SUPPORTING INFORMATION

A Hexylchloride-Based Catch-and-Release System for Chemical Proteomic Applications

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I. SUPPORTING FIGURES

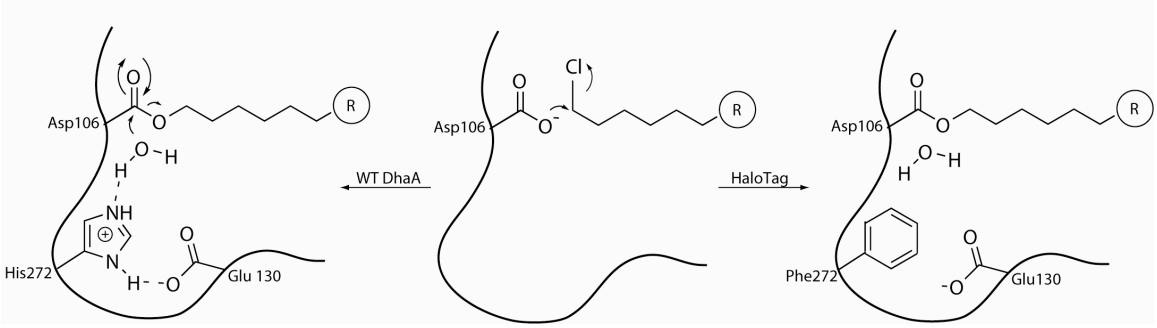


Figure S1. Reaction mechanism of WT DhaA and mutant HaloTag. DhaA catalyzes the nucleophilic displacement of chlorine from alkyl chains forming an ester intermediate. In the WT dehalogenase, His272 catalyzes the hydrolysis of the ester, regenerating the enzyme. In the mutant protein, HaloTag, Phe272 cannot activate water and the enzyme is irreversibly bound to its substrate. [1]

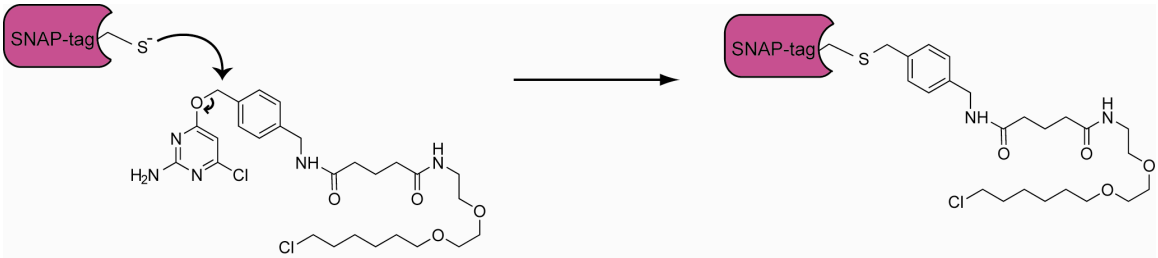


Figure S2. Generation of a hexylchloride-labeled protein. The self-labeling protein SNAP-tag (AGT) undergoes a selective reaction with the chloropyrimidine moiety of compound **2.** The result is a singly-labeled protein that displays the hexylchloride tag from its active site. The hexylchloride tag is directed out of the active site and is available for reaction with the enzyme HaloTag.

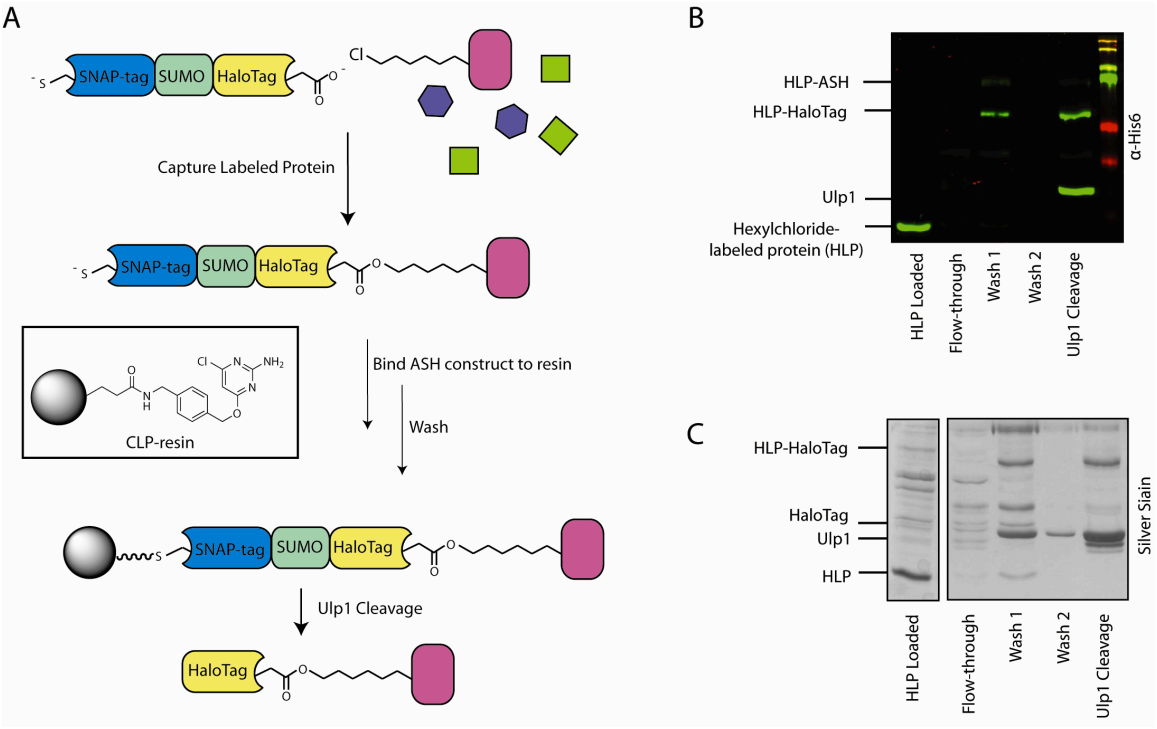


Figure S3. Selective catch-and-release of a hexylchloride-labeled protein in HeLa lysate. (A) Schematic for the catch-and-release of hexylchloride-labeled protein (HLPs). ASH is incubated with mammalian lysate supplemented with a protein, HLP, which contains a single hexylchloride tag (shown in pink). HaloTag selectively captures the HLP and then is incubated with CLP-resin. The SUMO protease, Ulp1, releases HaloTag (and any proteins captured by HaloTag) from the resin. (B) Western Blot analysis (anti-His6) of the catch-and-release experiment described in (A). Greater than 90% of the HLP is captured by HaloTag and immobilized on the resin and greater than 90% of the captured protein is released from the resin by Ulp1. (C) Silver stain analysis of the catch-and-release experiment described in (A). Only three protein bands, corresponding to the molecular weights of Ulp1, HaloTag, and the HLP/HaloTag conjugate, are present in the elution.

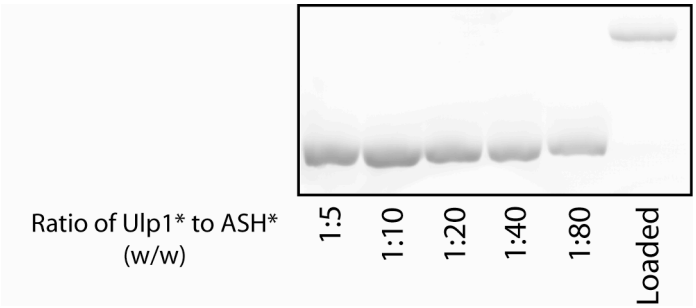


Figure S4. Selective cleavage of ASH* with Ulp1*. Greater than 85% of immobilized HaloTag was released from the resin using a 1:20 (w/w) ratio of Ulp1*: immobilized ASH*.

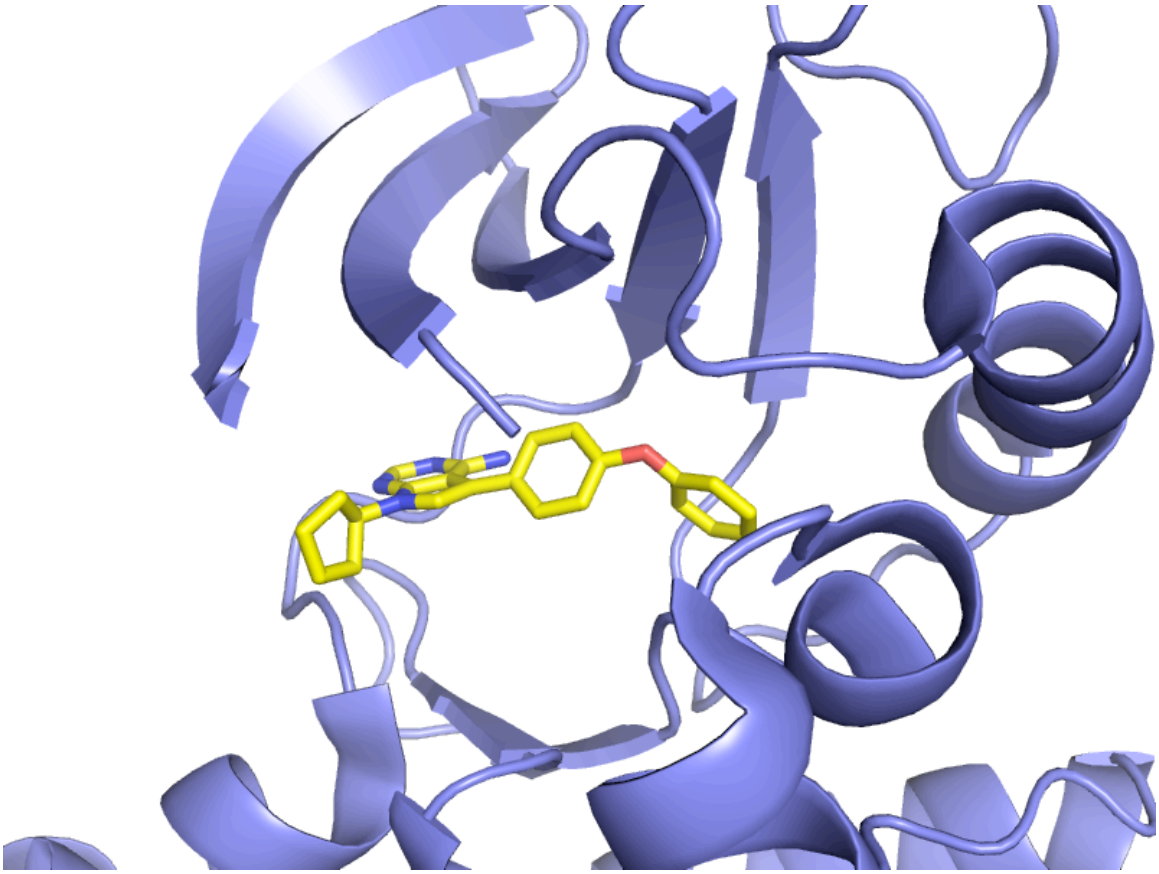


Figure S5. Crystal structure of BTK bound to **3** (PDB: 3GEN). Inhibitor **3** binds to BTK in the SRC/Cdk-like inactive conformation. In this conformation, the movement of the helix-αC out of the ATP-binding site creates a hydrophobic pocket occupied by the biphenyl ether of inhibitor **3**.

