Light-Mediated Liberation of Enzymatic Activity: "Small Molecule" Caged Protein Equivalents

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EXPERIMENTAL PROCEDURES

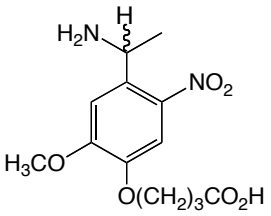
Materials and chemicals were obtained from Fisher and Aldrich, except for piperidine, 5-chloro-1-[bis(dimethylamino)methylene]-1H-benzotriazolium 3-oxide hexafluorophosphate (HCTU), 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt), N, N, N', N'-tetramethyl(succinimido) uranium tetrafluoroborate (TSTU), amino acids, Tentagel and Rink resins, which were obtained from Advanced

ChemTech, Novabiochem, Peptide International or Bachem. Human Src enzyme was purchased from Invitrogen. pUSE Src (wt) cDNA was purchased from Upstate Biotechnology. Anti-Src monoclonal antibody GD11 was purchased from Millipore. UltraLink iodoacetyl gel was purchased from Pierce.

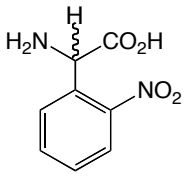
METHODS

Fluorescence assays were performed using a SpectraMAX Gemini EM plate reader (Molecular Device), and irradiation experiments utilized an Oriel Mercury Arc Lamp (Model 69907) equipped with a 360 nm colored glass filter (300 - 400 nm band pass) and an IR filter. Mass spectra by ESI (Electrospray Ionization) were acquired at the Mass Spectrometry Laboratory of the Albert Einstein College of Medicine or the Mass Spectrometry Laboratory of the University of North Carolina at Chapel Hill, Chemistry Department. High pressure liquid chromatography (HPLC) analysis was performed using a Waters 600 solvent delivery system and Waters Delta 600 controller with a 2996 Photodiode Array Detector. Analyses were carried out either on analytical (Altech Apollo C_{18} 5μ 4.6 X 250 mm) or preparative (Waters Atlantis dC_{18}, 19 X 100 mm) scales. Microscope imaging of fluorescent beads were performed using a Zeiss 510 Meta laser scanning confocal microscope, with 10x 0.3 NA objective and 488 nm laser line at the Michael Hooker Microscopy Facility of the University of North Carolina at Chapel Hill.

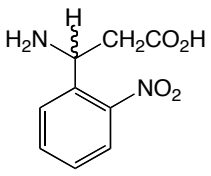
Fmoc protection of photolabile linkers II and III.



S-I



S-II



S-III

The amines of the α-amino acid (+/-) 2-nitrophenylglycine hydrochloride **S-II** (David, A. L.; Smith, D. R.; McCord, T. J. *J. Med. Chem.,* **1973***, 16(9),* 1043-5; Chang, C. Y.; Niblack, B.; Walker, B.; Bayley, H. *Chem. & Biol.* **1995**, *2(6),* 391-400) and the ß-amino acid (+/-) 3-amino-3-(2-nitrophenyl)propionic acid **S-III** (Alfa Aesar) were Fmoc protected using the following procedure: the amino acid (12.0 mmol) was suspended in aqueous sodium carbonate (10%, 25 mL) and acetone (12 mL) at 0 °C. Fmoc-chloride (3.24 g, 12.5 mmol) in acetone (40 mL) was slowly added over 20 min. After being stirred for an additional 1 h at 0 °C and 1 day at room temperature, the reaction mixture was poured into ice water (400 mL) and extracted with diethyl ether. The pH of the aqueous layer was adjusted to 1.0 by addition of 2N HCl. The precipitate was collected by filtration and dried *in vacuo* to yield the Fmoc-protected derivatives of **S-II** and **S-III**. Yield for **S-II** was 85%, ESIMS m/z calculated for $C_{23}H_{18}N_2O_6$ 418.40, found 419.2; yield for **S-III** was 95%, ESIMS m/z calculated for $C_{24}H_{20}N_2O_6$ 432.43, found 431.9. Fmoc-protected **S-I** was purchased from Novabiochem.

Synthesis of inhibitor peptides 1 - 2, 4 - 10.

