

Supplementary information

A dihydrouracil CRBN ligand mitigates IMiD associated safety liabilities in heterobifunctional targeted protein degrader

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Table of Contents

Chemistry general experimental.....	3
Details of tool compounds including the synthesis and characterization of intermediate and target compounds	5
LCMS traces of target compounds.....	16
NMR spectra of target compounds.....	29
Expression and purification of CRBN for the ITC experiments	35
ITC experiment.....	35
Determination of chemical stability at pH 1.0, 7.4 and 10.0	35
Expression and purification of CRBN for crystallographic studies.....	36
Crystallography	36
Supplementary figures.....	38
Supplementary note	49
References	51

Chemistry general experimental

Reagents and solvents (all anhydrous HPLC-grade) were obtained from commercial suppliers and used without any further purification unless otherwise stated. All reagents were weighed and handled in air unless otherwise stated. Brine refers to a saturated solution of NaCl. Concentration under reduced pressure refers to the use of a rotary evaporator. **Spectroscopy:** ^1H and ^{13}C NMR spectra were recorded using Bruker Avance III, Avance III HD or Avance III NEO spectrometers at a proton frequency of 300 MHz, or a Bruker Avance Neo spectrometer at a proton frequency of 500 MHz. All ^1H and ^{13}C NMR are quoted in ppm for measurement against TMS or residual solvent peaks as internal standards. Unless otherwise stated all experiments were carried out using DMSO- d_6 as solvent. ^1H NMR chemical shifts (δ) are given in ppm $\pm$ 0.01, and coupling constants (J) are given in Hz $\pm$ 0.1 Hz. The ^1H NMR spectra are reported as follows: δ /ppm (multiplicity, coupling constant(s) J /Hz, number of protons). Multiplicity is abbreviated as follows: s = singlet, br s = broad singlet, d = doublet, br d = broad doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, q = quartet, dq = doublet of quartets, quint = quintet, m = multiplet. ^{13}C NMR chemical shifts (δ) are given in ppm $\pm$ 0.1. **Mass spectrometry:** LC-MS experiments were performed using a Shimadzu LCMS-2020 with electrospray ionization in positive ion detection mode with 20ADXR pump, SIL-20ACXR autosampler, CTO-20AC column oven, M20A PDA Detector and LCMS 2020 MS detector. LC was run in two set ups: 1) Halo C18 column (2.0 μm 3.0*30 mm) in combination with a gradient (5-100% B in 1.2 min) of water and FA (0.1%) (A) and CH_3CN and FA (0.1%) (B) at a flow rate of 1.5 mL/min; 2) Poroshell HPH C18 column (2.7 μm 3.0*50 mm) in combination with a gradient (5-95% B in 2 min) of aqueous 46 mM ammonium carbonate/ammonia buffer at pH 10 (A) and MeCN (B) at a flow rate of 1.2 mL/min; 3) Halo C18 column (2.0 μm 3.0*30 mm) in combination with a gradient (5-95% B in 2 min) of water and TFA (0.05%) (A) and CH_3CN and TFA (0.05%) at a flow rate of 1.5 mL/min (B). The Column Oven (CTO-20AC) temperature was 40.0 $^\circ\text{C}$. The injection volume was 1 μL . PDA (SPD-M20A) detection was in the range 190–400 nm. The MS detector, which was configured with electrospray ionization as ionizable source; Acquisition mode: Scan; Nebulizing Gas Flow: 1.5 L/min; Drying Gas Flow: 15 L/min; Detector Voltage: Tuning Voltage $\pm$ 0.2 kV; DL Temperature: 250 $^\circ\text{C}$; Heat Block Temperature: 250 $^\circ\text{C}$; Scan Range: 90.00 - 900.00 m/z. **High resolution mass spectrometry:** Accurate mass data of samples were obtained using HRMS system with Waters Acquity I Class UPLC and Xevo G2-XS Q-TOF (Waters Corp., Milford, MA, USA). The samples were separated on reversed phase ACQUITY UPLC BEH C18 column (2.1*50 mm, 1.7 μm) using gradient elution with 0.1% FA in H_2O as mobile phase A and 0.1% FA in MeCN as mobile phase B. Acquity PDA Detector was in the range 210-400 nm. The injection volume was 0.1 μL . Analytes were separated by a gradient method. The column temperature was set at 40 $^\circ\text{C}$ and the flow rate at 0.4 mL/min. Instrument control and accurate mass data were processed using Masslynx software. The MS data were acquired in positive ionization mode with the following conditions: MS equipped with ESI, sensitivity mode, capillary voltage at 2.5 kV, sampling cone voltage at 40.0 V, source temperature at 100 $^\circ\text{C}$, cone gas flow at 50 L/h, desolvation gas flow at 600.0 (L/h), acquisition mass range of 50-1200 Da. **Flash column chromatography:** Chromatographic purification of products was accomplished with a CHEETAH[®] MP200 system with integrated UV detection, using column chromatography on (A) silica gels (40-60 μm), eluted with petroleum ether and ethyl acetate or dichloromethane and methanol or (B) C18 spherical (20-35 μm), eluted with water and acetonitrile with formic acid (0.1%) or ammonium formate (10 mmol) modifier. **Preparative HPLC:** Preparative HPLC was performed with a Waters MassLynx system with integrated MS detection and equipped with Prep C18 OBD 5 μm

30*150 mm columns from XBridge or Xselect CSH. Alternatively, Gilson GX-281 with integrated UV detection was used, equipped with either XBridge or Sunfire C18 10 μ m, 19*150 ID or 19*250 mm. As eluent (acidic) gradients of water/MeCN/FA acid (95/5/0.1) or water/0.05% TFA (A) and MeCN/MeOH (B) or 0.05% ammonia in water /10 mmol NH_4HCO_3 (A) and MeCN/MeOH (B) were applied. **Preparative chiral-HPLC:** Preparative chiral HPLC was performed with a Gilson GX-281 system with integrated UV detection and equipped with one of Chiralpak AS, AD, Chiralcel OD,OJ Chiralpak IA,IB,IC,ID,IE,IF,IG,IH columns (Daicel Chemical Industries, Ltd.) (R,R)-Whelk-O1, (S,S)-Whelk-O1 columns (Regis technologies, Inc.) CHIRAL Cellulose-SB, SC, SA columns (YMC Co., Ltd.) at different column size (250*20 mm, 250*30 mm) with noted percentage of either ethanol in hexane (%Et/Hex) or isopropanol in hexane (%IPA/Hex) as isocratic solvent systems. **Compound naming:** Compound names are those generated by ChemDraw 19.0. **Compound purity:** Purity for all tested compounds was assessed either via the analytical LC-MS method detailed above or via the HPLC method below. The purity of target compounds was >95% unless otherwise stated.

All HPLC analytical methods were performed on an Agilent 1200 system (Agilent, Santa Clara, CA) comprising 2 G1312B ultra-high-pressure binary pumps, a G1315C Diode Array detector, a G1316B Column Compartment, a G1379B Micro De-gasser, a G1367C Micro Well Plate Auto-sampler and a 35900E analog-to-digital converter. The eluent from the HPLC was split between an Agilent 6140 single quad mass spectrometer equipped with a multimode source and an ESA (ESA, Chelmsford, MA) Corona charged aerosol detector. HPLC reversed-phase separations were performed on a 50*2-mm Kinetex 2.6- μ m C18 column (Phenomenex, Torrance, CA) at a flow rate of 700 μ L per min using a gradient comprising (A) HPLC grade water (VWR) with 0.05% formic acid (Sigma Aldrich) and (B) HPLC grade acetonitrile (Honeywell) with 0.05% formic acid. The conditions for pump 1 were at time 0, A = 90%, with a linear gradient such that after 2 min, B = 100%, which was held for 0.5 min before returning to starting conditions. The gradient conditions for pump 2 were the opposite of pump 1 so as to combine the 2 solvent streams after the column and produce a constant 50:50 volume/volume mix of A and B at the detectors. The Agilent 6140 MS acquired from 100 to 1000 Da in sequential positive and negative ion modes with a total cycle time of 1 s, and the output from the Corona CAD (ESA) detector was acquired at a rate of 5 Hz through the analog-to-digital converter. The diode array detector (DAD) scanned from 220 to 300 nm at a rate of 20 Hz. All data were acquired into Chemstation Version B.03.01 (Agilent). The data from the DAD and CAD detectors were integrated automatically within Chemstation.

General chemistry abbreviations: The following abbreviations are used: Ac = acetyl; AcOH = acetic acid; Boc = *tert*-butyloxycarbonyl; Boc₂O = di-*tert*-butyl dicarbonate; DCE = 1,2-dichloroethane; DCM = dichloromethane; DIAD = diisopropyl azodicarboxylate; DIEA = *N,N*-Diisopropylethylamine; DMF = *N,N*-dimethylformamide; DMSO = dimethylsulfoxide; EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Ephos = dicyclohexyl(3-isopropoxy-2',4',6'-triisopropyl-[1,1'-biphenyl]-2-yl)phosphane; "Ephos Ph G4" = dicyclohexyl-[2-propan-2-yloxy-6-[2,4,6-tri(propan-2-yl)phenyl]phenyl]phosphonium; methanesulfonic acid; methyl-(2-phenylphenyl)azanide; palladium(2+) (CAS number: 2132978-44-8); ES = electrospray (in the context of mass spectrometry); EtOAc = ethyl acetate; FA = formic acid; h = hour(s); HATU = hexafluorophosphate azabenzotriazole tetramethyl uronium/O-(7-azabenzotriazol-1-yl)-*N,N,N'*,*N'*-tetramethyluronium hexafluorophosphate; HOBt = hydroxybenzotriazole;

HPLC = high performance liquid chromatography; m/z = mass-to-charge ratio in connection with mass spectrometry analysis; NMR = nuclear magnetic resonance; Petroleum ether = distilled petroleum of fraction 60-90 °C; Ph = phenyl; RT = room temperature (~18-25 °C); TFA = trifluoroacetic acid; TFE = 2,2,2-trifluoroethanol; THF = tetrahydrofuran.

Details of tool compounds including the synthesis and characterization of intermediate and target compounds

- Commercially available PROTACs were purchased from Tocris Bioscience (<http://www.tocris.com>):

Compound	TOCRIS Reference
BSJ-03-204	Cat. No. 6938/5
dBET1	Cat. No. 6327
dBRD9	Cat. No. 6606/5
ZXH-3-26	Cat. No. 6713

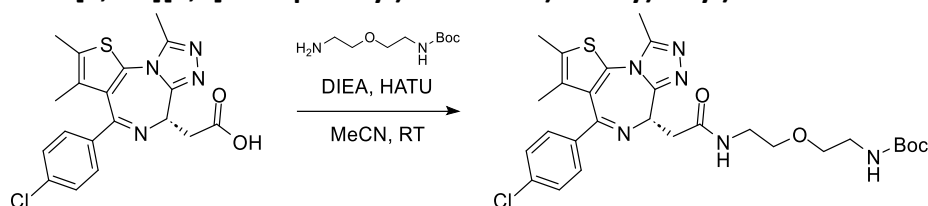
- Lenalidomide, pomalidomide, iberdomide and TMX-4116 are commercially available from MedChemExpress (<http://medchemexpress.com>):

Compound	Selleckchem Reference
Lenalidomide	Cat. No. HY-A0003
Pomalidomide	Cat. No. HY-10984
Iberdomide	Cat. No. HY-101291
TMX-4116	Cat. No. HY-145322

- The synthesis and characterization of **2**, **3**, **4**, **5**, **6**, **8** and **12** has been reported previously.¹ **1** is commercially available (CAS: 19144-80-0)

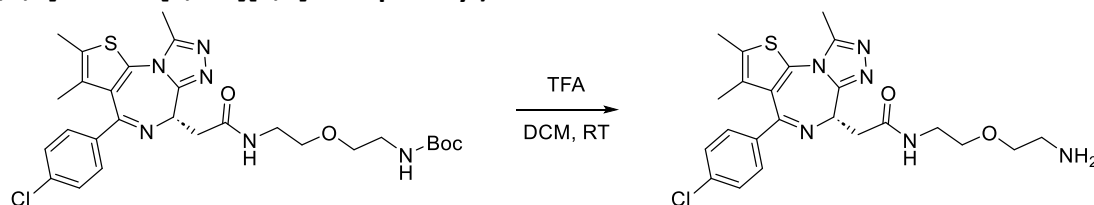
- **Synthesis and characterization of 7, 9, 10, 11, 13 and 14:**

***tert*-Butyl (S)-(2-(2-(2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)acetamido)ethoxy)ethyl)carbamate**



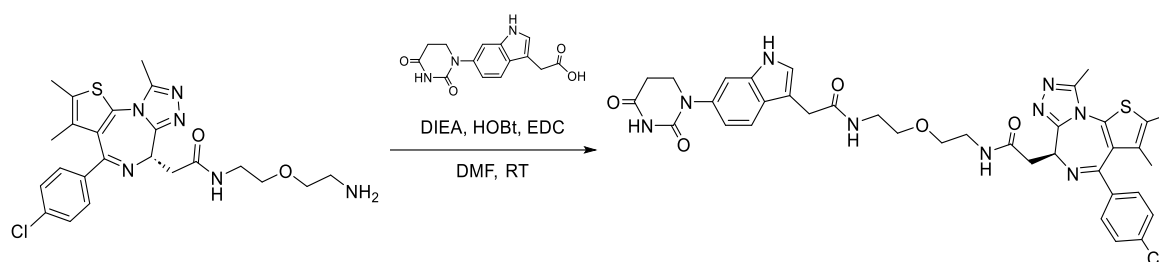
tert-Butyl (2-(2-aminoethoxy)ethyl)carbamate (1.146 g, 5.610 mmol) was added to a mixture of DIEA (1.307 mL, 7.483 mmol), HATU (1.707 g, 4.489 mmol) and (S)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)acetic acid (1.500 g, 3.742 mmol) in MeCN (30 mL). The resulting solution was stirred at RT for 2 h. The solvent was removed under reduced pressure and the crude product was purified by C18-flash chromatography, elution gradient 0 to 30% MeCN in water (containing 0.1% TFA). Fractions containing the desired compound were evaporated to dryness to afford the title compound (2.100 g, 96%) as a yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.37 (s, 9H), 1.62 (s, 3H), 2.41 (s, 3H), 2.59 (s, 3H), 3.04 – 3.31 (m, 6H), 3.38 – 3.49 (m, 4H), 4.50 (dd, *J* = 8.0, 5.8 Hz, 1H), 6.74 – 6.86 (m, 1H), 7.38 – 7.45 (2H, m), 7.46 – 7.52 (2H, m), 8.26 (t, *J* = 5.5 Hz, 1H); **MS (ESI)** *m/z* (ES⁺), [M+H]⁺ = 587.2.

(S)-*N*-(2-(2-(2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)acetamido)ethoxy)ethyl)carbamate



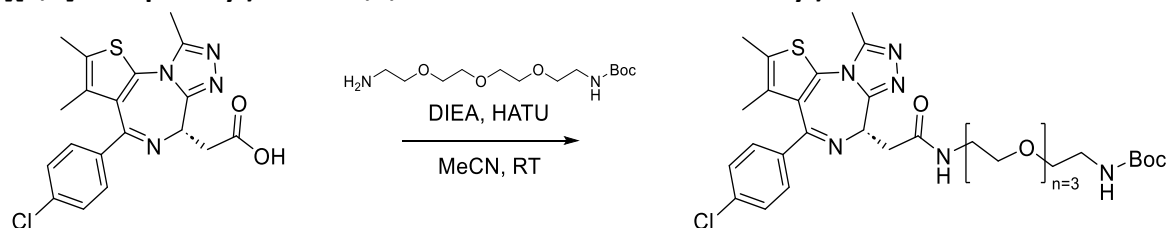
tert-Butyl (S)-(2-(2-(2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)acetamido)ethoxy)ethyl)carbamate (2.00 g, 3.41 mmol) was added to a solution of TFA (15 mL) and DCM (15 mL). The resulting solution was stirred at RT for 2 h. The solvent was removed under reduced pressure and the crude product was purified by C18-flash chromatography, elution gradient 0 to 50% MeOH in water (containing 0.1% HCl). Fractions containing the desired compound were evaporated to dryness to afford the title compound in the form of a hydrochloride salt (1.40 g, 79%) as a yellow solid. ¹H NMR (300 MHz, DMSO-*d*₆) δ 1.62 (s, 3H), 2.41 (s, 3H), 2.62 (s, 3H), 2.90 – 3.09 (m, 2H), 3.26 – 3.37 (m, 4H), 3.49 (t, *J* = 5.5 Hz, 2H), 3.64 (t, *J* = 5.1 Hz, 2H), 4.55 (t, *J* = 7.1 Hz, 1H), 7.40 – 7.46 (m, 2H), 7.47 – 7.53 (m, 2H), 8.17 (br s, 3H), 8.42 (t, *J* = 5.3 Hz, 1H); **MS (ESI)** *m/z* (ES⁺), [M+H]⁺ = 487.2.

(S)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3- α][1,4]diazepin-6-yl)-N-(2-(2-(2-(6-(2,4-dioxotetrahydropyrimidin-1(2H)-yl)-1H-indol-3-yl)acetamido)ethoxy)ethyl)acetamide (7)



2-(6-(2,4-dioxotetrahydropyrimidin-1(2H)-yl)-1H-indol-3-yl)acetic acid (30.0 mg, 0.104 mmol) was added to a mixture of (S)-N-(2-(2-aminoethoxy)ethyl)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3- α][1,4]diazepin-6-yl)acetamide, HCl (54.7 mg, 0.104 mmol), HOBT (19.2 mg, 0.125 mmol), EDC (24.0 mg, 0.125 mmol) and DIEA (36.5 μ L, 0.209 mmol) in DMF (2 mL). The resulting mixture was stirred at RT for 16 h and then purified directly by preparative HPLC (XSelect CSH Fluoro Phenyl column, 5 μ m, 30*150 mm) using decreasingly polar mixtures of water (containing 0.1% FA) and MeOH (elution gradient 52 to 62%). Fractions containing the desired compound were evaporated to dryness to afford the title compound (30.0 mg, 38%) as a white solid. $^1\text{H NMR}$ (600 MHz, DMSO- d_6) δ 1.61 (s, 3H), 2.40 (s, 3H), 2.59 (s, 3H), 2.70 (t, J = 6.7 Hz, 2H), 3.20 – 3.28 (m, 6H), 3.44 (t, J = 6.0 Hz, 4H), 3.51 (s, 2H), 3.76 (t, J = 6.7 Hz, 2H), 4.51 (dd, J = 7.9, 6.2 Hz, 1H), 6.91 (dd, J = 8.4, 1.9 Hz, 1H), 7.22 (d, J = 2.3 Hz, 1H), 7.26 (d, J = 1.7 Hz, 1H), 7.40 – 7.44 (m, 2H), 7.46 – 7.50 (m, 2H), 7.52 (d, J = 8.4 Hz, 1H), 7.92 (t, J = 5.6 Hz, 1H), 8.24 (t, J = 5.6 Hz, 1H), 10.23 (s, 1H), 10.92 (d, J = 1.9 Hz, 1H); $^{13}\text{C NMR}$ (151 MHz, DMSO- d_6) δ 10.89, 12.37, 13.78, 30.89, 32.29, 37.24, 38.27, 38.30, 45.20, 53.52, 68.52, 68.86, 108.76, 108.87, 116.66, 118.35, 124.37, 125.53, 128.17, 129.51, 129.75, 129.85, 130.45, 132.10, 135.11, 135.61, 135.65, 136.55, 149.65, 152.21, 155.14, 163.10, 169.73, 170.54, 170.64; **MS (ESI)** m/z (ES^+), $[\text{M}+\text{H}]^+ = 756.3$; **HRMS (ESI)** m/z calculated for $[\text{C}_{37}\text{H}_{39}\text{ClN}_9\text{O}_5\text{S}]^+ [\text{M}+\text{H}]^+ = 756.2478$, found = 756.2449.

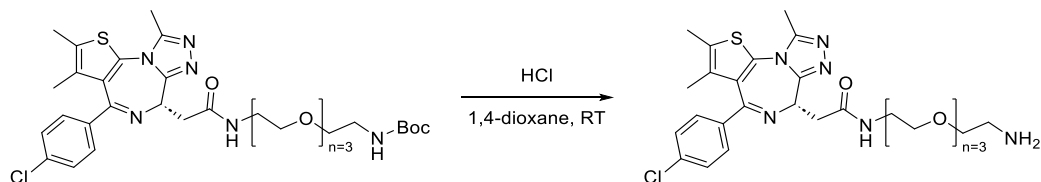
***tert*-Butyl (S)-(1-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3- α][1,4]diazepin-6-yl)-2-oxo-6,9,12-trioxa-3-azatetradecan-14-yl)carbamate**



tert-Butyl (2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)carbamate (1.422 g, 4.864 mmol) was added to a mixture of DIEA (1.133 mL, 6.487 mmol), HATU (1.480 g, 3.893 mmol) and (S)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3- α][1,4]diazepin-6-yl)acetic acid (1.300 g, 3.243 mmol) in MeCN (30 mL). The resulting solution was stirred at RT for 2 h. The solvent was removed under reduced pressure and the crude product was purified by C18-flash chromatography, elution gradient 0 to 30% MeCN in water (containing 0.1% TFA). Fractions containing the desired compound were evaporated to dryness to afford the title compound (2.050 g, 94%) as a yellow solid. $^1\text{H NMR}$ (300 MHz, DMSO) δ 1.36 (s, 9H), 1.62

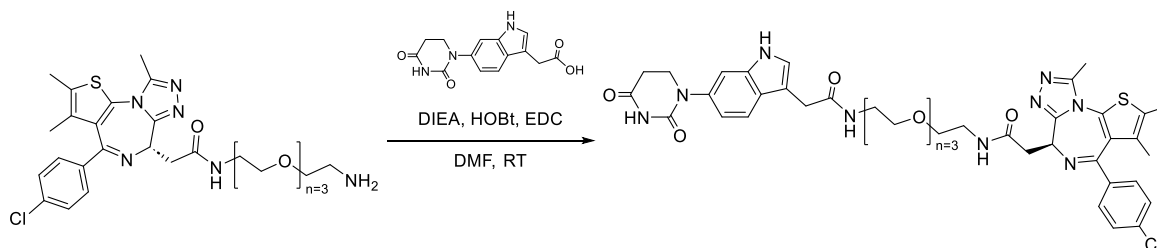
(s, 3H), 2.41 (s, 3H), 2.59 (s, 3H), 3.00 – 3.10 (m, 2H), 3.19 – 3.40 (m, 4H), 3.42 – 3.56 (m, 12H), 4.50 (dd, $J = 7.8, 6.1$ Hz, 1H), 6.70 – 6.81 (m, 1H), 7.38 – 7.47 (m, 2H), 7.44 – 7.53 (m, 2H), 8.27 (t, $J = 5.6$ Hz, 1H); **MS (ESI)** m/z (ES^+), $[M+H]^+ = 675.3$.

(S)-N-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)-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)acetamide



tert-Butyl (S)-(1-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)-2-oxo-6,9,12-trioxa-3-azatetradecan-14-yl)carbamate (2.00 g, 2.96 mmol) was added to a solution of HCl in 1,4-dioxane (4M, 30.0 mL, 120 mmol). The resulting solution was stirred at RT for 2h. The solvent was removed under reduced pressure and the crude product was purified by C18-flash chromatography, elution gradient 0 to 50% MeOH in water (containing 0.1% HCl). Fractions containing the desired compound were evaporated to dryness to afford the title compound in the form of a hydrochloride salt (1.50 g, 83%) as a yellow solid. ^1H NMR (300 MHz, DMSO- d_6 +water- d_2) δ 1.60 (s, 3H), 2.41 (s, 3H), 2.65 (s, 3H), 2.95 (t, $J = 5.2$ Hz, 2H), 3.17 – 3.35 (m, 4H), 3.45 (t, $J = 5.8$ Hz, 2H), 3.52 – 3.64 (m, 10H), 4.53 – 4.64 (m, 1H), 7.38 – 7.47 (m, 2H), 7.45 – 7.54 (m, 2H) - three exchangeable protons were deuterated; **MS (ESI)** m/z (ES^+), $[M+H]^+ = 575.3$.

