

Supporting Information For

Affinity-Based Probes Based on Type II Kinase Inhibitors

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I. Supplementary Figures

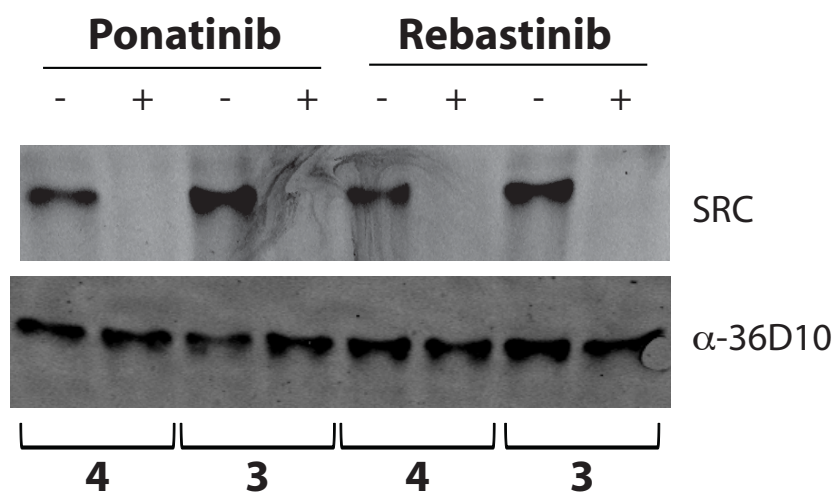


Figure S1: Type II inhibitors based on diverse scaffolds are able to prevent active site labeling of DFG-out adopting kinases. SRC kinase (90 nM) in mammalian cell lysate (0.2 mg/mL) was incubated with probes **3** (1 μ M) or **4** (1 μ M) in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of the type II inhibitors Ponatinib and Rebastinib (10 μ M). Ponatinib and Rebastinib are potent inhibitors of SRC kinase activity ($IC_{50} = < 10$ nM). All samples were irradiated with UV light for 15 minutes. Following irradiation, all samples were then tagged with rhodamine-azide, resolved by SDS-PAGE, and labeled proteins were detected with in-gel fluorescence scanning (top blots). Immunoblots were performed with a SFK-selective antibody (anti-36D10; Cell Signaling) to ensure equal amounts of kinase were present (bottom blots).

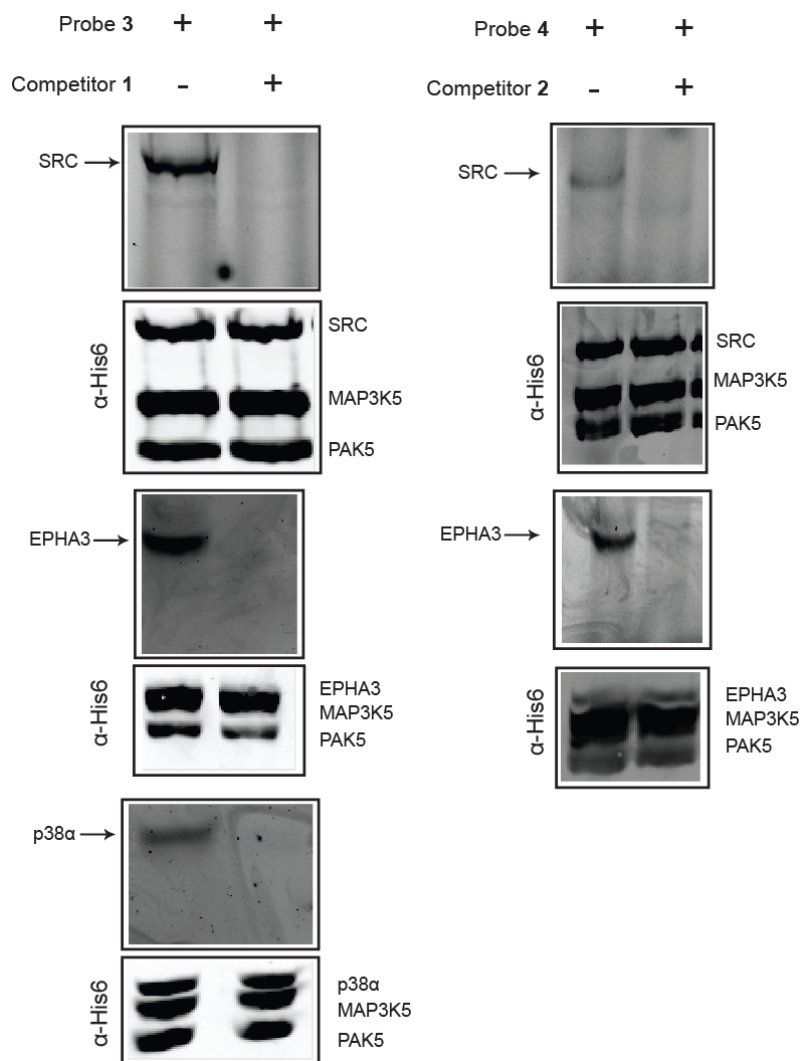


Figure S2: Labeling of DFG-out adopting kinases in the presence of kinases that have not been characterized in this inactive conformation. A DFG-out adopting kinase (90 nM) and two non-DFG-out adopting kinases (180 nM) that had been added to mammalian cell lysate (0.2 mg/mL) were incubated with probes **3** (1 μ M) or **4** (1 μ M) in the absence (lane 1) or presence (lane 2) of an active site competitor. Samples were then irradiated with UV light for 15 minutes. After photo-crosslinking, all samples were tagged with rhodamine-azide, resolved by SDS-PAGE, and labeled proteins were detected with in-gel fluorescence scanning. Immunoblots were performed with an anti-His6 tag antibody (Cell Signaling) to ensure equal amounts of kinase were present.

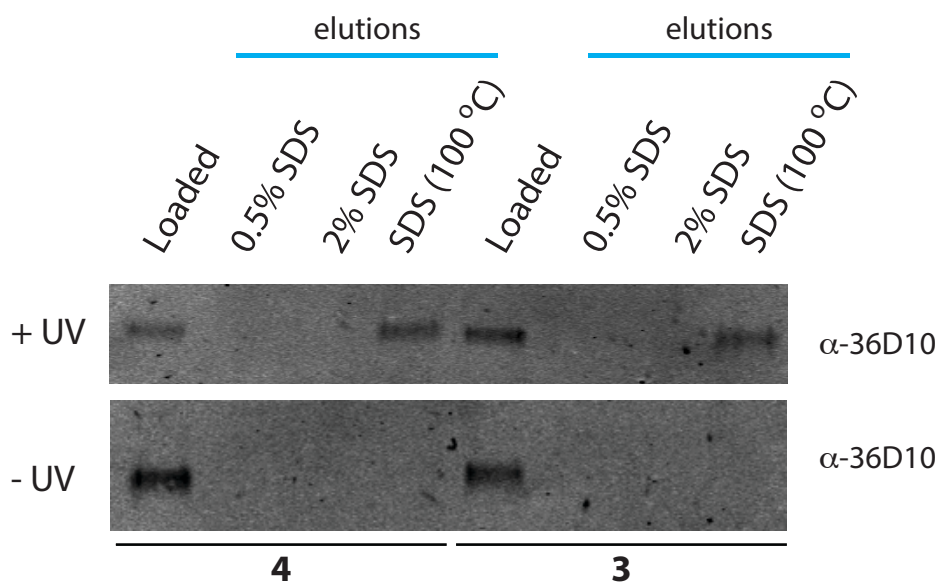


Figure S3: Model pull-down experiments to determine whether probes **3** and **4** non-covalently enrich kinase targets. Mammalian cell lysate supplemented with the tyrosine kinase HCK (90 nM) was incubated with probes **3** (1 μ M) or **4** (1 μ M). The samples shown in the top blot were irradiated with UV light, while those in the bottom blot were not. After photo-crosslinking (top blot) or incubation (bottom blot), samples were subjected to copper-mediated click chemistry with biotin-azide, and streptavidin-conjugated beads were used to enrich biotin-labeled proteins. The beads were then washed using the same protocol as in the SILAC experiments (1% SDS (3x) and 6M urea (3x)) and bound proteins were eluted with SDS. Eluted samples were resolved by SDS-PAGE and subjected to Western blot analysis with an anti-SFK antibody (α -36D10, Cell Signaling).

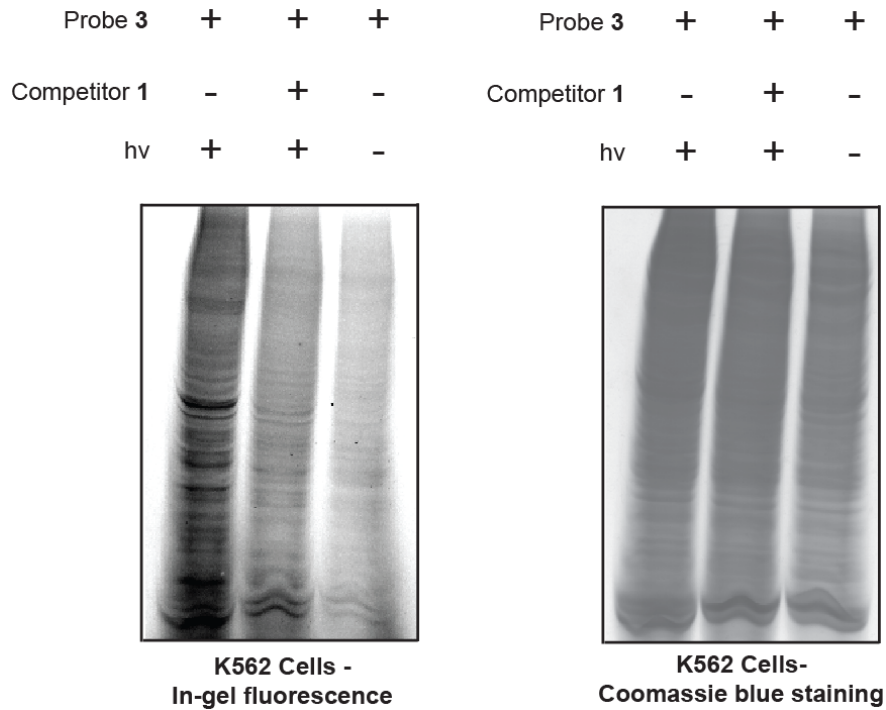


Figure S4: *In situ* labeling of K562 cells with probe 3. In lanes 1 and 2, K562 cells were incubated with probe 3 (1 μ M) in the presence or absence of competitor 1 (10 μ M) for 30 minutes and then irradiated with UV light for 7 minutes. Lysate obtained from these cells was conjugated to rhodamine-azide, resolved by SDS-PAGE, and labeled proteins were detected with in-gel fluorescence scanning. Lane 3 shows the results of an *in situ* labeling experiment performed without UV irradiation. A coomassie blue-stained gel showing protein loading is on the right.

