Supplemental Information

Phosphorylation-dependent Kinase-Substrate Crosslinking

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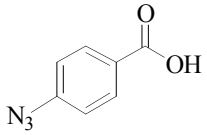
I. Materials. ATP was purchased from Calbiochem. PKA, CKII, Abl and α-casein were bought from New England Biolabs. CK2 and Abl peptide substrates (**12** and **13**) were purchased from Promega. 4-aminobenzoic acid, 2,2'-ethylenedioxy bis-(ethylamine), α-cyano-4-hydroxy-cinnamic acid (4HCCA), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), and N,N-dimethylformamide (DMF) were purchased from Acros. N-hydroxy succinimide (NHS) and t-boc-anhydride were bought from Fluka. Sodium azide and sodium nitrite were purchased from Fisher. Tris-base, ethylenediamine tetraacetic acid (EDTA) and sodium chloride were obtained from JT Baker. D_4-MeOH was purchased from Cambridge isotope lab, Inc. Dichloromethane (DCM) and ammonium hydroxide were purchased from Mallinchkrodt chem. Ethanol was obtained from Decon lab. HPLC grade acetonitrile was purchased from EMB. All the amino acids for peptide synthesis were purchased from Novabiochem. Flash chromatography was performed on silica gel, 200-400 mesh (Merck).

II. Instruments. ^{1}H NMR, ^{13}C NMR and ^{31}P NMR (Varian Mercury-400) and ESI MS (Waters ZQ2000) were used to authenticate all precursors as well as the final ATP-ArN_3 analog **3**. The peaks at δ 4.95 and 3.3 in the ^{1}H NMR and δ 58.0 in ^{13}C NMR are due to CD_3OD solvent. Likewise, the peaks at δ 1.3 and 3.2 in the ^{1}H NMR are due to ethanol solvent. The triethylamine counter ion in the final ATP-N_3 analog **3** is observed at δ 1.18 and 3.8 in the ^{1}H NMR and δ 8.7 and 46.0 in ^{13}C NMR. IR spectra were recorded on an FT/IR-460 plus (JASCO Co. Ltd.) spectrometer. Absorbances of intermediate compounds and the ATP-N_3 analog **3** were measured on UV-Vis spectrophotometer (HP 8452A Diode array spectrophotometer). High resolution mass spectra (HRMS) were obtained using LCT Premier XT, Waters. Quantitative mass spectrometric analyses of phosphopeptides resulting from the kinase reactions were performed using a MALDI-TOF MS (Bruker Ultraflex) with matrix, 4-hydroxy-α-cyanocinnamic acid (4HCCA). The peptides were purified using a Varian (*Prep Star*) HPLC.

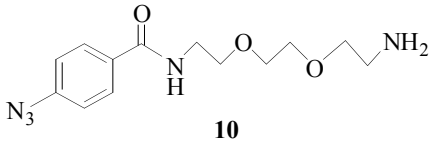
III. Synthesis of ATP-ArN_3 analog 2:



8

*(a) Synthesis of 4-azidobenzoic acid **8**:*[1]

To an ice cooled solution of 4-amino benzoic acid **7** (4 g, 29.1 mmol) in 40 mL of conc. hydrochloric acid, 150 mL of an aqueous solution of sodium nitrite (0.92g, 29.1 mmol) was added slowly. After 30 mins, a second aqueous solution (400 mL) of sodium azide (18.9 g, 291.7 mmol) was added at 0^0C. The reaction was incubated for an hour in room temperature. The product was extracted three times with EtOAc (100 mL). The organic phase was dried with anhydrous Na_2SO_4 and concentrated in vacuo to obtain brownish yellow crystals of 4-azido benzoic acid **8** (84%). The spectral data were consistent with the previously reported synthesis.[1]



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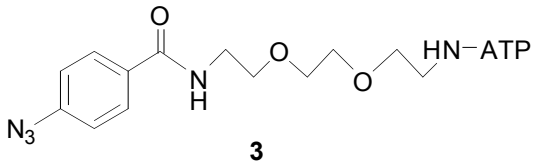
(b) Synthesis of N-(4-azido benzoyl)-2, 2'-ethylenedioxy bis-(ethylamine) ***10****:*

A mixture of 4-azido benzoic acid **8** (500 mg, 3 mmol) and NHS (348 mg, 3 mmol) was dissolved into 20 mL of a 1:1 mixture of DCM and DMF. After cooling to -78^{o}C, EDCI (385 mg, 4 mmol) was added, and the mixture was incubated overnight at room temperature. The DCM was evaporated *in vacuo* and the product was precipitated by adding 10 g of ice water. The solid product thus obtained was further recrystallized with ethyl acetate to obtain brownish crystals of the NHS-activated 4-azido benzoic acid (560 mg, 2.4mmol, 80%).

A solution of the NHS activated 4-azido benzoic acid (0.56 g, 2.4 mmol) in 50 mL of DCM was added dropwise over 30 mins into an ice-cooled solution of 2, 2'-ethylenedioxy bis-(ethylamine) **9** (1.8 mL, 12.5 mmol) in 200 mL of DCM. The reaction mixture was incubated overnight at room temperature in the absence of light. The solvent was removed *in vacuo* and the resulting mixture was purified with flash chromatography (4:2:0.1 DCM:EtOH:NH_4OH) to give 60% of the oily product **10** (422 g, 1.44 mmol). IR (neat, cm^{-1}): 3350, 2876, 1640, 1606, 1551, 1498, 1289, 1122. ^{1}H NMR (400 MHz, CD_3OD): δ 7.85 (d, 2H), 7.09 (d, 2H), 3.50-3.65 (m, 10H), 2.85 (s, 2H). ^{13}C NMR (400 MHz, CD_3OD): δ 39.7, 40.4, 69.4, 70.1, 70.6, 118.8, 129.1, 130.8, 143.6, 167.9. HRMS (MH^+) m/z calcd for $C_{13}H_{20}N_5O_3$: 294.1566, found: 294.1577.



