Staurosporine Tethered Peptide Ligands for cAMP-dependent Protein Kinase A (PKA): Optimization and Selectivity Profiling

Carolyn D. Shomin, Scott C. Meyer and Indraneel Ghosh*

Supplementary Information

Contents

Inhibitor Characterization: Mode of Inhibition

Kinetic experiments with PKA and the BL and cyclic peptide data were fit to the following models to determine the mode of inhibition and the inhibitor Ki.

$$v = \frac{V\max}{1 + \left(\frac{Km}{[S]}\right)\left(1 + \frac{I}{Ki}\right)}$$

Equation S1. Competitive Inhibition

$$v = \frac{V\max}{\left(1 + \frac{I}{Ki} + \frac{Km}{[S]}\right)}$$

Equation S2. Uncompetitive Inhibition

$$v = \frac{V\max}{\left(1 + \frac{I}{Ki}\right)\left(1 + \frac{Km}{[S]}\right)}$$

Equation S3. Noncompetitive Inhibition

$$v = \frac{V\max}{\left(\left(\frac{Km}{[S]}\right)\left(1 + \frac{I}{Ki}\right) + \left(1 + \frac{I}{\alpha Ki}\right)\right)}$$

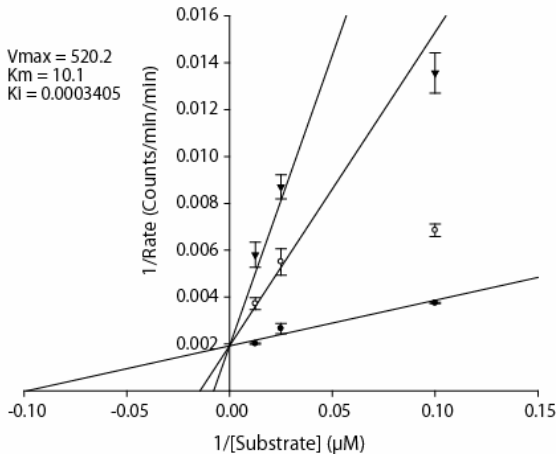
Equation S4. Mixed Inhibition

Where:
v = rate (Counts/min/min)
Km = substrate concentration at half maximal rate
Vmax = maximal rate
S = substrate (kemptide, LRRASLG)
Ki = inhibitor dissociation constant
α = constant

Kinetic Analysis of the initial PKA BL: Compound 1 with PKA to Determine the Mode of Inhibition

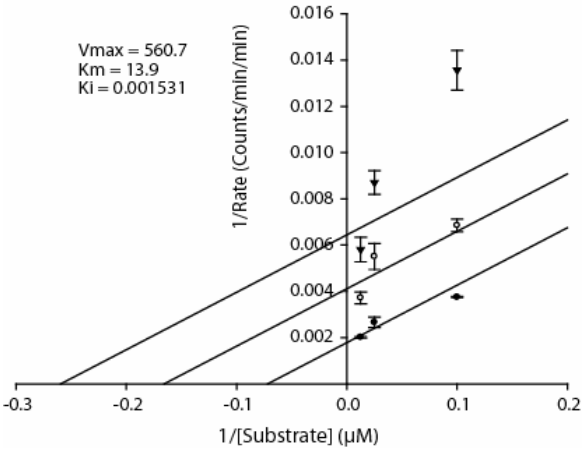
Competitive Inhibition

Parameters	
Vmax	520 ± 42
Km	10.1 ± 3.4
Ki	3.4e-4 ± 1.0e-4
Goodness of Fit	
Degrees of Freedom	15
AICc	143
R2	0.979
Sum of Squares	26,768
Sy.x	42.2



Uncompetitive Inhibition

Parameters	
Vmax	561 ± 43
Km	13.9 ± 3.7
Ki	1.51e-3 ± 2.3e-4
Goodness of Fit	
Degrees of Freedom	15
AICc	138
R2	0.984
Sum of Squares	20,826
Sy.x	37.3



Mixed Inhibition

Parameters	
Vmax	536 ± 35
Km	11.2 ± 2.9
Ki	1.48e-3 ± 8.9e-4
Alpha	1.56
Goodness of Fit	
Degrees of Freedom	14
AICc	136
R2	0.988
Sum of Squares	15,268
Sy.x	33.0

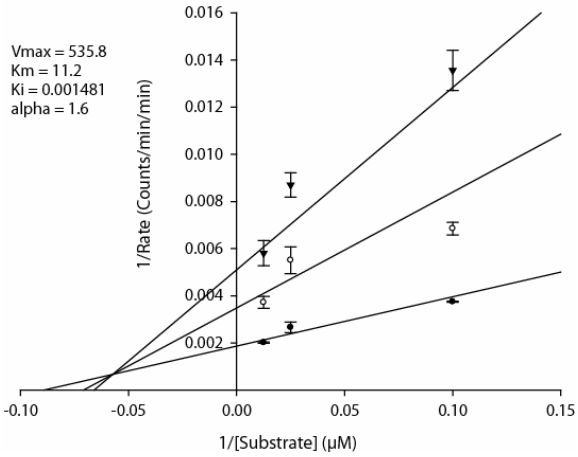


Figure S1. Linewear-Burk Analysis of the PKA Bivalent Ligand, **1**, Kinetic Experiments with PKA fit to competitive, uncompetitive and mixed inhibition.

