SUPPORTING INFORMATION

A Small-Molecule Activity-Based Probe for Monitoring Ubiquitin C-terminal Hydrolase L1 (UCHL1) Activity in Live Cells and Zebrafish Embryos

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Contents

Table S1: IC_{50} values[a]

Compound	DUB (concentration)	IC_{50} (μM)
6RK73	UCHL1 (1 nM)	0.23
6RK73	UCHL3 (0.01 nM)	235
6RK73	UCHL5 (1 nM)	>>100
6RK73	USP7 (1 nM)	68.8
6RK73	USP16 (2 nM)	>>100
6RK73	USP30 (10 nM)	9.8
6RK73	Papain (3 nM)	10.7
8RK64	UCHL1 (1 nM)	0.32
8RK64	UCHL3 (0.01 nM)	216
8RK64	UCHL5 (1 nM)	>>100
8RK59	UCHL1 (1 nM)	1.2
9RK15	UCHL1 (1 nM)	3.6
9RK87	UCHL1 (1 nM)	0.44
11RK72	UCHL1 (1 nM)	0.50
11RK73	UCHL1 (1 nM)	0.64

[a] After 30 min. of incubation of enzyme with inhibitor.

Figure S1

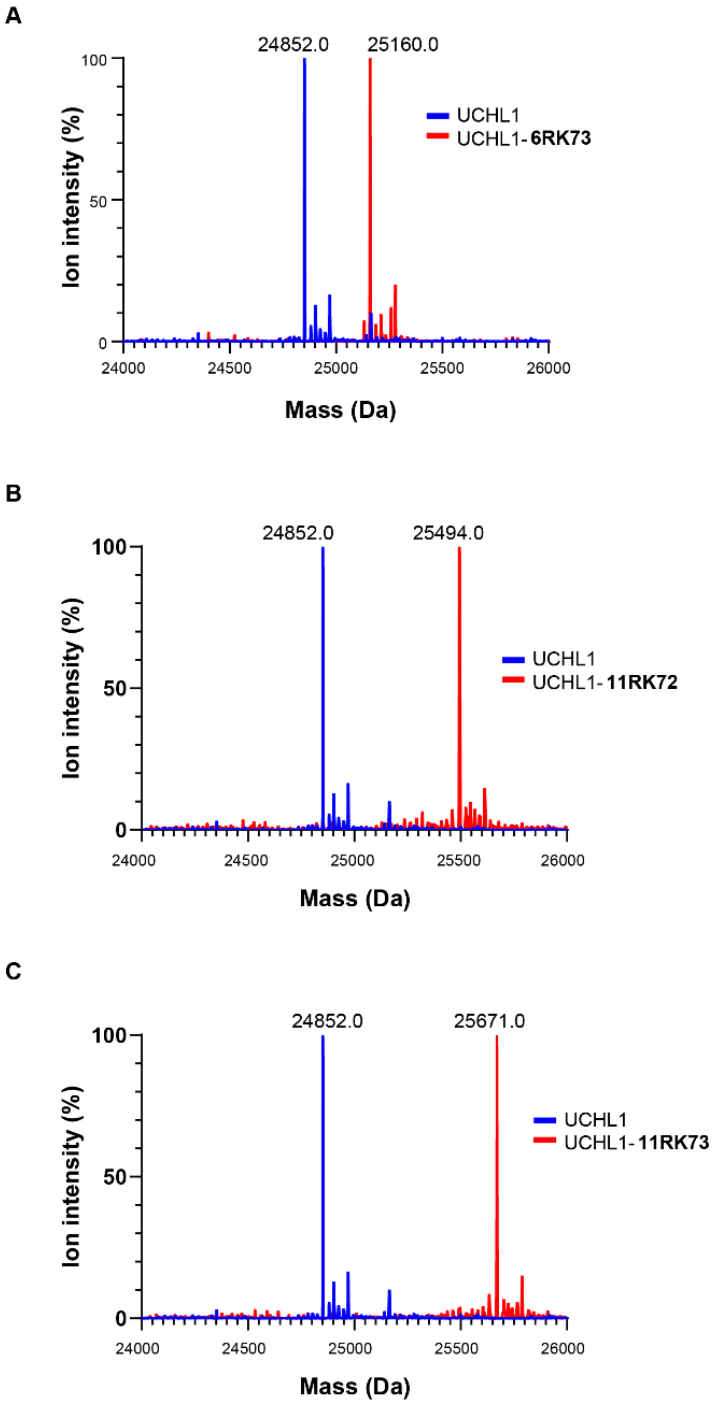


Figure S1. Deconvoluted mass spectra of UCHL1 before (blue) and after (red) reaction with A) **6RK73**, B) **11RK72** and C) **11RK73**. 1.4 µM UCHL1 was incubated with 140 µM final concentration **6RK73**, **11RK72** or **11RK73** for 30 min. at room temperature and analyzed by LC-MS.

Figure S2

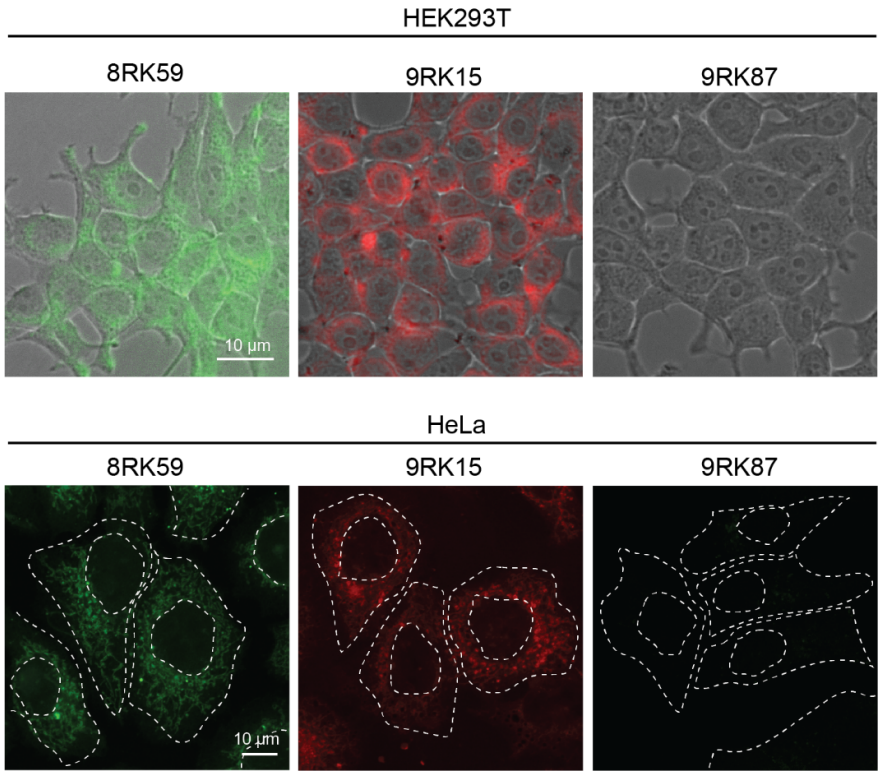
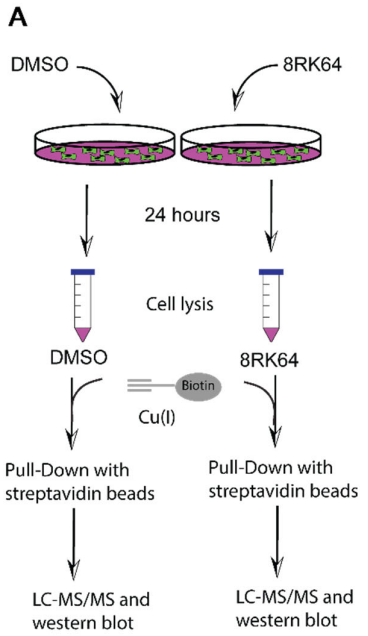
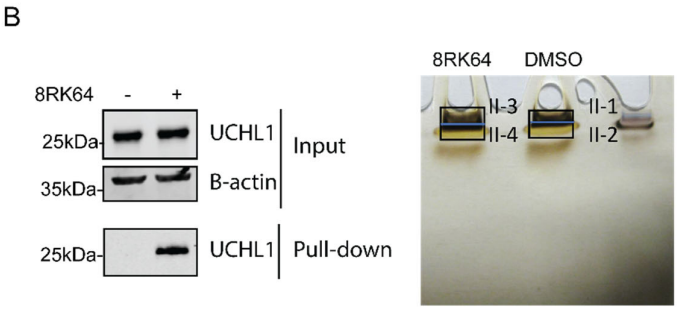


Figure S2. Cell permeability of **8RK59**, **9RK15**, and **9RK87** probes. HEK293T (top panel) and HeLa (bottom panel) cells were incubated with 5 µM final concentration of indicated probes for 24 hours at 37 °C. HEK293T cells were visualized using EVOS® FL Cell Imaging System. Nikon Plan Fluor 40×/0.75, infinity/0.17 objective was used. HeLa cells were fixed by 3.7% formaldehyde and then mounted using ProLong Gold antifade mounting medium with DAPI. Nuclei and cell boundaries are shown in dashed white lines. HeLa cells were imaged using Leica SP8 microscopes. HCX PL 63× 1.32 oil objectives and HyD detectors were used in confocal images.

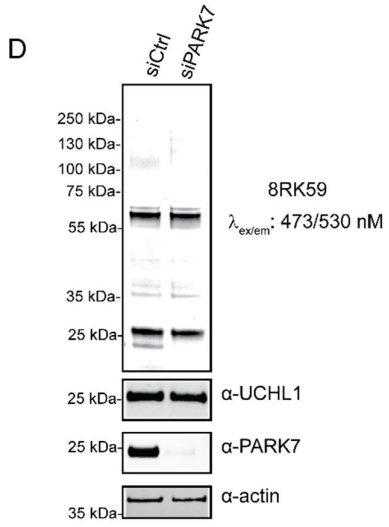
Figure S3

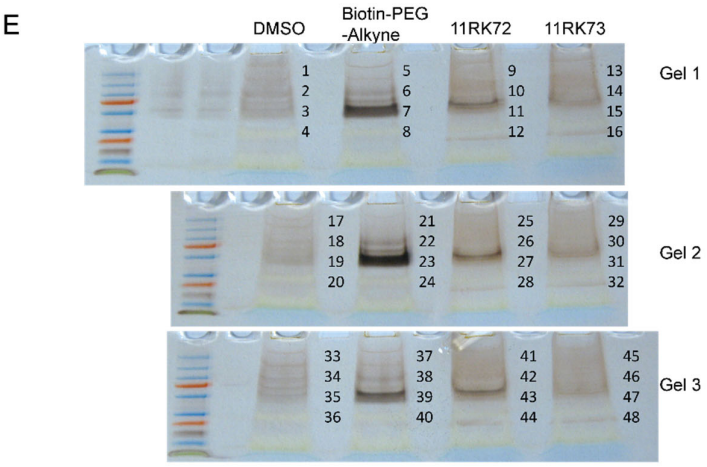




C

	Identified Proteins	MW	DMSO	8RK64
1	Ubiquitin carboxyl-terminal hydrolase isozyme L1 OS=Homo sapiens	25 kDa		15
2	Actin, cytoplasmic 2 OS=Homo sapiens GN=ACTG1	42 kDa	17	24
3	Protein DJ-1 OS=Homo sapiens GN=PARK7	20 kDa		4
4	Alpha-enolase OS=Homo sapiens GN=ENO1	47 kDa	18	8
5	Protease serine 1 OS=Homo sapiens GN=PRSS1	25 kDa	11	11
6	Tubulin alpha-1B chain OS=Homo sapiens GN=TUBA1B	50 kDa	9	12
7	Elongation factor 1-alpha 1 OS=Homo sapiens GN=EEF1A1	50 kDa	11	10
8	Elongation factor 2 OS=Homo sapiens GN=EEF2	95 kDa	8	12





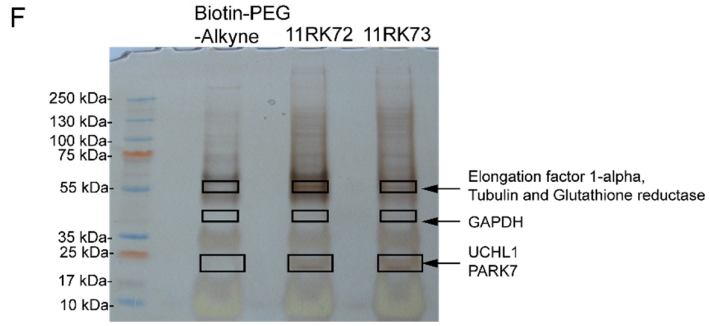


Figure S3. Pull-down and proteomics analysis either via a 1-step approach with biotin tagged-probes or via a 2-step labeling approach with click-chemistry. A) Schematic representation of 2-step labeling and pull-down approach. B) Western blot (left panel) and silver staining (right panel) of the samples obtained from 2-step labeling approach. Neutravidin beads from DMSO or **8RK64** treated samples were boiled in NuPAGE LDS sample buffer at 95 °C for 15 min. Proteins were run on 4-12% SDS-PAGE (1 cm for right panel) and either immunoblotted against PARK7, UCHL1 and Actin or stained using SilverQuest Silver Stain. The numbers on the gel indicate slices cut from the gel. C) List of the top eight proteins identified from proteomics experiment using 2-step labeling and pull-down approach Numbers represent the Total Spectrum Count. D) Confirmation of PARK7 labeling with **8RK59** via Fluorescent labeling and western blot analysis. Labeled proteins with **8RK59** were analyzed using in-gel fluorescence scanning followed by immunoblot against UCHL1. Actin is used as a loading control. E) Silver staining of three independent gels obtained from 1-step labeling approach. Related to Figure 4. Neutravidin beads from DMSO, Biotin-PEG_4-Alkyne, **11RK72** or **11RK73** treated samples were boiled in NuPAGE LDS sample buffer at 95 °C for 15 min. Proteins were run for 2 cm on 4-12% SDS-PAGE and stained using SilverQuest Silver Stain. The numbers on the gels indicate slices cut from the gels. F) Silver staining of the samples obtained from 1-step labeling approach**.** Neutravidin beads from Biotin-PEG_4-Alkyne, **11RK72** or **11RK73** treated samples were boiled in NuPAGE LDS sample buffer at 95 °C for 15 min. Proteins were run on 4-12% SDS-PAGE and stained using SilverQuest Silver Stain. Rectangles indicate the gel slices analyzed by LC-MS/MS.

Figure S4

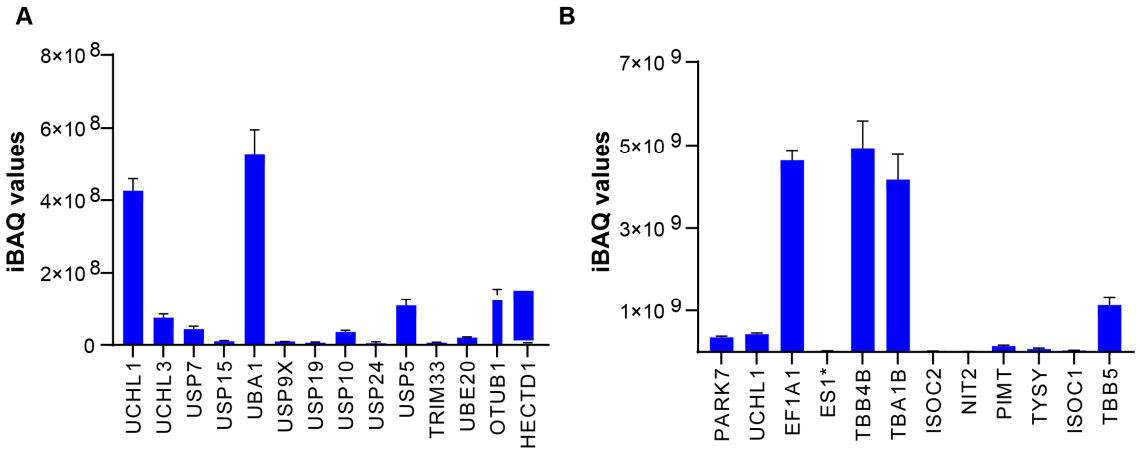


Figure S4. Reference intensities (iBAQ values) of proteins shown in Figure 4D,E in wild type HEK293T cells taken from the Maxquant protein groups output file from PRIDE data archive with entry number PDX015828. A) Enzymes related to the Ub system identified in the pull-down LC-MSMS experiment averaged over three replicates E) Top-12 highest ranked proteins from the pull-down LC-MSMS experiment averaged over three replicates. *ES1 has been annotated as Glutamine amidotransferase-like class 1 domain-containing protein 3A or 3B (GATD3A or GATD3B).