Diisopropylethylamine (DIPEA: 5 eq, 6.75 mmol, 0.53 g) was added to a suspension of Tentagel S COOH (90 μm, 5 g, 0.27 mmol/g, 1.35 mmol) in 15 mL of DMF containing TSTU (5.0 eq, 2.03 g). The mixture was shaken for 10~15 min at room temperature. Then a solution of cystamine dihydrochloride (10 eq, 13.5 mmol, 3.09 g) and DIEPA (20 eq, 27 mmol, 2.12 g) in 15 mL H_2O was carefully added. The mixture was shaken overnight and the resin subsequently washed with H_2O, DMF, and CH_2Cl_2 (each for 3 X 30 mL). The resulting resin had a free amine substitution of approximately 0.1 mmol/g. Peptides 1, 2, 4 - 10 were prepared using an Fmoc solid-phase peptide synthesis protocol. Fmoc-Tyr(PO(benzyloxy)OH)-OH was used for the incorporation of the phosphorylated tyrosine (pY) residue; Fmoc protected S-I – S-III were used as the photocleavable residues; the side chain of Glu was protected with t-Bu; the side chain of Fmoc-diaminopropionic acid (Dap) was protected with Adpoc (1-(1-Adamantyl)-1-methylethoxycarbonyl). After deprotection of the amino terminal Fmoc group, 3-methoxy-4-nitrobenzoic acid (5 eq, 2.5 mmol, 0.49 g) was attached

using HCTU (5eq, 1.05 g), HOBt (5 eq, 0.34 g), and DIPEA (10 eq, 0.58 g) in 20 mL DMF for 2 h at room temperature. The Adpoc group on the Dap side chain was then removed via exposure to 5% TFA in CH_2Cl_2 (twice), and 1,4,5,6,7,7-hexachloro-bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic acid was subsequently coupled to the free Dap side chain amine using HCTU, HOBt, DIPEA in DMF. Side chain protecting groups (benzyloxy group on pTyr residue and t-Bu group on Glu) were removed with 95% TFA, 2.5% TIS (triisopropylsilane), 2.5% H_2O for 4 h. The peptides were cleaved from the resin [10 mM dithiothreitol (DTT) in 100 mM Tris buffer, 3 times], and purified by preparative HPLC (Waters Atlantis dC_{18} 19 X 100 mm) using a binary solvent system (solvent A: 0.1% TFA/H_2O; solvent B: 0.1% TFA/CH_3CN) with a ratio of A:B that varied from 95:5 (0 min) to 70:30 (10 min) to 0:100 (30 min) and then changed in a linear fashion to 100:0 (45 min). ESIMS m/z peptide **1**: calculated for $C_{107}H_{138}Cl_6N_{20}O_{42}PS$ 2652.11, found 2653.3; peptide **2** calculated for $C_{68}H_{80}Cl_6N_{12}O_{24}S$ 1695.22, found 1695.5; peptide **4** calculated for $C_{117}H_{149}Cl_6N_{21}O_{46}PS$ 2861.30, found 2860.1; peptide **5** calculated for $C_{112}H_{139}Cl_6N_{21}O_{44}PS$ 2759.17, found 2760.0; peptide **6** calculated for $C_{113}H_{141}Cl_6N_{21}O_{44}PS$ 2773.20, found 2773.6; peptide **7** calculated for $C_{106}H_{135}Cl_6N_{21}O_{44}PS$ 2683.08, found 2682.3; peptide **8** calculated for $C_{111}H_{138}Cl_6N_{22}O_{44}PS$ 2760.16, found 2759.2; peptide **9** calculated for $C_{111}H_{138}Cl_6N_{22}O_{44}PS$ 2760.16, found 2759.8; peptide **10** calculated for $C_{117}H_{142}Cl_6N_{22}O_{44}PS$ 2836.26, found 2835.2. Peptide **3** was purchased from Sigma-Aldrich.

Synthesis of photocleavable inhibitor peptide 11.

Peptide **11** was synthesized on the NovaSyn TGR resin. Fmoc-Dap(Mtt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Tyr(PO(benzyloxy)OH)-OH, Fmoc-Cys(t-thiobutyl)-OH were used as the protected amino acids. The free N-terminus was acylated with 3-methoxy-4-nitrobenzoic acid. The methyltrityl (Mtt) group on the Dap residue was removed with 3% TFA in CH_2Cl_2 (twice), and 1,4,5,6,7,7-hexachloro-bicyclo[2.2.1]hept-5-ene-2,3-dicarboxylic acid was coupled using HCTU. The peptide was simultaneously cleaved from the resin (95% TFA, 2.5% TIS, 2.5% H_2O) along with side chain

(benzyloxy group on pTyr and t-Bu group on Glu) deprotection. The t-butylthio group on the Cys side chain was removed with 20 equiv. of DTT in 0.1 M ammonium bicarbonate under Ar. Finally peptide **11** was purified as described for peptides **1**, **2**, and **4** - **10**. ESIMS m/z calculated for $C_{111}H_{137}Cl_6N_{21}O_{44}PS$ 2745.15, found 2744.4.

