Supporting information

A cell permeable ATP analog for kinase catalyzed-biotinylation

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I. Materials

Spermine, pyruvate kinase/lactate dehydrogenase, NADH, phosphoenol pyruvic acid and alpha-cyano-4-hydroxycinnamic acid were purchased from Sigma Aldrich. ATP was purchased from MP Biomedicals. Dimethyl acetamide (DMAC), 2-(tert-butoxycarbonyloxyimino)-2-phenylacetonitrile (Boc-ON), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), N-hydroxy succinimide (NHS), and N,N,N',N'-Tetramethylethylenediamine for electrophoresis were purchased from Acros. The NHS-biotin ester was synthesized according to prior literature.[1] Sodium cyanoborohydride, formaldehyde, silica, DEAE sephadex A-25, and Ammonium persulfate (APS) for electrophoresis were bought from Fisher Scientific. Dichloromethane (DCM), acetic acid, hydrochloric acid, ammonium hydroxide, and HPLC grade acetonitrile were purchased from EMD. Ethanol was obtained from Decon lab. 40% Bis-acrylamide (37.5:1) for gel electrophoresis and Bradford reagent were purchases from Biorad. Myelin basic protein (MBP), F-12 media and trypan blue were bought from Invitrogen. PKA kinase was purchased from New England Biolabs. Antibiotic and Dulbecco's Phosphate Buffered Saline (DPBS) for cell culture were purchased from HyClone. 3-Hydroxypicolinic acid was bought from Fluka.

II. Instruments

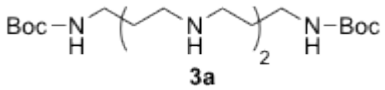
^{1}H NMR, ^{13}C NMR, ^{31}P NMR (Varian Mercury-400), and High resolution mass spectra (HRMS) (LCT Premier XT (Waters) were used to characterize the final APB analog. D_2O was observed at δ 4.63 in ^{1}H NMR. The peaks at δ 1.08 (t) and 3.01 in ^{1}H NMR, and δ 8.29 and 46.59 in ^{13}C NMR corresponded to the triethylamine counter ion. Absorbance of APB was measure by UV-Vis spectrophotometer (Shimadzu 2101 PC). A Lyophilizer (VirTis BT 3.3 EL Benchtop) and Speed-vac (ThermoSAVANT, SPD131 DDA) were used during synthesis of APB. RP-HPLC was performed with Waters 1525 binary HPLC pump, Waters 2998 photodiode array detector, and Reverse phase C-18 column (YMC America, INC 250×4.6 mm, 4µm, 8 nm). Bradford assay was done using a fluorimeter (GENios Plus Tecan). SDS-PAGE apparatus were bought from BioRad (Protean III). Protein transfer was performed using the Mini-Transblot Electrophoretic Transfer Cell apparatus from BioRad. SDS gels and PVDF membranes were visualized by a Typhoon 9210 scanner (Amersham Biosciences). Immunofluorescence images were visualized by Olympus fluorescence microscope (Model BX 41). Peptide masses was detected using MTP Plate (Bruker) and MALDI-TOF (Bruker Ultraflex).

III. Docking Studies

The crystal structure of PKA was downloaded from RCSB Protein Data Bank (pdb ID: 4DH1). The co-crystallized peptide, ATP, and water were deleted using Pymol 1.5.0.5 (Schrodinger, LLC). All hydrogen atoms, Gasteiger charges and merging non polar hydrogen were added by AutoDock Tools 1.5.6, followed by generation of pdbqt output file. The charge of Mg was changed from zero to +2 manually. A grid box with a spacing of 0.375 Å, size of 74 X 70 X 70, and coordinates for the center of the grid box (-9.145, 13.434, -21.018) were used. The grid map files required for docking calculations were generated by AutoGrid 4.2. APB was drawn in ChemBioDraw Ultra and MM2 energy minimization was done by Chem 3D Pro. AutoDock Tools 1.5.6 was used again to add hydrogens, compute Gasteiger charges, merge nonpolar hydrogens, choose torsions, and generate a pdbqt file. All acyclic bonds were rotatable except amide bonds. We then used AutoDock 4.2 to run docking calculations using the genetic algorithm, and a pdbqt file was generated. The pdbqt file for PKA was set as a rigid macromolecule and the genetic algorithm search parameters were set to 100 GA runs with a population size of 150, a maximum number of 2.5×10^5 energy evaluations, a maximum

number of 2.7 × 10^4 generations, a mutation rate of 0.2, and a crossover rate of 0.8. Default docking parameters were used and the output DLG file was converted to pdbqt extension. PyMOL 1.5.0.5 (Schrodinger, LLC) was used to create images in Figure 2 and S1.

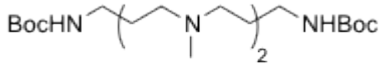
IV. Synthesis of ATP-polyamine-biotin (APB)



3a

Amine-protection of spermine: Synthesis of di-tert-butyl ((butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))dicarbamate (3a)

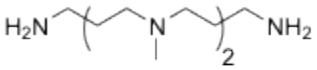
Compound **3a** was synthesized according to literature as follows.[2] Spermine (**3**, 1g, 4.9 mmol) was dissolved in THF (15 mL) at 0 °C. A solution of BOC-ON (2.42 g, 9.8 mmol) in THF (30 mL) was added drop wise under argon at 0 °C. After stirring at 0 ^{o}C for 2 minutes, the solution was stirred at room temperature for 1 hour. The reaction was quenched with saturated sodium carbonate (45 mL) and extracted with dichloromethane (135 mL). The organic layer was evaporated *in vacuo* and then the residue was purified by chromatography using silica and 5% ammonia in ethanol as eluting solvent to obtain **3a** as a white solid (1.7 g, 80% yield). Spectral characterization was consistent with prior literature.[2]



4

Synthesis of di-tert-butyl ((butane-1,4-diylbis(methylazanediyl))bis(propane-3,1-diyl))dicarbamate (4)

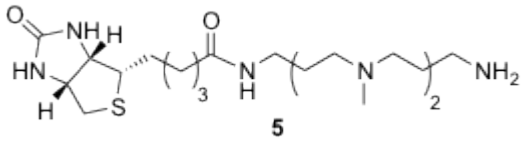
Compound **4** was synthesized according to literature[2]. Compound **3a** (1.7 g, 3.9 mmol) was dissolved in ethanol (45 mL) together with a 37% formaldehyde solution (17.3 mL, 213.1 mmol). Acetic acid (11.5 mL) was added, followed by sodium cyanoborohydride (4.3 g, 68.4 mmol). The reaction was stirred overnight and then quenched with saturated sodium carbonate solution until effervescence ceased, followed by extraction with dichlormethane (3 times). The organic layer was evaporated *in vacuo*. The residue was purified by chromatography using silica and 5% ammonia in ethanol as eluting solvent to obtain **4** as yellowish oil (1.8 mL, 98% yield). The spectral characterization was consistent with prior literature.[2]



4a

Synthesis N1,N1'-(butane-1,4-diyl)bis(N1-methylpropane-1,3-diamine) (4a)

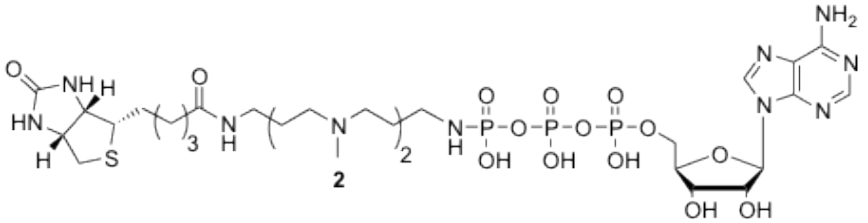
Compound **4a** was synthesized according to literature.[2] Compound **4** (1.83 g, 4.2 mmol) was dissolved in 1,4-dioxane (40 mL) at 0 °C, followed by addition of 4N HCl (40 mL). The reaction was stirred at room temperature for 4 hours and then the solvent was evaporated *in vacuo*. The residue was crystallized with methanol/ethylacetate to give white crystals of compound **4a** as a chloride salt (1.3 g, 90% yield). The spectral characterization was consistent with prior literature.[2]



