A Chemoproteomic Method for Profiling Inhibitor-Bound Kinase Complexes

Linglan Fang[†], Sujata Chakraborty[†], Emily M. Dieter[†], Zachary E. Potter[†], Chloe K. Lombard[†], and Dustin J. Maly[*,†,‡]

[†]Department of Chemistry and [‡] Biochemistry, University of Washington, Seattle, USA

[*]Email: djmaly@uw.edu

Table of Contents:

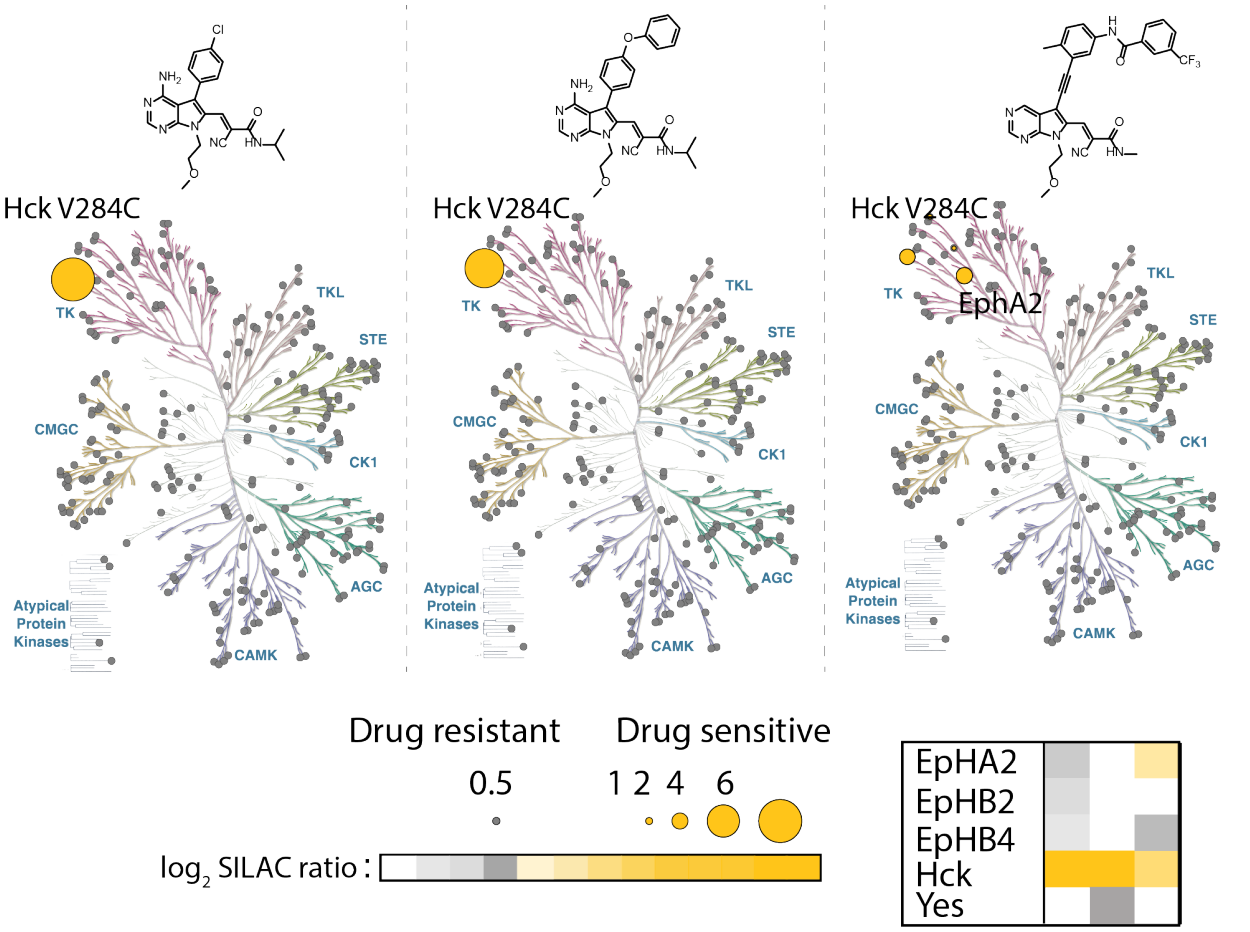


Figure S1. Phylogenetic trees showing the kinobead-based chemoproteomic profiling of CystIMATIK probes. Significantly competed kinases are shown as yellow circles with the size corresponding to the level of competition (larger circle, more competed). See the Supplementary Information of *Mol. Cell* **2019**, *74*, p. 393-408 for primary profiling data.

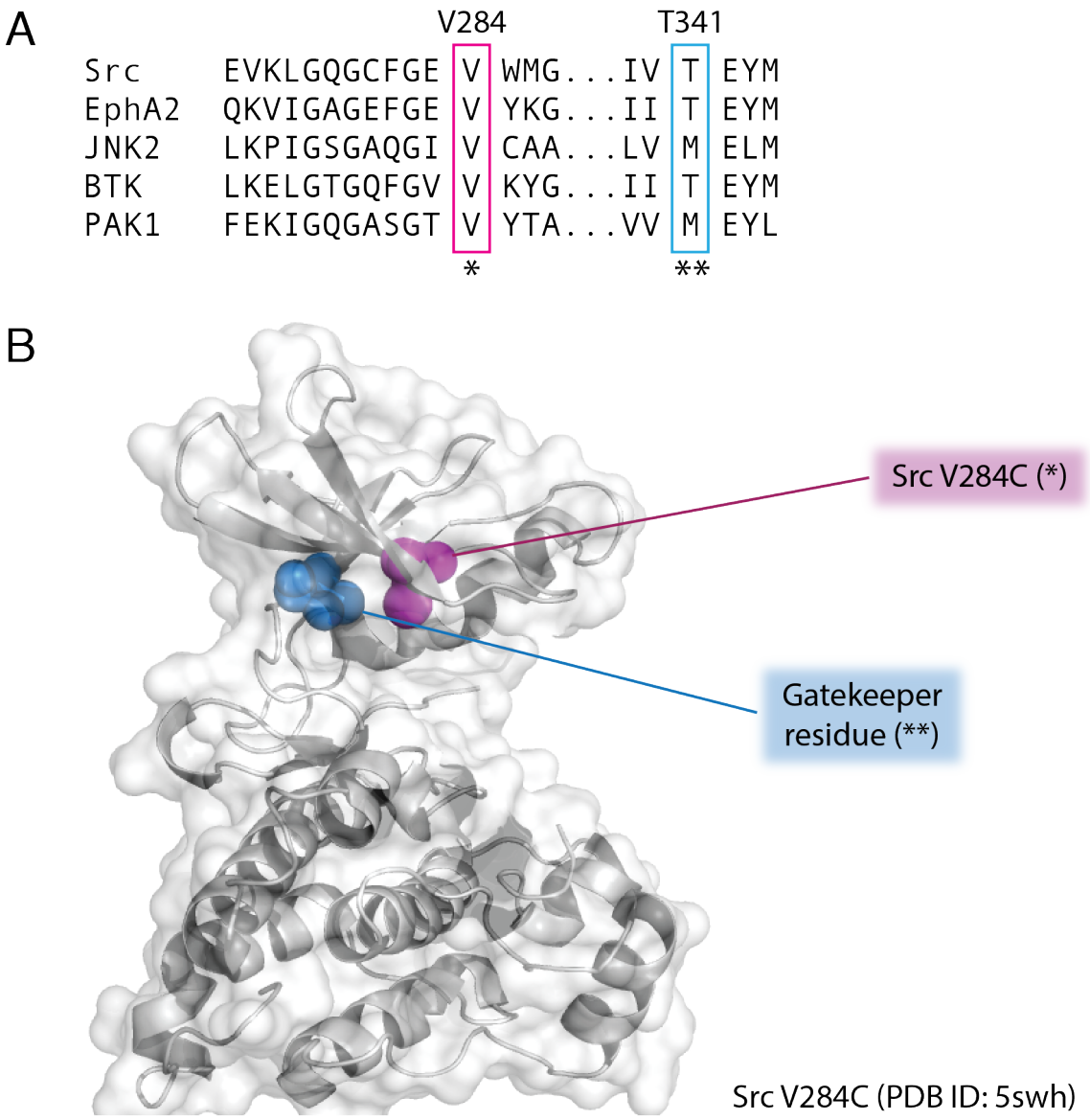
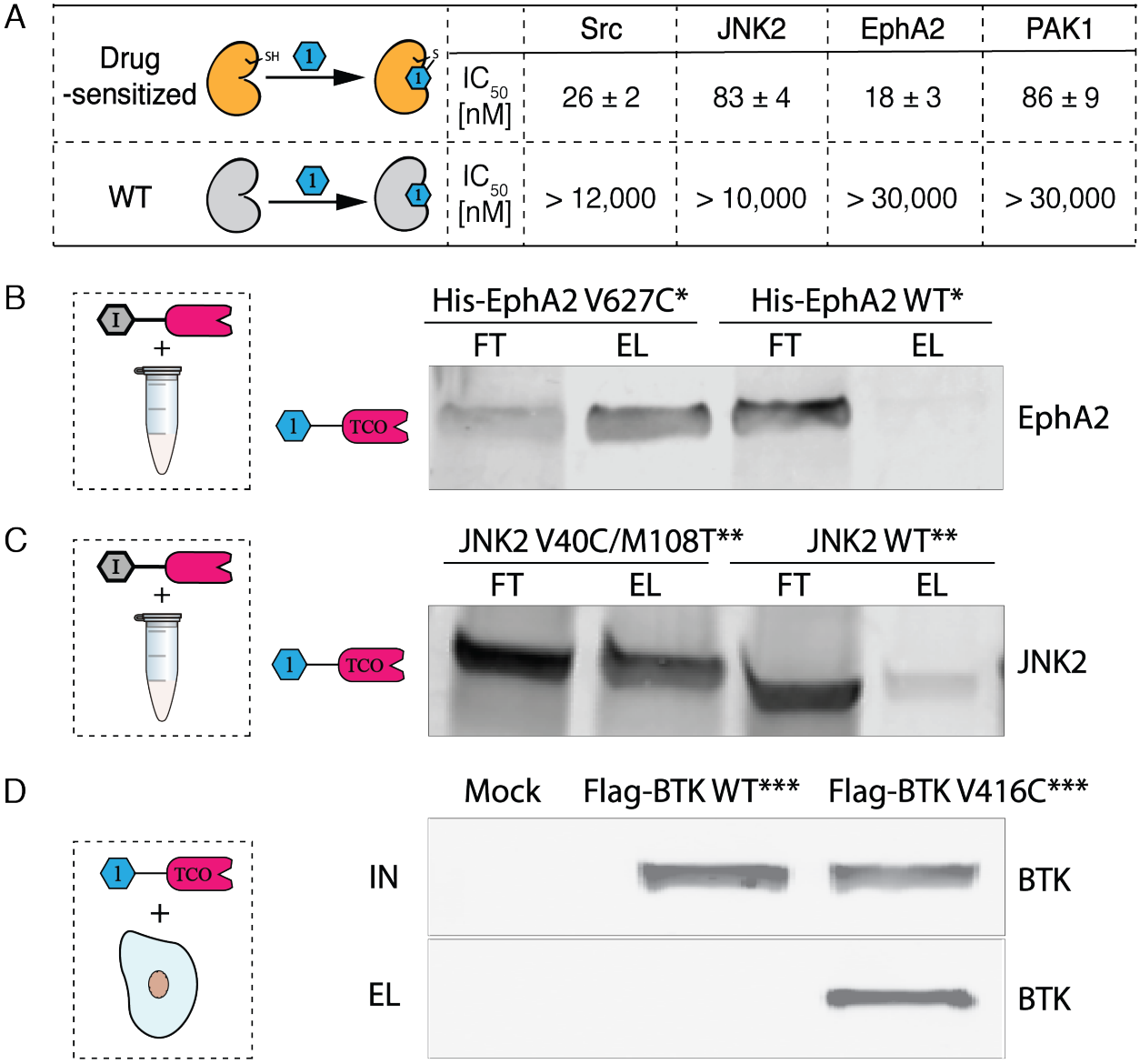


Figure S2. Sensitization of kinases to CystIMATIK probes. Introduction of a Cys at the residue homologous to V284 of Src and, if required, a Thr at the gatekeeper position sensitizes kinases to CystIMATIK probes. (A) Sequence alignment of the nonreceptor tyrosine kinases Src and BTK, the receptor tyrosine kinase Epha2, and the serine/threonine kinases PAK1 and JNK2. Residues at the position equivalent to V284 of Src are framed in magenta. Gatekeeper residues are framed in blue. Sequences used for sequence alignment are: PDB ID (Src): 2BDF; PDB ID (EphA2): 5I9Y; PDB ID (JNK2): 3E7O; PDB ID (BTK): 5FBO; PDB ID (PAK1): 2HY8. (B) X-ray crystal structure of the catalytic domain of Src V284C (PDB ID: 5SWH). The Cys284 residue is colored in magenta. The gatekeeper residue (T341) is colored in blue.



Notes: N-terminal His-tagged recombinant EphA2 (residue 590 - 876); ** Full-length reco--mbinant JNK2; *** N-terminal Flag-tagged full-length BTK*

Figure S3. Sensitization of diverse kinases to CystIMATIK probes **1** and **1-TCO**. (A) IC_{50} values of **1** against recombinant Src V284C, JNK2 V40C/M108T, EphA2 V627C and PAK1 V286C/M346T, and wild-type Src, JNK2, EphA2 and PAK1. IC_{50} values shown are mean ± SEM, $n = 3$. (B) **1-TCO** selectively enriches drug-sensitized EphA2 (V627C; residue 590 - 876) from cell lysates. HEK293 cell lysate containing either N-terminal His-tagged EphA2 V627C or EphA2 WT were treated with **1-TCO** (10 μM), incubated with tetrazine-conjugated sepharose beads (Tz-beads), and captured proteins were eluted under reducing and denaturing conditions. An anti-His Tag immunoblot of the flow through (FT) and elution (EL) are shown. (C) **1-TCO** selectively enriches drug-sensitized JNK2 (V40C/M108T) from cell lysates. HEK293 cell lysate containing either JNK2 V40C/M108T or JNK2 WT were treated with **1-TCO** (10 μM), incubated with Tz-beads, and captured proteins were eluted under reducing and denaturing conditions. An anti-JNK2

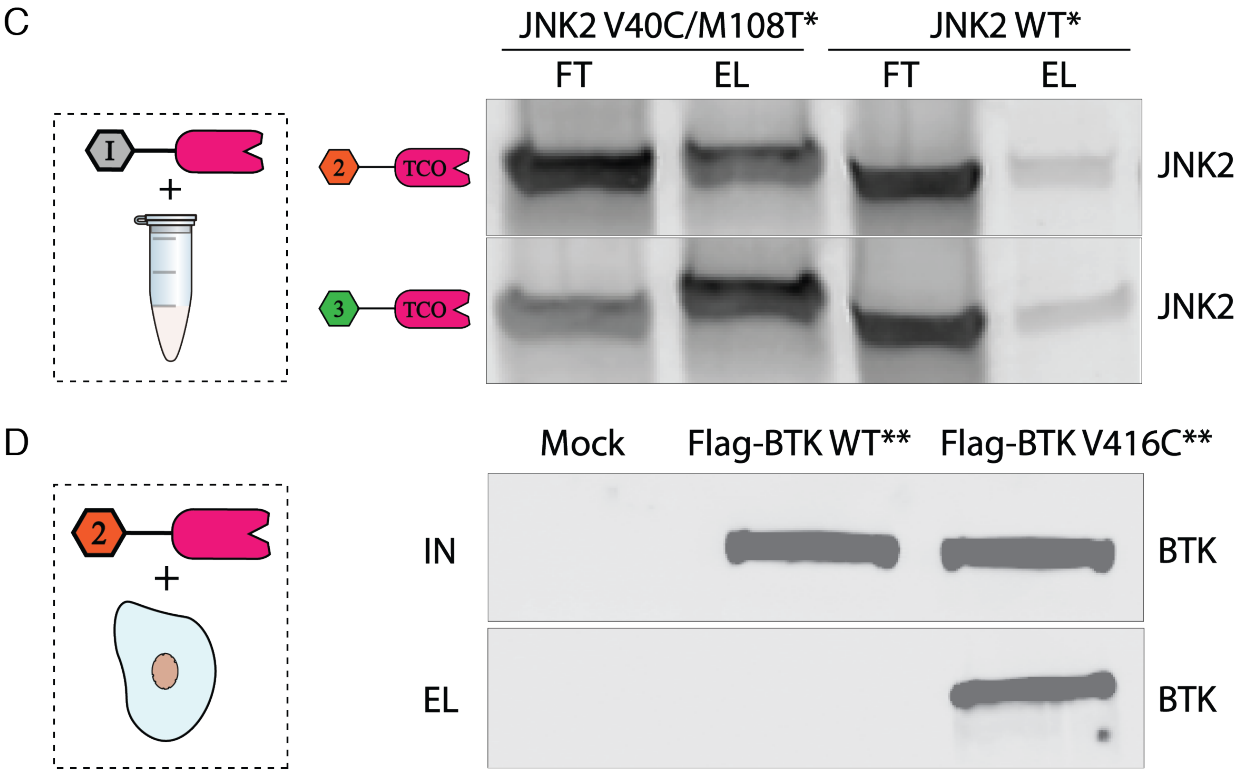
immunoblot of the flow through (FT) and elution (EL) are shown. (D) **1-TCO** selectively enriches drug-sensitized BTK (V416C) from cells. HEK293 cells transiently expressing either N-terminal Flag-tagged BTK (V416C) or BTK WT were treated with **1-TCO** (2.5 μM), lysed, and then incubated with Tz-beads. Captured proteins were eluted under reducing and denaturing conditions. An anti-DYKDDDDK Tag (Flag Tag) immunoblot of the input (IN) and elution (EL) are shown. Note: in Figure S3D, elution samples were 2-fold more concentrated than input samples.

A

		Src	JNK2	EphA2	PAK1
Drug -sensitized	IC_{50} [nM]	28 ± 3	180 ± 10	300 ± 30	151 ± 4
WT	IC_{50} [nM]	> 12,000	> 10,000	> 30,000	> 30,000

B

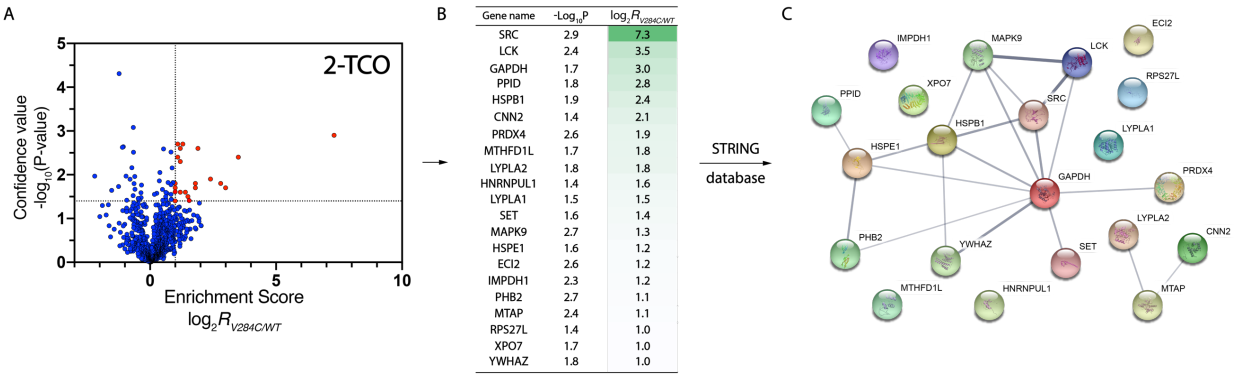
		Src	JNK2	EphA2	PAK1
Drug -sensitized	IC_{50} [nM]	39 ± 3	6.2 ± 0.2	32 ± 2	> 30,000
WT	IC_{50} [nM]	> 12,000	> 10,000	890 ± 60	> 30,000



Notes: ** Full-length recombinant JNK2; ** N-terminal Flag-tagged full-length BTK*

Figure S4. Sensitization of diverse kinases to conformation-selective CystIMATIK probes. (A) IC_{50} values of **2** against recombinant Src V284C, JNK2 V40C/M108T, EphA2 V627C and PAK1 V286C/M346T, and WT Src, JNK2, EphA2 and PAK1. Values shown are mean ± SEM, $n = 3$. (B) IC_{50} values of **3** against recombinant Src V284C, JNK2 V40C/M108T, EphA2 V627C and PAK1 V286C/M346T, and wild-type Src, JNK2, EphA2 and PAK1. Values shown are mean ± SEM, $n = 3$. (C) **2-TCO** and **3-TCO** selectively enrich drug-sensitized JNK2 (V40C/M108T) from

cell lysates. HEK293 cell lysate containing either JNK2 (V40C/M108T) or JNK2 WT were treated with **2-TCO** (10 μM) or **3-TCO** (10 μM), incubated with Tz-beads, and captured proteins were eluted under reducing and denaturing conditions. An anti-JNK2 immunoblot of the flow through (FT) and elution (EL) are shown. (D) **2-TCO** selectively enriches drug-sensitized BTK (V416C) from cells. HEK293 cells transiently expressing either N-terminal Flag-tagged BTK (V416C) or BTK WT were treated with **2-TCO** (2.5 μM), lysed, and then incubated with Tz-beads. Captured proteins were eluted under reducing and denaturing conditions. An anti-DYKDDDDK Tag (Flag Tag) immunoblot of the input (IN) and elution (EL) are shown. Note: in Figure S4D, elution samples were 2-fold more concentrated than input samples.



Gene name	-Log$_{10}$P	log$_2$R$_{V284C/WT}$
SRC	2.9	7.3
LCK	2.4	3.5
GAPDH	1.7	3.0
PPID	1.8	2.8
HSPB1	1.9	2.4
CNN2	1.4	2.1
PRDX4	2.6	1.9
MTHFD1L	1.7	1.8
LYPLA2	1.8	1.8
HNRNPUL1	1.4	1.6
LYPLA1	1.5	1.5
SET	1.6	1.4
MAPK9	2.7	1.3
HSPE1	1.6	1.2
ECI2	2.6	1.2
IMPDH1	2.3	1.2
PHB2	2.7	1.1
MTAP	2.4	1.1
RPS27L	1.4	1.0
XPO7	1.7	1.0
YWHAZ	1.8	1.0

Figure S5. Interrogation of growth-factor-stimulated **2-TCO**-bound Src's interactome with co-clickable precipitation. (A) Volcano plot showing the $\log_2$ transformed MaxQuant intensities of the label-free comparison of **2-TCO**-treated, growth factor-stimulated HeLa cells expressing Src V284C or Src WT (n=3). Statistically significant interactors for drug-sensitized Src were defined as protein hits with enrichment scores = $\log_2$[Intensity (drug-sensitized target)/Intensity (WT target)] ≥ 1.0 and confidence values = -$\log_{10}$(P-value) ≥ 1.4. The calculation was based on intensity values computed by MaxQuant. Missing protein intensity values were imputed by Perseus with a distribution downshift of 1.3 and a width of 0.2. Specific Src interactors are highlighted in red and non-specific binders are marked in blue. (B) Proteins that show statistically significant enrichment with **2-TCO** from Src V284C-expressing, growth factor-stimulated HeLa cells. The second column shows confidence values (-$\log_{10}$(P-value)) and the third column shows enrichment scores. (C) STRING network of the interactome of **2-TCO**-bound Src V284C. Significant interactors were visualized using the STRING database.[1, 2] Each circle (node) represents a member of the interactome. Each line between the nodes (edge) represents a detected protein-protein interaction (PPI) according to the STRING database.

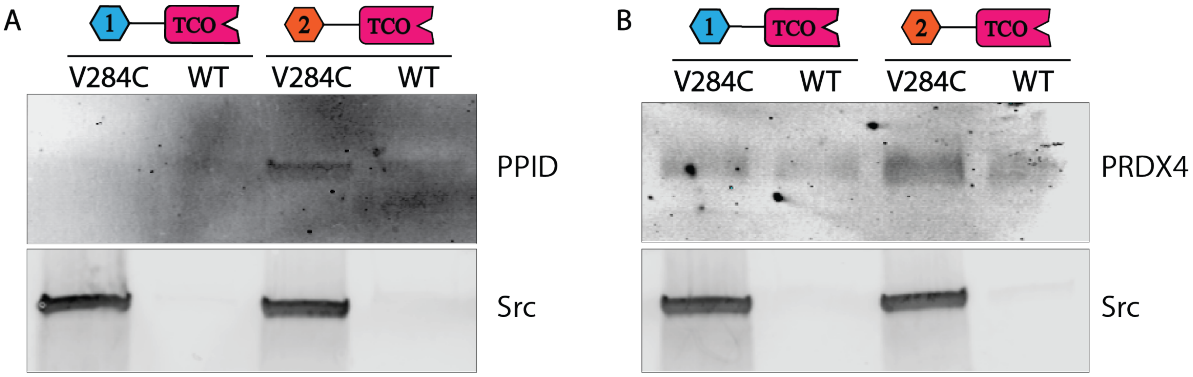


Figure S6. Interrogation of Src's interactome with co-clickable precipitation. (A) Western blot confirmation of PPID as an interactor with the **2-TCO**-bound Src V284C complex. An anti-PPID immunoblot of the elution (EL) is shown (*top*). An anti- Src immunoblot of the elution (EL) is also shown (*bottom*). (B) Western blot confirmation of PRDX4 as an interactor with the **2-TCO**-bound Src V284C complex. An anti-PRDX4 immunoblot of the elution (EL) is shown (*top*). An anti-Src immunoblot of the elution (EL) is also shown (*bottom*).

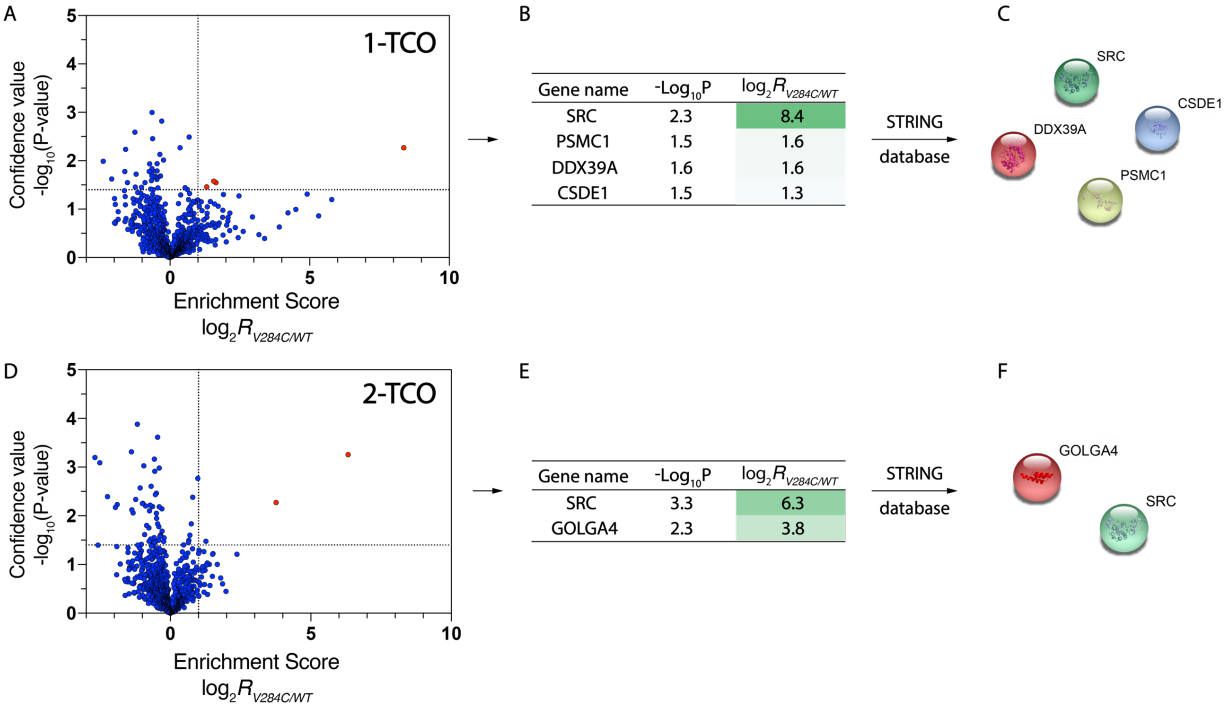
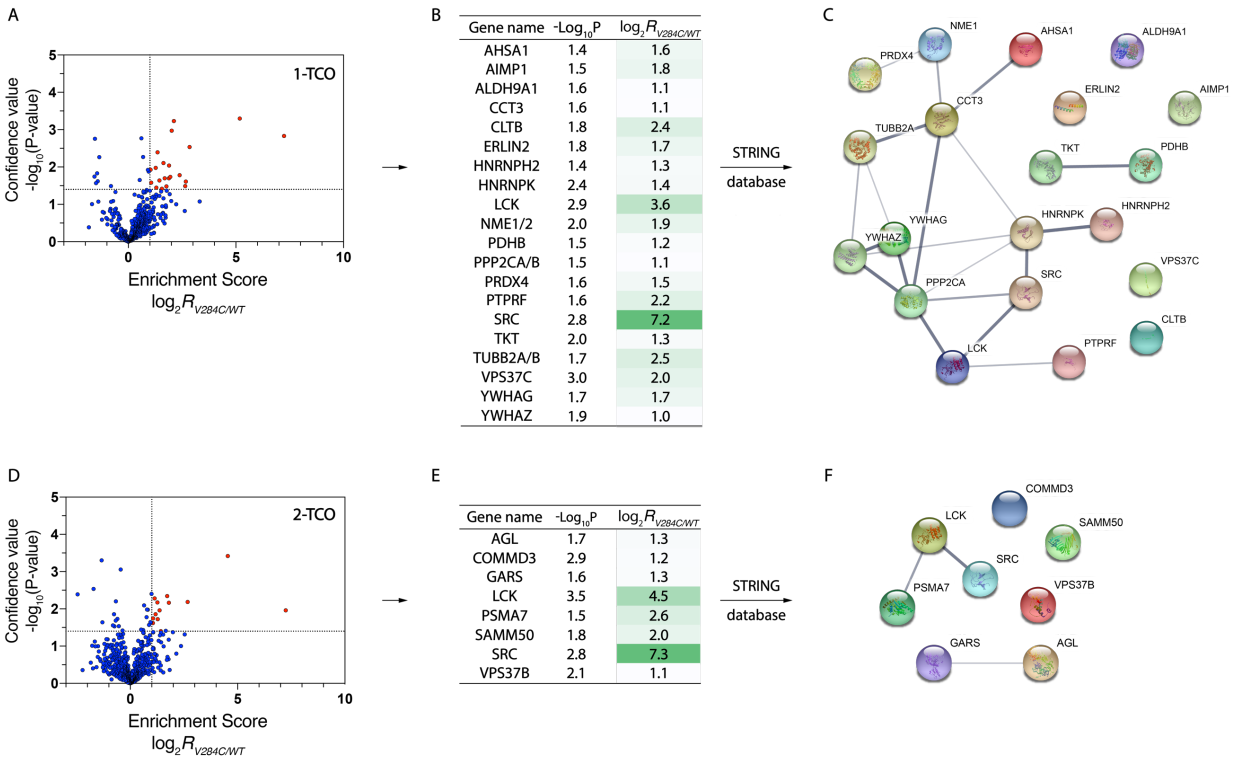


Figure S7. Characterization of **1-TCO**-bound and **2-TCO**-bound Src's interactome in serum-starved HeLa cells. (A, D) Volcano plot showing the $\log_2$ transformed MaxQuant intensities of the label-free comparison of **1-TCO**-treated (A, *top*) or **2-TCO**-treated (D, *bottom*), serum-starved HeLa cells expressing Src V284C or Src WT (n=3). Statistically significant interactors for drug-sensitized Src were defined as protein hits with enrichment scores = $\log_2$[Intensity (drug-sensitized target)/Intensity (WT target)] ≥ 1.0 and confidence values = $-\log_{10}$(P-value) ≥ 1.4. The calculation was based on intensity values computed by MaxQuant. Missing protein intensity values were imputed by Perseus with a distribution downshift of 1.3 and a width of 0.2. Specific Src interactors are highlighted in red and non-specific binders are marked in blue. (B, E) Proteins that significantly co-enriched with **1-TCO** (B, *top*) or **2-TCO** (E, *bottom*) from Src V284C-expressing, serum-starved HeLa cells. The second column shows confidence values ($-\log_{10}$(P-value)) and the third column shows enrichment scores. (C, F) STRING network of the interactome of **1-TCO**-bound (C, *top*) or **2-TCO**-bound (F, *bottom*) Src V284C. Significant interactors were visualized using the STRING database. Each circle (node) represents a member of the interactome.



