Assessing Different E3 Ligases for Small Molecule Induced Protein Ubiquitination and Degradation

Authors.

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Supporting Information

Supplementary Figures

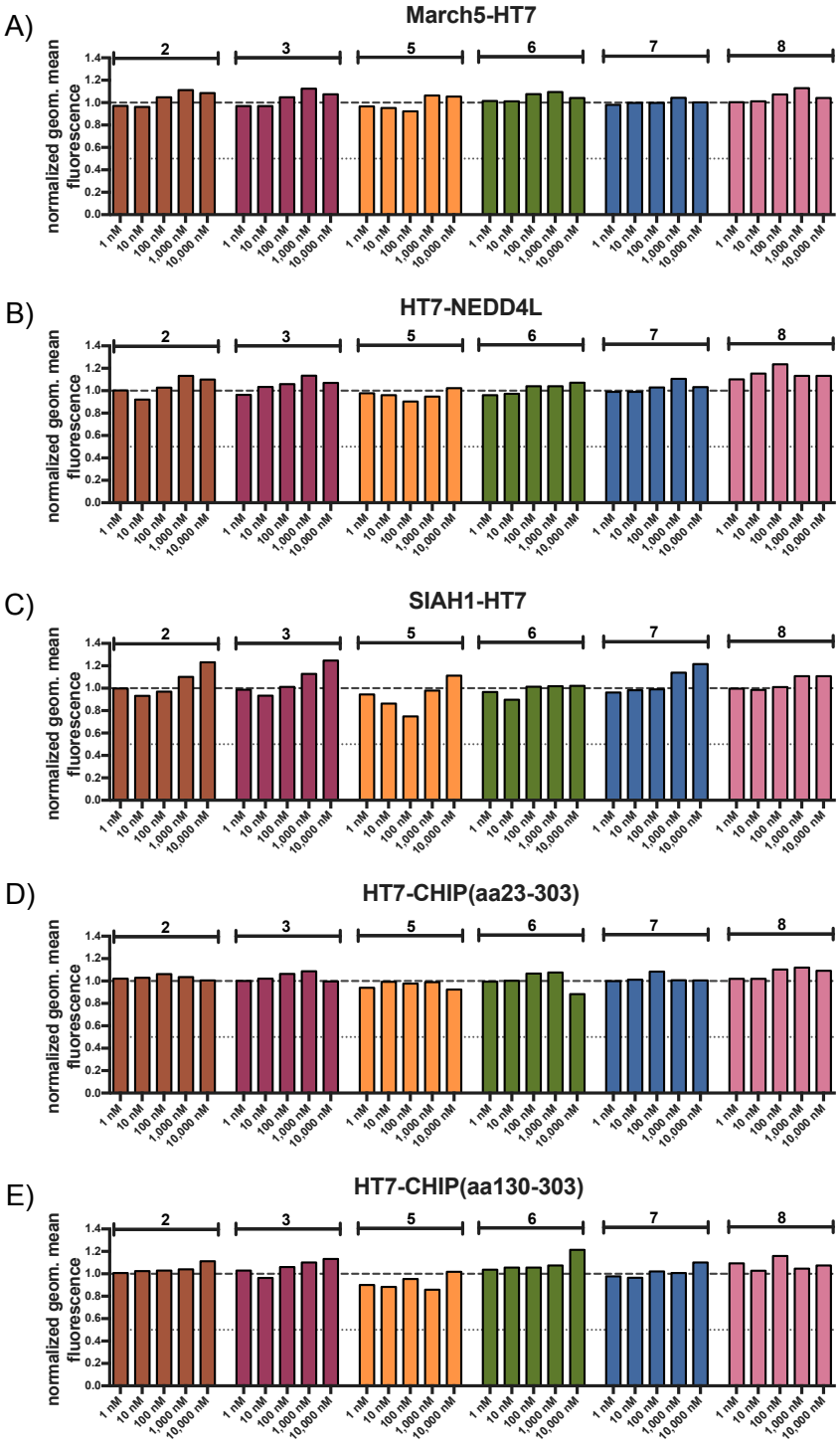


Figure S1. Flow-cytometry screening of E3 ligase constructs. Loss of fluorescence indicates degradation of the reporter substrate EGFP-FKBP. Each bar depicts the geometric mean fluorescence of ≥ 10.000 gated cells.

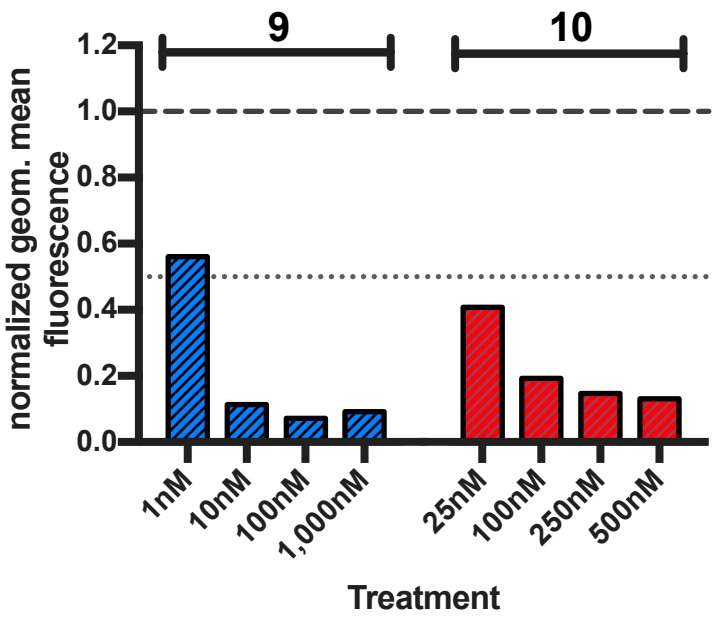


Figure S2. Flow-cytometry screening of PROTAC-recruited, endogenous E3 ligases. Loss of fluorescence indicates degradation of the reporter substrate EGFP-FKBP. Each bar depicts the geometric mean fluorescence of ≥ 10.000 gated cells.

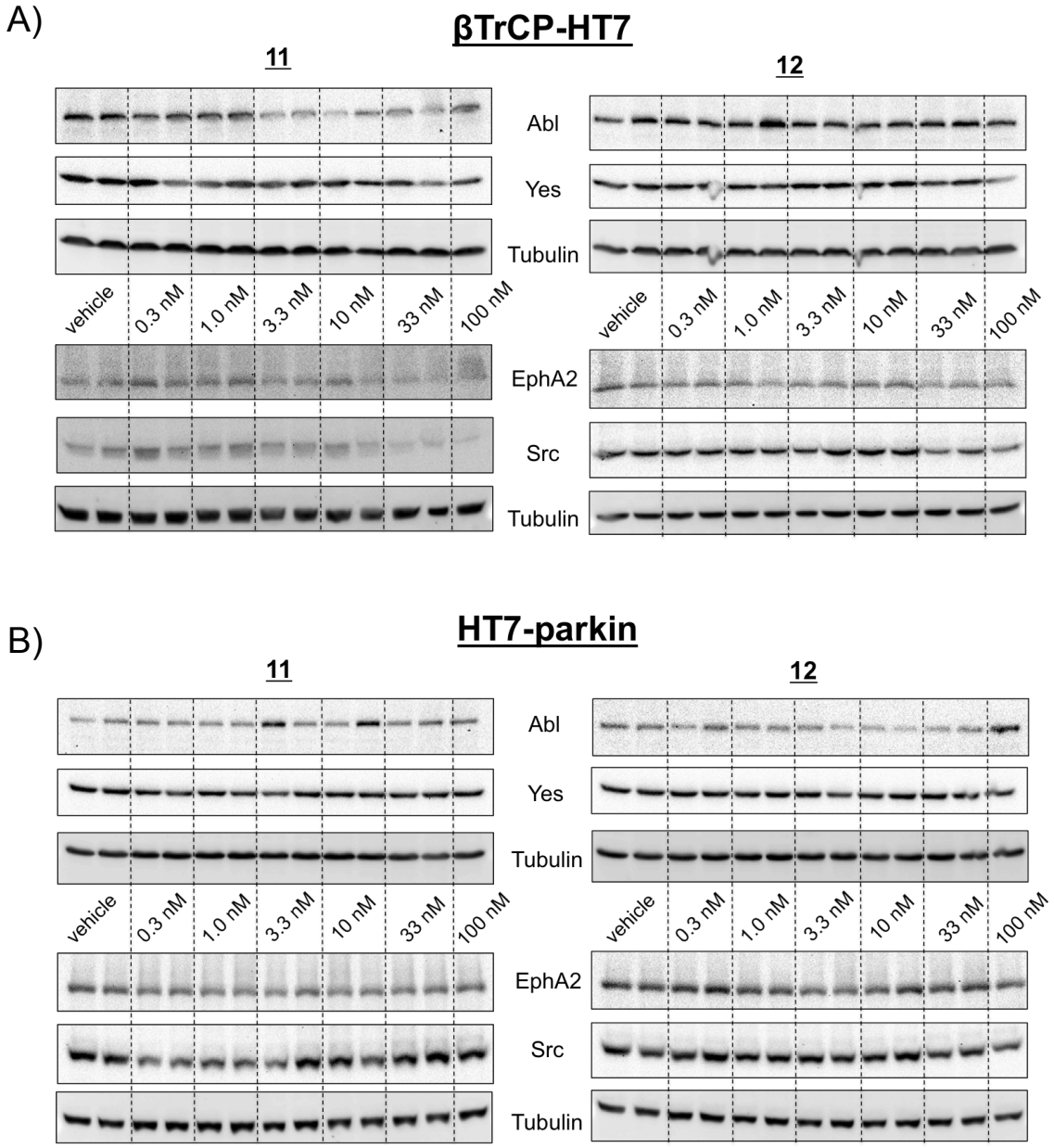


Figure S3. Immunoblots of cells expressing either βTRCP-HT7 or HT7-parkin, treated with dasatinib haloPROTACs **11** and **12**, respectively.

SUPPORTING INFORMATION

Experimental Section: Biology

Molecular Cloning

HaloTag 7 (HT7) was amplified via PCR from a pHTN-vector template (Promega) and terminal double HA-tags, as well as a (-Gly-Gly-Ser-Gly-)$_3$-linker were incorporated into the product. Both, N-terminal and C-terminal constructs of HT7 were each cloned into a pcDNA5/FRT/TO vector (Invitrogen) to create the plasmid-backbone for the insertion of the various E3 ligase constructs. E3 ubiquitin ligase constructs of CHIP, MARCH5, NEDD4L, parkin, and SIAH1 were cloned via PCR-amplification from human foreskin cDNA. The βTrCP template was a gift from Raymond Deshaies (California Institute of Technology). The use of EGFP-FKBP(F36V) expression vector and creation of stable cell lines thereof has been previously described.[1]

HA-tagged ubiquitin in a pRK5 expression vector (Addgene plasmid #17608), as well as the K48R mutant (Addgene plasmid #17604) and the lysine-dead K0 mutant (Addgene plasmid #17603) were gifts from Ted Dawson.[2] Ubiquitin mutants K6R, K11R, K27R, and K27/29R were gifts from Josef Kittler.[3] K33R and K63R mutants were created by site-directed mutagenesis on the pRK5-HA-ubiquitin.

Cell lines

Flp-In TREx 293 cells (Invitrogen)[4] were cultured in DMEM, supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO_2. Cells stably and constitutively expressing EGFP-FKBP(F36V)[1] were transfected with the respective HaloTag–E3 ligase construct in pcDNA5/FRT/TO vector (Invitrogen) together with a pOG44 Flp-Recombinase Expression vector (Invitrogen) in a 1:10 ratio, using Lipofectamine 2000 (Invitrogen) transfection reagent. Following transfection, Flp-In TREx 293 cells with pcDNA5/FRT/TO stably integrated into the recombination site were selected using 300 µg/mL hygromycin. Induction of HaloTag–E3 expression was achieved by supplementing the media with 1 µg/mL doxycycline and was tested by immunoblotting for the HA-tagged construct.

Flow Cytometry

Flow cytometry experiments were carried out as described before.[5, 6] Briefly, Flp-In TREx 293 cells stably expressing EGFP-FKBP and inducibly expressing one of the E3-HT7 fusion proteins, were subjected to treatment with PROTACs or haloPROTACs for 24 h. Cells were detached with trypsin solution and resuspended in DMEM. EGFP-conferred fluorescence was analyzed using a FACSCalibur flow cytometer (BD Biosciences). Results were quantified for mean geometric fluorescence using FlowJo software (FlowJo, LLC). All fluorescence signals were normalized to a vehicle (DMSO) only control, which was set to 1.0. Resulting data were plotted using Prism software (GraphPad Software, Inc).