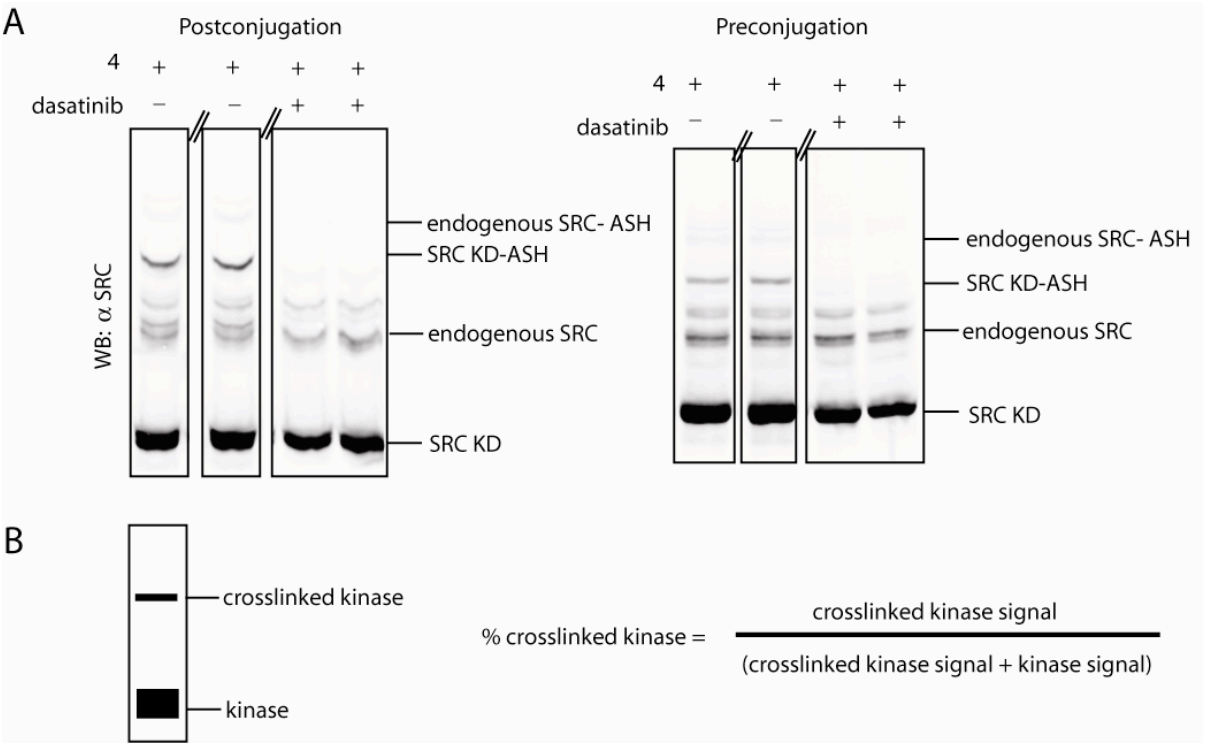


Figure S6. Crosslinking efficiency of Probe **4**. (A) Western blot analysis (anti-SRC) of the pre- and post-conjugation crosslinking to SRC. The molecular weight shift of SRC shows that approximately 10% of total SRC is crosslinked in the postconjugation method while less than 3% of SRC is crosslinked in the preconjugation method. (B) Equation for calculating percentage of crosslinked kinase.

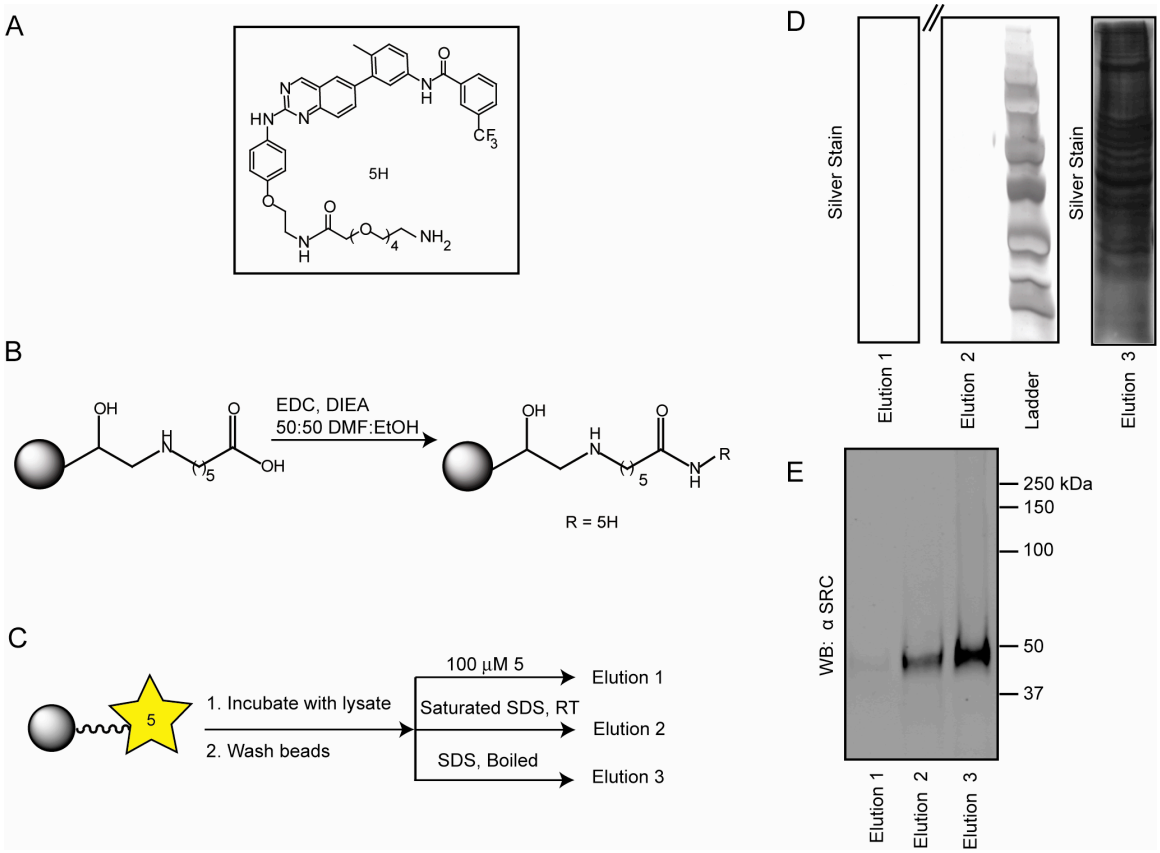


Figure S7. Synthesis and characterization of affinity matrix **5H**. (A) Structure of inhibitor **5H**. (B) Procedure for generating affinity matrix with inhibitor **5H**. (C) Overall strategy for the catch-and-release of lysate proteins with affinity matrix **5H**. HeLa lysate was incubated with the affinity matrix and then thoroughly washed. Three different elution conditions were used to release bound proteins. (D) Silver stain analysis of the catch-and-release experiment described in (C). Elution conditions 1 and 2 resulted in retention of bound proteins on the resin. Elution condition 3 resulted in release of many proteins from the resin. (E) Western blot analysis of the catch and release experiment described in (C) (lysate was supplemented with 10 nM SRC). Elution condition 3 (boiling the resin in SDS loading buffer) resulted in the greatest release of SRC from the resin.

II. SUPPORTING TABLES

Wash Conditions	Percent Cleavage
1 M NaCl	77%
0.5 mM EDTA	87%
pH 5	96%
pH 7	89%
pH 9	72%
6M urea	10%
0.1% SDS	1%
6M guanidine	6%
0.1% NP40	74%
0.1% Tween®20	87%

Table S1. Percent elution of fluorescently labeled HaloTag protein from CLP resin after stringent wash conditions. All wash conditions were performed in 50 mM Tris pH 7.5 and 100 mM NaCl unless otherwise noted.

	IC_{50} (nM)	
	6	**6control**
Src	**2.2**	**640**

Table S2. IC_{50} values for compounds **6** and **6control** against SRC.

	IC_{50} (nM)	
	5	**5control**
PTK2	**450**	**>10000**
EIF2AK2	**250**	**>10000**

Table S3. IC_{50} values for compounds **5** and **5control** against PTK2 and EIF2AK2.

III. SYNTHESIS

A. General Information

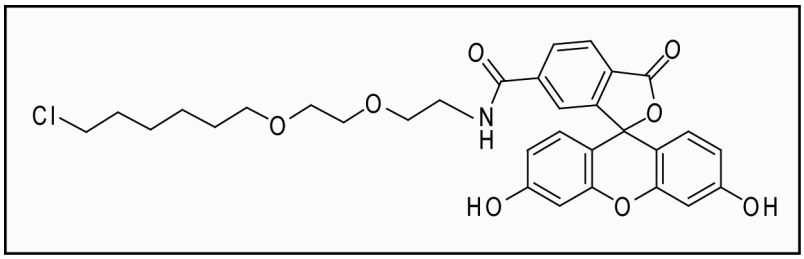
Unless otherwise noted, all reagents were obtained from commercial suppliers and used without further purification. ^{1}H-NMR spectra were obtained on a Bruker AV-300, AV-301, or Bruker 500 instrument at RT. Chemical shifts are reported in ppm, and coupling constants are reported in Hz. Mass spectrometry was performed on a Bruker Esquire Ion Trap MS instrument.

General HPLC Purification Conditions: Samples were injected on a preparatory reverse-phase C_{18} column (250 x 21 mm) run over 60 min. at 8 mL/min

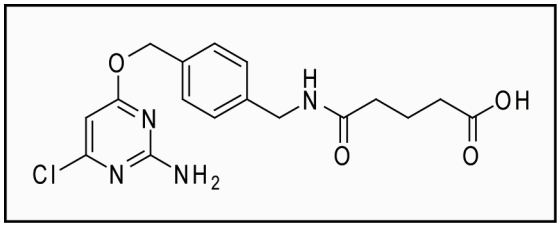
(Acetonitrile/Water–0.05% TFA gradient: 1:99 to 100:0). Purified products were detected by UV (254 nm).

General Analytical HPLC Conditions: General Analytical HPLC Conditions: C_{18} column (150 x 2.1 mm), Acetonitrile/Water-0.05% CF_3CO_2H gradient: 1:99 to 100:0 over 30 min. Methanol/Water–0.05% CF_3CO_2H gradient: 1:99 to 100:0 over 30 min. Flow rate = 1mL/min; 254 nM detection for 30 min.