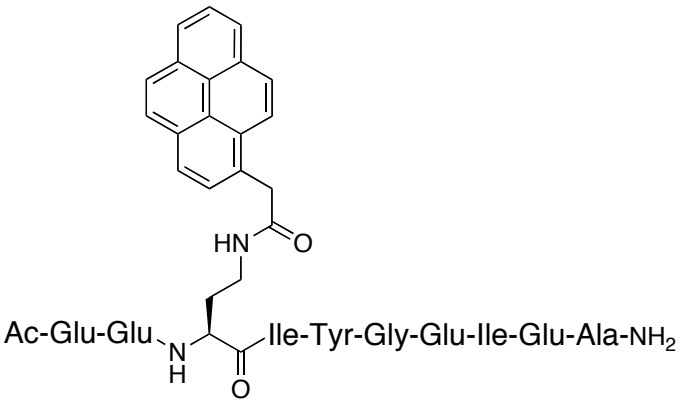
Fluorescent Src kinase assay.

IC_{50} values of inhibitors **1** – **2**, and **4** - **11** were determined as previously reported (Hah, J. M.; Sharma, V.; Li, H.; Lawrence, D. S. J. Amer. Chem. Soc. 2006, 128, 5996-7). The inhibitory efficacy of SH1 domain binding peptide 2 is essentially unchanged in the presence of an SH2 domain ligand (60 μM Ac-pTyr-Glu-Glu-Ile-Glu-amide). Bivalent inhibitors **4** – **11** possess the SH2 domain directed sequence, pTyr-Glu-Glu-Ile-Glu. As expected for an SH2 domain-dependent inhibitor, the inhibitory potency (IC_{50}) of **6** is progressively compromised in the presence of increasing concentrations of free SH2 domain ligand Ac-pTyr-Glu-Glu-Ile-Glu (Table **S-1**).

Table **S-1**. Influence of SH2 domain occupancy with pTyr-Glu-Glu-Ile-Glu on the IC_{50} value of bivalent inhibitor **6**.

[SH2 Ligand] present (μM)	**IC_{50} of inhibitor 6 (nM)**
320	325 ± 59
268	301 ± 43
139	267 ± 45
40	146 ± 20
5	55 ± 15
0.6	32 ± 8
0	18 ± 5

Photoactivation of the Src kinase via photocleavage of 6 as assessed by a continuous fluorescent assay with substrate S-1.