Gene name	-Log$_{10}$P	log$_2$R$_{V284C/WT}$
AHSA1	1.4	1.6
AIMP1	1.5	1.8
ALDH9A1	1.6	1.1
CCT3	1.6	1.1
CLTB	1.8	2.4
ERLIN2	1.8	1.7
HNRNPH2	1.4	1.3
HNRNPK	2.4	1.4
LCK	2.9	3.6
NME1/2	2.0	1.9
PDHB	1.5	1.2
PPP2CA/B	1.5	1.1
PRDX4	1.6	1.5
PTPRF	1.6	2.2
SRC	2.8	7.2
TKT	2.0	1.3
TUBB2A/B	1.7	2.5
VPS37C	3.0	2.0
YWHAG	1.7	1.7
YWHAZ	1.9	1.0

Gene name	-Log$_{10}$P	log$_2$R$_{V284C/WT}$
AGL	1.7	1.3
COMMD3	2.9	1.2
GARS	1.6	1.3
LCK	3.5	4.5
PSMA7	1.5	2.6
SAMM50	1.8	2.0
SRC	2.8	7.3
VPS37B	2.1	1.1

Figure S8. Characterization of **1-TCO**-bound and **2-TCO**-bound Src's interactome in Hela cells cultured in complete medium (DMEM). (A, D) Volcano plot showing the $\log_2$ transformed MaxQuant intensities of the label-free comparison of **1-TCO**-treated (A, *top*) or **2-TCO**-treated (D, *bottom*) HeLa cells expressing Src V284C or Src WT (n=3) cultured in complete medium (DMEM). Statistically significant interactors for drug-sensitized Src were defined as protein hits with enrichment scores = $\log_2$[Intensity (drug-sensitized target)/Intensity (WT target)] ≥ 1.0 and confidence values = $-\log_{10}$(P-value) ≥ 1.4. The calculation was based on intensity values computed by MaxQuant. Missing protein intensity values were imputed by Perseus with a distribution downshift of 1.3 and a width of 0.2. Specific Src interactors are highlighted in red and non-specific binders are marked in blue. (B, E) Proteins that significantly co-enriched with **1-TCO** (B, *top*) or **2-TCO** (E, *bottom*) from Src V284C-expressing HeLa cells cultured in complete medium (DMEM). The second column shows confidence values ($-\log_{10}$(P-value)) and the third column shows enrichment scores. (C, F) STRING network of the interactome of **1-TCO**-bound (C, *top*) or **2-TCO**-bound (F, *bottom*) Src V284C. Significant interactors were visualized using the STRING database. Each circle (node) represents a member of the interactome. Each line between the nodes (edge) represents a detected protein-protein interaction (PPI) according to the STRING database.

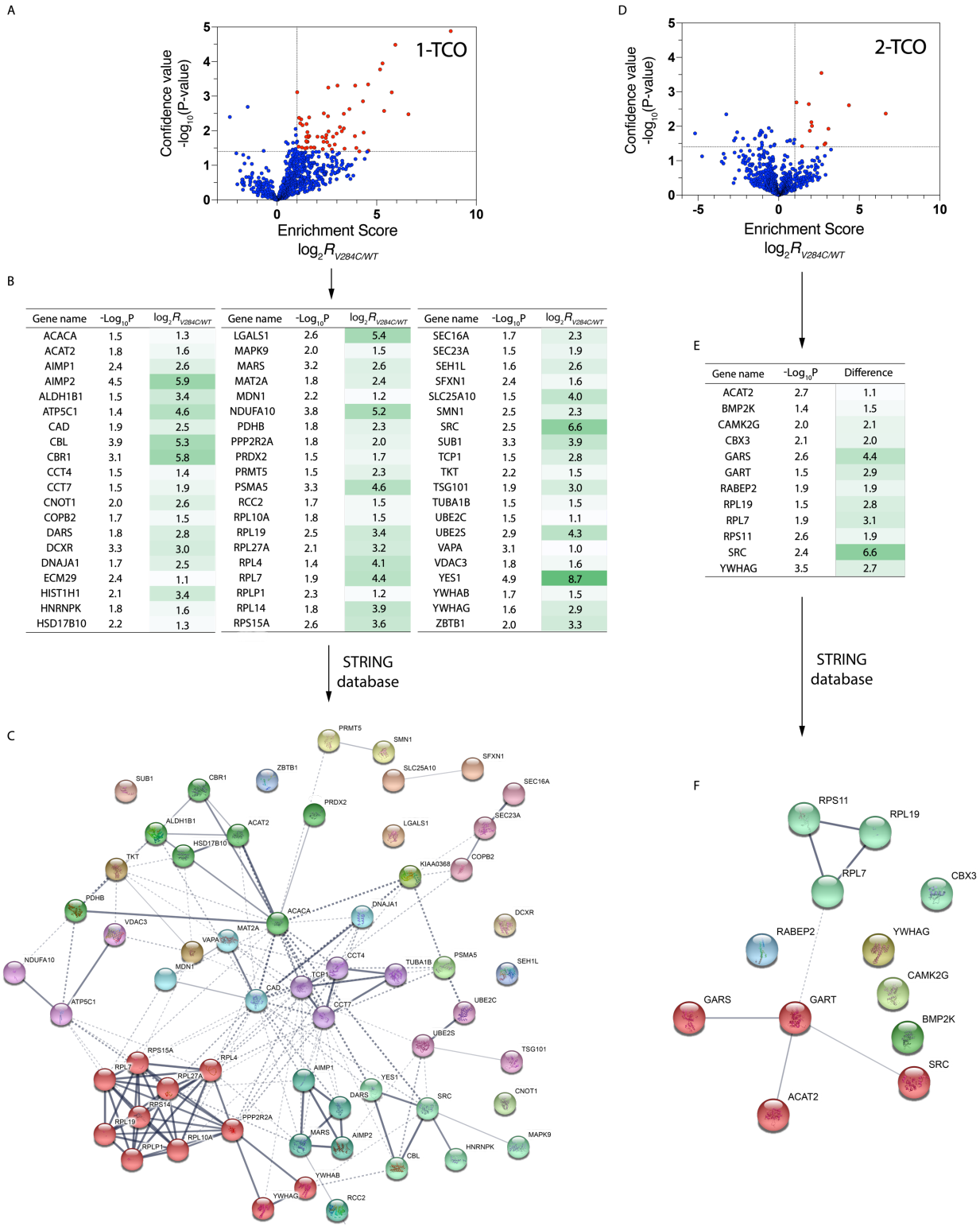


Figure S9. Characterization of **1-TCO**-bound and **2-TCO**-bound Src's interactome in HeLa cells plated on fibronectin. (A, D) Volcano plot showing the $\log_2$ transformed MaxQuant intensities of the label-free comparison of **1-TCO**-treated (A, *left*) or **2-TCO**-treated (D, *right*) HeLa cells expressing Src V284C or Src WT (n=3) plated on fibronectin. Statistically significant interactors for drug-sensitized Src were defined as protein hits with enrichment scores = $\log_2$[Intensity (drug-

sensitized target)/Intensity (WT target)] ≥ 1.0 and confidence values = $-\log_{10}$(P-value) ≥ 1.4. The calculation was based on intensity values computed by MaxQuant. Missing protein intensity values were imputed by Perseus with a distribution downshift of 1.3 and a width of 0.2. Specific Src interactors are highlighted in red and non-specific binders are marked in blue. (B, E) Proteins that significantly co-enriched with **1-TCO** (B, *left*) or **2-TCO** (E, *right*) from Src V284C-expressing HeLa cells plated on fibronectin. The second column shows confidence values ($-\log_{10}$(P-value)) and the third column shows enrichment scores. (C, F) Significant interactors were visualized using the STRING database. Each circle (node) represents a member of the interactome. Each line between the nodes (edge) represents a detected protein-protein interaction (PPI) according to the STRING database.

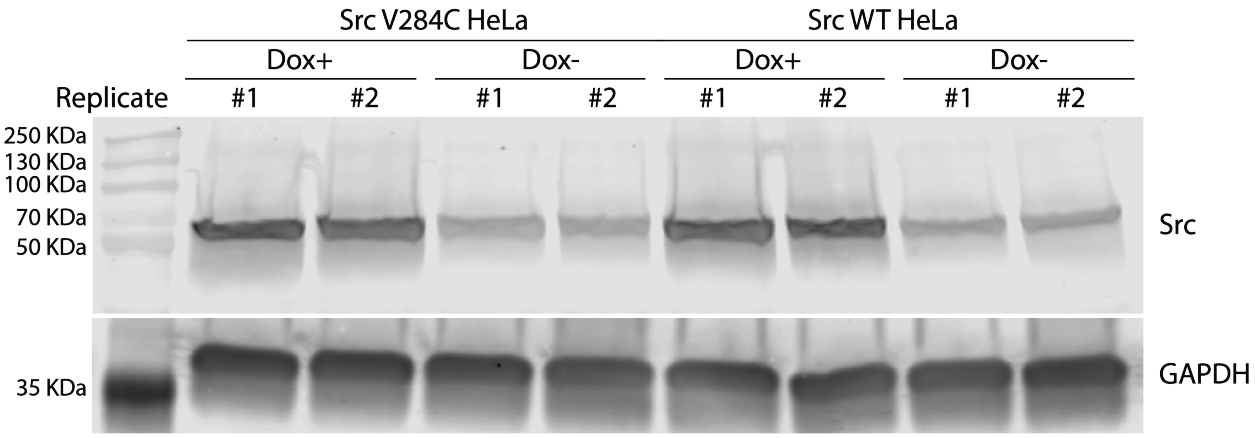


Figure S10. Anti-Src immunoblots of HeLa cells stably expressing Src V284C or Src WT (*n=2*). Src V284C and Src WT expression was induced with 1 μg/mL of doxycycline for 24 hours prior to performing PLA studies.

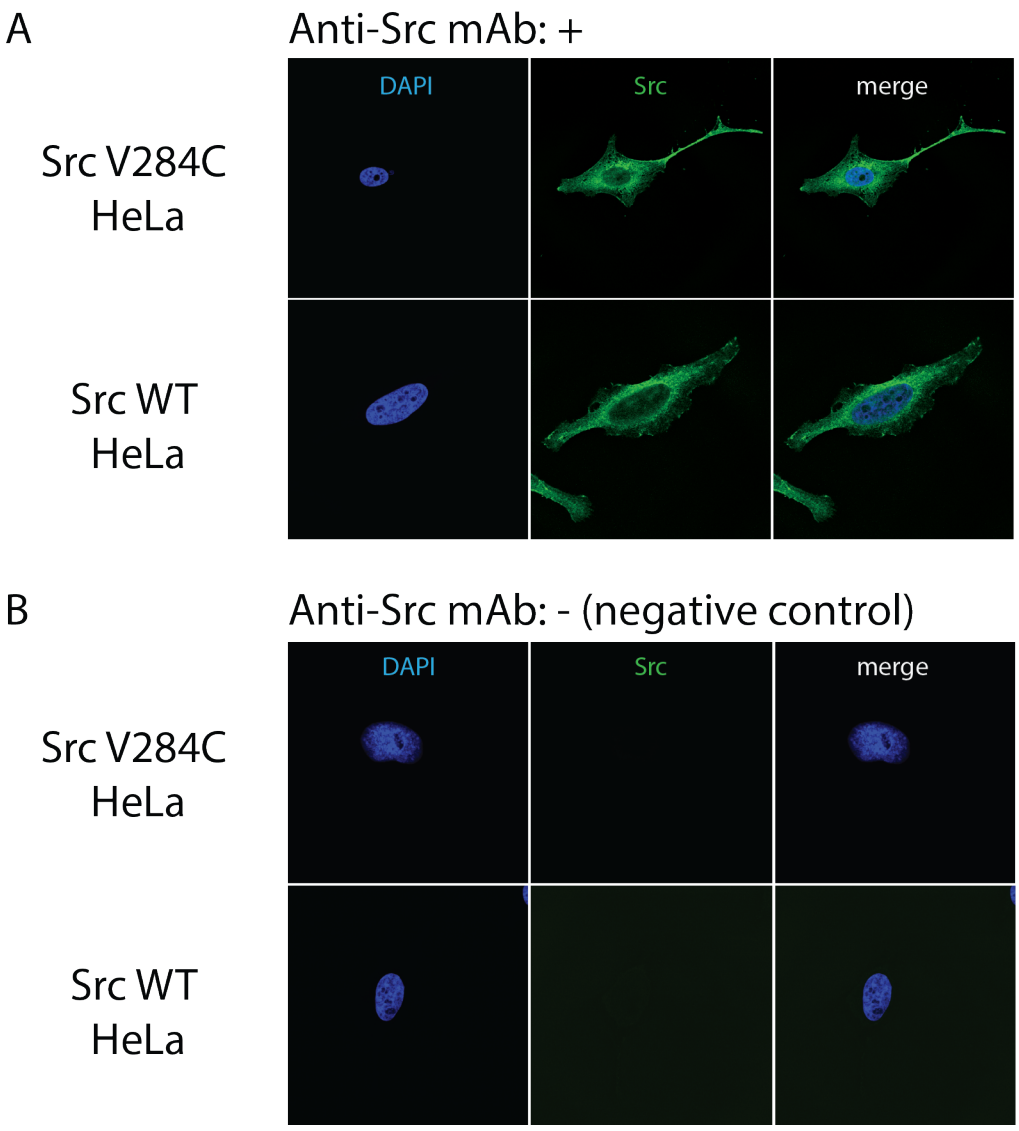


Figure S11. Cellular localization of Src V284C and Src WT in HeLa cells prior to treatment with TCO probes. (A) Immunofluorescent analysis of Src V284C (*top*) and Src WT (*bottom*) in HeLa cells. HeLa cells stably expressing Src V284C (*top*) or Src WT (*bottom*) were fixed and incubated with an anti-Src antibody, followed by a Mega 485-conjugated secondary antibody. The nucleus was visualized using DAPI. (B) Negative control of immunofluorescent analysis. HeLa cells stably expressing Src V284C (*top*) or Src WT (*bottom*) were fixed and incubated with only a Mega 485-conjugated secondary antibody. The nucleus was visualized using DAPI.

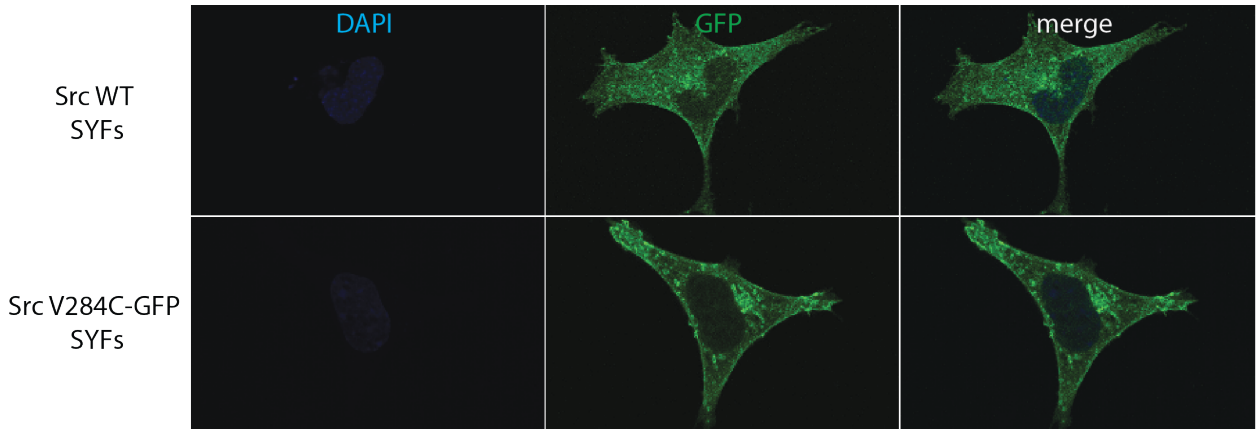


Figure S12. Cellular localization of C-terminal GFP-tagged full-length Src WT (*top*) and Src V284C (*bottom*) in Src/Yes/Fyn (-/-/-) fibroblast cells (SYFs). SYFs cells that transiently express Src WT-GFP (*top*) or Src V284C-GFP (*bottom*) were fixed with 4% para-formaldehyde. The nucleus was visualized using DAPI. GFP-tagged Src constructs were visualized under GFP channel (excitation: 488 nm; emission: 510 nm).

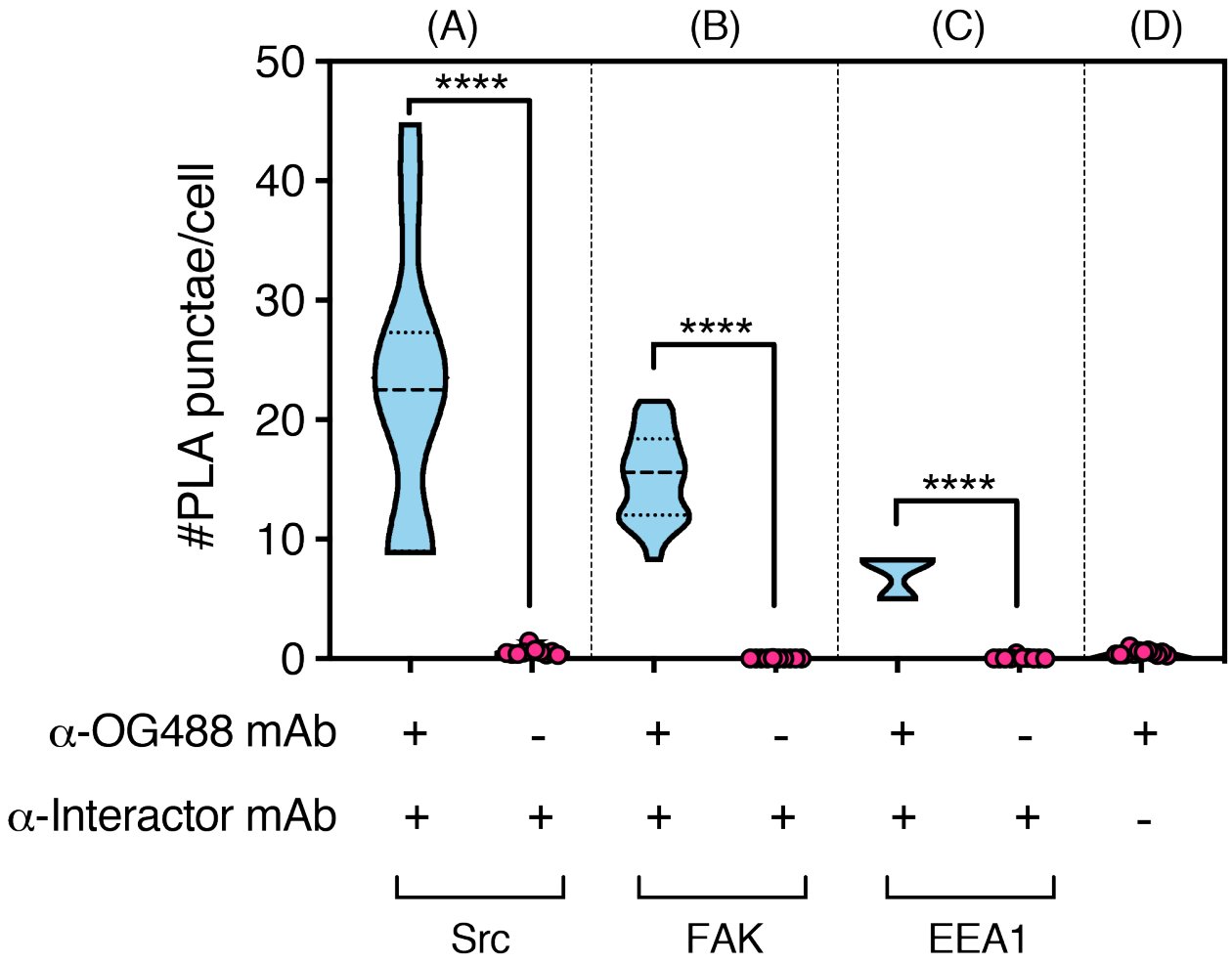


Figure S13. Technical negative controls of proximity ligation assays (PLAs) in HeLa cells treated by **1-TCO**. (A) Technical negative control of PLA for visualization of the probe-bound target. Quantified signals for the number of PLA-mediated fluorescent puncta observed per cell are shown in the violin plot. Values shown are the means of fluorescent puncta per cell from at least three independent experiments (number of total cells quantified for control experiments: *n*=308). (B) Technical negative control of the Src/FAK interaction PLA. Quantified signals for the number of PLA-mediated fluorescent puncta observed per cell are shown in the violin plot. Values shown are the means of fluorescent puncta per cell from at least three independent experiments. (number of total cells quantified for control experiments: *n*=464). (C) Technical negative control of the cellular organelle localization PLA. Quantified signals for the number of PLA-mediated fluorescent puncta observed per cell are shown in the violin plot. Values shown are the means of fluorescent puncta per cell from at least three independent experiments. (number of total cells quantified for control experiments: *n*=209). (D) Technical negative control of PLA with the anti-Oregon Green488 antibody. Quantified signals for the number of PLA-mediated fluorescent puncta observed per cell are shown in the violin plot. Values shown are the means of fluorescent puncta per cell from at least three independent experiments. (number of total cells quantified for control experiments: *n*=263).

Materials and Reagents

Lysis Buffer and Washing Buffer 1: [Tris]= 50 mM, pH= 7.8; [NaCl]= 120 mM; [NaF]= 10 mM; [Na_3VO_4]= 1 mM; [EDTA]= 1 mM; [IGEPAL CA-630]= 1% (by volume); Protease Inhibitor Tablet (Pierce)

Washing Buffer 2: [Tris]= 50 mM, pH= 7.8; [NaCl]= 120 mM; [EDTA]= 1 mM.

Denaturing Buffer: [Tris]= 50 mM, pH= 8.5; [Guanidinium Chloride]= 6 M; [TCEP]= 5 mM (add freshly); [CAM]= 10 mM (add freshly).

Pierce Protease Inhibitor Tablets (Thermo Scientific; Catalog number: A32963)

NHS-Activated Sepharose 4 Fast Flow (GE Healthcare; Catalog number: 17090601)

Pierce 660 nm Protein Assay (Thermo Scientific)

Lys-c Lysyl Endopeptidase, MS Grade (Wako)

MS Grade Trypsin (Pierce)

Grace Bio-labs CultureWell chambered coverglass (8 wells, well diam. × thickness 6 mm × 1 mm, well volume 15-30 µL; Sigma-Aldrich, cat. no. GBL103380)

Antibodies:

Anti-Src antibody (Cell Signaling Technology, cat. no. #2109)

Anti-FAK antibody (Cell Signaling Technology, cat. no. #71433)

Anti-c-Cbl antibody (Cell Signaling Technology, cat. no. #2747)

Anti-EGFR antibody (Cell Signaling Technology, cat. no. #4267)

Anti-EEA1 antibody (Cell Signaling Technology, cat. no. #3288)

Anti-Oregon Green488 antibody (Thermo Fisher, cat. no. A-11095)

Anti-His Tag antibody (Abcam, cat. no. G020)

Anti-DYKDDDDK Tag antibody (Cell Signaling Technology, cat. no. #2368)

Anti-SAPK/JNK antibody (Cell Signaling Technology, cat. no. #9252)

Anti-PPID antibody (ABclonal, cat. no. #A6949)

Anti-PRDX4 antibody (ABclonal, cat.no. #A1486)

Anti-GAPDH antibody (Abcam, cat. no. 9485)

Duolink *in situ* mounting medium with DAPI (Sigma-Aldrich, cat. no. DUO82040)

Duolink *in situ* PLA probe anti-rabbit PLUS (Sigma-Aldrich, cat. no. DUO92002)

Duolink *in situ* PLA probe anti-goat MINUS (Sigma-Aldrich, cat. no. DUO92006)

Duolink *in situ* detection reagents orange (Sigma-Aldrich, cat. no. DUO92007)

Duolink *in situ* wash buffers, Fluorescence (Sigma-Aldrich, cat. no. DUO82049)

Generation of Stable HeLa Cell Line

Doxycycline-inducible HeLa cells expressing Src V284C or Src WT were generated using Flp-In T-REx HeLa cells (a generous gift from Stephen S. Taylor, University of Manchester). Flp-In T-REx HeLa cells were co-transfected with Src V284C-pcDNA5/FRT/TO or Src WT-pcDNA5/FRT/TO vector, and pOG44 Flp recombinase vector (Thermo) (1:2= pcDNA5/FRT/TO:pOG) using Lipofectamine (1 μg of DNA: 3 μL of Lipofectamine). Stably transfected HeLa cells were selected with 200 μg/mL of hygromycin for 7–10 d. After induction with 1 μg/mL of doxycycline, expression of the desired transgenes was verified using Western blot analysis.

Inserted gene of Src V284C:

MGSNKSKPKDASQRRRSLEPAENVHGAGGGAFPASQTPSKPASADGHRGPSAAFAPAA
AEPKLFGGFNSSDTVTSPQRAGPLAGGVTTFVALYDYESRTETDLSFKKGERLQIVNNTE
GDWWLAHSLSTGQTGYIPSNYVAPSDSIQAEEWYFGKITRRESERLLLNAENPRGTFLV
RESETTKGAYCLSVSDFDNAKGLNVKHYKIRKLDSGGFYITSRTQFNSLQQLVAYYSKH
ADGLCHRLTTVCPTSKPQTQGLAKDAWEIPRESLRLEVKLGQGCFGECWMGTWNGTTR
VAIKTLKPGTMSPEAFLQEAQVMKKLRHEKLVQLYAVVSEEPIYIVTEYMSKGSLLDFL
KGETGKYLRLPQLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENLVCKVADFGL
ARLIEDNEYTARQGAKFPIKWTAPEAALYGRFTIKSDVWSFGILLTELTTKGRVPYPGMV
NREVLDQVERGYRMPCPPECPESLHDLMCQCWRKEPEERPTFEYLQAFLEDYFTSTEPQ
YQPGENL*

Inserted gene of Src WT:

MGSNKSKPKDASQRRRSLEPAENVHGAGGGAFPASQTPSKPASADGHRGPSAAFAPAA

AEPKLFGGFNSSDTVTSPQRAGPLAGGVTTFVALYDYESRTETDLSFKKGERLQIVNNTE

GDWWLAHSLSTGQTGYIPSNYVAPSDSIQAEEWYFGKITRRESERLLLNAENPRGTFLV

RESETTKGAYCLSVSDFDNAKGLNVKHYKIRKLDSGGFYITSRTQFNSLQQLVAYYSKH

ADGLCHRLTTVCPTSKPQTQGLAKDAWEIPRESLRLEVKLGQGCFGEVWMGTWNGTT

RVAIKTLKPGTMSPEAFLQEAQVMKKLRHEKLVQLYAVVSEEPIYIVTEYMSKGSLLDF

LKGETGKYLRLPQLVDMAAQIASGMAYVERMNYVHRDLRAANILVGENLVCKVADFG

LARLIEDNEYTARQGAKFPIKWTAPEAALYGRFTIKSDVWSFGILLTELTTKGRVPYPGM

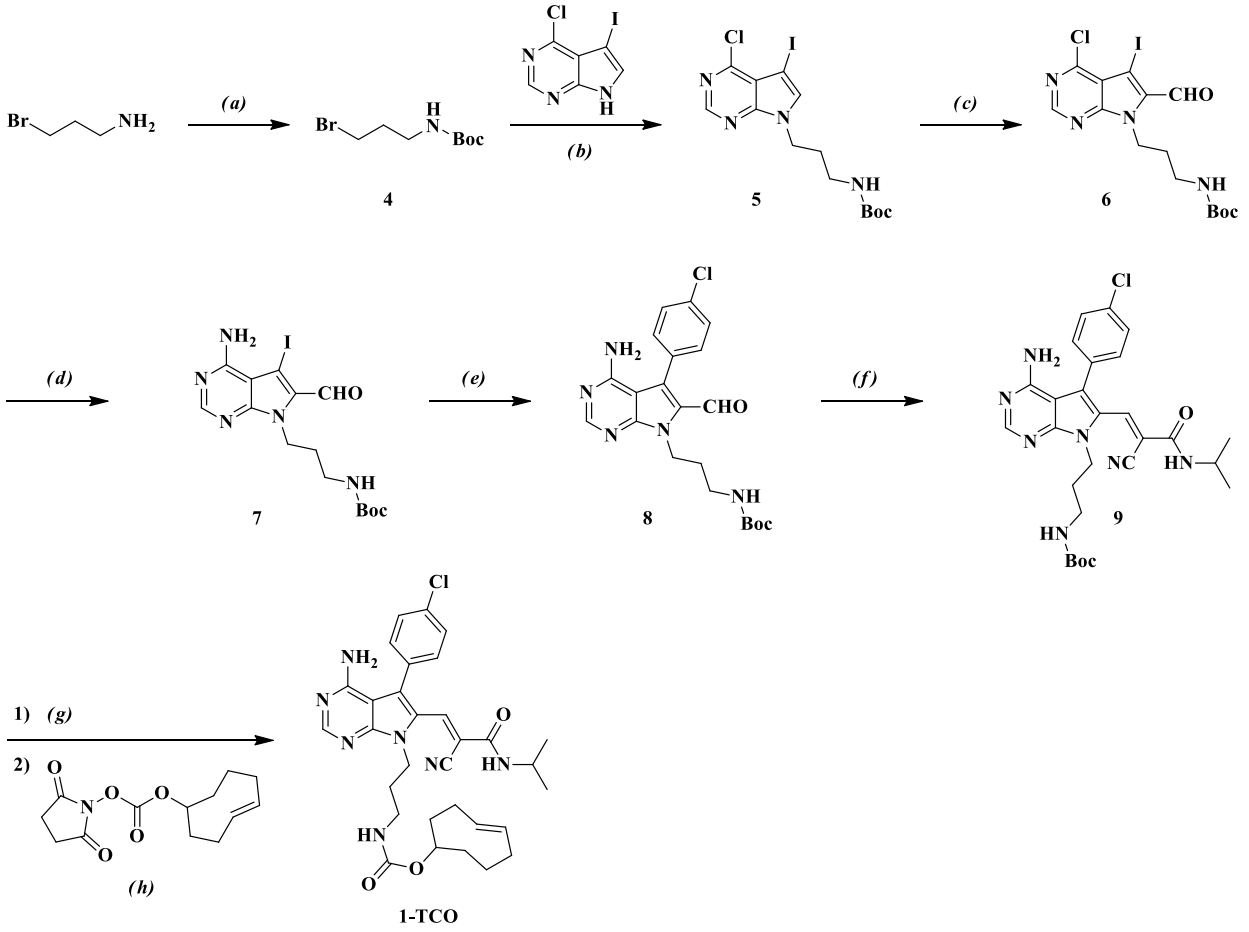
VNREVLDQVERGYRMPCPPECPESLHDLMCQCWRKEPEERPTFEYLQAFLEDYFTSTEP

QYQPGENL*

Synthesis of TCO probes (1-TCO, 2-TCO, and 3-TCO)

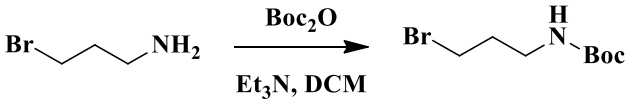
All chemicals purchased from commercial suppliers were used without further purification unless otherwise stated. Reactions were monitored with thin-layer chromatography (TLC) using silica gel 60 F254 coated glass plates (EM Sciences). Compound purification was performed with an IntelliFlash 280 automated flash chromatography system using pre-packed Varian SuperFlash silica gel columns (Hexane/EtOAc or CH_2Cl_2/MeOH gradient solvent). A Varian Dynamax Microsorb 100-5 C18 column (250 mm x 21.4 mm), eluting with H_2O/CH_3CN or H_2O/ MeOH gradient solvent (+0.05% TFA), was used for preparatory HPLC purification. The purity of all final compounds was determined by analytical HPLC with an Agilent ZORBAX SB-C18 (2.1 mm x 150 mm) or Varian Microsorb-MV 100-5 C18 column (4.6 mm x 150 mm), eluting with either H_2O/CH_3CN or H_2O/MeOH gradient solvent (+0.05% TFA). Elution was monitored by a UV detector at λ= 220 nm and λ= 254 nm, with all final compounds displaying >95% purity. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker 300 or 500 MHz NMR spectrometers at ambient temperature. Chemical shifts were reported in parts per million (ppm) and coupling constants in hertz (Hz). ^{1}H-NMR spectra were referenced to the residual solvent peaks as internal standards (7.26 ppm for $CDCl_3$, 2.50 ppm for d6-DMSO, and 3.34 ppm for CD_3OD). Mass spectra were recorded with a Bruker Esquire Liquid Chromatograph - Ion Trap Mass Spectrometer.

