Efficient targeted degradation via reversible and irreversible covalent PROTACs

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PROteolysis Targeting Chimeras (PROTACs) represent an exciting inhibitory modality with many advantages, including sub-stoichiometric degradation of targets. Their scope, though, is still limited to-date by the requirement for a sufficiently potent target binder. A solution that proved useful in tackling challenging targets is the use of electrophiles to allow irreversible binding to the target. However, such binding will negate the catalytic nature of PROTACs. Reversible covalent PROTACs offer the best of both worlds. They possess the potency and selectivity associated with the formation of the covalent bond, while being able to dissociate and regenerate once the protein target is degraded. Using Bruton’s tyrosine kinase (BTK) as a clinically relevant model system, we present a proof-of-concept for the first in class cyanoacrylamide reversible covalent PROTACs. We show efficient degradation with reversible covalent PROTACs, as well as their non-covalent and irreversible counterparts. The latter are amongst the most efficient PROTACs reported for BTK. They display single digit nM DC50, full degradation within 2-4 hours, proteome wide selectivity and show ~10-fold better inhibition of B cell activation than Ibrutinib. These examples refute the notion that covalent binders are not suitable as the basis for PROTACs, and may pave the way for the design of covalent PROTACs for a wide variety of challenging targets.
Efficient targeted degradation via reversible and irreversible covalent PROTACs

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Abstract

PROteolysis Targeting Chimeras ( PROTACs ) represent an exciting inhibitory modality with many advantages, including sub-stoichiometric degradation of targets. Their scope, though, is still limited to-date by the requirement for a sufficiently potent target binder. A solution that proved useful in tackling challenging targets is the use of electrophiles to allow irreversible binding to the target. However, such binding will negate the catalytic nature of PROTACs. Reversible covalent PROTACs offer the best of both worlds. They possess the potency and selectivity associated with the formation of the covalent bond, while being able to dissociate and regenerate once the protein target is degraded. Using Bruton’s tyrosine kinase ( BTK ) as a clinically relevant model system, we present a proof-of-concept for the first in class cyanoacrylamide reversible covalent PROTACs. We show efficient degradation with reversible covalent PROTACs, as well as their non-covalent and irreversible counterparts. The latter are amongst the most efficient PROTACs reported for BTK. They display single digit nM DC₅₀, full degradation within 2-4 hours, proteome wide selectivity and show ~10-fold better inhibition of B cell activation than Ibrutinib. These examples refute the notion that covalent binders are not suitable as the basis for PROTACs, and may pave the way for the design of covalent PROTACs for a wide variety of challenging targets.
PROteolysis TArgeting Chimeras (PROTACs) are receiving increasing attention as a new therapeutic modality, as was recently underscored by the first PROTAC, ARV-110, to enter clinical trials\(^1\). PROTACs are comprised of a protein target binding moiety, a linker and an E3 ubiquitin ligase binder\(^2,3\). Upon binding, the PROTAC induces the formation of a ternary complex between the target and E3 ligase\(^4,5,6,7\) resulting in the ubiquitination and degradation of the target. Compared to traditional inhibition of the target protein, targeted degradation has several important advantages, including the elimination of all levels of protein function, enhanced selectivity\(^8,9,10,11\), longer duration of action due to the need to resynthesize the target\(^12\), and degradation by sub-stoichiometric amounts of PROTAC\(^13\).

Efficient degradation typically requires high affinity binding to the target as well as optimized linker geometry, to optimize the ternary complex formation. However, many targets such as transcription factors\(^14,15\), protein-protein interfaces\(^16,17\), or challenging enzyme classes such as GTPases\(^18\), are recalcitrant to ligand discovery. This limits the applicability of PROTACs against such targets. A possible solution to this problem is to introduce an electrophile that will allow covalent binding to the target. However, irreversible binding may reduce potency by negating the catalytic nature of the PROTAC activity. While several covalent PROTACs have been developed and degrade their target successfully\(^19,20\), there are examples in which the introduction of irreversible binding reduces the potency of PROTACs\(^21,22\).

Theoretically, *reversible covalent* PROTACs can benefit from both the enhanced potency, selectivity, and long duration of action that accompany covalent bond formation\(^23,24,25\), without compromising the sub-stoichiometric activity of PROTACs. In this work we set out to test this hypothesis by the design of cyanoacrylamide-based reversible covalent PROTACs. To this end, we selected Bruton’s tyrosine kinase (BTK), which is an established target for non-covalent PROTACs\(^21,26,27,28,29,30\), and systematically tested a series of reversible covalent PROTACs along with their irreversible covalent and non-covalent PROTAC analogs.

We devised a modular scheme for the synthesis of cyanoacrylamide-based PROTACs (see methods and SI). Using this route, we synthesized a series of 12 reversible covalent PROTACs targeting Cysteine 481 in BTK (Supp. Table 1). These are based on the scaffold of the covalent BTK binder - Ibrutinib - as the protein targeting moiety\(^31\), and PEG-based linkers with varying length (Fig. 1). We used two approaches: in the first, we synthesized an alkyne-
functionalized BTK-binding cyanoacrylamide and an amine-functionalized E3 binder, and linked them in one-pot reactions using azide-PEG-NHS esters of varying lengths. In the second approach, we directly functionalized thalidomide with various PEGs and formed the cyanoacrylamide in a final condensation step.

We incubated K562 or Mino cells with 1 µM of the compounds for 24 hours and measured the abundance of BTK by western blot (Supp. Fig. 1). Compound PG27, based on a PEG6 linker displayed prominent levels of degradation in both cell lines and was therefore selected as a starting point for this study. Based on compound PG27 we synthesized additional compounds (Fig. 1), RG48 with a CH2 group replacing the oxygen nearest the β-carbon, their analogous acrylamides RG38 and RG52, and the non-covalent analog RG55.

![Figure 1. Structures of reversible covalent, irreversible covalent and non-covalent BTK PROTACs described in this study.](image-url)

We tested the ability of the compounds to bind and inhibit BTK using an in-vitro kinase activity assay (Fig. 2A) and intact protein LC-MS measurements (Fig. 2B; Supp. Fig. 2). All compounds in the series inhibited BTK with IC50s of 2-15 nM. The covalent compounds RG38 and RG48 showed higher potency than the non-covalent RG55, whereas Ibrutinib displayed the highest potency with an IC50 = 0.3 nM. LC-MS measurements indicated covalent labeling by all compounds except RG55, with RG38 and RG48 exhibiting the highest labeling rates, in accordance with the kinase activity assay results.
Figure 2: All PROTACs are potent BTK inhibitors \textit{in vitro}, and the cyanoacrylamides show slow dissociation kinetics.

A. \textit{In-vitro} kinase activity assay shows that the PROTACs are potent BTK inhibitors (1.2 nM BTK, 10 \(\mu\)M ATP).

B. Time course LC-MS binding assay (3 \(\mu\)M compound + 2 \(\mu\)M BTK at room temperature).

C. Ibrutinib competition assay, validates reversible binding by cyanoacrylamides. 40 \(\mu\)M Ibrutinib was added to the preformed complex, incubated at 37\(^\circ\)C, and the different species were quantified by LC-MS.

To test whether the formation of the covalent adducts is reversible and estimate the timescale of the exchange, we added 40 \(\mu\)M Ibrutinib to the samples after formation of the adducts and incubated at 37\(^\circ\)C. For the acrylamides RG38 and RG52, no Ibrutinib adduct was observed even after 28 hours of incubation. However, only 80-85\% of protein appeared to be labeled by the PROTACs (Fig. 2C). This may indicate that in fact RG38 and RG52 have stably labeled 100\% of the protein, thereby preventing Ibrutinib binding, but dissociation during the
separation or ionization process on the LC-MS may have generated the observed free protein peak.

In contrast, for the cyanoacrylamides, the addition of Ibrutinib led to immediate labeling of a large fraction of the protein (80% for PG27 and 20% for RG48; Fig. 2C), followed by gradual increased displacement of the PROTAC by Ibrutinib, confirming the reversibility of the cyanoacrylamide covalent binding. The exchange of the cyanoacrylamide was slow, on the order of 10-20 hours. The non-covalent PROTAC RG55 forms no covalent adduct and is rapidly exchanged by Ibrutinib (100% Ibrutinib labeling in 1 hour at room temperature; Supp. Fig. 2).

We next evaluated the ability of the compounds to induce degradation of BTK in human cell lines. We incubated Mino cells with the compounds and followed BTK levels after 24 hours by western blot (Fig. 3). The non-covalent PROTAC RG55 showed the highest degradation potency with DC50 = 1 nM. (Maximal degradation - Dmax = 100%) The irreversible acrylamides RG52 and RG38 followed closely (DC50 = 1.9 nM and 8.6 nM respectively; Dmax = 88% and 91% respectively) and were more potent than their cyanoacrylamide analogs RG48 (DC50 = 35 nM; Dmax = 66%) and PG27 (DC50 = 10 nM; Dmax = 68%; Supp. Fig. 3). Similar trends were observed in Ramos cells (Supp. Fig. 3A).

We followed the rate of BTK degradation facilitated by this compound series via a time course experiment in Ramos cells (Supp. Fig. 4). The rates of degradation correlated well with the DC50 observed after 24 hours, with RG55 and RG52 degrading BTK very rapidly within 2-4 hours, while RG48, RG38 and PG27 required 6-24 hours to reach maximum degradation.
Figure 3: Efficient BTK degradation in cells.

