Metabolically labile fumarate esters impart kinetic selectivity to irreversible inhibitors

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Figure S6. Competition experiments to characterize high-occupancy targets of 1 and 2. A. Ramos cells co-cultured with MetAP2-expressing HEK293T cells were treated with 1 or 2 (1 nM - 10 µM, 1 h) prior to treatment with 3 (200 nM, 1 h). Gel-based ABPP revealed concentration-dependent blockade of BTK reactivity with 3 by 1 and 2, with 1 displaying ~10-fold higher potency. B. Ramos cells co-cultured with hCES1-expressing HEK293T cells were treated with 1 or 2 (1 nM - 10 µM, 1 h) prior to treatment with 3 (200 nM, 1 h). Gel-based ABPP revealed that 2 inhibits BTK labeling by 3 with ~5-10-fold lower potency in the presence of hCES1. C. MS-based identification of high-occupancy targets of 1 and 2. Ramos cells co-cultured with MetAP2- or hCES1-expressing HEK293T cells were treated with inhibitor (1 or 2, 10 µM, 1 h) or DMSO followed by 3 (1 µM, 1 h) and then analyzed by ABPP-SILAC. ABPP-SILAC experiments were repeated in duplicate and targets with SILAC ratios > 4 (DMSO/inhibitor; dashed horizontal lines) were designated as high-occupancy inhibitor targets. SILAC ratios > 10 were designated as values of 10.
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Figure S8. Characterization of Ibrutinib probes in vivo by ABPP. A. Mice were treated intraperitoneally with 3, 4, or 6 (20 mg/kg) for 2 h, sacrificed per IACUC-approved protocols, and tissues (spleen, liver, lung, kidney) excised, separated into soluble and membrane proteomes, and analyzed by gel-based
ABPP. B. Mice were treated intraperitoneally with 1 or vehicle (20 mg/kg) for 2h prior to treatment with 3, 4, or 6 (20 mg/kg) for 2 h, sacrificed per IACUC-approved protocols, and tissues (liver and lung) excised, separated into soluble and membrane proteomes and analyzed by gel-based ABPP.
Figure S9. Stability of probes in mouse plasma. A. Plasma stability of compounds 3, 4, 6, and 8 in mouse plasma. Plasma was treated with indicated probe (0 – 60 min, 10 µM, 37 °C) prior to LC-MS/MS analysis to detect amount of probe remaining. Half-lives were calculated based on ln(2)/slope of the linear-regression of ln(% remaining) vs. time. B. Plasma stability of fumarate ester compounds 4 and 6 in mouse plasma following pretreatment with a CES inhibitor JZL184. Plasma was pretreated with JZL184 or DMSO vehicle (60 min, 10 µM, 37 °C) prior to treatment with indicated probe (0 – 60 min, 10 µM, 37 °C). LC-MS/MS analysis detected the amount of probe remaining. Half-lives were calculated based on ln(2)/slope of the linear-regression of ln(% remaining) vs. time.
Table S1. Proteomic data for ABPP-SILAC experiments.

Legend
Tab 1: Competition Experiment: DMSO v Compound 2 (10 µM, 1h), Probe 3 (1 µM, 1h), MetAP2+Ramos
Tab 2: Competition Experiment: DMSO v Compound 2 (10 µM, 1h), Probe 3 (1 µM, 1h), hCES1+Ramos
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**BIOLOGICAL METHODS**

**Cell Culture.**
HEK293T cells were cultured in DMEM media (Corning) enriched with 10% fetal bovine serum supplemented with Glutamine (2 mM) and Penicillin/Streptomycin (50 µg/mL). Ramos cells were cultured in RPMI media (Corning) enriched with 10% fetal bovine serum (Gemini) supplemented with Glutamine and Penicillin/Streptomycin. HEK293T cell lines stably expressing methionine aminopeptidase-2 (MetAP2), carboxylesterase-1 (hCES1) and carboxylesterase-2 (hCES2) were generated using lentiviral infection methods. All cell lines were maintained in a humidified incubator at 37 °C and 5.0% CO₂.