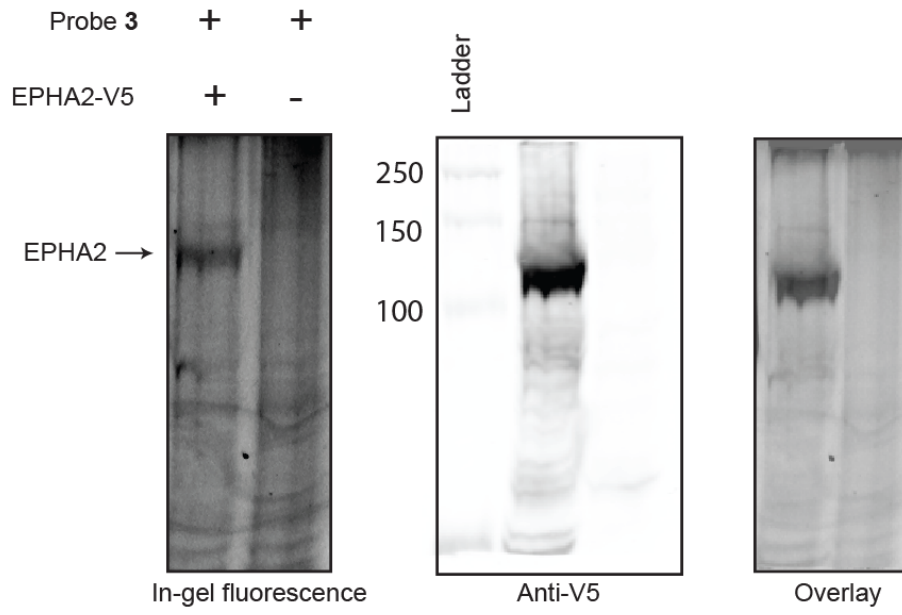


Figure S5: The entire gel shown in Figure 6B.

II. Supplementary Tables

Table S1. Data collection and refinement statistics

Data collection	
Space Group	P2 ₁
a, b, c (Å)	61.81, 49.04, 133.76
α , β , γ (°)	90.0, 101.4, 90.0
Resolution (Å)	50 – 2.00 (2.11-2.00)
Unique reflections	51438 (6324)
R _{pim}	0.054 (0.466)
mean I/ σ (I)	6.8 (1.5)
Completeness	96 (82)
Multiplicity	4.0 (3.4)
Refinement	
Resolution (Å)	2.00
Reflections (working set)	48774
Reflections (test set)	2620
R _{work} / R _{free}	0.223 / 0.246
# protein atoms	4313
# inhibitor atoms	84
# water molecules	81
# other atoms	21
TLS groups	Chain A: 24-78, 79-176, 177-213, 214-317 Chain B: 24-55, 56-176, 177-214, 215-317
RMS non-ideality	
bond length (Å)	0.011
bond angles (°)	1.393

Table S2. Proteins that are not kinases in A431 lysate.

Protein	Description	Peptide Count (3)	H/L Ratio (3)	Peptide Count (4)	H/L Ratio (4)
IPI00015161.1	PDE6D Retinal rod rhodopsin-sensitive cGMP 3',5'-cyclic phosphodiesterase subunit delta	10	10.5	12	> 50
IPI00909862.1	CDNA FLJ54951, HIGHLY SIMILAR TO EPOXIDE HYDROLASE 2	3	2.0	2	> 50
IPI00028946.2	ISOFORM 3 OF RETICULON-3	2	1.9	5	7.1
IPI00002557.1	Coatomer subunit gamma-2	2	7.9		
IPI00019407.1	NSDHL Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	6	2.4		
IPI00024502.2	ISOFORM 1 OF UBIQUILIN-4.	2	2.1		
IPI00024670.5	RECEPTOR EXPRESSION-	2	1.9		

	ENHANCING PROTEIN 5.				
IPI00026850.2	TSPO Translocator protein	5	2.1		
IPI00027444.1	LEUKOCYTE ELASTASE INHIBITOR	2	2.5		
IPI00030774.3	TBCD Isoform 4 of Tubulin-specific chaperone D	1	4.9		
IPI00103994.4	LARS Leucyl-tRNA synthetase, cytoplasmic	2	4.3		
IPI00104341.4	EPHX2 cDNA FLJ59619, highly similar to Epoxide hydrolase 2	17	2.0		
IPI00233885.1	UGT1A10 UDP-glucuronosyltransferase 1-10	2	4.0		
IPI00221178.1	ISOFORM 2 OF TUMOR PROTEIN D54.	12	1.9		
IPI00005981.9	TRANSGELIN-3			2	6.0
IPI00006250.4	ISOFORM 1 OF SHORT-CHAIN DEHYDROGENASE/REDUCTASE 3			1	20.3
IPI00008964.3	RAS-RELATED PROTEIN RAB-1B			1	4.3
IPI00011996.5	ISOFORM 1 OF UBIQUITIN-CONJUGATING ENZYME E2 Z			1	4.0
IPI00012575.1	PIRIN			63	5.2
IPI00013894.1	STRESS-INDUCED-PHOSPHOPROTEIN 1			6	12.5
IPI00014232.1	ADP-RIBOSYLATION FACTOR-LIKE PROTEIN 6-INTERACTING PROTEIN 1			3	4.6
IPI00016608.1	TRANSMEMBRANE EMP24 DOMAIN-CONTAINING PROTEIN 2			1	4.9
IPI00019912.3	PEROXISOMAL MULTIFUNCTIONAL ENZYME TYPE 2.			14	> 50
IPI00021475.1	RAS-RELATED PROTEIN RAB-33B			1	4.3
IPI00026850.2	TRANSLOCATOR PROTEIN			3	21.1
IPI00104341.4	CDNA FLJ59619, HIGHLY SIMILAR TO EPOXIDE HYDROLASE 2			7	> 50
IPI00106668.5	PERILIPIN-3 ISOFORM 3			33	6.7
IPI00171903.2	ISOFORM 1 OF HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN M.			115	5.0
IPI00185146.5	IMPORTIN-9.			1	5.1
IPI00218200.8	B-CELL RECEPTOR-ASSOCIATED PROTEIN 31			1	4.6
IPI00221178.1	ISOFORM 2 OF TUMOR PROTEIN D54			14	7.9
IPI00306825.3	ISOFORM 1 OF TUMOR PROTEIN D54.			5	6.3
IPI00329600.3	PROBABLE SACCHAROPINE DEHYDROGENASE.			1	9.0
IPI00410502.3	TRANSMEMBRANE PROTEIN 139			1	> 10
IPI00452747.6	SIGNAL PEPTIDASE COMPLEX SUBUNIT 2			1	4.8
IPI00555703.1	ISOFORM 1 OF TRANSMEMBRANE PROTEIN 41B			6	6.4
IPI00619958.3	ISOFORM 1 OF TUMOR PROTEIN D52			7	6.3

Table S3. Kinases enriched *in situ* with probe **3** in A431 cells.