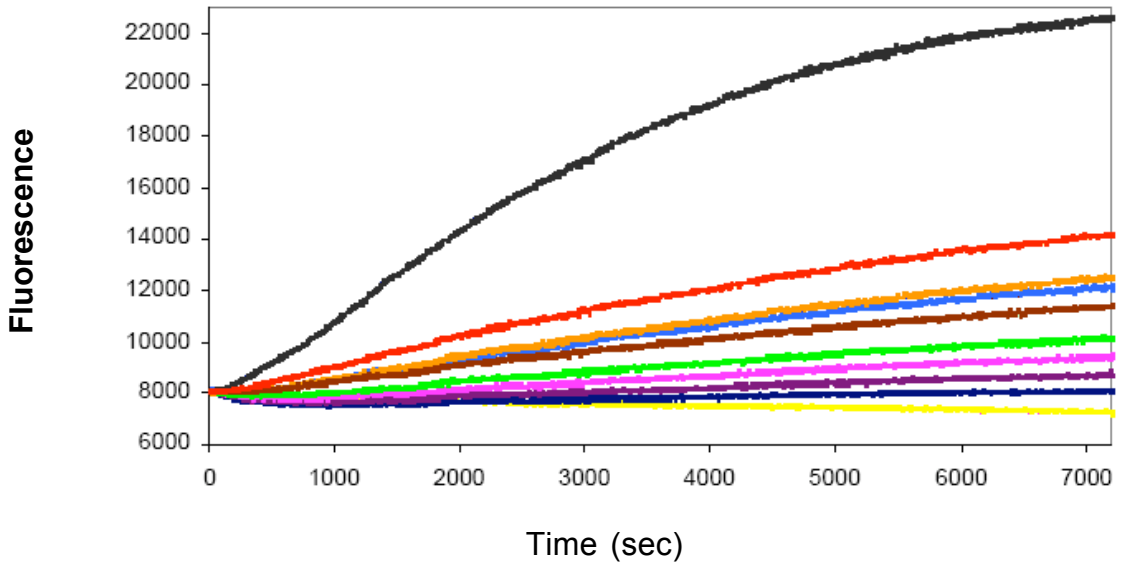


S-1

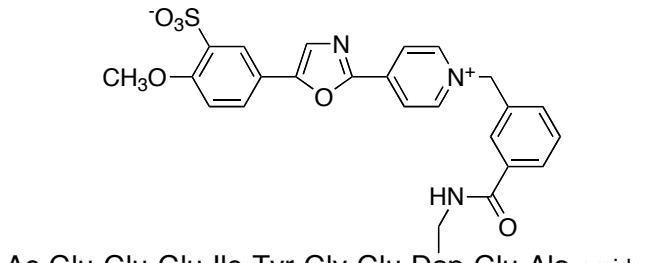
The bivalent inhibitor peptide **6** (1500 μL, 0.1 mM in 100 mM TBS) was placed in a 2 mL polypropylene microcentrifuge tube and cooled to 0 °C. The sample was irradiated at 200 W for 0, 1, 2, 4, 8, 15, 30, 60, or 120 min on ice. A 150 μL aliquot was removed, and 2.9 μL was diluted for analyzing inhibitory activity for Src as follows: To a 1.4 mL reaction buffer [53.6 mM Tris (pH 7.5), 5.36 mM $MgCl_2$, 1.07 mM $MnCl_2$, 2.14 mM DTT and 0.01 mg/mL BSA] was added 73 μL 300 nM Src stock solution (enzyme purchased from Invitrogen and aliquotted into 50 mM Tris buffer containing 10% glycerol) and 10.9 μL of 2.0 mM pyrene substrate **S-1** in aqueous solution. The reaction solution was aliquoted into 10 wells (one well per irradiation time) in a 96-well plate. 2.9 μL of previously irradiated bivalent inhibitor 6 was added into the wells and incubated for 5 min (in order to allow the pyrene fluorescence to stabilize). 5 μL of a 50 mM ATP aqueous solution was added into each well to initiate the phosphorylation reaction and the fluorescence of the solution was monitored on a SpectraMAX Gemini EM plate reader (λ_{ex} = 340 nm and λ_{em} = 380 nm). The final concentration was: 50 mM Tris, 15 μM pyrene substrate peptide, 2.0 μM inhibitor 6, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM DTT, 0.01 mg/mL BSA, 15 nM Src, and 1 mM ATP. The following control experiments were

performed: (i) no inhibitor, and (ii) in the presence of the monovalent inhibitor **2** (2 μM) and the SH2 domain ligand pYEEIE peptide **3** (2 μM). In Figure **S-1**, from top to bottom, the inhibitor present was as follows: black: no inhibitor; red: inhibitor **6** UV irradiated for 120 min; orange: UV 60 min; blue: UV 30 min; brown: UV 15 min; green: UV 8 min; pink: UV 4 min; purple: UV 2 min; indigo: UV 1 min; yellow: UV 0 min. Since the pyrene fluorophore is significantly quenched by vanadate ion, a mandatory phosphatase inhibitor for cell lysate studies, the cascade yellow labeled substrate S-2 was employed (Wang, Q.; Dai, Z.; Cahill, S. M.; Blumenstein, M.; Lawrence, D. S. *J. Amer. Chem. Soc.* 2006, *128*, 14016-7) for the time-dependant release of Src activity in cell lysates.

Figure S-1. Src kinase activity, in the presence of inhibitor **6**, as a function of irradiation time (black: no inhibitor; red: irradiated for 120 min; orange: irradiated for 60 min; blue: irradiated for 30 min; brown: irradiated for 15 min; green: irradiated for 8 min; pink: irradiated for 4 min; purple: irradiated for 2 min; indigo: irradiated for 1 min; yellow: irradiated for 0 min). The reaction was monitored by phosphorylation of the fluorescently responsive pyrene substrate **S-1**.



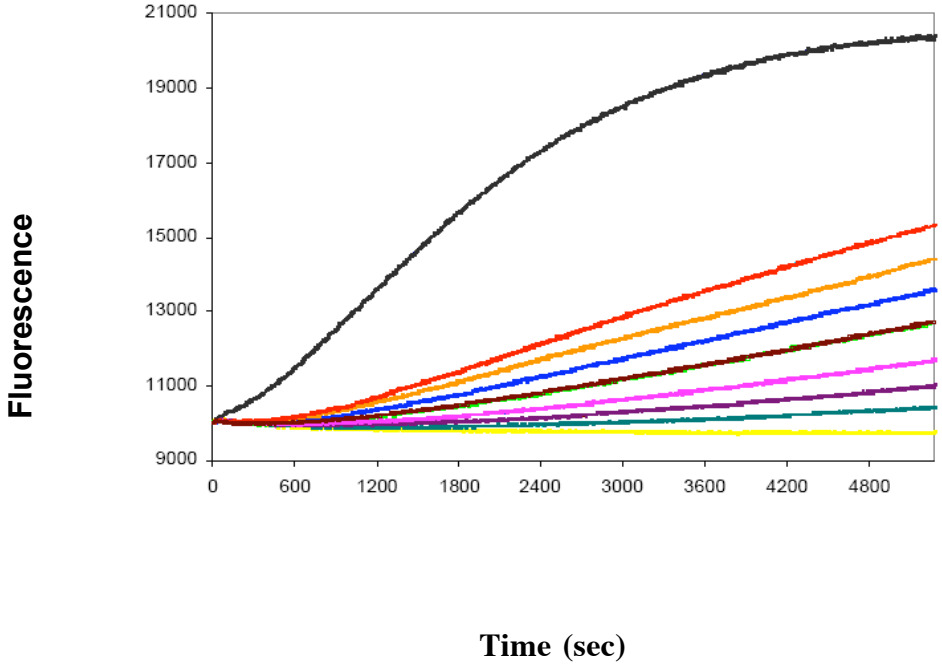
Photoactivation of the Src kinase via photocleavage of 6 as assessed by a continuous fluorescent assay with substrate S-2.