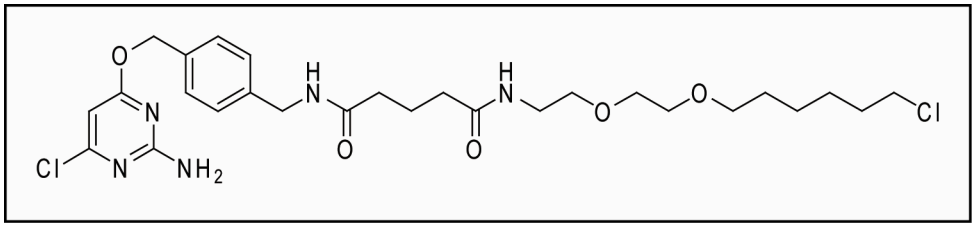
B. Synthesis of compounds 1, 2, 4, 5, 5control, 6, 6control, and 7



[1] Synthesized as previously reported .[1]

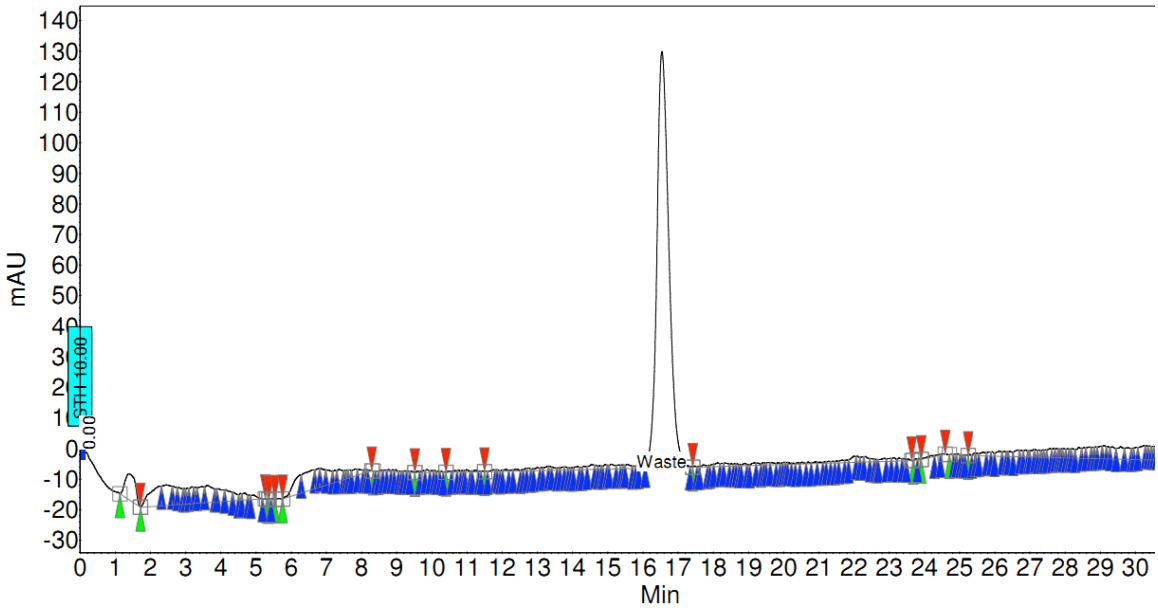


[2A] Synthesized as previously reported.[2]

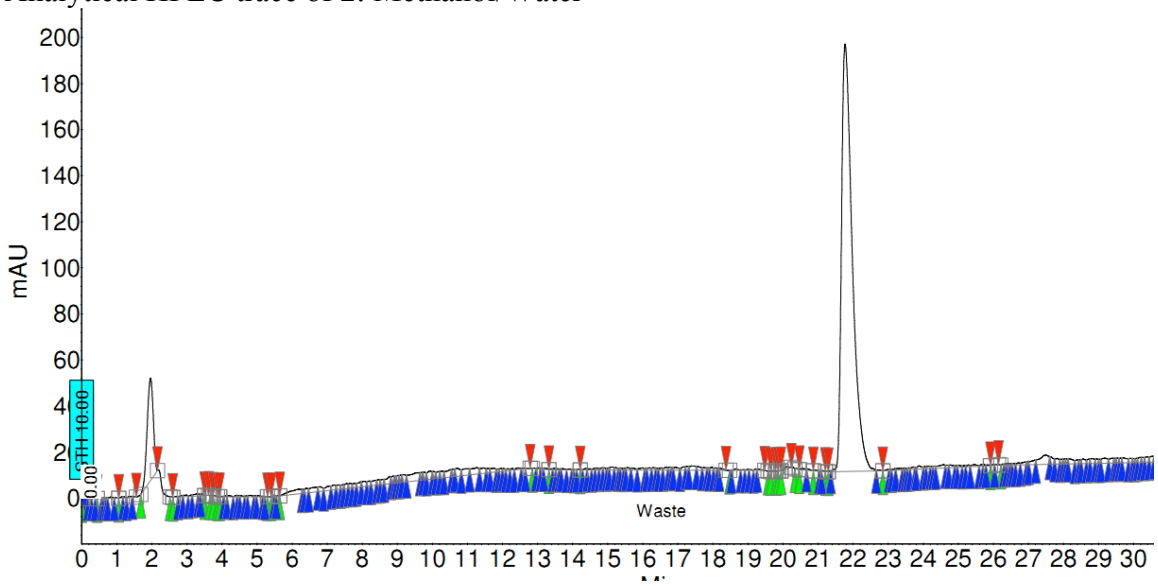


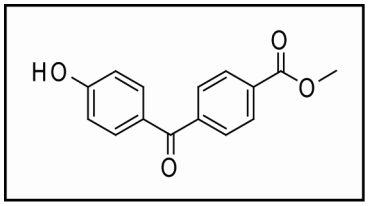
[2] 2A (5.0 mg, 0.013 mmol, 1 equiv.) and 2-(2-(6-chlorohexyloxy)ethoxy)ethanamine (8.9 mg, 0.026 mmol, 2 equiv.) were dissolved in DMF (80 µl) at 0 °C. HOBt (2.2 mg, 0.015 mmol, 1.1 equiv.), DIEA (8 µl, 0.046 mmol, 3.5 equiv.) and EDCI (3.7 mg, 0.014 mmol, 1.3 equiv.) were added sequentially and the reaction was warmed to RT overnight. The reaction was diluted in acetonitrile/water and purified using General HPLC Purification conditions. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.46 – 7.29 (m, 4H), 6.19 (s, 1H), 5.33 (s, 2H), 4.46 (d, J = 6.1 Hz, 2H), 3.67 – 3.51 (m, 8H), 3.50 – 3.34 (m, 4H), 2.34 – 2.24 (m, 2H), 2.11 – 1.97 (m, 2H), 1.88 – 1.73 (m, 2H), 1.66 – 1.29 (m, 8H). Calculated for $C_{27}H_{39}Cl_2N_5O_5$ ($M+H^+$): 584.2; found 584.4.

Analytical HPLC trace of **2**: Acetonitrile/Water

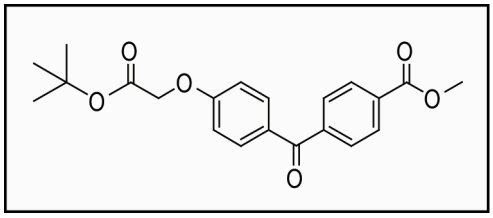


Analytical HPLC trace of **2**: Methanol/Water

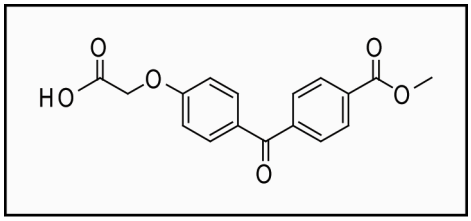




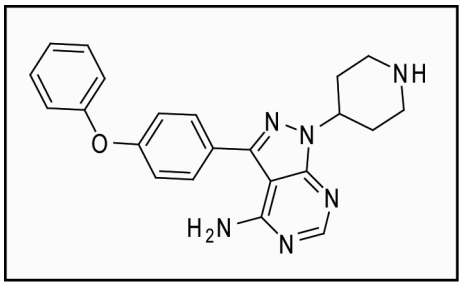
[4A] Synthesized as previously reported.[3]



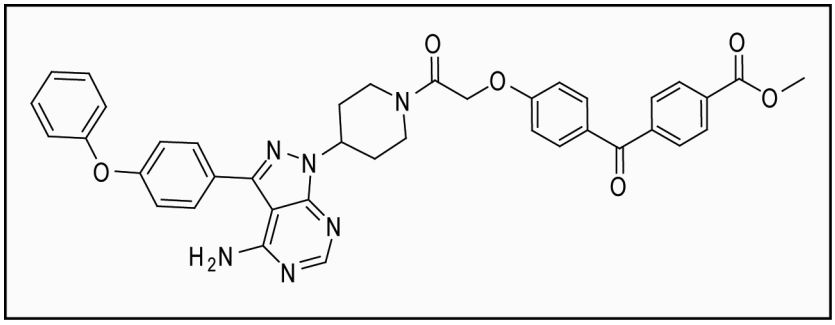
[4B] Under a nitrogen atmosphere, **4A** (284 mg, 1.11 mmol, 1 equiv.), tert-butyl bromoacetate (216 mg, 1.11 mmol, 1 equiv.), potassium carbonate (306 mg, 2.22 mmol, 2 equiv.), tetrabutylammonium iodide (36.9 mg, 0.100 mmol, 0.09 equiv.), and acetonitrile (2.3 mL) were combined, and the resulting mixture was heated to 70 °C. After 3 h, the reaction was cooled to 25 °C using an ice-water bath. The reaction was concentrated under reduced pressure. The concentrate was dissolved in dichloromethane, water was added, and the layers were separated. The organic layer was washed with water, dried over Na_2SO_4 and the solvent evaporated under reduced pressure to yield a pale yellow solid (91% yield) ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.08 (d, J = 9 Hz, 2H), 7.76 – 7.71 (m 4H), 6.90 (d, J = 9 Hz, 2H), 4.54 (s, 2H), 3.90 (s, 3H), 1.43 (s, 9H) Calculated for $C_{21}H_{22}O_6$ ($M+H^+$): 371.1; found 371.3.



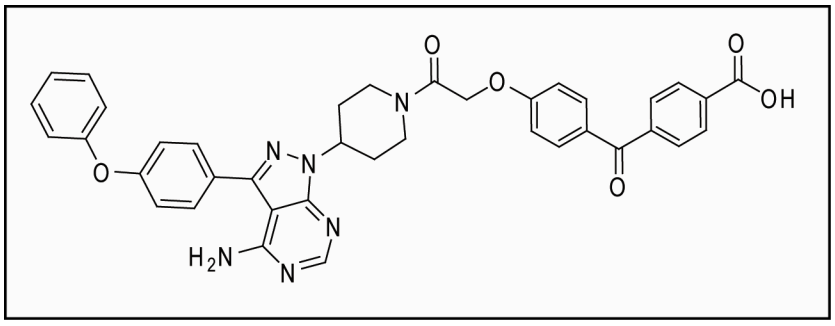
[4C] Under a nitrogen atmosphere, **4B** (373 mg, 1.01 mmol) and dichloromethane (1.9 mL) were combined at 0 °C. Trifluoroacetic acid (970 µl, 33% TFA) was added dropwise. The reaction was warmed to RT and stirred for 3 h. Toluene (1 mL) was added to the reaction and the resultant mixture was concentrated under vacuum. The product was used in the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.17 (d, J = 7.9 Hz, 2H), 7.88 – 7.81 (m, 4H), 7.04 (d, J = 9.1 Hz, 2H), 4.81 (s, 2H), 3.99 (s, 3H) Calculated for $C_{17}H_{14}O_6$ ($M+H^+$): 315.1; found 315.4.