Figure S5

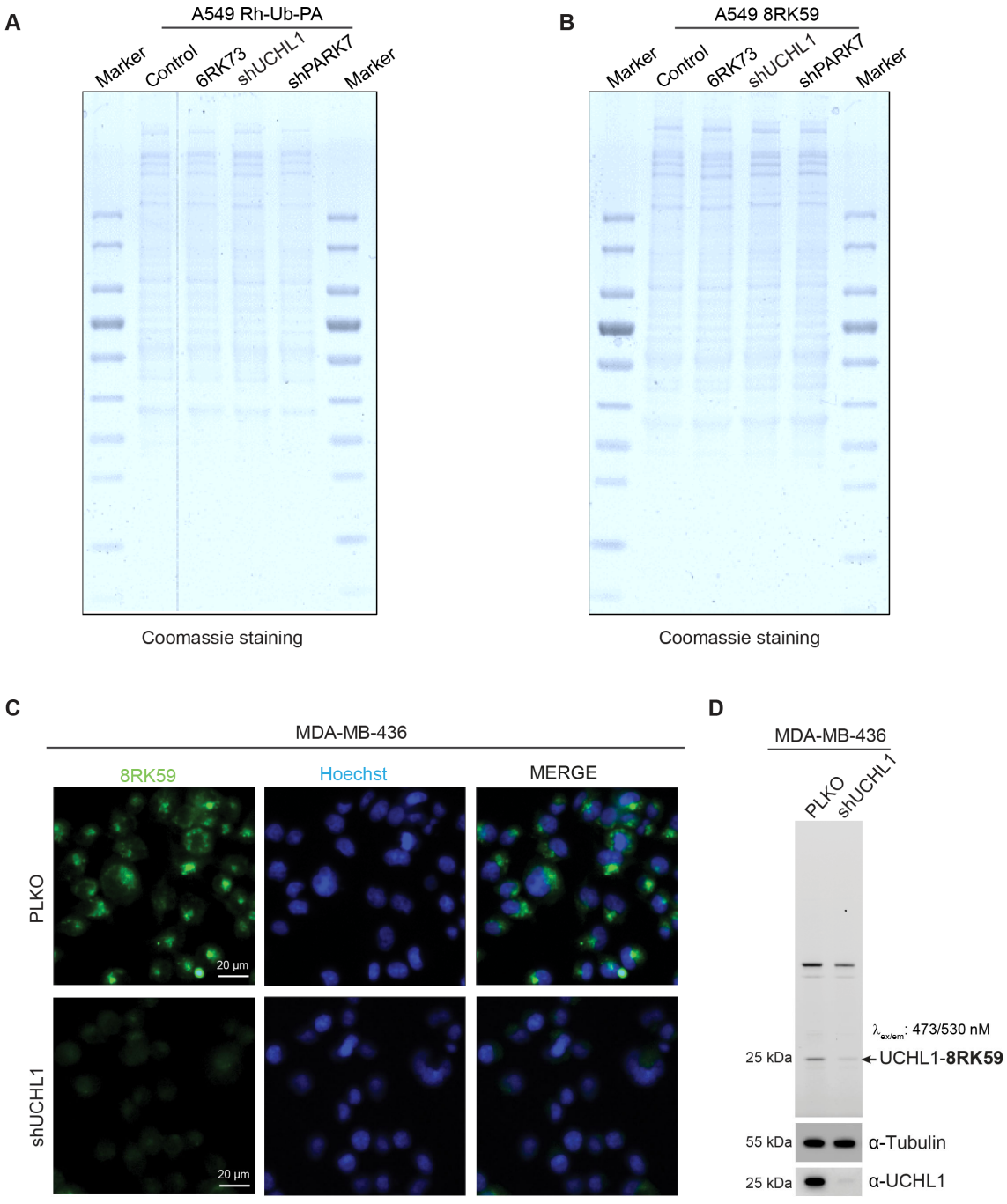


Figure S5. Probing UCHL1 activity in cells with **8RK59**. A) Coomassie staining of SDS-PAGE gel in which samples were analyzed for DUB activity in Control, **6RK73**, shUCHL1 and shPARK7 A549 cells with Rh-Ub-PA (this figure is complementary to Figure 5E). B) Coomassie staining of SDS-PAGE gel in which samples were analyzed for 5 µM **8RK59** labelled Control, **6RK73**, shUCHL1 and shPARK7 A549 cells in SDS-PAGE gel (this figure

is complementary to Figure 5F). C) Live-cell fluorescence imaging of 5 μM **8RK59** labelled PLKO and shUCHL1 MDA-MB-436 cells. Hoechst 33342 was used to stain the nuclei of the live cells. D) SDS-PAGE gel of endogenous UCHL1 activity in the lysate from **8RK59** labelled PLKO and shUCHL1 MDA-MB-436 cells (top panel). Western blotting was performed using UCHL1 antibody (bottom panel), and Tubulin levels were analyzed for loading controls (middle panel).

Figure S6

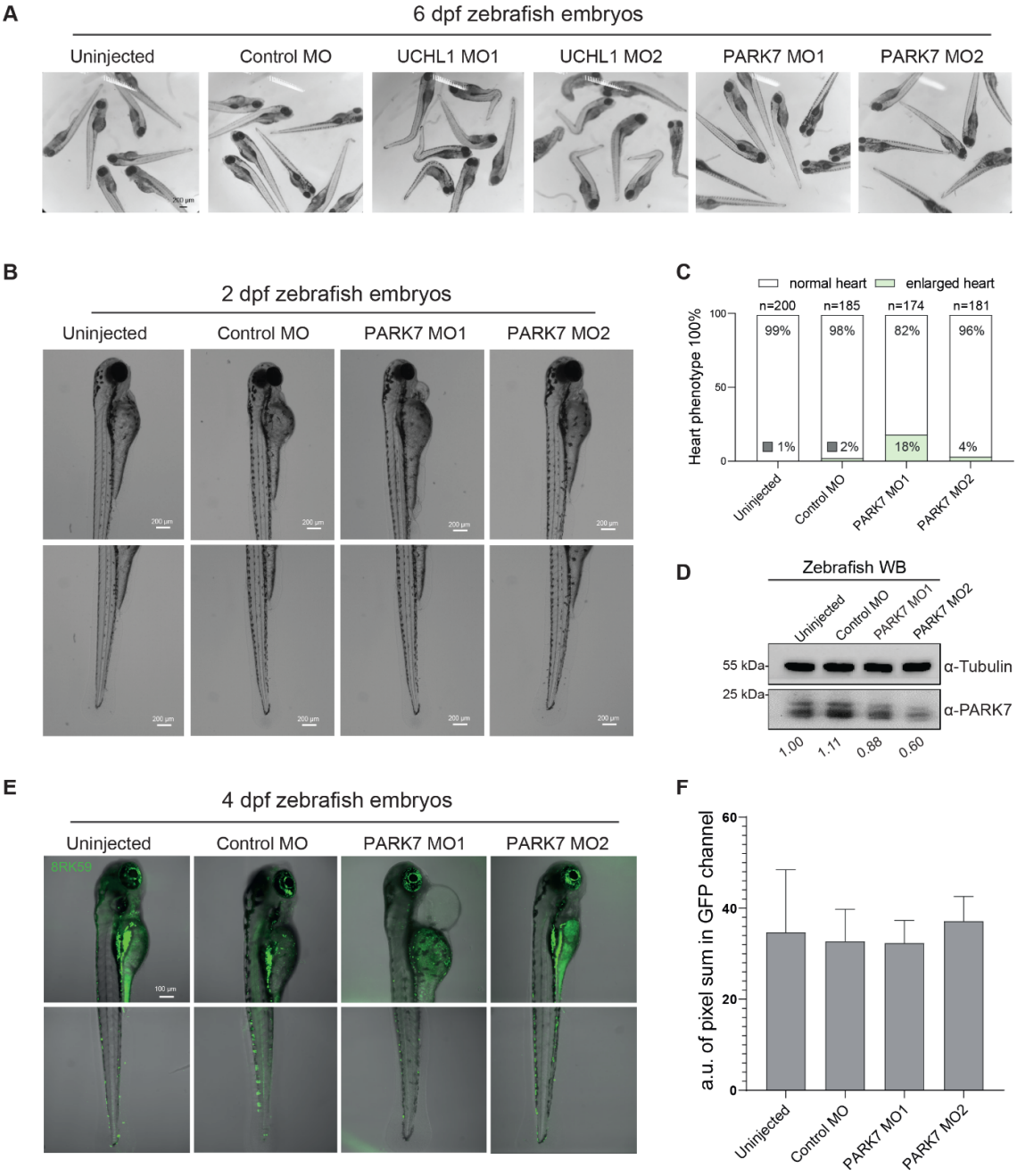


Figure S6. Probing UCHL1 activity with **8RK59** in genetically UCHL1 or PARK7 knockdown zebrafish embryos. A) Overview of 6 dpf zebrafish embryos without injection (Uninjected), injected with standard control morpholino (Control MO), injected with UCHL1 MO1, UCHL1 MO2, PARK7 MO1 and PARK7 MO2. B) Bright field images of 2 dpf zebrafish embryos with/without PARK7 MO injections. C) Quantification of abnormal enlarged heart phenotype in 2 dpf zebrafish embryos with/without PARK7 MO injections. D) WB of PARK7 in 2 dpf

zebrafish embryos with/without PARK7 MO injections. WB for Tubulin was included as a loading control. The expression level of PARK7 normalized to Tubulin are shown below. E) Probing UCHL1 activity with 5 μM **8RK59** in 4 dpf zebrafish embryos with/without PARK7 MO injections. F) The statistical analysis of **8RK59** signal in 4 dpf zebrafish embryos with/without PARK7 MO injections. The intensity of **8RK59** was measured in three zebrafish embryos of each group by calculating the pixel sum in the GFP channel of both the head and tail areas.

Figure S7

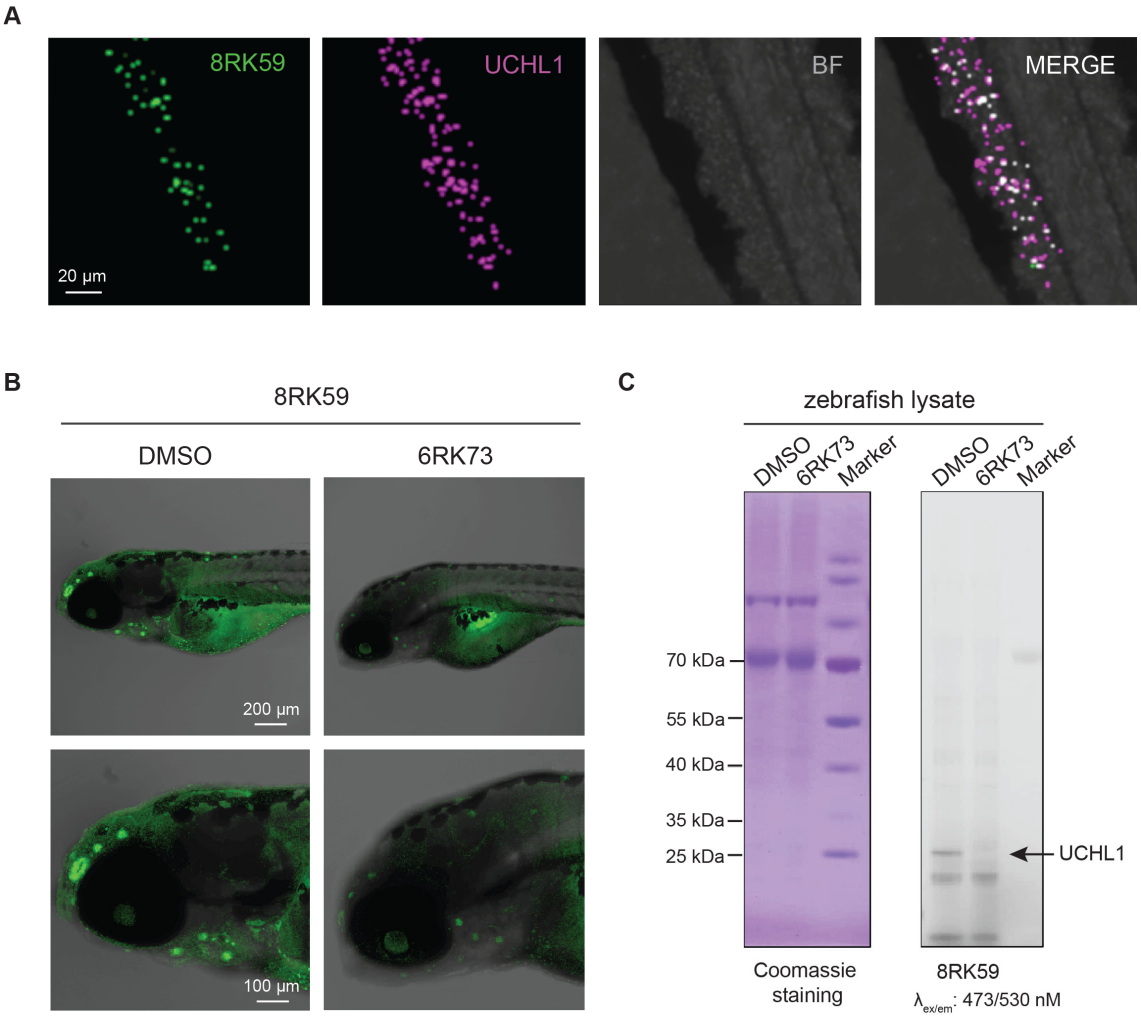


Figure S7. Monitoring UCHL1 activity in zebrafish embryos with **8RK59**. A) Immunofluorescent staining of UCHL1 in 5 µM **8RK59** labelled 6 dpf zebrafish embryo. Representative image of UCHL1 enriched spinal cord neurons in the tail of 6 dpf zebrafish embryo is shown. BF, bright field image. B) Monitoring UCHL1 activity changes in 5 µM **8RK59** labelled 6 dpf zebrafish embryos treated with/without UCHL1 activity inhibitor **6RK73**. C) SDS-PAGE gel of cell lysates from **8RK59** labelled 6 dpf zebrafish embryos treated with/without UCHL1 activity inhibitor **6RK73**. Coomassie staining was performed to control for loading (left panel). The band corresponding to UCHL1 is indicated with an arrow in the fluorescence scanned SDS-PAGE gel (right panel).

Compound synthesis

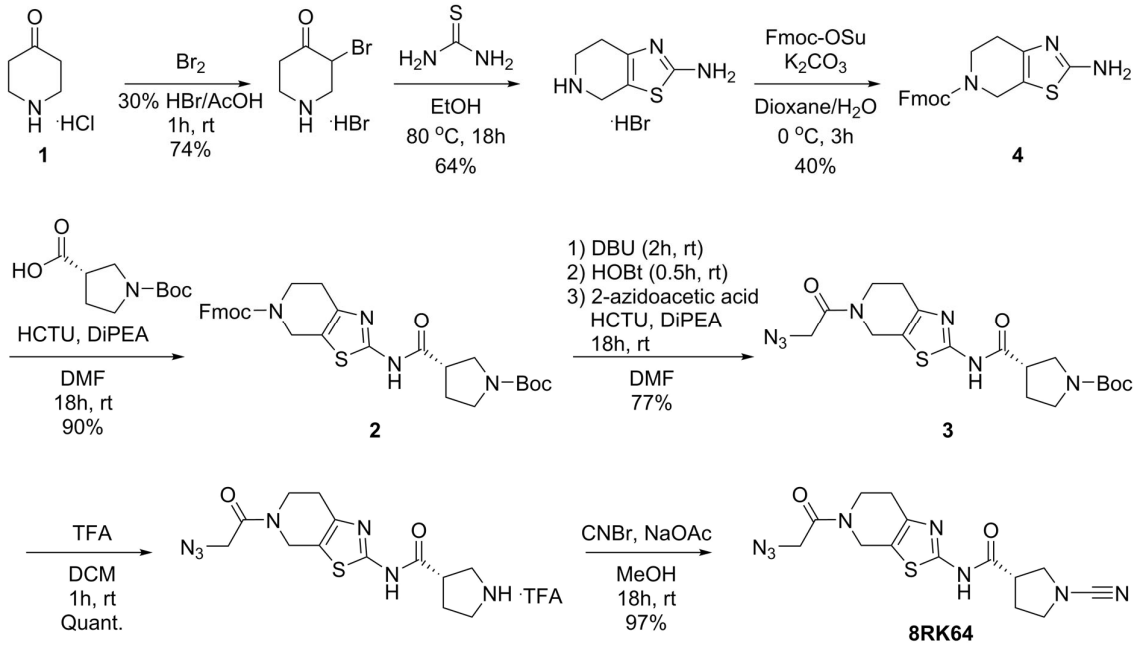
General

General reagents were purchased from Sigma-Aldrich, Biosolve, and Acros, and used as received. Solvents were purchased from Biosolve or Sigma-Aldrich. (*S*)-1-Boc-pyrrolidine-3-carboxylic acid (CAS number 140148-70-5, article number OR-5566) was purchased from Combi-Blocks Inc. BiotinPEG$_4$-alkyne (CAS number 1262681-31-1, article number PEG4950) was purchased from Iris Biotech GmbH). Thin Layer Chromatography (TLC) was performed on Merck aluminum sheets (pre-coated with silica gel 60 F_{254}). Compounds were visualized by UV adsorption (254 nm) and by using a solution of $KMnO_4$ (7.5 g L^{-1}) and K_2CO_3 (50 g L^{-1}) in H_2O or a solution of ninhydrin (15 g L^{-1}) in 3% AcOH/EtOH v/v. Compounds (unless stated otherwise) were purified by a Büchi Sepacore automatic flash chromatography system X10/X50. The Büchi Sepacore system was equipped with two Büchi pump modules C-605, a Büchi control unit C-620, Büchi fraction collector C-660 and a Büchi UV Photometer C-640. The silica columns were purchased at GraceResolv^{TM} and were packed with a grade of Davisil® silica. NMR spectra (^{1}H, ^{13}C) were recorded on a Bruker Ultrashield 300 MHz spectrometer at 298 K. Resonances are indicated with symbols 'd' (doublet), 's' (singlet), 't' (triplet) and 'm' (multiplet). Chemical shifts (δ) are given in ppm relative to $CDCl_3$, DMSO-d_6 or CD_3OD as an internal standard and coupling constants (*J*) are quoted in hertz (Hz). LC-MS measurements were performed on an LC-MS system equipped with a Waters 2795 Separation Module (Alliance HT), a Waters 2996 Photodiode Array Detector (190–750 nm), an Xbridge C18 column (2.1 × 100 mm, 3.5 µm) and an LCT ESI-Orthogonal Acceleration Time of Flight Mass Spectrometer. Samples were run using 2 mobile phases: A = 1% CH_3CN and 0.1% formic acid in H_2O and B = 1% H_2O and 0.1% formic acid in CH_3CN. Data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1. LC-MS Program: Waters Xbridge C18 column (2.1 × 100 mm, 3.5 µm); flow rate = 0.4 mL min^{-1}, runtime = 13 min, column T = 40 °C, mass detection: 100–1500 Da. Gradient: 0–0.4 min: 5% B; 0.4–9.0 min: 5% → 95% B; 9.0–11.2 min: 95% B; 11.2–11.3 min: 95% → 5% B; 11.3–13.00 min: 5% B. Electrospray Ionization (ESI) high-resolution mass spectrometry was carried on a Waters XEVO-G2 XS Q-TOF mass spectrometer equipped with an electrospray ion source in positive mode (capillary voltage: 3.0 kV, desolvation gas flow: 900 L h^{-1}, temperature: 60 °C) with a resolution R = 22,000 using 200 pg μL^{-1} Leu-Enk (*m/z* = 556.2771) as a "lock mass". Samples were run using 2 mobile phases: A = 0.1% formic acid in H_2O and B = 0.1% formic acid in CH_3CN on a Waters Acquity UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm); flow rate = 0.6 mL min^{-1},

runtime = 3.00 min, column T = 60 °C, mass detection: 50–1500 Da. Gradient: 0–0.15 min: 2% B; 0.15–1.85 min: 2% → 100% B; 1.85–2.05: 100% B; 2.05–2.10 min: 100% → 2% B; 2.10–3.00 min: 100% B. Data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1.