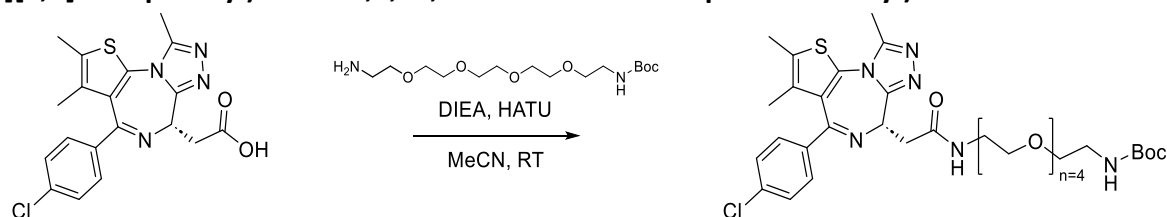
(S)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)-N-(1-(6-(2,4-dioxotetrahydropyrimidin-1(2H)-yl)-1H-indol-3-yl)-2-oxo-6,9,12-trioxa-3-azatetradecan-14-yl)acetamide (9)



2-(6-(2,4-dioxotetrahydropyrimidin-1(2H)-yl)-1H-indol-3-yl)acetic acid (30.0 mg, 0.104 mmol) was added to a mixture of (S)-N-(2-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)ethyl)-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)acetamide, HCl (63.9 mg, 0.104 mmol), HOBT (19.2 mg, 0.125 mmol), EDC (24.0 mg, 0.125 mmol) and DIEA (36.5 μ L, 0.209 mmol) in DMF (2 mL). The resulting mixture was stirred at RT for 16 h and then purified directly by preparative HPLC (XSelect CSH Fluoro Phenyl column, 5 μ m, 30*150 mm) using decreasingly polar mixtures of water (containing 0.1% FA) and MeOH (elution gradient 53 to 63%). Fractions containing the desired compound were evaporated to dryness to afford the title compound (30.0 mg, 34%) as a white solid. ^1H NMR (600 MHz, DMSO- d_6) δ 1.62 (s, 3H), 2.40 (s, 3H), 2.59 (s, 3H), 2.72 (t, $J = 6.7$ Hz, 2H), 3.16 – 3.31 (m, 6H), 3.40 (t, $J = 5.9$ Hz, 2H), 3.45 (t, $J = 5.9$ Hz, 2H), 3.46 – 3.58 (m, 10H), 3.77 (t, $J = 6.7$ Hz, 2H), 4.51 (dd, $J = 8.0, 6.1$ Hz, 1H), 6.92 (dd, $J = 8.4, 1.8$ Hz, 1H), 7.21 (d, $J = 2.2$ Hz, 1H), 7.27 (d, $J =$

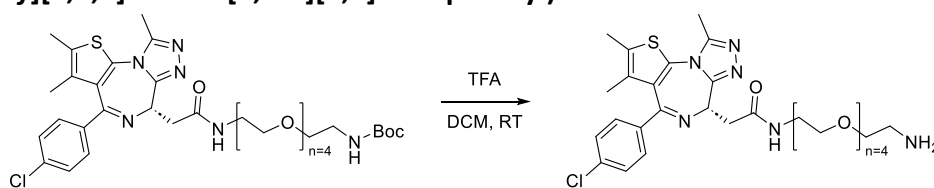
1.7 Hz, 1H), 7.40 – 7.45 (m, 2H), 7.46 – 7.50 (m, 2H), 7.52 (d, $J = 8.4$ Hz, 1H), 7.90 (t, $J = 5.6$ Hz, 1H), 8.25 (t, $J = 5.6$ Hz, 1H), 10.24 (s, 1H), 10.92 (d, $J = 1.6$ Hz, 1H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 11.25, 12.64, 14.00, 31.20, 32.59, 37.49, 38.60, 38.65, 45.51, 53.81, 69.09, 69.16, 69.54, 69.58, 69.70, 69.72, 108.98, 108.98, 116.90, 118.57, 124.63, 125.66, 128.42, 129.53, 129.79, 130.11, 130.66, 132.24, 135.18, 135.77, 135.94, 136.74, 149.76, 152.39, 155.08, 162.97, 169.64, 170.63, 170.69; **MS (ESI)** m/z (ES^+), $[\text{M}+\text{H}]^+ = 844.4$; **HRMS (ESI)** m/z calculated for $[\text{C}_{41}\text{H}_{47}\text{ClN}_9\text{O}_7\text{S}]^+ [\text{M}+\text{H}]^+ = 844.3002$, found = 844.2974

***tert*-Butyl (S)-(1-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)-2-oxo-6,9,12,15-tetraoxa-3-azaheptadecan-17-yl)carbamate**



tert-Butyl (14-amino-3,6,9,12-tetraoxatetradecyl)carbamate (1.636 g, 4.863 mmol) was added to a mixture of DIEA (1.133 mL, 6.487 mmol), HATU (1.480 g, 3.893 mmol) and (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 (1.300 g, 3.243 mmol) in MeCN (30 mL). The resulting solution was stirred at RT for 2 h. The solvent was removed under reduced pressure and the crude product was purified by C18-flash chromatography, elution gradient 0 to 30% MeCN in water (containing 0.1% TFA). Fractions containing the desired compound were evaporated to dryness to afford the title compound (2.200 g, 94%) as a yellow solid. ^1H NMR (300 MHz, DMSO- d_6) δ 1.36 (s, 9H), 1.62 (s, 3H), 2.41 (s, 3H), 2.59 (s, 3H), 2.98 – 3.11 (m, 2H), 3.18 – 3.40 (m, 4H), 3.41 – 3.57 (m, 16H), 4.50 (dd, $J = 8.0, 6.1$ Hz, 1H), 6.68 – 6.80 (m, 1H), 7.38 – 7.47 (m, 2H), 7.46 – 7.52 (m, 2H), 8.22 – 8.32 (m, 1H); **MS (ESI)** m/z (ES^+), $[\text{M}+\text{H}]^+ = 719.3$.

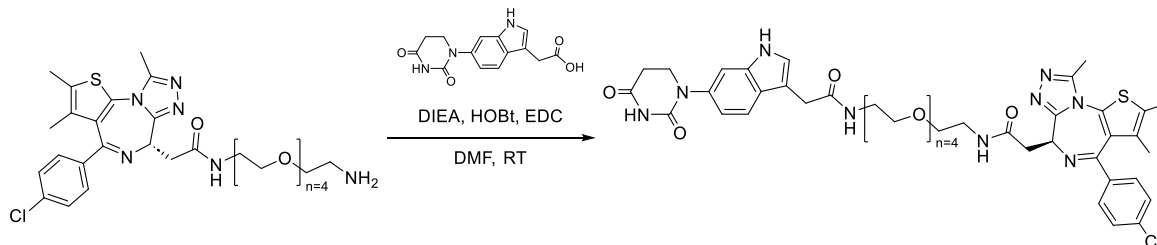
(S)-N-(14-amino-3,6,9,12-tetraoxatetradecyl)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)acetamide



tert-Butyl (S)-(1-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)-2-oxo-6,9,12,15-tetraoxa-3-azaheptadecan-17-yl)carbamate (2.10 g, 2.92 mmol) was added to a solution of TFA (15 mL) and DCM (15 mL). The resulting solution was stirred at RT for 2 h. The solvent was removed under reduced pressure and the crude product was purified by C18-flash chromatography, elution gradient 0 to 50% MeOH in water (containing 0.1% HCl). Fractions containing the desired compound were evaporated to dryness to afford the title compound in the form of a hydrochloride salt (1.50 g, 78%) as a yellow solid. ^1H NMR (300 MHz, DMSO- d_6) δ 1.62 (s, 3H), 2.42 (s, 3H), 2.61 (s, 3H), 2.87 – 3.03 (m, 2H), 3.14 – 3.36 (4H,m), 3.46 (t, $J = 5.9$ Hz, 2H), 3.50 – 3.67 (m, 14H), 4.49 – 4.56 (m, 1H),

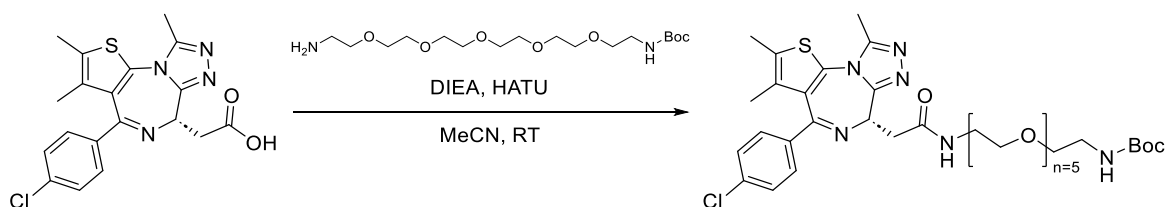
7.40 – 7.46 (m, 2H), 7.47 – 7.53 (m, 2H), 7.96 (s, 3H), 8.31 (t, $J = 5.5$ Hz, 1H); **MS (ESI)** m/z (ES^+), $[M+H]^+ = 619.3$.

(S)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3- α][1,4]diazepin-6-yl)-N-(1-(6-(2,4-dioxotetrahydropyrimidin-1(2H)-yl)-1H-indol-3-yl)-2-oxo-6,9,12,15-tetraoxa-3-azaheptadecan-17-yl)acetamide (10)



2-(6-(2,4-dioxotetrahydropyrimidin-1(2H)-yl)-1H-indol-3-yl)acetic acid (30.0 mg, 0.104 mmol) was added to a mixture of (S)-N-(14-amino-3,6,9,12-tetraoxatetradecyl)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3- α][1,4]diazepin-6-yl)acetamide, HCl (68.5 mg, 0.104 mmol), HOBT (19.2 mg, 0.125 mmol), EDC (24.0 mg, 0.125 mmol) and DIEA (36.5 μ L, 0.209 mmol) in DMF (2 mL). The resulting mixture was stirred at RT for 16 h and then purified directly by preparative HPLC (XSelect CSH Fluoro Phenyl column, 5 μ m, 30*150 mm) using decreasingly polar mixtures of water (containing 0.1% FA) and MeOH (elution gradient 53 to 63%). Fractions containing the desired compound were evaporated to dryness to afford the title compound (37.0 mg, 40%) as a tan solid. **^1H NMR** (600 MHz, DMSO- d_6) δ 1.62 (s, 3H), 2.40 (s, 3H), 2.59 (s, 3H), 2.72 (t, $J = 6.7$ Hz, 2H), 3.17 – 3.31 (m, 6H), 3.39 (t, $J = 5.9$ Hz, 2H), 3.45 (t, $J = 5.9$ Hz, 2H), 3.46 – 3.54 (m, 14H), 3.77 (t, $J = 6.7$ Hz, 2H), 4.51 (dd, $J = 8.0, 6.1$ Hz, 1H), 6.92 (dd, $J = 8.4, 1.7$ Hz, 1H), 7.21 (d, $J = 1.7$ Hz, 1H), 7.27 (d, $J = 1.6$ Hz, 1H), 7.40 – 7.44 (m, 2H), 7.46 – 7.50 (m, 2H), 7.52 (d, $J = 8.4$ Hz, 1H), 7.90 (t, $J = 5.6$ Hz, 1H), 8.25 (t, $J = 5.6$ Hz, 1H), 10.24 (s, 1H), 10.91 (d, $J = 1.4$ Hz, 1H); **^{13}C NMR** (151 MHz, DMSO- d_6) δ 11.25, 12.64, 14.01, 31.20, 32.59, 37.49, 38.60, 38.65, 45.51, 53.81, 69.08, 69.17, 69.54, 69.58, 69.69, 69.72, 69.75, 69.76, 108.98, 108.98, 116.90, 118.58, 124.63, 125.66, 128.42, 129.54, 129.79, 130.11, 130.66, 132.24, 135.18, 135.77, 135.94, 136.74, 149.76, 152.39, 155.08, 162.96, 169.64, 170.62, 170.69; **MS (ESI)** m/z (ES^+), $[M+H]^+ = 888.4$; **HRMS (ESI)** m/z calculated for $[C_{43}H_{51}ClN_9O_8S]^+$ $[M+H]^+ = 888.3264$, found = 888.3230.

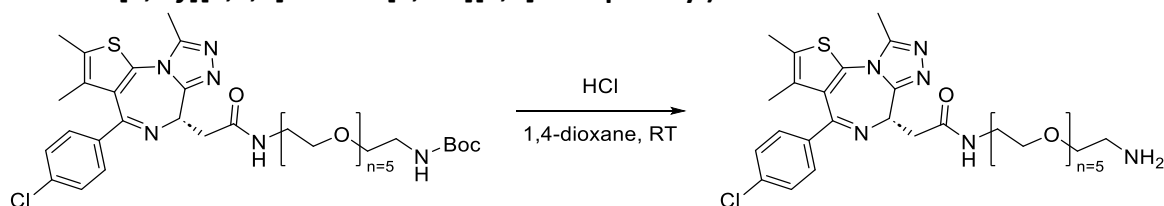
***tert*-Butyl (S)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3- α][1,4]diazepin-6-yl)-N-(1-(17-((tert-butoxycarbonyl)amino)-3,6,9,12,15-pentaoxaheptadecyl)-2-oxo-6,9,12,15-pentaoxa-3-azaicosan-20-yl)carbamate**



tert-Butyl (17-amino-3,6,9,12,15-pentaoxaheptadecyl)carbamate (1.42 g, 3.73 mmol) was added to a mixture of DIEA (871 μ L, 4.99 mmol), HATU (1.14 g, 3.00 mmol) and (S)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3- α][1,4]diazepin-6-yl)acetic acid (1.00 g, 2.49 mmol) in MeCN (30 mL). The resulting solution was stirred at RT for 2 h. The

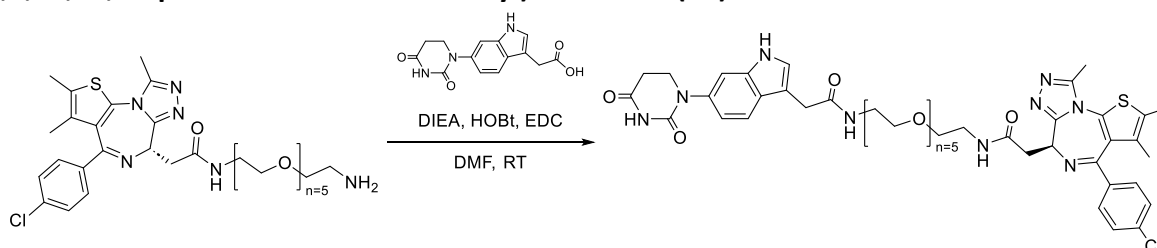
solvent was removed under reduced pressure and the crude product was purified by C18-flash chromatography, elution gradient 0 to 30% MeCN in water (containing 0.1% TFA). Fractions containing the desired compound were evaporated to dryness to afford the title compound (1.80 g, 95%) as a yellow solid. ¹H NMR (300 MHz, DMSO-d₆) δ 1.36 (s, 9H), 1.62 (s, 3H), 2.41 (s, 3H), 2.59 (s, 3H), 2.99 – 3.11 (m, 2H), 3.19 – 3.40 (m, 4H), 3.42 – 3.57 (m, 20H), 4.50 (dd, *J* = 8.0, 6.1 Hz, 1H), 6.69 – 6.79 (m, 1H), 7.37 – 7.47 (m, 2H), 7.44 – 7.53 (m, 2H), 8.27 (t, *J* = 5.5 Hz, 1H); **MS (ESI)** *m/z* (ES⁺), [M+H]⁺ = 763.3.

(S)-N-(17-amino-3,6,9,12,15-pentaoxaheptadecyl)-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)acetamide



tert-Butyl (S)-(1-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)-2-oxo-6,9,12,15,18-pentaoxa-3-azaicosan-20-yl)carbamate (1.75 g, 2.29 mmol) was added to a solution of HCl in 1,4-dioxane (4M, 30.0 mL, 120 mmol). The resulting solution was stirred at RT for 2h. The solvent was removed under reduced pressure and the crude product was purified by C18-flash chromatography, elution gradient 0 to 50% MeOH in water (containing 0.1% HCl). Fractions containing the desired compound were evaporated to dryness to afford the title compound in the form of a hydrochloride salt (1.40 g, 87%) as a yellow solid. ¹H NMR (300 MHz, DMSO-d₆+water-d₂) δ 1.60 (s, 3H), 2.40 (s, 3H), 2.63 (s, 3H), 2.95 (t, *J* = 5.1 Hz, 2H), 3.14 – 3.35 (4H, m), 3.40 – 3.62 (20H, m), 4.55 (dd, *J* = 8.0, 6.2 Hz, 1H), 7.38 – 7.45 (2H, m), 7.46 – 7.52 (2H, m) - three exchangeable protons were deuterated; **MS (ESI)** *m/z* (ES⁺), [M+H]⁺ = 663.3.

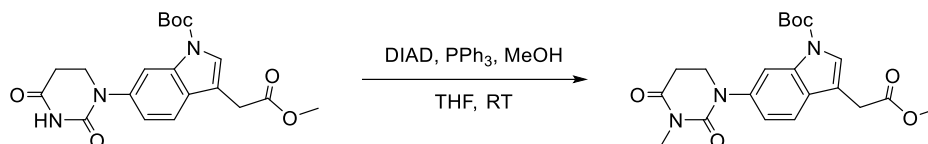
(S)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)-N-(1-(6-(2,4-dioxotetrahydropyrimidin-1(2H)-yl)-1H-indol-3-yl)-2-oxo-6,9,12,15,18-pentaoxa-3-azaicosan-20-yl)acetamide (11)



2-(6-(2,4-dioxotetrahydropyrimidin-1(2H)-yl)-1H-indol-3-yl)acetic acid (30.0 mg, 0.104 mmol) was added to a mixture (S)-N-(17-amino-3,6,9,12,15-pentaoxaheptadecyl)-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)acetamide, HCl (73.1 mg, 0.104 mmol), HOBT (19.2 mg, 0.125 mmol), EDC (24.0 mg, 0.125 mmol) and DIEA (36.5 μL, 0.209 mmol) in DMF (2 mL). The resulting mixture was stirred at RT for 16 h and then purified directly by preparative HPLC (XSelect CSH Fluoro Phenyl column, 5 μm, 30*150 mm) using decreasingly polar mixtures of water (containing 0.1% FA) and MeOH (elution gradient 55 to 65%). Fractions containing the desired compound were evaporated to

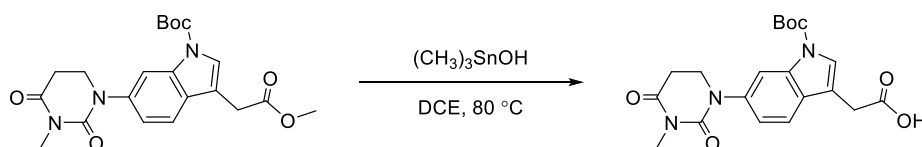
dryness to afford the title compound (43.0 mg, 44%) as a white solid. ^1H NMR (600 MHz, DMSO- d_6) δ 1.62 (s, 3H), 2.40 (s, 3H), 2.59 (s, 3H), 2.72 (t, J = 6.7 Hz, 2H), 3.18 – 3.29 (m, 6H), 3.39 (t, J = 5.9 Hz, 2H), 3.43 – 3.56 (m, 20H), 3.77 (t, J = 6.7 Hz, 2H), 4.50 (dd, J = 8.1, 6.0 Hz, 1H), 6.92 (dd, J = 8.4, 1.8 Hz, 1H), 7.21 (d, J = 2.2 Hz, 1H), 7.27 (d, J = 1.7 Hz, 1H), 7.40 – 7.45 (m, 2H), 7.46 – 7.50 (m, 2H), 7.52 (d, J = 8.4 Hz, 1H), 7.90 (t, J = 5.6 Hz, 1H), 8.25 (t, J = 5.6 Hz, 1H), 10.24 (s, 1H), 10.91 (d, J = 1.6 Hz, 1H); ^{13}C NMR (151 MHz, DMSO- d_6) δ 11.25, 12.64, 14.01, 31.20, 32.59, 37.48, 38.61, 38.65, 45.51, 53.81, 69.08, 69.17, 69.53, 69.58, 69.68, 69.71, 69.73 – 69.75, 69.76, 108.98, 108.98, 116.89, 118.57, 124.63, 125.66, 128.42, 129.54, 129.79, 130.11, 130.66, 132.24, 135.18, 135.77, 135.94, 136.74, 149.76, 152.39, 155.08, 162.96, 169.63, 170.62, 170.69; **MS (ESI)** m/z (ES^+), $[\text{M}+\text{H}]^+ = 932.5$; **HRMS (ESI)** m/z calculated for $[\text{C}_{45}\text{H}_{55}\text{ClN}_9\text{O}_9\text{S}]^+ [\text{M}+\text{H}]^+ = 932.3526$, found = 932.3488.

***tert*-Butyl 3-(2-methoxy-2-oxoethyl)-6-(3-methyl-2,4-dioxotetrahydropyrimidin-1(2*H*)-yl)-1*H*-indole-1-carboxylate**



Triphenylphosphane (147 mg, 0.560 mmol) was added to a mixture of DIAD (113 mg, 0.559 mmol), MeOH (15.1 μL , 0.373 mmol) and *tert*-butyl 6-(2,4-dioxotetrahydropyrimidin-1(2*H*)-yl)-3-(2-methoxy-2-oxoethyl)-1*H*-indole-1-carboxylate (CAS: 2766180-33-8, synthesised according to Borjesson, U. *et al.* Synthesis of heterocyclic substituted pyrimidine anticancer agents. WO2022069520 (2022),¹ 150 mg, 0.374 mmol) in THF (1 mL). The resulting mixture was stirred at RT for 17 h. The solvent was removed under reduced pressure and the crude product was purified by flash silica chromatography, elution gradient 40 to 80% EtOAc in petroleum ether. Fractions containing the desired compound were evaporated to dryness to afford the title compound (140 mg, 90%) as a yellow solid. ^1H NMR (400 MHz, DMSO- d_6) δ 1.62 (s, 9H), 2.86 (t, J = 6.7 Hz, 2H), 3.07 (s, 3H), 3.63 (s, 3H), 3.77 – 3.86 (m, 4H), 7.23 (dd, J = 8.4, 1.9 Hz, 1H), 7.56 (d, J = 8.4 Hz, 1H), 7.65 (s, 1H), 8.05 (br d, J = 1.1 Hz, 1H); ^{13}C NMR (101 MHz, DMSO) δ 27.26, 27.62, 29.80, 31.42, 44.09, 51.76, 83.85, 112.45, 113.58, 119.45, 120.88, 125.18, 128.08, 134.57, 139.46, 148.77, 152.99, 169.70, 171.06; **MS (ESI)** m/z (ES^+), $[\text{M}+\text{H}]^+ = 416.2$; **HRMS (ESI)** m/z calculated for $[\text{C}_{21}\text{H}_{26}\text{N}_3\text{O}_6]^+ [\text{M}+\text{H}]^+ = 416.1816$, found = 416.1818.