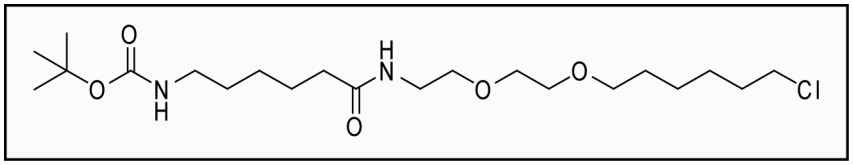
[4D] Synthesized as previously reported.[4]



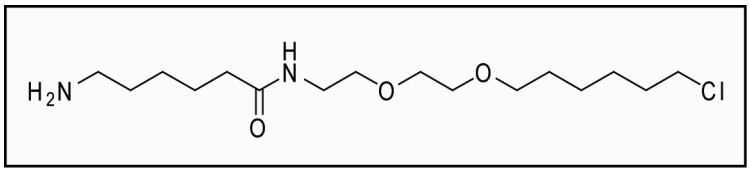
[4E] 4D (25.6 mg, 0.050 mmol, 1 equiv.) and **4C** (20.3 mg, 0.064 mmol, 1.3 equiv.) were dissolved in DMF (243 µl) at 0 °C. HOAt (8.8 mg, 0.064 mmol, 1.3 equiv.), DIEA (26 µl, 0.15 mmol, 3 equiv.) and EDCI (12.3 mg, 0.064 mmol, 1.3 equiv.) were added sequentially and the reaction was warmed to RT overnight. The reaction was diluted with ethyl acetate and washed with K_2CO_3 and saturated NH_4Cl. The product was then purified on silica, eluting with dichloromethane/methanol to yield the product (62%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.39 (s, 1H), 8.15 (d, *J* = 8.5 Hz, 2H), 7.82 (dd, *J* = 12.0, 8.7 Hz, 4H), 7.64 (d, *J* = 8.7 Hz, 2H), 7.45 – 7.37 (m, 2H), 7.24 – 7.02 (m, 7H), 5.08 – 5.04 (m, 1H), 4.86 (s, 2H), 4.78 – 4.74 (m, 1H), 4.22 – 4.18 (m, 1H), 3.98 (s, 3H), 3.41 – 3.37 (m, 1H), 3.00-2.95 (m, 1H), 2.46 – 2.07 (m, 4H). Calculated for $C_{39}H_{34}N_6O_6$ ($M+H^+$): 683.7; found 683.4.



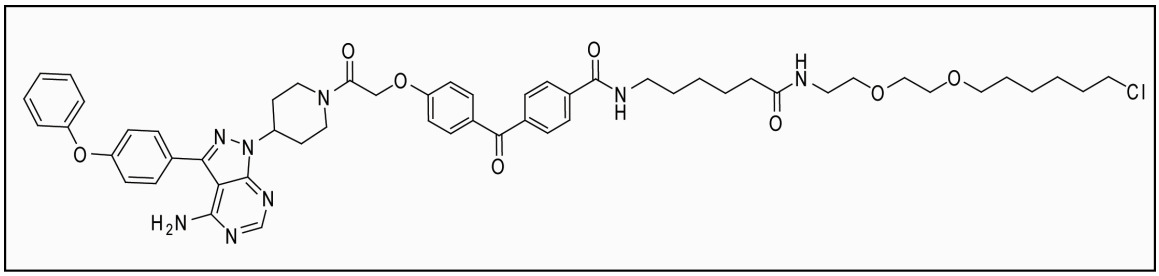
[**4F] 4E** (51.5 mg, 0.0756 mmol, 1 equiv.) was dissolved in dioxane. 2.2 equivalents of lithium hydroxide monohydrate (6.98 mg, 0.166 mmol) and 300 µl of water were added to the reaction mixture. The reaction was stirred at RT for 6 h. The reaction was taken up in water and washed with ethyl acetate. The aqueous layer was acidified with 3 M HCl and extracted with ethyl acetate. Extracted ethyl acetate layers were combined, dried with Na_2SO_4, and concentrated. The product was used in the next step without further purification. Calculated for $C_{38}H_{32}N_6O_6$ ($M+H^+$): 669.2; found 669.4.



[4G] 2-(2-(6-chlorohexyloxy)ethoxy)ethanamine (63 mg, 0.186 mmol, 1 equiv.) and Boc-6-aminohexanoic acid (56 mg, 0.242 mmol, 1.3 equiv.) were dissolved in DMF (93 µl) at 0 °C. HOAt (33 mg, 0.242 mmol, 1.3 equiv.), DIEA (97 µl, 0.558 mmol, 3 equiv.) and EDCI (46 mg, 0.242 mmol, 1.3 equiv.) were added sequentially and the reaction was warmed to RT overnight. The reaction was diluted in ethyl acetate and washed with K_2CO_3 and saturated NH_4Cl. The product was then purified on silica, eluting with dichloromethane/methanol to yield the product. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.69 – 3.56 (m, 6H), 3.56 – 3.44 (m, 4H), 3.19 – 3.07 (m, 2H), 2.24 – 2.16 (m, 2H), 2.00 – 1.89 (m, 2H), 1.85 – 1.75 (m, 2H), 1.74 – 1.63 (m, 6H), 1.46 (s, 9H), 1.42 – 1.32 (m, 6H). Calculated for $C_{21}H_{41}ClN_2O_5$ ($M+H^+$): 436.3; found ($M+Na^+$): 459.5.

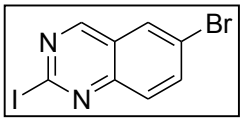


[4H] 4G (81 mg, 0.186 mmol) was dissolved in 30% TFA/DCM (9.3 mL) on ice. The reaction was stirred at RT for 3 h. Toluene (l mL) was added and the reaction was concentrated under reduced pressure. The reaction was diluted in acetonitrile/water and purified using General HPLC Purification conditions. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.71 – 3.49 (m, 10H), 3.50 – 3.34 (m, 4H), 2.21 (t, J = 7.0 Hz, 2H), 1.86 – 1.73 (m, 2H), 1.72 – 1.53 (m, 6H), 1.51 – 1.29 (m, 6H). Calculated for $C_{16}H_{33}ClN_2O_3$ ($M+H^+$): 337.2; found 337.5.

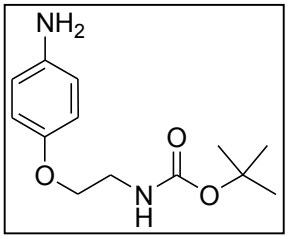


[4] **4F** (7.0 mg, 0.010 mmol, 1 equiv.) and **4H** (9.4 mg, 0.021 mg, 2 equiv.) were dissolved in DMF (52 µl) at 0 °C. HOAt (1.84 mg, 0.014 mmol, 1.3 equiv.), DIEA (6.4 µl, 0.031 mmol, 3 equiv.) and EDCI (2.6 mg, 0.014 mmol, 1.3 equiv.) were added sequentially and the reaction was warmed to RT overnight. The reaction was diluted in acetonitrile/water and purified using General HPLC Purification conditions to obtain 3.3 mg of **4**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.23 (s, 1H), 7.89 (d, J = 8.0 Hz, 2H), 7.82 (d, J = 8.6 Hz, 2H), 7.78 (d, J = 8.1 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H), 7.44 – 7.41 (m, 2H), 7.41 – 7.26 (m, 1H), 7.15 (d, J = 8.4 Hz, 2H), 7.09 (d, J = 7.9 Hz, 2H), 7.05 (d, J = 8.6 Hz, 2H), 4.85 (s, 2H), 3.66 – 3.44 (m, 19H), 2.42 – 2.27 (m, 2H), 2.23 (t, J = 7.0 Hz, 2H),

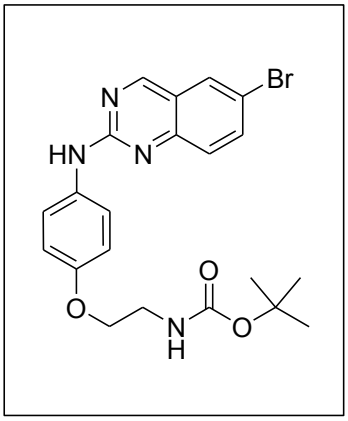
2.18 – 2.06 (m, 2H), 1.83 – 1.53 (m, 8H), 1.50 – 1.25 (m, 6H). Calculated for $C_{54}H_{63}ClN_8O_8$ ($M+H^+$): 987.5; found 987.6.



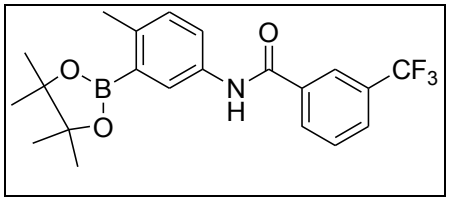
[5A] Synthesized as previously described.[5]



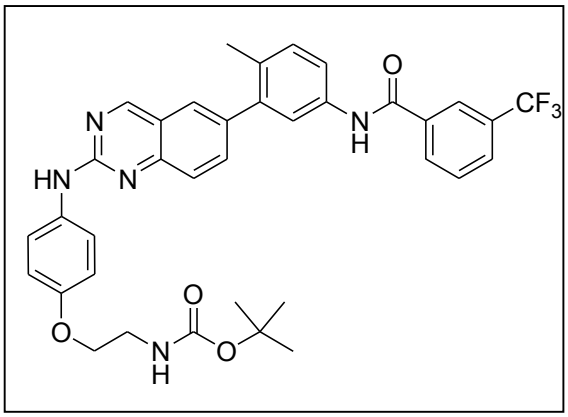
[5B] Synthesized as previously described.[6]



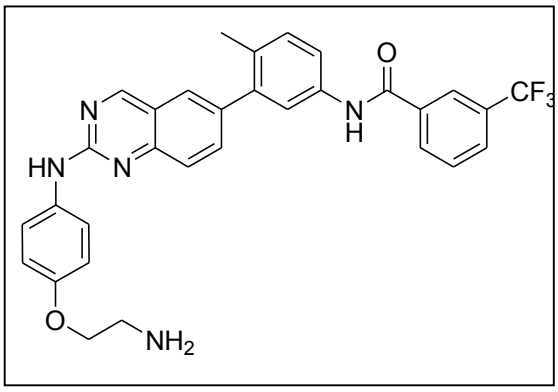
[5C] In a resealable Pyrex tube, **5A** (588.5 mg, 1.76 mmol) and **5B** (532 mg, 2.11 mmol) were dissolved in isopropanol (13.5 mL). TFA (262 µL, 3.52 mmol) was added and the tube was sealed. The suspension was stirred overnight at 70 °C. Triethylamine (1.5 mL) was added to neutralize the mixture and the mixture was concentrated. The residue was purified by column chromatography (50% ethyl acetate in hexanes) to afford 378.5 mg of compound **5C** (47% yield). ^{1}H NMR (300 MHz, $CDCl_3$) 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 (br s, 1H), 4.06 (t, J = 5.1 Hz, 2H), 3.59 – 3.53 (m, 2H), 1.48 (s, 9H). Calculated for $C_{21}H_{23}BrN_4O_3$ ($M+H^+$): 459.1; found 459.2.



[5D] 5-amino-2-methylphenylboronic acid pinacol ester (0.21 g, 0.86 mmol), 3-(trifluoromethyl)benzoic acid (0.21 g, 1.11 mmol), HOBt (0.17 g, 1.11 mmol), EDCI (0.21 g, 1.11 mmol) and DIEA (450 µL, 2.58 mmol) were dissolved in DMF (2.5 mL) and stirred overnight at RT. The crude mixture was diluted in ethyl acetate and washed with NH_4Cl and Na_2CO_3. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to afford 0.33 g of compound **5D** (96% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.14 (s, 1H), 8.07 (d, J = 6.0 Hz, 1H), 7.98 (d, J = 9.0 Hz, 1H), 7.84 – 7.81 (m, 2H), 7.68 – 7.62 (m, 2H), 7.23 (d, J = 9.0 Hz, 1H), 2.55 (s, 3H), 1.37 (s, 12H). Calculated for ($C_{21}H_{23}BF_3NO_3$) ($M+H^+$): 406.17; found 406.4.

