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Supplementary information

A chemical probe targeting the PWWP domain alters NSD2 nucleolar localization

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Supplementary Information

A chemical probe targeting the PWWP domain alters NSD2 nucleolar localization

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Notable compounds in this study are UNC6934 (**1**), MR837 (**2**), MRT866 (**3**), UNC7145 (**4**), UNC7096 (**5**).

Contents

Supplementary Table 1. Crystallographic data and refinement statistics

	NSD2-PWWP1+MRT866	NSD2-PWWP1+UNC6934
PDB Code	7MDN	6XCG
Data collection		
Space group	P1	P1
Cell dimensions		
a, *b*, *c* (Å)	68.3, 69.1, 80.2	49.6, 50.0, 51.2
α, β, γ (°)	75.5,79.4,60.5	91.4,91.9,118.3
Resolution (Å) (highest resolution shell)	48.81-2.42(2.51-2.42)	50.00-1.64(1.67-1.64)
Unique reflections	42507	49043
R_{merge}	5.4(60.2)	5.3(43.6)
$I/\sigma I$	8.9(1.5)	37.9(1.9)
Completeness(%)	90.4(91.9)	92.7(86.5)
Redundancy	2.1(2.1)	5.0(2.6)
CC(1/2)	0.998(0.701)	0.958(0.763)
Refinement		
Resolution (Å)	43.42-2.42	25.56-1.64
No. reflections (test set)	42500(2098)	47761(1168)
R_{work}/ R_{free} (%)	21.8/25.1	17.8/22.2
No. atoms		
Protein	7473	3180
Compound	184	99
Water	14	608
B-factors (Å^2)		
Protein	66.1	22.5
Compound	55.9	24.8
Water	41.7	31.1
RMSD		
Bond lengths (Å)	0.01	0.011
Bond angles (°)	1.06	1.434
Ramachandran plot % residues		
Favored	99.2	99.5
Additional allowed	0.8	0.5
Generously allowed	0	0.0
Disallowed	0	0.0

Supplementary Table 2. GPCR Selectivity Data

Compound	Receptor	Inhibition 1	Inhibition 2	Inhibition 3	Inhibition 4	Mean %	Ki (nM)
UNC6934	5-HT1A	23.89	1.72	-0.07	27.68	13.31	
UNC6934	5-HT1B	10.33	-2.42	-0.58	-3.75	0.9	
UNC6934	5-HT1D	6.2	15.98	10.6	12.16	11.24	
UNC6934	5-HT1E	2.09	9.97	-3.74	-11.49	-0.79	
UNC6934	5-HT2A	11.6	22.44	13.9	42.9	22.71	
UNC6934	5-HT2B	1.7	4.62	4.48	12.52	5.83	
UNC6934	5-HT2C	19.25	27.09	20.15	25.53	23.01	
UNC6934	5-HT3	8.3	-48.2	-23.33	1.49	-15.44	
UNC6934	5-HT5A	12.19	1.38	9.11	12.97	8.91	
UNC6934	5-HT6	8.82	15.47	-1.64	-15.59	1.77	
UNC6934	5-HT7A	-9.65	-16.68	1.5	-2.27	-6.78	
UNC6934	Alpha1A	20.43	18.61	-3.86	19.06	13.56	
UNC6934	Alpha1B	40.57	24.76	24.89	22.77	28.25	
UNC6934	Alpha1D	4.23	13.02	3.05	57.98	19.57	
UNC6934	Alpha2A	4.73	-24.1	-17.27	18.38	-4.57	
UNC6934	Alpha2B	9.74	2.91	11.02	29.31	13.25	
UNC6934	Alpha2C	19.32	4.02	4.71	30.21	14.57	
UNC6934	Beta1	-3.98	-3.07	-7.91	-20.94	-8.98	
UNC6934	Beta2	13.97	-5.56	82.85	6.37	24.41	
UNC6934	Beta3	30.07	24.21	-15.58	-2.12	9.15	
UNC6934	BZP Rat Brain Site	-4.03	-3.8	-17.77	-12.59	-9.55	
UNC6934	D1	6.69	3.77	-2.6	5.23	3.27	
UNC6934	D2	-4.48	-4.31	-10.72	-6.91	-6.61	
UNC6934	D3	11.52	11.17	20	23.53	16.56	
UNC6934	D4	-40.11	22.04	-4.7	10.84	-2.98	
UNC6934	D5	1.99	-18.91	-17.91	-11.61	-11.61	
UNC6934	DAT	58.31	37.73	29.93	50.18	44.04	
UNC6934	DOR	10.82	10.22	15.29	33.28	17.4	
UNC6934	GABAA	11.16	15.2	4.38	12.26	10.75	
UNC6934	H1	54.96	42.68	35.32	43.17	44.03	
UNC6934	H2	35.37	22.4	31.25	0	22.26	
UNC6934	H3	-32.92	-48.2	-14.64	-41.91	-34.42	>10,000
UNC6934	H4	5.69	0.91	2.24	-1.73	1.78	
UNC6934	KOR	-3.75	-8.91	11.91	0.42	-0.08	
UNC6934	M1	-10.22	-17.92	-17.51	9.88	-8.94	
UNC6934	M2	32.73	-6.4	-15.42	5.35	4.07	
UNC6934	M3	23.96	6.74	13.69	22.3	16.67	
UNC6934	M4	-32.86	-18.65	-29.66	-0.18	-20.34	
UNC6934	M5	15.73	-3.16	-25.62	-7.44	-5.12	
UNC6934	MOR	58.55	33.8	60.34	46.59	49.82	
UNC6934	NET	-19.48	-14.89	-34.9	-2.91	-18.05	

UNC6934	PBR	50.16	55.79	119.56	133.39	89.73	>10,000
UNC6934	SERT	59.39	55.69	62.25	62.37	59.93	1394
UNC6934	Sigma 1	12.15	30.62	23.33	40.83	26.73	
UNC6934	Sigma 2	10.74	-11.26	12.66	2.51	3.66	
UNC7145	5-HT1A	27.48	-5.07	4.92	15.9	10.81	
UNC7145	5-HT1B	12.5	10.83	-6.83	1.67	4.54	
UNC7145	5-HT1D	8.61	5.21	22.08	5.35	10.31	
UNC7145	5-HT1E	4.76	31.55	-6.03	1.84	8.03	
UNC7145	5-HT2A	10.51	-6.85	0.52	-29.4	-6.31	
UNC7145	5-HT2B	5.79	8.13	3.45	6.23	5.9	
UNC7145	5-HT2C	30.91	1.77	12.75	47.94	23.34	
UNC7145	5-HT3	-12.2	-17.82	-13.61	3.54	-10.02	
UNC7145	5-HT5A	-4.54	17.86	0.61	-0.42	3.38	
UNC7145	5-HT6	6.67	29.26	8.16	31.25	18.84	
UNC7145	5-HT7A	-14.02	3.04	-14.28	-5.79	-7.76	
UNC7145	Alpha1A	7.26	2.5	-3.18	-1.59	1.25	
UNC7145	Alpha1B	6.29	19.45	14.93	28.08	17.19	
UNC7145	Alpha1D	11.32	14.34	14.94	39.15	19.94	
UNC7145	Alpha2A	-14.24	-20.74	-0.26	15.24	-5	
UNC7145	Alpha2B	3.03	-3.23	8.47	31.28	9.89	
UNC7145	Alpha2C	28.82	8.6	-14.3	25.71	12.21	
UNC7145	Beta1	6.02	14.8	-52.14	-3.07	-8.6	
UNC7145	Beta2	1.49	40.27	70.37	8.27	30.1	
UNC7145	Beta3	5.37	-5.28	5.95	16.37	5.6	
UNC7145	BZP Rat Brain Site	5.44	-25.88	-7.41	-3.35	-7.8	
UNC7145	D1	-2.7	-1.76	-6.35	0.22	-2.65	
UNC7145	D2	-9.68	-12.97	-0.67	-9.68	-8.25	
UNC7145	D3	27.42	-50.29	36.25	34.83	12.05	
UNC7145	D4	1.99	8.49	-5.24	18.07	5.83	
UNC7145	D5	14.26	-9.29	3.65	-12.6	-1	
UNC7145	DAT	1.22	-15.04	4.54	6.87	-0.6	
UNC7145	DOR	19.03	14.81	54.54	42.46	32.71	
UNC7145	GABAA	3.46	10.06	13	10.8	9.33	
UNC7145	H1	58.15	74.1	54.96	72.14	64.84	>10,000
UNC7145	H2	15.1	-3.62	2.94	5.85	5.07	
UNC7145	H3	-5.06	-8.35	-33.22	0	-11.66	
UNC7145	H4	8.13	1.73	9.35	4.88	6.02	
UNC7145	KOR	-0.58	-9.08	3.25	-4.08	-2.62	
UNC7145	M1	-10.22	-9.47	7.29	-8.25	-5.16	
UNC7145	M2	3.1	-64.69	-15.74	0.85	-19.12	
UNC7145	M3	1.01	4.19	-4.98	9.48	2.43	
UNC7145	M4	45.8	9.36	-24.04	-34.16	-0.76	
UNC7145	M5	56.73	14.3	-2.81	-13.5	13.68	
UNC7145	MOR	67.07	60.61	74.77	61.99	66.11	7950

UNC7145	NET	-48.79	0	-30.44	4.22	-18.75	
UNC7145	PBR	67.57	91.65	117.51	134.92	102.91	>10,000
UNC7145	SERT	61.06	20.02	41.02	37.08	39.8	
UNC7145	Sigma 1	1.94	-1.94	4.37	39.85	11.06	
UNC7145	Sigma 2	12.51	-10.41	-24.56	9.2	-3.32	

Supplementary Table 3. Primer Sequences for pre-rRNA Expression Analysis

Target	Forward	Reverse	Reference
B2M	GAGTGCTGTCTCCATGTTTGATGT	AAGTTGCCAGCCCTCCTAGAG	57
pre-rRNA	GCCTTCTCTAGCGATCTGAGAG	CCATAACGGAGGCAGAGACA	58

Supplementary Table 4. Primer Sequences for ChIP & Gene Expression Analysis

Target	Forward	Reverse	Application
TBP	GGGCATTATTTGTGCACTGAGA	TAGCAGCACGGTATGAGCAACT	qPCR
BACE2	ACCATCCCCAAAGGCTTCAATA	GTCTCCAGAGAACTTGATGGCT	qPCR
CA2	TCACTTTCACTGGGGTTCACTT	CTGCACAGCTTTCCCAAAATCC	qPCR
CDC42	TGCAGTCACAGTTATGATTGGT	GTGGATAACTCAGCGGTCGTAA	qPCR
c-MYC	TTCTCTCCGTCCTCGGATTCTC	CCTGCCTCTTTTCCACAGAAAC	qPCR
TGFA	CCCTGGCTGTCCTTATCATCAC	GGCACCACTCACAGTGTTTTC	qPCR
DSG2	AGGGAGAGGATCTGTCCAAGAA	TGGCTCTGTAATCCCTTTTCCA	qPCR
ADAM9	ACTAGAGAAAGAAGAGAAGCCCC	ATATGCTCTTTTCCTTCAGCCT	qPCR
NEO1	ATTATCAGTGTGTGGCCACTGT	CCCAGCATAAACTGAGGAAGGT	qPCR
WASF3	AAAGTCACCCAGCTGGATTCAA	CTGGTCTTGGACTGTGGAACTT	qPCR
SERPINE2	CTCGCCATGGTGATGAGATACG	ACGATGGCCTTGTTGATCTTCT	qPCR
CDH2	GGGTCATCCCTCCAATCAACTT	ACCCGAGATGGGGTTGATAATG	qPCR
ITGB1	TACTCAGATCCAACCACAGCAG	AGGTCAATGGGATAGTCTTCAGC	qPCR
GPC1	CTGCTCGCTCCTTTGTGCAG	GAGCACAGTAGACCAGCTTCAT	qPCR
SNTB1	AAGGAGCATAGTACAGGGTTGC	CACTCCTGGTTTTTGTAGGTGC	qPCR
PFN2	ACAATGGACATCCGGACAAAGA	GACACCTTCCTTTCCCATGACT	qPCR
ID2	ACACGGATATCAGCATCCTGTC	CACACAGTGCTTTGCTGTCATT	qPCR
TWIST1	GCCGGAGACCTAGATGTCATTG	CGCCCTGTTTCTTTGAATTTGG	qPCR
ZYX	CCTCCCAGCTTCACCTATGC	ACCTCCTTCAGAGTCAGGGG	qPCR
CEACAM21	GTGTGAGGTCTCCAACCCAG	TTCGGAGGAGCAGGAAACAC	qPCR
RHOB	CCGAGGTGAAGCACTTCTGT	AGAGCACTCGAGGTAGTCGT	qPCR
CLDN3	GGGACTTCTACAACCCCGTG	TGGTGGCCGTGTACTTCTTC	qPCR
HOXB9	GTGTGAAGAACAAAGACAAGAG	CAGAGAGGGAGAAGAGAGAA	ChIP
HOXC4/8	CACCTCAGTCAATACCTCATATC	TCAGCGCTCATAAATGTCTC	ChIP
PLEKHA6	CTGTGGGCATAAGGGTTAAA	GAAGGGAGAACTGCTCAATC	ChIP