HPLC purifications were performed on a Waters preparative automated HPLC with mass detection. Samples were run using 3 mobile phases: A = H_2O, B = CH_3CN and C = 1% 4M NH_4OH in CH_3CN on a Xbridge PREP C18 column (5 µm 19 × 150 mm). Flowrate = 30 mL min^{-1}. Gradient: 0 – 2.5 min: 95% A, 5% B; 2.5 – 17.5 min: 5 → 40% B; 17.5 – 20.90 min: 40 → 95% B; 20.90 – 21.00 min: 95 → 5% B; 1 mL min^{-1} C was mixed throughout the whole run. Fractions containing the product were automatically collected based on observed mass (detection range 100–1500 Da) and UV-signal after which they were lyophilized to obtain the pure products.

Synthetic procedures



(9*H*-fluoren-9-yl)methyl 2-amino-6,7-dihydrothiazolo[5,4-*c*]pyridine-5(4*H*)-carboxylate (4). *Step 1*: 4-Piperidone monohydrate hydrochloride (10.0 g, 65.1 mmol, 1.0 eq.) was dissolved in AcOH (50 mL). 33% HBr in AcOH (0.82 mL) and bromine (1.67 mL, 32.55 mmol, 0.5 eq.) were added at rt. The resulting reaction mixture was stirred at rt for 1 hour. The reaction

mixture was concentrated *in vacuo* and the solids (white/yellow) were suspended in acetone (100 mL). The suspension was refluxed at 63 °C for 2 hours. The solid material was collected in a glass filter and the solids were washed with acetone under reduced pressure to obtain 3-bromopiperidin-4-one-hydrobromide as an off-white powder (12.5 g, 48.3 mmol). This material was not further purified and was used as such.

Step 2: 3-bromopiperidin-4-one-hydrobromide (12.5 g, 48.3 mmol, 1.0 eq.) was dissolved in EtOH (150 mL) and thiourea (3.67 g, 48.3 mmol, 1.0 eq.) was added. The resulting reaction mixture was heated at 80 °C for 18 hours. The formed precipitates were collected in a glass filter and were washed with EtOH under reduced pressure to yield 4,5,6,7-tetrahydrothiazolo[5,4-*c*]pyridine-2-amine hydrobromide as an off-white powder (7.4 g, 31.1 mmol).

Step 3: To a solution of 4,5,6,7-tetrahydrothiazolo[5,4-*c*]pyridine-2-amine hydrobromide (7.34 g, 31.1 mmol, 1.0 eq.) in 1,4-dioxane (45 mL) were added H_2O (75 mL) and K_2CO_3 (8.59 g, 62.1 mmol, 2.0 eq.). The resulting reaction mixture was cooled to 0 °C and Fmoc-OSu (15.72 g, 46.6 mmol, 1.5 eq.) was added. The reaction mixture was allowed to warm to rt and was stirred for 18 hours. The reaction mixture was poured into H_2O (500 mL) and the aqueous layer was extracted 3× with DCM. The combined organic layers were washed with BRINE (500 mL), dried over Na_2SO_4, filtered and concentrated. The resulting residue was purified by Büchi flash chromatography (DCM → 4% MeOH/DCM) to yield the titled compound as a white solid (4.43 g, 11.7 mmol, 38%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.90 (d, J = 7.4 Hz, 2*H*), 7.63 (s, 2*H*), 7.48 – 7.27 (m, 4*H*), 6.82 (s, 2*H*), 4.48 – 4.25 (m, 5*H*), 3.68 – 3.42 (m, 2*H*), 2.47 – 2.24 (m, 2*H*). HR-MS calculated for $C_{21}H_{19}N_3O_2S$ $[M+H]^+$ 378.1276, found 378.1269.

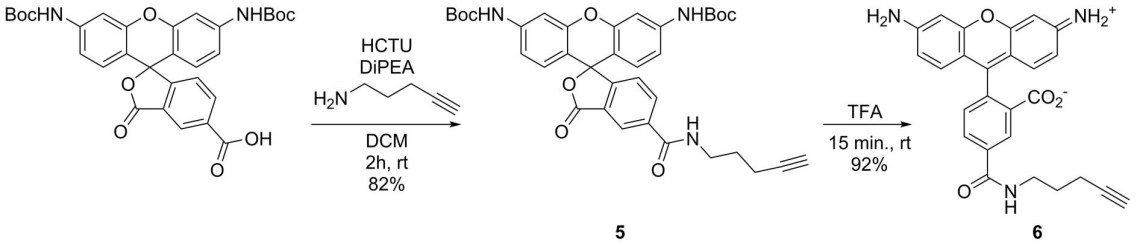
(9*H*-fluoren-9-yl)methyl (*S*)-2-(1-(*tert*-butoxycarbonyl)pyrrolidine-3-carboxamido)-6,7-dihydrothiazolo[5,4-*c*]pyridine-5(4*H*)-carboxylate (2). To a solution of (*S*)-1-Boc-1-pyrrolidine-3-carboxylic acid (3.4 g, 15.8 mmol, 1.5 eq) in DMF (80 mL) was added HCTU (6.54 g, 15.8 mmol, 1.5 eq) and DiPEA (5.5 mL, 31.6 mmol, 3.0 eq.) and the resulting reaction mixture was stirred for 10 min. at rt. Compound **4** (3.98 mmol, 10.5 mmol, 1.0 eq.) was added and the reaction mixture was stirred at rt for 18 hours. The solvents were evaporated under reduced pressure. The crude material was taken up in EtOAc (200 mL) and the organic layer was washed with 1M HCl (2× 100 mL), sat. aq. $NaHCO_3$ (2× 100 mL) and BRINE (100 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The resulting residue was purified by Büchi flash chromatography (DCM → 4% MeOH/DCM) to yield **2** as a white foam

(5.44 g, 9.5 mmol, 90%). ^{1}H NMR (300 MHz, $CDCl_3$-*d*) δ 9.82 (s, 1*H*), 7.77 (s, 2*H*), 7.58 (s, 2*H*), 7.41 (t, *J* = 7.4 Hz, 2*H*), 7.32 (t, *J* = 7.4 Hz, 2H), 4.72 – 4.45 (m, 4*H*), 4.29 (t, *J* = 6.6 Hz, 1*H*), 3.85 – 3.51 (m, 5*H*), 3.51 – 3.35 (m, 1*H*), 3.13 (s, 1*H*), 2.69 (s, 2*H*), 2.36 – 2.12 (s, 2*H*), 1.49 (s, 9*H*). ^{13}C NMR (75 MHz, $CDCl_3$-*d*) δ 170.2, 156.2, 155.3, 154.3, 143.8, 141.3, 127.8, 127.1, 124.9, 120.0, 79.8, 67.5, 48.3, 47.3, 45.4, 41.7, 28.5. HR-MS calculated for $C_{31}H_{34}N_4O_5S$ $[M+H]^+$ 575.2328, found 575.2336.

***Tert*-butyl(*S*)-3-((5-(2-azidoacetyl)-4,5,6,7-tetrahydrothiazolo[5,4-*c*]pyridin-2-yl) carbamoyl) pyrrolidine-1-carboxylate (3).** To a solution of **2** (500 mg, 0.87 mmol, 1.0 eq.) in DMF (10 mL) was added 1,8-Diazabicyclo[5.4.0]undec-7-ene (65 µL, 0.44 mmol, 0.5 eq.). The resulting reaction mixture was stirred at rt for 2.5 hours. Complete Fmoc removal was confirmed by TLC and LC-MS. 1-Hydroxybenzotriazole hydrate (176 mg, 1.3 mmol, 1.5 eq.) was added and the reaction mixture was stirred at rt for 30 min. In a separate round bottom flask 2-Azidoacetic acid (228 µL, 3.0 mmol, 3.5 eq.) was dissolved in DMF (5 mL) and HCTU (1.26 g, 3.0 mmol, 3.5 eq.) and DiPEA (758 µL, 4.4 mmol, 5.0 eq.) were added. The resulting reaction mixture was stirred at rt for 10 min. This reaction mixture was added to the first reaction mixture and the whole was stirred at rt for 18 hours. The reaction mixture was concentrated *in vacuo* and the crude material was taken up in EtOAc (100 mL). The organic layer was washed with 1M HCl (2× 100 mL), sat. aq. $NaHCO_3$ (2× 100 mL) and BRINE (100 mL). The 1M HCl phase was extracted with EtOAc (50 mL) and the sat. aq. $NaHCO_3$ phase was extracted with EtOAc (50 mL). All organic layers were combined, dried over Na_2SO_4, filtered and concentrated. The resulting residue was purified by Büchi flash chromatography (DCM → 5% MeOH/DCM) to yield **3** as a white foam (292 mg, 0.67 mmol, 77%). ^{1}H NMR (300 MHz, $CDCl_3$-*d*) (Mixture of rotamers) δ 4.70 (s, 1*H*), 4.47 (s, 1*H*), 3.99 (d, *J* = 8.8 Hz, 2*H*), 3.92 – 3.82 (m, 1*H*), 3.74 – 3.42 (m, 4*H*), 3.32 (dt, *J* = 10.7, 7.7 Hz, 1*H*), 3.20 – 3.03 (m, 1*H*), 2.78 – 2.70 (m, 1*H*), 2.70 – 2.61 (m, 1*H*), 2.29 – 2.04 (m, 2*H*), 1.38 (s, 9*H*). ^{13}C NMR (75 MHz, $CDCl_3$-*d*) (Mixture of rotamers) δ 166.4, 166.3, 156.6, 154.3, 143.8, 141.6, 119.4, 117.6, 79.8, 51.1, 51.0, 48.4, 45.4, 44.5, 43.6, 42.9, 42.7, 40.4, 40.2, 28.9, 28.5, 27.1, 26.2. HR-MS calculated for $C_{18}H_{25}N_7O_4S$ $[M+H]^+$ 436.1767, found 436.1755.

(*S*)-*N*-(5-(2-azidoacetyl)-4,5,6,7-tetrahydrothiazolo[5,4-*c*]pyridin-2-yl)-1-cyanopyrrolidine-3-carboxamide (8RK64). *Step 1:* To a solution of **3** (40.0 mg, 92 µmol, 1.0 eq.) in DCM (1.0 mL) was added TFA (1.0 mL). The resulting reaction mixture was stirred at

rt for 2 hours. The solvents were evaporated under reduced pressure and the residue was co-evaporated with DCM (3× 5.0 mL). This material was not further purified and was used as such. *Step 2:* The TFA-salt from Step 1 was dissolved in MeOH (1.0 mL) and NaOAc (38 mg, 0.46 mmol, 5.0 eq.) and cyanogen bromide (39 mg, 0.37 mmol, 4.0 eq.) were added. The resulting reaction mixture was stirred at rt for 18 hours. The solvents were evaporated under reduced pressure and the crude material was taken up in EtOAc (25 mL). The organic layer was washed with sat. $NaHCO_3$ (2× 25 mL) and BRINE (25 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The resulting residue was purified by Büchi flash chromatography (DCM → 5% MeOH/DCM) to yield **8RK64** as a white foam (32 mg, 89 µmol, 97%). ^{1}H NMR (300 MHz, $CDCl_3$-*d*) (Mixture of rotamers) δ 4.80 (s, 1*H*), 4.58 (s, 1*H*), 4.10 (d, *J* = 10.4 Hz, 2*H*), 4.01 – 3.94 (m, 1*H*), 3.75 (s, 1*H*), 3.72 (s, 2*H*), 3.69 – 3.60 (m, 1*H*), 3.59 – 3.47 (m, 1*H*), 3.39 – 3.23 (m, 1*H*), 2.89 – 2.81 (m, 1*H*), 2.81 – 2.72 (m, 1*H*), 2.31 (q, *J* = 7.2 Hz, 2*H*). ^{13}C NMR (75 MHz, $CDCl_3$-*d*) (Mixture of rotamers) δ 169.5, 169.2, 166.5, 166.4, 156.4, 156.3, 143.8, 141.7, 119.6, 117.9, 116.8, 52.6, 51.2, 51.0, 50.3, 44.2, 42.9, 42.7, 40.4, 40.3, 29.5. HR-MS calculated for $C_{14}H_{16}N_8O_2S$ $[M+H]^+$ 361.1195, found 361.1197.



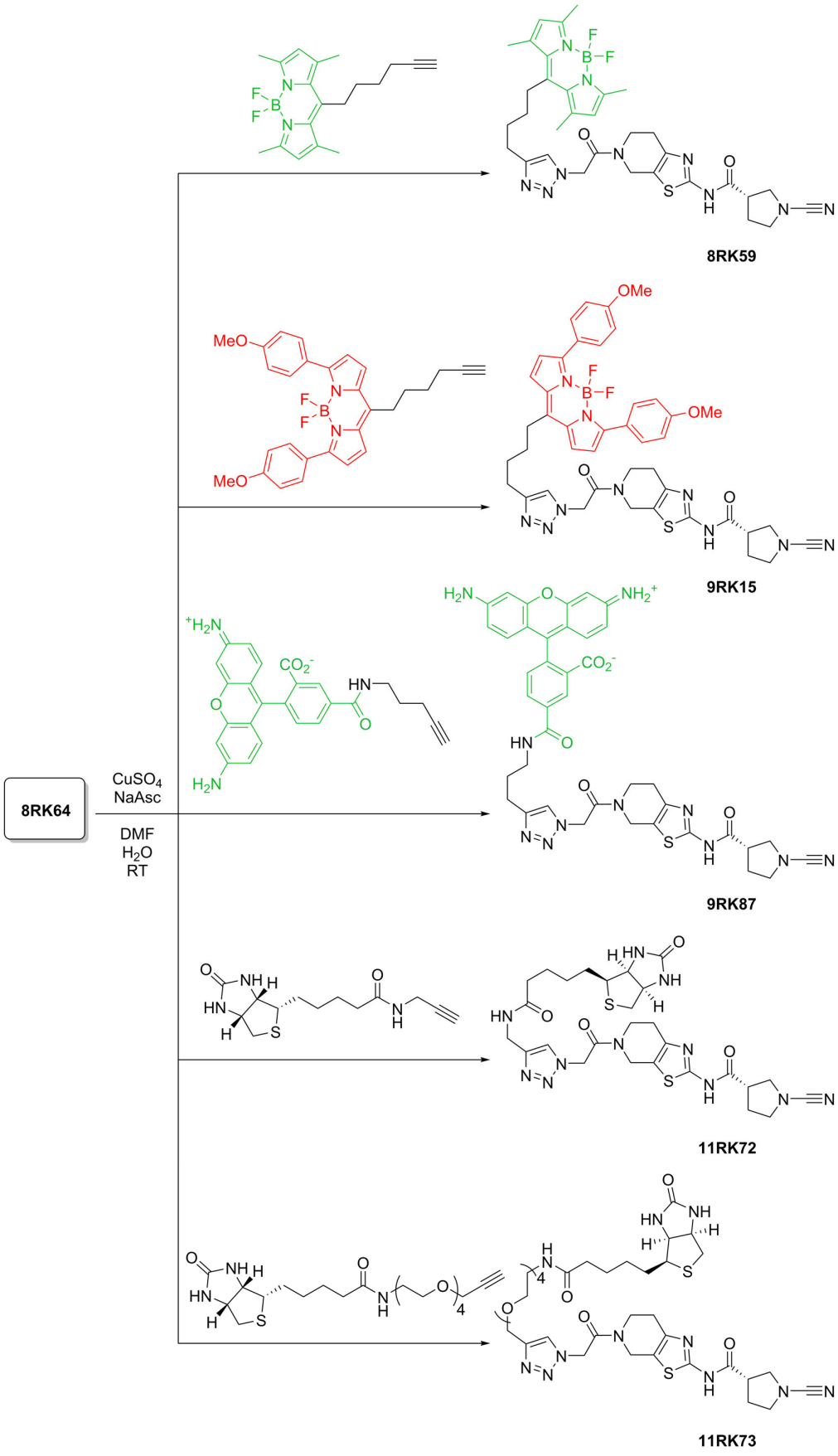
Di-*tert*-butyl (3-oxo-5-(pent-4-yn-1-ylcarbamoyl)-3*H*-spiro[isobenzofuran-1,9'-xanthene]-3',6'-diyl)dicarbamate (5). 3',6'-bis((*tert*-butoxycarbonyl)amino)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthene]-5-carboxylic acid[1] (127 mg, 0.22 mmol, 1.0 eq.) was dissolved in DCM (5 mL). To this were added DiPEA (153 µL, 0.88 mmol, 4.0 eq.), HCTU (137 mg, 0.33 mmol, 1.5 eq.) and pent-4-yn-1-amine (34 µL, 0.33 mmol, 1.5 eq.) and the mixture was stirred at rt until TLC indicated a complete conversion of starting material after 2 hours. The mixture was diluted with DCM (50 mL) and extracted with 1M HCl (2× 50 mL), sat. aq. $NaHCO_3$ (2× 50 mL) and BRINE (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The crude material was purified by Büchi flash chromatography (5 → 50% EtOAc/*n*-hept.) and the product was obtained as a colorless solid (115 mg, 0.18 mmol, 82%).

^{1}H NMR (300 MHz, $CDCl_3$-*d*) δ 8.4 (d, 1*H*), 8.2 (dd, *J* = 8.0, 1.6 Hz, 1*H*), 7.5 (d, *J* = 2.2 Hz, 2*H*), 7.2 (t, *J* = 5.8 Hz, 1*H*), 7.1 (dd, *J* = 8.0, 0.7 Hz, 1*H*), 7.0 (s, 2*H*), 6.9 (dd, *J* = 8.7, 2.2 Hz, 2*H*), 6.6 (d, *J* = 8.6 Hz, 2*H*), 3.6 (q, *J* = 6.5 Hz, 2*H*), 2.34 – 2.24 (m, 2*H*), 2.0 (t, *J* = 2.6 Hz, 1*H*), 1.9 (q, *J* = 6.8 Hz, 2*H*), 1.5 (s, 18*H*). ^{13}C NMR (75 MHz, $CDCl_3$-*d*) δ 168.9, 165.8, 155.4, 152.4, 151.8, 140.9, 136.6, 134.7, 128.2, 126.7, 124.3, 123.2, 114.3, 112.1, 106.2, 83.5, 81.1, 69.4, 39.6, 28.2, 27.8, 16.2. HR-MS calculated for $C_{36}H_{37}N_3O_8$ $[M+H]^+$ 640.2659, found 640.2654.