5

Synthesis of polyamine-biotin (using methylated spermine) (5)

Polyamine-biotin (**5**) was synthesized according to literature[3] with some modifications to the procedure. Methylated spermine (**4a**) (0.78 g, 2.5 mmol) was dissolved in a mixture of DMAC (25 mL) and DIPEA (3.3 mL) at 0 °C. A solution of NHS biotin ester[1] (350 mg, 1 mmol) in DMAC (30 mL) was added dropwise over an hour under argon and the reaction mixture was stirred overnight at room temperature, followed by addition of diethyl ether to precipitate the product. The precipitate was filtered and then purified by chromatography using silica and ethanol:THF:DCM: ammonia (4:4:2:1) as an eluting solvent. Polyamine-biotin (**5**) was obtained as oil (0.5 g, 44%). The spectral data was consistent with literature.[3]



2

Synthesis of ATP-polyamine-biotin (APB, 2)

The disodium salt of ATP (32 mg, 0.059 mmol) was dissolved in water (5 mL) and then the pH was adjusted to 7.0 with NaOH (0.5 M). EDCI (452.5 mg, 2.36 mmol) was added and the pH was readjusted to 5.6-5.8 with HCl (0.5 M). Polyamine-biotin (**5**) (114 mg, 0.25 mmol) in water (2 mL) was added and the pH was readjusted to 5.6-5.8 with HCl (0.5 M). The solution was stirred for 3 hours with control of pH at 5.6-5.8. The solution was brought to a pH of 8.5 uisng triethylamine and the product was separated by anion exchange column (A-25 sephadex) with 0.1-1 M triethyl ammonium carbonate buffer (pH 8.5) as eluent. Fractions containing APB were combined, lyophilized to dryness, and stored at -80 ^{o}C as a white solid (18.9 mg, 36%). Purity (95%) was assessed by RP-HPLC with triethyl ammonium acetate buffer (TEAAB, buffer A: 100 mM TEAAB in HPLC water) and acetonitrile (buffer B: 100% acetonitrile) using constant 5% buffer B for 10 minutes and then a gradient of 5% to 40% buffer B over 40 minutes. ^{1}H NMR (400 MHz, D_2O): δ 0.92 (4H, t), 1.24 (6H, t), 1.50-1.53 (3H, m), 1.66 (3H, m), 2.05 (1H, t), 2.45 (2H, t), 2.58 (3H, s), 2.71-2.90 (m, 9H), 3.15-3.21 (6H, q), 4.00 (2H, d), 4.18-4.21 (2H, m), 4.35-4.39 (2H, m), 5.91 (2H, d), 8.07 (1H, s), 8.37 (1H, s). ^{13}C NMR (100 MHz, D_2O): δ 25.1 (2), 25.9, 27.6, 27.9, 35.4 (2), 42.2, 44.7, 45.3, 45.4, 47.2 (2), 47.9 (2), 48.6 (2), 53.7 (2), 55.3, 60.1 (2), 61.9, 67.4, 86.5, 118.3, 123.7, 127.7, 139.9, 152.9, 167.6. ^{31}P NMR (162 MHz, D_2O): -1.8 (d), -11.8 (d), -23.1 (t). UV/Vis spectroscopy (H_2O): λ 260 nm. HRMS: $[M-1]^-$ for $C_{32}H_{58}N_{11}O_{14}P_3S$: calc. 944.3098, found 944.3046.

V. Synthesis of N-acetylated kemptide (AcLRRASLG)

Acetyl kemptide was synthesized by Fmoc based solid phase peptide solid according to literature.[4] The peptide was purified by RP-HPLC and characterized by MALDI-TOF, $[M+1]^+$ for $C_{34}H_{63}N_{13}O_{10}^+$: Calc. 814.4894, found 814.372.

VI. In vitro kinase-catalyzed biotinylation of MBP with PKA

Kinase-catalyzed biotinylation was performed by incubating ATP or APB analog (5 mM), PKA enzyme (5μg/mL, 500U), and myelin basic protein (MBP) (1 μg/μL) in the kinase buffer provided by the manufacturer (1X). The final volume for the reaction was 20 μL. The reaction mixture was incubated at 31 °C for 2 hours. Reactions without PKA and/or APB were conducted as control

experiments. Reactions in presence of the kinase inhibitor staursporine (1 μM final concentration) were also performed where inhibitor and PKA were preincubated for 30 minutes before addition of APB (Figure S11). Another control experiment was performed by adding TFA (50% final conc.), and incubating at 45 °C for 1 hr with shaking at 700 rpm to cleave the phosphoramidate bond. TFA was evaporated using speed vac, followed by neutralization of the remaining TFA with 1.5 M Tris base (pH=8.8, 10 μL). All reaction mixtures were separated by 16% SDS-PAGE and visualized with SYPRO® Ruby or transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Milipore) and visualized with streptavidin-Cy5 (Life Technologies).

VII. In vitro MS analysis of kinase-catalyzed biotinylation

APB analog (5 mM) was incubated with PKA enzyme (20μg/mL, 2000U) and N-acetylated kemptide (1 mM) in the kinase buffer provided by the manufacturer (1X). The final volume for the reaction was 10 μL. The reaction mixture was incubated at 31 °C for 2 hours. A reaction without PKA was conducted as a control experiment. The reactions were subsequently mixed with an equal volume (10 μL) of a saturated solution of a 1:1 mixture of 3-hydroxyalpha picolinic acid and alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile, then spotted on a MALDI plate. The spot was left to dry and analyzed by MALDI-TOF (Bruker). Phosphorylbiotinylated peptide masses were detected at high laser power.

VIII. *In vitro* quantification of kinase-catalyzed biotinylation of MBP with PKA

Kinase-catalyzed phosphorylation and biotinylation reactions were performed as in section V, followed by incubation with TFA (50% final conc.) for 1 hour at 45 ^{o}C with shaking at 700 rpm. The TFA incubation was necessary to cleave the phosphoramidate bond in the ATP-biotin products to create a phosphoprotein for quantitative analysis. TFA was evaporated using speed vac, followed by neutralization of the remaining TFA, as described in section V. The reaction mixtures were separated by SDS-PAGE (16%) gel electrophoresis and visualized by SYPRO® Ruby or Pro-Q diamond stain according to the manufacturer's instructions. Quantification of MBP phosphorylation on the Pro-Q diamond stained gel image was performed with ImageQuant 5.2 by drawing the same-sized rectangle on each MBP protein band. The MBP phosphorylation signal was background corrected by subtracting the signal after kinase reaction by the signal of untreated MBP. Percentage phosphorylation was calculated by dividing the background-corrected MBP phosphorylation signal in ATP-biotin or APB reactions by the signal in ATP reactions (set as 100% phosphorylation) and multiplying by 100.

IX. Kinetic analysis of APB with PKA

An NADH-dependent coupled assay was used to perform kinetic analysis, as previously described,[7] with some exceptions. The assay was performed using 0.5 mM NADH, 24 units/mL of pyruvate kinase, 36 units/mL of lactic acid dehydrogenase, PKA (2.5 μg/mL, 61 nM), ATP or ATP-biotin final concentrations of 0.5, 1, 3, 10, 30, and 100 μM, and absorbance at 360 nm taken every 30 second for 60 min. Kaleidagraph software (Synergy Software) and by non-linear regression analysis was used to obtain K_M and V_{max} values from the Michaelis-Mentor equation ($v = V_{max}*[S]/(K_M + [S])$, where v= rate of the reaction and [S]= substrate concentration). k_{cat} was calculated by dividing V_{max} by the concentration of PKA enzyme.

