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Supporting Information

69451 Weinheim, Germany

Substrate Activity Screening with Kinases: Discovery of Small-Molecule Substrate-Competitive c-Src Inhibitors**

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SUPPORTING INFORMATION

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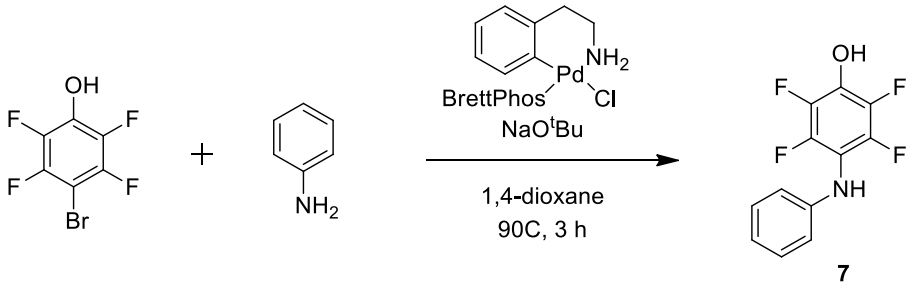
I. GENERAL SYNTHETIC METHODS

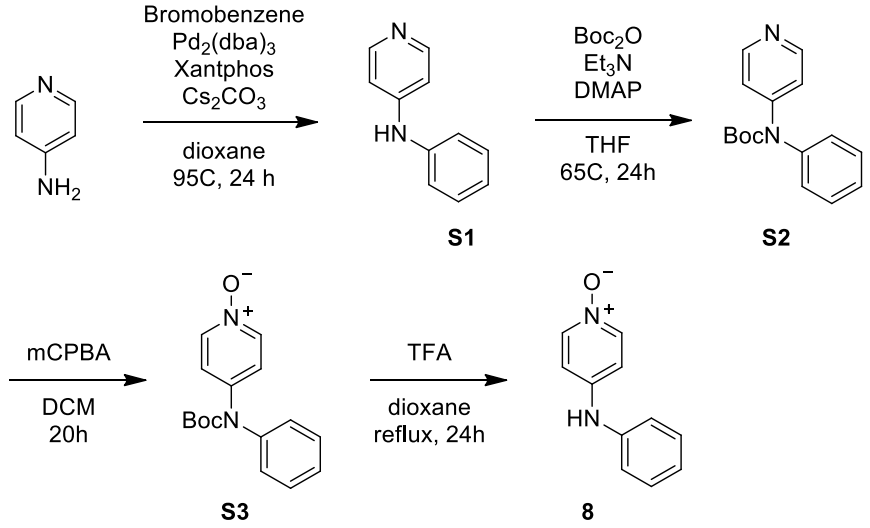
Unless otherwise noted, all reagents were obtained via commercial sources and used without further purification. ^{1}H, ^{13}C, and ^{19}F NMR spectra were measured with a Varian MR400 or Inova 500 spectrometer. Mass Spectrometry (HRMS) was carried out by the University of Michigan Mass Spectrometry Facility (J. Windak, director).

II. SYNTHESIS OF COMPOUNDS 7-12



7

2,3,5,6-tetrafluoro-4-(phenylamino)phenol (7). Compound **7** was prepared in a manner similar to that described by Maiti and Buchwald.[1] To an oven-dried 4 mL conical vial was added 4-bromo-2,3,5,6-tetrafluorophenol (123 mg, 0.5 mmol), aniline (55 µL, 0.6 mmol), sodium tert-butoxide (120 mg, 1.25 mmol), and BrettPhos palladacycle (0.8 mg, 0.001 mmol). The vial was flushed with N_2, then 1 mL anhydrous 1,4-dioxane was added. The reaction was stirred at 90 C for 3 h. After cooling to RT, the reaction was poured into a 10% aqueous citric acid solution (30 mL) and extracted with EtOAc (4 x 20 mL). The organic extracts were dried over $MgSO_4$ and the solvent was removed under reduced pressure. Purification by automated silica gel chromatography using a 6 → 60% EtOAc in hexanes gradient afforded **6** as a pale yellow crystalline solid (36 mg, 28% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28 – 7.14 (m, 2H), 6.96 – 6.86 (m, 1H), 6.74 (m, 2H), 5.51 (s, 1H), 5.23 (s, 1H). ^{19}F NMR (400 MHz, $CDCl_3$) δ -149.9 (dd, *J* = 23.7 Hz, 7.2 Hz, 2F), -163.8 (dd, *J* = 23.7 Hz, 7.2 Hz, 2F). HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{12}H_7F_4NO$, 258.0537; found 258.0533.



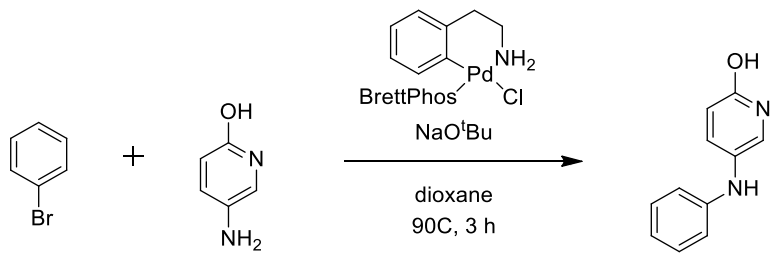
S1 **S2** **S3** **8**

***N*-phenylpyridin-4-amine (S1).** To an oven-dried vial was added 4-aminopyridine (113 mg, 1.2 mmol), bromobenzene (106 µL, 1.0 mmol), Cs_2CO_3 (814 mg, 2.5 mmol), Xantphos (58 mg, 0.1 mmol), and $Pd_2(dba)_3$ (46 mg, 0.05 mmol). The vial was flushed with N_2 for 5 min, then anhydrous 1,4-dioxane (4 mL) was added and the reaction was stirred at 95 C for 24 h. The reaction was cooled to room temperature, diluted with H_2O (30 mL), and extracted with EtOAc (3x20 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and the solvent was removed under reduced pressure. Purification by automated silica gel chromatography using a 0 → 10% MeOH in DCM gradient afforded **S1** as a light yellow solid (133 mg, 78% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.28 – 8.23 (m, 2H), 7.39 – 7.31 (m, 2H), 7.21 – 7.08 (m, 3H), 6.83 – 6.77 (m, 2H), 6.14 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 150.83, 149.91, 139.50, 129.52, 124.12, 121.63, 109.41. HRMS-ESI (*m*/*z*): $[M+H]^+$ calcd for $C_{11}H_{10}N_2$, 171.0917; found 171.0916.

Tert-butylphenyl(pyridin-4-yl)carbamate (S2). In a 25 mL roundbottom flask, **S1** (100 mg, 0.59 mmol) was dissolved in 6 mL anhydrous THF. TEA (160 µL, 1.2 mmol) was added followed by DMAP (0.7 mg, 0.006 mmol) and Boc_2O (154 mg, 0.71 mmol). The reaction was stirred at room temperature overnight then at 65 C for 24 h. The reaction was cooled to room temperature, diluted with H_2O (30 mL) and extracted with EtOAc (3x15 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and the solvent was removed under reduced pressure. Purification by automated silica gel chromatography using a 10 → 80% EtOAc in hexanes gradient afforded **S2** as a white solid (52 mg, 32% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.43 – 8.38 (m, 2H), 7.43 – 7.35 (m, 2H), 7.35 – 7.27 (m, 1H), 7.18 – 7.10 (m, 4H), 1.42 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.83, 150.11, 150.04, 140.99, 129.38, 128.30, 127.42, 117.78, 82.30, 28.04. HRMS-ESI (*m*/*z*): $[M+H]^+$ calcd for $C_{16}H_{18}N_2O_2$, 271.1441; found 271.1443.

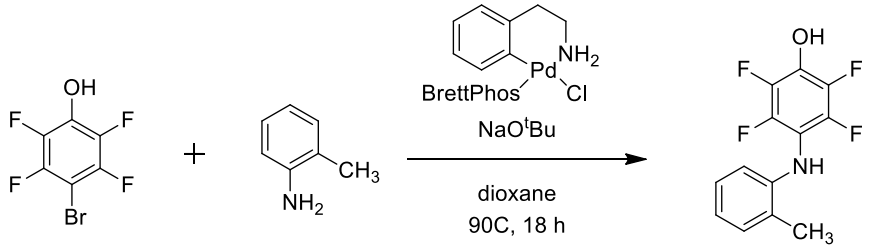
4-((tert-butoxycarbonyl)(phenyl)amino)pyridine 1-oxide (S3). S2 (41 mg, 0.15 mmol) was dissolved in 5 mL DCM then mCPBA (104 mg, 0.6 mmol) was added. The reaction was stirred at room temperature for 20 h. The reaction was diluted with 10 mL DCM and washed with 1 N NaOH (15 mL), and the organic layer was dried over MgSO4, filtered, and the solvent was removed under reduced pressure. Purification by automated silica gel chromatography using a 0 → 7% MeOH in DCM gradient afforded **S3** as a light yellow glass (38 mg, 89% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07 – 8.00 (m, 2H), 7.45 – 7.37 (m, 2H), 7.37 – 7.30 (m, 1H), 7.20 – 7.11 (m, 4H), 1.40 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.40, 141.55, 140.25, 138.70, 129.61, 127.98, 127.80, 119.80, 82.82, 27.91. HRMS-ESI (*m*/*z*): $[M+H]^+$ calcd for $C_{16}H_{18}N_2O_3$, 287.1390; found 287.1393.

4-(phenylamino)pyridine 1-oxide (8). In a dry 10 mL round bottom flask **S3** (30 mg, 0.10 mmol) was dissolved in 4 mL DCM then TFA (2 mmol) was added. The reaction was stirred at reflux for 24 h, then the solvent was removed under reduced pressure. Purification by automated silica gel chromatography using a 0 → 7% MeOH in DCM gradient afforded **8** as a light brown solid (8 mg, 41% yield). ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.08 (s, 1H), 8.01 – 7.95 (m, 2H), 7.35 (t, *J* = 7.8 Hz, 2H), 7.16 (d, *J* = 7.9 Hz, 2H), 7.04 (t, *J* = 7.4 Hz, 1H), 6.98 – 6.91 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 143.83, 140.00, 139.14, 129.54, 123.05, 119.99, 111.01. HRMS-ESI (*m*/*z*): $[M+H]^+$ calcd for $C_{11}H_{10}N_2O$, 187.0866; found 187.0866.



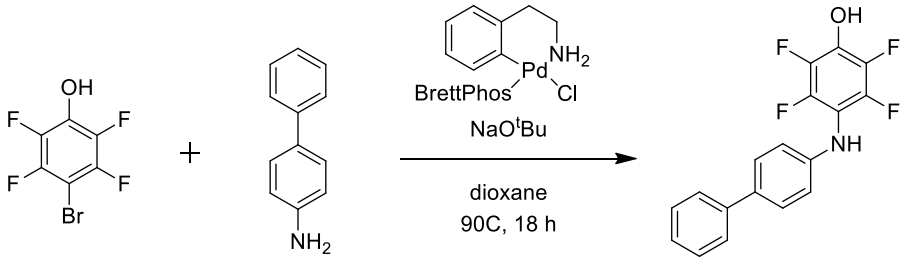
9

5-(phenylamino)pyridin-2(1H)-one (9). Compound **9** was prepared in a manner similar to that described by Maiti and Buchwald.[1] To an oven-dried 4 mL conical vial was added bromobenzene (53 µL, 0.5 mmol), 5-amino-2-hydroxypyridine (66 mg, 0.6 mmol), sodium tert-butoxide (120 mg, 1.25 mmol), and BrettPhos palladacycle (0.8 mg, 0.001 mmol). The vial was flushed with N_2, then 1 mL anhydrous 1,4-dioxane was added. The reaction was stirred at 90 C for 3 h. After cooling to RT, the reaction was poured into water (30 mL) and extracted with EtOAc (3 x 15 mL). The organic extracts were dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was dissolved in DMSO (2 mL) and purified by reverse-phase HPLC using a 5 → 95% acetonitrile in water gradient. Lyophilization afforded **9** as a pale brown solid (30 mg, 32% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.27 (s, 1H), 7.41 (s, 1H), 7.34 (dd, *J* = 9.6, 3.0 Hz, 1H), 7.19 – 7.07 (m, 3H), 6.71 – 6.61 (m, 3H), 6.36 (dd, *J* = 9.5, 0.7 Hz, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 159.89, 145.83, 139.90, 129.28, 127.54, 124.89, 118.90, 118.46, 114.31. HRMS-ESI (*m*/*z*): $[M+H]^+$ calcd for $C_{11}H_{10}N_2O$, 187.0866; found 187.0865.



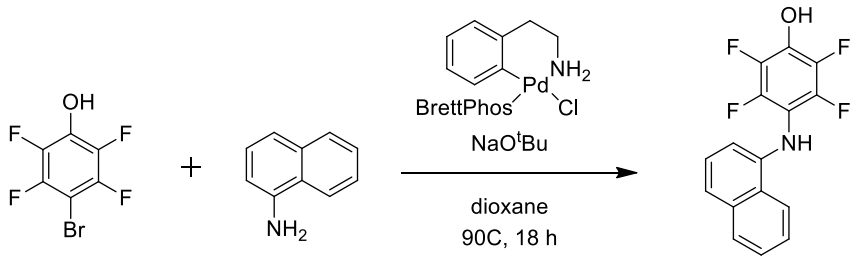
10

2,3,5,6-tetrafluoro-4-(o-tolylamino)phenol (10). Compound **10** was prepared in a manner similar to that described by Maiti and Buchwald.[1] To an oven-dried 4 mL conical vial was added 4-bromo-2,3,5,6-tetrafluorophenol (123 mg, 0.5 mmol), *o*-toluidine (64 µL, 0.6 mmol), sodium tert-butoxide (120 mg, 1.25 mmol), and BrettPhos palladacycle (0.8 mg, 0.001 mmol). The vial was flushed with N_2, then 1 mL anhydrous 1,4-dioxane was added. The reaction was stirred at 90 C for 18 h. After cooling to RT, the reaction was poured into a 10% aqueous citric acid solution (15 mL) and extracted with EtOAc (3 x 10 mL). The organic extracts were dried over $MgSO_4$ and the solvent was removed under reduced pressure. Purification by automated silica gel chromatography using a 6 → 50% EtOAc in hexanes gradient afforded **10** as purple crystalline solid (80 mg, 59% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20 – 7.03 (m, 2H), 6.86 (td, *J* = 7.4, 1.2 Hz, 1H), 6.55 (dq, *J* = 8.1, 1.9 Hz, 1H), 4.95 (s, 1H), 2.31 (s, 3H). ^{19}F NMR (400 MHz, $CDCl_3$) δ -150.6 (dd, *J* = 22.6 Hz, 6.6 Hz, 2F), -163.8 (dd, *J* = 22.6 Hz, 6.6 Hz, 2F). HRMS-ESI (*m*/*z*): $[M+H]^+$ calcd for $C_{13}H_9F_4NO$, 272.0693; found 272.0690.



11

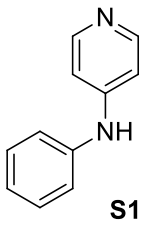
4-([1,1'-biphenyl]-4-ylamino)-2,3,5,6-tetrafluorophenol (11). Compound **11** was prepared in a manner similar to that described by Maiti and Buchwald.[1] To an oven-dried 4 mL conical vial was added 4-bromo-2,3,5,6-tetrafluorophenol (123 mg, 0.5 mmol), [1,1'-biphenyl]-4-amine (102 mg, 0.6 mmol), sodium tert-butoxide (120 mg, 1.25 mmol), and BrettPhos palladacycle (0.8 mg, 0.001 mmol). The vial was flushed with N_2, then 1 mL anhydrous 1,4-dioxane was added. The reaction was stirred at 90 C for 18 h. After cooling to RT, the reaction was poured into a 10% aqueous citric acid solution (15 mL) and extracted with EtOAc (3 x 10 mL). The organic extracts were dried over $MgSO_4$ and the solvent was removed under reduced pressure. Purification by automated silica gel chromatography using a 6 → 50% EtOAc in hexanes gradient afforded **11** as purple crystalline solid (105 mg, 63% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56 – 7.44 (m, 4H), 7.43 – 7.35 (m, 2H), 7.32 – 7.22 (m, 1H), 6.84 – 6.77 (m, 2H), 5.76 (s, 1H), 5.31 (s, 1H). ^{19}F NMR (400 MHz, $CDCl_3$) δ -149.8 (dd, *J* = 23.2 Hz, 7.6 Hz, 2F), -163.6 (dd, *J* = 23.2 Hz, 7.6 Hz, 2F). HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{16}H_9F_4NO$, 308.0693; found 308.0689.



12

2,3,5,6-tetrafluoro-4-(naphthalen-1-ylamino)phenol (12). Compound **12** was prepared in a manner similar to that described by Maiti and Buchwald.[1] To an oven-dried 4 mL conical vial was added 4-bromo-2,3,5,6-tetrafluorophenol (123 mg, 0.5 mmol), naphthalen-1-amine (86 mg, 0.6 mmol), sodium tert-butoxide (120 mg, 1.25 mmol), and BrettPhos palladacycle (0.8 mg, 0.001 mmol). The vial was flushed with N_2, then 1 mL anhydrous 1,4-dioxane was added. The reaction was stirred at 90 C for 18 h. After cooling to RT, the reaction was poured into a 10% aqueous citric acid solution (15 mL) and extracted with EtOAc (3 x 10 mL). The organic extracts were dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was dissolved in DMSO (2 mL) and purified by reverse-phase HPLC using a 5 → 95% acetonitrile in water gradient. Lyophilization afforded **12** as pale purple solid (12 mg, 8% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (m, 1H), 7.89 – 7.80 (m, 1H), 7.58 – 7.45 (m, 3H), 7.36 – 7.21 (m, 2H), 6.73 – 6.65 (m, 1H), 5.65 (s, 1H). ^{19}F NMR (400 MHz, $CDCl_3$) δ -151.0 (dd, *J* = 23.0 Hz, 5.8 Hz, 2F), -163.5 (dd, *J* = 23.0 Hz, 5.8 Hz, 2F). HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{18}H_{11}F_4NO$, 334.0850; found 334.0848.

III. SPECTRAL DATA FOR COMPOUNDS 7-12



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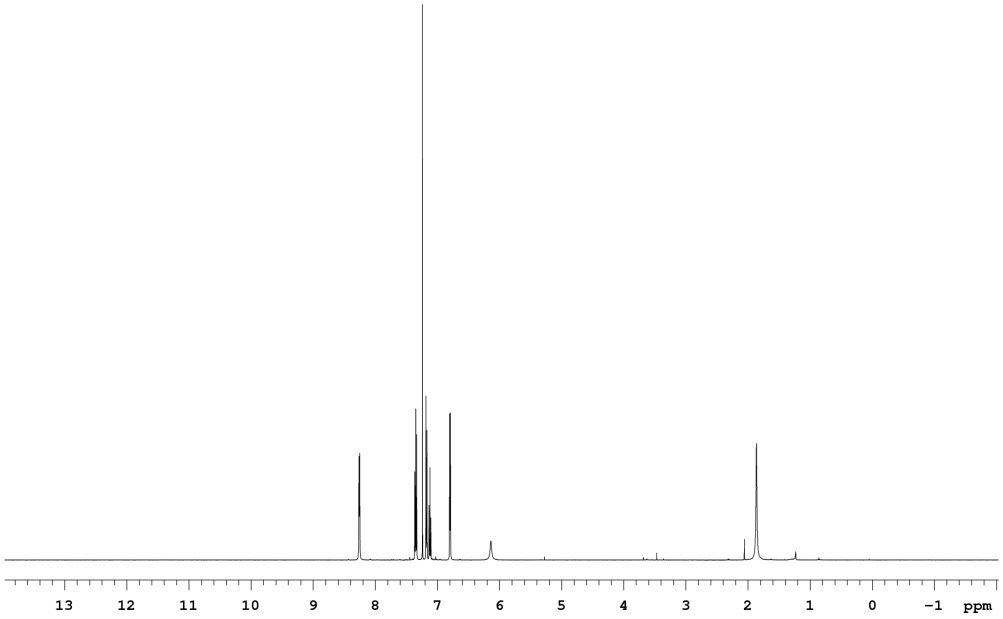

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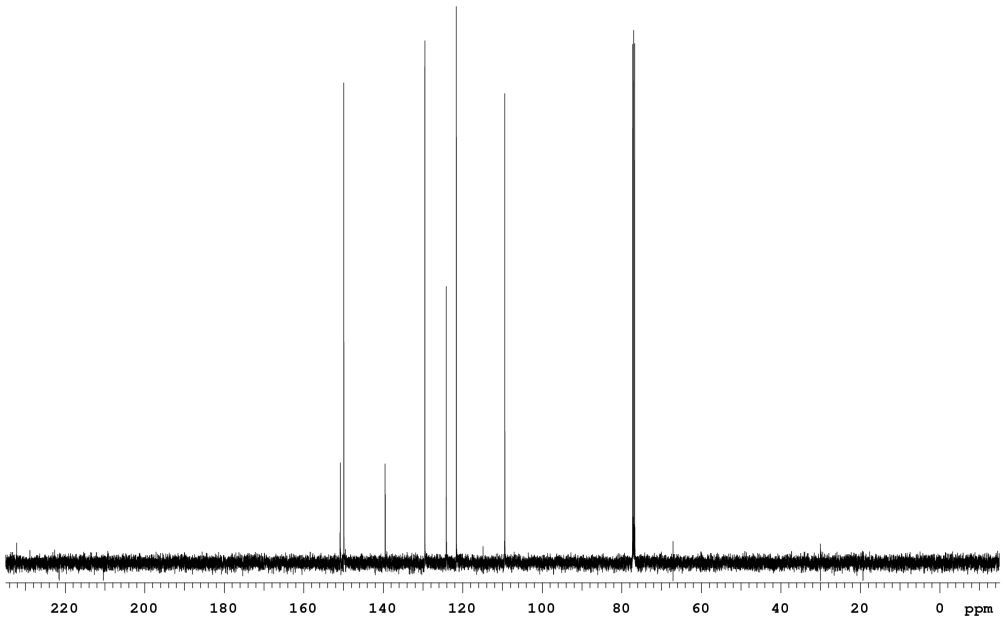

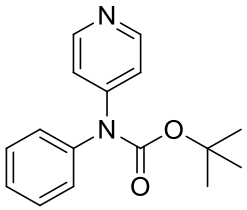

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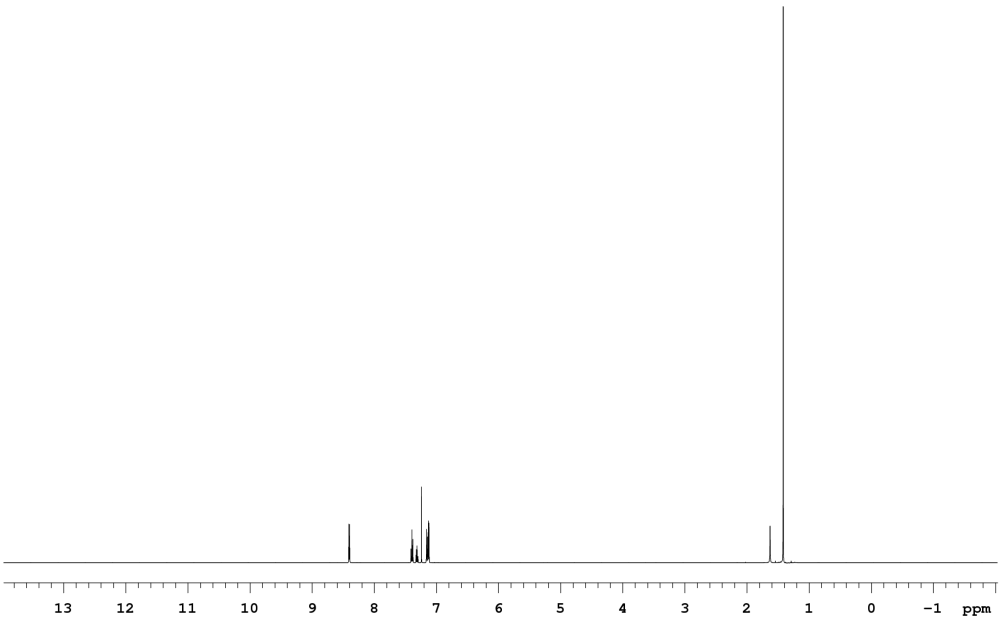
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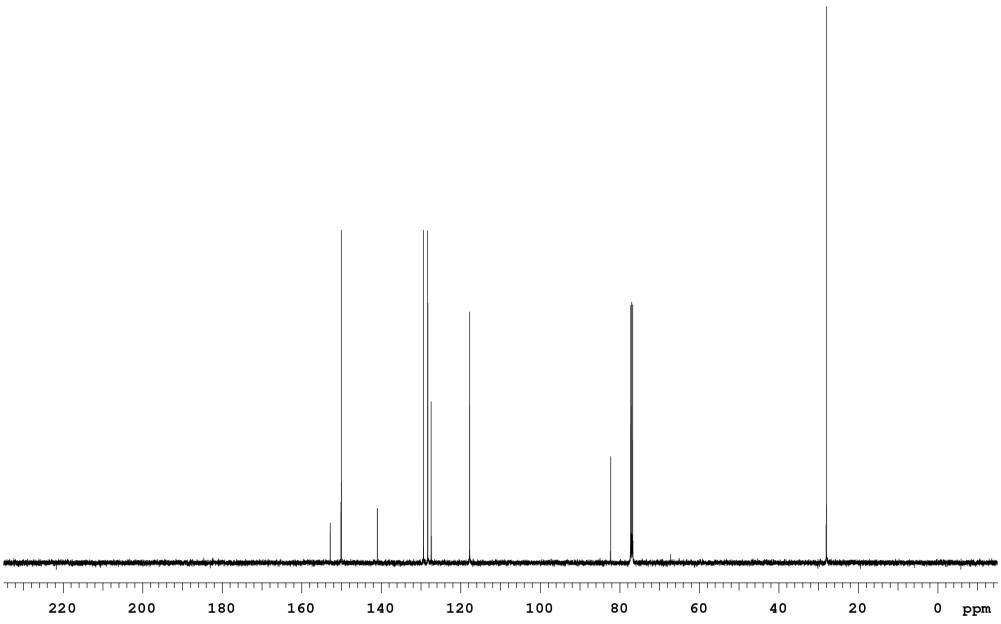
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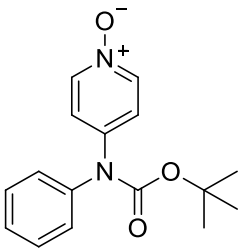