Ubiquitin Assay

For the purification of ubiquitinated EGFP-FKBP a protocol by Choo *et al*.[7] was modified. Cells were seeded and grown to confluency in the presence of 1 µg/mL doxycycline and were treated with PROTAC or mock control for 2 h (if not indicated otherwise). Subsequently, the cells were washed with chilled PBS and were lysed by the addition of ubiquitin lysis buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 2% SDS; 2 mM sodium orthovanidate; 50 mM NaF; 1 mM iodo-acetamide; 10 mM N-methylmaleimide; 50 µM PR-619 (Abcam); 5 mM dithiothreitol (DTT); 1 x cOmplete EDTA-free protease inhibitor cocktail (Sigma-Aldrich)). The lysate was transferred into a microcentrifuge tube and immediately boiled at 95 °C for 10 minutes to ensure inactivation of any deubiqutinases. Following a rapid cool-down on ice, the lysates were subjected to one second of sonication by a Branson Sonifyer 450, equipped with a microtip and set to constant output at 50% strength. Samples then were diluted 1:10 with dilution buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 2 mM EDTA; 1% Triton-X 100) and were rotated at 4 °C for 60 minutes. Subsequently, samples were centrifuged at 20,000 x g for 30 minutes, the supernatant was transferred into a fresh vial and protein concentrations were determined using a BCA-assay. Volumes of the diluted lysate supernatants were adjusted to yield equal amounts of protein (300 – 1,500 µg) for each set of samples. 2 µg of mouse anti-GFP antibody (Santa Cruz; sc-9996) were added and samples were rotated at 4 °C over night. Next day, 10 µL of dry bed volume of Protein G Sepharose Fast Flow (Sigma-Aldrich) beads were added and the samples were incubated rotating for 2,5 h at 4 °C. Following the incubation, beads were pelleted by centrifugation (1,000 x g for 5 min), washed once with 50 mM Tris, pH 8.0; 1 M NaCl; 1 mM EDTA; 1% Nonidet P-40; and, subsequently, twice with 50 mM Tris, pH 8.0; 150 mM NaCl.

Finally, beads were pelleted (1,000 x g for 5 min), all wash buffer was carefully removed and beads were resuspended in 20 µL of 1 x Laemmli sample buffer and boiled at 95 °C for 10 minutes. After centrifugation (5,000 x g for 5 min), the supernatant, containing the eluted protein in loading buffer was directly subjected to SDS-PAGE and subsequent immunoblotting.

For proteomic analyses, the final elution step – following the last wash – was replaced by the addition of one bed-volume of 0.1 M glycine (pH 2.0) to the beads. After incubation for 1 minute, beads were pelleted (1,000 x g for 5 min) and the supernatant was transferred into a fresh microcentrifuge tube and 5% (v/v) of 1.5 M Tris (pH 9.2) was added to neutralize the eluate. This extraction was repeated three times and eluates were pooled.

Immunoblotting

If not indicated otherwise, cells were seeded and grown to 80% confluency in the presence of 1 µg/mL doxycycline and were treated with PROTAC or control for 24 h. Subsequently, the cells were washed with chilled PBS and were lysed by the addition of lysis buffer (20 mM Tris, pH 7.5; 150 mM NaCl; 1% Triton-X 100; 1 x cOmplete EDTA-free protease inhibitor cocktail (Sigma-Aldrich)), followed by centrifugation at 16,000 x g for 10 minutes. Protein concentration of supernatants were determined via BCA-assay before addition of Laemmli sample buffer and boiling at 95 °C for 10 minutes. Equal amounts of protein were subjected to SDS-PAGE and subsequent electrophoretic transfer onto nitrocellulose membrane. Rabbit antibodies were purchased from Cell Signaling: HA-Tag (3724), ubiquitin (3933), EphA2 (6997), SRC (2123). Mouse antibodies were purchased from Santa Cruz: ABL (sc-23), YES (sc-48396). The tubulin antibody was coupled to Alexafluor488 and purchased from Millipore (16-232).

Proteomics

Briefly, IP elution in 100 mM glycine buffer (pH 7.5), containing approximately 10 µg of protein, as determined by NanoDrop (Peqlab), was dried down via SpeedVac, then reconstituted in 10 µL 8 M urea containing 0.4 M ammonium bicarbonate. To this, 1 µL 45 mM DTT was added to reduce the disulfide bonds at 37 ºC for 30 min and then 1 µL of 100 mM iodoacetamide (IAN) was added to alkylate the free sulfhydryl at room temperature for 30 minutes. Then 27 µL of water and 1 uL of a 0.5 µg/µL trypsin (Promega Inc.) solution was added and incubated at 37 ºC overnight for the enzyme digestion to take place. The digest solution was then desalted with a

Microspin (The Nest Group Inc.) and eluted peptides were dried and stored at -80 ºC until further analyzed. Dried peptides were reconstituted in Buffer A (100% water, 0.1% formic acid, and injected onto an Orbitrap Fusion (Thermo Fisher Scientific) LC MS/MS system equipped with a Waters nanoAcquity UPLC system, and uses a Waters Symmetry® C18 180 µm x 20 mm trap column and a 1.7 µm, 75 µm x 250 mm nanoAcquity™ UPLC™ column (37 ºC) for peptide separation. Trapping was done at 5 µl/min, 99% Buffer A for 3 minutes. Peptide separation was performed with a linear gradient over 140 minutes at a flow rate of 300 nL/min.

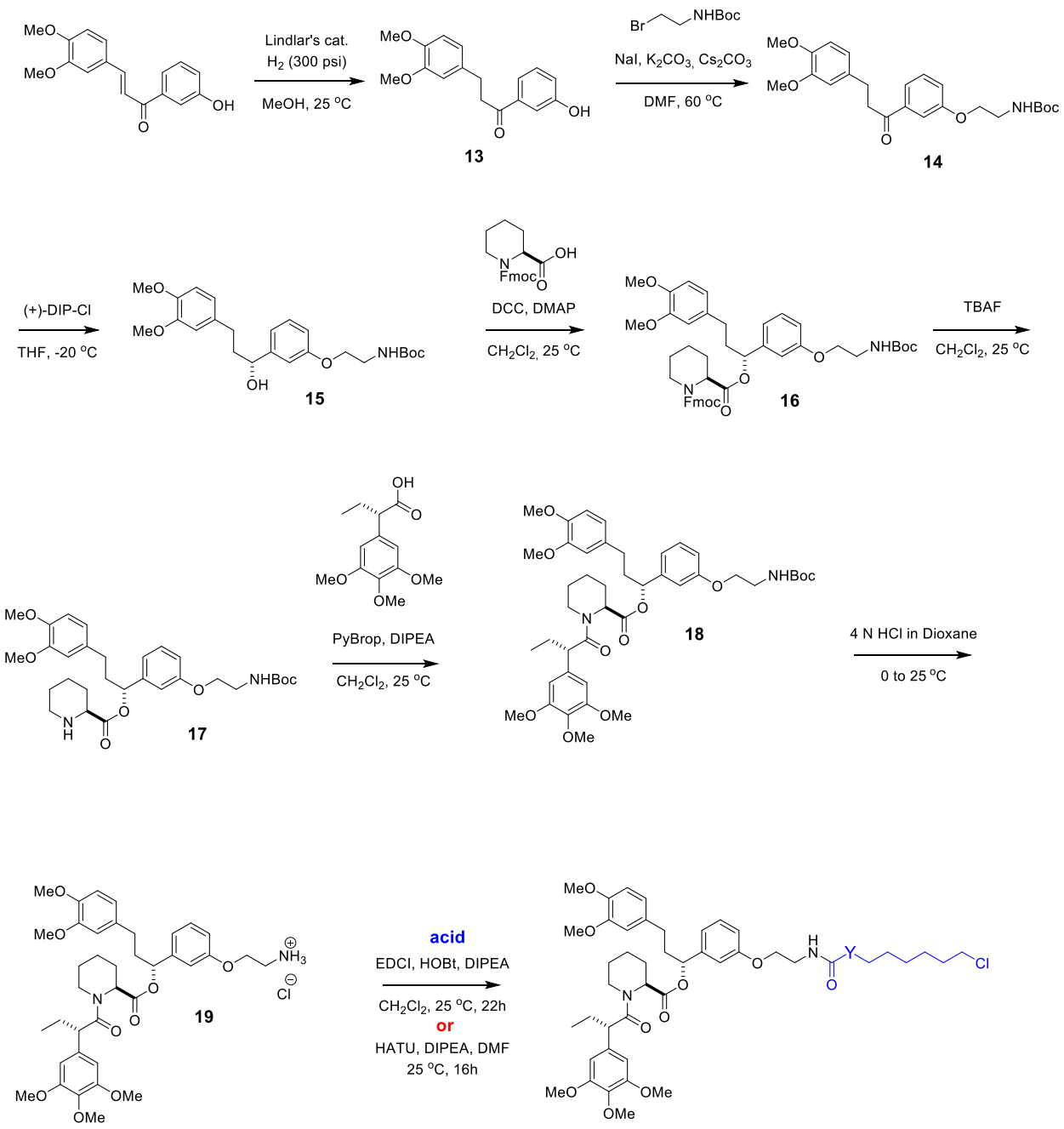
Precursor mass scans (300 to 1500 m/z range, target value 3E6, maximum ion injection times 45 ms) were acquired and followed by Higher energy Collisional Dissociation (HCD) based fragmentation (normalised collision energy 28). A resolution of 70,000 at m/z 200 was used for this MS1 scans, and up to 20 dynamically chosen, most abundant, precursor ions were fragmented (isolation window 1.7 m/z). The tandem MS/MS scans were acquired at a resolution of 17,500 at m/z 200 (target value 1E5, maximum ion injection times 100 ms).

Collected MS data were analyzed utilizing Mascot Distiller for peak picking, Mascot Search Engine for protein database search to identify proteins and their ubiquitinated site of modification on Lysine residue (via the added mass of a -GlyGly moiety).

Experimental Section: Chemistry

General comments

Unless otherwise indicated, common reagents or materials were obtained from commercial sources and used without further purification. Tetrahydrofuran (THF), Dimethylformamide (DMF) and Dichloromethane (CH_2Cl_2) were dried by a PureSolv^{TM} solvent drying system. Flash column chromatography was performed using silica gel 60 (230-400 mesh). Analytical thin layer chromatography (TLC) was carried out on Merck silica gel plates with QF-254 indicator and visualized by UV or $KMnO_4$. ^1H and ^{13}C NMR spectra were recorded on an Agilent DD_2 500 (500 MHz ^1H; 125 MHz ^{13}C) or Agilent DD_2 600 (600 MHz ^1H; 150 MHz ^{13}C) or Agilent DD_2 400 (400 MHz ^1H; 101 MHz ^{13}C) spectrometer at room temperature. Chemical shifts were reported in ppm relative to the residual $CDCl_3$ (δ 7.26 ppm ^1H; δ 77.00 ppm ^{13}C), CD_3OD (δ 4.87 ppm ^1H; δ 49.00 ppm ^{13}C), or d^6-DMSO (δ 2.50 ppm ^1H; δ 39.52 ppm ^{13}C). NMR chemical shifts were expressed in ppm relative to internal solvent peaks, and coupling constants were measured in Hz. (bs = broad signal). Only peaks of the major rotamer are reported. Mass spectra were obtained using electrospray ionization (ESI) on a time of flight (TOF) mass spectrometer. Analytical HPLC analyses were carried out on 250 x 4.6 mm C-18 column using gradient conditions (10 – 100% B, flow rate = 1.0 mL/min, 20 min). Preparative HPLC was carried out on 250 x 21.2 mm C-18 column using gradient conditions (10 – 100% B, flow rate = 10.0 mL/min, 20 min). The eluents used were: solvent A (H_2O with 0.1% TFA) and solvent B (CH_3CN with 0.1% TFA).

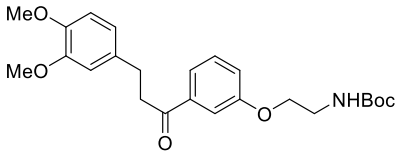


Supplementary Scheme 1. Synthesis of Ariad-ligand chloroalkanes.

Compound **13** was prepared according a previously reported procedure.[8]

The corresponding acid derivatives were synthesized according to a procedure previously described by Lai A. *et. al.*[9]

Synthesis of *tert*-butyl (2-(3-(3-(3,4-dimethoxyphenyl)propanoyl)phenoxy)ethyl)carbamate (**14**)

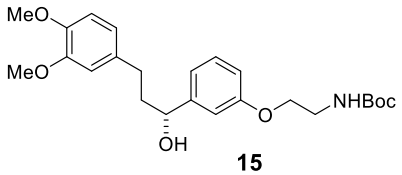


14
Chemical Formula: $C_{24}H_{31}NO_6$
Exact Mass: 429.2151
Molecular Weight: 429.5130

To a solution of **13** (330 mg, 1.2 mmol) and 1-*N*-Boc-2-bromoethylamine (312 mg, 1.4 mmol) in DMF (2.3 mL) were added NaI (8.7 mg, 0.058 mmol), K_2CO_3 (481 mg, 3.5 mmol), and Cs_2CO_3 (378 mg, 1.2 mmol) at 25 ^oC. The mixture was heated to 60 ^oC and then stirred for 14 h. After the reaction, the mixture was cooled to room temperature, poured into water and then extracted with ethyl acetate (x3). The combined organic layer was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. The crude residue was purified by column chromatography to afford **14** as brown oil (500 mg, quant).

