Design, synthesis, and in vitro evaluation of a fluorescently labeled irreversible inhibitor of the catalytic subunit of cAMP-dependent protein kinase (PKACα)

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Electronic Supplementary Information

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1. Table S1 – Kinase Panel Selectivity Data for myr-PKI(14-22)-NH_2 and inhibitor 1.

Kinase		% Inhibiton (at 1 uM)[a]	
Gene	**Protein**	**Irreversible Inhibitor 1**	**myr-PKI(14-22-NH2**
PRKACA	PKACα	97	88
ABL1	ABL1	-4	-17
AKT1	AKT1/PKBα	12	23
PRKA A1/B1/G1	AMPK α1/β1/γ1	6	6
CAMK1D	CaMKIδ	-20	12
CAMK4	CaMKIV	-1	9
CHEK1	CHK1	10	5
CHEK2	CHK2	1	6
ERBB1	EGFR	-2	-2
ERBB4	HER4	-4	-6
FLT1	VEGFR1	-5	-13
KDR	VEGFR2	10	9
MAPK1	ERK2	7	3
MAPK3	ERK1	0	2
MAPK14	p38α	2	7
MAPKAPK2	MAPKAPK2	14	-13
MARK1	MARK1	2	7
MET	c-Met	8	11
PDGFRA	PDGFRα	-2	-6
PDPK1	PDK1	6	-1
PRKCA	PKCα	13	12
PRKCQ	PKCθ	-1	-1
PRKG1	PKGα	6	5
PTK2	FAK1	0	4
ROCK1	ROCK1	0	10
RPS6KA1	RSK1	42	11
RPS6KA5	MSK1	40	42
RPS6KB1	p70S6Kα	-7	3
SGK1	SGK1	12	3
SRC	c-Src	-1	12

a. Data from Life Technologies SelectScreen Kinase Profiling Service.

2. Figure S1 – Sequence Alignment of Kinases with an Activation Loop Cysteine

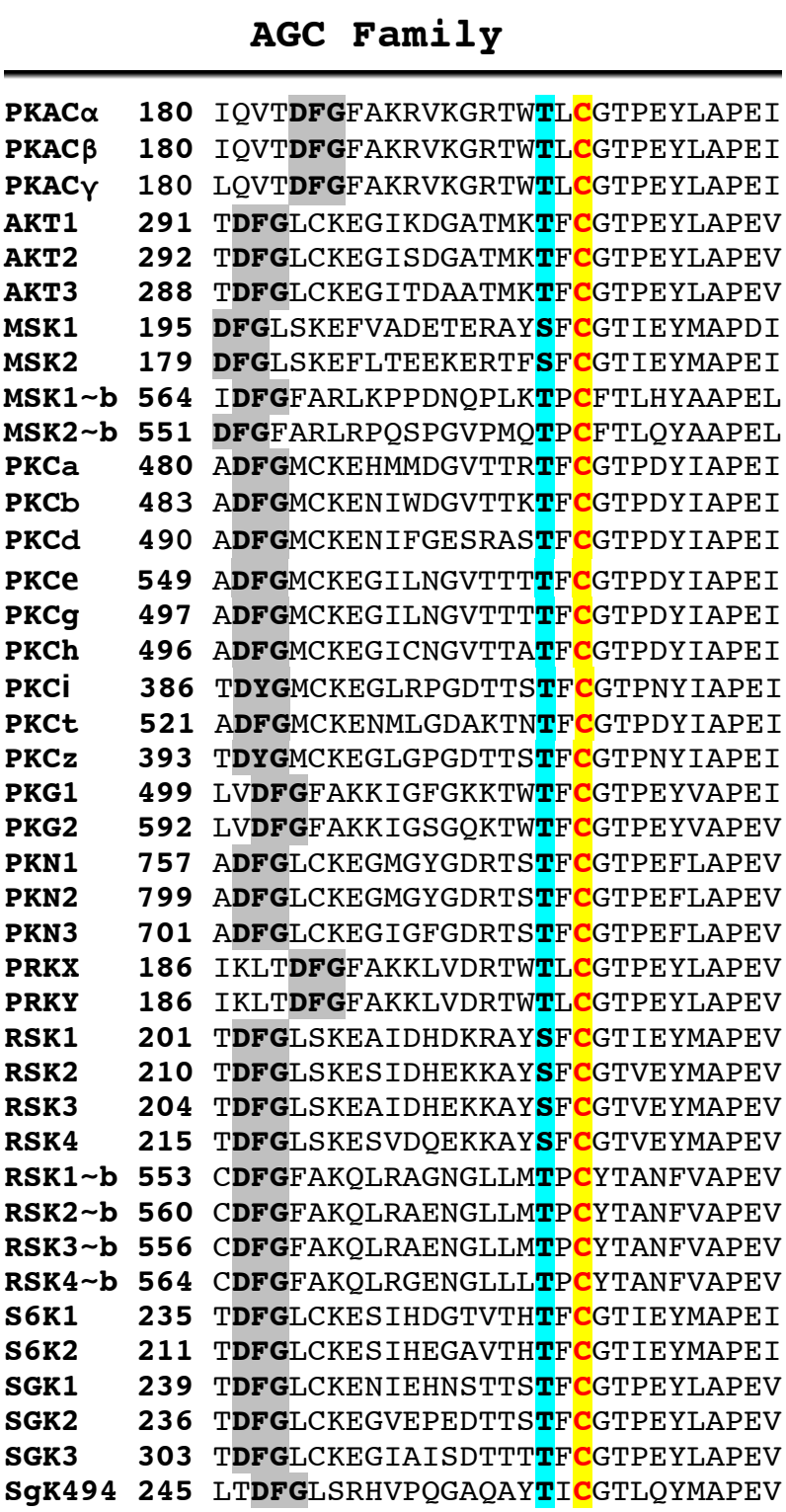

Figure S1. Kinases that contain an activation loop cysteine in the same position as PKACα C199. To identify these kinases, a sequence alignment of the human kinome was carried out in ClustalX 2.1 (http://www.clustal.org/clustal2/) using kinase domain sequences from the KinBase database (http://kinase.com/kinbase/FastaFiles/Human_kinase_domain.fasta). The sequences were uploaded into ClustalX and a Complete Alignment performed. Kinases with an activation loop that contain a cysteine residue in the same position as in PKACα (Cys199) were identified. This cysteine (red text, highlighted yellow) as well as the activation loop threonine/serine (highlighted cyan) and the DFG loop for each kinase (highlighted grey) are shown.