ATF7	CTGTCCTCTTGTCCTTGTTT	GACTTCGCAGATCAGTAAGAG	ChIP
HMCN1	CATGCCTGGAGAGATTCTATTT	TCTCCGGTGTCTGCTTTA	ChIP
SKAP1	CACCTCAGTCAATACCTCATATC	TCAGCGCTCATAAATGTCTC	ChIP
FOS	CCTCACCCTTTCGGAGTC	CCTCCTGCCAATGCTCT	ChIP
BACE2	CTAGCACATACCGCTCCAA	GATGGTGACGAGGTCTTCC	ChIP
CA2	AGCACTGGCATAAGGACTTC	CTTCAGGGAAGGGTCATACTTG	ChIP
CDC42	CCTCCAACCTGATGACCTATTC	GGAAGTCAGCTAAGAAACTAGGG	ChIP
c-MYC	GGTGCAGCCGTATTTCTACT	AGCAGCTCGAATTTCTTCCA	ChIP
TGFA	GTGATGGCCTGCTTCTTCT	TGCCATTCTGGGTACGTTG	ChIP
DSG2	GTATCTCTGGAGCCTGCTTATC	CTCTCTGTCCAAGGTAACACTG	ChIP
ADAM9	CTTTCAACAGACCTCACATCTTTC	GGGCTTCTCTTCTTTCTCTAGTT	ChIP
NEO1	GGAGTTCAGGCTTCCATTCT	GGGAGTCTGTAATCTTCTGGTG	ChIP
WASF3	GGGTCTTCTCTTTCGTCCTC	GTCGCTTCGAGCATCACT	ChIP
SERPINE2	CGAGAGACAGAGGATTGAAGTG	CTTGGTGGAAGGAACCATGA	ChIP
CDH2	TTTCCTGGGTCTCTTTGTCTTG	GGATGAAGATGGCATGGTGTAT	ChIP
ITGB1	GCTGTGGTTGGATCTGAGTAA	CCCAGAGGCTCCAAAGATATAAA	ChIP
GPC1	TGCCTGATGACTACCTGGA	GCACAAAGGAGCGAGCA	ChIP
SNTB1	TCTCTGTGGAAGGAGAAGGA	CGAGATTGGGTGGGAAACA	ChIP
PDN2	GCAGTCACCATCGACGTATAG	CCGGGAAGGTTTCTTTACCA	ChIP
GAS1	GTCATCGTAGTCCTCATCGTAG	GAGTCGGTCAAGGAGAACAT	ChIP
ID2	CGATGAGCCTGCTATACAACAT	TCCATCTTGCTCACCTTCTTG	ChIP
TWIST1	AGCTTGCCATCTTGGAGTC	AGCTGAGCAAGATTCAGACC	ChIP
rDNA -1kb	CCGTGGGTTGTCTTCTGAC	AAGCGAAACCGTGAGTCG	ChIP
rDNA 5ETS	CCTCCAGTGGTTGTCGACTT	GAACGACACACCACCGTTC	ChIP
rDNA ITS1	CCCGTGGTGTGAAACCTTC	AAGAGGAGAGGGGGTTGC	ChIP
rDNA IGS	GTGTGCCTCCGTCTTCTCTC	GTCAAGGGGCTATGCCATC	ChIP

Synthetic Procedures:

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 (<10 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. 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. 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. Analytical LCMS (at 254 nm) was used to establish the purity of targeted compounds. HRMS collected using an Agilent 6545 QTOF instrument. 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)

^{1}H 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 (^{1}H) and 77.1 ppm (^{13}C), DMSO-d_6 referenced at 2.50 (^{1}H) and 39.5 ppm (^{13}C), acetone-d_6 referenced at 2.05 (^{1}H) and 29.8 ppm (^{13}C), and MeOD-d_4 referenced at 3.31 (^{1}H) and 49.0 ppm (^{13}C). All compounds that were evaluated in biochemical and biophysical assays had >95% purity as determined by ^{1}H NMR or LCMS (spectra provided in Supplementary Note).

High-Resolution Mass Spectrometry

Samples were prepared by dilution with 600 µL of acetonitrile. Samples were further diluted using 100 µL of the compound solution in 900 µL of acetonitrile, regardless of the original dilution solvent. 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 0.6 mL/min. Electrospray source conditions were set as: spray voltage 3.0 kV, sheath gas (nitrogen) 60 arb, auxillary 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.

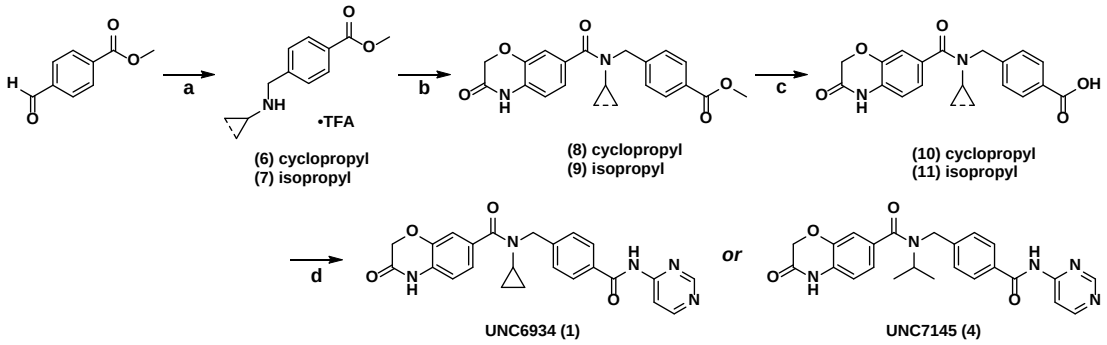
Separations were conducted on a Waters Acquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm particle size). LC conditions were set at 95% water with 0.1% formic acid (A) ramped linearly over 5 mins to 100% acetonitrile with 0.1% formic acid (B) and held until 6 mins. At 7 mins the gradient was switched back to 95% A and allowed to re-equilibrate until 9 mins. The 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

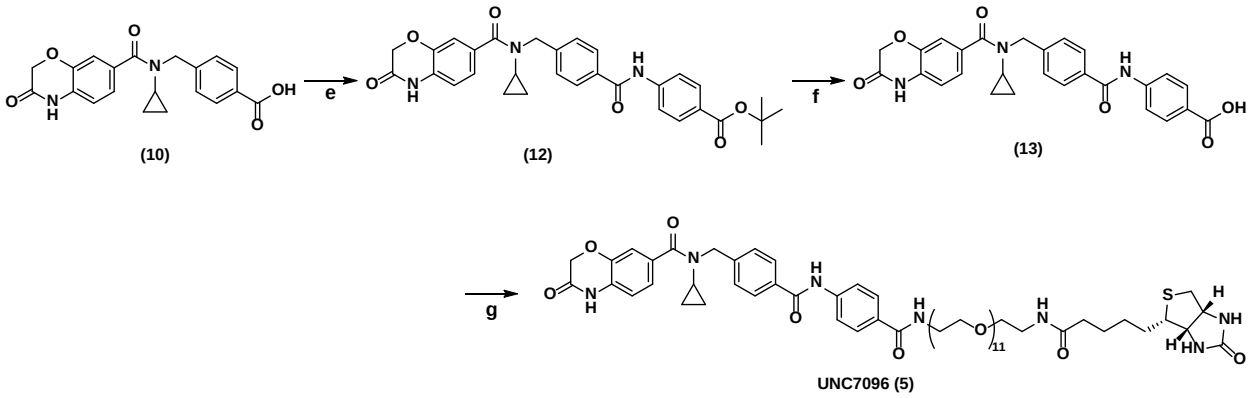
DCM	Dichloromethane
DIPEA	*N*,*N*-Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
EDC	*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride
HOAt	1-Hydroxy-7-azabenzotriazole
LCMS	Liquid Chromatography-Mass Spectrometry
NMR	Nuclear Magnetic Resonance

TCFH	Chloro-*N*,*N*,*N*′,*N*′-tetramethylformamidinium hexafluorophosphate
TFA	2,2,2-Trifluoroacetic acid
THF	Tetrahydrofuran

Synthetic Schemes

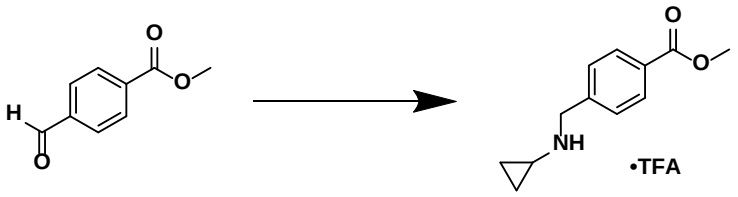


Scheme 1: Synthesis of probe compounds. Reagents and conditions: a) i. (cyclo/iso)propylamine, MeOH ii. $NaBH_4$; b) 3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7- carboxylic acid, EDC, HOAt, triethylamine, MeCN; c) LiOH•H_2O; d) 4-aminopyrimidine, 1-methylimidazole, TCFH[1].



Scheme 2: **Synthesis of biotinylated tool compound UNC7096.** Reagents and conditions: e) *tert*-butyl 4-aminobenzoate, EDC, DMAP, DMF; f) TFA, DCM; g) Biotin-PEG11-amine, EDC, HOAt, DIPEA, DMF.

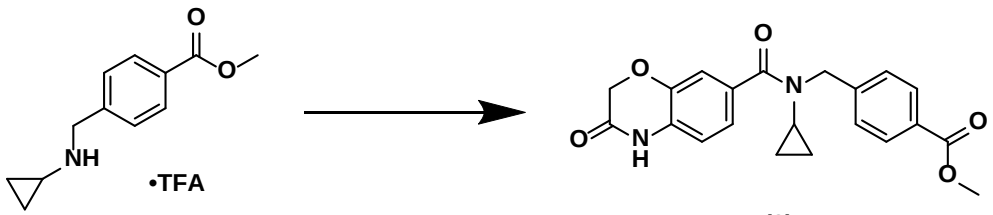
methyl 4-((cyclopropylamino)methyl)benzoate trifluoroacetate salt (6)



(6)

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 (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 filtered and washed rigorously with diethyl ether to provide **6** (1.586 g, 4.976 mmol, 82%) as a fluffy white powder. ^{1}H NMR (400 MHz, Methanol-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), 2.8–2.75 (m, 1H), 0.96–0.85 (m, 4H); ^{13}C NMR (101 MHz, Methanol-d_4) δ 167.74, 137.52, 132.47, 131.24, 131.19, 52.84, 52.36, 31.25, 4.27.

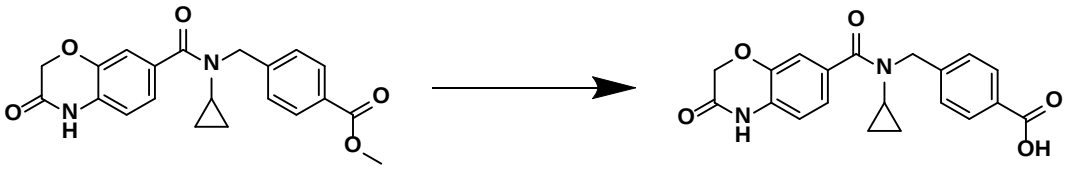
methyl 4-((N-cyclopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamido)methyl)benzoate (8)



(6) **(8)**

To a flask was added 3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxylic acid (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 **6** (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 (0-100% ethyl acetate in DCM) provided **8** (292.7 mg, 769.5 µmol, 74.3%) as a white solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.43 (s, 1H), 8.02 (d, *J*=8.3 Hz, 2H), 7.39 (d, *J*=7.9 Hz, 2H), 7.19–7.12 (m, 2H), 6.85 (d, *J*=7.8 Hz, 1H), 4.77 (s, 2H), 4.62 (s, 2H), 3.91 (s, 3H), 2.62 (tt, *J*=6.9, 4.0 Hz, 1H), 0.67–0.55 (m, 2H), 0.55–0.45 (m, 2H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 167.00, 166.05, 143.15, 132.88, 130.15, 129.83, 129.45, 127.96, 127.69, 122.40, 116.35, 115.76, 67.27, 52.28, 27.02, 10.15. 1 aromatic and 1 aliphatic C not observed.

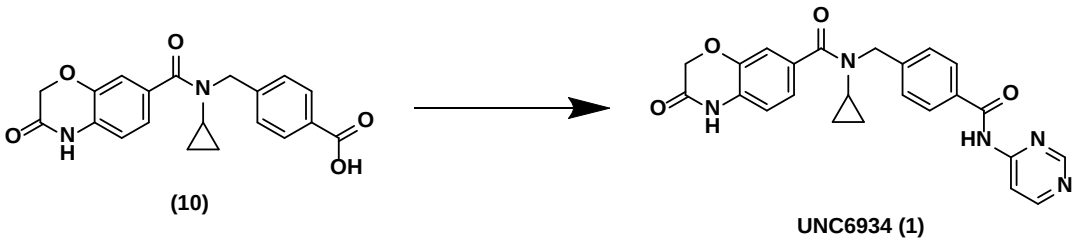
4-((N-cyclopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamido)methyl)benzoic acid (10)



(8) **(10)**

To a round-bottom flask was added **8** (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 $KHSO_4$, and the precipitate produced was filtered off, washed with cold water, and dried to provide **10** (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).