MS (APCI) m/z: 430.5 $[M+H]^+$

Synthesis of (*R*)-*tert*-butyl (2-(3-(3-(3,4-dimethoxyphenyl)-1-hydroxypropyl)phenoxy)ethyl)carbamate (**15**)

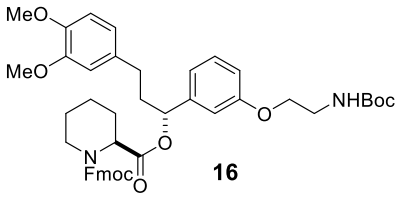


15
Chemical Formula: $C_{24}H_{33}NO_6$
Exact Mass: 431.2308
Molecular Weight: 431.5290

To a solution of **14** (500 mg, 1.2 mmol) in dry THF (1.2 mL) was added a solution of (+)-DIP-Cl (566 mg, 1.8 mmol) in dry THF (1.3 mL) at -20 ^oC. The mixture was stirred at -20 ^oC for 24 h, and then concentrated under reduced pressure. The resulting mixture was diluted with diethyl ether (0.4 mL), and diethanolamine (1.0 mL) was added to the solution at 25 ^oC. The mixture was stirred at room temperature for 6 h and then filtered through celite, and washed with ethyl acetate. The filtrate was concentrated under reduced pressure and then the crude residue was purified by column chromatography to afford **15** as a colorless oil (417 mg, 82 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 (t, J = 5.0 Hz, 1H), 6.92 (d, J = 9.9 Hz, 1H), 6.91 (s, 1H), 6.81-6.77 (m, 2H), 6.72 (d, J = 10.0 Hz, 1H), 6.71 (s, 1H), 5.01 (brs, 1H), 4.65 (dd, J = 8.5, 5.2 Hz, 1H), 4.01 (t, J = 5.1 Hz, 2H), 3.52-3.51 (m, 2H), 3.85 (s, 3H), 3.86 (s, 3H), 2.70-2.60 (m, 2H), 2.10-1.94 (m, 3H), 1.45 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 158.4, 155.7, 148.5, 146.9, 146.6, 134.3, 129.2, 120.0, 118.4, 113.0, 111.8, 111.6, 111.1, 79.2, 73.1, 66.7, 60.2, 55.6, 55.5, 40.5, 39.8, 31.4, 28.1(3). **MS** (APCI) m/z: 432.5 $[M+H]^+$

Synthesis of (*S*)-1-((9*H*-fluoren-9-yl)methyl)-2-((*R*)-1-(3-(2-((*tert*-butoxycarbonyl)amino)ethoxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl) piperidine-1,2-dicarboxylate (**16**)



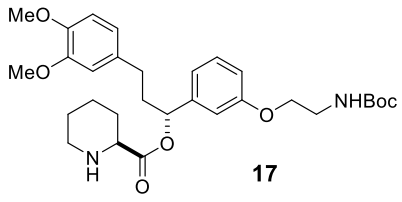
16

Chemical Formula: $C_{45}H_{52}N_2O_9$
Exact Mass: 764.3673
Molecular Weight: 764.9160

To a solution of **14** (380 mg, 0.88 mmol) and Fmoc-L-pipecolic acid (340 mg, 0.97 mmol) in CH_2Cl_2 (3.5 mL) were added DMAP (12 mg, 0.097 mmol) and DCC (200 mg, 0.97 mmol) at room temperature. The mixture was stirred for 12 h, and then concentrated under reduced pressure. The mixture was dissolved in diethyl ether, and filtered through celite. The filtrate was concentrated and then purified by column chromatography to afford **16** as a white foam (591mg, 88 %).

^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.70 (d, J = 2.1 Hz, 1H), 7.67 (d, J = 18.3 Hz, 1H), 7.53 (t, J = 5.6 Hz, 1H), 7.50-7.35 (m, 3H), 7.33-7.28 (m, 1H), 7.25-7.12 (m, 2H), 6.84 (d, J = 7.6 Hz, 1H), 6.77 (s, 1H), 6.70 (dd, J = 31.4, 8.2 Hz, 1H), 6.67 (d, J = 8.2 Hz, 1H), 6.58-6.61 (m, 1H), 5.68-5.67 (m, 1H), 4.96-4.95 (m, 1H), 4.49-4.32 (m, 2H), 4.27-4.25 (m, 1H), 4.20-4.01 (m, 2H), 4.00 (m, 1H), 3.43 (brs, 1H), 3.36 (brs, 1H), 3.17-3.12 (m, 1H), 3.05-2.95 (m, 1H), 2.64-2.41 (m, 2H), 2.34-2.28 (m, 1H), 2.26-2.17 (m, 1H), 2.06-1.98 (m, 1H), 1.75-1.54 (m, 4H), 1.45 (s, 9H). **^{13}C NMR** (125 MHz, $CDCl_3$) δ = 171.0. 158.7, 156.4, 156.0, 148.9, 147.4, 144.1, 143.9, 141.8, 141.6, 141.3, 133.5, 129.8(2), 127.7(2), 127.1(3), 125.1(2), 120.1, 119.3, 113.8, 113.1, 111.7, 111.3, 79.5, 76.3, 67.8, 67.1, 55.9, 55.8, 47.2, 41.9, 40.1, 38.1, 31.2, 28.4(3), 26.9, 24.8, 20.9. **MS** (APCI) m/z: 765.7 $[M+H]^+$

Synthesis of (*S*)-(*R*)-1-(3-(2-((*tert*-butoxycarbonyl)amino)ethoxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl-piperidine-2-carboxylate (**17**)



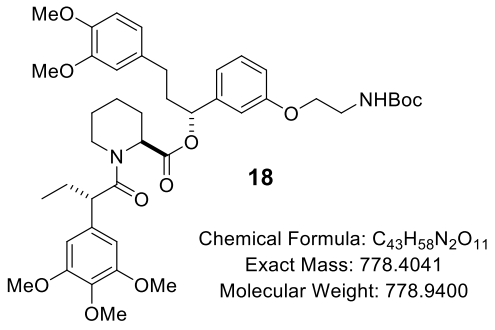
17

Chemical Formula: $C_{30}H_{42}N_2O_7$
Exact Mass: 542.2992
Molecular Weight: 542.6730

To a solution of **16** (300 mg, 0.4 mmol) in CH_2Cl_2 (8.0 mL) was added piperidine (0.4 mL) at 0 ^oC. The mixture was stirred at room temperature for 14 h. After the reaction, the resulting mixture was concentrated under reduced pressure. The crude residue was purified by column chromatography to afford **17** as a colorless oil (217 mg, 69 %).

^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.25-7.22 (m, 1), 6.93 (d, J = 7.7 Hz, 1H), 6.86 (s, 1H), 6.82-6.76 (m, 3H), 6.68-6.62 (m, 2H), 5.76 (t, J = 2.3 Hz, 1H), 5.00 (brs, 1H), 4.01-3.99 (m, 2H), 3.57-3.55 (m, 1H), 3.56-3.51 (m, 2H), 3.03-2.98 (m, 1H), 2.78-2.70 (m, 1H), 2.61-2.52 (m, 2H), 2.27-2.19 (m, 1H), 2.09-1.95 (m, 1H), 1.93-1.79 (m, 2H), 1. 75-1.60 (m, 4H), 1.44 (s, 3H). **^{13}C NMR** (125 MHz, $CDCl_3$) δ = 172.7, 158.4, 155.63, 148.6, 147.1, 141.9, 133.4, 129.3, 119.9, 118.9, 113.3, 112.7, 111.5, 111.1, 79.3, 16.7, 66.9, 63.2, 55.7, 55.6, 48.4, 39.8, 37.8, 31.2, 28.1(3), 25.2, 22.2, 14.9. **MS** (APCI) m/z: 543.7 $[M+H]^+$

Synthesis of (*S*)-(*R*)-1-(3-(2-((*tert*-butoxycarbonyl)amino)ethoxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl-1-((*S*)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate (**18**)



18

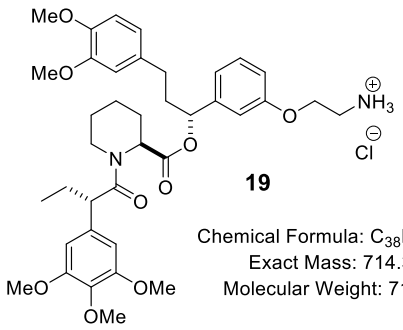
Chemical Formula: $C_{43}H_{58}N_2O_{11}$
Exact Mass: 778.4041
Molecular Weight: 778.9400

To a solution of **17** (150 mg, 0.28 mmol) and (*S*)-2-)3,4,5-trimethoxyphenyl)butyric acid (141 mg, 0.55 mmol) in CH_2Cl_2 (2.8 mL) was added PyBrop (258 mg, 0.55 mmol) and DIPEA

(0.24 mL, 1.4 mmol) at room temperature. The mixture was stirred for 18 h. After the reaction was complete, the resulting mixture was diluted with ethyl acetate, washed with sat. NH_4Cl (aq), sat. $NaHCO_3$ (aq), brine, and then dried over $MgSO_4$. The crude residue was purified by column chromatography to afford **18** as a colorless oil (118 mg, 55 %).

MS (APCI) m/z: 779.6 $[M+H]^+$

Synthesis of (*S*)-(*R*)-1-(3-(2-aminoethoxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl-1-((*S*)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate.HCl (**19**)



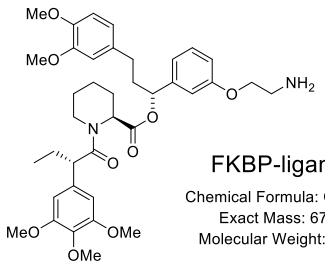
19

Chemical Formula: $C_{38}H_{51}ClN_2O_9$
Exact Mass: 714.3283
Molecular Weight: 715.2810

To a solution of **18** (107 mg, 0.14 mmol) in CH_2Cl_2 (0.7 mL) was added 4*N* HCl (dioxane, 0.45 mL, 1.8 mmol) at 0 ^oC. The mixture was warmed to room temperature and stirred for 2.5 h. After the reaction, the mixture was concentrated under reduce pressure. Compound **19** was used in the next step without further purification. (HCl salt, 107 mg, quant).

^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.09 (t, J = 7.9 Hz, 1H), 6.72-6.70 (m, 3H), 6.57 (d, J = 3.1 Hz, 1H), 6.57 (s, 1H), 6.53 (d, J = 7.7 Hz, 1H), 6.34 (s, 2H), 5.54 (t, J = 2.3 Hz, 1H), 5.40 (d, J = 4.6 Hz, 1H), 4.69-4.57 (m, 1H), 3.93-3.90 (m, 2H), 3.92-3.90 (m, 11H), 3.78 (s, 6H), 3,63 (t, J = 1.7 Hz, 1H), 3.03-3.00 (m, 2H), 2.84-2.73 (m, 1H), 2.61-2.39 (m, 2H), 2.32-2.22 (m, 1H), 2.14-1.99 (m, 2H), 1.98-1.87 (m, 1H), 1.72-1.60 (m, 6H), 0/83 (t, J = 7.3 Hz, 3H). **^{13}C NMR** (125 MHz, $CDCl_3$) δ = 172.6, 170.6, 158.9, 153.2(2), 148.9, 147.3, 141.9, 136.7, 135.4, 133.5, 129.5, 1201., 118.6, 113.8, 112.7, 111.7, 111.3, 105.0(2), 76.9, 70.1, 60.7, 56.3, 56.0(4), 51.2, 43.4, 41.5, 38.1, 30.1, 26.8, 25.4, 22.7, 18.4, 14.1. **MS** (APCI) m/z: 678.7 $[M+H]^+$

Synthesis of (*S*)-(*R*)-1-(3-(2-aminoethoxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl 1-((S)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate (FKBP-ligand **1**)



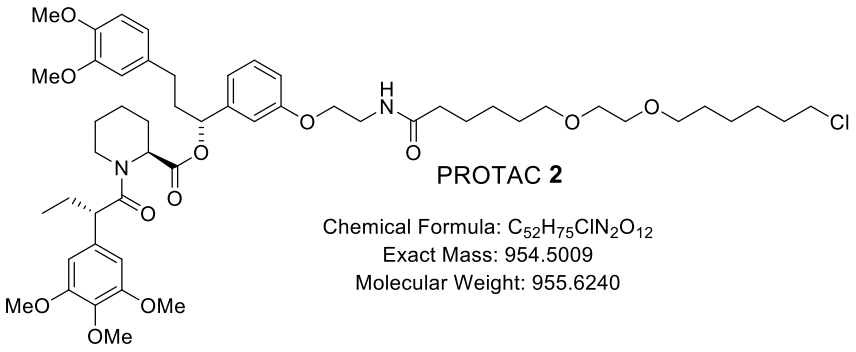
FKBP-ligand **1**

Chemical Formula: $C_{38}H_{50}N_2O_9$
Exact Mass: 678.3516
Molecular Weight: 678.8230

A solution of **18** (30 mg, 0.04 mmol) in a mixture of CH_2Cl_2 (1 mL) and TFA (0.5 mL) was stirred at room temperature for 2 h. The reaction mixture was concentrated under reduce pressure and the crude purified by Prep TLC (DCM/MeOH/ammonia: 60/10/1, v/v/v) to give 21.4 mg, 79% of FKBP-ligand **1** as a yellow oil.