2-(6-amino-3-iminio-3*H*-xanthen-9-yl)-5-(pent-4-yn-1-ylcarbamoyl)benzoate (6). Compound **5** (115 mg, 0.18 mmol) was dissolved in DCM (3 mL) after which TFA (3 mL) was added. The mixture was stirred at rt until TLC and LC-MS analysis indicated a complete reaction. Toluene (15 mL) was added and the reaction was concentrated under reduced pressure, followed by co-evaporation with toluene (2×). The product was dissolved in H_2O/CH_3CN/AcOH 1:1:0.1 v/v/v (10 mL) and lyophilized, which yielded the product as a red/brown solid (91 mg, 0.16 mmol, 92%). ^{1}H NMR (300 MHz, MeOD-d_4) δ 8.76 (d, *J* = 1.8 Hz, 1*H*), 8.24 (dd, *J* = 7.9, 1.9 Hz, 1*H*), 7.50 (d, *J* = 8.0 Hz, 1*H*), 7.03 (d, *J* = 9.6 Hz, 2*H*), 6.79 (m, 4*H*), 3.66 – 3.49 (m, 2*H*), 2.42 – 2.21 (m, 3*H*), 1.98 – 1.92 (m, 2*H*). ^{13}C NMR (75 MHz, MeOD-d_4) δ 168.3, 167.5, 161.2, 161.0, 159.6, 138.1, 137.6, 132.9, 132.7, 132.2, 131.9, 131.2, 117.9, 114.7, 98.4, 84.2, 70.1, 40.4, 29.4, 16.9. HR-MS calculated for $C_{26}H_{21}N_3O_4$ $[M+H]^+$ 440.1610, found 440.1627.

General procedure for click chemistry

Compound **8RK64** (1.0 eq.) and the appropriate alkyne (1.2 eq.) were dissolved in dry DMF (1-2 mL). Argon was bubbled through the reaction mixture for 30 min. An aqueous solution of $CuSO_4{\cdot}5H_2O$ (100 µL, 0.50 eq.) and an aqueous solution of sodium ascorbate (100 µL, 0.75 eq.) were added. The aqueous solutions of sodium ascorbate and $CuSO_4.5H_2O$ were prepared in 5.0 mL volume and degassed for 30 min. with argon bubbling. After addition of sodium ascorbate and $CuSO_4{\cdot}5H_2O$ the resulting reaction mixture was stirred at rt for 2-18 hours, concentrated under reduced pressure and purified as described. In case necessary, excess alkyne was reacted with 2-Azidoacetic acid for ease of purification.


8RK64
CuSO4
NaAsc
DMF
H2O
RT
8RK59
9RK15
9RK87
11RK72
11RK73

BodipyFL probe 8RK59. Synthesized according to the general procedure for click chemistry using **8RK64** (25.0 mg, 69 µmol) and BodipyFL-alkyne[2] (27.3 mg, 83 µmol). The crude material was taken up in DCM (20 mL). The organic layer was washed with BRINE (10 mL), dried over Na_2SO_4, filtered and concentrated. The resulting residue was purified by Büchi flash chromatography (DCM → 5% MeOH/DCM) to yield **8RK59** as a red solid (10 mg, 14 µmol, 21%). ^{1}H NMR (300 MHz, DMSO-*d*$_6$) (Mixture of rotamers) δ 12.37 – 12.22 (m, 1*H*), 7.81 (d, *J* = 1.9 Hz, 1*H*), 6.26 (s, 2*H*), 5.54 (d, *J* = 20.1 Hz, 2*H*), 4.73 (d, *J* = 36.4 Hz, 2*H*), 3.85 (m, 2*H*), 3.72 – 3.43 (m, 4*H*), 3.07 – 2.95 (m, 2*H*), 2.89 – 2.67 (m, 4*H*), 2.43 (m, 12*H*), 2.30 – 2.01 (m, 3*H*), 1.87 (m, 2*H*), 1.67 (d, 2*H*). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) (Mixture of rotamers) δ 170.8, 165.5, 165.3, 157.6, 156.5, 153.5, 147.1, 146.6, 143.6, 143.2, 141.3, 131.1, 124.2, 122.1, 118.6, 118.3, 117.5, 52.5, 51.3, 51.1, 50.4, 48.5, 43.5, 42.6, 42.2, 31.2, 29.9, 29.7, 28.1, 27.1, 26.3, 25.0, 16.3, 14.4. HR-MS calculated for $C_{33}H_{39}BF_2N_{10}O_2S$ $[M\text{-}F]^+$ 669.3061, found 669.3063.

BodipyTMR probe 9RK15. Synthesized according to the general procedure for click chemistry using **8RK64** (24.0 mg, 67 µmol) and BodipyTMR-alkyne[2] (39.0 mg, 81 µmol). The crude material was taken up in EtOAc (30 mL). The organic layer was washed with H_2O (10 mL), dried over Na_2SO_4, filtered and concentrated. The resulting residue was purified by Büchi flash chromatography (DCM → 5% MeOH/DCM) to yield **9RK15** as a red solid (17.7 mg, 21 µmol, 31%). ^{1}H NMR (300 MHz, DMSO-*d*$_6$) (Mixture of rotamers) δ 12.27 (d, *J* = 10.7 Hz, 1*H*), 7.76 – 7.86 (m, 5*H*), 7.68 – 7.62 (m, 2*H*), 7.05 – 6.98 (m, 4*H*), 6.80 (d, *J* = 4.3 Hz, 2*H*), 5.51 (d, *J* = 20.0 Hz, 2*H*), 4.70 (d, *J* = 34.1 Hz, 2*H*), 3.85 – 3.77 (m, 8*H*), 3.63 – 3.57 (m, 1*H*), 3.57 – 3.49 (m, 1*H*), 3.49 – 3.40 (m, 2*H*), 3.16 – 3.03 (m, 2*H*), 2.85 – 2.77 (s, 1*H*), 2.77 – 2.61 (m, 3*H*), 2.30 – 1.97 (m, 3*H*), 1.79 (m, 2.30 – 1.97, 4*H*). ^{13}C NMR (75 MHz, DMSO-*d*$_6$) (Mixture of rotamers) δ 170.9, 170.8, 165.5, 165.3, 160.7, 157.0, 156.6, 156.5, 146.7, 146.6, 143.6, 143.2, 136.2, 131.2, 125.1, 124.1, 120.7, 118.6, 118.3, 117.5, 114.2, 73.5, 72.7, 70.2, 55.7, 52.5, 51.3, 51.1, 50.4, 43.5, 42.6, 42.2, 33.7, 30.0, 29.7, 29.5, 29.2, 27.1, 26.3, 25.1. HR-MS calculated for $C_{43}H_{43}BF_2N_{10}O_4S$ $[M\text{-}F]^+$ 825.3274, found 825.3346.

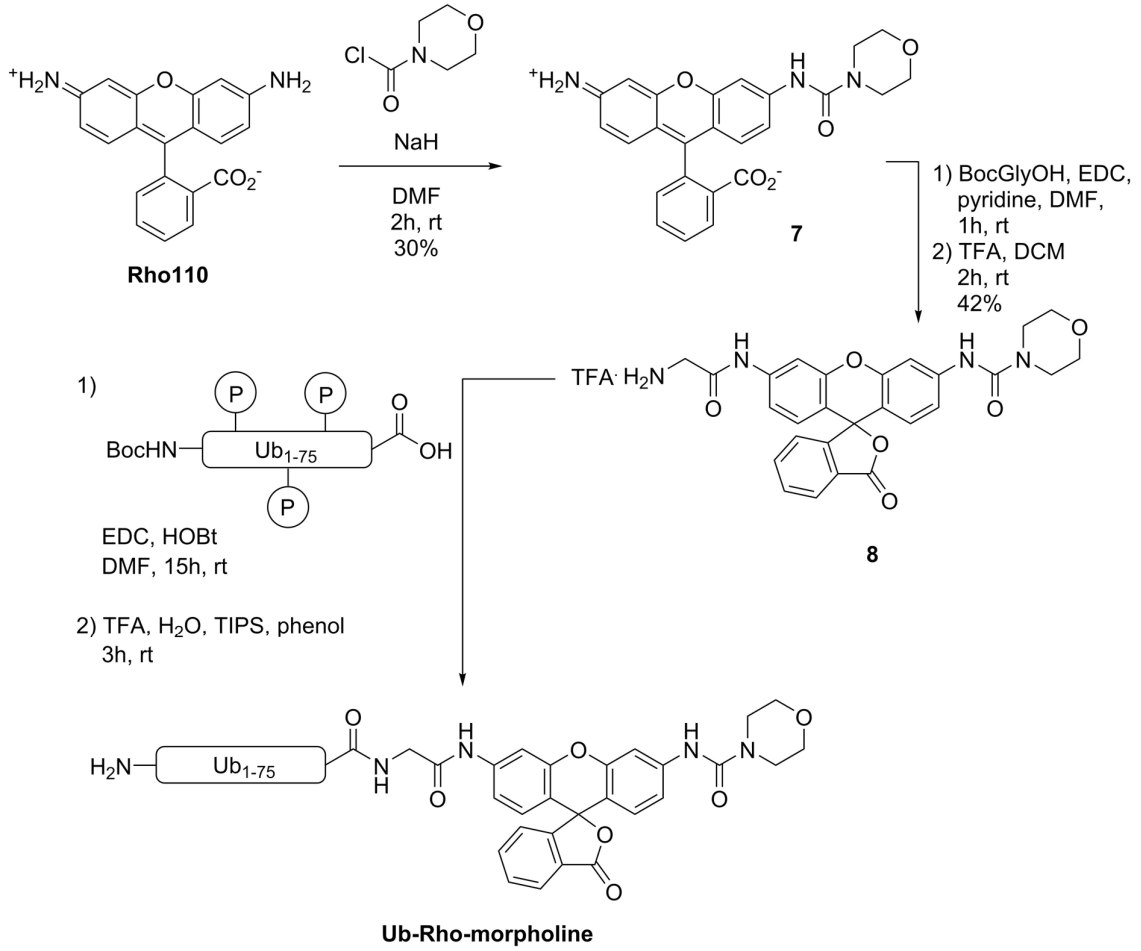
Rhodamine probe 9RK87. Synthesized according to the general procedure for click chemistry using **8RK64** (18.57 mg, 52 µmol) and **6** (39.17 mg, 62 µmol). The crude material was purified by preparative HPLC to yield **9RK87** as a red solid (3.56 mg, 4.45 µmol, 9%). ^{1}H NMR (300 MHz, DMSO-*d*$_6$) (Mixture of rotamers) δ 12.27 (m, 1*H*), 8.88 (t, *J* = 5.6 Hz, 1*H*), 8.44 (s, 1*H*),

8.24 (dd, *J* = 8.0, 1.5 Hz, 1*H*), 7.82 (d, *J* = 2.9 Hz, 1*H*), 7.33 (d, *J* = 8.0 Hz, 1*H*), 6.44 – 6.26 (m, 5*H*), 5.73 – 5.47 (m, 5*H*), 4.71 (d, *J* = 37.0 Hz, 2*H*), 3.83 (d, *J* = 5.3 Hz, 2*H*), 3.68 – 3.57 (m, 1*H*), 3.54 (dd, *J* = 6.1, 3.7 Hz, 1*H*), 3.48 – 3.36 (m, 5*H*), 2.86 – 2.78 (m, 1*H*), 2.78 – 2.62 (m, 3*H*), 2.25 – 2.12 (m, 1*H*), 2.12 – 1.99 (m, 1*H*), 1.98 – 1.84 (m, 2*H*). HR-MS calculated for $C_{40}H_{37}N_{11}O_6S$ $[M+H]^+$ 800.2727, found 800.2749.

Biotin probe 11RK72. Synthesized according to the general procedure for click chemistry using **8RK64** (13.0 mg, 36 µmol) and Biotin-alkyne[3] (12.20 mg, 43 µmol). The crude material was purified by preparative HPLC to yield **11RK72** as a white solid (7.23 mg, 11.3 µmol, 31%). HR-MS calculated for $C_{27}H_{35}N_{11}O_4S_2$ $[M+H]^+$ 642.2393, found 642.2393.

Biotin-PEG$_4$ probe 11RK73. Synthesized according to the general procedure for click chemistry using **8RK64** (13.0 mg, 36 µmol) and BiotinPEG$_4$-alkyne (19.8 mg, 43 µmol). The crude material was purified by preparative HPLC to yield **11RK73** as a white solid (5.24 mg, 6.4 µmol, 18%). HR-MS calculated for $C_{35}H_{51}N_{11}O_8S_2$ $[M+H]^+$ 818.3442, found 818.3470.

Synthesis of Ub-Rho-morpholine



2-(3-iminio-6-(morpholine-4-carboxamido)-3*H*-xanthen-9-yl)benzoate (7).[4]

Rhodamine110 (500 mg, 1.36 mmol, 1.0 eq.) was dissolved in anhydrous DMF (50 mL) and flushed with argon. NaH (60% w/w in mineral oil, 114 mg, 2.86 mmol, 2.1 eq.) was carefully added in 3 portions and the solution was stirred for 1 hour at rt. 4-Morpholinecarbonyl chloride (156 µL, 1.36 mmol, 1.0 eq.) was added dropwise and the mixture was stirred for another 3 hours. LC-MS analysis indicated the formation of a mixture of unreacted, 1× and 2× morpholinecarbonyl-coupled rhodamine. The mixture was concentrated to dryness under reduced pressure and purified by Büchi flash chromatography (1% AcOH/DCM → 1% AcOH/ (10% MeOH/DCM)) to yield the titled compound as an orange solid (180 mg, 0.40 mmol, 30%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.94 (d, J = 6.5 Hz, 1H), 7.65 – 7.52 (m, 2H), 7.48 (s, 1H), 7.40 (d, J = 2.0 Hz, 1H), 7.08 (d, J = 6.7 Hz, 1H), 6.85 (dd, J = 8.6, 2.1 Hz, 1H), 6.49 (dd, J = 12.4, 8.6 Hz, 2H), 6.38 (d, J = 2.2 Hz, 1H), 6.27 (dd, J = 8.5, 2.2 Hz, 1H), 3.58 (t, J = 4.8

Hz, 4H), 3.41 (d, J = 4.9 Hz, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.34, 155.11, 152.90, 151.98, 149.67, 141.76, 135.11, 129.76, 129.04, 128.13, 127.17, 125.03, 124.44, 115.75, 113.04, 111.88, 108.16, 107.67, 101.33, 66.52, 44.32, 43.59.

***N*-(3'-(2-aminoacetamido)-3-oxo-3*H*-spiro[isobenzofuran-1,9'-xanthen]-6'-yl)morpholine-4-carboxamide TFA salt (8).** Compound **7** (158 mg, 0.35 mmol, 1.0 eq.) was dissolved in a mixture of anhydrous DMF (3 mL) and pyridine (2 mL) and flushed with argon. EDC·HCl (135 mg, 0.70 mmol, 2.0 eq.) and 1-Hydroxybenzotriazole hydrate (123 mg, 0.70 mmol, 2.0 eq.) were added and the mixture was stirred at rt for 1 hour after which LC-MS analysis indicated complete conversion. The mixture was concentrated under reduced pressure, dissolved in DCM (20 mL) and extracted with 1M HCl (2× 20 mL) and BRINE (20 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated and the resulting white solid was dissolved in a 2:1 v/v mixture of DCM/TFA (5 mL) and stirred for 2 hours at rt until LC-MS analysis indicated complete consumption of starting material. The mixture was concentrated under reduced pressure, co-evaporated with (DCE 3× 20 mL) and purified by Büchi flash chromatography (DCM → 15% MeOH/DCM)) to yield the titled compound as a white solid (74 mg, 0.15 mmol, 42%). ^{1}H NMR (300 MHz, MeOD-d_4) δ 7.99 (dd, J = 7.2, 0.9 Hz, 1H), 7.74 (d, J = 2.0 Hz, 1H), 7.69 (ddd, J = 9.0, 7.2, 1.3 Hz, 2H), 7.48 (d, J = 2.1 Hz, 1H), 7.13 (ddd, J = 8.6, 5.2, 1.5 Hz, 2H), 7.05 (dd, J = 8.7, 2.2 Hz, 1H), 6.67 (d, J = 8.6 Hz, 1H), 6.61 (d, J = 8.7 Hz, 1H), 3.67 (dd, J = 5.7, 3.9 Hz, 4H), 3.49 (dd, J = 5.7, 4.0 Hz, 4H), 3.35 (s, 2H). ^{13}C NMR (75 MHz, Methanol-d_4) δ 171.32, 157.26, 154.32, 152.96, 152.83, 143.63, 141.73, 136.71, 131.23, 129.43, 129.02, 127.61, 125.89, 125.13, 117.24, 116.42, 115.36, 113.85, 108.56, 108.40, 84.47, 67.61, 45.58, 44.85.

Ub-Rho-morpholine. Step 1: Sidechain-protected Boc-Ub$_{1-75}$-COOH (~20 μmol) was obtained by Fmoc SPPS on trityl-resin as described in literature.[5] It was dissolved in DMF (4 mL) and to this were added EDC·HCl (10.9 mg, 60 μmol 3.0 eq.), HOBt (7.7 mg, 60 μmol, 3.0 eq.) and compound **8** (28.1 mg, 60 μmol, 3.0 eq.) and the mixture was stirred at rt for 15 hours before being concentrated to dryness under reduced pressure. The resulting residue was dissolved in a mixture of 60:30:10 v/v/v H_2O/CH_3CN/AcOH (5 mL) and lyophilized.

Step2: The resulting residue from step 1 was dissolved in a mixture of 90.5/5/2/2.5 v/v/v/v TFA/H_2O/TIPS/phenol (5 mL) and stirred for 3 hours at rt. The protein was precipitated from ice-cold Et_2O/*n*-pentane (3/1; v/v; 20 mL). The solution was centrifuged and Et_2O/*n*-pentane

(supernatant) was removed. The pellet was washed with Et_2O (20 mL), the solution was vortexed, the suspension was centrifuged and Et_2O was removed. The wash step was repeated twice. The pellet was dissolved in H_2O/CH_3CN/formic acid (65/25/10; v/v/v; 10 mL) and lyophilized. The protein was subsequently purified using RP-HPLC and the product was obtained as a white solid (99.7 mg, 11 μmol, 55%). LC-MS (6 min. run): R_t (min.) 1.87; deconvoluted mass: 8973.0.

