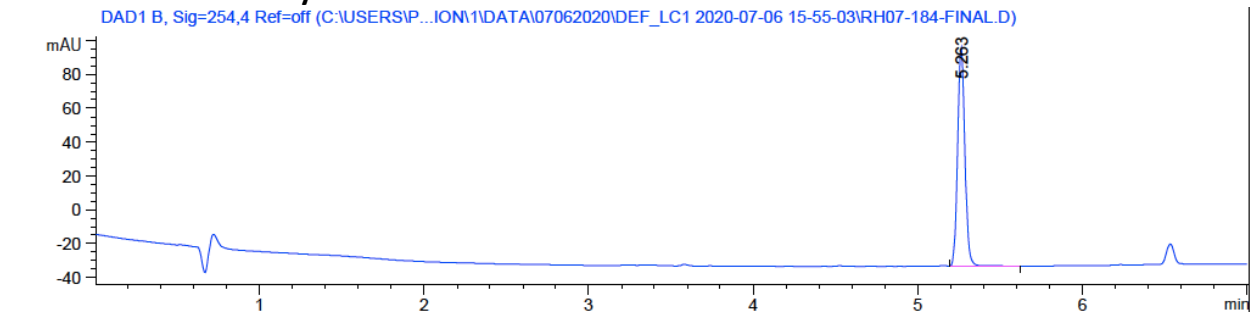
UNC7896 – LCMS Analysis



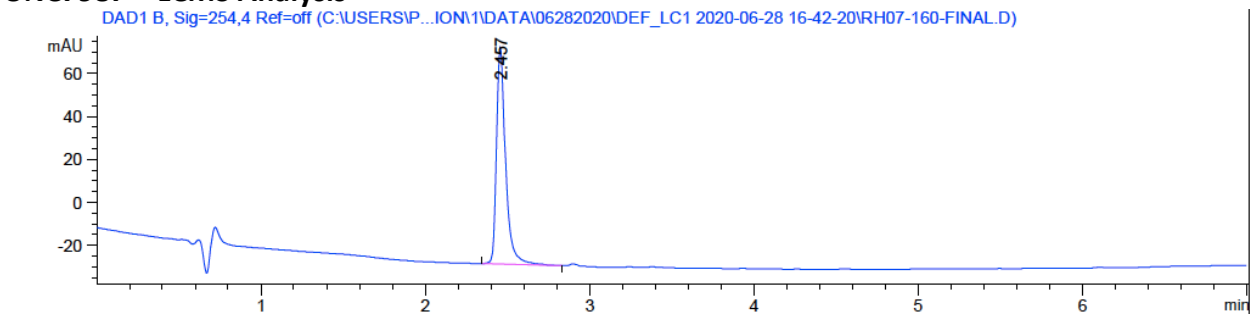
UNC7746 – LCMS Analysis



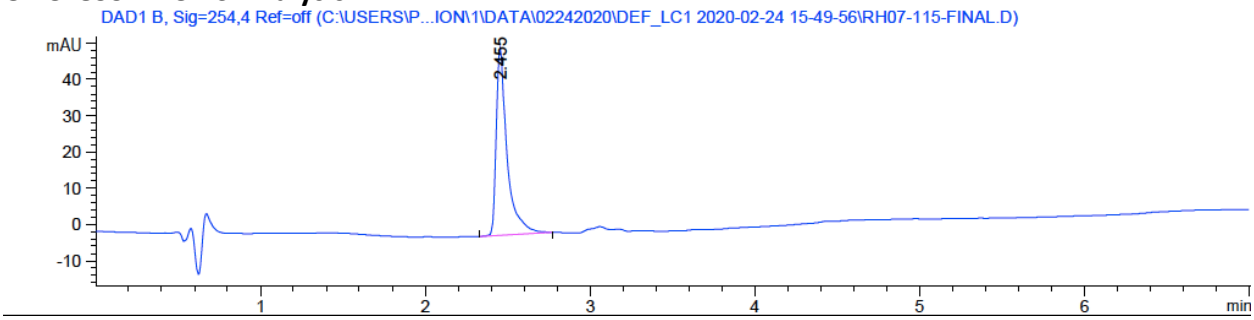
UNC8012 – LCMS Analysis



