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Chemically reprogramming the phospho-transfer reaction to crosslink protein kinases to their substrates.

by
Allison Wong

DISSERTATION

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DOCTOR OF PHILOSOPHY

in

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in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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By

Allison W. Wong

Dedicated to my father, John Wong, and in memory of my mother and grandmother, Katherine Wong and Evelyn Wong. Without your love and support this work would not be possible.

Chemically reprogramming the phospho-transfer reaction to crosslink protein kinases to their substrates

By Allison Wong

Abstract

The proteomic mapping of enzyme-substrate interactions is challenged by their transient nature. A method to capture interacting protein kinases in complexes with a single substrate of interest would provide a new tool for mapping kinase signaling networks. Here we describe a nucleotide based substrate analog capable of reprogramming the wild-type phosphoryl-transfer reaction to produce a kinase-acrylamide based thioether crosslink to mutant substrates with a cysteine nucleophile substituted at the native phosphorylation site. A previously reported ATP-based methacrylate crosslinker (ATP-MA) was capable of mediating kinase crosslinking to short peptides but not protein substrates. Exploration of structural variants of ATP-MA to enable crosslinking of protein substrates to kinases led to the discovery that an ADP-based methacrylate (ADP-MA) crosslinker was superior to the ATP scaffold at crosslinking *in vitro*. The improved efficiency of ADP-MA over ATP-MA is due to reduced inhibition of the second step of the kinase-substrate crosslinking reaction by the product of the first step of the reaction. The new probe, ADP-MA, demonstrated enhanced *in vitro* crosslinking between the Src tyrosine kinase and its substrate Cortactin in a phosphorylation site specific manner. The kinase-substrate crosslinking reaction can be carried out in a complex mammalian cell lysate setting, although the low abundance of endogenous kinases remains a significant challenge for efficient capture.

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Chapter 1.

Introduction: Covalently stabilizing the kinase-substrate complex to map regulatory networks.

1.1 Developing new tools to characterize kinase-substrate networks.

Posttranslational modifications (PTM) of proteins are essential modulators of cellular protein function. Protein phosphorylation, or the enzyme-catalyzed transfer of a phosphate group from a nucleotide triphosphate to an amino acid side chain, is the most common and well studied PTM in signal transduction.¹ Through phosphorylation, protein kinases broadly coordinate cellular signaling processes that are essential for eukaryotes.² Despite the significance of phosphorylation and the breadth and scope of large-scale proteomic efforts to identify and study phosphorylation sites, proteomic profiling generally cannot capture the transient enzyme-substrate interactions that place and remove PTMs.³ Consequently, much effort has been made to contextualize PTMs by developing tools to characterize the enzymes that regulate them.

Activity based protein profiling (ABPP) describes a class of chemical tools which profile the functional status of enzymes in a given family.⁴⁻⁶ ABPP uses covalent probes that are targeted to the active sites of enzymes in a family and provide a purification handle. Inactive enzymes that cannot bind probe to the active site are excluded from subsequent affinity purification and profiling. While ABPP is a powerful tool for assessing the functional status and output of enzymes, we developed a way to not only identify active enzymes but to capture the enzymes as they bind to a specific substrate, a method we term substrate-based protein profiling (SBPP).

This thesis focuses on the specific problem of identifying the kinase(s) that modify a specific phosphorylation site of interest. Over 90% of phosphorylation sites are ‘orphan’ sites for which the kinase is unknown (about 225,000 of 240,000 sites in 2014).⁷ Currently, there is no unified approach for identifying the kinase responsible for a given phosphorylation or site. The lack of tools to deorphan phosphorylation sites has many consequences. From a cell biology perspective, phosphorylation is intimately involved in signaling regulation,⁸ and it is difficult to understand the functional significance of a phosphorylation site without the ability to perturb the regulatory kinase. Genetic manipulation of the substrate, for example the introduction of phosphomimetic and phosphorylation dead mutations, is a powerful approach, but lacks temporal regulation and sometimes does not accurately recapitulate the phosphorylation phenotype.

From a translational perspective, protein kinases represent a class of enzymes that are highly druggable by small molecules.⁹ ‘Undruggable’ protein targets like transcription factors are frequently regulated by phosphorylation.¹⁰ Understanding which kinases are involved could facilitate the identification of new kinase drug targets for diseases like cancer, which are frequently driven by ‘undruggable’ aberrant signaling processes.

Some existing approaches to deorphaning phosphorylation sites include an *in vitro* screen of all 518 protein kinases, genetic or pharmacologic inhibition *in vivo*, or complementation.^{11,12} At present, an *in vitro* screen is impractical given the difficulty of expressing many human protein kinases heterologously in their active form. Furthermore, most activity-based assays require artificially high concentrations of kinase and lack native effectors. Genetic inhibition is an attractive alternative given that siRNA and CRISPRi constructs can be made for every individual kinase; however, due to the high degree of interconnectedness and degeneracy of kinase pathways, kinase knockdown can often have significant indirect effects on the phosphorylation of the proteome. In addition to indirect effects, pharmacologic inhibition faces

significant challenges in that the majority of kinases lack a specific inhibitor and many specific inhibitors also inhibit off-target or phylogenetically similar kinases to some degree.¹³ Finally, the low affinity of kinases for their phosphorylated substrates has stymied biological complementation approaches.¹² Accordingly, we looked to chemical genetic methods for capturing the kinase-substrate complex, inspired by ABPP.

We propose a set of chemical tools for specifically and covalently stabilizing the kinase-substrate interaction so that it can be studied. These tools target both the kinase and a modified ‘bait’ substrate in two chemical reaction steps (Figure 1.1.1). Analogously to ABPP approaches, in the first step we target an essential active site lysine residue conserved in protein kinases (Figure 1.1.1 Panel B). The modification of the active site lysine consists of the acylation of the primary amine, producing an electrophilic acrylamide close to the phosphorylation site. We position a nucleophile in the substrate phosphorylation site by mutating the native serine/threonine/tyrosine to a cysteine residue. In the second step, the cysteine thiol subsequently modifies the acrylamide via Michael addition (Figure 1.1.1 Panel C), forming a thioether (Panel D). In Chapters 1-4 this specific approach is discussed, whereas in Chapter 5 this method is generalized to describe the delivery of a biorthogonal reaction pair to the kinase-substrate interface.

Unlike ABPP, a particular challenge for our method is that the positioning and reactivity of this moiety, an affinity dependent and substrate-specific crosslinker, is critical for targeting the introduced mutation at the substrate phosphorylation site in the second step. In particular, the two reaction steps must be sufficiently reactive to efficiently label the desired kinase and substrate amino acid residues, but not so reactive as to be quenched, for example by interactions with nucleophiles in unrelated proteins. Because the substrate and phosphorylation site are known beforehand, we can express the substrate with a tag for enrichment as well as introduce

mutations at or nearby the phosphorylation site for specific chemical reactivity. Our goal is to stabilize the kinase-substrate interaction in order to facilitate unbiased approaches for identifying the kinases interacting with a specific phosphorylation site on a substrate of interest.

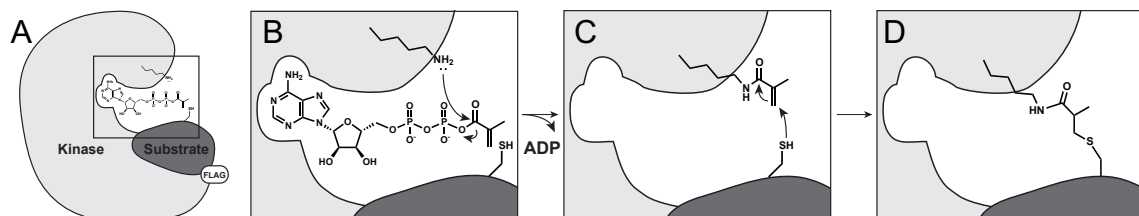


Figure 1.1.1. Schematic representation of the kinase-substrate crosslinking reaction.

1.2 Previous approaches and crosslinkers to stabilize the kinase-substrate interaction were inefficient in protein lysate or with protein substrates.

Our laboratory previously reported several efforts to covalently stabilize the kinase-substrate complex. The first of these involved a three-component system capable of covalently linking kinase to a sequence optimized substrate peptide *in vitro* (Figure 1.2.1).¹⁴ In this system the probe consisted of an adenosine scaffold for binding affinity linked to an aromatic o-phthaldialdehyde which reacted with a conserved kinase lysine and an introduced substrate residue. To gain selective reactivity for the substrate peptide of interest, the substrate phosphorylation site serine, threonine, or tyrosine was mutated to a cysteine residue. This reaction produced sufficient crosslinked product for detection on western blot *in vitro*, but not when performed in the presence of protein lysate. Furthermore, the reaction did not proceed appreciably when monitored by LC-MS.

A second generation probe sought to improve upon the first probe by increasing its affinity for the kinase active site (Figure 1.2.2).¹⁵ Rather than adenosine, this probe consisted of a promiscuous kinase active site inhibitor scaffold with the same o-phthaldialdehyde covalent ‘warhead’. However, despite this modification, the reaction still did not proceed appreciably in the presence of lysate or when monitored by LC-MS.

A third generation probe modified the highly reactive o-phthaldialdehyde warhead by replacing it with a less reactive thiophene-2,3-dicarboxaldehyde moiety (Figure 1.2.3).¹⁵ Due to its decreased reactivity, this probe could mediate kinase-peptide crosslinking in the presence of protein lysate detectable by western blot; however, reaction efficiency remained very low.

Finally, a subsequent probe based on an ATP scaffold, closely related to the main focus of my work described in this thesis, used a two-step mechanism in which a conserved catalytic

lysine in the kinase was covalently modified, forming an electrophilic acrylamide for reaction with the cysteine mutant substrate (Figure 1.2.4).¹⁶ This approach significantly improved the yield of the crosslinking reaction such that crosslinking could be observed in the presence of protein lysate. However, the *in vitro* yield of kinase-peptide crosslinking with this probe in a model system was limited to about 30%, limiting the application of this probe for unbiased kinase-substrate detection.

Others have reported a kinase-catalyzed transfer of a photocrosslinker residue (K-CLASP) by modifying the γ -phosphate of ATP with a non-hydrolyzable linker to the photocrosslinkers phenyl azide and benzophenone (Figure 1.2.5).^{17,18} An advantage of this approach is that it requires no mutation of the substrate or kinase of interest. However, this approach has some limitations, most notably that the reaction has no specificity for the particular substrate phosphorylation site of interest or its kinase, and that the photocrosslinker-phosphorylated substrate can diffuse away from the kinase before photocrosslinking takes place, resulting in low efficiency of crosslinking to the kinase of interest and potential off-target crosslinking to abundant proteins.

Here we demonstrate an ADP scaffolded probe for which the *in vitro* crosslinking between kinase and peptide produced quantitative crosslinked product. For the first time we demonstrate crosslinking between kinase and its protein substrate in the presence of protein lysate. Finally, we evaluate our crosslinker probe using a MS/MS approach to detect kinase.

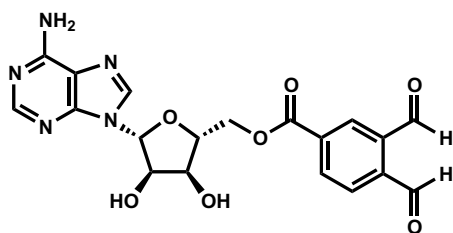


Figure 1.2.1

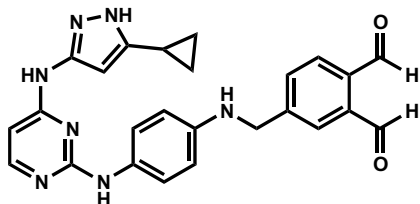


Figure 1.2.2

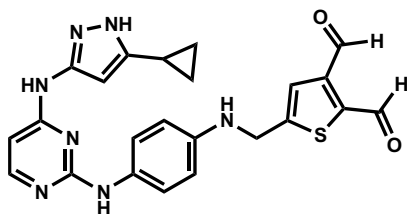


Figure 1.2.3

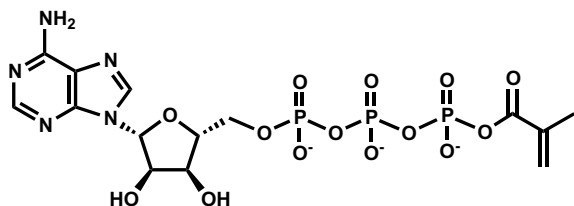


Figure 1.2.4

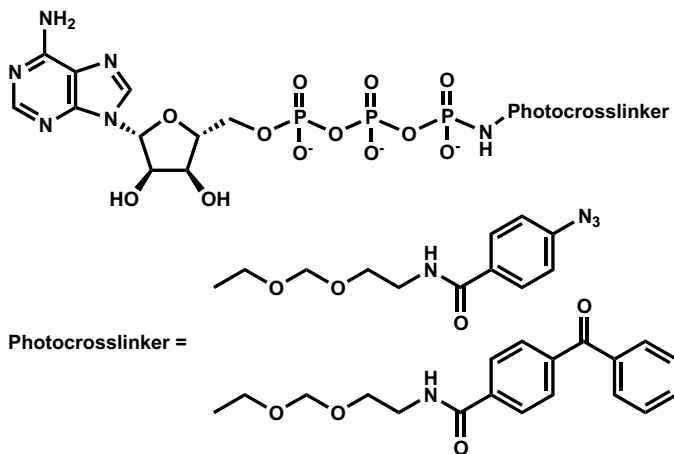


Figure 1.2.5

Figure 1.2.1, 1.2.2, 1.2.3, 1.2.4, 1.2.5. Structures of previously published crosslinkers.

Chapter 2

Synthesis, purification, and chemical characterization of nucleotide-scaffolded probes.

2.1 Synthesis of nucleotide-scaffolded probes.

Nucleotides are essential molecules for biologic systems that play central roles in diverse and critical functions like cellular energy storage, metabolic signaling, and the genetic code. Nucleotides consist of a nitrogen-containing heterocyclic base, a sugar, and one or more phosphate groups. They are ubiquitous in all forms of life in various forms, including polymers like DNA and RNA, monomers including nucleolipids, cyclic nucleotides, and dinucleotides. Nucleotides are of such importance that synthetic nucleotide derivatives have been used both as chemical probes for mechanistic understanding of biologic systems as well as therapies for various diseases. Briefly, some examples of important applications of synthetic nucleotide derivatives include Sanger sequencing and early genomic analysis as well as the development of antivirals like acyclovir and zidovudine.¹⁹⁻²² Nucleotide derivatives can be functionalized at the nitrogenous base, such as in analog-sensitive approaches, at the hydroxyl groups in the sugar ring, such as in fluorophores, or at the phosphate, as described below.^{23,24} In this thesis I will focus on the synthesis of phosphate esters of monomeric nucleotide derivatives: specifically, adenosine mono-, di-, and tri-phosphate derivatives and their relevance to kinase signaling.

The synthesis and purification of nucleotide derivatives is challenging due to the polarity of their phosphate side chains, which are difficult to resolve by chromatography. Furthermore, the esterification of nucleotide phosphates produces chemically similar constitutional isomers due to the low pK_a values of multiple phosphate monoester hydroxyl groups. The synthesis of

gamma-phosphate modified ATP has been previously described by our research laboratory as well as others.^{16-18,25-27}

We took a general approach to synthesize nucleotide-scaffolded probes in which we incubated the triethylammonium salt of ADP or ATP with either the symmetric or a sterically hindered mixed anhydride of the desired functional group. The synthesis of the nucleotide triethylammonium salt was necessary to increase the solubility of ADP or ATP in organic solvent. This approach was found to be generalizable to make other probes not described in this thesis. Due to its reduced polarity relative to ADP and ATP, AMP was soluble in organic solvent without the synthesis of the triethylammonium salt and all AMP scaffolded probes were made by the direct reaction of AMP sodium salt with anhydride. All AMP scaffolded probes described in this thesis were made by Krister Barkovich and protocols for their synthesis are previously reported (Krister Barkovich, PhD thesis, UCSF, 2018, Proquest).

We attempted to make ATP- and ADP- scaffolded probes using base-catalyzed 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) activation but found that this approach primarily formed multiply modified species including extensive modification of nonphosphate reactive groups. We did not try approaches that relied on coupling nucleoside to phosphoester.²⁸

We further developed an improved two-step protocol for the isolation of nucleotide-scaffolded probes and applied it in order to separate positional isomers. The synthesis of ADP- and ATP-scaffolded probes produces constitutional isomers (Figure 2.1.1) which differ in their reactivity toward kinase (Figure 2.1.2). We required a purification approach that would remove unreacted starting material and multiply modified side products as well as isolate isomers. We first used anion exchange to remove unreacted starting material, followed by purification on a styrene-divinylbenzene column to separate isomers and side products as well as remove excess triethylammonium, isolating an active isomer in high purity.²⁹

Based on NMR assignment, the isomers of ADP-MA are presumed to be constitutional isomers in which the α - or β - phosphate is substituted. We found the isomer that was unreactive to Src kinase to be α -substituted ADP-MA based on MS fragmentation. The α -substituted ADP-MA should have a unique fragment corresponding to the loss of a single phosphate but not the methacrylate, whereas this fragment is not possible in β -substituted ADP-MA because loss of the terminal phosphate would result in loss of the methacrylate as well. We examined a 1:1 mixture of ADP-MA active and inactive isomers by LC-MS and observed a mass corresponding to ADP-MA minus one phosphate, $C_{14}H_{17}N_5O_8P^-$ of 414.082 in the inactive isomer peak at 0.66, but not in the active isomer peak at 0.40 (Figure 2.1.3). We additionally performed NOESY on both isomers hoping to observe interactions between the methacrylate and the sugar substituents to further confirm the identity of the α - and β - substituted isomers and did not observe significant differences between the two spectra (Figure 2.1.8, Figure 2.1.11).