A. Western blot evaluation of BTK levels in Mino cells in response to various concentrations of RG48, RG52, RG55.

B. Quantification of BTK levels in (A) by normalization to the β-actin house-keeping gene in Mino cells. DC₅₀ and D₉₅ were calculated by fitting the data to a second order polynomial using Prism software.

To validate the mechanism of PROTAC mediated degradation of BTK, Mino cells were pre-treated for 2 hours with either Ibrutinib or thalidomide-OH, and subsequently treated with the PROTACs for an additional 24 hours. Both Ibrutinib pre-treatment as well as thalidomide-OH, hindered BTK degradation (Fig. 4A). In contrast to the covalent PROTACs, degradation by the non-covalent RG55 was only slightly hindered by thalidomide. In addition, a methylated thalidomide analog of PG27, no longer able to bind CRBN, lost all activity (Supp. Fig. 5),
further suggesting CRBN mediated degradation. We treated Mino cells with Bortezomib, a proteasome inhibitor\textsuperscript{32}, for 1 hour before treatment with the PROTACs and assessed BTK levels after an additional 4 hours. Bortezomib significantly inhibited degradation, suggesting proteasome-dependent degradation (Fig. 4B).

![Image](image.png)

**Figure 4:** PROTAC mediated BTK degradation is hindered by Ibrutinib, thalidomide and by proteasome inhibition.

A. Mino cells were either pre-treated for 2 hours with Ibrutinib/thalidomide-OH or untreated, before treatment with a BTK PROTAC for 24 hours, subsequently BTK levels were measured via western blot.

B. Mino cells were treated for 1 hour with Bortezomib to inhibit proteasome dependent degradation, then PROTACs were added for 4 hours, followed by measuring BTK levels via western blot.

To assess their proteomic selectivity, we incubated the PROTACs at 50 or 100 nM for 24 hours with Ramos cells, and followed the change in protein abundance via quantitative label-free proteomics (Fig. 5, Supp. Fig. 6). This revealed that the levels of BTK, CSK and to a lower extent LYN were significantly reduced, by up to 7-8 fold in PROTAC treated cells compared to DMSO control. CSK and LYN are known off-targets of Ibrutinib\textsuperscript{31}. No other significant off-targets were detected consistently (Supp. Dataset 1). The proteomics results
correlated with the western blot analysis, with **RG48** being the least potent (~2 fold degradation), followed by the more potent irreversible covalent **RG38** and **RG52** (4-8 fold) and the noncovalent **RG55** being the most potent (close to 16-fold reduction). We note that CSK, to which Ibrutinib binds reversibly, is degraded to a similar extent by **RG38**, **RG52** and **RG55**.

![Proteomic analysis reveals high selectivity for both covalent and non-covalent BTK PROTACs.](image)

**Figure 5.** Proteomic analysis reveals high selectivity for both covalent and non-covalent BTK PROTACs. Ramos cells were incubated with each PROTAC (100nM) or DMSO in quadruplicates for 24h, and were then subjected to label-free quantitative proteomics analysis. Each graph plots the Log2 fold-change of proteins in the treated samples compared to the DMSO controls (X-axis) vs. the -log(p-value) of that comparison in a student's T-test (Y-axis).

Lastly, we assessed the ability of the PROTACs to abrogate the activation of primary mouse B cells in response to B cell receptor stimulation. For this purpose, primary B cells were treated with anti-IgM for 18 hours, followed by staining for CD86, a B cell activation surface marker (Fig. 6). The inhibition of B cell activation correlated well with the BTK degradation activity, with **RG55** and **RG52** showing the strongest effect, with half maximal
effective concentration (EC$_{50}$) values of 56 nM. The acrylamide RG38 was less active with EC$_{50} = 172$ nM, while the cyanoacrylamides RG48 and PG27 displayed EC$_{50}$’s of 486 nM and > 1 µM, respectively. The most active PROTACs RG55, RG52 and RG38 displayed superior inhibition compared to Ibrutinib (EC$_{50} = 344$ nM), underscoring the benefit of targeted degradation compared to inhibition alone.

**Figure 6: PROTACs inhibit B cell receptor signaling more potently than Ibrutinib.**

Dose response curves for B cell response after anti-IgM induced activation and treatment with BTK PROTACs or Ibrutinib for 24 hours. The Y-axis shows normalized CD86 Mean fluorescence intensity, where 100% activation is cells stimulated with anti-IgM, and 0% activation is unstimulated cells.

Several studies characterized the reversible covalent binding of cyanoacrylamides$^{23,25}$ and we have shown that the cyanoacrylamide PROTACs do indeed bind reversibly (Fig. 2). If the reversibility of binding underlies the improved potency of the non-covalent PROTAC compared to the covalent PROTACs, it raises the question - why are the cyanoacrylamide PROTACs not superior to their acrylamide counterparts? The covalent PROTACs did not inhibit BTK more potently than the non-covalent RG55 in the activity assay, unlike Ibrutinib, that was 10-fold more active than any of the PROTACs. This likely reflects lower reactivity of the substituted acrylamides in the PROTACs and/or additional steric hindrance. In the timescale we observed for PROTAC-induced degradation of 2-4 hours (Supp. Fig. 4), the covalent PROTACs are not better inhibitors of BTK, and therefore differences of binding to BTK are unlikely to be the major factor contributing to the difference in potencies.
Both RG48 and RG55 bind BTK reversibly and inhibit it with similar potencies, yet display a significant difference in degradation potency. We suggest three hypotheses for this discrepancy: First, the non-covalent RG55 has a much less rigid linker than RG52 and RG48, with free rotation around the bond proximal to the amide linkage. This flexibility may aid the PROTAC in adopting the optimal configuration for the ternary complex formation and for ubiquitination, which may also explain the ability of RG55 to compete with thalidomide (Fig. 4A) compared to the other PROTACs. Second, the non-covalent RG55 has a rapid binding and dissociation equilibrium – in the presence of preincubated RG55, Ibrutinib labels BTK fully within 1 hour (Supp. Fig. 2). Therefore, RG55 can bind BTK in the cell, induce the formation of the ternary complex to induce ubiquitination, and quickly dissociate to bind more BTK molecules, even before the ubiquitinated BTK undergoes proteasomal degradation. The cyanoacrylamides PG27 and RG48 dissociate in timescales of 10-20 hours, similar to the residence times observed for other cyanoacrylamide inhibitors\(^{30}\). Therefore, they can only be recycled after the bound BTK molecule has been degraded, resulting in less efficient catalysis. Lastly, inverted cyanoacrylamides as we describe may suffer from stability issues in cells. This might explain the discrepancy between their in vitro and cellular activity, and warrants further investigation.

The benefits of covalent PROTACs might be more evident when targeting more challenging binding pockets, for which no high affinity ligand is available. The fact that the non-covalent BTK inhibitor is equipotent with both reversible and irreversible covalent binders might hinder the typical increase in potency that is associated with the formation of a covalent bond. In conclusion, we demonstrate that a covalent PROTAC can degrade BTK efficiently at low nM concentrations, refuting the perception that irreversible PROTACs are inefficient. Moreover, we report the first cyanoacrylamide reversible covalent PROTACs. These will further broaden the scope of covalent PROTACs in the future.
Methods:

General outline of reversible covalent PROTAC synthesis

To synthesize reversible covalent PROTACs, we prepared PEG-monotosylates of different lengths and coupled them to 4-OH-thalidomide to generate thalidomide-PEG-OH constructs (Supplementary Material). These were oxidized to aldehydes, followed by an aldol condensation with the BTK inhibitor cyanoacetate to generate the cyanoacrylates. We also found that during the condensation the ether linkage nearest the cyanoacrylate was frequently cleaved, as observed by LC/MS measurements. To increase the yields and stability of the products, for later compounds we attempted replacing the last ether linkage with a CH$_2$ group. This slowed down the condensation, reduced unwanted side reactions and increased the yield, and eventually also improved the potency of the compounds (see Supp. Material for synthesis procedures). $^1$H and $^{13}$C NMR spectra were recorded on a 11.7T Bruker AVANCE III HD spectrometers. Chemical shifts are reported in ppm on the $\delta$ scale downfield from TMS and are calibrated according to the deuterated solvents (see supplementary material).

In-vitro Activity Assays for BTK (Carried out by Nanosyn, Santa Clara, CA)

Test compounds were diluted in DMSO to a final concentration that ranged from 2 $\mu$M to 11.3 pM, while final concentration of DMSO in all assays was kept at 1%. The compounds were incubated with BTK for 2 hours at room temperature before adding substrate. Reference compound, Staurosporine, was tested in a similar manner. 1.2 nM BTK was incubated with its substrate in a 2x buffer comprising 100 mM HEPES pH = 7.5, 10 mM MgCl$_2$, 2 mM DTT, 0.1% BSA, 0.01% Triton X-100, 20 $\mu$M Sodium Orthovanadate and 20 $\mu$M Beta-Glycerophosphate for 2 hours at room temperature, then added with 10 $\mu$M ATP.

