Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

A Caged E3 Ligase Ligand for PROTAC-mediated Protein Degradation with Light

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1. Supplementary figures

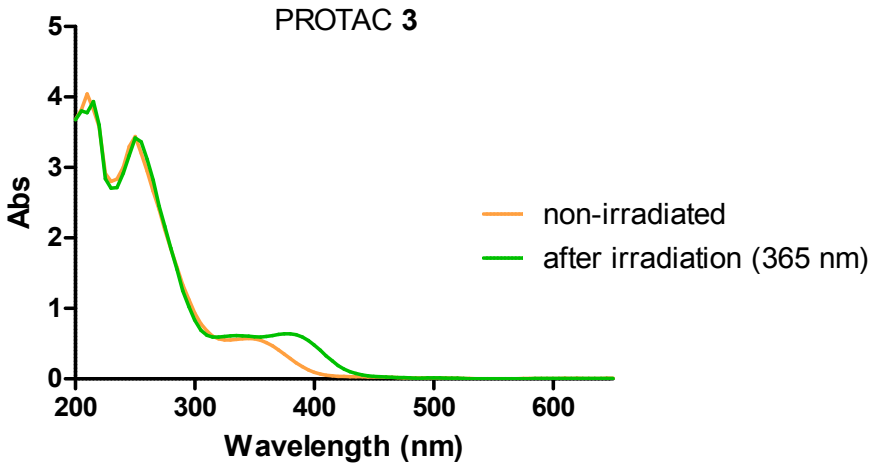


Figure S1 - UV-Vis spectrum of caged PROTAC **3**. A 200 µM solution of **3** in acetonitrile was prepared. Spectrum was recorded before and after a three-minute irradiation time at 365 nm under a 25 mW LED.

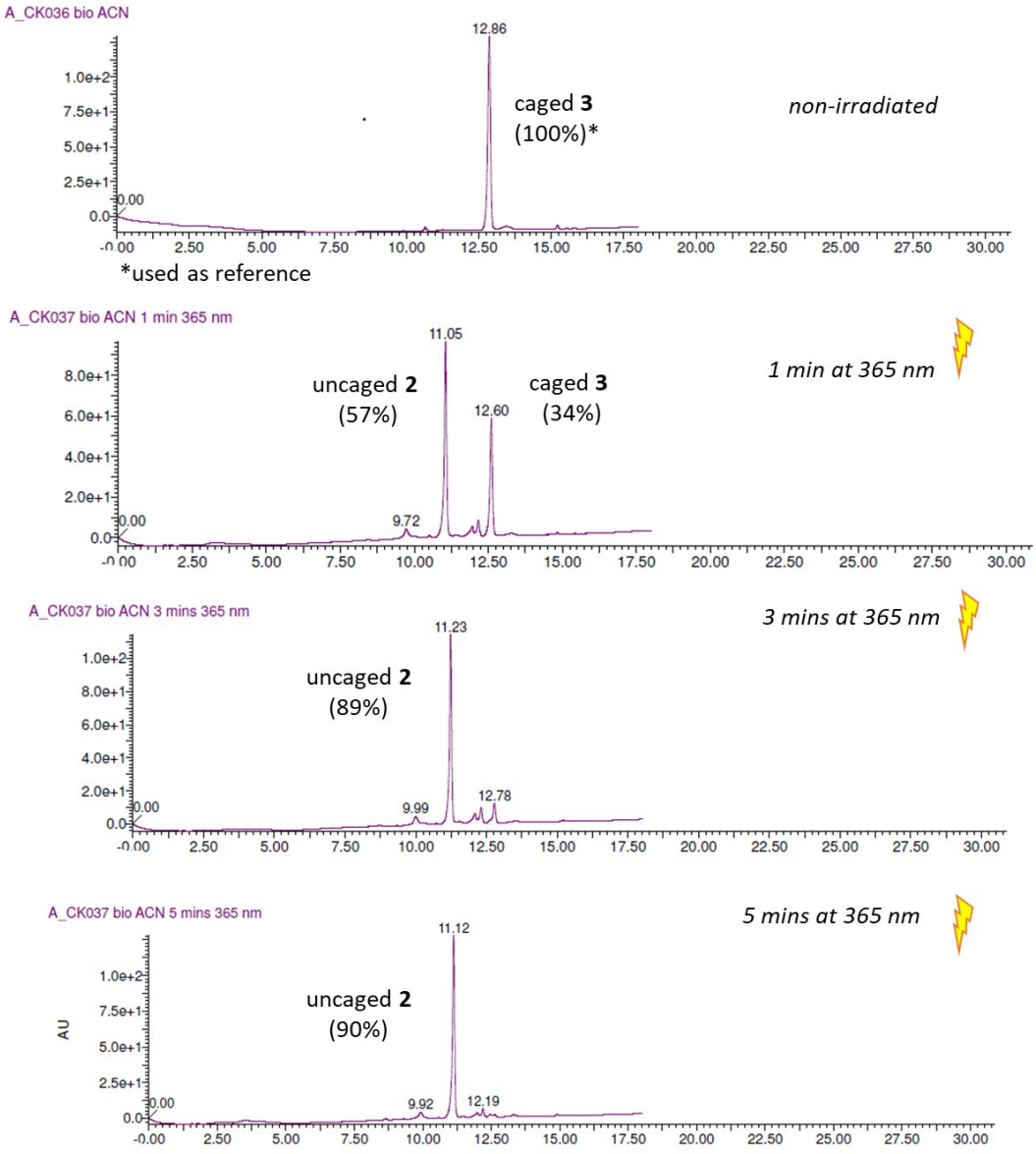


Figure S2 - LCMS profile after uncaging of PROTAC **3.** A 50 µM solution of PROTAC **3** in acetonitrile-water (1:1) was irradiated for 1 min, 3 mins and 5 mins at 365 nm. AUC was extracted from the chromatogram UV trace.

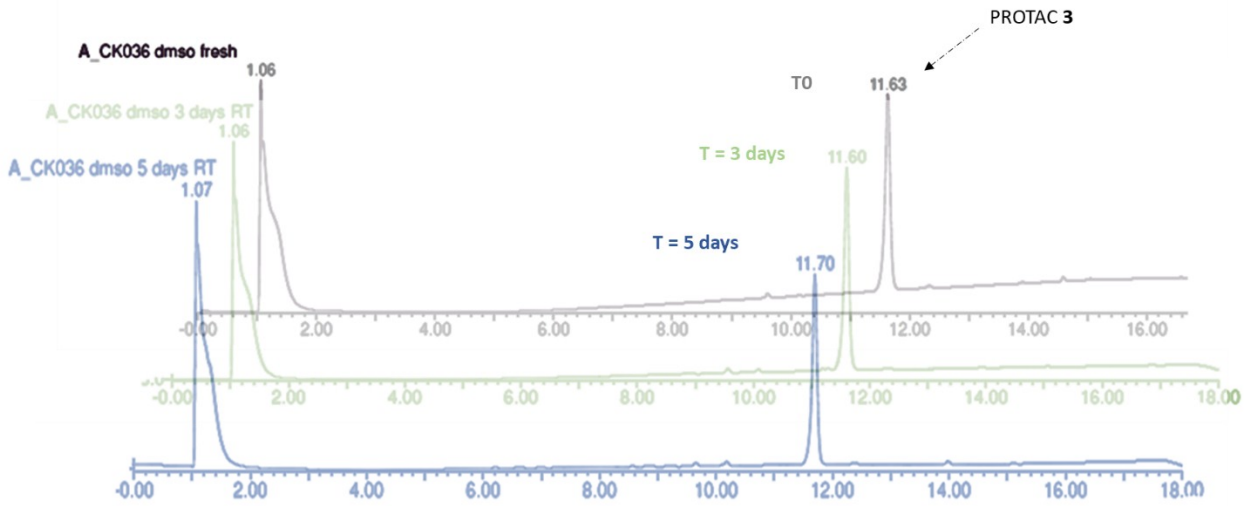


Figure S3 - Caged PROTAC **3** stability in DMSO. A fresh solution of PROTAC **3** in DMSO (100 µM) was prepared and analyzed by LCMS at t0. The solution was kept at room temperature and protected from light. It was re-analyzed after 3 and 5 days. Chromatogram UV trace is represented.

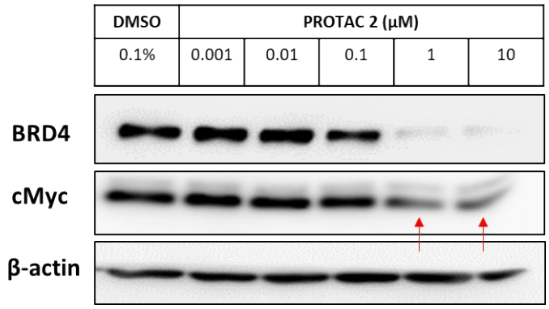


Figure S4 - c-Myc Western blot after treatment with PROTAC **2.** HeLa cells were treated with uncaged PROTAC **2** for 24 h. Cells were lysed, and lysates collected for Western blot analysis. c-Myc depletion can be observed from 1 µM and above[1].

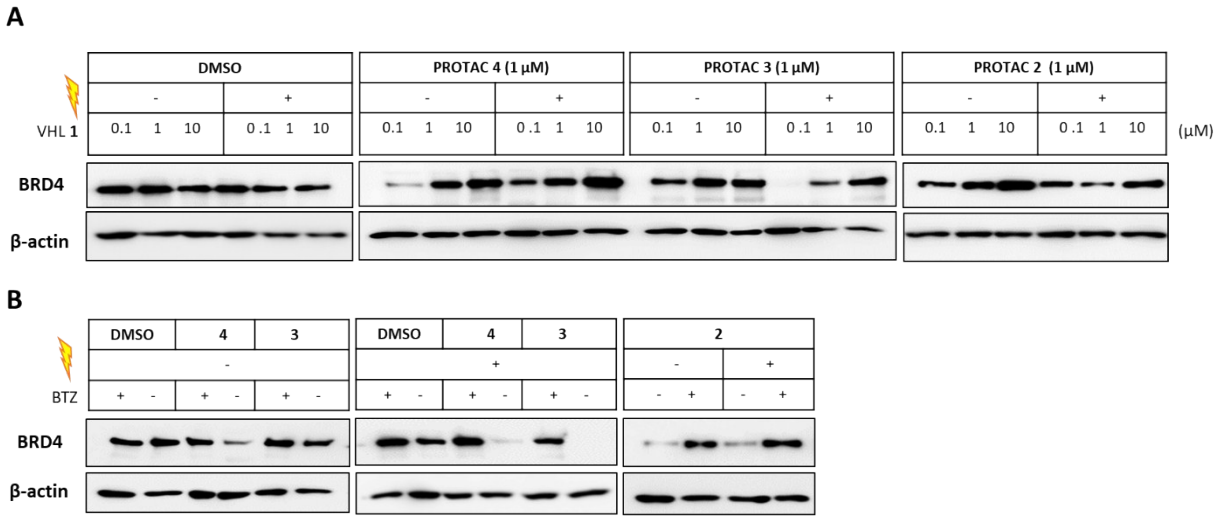


Figure S5 - Evaluation of caged PROTAC mode of action. (A) VHL-dependency: HeLa cells were pre-incubated with VHL ligand **1** (0.1, 1 and 10 µM) for 2 h, then treated with DMSO vehicle 0.1% (v/v), PROTAC **4**, **3** or **2** (1 µM) for 2 h before irradiation, followed by cell lysis and Western blot analysis. (B) Proteasome-dependency: HeLa cells were pre-incubated with proteasome inhibitor bortezomib (BTZ, 10 µM) for 2 h, then treated with DMSO vehicle 0.1% (v/v), PROTAC **4**, **3** or **2** (1 µM) for 2 h before irradiation followed by cell lysis and Western blot analysis.

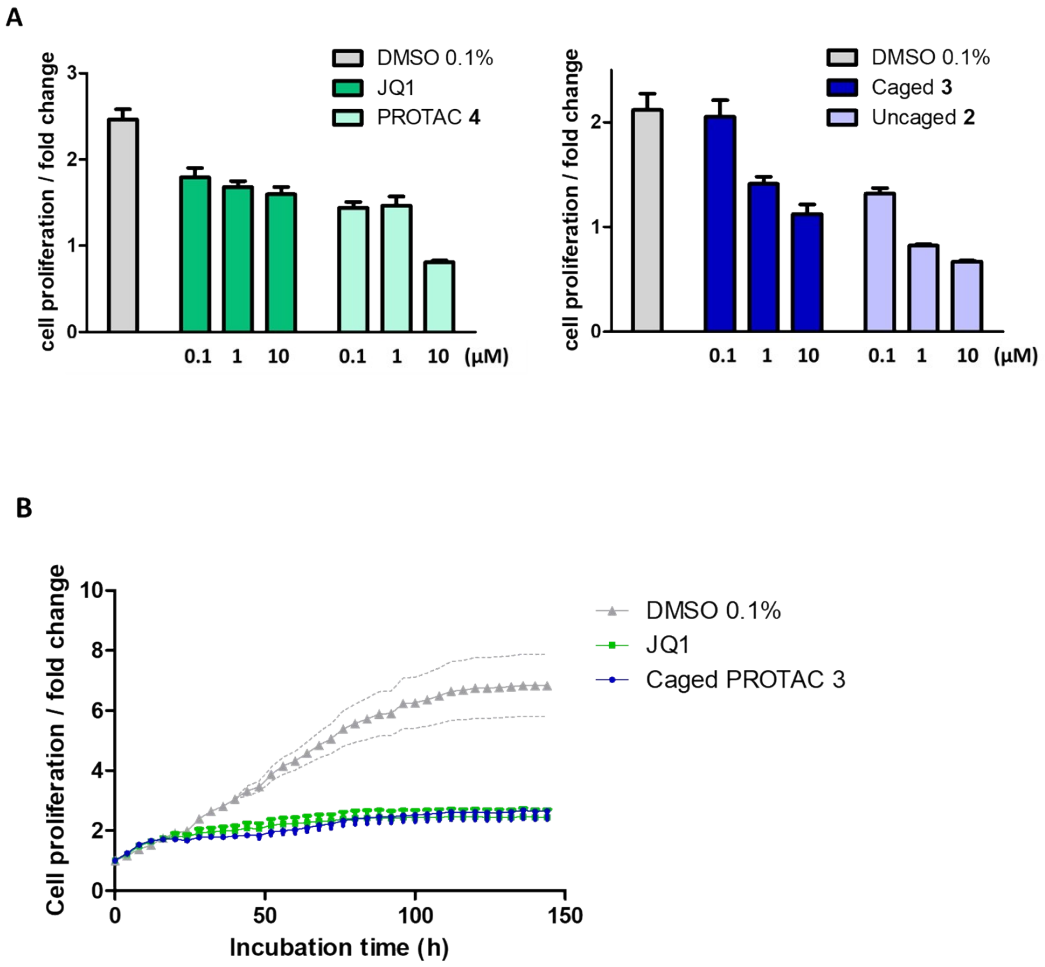


Figure S6 - Effect of JQ1, PROTAC **2**, **3**, and **4** on cell proliferation without irradiation (A) HeLa cells were treated with DMSO, JQ1, PROTAC **2, 3** or **4** for 24 h. Cell proliferation was monitored via live-cell microscopy (IncuCyte S3). Both uncaged PROTAC **2** and caged PROTAC **3** affected cell proliferation through BRD4 degradation and BRD4 inhibition, respectively. (B) HeLa cells were treated with DMSO, JQ1 (1 µM) or PROTAC **3** (1 µM) without irradiation. Cell count was followed over 6 days, normalized to cell count at t0, and fold changes plotted.

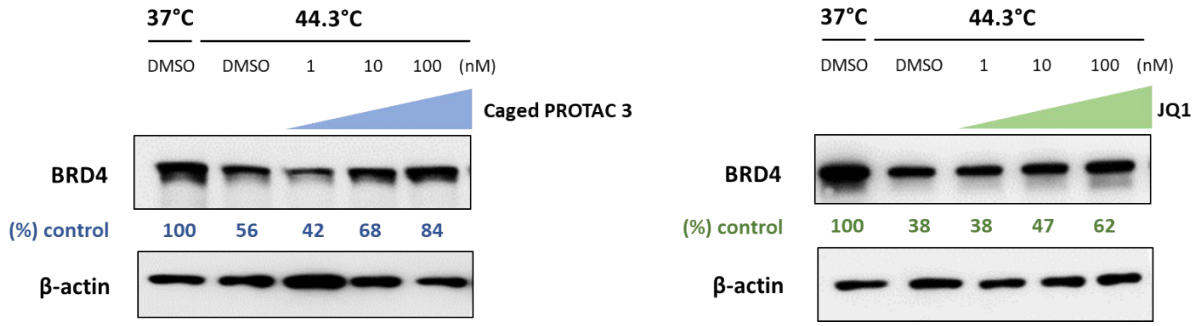


Figure S7 - Evaluation of ligand-induced thermal stabilization of BRD4 by CETSA (Cellular Thermal Shift Assay)[2]. Hela cells were treated with caged PROTAC **3** or JQ1 for 18 h. After collection of the intact cells, heat treatment was performed at 44.3°C (melting temperature previously obtained) followed by cell lysis. The recovered soluble fractions were analyzed by Western blot.

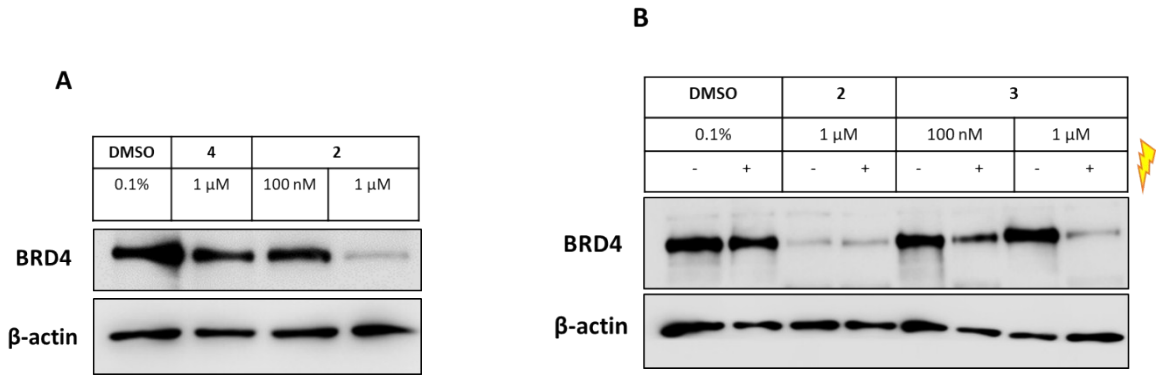


Figure S8 - Evaluation of PROTAC **2**, **3** or **4** in HEK293 cells. (A) HEK293 cells were treated with DMSO, PROTAC **4** or uncaged PROTAC **2** for 24 h. Cells were lysed, and lysates collected for Western blot analysis. (B) Effect of irradiation: HEK293 cells were incubated with DMSO, PROTAC **2** or PROTAC **3** for 2 h then irradiated for 1 min. After 24 h, lysates were collected for Western blot analysis.