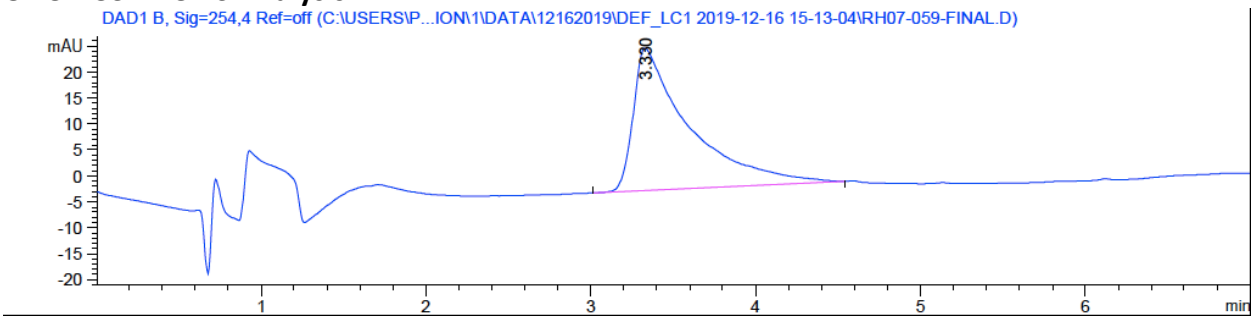
UNC7987 – LCMS Analysis



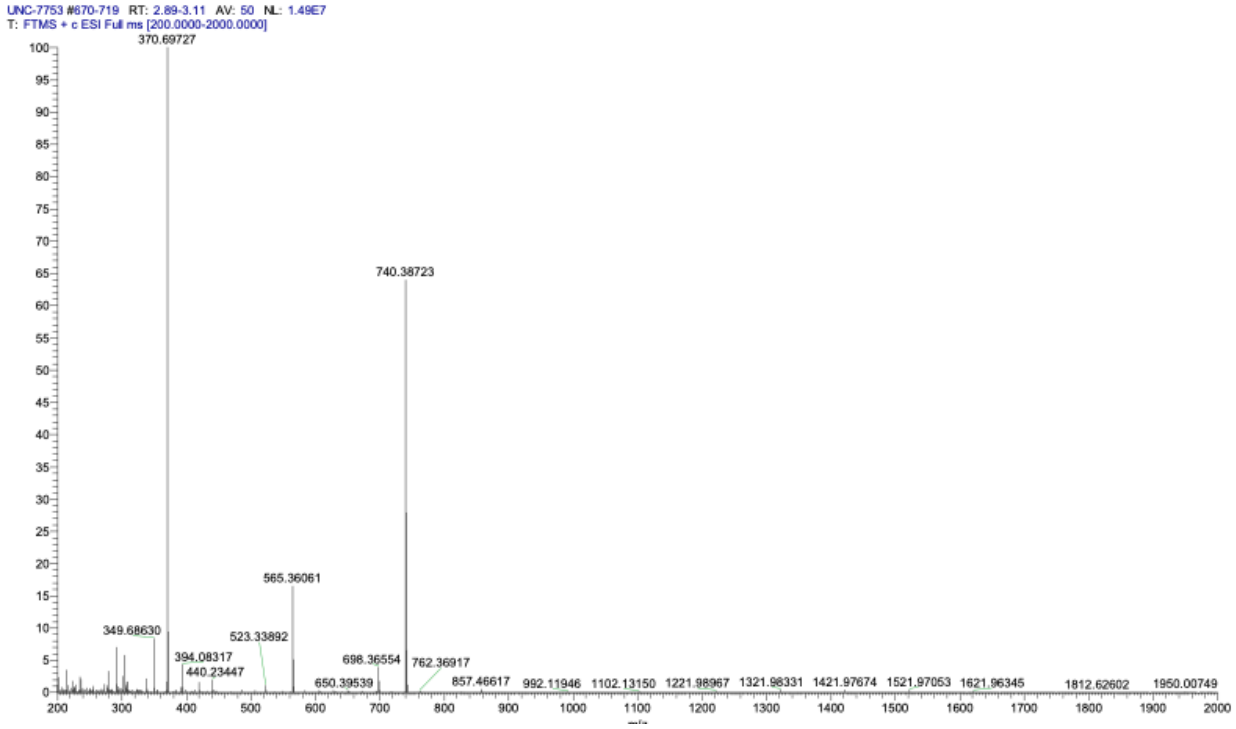
UNC7899 – LCMS Analysis



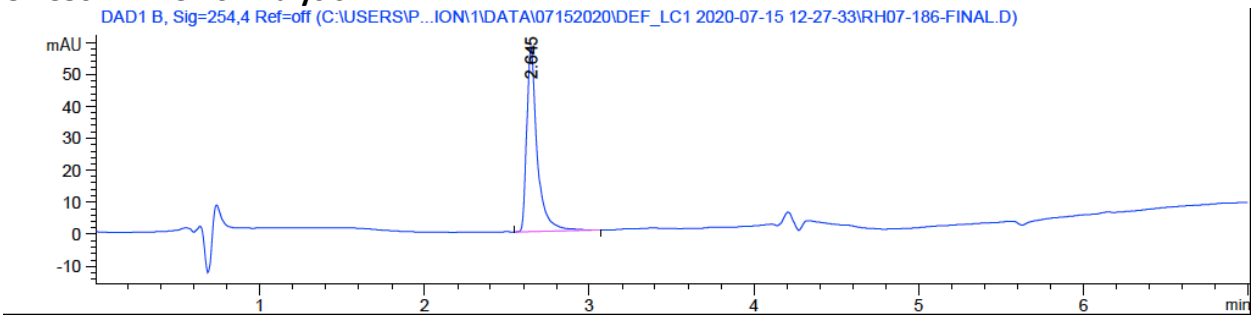
UNC7753 – LCMS Analysis



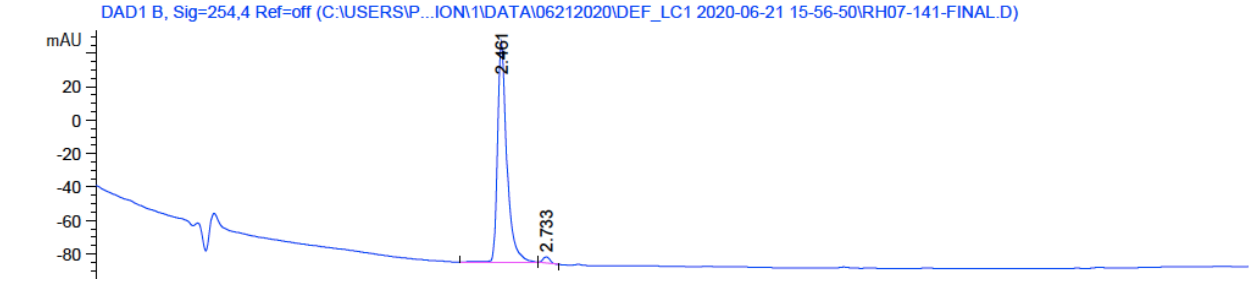