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(d) Synthesis of ATP-[γ]-N-(4-azido benzoyl)-2,2'-ethylenedioxy bis-(ethylamine) ***3:****[2]*

ATP·2Na (27 mg, 0.05 mmol) was dissolved into 5 mL of water and the pH was adjusted to 7.0 with 1N HCl. EDCI (380 mg, 2 mmol) was added and the pH was adjusted to 5.6-5.8 followed by addition of 1 mL water. A solution of N-(4-azido benzoyl)-2, 2'-ethylenedioxy bis-(ethylamine) **10** (590 mg, 2 mmol) in 0.5N hydrochloric acid (1 mL) was added to the ATP mixture and the reaction was incubated for 2 hours under the controlled pH 5.6-5.8. The reaction mixture was brought to pH 8.5 using 1M triethyl ammonium bicarbonate buffer (TEAB) (pH 8.5) and purified using an A-25 sephadex anion exchange column (Aldrich) with 0.1M-1M TEAB buffer solution (pH 8.5) as eluent. The purified product was lyophilized to dryness to obtain 20% of ATP-N_3 **3** as a white TEA salt (9.8 mg, 0.01 mmol). The product thus obtained was dissolved into methanol and stored at -20^{0}C for several months. UV (MeOH): λ 263 nm. ^{1}H NMR (400 MHz, CD_3OD): δ 8.58 (s, 1H), 8.17 (s, 1H), 7.87 (d, 2H), 7.12 (d, 2H), 6.08 (d, 1H), 4.54 (t, 1H), 4.18 (t, 1H), 4.23-4.30 (m, 3H), 3.55-3.63 (m, 12H). ^{13}C NMR (400 MHz, CD_3OD): δ 39.8, 41.5, 65.2, 69.4, 69.9, 70.1, 70.6, 71.9, 75.0, 84.5, 87.6, 118.7, 129.1, 130.9, 140.1, 143.5, 149.7, 152.6, 156.0, 168.0. ^{31}P NMR (400 MHz, CD_3OD): δ -0.19 (d, γ-P), -9.95 (d, α-P), -20.63 (t, β-P). HRMS (MH^+) m/z calcd for $C_{23}H_{33}N_{10}O_{15}P_3$: 781.1340, found: 781.1230.

IV. Solid-phase syntheses of peptides:*[3,6]*

PKA substrate peptide **9** (Ac-LRRTSIIGT), rhodamine B-labeled CK2 substrate peptide **14** (Rho-RRREEETEEEE, Rho-**14)**, and 5(6)-carboxy-X-rhodamine (ROX)-labeled Abl substrate peptide **13** (ROX-EAIYAAPFAKKK, ROX-**13)** were synthesized by Fmoc based solid phase peptide synthesis (SPPS), as

described previously.[3,6] For peptides ROX-**13** and Rho-**14**, the fluorophores were attached to the amino terminus as the final coupling step, as previously described.[6] Specifically, the solid phase-bound CK2 peptide **14** (RRREEETEEEE) and **13** (EAIYAAPFAKKK) were coupled with rhodamine B and ROX, respectively. ROX-NHS (0.45 mmol, 3 eq., Acros) was coupled with peptide **13** directly, while Rhodamine B (0.45mmol, 3 eq., Acros) was activated with HOBt (0.45 mmol, 3 eq.) and N, N'-diisopropylcarbodiimide (0.45 mmol, 3 eq.) before coupling with peptide **14**.[6] Rho-**14** was purified by HPLC using a gradient of 100% Buffer A to 60% Buffer A over 5 minutes, followed by 60% Buffer A to 5% Buffer A over 25 minutes (Buffer A= 99.9% water with 0.1% trifluoroacetic acid; Buffer B = 100% acetonitrile) at 3 mL/min and monitoring at 214 nm. Rho-**14** had a retention time of 11.5 min and was authenticated by MALDI-TOF (M-H^+ calculated for $C_{85}H_{123}N_{22}O_{29}^+$= 1916.88 and observed=1916.78). Likewise, ROX-**13** was purified using a gradient of 100% Buffer A for 5 minutes followed by 100% Buffer A to 60% buffer A over 7 minutes and 60% Buffer A to 5% Buffer A over 8 minutes at a rate of 3 mL/min and monitoring at 260 nm. ROX **13** had a retention time of 17 min and was authenticated by MALDI-TOF (M-H^+ calculated for $C_{97}H_{131}N_{17}O_{20}^+$ = 1852.97 and observed=1852.55).[6]

V. Kinase-catalyzed labeling and mass spectrometric analyses: [3,4]

For quantitative mass spectrometric analysis, kinase-catalyzed labeling was performed as described previously.[4,6] Each reaction mixture contained ATP **1** or ATP-ArN_3 **3** (1.3 mM) and either the PKA substrate peptide **11** (33.3 μM), CK2 substrate peptide **12** (33.3 μM) or Abl substrate peptide **13** (33.3 μM). Into each reaction mixture, PKA (26.67 units/μL), CK2 (9 units/μL), or Abl (9 units/μL) were added. Kinase reactions were carried out in kinase reaction buffer (for PKA: 4.8 mM Tris-HCl, 26.2 mM NaCl, 3.7 mM KCl, 0.15 mM DTT, 0.2 mM EDTA, 0.007%Triton X-100 pH 7.5 @ 25°C; CK2 and Abl: 2.9 mM Tris-HCl, 15.7 mM NaCl, 2.2 mM KCl, 0.09 mM DTT, 0.13 mM EDTA, 0.004%Triton X-100 pH 7.5 @ 25°C). The final volume for the PKA reaction was 7.5 μL while that for the CK2 and Abl reactions were 4.5 μL. The reaction mixtures were incubated for 2 hours at 30°C. The products were analyzed by quantitative MS analysis after differential isotopic esterification, as described previously. [4,6] Briefly, the enzymatic reaction products were esterified by adding acetyl chloride (30 μL) and 200 μL of either d_4-methanol (for the ATP reaction) or d_0-methanol (for the ATP-ArN_3 reaction). The reaction mixtures were incubated at room temperature for 3 hours and the acidic solution was evaporated using a ThermoSavant Speedvac. The two isotopically differentiated reaction products were combined in 4 μL of water. For MALDI-TOF MS analysis, the resultant solution was mixed with 10 μL of a saturated solution of 4-HCCA in 1:1 acetonitrile:water containing 0.1% TFA. The mixture (2 μL) was spotted into a MALDI plate (Standard 384 MTP, Bruker) and analyzed using MALDI-TOF instrument (Bruker Ultraflex). A single representative experiment for each kinase reaction is shown in Figures S1, S2 and S3. The mean percentage conversion from at least three trials is reported in the text (Table 1).