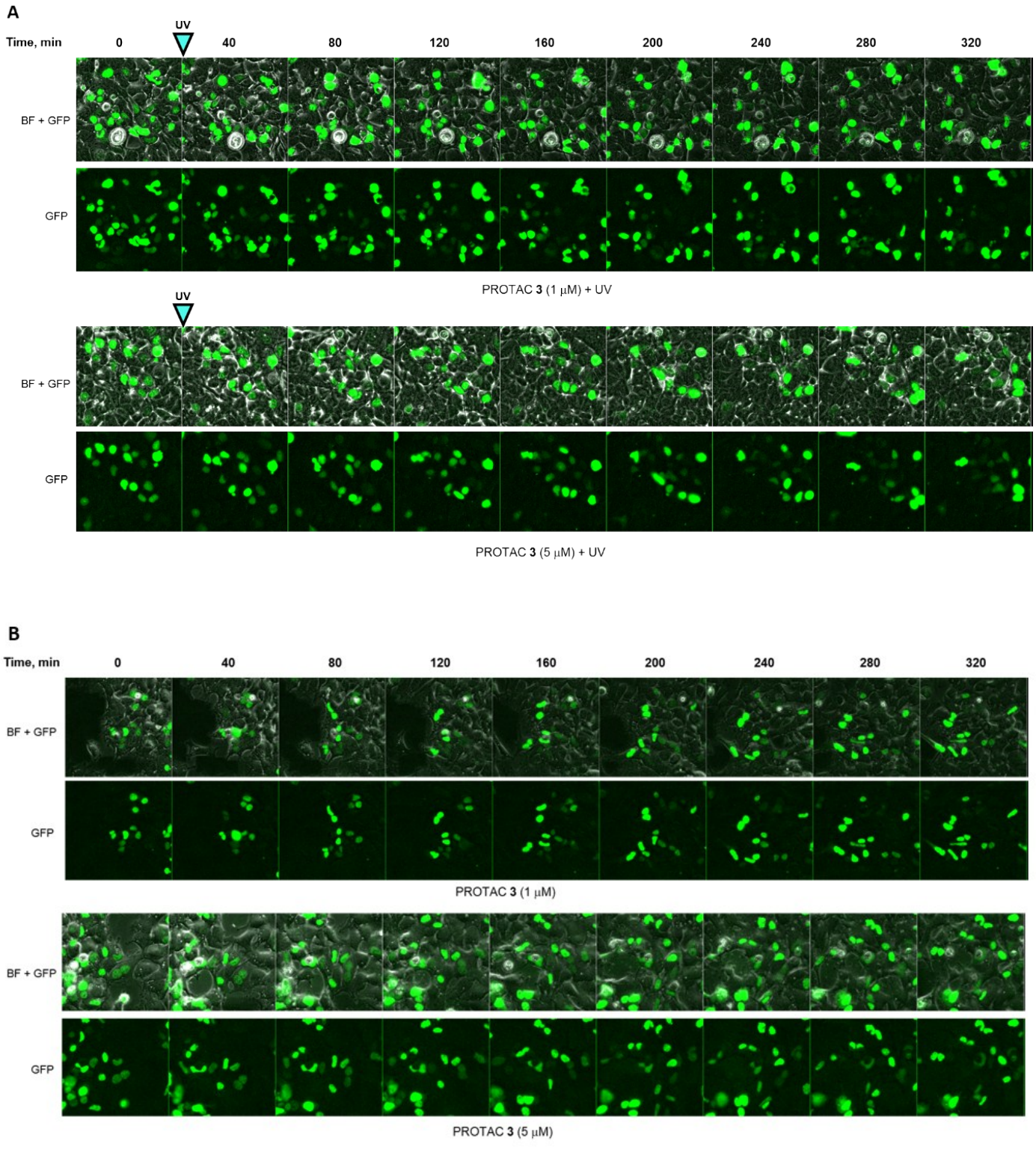


Figure S9 - Live cell fluorescence imaging of HEK293 cells transfected with pEGFP-BRD4-C1 plasmid. (A) Cells were incubated with 1 or 5 μM PROTAC **3** for 1 h, UV irradiated for 60 seconds and imaged over 5 h 20 mins. (B) Cells were treated with 1 or 5 μM PROTAC **3** and imaged over 5 h 20 mins.

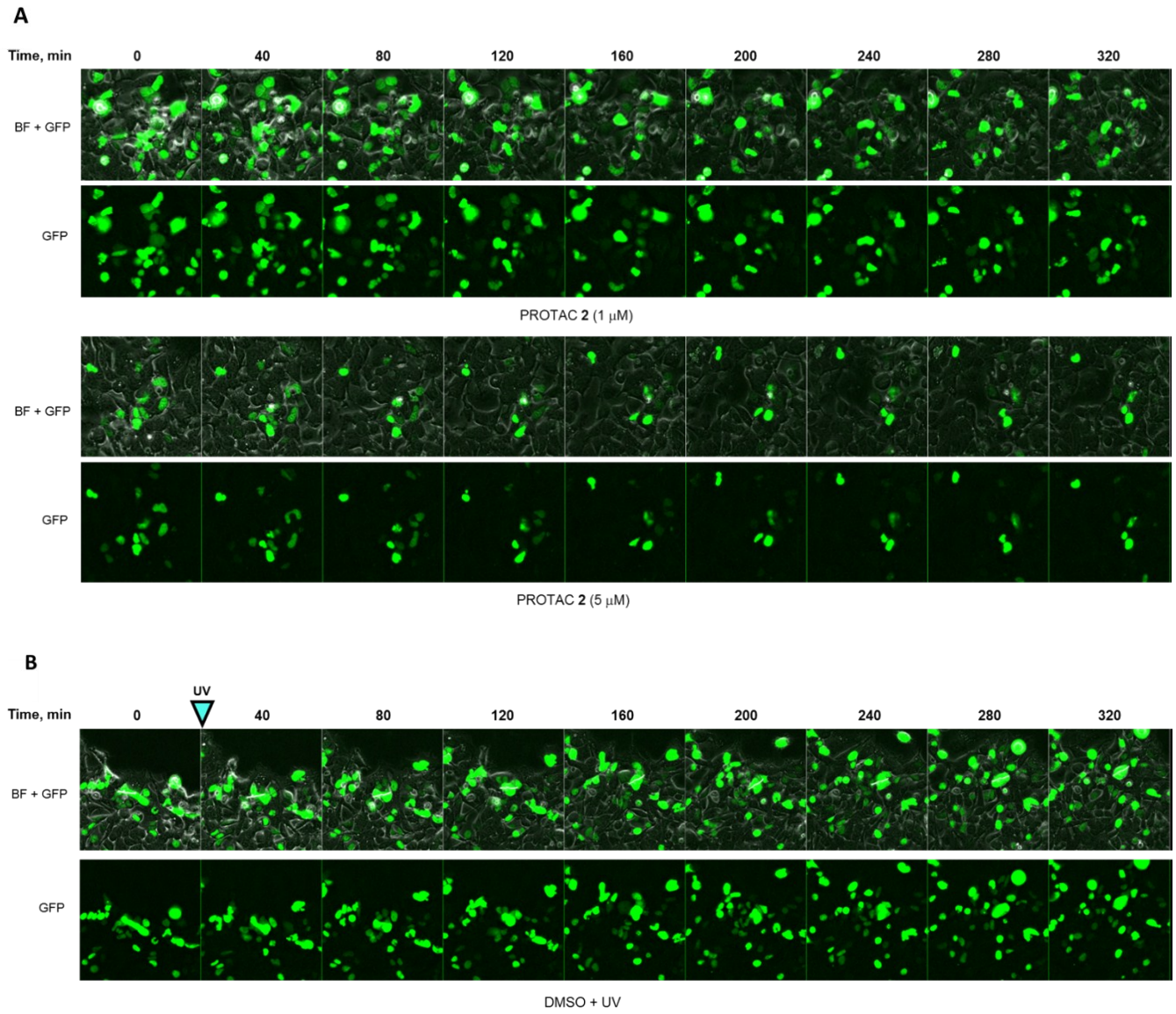


Figure S10. Live-cell fluorescence imaging of HEK293 cells transfected with pEGFP-BRD4-C1 plasmid (A) Cells were treated with 1 or 5 μM PROTAC **2** and imaged over 5 h 20 mins. (B) Cells were treated with DMSO (0.1% v/v), UV irradiated for 60 seconds and imaged over 5 h 20 mins.

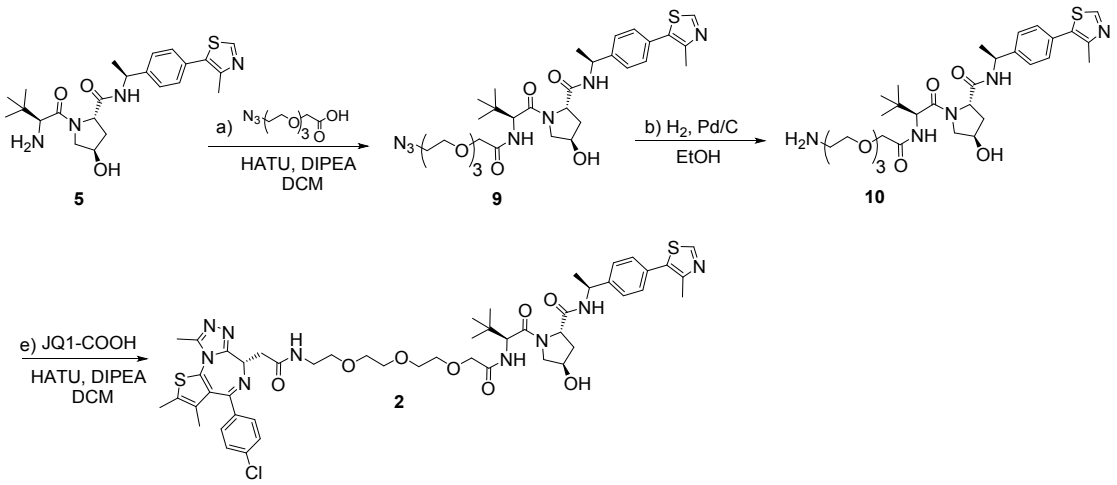
2. Chemical Synthesis

a. General methods

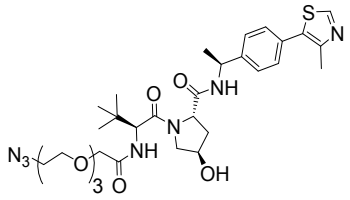
All reagents and solvents were purchased from commercial sources and used as supplied unless otherwise indicated. Pan-bromodomain inhibitor JQ1 and VHL E3 ligase ligand **5** were provided by GlaxoSmithKline Medicines Research Centre, Stevenage. Reactions were monitored by thin-layer chromatography (TLC) using Merck silica gel 60 F254 plates (0.25 mm). TLC plates were visualized using UV light (254 nm) and / or by using the appropriate TLC stain. Flash column chromatography was performed using silica gel Geduran® Si 60 (40-63 µm) silica gel (Merck). All compounds bearing the photocleavable 4,5-dimethoxy-2-nitrobenzyl (DMNB) group were protected from ambient light during synthesis and purification by turning off the lights of the fume cupboard and were covered with aluminum foil after isolation.
Final compound's purity was determined by their LC-MS spectra recorded on a Waters high performance liquid chromatography (HPLC) system, including a 2767 autosampler, 515 pump and a 3100-electrospray ionization (ESI) mass spectrometer, using MassLynx 4.1 software. Compounds were separated on a 4.6 mm × 100 mm analytical Waters XBridge C18 column using the following gradient: 20-98% over 12 min then 98% acetonitrile for 3 mins. UV-Vis spectrum of PROTAC **3** was recorded on a Cary 60 UV vis spectrometer (wavelength range: 190 – 1100 nm, resolution: 1.5 nm, Light source: Xenon Flash Lamp (80 Hz)).
Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV-400 (400 Hz) instrument and in deuterated solvents. Chemical shifts (δ) are quoted in ppm (parts per million) to the nearest 0.01 ppm downfield from tetramethylsilane, referenced to residual solvent signals. Coupling constants (J) are given to the nearest 0.1 Hz. The following abbreviations are used to indicate signal multiplicity: s: singlet, d: doublet, dd: doublet of doublet, t: triplet, q: quartet, m: multiplet and br: broad. Spectra were analysed using MestReNova 12 software.

b. Synthetic procedures

Scheme S1 - Synthesis of uncaged PROTAC **2**



a) Azido-PEG3-acetic acid, HATU, DIPEA, DCM, RT, 16h (36%) b) H_2, Pd/C 10%, EtOH, RT, 3h (70%); c) JQ1-COOH, HATU, DIPEA, DCM, RT, h (54%)

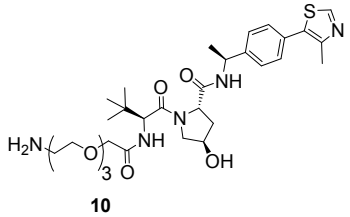


9

(2S,4R)-1-((S)-14-azido-2-(tert-butyl)-4-oxo-6,9,12-trioxa-3-azatetradecanoyl)-4-hydroxy-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (9)

2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetic acid (26 mg, 0.112 mmol, 1.0 eq) was dissolved in DCM (3 ml) along with HATU (55 mg, 0.15 mmol, 1.3 eq) and DIPEA (0.45 mmol, 76 µl, 4.0 eq). The amine **5** (hydrochloride salt, 50 mg, 0.11 mmol, 1.0 eq) was then added and the solution obtained was stirred at room temperature for 16 h. The reaction mixture was subsequently washed twice with water (2 * 3 ml). The recovered organic layer was dried over sodium sulfate, filtered and concentrated in vacuo. The residue was purified by silica gel flash chromatography (2-10% MeOH in DCM) to afford **9** as a white solid (26.5 mg, 36 %).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.70 (s, 1H), 7.50 (d, *J* = 7.8 Hz, 1H), 7.45 – 7.33 (m, 6H), 5.13 – 5.07 (m, 1H), 4.76 (t, *J* = 7.8 Hz, 1H), 4.56 (d, *J* = 8.6 Hz, 1H), 4.53 (br.s, 1H), 4.14 – 4.08 (m, 1H), 4.04 (d, *J* = 5.4 Hz, 2H), 3.73 – 3.69 (m, 8H), 3.69 – 3.66 (m, 2H), 3.63 (dd, *J* = 11.3, 3.8 Hz, 1H), 3.40 (dd, *J* = 5.6, 4.5 Hz, 2H), 2.60 – 2.50 (m, 4H), 2.07 (ddt, *J* = 13.5, 8.3, 1.9 Hz, 1H), 1.50 (d, *J* = 7.0 Hz, 3H), 1.09 (s, 9H). **^{13}C NMR** (101 MHz, Chloroform-*d*) δ 171.57, 170.56, 169.64, 150.31, 148.45, 143.19, 131.62, 130.85, 129.56, 126.43, 71.17, 70.71, 70.55, 70.35, 70.07, 58.35, 57.20, 56.59, 50.69, 48.87, 35.37, 35.00, 26.49, 22.27, 16.08. **MS** (ESI) *m/z* 660 $(M+H)^+$. **HRMS** (ESI) m/z: calculated for $C_{31}H_{46}N_7O_7S^+$, 660.3179; found 660.3181.

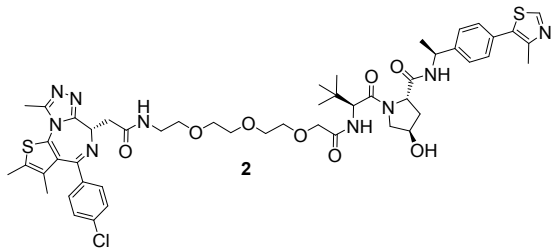


10

(2S,4R)-1-((S)-14-amino-2-(tert-butyl)-4-oxo-6,9,12-trioxa-3-azatetradecanoyl)-4-hydroxy-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (10)

The azide **9** (145 mg, 0.22 mmol) was dissolved in ethanol (5 ml) and was added palladium 10% on carbon (14.5 mg, 10% w/w). After 3 cycles of degassing and purging with nitrogen gas, the solution was placed under hydrogen gas for 3 h at room temperature. It was then filtered over celite and the recovered solution concentrated in vacuo and further dried under high vacuum to give a sticky colorless gum (96 mg, 70%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.70 (s, 1H), 8.57 (d, *J* = 7.9 Hz, 1H), 7.49 – 7.33 (m, 5H), 5.20 – 5.06 (m, 1H), 4.78 (t, *J* = 8.6 Hz, 1H), 4.72 (d, *J* = 9.6 Hz, 1H), 4.43 (s, 1H), 4.21 (d, *J* = 15.6 Hz, 1H), 4.08 – 3.95 (m, 2H), 3.90 – 3.56 (m, 12H), 3.40 – 3.11 (m, 1H), 2.55 (s, 3H), 2.29 (dd, *J* = 13.4, 7.5 Hz, 1H), 2.08 – 1.92 (m, 1H), 1.52 (d, *J* = 7.0 Hz, 3H), 1.08 (s, 9H). **^{13}C NMR** (101 MHz, Chloroform-*d*) δ 171.19, 170.46, 170.15, 150.16, 148.36, 143.91, 131.79, 130.47, 129.43, 126.38, 70.59, 70.27, 69.92, 69.84, 69.53, 66.55, 59.09, 57.24, 56.82, 48.84, 39.95, 37.68, 36.08, 26.45, 22.59, 16.11. **MS** (ESI) *m/z* 634 $(M+H)^+$. **HRMS** (ESI) m/z: calculated for $C_{31}H_{48}N_5O_7S^+$, 634.3274; found 634.3274.



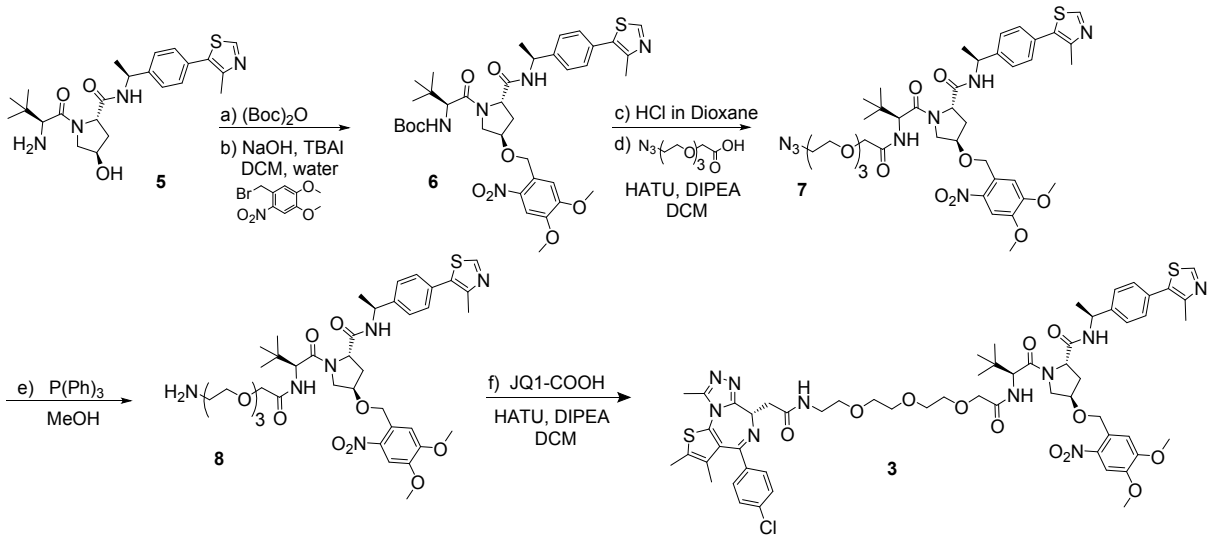
2

(2S,4R)-1-((S)-2-(tert-butyl)-17-((S)-4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)-4,16-dioxo-6,9,12-trioxa-3,15-diazaheptadecanoyl)-4-hydroxy-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (2)

JQ1 carboxylic acid (JQ1-COOH, 24 mg, 0.06 mmol, 1.0 eq) was dissolved in DCM (4 ml) along with HATU (30 mg, 0.078 mmol, 1.3 eq) and DIPEA (20 µl, 0.12 mmol, 2.0 eq). The amine **10** (38 mg, 0.06 mmol, 1.0 eq) was then added and the solution obtained was stirred at room temperature for 16 h. The reaction mixture was subsequently washed twice with water (2 x 3 ml). The recovered organic layer was dried over sodium sulfate, filtered and concentrated in vacuo. The residue recovered was purified by silica gel flash chromatography (2-8% MeOH in DCM) to afford **2** as a white solid (33 mg, 54%).