(continued on next page)

Figure S1 (continued)

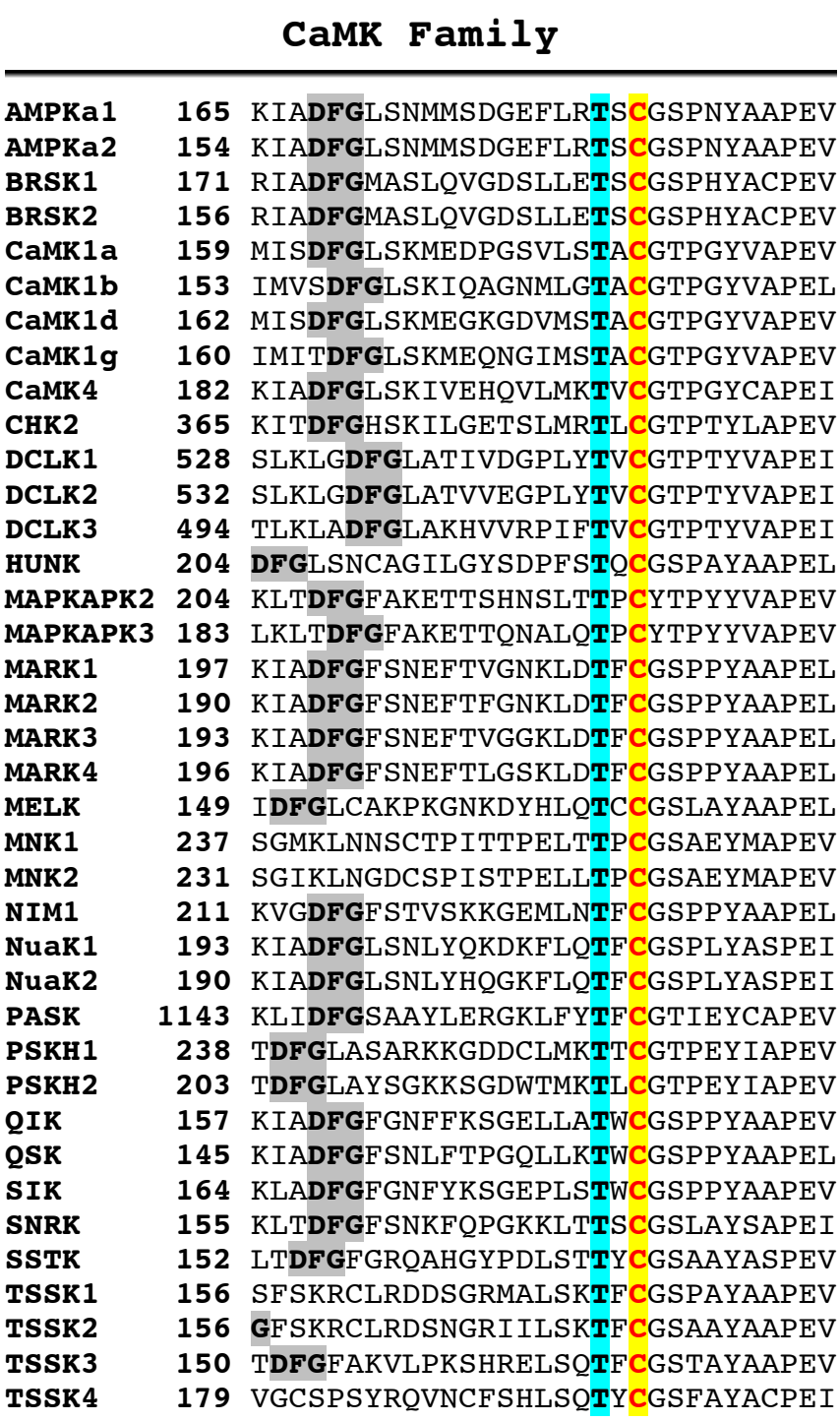

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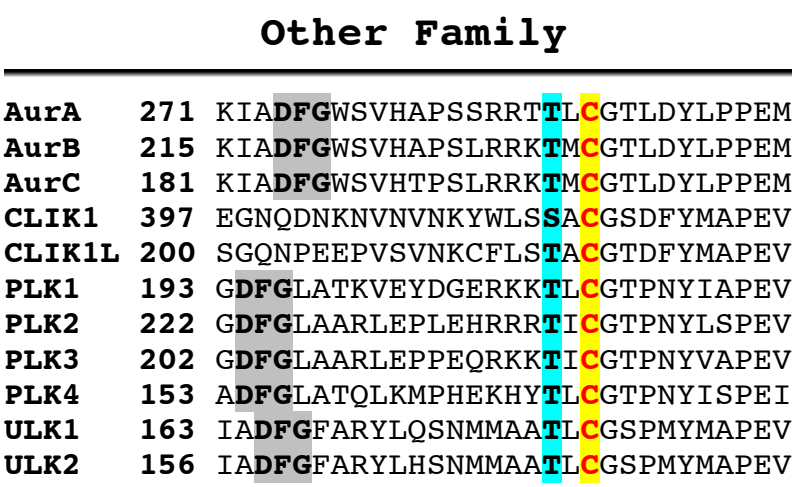
Figure S1 (continued)



3. Table S2 – Activation Loop Cysteine Residues in Kinases

AGC Family	
Kinase	**Activation Loop Cysteine**
PKACα	Cys199
PKACβ	Cys199
PKACγ	Cys199
AKT1	Cys310
AKT2	Cys311
AKT3	Cys307
MSK1	Cys214
MSK2	Cys198
MSK1~b	Cys583
MSK2~b	Cys570
PKCa	Cys499
PKCb	Cys502
PKCd	Cys509
PKCe	Cys568
PKCg	Cys516
PKCh	Cys515
PKCi	Cys405
PKCt	Cys540
PKCz	Cys412
PKG1	Cys518
PKG2	Cys611
PKN1	Cys776
PKN2	Cys818
PKN3	Cys720
PRKX	Cys205
PRKY	Cys205
RSK1	Cys220
RSK2	Cys229
RSK3	Cys223
RSK4	Cys234
RSK1~b	Cys572
RSK2~b	Cys579
RSK3~b	Cys575
RSK4~b	Cys583
S6K1	Cys254
S6K2	Cys230
SGK1	Cys258
SGK2	Cys255
SGK3	Cys322
SgK494	Cys264