VI. Photocrosslinking reaction:

Crosslinking with fluorophore-labeled peptide substrates:

For the crosslinking experiments, CK2 (50 units/μL, 0.7 μM) was incubated with Rho-**14** (200 μM). Likewise, Abl (20 units/μL, 44.4 nM) was crosslinked with ROX-**13** (200 μM). The reactions were carried out in the presence of either ATP **1** or ATP-ArN_3 **3** (1.3 mM) in a kinase buffer (For CK2: 24.5 mM Tris-HCl, 10 mM $MgCl_2$ 35 mM NaCl, 50 mM KCl, 0.2 mM DTT, 0.2 mM EDTA, 0.01%Triton X-100 pH 7.5 @ 25°C; For Abl : 5 mM HEPES, 52.5 mM Tri-HCl, 10 mM NaCl, 10 mM $MgCl_2$, 0.02 mM Na_2EDTA, 1 mM EGTA, 2.1 mM DTT, 5% glycerol, 0.02% Brij 35, pH7.5 @ 25°C) for 2 hrs at 30°C with or without exposure to UV light (365 nm) using with a handheld lamp (UV-entela, model UVGL-25). The final

volumes of the reactions were 10 μL. The presence of a phosphoramidate bond in the crosslinked product was confirmed by acid-mediated cleavage of the complex by adding 10 μL of TFA to the reaction mixture (final concentration of 50% TFA) and incubating at 16^0C for 3 hours. The acidic reaction mixture was neutralized with Tris buffer (pH=12) before gel analysis. The ATP competition reaction was performed by adding ATP (2.6 mM) into the above mentioned crosslinking reaction mixture. Likewise, a substrate competition experiment was performed with the CK2 reactions by including additional unlabeled CK2 peptide **12** (200 μM). For reactions with heat-denatured CK2, the enzyme was incubated at 95°C for 5 min and cooled on ice before use. As a negative control, the inappropriate Abl substrate peptide ROX-**13** (200 μM) was incubated under UV with CK2 in presence of ATP-ArN_3, as described. Proteins were separated by SDS-PAGE and visualized by in-gel scanning with a Typhoon 9210 phosphoimager using the 532 nm laser (Green) with emission filter 610 BP 30 (deep purple). The total protein content was also detected using either Coomassie (NuSpec) or silver staining. The gel images in Figure 2 and S4 are representative of at least 3 trials.

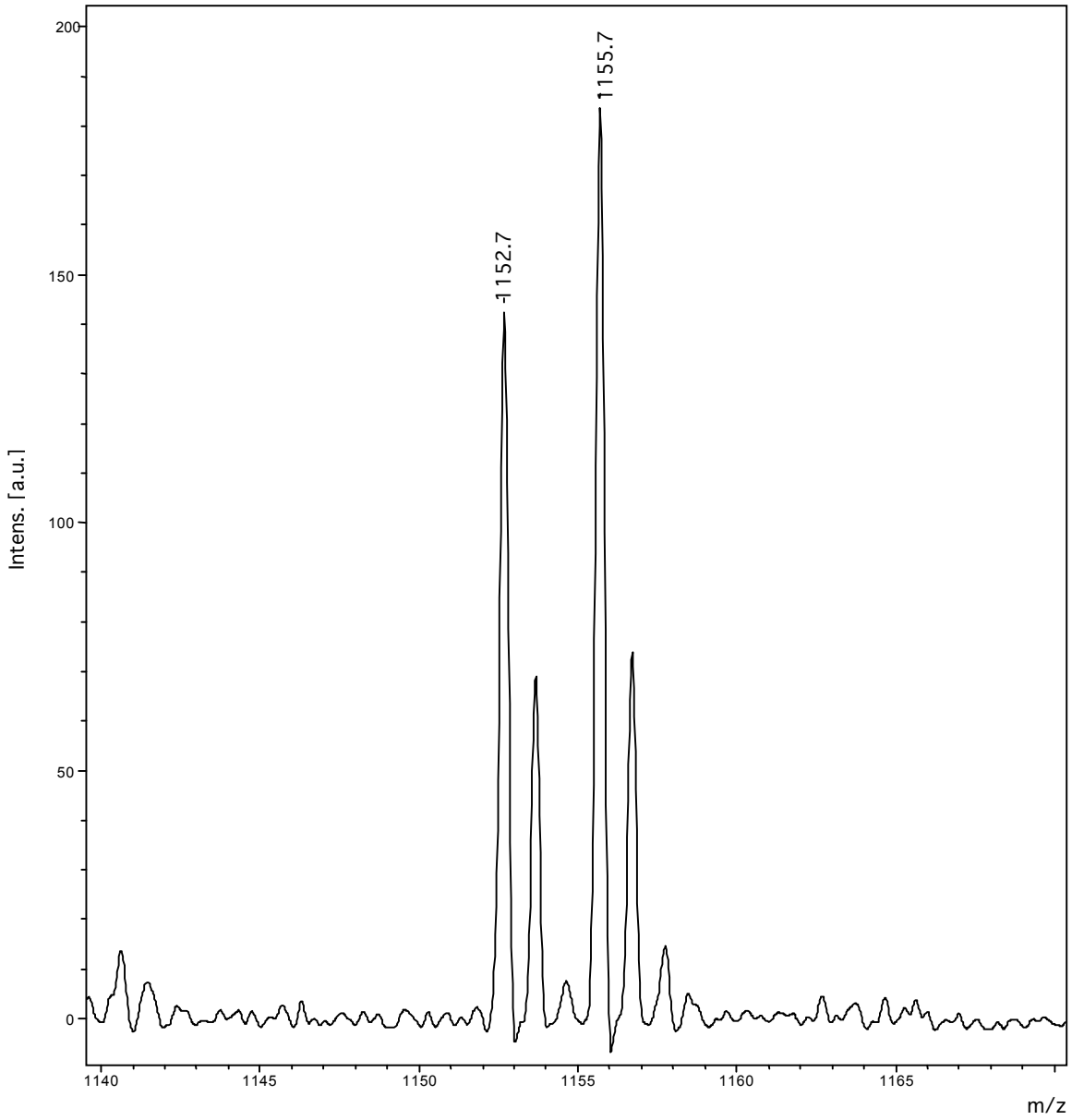
Crosslinking with full length protein, α-casein:

CK2 (5 units/μL, 0.7 μM) was incubated with α-casein (100 μM) and ATP **1** or ATP-ArN_3 **3** (1 mM) in kinase reaction buffer (24.5 mM Tris-HCl, 10 mM $MgCl_2$ 35 mM NaCl, 50 mM KCl, 0.2 mM DTT, 0.2 mM EDTA, 0.01%Triton X-100 pH 7.5 @ 25°C). The final volume of the reaction was 10 μL. The reaction mixture was incubated for 2 hrs at 30°C. A crosslinked protein was obtained by simultaneously irradiating (365 nm) the reaction mixtures with a handheld UV lamp (UV-entela, model UVGL-25). The presence of a phosphoramidate bond in the crosslinked product was confirmed by acid-mediated cleavage of the complex as described above. The products were separated by SDS-PAGE and the proteins were visualized with coomassie stain (NuSpec) or by western blotting with an anti-CK2 antibody (Millipore) after transfer onto PVDF membrane (Immobilon-P^{SQ}). The images in Figure 3 of the text are representative of at least 3 trials.

VII. References:

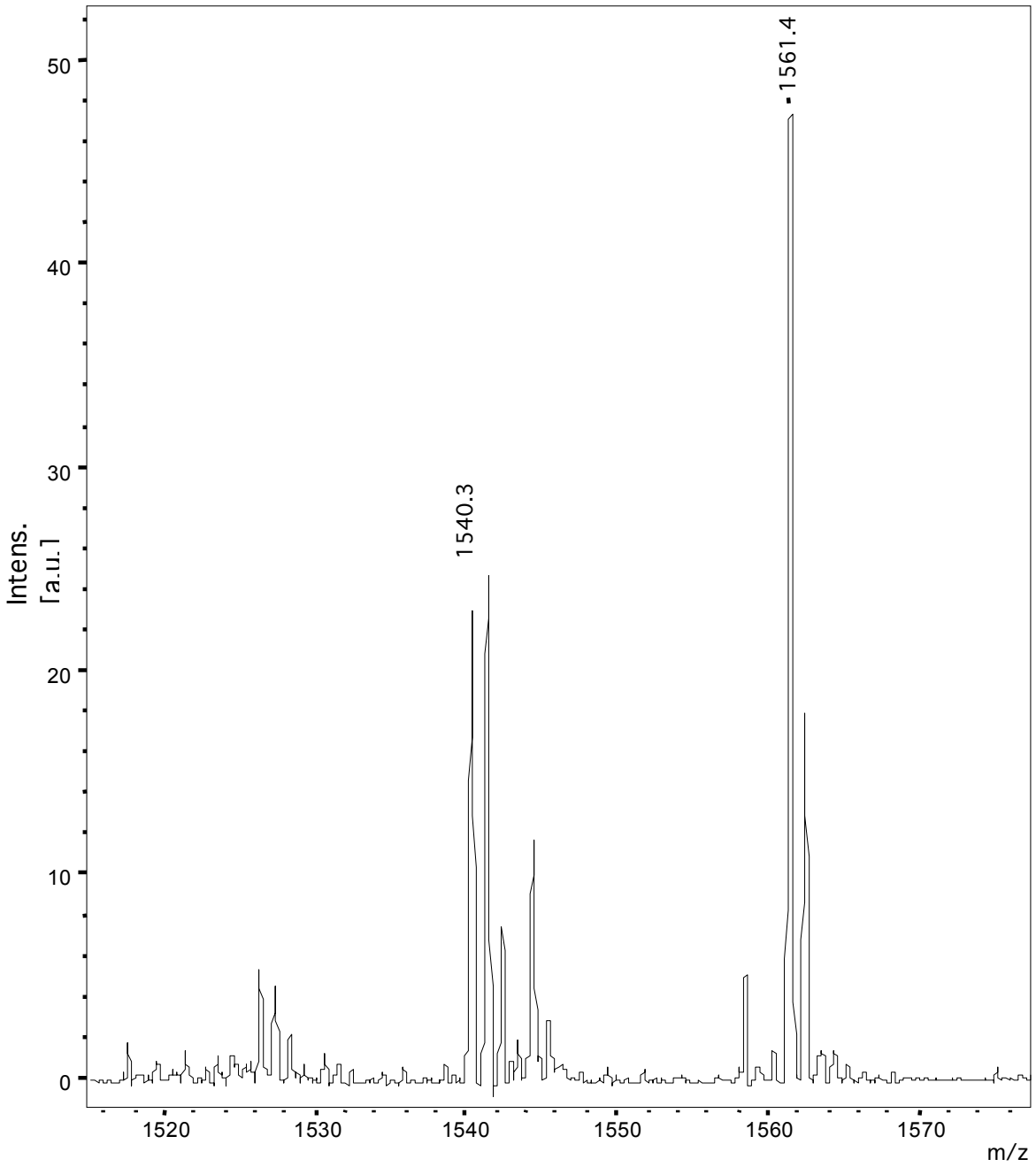
[1] Yan Siong, Dan Bernardi, Stacie Bratton, Michael D. Ward, Eric Battaglia, Moshe Finel, Richard R. Drake and Anna Radominska-Pandya, *Biochemistry*, **2006**, *45*, 2322.
[2] L.R. Yarbrough, Joseph G. Schlageck and Michael Baughman, *J. Bio. Chem*. **1979**, *254*, 12069.
[3] Mangalika Warthaka, Mary K.H. Pflum, *ACS Chem. Biol.*, **2006**, *11*, 697.
[4] Keith D. Green, Mary K.H. Pflum, *J. Am chem. Soc*. **2008**, *129*, 10.
[5] Keykavaos Parang, Jeffrey A. Kohn, Adrian Saldanha, Philip A Cole, *FEBS Letters* **2002**, *520*, 156.
[6] Keith D. Green and Mary Kay H. Pflum, *ChemBioChem* **2009**, *10*(2), 234-237.