References

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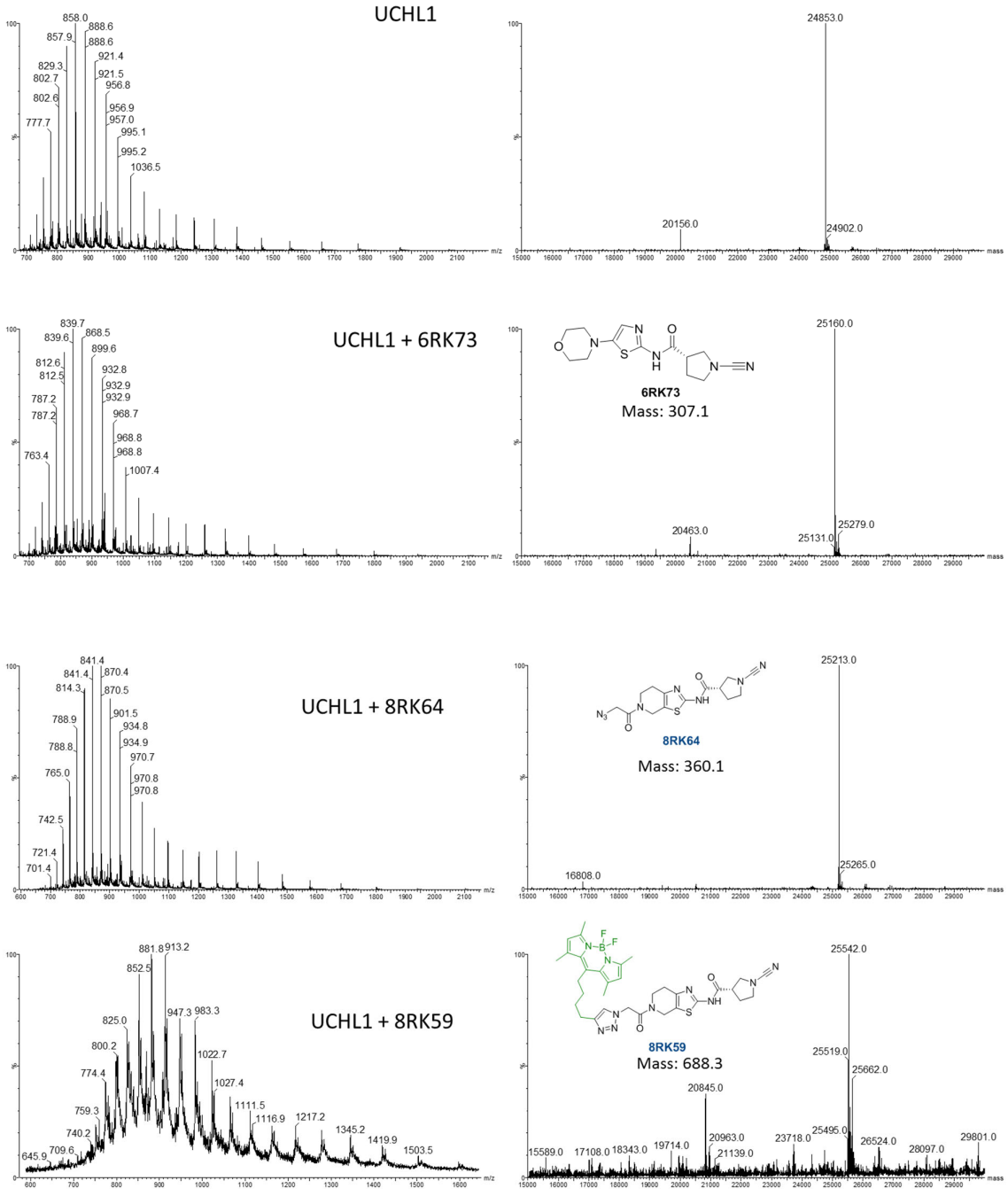
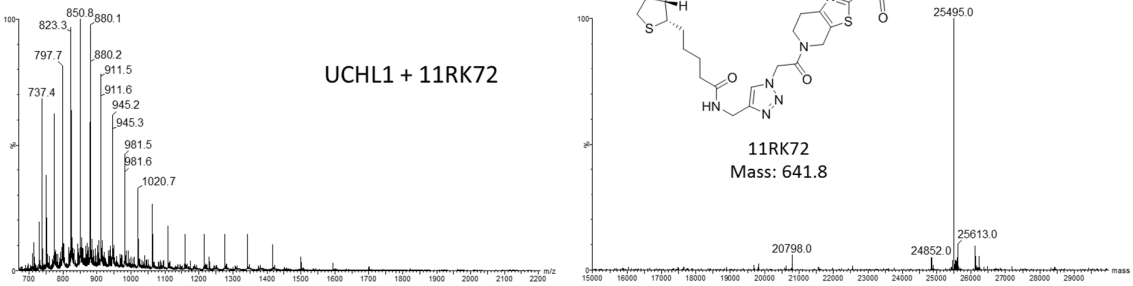
MS analysis of covalent complex formation between UCHL1 and inhibitors/probes




UCHL1 + 11RK72
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797.7
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911.5
737.4
911.6
945.2
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981.5
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m/z
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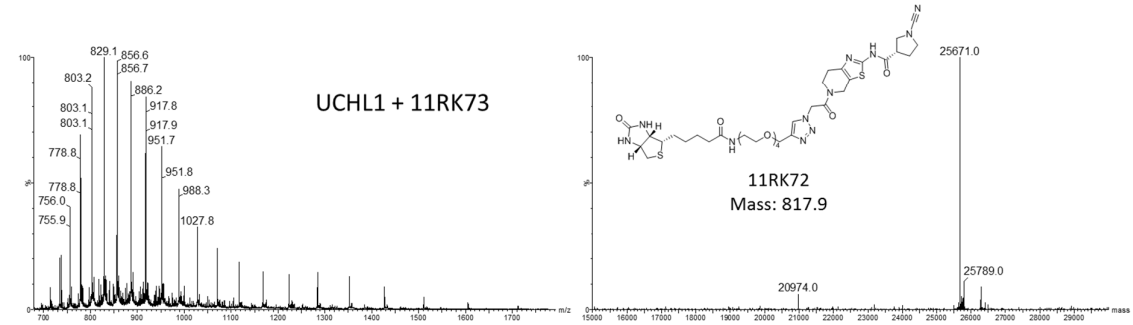
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778.8
951.8
778.8
988.3
756.0
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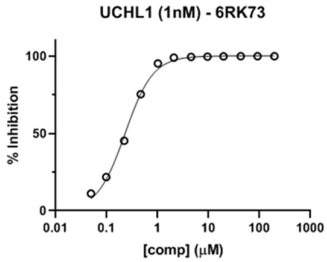
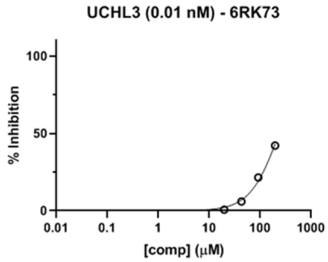
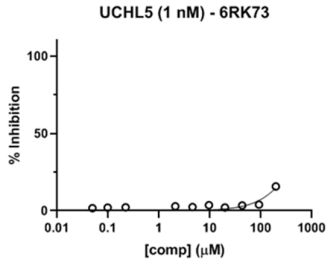
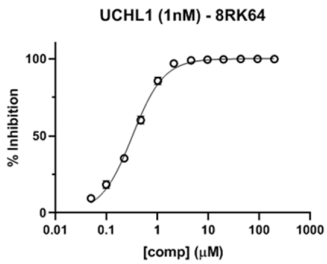
mass

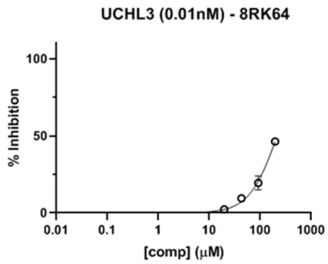
IC_{50} curves


UCHL1 (1nM) - 6RK73
% Inhibition
[comp] (μM)


UCHL3 (0.01 nM) - 6RK73
% Inhibition
[comp] (μM)


UCHL5 (1 nM) - 6RK73
% Inhibition
[comp] (μM)


UCHL1 (1nM) - 8RK64
% Inhibition
[comp] (μM)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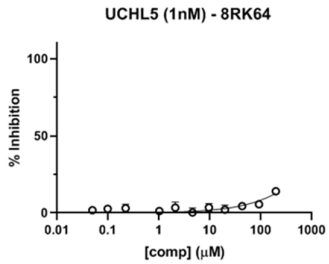
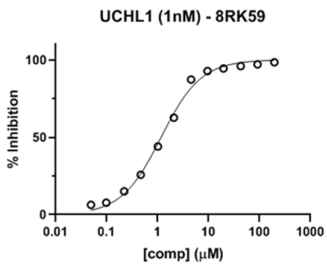
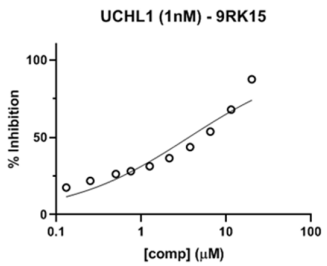
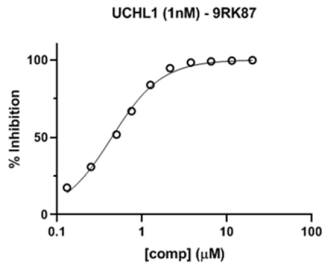
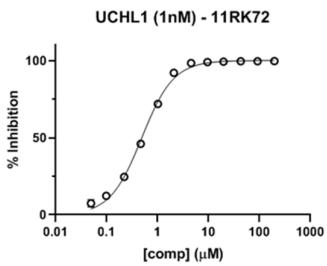
% Inhibition
[comp] (μM)

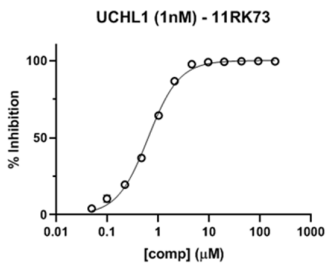

UCHL5 (1nM) - 8RK64
% Inhibition
[comp] (μM)

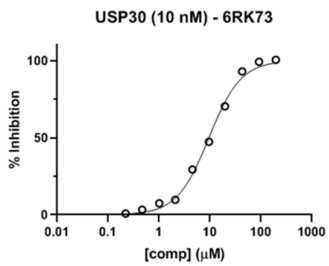

UCHL1 (1nM) - 8RK59
% Inhibition
[comp] (μM)

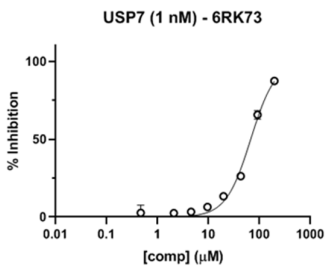

UCHL1 (1nM) - 9RK15
% Inhibition
[comp] (μM)

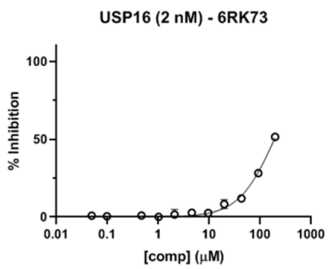

UCHL1 (1nM) - 9RK87
% Inhibition
[comp] (μM)

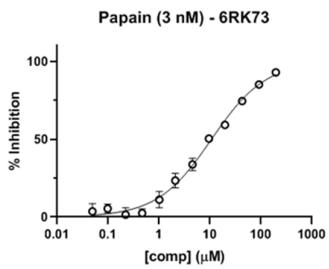

UCHL1 (1nM) - 11RK72
% Inhibition
[comp] (μM)


UCHL1 (1nM) - 11RK73
% Inhibition
[comp] (μM)


USP30 (10 nM) - 6RK73
% Inhibition
[comp] (μM)

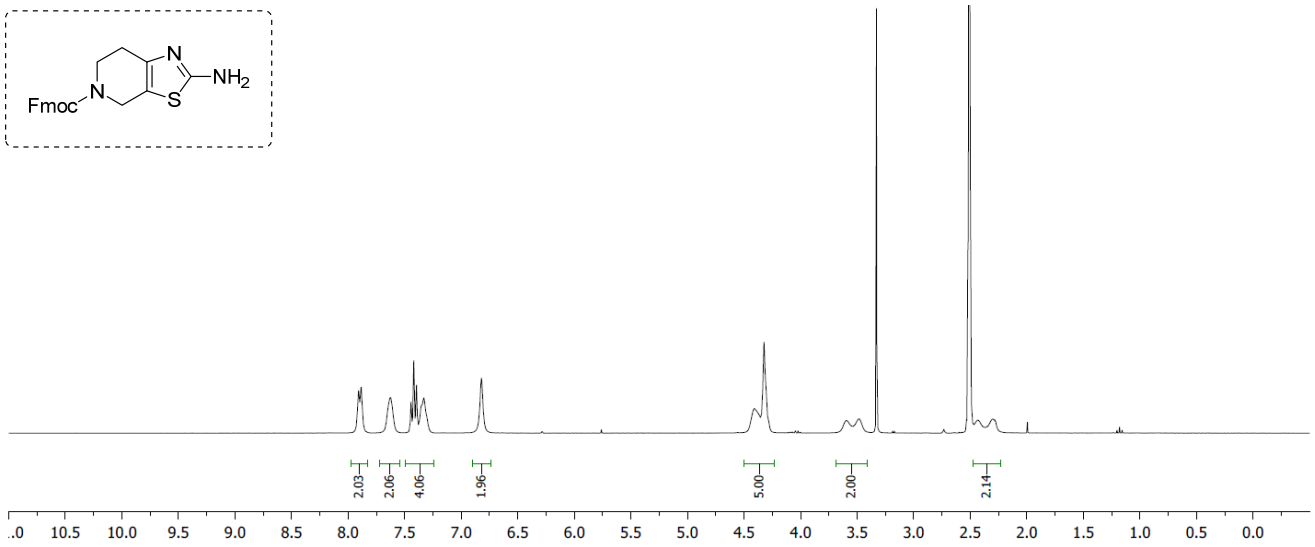

USP7 (1 nM) - 6RK73
% Inhibition
[comp] (μM)


USP16 (2 nM) - 6RK73
% Inhibition
[comp] (μM)

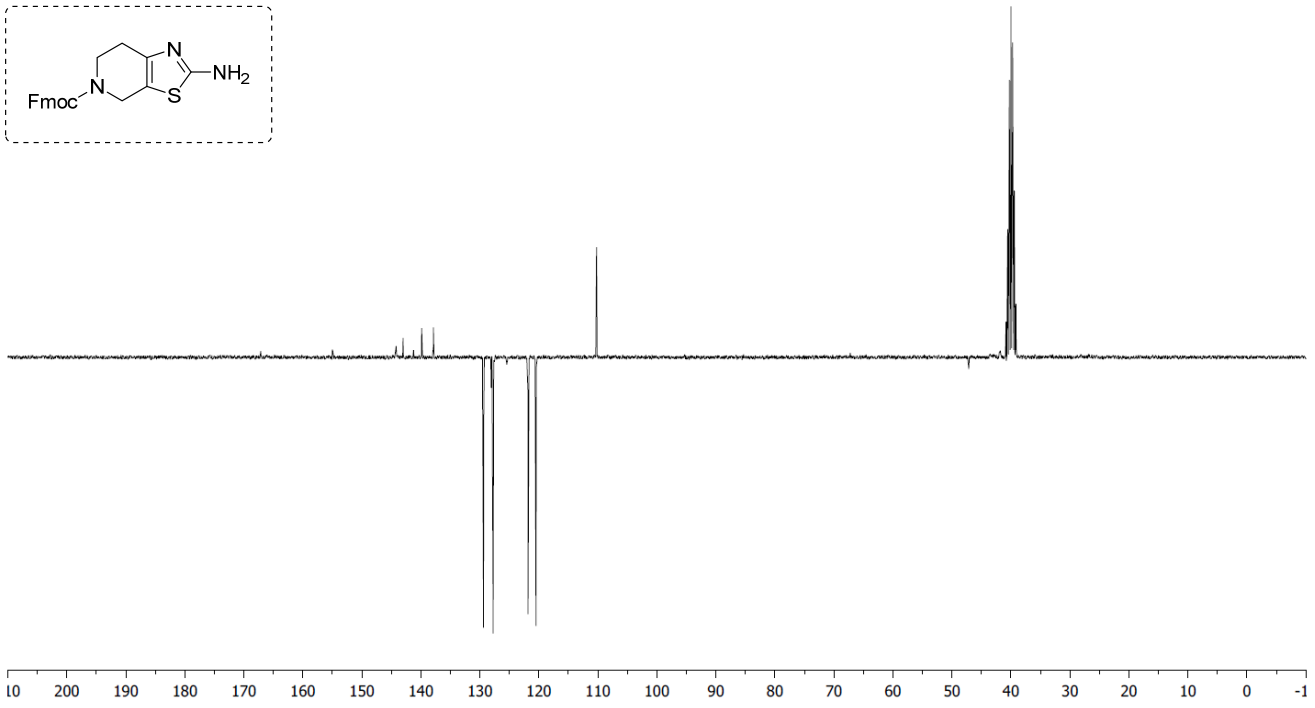

Papain (3 nM) - 6RK73
% Inhibition
[comp] (μM)

NMR & LC-MS analysis of synthesized compounds

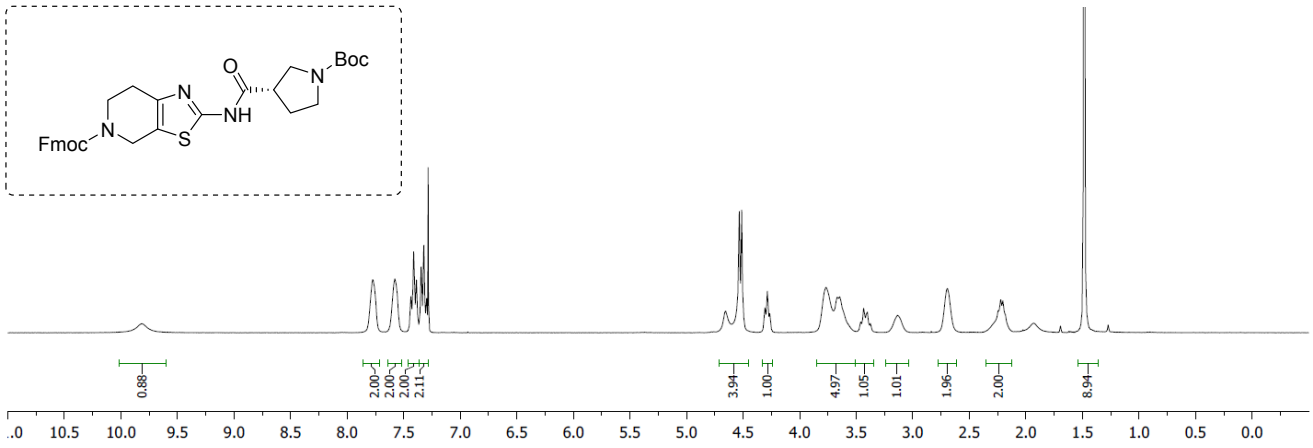
Compound 4: ^{1}H NMR (300.17 MHz, DMSO)