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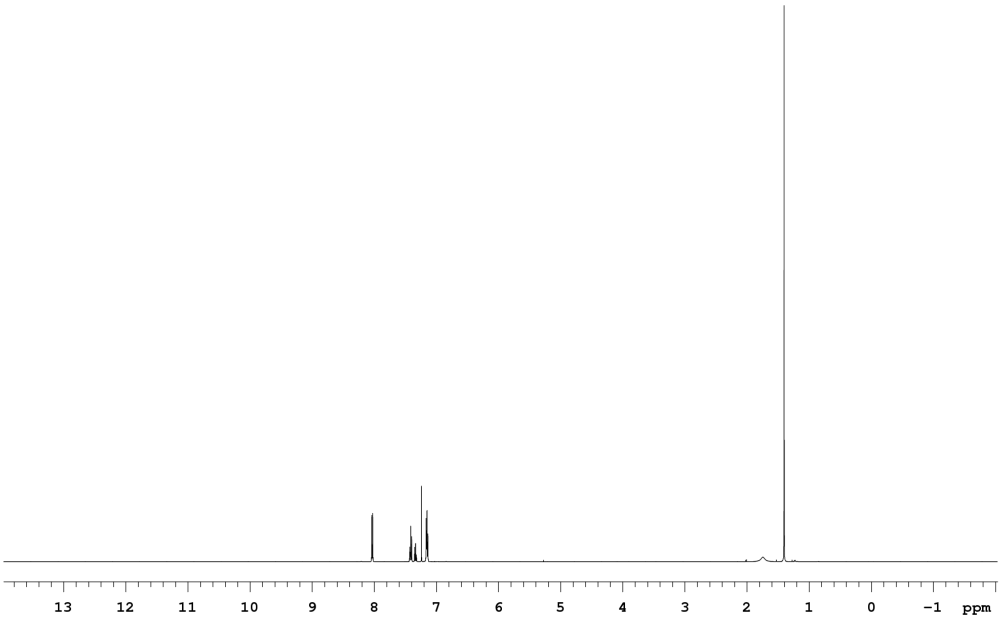
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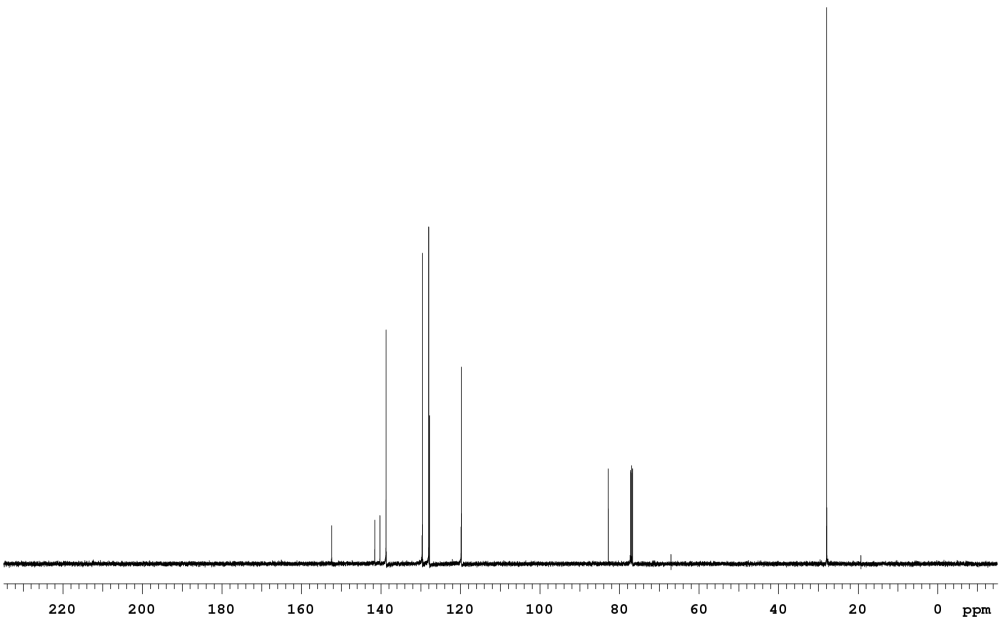
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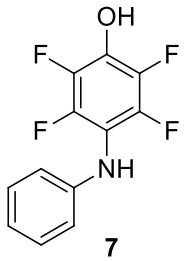

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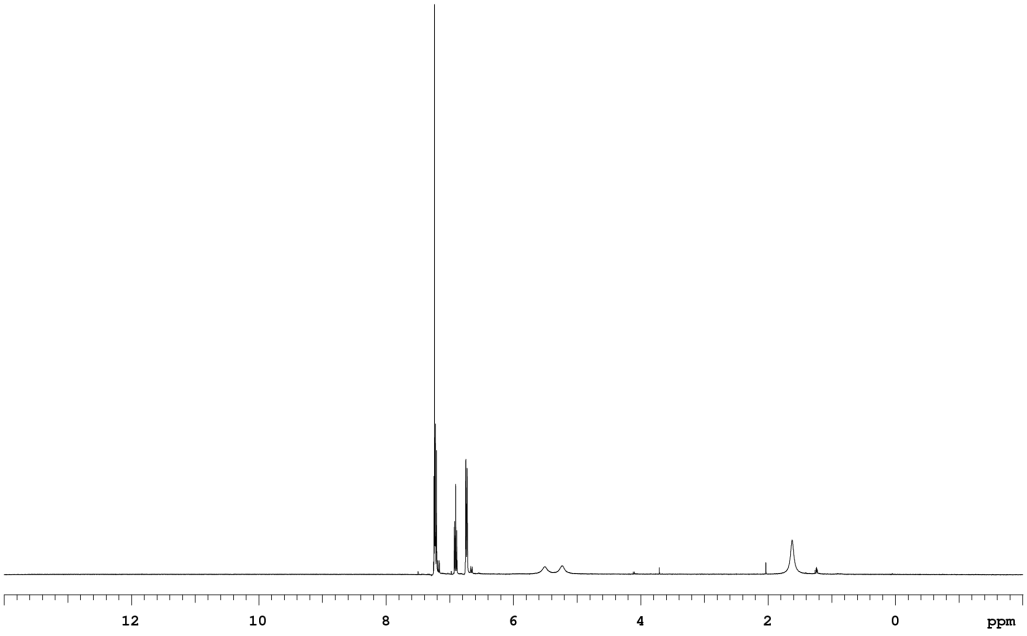

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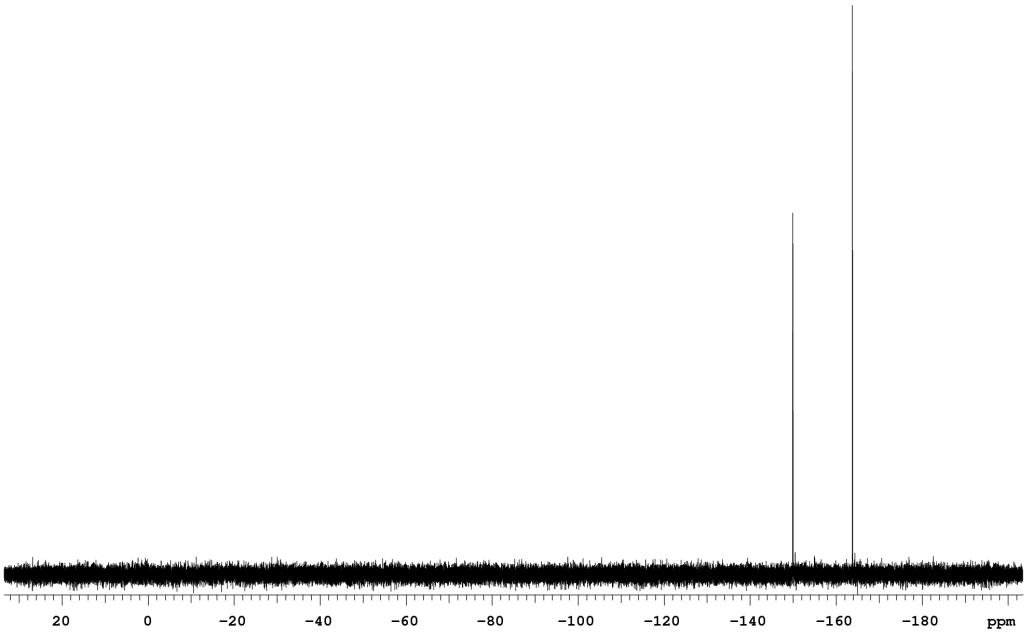

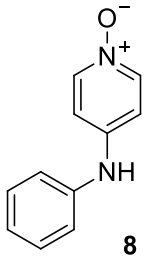
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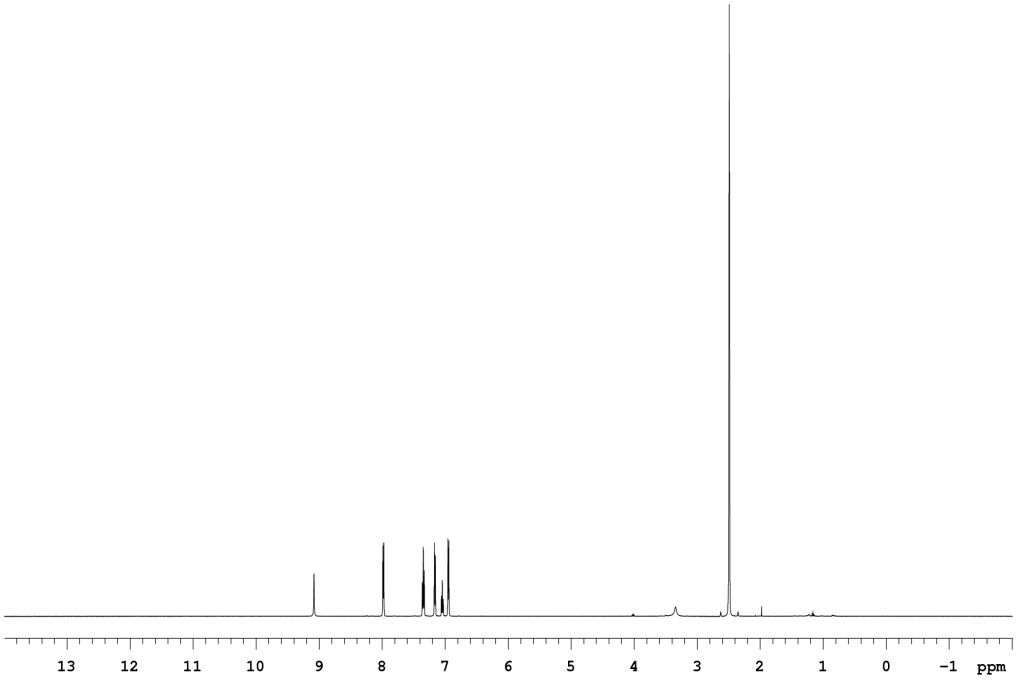
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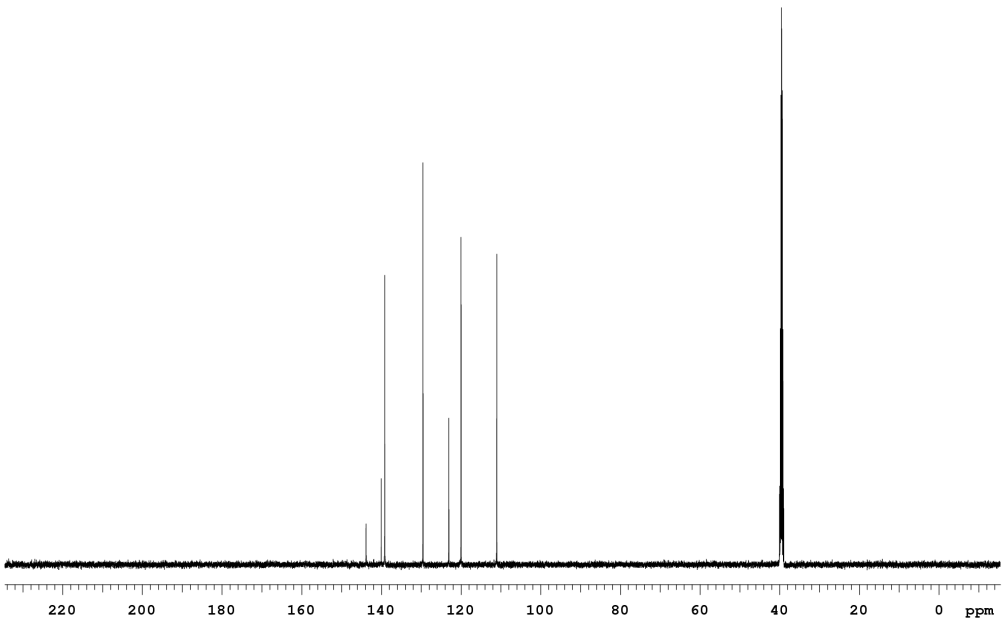
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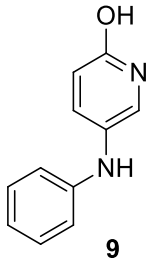
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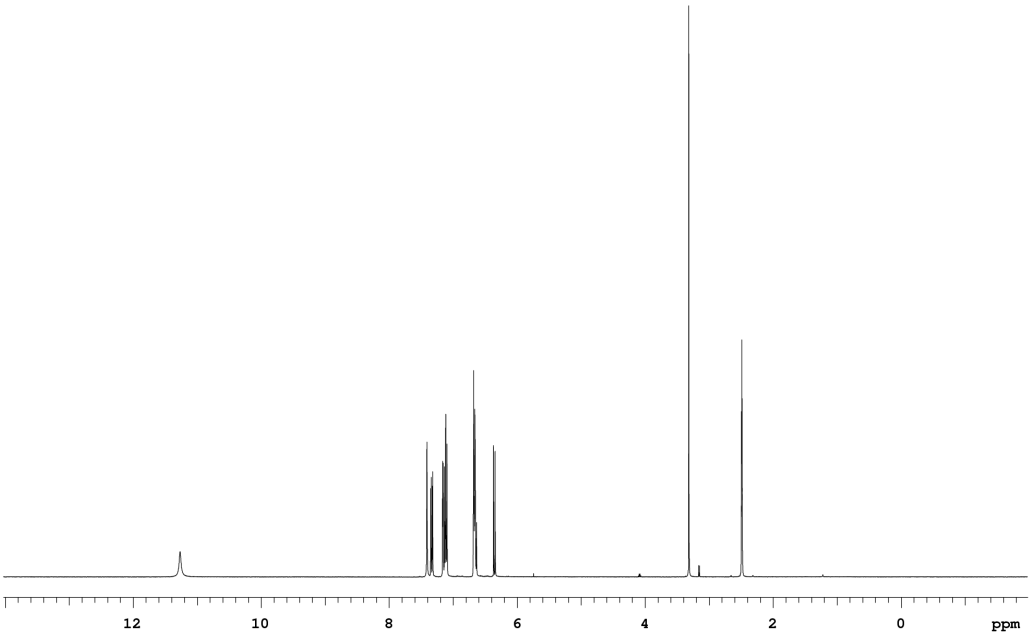
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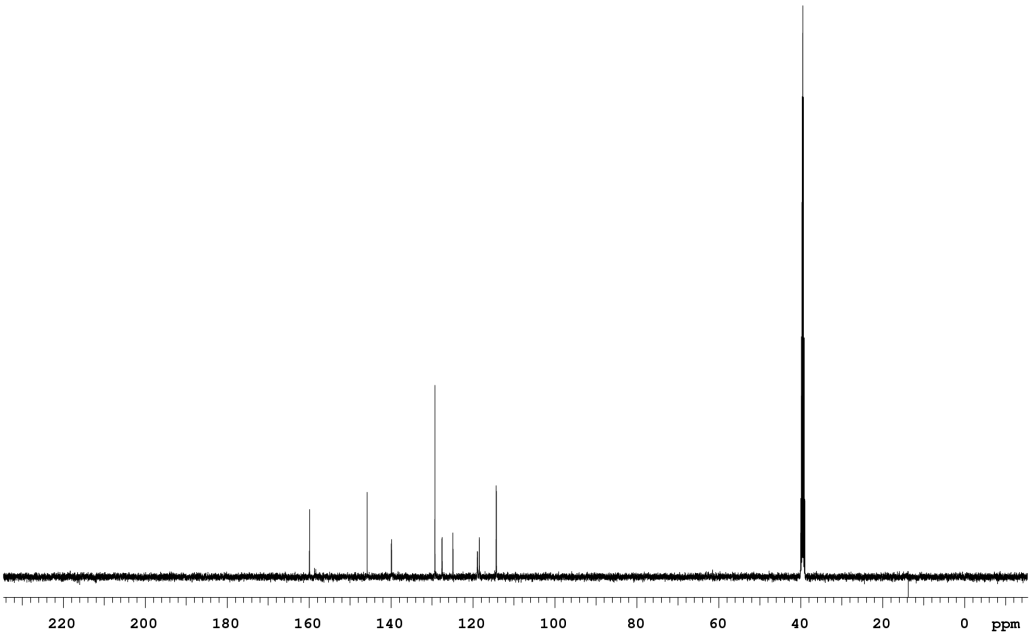
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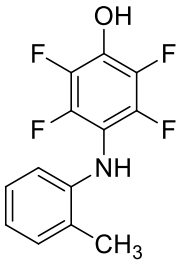
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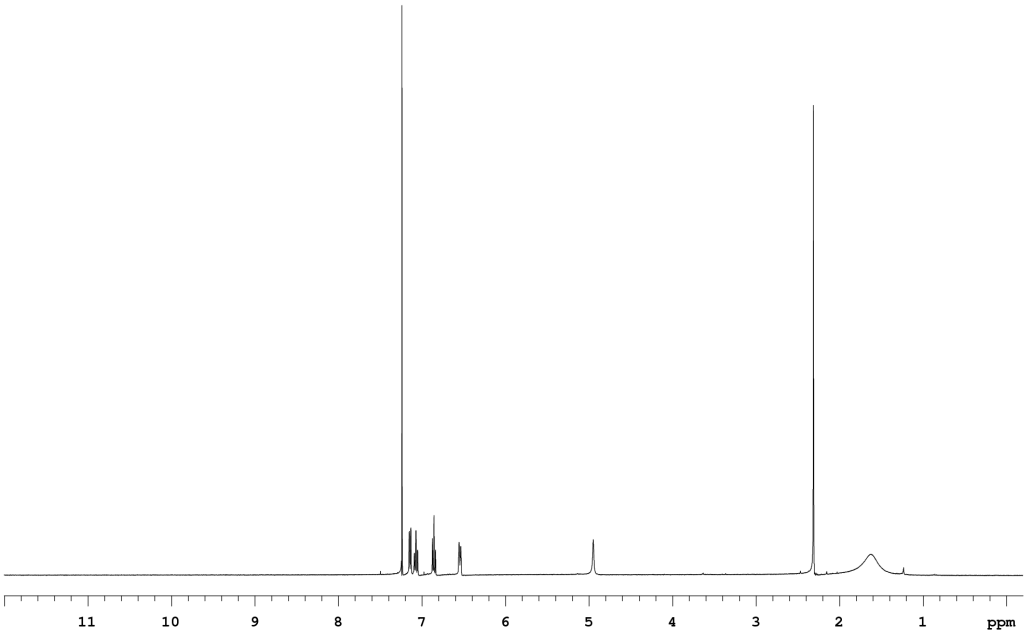
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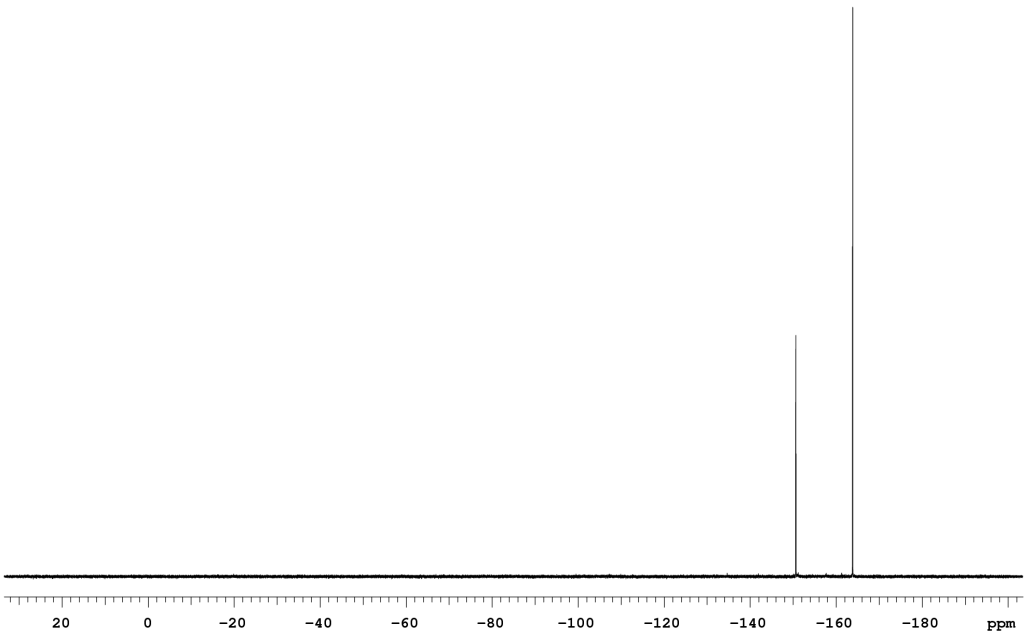
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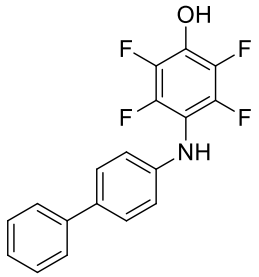
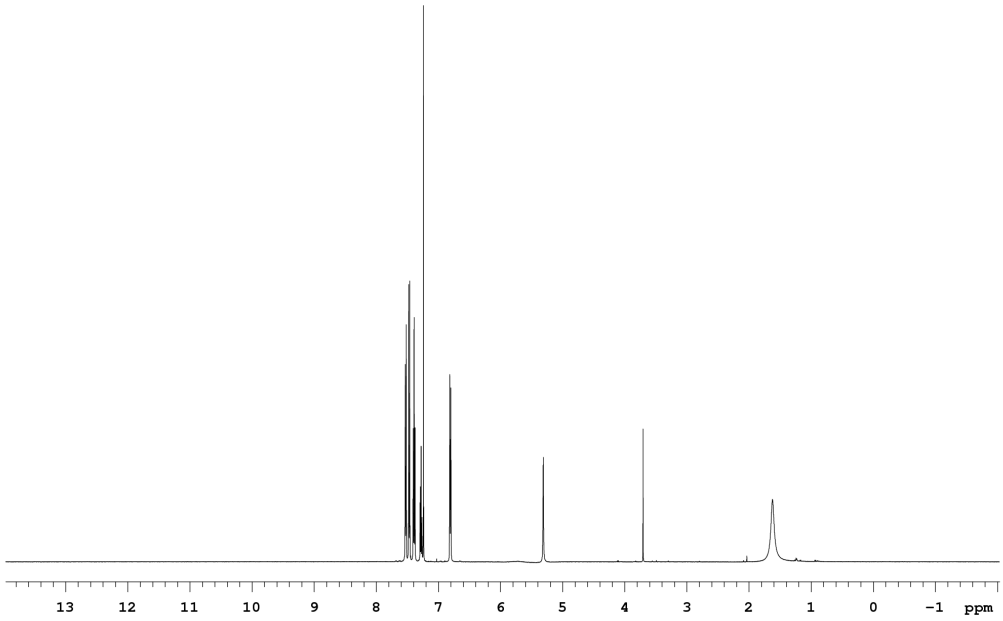
ppm