Kinetic Analysis of the selected cyclic peptide (CTFRVFGCG), **1b**, with PKA to determine the mode of inhibition

Noncompetitive Inhibition

Parameters	
Vmax	973 ± 65
Km	13.3 ± 3.3
Ki	85.6 ± 16.0
Goodness of Fit	
Degrees of Freedom	15
AICc	157
R2	0.991
Sum of Squares	59,030
Sy.x	62.7

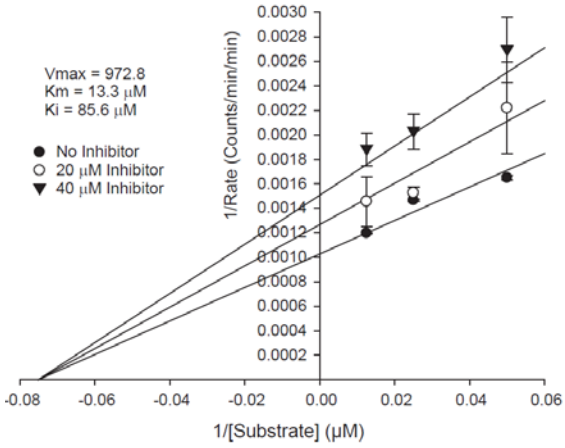


Figure S2. Noncompetitive Inhibition: Best Fit Linewear-Burk Analysis of the Cyclic Peptide Kinetic Experiments with PKA.

The kinetic experiments with the PKA bivalent inhibitor showed a best fit to noncompetitive inhibition suggesting that the bivalent inhibitor does not bind in the peptide substrate binding site. To determine the mode of inhibition of the cyclic peptide alone, kinetic kinase assays of the cyclic peptide, CTFRVFGCG, were performed. The cyclic peptide kinetic experiments analysis best fit to noncompetitive inhibition supporting the results of the bivalent inhibitor kinetic analysis. The K_i of 86 μM and the K_m of 13.3 for kemptide was similar to literature values, K_m for kemptide = 16 μM, and previous experiments with the IC_{50} of the cyclic peptide = 57 μM.

Kinetic Kinase Assays Kinetic assays were performed in duplicate. In a 60 μl final volume, [γ-^{32}P]ATP (30 μM) initiated the reaction with 0.52 nM PKA and Kemptide (LRRASLG, 20, 40, and 80 μM) in the presence of the cyclic peptide (0, 20 and 40 μM) in PKA Assay Buffer (40 mM Tris, 20 mM Mg Acetate, pH 7.4) with 0.01% BSA and 2.5% DMSO. At 7 minute intervals, 10 μl of the reaction mixture was spotted on P81 phosphocellulose paper. The samples were washed three times in 500 ml of 0.85% phosphoric acid and once in 500 ml of ethanol for 5 minutes each. The amount of ^{32}P labeling of the peptide substrate was quantified using a Beckman LS 6000IC liquid scintillation counter.

IC_{50}

Increased Stability of the Amide Isostere PKA BL, 7, relative to the optimized PKA BL, 4