Protein	H/L Peptide Ratio	# of Peptides Identified	Kinase Family
EPHA2	9.4	77	Tyrosine
EPHB2	67	27	Tyrosine
GAK	3.5	4	CMGC
SRC	4.1	4	Tyrosine
ADCK4	2.7	4	Atypical
EPHB3	32	17	Tyrosine
p38α	8.3	1	CMGC
YES	5.5	1	Tyrosine

III. Synthesis of 3 and 4

a) General Information

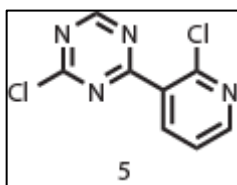
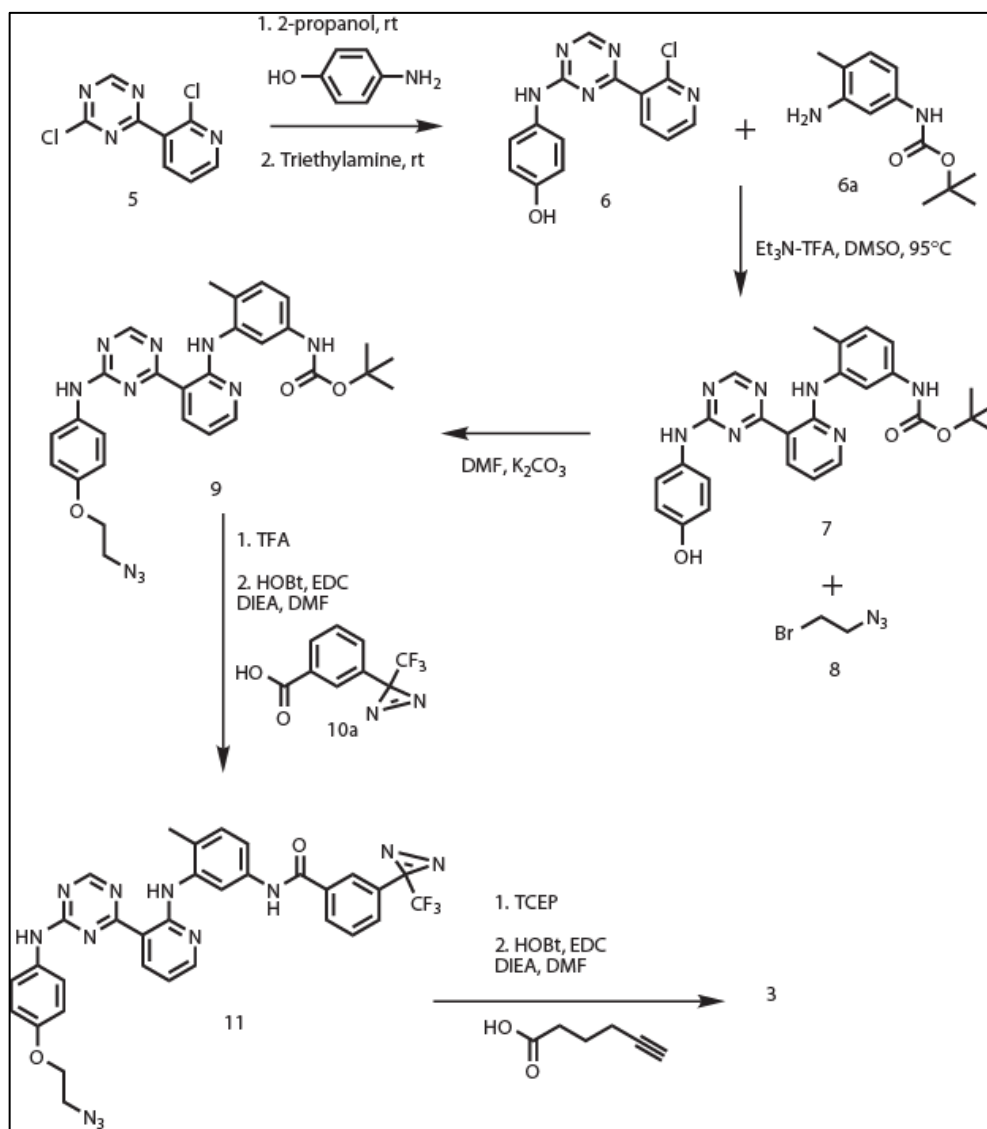
All reagents were purchased from commercial sources without further purification, unless otherwise noted. ¹H-NMR spectra were taken on a Bruker AV300 or AV301 and ¹H resonances are referenced to CDCl₃-d₁ (7.26 ppm), DMSO-d₆ (2.54 ppm) or CD₃OD-d₄ (3.34). Chemical shifts and coupling constants are reported in ppm and Hz respectively. Mass spectrometry was performed on a Bruker Esquire Ion Trap. All final compounds were purified using reverse phase chromatography using the following conditions:

- i. **General HPLC Purification Conditions:** C₁₈ column (250 x 21 mm), CH₃CN/H₂O–0.1% CF₃CO₂H = 1:99 to 100:0 over 60-78 minutes; 8 mL/min; 254 nm detection for 60-78 minutes.
- ii. **Analytical HPLC Conditions:**
 - o *Analytical conditions A:* [C₁₈ (150 x 4.6 mm), CH₃CN/H₂O–0.1% CF₃CO₂H = 1:99 to 100:0 over 30-33 minutes; 1 mL/min; 220 and 254 nm detection for 30-33 minutes].
 - o *Analytical conditions B:* [C₁₈ (150 x 4.6 mm), CH₃OH/H₂O–0.1% CF₃CO₂H = 1:99 to 100:0 over 30-33 minutes; 1 mL/min; 220 and 254 nm detection for 30-33 minutes].

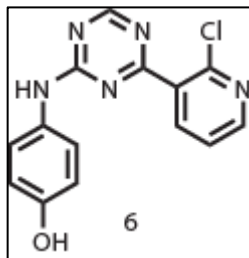
The purity of **3** and **4** were determined to be >95% by analytical HPLC.

b) Synthesis of compounds 1 and 2: Compounds **1** and **2** were synthesized according to a previously described protocol.^{1,2}

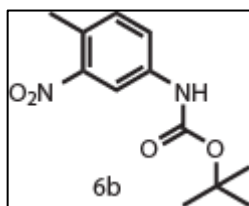
c) Synthetic Scheme for 3



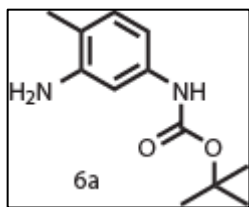
[5] Compound 5 was prepared using a previously described procedure.¹



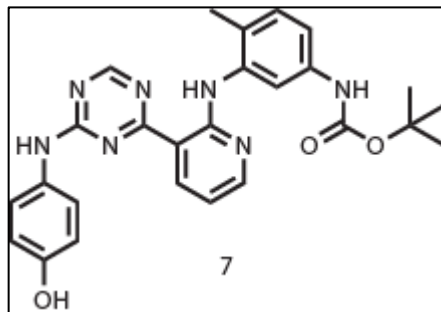
[6] Compound **5** (1 equivalent) and 4-aminophenol (1.1 equivalent) were dissolved in 2-propanol (0.04 M) and stirred at room temperature overnight. Then triethylamine (2 equivalents) was added to the reaction and stirred overnight at room temperature. The reaction was concentrated *in vacuo* and triturated with *t*-BuOMe to afford **6** as a triethylamine salt. ¹H NMR (300 MHz, DMSO-*d*₆) δ 10.25 (s, 1H), 9.35 (d, *J* = 8.1 Hz, 1H), 8.80 (s, 1H), 8.57 (s, 1H) 8.20 (broad s, 1H), 7.61 (m, 1H), 7.47 (m, 2H), 6.75 (t, *J* = 8.4 Hz, 2H); MS *m/z* (C₁₄H₁₀ClN₅O) calc'd = 299.06, observed: (M+H⁺) = 300.7



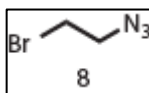
[6b] 4-methyl-3-nitrobenzamine (500 mg, 3.3 mmol) was dissolved in THF (1.3 mL) and di-*tert*-butyl dicarbonate (870 mg, 4.0 mmol) was added dropwise. The reaction was refluxed overnight. The crude material was then diluted with ethyl acetate, which was then washed with NH₄Cl (3X). The organic layer was concentrated *in vacuo* to obtain 820 mg (99% yield) of **6b**. ¹H NMR (300 MHz, DMSO-*d*₆) δ 9.75 (s, 1H), 8.22 (d, *J* = 3.0 Hz, 1H), 7.58 (dd, *J* = 3.0 Hz, *J* = 9.0 Hz, 1H), 7.38 (m, 1H), 2.44 (s, 3H), 1.49 (s, 9H).



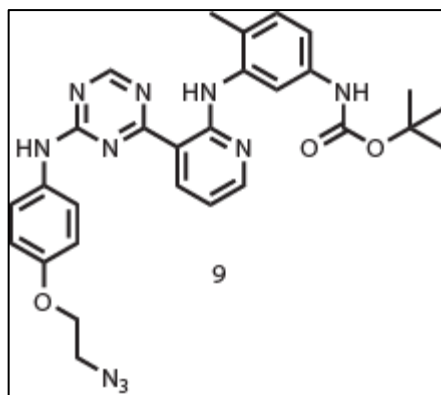
[6a] Compound **6b** (820 mg, 3.3 mmol) was dissolved in ethanol (20 mL) and Pd/C (3%, 165 mg) was added. The reaction mixture was stirred under H₂ gas overnight at room temperature. The crude material was filtered through Celite and purified by column chromatography (10%-15% ethyl acetate in hexanes) to obtain 480 mg (65% yield) of **6a**. ¹H NMR (300 MHz, DMSO-*d*₆) δ 8.90 (s, 1H), 6.82 (s, 1H), 6.73 (d, *J* = 9.0 Hz, 1H), 6.48 (dd, *J* = 3.0 Hz, *J* = 9.0 Hz, 1H), 4.72 (s, 2H), 1.96 (s, 3H), 1.45 (s, 9H).



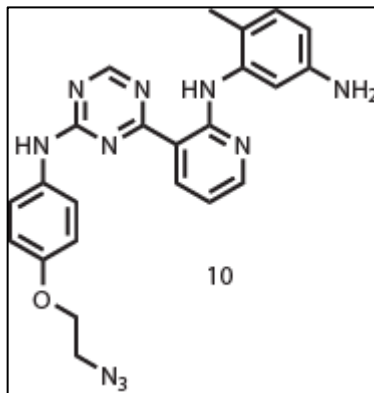
[7] Compound **6** (137 mg, 0.455 mmol) and tert-butyl 3-amino-4-methylphenylcarbamate (213 mg, 0.96 mmol) and a drop of triethylamine-TFA salt were dissolved in DMSO (0.4 mL) and stirred overnight at 95°C. The crude product was purified by column chromatography (30% ethyl acetate in hexanes) to afford 90 mg (41% yield) of **7**. $^1\text{H NMR}$ (300 MHz, $\text{DMSO-}d_6$) δ 10.17 (s, 1H), 9.26 (m, 2H), 8.78 (m, 2H), 8.25 (s, 1H), 7.94 (m, 1H), 7.43 (m, 2H), 7.13 (m, 2H), 6.86 (m, 1H), 6.73 (m, 2H), 2.19 (s, 3H), 1.46 (s, 9H). MS m/z ($\text{C}_{26}\text{H}_{27}\text{N}_7\text{O}_3$) calc'd = 485.2, observed: ($\text{M}+\text{H}^+$) = 486.5.



