

SUPPORTING INFORMATION

Bivalent inhibitors of c-Src tyrosine kinase that bind a regulatory domain

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I. SUPPLEMENTARY FIGURES FOR MAIN TEXT

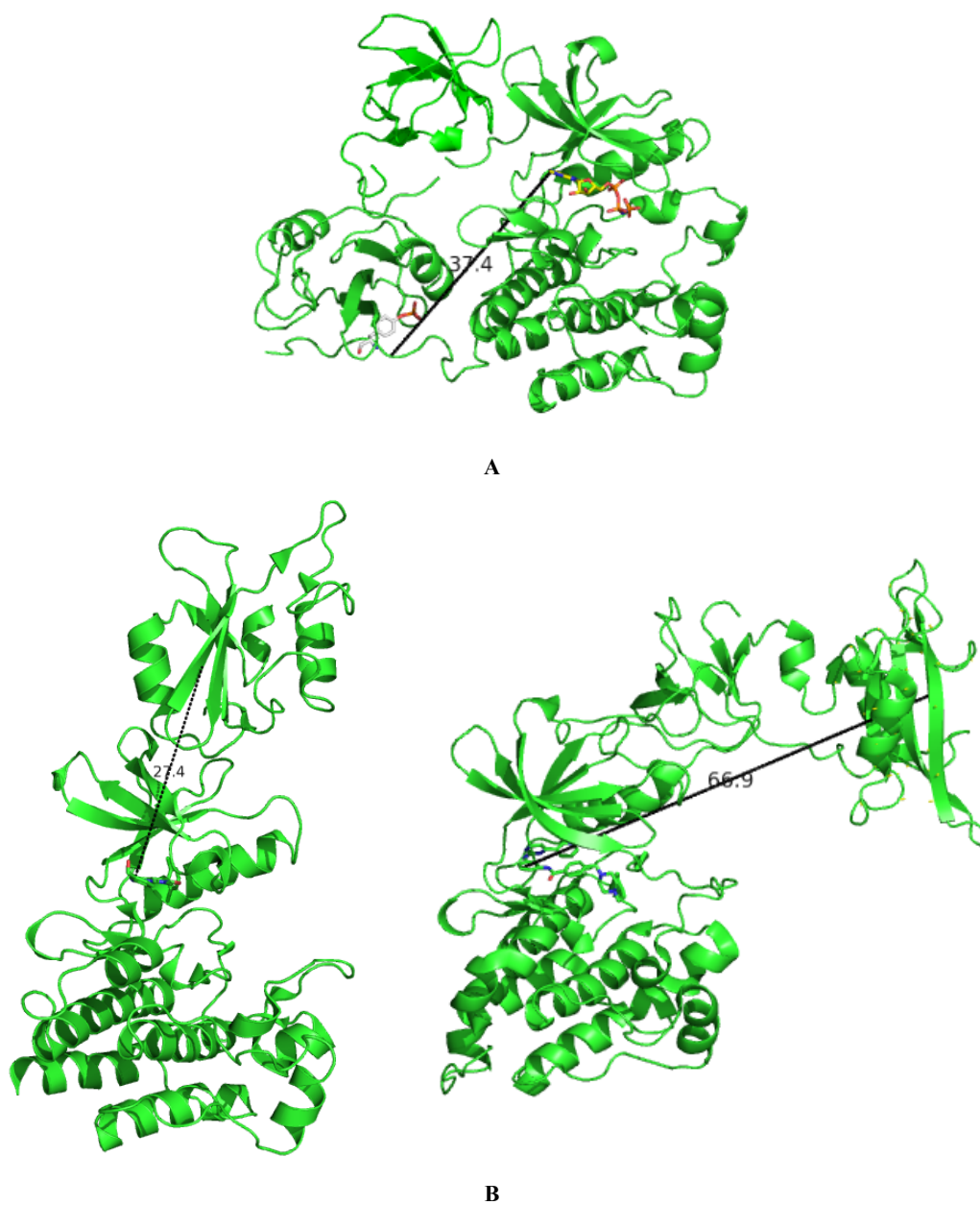


Figure S1: Crystal structures showing measurements between the ATP-pocket and SH2 domain of protein tyrosine kinases in various conformations. **A)** Closed/inactive conformation (1.91 Å, PDB code: 2SRC) **B)** Open/active conformations exhibiting a variety of distances between the kinase domain and SH2 domain ranging from 27 Å (3.42 Å, PDB code: 1OPL) to 67 Å (1.5 Å, PDB code: 1Y57)

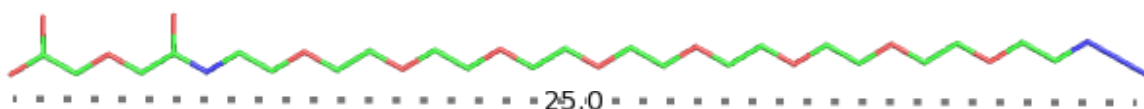


Figure S2: PyMOL measurement in Angstroms of the PEG linker used to create 2 and bivalent inhibitors 3, 4, and 5.

II. FULL AUTHOR LIST FOR THE REFERENCES FROM THE ARTICLE

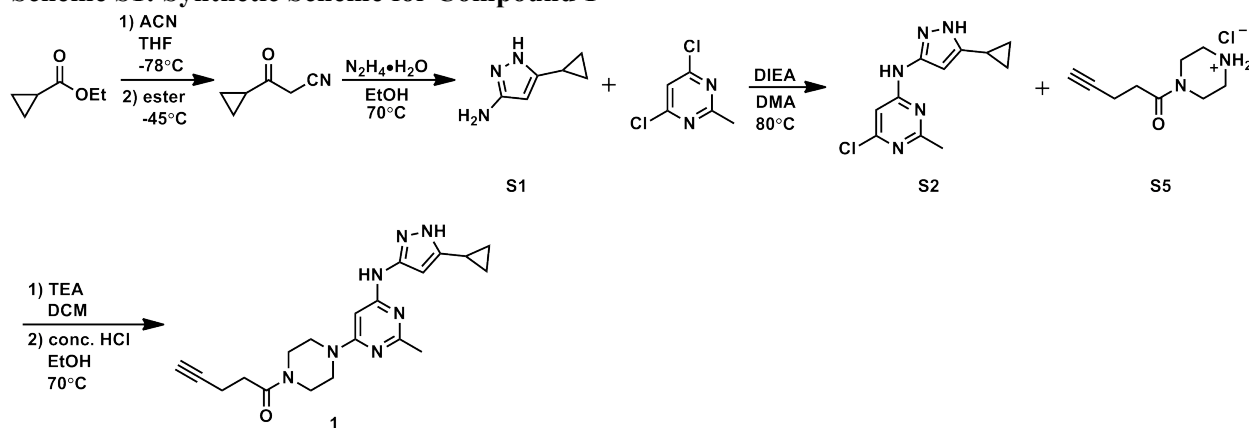
(6) Fabian, M. A., Biggs, W. H., Treiber, D. K., Atteridge, C. E., Azimioara, M. D., Benedetti, M. G., Carter, T. A., Ciceri, P., Edeen, P. T., Floyd, M., Ford, J. M., Galvin, M., Gerlach, J. L., Grotzfeld, R. M., Herrgard, S., Insko, D. E., Insko, M. A., Lai, A. G., Lélias, J.-M., Mehta, S. A., Milanov, Z. V., Velasco, A. M., Wodicka, L. M., Patel, H. K., Zarrinkar, P. P., and Lockhart, D. J. (2005) A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat. Biotechnol.* **23**, 329–336.

(26) Zhou, S., Shoelson, S., Chaudhuri, M., Gish, G., Pawson, T., Haser, W., King, F., Roberts, T., Ratnofsky, S., Lechleider, R., Neel, B., Birge, R., Fajardo, J., Chou, M., Hanafusa, H., Schaffhausen, B., and Cantley, L. (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**, 767–778.

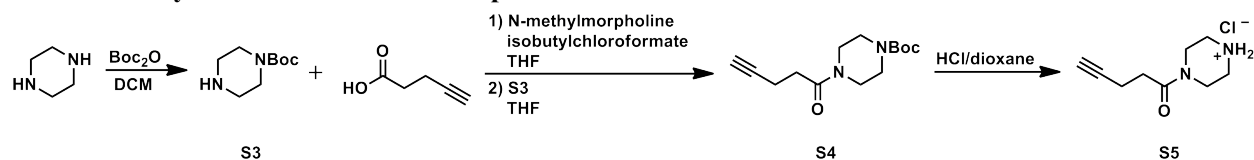
II. SYNTHESIS OF COMPOUNDS S1-5.

General Methods: Unless otherwise noted, all reagents were obtained via commercial sources and used without further purification. Abbreviations used are as follows: saturated (sat.), dimethylformamide (DMF), dimethylacetamide (DMA), oxalyl chloride (COCl)₂ sodium hydride (NaH), potassium carbonate (K₂CO₃), sodium bicarbonate (bicarb), hydrochloric acid or hydrogen chloride (HCl), sodium hydroxide (NaOH), ammonium chloride (NH₄Cl), sodium sulfate (Na₂SO₄), CuI (copper iodide), N,N-Diisopropylethylamine (DIEA), triethylamine (TEA), methanol (MeOH), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), Fluorenylmethyloxycarbonyl (Fmoc), N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), bromotrimethylsilane (TMS-Br), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), O-(2-Azidoethyl)-O'-(N-diglycolyl-2-aminoethyl)-heptaethyleneglycol (N₃-PEG₇-CO₂H), Dibenzocyclooctyne-amine (DBCO-amine), N-Methyl-2-pyrrolidone (NMP), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), Trimethylsilyl chloride (TMS-Cl), ethyl acetate (EtOAc), ethanol (EtOH). Tetrahydrofuran (THF) and dichloromethane (DCM) were dried over alumina under a nitrogen atmosphere. All ¹H NMR spectra were measured with a Varian MR400 or Inova 500 spectrometer. Mass Spectrometry (HRMS) was carried out by the University of Michigan Mass Spectrometry Facility (J. Windak, director).

Scheme S1: Synthetic Scheme for Compound 1



Scheme S2: Synthetic Scheme for Compound S5



Synthesis of S1: This compound was prepared as previously reported.¹

Synthesis of S2: Compound **S1** (997.4 mg, 8.10 mmol) was combined with 2,6-dichloro-4-methylpyrimidine (1.3216 g, 8.11 mmol) in an oven-dried round bottom flask. DMA (20.0 mL) was added followed by DIEA (2.10 mL, 12.02 mmol) and the reaction was stirred at 80°C overnight. The reaction was then brought up in EtOAc and washed 3 times with sat. NH_4Cl . After drying over Na_2SO_4 , the product was purified by silica gel chromatography using a Biotage Isolera One (linear gradient 40 \rightarrow 90% EtOAc in hexanes). The pure fractions were combined and the solvent was removed under reduced pressure to yield **S2** as a yellow solid (690 mg, 34% yield). **Spectral data.** ^1H NMR (500 MHz, Chloroform-*d*) δ 9.82 (s, 1H), 7.50 (s, 1H), 7.08 (s, 1H), 5.84 (s, 1H), 2.56 (s, 3H), 1.87 (tt, J = 8.4, 5.0 Hz, 1H), 1.04 – 0.99 (m, 2H), 0.76 (dt, J = 6.6, 4.8 Hz, 2H); ^{13}C NMR (100 MHz, dmsO) δ 167.81, 160.80, 148.28, 146.32, 101.48, 92.70, 25.69, 8.18, 7.13; HRMS-ESI (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{11}\text{H}_{12}\text{ClN}_5$ 250.0854; found 250.0853.

Synthesis of S3: This compound was prepared as previously reported.²

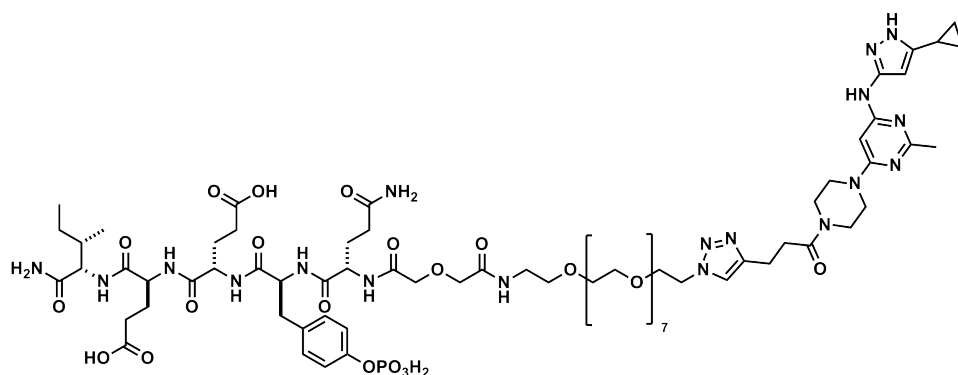
Synthesis of S4: 4-pentynoic acid (392.9 mg, 4.01 mmol) was added to an oven-dried flask. Anhydrous THF (2.70 mL) was then added to the flask followed by N-methylmorpholine (0.44 mL, 4.03 mmol). Isobutyl chloroformate (0.52 mL, 4.03 mmol) was then added dropwise and the reaction was stirred for 30 minutes. The reaction was then filtered and washed with THF. The filtrate was then added to a solution of **S3** (481.6 mg, 2.59 mmol) in anhydrous THF (2.7 mmol), and the reaction was stirred for 2 hrs. Upon completion, the solvent was removed under reduced pressure. The resulting residue was brought up in EtOAc and washed one time each with sat. bicarb, 10% citrate, and brine. After drying over Na_2SO_4 , the product was purified by silica gel chromatography using a Biotage Isolera One (linear gradient 30 \rightarrow 80% EtOAc in hexanes). The pure fractions were combined and the solvent was removed under reduced pressure to yield **S4** as a white solid (690 mg, 97% yield). **Spectral data.** ^1H NMR (500 MHz, Chloroform-*d*) δ 3.60 (t, J = 5.4 Hz, 2H), 3.43 (d, J = 22.3 Hz, 6H), 2.60 – 2.53 (m, 4H), 1.98 (q, J = 2.5 Hz, 1H), 1.47 (s, 9H); ^{13}C NMR (126 MHz, cdCl_3) δ 188.00, 169.54, 83.30, 80.36, 68.87, 45.21, 41.51, 32.18, 28.36, 14.49; HRMS-ESI (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{14}\text{H}_{22}\text{N}_2\text{O}_3$ 267.1703; found 267.1705, $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{14}\text{H}_{22}\text{N}_2\text{O}_3$ 289.1523; found 289.1522.