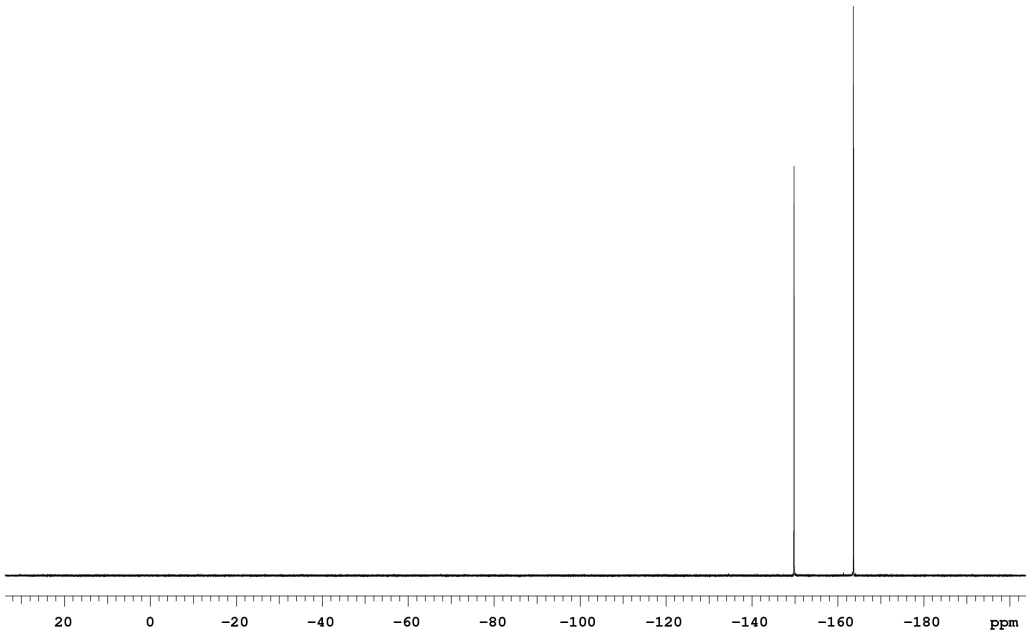

OH
F
F
F
F
NH

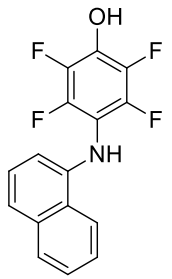
11

^{1}H:


13
12
11
10
9
8
7
6
5
4
3
2
1
0
−1
ppm

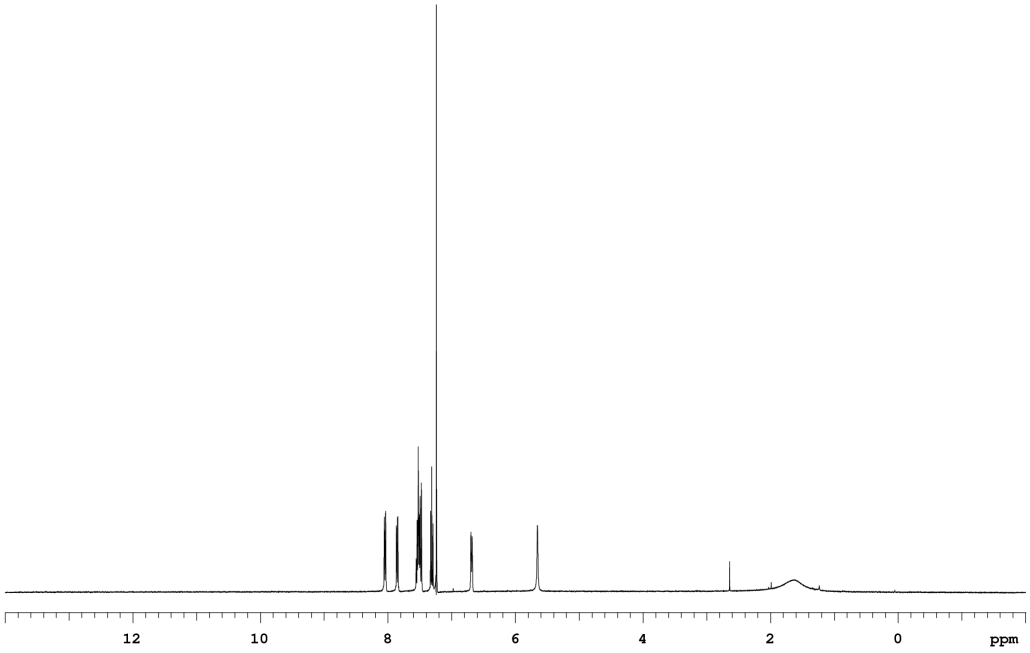
^{19}F:


20
0
−20
−40
−60
−80
−100
−120
−140
−160
−180
ppm

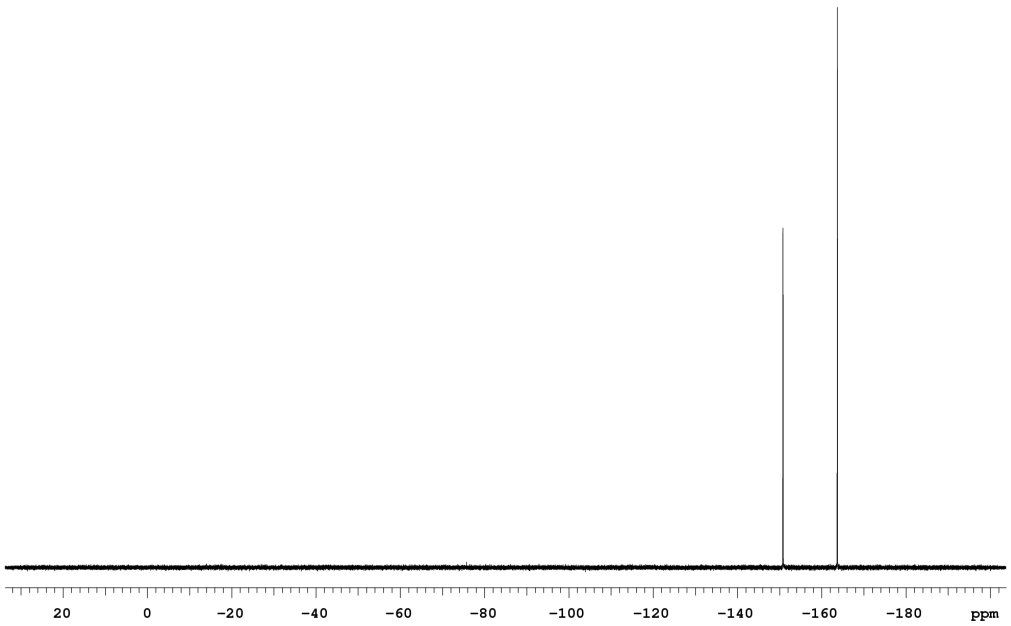


12

^{1}H:

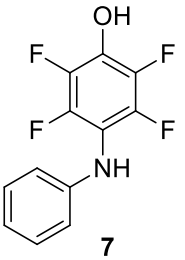


^{19}F:

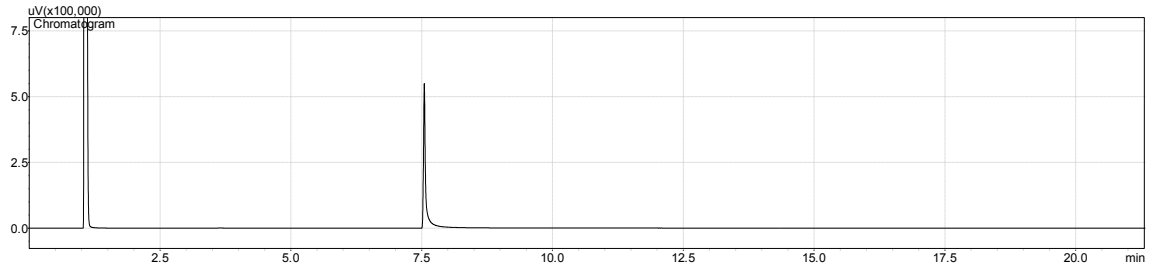


IV. GAS CHROMATOGRAPHY TRACES FOR COMPOUNDS 7-12

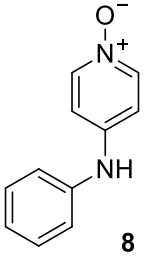
General procedure. Gas chromatography was carried out using a Shimadzu GC 2010 containing a Shimadzu SHRX5 (crossbound 5% diphenyl–95% dimethyl polysiloxane; 15 m, 0.25 mm ID, 0.25µm df) column.



7



Peak	Retention Time	Area	% Area
1	3.636	513.3	0.3
2	7.550	1672609.0	99.7



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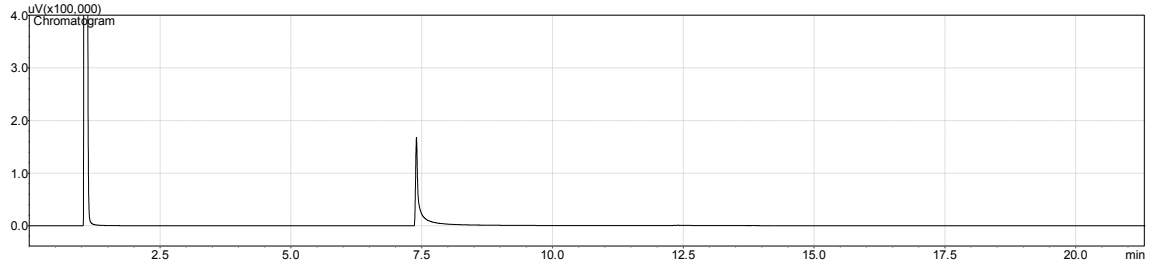
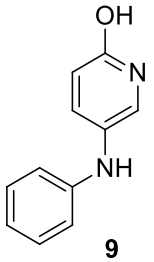


Peak	Retention Time	Area	% Area
1	7.399	835686.0	99.6
2	12.400	3050.1	0.4



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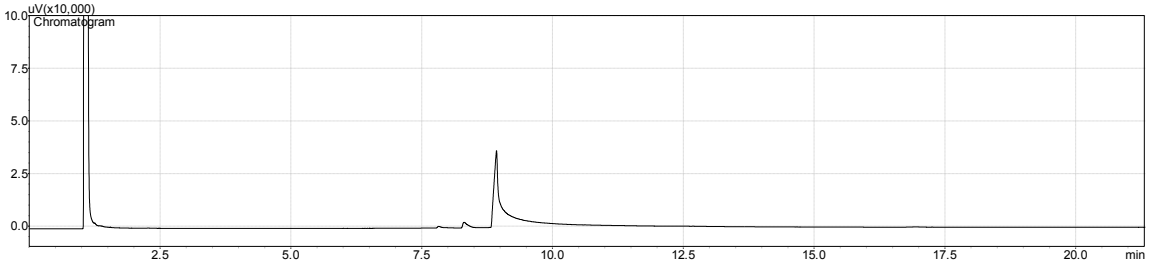
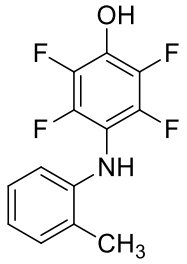


Peak	Retention Time	Area	% Area
1	7.826	3767.6	0.8
2	8.311	18333.3	4.0
3	8.929	440013.5	95.2



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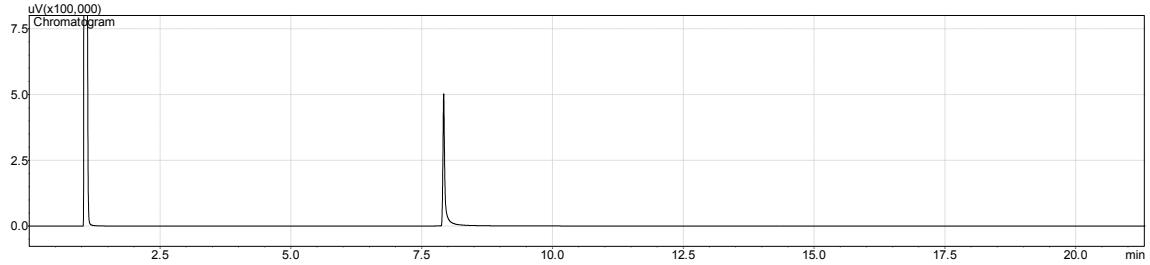
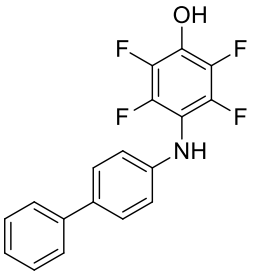


Peak	Retention Time	Area	% Area
1	7.920	1454029.6	100.0



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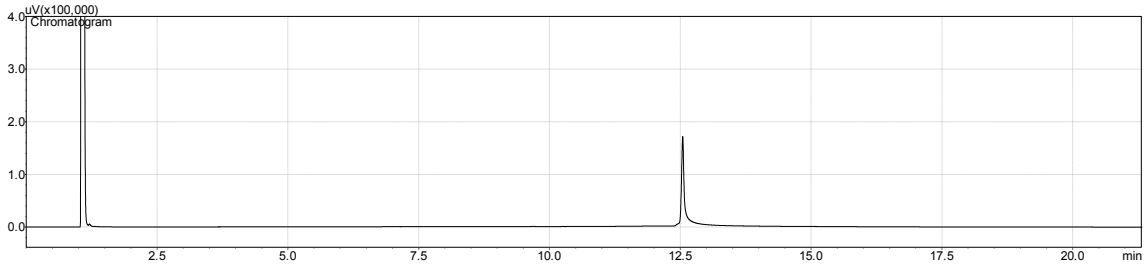
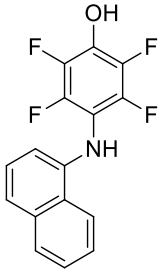
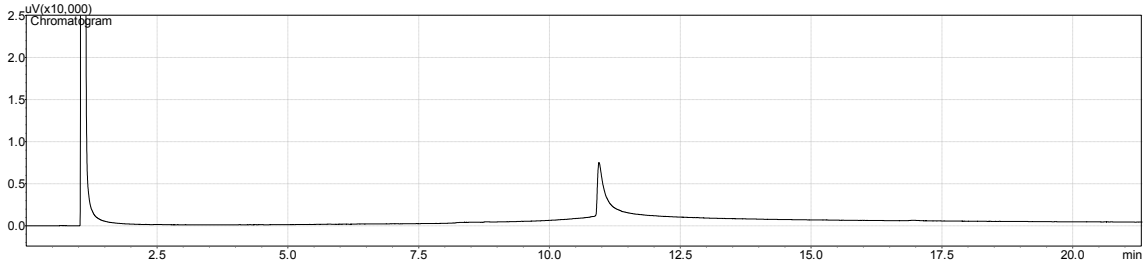


Peak	Retention Time	Area	% Area
1	6.927	1071.8	0.1
2	9.672	1024.6	0.1
3	12.546	789498.5	99.7



12



Peak	Retention Time	Area	% Area
1	10.945	71352.0	100.0

V. BIOCHEMICAL SUBSTRATE IDENTIFICATION ASSAYS

Single concentration screening of phenolic compounds as substrates for c-Src. Substrate identification assays were performed using an ADP-Glo™ assay kit (Promega). Compounds were solubilized in 100% DMSO to 0.2M then subsequently diluted into ADP-Glo Kinase buffer (40 mM Tris pH 7.5, 20 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin) to a working stock concentration of 167 µM (1.7% DMSO). 3 µl of the compound solution was added to the wells of a 384-well PerkinElmer Optiplate followed by the addition of 2 µl of kinase/ATP mix (250 nM c-Src, 250 µM ATP in ADP-Glo Kinase buffer). The plate was then sealed and pulse-spun in a table top centrifuge for 10 seconds to thoroughly mix the well contents. The final concentrations in the well were: 100 µM compound (1.0% DMSO), 100 nM c-Src, and 100 µM ATP. The peptide substrate (Ac-AIYAA-NH_2) was used as a control substrate to verify kinase activity.[2] Blank wells containing the reaction mixture without substrate were included as well. The kinase reaction was allowed to proceed at room temperature for 30 minutes after which the reaction was stopped by the addition of 5 µl/well of ADP-Glo Reagent (Promega). The contents were mixed by centrifugation as above and an additional room temperature 40 minute incubation was followed per the Promega protocol. 10 µl/well of Kinase Detection Reagent (Promega) was added to each well, the plate was spun as described to mix the contents, and an additional 30 minute room temperature incubation was performed per the Promega protocol. Luminescence was read in a Biotek Synergy 4 multimode plate reader and the % ADP formed in the well was calculated against a standard curve using a ratio of ATP/ADP as described in the Promega protocol under the same incubation conditions as the kinase reaction.

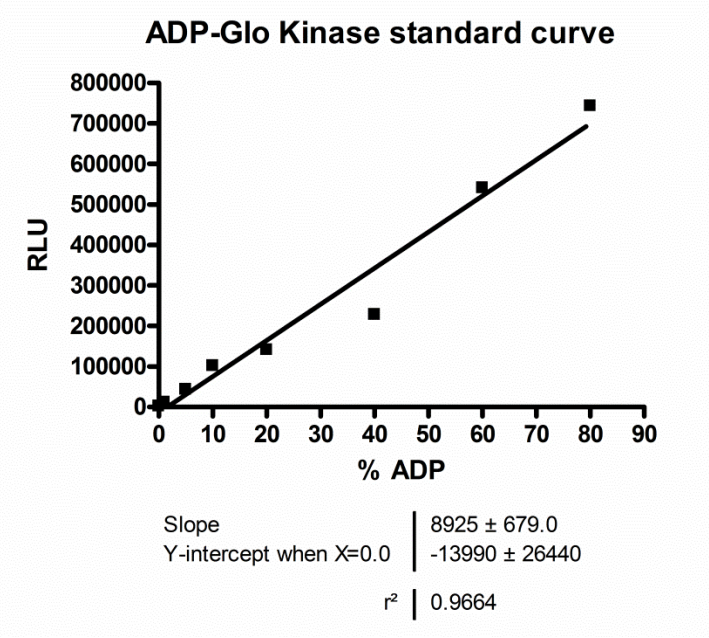
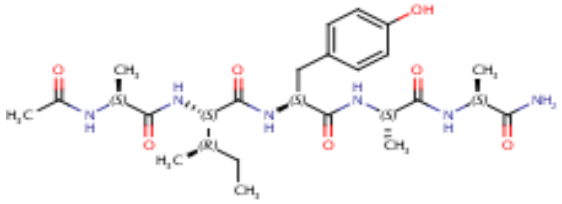
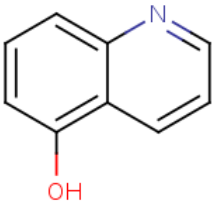
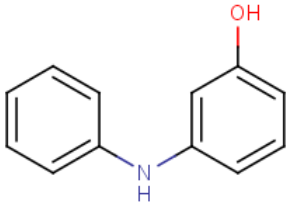
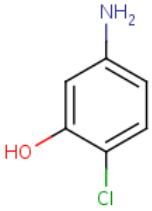
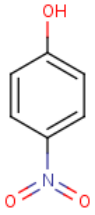
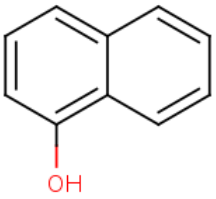
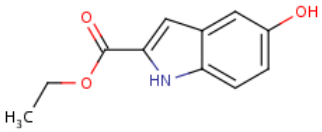
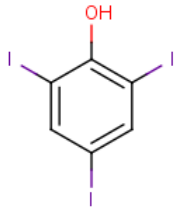
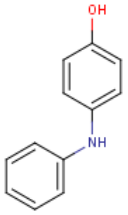
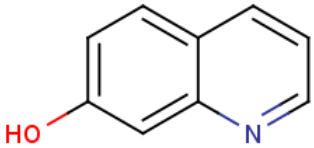
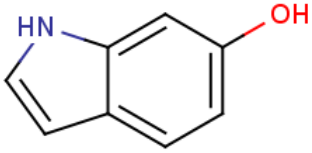
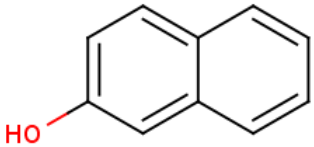
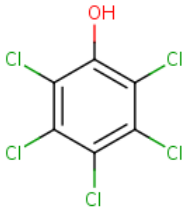
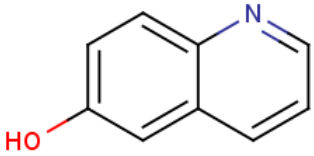
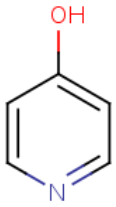
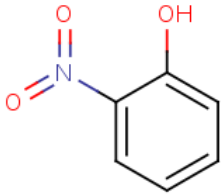
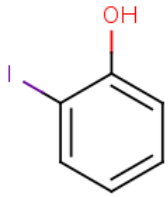
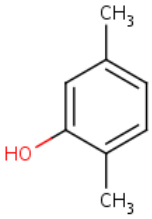
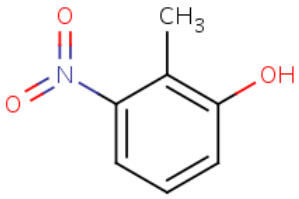
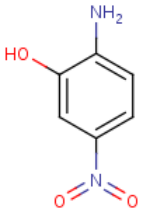
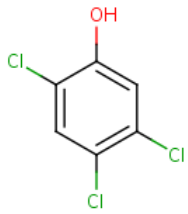
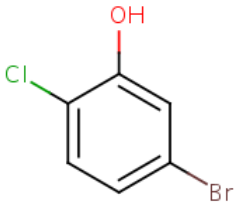
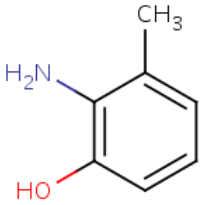
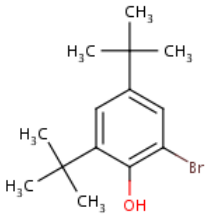
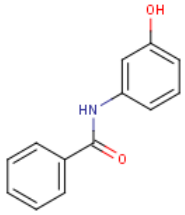
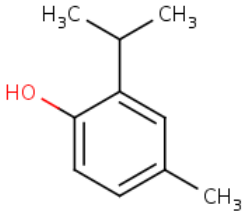
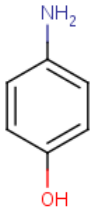


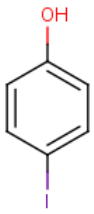
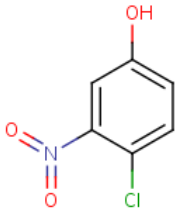
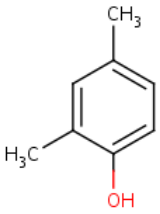
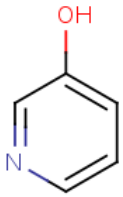
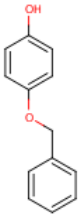
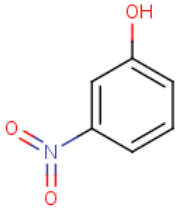
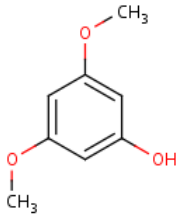
Table S1. Average percent ADP generated by c-Src after 30 minutes in the presence of small molecule phenols.