Synthesis and purification of nucleotide-scaffolded probes

Anion exchange with TEAA gradient

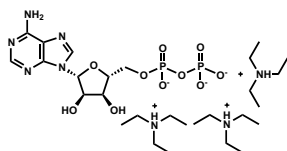
Nucleotide-MA reaction mixture was suspended in 1 mL H_2O and centrifuged for 5 min at 20817 rpm in a tabletop centrifuge. Due to the presence of excess TEA in synthesis, pH was measured and found to be basic. A 5 mL HiTrapQ column (Q Sepharose FF, 6% crosslinked) (17-1154-01, GE Healthcare) was used on a Waters Acquity FPLC instrument with a 4.5 mL/min flow rate and elution volume of 51 mL with a gradient of 15%-100% 0.05-0.75 M TEAA, pH 7. A flow-through peak and three large peaks corresponding to multiply alkylated ADP, singly alkylated ADP, and unreacted ADP were observed in this order, at roughly 25%, 30%, and 50% 0.75 M TEAA, pH 7 respectively (Figure 2.1.4). (Additional unreacted ADP eluted when the column was washed with 100% 0.75 M TEAA, pH 7 and was detected as a 4th peak.) Singly

alkylated ADP eluted at 32% Solution B with some contamination of multiply alkylated product. Fractions containing the compound of interest were pooled, frozen, and lyophilized. (Excess triethylammonium acetate was removed in the next purification step.)

MCI gel purification

3 mL of MCI CHP20P gel (400-600 Å mean pore size, ~1.30 mL/g pore volume, 75-150 µm particle size, 13629-U, Sigma-Aldrich) was rehydrated in 30% MeOH overnight. MCI was transferred to a disposable column and washed with 1 column volume H₂O 5 times. Lyophilized fractions from anion exchange containing ADP-MA were suspended in 1 mL H₂O and loaded onto the column. Fractions were eluted from the column using H₂O and collected in a receiving plate. H₂O alone was sufficient to elute compounds in this order: ADP (if any, usually fully removed by anion exchange), ADP-MA β-substituted (active) isomer, and ADP-MA α-substituted (inactive) isomer. No reaction between Src and ADP-MA inactive isomer was observed at extended time points by LC-MS (Figure 3.1.2). Elution with 10-100% ACN removed multiply alkylated products from the gel. Pure fractions containing ADP-MA active isomer were pooled and lyophilized and verified by LC-MS and NMR. ADP-MA concentration for biological assays was determined by A260 reading on Nanodrop ($\epsilon=15.4 \times 10^3 \text{ cm}^{-1}\text{M}^{-1}$).

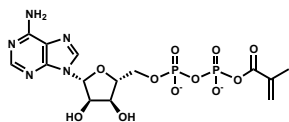
Synthesis of ADP-triethylammonium salt



Dowex 50W-8X (10 g, H⁺ form 100-200 mesh, 217514-100G, Sigma-Aldrich) was soaked in saturated TEA/H₂O solution (100 mL, 7.6 v/v%) for 1 h. Slurry was transferred to a column and rinsed with 1 mL H₂O. ADP • 3Na (0.8 g, A2754-1G, Sigma-Aldrich) was dissolved in 1 mL

H₂O and applied to stoppered column. After 30 minutes, flowthrough was collected and column was eluted 3 times with 5 mL H₂O. Flowthrough and eluate were pooled, frozen, and lyophilized in aliquots. Product was carried forward without characterization. Product was an off-white oily solid.

Synthesis of ADP-methacrylate (ADP-MA)



In a dry scintillation vial under Ar, ADP-TEA₃ (0.419 g, 0.571 mmol) was suspended in a mixture of dry 6:1:1 DMSO: DMF: Dioxane (8 mL total solvent). To this mixture dry TEA (79.6 μ L, 1 equiv) was added. Methacrylic anhydride (94%, 276685-500ML, Sigma-Aldrich) (84.6 μ L, 1 equiv) was added dropwise with stirring. The reaction mixture was stirred for 72 h at room temperature and solvent was removed for purification (as above). Product was a white solid.

Characterization of ADP-MA β -phosphate substituted active isomer:

¹H NMR (400 MHz, D₂O) δ 8.51 (s, 1H), 8.28 (s, 1H), 6.17 (s, 1H), 6.13 (d, J = 8.62 Hz, 1H), 5.67 (s, 1H), 4.77 (m, 1H), 4.53 (m, 1H), 4.39 (m, 1H), 4.23 (m, 2H), 3.20 (qd, J = 7.3, 1.9 Hz, 12H), 1.80 (s, 3H), 1.27 (td, J = 7.3, 1.8 Hz, 18H). (Figure 2.1.5)

¹³C NMR (100 MHz, D₂O) δ 165.45, 155.30, 152.03, 149.82, 141.19, 136.15, 130.10, 119.43, 87.87, 84.88, 75.12, 71.28, 66.15, 47.51, 17.90, 9.07. (Figure 2.1.6)

³¹P NMR (162 MHz, D₂O) δ -11.88, -12.00 (d, 1P), -19.39, -19.53 (d, 1P). (Figure 2.1.7)

Heteronuclear NOE (Figure 2.1.8)

HRMS m/z calculated for ADP-MA: C₁₄H₁₉N₅O₁₁P₂⁻[M-H⁺]: expected 494.0483, found 494.0483.

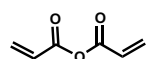
Characterization of ADP-MA α -phosphate substituted inactive isomer:

^1H NMR (400 MHz, D_2O) δ 8.59 (s, 1H), 8.32 (s, 1H), 6.21 (s, 1H), 6.17 (d, 1H), 5.74 (s, 1H), 4.97 (m, 1H), 4.78 (m, 1H), 4.56 (m, 1H), 4.18 (m, 2H), 3.12 (m, 10H), 1.91 (s, 3H), 1.19 (m, 15H). (Figure 2.1.9)

^{31}P NMR (162 MHz, D_2O) δ -10.68, -10.80 (d, 1P), -11.13, -11.28 (d, 1P). (Figure 2.1.10)

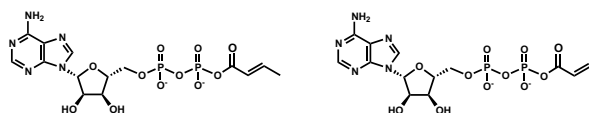
Heteronuclear NOE (Figure 2.1.11)

Synthesis of acrylic anhydride



A dry scintillation vial was charged with acrylic acid under Ar(99%, 147230, Sigma-Aldrich) (0.70 g, 9.7 mmol) in THF. Triethylamine (99.5%, 471283-100 ML, Sigma-Aldrich)(1.354 mL, 1 equiv) was added with stirring at -78 °C. Acryloyl chloride (97%, 549797-5G, Sigma-Aldrich) (0.785 mL, 1 equiv) was added dropwise and the mixture was allowed to warm to room temperature over 10 minutes. Solvent was removed *in vacuo* at room temperature on a rotary evaporator for 30 minutes at approximately 50 mbarr and the crude residue was carried forward without characterization. Product is volatile. Some evaporative loss was noted and prolonged vacuum was avoided.

Synthesis of ADP-acrylate (ADP-A) and ADP-crotonate (ADP-CA)



As ADP-MA, but using 1.25 equivalents acrylic anhydride (synthesis listed above) and 2 equivalents commercially available crotonic anhydride (98%, C0420, Tokyo Chemical Industry Co.) respectively. The reactions were monitored by LC-MS and the formation of ADP-A was

complete within 5 minutes, whereas ADP-CA proceeded slowly over several days. ADP-A was not fully characterized. Products were white solids.

Characterization of ADP-CA:

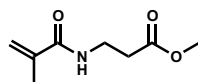
¹H NMR (400 MHz, D₂O) δ 8.40 (s, 1H), 8.14 (s, 1H), 6.96 (m, 1H), 6.02 (d, *J* = 5.9 Hz, 1H), 5.65 (d, 1H, *J* = 15.4 Hz), 4.68 (m, 1H), 4.43 (m, 1H), 4.30 (m, 1H), 4.14 (m, 2H), 3.09 (q, *J* = 7.3 Hz, 12H), 1.65 (d, 3H, *J* = 6.9 Hz), 1.21 (t, *J* = 7.3 Hz, 18H). (Figure 2.1.12)

¹³C NMR (100 MHz, D₂O) δ 163.61, 154.28, 151.15, 150.65, 148.80, 140.25, 121.10, 118.38, 86.98, 84.00, 74.35, 70.42, 65.31, 46.60, 17.41, 8.18. (Figure 2.1.13)

³¹P NMR (162 MHz, D₂O) δ -11.84, -11.98 (d, 1P), -19.49, -19.63 (d, 1P). (Figure 2.1.14)

HRMS *m/z* calculated for ADP-CA: C₁₄H₁₉N₅O₁₁P₂⁻[M⁻H⁺]: expected 494.0483, observed 494.0593.

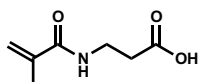
Synthesis of ethyl 2-(2-methylprop-2-enoylamino)acetate (β-ala-methacrylic acetate)



A round bottom flask was charged with ethyl 3-aminopropanoate (0.75 g, 4.8 mmol) in acetonitrile. To the flask triethylamine (1.36 mL, 2 equiv) was added. Methacrylic anhydride (94%, 276685-500 ML, Sigma-Aldrich) (0.905 mL, 1.25 equiv) was added dropwise with stirring. The reaction was stirred at room temperature and monitored by TLC with a mobile phase of 1:1 EtOAc: hexanes. Spots were visualized using KMnO₄ solution. Upon consumption of the starting material as indicated by TLC, the mixture was concentrated to dryness and the residue was suspended in 1% aqueous HCl solution and the resulting mixture was extracted 3 times with 5 mL EtOAc. The combined organic layers were extracted 3 times with 5 mL saturated sodium bicarbonate solution and 1 time with saturated brine solution. The organics

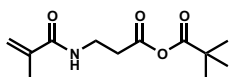
were separated and dried over Na₂SO₄, filtered, and the filtrate was concentrated. The crude solid was purified by flash column chromatography eluting with a gradient of 0-15-50-100% EtOAc in hexanes. The desired fractions were pooled and concentrated to dryness *in vacuo*. Product was a clear oil, 0.559 g, 62% yield.

Synthesis of 2-(2-methylprop-2-enoylamino)acetic acid (β -ala-methacrylic acid)



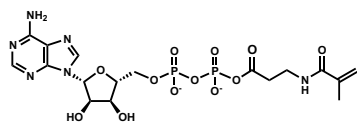
Ethyl 2-(2-methylprop-2-enoylamino)acetate (74.5 mg, 0.44 mmol) was suspended in 1 mL of 1:1 THF:NaOH solution (26 mg, 1.5 equiv in 0.5 mL water) at 0 °C. After 1 h, reaction was neutralized dropwise with dilute aqueous HCl solution. Solvent was removed *in vacuo* and the residue was extracted with ethyl acetate. The product was combined with another reaction and carried forward without characterization.

Synthesis of mixed pivaloyl- β -ala-methacrylic anhydride



2-(2-methylprop-2-enoylamino)acetic acid (42 mg, 0.29 mmol) was suspended in dry acetonitrile under Ar. Triethylamine (0.04 mL, 1 equiv) was added and pivaloyl chloride (99%, T72605-100ML, Sigma-Aldrich) (0.034 mL, 0.95 equiv) was added dropwise with stirring. Solvent was removed *in vacuo* and NMR of product was taken in CDCl₃.

Synthesis of ADP- β -alanine-methacrylate (ADP- β -ala-MA)



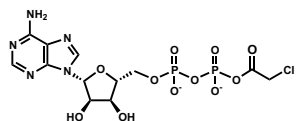
In a dry scintillation vial under Ar, ADP-TEA was suspended in a mixture of dry 6:1:1 DMSO: DMF: Dioxane (8 mL total solvent). To this mixture dry TEA (1 equiv) was added. pivaloyl- β -ala-methacrylic anhydride (2 equiv) was added dropwise with stirring. The reaction was stirred at room temperature and monitored by LC-MS. Upon completion the mixture was concentrated and product was purified as above.

Characterization of ADP- β -alaMA

^1H NMR (400 MHz, D_2O) δ 8.42 (s, 1H), 8.15 (s, 1H), 6.01 (m, 1H), 5.57 (m, 1H), 5.51, (s, 1H), 5.33 (m, 1H), 5.26 (m, 1H) 4.64 (m, 2H), 4.43 (m, 1H), 4.30 (m, 1H), 4.14 (m, 2H), 3.39 (m, 3H), 3.09 (m, 14H), 2.62 (m, 6H), 2.45 (m, 2H) 1.81 (m, 2H), 1.74 (m, 3H), 1.18 (m, 22H). (Figure 2.1.15)

^{31}P NMR (162 MHz, D_2O) δ -11.82, -11.96 (d, 1P), -19.97, -20.11 (d, 1P). (Figure 2.1.16)

Synthesis of ADP- α -chloroacrylate (ADP- α -ClA)



In a dry scintillation vial under Ar, ADP-TEA was suspended in a mixture of dry 6:1:1 DMSO: DMF: Dioxane (8 mL total solvent). Chloroacetyl chloride (98%, A15846, Alfa Aesar) (0.8 equiv) was added dropwise with stirring. After addition, solvent was immediately removed *in vacuo*. Product was not fully characterized.

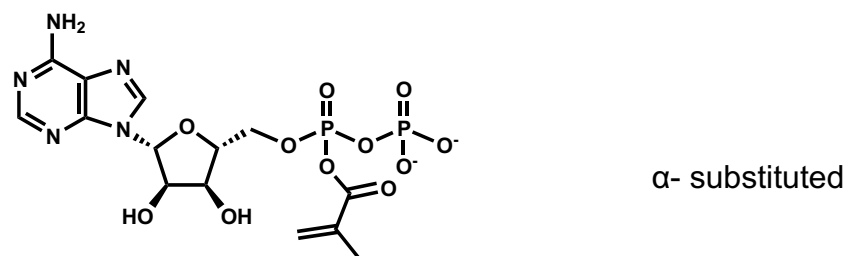
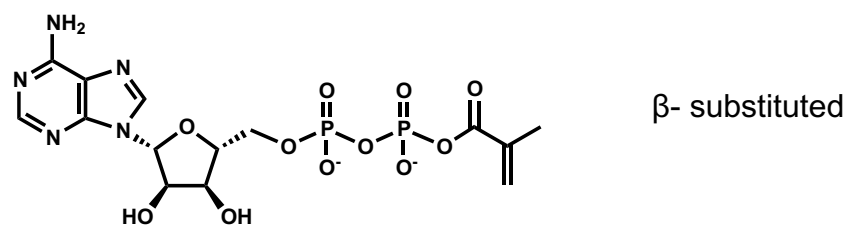


Figure 2.1.1. Constitutional isomers of ADP-MA.

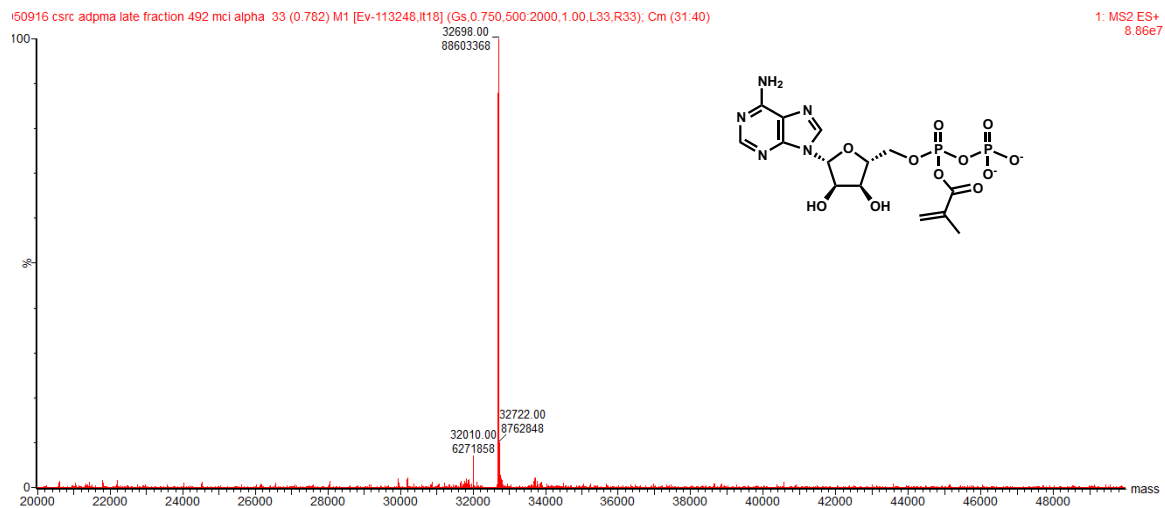


Figure 2.1.2. No reaction is observed between Src and ADP-MA inactive isomer. Parent mass of Src is 32698 Da, expected methacrylamide adduct is $\Delta 69$. Inactive isomer is presumed to be the α -substituted isomer.