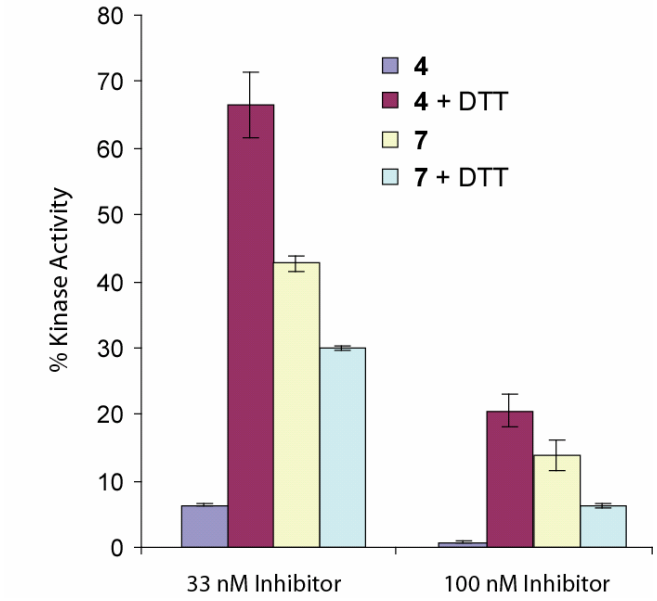


Figure S3. The Effect of Reducing Environments: DTT Assays of the Disulfide and Amide Isostere Bivalent Inhibitors. The PKA bivalent inhibitor contains a disulfide (cyclic peptide) that is susceptible to reduction, which was verified in the kinase assays with 7.5 mM DTT. An amide isostere was synthesized to increase the stability of the bivalent inhibitor and hence its utility. The DTT assays indicated a loss of potency of the disulfide bivalent inhibitor (> 10-fold) whereas the amide isostere was impervious to the reducing conditions.

DTT Assays Kinase assays were performed in triplicate. In a 30 µl final volume, [γ-^{32}P]ATP (30 µM) initiated the reaction with 2.6 nM PKA and Kemptide (LRRASLG, 30 µM) in PKA Assay Buffer (40 mM Tris, 20 mM Mg Acetate, pH 7.4) with 7.5 mM DTT, 0.01% BSA and 2.5% DMSO. The reaction was quenched with 15 µl of 1.8% Phosphoric Acid and 30 µl of the reaction mixture was spotted on P81 phosphocellulose paper. The samples were washed three times in 500 ml of 0.85% Phosphoric Acid for 5 minutes. The samples were washed once in 500 ml of ethanol. The amount of ^{32}P labeling of the peptide substrate was quantified using a Beckman LS 6000IC liquid scintillation counter. Data were normalized to reactions containing no inhibitors, which were run in triplicate.

Determining Staurosporine Warhead with a Short Linker (1a) Affinity for PKA

In previous work, we evaluated the staurosporine derivative (CarboxyStaur) for activity against PKA. However, the staurosporine with a short linker provides a better control both for comparison to the BL and as a screening compound. Through the addition of a linker, any negative or positive effects that might be present in the generated BLs can be identified with the staurosporine derivative with a short linker, **1a**, via screening that would be missed with the staurosporine free acid (CarboxyStaur) first compound. The staurosporine derivative plus linker was assayed for activity against PKA (IC_{50} = 159 nM). This compound, **1a**, was not significantly different than the first staurosporine derivative (CarboxyStaur, IC_{50} = 243 nM) tested.

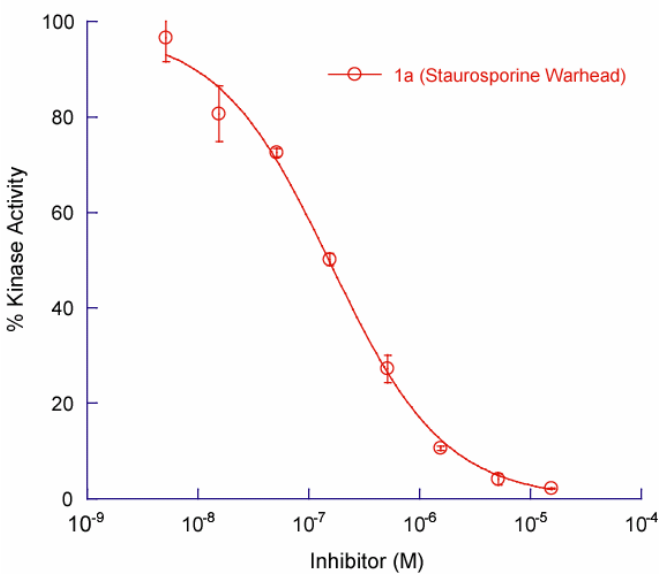


Figure S4. PKA assay to determine IC_{50} of the modified staurosporine derivative (Warhead) **1a**. At 2.6 nM PKA the IC_{50} of the staurosporine derivative is 159 nM.

Determining the Selectivity of the Kinase Ligands Against a Large Kinase Panel (90 kinases)