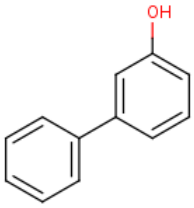
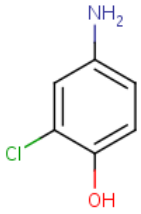
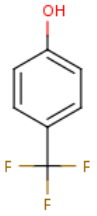
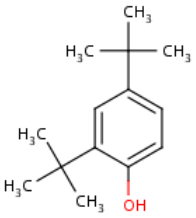
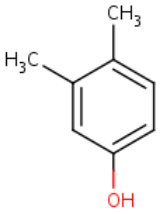
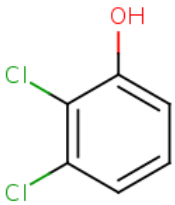
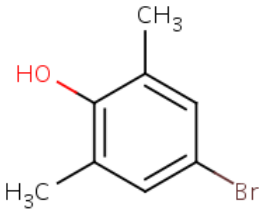
Name	Structure	% ADP (100 µM)
Positive Control		12.8 ± 5.2 (n=16)
P-S1 (**1**)		7.6 ± 4.1 (n=3)
P-S2 (**2**)		5.0 ± 3.4 (n=3)
P-S3 (**3**)		4.6 ± 4.8 (n=4)
P-S4		4.4 ± 6.2 (n=2)
P-S5 (**4**)		4.3 ± 2.5 (n=3)
P-S6		3.6 ± 5.6 (n=4)

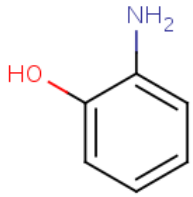
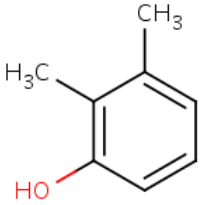
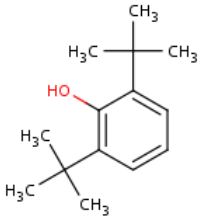
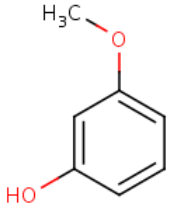
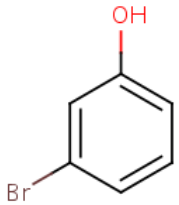
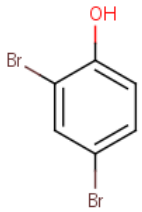
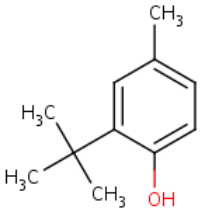
P-S7		2.8 ± 1.6 (n=2)
P-S8 (**5**)		2.6 ± 4.8 (n=3)
P-S9 (**6**)		2.5 ± 5.5 (n=4)
P-S10		1.4 ± 2.6 (n=2)
P-S11		1.3 ± 1.7 (n=2)
P-S12		1.0 ± 4.1 (n=3)
P-S13		1.0 ± 0.2 (n=2)

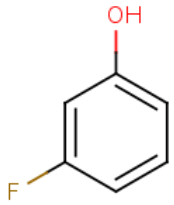
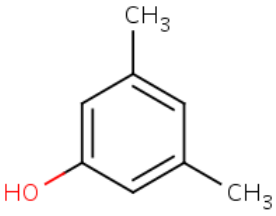
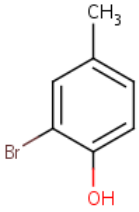
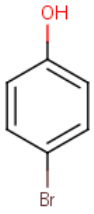
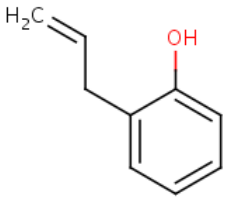
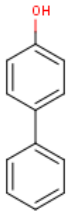
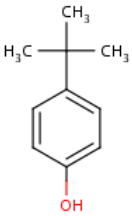
P-S14		0.8 ± 2.3 (n=2)
P-S15		0.4 ± 1.5 (n=2)
P-S16		0.1 ± 0.7 (n=2)
P-S17		0.0 ± 0.4 (n=2)
P-S18		-0.2 ± 0.2 (n=2)
P-S19		-0.2 ± 0.6 (n=2)
P-S20		-0.2 ± 0.6 (n=2)

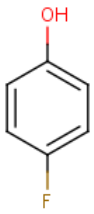
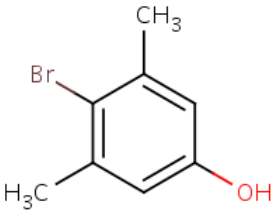
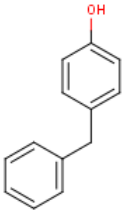
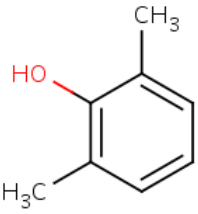
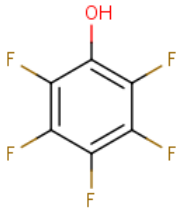
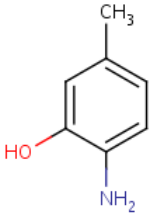
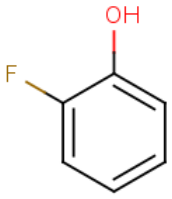
P-S21		-0.2 ± 0.2 (n=2)
P-S22		-0.3 ± 0.3 (n=2)
P-S23		-0.4 ± 1.5 (n=2)
P-S24		-0.4 ± 1.0 (n=2)
P-S25		-0.5 ± 1.0 (n=2)
P-S26		-0.6 ± 0.7 (n=2)
P-S27		-0.6 ± 0.2 (n=2)

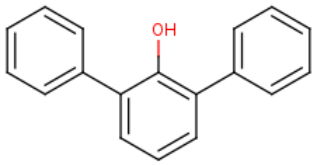
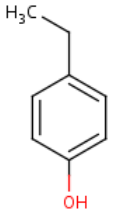
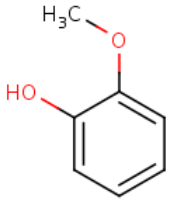
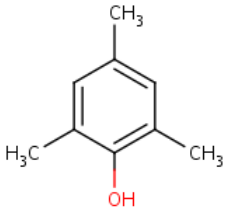
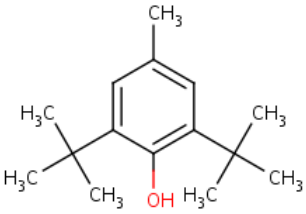
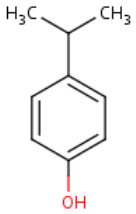
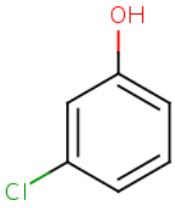
P-S28		-0.7 ± 0.2 (n=2)
P-S29		-0.8 ± 1.1 (n=2)
P-S30		-0.9 ± 0.4 (n=2)
P-S31		-0.9 ± 0.1 (n=2)
P-S32		-0.9 ± 0.5 (n=2)
P-S33		-1.0 ± 0.4 (n=2)
P-S34		-1.0 ± 1.6 (n=2)

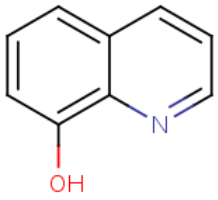
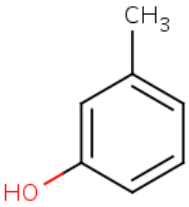
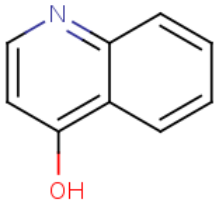
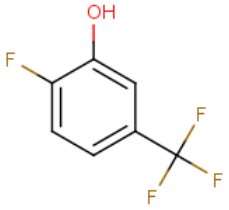
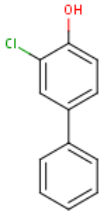
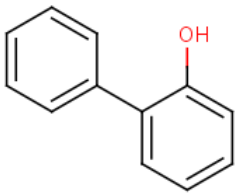
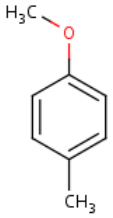
P-S35		-1.0 ± 0.3 (n=2)
P-S36		-1.0 ± 1.0 (n=2)
P-S37		-1.1 ± 0.3 (n=2)
P-S38		-1.1 ± 0.5 (n=2)
P-S39		-1.2 ± 1.3 (n=2)
P-S40		-1.2 ± 1.3 (n=2)
P-S41		-1.2 ± 0.5 (n=2)

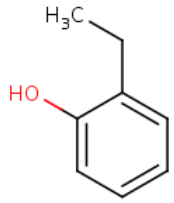
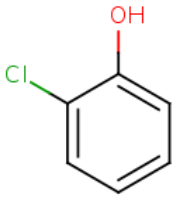
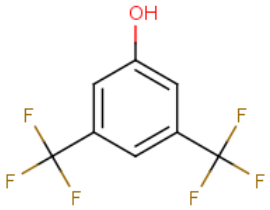
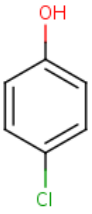
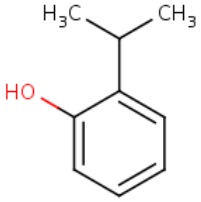
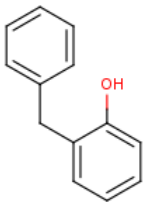
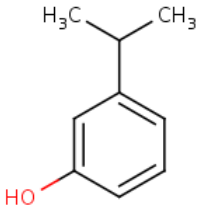
P-S42		-1.3 ± 0.2 (n=2)
P-S43		-1.3 ± 1.3 (n=2)
P-S44		-1.3 ± 0.7 (n=2)
P-S45		-1.3 ± 1.5 (n=2)
P-S46		-1.4 ± 0.3 (n=2)
P-S47		-1.4 ± 0.0 (n=2)
P-S48		-1.4 ± 0.5 (n=2)

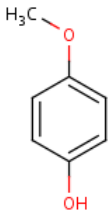
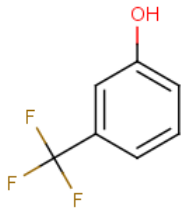
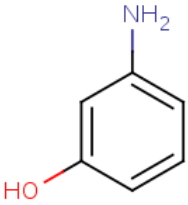
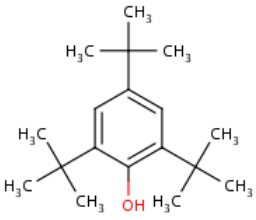
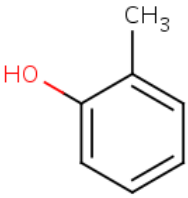
P-S49		-1.4 ± 0.9 (n=2)
P-S50		-1.5 ± 0.2 (n=2)
P-S51		-1.6 ± 0.3 (n=4)
P-S52		-1.6 ± 1.1 (n=2)
P-S53		-1.7 ± 0.7 (n=2)
P-S54		-1.9 ± 0.2 (n=2)
P-S55		-1.9 ± 0.5 (n=2)

P-S56		-1.9 ± 0.3 (n=2)
P-S57		-1.9 ± 1.3 (n=2)
P-S58		-2.0 ± 1.0 (n=2)
P-S59		-2.0 ± 1.2 (n=2)
P-S60		-2.1 ± 0.8 (n=2)
P-S61		-2.2 ± 0.2 (n=2)
P-S62		-2.2 ± 0.4 (n=2)

P-S63		-2.3 ± 0.3 (n=2)
P-S64		-2.3 ± 0.8 (n=2)
P-S65		-2.4 ± 0.2 (n=2)
P-S66		-2.5 ± 0.3 (n=2)
P-S67		-2.5 ± 0.4 (n=2)
P-S68		-2.6 ± 0.0 (n=2)
P-S69		-2.7 ± 0.7 (n=2)

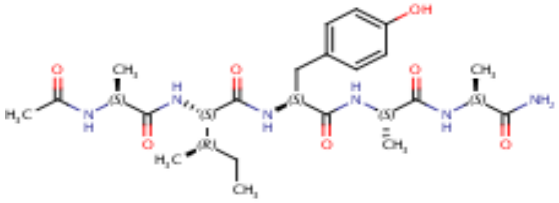
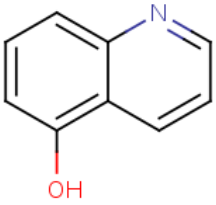
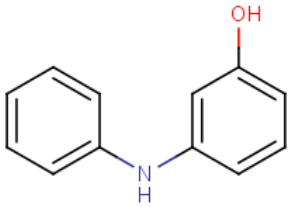
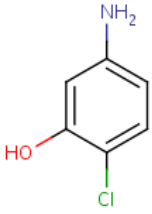
P-S70		-2.6 ± 0.6 (n=2)
P-S71		-2.7 ± 0.2 (n=2)
P-S72		-2.7 ± 0.0 (n=2)
P-S73		-2.7 ± 0.7 (n=2)
P-S74		-2.8 ± 0.6 (n=2)
P-S75		-2.8 ± 0.1 (n=2)
P-S76		-2.8 ± 1.3 (n=2)

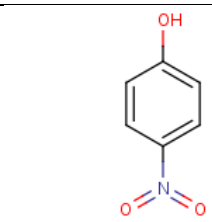
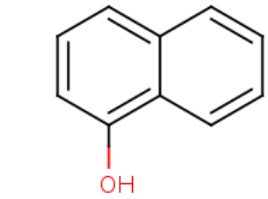
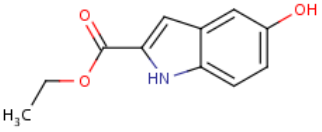
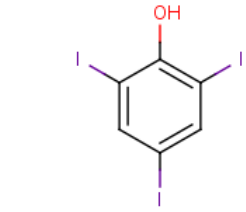
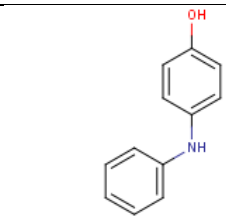
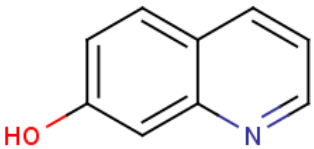
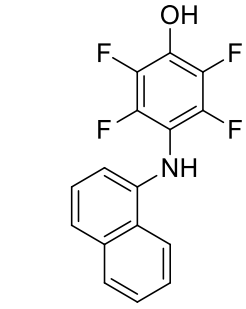
P-S77		-2.8 ± 0.5 (n=2)
P-S78		-2.8 ± 0.6 (n=2)
P-S79		-2.9 ± 0.7 (n=2)
P-S80		-2.9 ± 1.8 (n=2)
P-S81		-2.9 ± 0.6 (n=2)
P-S82		-3.0 ± 1.5 (n=2)
P-S83		-3.0 ± 0.6 (n=2)

P-S84		-3.1 ± 0.3 (n=2)
P-S85		-3.1 ± 0.3 (n=2)
P-S86		-3.1 ± 0.2 (n=2)
P-S87		-3.3 ± 0.9 (n=2)
P-S88		-3.3 ± 0.8 (n=2)

***K_M* determination for phenolic c-Src substrates.** The K_M values for the phenolic substrates were determined using an ADP-Glo™ assay kit (Promega). The compounds were supplied as 100% DMSO solutions at 200 mM and were diluted to 2.5 mM into ADP-Glo buffer (40 mM Tris, pH 7.5, 20 mM $MgCl_2$, 0.1 mg/ml BSA, 0.1 mM Na_3VO_4, 0.01% Triton X-100). 1:1 dilutions were done in ADP-Glo buffer containing 1.25% DMSO and 2 µl of the solutions (in triplicate) were then added to wells of a 384-well PerkinElmer Optiplate. The kinase and ATP solution was prepared in ADP-Glo buffer to 2.5X the final concentrations desired in the assay and 3 µl was added to the wells containing the compound. The final concentrations used in the assay were: ATP = 100 µM, Src and Hck = 30 nM, Abl = 150 nM. The solutions were mixed in the plate by a brief centrifugation and allowed to incubate for 30 minutes at room temperature. 5 µl of ADP-Glo Reagent was added to each well and mixed by a brief centrifugation to terminate the kinase reaction. The plate was then incubated an additional 40 minutes at room temperature. The luciferase signal was generated by adding 10 µl per well of Kinase Detection Reagent and mixing by a brief centrifugation and incubation for 30 minutes at room temperature. Luminescence was measured in a Biotek synergy 4 multimode plate reader. ATP conversion was determined against a standard curve of ATP/ADP using the ADP-Glo workup conditions listed above.

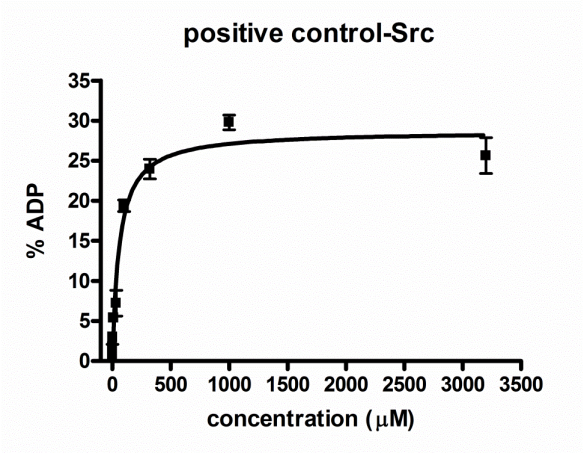
Table S2. K_M values for small molecule phenols with c-Src, Hck, and c-Abl.

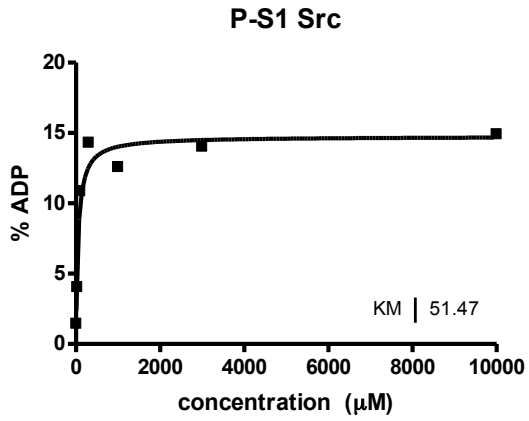
Name	Structure	K_M c-Src	V_{max} c-Src (%ADP)	K_M Hck	K_M c-Abl
Positive Control		60 µM	29	ND[a]	ND[a]
P-S1 (**1**)		52 µM	15	124 µM	>1 mM
P-S2 (**2**)		370 µM	6.5	316 µM	>1 mM
P-S3 (**3**)		15 µM	10	ND[a]	ND[a]

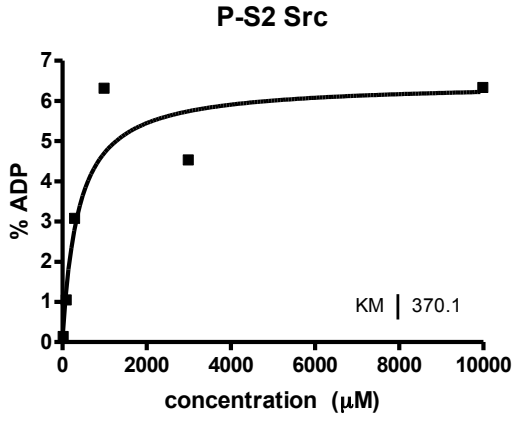
P-S4		56 µM	3.2	>1 mM	>1 mM
P-S5 (**4**)		522 µM	19	169 µM	>1 mM
P-S6		>10 mM	ND	ND[a]	ND[a]
P-S7		32 µM	2.5	>1 mM	>1 mM
P-S8 (**5**)		120 µM	7.3	137 µM	>1 mM
P-S9 (**6**)		33 µM	14	ND[a]	ND[a]
12		>1 mM	0	ND[a]	ND[a]

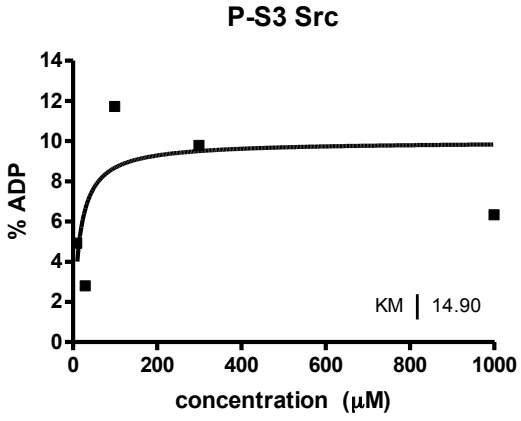
[a] K_M value was not determined.

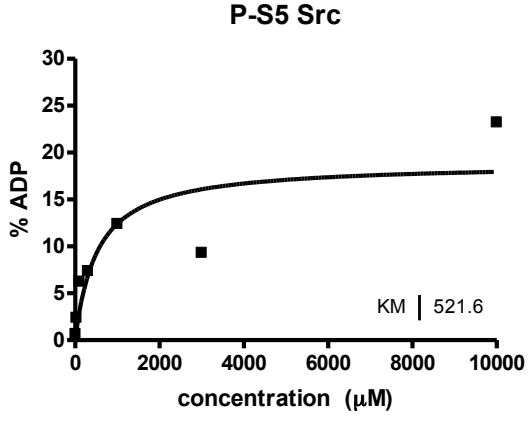
Analytical data for K_M determinations. The K_M curve for substrates **P-S1** – **P-S9** with c-Src kinase domain is shown below. Inhibitor **12** was also evaluated as a c-Src substrate and the data is shown below. K_M curves for **P-S1**, **P-S2**, **P-S5**, and **P-S8** with Hck kinase domain are also shown. Each data point was determined in triplicate and is shown as the mean ± standard error. Curve fitting was done using Graphpad Prism 4 software using nonlinear curve fitting parameters.

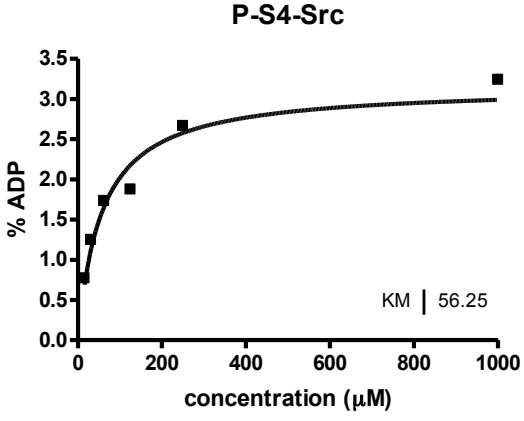


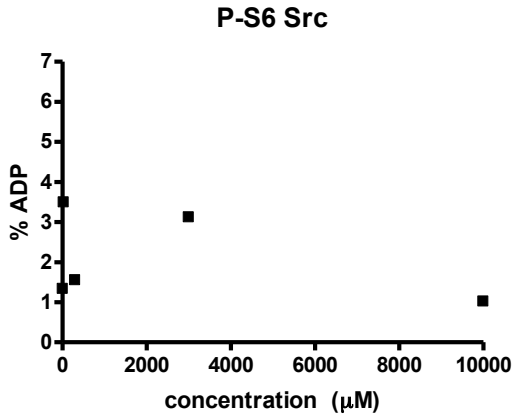


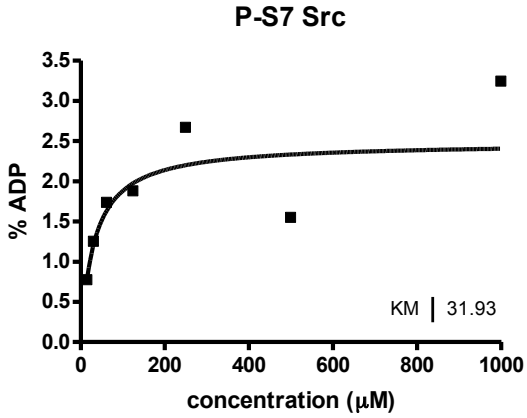


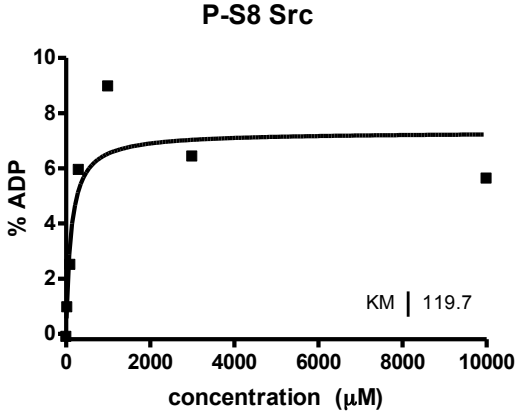


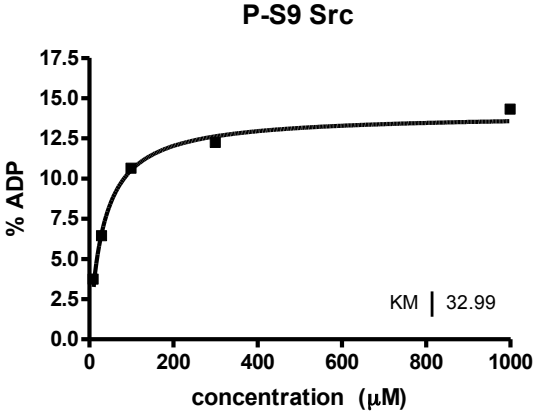


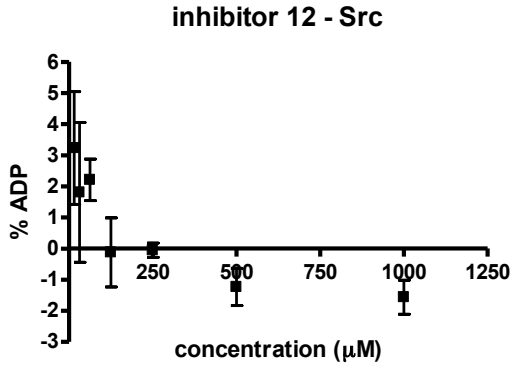


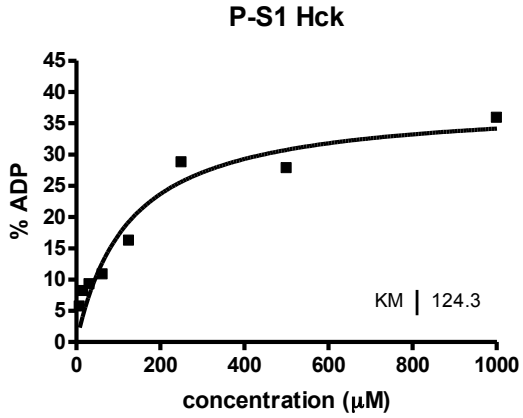

P-S6 Src
% ADP
concentration (μM)

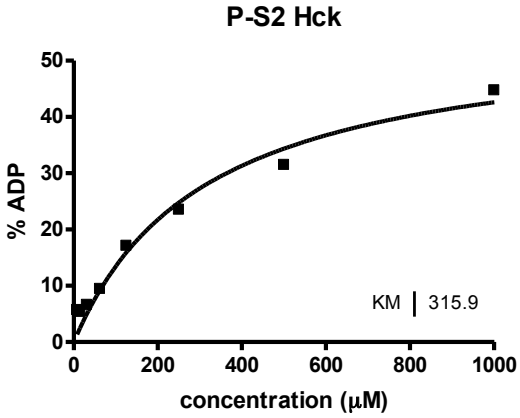

P-S7 Src
% ADP
KM | 31.93
concentration (μM)