CAMK Family	
Kinase	**Activation Loop Cysteine**
AMPKa1	Cys185
AMPKa2	Cys174
BRSK1	Cys191
BRSK2	Cys176
CaMK1a	Cys179
CaMK1b	Cys173
CaMK1d	Cys182
CaMK1g	Cys180
CaMK4	Cys202
CHK2	Cys385
DCLK1	Cys548
DCLK2	Cys552
DCLK3	Cys514
HUNK	Cys224
MAPKAPK2	Cys224
MAPKAPK3	Cys203
MARK1	Cys217
MARK2	Cys210
MARK3	Cys213
MARK4	Cys216
MELK	Cys169
MNK1	Cys257
MNK2	Cys251
NIM1	Cys231
NuaK1	Cys213
NuaK2	Cys210
PASK	Cys1163
PSKH1	Cys258
PSKH2	Cys223
QIK	Cys177
QSK	Cys165
SIK	Cys184
SNRK	Cys175
SSTK	Cys172
TSSK1	Cys176
TSSK2	Cys176
TSSK3	Cys170
TSSK4	Cys199

Other Family	
Kinase	**Activation Loop Cysteine**
AurA	Cys290
AurB	Cys234
AurC	Cys200
CLIK1	Cys416
CLIK1L	Cys219
PLK1	Cys212
PLK2	Cys241
PLK3	Cys221
PLK4	Cys172
ULK1	Cys182
ULK2	Cys175

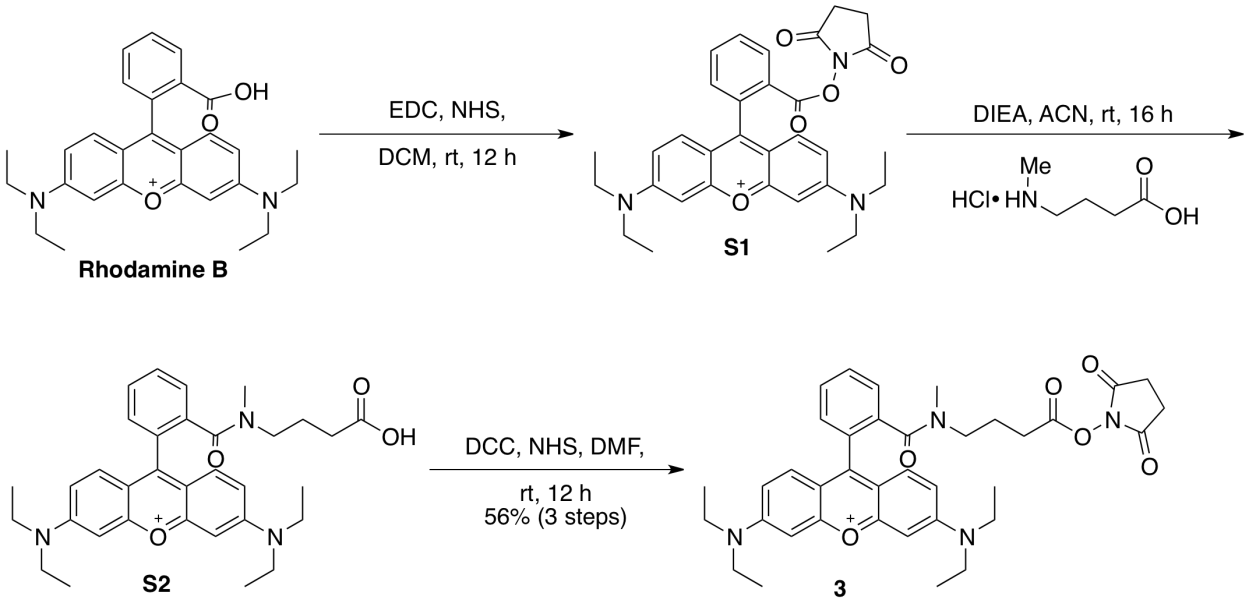
4. General Chemical Methods

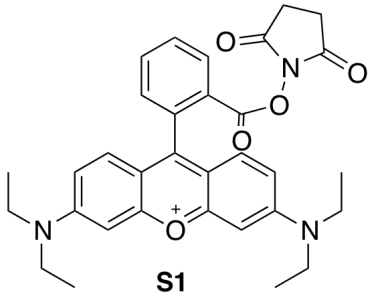
Chemical reagents and solvents were purchased from Sigma-Aldrich (MO, USA), Alfa-Aesar (MA, USA), and Fisher Scientific (PA, USA). Amino acids and coupling reagents were purchased from Chem-Impex. Analytical Thin Layer Chromatography (TLC) was performed using silica gel GHLF plates (Analtech Inc., DE, USA). Flash Chromatography was performed on TELEDYN ISCO CombiFlash® Rf instrument using RediSep Rf Normal-phase Flash Columns (4g, 12g, 24g, or 40g). ^{1}H NMR and ^{13}C NMR were recorded on a Bruker Topspin 400MHz using Chloroform-d and deuterated DMSO. All chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform and DMSO residual peaks at 7.26 and 2.50 respectively (^{1}H) and 77.16 and 39.52 respectively (^{13}C). The multiplicity (s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = muliplet), coupling constants(s) (Hz). Electrospray ionization (ESI) mass spectra were obtained from Perkin Elmer Flexar UPLC/AxION2 TOF Mass Spectrometer. Matrix-Assisted Laser Desorption/Ionization (MALDI) spectra were obtained from Voyager DE-Pro™ MALDI TOF Mass Spectrometer.

5. Synthesis of Inhibitor 1

5a. Synthesis of Rhodamine-NHS Ester 3

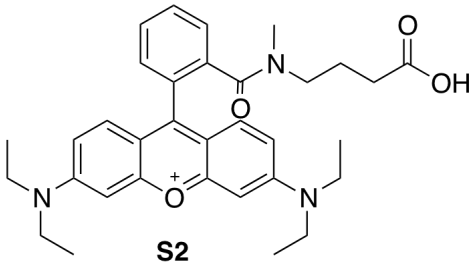
Rhodamine-NHS **3** was prepared following a previously reported method.[1,2] Characterization data were consistent with previously reported data.