Table SI. Screening Data of the Kinase Ligands: **4**, **7**, and **1a**

Ambit Gene Symbol	**4** (200 nM)	**7** (50 nM)	**1a** (500 nM)
ABL1	100	94	68
ABL1(E255K)	100	97	92
ABL1(T315I)	78	100	35
ACVR1B	95	100	87
ADCK3	96	91	91
AKT1	100	100	100
AKT2	100	100	37
ALK	60	92	5.2
AURKA	19	63	4.2
AURKB	5.8	33	0
AXL	58	81	8
BMPR2	93	99	100
BRAF	100	96	100
BRAF(V600E)	100	84	94
BTK	62	98	31
CDK11	87	100	61
CDK2	100	100	69
CDK3	100	100	87
CDK7	74	98	100
CDK9	100	99	92
CHEK1	60	91	54
CSF1R	44	83	22
CSNK1D	100	100	89
CSNK1G2	100	100	99
DCAMKL1	100	95	100
DYRK1B	14	79	5.6
EGFR	96	87	54
EGFR(L858R)	86	100	53
EPHA2	100	99	80
ERBB2	100	100	95
ERBB4	100	100	70
ERK1	100	100	100
PTK2	91	100	65
FGFR2	66	86	27
FGFR3	85	100	43
FLT3	1	7.4	0.35
GSK3B	100	74	94
IGF1R	100	100	82
INSR	100	92	55
JAK2(Kin.Dom.2)	4	42	2.4
JAK3(Kin.Dom.2)	4.6	16	3.6
JNK1	83	100	52
JNK2	89	88	87

JNK3	65	85	55
KIT	39	85	7.4
KIT(D816V)	0.65	6.5	1
KIT(V559D,T670I)	4.1	59	4.8
LKB1	81	100	41
MAP3K4	94	96	95
MAPKAPK2	100	100	100
MARK3	18	61	16
MEK1	100	95	100
MEK2	100	87	86
MET	77	100	46
MKNK1	96	100	82
MKNK2	57	100	39
MLK1	0.65	5.5	0.55
p38-alpha	89	95	93
p38-beta	100	100	100
PAK1	14	66	4
PAK2	36	72	14
PAK4	40	90	34
PCTK1	94	98	75
PDGFRA	41	100	7
PDGFRB	3.2	23	0.05
PDPK1	16	48	18
PIK3C2B	100	100	98
PIK3CA	89	100	100
PIK3CG	100	91	15
PIM1	53	79	20
PIM2	100	100	100
PIM3	26	66	10
PKAC-alpha	19	10	52
PLK1	95	100	85
PLK3	100	98	100
PLK4	4.9	5.7	3.6
PRKCE	0	0	0.6
RAF1	89	100	73
RET	40	94	5.6
RPS6KA3(Kin.Dom.1)	100	100	44
SNARK	0.45	1.8	3.2
SRC	100	100	72
TGFBR1	100	100	75
TIE2	65	100	38
TRKA	57	98	13
TSSK1	17	100	2.6
TYK2(Kin.Dom.2)	62	100	19
VEGFR2	73	91	36
YANK3	100	100	100
ZAP70	16	57	2.2

The staurosporine derivative, PKA bivalent inhibitors (disulfide and amide isostere) were screened against 90 kinases (Ambit Biosciences) to determine a kinase inhibition profile for the compounds. The staurosporine derivative, was screened to see what enhancement if any was observed with the PKA bivalent inhibitors, but most importantly the kinase inhibition profile for the staurosporine reveals what kinases may be targeted using this compound as the warhead in our strategy. Clearly the staurosporine derivative is not as potent as staurosporine, but still retains a non-selective profile thus is could be used as a warhead in targeting other kinases. The profile of the disulfide is non-selective possibly due to the reduction of the disulfide bond due to assay conditions (7.5 mM DTT), which was confirmed in kinase assays with 7.5 mM DTT (above). To increase stability an amide isostere bivalent inhibitor was synthesized and screened. The profile of the amide bivalent inhibitor demonstates enhanced affinity for PKA, the target kinase, without the non-selectivity observed with the disulfide bivalent inhibitor.

Analysis of the Selectivity of the Staurosporine Warhead with Linker (St3BA) Relative to Staurosporine

Table SII. Kinases (from 90 kinase panel) that have Kinase-Staurosporine Co-Crsytal Structures

Kinase	PDB Code	Interacting Residue(s)*	Binding Relative to Staurosporine	Reference
PKA	1STC	E127, E170	(-) 14-fold worse	Prade et al
CHK1	1NVR	E91, E134	(-) 84-fold worse	Zhao et al
PDK1	1OKY	E166, E209	(-) 52-fold worse	Komander et al
CDK2	1AQ1	D86, Q131	(-) 50-fold worse	Lawrie et al
PIM1	1YHS	D128	(-) 31-fold worse	Jacobs et al
GSK3B	1Q3D	--	(-) 7-fold worse	Bertrand et al
EGFR	2ITW	--	No change	Yun et al
JAK3	1YVJ	R953	No Change	Boggon et al
ZAP70	1U59	R465	(+) 3-fold better	Jin et al
PIK3CG	1E8Z	T886, D964	(+) 6-fold better	Walker et al