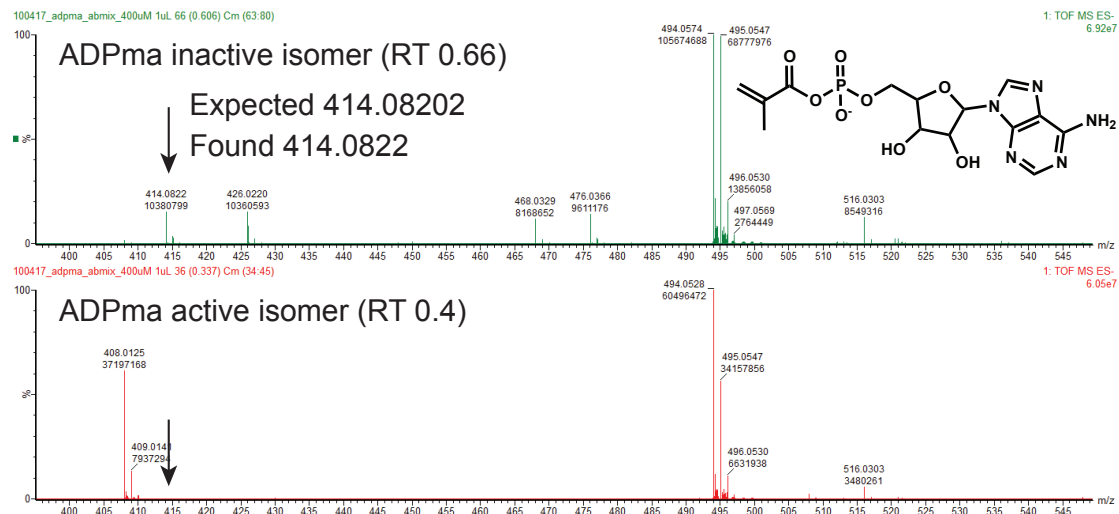


Figure 2.1.3. LC-MS spectrum of 1:1 mixture of ADP-MA active and inactive isomer. Inactive isomer contains a fragment corresponding to loss of the terminal phosphate.

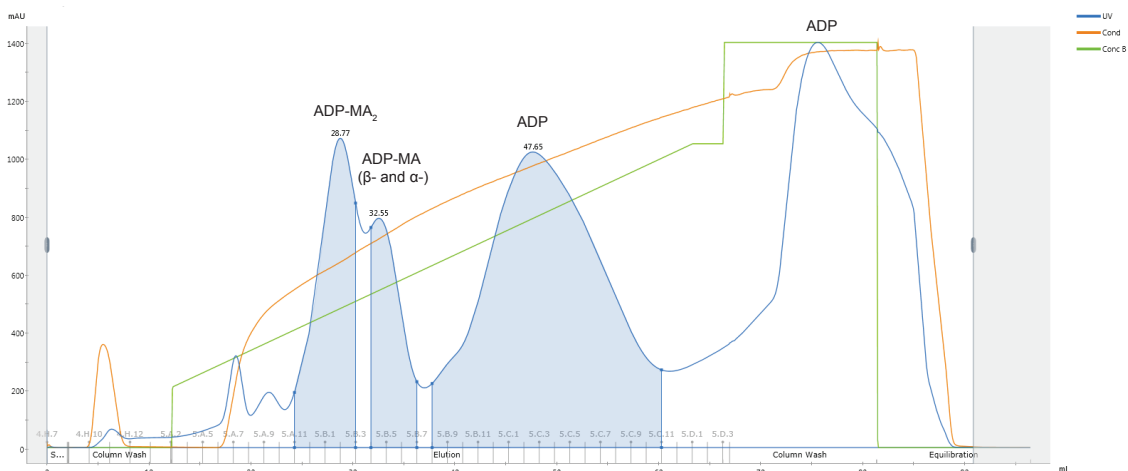


Figure 2.1.4. Anion exchange purification of ADP-MA reaction mixture. A 5 mL HiTrapQ column (Q Sepharose FF, 6% crosslinked)(17-1154-01, GE Healthcare) was used on a Waters Acquity FPLC instrument with a 4.5 mL/min flow rate and elution volume of 51 mL with a gradient of 15%-100% 0.05-0.75 M TEAA, pH 7.

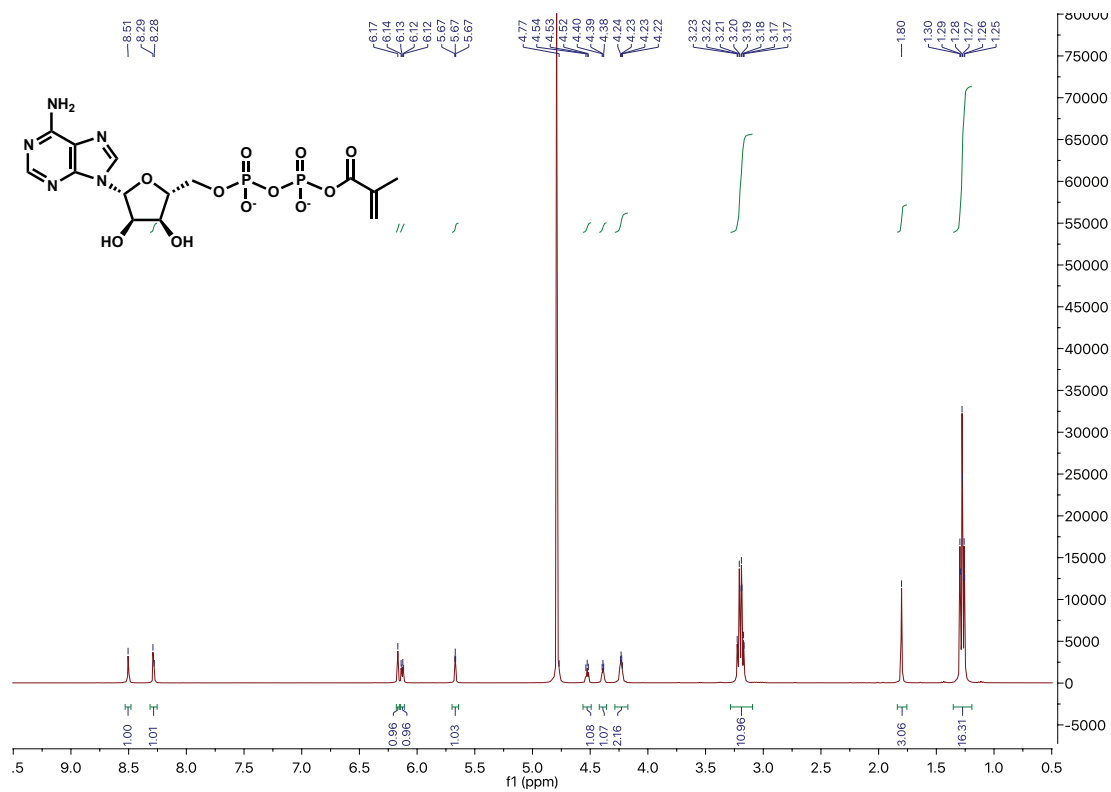


Figure 2.1.5. ^1H NMR (400 MHz, D_2O) of ADP-MA β -substituted active isomer.

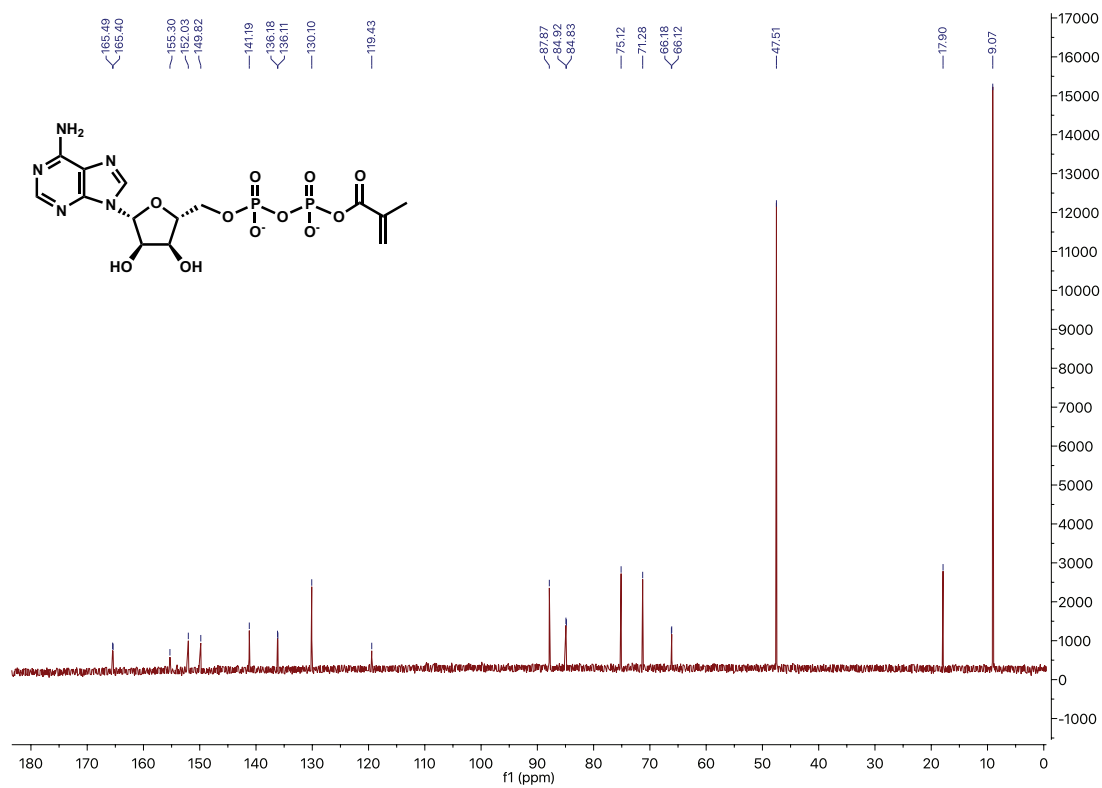


Figure 2.1.6. ^{13}C NMR (400 MHz, D_2O) of β -substituted ADP-MA active isomer.

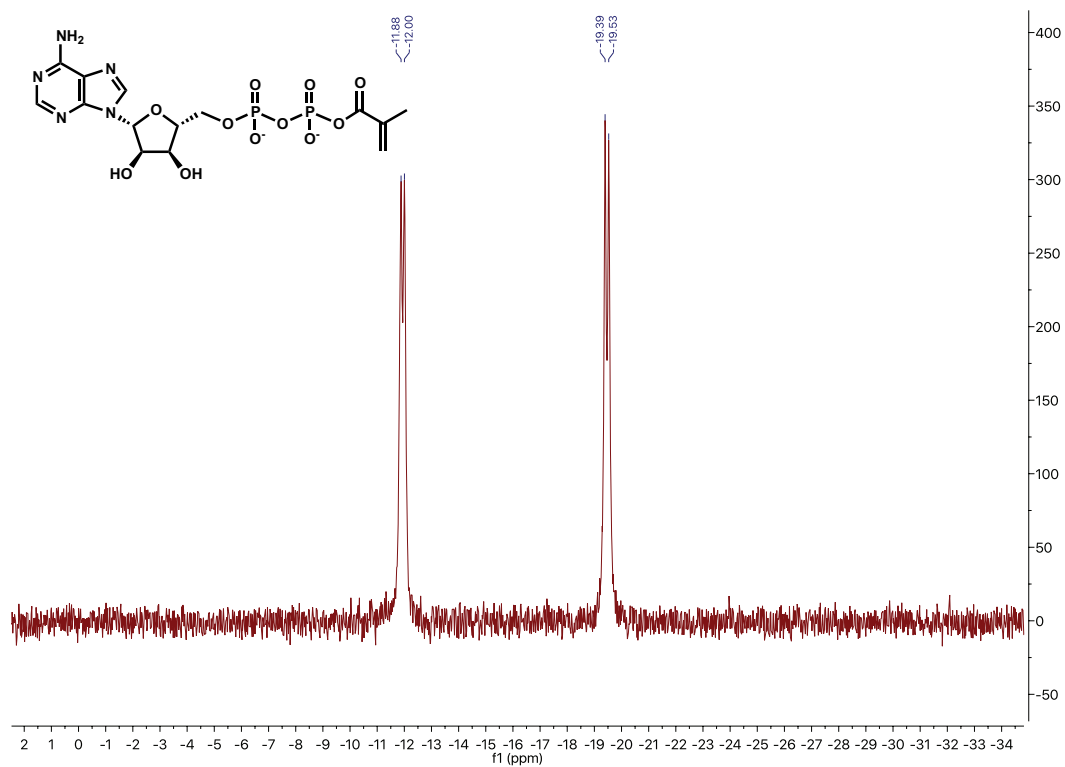


Figure 2.1.7. ^{31}P NMR (400 MHz, D_2O) of β -substituted ADP-MA active isomer

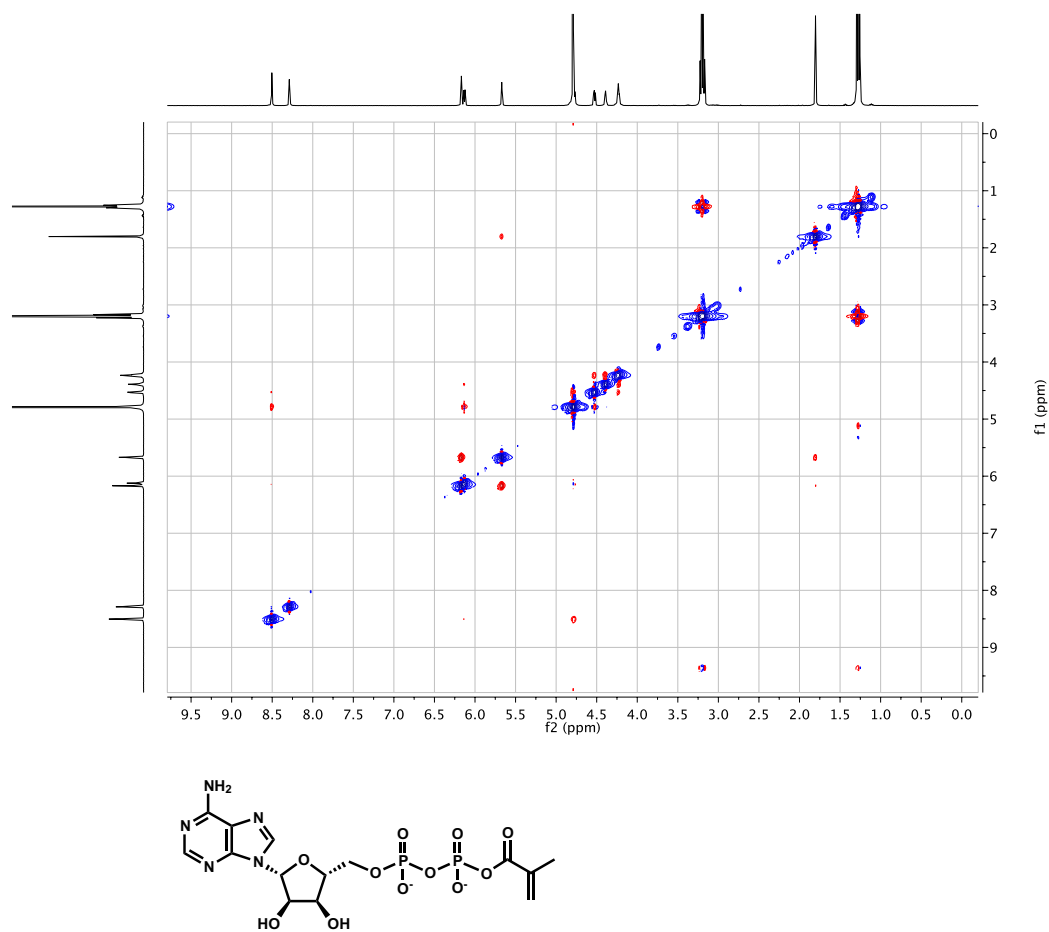


Figure 2.1.8. (400 MHz, D₂O) NOE of β -substituted ADP-MA active isomer.