Western Blotting

Ramos (ATCC, CRL-1596), Mino (ATCC, CRL-3000) or K562 (NCI-60) cell lines were counted and diluted to $10^6$ cell/ml, using 1 mL per well in a 24-well plate. Cells were incubated with 1% DMSO or compound in indicated concentrations for 24 hours unless indicated differently or cells were left untreated. Lysates were prepared as previously described$^{27}$, and samples were measured for total protein quantification by Bicinchoninic Acid (BCA) assay (#23225 ThermoFisher Scientific), supplemented with 4x loading buffer including 20 mM DTT, heated to 70°C for 10 minutes and loaded into 4% SDS-PAGE gel, run for 45 minutes at 140 mV, then transferred into nitrocellulose membrane (Biorad) using Trans-Blot Turbo transfer system (Biorad). Membrane was stained with Ponceau (Sigma) to validate
transfer for 10 minutes in gentle agitation then de-stained for 1 hour with MQ water. Membrane was blocked with Licor blocking buffer (LIC927-70001) for 1 hour, washed three times for 5 minutes with TBS-T and incubated with primary antibody BTK (D3H5) Rabbit mAb (CST; 8547 S) overnight (16 hours) at 4°C, washed three times for 5 minutes with TBS-T and incubated with primary antibody β-actin (CST; 3700) for 1 hour at 25°C. Membrane was washed three times for 5 minutes with TBS-T and incubated with a fluorescent secondary antibodies Anti-Mouse IgG Atto 488 (Sigma; 62197) and Anti-Rabbit-IgG Atto 647N (Sigma; 40839) for 1 hour, then washed three times for 5 minutes with TBS-T, dried and immediately imaged and analysed using Licor odyssey CLx. Prism (GraphPad) software was used to calculate degradation levels, and we used second order polynomial fit to estimate DC50 and Dmax values.

**Expression and Purification of Recombinant BTK**

The expression and purification of human BTK kinase domain (residues 387–659) was based on the method used by Bradshaw JM. et al. The kinase domain was inserted into pFastBac-1 with an N-terminal 6×-His tag followed by a TEV protease cleavage site (The plasmid was a gift from Dr. Ville Paavilainen, University of Helsinki). Viruses were produced in Sf9 cells and expression of the BTK kinase domain was subsequently induced in Tni insect cells by infection of 2 L of cultured cells with 1:200 mL virus solution such that cell growth was terminated after 3 days. The cells were collected by centrifugation (800 g for 15 minutes), and the pellet was resuspended in 50 mL lysis buffer (10 mM Hepes, pH 7.5, 400 mM NaCl, 1.5 mM DTT) supplemented with 1× protease inhibitor cocktail (Roche). The cells were lysed by five passages through a cell homogenizer. The cellular debris was pelleted by centrifugation (30,000 g for 30 minutes). The protein was bound in batch to nickel–nitrilotriacetic acid agarose beads in binding buffer (lysis buffer supplemented with 20 mM imidazole) for 4 hours at 4°C. The beads were washed with additional binding buffer (four 5 mL washes), and the protein was eluted with four 0.5 mL portions of elution buffer (lysis buffer supplemented with 300 mM imidazole). The His tag was cleaved by the addition of TEV protease with concomitant dialysis overnight into cleavage buffer (50 mM Tris, pH 8.0, 0.5 mM EDTA, 1 mM DTT) at 4°C. The resulting soluble protein was passed over Ni-NTA beads to remove the protease and purified further by gel filtration on a HiLoad_16/60_Superdex_75 (GE Healthcare) column equilibrated with 20 mM Tris, pH 8.0, 50 mM NaCl, 1 mM DTT. The pure protein was then flash-frozen in liquid nitrogen and stored at −80 °C.
C57BL/6 mice

C57BL/6 mice were purchased from Harlan (Rehovot, Israel) and maintained in a pathogen-free facility, experiments were carried on 8-12 weeks old male mice. All experiments with mice were approved by the Weizmann Institute Animal Care and Use Committee.

B Cell Activation

Splenic cells from C57BL/6 mice were isolated by forcing spleen tissue through mesh into PBS containing 2% fetal calf serum and 1 mM EDTA and red blood cells were depleted by lysis buffer. Cells were cultured in 96-well U-bottom dishes (1x10^6 cells/mL in RPMI 10% FCS) and incubated with BTK PROTACs or Ibrutinib in different concentrations (1 µM, 100 nM, 10 nM, 1 nM, 0.1 nM) for 24 hours at 37° in 5% humidified CO₂. Following a 24 hours incubation, cells were stimulated with anti-IgM overnight (5 µg/mL, Sigma-Aldrich). Subsequently, cells were stained with anti-B220 (clone RA3-6B2, Biolegend) and anti-CD86 (clone GL-1, Biolegend) antibodies for 30 minutes at 4°C. Single cell suspensions were analyzed by a flow cytometer (CytoFlex, Beckman Coulter).

Proteomics

Sample preparation: 10^6 Ramos cells were treated in four replicates with either compound or DMSO for 24 hours. Cells were then washed twice by centrifuge at 200 rcf, 4°C for 5 minutes, removing the supernatant and washed with ice cold PBS. Samples were then centrifuged at 200 rcf, 4°C for 5 minutes, then supernatant was removed and samples were frozen at -80°C. Samples were dispersed in 75 µl of 50 mM ammonium bicarbonate, and transferred to 1.8 ml glass vials. 75 µl of 10% SDS in 50 mM ammonium bicarbonate were added and the samples were heated to 96 °C for 6 minutes. The samples were sonicated thoroughly in a sonication bath until the DNA was sheared as indicated by reduction in viscosity to level enabling easy pipettation. Total protein concentration was estimated using BCA assay and 30 µg from each sample was taken for the experiment.

The samples were reduced by the addition of 1/20 volume of 100 mM DTT and heating to 60 °C for 45 minutes. The samples were cooled to room temperature, and 1/20 volume of 200 mM iodoacetamide was added and the reaction was performed in the dark for 30 minutes.

At this point, 1/10 volume of 12% phosphoric acid was added, followed by 6 volume of 90% methanol in 50 mM ammonium bicarbonate. The samples were loaded on S-trap Micro columns (Protify), and washed 3 times with 90% methanol in 50 mM ammonium bicarbonate.
The columns were spun without washing to dry the methanol. Then, 1 µg of trypsin (Promga) in 20 µl of 50 mM ammonium bicarbonate was added to each column, and they were incubated at 47°C for 1.5 hours. Then, 40 µl of 50 mM ammonium bicarbonate was added and the sample was eluted and another 0.5 µg of trypsin was added, followed by overnight incubation at 37°C. The columns themselves were further eluted with 40 µl 0.2% formic acid in water, followed by 40 µl of 0.2% formic acid in 50% acetonitrile:water, to elute strongly bound peptides. This elution was stored at 4°C and combined the next day with the first elution. The samples were then dried by SpeedVac and analyzed.

Liquid chromatography: ULC/MS grade solvents were used for all chromatographic steps. Each sample was loaded using split-less nano-Ultra Performance Liquid Chromatography (10 kpsi nanoAcquity; Waters, Milford, MA, USA). The mobile phase was: A) H2O + 0.1% formic acid and B) acetonitrile + 0.1% formic acid. Desalting of the samples was performed online using a reversed-phase Symmetry C18 trapping column (180 µm internal diameter, 20 mm length, 5 µm particle size; Waters). The peptides were then separated using a T3 HSS nano-column (75 µm internal diameter, 250 mm length, 1.8 µm particle size; Waters) at 0.35 µL/min. Peptides were eluted from the column into the mass spectrometer using the following gradient: 4% to 30% B in 150 minutes, 35% to 90% B in 5 minutes, maintained at 90% for 5 minutes and then back to initial conditions.

Mass Spectrometry: The nanoUPLC was coupled online through a nanoESI emitter (10 µm tip; New Objective; Woburn, MA, USA) to a tribrid Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) using a PicoView nanospray apparatus (New Objective). Data was acquired in data dependent acquisition (DDA) mode, using a top-speed method with maximum cycle time of 3 sec. MS1 resolution was set to 120,000 (at 200 m/z), mass range of 375-1650 m/z, AGC of 4e5 and maximum injection time was set to 50 msec. MS2 was performed by HCD in the Orbitrap with resolution set to 15,000, quadrupole isolation 1 Th, AGC of 5e4, and maximum injection time of 100 msec.
Author Contributions

N.L. directed the study. R.G., A.S., P.G. and N.L. designed the experiments and wrote the paper. R.G., P.G., and E.L. designed and synthesized the compounds. A.S. performed western blotting and competition assays. N.G. and Z.S. designed and performed the primary mouse B cell experiments. R.G. performed and analyzed the proteomics experiments and reversibility assay via LC/MS. R.G., P.G., L.A. and E.L. analyzed molecules by nuclear magnetic resonance. T. U. and S. A. expressed and purified recombinant BTK. All authors contributed to the final form of the manuscript.

