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Supplementary Information for

Delineating the Role of Cooperativity in the Design of Potent PROTACs for BTK

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Supplementary Information Text

METHODS

Cell culture and immunoblotting Ramos cells (ATCC) were cultured in RPMI1640, supplemented with L-glutamine and 10% FBS whereas THP-1 cells (ATCC) were cultured in the same media additionally supplemented with 50 µM beta-mercaptoethanol and Pen/Strep. Cells were grown at 37 °C with 5% CO_2. For time-dependent monitoring of BTK degradation, 20 mL Ramos cells ($1.0x10^6$cells/mL) were incubated with 1 µL 2 mM PROTAC (**9**) ($[PROTAC(\mathbf{9})]_{final}$=0.1 µM, $[DMSO]_{final}$=0.005%). At the indicated timepoints, 1mL cells were collected, spun down and washed with 1xPBS. The pellet was then resuspended in 75 µL lysis buffer (150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 1mM ethylene diamine tetraacetic acid, 1 mM phenyl methyl sulfonyl fluoride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, protease inhibitor cocktail, benzonase), incubated on ice for 10mins, clarified of cellular debris through centrifugation (15,000g, 3mins, 4 °C) and the supernatant was subjected to total protein quantification using the Bicinchoninic Acid (BCA) assay (#23225 ThermoFisher Scientific, Philadelphia, PA). For washout studies, following compound incubation for 24 h (same cell volume, density and compound amounts as above), cells were washed 3x with ice-cold PBS, incubated with 20 mL fresh media, and 1mL cells were harvested at the indicated timepoints for Western Blot analysis. For dose-dependent cellular degradation studies of BTK, 1.5 mL of $1.5x10^6$cells/mL (either Ramos or THP-1) were plated in each well of a 12-well plate and supplemented with 500 uL media

containing 4x compound ($[compound]_{final}$=1 nM-30 µM, 3-fold serial dilution). Cells were incubated at 37 °C / 5% CO_2 for 24h and lysates were collected and analyzed as described below. For outcompetition studies, PROTAC (**9**) and either/both BTK/CRBN ligands were added to cells at the same time and incubated for 24 h. For subcellular fractionation studies, aimed at determining sub-cellular localization of target and various ligases, 10 mL of either Ramos or THP-1 cells ($1.0x10^6$cells/ml) were used (Subcellular Protein Fractionation Kit #78840, ThermoFisher Scientific, Philadelphia, PA).

5 µL of 0.2 µg/µL total protein lysate was loaded onto a 12-230kDa Wes assay plate (ProteinSimple, San Jose, CA) where 400nL sample was withdrawn through a capillary, subjected to electrophoretic separation of proteins by size and followed by HRP-based detection of proteins of interest using an HRP-conjugated secondary antibody and the following primary antibodies:

anti-BTK (1:50 dilution, #8547 Cell Signalling Technology, Boston, MA),

anti -Vinculin (1:200 dilution, #700062 Fisher Healthcare, Houston, TX),

anti -Actin (1:200 dilution, #4970 Cell Signalling Technology, Boston, MA),

anti -CRBN (1:50 dilution, #NB100-91810 Novus Biologicals, Littleton, CO),

anti -VHL (1:50 dilution, #68547 Cell Signalling Technology, Boston, MA) and

anti -XIAP (1:50 dilution, #2042 Cell Signalling Technology, Boston, MA).

To facilitate data analysis, BTK and vinculin (or actin) were multiplexed and BTK concentrations were normalized to vinculin (or actin) loading controls. To allow comparison of PROTAC efficacy among different cell lines, DCmax and

DC50 were respectively defined as the concentration of PROTAC required for maximal or 50% BTK degradation, in line with previous definitions [1].

BTK and CRBN Purification Human BTK kinase domain (384-659) was subcloned into pRSETA and recombinantly expressed as an N-terminal His_6-SUMO-TEV and C-terminal BAP (Biotin Acceptor Peptide, GLNDIFEAQKIEWHE) fusion protein in *E. coli*. Cells were grown in TB and induced with 0.6 mM IPTG/L culture overnight at 18 °C. Cell pellets were resuspended in Buffer A (50 mM Tris pH 8.0, 10% glycerol, 150 mM NaCl, 1mM TCEP, 20 mM imidazole) supplemented with 8 tablets of Roche Complete EDTA-free Protease Inhibitors and 16uL benzonase, and lysed with three passes in microfluidizer at 18,000 psi. Supernatant was clarified via centrifugation at 10,000rpm for 30 mins and further filtered using VacuCap 90 (0.8/0.2 µm membrane; Pall Life Sciences, Ann Arbor, MI) before loading onto HisTrap HP 5 mL column (GE Lifesciences, Piscataway, NJ) and eluting at 2 mL/min using a gradient of 0-100% Buffer B (same as Buffer A but with 300 mM imidazole) over 30 mins. Pooled protein was first dialyzed overnight against Buffer C (50 mM Tris pH 8.0, 3% glycerol, 75 mM NaCl, 1 mM TCEP), then biotinylated for 6 h at RT according to manufacturer's instructions (BirA Biotin Ligase Kit, Avidity LLC, Aurora, CO), and lastly ran through a second nickel column to remove ATP, BirA and biotin. Pooled fractions were TEV-cleaved overnight in 4°C – while dialyzing against Buffer C – followed by dephosphorylation with Lambda Phosphatase (LPP) and 1 mM $MnCl_2$ for 4h at RT and ran through a 3rd nickel column to

remove His_6 tag, TEV and LPP. BAP-BTK was further run through gel filtration and Q columns, dialyzed in storage Buffer D (50 mM Tris pH 8.0, 10% glycerol, 150 mM NaCl, 1 mM TCEP) and sent for intact Mass Spectrometry which confirmed BAP-BTK to be dephosphorylated, TEV-cleaved and biotinylated.

E. coli cell paste containing the overexpressed recombinant His-BAP-CRBN (319-442) protein was resuspended in 3 volumes of buffer A (50 mm Tris pH 7.5, 10% (v/v) glycerol, 500 mm NaCl, 1 mm TCEP, 10 mM imidazole, Complete™ EDTA-free protease inhibitor (1 tablet per 50 ml of buffer, Roche), and benzonase (2 uL per 50 ml of buffer, Sigma). Cells were lysed with 3 passes on a microfluidizer (pressure at 18,000 psi). The lysate was clarified by centrifugation at 4°C for 30 min with a F12-6x500 LEX rotor (ThermoFisher) at 10,000 rpm. The supernatant was decanted and filtered using a VacuCap 90 bottle top filtration device (0.8/0.2 um membrane, Pall). The sample was applied using a NCG chromatography system (Bio-Rad) to a 5 mL HisTrap FF crude column (GE Healthcare) that was pre-equilibrated with buffer A. The column was washed with buffer A until a steady baseline was achieved. The column was step eluted with 100% buffer B (50 mm Tris pH 7.5, 10% (v/v) glycerol, 500 mM NaCl, 1 mM TCEP, and 500 mM imidazole). A pre-equilibrated HiPrep 26/10 Desalting column (GE Healthcare) was used to exchange the pooled peak fractions into Buffer C (50 mM Tris pH 8.0, 10% (v/v) glycerol, 20 mM NaCl, and 1 mM TCEP). The resulting peak fractions were put over a 5 mL Q FF column (GE Healthcare) equlibrated in Buffer C. The column was eluted with a 0-50% gradient of Buffer D (50 mM Tris pH 8.0, 10% (v/v) glycerol, 1000 mM NaCl, and

1 mM TCEP) with a 10 mM increase of NaCl per column volume. The peak fractions were dialyzed into 2L of Buffer E (50 mM Tris pH 8.0, 3% glycerol, 75 mM NaCl, and 0.5 mM TCEP. The protein was biotinylated by adding one tube of BirA and one tube of Biomix B (Avidity) for 3 hours at room temperature. The protein was then concentrated to 5 mL using a Amicon Ultrafree-15 centrifugation unit (10K MWCO, Millipore) and injected onto a pre-equilibrated HiLoad 16/60 Superdex 75 column in Buffer F (50 mM HEPES pH 7.5, 10% (v/v) glycerol, 150 mM NaCl, and 1 mM TCEP). The peak fractions were pooled and concentrated again to approximately 1-2 mg/mL. The biotinylation of the protein was confirmed by mass spectrometry. FL Flag-tagged CRBN and FL His-tagged DDB1 (CRBN/DDB1) were cloned into pFastbac1, transposed into DH10Bac cells, and used to generate baculovirus. SF9 cells were co-infected with CRBN and DDB1 and expression was allowed to proceed for 48-72 hours before harvest. Protein was purified by Flag and His affinity chromatography, followed by size-exclusion chromatography. CRBN/DDB1 was used for preliminary and confirmatory studies.

TR-FRET Ternary Complex Formation Assay 800 nM (4x) biotinylated BTK was labelled with 80 nM Streptavidin-tagged Terbium Cryptate Lumi4-Tb (#610SATLB, Cisbio, Bedford, MA) whereas 2000 nM (4x) biotinylated CRBN was labelled with 1000 nM (4x) Streptavidin-tagged XLA665 (#610SAXLB, Cisbio, Bedford, MA). Proteins and their respective labels were incubated for 30 mins prior to quenching with 1 mM streptavidin. Dose-response curves were

obtained by adding increasing concentrations of PROTACs spanning 0.11nM – 100μM range (0.11, 0.27, 0.67, 1.68, 4.19, 10.49, 26.21, 65.54, 183.84, 409.60, 1024.00, 2560.00, 6400.00, 16000.00, 40000.00, 100000.00nM) to donor-BTK ($[BTK]_{final}$ = 200nM, 5μL/well) and acceptor-CRBN ($[CRBN]_{final}$ = 500nM, 5μL/well) conjugates in 20μL final assay volume in a 384-well low volume plate (Greiner black polystyrene flat bottom wells, #M3686, Sigma-Aldrich, St. Louis, MO) in a buffer containing 50mM Tris pH7.5, 150mM NaCl, 0.005% P20, 10% Glycerol, 2% DMSO. All components were incubated for 30 mins prior to detection using a Tecan microplate reader. Following Tb excitation at 340 nm (excitation bandwidth 20 nm), a 60 μs time delay was introduced to reduce background fluorescence, prior to recording long-lived fluorescence at the donor (Tb, 620 nm) and acceptor (XLA665, 665 nm) emission wavelengths (emission bandwidth 10 nm). The resulting data are reported as a 665/620 ratio and normalized to the maxima of PROTAC (**9**) in order to have a point of reference when comparing various PROTACs' ability to form ternary complex.

SPR for binary interactions On a Biacore SA Chip, BAP-BTK was immobilized on flow cells Fc2 and Fc3 at 4300RU and 4000RU respectively, while Fc4 was used as reference. Similarly, in a duplicate set of experiments, BAP-CRBN was immobilized on Fc2 and Fc3 and 4600RU and 4200RU respectively. The highest concentration of each compound tested was 90 μM (for CRBN binding studies) or 10 μM (for BTK binding studies) in a 3-fold dilution series in running buffer 50 mM Tris pH7.5, 150 mM NaCl, 0.005% P20, 10% Glycerol, 2% DMSO. Care was

taken to match DMSO % across all compound concentration points – compounds were stored in 100% DMSO at 30 mM concentrations. All biosensor data processing and analysis was performed using Scrubber 2.0 (BioLogic Software). For kinetic analysis, data were locally fitted to a 1:1 interaction model.

SPR for ternary interactions Experiments were performed on a Biacore^{TM} 3000 instrument (GE Healthcare). Biotinylated Bap-tagged CRBN was captured onto a streptavidin sensor chip to levels ranging from 1500-4500 RU. Compound binding experiments were performed in 25 mM Tris, pH 7.5, 150 mM NaCl, 0.01% P20, and 2% DMSO at 25ºC. Binding affinity for PROTAC (**10**) to CRBN was determined by injecting compound alone. Samples were tested with N = 2 using the following concentrations: 20 µM, 10 µM, 5 µM, 2.5 µM, 1.25 µM (experiment 1) and 10 µM, 5 µM, 2.5 µM, 1.25 µM, 0.625 µM, 0.312 µM (experiment 2). Binding affinity for the ternary complex formed between CRBN, PROTAC (**10**) and BTK was determined by injecting BTK + PROTAC (**10**) together at equal concentrations. Samples were tested with N = 2 using the following concentrations: 20 µM, 10 µM, 5 µM, 2.5 µM, 1.25 µM (experiment 1) and 10 µM, 5 µM, 2.5 µM, 1.25 µM, 0.625 µM, 0.312 µM (experiment 2). Compounds were injected at a flow rate of 50 uL/min, total contact time of 120 s, and dissociation times of 200 s. Binding responses were processed using Scrubber 2 (BioLogic Software Pty Ltd) to zero, x-aligned, double referenced and corrected for excluded volume effects of DMSO in the data.

Ternary Complex model and alpha calculations In order to calculate theoretical curves in a non-cooperative system, α=1, the model from Douglass *et al*. ([2], equation (S13), also reproduced below) was employed.

$$\frac{1}{\alpha}\Bigg([ABC]^5(\alpha-1)\alpha^2 - [ABC]^4\alpha\begin{pmatrix}\alpha^2(2[A]_t+[B]_t+2[C]_t)+\\ 2\alpha(K_{AB}+K_{BC}-[A]_t-[C]_t)-\\ 2(K_{AB}+K_{BC})\end{pmatrix}+$$
$$[ABC]^3\Bigg(\alpha^3\begin{pmatrix}[A]^2_t+\\ 2[A]_t([B]_t+2[C]_t)+\\ [C]_t(2[B]_t+[C]_t)\end{pmatrix}+$$
$$\alpha^2\begin{pmatrix}-[A]_t([B]_t+3[C]_t-2K_{AB}-3K_{BC})+\\ K_{BC}([B]_t+2[C]_t+K_{AB})-\\ [A]^2_t+[B]^2_t-[B]_t[C]_t-[C]^2_t+\\ [B]_tK_{AB}+3[C]_tK_{AB}\end{pmatrix}+$$
$$\alpha\begin{pmatrix}K^2_{BC}-\\ 2K_{BC}([A]_t+[C]_t+K_{AB})+\\ K_{AB}(K_{AB}-2([A]_t+[C]_t))\end{pmatrix}-(K_{AB}-K_{BC})^2\Bigg)-$$
$$[ABC]^2\alpha\Bigg(\alpha^2([B]_t[C]^2_t+2[A]_t[C]_t(2[B]_t+[C]_t)+[A]^2_t([B]_t+2[C]_t))-$$
$$\begin{pmatrix}[A]_t([B]_t+[C]_t)+\\ [C]_t([C]_t-[B]_t-K_{AB})\end{pmatrix}K_{AB}-\begin{pmatrix}[A]^2_t+[C]_t([B]_t+K_{AB})+\\ [A]_t([C]_t-[B]_t+K_{AB})\end{pmatrix}K_{BC}+$$
$$\alpha\Bigg(-[A]^2_t([B]_t+[C]_t-K_{BC})+$$
$$[A]_t\begin{pmatrix}[B]^2_t+\\ [B]_t(K_{AB}+K_{BC}-2[C]_t)-\\ [C]^2_t+3[C]_t(K_{AB}+K_{BC})+\\ K_{AB}K_{BC}\end{pmatrix}+$$
$$[C]_t\begin{pmatrix}[B]^2_t+\\ [B]_t(K_{AB}+K_{BC}-[C]_t)+\\ K_{AB}([C]_t+K_{BC})\end{pmatrix}\Bigg)+$$
$$[A]_tK^2_{BC}\Bigg)+$$
$$[ABC]\alpha^2[A]_t[C]_t\begin{pmatrix}[B]^2_t+\\ [B]_t(2\alpha[C]_t+K_{AB}+K_{BC}-[C]_t)+\\ [A]_t((2\alpha-1)[B]_t+\alpha[C]_t+K_{BC})+\\ K_{AB}([C]_t+K_{BC})\end{pmatrix}-\alpha^3[A]^2_t[B]_t[C]^2_t\Bigg)=0$$

(1)

For a non-cooperative system (α=1), the equation is simplified to Equation S61 in the Douglass et al. paper [2], also reproduced below (equation 2):

$$[ABC] = \frac{\left(\frac{[A]_t+[B]_t+K_{AB}- \sqrt{([A]_t+[B]_t+K_{AB})^2 -4[A]_t+[B]_t}}{2}\right)\left(\frac{[B]_t+[C]_t+K_{BC}- \sqrt{([B]_t+[C]_t+K_{BC})^2 -4[B]_t+[C]_t}}{2}\right)}{[B]_t}$$

(2)

In a first set of experiments, the following values were substituted in equation 2: K_{AB} and K_{BC} were the binary K_D values measured by SPR and reported in **Fig.3(b)** and **Fig. S5**, A_t and C_t were 200 nM and 500 nM and respectively reflected the concentration of BTK and CRBN used in TR-FRET experiments whereas B_t, being the PROTAC concentration, was variable spanning 0.11nM – 100μM range (0.11, 0.27, 0.67, 1.68, 4.19, 10.49, 26.21, 65.54, 183.84, 409.60, 1024.00, 2560.00, 6400.00, 16000.00, 40000.00, 100000.00nM). We used equation (2) to graph the curves shown in **Fig. S6(a)**, middle. To allow for comparison between different PROTACs, data was shown relative to PROTAC (**9**) whose maxima is normalized to 1.

Comparing the relative values of ABC between PROTACs to the results of the relative TR-FRET signals, we could see that the model reasonably predicted a relationship between longer PROTACs (**6-11**) and ternary complex formation without the need for cooperativity. However, the model was suboptimal when describing curves involving PROTACs (**1-5**) and we wanted to see explicitly how incorporating cooperativity would change the way the model fit the experimental

data. In order to do this, we used equation 1 (an implicit function of both α and ABC) together with the known values of A_t, B_t, C_t, K_{AB} and K_{BC}. Namely, we sought to minimize the SSE (Sum of the Squared Error) between the TR-FRET curve and the ABC curve (generated with equation 1) by varying α using built-in MATLAB functions. In doing so, we found an α for each PROTAC that led to the best fit of the Spiegel model to our data. The α that yielded such minimum SSE was taken to be an estimate of cooperativity in the system. α was also the only parameter that we solved for and its value is shown in **Fig.3(c).**

Computational Modeling A computational workflow was developed, that given a target, E3 ligase, and PROTAC, will generate an ensemble of multiple possible ternary complexes**.** Ternary Complexes Model Construction**:** the 3D ligand structures from corresponding crystal structures in PDB for target and E3 ligase were used to construct PROTAC molecules. Proteins were prepared for modeling using the Protein Preparation workflow in Maestro [3]. Linkers were added by using Pfizer internal 3D model builder tool, and PROTAC molecules were minimized with both BTK binder and E3 binder fragments frozen to their initial conformations. Conformation Sampling**:** PROTACs were further minimized with the OPLS-2005 force field prior to the constrained and restrained conformational searches carried out using MacroModel in Maestro (Schrödinger, NY). Atoms of target binding portion of PROTAC were restrained to their binding positions while all torsion angles in E3 ligand portion were constrained to its binding conformation with a force constant 100 kJ/mol. The mixed torsional and

low-mode sampling was employed with 36 steps per rotational bonds. A 200 kJ/mol energy window was used to allow a larger set of structurally diverse conformers to be saved for further filtering. Ternary Complex Model Identification**:** A python script was developed with OEChem TK (OEChem Toolkit, 2016) that processes the ensemble of solutions generated in the conformational search at multiple steps to either reject or retain conformations as per a steric scoring scheme. Clashes between atoms of protein-protein and protein-PROTAC were recognized, evaluated, and penalized. First, PROTAC conformations that sterically clash with target protein BTK are rejected. In the E3 complex structure (for example, PDB code: 4CI3, 4W9F for CRBN and VHL, respectively) we used the E3 ligand structure for substructure searching/mapping and its coordinates for defining the transformational matrix for structural overlaying. The defined transformational matrix was then applied to E3 ligase protein structure. The removal of conformations of PROTAC that sterically clash with E3 or those have steric clashes between BTK and CRBN was then performed. The resultant set of ternary complexes of BTK-PROTAC-CRBN is then recorded as likely structurally compatible solutions of possible ternary complexes. A simple count of the number of possible solutions obtained was then prioritized over other comparable systems that had fewer or no possible solution.

MS/MS Sequence coverage of BAP-BTK For tandem mass spectrometry (MS/MS) sequence coverage experiment, the purified protein (39 µM) was

diluted to 10 µM using a buffer composed of 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 (referred to as H_2O buffer hereafter). The buffer for HDX on-exchange experiments had the same composition except H_2O was replaced with D_2O (99.9%) (referred to as D_2O buffer hereafter). A total of 5 µL of 10 µM BAP-BTK protein solution was manually mixed with 20 µL of the H_2O buffer and 25 µL of the quench solution containing 3 M urea and 1% (v/v) TFA. The above 50 µL protein sample was then digested online by passing through an immobilized pepsin-coupled column (2.1 mm i.d. x 30 mm, NovaBioAssays, Woburn, MA) and the digest was subjected to high performance liquid chromatography (HPLC) analysis using a trap column (Hypersil Gold C8 5 µm packing, 10 x 1 mm drop-in guard, Thermo Fisher Scientific, San Jose, CA), an analytical column (Betasil C18 5 µm packing, 50 x 1 mm, Thermo Fisher Scientific, San Jose, CA) and 1/16" o.d. x 75 µm i.d. PEEKsil™ tubing (IDEX Health & Science, Oak Harbor, WA). Sample loading, digestion, and desalting were driven by a capillary-scale HPLC pump (Agilent 1100 series, G1312A binary pump, Santa Clara, CA), while gradient elution was performed with a separate HPLC pump of the same made and model. For HPLC, buffer A was H_2O containing 0.1% (v/v) formic acid, buffer B was acetonitrile containing 0.1% (v/v) formic acid. The flow rate was 50 µL/min with a gradient from 0.5% to 40% B over 60 min. The mobile phase for the immobilized pepsin-coupled column was buffer A and the flow rate was 50 µL/min. MS and collision-induced dissociation (CID) MS/MS spectra were acquired in positive ion mode on an Orbitrap Velos Pro hybrid ion trap-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an

ESI source operated at capillary temperature of 250 °C and spray voltage of 4 kV. Mass spectra were acquired for m/z 300-2000 using a maximum injection time of 10 ms and automatic gain control (AGC) target value of 1 x 10^6. MS/MS spectra were acquired using data-dependent acquisition with dynamic exclusion where the top 10 most abundant ions in each scan were selected and subjected to CID. MS/MS spectra were acquired at an activation Q value of 0.25, activation time of 30 ms, normalized collision energy of 35, isolation width of 2 m/z, AGC target value of 1 x 10^4 and maximum injection time of 100 ms. Each MS scan consisted of 1 microscan under normal scan mode and each MS/MS scan was the average of 4 microscans under normal scan mode.

The resulting spectra were searched against the BAP-BTK sequence using MASCOT (version 2.5.1, Matrix Science, London, UK) with peptide mass tolerance of ± 10 ppm and fragment mass tolerance of ± 0.8 Da. Peptides with an ion score of greater than 20 and mass error of less than 3 ppm were included in the peptide set used for HDX. BAP-CRBN sequence was similarly determined.

HDX analysis Before each HDX experiment, the 10 µM BAP-BTK samples were made by diluting 30.8 µL 39 µM BAP-BTK stock using 86.8 µL H_2O buffer with 3.5% glycerol, 2.4 µL DMSO or 500 µM compound (to yield a final compound concentration of 10 µM). The 10 µM BAP-BTK and 15 µM CRBN samples were made by diluting 30.8 µL 39 µM BAP-BTK stock and 31.4 µL 57.4 µM CRBN stock using 55.4 µL H_2O buffer with 3.5% glycerol, 2.4 µL DMSO or 500 µM compound (to yield a final compound concentration of 10 µM). The protein

samples were allowed to equilibrate at 4 °C for 1 h before HDX analysis. For HDX MS experiments, 5 µL protein solution was mixed with 20 µL of the D_2O buffer (final D_2O content was 80%), or H_2O buffer for 0 s controls, and incubated for various lengths of time at 4 °C, before being added to 25 µL quench solution. Deuterated protein was then digested online using the same procedure as described above. The pepsin column, trap column and analytical column utilized in the HDX MS experiments were the same as in the MS/MS sequence coverage experiment. HPLC was performed with a flow rate of 50 µL/min, and a gradient proceeded by a 0.5% to 10% B over 0.2 min ramp followed by a 10% to 30% B gradient over 5.3 min. MS spectra were acquired in the range of m/z 300-1700 in positive ion mode on the Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an ESI source operated at capillary temperature of 250 °C and spray voltage of 4 kV. Protein and quench solutions, trap and analytical columns were kept at 4 °C inside of the chromatography refrigerator (Isotemp^{TM}, Fisher Scientific, Waltham, MA). The online pepsin column was kept at 15 °C using a temperature controller (Analytical Sales & Services, Pompton Plains, NJ). HDX incubation was performed at 4 °C for 6 different time points: 10, 30, 60, 300, 900, and 3600 s. The experiments were performed at random order and the data for each on-exchange time point were obtained in 3 replicates. All HDX data were normalized to 100% D_2O content, corrected for an estimated average deuterium recovery of 70%, and processed using the HDX WorkBench version 3.3 [5].

Sample preparation and TMT labelling After compound treatment, Ramos cells were washed repeatedly with PBS and lysed with lysis solution (8 M urea, 100 mM Tris.HCl pH 8.0, proteasome and phosphatase inhibitors). For the rat tissues, extracted proteins were first precipitated with 20°C acetone overnight before being re-dissolved in urea lysis solution. Lysates were then sonicated for 3 minutes using a Qsonica XL-2000 series. Proteins were reduced and alkylated with (dithiothreitol) DTT and (iodoacetamide) IAA respectively. Urea concentration of the lysate solution was reduced to 4 M by adding 100 mM ABC buffer. Proteins were digested first with LysC for overnight at room temperature and followed with trypsin digestion for another 12 hours at 37°C. Digestion process was stopped by acidifying with TFA. Tryptic peptides were desalted with Sep-Pak C-18 (Waters, Milford, MA). Desalted peptides were quantified and chemically labeled with Tandem Mass Tag (TMT) 10-plex isobaric reagents (ThermoFischer Scientific, Waltham, MA) according to the manufacturer provided protocols [6]. Chemically labelled peptides from different experimental treatment times were combined and fractionated using high pH reversed phase chromatography with a 4.6 x 250 mm Xbridge C-18 column (Waters, Milford, MA) connected to an Åkta FPLC system (GE Healthcare, Pittsburg, PA) [7, 8]. Eluting chemically labelled TMT peptides were collected and pooled to give 20 fractions.

LC/MS/MS were performed using a Waters Nano Equity LC coupled to a Lumos Orbitrap mass analyzer. Peptide separation was carried out with an Easy-Spray 50cm column prepacked with 2µM C-18 resin (ThermoFisher, Waltham, MA).

Mass spectrometric analyses were carried out in positive ESI automatically switching between survey (MS) and fragmentation (MS/MS) modes for every 3 seconds. Both survey and fragmentation spectral scans were acquired in the Orbitrap analyzer, with resolution preset at R = 60,000 and R= 50,000, respectively. The most intense spectral peaks with assigned charge states of ≥ 2 were fragmented by higher energy collision dissociation (HCD) at a threshold 38 NCE. Isolation window was set at 0.7 m/z while the dynamic exclusion was set at 90 s. Automatic Gain Control (AGC) target was set for 3.0x105 ions with a maximum injection time of 200 ms.

MS data processing Protein identification and quantitation were performed using MaxQuant (1.5.5.1) computational platform [9]. Peptide identification was carried out using Andromeda search engine [10] by querying the Uniprot human or rat fastas. Report ion MS2 with TMT-10 plex default option was selected. LysC and trypsin were selected as digestion enzymes. Variable modifications were acetyl (protein-N-term), and oxidation (M) while Carbamidomethyl (C) was selected as fixed modification. Mass deviation thresholds for first search and main searches were 20 ppm and 10 ppm, respectively. False Discovery Rate (FDR) was set at 0.05 for both Peptide-Spectrum-Match (PSM) and protein. Search results were subsequently imported to Perseus (1.5.6.0) [11] for further bioinformatics analyses. Proteins that were categorized as reverse and potential contaminants were first removed. Log2 transformed were then performed for

entries for each of the replicates. Normalization was performed by subtracting the median. T-tests were performed with both sides and FDR value was set to 0.05. Absolute protein concentrations (pmol/mg total protein) in Ramos cells and Rat tissues were determined by The Total Protein Approach (TPA) [12]. The corrected signals corresponding to proteins from the control samples were used to compute protein concentrations. Data and search files will be deposited in the ProteomeXchange via the PRIDE database.

Animals Male Wistar-Hannover rats (weighing 240–350 g) with surgically implanted vascular cannulas in the carotid artery were purchased from Taconic Biosciences (Hudson, NY). Rats were housed one per cage in an American Animal Association Laboratory Animal Care accredited facility and maintained under standard conditions of temperature (22 °C ± 2°C), relative humidity (50%) and light and dark cycle (12/12 hours). Rats were allowed to acclimate to their environment for 1 week. Subcutaneous (SC) dosed rats were fasted overnight prior to dosing and had access to water ad libitum. On the day of study, food was with- held for 4 hours after administration of the initial dose. All animal experiments were in accordance with the animal care and use committee of Pfizer Inc.

Protocol for tissue exposure studies in rat The pharmacokinetic profile of PROTAC (**10**) following SC administration was evaluated in Wistar-Hannover rats (240–350 g). The animals were randomly distributed into five experimental

groups (n=2). SC dosed animals were fasted overnight prior to drug administration with free access to water. PROTAC (**10**) or vehicle only were formulated in a non-aqueous vehicle. The SC treatment groups were dosed with a single dose of 0, 0.35, 35, 175 mg/kg; or 175 mg/kg at 0hr and 24hr via mid back. The injection volume of was 1 or 5 mL/kg. Blood samples (0.25 mL) were withdrawn from the carotid artery predose, and at the following time points after dosing, 0.25, 0.5, 1, 2, 4, 7, 24 hrs for groups 0.35, 35, and 175 mg/kg, and up to 48 hr for group 175 mg/kg when given at 0 and 24hr, for PROTAC (**10**) bioanalysis. After each blood draw, a volume of sterile normal saline (0.9% sodium chloride, USP containing 100 unit/mL heparin) equivalent to the volume of blood drawn was injected via carotid artery cannula to maintain a constant blood volume (0.25 mL). All blood samples were immediately centrifuged at 3000 rpm for 10 minutes. The resultant plasma was stored at –70°C until PROTAC (**10**) Liquid Chromatography-Mass Spectrometry (LC/MS/MS) analysis. Pancreas, lung, and spleen tissues were also harvested at the 24 hrs timepoint for the 0.35, 35, and 175 mg/kg groups at the end of the in-life study and 48hrs for the 175 mg/kg twice dosing group. Tissues were rinsed with saline and matted dry then divided in collection tubes (one half for exposure analysis and other half for BTK analysis). They were kept frozen on dry ice or -80°C. The exposure evaluation of PROTAC (**10**) was performed using Watson v.7.5 (ThermoFisher Scientific, Philadelphia, PA).

For tissue BTK anaylsis; lung and spleen tissues were thawed on ice and placed into Eppendorf tubes with BTK lysis buffer (2 ml per 1 gm tissue). Tissues

were homogenized with a mini beadbeater using 2.0 mm zircona beads for 2 mins (Biospec Products, Bartlesville, OK) in lysis buffer (150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 1 mM ethylene diamine tetraacetic acid, 1 mM phenyl methyl sulfonyl fluoride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, protease inhibitor cocktail, benzonase) and the homogenates were centrifuged (15,000g, 10 mins, 4 °C) and transferred to new tubes. Bicinchoninic Acid (BCA) assay (ThermoFisher Scientific, Philadelphia, PA) was used to quantify total protein. 5 µL of 0.2 µg/µL total protein lysate from each tissue sample collected, was loaded onto Wes (ProteinSimple, San Jose, CA) and blotted for BTK (1:50 dilution, #8547 Cell Signalling Technology, Boston, MA) and Vinculin (1:200 dilution, #NC0769989 Fisher Healthcare, Houston, TX).

LC/MS/MS analysis of PROTAC (**10**) LC/MS/MS analysis was carried out for PROTAC (**10**) using a Ultra performance liquid chromatography (UPLC) Acquity system (Waters, Milford, MA) interfaced to an API 6500 quadrupole tandem mass spectrometer (Applied Biosystems/ MDS Sciex Inc., Ontario, Canada).

A 20 µL aliquot of plasma/tissue sample (including dilutions) was precipitated with 100 µL of acetonitrile containing internal standard. The tissues were homogenized using a 1:4 dilution in 60:40 isopropyl:water with a mini beadbeater using 2.0 mm zircona and steel beads for 2 mins (Biospec Products, Bartlesville, OK). All samples were extracted using a mixed matrix approach where equal aliquots of samples and standards (plasma or homogenate) were subjected to protein preceipitation with acetonitrile containing internal stardard.

Samples were then centrifuged at 3000 rpm with a standard laboratory centrifuge (Beckman Coulter, Brea, CA). The supernatant (50 µL) was collected in a clean 96-well collection plate which also contained 150 µL of water. The samples were then vortexed for 1 min and 10 µL was injected onto LC/MS/MS system for analysis. PROTAC (**10**) and internal standard were separated on a Waters Acquity UPLC HSS T3 Column (2.1×50 mm, 1.8 µm) (Mildford, MA) by gradient elution. Diclofenac was the internal standard used for the in vivo work performed. The mobile phase consisted of solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The gradient was as follows: solvent B was held at 5% for 0.3 minute, linearly ramped from 5% to 95% in 2.0 minutes, held for 0.3 mintues, and then immediately brought back to 5% for re-equilibration for 0.3 minute. A flow rate of 0.65 mL/min was used. The mass spectrometer was operated in positive ion ESI mode for the detection of PROTAC (**10**) and the internal standard (diclofenac). Multiple reaction monitoring was performed with the transitions m/z 489.2→172.1 for PROTAC (**10**) and m/z 296.1→214 for diclofenac. All raw data were processed using Analyst Software V. 1.6.2. (Applied Biosystems/MDS Sciex Inc., Ontario, Canada).

The PK profile at increasing doses of PROTAC (**10**) (**Fig. 5d**) suggests sustained release at 35 and 175 mg/kg dose with plateauing of levels beyond 9 hours indicating change in release kinetics. Drug forms a reservoir at the injection site and slowly releases providing sustained release. Typically for a lipophilic molecule such as PROTAC (**10**), the primary mechanism for release is

believed to be partitioning of drug from the non-aqueous vehicle at the injection site into lymphatics [13].

Native State Mass Spectrometry (Native Electrospray Ionization (ESI)) The purified protein samples were buffer exchanged to 200 mM ammonium acetate buffer (pH = 7.5) using microcentrifuge filter units (Millipore, Bedford, MA). The two protein samples were then mixed with PROTAC (**10**) and incubated for 1-2 hours at room temperature before MS measurements. ~ 5 µL sample was loaded into an offline electrospray capillary (GlassTip 4 µm i.d.; New Objective, Woburn, MA), and high voltage 1.5–2.3 kV was applied together with a low pressure gas at the back end of the capillary to start and maintain a stable nanoelectrospray. The hybrid ion-mobility quadrupole time-of flight mass spectrometer (SYNAPT G2 HDMS; Waters, Milford, MA) was used and operated in the sensitivity mode with ESI source parameters tuned to preserve the binary and ternary complexes intact and maintain sensitivity. Parameters used include source temperature 40 °C, sampling cone 80 V, trap potential 5V, and backing pressure of 5–6 mbar. The instrument was externally calibrated using CsI solution for up to m/z 8000, and the data processing was performed using MassLynx (ver. 4.1, Waters).