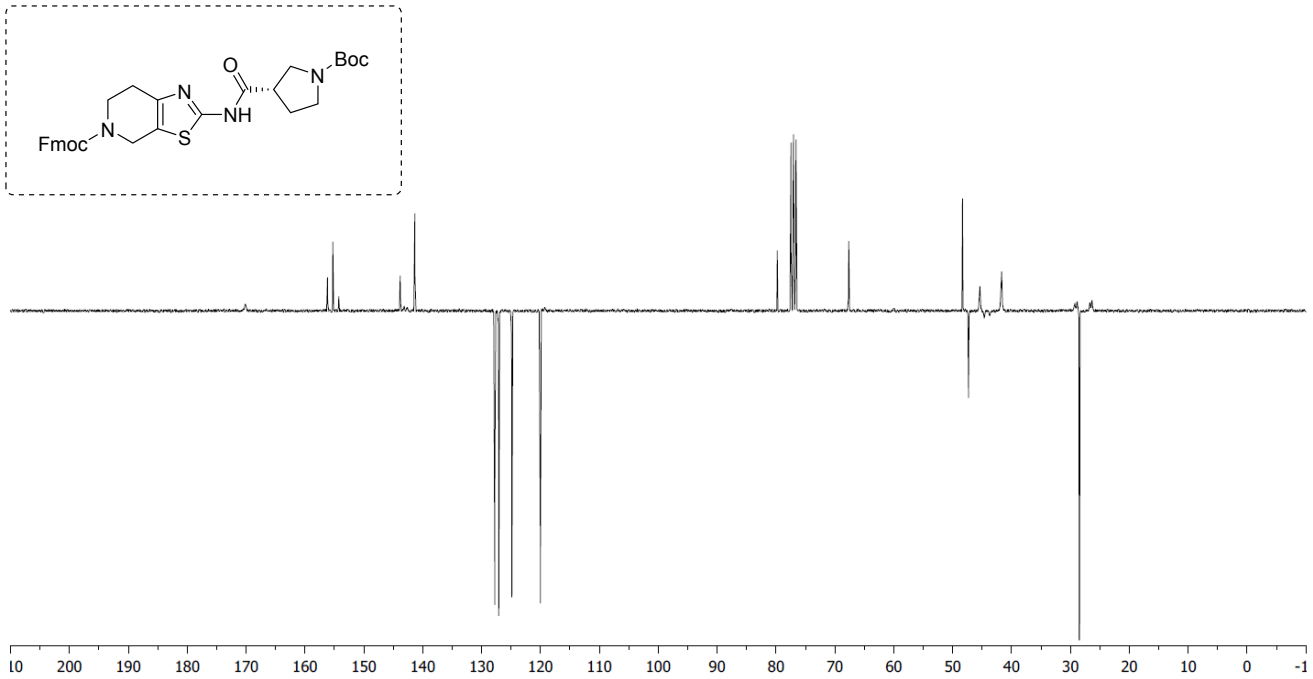
Compound 4: ^{13}C NMR (75.47 MHz, DMSO)



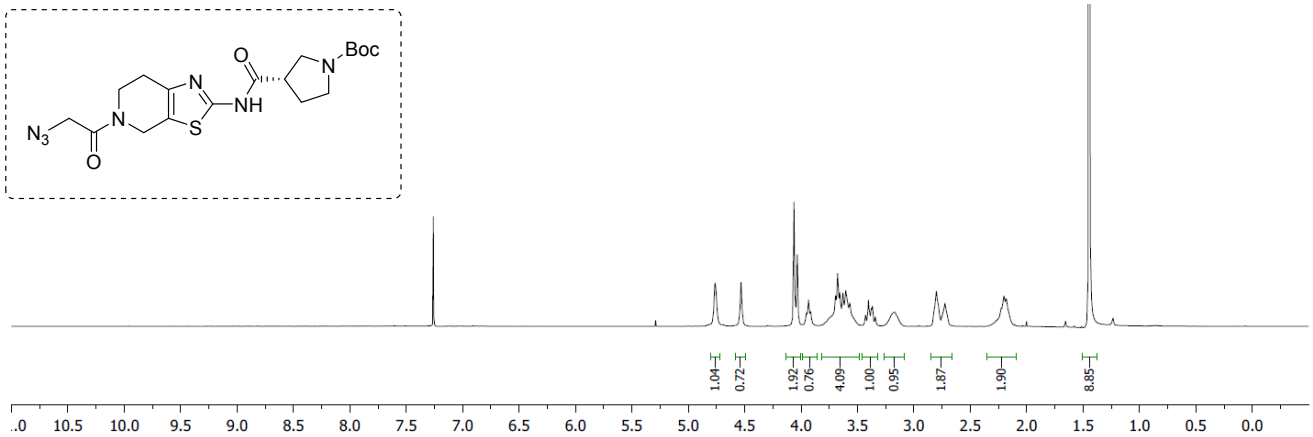
Compound 2: ^{1}H NMR (300.17 MHz, $CDCl_3$)



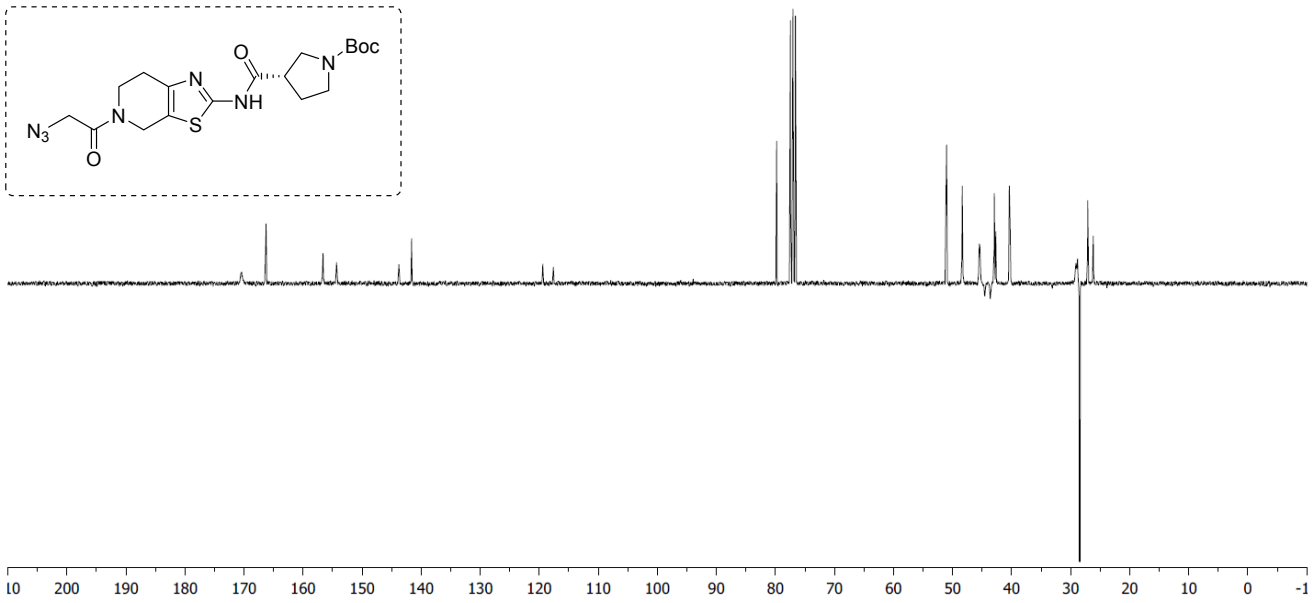
Compound 2: ^{13}C NMR (75.47 MHz, $CDCl_3$)



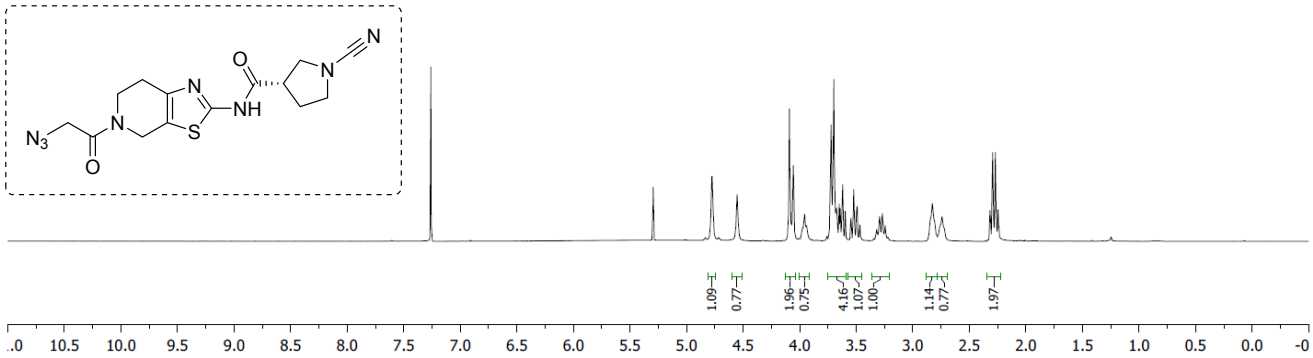
Compound 3: ^{1}H NMR (300.17 MHz, $CDCl_3$)



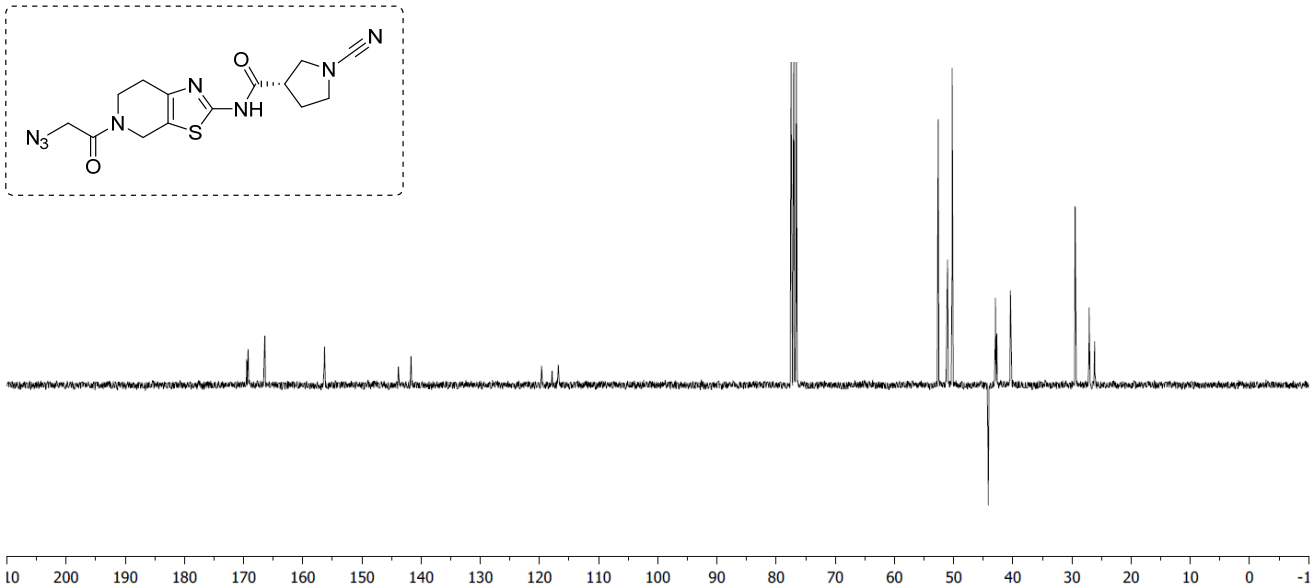
Compound 3: ^{13}C NMR (75.47 MHz, $CDCl_3$)



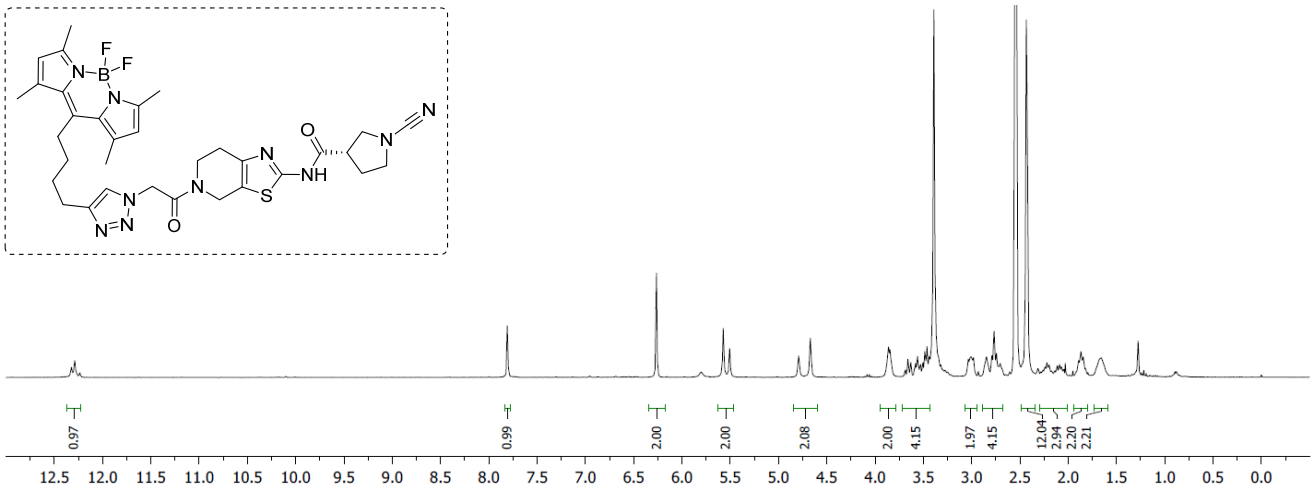
Compound 8RK64: ^{1}H NMR (300.17 MHz, $CDCl_3$)



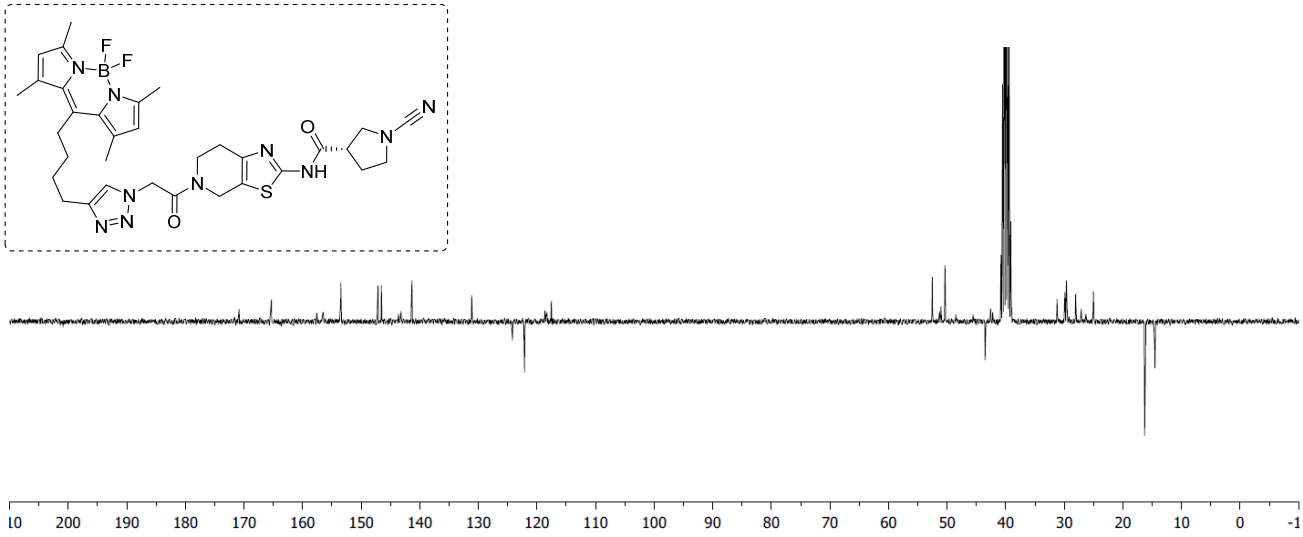
Compound 8RK64: ^{13}C NMR (75.47 MHz, $CDCl_3$)



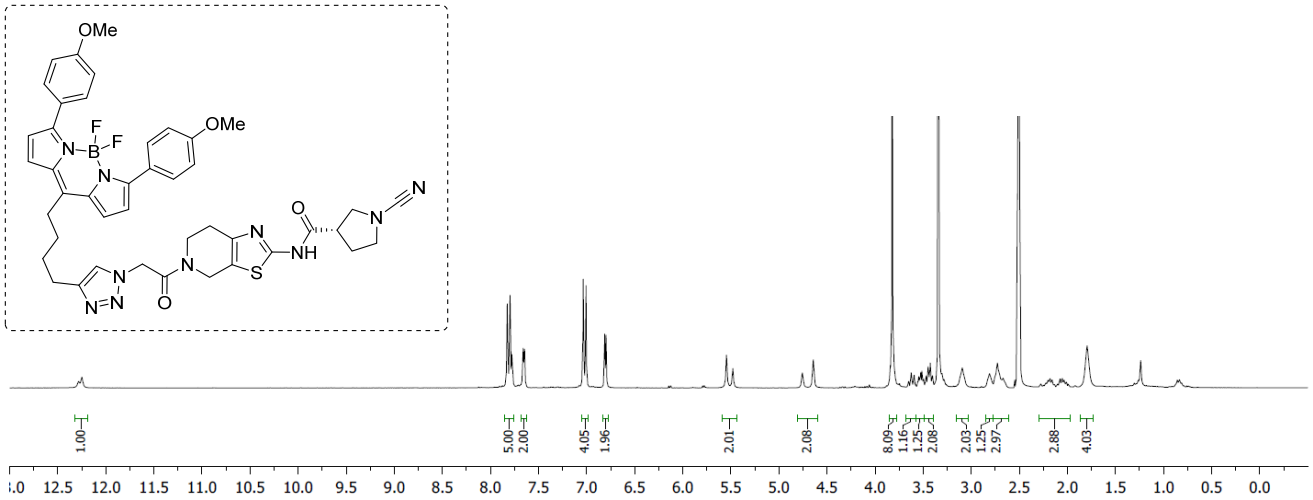
Compound 8RK59: ^{1}H NMR (300.17 MHz, DMSO)



