SUPPORTING INFORMATION

Exquisitely Specific Bisubstrate Inhibitors of c-Src Kinase

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I. SUPPLEMENTAL FIGURES

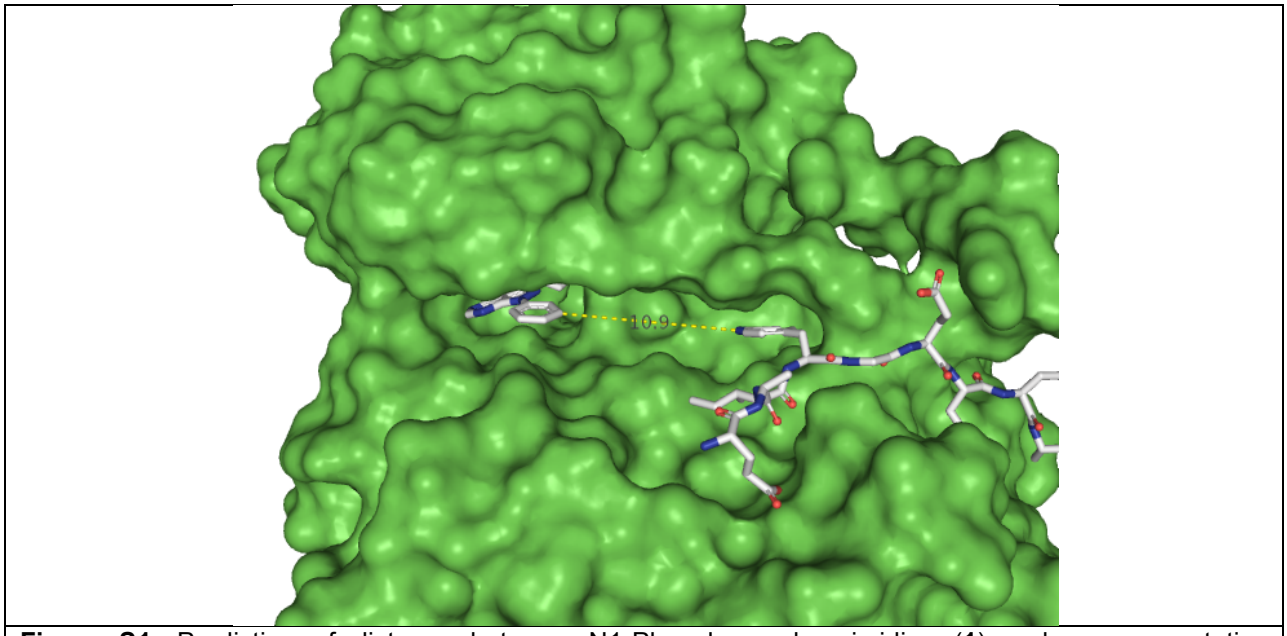


Figure S1. Prediction of distance between N1-Phenylpyrazolopyrimidine (**1**) and a representative substrate peptide bound to a kinase based upon a molecular model. Pyrazolopyrimidine binding was modeled based using a structure of PP2 bound to c-Src kinase (PDB 3GEQ). Peptide substrate binding was visualized using a structure of a bisubstrate inhibitor bound to c-Abl (PDB 2G1T). 3GEQ was aligned with 2G1T using PyMol. In this representation, only the peptidic portion of the inhibitor in 2G1T is shown for clarity. Rendering and distance measurement was performed using PyMol.

Compound	Linker length (n =)	c-Src IC_{50} (nM)
S8	3	159
4	5	< 30
S9	7	121

Table S1. Optimization of linker length for a c-Src bisubstrate inhibitor. Inhibitors were characterized using a continuous fluorescence assay. Assay conditions: 5 mM ATP, 45 μM peptide substrate.

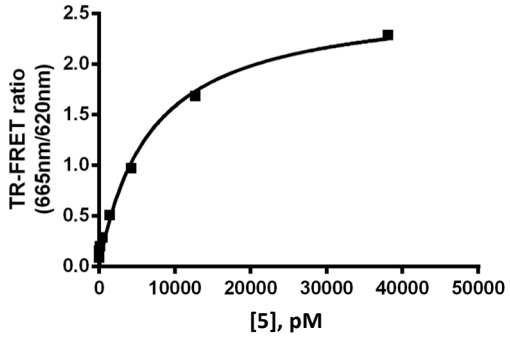
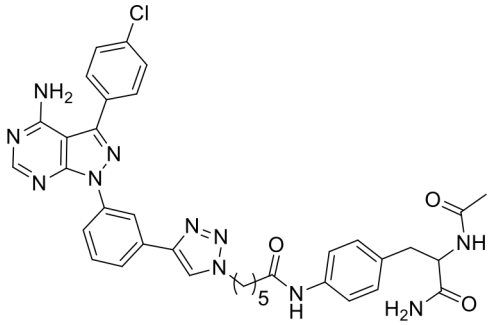


Figure S2. Measurement of the affinity of a fluorescently labeled bisubstrate probe **5** for c-Src. Binding was determined using a TR-FRET assay. A representative run is shown. The probe was found to have an affinity of K_d = 6.3 ± 1.6 nM.

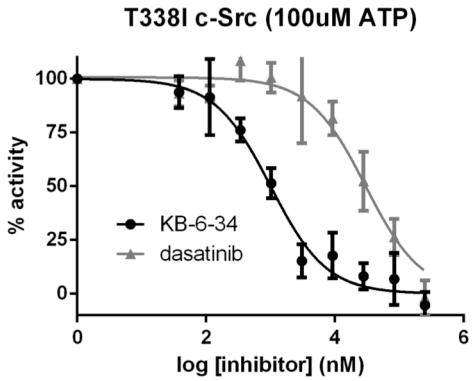


4

IC_{50} (nM)

c-Src	Hck	Abl
1,182	555	1,134

Figure S3. Structure and biochemical characterization of a truncated c-Src bisubstrate inhibitor **4**. Inhibitors were characterized using a continuous fluorescence assay. Assay conditions: 100 μM ATP, 45 μM peptide substrate.



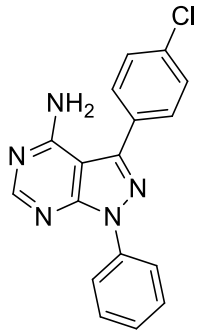
	3	dasatinib
EC50	1009	29699

Figure S4. ATP-competitive vs. bisubstrate-competitive inhibition of a clinically observed resistance mutation (T338I). IC_{50}s were determined using the activity assay described in the Experimental section. In Table legend, KB-6-34 is bisubstrate inhibitor **3**.

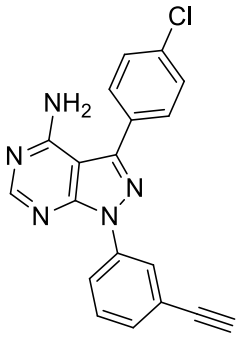
II. GENERAL SYNTHETIC METHODS.

Unless otherwise noted, all reagents were obtained via commercial sources and used without further purification. Mass Spectrometry (HRMS) was carried out by the University of Michigan Mass Spectrometry Facility (J. Windak, director).

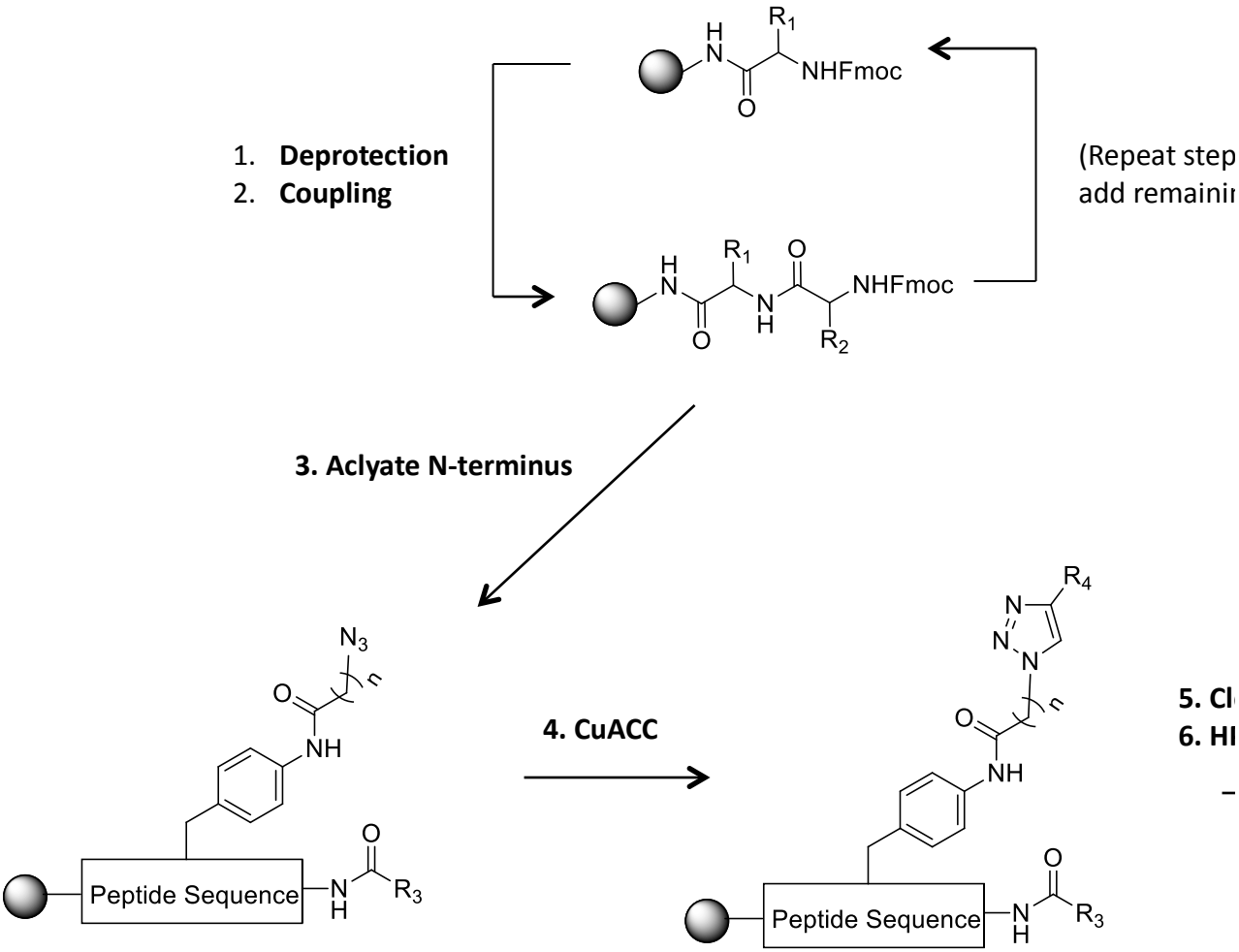
III. SYNTHESIS OF COMPOUNDS 1–6



Synthesis of **1**: **1** was synthesized as previously described.[1]