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1 reversible non-covalent

2 irreversible covalent

3 reversible covalent
Efficient targeted degradation via reversible and irreversible covalent PROTACs

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Supplementary Table 1: Reversible covalent BTK PROTAC library

| Compound | E3 binder | Linker size (n=) |
|----------|-----------|-----------------|
| PG15     | R1 = CRBN | 3               |
| RGA 065a | R1 = CRBN | 4               |
| PG37     | R1 = CRBN | 4               |
| PG27     | R1 = CRBN | 5               |
| RG10     | R2 = CRBN | 2               |
| RG11     | R2 = CRBN | 3               |
| RG12     | R2 = CRBN | 4               |
| RG8      | R2 = CRBN | 6               |
| RG13     | R2 = VHL  | 2               |
| RG14     | R2 = VHL  | 3               |
| RG15     | R2 = VHL  | 4               |
| RG16     | R2 = VHL  | 6               |
Supplementary Figures

**Supplementary Figure 1:** Library screen for BTK PROTACs highlights PG27 as prominent PROTAC.

**A.** Chemical structure of PI3I, a previously reported non-covalent BTK PROTAC.

**B.** Western blot of Mino cells treated with the reversible covalent library of BTK PROTACs.

**C.** Western blot of K562 cells treated with the reversible covalent library of BTK PROTACs.
Supplementary Figure 2: Kinetic studies of BTK labeling using LC/MS

BTK was incubated with 3 μM compound for 2 hours at 25°C, followed by addition of Ibrutinib and incubation at indicated times and temperatures. Samples were analyzed before and after addition of Ibrutinib by intact LC/MS at indicated times.
Supplementary Figure 3: BTK degradation by PROTACs

A. Western blot depicting a full dose-response curve for PROTACs in Ramos cell lines.

B. Western blot depicting a full dose-response curve for PROTACs in Mino cell lines.

Both experiments are after 24h incubations with the PROTACs.
Supplementary Figure 4: BTK degradation time dependency.
Ramos cells were treated with 100 nM of PROTAC or DMSO for indicated times, subsequently harvested for BTK levels measurement via western blot.
Supplementary Figure 5: Reversible covalent PROTAC induced degradation is mediated by CRBN.

A. Structure of RG32, an analog of PG27, with a methylated (blue) CRBN binder, that should abrogate CRBN binding.

B. *In-vitro* kinase assay in the presence of Ibrutinib or RG32, shows that BTK binding is not hampered by this methylation (compare to RG27 in Fig. 2A)

C. Western blot depicting a full dose-response curve for RG32 in Mino cell lines, shows no apparent degradation.
Supplementary Figure 6: Proteomics analysis reveals high selectivity for both covalent and non-covalent BTK PROTACs. Ramos cells were incubated with each PROTAC (50nM) or DMSO in quadruplicates for 24h, and were then subjected to label-free quantitative proteomics analysis. Each graph plots the Log₂ fold-change of proteins in the treated samples compared to the DMSO controls (X-axis) vs. the -log(p-value) of that comparison in a student's T-test (Y-axis).
Efficient targeted degradation via reversible and irreversible covalent PROTACs

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Keywords: PROTACs, reversible covalent, cyanoacrylamides, BTK, targeted degradation
Materials and Methods:

Materials: were purchased from the following vendors
- BTK inhibitor (free amine), CAS 1022150-12-4: purchased from BLD pharmatech
- Deuterated solvents: Cambridge isotope laboratories
- Non-modified polyethylene glycol: Broadpharm
- Polyethylene glycol (azide-NHS ester): BiochemPEG
- Solvents: Sigma Aldrich
- Other reagents – Sigma Aldrich

Chemistry:

The synthesis of the compounds used in this study is outlined in scheme 1.
Scheme 1: synthetic scheme: (a) cyanoacetic acid/EDC/DIEA, DCM, RT (b) Na₂CO₃ / DMF, 100 °C (c) 5% TFA/methanol, RT (d) Dess Martin Periodinane / DCM, 50°C (e) Na₂CO₃ / pyridine, 50 °C (f) acrylic acid + Grubbs 2nd gen./THF, 50 °C (g) EDC/DIEA/Hobt in DCM, RT

(R)-3-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-3-oxopropanenitrile:

Cyanoacetic acid (1.94 mmol, 165 mg), was dissolved in 10 ml dry dichloromethane. The reaction was cooled on ice and 3.2 mmol EDC hydrochloride (614 mg) and 7.7 mmol of triethylamine (1.07 ml) was added. After 10 minutes 1.29 mmol BTK inhibitor free amine (498 mg) was added and the reaction was warmed to room temperature. After 2 hours the reaction was washed twice with 1M HCl, followed by two washes with 5% NaHCO₃, drying over sodium sulfate and evaporating. This yielded 1 as a light brown low density powder, 510 mg (yield 95%).

HR-MS (m/z): Calculated: 453.19; Found: 454.1998 [M+H]⁺, 476.1815 [M+Na]⁺.

¹H NMR (500 MHz, Acetonitrile-d3) δ 10.95 (br s, 1H), 8.35 (s, 1H, minor rotamer set A), 8.33 (s, 1H, major rotamer set A), 7.69 – 7.65 (m, 2H), 7.46 (dd, J = 8.6, 7.3 Hz, 1H), 7.25 – 7.20 (m, 2H), 7.15 (d, J = 7.3 Hz, 1H), 6.73 (br s, 1H), 4.99 (tt, J = 9.0, 4.5 Hz, 1H, minor rotamer set B), 4.87 (tt, J = 10.5, 4.3 Hz, 1H, major rotamer set B), 4.59 – 4.53 (m, 1H, major rotamer set C), 4.11 (dt, J = 13.3, 4.5 Hz, 1H, minor rotamer set C), 3.91 – 3.60 (m, 4H), 3.35 – 3.15 (m, 2H), 2.37 – 2.18 (m, 4H, major rotamer set D), 2.03 – 1.87 (m, 4H, minor rotamer set D), 1.85 – 1.74 (m, 1H, major rotamer set E), 1.69 (m, 1H, minor rotamer set E).
$^{13}$C NMR (126 MHz, Acetonitrile-d3) $\delta$ 162.3, 161.6, 161.5, 158.8, 158.8, 156.4, 153.7, 153.6, 151.8, 146.7, 146.4, 130.1, 130.1, 130.1, 126.2, 126.1, 124.1, 124.1, 119.5, 119.5, 119.3, 117.3, 115.2, 97.3, 53.2, 52.7, 49.2, 45.9, 45.8, 42.0, 29.5, 29.2, 25.1, 25.0, 23.9, 22.6.

Approximated ratio of amide rotamers from 1H-NMR integral ratios: 63:37

2-(2,6-dioxopiperidin-3-yl)-4-hydroxyisoindole-1,3-dione:

\[
\text{HNCO\textsubscript{4}NH\textsubscript{3}Cl}^{-} + \text{O-CNCO} \xrightarrow{\text{Na\textsuperscript{+}CH\textsubscript{3}COO\textsuperscript{-} / CH\textsubscript{3}COOH}} \text{O-CNCO}
\]

1 g of 2,6-dioxopiperidin-3-aminium (6.1 mmol) and 1 g of 4-hydroxyisobenzofuran-1,3-dione (1 eq.) were dissolved in acetic acid with 2 equivalents of sodium acetate (1 g) and refluxed (110 °C) for 12 hours. The solvent was evaporated under reduced pressure, and the residue was washed with ice-cold water and lyophilized. The product was obtained as a brown powder, 1.51 g (90% yield).

HR-MS (m/z): Calculated: 274.06; Found: 297.0492 [M+Na$^+$].

$^1$H NMR (500 MHz, DMSO-d6) $\delta$ 11.18 (s, 1H), 11.09 (s, 1H), 7.66 (t, J = 7.8 Hz, 1H), 7.33 (d, J = 7.1 Hz, 1H), 7.26 (d, J = 8.4 Hz, 1H), 5.08 (dd, J = 12.8, 5.4 Hz, 1H), 2.89 (ddd, J = 17.0, 13.8, 5.4 Hz, 1H), 2.66 – 2.42 (m, 2H, overlaps with residual DMSO), 2.06 – 1.98 (m, 1H).

$^{13}$C NMR (126 MHz, DMSO-d6) $\delta$ 173.3, 170.5, 167.5, 166.3, 155.9, 136.9, 133.6, 124.0, 114.8, 114.8, 49.1, 40.5, 40.3, 40.2, 40.0, 39.8, 39.7, 39.5, 31.4, 22.5.

17-hydroxy-3,6,9,12,15-pentaoxaheptadecyl 4-methylbenzenesulfonate:
Hexaethyleneglycol (25 g, 90 mmol, 1.0 equiv.) was dissolved in DCM (150 mL), followed by the addition of pyridine (3.62 mL, 45 mmol, 0.5 equiv.). To the stirred solution was added tosyl chloride (8.58 g, 45 mmol, 0.5 equiv.) in four batches, each 15 min apart. After the last addition, the mixture was stirred for 18 h. The reaction mixture was thoroughly freed from DCM using a rotary evaporator. The resulting residue was treated with 0.1 M HCl (150 mL). The mixture was extracted twice with hexane (50 mL each) to remove excess tosyl chloride. The aqueous layer was washed with three portions of diethyl ether (20 mL each) to remove the ditosylated byproduct, and the extracts were checked by TLC to avoid premature extraction of the monotosylate. The aqueous layer was extracted with three portions of DCM (50 mL each). The combined organic layers were washed with 0.1 M HCl (50 mL), dried over MgSO₄, filtered and freed from solvent by rotary evaporation to give the product as a colourless oil (13.2 g weighed mass, 28.71 mmol based on 95% purity by LC/MS, 32%). Monotosylated PEGs of other length were synthesized using analogous procedures.

HR-MS (m/z): Calculated: 436.18; Found: 437.1845 [M+H]⁺, 459.1674 [M+Na]⁺

¹H NMR (500 MHz, Chloroform-d) δ 7.81 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 7.9 Hz, 2H), 4.17 (t, J = 4.8 Hz, 2H), 3.77 – 3.58 (m, 22H), 3.37 (br s, 1H), 2.46 (s, 3H).

¹³C NMR (126 MHz, Chloroform-d) δ 144.8, 133.0, 129.8, 128.0, 72.5, 70.7, 70.6, 70.5, 70.5, 70.3, 69.3, 68.7, 61.7, 21.7.