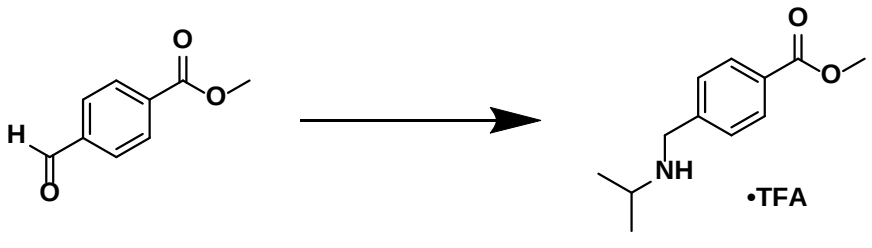
N-cyclopropyl-3-oxo-N-(4-(pyrimidin-4-ylcarbamoyl)benzyl)-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamide (UNC6934 (1))



(10) UNC6934 (1)

To a 2-dram vial with a stirbar was added pyrimidin-4-amine (50.6 mg, 1.3 Eq, 532 µmol), **10** (150 mg, 1 Eq, 409 µmol), 1-methylimidazole (118 mg, 114 µL, 3.5 Eq, 1.43 mmol), THF (1.5 mL), and finally TCFH (149 mg, 1.3 Eq, 532 µmol). The reaction was left to stir at room temperature for 24 hours, then quenched with 25 mL of distilled water and extracted 7 times with 25 mL portions of ethyl acetate. The combined organic layers were extracted once with 15 mL of saturated sodium bicarbonate (for recovery of the starting acid), then washed twice with water, once with saturated sodium bicarbonate, and once with brine, and finally dried over anhydrous sodium sulfate and concentrated to a yellow oil. The oil was dry-loaded on Celite and purified by normal phase chromatography over silica (0-10% methanol in DCM) to yield UNC6934 **(1)** (106 mg, 239 µmol, 58.4%) as an off-white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.21 (s, 1H), 10.87 (s, 1H), 8.95 (d, *J*=1.4 Hz, 1H), 8.72 (d, *J*=5.8 Hz, 1H), 8.21 (dd, *J*=5.8, 1.3 Hz, 1H), 8.03 (d, *J*=8.3 Hz, 2H), 7.45 (d, *J*=8.0 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), 2.88–2.77 (m, 1H), 0.61–0.50 (m, 2H), 0.50–0.41 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 166.90, 164.85, 158.30, 158.26, 158.17, 143.21, 142.48, 132.03, 131.73, 128.63, 128.40, 127.16, 121.90, 115.39, 115.14, 110.65, 66.73, 9.54. 1 aromatic C and 2 aliphatic C not observed; HRMS (*m/z):* $[M+H]^+$ calculated for $C_{24}H_{22}N_5O_4$, 444.16663; found, 444.16638.

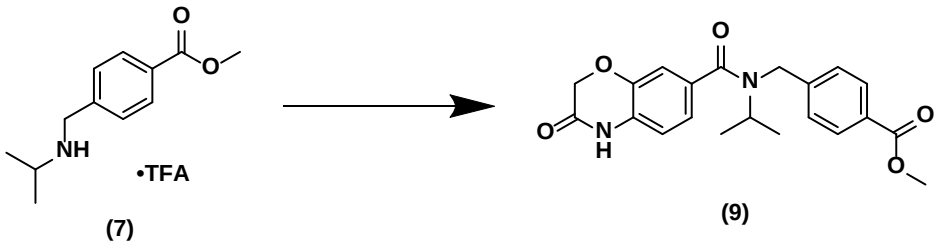
methyl 4-((isopropylamino)methyl)benzoate trifluoroacetate salt (7)



(7)

To a 50 mL flask equipped with a stir bar was added methyl 4-formylbenzoate (0.50 g, 1 Eq, 3.0 mmol) and methanol (5 mL), followed by isopropylamine (0.18 g, 0.26 mL, 1 Eq, 3.0 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 (0.23 g, 2 Eq, 6.1 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 (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 (0.52 g, 0.35 mL, 1.5 Eq, 4.6 mmol) was added dropwise. A voluminous white solid formed, which was filtered and washed rigorously with diethyl ether to provide **7** (773.4 mg, 2.407 mmol, 79%) as a fluffy white powder. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.10 (d, J=8.4 Hz, 2H), 7.62 (d, J=8.6 Hz, 2H), 4.29 (s, 2H), 3.92 (s, 3H), 3.53–3.42 (m, 1H), 1.41 (d, J=6.6 Hz, 6H); ^{13}C NMR (100 MHz, Methanol-d_4) δ 167.75, 137.93, 132.38, 131.23, 131.01, 52.83, 52.23, 49.15, 19.21.

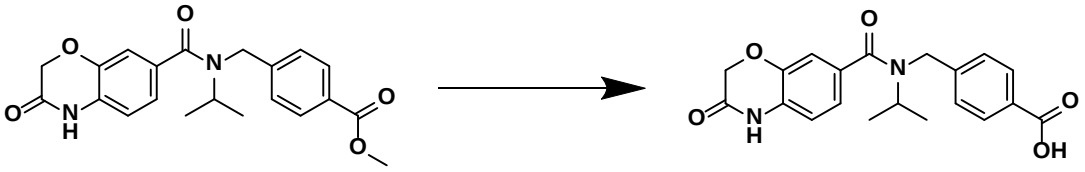
methyl 4-((N-isopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamido)methyl)benzoate (9)



To a scintillation vial was added 3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-7-carboxylic acid (50 mg, 1 Eq, 0.26 mmol), EDC (87 mg, 1.75 Eq, 0.45 mmol), HOAt (70 mg, 2 Eq, 0.52 mmol), and acetonitrile (2.0 mL). The reaction was left to stir at room temperature for 30 minutes. To the vial was then added **7** (0.10 g, 1.2 Eq, 0.31 mmol), followed by triethylamine (0.10 g, 0.14 mL, 4 Eq, 1.0 mmol), and the reaction was left to stir overnight. The next day, the volatiles were removed under reduced pressure and the residue 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 once with 0.5 M citric acid, once with water, once with saturated sodium bicarbonate, and once with brine, then dried over sodium sulfate and concentrated to an off-white residue. Normal phase chromatography over silica gel (0-100% ethyl acetate in DCM) provided **9** (90.1 mg, 236 µmol, 91%) as a white solid. ^{1}H NMR (400 MHz, Chloroform-

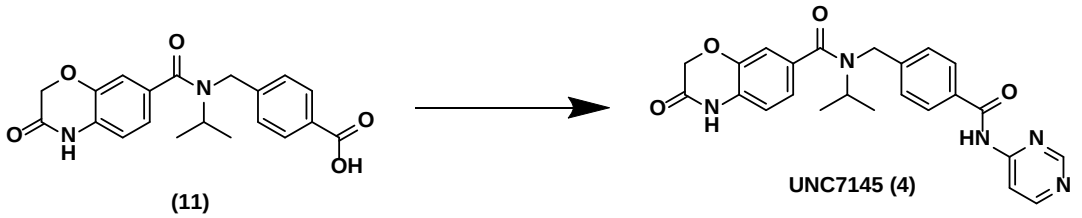
d) δ 9.58 (s, 1H), 8.01–7.94 (m, 2H), 7.36 (br s, 2H), 7.01 (d, *J*=15.7 Hz, 2H), 6.84 (br s, 1H), 4.65 (s, 2H), 4.61 (s, 2H), 4.21 (s, 1H), 3.89 (s, 3H), 1.13 (d, *J*=6.6 Hz, 6H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ 171.38, 167.02, 165.94, 144.52, 143.57, 132.68, 129.94, 128.97, 127.48, 126.93, 121.00, 116.31, 115.19, 67.21, 52.21, 21.43, 14.29. 1 aliphatic C not observed.

4-((N-isopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamido)methyl)benzoic acid (11)



To a round-bottom flask was added **9** (405 mg, 1 Eq, 1.06 mmol), 1,4-dioxane (6 mL), and lithium hydroxide hydrate (15% in water, 1.5 mL, 5 Eq, 5.30 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 $KHSO_4$, and the precipitate produced was filtered off, washed with cold water, and dried to provide **11** (319.8 mg, 868.1 µmol, 82.0%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.85 (s, 1H), 7.89 (d, *J*=8.2 Hz, 2H), 7.41 (d, *J*=7.7 Hz, 2H), 7.03 (s, 2H), 6.99–6.89 (m, 1H), 4.61 (s, 4H), 4.08 (s, 1H), 1.08 (d, *J*=6.6 Hz, 6H).

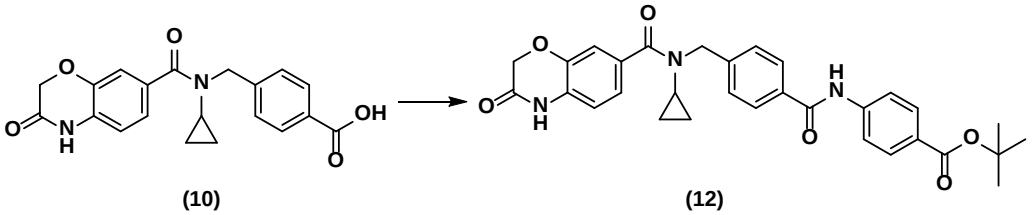
N-isopropyl-3-oxo-N-(4-(pyrimidin-4-ylcarbamoyl)benzyl)-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamide (UNC7145(4))



To a 2-dram vial with a stirbar was added pyrimidin-4-amine (33.6 mg, 1.3 Eq, 353 µmol), **11** (100 mg, 1 Eq, 271 µmol), 1-methylimidazole (78.0 mg, 75.7 µL, 3.5 Eq, 950 µmol), acetonitrile (1.0 mL), and finally TCFH (99.0 mg, 1.3 Eq, 353 µmol). The reaction was heated to 40 °C for 16 hours, then quenched with 25 mL of distilled water and extracted 6 times with 25 mL portions of ethyl acetate. The combined organic layers were extracted once with 15 mL of saturated sodium bicarbonate (for recovery of the starting

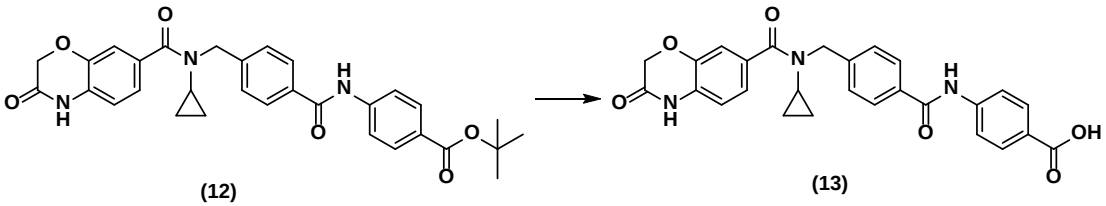
acid), then washed twice with water, once with saturated sodium bicarbonate, and once with brine, and finally dried over anhydrous sodium sulfate and concentrated to a yellow oil. The oil was dry-loaded on Celite and purified by normal phase chromatography over silica (0-10% methanol in DCM) to yield UNC7145 **(4)** (52 mg, 0.12 mmol, 43%) as an off-white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.18 (s, 1H), 10.86 (s, 1H), 8.95 (d, *J*=1.3 Hz, 1H), 8.71 (d, *J*=5.8 Hz, 1H), 8.21 (dd, *J*=5.8, 1.3 Hz, 1H), 7.98 (d, *J*=8.4 Hz, 2H), 7.51–7.39 (m, 2H), 7.10–6.99 (m, 2H), 6.96 (s, 1H), 4.69–4.54 (m, 4H), 4.16–4.03 (m, 1H), 1.10 (d, *J* = 6.6 Hz, 6H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.23, 166.97, 164.78, 158.29, 158.27, 158.21, 144.61, 143.00, 131.76, 131.58, 128.36, 128.16, 126.58, 120.54, 115.76, 114.27, 110.67, 66.76, 50.47, 42.82, 20.74; HRMS (*m/z):* $[M+H]^+$ calculated for $C_{24}H_{24}N_5O_4$, 446.18228; found, 446.18216.

tert-butyl 4-(4-((N-cyclopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamido)methyl)benzamido)benzoate (12)



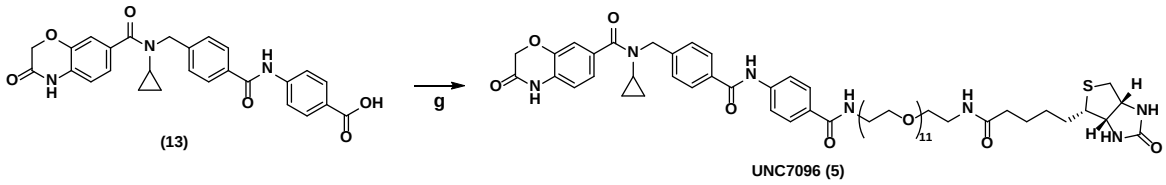
To a scintillation vial was added **10** (18 mg, 1 Eq, 50 µmol), *tert*-butyl 4-aminobenzoate (19 mg, 2 Eq, 0.10 mmol), EDC (19 mg, 2 Eq, 0.10 mmol), DMAP (12 mg, 2 Eq, 0.10 mmol), and DMF (0.2 mL). The reaction was heated to 50 ˚C and stirred overnight. The next day, the reaction was partitioned between 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 (0-100% ethyl acetate in DCM) provided **12** (20 mg, 37 µmol, 74%) as a white solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.25 (s, 1H), 8.83 (s, 1H), 7.94 (d, *J*=8.7 Hz, 2H), 7.84 (d, *J*=8.3 Hz, 2H), 7.77 (d, *J*=8.8 Hz, 2H), 7.31 (d, *J*=7.9 Hz, 2H), 7.09 (s, 1H), 7.07 (d, *J*=8.1 Hz, 1H), 6.79 (d, *J*=8.0 Hz, 1H), 4.75 (s, 2H), 4.59 (s, 2H), 2.63 (tt, *J*=7.0, 4.0 Hz, 1H), 1.59 (s, 9H), 0.66–0.56 (m, 2H), 0.56–0.44 (m, 2H); ^{13}C NMR (100 MHz, Chloroform-*d*) δ 165.95, 165.68, 165.60, 143.15, 142.29, 141.99, 134.02, 132.57, 130.65, 127.95, 127.84, 127.64, 122.21, 119.43, 116.13, 115.81, 81.09, 67.29, 28.37. 2 aromatic C and 3 aliphatic C not observed.