X. Hela cells lysis procedure

Hela cells (National Cell Culture Center, Biovest) (20×10^6) were lysed in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, and 1X protease inhibitor cocktail (GenDepot); 1 mL) with rotation for an hour at 4°C. Cell debris was removed by centrifugation at

13,200 rpm for 10 minutes at 4 ^{o}C. The supernatant was collected and protein concentration was determined by Bradford assay. Protein content was brought to a working concentration (10 mg/mL) using lysis buffer before storage at -80 ^{o}C.

XI. Kinase-catalyzed biotinylation of Hela cell lysates

APB (5 mM) or ATP-biotin (5 mM) was incubated with HeLa cell lysates (4 μg/μL) at 30°C for 2 hours. The final volume of the reaction was 20 μL. Heat-inactivated Hela cell lysates were produced by heating at 95°C for 5 min before adding APB. NHS-biotin (0.5 mM) was incubated with cell lysates as a positive biotinylation control reaction. As a control to cleave the phosphoramidate bond, TFA (50% final conc.) was added after reaction and incubated for 1 hour at 31°C with shaking at 800 rpm, followed by evaporation using speed vac, and neutralization by adding 1.5 M Tris base (pH=8.8, 10 μL). The reaction mixtures were separated by SDS-PAGE (10%) and visualized by SYPRO® Ruby or transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Milipore) and visualized with streptavidin-Cy5 (Life Technologies).

XII. Kinase-catalyzed biotinylation of Hela cells

Hela cells (200,000 cells) were added to 12-well plates and allowed to grow for 48 h in F-12 media containing 10% FBS, penicillin (9 units), and streptomycin (9 units) at 37°C in a 5% CO_2 environment. The cells were then incubated with ATP-biotin (5 mM) or APB (5 mM) dissolved in F-12 growth media (400mL) for 1 hr under the same growth conditions as above. As a control, one well was preincubated with staurosporine (1 μM final concentration) in growth media for 1 hour before adding media containing APB. The media was removed and cells were washed with DPBS (400 μL) two times, scraped, and collected by centrifugation at 1000 rpm for 5 min at 0°C. Cell pellets were lysed in lysis buffer (31 μL) on ice for 30 minutes and then lysates were collected as in section VII. The lysate mixtures were separated by 10 % SDS-PAGE and visualized by SYPRO® Ruby or transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Milipore) and visualized with streptavidin-Cy5 (Life Technologies).

XIII. Cell viability assay[5]

Hela cells (100,000) were incubated in a 24 well plate and allowed to grow for 48 h in F-12 media containing 10% FBS, penicillin (9 units), and streptomycin (9 units) at 37°C in a 5% CO_2 environment. APB (1, 5, 10, 15, 20, 30 mM) in media (200 uL) was added to cells and cells were incubated for an hour. The media was removed and cells were washed twice with DPBS (400uL), followed by trypsinization. Cells were collected by centrifugation at 4 ^{o}C at 1000 rpm for 5 minutes, followed by washing twice with DPBS (100 uL). Cells were resuspended and an equal volume of cell suspension was mixed with trypan blue (0.4%) and counted using hemocytometer. As a control, untreated cells were counted in one well after subjecting them to the same washing conditions. Percentage viability was calculated by dividing the number of treated live cells by untreated ones. Kaleidagraph software (Synergy Software) was used to calculate EC_{50} of APB. The results are from three independent trials.

IVX. Fluorescence microscopy

Hela cells (80,000) were grown on a cover slip in a 24 well plate overnight in F-12 media (500 μL) containing 10% FBS, penicillin (9 units), and streptomycin (9 units) at 37°C in a 5% CO_2 environment until 80% confluency. The cells were then incubated in serum free media for 2 hours, followed by incubation with APB (5 mM) in serum free media (500 μL) for 1 hour. Cells were washed with DPBS (500μL) 3 times, followed by 1X PBS (500 μL, 137mM NaCl, 27 KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) three times. Cells were incubate with paraformaldehyde (4%, 500 μL)

for 20 minutes at room temperature, then washed wit 1X PBS (500 μL) three times. Triton-X100 (0.2 % in PBS) was added and incubated with cells for 5 minutes at room temperature, followed by washing with PBS (500 μL) 3 times each for 5 min. Blocking buffer (2% BSA, 2% normal goat serum, and 0.2 % gelatin in 1X PBS) was incubated with cells for 1 hour at room temperature. Streptavidin Cy5 (10 ug/mL) in blocking buffer (50 μL) was added and incubated with cells for 30 minutes. Cells were washed with 0.1% BSA in 1X PBS (500μL) 3 times each for 5 minutes. Cells were incubated with DAPI (100 μg/mL) for 5 minutes, followed by washing with 1X PBS (500 μL) 3 times each for 5 min. The cover slips were washed in water then installed on a glass slide containing Mowiol mounting solution (50 μL, Sigma Aldrich) saturated with DABCO (Sigma Aldrich). The slide was incubate at 31 ^{o}C for 30 minutes, and then kept at 4 ^{o}C until microscopy. Fluorescence photos were generated on an Olympus fluorescence microscope using laser wavelengths corresponding to DAPI (350 nm) and Cy5 (650nm). DAPI photos were generated at 10 ms and Cy5 at 200 ms. Merged images were created using Adobe Photoshop.