LC/MS $[M+ H]^+$ for $C_{38}H_{51}N_2O_9$ calculated: 679.4; found: 679.2 $[M+ H]^+$.

Synthesis of (*S*)-(*R*)-1-(3-(2-(6-(2-((6-chlorohexyl)oxy)ethoxy)hexanamido)ethoxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl 1-((*S*)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate (PROTAC **2**)



PROTAC **2**

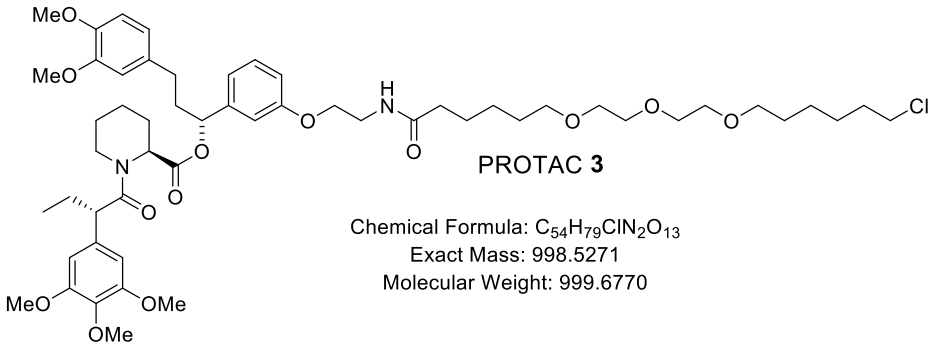
Chemical Formula: $C_{52}H_{75}ClN_2O_{12}$
Exact Mass: 954.5009
Molecular Weight: 955.6240

To a solution of 6-[2-(6-chlorohexoxy)ethoxy]hexanoic acid (5.53 mg, 0.02 mmol) in DMF (1 mL) was added HATU (11.9 mg, 0.031 mmol) and the resulting solution stirred for 10 min. at room temperature after which **19** (10.62 mg, 0.02 mmol) and DIEA (0.013 mL, 0.078 mmol) were added respectively. The resulting mixture was stirred at room temperature for 16 h. The product was extracted with ethyl acetate (2x). The combined organic layer was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. The crude residue was purified by prep TLC (DCM/MeOH 90:10) to yield 6.7 mg (44.8%) of PROTAC **2** as colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.15 (t, *J* = 7.9 Hz, 1H), 6.96 – 6.72 (m, 3H), 6.71 – 6.59 (m, 4H), 6.40 (d, *J* = 6.2 Hz, 2H), 6.27 – 6.06 (m, 1H), 5.60 (dd, *J* = 8.2, 5.5 Hz, 1H), 5.45 (d, *J* = 5.6 Hz, 1H), 4.01 (h, *J* = 6.9, 6.0 Hz, 2H), 3.86 – 3.82 (m, 8H), 3.78 (s, 3H), 3.74 – 3.61 (m, 14H),

3.52 (d, *J* = 12.7 Hz, 6H), 3.43 (q, *J* = 6.2 Hz, 4H), 2.50 (dddd, *J* = 48.1, 14.7, 9.7, 6.7 Hz, 2H), 2.33 – 2.18 (m, 4H), 2.12 – 1.89 (m, 3H), 1.79 – 1.56 (m, 10H), 1.47 – 1.23 (m, 6H), 0.96 – 0.79 (m, 3H). **LC/M**S [M+ H]$^+$ for $C_{52}H_{76}ClN_2O_{12}$ calculated: 956.6; found: 956.3 [M+ H]$^+$.

Synthesis of (*S*)-(*R*)-1-(3-((22-chloro-4-oxo-10,13,16-trioxa-3-azadocosyl)oxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl 1-((*S*)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate (PROTAC **3**)



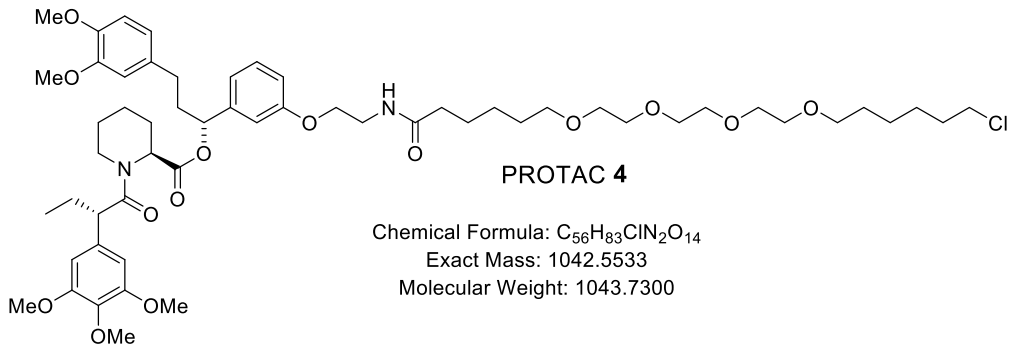
PROTAC **3**

Chemical Formula: $C_{54}H_{79}ClN_2O_{13}$
Exact Mass: 998.5271
Molecular Weight: 999.6770

To a solution of 6-[2-[2-(6-chlorohexoxy)ethoxy]ethoxy]hexanoic acid (6.36 mg, 0.02 mmol) in DMF (1 mL) was added HATU (11.9 mg, 0.031 mmol) and the resulting solution stirred for 10 min. at room temperature after which **19** (10.62 mg, 0.02 mmol) and DIEA (0.013 mL, 0.078 mmol) were added respectively. The resulting mixture was stirred at room temperature for 16 h. The product was extracted with ethyl acetate (2x). The combined organic layer was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. The crude residue was purified by prep TLC (DCM/MeOH 90:10) to yield (9.2 mg, 52%) of PROTAC **3** as colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.15 (t, *J* = 7.9 Hz, 1H), 6.97 – 6.72 (m, 3H), 6.71 – 6.59 (m, 4H), 6.40 (d, *J* = 4.5 Hz, 2H), 6.17 (dt, *J* = 40.9, 5.8 Hz, 1H), 5.60 (dd, *J* = 8.2, 5.5 Hz, 1H), 5.46 (d, *J* = 5.4 Hz, 1H), 4.02 (dt, *J* = 11.0, 5.1 Hz, 2H), 3.91 – 3.81 (m, 9H), 3.78 (s, 2H), 3.74 – 3.60 (m, 17H), 3.55 (ddd, *J* = 14.5, 7.7, 5.0 Hz, 6H), 3.43 (q, *J* = 6.4 Hz, 4H), 2.51 (m, 2H), 2.35 – 2.17 (m, 4H), 2.15 – 1.88 (m, 2H), 1.80 – 1.52 (m, 13H), 1.49 – 1.23 (m, 6H), 0.87 (m, 3H). **LC/MS** [M+ H]$^+$ for $C_{54}H_{80}ClN_2O_{13}$ calculated: 1000.6; found: 1000.4 [M+ H]$^+$.

Synthesis of (*S*)-(*R*)-1-(3-((25-chloro-4-oxo-10,13,16,19-tetraoxa-3-azapentacosyl)oxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl 1-((S)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate (PROTAC **4**)



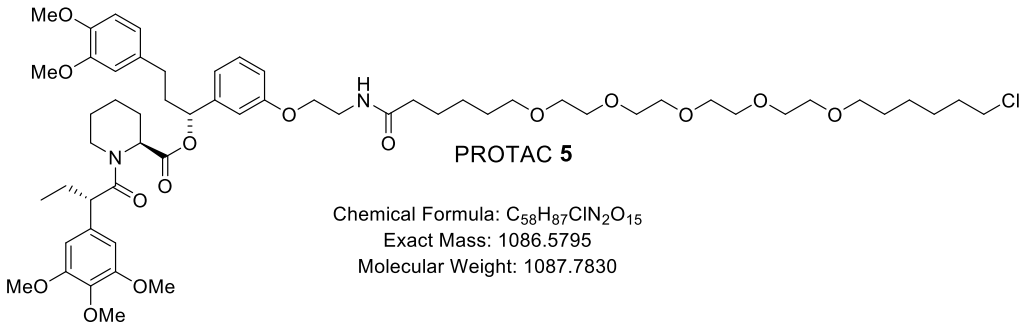
PROTAC **4**

Chemical Formula: $C_{56}H_{83}ClN_2O_{14}$
Exact Mass: 1042.5533
Molecular Weight: 1043.7300

To a solution of 6-[2-[2-[2-(6-chlorohexoxy)ethoxy]ethoxy]ethoxy]hexanoic acid (7.2 mg, 0.02 mmol) in DMF (1 mL) was added HATU (11.9 mg, 0.031 mmol) and the resulting solution stirred for 10 min. at room temperature after which **19** (10.62 mg, 0.02 mmol) and DIEA (0.013 mL, 0.078 mmol) were added respectively. The resulting mixture was stirred at room temperature for 16 h. The product was extracted with ethyl acetate (2x). The combined organic layer was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. The crude residue was purified by prep TLC (DCM/MeOH 90:10) to yield 8.9 mg (54.5%) of PROTAC **4** as colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.14 (t, *J* = 7.9 Hz, 1H), 6.96 – 6.72 (m, 3H), 6.70 – 6.60 (m, 4H), 6.44 – 6.38 (m, 2H), 6.28 – 6.09 (m, 1H), 5.60 (dd, *J* = 8.1, 5.5 Hz, 1H), 5.45 (d, *J* = 5.5 Hz, 1H), 3.87 – 3.82 (m, 8H), 3.77 (s, 2H), 3.68 – 3.60 (m, 24H), 3.58 – 3.49 (m, 6H), 3.43 (q, *J* = 6.7 Hz, 4H), 2.61 – 2.41 (m, 2H), 2.34 – 2.18 (m, 4H), 2.13 – 1.89 (m, 2H), 1.80 – 1.52 (m, 12H), 1.36 (m, 8H), 0.94 – 0.76 (m, 3H). **LC/MS** $[M+H]^+$ for $C_{56}H_{84}ClN_2O_{14}$ calculated: 1043.7; found: 1043.5 $[M+H]^+$.

Synthesis of (*S*)-(*R*)-1-(3-((28-chloro-4-oxo-10,13,16,19,22-pentaoxa-3-azaoctacosyl)oxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl 1-((S)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate (PROTAC **5**)



PROTAC **5**

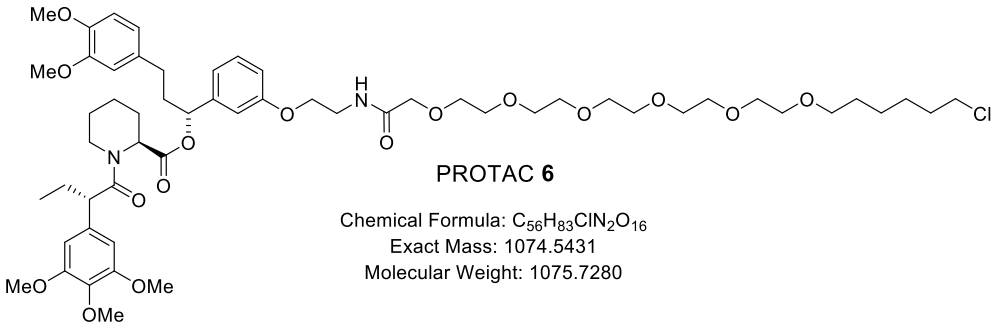
Chemical Formula: $C_{58}H_{87}ClN_2O_{15}$
Exact Mass: 1086.5795
Molecular Weight: 1087.7830

To a solution of 6-[2-[2-[2-[2-(6-chlorohexoxy)ethoxy]ethoxy]ethoxy]ethoxy]hexanoic acid (8.0 mg, 0.02 mmol) in DMF (1 mL) was added HATU (11.9 mg, 0.031 mmol) and the resulting solution stirred for 10 min. at room temperature after which **19** (10.62 mg, 0.02 mmol) and DIEA (0.013 mL, 0.078 mmol) were added respectively. The resulting mixture was stirred at room temperature for 16 h. The product was extracted with ethyl acetate (2x). The combined organic layer was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. The crude residue was purified by prep TLC (DCM/MeOH 90:10) to yield 8.4 mg (49.4%) of PROTAC **5** as colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.14 (t, *J* = 7.9 Hz, 1H), 6.96 – 6.73 (m, 3H), 6.70 – 6.59 (m, 4H), 6.43 – 6.36 (m, 2H), 6.26 – 6.09 (m, 1H), 5.60 (dd, *J* = 8.2, 5.5 Hz, 1H), 5.45 (d, *J* = 5.6 Hz, 1H), 4.05 – 3.99 (m, 2H), 3.87 – 3.81 (m, 8H), 3.78 (s, 3H), 3.74 – 3.59 (m, 30H), 3.56 (dd, *J* = 8.1, 5.0 Hz, 4H), 3.43 (dt, *J* = 9.3, 6.7 Hz, 4H), 2.59 – 2.40 (m, 2H), 2.31 – 2.18 (m, 4H), 2.10 – 1.91 (m, 2H), 1.80 – 1.54 (m, 8H), 1.48 – 1.23 (m, 6H), 0.93 – 0.80 (m, 3H).