4-(4-((N-cyclopropyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamido)methyl)benzamido)benzoic acid (13)



To a vial containing **12** (155 mg, 1 Eq, 286 µmol) was added DCM (1.3 mL) and TFA (489 mg, 331 µL, 15 Eq, 4.29 mmol), which were stirred at room temperature overnight. The next day, the volatiles were removed *in vacuo* to provide **13** (144 mg, 297 µmol, quantitative) as a tan solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.87 (s, 1H), 10.52 (s, 1H), 7.98–7.89 (m, 6H), 7.47 (d, *J*=8.1 Hz, 2H), 7.18 (d, *J*=8.3 Hz, 1H), 7.15 (d, *J*=1.8 Hz, 1H), 6.93 (d, *J*=8.1 Hz, 1H), 4.72 (s, 2H), 4.62 (s, 2H), 2.84–2.75 (m, 1H), 0.59–0.51 (m, 2H), 0.51–0.43 (m, 2H).

N-cyclopropyl-3-oxo-N-(4-((4-((37-oxo-41-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-3,6,9,12,15,18,21,24,27,30,33-undecaoxa-36-azahentetracontyl)carbamoyl)phenyl)carbamoyl)benzyl)-3,4-dihydro-2H-benzo[b][1,4]oxazine-7-carboxamide (UNC7096 (5))



To a scintillation vial was added **13** (3.1 mg, 1 Eq, 6.5 µmol), EDC (2.5 mg, 2 Eq, 13 µmol), HOAt (1.8 mg, 2 Eq, 13 µmol), and DMF (0.2 mL). The reaction was left to stir at room temperature for 30 minutes. To the vial was then added Biotin-PEG11-amine (5.0 mg, 1 Eq, 6.5 µmol) dissolved in DMF (0.2 mL), followed by DIPEA (2.5 mg, 3.4 µL, 3 Eq, 19 µmol), and the reaction was left to stir overnight. The next day, the reaction was diluted with distilled water and purified directly by reverse phase chromatography (10-100% methanol in water + 0.1% TFA) and lyophilized to provide UNC7096 (**5**) (6.51 mg, 5.26 µmol, 81%) as a white hygroscopic solid. ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.97 (d, *J*=8.3 Hz, 2H), 7.90–7.83 (m, 4H), 7.51 (d, *J*=7.9 Hz, 2H), 7.20 (d, *J*=8.6 Hz, 1H), 7.18–7.16 (m, 1H), 6.97 (d, *J*=8.1 Hz, 1H), 4.83 (s, 2H), 4.63 (s, 2H), 4.48 (ddd, *J*=7.9, 5.0, 0.9 Hz, 1H), 4.29 (dd, *J*=7.9, 4.5 Hz, 1H), 3.71–3.55 (m, 40H), 3.53 (t, *J*=5.4 Hz, 2H), 3.37–3.33 (m, 2H), 3.19 (dt, *J*=9.8, 5.3 Hz, 1H), 2.92 (dd, *J*=12.8, 5.0 Hz, 1H), 2.86–2.80 (m, 1H), 2.70 (d, *J*=12.8 Hz, 1H), 2.21 (t, *J*=7.4 Hz, 2H), 1.78–1.53 (m, 5H), 1.43 (p, *J*=7.6 Hz, 2H),

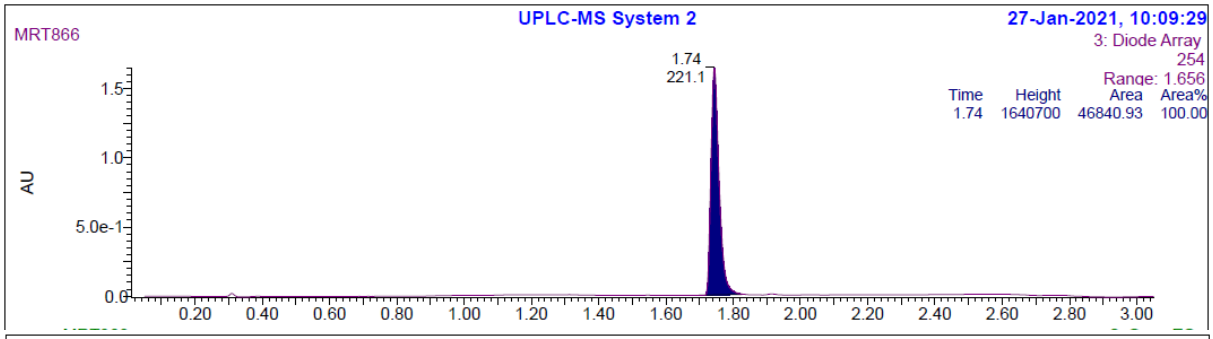
0.66 (d, *J*=6.5 Hz, 2H), 0.57 (s, 2H). PEG group underintegrates slightly; LRMS (*m/z):* $[M+H]^+$ calculated for $C_{61}H_{88}N_7O_{18}S$, 1238.59; found, 1238.40**;** HRMS (m/z): [M+H]+ calculated for $C_{61}H_{88}N_7O_{18}S$, 1238.5901; found, 1238.5924.

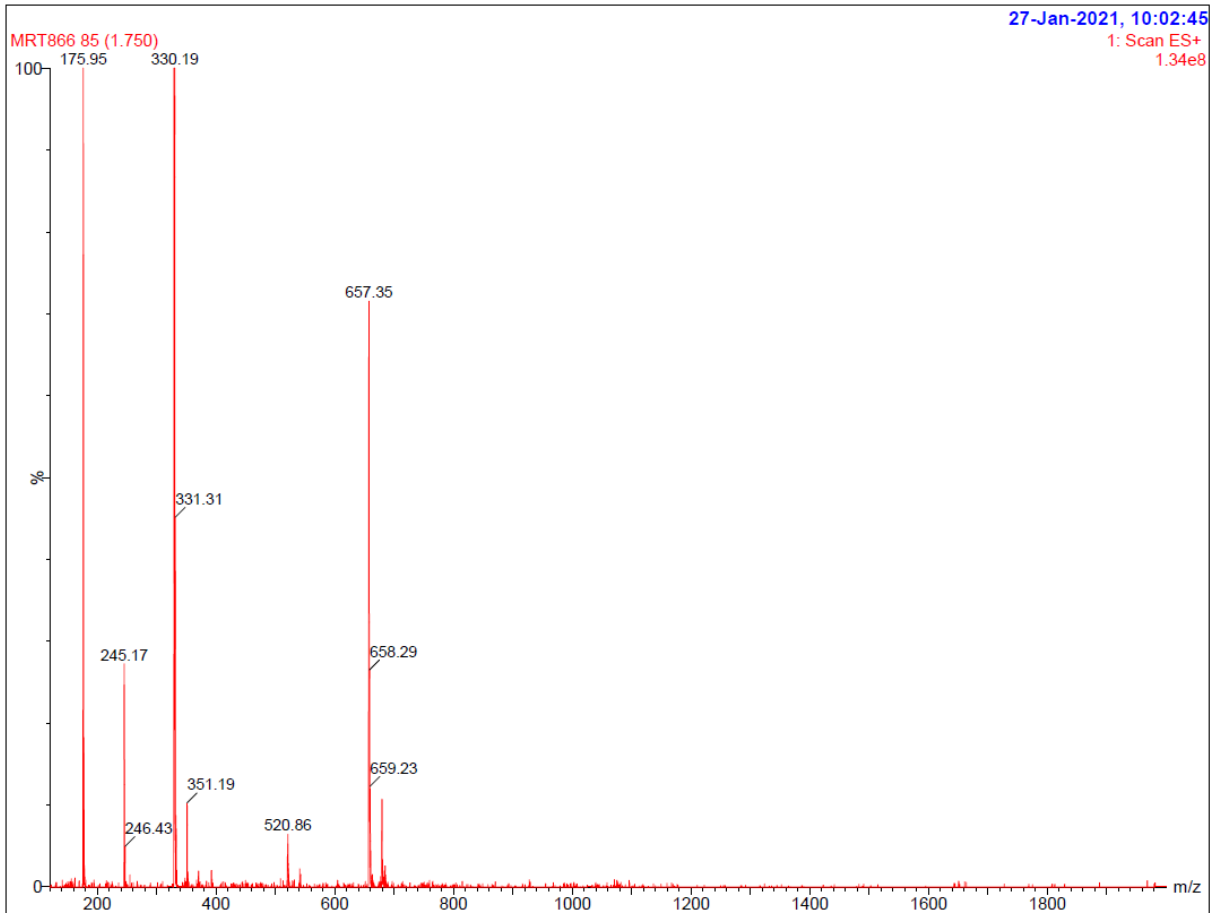
References

1. Beutner, G. L. *et al.* TCFH-NMI: Direct Access to N-Acyl Imidazoliums for Challenging Amide Bond Formations. *Org. Lett.* **20**, 4218–4222 (2018).

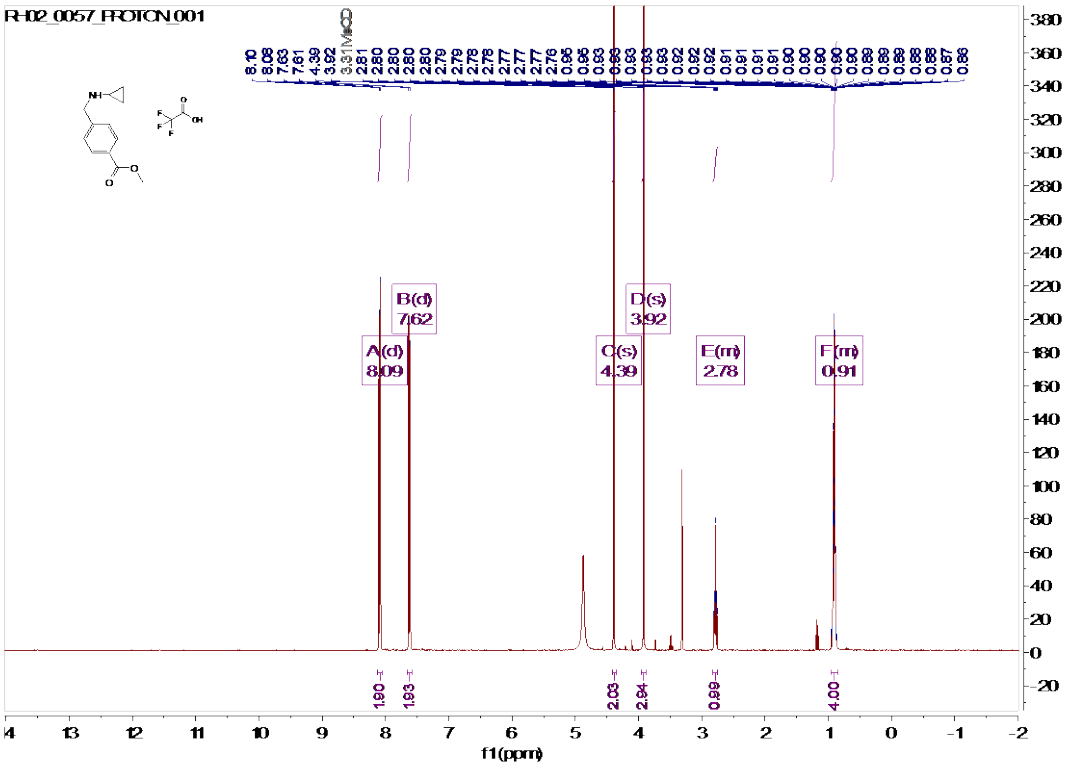
Compound Spectra and QC data:

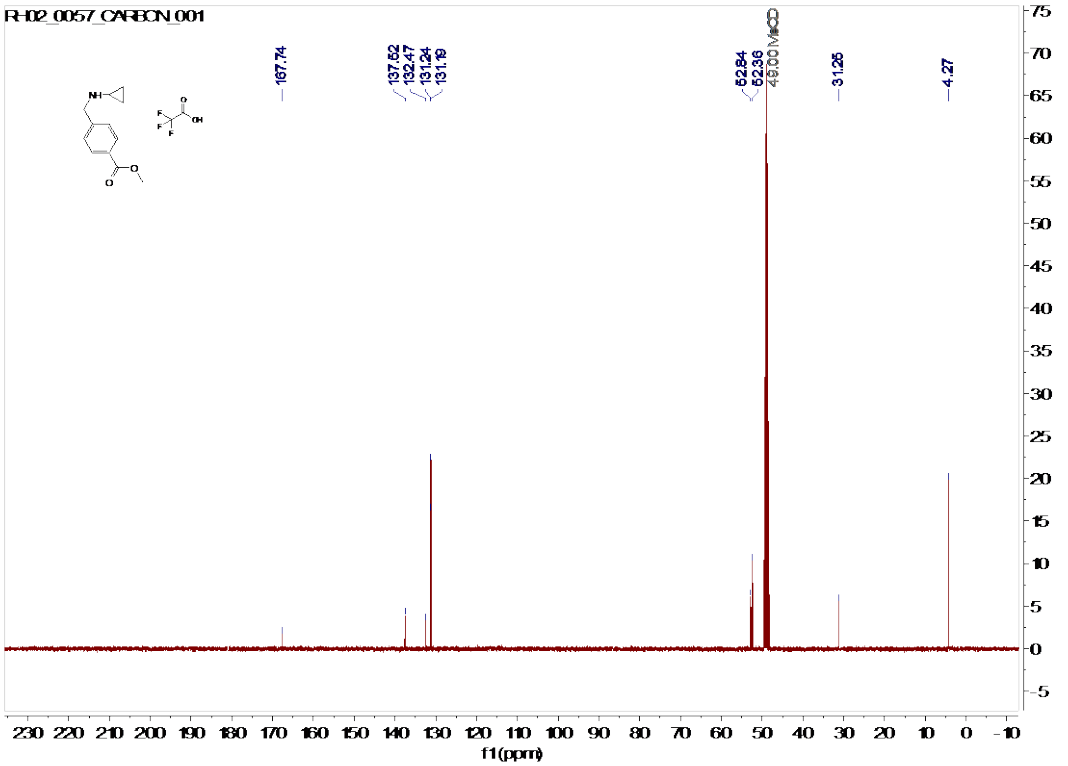
MRT866 (2) Purchased from Enamine (Cat. No. Z357319716)