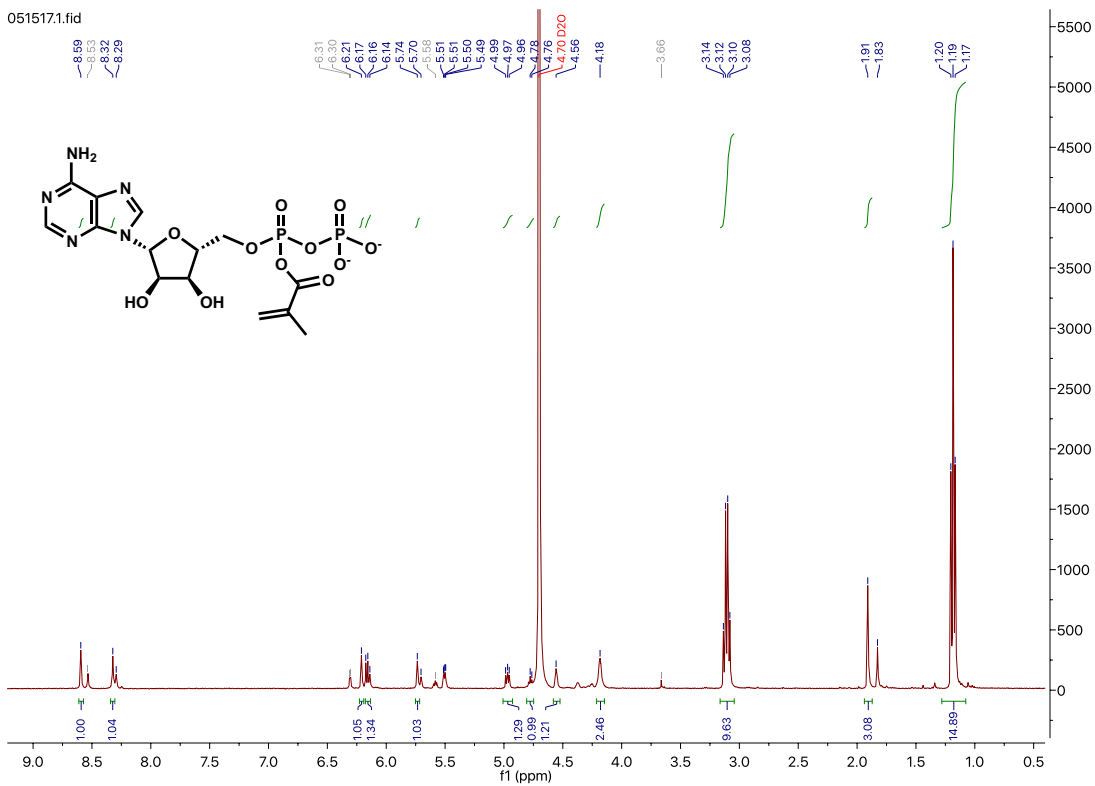


Figure 2.1.9. ¹H NMR (400 MHz, D₂O) of α -substituted ADP-MA inactive isomer.

012416 adpma late peaks mci.1.fid

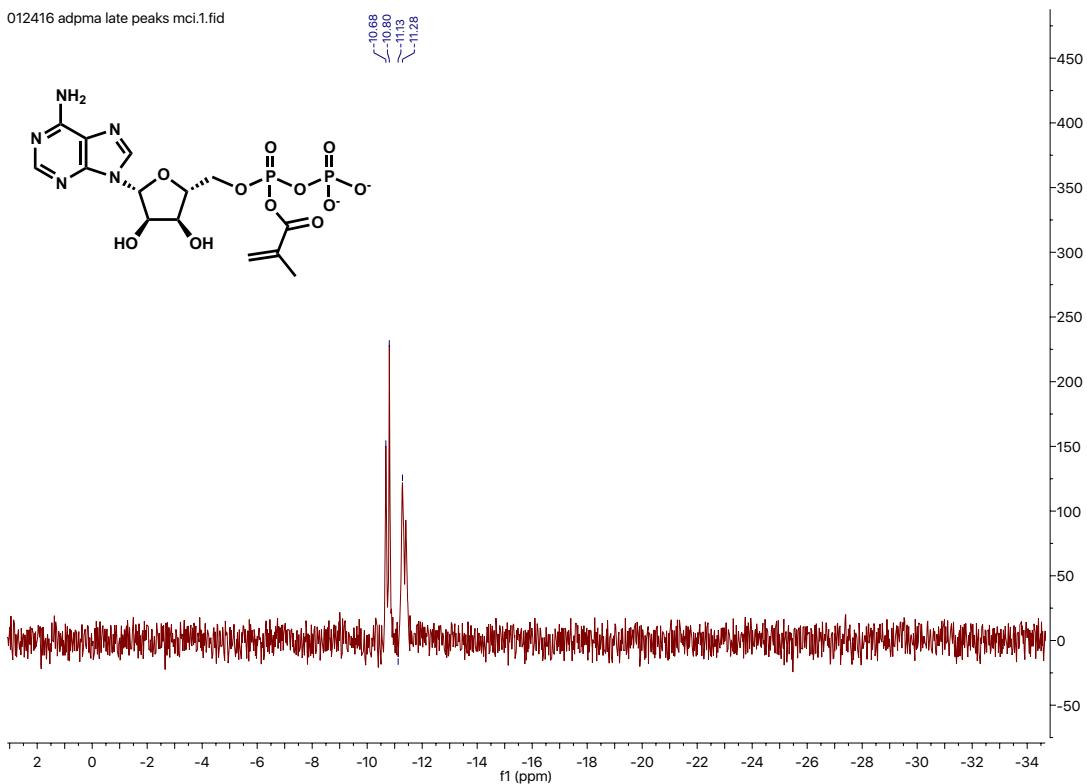


Figure 2.1.10. ^{31}P NMR (400 MHz, D_2O) of α -substituted ADP-MA inactive isomer.

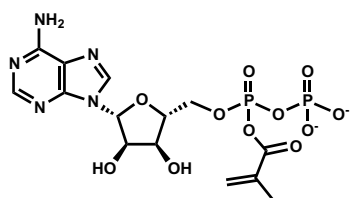
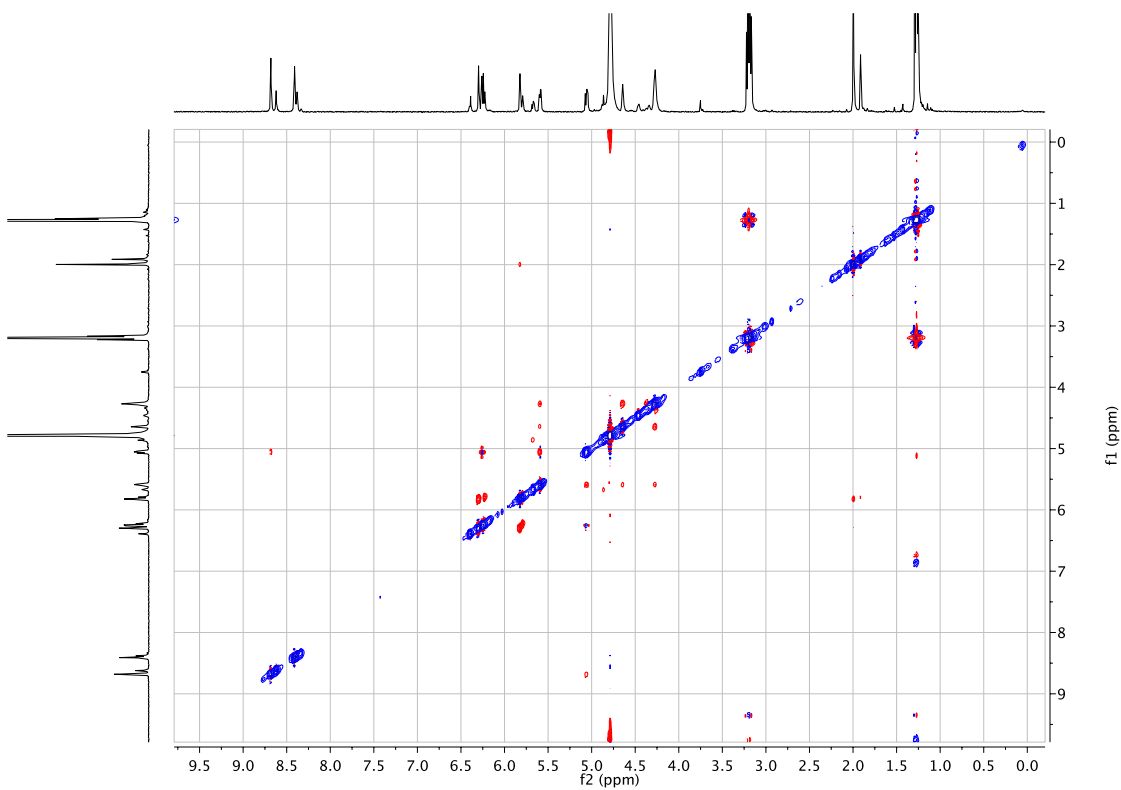


Figure 2.1.11. (400 MHz, D₂O) Heteronuclear NOE of α -substituted ADP-MA inactive isomer.

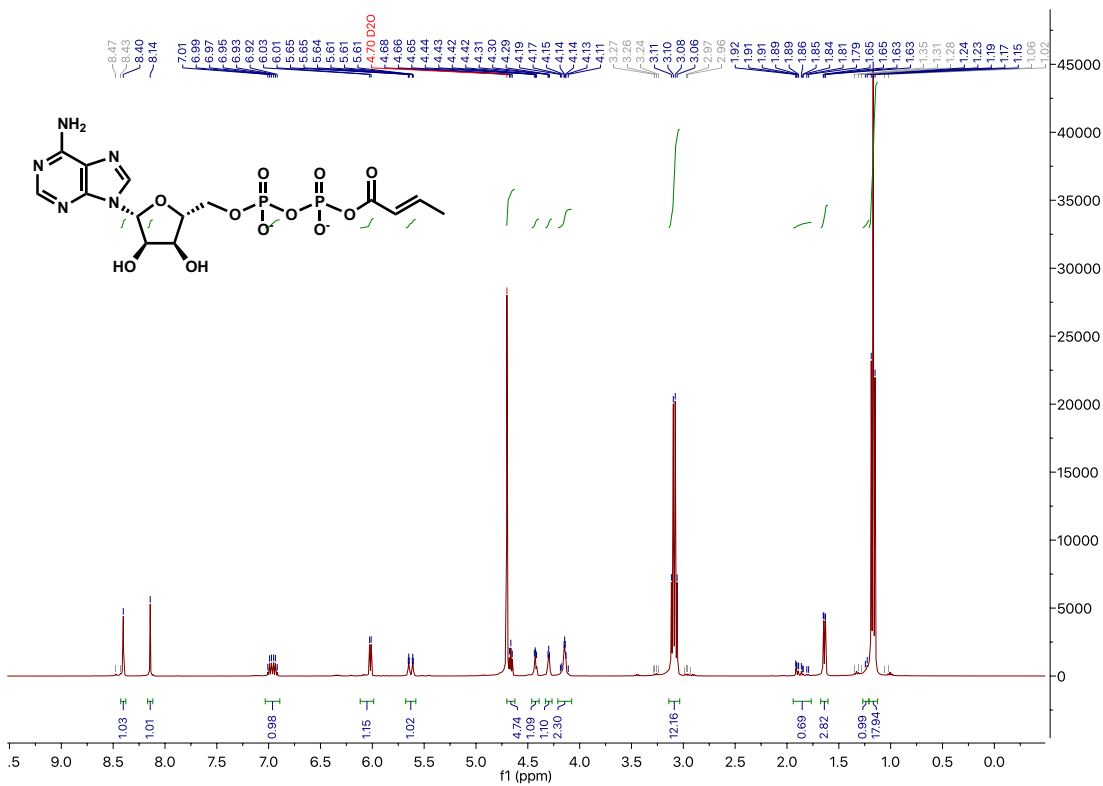


Figure 2.1.12. ¹H NMR (400 MHz, D₂O) of ADP-CA active isomer.

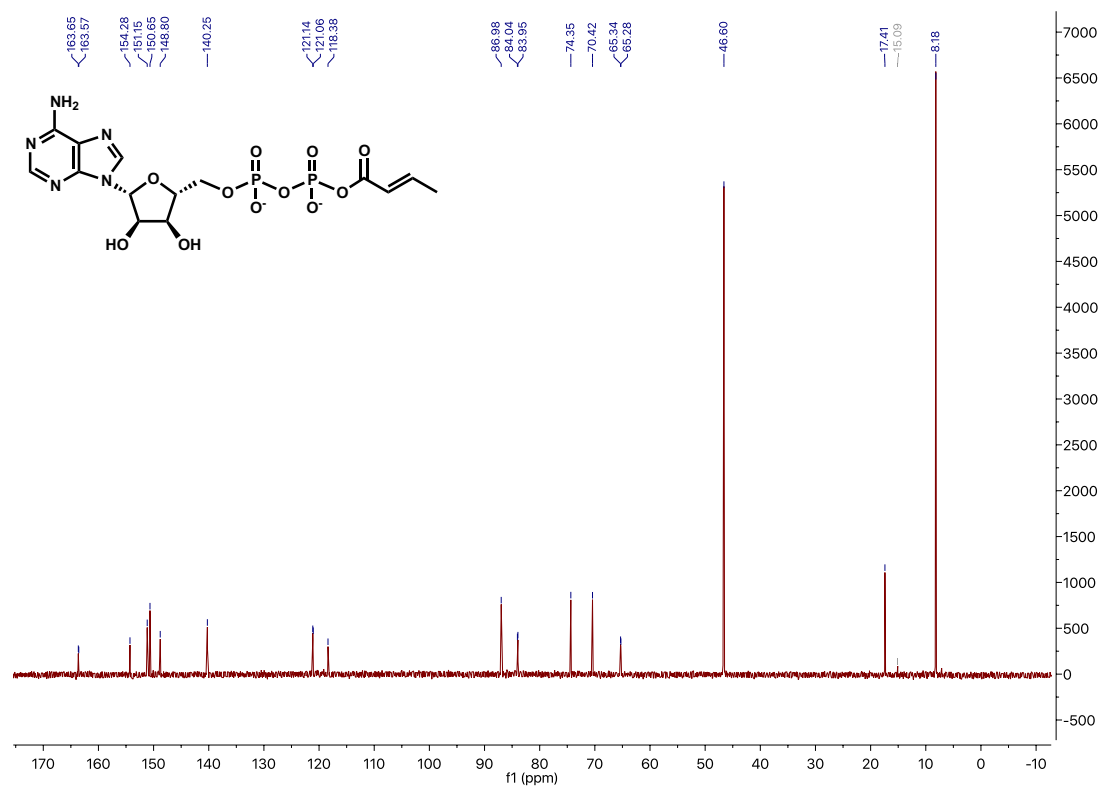


Figure 2.1.13. ¹³C NMR (400 MHz, D₂O) of ADP-CA active isomer.

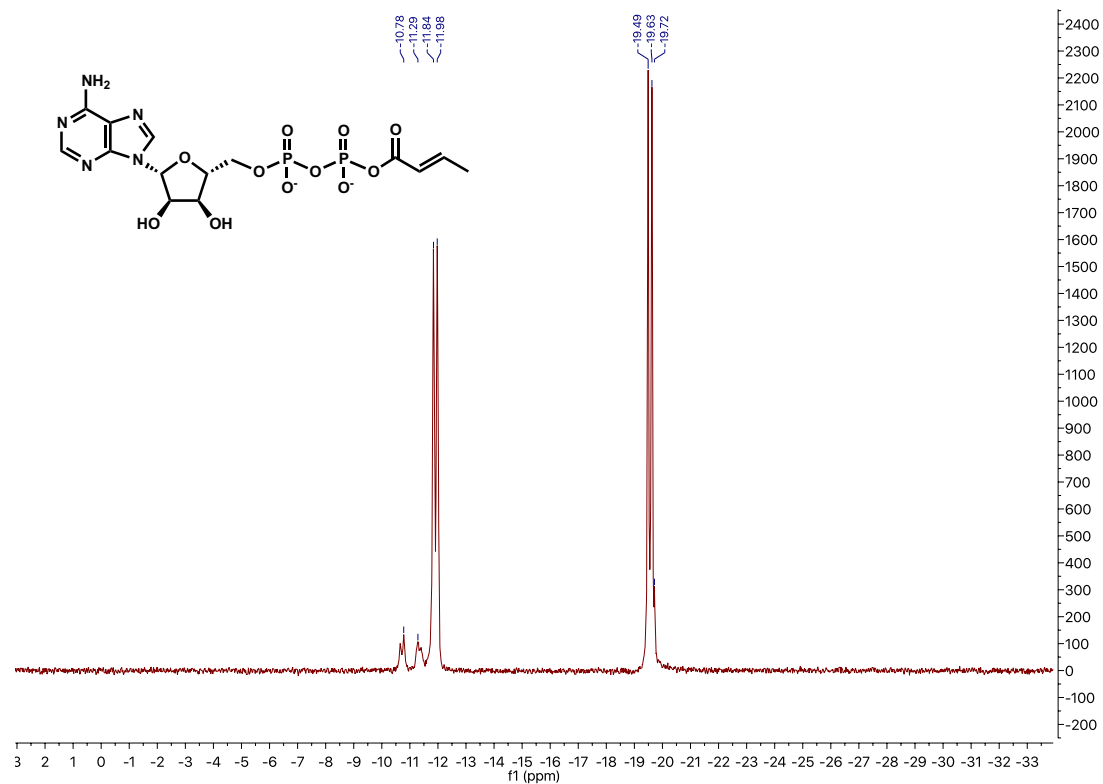


Figure 2.1.14. ³¹P NMR (400 MHz, D₂O) of ADP-CA active isomer.