Compound synthesis and characterization

BTK compound reference: Springer, J. R. U.S. Patent 61,721,920, 2012.

VHL compound reference: Borzilleri, R. M. US Patent 61,702,384, 2012.

IAP compound reference: Crews. C. M. US Patent 61,585,769, 2012.

General Carbonate Procedure:

Method A: 9-Fluorenylmethyl pentafluorophenyl carbonate (1 eq) was added to a solution of phthalimide alcohol (1 eq), and trimethylamine (30 eq) in tetrahydrofuran (0.2 M) and the reaction was stirred at rt for 1 h. The reaction turned to a purple color during this time. To the reaction was added 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (1 eq) and the reaction was stirred at rt until complete conversion was achieved as monitored by TLC and/or LC. The reaction was quenched with sat. aqueous sodium bicarbonate and extracted with dichloromethane. The organic layers were combined, dried, filtered, and concentrated *in vacuo*. The crude material was purified via flash chromatography to afford the desired product.

Method B: 9-Fluorenylmethyl pentafluorophenyl carbonate (1 eq) was added to a solution of azide alcohol (1 eq), and diisopropylethylamine (22 eq) in tetrahydrofuran (0.2 M) and the reaction was stirred at rt for 1 h. The reaction turned to a purple color during this time. To the reaction was added 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (1 eq) and the reaction was stirred at rt until complete conversion was achieved as monitored by TLC and/or LC. The reaction was quenched with sat. aqueous sodium bicarbonate and extracted with dichloromethane. The

organic layers were combined, dried, filtered, and concentrated *in vacuo*. The crude material was purified via flash chromatography to afford the desired product.

General Phthalimide Deprotection:

Hydrazine hydrate (1 eq) was added to a solution of phthalimide (1 eq) in dioxane (0.08 M) and the reaction was stirred at 50 ˚C until complete conversion was achieved as monitored by TLC and/or LC. The reaction mixture was concentrated *in vacuo*. The desired product was purified via trituration, and/or chromatography.

General SnAr Procedure:

Amine (1 eq) was added to a solution of 2-(2,6-dioxopiperidin-3-yl)-4-fluoro-1*H*-isoindole-1,3(2*H*)-dione (1 eq) and diisopropylethylamine (3 eq) in dimethyl sulfoxide (0.15 M). The reaction was stirred at 110 ˚C until complete conversion was achieved as monitored by TLC and/or LC. The reaction was cooled to rt and the crude material was purified via prep HPLC or C18 flash chromatography to afford the desired product.

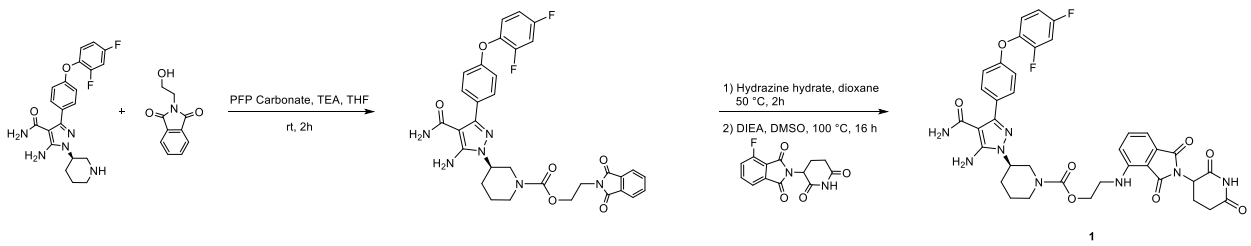
General Amide Coupling:

5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (1 eq) was added to a solution of phthalimide acid (1eq), HATU (1.5 eq), and diisopropylethylamine (5 eq) in dimethylformamide (0.1 M). The reaction was stirred at rt until complete conversion was achieved as monitored by TLC and/or LC. The reaction was extracted with ethyl acetate and washed with brine. The combined organic layers were combined, dried, filtered, and concentrated *in vacuo*. The crude material was purified via flash chromatography to afford the desired product.

General Staudinger Reaction:

Triphenyl phosphine (1.5 eq) was added to a solution of azide (1 eq) in tetrahydrofuran (0.1 M) followed by addition of water (1 eq). The reaction was stirred at rt until complete conversion was achieved as monitored by TLC and/or LC. The reaction was extracted between 1N hydrochloric acid and ethyl acetate. The aqueous layer was basified to pH = 14 with a 50% aqueous sodium hydroxide solution and extracted with ethyl acetate. This organic layer was dried, filtered, and concentrated *in vacuo* to yield the crude desired product which was used without further purification.

Preparation of 2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (1).



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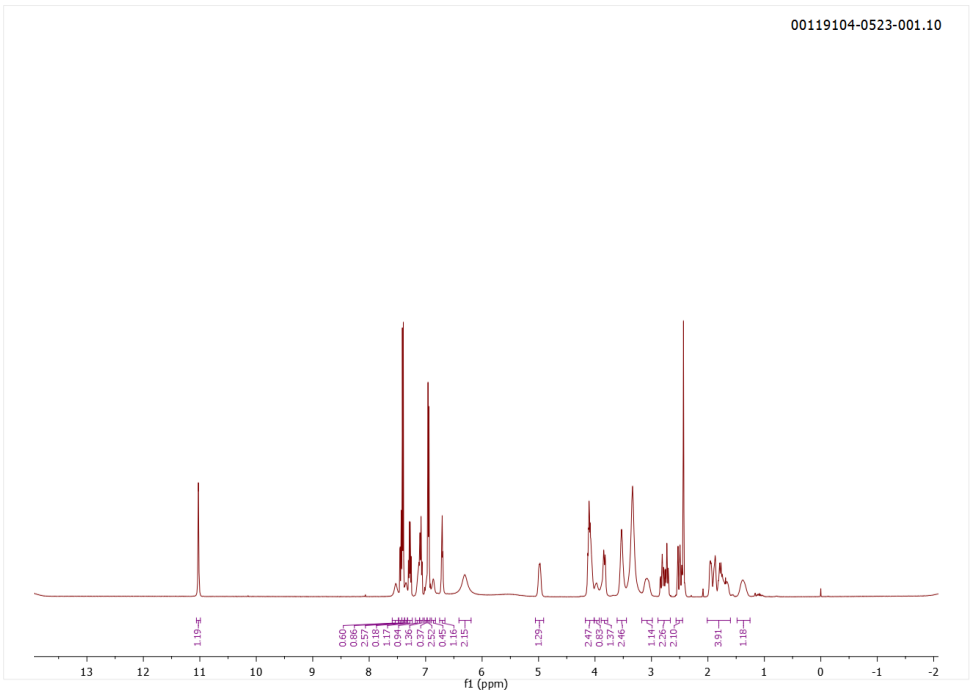
2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. The title compound (138 mg, 60%) was obtained as a white solid from 2-(2-hydroxyethyl)-1*H*-isoindole-1,3(2*H*)-dione using a procedure analogous to that described in the Carbonate Procedure, Method A. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.84 (s, 4H), 7.56 – 7.50 (m, 1H), 7.50 – 7.44 (m, 2H), 7.36 (td, *J* = 9.2, 5.6 Hz, 1H), 7.19 – 7.12 (m, 1H), 7.03 (d, *J* = 8.2 Hz, 2H), 6.27 (d, *J* = 49.4 Hz, 2H), 4.25 – 4.15 (m, 1H), 4.11 (tt, *J* = 10.7, 4.2 Hz, 1H), 3.95 (s, 2H), 3.85 (s, 3H), 3.10 (s, 1H), 2.73 (s, 1H), 1.93 (t, *J* = 7.7 Hz, 1H), 1.84 (qd, *J* = 12.6, 3.8 Hz, 1H), 1.73 (s, 1H), 1.41 (q, *J* = 13.1, 12.1 Hz, 1H). LCMS: m/z 631.3 $[M+H]^+$.

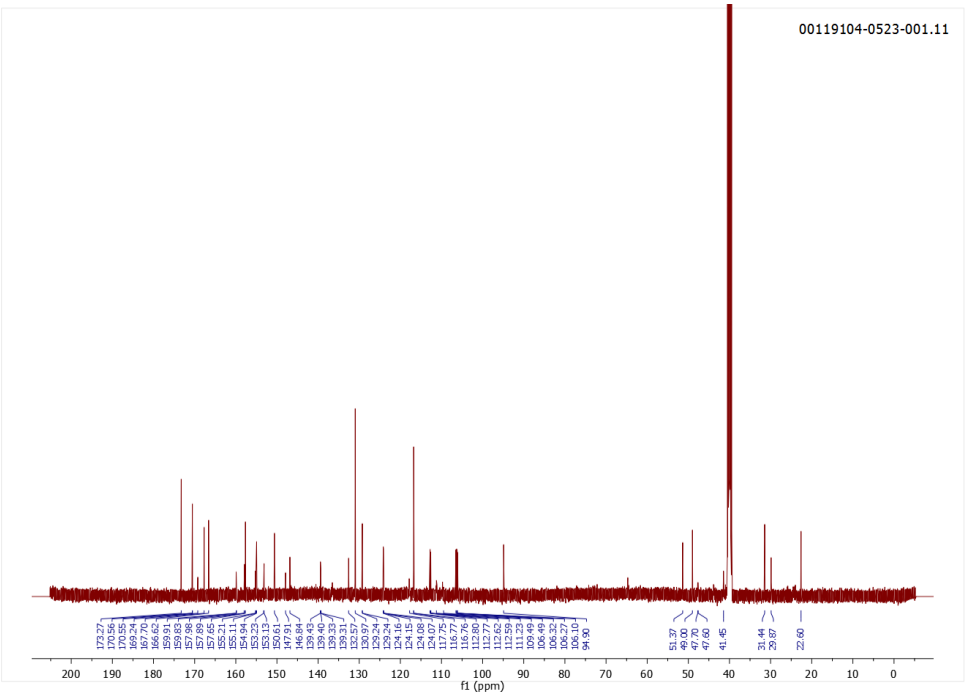
2-aminoethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. The title compound (60 mg, 70%) was obtained as a white solid from 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the Phthalimide Deprotection Procedure. LCMS: m/z 523.0 $[M+Na]^+$.

2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (1). The title compound (44 mg, 48%) was obtained as a yellow solid from 2-aminoethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the SnAr Procedure. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 11.02 (s, 1H), 7.53 (s, 0.5H), 7.48 – 7.42 (m, 1H), 7.40 (d, *J* = 8.4 Hz, 2H), 7.35 (s, 0.5H), 7.28 (td, *J* = 9.2, 5.6 Hz, 1H), 7.18 – 7.11 (m, 1H), 7.11 – 7.05 (m, 1H), 7.02 – 6.97 (m, 0.5H), 6.95 (d, 2H), 6.86 (s, 0.5H), 6.71 (t, *J* = 6.4 Hz, 1H), 6.31 (s, 2H), 4.98 (dd, *J* = 12.8, 5.9 Hz, 1H), 4.17 – 4.01 (m, 3H), 3.98 (s, 1H), 3.84 (d, *J* = 13.2 Hz, 1H), 3.53 (s, 2H), 3.08 (s, 1H), 2.88 – 2.67 (m, 2H), 2.56 – 2.45 (m, 2H), 2.01 – 1.60 (m, 4H), 1.38 (s, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) (observed) δ 173.27, 170.56, 169.24, 167.70, 166.62, 158.90 (dd, *J* = 243.6, 10.7 Hz), 157.65, 154.94, 154.17 (dd, *J* = 250.4, 12.9 Hz), 150.61, 147.91, 146.84, 139.37 (dd, *J* = 11.7, 3.7 Hz), 132.57, 130.97, 129.24, 129.24, 124.11 (dd, *J* = 10.0, 1.9 Hz), 117.75, 116.77, 112.69 (dd, *J* = 23.0, 3.8 Hz), 111.23, 109.49, 106.29 (dd, *J* = 27.5, 22.3 Hz), 94.90, 51.37, 49.00, 47.70, 41.45, 31.44, 29.87, 22.60. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.28, -126.18. LCMS: m/z 757.0 $[M+H]^+$.

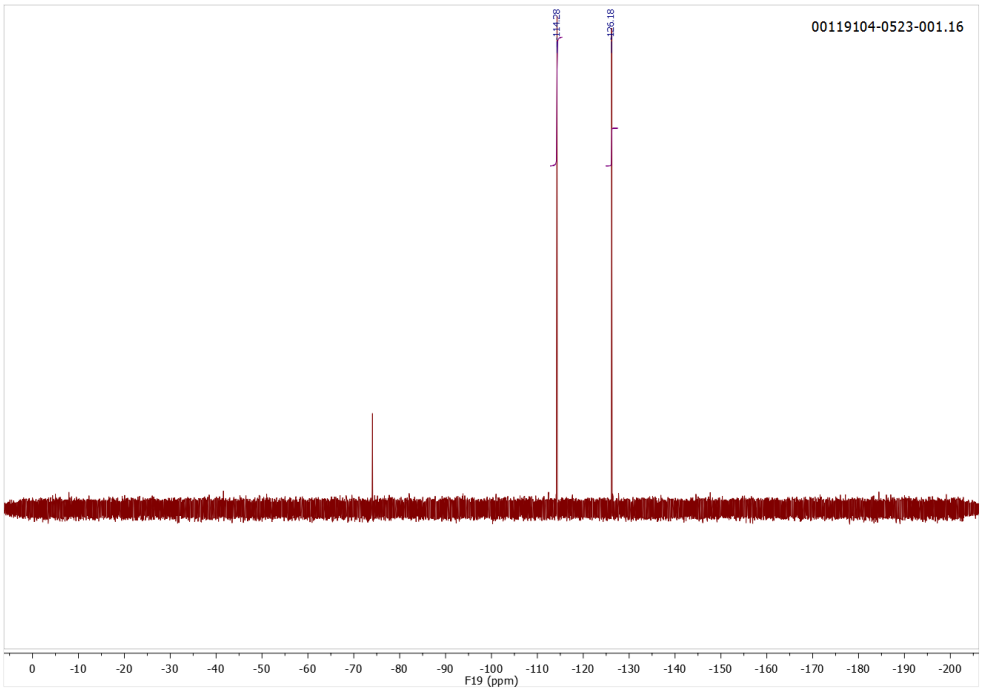
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



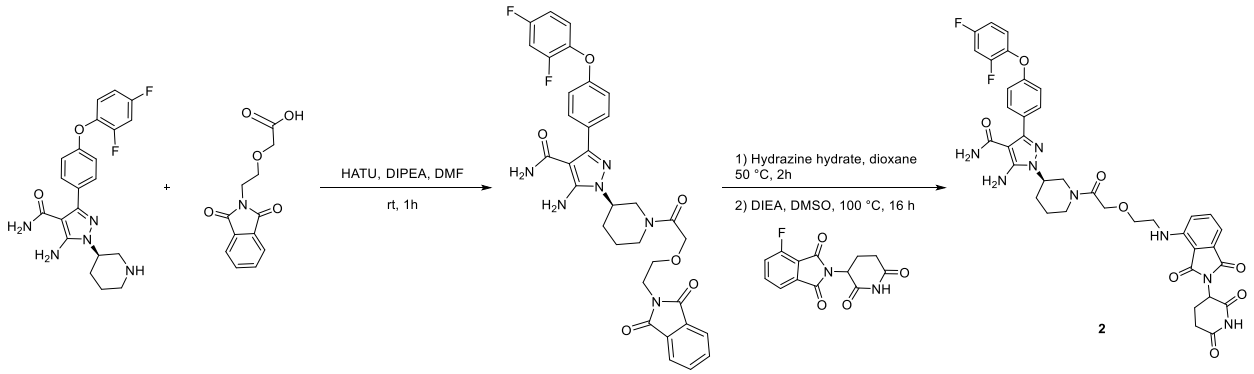
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-{(3*R*)-1-[(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}ethoxy)acetyl]piperidin-3-yl}-1*H*-pyrazole-4-carboxamide (2).



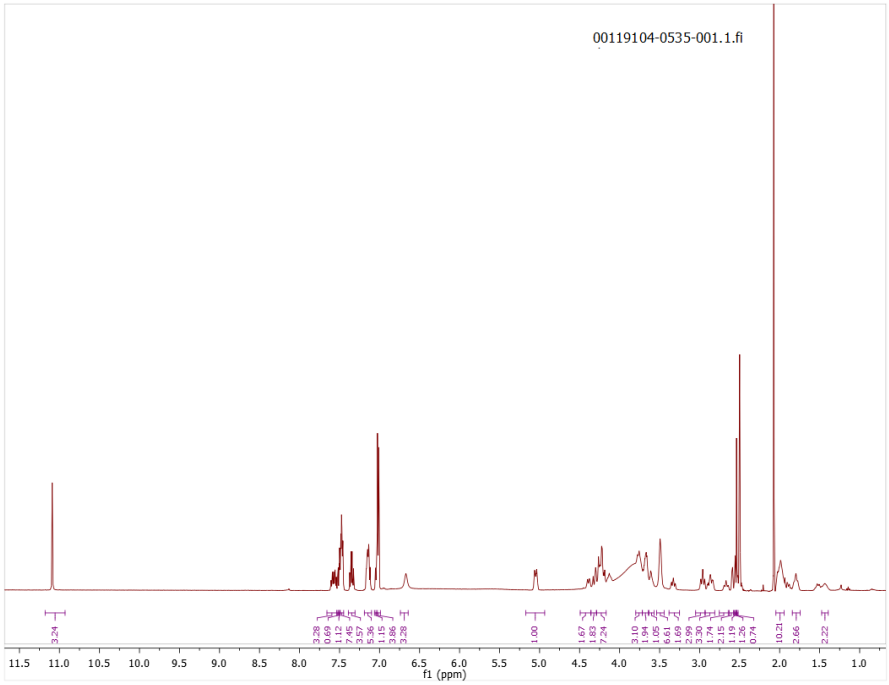
5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-1-{[2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethoxy]acetyl}piperidin-3-yl]-1*H*-pyrazole-4-carboxamide. The title compound (227 mg, 58%) was obtained as a solid from [2-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)ethoxy]acetic acid using a procedure analogous to that described in the Amide Coupling. LCMS: m/z 645.2 $[M+H]^+$.

5-amino-1-{(3*R*)-1-[(2-aminoethoxy)acetyl]piperidin-3-yl}-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazole-4-carboxamide. The title compound (49 mg, 78%) was obtained as a white solid from 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-1-{[2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethoxy]acetyl}piperidin-3-yl]-1*H*-pyrazole-4-carboxamide using a procedure analogous to that described in the Phthalimide Deprotection Procedure. LCMS: m/z 515.1 $[M+H]^+$.

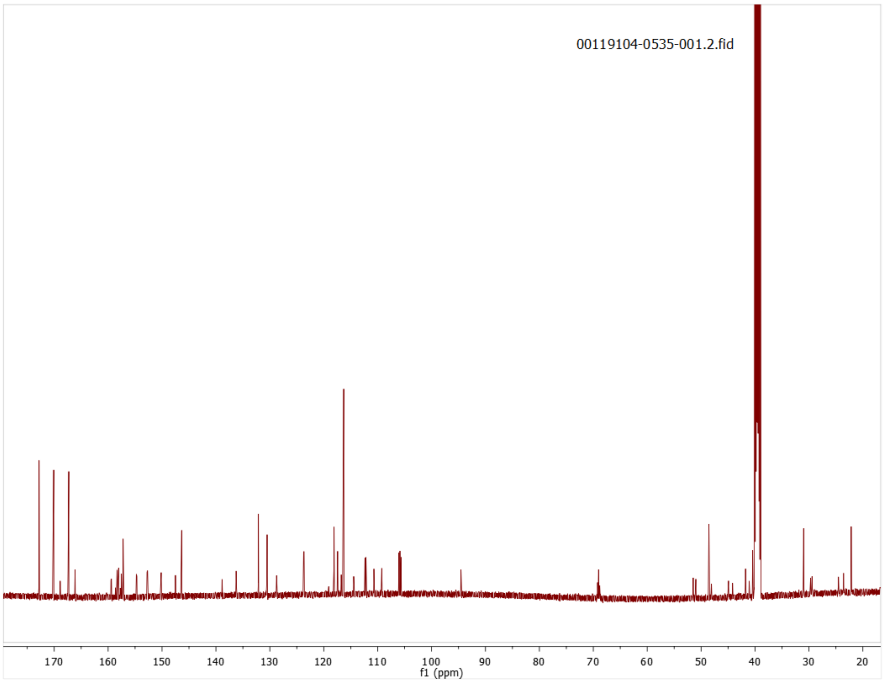
5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-{(3*R*)-1-[(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-

yl]amino}ethoxy)acetyl]piperidin-3-yl}-1*H*-pyrazole-4-carboxamide (2). The title compound (21 mg, 29%) was obtained as a yellow solid from 5-amino-1-{(3*R*)-1-[(2-aminoethoxy)acetyl]piperidin-3-yl}-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazole-4-carboxamide using a procedure analogous to that described in the SnAr Procedure. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.09 (s, 3H), 7.66 – 7.53 (m, 3H), 7.52 (d, *J* = 3.0 Hz, 1H), 7.50 (t, *J* = 2.7 Hz, 1H), 7.47 (td, *J* = 5.9, 2.1 Hz, 7H), 7.35 (td, *J* = 9.2, 5.6 Hz, 4H), 7.19 – 7.10 (m, 5H), 7.04 (d, *J* = 6.8 Hz, 1H), 7.03 (s, 4H), 6.67 (s, 3H), 5.05 (d, *J* = 12.6 Hz, 1H), 4.39 (d, *J* = 12.9 Hz, 2H), 4.36 – 4.29 (m, 2H), 4.29 – 4.17 (m, 7H), 3.77 (d, *J* = 11.4 Hz, 3H), 3.72 – 3.64 (m, 2H), 3.61 (q, *J* = 5.1, 4.7 Hz, 1H), 3.49 (s, 7H), 3.33 (t, *J* = 11.8 Hz, 2H), 2.96 (t, *J* = 12.0 Hz, 3H), 2.93 – 2.81 (m, 3H), 2.67 (t, *J* = 12.7 Hz, 2H), 2.59 (d, *J* = 3.1 Hz, 2H), 2.56 (s, 1H), 2.54 (s, 1H), 2.53 (d, *J* = 4.4 Hz, 1H), 1.99 (s, 10H), 1.80 (s, 3H), 1.44 (s, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.81, 170.09, 168.89, 168.85, 167.43, 167.37, 167.30, 166.15, 166.12, 158.46 (dd, *J* = 243.6, 10.9 Hz), 158.34, 158.05, 157.22, 153.73 (dd, *J* = 249.6, 13.0 Hz), 150.16, 150.10, 147.51, 147.46, 146.36, 138.90 (dd, *J* = 12.2, 3.7 Hz), 136.25, 136.21, 132.12, 130.54, 130.51, 128.78, 128.75, 123.69 (dd, *J* = 10.1, 2.0 Hz), 118.09, 117.43, 116.76, 116.30, 114.44, 112.25 (dd, *J* = 22.9, 3.8 Hz), 109.25, 105.85 (dd, *J* = 27.5, 22.5 Hz), 94.55, 94.49, 69.21, 69.02, 68.83, 51.46, 50.98, 48.56, 48.08, 44.91, 44.17, 41.74, 41.08, 40.43, 30.98, 29.68, 29.42, 24.52, 23.57, 22.16. ^{19}F NMR (471 MHz, DMSO) δ -114.29, -126.18. LCMS: m/z 771.1 $[M+H]^+$.

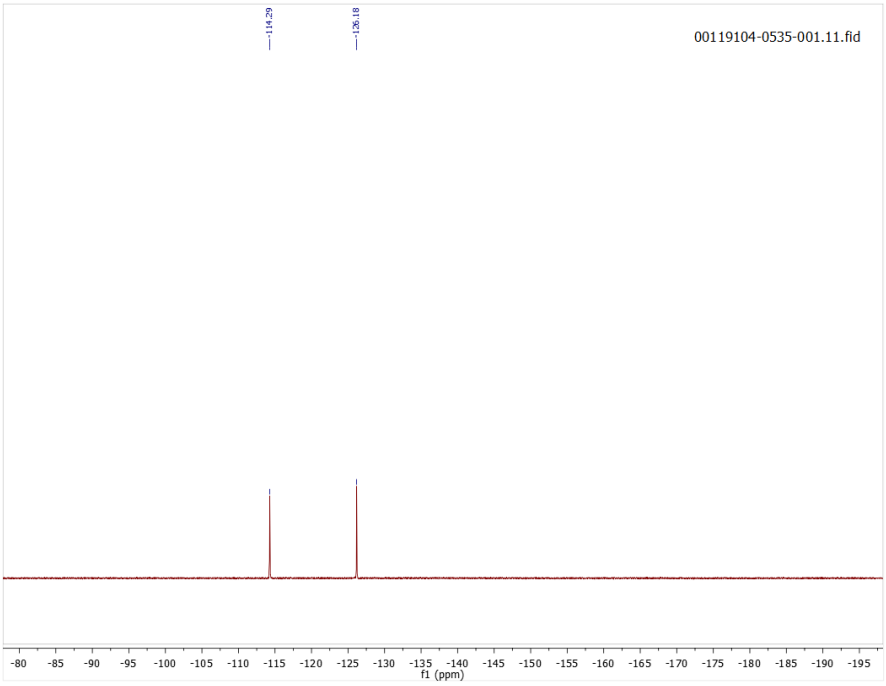
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



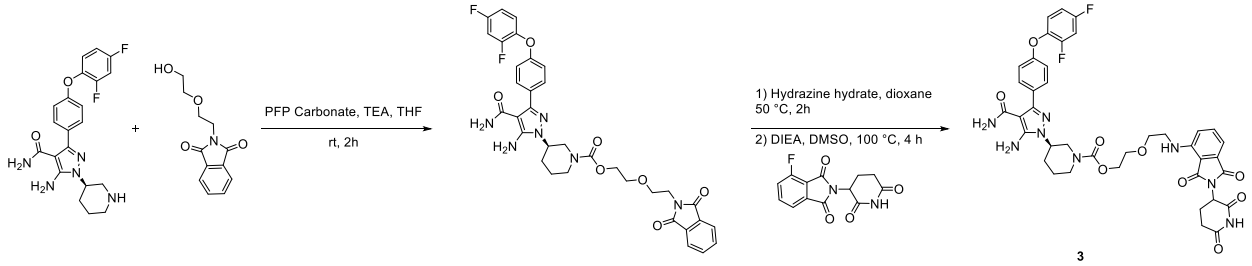
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 2-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}ethoxy)ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (3).



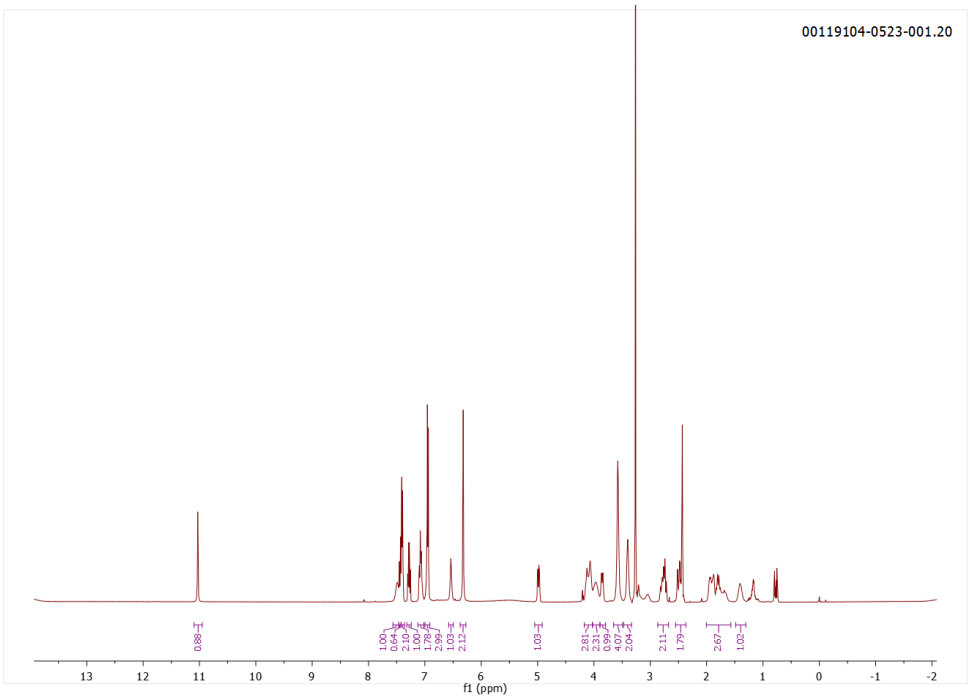
2-[2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethoxy]ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. The title compound (160 mg, 65%) was obtained as a white solid from 2-[2-(2-hydroxyethoxy)ethyl]-1*H*-isoindole-1,3(2*H*)-dione using a procedure analogous to that described in the Carbonate Procedure, Method A. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.84 (d, *J* = 22.6 Hz, 4H), 7.50 (ddt, *J* = 12.2, 8.8, 3.3 Hz, 3H), 7.36 (td, *J* = 9.2, 5.5 Hz, 1H), 7.20 – 7.11 (m, 1H), 7.07 – 6.99 (m, 2H), 6.38 (d, *J* = 10.5 Hz, 2H), 4.26 – 3.90 (m, 4H), 3.90 – 3.53 (m, 7H), 3.10 (d, *J* = 57.9 Hz, 1H), 2.71 (s, 1H), 1.93 (s, 1H), 1.84 (d, *J* = 12.6 Hz, 1H), 1.69 (s, 1H), 1.43 (s, 1H). LCMS: m/z 675.2 $[M+H]^+$.

2-(2-aminoethoxy)ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. The title compound (83 mg, quant.) was obtained as a colorless oil from 2-[2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethoxy]ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a

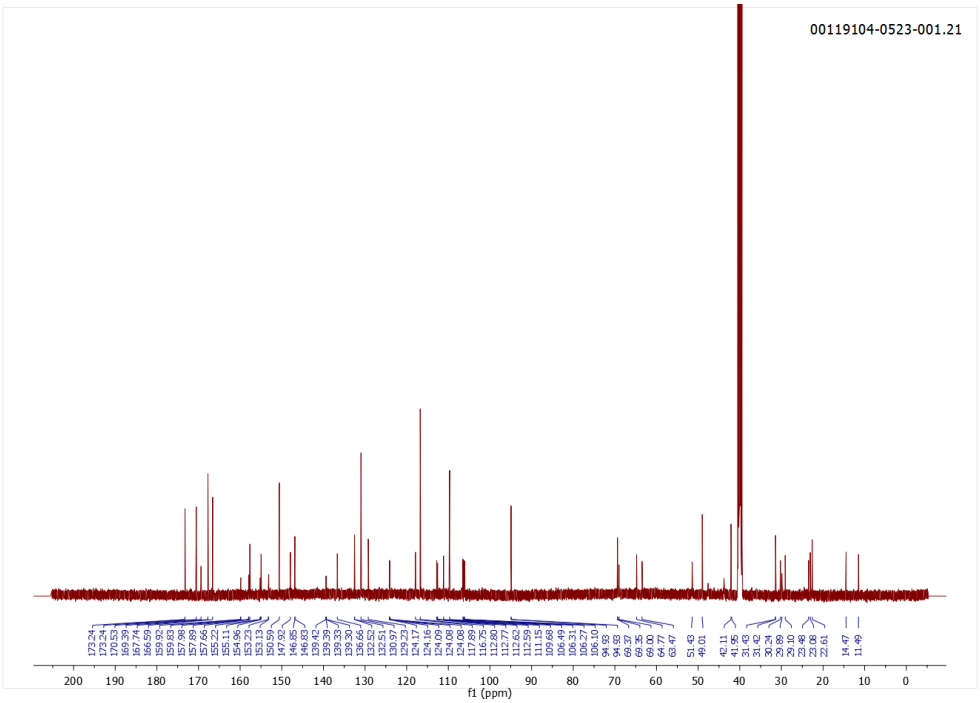
procedure analogous to that described in the Phthalimide Deprotection Procedure. LCMS: m/z 545.1 $[M+H]^+$.

2-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}ethoxy)ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (3). The title compound (42 mg, 26%) was obtained as a solid from 2-(2-aminoethoxy)ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the SnAr Procedure. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 11.03 (s, 1H), 7.49 (s, 1H), 7.46 – 7.42 (m, 1H), 7.42 – 7.36 (m, 2H), 7.28 (td, *J* = 9.2, 5.6 Hz, 1H), 7.08 (tdd, *J* = 9.2, 3.2, 1.7 Hz, 2H), 7.00 – 6.91 (m, 2H), 6.54 (t, *J* = 5.9 Hz, 1H), 6.32 (s, 2H), 4.98 (ddd, *J* = 13.1, 5.5, 2.2 Hz, 1H), 4.10 (d, *J* = 28.6 Hz, 3H), 3.97 (s, 2H), 3.85 (d, *J* = 12.9 Hz, 1H), 3.58 (s, 4H), 3.40 (s, 2H), 2.87 – 2.68 (m, 2H), 2.55 – 2.37 (m, 3H), 1.82 (ddd, *J* = 59.2, 40.5, 32.8 Hz, 4H), 1.41 (s, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) (observed) δ 173.24, 173.24, 170.53, 169.39, 167.74, 166.59, 158.90 (dd, *J* = 243.7, 10.7 Hz), 157.66, 155.16 (dd, *J* = 249.3, 13.1 Hz), 154.96, 150.59, 147.92, 146.85, 146.83, 139.36 (dd, *J* = 11.5, 3.8 Hz), 132.52, 130.97, 129.23, 124.12 (dd, *J* = 9.9, 1.9 Hz), 116.75, 112.69 (dd, *J* = 23.0, 3.7 Hz), 109.68, 106.29 (dd, *J* = 27.5, 22.4 Hz), 94.93, 94.93, 69.37, 69.35, 69.00, 64.77, 63.47, 51.43, 49.01, 42.11, 41.95, 30.24, 29.10, 23.08, 22.61. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.28, -126.17. LCMS: m/z 801.2 $[M+H]^+$.