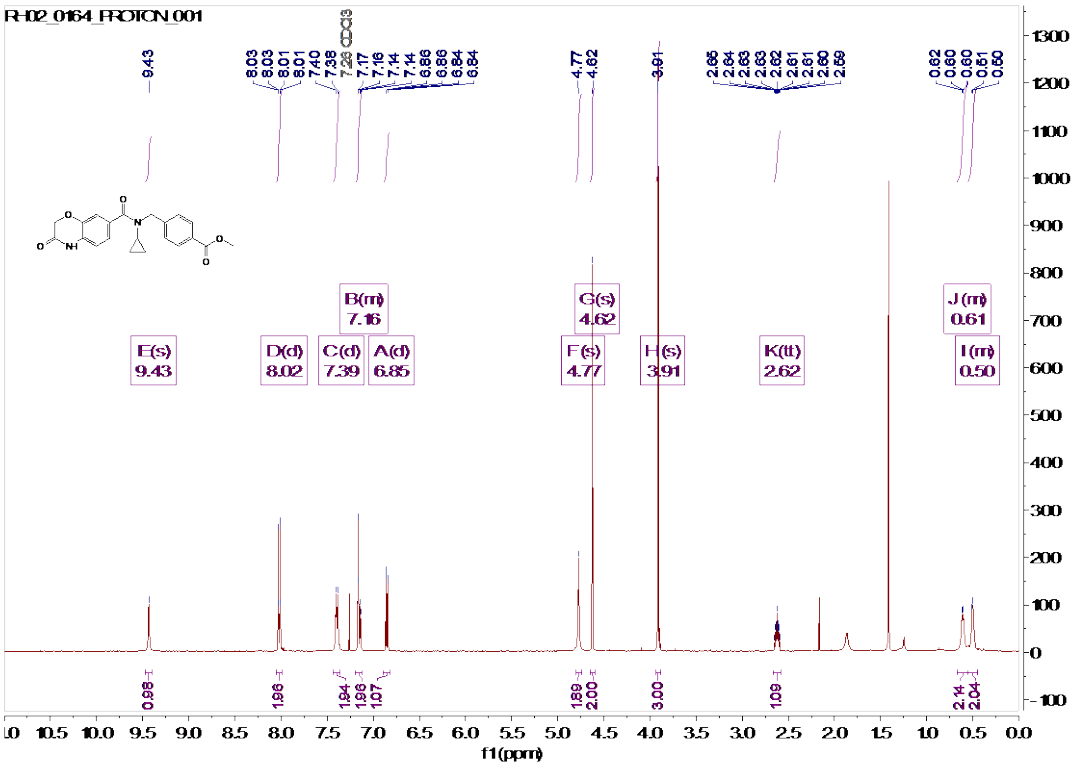


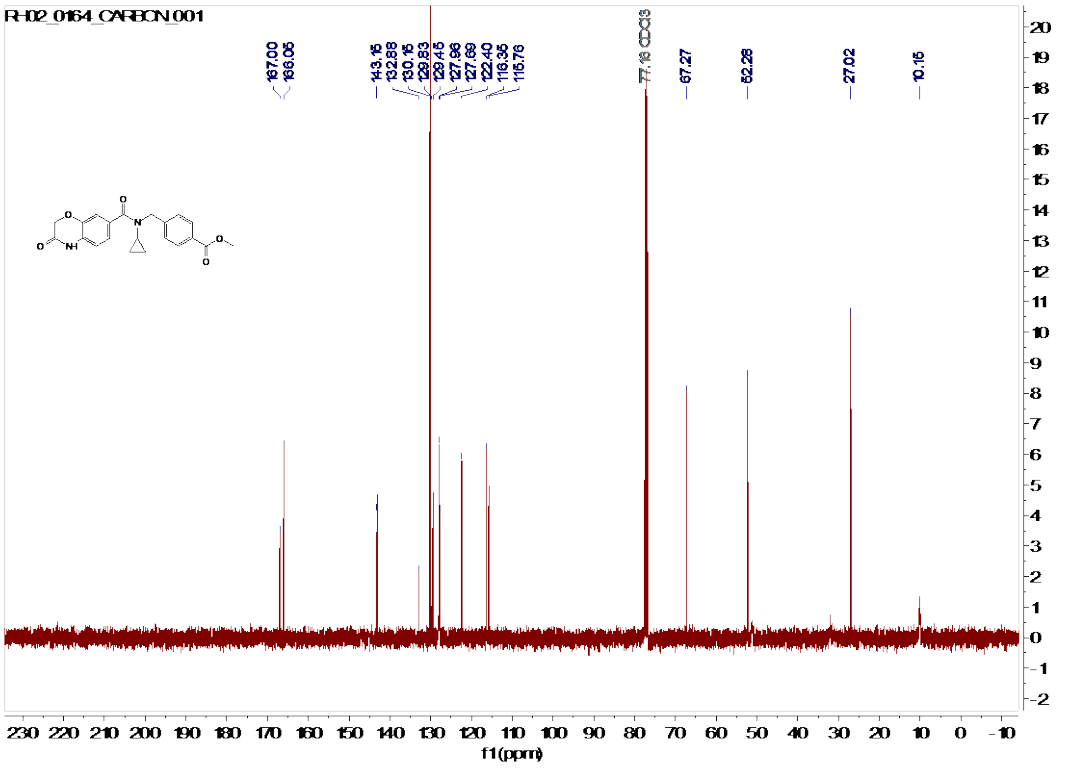
(6): ^{1}H-NMR and ^{13}C-NMR in CD_3OD



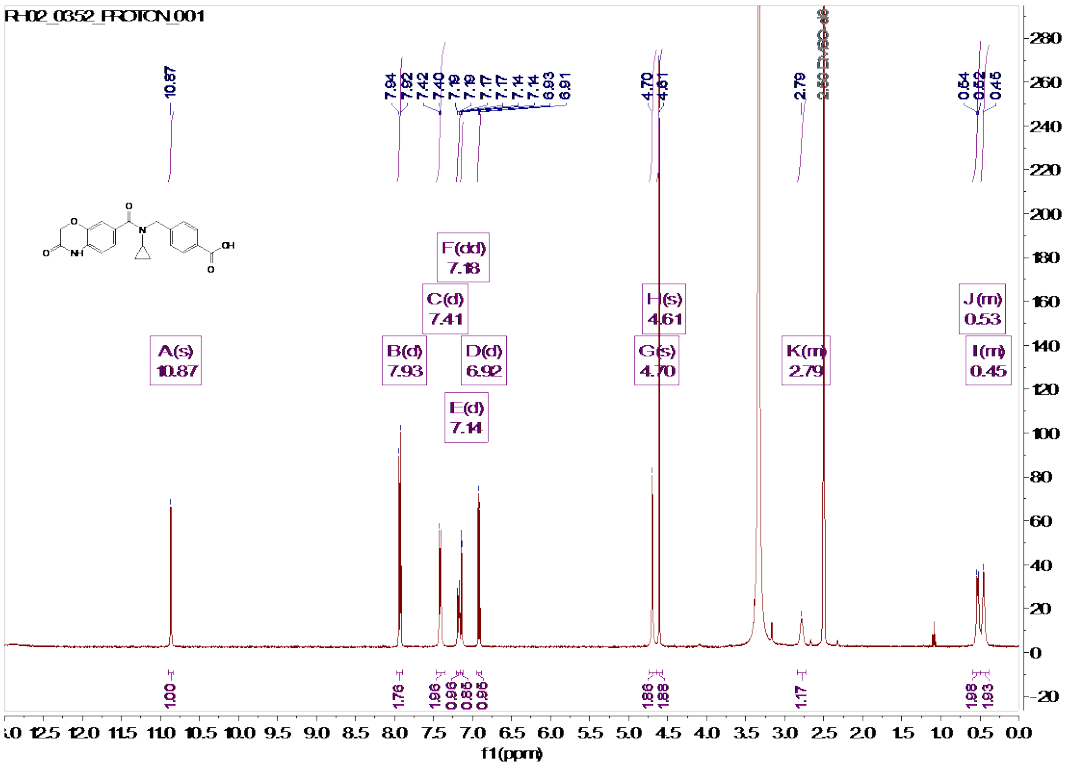


(8): ^{1}H-NMR and ^{13}C-NMR in $CDCl_3$

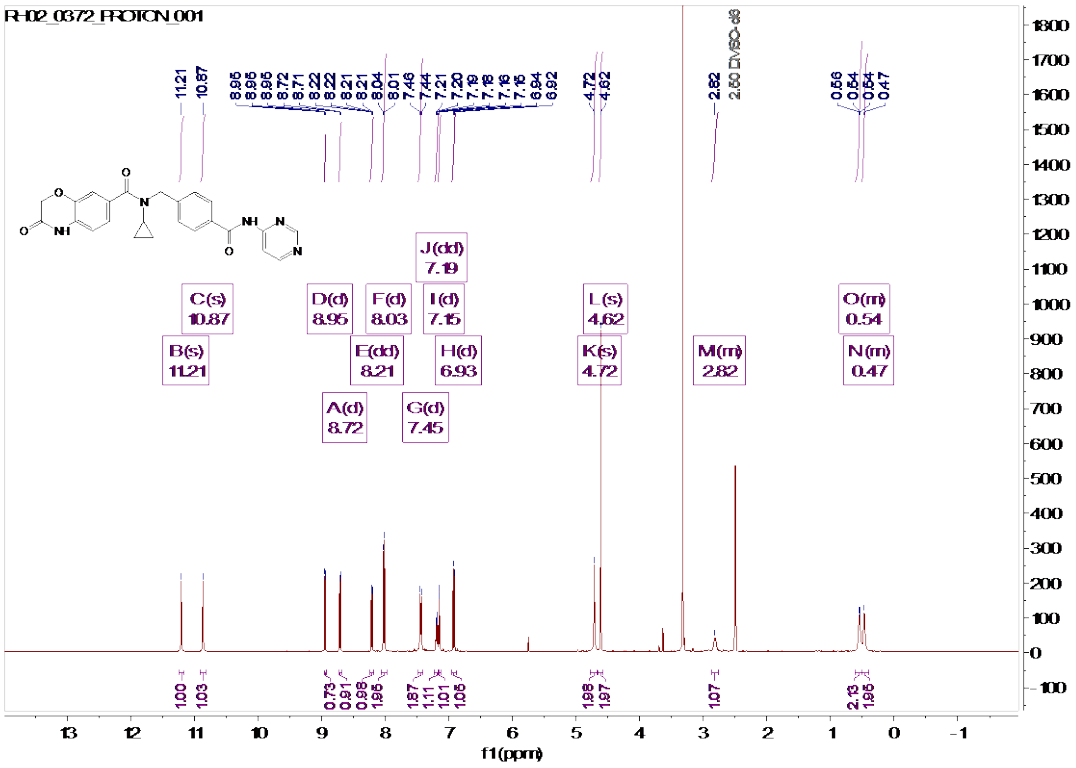


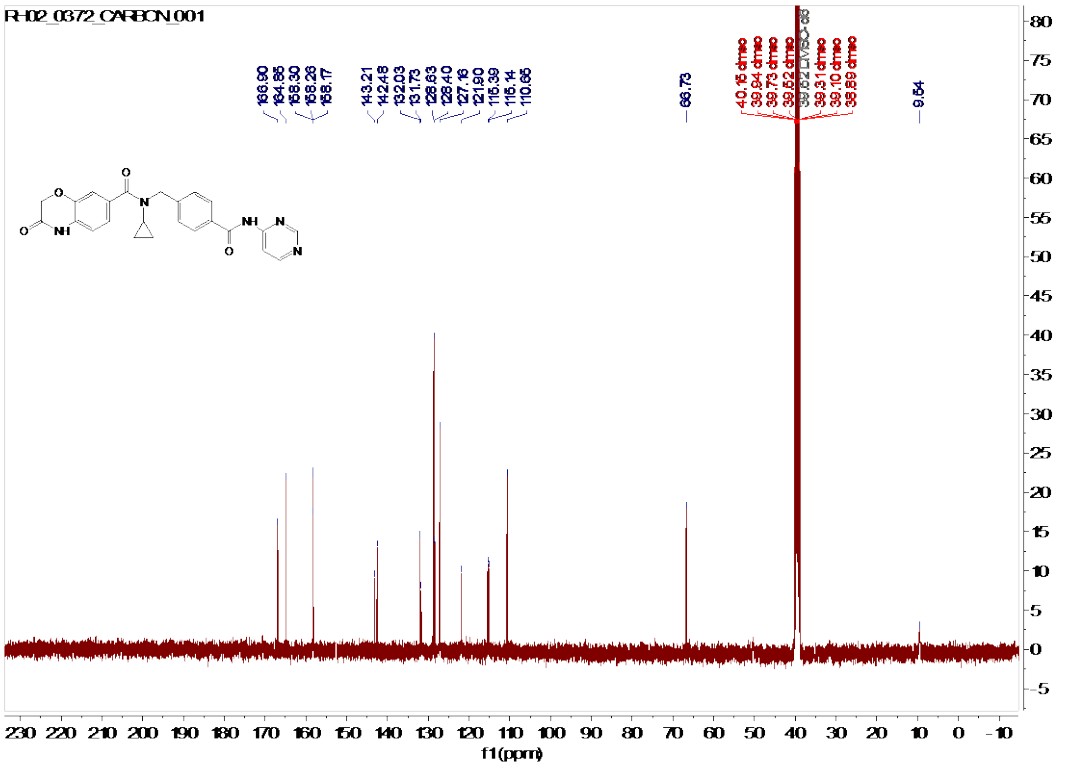


(10): ^{1}H-NMR in DMSO-d_6

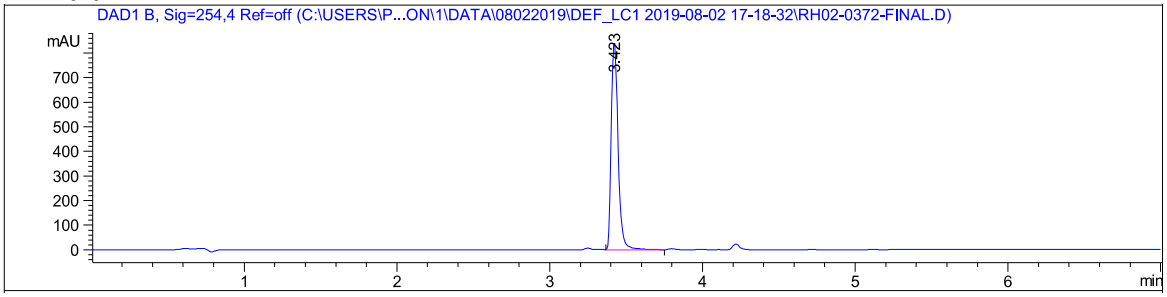