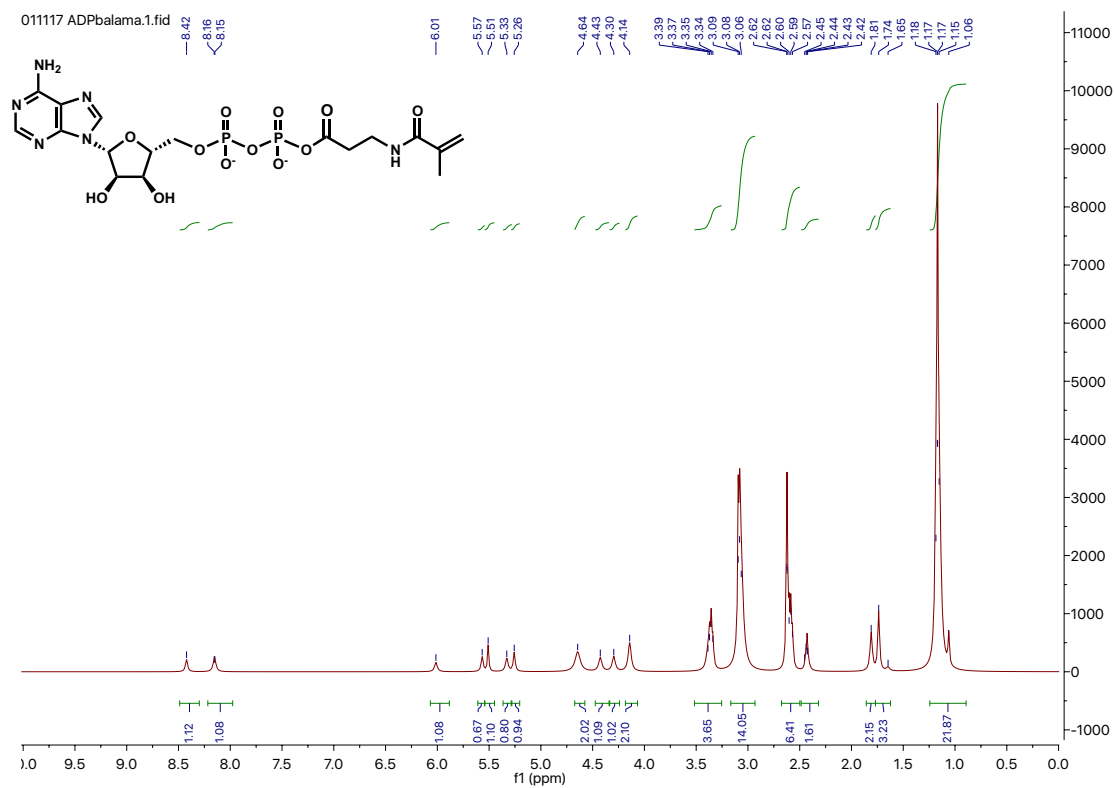


Figure 2.1.15. ^1H NMR (400 MHz, D_2O) of ADP- β -alaMA.

011117 ADPbalama phosph.1.fid

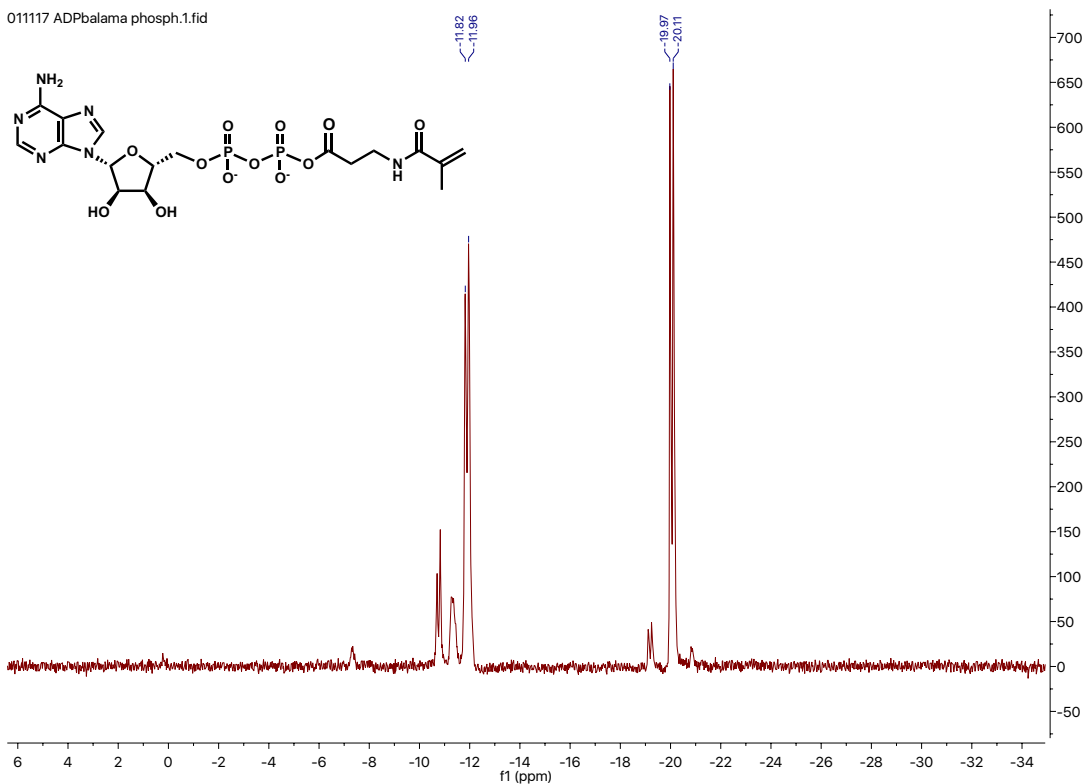


Figure 2.1.16. ^{31}P NMR (400 MHz, D_2O) of ADP- β -alaMA.

Chapter 3

A substrate based protein profiling approach for crosslinking kinases to Cys mutant substrates.

3.1 A substrate based protein profiling approach delivers a Cys reactive electrophile to kinase Lys.

Acrylamides are affinity-dependent electrophiles that have been successfully applied in the development of covalent drugs due to their limited reactivity with free cysteines and efficient reactivity with cysteines proximal to the bound ligand.³⁰ We previously described a methacrylate-modified nucleotide probe, ATP-MA,¹⁶ inspired by the activity based probes made by ActivX Biosciences.^{26,27} This probe delivered a Cys reactive electrophile to the kinase active site and selectively crosslinked a Cys mutant peptide substrate in the presence of a complex protein lysate, but was unable to demonstrate quantitative crosslinking or crosslinking to a protein substrate. To explore structural modifications that might improve crosslinking, we modified the scaffolding nucleotide to form a new probe, ADP-MA (Figure 1.1.1).

In ATP-MA, the gamma phosphate of ATP is modified with a methacrylate group. Upon ATP binding to the active site, this mixed anhydride is attacked by a conserved kinase lysine, releasing ATP and forming a methyl-substituted acrylamide. To improve upon our ATP scaffolded crosslinker, we considered the geometry of the kinase active site.³¹ We observed that the conserved kinase lysine is positioned proximally to the alpha and beta phosphates of ATP in general and postulated that modifying ADP rather than ATP might facilitate transfer of the methacrylate to the desired lysine. We isolated ADP-MA in high purity and tested it in the model kinase Src and observed single labeling of Src by LC-MS analysis even in the presence of excess

probe (100 equiv, observed $\Delta 72$, expected $\Delta 69$) (Figure 3.1.1). By comparison, ATP-MA also labeled Src once by LC-MS with a shift of $\Delta 70$ (expected $\Delta 69$), but with formation of side products $\Delta 149$, probably corresponding to double addition, and $\Delta 585$, discussed further below (Figure 3.1.2).

We characterized the kinetics of the first step of the reaction of ADP-MA and ATP-MA, the formation of methylacrylamide in the kinase active site (Figure 3.1.3). From the formation of Src-MA by LC-MS we found the k_{inact}/K_I of ADP-MA to be $45 \pm 2 \text{ M}^{-1}\text{s}^{-1}$ at 4 °C and that of ATP-MA to be $129 \pm 8 \text{ M}^{-1}\text{s}^{-1}$ (Figure 3.1.3 Panel C). The correlation between k_{obs} vs [ADP-MA] remained linear at all probe concentrations tested (up to 50 μM), from which we inferred that K_I is greater than 50 μM . This lower bound for K_I is consistent with the previously reported K_m of ATP for Src (51-169 μM). The k_{inact}/K_I of ADP-MA is approximately an order of magnitude higher than that of general covalent lysine targeted kinase inhibitors such as 5'-(4-fluorosulfonylbenzoyl) adenosine,³² but three to five orders of magnitude lower than the epidermal growth factor receptor-targeting covalent kinase inhibitors, which modify cysteine.³³

We additionally tested the probes ADP-acrylate (ADP-A), ADP-crotonate (ADP-CA), and ADP- β -alanine-methacrylate (ADP- β -alaMA), and ADP- α -chloroacrylate (ADP- α -ClA) to see if these probes behaved similarly in the first reaction step with model kinase Src (Figure 3.1.4). We found that at 4 °C, 50 μM ADP-A labeled Src through a Src cysteine via the electrophile, resulting in a shift of the mass of Src of $\sim\Delta 479$, the mass of the whole probe, rather than the $\Delta 55$ mass from the formation of the acrylamide alone through the Src lysine (Figure 3.1.5, observed $\Delta 490$). We found that the $\Delta 490$ shift was modifiable to $\Delta 89$ with the addition of hydroxylamine, approximately consistent with addition through Cys and subsequent cleavage of the mixed anhydride through hydroxylamine addition (expected $\Delta 87$) (Figure 3.1.6). To our surprise, we consistently found that despite the increased steric bulk on the Michael acceptor site,

ADP-CA also preferentially labeled Src through Cys, resulting in $\sim\Delta 493$, rather than the expected $\Delta 68$ (observed $\Delta 498$) (Figure 3.1.7). This adduct was also cleavable with hydroxylamine to $\sim\Delta 101$ (observed $\Delta 89$), further confirming addition through the electrophile. We found that ADP- β -alaMA formed an adduct with the shift of $\sim\Delta 140$ (observed $\Delta 136$) consistent with formation of the acrylamide (Figure 3.1.8). A small ($\sim 10\%$ of parent) peak was observed which could correspond to addition through the electrophile or could be noise (expected $\Delta 564$, observed $\Delta 530$). Finally, we found that ADP- α -CIA formed two products consistent with single and double addition (expected $\Delta 76.5$, observed $\Delta 79$, $\Delta 155$) (Figure 3.1.9). Interestingly, we did not observe addition to kinase through the electrophile only. We thought this observation is possibly due to the shorter length of ADP- α -CIA given that the alpha-chloroacrylate electrophile would be expected to be more reactive than the acrylates tested in this series. An alternative explanation is that ADP- α -CIA mediates intramolecular crosslinking to both Lys and Cys present in Src. However, because we were unable to observe successful crosslinking with ADP- α -CIA between Src and cysteine-containing peptide, we did not investigate this reaction further. We observed addition through the electrophile in the reaction of Src with ATP-MA as well ($\Delta 585$ minor product, expected $\Delta 572$) and we observed ADP-MA addition through the electrophile in the reaction of Src with ADP-MA as a minor product at room temperature in excess of 200 equivalents probe (data not shown).



Figure 3.1.1. LC-MS MaxEnt spectrum of Src with ADP-MA. Expected methacrylamide shift is $\Delta 69$; observed shift $\Delta 72$. Unless otherwise noted, spectra were obtained on a Waters Acquity UPLC/ESI-TQD with a 2.1 X 50 mm Acquity UPLC BEH300 C4 column and Src concentration was 4 μM .

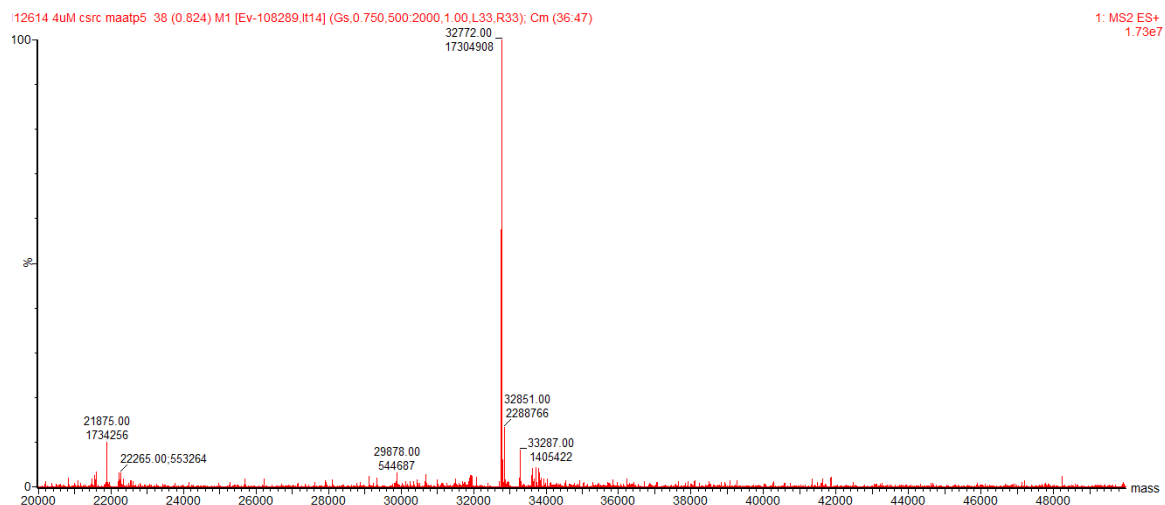


Figure 3.1.2. LC-MS MaxEnt spectrum of Src with ATP-MA. Expected methacrylamide shift is $\Delta 69$; observed shifts $\Delta 70$ and $\Delta 149$, $\sim 10\%$ of parent. Expected Cys addition is $\Delta 572$; observed shift $\Delta 585$.

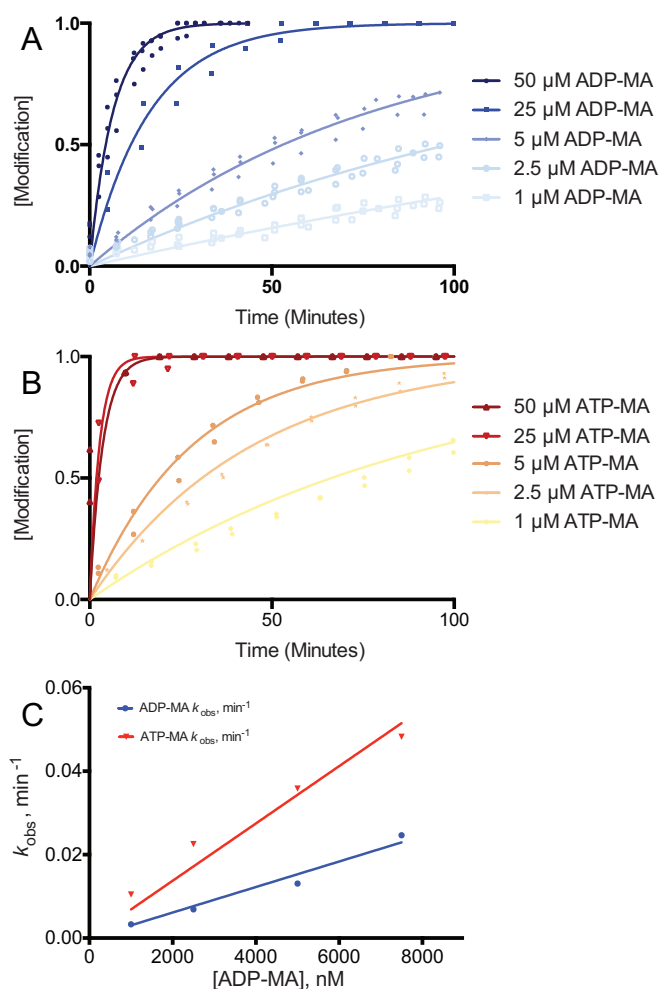
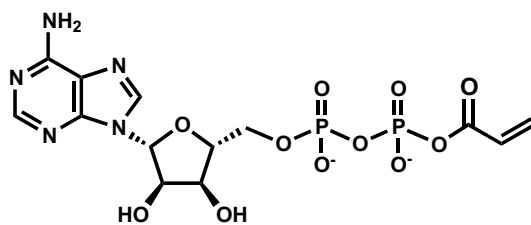
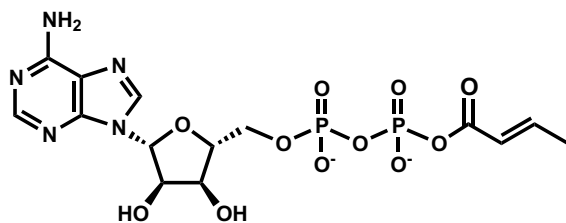


Figure 3.1.3. Characterization of covalent kinetics of reaction of Src with ADP-MA and ATP-MA by LC-MS at 4 °C. Time course of formation of Src-MA with A. ADP-MA and B. ATP-MA. C. K_{obs} vs [probe]. Src concentration was 100 nM and buffer components were 25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM MgCl_2 .