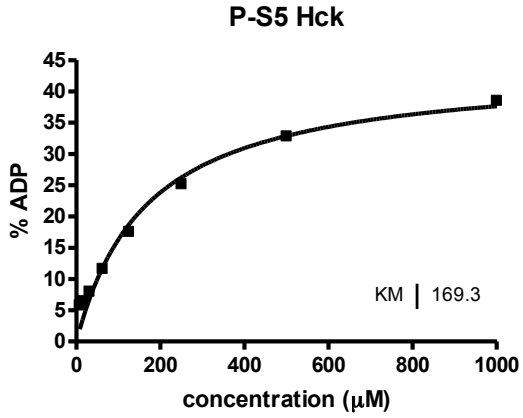

P-S8 Src
% ADP
KM | 119.7
concentration (μM)

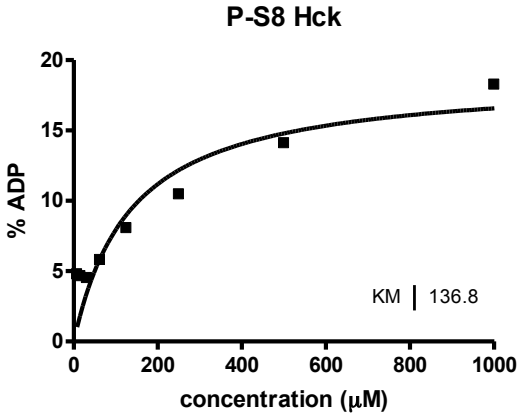

P-S9 Src
% ADP
KM | 32.99
concentration (μM)


inhibitor 12 - Src
% ADP
concentration (μM)

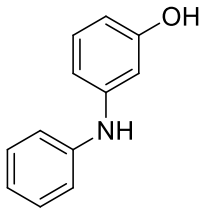

P-S1 Hck
% ADP
concentration (μM)
KM | 124.3


P-S2 Hck
% ADP
concentration (μM)
KM | 315.9

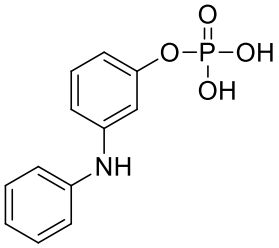

P-S5 Hck
% ADP
concentration (μM)
KM | 169.3


P-S8 Hck
% ADP
concentration (μM)
KM | 136.8

Analytical data for confirmation of c-Src mediated phosphorylation. The phosphorylation of phenol **P-S2** by c-Src was monitored by analytical reverse phase HPLC (Zorbax Eclipse Plus C18 4.6 x 75 mm, 3.5 μm column) using an acetonitrile in water (+0.1% TFA) gradient (5% ACN for 3 min, then 5→70% ACN over 10 min). The enzymatic reaction had a final concentration 200 μM **P-S2**, 200 μM ATP, 6 μM c-Src, 100 mM Tris buffer (pH 8), and 10 mM $MgCl_2$. The phosphate **P-S2-phos** was prepared as described by Soellner *et al* and used as a standard.[3]

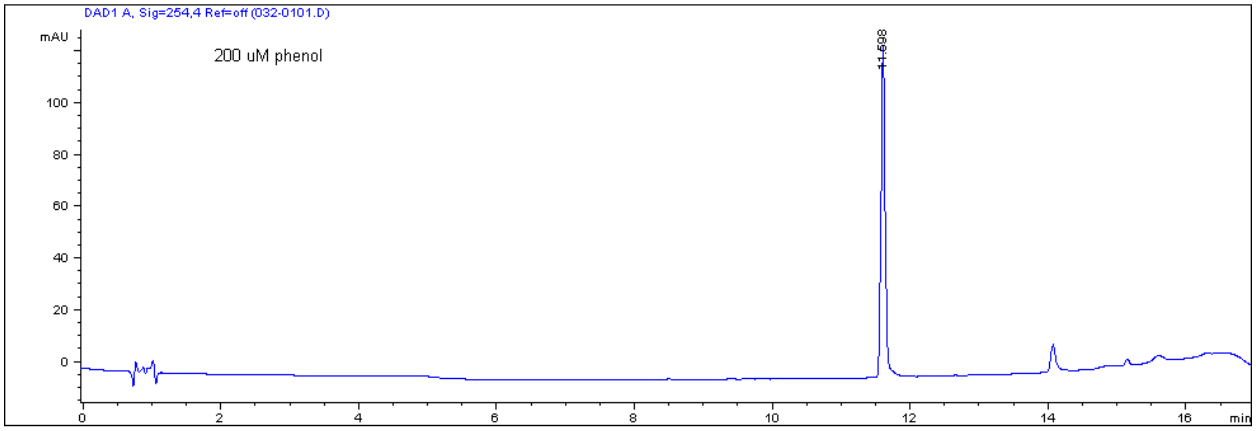


P-S2

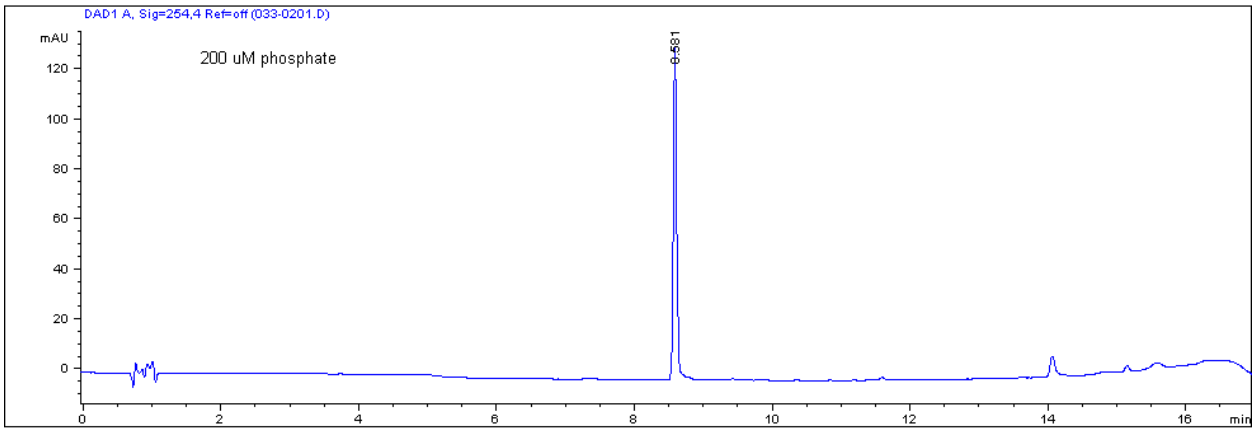


P-S2-phos

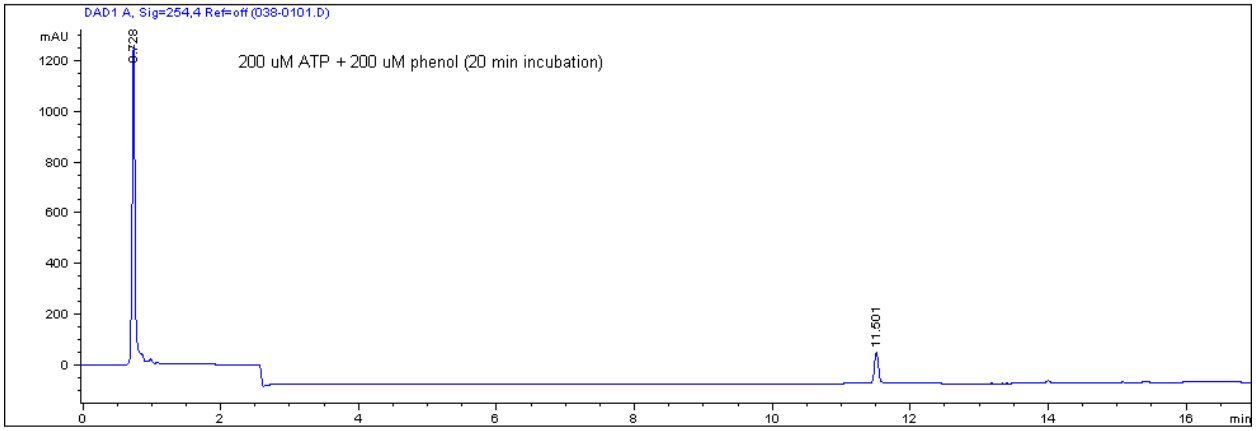
200 μM P-S2



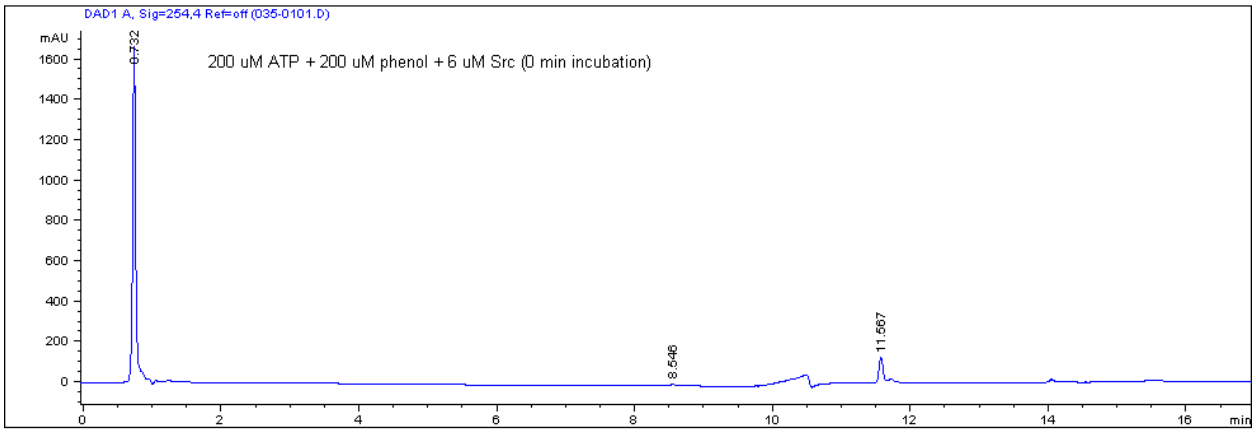
200 μM of P-S2-phos



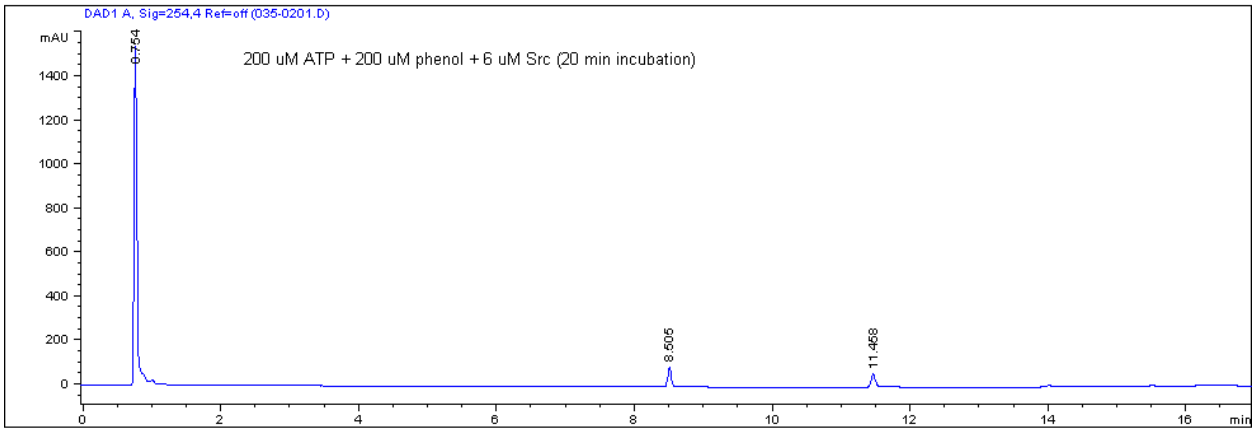
20 min incubation: 200 µM P-S2 and 200 µM ATP



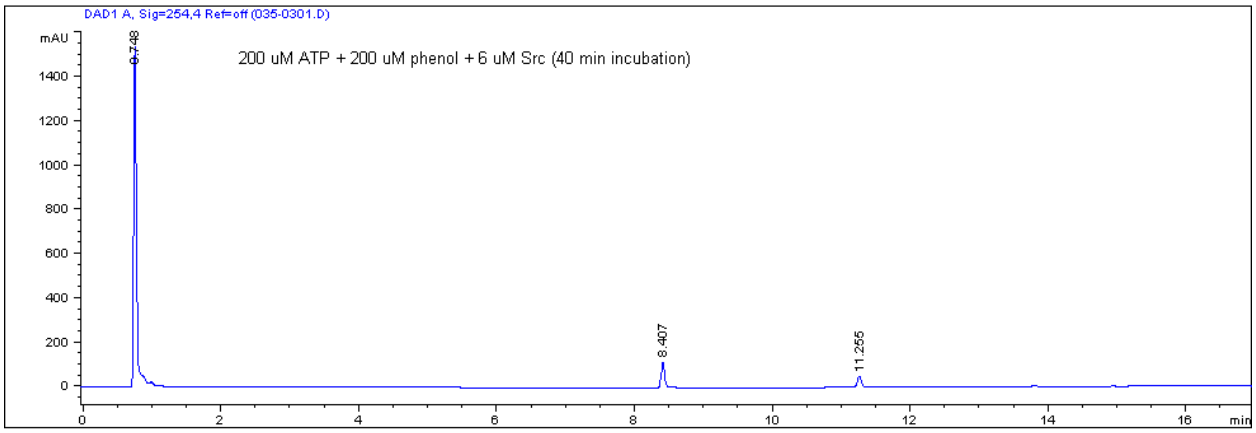
0 min incubation: 200 µM P-S2, 200 µM ATP, and 6 µM c-Src



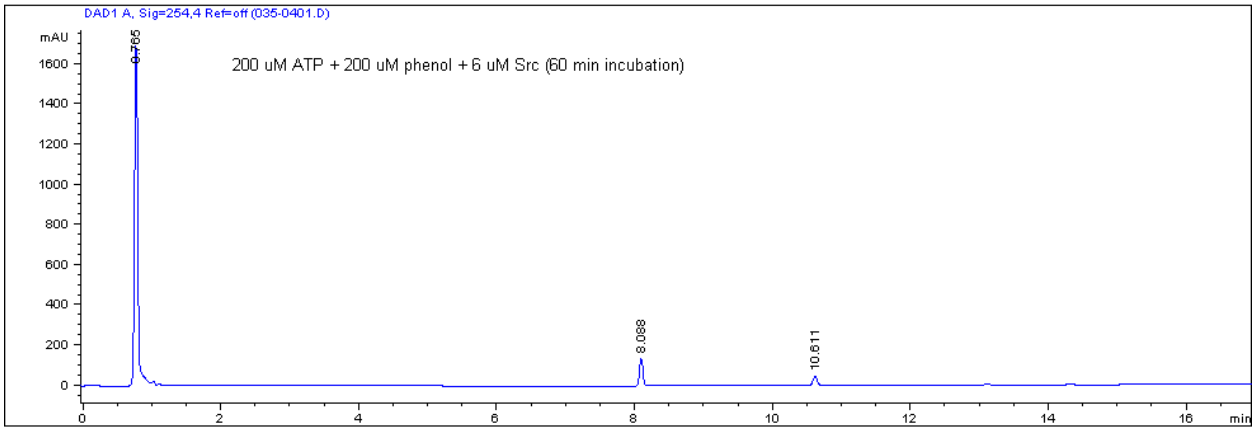
20 min incubation: 200 µM P-S2, 200 µM ATP, and 6 µM c-Src



40 min incubation: 200 µM P-S2, 200 µM ATP, and 6 µM c-Src



60 min incubation: 200 µM P-S2, 200 µM ATP, and 6 µM c-Src



VI. BIOCHEMICAL CHARACTERIZATIONS OF COMPOUNDS 7-12

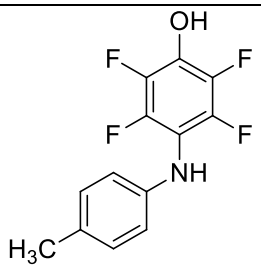
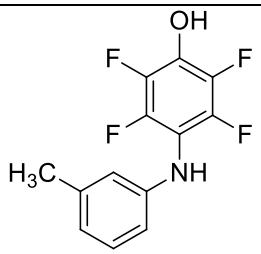
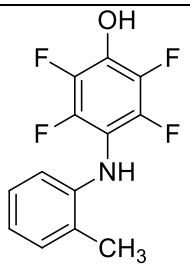
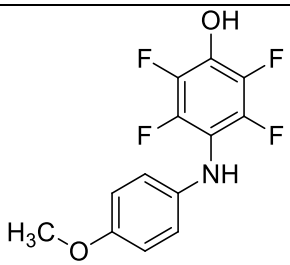
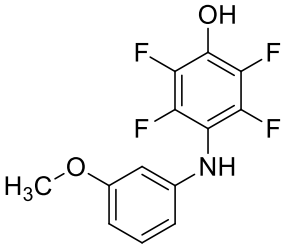
General procedures. Black, opaque-bottom 96 well plates were purchased from Nunc. c-Src, c-Abl, and Hck were expressed in *E. coli* using previously published procedures.[4] Chicken c-Src numbering is used unless otherwise noted. Blk, Fgr, Frk, Fyn A, Lck, and Lyn A were purchased from SignalChem. Data was obtained using a Molecular Devices SpectraMax M5 plate reader. Curve fitting was done using Graphpad Prism 4 software.

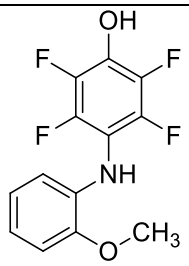
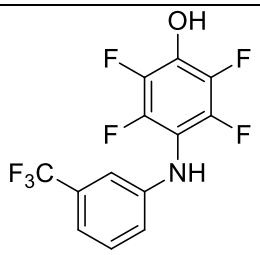
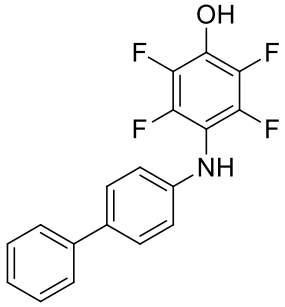
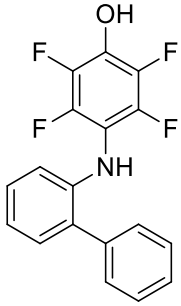
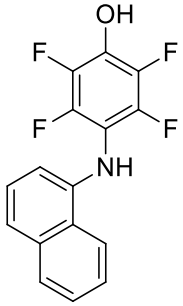
General procedure for determination of inhibitor K_i. A continuous fluorescence assay was used to determine K_i.[5] Reaction volumes of 100 µL were used in 96-well plates. 85 µL of enzyme in buffer mix was added to each well followed by 2.5 µL of the appropriate inhibitor dilution (typically 80, 40, 20, 10, 5, 2.5, 1.25, 0.625 mM in DMSO) and 2.5 µL of a substrate peptide solution ("compound 3" as described in Wang *et al.*, typically 1.8 mM in DMSO). The reaction was initiated with 10 µL of ATP (10 mM in water), and reaction progress was immediately monitored at 405 nm (ex. 340 nm) for 10 minutes. Reactions had final concentrations of 1 mM ATP, 100 µM Na_3VO_4, 100mM Tris buffer (pH 8), 10 mM $MgCl_2$, and 0.01% Triton X-100. Final concentrations of enzyme and "substrate 3" for each kinase are as follows: c-Src kinase domain, 3 domain (3D) c-Src, phosphorylated (pY416) c-Src kinase domain, double mutant R388A/A390R c-Src kinase domain, Hck kinase domain assays, and Yes kinase domain reactions had a final concentration of 30 nM enzyme and 45 µM peptide substrate. T338M c-Src kinase domain reactions had a final concentration of 30 nM enzyme and 20 µM peptide substrate. c-Abl kinase domain reactions had a final concentration of 120 nM enzyme and 120 µM peptide substrate. 3D Blk reactions had a final concentration of 30 nM enzyme and 85 µM peptide substrate. 3D Fgr reactions had a final concentration of 30 nM enzyme and 54 µM peptide substrate. 3D Frk reactions had a final concentration of 30 nM enzyme and 165 µM peptide substrate. 3D Fyn A reactions had a final concentration of 30 nM enzyme and 69 µM peptide substrate. 3D Lck reactions had a final concentration of 30 nM enzyme and 96 µM peptide substrate. 3D Lyn A reactions had a final concentration of 30 nM enzyme and 140 µM peptide substrate. The initial rate data collected was used for determination of K_i values. For K_i determination, the kinetic values were obtained directly from nonlinear regression of substrate-velocity curves in the presence of various concentrations of the inhibitor. The K_M values used for "substrate 3" were obtained from Wang *et al* or were determined as described below (see "General procedure for determination of "substrate 3" K_M").[5]

For biochemical evaluation of PP2, the protocol above was followed with modifications to the ATP concentration. c-Src, Blk, Fgr, Frk, Fyn A, Lck, and Lyn A reactions had a final concentration of 300 µM ATP. c-Abl and Hck reactions had a final concentration of 100 µM ATP, and Yes reactions had a final concentration of 500 µM ATP. The K_M values used for ATP were obtained from Carna Biosciences or were determined as described below (see "General procedure for determination of ATP K_M").[6]

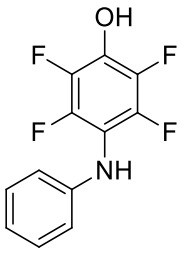
Focused library. A focused library of 10 inhibitors was prepared and evaluated using the continuous fluorescence assay described above. Each inhibitor K_i value was determined using at least 3 independent experiments which were averaged to give an average K_i value ± standard deviation.

Table S3. c-Src K_i values for a focused library of analogues of compound **7**.

Name	Structure	c-Src K_i (μM)
S4		549 ± 57
S5		406 ± 67
10		127 ± 11
S6		229 ± 49
S7		399 ± 92

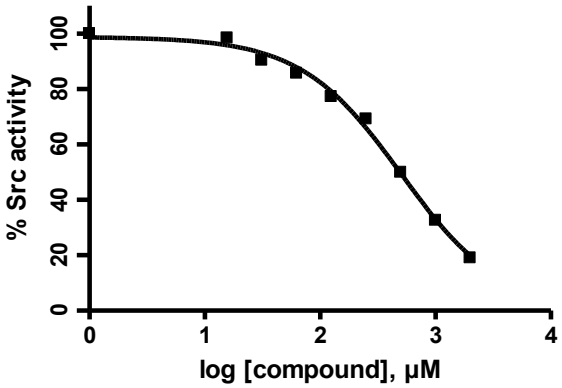
S8		443 ± 13
S9		318 ± 16
11		80 ± 6
S10		257 ± 8
12		16 ± 1

Analytical data for K_i determinations. Each inhibitor K_i value was determined using at least 3 independent experiments (unless otherwise noted) which were averaged to give an average K_i value ± standard deviation. A representative curve is shown for inhibitors **7-12** and **PP2** with c-Src kinase domain, Hck kinase domain, and c-Abl kinase domain. For inhibitor **12** additional representative curves are shown for Yes kinase domain, 3 domain (3D) c-Src, phosphorylated (pY416) c-Src kinase domain, T338M c-Src kinase domain, double mutant R388A/A390R c-Src kinase domain, 3D Blk, 3D Fgr, 3D Frk, 3D Fyn A, 3D Lck, and 3D Lyn A. For PP2, additional representative curves are shown for Yes kinase domain, 3D Blk, 3D Fgr, 3D Frk, 3D Fyn A, 3D Lck, and 3D Lyn A.