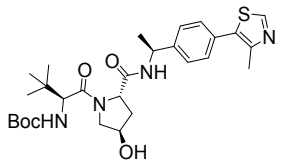
^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.68 (s, 1H), 7.97 (t, *J* = 5.6 Hz, 1H), 7.45 (d, *J* = 7.8 Hz, 1H), 7.43 – 7.28 (m, 10H), 5.13 – 5.03 (m, 1H), 4.82 (t, *J* = 7.8 Hz, 1H), 4.73 (d, *J* = 9.3 Hz, 1H), 4.68 (dd, *J* = 8.8, 5.2 Hz, 1H), 4.44 (br.s, 1H), 4.35 (d, *J* = 15.9 Hz, 1H), 4.15 (d, *J* = 15.9 Hz, 1H), 4.11 (br.s, 1H), 3.77 – 3.59 (m, 10H), 3.58 – 3.49 (m, 2H), 3.39 – 3.31 (m, 2H), 2.63 (s, 3H), 2.52 (s, 3H), 2.49 – 2.42 (m, 1H), 2.39 (s, 3H), 2.12 – 2.05 (m, 1H), 1.67 (s, 3H), 1.46 (d, *J* = 6.9 Hz, 3H), 1.07 (s, 9H). **^{13}C NMR** (126 MHz, Chloroform-*d*) δ 171.40, 170.94, 170.85, 170.04, 163.81, 162.16, 155.82, 150.29, 149.78, 148.33, 143.34, 136.70, 136.49, 131.80, 131.70, 131.06, 130.92, 130.76, 130.64, 129.97, 129.48, 128.70, 127.90, 126.44, 77.00, 71.72, 70.83, 70.49, 70.30, 70.11, 69.87, 58.62, 56.99, 56.69, 54.10, 48.76, 39.78, 38.05, 35.62, 35.49, 26.48, 22.19, 16.02, 14.43, 13.10, 11.73. **MS** (ESI) *m/z* 1016 $(M+H)^+$. **HRMS** (ESI) m/z: calculated for $C_{50}H_{63}N_9O_8S_2Cl^+$, 1016.3930; found 1016.3928.

Scheme S2 - Synthesis of caged PROTAC **3**



5 **6** **7** **8** **3**

a) Boc anhydride, triethylamine, DCM, RT, 16 h (77%); b) DMNB-Br, TBAI, 50% aq. NaOH, DCM, RT, 2h (53%); c) HCl 4M in Dioxane, RT, 4 h (quant.); d) Azido-PEG3-acetic acid, HATU, DIPEA, DCM, RT, (41%); e) $P(Ph)_3$, MeOH, 50˚C, 8h; f) JQ1-COOH, HATU, DIPEA, DCM, RT, h (41% over 2 steps)

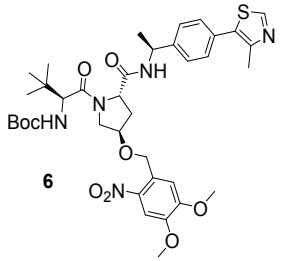


6a

tert-butyl ((S)-1-((2S,4R)-4-hydroxy-2-(((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)carbamoyl)pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamate (6a)

Amine **5** (hydrochloride salt, 400 mg, 0.90 mmol, 1.0 eq) was dissolved in DCM (5 ml) and triethylamine (0.57 ml, 4.04 mmol, 4.5 eq) was added. A solution of di-*tert*-butyl dicarbonate (340 mg, 1.56 mmol, 1.75 eq) in DCM (5 ml) was then slowly added to the amine and the mixture was stirred at room temperature for 16 h. The crude mixture was subsequently washed with water (3 x 3 ml). The recovered organic layer was dried over sodium sulfate, filtered and concentrated in vacuo to afford **6a** as a white solid (377 mg, 77%).

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.98 (s, 1H), 8.40 (d, *J* = 7.7 Hz, 1H), 7.43 (d, *J* = 8.4 Hz, 1H), 7.37 (d, *J* = 8.4 Hz, 2H), 6.40 (d, *J* = 9.3 Hz, 1H), 5.11 (d, *J* = 3.6 Hz, 1H), 4.90 (t, *J* = 7.2 Hz, 1H), 4.45 (t, *J* = 8.1 Hz, 1H), 4.28 (br.s, 1H), 4.14 (d, *J* = 9.3 Hz, 1H), 3.66 – 3.49 (m, 2H), 2.45 (s, 3H), 2.03 (ddd, *J* = 11.5, 8.0, 3.0 Hz, 1H), 1.84 – 1.72 (m, 1H), 1.44 – 1.31 (m, 12H), 0.93 (s, 9H). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 171.05, 170.15, 155.79, 151.93, 148.21, 145.21, 131.58, 130.13, 129.28, 126.79, 78.56, 69.25, 58.99, 58.88, 56.74, 48.20, 40.63, 38.18, 35.83, 28.66, 27.82, 26.80, 26.69, 22.96, 16.46. **MS** (ESI) *m/z* 545 $(M+H)^+$. **HRMS** (ESI) m/z: calculated for $C_{28}H_{41}N_4O_5S^+$, 545.2798; found 545.2798.

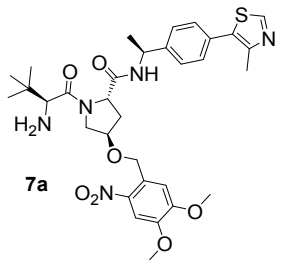


6

tert-butyl((S)-1-((2S,4R)-4-((4,5-dimethoxy-2-nitrobenzyl)oxy)-2-(((S)-1-(4-(4-methylthiazol-5 yl)phenyl)ethyl)carbamoyl) pyrrolidin-1-yl)-3,3-dimethyl-1-oxobutan-2-yl)carbamate (6).[3]

Intermediate **6a** (160 mg, 0.29 mmol, 1.0 eq) and tetrabutylammonium iodide (22 mg, 0.059 mmol, 0.2 eq) were dissolved in DCM (8 ml). 4,5-Dimethoxy-2-nitrobenzyl bromide (89 mg, 0.32 mmol, 1.1 eq) was then added followed by aqueous sodium hydroxide 50% (2 ml). The biphasic mixture was stirred at room temperature and was protected from ambient light (fume cupboard lights off). Completion of the reaction was monitored by TLC. After 2 h, the aqueous layer (bottom layer) was removed with a pipette and the remaining organic layer was washed with water (5 x 6 ml). The recovered organic layer was dried over sodium sulfate, filtered and concentrated in vacuo. The residue recovered was then purified by silica gel flash chromatography (40-80% ethyl acetate in n-hexane) to afford **6** as a pale yellow solid (116 mg, 53%)

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.69 (s, 1H), 7.69 (s, 1H), 7.45 – 7.34 (m, 4H), 7.18 (s, 1H), 5.23 (d, *J* = 9.8 Hz, 1H), 5.10 – 5.02 (m, 1H), 4.94 (d, *J* = 4.7 Hz, 2H), 4.76 (dd, *J* = 8.2, 6.0 Hz, 1H), 4.44 (t, *J* = 4.5 Hz, 1H), 4.34 (d, *J* = 9.7 Hz, 1H), 4.27 – 4.21 (m, 1H), 4.13 (dd, *J* = 11.2, 3.4 Hz, 1H), 3.97 (s, 3H), 3.94 (s, 3H), 3.75 (dd, *J* = 11.0, 4.9 Hz, 1H), 3.71 – 3.66 (m, 1H), 3.64 (s, 1H), 2.71 (dt, *J* = 13.1, 5.5 Hz, 1H), 2.53 (s, 3H), 2.16 (ddd, *J* = 12.9, 8.2, 4.4 Hz, 1H), 1.78 (br.s, 2H), 1.67 – 1.51 (m, 1H), 1.47 (d, *J* = 6.8 Hz, 3H), 1.33 (s, 9H), 1.29 – 1.23 (m, 1H), 1.03 (s, 9H). **^{13}C NMR** (101 MHz, Chloroform-*d*) δ 172.86, 169.30, 155.55, 153.75, 150.34, 147.62, 143.07, 139.04, 130.91, 130.23, 129.61, 126.46, 109.38, 107.88, 79.73, 76.72, 70.55, 69.35, 67.94, 63.04, 58.62, 58.53, 56.48, 56.37, 53.17, 48.98, 47.19, 35.71, 32.55, 31.73, 29.58, 28.19, 26.39, 25.45, 22.64, 22.21, 16.06, 13.97, 11.81. **MS** (ESI) *m/z* 740 $(M+H)^+$. **HRMS** (ESI) m/z: calculated for $C_{37}H_{50}N_5O_9S^+$, 740.3329; found 740.3329.



7a

(2S,4R)-1-((S)-2-amino-3,3-dimethylbutanoyl)-4-((4,5-dimethoxy-2-nitrobenzyl)oxy)-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (7a)

Caged intermediate **6** (115 mg, 0.15 mmol, 1.0 eq) was dissolved in DCM (5 ml) and HCl 4M in Dioxane (0.19 ml, 0.78 mmol, 5.0 eq) was added. The solution was stirred at room temperature for 4 h and was protected from ambient light (fume cupboard lights off).The crude was then concentrated in vacuo to dryness to give **7a** as pale brown solid (105 mg, quant.).

^{1}H NMR (400 MHz, Methanol-d_4) δ 10.00 (s, 1H), 7.70 (s, 1H), 7.60 – 7.49 (m, 4H), 7.27 (s, 1H), 5.02 (d, *J* = 7.0 Hz, 1H), 4.97 (d, *J* = 13.4 Hz, 1H), 4.86 (d, *J* = 13.8 Hz, 1H), 4.69 (dd, *J* = 9.6, 7.6 Hz, 1H), 4.34 (t, *J* = 4.3 Hz, 1H), 4.17 (s, 1H), 4.15 (br.s, 1H), 3.97 (s, 3H), 3.90 (s, 3H), 3.69 (dd, *J* = 11.6, 3.5 Hz, 1H), 3.35 (s, 1H), 2.62 (s, 3H), 2.55 (dd, *J* = 13.5, 7.7 Hz, 1H), 1.98 (ddd, *J* = 13.7, 9.6, 4.3 Hz, 1H), 1.52 (d, *J* = 7.0 Hz, 3H), 1.16 (s, 9H). **^{13}C NMR** (101 MHz, Methanol-d_4) δ 172.68, 168.53, 156.62, 155.01, 149.73, 147.94, 142.43, 141.51, 137.47, 130.68, 129.83, 128.20, 128.01, 112.60, 109.39, 79.50, 69.01, 60.94, 60.49, 57.06, 56.85, 55.01, 50.30, 36.34, 35.87, 26.68, 22.43, 13.05 **MS** (ESI) *m/z* 640 $(M+H)^+$. **HRMS** (ESI) m/z: calculated for $C_{32}H_{42}N_5O_7S^+$, 640.2805; found 640.2811.

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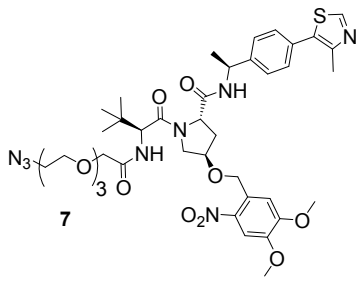
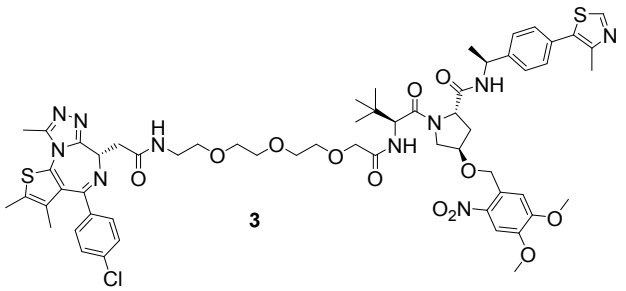


7

(2S,4R)-1-((S)-14-azido-2-(tert-butyl)-4-oxo-6,9,12-trioxa-3-azatetradecanoyl)-4-((4,5-dimethoxy-2-nitrobenzyl)oxy)-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (7)

2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)acetic acid (10 mg, 0.043 mmol, 1.2 eq) was dissolved in DCM (3 ml) along with HATU (17 mg, 0.044 mmol, 1.3 eq) and DIPEA (0.17 mmol, 29 µl, 5.0 eq). The amine **6** (hydrochloride salt, 25 mg, 0.034 mmol, 1.0 eq) was then added and the solution obtained was stirred at room temperature for 16 h (fume cupboard lights off). The reaction mixture was subsequently washed twice with water (2 x 3 ml). The recovered organic layer was dried over sodium sulfate, filtered and concentrated in vacuo. The residue was purified by silica gel flash chromatography (30-90% ethyl acetate in n-hexane) to afford **7** as a beige solid (12 mg, 41%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.74 (s, 1H), 7.69 (s, 1H), 7.41 (d, *J* = 8.7 Hz, 2H), 7.37 (d, *J* = 8.3 Hz, 2H), 7.30 (d, *J* = 7.7 Hz, 1H), 7.18 (s, 1H), 5.12 – 5.00 (m, 1H), 4.91 (q, *J* = 13.6 Hz, 2H), 4.77 – 4.67 (m, 2H), 4.45 (t, *J* = 4.4 Hz, 1H), 4.15 (dd, *J* = 10.8, 3.5 Hz, 1H), 4.01 (d, *J* = 15.5 Hz, 1H), 3.97 (s, 3H), 3.94 (s, 3H), 3.88 (d, *J* = 15.5 Hz, 1H), 3.78 (dd, *J* = 11.0, 4.9 Hz, 1H), 3.72 – 3.64 (m, 10H), 3.39 (t, *J* = 3.9 Hz, 2H), 2.80 (s, 2H), 2.71 – 2.60 (m, 1H), 2.54 (s, 3H), 2.17 (ddd, *J* = 11.0, 8.3, 4.3 Hz, 1H), 1.99 – 1.87 (m, 4H), 1.48 (d, *J* = 7.5 Hz, 3H), 1.06 (s, 9H). **^{13}C NMR** (101 MHz, Chloroform-*d*) δ 171.71, 169.56, 169.42, 153.76, 150.60, 147.68, 143.25, 139.14, 130.01, 129.60, 126.50, 109.74, 107.92, 71.07, 70.67, 70.47, 70.30, 70.02, 68.07, 58.63, 56.56, 56.50, 56.37, 53.28, 50.68, 48.96, 38.63, 35.68, 32.63, 26.45, 22.16, 15.89. **MS** (ESI) *m/z* 855 $(M+H)^+$. **HRMS** (ESI) m/z: calculated for $C_{40}H_{54}N_8O_{11}NaS^+$ (Na adduct), 877.3530; found 877.3550.



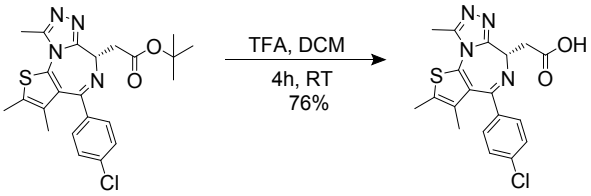
3

(2S,4R)-1-((S)-2-(tert-butyl)-17-((S)-4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)-4,16-dioxo-6,9,12-trioxa-3,15-diazaheptadecanoyl)-4-((4,5-dimethoxy-2-nitrobenzyl)oxy)-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (3)

Intermediate **7** (10 mg, 0.012 mmol, 1.0 eq) was dissolved in MeOH (2 ml) and triphenylphosphine (4 mg, 0.015 mmol, 1.25 eq) was added. The solution obtained was stirred at 50˚C for 8 h under N_2. The crude mixture was then concentrated in vacuo and the residue redissolved in DCM (3 ml) along with HATU (6 mg, 0.016 mmol, 1.3 eq) and DIPEA (0.024 mmol, 4.2 µl, 2.0 eq). JQ1-COOH (5 mg, 0.012 mmol, 1.0 eq) was then added and the solution obtained was stirred at room temperature for 16 h (fume cupboard lights off). The reaction mixture was subsequently washed twice with water (2 x 3 ml). The recovered organic layer was dried over sodium sulfate, filtered and concentrated in vacuo. The residue was purified by preparative HPLC (20-98% acetonitrile in water with 0.1% formic acid) to afford **3** as a white solid (6 mg, 41% over 2 steps).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.71 (s, 1H), 7.73 – 7.58 (m, 2H), 7.44 – 7.29 (m, 9H), 7.17 (s, 1H), 5.12 – 5.02 (m, 1H), 4.87 (d, *J* = 1.3 Hz, 2H), 4.84 – 4.78 (m, 1H), 4.72 (d, *J* = 9.4 Hz, 1H), 4.44 – 4.37 (m, 1H), 4.19 (d, *J* = 11.6 Hz, 1H), 4.09 (d, *J* = 15.6 Hz, 1H), 4.00 – 3.94 (m, 4H), 3.93 (s, 3H), 3.82 (dd, *J* = 11.0, 4.7 Hz, 1H), 3.75 – 3.63 (m, 8H), 3.61 – 3.55 (m, 2H), 3.51 – 3.41 (m, 4H), 2.66 (s, 3H), 2.52 (s, 4H), 2.40 (s, 3H), 2.27 (td, *J* = 10.3, 9.2, 4.9 Hz, 1H), 1.67 (s, 3H), 1.45 (d, *J* = 7.0 Hz, 3H), 1.08 (s, 9H). **^{13}C NMR** (101 MHz, Chloroform-*d*) δ 171.37, 170.61, 169.87, 169.82, 155.45, 153.77, 150.35, 149.98, 148.03, 147.62, 143.51, 139.06, 131.23, 130.44, 130.10, 130.04, 129.47, 128.79, 126.50, 109.75, 107.87, 70.90, 70.62, 70.58, 70.42, 70.30, 69.86, 67.94, 58.75, 56.62, 56.56, 56.34, 54.05, 53.49, 48.91, 39.54, 35.62, 33.25, 26.49, 22.15, 15.91, 14.42, 13.14, 11.77. **MS** (ESI) *m/z* 1211 $(M+H)^+$. **HRMS** (ESI) m/z: calculated for $C_{59}H_{72}N_{10}O_{12}S_2Cl^+$, 1211.4461; found 1211.4481.

Synthesis of JQ1 carboxylic acid



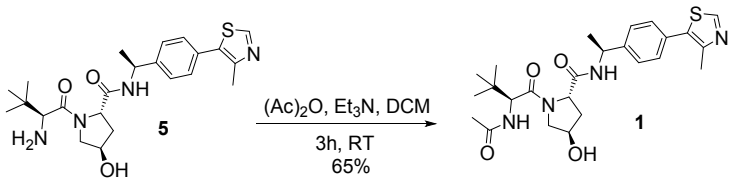
JQ1-COOH

(S)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)acetic acid (JQ1-COOH).

(+)-JQ1 (300 mg, 0.66 mmol, 1.0 eq) was dissolved in DCM (8 ml) and was added trifluoroacetic acid (1.5 ml, 19.8 mmol, 30 eq). The solution was stirred at room temperature for 4 h.The crude was then concentrated multiple times in vacuo to dryness to give **JQ1-COOH** as a white solid (200 mg, 76%).

^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.86 (s, 3H), 7.45 (d, *J* = 8.6 Hz, 2H), 7.38 (d, *J* = 8.8 Hz, 2H), 4.67 (t, *J* = 7.0 Hz, 1H), 3.70 (dd, *J* = 17.2, 6.9 Hz, 1H), 3.60 (dd, *J* = 17.2, 7.1 Hz, 1H), 2.82 (s, 3H), 2.46 (s, 3H), 1.73 (s, 3H). **^{13}C NMR** (101 MHz, Chloroform-*d*) δ 173.80, 164.85, 160.18, 154.93, 150.43, 137.58, 135.59, 132.57, 131.39, 131.14, 130.96, 130.04, 128.91, 53.26, 36.13, 14.41, 13.20, 11.28. **HRMS** (ESI) m/z: calculated for $C_{19}H_{18}N_4O_2SCl^+$, 401.0839; found 401.0844.