Differences in relaxation times between Aryl-H and Alkyl-H lead to incongruent integration results between these two signal sets.

17-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)-3,6,9,12,15-pentaoxaheptadecanal:
150 mg of 2 (0.547 mmol) were dissolved in 1 ml of dry DMF. 5 equivalents of sodium carbonate (290 mg) were added, and the solution was heated to 100°C under argon. 1.5 equivalents of 3 (358 mg) were dissolved in 250 µl dry DMF and added dropwise to the hot solution while stirring. The reaction was stirred for 3.5 hours under argon. The reaction was stopped with the addition of 1 ml acetic acid, and the solvent was evaporated under reduced pressure at 75°C. The residue was dissolved in 20%:80% acetonitrile:water (v/v) + 0.1% trifluoroacetic acid and purified by reverse phase HPLC, yielding 212 mg of 4 (71% yield, HR-MS (m/z): Calculated: 538.22; Found: 539.2241 [M+H]^+, 561.2076 [M+Na]^+). Then, 90 mg of 4 (0.167 mmol) were dissolved in 2 ml DCM and stirred on ice. 2.2 equivalents of Dess Martin Periodinane (156 mg) were added and the reaction stirred on ice for 15 minutes, followed by 2 hours at room temperature. The resulting solution was filtered and evaporated, and the aldehyde 5 was separated from unoxidized alcohol and further oxidized species by HPLC, yielding 23.7 mg of 5 (26% yield) as a highly viscous pale oil (HR-MS (m/z): Calculated: 536.20; Found: 537.2076 [M+H]^+, 559.1913 [M+Na]^+, 577.2012 [M+Na+H2O]^+).

(E)-18-((S)-3-(4-amino-3-(4-phenoxypyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidine-1-carbonyl)-1-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy)-3,6,9,12,15-pentaaxanadec-17-ene-19-nitrile:

22 mg of 1 (0.0486 mmol) were dissolved in 10 ml of pyridine, and 2.4 mg of sodium carbonate (0.0226 mmol) were added under argon. The aldehyde 5 (30.3 mg, 0.057 mmol)
was dissolved in pyridine and added to the reaction, which was stirred at 50°C for 3.5 hours. Dilute reaction conditions and limited reaction times were essential to avoid reaction of condensation products with 1 to give higher molecular weight adducts. The reaction was stopped by addition of 25 mg citric acid and evaporation of the solvent, followed by purification using HPLC. Obtained 6 as a white powder, 12 mg (25% yield).

HR-MS (m/z): Calculated: 972.3814; Found: 972.3887 [M+H]+, 97-94.3701 [M+Na]+

1H NMR (500 MHz, DMSO-d6) δ 11.11 (s, 1H, NH), 8.40 – 8.27 (m, 1H Ibrutinib-amine-aryl-H), 7.80 (td, J = 7.9, 2.6 Hz, 1H, Thalidomide-aryl-H), 7.67 (d, J = 8.1 Hz, 2H), 7.52 (dd, J = 8.6, 5.1 Hz, 1H, Thalidomide-aryl-H), 7.45 (m, 3H), 7.25 – 7.07 (m, 5H, Ph-O-Ibrutinib amine), 6.86 – 6.24 (m, 1H, Alkenyl-CH), 5.25 – 1.38 (complicated aliphatic region, 36H). Mixture of E/Z diastereomers and amide bond rotamers. Heteroaryl-NH2 could not be detected.

13C NMR (126 MHz, DMSO-d6) δ 173.2, 170.4, 167.3, 165.7, 164.1, 158.8, 158.5, 157.8, 156.7, 156.3, 153.6, 150.2, 137.4, 133.7, 130.6, 127.7, 124.3, 120.5, 119.5, 119.4, 116.8, 115.9, 97.6, 70.6, 70.3, 70.3, 70.2, 70.2, 69.3, 69.1, 52.7, 49.2, 46.5, 46.0, 31.4, 29.6, 24.3, 22.5. Not all 13C signals could be detected and/or overlapped due to the presence of a hexaethyleneglycol chain.

\[
\text{3,6,9,12,15-pentaoxaoctadec-17-en-1-yl 4-methylbenzenesulfonate:}
\]

\[
\begin{align*}
\text{SO}_3\text{O} & \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{H} + \\
\text{Br} & \quad \text{CH} \quad \text{CH} \quad \text{CH} \quad \text{CH} \\
\text{NaH/THF} & \quad \text{NaH/THF} \\
\text{SO}_3\text{O} & \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{H}
\end{align*}
\]

500 mg of Tos-PEG5-OH (1.29 mmol) was dissolved in dry THF under argon. 2.5 equivalents of allyl bromide were added. The reaction was cooled on ice, and 1.5 equivalents of sodium hydride were added. The solution turned cloudy due to precipitation of sodium bromide. After 20 the reaction was heated to room temperature under argon for 1 hour. TLC using 3% methanol in chloroform confirmed reaction was complete. The reaction was quenched with 1 M HCl, the THF was evaporated in vacuo, and the product was extracted 3 times with dichloromethane, washed with 5% NaHCO3, dried over sodium sulfate, and
purified using silica gel chromatography using a gradient of 0 to 10% methanol in chloroform. Obtained the product as a bright yellow oil, 140 mg (yield 25%).

HR-MS (m/z): Calculated: 432.18; Found: 433.1888 [M+H]+, 455.1718 [M+Na]+.

1H NMR (500 MHz, Chloroform-d) δ 7.81 (d, J = 8.2 Hz, 2H), 7.35 (d, J = 8.0 Hz, 2H), 5.92 (ddt, J = 17.4, 10.4, 5.7 Hz, 1H), 5.28 (dd, J = 17.2, 1.6 Hz, 1H), 5.18 (dd, J = 10.4, 1.5 Hz, 1H), 4.19 – 4.15 (m, 2H), 4.03 (dt, J = 5.7, 1.5 Hz, 2H), 3.72 – 3.57 (m, 18H), 2.46 (s, 3H).

13C NMR (126 MHz, Chloroform-d) δ 144.8, 134.8, 133.0, 129.8, 128.0, 117.1, 107.6, 106.4, 77.3, 72.2, 70.7, 70.7, 70.6, 70.6, 70.5, 70.5, 69.4, 69.2, 68.7, 67.7, 67.4, 29.5, 29.5, 23.8, 23.8, 21.7.

Differences in relaxation times between Aryl-H, Alkenyl-H and Alkyl-H lead to incongruent integration results between these three signal sets.

\[(E)-1-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindoindolin-4-yl)oxy)-3,6,9,12,15-pentaoxanonadec-17-en-19-oic acid :\]

37.5 mg of 2 (0.137 mmol) were dissolved in dry DMF under argon. 5 equivalents of sodium carbonate (69 mg) were added and the solution was heated to 100°C. The tosylate 7 (1.2 equivalents, 71 mg) was dissolved in DMF and added dropwise to the solution. The reaction proceeded for 2 hours under argon, neutralized with 0.5 ml of acetic acid, evaporated and purified by HPLC to yield 8 as a pale, viscous oil (51 mg, 70% yield, HR-MS (m/z): Calculated: 534.32; Found: 535.2280 [M+H]+, 557.2117 [M+Na]+). Then 24 mg of 8 (0.045 mmol) was dissolved in dry THF, and 30 equivalents of acrylic acid (92.5 µl) were added. 0.05 equivalents of Grubbs catalyst 2nd generation was added, and the reaction proceeded under argon at 40°C. The solvent was evaporated and the acid 9 was purified by HPLC as a white powder (10.8 mg, 42 % yield).
HR-MS (m/z): Calculated: 578.21; Found: 601.2010 [M+Na]+.

$^1$H NMR (500 MHz, DMSO-d6) δ 12.32 (s, 1H), 11.10 (s, 1H), 7.82 (dd, J = 8.5, 7.2 Hz, 1H), 7.54 (d, J = 8.5 Hz, 1H), 7.46 (d, J = 7.2 Hz, 1H), 6.81 (dt, J = 15.7, 4.2 Hz, 1H, major isomer set A), 6.33 (dt, J= 11.7, 5.0 Hz, 1H, minor isomer set A), 5.93 (dt, J = 15.7, 2.0 Hz, 1H, major isomer set B), 5.77 (dt, J= 11.7, 2.3 Hz, 1H, minor isomer set B), 5.09 (dd, J = 12.8, 5.4 Hz, 1H), 4.39 – 4.32 (m, 2H), 4.17 – 4.11 (m, 2H), 3.84 – 3.78 (m, 2H), 3.67 – 3.62 (m, 2H), 3.56 – 3.50 (m, 14H), 2.94 – 2.84 (m, 1H), 2.65 – 2.52 (m, 2H), 2.07 – 2.00 (m, 1H).

$^{13}$C NMR (126 MHz, DMSO-d6 δ 173.24, 170.39, 167.31, 165.72, 156.31, 145.27, 137.44, 133.71, 121.57, 120.51, 116.79, 115.85, 70.63, 70.31, 70.29, 70.24, 70.21, 70.18, 70.07, 69.32, 69.30, 69.15, 49.22, 31.42, 22.47.

(Mixture of isomers).