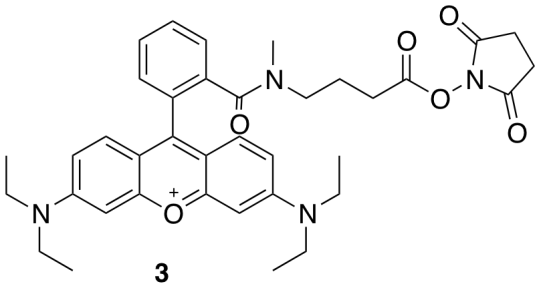
S1

3,6-bis(diethylamino)-9-(2-(((2,5-dioxopyrrolidin-1-yl)oxy)carbonyl)phenyl)xanthylium (S1): To a solution of rhodamine B (5.0 g, 10.44 mmol) and EDC (3.0 g, 15.66 mmol) in DCM (250 mL) was added N-hydroxysuccinimide (1.3 g, 11.48 mmol). The reaction was stirred at ambient temperature for 12 hr. Solvent was removed and the product was purified via flash chromatography (silica DCM:MeOH 9:1). Yield 75% (4.3 g). Analytical data (^{1}H NMR, ^{13}C NMR, mass spec) matched the previously reported data.[1,2]



S2

9-(2-((3-carboxypropyl)(methyl)carbamoyl)phenyl)-3,6-bis(diethylamino)xanthylium (S2): To a solution of **S1** (2.9 g, 5.29 mmol) and DIEA (2.7 g, 21.16 mmol) in ACN (52.9 mL) was added 4-methylaminebutyric acid hydrochloride (1.2 g, 7.94 mmol). The reaction was stirred at ambient temperature for 16 hr. Solvent was removed under vacuum and product was isolated by extraction ($H_2O/CHCl_3$). The organic layers were combined, dried, and taken directly to next step. Yield 88%. MS (MALDI) $C_{32}H_{40}N_3O_4$ Expected: 542.3013, Found 542.5626. Analytical data (^{1}H NMR, mass spec) matched the previously reported data.[2]



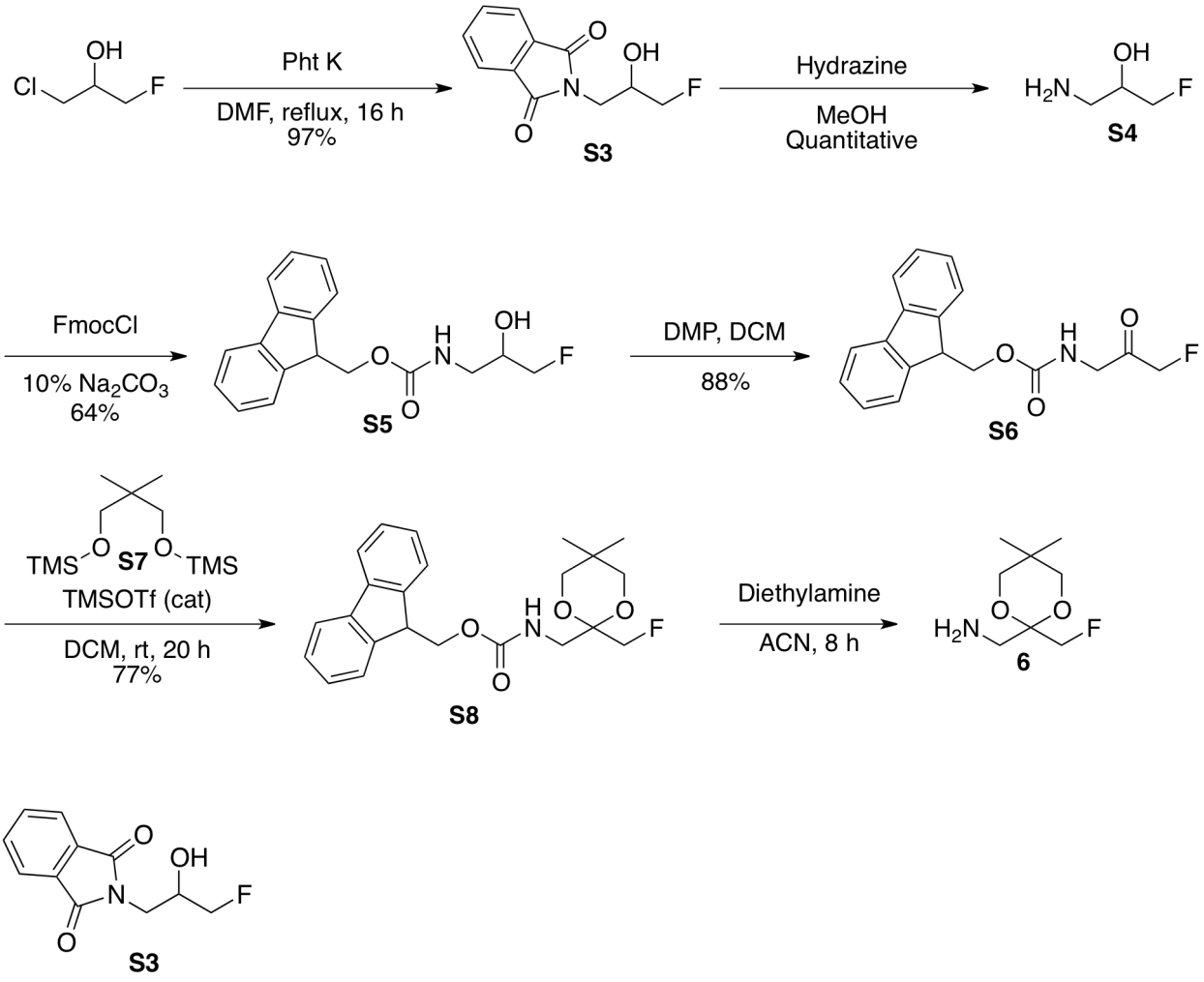
3

3,6-bis(diethylamino)-9-(2-((4-((2,5-dioxopyrrolidin-1-yl)oxy)-4-oxobutyl)(methyl) carbamoyl)phenyl)xanthylium (3): To a solution of **S2** (2.2 g, 4.00 mmol) and DCC (1.1 g, 5.2 mmol) in DMF (35 mL) was added N-hydroxysuccinimide (0.5 g, 4.00 mmol). The reaction was stirred at ambient temperature for 16 hr. The filtrate was collected then triturated with diethyl ether, centrifuged, and solvent decanted. The product, collected in the bottom of the centrifuge tube as an oil, was taken to next step. Yield 86%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 (s, 1H), 7.65 (m, 2H), 7.52 (m, 1H), 7.34 (m, 1H), 7.22 (d, 1H J = 9.5 Hz), 6.94 (d, 2H J = 9.6 Hz), 6.76

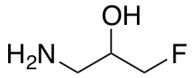
(s, 2H), 3.61 (m, 8H), 3.27 (t, 2H J = 7.0 Hz), 2.92 (s, 3H) 2.85 (m, 6H), 2.17 (t, 2H J = 7.0 Hz), 1.29 (t, 12H J = 7.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.27, 169.21, 168.51, 167.77, 162.51, 157.61, 155.66, 136.16, 131.93, 130.03, 127.37, 114.04, 96.47, 53.85, 46.12, 42.22, 37.76, 36.45, 31.36, 27.90, 25.60, 21.39, 18.61, 17.31, 12.60. MS (MALDI) $C_{37}H_{43}N_4O_6$ Expected: 639.3177 Found: 639.8263.