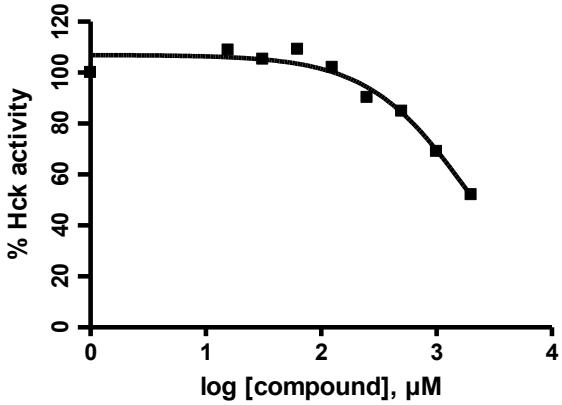


7

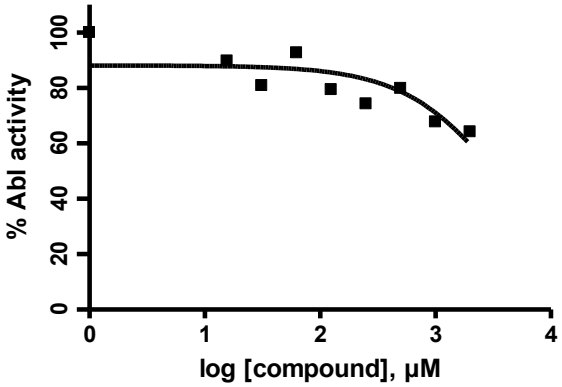
c-Src K_i = 257 ± 28 μM

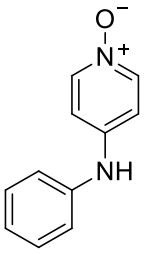


Hck K_i = 1015 ± 165 μM



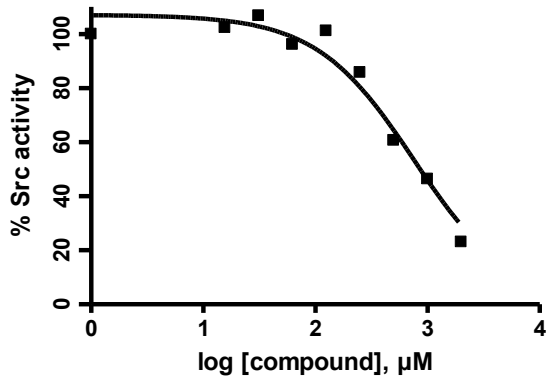
c-Abl K_i >1170 μM



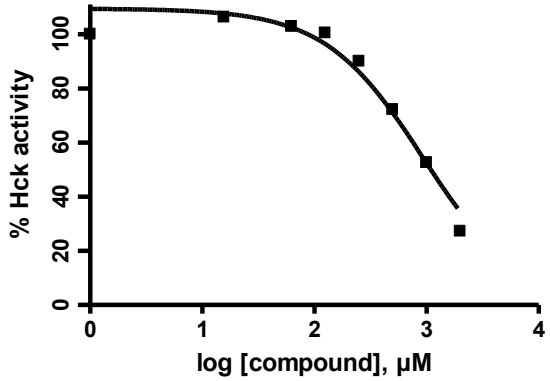


8

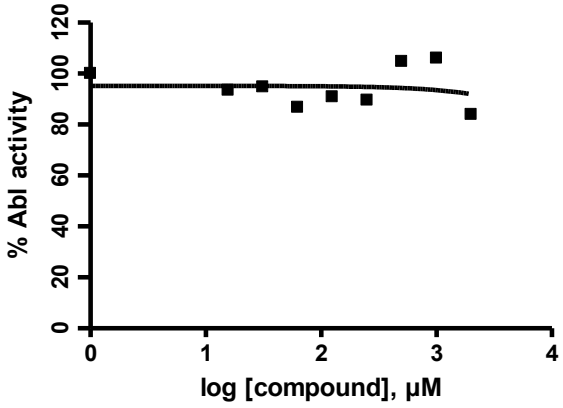
c-Src K_i = 478 ± 82 µM

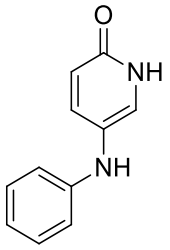


Hck K_i = 517 ± 32 µM



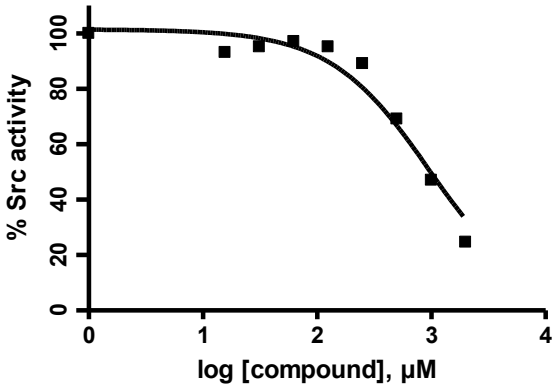
c-Abl K_i >1170 µM



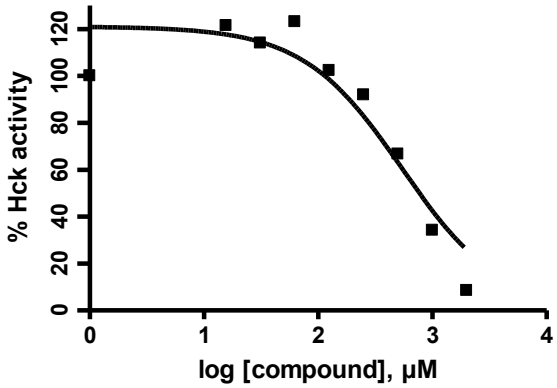


9

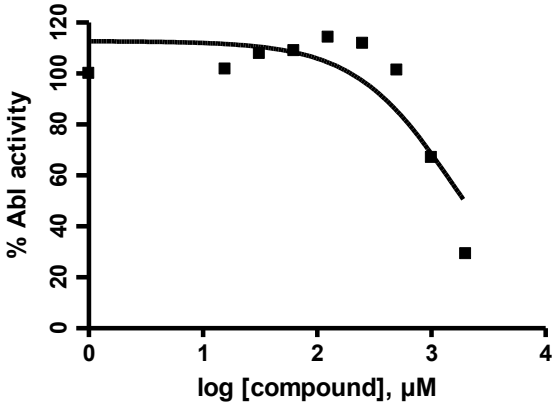
c-Src K_i = 552 ± 9 μM

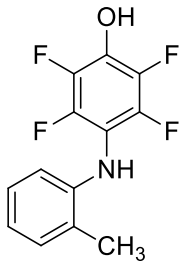


Hck K_i = 318 ± 17 μM



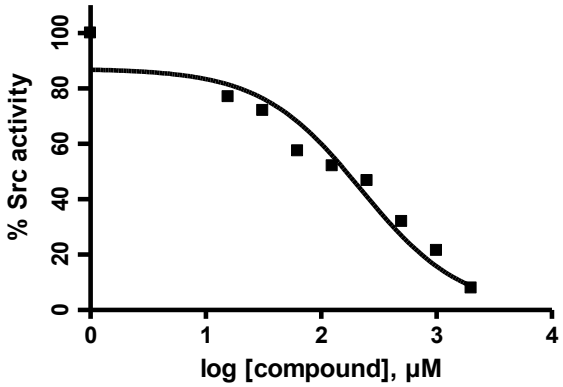
c-Abl K_i = 1046 ± 176 μM



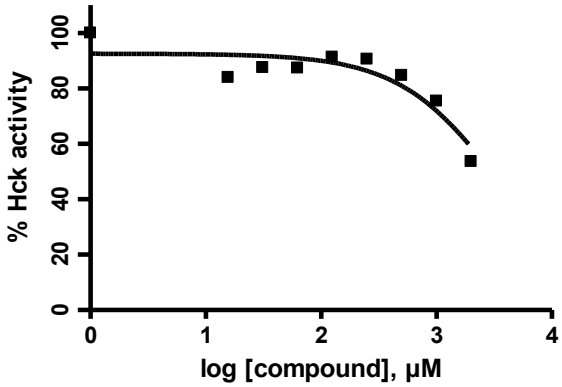


10

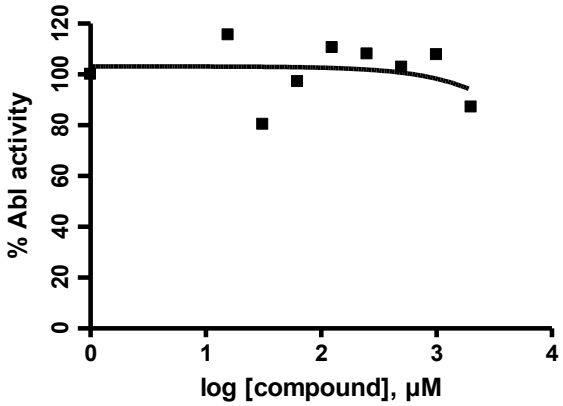
c-Src K_i = 127 ± 11 µM

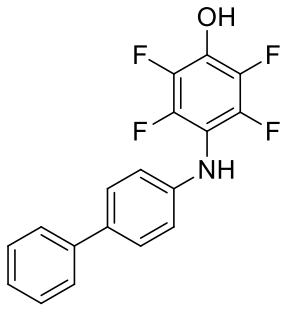


Hck K_i >1080 µM



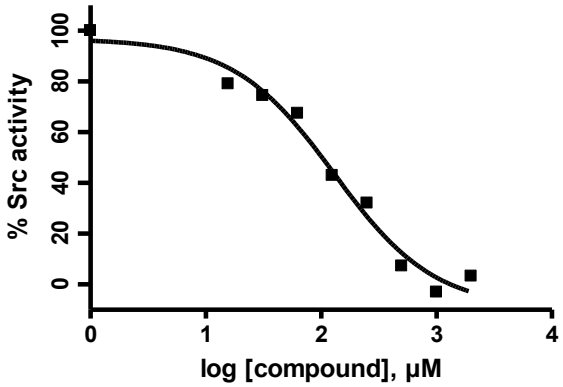
c-Abl K_i >1170 µM



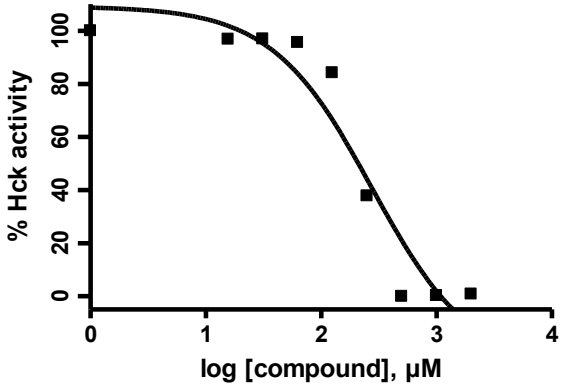


11

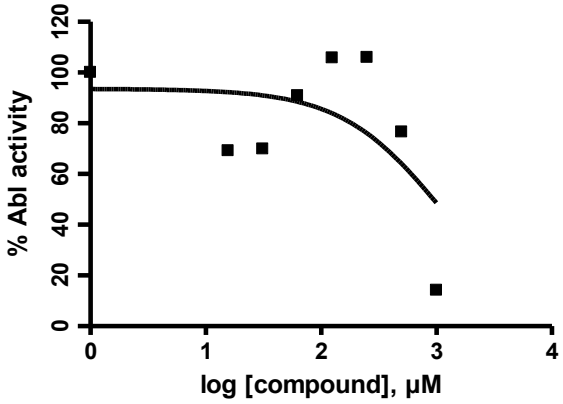
c-Src K_i = 80 ± 6 µM

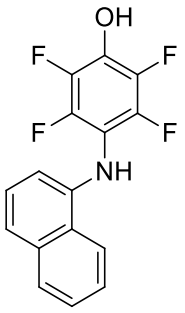


Hck K_i = 149 ± 6 µM



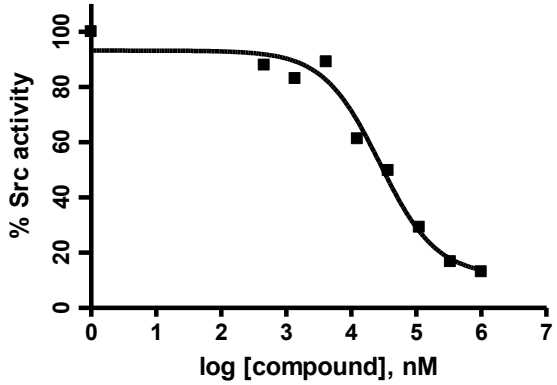
c-Abl K_i = 549 ± 138 µM



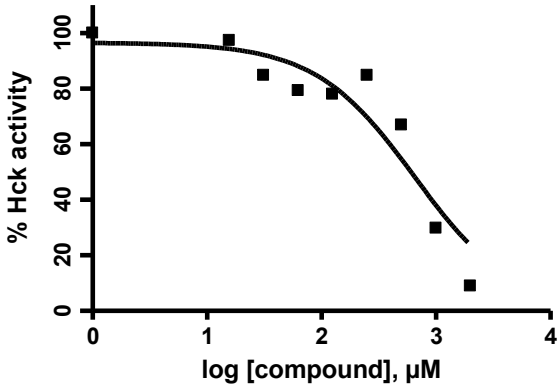


12

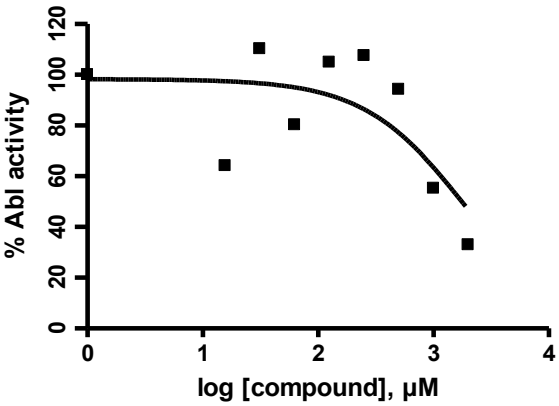
c-Src K_i = 16 ± 1 µM



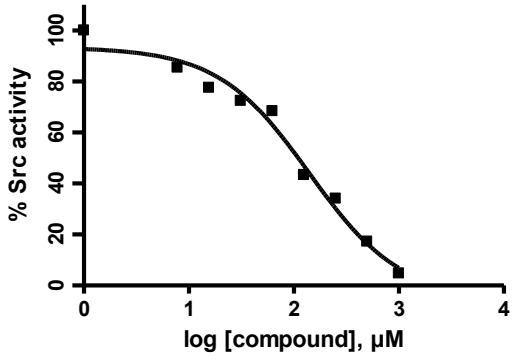
Hck K_i = 325 ± 30 µM



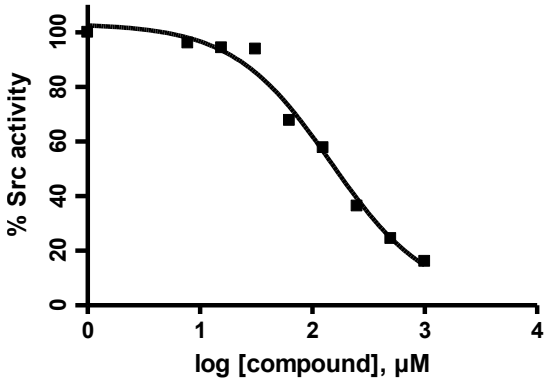
c-Abl K_i = 1067 ± 385 µM



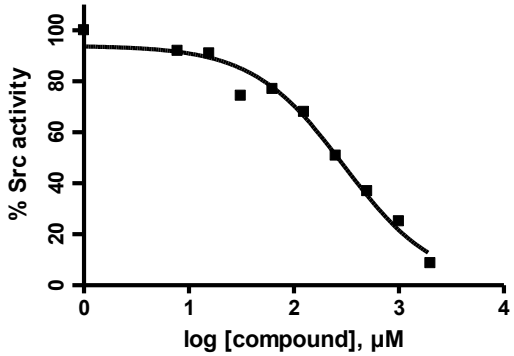
pY416 c-Src K_i = 73 ± 9 µM



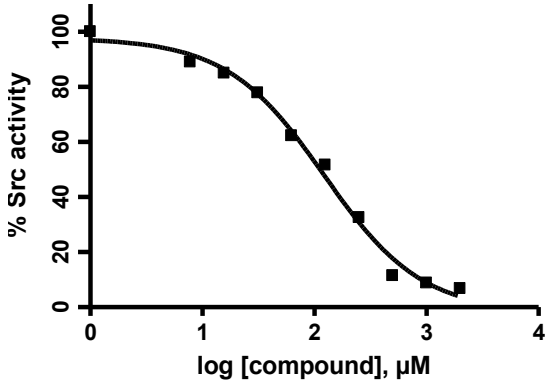
T338M c-Src K_i = 75 ± 15 μM



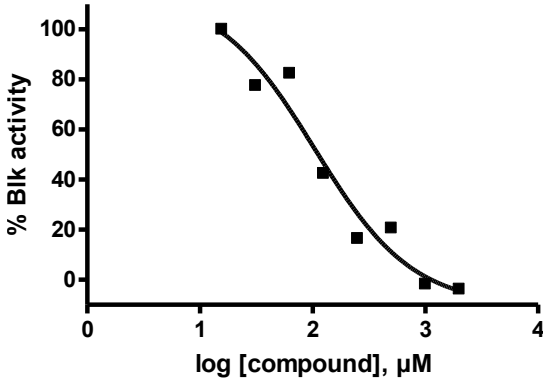
R388A/A390R c-Src K_i = 184 ± 53 μM



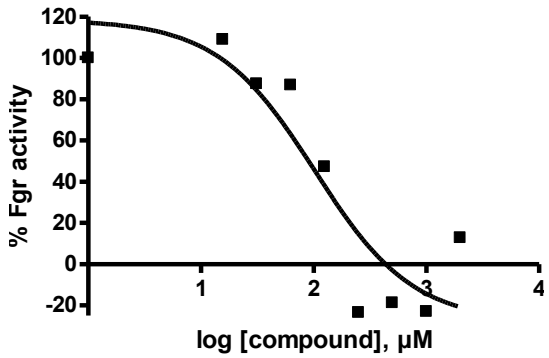
Yes K_i = 82 ± 16 μM



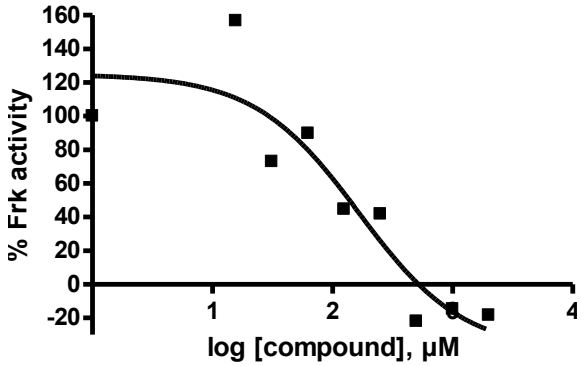
Blk K_i = 52 μM (n = 1)



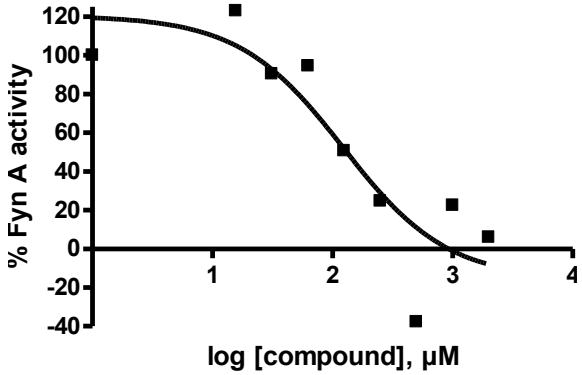
Fgr K_i = 51 μM (n = 1)



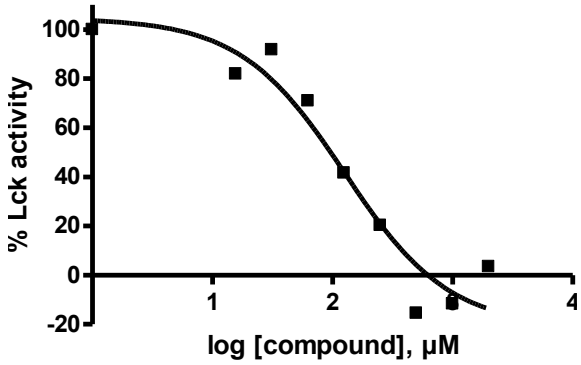
Frk K_i = 83 µM (n = 1)



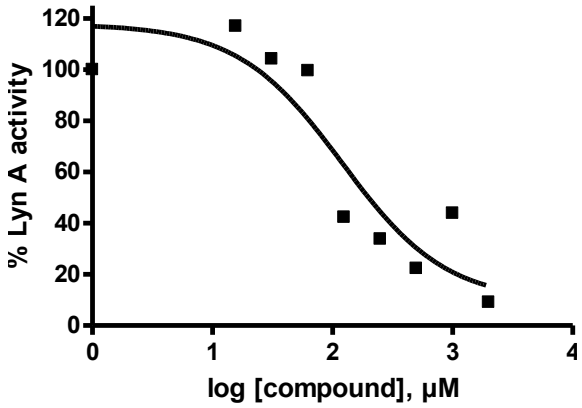
Fyn A K_i = 61 µM (n = 1)

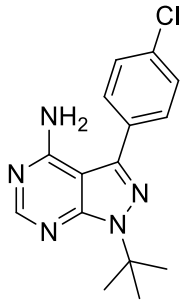


Lck K_i = 63 µM (n = 1)



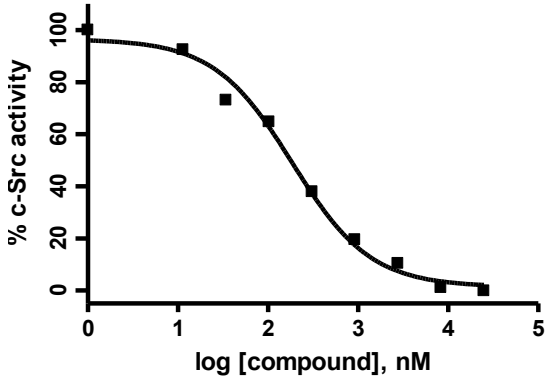
Lyn A K_i = 60 µM (n = 1)



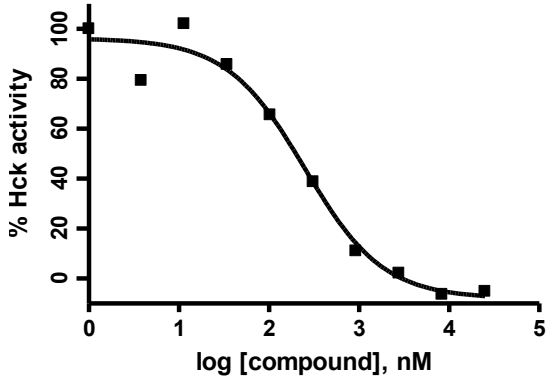


PP2

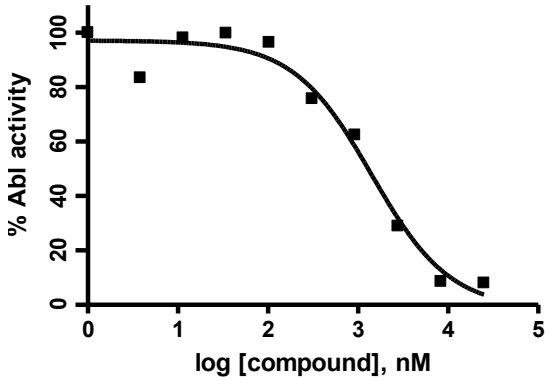
c-Src K_i = 45 ± 1 nM



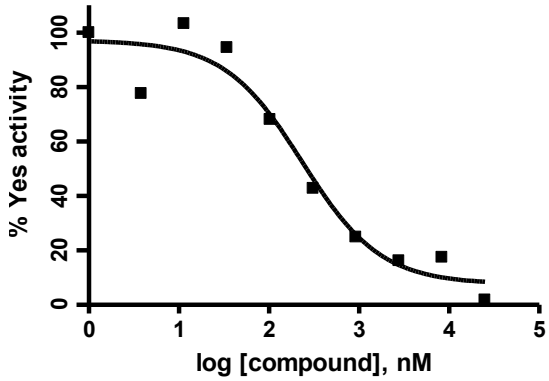
Hck K_i = 88 ± 5 nM



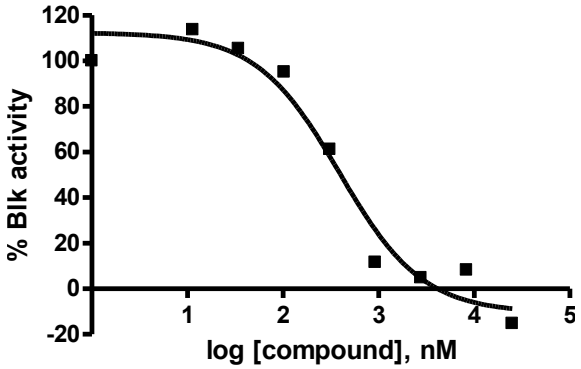
c-Abl K_i = 387 ± 17 nM



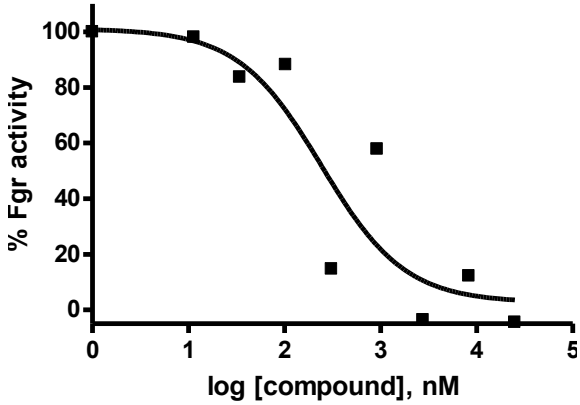
Yes K_i = 46 ± 11 nM



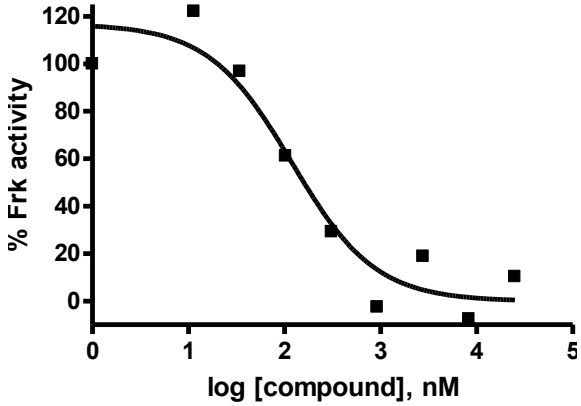
Blk K_i = 67 nM (n = 1)



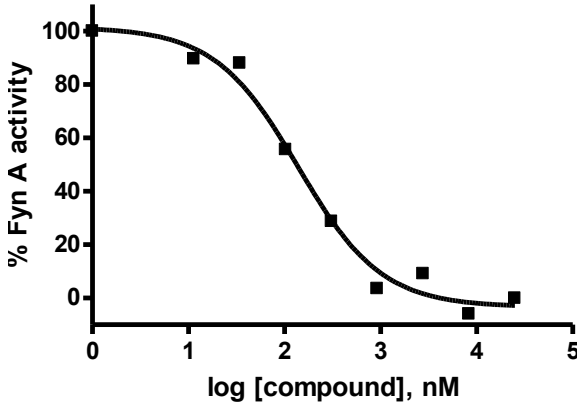
Fgr K_i = 25 nM (n = 1)



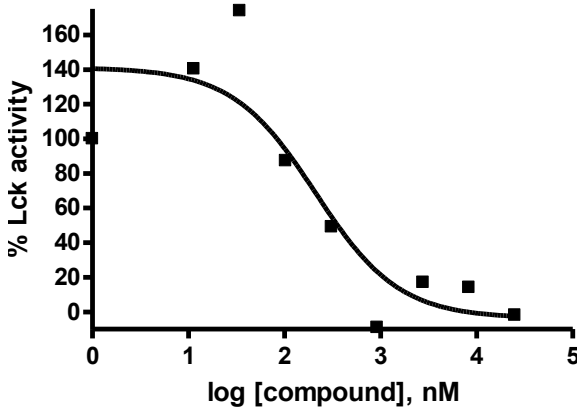
Frk K_i = 20 nM (n = 1)



Fyn A K_i = 20 nM (n = 1)



Lck K_i = 9 nM (n = 1)



Lyn A K_i = 16 nM (n = 1)

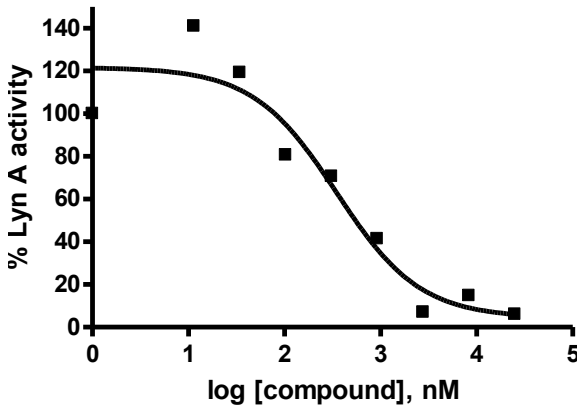


Table of selectivity data for compound 12 and PP2 with Src family kinases.