LC/MS $[M+H]^+$ for $C_{58}H_{88}ClN_2O_{15}$ calculated: 1088.7; found: 1089.2 $[M+H]^+$.

Synthesis of (*S*)-(*R*)-1-(3-((27-chloro-4-oxo-6,9,12,15,18,21-hexaoxa-3-azaheptacosyl)oxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl 1-((*S*)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate (PROTAC **6**)



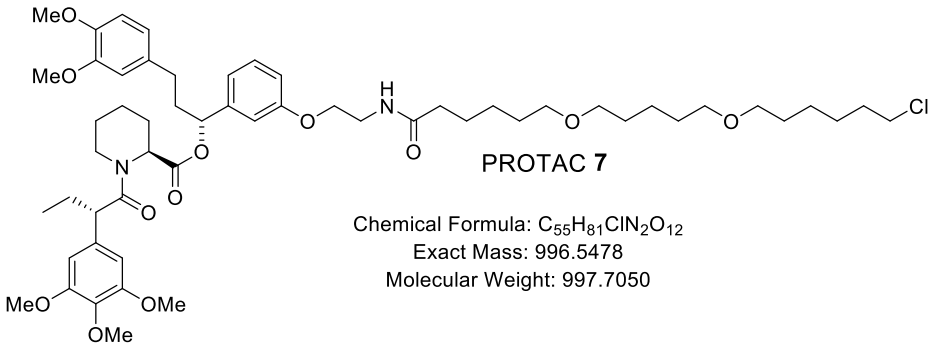
PROTAC **6**

Chemical Formula: $C_{56}H_{83}ClN_2O_{16}$
Exact Mass: 1074.5431
Molecular Weight: 1075.7280

To a solution of acid (10 mg, 0.024 mmol) in CH_2Cl_2 (0.16 mL) were added EDCI (5.1 mg, 0.065 mmol), HOBt (3.4 mg, 0.053 mmol), **19** (18 mg, 0.027 mmol) and DIPEA (0.005 mL, 0.031 mmol) at 0 ^oC. The mixture was stirred at room temperature for 22 h. After the reaction, the mixture was poured with 10% citric acid, and then extracted with ethyl acetate (x3). The combined organic layer was washed with sat. $NaHCO_3$, brine, dried over $MgSO_4$, and then concentrated under reduced pressure. The crude residue was purified by column chromatography to afford PROTAC **6** (5 mg, 19 %) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.11 (t, J = 7.9 Hz, 1H), 6.77-6.72 (m, 2H), 6.63 (d, J = 9.2 Hz, 1H), 6.62 (s, 1H), 6.57 (d, J = 7.7 Hz, 1H), 6.40 (s, 2H), 5.52 (t, J = 2.2 Hz, 1H), 5.93 (m, 1H), 4.04-4.01 (m, 2H), 3.99 (s, 2H), 3.84-3.80 (m, 9H), 3.67 (s, 6H), 3.65-3.59 (m, 22H), 3.56-3.53 (m, 3H), 3.51 (t, J = 6.7 Hz, 2H), 3.42 (t, J = 6.6 Hz, 2H), 2.79-2.73 (m, 1H), 2.59-2.38 (m, 2H), 2.35-2.28 (m, 1H), 2.15-2.03 (m, 2H), 1.96-1.87 (m, 1H), 1.76-1.72 (m, 2H), 1.69-1.66 (m, 2H), 1.61-1.59 (m, 4H), 1.58-1.55 (m, 4H), 1.44-1.39 (m, 2H), 1.37 (m, 2H), 0.88 (t, J = 7.3 Hz, 3H).
LC/MS $[M+H]^+$ for $C_{56}H_{84}ClN_2O_{16}$ calculated: 1075.5; found: 1076.1 $[M+H]^+$.

Synthesis of (*S*)-(*R*)-1-(3-(2-(6-((5-((6-chlorohexyl)oxy)pentyl)oxy)hexanamido)ethoxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl 1-((*S*)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate (PROTAC **7**)



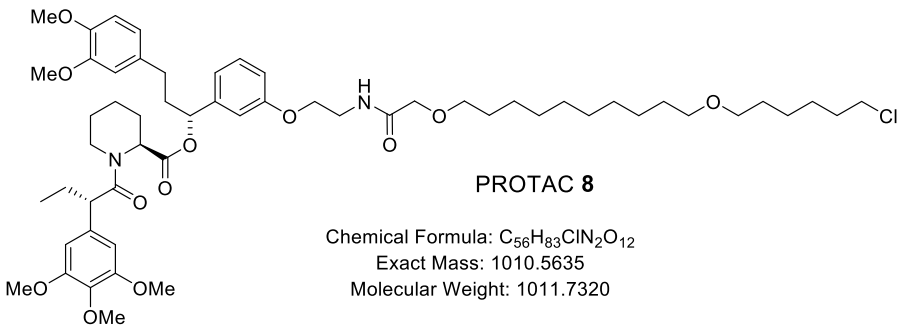
PROTAC **7**

Chemical Formula: $C_{55}H_{81}ClN_2O_{12}$
Exact Mass: 996.5478
Molecular Weight: 997.7050

To a solution of 6-[5-(6-chlorohexoxy)pentoxy]hexanoic acid (3.35 mg, 0.01 mmol) in DMF (1 mL) was added HATU (6.3 mg, 0.02 mmol) and the resulting solution stirred for 10 min. at room temperature after which **19** (5.62 mg, 0.01 mmol) and DIEA (0.01 mL, 0.04 mmol) were added respectively. The resulting mixture was stirred at room temperature for 16 h. The product was extracted with ethyl acetate (2x). The combined organic layer was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. The crude residue was purified by prep TLC (DCM/MeOH 90:10) to yield 5.3 mg (50%) of PROTAC **7** as Colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.16 (t, *J* = 7.9 Hz, 1H), 6.88 – 6.73 (m, 3H), 6.72 – 6.61 (m, 4H), 6.44 – 6.39 (m, 2H), 6.27 – 6.08 (m, 1H), 5.61 (dd, *J* = 8.2, 5.5 Hz, 1H), 5.46 (d, *J* = 5.5 Hz, 1H), 4.07 – 4.00 (m, 2H), 3.87 – 3.83 (m, 9H), 3.79 (s, 3H), 3.74 – 3.56 (m, 8H), 3.53 (td, *J* = 6.7, 2.5 Hz, 2H), 3.47 – 3.35 (m, 6H), 2.51 (dddd, *J* = 47.7, 14.3, 9.4, 6.3 Hz, 2H), 2.34 – 2.18 (m, 4H), 2.14 – 1.88 (m, 3H), 1.80 – 1.53 (m, 14H), 1.46 – 1.25 (m, 10H), 0.95 – 0.81 (m, 3H).
LC/MS [[M+ H]$^+$ for $C_{55}H_{82}ClN_2O_{12}$ calculated: 998.6; found: 998.2 [M+ H]$^+$.

Synthesis of (*S*)-(*R*)-1-(3-(2-(2-((10-((6-chlorohexyl)oxy)decyl)oxy)acetamido)ethoxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl 1-((S)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate (PROTAC **8**)

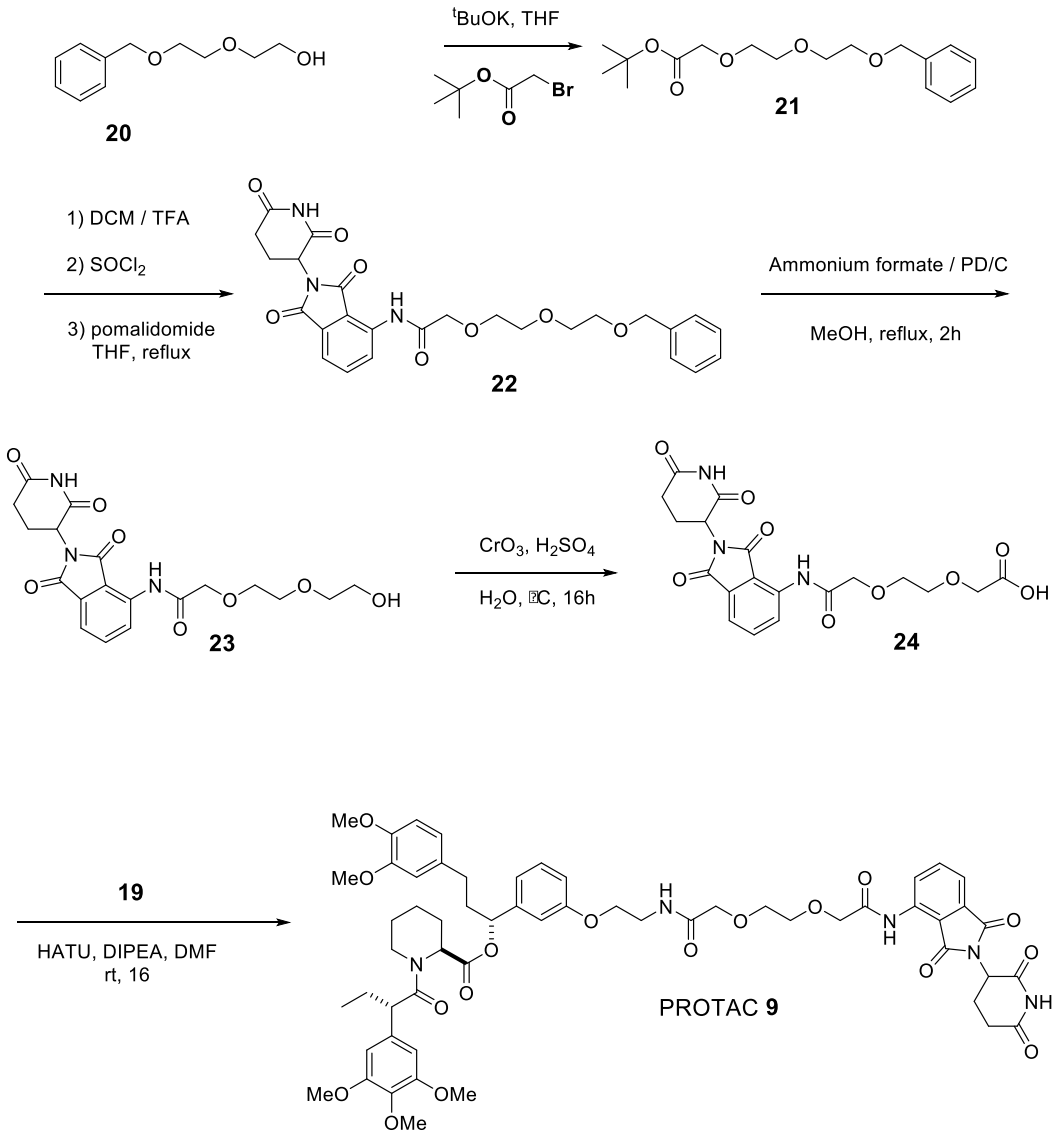


PROTAC **8**

Chemical Formula: $C_{56}H_{83}ClN_2O_{12}$
Exact Mass: 1010.5635
Molecular Weight: 1011.7320

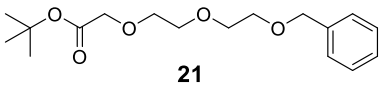
To a solution of 2-((10-((6-chlorohexyl)oxy)decyl)oxy)acetic acid (7.3 mg, 0.02 mmol) in DMF (1 mL) was added HATU (16.1 mg, 0.04 mmol) and the resulting solution stirred for 10 min. at room temperature after which **19** (10.6 mg, 0.02 mmol) and DIEA (0.013 mL, 0.078 mmol) were added respectively. The resulting mixture was stirred at room temperature for 16 h. The product was extracted with ethyl acetate (2x). The combined organic layer was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. The crude residue was purified by prep TLC (DCM/MeOH 90:10) to yield 10.6 mg (52.6%) of PROTAC **8** as colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.13 (t, *J* = 7.7 Hz, 1H), 7.02 – 6.90 (m, 2H), 6.77 – 6.71 (m, 3H), 6.65 – 6.57 (m, 3H), 6.40 – 6.36 (m, 2H), 5.59 (dd, *J* = 8.1, 5.5 Hz, 1H), 5.44 (d, *J* = 5.4 Hz, 1H), 4.07 – 4.00 (m, 2H), 3.92 (d, *J* = 2.5 Hz, 2H), 3.88 – 3.78 (m, 11H), 3.76 (s, 3H), 3.66 (s, 6H), 3.59 – 3.41 (m, 4H), 3.36 (td, *J* = 6.6, 4.6 Hz, 4H), 2.58 – 2.41 (m, 2H), 2.11 – 1.91 (m, 3H), 1.76 – 1.65 (m, 4H), 1.62 – 1.49 (m, 5H), 1.48 – 1.21 (m, 23H), 0.92 – 0.78 (m, 3H).
LC/MS $[M+H]^+$ for $C_{56}H_{83}ClN_2O_{12}$ calculated: 1011.7; found: 1011.3 $[M+H]^+$.