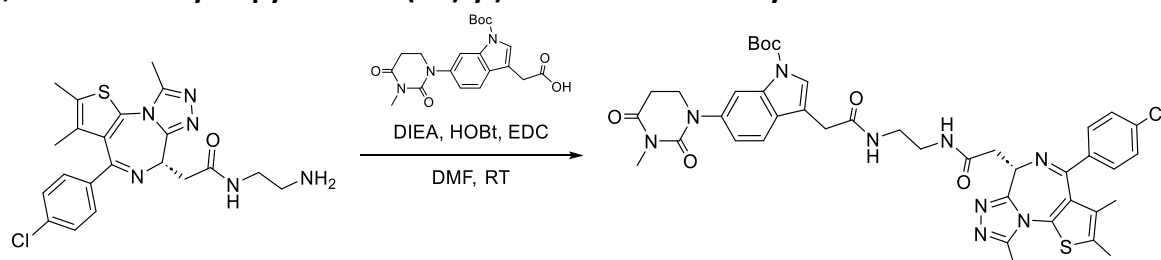
2-(1-(*tert*-Butoxycarbonyl)-6-(3-methyl-2,4-dioxotetrahydropyrimidin-1(2*H*)-yl)-1*H*-indol-3-yl)acetic acid



Trimethyl tin hydroxide (225 mg, 1.24 mmol) was added to a solution of *tert*-butyl 6-(2,4-dioxotetrahydropyrimidin-1(2*H*)-yl)-3-(2-methoxy-2-oxoethyl)-1*H*-indole-1-carboxylate (100 mg, 0.249 mmol) in DCE (2 mL). The resulting mixture was stirred at 80 $^{\circ}\text{C}$ for 17 h. The solvent was removed under reduced pressure and the crude product was purified by flash silica chromatography, elution gradient 5 to 15% MeOH in DCM. Fractions containing the desired compound were evaporated to dryness to afford the title compound (43.0 mg, 43%) as a

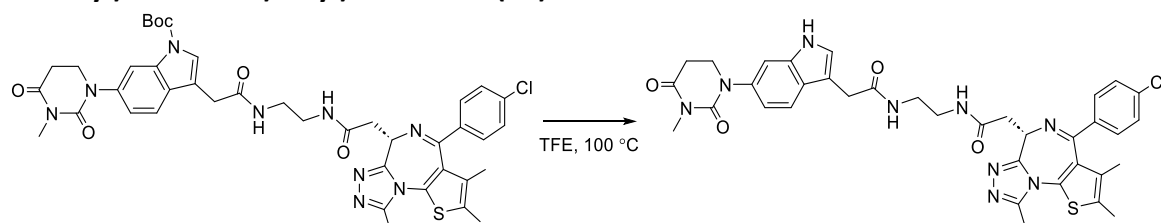
white solid. **¹H NMR** (400 MHz, DMSO) δ 1.62 (s, 9H), 2.86 (t, J = 6.6 Hz, 2H), 3.07 (s, 3H), 3.71 (s, 2H), 3.81 (t, J = 6.6 Hz, 2H), 7.22 (dd, J = 8.4, 1.6 Hz, 1H), 7.56 (d, J = 8.4 Hz, 1H), 7.62 (s, 1H), 8.05 (s, 1H), 12.45 (br s, 1H); **¹³C NMR** (101 MHz, DMSO) δ 27.27, 27.64, 30.27, 31.44, 44.12, 83.76, 112.44, 114.30, 119.52, 120.80, 124.96, 128.30, 134.59, 139.39, 148.83, 153.00, 169.71, 172.14; **MS (ESI)** m/z (ES^+), $[M+H]^+ = 402.1$; **HRMS (ESI)** m/z calculated for $[C_{20}H_{24}N_3O_6]^+$ $[M+H]^+ = 402.1660$, found = 402.1661.

tert-Butyl (S)-3-(2-((2-(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)acetamido)ethyl)amino)-2-oxoethyl)-6-(3-methyl-2,4-dioxotetrahydropyrimidin-1(2H)-yl)-1H-indole-1-carboxylate



DIEA (52.0 μ L, 0.298 mmol) was added to a mixture of 2-(1-(*tert*-butoxycarbonyl)-6-(3-methyl-2,4-dioxotetrahydropyrimidin-1(2H)-yl)-1H-indol-3-yl)acetic acid (30.0 mg, 0.0747 mmol), (*S*)-*N*-(2-aminoethyl)-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)acetamide, HCl (CAS: 3083250-30-7, synthesised according to Borjesson, U. *et al.* Synthesis of heterocyclic substituted pyrimidine anticancer agents. WO2022069520 (2022),¹ 35.8 mg, 0.0747 mmol), HOBt (22.9 mg, 0.150 mmol), EDC (28.7 mg, 0.150 mmol) in DMF (2 mL). The resulting mixture was stirred at RT for 17 h. The reaction mixture was quenched with water (5 mL), extracted with EtOAc (2 x 5 mL) and washed with saturated brine (3 x 10 mL). The organic layer was dried over Na_2SO_4 , filtered and evaporated to afford the impure (54% pure by LCMS) title compound (60.0 mg) as a yellow oil. **MS (ESI)** m/z (ES^+), $[M+H]^+ = 826.4$.

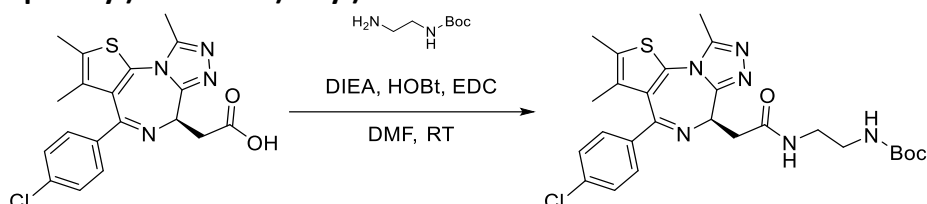
(S)-2-(4-(4-Chlorophenyl)-2,3,9-trimethyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepin-6-yl)-N-(2-(2-(6-(3-methyl-2,4-dioxotetrahydropyrimidin-1(2H)-yl)-1H-indol-3-yl)acetamido)ethyl)acetamide (13)



tert-butyl (S)-3-(2-((2-(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)acetamido)ethyl)amino)-2-oxoethyl)-6-(3-methyl-2,4-dioxotetrahydropyrimidin-1(2H)-yl)-1H-indole-1-carboxylate (50.0 mg, 0.0605 mmol; material assumed pure for the purposes of reagent equivalent calculation) was dissolved in TFE (2 mL) and sealed into a microwave tube. The reaction was heated to 120 °C for 1 h in a microwave reactor and then cooled to RT. The resulting mixture was purified directly by preparative HPLC (Xselect CSH OBD column, 5 μ m, 30*150 mm) using decreasingly polar

mixtures of water (containing 0.1% FA) and MeCN (elution gradient 28 to 50%). Fractions containing the desired compound were evaporated to dryness to afford the title compound (28.0 mg, 61% over two steps) as a white solid. **¹H NMR** (600 MHz, DMSO-*d*₆) δ 1.62 (s, 3H), 2.41 (s, 3H), 2.59 (s, 3H), 2.84 (t, *J* = 6.7 Hz, 2H), 3.05 (s, 3H), 3.13 – 3.27 (m, 6H), 3.50 (s, 2H), 3.75 (t, *J* = 6.7 Hz, 2H), 4.51 (t, *J* = 7.1 Hz, 1H), 6.93 (dd, *J* = 8.4, 1.8 Hz, 1H), 7.23 (d, *J* = 2.2 Hz, 1H), 7.28 (d, *J* = 1.6 Hz, 1H), 7.40 – 7.45 (m, 2H), 7.45 – 7.50 (m, 2H), 7.53 (d, *J* = 8.4 Hz, 1H), 7.90 (t, *J* = 4.9 Hz, 1H), 8.22 (t, *J* = 4.8 Hz, 1H), 10.95 (d, *J* = 1.6 Hz, 1H); **¹³C NMR** (151 MHz, DMSO-*d*₆) δ 11.26, 12.64, 14.01, 27.24, 31.48, 32.69, 37.63, 38.38, 38.53, 44.45, 53.77, 108.86, 109.06, 116.99, 118.58, 124.77, 125.76, 128.44, 129.55, 129.82, 130.12, 130.67, 132.23, 135.18, 135.77, 136.52, 136.73, 149.79, 152.97, 155.10, 163.00, 169.71, 169.80, 170.76; **MS (ESI)** *m/z* (ES⁺), [M+H]⁺ = 726.3; **HRMS (ESI)** *m/z* calculated for [C₃₆H₃₇ClN₉O₄S]⁺ [M+H]⁺ = 726.2372, found = 726.2344.

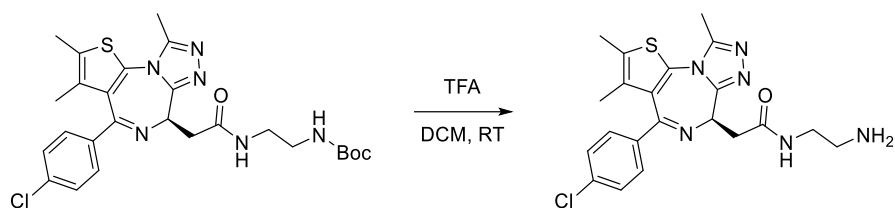
***tert*-Butyl (R)-(2-(2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)acetamido)ethyl)carbamate¹**



DIEA (523 μ L, 2.99 mmol) was added to a mixture of *tert*-butyl (2-aminoethyl)carbamate (120 mg, 0.749 mmol), HOBT (344 mg, 2.25 mmol), EDC (430 mg, 2.25 mmol) and (R)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)acetic acid (300 mg, 0.748 mmol) in DMF (5 mL). The resulting solution was stirred at RT for 16 h. The solvent was removed under reduced pressure and the crude product was purified by C18-flash chromatography, elution gradient 0 to 50% MeCN in water (0.1% NH₄HCO₃). Fractions containing the title compound were evaporated to dryness to afford the impure (69% pure by LCMS) title compound (350 mg) as a yellow oil. **MS (ESI)** *m/z* (ES⁺), [M+H]⁺ = 543.3.

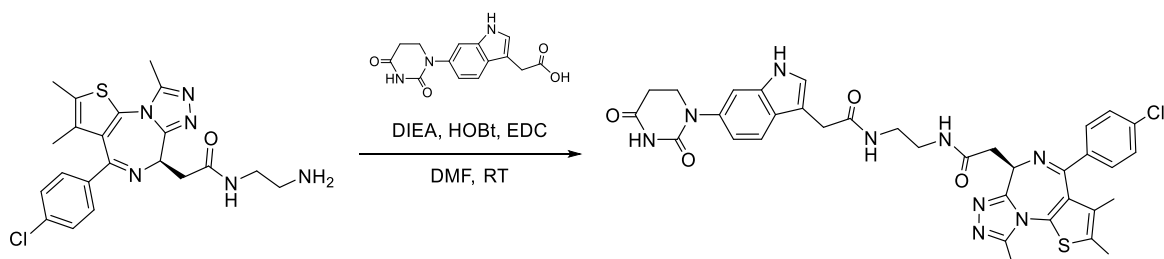
(R)-N-(2-aminoethyl)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)acetamide

¹ The title compound was also made on a smaller scale using HATU as the amide coupling reagent: *tert*-Butyl (2-aminoethyl)carbamate (48.0 mg, 0.300 mmol) was added to a mixture of DIEA (87.0 μ L, 0.498 mmol), HATU (114 mg, 0.300 mmol) and (R)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)acetic acid (100 mg, 0.249 mmol) in MeCN (2 mL). The resulting solution was stirred at RT for 2 h. The solvent was removed under reduced pressure and the crude product was purified by preparative HPLC (Xbridge Prep C18 OBD column, 5 μ m, 19*250 mm) using decreasingly polar mixtures of water (containing 0.1% NH₄HCO₃) and MeCN (elution gradient 45 to 57%). Fractions containing the desired compound were evaporated to dryness to afford the title compound (42.0 mg, 31%) as a pale yellow solid. **¹H NMR** (400 MHz, DMSO-*d*₆) δ 1.37 (s, 9H), 1.63 (s, 3H), 2.41 (s, 3H), 2.59 (s, 3H), 2.97 – 3.07 (m, 2H), 3.06 – 3.31 (m, 4H), 4.47 – 4.55 (m, 1H), 6.78 (t, *J* = 5.0 Hz, 1H), 7.39 – 7.46 (m, 2H), 7.45 – 7.51 (m, 2H), 8.22 (t, *J* = 5.3 Hz, 1H); **¹³C NMR** (101 MHz, DMSO-*d*₆) δ 11.25, 12.63, 14.01, 28.18, 37.60, 38.72, 53.76, 77.63, 128.43, 129.55, 129.81, 130.11, 130.65, 132.23, 135.17, 136.74, 149.76, 155.09, 155.58, 162.97, 169.70 (one carbon peak is obscured by the solvent's multiplet); **MS (ESI)** *m/z* (ES⁺), [M+H]⁺ = 543.2; **HRMS (ESI)** *m/z* calculated for [C₂₆H₃₂ClN₆O₃S]⁺ [M+H]⁺ = 543.1940, found = 543.1946.



TFA (2.3 mL) was added to a solution of *tert*-butyl (*R*)-(2-(2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)acetamido)ethyl)carbamate (330 mg, 0.608 mmol; material assumed pure for the purposes of reagent equivalent calculation) in DCM (10 mL). The resulting solution was stirred at RT for 2h. The solvent was removed under reduced pressure to afford the title compound (200 mg) as a brown oil. This material was used in the next step without further purification. **MS (ESI)** m/z (ES^+), $[M+H]^+ = 443.2$.

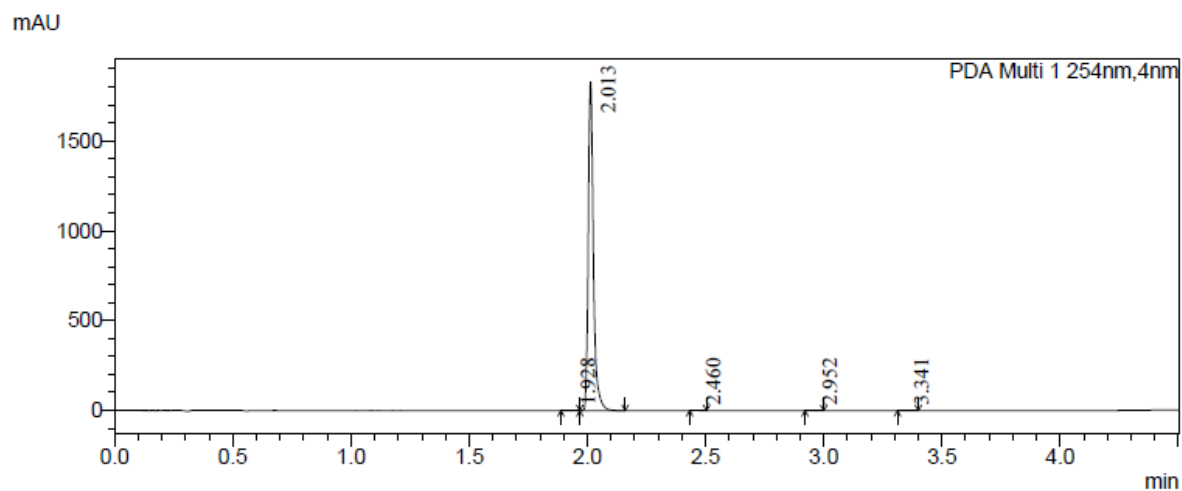
(*R*)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)-*N*-(2-(2-(6-(2,4-dioxotetrahydropyrimidin-1(2*H*)-yl)-1*H*-indol-3-yl)acetamido)ethyl)acetamide (14)



DIEA (158 μ L, 0.905 mmol) was added to a mixture of 2-(2-(6-(2,4-Dioxotetrahydropyrimidin-1(2*H*)-yl)-1*H*-indol-3-yl)acetic acid (64.9 mg, 0.226 mmol), (*R*)-*N*-(2-aminoethyl)-2-(4-(4-chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl)acetamide, TFA (126 mg, 0.226 mmol; material assumed pure for the purposes of reagent equivalent calculation), HOBT (104 mg, 0.679 mmol), EDC (130 mg, 0.678 mmol) in DMF (5 mL). The resulting mixture was stirred at RT for 16 h and then purified directly by preparative HPLC (XSelect CSH Fluoro Phenyl column, 5 μ m, 30*250 mm) using decreasingly polar mixtures of water (containing 0.1% FA) and MeCN (elution gradient 30 to 40%). Fractions containing the desired compound were evaporated to dryness to afford the title compound (28.0 mg, 9% over three steps) as a white solid. **^1H NMR** (600 MHz, DMSO- d_6) δ 1.62 (s, 3H), 2.41 (s, 3H), 2.59 (s, 3H), 2.71 (t, $J = 6.7$ Hz, 2H), 3.13 – 3.20 (m, 4H), 3.23 (dd, $J = 7.0, 3.5$ Hz, 2H), 3.50 (s, 2H), 3.77 (t, $J = 6.7$ Hz, 2H), 4.51 (t, $J = 7.1$ Hz, 1H), 6.92 (dd, $J = 8.4, 1.8$ Hz, 1H), 7.23 (d, $J = 2.2$ Hz, 1H), 7.27 (d, $J = 1.6$ Hz, 1H), 7.40 – 7.44 (m, 2H), 7.45 – 7.50 (m, 2H), 7.52 (d, $J = 8.4$ Hz, 1H), 7.86 – 7.92 (m, 1H), 8.19 – 8.25 (m, 1H), 10.24 (s, 1H), 10.93 (d, $J = 1.5$ Hz, 1H); **^{13}C NMR** (151 MHz, DMSO- d_6) δ 11.26, 12.65, 14.01, 31.21, 32.69, 37.63, 38.38, 38.54, 45.51, 53.77, 108.83, 109.04, 116.93, 118.52, 124.70, 125.67, 128.44, 129.55, 129.82, 130.12, 130.67, 132.23, 135.18, 135.77, 135.94, 136.73, 149.80, 152.39, 155.10, 163.01, 169.71, 170.70, 170.76; **MS (ESI)** m/z (ES^+), $M^+ = 712.3$; **HRMS (ESI)** m/z calculated for $[C_{35}H_{35}ClN_9O_4S]^+$ $[M+H]^+ = 712.2216$, found = 712.2180.

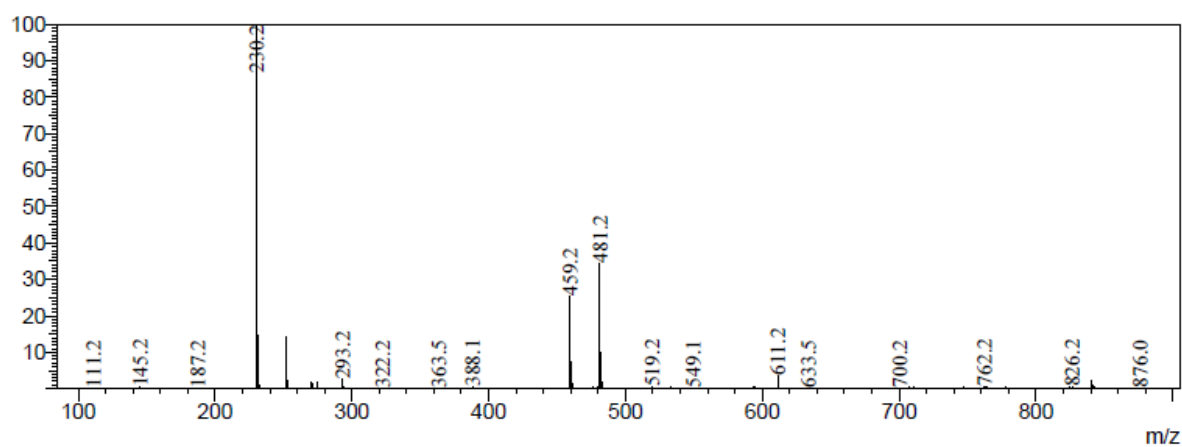
LCMS traces of target compounds

LCMS trace of **2**:



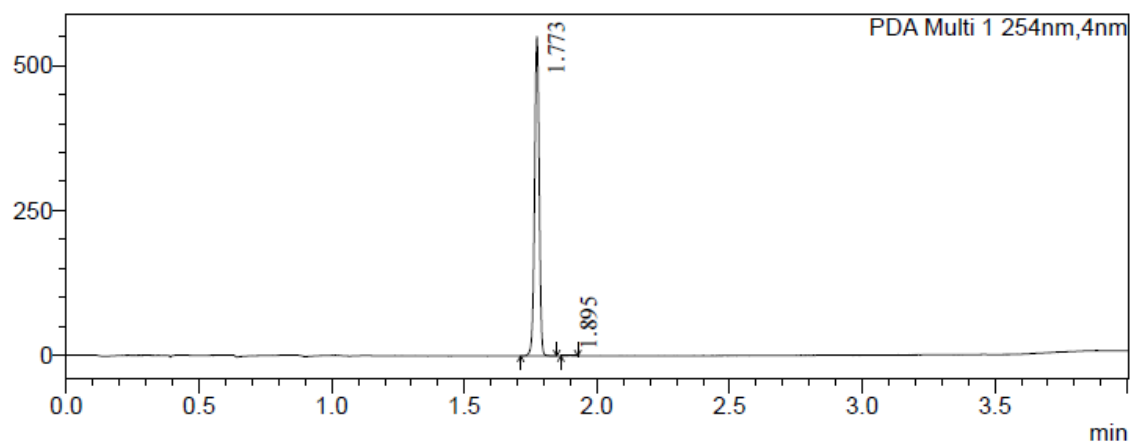
Peak Table
PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.928	428	0.024	1100	0.040
2	2.013	1771470	99.773	2772634	99.759
3	2.460	2176	0.123	3241	0.117
4	2.952	1082	0.061	1810	0.065
5	3.341	346	0.019	537	0.019
Total		1775502	100.000	2779323	100.000



LCMS trace of **3**:

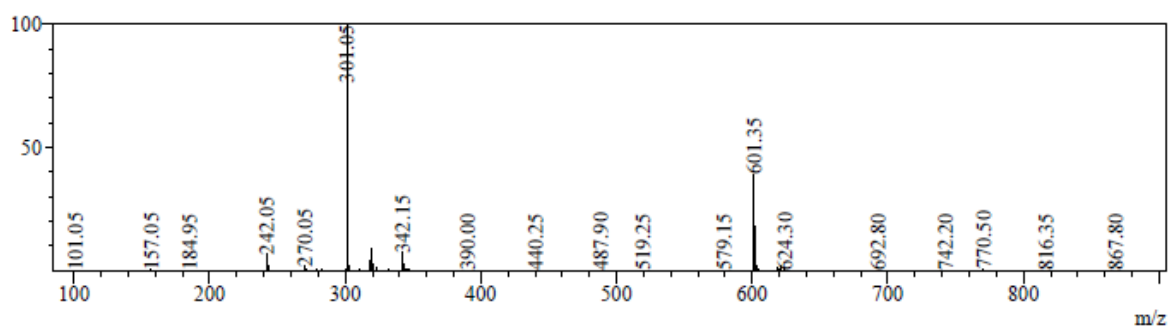
mAU



Peak Table

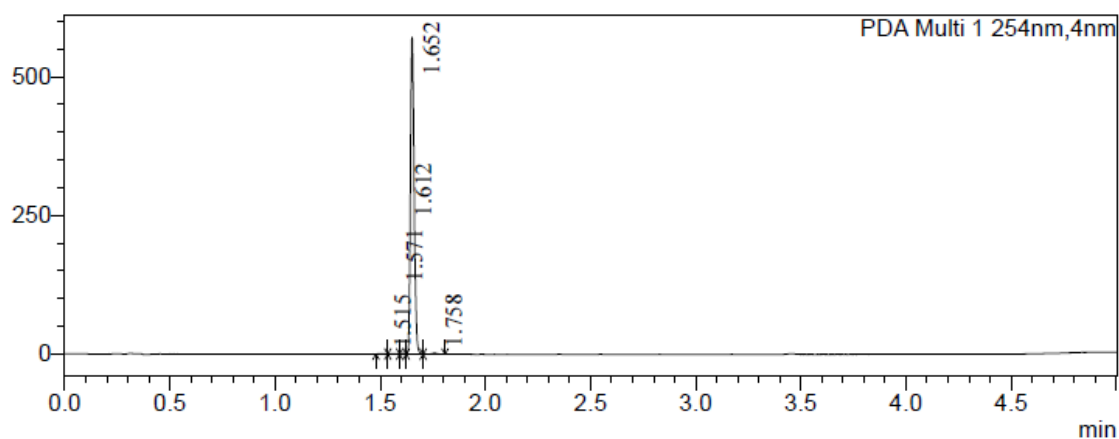
PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.773	526864	99.922	668477	99.877
2	1.895	413	0.078	823	0.123
Total		527277	100.000	669300	100.000