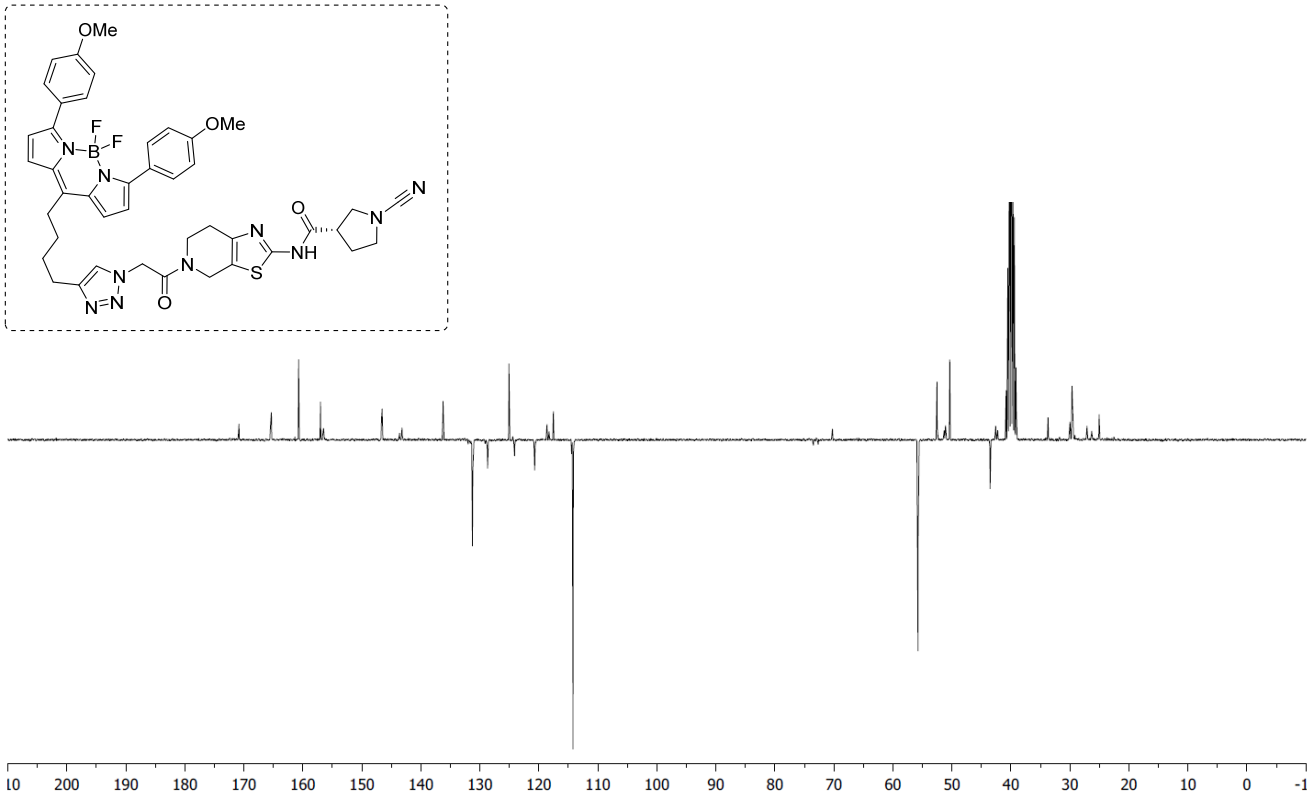
Compound 8RK59: ^{13}C NMR (75.47 MHz, DMSO)



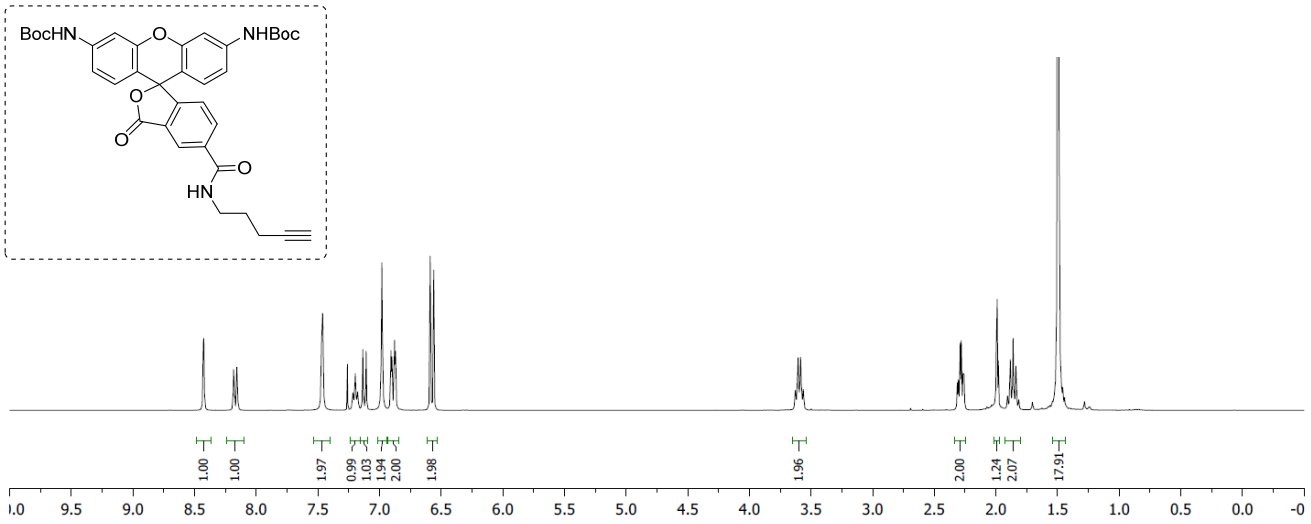
Compound 9RK15: ^{1}H NMR (300.17 MHz, DMSO)



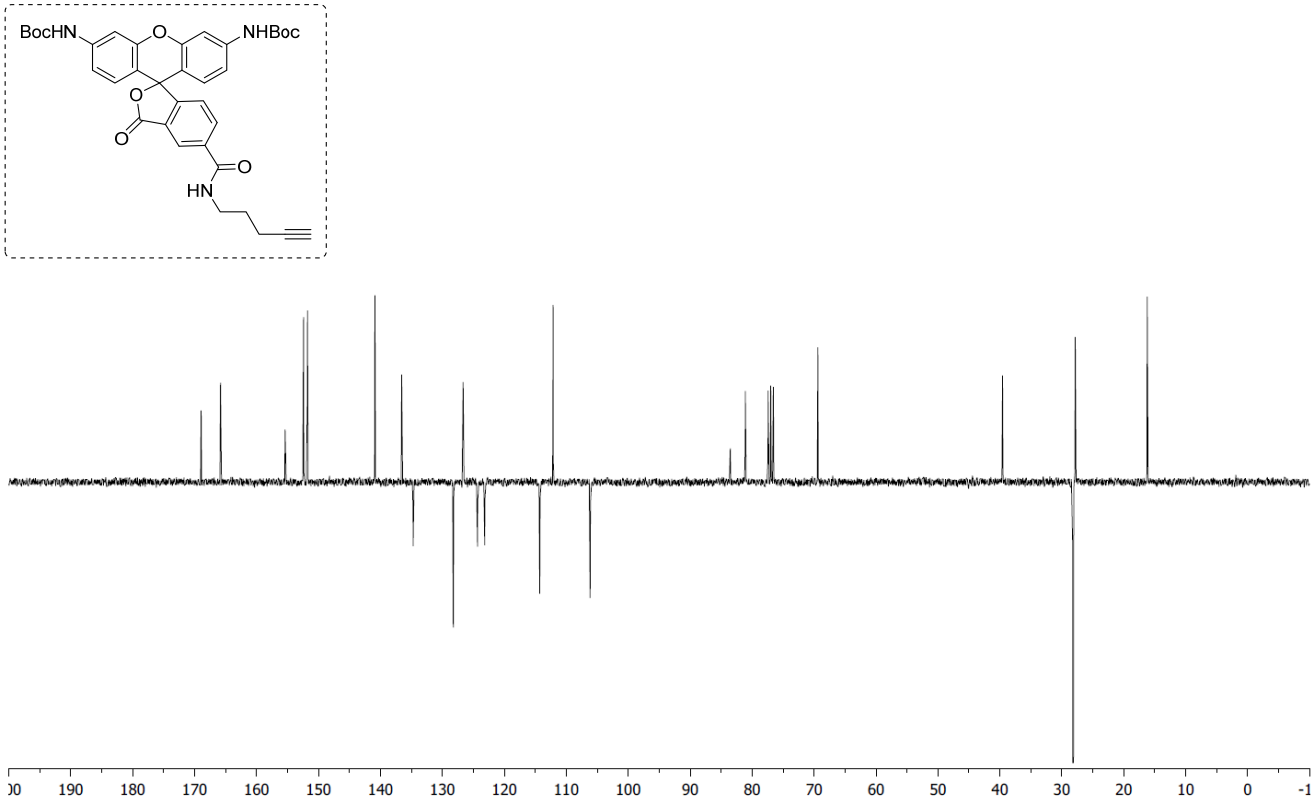
Compound 9RK15: ^{13}C NMR (75.47 MHz, DMSO)



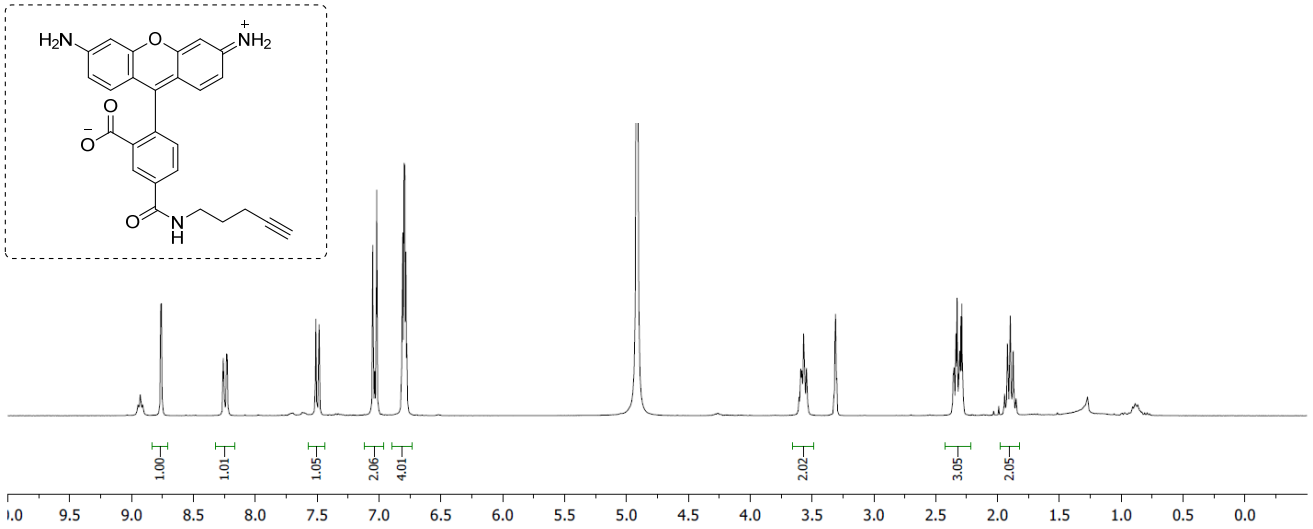
Compound 5: ^{1}H NMR (300.17 MHz, $CDCl_3$)



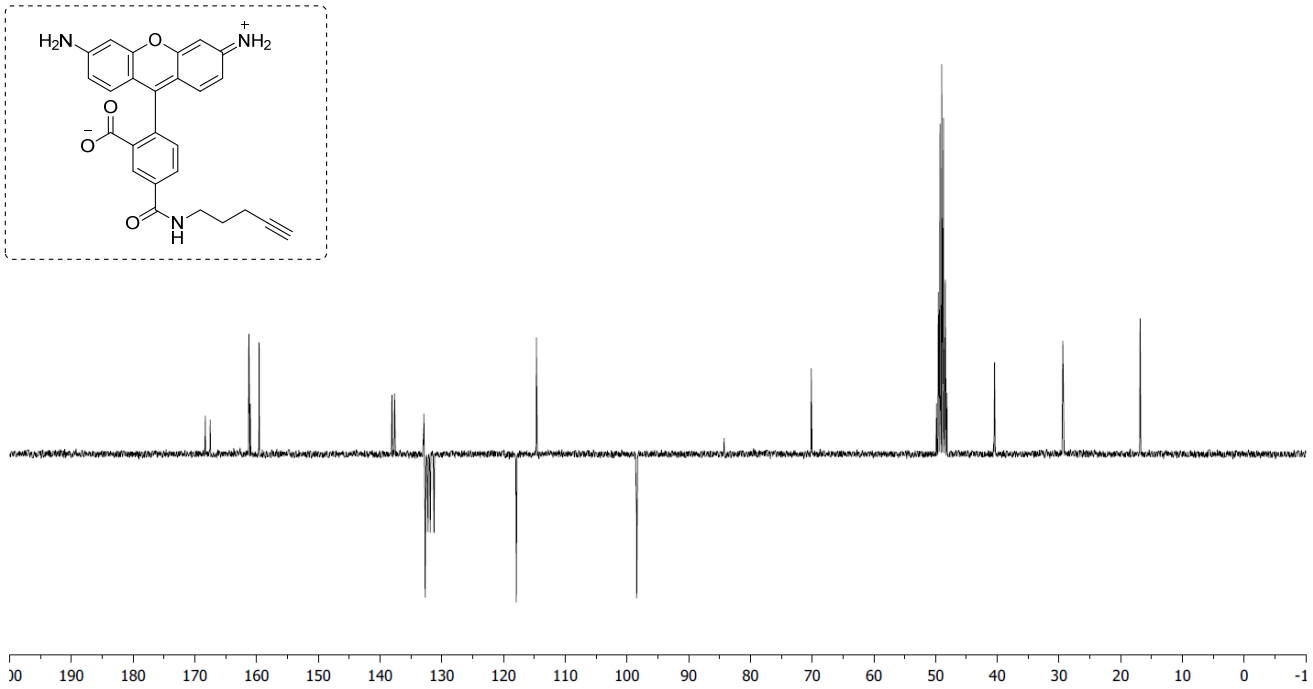
Compound 5: ^{13}C NMR (75.47 MHz, $CDCl_3$)



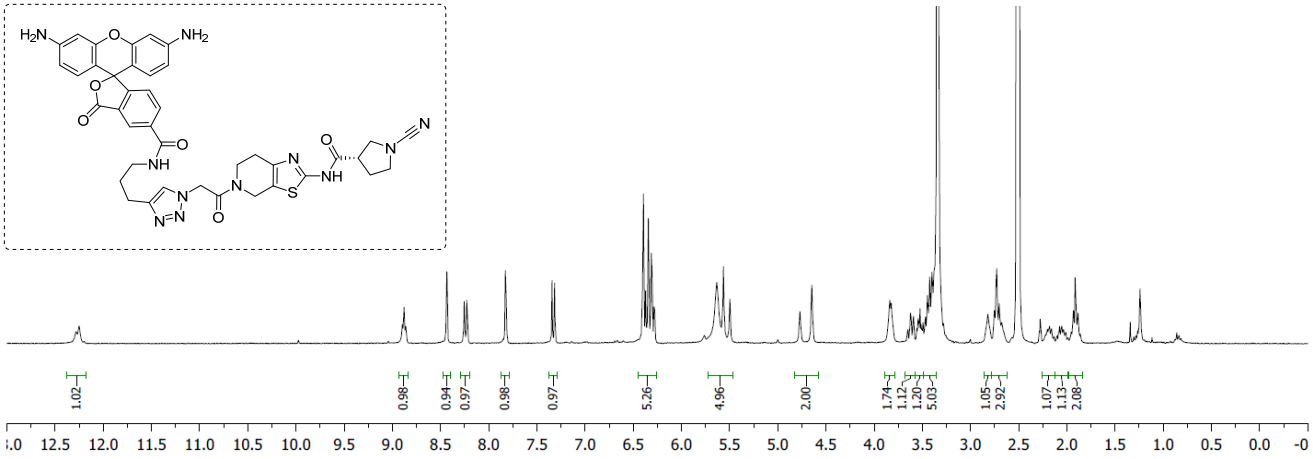
Compound 6: ^{1}H NMR (300.17 MHz, MeOD)



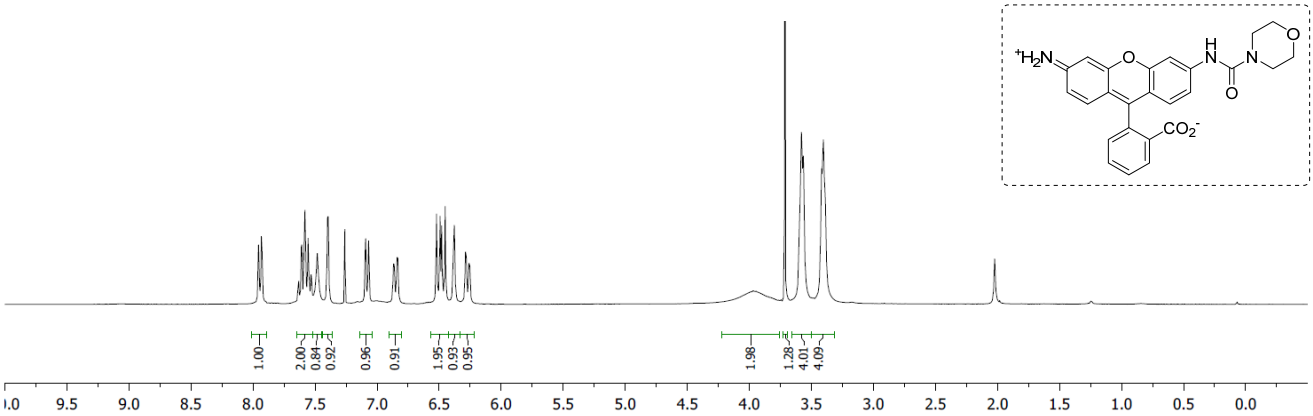
Compound 6: ^{13}C NMR (75.47 MHz, MeOD)