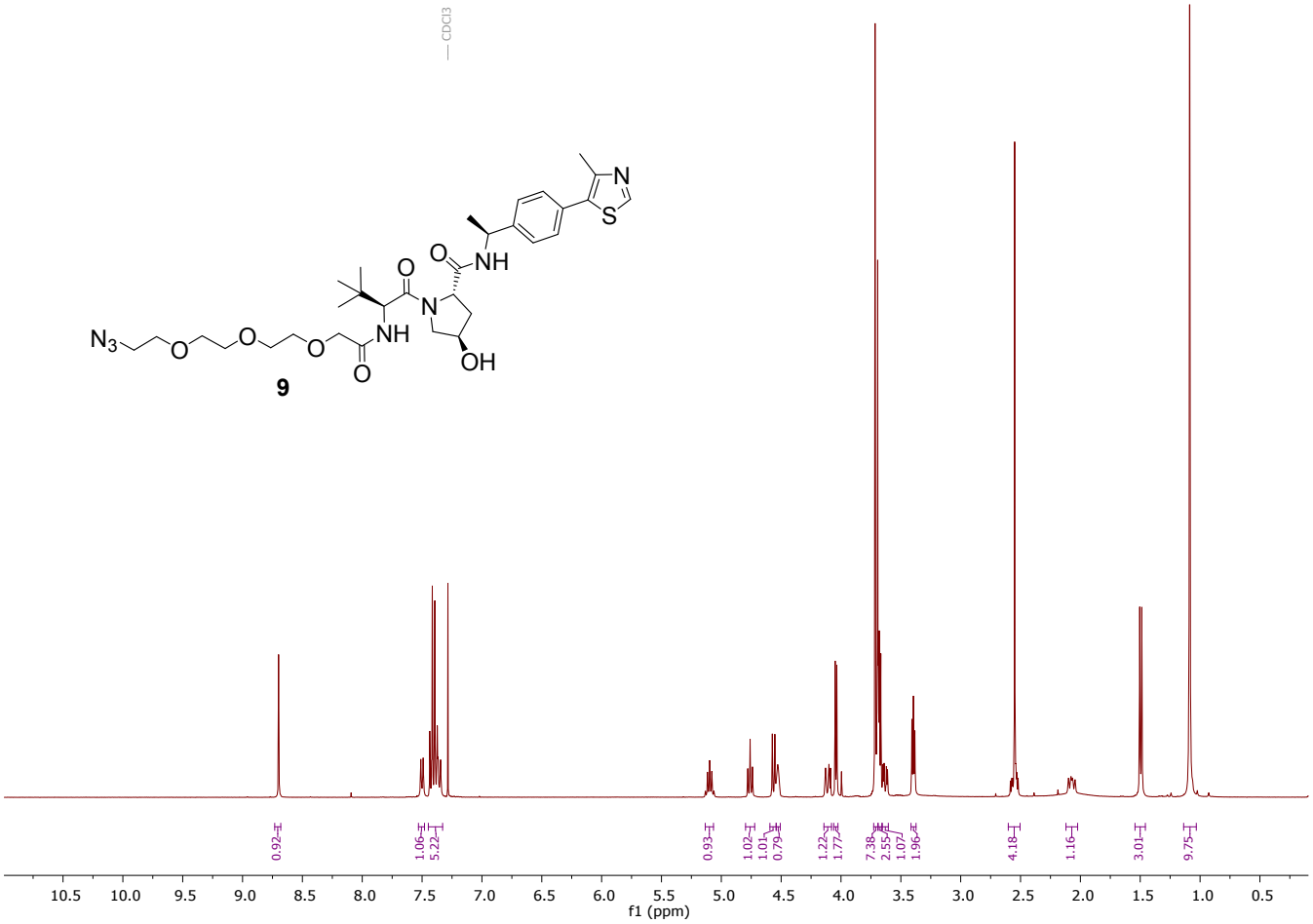
Synthesis of VHL ligand **1**



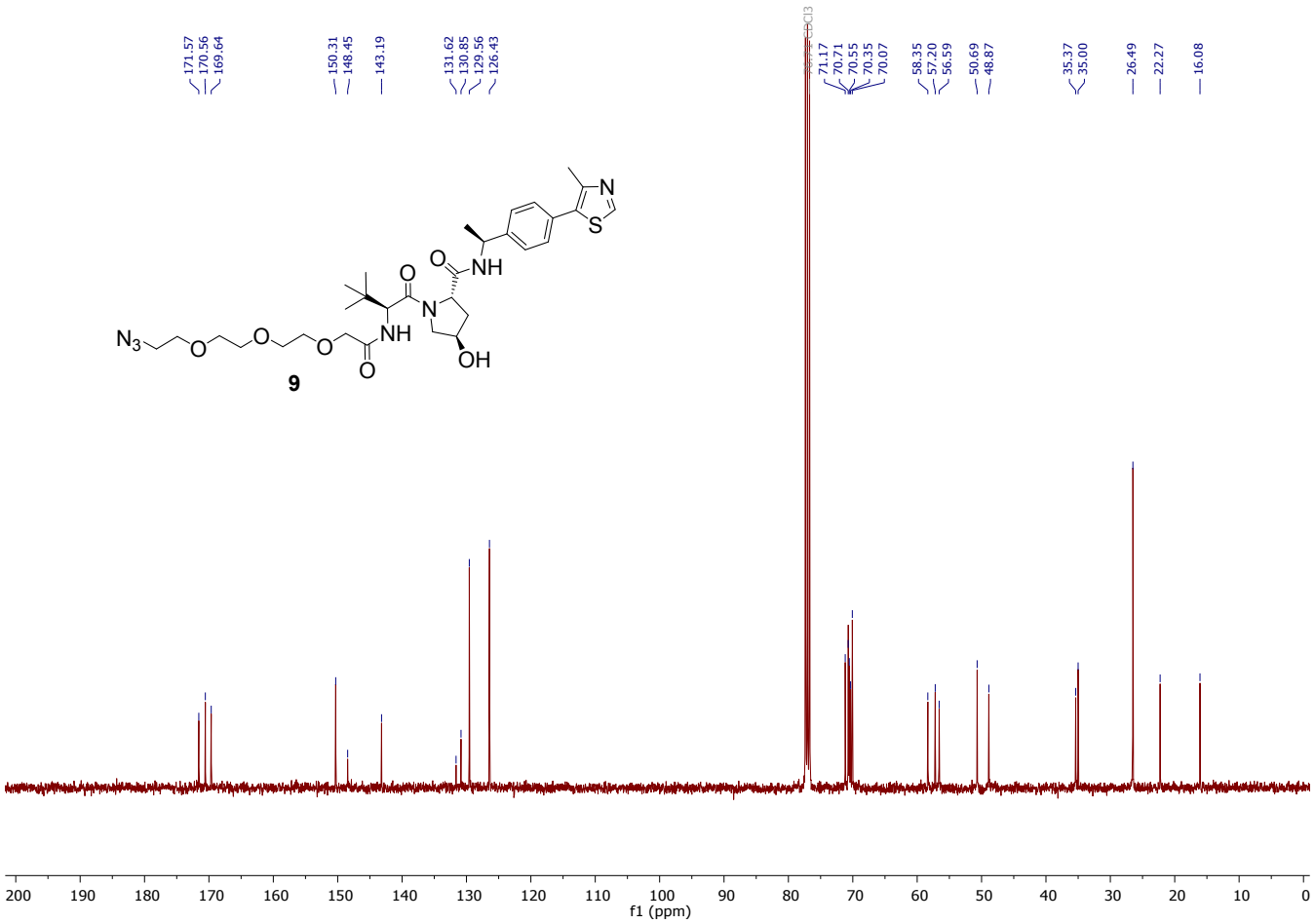
(2S,4R)-1-((S)-2-acetamido-3,3-dimethylbutanoyl)-4-hydroxy-N-((S)-1-(4-(4-methylthiazol-5-yl)phenyl)ethyl)pyrrolidine-2-carboxamide (1). Acetylation was performed according to a reported procedure.[4] Amine **5** (hydrochloride salt, 50 mg, 0.11 mmol, 1.0 eq) was dissolved in DCM (5 ml) and triethylamine (63 µl, 0.44 mmol, 4.0 eq) was added followed by acetic anhydride (16 µl, 0.17 mmol, 1.5 eq). The mixture was stirred at room temperature for 3 h and was subsequently washed with water (3 x 3 ml). The recovered organic layer was dried over sodium sulfate, filtered and concentrated in vacuo. The residue obtained was then purified by silica gel flash chromatography (60-90% ethyl acetate in n-hexane) to afford **1** as a white solid (35 mg, 65%)
^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.71 (s, 1H), 7.49 (d, *J* = 7.9 Hz, 1H), 7.46 – 7.36 (m, 4H), 6.30 (d, *J* = 8.8 Hz, 1H), 5.16 – 5.05 (m, 1H), 4.71 (t, *J* = 8.0 Hz, 1H), 4.57 (d, *J* = 8.9 Hz, 1H), 4.52 (br.s, 1H), 4.09 (dt, *J* = 11.5, 1.8 Hz, 1H), 3.62 (dd, *J* = 11.4, 3.7 Hz, 1H), 2.55 (s, 3H), 2.48 (ddd, *J* = 13.0, 7.9, 4.6 Hz, 1H), 2.09 (ddt, *J* = 13.5, 8.0, 1.9 Hz, 1H), 2.00 (s, 3H), 1.50 (d, *J* = 7.0 Hz, 3H), 1.06 (s, 9H). **^{13}C NMR** (101 MHz, Chloroform-*d*) δ 171.94, 170.85, 169.73, 150.37, 148.41, 143.18, 131.61, 130.87, 129.58, 126.48, 70.00, 58.59, 57.72, 56.78, 48.84, 35.59, 35.09, 26.49, 23.09, 22.17, 16.05. **MS** (ESI) *m/z* 487 $(M+H)^+$. **HRMS** (ESI) m/z: calculated for $C_{25}H_{35}N_4O_4S^+$, 487.2379; found 487.2367. .

c. NMR spectra

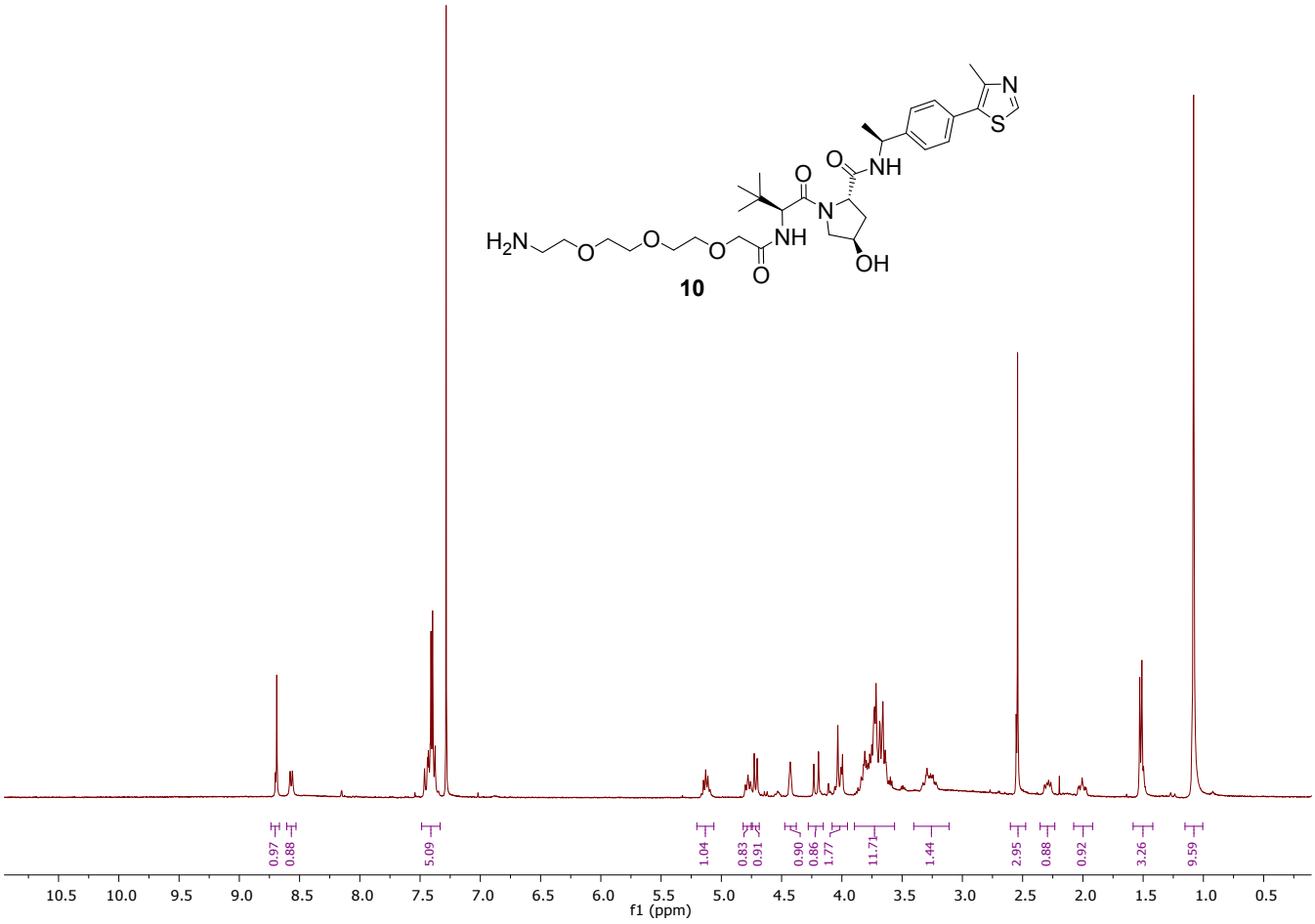
^{1}H NMR compound **9** (Chloroform-*d*)



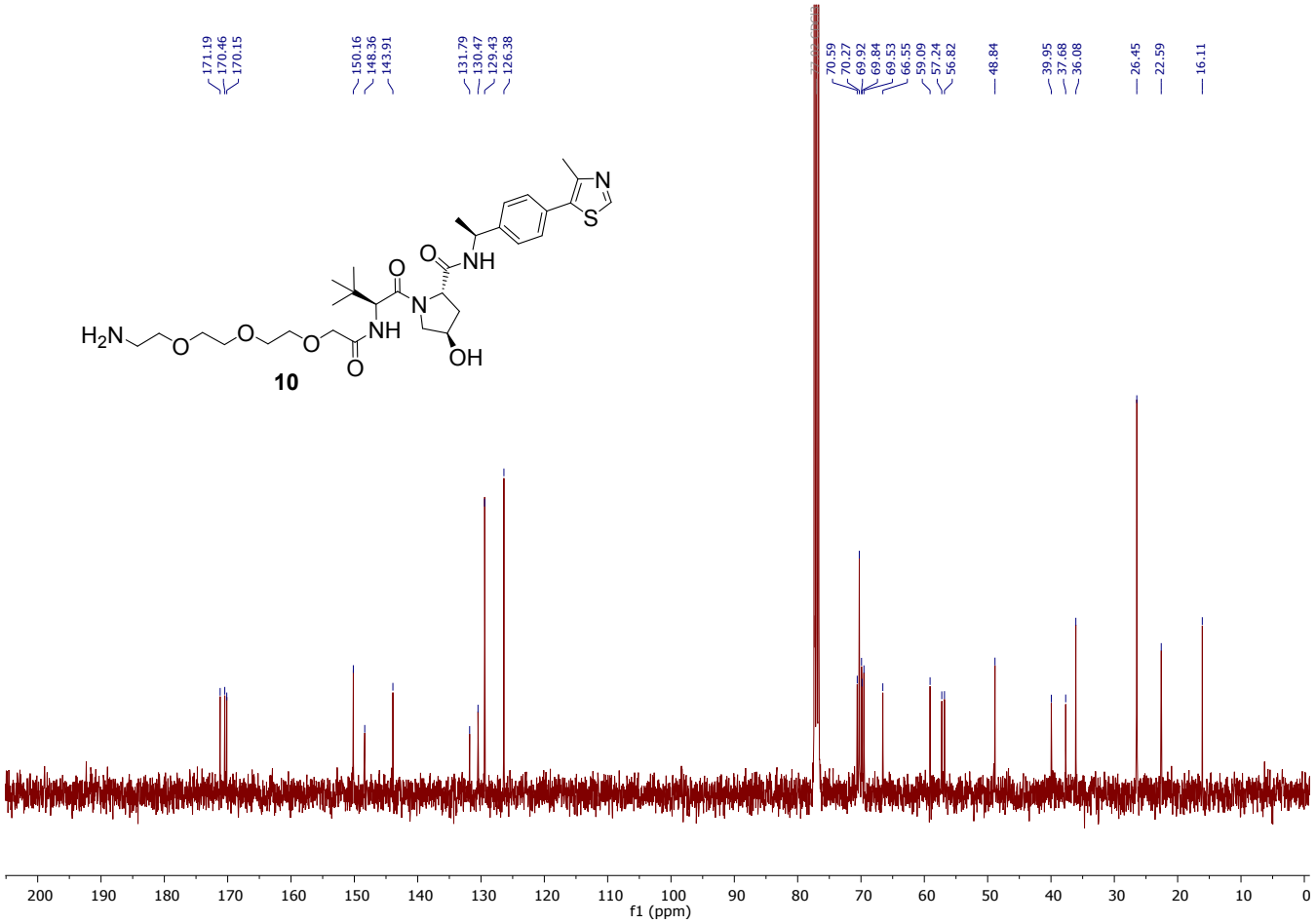
^{13}C NMR compound **9**



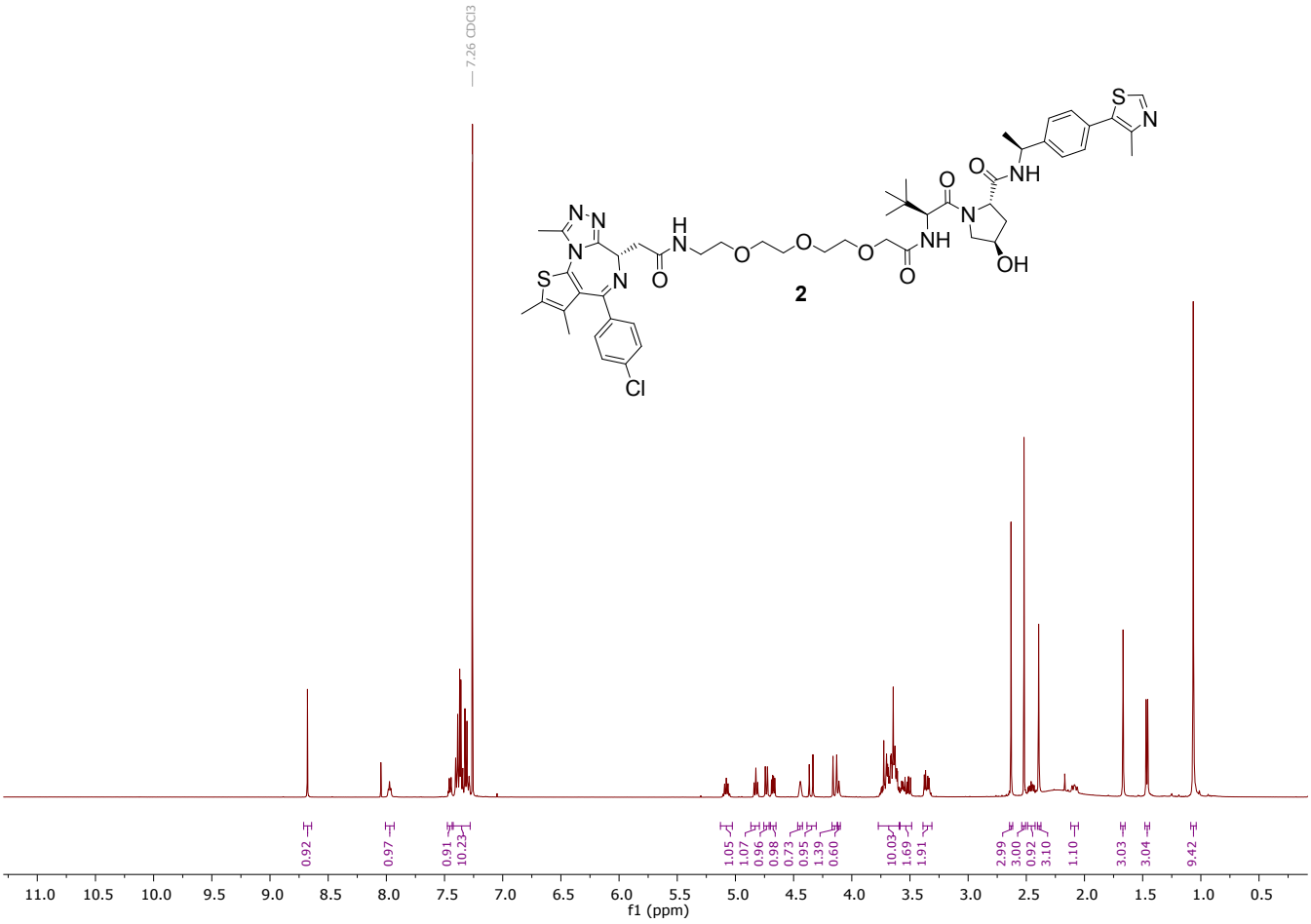
^{1}H NMR compound **10** (Chloroform-*d*)