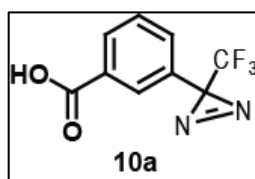
[8] Dibromoethane (500 mg, 2.7 mmol) was suspended in DMF (2 mL) and sodium azide (1.6 mmol) was added portion-wise over a period of 4 h. The reaction was then diluted in ether and the organic layer was washed with H_2O (3X). The organic layer was dried with sodium sulfate and concentrated *in vacuo* to yield **8**. $^1\text{H NMR}$ (300 MHz, CDCl_3 -*d*₁) δ 3.66-3.54 (m, 2H), 3.45-3.33 (m, 2H).



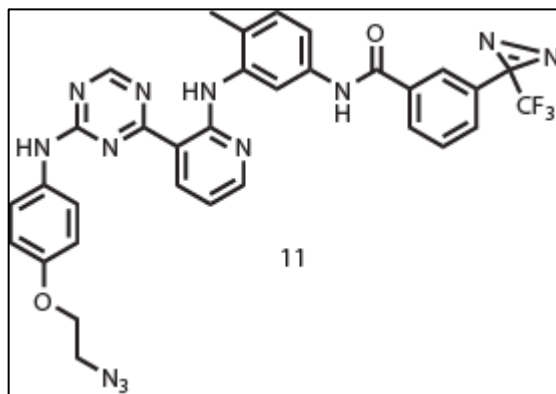
[9] Compound **7** (25 mg, 0.05 mmol), compound **8** (61 mg, 0.4 mmol) and K_2CO_3 (21 mg, 0.15 mmol) were suspended in DMF (0.1 mL) and stirred at room temperature overnight. The crude material was taken up in ethyl acetate and the organic layer was washed with a saturated K_2CO_3 solution (3X). The organic layer was dried with sodium sulfate and concentrated *in vacuo*. The crude product was purified with column chromatography (gradient 20-40% ethyl acetate in hexanes) to afford 18 mg (65% yield) of **9**. $^1\text{H NMR}$ (300 MHz, CDCl_3) δ 8.73 (m, 1H), 8.65 (s, 1H), 8.32 – 8.15 (m, 1H), 7.75 – 7.63 (m, 1H), 7.37 – 7.25 (m, 3H), 7.09 (m, 2H), 6.73 (m, 3H), 6.42 (s, 1H), 3.97 (m, 2H), 3.53 (m, 2H), 2.13 (s, 3H), 1.44 (s, 9H). MS m/z ($\text{C}_{28}\text{H}_{30}\text{N}_{10}\text{O}_3$) calc'd = 554.3, observed: ($\text{M}+\text{H}^+$) = 555.5



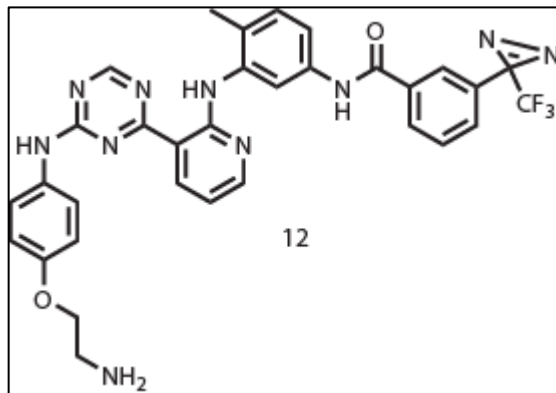
[10] Compound **9** (18 mg, 0.03 mmol) was dissolved in 30% TFA in CH_2Cl_2 (14 mL) and stirred at room temperature for 1 h. The reaction was then concentrated and resuspended in ethyl acetate. The organic layer was washed with saturated K_2CO_3 solution, dried over sodium sulfate and concentrated *in vacuo* to afford a quantitative yield of **10**. MS m/z ($\text{C}_{23}\text{H}_{22}\text{N}_{10}\text{O}$) calc'd = 454.2, observed: $(\text{M}+\text{H}^+) = 455.5$.



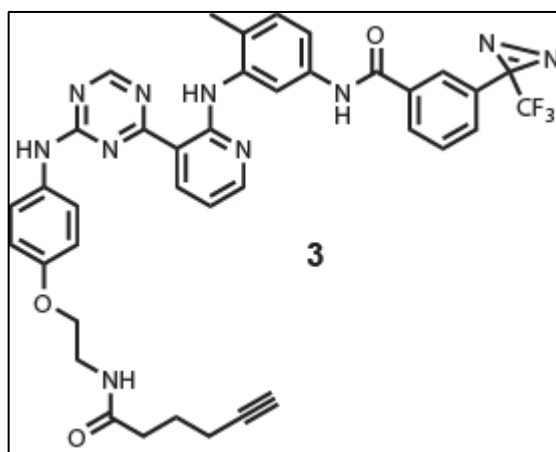
[10a] 3-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoic acid was prepared using a previously described protocol.³



[11] Compound **10** (15 mg, 0.03 mmol), 3-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoic acid (**10a**) (10 mg, 0.04 mmol), EDCI (8 mg, 0.04 mmol), HOBt (6.6 mg, 0.04 mmol) and DIEA (8.5 mg, 0.06 mmol) were suspended in DMF (0.22 mL) and stirred overnight at room temperature. The reaction was taken up in ethyl acetate and washed with saturated K_2CO_3 solution (3X). The organic layer was dried over sodium sulfate and concentrated *in vacuo* to afford compound **11**. MS m/z ($\text{C}_{32}\text{H}_{25}\text{F}_3\text{N}_{12}\text{O}_2$) calc'd = 666.2, observed: $(\text{M}+\text{H}^+) = 667.5$



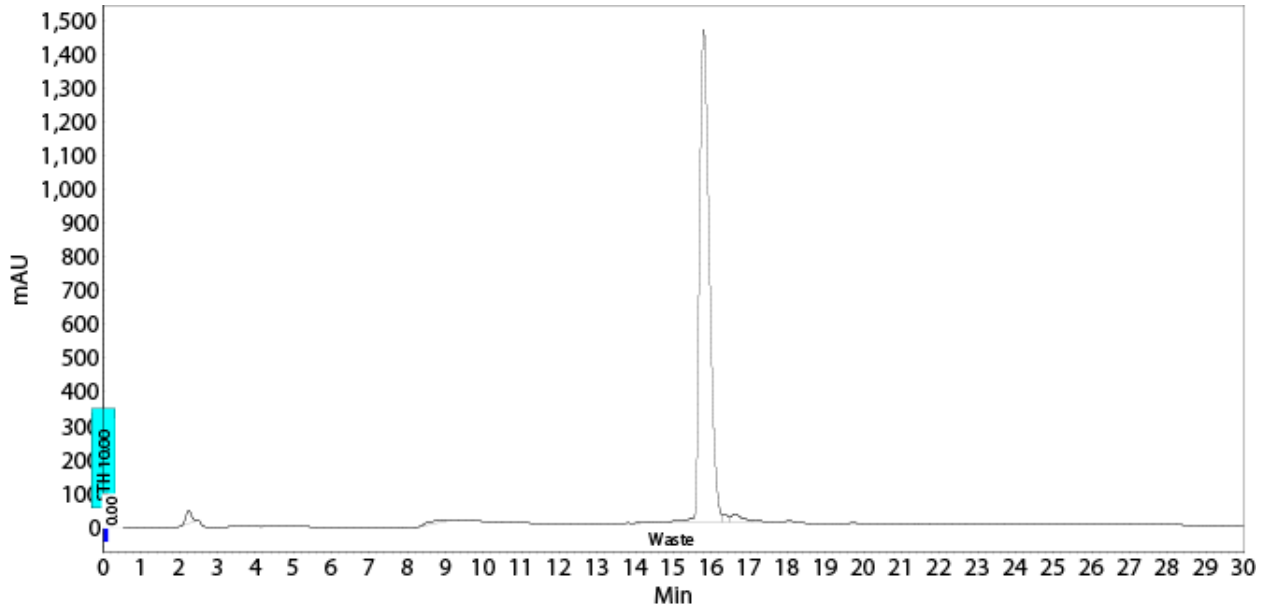
[12] Compound **11** (22 mg, 0.03 mmol) and TCEP (8.3 mg, 0.033 mmol) were dissolved in methanol (0.33 mL) and stirred at room temperature for 2 h. The crude material was concentrated *in vacuo* and purified using reverse phase chromatography using **General HPLC Purification Conditions**. MS *m/z* ($C_{32}H_{27}F_3N_{10}O_2$) calc'd = 640.2, observed: ($M+H^+$) = 641.5