5b. Synthesis of protected glycine fluoromethylketone 6

The fluoromethyl ketone fragment was synthesized according to a previously reported route.[3]

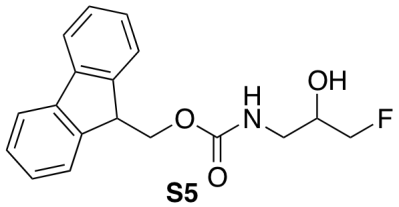


2-(3-fluoro-2-hydroxypropyl)isoindoline-1,3-dione (S3). 1-Chloro-3-fluoro-2-propanol (0.87 mL), potassium phthalamide (1.76 g), and DMF (10 mL) were added to a round bottom flask and heated to reflux for 16 hr. The reaction mixture was concentrated to an oil, triturated with ether, and the precipitate collected. The crude product was purified by silica gel chromatography to obtain the desired product (1.34 g, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.8 (m, 2H), 7.7 (m, 2H), 4.5 (m, 1H), 4.4 (m, 1H) 4.1 (m, 1H), 3.8 (m, 1.5H), 3.1 (s, 0.5H), 2.9 (s, 1H), 2.8 (s, 1H), 2.0 (s, 0.5H), 1.2 (m, 1H). HRMS (ESI) $C_{11}H_{11}FNO_3$ $[M+H]^+$ Expected: 224.0645, Found 224.0716.



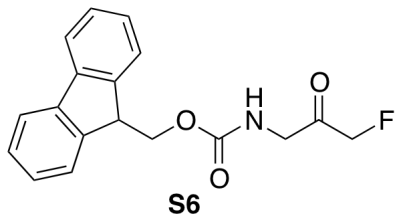
S4

1-amino-3-fluoropropan-2-ol (S4). To a solution of **S3** (1.12g, 5 mmol) in MeOH (100 mL) was added hydrazine hydrate (0.23 mL, 0.95 equiv) and the reaction mixture was stirred at ambient temperature for 20 h, during which time a precipitate formed. Conc. aq. HCl (1mL) was added and the reaction mixture stirred 3 hours. The precipitate was then removed by filtration and the MeOH was removed *in vacuo*. The crude oil (quantitative yield) was taken forward directly to next step.



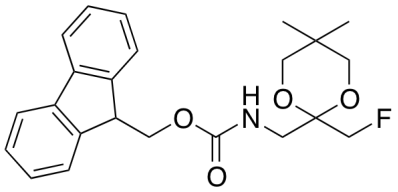
S5

(9H-fluoren-9-yl)methyl (3-fluoro-2-hydroxypropyl)carbamate (S5). Crude **S4** was dissolved in water (~30 mL), cooled to 0°C, and made basic by addition of 10% Na_2CO_3 (~30 mL). To the solution was added Fmoc-chloride (1.102g) dissolved in dioxane (50 mL). This solution was allowed to reach 16 hr, after which the dioxane was removed under vacuum and crude product was extracted with EtOAc. The organic layer was washed with saturated NaCl solution and solvent removed *in vacuo*. The crude product was purified by silica gel chromatography to obtain the desired product (64%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.8 (d, 2H J=7.52), 7.6 (m, 2H), 7.4 (m, 2H), 7.3 (m, 2H), 4.4 (m, 2H), 4.1 (m, 1H), 2.9 (s, 4H), 1.3 (s, 2H). HRMS (ESI) $C_{18}H_{18}FNO_3$ $[M+Na]^+$ Expected: 338.1168, Found 338.1172.



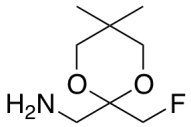
S6

(9H-fluoren-9-yl)methyl (3-fluoro-2-oxopropyl)carbamate (S6). Alcohol **S5** (81.33 mg, 0.26 mmol) was taken up in DCM (5 mL) and to this solution was added Dess-Martin periodinane (275.69 mg, 0.65 mmol). The reaction monitored by TLC until judged to be complete, at which time the solvent removed *in vacuo*. The crude product was purified by silica gel chromatography to obtain the desired product (88%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.8 (d, 2H J=7.52), 7.6 (m, 2H), 7.4 (m, 2H), 7.3 (m, 2H), 5.4 (s, 1H), 5.0 (s, 1H), 4.9 (s, 1H), 4.4 (m, 2H), 4.3 (s,2H), 4.2 (m, 1H), 1.2 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 202.2, 156.3, 143.9, 141.4, 127.9, 127.2, 125.0, 120.1, 84.1, 67.2, 48.2, 47.3. HRMS (ESI) $C_{18}H_{16}FNO_3$ $[M+Na]^+$ Expected: 336.1012, Found 336.1017.



S8

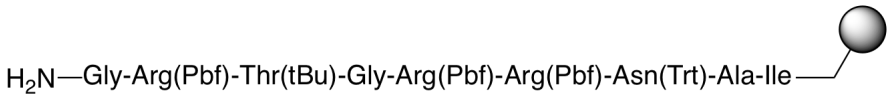
(9H-fluoren-9-yl)methyl ((2-(fluoromethyl)-5,5-dimethyl-1,3-dioxan-2-yl)methyl)carbamate (S7). Ketone **S6** (61.24 mg, 0.20 mmol) was taken up in DCM (7 mL) and to the solution was added TMS protected diol **S7**[3] (69.58 mg, 0.28 mmol). The solution cooled to 0°C and to the reaction TMSOTf (catalytic, 0.02 mmol) was added. The reaction warmed to ambient temperature and stirred for 16 hr, after which the solvent was removed *in vacuo*. The crude product was purified over silica gel chromatography to obtain the desired product (77%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.8 (d, 2HJ=7.52), 7.6 (m, 2H), 7.4 (m, 2H), 7.3 (m, 2H), 5.4 (s, 1H), 5.0 (s, 1H), 4.9 (s, 1H), 4.4 (m, 2H),4.3 (s, 2H), 4.2 (m, 1H), 1.2 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 202.2, 156.3, 143.9, 141.4,127.9, 127.2, 125.0, 120.1, 84.1, 67.2, 48.2, 47.3. HRMS (ESI) $C_{23}H_{26}FNO_3$ $[M+Na]^+$ Expected: 422.1744, Found 422.1751.
[NOTE: In our hands, this penultimate intermediate is stable at 4 °C for at least 3 months.]