Synthesis of 1-TCO



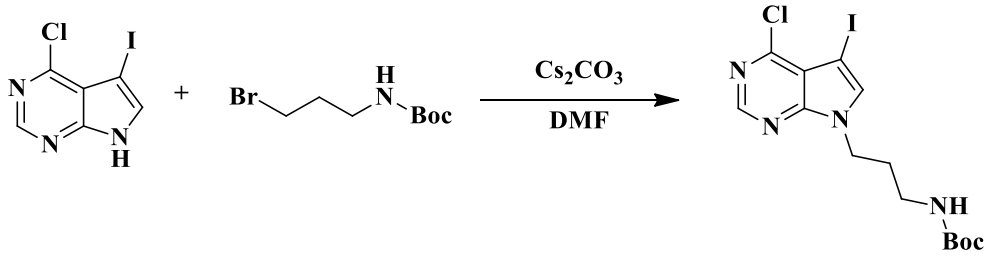
Reaction scheme for **1-TCO**: (a) Boc_2O, Et_3N, DCM, 0 °C-rt, 95%; (b) Cs_2CO_3, DMF, 50 °C, 14 hours, 59%; (c) Diisopropylamine, *n*-BuLi, ethyl formate, THF, -78 °C-rt, overnight, 22%; (d) Ammonium hydroxide, 1,4-Dioxane, 40 °C, 3 hours, 43%; (e) 4-Chlorophenylboronic acid, K_3PO_4, $Pd(dppf)_2Cl_2$, 1,4-Dioxane, H_2O, 90 °C, 130 mins (microwave), 74%; (f) 2-Cyano-*N*-isopropylacetamide, DBU, THF, rt, 6 hours, 50%; (g) TFA, DCM, rt, 30 mins; (h) DIPEA, THF, rt, overnight, 60%.

***Tert*-butyl (3-bromopropyl)carbamate (4)**



3-Bromopropylamine hydrobromide (5.3 g, 24 mmol, 1.0 equiv.) and triethylamine (2.7 g, 26 mmol, 3.6 mL, 1.1 equiv.) were dissolved in anhydrous dichloromethane (50 mL) and cooled to 0˚C in a 250-mL round bottom flask under nitrogen. A solution of di-*tert*-butyl dicarbonate (5.3 g, 24 mmol, 1.0 equiv.) in dichloromethane (40 mL) was added dropwise to the above solution over 30 minutes. The resulting reaction mixture was allowed to warm to room temperature overnight. The reaction was then quenched with aqueous sodium hydroxide solution (20 mL, 5% by weight). The organic phase was washed with water (20 mL), brine (20 mL) and then dried over anhydrous sodium sulfate. The solvent was removed in vacuo and afforded *tert*-butyl (3-bromopropyl)carbamate as a white solid (5.4 g, 95%). The crude product was used in further steps without purification. ^{1}H-NMR (300 MHz, $CDCl_3$) δ= 4.64 (s, 1H), 3.44 (t, J = 6.5 Hz, 2H), 3.33 – 3.22 (m, 2H), 2.12 – 1.99 (m, 2H), 1.45 (s, 9H); MS (ESI, m/z) calculated for $C_8H_{16}BrNO_2$ 237.0, $[M+H]^+$ found 238.8.

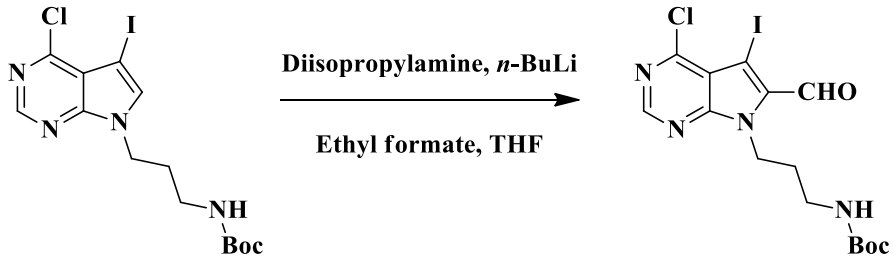
***Tert*-butyl (3-(4-chloro-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (5)**



4-Chloro-5-iodo-7H-pyrrolo[2,3-d]pyrimidine (1.7 g, 6.2 mmol, 1.0 equiv.), *tert*-butyl (3-bromopropyl)carbamate (2.0 g, 8.4 mmol, 1.4 equiv.) and cesium carbonate (6.7 g, 12.4 mmol, 2.0

equiv.) were dissolved in dimethylformamide (20 mL) in a 100-mL round bottom flask under nitrogen. The reaction mixture was heated at 50 ˚C for 14 hours. The solvent was then removed in *vacuo* and the resulting solid residue was redissolved in ethyl acetate (200 mL). The organic phase was washed with aqueous ammonium chloride solution (40 mL), aqueous sodium bicarbonate solution (40 mL), brine (40 mL), dried over anhydrous sodium sulfate and concentrated in *vacuo*. Purification with Flash chromatography afforded *tert*-butyl (3-(4-chloro-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a pale yellow solid (1.6 g, 59%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ= 8.62 (s, 1H), 7.45 (d, J = 14.2 Hz, 1H), 5.01 (s, 1H), 4.33 (t, J = 6.6 Hz, 2H), 3.11 – 3.03 (m, 2H), 2.06 – 1.97 (m, 2H), 1.45 (s, 9H); MS (ESI, m/z) calculated for $C_{14}H_{18}ClIN_4O_2$ 436.0, $[M+H]^+$ found 437.1.

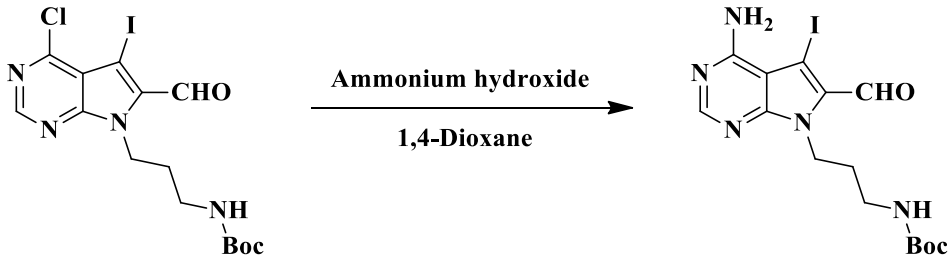
Tert-butyl (3-(4-chloro-6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (6)



In a flame-dried 100-mL round bottom flask, diisopropylamine (228 mg, 316 µL, 2.25 mmol, 1.75 equiv.) was dissolved in dry THF (10 mL) and cooled to -78˚C under nitrogen. *n*-BuLi solution (2.5 M in hexane, 901 µL, 2.25 mmol, 1.75 equiv.) was added dropwise to the above solution at -78 ˚C. The reaction was stirred at 0 ˚C for 1 hour and then cooled to -78 ˚C. A solution of *tert*-butyl (3-(4-chloro-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (562 mg, 1.29 mmol, 1.00 equiv.) in dry THF (5 mL) was added dropwise at -78 ˚C. After 1 hour, ethyl formate

(200 mg, 218 µL, 2.70 mmol, 2.10 equiv.) was added dropwise at -78 ˚C. The reaction mixture was allowed to warm up to room temperature overnight. The reaction was quenched by adding aqueous ammonium chloride solution (2 mL) and diluted with ethyl acetate (150 mL). The organic phase was washed with aqueous sodium bicarbonate solution (20 mL), brine (20 mL), dried over anhydrous sodium sulfate and concentrated in *vacuo*. Purification by flash chromatography on silica gel (EtOAc:Hexane= 0:100 to EtOAc: Hexane= 1:2) afforded *tert*-butyl (3-(4-chloro-6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a pale brown powder (130 mg, 22%; R_f= 0.57 in EtOAc:Hexane= 3:2). ^{1}H -NMR (300 MHz, $CDCl_3$) δ= 10.08 (s, 1H), 8.77 (s, 1H), 5.12 (s, 1H), 4.72 (t, *J* = 6.6 Hz, 2H), 3.14 – 2.96 (m, 2H), 2.03 – 1.89 (m, 2H), 1.45 (s, 9H); MS (ESI, m/z) calculated for $C_{15}H_{18}ClIN_4O_3$ 464.0, $[M+H]^+$ found 465.0.

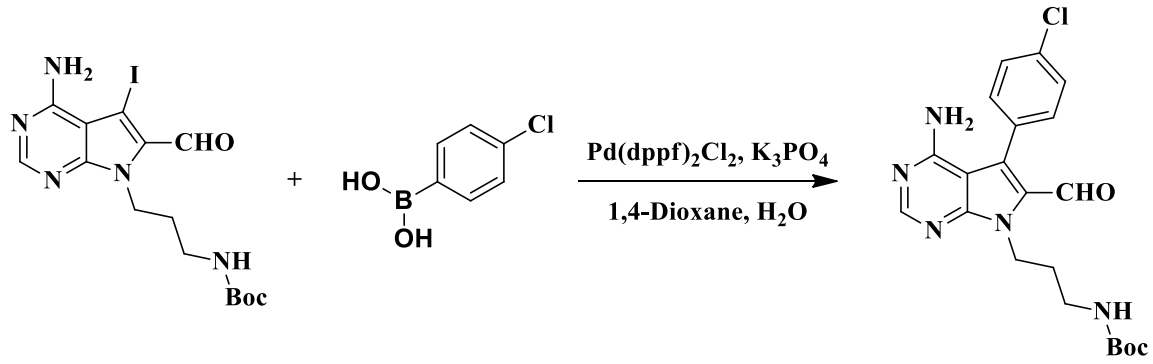
***Tert*-butyl (3-(4-amino-6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (7)**



In a 10-mL pressure tube, *tert*-butyl (3-(4-chloro-6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (130 mg, 0.28 mmol, 1.0 equiv.) was dissovled in 1,4-Dioxane (1.2 mL). To it was added aqueous ammonium hydroxide solution (1.2 mL, 28.0 %- 30.0 % NH_3 basis). The resulting solution was heated at 40 ˚C for 3 hours and then diluted with ethyl acetate (40 mL). The organic phase was washed with 1 M hydrochloride aqueous solution, sodium bicarbonate, brine and dried over anhydrous sodium sulfate. Purification on silica gel using a gradiant of MeOH:

DCM= 0:100 to MeOH: DCM= 10:90 afforded *tert*-butyl (3-(4-amino-6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a pale brown solid (54 mg, 43%; R_f= 0.51 in MeOH:DCM= 1:9). ^{1}H-NMR (300 MHz, $CDCl_3$) δ= 9.82 (s, 1H), 8.38 (s, 1H), 6.04 (s, 2H), 5.50 (s, 1H), 4.63 (t, J = 6.4 Hz, 2H), 3.07 – 2.91 (m, 2H), 2.01 – 1.86 (m, 2H), 1.45 (s, 9H); MS (ESI, m/z) calculated for $C_{15}H_{20}IN_5O_3$ 445.1, $[M+H]^+$ found 446.3.

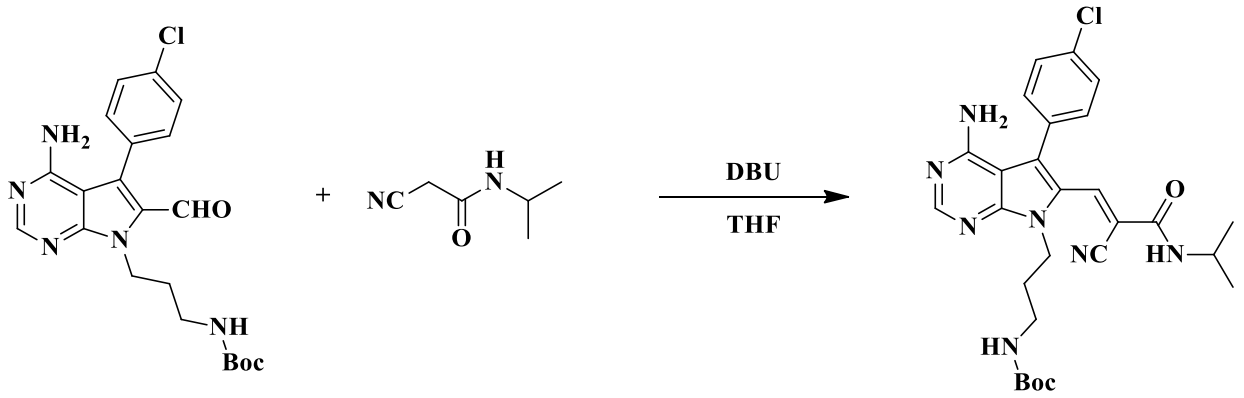
Tert-butyl(3-(4-amino-5-(4-chlorophenyl)-6-formyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (8)



In a 10-mL microwave reaction tube, tert-butyl (3-(4-amino-6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (54 mg, 0.12 mmol, 1.0 equiv.), 4-chlorophenylboronic acid (28 mg, 0.18 mmol, 1.5 equiv.), potassium phosphate (77 mg, 0.36 mmol, 3.0 equiv.) and [1,1′-Bis(diphenylphosphino)ferrocene]dichloropalladium(II) (9.9 mg, 0.012 mmol, 0.1 equiv.) were dissolved in a mixture of 1,4-Dioxane (1.0 mL) and water (0.25 mL). The resulting mixture was heated in a microwave reactor at 90 ˚C for 130 mins. The reaction was then quenched with saturated NH_4Cl solution (1 mL) and diluted with ethyl acetate (30 mL). The organic phase was washed with saturated NaHCO3 solution (5 mL), brine (5 mL), dried over anhydrous Na_2SO_4 and concentrated in *vacuo*. Purification using flash chromatography on silica gel with a EtOAc/Hexane gradient (0:100 to 90:10) afforded *tert*-butyl (3-(4-amino-5-(4-chlorophenyl)-6-formyl-7*H*-

pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a pale yellow solid (39 mg, 74%; Rf= 0.31 in EtOAc:Hexane= 9:1).^{1}H-NMR (300 MHz, $CDCl_3$) δ= 9.60 (s, 1H), 8.41 (s, 1H), 7.58 – 7.42 (m, 4H), 5.62 (s, 1H), 5.32 (s, 2H), 4.70 (t, *J* = 6.3 Hz, 2H), 3.19 – 2.94 (m, 2H), 2.06 – 1.96 (m, 2H), 1.46 (s, 9H); MS (ESI, m/z) calculated for $C_{21}H_{24}ClN_5O_3$ 429.2, $[M+H]^+$ found 430.1.

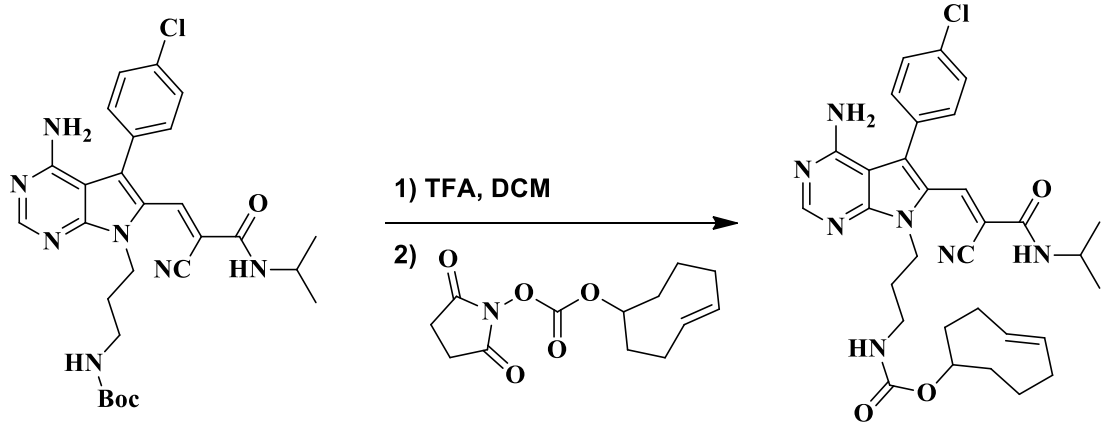
(*Z*)- or (*E*)-*tert*-butyl (3-(4-amino-5-(4-chlorophenyl)-6-(2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (9)



Tert-butyl (3-(4-amino-5-(4-chlorophenyl)-6-formyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (20 mg, 0.048 mmol, 1.0 equiv.) and 2-cyano-*N*-isopropylacetamide (9.2 mg, 0.072 mmol, 1.5 equiv.) were dissolved in tetrahydrofuran (300 μL). To the above solution was added 1,8-diazabicyclo[5.4.0]undec-7-ene (15 mg, 14 μL, 0.096 mmol, 2.0 equiv). The reaction mixture was stirred at room temperature for 6 hours. The reaction was then quenched with saturated NH_4Cl solution (1 mL) and diluted with ethyl acetate (30 mL). The organic layer was washed with saturated NaHCO3 solution (5 mL), brine (5 mL), dried over anhydrous Na_2SO_4 and concentrated in *vacuo*. Purification using preparative thin-layer chromatography developed by pure EtOAc rendered (*Z*)- or (*E*)-*tert*-butyl (3-(4-amino-5-(4-chlorophenyl)-6-(2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a

bright yellow powder (13 mg, 50%; Rf [(E)- and (Z)-isomers)]= 0.25 in EtOAc:Hexane= 9:1). ^{1}H-NMR (300 MHz, MeOD) δ= 8.30 – 8.18 (m, 3H), 7.70 (s, 1H), 7.64 – 7.36 (m, 8H), 6.68 (s, 2H), 5.53 (s, 2H), 5.16 (s, 4H), 4.43 (t, *J* = 6.9 Hz, 2H), 4.34 (t, *J* = 7.0 Hz, 2H), 4.19 – 4.04 (m, 1H), 3.71 – 3.54 (m, 1H), 3.15 – 2.97 (m, 4H), 2.06 – 1.92 (m, 4H), 1.46 (s, 9H), 1.33 (s, 9H), 1.24 (d, *J* = 6.6 Hz, 6H), 1.05 (d, *J* = 6.6 Hz, 6H); MS (ESI, m/z) calculated for $C_{27}H_{32}ClN_7O_3$ 537.2, $[M+H]^+$ found 538.1.

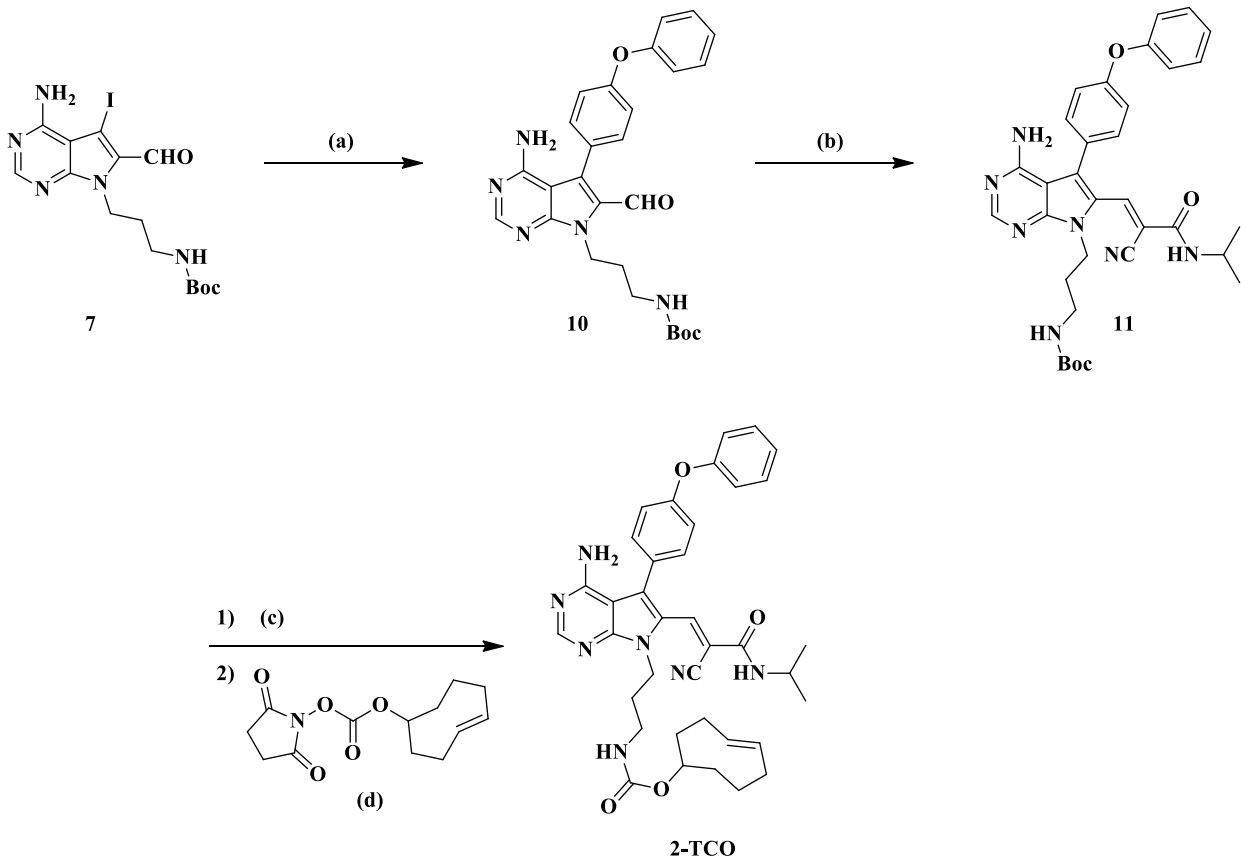
(*E*)-cyclooct-4-en-1-yl (3-(4-amino-5-(4-chlorophenyl)-6-((*E*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate or (*E*)-cyclooct-4-en-1-yl (3-(4-amino-5-(4-chlorophenyl)-6-((*Z*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (1-TCO)



(*Z*)- or (*E*)-*tert*-butyl (3-(4-amino-5-(4-chlorophenyl)-6-(2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (13 mg, 0.024 mmol, 1.0 equiv.) was dissolved in a mixture of trifluoacetic acid (0.30 mL) and dichloromethane (0.70 mL). The reaction solution was stirred at room temperature for 30 mins and concentrated in *vacuo*. The solid residue was dissolved in anhydrous tetrahydrofuran (400 μL). Then diisopropylethylamine (7.8 mg, 11 μL, 0.060 mmol, 2.5 equiv.) and (*E*)-cyclooct-4-en-1-yl (2,5-dioxopyrrolidin-1-yl)

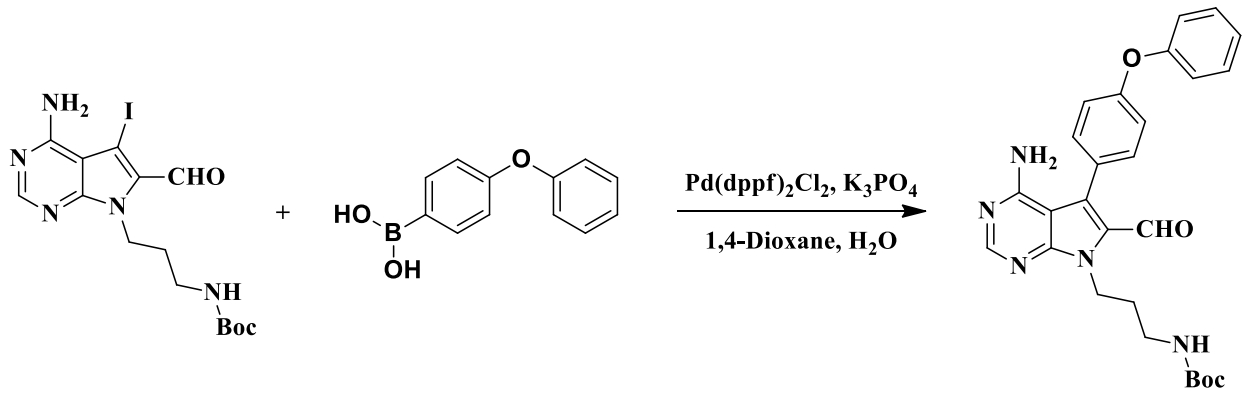
carbonate (8.4 mg, 0.031 mmol, 1.3 equiv.) were added to the above solution sequentially. The resulting mixture was stirred at room temperature overnight and quenched with water (1 mL) and diluted with ethyl acetate (30 mL). The organic layer was washed with saturated $NaHCO_3$ solution (5 mL), brine (5 mL), dried over anhydrous Na_2SO_4 and concentrated in *vacuo*. Purification using flash chromatography with a EtOAc/Hexane gradient (0:100 to 90:10) afforded a mixture of (*E*)-cyclooct-4-en-1-yl (3-(4-amino-5-(4-chlorophenyl)-6-((*E*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate or (*E*)-cyclooct-4-en-1-yl (3-(4-amino-5-(4-chlorophenyl)-6-((*Z*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a bright yellow powder (8.6 mg, 60% for 2 steps; R_f [(*E*)- and (*Z*)-isomers)]= 0.33 or 0.26 in EtOAc:Hexane= 9:1) ^{1}H-NMR (300 MHz, MeOD) δ= 8.26 – 8.16 (m, 3H), 7.66 (s, 1H), 7.60 – 7.34 (m, 8H), 6.77 (s, 2H), 5.76 – 5.36 (m, 4H), 5.13 (s, 4H), 4.45 – 4.21 (m, 4H), 4.18 – 4.00 (m, 1H), 3.70 – 3.49 (m, 1H), 3.17 – 2.92 (m, 4H), 2.43 – 2.27 (m, 4H), 2.27 – 0.94 (m, 32H), 1.00 – 0.82 (m, 4H); MS (ESI, m/z) calculated for $C_{31}H_{36}ClN_7O_3H$ (M+H) 590.2641, $[M+H]^+$ (HRMS) found 590.2633; HPLC purity: 97.9% (UV detector wavelength: 254 nm).

Synthesis of 2-TCO



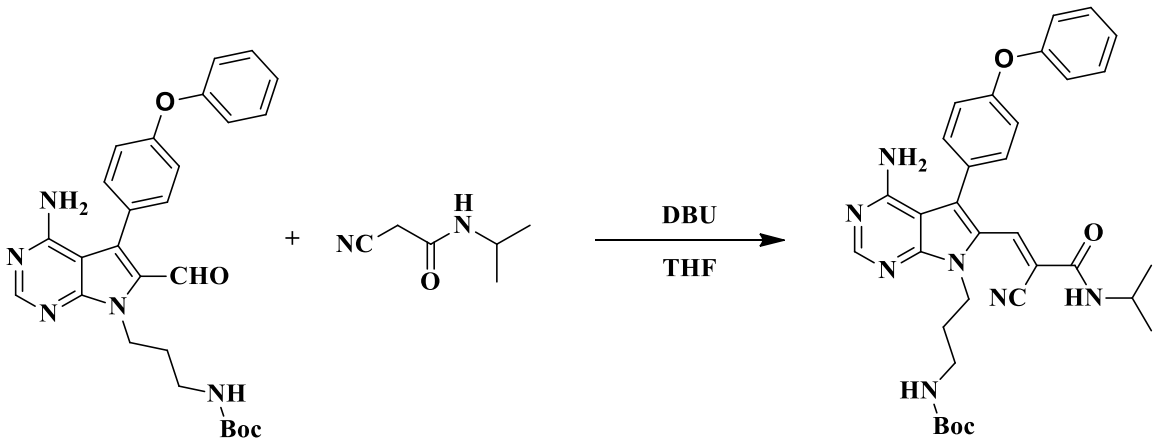
Reaction scheme for **2-TCO**: (a) 4-Phenoxyphenylboronic acid, K_3PO_4, $Pd(dppf)_2Cl_2$, 1,4-Dioxane, H_2O, 90 °C, 150 mins (microwave), 72%; (b) 2-Cyano-*N*-isopropylacetamide, DBU, THF, rt, 80 mins, 57%; (c) TFA, DCM, rt, 30 mins; (d) DIPEA, THF, rt, overnight, 44%.