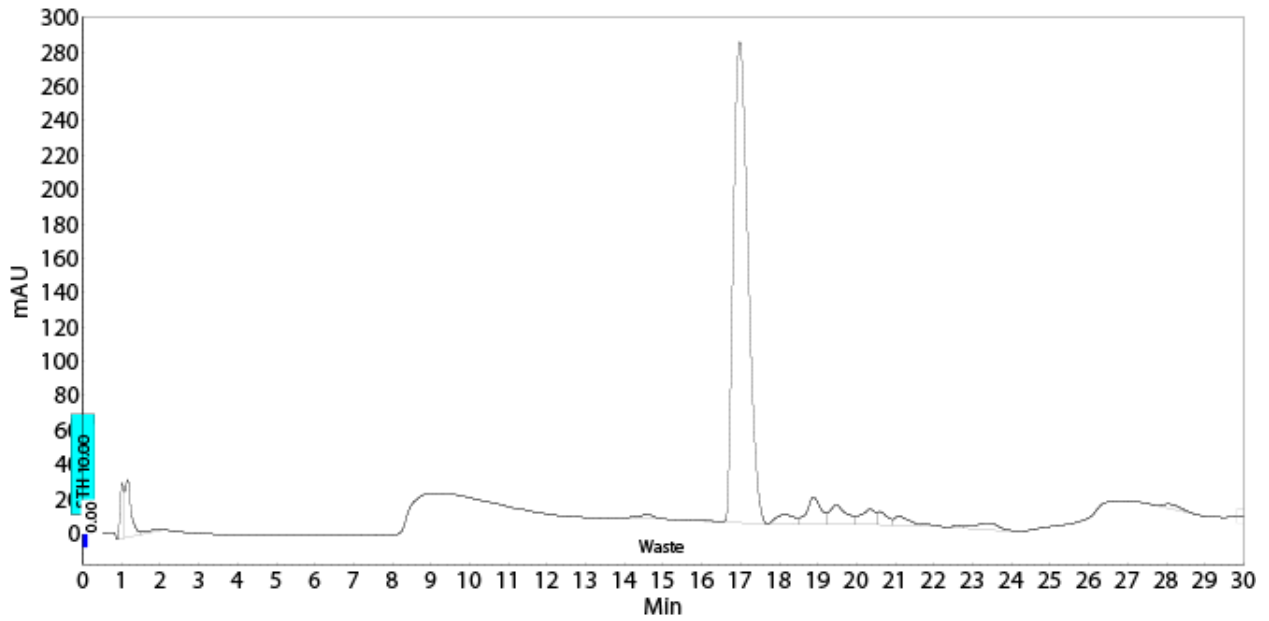
[3] Compound **12** (8 mg, 0.012 mmol), 5-hexynoic acid (4.2 mg, 0.04 mmol), HOBt (5.7 mg, 0.04 mmol), EDCI (7.2 mg, 0.04 mmol) and DIEA (6.5 mg, 0.05 mmol) were dissolved in DMF (0.12 mL) and stirred overnight at room temperature. The crude material was diluted in acetonitrile/water mixture and purified using reverse phase HPLC (**General HPLC Purification Conditions**) to obtain 6.4 mg (70% yield) of **3**. 1H NMR (300 MHz, $DMSO-d_6$) δ 10.35-10.30 (m, 2H), 8.84 (m, 2H), 8.39 – 8.18 (m, 2H), 8.19 – 8.03 (m, 2H), 7.86 – 7.63 (m, 2H), 7.63 – 7.40 (m, 4H), 7.25 – 7.19 (m, 1H), 7.07 – 6.77 (m, 3H) 3.86 (m, 2H), 3.38 (m, 2H), 2.32 – 2.12 (m, 5H), 2.08 (s, 3H), 1.75 – 1.60 (m, 2H). MS *m/z* ($C_{38}H_{33}F_3N_{10}O_3$) calc'd = 734.3, observed: ($M+H^+$) = 735.3.

Analytical Condition A

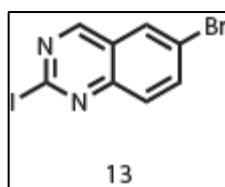
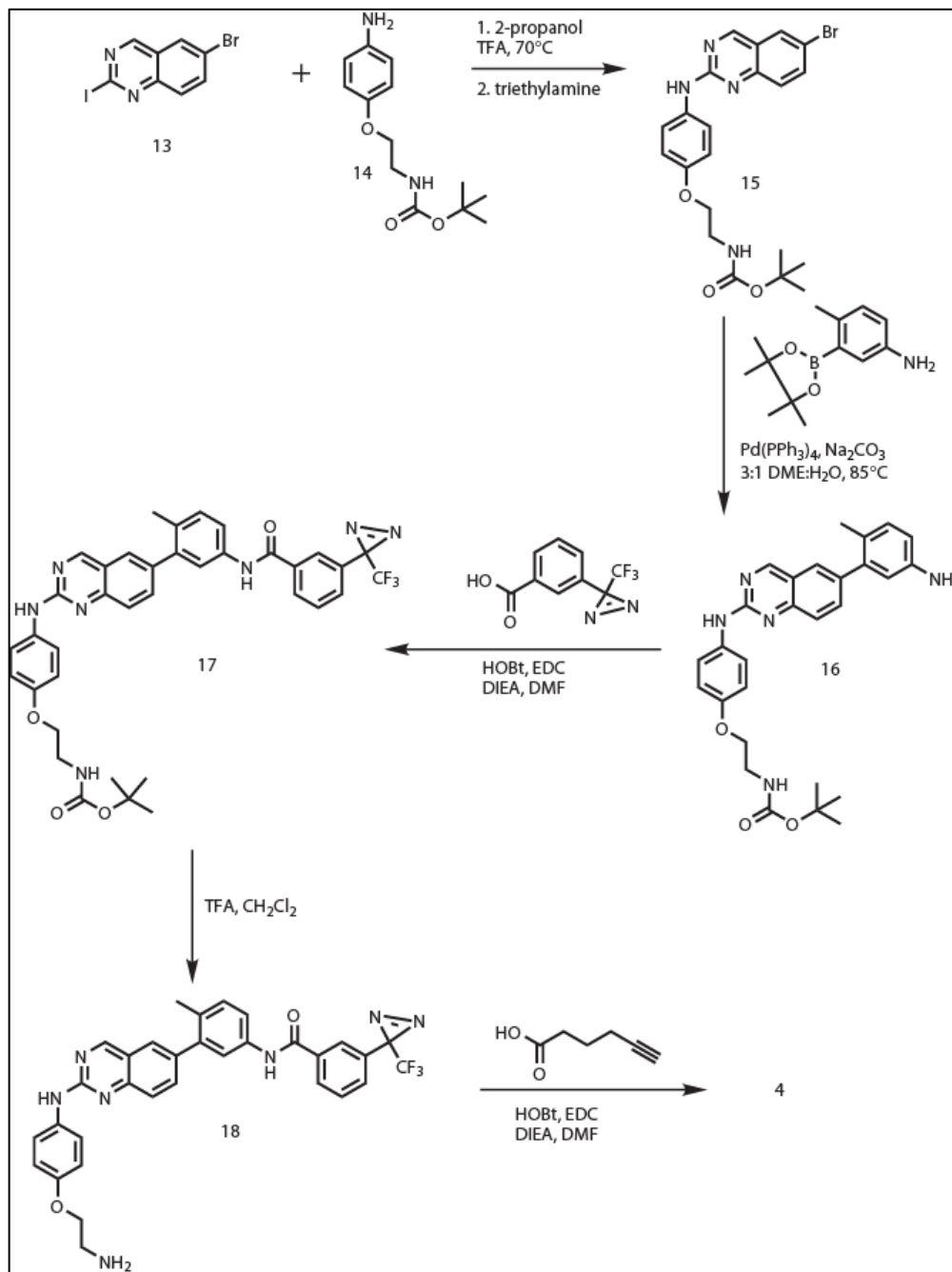
Calculated purity: >95%



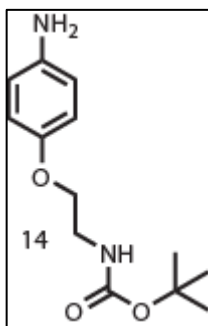
Analytical Condition B



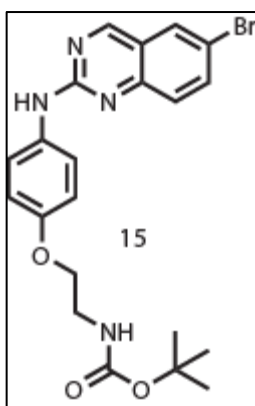
d) Synthetic scheme for 4



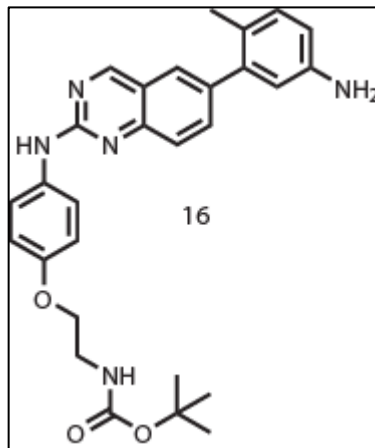
[13] Compound **13** was prepared using a previously described procedure.²



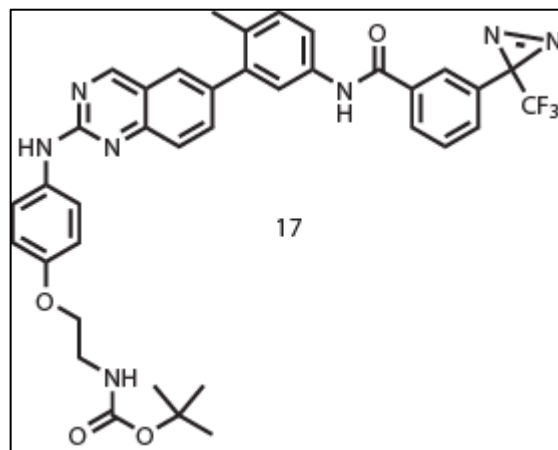
[14] Compound **14** was prepared using a previously described procedure.⁴