UNC6934 (1): ^{1}H-NMR and ^{13}C-NMR in DMSO-d_6

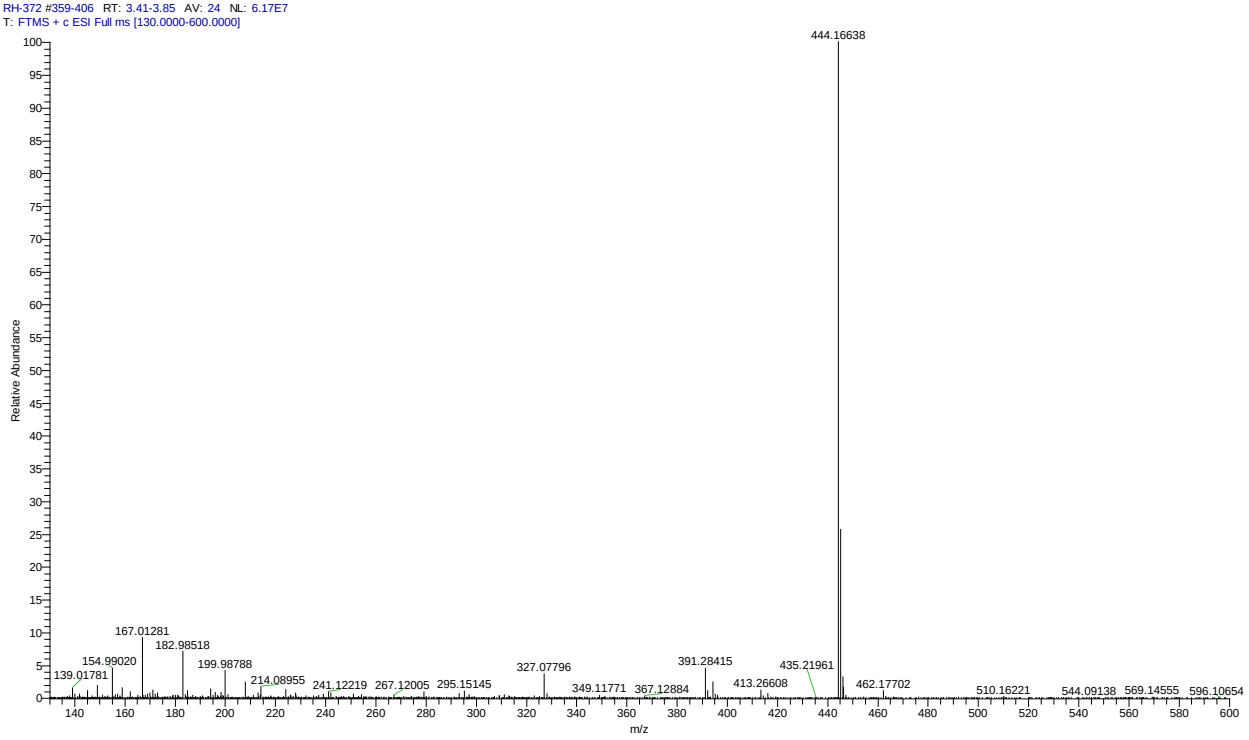




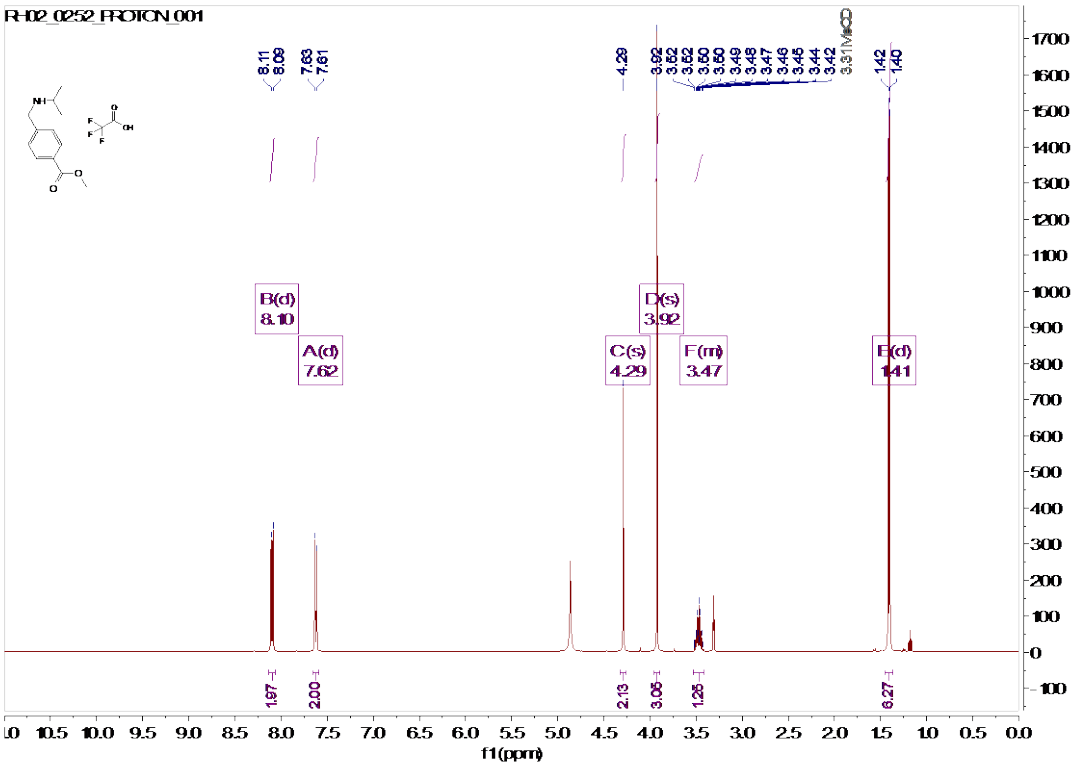
UNC6934 (1): LCMS trace

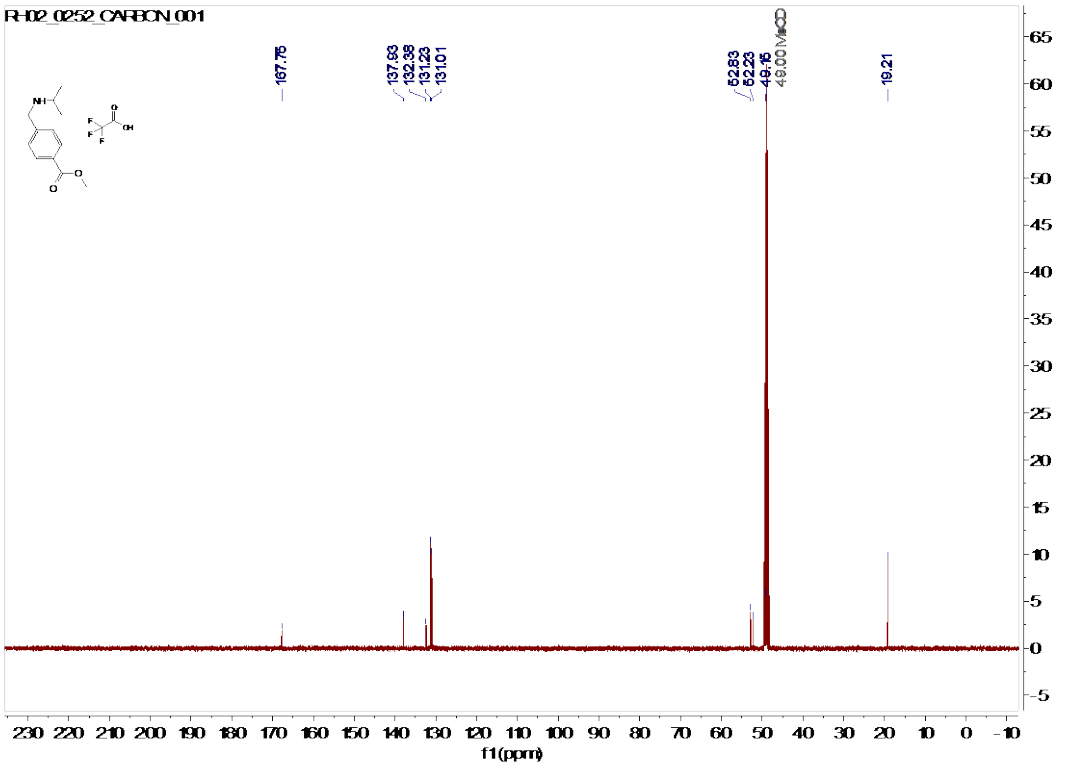


UNC6934 (1): HRMS spectrum

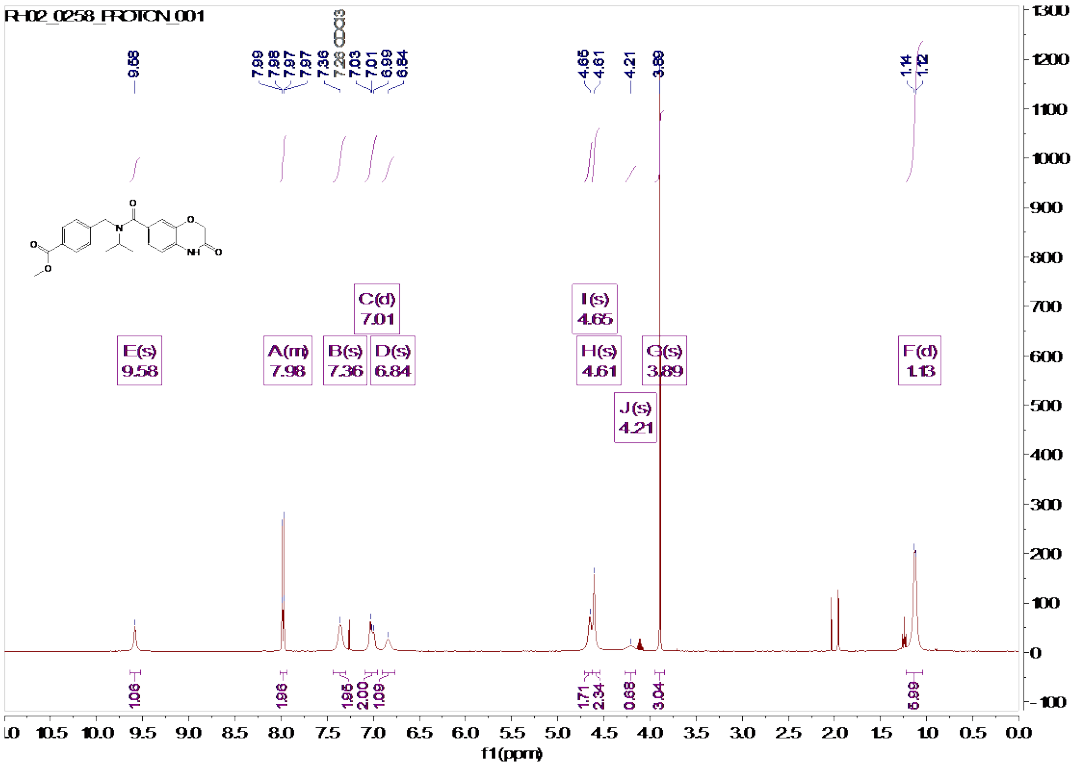


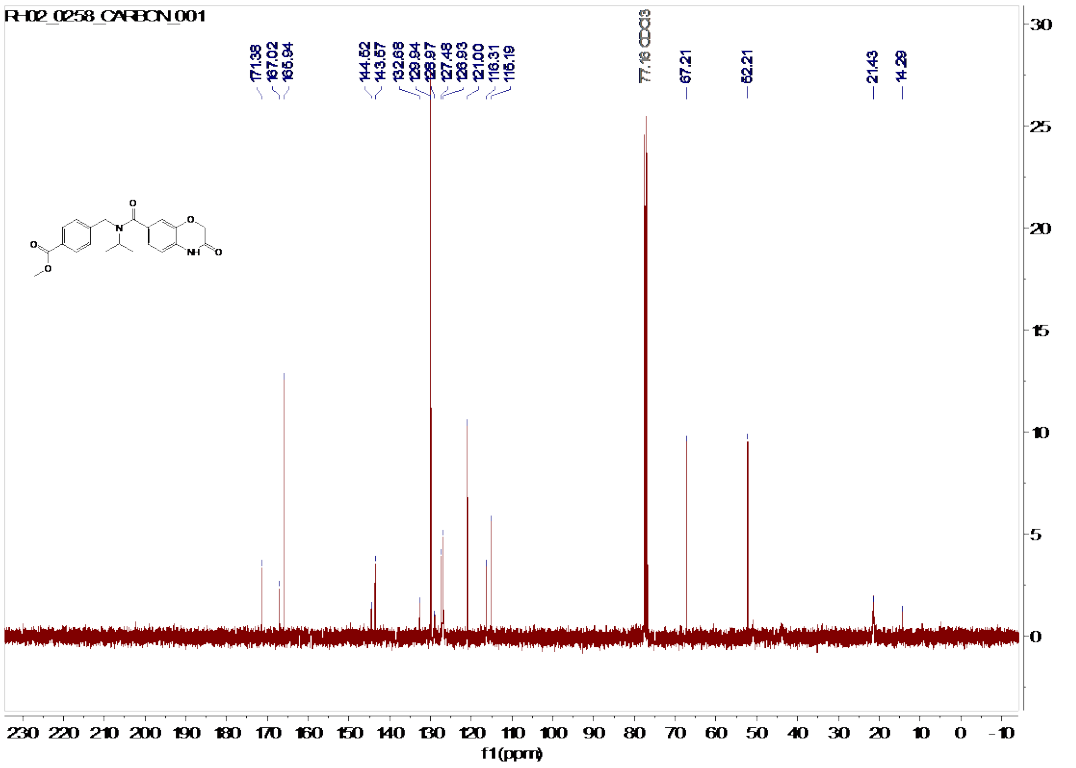
(7): ^{1}H-NMR and ^{13}C-NMR in CD_3OD