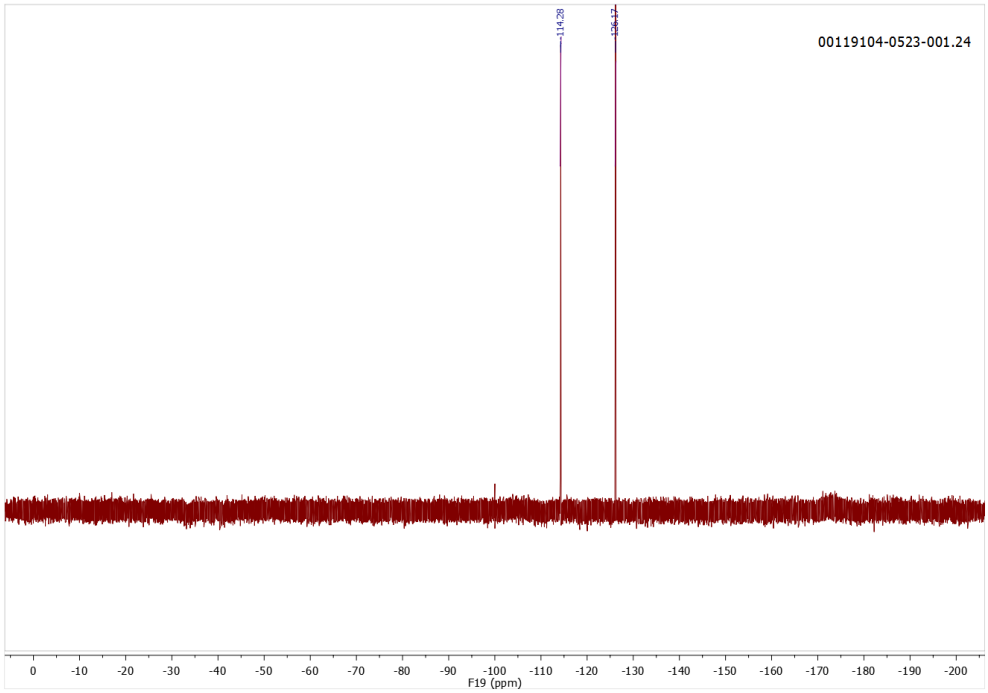
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)

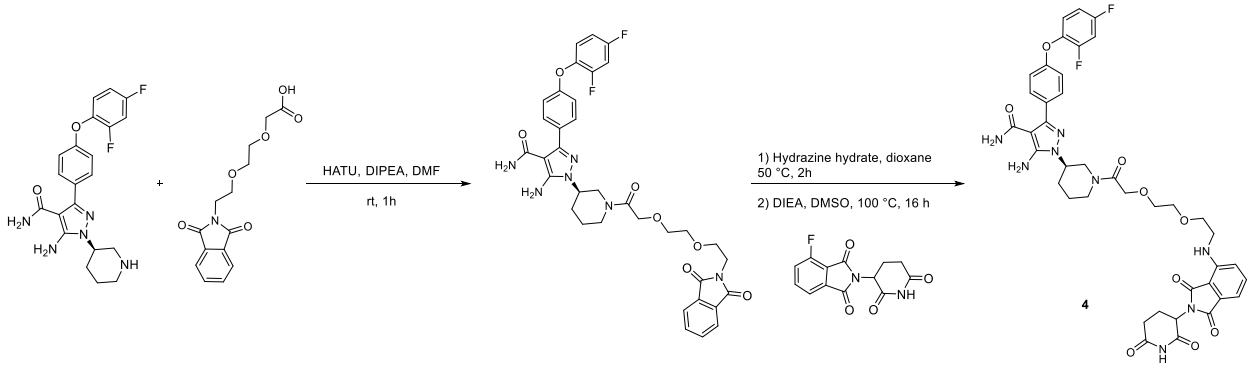


^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-1-{[2-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-

yl]amino}ethoxy)ethoxy]acetyl}piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (4).

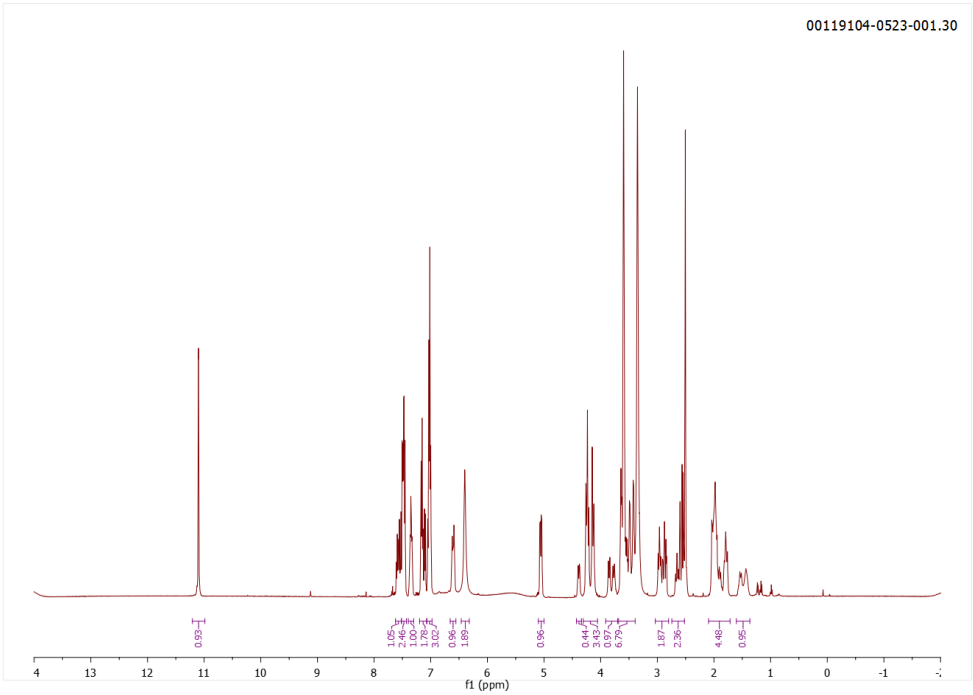


5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-1-({2-[2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethoxy]ethoxy}acetyl)piperidin-3-yl]-1*H*-pyrazole-4-carboxamide. The title compound (106 mg, 39%) was obtained as an oil from {2-[2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethoxy]ethoxy}acetic acid using a procedure analogous to that described in the Amide Coupling. LCMS: m/z 689.3 $[M+H]^+$.

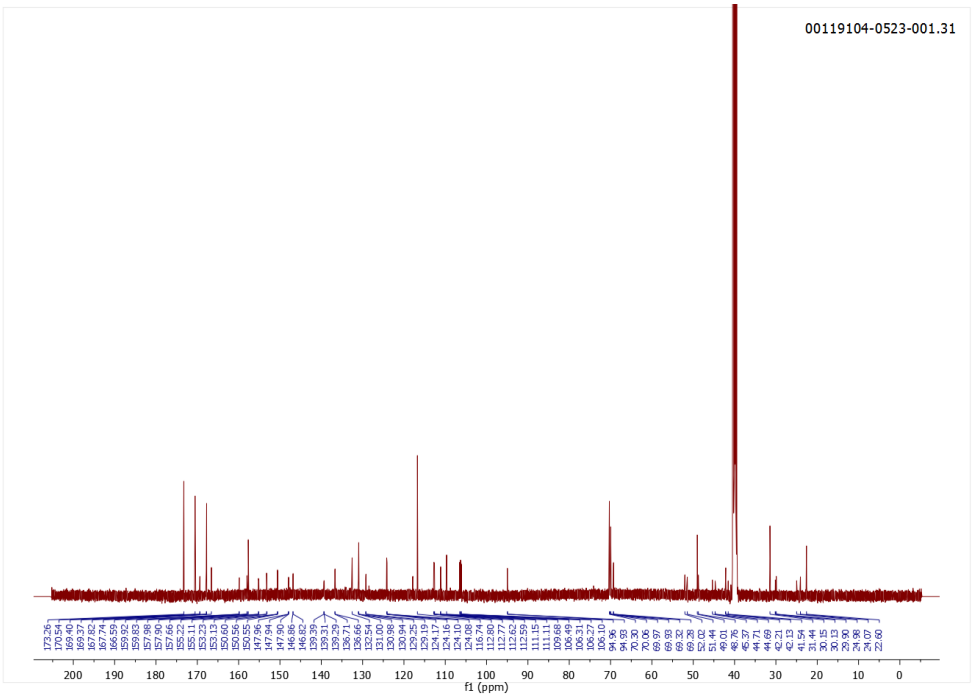
5-amino-1-[(3*R*)-1-{[2-(2-aminoethoxy)ethoxy]acetyl}piperidin-3-yl]-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazole-4-carboxamide. The title compound (69 mg, quant.) was obtained as an oil from 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-1-({2-[2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethoxy]ethoxy}acetyl)piperidin-3-yl]-1*H*-pyrazole-4-carboxamide using a procedure analogous to that described in the Phthalimide Deprotection Procedure. LCMS: m/z 559.1 $[M+H]^+$.

5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-1-{[2-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}ethoxy)ethoxy]acetyl}piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (4). The title compound (35 mg, 30%) was obtained as a solid from 5-amino-1-[(3*R*)-1-{[2-(2-aminoethoxy)ethoxy]acetyl}piperidin-3-yl]-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazole-4-carboxamide using a procedure analogous to that described in the SnAr Procedure. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 11.10 (s, 1H), 7.62 – 7.52 (m, 1H), 7.52 – 7.43 (m, 3H), 7.35 (ddt, *J* = 14.8, 9.5, 5.7 Hz, 1H), 7.20 – 7.07 (m, 2H), 7.03 (q, *J* = 9.6, 8.5 Hz, 3H), 6.60 (dt, *J* = 15.2, 5.9 Hz, 1H), 6.40 (s, 2H), 5.06 (dd, *J* = 12.8, 5.3 Hz, 1H), 4.39 (dd, *J* = 12.5, 4.1 Hz, 1H), 4.31 – 4.06 (m, 4H), 3.81 (dd, *J* = 40.6, 13.3 Hz, 1H), 3.68 – 3.39 (m, 7H), 3.04 – 2.80 (m, 2H), 2.74 – 2.52 (m, 2H), 2.10 – 1.72 (m, 4H), 1.61 – 1.37 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) (observed) δ 173.26, 170.54, 169.37, 167.82, 167.74, 166.59, 158.91 (dd, *J* = 243.6, 10.9 Hz), 157.66, 154.17 (dd, *J* = 249.5, 13.0 Hz), 150.60, 150.55, 147.96, 147.90, 146.86, 146.82, 136.69 (d, *J* = 5.9 Hz), 132.54, 131.00, 130.94, 129.25, 129.19, 124.13 (dd, *J* = 9.9, 1.9 Hz), 116.74, 112.69 (dd, *J* = 23.0, 3.7 Hz), 111.15, 111.11, 109.68, 106.29 (dd, *J* = 27.5, 22.4 Hz), 94.96, 94.93, 70.30, 70.06, 69.97, 69.93, 69.32, 69.28, 52.02, 51.44, 49.01, 48.76, 45.37, 44.69, 42.21, 42.13, 41.54, 31.44, 30.13, 29.90, 24.98, 24.07, 22.60. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.27, -126.17. LCMS: m/z 815.1 $[M+H]^+$.

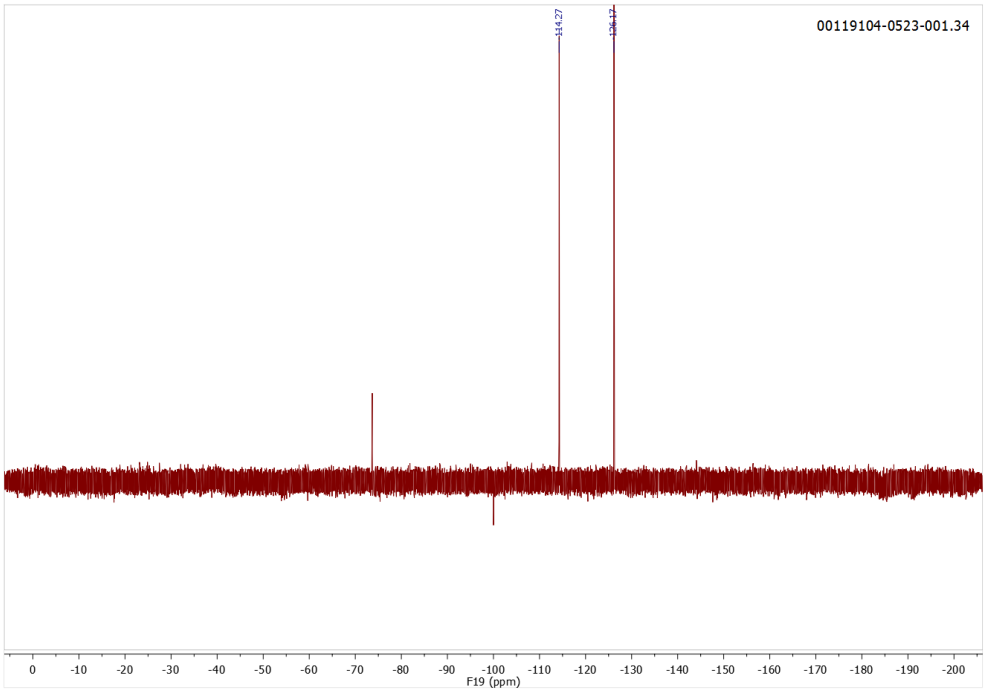
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



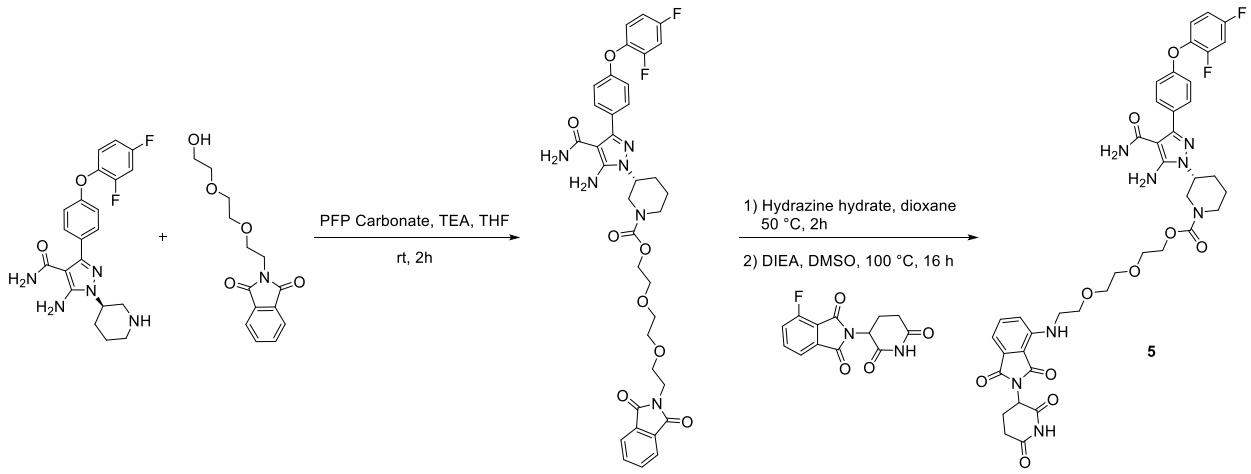
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 2-[2-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}ethoxy)ethoxy]ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (5).



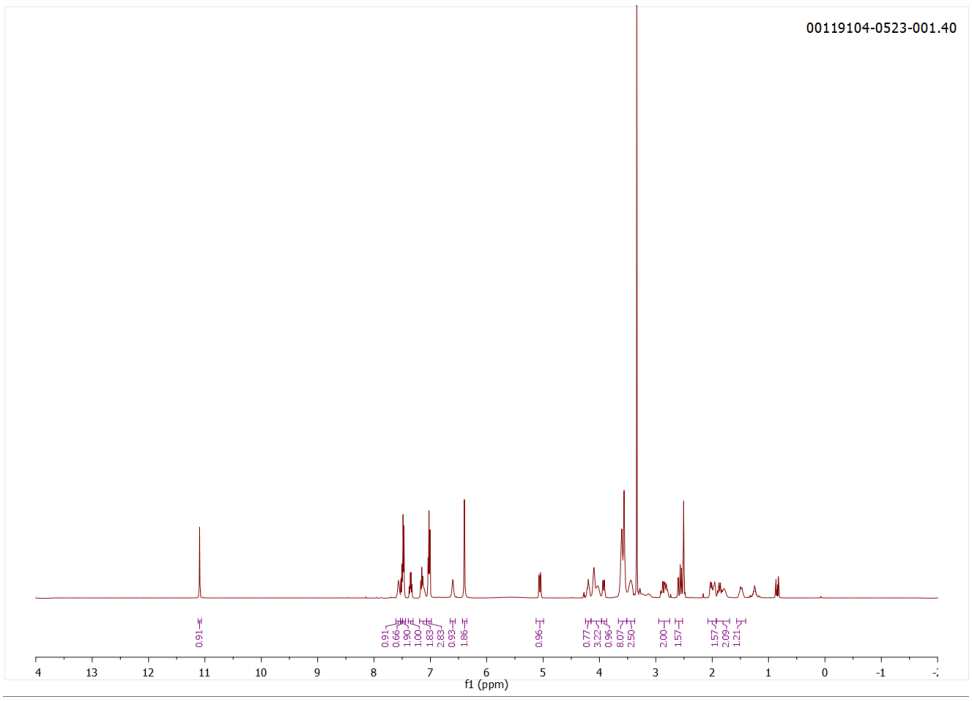
2-{2-[2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethoxy]ethoxy}ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. The title compound (137 mg, 53%) was obtained as a white solid from 2-{2-[2-(2-hydroxyethoxy)ethoxy]ethyl}-1*H*-isoindole-1,3(2*H*)-dione using a procedure analogous to that described in the Carbonate Procedure, Method A. ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.86 (ddt, *J* = 13.7, 5.7, 2.7 Hz, 4H), 7.56 – 7.50 (m, 1H), 7.50 – 7.45 (m, 2H), 7.35 (td, *J* = 9.2, 5.6 Hz, 1H), 7.16 (tdd, *J* = 9.0, 3.0, 1.6 Hz, 1H), 7.06 – 6.98 (m, 2H), 6.39 (s, 2H), 4.19 (dq, *J* = 10.3, 5.4, 4.2 Hz, 1H), 4.09 – 3.95 (m, 3H), 3.92 (d, *J* = 13.2 Hz, 1H), 3.79 – 3.69 (m, 2H), 3.62 (d, *J* = 5.9 Hz, 2H), 3.58 – 3.43 (m, 6H), 2.88 – 2.77 (m, 1H), 1.97 (d, *J* = 12.4 Hz, 1H), 1.93 – 1.82 (m, 1H), 1.80 (s, 2H), 1.48 (d, *J* = 13.0 Hz, 1H). LCMS: m/z 719.3 $[M+H]^+$.

2-[2-(2-aminoethoxy)ethoxy]ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. The title compound (58 mg, quant.) was obtained as an oil from 2-{2-[2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethoxy]ethoxy}ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the Phthalimide Deprotection Procedure. LCMS: m/z 589.0 $[M+H]^+$.

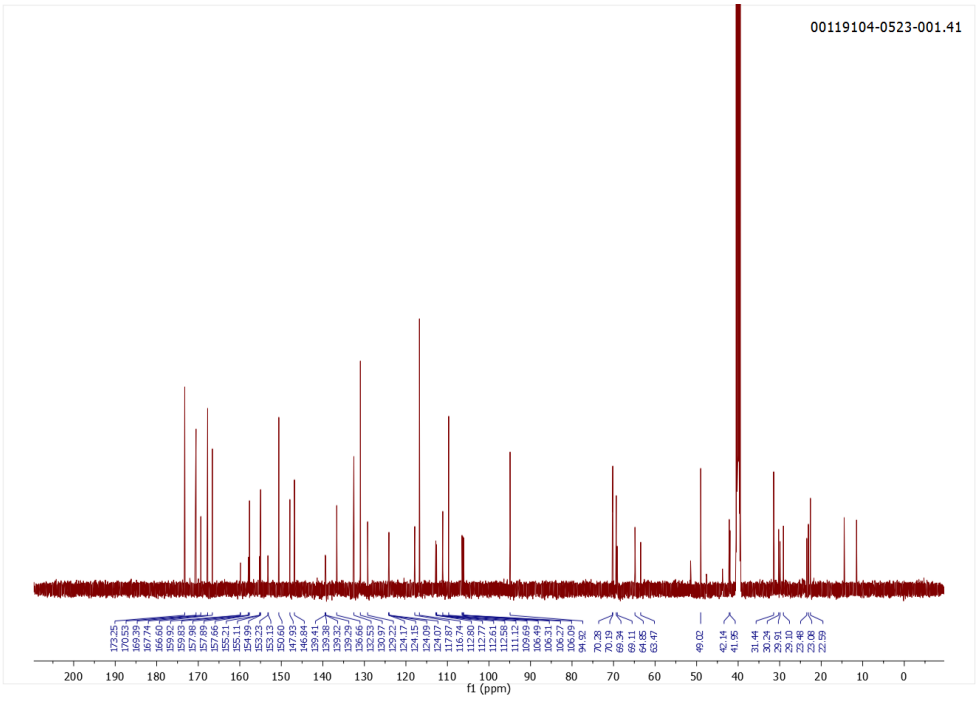
2-[2-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}ethoxy)ethoxy]ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-

difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (5). The title compound (34 mg, 26%) was obtained as a solid from 2-[2-(2-aminoethoxy)ethoxy]ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the SnAr Procedure. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 11.09 (s, 1H), 7.61 – 7.53 (m, 1H), 7.53 – 7.49 (m, 1H), 7.49 – 7.44 (m, 2H), 7.35 (td, *J* = 9.2, 5.6 Hz, 1H), 7.19 – 7.07 (m, 2H), 7.02 (dd, *J* = 9.8, 7.8 Hz, 3H), 6.60 (s, 1H), 6.40 (s, 2H), 5.06 (dd, *J* = 12.8, 5.4 Hz, 1H), 4.20 (dt, *J* = 10.6, 5.9 Hz, 1H), 4.07 (d, *J* = 35.3 Hz, 3H), 3.93 (d, *J* = 13.0 Hz, 1H), 3.59 (d, *J* = 23.8 Hz, 8H), 3.44 (s, 3H), 2.95 – 2.75 (m, 2H), 2.66 – 2.53 (m, 2H), 2.08 – 1.93 (m, 2H), 1.93 – 1.69 (m, 2H), 1.48 (d, *J* = 12.7 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) (observed) δ 173.25, 170.53, 169.39, 167.74, 166.60, 158.90 (dd, *J* = 243.6, 10.9 Hz), 157.66, 154.99, 154.17 (dd, *J* = 249.0, 12.9 Hz), 150.60, 147.93, 146.84, 139.35 (dd, *J* = 11.7, 3.7 Hz), 136.66, 132.53, 130.97, 129.22, 124.12 (dd, *J* = 10.0, 2.0 Hz), 117.87, 116.74, 112.69 (dd, *J* = 22.9, 3.8 Hz), 111.12, 109.69, 106.29 (dd, *J* = 27.4, 22.4 Hz), 94.92, 70.28, 70.19, 69.34, 69.11, 64.85, 63.47, 49.02, 42.14, 41.95, 31.44, 30.24, 29.91, 29.10, 23.48, 23.08, 22.59. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.26, -126.17. LCMS: m/z 845.1 $[M+H]^+$.

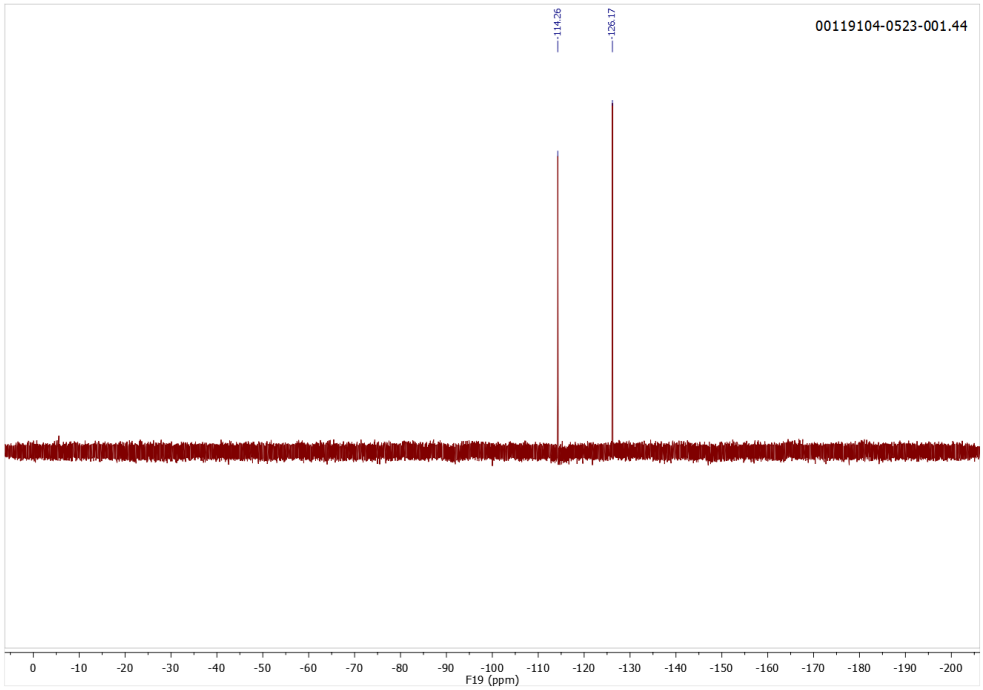
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



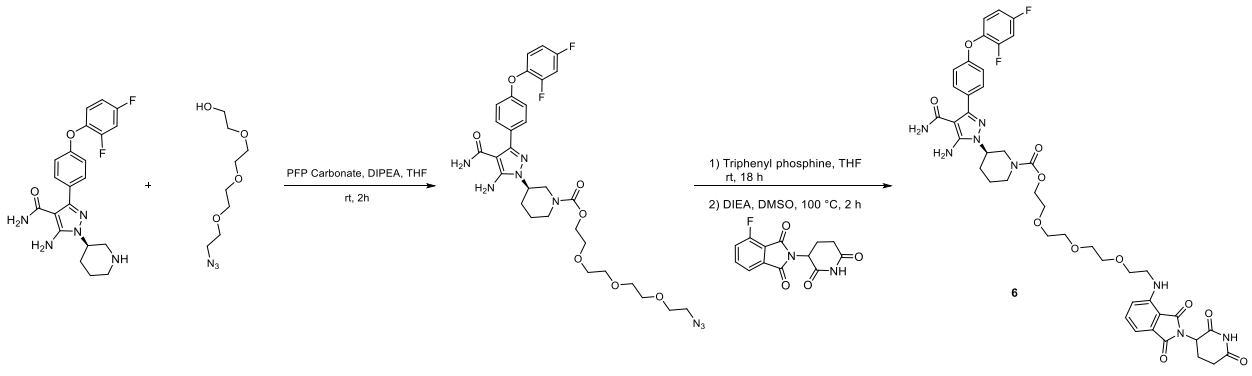
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 2-{2-[2-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}ethoxy)ethoxy]ethoxy}ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (6).



2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate.

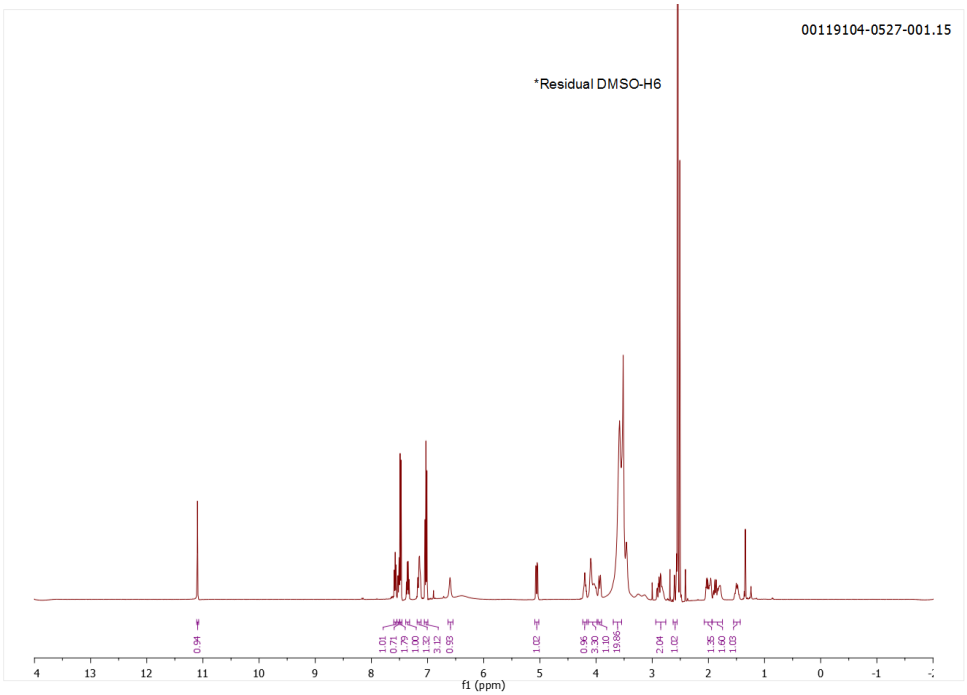
The title compound (252 mg, 79%) was obtained as a clear, colorless, viscous oil from 2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}ethanol using a procedure analogous to that described in the Carbonate Procedure, Method B. LCMS: m/z 660.0 $[M+H]^+$.

2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy}ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate.
The title compound (180 mg, 74%) was obtained as a clear, colorless, viscous oil from 2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the Staudinger Reaction. LCMS: m/z 633.7 $[M+H]^+$.

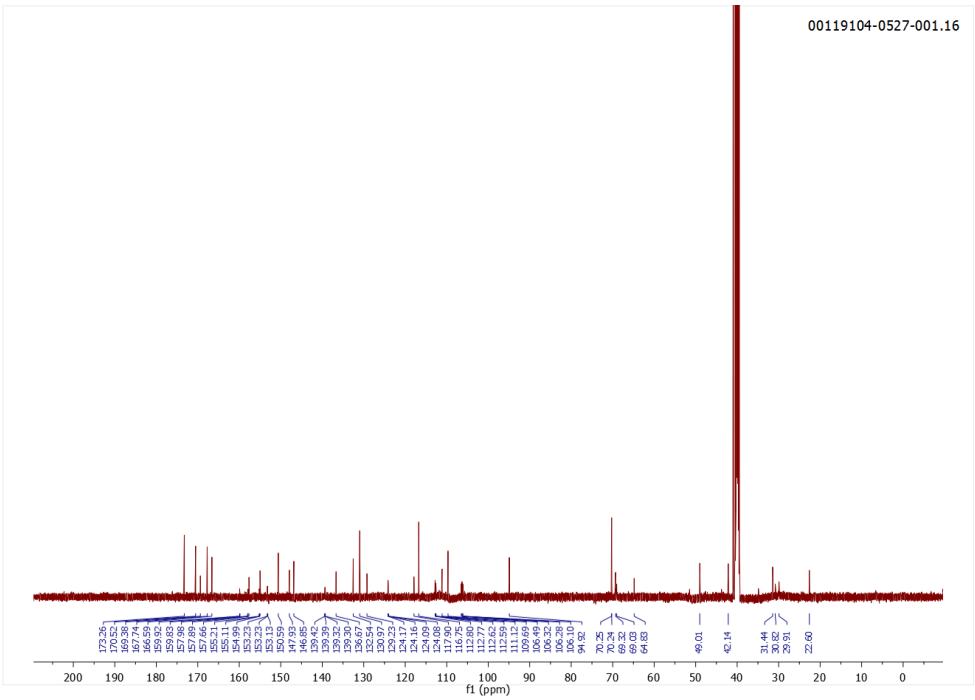
2-{2-[2-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}ethoxy)ethoxy]ethoxy}ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (6). The title compound (36 mg, 14%) was obtained as a solid from 2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy}ethyl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the SnAr Procedure. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 11.10 (s, 1H), 7.60 – 7.55 (m, 1H), 7.55 – 7.50 (m, 1H), 7.48 (d, *J* = 8.8 Hz, 2H), 7.35 (td, *J* = 9.2, 5.6 Hz, 1H), 7.19 – 7.12 (m, 2H),

7.06 – 6.99 (m, 3H), 6.60 (s, 1H), 5.06 (dd, *J* = 12.7, 5.4 Hz, 1H), 4.20 (tt, *J* = 10.2, 4.3 Hz, 1H), 4.06 (d, *J* = 30.5 Hz, 3H), 3.93 (d, *J* = 13.0 Hz, 1H), 3.58 (s, 15H), 2.94 – 2.76 (m, 2H), 2.63 – 2.56 (m, 2H), 2.07 – 1.94 (m, 2H), 1.93 – 1.75 (m, 2H), 1.49 (d, *J* = 13.2 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) (observed) δ 173.26, 170.52, 169.38, 167.74, 166.59, 158.90 (dd, *J* = 243.0, 11.1 Hz), 157.66, 154.99, 153.98 (dd, *J* = 249.7, 13.0 Hz), 150.59, 147.93, 146.85, 139.36 (dd, *J* = 11.7, 3.8 Hz), 136.67, 132.54, 130.97, 129.23, 124.12 (dd, *J* = 10.1, 1.8 Hz), 117.90, 116.75, 112.70 (dd, *J* = 23.0, 3.7 Hz), 111.12, 109.69, 106.30 (dd, *J* = 27.7, 22.3 Hz), 94.92, 70.25, 69.32, 69.03, 64.83, 49.01, 42.14, 31.44, 30.82, 29.91, 22.60. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.29, -126.18. LCMS: m/z 889.5 $[M+H]^+$.

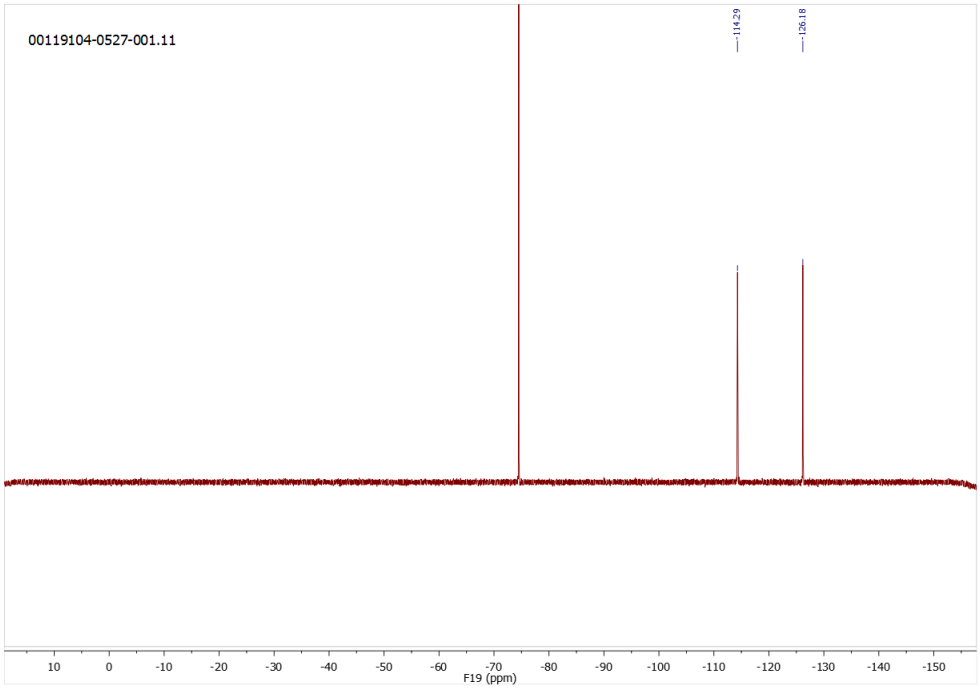
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



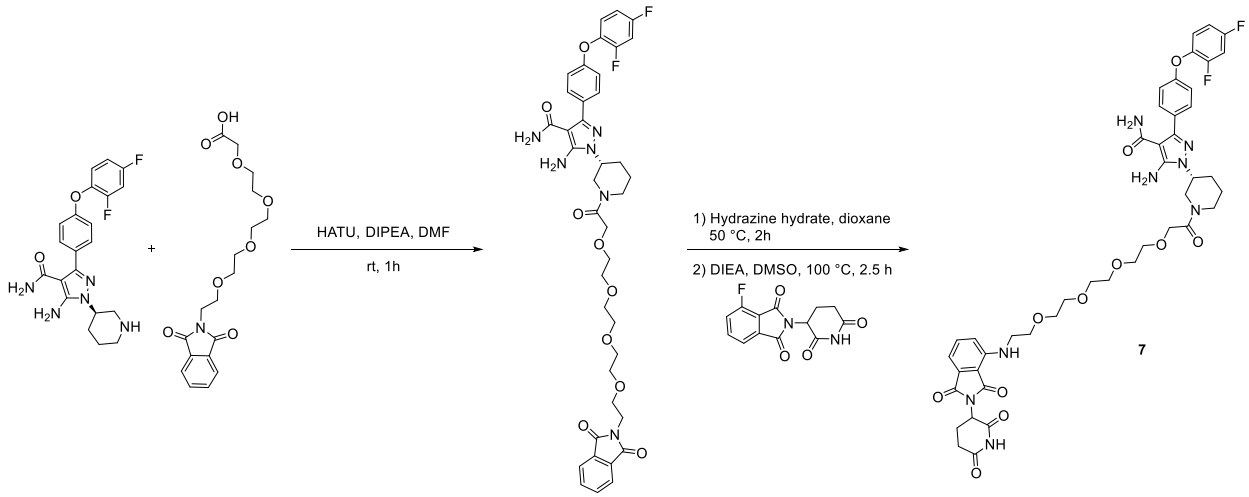
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-1-(14-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}-3,6,9,12-tetraoxatetradecan-1-oyl)piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (7).