Synthesis of **2**: **2** was synthesized as previously described.[5]



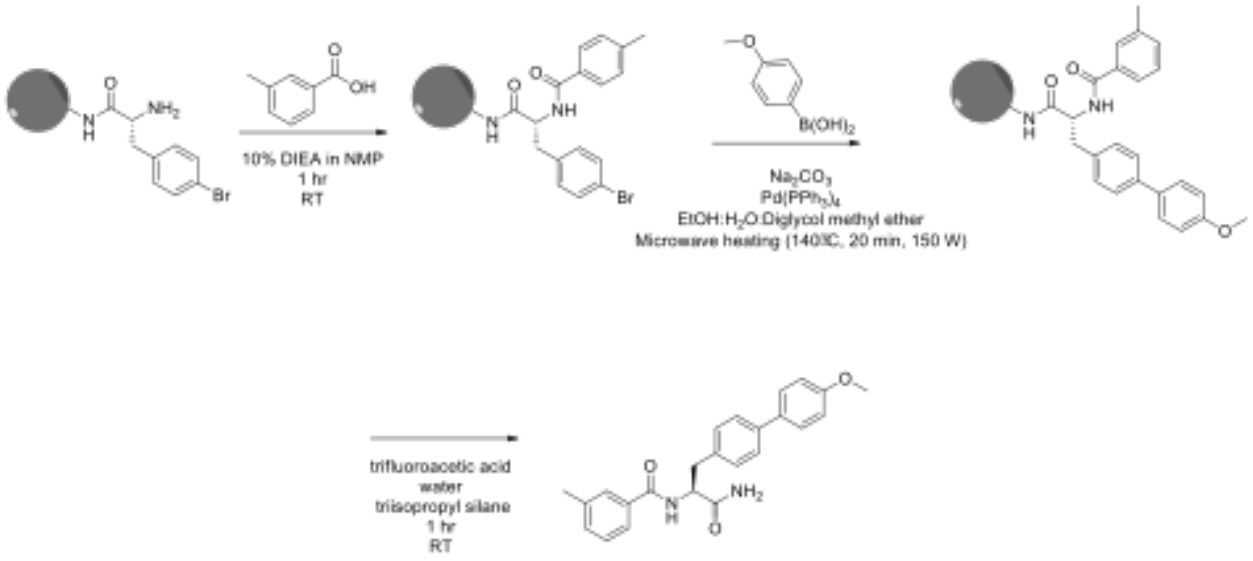
Scheme S1. General synthetic scheme for bisubstrate competitive kinase inhibitors using solid phase peptide synthesis.

Peptide synthesis: Standard solid phase Fmoc peptide synthesis using rink amide resin was performed. Briefly, to a 10 ml peptide synthesis vessel was added 0.1 mmol of rink amide resin and 4 ml of deprotection solution (20% piperidine in NMP). The reaction was sealed and agitated for 30 min. The reaction solution was then removed via filtration and the resin was rinsed three times with NMP. Separately, a solution of 0.3 mmol amino acid and 0.3 mmol HBTU in activator solution (5 % DIEA in NMP) was prepared; this solution was added to the vessel loaded with the pre-swelled rink amide resin. The vessel was sealed and agitated using a mechanical shaker for 30 min. The reaction solution was removed via filtration and the crude resin was rinsed three times with NMP. To the vessel containing the crude resin was added 4 ml deprotection solution (20% piperidine in NMP). The vessel was sealed and agitated using a mechanical shaker for 30 min. The reaction solution was drained and the crude resin was rinsed three times using NMP. The coupling-deprotection sequence was repeated with the amino acids necessary to afford the final desired peptides. After the final Fmoc

deprotection, the terminal amine was acetylated using “Cap-mix A” (80% tetrahydrofuran, 10% acetic anhydride, 10% pyridine).

Solid phase copper-catalyzed cycloadditions: To a 10 mL peptide synthesis vessel which was loaded with 0.1 mmol of resin (prepared as described in previous step) was added 0.3 mmol copper (I) iodide and 4 ml of a 25% diisopropylamine solution in NMP. The reaction mixture was then agitated overnight at room temperature using a mechanical shaker. The reaction solution was drained and the crude resin was rinsed three times respectively using water, then NMP, and finally chloroform. The products were cleaved using a trifluoroacetic acid (TFA) solution (90% TFA, 5% water, 5% triisopropylsilane). TFA was then removed under reduced pressure. The crude reaction mixture was then dissolved in DMSO and purified using reverse phase HPLC (10% -> 85% acetonitrile in water).

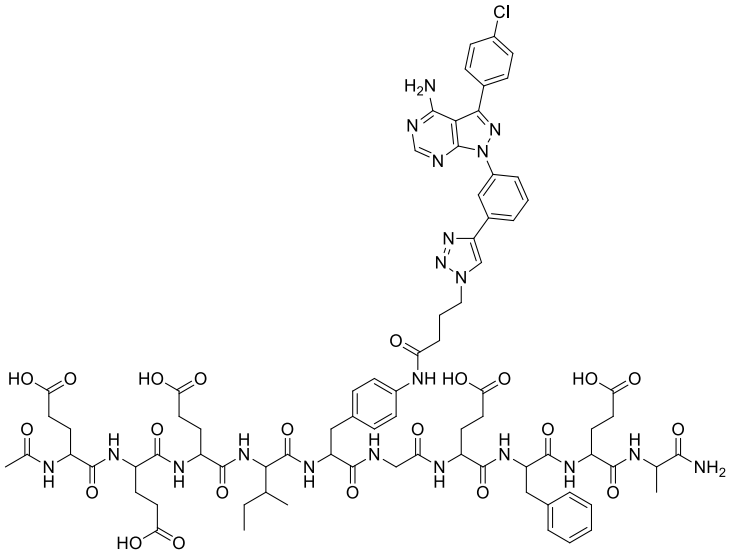
Synthesis of Probe 5. The general protocol for peptide synthesis was followed with the exception being that a dye containing an acid handle for coupling was used to Cap the N-terminus.



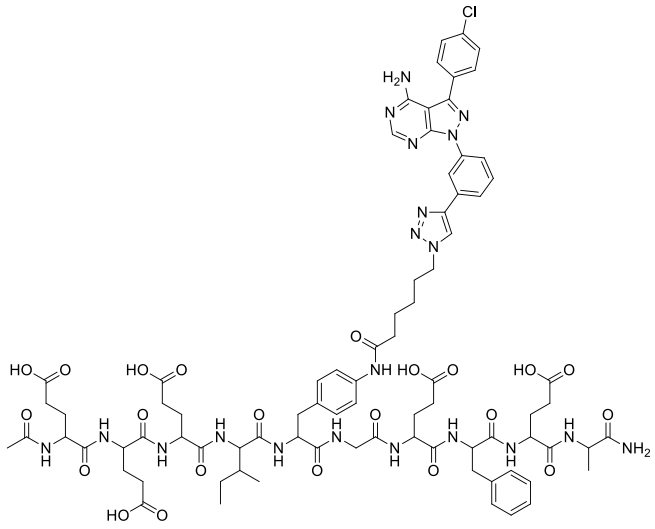
Synthesis of **6**. Compound **6** was separated from resin using cleavage conditions (trifluoroacetic acid 3.8 mL, water 0.1 mL, triisopropyl silane 0.1 mL) for 1 hour with intermittent stirring. The resulting solution was filtered, collected and the solvent was removed under reduced pressure. The resulting oil was purified via reverse-phase chromatography (linear gradient of 5 → 95% CH_3CN (0.1% HOAc) in H_2O (0.1% HOAc)) to yield **BiPH 250** (2.0 mg, <1% yield). **Spectral data**. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.44 (d, J = 8.4 Hz, 1H), 7.66 – 7.42 (m, 6H), 7.41 – 7.24 (m, 4H), 7.11 (d, J = 2.5 Hz, 2H), 7.01 – 6.94 (m, 2H), 4.66 (ddd, J = 10.5, 8.4, 4.2 Hz, 1H), 3.77 (s, 3H), 3.20 – 3.08 (m, 1H), 3.08 – 2.97 (m, 1H), 2.34 (s, 3H). ^{13}C NMR (500 MHz, DMSO-d_6) δ 173.82,

166.76, 159.14, 138.10, 137.86, 137.53, 134.54, 132.73, 132.24, 128.51, 128.40, 127.98, 126.22, 124.99, 114.72, 55.58, 55.16, 37.27, 21.39. HRMS-ESI (*m/z*): $[M+H]^+$ calcd for $C_{24}H_{24}N_2O_3$ 389.1860; found 389.1862.

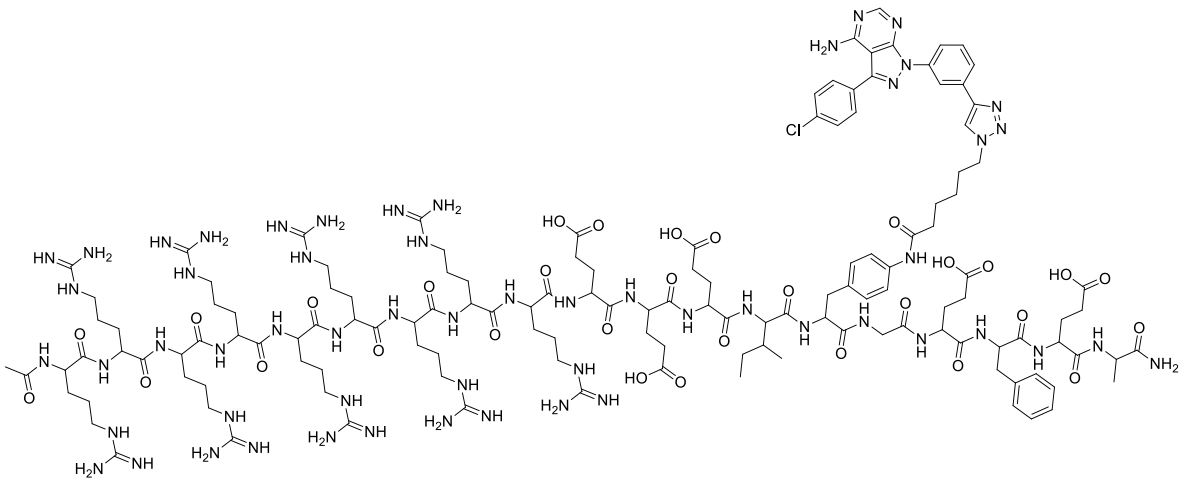
IV. ANALYTICAL DATA FOR COMPOUNDS 3–6 and S8–S11.