***Tert*-butyl (3-(4-amino-6-formyl-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (10)**



In a 10-mL microwave reaction tube, *tert*-butyl (3-(4-amino-6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (72 mg, 0.16 mmol, 1.0 equiv.), 4-phenoxyphenylboronic acid (52 mg, 0.24 mmol, 1.5 equiv.), potassium phosphate (100 mg, 0.49 mmol, 3.0 equiv.) and [1,1′-Bis(diphenylphosphino)ferrocene]dichloropalladium(II) (13 mg, 0.016 mmol, 0.1 equiv.) were dissolved in a mixture of 1,4-Dioxane (1.3 mL) and water (0.33 mL). The resulting mixture was heated in a microwave reactor at 90 ˚C for 150 mins. The reaction was quenched with saturated NH_4Cl solution (1 mL) and diluted with ethyl acetate (30 mL). The organic phase was washed with saturated NaHCO3 solution (5 mL), brine (5 mL), dried over anhydrous Na_2SO_4 and concentrated in *vacuo*. Purification using flash chromatography on silica gel with a EtOAc/Hexane gradient (0:100 to 90:10) afforded *tert*-butyl (3-(4-amino-6-formyl-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a pale yellow solid (57 mg, 72%; R_f= 0.87 in EtOAc:Hexane= 9:1). ^{1}H-NMR (500 MHz, $CDCl_3$) δ= 9.64 (s, 1H), 8.42 (s, 1H), 7.48 – 7.38 (m, 4H), 7.21 (t, J = 7.4 Hz, 1H), 7.16 – 7.09 (m, 4H), 5.68 (s, 1H), 5.35 (s, 2H), 4.69 (t, J = 6.3 Hz, 2H), 3.10 – 2.98 (m, 2H), 2.05 – 1.96 (m, 2H), 1.46 (s, 9H); MS (ESI, m/z) calculated for $C_{27}H_{29}N_5O_4$ 487.2, $[M+H]^+$ found 488.5.

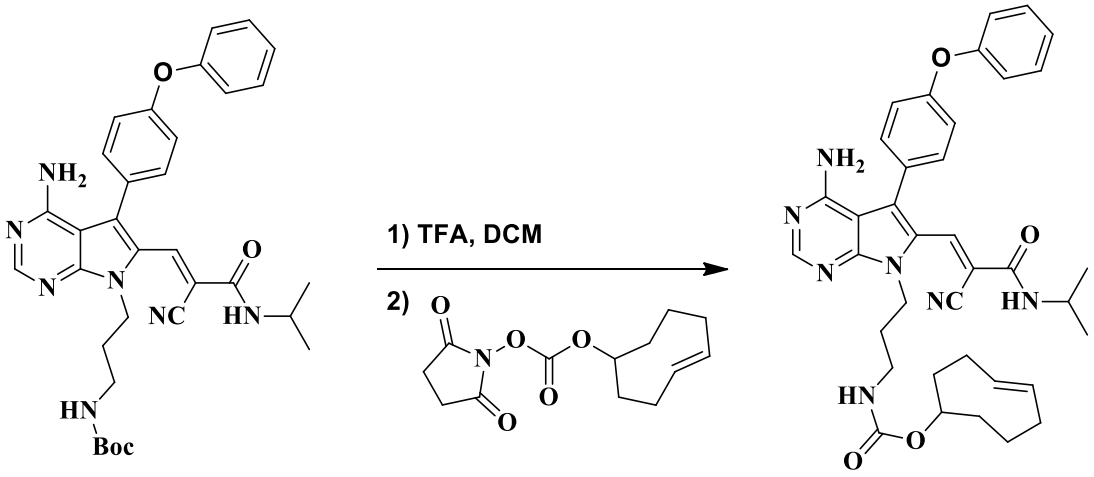
(*Z*)- or (*E*)-tert-butyl (3-(4-amino-6-(2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (11)



Tert-butyl (3-(4-amino-6-formyl-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (57 mg, 0.095 mmol, 1.0 equiv.) and 2-cyano-*N*-isopropylacetamide (18 mg, 0.14 mmol, 1.5 equiv.) were dissolved in dry THF (600 µL mL). 1,8-Diazabicyclo[5.4.0]undec-7-ene (29 mg, 28 µL, 0.19 mmol, 2.0 equiv.) was added and the resulting solution was stirred at room temperature for 80 mins. The reaction mixture was then diluted with EtOAc (30 mL) and quenched with saturated aqueous NH_4Cl solution. The organic phase was washed with saturated $NaHCO_3$ solution (5 mL), brine (5 mL), dried over anhydrous Na_2SO_4. Purification with preparative thin-layer chromatography afforded a mixture of (*Z*)- or (*E*)-tert-butyl (3-(4-amino-6-(2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a bright yellow powder (32 mg, 57%; Rf [(E) and (Z)-isomers)]= 0.26 in EtOAc:Hexane= 9:1). ^{1}H-NMR (300 MHz, MeOD) δ= 8.25 – 8.19 (m, 3H), 7.65 (s, 1H), 7.51 – 7.34 (m, 8H), 7.24 – 7.05 (m, 10H), 5.14 (s, 2H), 4.48 – 4.38 (m, 2H), 4.31 (t, *J* = 7.0 Hz, 2H), 4.15 – 4.07 (m, 1H), 3.74 – 3.62 (m, 1H), 3.13 – 2.94 (m, 4H), 2.04 – 1.85 (m,

4H), 1.44 (s, 18H), 1.23 (d, *J* = 6.6 Hz, 6H), 1.07 (d, *J* = 6.5 Hz, 6H); MS (ESI, m/z) calculated for $C_{33}H_{37}N_7O_4$ 595.3, $[M+H]^+$ found 597.4.

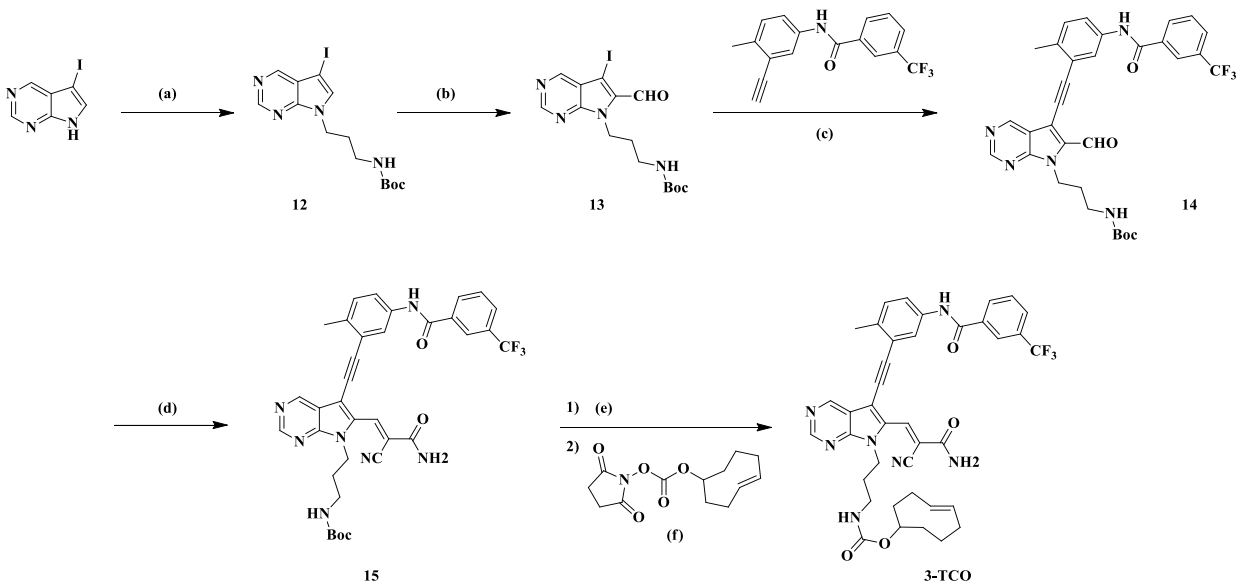
(*E*)-cyclooct-4-en-1-yl (3-(4-amino-6-((*E*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate or (*E*)-cyclooct-4-en-1-yl (3-(4-amino-6-((*Z*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (2-TCO)



A mixture of (*Z*)- or (*E*)-tert-butyl (3-(4-amino-6-(2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (24 mg, 0.034 mmol, 1.0 equiv.) was dissolved in a mixture of trifluoacetic acid (0.60 mL) and dichloromethane (1.4 mL). The resulting soluting was stirred at room temperature for 30 mins and concentrated in vacuo. The solid residue was dissolved in anhydrous tetrahydrofuran (2 mL), and to it was added diisopropylethylamine (22 mg, 30 μL, 0.17 mmol, 5.0 equiv.) and (*E*)-cyclooct-4-en-1-yl (2,5-dioxopyrrolidin-1-yl) carbonate (12 mg, 0.044 mmol, 1.3 equiv.) sequentially. The resulting mixture was stirred at room temperature overnight and quenched with water (1 mL) and diluted with ethyl acetate (30 mL). The organic layer was washed with saturated NaHCO3 solution (5

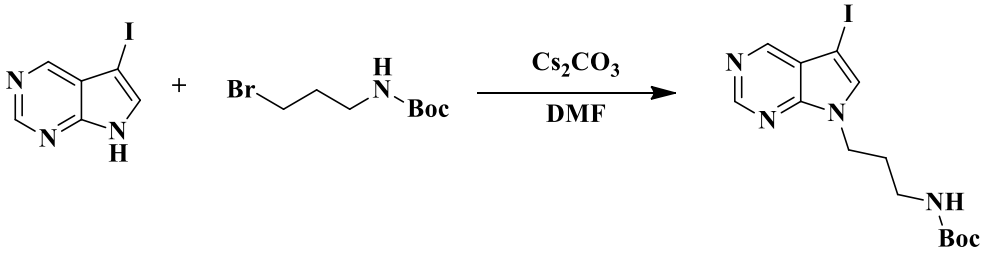
mL), brine (5 mL), dried over anhydrous Na2SO4. The crude product was purified using flash chromatography with a MeOH/EtOAc gradient (0:100 to 10:90). A mixture of isomers of (*E*)-cyclooct-4-en-1-yl (3-(4-amino-6-((*E*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate or (*E*)-cyclooct-4-en-1-yl (3-(4-amino-6-((*Z*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate was rendered as a bright yellow powder (9.6 mg, 44% for 2 steps; Rf [(E) and (Z)-isomers)]= 0.27 in EtOAc:Hexane= 3:2) ^{1}H-NMR (300 MHz, MeOD) δ= 8.26 – 8.13 (m, 3H), 7.60 (s, 1H), 7.50 – 7.32 (m, 8H), 7.23 – 7.05 (m, 10H), 5.52 (t, 4H), 4.40 (t, J = 6.8 Hz, 2H), 4.27 (t, J = 6.8 Hz, 2H), 4.19 – 4.02 (m, 1H), 3.77 – 3.59 (m, 1H), 3.27 – 3.14 (m, 2H), 3.14 – 2.93 (m, 4H), 2.42 – 2.22 (m, 4H), 2.22 – 0.97 (m, 32H), 0.97 – 0.81 (m, 4H); MS (ESI, m/z) calculated for $C_{37}H_{41}N_7O_4H$ (M+H) 648.3293, $[M+H]^+$ (HRMS) found 648.3279; HPLC purity: 99.0% (UV detector wavelength: 254 nm)

Synthesis of 3-TCO



Reaction scheme for **3-TCO**: (a) Cs_2CO_3, DMF, 50 °C, overnight, 73%; (b) Diisopropylamine, *n*-BuLi, ethyl formate, THF, -78 °C-rt, overnight, 32%; (c) *N*-(3-ethynyl-4-methylphenyl)-3-(trifluoromethyl)-benzamide, $Pd(PPh_3)_2Cl_2$, CuI, DMF, 50 °C, overnight, 39%; (f) Cyanoacetamide, DBU, THF, rt, 25 mins, 44%; (g) TFA, DCM, rt, 30 mins; (h) DIPEA, THF, rt, 30 mins, 59%.

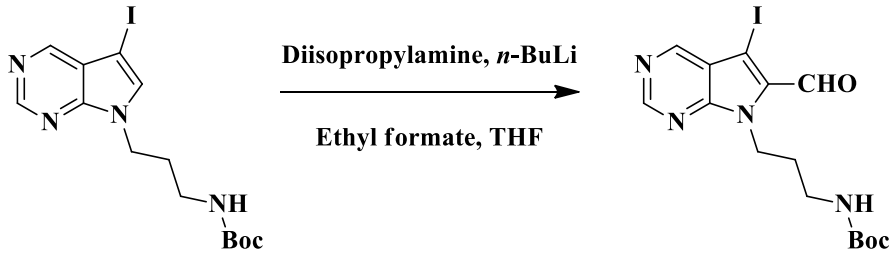
***Tert*-butyl (3-(5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (12)**



5-Iodo-7*H*-pyrrolo[2,3-*d*]pyrimidine (2.0 g, 8.2 mmol, 1.0 equiv.), *tert*-butyl (3-bromopropyl)carbamate (2.5 g, 11 mmol, 1.3 equiv.) and cesium carbonate (8.8 g, 16 mmol, 2.0 equiv.) were dissolved in dimethylformamide (20 mL) in a 100-mL round bottom flask. The

reaction mixture was heated at 50 ˚C overnight. The solvent was then removed in *vacuo*. The crude compound was redissolved in ethyl acetate (200 mL) and washed with saturated NH_4Cl solution (40 mL), aqueous $NaHCO_3$ solution (40 mL), brine (40 mL), dried over anhydrous Na_2SO_4. Purification with Flash chromatography afforded *tert*-butyl (3-(5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a pale brown solid (2.4 g, 73%; Rf= 0.20 in EtOAc:Hexane= 1:2). ^{1}H-NMR (300 MHz, $CDCl_3$) δ= 8.81 (s, 1H), 8.66 (s, 1H), 7.16 (s, 1H), 5.02 (s, 1H), 4.25 (t, *J* = 6.5 Hz, 2H), 3.13 – 2.82 (m, 2H), 2.09 – 1.82 (m, 2H), 1.36 (s, 9H); MS (ESI, m/z) calculated for $C_{14}H_{19}IN_4O_2$ 402.1, $[M+H]^+$ found 403.1.

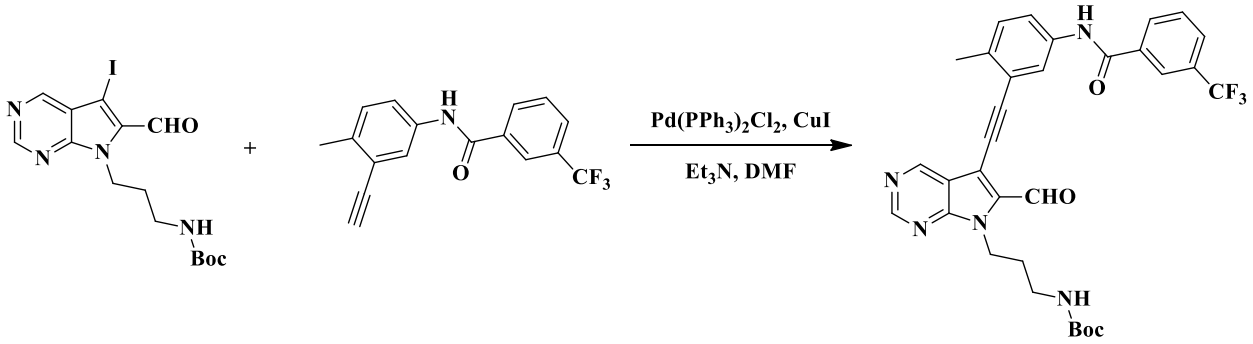
***Tert*-butyl (3-(6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (13)**



Diisopropylamine (490 mg, 680 µL, 4.9 mmol, 2.7 equiv.) was dissolved in dry THF (6 mL) and cooled to -78°C. A solution of *n*-BuLi (2.5 M in hexane, 1.8 mL, 4.5 mmol, 2.5 equiv.) was added dropwise to the above solution at -78 ˚C. The solution was stirred at 0 °C for 1 hour and then cooled to -78 °C. A solution of *tert*-butyl (3-(5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (730 mg, 1.8 mmol, 1.0 equiv., dissolved in 3 mL dry THF) was added dropwise and the reaction mixture was then stirred at -78 °C for 1 hour. A solution of ethyl formate (536 mg, 580 µL, 7.2 mmol, 4.0 equiv., dissolved in 3 mL dry THF) was then added dropwise at -78°C. The reaction was allowed to warm to room temperature overnight. After reaction, saturated NH4Cl solution (2 mL) was added and the resulting mixture was diluted with ethyl acetate (200

mL). The organic layer was washed with saturated aqueous NaHCO3 solution, brine and dried over anhydrous Na2SO4. Purification by flash chromatography on silica gel (EtOAc: Hexane= 0:100 to EtOAc: Hexane= 3:2) afforded *tert*-butyl (3-(6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a pale brown solid (250 mg, 32%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ= 10.02 (s, 1H), 9.07 (s, 1H), 8.98 (s, 1H), 5.22 (s, 1H), 4.73 (t, *J* = 6.6 Hz, 2H), 3.13 – 2.96 (m, 2H), 2.10 – 1.91 (m, 2H), 1.45 (s, 9H). MS (ESI, m/z) calculated for $C_{15}H_{19}IN_4O_3$ 430.1, $[M+H]^+$ found 431.0.

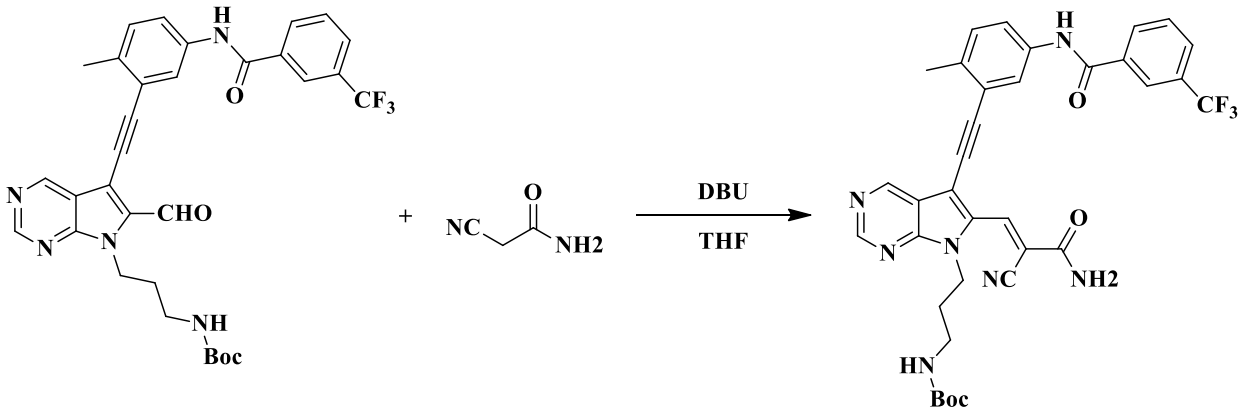
Tert-butyl (3-(6-formyl-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (14)



N-(3-ethynyl-4-methylphenyl)-3-(trifluoromethyl)-benzamide was synthesized according to previous reported (ref. SFK paper). *Tert*-butyl (3-(6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (120 mg, 0.28 mmol, 1.0 equiv.) was dissovled in anhydrous DMF (3.5 mL) under nitrogen. Triethylamine (110 mg, 153 μL, 1.1 mmol, 4.0 equiv.), N-(3-ethynyl-4-methylphenyl)-3-(trifluoromethyl)benzamide (130 mg, 0.42 mmol, 1.5 equiv.), bis(triphenylphosphine)palladium(II) dichloride (9.8 mg, 0.014 mmol, 0.05 equiv.) and copper (I) iodide (5.3 mg, 0.028 mmol, 0.1 equiv.) were added to the above solution sequentially. The

reaction was heated at 50 ˚C for overnight and then quenched with saturated NH4Cl (1 mL). The resulting mixture was diluted with ethyl acetate (200 mL) and the organic phase was washed with saturated NaHCO3 (30 mL), brine (30 mL) and then dried over anhydrous Na2SO4. Purification by flash chromatography on silica gel using a gradient of EtOAc/Hexane (0:100 to 60:40) afforded *tert*-butyl (3-(6-formyl-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl) ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a pale brown solid (65 mg, 39%; Rf= 0.42 in EtOAc:Hexane= 3:2). ^{1}H-NMR (500 MHz, $CDCl_3$) δ= 10.26 (s, 1H), 9.19 (s, 1H), 9.02 (s, 1H), 8.72 (s, 1H), 8.16 (s, 1H), 8.10 (d, *J* = 7.8 Hz, 1H), 7.92 (s, 1H), 7.76 (d, *J* = 7.7 Hz, 1H), 7.58 (d, *J* = 6.6 Hz, 1H), 7.21 (d, *J* = 8.3 Hz, 1H), 5.33 (s, 1H), 4.68 (t, *J* = 6.0 Hz, 2H), 3.12 – 2.95 (m, 2H), 2.48 (s, 3H), 2.02 – 1.91 (m, 2H), 1.43 (s, 9H). MS (ESI, m/z) calculated for $C_{32}H_{30}F_3N_5O_4$ 605.2, $[M+H]^+$ found 606.2.

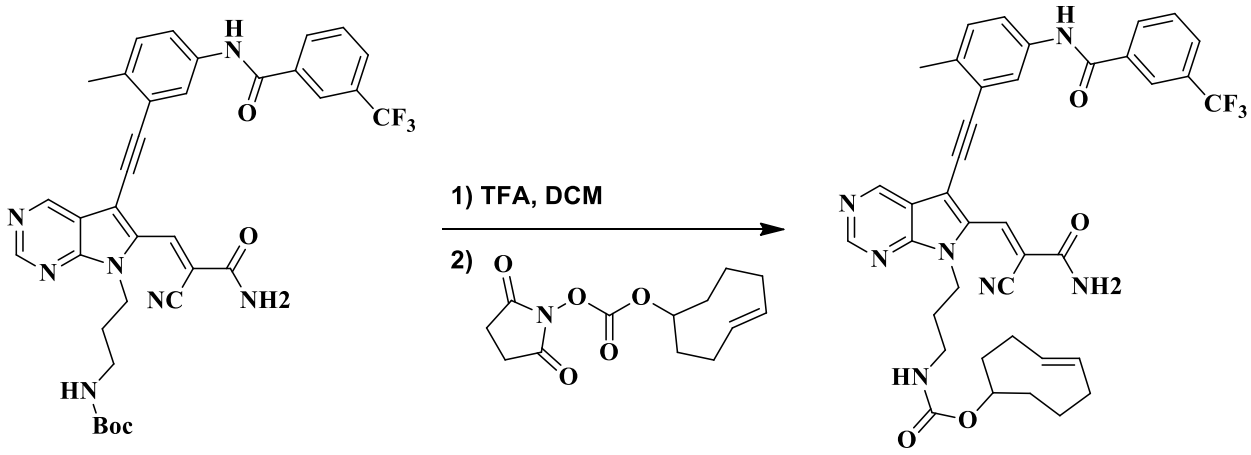
(*Z*)- or (*E*)-*tert*-butyl (3-(6-(3-amino-2-cyano-3-oxoprop-1-en-1-yl)-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (15)



Tert-butyl (3-(6-formyl-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (33 mg, 0.054 mmol, 1.0 equiv.) and

cyanoacetamide (5.4 mg, 0.065 mmol, 1.2 equiv.) were dissolved in anhydrous THF (1.0 mL). 1,8-Diazabicyclo[5.4.0]undec-7-ene (16 mg, 16 µL, 0.11 mmol, 2.0 equiv.) was added and the resulting mixture was stirred at room temperature for 25 mins. The reaction was quenched with saturated NH_4Cl solution and diluted with EtOAc (20 mL). The organic phase was washed with saturated $NaHCO_3$ (4 mL), brine (4 mL) and dried over anhydrous Na_2SO_4. Purification using preparative thin-layer chromatography developed with pure ethyl acetate afforded a mixture of (*Z*)- or (*E*)-*tert*-butyl (3-(6-(3-amino-2-cyano-3-oxoprop-1-en-1-yl)-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a bright yellow solid powder (16 mg, 44%; R_f [(*E*) and (*Z*)-isomers)]= 0.48 in MeOH:DCM= 1:9).^{1}H-NMR (300 MHz, MeOD) δ= 9.18 (s, 1H), 9.06 (s, 1H), 8.96 (s, 1H), 8.90 (s, 1H), 8.42 (s, 2H), 8.28 (s, 2H), 8.22 (d, *J* = 7.8 Hz, 2H), 8.07 (s, 1H), 8.06 (s, 1H), 7.92 (d, *J* = 6.7 Hz, 1H), 7.89 (d, *J* = 6.7 Hz, 1H), 7.79 – 7.69 (m, 2H), 7.68 – 7.59 (m, 2H), 7.29 (d, *J* = 8.4 Hz, 2H), 5.12 (s, 2H), 4.58 – 4.38 (m, 4H), 3.13 – 2.98 (m, 4H), 2.54 (s, 6H), 2.11 – 1.91 (m, 4H), 1.42 (s, 18H); MS (ESI, m/z) calculated for $C_{35}H_{32}F_3N_7O_4$ 671.3, $[M+H]^+$ found 672.2.

(*E*)-cyclooct-4-en-1-yl (3-(6-((*E*)-3-amino-2-cyano-3-oxoprop-1-en-1-yl)-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate or (*E*)-cyclooct-4-en-1-yl (3-(6-((*Z*)-3-amino-2-cyano-3-oxoprop-1-en-1-yl)-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (3-TCO)



A mixture of (*Z*)- or (*E*)-*tert*-butyl (3-(6-(3-amino-2-cyano-3-oxoprop-1-en-1-yl)-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (16 mg, 0.024 mmol, 1.0 equiv.) was dissolved in a mixture of trifluoacetic acid (0.3 mL) and dichloromethane (0.7 mL). The reaction solution was stirred at room temperature for 30 mins and the solvent was removed in *vacuo*. The solid residue was dissolved in anhydrous tetrahydrofuran (1 mL). To the above solution was added diisopropylethylamine (22 mg, 30 µL, 0.17 mmol, 5.0 equiv.) and (*E*)-cyclooct-4-en-1-yl (2,5-dioxopyrrolidin-1-yl) carbonate (12 mg, 0.044 mmol, 1.3 equiv.) sequentially. The reaction was quenched with water (1 mL) after maintaining at room temperature overnight. The resulting mixture was then diluted with ethyl acetate (30 mL). The organic phase washed with saturated $NaHCO_3$ solution (5 mL), brine (5 mL), dried over anhydrous Na_2SO_4. Purification using flash chromatography on silica gel with a EtOAc/Hexane gradient (0:100 to 100:0) afforded a mixture of (*E*)-cyclooct-4-en-1-yl (3-(6-

((*E*)-3-amino-2-cyano-3-oxoprop-1-en-1-yl)-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate or (*E*)-cyclooct-4-en-1-yl (3-(6-((*Z*)-3-amino-2-cyano-3-oxoprop-1-en-1-yl)-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate as a bright yellow powder (10 mg, 59% for 2 steps; Rf [(E) and (Z)-isomers)]= 0.50 in pure EtOAc) ^{1}H-NMR (500 MHz, MeOD) δ= 9.22 (s, 1H), 9.10 (s, 1H), 8.99 (s, 1H), 8.94 (s, 1H), 8.45 (s, 2H), 8.32 (s, 2H), 8.26 (d, *J* = 6.9 Hz, 2H), 8.13 (s, 1H), 7.99 (s, 1H), 7.94 (d, *J* = 6.0 Hz, 2H), 7.85 – 7.73 (m, 2H), 7.67 (d, *J* = 7.7 Hz, 2H), 7.34 (d, *J* = 8.0 Hz, 2H), 5.70 – 5.56 (m, 2H), 5.55 – 5.44 (m, 2H), 5.25 (s, 4H), 5.16 (s, 2H), 4.52 (s, 4H), 4.30 (s, 2H), 3.60 – 3.45 (m, 2H), 3.29 – 3.20 (m, 2H), 3.17 – 3.10 (m, 2H), 2.58 (s, 6H), 2.43 – 0.72 (m, 20H); MS (ESI, m/z) calculated for $C_{39}H_{36}F_3N_7O_4H$ (M+H) 724.2854, $[M+H]^+$ (HRMS) found 724.2842; HPLC purity: 98.2% (UV detector wavelength: 254 nm)

Expression and Purification of recombinant proteins

Recombinant Src expresssion

N-terminal His-tagged Src V284C and Src WT (residue 87-536) were expressed and purified accordingly to the Supplementary Information of *Mol. Cell* **2019**, *74*, p. 393-408 for primary profiling data.

Recombinant JNK2 expression

His-TEV+LIC-JNK2α2 WT and His-TEV+LIC-JNK2α2 V40C/M108T were expressed in *Escherichia coli* BL21(DE3) cells in LB Miller broth. JNK2α2 constructs were expressed in their inactive forms. Cells were grown to OD600 ~0.5 at 37 °C. The temperature was reduced to 18 °C and cells were induced 30 min after temperature reduction with 0.4 M isopropyl β-D-thiogalactoside. Expression occurred at 18 °C overnight. All purification steps were carried out at 4 °C. Cells were pelleted in 1L bottles via centrifugation at 6000 rpm. Cells were lysed with sonication in 2 mL/gram pellet weight of wash/lysis buffer consisting of 50 mM HEPES (pH = 7.5), 300 mM NaCl, 20 mM imidazole, 10% glycerol and 1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at 10000 g for 20 min and the supernatant was allowed to batch bind for 60 min with 0.4 ml/L cell culture of Ni-NTA (Ni-nitrilotriacetate) resin. The resin was collected by centrifugation at 500 g for 5 min and washed with 20 mL of wash/lysis buffer per liter of culture. The wash step was repeated three times. The protein was eluted using ~5 mL of elution buffer (50 mM HEPES (pH 7.5), 300 mM NaCl, 200 mM imidazole and 10% glycerol) per liter of culture. Then, the eluate was dialyzed against 50 mM HEPES (pH = 7.5), 200 mM NaCl, 5% glycerol and 1 mM dithiothreitol (DTT) overnight. WT His-TEV+LIC-JNK2α2 was treated with TEV during

O.N. dialysis (2:25 wt:wt TEV:JNK2). The next day, the TEV cleaved WT JNK2α2 was allowed to bind to 1 mL of Ni-NTA slurry for 1 hr to remove the cleaved Hig tag and TEV. Cleaved JNK2α2 was rinsed from the resin with wash/lysis buffer. Constructs were dialyzed two additional times. The aliquoted proteins were flash-frozen and stored at −80 °C. Protein concentrations were determined using in-gel BSA standards.