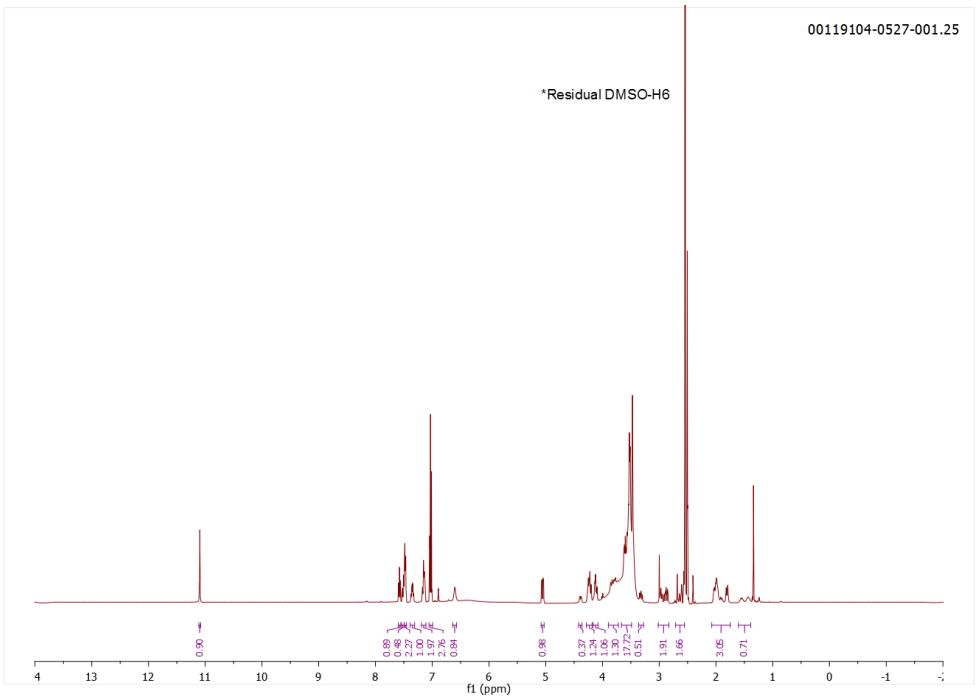
5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-{(3*R*)-1-[14-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-3,6,9,12-tetraoxatetradecan-1-oyl]piperidin-3-yl}-1*H*-pyrazole-4-carboxamide. The title compound (215 mg, 82%) was obtained as a clear, colorless, viscous oil from 14-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-3,6,9,12-tetraoxatetradecan-1-oic acid using a procedure analogous to that described in the Amide Coupling. LCMS: m/z 777.8 $[M+H]^+$.

5-amino-1-[(3*R*)-1-(14-amino-3,6,9,12-tetraoxatetradecan-1-oyl)piperidin-3-yl]-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazole-4-carboxamide. The title compound (179 mg, quant.) was obtained as a clear, colorless, viscous oil from 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-{(3*R*)-1-[14-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-3,6,9,12-tetraoxatetradecan-1-oyl]piperidin-3-yl}-1*H*-pyrazole-4-carboxamide using a procedure analogous to that described in the Phthalimide Deprotection Procedure. LCMS: m/z 647.7 $[M+H]^+$.

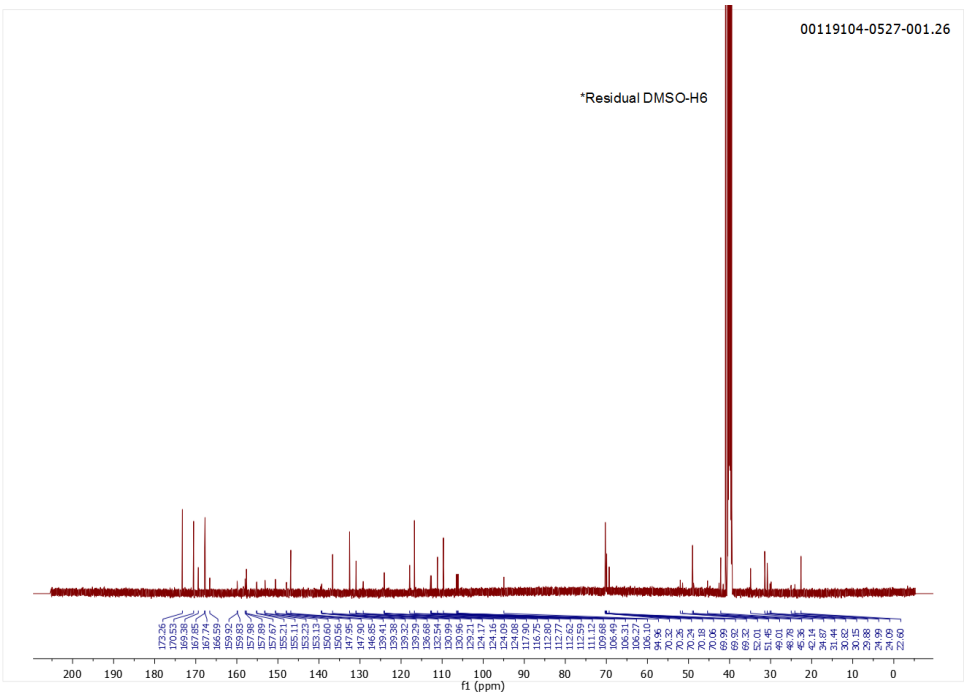
5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-1-(14-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}-3,6,9,12-tetraoxatetradecan-1-oyl)piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (7). The title compound (2 mg, 2%) was obtained as a solid from 5-amino-1-[(3*R*)-1-(14-amino-3,6,9,12-tetraoxatetradecan-1-oyl)piperidin-3-yl]-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazole-4-carboxamide using a procedure analogous to that described in the SnAr Procedure. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 11.10 (s, 1H), 7.60 – 7.55 (m, 1H), 7.54 – 7.49 (m, 1H), 7.48

(dd, *J* = 8.3, 3.1 Hz, 2H), 7.39 – 7.31 (m, 1H), 7.15 (td, *J* = 9.3, 8.2, 3.4 Hz, 2H), 7.03 (t, *J* = 8.1 Hz, 3H), 6.60 (s, 1H), 5.06 (dd, *J* = 12.8, 5.4 Hz, 1H), 4.39 (d, *J* = 12.5 Hz, 1H), 4.22 (t, *J* = 12.5 Hz, 1H), 4.11 (dd, *J* = 13.8, 5.7 Hz, 1H), 3.81 (dd, *J* = 28.0, 12.9 Hz, 1H), 3.67 – 3.49 (m, 18H), 3.36 – 3.27 (m, 1H), 3.02 – 2.83 (m, 2H), 2.71 – 2.56 (m, 2H), 2.07 – 1.75 (m, 4H), 1.60 – 1.39 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) (observed) δ 173.26, 170.53, 169.38, 167.85, 167.74, 166.59, 158.91 (dd, *J* = 243.6, 10.8 Hz), 157.67, 154.17 (dd, *J* = 249.6, 12.9 Hz), 150.60, 150.56, 147.95, 147.90, 146.85, 139.35 (dd, *J* = 11.1, 3.5 Hz), 136.68, 132.54, 130.99, 130.96, 129.21, 124.13 (dd, *J* = 10.0, 1.9 Hz), 117.90, 116.75, 112.69 (dd, *J* = 22.9, 3.7 Hz), 111.12, 109.68, 106.29 (dd, *J* = 27.6, 22.5 Hz), 94.96, 70.32, 70.24, 70.18, 70.06, 69.99, 69.92, 69.32, 52.01, 51.45, 49.01, 48.78, 45.36, 42.14, 34.87, 31.44, 30.82, 30.15, 29.88, 24.99, 24.09, 22.60. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.28, -126.18. LCMS: m/z 903.5 $[M+H]^+$.

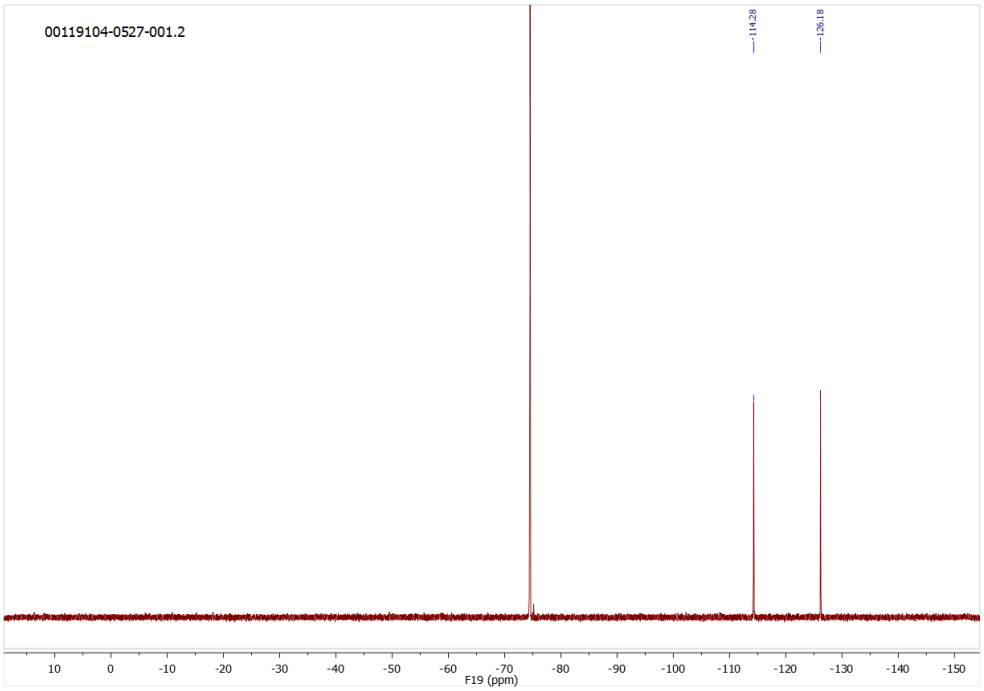
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



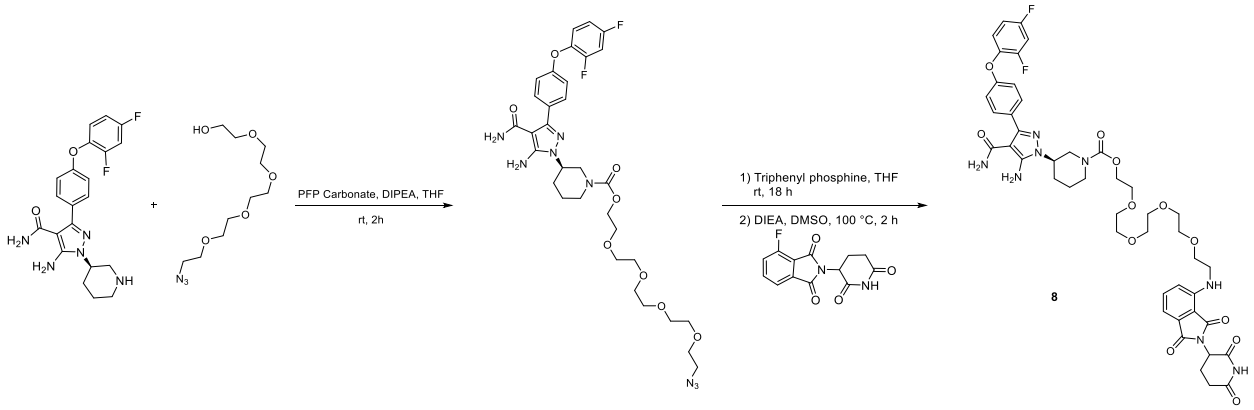
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 14-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}-3,6,9,12-tetraoxatetradec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (8).



14-azido-3,6,9,12-tetraoxatetradec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. The title compound (183 mg, 27%) was obtained as a colorless oil from 14-azido-

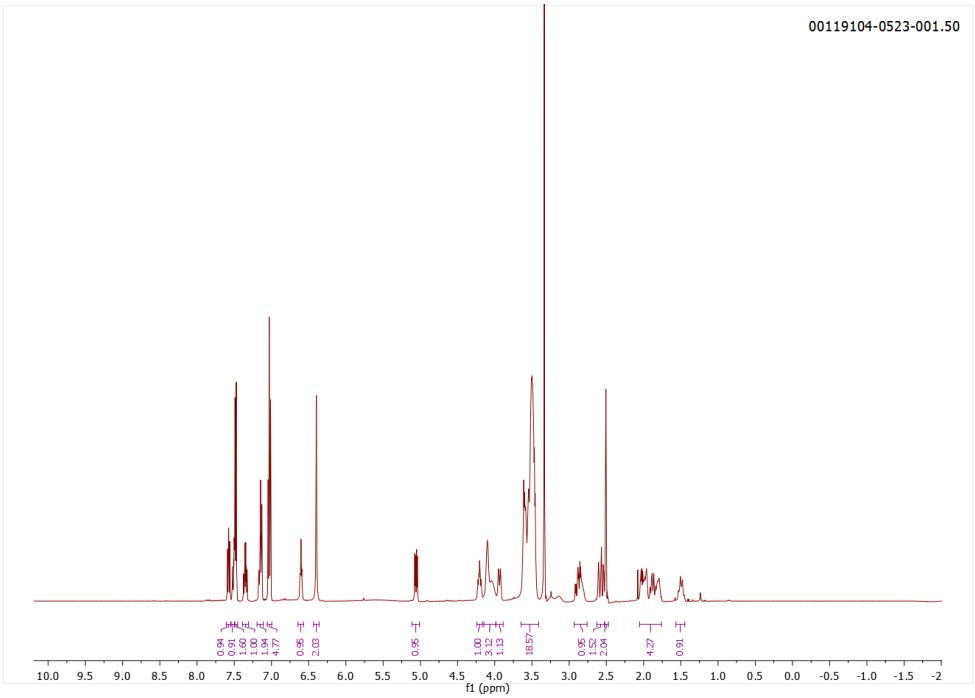
3,6,9,12-tetraoxatetradecan-1-ol using a procedure analogous to that described in the Carbonate Procedure, Method B. LCMS: m/z 703.7 $[M+H]^+$.

14-amino-3,6,9,12-tetraoxatetradec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. The title compound (175 mg, quant.) was obtained as a clear, colorless, viscous oil from 14-azido-3,6,9,12-tetraoxatetradec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the Staudinger Reaction. LCMS: m/z 677.7 $[M+H]^+$.

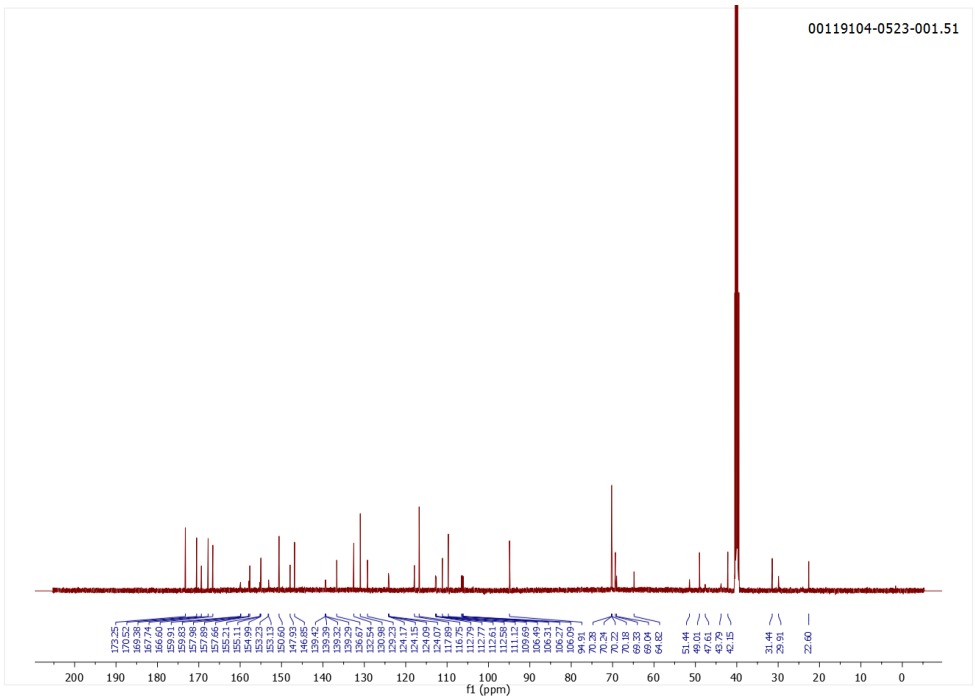
14-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}-3,6,9,12-tetraoxatetradec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (8). The title compound (92 mg, 76%) was obtained as a solid from 14-amino-3,6,9,12-tetraoxatetradec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the SnAr Procedure. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.10 (s, 1H), 7.58 (dd, *J* = 8.6, 7.1 Hz, 1H), 7.54 – 7.50 (m, 1H), 7.48 (d, *J* = 8.7 Hz, 2H), 7.35 (td, *J* = 9.2, 5.6 Hz, 1H), 7.19 – 7.11 (m, 2H), 7.06 – 7.00 (m, 5H), 6.60 (t, *J* = 5.8 Hz, 1H), 6.40 (s, 2H), 5.06 (dd, *J* = 12.7, 5.4 Hz, 1H), 4.20 (tt, *J* = 10.6, 4.2 Hz, 1H), 4.07 (d, *J* = 34.3 Hz, 3H), 3.94 (d, *J* = 13.1 Hz, 1H), 3.65 – 3.41 (m, 18H), 2.88 (ddd, *J* = 16.8, 13.7, 5.4 Hz, 1H), 2.63 –

2.53 (m, 2H), 2.51 (p, *J* = 2.0 Hz, 2H), 2.05 – 1.76 (m, 4H), 1.49 (dd, *J* = 10.6, 6.6 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 173.25, 170.52, 169.38, 167.74, 166.60, 158.90 (dd, *J* = 243.6, 10.8 Hz), 157.66, 154.99, 154.52 (dd, *J* = 250.8, 12.9 Hz), 150.60, 147.93, 146.85, 139.36 (dd, *J* = 11.6, 3.8 Hz), 136.67, 132.54, 130.98, 129.23, 124.12 (dd, *J* = 10.0, 2.0 Hz), 117.89, 116.75, 112.69 (dd, *J* = 23.0, 3.6 Hz), 111.12, 109.69, 106.29 (dd, *J* = 27.5, 22.4 Hz), 94.91, 70.28, 70.24, 70.18, 69.33, 69.04, 64.82, 51.44, 49.01, 47.61, 43.79, 42.15, 31.44, 29.91, 22.60. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.28, -126.18. LCMS: m/z 933.6 $[M+H]^+$.

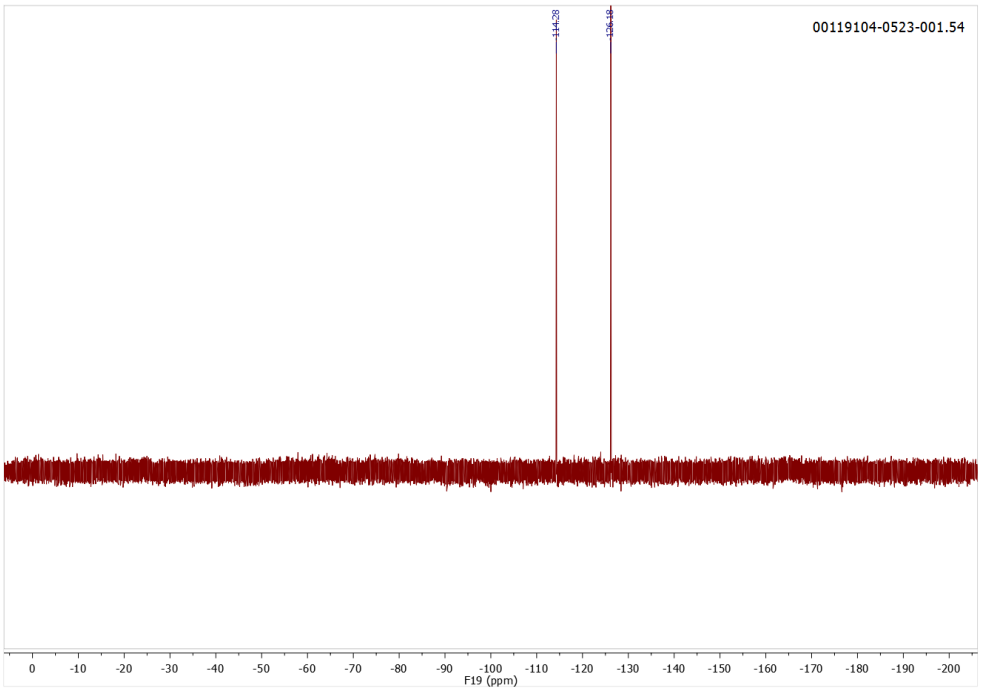
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



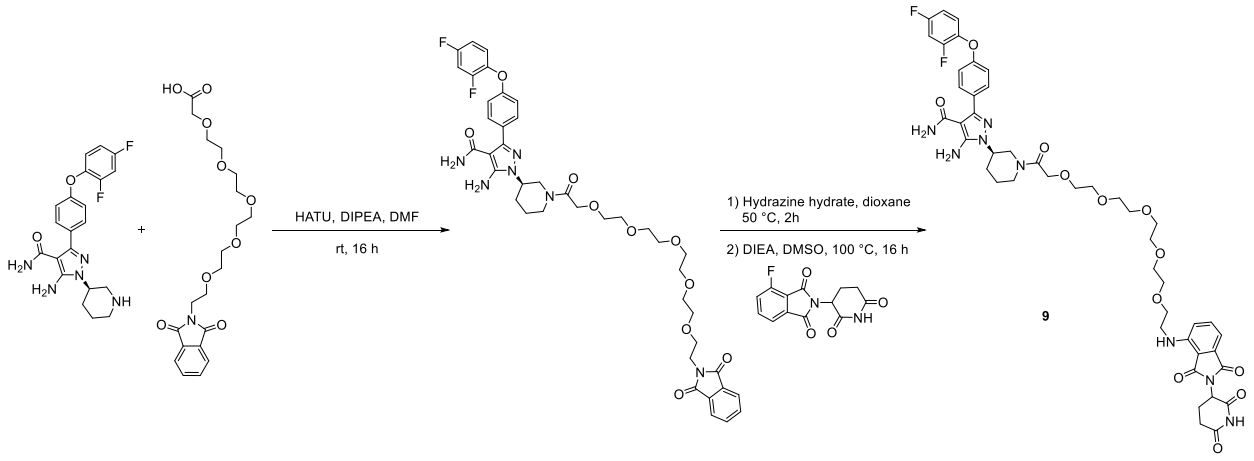
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-1-(17-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}-3,6,9,12,15-pentaoxaheptadecan-1-oyl)piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (9).



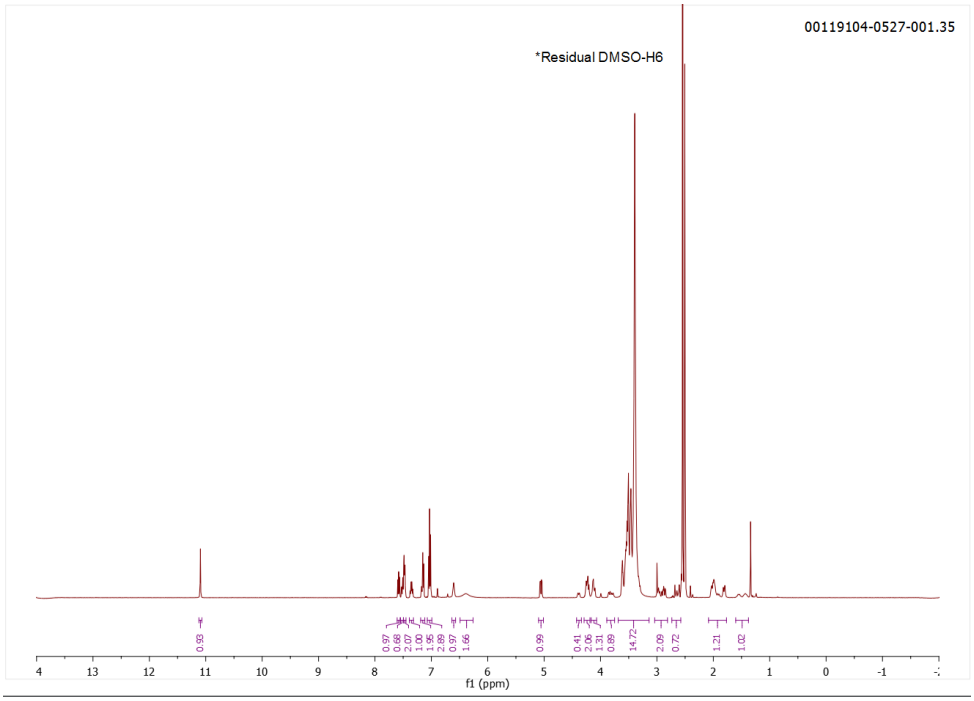
5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-{(3*R*)-1-[17-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-3,6,9,12,15-pentaoxaheptadecan-1-oyl]piperidin-3-yl}-1*H*-pyrazole-4-carboxamide. The title compound (75 mg, 27%) was obtained as an oil from 17-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-3,6,9,12,15-pentaoxaheptadecan-1-oic acid using a procedure analogous to that described in the Amide Coupling. LCMS: m/z 821.4 $[M+H]^+$.

5-amino-1-[(3*R*)-1-(17-amino-3,6,9,12,15-pentaoxaheptadecan-1-oyl)piperidin-3-yl]-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazole-4-carboxamide. The title compound (63 mg, quant.) was obtained as an oil from 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-{(3*R*)-1-[17-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-3,6,9,12,15-pentaoxaheptadecan-1-oyl]piperidin-3-yl}-1*H*-pyrazole-4-carboxamide using a procedure analogous to that described in the Phthalimide Deprotection Procedure. LCMS: m/z 691.1 $[M+H]^+$.

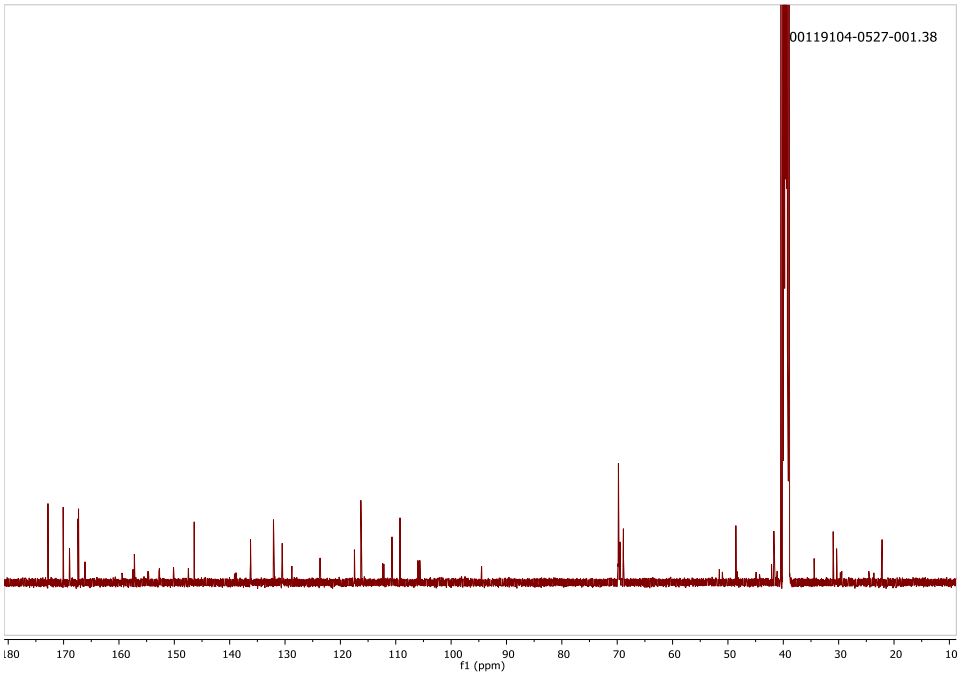
5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-1-(17-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}-3,6,9,12,15-pentaoxaheptadecan-1-oyl)piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (9). The title compound (29 mg, 21%) was obtained as a solid from 5-amino-1-[(3*R*)-1-(17-amino-3,6,9,12,15-pentaoxaheptadecan-1-oyl)piperidin-3-yl]-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazole-4-carboxamide using a procedure analogous to that described in the SnAr Procedure. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 11.10 (s, 1H), 7.58 (dd, *J* = 8.6, 7.1 Hz, 1H),

7.55 – 7.50 (m, 1H), 7.48 (dd, *J* = 8.8, 3.1 Hz, 2H), 7.35 (td, *J* = 9.2, 5.6 Hz, 1H), 7.19 – 7.12 (m, 2H), 7.03 (t, *J* = 8.3 Hz, 3H), 6.60 (s, 1H), 6.39 (s, 2H), 5.06 (dd, *J* = 12.7, 5.4 Hz, 1H), 4.39 (d, *J* = 12.5 Hz, 1H), 4.23 (dd, *J* = 14.0, 9.9 Hz, 2H), 4.12 (dd, *J* = 13.7, 5.9 Hz, 1H), 3.82 (dd, *J* = 26.3, 13.2 Hz, 1H), 3.70 – 3.20 (m, 24H), 3.04 – 2.81 (m, 2H), 2.74 – 2.58 (m, 1H), 2.09 – 1.77 (m, 2H), 1.60 – 1.38 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 172.82, 170.08, 168.93, 167.41, 167.30, 166.13, 158.46 (dd, *J* = 243.5, 10.8 Hz), 157.22, 153.73 (dd, *J* = 249.5, 12.9 Hz), 150.15, 150.11, 147.50, 147.46, 146.41, 138.90 (dd, *J* = 11.7, 3.9 Hz), 136.24, 132.09, 130.51, 128.79, 128.76, 123.69 (dd, *J* = 9.8, 1.7 Hz), 117.47, 116.30, 112.25 (dd, *J* = 22.9, 3.7 Hz), 110.68, 109.23, 105.85 (dd, *J* = 27.5, 22.4 Hz), 94.51, 69.85, 69.78, 69.70, 69.61, 69.53, 69.48, 68.88, 51.56, 51.01, 48.56, 48.34, 44.93, 44.30, 42.12, 41.70, 41.10, 34.43, 30.99, 30.37, 29.70, 29.44, 24.55, 23.64, 22.16.^{19}F NMR (471 MHz, DMSO-d_6) δ -114.27, -126.18. LCMS: m/z 947.1 $[M+H]^+$.

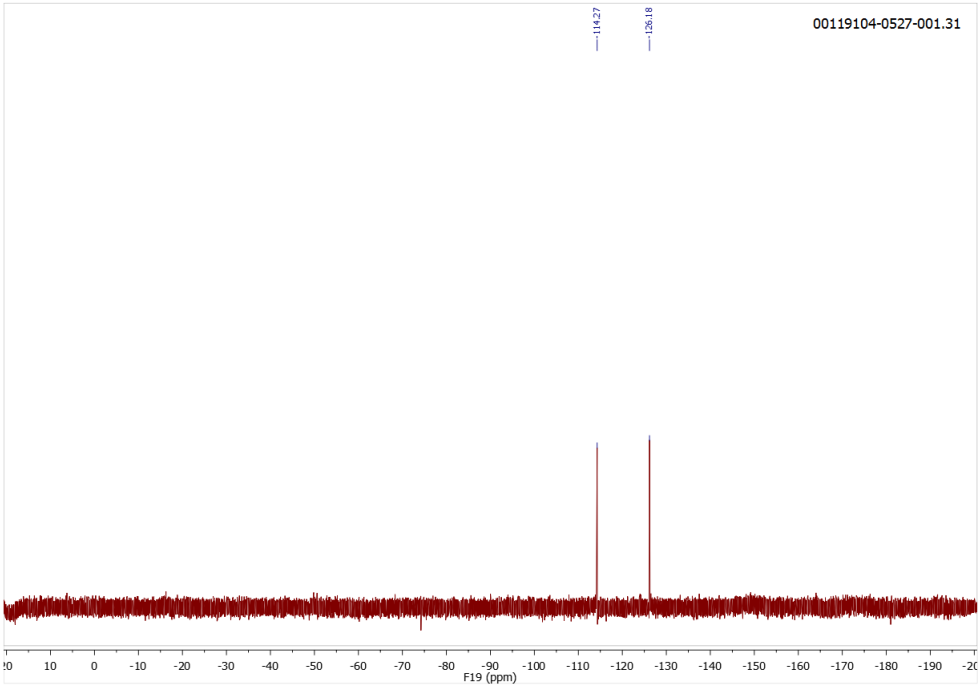
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



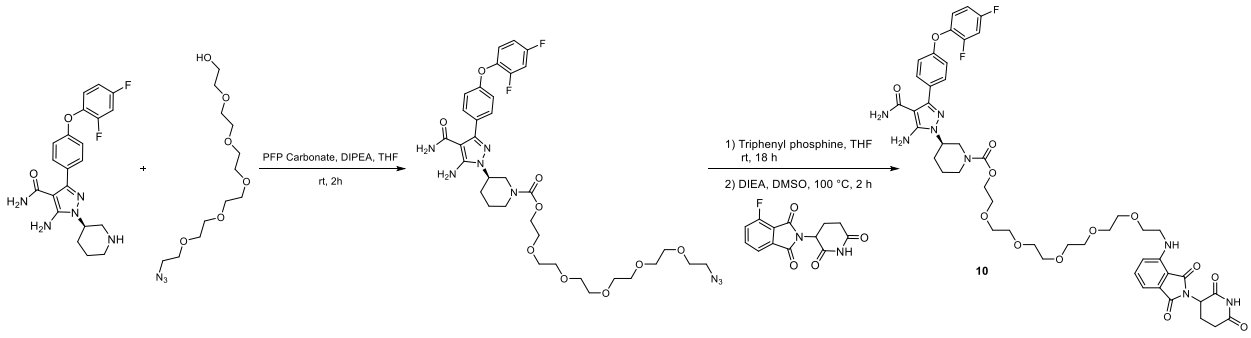
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 17-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}-3,6,9,12,15-pentaoxaheptadec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (10).