S-2

The bivalent inhibitor peptide **6** was UV irradiated in the same fashion as described above. A 150 μL aliquot was removed at various irradiation times, and 1.5 μL was diluted for analyzing inhibitory activity for Src kinase as follows: To a 1.4 mL reaction buffer [53.6 mM Tris (pH 7.5), 5.36 mM $MgCl_2$, 1.07 mM $MnCl_2$, 2.14 mM DTT and 0.01 mg/mL BSA] was added 73 μL 300 nM Src stock solution and 17.3 μL 3.6 mM cascade yellow substrate **S-2** in aqueous solution. The reaction solution was aliquoted into 10 wells (one well per irradiation time) in a 96-well plate. 1.5 μL of previously irradiated bivalent inhibitor **6** was added into the wells and incubated for 5 min (in order to allow the cascade yellow fluorescence to stabilize). 5 μL of a 50 mM ATP aqueous solution was added into each well to initiate the phosphorylation reaction and the fluorescence of the solution was monitored on a SpectraMAX Gemini EM plate reader (λ_{ex} = 400 nm and λ_{em} = 535 nm). The final concentration was: 50 mM Tris, 42 μM cascade yellow substrate peptide, 1.0 μM inhibitor **6**, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM DTT, 0.01 mg/mL BSA, 15 nM Src, and 1 mM ATP. The following control experiments were performed: (i) no inhibitor, and (ii) in the presence of the monovalent inhibitor **2** (1 μM) and the SH2 domain ligand pYEEIE peptide **3** (1 μM). In Figure **S-2**, from top to bottom, the inhibitor present was as follows: black: no inhibitor; red: UV irradiated for 120 min; orange: 60 min; blue: 30 min; brown: 15 min; green: 8 min; pink: 4 min; purple: 2 min; indigo: 1 min; yellow: 0 min.

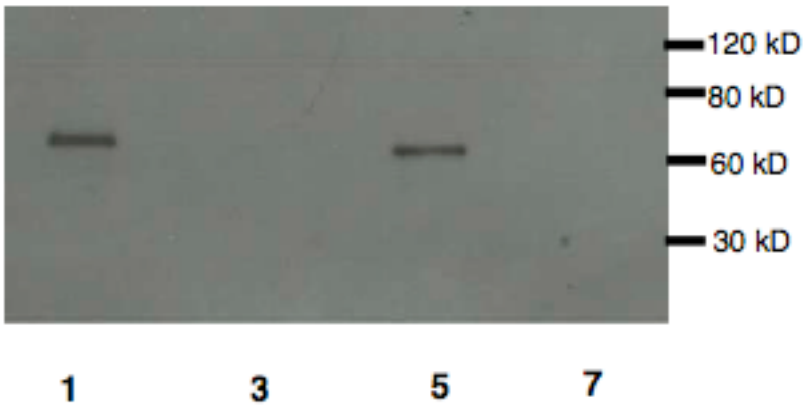
Figure S-2. Src kinase activity, in the presence of inhibitor **6**, as a function of irradiation time (black: no inhibitor; red: UV irradiated for 120 min; orange: irradiated for 60 min; blue: irradiated for 30 min; brown: irradiated for 15 min; green: irradiated for 8 min; pink: irradiated for 4 min; purple: irradiated for 2 min; indigo: irradiated for 1 min; yellow: irradiated for 0 min.). The reaction was monitored by phosphorylation of the fluorescently responsive cascade yellow substrate **S-2**.



Overexpression of wild type Src in COS-1 cells.

1 - 2 x 10^5 COS-1 cells were seeded in a six well tissue culture plate in 2 mL of DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% FBS (fetal calf serum). The cells were incubated for 18 - 24 h at 37 °C in a CO_2 humidified incubator until the cells were 40 - 60% confluent. 1 - 2 μg of pUSE Src (wild type) cDNA was diluted into 100 μL serum free DMEM in a sterile tube. In a second tube, 20 μL of lipofectin 2000 reagent (Invitrogen) was diluted into 100 μL serum free DMEM. The solutions in both tubes were allowed to stand at room temperature for 30 - 45 min, combined, mixed gently and incubated for another 10 - 15 min. COS-1 cells were washed once with 2 mL of serum free DMEM. For each transfection, 0.8 mL serum-free DMEM was added into each tube containing the lipofectin reagent-DNA complex, mixed well, and overlayed onto cells. After 5 h of incubation at 37 °C in a CO_2 humidified incubator, the DNA-containing medium was removed and replaced with 2 mL of DMEM supplemented with 10% FBS. The cells were incubated for additional 48 - 72 h, then washed twice with PBS and collected by scraping off the plate. The cells were lysed in a buffer containing 0.9% NP40, 0.9 mM EDTA, and 50 mM Tris for 30 min at 4 °C. Overexpression of wild type Src was verified by western blot analysis (Figure **S-3**) as follows: The lysate of the transiently transfected COS-1 cells was resolved by electrophoresis, transferred to a PVDF membrane, and probed with anti-Src monoclonal antibody (Invitrogen). Proteins were visualized using a goat anti-mouse secondary antibody conjugated to HRP and a chemiluminescence detection system (Amersham).