4-(((E)-19-((R)-3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-19-oxo-3,6,9,12,15-pentaoxanonadec-17-en-1-yl)oxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione:

3 mg of 9 (5.19 µmol) was dissolved in dry DCM on ice. 1.5 equivalents of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.5 mg) and 3 µl diisopropylethylamine were added. After 5 minutes, 2 equivalents of the BTK inhibitor amine (4 mg) were added, and the reaction proceeded for 1 hour at room temperature. The solvent was evaporated and the product 10 was purified by HPLC as a white powder, 2.4 mg (50% yield).

HR-MS (m/z): Calculated: 946.39; Found: 947.3911 [M+H]+, 969.3732 [M+Na]+.
$^1$H NMR (500 MHz, DMSO-$d_6$) δ 11.11 (s, 1H), 8.45 (s, 1H), 7.80 (t, J = 7.9 Hz, 1H), 7.70 – 7.63 (m, 2H), 7.53 (d, J = 8.6 Hz, 1H), 7.48 – 7.41 (m, 3H), 7.24 – 7.19 (m, 1H), 7.19 – 7.10 (m, 4H), 6.68 (s, 1H), 6.61 – 6.42 (m, 1H), 5.09 (dd, J = 12.8, 5.4 Hz, 1H), 4.84 – 4.64 (m, 1H), 4.59 – 4.48 (m, 1H), 4.40 – 4.28 (m, 2H), 4.19 – 3.96 (m, 4H), 3.85 – 3.73 (m, 3H), 3.69 – 3.60 (m, 3H), 3.58 – 3.38 (m, 16H), 2.94 – 2.82 (m, 1H), 2.65 – 2.55 (m, 1H), 2.31 – 2.19 (m, 1H), 2.19 – 2.09 (m, 1H), 2.07 – 1.88 (m, 2H), 1.67 – 1.52 (m, 1H).

$^{13}$C NMR (126 MHz, DMSO-$d_6$ δ 173.23, 170.38, 167.26, 165.71, 164.79, 158.69, 157.94, 156.62, 156.29, 142.11, 141.62, 137.42, 133.69, 130.62, 124.36, 120.85, 120.49, 119.52, 119.45, 117.48, 116.77, 115.84, 115.15, 97.54, 70.62, 70.29, 70.22, 70.18, 69.93, 69.78, 69.68, 69.32, 69.15, 53.41, 52.86, 49.74, 49.21, 46.16, 45.63, 42.09, 31.41, 29.98, 29.54, 25.24, 23.49, 22.46 (2 unaccounted for carbon peaks indicating some impurities)

2-((tert-butoxycarbonyl)amino)ethyl 4-methylbenzenesulfonate:

![Chemical structure](image)

1 g of Boc-ethanolamine (6.2 mmol) was dissolved in dry DCM on ice, under argon. 2.4 g of tosyl chloride (12.4 mmol) and 4.3 ml of triethylamine (31 mmol) were added, and the reaction proceeded at room temperature for 3 hours. The reaction was quenched with ice, extracted three times with DCM, washed with 1N HCl, 5% sodium bicarbonate and water, dried over sodium sulfate and dried. The residue was purified using flash chromatography using a gradient of 0-10% methanol in chloroform, yielding a waxy solid, 1 g (50%).

$^1$H NMR (500 MHz, Chloroform-d) δ 7.81 (d, J = 8.3 Hz, 2H), 7.37 (d, J = 8.0 Hz, 2H), 4.08 (t, J = 5.1 Hz, 2H), 3.45 – 3.32 (m, 2H), 2.47 (s, 3H), 1.42 (s, 9H).

$^{13}$C NMR (126 MHz, Chloroform-d) δ 145.0, 132.7, 123.0, 127.9, 79.8, 77.2, 69.5, 39.8, 28.3, 21.7.
2-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)ethan-1-aminium:

2 was reacted with 11 similar to the procedure for 3 and 8. The product was HPLC purified, deprotected with 25% trifluoroacetic acid in DCM for 1 hour, and air-dried. The residue was lyophilized from 40%:40%:20% acetonitrile:water:acetic acid to yield the acetate salt, yield 50%.

$^1$H NMR (500 MHz, DMSO-d6) δ 11.12 (br s, 1H), 8.05 (br s, 3H), 7.87 (dd, J = 8.5, 7.3 Hz, 1H), 7.56 (dd, J = 16.1, 7.9 Hz, 2H), 5.11 (dd, J = 12.8, 5.4 Hz, 1H), 4.45 (t, J = 5.2 Hz, 2H), 3.28 (d, J = 10.8 Hz, 2H), 2.90 (ddd, J = 17.0, 13.9, 5.4 Hz, 1H), 2.65 – 2.53 (m, 2H), 2.04 (dtd, J = 13.1, 5.4, 2.3 Hz, 1H).

$^{13}$C NMR (126 MHz, DMSO-d6) δ 173.24, 170.34, 167.16, 165.70, 155.50, 137.58, 133.73, 121.18, 118.50, 117.54, 116.72, 66.58, 49.27, 38.60, 31.40, 22.49, 21.51.

(contains residual water).

(S,Z)-2-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidine-1-carbonyl)hept-2-en-6-yenitrile:
40 mg BTK cyanoacetate were reacted with 30 mg 4-pentynal (4 eq) and 0.5 equivalents potassium carbonate in 4 ml pyridine at 50 C. after 6 hours acetic acid was added, the solvent was evaporated and the product was purified by HPLC as a white powder, 15 mg (33%).

**Description of assembly of PROTAC series using combined click and amidation reactions:**

Stock solutions were prepared in DMSO as follows: 12, 200 mM; VHL inhibitor hydrochloride (Molport), 200 mM; bifunctional PEG azide/NHS esters, 200 mM; Triethylamine, 0.5 M; 13, 200 mM; sodium ascorbate, 200 mM in water, dissolved fresh; TBTA-copper complex, 50 mM in 1:1 DMSO water (prepared by mixing equal volumes of 100 mM Copper sulfate in water and 100 mM TBTA in DMSO).

1. Reaction 1: 2 µmol of E3 ligase binder with 2 µmol of PEG reagents with 2.5 equivalents of triethylamine in DMSO (10 µl of each stock solution). Reactions were typically complete within 1 hour and monitored by LCMS.

2. Reaction 2: Mixed entire amidation reaction (30 µl of 67 mM) with 2.2 µmol 13 (11 µl), add 4 µmol of sodium ascorbate (20 µl stock), 30 µl water, 100 µl tertbutanol, and 0.6 µl TBTA:copper complex (12 µl stock). Reaction was complete after 2 hours at room temperature.

Products were purified by HPLC, yielding 1-1.9 mg, 50-80% yield.

**2-((5-bromopentyl)oxy)tetrahydro-2H-pyran:**
1 g of 5-bromopentanol (6.15 mmol) was dissolved in 20 ml dry DCM and cooled to 0°C on ice. 53 mg of Toluenesulfonic acidhydrate was added, followed by dropwise addition of 0.78 ml 3,4-dihydro-2H-pyran (1.5 equivalents). Reaction proceeded on ice for 12 hours, stopped by addition of aqueous sodium bicarbonate, extracted with DCM, dried over sodium sulfate and evaporated. The residue was purified by flash chromatography hexane:diethyl ether 30:1. The product was obtained as a clear viscous liquid, 978 mg (63% yield).

$^1$H NMR (500 MHz, Chloroform-d) $\delta$ 4.59 (dd, $J = 4.4, 2.7$ Hz, 1H), 3.88 (ddd, $J = 11.0, 7.6, 3.2$ Hz, 1H), 3.77 (dt, $J = 9.7, 6.6$ Hz, 1H), 3.55 – 3.50 (m, 1H), 3.47 – 3.38 (m, 3H), 1.92 (p, $J = 7.0$ Hz, 2H), 1.88 – 1.81 (m, 1H), 1.76 – 1.71 (m, 1H), 1.69 – 1.50 (m, 8H).

$^{13}$C NMR (126 MHz, Chloroform-d) $\delta$ 98.9, 67.2, 62.4, 33.8, 32.6, 30.8, 28.9, 25.5, 25.0, 19.7.

17-((tetrahydro-2H-pyran-2-yl)oxy)-3,6,9,12-tetraoxaheptadecyl 4-methylbenzenesulfonate:

8.13 mmol tetraethylene glycol (1.58 g) were dissolved in dry THF and added slowly at room temperature to a stirring suspension of sodium hydride (327 mg slurry, 8.13 mmol). After 15 minutes at room temperature the reaction was heated to reflux and a solution of 611 mg of 14 (2.42 mmol) in THF was added dropwise. The reflux was continued to reflux for 2.5 hours. The reaction was cooled to room temperature, water was added and the THF was evaporated. The product was extracted with DCM, washed with brine, dried over sodium sulfate and evaporated, yielding 620 mg of crude product (dark yellow oil), which was used in the
following step without purification. The product was dissolved in dry DCM on ice and 3 equivalents of pyridine (412 µl) were added. 1.5 equivalents of Tosyl chloride (487 mg) were added in portions while stirring, and the reaction was warmed to room temperature and proceeded for 12 hours. The solvents was then evaporated, the residue was dispersed in a solution of 5% sodium bicarbonate in water, extracted with DCM, washed with brine and dried over sodium sulfate. The product was purified using flash chromatography using 0-10% methanol in chloroform, yielding 15 as a yellow oil, 405 mg (32%). Care was taken to avoid an acidic environment during the reactions and workups to avoid deprotection of the THP group.