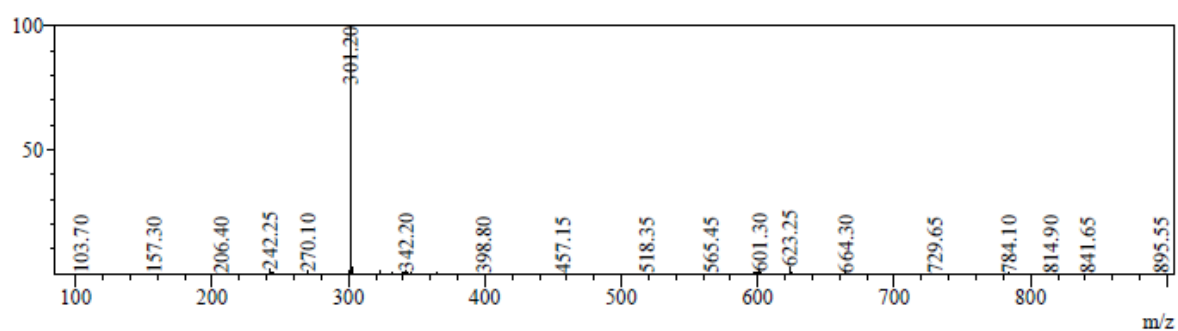
LCMS trace of **4**:

mAU



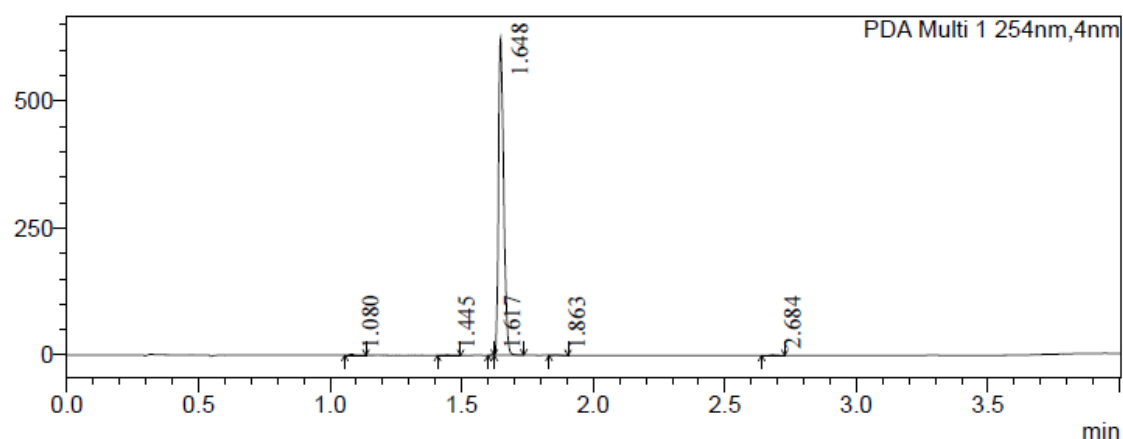
Peak Table

PDA Ch1 254nm						
Peak#	Ret. Time	Height	Height%	Area	Area%	
1	1.515	774	0.140	1579	0.225	
2	1.571	675	0.123	1755	0.250	
3	1.612	1167	0.212	1510	0.215	
4	1.652	546621	99.151	692108	98.724	
5	1.758	2061	0.374	4104	0.585	
Total		551299	100.000	701056	100.000	



LCMS trace of 5:

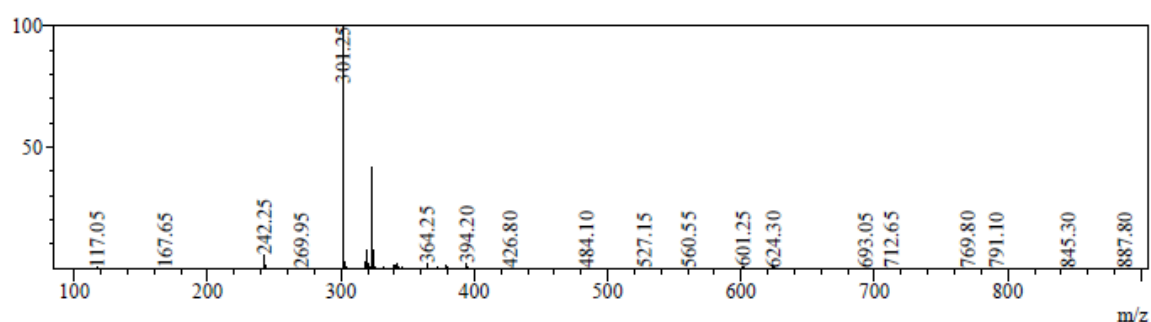
mAU



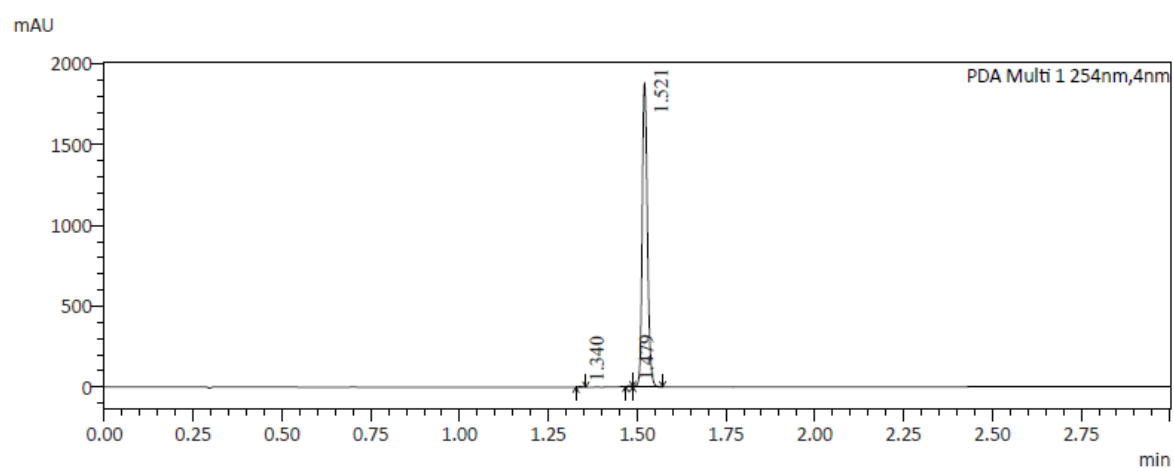
Peak Table

PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.080	1620	0.287	2589	0.301
2	1.445	1002	0.178	1634	0.190
3	1.617	929	0.165	1243	0.145
4	1.648	559291	99.111	852223	99.116
5	1.863	453	0.080	553	0.064
6	2.684	1015	0.180	1581	0.184
Total		564309	100.000	859821	100.000



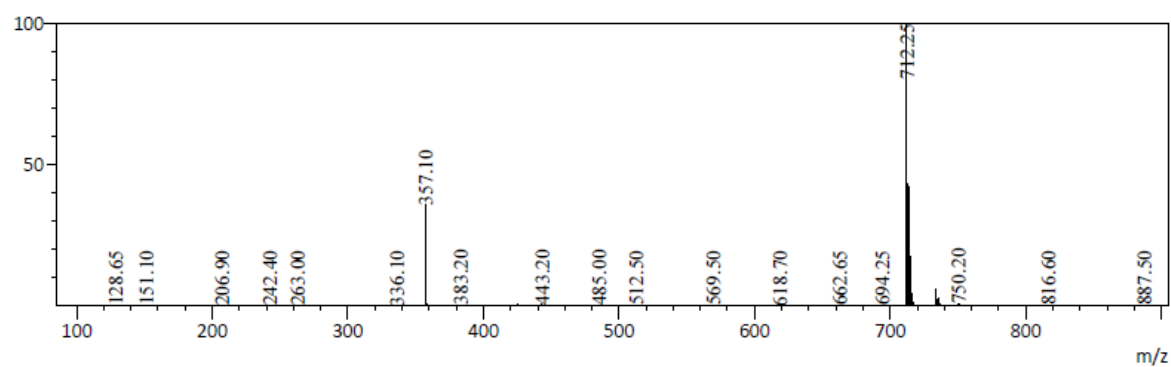
LCMS trace of **6**:



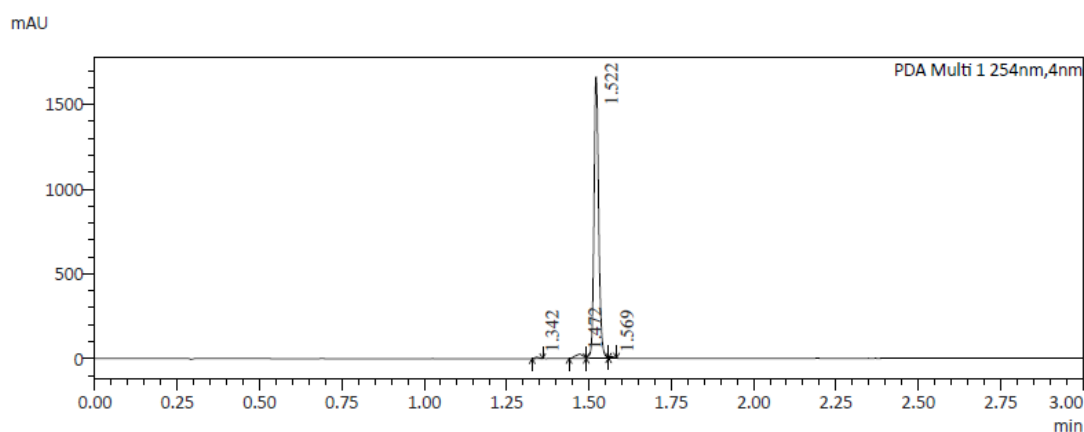
Peak Table

PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.340	3067	0.168	2474	0.127
2	1.479	4773	0.262	4142	0.213
3	1.521	1812367	99.569	1934265	99.659
Total		1820207	100.000	1940881	100.000



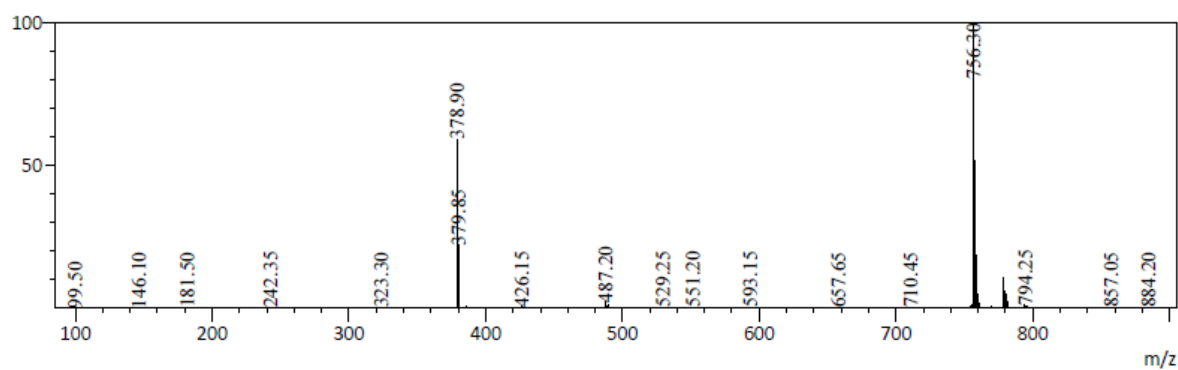
LCMS trace of 7:



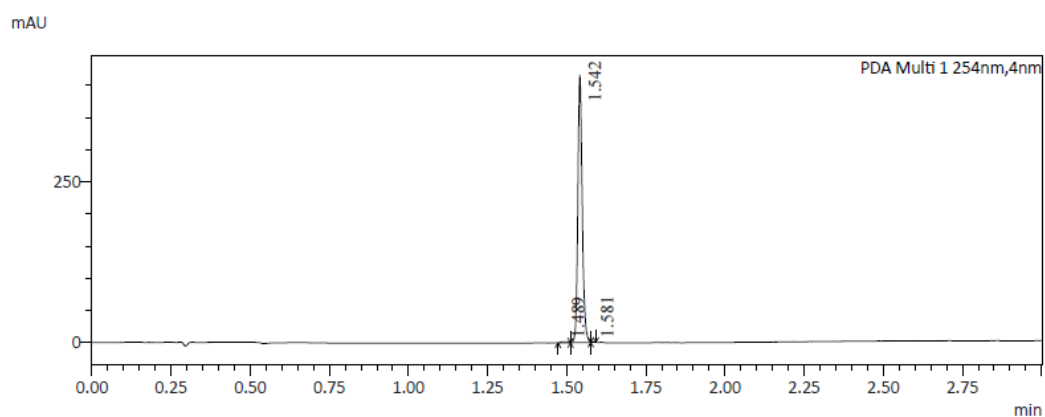
Peak Table

PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.342	8935	0.532	7434	0.437
2	1.472	24991	1.487	40889	2.402
3	1.522	1639115	97.507	1645943	96.701
4	1.569	7974	0.474	7825	0.460
Total		1681015	100.000	1702090	100.000

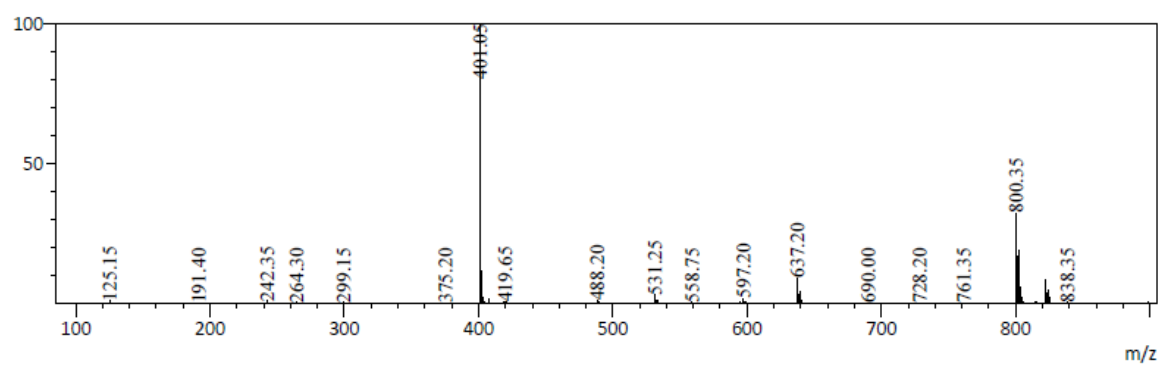


LCMS trace of **8**:



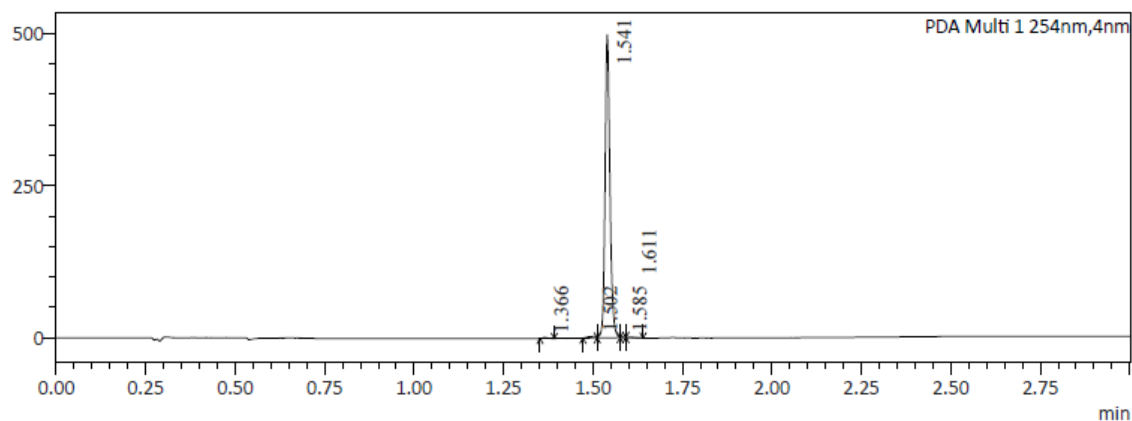
Peak Table

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.489	1643	0.408	2885	0.708
2	1.542	400369	99.335	404315	99.171
3	1.581	1039	0.258	495	0.121
Total		403051	100.000	407695	100.000



LCMS trace of 9:

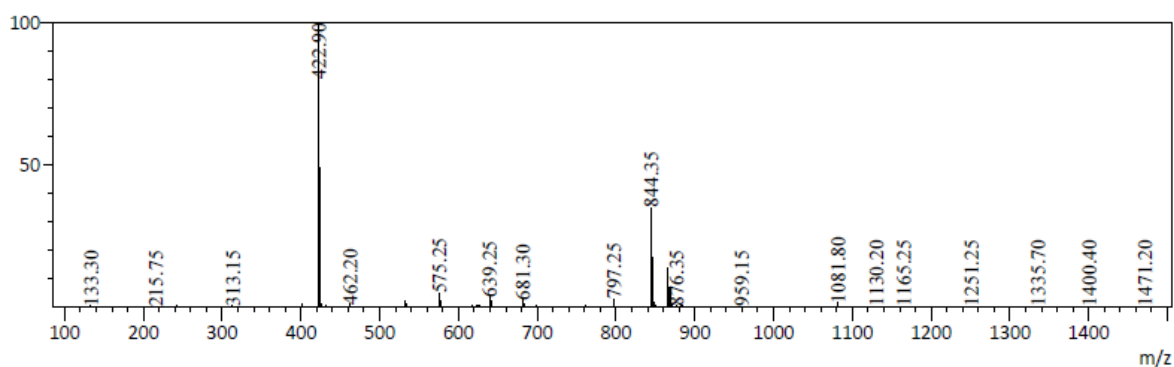
mAU



Peak Table

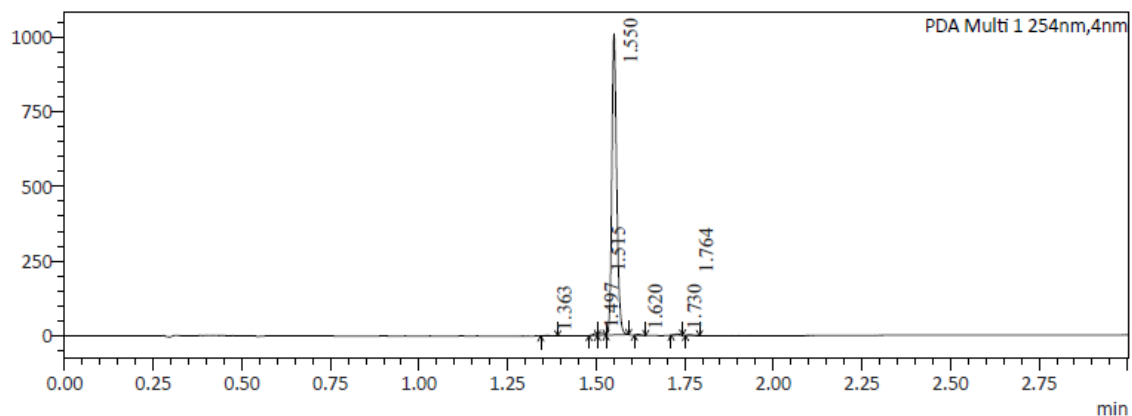
PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.366	1498	0.322	1420	0.297
2	1.502	2547	0.548	4469	0.934
3	1.541	459669	98.821	471992	98.633
4	1.585	838	0.180	436	0.091
5	1.611	602	0.129	215	0.045
Total		465154	100.000	478532	100.000



LCMS trace of **10**:

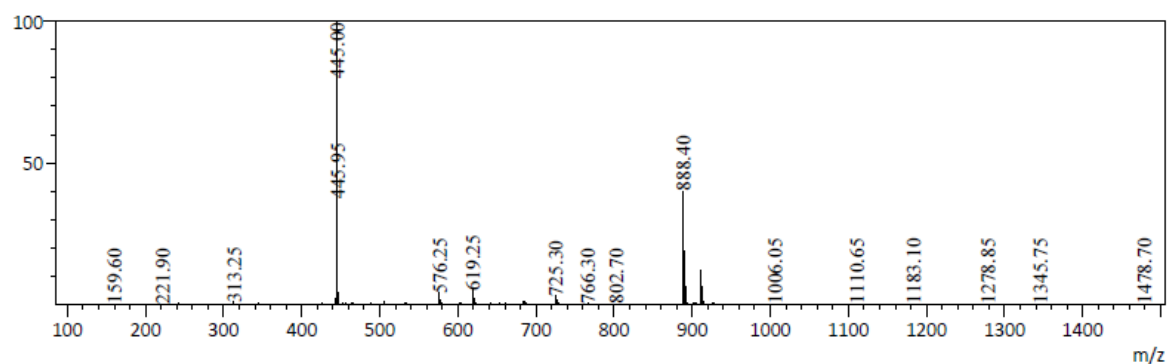
mAU



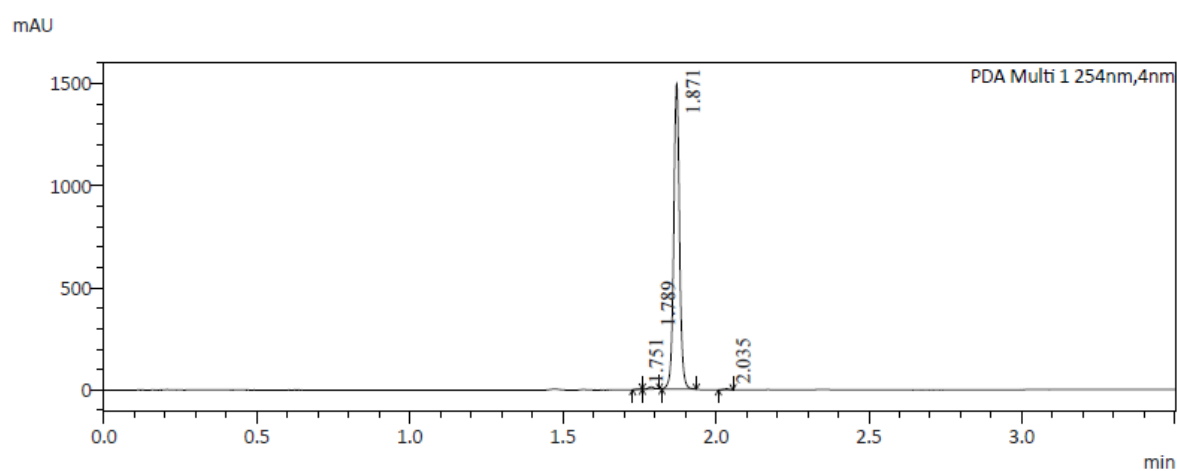
Peak Table

PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.363	1716	0.189	1957	0.199
2	1.497	3931	0.432	5366	0.545
3	1.515	6568	0.722	8274	0.840
4	1.550	888211	97.705	960928	97.611
5	1.620	3015	0.332	2545	0.259
6	1.730	3103	0.341	3227	0.328
7	1.764	2533	0.279	2153	0.219
Total		909078	100.000	984450	100.000