**In Vitro proteome labeling.**
Cells were collected, washed with PBS and pelleted (1000 x g, 5 min, ºC). Cell pellets were then resuspended in ice-cold PBS and sonicated (2 x 6 pulses, 20% power, 40% pulse rate). Lysed proteome was then fractionated by ultracentrifugation (100,000 x g, 45 min, 4 ºC) to separate membrane and soluble proteome. Protein concentration of each fraction was determined by DC Assay (Bio-Rad) and further diluted to 1 mg/mL prior to treatment with desired probe for 1h (1000x stock in DMSO). Treated proteome was then subjected to CuAAC as described below.

**Dual cell culture system.**
HEK293T cells expressing MetAP, hCES1 or hCES2 were plated 16h prior to treatment to reach a final number of 250,000/ 10 mL for gel-based experiments or 2,000,000/10 mL for proteomics experiments. Just before treatment time, the media is removed, and replaced with media containing Ramos cells (1.5 million for gel-based experiments, 12 million for proteomics experiments) to be subjected to in situ downstream experiments with indicated probes described below. Ratios of HEK293:Ramos can be adjusted to reach desired amount of hCES1-mediated metabolism.

**In situ cell labeling – ABPP experiments.**
Cells plated in the dual cell culture system were treated with indicated probe in RPMI media containing 10% FBS and penicillin/streptomycin for 1 – 24 h. Upon treatment completion, cells were scraped to free adherent HEK293T cells from the plate into the suspension Ramos cells and transferred to a conical tube. Cells were pelleted (1,000 x g, 5 min, 4 ºC), washed with PBS (2x, 1,000 x g, 5 min, 4 ºC) and repelleted. Washed pellets were resuspended in ice-cold PBS and sonicated (2 x 6 pulses, 20% power, 40% pulse rate). Lysed proteome was then fractionated by ultracentrifugation (100,000 x g, 45 min, 4 ºC) to separate membrane and soluble proteome. Protein concentration of each fraction was determined by DC Assay (Bio-Rad) and further diluted to 1 mg/mL prior to CuAAC.

**In situ cell labeling – competition ABPP experiments.**
Cells plated in the dual cell culture system were treated with indicated inhibitor (1 nM – 10 µM, 1h) in RPMI media containing 10% FBS and penicillin/streptomycin for 1 h. Probe was then added (200 nM for gel-based experiments, 1 µM for proteomics experiments, 1h). Upon treatment completion, cells were scraped to free adherent HEK293T cells from the plate into the suspension Ramos cells and transferred to a conical tube. Cells were pelleted (1,000 x g, 5 min, 4 ºC), washed with PBS (2x, 1,000 x g, 5 min, 4 ºC) and repelleted. Washed pellets were resuspended in ice-cold PBS and sonicated (2 x 6 pulses, 20% power, 40% pulse rate). Lysed proteome was then fractionated by ultracentrifugation (100,000 x g, 45 min, 4 ºC) to separate membrane and soluble proteome. Protein concentration of each fraction was determined by DC Assay (Bio-Rad) and further diluted to 1 mg/mL prior to CuAAC.
**In vivo labeling.**

Compounds were resuspended in a vehicle 17:2:1 (v/v/v) solution of PBS/ Cremophor/Ethanol (stock concentration 2 mg/mL). C57BL/6 mice were injected intraperitoneally with Ibrutinib (1) or vehicle (20 mg/kg, 2h) prior to treatment with indicated probe (20 mg/kg, 2h). Mice were anesthetized with isoflurane and sacrificed by cervical dislocation in accordance with IACUC protocol. Tissues were harvested, washed with ice-cold PBS, weighed and diced into small pieces. Tissues were homogenized using a NextAdvance bullet blender according manufacturer’s protocol for each tissue. The resulting homogenate was allowed to rest for 5 min to allow beads to settle and supernatant removed. The supernatant was further fractionated by ultra-centrifugation (100,000 x g, 45 min, 4 ºC) to separate membrane and soluble proteome. Protein concentration of each fraction was determined by DC Assay (Bio-Rad) and further diluted to 1 mg/mL prior to CuAAC.