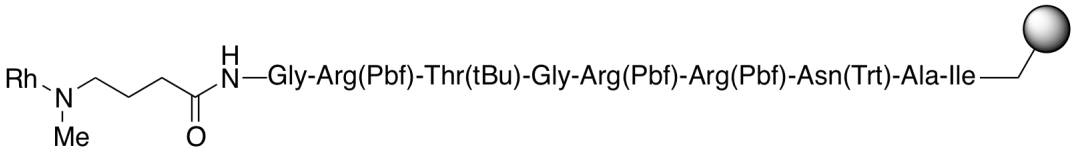
6

(2-(fluoromethyl)-5,5-dimethyl-1,3-dioxan-2-yl)methanamine (6). Fmoc carbamate **S8** (52 mg) was taken up in acetonitrile (3 mL) and to the solution was added diethyl amine (0.23 mL) The reaction was monitored by TLC and upon completion, the solvent removed under vacuum. The crude reaction mixture used directly and quickly in the subsequent peptide-coupling step to avoid degradation or decomposition.

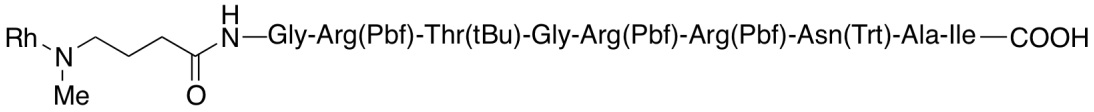
5c. Peptide Synthesis and Assembly of Inhibitor 1



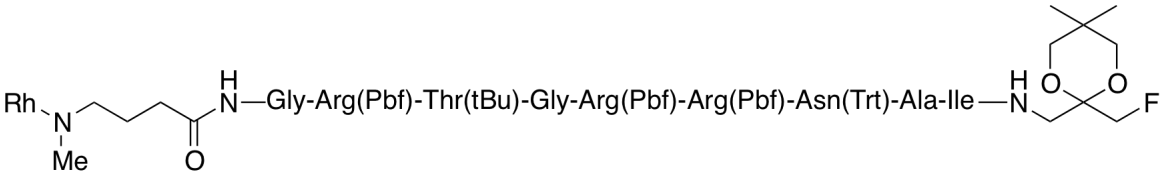
PKI(14-22) on 2-Cl-trityl Resin (2). 2-Chlorotrityl Chloride (2-CTC) resin preloaded with Ile was purchased from ChemImpex (200-400 mesh, Catalog #03472). Peptide couplings were carried out manually or on a CEM Liberty Blue microwave peptide synthesizer via standard Fmoc amino acid protection chemistry. Amino acids were activated and added to resin with (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and diisopropylethylamin (DIEA). The resin was swelled with DCM (5 min) then DMF (5 min). All amino acids were added by the general procedure that follows (with the exception of Arg, which underwent double couplings): Fmoc deprotection (20% piperidine in DMF, 3 x 5 min), wash with DMF 3x, amino acid coupling (PyBOP,DIEA 0.1 M in DMF, 1 hr, X2 for Arg), wash with DMF 3x, acetyl cap (pyridine:acetic anhydride 20:20, 0.15 M in DMF, 30 min). After the final deprotection (Gly 14) with 20% piperidine in DMF, the resin-bound product was taken directly to the next step.



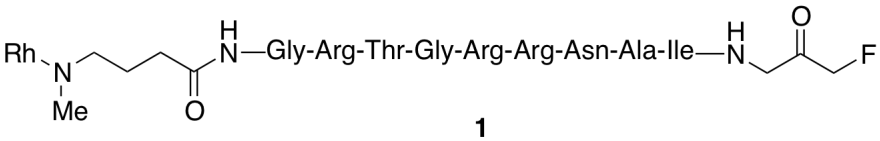
Rhodamine-PKI(14-22) on 2-Cl-trityl Resin (4). To the peptide on resin was added **3** (6 eq) and TEA (10 eq) in DMF (0.05 M of **3**). The reaction was stirred for 3 h, washed with DMF 5x, then DCM 5x. The resin-bound product was taken directly to the next step.



Rhodamine-PKI(14-22)-OH (5). Rhodamine-PKI(14-22)-OH (**5**) was cleaved from the resin with 2% TFA in DCM (this preserves the side chain protecting groups) and the solvent was removed under vacuum. The product was taken on directly to the next step.



Intermediate 7. Rhodamine-PKI(14-22)-OH **5** (0.05 mmol) was taken up in DCM (5 mL). PyBOP (0.065 mmol), DIEA (0.2 mmol), and **6** (0.065 mmol) were added to the solution. The reaction was stirred for 16 h. Solvent was removed and the product purified on a silica gel gravity column (loaded with DCM, eluted with 20% MeOH/DCM). MS (MALDI) $C_{142}H_{195}FN_{23}O_{25}S_3$ Expected: 2737.3841, Found 2737.3524.



1

Inhibitor 1. Intermediate **7** was globally deprotected with 95:2.5:2.5 TFA:TIS:H_2O (5 mL) for 1 h and then triturate with ether to afford crude product. The product purified on HPLC (95:5 to 75:25 H_2O:ACN over 20 minutes). MS (MALDI) $C_{75}H_{115}FN_{23}O_{15}$ Expected: 1596.8927, Found 1596.8413.

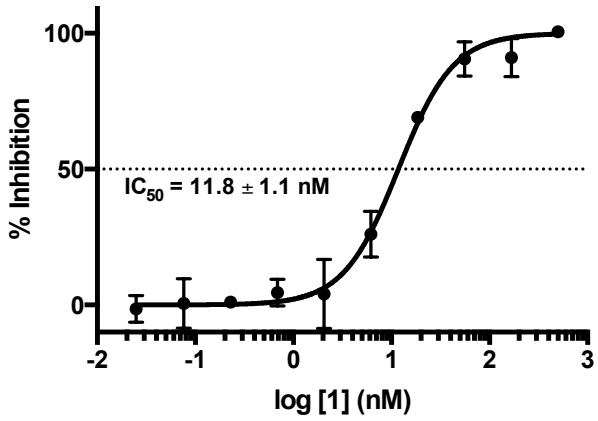
6. Protein Production and Purification

PKACα was produced using a modification of the procedure of Taylor and co-workers.[4] For our work, we constructed a PKACα/PDK1 co-expression system in *E. coli* to ensure that the PKACα was fully phosphorylated at Thr197. The PKACα plasmid was purchased from Addgene (Plasmid #14921) and used as received. The initial PDK1 plasmid was a gift from Justin Rettenmaier, Wells Lab, UCSF. The PDK1 gene was moved into the pRSFDuet vector, which is selected for with kanamycin, in order to facilitate development of the co-expression system. The plasmid carrying the mutation for expression of C199A-PKACα variant protein was prepared using a QuikChange II Kit.