Recombinant PAK1 expression

PAK1 V286C/M346T (residues 250-547) was cloned into the bacterial expression plasmid pMCSG7 as a N-terminal His-SUMO-tagged construct. The construct was transformed in *Escherichia coli* BL21(DE3) cells and plated on ampicillin selective plates. A single colony was picked and grown in an overnight culture of 50 mL of LB broth containing ampicillin. An 1 L culture was inoculated with the starter culture, grown to an OD600 of 0.8. The protein expression was then induced with 0.2 M IPTG at 18 ˚C. The next day, cells were harvested by centrifugation, lysed by sonication in lysis buffer (50 mM HEPES, pH = 8.0, 300 mM NaCl, 1 mM PMSF, 0.1% Triton-X, 20 mM imidazole), and centrifuged for 45 minutes at 10,000 rpm. Cleared lysates were incubated with 0.5 mL Ni-NTA resin (Thermo) for 1 h. The supernatant was then discarded, and beads were washed with 20 mL lysis buffer before bound protein was eluted with lysis buffer containing 300 mM imidazole. The eluted PAK1 V286C/M346T protein was dialyzed overnight at 4 ºC in dialysis buffer (50 mM Tris, pH = 8.0, 150 mM NaCl, 5% glycerol, 1 mM DTT). PAK1 WT was purchased from SignalChem (cat. no. P02-10BG-05).

Recombinant EphA2 expression

The genes of N-terminal His-tagged EphA2 V627C and EphA2 WT (residue 590-876) were cloned into the bacterial expression plasmid pMCSG7. The construct was transformed in *Escherichia coli* BL21(DE3) cells and plated on ampicillin selective plates. A single colony was picked and grown in an overnight culture of 50 mL of LB broth containing ampicillin. The starting culture was then transferred to an one liter LB corth, grown to an OD600 of 1.2. Next, protein expression was induced with 0.2 M IPTG at 18 ˚C. The next day, cells were harvested by centrifugation, lysed by sonication in lysis buffer (50 mM HEPES, 300 mM NaCl, 1 mM PMSF, 0.1% Triton-X, 20 mM imidazole, pH 8.0), and centrifuged for 45 min at 10,000 rpm. Cleared lysates were incubated with 0.5 mL Ni-NTA resin (Thermo) for 1 h. The supernatant was then discarded, and beads were washed with 20 mL lysis buffer before the bound protein was eluted with lysis buffer containing 300 mM imidazole. The Eluted His-tagged recombinant EphA2 proteins were dialyzed overnight at 4 ºC in dialysis buffer (50 mM Tris, pH = 8.0, 150 mM NaCl, 5% glycerol, 1 mM DTT).

Protocol of activity assays to determine *Ki* values of 1, 2, 3 and TCO probes.

Src activity assay

Enzyme inhibition of Src V284C-variant or wild-type recombinant Src constructs (residue 87-536) was determined using a fluorogenic assay using a self-reporting Src kinase substrate EEEIYGE-(DAP-Pyrene)-EA. **1**, **2**, **3**, or TCO-conjugated probes (3-fold dilution, initial concentration of probe in reaction= 30 μM) were assayed in triplicate against 12.5 nM of V284C-Src-3D or 7.5 nM of WT Src-3D in kinase reaction buffer ([HEPES]= 75 mM, pH= 8.0, [$MgCl_2$]= 15 mM, [EGTA]= 3.75 mM, [NaCl]= 150 mM, [BSA]= 0.2 mg/mL and [Na_3VO_4]= 750 nM). Src was incubated with inhibitors and ATP (1 mM) for 30 mins in a Corning® 384 well microplate (Low flange; product number: CLS3573). Then 20 μM of fluorogenic substrate solution in kinase reaction buffer was added and incubated for 120 mins. Fluorescent emission readouts were measured at wavelength of 405 nm when excited at 344 nm using a Perkin Elmer Envision Plate Reader. Percentage of inhibition was calculated and IC50 values were determined using One site – Fit logIC_{50} model in GraphPad software Prism 7.

PAK1 activity assay

(*Pak1*) Pak1 WT and Pak1 V286C/M346T catalytic domains were catalytically active after expression in *E. coli* and purification. Pak1 WT (18 nM) and Pak1 V284C/M344T (35 nM) were incubated with **1**, **2** or **3** (initial concentration = 10 μM, 3-fold serial dilutions, 10 data points), 4 μM cold ATP, and 0.007 μCi/μL γ^{32}P-ATP for 30 min in assay buffer (76 mM HEPES, pH = 7.5, 5 mM $MgCl_2$, 150 mM NaCl, 3.8 mM EGTA, 0.2 mg/mL BSA, 150 uM Na_3VO_4, 1 mM BME) then PAKtide (sequence:RRRLSFAEPG) (final concentration = 0.2 mg/mL) was added. After

incubating for 2-4 h, 4.6 µL of the reaction mixture were spotted onto phosphocellulose. Membranes were washed with 0.05% phosphoric acid (3x 10 min wash) and air-dried, and the radioactivity was determined by phosphor-imaging with a GE Typhoon FLA 9000 phosphor-scanner. Data was analyzed using GraphPad Prism software, and IC_{50} values were determined using "One-site fit logIC_{50}" option. All assays were performed in triplicate.

JNK2 activity assay

Activated JNKs were prepared and activated using purified, activated MKK4α. 2.5 µM JNK2 was pre-activated with 150 nM MKK4α for 1 hour at RT in (50 mM Tris/HCl, pH = 7.5, 0.01% (v/v) Tween 20, 10 mM MgCl2, 2 mM DTT, 1 mM EGTA, 0.1 mg/mL BSA) with 400 µM ATP.

To get an inhibitor dose response curve, an enzyme titration for JNK2 was first carried out in order to determine the linear working concentration of the enzyme. Inhibitors (initial concentration = 10 µM, 3-fold serial dilutions, 9 data points) were assayed in triplicate against kinases (JNK2 WT = 6.25 nM and JNK2 V40C/M108T = 0.5 nM; in assay buffer (50 mM Tris/HCl, pH = 7.5, 0.01% (v/v) Tween 20, 10 mM $MgCl_2$, 0.1 mM DTT, 1 mM EGTA, 0.1 mg/mL BSA). JNK2 was incubated with inhibitors 4 µM cold ATP, and 0.007 uCi/uL ATP-[γ^{32}P] for 30 mins in a 96-well plate. To initiate the reaction, myelin basic protein (MBP) was added, at a final concentration of 0.2 mg/mL. The reaction mixture was incubated in a volume of 30.2 µL per well, at room temperature, for 2 hours for JNK2 WT and 4 h for JNK2 V40C/M108T. To quench the reaction, 4.6 µl of the reaction mixture were spotted onto nitrocellulose, the membranes washed 3 times with 0.5% phosphoric acid and dried with acetone. Membranes were wrapped in plastic wrap and placed in a phosphor screen for autoradiography and exposed overnight. Phospho imaging was performed using a Typhoon FL 9000 instrument (GE Healthcare, Pittsburgh, PA). Data was

analyzed using GraphPad Prism software, and IC_{50} values were determined using "One-site fit logIC_{50}" option. All assays were performed in triplicate.

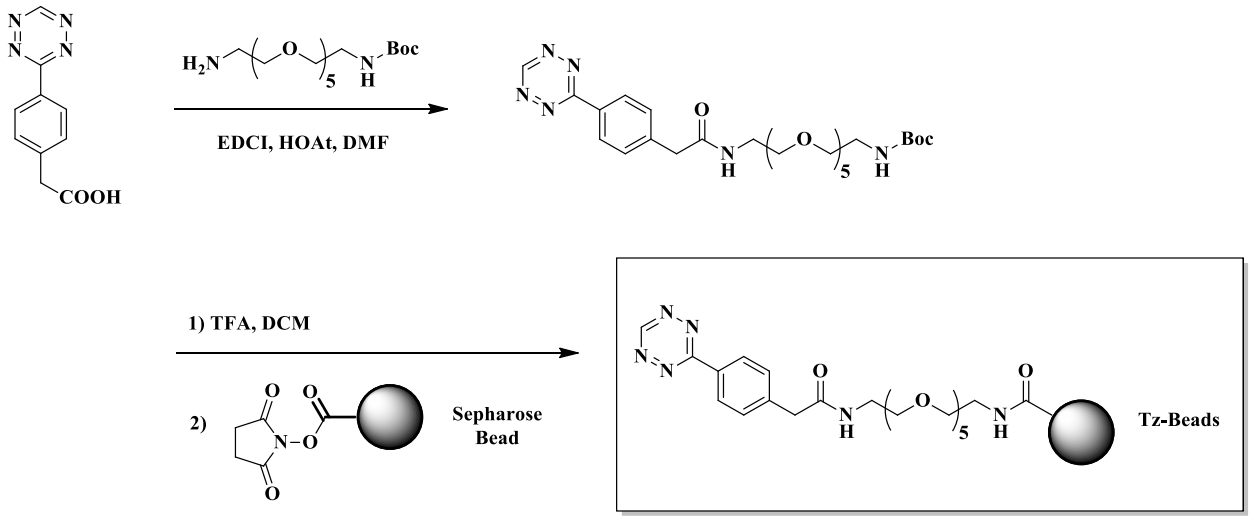
EphA2 activity assay

2.5 nM of recombinant EphA2 (residue 590-876) was incubated with inhibitors (initial concentration = 30 μM, 3-fold serial dilutions, 10 data points) for 30 minutes at room temperature in reaction buffer ([HEPES]= 50 mM, pH= 7.5, [$MgCl_2$]= 10 mM, [EGTA]= 1 mM, Tween-20= 0.1%, [DTT]= 1mM, BSA= 0.5 mg/mL). The reaction mixture was then incubated with 0.0067 mCi/mL of $^{32}\gamma$-ATP and 2 mM of cold ATP at room temperature for 30 minutes. EphA2 substrate Poly Glu:Tyr= 4:1 (0.5 mg/mL) was added and the reaction was incubated at room temperature for 4 hours. The reaction was terminated by spotting 4.6 μL of the reaction mixture onto a phosphocellulose membrane. The membrane was washed with 0.05% phosphor5ic acid three times (10 mins each) and dried by air. The concentration of radioactively labeled EphA2 substrate was determined by phosphor-imaging with a GE Typhoon FLA 9000 phosphor-scanner. The image was processed and quantified with Image StudioLite software, and data were analyzed using GraphPad Prism, and IC_{50} values were determined using "One-site fit logIC_{50}" option. All assays were performed in triplicate.

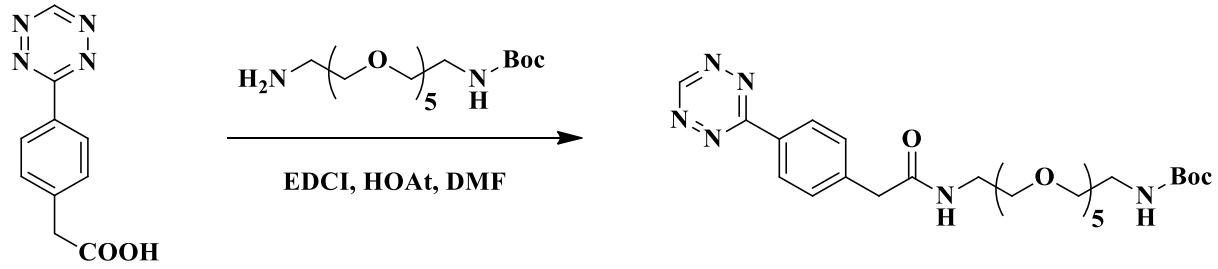
SH3 domain pulldown assay: 10 μL of a 50% slurry (by volume) of O^6-benzylguanine-containing sepharose beads were washed with immobilization buffer ([Tris]= 50 mM, pH= 7.5, [NaCl]= 100 mM, [DTT]= 1 mM, [BSA]=0.2 mg/mL) (10 bed volumes for 3 times). The washed beads were incubated with 50 μL of SNAP-tagged-polyproline peptide (VSLARRPLPPLP) (8 μM) on an end-to-end rotator at ambient temperature for 1 hour. After immobilization of the polyproline peptide, the beads were washed with immobilization buffer (10-bed volumes for 3 times).

Meanwhile, a saturating amount of **1-TCO**, **2-TCO**, or **3-TCO** (3 μM or 5 μM) was added to 50 μL of a solution in immobilization buffer containing 25 nM of recombinant TAMRA-conjugated Src V284C.[3,4] The solution was incubated at ambient temperature for 30 mins before being added to the polyproline peptide beads. After incubating at ambient temperature for 1 hour, the flow-through was collected and the beads were washed with immobilization buffer (10-bed volumes for 3 times). The bead-bound Src was eluted by boiling the beads in 50 μL of 1x SDS loading buffer at 95 ˚C for 10 mins. Input (IN) and elution (EL) were processed using SDS-PAGE electrophoresis and imaged on GE-Typhoon FLA 9000. Intensities of rhodamine fluorescence for each band was quantified using ImageStudio™ Lite. Percentage of Src bound to the polyproline-containing beads= Intensity(EL)/[Intensity(IN)+Intensity(EL)] × 100% was plotted using GraphPad software Prism 7.

Generation of Tetrazine-Beads



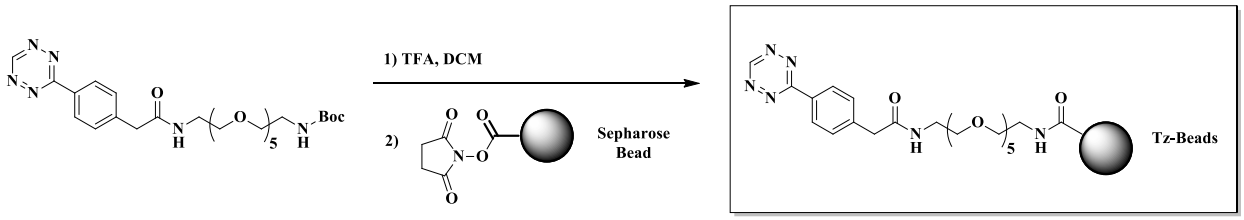
***Tert*-butyl (1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-6,9,12,15,18-pentaoxa-3-azaicosan-20-yl)carbamate**



2-(4-(1,2,4,5-tetrazin-3-yl)phenyl)acetic acid (50 mg, 0.23 mmol, 1.0 equiv.), *tert*-butyl (17-amino-3,6,9,12,15-pentaoxaheptadecyl)carbamate (114 mg, 0.30 mmol, 1.3 equiv.) and 1-Hydroxy-7-azabenzotriazole (41 mg, 0.30 mmol, 1.3 equiv.) were dissolved in anhydrous DMF (3 mL). The solution was cooled to 0 ˚C and to it was added *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (58 mg, 0.30 mmol, 1.3 equiv.). The reaction was allowed to warm to room temperature overnight. The solvent was removed in *vacuo* and the solid residue was dissolved in ethyl acetate (200 mL). The organic phase was washed with saturated KH_2PO_4 solution (30 mL), saturated $NaHCO_3$ solution (30 mL), brine (30 mL) and dried over anhydrous

Na_2SO_4. Purification by flash chromatography on silica gel with a MeOH/EtOAc gradient (0:100 to 20:80) afforded *tert*-butyl (1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-6,9,12,15,18-pentaoxa-3-azaicosan-20-yl)carbamate as a bright pink solid (103 mg, 77%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ= 10.22 (s, 1H), 8.59 (d, *J* = 7.9 Hz, 2H), 7.56 (d, *J* = 7.8 Hz, 2H), 6.69 (s, 1H), 5.12 (s, 1H), 3.68 (s, 2H), 3.67 – 3.43 (m, 22H), 3.32 – 3.26 (m, 2H), 1.43 (s, 9H); MS (ESI, m/z) calculated for $C_{27}H_{42}N_6O_8$ 578.3, $[M+H]^+$ found 479.5 (cleavage product of Boc-protection group - $COOC(CH_3)_3$); HPLC purity: 99.0% (UV detector wavelength: 254 nm)

Immobilization of tetrazine



Tert-butyl (1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-6,9,12,15,18-pentaoxa-3-azaicosan-20-yl)carbamate (22 mg, 0.037 mmol, 1.0 equiv.) was dissolved in a mixture of trifluoroacetic acid (0.60 mL) and dichloromethane (1.4 mL). The solution was maintained at room temperature for 30 mins. After reaction, the solvent was removed in *vacuo* and the residue was dissolved in a mixture of EtOH (1.0 mL) and DMF (1.0 mL) as coupling solution. Meanwhile, a 50% slurry of NHS-activated Fast Flow sepharose beads (1.0 mL) were quickly washed three times with a mixed solvent consisting of EtOH and DMF (EtOH:DMF=1:1; use 1.0 mL for each wash) in 2-mL Pierce Centrifuge Columns (Thermo Scientific). The drained beads were resuspended in the coupling solution (2 mL) and to it was added triethylamine (50 μL). The pH was of the supernatant was tested and adjusted to 7-8 with triethylamine if necessary. The reaction was agitated on a tube rotator at 4 ˚C overnight. On the second day, the beads were drained and washed three times with

a mixed solvent consisting of EtOH and DMF (EtOH:DMF=1:1; use 1.0 mL for each wash), followed by three quick washes with an acid solution ([total CH3COOH/NaOOCCH3]= 0.1 M, [NaCl]= 0.5 M, pH=4.0) and a basic solution ([Tris-HCl]= 0.1 M, [NaCl]= 0.5 M, pH= 8.0) (1 mL for each wash). The beads were then washed three times with 20% EtOH in water (1 mL for each wash) and stored as 50% slurry in 20% EtOH in water.

Generation of sepharose beads for pre-clearing lysates

1 mL of 50% slurry of NHS-activated Fast Flow sepharose beads were quickly washed three times with a mixed solvent consisting of EtOH and DMF (EtOH:DMF=1:1; use 1.0 mL for each wash) in 2-mL Pierce Centrifuge Columns (Thermo Scientific). The washed beads were resuspended in a mixture of EtOH (1 mL) and DMF (1 mL) containing 150 mM ethanolamine. The reaction mixture was agitated on a tube rotator at 4˚C overnight. The reaction was agitated on a tube rotator at 4 ˚C overnight. On the second day, the beads were drained and washed three times with a mixed solvent consisting of EtOH and DMF (EtOH:DMF=1:1; use 1.0 mL for each wash), followed by three quick washes with an acid solution ([total CH3COOH/NaOOCCH3]= 0.1 M, [NaCl]= 0.5 M, pH=4.0) and a basic solution ([Tris-HCl]= 0.1 M, [NaCl]= 0.5 M, pH= 8.0) (1 mL for each wash). The beads were then washed three times with 20% EtOH in water (1 mL for each wash) and stored as 50% slurry in 20% EtOH in water.

Procedure for Enriching Probe-Bound Target in Cell Lysates

Cell culture and generation of cell lysates: HEK293 cells were grown on 10-cm plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (by volume) Fetal Bovine Serum (FBS) at 37 ˚C in a humidified incubator containing 5% CO_2 until 90% confluence. Cells were washed with ice-cold DPBS once and lysed with 500 μL of ice-cold lysis buffer. Cell lysates were centrifuged at 17,000 × g for 10 minutes at 4 ˚C and were quantified using Pierce™ 660nm Protein Assay Reagent and diluted to 1.5 mg/mL with ice-cold lysis buffer.

TCO probe treatment: Recombinant 3D construct of Src V284C or Src WT were added to the diluted HEK293 cell lysates to a final concentration of 100 nM. 1 μL of **1-TCO**, **2-TCO**, or **3-TCO** solution (100× solution in DMSO) was added to 100 μL of the lysate (DMSO%= 1% in final solution). Lysates mixture was incubated with **TCO** probe for 30 minutes at 4 ˚C on an end-to-end rotator.

Capture probe-bound target using Tz-Beads: 5 μL (volume of drained beads) of Tz-Beads were added to a 200 μL tube and washed with ice-cold lysis buffer (three times; 100 μL for each wash). 100 μL of TCO probe-treated lysates mixture was added to the washed Tz-Beads and was gently incubated on an end-to-end rotator for 30 mins (or the indicated amount of time in Figure 3G) at 4 ˚C. After incubation with Tz-Beads, the flow-through was quickly removed by aspiration. The beads were then washed for three times with ice-cold Washing Buffer **1** (100 μL for each wash) by gently inverting the tube for four times. The probe-bound target should be bound to Tz-Beads by the end of this step.

Elute enriched probe-bound target and Western blot analysis: The sepharose matrix was incubated with 100 μL of Lysis Buffer containing 2% SDS (by weight) for 20 mins at 25 ˚C with agitation. The eluted proteins were separated using SDS-PAGE gel electrophoresis, transferred

onto nitrocellulose membranes and immunoblotted with corresponding primary antibodies. The nitrocellulose membranes were imaged on LI-COR Odyssey IR imager.

Enrichment of probe-bound Src kinase complex from live mammalian cell

Cell culture and TCO probe treatment:

(**Condition *a*: Serum starvation**) Hela cells that stably overexpressed full-length wild-type Src and V284C variant of Src were grown on 10-cm plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (by volume) Fetal Bovine Serum (FBS) at 37 ˚C in a humidified incubator containing 5% CO_2 until 90% confluence, and was serum-starved for 24 hours prior to the assay. Expression of Src WT and Src V284C was induced by addition of Doxycycline (1 μg/mL) 24 hours prior to the assay. Click probes were added to the cells by replacing medium with fresh DMEM (5 mL) containing **1-TCO** (5 μM; DMSO%= 0.1% in final medium) or **2-TCO** (2.5 μM; DMSO%= 0.1% in final medium). Cells were incubated with **1-TCO** or **2-TCO** for 30 minutes at 37 ˚C in the incubator and the medium was removed immediately after incubation. The cells were quickly washed with ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS) (5 mL, one time) and immediately lysed with ice-cold Lysis Buffer (500 μL). Cells were scrapped into pre-chilled 1.5-mL Eppendorf tubes and were centrifuged at 17,000 × g for 20 minutes at 4 ˚C. The supernatant of lysates was collected, and protein concentration was measured using Pierce 660 nm Protein Assay. The protein concentration of lysates was diluted to 1.5 mg/mL with ice-cold lysis buffer.

(**Condition *b*: Complete medium (DMEM)**) Hela cells that stably overexpressed full-length wild-type Src and V284C variant of Src were grown on 10-cm plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (by volume) Fetal Bovine Serum (FBS) at 37 ˚C in a humidified incubator containing 5% CO_2 until 90% confluence. Expression of Src WT and Src V284C was induced by addition of Doxycycline (1 μg/mL) 24 hours prior to the assay. Click probes were added to the cells by replacing medium with fresh DMEM (5 mL) containing 10%

FBS and **1-TCO** (5 μM; DMSO%= 0.1% in final medium) or **2-TCO** (2.5 μM; DMSO%= 0.1% in final medium). Cells were incubated with **1-TCO** or **2-TCO** for 30 minutes at 37 ˚C in the incubator and the medium was removed immediately after incubation. The cells were quickly washed with ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS) (5 mL, one time) and immediately lysed with ice-cold Lysis Buffer (500 μL). Cells were scrapped into pre-chilled 1.5-mL Eppendorf tubes and were centrifuged at 17,000 × g for 20 minutes at 4 ˚C. The supernatant of lysates was collected, and protein concentration was measured using Pierce 660 nm Protein Assay. The protein concentration of lysates was diluted to 1.5 mg/mL with ice-cold lysis buffer.

(**Condition *c*: Growth factor**) Hela cells that stably overexpressed full-length wild-type Src and V284C variant of Src were grown on 10-cm plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (by volume) Fetal Bovine Serum (FBS) at 37 ˚C in a humidified incubator containing 5% CO_2 until 90% confluence, and was serum-starved for 24 hours prior to the assay. Expression of Src WT and Src V284C was induced by addition of Doxycycline (1 μg/mL) 24 hours prior to the assay. Click probes were added to the cells by replacing medium with fresh DMEM (5 mL) containing 10% FBS and **1-TCO** (5 μM; DMSO%= 0.1% in final medium) or **2-TCO** (2.5 μM; DMSO%= 0.1% in final medium). Cells were incubated with **1-TCO** or **2-TCO** for 30 minutes at 37 ˚C in the incubator. The medium was then immediately replaced with 5 mL of DMEM containing 10% FBS and EGF (100 ng/mL). After 10 minutes, the medium was removed immediately. The cells were quickly washed with ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS) (5 mL, one time) and immediately lysed with ice-cold Lysis Buffer (500 μL). Cells were scrapped into pre-chilled 1.5-mL Eppendorf tubes and were centrifuged at 17,000 × g for 20 minutes at 4 ˚C. The supernatant of lysates was collected, and protein concentration

was measured using Pierce 660 nm Protein Assay. The protein concentration of lysates was diluted to 1.5 mg/mL with ice-cold lysis buffer.

(**Condition *d*: Fibronectin**) 10-cm petri dish plates (Petri Dishes with Clear Lid. Thermo Fisher, catalog No. FB0875712) were treated with 5 mL of 10 μg/mL of Fibronectin solution in DPBS for 1 hour at 37 ˚C. The plates were washed with DPBS once before usage. Hela cells that stably overexpressed full-length wild-type Src and V284C variant of Src were grown on 10-cm fibronectin-coated plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (by volume) Fetal Bovine Serum (FBS) at 37 ˚C in a humidified incubator containing 5% CO_2 until 90% confluence. Expression of Src WT and Src V284C was induced by addition of Doxycycline (1 μg/mL) 24 hours prior to the assay. Click probes were added to the cells by replacing medium with fresh DMEM (5 mL) containing 10% FBS and **1-TCO** (5 μM; DMSO%= 0.1% in final medium) or **2-TCO** (2.5 μM; DMSO%= 0.1% in final medium). Cells were incubated with click probes for 30 minutes at 37 ˚C in the incubator. The cells were quickly washed with ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS) (5 mL, one time) and immediately lysed with ice-cold Lysis Buffer (500 μL). Cells were scrapped into pre-chilled 1.5-mL Eppendorf tubes and were centrifuged at 17,000 × g for 20 minutes at 4 ˚C. The supernatant of lysates was collected, and protein concentration was measured using Pierce 660 nm Protein Assay. The protein concentration of lysates was diluted to 1.5 mg/mL with ice-cold lysis buffer.

Capture probe-bound kinase complexes using Tz-Beads: For each co-clickable precipitation experiment, 15 μL (volume of drained beads) of Tz-Beads were added to a 1.5-mL Eppendorf tube and were washed with ice-cold lysis buffer (three times; 400 μL for each wash). 750 μg of protein lysates (1.5 mg/mL) was added to Tz-Beads (500 μL) and was gently incubated on an end-to-end rotator for 30 mins at 4 ˚C. To reduce background contaminants, lysates were pre-cleared by

incubating with 15 μL (volume of drained beads) of sepharose beads coupled to ethanolamine for 1 hour at 4 ˚C prior to incubation with Tz-beads. After incubation with Tz-Beads, the flow-through was quickly removed by aspiration. The beads were then washed with ice-cold Washing Buffer **1** by gently inverting the tube for four times (three times; 400 μL for each wash), followed by washing with Washing Buffer **2** by gently inverting the tube for four times (three times; 400 μL for each wash). The bait protein and its interactors should be enriched and bound to the Tz-Beads by the end of this step.

Characterization of kinase complex using Western-blot analysis: To detect the components of proteins enriched by Tz-Beads using Western-blot analysis, the beads were incubated with Lysis Buffer containing 2% SDS (by weight) for 20 mins (three times; 40 μL for each elution) at 25 ˚C with frequent agitation. The elutions were collected. The eluted proteins were processed using SDS-PAGE gel electrophoresis, transferred onto nitrocellulose membranes and immunoblotted with corresponding primary antibodies. The western-blots were imaged on LI-COR Odyssey IR imager.

On-bead tryptic digestion of kinase complexes for tandem mass spectrometry analysis: To detect enriched bait and interactors using LC/MS, the washed beads were resuspended in 50 μL of Denaturing Buffer and were boiled at 95 ˚C for 5 minutes. After cooling down, 100 mM TEAB (50 μL) was added. The pH value was adjusted to 8 to 9 by adding NaOH (1 M). Lys-c Lysyl Endopeptidase (1 μg) was then added and incubated with the beads at 37 ˚C for 2 hours in a thermal mixer agitating at 1,400 rpm. After incubation, the suspension was diluted with 100 mM TEAB (100 μL) and MS Grade Trypsin (1 μg) was added. pH was adjusted to 8 to 9. The beads were incubated with Trypsin overnight at 37 ˚C in a thermal mixer agitating at 1,400 rpm. After incubation, Buffer A (200 μL) was added to the beads and trypsin was quenched by adjusting the

pH to 2 to 3 using formic acid. The resulting supernatant was desalted on C18 StageTip and washed once by buffer A (50 μL). The tryptic peptides were eluted off the C18 StageTip using buffer B (50 μL). The eluted solution was dried in speed-vac and then reconstituted with buffer A (20 μL) for LC/MS analysis.

Proteomic Data Acquisition, Process and Visualization of Target Interactome.

Proteomic Data Acquisition: Peptides were separated on a nanoAcquity UPLC instrument with 12 cm long fused silica capillary column (Polymicro Technologies Flexible Fused Silica Capillary Tubing, Inner Diameter 75 μm, Outer Diameter 375 μm, cat. no. 1068150019) made in-house with a laser puller and packed with 5 μm 120 Å reversed phase C18 beads (ReproSil-Pur 120 C18-AQ, Dr. Maisch GmbH HPLC, cat. no. r15.aq.). The LC gradient was 90 min long with 10-35% B at 200 nL/min. LC solvent A was 0.1% acetic acid and LC solvent B was 0.1% acetic acid, 99.9% acetonitrile. MS data were collected with a Thermo Scientific Orbitrap Fusion Tribrid mass spectrometer.

Proteomic Data Process: Raw data were analyzed using MaxQuant and search engine Andromeda using the following default settings: Multiplicity was set to 1; Digestion was set to trypsin/P; Variable modifications were set to Oxidation (M) and Acetyl (Protein N-term); LFQ minimal ratio count was set to 2; LFQ minimal number of neighbors set to 3; LFQ average number of neighbors set to 6; MS/MS spectra were searched against the UniProt human database (updated July 22nd, 2015); False discovery rate (FDR) was set to 0.01; First search and MS/MS search mass tolerance was set to 10 ppm; The data in the Protein Groups result table was processed with Perseus software. MaxQuant intensity values of identified proteins were used for all downstream analysis. We only consider proteins identified if MaxQuant was able to compute corresponding protein intensity values. Identified proteins that were classified as "only identified by site", "reverse", or "contaminant" were filtered and removed from the table. Proteins with 1 or fewer peptide counts were filtered and removed. All intensity values were transformed based on log2 algorithm. Missing protein intensity values for identified proteins were imputed using Perseus by sampling from a distribution downshifted by 1.3 and having a width of 0.2[5, 6]. Imputed values are reported in the

SI-Excel file 1. P-value was calculated using two-tailed, two-sample T-test. Enrichment scores ($\log_2$[intensity (Src V284C-expressing)/intensity (Src WT-expressing)]) were calculated using the relative intensity values from Src V284C- and WT-expressing cells with confidence values (-$\log_{10}$(P-value)) for each identified protein. We defined significant interactors as protein hits with an enrichment score ≥ 1.0 and a confidence value ≥ 1.4.