\[ ^{1}H \text{NMR (500 MHz, Chloroform-d)} \delta 7.81 (d, J = 8.2 Hz, 2H), 7.36 (d, J = 8.0 Hz, 2H), 4.58 (dd, J = 4.5, 2.7 Hz, 1H), 4.26 – 4.09 (m, 2H), 3.88 (ddd, J = 11.0, 7.6, 3.2 Hz, 1H), 3.78 – 3.32 (m, 18H), 2.46 (s, 3H), 1.90 – 1.33 (m, 13H). \]

\[ ^{13}C \text{NMR (126 MHz, Chloroform-d)} \delta 144.8, 133.0, 129.8, 127.9, 98.9, 71.4, 70.8, 70.6, 70.6, 70.5, 70.1, 69.2, 68.7, 67.5, 62.4, 30.8, 29.6, 29.5, 25.5, 22.8, 21.7, 19.7. \]

\[ 1-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)-3,6,9,12-tetraoxaheptadecan-17-ol: \]

124 mg of 15 (0.24 mmol) was reacted with 2 using the same conditions was described for 4 and 8. Following evaporation of the DMF, the residue was dissolved in methanol + 5% trifluoroacetic acid and stirred at room temperature. Full removal of the THP protecting group (as determined by LC-MS) was achieved within 1.5 hours. The solvent was evaporated and the free alcohol was purified using HPLC as a pale, viscous oil, 85 mg (66% yield). 72 mg of the alcohol (0.134 mmol) was dissolved in DCM and oxidized with 0.16 mmol of dess martin periodinane (68 mg) at 50°C for 2 hours. The solvent was evaporated and the residue
was dissolved in 20\%:80\% acetonitrile:water + 0.1\% TFA, filtered and purified using HPLC, yielding 53.6 mg of the purified aldehyde 16 (75\% yield) as a pale, highly viscous oil.

HR-MS (m/z): Calculated: 534.22; Found: 557.2118 [M+Na]⁺.

1H NMR (500 MHz, DMSO-d6) δ 11.11 (s, 1H), 9.64 – 9.66 (t, J = 1.42 Hz, 1H), 7.89 – 7.72 (m, 1H), 7.54 (d, J = 8.8 Hz, 1H), 7.46 (d, J = 7.3 Hz, 1H), 5.09 (dd, J = 12.9, 5.4 Hz, 1H), 4.35 (dd, J = 5.7, 3.5 Hz, 2H), 3.81 (dd, J = 5.6, 3.3 Hz, 2H), 3.70 – 3.61 (m, 2H), 3.58 – 3.52 (m, 2H), 3.52 – 3.40 (m, 10H), 3.36 (t, J = 6.1 Hz, 2H), 2.89 (ddd, J = 17.1, 13.8, 5.4 Hz, 1H), 2.66 – 2.52 (m, 2H), 2.09 – 1.96 (m, 1H), 1.61 – 1.38 (m, 4H).

13C NMR (126 MHz, DMSO-d6) δ 203.89, 173.23, 170.38, 167.27, 165.71, 156.30, 137.44, 133.70, 120.51, 115.85, 70.64, 70.35, 70.30, 70.25, 70.21, 69.90, 69.33, 69.15, 49.22, 43.17, 31.42, 29.00, 22.47, 18.87.

(Z)-18-((S)-3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidine-1-carbonyl)-1-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)-3,6,9,12-tetraoxanonadec-17-ene-19-nitrile

43 mg of 16 (0.08 mmol) were dissolved in 1 ml of pyridine, and 36 mg of 1 (0.08 mmol) were also dissolved in 1 ml of pyridine. 4.24 mg of sodium carbonate (0.04 mmol) were added to the solution of 1, mixed RT for 5 minutes, and then the aldehyde was added. Reaction proceeded under argon at 50 °C for 16 hours. Citric acid was added to neutralize the base and the solvent was evaporated. The product was purified by HPLC, giving 17 as a white powder, 19 mg (25\% yield).
HR-MS (m/z): Calculated: 969.40; Found: 970.4078 [M+H]+, 992.3896 [M+Na]+.

1H NMR (500 MHz, DMSO-d6) δ 11.11 (s, 1H), 8.45 – 8.37 (m, 1H), 7.83 – 7.77 (m, 1H), 7.67 (d, J = 8.5 Hz, 2H), 7.53 (d, J = 8.5 Hz, 1H), 7.45 (t, J = 7.7 Hz, 3H), 7.24 – 7.09 (m, 5H), 7.07 – 6.81 (m, 1H), 5.09 (dd, J = 12.8, 5.4 Hz, 1H), 4.94 – 4.77 (m, 1H), 4.34 (t, J = 4.5 Hz, 2H), 3.80 (t, J = 4.4 Hz, 2H), 3.68 – 3.62 (m, 2H), 3.59 – 3.10 (m, 15H), 2.89 (dd, J = 17.0, 13.8, 5.4 Hz, 1H), 2.64 – 2.47 (m, 3H), 2.46 – 2.12 (m, 4H), 2.02 (dd, J = 12.8, 5.4, 2.7 Hz, 2H), 1.77 – 1.60 (m, 1H), 1.56 – 1.35 (m, 4H).

13C NMR (126 MHz, DMSO-d6) δ 173.2, 170.4, 167.3, 165.7, 162.1, 159.2, 158.9, 158.0, 156.6, 156.3, 153.3, 145.2, 137.4, 133.7, 130.6, 127.4, 124.4, 120.5, 119.5, 119.5, 117.5, 116.8, 115.8, 115.2, 115.1, 110.9, 97.6, 70.6, 70.3, 70.2, 69.9, 69.3, 69.2, 49.2, 31.5, 31.4, 29.1, 24.5, 22.5.

3,6,9,12-tetraoxaoctadec-17-en-1-yl 4-methylbenzenesulfonate:

10 mmol PEG4 (1.94 g) was dissolved in dry THF and added dropwise to a suspension of 400 mg NaH/oil dispersion in dry THF at room temperature (400 mg NaH in THF) under nitrogen. After 30 minutes the reaction was heated to reflux and 3 mmol 6-bromo-1-hexene (489 mg) dissolved in THF was added to the solution while it refluxed. After 2 hours the reaction was cooled, neutralized with citric acid and water and the THF was evaporated. The product was extracted 3 times with DCM, washed with brine, dried over sodium sulfate and evaporated, resulting in 550 mg of a brown oil, which was used without purification for the next step. The oil was dissolved in DCM, cooled on ice and 6 mmol pyridine (484 mg) was added. 3 mmol of tosyl chloride (576 mg) was added in portions, and after 30 minutes the reaction was warmed to room temperature and proceeded for 12 hours. The solution was
evaporated, and the residue was dispersed in water and extracted with DCM. The organic phase was washed with brine, dried over sodium sulfate, and the crude material was purified using flash chromatography using a gradient of 0-6% methanol in chloroform. The product 18 was obtained as a light brown oil, 400 mg (31% yield).

\[ ^1H \text{NMR (500 MHz, Chloroform-d) } \delta 7.82 (d, J = 8.3 \text{ Hz}, 2H), 7.36 (d, J = 8.0 \text{ Hz}, 2H), 5.82 (ddt, J = 16.9, 10.2, 6.7 \text{ Hz}, 1H), 5.02 (dd, J = 17.2, 1.7 \text{ Hz}, 1H), 4.96 (d, J = 10.3 \text{ Hz}, 1H), 4.20 - 4.16 (m, 2H), 3.73 - 3.57 (m, 14H), 3.47 (t, J = 6.7 \text{ Hz}, 2H), 2.47 (s, 3H), 2.11 - 2.04 (m, 2H), 1.65 - 1.57 (m, 2H), 1.50 - 1.40 (m, 2H). \]

\[ ^{13}C \text{ NMR (126 MHz, Chloroform-d) } \delta 144.8, 138.8, 133.0, 129.8, 128.0, 128.0, 114.5, 71.3, 70.8, 70.6, 70.6, 70.5, 70.1, 69.2, 68.7, 33.5, 29.1, 25.4, 21.7. \]

HR-MS (m/z): Calculated: 430.20; Found: 453.1932 [M+Na]^+, 469.1673 [M+K]^+.

\((E)-1-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)-3,6,9,12-tetraoxanonadec-17-en-19-oic acid:\]

94 mg of 18 (0.219 mmol) was reacted with 50 mg 2 (0.182 mmol) as described before. The product was purified by HPLC. 49.4 mg product (0.092 mmol) was dissolved in 1.5 ml dry THF and mixed with 30 equivalents of acrylic acid (190.4 µl). 0.05 equivalents of Grubbs catalysis 2nd generation (4 mg) was adduct and the reaction was performed at 50°C. The solvent was evaporated and the resulting acid was purified by HPLC as a yellow oil, 46.2 mg (37% yield).