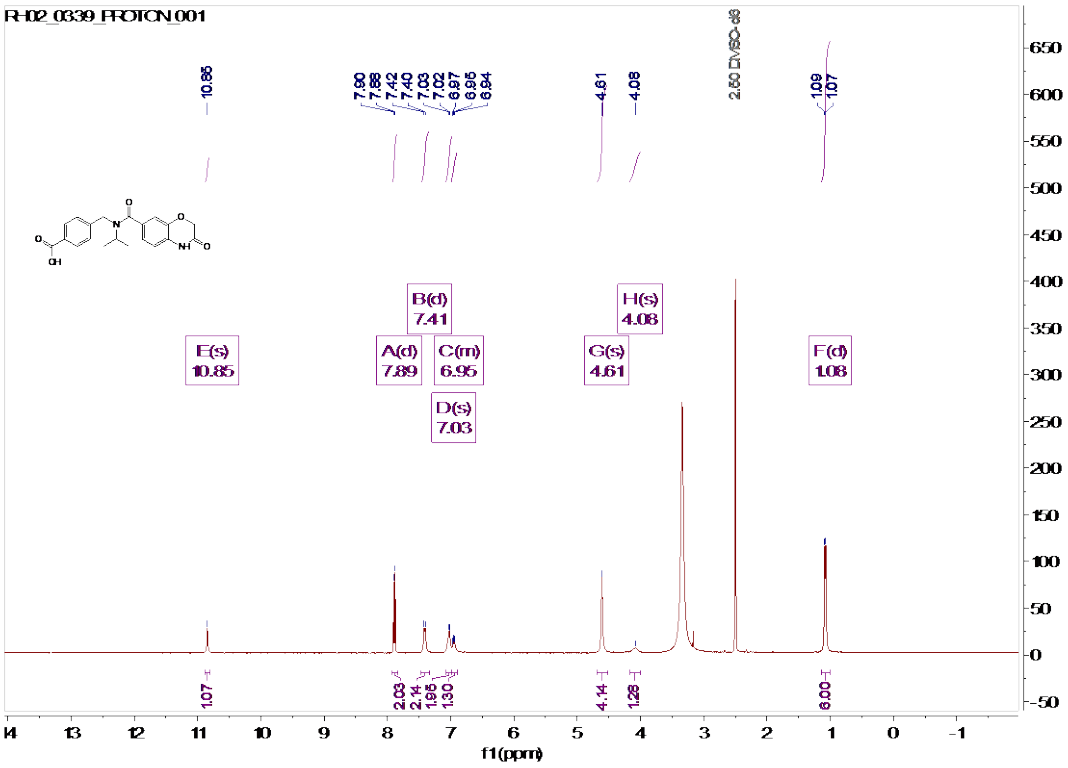


(9): ^{1}H-NMR and ^{13}C-NMR in $CDCl_3$

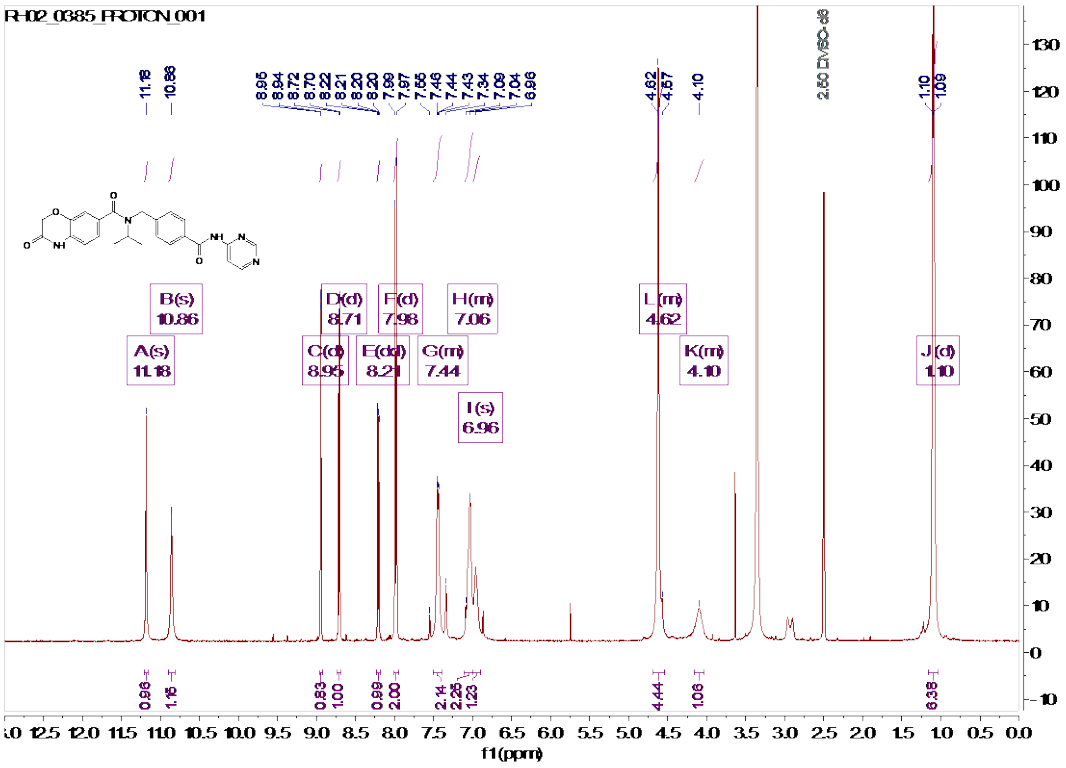


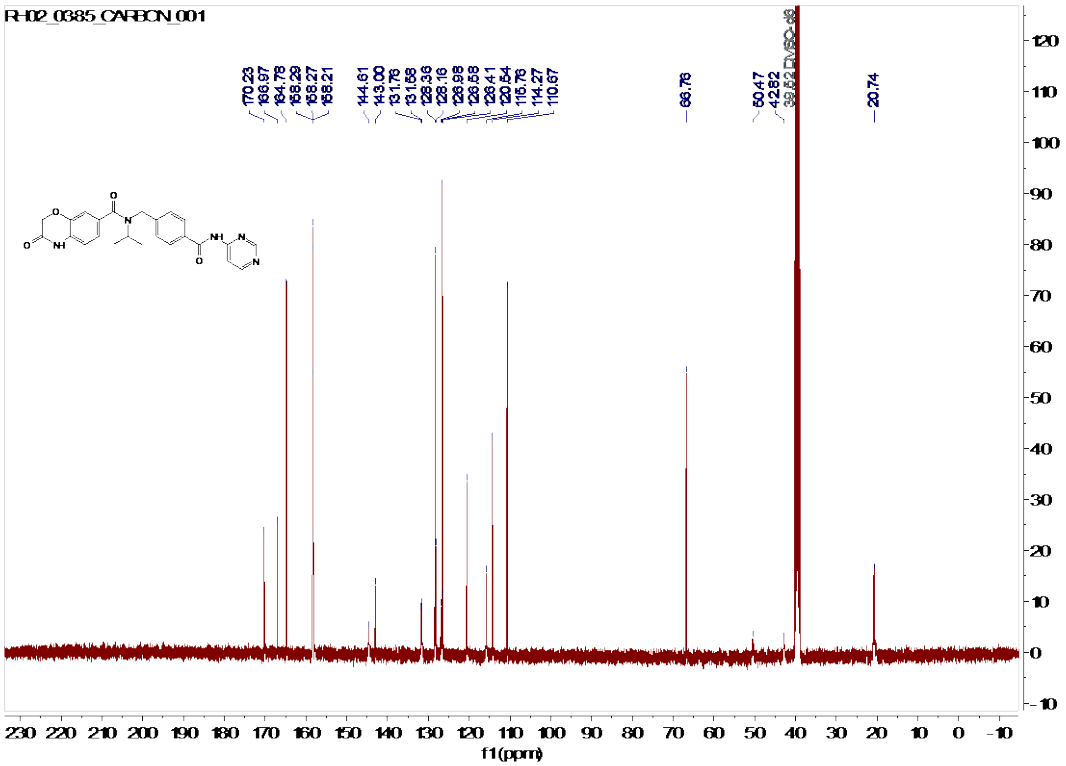


(11): ^{1}H-NMR and in DMSO-d_6

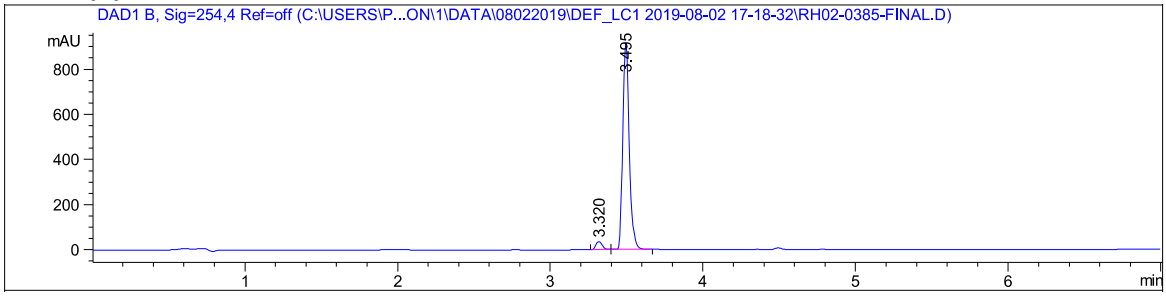


UNC7145 (4): ^{1}H-NMR and ^{13}C-NMR in DMSO-d_6

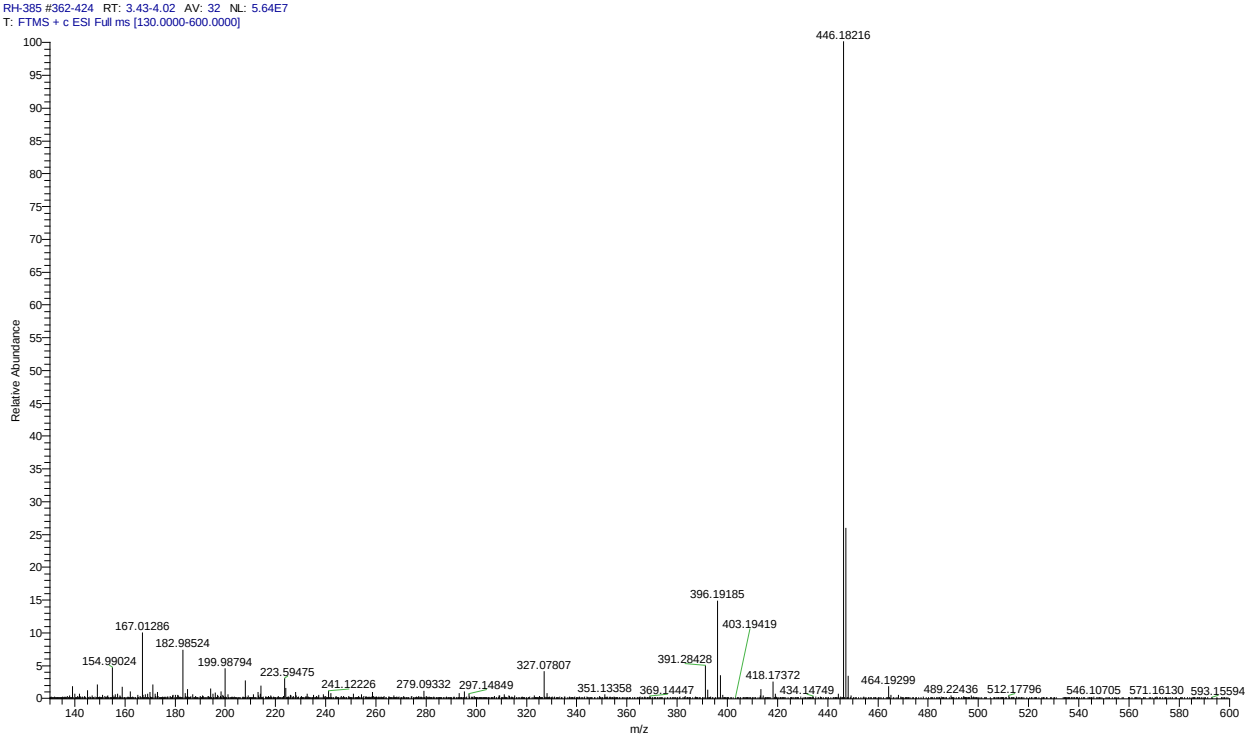




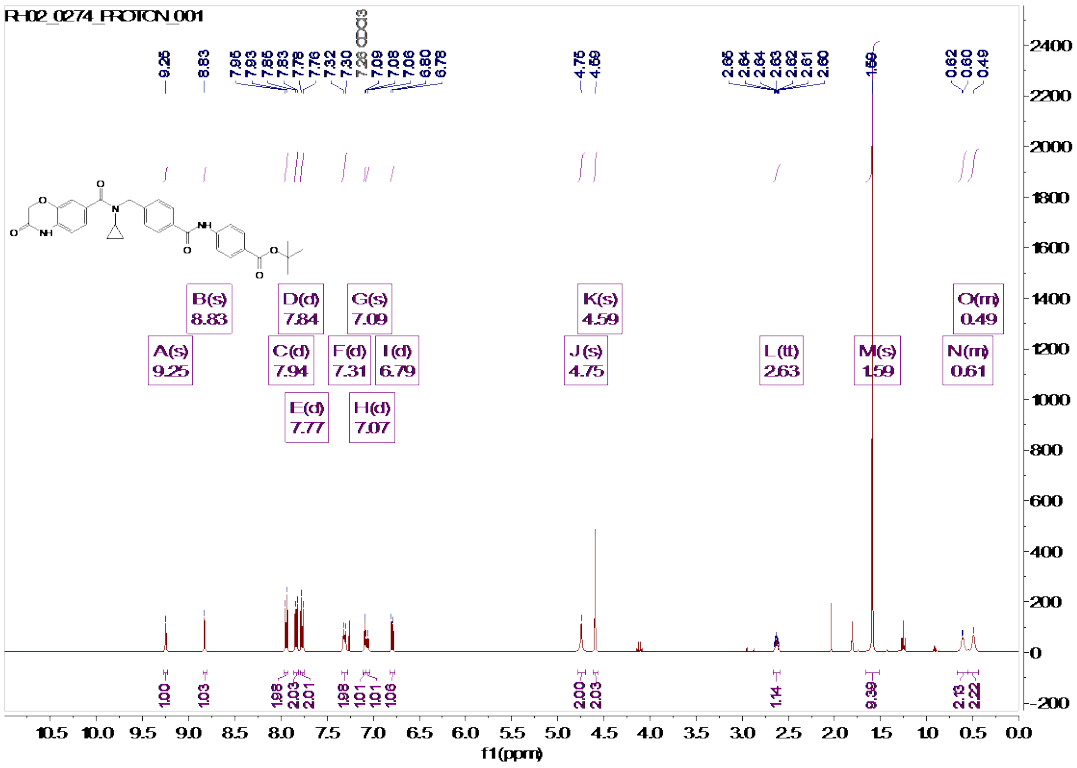