VIII. Supporting Data:



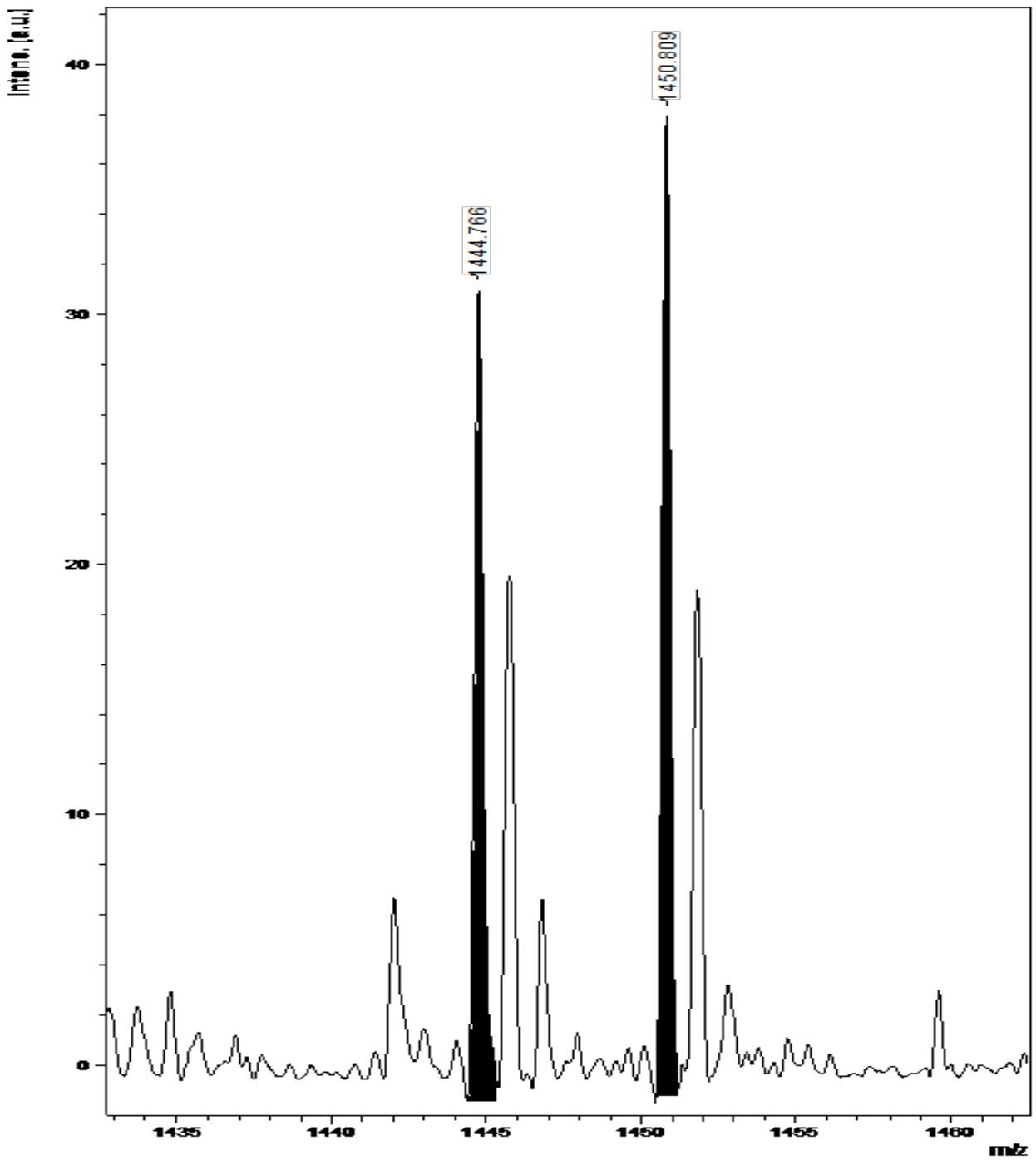
m/z	SN	Quality Fac.	Res.	Intens.	Area
1152.7	30.7	6764	3876	142.35	44
1155.7	39.5	8817	3910	183.55	55

Figure S1: Quantitative MALDI-TOF MS of peptide substrate **11** (Ac-LRRTSIIGT) with PKA and either ATP **1** or ATP-ArN_3 **3.** The peak at m/z 1152.7 corresponds to methylated phosphopeptide after reaction with ATP-ArN_3 and acidic cleavage of phosphoramide bond, while the peak at m/z 1155.77 corresponds to the deuterated phosphopeptide after phosphorylation with ATP. Percent conversion of this single trial is 80 %.