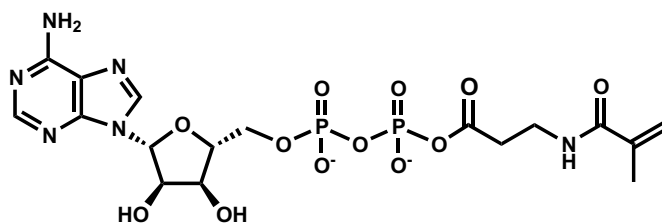
ADP-A



ADP-CA



ADP- β -alaMA



ADP- α -CIA

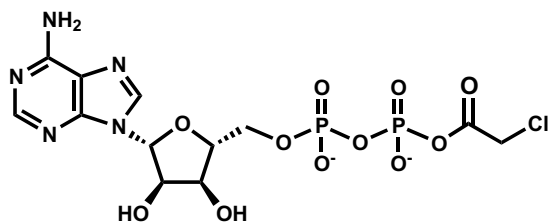


Figure 3.1.4. Structures of ADP-A, ADP-CA, ADP- β -alaMA, and ADP- α -CIA.

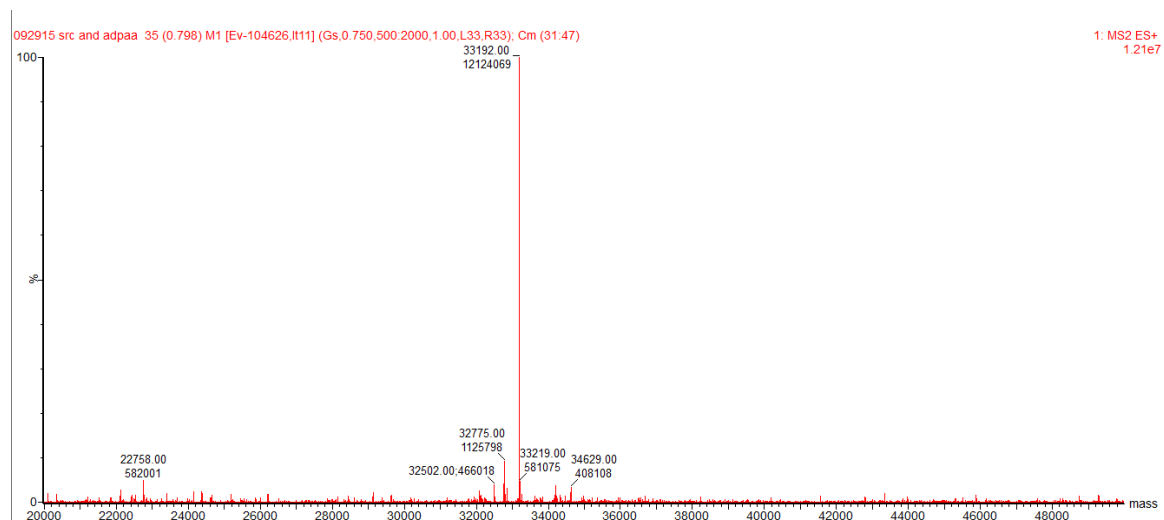


Figure 3.1.5. LC-MS MaxEnt spectrum of Src with ADP-A. Src alone observed mass is 32702; expected Cys addition through electrophile shift is $\Delta 479$, observed $\Delta 490$.

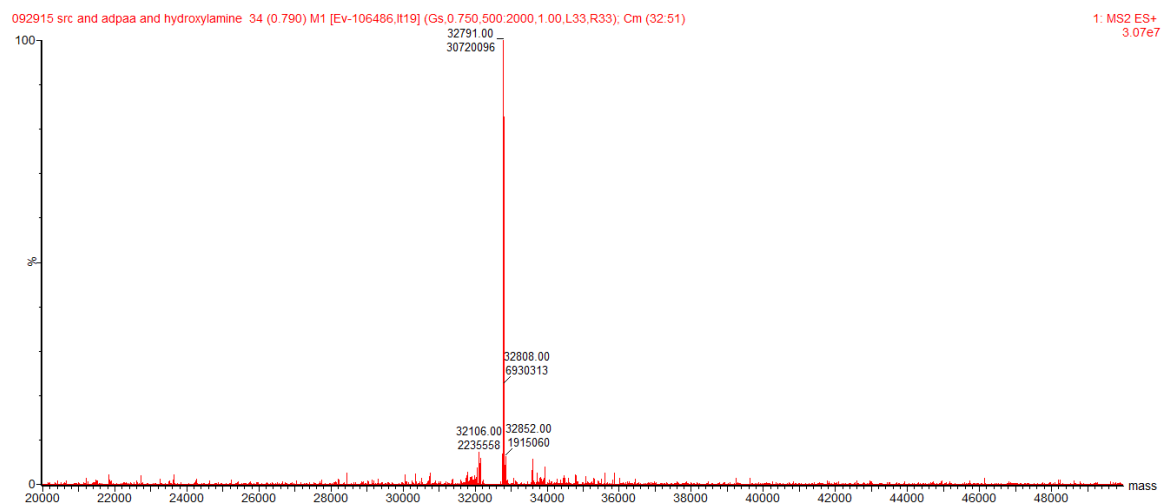


Figure 3.1.6. LC-MS MaxEnt spectrum of Src with ADP-A followed by hydroxylamine. Expected Src + C₃H₃ONHOH shift is $\Delta 87$; observed shift $\Delta 89$.

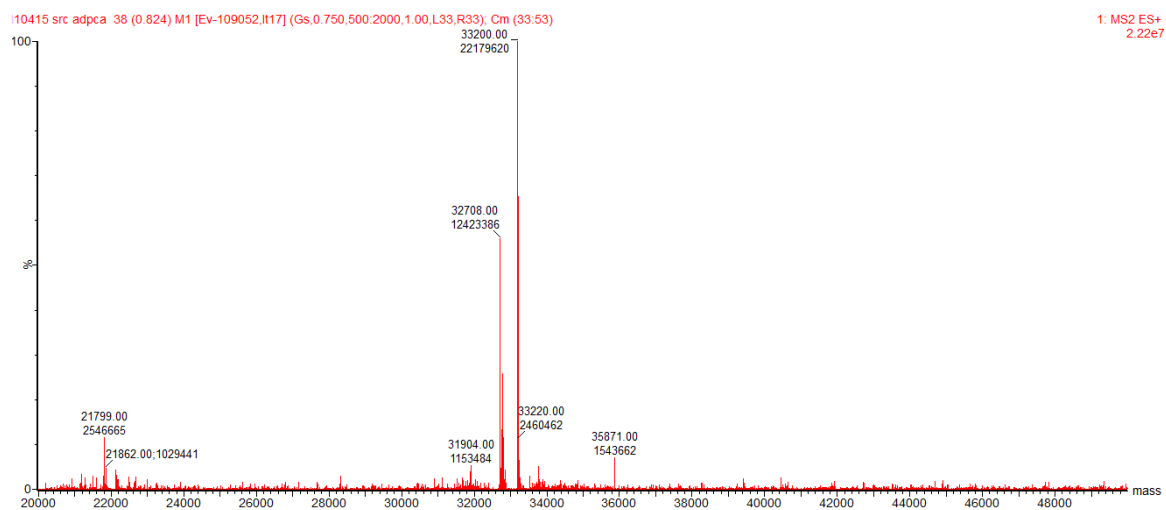


Figure 3.1.7. LC-MS MaxEnt spectrum of Src with ADP-CA. Expected $\Delta 68$ for CA shift, expected Cys addition $\Delta 493$; observed shift predominantly $\Delta 498$ with some unmodified Src.

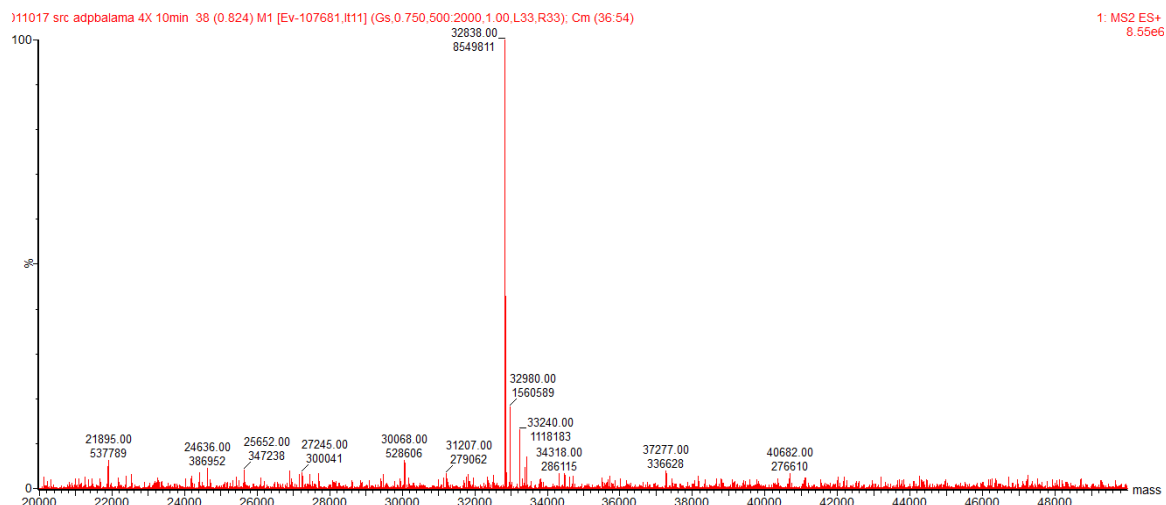


Figure 3.1.8. LC-MS MaxEnt spectrum of Src with ADP- β -alama. Expected Src + β -alama shift is $\Delta 140$; observed shift $\Delta 136$. Expected Cys addition is $\Delta 564$; a small peak at $\Delta 538$ was observed which could correspond to minor product formation.

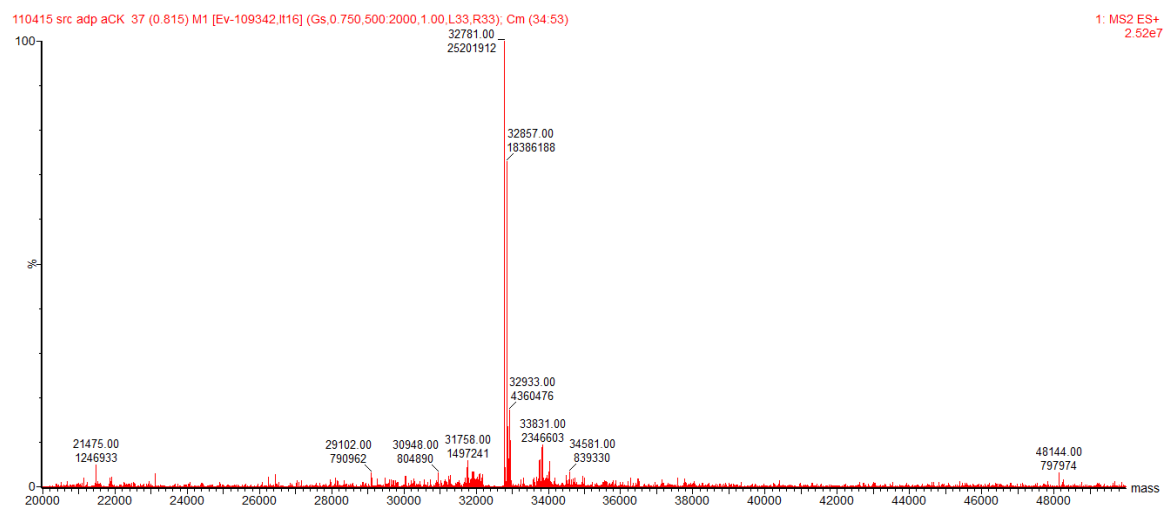


Figure 3.1.9. LC-MS MaxEnt spectrum of Src with ADP- α -ClA. Expected Src + α -ClA shift is $\Delta 76.5$; observed shift $\Delta 79$ and $\Delta 155$, consistent with possible double addition.

3.2 ADP-MA mediates crosslinking in several peptide substrate-kinase systems.

Following successful labeling of kinase with the electrophilic adduct, we looked to evaluate kinase-substrate crosslinking using a model system, Src kinase and its peptide Cys-Srctide. (For peptide tags, sequences, and molecular weights, see Table 3.2.1). We considered that successful labeling of kinase with the electrophile would be necessary but not sufficient for crosslinking, given that the probe must first covalently react with kinase and then position the electrophile in proximity to the Cys mutant phosphorylation site for covalent reaction with substrate.

Briefly, we found that ADP-MA was able to mediate crosslinking between Src and Cys-Srctide to 100% conversion by LC-MS (Figure 3.2.2). However, we found that ATP-MA mediated Src-Srctide crosslinking under multiple concentrations of peptide and the same reaction conditions did not crosslink as cleanly or as quickly as ADP-MA (Figure 3.2.3). We were puzzled by this finding given that the reaction of Src with ATP-MA proceeds to completion and the chemical composition of the adduct should be identical to that of ADP-MA. Further experiments that address this discrepancy are discussed in Chapter 4.

We also used Src-Cys-Srctide crosslinking to evaluate the other ADP-scaffolded crosslinkers discussed in 3.1 if addition was not exclusively through the electrophile. ADP-CA could crosslink FLAG-Cys-Srctide as a minor product (<8% of parent), but the majority of the product observed was the Cys-addition side product (Figure 3.2.4) in which a Cys native to Src, rather than the substrate Cys, performs Michael addition.

In contrast, ADP- β -alaMA labeled Src with the acrylamide to completion, but no crosslinking was observed after incubation with 260 μ M FLAG-Cys-Srctide for 1 hour (Figure 3.2.5). This observation suggests that the β -alamethacrylamide labeled Src is not positioned

optimally for reaction with the phosphorylation site Cys mutant. It is possible that the longer β -alamethacrylamide was better positioned for intramolecular reaction with Src cysteine. We did not evaluate this possibility, but this hypothesis could be tested by evaluating ADP- β -alaMA mediated crosslinking between Cys-Src tide and mutant Src C277Q in which the most likely reactive cysteine has been mutated to an unreactive amino acid.

Similarly, ADP- α -CIA labeled Src with the α -chloroacetamide to completion with some double addition product, but no peptide crosslinked product was observed (Figure 3.2.6).

Finally, we also tested the Ser/Thr kinase Aurora A with its peptide AurAtide. We found that the product of the crosslinking reaction by LC-MS was difficult to deconvolute using MaxEnt software, suggesting that the reaction proceeded to multiple products. However, we observed ADP-MA mediated crosslinking by western blot (Figure 3.2.7). I will describe further evaluation of Aurora A labeling in Chapter 3.4.

Name	Tag	Sequence	MW (Da)
Biotin-Cys-AurAtide	Biotin	ALKRACLGAA	1197
Biotin-Cys-AurAtide	Biotin	ALKRACLGAA	1197
Biotin-Cys-Srctide	Biotin	AEEAcICGEFRRR	1407
FLAG-Cys-AurAtide	DYKDDDDK	ALKRACLGAA	1968
FN3-Srctide	Biotin	EEI(Y*)GEFRRR	1606
FN3-SRPKtide	Biotin	PRSR(Y*)PVSR	1455
FN3-AurAtide	Biotin	ALKRA(Y*)LGAA	1285
FLAG-Cys-Srctide	DYKDDDDK	EEICGEFRRR	2289
FLAG-Cys-AKTtide	DYKDDDDK	RPRTSCF	1861
FLAG-Cys-CK2tide	DYKDDDDK	RRADDCDDDD	2190
FLAG-Tyr-Srctide	DYKDDDDK	EEIYGEFRRR	2349
FLAG-Ser-AKTtide	DYKDDDDK	RPRTSSF	1845
FLAG-Ser-CK2tide	DYKDDDDK	RRADDSDDDD	2174
FLAG-Tyr-AKTtide	DYKDDDDK	RPRTSYF	1921
FLAG-Tyr-CK2tide	DYKDDDDK	RRADDYDDDD	2250
Cys-Srctide	None	EEICGEFRRR	1294
Cys-AKTtide	None	RPRTSCF	866.5
Cys-CK2tide	None	RRADDCDDDD	1195
FLAG-Ser-Srctide	DYKDDDDK	EEISGEFRRR	2273
FLAG-Cys-Corttide	DYKDDDDK	SPVCEDAASF	2020
FLAG-Tyr-Corttide	DYKDDDDK	SPVYEDAASF	2080
FLAG-Ser-Corttide	DYKDDDDK	SPVSEDAASF	2004

Table 3.2.1. Reference table of all peptides used to evaluate crosslinking and phosphorylation.