To express the fully-phosphorylated PKACα, competent *E. coli* cells were transformed with wild-type or C199A PKACα plasmids and the pRSFDuet-PDK1 plasmid, selecting with Amp/Kan. Cells were grown in auto induction media at 37ºC for 16 hr. Following growth, cells were spun down at 6000 rpm for 10 min, lysed into buffer (25 mM tris, pH 8, 300 mM NaCl, and 10 mM imidazole) allowed to equilibrate for 30 min at ambient temperature. Debris was removed by centrifugation at 20,000 rpm for 30 min and the soluble fraction was decanted onto nickel column. Following loading, the nickel column was washed with 5 column volumes of buffer (25 mM tris, pH8, 300 mM NaCl, and 10 mM imidazole), pure PKACα was eluted with 100 mM imidazole.

7. Inhibition of PKACα Activity by 1 (IC_{50})

The assay for inhibition of PKACα was performed using Z′-LYTE Kinase Assay Kit – Ser/Thr 1 Peptide (Invitrogen, NY, USA) in non-binding low-volume 384-well plates (Cat. No. 3676, Corning, NY, USA) according to the manufacturer's instructions. All the reagents were diluted in kinase buffer (50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM $MgCl_2$, 1 mM EGTA). The compounds were prepared as 10 mM solutions in DMSO and diluted to 0.8% final DMSO concentration in the assay. A typical 10 µL assay contained 0.3 ng of PKAα, 4 µM ATP and 2 µM peptide substrate in kinase buffer in the presence/absence of tested compounds. The assays were incubated at room temperature for 1 h at which time development reagent (5 µL) was then added and the assay mixture was incubated at room temperature for a further 1 h. The kinase reaction was terminated by the addition of kit stop reagent (5 µL). The fluorescence signal was measured using Flexstation 3 microplate reader (Molecular Devices, CA, USA) using an excitation wavelength of 410 nm and emission wavelengths of 458 nm and 522 nm. The percent inhibition was calculated according to the kit guidelines. myr-PKI(14-22) (Invitrogen) was used as a control.



8. Kinase Panel for Inhibitor 1 and myr-PKI(14-22) (Single Dose @ 1 µM)

Inhibitor **1** (75 µL of a 100 µM (100x) stock) and myr-PKI(14-22) (75 µL of a 100 µM (100x) stock) were submitted for the SelectScreen Kinase Profiling Service (Thermo/Life Technologies) for testing against 30 kinases (including PKACα) at a single dose of 1 µM. Data are the mean of duplicate runs. For each kinase, the assay was run so that the final concentration of ATP = Km_{app} for the kinase tested. Detailed information on the assay protocols may be found on Thermo/Life Technologies' web site at http://www.lifetechnologies.com/drugdiscovery.

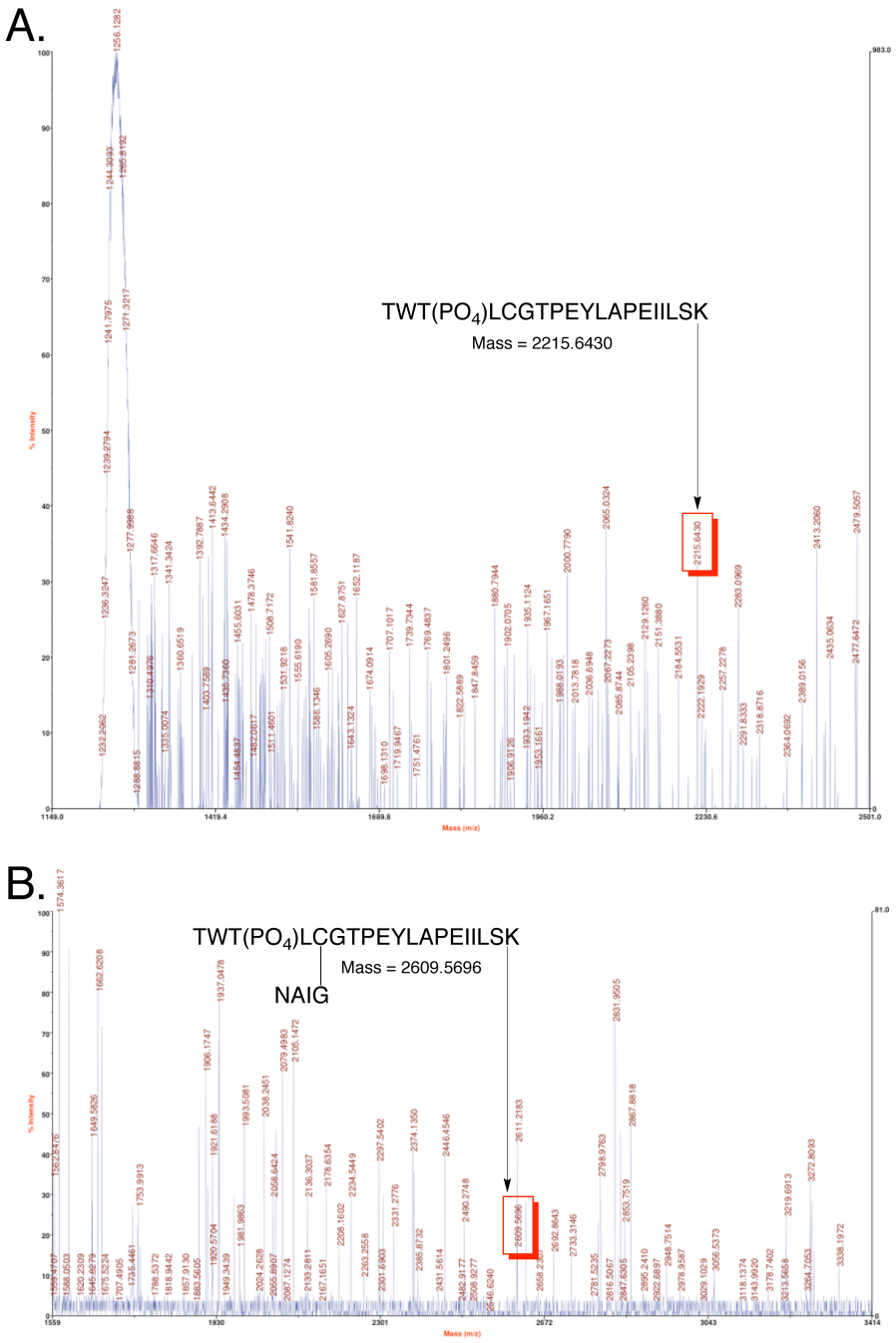
9. *In Vitro* Labeling of wild-type and C199A-PKACα by Inhibitor 1

Recombinant proteins (wt or C199A, 2 µg, final concentration 1.2 µM) and inhibitor/vehicle (final concentration of 2.3 µM) were diluted with 25 mM phosphate buffer (pH 7.2) to a final reaction volume of 40 µL and incubated for 1 hr. SDS-page sample buffer (10 µL of a 5x stock) was added and the reactions boiled for 5 min to stop the labeling reaction and denature the proteins, which were then separated on a 10% SDS-PAGE gel. Fluorescent imaging was performed on an Amersham Imager 600 (AI600) instrument. The gel was then stained with coomassie and imaged on the AI600.