Synthesis of S5: **S4** (266.2 mg, 0.98 mmol) was added to an oven-dried flask. Anhydrous 1,4-dioxane (0.62 mL) was added followed by 4 N HCl in dioxane (2.30 mL, 9.2 mmol). The resulting solution was stirred for 1.5 hrs. The solvent was then removed under reduced pressure. The resulting residue was carried forward crude without purification assuming theoretical yield.

Synthesis of 1: **S5** (199.5 mg, 0.98 mmol) was added to an oven-dried flask. DCM (10.0 mL) was added followed by TEA (0.15 mL, 1.08 mmol). The resulting mixture was stirred for 0.5 hrs and then washed once with 10% K_2CO_3 . The solvent was then removed under reduced pressure. **S2** was added to the flask containing the resulting residue and brought up in EtOH (3.0 mL). Concentrated HCl was added and the reaction was refluxed overnight. Upon completion, the EtOH was removed under reduced pressure. The resulting residue was brought up in EtOAc and washed 3 times each with bicarb and brine. After drying over Na_2SO_4 , the product was purified by silica gel chromatography using a Biotage Isolera One (linear gradient 0 \rightarrow 5% MeOH in EtOAc). The pure fractions were combined and the solvent was removed under reduced pressure to yield **1** as a white solid (100 mg, 54% yield). **Spectral data.** ^1H NMR (500 MHz,

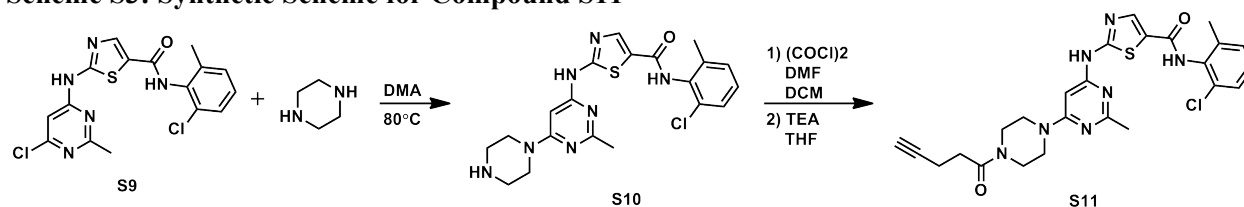
CCOP(=O)(OCC)c1ccc(cc1)C(=O)NCC(=O)OCc2c3ccccc3c4ccccc24[illegible]CC(C)[C@H](C(=O)N)C(=O)N[C@@H](CC(=O)O)C(=O)N[C@@H](CC(=O)O)C(=O)N[C@@H](CC(=O)N)C(=O)OCCOC(=O)NCCOC([C@H]1CCOC1)7CCN2C=CN=C(C2)CC(=O)N3CCN(CC3)C4=CN=C(NC5=CC=NC=C5C)C=C4

S5



Synthesis of 3: **S8** (48.5 mg, 0.028 mmol) was added to an oven-dried flask. DCM (0.5 mL) was then added followed by TMS-Br (0.5 mL) and the reaction mixture was stirred overnight. The solvent was then removed under reduced pressure. The reaction mixture was then suspended in water (0.5 mL) and stirred for 2 hrs. The reaction was purified by reverse-phase HPLC using a linear 5→95% acetonitrile + 0.1% TFA in water gradient. Lyophilization afforded **3** as a white powder (5.5 mg, 12% yield). **Spectral data.** HRMS-ESI (*m/z*): [*M*] calcd for C₇₂H₁₁₁N₁₈O₂₆P 1674.7649; found after resolved isotope deconvolution 1674.7648.

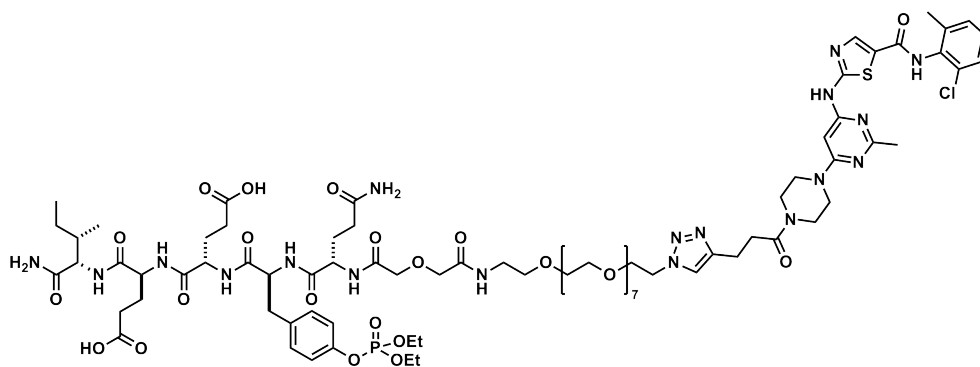
Scheme S3: Synthetic Scheme for Compound S11



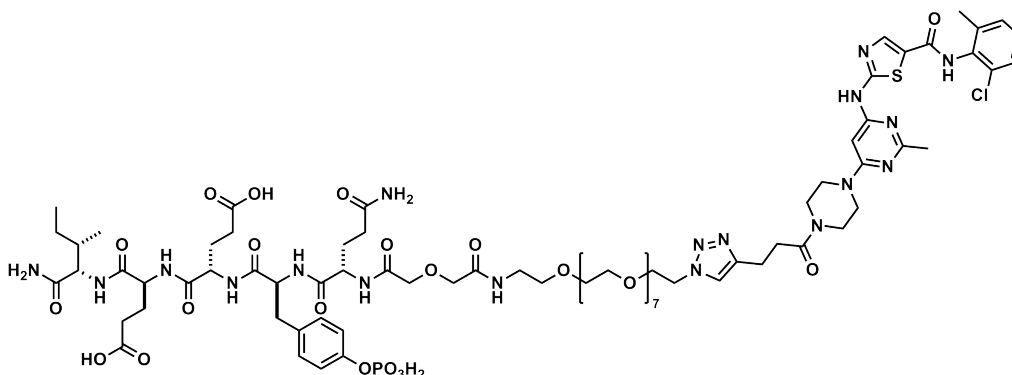
Synthesis of S9: This compound was prepared as previously reported.⁴

Synthesis of S10: **S9** (76.1 mg, 0.19 mmol) was added to an oven-dried flask. Anhydrous DMA (0.38 mL) was added followed by piperazine (112.8 mg, 0.95 mmol). The resulting solution was stirred at 80°C for 2.5 hrs. After completion, the reaction was cooled to room temperature and water was added to precipitate the product. The resulting residue was filtered and carried forward crude without purification.

Synthesis of S11: (COCl)₂ (0.02 mL, 0.23 mmol) was added to a stirred solution of 4-pentynoic acid (15.1 mg, 0.075 mmol) in anhydrous DCM (0.23 mL). Catalytic DMF (1 drop) was added and the reaction was allowed to stir for 2 hrs. The solvent was then removed under reduced pressure. The resulting residue was then dissolved in anhydrous THF (0.5 mL). **S10** (41.8 mg, 0.09 mmol) and TEA (0.02 mL, 0.14 mmol) were added and the reaction was allowed to stir overnight. The solvent was then removed under reduced pressure. The resulting residue was brought up in EtOAc and washed with 10% citrate, sat. bicarb, and brine. After drying over Na₂SO₄, the product was purified by silica gel chromatography using a Biotage Isolera One (linear gradient 50 → 100% EtOAc in Hexanes). The peak fractions were combined and the solvent was removed under reduced pressure. The product was not pure and was further purified by reverse-phase HPLC using a linear 30→90% acetonitrile + 0.1% TFA in water gradient. Lyophilization afforded **4** as a white solid (3.2 mg, 8.1% yield). **Spectral Data.** ¹H NMR (500 MHz, DMSO-*d*₆) δ 11.53 (s, 1H), 9.89 (s, 1H), 8.22 (s, 1H), 7.40 (d, *J* = 7.7 Hz, 1H), 7.27 (dt, *J* = 15.4, 7.5 Hz, 2H), 6.06 (s, 1H), 3.56 (d, *J* = 6.5 Hz, 8H), 2.78 (t, *J* = 2.7 Hz, 1H), 2.59 (t, *J* = 7.4 Hz, 2H), 2.42 (s, 3H), 2.38 (td, *J* = 7.5, 7.1, 2.6 Hz, 2H), 2.23 (s, 3H); ¹³C NMR (176 MHz, dmsol) δ 169.58, 165.61, 162.94, 162.59, 160.36, 157.40, 141.24, 139.25, 133.94, 132.86, 129.46, 128.62, 127.44, 84.53, 83.21, 71.71, 44.48, 43.91, 43.66, 42.55, 31.82, 25.99, 18.74, 14.30; HRMS-ESI (*m/z*): [*M* + H]⁺ calcd for C₂₅H₂₆ClN₇O₂S 524.1630; found 524.1625.

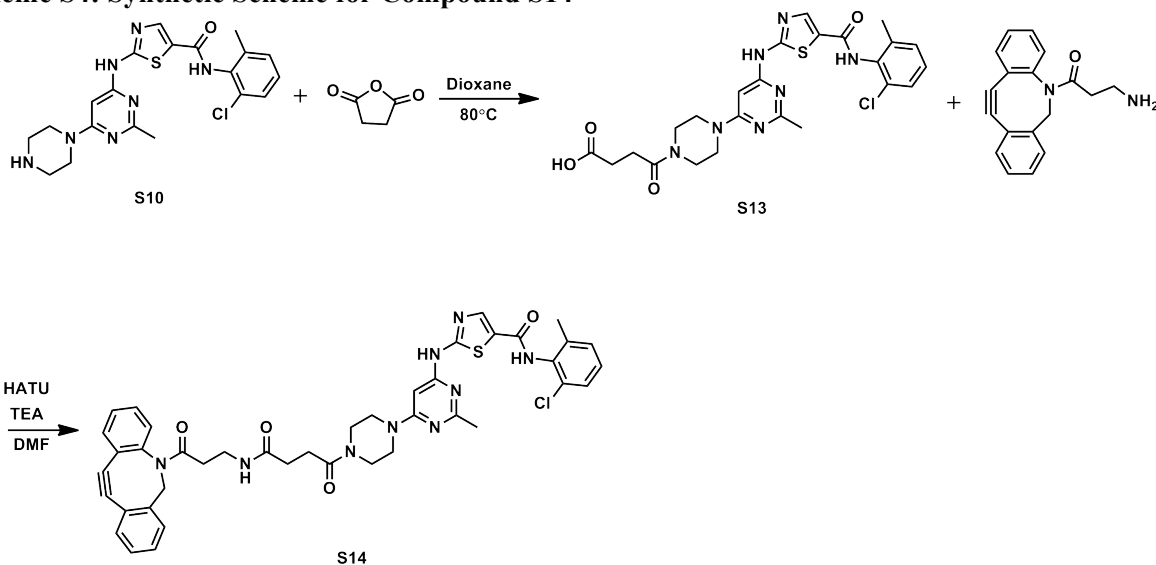


Synthesis of S12: **2** (20.3 mg, 0.015 mmol), CuI (5.8 mg, 0.03 mmol) and **S11** (6.3 mg, 0.012 mmol) were added to an oven-dried flask. DMF (0.24 mL) was added followed by TEA (0.01 mL, 0.07 mmol). The reaction mixture was stirred at 45°C overnight. The reaction was purified by reverse-phase HPLC using a linear 20→80% acetonitrile + 0.1% TFA in water gradient. Lyophilization afforded **S12** as a white solid (4.0 mg, 18% yield). **Spectral data.** MS-ESI (m/z): $[M + 2H]^+$ calcd for $C_{81}H_{120}ClN_{18}O_{27}PS$, 938.39; found 938.08.



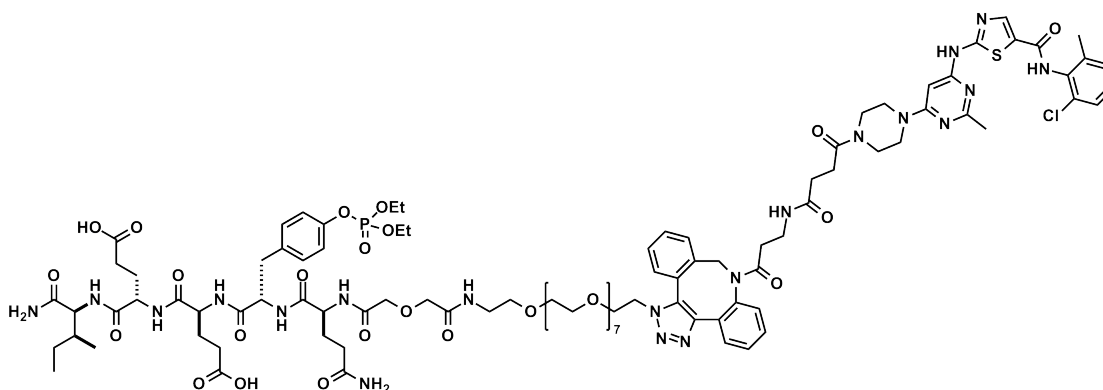
Synthesis of 4: **S12** (4.0 mg, 0.002 mmol) was added to an oven-dried flask. ACN (0.5 mL) was then added followed by TMS-Br (0.5 mL) and reaction mixture was stirred overnight. Water (0.5 mL) was then added and the reaction was again stirred overnight. The solvent was then removed under reduced pressure. The reaction was purified by reverse-phase HPLC using a linear 20→80% acetonitrile + 0.1% TFA in water gradient. Lyophilization afforded **4** as a white powder (0.5 mg, 14% yield). **Spectral data.** HRMS-ESI (m/z): $[M]$ calcd for $C_{77}H_{112}ClN_{18}O_{27}PS$ 1818.7091; found after resolved isotope deconvolution 1818.7093.