XV. Supporting data

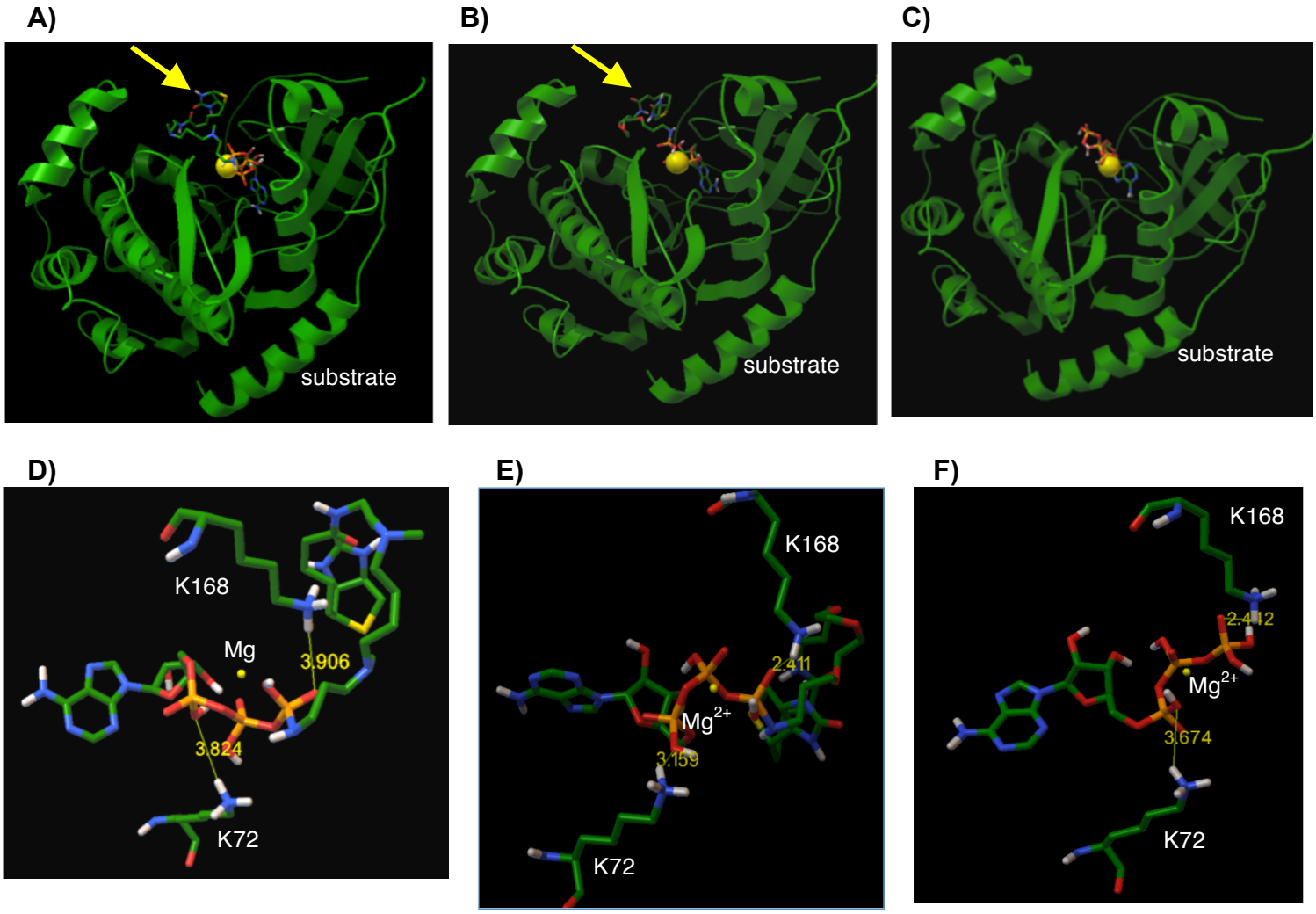


Figure S1: Docking of APB (A), ATP-biotin (A), and ATP (B) into the crystal structure of the catalytic active site of PKA kinase (green) co-crystallized with peptide substrate (pdb: 4DH1)[6] using Autodock4.2[7]. The lowest energy binding pose is shown. An arrow points to the biotin group of APB and ATP-biotin in parts A and B. (D, E, and F) An enlarged view of the interaction of APB (D), ATP-biotin (E) and ATP (F) with the catalytic Mg^{2+} metal (yellow orb) and amino acids K72 and K168 of PKA. The γ-phosphates of ABP, ATP-biotin, and ATP are positioned in a close proximity to the co-crystallized peptide (A, B, and C) and catalytic K168 (D, E, and F). The atoms of ABP, ATP-biotin, and ATP are color-coded (C = green; H grey; N = blue; O = red; p = orange). For clarity, the atomic radii of Mg^{2+} were reduced to 0.5 Å in D-F. Parts A and D are the same images in Figure 2 of the manuscript.

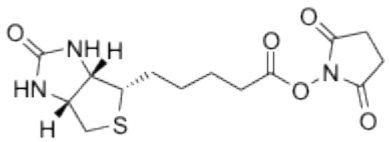


Figure S2: The structure of NHS-biotin used in the synthesis of APB and the studies in Figure 4.

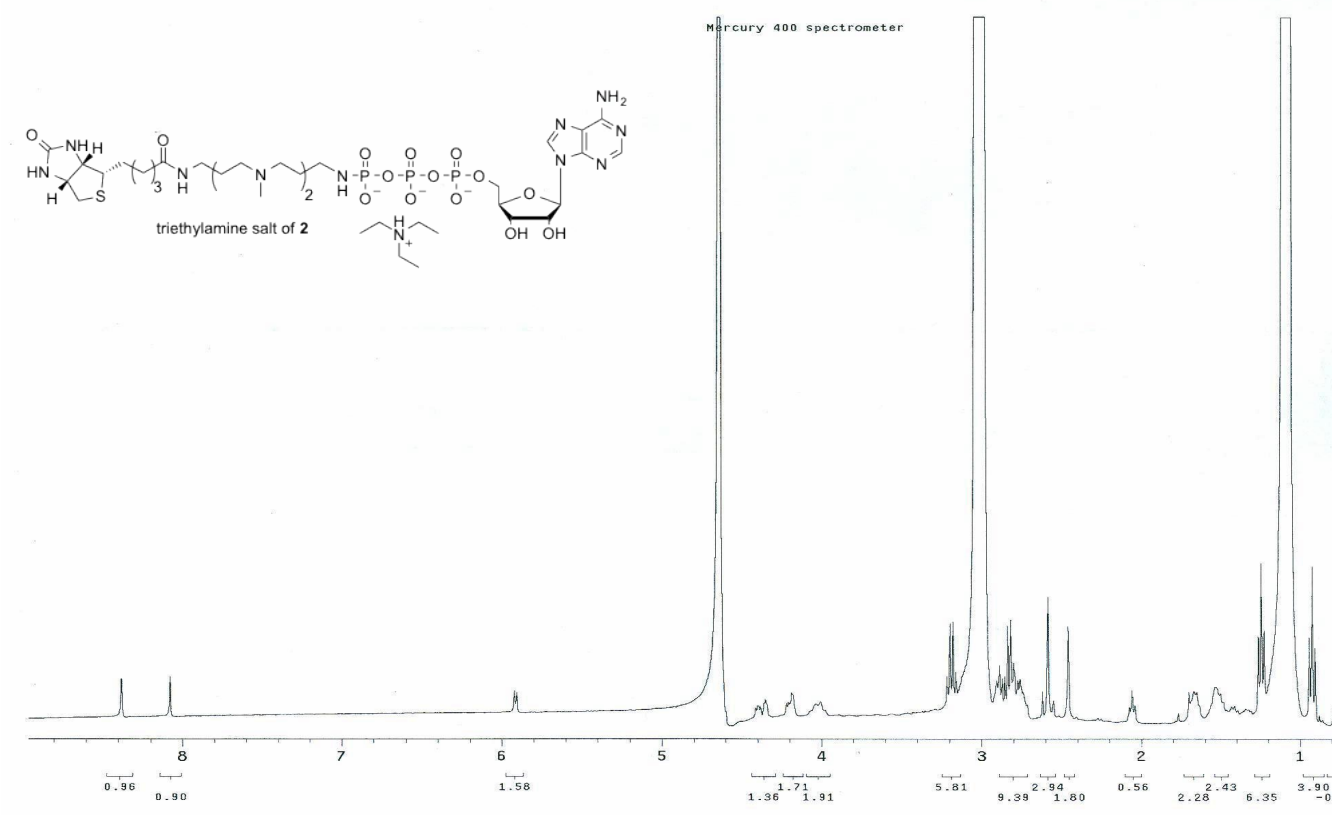


Figure S3: ^{1}H NMR of the triethyl amine salt of ATP-polyamine-biotin (APB) recorded in D_2O at 400 MHz.