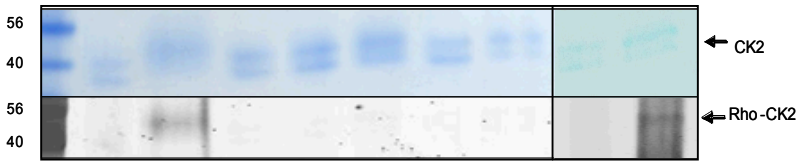
m/z	SN	Quality Fac.	Res.	Intens.	Area
1540.3	19.6	1215	4738	23.03	8
1561.4	40.4	2302	5166	47.39	15

Figure S2: Quantitative MALDI-TOF MS of peptide substrate **12** (RRREEE**T**EEE) with CKII and either ATP **1** or ATP-ArN_3 **3.** The peak at m/z 1540.3 corresponds to heptamethylated phosphopeptide after reaction with ATP-ArN_3 and acidic cleavage of the phosphoramide bond, while the peak at m/z 1561.4 corresponds to the deuterated heptamethylated phosphopeptide after phosphorylation with ATP. Percent conversion of this single trial is 53.3%.



m/z	SN	Quality Fac.	Res.	Intens.	Area
1444.766	14.0	1405	4770	30.94	10
1450.809	17.1	1365	4822	37.95	11

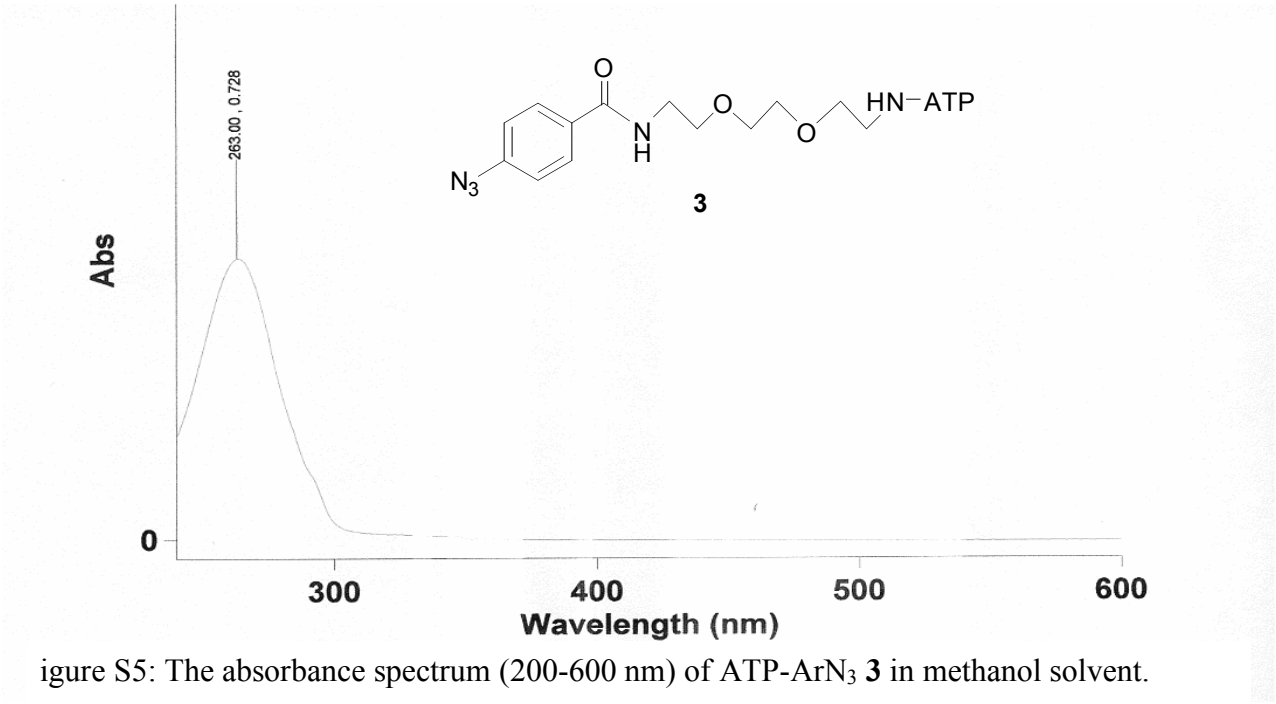
Figure S3: Quantitative MALDI-TOF MS of peptide substrate **13** (EAI**Y**AAPFAKKK) with Abl and either ATP **1** or ATP-ArN_3 **3.** The peak at m/z 1444.76 corresponds to the dimethylated phosphopeptide after reaction with ATP-ArN_3 and acidic cleavage of the phosphoramide bond, while the peak at m/z 1450.80 corresponds to the deuterated phosphopeptide after phosphorylation with ATP. Percent conversion of this single trial is 90.9 %.



	1	2	3	4	5	6	7	8	9
CK2	+	+	+	+	+	+	-	+	+
Rho **14**	+	+	+	+	+	+	+	-	+
ATP	+	-	+	+	+	-	-	-	-
ATP-ArN_3	-	+	-	+	-	-	+	+	+
UV	+	+	+	+	+	+	+	+	+
acid	-	-	+	-	-	-	-	-	-
Peptide **12**	-	-	-	-	+	-	-	-	-
denatured CK2	-	-	-	-	-	-	+	-	-
ROX **13**	-	-	-	-	-	-	-	+	-

Figure S4: Additional controls for the kinase-catalyzed photocrosslinking of peptide substrates. CK2 kinase was incubated with rhodamine-labeled CK2 substrate peptide **14** (Rho-**14**) and the components indicated in the presence of UV light (UV, 365 nm) before separation by SDS-PAGE and visualization by in-gel fluorescence scanning (bottom) or coomassie staining (top). The molecular weight markers are indicated to the left. Lanes 4 and 9 are identical reactions. Lanes 1-4 are identical to lanes 5-8 in Figure 2a of the manuscript. As additional controls, reactions in the presence of competitive, unlabeled peptide **12** (lane 5), in the absence of ATP analog (lane 6), in the presence of heat denatured CK2 kinase (lane 7), or with the inappropriate Abl substrate ROX-**13** (lane 8) resulted in no fluorescence labeling of CK2. This gel is representative of at least three independent experiments.