Supplementary Scheme 2. Synthesis of pomalidomide coupled Ariad ligand PROTAC **9**.

Synthesis of *tert*-butyl 2-(2-(2-(benzyloxy)ethoxy)ethoxy)acetate (**21**)

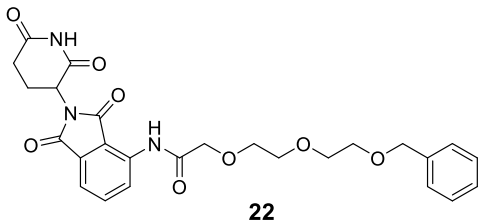


21

Chemical Formula: $C_{17}H_{26}O_5$
Exact Mass: 310.1780
Molecular Weight: 310.3900

To a solution of KOtBu (0.86 g, 8.0 mmol) in THF (40 mL) at 0 °C was added **20** (1.47 mL, 7.7 mmol). The clear resulting solution was heated at 40 °C for 30 min. Then the mixture was cooled down to 0 °C and *tert*-butyl 2-bromoacetate (1.1 mL, 7.7 mmol) was added in one portion. The resulting solution was stirred for 1 h at 0 °C and then 8 h at room temperature. After dilution with ethyl acetate (250 mL) and water (250 mL), the aqueous phase was extracted with ethyl acetate (3 x 100 mL). The combined organic fractions were washed with brine and dried over $MgSO_4$. After concentration, the crude material was subjected to column chromatography on silica gel (hexane/ethyl acetate 5:1) to give 1.4 g (59%) of **21** as colorless oil.
^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.40 – 7.21 (m, 5H), 4.56 (d, *J* = 1.2 Hz, 2H), 4.02 (d, *J* = 1.3 Hz, 2H), 3.76 – 3.58 (m, 9H), 1.47 (d, *J* = 1.3 Hz, 9H).

Synthesis of 2-(2-(2-(benzyloxy)ethoxy)ethoxy)-*N*-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)acetamide (**22**)

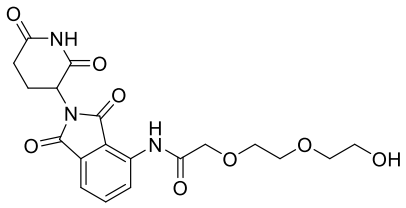


22

Chemical Formula: $C_{26}H_{27}N_3O_8$
Exact Mass: 509.1798
Molecular Weight: 509.5150

1.4 g (4.5 mmol) of **21** were dissolved in a mixture of TFA (1 mL) and DCM (3 mL). The resulting solution was stirred at room temperature for 2 h. The volatiles were evaporated and the residue dissolved in $SOCl_2$ and heated at 60 °C for 1h. The solvent was evaporated and the crude product dissolved in dry THF (50 mL). To this solution was added pomalidomide (1.2 g, 4.5 mmol). The resulting mixture was refluxed for 16 h. After cooling to room temperature, it was filtered through celite to give 1.9 g of **22** which was carried to the next step without further purification.

Synthesis of *N*-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)-2-(2-(2-hydroxyethoxy)ethoxy)acetamide (**23**)



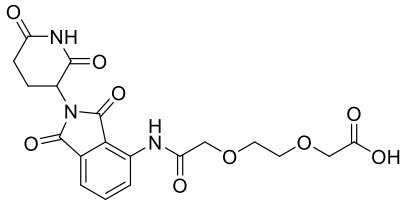
23

Chemical Formula: $C_{19}H_{21}N_3O_8$
Exact Mass: 419.1329
Molecular Weight: 419.3900

To a solution of **22** (50 mg, 0.1 mmol) in methanol 5 mL was added ammonium formate (6.19 mg, 0.1 mmol) and Pd/C (10%, 10.44 mg, 0.01 mmol). The resulting mixture was heated to reflux for 2h. The reaction was filtered over celite and the filtrate evaporated under reduced pressure and purified via column chromatography (hexane/ethyl acetate 5:1) to give 26 mg (60.7%) of **23** as a yellow solid.

^{1}H NMR (500 MHz, DMSO) δ = 11.12 (s, 1H), 10.34 (s, 1H), 8.70 (d, *J* = 8.5 Hz, 1H), 7.85 (t, *J* = 7.9 Hz, 1H), 7.61 (d, *J* = 7.3 Hz, 1H), 5.14 (dd, *J* = 12.8, 5.4 Hz, 1H), 4.51 (t, *J* = 5.3 Hz, 1H), 4.19 (s, 2H), 3.74 (dd, *J* = 5.8, 3.6 Hz, 2H), 3.64 (dd, *J* = 9.7, 5.5 Hz, 2H), 3.44 (dq, *J* = 13.8, 4.8 Hz, 4H), 2.87 (ddd, *J* = 18.3, 13.9, 5.4 Hz, 1H), 2.63 – 2.52 (m, 2H), 2.09 – 2.01 (m, 1H). **LC/MS** $[M+H]^+$ for $C_{19}H_{22}N_3O_8$ calculated: 419.4; found: 419.6 $[M+H]^+$.

Synthesis of 2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)-2-oxoethoxy)ethoxy)acetic acid (**24**)



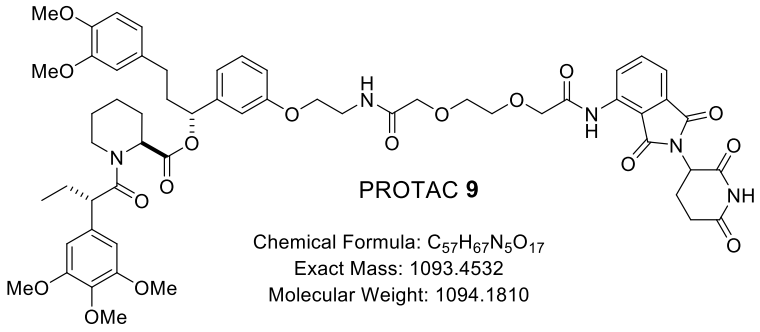
24

Chemical Formula: $C_{19}H_{19}N_3O_9$
Exact Mass: 433.1121
Molecular Weight: 433.3730

To a solution of **23** (0.02 mL, 0.05 mmol) in acetone (5 mL) was added jone`s reagent mixture of H_2SO_4 (0.13 mL, 2.35 mmol) and CrO_3 (4.77 mg, 0.05 mmol) in water (2 mL) at 0 °C. The mixture was stirred for 16 h. After the reaction was complete, the mixture was quenched with isopropanol (to reduce the toxic Chromium (VI)). The resulting solution was extracted with ethyl

acetate (x3). The combined organic phase was dried over $MgSO_4$. After completely removing the solvent, the crude product was used to the next step without further purification.

Synthesis of (2*S*)-(1*R*)-3-(3,4-dimethoxyphenyl)-1-(3-(2-(2-(2-(2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)amino)-2-oxoethoxy)ethoxy)acetamido)ethoxy)phenyl)propyl 1-((S)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate (PROTAC **9**)

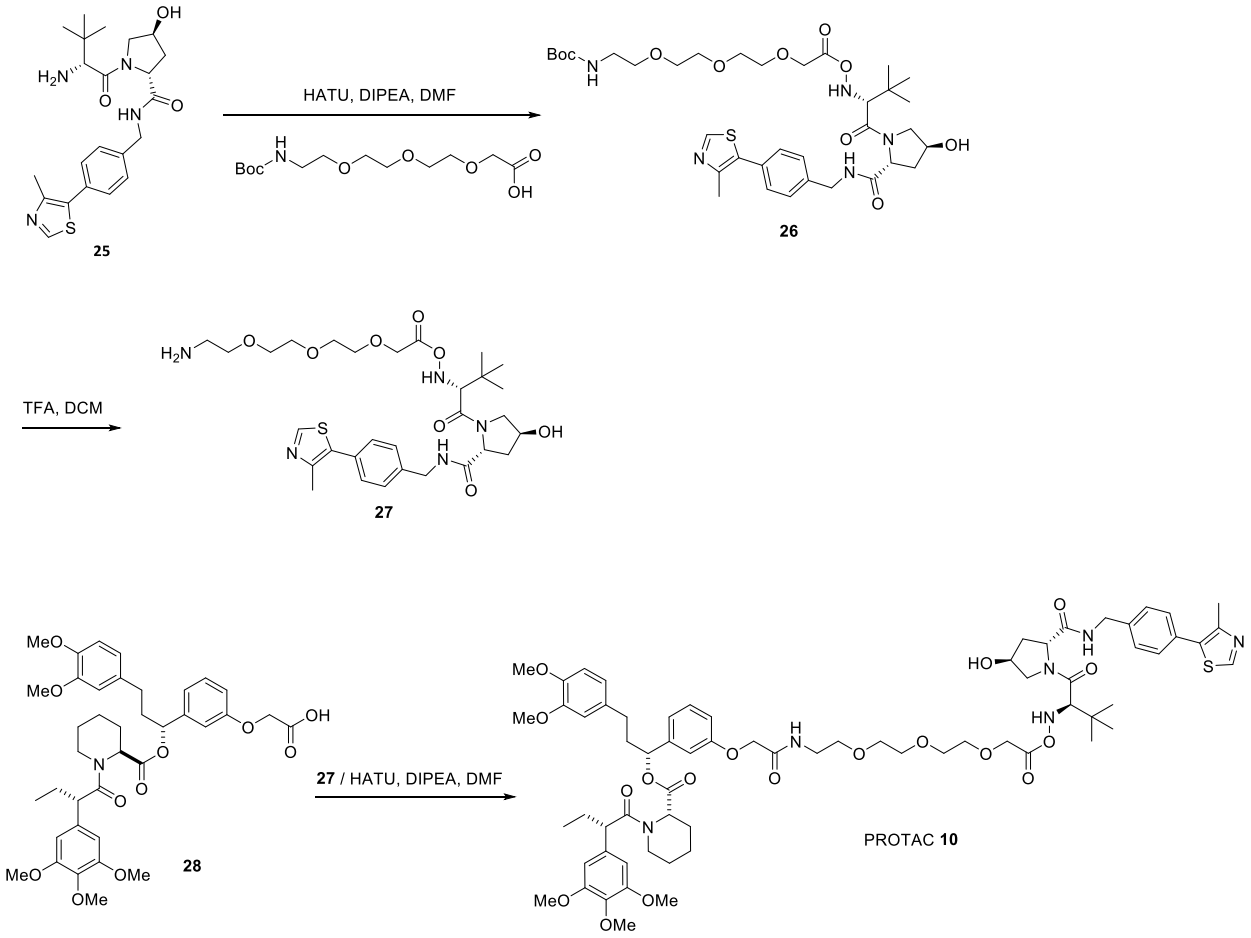


PROTAC **9**

Chemical Formula: $C_{57}H_{67}N_5O_{17}$
Exact Mass: 1093.4532
Molecular Weight: 1094.1810

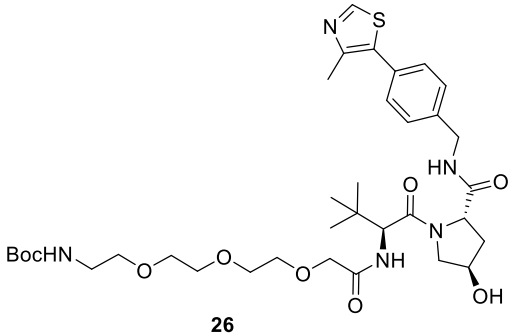
To a solution of **24** (15 mg, 0.03 mmol) was added HATU (26.32 mg, 0.07 mmol) and the resulting solution stirred for 10 minutes at room temperature after which **19** (23.5 mg, 0.03 mmol) and DIEA (0.03 mL, 0.17 mmol) were added respectively. The resulting mixture was stirred at room temperature for 16 h. The product was extracted with ethyl acetate (2x). The combined organic layer was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. The crude residue was purified by Prep TLC (DCM/MeOH/ammonia: 60/10/1) to yield 11.9 mg (46.0%) of PROTAC **9**.