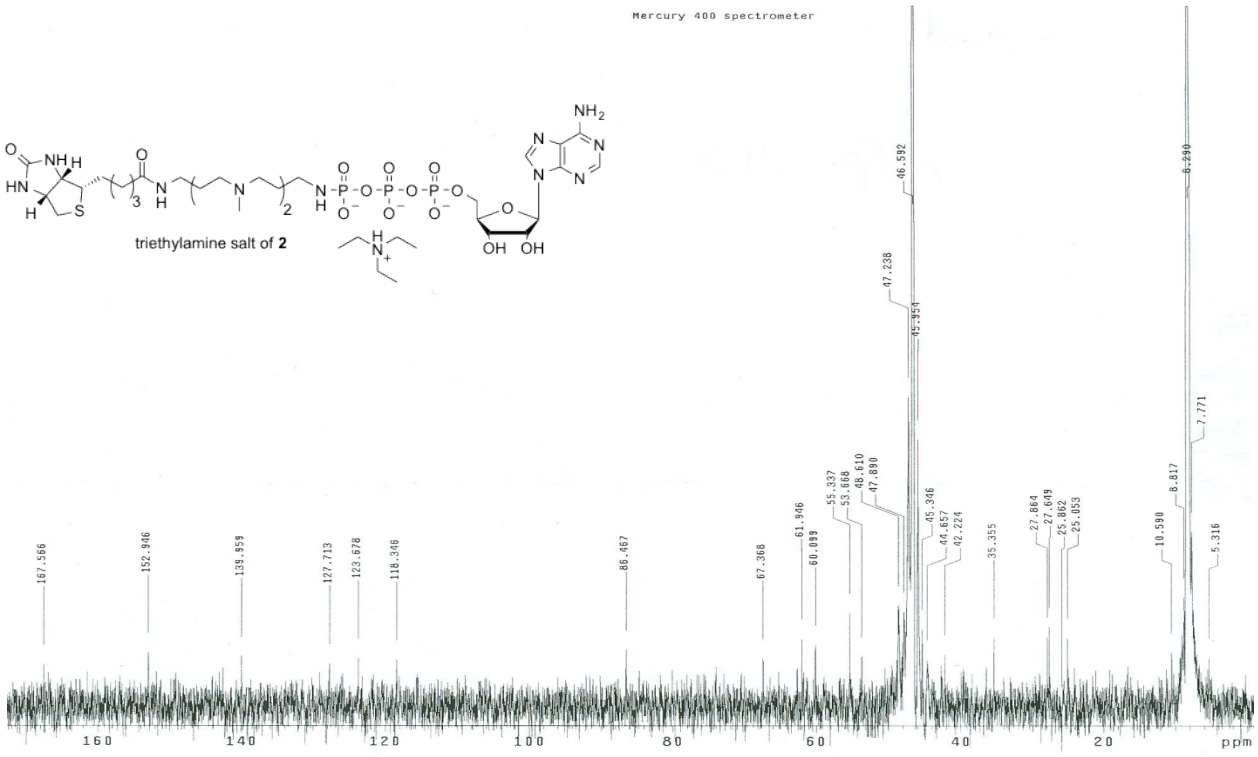


Figure S4: ^{13}C NMR of the triethyl amine salt of ATP-polyamine-biotin (APB) recorded in D_2O at 100 MHz.

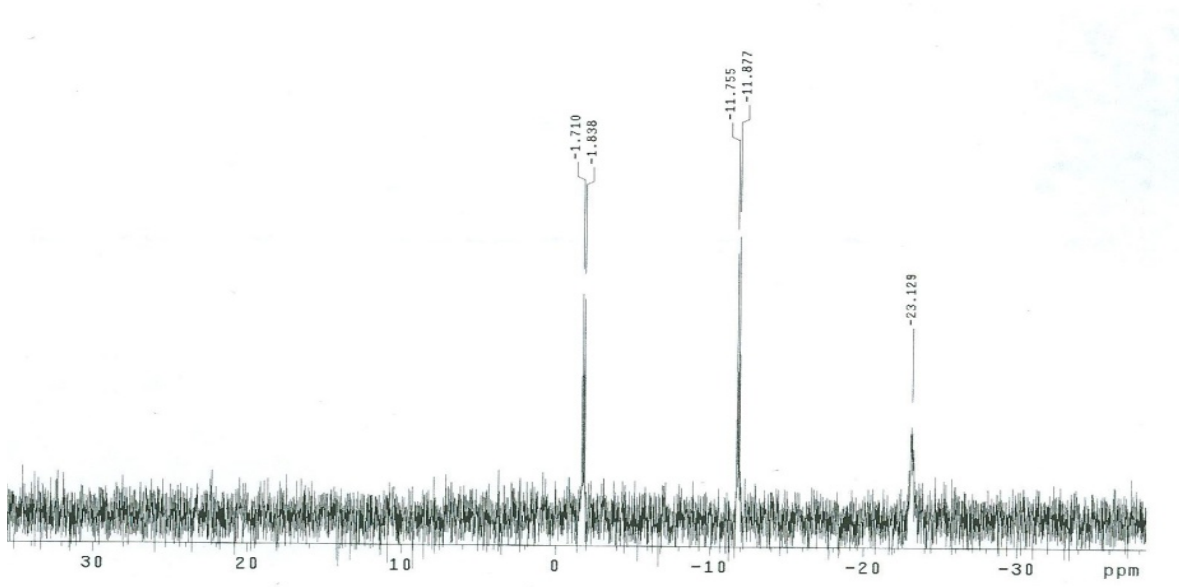


Figure S5: ^{31}P NMR of the triethylamine salt of ATP-polyamine-biotin (APB) recorded in D_2O at 162 MHz.

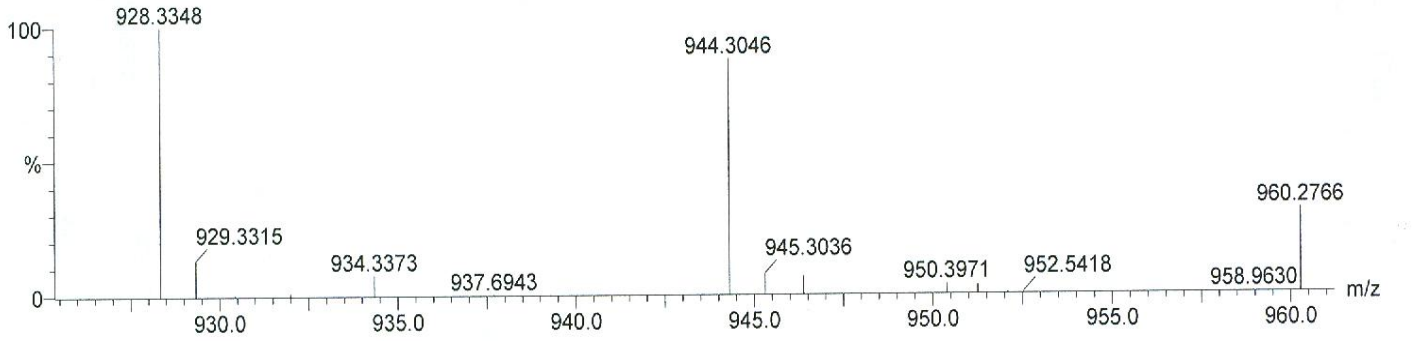


Figure S6: Electrospray ionization (ESI) negative mode high resolution mass spectrum (HRMS) of ATP-polyamine-biotin (APB) recorded in acetonitrile. Calculated $[M-H]^{-1}$ for $C_{32}H_{58}N_{11}O_{14}P_3S$: 944.3098; Observed: 944.3046

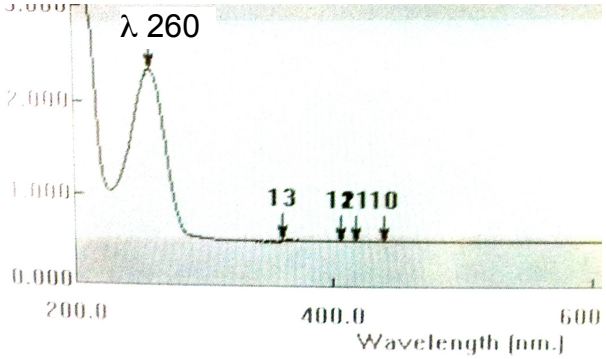
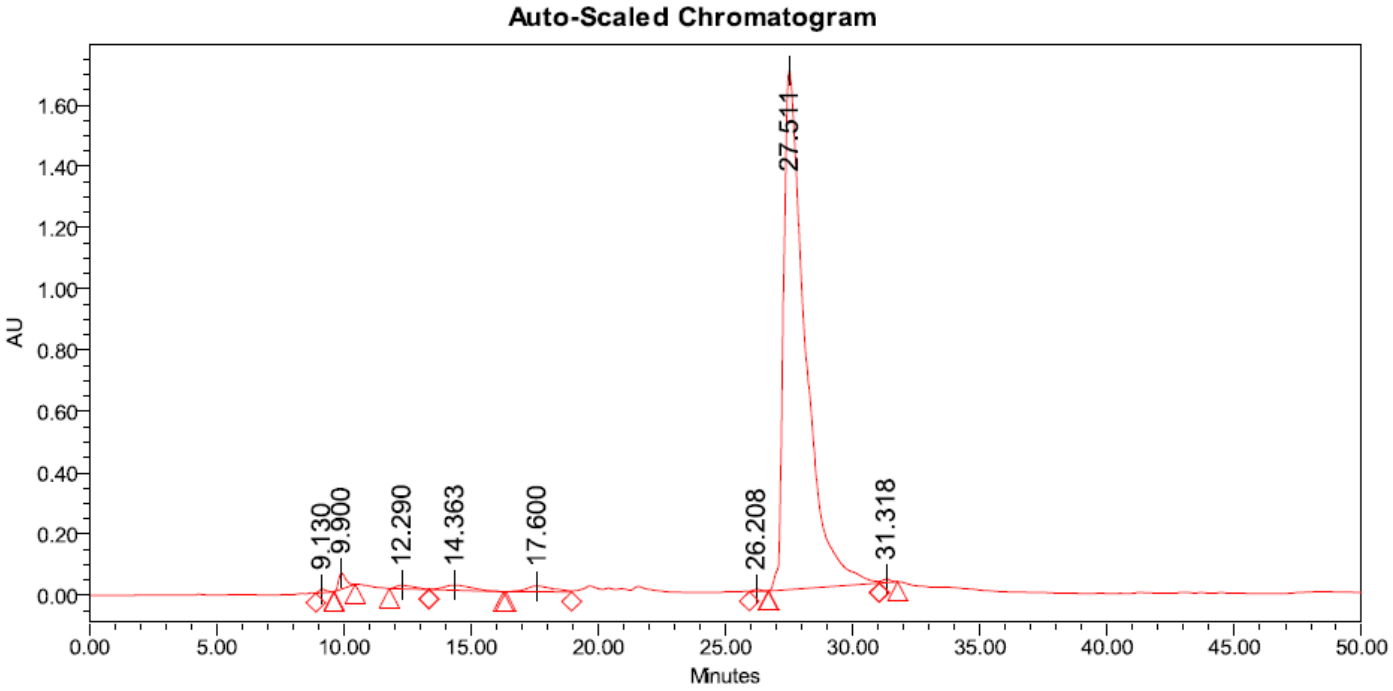