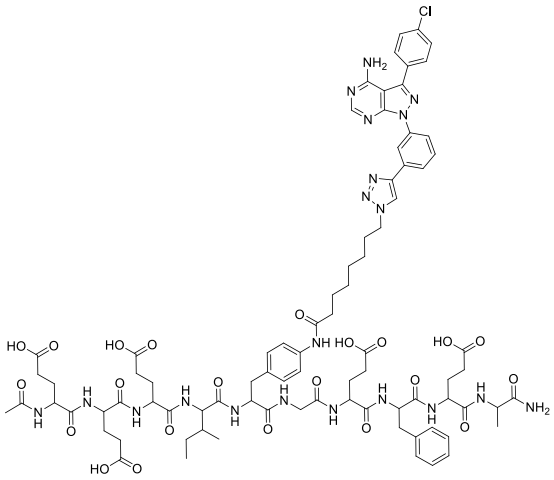
Spectral data for compound S8. HRMS-QTOF ESI+ (*m/z*): $[M + H]^+$ calcd for $C_{79}H_{95}ClN_{20}O_{22}$, 1711.6691; found 1711.6655.



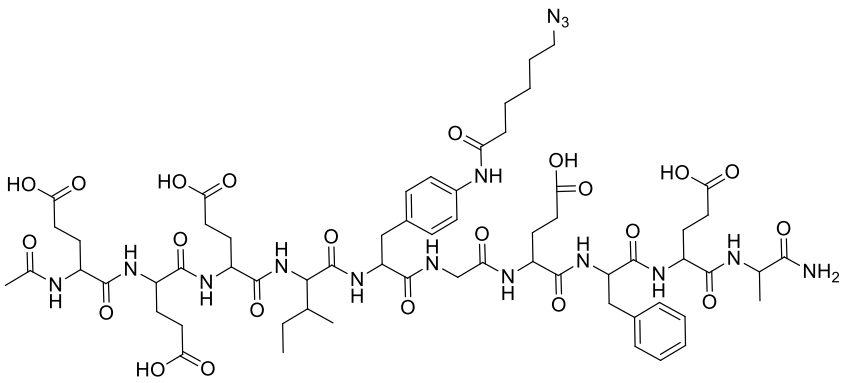
Spectral data for compound 3. HRMS-QTOF ESI+ (*m/z*): $[M + 2H]^{++}$ calcd for $C_{81}H_{99}ClN_{20}O_{22}$, 870.3538; found 870.3510.



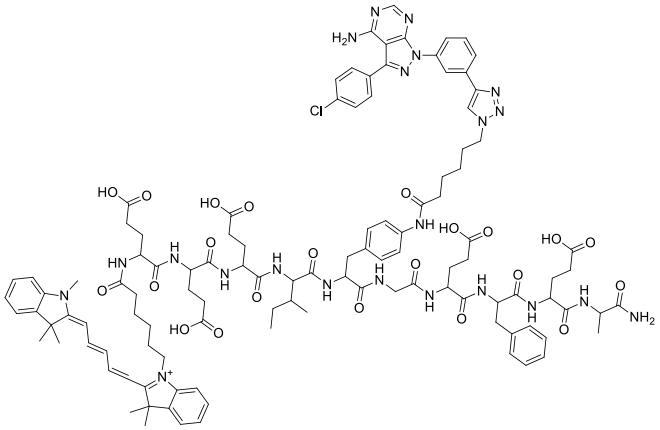
Spectral data for compound 7. HRMS-QTOF ESI+ (*m/z*): $[M + H]^+$ calcd for $C_{121}H_{182}ClN_{48}O_{28}$, 2790.3981, found 2790.5580.



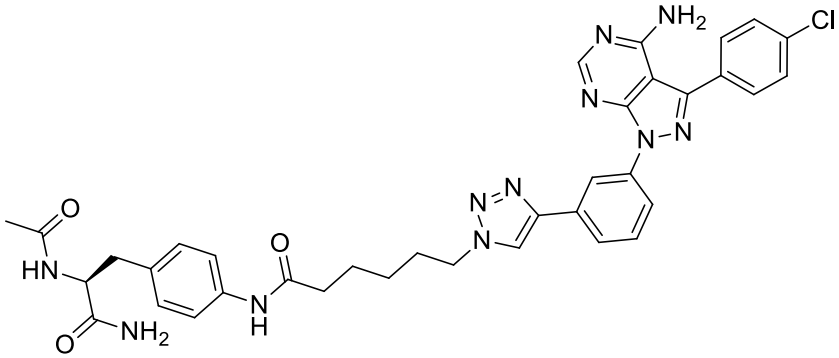
Spectral data for compound S9. HRMS-QTOF ESI+ (*m/z*): $[M + 2H]^{++}$ calcd for $C_{83}H_{103}ClN_{20}O_{22}$, 884.3695; found 884.3687.



Spectral data for compound S10. HRMS-QTOF ESI+ (*m/z*): $[M + H]^+$ calcd for $C_{62}H_{87}N_{15}O_{22}$, 1394.6223; found 1394.6251.



Spectral data for compound 5. HRMS-QTOF ESI+ (*m/z*): $[M + 2H]^{++}$ calcd for C111H133ClN22O22, 1081.9939; found 1081.9893.



Spectral data for compound 4. HRMS-QTOF ESI+ (*m/z*): $[M + H]^+$ calcd for $C_{36}H_{36}ClN_{11}O_3$, 706.2764, found 706.2755.

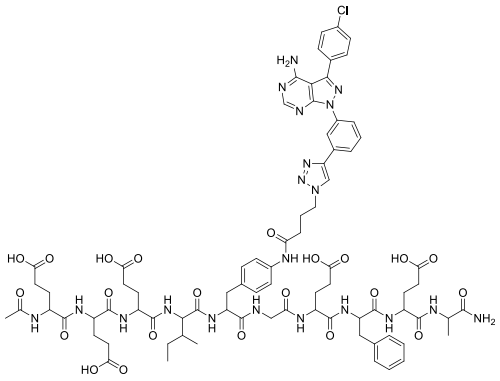
V. BIOCHEMICAL CHARACTERIZATION

A. General procedure for determination of inhibitor IC_{50}.

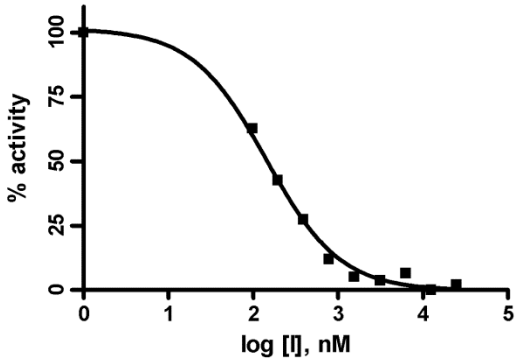
A continuous fluorescence assay[15] was used to determine IC_{50}. Reaction volumes of 100 µL were used in 96-well plates. 85 µL of enzyme in buffer was added to each well. 2.5 µL of the appropriate inhibitor dilution (typically 5000, 1666, 555, 185, 61, 20, 6.8, 2.2, 0.76, 0 µM in DMSO) was then added. 2.5 µL of a substrate peptide ("compound 3" as described in Wang et al[16]) solution (1.8 mM in DMSO) was added. The reaction was initiated with 10 µL of ATP (1 mM in water), and reaction progress was immediately monitored at 405 nm (ex. 340 nm) for 10 minutes. Reactions had final concentrations of 30 nM enzyme, 45 µM peptide substrate, 100 µM Na_3VO_4, 100 mM Tris buffer (pH 8), 10 mM $MgCl_2$, 0.01% Triton X-100; the concentration of ATP varied from 0.1 – 5 mM, and the concentrations are specified for each experiment in the following section. The initial rate data collected was used for determination of IC_{50} values. For IC_{50} determination, the kinetic values were obtained directly from nonlinear regression of substrate-velocity curves in the presence of various concentrations of the inhibitor. The equation Y=Bottom + (Top-Bottom)/(1+10^(X-LogEC50)) was used in the nonlinear regression.

B. Analytical data for c-Src IC_{50} determination.

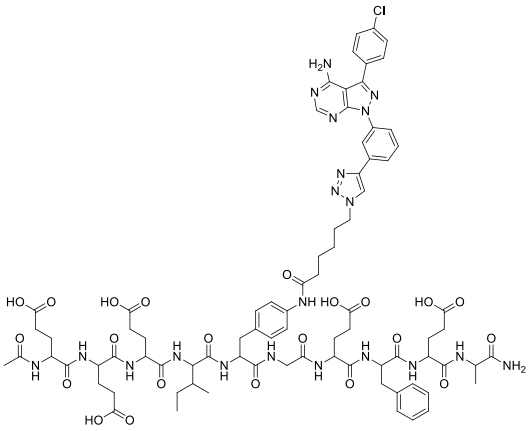
Each inhibitor IC_{50} value was determined using at least three independent experiments; a representative inhibition curve is shown.