^{1}H NMR (500 MHz, $CDCl_3$) δ = 10.49 (s, 1H), 8.80 (d, *J* = 8.4 Hz, 1H), 7.69 (t, *J* = 7.9 Hz, 1H), 7.55 (dd, *J* = 7.4, 1.6 Hz, 1H), 7.22 – 7.06 (m, 2H), 6.84 – 6.61 (m, 5H), 6.51 – 6.44 (m, 1H), 6.43 – 6.36 (m, 2H), 5.81 – 5.52 (m, 1H), 5.50 – 5.42 (m, 1H), 4.94 (ddd, *J* = 12.0, 7.4, 4.8 Hz, 1H), 4.20 – 3.97 (m, 6H), 3.90 – 3.75 (m, 18H), 3.71 – 3.63 (m, 5H), 2.62 – 2.40 (m, 2H), 2.27 – 1.91 (m, 5H), 1.75 – 1.57 (m, 4H), 1.44 – 1.22 (m, 2H), 0.95 – 0.79 (m, 3H). **LC/MS** $[M+H]^+$ for $C_{57}H_{68}N_5O_{17}$ calculated: 1094.2; found: 1094.5 $[M+H]^+$.



Supplementary Scheme 3. Synthesis of VHL coupled Ariad ligand PROTAC **10**.

Synthesis of tert-butyl ((*S*)-13-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidine-1-carbonyl)-14,14-dimethyl-11-oxo-3,6,9-trioxa-12-azapentadecyl)carbamate (**26)**



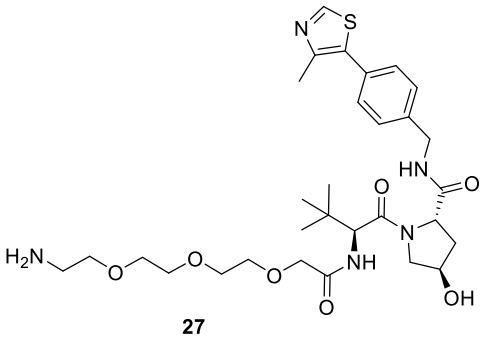
26

Chemical Formula: $C_{35}H_{53}N_5O_9S$
Exact Mass: 719.3564
Molecular Weight: 719.8950

To a solution of (2*R*,4*S*)-1-((*R*)-2-amino-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (**25**) (86 mg, 0.2 mmol) was added HATU (101 mg, 0.26 mmol) and the resulting solution stirred for 10 minutes at room temperature after which 2,2-dimethyl-4-oxo-3,8,11,14-tetraoxa-5-azahexadecan-16-oic acid (86 mg, 0.26 mmol) and DIEA (0.16 mL, 0.92 mmol) were added respectively. The resulting mixture was stirred at room temperature for 16 h. The product was extracted with ethyl acetate (2x). The combined organic layer was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. The crude residue was purified by Prep TLC (DCM/MeOH/ammonia: 60/10/1) to yield 93.5 mg (65.0%) of **26**.

^{1}H NMR (500 MHz, $CDCl_3$) δ = 8.6 (s, 1H), 7.3 (m, 4H), 4.5 (m, 3H), 4.3 (m, 1H), 4.0 (m, 2H), 3.6 (m, 8H), 3.5 (m, 2H), 3.2 (m, 2H), 2.6 (m, 1H), 2.5 (s, 3H), 2.3 (m, 1H), 2.2 (m, 3H), 1.4 (s, 9H), 1.0 (m, 9H).

Synthesis of (2*R*,4*S*)-1-((*R*)-15-amino-2-(tert-butyl)-5-oxo-4,7,10,13-tetraoxa-3-azapentadecanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (**27)**

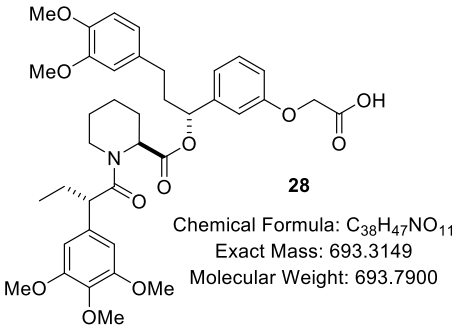


27

Chemical Formula: $C_{35}H_{53}N_5O_9S$
Exact Mass: 719.3564
Molecular Weight: 719.8950

Compound **26** (93.5 mg, 0.13 mmol) was dissolved in a 1:1 (v/v) mixture of Dichloromethane and TFA (10 mL) and stirred for 16 h at room temperature. The solvent was removed under reduced pressure and compound **27** was used without further purification.

Synthesis of 2-(3-((*R*)-3-(3,4-dimethoxyphenyl)-1-(((*S*)-1-((*S*)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carbonyl)oxy)propyl)phenoxy)acetic acid (**28**)

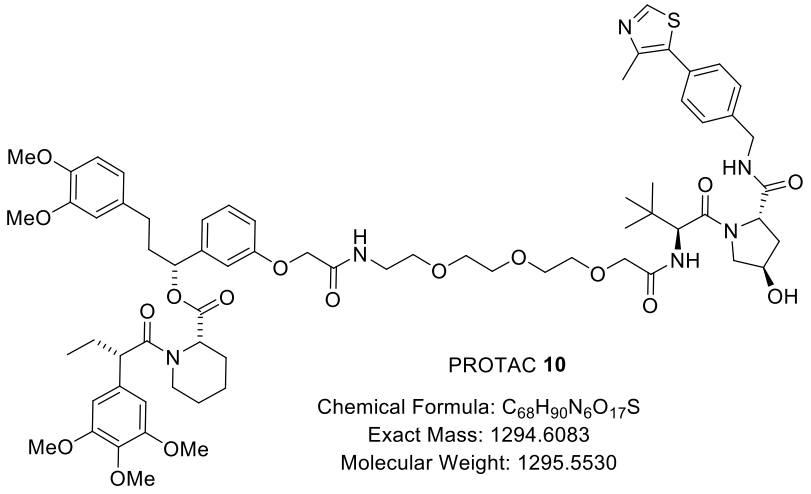


28

Chemical Formula: $C_{38}H_{47}NO_{11}$
Exact Mass: 693.3149
Molecular Weight: 693.7900

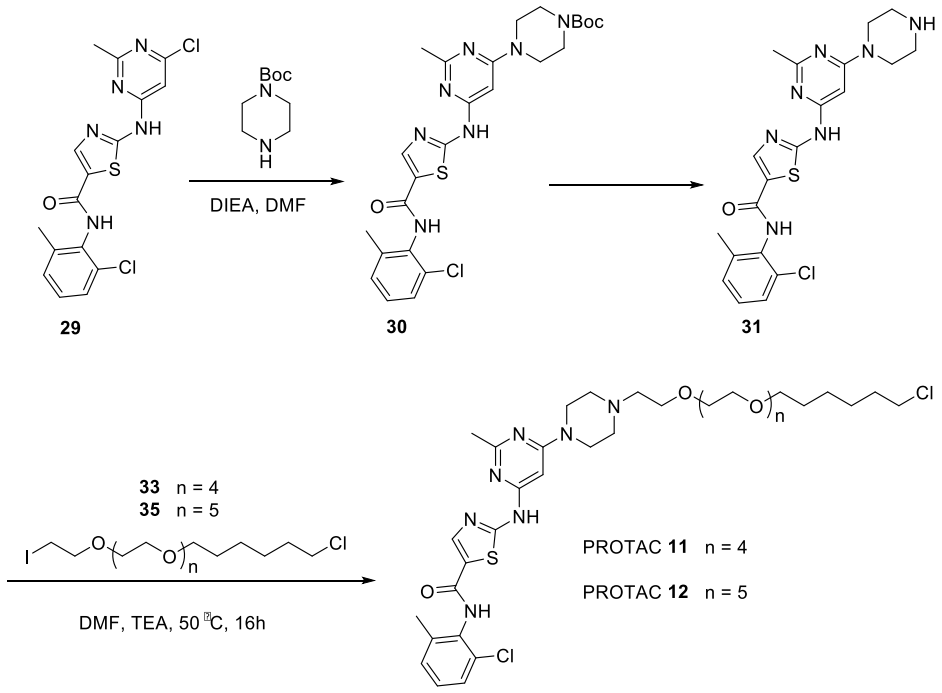
Synthesis of compound **28** has been previously described.[1]

Synthesis of (*R*)-3-(3,4-dimethoxyphenyl)-1-(3-(((*S*)-16-((2*S*,4*R*)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidine-1-carbonyl)-17,17-dimethyl-4,14-dioxo-6,9,12-trioxa-3,15-diazaoctadecyl)oxy)phenyl)propyl (*S*)-1-((*S*)-2-(3,4,5-trimethoxyphenyl)butanoyl)piperidine-2-carboxylate (PROTAC **10)**



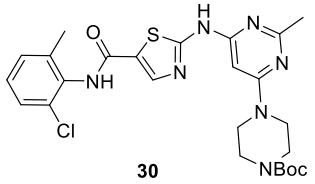
To a solution of **27** (9.5 mg, 0.014 mmol) was added HATU (5.8 mg, 0.015 mmol) and the resulting solution stirred for 10 minutes at room temperature after which **28** (14 mg, 0.019 mmol) and DIEA (0.006 mL, 0.03 mmol) were added respectively. The resulting mixture was stirred at room temperature for 16 h. The product was extracted with ethyl acetate (2x). The combined organic layer was washed with brine, dried over $MgSO_4$, and then concentrated under reduced pressure. The crude residue was purified by Prep TLC (DCM/MeOH/ammonia: 60/10/1) to yield 2.5 mg (14.0%) of PROTAC **10**.

^{1}H NMR (500 MHz, $CDCl_3$) δ = 9.1 (s, 1H), 7.4 (m, 1H), 7.3 (m, 3H), 6.8 (m, 1H), 6.7 (m, 2H), 6.6 (m, 2H), 6.3 (m, 2H), 5.5 (m, 1H), 5.3 (m, 1H), 4.7 (m, 1H), 4.5 (m, 2H), 4.4 (m, 2H), 4.3 (m, 1H), 4.0 (m, 12H), 3.8 (m, 13H), 3.5 (m, 14H), 2.8 (m, 1H), 2.5 (s, 3H), 2.2 (m, 3H), 1.9 (m, 2H), 1.8 (m, 1H), 1.5 (m, 3H), 1.3 (m, 1H), 1.2 (m, 3H), 0.9 (m, 6H), 0.8 (m, 4H). **LC/MS** $[M+H]^+$ for $C_{68}H_{91}N_6O_{17}S^+$ calculated: 1295.6156; found: 1295.6656 $[M+H]^+$.



Supplementary Scheme 4. Synthesis of dasatinib chloroalkanes.

Synthesis of *tert*-butyl 4-(6-((5-((2-chloro-6-methylphenyl)carbamoyl)thiazol-2-yl)amino)-2-methylpyrimidin-4-yl)piperazine-1-carboxylate (**30**)



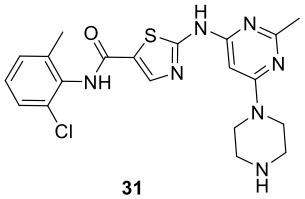
30

Chemical Formula: $C_{25}H_{30}ClN_7O_3S$
Exact Mass: 543.1819
Molecular Weight: 544.0710

To a solution of 4*N*-(2-chloro-6-methyl-phenyl)-2-[(6-chloro-2-methyl-pyrimidin-4-yl)amino]thiazole-5-carboxamide (**29**) (500 mg, 1.27 mmol, 1.0 eq.) and DIEA (562 µL, 3.17 mmol, 2.5 eq.) in DMF (3 mL) was added *tert*-butyl piperazine-1-carboxylate (283.43 mg, 1.52 mmol, 1.2 eq.) and the resulting mixture stirred for 16 h at 110 °C. The reaction mixture was cooled to room temperature and the product precipitated by the addition of water (10 mL). The crude product was filtered off and dried until constant weight to yield 800 mg (115%) and was used without further purification.

LC/MS $[M+H]^+$ for $C_{25}H_{31}ClN_7O_3S^+$ calculated: 544.1892; found = 544.2046 $[M+H]^+$.

Synthesis of *N*-(2-chloro-6-methylphenyl)-2-((2-methyl-6-(piperazin-1-yl)pyrimidin-4-yl)amino)thiazole-5-carboxamide (**31**)



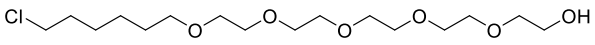
31

Chemical Formula: $C_{20}H_{22}ClN_7OS$
Exact Mass: 443.1295
Molecular Weight: 443.9540

Compound **30** (690 mg, 1,27 mmol, 1.0 eq) was dissolved in a 1:1 (v/v) mixture of Dichloromethane and TFA (10 mL) and stirred for 16 h at room temperature. The solvent was removed under reduced pressure and the crude taken up in DCM. After addition of sat. $NaHCO_3$ the pure product precipitated and filtered off. 436 mg (78%) of compound **31** were obtained as a brown solid.