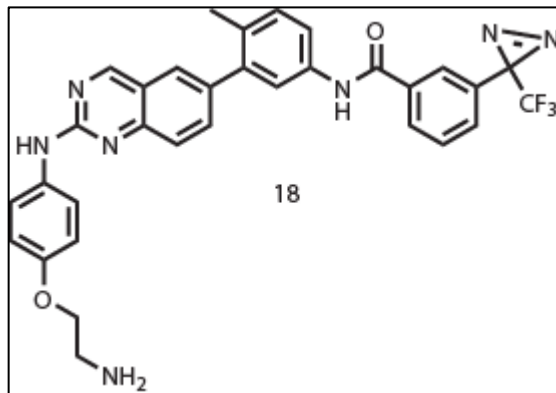
[15] In a resealable Pyrex tube, compound **13** (589 mg, 1.76 mmol) and compound **14** (532 mg, 2.11 mmol) were dissolved in 2-propanol (13.5 mL). Trifluoroacetic acid (0.3 mL, 3.52 mmol) was added and the tube was sealed. The suspension was stirred overnight at 70°C. Triethylamine (1.5mL) was then added to neutralize the mixture and the mixture was concentrated *in vacuo*. The residue was purified by column chromatography (50% ethyl acetate in hexanes) to afford 379 mg of compound **15** (47% yield). ¹H NMR (300 MHz, CDCl₃-d₁) δ 9.17 (s, 1H), 8.01 – 7.94 (m, 2H), 7.75 (d, *J* = 9.0 Hz, 1H), 7.57 (d, *J* = 9.0 Hz, 2H), 6.98 – 6.92 (m, 2H), 5.00 (broad s, 1H), 4.06 (t, *J* = 5.1 Hz, 2H), 3.59 – 3.53 (m, 2H), 1.48 (s, 9H). MS *m/z* (C₂₁H₂₃BrN₄O₃) calc'd = 458.1, observed: (M+H⁺) = 459.2



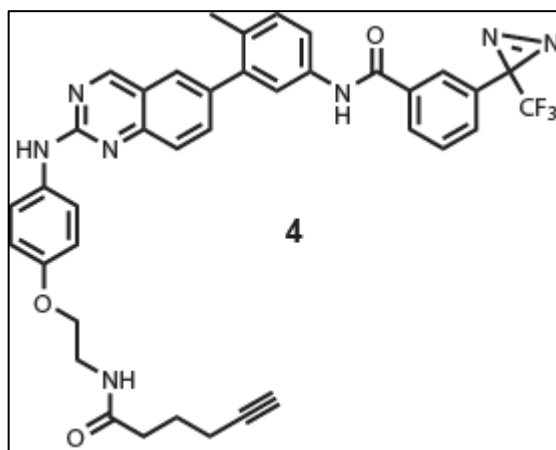
[16] A mixture of compound **15** (83 mg, 0.18 mmol), 5-Amino-2-methylphenylboronic acid pinacol ester (48 mg, 0.20 mmol), tetrakis(triphenylphosphine)palladium (6.4 mg, 5.4 μmol) and sodium carbonate (42 mg, 0.40 mmol) were dissolved in a 3:1 mixture of DME/water (0.71 mL). The mixture was heated overnight at 85°C. The crude mixture was cooled to room temperature, diluted with ethyl acetate and washed with water and brine. The organic layer was dried over sodium sulfate, concentrated *in vacuo* and the resultant product **16** was used in the next step without further purification. ^1H NMR (300 MHz, CDCl_3-d_1) δ 9.09 (m, 1H), 7.77 – 7.72 (m, 3H), 7.66 – 7.65 (m, 1H), 7.20-7.17 (m, 1H), 7.14-7.09 (m, 1H), 6.99 – 6.94 (m, 2H), 6.71-6.67 (m, 2H), 5.03 (s, 2H), 4.06 (t, $J = 3.0$ Hz, 2H), 3.61-3.54 (m, 2H), 2.21 (m, 3H), 1.50 – 1.48 (m, 9H). MS m/z ($\text{C}_{28}\text{H}_{31}\text{N}_5\text{O}_3$) calc'd = 485.2, observed: ($\text{M}+\text{H}^+$) = 486.5



[17] Compound **16** (35.2 mg, 0.072 mmol), 3-(3-(trifluoromethyl)-3H-diazirin-3-yl) benzoic acid (20 mg, 0.086 mmol), HOBt (14.9 mg, 0.094 mmol), EDCI (18.3 mg, 0.094 mmol) and DIEA (38 μL , 0.216 mmol) were dissolved in DMF (0.2 mL) and stirred overnight at room temperature. The crude mixture was diluted in ethyl acetate and washed with NH_4Cl and Na_2CO_3 . The organic layer was dried over sodium sulfate and concentrated *in vacuo* to yield compound **17**. MS m/z ($\text{C}_{37}\text{H}_{34}\text{F}_3\text{N}_7\text{O}_4$) calc'd = 697.3, observed: ($\text{M}+\text{H}^+$) = 698.4



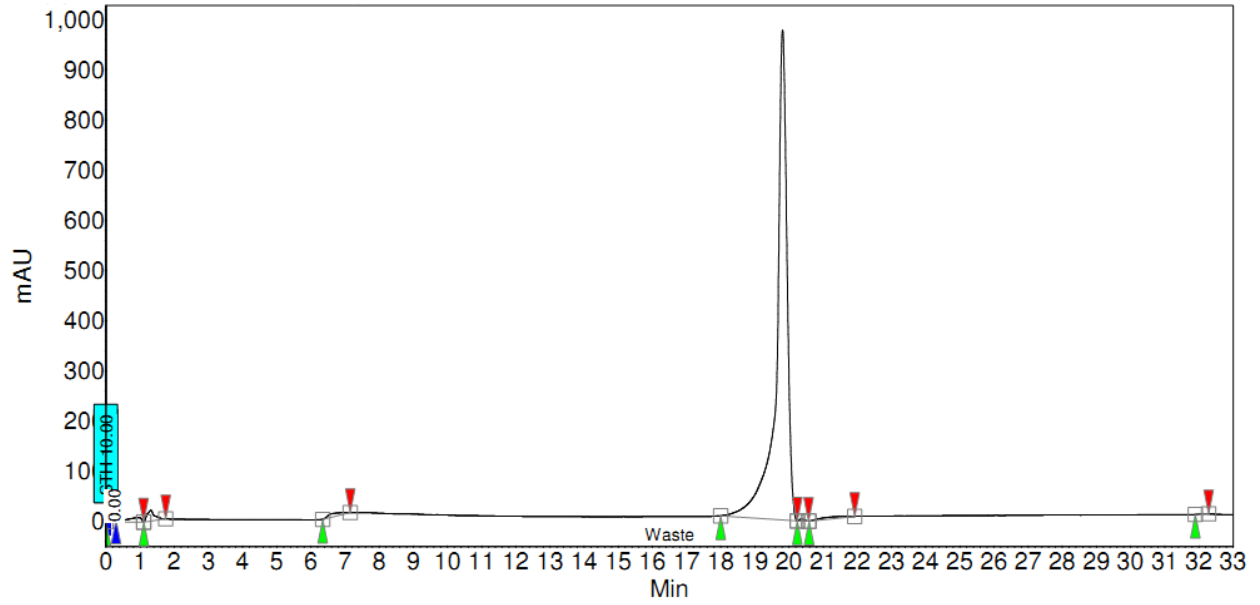
[18] Compound **17** (35.2 mg, 0.072 mmol) was dissolved in CH_2Cl_2 (1.25 mL) and TFA (0.52 mL) and stirred for 3 h at room temperature. The reaction was concentrated and purified by reverse phase HPLC (**General HPLC Purification Conditions**) to obtain 23 mg (54% yield after 2 steps) of the desired product **18**. ^1H NMR (300 MHz, $\text{CD}_3\text{OD}-d_4$) δ 9.30 (s, 1H), 8.09 – 8.05 (m, 1H), 7.92 – 7.86 (m, 1H), 7.79 – 7.73 (m, 3H), 7.70 – 7.62 (m, 2H), 7.59 – 7.48 (m, 2H), 7.43 – 7.40 (m, 1H), 7.37 – 7.30 (m, 2H), 7.23 – 7.08 (m, 2H), 4.33 – 4.26 (m, 2H), 3.47 – 3.39 (m, 2H), 3.32 – 3.28 (m, 3H). MS m/z ($\text{C}_{32}\text{H}_{26}\text{F}_3\text{N}_7\text{O}_2$) calc'd = 597.2, observed: $(\text{M}+\text{H}^+)$ = 598.5



[4] Compound **18** (10.4 mg, 0.017 mmol), 5-hexynoic acid (2.52 μL , 0.022 mmol), HOBt (3.5 mg, 0.022 mmol), EDCI (4.3 mg, 0.022 mmol) and DIEA (12 μL , 0.068 mmol) were dissolved in DMF (0.12 mL) and stirred overnight at room temperature. The crude material was diluted in acetonitrile/water mixture and purified by reverse phase HPLC (**General HPLC Purification Conditions**) to obtain 7.9 mg (65% yield) of compound **4**. ^1H NMR (300 MHz, $\text{CD}_3\text{OD}-d_4$) δ 9.31 (m, 1H), 8.05 (d, $J = 6.0$ Hz, 1H), 7.93-7.86 (m, 1H), 7.75-7.71 (m, 2H), 7.66-7.53 (m, 4H), 7.50-7.44 (m, 2H), 7.36-7.27 (m, 2H), 7.14-7.02 (m, 2H), 4.13 – 4.07 (m, 2H), 3.63 – 3.58 (m, 2H), 2.38 – 2.20 (m, 8H), 1.85 – 1.79 (m, 2H). MS m/z ($\text{C}_{38}\text{H}_{32}\text{F}_3\text{N}_7\text{O}_3$) calc'd = 691.3, observed: $(\text{M}+\text{H}^+)$ = 692.5