S8



Avg IC_{50} = 159 ± 28 nM
[ATP] = 5 mM



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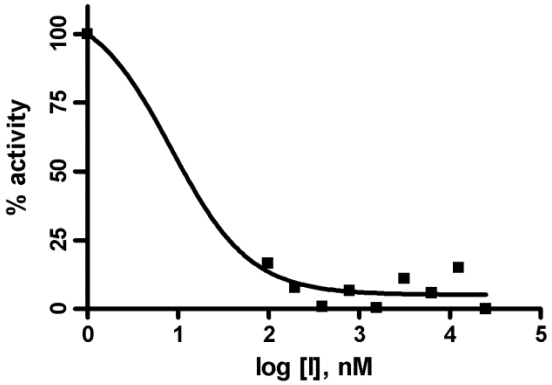
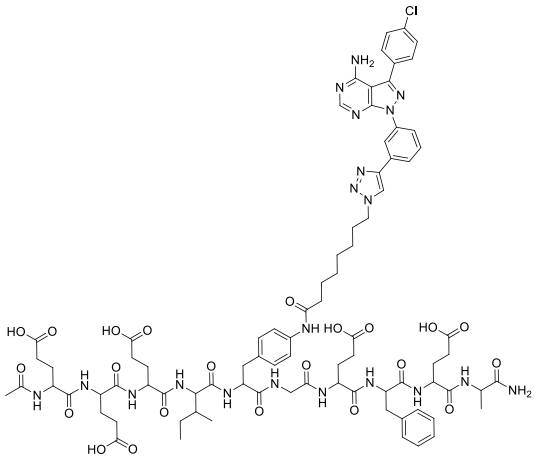
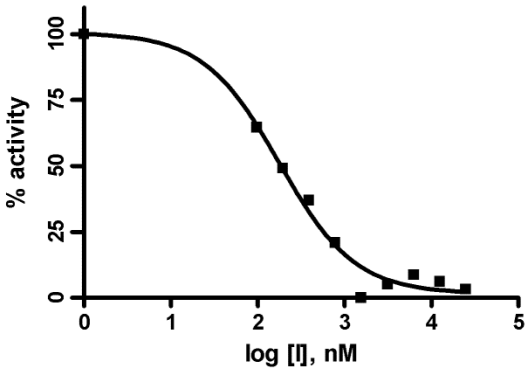


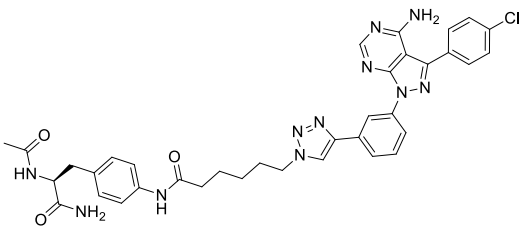
Avg IC_{50} ≤ 8.4 nM
[ATP] = 5 mM



S9



Avg IC_{50} = 121 ± 50 nM
[ATP] = 5 mM



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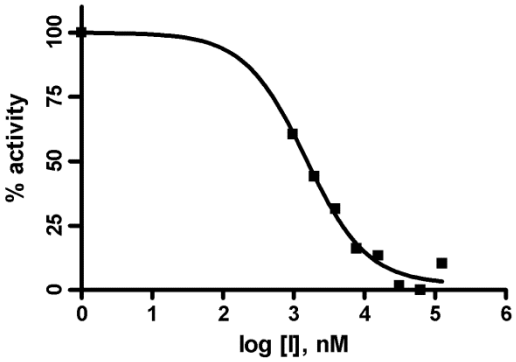
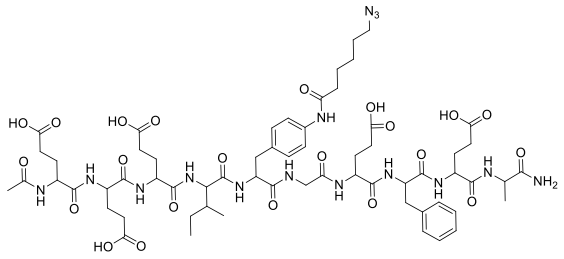
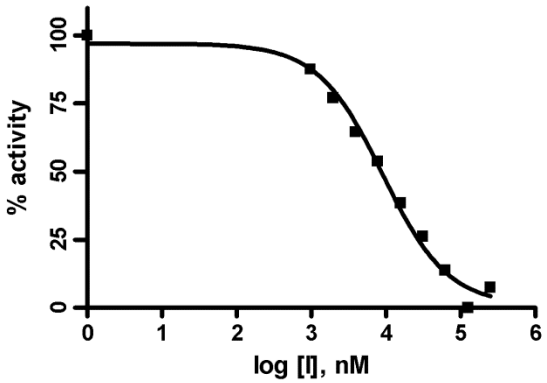


Avg IC_{50} = 1,182 ± 275 nM
[ATP] = 100 μM

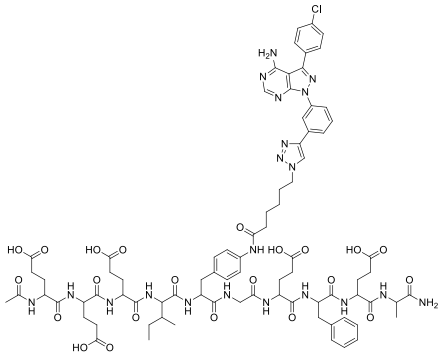


S10



Avg IC_{50} = 8,372 ± 861 nM
[ATP] = 5 mM

C. Analytical data for T338I c-Src IC_{50} determination. Each inhibitor IC_{50} value was determined using at least three independent experiments; a representative inhibition curve is shown.



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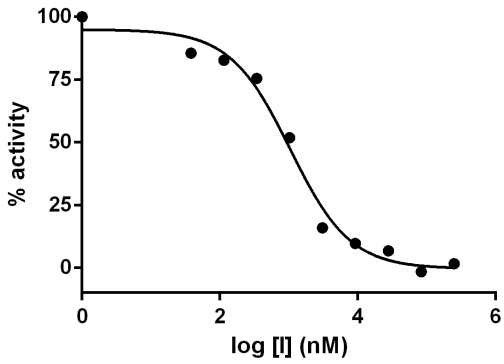
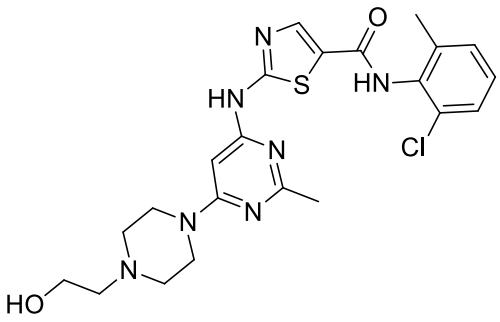
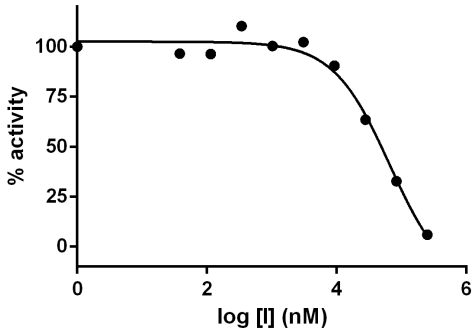


Avg IC_{50} = 953 ± 82 nM
[ATP] = 100 μM

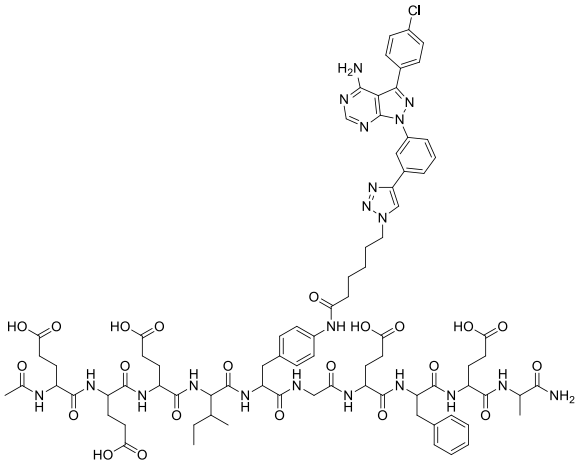


dasatinib



Avg IC_{50} = 50,395 ± 18,520 nM
[ATP] = 100 μM

D. Analytical data for Hck IC_{50} determination. Each inhibitor IC_{50} value was determined using at least three independent experiments; a representative inhibition curve is shown.



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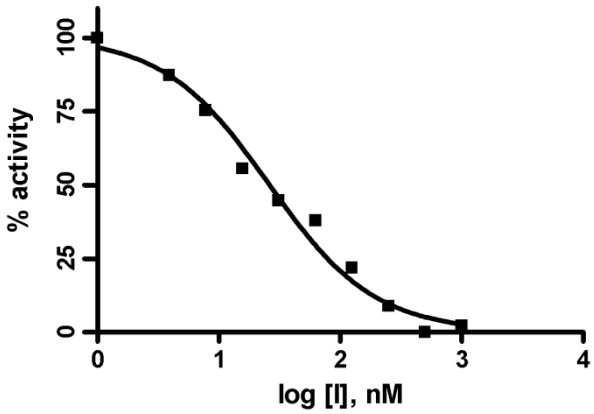
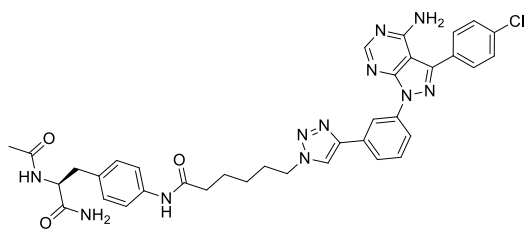


Avg IC_{50} = 37 ± 14 nM
[ATP] = 5 mM



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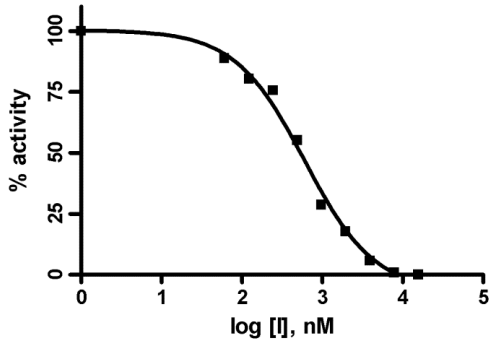
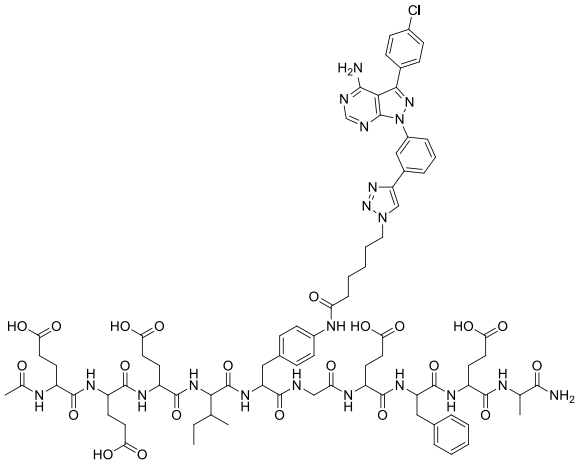
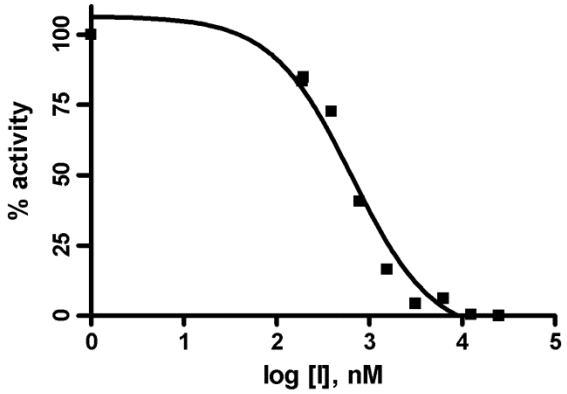