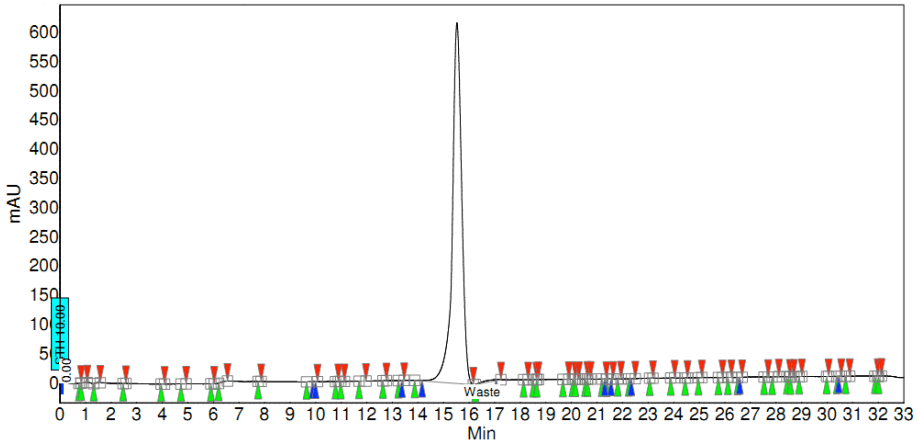


[5E] A mixture of compound **5C** (120 mg, 0.26 mmol), compound **5D** (127 mg, 0.31 mmol), tetrakis(triphenylphosphine)palladium (9.3 mg, 7.8 µmol) and sodium carbonate (60.9 mg, 0.57 mmol) was dissolved in a 3:1 mixture of DME/water (1 mL). The mixture was heated overnight at 85 °C. The crude mixture was cooled to RT, diluted with ethyl acetate and washed with water and brine. The organic layer was dried over Na_2SO_4, concentrated *in vacuo* and the resultant crude product was purified by column chromatography (5% methanol in dichloromethane) to afford 132 mg of compound **5E** (77 % yield). ^{1}H NMR (300 MHz, Methanol-d_4) δ 9.03 (s, 1H), 8.23 – 8.14 (m, 2H), 7.85 – 7.80 (m, 1H), 7.71 – 7.62 (m, 7H), 7.26 – 7.20 (m, 1H), 6.89 – 6.86 (m, 2H), 6.71 – 6.67 (m, 1H), 3.98 – 3.85 (m, 2H), 3.42 – 3.37 (m, 2H), 2.23 – 2.15 (m, 3H), 1.42 (d, J = 3.0 Hz, 9H). Calculated for ($C_{36}H_{34}F_3N_5O_4$) ($M+H^+$): 658.26; found 658.4.

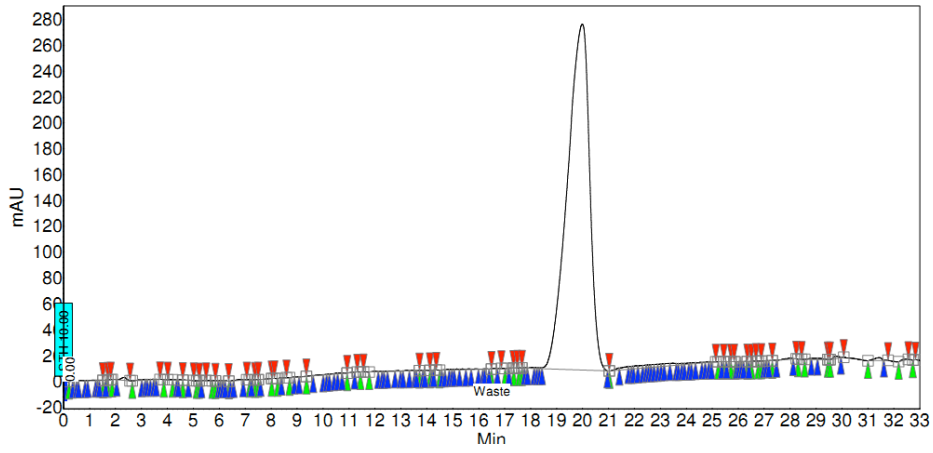


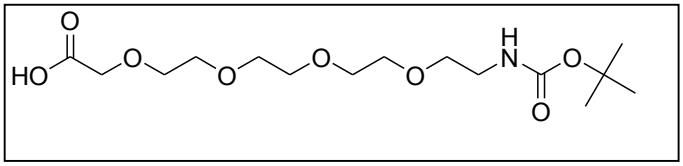
[5] Compound **5E** (40 mg, 0.062 mmol) was dissolved in CH_2Cl_2 (1 mL) and TFA (440 µL) and stirred for 3 h at RT. The reaction was concentrated and purified by reverse phase chromatography (HPLC) to obtain 20 mg (58 % yield) of the desired product **5**. ^{1}H NMR (300 MHz, Methanol-d_4) δ 9.27 (s, 1H), 8.28 – 8.21 (m, 2H), 7.93 – 7.85 (m, 2H), 7.80 – 7.73 (m, 4H), 7.67 – 7.48 (m, 2H), 7.43 – 7.28 (m, 2H), 7.21 (d, *J* = 8.9 Hz, 1H), 7.08 (d, *J* = 9.1 Hz, 1H), 5.50 (s, 1H), 4.33 – 4.26 (m, 2H), 3.44 – 3.37 (m, 2H), 2.32 – 2.28 (m, 3H). Calculated for ($C_{31}H_{26}F_3N_5O_2$) ($M+H^+$): 558.2; found 558.4.

Analytical HPLC trace of **5**: Acetonitrile/Water

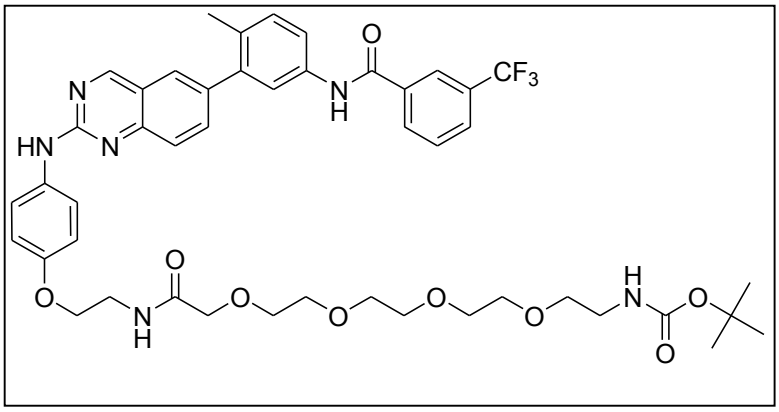


Analytical HPLC trace of **5**: Methanol/Water

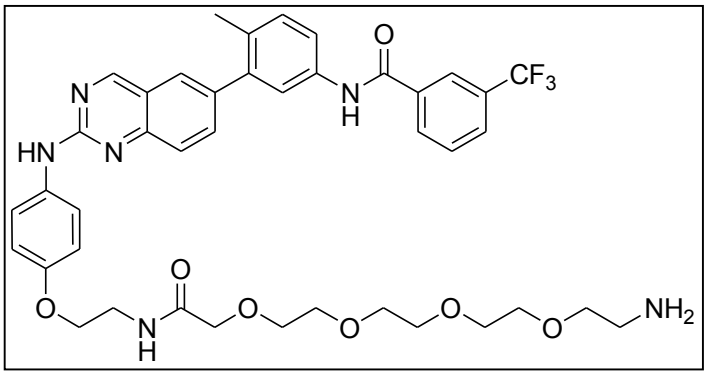




[5F] Synthesized as previously reported.[6]

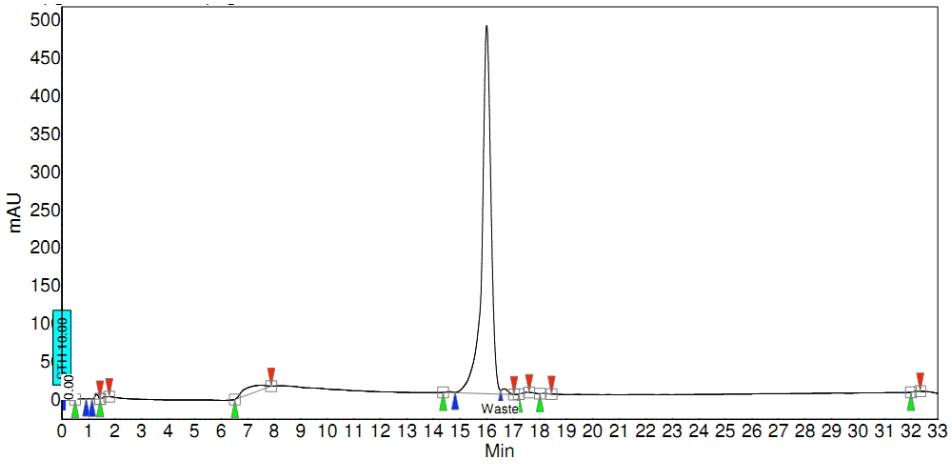


[5G] Compound **5** (62 mg, 0.112 mmol), compound **5F** (51 mg, 0.146 mmol), HOBt (23 mg, 0.146 mmol), EDCI (28.5 mg, 0.146 mmol) and DIEA (60 µL, 0.336 mmol) were dissolved in DMF (330 µL) and stirred overnight at RT. The crude mixture was diluted in ethyl acetate and washed with NH_4Cl and Na_2CO_3. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to obtain compound **5G**, which was used in the next step without further purification. Calculated for ($C_{46}H_{53}F_3N_6O_9$) ($M+H^+$): 891.4; found ($M+Na^+$): 913.4.

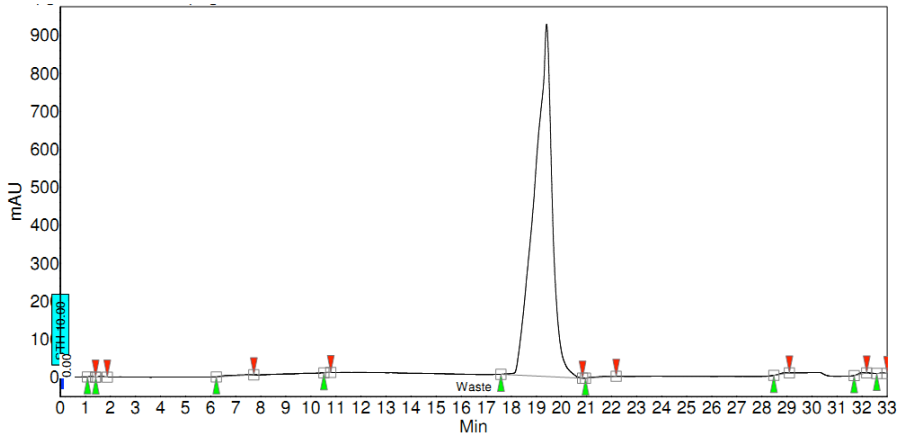


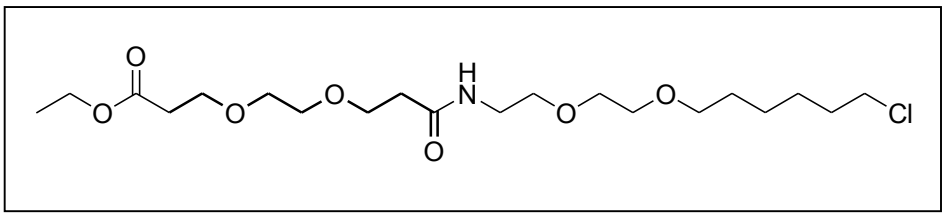
[5H] Compound **5G** (0.112 mmol) was dissolved in CH_2Cl_2 (1.9 mL) and TFA (0.8 mL) and stirred for 5 h at RT. The reaction was concentrated and purified by reverse phase chromatography (HPLC) to obtain 17.4 mg (20% yield) of the desired product **5H**. ^1H NMR (300 MHz, Methanol-d_4) δ 9.26 (s, 1H), 8.28 – 8.21 (m, 2H), 7.92 – 7.84 (m, 2H), 7.77 – 7.43 (m, 3H), 7.40 – 7.26 (m, 3H), 7.25 – 7.09 (m, 2H), 7.14 (d, J = 8.4 Hz, 1H), 7.02 (d, J = 8.8 Hz, 1H), 5.80 (s, 1H), 4.20 – 4.01 (m, 4H), 3.77 – 3.60 (m, 16H), 3.17 – 3.10 (m, 2H), 2.32 – 2.28 (m, 3H). Calculated for ($C_{41}H_{45}F_3N_6O_7$) ($M+H^+$): 791.3; found 791.6.