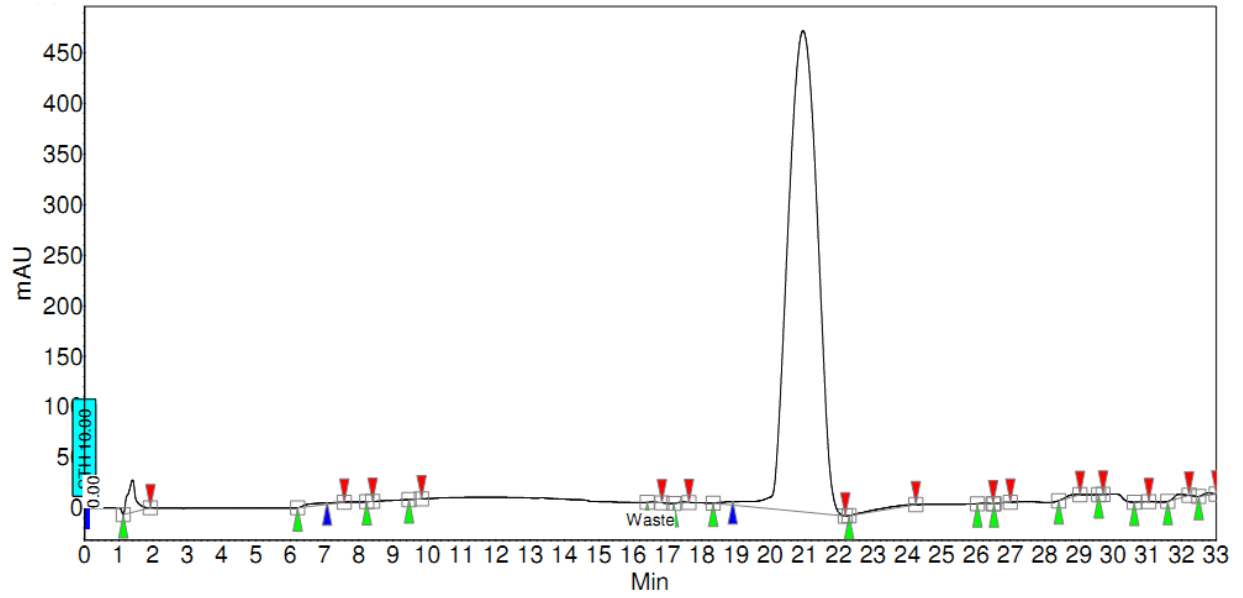
Analytical Condition A

Calculated purity: > 95%



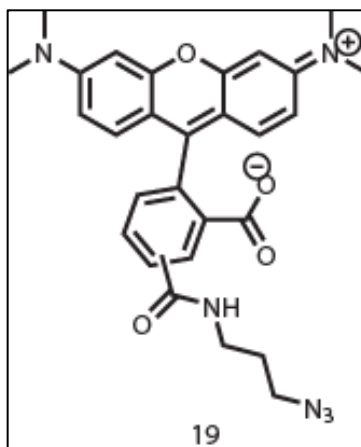
Analytical Condition B

Calculate purity: > 95%

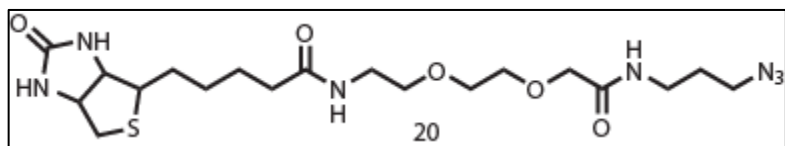


IV. Structures of rhodamine-azide (19) and biotin-azide (20)

[19] Compound **19** was prepared using a previously described protocol.⁵



[20] Compound **20** was prepared using a previously described protocol.⁶



V) Mammalian Lysate Preparation

Cells were suspended in 50 mM Tris (pH = 7.4), 150 mM NaCl, containing 1 mM PMSF and protease inhibitor cocktail (roche) and lysed using a dounce homogenizer. Samples were centrifuged at 14,000 g to remove cell debris. Protein samples were quantified using Bradford assay.

VI) Protein Kinase Activity Assays

In vitro activity assays for ABL, CSK, HCK, IRAK4, LCK, LOK, MAP3K5, p38 α , PAK4, PAK5, PKA and SRC were performed using previously published protocols.⁴

EphA3: Inhibitors (initial concentration = 10 μ M, 3-fold serial dilutions down to 0.2 nM) were assayed in triplicate or quadruplicate against His6-EphA3 (final concentration = 10 nM) in assay buffer containing 30 mM HEPES, pH = 7.5, 38 mM MgCl₂, 630 μ M EGTA, 2 mM Na₃VO₄, 3 μ M ATP, 40 μ g/ml BSA, γ ³²P ATP (0.2 μ Ci/well) and myelin basic protein as substrate (final concentration = 200 μ g/mL). The final volume of each assay well was 30 μ L. The enzymatic reaction was run at room temperature for 2 h and then terminated by spotting 4.5 μ L of the reaction mixture onto a phosphocellulose membrane. Membranes were washed with 0.5% phosphoric acid (4X, 10 minutes each wash), dried and the radioactivity was determined by phosphorimaging with a GE Typhoon FLA9000 scanner. The scanned membranes were quantified with ImageQuant and converted to percent inhibition. Data was analyzed using Prism Graphpad software and IC₅₀ values were determined using non-linear regression analysis.

p38 δ : Catalytic domain of wild type His6-p38 δ (2 μ M) was activated with excess MKK6 in buffer containing 50 mM MOPS, pH 7.4, 10 mM MgCl₂, 1 mM DTT, 0.001% (v/v) Tween 20, 100 ng BSA and 670 μ M ATP. Activation reaction was incubated for 1 h at room temperature and used directly in the activity assay. Inhibitors (concentrations = 10 μ M, 1 μ M, and 100 nM) were assayed in duplicate against the catalytic domain of wild type activated His6-p38 δ (final concentration = 20 nM) in assay buffer containing 33.5 mM HEPES, pH = 7.5, 6.7 mM MgCl₂, 1.7 mM EGTA, 67 mM NaCl, 2 mM Na₃VO₄, 3 μ M ATP, γ ³²P ATP (0.2 μ Ci/well) and myelin basic protein as substrate (final concentration 200 μ g/mL). The final volume of each assay well was 30 μ L. The enzymatic reaction was run at room temperature for 2.5 h and then terminated by spotting 4.5 μ L of the reaction mixture onto a phosphocellulose membrane. Membranes were washed with 0.5% phosphoric acid (4X, 10 minutes each wash), dried and the radioactivity was determined by phosphorimaging with a GE Typhoon FLA9000 scanner. The scanned membranes were quantified with ImageQuant and converted to percent inhibition. Data was analyzed using Prism Graphpad software and IC₅₀ values were determined using non-linear regression analysis.

VII) *In vitro* crosslinking assay with probes 3 and 4

His6-tagged kinases (90 nM) were added to PBS containing 0.2-mg/mL mammalian lysate. This sample was aliquoted (84 μ L) into 96-well, U-bottom plates. DMSO (2 μ L) was added to crosslinking wells while 10 μ M competitor (**1**, **2**, Ponatinib, or Rebastinib) was added to competition wells. Samples were incubated for 15 minutes at room temperature. 1 μ M probe (**3** or **4**) was then added into each well and incubated for 1 h at room temperature. The final concentration of DMSO was 4% in each well. Samples were irradiated at 365 nm with a Spectroline ENF-260C UV lamp by placing the lamp directly over the plate (0°C, 15 minutes).

The no light control sample was not irradiated. After UV irradiation, rhodamine-azide was conjugated to all samples using the following click chemistry conditions: 50 μ M rhodamine-azide, 1 mM TCEP (freshly prepared), 100 μ M TBTA, and 1 mM CuSO₄ (freshly prepared). The final volume of the reaction was 100 μ L. Reactions were incubated for 1 h at room temperature and quenched with addition of 50 μ L of 3X SDS loading buffer. Samples were resolved on SDS-PAGE and visualized using GE Typhoon FLA9000 fluorescence scanner. The same gel was transferred onto a nitrocellulose membrane and probed with α -His6 antibody (Cell Signaling) for detection of protein loaded in each lane.

VIII) *In vitro* crosslinking of inactive and activated HCK constructs with probe 3

A His6-tagged HCK construct (50 or 100 nM) was added to PBS containing 0.2 mg/mL mammalian lysate. This sample was aliquoted (84 μ L) into 96-well, U-bottom plates. 1 μ M of probe **3** was added into each well and incubated for 1 h at room temperature. The final concentration of DMSO was 4% in each well. Samples were irradiated at 365 nm with a Spectroline ENF-260C UV lamp by placing the lamp directly over the plate (0°C, 15 minutes). After UV irradiation, rhodamine-azide was conjugated to all samples using the following click chemistry conditions: 100 μ M rhodamine-azide, 1 mM DTT (freshly prepared), 250 μ M BTAA, and 250 μ M CuBr (freshly prepared). The final volume of the reaction was 100 μ L. Reactions were incubated for 1 h at room temperature and quenched with addition of 50 μ L of 3X SDS loading buffer. Samples were resolved on SDS-PAGE and visualized/quantified using a GE Typhoon FLA9000 fluorescence scanner. The same gel was transferred onto a nitrocellulose membrane and probed with α -His6 antibody (Cell Signaling) for detection of protein loaded in each lane.