*Methylamino group on Staurosporine Hydrogen Bonds to these Residues

The kinase inhibition profile for the staurosporine derivative (warhead, **1a**) was diminished relative to staurosporine for about half of the kinases screened (45). This decrease in binding may be due to the loss of the aminomethyl (staurosporine) hydrogen bonding interactions that are observed in the co-crystal structures referenced in Table SII. The staurosporine warhead is derivatized at this position resulting in an amide. For the kinase structures that hydrogen bond to the aminomethyl group of staurosporine through an aspartic or glutamic acid the affinity for the staurosporine derivative (**1a**) is diminished on the order of 7 to 84-fold worse. The only exception is PIK3CG (6-fold improvement), which has T886 and D964 as interacting residues and it is possible that the amide group still interacts with T886. Additionally, if there are no observable hydrogen bonding interactions or the interacting residues are arginines there appears to be no diminished affinity as for these kinases there was either no change or a modest increase in affinity (3-fold, ZAP70). The only exception to this is GSK3B, for which there is a 7-fold decrease in affinity despite the lack of any observable hydrogen bonding interactions. This may be due to a steric effect of derivatization at the aminomethyl group. Clearly, the change in hydrogen bonding potential of the staurosporine derivative relative to staurosporine is influential in the affinity of the derivative for kinases.

Selectivity Enhancement of the PKA BL (4)

To determine the contribution of the cyclic peptide to the selectivity profile of the PKA BL (**4**), the data from screening compound **4** against a panel of 90 kinases was normalized to the staurosporine derivative plus linker (**1a**). From this analysis of the data, the effect of staurosporine on the selectivity profile is accounted for and the effect of the addition of the cyclic peptide is pronounced. For PKA, an enhancement is expected for the BL relative to the warhead alone. Indeed this is observed for PKA with a >2-fold enhancement for the BL.

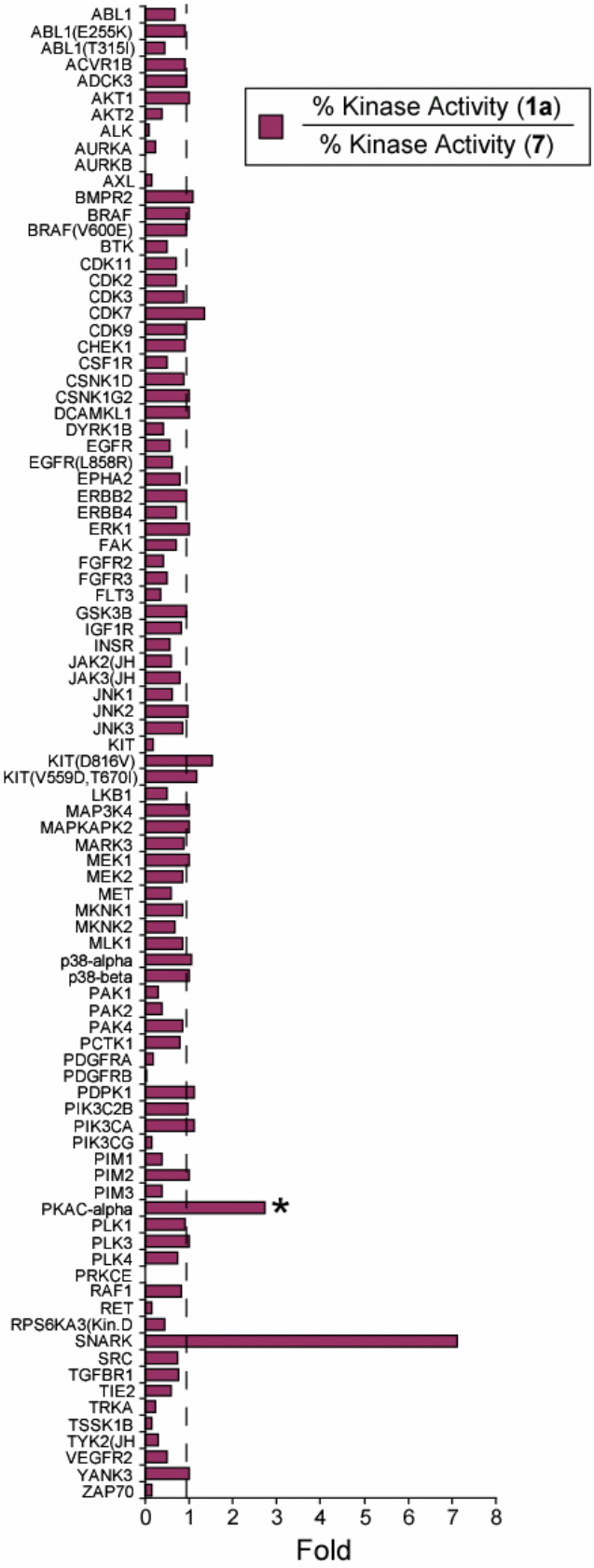


Figure S5. The PKA BL (**4**)selectivity relative to the staurosporine warhead (**1a**). The BL appears selective for PKA (>2-fold), with the exception of SNARK (7-fold).