17-azido-3,6,9,12,15-pentaoxaheptadec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate.

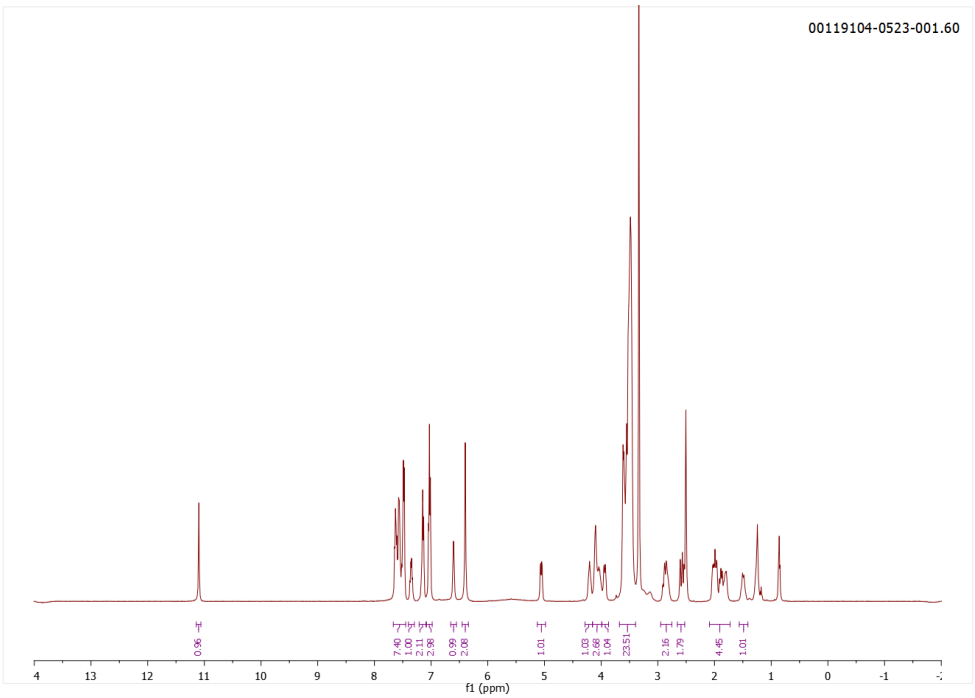
The title compound (2650 mg, 98%) was obtained as a clear, light tan, viscous oil from 17-azido-3,6,9,12,15-pentaoxaheptadecan-1-ol using a procedure analogous to that described in the Carbonate Procedure, Method B. LCMS: m/z 747.7 $[M+H]^+$.

17-amino-3,6,9,12,15-pentaoxaheptadec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. The title compound (1550 mg, 61%) was obtained as a clear, near-colorless, viscous oil from 17-azido-3,6,9,12,15-pentaoxaheptadec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the Staudinger Reaction. LCMS: m/z 721.7 $[M+H]^+$.

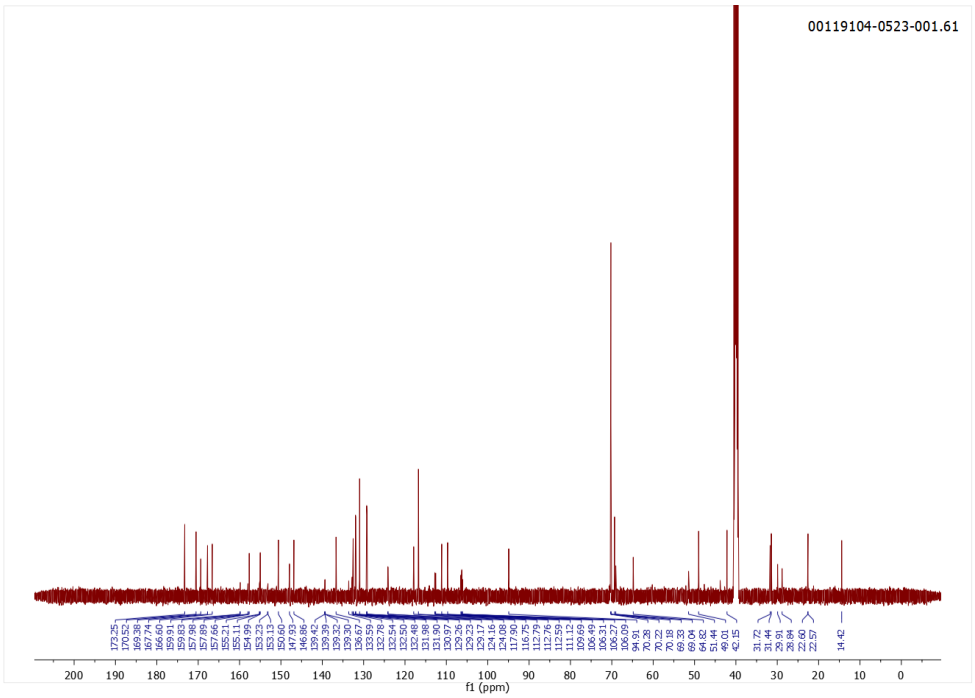
17-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}-3,6,9,12,15-pentaoxaheptadec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (10). The title compound (371 mg, 13%) was obtained as a bright yellow solid from 17-amino-3,6,9,12,15-pentaoxaheptadec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the SnAr Procedure. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 11.10 (s, 1H), 7.67 – 7.45 (m, 7H), 7.35 (td, *J* = 9.2, 5.5 Hz, 1H), 7.14 (d, *J* = 8.8 Hz, 2H), 7.03 (t, *J* = 8.4 Hz, 3H), 6.60 (t, *J* = 5.9 Hz, 1H), 6.40 (s, 2H), 5.06 (dd, *J* = 12.7, 5.4 Hz, 1H), 4.20 (dt, *J* = 11.0, 5.9 Hz, 1H), 4.06 (d, *J* = 38.4

Hz, 3H), 3.94 (d, *J* = 13.0 Hz, 1H), 3.68 – 3.39 (m, 23H), 2.95 – 2.75 (m, 2H), 2.56 (dd, *J* = 25.2, 12.2 Hz, 2H), 2.09 – 1.72 (m, 4H), 1.49 (d, *J* = 13.1 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.52, 169.38, 167.74, 166.60, 158.84 (dd, *J* = 243.5, 10.4 Hz), 157.66, 155.16 (dd, *J* = 250.5, 13.4 Hz), 154.99, 150.60, 147.93, 146.86, 139.50 – 139.15 (m), 136.67, 132.54, 132.50, 132.48, 131.98, 131.90, 130.97, 129.26, 129.23, 129.17, 124.13 (d, *J* = 10.3 Hz), 117.90, 116.75, 112.82 – 112.54 (m), 111.12, 109.69, 106.28 (dd, *J* = 27.7, 22.5 Hz), 94.91, 70.28, 70.22, 70.18, 69.33, 69.04, 64.82, 51.44, 49.01, 42.15, 31.72, 31.44, 29.91, 28.84, 22.60, 22.57. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.25, -126.17. LCMS: m/z 977.4 $[M+H]^+$.

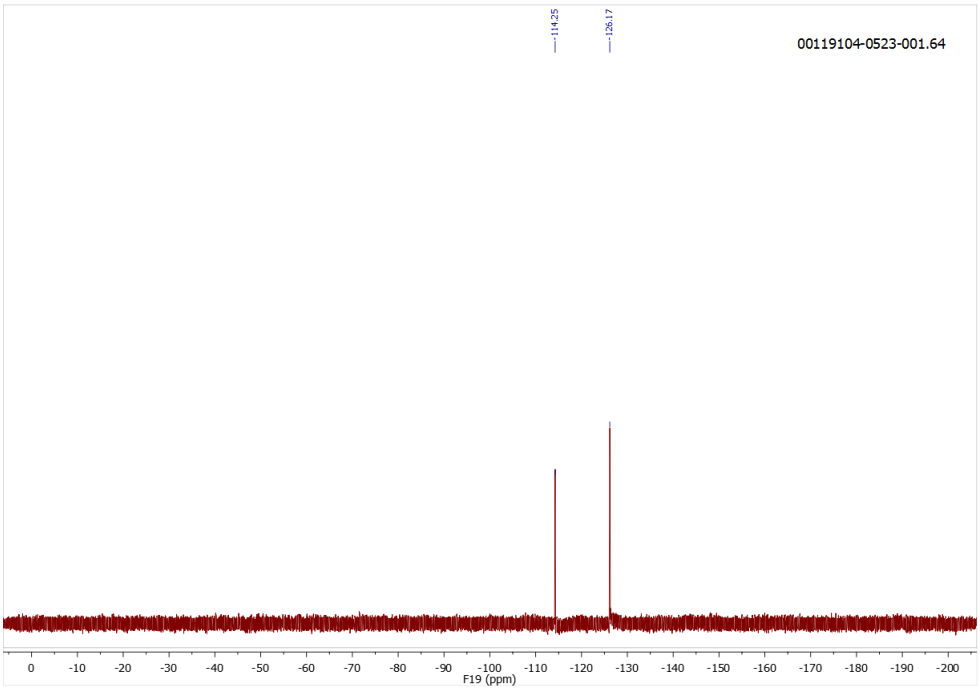
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



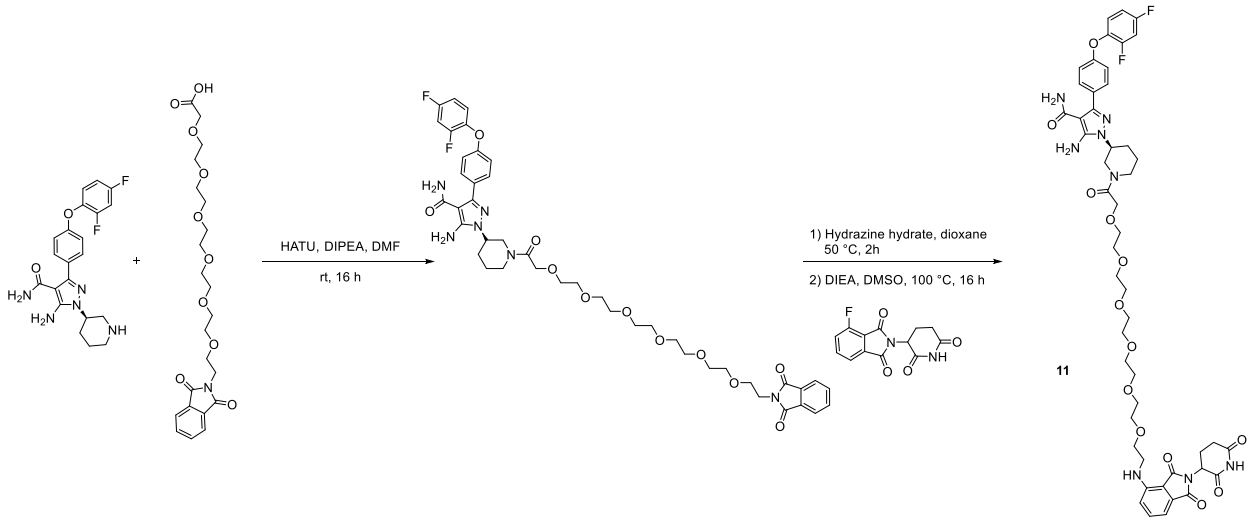
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*S*)-1-(20-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}-3,6,9,12,15,18-hexaoxaicosan-1-oyl)piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (11).



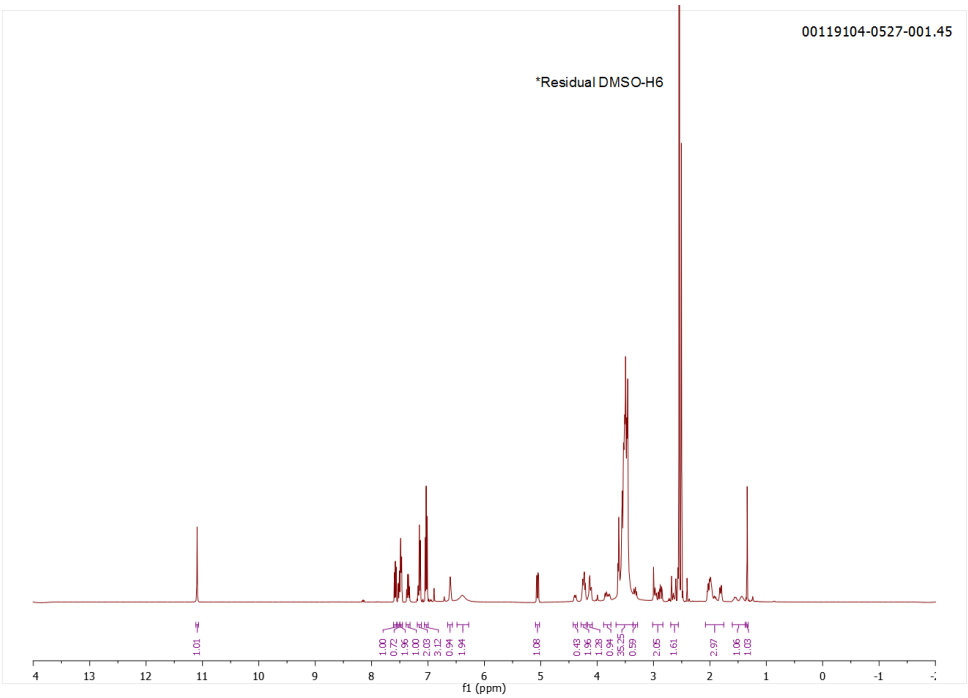
5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-{(3*R*)-1-[20-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-3,6,9,12,15,18-hexaoxaicosan-1-oyl]piperidin-3-yl}-1*H*-pyrazole-4-carboxamide. The title compound (210 mg, 76%) was obtained as an oil from 20-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-3,6,9,12,15,18-hexaoxaicosan-1-oic acid using a procedure analogous to that described in the Amide Coupling. LCMS: m/z 865.1 $[M+H]^+$.

5-amino-1-[(3*R*)-1-(20-amino-3,6,9,12,15,18-hexaoxaicosan-1-oyl)piperidin-3-yl]-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazole-4-carboxamide. The title compound (130 mg, 73%) was obtained as a light brown gum from 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-{(3*R*)-1-[20-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-3,6,9,12,15,18-hexaoxaicosan-1-oyl]piperidin-3-yl}-1*H*-pyrazole-4-carboxamide using a procedure analogous to that described in the Phthalimide Deprotection Procedure. LCMS: m/z 735.8 $[M+H]^+$.

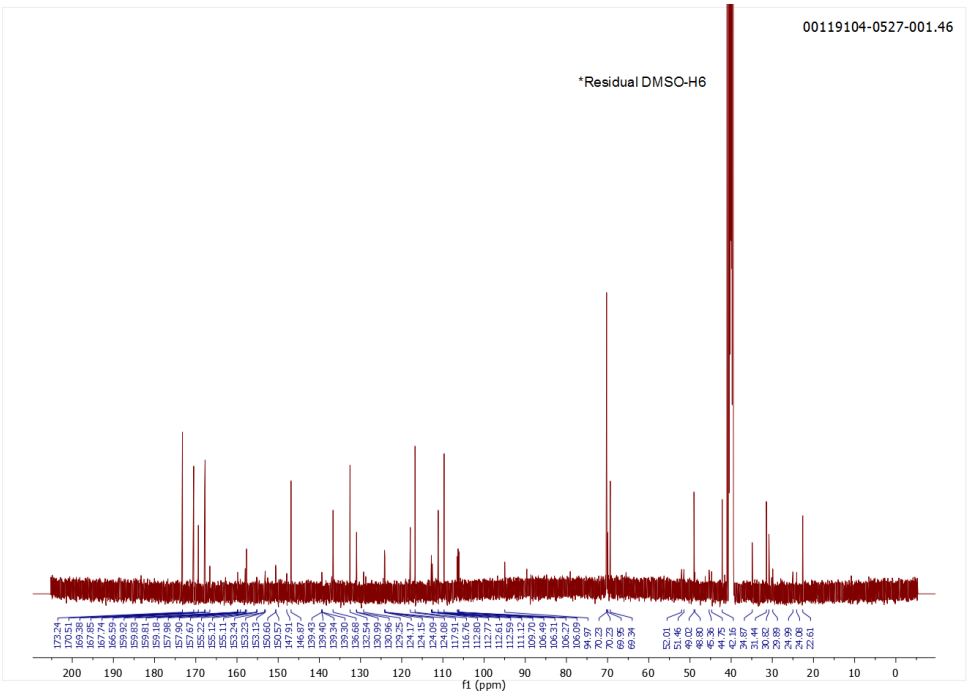
5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*S*)-1-(20-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}-3,6,9,12,15,18-hexaoxaicosan-1-oyl)piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (11). The title compound (17 mg, 10%) was obtained as a solid from 5-amino-1-[(3*R*)-1-(20-amino-3,6,9,12,15,18-hexaoxaicosan-1-oyl)piperidin-3-yl]-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazole-4-carboxamide using a procedure analogous to that described in the SnAr Procedure. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 11.10 (s, 1H), 7.58 (dd, *J* = 8.6, 7.0 Hz, 1H), 7.55 –

7.50 (m, 1H), 7.48 (dd, *J* = 8.6, 3.2 Hz, 2H), 7.35 (td, *J* = 9.2, 5.6 Hz, 1H), 7.19 – 7.12 (m, 2H), 7.06 – 7.00 (m, 3H), 6.60 (d, *J* = 6.1 Hz, 1H), 6.39 (s, 2H), 5.06 (dd, *J* = 12.8, 5.4 Hz, 1H), 4.39 (d, *J* = 12.5 Hz, 1H), 4.23 (dd, *J* = 14.0, 9.0 Hz, 2H), 4.12 (dd, *J* = 13.7, 5.5 Hz, 1H), 3.82 (dd, *J* = 26.3, 13.2 Hz, 1H), 3.67 – 3.36 (m, 24H), 3.32 (t, *J* = 11.8 Hz, 1H), 3.02 – 2.83 (m, 2H), 2.69 – 2.56 (m, 2H), 2.08 – 1.75 (m, 3H), 1.60 – 1.38 (m, 1H), 1.34 (s, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) (observed) δ 173.24, 170.51, 169.38, 167.85, 167.74, 166.59, 158.91 (dd, *J* = 243.4, 10.8 Hz), 157.67, 155.30 – 153.05 (m), 150.60, 150.57, 147.91, 146.87, 139.37 (dd, *J* = 11.8, 4.3 Hz), 136.68, 132.54, 130.99, 130.96, 129.25, 124.12 (dd, *J* = 9.9, 1.9 Hz), 117.91, 116.76, 112.69 (dd, *J* = 22.9, 3.7 Hz), 111.12, 109.70, 106.29 (dd, *J* = 27.6, 22.3 Hz), 94.97, 70.23, 69.95, 69.34, 52.01, 51.46, 49.02, 48.80, 45.36, 44.75, 42.16, 34.87, 31.44, 30.82, 29.89, 24.99, 24.08, 22.61. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.28, -126.18. LCMS: m/z 991.7 $[M+H]^+$.

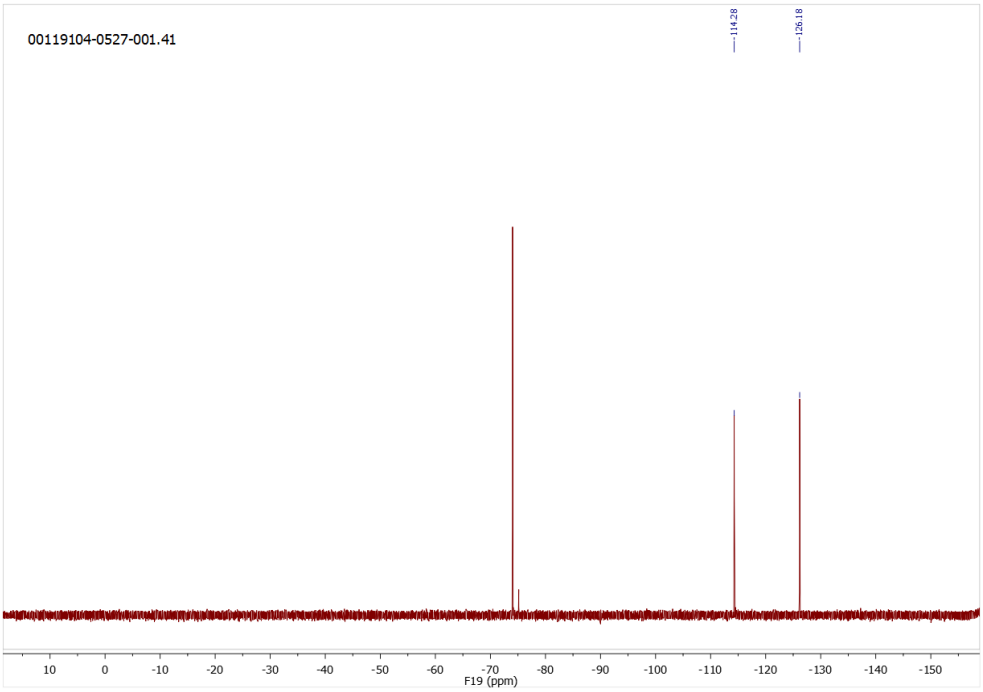
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



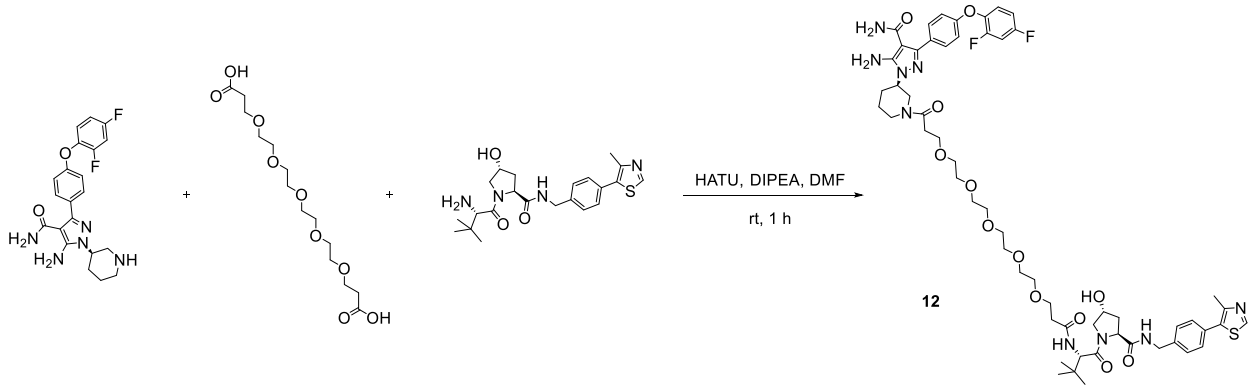
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



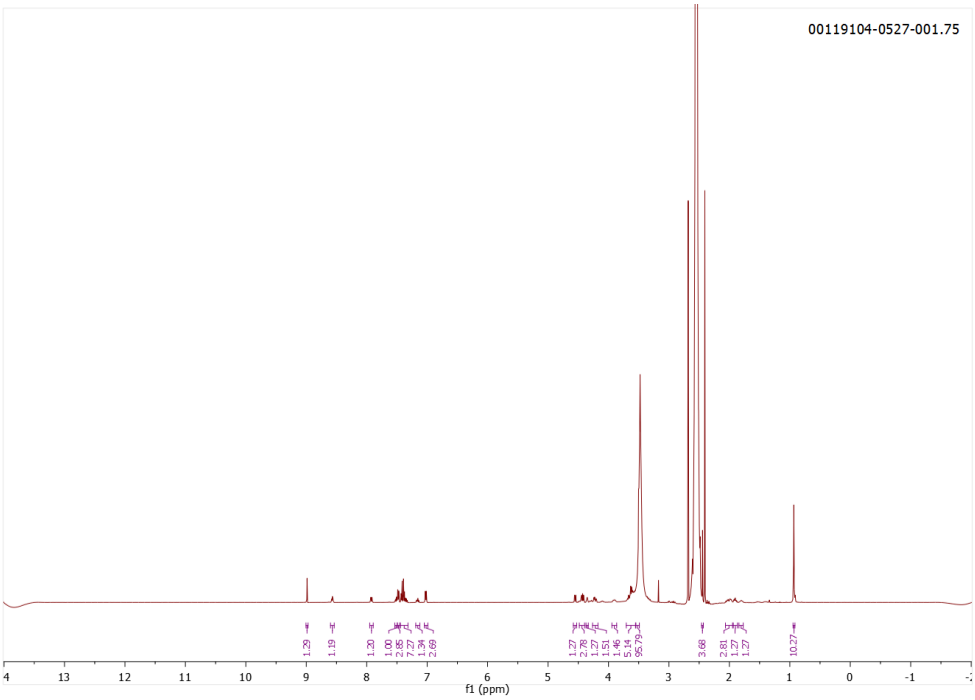
Preparation of *N*-{19-[(3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidin-1-yl]-19-oxo-4,7,10,13,16-pentaoxanonadecan-1-oyl}-3-methyl-L-valyl-(4*R*)-4-hydroxy-*N*-[4-(4-methyl-1,3-thiazol-5-yl)benzyl]-L-prolinamide (12).



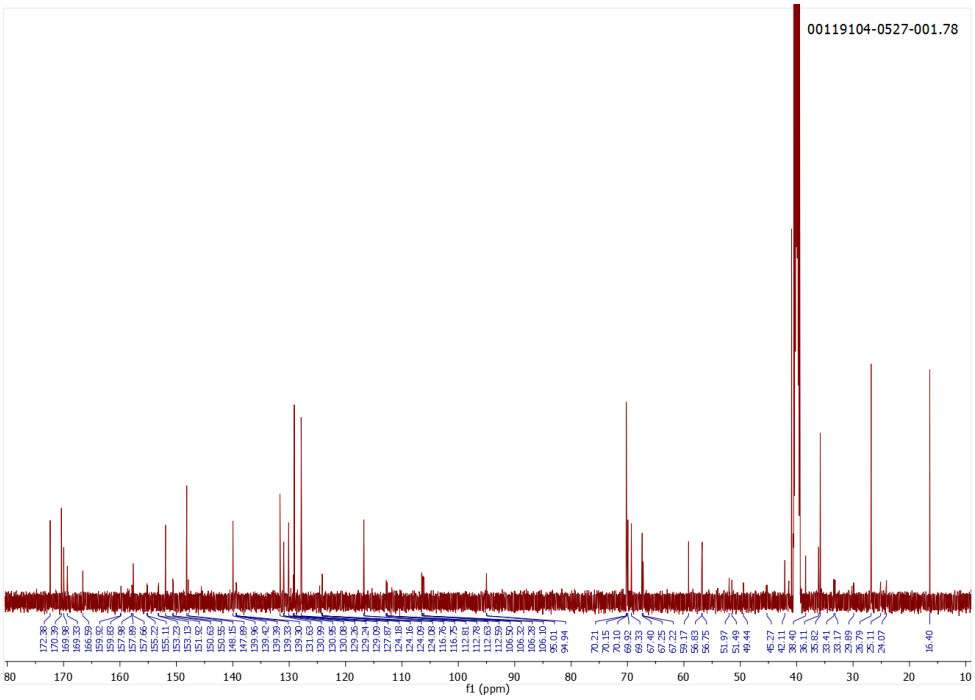
HATU (76 mg, 1 eq) was added to a solution of 4,7,10,13,16-pentaoxanonadecane-1,19-dioic acid (68 mg, 1 eq), 3-methyl-L-valyl-(4*R*)-4-hydroxy-*N*-[4-(4-methyl-1,3-thiazol-5-yl)benzyl]-L-prolinamide (94 mg, 1 eq), and diisopropylethylamine (70 µL, 2 eq) in dimethylformamide (0.4 mL, 0.5 M). The reaction was stirred at rt for 30 min. To the reaction was added 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (99 mg, 1.2 eq), diisopropylethylamine (70 µL, 2 eq), and HATU (84 mg, 1.1 eq) sequentially. The reaction was stirred at rt for 20 min. The reaction was extracted with dichloromethane and washed with water. The combined organic layers were combined, dried, filtered, and concentrated *in vacuo*. The crude material was purified via flash chromatography and prep HPLC to afford the desired product *N*-{19-[(3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidin-1-yl]-19-oxo-4,7,10,13,16-pentaoxanonadecan-1-oyl}-3-methyl-L-valyl-(4*R*)-4-hydroxy-*N*-[4-(4-methyl-1,3-thiazol-5-yl)benzyl]-L-prolinamide (12) (18 mg, 8%) as a solid. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 8.99 (s, 1H), 8.57 (t, *J* = 6.1 Hz, 1H), 7.92 (d, *J* = 9.3 Hz, 1H), 7.54 – 7.49 (m, 1H), 7.49 – 7.45 (m, 3H), 7.44 – 7.32 (m, 7H), 7.15 (tdd, *J* = 9.1, 3.1, 1.6 Hz, 1H), 7.05 – 6.99 (m, 3H), 4.55 (d, *J* = 9.4 Hz, 1H), 4.48 – 4.40 (m, 3H), 4.37 – 4.33 (m, 1H), 4.22 (dd, *J* = 15.8, 5.5 Hz, 2H), 3.94 – 3.86 (m, 1H), 3.71 – 3.55 (m, 5H), 3.49 (s, 22H), 2.44 (s, 3H), 2.06 – 1.95 (m, 3H), 1.90 (ddd, *J* = 12.9, 8.4, 4.6 Hz, 1H), 1.79 (d, *J* = 12.5 Hz, 1H), 0.93 (s, 9H). ^{13}C NMR (126 MHz, DMSO-d_6) (observed) δ 172.38, 170.39, 169.98, 169.33, 166.59, 158.91 (dd, *J* = 243.6, 10.9 Hz), 157.66, 154.17 (dd, *J* = 248.9, 9.3 Hz),

151.92, 150.63, 150.55, 148.15, 147.89, 139.96, 139.36 (dd, *J* = 11.3, 3.9 Hz), 131.63, 130.99, 130.95, 130.08, 129.26, 129.24, 129.09, 127.87, 124.13 (dd, *J* = 10.5, 1.4 Hz), 116.75, 112.70 (dd, *J* = 23.1, 4.3 Hz), 106.30 (dd, *J* = 27.5, 22.4 Hz), 95.01, 94.94, 70.21, 70.15, 70.10, 69.92, 69.33, 67.40, 67.25, 67.22, 59.17, 56.83, 56.75, 51.97, 51.49, 49.44, 45.27, 42.11, 38.40, 36.11, 35.82, 33.41, 33.17, 29.89, 26.79, 25.11, 24.07, 16.40. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.26, -126.16. LCMS: m/z 1146.9 $[M+H]^+$.

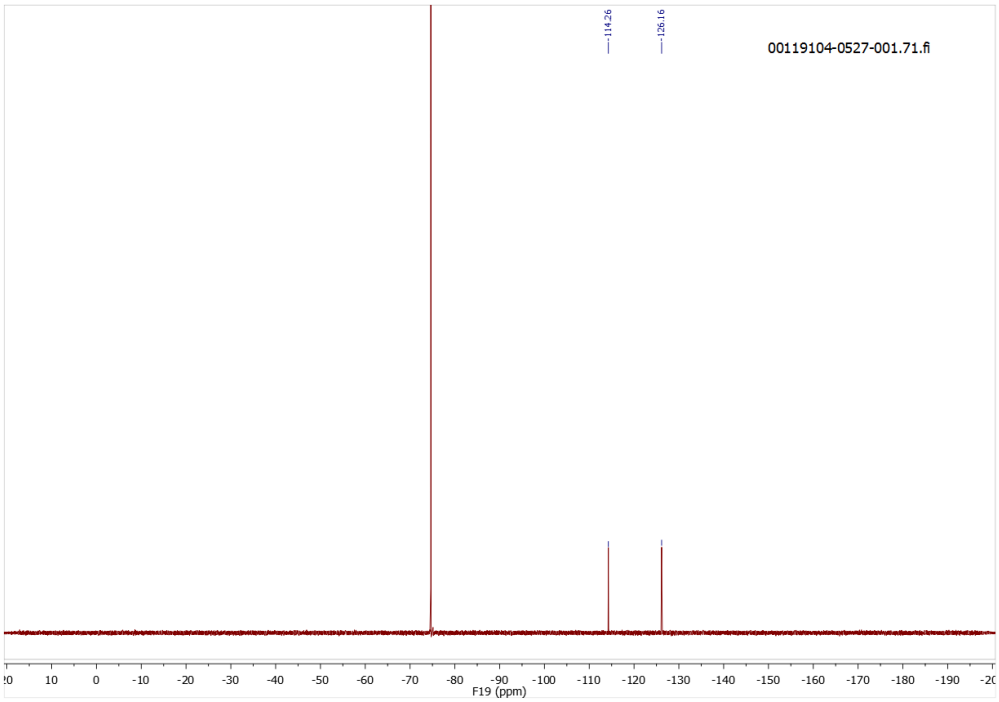
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



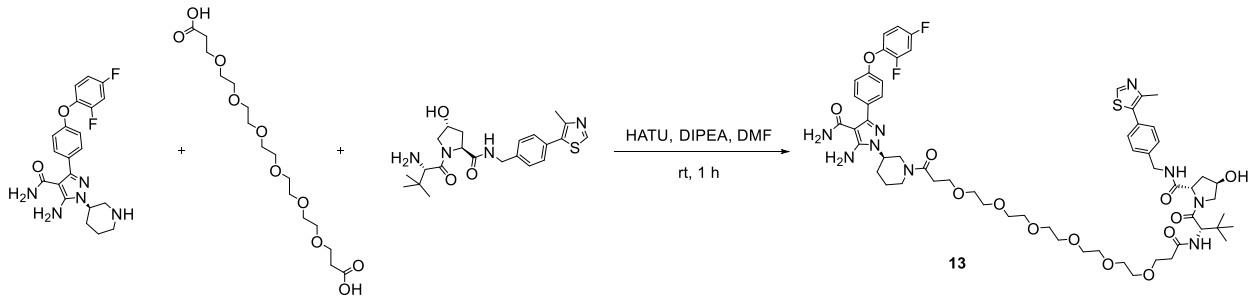
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



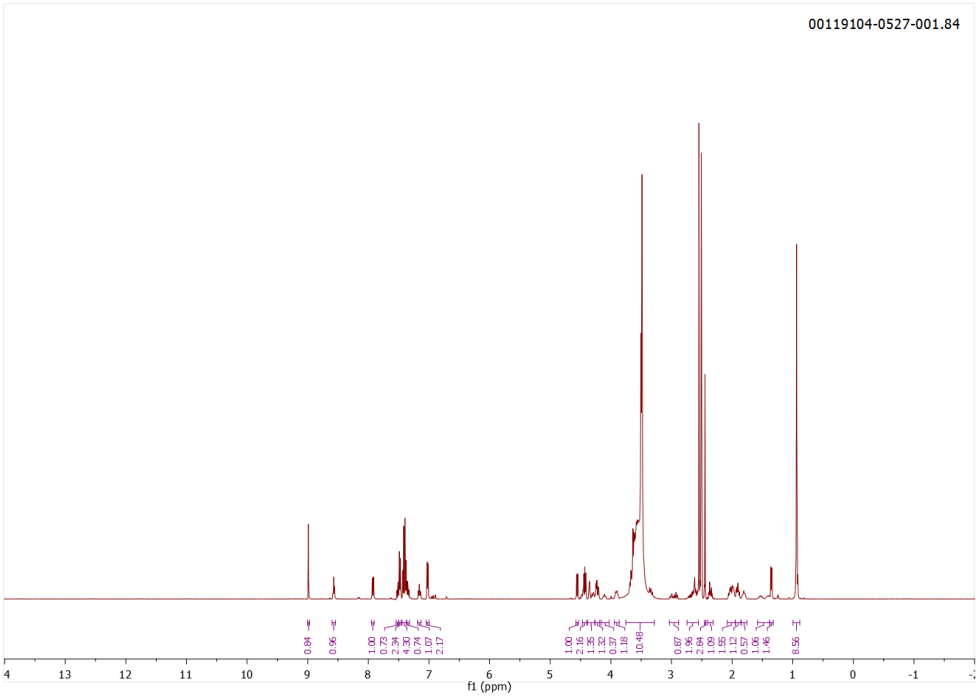
Preparation of *N*-{22-[(3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidin-1-yl]-22-oxo-4,7,10,13,16,19-hexaoxadocosan-1-oyl}-3-methyl-L-valyl-(4*R*)-4-hydroxy-*N*-[4-(4-methyl-1,3-thiazol-5-yl)benzyl]-L-prolinamide (13).