HR-MS (m/z): Calculated: 576.23; Found: 577.2393 [M+H]^+, 599.2224 [M+Na]^+.
\[ ^1H \text{NMR} (500 \text{ MHz, DMSO-d}_6) \delta 11.11 (s, 1H), 7.82 (dd, J = 8.5, 7.2 \text{ Hz}, 1H), 7.54 (d, J = 8.5 \text{ Hz}, 1H), 7.46 (d, J = 7.2 \text{ Hz}, 1H), 6.80 (dt, J = 15.5, 6.9 \text{ Hz}, 1H), 5.75 (d, J = 15.6 \text{ Hz}, 1H), 5.09 (dd, J = 12.8, 5.4 \text{ Hz}, 1H), 4.35 (dd, J = 5.6, 3.5 \text{ Hz}, 2H), 3.83 – 3.79 (m, 2H), 3.65 (dd, J = 5.8, 3.7 \text{ Hz}, 2H), 3.63 – 3.58 (m, 4H), 3.54 (dd, J = 5.9, 3.7 \text{ Hz}, 2H), 3.46 – 3.43 (m, 2H), 3.37 (t, J = 6.1 \text{ Hz}, 2H), 2.89 (ddd, J = 17.0, 13.8, 5.4 \text{ Hz}, 1H), 2.63 – 2.56 (m, 1H), 2.19 – 2.15 (m, 2H), 2.06 – 1.98 (m, 1H), 1.81 – 1.72 (m, 5H), 1.53 – 1.38 (m, 4H).

\[ ^{13}C \text{NMR} (126 \text{ MHz, DMSO-d}_6) \delta 173.2, 170.4, 167.3, 165.7, 156.3, 139.1, 137.4, 133.7, 120.5, 116.8, 115.8, 115.2, 70.6, 70.5, 70.3, 70.3, 70.2, 69.9, 69.3, 69.2, 67.5, 49.2, 33.4, 31.4, 29.1, 25.6, 25.4, 22.5.

\[ 4-(((E)-19-((S)-3-(4-amino-3-(4-phenoxyphe ny l)-1H-p yrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-19-oxo-3,6,9,12-tetraoxanadec-17-en-1-yl)oxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione:

16.5 mg of 19 (0.0286 mmol) were dissolved in DCM and stirred on ice. 1.5 equivalents of EDC (5.5 mg) and 5 equivalents of DIEA (25 \mu l) were added, and after 10 minutes, 2 equivalents of the BTK inhibitor amine (22 mg) were added. The reaction proceeded at room temperature for 90 minutes, and then the solvent was evaporated and the product 20 was isolated using HPLC as a white powder, 12 mg (44% yield).

HR-MS (m/z): Calculated: 944.41; Found: 945.4130 [M+H]^+, 967.3942 [M+Na]^+

\[ ^1H \text{NMR (500 MHz, DMSO-d}_6) \delta 11.11 (s, 1H), 8.39 (d, J = 6.1 \text{ Hz}, 1H), 7.80 (t, J = 7.9 \text{ Hz}, 1H), 7.66 (d, J = 8.0 \text{ Hz}, 2H), 7.53 (d, J = 8.5 \text{ Hz}, 1H), 7.45 (t, J = 7.7 \text{ Hz}, 3H), 7.24 – 7.06 \text{ ppm.} \]
(m, 4H), 6.74 – 6.22 (m, 2H), 5.09 (dd, J = 12.8, 5.4 Hz, 1H), 4.80 – 4.47 (m, 1H), 4.40 – 4.31 (m, 2H), 4.22 – 4.02 (m, 2H), 3.80 (d, J = 4.1 Hz, 2H), 3.64 (d, J = 4.6 Hz, 2H), 3.56 – 2.99 (m, 16H), 2.89 (ddd, J = 16.9, 13.9, 5.4 Hz, 1H), 2.63 – 2.54 (m, 1H), 2.34 – 1.80 (m, 7H), 1.66 – 1.21 (m, 6H).

$^{13}$C NMR (126 MHz, DMSO-d$_6$) $\delta$ 173.2, 170.4, 167.3, 165.7, 165.2, 157.9, 156.7, 156.3, 146.1, 137.4, 133.7, 130.62, 124.4, 121.1, 120.5, 119.5, 119.5, 116.8, 115.8, 110.9, 97.6, 70.6, 70.4, 70.3, 70.2, 70.2, 69.9, 69.3, 69.2, 49.2, 46.2, 31.8, 31.4, 29.2, 24.9, 22.5. Not all $^{13}$C signals could be detected.

*$^{13}$NMR (500 MHz, Chloroform-d) $\delta$ 7.82 (d, J= 8.2 Hz, 2H), 7.36 (d, J = 8.0 Hz, 2H), 3.71 (t, J = 4.9 Hz, 2H), 3.69 – 3.62 (m, 8H), 3.62 – 3.57 (m, 6H), 3.47 (t, J = 6.5 Hz, 2H), 2.47 (s, 3H), 2.36 (t, J = 7.4 Hz, 2H), 1.68 – 1.58 (m, 4H), 1.41 – 1.36 (m, 4H).

8.13 mmol of tetraethylene glycol (1.58 g) were dissolved in dry THF and added to a slurry of sodium hydride mineral oil dispersion in THF at room temperature (423 mg). After 15 minutes the reaction was warmed to reflux and a solution of 511 mg 7-bromoheptanoic acid (2.42 mmol) in THF was added dropwise, and refluxed for 2 hours. The reaction was stopped by adding water and evaporating the THF. HCl was added to acidify the sample, and the sample was extracted with DCM, washed with brine, dried over sodium sulfate, and evaporated, giving 667 mg of red oil. The product was tosylated as described for 17 and 15, yielding 47.6 mg of the acid 21 (yield 4%).

HR-MS (m/z): Calculated: 476.21; Found: 499.1986 [M+Na]$^+$, 515.1710 [M+K]$^+$. 

$^{13}$C NMR (126 MHz, Chloroform-d) $\delta$ 177.86, 144.78, 133.01, 129.82, 127.99, 71.25, 70.74, 70.64, 70.61, 70.55, 70.50, 70.10, 69.24, 68.69, 33.68, 29.34, 28.77, 25.70, 24.61, 21.65.
(S)-3-(4-phenoxyphenyl)-1-(piperidin-3-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine 1-((2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisindolin-4-yl)oxy)-3,6,9,12-tetraoxanonadecan-19-oate:

36 mg of 2 (0.131 mmol) as dissolved in dry DMF and 70 mg of sodium carbonate was added. The reaction was warmed to 100°C under nitrogen and a solution of 2 mg 21 (0.088 mmol) in DMF was added dropwise. The reaction continued for 2 hours, and the product was purified by HPLC, yielding 9 mg of 22 as a yellow oil (18% yield).

HR-MS (m/z): Calculated: 578.25; Found: 579.2552 [M+H]⁺, 601.2380 [M+Na]⁺.

4-((19-((S)-3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-19-oxo-3,6,9,12-tetraoxanonadecyl)oxy)-2-(2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione

4.5 mg of 22 (7.78 µmol) was dissolved in DCM and cooled on ice. 2 equivalents of EDC (3 mg), 2 equivalents of hydroxybenzotriazole (2.1 mg) and 4.5 equivalents of DIEA (4.7 µl) were added, and the reaction proceeded for 10 minutes on ice. Then 3 equivalents of the BTK inhibitor amine (7.5 mg) were added, and the reaction proceeded for 5 hours at room temperature. The product 23 was purified by HPLC and obtained as a white powder, 6.5 mg (88% yield).
HR-MS (m/z): Calculated: 946.42; Found: 947.4299 [M+H]+, 969.4115 [M+Na]+.

1H NMR (500 MHz, DMSO-d6) δ 11.11 (s, 1H), 8.38 (d, J = 3.7 Hz, 1H), 7.80 (t, 1H), 7.67 (dd, J = 8.5, 3.6 Hz, 2H), 7.53 (dd, J = 8.7, 3.6 Hz, 1H), 7.49 – 7.42 (m, 3H), 7.20 (t, J = 7.4 Hz, 1H), 7.19 – 7.15 (m, 2H), 7.15 – 7.10 (m, 2H), 5.09 (dd, J = 12.7, 5.3 Hz, 1H), 4.81 – 4.72 (m, 1H, minor rotamer set A), 4.70 – 4.62 (m, 1H, major rotamer set A), 4.51 (dd, J = 12.7, 4.2 Hz, 1H, major rotamer set B), 4.38 – 4.29 (m, 2H), 4.19 (d, J = 12.9 Hz, 1H, minor rotamer set C), 4.03 (dd, J = 13.1, 3.8 Hz, 1H, minor rotamer set B), 3.87 (d, J = 13.6 Hz, 1H, major rotamer set C), 3.84 – 3.75 (m, 2H), 3.70 – 3.61 (m, 2H), 3.56 – 3.52 (m, 2H), 3.52 – 3.40 (m, 8H), 3.38 – 3.29 (m, 2H), 3.18 – 3.09 (m, 1H), 2.94 – 2.83 (m, 2H), 2.66 – 2.53 (m, 2H), 2.40 – 2.08 (m, 4H), 2.07 – 1.98 (m, 1H), 1.97 – 1.84 (m, 1H), 1.68 – 1.58 (m, 1H, major rotamer set D), 1.56 – 1.36 (m, 5H), 1.33 – 1.16 (m, 5H).

13C NMR (126 MHz, DMSO-d6) δ 173.23, 171.33, 171.14, 170.37, 167.26, 165.71, 158.83, 158.55, 157.91, 157.86, 156.64, 156.30, 153.60, 153.24, 144.96, 144.62, 137.43, 133.70, 130.62, 130.55, 127.68, 127.55, 124.36, 120.50, 119.51, 119.45, 117.52, 116.78, 115.84, 115.19, 97.65, 97.52, 70.73, 70.63, 70.30, 70.24, 70.22, 69.93, 69.91, 69.33, 69.15, 53.44, 52.88, 49.69, 49.21, 45.71, 45.42, 41.48, 32.78, 31.42, 29.98, 29.78, 29.75, 29.60, 29.56, 29.10, 25.99, 25.91, 25.28, 25.03, 23.88, 22.47.
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