UNC7753 – HRMS Analysis



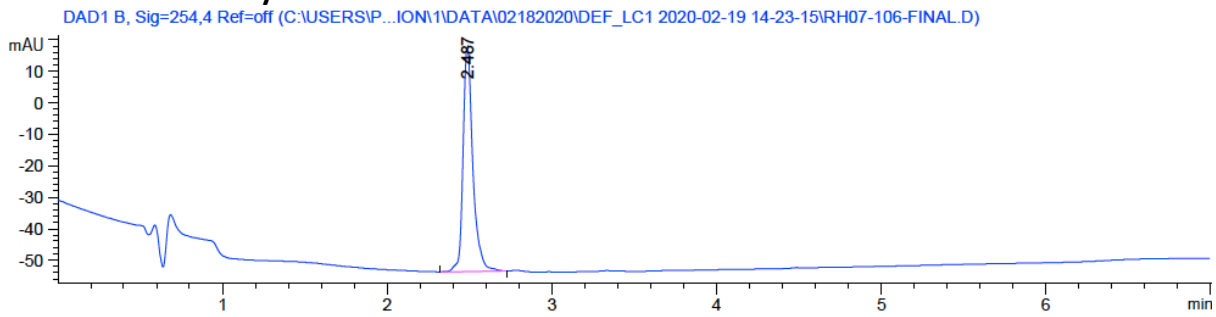
UNC8014 – LCMS Analysis



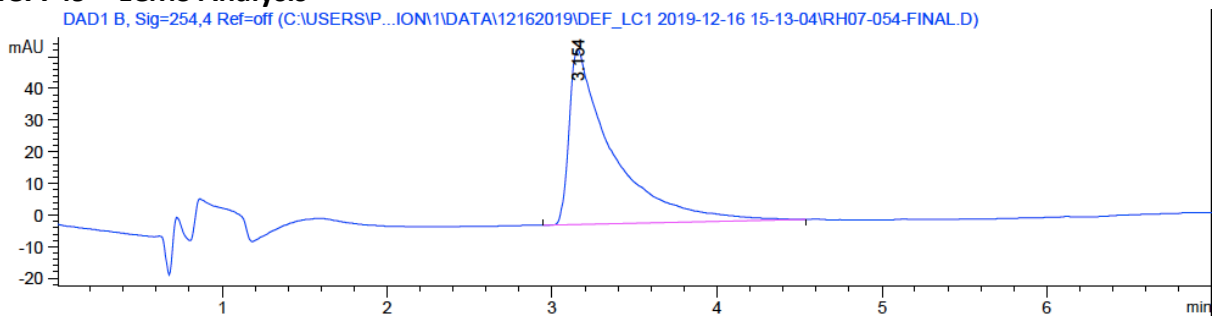
UNC7985 – LCMS Analysis



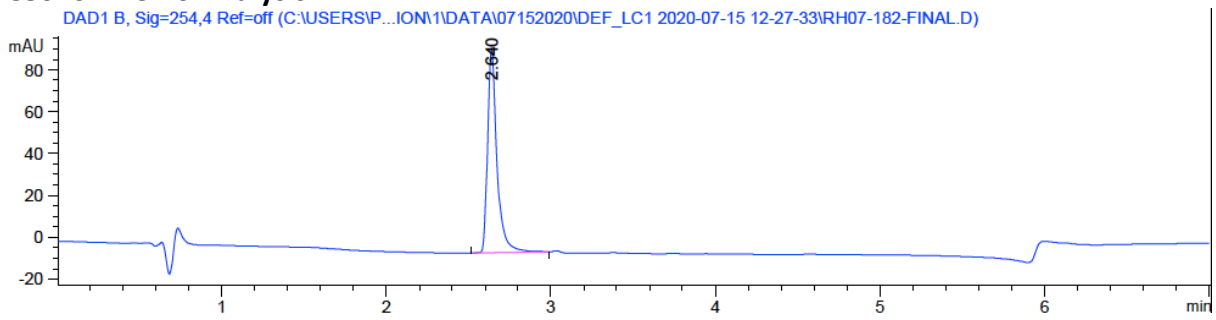
UNC7895 – LCMS Analysis