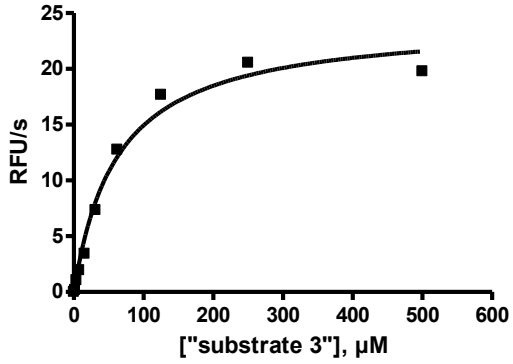
Kinase	Compound 12 Ki (uM)	Compound 12 Selectivity	PP2 Ki (nM)	PP2 Selectivity
Src	16	1	45	1
Hck	325	20.3	88	2.0
Yes	82	5.1	46	1.0
Blk	52	3.3	67	1.5
Fgr	51	3.2	25	0.6
Frk	83	5.2	20	0.4
Fyn A	61	3.8	20	0.4
Lck	63	3.9	9	0.2
Lyn A	60	3.8	16	0.4

General procedure for determination of "substrate 3" K_M. A continuous fluorescence assay was used to determine K_M for "substrate 3" described in Wang *et al*.[5] Reaction volumes of 100 µL were used in 96-well plates. 85 µL of enzyme in buffer mix was added to each well followed by 2.5 µL of the appropriate dilution of "substrate 3" (typically 20, 10, 5, 2.5, 1.25, 0.625, 0.31, 0.16, 0.078, and 0 mM in DMSO) and 2.5 µL of DMSO. The reaction was initiated with 10 µL of ATP (10 mM in water), and reaction progress was immediately monitored at 405 nm (ex. 340 nm) for 10 minutes. Reactions had final concentrations of 1 mM ATP, 100 µM Na_3VO_4, 100mM Tris buffer (pH 8), 10 mM $MgCl_2$, and 0.01% Triton X-100. c-Src, Hck, Yes, Blk, and Frk reactions had a final concentration of 30 nM enzyme, and c-Abl reactions had a final concentration of 120 nM enzyme. The initial rate data collected was used for determination of K_M values. For K_M determination, the kinetic values were obtained directly from nonlinear regression of substrate-velocity curves.

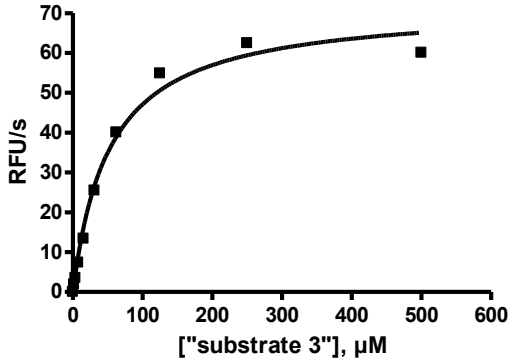
General procedure for determination of ATP K_M. A continuous fluorescence assay was used to determine K_M for ATP described in Wang *et al*.[5] Reaction volumes of 100 µL were used in 96-well plates. 85 µL of enzyme in buffer mix was added to each well followed by 2.5 µL of "substrate 3" (1.8 mM in DMSO) and 2.5 µL of DMSO. The reaction was initiated with 10 µL of ATP (typically 10, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.078, 0.039, and 0 mM in water), and reaction progress was immediately monitored at 405 nm (ex. 340 nm) for 10 minutes. Reactions had final concentrations of 45 µM "substrate 3", 100 µM Na_3VO_4, 100mM Tris buffer (pH 8), 10 mM $MgCl_2$, and 0.01% Triton X-100. c-Src, Hck, and Yes reactions had a final concentration of 30 nM enzyme, and c-Abl reactions had a final concentration of 120 nM enzyme. The initial rate data collected was used for determination of K_M values. For K_M determination, the kinetic values were obtained directly from nonlinear regression of substrate-velocity curves.

Analytical data for K_M determinations. The "substrate 3" and ATP K_M values were determined using at least 3 independent experiments which were averaged to give an average K_M value ± standard deviation. A representative curve for "substrate 3" is shown for c-Src kinase domain, 3-domain (3D) c-Src, phosphorylated (pY416) c-Src kinase domain, T338M c-Src kinase domain, double mutant R388A/A390R c-Src kinase domain, Hck kinase domain, Yes kinase domain, 3D Blk, 3D Frk, and c-Abl kinase domain. A representative curve for ATP is shown with c-Abl kinase domain, Hck kinase domain, and Yes kinase domain. For analytical data for ATP with c-Src kinase domain see Kwarcinski *et al*.[7] For "substrate 3" with Fgr, Fyn A, Lck, and Lyn A the K_M values reported in Wang *et al*. were used.[5] For ATP with Blk, Fgr, Frk, Fyn A, Lck, and Lyn A the K_M values reported by Carna Biosciences were used.[6]

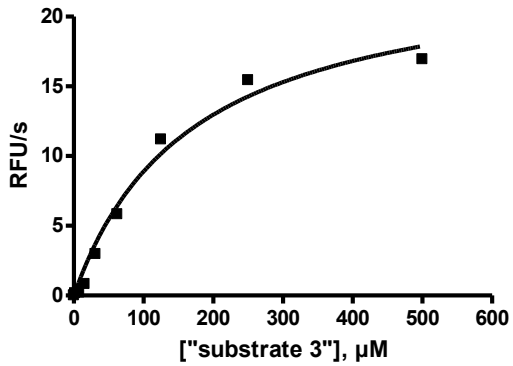
"substrate 3" c-Src K_M = 61 ± 4 µM



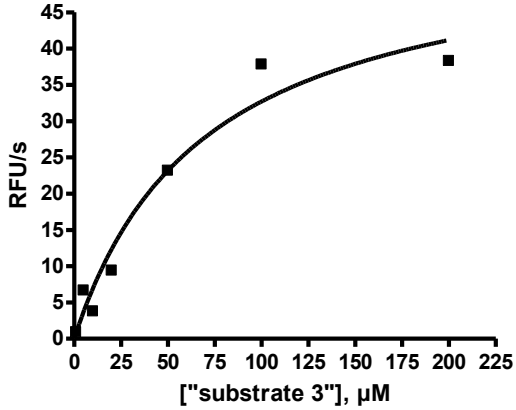
"substrate 3" Hck K_M = 53 ± 2 µM



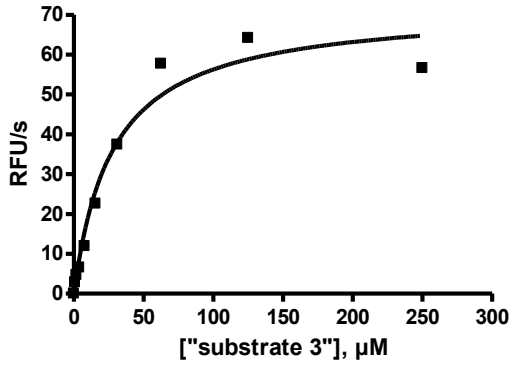
"substrate 3" c-Abl K_M = 168 ± 19 μM



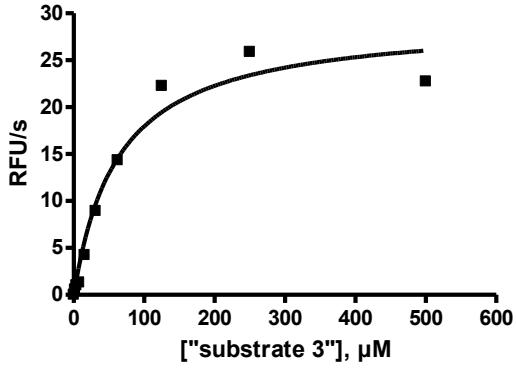
"substrate 3" R388A/A390R c-Src K_M = 70 ± 17 μM



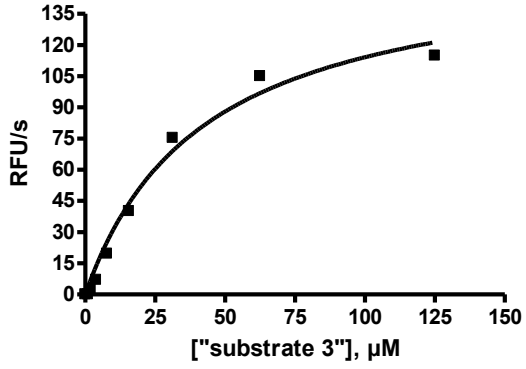
"substrate 3" T338M c-Src K_M = 26 ± 8 μM



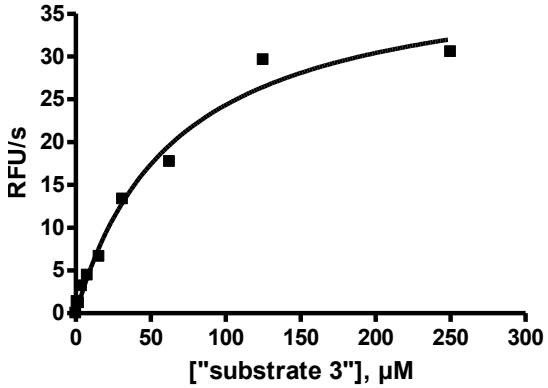
"substrate 3" 3D c-Src K_M = 64 ± 4 μM



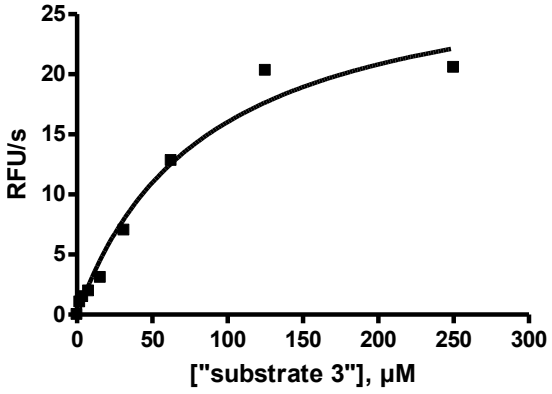
"substrate 3" pY416 Src K_M = 43 ± 3 μM



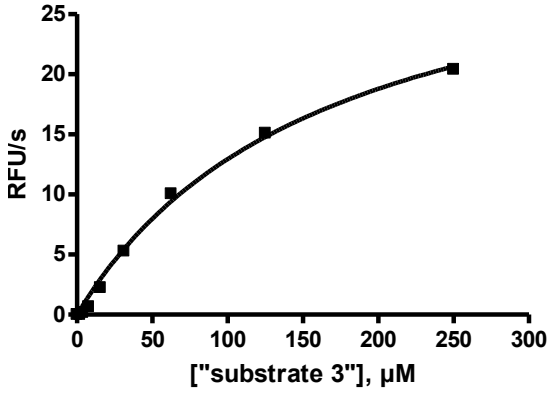
"substrate 3" Yes K_M = 62 ± 6 μM



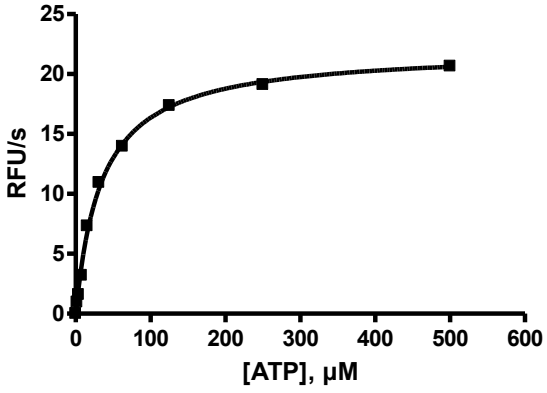
"substrate 3" Blk K_M = 85 µM (n = 1)



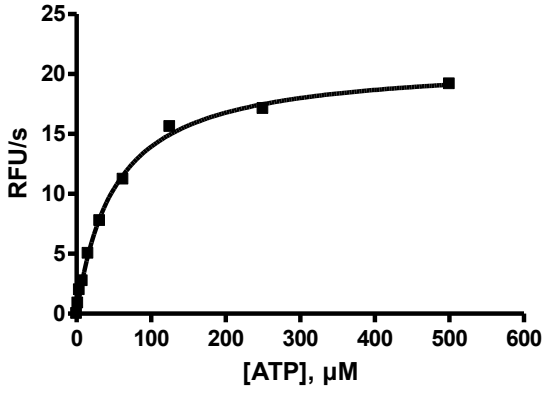
"substrate 3" Frk K_M = 165 µM (n = 1)



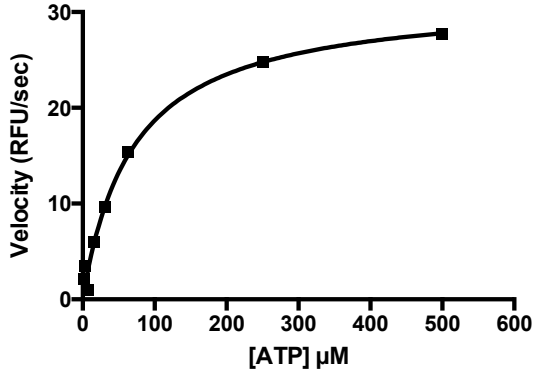
ATP c-Abl K_M = 37 ± 6 µM



ATP Hck K_M = 50 ± 3 µM



ATP Yes K_M = 105 ± 21 µM

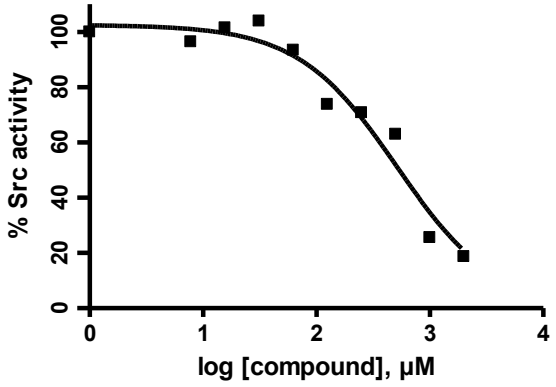


Changes in IC_{50} for compounds 7-9 and 12 at variable substrate and ATP concentrations. The continuous fluorescence assay described previously was used to determine IC_{50} for inhibitors at higher "substrate 3" concentration and at lower ATP concentration with c-Src.[5] For higher "substrate 3" conditions the reactions had final concentrations of 30 nM c-Src, 500 µM "substrate 3", 1 mM ATP, 100 µM Na_3VO_4, 100mM Tris buffer (pH 8), 10 mM $MgCl_2$, and 0.01% Triton X-100. For lower ATP conditions the reactions had final concentrations of 30 nM c-Src, 45 µM "substrate 3", 100 µM ATP, 100 µM Na_3VO_4, 100mM Tris buffer (pH 8), 10 mM $MgCl_2$, and 0.01% Triton X-100. The initial rate data collected was used for determination of IC_{50} values, which were obtained directly from nonlinear regression of substrate-velocity curves in the presence of various concentrations of the inhibitor. The IC_{50} value for inhibitors under each set of conditions was determined using at least 3 independent experiments which were averaged to give an average IC_{50} ± standard deviation. Representative curves for compound **12** are shown for each set of conditions.

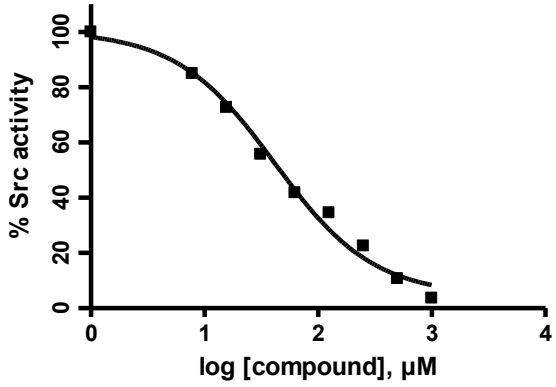
Table S4. c-Src IC_{50} values under standard and high "substrate 3" conditions.

Compound	IC_{50} under standard conditions	IC_{50} under high substrate conditions
7	436 ± 60 µM	>2000 µM
8	830 ± 143 µM	1520 ± 440 µM
9	958 ± 16 µM	1742 ± 614 µM
12	27 ± 2 µM	557 ± 149 µM

12 with high "substrate 3" conditions
IC_{50} = 557 ± 149 µM



12 with low ATP conditions IC_{50} = 44 ± 4 µM



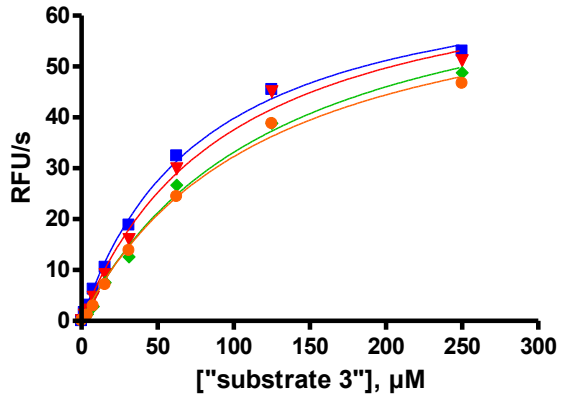
General procedure for Lineweaver-Burk analysis of compound 12. A continuous fluorescence assay was used to determine initial velocities for ATP and "substrate 3" described in Wang *et al* with c-Src in the presence of compound **12**.[5]

For "substrate 3", reaction volumes of 100 µL were used in 96-well plates. 85 µL of enzyme in buffer mix was added to each well followed by 2.5 µL of the appropriate dilution of "substrate 3" (typically 20, 10, 5, 2.5, 1.25, 0.625, 0.31, 0.16, 0.078, and 0 mM in DMSO) and 2.5 µL of compound **12** (0.4, 1.2, or 2.4 mM in DMSO). The reaction was initiated with 10 µL of ATP (5 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 c-Src, 500 µM ATP, 100 µM Na_3VO_4, 100 mM Tris buffer (pH 8), 10 mM $MgCl_2$, and 0.01% Triton X-100. For K_M determination, the kinetic values were obtained directly from nonlinear regression of substrate-velocity curves.

For ATP, reaction volumes of 100 µL were used in 96-well plates. 85 µL of enzyme in buffer mix was added to each well followed by 10 µL of the appropriate dilution of ATP (typically 5, 2.5, 1.25, 0.625, 0.31, 0.16, 0.078, 0.039, 0.020, and 0 mM in water) and 2.5 µL of compound **12** (0.4, 1.2, or 2.4 mM in DMSO). The reaction was initiated with 2.5 µL of "substrate 3" (1.8 mM in DMSO), and reaction progress was immediately monitored at 405 nm (ex. 340 nm) for 10 minutes. Reactions had final concentrations of 30 nM c-Src, 45 µM "substrate 3", 100 µM Na_3VO_4, 100 mM Tris buffer (pH 8), 10 mM $MgCl_2$, and 0.01% Triton X-100. For K_M determination, the kinetic values were obtained directly from nonlinear regression of substrate-velocity curves.

Analytical data for K_M and Lineweaver-Burk analysis. The initial velocities for "substrate 3" or ATP in the presence of inhibitor **12** were determined (n = 4), and the average velocities were used to generate substrate-velocity curves. Lineweaver-Burk plots were generated by plotting the reciprocal of the average initial rates as a function of 1/["substrate 3"] or 1/[ATP] and performing linear regression.

Substrate K_M and V_{max} at varied inhibitor concentrations:



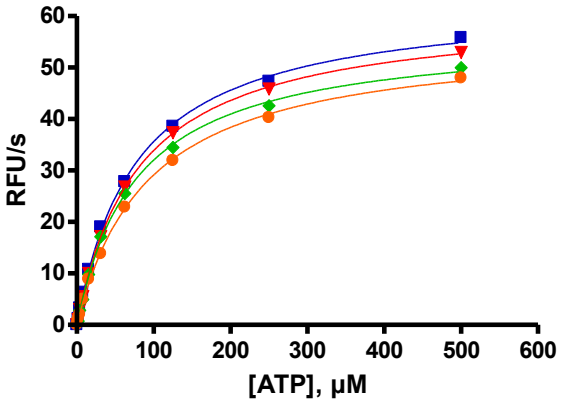
	0 µM 12	10 µM 12	30 µM 12	60 µM 12
Michaelis-Menten Best-fit values				
VMAX	71.69	73.57	75.22	71.18
KM	80.20	96.00	127.1	120.5

Global fit data for substrate K_M data:

	R^2
competitive	0.993
non-competitive	0.891
uncompetitive	0.888

Both the global fit and K_M / V_{max} data are most consistent with a substrate-competitive mode of action.

ATP K_M and V_{max} at varied inhibitor concentrations:



	0 µM 12	10 µM 12	30 µM 12	60 µM 12
Michaelis-Menten Best-fit values				
VMAX	63.41	61.14	56.82	56.20
KM	78.13	80.43	77.48	92.93

Global fit data for ATP K_M data:

	R^2
competitive	0.897
non-competitive	0.999
uncompetitive	0.897

Both the global fit and K_M / V_{max} data are most consistent with an ATP-non-competitive mode of action.

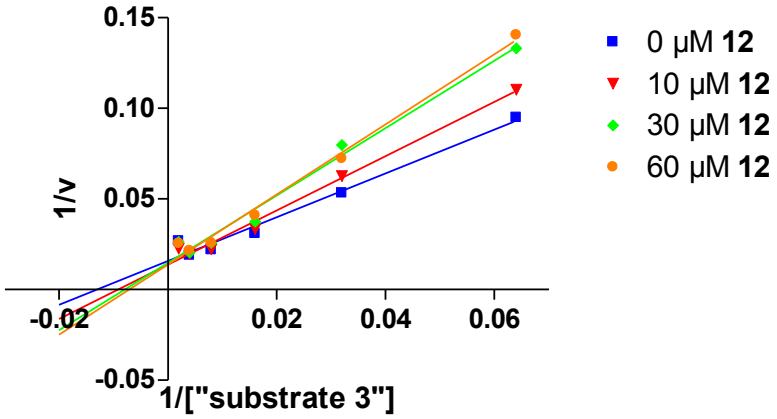


Figure S1. Lineweaver-Burk plot for compound **12** versus "substrate 3" peptide. The lines intersect on the Y-axis demonstrating that compound **12** is competitive with the peptide substrate.