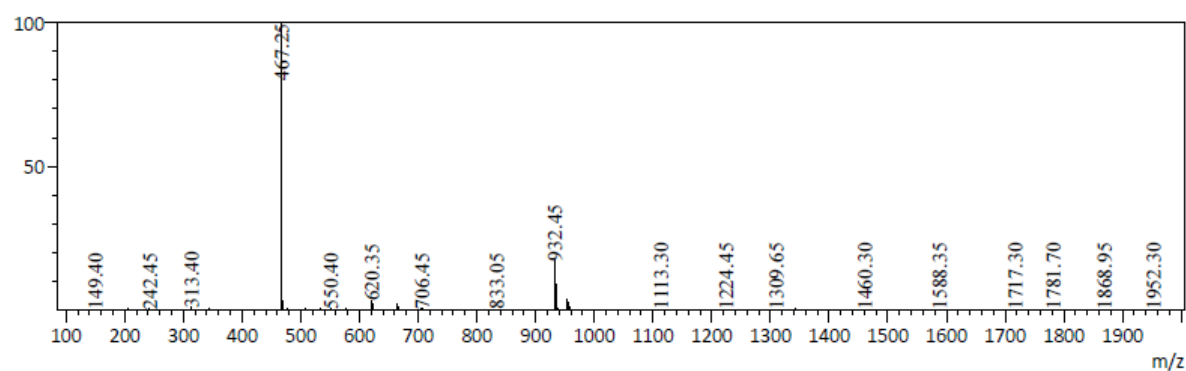
LCMS trace of **11**:



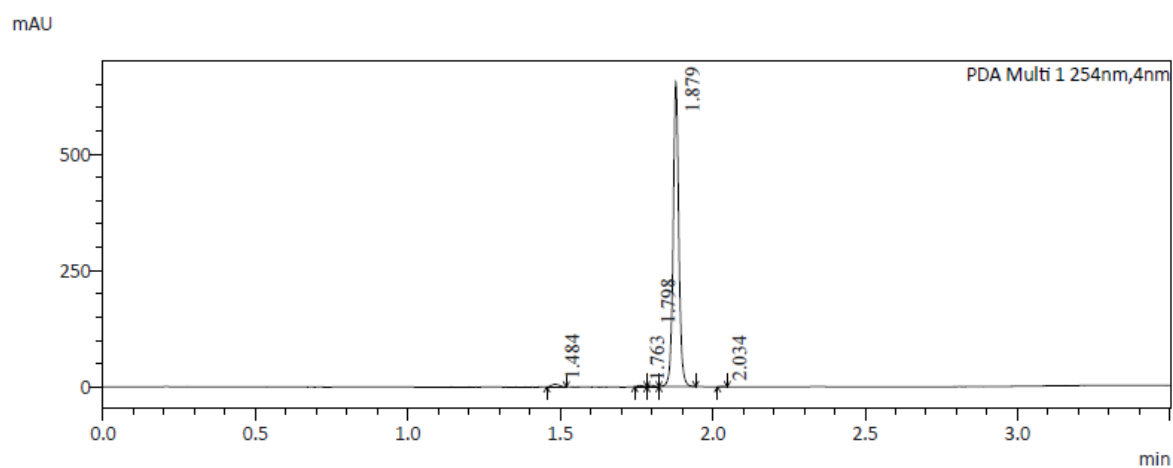
Peak Table

PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.751	1441	0.104	1660	0.083
2	1.789	9859	0.712	10421	0.518
3	1.871	1369619	98.845	1994475	99.117
4	2.035	4707	0.340	5692	0.283
Total		1385626	100.000	2012249	100.000

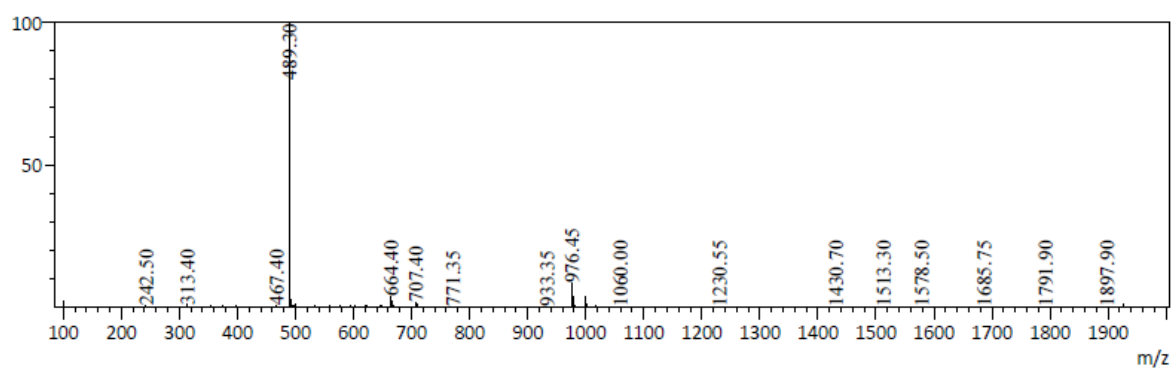


LCMS trace of **12**:

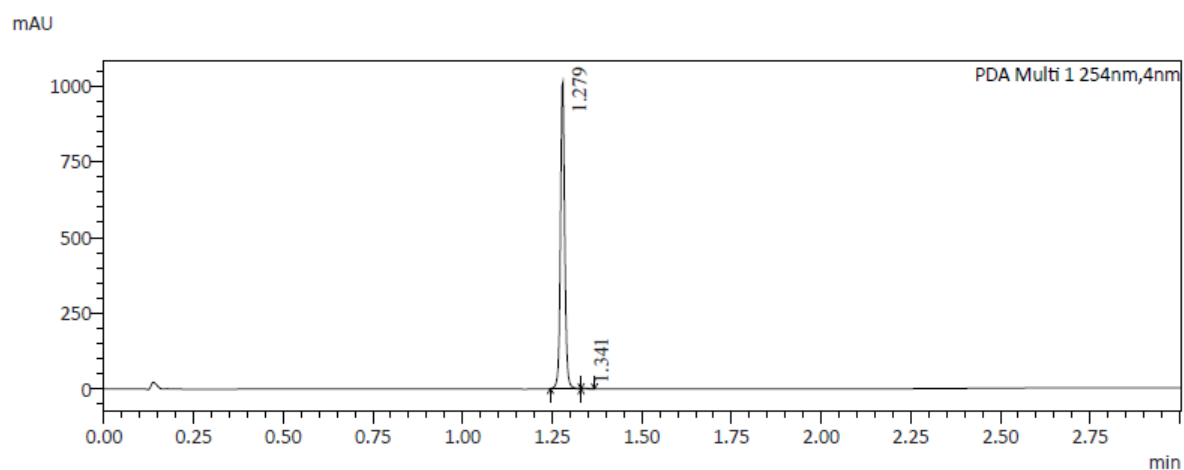


Peak Table
PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.484	5539	0.912	10262	1.173
2	1.763	2351	0.387	3460	0.396
3	1.798	2862	0.471	3969	0.454
4	1.879	595729	98.113	856004	97.886
5	2.034	706	0.116	799	0.091
Total		607188	100.000	874494	100.000



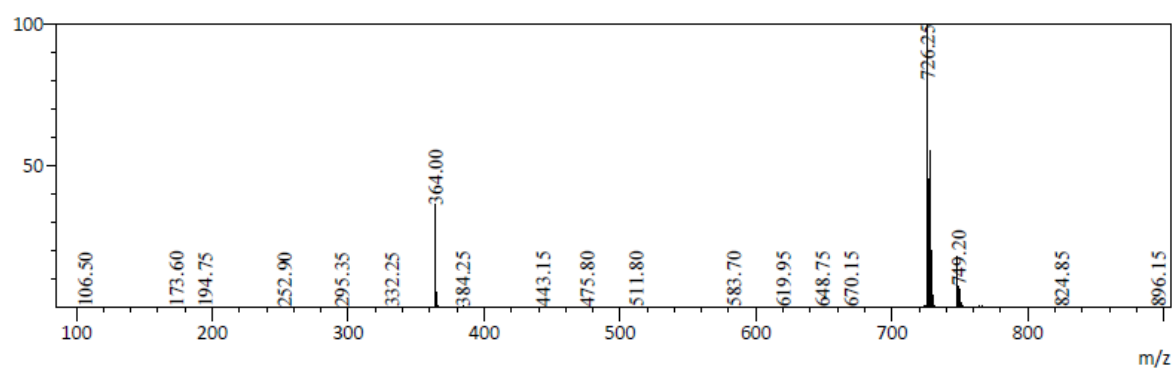
LCMS trace of **13**:



Peak Table

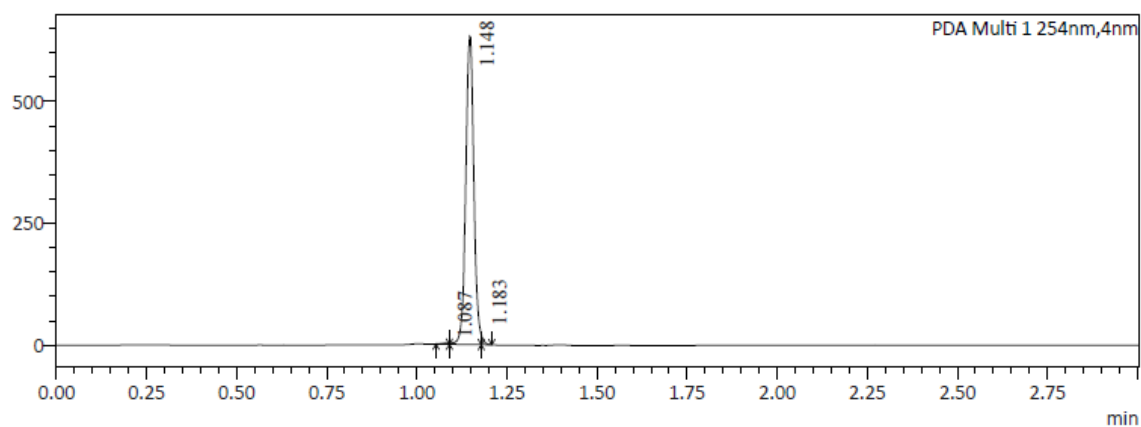
PDA Ch1 254nm

Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.279	997105	99.767	835762	99.743
2	1.341	2325	0.233	2151	0.257
Total		999430	100.000	837913	100.000



LCMS trace of **14**:

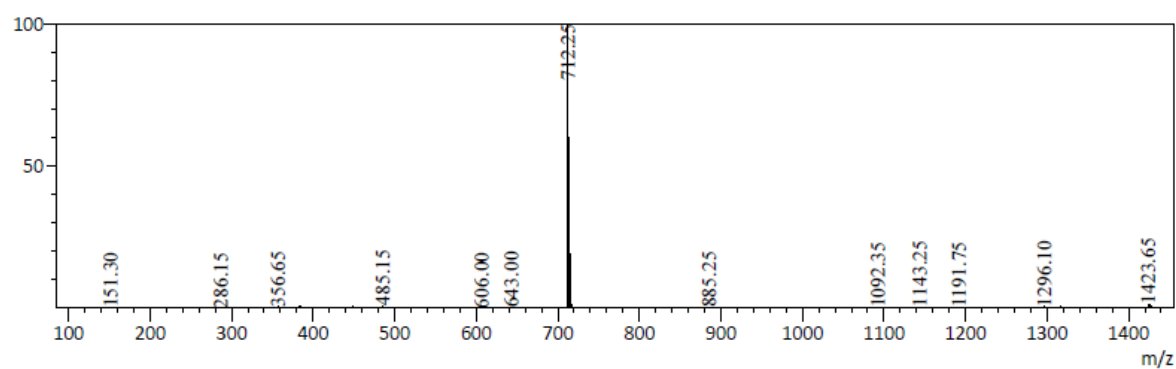
mAU



Peak Table

PDA Ch1 254nm

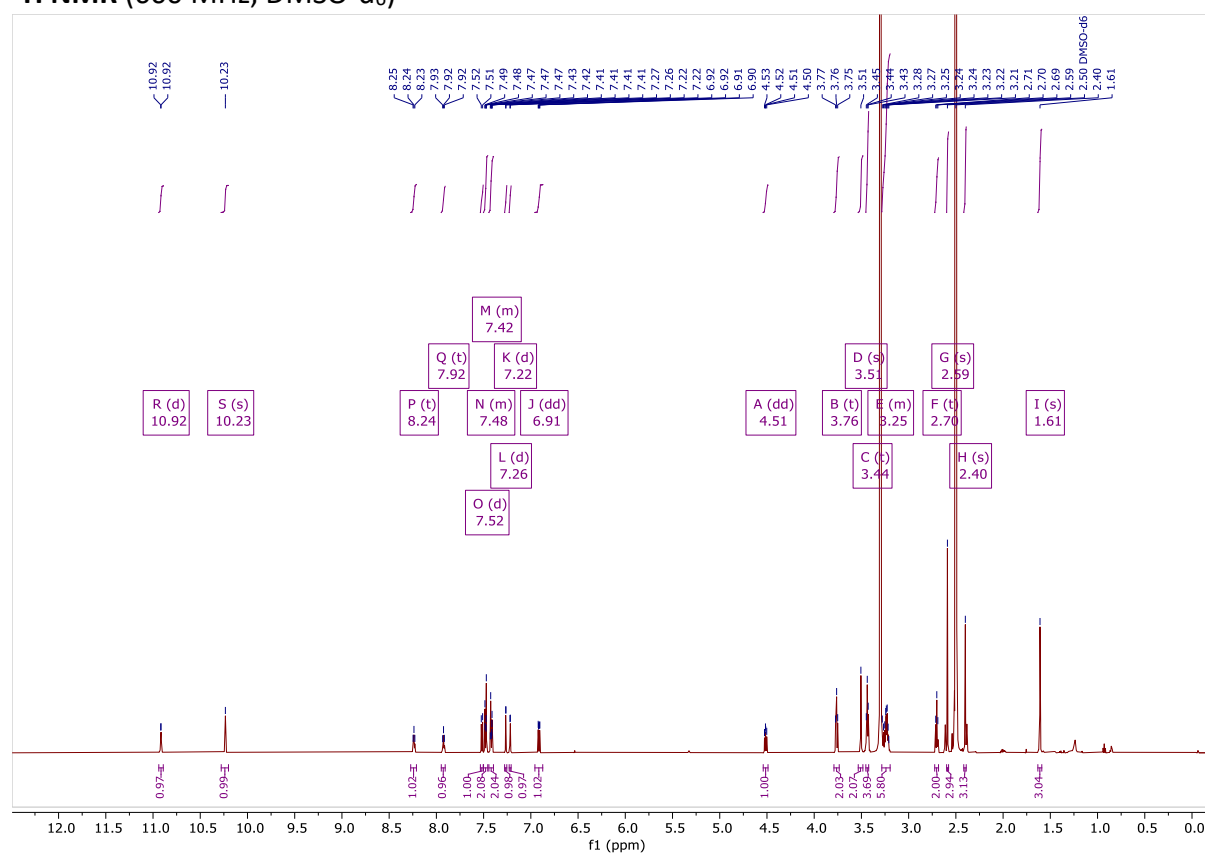
Peak#	Ret. Time	Height	Height%	Area	Area%
1	1.087	2559	0.394	3897	0.388
2	1.148	632150	97.346	992398	98.905
3	1.183	14674	2.260	7088	0.706
Total		649383	100.000	1003383	100.000



NMR spectra of target compounds

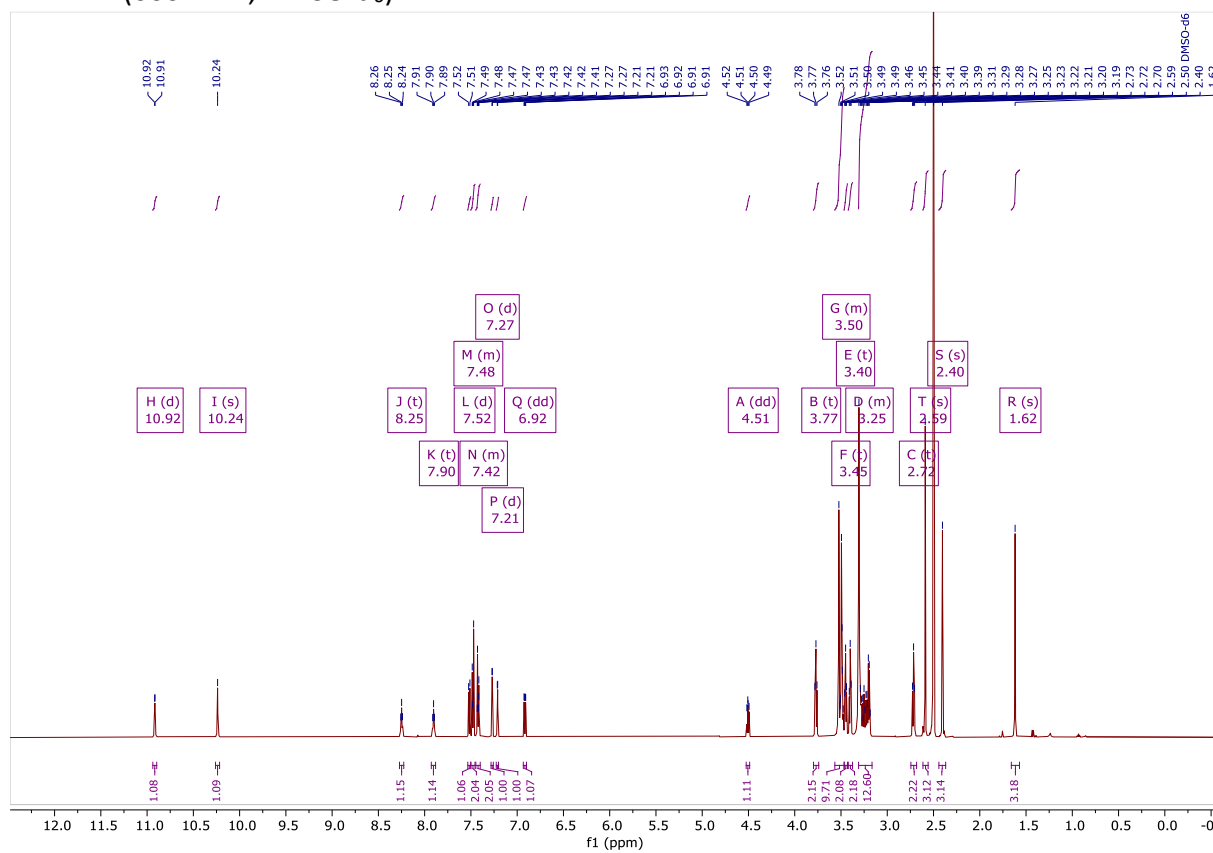
NMR spectra of **7**:

^1H NMR (600 MHz, DMSO- d_6)

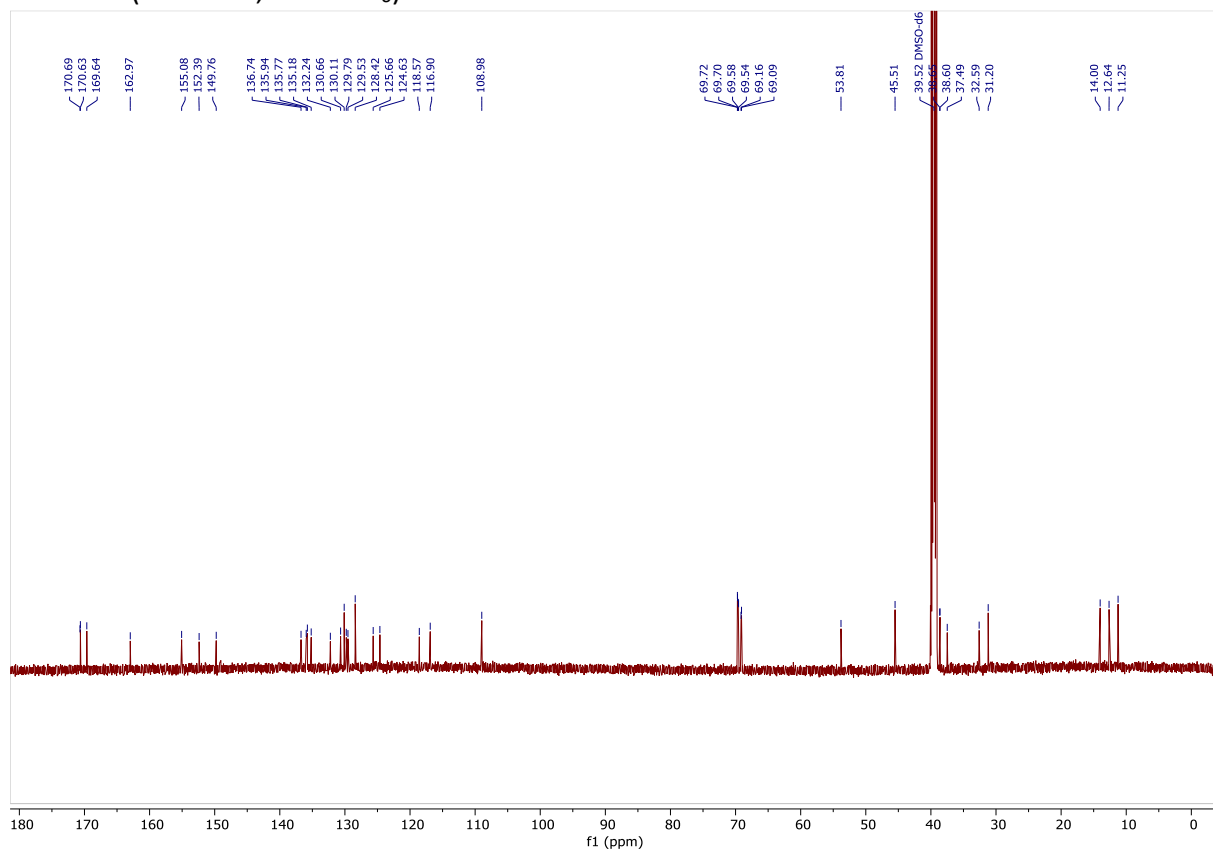


NMR spectra of **9**:

¹H NMR (600 MHz, DMSO-d₆)