**Preparation of proteome and Cu(I)-Catalyzed [3 + 2] Azide-Alkyn Cycloaddition (CuAAC).**

Cell lysate (25 µg) was diluted with PBS to obtain a desired concentration of 1 µg µL⁻¹. Newly-made click chemistry cocktail (3 µL) was added to each sample [alkynyl-rhodamine tag (25 µM, 1.25 mM stock solution in DMSO); tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (1 mM, 50 mM freshly prepared stock solution in water); tris[(1-benzyl-1-H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (100 µM, 1.7 mM stock solution in 3:1 tertbutanol:DMSO); CuSO₄·5H₂O (1 mM, 50 mM freshly prepared stock solution in water) for a total reaction volume of 28 µL. The reaction was gently vortexed and allowed to sit at room temperature for 1 h. Upon completion, 10 µL of 4x loading buffer (40% glycerol, 0.4% bromophenol blue, 2.8% β-mercaptoethanol, pH 6.8) was then added and 20 µg of protein was then loaded per lane for SDS-PAGE separation on a 10% Tris-glycine gel.

**In-gel Fluorescence Scanning.**

Following SDS-PAGE separation, gels were scanned on a ChemiDoc MP (Bio-Rad) using the green epi illumination excitation light source and a 605/50 filter.

**Cysteine Reactivity Assay.**

L-cysteine reduced was diluted to a final concentration of 250 µM in assay buffer (100 mM Tris pH 8.8, 50% methanol as a cosolvent). In triplicate, 100 µL of L-cysteine (250 µM) in assay buffer was added to each well in a clear, flat-bottom 96-well plate (Costar, Corning). The indicated probe (DMSO stock, final concentration 500 µM) or DMSO vehicle was added to each sample and incubated at room temperature for 0-60 min. Upon incubation completion, Ellman’s reagent (5 µL, 100 mM stock in DMSO, final concentration 5 mM) was added and the reaction incubated at room temperature for 10 minutes. Absorbance was measured at 412 nM on a plate reader (CLARIOstar, BMG LABTECH). Concentration of cysteine was determined by concentration curve. Second-order rate constants were determined according to previous reports.

**MS-based Hydrolysis Assay.**

HEK293T cells stable expressing MetAP, hCES1 or hCES2 were plated 24 h prior to treatment in 6-well plates (500,000 cells/well). At treatment time, media was removed and replaced with media containing 1 or 2 or DMSO (10 µM). Cells were incubated for 1h. At experimental endpoint, media was aspirated, and cells were washed with PBS (1 mL). Methanol spiked with 3.33 µM 3 and 4 was added to each well (600 µL). Protein was scraped and transferred to a 2 dram vial. Chloroform (1.4 mL) was then added to the sample to a final volume of 2 mL. Precipitate was pelleted by centrifugation (2000 x g, 15 min), and supernatant was transferred to 1 dram vial. Samples were then dried down and stored at -80 ºC prior to analysis.
Samples were prepared in quintuplicate for each probe with each cell type. Metabolites were separated by reverse phase chromatography (Phenomenex C18) on an Agilent G6410B tandem mass spectrometer with ESI source. Buffer A was composed of 95:5 (v/v) H₂O:CH₃CN supplemented with 1% formic acid. Buffer B was composed of 100% CH₃CN supplemented with 1% formic acid. The flow rate started at 0.1 mL/min for 5 min at 100% A. Following the isocratic equilibration, flow rate increased to 0.4 mL/min and moved from 0 to 100% B from 5.01 to 20 min in a linear gradient. The flow rate was then increased to 0.5 mL/min and 100% B ran for 5 min. A linear gradient then returned the solvent to 100% A over 5 min. For each run the injection volume was 10 µL. The mass spectrometer was run in MRM mode, monitoring the transition of m/z for each analyte below:

<table>
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<th>Analyte</th>
<th>Precursor Ion</th>
<th>Product Ion</th>
<th>Fragmentor</th>
<th>Collision Energy</th>
<th>Polarity</th>
<th>Retention Time (min)</th>
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<td>100</td>
<td>25</td>
<td>Positive</td>
<td>17.2</td>
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</table>

Metabolites were quantified by measuring the area under the peak in comparison to internal probe standards (3 and 4) at known concentrations. Experiments performed with DMSO-treated control cells revealed < 10% signals for the measured m/z values for indicated compounds.