Summary of the activity of all PKA targeted compounds

Table SIII. Activity of PKA targeted Compounds

Compound	IC_{50}
2	2.1 nM
3	< 0.52 nM
4	< 0.52 nM
5	0.65 nM
1a	159 nM
7	12.6 nM
6	2.9 uM
1b	57 uM
6a	> 1 mM

Characterization of Synthesized Compounds

All compounds synthesized by solid phase peptide synthesis using standard fmoc chemistry, HPLC purified (gradient 20-70% in 40 minutes for BLs, 0-40% in 40 minutes for cyclic peptides, 0-100% in 100 minutes for biotinylated compounds), verified by mass spectrometry (MALDI,), concentration determined by staurosporine absorbance (λmax = 294 nM, ε = 57.38 mM-1) and confirmed by amino acid analysis (Keck Facility, Yale).

Table SIV. Mass Spectrometry Data for Compounds

Compound	Expected m/z	Found m/z
2	1748	1748.4
3	1890	1889.9
4	2032	2032.3
5	2174	2174.5
6	1746	1746.6
7	1954.5	1954.7
1a	778.9	779.3

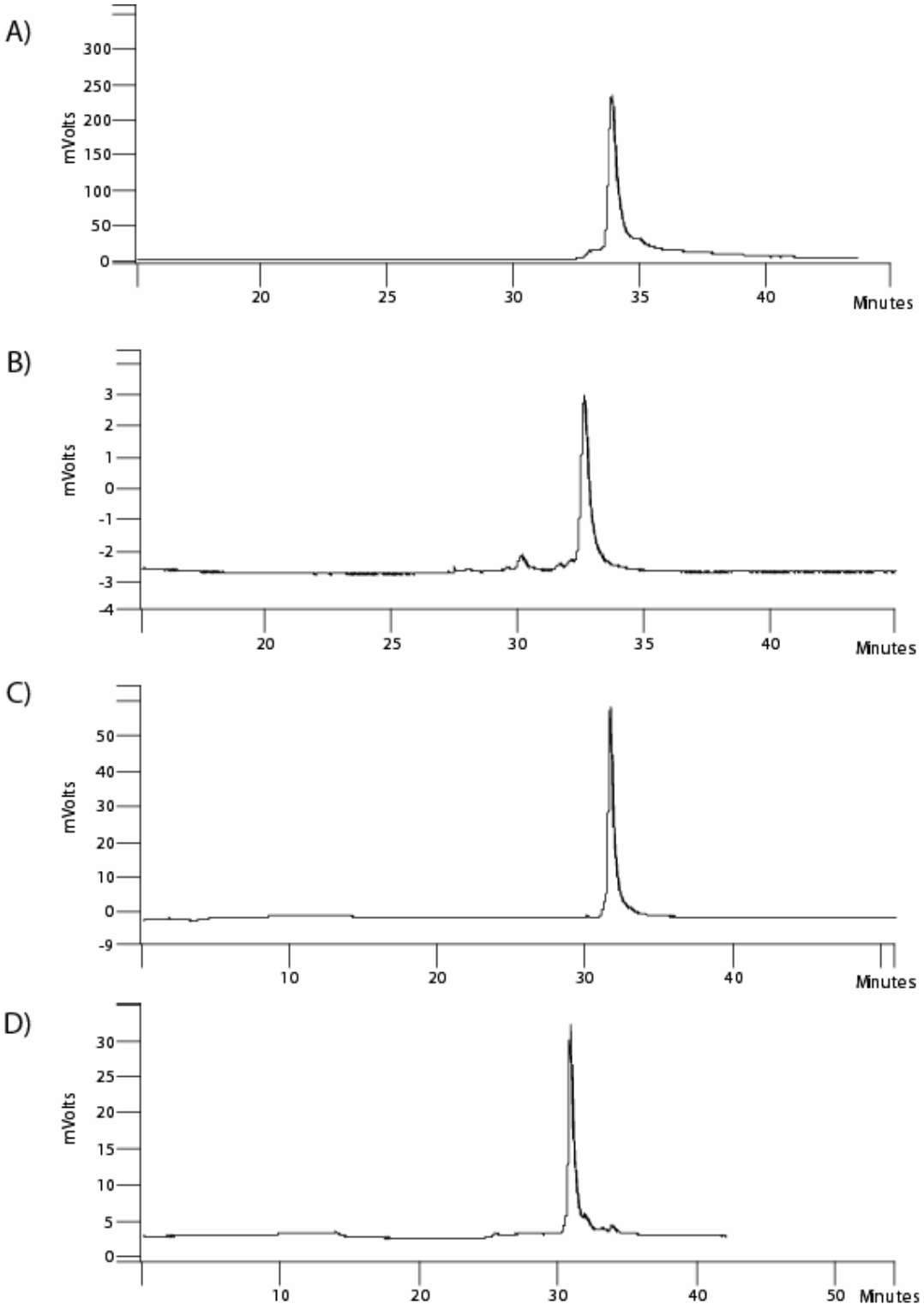


Figure S6. HPLC traces of PKA BLs of Varying Linker Composition. A) HPLC trace of compound **2**. B) HPLC trace of compound **3**. C) HPLC trace of compound **4**. D) HPLC trace of compound **5**.