Figure S-3. Western blot of wild type Src overexpressed in COS-1 cells. Lane 1: Commercially available wild type Src (Invitrogen, 27 ng); Lane 3: mock-transfected pUSE vector in COS-1 cells (~5 x 10^5 cells/lane); Lane 5: transient transfected wild type Src in COS-1 cells (~5 x 10^5 cells/lane). Membrane was blotted with anti-Src monoclonal antibody (Millipore).

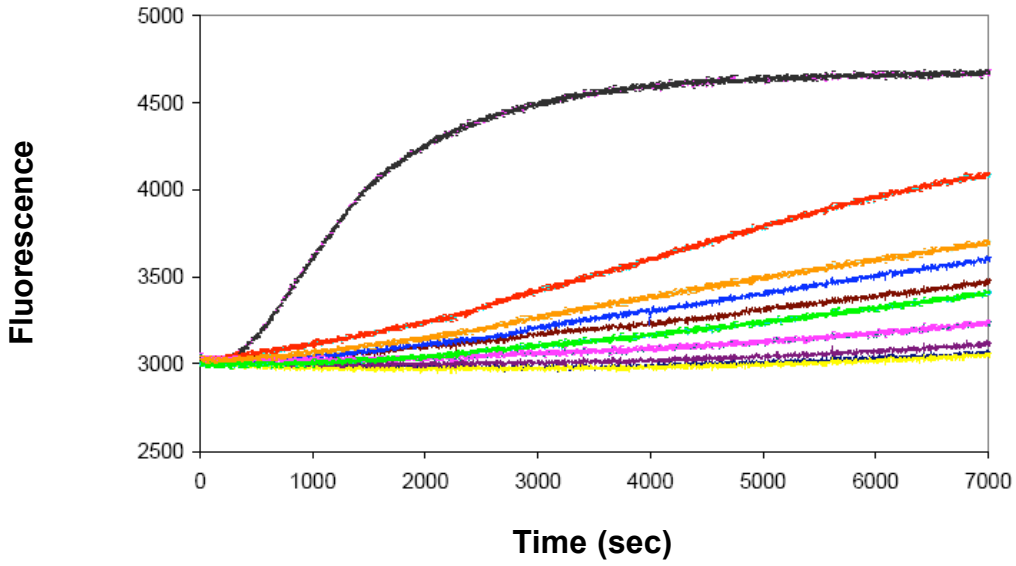


Photoactivation of the Src kinase in cell lysates via photocleavage of 6 as assessed by a continuous fluorescent assay with substrate S-2.

The bivalent inhibitor peptide **6** was UV irradiated in the same fashion as previously described. A 150 μL aliquot was removed at various irradiation times, and 3.2 μL was diluted for analyzing inhibitory activity for Src as follows: To a 1.4 mL reaction buffer [53.6 mM Tris (pH 7.5), 5.36 mM $MgCl_2$, 1.07 mM $MnCl_2$, 2.14 mM DTT and 0.01 mg/mL BSA] was added 50 μL COS-1 cell lysate overexpressing wild type Src (~5 X 10^6 cells in presence of 1% phosphatase inhibitor cocktail) and 12 μL 3.6 mM cascade yellow substrate aqueous solution. The reaction solution was aliquoted into 10 wells (one well per irradiation time) in a 96-well plate. 3.2 μL of previously irradiated bivalent inhibitor **6** was added into the wells and incubated for 3 min (in order to allow the cascade yellow **S-2** fluorescence to stabilize). 5 μL of a 50 mM ATP aqueous solution was added into each well to initiate the phosphorylation reaction and the fluorescence of the solution was monitored on a SpectraMAX

Gemini EM plate reader (λ_{ex} = 400 nm and λ_{em} = 535 nm). The final concentration was: 50 mM Tris, 28.8 μM cascade yellow substrate peptide **S-2**, 2.0 μM inhibitor 6, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM DTT, 0.01 mg/mL BSA, 15 nM Src, and 1 mM ATP. The following control experiments were performed: (i) no inhibitor, and (ii) in the presence of the monovalent inhibitor **2** (2 μM) and the SH2 domain ligand pYEEIE peptide **3** (2 μM) (see Figure **S-4**).