**Plasma Stability Assay**

Mouse plasma was treated with compounds 3, 4, 6, 8 with shaking for 0 – 60 min (10 µM, 37 °C). Upon incubation completion, samples were transferred to a vial containing 4x volume of ice-cold acetonitrile containing carbamazepine as an internal standard to quench any esterase activity. The remaining concentration for each compound was determined by LC-MS/MS (ABSciex 5500) and expressed as percent remaining by relating the ratio of the peak area of the sample/IS at individual time points to the ratio for the 0 min sample. The percent remaining at individual time points was transformed as natural log and plotted as ln(% remaining) vs. time. half-life was determined as half-life = ln(2)/-slope. The involvement of esterases was demonstrated by the use of an esterase inhibitor, JZL184.² Samples were preincubated with or without JZL184 (10 µM, 37 °C, 1h) prior to treatment with the indicated compound for the appropriate time. Formation of the hydrolytic product of 4 and 6, compound 8, was also monitored.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mass</th>
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<th>Q3</th>
<th>DP</th>
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**ABPP-SILAC Sample preparation.**

All SILAC experiments were conducted using isotopically labelled HEK293T stably expressing MetAP or hCES1 and Ramos (generated by 6 passages in light (100 µg/mL L-arginine and L-lysine) or heavy (100 µg/mL ¹³C₆¹⁵N₄ L-arginine and ¹³C₆¹⁵N₂ L-lysine, Sigma-Aldrich) media (DMEM for HEK293T and RPMI-1640 for Ramos (ThermoScientific), supplemented with 10% dialyzed FBS, penicillin/streptomycin and glutamine). Light Ramos (12 million, 10 mL) were plated on light HEK293T
expressing MetAP or hCES1 (2 million) and treated with 3, 4 or DMSO vehicle (1 µM, 1 or 24 h). Upon treatment completion, cells were scraped to free adherent HEK293T cells from the plate into the suspension Ramos cells and transferred to a conical tube. Cells were pelleted (1,000 x g, 5 min, 4 °C), washed with PBS (2x, 1,000 x g, 5 min, 4 °C) and repelleted. Washed pellets were resuspended in ice-cold PBS and sonicated (2 x 6 pulses, 20% power, 40% pulse rate). Lysed proteome was then fractionated by ultracentrifugation (100,000 x g, 45 min, 4 °C) to separate membrane and soluble proteome. Protein concentration of each fraction was determined by DC Assay (Bio-Rad) and further diluted to 2 mg/mL. Each heavy and light pair was then combined (500 µL, 1 mg protein each) to a final volume of 1 mL. Each CuAAC reagent was added stepwise to the sample [biotin-azide (10 µL, 100 µM final concentration, 5 mM stock solution in DMSO); tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (20 µL, final concentration 1 mM, 50 mM freshly prepared stock solution in water); tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (60 µL, final concentration 100 µM, 1.7 mM stock solution in 3:1 tertbutanol:DMSO); CuSO4•5H2O (20 µL, final concentration 1 mM, 50 mM freshly prepared stock solution in water)]. The reaction was gently vortexed and allowed to sit at room temperature for 1 h.

Following reaction completion, the reaction was quenched with 2 mL ice-cold methanol and vortexed. Chloroform was then added (500 µL) and ice-cold water (1 mL). The mixture was vortexed into a milky solution and centrifuged to generate a protein disc at the organic/aqueous interface (5,000 x g, 15 min, 4 °C). The organic layer was removed from the bottom, leaving the disc to stick to the side of the tube and allowing the aqueous to be decanted. The pellet was washed with 1:1 Methanol:Chloroform (2x) and sonicated back in to ice-cold Methanol (2 mL). The precipitated protein was allowed to sit at -80 °C for 16 h. Ice-cold methanol (500 µL) was then added and protein pelleted (5,000 x g, 15 min, 4 °C). Solvents were removed and the pellet allowed to air dry briefly.