IX) Model pull-down experiments with HCK in mammalian cell lysate

HCK (90 nM) was added to PBS containing 0.2 mg/mL mammalian lysate. This sample was aliquoted (84 μ L) into 96-well, U-bottom plates. 1 μ M of probes **3** or **4** were added into each well and incubated for 1 h at room temperature. One set of samples were irradiated with IV light for 10 minutes, while the other set was incubated in the dark. Following irradiation or incubation, samples were tagged with biotin-azide using the following click chemistry conditions: 50 μ M biotin-azide, 1 mM TCEP (freshly prepared), 100 μ M TBTA, and 1 mM CuSO₄ (freshly prepared). Reactions were incubated at room temperature for 1 h. Protein was precipitated using methanol/CHCl₃ and resolubilized into 50 μ L of 2.5% SDS in PBS. Samples were then diluted to 0.2% SDS and incubated with 20 μ L of pre-washed streptavidin beads (Pierce) for 1.5 h. Beads were sequentially washed with 20 bed volumes of 1% SDS in PBS (3X), 6 M Urea (3X) and PBS (3X). Bound proteins were eluted with SDS (first elution = 0.5% SDS; second elution = 2% SDS; third elution = saturated SDS (100 °C)). The eluted samples (and a loading control) were resolved by SDS-PAGE, transferred to nitrocellulose and probed with a SRC-family kinase-specific antibody (anti-36D10, Cell Signaling).

X) Mass spectrometric analysis of biotin enriched kinases from *in vitro* labeling performed with probes 3 and 4

A431 cells were grown in DMEM containing either ¹³C₆-Lys/¹³C₆-Arg (heavy) or natural abundance Lys and Arg (light) and antibiotics (streptomycin and penicillin). Heavy amino acid incorporation was confirmed by mass spectrometry (>95% labeling). Cells combined from three

25-cm confluent plates (>95%) grown in either $^{13}\text{C}_6$ -Lys/ $^{13}\text{C}_6$ -Arg or regular Lys/Arg were suspended in PBS containing protease cocktail inhibitor (Roche) and lysed via sonication. Cleared lysate from both samples were quantified using Bradford assay (2.5 mg/mL protein). Both samples (4 mL each) were aliquoted into 200 μL aliquots into 96-well U-bottom plates. “Light” samples were incubated with 10 μM competitor **1** or **2** while the “heavy” samples were incubated with DMSO for 15 minutes at room temperature. All samples were then incubated with 1 μM crosslinking probe **3** or **4** for 1 h at room temperature. The final concentration of DMSO was 4% in each well. Samples were irradiated on ice for 15 minutes. Light and heavy samples were pooled, aliquoted into 500 μL volume and tagged with biotin-azide using the following click chemistry conditions: 50 μM biotin-azide, 1 mM TCEP (freshly prepared), 100 μM TBTA, and 1 mM CuSO_4 (freshly prepared). The final volume of each reaction aliquot was 570 μL . Reactions were incubated at room temperature for 1 h. Protein was precipitated using methanol/ CHCl_3 method and resolubilized into 650 μL of 2.5% SDS in PBS. Fractions were pooled and the SDS concentration was diluted to 0.5% until further use. For enrichment of tagged proteins, sample was further diluted to 0.2% SDS and incubated with 100 μL of pre-washed streptavidin beads (Pierce) for 1.5 h. To remove unbound proteins, beads were sequentially washed with 20 bed volumes of 1% SDS in PBS (3X), 6 M Urea (3X) and PBS (3X). Beads were transferred into a 15-mL falcon tube and resuspended in 20 bed volumes of 6 M Urea. Bound proteins were reduced with 10 mM DTT for 30 minutes at 55°C and alkylated in the dark with 25 mM iodoacetamide for 30 minutes. Excess iodoacetamide was quenched with 10 mM DTT for 15 minutes. Beads were washed with PBS and transferred to a spin column. For protein digestion, beads were incubated with 500 μL of 2 M urea, 1.4 mM CaCl_2 , and trypsin overnight at 37°C. Digested sample was collected into a microcentrifuge tube the next day. An additional 500 μL volume of 2 M urea was added to the beads and collected into the same tube. Tryptic peptide sample was acidified using 10% TFA solution and desalted using a Sep-Pak C18 column before mass spectrometric analysis. Mass spectra were searched against the human IPI database supplemented with human kinases from Kinase.com using the Sequest algorithm. Peptide hits were filtered to a 1% false-discovery rate at the protein level, and quantified using in-house software. To determine which proteins were labeled specifically, test data consisting of two control conditions mixed in equal ratios was used. Analyzing this data set using the same filtering criteria as our experimental data resulted in 0.5% of measurements having a greater than two fold change. Conversely, under experimental conditions we observed a statistically significant enrichment of measurements having a greater than two fold change (average of all experimental results was 6.1%, $p < 0.001$).

XI) *In situ* protein labeling with probe 3

A431 cells were grown in DMEM supplemented with 10% FBS and antibiotics (streptomycin and penicillin). 2.5×10^6 cells were plated into three 6-cm plates and grown for 48 h at which point plates were >95% confluent. The growth medium was aspirated and cells were washed with 1.5 mL PBS (1X). 1 mL PBS was added to each plate. The competition plate was incubated with 10 μM competitor while the crosslinking and no light plates were incubated with DMSO (2.5 μL) for 10 minutes at 37°C. Each plate was then incubated with 1 μM probe for 30 minutes at 37°C. The final concentration of DMSO per plate was 0.5%. Before irradiation, fresh PBS (1 mL) was added to crosslinking and no light control plates while PBS (1 mL) with 10 μM competitor was added to the competition plate. All plates were irradiated for 7 minutes at 37°C

except the no light control which was kept in the dark. Cells from each plate were scraped and collected into microcentrifuge tubes. Samples were spun down at 14,000 g for 10 minutes at 4°C and the PBS was removed. Cell pellets were washed with cold PBS (1X), spun down and the PBS was removed. To lyse, cells were resuspended in 50 μ L of buffer A (0.05% SDS, 5 mM MgCl₂, 10 mM triethanolamine, pH= 7.4) with 1 mM PMSF, protease cocktail inhibitor (roche) and Benzonase (0.5 μ L) (Sigma Aldrich) and incubated for 20 minutes on ice. 150 μ L of buffer B (4% SDS, 50 mM triethanolamine, pH= 7.4, 150 mM NaCl) was added to the cells and briefly vortexed. Samples were centrifuged at 14,000 g for 10 minutes to obtain cleared lysate. Proteins were quantified using BCA assay. Equal amounts of proteins from each sample were conjugated to rhodamine-azide using the following click chemistry conditions: 100 μ M rhodamine-azide, 1 mM TCEP (freshly prepared), 100 μ M TBTA, and 1 mM CuSO₄ (freshly prepared). The final volume of each reaction was 200 μ L. Reactions were incubated for 1 h at room temperature. Proteins were precipitated using MeOH/CHCl₃ method and the resulting protein pellet was resolubilized in SDS loading buffer. Samples were resolved on SDS-PAGE and visualized using a GE Typhoon FLA9000 fluorescence scanner.

XII) Mass spectrometric analysis of biotin enriched kinases from *in situ* labeling

2.5 X 10⁶ cells were grown in 6-cm culture plates supplemented with either ¹³C₆-Lys or regular Lys for ~48 h. Heavy amino acid incorporation was confirmed with mass spectrometry (> 95%). Ten culture plates grown in regular Lys were incubated with 10 μ M competitor while another ten culture plates grown in ¹³C₆-Lys were incubated with DMSO for 10 minutes. All plates were then incubated with 1 μ M probe for 30 minutes. The final concentration of DMSO in each plate was 0.5%. Cells were subjected to standard irradiation and lysis protocol as described above. Before click chemistry, equal amounts of lysate samples from both experimental conditions were mixed together. Photo-crosslinked proteins were tagged with biotin-azide in 500 μ L aliquots using the following click chemistry conditions: 50 μ M biotin-azide, 1 mM TCEP (freshly prepared), 100 μ M TBTA, and 1 mM CuSO₄ (freshly prepared). Proteins were precipitated using MeOH/CHCl₃ method and pellets were resolubilized in 650 μ L of 2.5% SDS in PBS. Samples were pooled into a falcon tube and subjected to streptavidin bead enrichment as described above. Protein digestion and peptide identification was performed as described above.

XIII) Crystallography

Crystals were grown at 4°C by vapor diffusion from a sitting drop containing 1 μ L protein solution (13.5 mg/mL protein in standard buffer to which compound **1** in 10% DMSO was added to yield a net concentration of 1 mM) plus 0.5 μ L crystallization buffer (50% PEG 300, 100 mM sodium cacodylate, pH 6.25, 240 mM CaOAc). Diffraction images were measured at SSRL beamline 9-2 at an X-ray wavelength of 0.9795Å and processed using Mosflm and the CCP4 program suite. The initial structural model was found by molecular replacement using PDB entry 2J51 as a probe and then subjected to automated rebuilding in ARP/wARP.⁷ The model was completed by alternating rounds of manual fitting in Coot and automated refinement in Refmac. One PEG molecule and five partially occupied calcium sites were explicitly included in the model. The identification of calcium sites was aided by inspection of an anomalous difference Fourier map. Residual electron density remained in the vicinity of the calcium sites, suggesting

the presence of additional unmodeled calcium atoms. A segmented TLS model describing atomic displacements was generated using TLSMD.⁸ The final protein geometry was validated using Molprobity, which reported 98% of the residues as lying in favorable regions of ϕ/ψ space, with no outliers. The final model has been deposited with the PDB as entry 4EQU.

XIV) Supplemental References

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