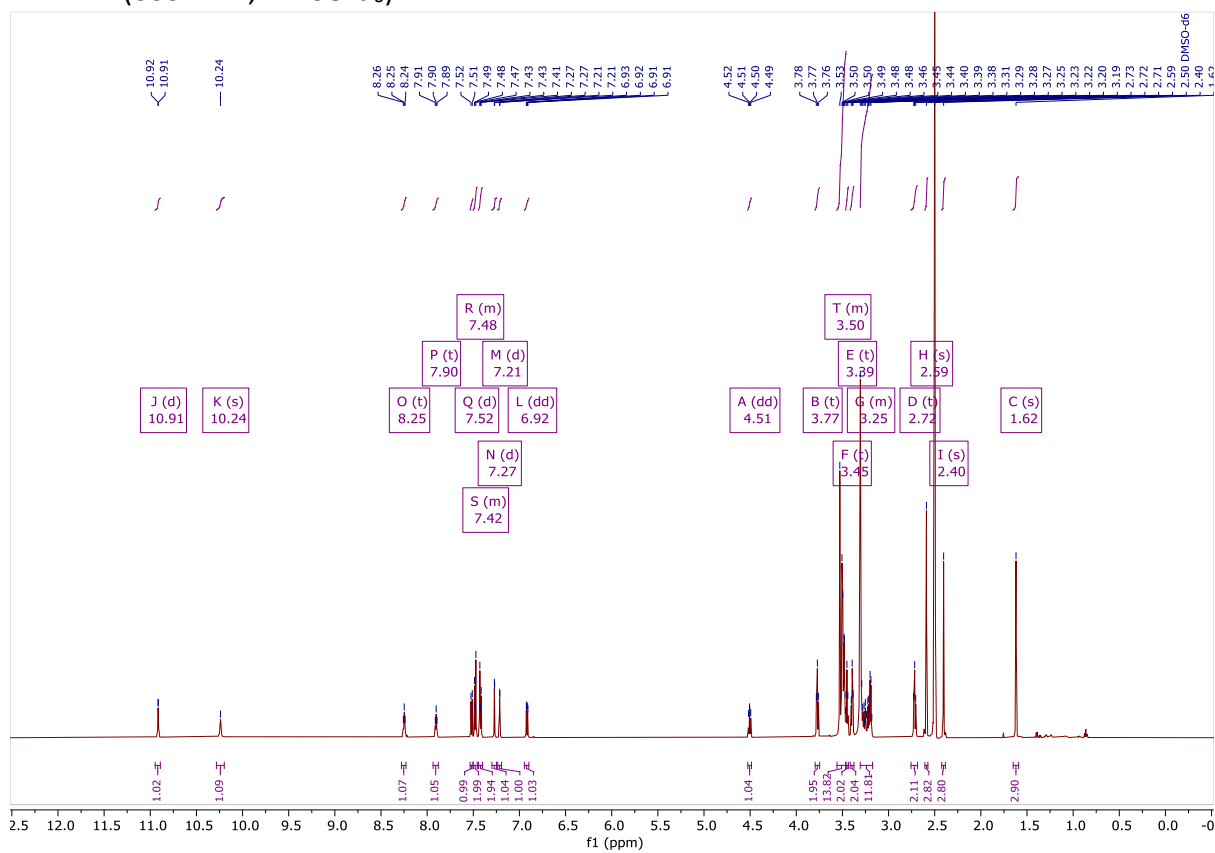


¹³C NMR (151 MHz, DMSO-d₆)

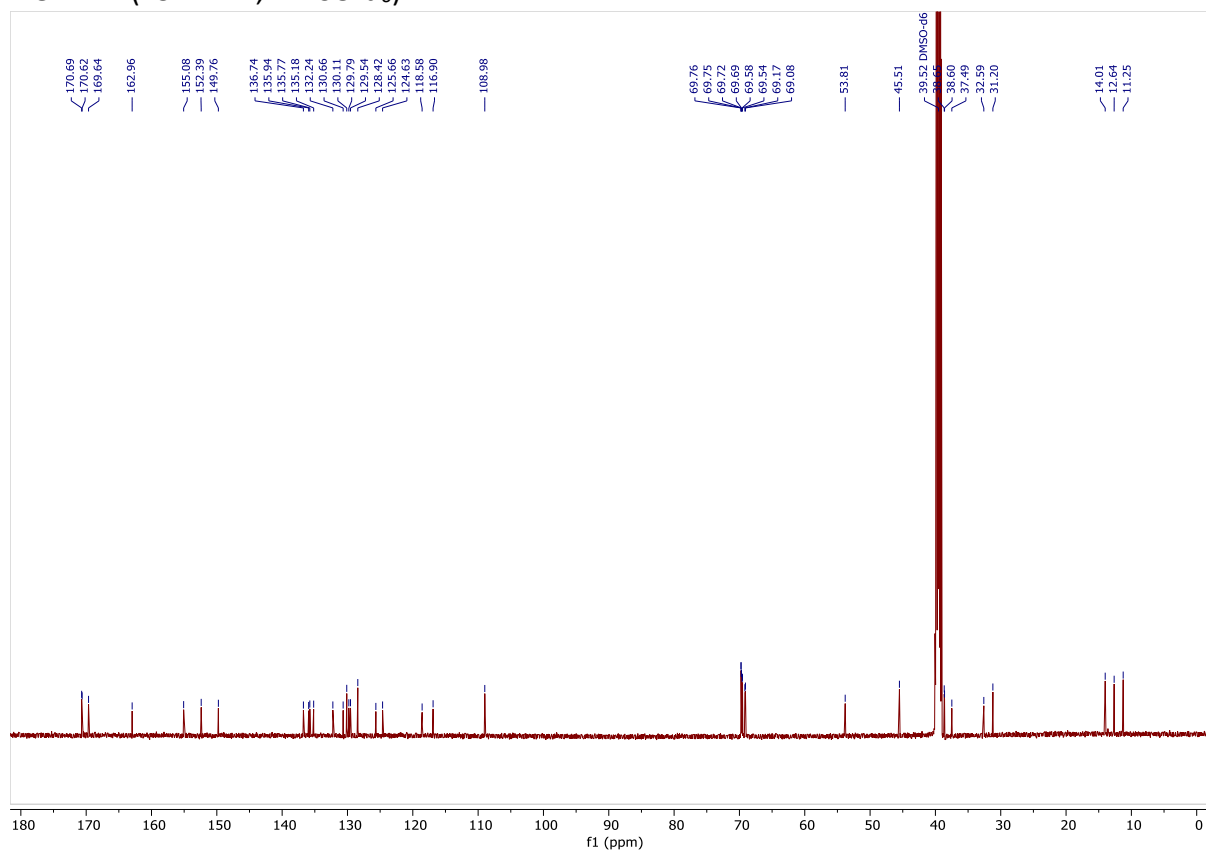


NMR spectra of **10**:

¹H NMR (600 MHz, DMSO-d₆)

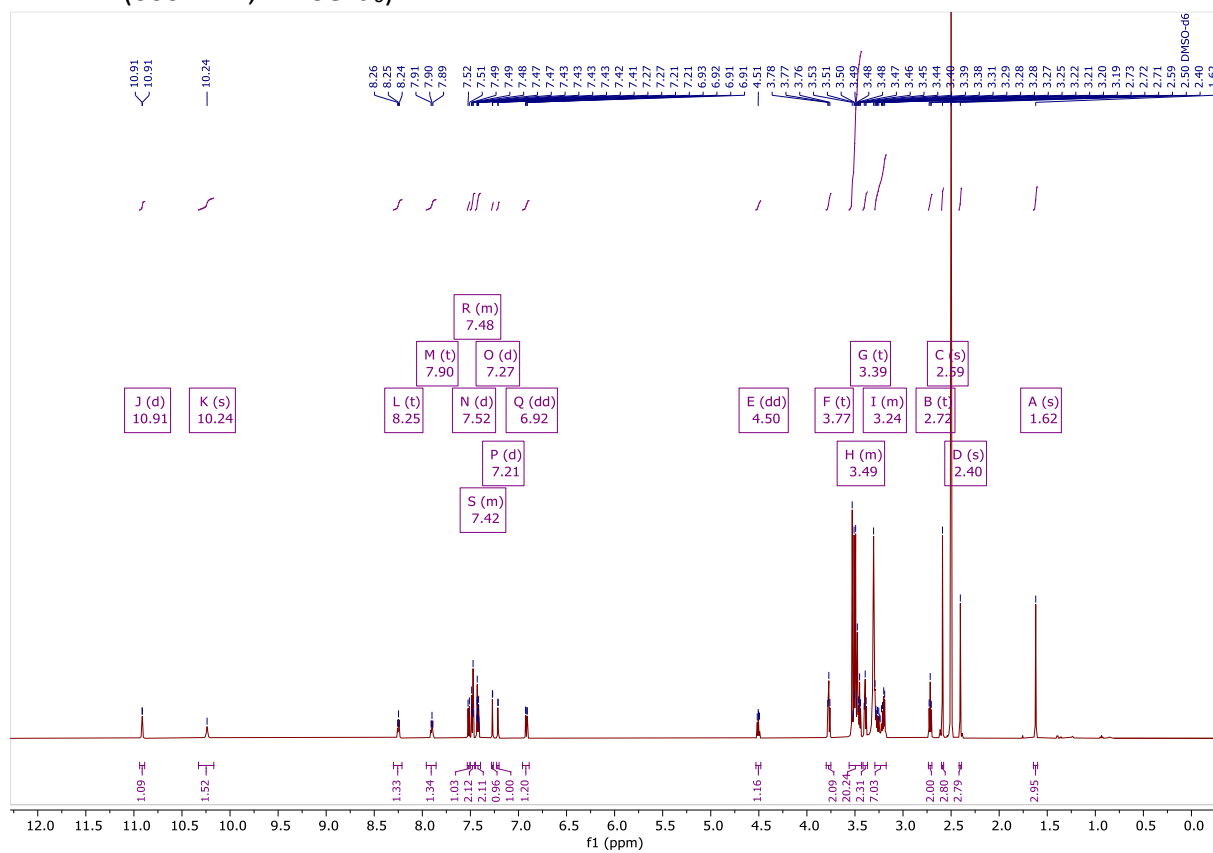


¹³C NMR (151 MHz, DMSO-d₆)

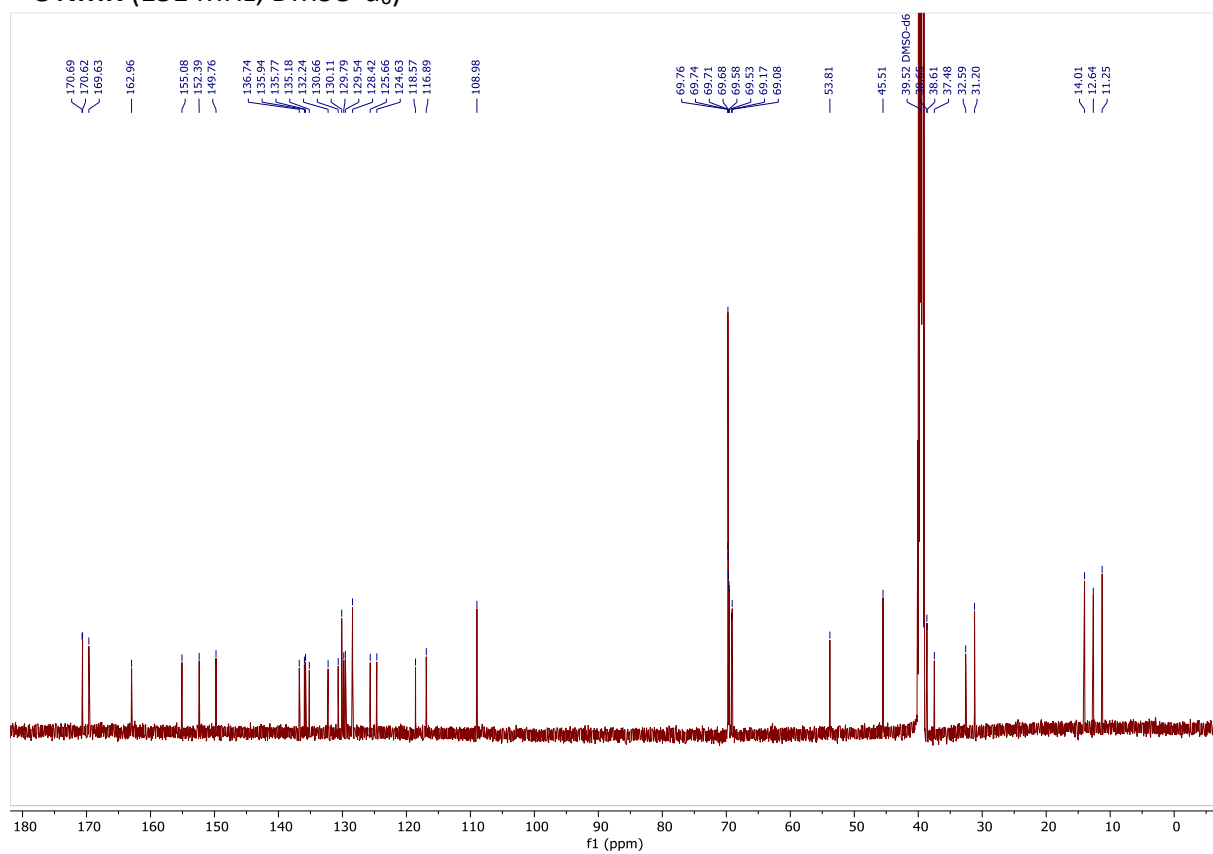


NMR spectra of **11**:

¹H NMR (600 MHz, DMSO-d₆)

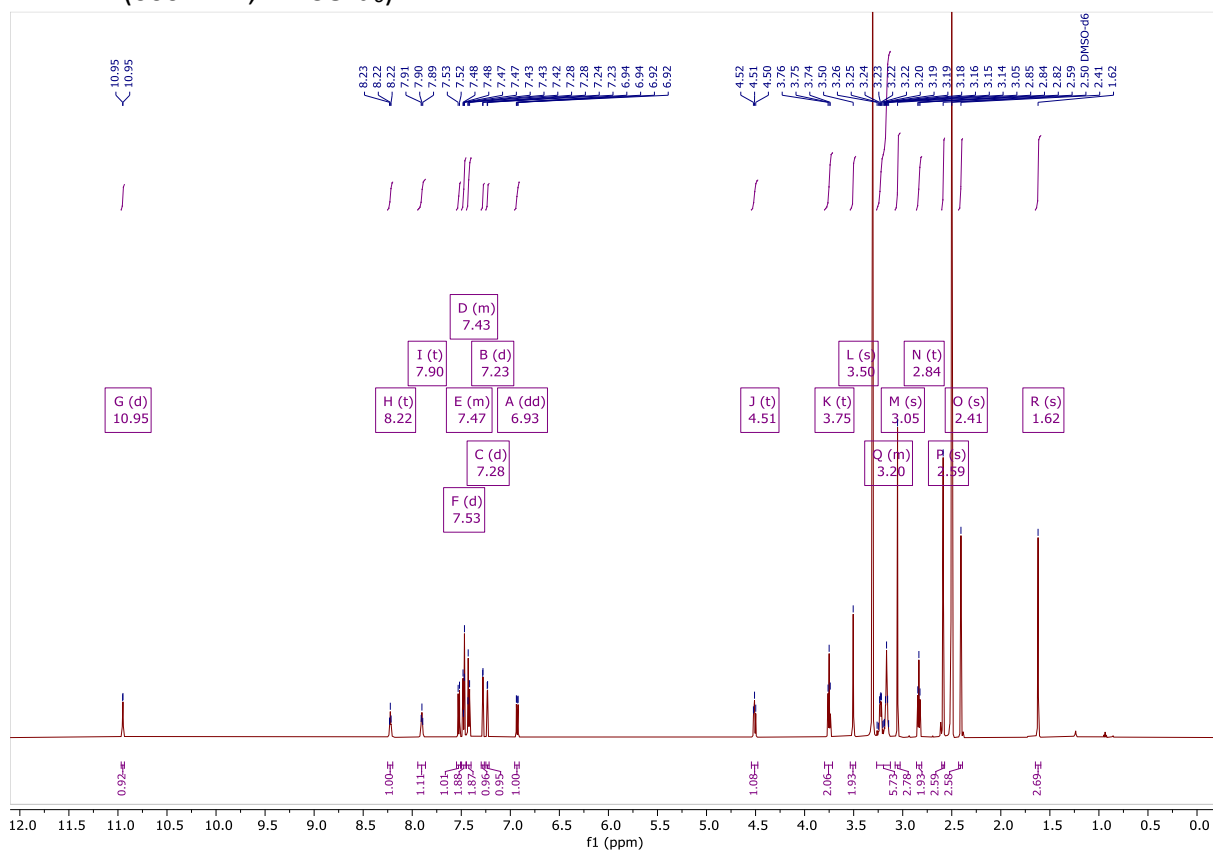


¹³C NMR (151 MHz, DMSO-d₆)

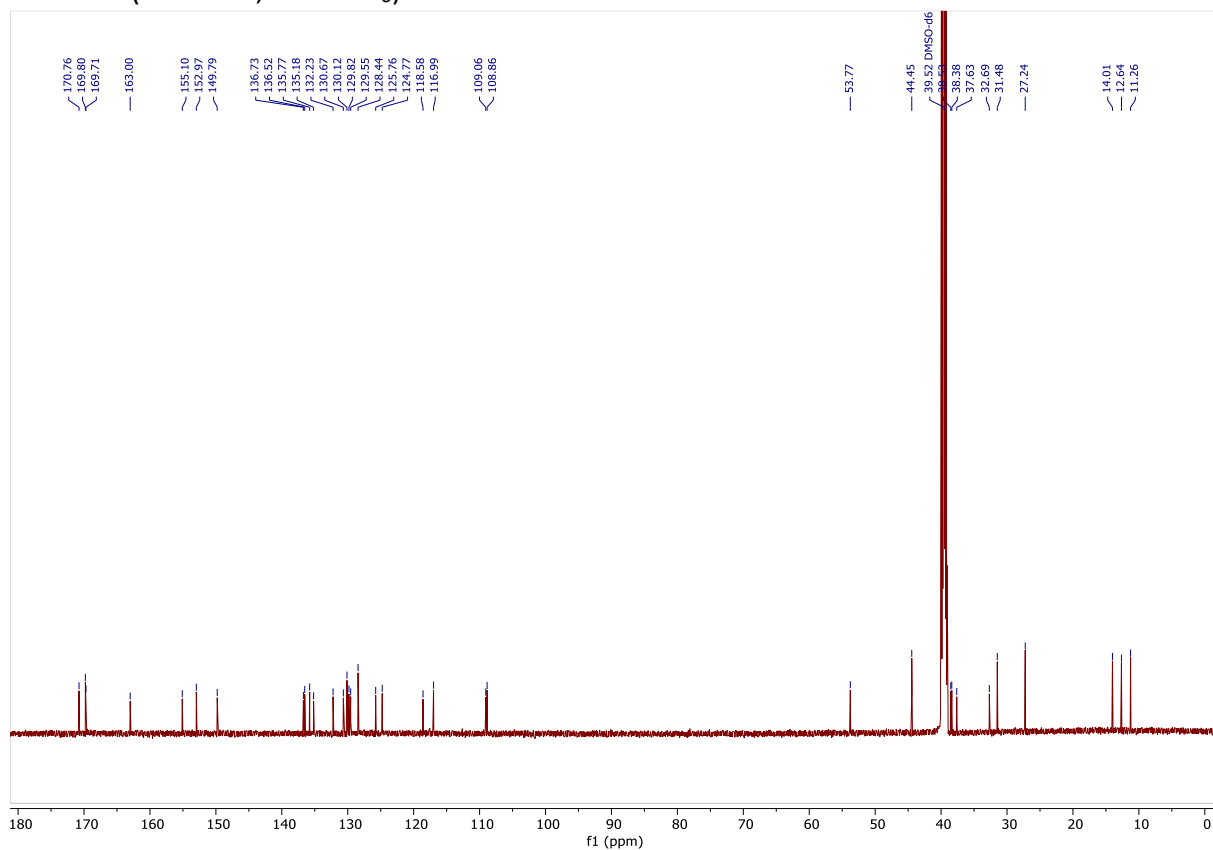


NMR spectra of **13**:

¹H NMR (600 MHz, DMSO-d₆)

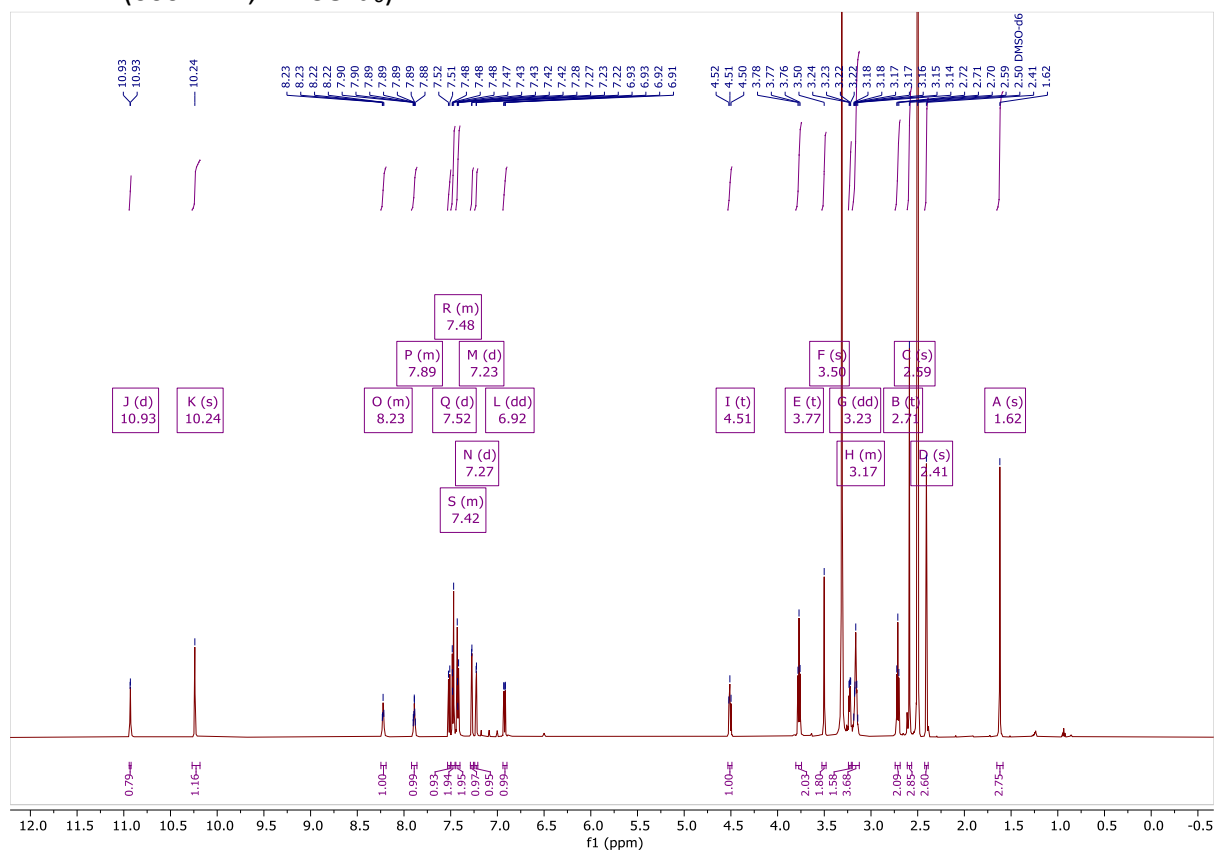


¹³C NMR (151 MHz, DMSO-d₆)