Visualization of Target Interactome: The list of gene names for significant Src interactors were uploaded to STRING: functional protein association networks database (version 10.5). The organism was set to *Homo sapiens*. The meaning of network edges was set to the confidence of the interaction, where the line thickness indicates the strength of data support as calculated by the STRING network database.[1] The edge confidence was indicated in Figure 4E. The minimum required interaction score is 0.400. The colored nodes represent query proteins. Each node represents all the proteins produced by a single, protein-coding gene locus. The splice isoforms or post-translational modifications are collapsed into the same node.

Clickable Proximity Ligation Assays (PLA).

Cell seeding: (for Figure 6B and Figure 6C) 40 μL of 0.8×10^5 cells/mL of Hela cells expressing Src-V284C or Src-WT were plated in Grace Bio-labs CultureWell chambered coverglass (8 wells, well diam. × thickness 6 mm × 1 mm, well volume 15-30 μL; Sigma-Aldrich; Catalog number: GBL103380) in DMEM supplemented with 10% FBS. The cells were then incubated until reaching 30-40% confluency in a humidified incubator at 37 ˚C containing 5% CO_2; **(for Figure 6D)** Prior to cell seeding, chambered coverglass was incubated with 40 μL of 1 mg/mL Fibronectin solution for 30 minutes at 37 ˚C for 30 mins and then rinsed with DPBS once. 40 μL of 0.8×10^5 cells/mL of Hela cells expressing Src-V284C or Src-WT were plated in Grace Bio-labs CultureWell chambered coverglass (8 wells, well diam. × thickness 6 mm × 1 mm, well volume 15-30 μL; Sigma-Aldrich; Catalog number: GBL103380) in DMEM supplemented with 10% FBS. The cells were then incubated until reaching 30-40% confluency in a humidified incubator at 37 ˚C containing 5% CO_2

Probe treatment: Cells were treated with 50 μL of 1-TCO (5 μM), 2-TCO (2.5 μM) or 3-TCO (10 μM) in DMEM supplemented with 10% FBS for 30 minutes.

Cell fixation and permeabilization: After incubation, the cells were gently washed with warm DPBS (40 μL) once and fixed with 40 μL of 4% paraformaldehyde for 15 minutes at room temperature. To quench the remaining para-formaldehyde, cells were then incubated with 40 μL of 125 mM of Glycine in DPBS for 5 mins. After washing with DBPS once, the cells were permeabilized using DPBS containing 0.75% Tween-20 and 0.75% Igepal CA-630 for 60 minutes at room temperature in a coplin jar on a shaker. The cells were then washed with DBPS once for 5 minutes at room temperature.

Click chemistry: Cells were treated with 40 μL of 500 nM Tetrazine-conjugated OregonGreen488 in DPBS for 10 minutes at room temperature. After the click chemistry reaction, the reaction solution was removed from chambered coverglass and the cells were washed with DPBS for 5 minutes. The washing step was repeated for six times in total.

Blocking and treatment with primary antibodies: Cells were blocked with blocking buffer (Sigma Duolink buffer) at 37 ˚C for 1 hour. After blocking, blocking buffer was aspirated and cells were then treated with 40 μL of a solution consisted of primary antibodies in Antibody Dilution Buffer (Sigma Duolink) for OregonGreen488 (anti-fluorescein/OregonGreen goat IgG fraction; Life technologies; Catalog number: A11095) and Src(anti-Src (36D10) Rabbit mAb; Cell Signaling Technology; Catalog #: 2109), EEA1 (anti-EEA1 (C45B10) Rabbit mAb; Cell Signaling Technology; Catalog #: 3288) or FAK (anti-FAK (D5O7U) XP Rabbit mAb; Cell Signaling Technology; Catalog #: 71433) at 4 ˚C overnight. After incubation, the cells were washed with Wash Buffer A (Sigma Duolink) for 5 minutes twice in a coplin jar on a shaker.

Treatment with secondary antibodies and proximity ligation: Cells were then treated with 40 μL of a solution containing Duolink in situ rabbit PLUS probes and Duolink in situ goat MINUS probes in Antibody Dilution Buffer at 37 ˚C for 1 hour. After incubation, the cells were washed with Wash Buffer A for 5 minutes for three times. 40 μL of Ligation solution containing DNA ligase in ligation buffer was added to the cells. Cells were incubated with ligase for 30 minutes at 37 ˚C. After DNA ligation, cells were washed with Wash Buffer A for 5 minutes twice. For rolling cycle amplification, cells were treated with 40 μL of amplification buffer consisted of Polymerase and polymerase buffer (Sigma Duolink In Situ Detection Reagents Orange; Catalog #: DUO92007) for exactly 100 minutes at 37 ˚C. After incubation, the cells were washed with Wash Buffer B for 10 minutes twice at room temperature in a coplin jar on a shaker. The excess wash

solution was removed. To stain nuclei, one drop of the Duolink In Situ mounting media containing DAPI was added to each well and the coverglass was mounted to a glass coverslip.

Imaging, data acquisition, and analysis: Cells were imaged using a Leica SP8X Confocal microscope equipped with a 63X objective (oil objective). Nuclei were imaged using laser excitation at 405 nm with 5% laser power. PLA puncta were imaged using laser excitation at 554 nm with 10% laser power. The number of PLA punctae was quantified using ImageJ software as following: Firstly, images were smoothened. Secondly, threshold was manually selected to discriminate PLA puncta from background fluorescence. Once selected, the threshold was applied uniformly to all images in the same sample set. The number of PLA puncta in each image was quantified using "Analyze Particles" function with the particle size of "0.75 μm^2-200 μm^2". The number of cells in each image was quantified using the number of nucleus. As an assay readout, the number of PLA puncta per cell was calculated. All data were visualized in violin plots using GraphPad Prism software.

Reference:

1. Szklarczyk, D.; Franceschini, A.; Wyder, S.; Forslund, K.; Heller, D.; Huerta-Cepas, J.; Simonovic, M.; Roth, A.; Santos, A.; Tsafou, K. P.; Kuhn, M.; Bork, P.; Jensen, L. J.; von Mering, C., STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res* **2015,** *43* (Database issue), D447-52.

2. Szklarczyk, D.; Morris, J. H.; Cook, H.; Kuhn, M.; Wyder, S.; Simonovic, M.; Santos, A.; Doncheva, N. T.; Roth, A.; Bork, P.; Jensen, L. J.; von Mering, C., The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res* **2017,** *45* (D1), D362-D368.

3. Lombard, C. K.; Davis, A. L.; Inukai, T.; Maly, D. J., Allosteric Modulation of JNK Docking Site Interactions with ATP-Competitive Inhibitors. *Biochemistry* **2018,** *57* (40), 5897-5909.

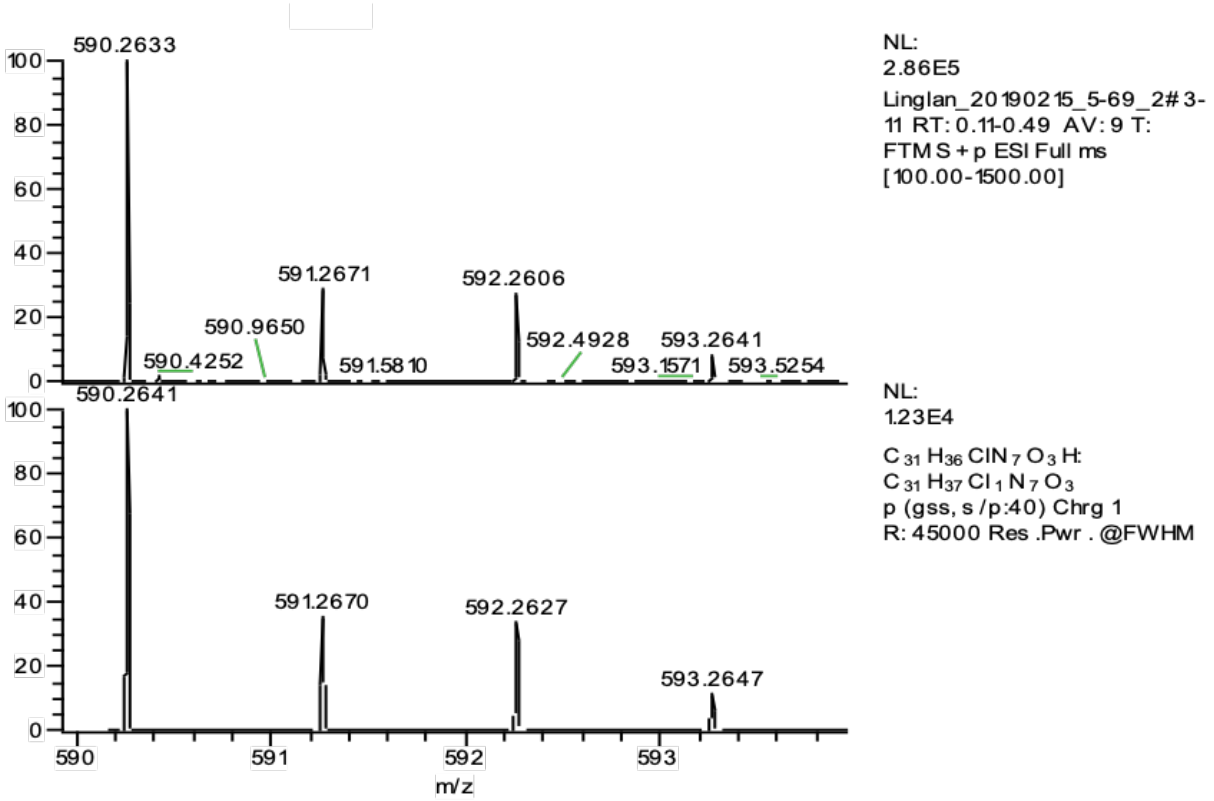
4. Chakraborty, S.; Inukai, T.; Fang, L.; Golkowski, M.; Maly, D. J., Targeting Dynamic ATP-Binding Site Features Allows Discrimination between Highly Homologous Protein Kinases. *ACS Chemical Biology* **2019**, *14* (6), 1249-1259.

5. Tyanova, S.; Temu, T.; Sinitcyn, P.; Carlson, A.; Hein, M. Y.; Geiger, T.; Mann, M.; Cox, J., The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* **2016,** *13* (9), 731-40.

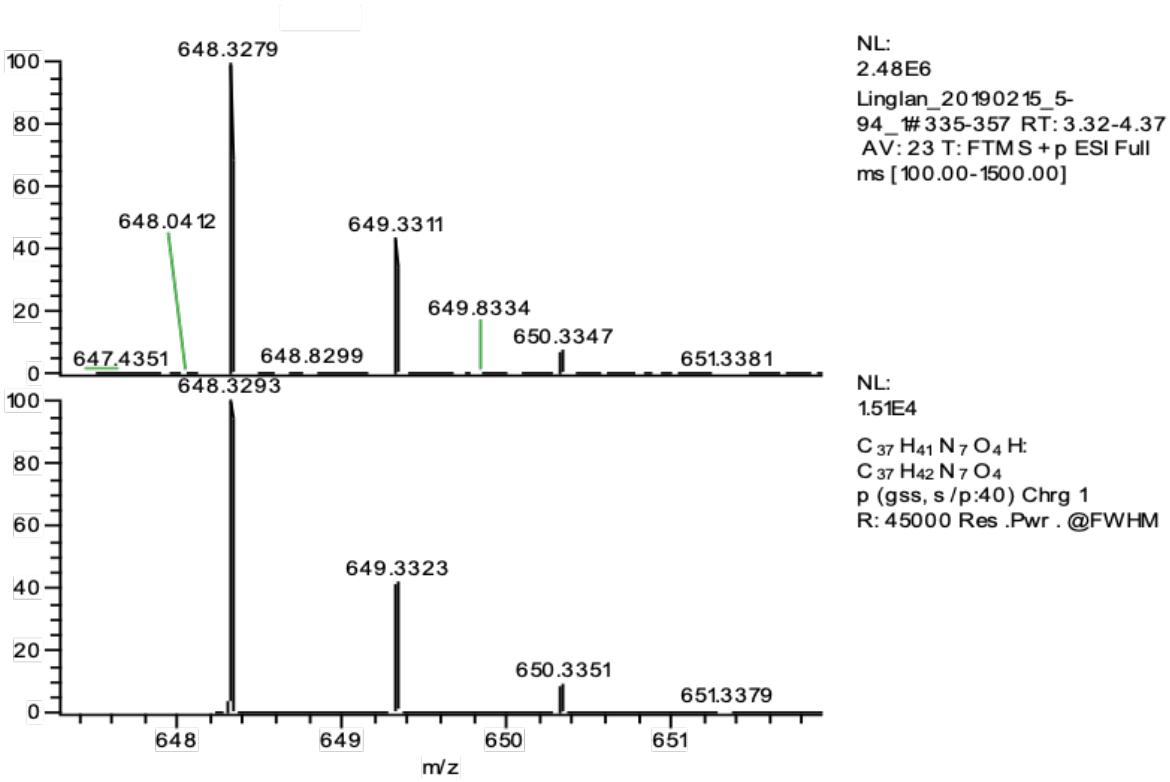
6. Golkowski, M.; Vidadala, R. S.; Lombard, C. K.; Suh, H. W.; Maly, D. J.; Ong, S. E., Kinobead and Single-Shot LC-MS Profiling Identifies Selective PKD Inhibitors. *J Proteome Res* **2017,** *16* (3), 1216-1227.

HRMS data:

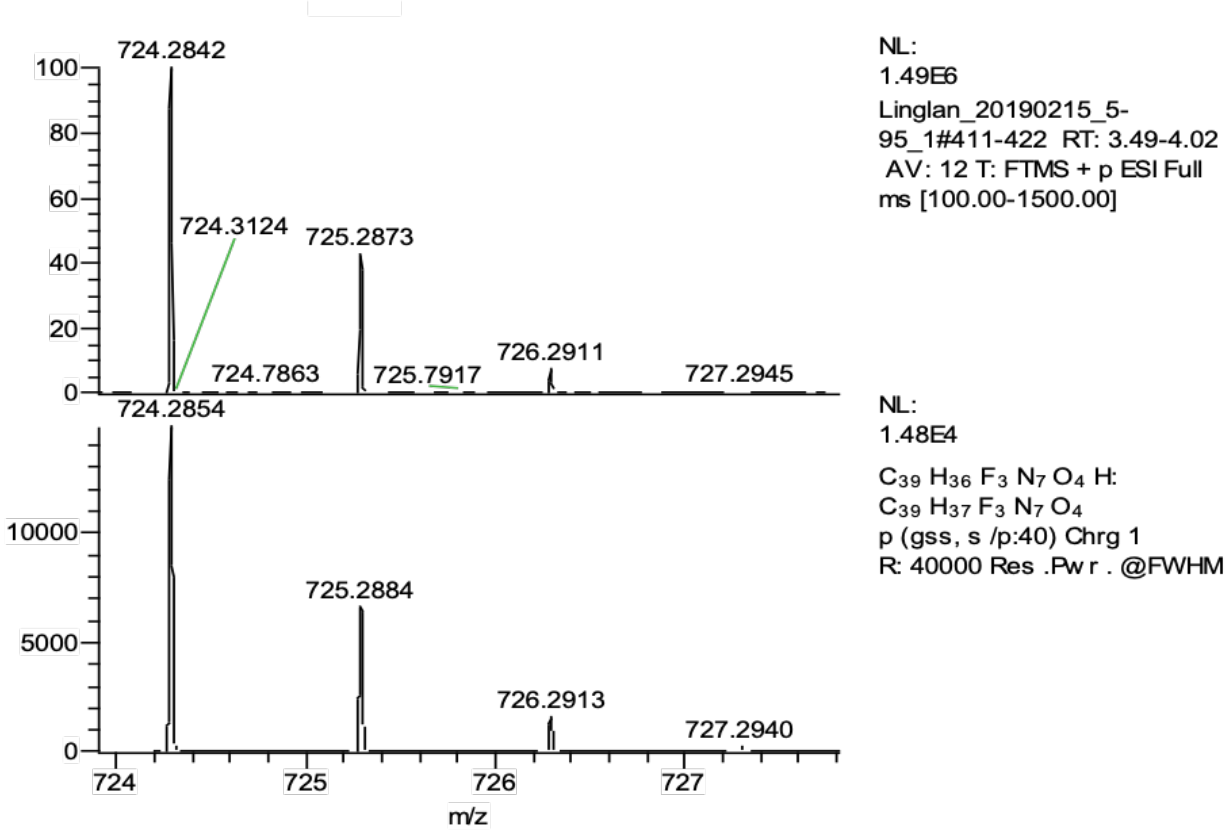
(*E*)-cyclooct-4-en-1-yl (3-(4-amino-5-(4-chlorophenyl)-6-((*E*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate or (*E*)-cyclooct-4-en-1-yl (3-(4-amino-5-(4-chlorophenyl)-6-((*Z*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (1-TCO)



(*E*)-cyclooct-4-en-1-yl (3-(4-amino-6-((*E*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate or (*E*)-cyclooct-4-en-1-yl (3-(4-amino-6-((*Z*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (2-TCO)

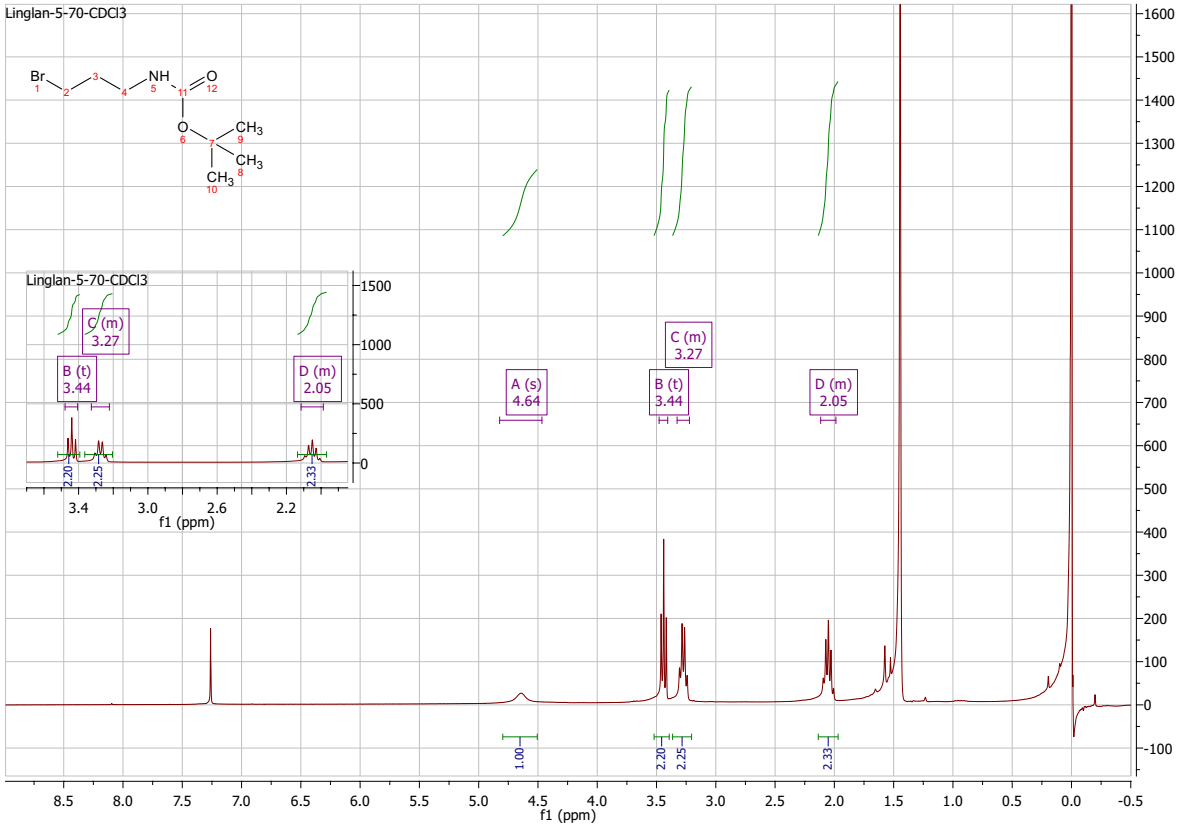


(*E*)-cyclooct-4-en-1-yl (3-(6-((*E*)-3-amino-2-cyano-3-oxoprop-1-en-1-yl)-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate or (*E*)-cyclooct-4-en-1-yl (3-(6-((*Z*)-3-amino-2-cyano-3-oxoprop-1-en-1-yl)-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (3-TCO)

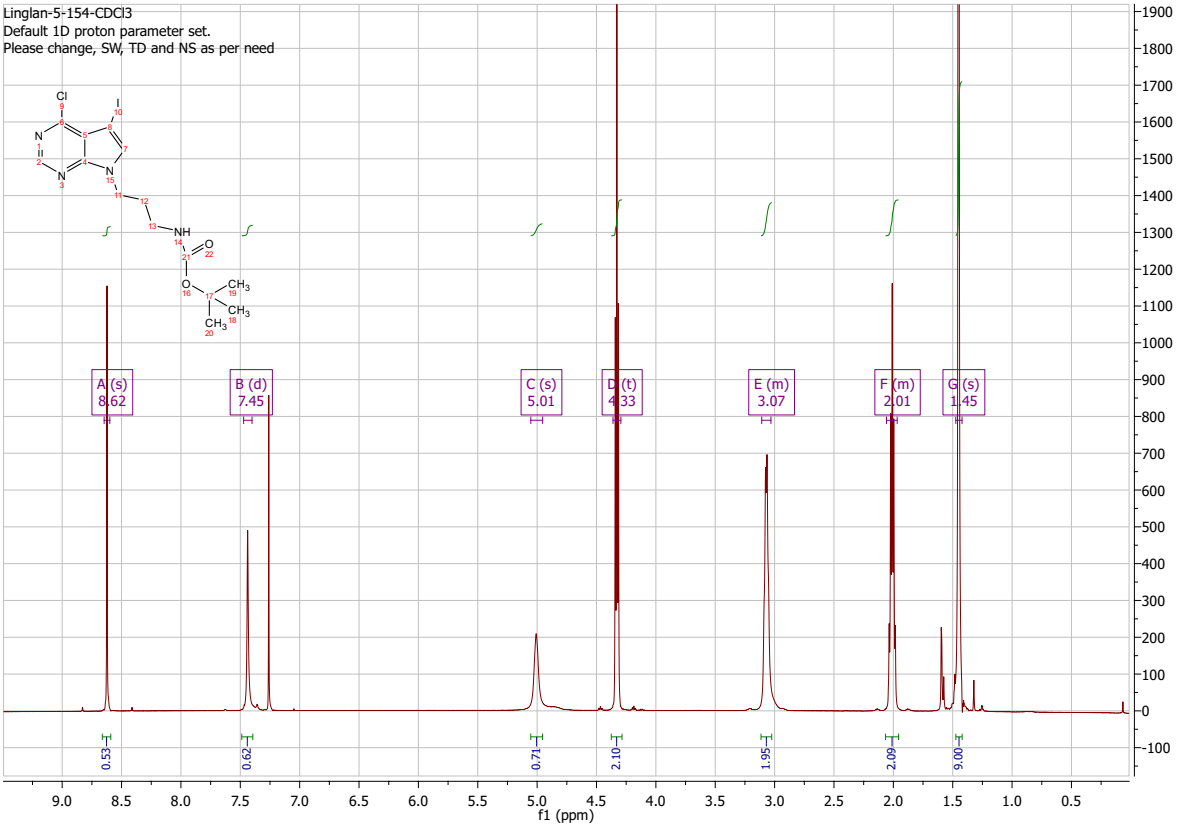


NMR spectra:

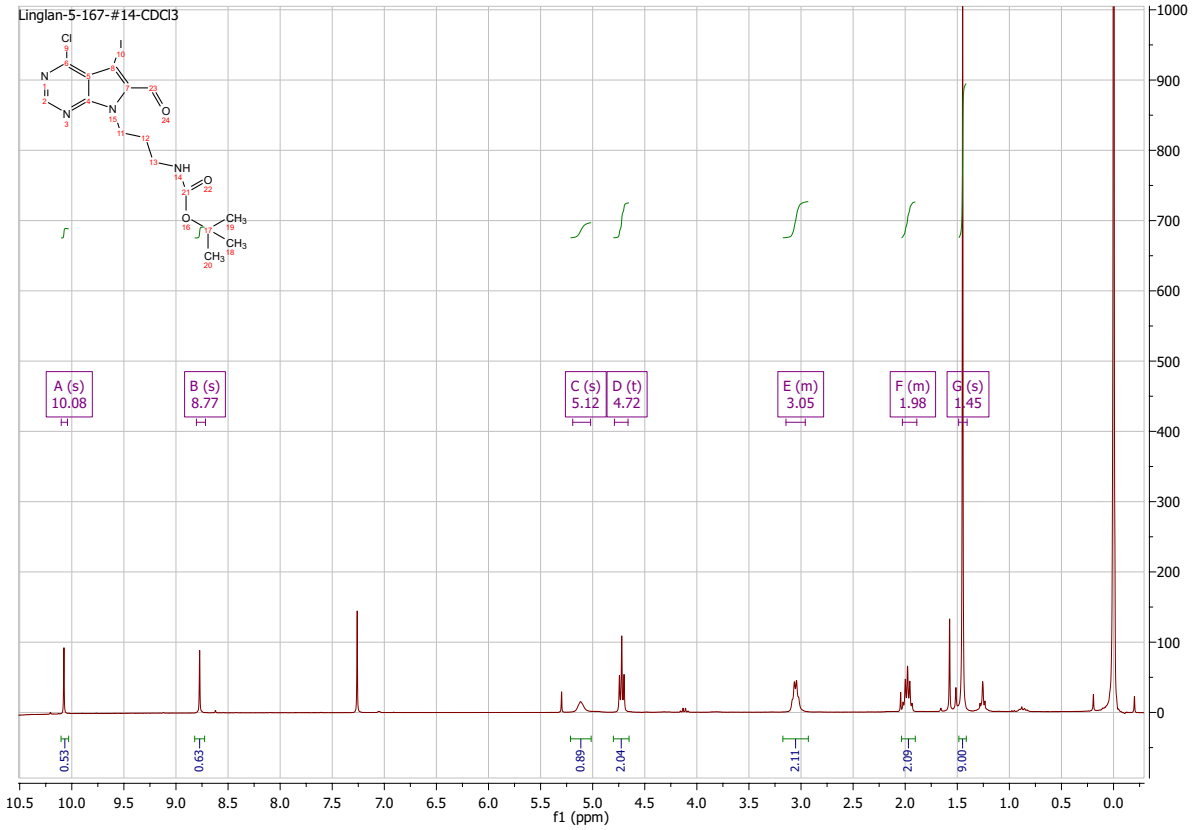
Tert-butyl (3-bromopropyl)carbamate (4)



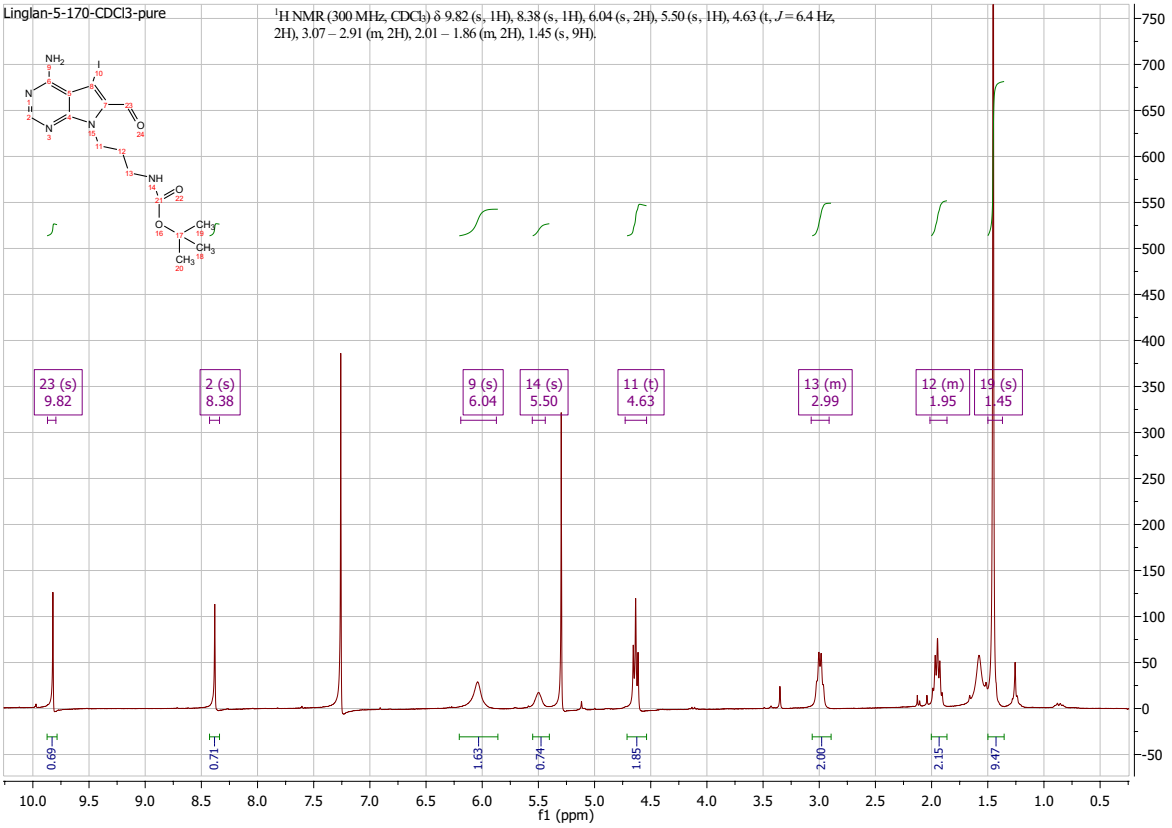
Tert-butyl (3-(4-chloro-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (5)