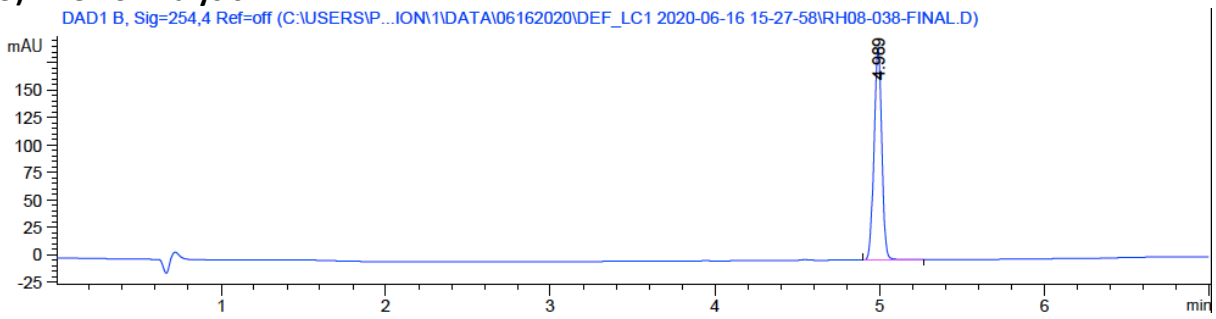
UNC7749 – LCMS Analysis



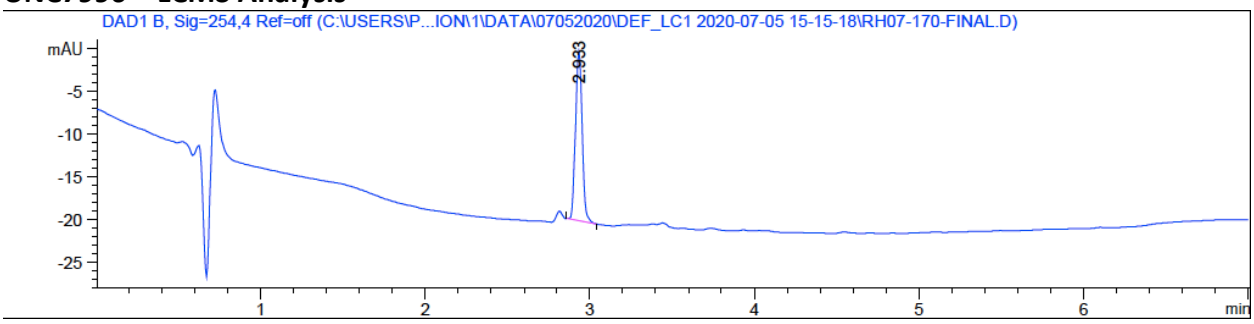
UNC8010 – LCMS Analysis



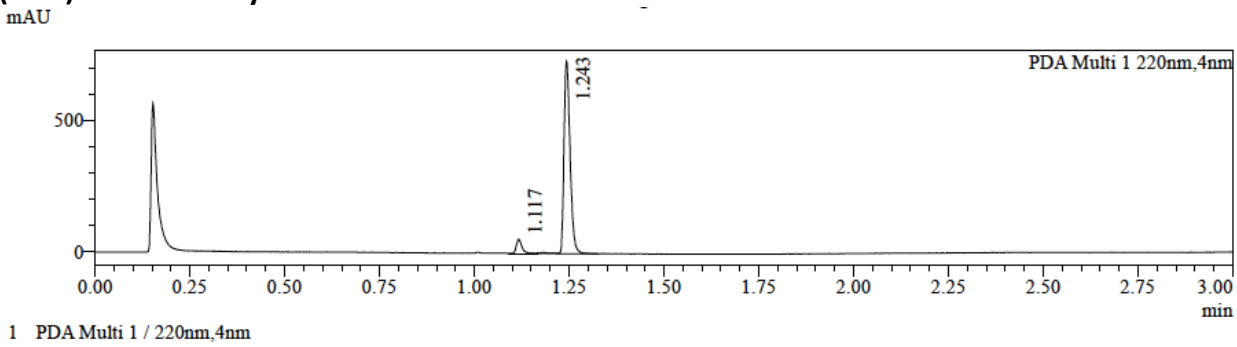
(S-9) – LCMS Analysis