Scheme S4: Synthetic Scheme for Compound S14

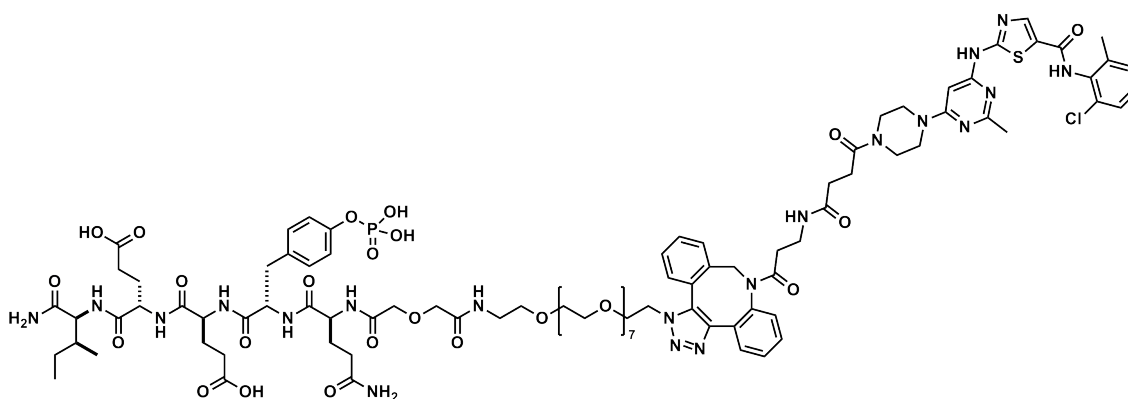


Synthesis of S13: **S10** (95.2 mg, 0.21 mmol) was added to an oven-dried flask. Dioxane (1.0 mL) was added followed by succinic anhydride (21.0 mg, 0.21 mmol). The resulting solution was stirred at 80°C overnight. After completion, the reaction was cooled to room temperature. The resulting residue was filtered to afford **S13** as a tan solid (83.0 mg, 73% yield). **Spectral Data.** ^1H NMR (500 MHz, $\text{DMSO-}d_6$) δ 11.95 (s, 1H), 11.53 (s, 1H), 9.89 (s, 1H), 8.23 (s, 1H), 7.40 (d, $J = 7.6$ Hz, 1H), 7.27 (dt, $J = 15.3, 7.5$ Hz, 2H), 6.06 (s, 1H), 3.56 (dd, $J = 17.6, 11.5$ Hz, 8H), 2.58 (t, $J = 6.6$ Hz, 2H), 2.45 (t, $J = 6.7$ Hz, 2H), 2.42 (s, 3H), 2.24 (s, 3H). ^{13}C NMR (100 MHz, dmso) δ 174.36, 170.24, 165.62, 162.93, 162.63, 160.32, 157.37, 141.25, 139.24, 133.93, 132.85, 129.45, 128.30, 127.43, 126.17, 83.17, 66.77, 44.39, 43.82, 43.62, 29.33, 27.92, 26.01, 18.73. MS-ESI (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{24}\text{H}_{26}\text{ClN}_7\text{O}_4\text{S}$ 544.1528; found 544.1522.

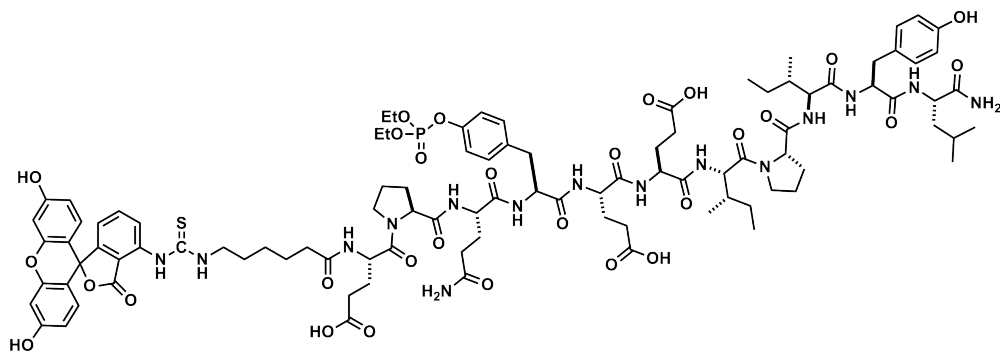
Synthesis of S14: **S13** (49.0 mg, 0.09 mmol) and HATU (45.5 mg, 0.12 mmol) were added to an oven-dried flask. DMF (0.92 mL) and TEA (0.04 mL, 0.28 mmol) were added and the reaction was allowed to stir for 10 min. After pre-activation of the acid, DBCO-amine (23.7 mg, 0.09 mmol) was added and the reaction was stirred overnight. After completion, the reaction was cooled to room temperature and water was added to precipitate the product. Filtration afforded **S14** as a tan solid (59.5 mg, 81% yield). **Spectral Data.** ^1H NMR (700 MHz, $\text{DMSO-}d_6$) δ 11.50 (s, 1H), 9.87 (s, 1H), 8.22 (s, 1H), 7.67 (t, $J = 5.7$ Hz, 1H), 7.61 (d, $J = 7.6$ Hz, 1H), 7.60-7.55 (m, 1H), 7.49-7.46 (m, 1H), 7.45-7.41 (m, 1H), 7.37 (td, $J = 7.6, 1.6$ Hz, 2H), 7.33 (t, $J = 7.5$ Hz, 1H), 7.29-7.25 (m, 2H), 7.23 (t, $J = 7.7$ Hz, 1H), 6.05 (s, 1H), 5.02 (d, $J = 14.0$ Hz, 1H), 3.61 (d, $J = 14.0$ Hz, 1H), 3.58-3.44 (m, 9H), 3.12-3.05 (m, 1H), 2.93 (ddt, $J = 14.0, 8.8, 5.7$ Hz, 1H), 2.41 (s, 3H), 2.22 (s, 3H). ^{13}C NMR (176 MHz, dmso) δ 171.64, 170.54, 165.64, 162.97, 162.66, 160.36, 157.40, 151.87, 148.81, 141.27, 139.26, 133.96, 132.88, 130.00, 129.49, 129.41, 128.64, 128.60, 128.46, 128.16, 127.44, 127.24, 126.20, 125.64, 122.91, 121.86, 114.73, 108.53, 83.20, 55.28, 44.49, 43.88, 43.63, 42.67, 41.33, 35.49, 34.64, 30.68, 28.22, 26.02, 25.97, 18.76. MS-ESI (m/z): $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{42}\text{H}_{40}\text{ClN}_9\text{O}_4\text{S}$ 802.2685; found 802.2682.



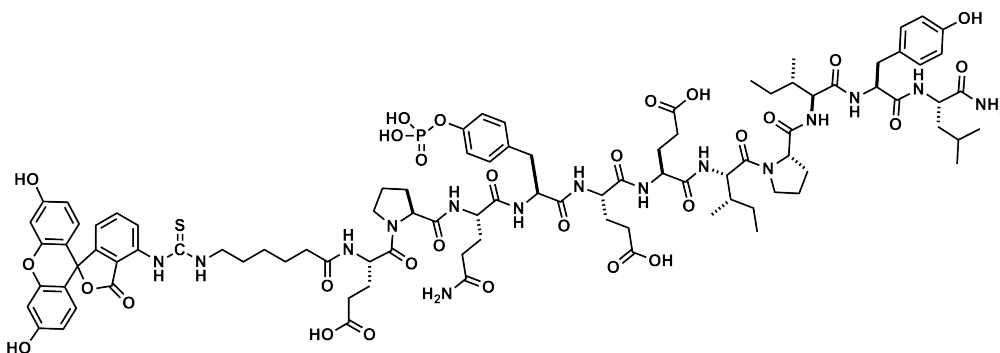
Synthesis of S15: **S14** (59.5 mg, 0.074 mmol) was stirred in DMF (0.30 mL). **2** was dissolved in DMF to make a 0.125 M solution. **2** in DMF (0.60 mL, 0.075 mmol) was added to **S14** in DMF. The reaction mixture was stirred overnight. The crude reaction was purified by reverse-phase HPLC using a linear 20→80% acetonitrile + 0.1% TFA in water gradient. Lyophilization afforded **S15** as a white solid (3.7 mg, 2.3% yield). **Spectral data.** MS-ESI (m/z): [M - H]⁻ calcd for C₉₈H₁₃₅ClN₂₀O₂₉PS, 2151.8700; found 2151.8681.



Synthesis of 5: **S15** (3.7 mg, 0.0017 mmol) was added to an oven-dried flask. ACN (0.5 mL) was then added followed by TMS-Br (0.5 mL) and reaction mixture was stirred overnight. Water (0.5 mL) was then added and the reaction was again stirred for an hour. The solvent was then removed under reduced pressure. The reaction was purified by reverse-phase HPLC using a linear 20→80% acetonitrile + 0.1% TFA in water gradient. Lyophilization afforded **5** as a white powder (0.9 mg, 24% yield). **Spectral data.** HRMS-ESI (m/z): [M - H]⁻ calcd for C₉₄H₁₂₆ClN₂₀O₂₉PS, 2095.8007; found 2095.8074.



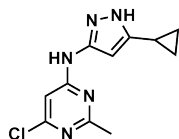
Synthesis of S16: Compound **S16** was synthesized on 0.2 mmol scale on Rink Amide AM resin (0.47 mmol/g) using standard Fmoc solid-phase peptide synthesis. Couplings were performed using DIEA and HBTU in NMP and were allowed to proceed for 30 minutes at room temperature. Fmoc deprotections were performed using piperidine in NMP and were allowed to proceed for 15 min at room temperature. The completeness of each coupling and deprotection was monitored by the Kaiser test. After deprotection of the final amino acid in the sequence, the peptide was capped by coupling on FITC (4 mg, 0.010 mmol) using DIEA in DMF (1 mL). Cleavage of the peptide from the resin with concomitant removal of all side chain-protecting groups was accomplished with 6 mL of a 95% TFA, 2.5% H₂O, and 2.5% TIPS solution for 45 minutes twice. The TFA was mostly removed under reduced pressure. The product was precipitated in ether and carried forward crude without purification assuming theoretical yield.



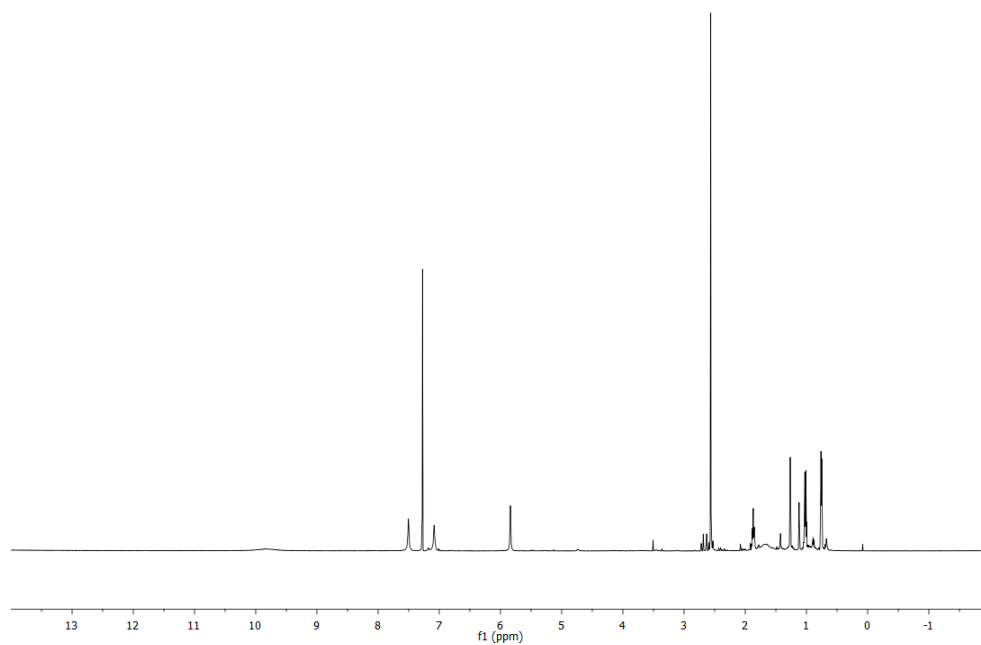
Synthesis of S17: **S16** was added to an oven-dried flask. ACN (0.5 mL) was then added followed by TMS-Br (0.5 mL) and reaction mixture was stirred overnight. Water (0.5 mL) was then added and the reaction was again stirred for an hour. The solvent was then removed under reduced pressure. The reaction was purified by reverse-phase HPLC using a linear 5→80% acetonitrile + 0.1% TFA in water gradient. Lyophilization afforded **S17** as a white powder (2.2 mg, 0.56% yield). **Spectral data.** HRMS-ESI (m/z): [M - H]⁻ calcd for C₉₃H₁₂₀N₁₅O₂₉PS 1972.7762; found 1972.7802.