IX. Compound Characterization



igure S5: The absorbance spectrum (200-600 nm) of ATP-ArN_3 **3** in methanol solvent.

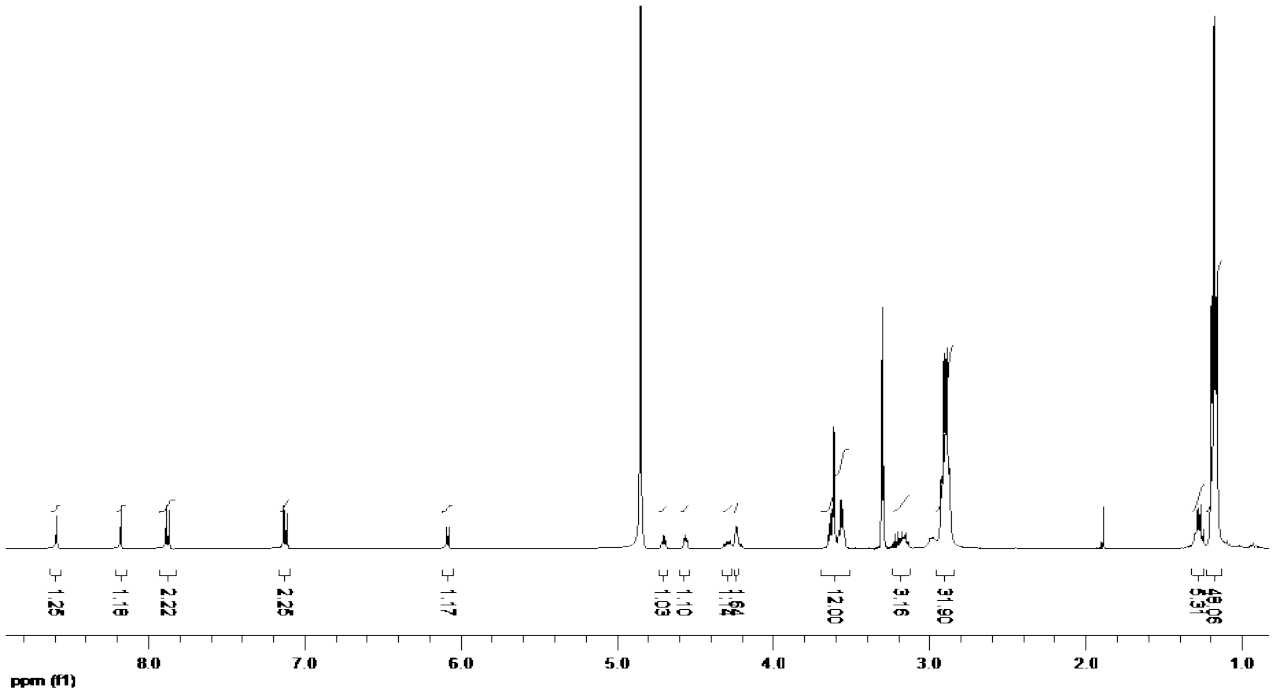


Figure S6: ^{1}H-NMR of ATP-ArN_3 **3** recorded in CD_3OD solvent.

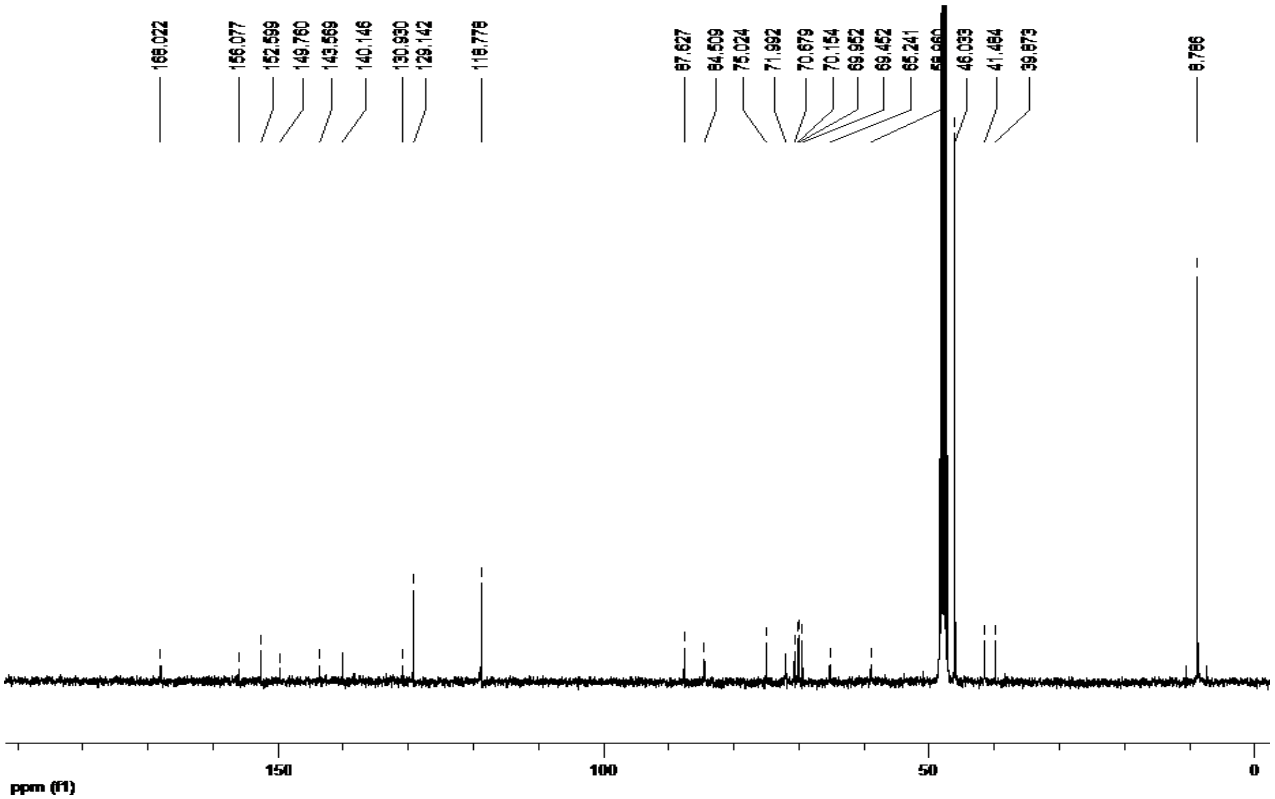


Figure S7: ^{13}C-NMR of ATP-ArN_3 **3** recorded in CD_3OD solvent.