Avg IC_{50} = 555 ± 73 nM
[ATP] = 100 μM

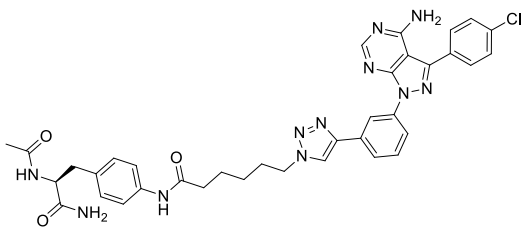
E. Analytical data for c-Abl IC_{50} determination. Each inhibitor IC_{50} value was determined using at least three independent experiments; a representative inhibition curve is shown.



3



Avg IC_{50} = 1,168 ± 731 nM
[ATP] = 5 mM



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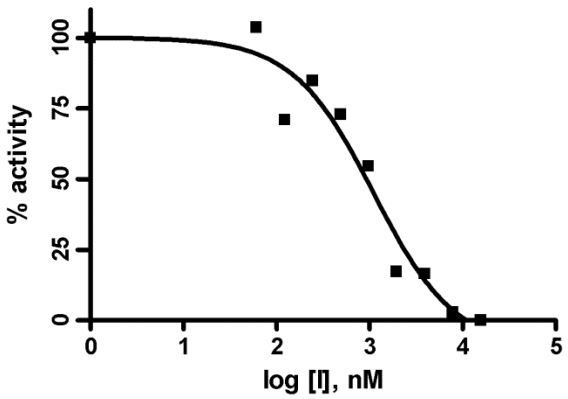
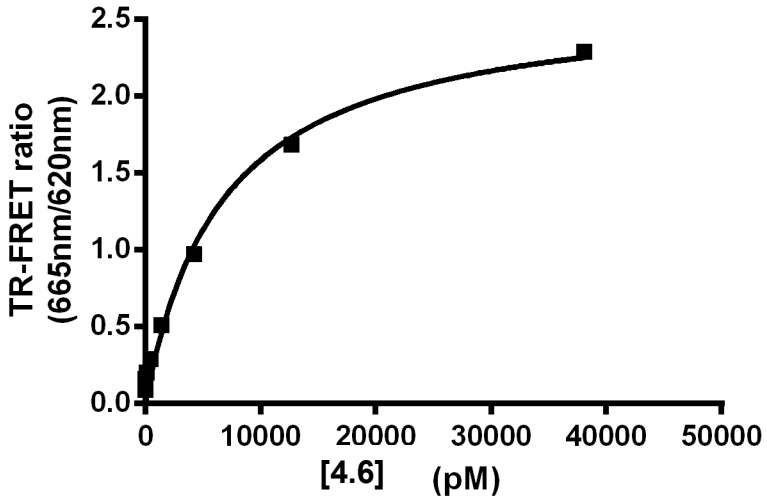


Avg IC_{50} = 1,134 ± 374 nM
[ATP] = 100 μM

F. General procedure for determination of K_d for Cy-5 labeled bisubstrate inhibitor (5).

A Time Resolved FRET (TR-FRET) assay was used to determine K_d. Well volumes of 20 µL were used in 384-well plates. 10 µL of a solution of 6xHis-enzyme and Eu3+ labeled Anti-6xHis antibody in buffer was added to each well. 5 µL of the appropriate probe dilution in buffer (typically 5000, 1666, 555, 185, 61, 20, 6.8, 2.2, 0.76, 0 nM in buffer) was then added. 5 µl buffer was then added. The plate was then centrifuged for 30 seconds at 4,000 rpm. The plate was then allowed to incubate at room temperature for 30 minutes in an area devoid of light. TR-FRET emission was then determined respectively at 650 and 620 nM (ex. 360 ± 40 nm). A 570 ± 100 nm emission filter was used. 20 measurements were collected per data point with 100 µsec of delay time and 200 µsec of data collection. Wells had final concentrations of 5 nM enzyme, 2 nM Eu3+ labeled Anti-6xHis antibody, 50 mM Tris buffer (pH 8.0), 100 mM NaCl, 0.1% BSA, an varying concentrations of probe **5**,. For K_d determination, the kinetic values were obtained directly from nonlinear regression of probe-binding curves. The equation Y=Bmax*X/(Kd+X) was used in the nonlinear regression.

G. Analytical data for c-Src Kd determination of 5.



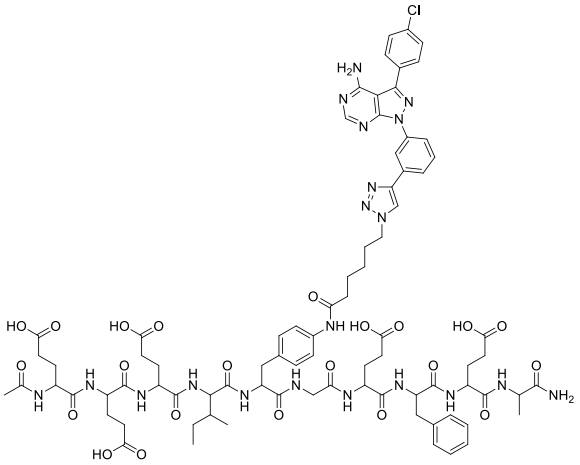
Probe **5** Avg K_d = 6.3 ± 1.6 nM

H. General procedure for competition-assay based determination of inhibitor K_d.

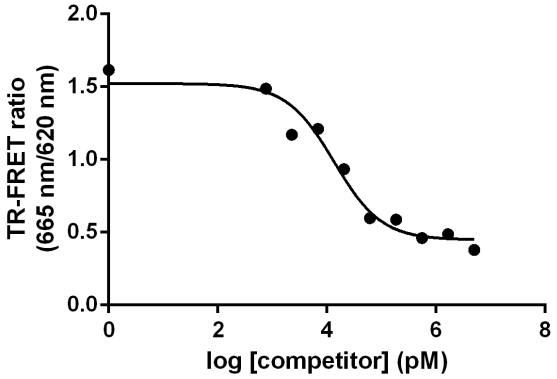
A Time Resolved FRET (TR-FRET) competition assay was used to determine K_d. Reaction volumes of 20 µL were used in 384-well plates. 10 µL of a solution of 6xHis-enzyme and Eu3+ labeled Anti-6xHis antibody in buffer was added to each well. 5 µL solution of probe **5** in buffer was added (final concentrations for individual experiments

are specified below). 5 µL of the appropriate inhibitor dilution (typically 5000, 1666, 555, 185, 61, 20, 6.8, 2.2, 0.76, 0 nM in buffer) was then added. The plate was then centrifuged for 30 seconds at 4,000 rpm. The plate was then allowed to incubate at room temperature for 30 minutes in an area devoid of light. TR-FRET emission was then determined respectively at 650 and 620 nM (ex. 360 ± 40 nm). A 570 ± 100 nm emission filter was used. 20 measurements were collected per data point with 100 µsec of delay time and 200 µsec of data collection. Reactions had final concentrations of 5 nM enzyme, 2 nM Eu3+ labeled Anti-6xHis antibody, 50 mM Tris buffer (pH 8.0), 100 mM NaCl, 0.1% BSA. For K_d determination, the kinetic values were obtained directly from nonlinear regression of probe-binding curves in the presence of various concentrations of the competing ligand. The equation Y=Bottom + (Top-Bottom)/(1+10^(X-LogEC50)) was used in the nonlinear regression.

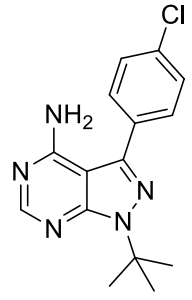
I. Analytical data for c-Src inhibitor K_d determination using probe 5. Each ligand EC_{50} value was determined using at least three independent experiments; a representative binding curve is shown.



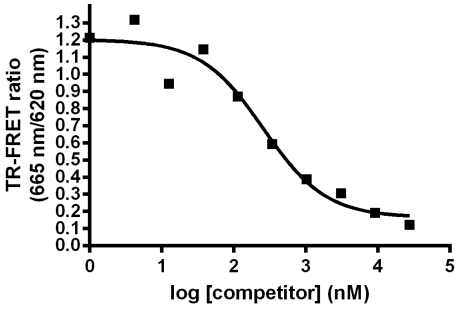
3



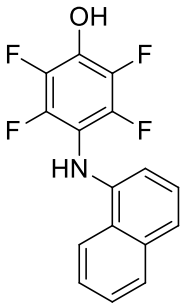
Avg K_d = 280 ± 76 pM
[fluorescent probe **5**] = 400 nM