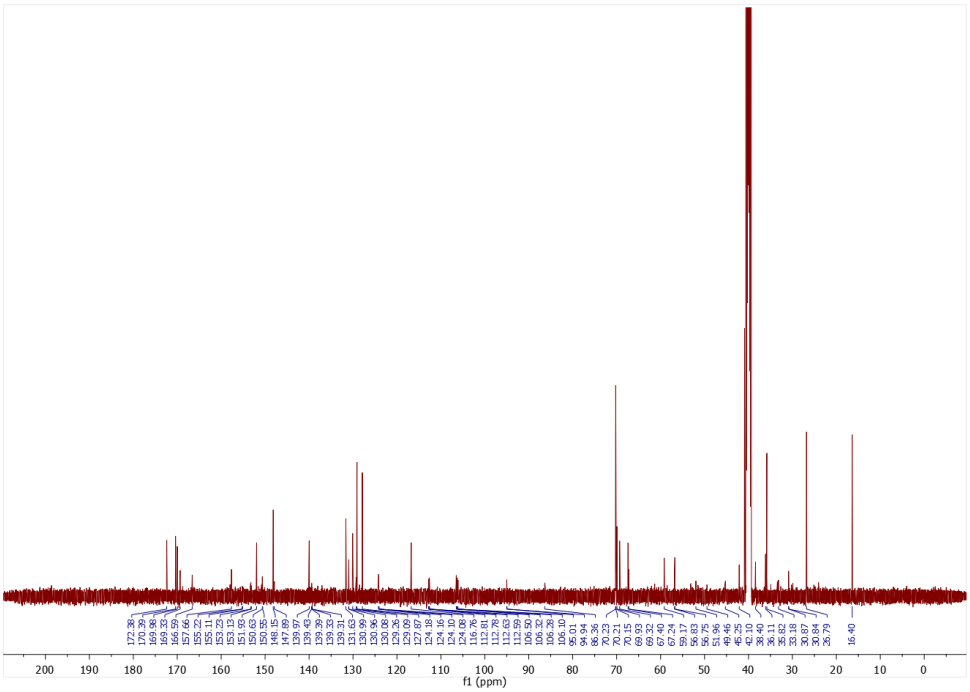
HATU (38 mg, 1 eq) was added to a solution of 4,7,10,13,16,19-hexaoxadocosane-1,22-dioic acid (38 mg, 1 eq), 3-methyl-L-valyl-(4*R*)-4-hydroxy-*N*-[4-(4-methyl-1,3-thiazol-5-yl)benzyl]-L-prolinamide (47 mg, 1 eq), and diisopropylethylamine (35 µL, 2 eq) in dimethylformamide (0.2 mL, 0.5 M). The reaction was stirred at rt for 15 min. To the reaction was added 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (46 mg, 1.1 eq), diisopropylethylamine (35 µL, 2 eq), and HATU (42 mg, 1.1 eq) sequentially. The reaction was stirred at rt for 15 min. The reaction was extracted with dichloromethane and washed with water. The combined organic layers were combined, dried, filtered, and concentrated *in vacuo*. The crude material was purified via flash chromatography and prep HPLC to afford the desired product *N*-{22-[(3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidin-1-yl]-22-oxo-4,7,10,13,16,19-

hexaoxadocosan-1-oyl}-3-methyl-L-valyl-(4*R*)-4-hydroxy-*N*-[4-(4-methyl-1,3-thiazol-5-yl)benzyl]-L-prolinamide (13) (28 mg, 24%) as a solid. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 8.99 (s, 1H), 8.57 (t, *J* = 6.1 Hz, 1H), 7.93 (d, *J* = 9.4 Hz, 1H), 7.54 – 7.50 (m, 1H), 7.48 (dd, *J* = 8.7, 1.8 Hz, 2H), 7.44 – 7.37 (m, 4H), 7.35 (dd, *J* = 9.3, 5.6 Hz, 1H), 7.19 – 7.13 (m, 1H), 7.05 – 7.00 (m, 2H), 4.56 (d, *J* = 9.3 Hz, 1H), 4.47 – 4.40 (m, 2H), 4.32 (d, *J* = 34.6 Hz, 1H), 4.22 (dd, *J* = 15.8, 5.5 Hz, 1H), 4.09 (d, *J* = 10.9 Hz, 1H), 3.91 (d, *J* = 13.1 Hz, 1H), 3.75 – 3.28 (m, 29H), 2.96 (dt, *J* = 33.4, 12.1 Hz, 1H), 2.74 – 2.56 (m, 2H), 2.45 (s, 3H), 2.36 (dt, *J* = 14.7, 6.2 Hz, 1H), 2.08 – 1.95 (m, 2H), 1.91 (ddd, *J* = 12.9, 8.4, 4.7 Hz, 1H), 1.81 (s, 1H), 1.46 (dd, *J* = 64.4, 13.5 Hz, 1H), 1.38 – 1.32 (m, 1H), 0.94 (s, 9H). ^{13}C NMR (126 MHz, DMSO-d_6) (observed) δ 172.38, 170.39, 169.98, 169.33, 166.59, 157.66, 154.17 (dd, *J* = 251.9, 13.9 Hz), 151.93, 150.63, 150.55, 148.15, 147.89, 139.97, 139.36 (dd, *J* = 11.6, 3.5 Hz), 131.63, 130.99, 130.96, 130.08, 129.26, 129.09, 127.87, 124.13 (dd, *J* = 10.0, 1.6 Hz), 116.76, 112.70 (dd, *J* = 22.8, 3.9 Hz), 106.30 (dd, *J* = 27.4, 22.4 Hz), 95.01, 94.94, 86.36, 70.23, 70.21, 70.15, 69.93, 69.32, 67.40, 67.24, 59.17, 56.83, 56.75, 51.96, 49.46, 45.25, 42.10, 38.40, 36.11, 35.82, 33.18, 30.87, 30.84, 26.79, 16.40. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.20, -126.13. LCMS: m/z 1190.7 $[M+H]^+$.

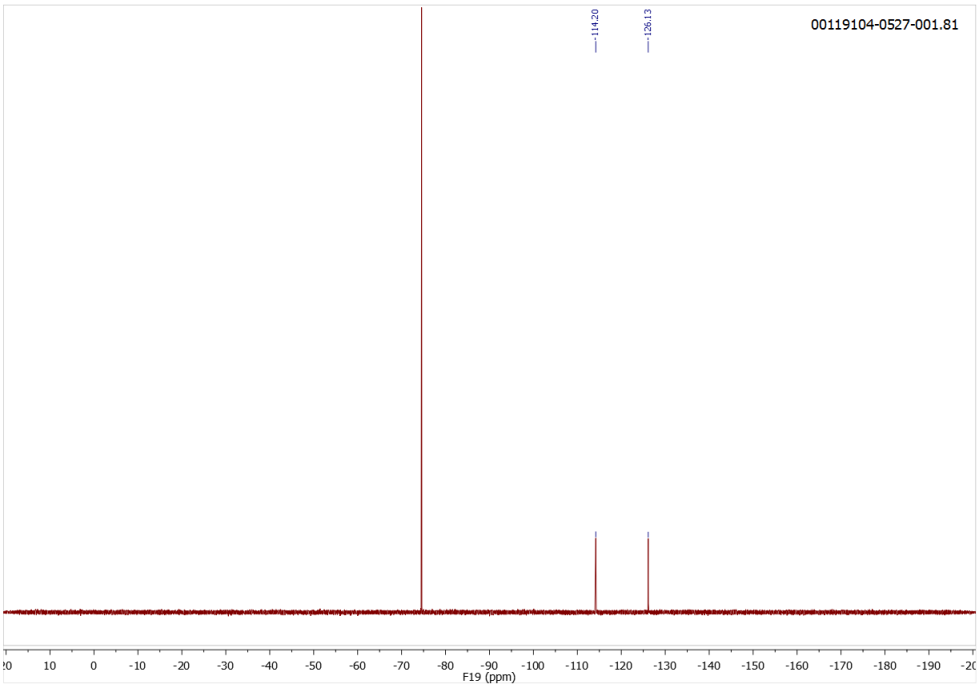
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



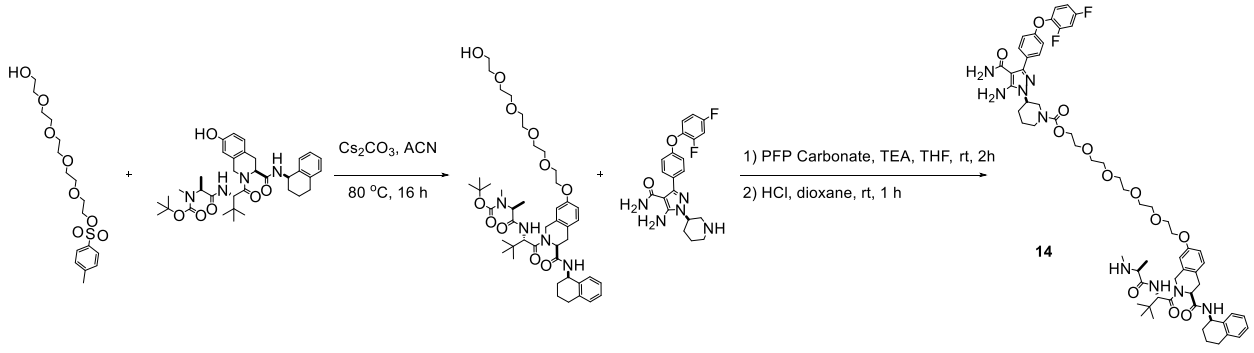
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 14-({(3*S*)-2-(*N*-methyl-L-alanyl-3-methyl-L-valyl)-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-1,2,3,4-tetrahydroisoquinolin-7-yl}oxy)-3,6,9,12-tetraoxatetradec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (14).



***tert*-butyl [(2*S*)-1-({(2*S*)-1-[(3*S*)-7-[(14-hydroxy-3,6,9,12-tetraoxatetradec-1-yl)oxy]-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-3,4-dihydroisoquinolin-2(1*H*)-yl]-3,3-dimethyl-1-oxobutan-2-yl}amino)-1-**

oxopropan-2-yl]methylcarbamate. *Tert*-butyl [(2*S*)-1-({(2*S*)-1-[(3*S*)-7-hydroxy-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-3,4-dihydroisoquinolin-2(1*H*)-yl]-3,3-dimethyl-1-oxobutan-2-yl}amino)-1-oxopropan-2-yl]methylcarbamate (50 mg, 1 eq) was added to a mixture of 14-hydroxy-3,6,9,12-tetraoxatetradec-1-yl 4-methylbenzenesulfonate (63 mg, 2 eq), and cesium carbonate (66 mg, 2.5 eq) in acetonitrile (2 mL, 0.04 M). The reaction was stirred at 80 ˚C for 16 h. The reaction was extracted with dichloromethane and washed with water. The combined organic layers were combined, dried, filtered, and concentrated *in vacuo*. The crude material was purified via flash chromatography with an eluate of 25% methanol in ethyl acetate to afford the title compound (57 mg, 84%) as a clear, colorless oil. LCMS: m/z 842.2 $[M+H]^+$.

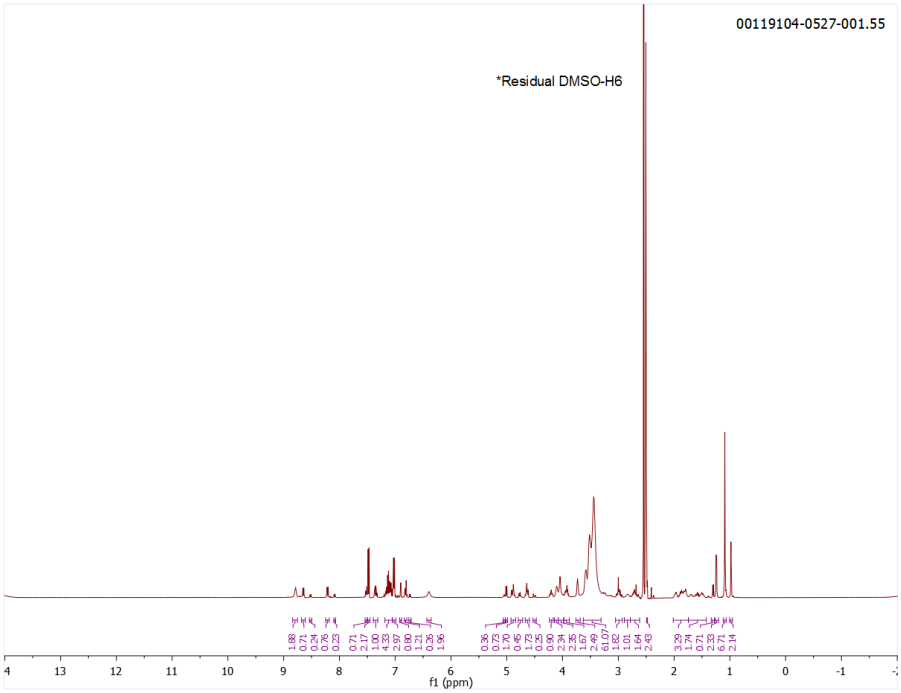
14-({(3*S*)-2-[*N*-(*tert*-butoxycarbonyl)-*N*-methyl-L-alanyl-3-methyl-L-valyl]-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-1,2,3,4-tetrahydroisoquinolin-7-yl}oxy)-3,6,9,12-tetraoxatetradec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. 9-Fluorenylmethyl pentafluorophenyl carbonate (26 mg, 1 eq) was added to a solution of *tert*-butyl [(2*S*)-1-({(2*S*)-1-[(3*S*)-7-[(14-hydroxy-3,6,9,12-tetraoxatetradec-1-yl)oxy]-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-3,4-dihydroisoquinolin-2(1*H*)-yl]-3,3-dimethyl-1-oxobutan-2-yl}amino)-1-oxopropan-2-yl]methylcarbamate (56 mg, 1 eq), and trimethylamine (1 mL, 100 eq) in tetrahydrofuran (2 mL, 0.03 M) and the reaction was stirred at rt for 1 h. The reaction turned to a purple color during this

time. To the reaction was added 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (28 mg, 1 eq) and the reaction was stirred at rt for 1 h. The reaction was quenched with sat. aqueous sodium bicarbonate and extracted with dichloromethane. The organic layers were combined, dried, filtered, and concentrated *in vacuo*. The crude material was purified via flash chromatography to afford the title compound (85 mg, quant.) as a clear, colorless oil. LCMS: m/z 1281.3 $[M+H]^+$.

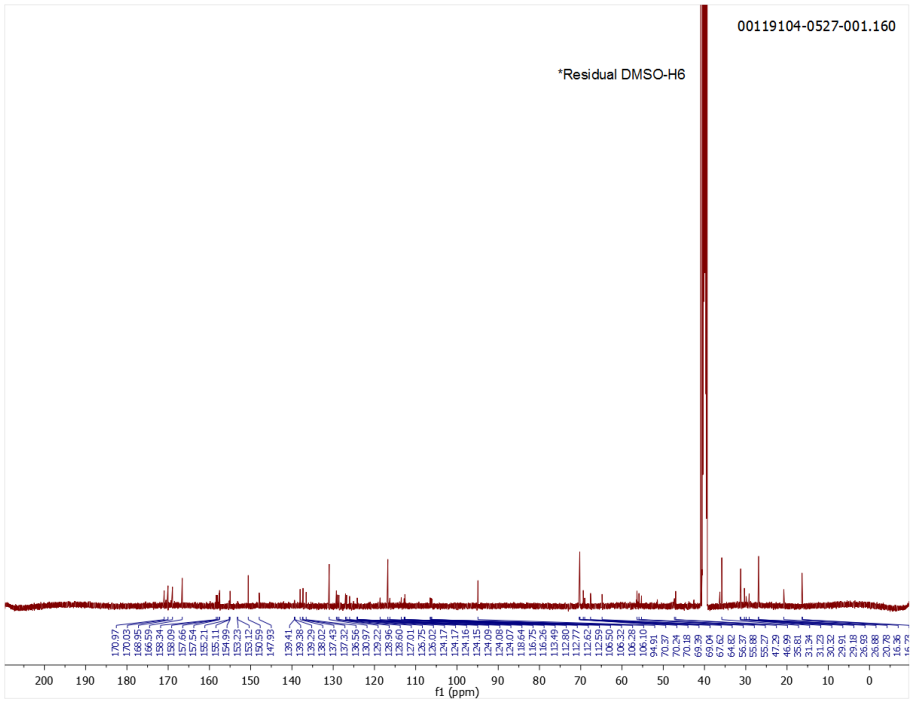
14-({(3*S*)-2-(*N*-methyl-L-alanyl-3-methyl-L-valyl)-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-1,2,3,4-tetrahydroisoquinolin-7-yl}oxy)-3,6,9,12-tetraoxatetradec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (14). A 4N solution of hydrochloric acid in dioxane (1 mL, 60 eq) was added to a solution of 14-({(3*S*)-2-[*N*-(*tert*-butoxycarbonyl)-*N*-methyl-L-alanyl-3-methyl-L-valyl]-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-1,2,3,4-tetrahydroisoquinolin-7-yl}oxy)-3,6,9,12-tetraoxatetradec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (85 mg, 1 eq) in dioxane (1 mL, 0.07 M). The reaction was stirred at rt for 1 h. The solvent was removed *in vacuo* and the crude product was purified via prep HPLC to afford the title compound (10 mg, 13%) as a solid. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 8.79 (s, 2H), 8.65 (d, *J* = 8.8 Hz, 0.75H), 8.52 (d, *J* = 8.8 Hz, 0.25H), 8.21 (d, *J* = 8.8 Hz, 0.75H), 8.09 (d, *J* = 8.3 Hz, 0.25H), 7.54 – 7.50 (m, 1H), 7.50 – 7.45 (m, 2H), 7.35 (td, *J* = 9.2, 5.7 Hz, 1H), 7.19 – 7.05 (m, 4H), 7.05

– 6.99 (m, 3H), 6.90 (d, *J* = 2.5 Hz, 1H), 6.84 – 6.79 (m, 0.75H), 6.74 (d, *J* = 7.7 Hz, 0.25H), 6.40 (s, 2H), 5.04 (d, *J* = 5.1 Hz, 0.25H), 5.01 (d, *J* = 8.9 Hz, 0.75H), 4.92 – 4.84 (m, 1.75H), 4.76 (t, *J* = 7.9 Hz, 0.25H), 4.67 – 4.59 (m, 1.75H), 4.51 (d, *J* = 17.1 Hz, 0.25H), 4.20 (dq, *J* = 10.2, 5.2, 4.2 Hz, 1H), 4.10 (s, 2H), 4.05 (d, *J* = 5.0 Hz, 2H), 3.93 (t, *J* = 10.1 Hz, 2H), 3.73 (s, 2H), 3.48 (d, *J* = 39.2 Hz, 20H), 3.05 – 2.93 (m, 2H), 2.83 (s, 1H), 2.79 – 2.62 (m, 2H), 2.49 (d, *J* = 5.5 Hz, 3H), 2.02 – 1.74 (m, 3H), 1.74 – 1.42 (m, 2H), 1.30 (d, *J* = 6.8 Hz, 1H), 1.24 (d, *J* = 6.9 Hz, 2H), 1.09 (s, 7H), 0.98 (s, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) (observed) δ 170.97, 170.03, 168.95, 166.59, 158.34, 158.09, 157.66, 157.54, 155.16 (dd, *J* = 249.1, 12.8 Hz), 154.99, 150.59, 147.93, 139.35 (dd, *J* = 11.8, 4.0 Hz), 138.02, 137.43, 137.32, 136.56, 130.97, 129.22, 128.96, 128.60, 127.01, 126.75, 126.02, 124.12 (dd, *J* = 10.4, 2.0 Hz), 118.64, 116.75, 116.26, 113.49, 112.70 (dd, *J* = 23.3, 3.4 Hz), 106.30 (dd, *J* = 27.4, 22.6 Hz), 94.91, 70.37, 70.24, 70.18, 69.39, 69.04, 67.62, 64.82, 56.37, 55.88, 55.27, 47.29, 46.99, 35.81, 31.34, 31.23, 30.32, 29.91, 29.18, 26.93, 26.88, 20.78, 16.36, 16.23. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.28, -126.18. LCMS: m/z 1181.3 $[M+H]^+$.

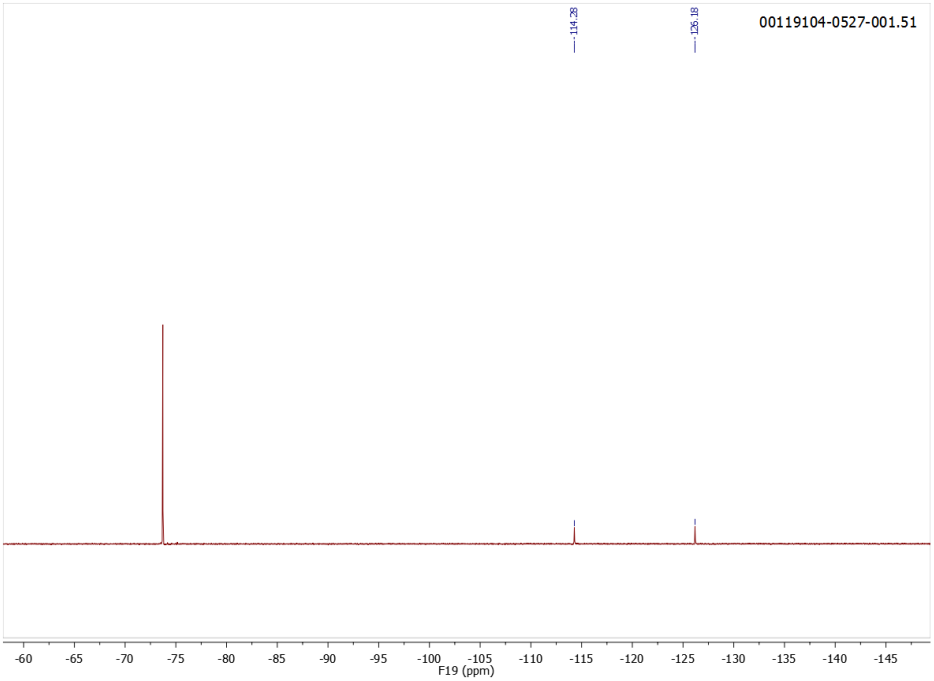
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



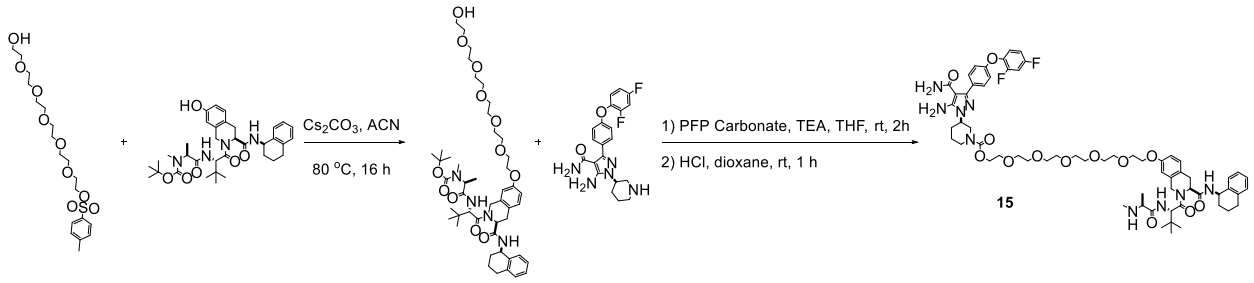
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 17-({(3*S*)-2-(*N*-methyl-L-alanyl-3-methyl-L-valyl)-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-1,2,3,4-tetrahydroisoquinolin-7-yl}oxy)-3,6,9,12,15-pentaoxaheptadec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (15).



***tert*-butyl [(2*S*)-1-({(2*S*)-1-[(3*S*)-7-[(17-hydroxy-3,6,9,12,15-pentaoxaheptadec-1-yl)oxy]-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-**

ylcarbamoyl]-3,4-dihydroisoquinolin-2(1*H*)-yl]-3,3-dimethyl-1-oxobutan-2-yl}amino)-1-oxopropan-2-yl]methylcarbamate. *Tert*-butyl [(2*S*)-1-({(2*S*)-1-[(3*S*)-7-hydroxy-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-3,4-dihydroisoquinolin-2(1*H*)-yl]-3,3-dimethyl-1-oxobutan-2-yl}amino)-1-oxopropan-2-yl]methylcarbamate (50 mg, 1 eq) was added to a mixture of 17-hydroxy-3,6,9,12,15-pentaoxaheptadec-1-yl 4-methylbenzenesulfonate (70 mg, 2 eq), and cesium carbonate (66 mg, 2.5 eq) in acetonitrile (2 mL, 0.04 M). The reaction was stirred at 80 ˚C for 16 h. The reaction was extracted with dichloromethane and washed with water. The combined organic layers were combined, dried, filtered, and concentrated *in vacuo*. The crude material was purified via flash chromatography with an eluate of 25% methanol in ethyl acetate to afford the title compound (57 mg, 84%) as a clear, colorless oil. LCMS: m/z 886.0 $[M+H]^+$.

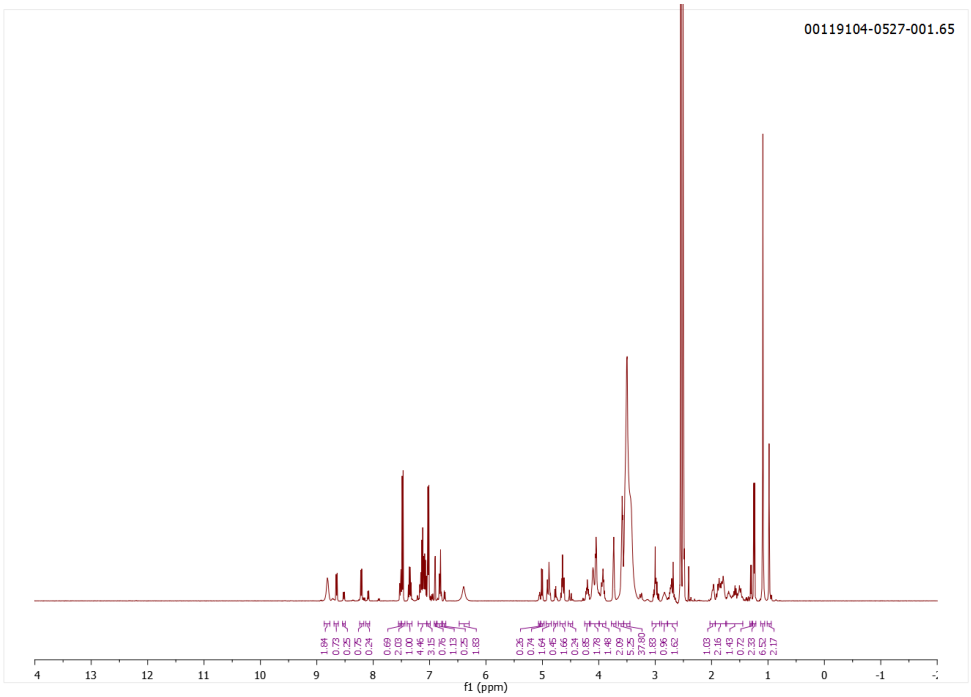
17-({(3*S*)-2-[*N*-(*tert*-butoxycarbonyl)-*N*-methyl-L-alanyl-3-methyl-L-valyl]-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-1,2,3,4-tetrahydroisoquinolin-7-yl}oxy)-3,6,9,12,15-pentaoxaheptadec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. 9-Fluorenylmethyl pentafluorophenyl carbonate (33 mg, 1 eq) was added to a solution of *tert*-butyl [(2*S*)-1-({(2*S*)-1-[(3*S*)-7-[(17-hydroxy-3,6,9,12,15-pentaoxaheptadec-1-yl)oxy]-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-3,4-dihydroisoquinolin-2(1*H*)-yl]-3,3-dimethyl-1-oxobutan-2-yl}amino)-1-oxopropan-2-yl]methylcarbamate (70 mg, 1

eq), and trimethylamine (1 mL, 90 eq) in tetrahydrofuran (2 mL, 0.04 M) and the reaction was stirred at rt for 1 h. The reaction turned to a purple color during this time. To the reaction was added 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (31 mg, 1 eq) and the reaction was stirred at rt for 1 h. The reaction was quenched with sat. aqueous sodium bicarbonate and extracted with dichloromethane. The organic layers were combined, dried, filtered, and concentrated *in vacuo*. The crude material was purified via flash chromatography to afford the title compound (100 mg, 95%) as a clear, colorless oil. LCMS: m/z 1325.3 $[M+H]^+$.

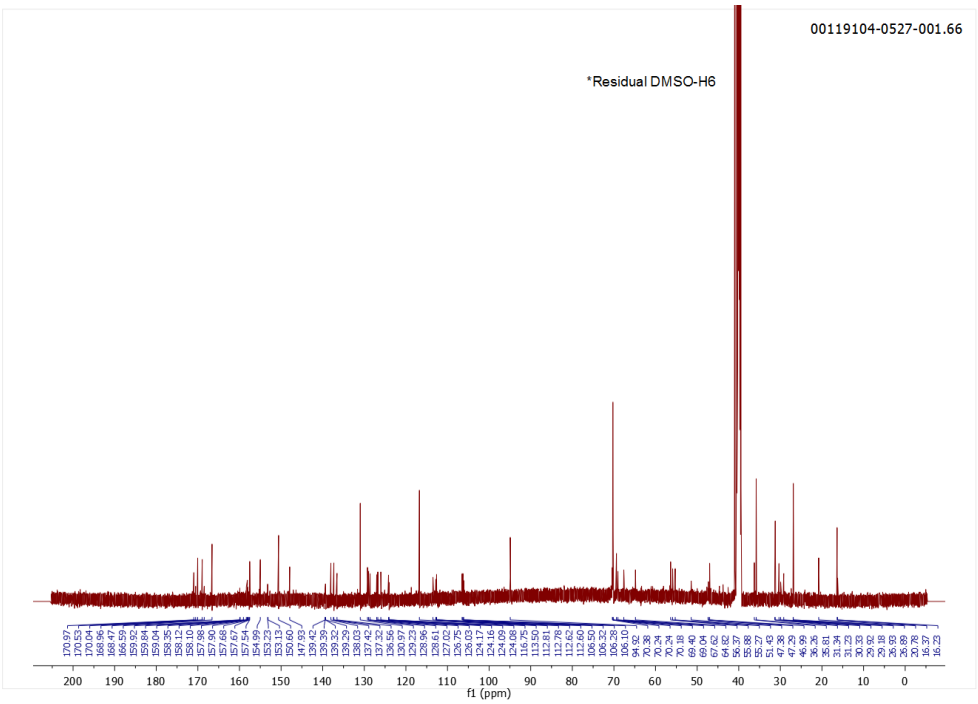
17-({(3*S*)-2-(*N*-methyl-L-alanyl-3-methyl-L-valyl)-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-1,2,3,4-tetrahydroisoquinolin-7-yl}oxy)-3,6,9,12,15-pentaoxaheptadec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (15). A 4N solution of hydrochloric acid in dioxane (1 mL, 50 eq) was added to a solution of 17-({(3*S*)-2-[*N*-(*tert*-butoxycarbonyl)-*N*-methyl-L-alanyl-3-methyl-L-valyl]-3-[(1*R*)-1,2,3,4-tetrahydronaphthalen-1-ylcarbamoyl]-1,2,3,4-tetrahydroisoquinolin-7-yl}oxy)-3,6,9,12,15-pentaoxaheptadec-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (100 mg, 1 eq) in dioxane (1 mL, 0.08 M). The reaction was stirred at rt for 1 h. The solvent was removed *in vacuo* and the crude product was purified via prep HPLC to afford the title compound (6 mg, 6%) as a solid. ^{1}H NMR (500 MHz, DMSO-d_6) (observed) δ 8.81 (s, 2H), 8.65 (d, *J* = 8.8 Hz,

0.75H), 8.52 (d, *J* = 8.8 Hz, 0.25H), 8.21 (d, *J* = 8.8 Hz, 0.75H), 8.09 (d, *J* = 8.3 Hz, 0.25H), 7.55 – 7.50 (m, 1H), 7.50 – 7.44 (m, 2H), 7.35 (td, *J* = 9.2, 5.6 Hz, 1H), 7.20 – 7.05 (m, 4H), 7.03 (dd, *J* = 7.9, 5.6 Hz, 3H), 6.90 (d, *J* = 2.5 Hz, 0.75H), 6.87 – 6.79 (m, 1H), 6.73 (d, *J* = 7.7 Hz, 0.25H), 6.40 (s, 2H), 5.05 (t, *J* = 4.6 Hz, 0.25H), 5.01 (d, *J* = 8.9 Hz, 0.75H), 4.93 – 4.84 (m, 2H), 4.76 (t, *J* = 7.9 Hz, 0.5H), 4.69 – 4.60 (m, 1.5H), 4.50 (d, *J* = 17.2 Hz, 0.25H), 4.20 (dq, *J* = 10.2, 5.2, 4.2 Hz, 1H), 4.05 (t, *J* = 4.5 Hz, 2H), 3.93 (t, *J* = 9.6 Hz, 1H), 3.73 (t, *J* = 4.8 Hz, 2H), 3.65 – 3.56 (m, 5H), 3.56 – 3.45 (m, 24H), 3.06 – 2.92 (m, 2H), 2.83 (s, 1H), 2.78 – 2.61 (m, 2H), 1.98 (d, *J* = 12.3 Hz, 1H), 1.94 – 1.75 (m, 2H), 1.53 (dd, *J* = 40.7, 11.2 Hz, 1H), 1.30 (d, *J* = 6.9 Hz, 1H), 1.24 (d, *J* = 6.8 Hz, 2H), 1.09 (s, 7H), 0.98 (s, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) (observed) δ 170.97, 170.53, 170.04, 168.96, 168.47, 166.59, 159.23 (dd, *J* = 244.1, 11.1 Hz), 158.35, 158.10, 157.68, 157.67, 157.54, 154.99, 153.18 (d, *J* = 12.7 Hz), 150.60, 147.93, 139.36 (dd, *J* = 11.7, 3.6 Hz), 138.03, 137.42, 137.32, 136.56, 130.97, 129.23, 128.96, 128.61, 127.02, 126.75, 126.03, 124.12 (dd, *J* = 10.0, 1.7 Hz), 116.75, 113.50, 112.70 (dd, *J* = 22.5, 3.2 Hz), 106.30 (dd, *J* = 27.5, 22.4 Hz), 94.92, 70.38, 70.24, 70.18, 69.40, 69.04, 67.62, 64.82, 56.37, 55.88, 55.27, 51.43, 47.38, 47.29, 46.99, 36.26, 35.81, 31.34, 31.23, 30.33, 29.92, 29.18, 26.93, 26.89, 20.78, 16.37, 16.23. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.26, -126.19. LCMS: m/z 1224.7 $[M+H]^+$.