Figure **S-4**. Src kinase activity, in the presence of inhibitor **6**, as a function of irradiation time (black: no inhibitor; red: irradiated 120 min; orange: irradiated 60 min; blue: irradiated 30 min; brown: irradiated 15 min; green: irradiated 8 min; pink: irradiated 4 min; purple: irradiated 2 min; indigo: irradiated 1 min; yellow: irradiated 0 min). The reaction was monitored by phosphorylation of the fluorescently responsive cascade yellow substrate **S-2**.



Photorelease of Src kinase using the photocleavable peptide 11-UltraLink bead conjugate.

Bivalent inhibitor peptide **11** (8 mg) was reacted with 150 μL settled UltraLink iodoacetyl beads (Pierce) in a buffer containing 50 mM Tris, 5 mM EDTA, pH 8.5 in the presence of 25 mM tris-(2-carboxyethyl)phosphine (TCEP) at room temperature overnight. The beads were subsequently exposed to 50 mM cysteine (twice) and then blocked with 1% prionex (Sigma-Aldrich) in PBS for 1 h each. They were washed with PBS and then with Src assay buffer containing 50 mM Tris, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM DTT, and 0.01 mg/mL BSA. Beads modified with peptide **11** and beads modified with cysteine only were partially transferred into separate glass vials. Whole mouse serum was added to a concentration of 0.5% and the samples were irradiated under UV light at 0 °C for 40 min. The beads were then washed thoroughly with TBS (5 x 1 mL) and reblocked with 1% prionex in TBS for half an hour. 10 μL of non-UV treated, UV treated peptide **11**-loaded beads, as well as cysteine-modified control beads were each incubated with 30 nM Src and 10 μM pyrene substrate in kinase assay buffer for 20 min at 0 °C. The supernatants were transferred into a 96 well plate. ATP stock solution was added to a final concentration of 5 mM to initiate the phosphorylation reaction. The continuous fluorescent assay was monitored on a SpectraMAX Gemini EM plate reader (Figure **S-5**) using peptide **S-1**. The beads were then washed with TBS (4 x 1 mL). Src activity is essentially eliminated after incubation with bivalent inhibitor **11** loaded beads (Figure **S-5**). However, illumination of those beads released approximately 45% of original Src activity. To directly image the captured Src enzyme on the beads, a mouse monoclonal antibody to Src (GD11 from Millipore) was labeled with Alexa Fluo488 using a monoclonal antibody labeling kit (Invitrogen). The degree of labeling was determined to be 4 moles of dye per mole of antibody. The beads were incubated with labeled antibody (1:100 dilution in TBS in the presence of 1% mouse serum) for 1 h, washed (4 x 1 mL), and imaged (Figure **2**) on a Zeiss 510 Meta laser scanning confocal microscope, with a 10x 0.3 NA objective at the 488 nm laser line. To validate the linear relationship between Src enzyme captured on the beads and fluorescence intensity imaged, bivalent inhibitor peptide **11** loaded beads were incubated with various

concentrations of Src ranging from 2.5 μM to 40 μM. The beads were subsequently imaged after incubation with the Alexa labeled antibody (Figure **S-6**). A standard curve correlating "quantity of Src enzyme on the beads" with "fluorescence intensity of the beads" is depicted in Figure **S-7**. According to Figure **S-7**, beads on the right panel (UV irradiated beads) of Figure **2** has 50% of src kinase bound compared to left panel (no UV irradiated beads) when Src concentration was 30 nM.

Figure S-5. Supernatent Src kinase activity upon incubation with bivalent inhibitor peptide **11** modified UltraLink beads, UV irradiated peptide **11** modified beads, and cysteine modified control beads. Red curve: supernatant Src activity upon incubation of 30 nM Src with bivalent inhibitor peptide **11** modified UltraLink beads (no UV irradiation); blue curve: supernatant Src activity upon incubation of 30 nM Src with bivalent inhibitor peptide **11** modified UltraLink beads (UV irradiated for 40 min at 0 °C); yellow curve: supernatant Src activity upon incubation of 30 nM Src with cysteine modified UltraLink beads (UV irradiated for 40 min at 0 °C).