UNC7145 (4): LCMS trace

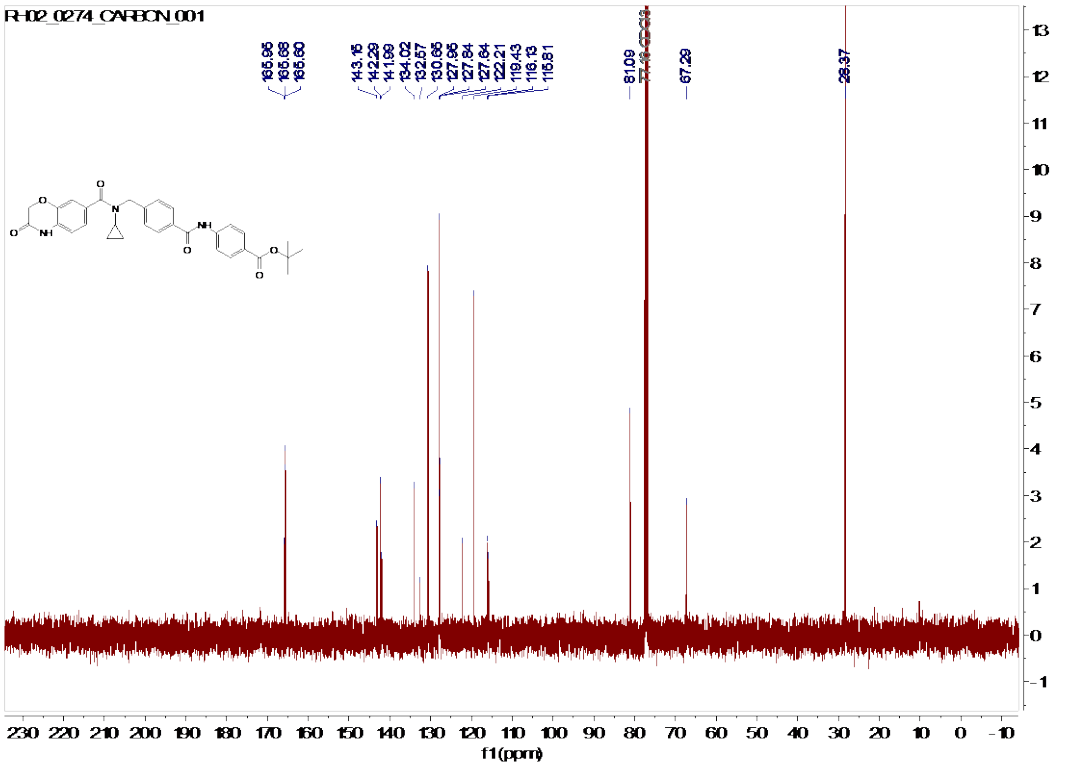


UNC7145 (4): HRMS spectrum

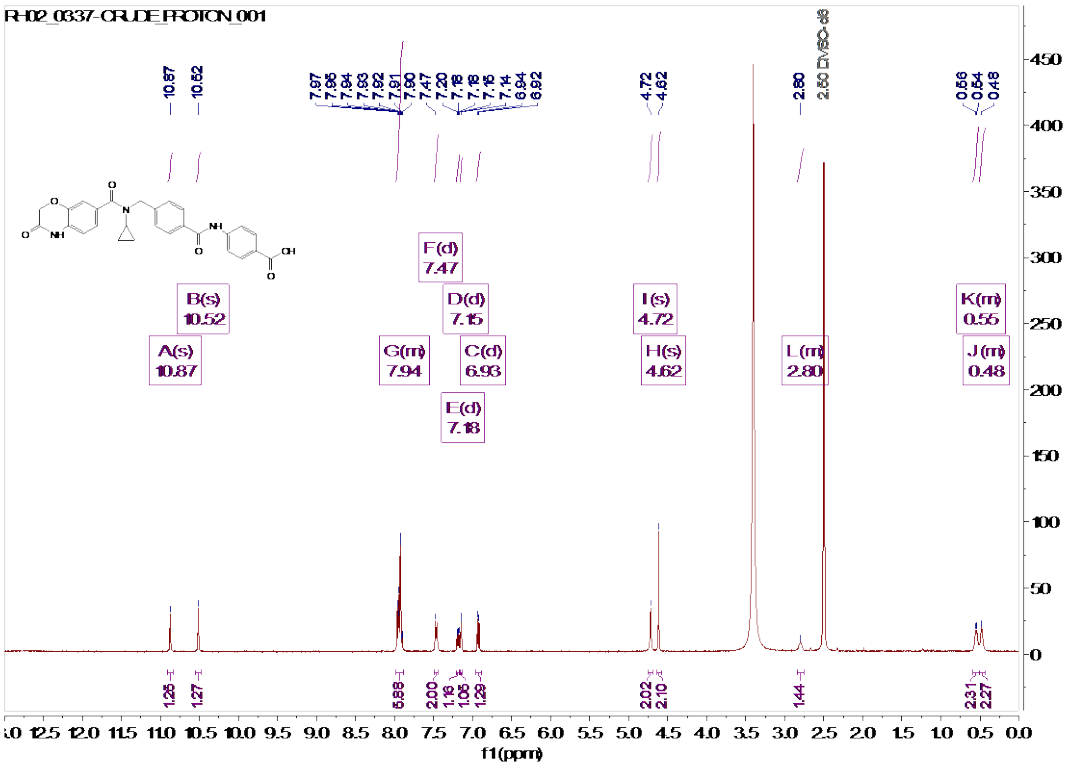


(12): ^{1}H-NMR and ^{13}C-NMR in $CDCl_3$

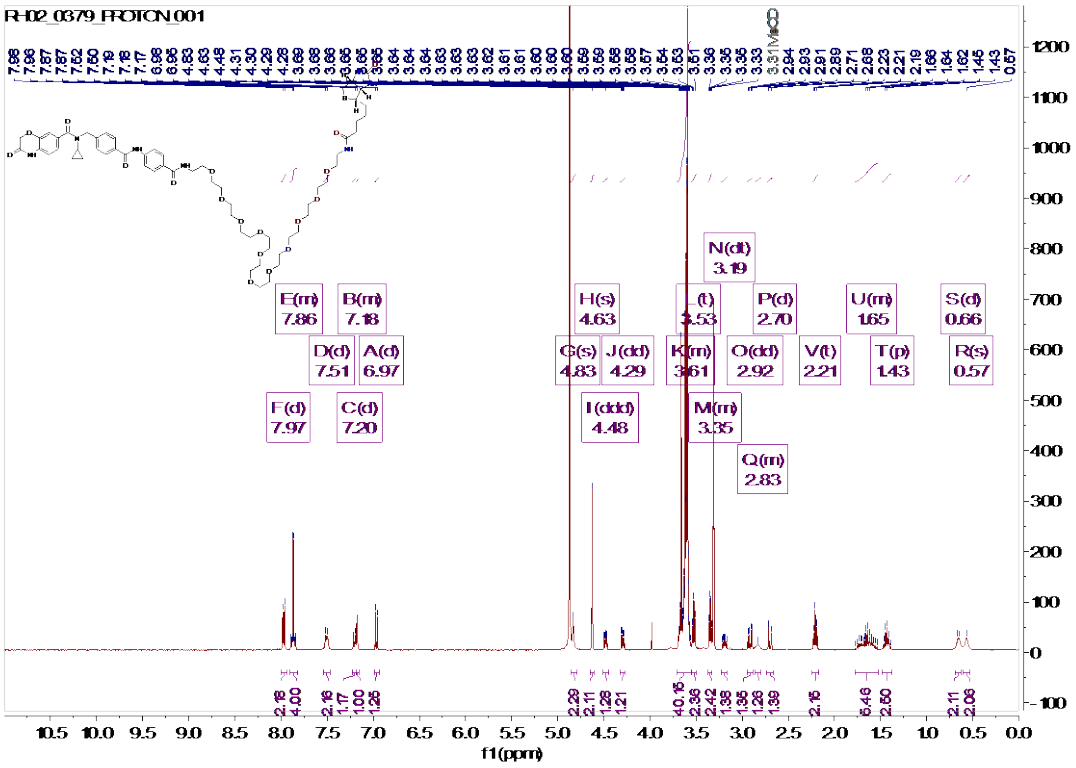




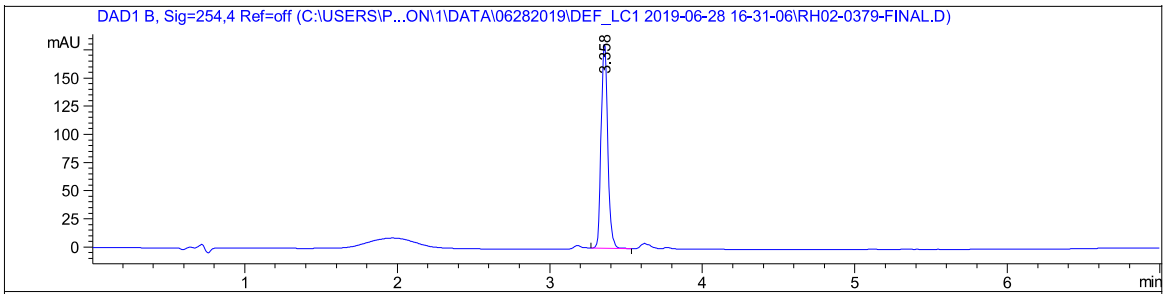
(13): ^{1}H-NMR in DMSO-d_6



UNC7096 (5): ^{1}H-NMR in CD_3OD



UNC7096 (5): LCMS trace



UNC7096 (5): HRMS