^{1}H NMR (400 MHz, DMSO) δ = 8.26 (s, 1H), 7.39 (d, J = 7.5 Hz, 1H), 7.27 (q, J = 7.3 Hz, 2H), 6.06 (s, 1H), 3.45 (t, J = 4.9 Hz, 4H), 2.74 (d, J = 5.1 Hz, 4H), 2.40 (s, 3H), 2.24 (s, 3H). **^{13}C NMR** (101 MHz, DMSO) δ = 165.1, 162.5, 162.5, 159.9, 156.9, 140.9, 138.8, 133.6, 132.4, 129.0, 128.1, 127.0, 125.6, 82.5, 45.2, 44.7, 25.6, 18.3. **LC/MS** $[M+ H]^+$ for $C_{20}H_{23}ClN_7OS^+$ calculated: 444.1368; found = 444.1402 $[M+H]^+$.

Synthesis of 21-chloro-3,6,9,12,15-pentaoxahenicosan-1-ol (**32**)

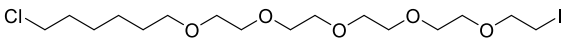


32

Chemical Formula: $C_{16}H_{33}ClO_6$
Exact Mass: 356.1966
Molecular Weight: 356.8840

To a solution of the pentaehtylene glycol (4.3 mL, 20.28 mmol, 5.0 eq.) in a mixture of DMF and THF (1:1, v/v; 40 mL) was added portion wise NaH (488 mg, 12.17 mmol, 3.0 eq) at 0 °C. After 40 min. 1-chloro-6-iodo-hexane (0,62 mL, 4.06 mmol, 1.0 eq.) was added, the reaction mixture was allowed to warm to room temperature and stirred for additional 16 h at room temperature. The reaction was quenched with water, diluted with 1M HCl (30 mL), and extracted with ethyl acetate (3x 40 mL). The combined organic layers were washed with brine (40 mL), dried over $MgSO_4$ and the solvent removed under reduced pressure. The crude product (1156 mg, 80%) was used without further purification.

Synthesis of 21-chloro-1-iodo-3,6,9,12,15-pentaoxahenicosane (**33**)



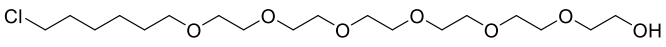
33

Chemical Formula: $C_{16}H_{32}ClIO_5$
Exact Mass: 466.0983
Molecular Weight: 466.7815

To a suspension of triphenylphosphine (265 mg, 1.01 mmol, 1.2 eq.), imidazole (68.7 mg, 1.01 mmol, 1.2 eq.) and iodine (320 mg, 1.26 mmol, 1.5 eq.) in dry tetrahydrofuran (10 mL) crude compound **32** (300 mg, 0.84 mmol, 1.0 eq.) was added dropwise at room temperature. After stirring at room temperature for 2 h (TLC control) the reaction mixture was filtered to remove the white precipitate. Afterwards, the solvent was removed under reduced pressure and the crude mixture purified by flash chromatography (20 – 100% ethyl acetate in hexanes) to yield 295 mg (76%) of pure product **33**.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.75 (t, J = 6.9 Hz, 2H), 3.68 – 3.62 (m, 14H), 3.57 (dd, J = 5.8, 3.6 Hz, 2H), 3.52 (t, J = 6.7 Hz, 2H), 3.45 (t, J = 6.6 Hz, 2H), 3.25 (t, J = 6.9 Hz, 2H), 1.77 (p, J = 6.9 Hz, 2H), 1.59 (p, J = 6.8 Hz, 2H), 1.49 – 1.30 (m, 4H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ = 72.1, 71.4, 70.8, 70.8, 70.7, 70.4, 70.3, 45.2, 32.7, 29.6, 26.8, 25.6, 3.1.

Synthesis of 24-chloro-3,6,9,12,15,18-hexaoxatetracosan-1-ol (**34**)



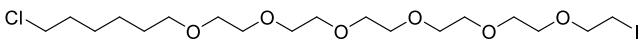
34

Chemical Formula: $C_{18}H_{37}ClO_7$
Exact Mass: 400.2228
Molecular Weight: 400.9370

To a solution of the hexaehtylene glycol (5.1 mL, 20.28 mmol, 5.0 eq.) in a mixture of DMF and THF (1:1, v/v; 40 mL) was added portion wise NaH (488 mg, 12.17 mmol, 3.0 eq) at 0 °C. After 40 min. 1-chloro-6-iodo-hexane (0,62 mL, 4.06 mmol, 1.0 eq.) was added, the reaction mixture was allowed to warm to room temperature and stirred for additional 16 h at room temperature. The reaction was quenched with water, diluted with 1M HCl (30 mL), and extracted with ethyl acetate (3x 40 mL). The combined organic layers were washed with brine (40 mL), dried over $MgSO_4$ and the solvent removed under reduced pressure. The crude product was purified by flash chromatography (20 – 100% ethyl acetate in hexanes) to yield 381 mg (24%) of pure product **34**.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.72 (dd, J = 5.4, 3.6 Hz, 2H), 3.72 – 3.58 (m, 20H), 3.59 – 3.55 (m, 2H), 3.53 (t, J = 6.7 Hz, 2H), 3.45 (td, J = 6.6, 1.4 Hz, 2H), 1.83 – 1.69 (m, 2H), 1.59 (p, J = 6.8 Hz, 2H), 1.50 – 1.32 (m, 4H). **^{13}C NMR** (101 MHz, CDCl3) δ = 72.6, 71.4, 71.3, 70.8, 70.7, 70.7, 70.5, 70.3, 70.2, 61.9, 45.2, 32.7, 29.6, 26.9, 25.6.

Synthesis of 24-chloro-1-iodo-3,6,9,12,15,18-hexaoxatetracosane (**35**)



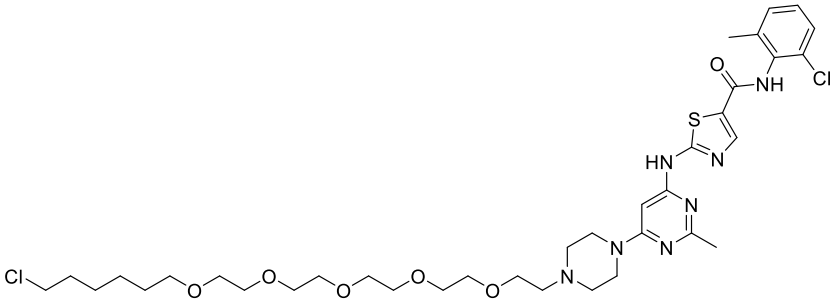
35

Chemical Formula: $C_{18}H_{36}ClIO_6$
Exact Mass: 510.1245
Molecular Weight: 510.8345

To a suspension of triphenylphosphine (78.5 mg, 0.3 mmol, 1.2 eq.), imidazole (20.4 mg, 0.3 mmol, 1.2 eq.) and iodine (95 mg, 0.38 mmol, 1.5 eq.) in dry tetrahydrofuran (5 mL) compound **34** (100 mg, 0.25 mmol, 1.0 eq.) was added dropwise at room temperature. After stirring at room temperature for 2 h (TLC control) the reaction mixture was filtered to remove the white precipitate. Afterwards, the solvent was removed under reduced pressure and the crude mixture purified by flash chromatography (20 – 100% ethyl acetate in hexanes) to yield 66.5 mg (52%) of pure product **35**.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.76 (t, J = 6.9 Hz, 2H), 3.66 (d, J = 1.7 Hz, 18H), 3.61 – 3.56 (m, 2H), 3.53 (t, J = 6.7 Hz, 2H), 3.46 (td, J = 6.7, 1.3 Hz, 2H), 3.26 (t, J = 6.9 Hz, 2H), 1.82 – 1.72 (m, 2H), 1.62 – 1.56 (m, 2H), 1.48 – 1.33 (m, 4H). **^{13}C NMR** (101 MHz, CDCl3) δ = 72.1, 71.4, 70.8, 70.7, 70.7, 70.4, 70.3, 45.2, 32.7, 29.6, 26.9, 25.6, 3.1.

Synthesis of 2-((6-(4-(21-chloro-3,6,9,12,15-pentaoxahenicosyl)piperazin-1-yl)-2-methylpyrimidin-4-yl)amino)-*N*-(2-chloro-6-methylphenyl)thiazole-5-carboxamide (PROTAC **11**)



PROTAC **11**

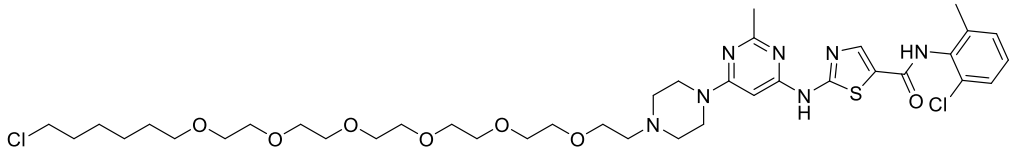
Chemical Formula: $C_{36}H_{53}Cl_2N_7O_6S$
Exact Mass: 781.3155
Molecular Weight: 782.8230

To a solution of compound **27** (30 mg, 0,068 mmol, 1.0 eq.) and **29** (47,31 mg, 0.0748 mmol, 1.1 eq.) in DMF (2 mL) was added TEA (0.8 mL) and the resulting solution stirred for 16 h at 65 °C. The reaction mixture was cooled to room temperature and ethyl acetate (40 mL) was added. The organic phase was washed with water and sat. $NaHCO_3$ (1:1 v/v; 3x 40mL) and brine (40 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure and

the crude product purified by preparative TLC (DCM/MeOH/NH_4OH, 94.83/4.7/0.47) to yield 6.2 mg (12%) of pure product PROTAC **11**.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.35 (s, 1H), 7.99 (s, 1H), 7.37 (s, 1H), 7.31 (dd, J = 7.5, 2.0 Hz, 1H), 7.22 – 7.12 (m, 2H), 5.85 (s, 1H), 3.79 – 3.68 (m, 4H), 3.63 (m, 20H), 3.51 (t, J = 6.7 Hz, 2H), 3.45 (t, J = 6.7 Hz, 2H), 2.68 (s, 4H), 2.51 (s, 3H), 2.35 (s, 3H), 1.75 (p, J = 6.9 Hz, 2H), 1.61 – 1.54 (m, 2H), 1.45 – 1.32 (m, 4H). **LC/MS** $[M+H]^+$ for $C_{36}H_{54}Cl_2N_7O_6S^+$ = 782.3228; found calculated: 782.3667 $[M+H]^+$; 391.6781 $[M+2H]^{2+}$.

Synthesis of 2-((6-(4-(24-chloro-3,6,9,12,15,18-hexaoxatetracosyl)piperazin-1-yl)-2-methylpyrimidin-4-yl)amino)-*N*-(2-chloro-6-methylphenyl)thiazole-5-carboxamide (PROTAC **12**)



PROTAC **12**
Chemical Formula: $C_{38}H_{57}Cl_2N_7O_7S$
Exact Mass: 825.3417
Molecular Weight: 826.8760

To a solution of compound **27** (44.3mg, 0,1 mmol, 1.2 eq.) and **31** (42.5 mg, 0.083 mmol, 1.0 eq.) in DMF (2 mL) was added TEA (0.8 mL) and the resulting solution stirred for 16 h at 65 °C. The reaction mixture was cooled to room temperature and ethyl acetate (40 mL) was added. The organic phase was washed with water and sat. $NaHCO_3$ (1:1 v/v; 3x 40mL) and brine (40 mL) and dried over $MgSO_4$. The solvent was removed under reduced pressure and the crude product purified by preparative TLC (DCM/MeOH/NH_4OH, 94.83/4.7/0.47) to yield 7.2 mg (11%) of pure PROTAC **12**.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.41 (s, 1H), 7.99 (s, 1H), 7.38 (s, 1H), 7.31 (d, J = 7.5 Hz, 1H), 7.22 – 7.14 (m, 2H), 5.87 (s, 1H), 3.82 – 3.69 (m, 4H), 3.69 – 3.60 (m, 22H), 3.57 (t, J = 4.8 Hz, 2H), 3.51 (t, J = 6.7 Hz, 2H), 3.43 (t, J = 6.6 Hz, 2H), 2.81 – 2.61 (m, 4H), 2.51 (s, 3H), 2.35 (s, 3H), 1.75 (p, J = 6.9 Hz, 2H), 1.56 (p, J = 6.9 Hz, 2H), 1.45 – 1.31 (m, 4H). **LC/MS** $[M+H]^+$ for $C_{38}H_{58}Cl_2N_7O_7S^+$ calculated: 826.3490; found = 826.3782 $[M+H]^+$; 413.6860 $[M+2H]^{2+}$.

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