To each sample was freshly prepared 6 M Urea in PBS (500 µL). A solution of freshly prepared 1:1 TCEP (200 mM in water stock) and K2CO3 (600 mM in water stock) was added to each sample (50 µL) followed by addition of 10% SDS in PBS (10 µL). The protein pellet was then resuspended by sonication and the resulting mixture incubated with shaking (37 °C, 0.5 h). Following incubation, IA (70 µL, 400 mM in water stock) was added, and the mixture was allowed to stand in the dark for 0.5 h. Upon completion of alkylation, reaction was quenched with 10% SDS in PBS (130 µL). Sample was further diluted with PBS (5.5 mL). The diluted proteome was then sonicated briefly and centrifuged (5000 x g, 2 min) to remove any leftover particulate.

Avidin beads (pre-washed 2x with 10 mL PBS) were added to each sample (100 µL), and the samples were rotated (room temperature, 2h). Beads were then pelleted (1600 x g, 2 min) and supernatant removed by aspiration. Beads were washed with 0.2% SDS in PBS (1x, 10 mL), PBS (2x, 10 mL) and water (1x, 10 mL) with pelleting after each wash (2600 x g, 2 min). Beads were then transferred to safe-lock tube (Eppendorf) in water (2x, 500 µL). Beads were then resuspended in freshly prepared 2 M Urea in 25 mM ammonium bicarbonate (200 µL). To a vial of trypsin (20 µg) was added trypsin buffer (40 µL, provided by Promega) and 100 mM CaCl2 (20 µL). Trypsin was then added to each sample (6 µL) and samples digested with shaking (16 – 18 h, 37 °C).

Following digestion, samples were transferred to a Bio-Spin column (Bio-Rad) and spun (0.8 x g, 0.5 min). The beads were further washed with PBS (100 µL) and spun (0.8 x g, 0.5 min). Formic acid was then added to eluent (16 µL, 5% final concentration), and the sample stored at -20 °C until LC-MS/MS analysis.
LC-MS/MS Proteomic Analysis.  
The MS2 spectra data were extracted from the raw file using RAW Xtractor (version 1.9.9.2; available at http://fields.scripps.edu/yates/wp/?page_id=17). MS2 spectra data were searched using the ProLuCID algorithm (publicly available at http://fields.scripps.edu/yates/wp/?page_id=17) using a reverse concatenated, nonredundant variant of the Human UniProt database (release-2012_11). Searches included methionine oxidation as a differential modification (+15.9949 M) and mass shifts of SILAC labeled amino acids (+10.0083 R, +8.0142 K) and no enzyme specificity. Peptides were required to have at least one tryptic terminus and unlimited missed cleavage sites. 2 peptide identifications were required for each protein. ProLuCID data was filtered through DTASelect (version 2.0) to achieve a peptide false-positive rate below 1%. Ratios of heavy/light or light/heavy (alternated to account for potential differences in protein expression between the heavy and light proteomes) peaks were calculated using in-house CIMAGE software. Reported SILAC ratios (R values) were represent a median of all quantified peptides per protein across all replicates. The mean R values and standard deviation were also calculated across all biological replicates.

All ABPP-SILAC experiments were repeated in at least duplicate and combined datasets subjected to the following filtration criteria:

1. Proteins were required to have been quantified in two separate biological experiments
2. Proteins containing 'Keratin', 'Statherin', 'Serum albumin', 'Hornerin', 'Trypsin' containing were dropped.
3. The number of unique quantified peptides was generated per protein from the combined datasets.
4. Proteins had to contain ≥2 unique quantified filtered peptides (uqfp) and ≥4 quantified peptides (noqp)
5. Proteins subjected to SD filtration in order to validate median ratio.
6. Manual inspection of high-ratio targets after previous curation steps to remove any remaining low quality elution profiles.

See SupplementaryTable1 for ABPP-SILAC data.
SYNTHETIC METHODS AND COMPOUND CHARACTERIZATION

General information
All reagents used for chemical synthesis were purchased from Sigma-Aldrich, Alfa Aesar or EMD Millipore unless otherwise specified and used without further purification. All anhydrous reactions were performed under nitrogen atmosphere. Analytical thin-layer chromatography (TLC) was conducted on EMD Silica Gel 60 F_{254} plates with detection by anisaldehyde or UB. For preparative TLC chromatography, Analtech Uniplates F_{254} silica plates with a layer thickness of 500 - 1500 µm silica gel matrix depending on amount of crude material. ^1^H spectra were obtained at 400 or 500 MHz on a Bruker AV400 or DRX500. Chemical shifts are recorded in ppm (δ) relative to solvent. Coupling constants (J) are reported in Hz. ^1^C spectra were obtained at 151 MHz on the Bruker DRX600 equipped with a 5mm DCH cryoprobe.