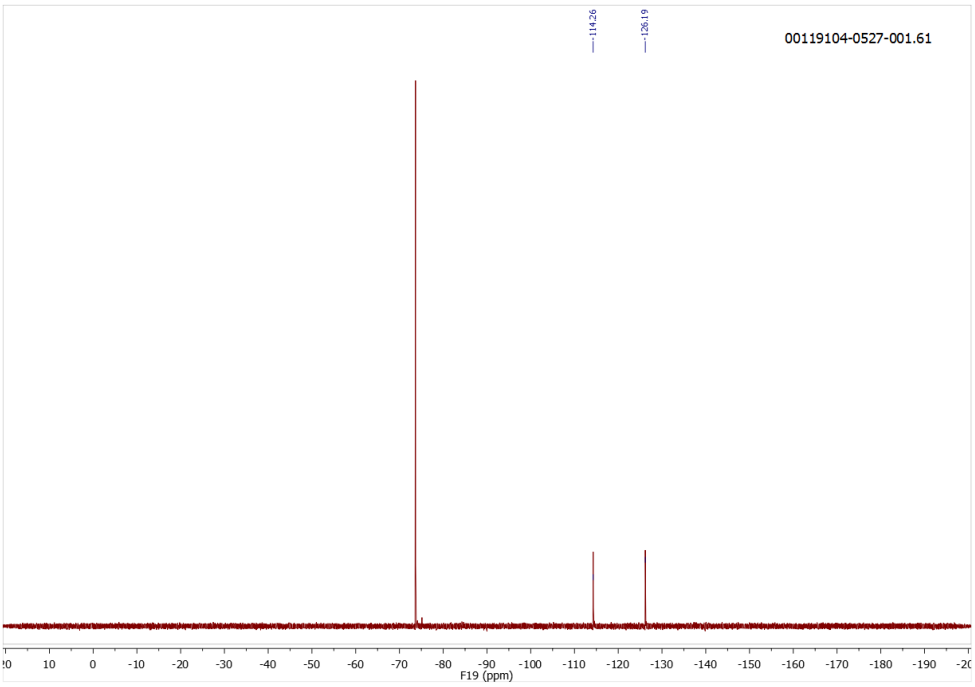
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



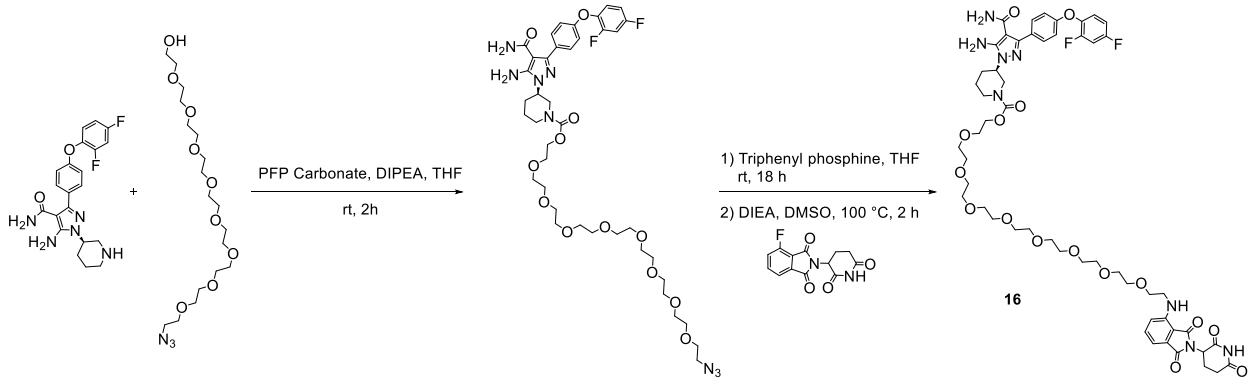
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



Preparation of 26-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}-3,6,9,12,15,18,21,24-octaoxahexacos-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (16).



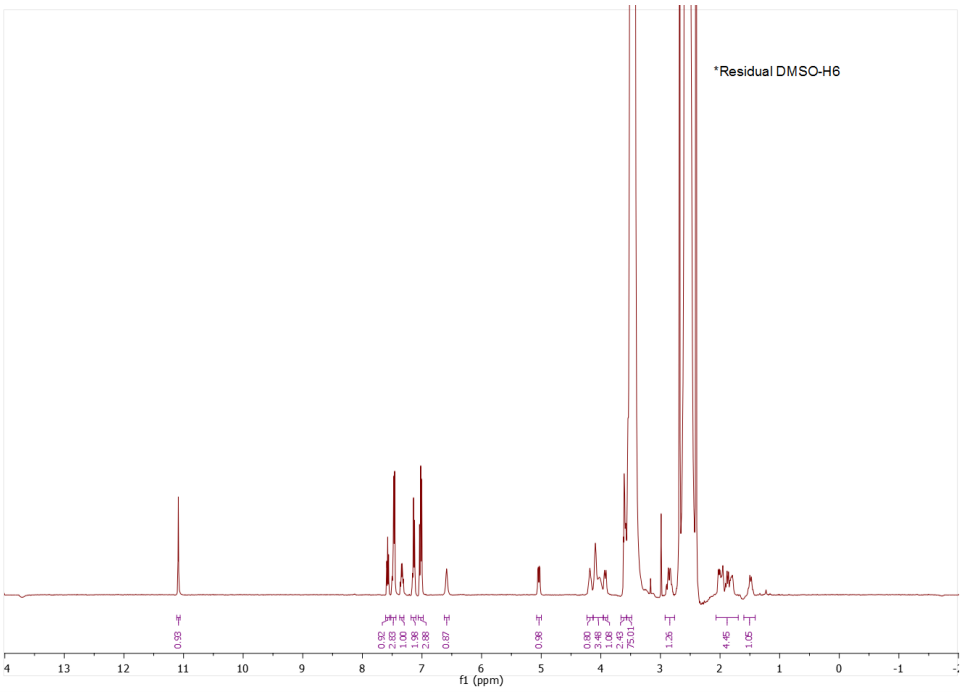
26-azido-3,6,9,12,15,18,21,24-octaoxahexacos-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. The title compound (220 mg, 83%) was obtained as an oil from 26-azido-3,6,9,12,15,18,21,24-octaoxahexacosan-1-ol using a procedure analogous to that described in the Carbonate Procedure, Method B. LCMS: m/z 880.0 $[M+H]^+$.

26-amino-3,6,9,12,15,18,21,24-octaoxahexacos-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate. The title compound (183 mg, 86%) was obtained as an oil from 26-azido-3,6,9,12,15,18,21,24-octaoxahexacos-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the Staudinger Reaction. LCMS: m/z 854.6 $[M+H]^+$.

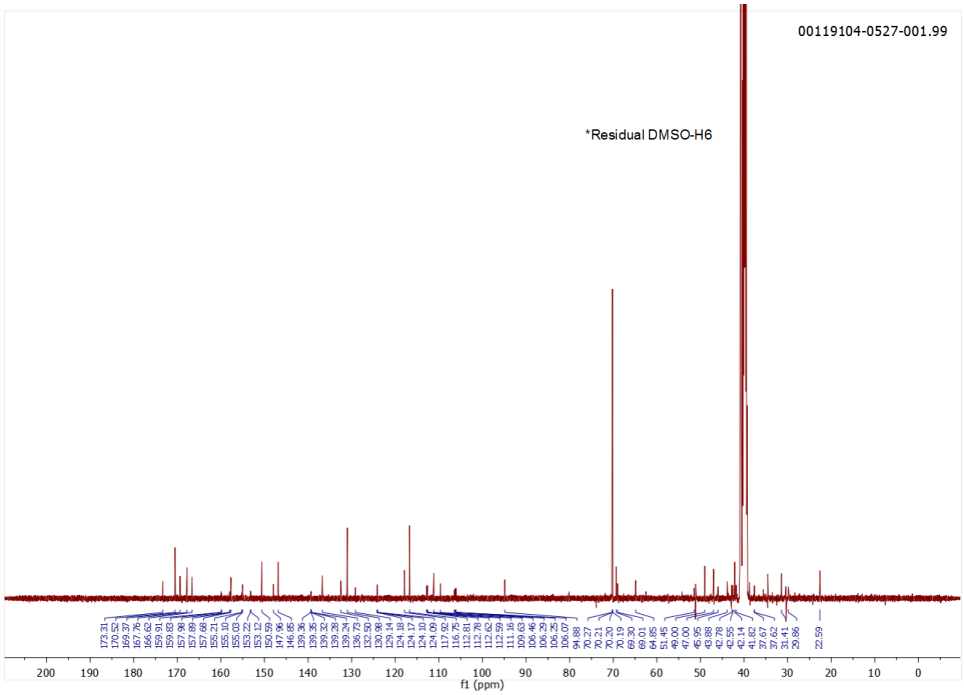
26-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxo-2,3-dihydro-1*H*-isoindol-4-yl]amino}-3,6,9,12,15,18,21,24-octaoxahexacos-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate (16). The title compound (13 mg, 11%) was obtained as a solid from 26-amino-3,6,9,12,15,18,21,24-octaoxahexacos-1-yl (3*R*)-3-{5-amino-4-carbamoyl-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazol-1-yl}piperidine-1-carboxylate using a procedure analogous to that described in the SnAr Procedure. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.09 (s, 1H), 7.58 (t, *J* = 7.8 Hz, 1H), 7.47 (d, *J* = 8.5 Hz, 3H), 7.34 (td, *J* = 9.0, 5.5 Hz, 1H), 7.13 (d, *J* = 8.7 Hz,

2H), 7.02 (dd, *J* = 11.0, 7.7 Hz, 3H), 6.59 (s, 1H), 5.04 (dd, *J* = 13.0, 5.4 Hz, 1H), 4.18 (s, 1H), 4.05 (d, *J* = 36.4 Hz, 3H), 3.93 (d, *J* = 13.2 Hz, 1H), 3.60 (q, *J* = 8.5, 7.0 Hz, 2H), 3.49 (s, 36H), 2.92 – 2.76 (m, 1H), 2.07 – 1.69 (m, 4H), 1.49 (d, *J* = 13.3 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) (observed) δ 173.31, 170.52, 169.37, 167.76, 166.62, 158.90 (dd, *J* = 243.8, 11.0 Hz), 157.68, 155.03, 154.51 (dd, *J* = 249.1, 13.1 Hz), 150.59, 147.96, 146.85, 139.30 (dd, *J* = 10.8, 4.9 Hz), 136.73, 132.50, 130.98, 129.14, 124.14 (dd, *J* = 9.9, 2.0 Hz), 117.92, 116.75, 112.70 (dd, *J* = 22.9, 3.7 Hz), 111.16, 109.63, 106.27 (dd, *J* = 27.4, 22.5 Hz), 94.88, 70.27, 70.21, 70.19, 69.30, 69.01, 64.85, 51.45, 49.00, 47.00, 45.95, 43.88, 42.55, 42.14, 41.82, 37.67, 37.62, 31.41, 29.86, 22.59. ^{19}F NMR (471 MHz, DMSO-d_6) δ -114.26, -126.16. LCMS: m/z 1109.8 $[M+H]^+$.

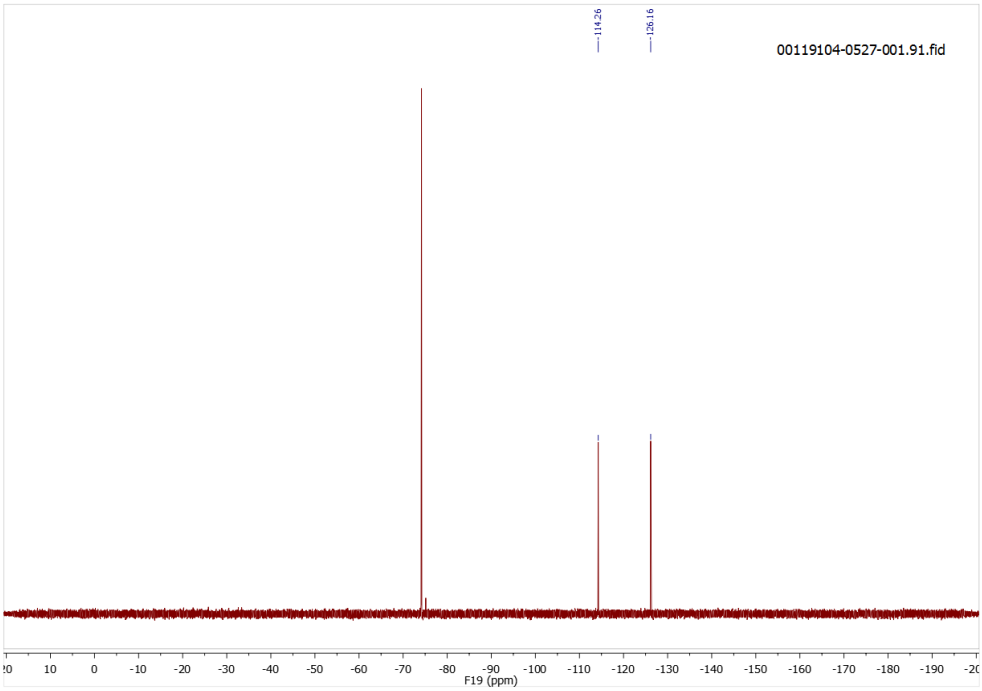
^{1}H NMR (500 MHz, DMSO-d_6 at 25°C)



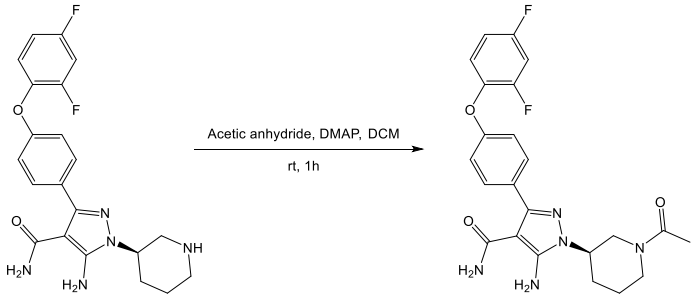
^{13}C NMR (126 MHz, DMSO-d_6 at 25°C)



^{19}F NMR (471 MHz, DMSO-d_6 at 25°C)



General procedure for BTK ligand. 1-[(3*R*)-1-acetylpiperidin-3-yl]-5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1*H*-pyrazole-4-carboxamide

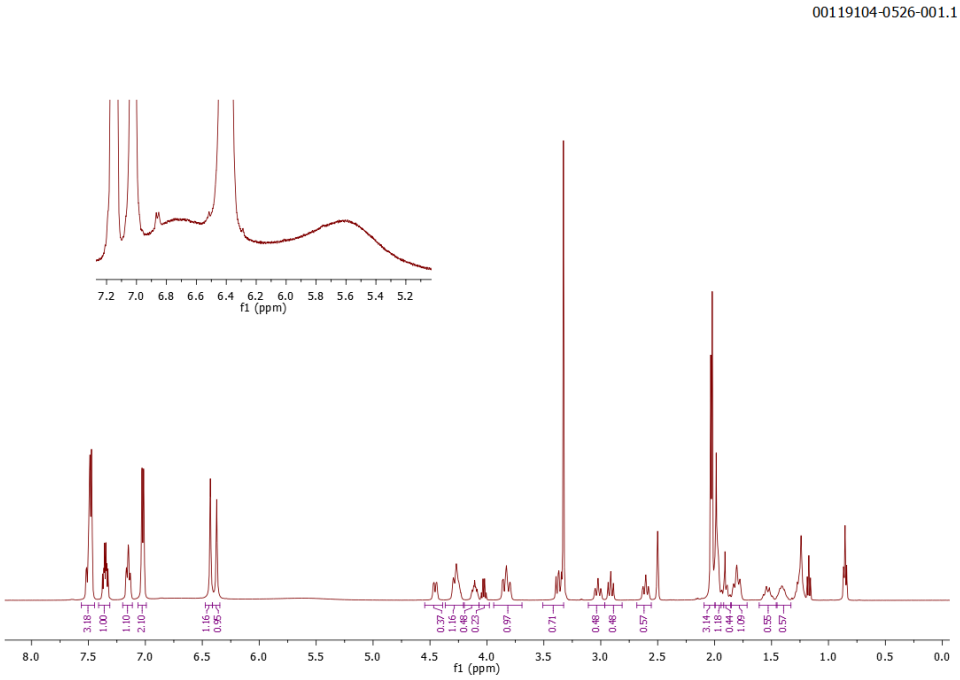


17

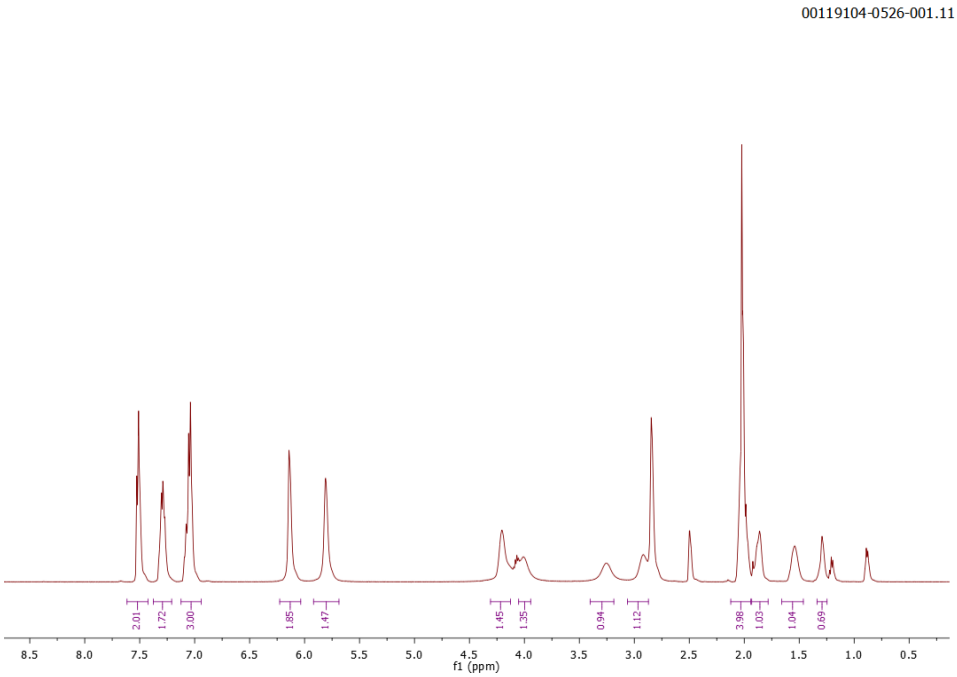
To a solution of 5-amino-3-[4-(2,4-difluorophenoxy)phenyl]-1-[(3*R*)-piperidin-3-yl]-1*H*-pyrazole-4-carboxamide (0.48 mmol, 1.0 eq) in CH_2Cl_2 (0.05 M) were added acetic anhydride (0.51 mmol, 1.05 eq), and DMAP (0.048 mmol, 0.1 eq). The mixture was stirred for 1 h at rt. Upon reaction completion, the solvent was reduced to ¼ volume and the crude product was purified by flash chromatography (0:100 to 10:90 methanol:ethyl acetate) to yield 1-[(3*R*)-1-acetylpiperidin-3-yl]-5-amino-3-[4-(2,4-

difluorophenoxy)phenyl]-1*H*-pyrazole-4-carboxamide, **17** (205 mg, 0.45 mmol, 93% yield). ^{1}H NMR (500 MHz, DMSO-d_6 at 25^{o}C) (observed) δ 7.56 – 7.45 (m, 3H), 7.35 (td, *J* = 9.2, 5.6 Hz, 1H), 7.20 – 7.11 (m, 1H), 7.02 (dd, *J* = 8.7, 2.2 Hz, 2H), 6.71 (br s, 1H), 6.43 (s, 1H), 6.37 (s, 1H), 5.25 (br s, 1H), 4.45 (dd, *J* = 12.2, 4.6 Hz, 0.5 H), 4.37 – 4.21 (m, 1H), 4.11 (tt, *J* = 10.8, 4.2 Hz, 0.5 H), 4.03 (q, *J* = 7.1 Hz, 0H), 3.94 – 3.69 (m, 1H), 3.37 (dd, *J* = 13.3, 10.4 Hz, 1H), 3.02 (td, *J* = 13.2, 2.7 Hz, 0.5 H), 2.91 (t, *J* = 11.6 Hz, 0.5 H), 2.60 (td, *J* = 12.6, 2.9 Hz, 0.5 H), 2.03 (d, *J* = 6.8 Hz, 3H), 2.00 – 1.92 (m, 1H), 1.94 – 1.86 (m, 0.5 H), 1.81 (tt, *J* = 13.9, 3.3 Hz, 1H), 1.53 (qt, *J* = 13.4, 13.1, 4.1, 3.5 Hz, 0.5 H), 1.41 (dq, *J* = 17.6, 5.4 Hz, 0.5 H). ^{1}H NMR (500 MHz, DMSO-d_6 at 125 ^{o}C) (rotamers resolved) δ 7.62 – 7.42 (m, 2H), 7.30 (dq, *J* = 9.3, 5.2 Hz, 2H), 7.05 (m, 3H), 6.14 (s, 2H), 5.81 (s, 1H), 4.20 (s, 1H), 4.01 (s, 1H), 3.25 (s, 1H), 2.92 (s, 1H), 2.12 – 1.94 (m, 4H), 1.93 – 1.78 (m, 1H), 1.55 (br s, 1H), 1.29 (br s, 1H). ^{13}C NMR (126 MHz, DMSO-d_6 at 25 ^{o}C) (observed) δ 168.37, 168.31, 166.15, 166.13, 158.44 (dd, *J* = 243.5, 10.8 Hz), 157.20, 153.71 (dd, *J* = 249.6, 13.1 Hz), 150.17, 150.08, 147.47, 147.45, 138.91 (dd, *J* = 11.6, 3.8 Hz), 130.52, 130.49, 128.78, 123.65 (d, *J* = 9.4 Hz), 116.30, 116.29, 112.21 (dd, *J* = 22.9, 3.6 Hz), 105.82 (dd, *J* = 27.5, 22.5 Hz), 94.52, 94.46, 51.46, 51.02, 49.77, 45.67, 44.69, 40.75, 31.25, 29.69, 29.40, 28.36, 24.62, 23.61, 22.09, 21.39, 21.32. ^{13}C NMR (126 MHz, DMSO-d_6 at 125 ^{o}C) (rotamers resolved) δ 167.73, 165.53, 157.86 (dd, *J* = 244.4, 9.8 Hz), 156.72, 153.22 (dd, *J* = 250.0, 12.6 Hz), 149.61, 146.94, 138.90 (d, *J* = 11.7 Hz), 129.82, 128.78, 122.65 (d, *J* = 10.2 Hz), 116.03, 111.20 (dt, *J* = 23.0, 3.2 Hz), 104.83 (dd, *J* = 27.4, 22.5 Hz), 94.80, 51.03, 47.06 (br), 42.65 (br), 28.59, 23.14, 20.21. ^{19}F NMR (471 MHz, DMSO at 25^{o}C) δ -114.28, -126.19. LCMS: m/z 456.5 $[M+H]^{+}$.

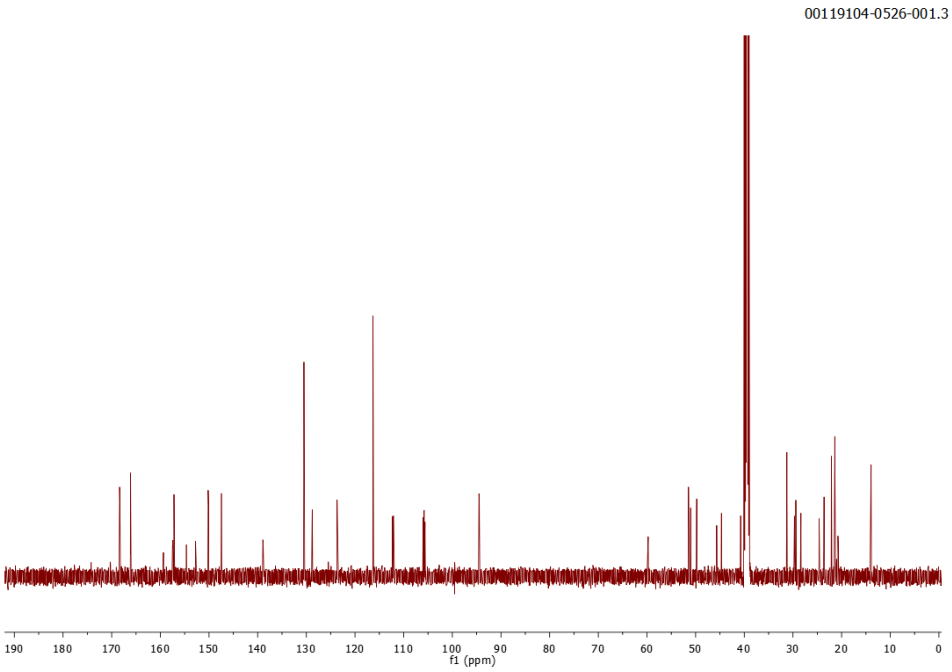
^{1}H NMR (500 MHz, DMSO-d_6 at 125^{o}C)



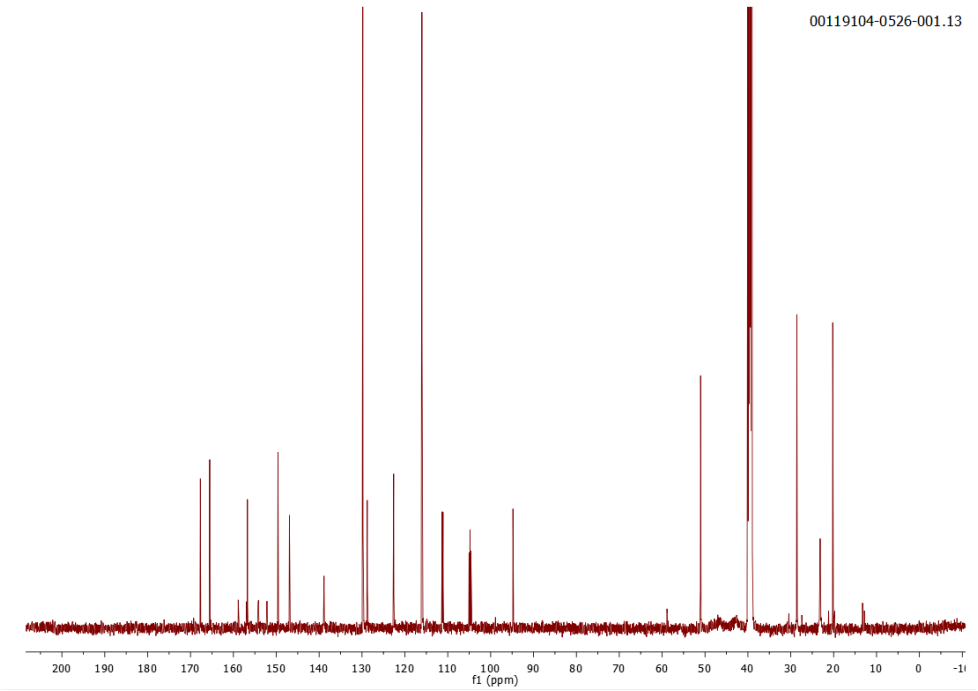
^{1}H NMR (500 MHz, DMSO-d_6 at 25 ^{o}C)



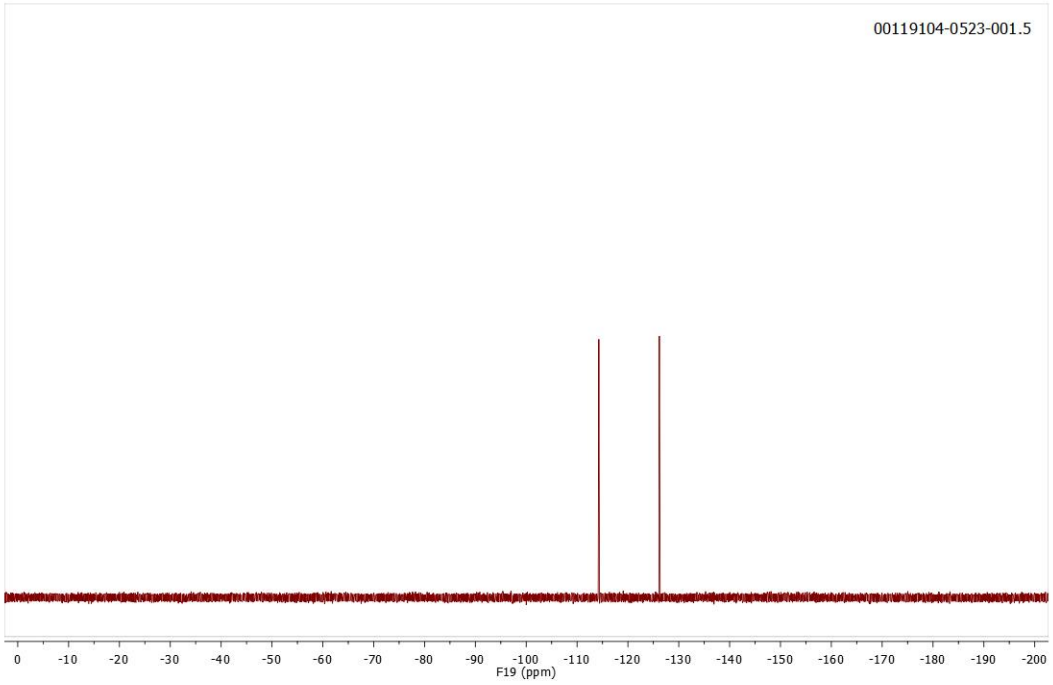
^{13}C NMR (126 MHz, DMSO-d_6 at 125 ^{o}C)



^{13}C NMR (126 MHz, DMSO-d_6 at 25 ^{o}C)



^{19}F NMR (471 MHz, DMSO at 25^{o}C)



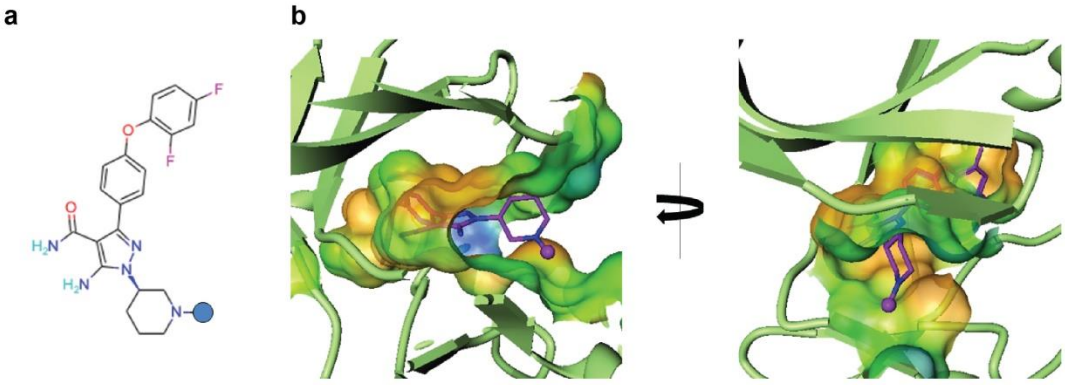


Figure S1 I Modeled conformation of BTK binder. (**a**) Scaffold of BTK binding moiety used for modeling into ATP pocket of BTK. (**b**) Modeled pose of ligand shown in two rotated views. Sphere denotes approximate vector of N-linked substituent of the piperidine.

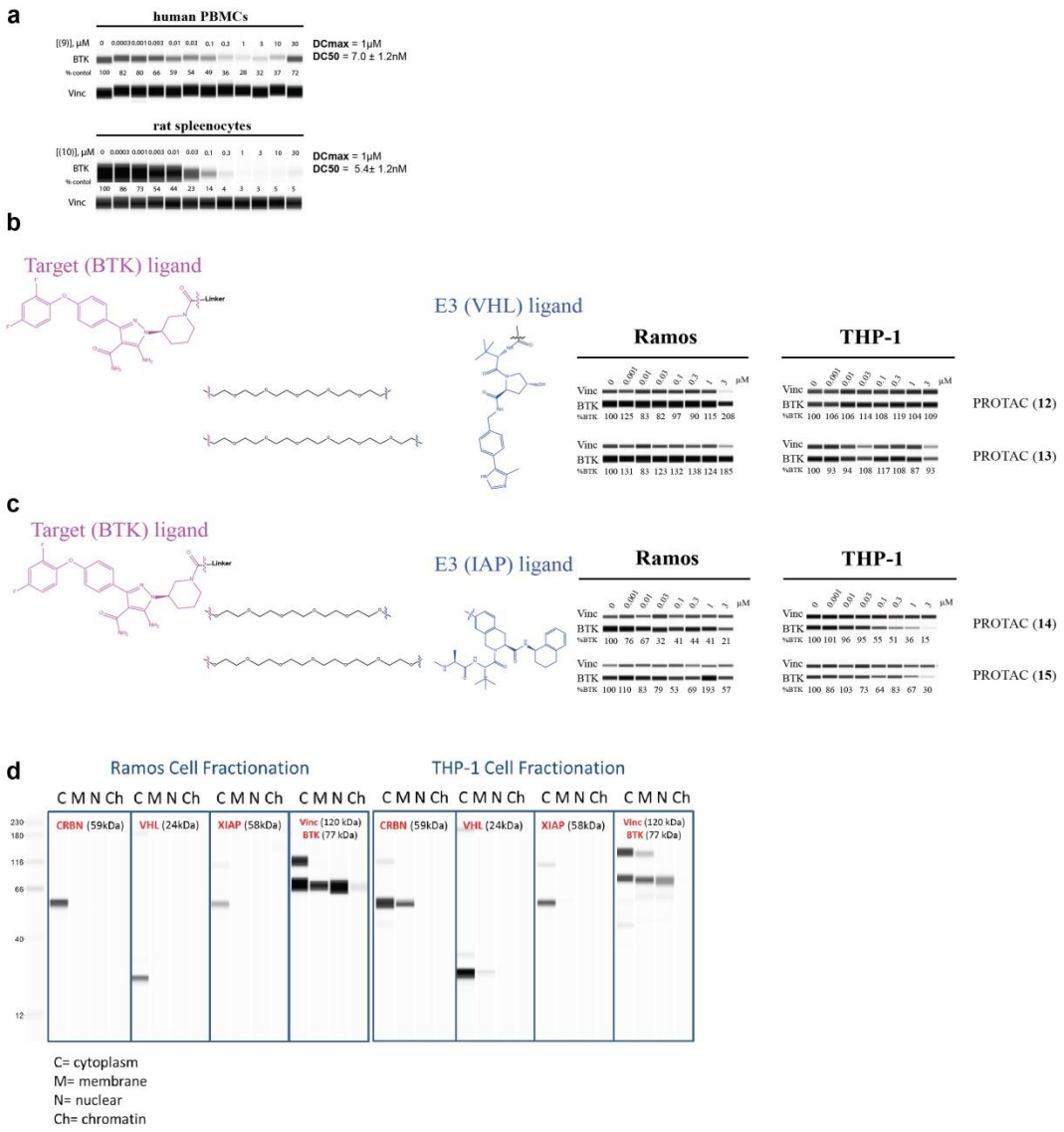


Figure S2 I PROTAC (**9**) degrades BTK in primary cultures and in an E3 ligase dependent manner. (**a**) Dose-dependent degradation of BTK in primary cell lines - Peripheral Blood Mononuclear Cells (PMBCs) and spleenocytes - by PROTACs (**9**) and (**10**). BTK degradation is inefficient when either VHL (**b**) or IAP (**c**) are recruited instead of CRBN, despite all proteins co-localizing in similar cellular compartments whether in Ramos or THP-1 cells (**d**). For dosing experiments, cells were incubated for 24 h with compound and lysates were blotted for BTK and various indicated proteins.