Figure S7: UV-VIS spectrum of ATP-polyamine-biotin (APB) recorded in water. The APB absorbance appears at λ 260 nm



Peak Results

	RT	Area	Height	% Area
1	9.130	227259	11719	0.21
2	9.900	1065184	52685	1.00
3	12.290	558650	11098	0.53
4	14.363	1320540	16439	1.24
5	17.600	1390379	19014	1.31
6	26.208	145197	6432	0.14
7	27.511	101123948	1693568	95.31
8	31.318	264478	11226	0.25

Figure S8: HPLC analysis of ATP-polyamine-biotin (APB) purity. ATP-polyamine-biotin appears at 27.511 minutes and is 95% pure.

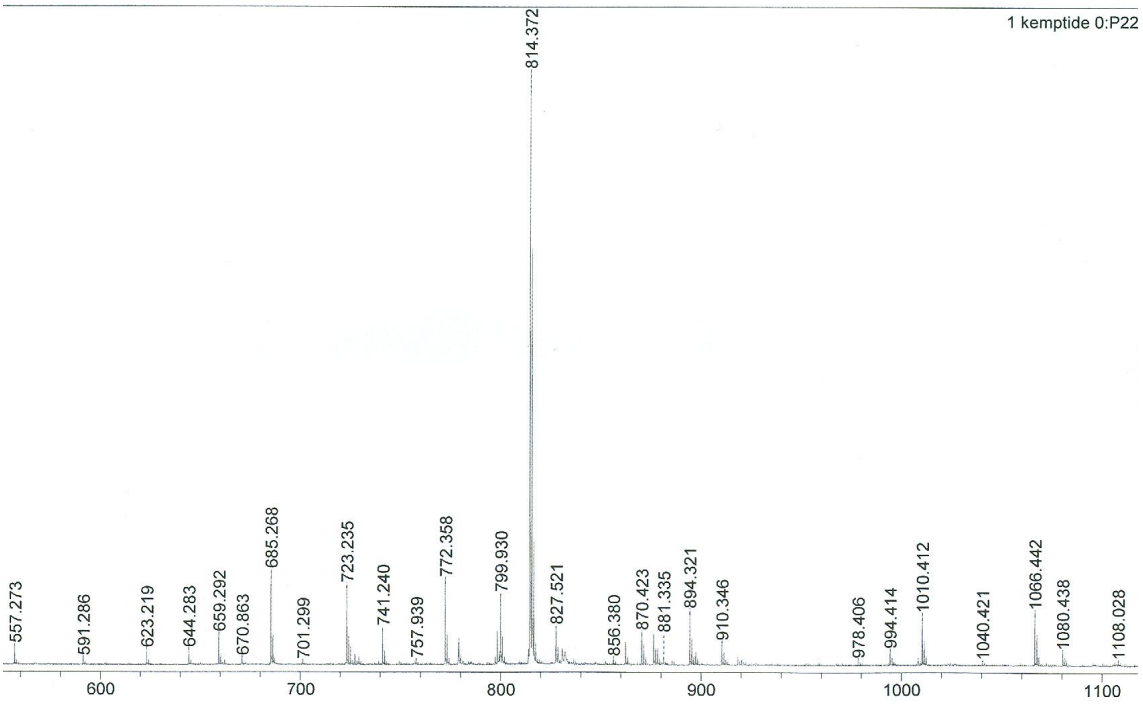


Figure S9: MALDI-ToF spectrum in the positive ion mode of N-acetylated kemptide peptide (AcLRRASLG). Calculated $[M+H]^{+1}$ for $C_{34}H_{63}N_{13}O_{10}^{+}$: 814.4894; Observed: 814.372

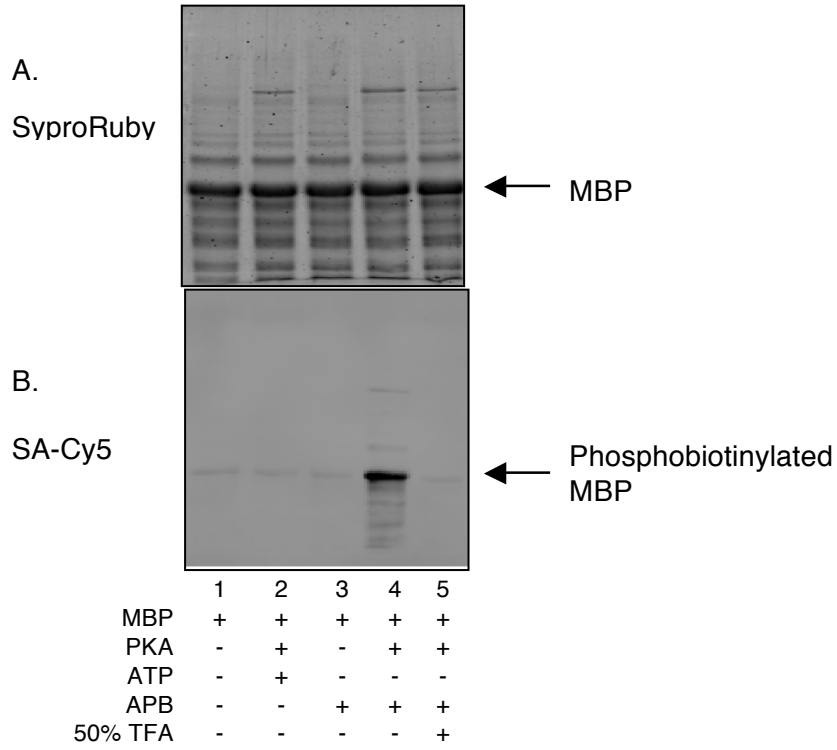


Figure S10: Full gel images of kinase-catalyzed biotinylation with MBP, PKA, and APB, which is shown in Figure 3A of the manuscript. The labeled mixtures were separated by SDS-PAGE and visualized by SYPRO® Ruby to see total proteins (A) or streptavdine Cy-5 (SA-Cy5, Life Technologies) to detect biotinylation (B). TFA (50%) was added after biotinylation labeling to assure biotinylation via an acid-labile phosphoramidate bond (lane 5). The images are representative of at least three independent trials.