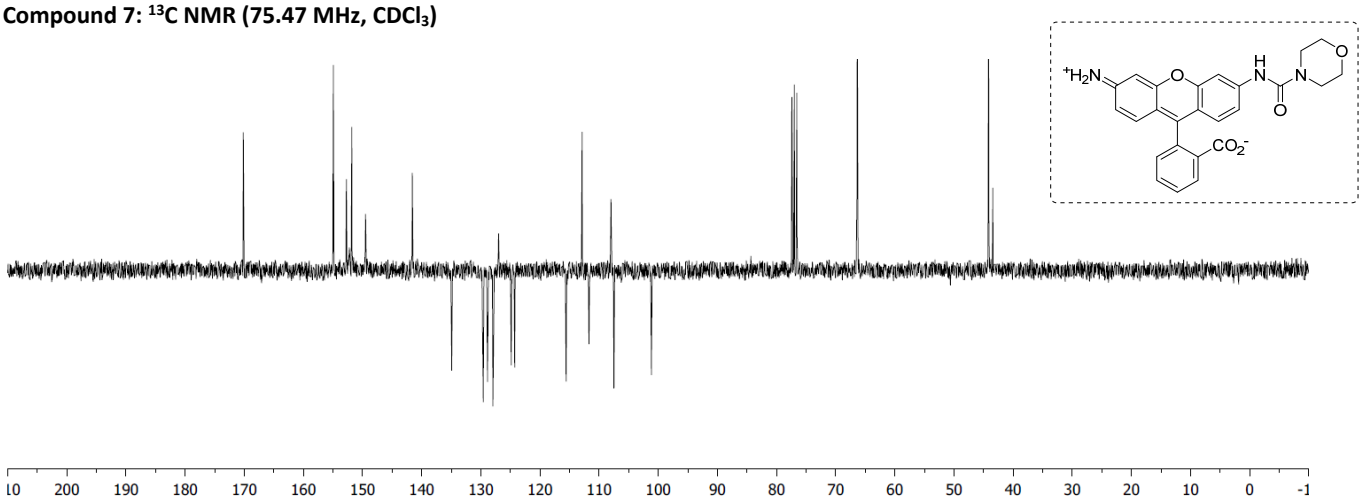
Compound 9RK87: ^{1}H NMR (300.17 MHz, DMSO)



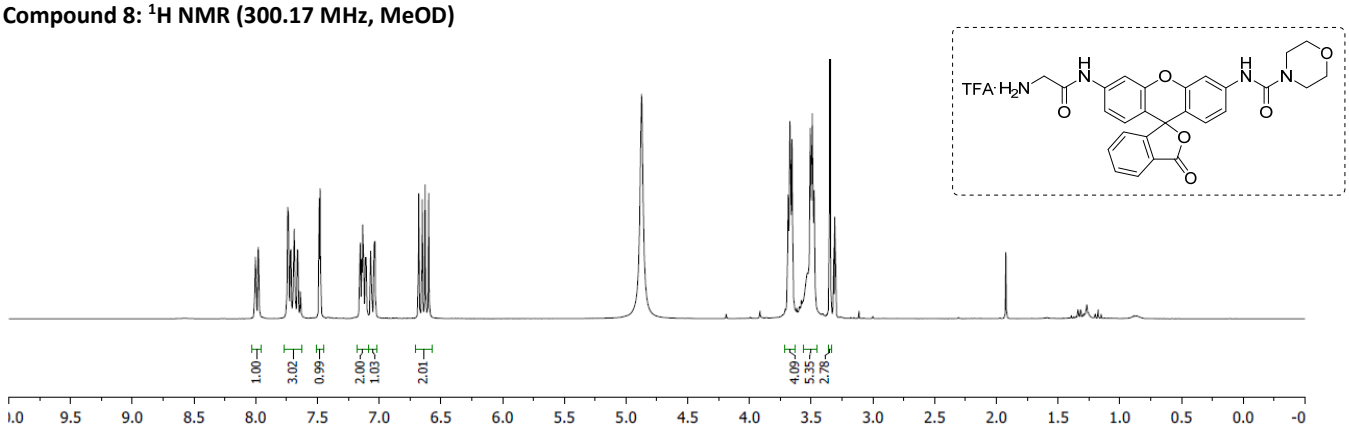
Compound 7: ^{1}H NMR (300.17 MHz, $CDCl_3$)



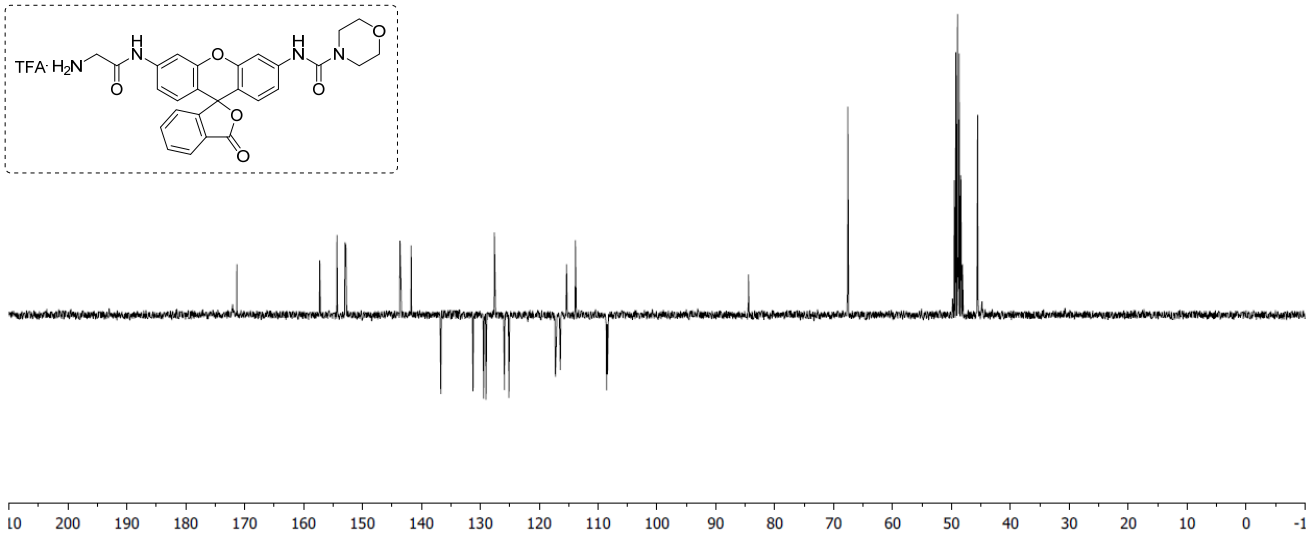
Compound 7: ^{13}C NMR (75.47 MHz, $CDCl_3$)



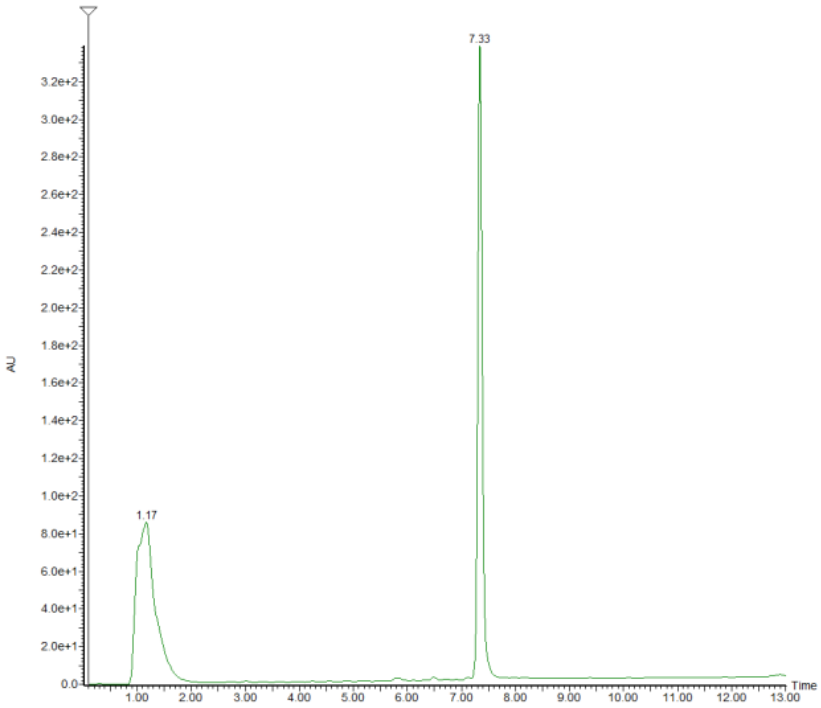
Compound 8: ^{1}H NMR (300.17 MHz, MeOD)

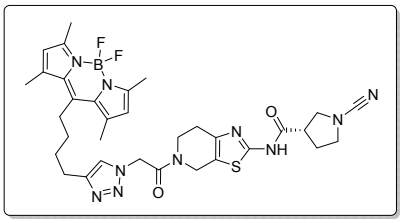


Compound 8: ^{13}C NMR (75.47 MHz, MeOD)

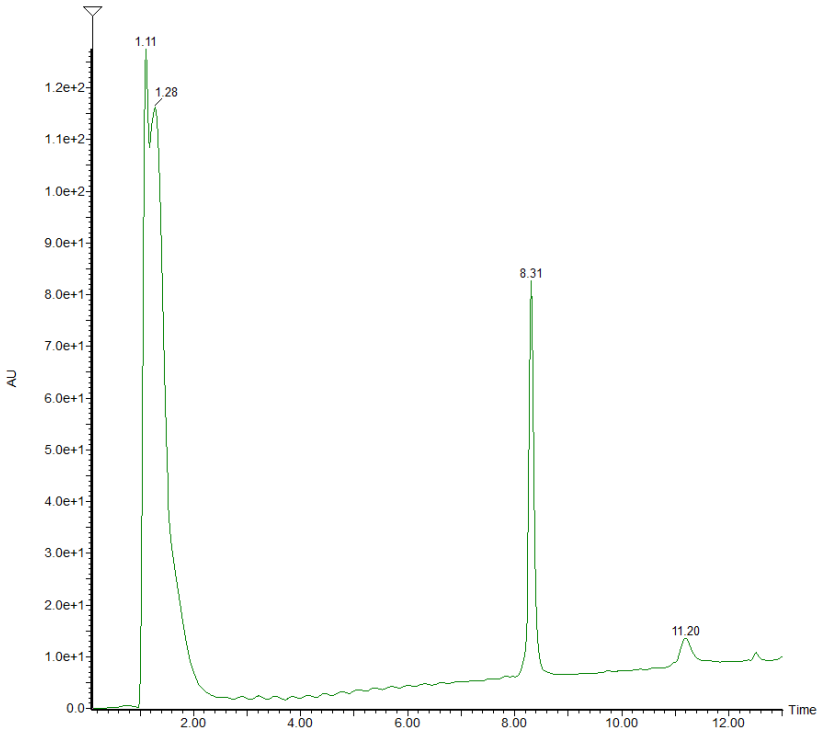


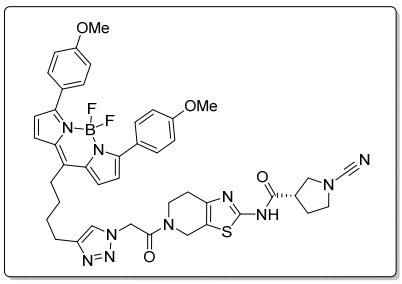
Compound 8RK59: UV Trace.



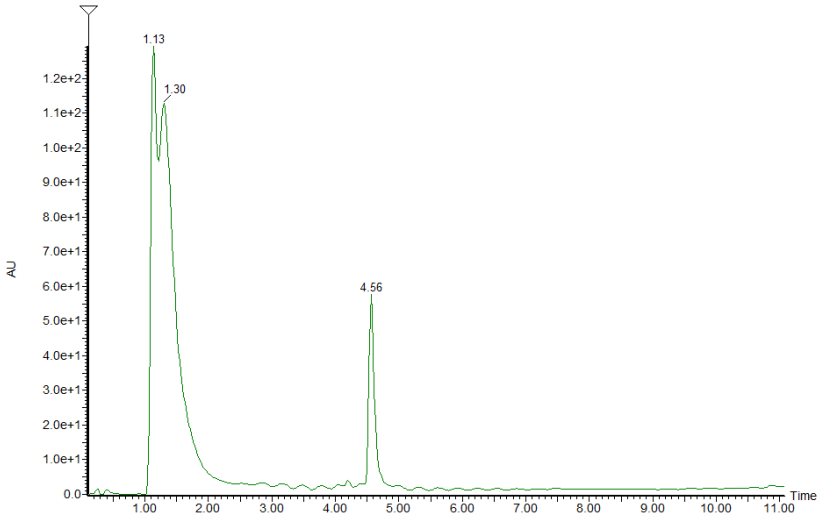


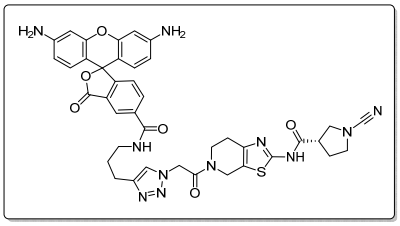
Compound 9RK15: UV Trace.



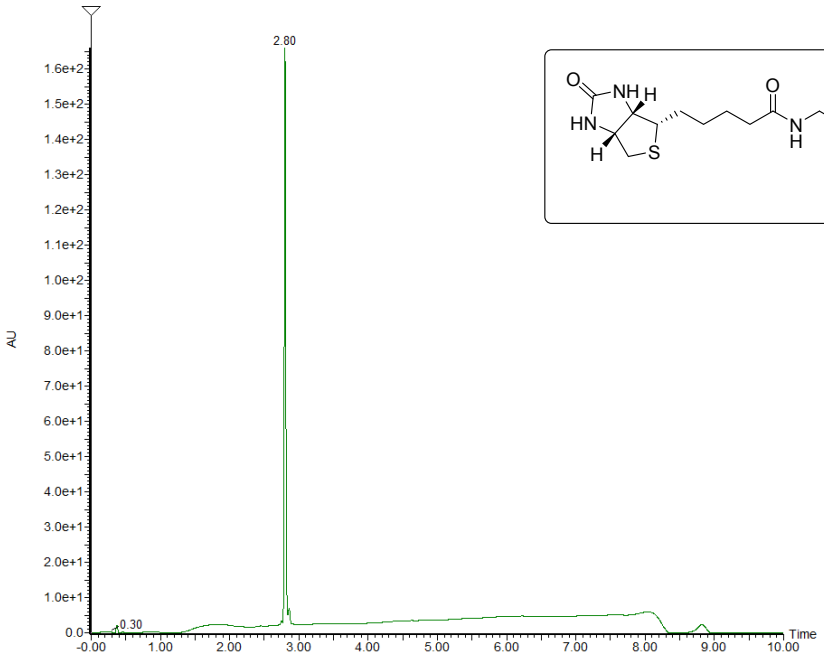


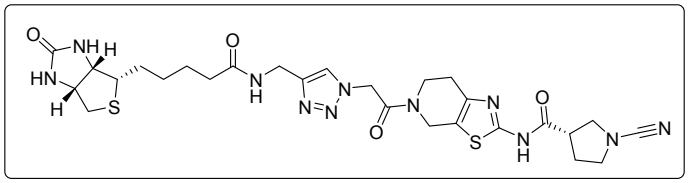
Compound 9RK87: UV Trace.



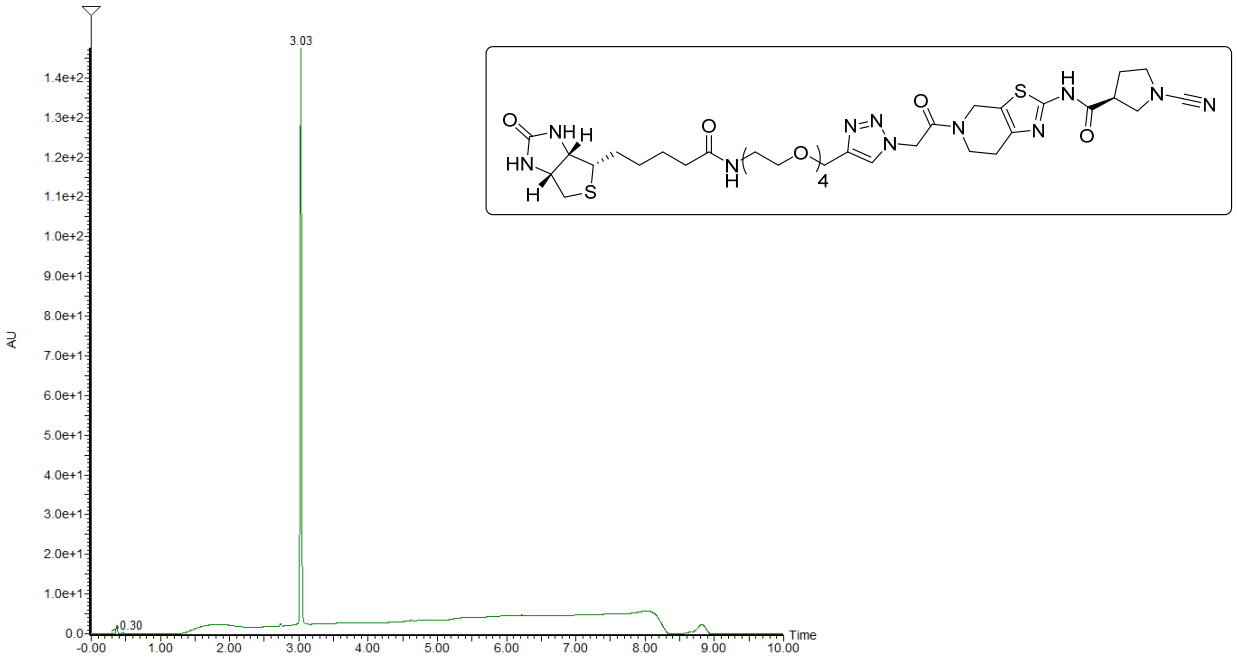


Compound 11RK72: UV Trace.





Compound 11RK73: UV Trace.



Ub-Rho-morpholine LC-MS