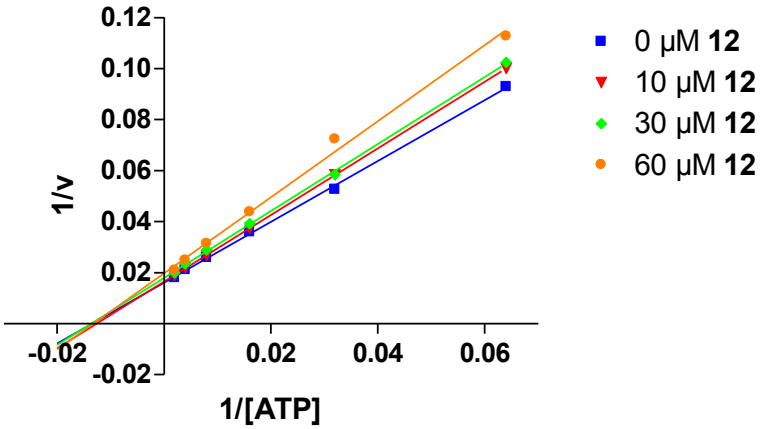
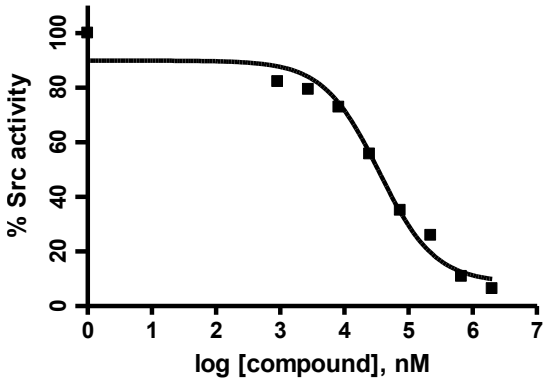


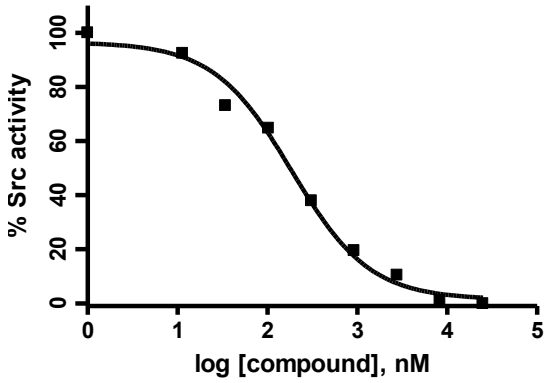
Figure S2. Lineweaver-Burk plot for compound **12** versus ATP. The lines intersect on the X-axis demonstrating that compound **12** is noncompetitive with the ATP.

Combination studies of compound 12 with PP2 and PP5. The continuous fluorescence assay described previously was used to determine IC_{50} for **12**, PP2, and PP5 with c-Src.[5] Reactions had final concentrations of 30 nM c-Src, 45 µM "substrate 3", 300 µM ATP, 100 µM Na_3VO_4, 100mM Tris buffer (pH 8), 10 mM $MgCl_2$, and 0.01% Triton X-100. The initial rate data collected was used for determination of IC_{50} values, which were obtained directly from nonlinear regression of substrate-velocity curves in the presence of various concentrations of the inhibitor. Each inhibitor IC_{50} value was determined using at least 3 independent experiments which were averaged to give an average IC_{50} ± standard deviation. A representative curve is shown for each inhibitor with c-Src.

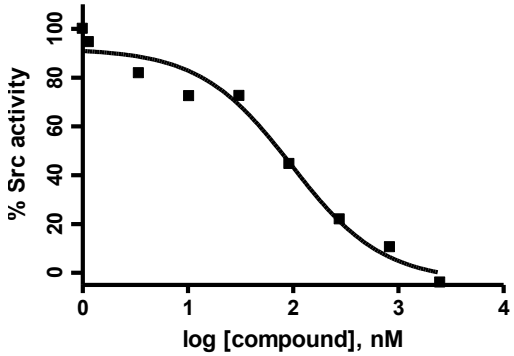
Inhibitor **12** c-Src IC_{50} = 28 ± 14 µM



PP2 c-Src IC_{50} = 181 ± 6 nM



PP5 c-Src IC_{50} = 98 ± 17 nM



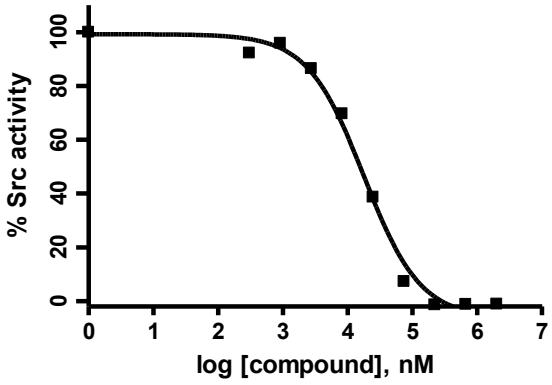
To determine if additive or synergistic effects occurred when compound **12** was combined with ATP-competitive inhibitors, single point assays of the inhibitors at IC_{35} (20 µM inhibitor **12**, 100 nM PP2, and 50 nM PP5) were carried out using the procedure described above. Initial velocity was measured for **12**, PP2, and PP5 alone as well as for combinations of **12** + PP2, **12** + PP5, and PP2 + PP5. The ratio of the initial velocity in the presence of inhibitor(s) and the initial velocity in the presence of no inhibitor (DMSO control) was used to calculate percent activity remaining, and percent inhibition was calculated as 100 - percent activity remaining. The percent inhibition for each inhibitor and combination of inhibitors was determined using at least 6 independent experiments which were averaged to give an average percent inhibition ± standard deviation. The predicted additivity was determined using the Bliss equation [predicted additivity = (eA + eB) – (eA * eB)].[8]

Table S5. Percent c-Src inhibition for single inhibitors and combinations of inhibitors.

Inhibitor(s)	c-Src Inhibition (%)	Predicted Additivity (%)
100 nM PP2	34 ± 6	NA
50 nM PP5	32 ± 8	NA
20 μM 12	36 ± 10	NA
100 nM PP2 + 50 nM PP5	50 ± 3	55
100 nM PP2 + 20 μM 12	72 ± 6	58
50 nM PP5 + 20 μM 12	88 ± 5	56

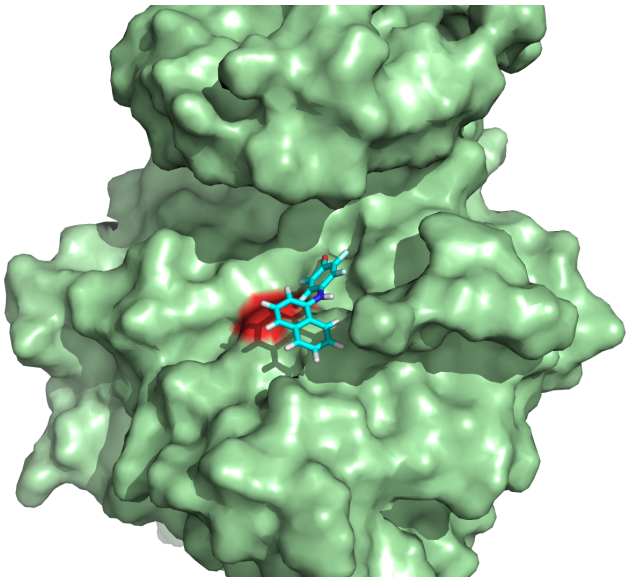
Time dependence control for compound 12. The continuous fluorescence assay described previously was used to determine IC_{50} for compound **12** with c-Src following a 2 hour incubation period.[5] 85 µL of enzyme in buffer mix was added to each well followed by 2.5 µL of the appropriate inhibitor dilution, and the mixture was incubated for 2 hours at room temperature. Following the incubation period, 2.5 µL of "substrate 3" was added, the reaction was initiated with 10 µL of ATP, and reaction progress was immediately monitored. Reactions had final concentrations of 30 nM c-Src, 45 µM "substrate 3", 1 mM ATP, 100 µM Na_3VO_4, 100 mM Tris buffer (pH 8), 10 mM $MgCl_2$, and 0.01% Triton X-100. The initial rate data collected was used for determination of K_i values, which were obtained directly from nonlinear regression of substrate-velocity curves in the presence of various concentrations of the inhibitor. The compound **12** K_i value was determined using 3 independent experiments which were averaged to give an average K_i ± standard deviation. A representative curve is shown.

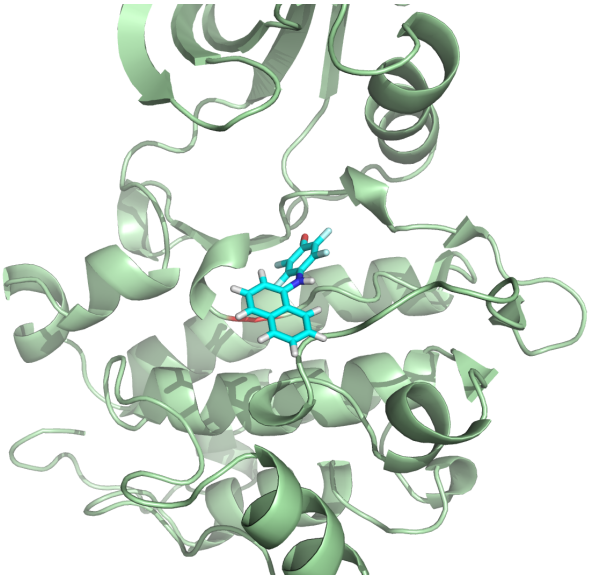
2 h incubation c-Src K_i = 9 ± 2 µM



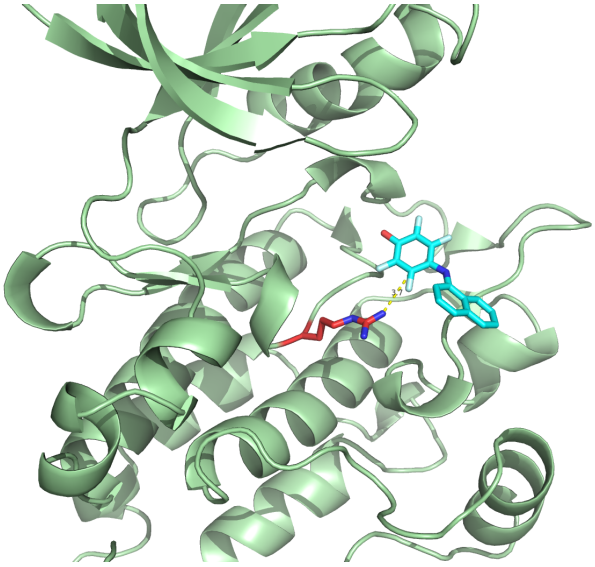
VII. COMPUTATIONAL DOCKING MODEL

Induced fit molecular docking. The induced fit docking (IFD) workflow from the Schrodinger Suite of Programs was used to flexibly dock SUB1 into four Src kinase structures (PDB IDs: 1YI6, 1Y57, 2BDF, 2SRC). Prime, also from the Schrodinger Suite Programs, was used to build in the activation loop of 2BDF using 3DQW as the template applying default parameters. Default parameters were used for IFD the docking score/glide gscore along with visual inspection was employed to determine if a binding pose was reasonable. Images for the top scoring complex in 2SRC are shown below with Arg388 colored red.

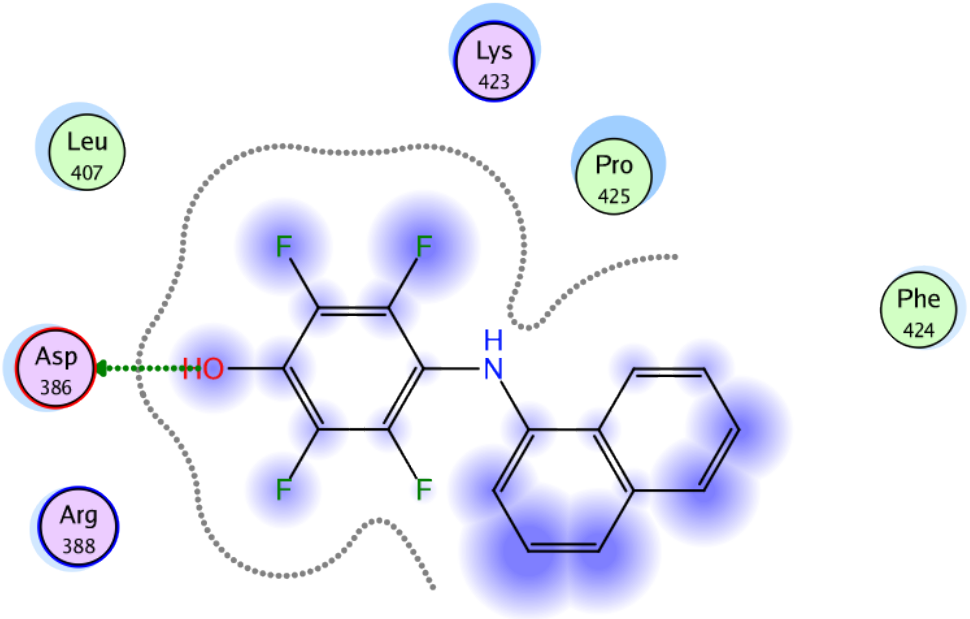




Contact distance between Arg388 and inhibitor **12** is 3.7 Å:



Ligand interactions between inhibitor **12** and c-Src:

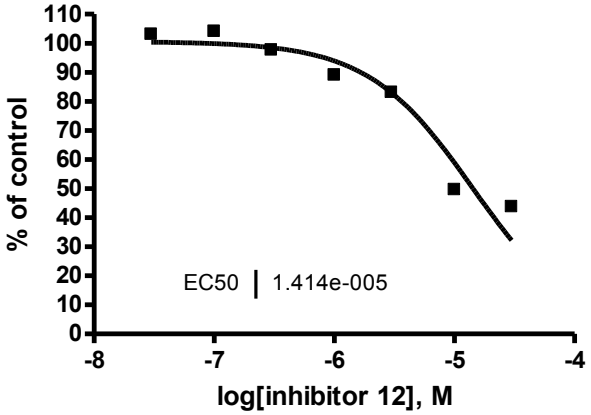


VIII. KINASE MUTANT PRODUCTION

Production of c-Src R388A/A390R double mutant. Chicken c-Src kinase domain in pET28a, modified with a TEV protease cleavable N-terminal 6x-His tag, was prepared as previously reported.[4] The desired mutations were added to this plasmid using iterative rounds of mutagenesis using the Agilent QuikChange II kit. The plasmid was transformed by electroporation into Bl21DE3 electrocompetent cells containing YopH in pCDFDuet-1. Cell growth, expression, and protein purification were performed using modified literature protocols for expression of wild-type c-Src kinase domain.[4]

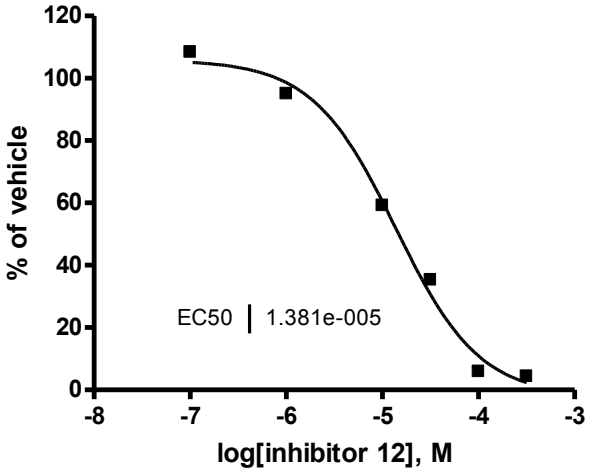
IX. *IN CELLULO* CHARACTERIZATION OF COMPOUND 12

c-Src autophosphorylation. This assay was performed by ProQinase GmbH (Freiburg, Germany). Murine embryonal fibroblast (MEF) cells were used that express a high level of exogenously introduced full-length Src. The high Src expression level results in a constitutive tyrosine autophosphorylation of Src at Tyr416. MEF-SRC cells were plated in DMEM supplemented with 10% FCS in multiwell cell culture plates. Compound incubation was done in serum-free medium. Quantification of Src phosphorylation was assessed in 96-well plates via ELISA using a phospho-Src specific antibody and a secondary detection antibody. Raw data were converted into percent phosphorylation and the IC_{50} value was determined using GraphPad Prism software. Each concentration has n = 2 data points and the graph below represents the average at each concentration.

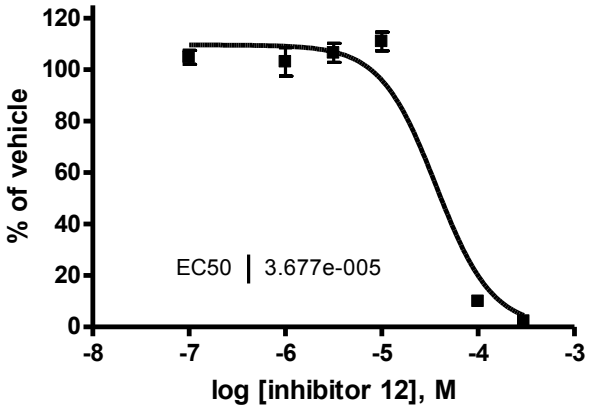


Cancer cell growth inhibition assays. SK-BR-3 (ATCC® HTB-30™), MCF7 (ATCC® HTB-22™), T47D (ATCC® HTB-133™) and HT-29 (ATCC® HTB-38™) cells were grown and maintained using complete growth media (DMEM for SK-BR-3, MCF7, and T47D and McCoy's 5a Modified Medium for HT-29, supplemented with 10% fetal bovine serum) in a 37°C, 5% CO_2, humidified air incubator. Cells were then plated into 96 well plates in complete growth media (100 µl/well) at a concentration of 5000 cells per well and allowed to attach overnight. The cells were dosed with compound at 1% DMSO in media then cultured for 72 hours prior to addition of 10 µl/well of Cell Proliferation Reagent WST-1 (Roche) for SK-BR-3 and HT-29. The absorbance at 450 and 630 nm was read on a Biotek Synergy 4 multimode reader after 1 hour 37°C incubation and the delta (A_{450}-A_{630}) was plotted against the average of the vehicle-treated wells. Life Technologies CyQUANT direct cell proliferation assay was used for MCF7 and T47D. This general procedure was followed for all cell lines with the following differences: Prior to dosing the SK-BR-3 cells, the complete growth media was removed by aspiration and replaced with compound at 1% DMSO in base media, 0.5% fetal bovine serum, and 60 ng/ml epidermal growth factor in DMEM (100 µl/well). HT-29, MCF7, and T47D cells were dosed by addition of 1 µl of 100X compound in 100% DMSO to complete growth media.

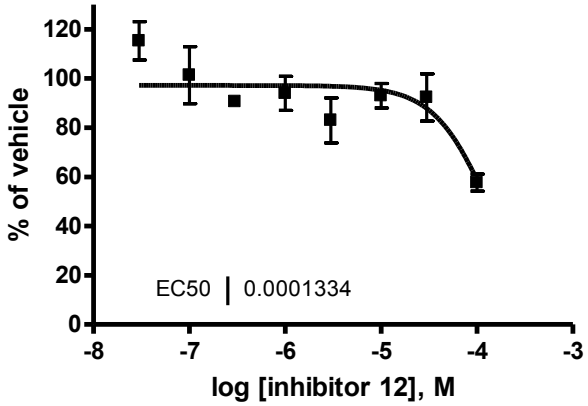
SK-BR-3 GI_{50} = 14 µM



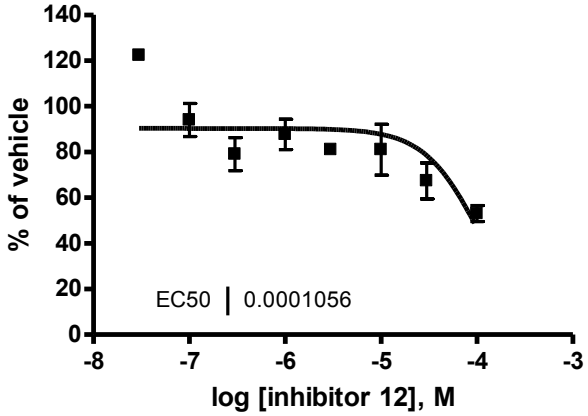
HT-29 GI_{50} = 37 µM



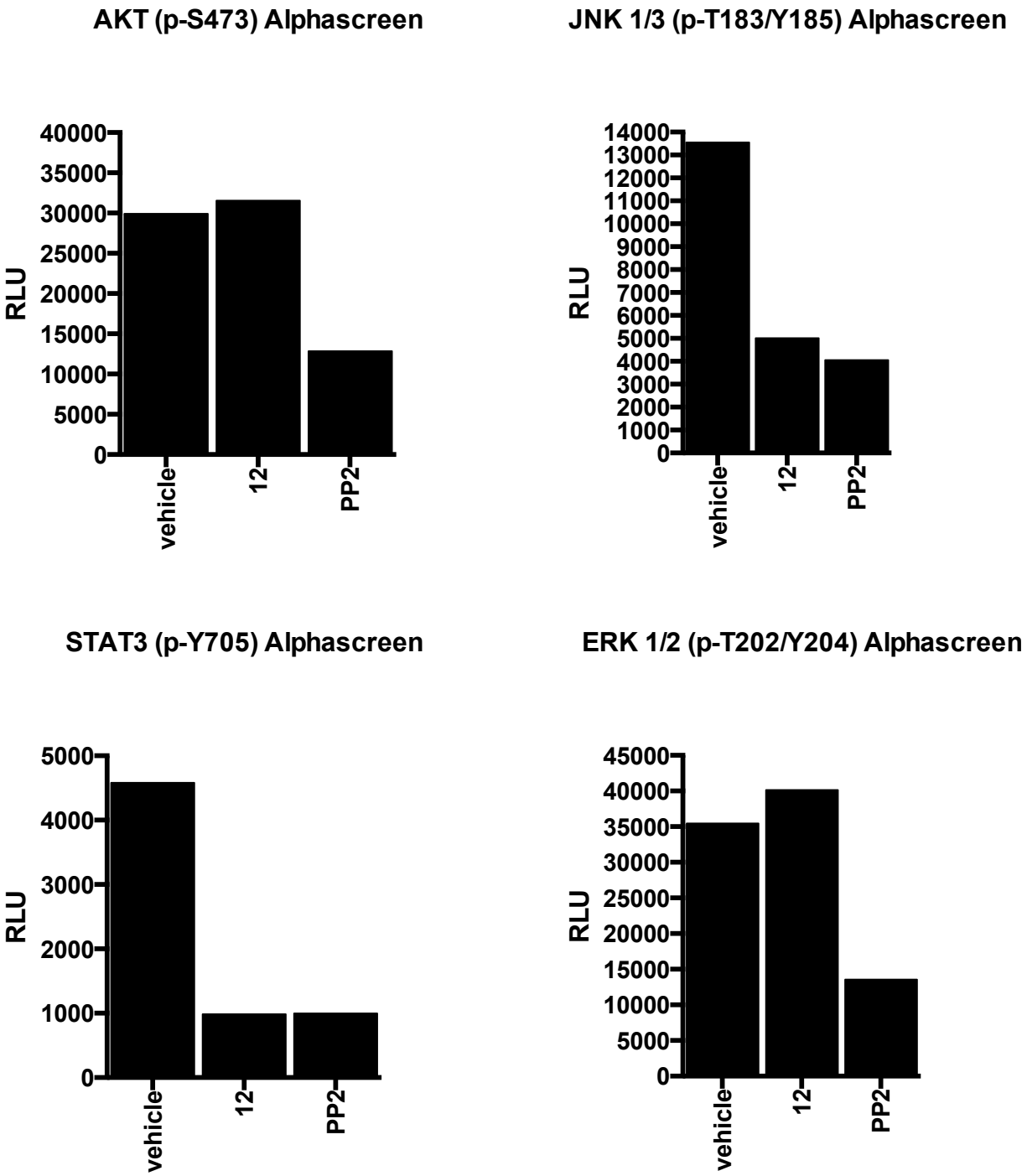
T47D GI_{50} = 133 µM



MCF7 GI_{50} = 106 µM



AlphaScreen SureFire Cell Signaling Assays. SK-BR-3 cells (ATCC) were plated in 96-well plates at a density of 1.0–2.0 x 10^4 cells per well. The cells were grown to 80-90% confluency prior to overnight serum-starvation in DMEM, 0.1% BSA. The serum-free media was then removed and replaced with DMEM containing 10 μM compound **12** (or PP2) in 1% DMSO. The cells were incubated for 60 min prior to addition of EGF (Sigma Aldrich). After incubation, the media was removed and 50 μL AlphaScreen lysis buffer (PerkinElmer) was added to each well. The lysates were analyzed using the AlphaScreen SureFire assay kit (PerkinElmer) according to the manufacturer's protocol. For each compound, n = 3.



X. REFERENCES

1. Maiti, D.; Buchwald, S. L. *J. Am. Chem. Soc.* **2009**, *131*, 17423.
2. Songyang, Z.; Carraway III, K. L.; Eck, M. J.; Harrison, S. C.; Feldman, R. A.; Mohammadi, M.; Schlessinger, J.; Hubbard, S. R.; Smith, D. P.; Eng, C.; Lorenzo, M. J.; Ponder, B. A. J.; Mayer, B. J.; Cantley, L. C. *Nature* **1995**, *373*, 536.
3. Soellner, M. B.; Rawls, K. A.; Grundner, C.; Alber, T.; Ellman, J. A. *J. Am. Chem. Soc.* **2007**, *129*, 9613.
4. Seeliger, M.A.; Young, M.; Henderson, M. N.; Pellicena, P.; King, D.S.; Falick, A. M.; Kuriyan, J. *Protein Sci.* **2005**, *14*, 3135.
5. Wang, Q.; Cahill, S. M.; Blumenstein, M.; Lawrence, D. S. *J. Am. Chem. Soc.* **2006**, *128*, 1808.
6. Carna Biosciences, <http://www.carnabio.com/output/pdf/ProfilingProfilingBook_en.pdf>.
7. Kwarcinski, F. E.; Fox, C. C.; Steffey, M. E.; Soellner, M. B. *ACS Chem. Biol.* **2012**, *7*, 1910.
8. Berenbaum, M. C. *Adv. Cancer Res.* **1981**, *35*, 269.