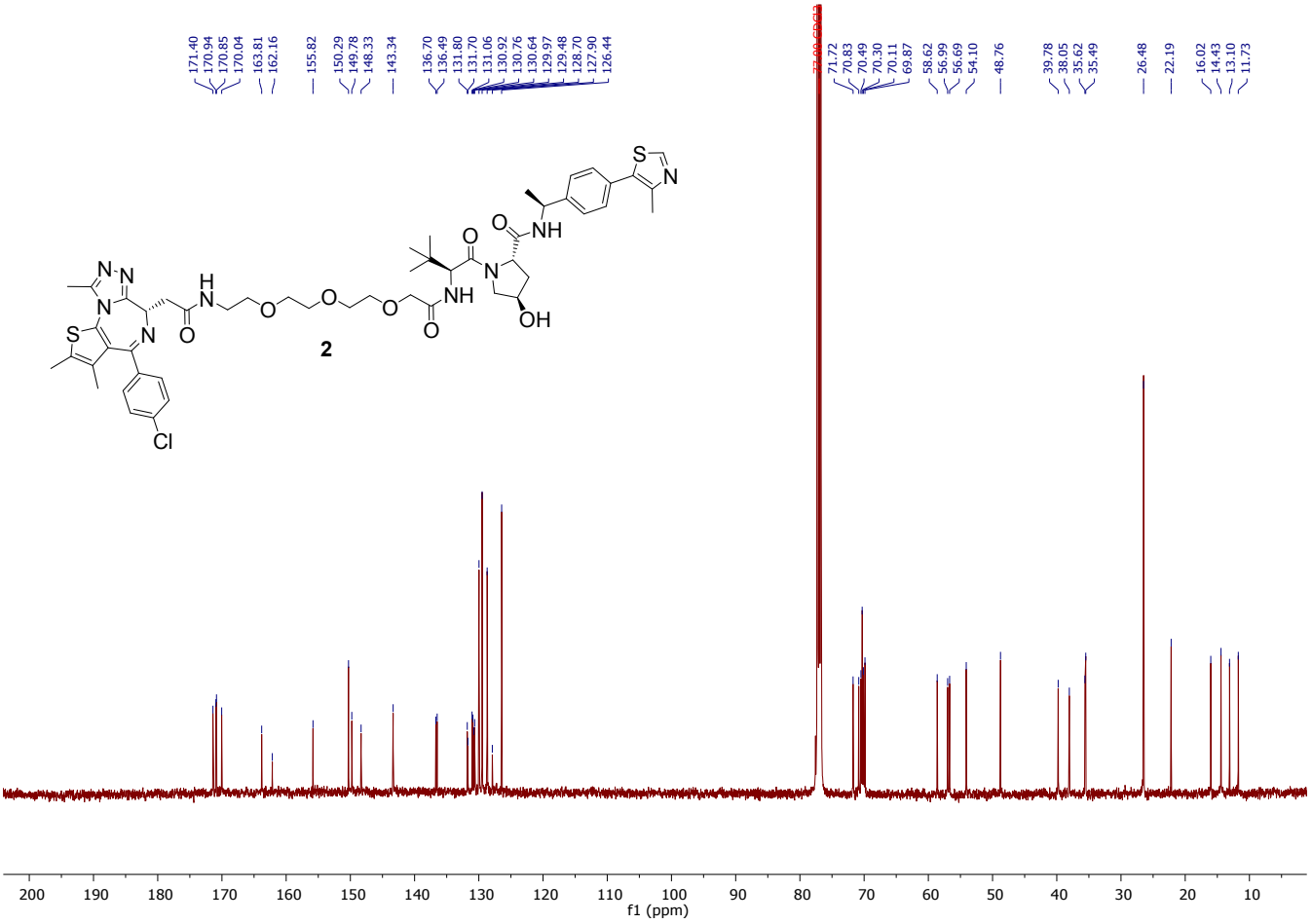
^{13}C NMR compound **10**



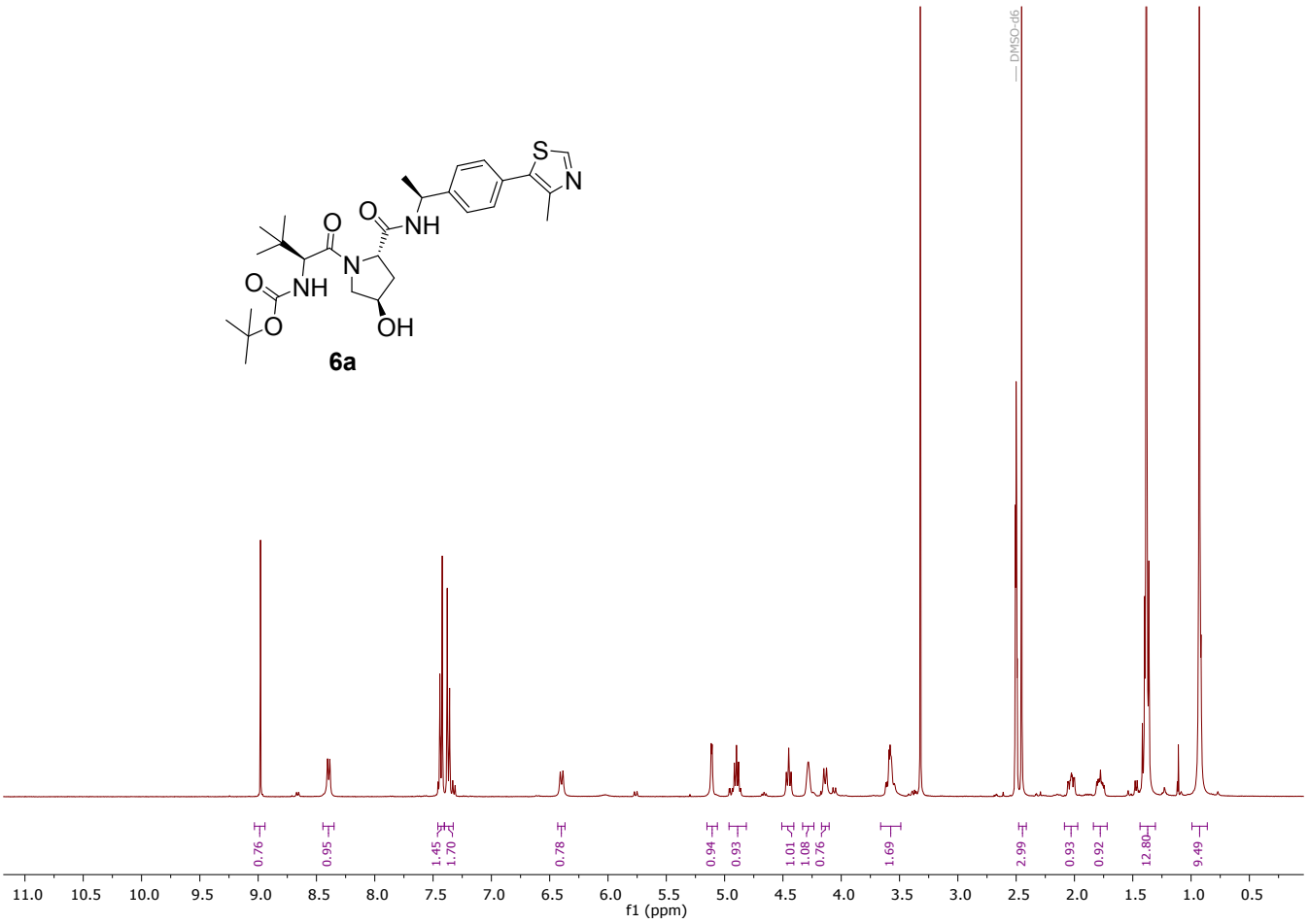
^{1}H NMR compound **2** (Chloroform-*d*)



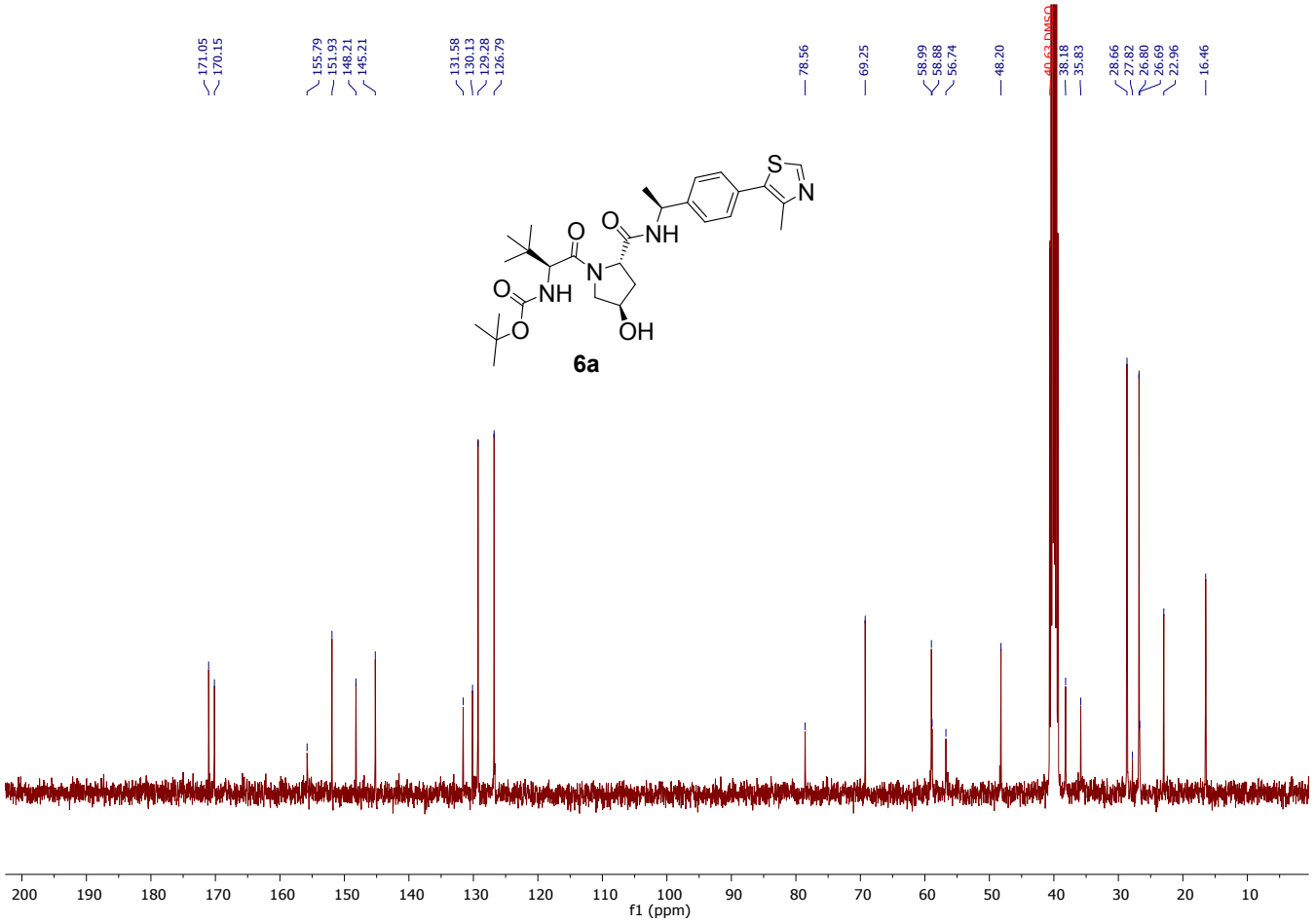
^{13}C NMR compound **2**



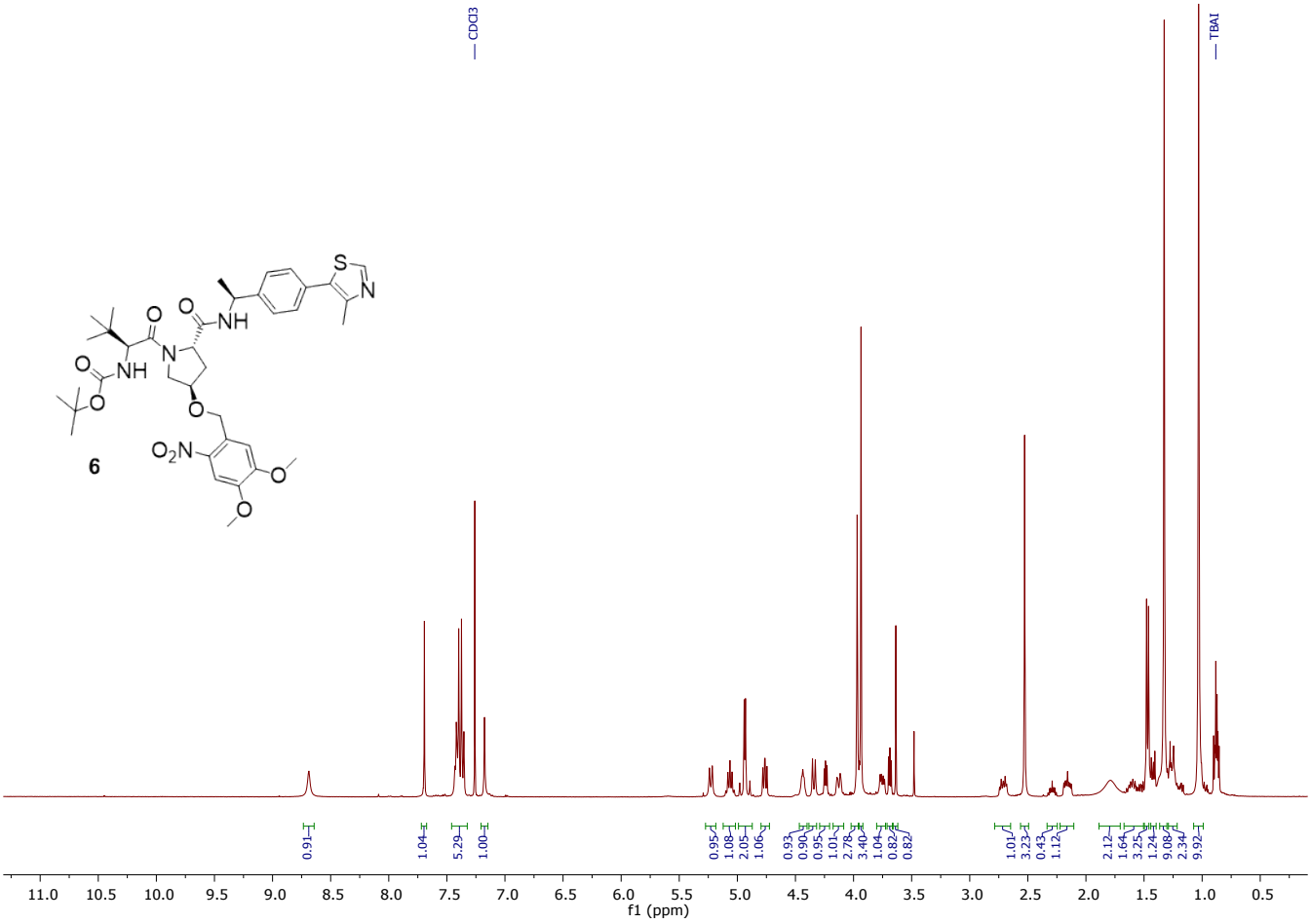
^{1}H NMR compound **6a** (DMSO-d_6)