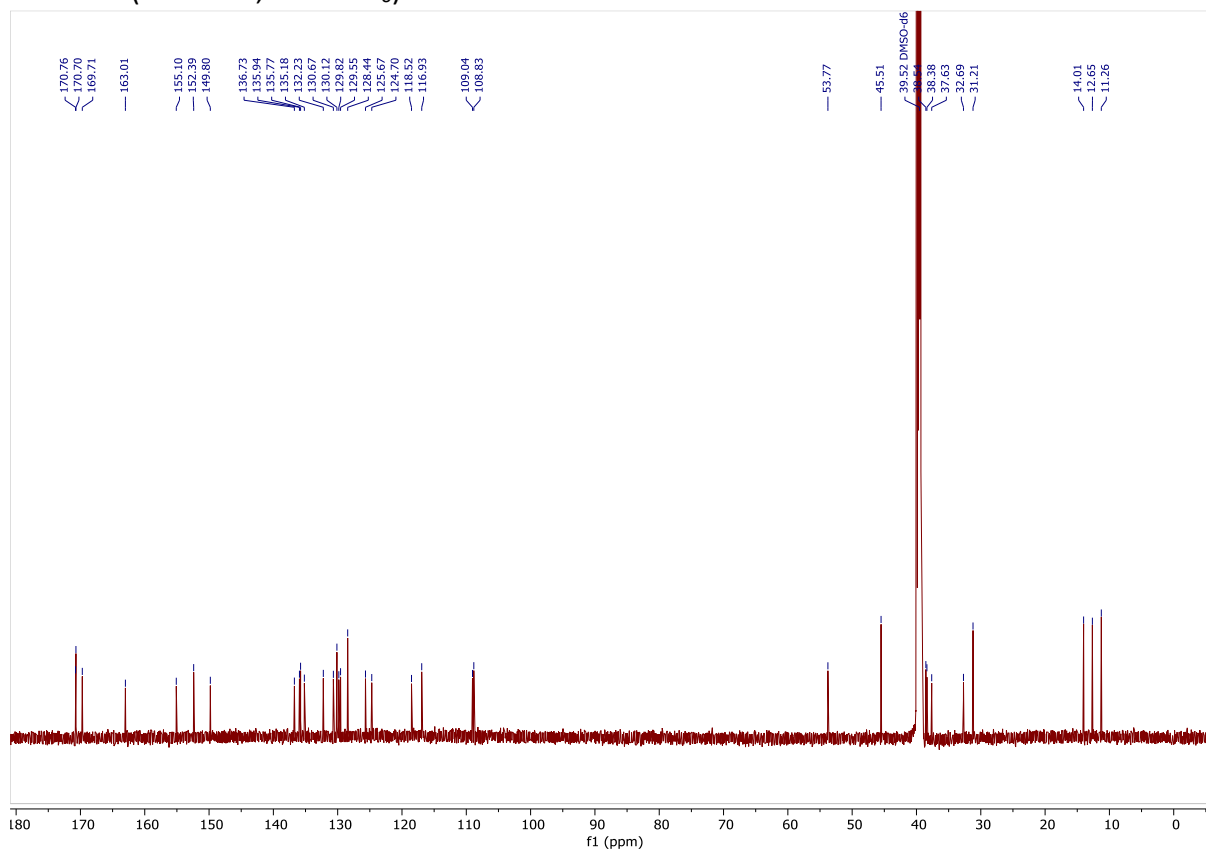


NMR spectra of **14**:

¹H NMR (600 MHz, DMSO-d₆)



¹³C NMR (151 MHz, DMSO-d₆)



Expression and purification of CRBN for the ITC experiments

The protein construct ZZ-6his-thrombin-hCereblon 40-442::6His-TEV-hDDB1-deltapropeller was used in the ITC experiments. The expression plasmid of this construct was synthesized by GenScript and cloned into the pFastbac1 vector. The protein was expressed in Sf21 insect cells for 48 h using baculo virus. For purification, 160 g insect cells were resuspended in lysis buffer (50 mM Tris-Cl pH 8, 200 mM NaCl, 20 mM Imidazole, 10 % Glycerol, 1 mM DTT, Protease inhibitors (1 tablet/ 50 mL) and lysed for 1 min using Turrax. The lysate was centrifuged at 30000 x g for 45 min and the supernatant incubated with equilibrated Ni-NTA slurry (Qiagen) overnight on a roller table at 4 °C. The resin was collected and packed on a XK50 column and washed until baseline using wash buffer (50 mM Tris-Cl pH 8, 20 mM Imidazole, 200 mM NaCl, 10 % Glycerol, 1 mM DTT) on an FPLC system. The protein was eluted using imidazole (50 mM Tris-Cl pH 8, 200 mM NaCl, 300 mM Imidazole, 1 mM DTT). Digestion of the protein with thrombin and TEV was done simultaneously during dialysis overnight at 4 °C. Dialysis was performed in Snake skin (ThermoFisher) in 5 L buffer. The digest mix was passed over a HisTrap column (GE Healthcare) for tag and protease removal and the flow through collected. The digested protein was concentrated using PALL 10K spin filter followed by size exclusion chromatography (GE Healthcare 26/60 column) (10mM HEPES pH 7.5, 240 mM NaCl, 1 mM DTT). Selected peak fractions were pooled and checked for its quality using SDS-Page, MS and SEC-MALS.

ITC experiment

ITC experiments were performed using a MicroCal™ iTC200 System (Malven Panalytical). All experiments were carried out in 10 mM Hepes pH 7.5, 150 mM NaCl, 0.5 mM TCEP and 2% DMSO. Extensive dialysis was done to buffer match both components before the experiment. In the ITC setup, 40 μ M CRBN/DDB1 construct (co-expressed using different vectors) was put in the ITC cell and 200 μ M of the compound was in the syringe. Heat changes of the reaction were recorded at 25 °C over the course of 19 injections (1 x 0.5 μ L then 18 x 2 μ L) and the data analyzed in MicroCal PEAQ-ITC Analysis Software version 1.41 (Malvern Panalytical). Data was referenced through subtraction of the heat changes when injecting the compound into buffer and baseline correction. The data was fitted using the “one set of sites model”. Experiments were performed with n=1.

Obtained parameters:

N (sites) = 0.602

KD (M) = 216e-9

ΔH (kcal/mol) = 3.30

Offset (kcal/mol) = -0.505

ΔG (kcal/mol) = -9.10

-T ΔS (kcal/mol) = -12.4

Determination of chemical stability at pH 1.0, 7.4 and 10.0

Buffer solutions (975 μ L) at pH 1.0, 7.4 and 10 were pre-incubated at 70 °C for 20 min before

addition of compound (25 μ L) to give a final compound concentration of 25 μ M in 2.5% DMSO. Solutions were vortexed at 3000 rpm for 1 min prior to incubation at 70 °C at 300 rpm on an Eppendorf Thermomixer comfort plate shaker. Aliquots of 150 μ L of the solution at each pH were transferred at 0, 2, 4, 8 and 24 hours to 200 μ L clear polypropylene tubes and analyzed directly by injection on a generic LC/UV/MS. Compound stability half-life was determined by following loss of parent compound over time and fitting to first order kinetics. An extrapolated half-life at 25 °C was determined by assuming a factor of 2 decline in reaction rate for each 10 °C reduction in temperature.

Expression and purification of CRBN for crystallographic studies

The gene encoding residues 18-124 of CRBN from *Magnetospirillum gryphiswaldense* with the following mutations – A52H, M54Y, F56H, F77H, Y101F and R117A – was synthesized, codon-optimized for expression in *Escherichia coli* and cloned into a pET24a vector along with an N-terminal hexa-histidine tag followed by a cleavage site of the TEV (Tobacco Etch Virus) protease (GeneArt, ThermoFisher). The protein was expressed in NEB BL21 phage resistant cells in TB media. Cells were induced with 0.1 mM IPTG and grown overnight at 18 °C. Cells were pelleted and resuspended in lysis buffer (40 mM HEPES pH 8.0, 500 mM NaCl, 1 mM TCEP, 20 mM imidazole, 5% glycerol, 1 mL/200 mL protease inhibitor (Expedeon), 1.5 μ L/10 mL Nuclease A) and passed twice through a homogenizer at 10-15 kpsi. Cells were then centrifuged for 1 hour at 4 °C at 16000 rpm in a Beckman JLA-16.250 rotor. After centrifugation, the supernatant was added to NiNTA resin equilibrated with lysis buffer and left overnight at 4 °C. Histidine tagged proteins were eluted with 40 mM HEPES pH 8.0, 500 mM NaCl, 1 mM TCEP, 400 mM imidazole, 5% glycerol. The eluate was dialysed against 40 mM HEPES pH 7.5, 200 mM NaCl, 1 mM TCEP, 15 mM imidazole, 5% glycerol. The histidine tag was cleaved overnight at 4 °C by TEV protease and the removed using Ni-NTA agarose beads. The flow through was concentrated and applied to a Superdex 75 16/60 column equilibrated in 40 mM HEPES pH7.5, 200 mM NaCl, 1 mM TCEP. Peak fractions were collected and the protein was concentrated to 8.7 mg/mL using a 10k MWCO centricon.

Crystallography

For crystallisation trials, purified, recombinant cereblon protein from the bacterial strain *Magnetospirillum gryphiswaldense*^{2,3} (uniprot entry: A4TVL0) with the following humanizing mutations – A52H, M54Y, F56H, F77H, Y101F and R117A – was used at a concentration of 8.7 mg/ml. Prior to crystallisation trials, compound **2** was added to this protein to a final concentration of 2 mM from a 100 mM stock in 100 % DMSO. Crystals were grown in standard sitting drops composed of 100 nL of the protein-ligand complex plus 100 nL of a crystallisation reagent composed of 10 % PEG8000, 8 % ethylene glycol and 100 mM Na-HEPES buffer (pH 7.5). Crystals were frozen in liquid nitrogen and diffraction data were collected on beamline I04 at the Diamond Light Source synchrotron. Data were processed using autoproc⁴ and the structure solved by molecular replacement using a publicly available search model (PDB entry 4V30³) using Phaser⁵ from the CCP4 suite.⁶ Model building and refinement were performed

in Coot⁷ and Buster (Global Phasing Ltd.), respectively. Structure factors and atomic coordinates have been deposited in the Protein Data Bank with the following accession code: 9GJQ. Data collection and refinement statistics can be found in the table below and the Protein Data Bank.

Data collection and refinement statistics for X-ray crystal structure of CRBN from *Magnetospirillum gryphiswaldense* bound by compound **2**.

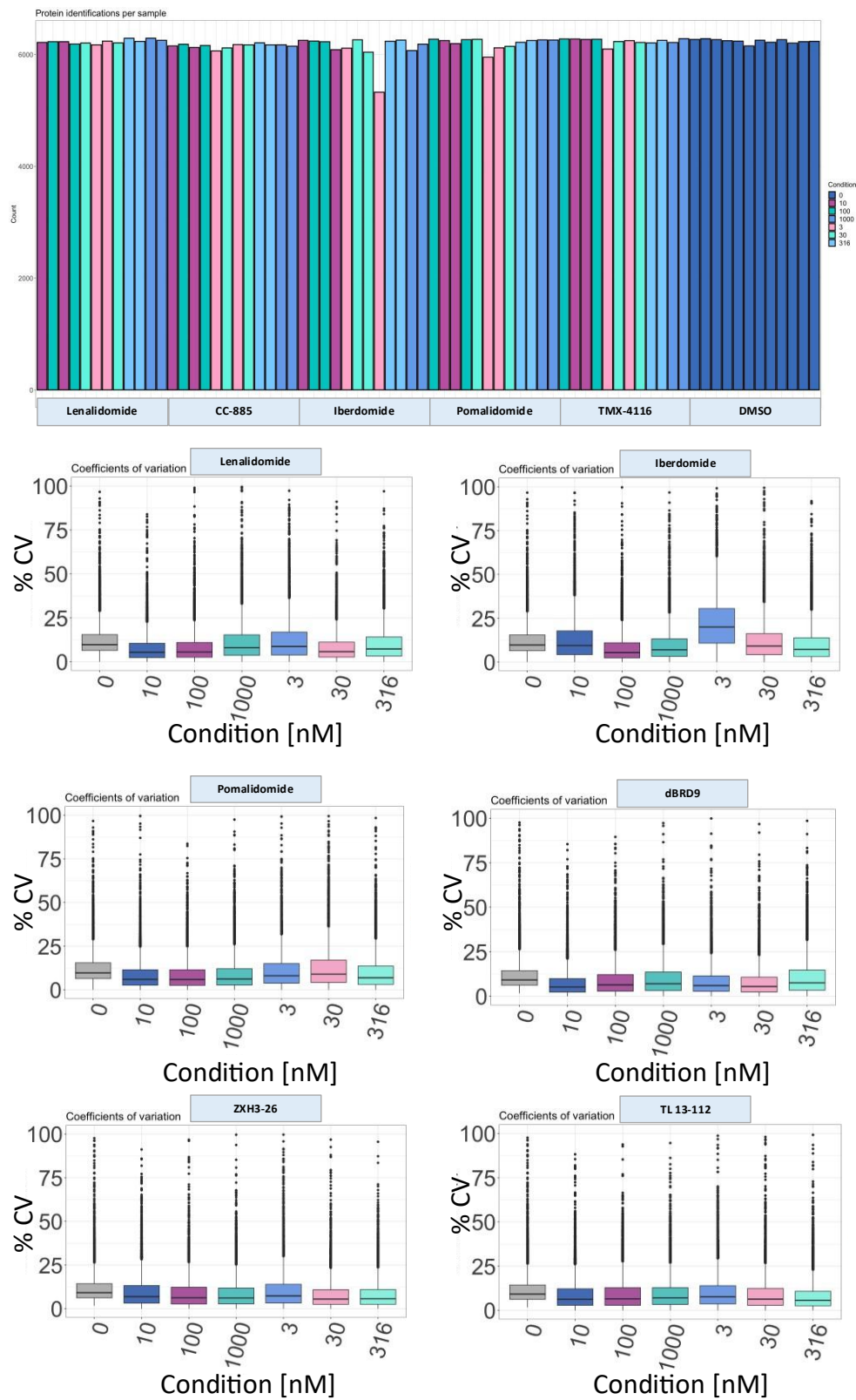
Data collection	
Space group	C222 ₁
a, b, c (Å)	51.78, 82.31, 52.45
α, β, γ (°)	90.00, 90.00, 90.00
Resolution (Å)	43.83 – 1.39 (1.47 – 1.39)
R _{meas} (%)	8.9 (98.0)
CC ½ (%)	99.9 (73.8)
I / σ	12.6 (1.8)
Reflections (total)	140677
Reflections (unique)	18772
Completeness (spherical) (%)	82.4 (29.1)
Completeness (ellipsoidal) (%)	94.2 (64.1)
Redundancy	7.5 (7.6)
Refinement	
Resolution (Å)	43.83 – 1.39
R _{work} / R _{free} (%)	19.99 / 20.87
Protein atoms	836
Waters	116
Overall B-factor (Å ²)	16.93
R.m.s. deviations	
Bond-lengths (Å)	0.011
Bond-angles (°)	1.07
PDB code	9GJQ

Values in brackets refer to high resolution shell.

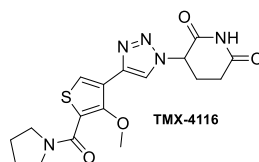
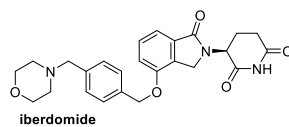
Supplementary figures

Supplementary Figure 1. High-throughput proteomics for protein degradation screening

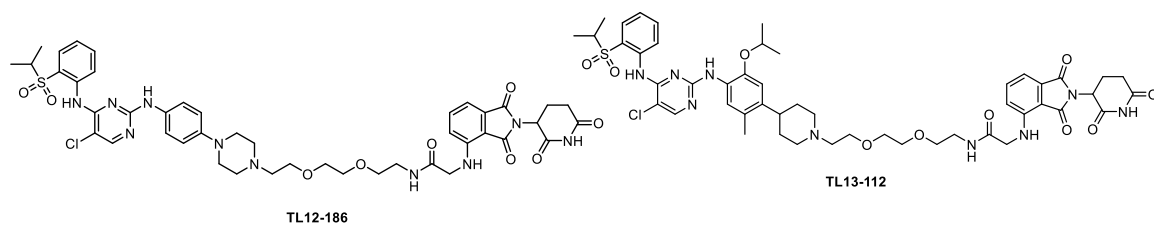
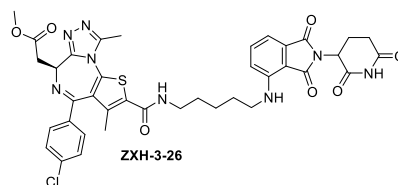
Protein identification rates across concentrations. Related to Figure 1.



IMiDs



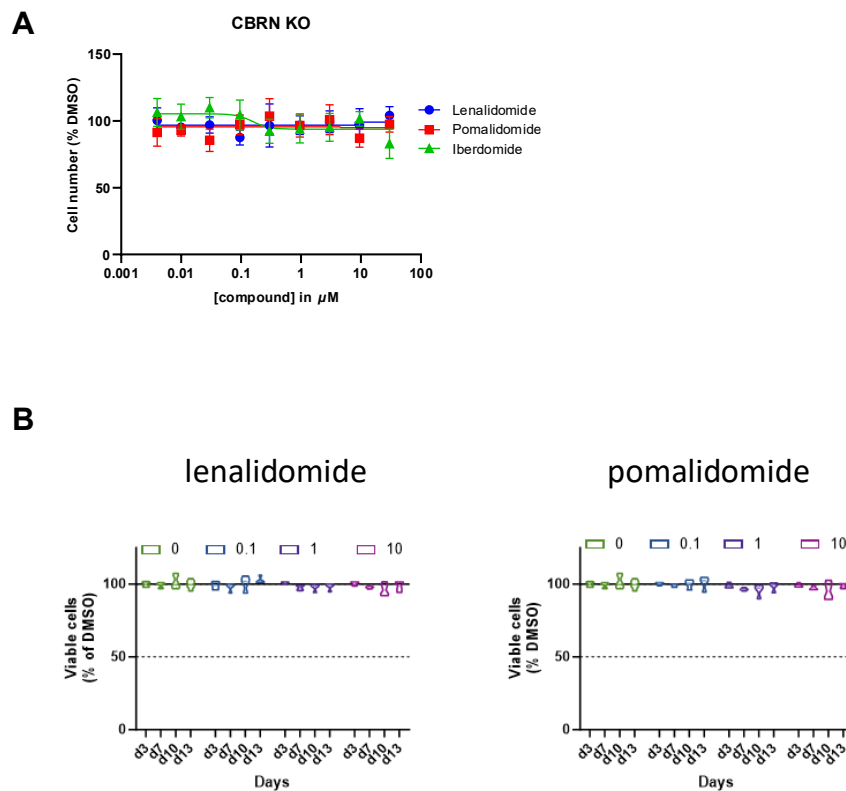
PROTACS



Supplementary Figure 3. Hematopoietic stem and progenitor cells viability in 3D static culture over 13 days

(A) HSPC proliferation assay under lenalidomide, pomalidomide or iberdomide treatment after 5 days of dosing in CBRN knock-out cells. N=3 experiments per compound

(B) HSPC viability under lenalidomide and pomalidomide treatment (0.1, 1, 10 μ M) over 13 days in 3D static culture, normalized to time matched controls. N=4 replicates per treatment condition. Related to Figure 2.



Supplementary Figure 4. Lenalidomide causes lymphopenia in *Crbn* I391V CRISPR knock-in mice

(A) Schematic representation of the *Crbn* I391V CRISPR knock-in strategy and Sanger sequencing of *Crbn*^{I391I} wild type and *Crbn*^{I391V} homozygous mice. The mouse *Crbn* locus was modified directly in zygotes by electroporation with a Ribonucleoprotein (RNP) complex together with a single stranded oligo donor harbouring the point mutation of interest. The founder mice were verified by sequencing and bred to wild-type C57Bl6/N mice for germline transmission. Breeding with heterozygous siblings allowed for the generation of homozygous *Crbn*^{I391V}. Silent point mutations L395L AAG→AAA and I396I, ATC→ATT (stars/★) from oligo donor are not transferred to *Crbn* I391V mouse. Corresponding amino acid change shown below sequencing trace. Generated by Biorender.

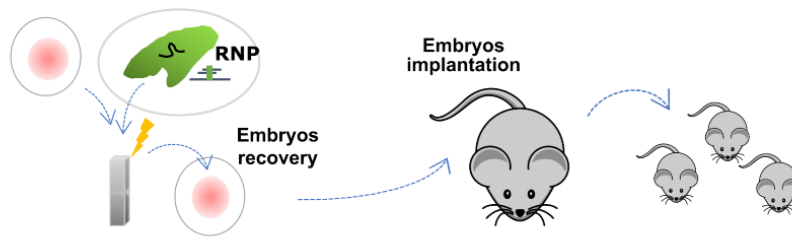
(B) SDS-PAGE analysis of Ikaros and Aiolos degradation in the spleen of wild-type (WT) or *Crbn* transgenic (I391V) animals by lenalidomide treatment after 6 h of dosing at 100 mg/kg.

(C) Quantification of Ikaros and Aiolos degradation after 21 days of lenalidomide treatment at 30 or 100 mg/kg in the spleen of WT and *Crbn* transgenic (I391V) animals (minimum of 4 animals per group).

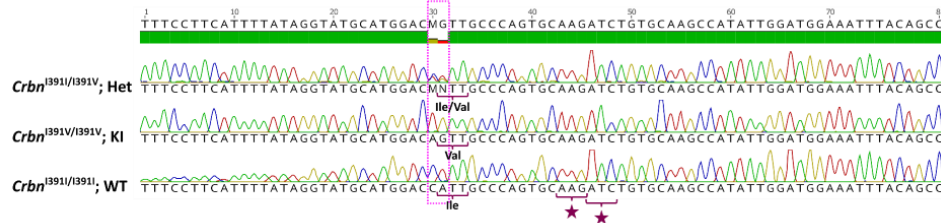
(D) Peripheral blood count (lymphocytes and white blood cells) graphs in WT or *Crbn* transgenic (I391V) animals treated with lenalidomide for 21 days at 30 or 100 mg/kg (minimum of 4 animals per group).

Related to Figure 3.

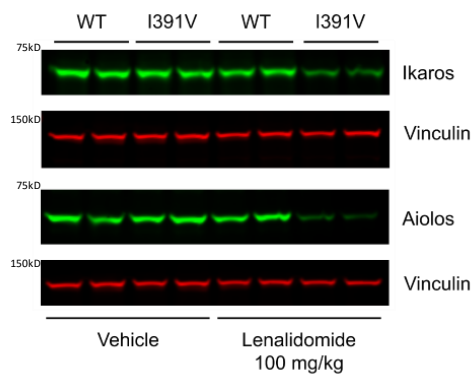
A



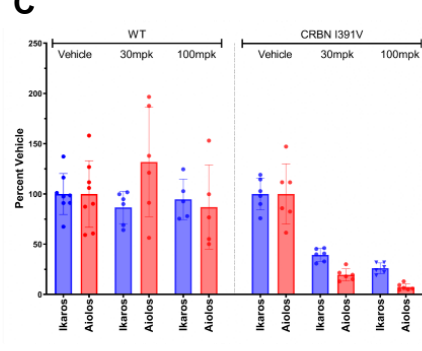
Born pups sequencing validation:



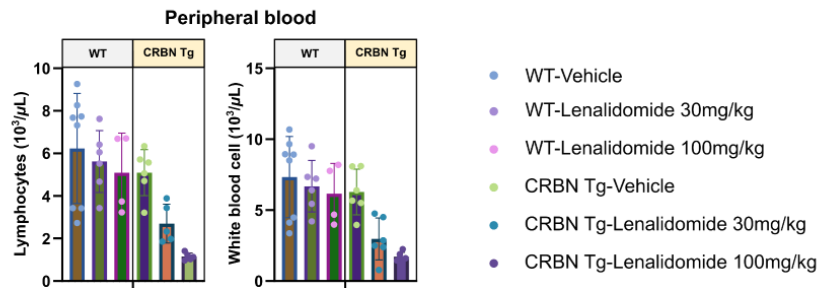
B



C



D



Supplementary Figure 5. (A) HTRF assay curves for compound 1 and 2; (B) and (C) ITC experiments of compound 2 binding to CRBN/DDB1 fitted to a “one set of sites model”

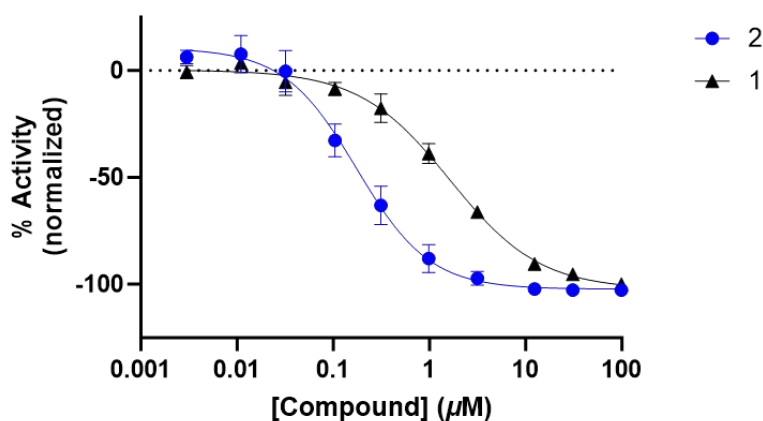
(A) Homogeneous time resolved fluorescence (HTRF) curves for compound 1 and compound 2, to accompany data shown in Figure 3A. Raw data is normalized to DMSO (0% activity) and 10 μM Pomalidomide (-100% Activity). Experiments with compound 2 were performed with $n=4$. Experiments with compound 1 were performed with $n=2$.