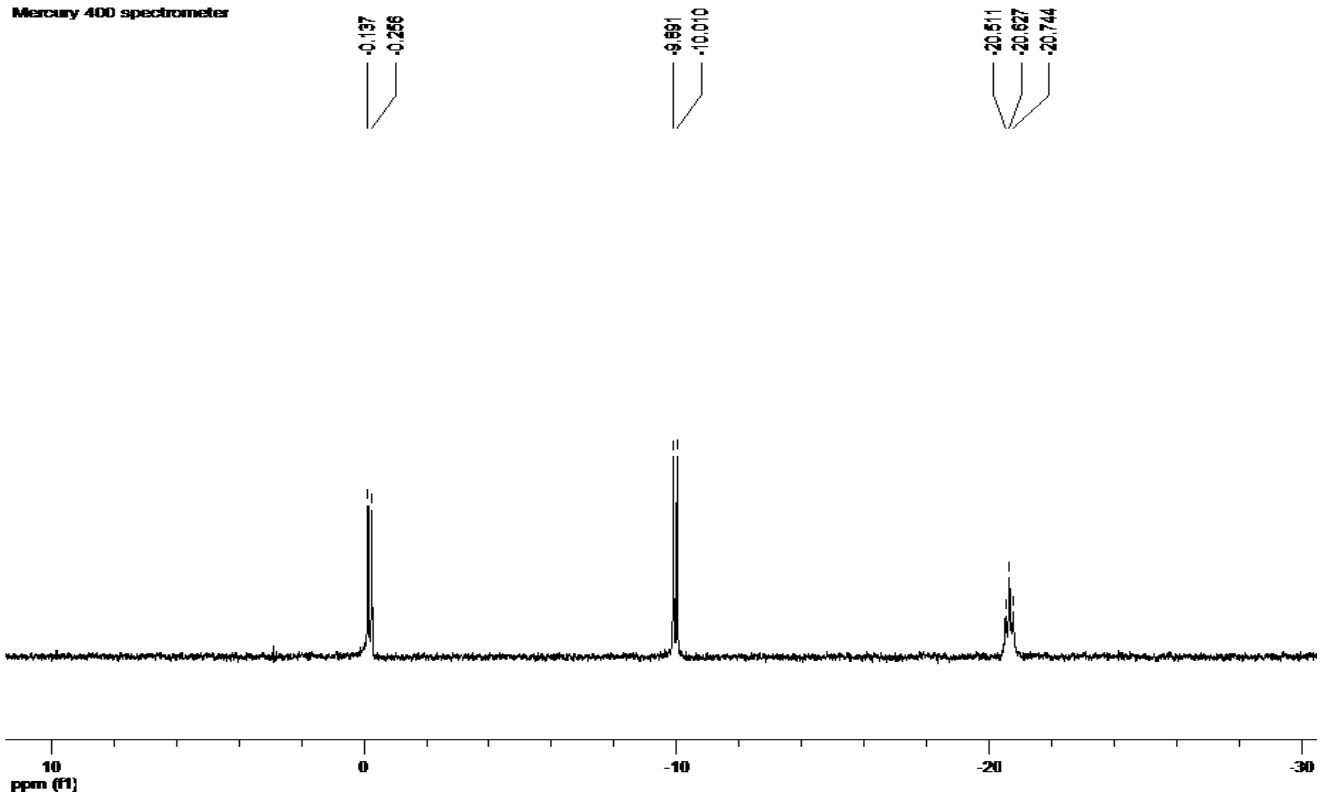
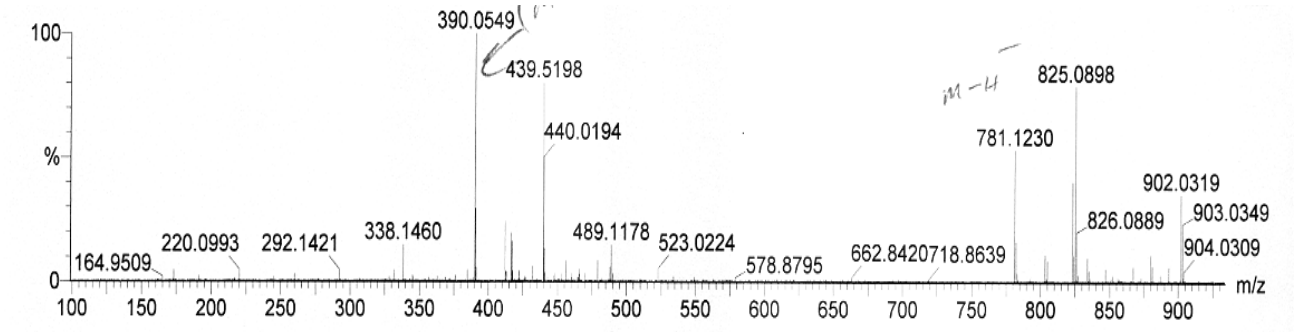


Figure S8: ^{31}P-NMR of ATP-ArN_3 **3** recorded in CD_3OD solvent.



Minimum:				-1.5			
Maximum:		5.0	5.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
781.1230	781.1262	-3.2	-4.1	14.5	91.4	0.0	C23 H32 N10 O15 P3

Figure S9: Exact mass of ATP-ArN_3 **3** recorded with methanol solvent.