Synthesis of inhibitors and probes.
Compounds 1 and 3 were synthesized according to literature procedure.^3^,^4^ 3 is available through Sigma Aldrich (Item #PZ0242).

**Compound 2:** methyl (R,E)-4-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-4-oxobut-2-enoate
(R)-tert-butyl-3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidine-1-carboxylate was synthesized according to literature procedure (9).^4^ Compound 9 (26 mg, 0.0673 mmol) was dried overnight under vacuum prior to resuspension in anhydrous DMF (1 mL). Diisopropylethylamine (35 µL, 0.2019 mmol, 3 eq), monomethylfumarate (9.6 mg, 0.074 mmol, 1.1 eq) and propylphosphonic anhydride solution (50% by weight in DMF, 60 µL, 0.088 mmol, 1.3 eq) were added. The mixture was warmed to 50 °C and allowed to stir for 2 days after which time the reaction was determined to be complete by TLC (5% MeOH in CH2Cl2). The crude reaction was dried down from CH2Cl2 under reduced pressure to remove DMF and dried under vacuum overnight. Crude was then resuspended in 5% MeOH in CH2Cl2 and purified by preparative TLC to yield the final product 2 (10.4 mg, 0.0289 mmol, 43% yield). ^1^H NMR (600 MHz, CDCl3) δ 8.37 (d, J = 8.3 Hz, 1H), 7.69 – 7.63 (m, 2H), 7.48 (d, J = 15.4 Hz, 0.5H), 7.41 (ddd, J = 8.9, 7.3, 1.8 Hz, 2H), 7.37 (d, J = 15.3 Hz, 0.5H), 7.23 – 7.15 (m, 3H), 7.13 – 7.09 (m, 2H), 6.77 (dd, J = 53.9, 15.4 Hz, 1H), 5.79 (br s, 2H), 4.96 – 4.82 (m, 1.5H), 4.45 (d, J = 13.3 Hz, 0.5H), 4.14 (dd, J = 13.5, 4.1 Hz, 0.5H), 4.03 (d, J = 13.8 Hz, 0.5H), 3.94 (dd, J = 13.4, 9.7 Hz, 0.5H), 3.80 (d, J = 41.4 Hz, 3H), 3.48 (dd, J = 12.8, 10.7 Hz, 0.5H), 3.32 – 3.23 (m, 0.5H), 3.12 (td, J = 12.2, 10.9, 3.2 Hz, 0.5H), 2.50 – 2.34 (m, 1.5H), 2.33 – 2.24 (m, 1H), 2.07 (td, J = 14.5, 13.1, 4.0 Hz, 1H), 1.77 (qd, J = 10.0, 9.3, 6.1 Hz, 1H), 1.66 (dq, J = 14.8, 7.3 Hz, 0.5H). ^1^C NMR (151 MHz, CDCl3) δ 165.68, 163.40, 158.19, 157.41, 155.83, 155.23, 153.83, 143.65, 133.63, 130.48, 129.47, 127.24, 123.63, 119.11, 118.67, 98.17, 52.83, 51.86, 49.61,
45.71, 41.96, 31.46, 29.77, 29.23, 24.78, 23.08, 22.13, 13.67. HRMS (m/z) calculated for C_{27}H_{27}N_{6}O_{4} [M+H]^+ 499.2088; found 499.2090.