Analytical HPLC trace of **5H**: Acetonitrile/Water



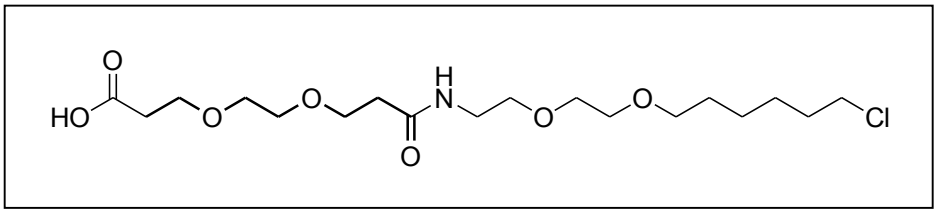
Analytical HPLC trace of **5H**: Methanol/Water



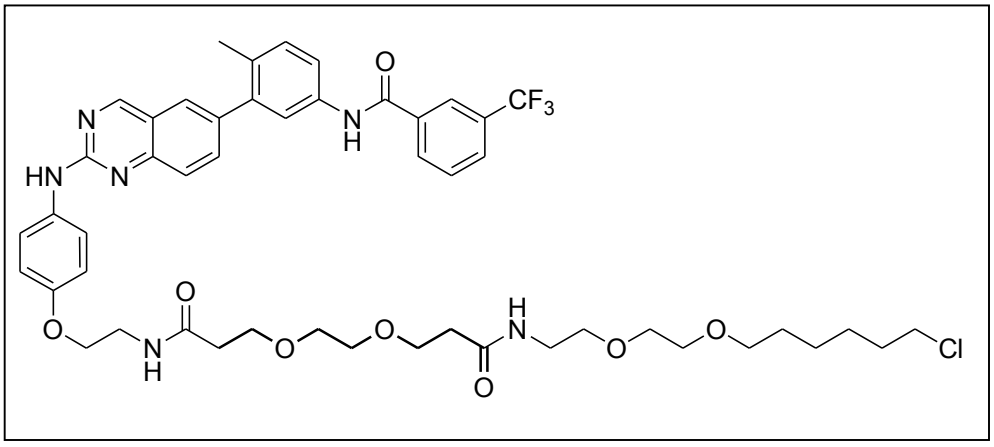


[6A] 2-(2-(6-chlorohexyloxy)ethoxy)ethanamine (74.6 mg, 0.33 mmol), 3-(2-(3-ethoxy-3-oxopropoxy)ethoxy)propanoic acid (101.6 mg, 0.43 mmol), HOBt (68.7 mg, 0.43 mmol), EDCI (84.9 mg, 0.43 mmol) and DIEA (180 µL, 1.0 mmol) were dissolved in DMF (980 µL) and stirred overnight at RT. The crude mixture was diluted in ethyl acetate and washed with NH_4Cl and Na_2CO_3. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to afford 106 mg of compound **6A** (73% yield). ^{1}H NMR (300 MHz, Methanol-d_4) δ 4.04 (q, *J* = 6.0 Hz, 2H), 3.70 – 3.58 (m, 4H), 3.56 – 3.40 (m,

12H), 3.38 – 3.29 (m, 4H), 2.51 – 2.46 (m, 2H), 2.38 – 2.34 (m, 2H), 1.70 – 1.63 (m, 2H), 1.53 – 1.48 (m, 2H), 1.39 – 1.24 (m, 4H) 1.17 (t, J = 6.0 Hz, 3H). Calculated for ($C_{20}H_{38}ClNO_7$) ($M+H^+$): 440.2; found 440.4.

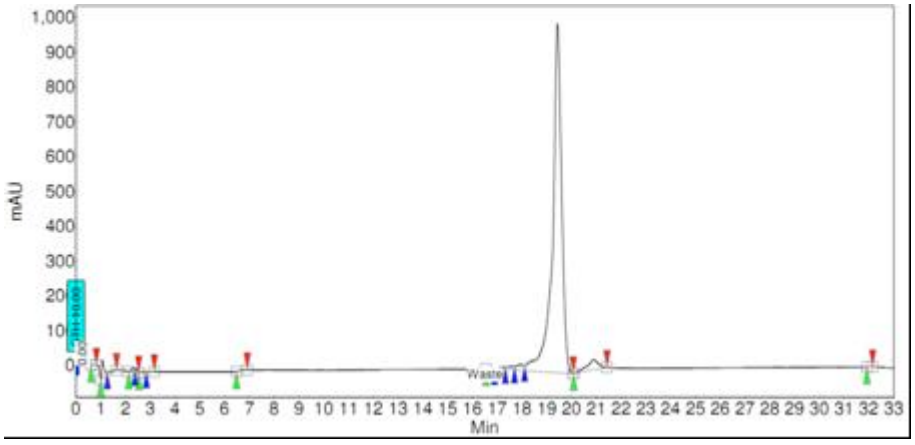


[6B] Compound **6A** (106 mg, 0.24 mmol) and $LiOH{\cdot}H_2O$ (30.5 mg, 0.73 mmol) was dissolved in a 1:1 mixture of THF/water (2.4 mL) and stirred for 2.5 h at RT. The reaction was concentrated and the pH was adjusted to 2 with 5 M HCl. The aqueous layer was extracted with ethyl acetate (4X), dried over Na_2SO_4 and concentrated *in vacuo* to afford 95.6 mg of compound **6B** (96% yield). Calculated for ($C_{18}H_{34}ClNO_7$) ($M+H^+$): 412.2; found 412.8.

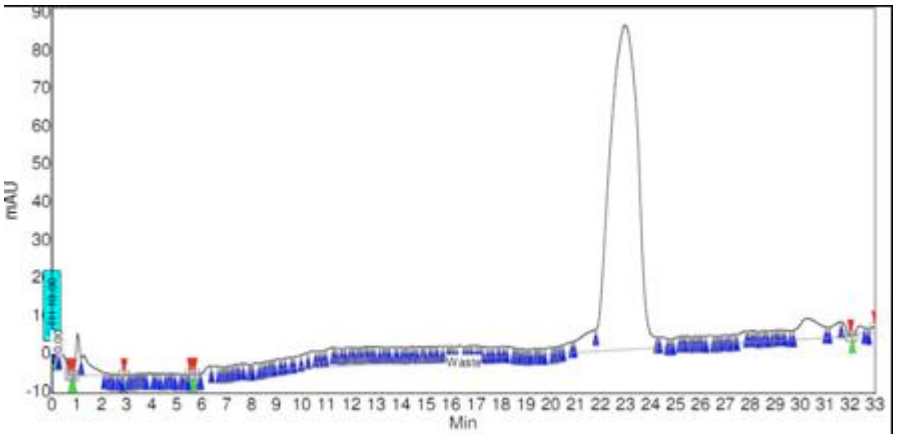


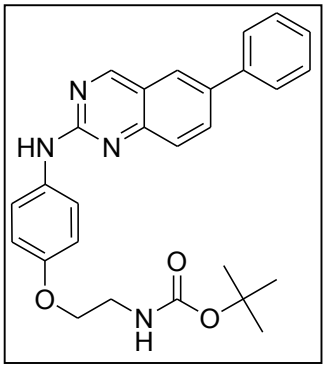
[6] Compound **5** (18.4 mg, 0.033 mmol), compound **6B** (17.6 mg, 0.043 mmol), HOBt (6.8 mg, 0.043 mmol), EDCI (8.4 mg, 0.043 mmol) and DIEA (17 µL, 0.099 mmol) were dissolved in DMF (100 µL) and stirred overnight at RT. The reaction was concentrated and purified by reverse phase chromatography (HPLC) to obtain 14.1 mg (45% yield) of the desired product **6**. ^{1}H NMR (300 MHz, Methanol-d_4) δ 9.37 (s, 1H), 8.24 (s, 1H), 8.19 (d, J = 6.0 Hz, 1H), 7.98 – 7.87 (m, 2H), 7.78 – 7.68 (m, 2H), 7.63 – 7.45 (m, 4H), 7.35 – 7.27 (m, 3H), 7.13 – 7.03 (m, 2H), 4.12 – 4.08 (m, 2H), 3.74 – 3.66 (m, 3H), 3.62 – 3.48 (m, 14H), 3.46 – 3.42 (m, 2H), 3.36 – 3.30 (m, 2H), 2.49 – 2.39 (m, 4H), 2.29 – 2.25 (m, 3H), 1.85 – 1.67 (m, 2H), 1.64 – 1.51 (m, 2H), 1.49 – 1.28 (m, 4H). Calculated for ($C_{49}H_{58}ClF_3N_6O_8$) ($M+H^+$): 951.4; found 951.7.

Analytical HPLC trace of **6**: Acetonitrile/Water



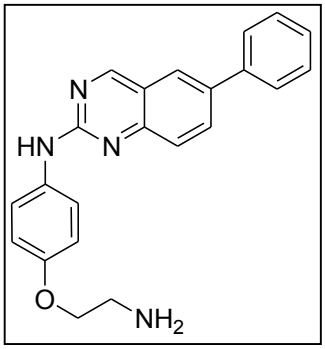
Analytical HPLC trace of **6**: Methanol/Water



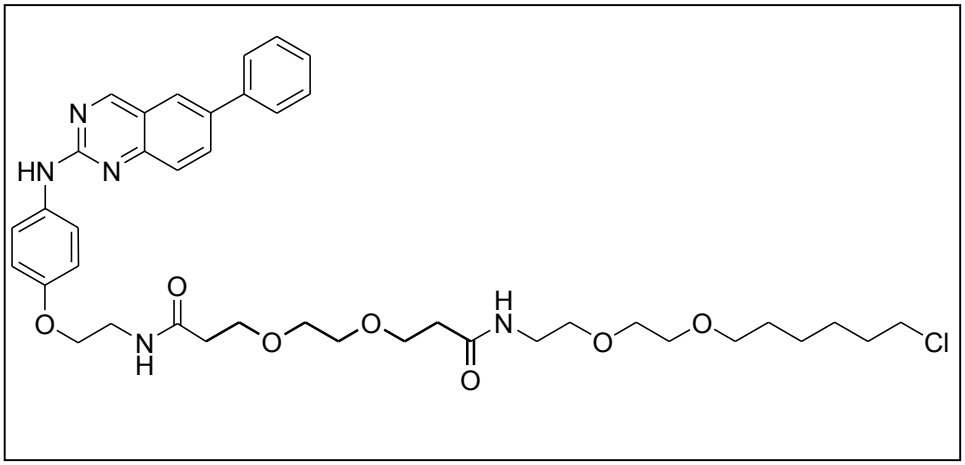


[5control A] A mixture of compound **5C** (45.1 mg, 0.098 mmol), phenylboronic acid pinacol ester (14.7 mg, 0..118 mmol), tetrakis(triphenylphosphine)palladium (3.5 mg, 3.0 µmol) and sodium carbonate (23 mg, 0.217 mmol) was dissolved in a 3:1 mixture of DME/water (390 µL). The mixture was heated overnight at 85 °C. The crude mixture was cooled to RT, diluted with ethyl acetate and washed with water and brine. The organic layer was dried over Na_2SO_4, concentrated *in vacuo* and the resultant crude product was

purified by column chromatography (50% ethyl acetate in hexanes) to afford 44 mg of compound **5control A** (98 % yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.16 (s, 1H), 8.06 (dd, *J* = 6.0 Hz, *J* = 3.0 Hz, 1H), 7.94 (d, *J* = 3.0 Hz, 1H), 7.81 (d, *J* = 9.0 Hz, 1H), 7.74 – 7.69 (m, 4H), 7.52 (t, *J* = 9.0 Hz, 2H), 7.44 – 7.39 (m, 1H), 6.96 (d, *J* = 9.0 Hz, 2H), 4.07 (t, *J* = 6.0 Hz, 2H), 3.60 – 3.54 (m, 2H), 1.49 (s, 9H). Calculated for ($C_{27}H_{28}N_4O_3$) ($M+H^+$): 457.2; found 457.4.