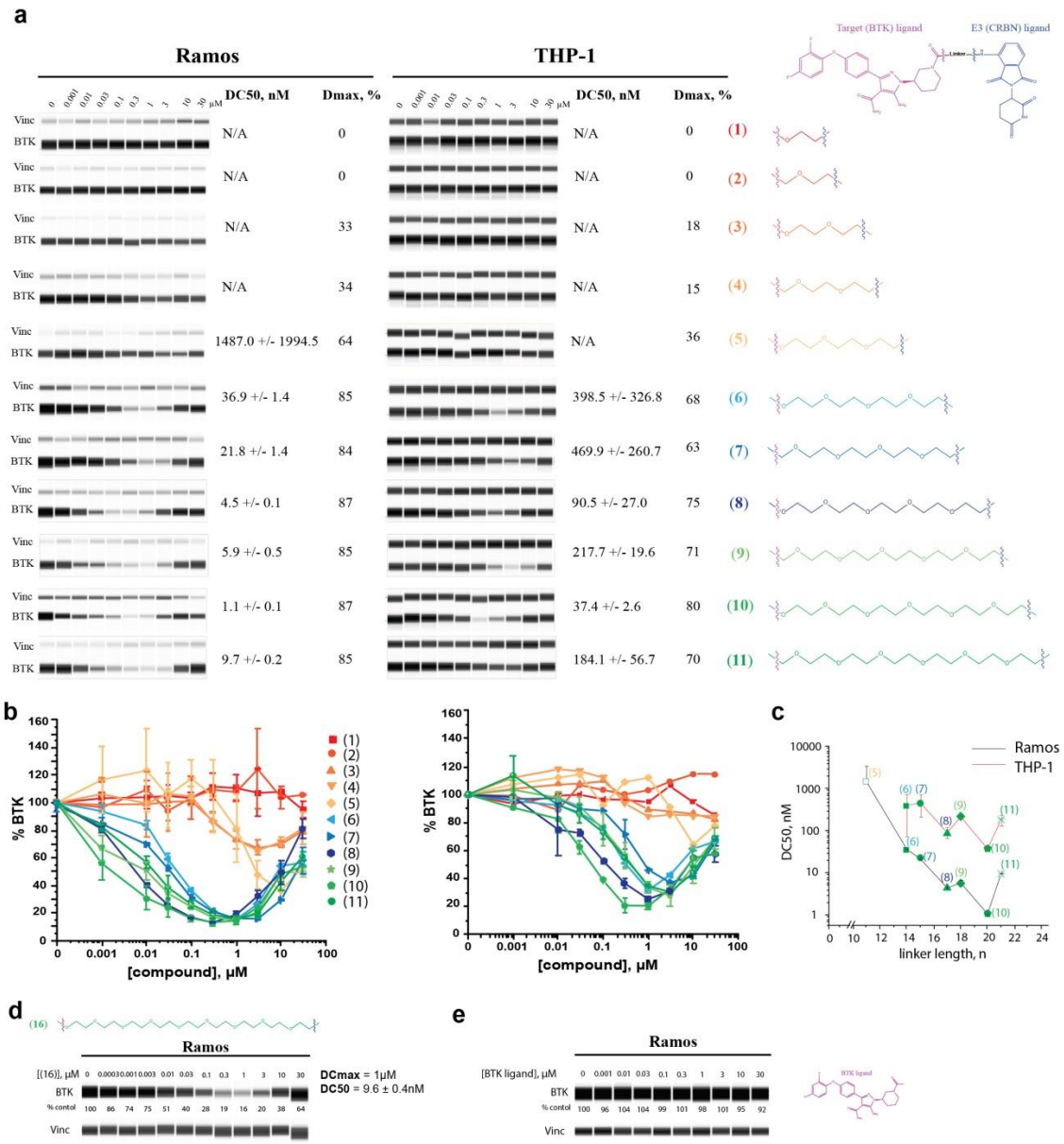


Figure S3 I Longer linkers allow for efficacious BTK degradation whether in Ramos or THP-1 cells. (**a**) Raw data of BTK degradation in Ramos (left) and THP-1 (middle) cells with their corresponding DC50s and Dmax (determined at 24 hour timepoint). (**b**) BTK signal was quantified and normalized to Vinc loading control for either Ramos (left) or THP-1 (right) cells. (**c**) A change of 2 atoms

when using PROTAC (**5**) vs. (**6**) results in vast improvement in BTK degradation efficiency and tightening of DC50 by more than an order of magnitude. Result is consistent in THP-1 cells although DC50 of PROTAC (**5**) is too weak to be quantified. Further relief of steric clashes with linkers as long as 29 atoms (**d**) does not lead to additional gain in BTK degradation efficacy. Parent binder alone (**e**) does not cause BTK degradation.

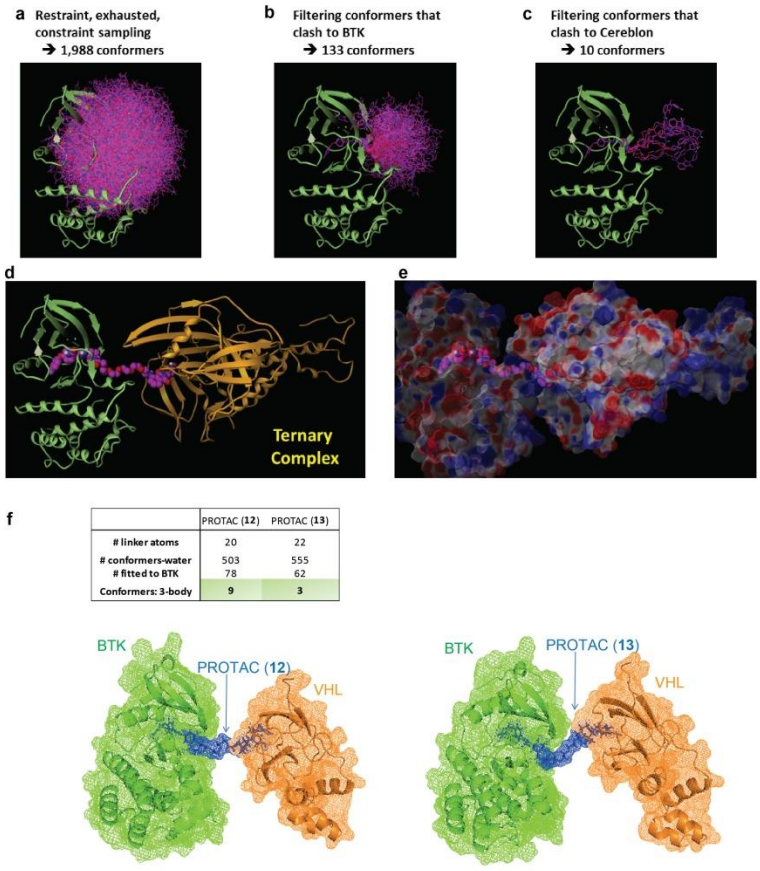
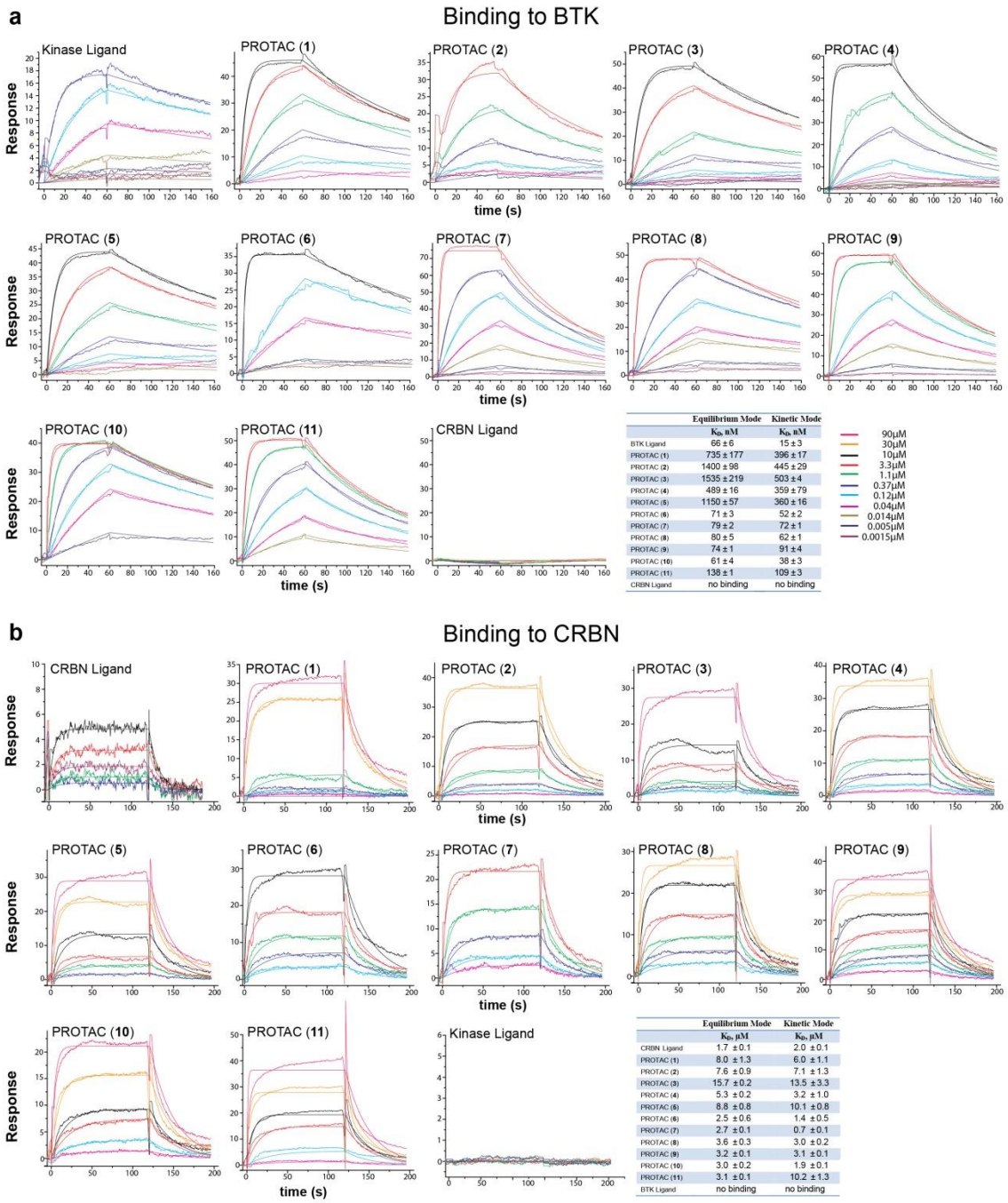


Figure S4 I Computational Modeling Workflow - hypothesizes that systems that can generate multiple possible solutions are likely to form at least one successful ternary complex available for ubiquitination. (**a**) Conformational sampling of the PROTAC compound by keeping the coordinates of the target binder piece fixed and torsions of E3 ligase binder piece fixed (**b**) PROTAC conformations that clash with target protein are rejected (**c**) E3 ligase is transposed and conformations of PROTAC that clash with E3 ligase or solutions that have steric clashes between target protein and E3 ligase, are rejected. A representative example of a successful ternary complex involving PROTAC (**9**) is shown in cartoon (**d**) or space-fill mode (**e**). (**f**) Ternary complex modeling for BTK-VHL using PROTAC (**12**, **13**) with representative poses shown below.



	Equilibrium Mode K_D, nM	Kinetic Mode K_D, nM
BTK Ligand	66 ± 6	15 ± 3
PROTAC (1)	735 ± 177	396 ± 17
PROTAC (2)	1400 ± 98	445 ± 29
PROTAC (3)	1535 ± 219	503 ± 4
PROTAC (4)	489 ± 16	359 ± 79
PROTAC (5)	1150 ± 57	360 ± 16
PROTAC (6)	71 ± 3	52 ± 2
PROTAC (7)	79 ± 2	72 ± 1
PROTAC (8)	80 ± 5	62 ± 1
PROTAC (9)	74 ± 1	91 ± 4
PROTAC (10)	61 ± 4	38 ± 3
PROTAC (11)	138 ± 1	109 ± 3
CRBN Ligand	no binding	no binding

	Equilibrium Mode K_D, μM	Kinetic Mode K_D, μM
CRBN Ligand	1.7 ± 0.1	2.0 ± 0.1
PROTAC (1)	8.0 ± 1.3	6.0 ± 1.1
PROTAC (2)	7.6 ± 0.9	7.1 ± 1.3
PROTAC (3)	15.7 ± 0.2	13.5 ± 3.3
PROTAC (4)	5.3 ± 0.2	3.2 ± 1.0
PROTAC (5)	8.8 ± 0.8	10.1 ± 0.8
PROTAC (6)	2.5 ± 0.6	1.4 ± 0.5
PROTAC (7)	2.7 ± 0.1	0.7 ± 0.1
PROTAC (8)	3.6 ± 0.3	3.0 ± 0.2
PROTAC (9)	3.2 ± 0.1	3.1 ± 0.1
PROTAC (10)	3.0 ± 0.2	1.9 ± 0.1
PROTAC (11)	3.1 ± 0.1	10.2 ± 1.3
BTK Ligand	no binding	no binding

Figure S5 I Raw sensorgrams of PROTACs and parent molecules binding to BTK (**a**) or CRBN (**b**). Calculated K_D's using either the equilibrium or the kinetic binding modes are shown in the tables adjacent to the raw data. Error bars represent the standard error in n=2 measurements.

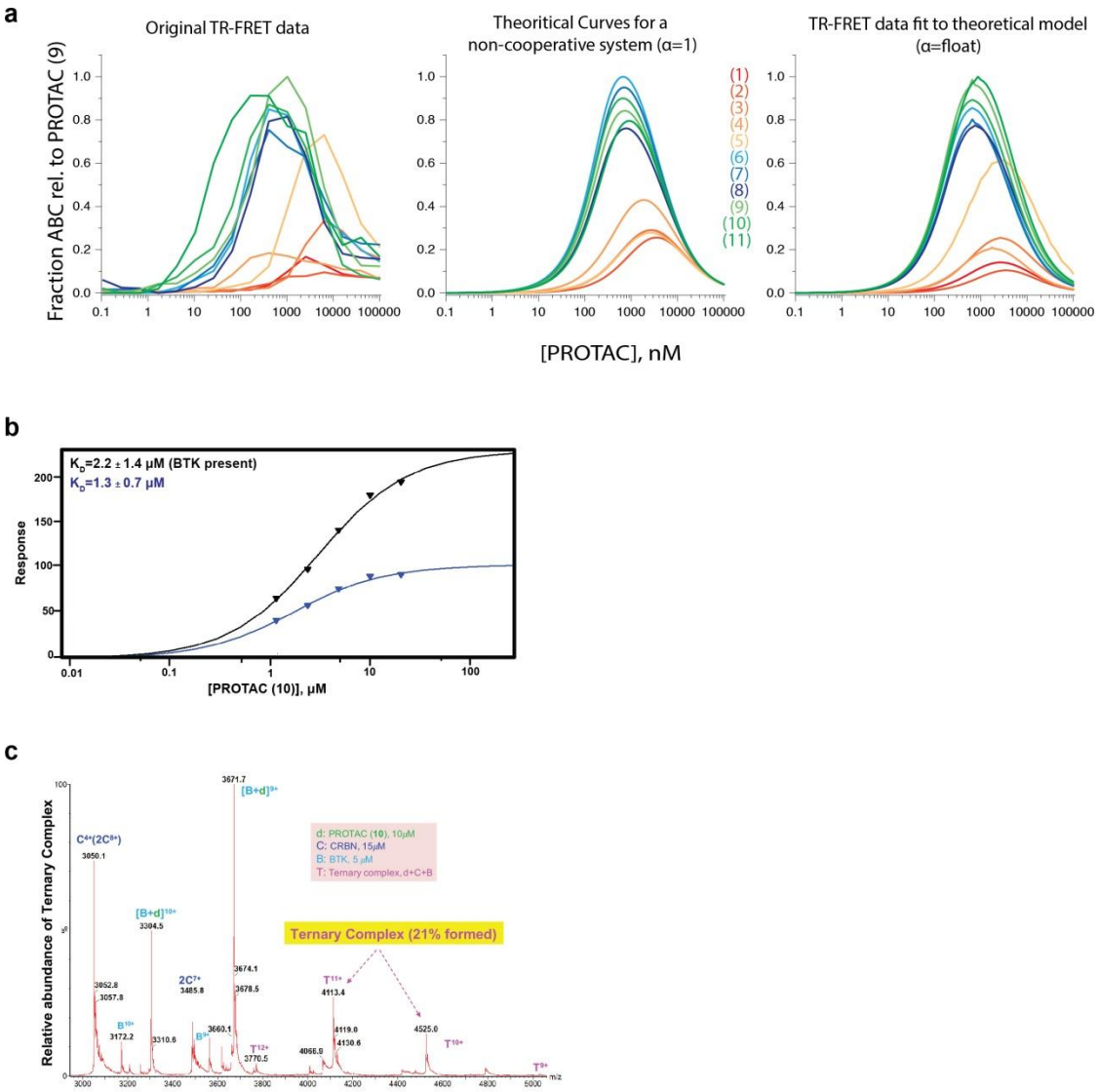


Figure S6 I Negative cooperativity dominates ternary complex formation models for inefficacious PROTACs, whereas efficacious PROTACs do not rely on cooperativity for ternary complex formation. (**a**) Left: TR-FRET data shown relative to PROTAC (**9**) whose maxima is set to 1. TR-FRET data fit to Equation (1) where α=1 (center) or α was allowed to vary until the sum of the square of

errors between the experimental and theoretical curves were minimized (right). (**b**) SPR binding affinity of PROTAC (**10**) to CRBN in the absence and presence of BTK. (**c**) Native State Mass Spectrometry based detection of {BTK-PROTAC(**10**)-CRBN} ternary complex. Experiment run at 5 μM BTK, 10 μM PROTAC(**10**), and 15 μM CRBN. Approximately 21% ternary complex is detected (relative to BTK), consistent with absence of positive cooperativity.

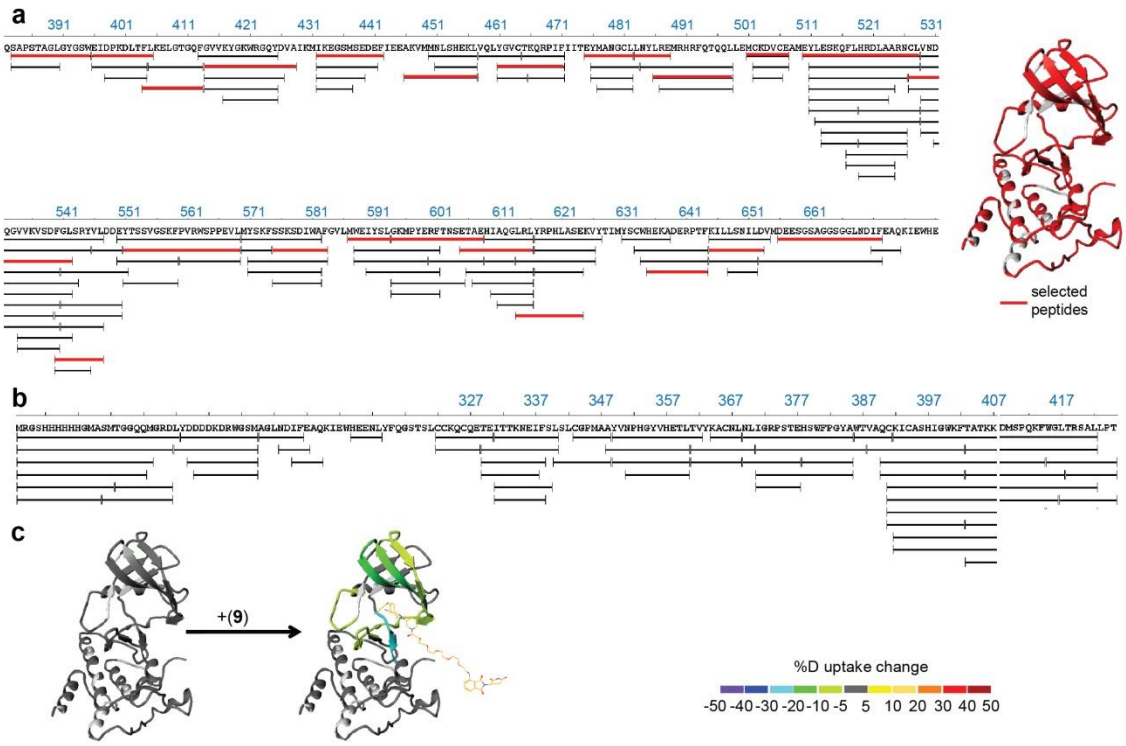


Figure S7 I Lack of a single, rigid, stable ternary complex as reported by HDX-MS. Peptide sequence coverage map for BTK (**a**) and CRBN (**b**). Lines below the sequence indicate peptides that were identified by MS/MS with Mascot ion score >20 and generated quantifiable, reproducible HDX data at all time points (10, 30, 60, 300, 900 and 3600s) in both apo and ligand bound states of BTK or CRBN. Numbers represent sequence alignment with the wild-type human BTK or CRBN. Peptides selected in the core set are highlighted in red and mapped to the 3D structure of BTK kinase domain. (**c**) HDX perturbation data mapped onto the 3D structure of BTK kinase domain. A color gradient is used to represent the average deuterium uptake differences across all 6 time points between apo BTK (left) and in the presence of PROTAC (**9**) (right). White indicates regions not covered by the core peptide set.

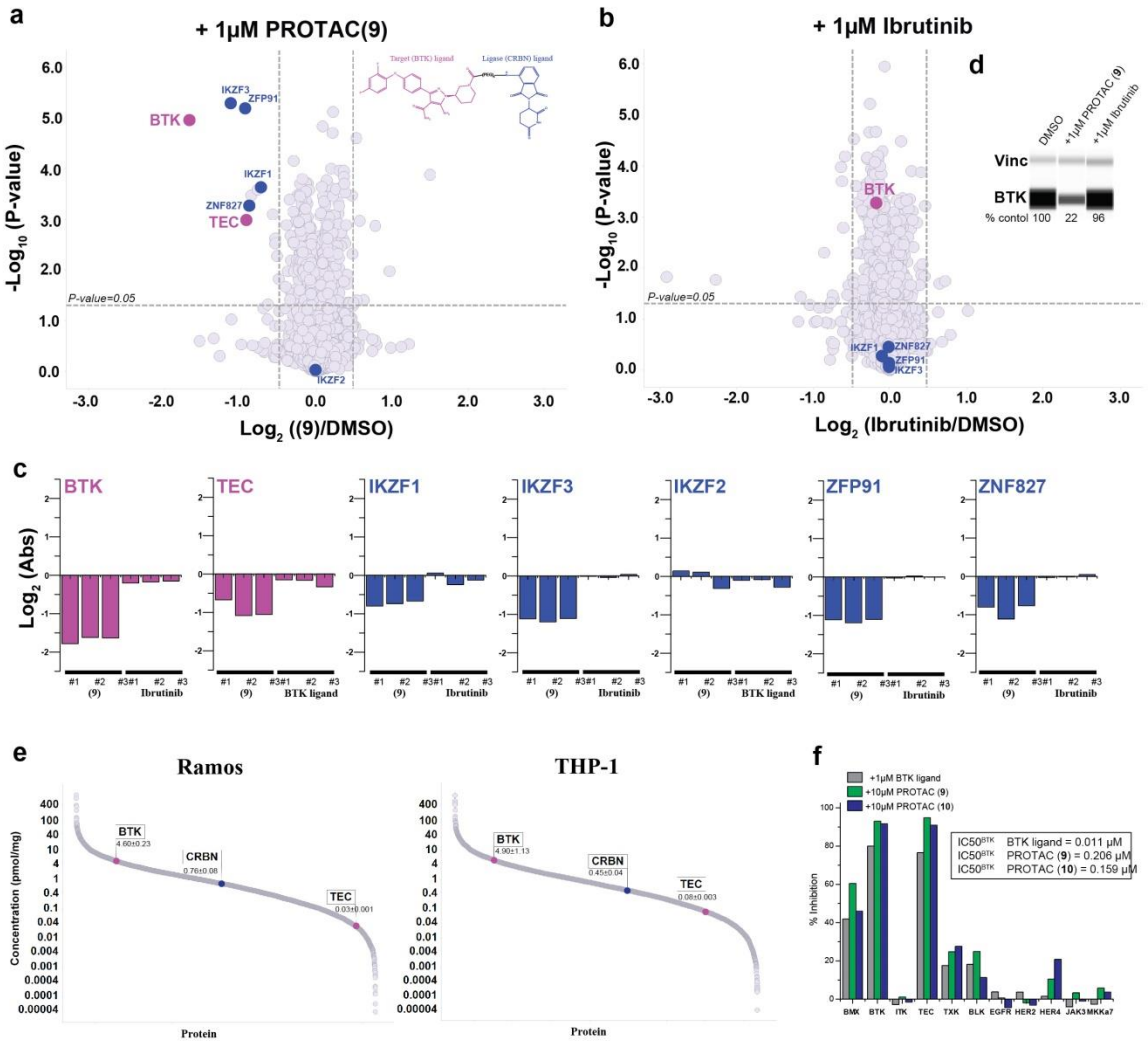


Figure S8 I PROTAC (**9**) selectively degrades BTK. (**a**) PROTAC (**9**) degrades BTK and IMiD-dependent proteins, in a similar manner to PROTAC (**10**), whereas (**b**) Ibrutinib, does not degrade any of these proteins. Similar to **Figure 4**, volcano plots show significantly degraded proteins to lie in the upper left quadrant – above the p=0.05 significance line and to the left of the non-axial vertical line that notes -1.4-fold change. Proteins levels are shown relative to DMSO controls. Data was collected in triplicates and Ramos cells were incubated for 24 h with either compound (+1 μM PROTAC (**9**) or +1 μM Ibrutinib)

or DMSO. Only proteins with at least one uniquely identified peptide are shown out of a total of 8055 proteins that were identified. (**c**) Absolute protein levels, as compared to DMSO controls, shown for each MS replica. Lowly expressed TEC and IKZF2 were not detected in these experiments. (**d**) Half of the MS lysates used in these experiments were saved for Western Blots. Representative of 1 of 3 MS replicates showing degradation of BTK by PROTAC (**9**), but not Ibrutinib. (**e**) TPA-quantified BTK, CRBN, TEC levels in Ramos and THP-1 cells allowing for relative comparison of available protein for ternary complex formation across cell lines. (**f**) Partial Carna kinase panel on inhibition of BTK-family and cysteinome kinases by either the BTK ligand or PROTACs(**9**) or (**10**) showing complete alignment of BTK ligand and PROTAC selectivity.

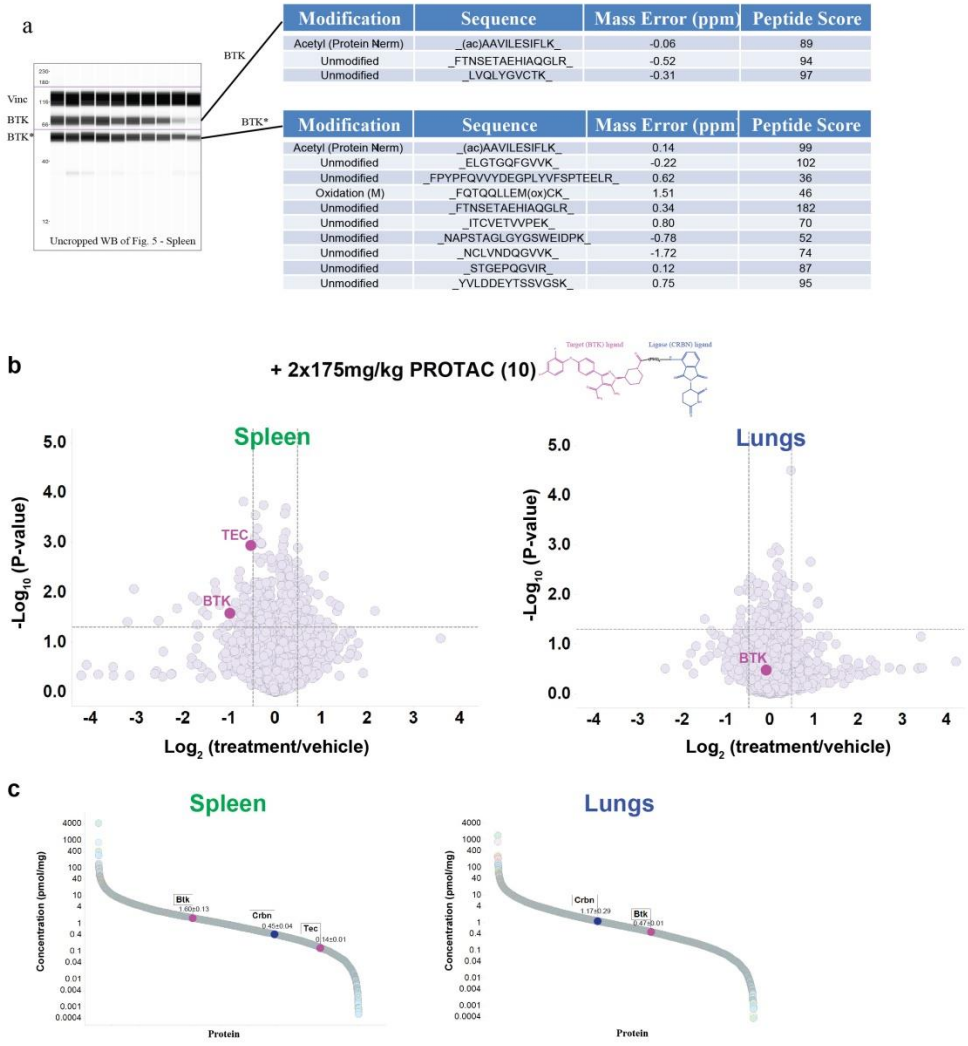


Figure S9 I PROTAC (**10**) selectively degrades BTK *in vivo*. (**a**) Uncropped western blot of *in vivo* dosing experiments from **Figure 5a**, shows the presence of two bands, both confirmed to be BTK by MS analysis. (**b**) Similar to **Figure S8**, volcano plots show affected protein levels in two animals treated with 2x175 mg/ml PROTAC (**10**) for 48 hr as compared to vehicle controls, with relative spleen (left) and lungs (right) protein levels shown. Only proteins with at least one uniquely identified peptide are shown. (**c**) Absolute proteins levels as compared to vehicle controls for the same experiment setup as in (**b**). TEC was only detected in the spleen but not the lungs samples.

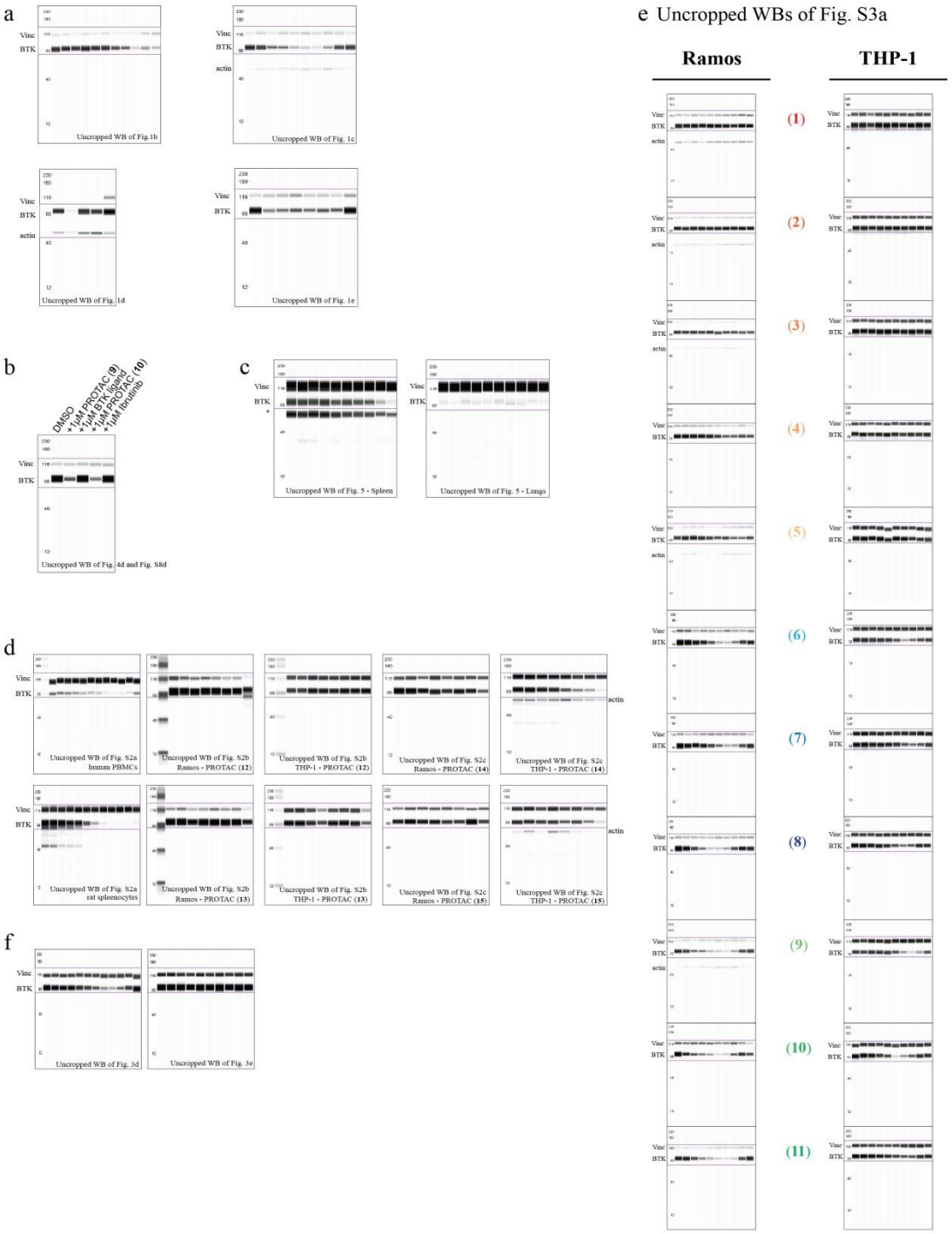


Figure S10 | Uncropped western blots of indicated figures.

Supplementary References:

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