**Compound 4:** methyl (R,E)-4-(3-(4-amino-3-(4-(3-ethynylphenoxy)phenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-4-oxobut-2-enoate (R)-3-(4-(3-ethynylphenoxy)phenyl)-1-(piperidin-3-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (10) was synthesized according to literature procedure. Compound 10 (37 mg, 0.090 mmol, 1 eq) was dried overnight under vacuum prior to resuspension in anhydrous DMF (2 mL). Diisopropylethylamine (47 µL, 0.270 mmol, 3 eq), monomethylfumarate (14 mg, 0.011 mmol, 1.1 eq) and propylphosphonic anhydride solution (50% by weight in DMF, 15. µL, 0.024 mmol, 1.3 eq) were added. The mixture was warmed to 50 °C and allowed to stir for 2 days after which time the reaction was determined to be complete by TLC (5% MeOH in CH_{2}Cl_{2}). The crude reaction was dried down from CH_{2}Cl_{2} under reduced pressure to remove DMF and dried under vacuum overnight. Crude was then resuspended in 5% MeOH in CH_{2}Cl_{2} and purified by preparative TLC to yield the final product 5 (26 mg, 0.46 mmol, 51% yield). ¹H NMR (500 MHz, CDCl₃) δ 8.45 (d, J = 5.4 Hz, 1H), 7.81 – 7.70 (m, 2H), 7.54 (d, J = 15.3 Hz, 0.5H), 7.46 – 7.36 (m, 2.5H), 7.29 – 7.22 (m, 3H), 7.17 (d, J = 8.1 Hz, 1H), 6.83 (dd, J = 44.1, 15.4 Hz, 1H), 5.65 (br s, 2H), 5.04 – 4.88 (m, 1.5H), 4.51 (d, J = 13.2 Hz, 0.5H), 4.20 (d, J = 12.8 Hz, 0.5H), 4.09 (d, J = 13.7 Hz, 0.5H), 4.00 (dd, J = 13.4, 9.7 Hz, 0.5H), 3.86 (d, J = 34.0 Hz, 3H), 3.54 (dd, J = 12.7, 10.3 Hz, 0.5H), 3.34 (t, J = 13.0 Hz, 0.5H), 3.18 (s, 1.5H), 2.46 (dt, J = 32.7, 10.1 Hz, 1H), 2.35 (dd, J = 13.3, 4.7 Hz, 1H), 2.20 – 1.60 (m, 2H). ¹³C NMR (151 MHz, CDCl₃) δ 165.68, 163.39, 157.53, 157.37, 156.89, 155.33, 153.88, 143.48, 133.63, 130.49, 129.62, 127.82, 127.30, 122.18, 122.15, 119.69, 119.07, 98.18, 82.29, 77.56, 52.86, 51.88, 49.61, 45.70, 41.95, 31.47, 29.24, 24.78, 23.09, 22.24, 13.67. HRMS (m/z) calculated for C_{32}H_{27}N_{6}O_{4} [M+H]^+ 523.2088; found 523.2087.

**Compound 6:** isopropyl (R,E)-4-(3-(4-amino-3-(4-(3-ethynylphenoxy)phenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-4-oxobut-2-enoate (R)-3-(4-(3-ethynylphenoxy)phenyl)-1-(piperidin-3-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (10) was synthesized according to literature procedure. Compound 10 (7.5 mg, 0.0183 mmol, 1 eq) was dried under reduced pressure to remove DMF and dried under vacuum overnight. Crude was then resuspended in 5% MeOH in CH_{2}Cl_{2} and purified by preparative TLC to yield the final product 6 (7.5 mg, 0.0183 mmol, 1 eq) δ 8.38 (d, J = 4.0 Hz, 1H), 7.70 – 7.63 (m, 2H), 7.42 (d, J = 15.4 Hz, 0.5H), 7.37 – 7.27 (m, 2.5H), 7.22 – 7.12 (m, 3H), 7.09 (d, J = 8.1 Hz, 1H), 6.71 (dd, J = 32.2, 15.4 Hz, 1H), 5.52 (s, 2H), 5.09 (dp, J = 32.5, 6.2 Hz, 1H), 4.87 (dt, J = 28.9, 13.2 Hz, 1.5H), 4.48 (d, J = 13.3 Hz, 0.5H), 4.15 (d, J = 12.9 Hz, 0.5H), 4.01 (d, J = 13.7 Hz, 0.5H), 3.89 (dd, J = 13.3, 10.0 Hz, 0.5H), 3.51 – 3.42 (m, 0.5H), 3.25 (t, J = 12.8 Hz, 0.5H), 3.10 (s, 1H), 3.03 (t, J = 12.2 Hz, 0.5H), 2.47 – 2.30 (m, 1H), 2.27 (dd, J = 13.1, 4.5 Hz, 1H), 2.08 – 1.99 (m, 1H), 1.75 (t, J = 12.8 Hz, 1H), 1.62 (s, 3H), 1.30 (dd, J = 6.3, 2.8 Hz, 3H). ¹³C NMR (126 MHz, DMSO) δ 164.76, 163.63, 157.52, 157.33, 155.90, 155.33, 153.90, 143.45, 133.20, 131.40, 129.60, 127.85, 127.30, 123.36, 122.15, 119.69, 119.07, 98.20, 82.29, 77.55, 68.32, 52.90, 51.91, 49.60, 45.64, 41.95, 31.38, 29.88, 29.25, 24.87, 23.09, 22.17, 21.31, 13.71. HRMS (m/z) calculated for C_{31}H_{31}N_{6}O_{4} [M+H]^+ 551.2401; found 551.2402.
**Compound 7:** ethyl (R,E)-4-(3-(4-amino-3-(4-(3-ethynylphenoxy)phenyl))-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-4-oxobut-2-enoate