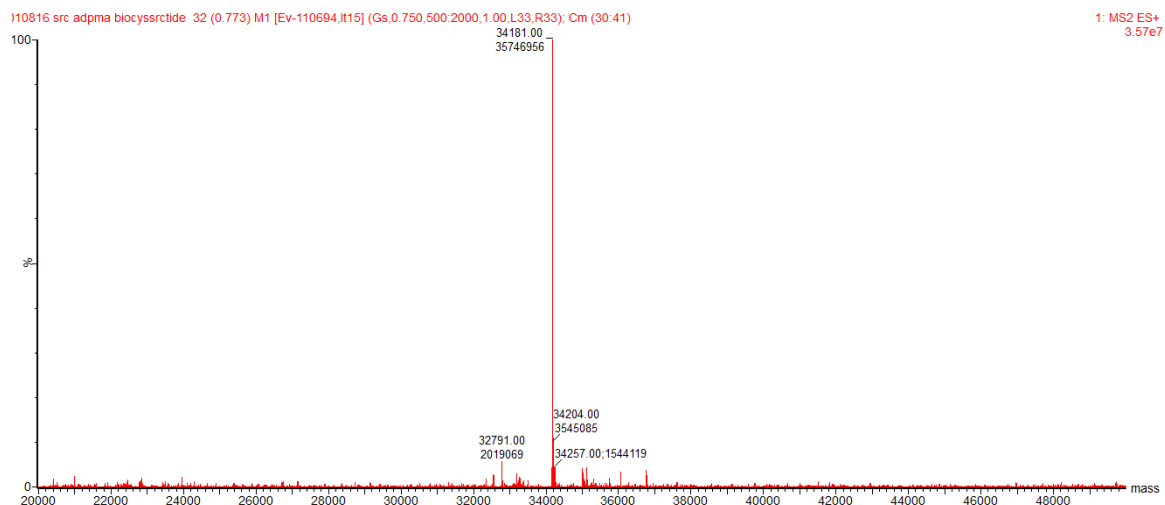


Figure 3.2.2. LC-MS MaxEnt spectrum of Src with ADP-MA and Biotin-Cys-Srctide. Crosslinking proceeds to completion. Src-MA-Srctide expected shift is $\Delta 1476$, observed $\Delta 1479$. Src was 5 μM , Biotin-Cys-Srctide was 500 μM , and incubation was for 90 minutes at 4 $^{\circ}\text{C}$.

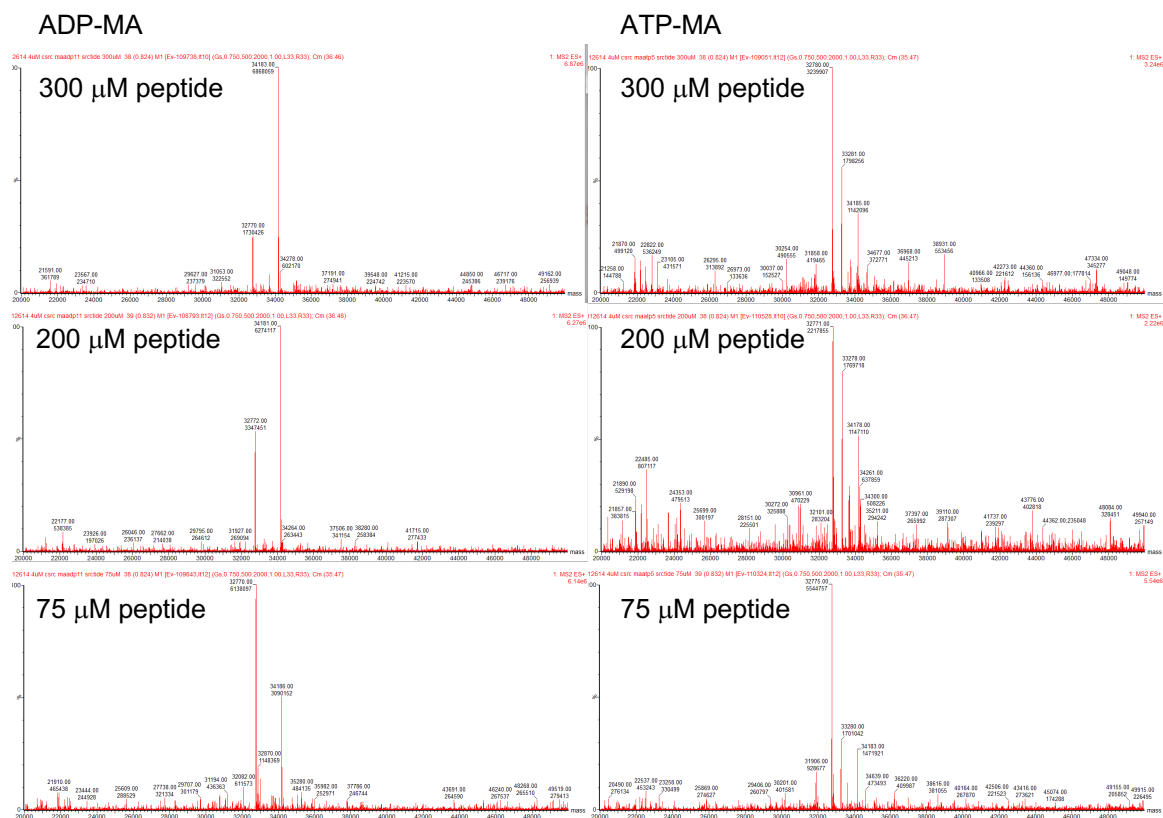


Figure 3.2.3. LC-MS MaxEnt spectrum of 4 μ M Src with Biotin-Cys-Srcptide and 500 μ M ADP-MA (left) vs ATP-MA (right) at 300 μ M, 200 μ M, and 75 μ M peptide. Src-MA-Srcptide expected shift is $\Delta 1476$, observed $\Delta 1477$ -1485.

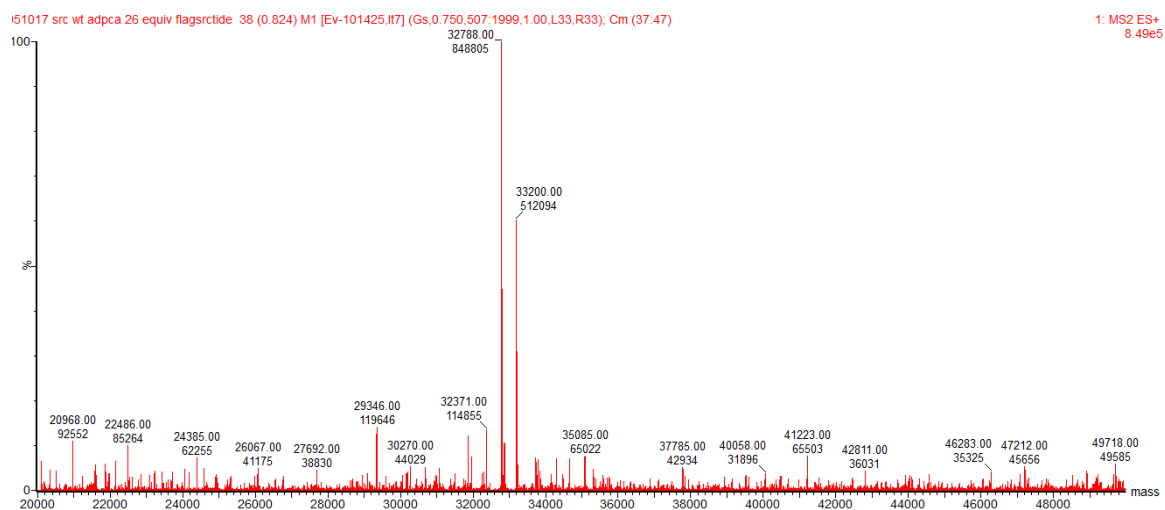


Figure 3.2.4. LC-MS MaxEnt spectrum of 1 μ M Src with 260 μ M FLAG-Cys-Srctide and ADP-CA. Src-CA-FLAG-Srctide expected shift is $\Delta 2358$, minor product of $\Delta 2383$ observed ($>8\%$ parent).

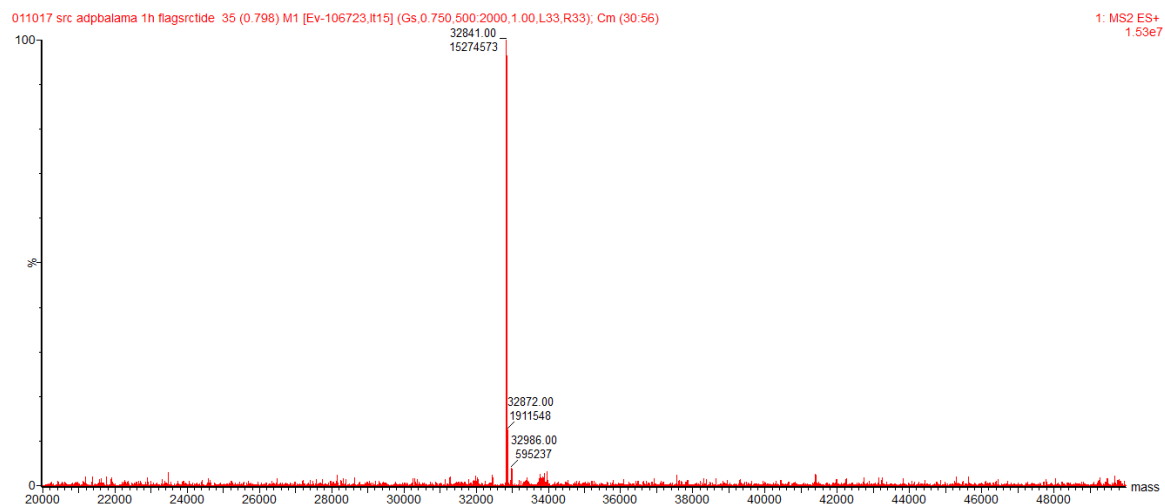


Figure 3.2.5. LC-MS MaxEnt spectrum of 4 μ M Src with 260 μ M FLAG-Cys-Srctide and ADP- β -alaMA. Acrylamide formation proceeded to completion; expected $\Delta 140$, observed $\Delta 139$. No crosslinked peptide product was observed.

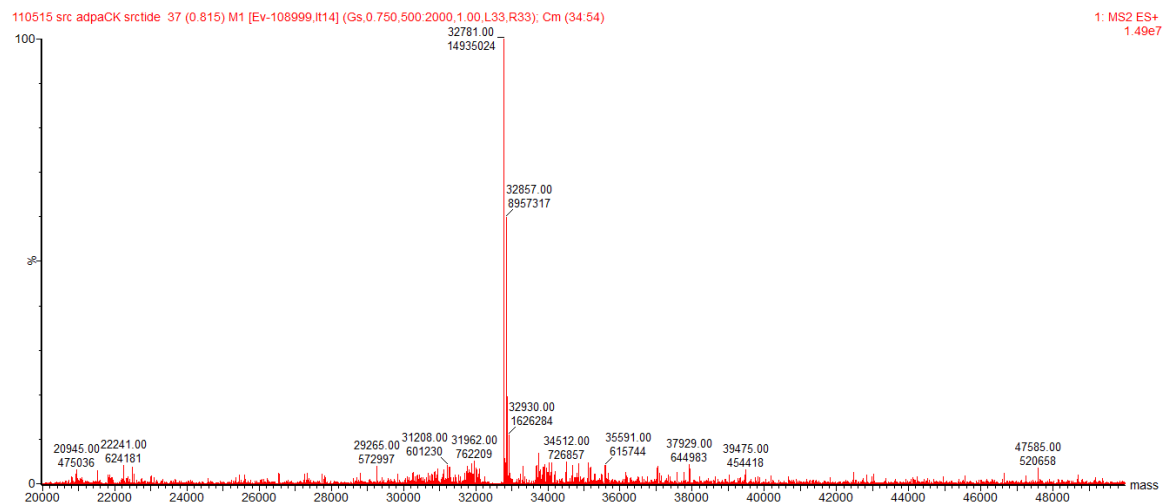


Figure 3.2.6. LC-MS MaxEnt spectrum of 4 μ M Src with 100 μ M FLAG-Cys-Src tide and ADP- α -CIA. Formation of the α -chloroacetamide was observed as the majority product (expected $\Delta 76.5$, observed $\Delta 79$) with double addition as a minor product. Formation of the crosslinked product was not observed (expected $\Delta 2330$).

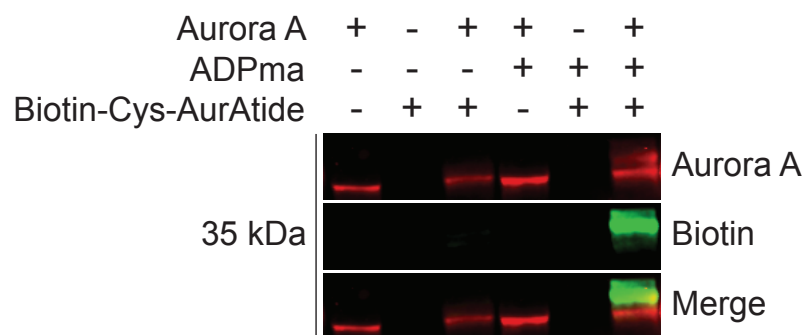


Figure 3.2.7. Western blot analysis of ADP-MA mediated Biotin-Cys-AurAtide-Aurora A crosslinking. Aurora A was 1 μ g/3 μ M, Biotin-Cys-AurAtide 6 μ M, 4 h, 4 $^{\circ}$ C. Buffer: Tris 50 mM pH 8, NaCl 150 mM. Order of addition in all crosslinking samples is buffer, substrate, kinase, crosslinker unless otherwise noted.

3.3 Characterizing AMP-scaffolded probes.

In addition to ADP- and ATP-scaffolded probes, we also considered the corresponding AMP-based compounds. AMP derivatives are generally simpler to synthesize and purify relative to their ADP- and ATP- counterparts due to their increased solubility in nonpolar solvents and fewer possible constitutional isomers. However, many kinases have lower affinity for AMP relative to ATP and ADP and therefore the AMP-based probes are expected to bind more weakly.

First, we considered the AMP analogs of ADP-A, ADP-CA, and ADP-MA and tested their reactivity to Src by LC-MS. All reactions took place significantly slower than the corresponding reaction with the ADP analog. Surprisingly, at 110 minutes at 4 °C, AMP-acrylate (AMP-A) with Src had completely proceeded to the Lys-modified acrylamide product. Unlike ADP-A, which labeled an endogenous Cys through the electrophile resulting in a mass shift of the full crosslinker mass that is reactive with hydroxylamine, we did not observe evidence of Cys labeling through the electrophile for AMP-A (Figure 3.3.1, expected shift $\Delta 55$, observed $\Delta 53$). However, after 2 hours at room temperature, AMP-A did not mediate crosslinking to 50 μ M Cys-Src-tide (Figure 3.3.2). Given that the unsubstituted acrylate should be expected to be significantly more reactive than the methacrylate, we hypothesize that intramolecular crosslinking to Src Cys is competing for formation of the intermolecular crosslinking product.

AMP-crotonate (AMP-CA) did not react appreciably with Src at 110 minutes at 4 °C (Figure 3.3.3), but after 2 hours at room temperature some formation of the crotonylamide was observed; however, no crosslinking to Cys-Src-tide was appreciated (Figure 3.3.4). Finally, AMP-methacrylate (AMP-MA) did not react with Src at 110 minutes at 4 °C (Figure 3.3.5), but after 2

hours at room temperature some degradation and some formation of the methacrylamide was observed; however, no crosslinking to Cys-Src³ was observed (Figure 3.3.6).

We hypothesized that the differing reactivities of AMP-acrylates compared to ADP and ATP probes could be largely attributed to the decreased affinity of Src for AMP. One other consideration is that we obtained a quadrupole time-of-flight LC-MS instrument (Waters Xevo G2-XS QToF) requiring lower concentration of protein than the older triple quadrupole machine (Waters Acquity UPLC/ESI-TQD); these experiments were run at 100-500 nM rather than 4 μ M Src. Nonetheless, we had also run AMP-MA on the original instrument with similar results to those obtained on the new instrument (data not shown), so we considered it likely that AMP derivatives were worse crosslinkers in general than their corresponding ADP scaffolded probes.