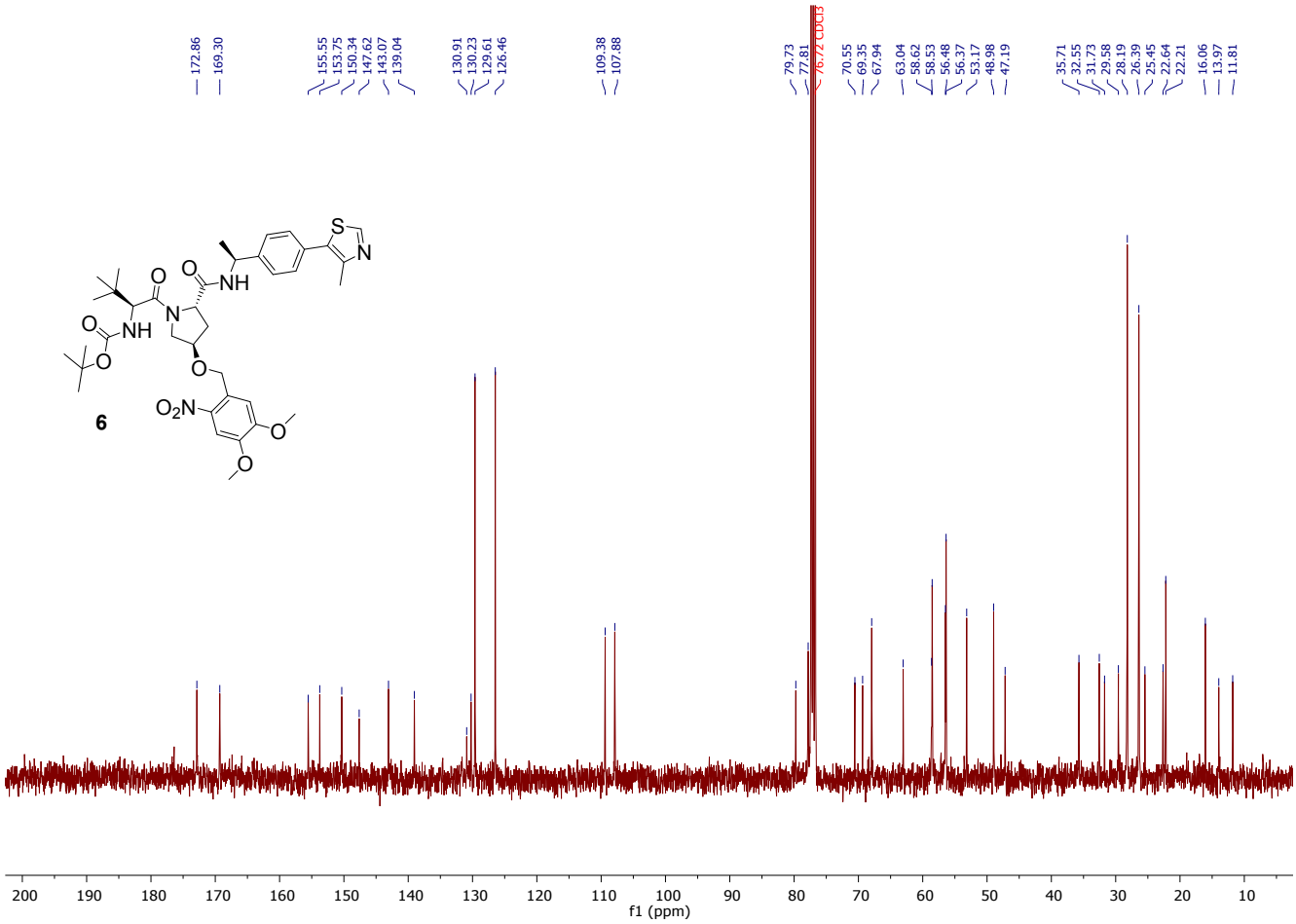
^{13}C NMR compound **6a**



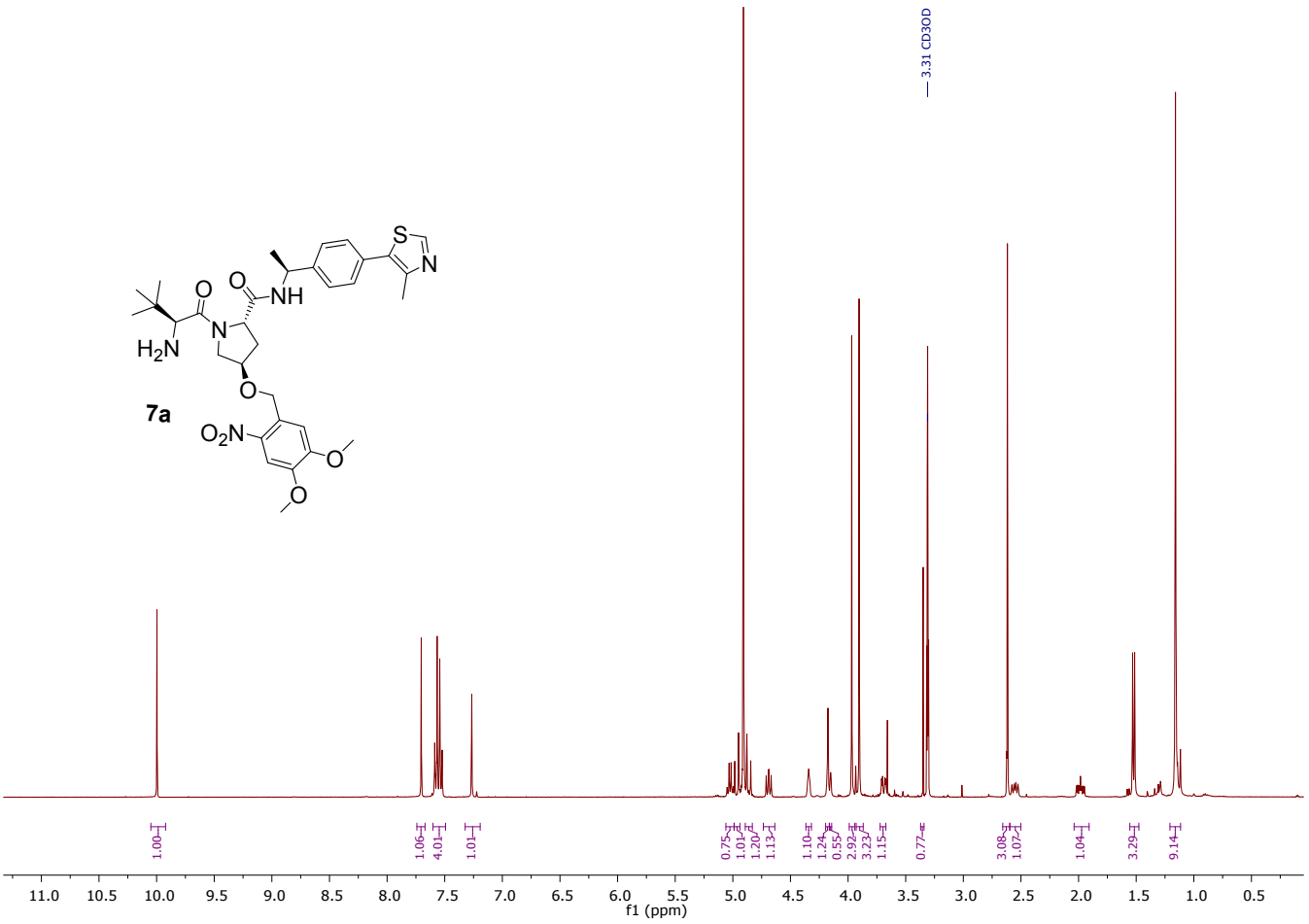
^{1}H NMR compound **6** (Chloroform-*d*)



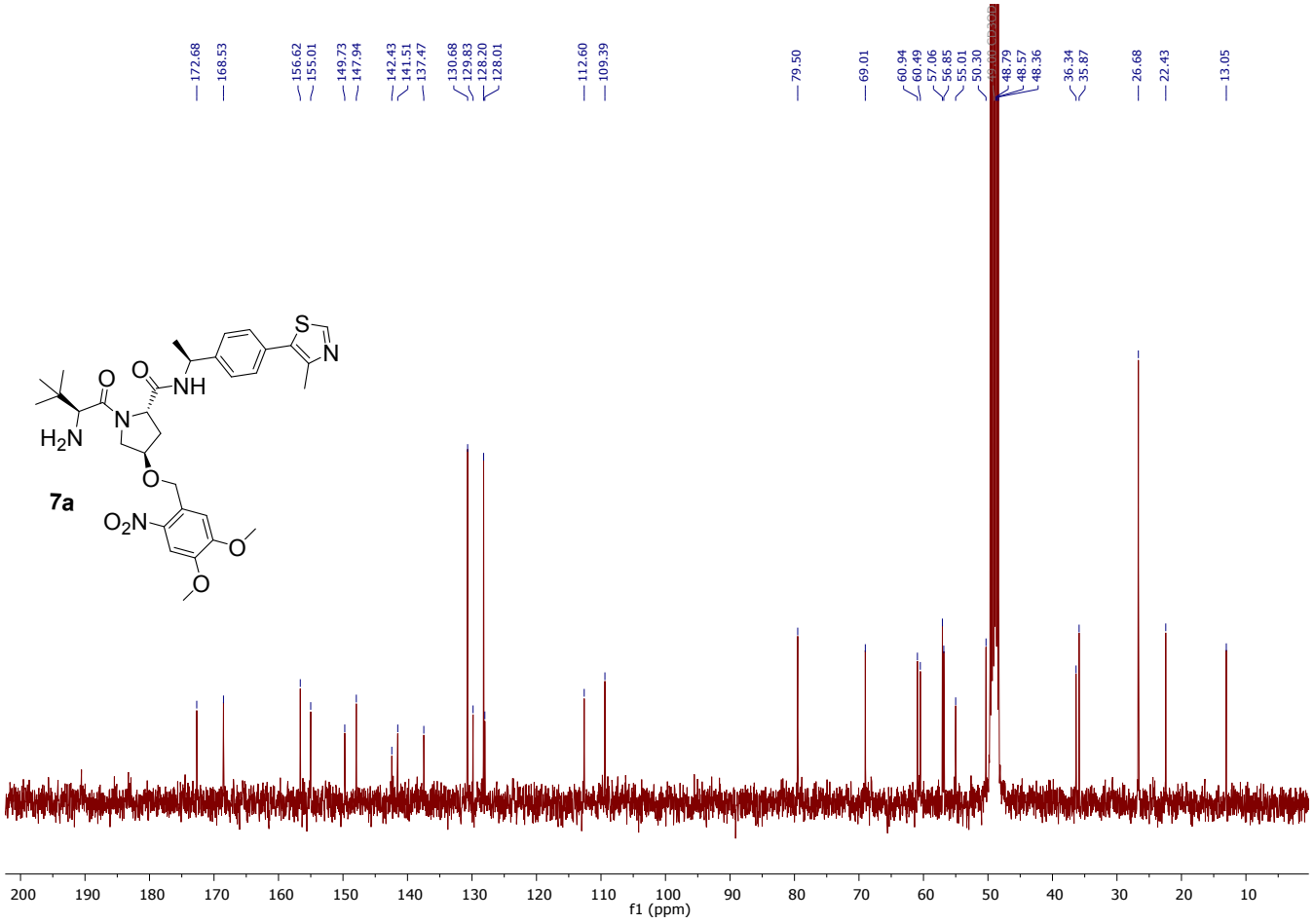
^{13}C NMR compound **6**



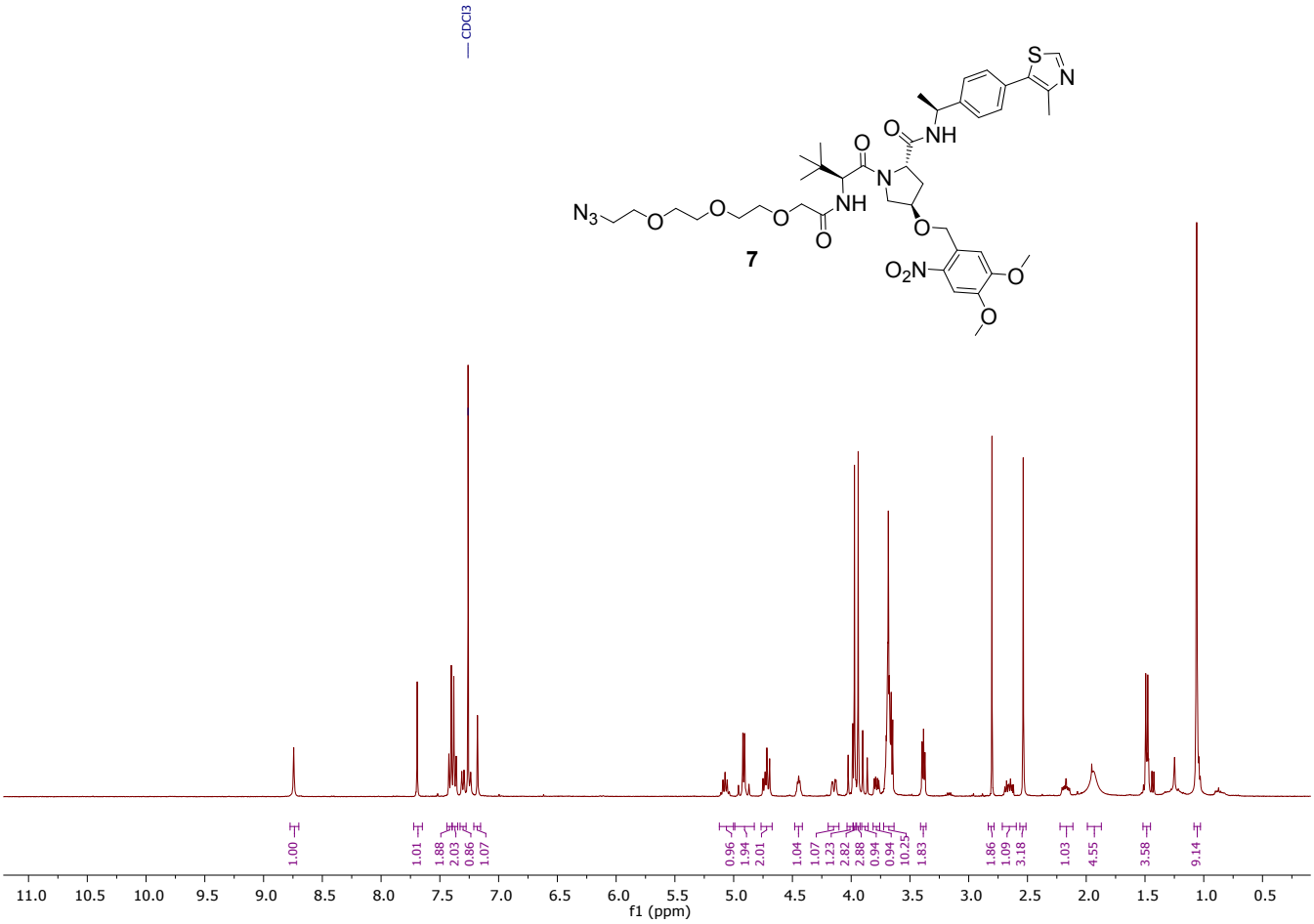
^{1}H NMR compound **7a** (Methanol-d_4)



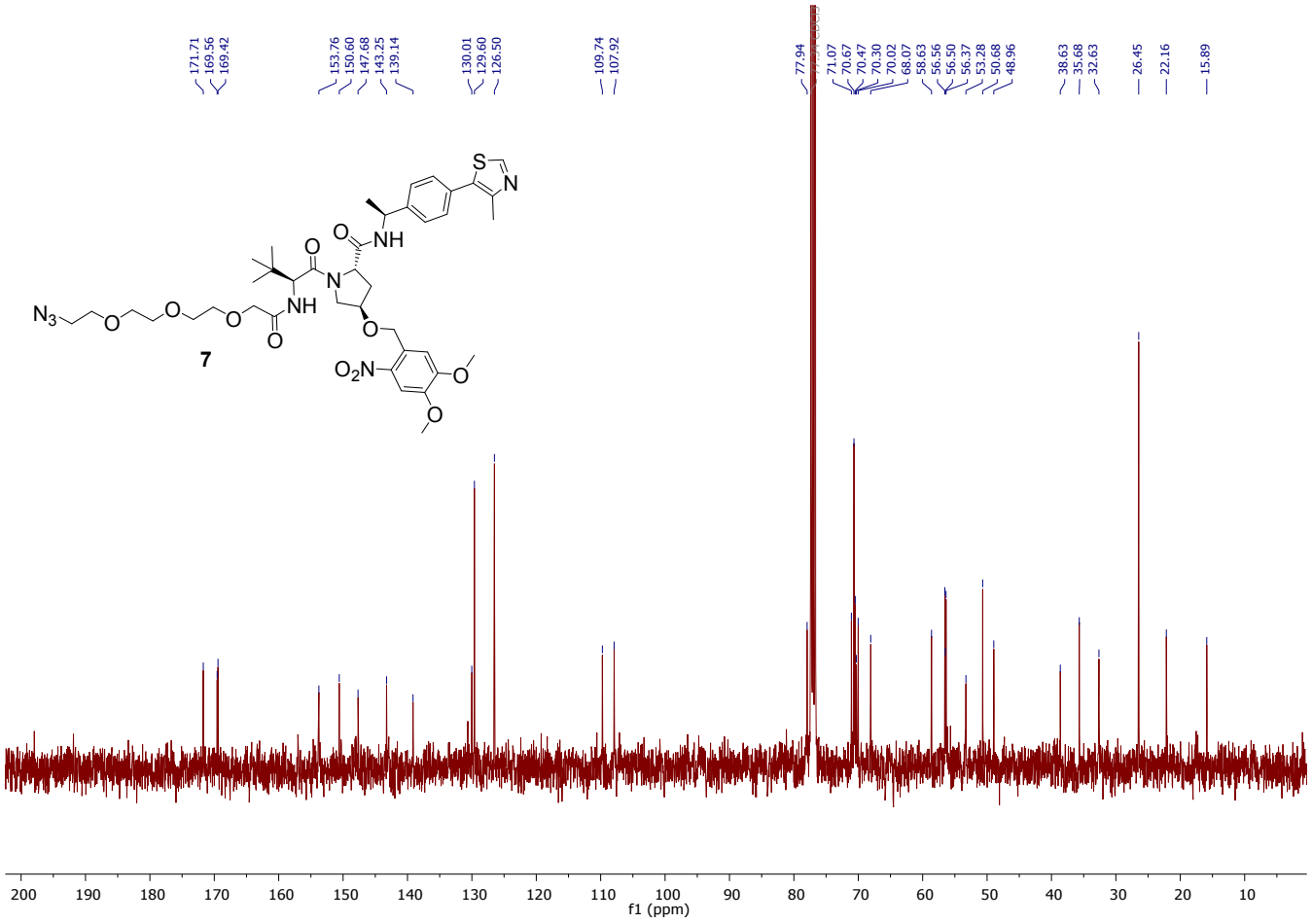
^{13}C NMR compound **7a**



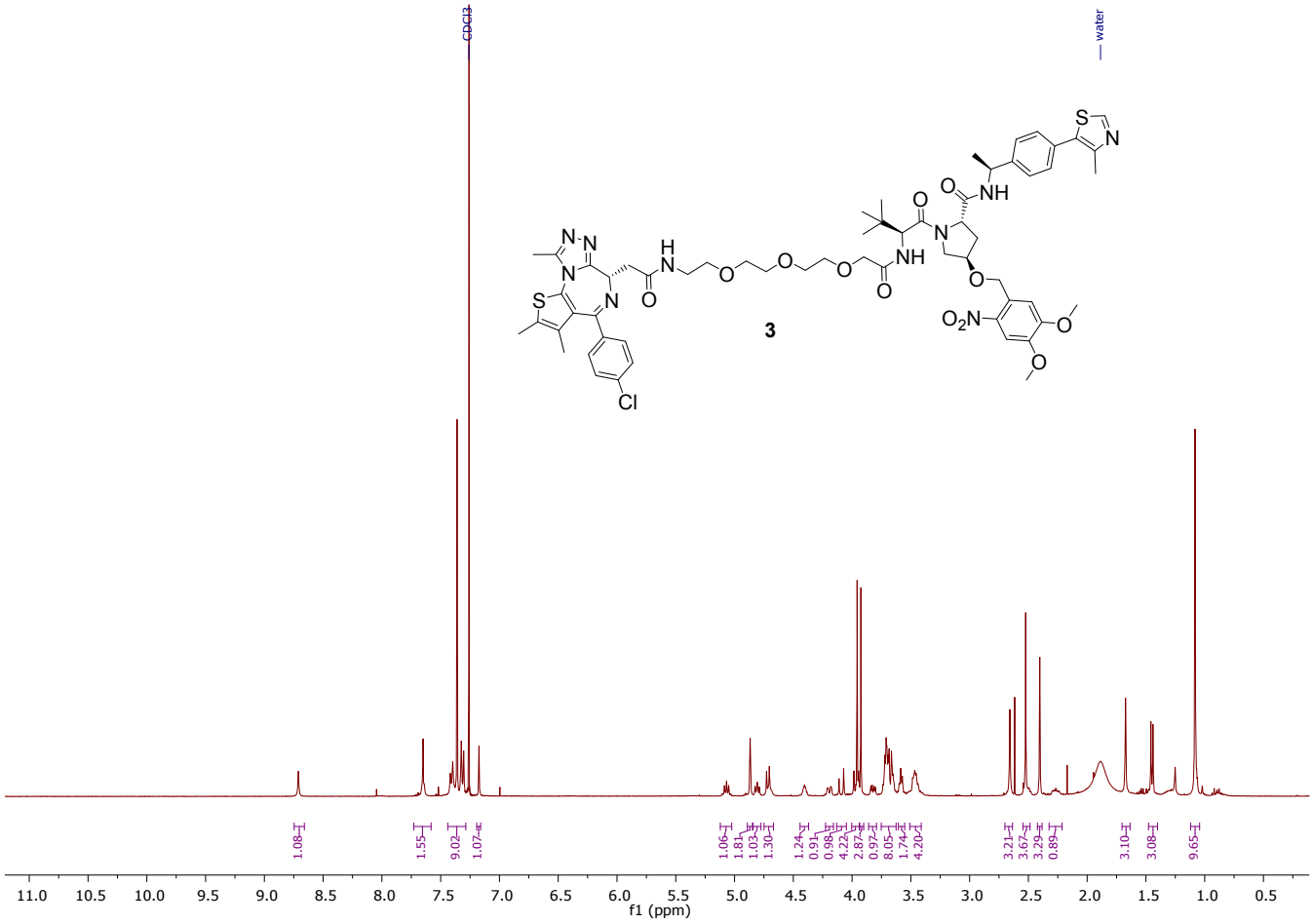
^{1}H NMR compound **7** (Chloroform-*d*)