9. Mass Spectrometry Experiments

9a. Whole fragment MALDI analysis

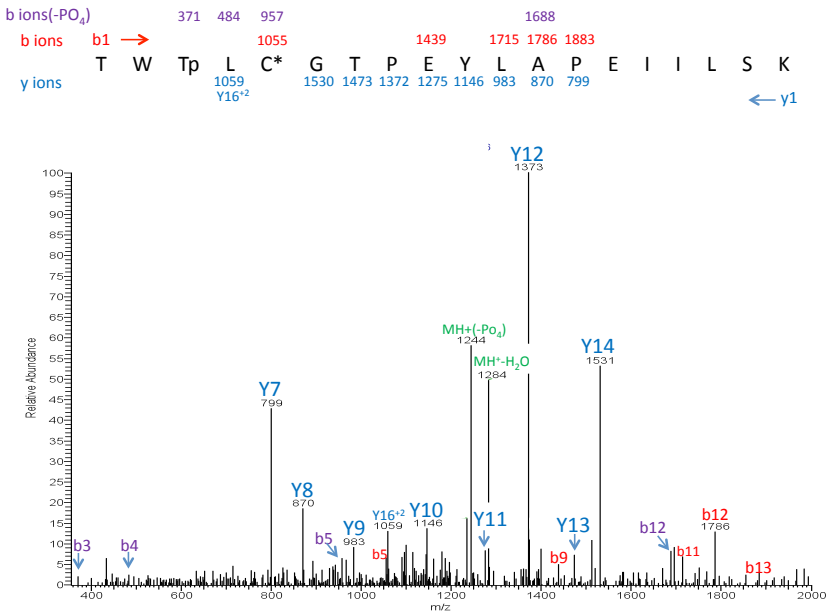
PKACα incubated with **1** or control (untreated PKACα) were exchanged into lysis buffer (25 mM Tris pH 7.6, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 10 mM DTT) and trypsin was added at a ratio of 1:20 trypsin to protein. The resulting solution was incubated at 37 ºC for 24 hr. The sample was analyzed by MALDI mass spectrometry with CHCA matrix (Sigma) on a Voyager DE-Pro™ MALDI TOF Mass Spectrometer.



MALDI Spectra for untreated PKACα control (A.) and PKACα treated with Inhibitor 1 (B.).

9b. Sequencing ESI MS/MS

PKACα incubated with **1** or control (untreated PKACα) were buffer exchanged on a Sephadex G-50 (Sigma) column into 100ul of 100 mM ammonium bicarbonate pH 7.4 (Sigma). The protein was then digested for 2 hr at 37 ºC with proteomics grade Trypsin (Promega) at a ratio of 1:50 trypsin to protein. The mixture of fragment peptides (10%, ~2.5 µg) were loaded on a self-packed fused silica (Polymicro Technologies) trap column (360 micron o.d. x 100 micron i.d.) with a Kasil frit packed with 5-15 micron irregular phenyl C-18 YMC packing. The trap column was connected to an analytical column (360 micron x 50 micron) with a fritted tip at 5 micron or less (New Objective) packed with 5 µm phenyl C-18 YMC packing. Peptides were trapped and then eluted into a Thermo Finnigan LCQ deca XP max mass spectrometer with an acetonitrile gradient from 0 % to 80 % over 2 hr at a flow rate of between 50-150 nL/minute. The mass spectrometer was operated in data dependent mode. First a MS scan from mass 300-1600 m/z was collected to determine the mass of peptides eluting at that time, then the top five most abundant masses were fragmented into MS/MS scans and placed on an exclusion list. This sequence MS followed by 5 MS/MS scans with exclusion was repeated throughout the 2 hr gradient. The approximately 5000 MS2 scans were then searched in Sequest using a single protein database for PKACα and additional variable modifications including oxidized Met, and phosphorylated Ser, Thr, Tyr and Cys modification of 370 Da (corresponds to the Asn-Ala-Ile-Gly amino acids of **1**). A Xcoor cut off of (1.25, 1.75, 2.25) for (+1, +2, +3) peptide charge tates was applied.



Results: Analysis of the modified sample yielded coverage of 73.24 % of the PKACα sequence as mapped by high confidence identification of tryptic peptides. The peptide containing Cys199 was observed with the appropriate mass change to confirm modification with the Asn-Ala-Ile-Gly amino acids of **1** with a high confidence (Xcoor of 4.075).

10. Kinetics Analysis of PKACα Inhibition by 1

Kinetics experiments were performed in assay buffer containing 50 mM MOPS (pH 7.2), 10 mM $MgCl_2$, 500 μM ATP, 1 mM phosphoenolpyruvate, 300 μM NADH, 85.5 Units of lactate dehydrogenase, 60 Units pyruvate kinase, 0.1 mg/mL BSA, and 100 μM DTT in the presence of inhibitor **1** (0 - 10 μM). To each well of a 96-well plate (Greiner) were added 50 μL of assay buffer, 10 μL of inhibitor, 10 μL of Kemptide (250 μM final concentration), and lastly 10 μL of PKACα (30 nM final concentration) was added to initiate the reactions. Once all reagents were added, the assay was run at 30 ˚C for 90 minutes collecting absorbance readings at 340 nm every 20 seconds on a FlexStation 3 plate reader (Molecular Devices). The data obtained were fit to Eq. (1)[5] using Prism 6 (Graphpad).

$$[P] = v_i\,[1-\exp(-k_{obs}*t)]/k_{obs} \qquad \text{(Eq. 1)}$$

where v_i is the initial velocity, k_{obs} is the apparent pseudo-first-order rate constant, and [P] refers to the concentration of phosphorylated Kemptide produced during the reaction process. In order to obtain k_{inact} and K_I, the apparent k_{obs}'s were multiplied by the transformation $(1 + [S]/Km)$[5] to obtain the pseudo-first-order rate constant, kobs, and these values were plotted versus inhibitor **1** concentrations and fit to Eq. (2)[5] using Prism 6 (Graphpad).

$$k_{obs} = k_{inact}[\text{inhibitor } \mathbf{1}]/(K_I+[\text{inhibitor } \mathbf{1}]) \qquad \text{(Eq. 2)}$$

11. References

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