III. SPECTRAL DATA FOR COMPOUNDS S2-4.

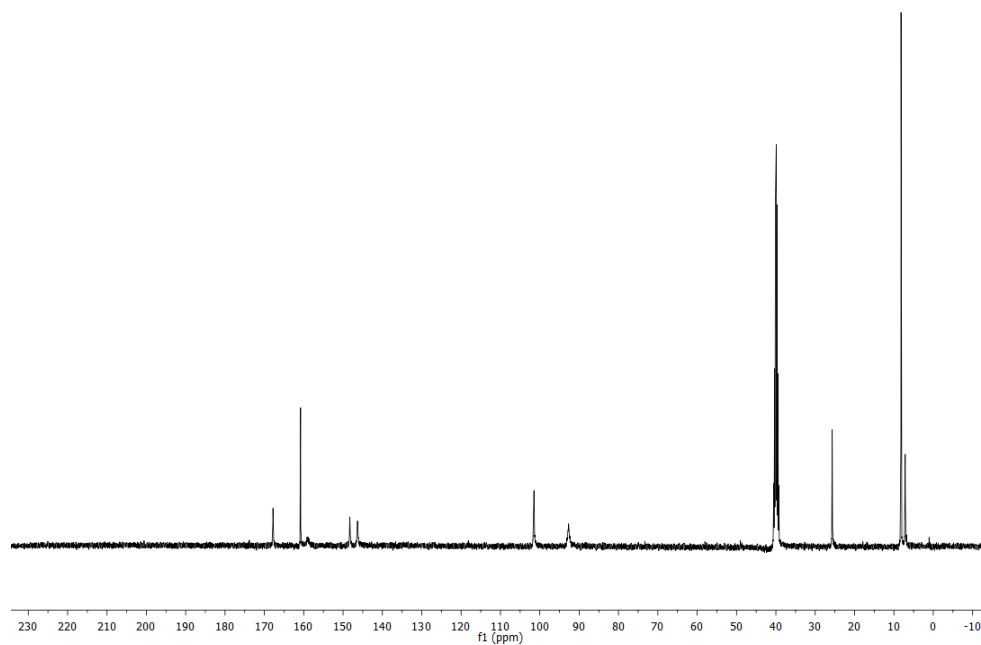
S2:



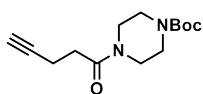
S2 ^1H :



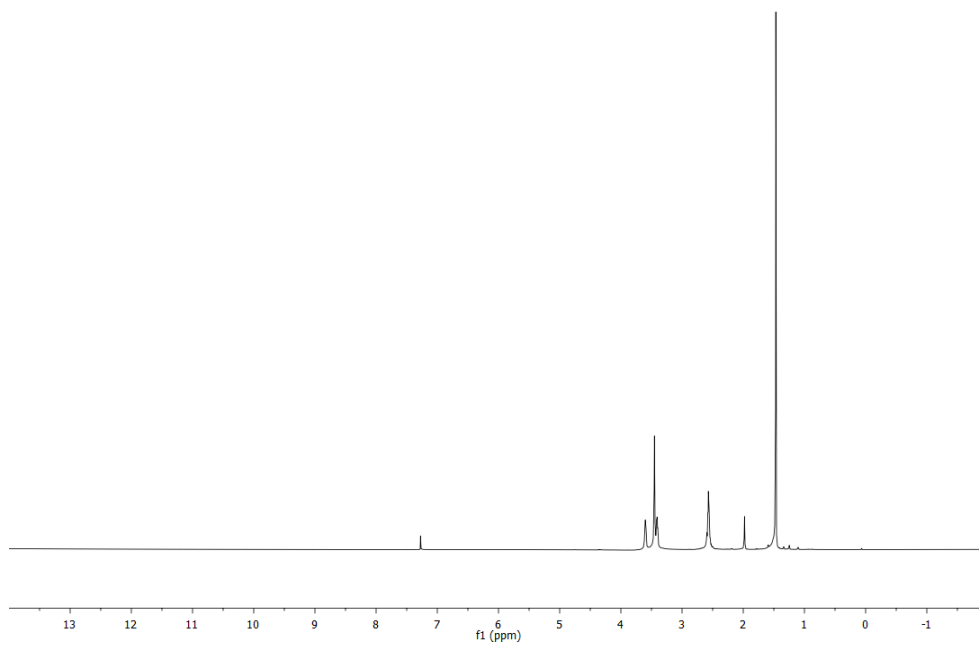
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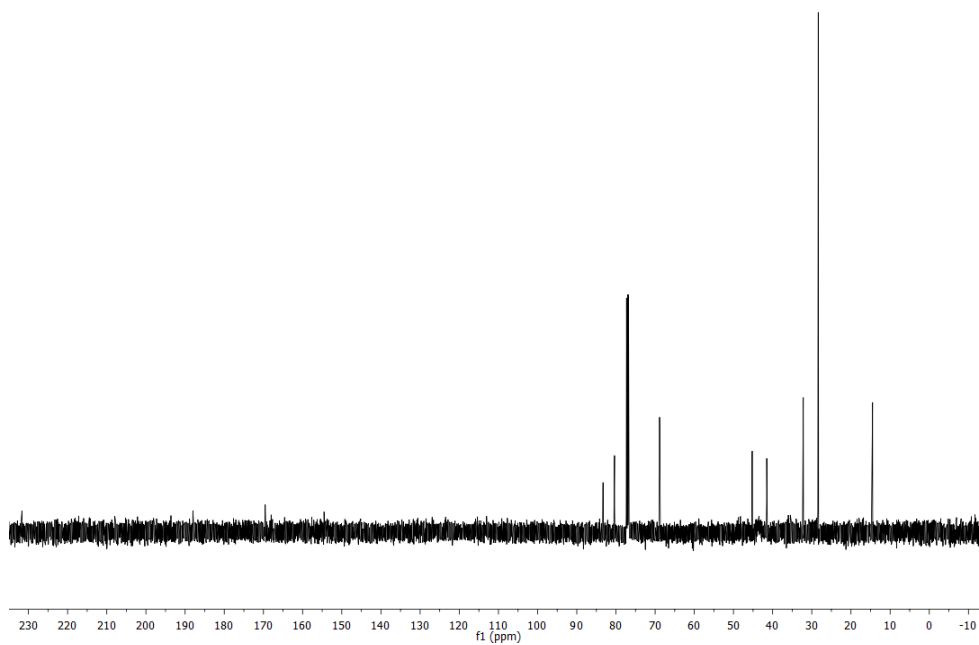
S4:



S4 ^1H :



S4 ^{13}C :

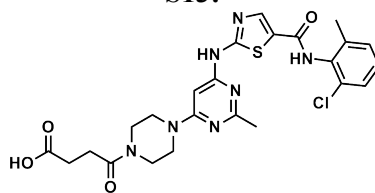


S12

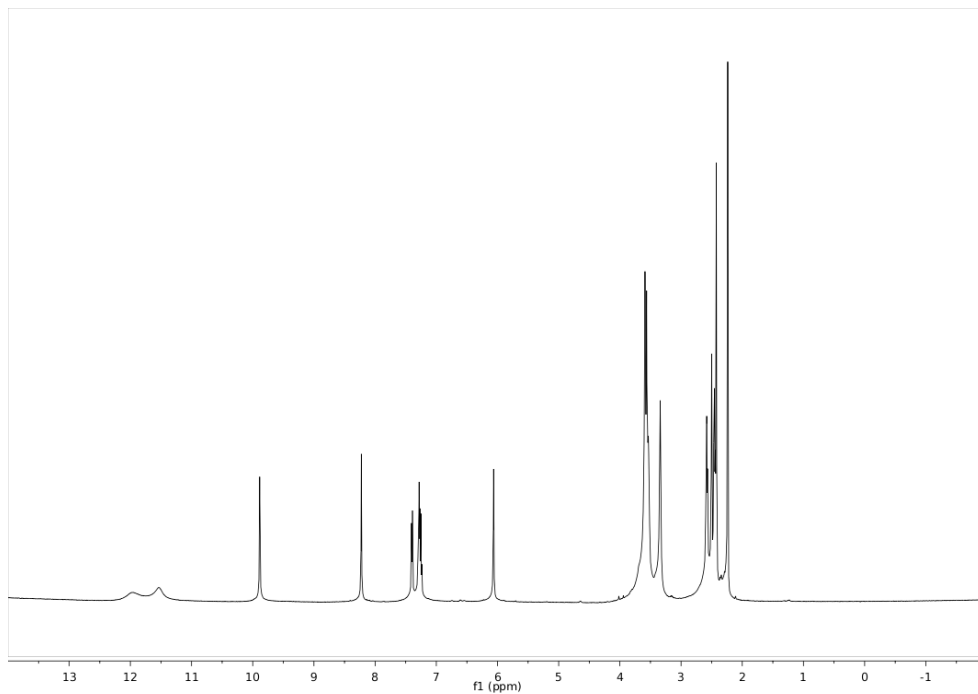
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S13

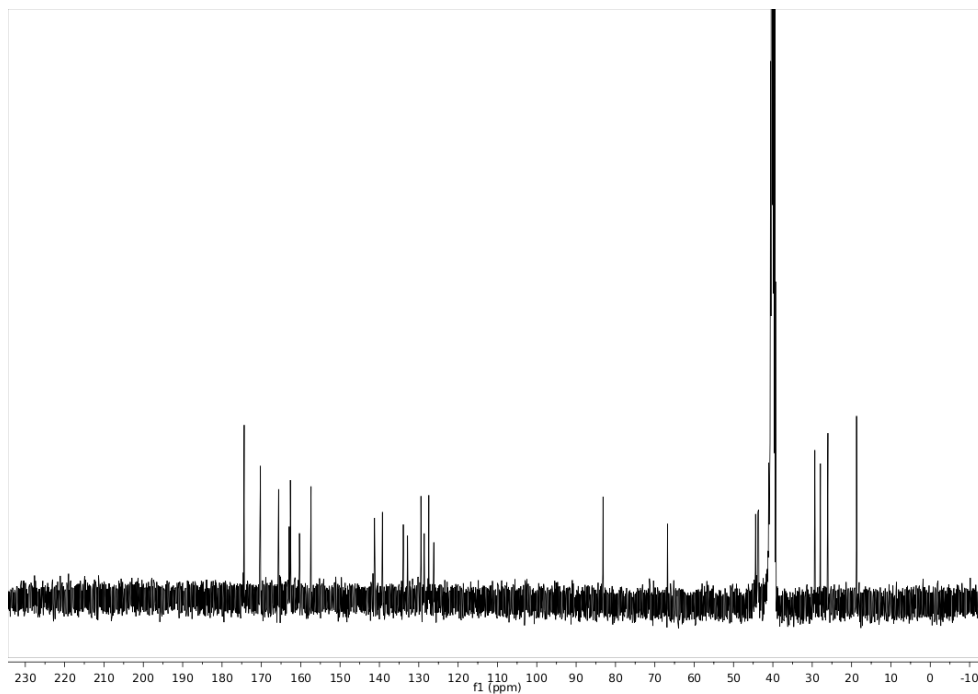
S13:



S13 ^1H :

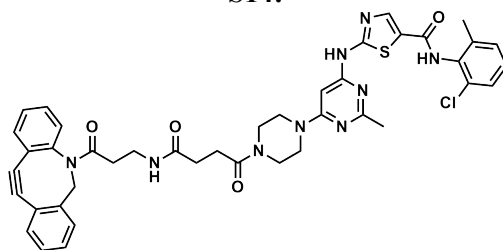


S13 ^{13}C :

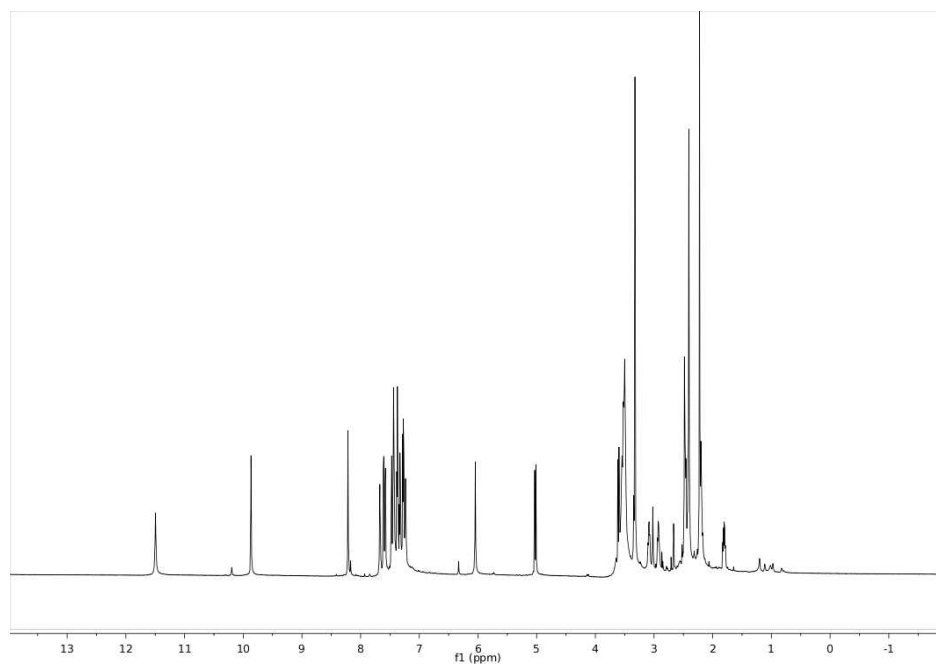


S14

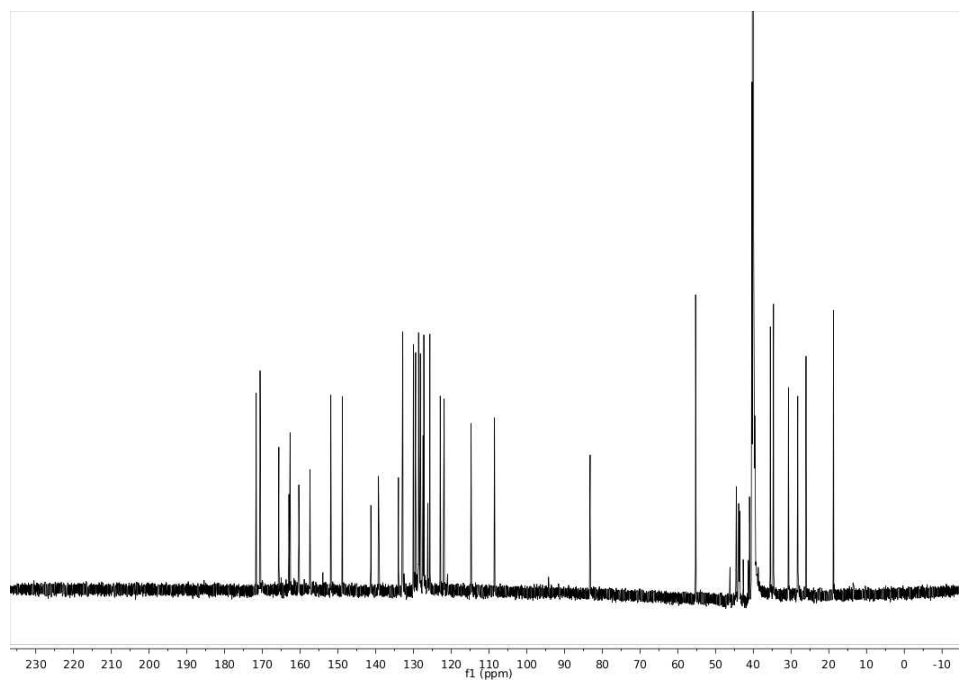
S14:



S14 ^1H :

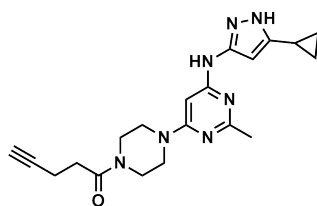


S14 ^{13}C :

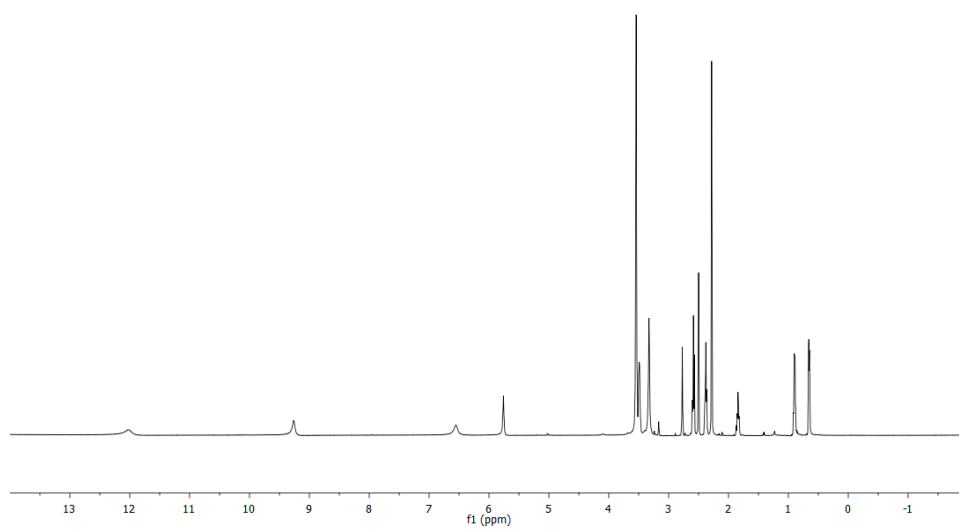


S15

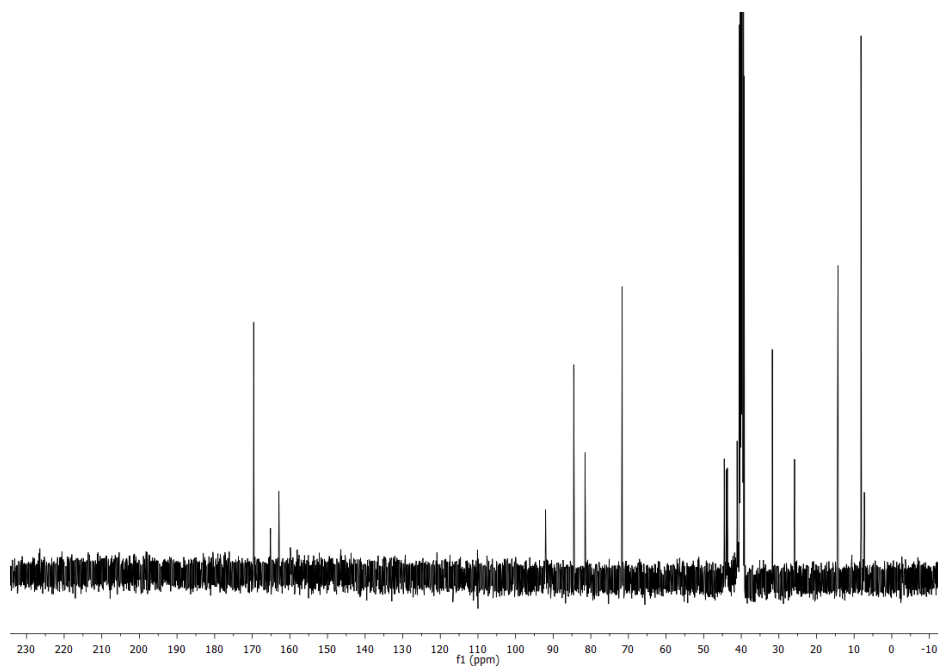
1:



^1H :



^{13}C :

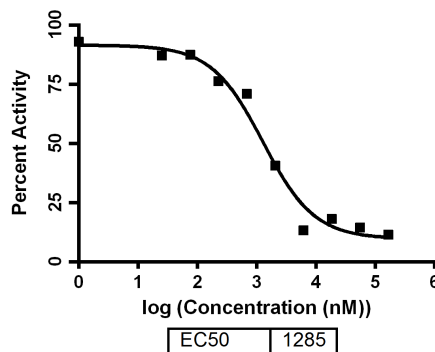
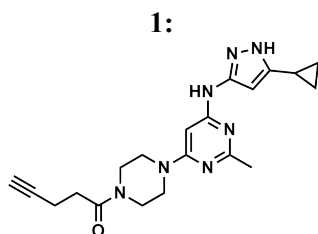


IV. BIOCHEMICAL CHARACTERIZATION

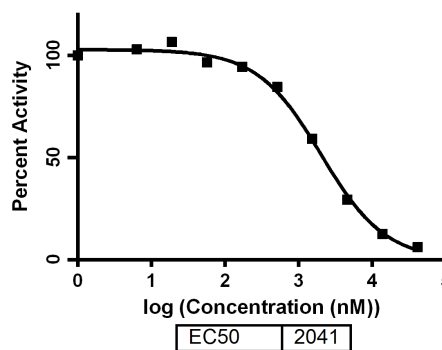
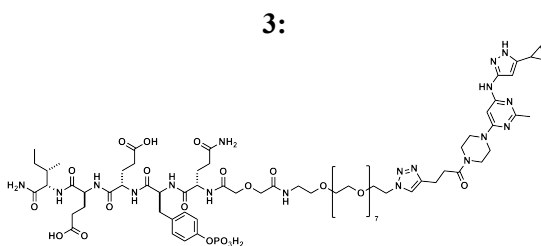
General procedures. Black, opaque-bottom 96 well plates were purchased from Nunc. All proteins were expressed in *E. coli* using previously published procedures.⁵ Data were obtained using Biotek Synergy Mx and Biotek Synergy 4 plate readers. Curve fitting was done using Graphpad Prism 6 software.

General procedure for determination of inhibitor IC₅₀ against Src Family Kinases. A continuous fluorescence assay was used to determine IC₅₀.⁶ The reaction volume was 100 μ L consisting of 85 μ L of enzyme in buffer, 2.5 μ L of the appropriate inhibitor dilution (typically 10000, 3333.33, 1111.11, 370.37, 123.46, 41.15, 13.72, 4.57, 1.52, and 0 μ M in DMSO), and 2.5 μ L (1.8 mM in DMSO) of a substrate peptide (“compound 3” as described in Wang et al.)⁶. The reaction was initiated with 10 μ L of ATP (1 mM in water), and reaction progress was immediately monitored at 405 nm (ex. 340 nm) for 10 minutes. Reactions had final concentrations of 30 nM enzyme, 45 μ M peptide substrate, 100 μ M ATP, 100 μ M Na₃VO₄, 100 mM Tris buffer (pH 8), 10 mM MgCl₂, 0.01% Triton X-100 unless otherwise noted. The initial rate data collected was used for determination of IC₅₀ values. For IC₅₀ determination, the kinetic values were obtained directly from nonlinear regression of substrate-velocity curves in the presence of various concentrations of the inhibitor. The equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{X - \text{LogEC50}})$, $X = \log(\text{concentration})$ and $Y = \text{binding}$; was used in the nonlinear regression unless otherwise noted.

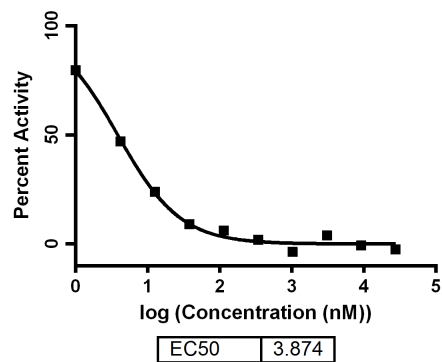
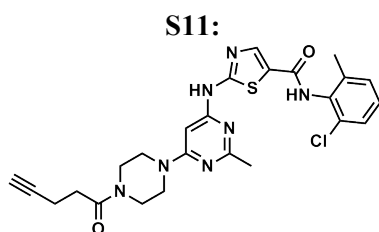
Analytical data for KD c-Src IC₅₀ determinations. An IC₅₀ value was determined for each inhibitor using at least three independent experiments. The individual IC₅₀ values for the three runs were then averaged to provide a standard deviation. A representative inhibition curve of a single run is shown.



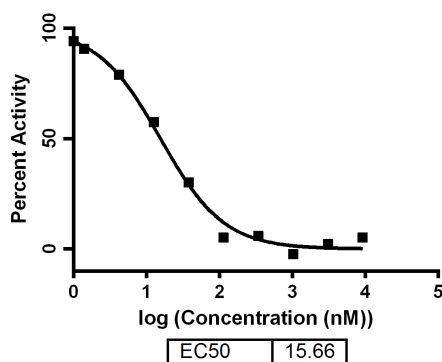
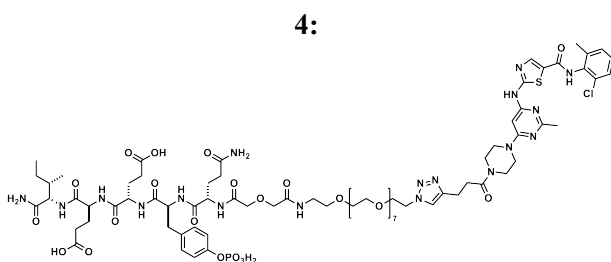
Avg IC₅₀ = 1.3 \pm 0.5 μ M



Avg IC₅₀ = 2.1 \pm 0.3 μ M

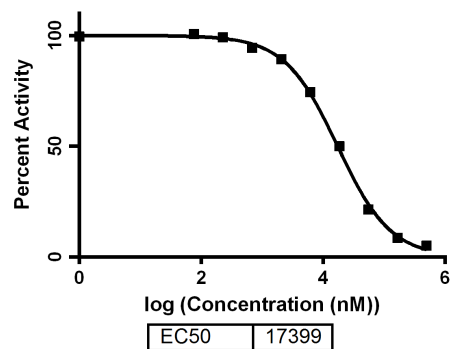
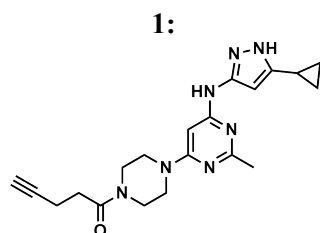


Avg IC₅₀ < 10 nM
 [ATP] = 5 mM
 [Src] = 10 nM

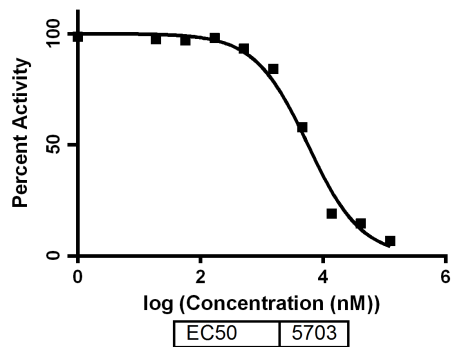
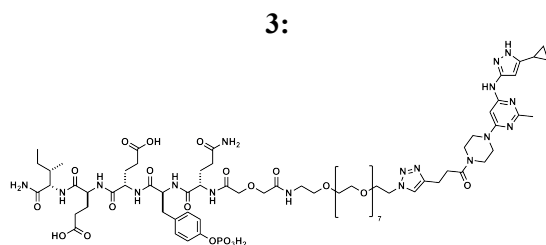


Avg IC₅₀ < 10 nM
 [ATP] = 5 mM
 [Src] = 10 nM

Analytical data for KD Hck IC₅₀ determination. An IC₅₀ value was determined for each inhibitor using at least three independent experiments. The individual IC₅₀ values for the three runs were then averaged to provide a standard deviation. A representative inhibition curve of a single run is shown.