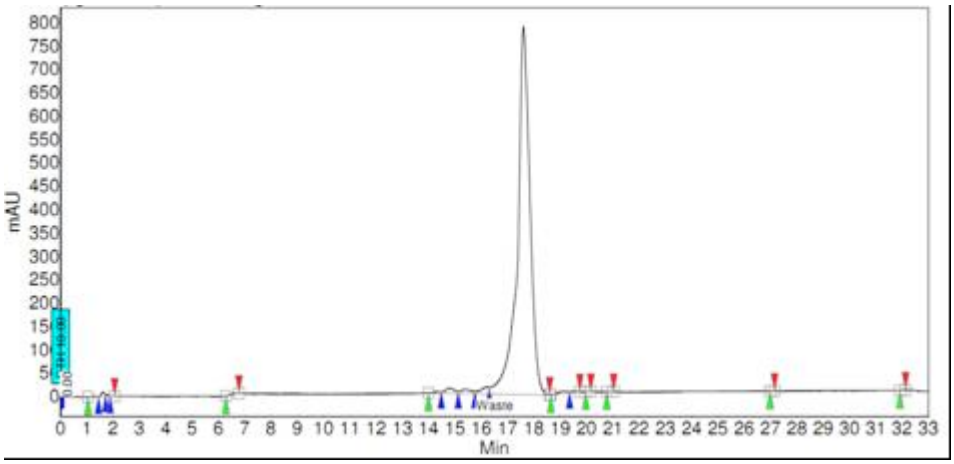
[5control B] Compound **5control A (**0.098 mmol) was dissolved in CH_2Cl_2 (1.6 mL) and TFA (0.7 mL) and stirred for 3 h at RT. The reaction was concentrated to obtain 34.1 mg (99% yield) of the desired product **5control B**. Crude product was azeotroped with toluene and used in the next step without further purification. Calculated for ($C_{22}H_{20}N_4O$) ($M+H^+$): 357.2; found 357.5.



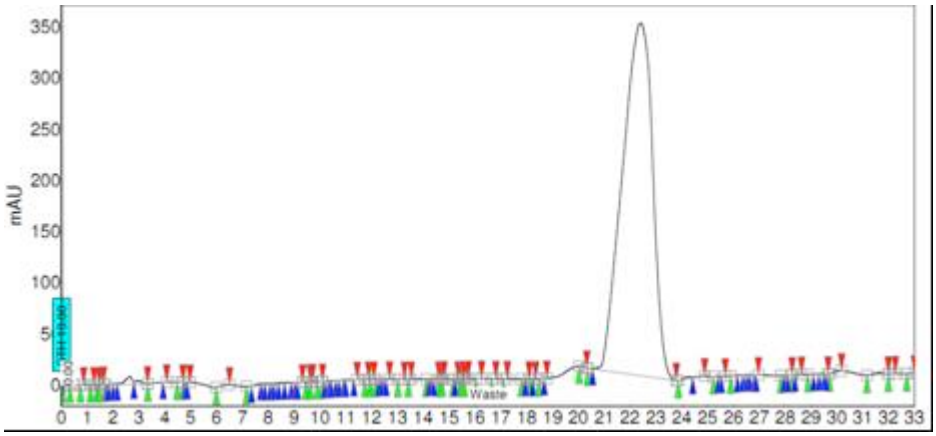
[6control] Compound **5control B** (26.3 mg, 0.074 mmol), **6B** (39.6 mg, 0.096 mmol), HOBt (15.2 mg, 0.096 mmol), EDCI (18.8 mg, 0.096 mmol) and DIEA (39 µL, 0.222 mmol) were dissolved in DMF (220 µL) and stirred overnight at RT. The reaction was concentrated and purified by reverse phase chromatography (HPLC) to obtain 26.0 mg (47% yield) of the desired product **6control**. ^{1}H NMR (300 MHz, Methanol-d_4) δ 9.22 (s, 1H), 8.31 – 8.23 (m, 1H), 8.12 – 7.97 (m, 2H), 7.81 – 7.64 (m, 4H), 7.54 – 7.45 (m, 2H), 7.43 – 7.36 (m, 1H), 7.02 – 6.91 (m, 2H), 4.12 – 4.02 (m, 2H), 3.79 – 3.67 (m, 4H), 3.66 – 3.50 (m, 14H), 3.49 – 3.42 (m, 2H), 3.39 – 3.37 (m, 2H), 2.51 (t, *J* = 6.0 Hz, 2H), 2.44

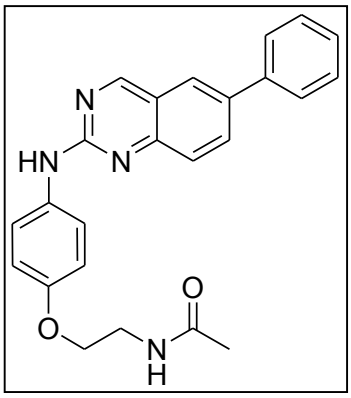
(t, J = 6.0 Hz, 2H), 1.79 – 1.67 (m, 2H), 1.63 – 1.51 (m, 2H), 1.47 – 1.30 (m, 4H). Calculated for ($C_{40}H_{52}ClN_5O_7$) ($M+H^+$): 750.4; found 750.5.

Analytical HPLC trace of **6control**: Acetonitrile/Water

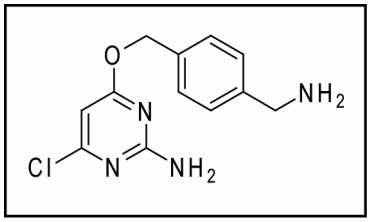


Analytical HPLC trace of **6control**: Methanol/Water





[**5control] 5control B** (6.3 mg, 0.0177 mmol) was dissolved in acetic anhydride (0.35 mL). Triethylamine (10 µL, 0.0708 mmol) was added and the reaction was stirred at RT for 2.5 h. The reaction was concentrated and purified by reverse phase chromatography (HPLC). ^{1}H NMR (300 MHz, Methanol-d_4) δ 9.15 (s, 1H), 8.04 (dd, J = 6.0 Hz, J = 3.0 Hz, 1H), 7.94 (d, J = 3.0 Hz, 1H), 7.86 – 7.63 (m, 5H), 7.52 (t, J = 9.0 Hz, 2H), 7.44 – 7.39 (m, 1H), 6.97 (d, J = 9.0 Hz, 2H), 4.08 (t, J = 6.0 Hz, 2H), 3.75 – 3.65 (m, 2H), 2.04 (s, 3H). Calculated for ($C_{24}H_{22}N_4O_2$) ($M+H^+$): 399.2; found 399.6.



[7] Synthesized as previously described.[2]

C. Generation of Chloropyrimidine (CLP) Resin

0.5 mL of NHS-activated Sepharose™ 4 Fast Flow (GE Healthcare) was washed with 10 bed volumes of 1:1 DMF/EtOH. 50 µL of 0.75 mM **7** was then added to the resin followed by 5 µL of DIEA. The reaction mixture was rotated overnight at RT, then drained and the resin washed with 2 bed volumes of DMF/EtOH. Unreacted NHS-activated esters were capped by incubating the resin with 0.1 M Tris-HCl, pH 8.5 overnight. The resin was washed with 10 bed volumes of Wash Buffer A (0.1 M Tris-HCl, pH 8.5) then Wash Buffer B (0.1 M sodium acetate, pH 5, 0.5 M NaCl). This wash cycle was repeated 3 times followed by 10 bed volumes of 20% EtOH (1x). 0.5 mL of 20% EtOH was added to the resin resulting in a 50% slurry for storage at 4 °C.

IV. PROTEIN EXPRESSION AND PURIFICATION

A. ASH-Fusion Protein Design, Expression, and Purification

ASH-Fusion Protein Design

The AGT gene from the SNAP source plasmid pss26b (Covalys) was amplified using primers that included a 3' end complementary to the 5' end of SUMO. The SUMO gene was amplified from a plasmid containing the SMT3/SUMO gene (Plasmid 16092: pT-35 Addgene) with primers that included a 5' end complementary to the 3' end of AGT and a 3' end complementary to the 5' end of HaloTag. The HaloTag gene from the pFC8K

vector (Promega) was amplified using primers that included a 5' end complementary to the 3' end of SUMO. The two double-stranded DNA fragments from AGT and SUMO containing complementary overlapping regions were fused over 35 cycles using the LIC AGT Fwd and SUMO HaloTag Rev primers to give the AGT-SUMO construct. Likewise, the double-stranded AGT-SUMO fusion product and the HaloTag product were fused over 35 cycles using the LIC Fwd and LIC Rev primers to give the final construct AGT-SUMO-HaloTag (ASH). The complete construct was then incorporated into the vector pMCSG7 (Midwest Center for Structural Genomics) by ligation independent cloning.

Expression of ASH and ASH* Fusion Proteins

Single colonies of BL21(DE3) cells transformed with the ASH plasmid were grown overnight in 6 mL LB supplemented with ampicillin (100 μg/mL) at 37 °C. Overnight cultures were then centrifuged at 3000 x g for 6 min. at 4 °C. The media was decanted and the cell pellet was resuspended in 2 mL of LB and transferred to 1 L LB (100 μg/mL ampicillin). The bacterial culture was grown at 37 °C to an OD_{600nm} of 0.8-1.0. The temperature was then decreased to 18 °C before expression of ASH was induced with 1 mM IPTG. The culture was grown for an additional 16 h and then was centrifuged at 5000 x g for 20 min.. Cell pellets were stored at -80 °C until purification.

Purification of ASH and ASH* Fusion Proteins

Cell pellets from 250 mL cultures were re-suspended in His6 Wash Buffer (50 mM HEPES, 10 mM imidazole, pH 7.5) and PMSF (100 μg/mL) was added. Cells were lysed by sonication and centrifuged at 10,000 rpm for 20 min. at 4 °C to clear the lysate. The cleared lysate was then added to Promega HisLink^{TM} Protein Purification resin and rotated at 4 °C for 30 min. The resin was washed with His6 Wash Buffer (3X) and then the protein was eluted with His6 Elution Buffer (100 mM HEPES, 600 mM imidazole, pH 7.5). The collected fractions were then dialyzed into storage buffer (50 mM HEPES, 1 mM DTT, pH 7.5) and concentrated.