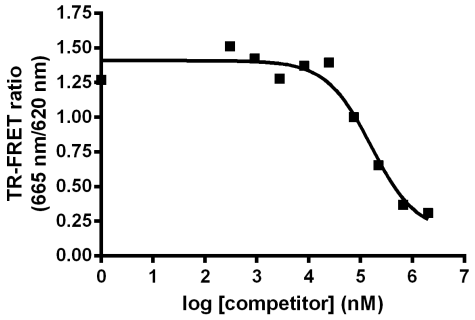
PP2



Avg K_d = 22 ± 7 nM
[fluorescent probe **5**] = 40 nM

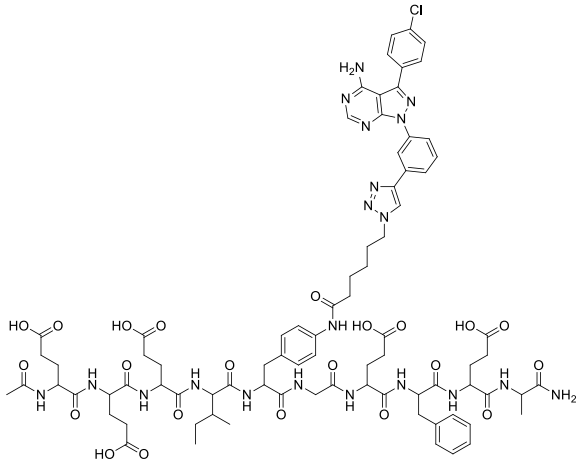


MEB-SCI

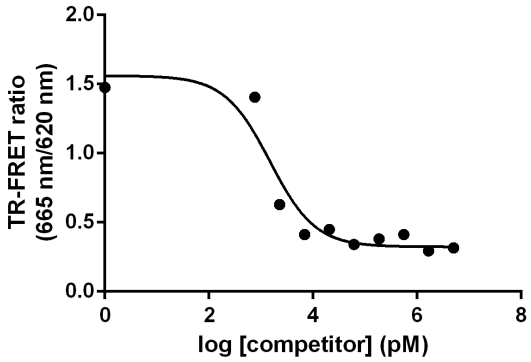


Avg K_d = 15,567 ± 4,304 nM
[fluorescent probe **5**] = 40 nM

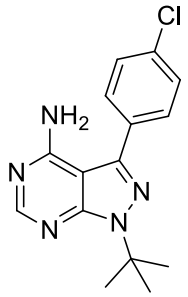
J. Analytical data for c-Src inhibitor K_d determination using kinase Tracer 236. A modified protocol which employed kinase Tracer 236 (final concentrations for individual experiments are specified below)**.** Each ligand EC_{50} value was determined using at least three independent experiments; a representative binding curve is shown.



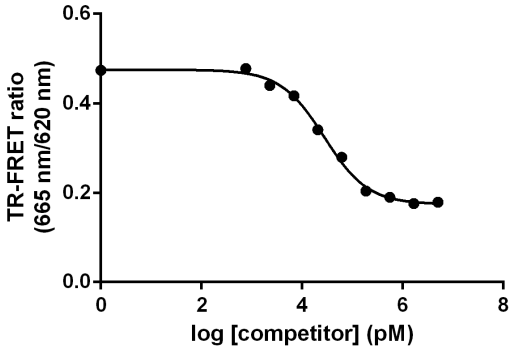
3



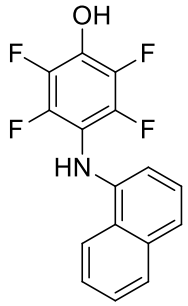
Avg K_d = 187 pM
[tracer 236] = 250 nM



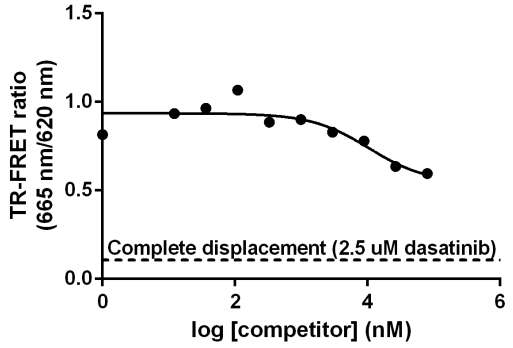
PP2



Avg K_d = 8.9 ± 2.7 nM
[tracer 236] = 50 nM



MEB-SCI



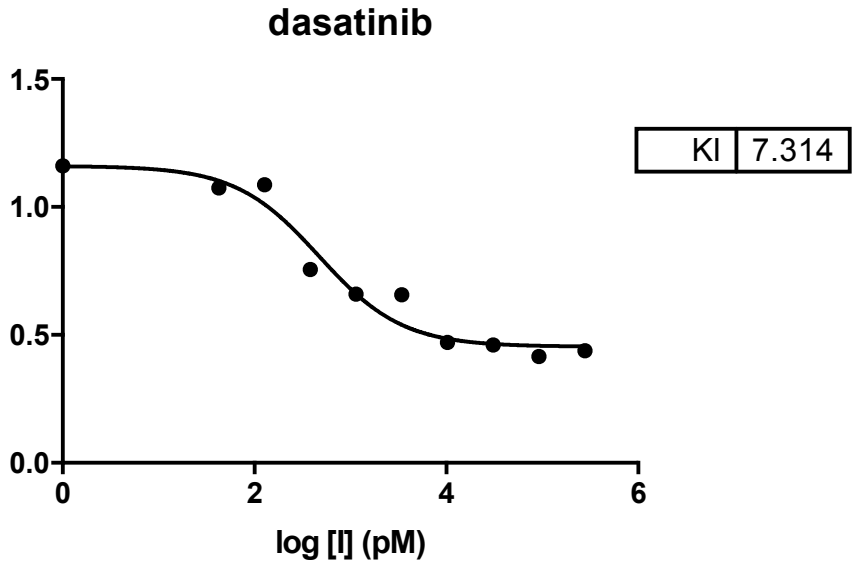
Avg K_d > 330,000
[tracer 236] = 50 nM

K. Time-dependent TR-FRET data using 5:

30 minute incubation, avg K_d = 187 pM

120 minute incubation, avg K_d = 210 pM

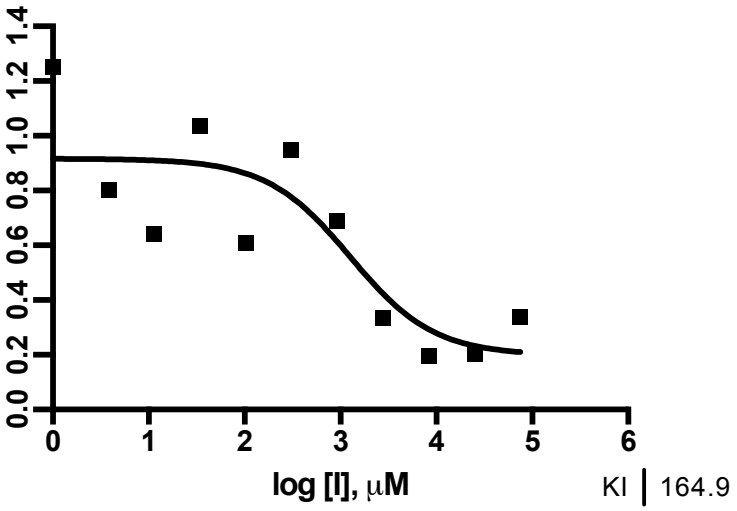
L. K_d determination for a known tight-binder of c-Src using 5 in TR-FRET assays:



Avg K_d = 7.1 ± 1.8 pM
[tracer **5**] = 400 nM

Literature value for thermodynamic K_d for dasatinib = 16 ± 1.0 pM (ref: *J. Med. Chem.*, **2004**, *47*, 6658–6661.

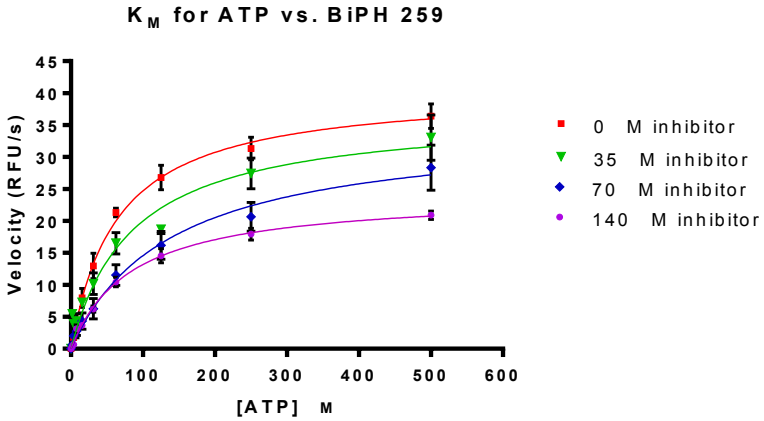
M. Analytical data for non-ATP-competitive inhibitor 6. Each ligand EC_{50} value was determined using at least three independent experiments; a representative binding curve is shown.



Best-fit values	
Bottom	0.1983
Top	0.9166
LogEC50	3.102
EC50	1264
KI	164.9
Ligand (Constant)	40.00
Kd (Constant)	6.000

Avg K_d for **6** = 114 +/- 38 nM
[tracer 5] = 40 nM

N. Analytical data for c-Src Kinase Domain ATP Lineweaver-Burk analysis of inhibitor. Each inhibitor K_M value was determined using at least 4 independent experiments; a representative K_M curve is shown. In the Figure below, "BiPH 259" refers to compound **6**.



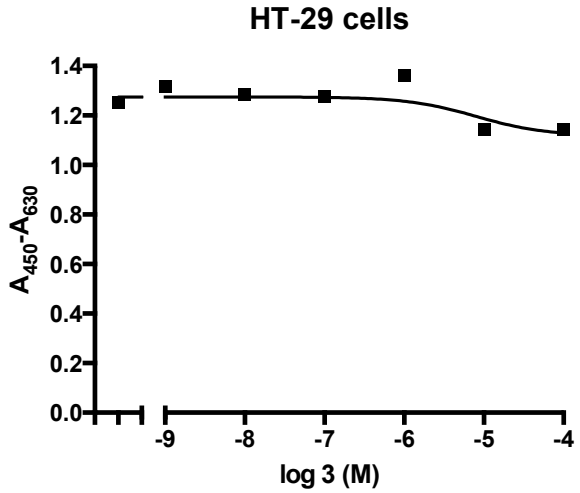
	0 μM inhibitor	35 μM inhibitor	70 μM inhibitor	140 μM inhibitor
Best-fit values				
Vmax	40.60	37.01	34.74	24.25
Km	64.35	85.25	138.7	84.23
Std. Error				
Vmax	1.649	2.527	3.386	0.8552
Km	8.113	16.75	33.78	8.635
95% Confidence Intervals				
Vmax	37.24 to 43.96	31.87 to 42.14	27.85 to 41.63	22.51 to 25.99
Km	47.84 to 80.87	51.18 to 119.3	69.93 to 207.5	66.69 to 101.8