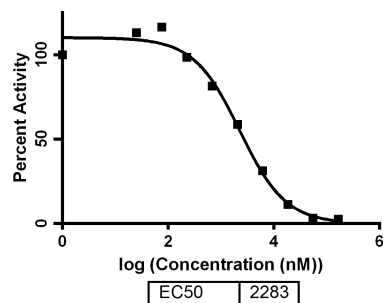
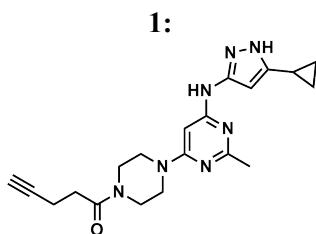


Avg IC₅₀ = 20 ± 3 μM

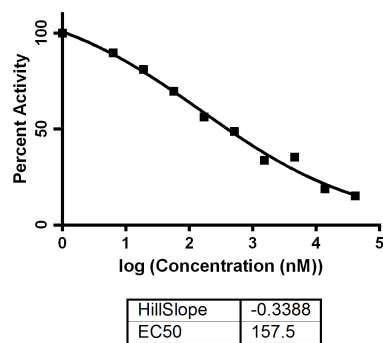
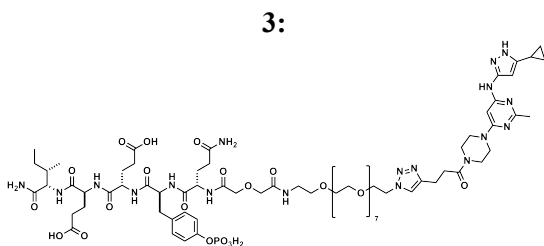


Avg IC₅₀ = 6 ± 1 μM

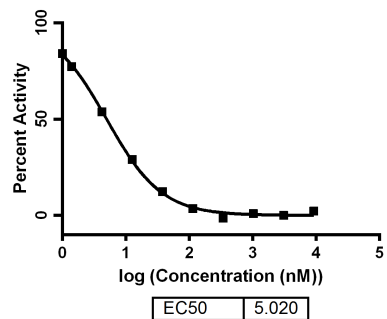
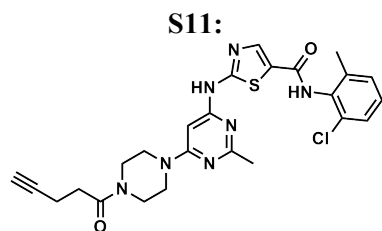
Analytical data for 3D Src IC₅₀ determination. An IC₅₀ value was determined for each inhibitor using at least three independent experiments. The individual IC₅₀ values for the three runs were then averaged to provide a standard deviation. A representative inhibition curve of a single run is shown. The IC₅₀ curve for **3** was fit using the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope}))})$, $X = \log(\text{concentration})$ and $Y = \text{binding}$.



Avg IC₅₀ = 2.9 ± 0.6 μM



Avg IC₅₀ = 160 ± 4 nM

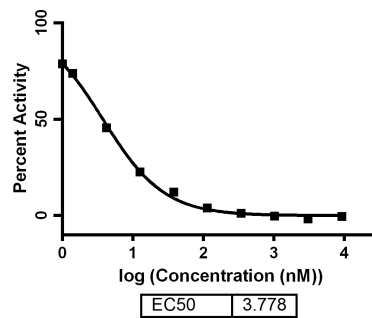
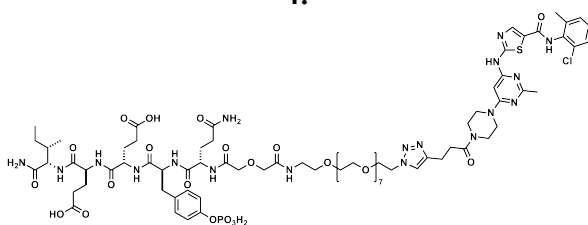


Avg IC₅₀ < 10 nM

[ATP] = 5 mM

[Src] = 10 nM

4:

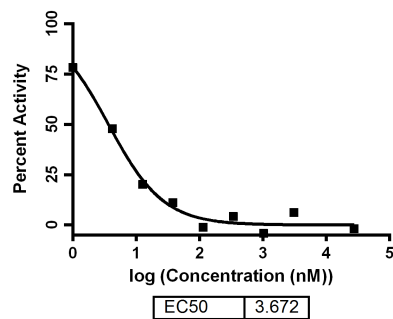
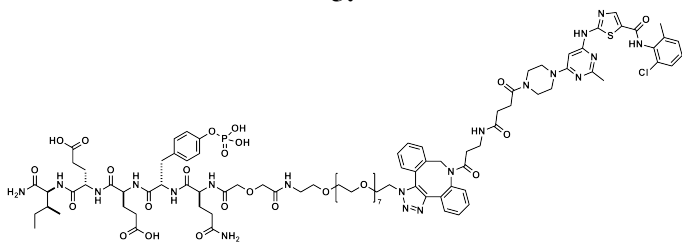


Avg IC₅₀ < 10 nM

[ATP] = 5 mM

[Src] = 10 nM

5:

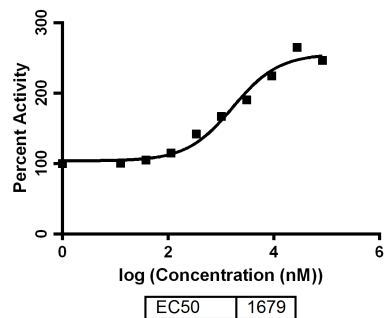


Avg IC₅₀ < 10 nM

[ATP] = 5 mM

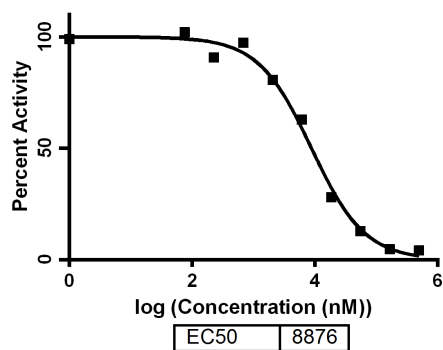
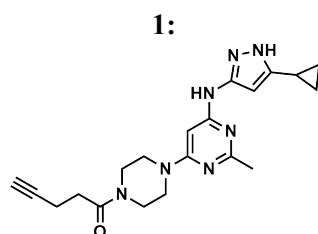
[Src] = 10 nM

SH2 Domain Pentapeptide:
H₂N-Q-pY-E-E-I-CONH₂

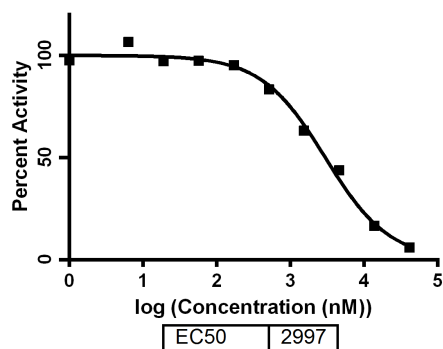
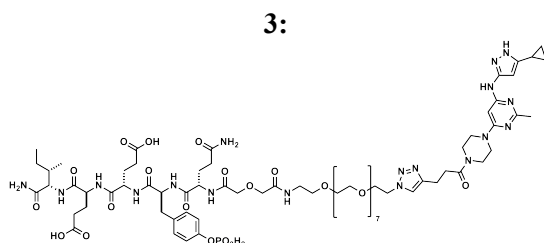


Avg EC₅₀ (activation) = 1.6 ± 0.3 μM

Analytical data for 3D Hck IC₅₀ determination. An IC₅₀ value was determined for each inhibitor using at least three independent experiments. The individual IC₅₀ values for the three runs were then averaged to provide a standard deviation. A representative inhibition curve of a single run is shown. The IC₅₀ curve for **3** was fit using the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope}))}$, X = log(concentration) and Y = binding.

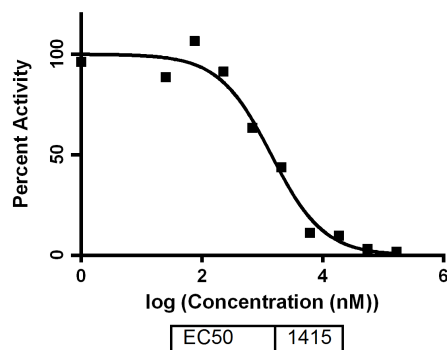
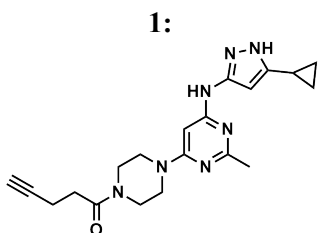


Avg IC₅₀ = 8.9 ± 0.5 μM



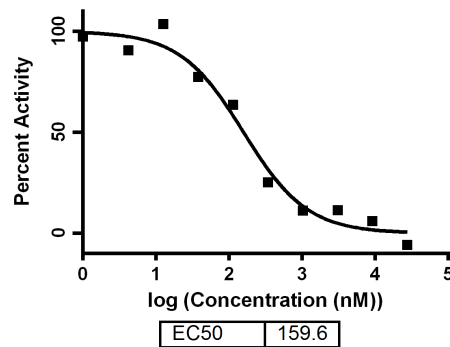
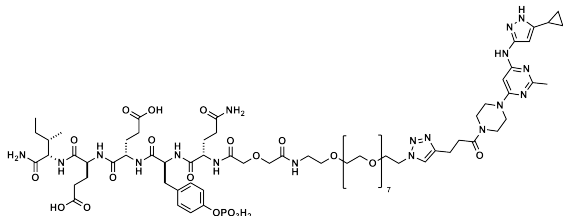
Avg IC₅₀ = 2.8 ± 0.7 μM

Analytical data for T338M 3D Src IC₅₀ determination. An IC₅₀ value was determined for each inhibitor using at least three independent experiments. The individual IC₅₀ values for the three runs were then averaged. A representative inhibition curve of a single run is shown.



Avg IC₅₀ = 1.2 ± 0.3 μM

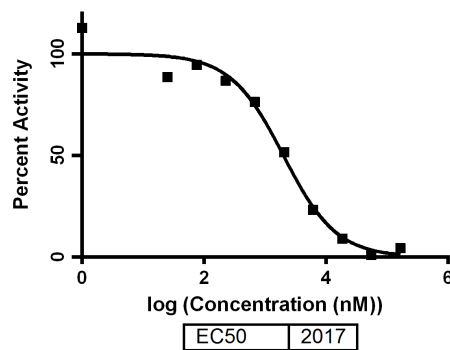
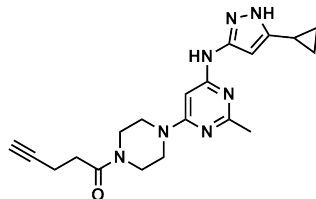
3:



Avg IC₅₀ = 189 ± 8 nM

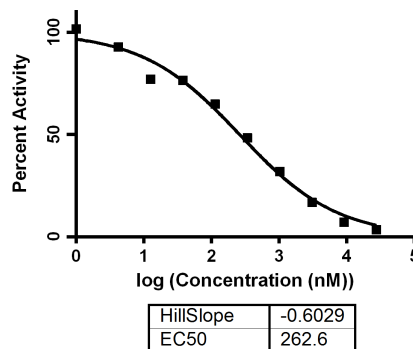
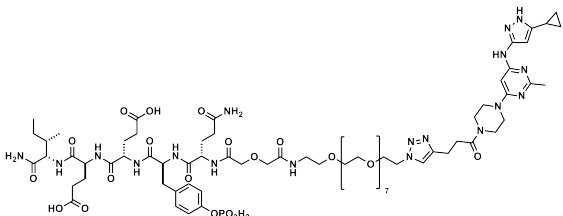
Analytical data for P-Y416 3D Src IC₅₀ determination. An IC₅₀ value was determined for each inhibitor using at least three independent experiments. The individual IC₅₀ values for the three runs were then averaged to provide a standard deviation. A representative inhibition curve of a single run is shown. The IC₅₀ curve for **3** was fit using the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope}))})$, $X = \log(\text{concentration})$ and $Y = \text{binding}$.

1:



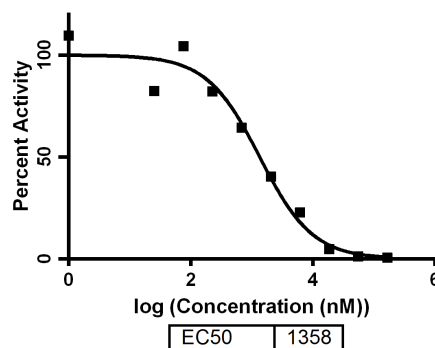
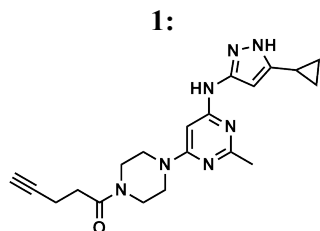
Avg IC₅₀ = 2.4 ± 0.6 μM

3:

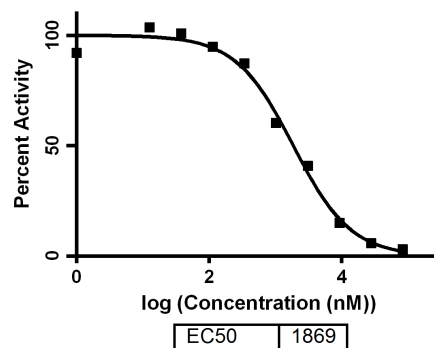
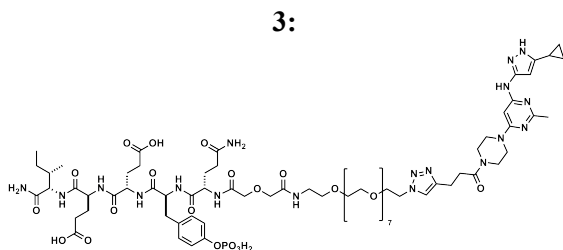


Avg IC₅₀ = 240 ± 100 nM

Analytical data for P~Y527 3D Src IC₅₀ determination. An IC₅₀ value was determined for each inhibitor using at least three independent experiments. The individual IC₅₀ values for the three runs were then averaged to provide a standard deviation. A representative inhibition curve of a single run is shown.