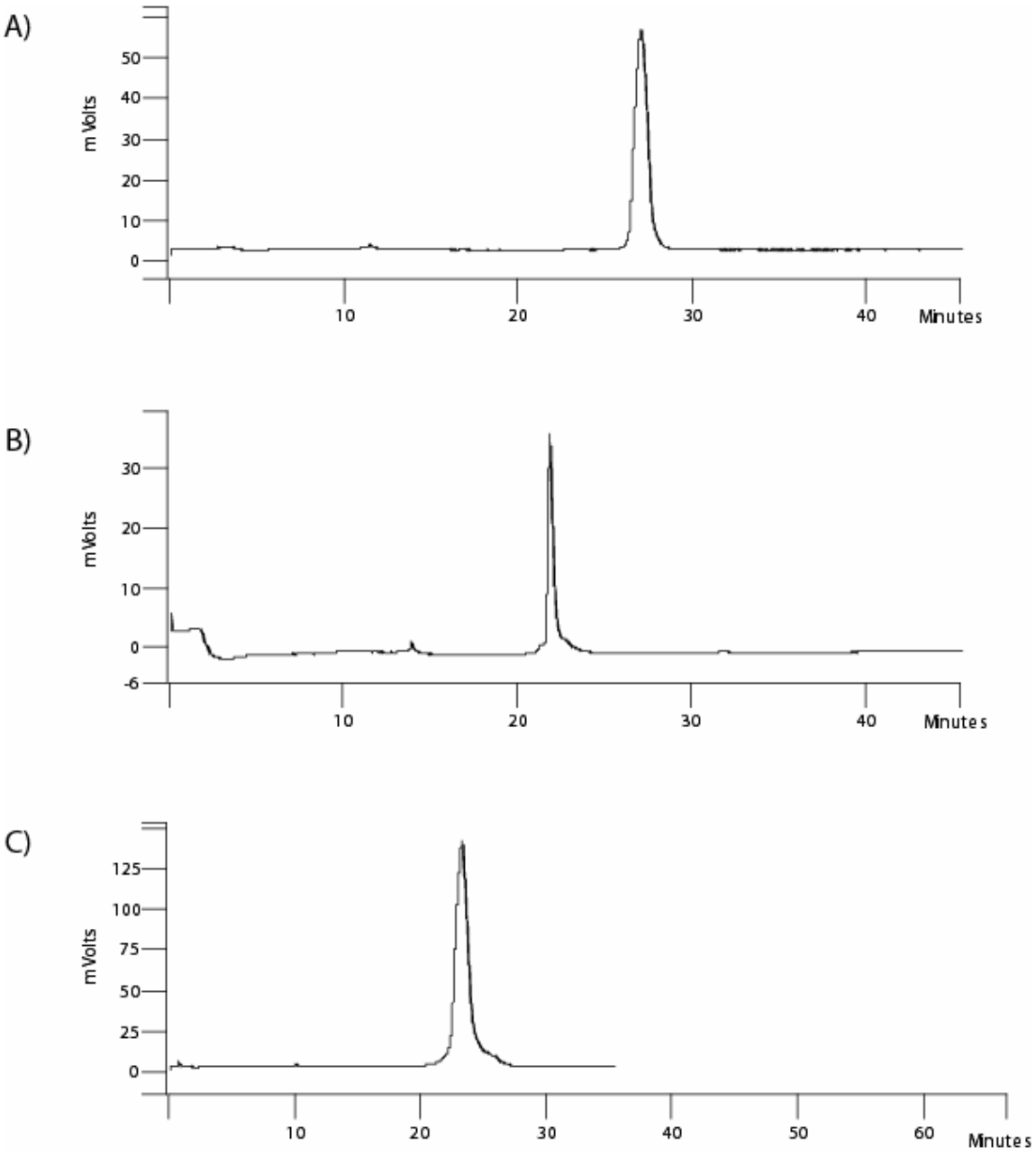


Figure S7. HPLC traces of the staurosporine warhead (**1a**, A), the adensosine PKA BL (**6**, B) and the amide PKA BL (**7**, C).