VI. CELLULAR CHARACTERIZATION

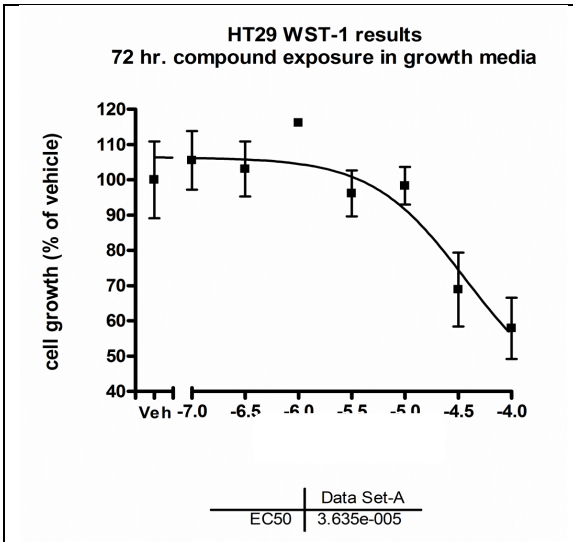
General procedure:

1. **Cell culture and seeding**: Cells are dispersed from flasks and collected by centrifugation (125xg for 5 minutes at room temperature). An aliquot of the resuspended cells is mixed with trypan blue solution and the cell number is quantified using a hemacytometer. In general, depending on the growth rate of the untreated cells, the cells will be plated at 5.0 – 7.5 x 10^3 cells per well. 100 µL of the cell mixture will be added to each well so the concentration should be 10X the cells per well in cells per mL. The cells are plated into sterile, clear bottom 96 well plates and cultured under normal growth conditions overnight prior to dosing with compound.
2. **Dosing**: The 100% DMSO compound stocks need to be prepared to 100X the final concentration that is desired in the assay. 3 µL of the DMSO stock solution is then added to 297 µL of the cell growth media to give a DMSO concentration of 1%. The cell media is removed by aspiration for adherent cells and replaced with 100 µL per well of the cell growth media containing the compound. In general each compound concentration is dosed in triplicate wells. The plates are returned to normal culture conditions for 24 – 72 hours.
3. **Assay**: After the required incubation period the plates are removed from the incubator and 10 µL per well of WST-1 reagent is added. The plates are returned to the incubator and the color change is visually monitored for 0.5 – 2 hours. When sufficient color change has occurred the plates are shaken on a plate shaker for 60 seconds and read in the appropriate plate reader.
4. **Data Analysis**: The reference absorbance reading is subtracted from the formazan absorbance and the data is plotted as a percentage of the vehicle (1% DMSO alone). Data analysis and curve fitting was performed using Graphpad Prism. For each cell line, there were n = 3 data points for each concentration. Each dose response curve was performed at least twice, providing n ≥ 6 for each data point.

HT-29 proliferation vs 3:

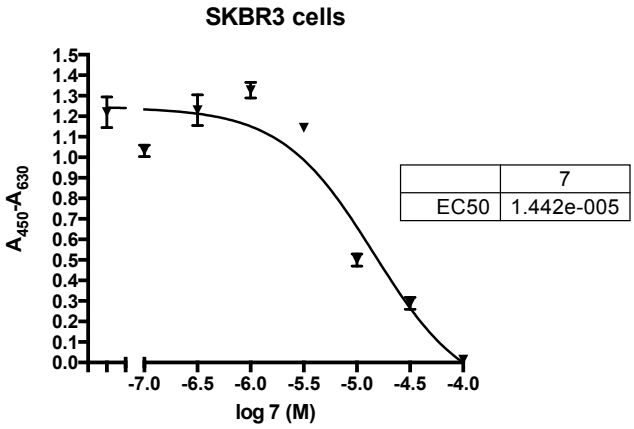


HT-29 proliferation vs 7:



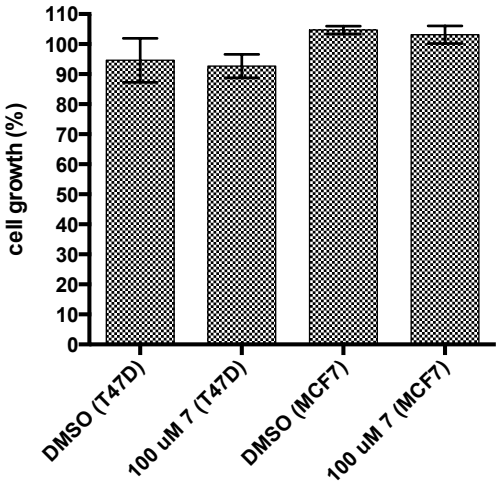
Treatment of HT-29 colon cancer cells with **7**

SKBR3 proliferation vs 7:

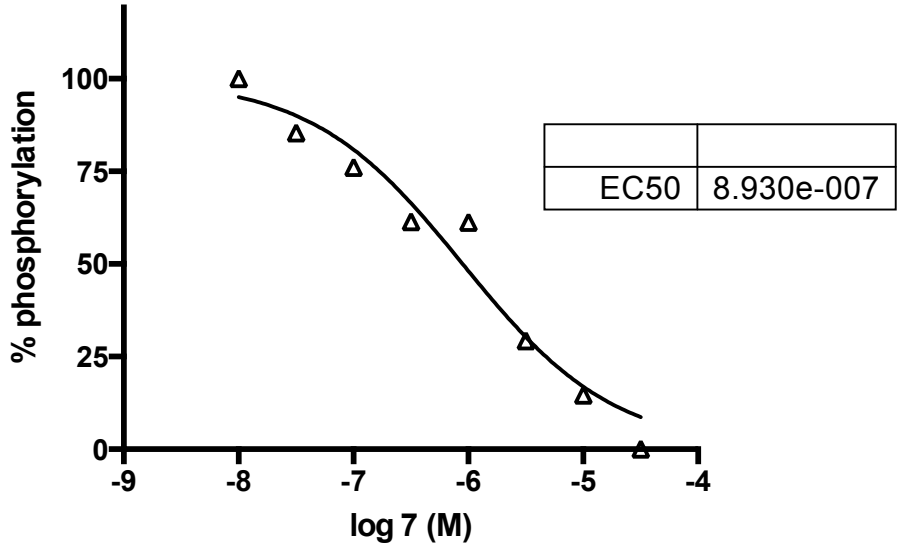


	7
EC50	1.442e-005

Proliferation of non-Src dependent cancer cells lines (T47D and MCF7). DMSO vs control:



c-Src autophosphorylation with 7. 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.

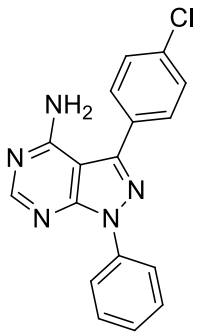


VII. KINOME PROFILING

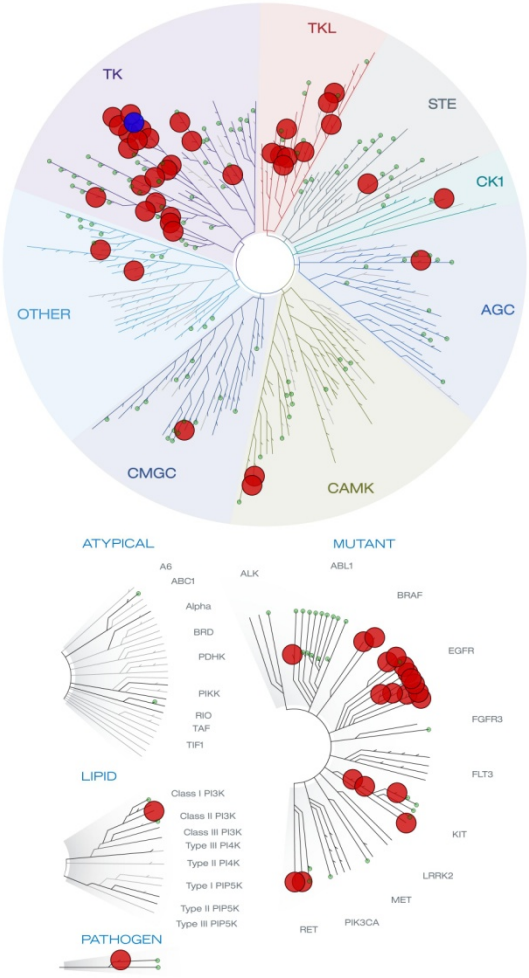
KINOMEscan profiling

Kinome profiling for compound **1** was performed by KINOMEscan (DiscoveRx, Fremont, CA). The compound was profiled at a concentration of 10 μM.

A. 1 TREEspot analysis:



1



Percent Control

≤ 35%

> 35%

B. S-Score for 1:

S(35) = 0.252

C. Tabulated data for compound 1:

KINOMEscan Gene Symbol	% control
ABL1(E255K)-phosphorylated	36
ABL1(F317I)-nonphosphorylated	100
ABL1(F317I)-phosphorylated	76
ABL1(F317L)-nonphosphorylated	100
ABL1(F317L)-phosphorylated	44
ABL1(H396P)-nonphosphorylated	20
ABL1(H396P)-phosphorylated	52
ABL1(M351T)-phosphorylated	90
ABL1(Q252H)-nonphosphorylated	73
ABL1(Q252H)-phosphorylated	51
ABL1(T315I)-nonphosphorylated	100
ABL1(T315I)-phosphorylated	87
ABL1(Y253F)-phosphorylated	57
ABL1-nonphosphorylated	59
ABL1-phosphorylated	49
ABL2	27
ACVR1	20
ACVR1B	62
ACVR2A	50
ACVR2B	8.6
ACVRL1	15
ADCK3	64
AKT1	99
AKT2	99
ALK	100
AURKA	90
AURKB	94
AXL	69
BLK	0.75
BMPR2	100
BMX	37
BRAF	22
BRAF(V600E)	18
BRK	3.2
BTK	66
CDK11	44
CDK2	83
CDK3	100
CDK7	97
CDK9	76
CHEK1	100
CSF1R	40
CSK	92
CSNK1D	85
CSNK1E	0.35
CSNK1G2	78
DCAMKL1	99
DDR1	28
DDR2	61
DMPK	81
DMPK2	21
DYRK1B	79
EGFR	4.4
EGFR(E746-A750del)	8.2
EGFR(G719C)	0.45
EGFR(G719S)	1.6
EGFR(L747-E749del, A750P)	6.4
EGFR(L747-S752del, P753S)	6.2
EGFR(L747-T751del,Sins)	6.6
EGFR(L858R)	9.6
EGFR(L858R,T790M)	80
EGFR(L861Q)	5.7
EGFR(S752-I759del)	6.2
EGFR(T790M)	33
EPHA1	13
EPHA2	51
EPHA3	66
EPHA4	44
EPHA5	54
EPHA6	64
EPHA7	100
EPHA8	15
EPHB1	75
EPHB2	66
EPHB3	16
EPHB4	60
EPHB6	31
ERBB2	56
ERBB3	48
ERBB4	31
ERK1	92
FAK	92
FGFR1	92
FGFR2	86
FGFR3	100