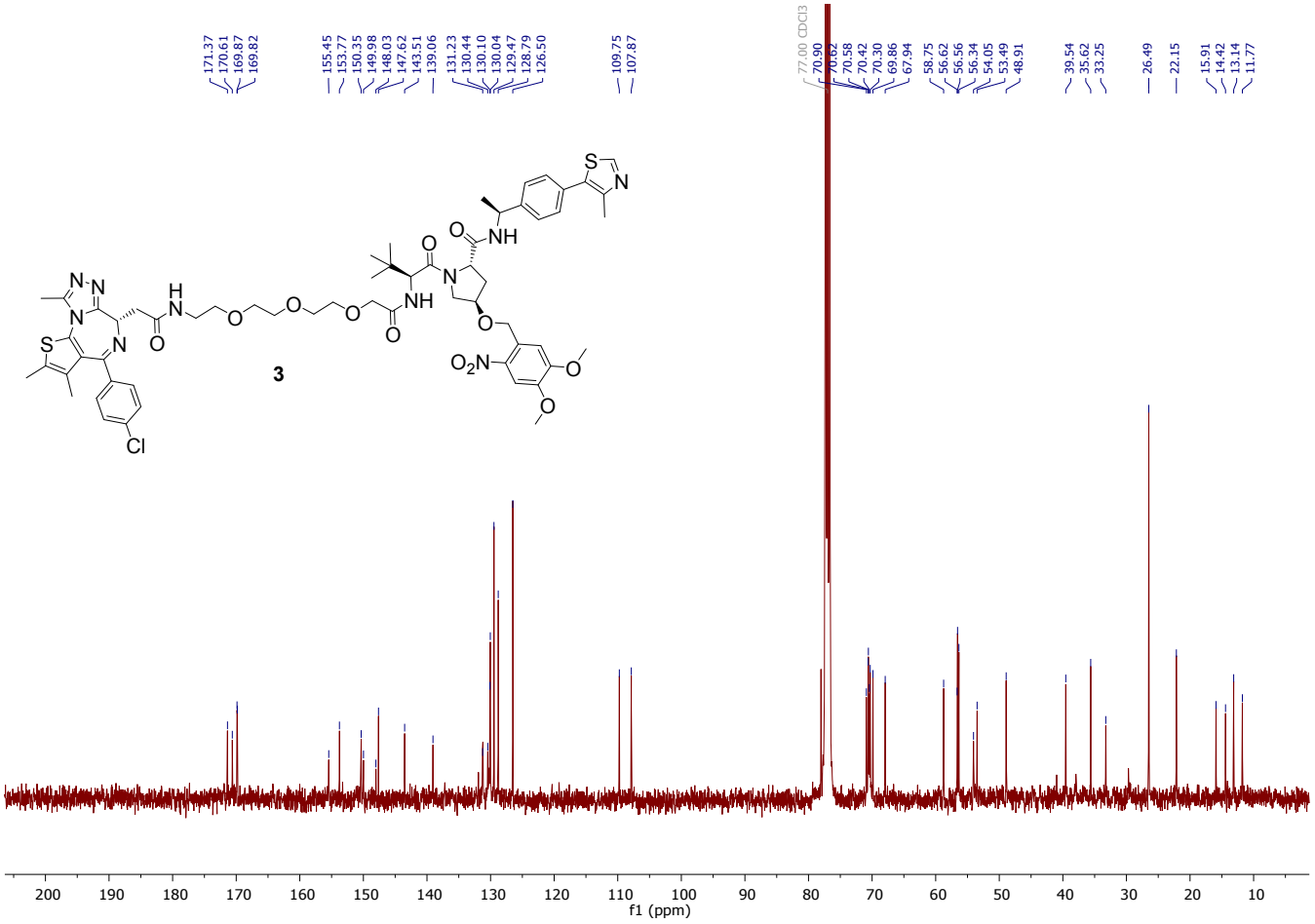
^{13}C NMR compound **7**



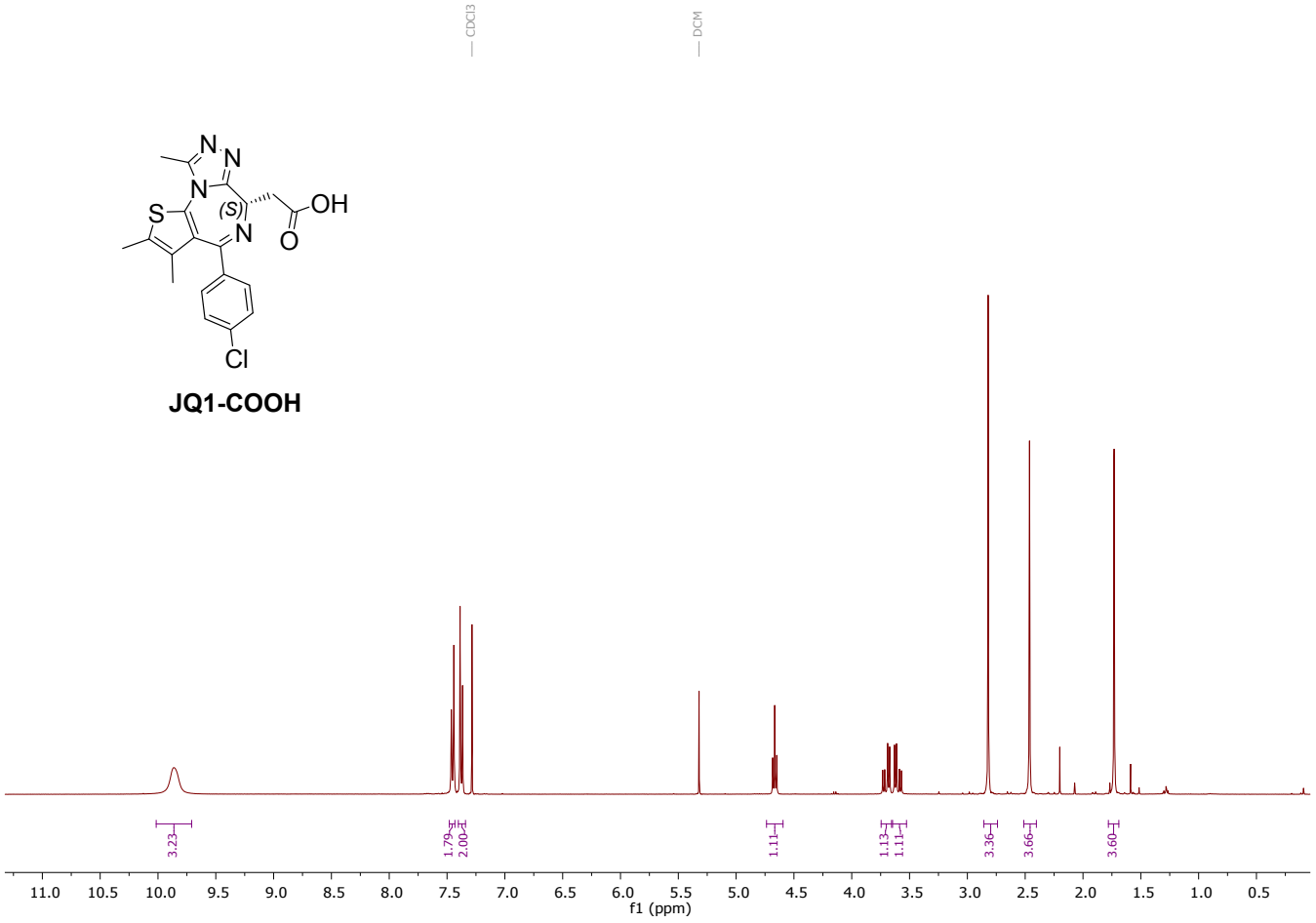
^{1}H NMR compound **3** (Chloroform-*d*)



^{13}C NMR compound **3**

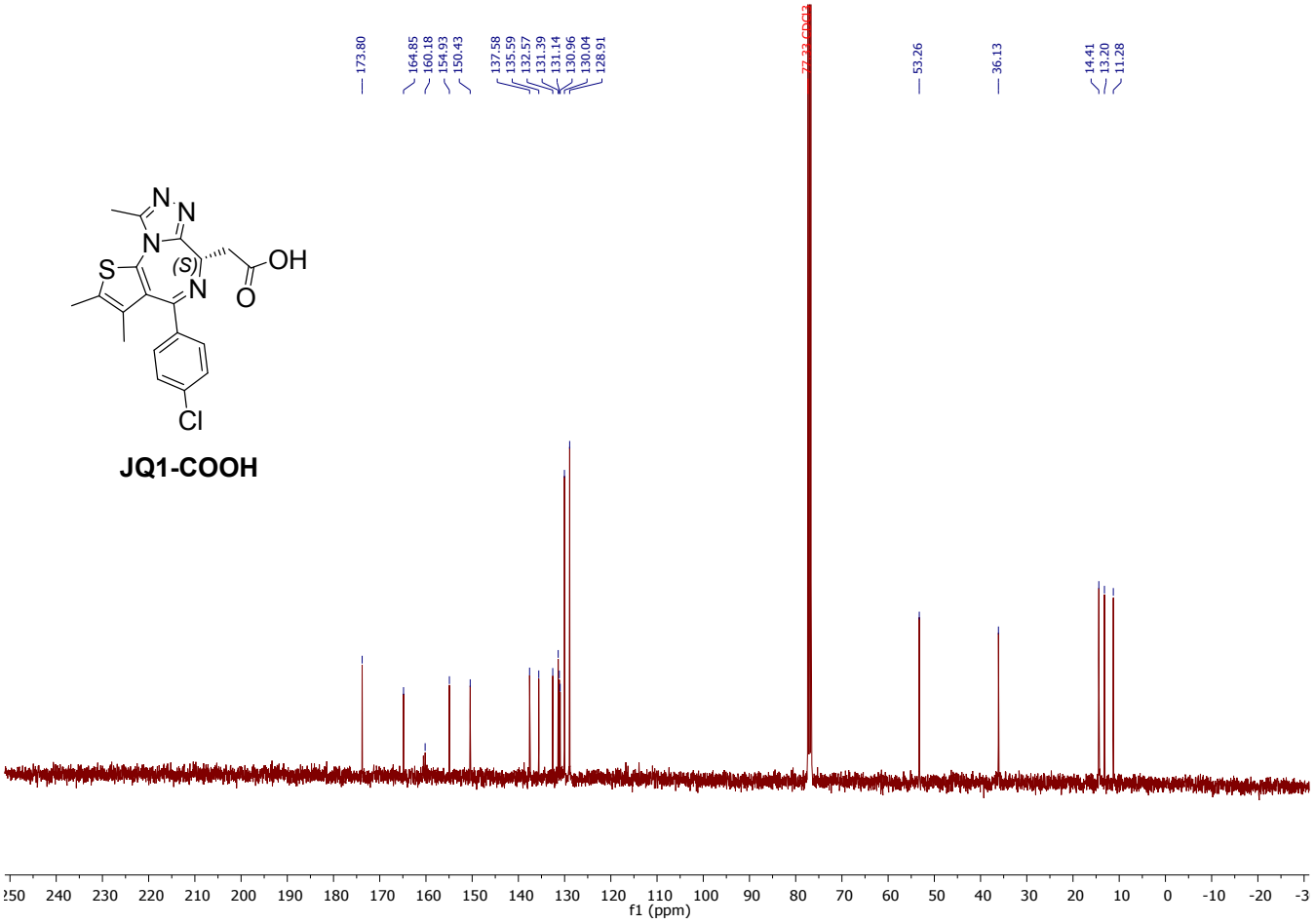


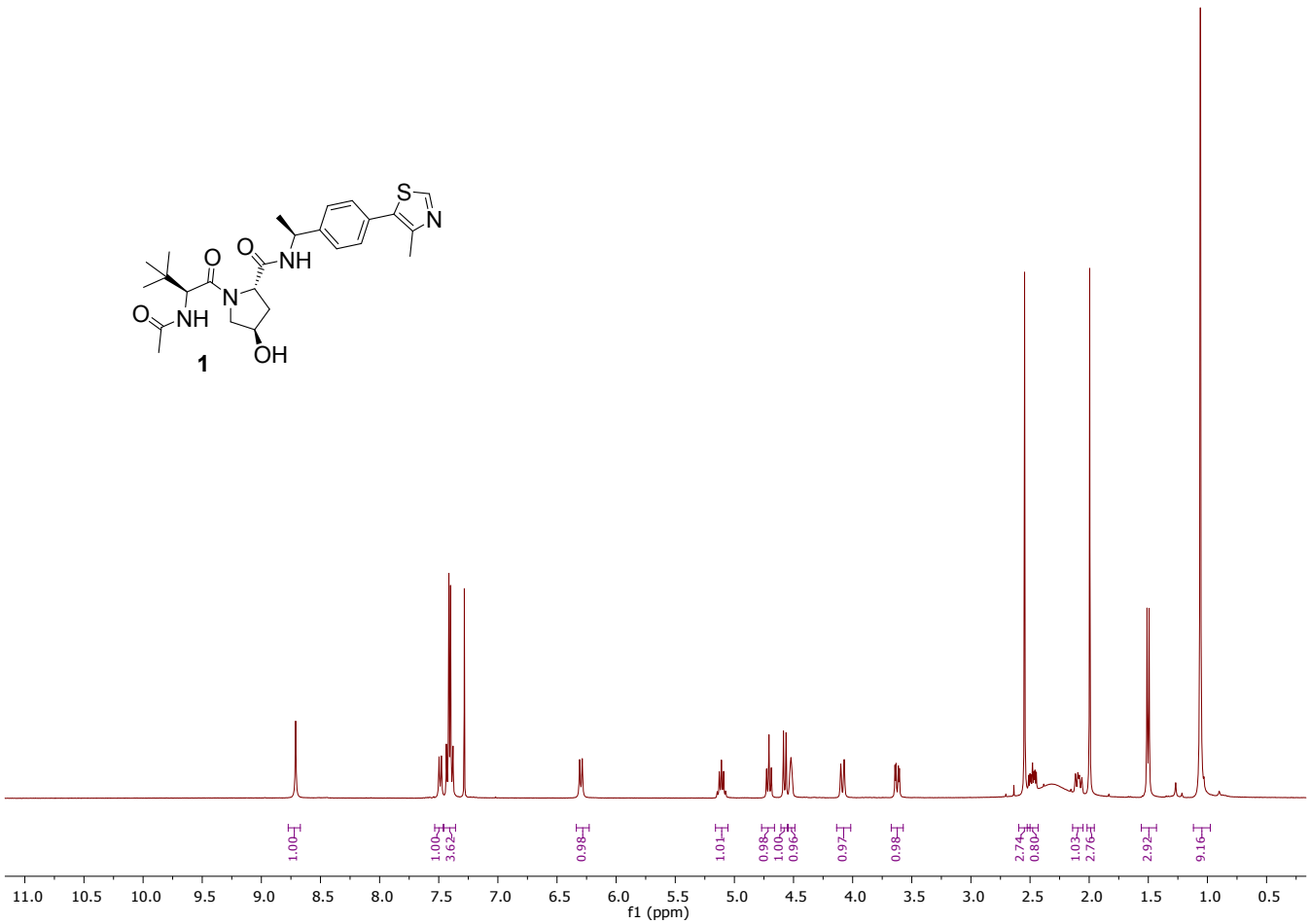
^{1}H NMR compound **JQ1-COOH** (Chloroform-*d*)



JQ1-COOH

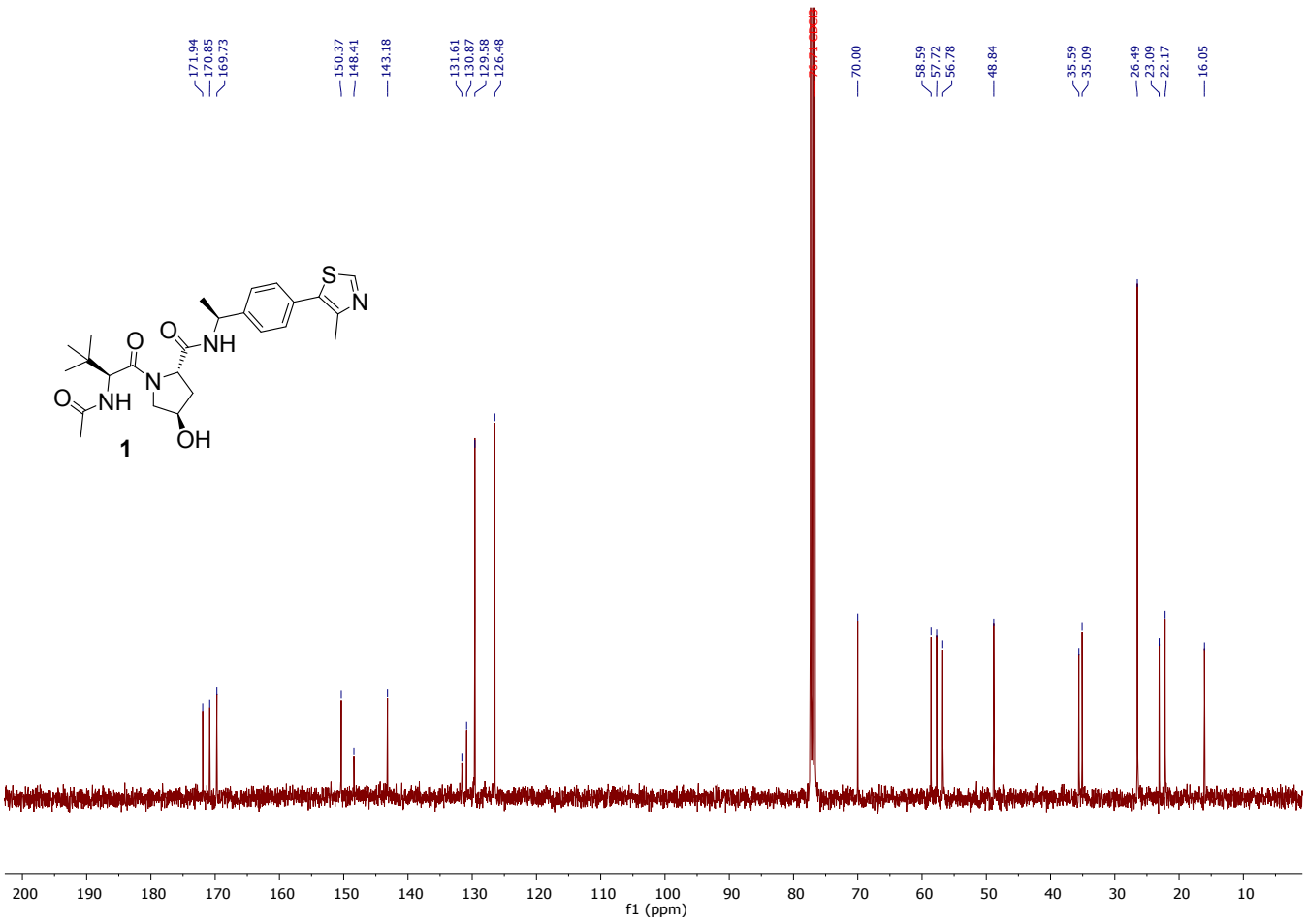
^{13}C NMR compound **JQ1-COOH**





^{1}H NMR compound **1** (Chloroform-*d*)

^{13}C NMR compound **1**



3. Biological methods

a. General methods

Reagents
All reagents were purchased from commercial sources and used as supplied unless otherwise indicated. Reagents for the radio immunoprecipitation assay buffer (RIPA lysis buffer) were purchased from Sigma Aldrich and include sodium deoxycholate, Triton X-100 and SDS 10%. Tris base and sodium chloride were purchased from VWR. DMEM media and heat deactivated fetal bovine serum (FBS) were purchased from Merck and Gibco, Life Technologies respectively. Complete Mini EDTA free Protease inhibitor cocktail was purchased from Sigma. Benzonase Nuclease was purchased from Sigma Aldrich,

Lysis buffer composition (20 ml final volume)
NaCl (2 ml, 750 mM, final concentration 75 mM), sodium deoxycholate (1 ml, 5%, final concentration 0.25%), Triton X-100 (100 µl, final concentration 0.5%), SDS 10% (100 µl, final concentration 0.05%), 16.8 ml of Milli-Q purified water. Benzonase (4 µl, 250 units per µl).