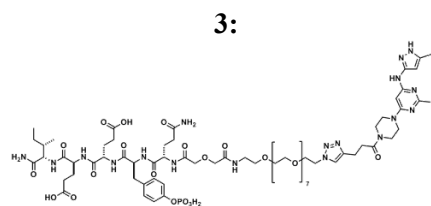
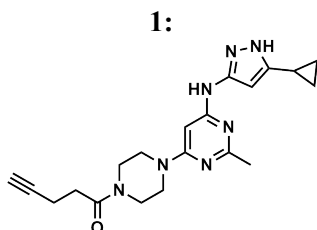
Avg IC₅₀ = 1.38 ± 0.08 μM



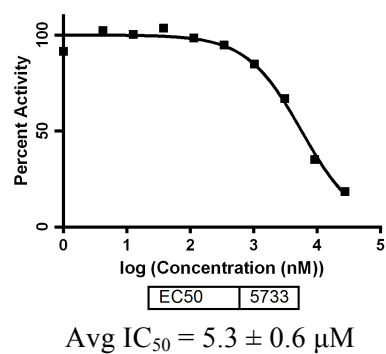
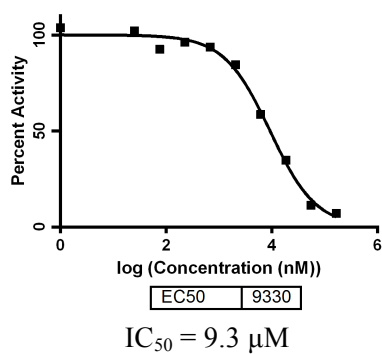
Avg IC₅₀ = 1.8 ± 0.1 μM

Analytical data for IC₅₀ Determination Using a Panel of 3D Src Family Kinases. The IC₅₀ value for compound **1** was determined using one experiment, and the IC₅₀ value for compound **3** was determined using two independent experiments which were then averaged for the final IC₅₀ value. A representative inhibition curve is shown.

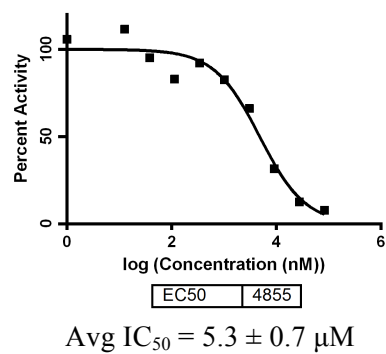
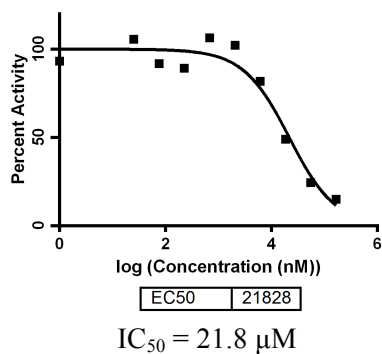
**Src Family
Kinase**



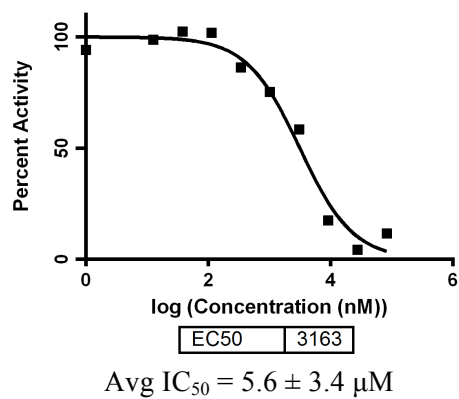
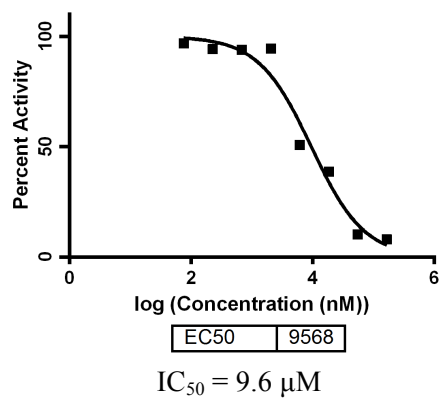
Lck



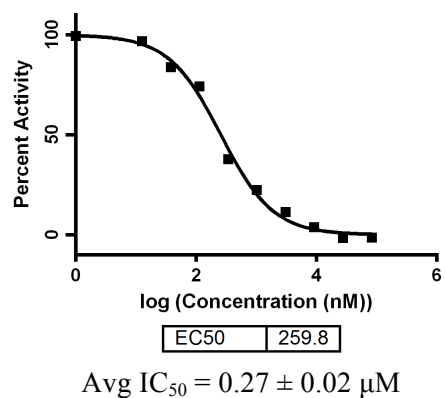
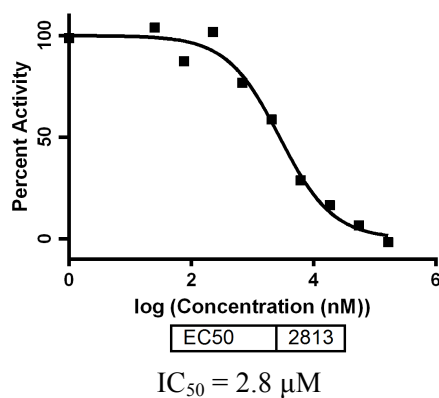
Blk



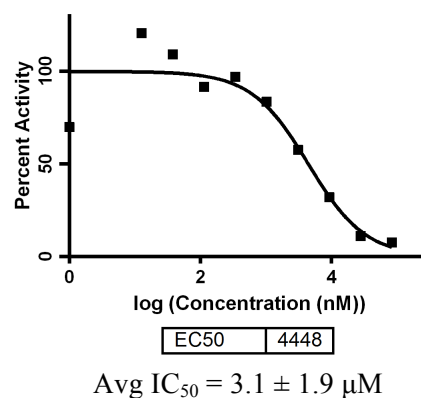
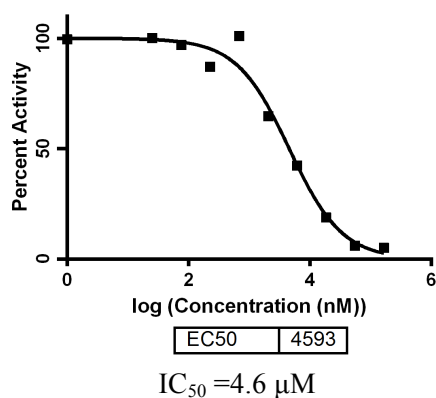
Frk



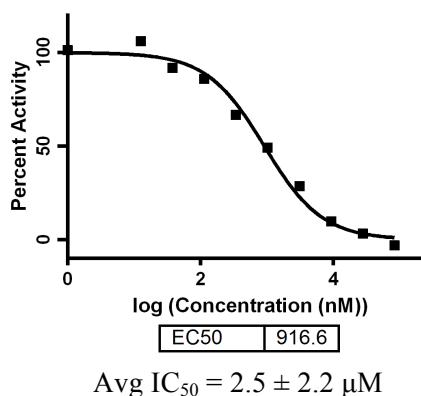
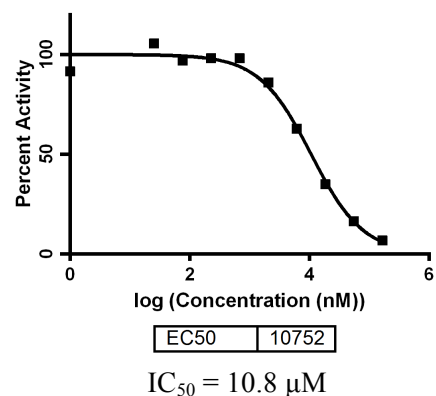
Fyn



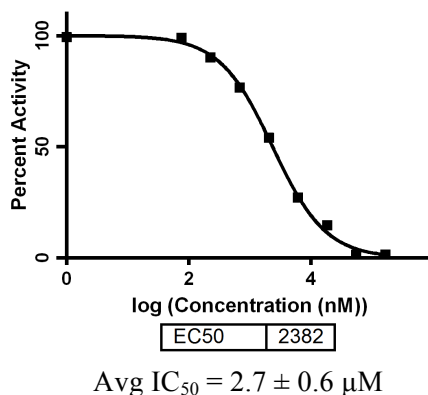
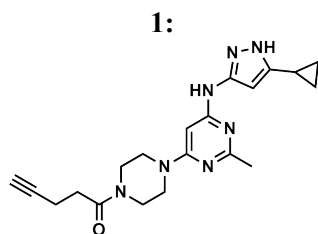
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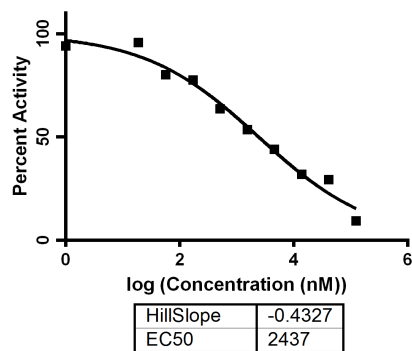
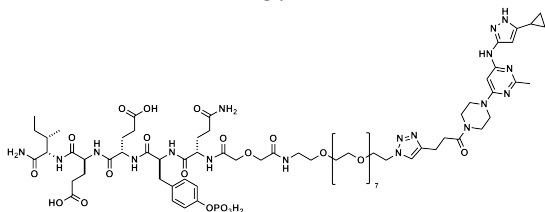
Lyn



Analytical data for 3D Src SH2-engaged IC_{50} determination. An IC_{50} value was determined for each inhibitor using at least three independent experiments. The individual IC_{50} values for the three runs were then averaged. A representative inhibition curve of a single run is shown. These data were obtained using 100 nM Src instead of 30 nM as described above. The IC_{50} curve for **3** was fit using the equation $Y = Bottom + (Top - Bottom) / (1 + 10^{((LogEC50 - X) * HillSlope)})$, $X = \log(\text{concentration})$ and $Y = \text{binding}$.



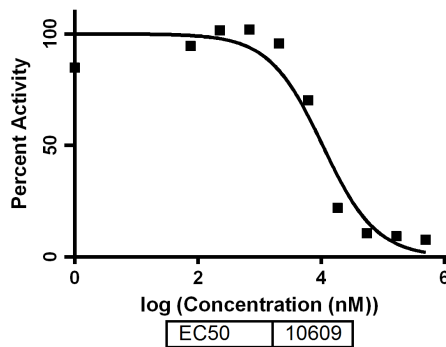
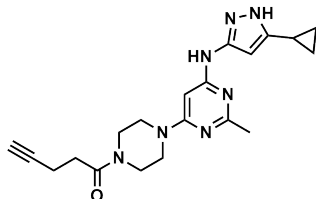
3:



Avg IC₅₀ = 2.2 ± 0.8 μM

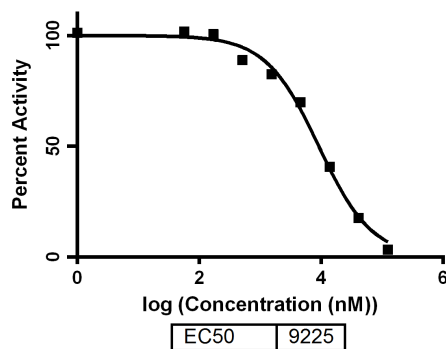
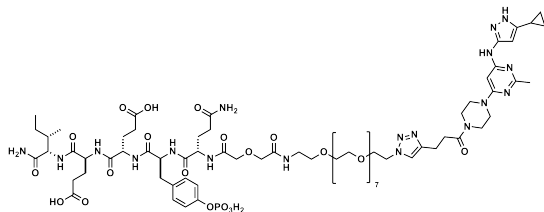
Analytical data for 3D Hck SH2-engaged IC₅₀ determination. An IC₅₀ value was determined for each inhibitor using at least three independent experiments (unless otherwise noted). The individual IC₅₀ values for the three runs were then averaged. A representative inhibition curve of a single run is shown. The IC₅₀ curve for **3** was fit using the equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC50} - X) * \text{HillSlope}))}$, $X = \log(\text{concentration})$ and $Y = \text{binding}$.

1:



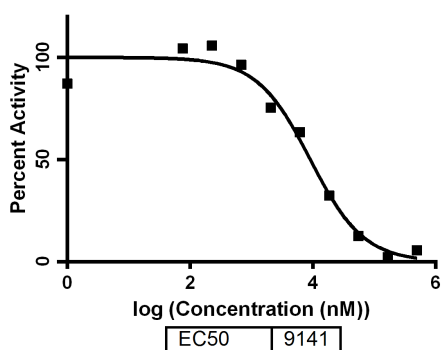
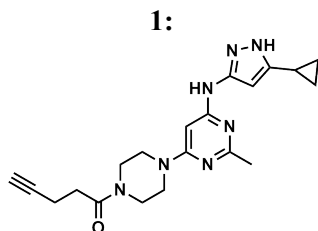
Avg IC₅₀ = 9 ± 2 μM
(n=2)

3:

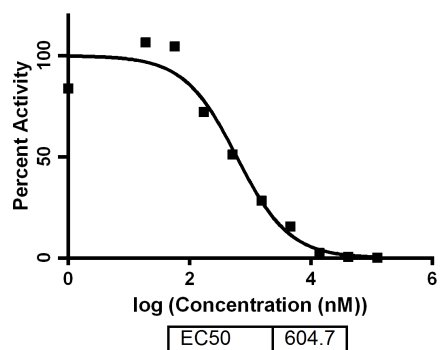
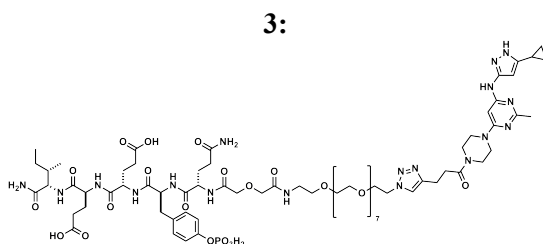


Avg IC₅₀ = 10 ± 3 μM

Analytical data for 3D Hck SH3-engaged IC₅₀ determination. An IC₅₀ value was determined for each inhibitor using at least three independent experiments. The individual IC₅₀ values for the three runs were then averaged. A representative inhibition curve of a single run is shown.