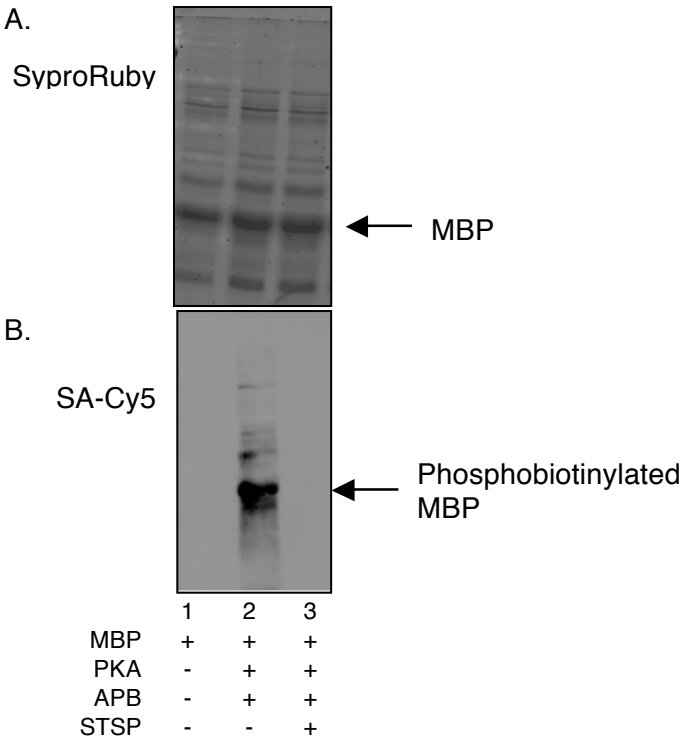
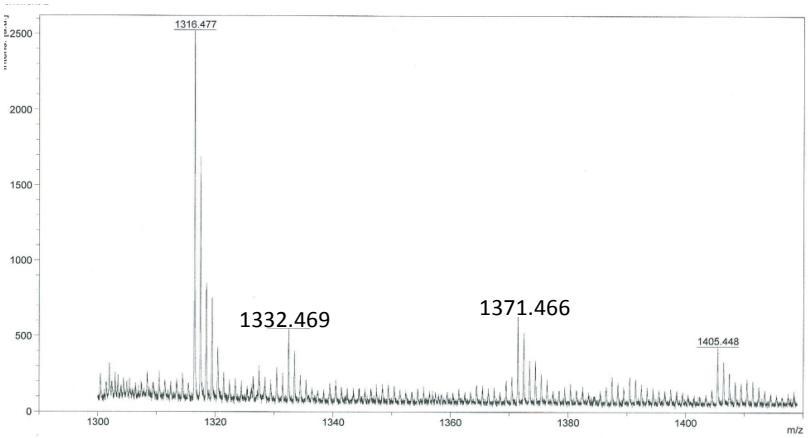


Figure S11: Gel images of kinase-catalyzed biotinylation with MBP, PKA, and APB in presence and absence of 1 μM staursporine (STSP). The labeled mixtures were separated by SDS-PAGE and visualized by SYPRO® Ruby to observe total proteins (A) or streptavidin Cy-5 (SA-Cy5, Life Technologies) to detect biotinylation (B). The images are representative of at least three independent trials.

A.



B.

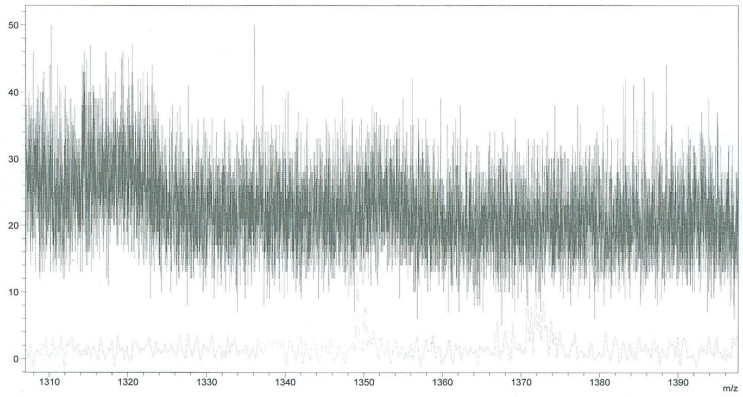
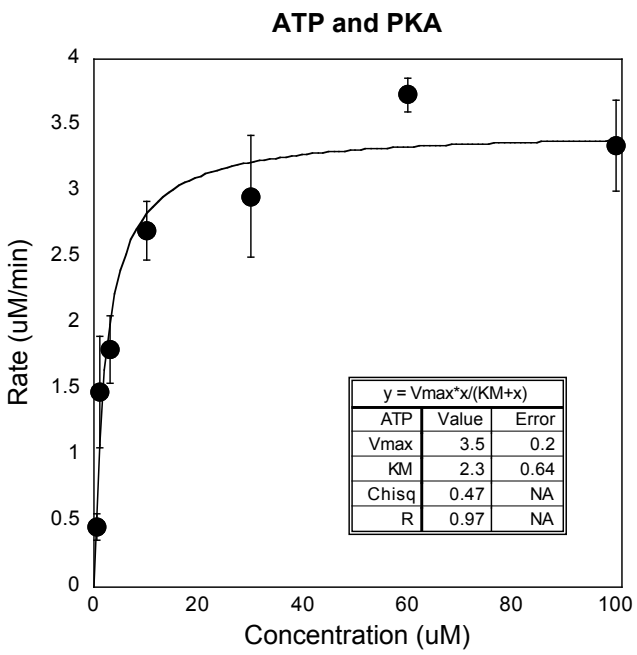
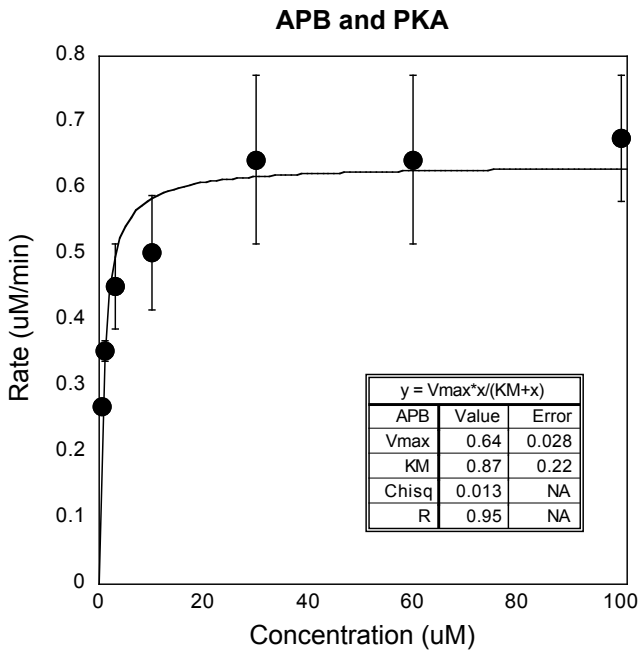


Figure S12: MALDI-TOF spectra of a kinase-catalyzed phosphobiotinylation reaction of N-acetylated kemptide with PKA in positive ion mode. A) Reaction in the presence of APB: ($[M+H]^+$ for $C_{56}H_{107}N_{19}O_{14}PS$, Calculated: 1332.7698; Observed: 1332.469; $[M+K]^+$ for $C_{56}H_{106}KN_{19}O_{14}PS$, Calculated: 1371.7151; Observed: 1371.466 B) Reaction in the absence of APB showing no product peaks in the same m/z range.

A



B



C

	ATP	APB
K_M (μM)	2.3 ± 0.6	0.87 ± 0.21
V_{max} (μM/min)	3.5 ± 0.2	0.64 ± 0.02
k_{cat} (s^{-1})	1.2± 0.1	0.22± 0.01
k_{cat}/K_M ($\mu M\ s)^{-1}$	0.52	0.25

Figure S13: Michaelis-Menton curve fits for reactions containing PKA kinase and either ATP (A) or APB (B) as the cosubstrate, with kinetic analysis comparison between ATP and APB in the table (C).