Cell culture
HeLa cells and HEK293 cells were cultured in low-glucose Dulbecco's Modified Medium (DMEM, Merck) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco). All cells were maintained in a humidified incubator at 37°C and 5% CO_2. Cells were seeded on plates at least 24 h before experiment.

Western blot analysis
After cell treatment, the media was aspirated, and the tissue layer washed with 0.5 ml of PBS. The cells were then added 150 µl of cold RIPA-buffer containing protease inhibitor cocktail and were detached using a cell scraper. After collection of the lysates on ice, centrifugation at 4 °C (12,000 g, 15 mins) allowed the recovery of supernatant. The protein concentration was measured with a Bio-Rad *DC* Protein Assay. 15-20 µg of protein extracts were fractionated by SDS-PAGE and transferred to PVDF membranes which were then blocked with 5% skim milk in Tris-buffered saline with 0.1 % Tween-20 (TBS-T). Subsequent incubation with primary antibodies Anti-BRD4 (Cell Signaling, (E2A7X) Rabbit mAb #13440), Anti-c-myc (Abcam, ab32072) and Anti-beta Actin (Abcam, (ab8227)) was conducted overnight at 4 °C. After washes with TBS-T (3 x 10 mins), the appropriate secondary antibodies (Goat Anti-Rabbit IgG H&L (HRP) (ab205718)) was incubated for 1 h. Finally, the bands were detected by western fluorescent detection reagent (Merck™ Luminata™ Western HRP Chemiluminescence) and imaged within the ImageQuant LAS 4000 series.

b. Experimental procedures

Testing of PROTAC 4 and 2
HeLa cells were seeded in 6-well plates with a density of 0.6 x 10^6 cells per well in 2.0 ml growth medium (low-glucose DMEM). 24 h after settling, 2 ml solutions of PROTAC **2** and **4** in growth medium were prepared in serial dilution (10 µM to 1 nM) from 10 mM DMSO stocks. After aspiration of the media, cells were treated with PROTACs for 24 h in the incubator (37 °C, 5% CO2). Cells were lysed according to the protocol described above.

Testing of PROTAC 2, 3 and 4 (with irradiation)
HeLa cells were seeded in 6-well plates with a density of 0.6 x 10^6 cells per well in 2.0 ml growth medium (low-glucose DMEM). 24 h after settling, 2 ml solutions of PROTAC **3** in growth medium were prepared in serial dilution (1 µM to 10 nM) from a 10 mM DMSO stock.
PROTAC **2** and **4** were tested at 1 µM. After aspiration of the media, cells were treated with PROTACs for 2 h in the incubator (37 °C, 5% CO2) prior to irradiation for 60 seconds at 80 mm from a 25 mW 365 nm LED. Cells were further kept in the incubator for 22h and were finally lysed according to the protocol described above.

Time course experiment with PROTAC 2, 3 and 4
Cells were seeded and treated as above. For PROTAC **2** and **4** (1 µM) , the time course started immediately after incubation with the compounds. For PROTAC **3** (1 µM), the time course started after irradiation for 60 seconds at 365 nm. Cells were lysed at 60 min intervals to evaluate onset of BRD4 degradation.

VHL-dependency experiment
HeLa cells were seeded in 6-well plates with a density of 0.6 x 10^6 cells per well in 2.0 ml growth medium (low-glucose DMEM). 24 h after settling, cells were treated with rising concentrations of VHL ligand **1** (0.1 to 10 µM) for 2 h then were treated with DMSO vehicle 0.1% (v/v), PROTAC **4**, **3** or **2** (1 µM) for 2 h before irradiation for 60 seconds at 365 nm. Cells were further kept in the incubator (37 °C, 5% CO2) for another 20 h belore lysis.

Proteasome-dependency experiment
HeLa cells were seeded in 6-well plates with a density of 0.6 x 10^6 cells per well in 2.0 ml growth medium (low-glucose DMEM). 24 h after settling, cells were treated with proteasome inhibitor bortezomib (BTZ**,** 10 µM) for 2 h then were treated with DMSO vehicle 0.1% (v/v), PROTAC **4**, **3** or **2** (1 µM) for 2 h prior to irradiation for 60 seconds at 365 nm. Cells were further kept in the incubator (37 °C, 5% CO2) for another 20 h belore lysis.

Washout experiment
HeLa cells were seeded in 6-well plates with a density of 0.6 x 10^6 cells per well in 2.0 ml growth medium (low-glucose DMEM). 24 h after settling, cells were treated with PROTAC **2, 3** or **4** (1 µM) for 2 h then cells were washed with PBS (3 x 0.5 ml) prior to irradiation for 60 seconds at 365 nm. Cells were further kept in the incubator for 6 h and were finally lysed according to the protocol described above.

Cell proliferation experiment
HeLa cells were seeded in 96-well plates with a density of 0.5 x 10^4 cells per well in 0.2 ml growth medium (low-glucose DMEM). 24 h after settling, cells were treated with DMSO, JQ1, PROTAC **2, 3** or **4** for 1 h 30 mins then cells were irradiated for 60 seconds at 365 nm. Cell proliferation was monitored over 6 days via live-cell microscopy using the IncuCyte S3. Values were normalized to cell count at T0 and fold changes were plotted.

CETSA experiment
Tm determination: HeLa cells were seeded in a T75 flask (triplicate). After reaching about 90% confluency, cells were collected (detachment with trypsin) and pelleted by centrifugation. The pellet was washed with PBS twice then resuspended in PBS containing a protease inhibitor cocktail. The sample obtained was divided into twelve aliquots of 100 µl each and individual aliquots were heated for 3 mins within a PCR tube in a thermo cycler following a temperature gradient (12 temperatures from 37.6°C to 66,9°C). After cooling, the samples were subjected to a freeze-thaw cycle (3 times) to lyse the cells. A centrifugation step allowed the recovery of the supernatant from the cell debris. The lysates were subsequently analysed by Western Blot. Gel quantification with ImageJ yielded the melting temperature Tm = 44.3°C.
Isothermal dose response CETSA: HeLa cells were seeded in 6-well plates with a density of 0.6 x 10^6 cells per well in 2.0 ml growth medium (low-glucose DMEM). 24 h after settling, cells were treated with PROTAC **3** or JQ1 for 18h. Cells were detached as per above and the resulting aliquots were heated at 44.3°C. Following lysis and centrifugation, the soluble fractions obtained were analyzed by Western blot.

Generation of pEGFP-BRD4-C1 plasmid
The BRD4 gene was cut out from NanoLuc®-BRD4 plasmid (pFN31K-BRD4, kind gift from Promega, UK) using SalI, XhoI, ClaI and SpeI restriction enzymes and isolated by agarose gel purification. The pEGFP-C1 vector (kind gift from Dr Cory Antonio Ocasio (Francis Crick Institute, London, UK) was linearized by PCR using primers tacccggggatcctctagag*tcgac*GCCATACCACATTTGTAGAGG (forward, overlaps with SalI restriction site) and agacatggcgatcgcgccg*ctcgaggctct*CTTGTACAGCTCGTCCATGC (reverse, overlaps with XhoI restriction site). The N-terminally tagged GFP-BRD4 fusion sequence was assembled with NEBuilder® HIFI DNA Assembly Master Mix (New England Biolabs, MA).

Live-cell fluorescence imaging and quantification
HEK293 cells were seeded into 12-well plate coverslips (Mattek, P12G-1.5-14-F) and incubated overnight in DMEM (Gibco) (with 10% FCS). Cells were then transfected with 100 ng of pEGFP-BRD4-C1 plasmid (see above) per well with 300 ng of empty vector and 1.2 µL of Lipofectamine 2000 (Invitrogen) in Opti-MEM® (Gibco) following the manufacturer's protocol. After 2 h post-transfection, medium was removed and replaced with fresh DMEM (with 10% FCS). After 24 h post-transfection, cells were treated with PROTACs at various concentrations and immediately imaged using a Nikon Eclipse Ti2 inverted microscope (Nikon, Tokyo, Japan) equipped with 20x 0.75NA Ph2 objective and controlled with Micro-Manager v2.0 software (Open-Imaging). For uncaging, cells were incubated with PROTAC **3** or DMSO for 1 h, imaged at 0 time point, irradiated with a UV light box at 365 nm for 60 seconds and then imaged after irradiation every 20 min. GFP fluorescence was monitored using LED GFP 470/24 (excitation) and ET525_50m (emission) standard filters over 6 hours with 20 min interval between image captures. Image processing, analysis and quantification was performed using ImageJ (NIH) and GraphPad Prism. Upon background subtraction, a group of cells was selected, and snapshots generated at given time intervals. For quantification, a fluorescence intensity of 10 individual cells was measured over time and divided by fluorescence intensity for each cell at 0 time. The mean fluorescence intensity with SEM was plotted on a graph at given time points.

c. Original gels

Figure 3A – PROTAC **4**

BRD4 **β-actin**

Figure 3A – PROTAC **2**

BRD4 **β-actin**

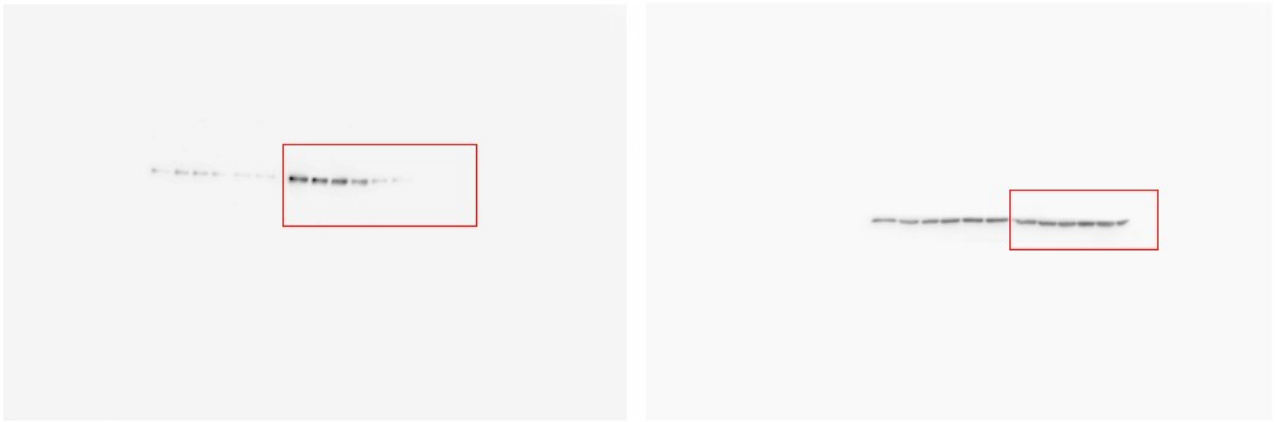


Figure 3B

BRD4 - DMSO, PROTAC **4,** PROTAC **2**

BRD4 - PROTAC **3**

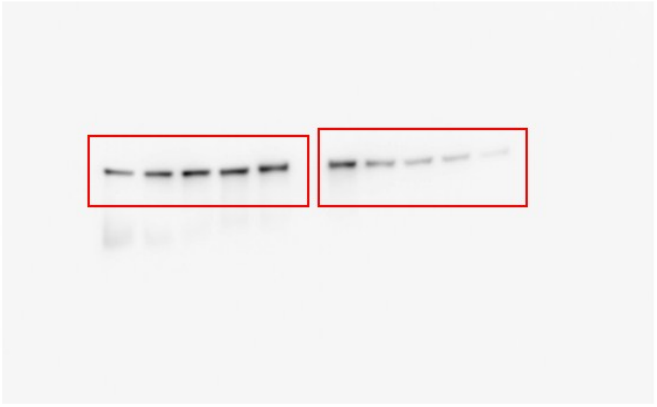
Figure 3B

β-actin – DMSO, PROTAC **4**, PROTAC **2**

β-actin – PROTAC **3**

Figure 3C

BRD4 DMSO PROTAC **4** (1 μM)



β-actin DMSO PROTAC **4** (1 μM)

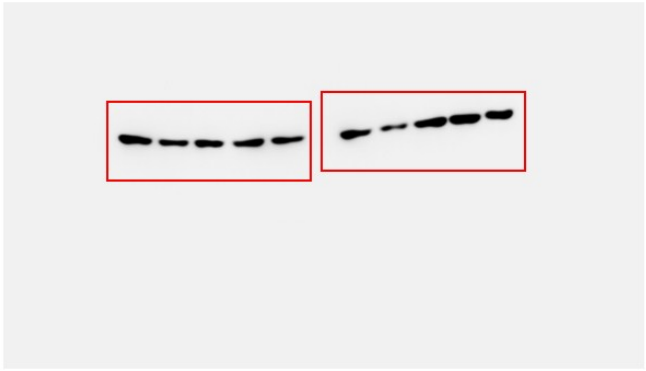


Figure 3C

BRD4 PROTAC **2** (1 μM) PROTAC **3** (1 μM)

β-actin PROTAC **2** (1 μM) PROTAC **3** (1 μM)

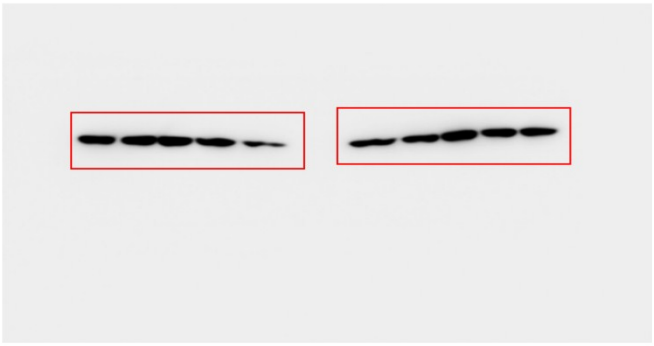


Figure S5A

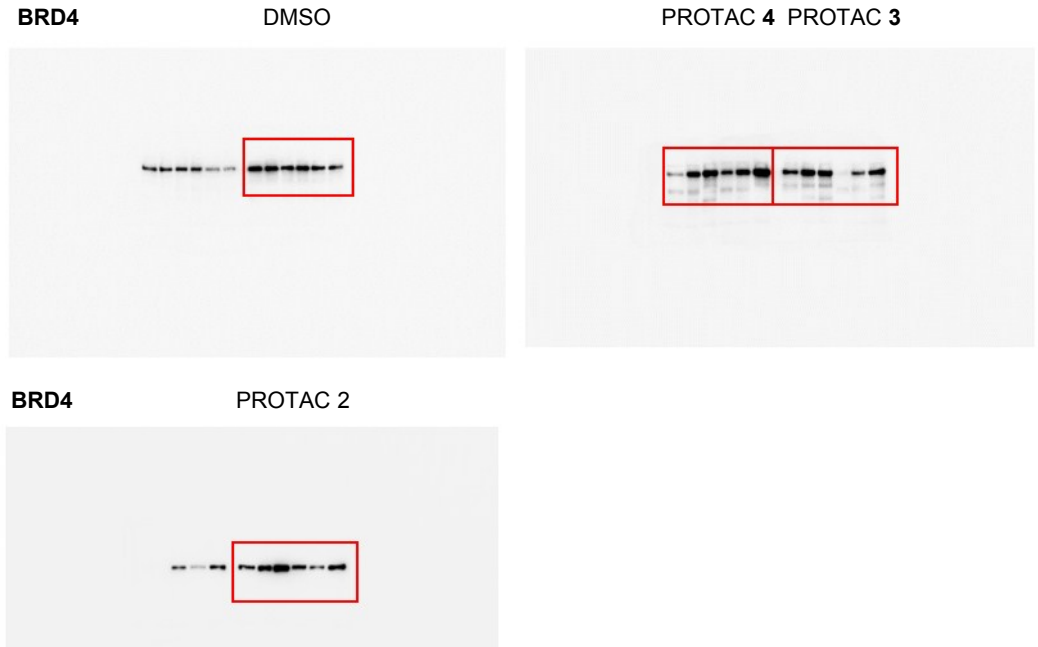

BRD4
DMSO
PROTAC 4
PROTAC 3
BRD4
PROTAC 2
β-actin
DMSO
PROTAC 4
PROTAC 3
β-actin
PROTAC 2

Figure S5B

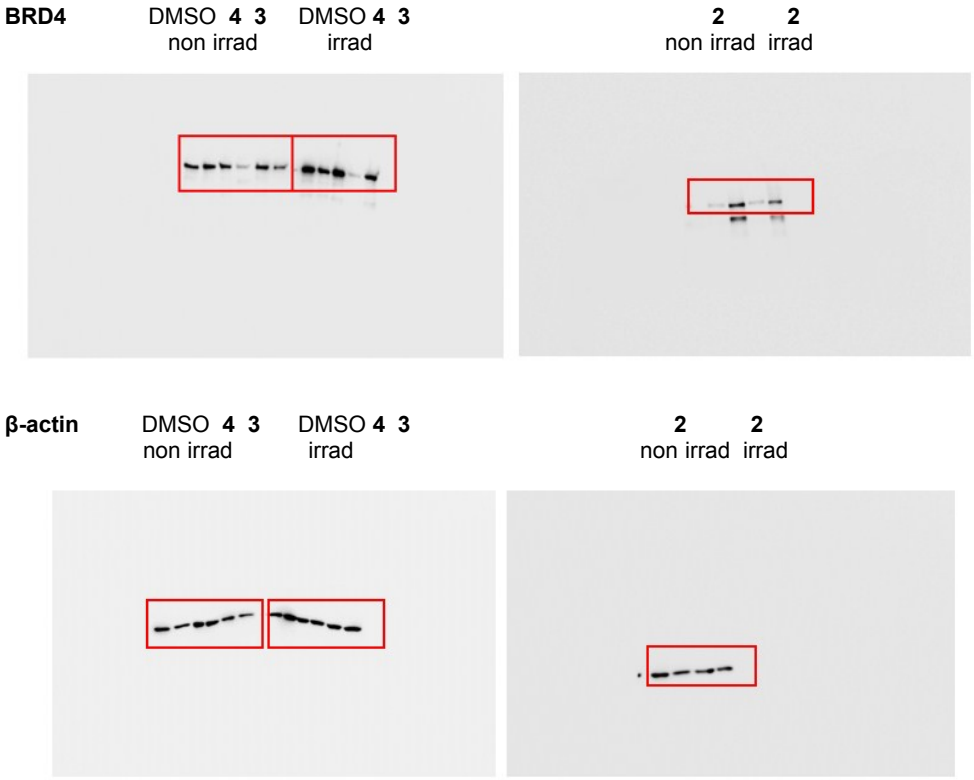
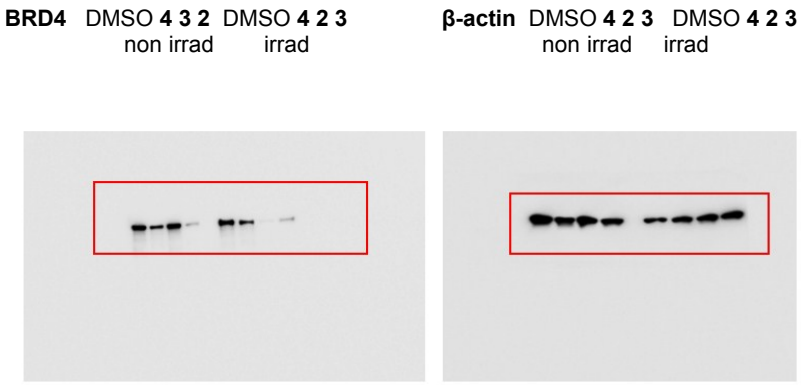


Figure 3D



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5. Author contributions

M.M., M.D.R. and E.W.T. conceptualized the caged E3 ligase ligand idea. C.S.K. synthesized the compounds and conducted the biological evaluation. M.M.S. generated pEGP-BRD4-C1 plasmid for transfection and conducted the live cell fluorescence imaging experiment. C.N.S. designed the LED set up to perform the irradiation of samples and cells. M.M. and J.D.H. provided supervisory support. E.W.T. secured funding and directed the study. C.S.K. and E.W.T. wrote the manuscript with input from all authors.

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