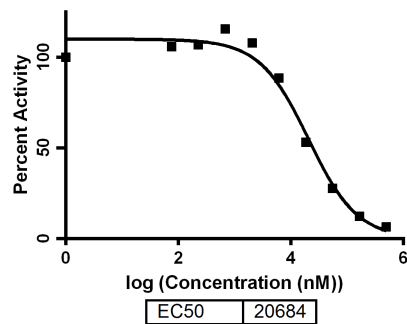
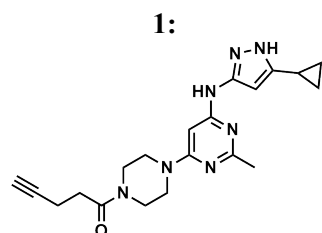
Avg IC₅₀ = 9.3 ± 0.3 μM



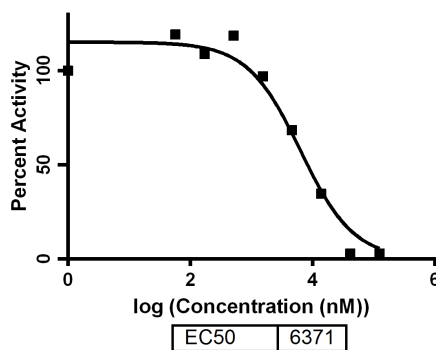
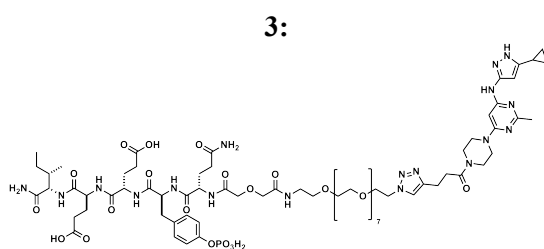
Avg IC₅₀ = 609 ± 62 nM

General procedure for determination of inhibitor IC₅₀ using c-Abl. A continuous fluorescence assay was used to determine IC₅₀.⁶ The reaction volume was 100 μL consisting of 85 μL of enzyme in buffer, 2.5 μL of the appropriate inhibitor dilution (typically 10000, 3333.33, 1111.11, 370.37, 123.46, 41.15, 13.72, 4.57, 1.52, and 0 μM in DMSO), and 2.5 μL (2.4 mM in DMSO) of a substrate peptide⁷. The reaction was initiated with 10 μL of ATP (1 mM in water), and reaction progress was immediately monitored at 405 nm (ex. 340 nm) for 10 minutes. Reactions had final concentrations of 30 nM enzyme, 60 μM peptide substrate, 100 μM ATP, 100 μM Na₃VO₄, 100 mM Tris buffer (pH 8), 10 mM MgCl₂, 0.01% Triton X-100. The initial rate data collected was used for determination of IC₅₀ values. For IC₅₀ determination, the kinetic values were obtained directly from nonlinear regression of substrate-velocity curves in the presence of various concentrations of the inhibitor. The equation $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{X - \text{LogEC50}})$, $X = \log(\text{concentration})$ and $Y = \text{binding}$; was used in the nonlinear regression.

Analytical data for KD c-Abl IC₅₀ determination. An IC₅₀ value was determined for each inhibitor using at least three independent experiments. The individual IC₅₀ values for the three runs were then averaged. A representative inhibition curve of a single run is shown.

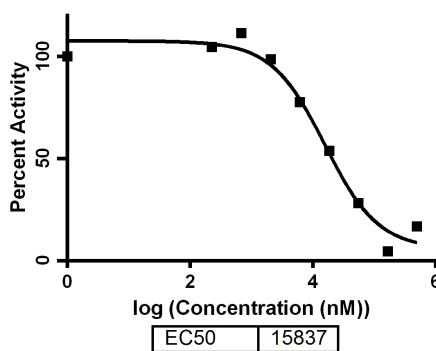
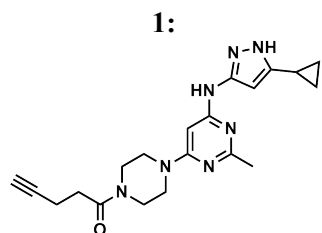


Avg IC₅₀ = 20 ± 2 μM



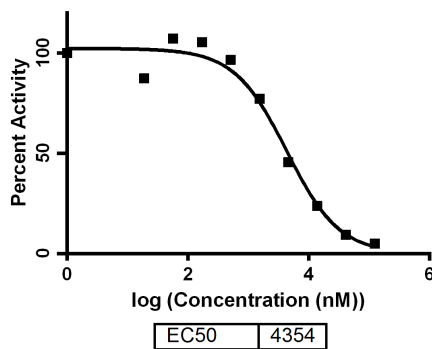
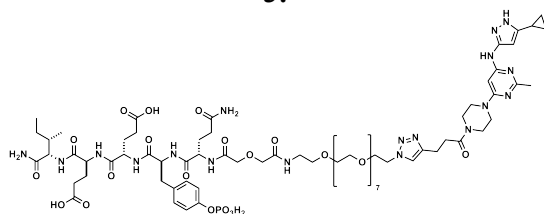
Avg IC₅₀ = 6.1 ± 0.7 μM

Analytical data for 3D Abl IC₅₀ determination. An IC₅₀ value was determined for each inhibitor using at least three independent experiments. The individual IC₅₀ values for the three runs were then averaged. A representative inhibition curve of a single run is shown. These data were obtained using 60 nM Abl instead of 30 nM as described above.



Avg IC₅₀ = 15 ± 4 μM

3:

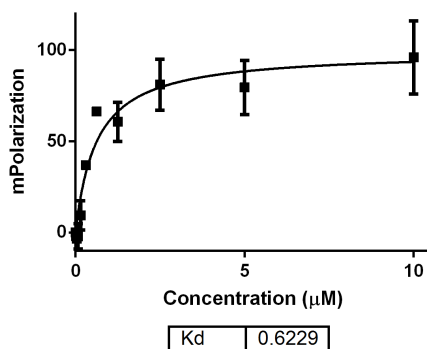


Avg IC₅₀ = 4.1 ± 0.4 μM

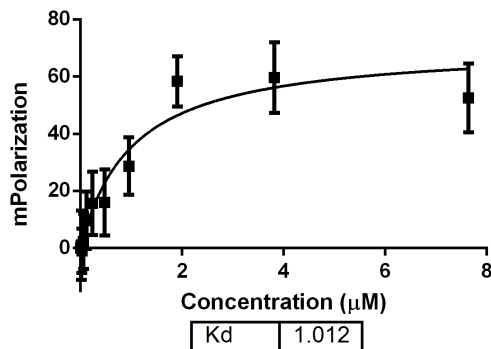
General procedure for determination of SH2 Peptide (S17) K_d. A fluorescence polarization assay was used to determine K_d of peptide S17. The reaction volume was 50 μL consisting of 49 μL of enzyme dilutions in buffer (50 mM Tris buffer (pH 8), 10 mM NaCl, 5% Glycerol, 1 mM DTT) and 1.0 μL of S17 (10 mM in DMSO). The protein was plated to provide 2x dilutions. The peptide was then added and the plate was incubated on ice for 1 hr. After 1 hr, fluorescence polarization was monitored at 528 nm (ex. 485 nm). Reactions had final concentrations of 200 nM S17. For K_d determination, the mPolarization values were plotted vs. varying protein concentrations. The equation $Y = B_{max} * X / (K_d + X)$, X = Protein concentration and Y = FP response; was used in the nonlinear regression.

Analytical data for SH2 Peptide (S17) K_d. Each K_d value was determined using at least three independent experiments. A representative inhibition curve is shown.

3D Src
Avg K_d = 0.73 ± 0.1 μM

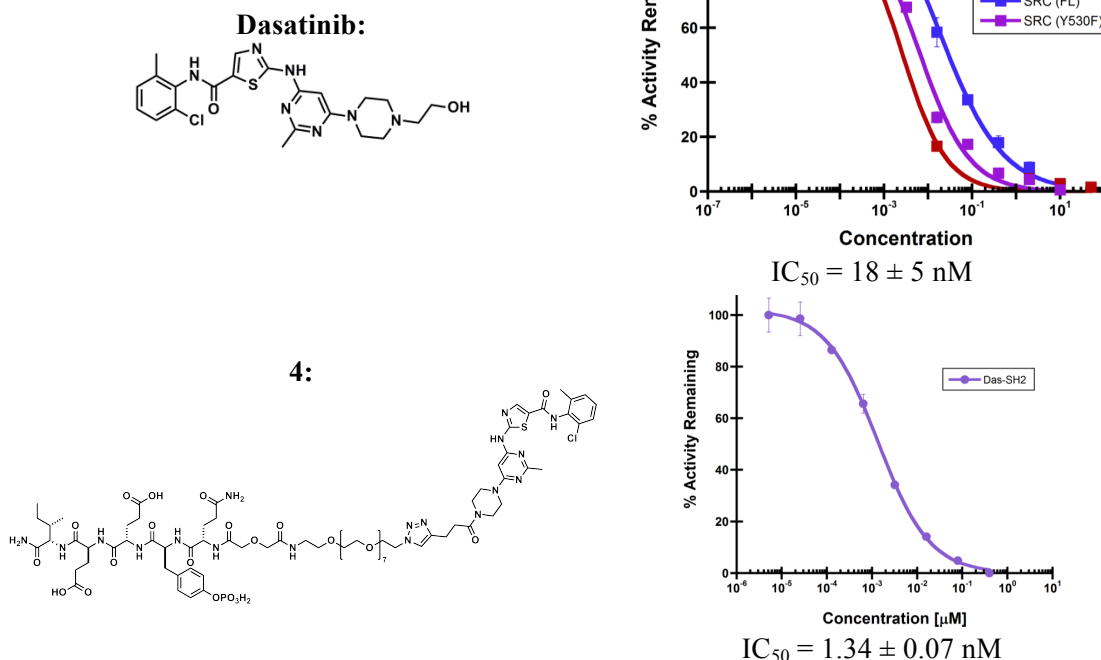


3D Hck
Avg K_d = 1.0 ± 0.3 μM



V. IC₅₀ DETERMINATION USING KINASE SEEKER, LUCEOME BIOTECHNOLOGIES^{8,9}

Analytical data for 3D Src IC₅₀ determination



VI. REFERENCES

- (1) Jung, F. H., Morgentin, R. R., and Ple, P. (2007) Quinoline derivatives as platelet-derived growth factor inhibitors, their preparation, pharmaceutical compositions, and use in the treatment of cancer. *PCT Int. Appl.* 1–154.
- (2) Moussa, I. A., Banister, S. D., Beinat, C., Giboureau, N., Reynolds, A. J., and Kassiou, M. (2010) Design, synthesis, and structure-affinity relationships of regioisomeric N-benzyl alkyl ether piperazine derivatives as σ -1 receptor ligands. *J. Med. Chem.* 53, 6228–6239.
- (3) Wang, H., Yang, C., Wang, L., Kong, D., Zhang, Y., and Yang, Z. (2011) Self-assembled nanospheres as a novel delivery system for taxol: a molecular hydrogel with nanosphere morphology. *Chem. Commun.* 47, 4439–4441.
- (4) Chen, B.-C., Droghini, R., Lajeunesse, J., Dimarco, J. D., Galella, M., and Chidamdaram, R. (2005) Process for Preparing 2-aminothiazole-5-aromatic carboxamides as Kinase Inhibitors. US.
- (5) Seeliger, M. A., Young, M., Henderson, M. N., Pellicena, P., King, D. S., Falick, A. M., and Kuriyan, J. (2005) High yield bacterial expression of active c-Abl and c-Src tyrosine kinases. *Protein Sci.* 14, 3135–3139.
- (6) Wang, Q., Cahill, S. M., Blumenstein, M., and Lawrence, D. S. (2006) Self-reporting fluorescent substrates of protein tyrosine kinases. *J. Am. Chem. Soc.* 128, 1808–1809.
- (7) Rabuck, J. N., Hyung, S.-J., Ko, K. S., Fox, C. C., Soellner, M. B., and Ruotolo, B. T. (2013) Activation state-selective kinase inhibitor assay based on ion mobility-mass spectrometry. *Anal. Chem.* 85, 6995–7002.
- (8) Jester, B. W., Cox, K. J., Gaj, A., Shomin, C. D., Porter, J. R., and Ghosh, I. (2010) A Coiled-Coil Enabled Split-Luciferase Three-Hybrid System: Applied Toward Profiling Inhibitors of Protein Kinases. *J. Am. Chem. Soc.* 132, 11727–11735.
- (9) Jester, B. W., Gaj, A., Shomin, C. D., Cox, K. J., and Ghosh, I. (2012) Testing the Promiscuity of Commercial Kinase Inhibitors Against the AGC Kinase Group Using a Split-luciferase Screen. *J. Med. Chem.* 55, 1526–1537.