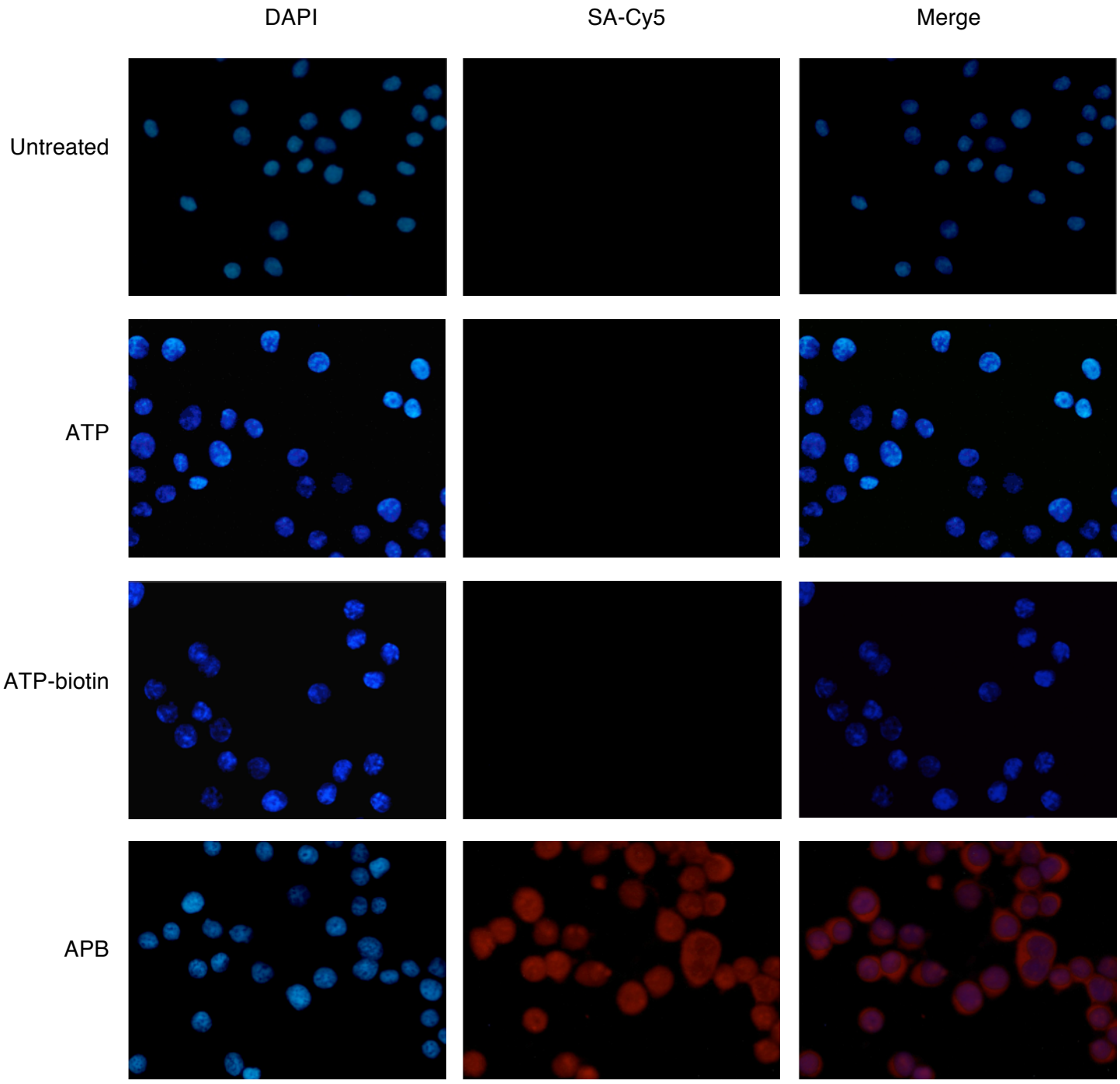


Figure S14: Microscopy studies of ATP-biotin or APB cell permeability. HeLa cells were untreated or treated with ATP, ATP-biotin or APB before visualization with the nuclear stain DAPI or biotin stain streptavidin-Cy5 (SA-Cy5). The merged images of DAPI and SA-Cy5 show that APB is found in both the nucleus and cytoplasm. This figure includes enlarged images also shown in Figure 4B.

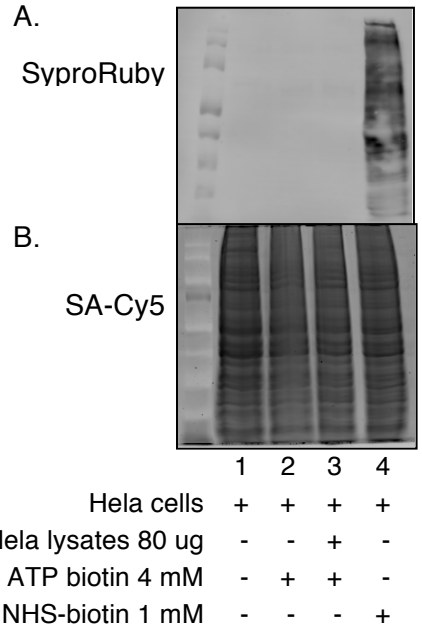
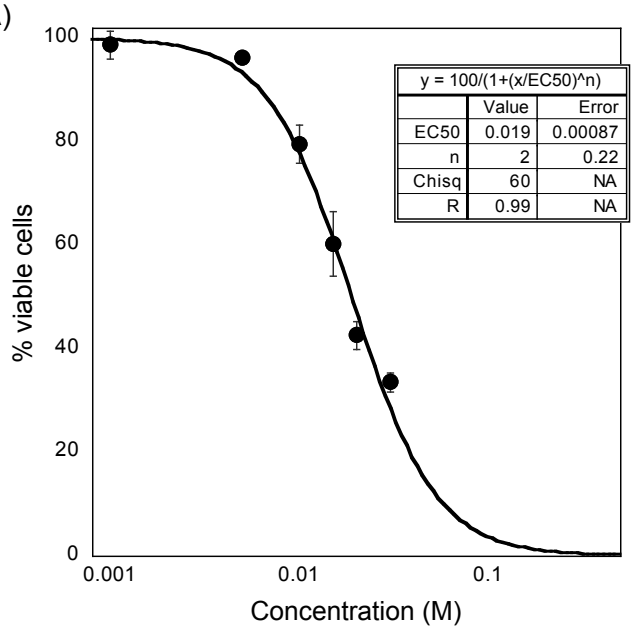


Figure S15: Gel images of kinase-catalyzed biotinylation of live HeLa cells with impermeable ATP-biotin analog (4 mM final concentration) in presence and absence of exogenous HeLa lysates (80 µg), which contain kinase activity. As a control for the presence of cell surface proteins, HeLa cells were incubated with NHS-biotin (Figure S2, 1 mM final concentration, lane 4). The cells were washed, lysated, and protein mixtures were separated by SDS-PAGE and visualized by SYPRO® Ruby to observe total proteins (A) or streptavidin Cy5 (SA-Cy5, Life Technologies) to detect biotinylation (B). Biotin signal is absent with ATP-biotin in the presence or absence of the kinase activity in lysates (panel A, lanes 2 and 3), indicating no biotinylation of cell surface proteins. The images are representative of at least three independent trials.

A)



B)

Concentration (mM)	% cell viability
0.0	100%
1.0	99 ± 3 %
5.0	96 ± 1 %
10	80 ± 4 %
15	60 ± 6 %
20	43 ± 3 %
30	34 ± 2 %

Figure S16: Cell viability assays with varying concentrations of APB. A) Dose response curve of APB with 1, 5, 10, 15, 20, 30 mM final concentrations. The EC_{50} of APB is 19 ± 1 mM. B) Table with the percentage viablity data plotted in part A. The concentration of APB used in kinase-catalyzed labeling of cells was 5 mM.

XVI. References

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