(R)-3-(4-(3-ethynylphenoxy)phenyl)-1-(piperidin-3-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (10) was synthesized according to literature procedure. Compound 10 (7.5 mg, 0.0183 mmol, 1 eq) was dried overnight under vacuum prior to resuspension in anhydrous DMF (0.250 mL). Diisopropylethylamine (9.6 µL, 0.0548 mmol, 3 eq), monoethylfumarate (3 mg, 0.020 mmol, 1.1 eq) and propylphosphonic anhydride solution (50% by weight in DMF, 15 µL, 0.024 mmol, 1.3 eq) were added. The mixture was allowed to stir for 16 h after which time the reaction was determined to be complete by TLC (5% MeOH in CH₂Cl₂). The crude reaction was dried down from CH₂Cl₂ under reduced pressure to remove DMF and dried under vacuum overnight. Crude was then resuspended in 5% MeOH in CH₂Cl₂ and purified by preparative TLC to yield the final product 6 (7.6 mg, 0.0142 mmol, 77% yield).

**1H NMR (500 MHz, CDCl₃)** δ 8.46 (d, J = 3.7 Hz, 1H), 7.81 – 7.70 (m, 2H), 7.58 – 7.35 (m, 3H), 7.31 – 7.22 (m, 3H), 7.22 – 7.12 (m, 1H), 6.82 (dd, J = 38.9, 15.4 Hz, 1H), 5.59 (br s, 2H), 5.07 – 4.89 (m, 1.5H), 4.54 (d, J = 13.4 Hz, 0.5H), 4.32 (dq, J = 32.9, 7.1 Hz, 2H), 4.22 (d, J = 12.6 Hz, 0.5H), 4.11 (s, 0.5H), 3.99 (dd, J = 13.4, 9.8 Hz, 0.5H), 3.60 – 3.49 (m, 0.5H), 3.34 (t, J = 12.9 Hz, 0.5H), 3.18 (s, 1.5H), 2.47 (dd, J = 31.9, 10.5 Hz, 1H), 2.35 (dd, J = 13.0, 4.7 Hz, 1H), 2.13 (d, J = 13.7 Hz, 1H), 1.82 (d, J = 12.2 Hz, 1H), 1.69 (m, 3H).

**13C NMR (151 MHz, CDCl₃)** δ 165.24, 157.52, 157.25, 157.25, 155.90, 155.49, 153.94, 143.41, 133.32, 130.93, 129.60, 127.87, 127.25, 123.36, 122.18, 119.70, 119.07, 98.20, 82.29, 77.54, 60.73, 52.88, 51.90, 49.61, 45.71, 41.91, 29.79, 29.38, 29.25, 24.79, 23.14, 13.70. HRMS (m/z) calculated for C₃₀H₂₉N₆O₄ [M+H]⁺ 537.2245; found 537.2248.
Compound 2 $^1$H
Compound 4 $^1$H
Compound 7 $^1$H
Compound $^{13}$C

References