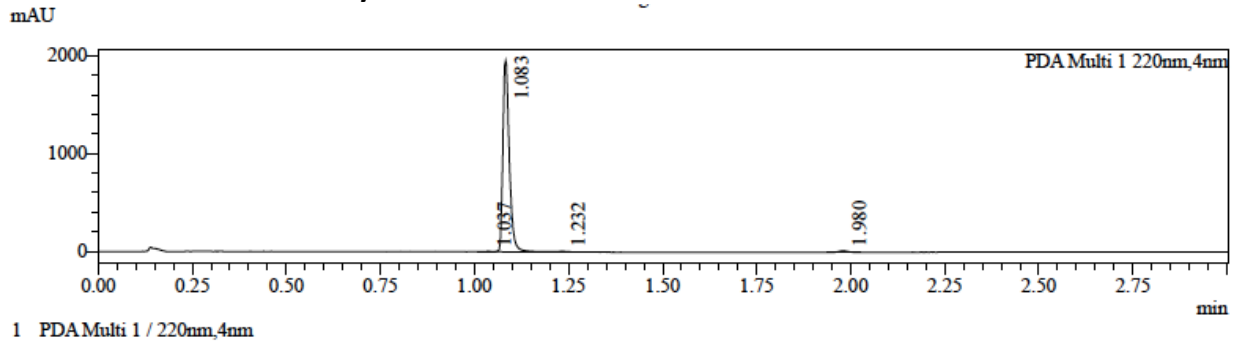
UNC7996 – LCMS Analysis



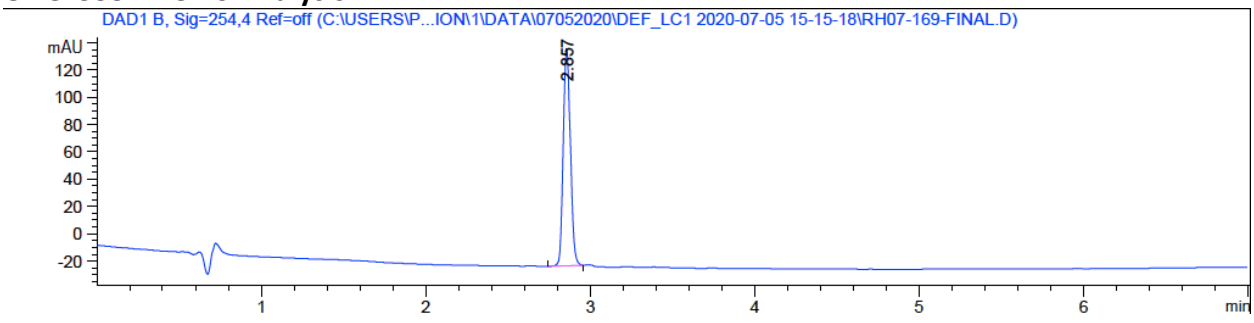
(S-17) – LCMS Analysis



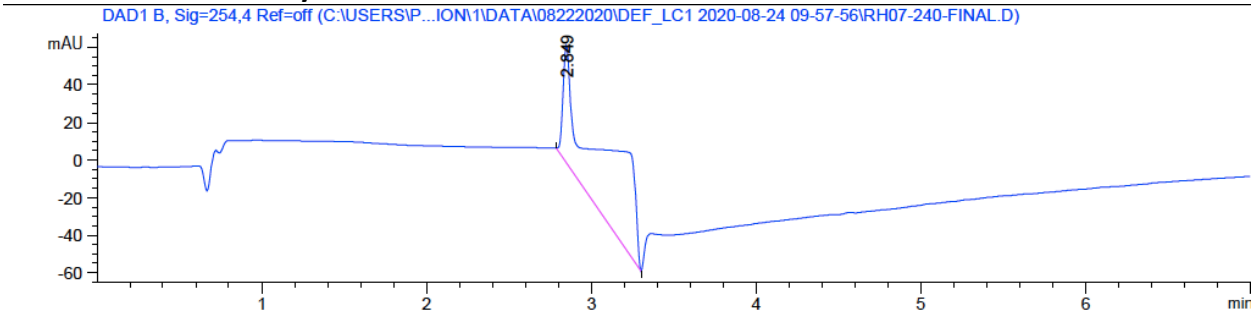
UNC10302685 – LCMS Analysis



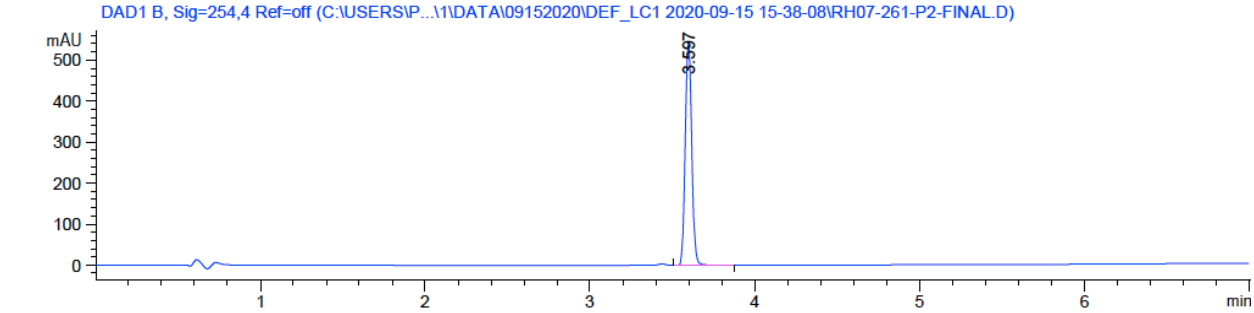