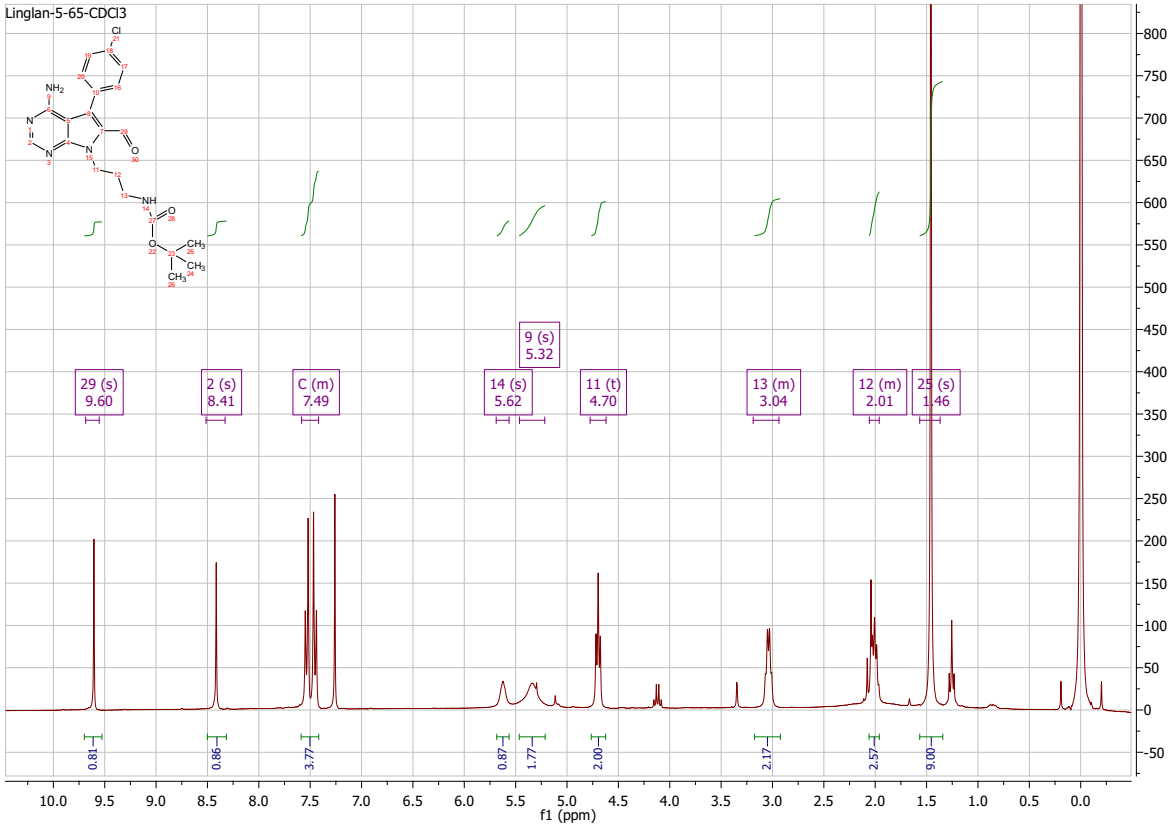
***Tert*-butyl (3-(4-chloro-6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (6)**



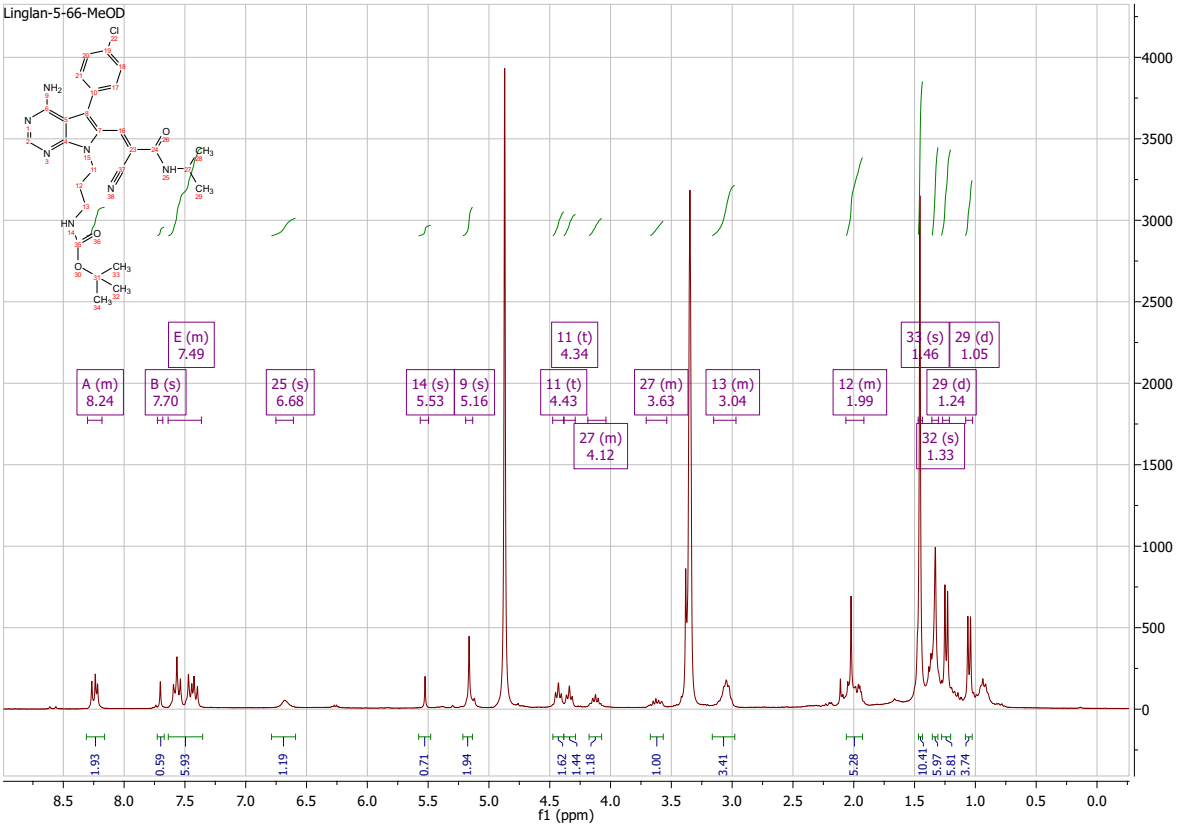
***Tert*-butyl (3-(4-amino-6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (7)**



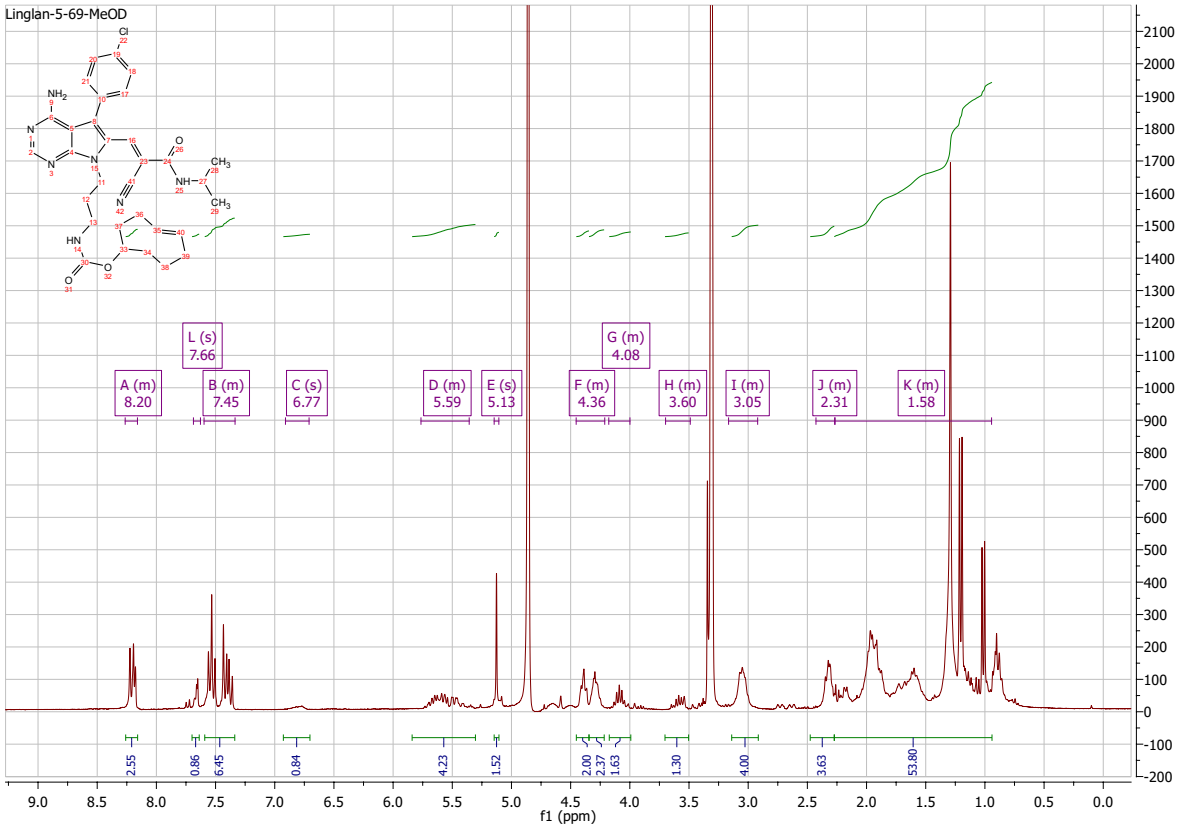
***Tert*-butyl (3-(4-amino-5-(4-chlorophenyl)-6-formyl-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (8)**



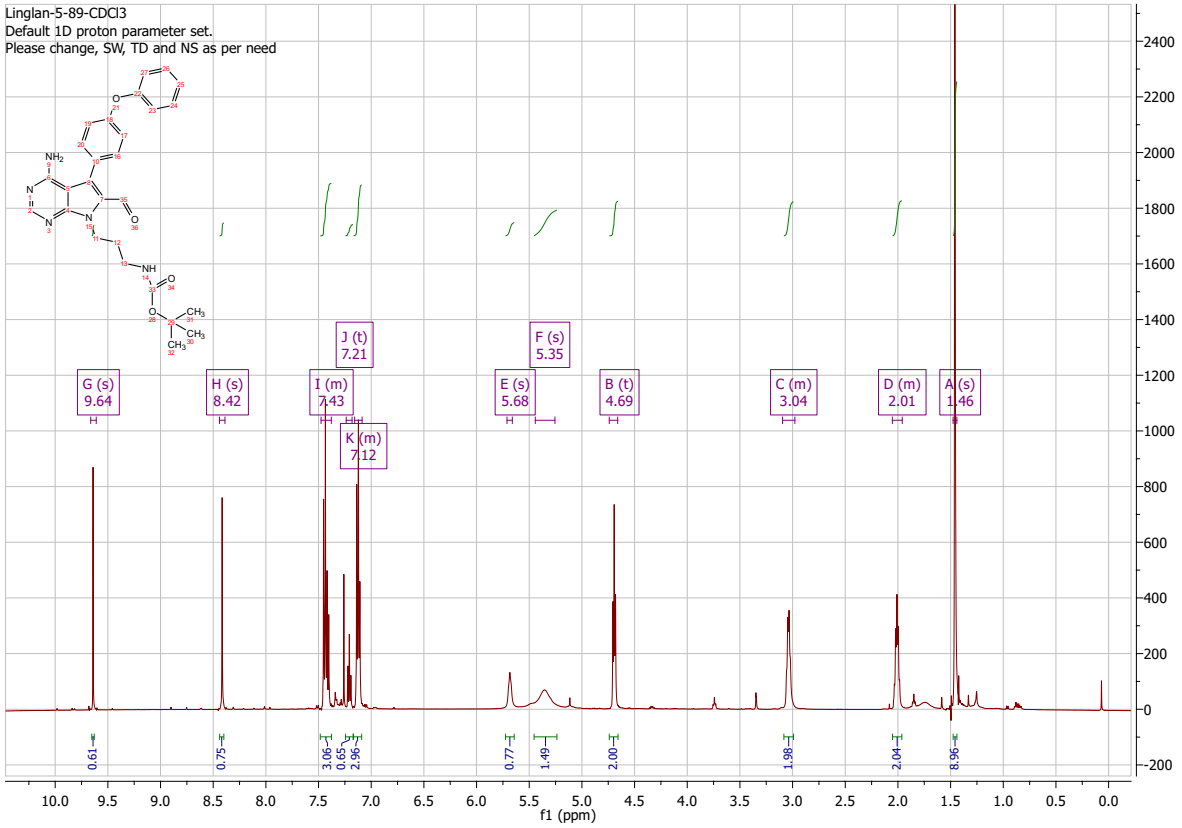
(*Z*)- or (*E*)-*tert*-butyl (3-(4-amino-5-(4-chlorophenyl)-6-(2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (9)



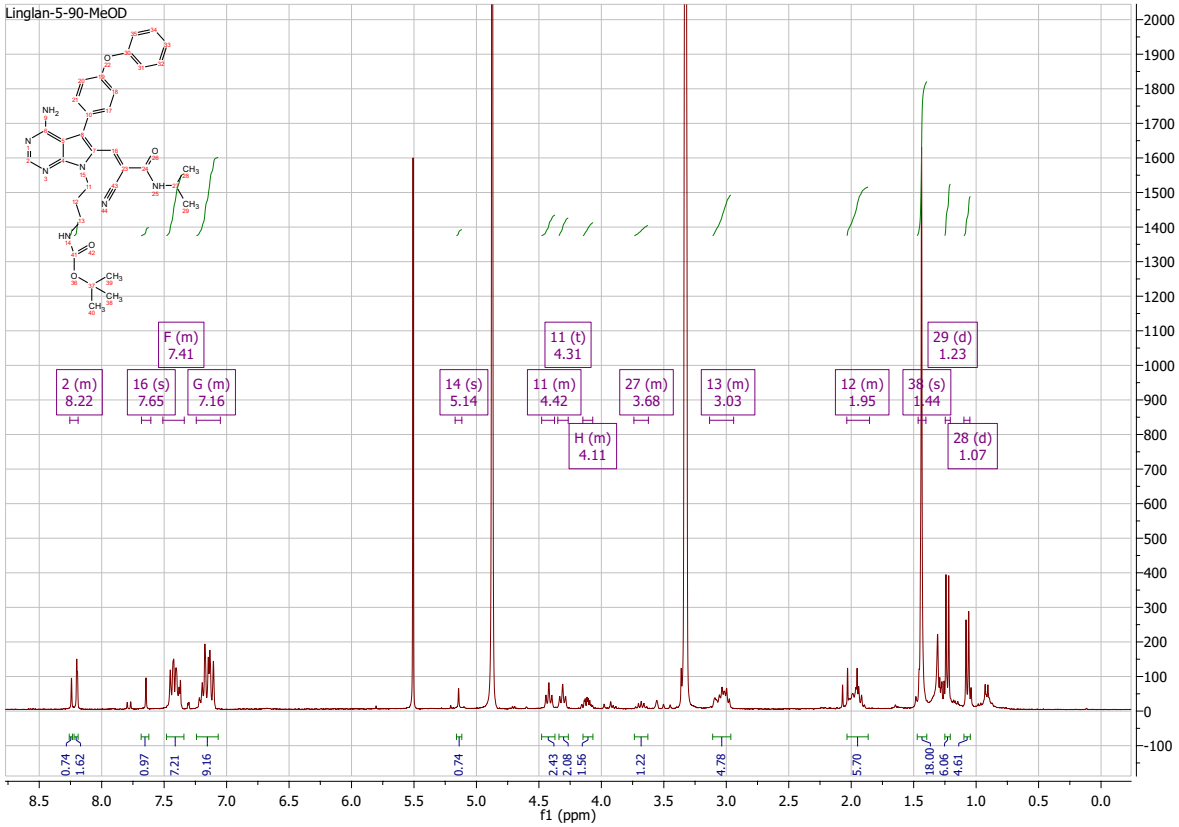
(*E*)-cyclooct-4-en-1-yl (3-(4-amino-5-(4-chlorophenyl)-6-((*E*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate or (*E*)-cyclooct-4-en-1-yl (3-(4-amino-5-(4-chlorophenyl)-6-((*Z*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (1-TCO)



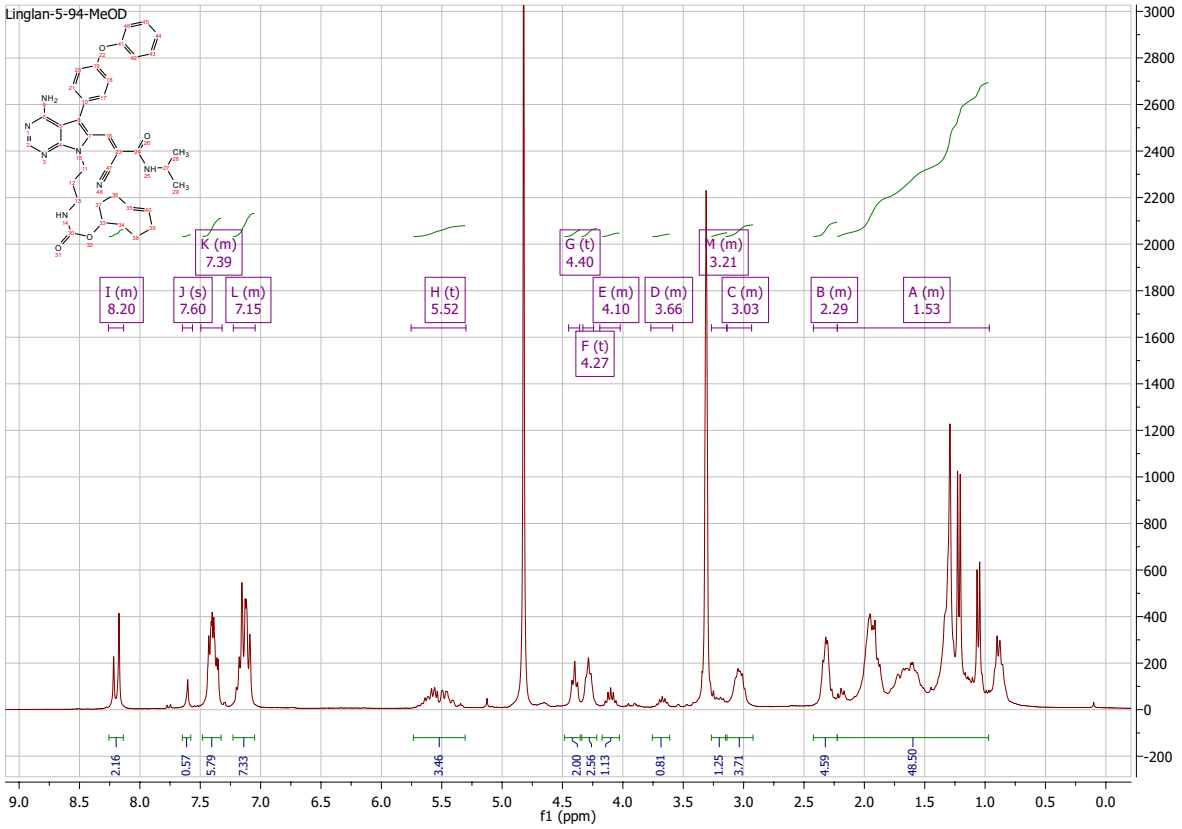
***Tert*-butyl (3-(4-amino-6-formyl-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (10)**



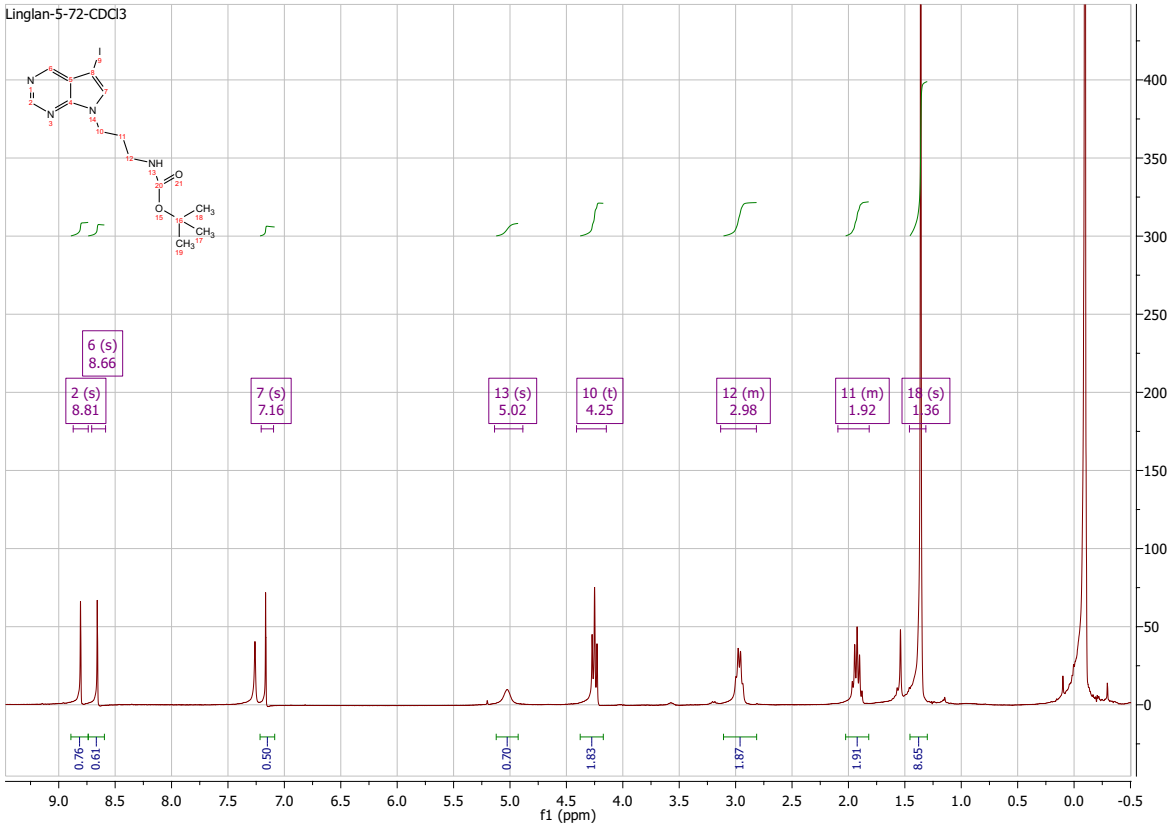
(*Z*)- or (*E*)-tert-butyl (3-(4-amino-6-(2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (11)



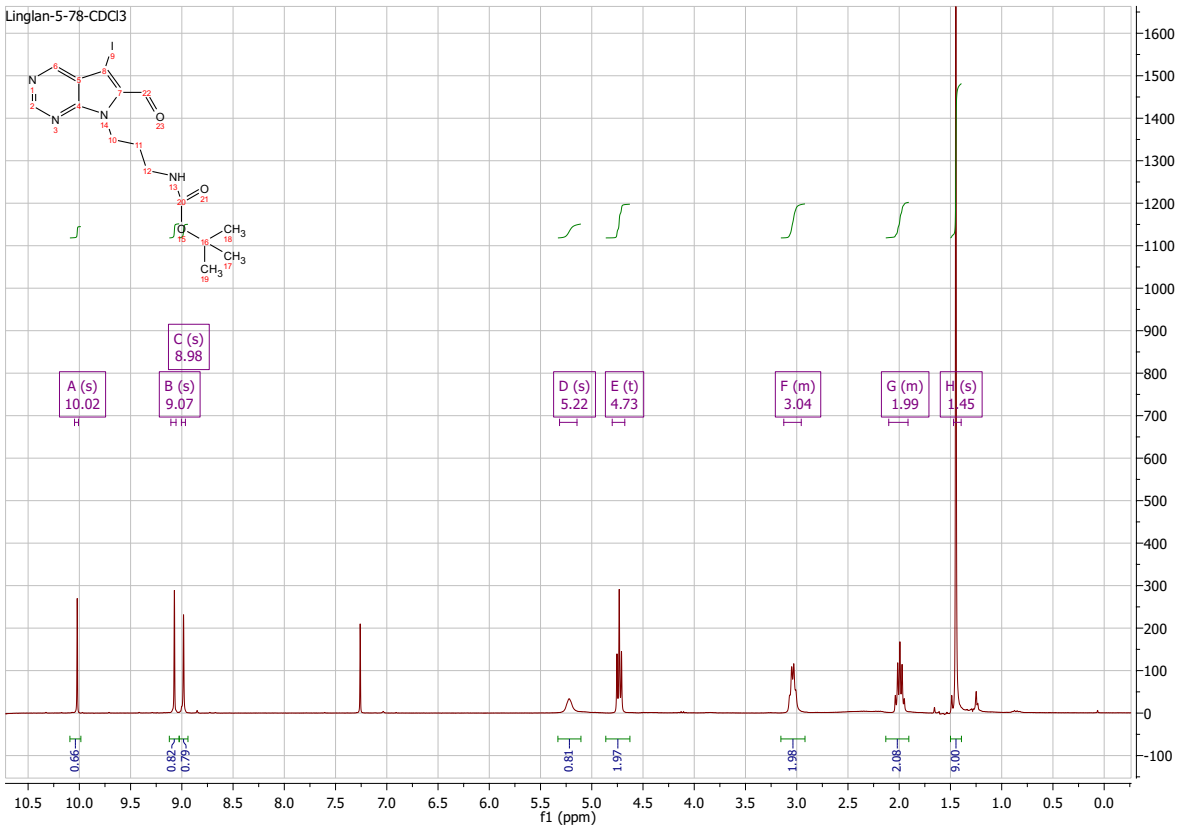
(*E*)-cyclooct-4-en-1-yl (3-(4-amino-6-((*E*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate or (*E*)-cyclooct-4-en-1-yl (3-(4-amino-6-((*Z*)-2-cyano-3-(isopropylamino)-3-oxoprop-1-en-1-yl)-5-(4-phenoxyphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (2-TCO)



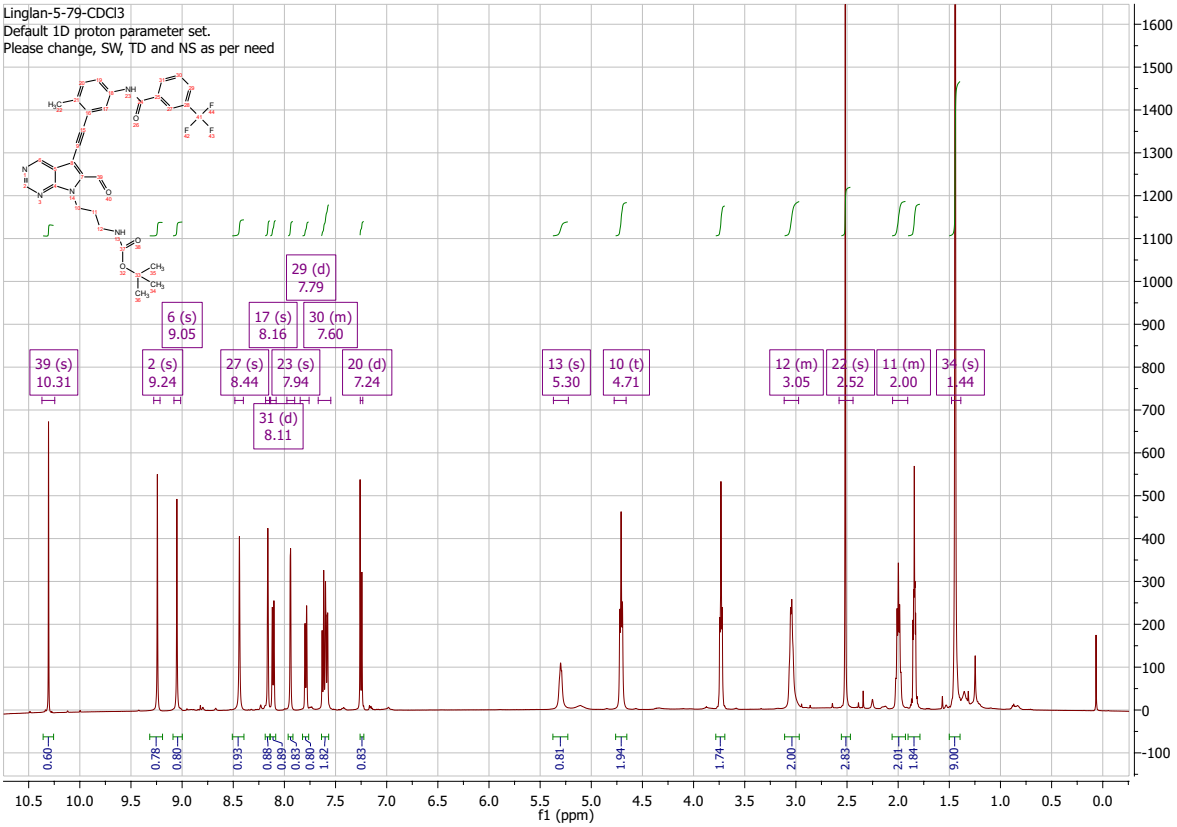
Tert-butyl (3-(5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (12)



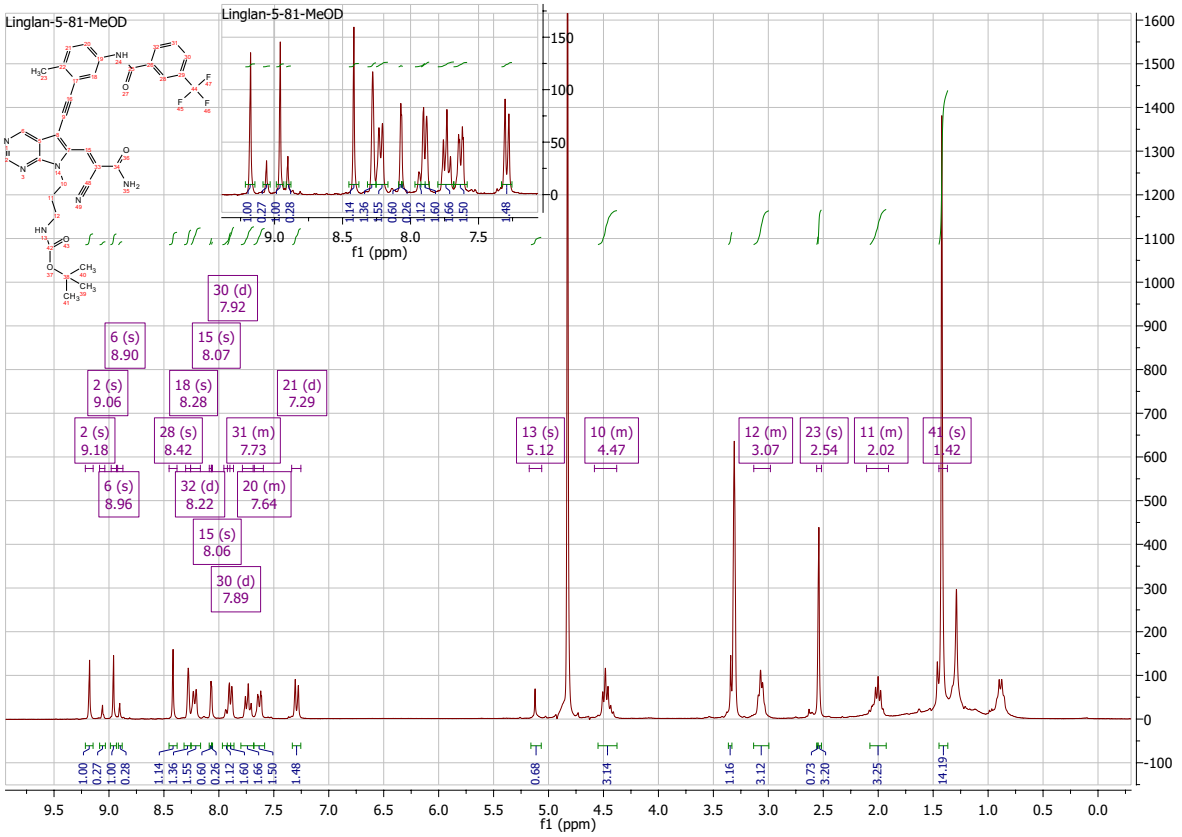
Tert-butyl (3-(6-formyl-5-iodo-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (13)