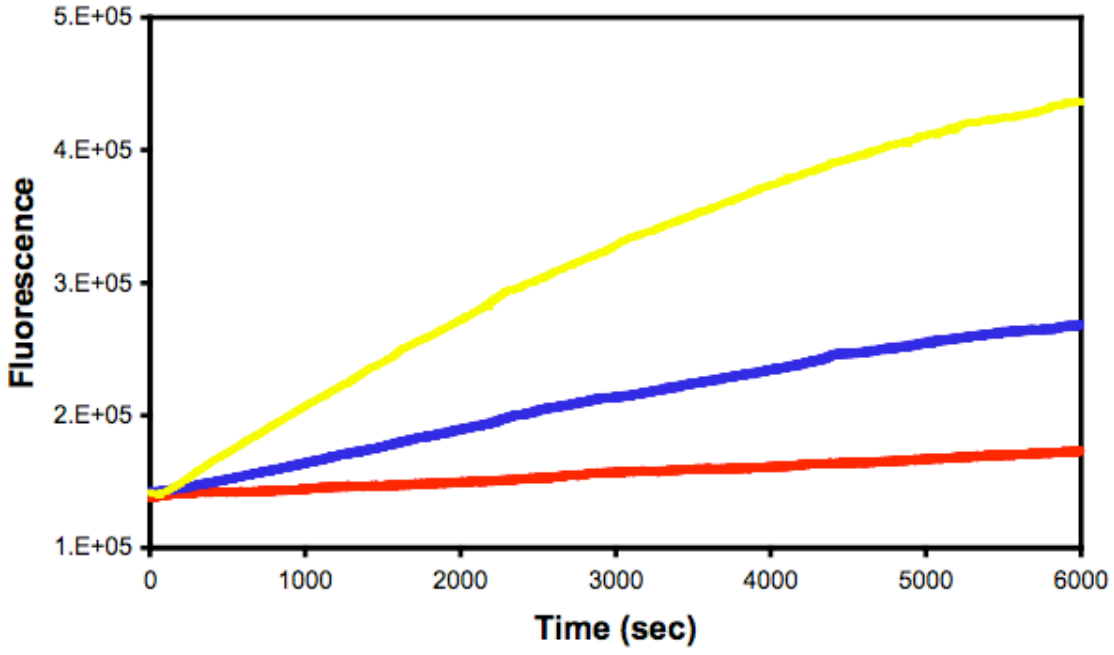
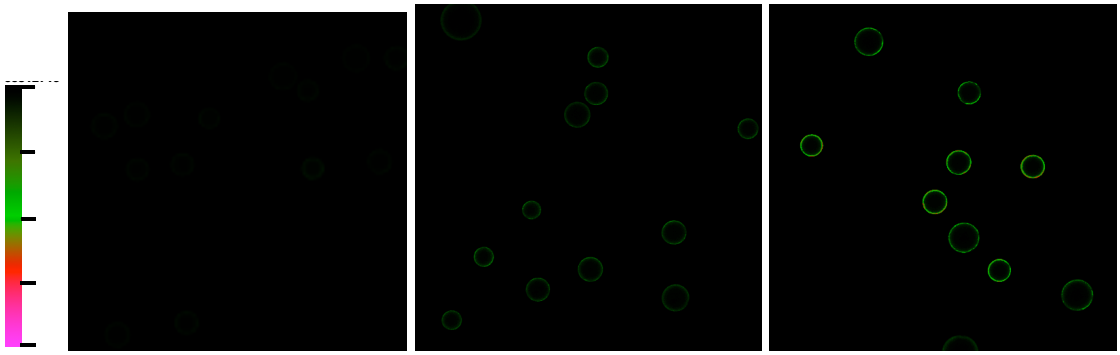
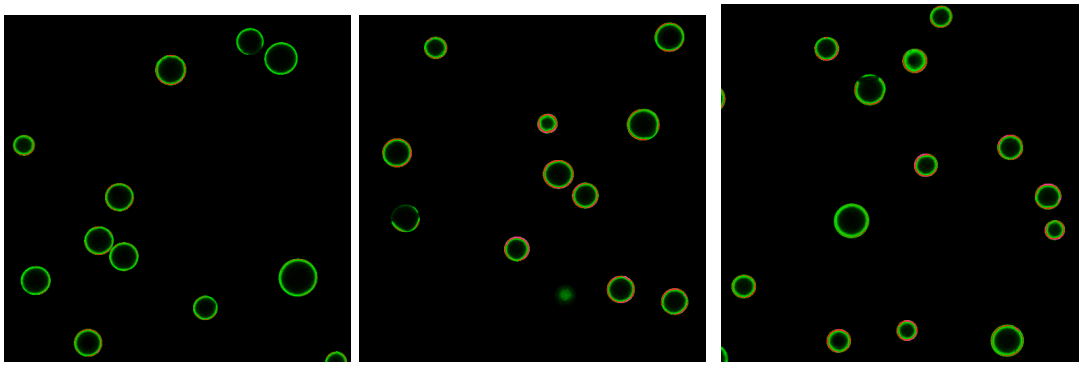


Figure S-6. Fluorescence images of beads in presence of increasing concentration of Src enzyme (2.5 nM, 5 nM, 10 nM, 20 nM 30 nM and 40 nM) and imaging antibody (1:100 dilution in TBS in presence of 1% mouse serum).



[Src]= 2.5 nM [Src] = 5 nM [Src] = 10 nM



[Src] = 20 nM [Src] = 30 nM [Src] = 40 nM

Figure S-7. Standard binding curve derived from the fluorescence as a function of [Src] data from Figure **S-6**.