UNC7995 – LCMS Analysis



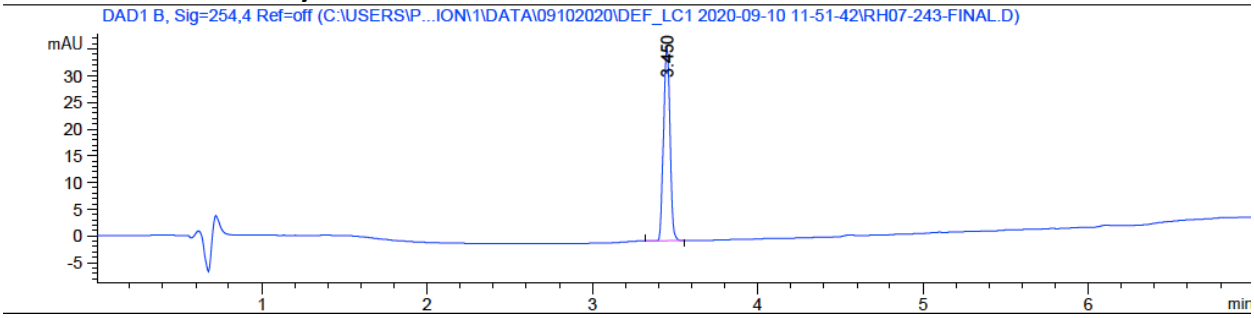
UNC8148 – LCMS Analysis



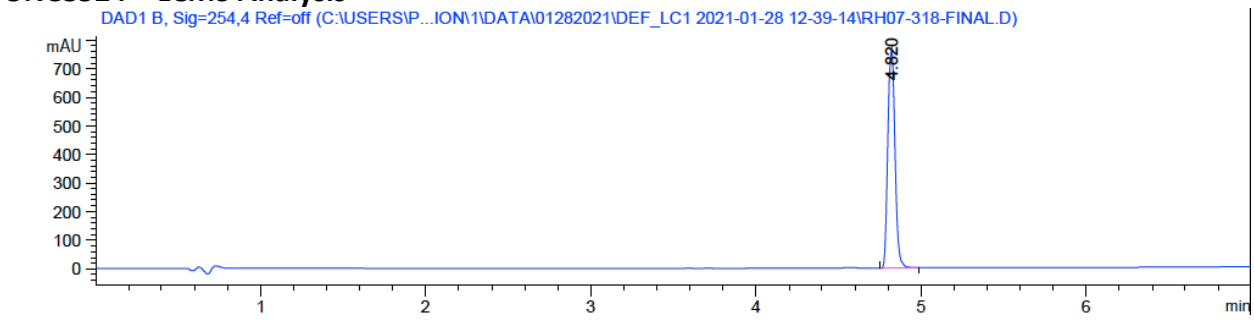
UNC8172 – LCMS Analysis



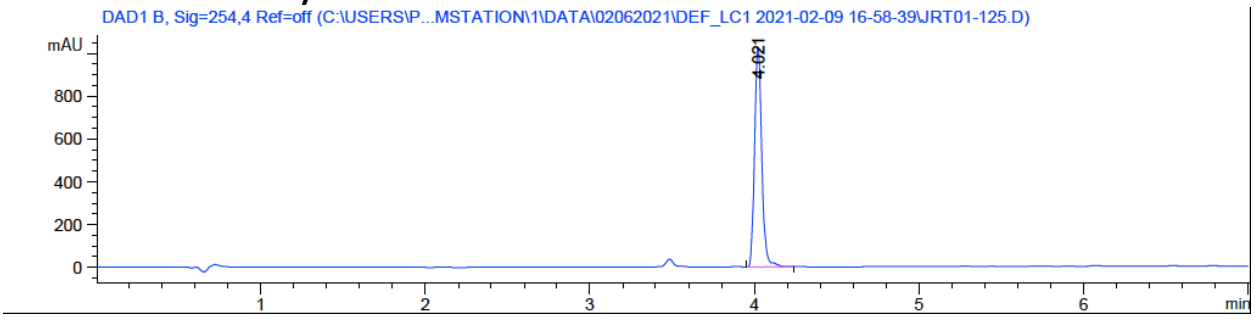