Table SV. Amino Acid Analysis of Compounds (Keck Facility, Yale University)

Compound	Arg	Gly	Thr	Val	Phe	Asx
2	0.922 (1)	2.21 (2)	0.863 (1)	0.938 (1)	2.07 (2)	
3	0.715 (1)	2.23 (2)	0.761 (1)	0.891 (1)	0.747 (2)	
4	0.952 (1)	2.16 (2)	0.841 (1)	0.998 (1)	2.05 (2)	
5	0.955 (1)	2.16 (2)	0.858 (1)	0.979 (1)	2.05 (2)	
6	0.971 (1)	2.48 (2)	0.834 (1)	0.904 (1)	1.82 (2)	
7	0.572 (1)	3.22 (1)	0.527 (1)	0.552 (1)	1.54 (2)	0.583

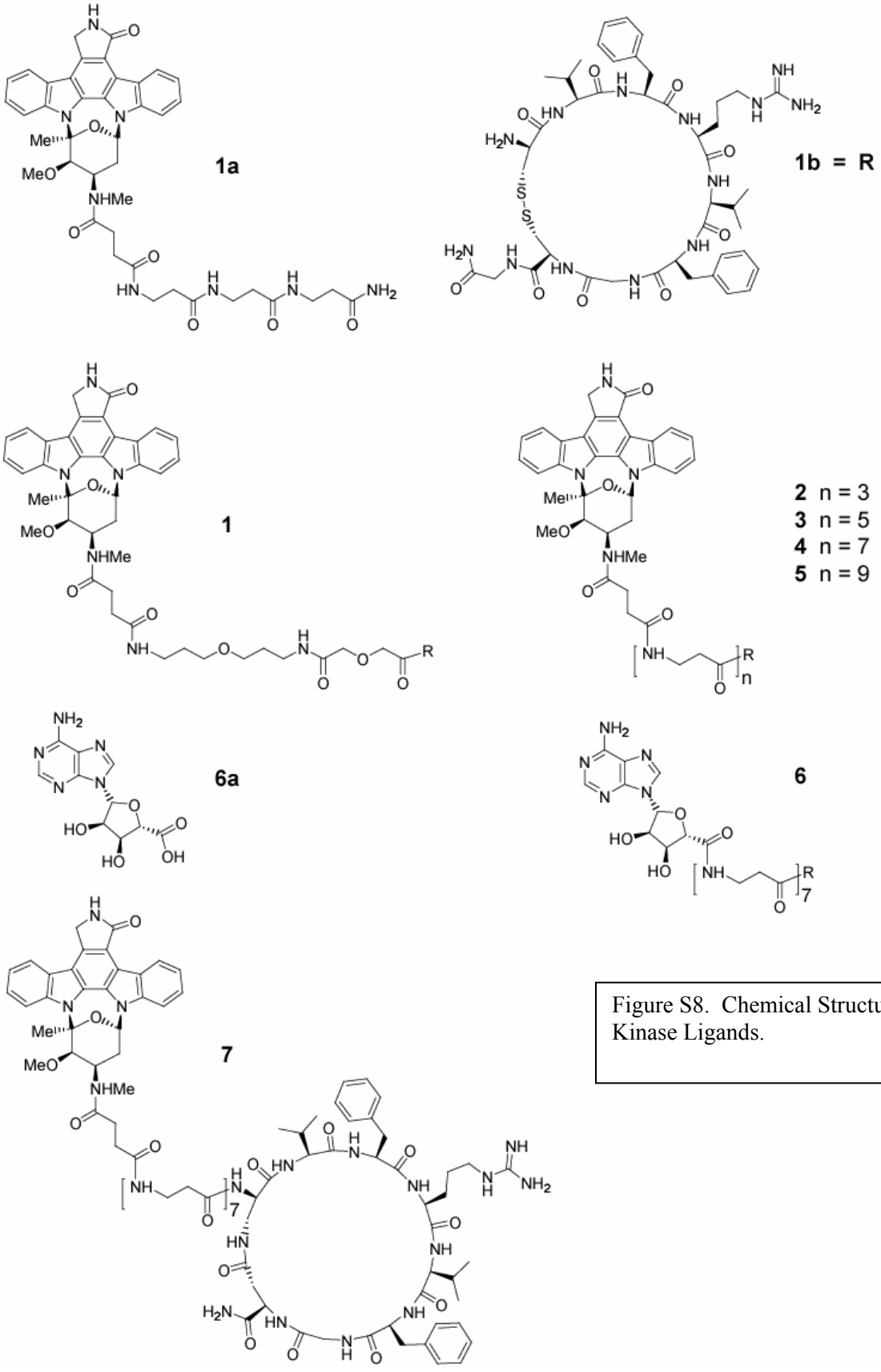


Figure S8. Chemical Structures of Kinase Ligands.