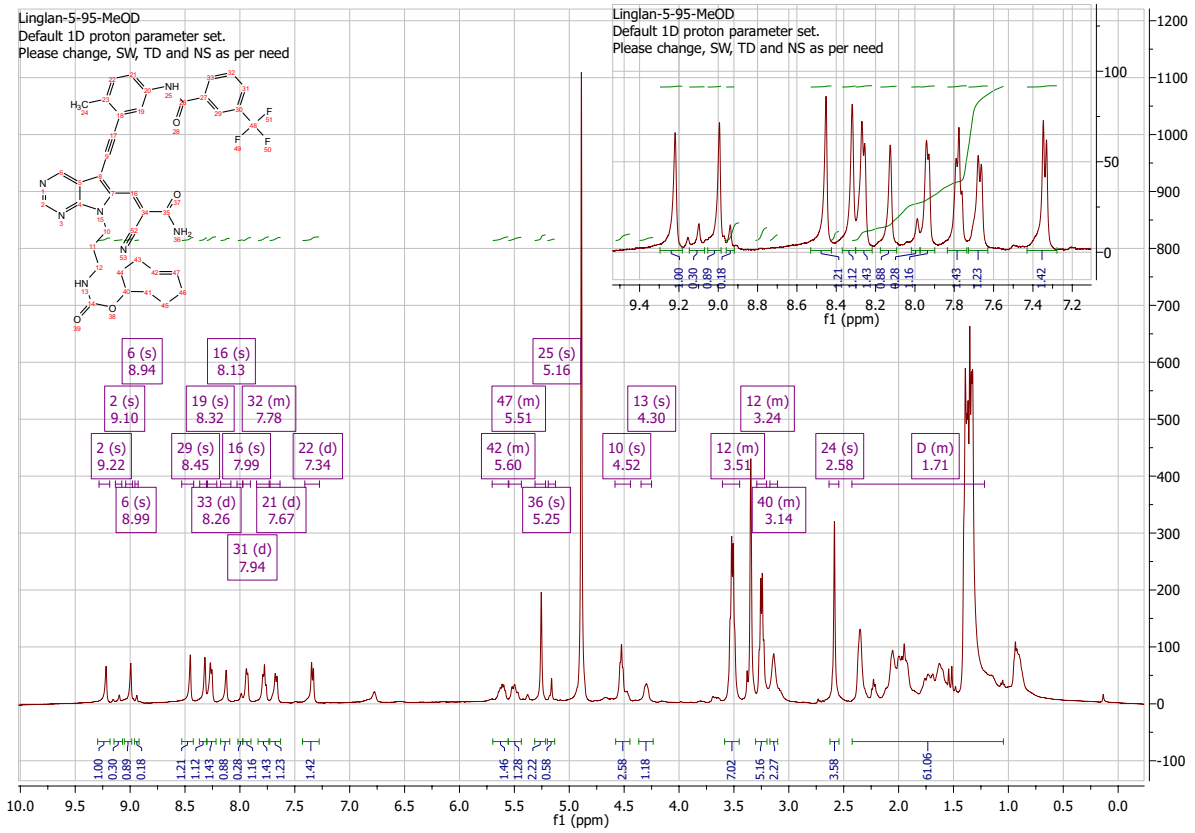
***Tert*-butyl (3-(6-formyl-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (14)**



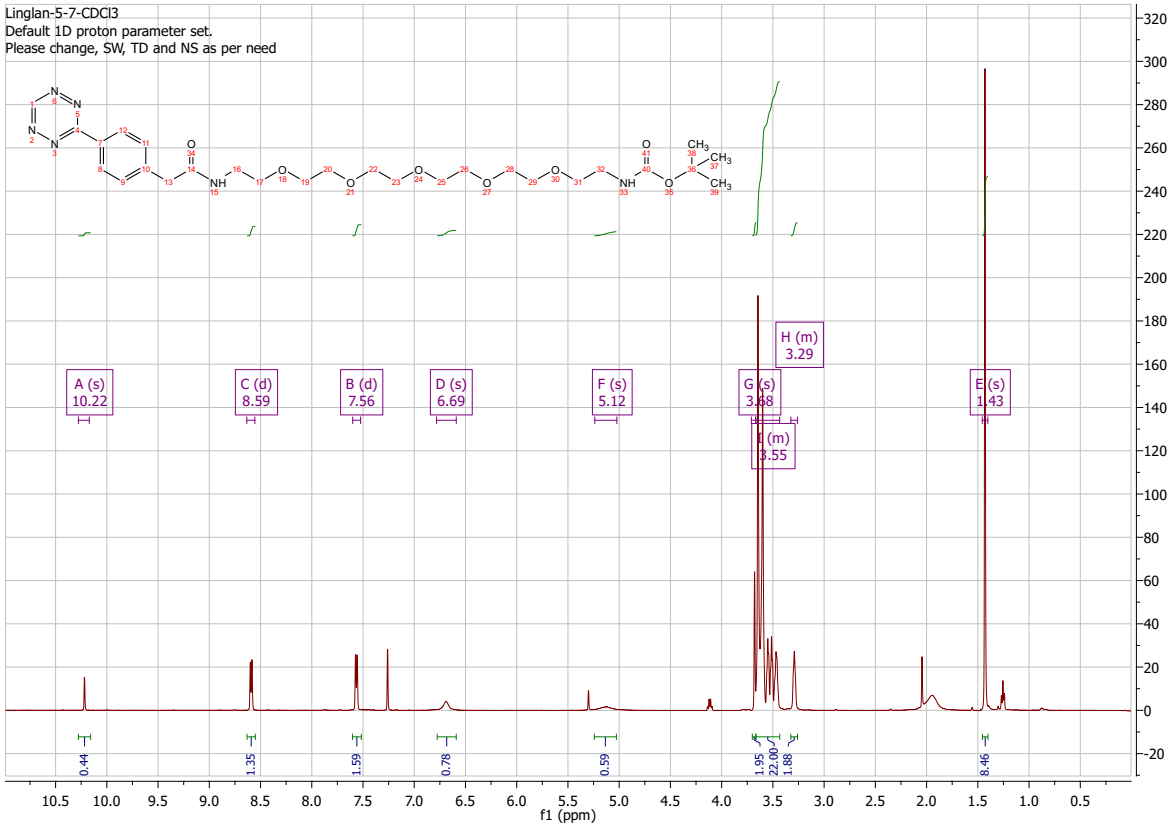
(*Z*)- or (*E*)-*tert*-butyl (3-(6-(3-amino-2-cyano-3-oxoprop-1-en-1-yl)-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (15)



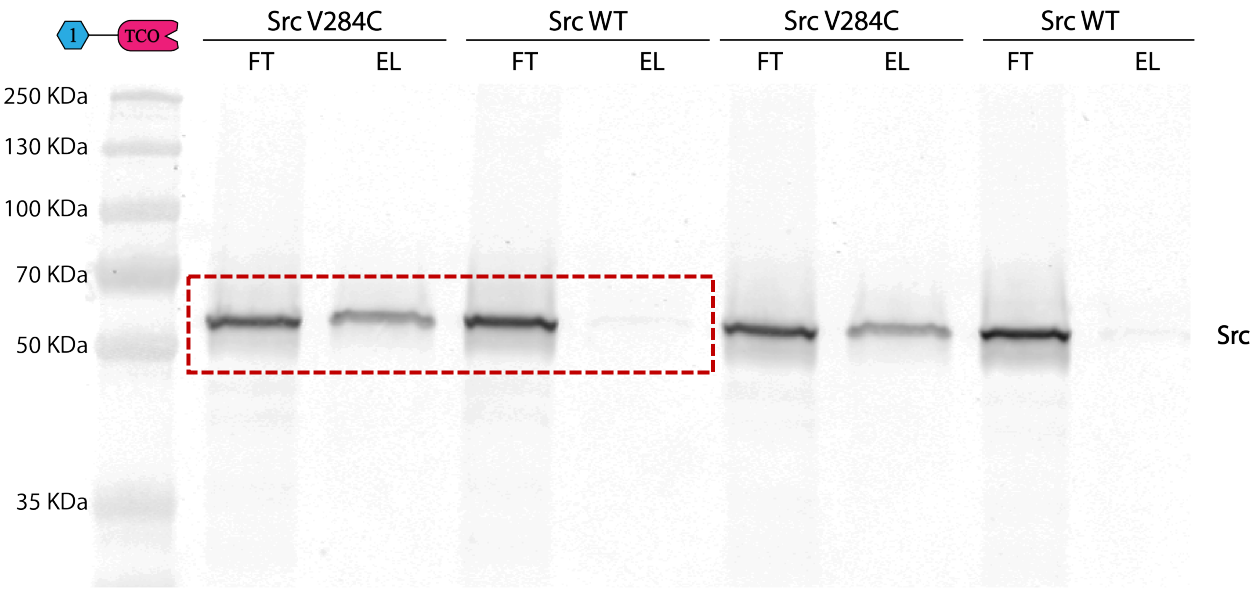
(*E*)-cyclooct-4-en-1-yl (3-(6-((*E*)-3-amino-2-cyano-3-oxoprop-1-en-1-yl)-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate or (*E*)-cyclooct-4-en-1-yl (3-(6-((*Z*)-3-amino-2-cyano-3-oxoprop-1-en-1-yl)-5-((2-methyl-5-(3-(trifluoromethyl)benzamido)phenyl)ethynyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)propyl)carbamate (3-TCO)



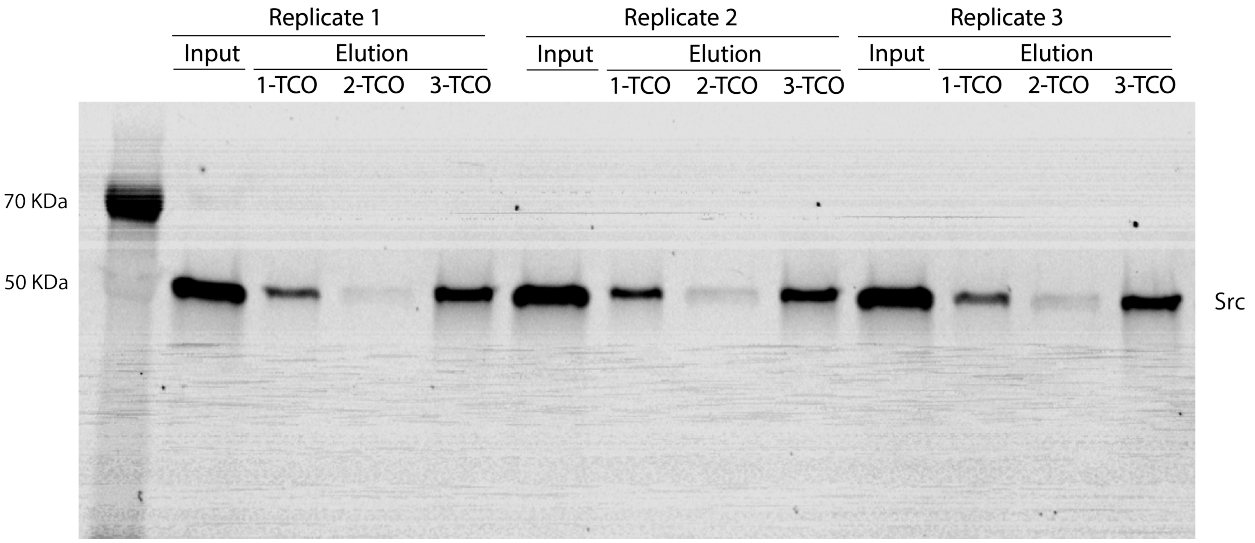
***Tert*-butyl (1-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-2-oxo-6,9,12,15,18-pentaoxa-3-azaicosan-20-yl)carbamate**



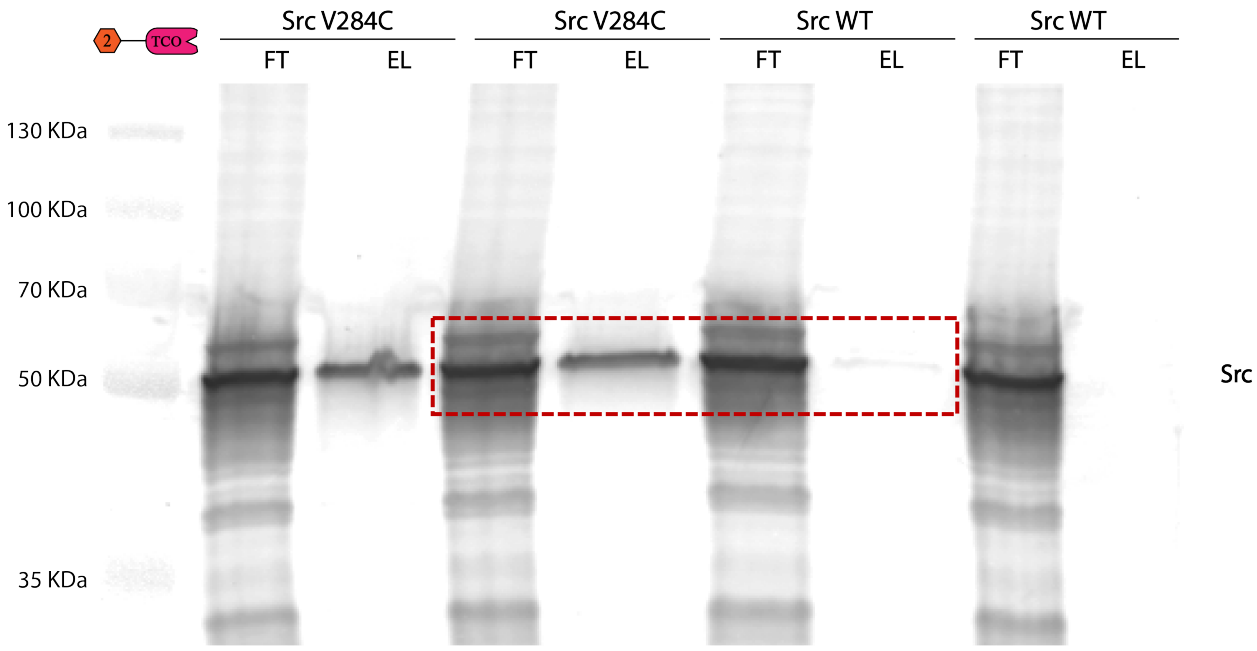
Immunoblots for Figure 2E (contain protein ladders)



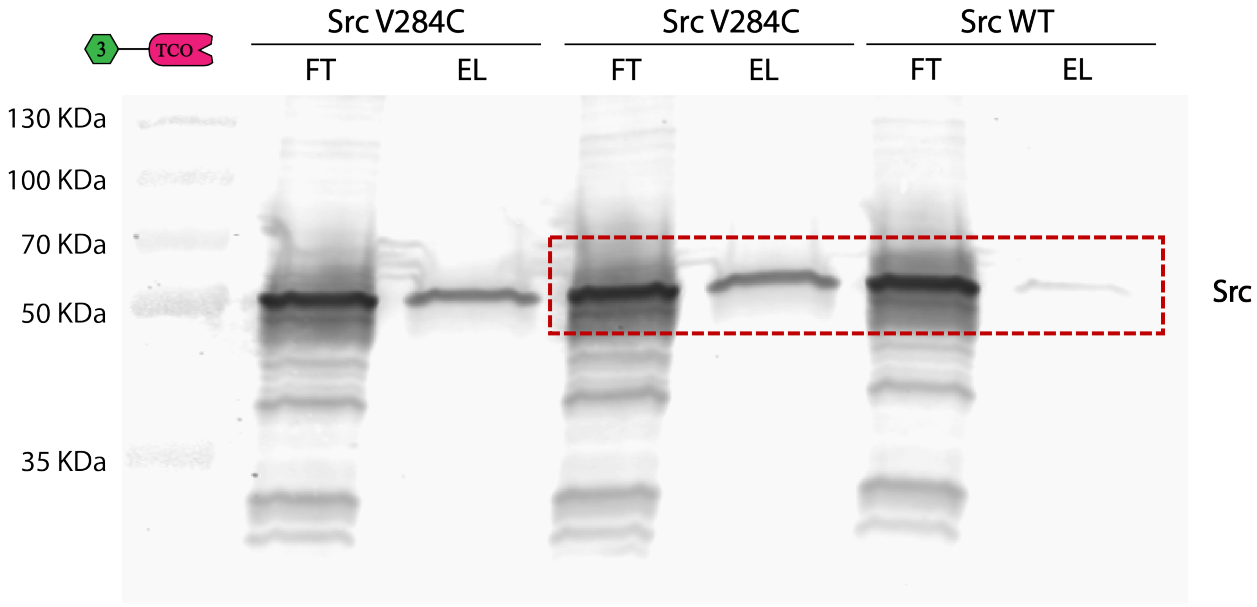
Immunoblots for Figure 3C (contain protein ladders)



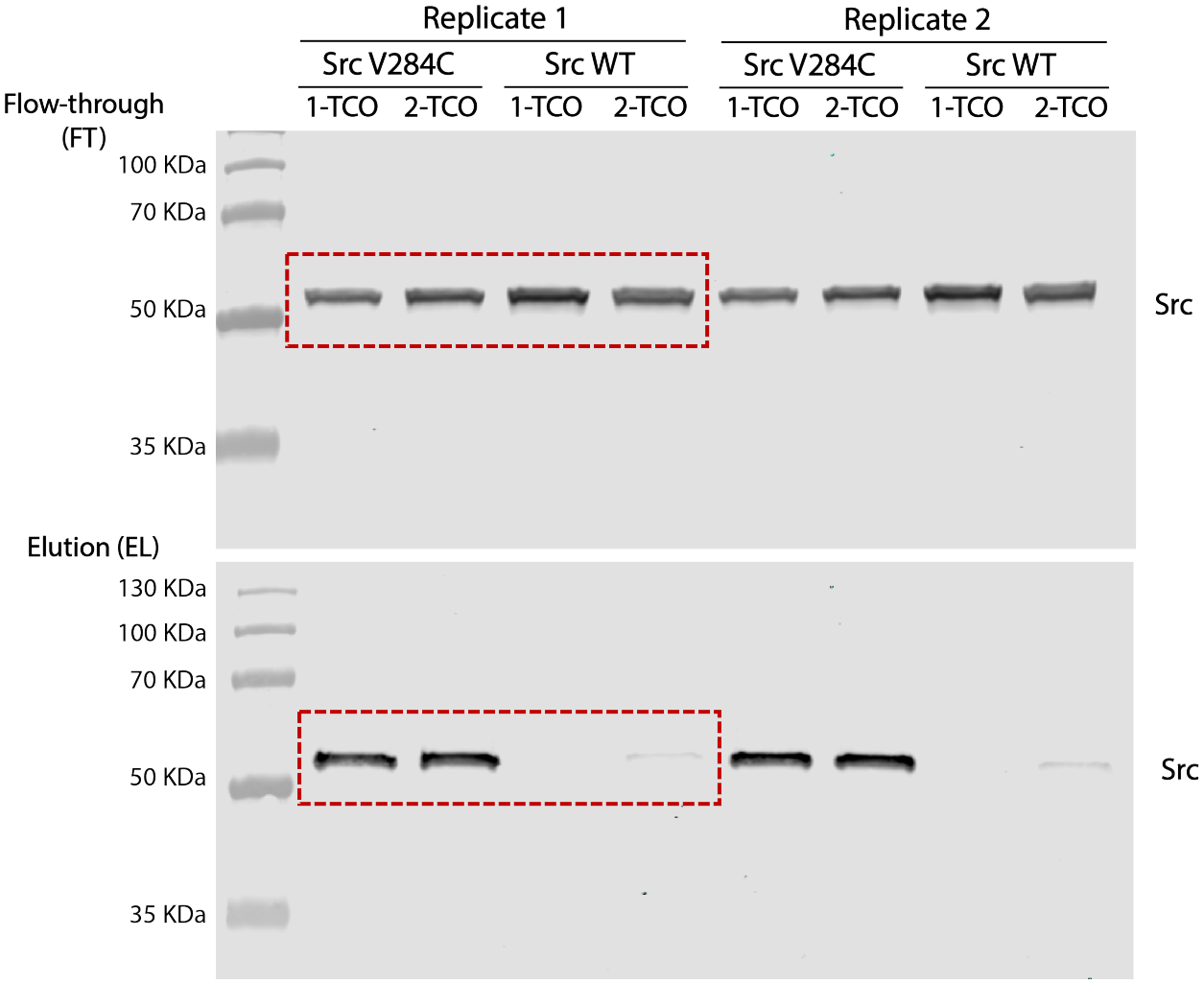
Immunoblots for Figure 3D (*top*) (contain protein ladders)



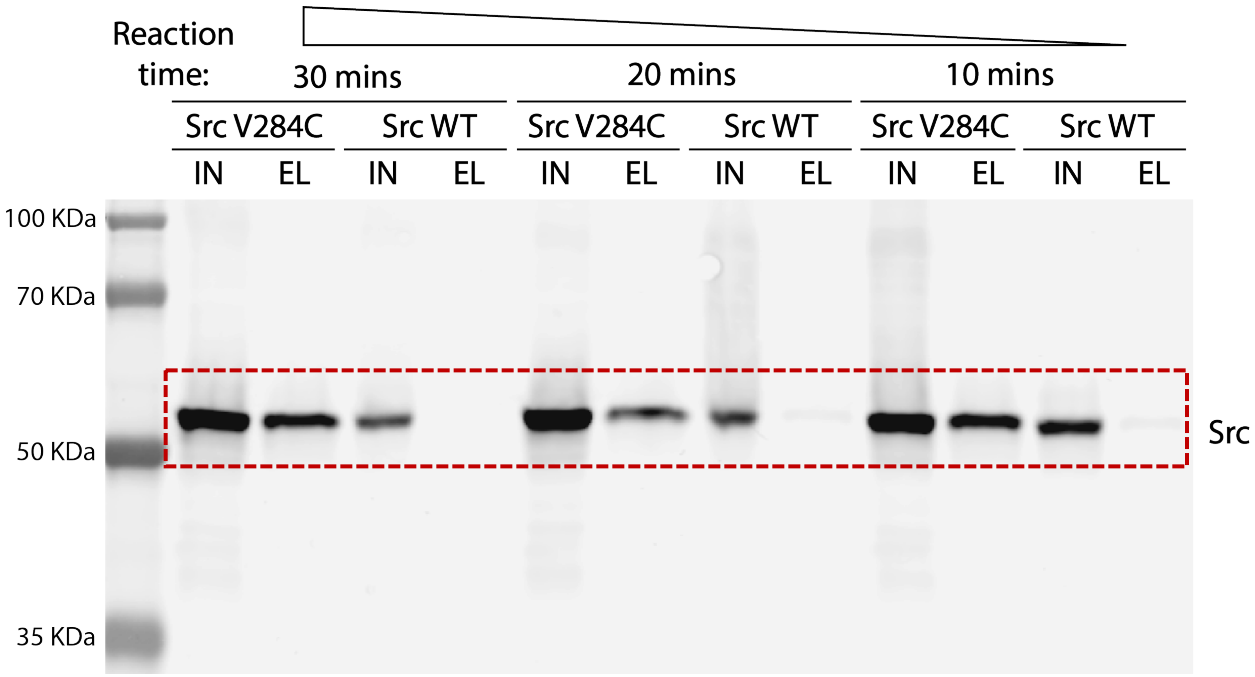
Immunoblots for Figure 3D (*bottom*) (contain protein ladders)



Immunoblots for Figure 3F (contain protein ladders)

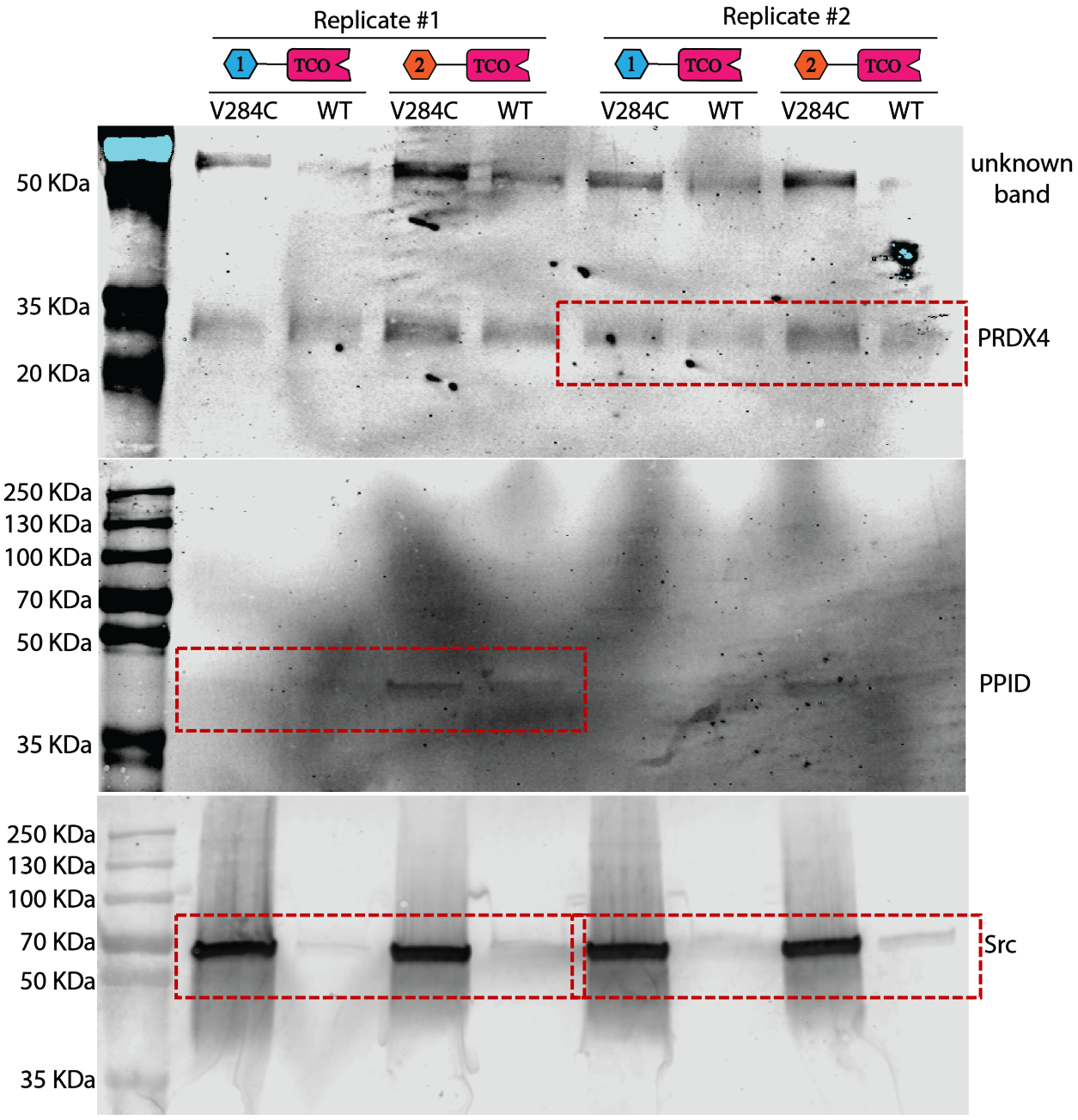


Immunoblots for Figure 3G (contain protein ladders)

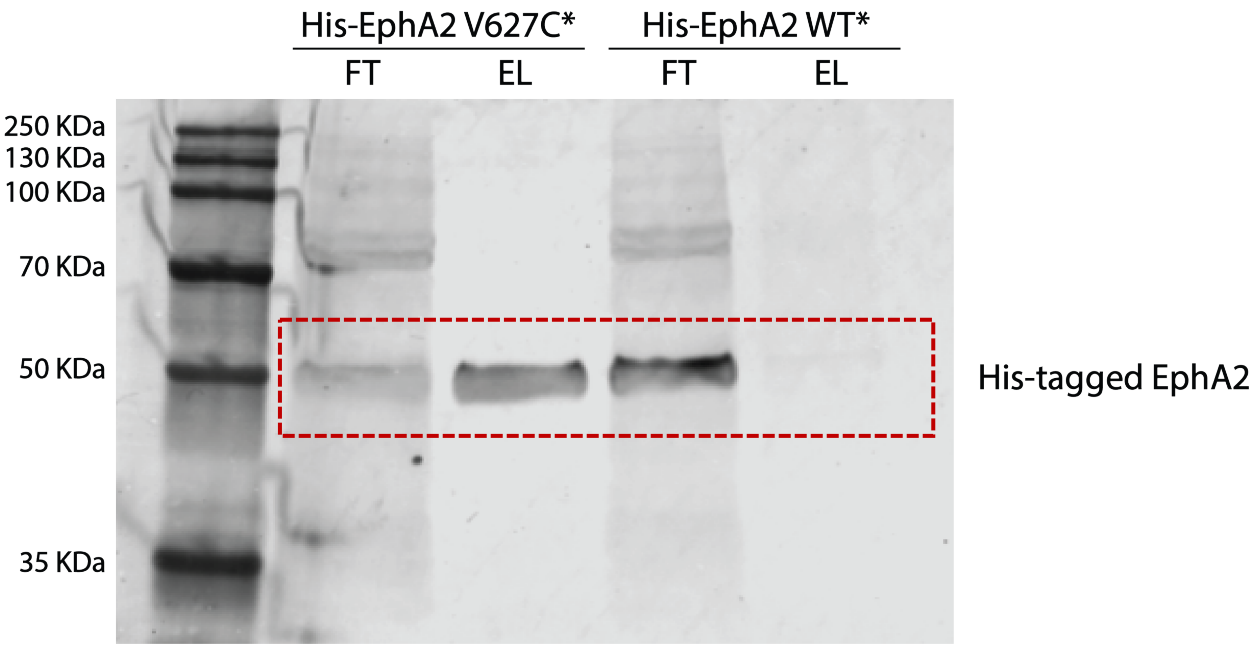


In vitro activity assay for determining IC50 values of 1, 2 and 3 against Src, EphA2 and JNK2.

Immunoblots for Figure S6



Immunoblots for Figure S3B



Immunoblots for Figure S3C, S4C