UNC8151 – LCMS Analysis



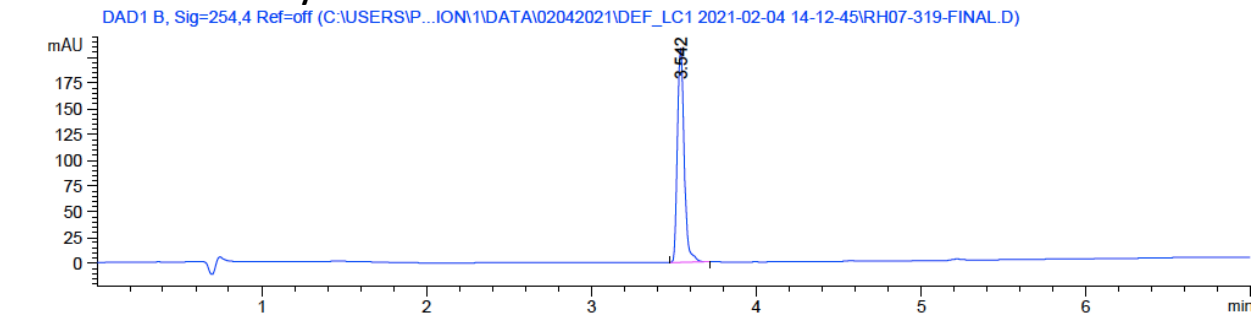
UNC8524 – LCMS Analysis



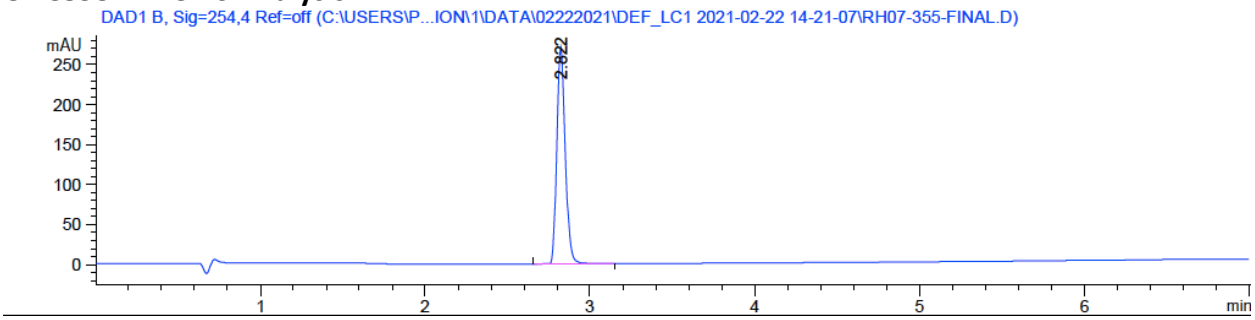
UNC8515 – LCMS Analysis



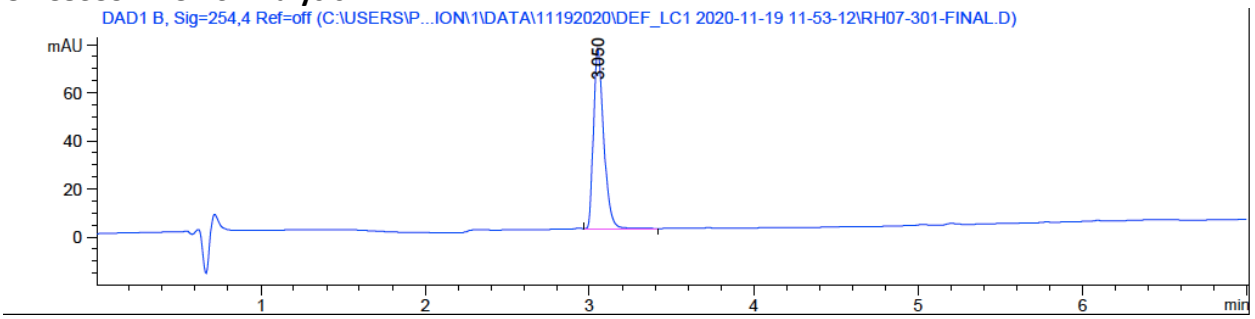
UNC8526 – LCMS Analysis