B. Ulp1 Expression and Purification

Ulp1 and Ulp1* Expression

The gene encoding for Ulp1 (GenScript) was cloned into the vector pMCSG7 (Midwest Center for Structural Genomics). Single colonies of BL21(DE3) cells transformed with the Ulp1 plasmid were grown overnight in 6 mL of LB supplemented with ampicillin (100 μg/mL) at 37^{o} C. Overnight cultures were then centrifuged at 3000 x g for 6 min. at 4 °C. The media was decanted and the cell pellet was resuspended in 2 mL of LB and transferred to 1 L LB (100 μg/mL ampicillin). The bacterial culture was grown at 37 °C to an OD_{600nm} of 0.6 – 0.8. The temperature was then decreased to 25 °C before expression of Ulp1 was induced with 1 mM IPTG. The culture was grown for an additional 4 h and then was centrifuged at 5000 x g for 20 min. Cell pellets were stored at -80 °C until purification.

Ulp1 and Ulp1* Purification

Cell pellets from 250 mL cultures were re-suspended in His6 Wash Buffer (50 mM HEPES, 10 mM imidazole, pH 7.5) and PMSF (100 μg/mL) was added. Cells were lysed

by sonication and centrifuged at 10,000 rpm for 20 min at 4 °C to clear the lysate. The cleared lysate was then added to Promega HisLink^{TM} Protein Purification resin and rotated at 4 °C for 30 min. The resin was washed with His6 Wash Buffer (3X) and then the protein was eluted with His6 Elution Buffer (100 mM HEPES, 600 mM imidazole, pH 7.5). The collected fractions were then dialyzed into storage buffer (75 mM Tris (pH 8.0), 1 mM DTT, 2 mM EDTA) and concentrated.

C. ASH* Primer Design

SUMO mutations (R64T and R71E) were generated using site-directed mutagenesis (Stratagene).

Fwd R64T: 5' – GAA ATG GAC TCC TTA ACC TTC TTG TAC GAC GGT – 3'
Rev R64T: 5' – ACC GTC GTA CAA GAA GGT TAA GGA GTC CAT TTC – 3'

Fwd R71E: 5' – TTG TAC GAC GGT ATT GAA ATT CAA GCT GAT CAG – 3'
Rev R71E: 5' – CTG ATC AGC TTG AAT TTC AAT ACC GTC GTA CAA – 3'

D. Ulp1* Design

Ulp1 mutations (D451S, T452G, and E455S) were generated using two sequential site-directed mutagenesis reactions (Stratagene).

Ulp1 QC Fwd1: 5' – CCG CGT CGC TGG CTG AAT AGT GGC ATC ATC GAA TTT TTC ATG – 3'
Ulp1 QC Rev1: 5' – CAT GAA AAA TTC GAT GAT GCC ACT ATT CAG CCA GCG ACG CGG – 3'

Ulp1 QC Fwd2: 5' – CTG AAT AGT GGC ATC ATC AGT TTT TTC ATG AAA TAC ATC – 3'
Ulp1 QC Rev2: 5' – GAT GTA TTT CAT GAA AAA ACT GAT GAT GCC ACT ATT CAG – 3'

E. SRC Expression and Purification

The catalytic domain of chicken c-SRC (SRC KD, residues 251-533) and c-SRC that contains the SH1, SH2 and SH3 domains (SRC) were expressed and purified using a previously published procedure.[7] This method generates unphosphorylated SRC.

V. MAMMALIAN CELL CULTURE

A. Cell culture

HeLa cells were cultured in DMEM-Low Glucose media supplemented with 10% fetal bovine serum. COS-7 cells were cultured in DMEM-High Glucose media supplemented with 10% fetal bovine serum. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

B. Transfection Protocol
Cells were transfected with mammalian-optimized genes in pDEST26 (Invitrogen) mammalian expression vector using FuGene HD transfection reagent (Promega). 2 x 10^5 cells were plated in each well of a 12 well plate. Cells were grown overnight to 50-70% confluency then washed with serum free media and incubated with 1 mL of Opti-MEM Media (Gibco). Each well of cells received 2 μg of DNA and 6 μL of FuGene reagent in 100 μL of Opti-MEM Media. Cells were incubated for 24 h to allow for protein expression.

C. Mammalian Cell Lysate
COS-7 cells or HeLa cells were grown to confluency in 15 cm plates and pelleted. Five pellets were then combined and resuspended in 1.5 mL of cell lysis buffer (50 mM Tris, 100 mM NaCl, 1 mM PMSF). The cells were lysed using a Dounce homogenizer and cleared via centrifugation for 20 min at 16,000 rpm. The lysate concentration was determined using a Bradford assay.

VI. *IN VITRO* PULLDOWN METHODS

A. Generation of a singly-labeled hexylchloride protein
Purified SNAP-tag (15 μM) was incubated with **2** (22.5 μM) in labeling buffer (50 mM Tris buffer, pH = 7.5, 100 mM NaCl, 0.1% Tween 20 and 1 mM DTT) for 1.5 h at RT. The singly-labeled SNAP-tag was then separated from the unconjugated labeling reagent **2** by running the reaction mixture through a Bio-Rad Micro Bio-Spin© column equilibrated with labeling buffer.

B. Crosslinking Procedures
Preconjugation:
All crosslinking experiments were performed in a 96-well, U-bottom plate. Purified SRC was diluted in PBS to 25 μL at a concentration of 100 nM with a concentration of 2 mg/mL mammalian cell lysate. ASH*, covalently labeled with **4**, was added at a final concentration of 500 nM, such that the final volume per well was 50 μL ($[kinase]_f$ = 50 nM). Dasatinib was added to control wells only at a final concentration of 10 μM. Samples were irradiated at 365 nm on ice for 10 min by placing a Spectroline ENF-260C UV lamp directly on top of the plate**.** Samples were run on 10% SDS-PAGE gels and immunoblotted (Src (36D10) antibody, Cell Signaling). The scanned blots were quantified with Li-cor Odyssey software to determine crosslinking efficiency.

Postconjugation:
Purified SRC was diluted in PBS to 50 μL at a concentration of 50 nM with a concentration of 1 mg/mL mammalian cell lysate. Compound **4**, was added at a final concentration of 500 nM. Dasatinib was added to control wells only at a final concentration of 10 μM. Samples were irradiated at 365 nm on ice for 10 min by placing a Spectroline ENF-260C UV lamp directly on top of the plate**.** After irradiation, 1 μM ASH* was added to the crosslinked sample and incubated at RT for 1 h. Samples were run on 10% SDS-PAGE gels and immunoblotted (SRC (36D10) antibody, Cell

Signaling). The scanned blots were quantified with Li-cor Odyssey software to determine crosslinking efficiency.

VII. AFFINITY RESIN METHODS

A. Generation of Affinity Resin

Affinity matrix **5H** was generated from ECH Sepharose resin using a previously published procedure [6] (Figure S5).

B. General method for affinity resin catch-and-release experiments

HeLa lysate (500 µg of protein) was added to 25 µL of affinity resin and incubated for 2 h at 4 °C. The resin was then washed 4X by incubating with 10 bed volumes of Wash Buffer (50 mM HEPES, pH 7.5, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, and 100 mM NaCl) for 30 min at 4 °C. After the final wash, one of three elution conditions was used: (1.) Incubation with 200 µL of free inhibitor, **5**, at a final concentration of 10 µM for 30 min at RT; (2.) 100 µL of saturated SDS in binding buffer was added to the resin and incubated for 10 min at RT. The eluted protein was collected and this process was repeated once; (3.) 200 µL 1X SDS Loading Buffer was added to the resin and boiled for 20 min. Samples were run on 10% SDS-PAGE gels and either silver stained (Invitrogen SilverXpress Staining Kit) or immunoblotted (Src (36D10) antibody, Cell Signaling). The scanned blots were quantified with Li-cor Odyssey software to determine elution efficiency.

C. Enrichment of endogenous kinases from HeLa lysate

ASH* (0.2 mg) was immobilized on CLP resin and then incubated with 200 µL of **6** (final concentration = 8.5 µM) or **6control** (final concentration = 8.5 µM) in Buffer A (50 mM Tris, pH=7.5, 100 mM NaCl) for 1 h at RT. Excess small molecule was removed through a series of washes with Buffer A. HeLa lysate was then incubated with the resin for 2 h at RT. After a series of washes with Buffer B (50 mM Tris, pH=8.0, 300 mM NaCl, 0.1% Tween), the beads were incubated with Ulp1* (1:20 mass ratio Ulp1*:ASH*). Eluted samples were separated on 10% SDS-PAGE gels. Samples were then subjected to the Mass Spectrometry protocol described below.

VIII. ACTIVITY ASSAYS

In vitro activity assays for ABL, CSK, HCK, IRAK4, LCK, STK10, MAP3K5, p38α, PAK4, PAK5, EPHA3 and SRC were performed using previously published protocols.[8]

PTK2:

Inhibitors (initial concentration = 10 µM, 3-fold serial dilutions down to 0.2 nM) were assayed in triplicate against PTK2 (Invitrogen) (final concentration = 1 ng/µL) in assay buffer containing 50 mM HEPES, pH = 7.5, 60 mM MgCl2, 1 mM EGTA, 2 mM Na_3VO_4, 100 µg/mL BSA, γ^{32}P ATP (0.2 µCi/well) and poly(Glu, Tyr) (Sigma) as substrate (final concentration = 200 µg/mL). The final volume of each assay well was 30 µL. The enzymatic reaction was run at RT for 2 h and then terminated by spotting 4.6 µL of the reaction mixture onto a nitrocellulose membrane. Membranes were washed with

0.5% phosphoric acid (4 x 5 min 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 IC_{50} values were determined using non-linear regression analysis.

EIF2AK2:
Inhibitors (initial concentration = 10 μM, 3-fold serial dilutions down to 0.2 nM) were assayed in triplicate against EIF2AK2 (Invitrogen) (final concentration = 165 pg/μL) in assay buffer containing 50 mM HEPES, pH = 7.5, 60 mM MgCl2, 1 mM EGTA, 2 mM Na_3VO_4, 100 μg/mL BSA, γ32P ATP (0.2 μCi/well) and myelin basic protein as substrate (final concentration = 200 μg/mL). The final volume of each assay well was 30 μL. The enzymatic reaction was run at RT for 100 min and then terminated by spotting 4.6 μL of the reaction mixture onto a phosphocellulose membrane. Membranes were washed with 0.5% phosphoric acid (4 x 5 min 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 IC_{50} values were determined using non-linear regression analysis.

IX. MASS SPECTROMETRY

A. In-gel trypsin digest of enriched DFG-out proteins

Each lane of a 10% SDS-PAGE gel above 30 kDa was divided into three sections. Each section was excised from the gel and placed in 1.5 mL eppendorf tubes. Each gel slice was washed three times: first with 500 μL of 100 mM ammonium bicarbonate by rotating for 15 min at RT then with 500 μL acetonitrile (15 min, RT). Gel slices were then speedvaced to dryness and rehydrated on ice for 45 min with 50 μL of a trypsin solution (20 μg dissolved in 1 mL 50 mM ammonium bicarbonate) (Sigma, Proteomics Grade, BioReagent, Dimethylated). 50 mM ammonium bicarbonate was then added to cover the expanded gel slice and then the gel slice was incubated overnight at 37 °C.

B. Mass Spectrometry Analysis

Samples were submitted to Mass Spectrometry Center at the University of Washington, School of Pharmacy. Data were generated with an LTQ Velos (Thermo Scientific).

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