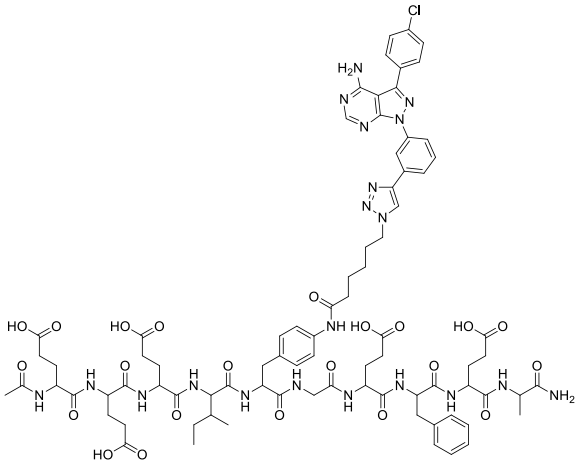
FGFR4	100	MET	100
FGR	5.2	MKNK1	100
FLT1	83	MKNK2	100
FRK	5.8	MLK1	96
FYN	7	MRCKA	55
GAK	4.7	MRCKB	70
GCN2(Kin.Dom.2,S808G)	90	MST4	80
GSK3B	100	NLK	13
HCK	3.3	p38-alpha	90
IGF1R	100	p38-beta	73
IKK-alpha	100	PAK1	100
IKK-beta	100	PAK2	78
INSR	81	PAK4	99
JAK2(JH1domain-catalytic)	97	PCTK1	100
JAK3(JH1domain-catalytic)	92	PDGFRA	38
JNK1	94	PDGFRB	0.5
JNK2	84	PDPK1	96
JNK3	91	PFCDPK1(P.falciparum)	13
KIT	1	PFPK5(P.falciparum)	80
KIT(A829P)	86	PIK3C2B	100
KIT(D816H)	96	PIK3CA	91
KIT(D816V)	6.8	PIK3CG	12
KIT(L576P)	1.8	PIM1	100
KIT(V559D)	0.5	PIM2	77
KIT(V559D,T670I)	79	PIM3	100
KIT(V559D,V654A)	37	PKAC-alpha	37
KIT-autoinhibited	89	PKMYT1	65
LCK	0.5	PKNB(M.tuberculosis)	100
LIMK1	77	PLK1	100
LIMK2	85	PLK3	91
LKB1	88	PLK4	67
LOK	81	PRKCE	100
LYN	21	RAF1	20
MAP3K4	89	RET	7.6
MAP4K2	94	RET(M918T)	3.6
MAP4K3	96	RET(V804L)	82
MAP4K4	97	RET(V804M)	97
MAP4K5	100	RIOK2	100
MAPKAPK2	100	RIPK2	0.25
MARK3	83	ROCK1	77
MEK1	65	ROCK2	83
MEK2	65	RSK2(Kin.Dom.1-N-terminal)	79
MEK3	96	SIK	12
MEK4	100	SIK2	18
MEK5	7.9	SLK	77

SNARK	79	TNNI3K	75
SRC	0.15	TRKA	69
SRMS	74	TSSK1B	91
SRPK3	100	TXK	1.1
STK36	33	TYK2(JH1domain-catalytic)	100
SYK	80	ULK2	98
TEC	100	VEGFR2	100
TESK1	23	WEE1	100
TGFBR1	68	YANK3	78
TGFBR2	3.1	YES	11
TIE2	89	ZAK	33
TNIK	83	ZAP70	100
TNK2	52		

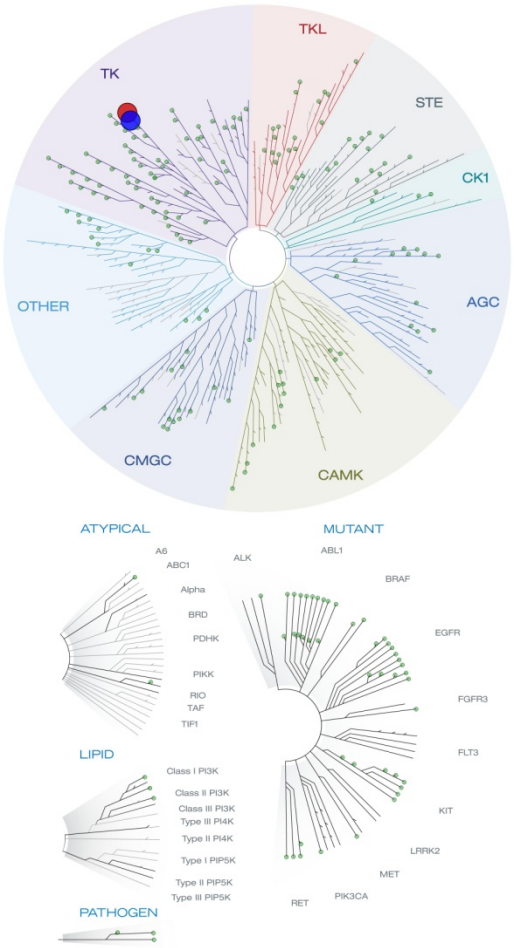
Luceome selectivity profiling

Kinome profiling for compound **3** was performed by KinaseSeeker™ (Luceome Biotechnologies, Tucson, AZ). The compound was profiled at a concentration of 115 nM.

A. Compound 3 TREEspot analysis:



4.3



Percent Control

≤ 35%

> 35%

B. S-Scores for 3:

S(35) = 0.010

C. Tabulated Luceome selectivity profiling data for compound 3:

Kinase	Family	% Activity Remaining
ABL1	TK	100.0
ABL2	TK	96.5
AKT1	AGC	100.0
AKT1(FL)	AGC	96.8
AKT2	AGC	98.4
AKT2(S474A)	AGC	94.7
AKT2(S474D)	AGC	94.9
AKT2(T309A,S474A)	AGC	94.4
AKT2(T309D,S474D)	AGC	90.9
AKT3	AGC	100.0
AMPK-a1	CAMK	99.7
AMPK-a2	CAMK	100.0
AURKA	Other	100.0
AURKB	Other	100.0
AURKC	Other	96.3
AXL	TK	100.0
BIKE	Other	96.5
BLK	TK	42.4
BTK	TK	100.0
CAMK1	CAMK	99.9
CAMK1D	CAMK	99.1
CAMK1G	CAMK	100.0
CAMK2A	CAMK	100.0
CAMK2B	CAMK	97.1
CAMK2D	CAMK	100.0
CAMKK1	Other	100.0
CAMKK2	Other	100.0
CHEK1	CAMK	96.9
CK1D	CK1	100.0
CLK1	CMGC	100.0
CLK2	CMGC	100.0
CSK	TK	95.3
DAPK1	CAMK	100.0
DAPK2	CAMK	100.0
DAPK3	CAMK	97.4
DDR1	TK	95.0
DDR2	TK	96.3
DMPK	AGC	100.0
EPHA1	TK	100.0
EPHA2	TK	100.0
EPHA3	TK	100.0
EPHA4	TK	100.0
EPHB2	TK	100.0
EPHB3	TK	90.3
EPHB4	TK	98.9
FGFR2	TK	100.0
FLT1	TK	100.0
FLT2	TK	92.5
FLT3	TK	100.0
FYN	TK	37.7
GSK3a	CMGC	100.0
HCK	TK	54.7
IGF1R	TK	92.1
IKK-e	Other	97.3
INSR	TK	100.0
ITK	TK	100.0
LIMK1	TKL	100.0
LYN	TK	78.4
MARK1	CAMK	90.1
MARK2	CAMK	100.0
MARK3	CAMK	100.0
MARK4	CAMK	100.0
MELK	CAMK	100.0
MET	TK	100.0
MLK1	TKL	100.0
MLK3	TKL	100.0
MST2	STE	81.0
MUSK	TK	100.0
MYLK	CAMK	88.0
MYLK2	CAMK	100.0
p38-g	CMGC	97.0
PAK1	STE	100.0
PAK1(T423A)	STE	100.0
PAK1(T423E)	STE	99.2
PDGFRA	TK	100.0
PDGFRB	TK	100.0
PDK1	AGC	100.0
PHKG1	CAMK	90.0
PIM1	CAMK	88.3
PIM2	CAMK	100.0
PKAC-a	AGC	98.9
PKAC-b	AGC	100.0
PKC-d	AGC	94.9
PKC-e	AGC	98.7
PKC-g	AGC	100.0

PKC-h	AGC	100.0
PKC-t	AGC	84.0
PRKD2	CAMK	100.0
PRKD3	CAMK	93.3
PKG1	AGC	100.0
PKN3	AGC	74.7
PLK4	Other	96.5
PKX	AGC	100.0
PTK2	TK	100.0
PTK2B	TK	92.7
PTK6	TK	76.7
RET	TK	91.9
RIPK2	TK	95.5
RPS6KA1/RSK1	AGC	94.4
RPS6KA2/RSK3	AGC	100.0
RPS6KA3/RSK2	AGC	100.0
RPS6KA4/MSK2	AGC	82.4
RPS6KA5/MSK1	AGC	98.0
RPS6KA6/RSK4	AGC	98.8
SGK2	AGC	95.1
SGK3	AGC	100.0
SNF1LK	CAMK	87.7
SNF1LK2	CAMK	84.9
SIK3	CAMK	96.3
SLK	STE	100.0
SNARK	CAMK	77.1
SRC	TK	15.9
STK16	Other	78.5
STK33	CAMK	97.5
SYK	TK	98.7
TBK1	Other	100.0
TEC	TK	100.0
TESK1	TKL	100.0
TESK2	TKL	85.7
TIE1	TK	100.0
TIE2	TK	95.0
TNK2	TK	100.0
TNNI3K	TKL	97.6
TRKB	TK	97.0
TRKC	TK	100.0
TKX	TK	63.4
VEGFR2	TK	100.0
YANK2	AGC	100.0
YES1	TK	17.4
YSK1	STE	88.2

VIII. REFERENCES

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