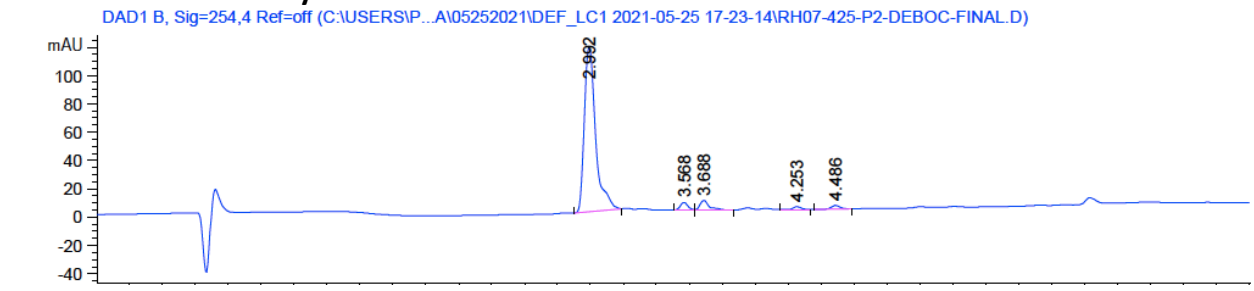
UNC8582 – LCMS Analysis



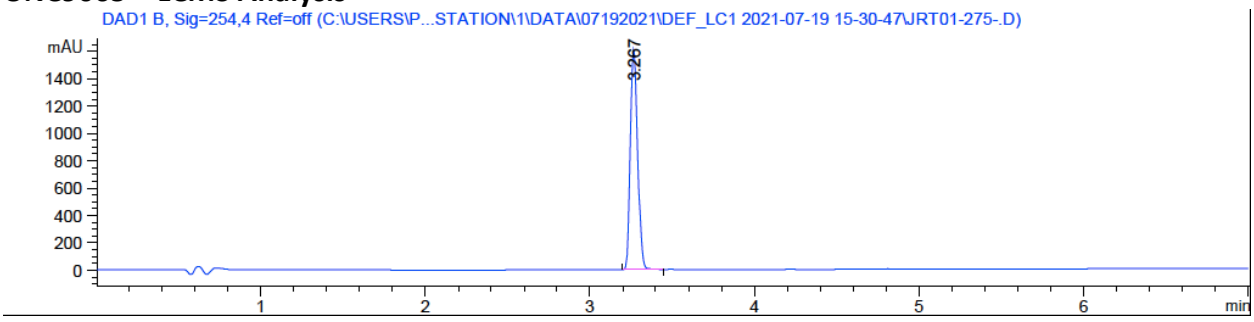
UNC8359 – LCMS Analysis



UNC8863 – LCMS Analysis



UNC9065 – LCMS Analysis



UNC8877 – LCMS Analysis