(B) Compound 2 (200 μM) titrated into CRBN (40 μM).

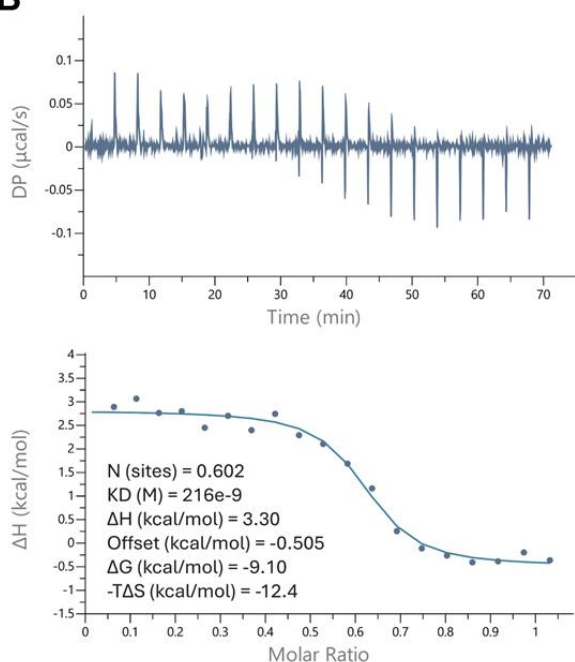
(C) compound 2 (200 μM) titrated into buffer. Experiments were performed with $n=1$.

Related to Figure 3.

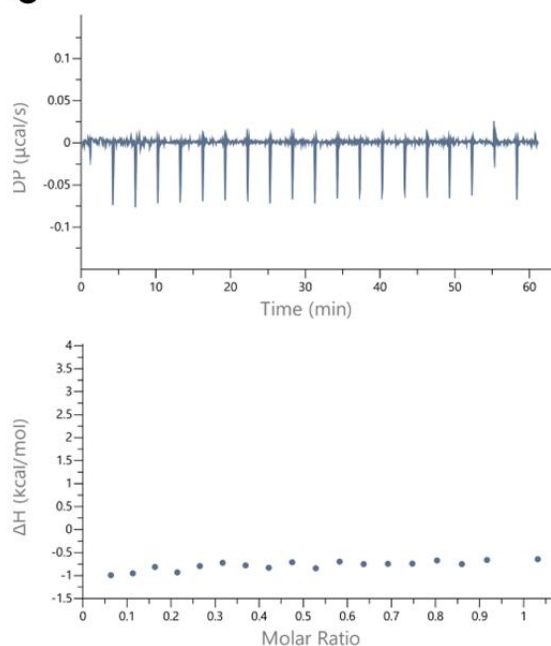
A



B



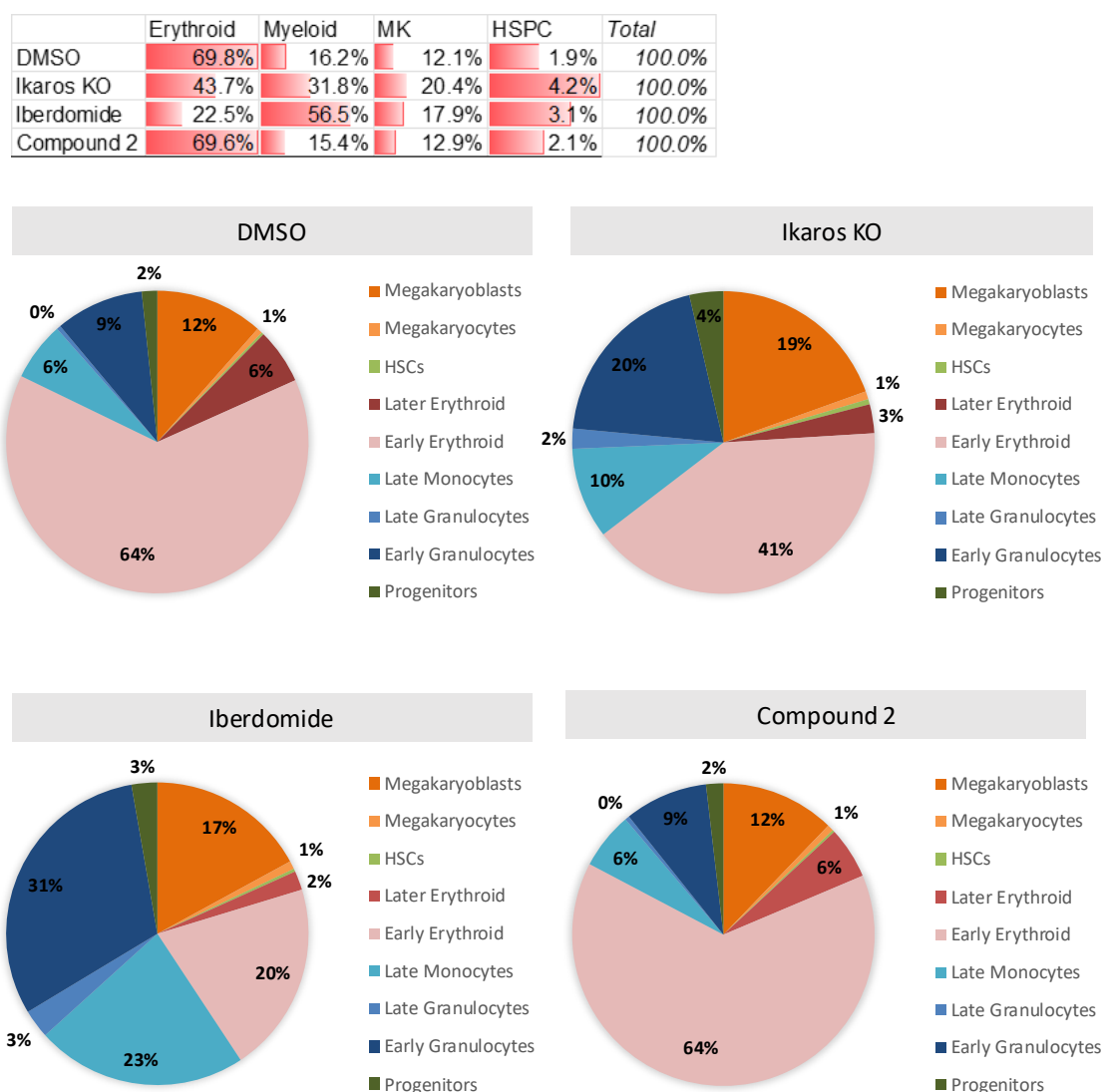
C



Supplementary Figure 6. Ikaros knock-out, Iberdomide and compound 2 treatment on hematopoietic stem and progenitor cell differentiation

Table and pie charts showing distribution of bone-marrow populations of cells upon differentiation into the erythroid lineages in the absence of Ikaros (Ikaros KO), in the presence of 10 μ M Iberdomide or 10 μ M of compound 2 using flow cytometry (Cytek Aurora™ 5L). n=5 technical replicates pooled for the analysis. Cell differentiation assay performance was assessed using two independent primary donors, the figure displays data from only one of the donor and therefore biological replicates is n=1. MK: megakaryocytes, HSPC: hematopoietic stem and progenitor cells.

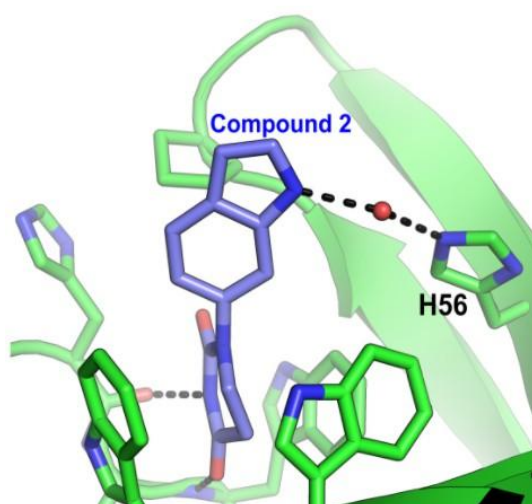
Related to Figure 4.



Supplementary Figure 7. Crystal structure of CRBN bound by compound 2

Structure of compound 2 bound to CRBN with the NH of the indole that makes a water-mediated interaction with H56.

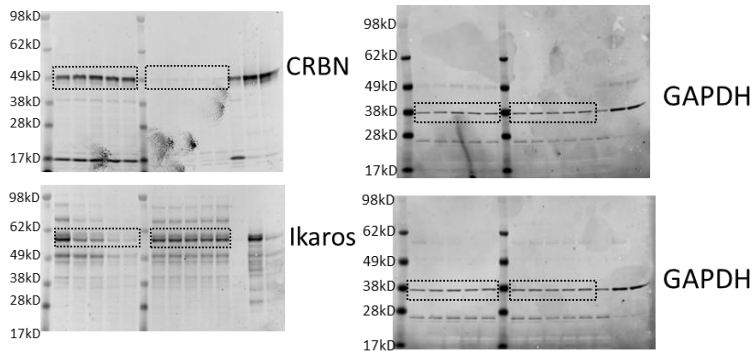
Related to Figure 6.



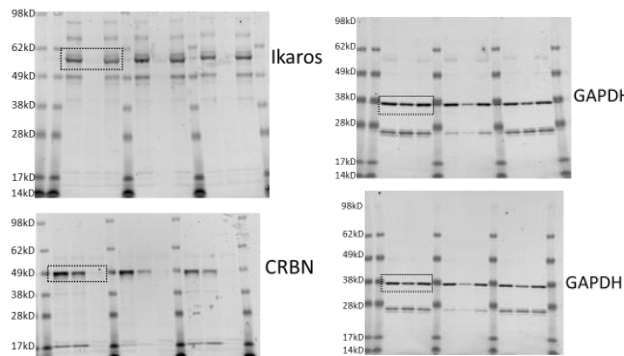
Supplementary Figure 8. Uncropped western blots

Related to Figures 2B, 2D, 3C and 3E.

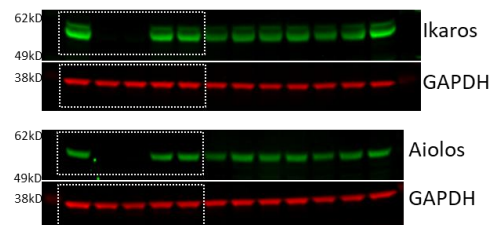
Related to Figure 2B



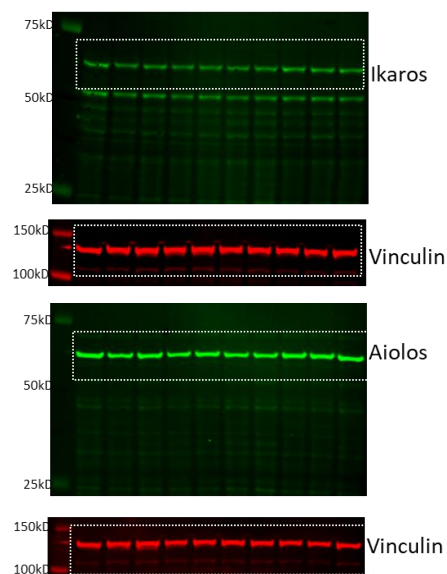
Related to Figure 2D



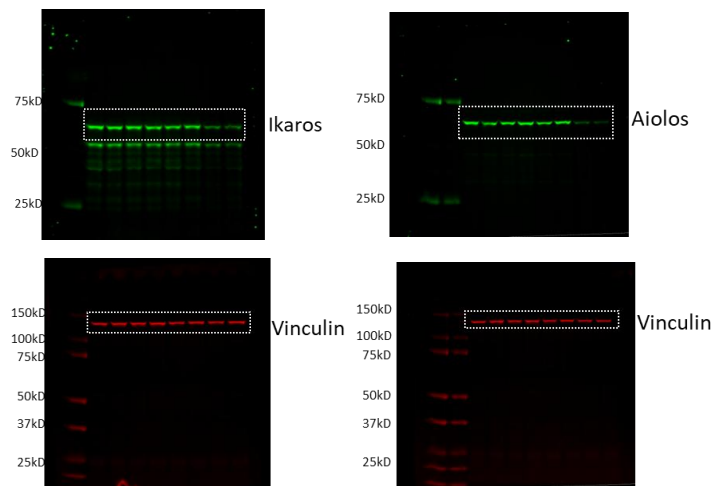
Related to Figure 3C



Related to Figure 3E



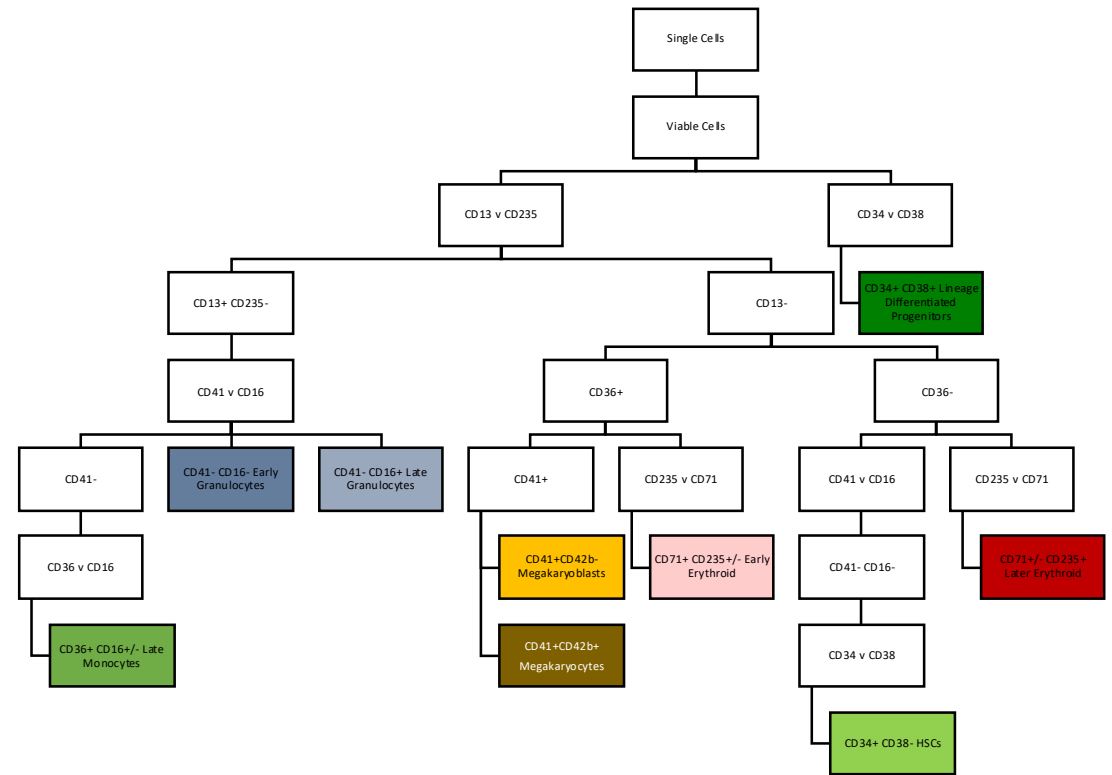
Related to Supplementary Figure 4B



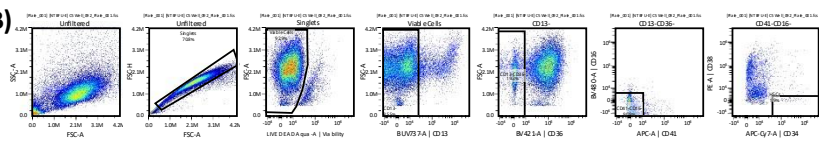
Supplementary Figure 9. Spectral flow cytometry gating strategy

(A) schematic and (B-J) with an illustrative sample. Spectral data was unmixed using single autofluorescence extraction (SpectroFlo software), and gates were defined using fluorescence minus one controls in OMIQ software to assess (B) CD13-CD36-CD41-CD16-CD38-CD34+ HSPC differentiation into (C) CD38+CD34+ lineage differentiated progenitors, (D) CD13-CD36+CD71+ early and (E) CD13-CD36-CD235a+ later erythrocytes, (F) CD13-CD41+CD42b- megakaryoblasts, (G) CD13-CD41+CD42b+ megakaryocytes, (H) CD13+CD235a-CD41-CD16- early and (I) CD13+CD235a-CD41-CD16+ later granulocytes, and (J) CD13+CD235a-CD41-CD36+ later monocytes.

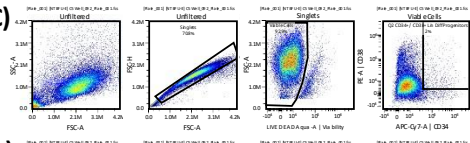
(A)



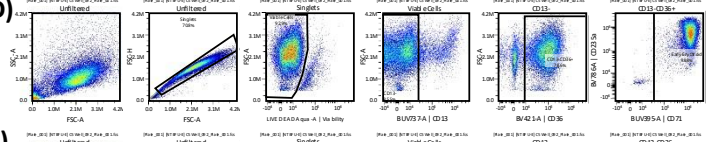
(B)



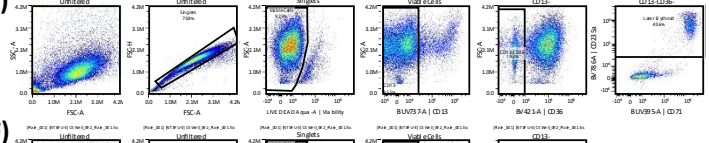
(C)



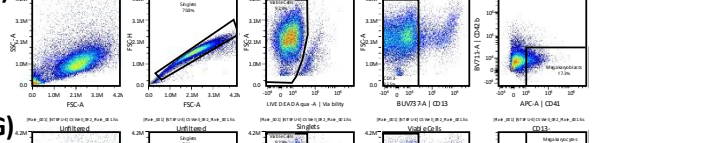
(D)



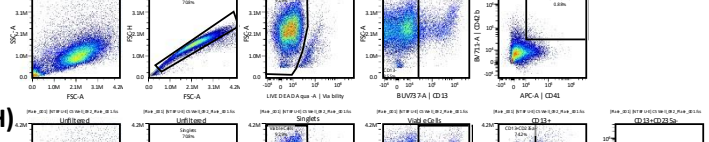
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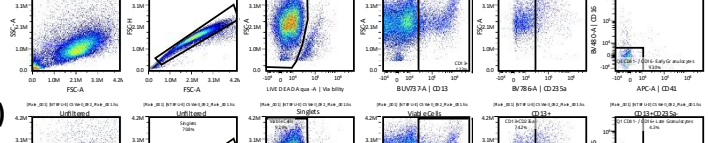
(F)



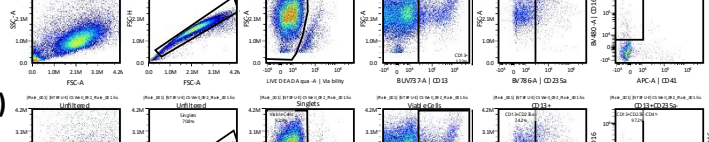
(G)



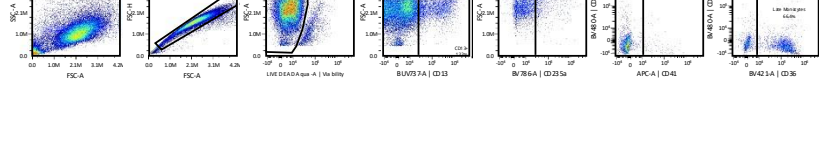
(H)



(I)



(J)



Supplementary note



Ikaros knock-out and Iberdomide treatment affect hematopoietic stem and progenitor cell differentiation

Pie charts showing distribution of 8 populations of cells upon differentiation into either the erythroid (BFU media) or myeloid (GM) lineages in the absence of Ikaros (Ikaros KO) or in the presence of 0.1 μ M Iberdomide (Iberdomide). n=5 technical replicates pooled for the analysis using a different donor than for Supplementary Figure 6 and a BD LSR Fortessa (BD Biosciences). HSC: hematopoietic stem cells.

Sample name	media	Sample_Quality	All Cells/Single Cells	All Cells/Single Cells/Non Viable Cells	All Cells/Single Cells/Viable Cells	% viability
BFU media_CRISP Ikaros	BFU	CRISP Ikaros	26922	2280	24641	0.915273754
BFU media DMSO	BFU	Mock DMSO	24280	1278	23000	0.947281713
BFU media Iber	BFU	Mock Iber	27455	2180	25275	0.920597341
GM media_CRISP Ikaros	GM	CRISP Ikaros	27564	2291	25272	0.916848063
GM media DMSO	GM	Mock DMSO	28069	1729	26338	0.938330543
GM media Iber	GM	Mock Iber	27253	3359	23891	0.876637434

% viability	EdU- Non Proliferating	Megakaryocytes	HSCs	Later Erythroid	Early Erythroid	Late Monocytes	Late Granulocytes	Early Granulocytes	Progenitors	SUM
0.915273754	14809	2259	2188	1847	9500	4282	2200	2907	174	25357
0.947281713	11223	519	882	1267	14128	2957	1224	2514	196	23687
0.920597341	15831	4986	1691	1440	10428	5180	2111	3690	77	29603
0.938548948	14290	4203	1088	1448	12544	4542	2613	2348	48	28834
0.916848063	19253	1170	4028	143	1066	7985	3238	7040	159	24829
0.938330543	20198	284	3009	68	2033	12086	3281	11294	362	32417
0.876637434	19014	1094	2998	59	1583	8738	3366	7001	54	24893
0.881205042	19266	754	2887	57	1837	10622	4745	7631	69	28602

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- 2 Boichenko, I. *et al.* Chemical Ligand Space of Cereblon. *ACS omega* **3**, 11163-11171 (2018). <https://doi.org/10.1021/acsomega.8b00959>
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- 4 Vonrhein, C. *et al.* Data processing and analysis with the autoPROC toolbox. *Acta Crystallogr. D* **67**, 293-302 (2011). <https://doi.org/10.1107/s0907444911007773>
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