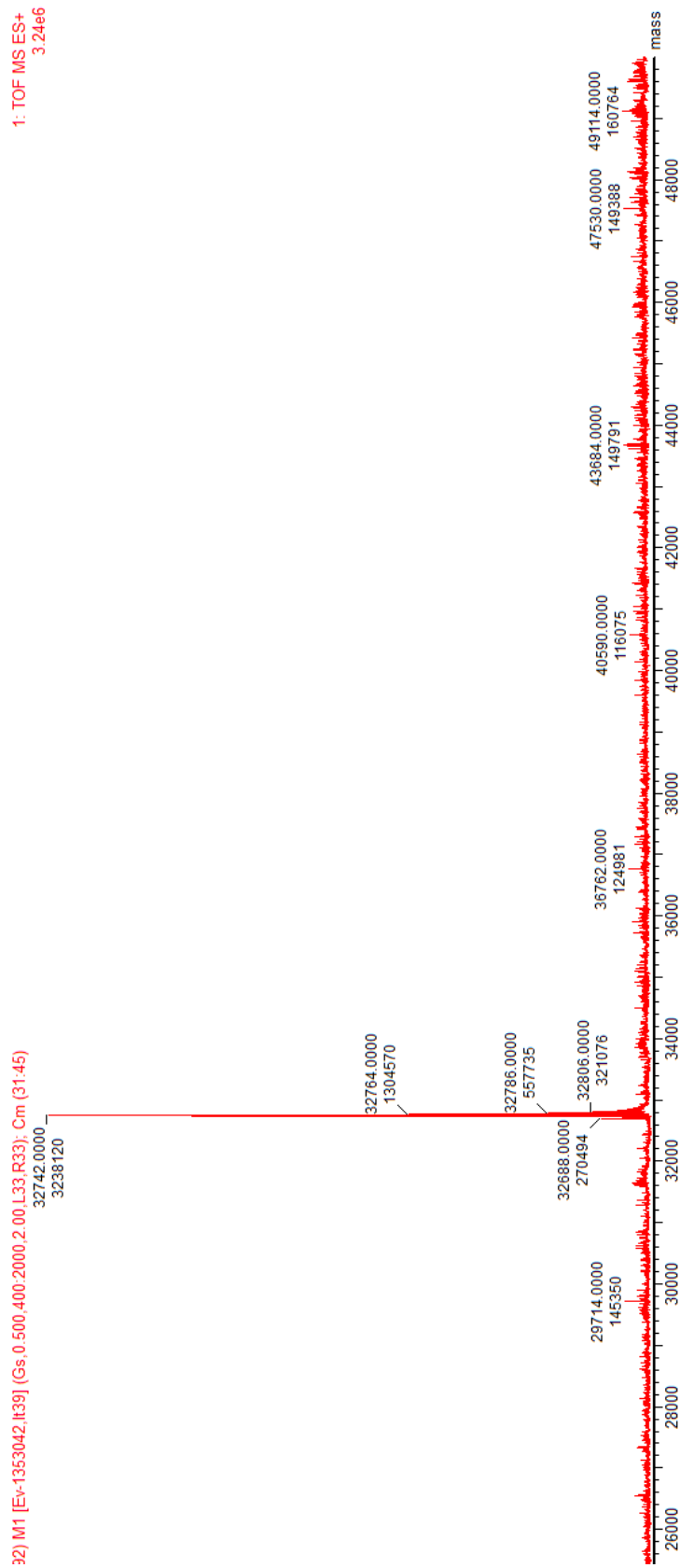


Figure 3.3.1. LC-MS MaxEnt spectrum of 100 nM Src with 5 μ M AMP-A. Src parent mass is 32689 Da. Acrylamide expected shift $\Delta 53$, observed $\Delta 53$. This spectrum was obtained with a Waters Xevo G2-XS QToF with a 2.1 X 50 mm Acquity UPLC BEH300 C4 column rather than the Waters Acquity. All subsequent spectra were run on the Waters Xevo unless otherwise noted. Due to increased machine sensitivity, kinase is at 100-500 nM concentration rather than 4 μ M.

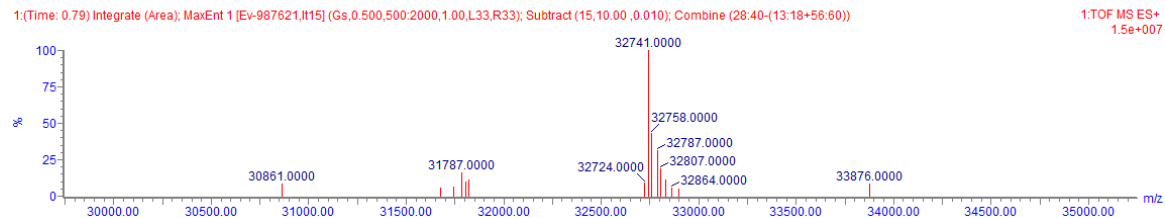


Figure 3.3.2. LC-MS MaxEnt spectrum of 100 nM Src with 5 μ M AMP-A and 50 μ M Cys-Srctide for 120 minutes at room temperature. Src-A-Srctide expected shift is $\Delta 1350$. Crosslinking was not observed.

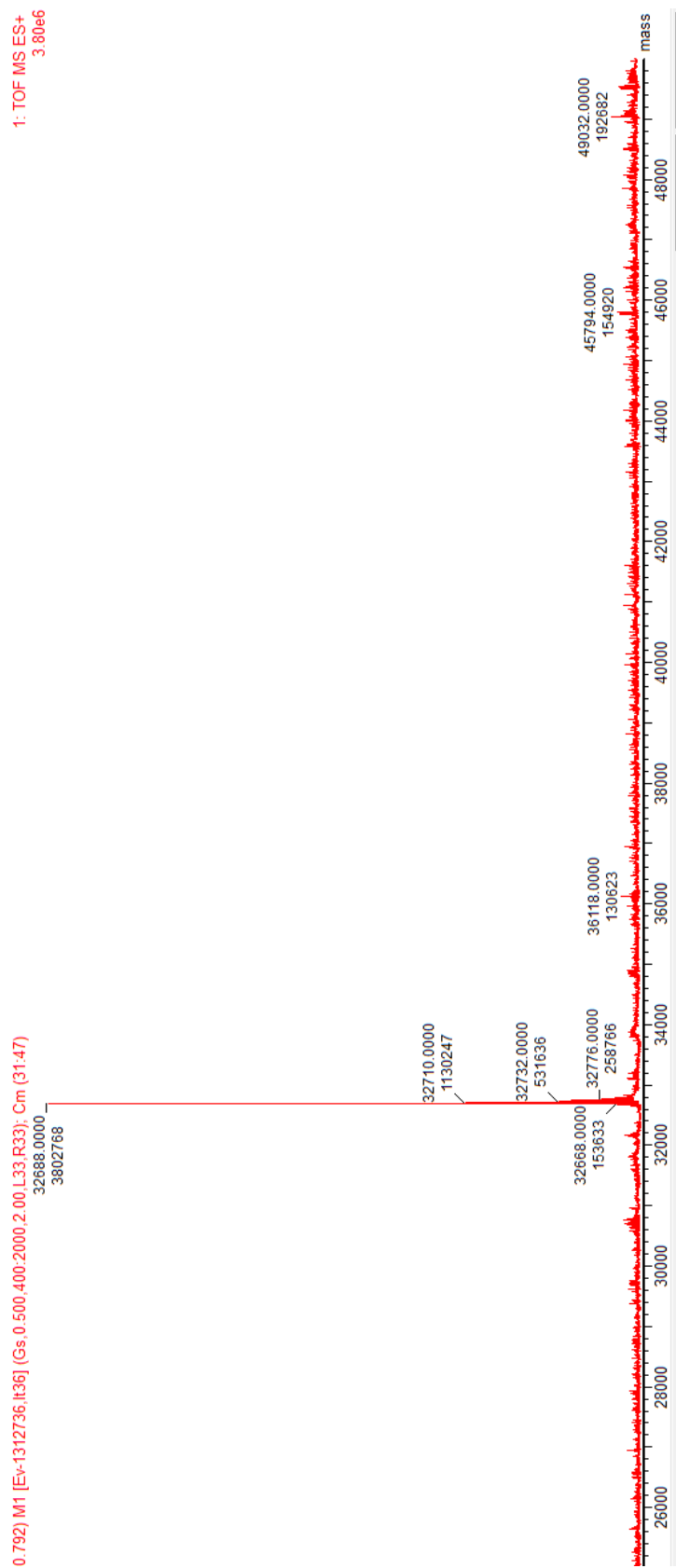


Figure 3.3.3. LC-MS MaxEnt spectrum of 100 nM Src with 5 μ M AMP-CA. Src parent mass is 32688 Da. Crotonylamide expected shift $\Delta 69$, observed no modification from parent.

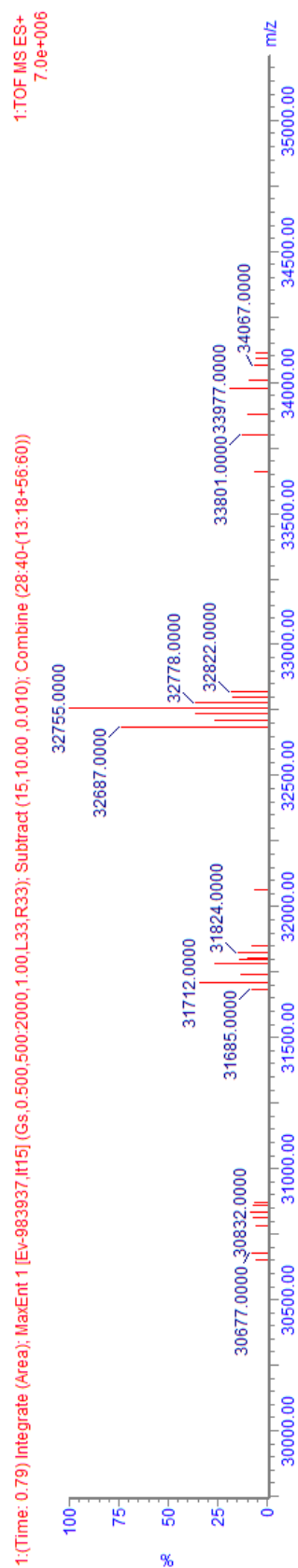


Figure 3.3.4. LC-MS MaxEnt spectrum of 100 nM Src with 5 μ M AMP-CA and 50 μ M Cys-Src tide. After 120 minutes at room temperature we observed some formation of crotonylamide, expected $\Delta 69$, observed $\Delta 68$. Src-CA-Src tide expected shift is $\Delta 1364$. Crosslinking was not observed.

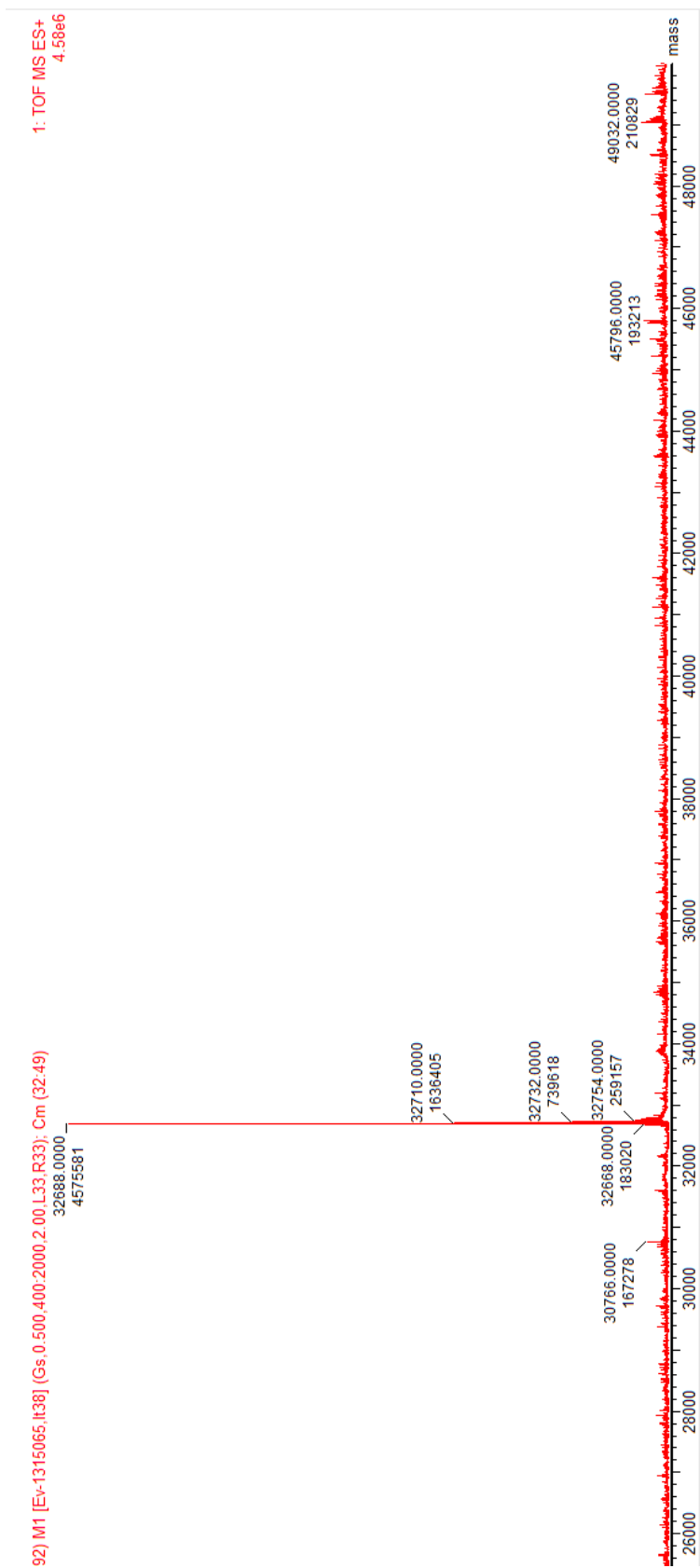


Figure 3.3.5. LC-MS MaxEnt spectrum of 100 nM Src with 5 μ M AMP-MA. Src parent mass is 32688 Da. Methacrylamide expected shift $\Delta 69$; we observed no modification from parent.

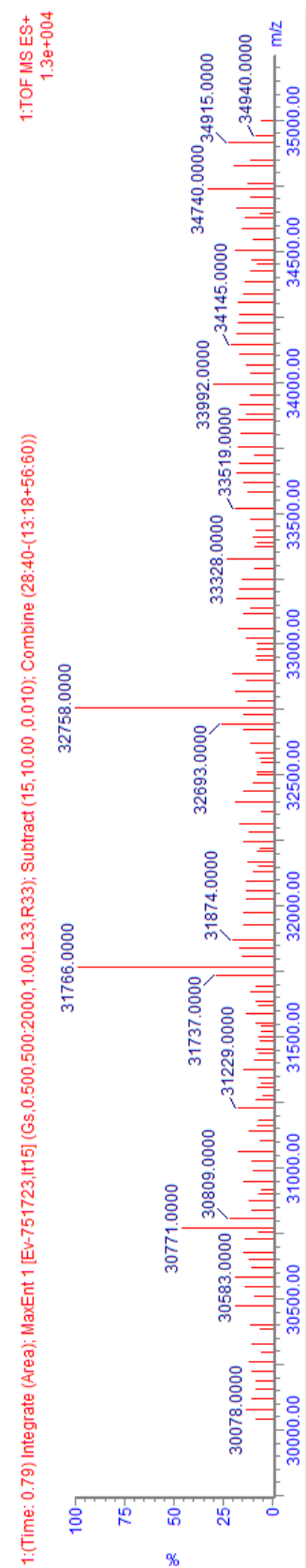


Figure 3.3.6. LC-MS MaxEnt spectrum of 100 nM Src with 5 μ M AMP-MA and 50 μ M Cys-Srcide. After 120 minutes at room temperature we observed some protein decomposition to lower masses as well as formation of some methacrylamide, expected $\Delta 68$, observed $\Delta 68$. Src-MA-Srcide expected shift is $\Delta 1364$. Crosslinking was not observed. Formation of a $\Delta 2051$ peak of unknown identity was observed.

3.4 Characterizing the Src and Aurora A labeling sites of ATP-MA and ADP-MA.

Given the differing presentation of the mixed anhydride bond between ATP-MA and ADP-MA but the identical single mass modification predominantly observed by LC-MS, we investigated the identity of the modified Lys to ascertain whether its identity was the same between the two probes. To answer this question, we labeled Src with either ADP-MA or ATP-MA, confirmed single labeling by LC-MS, digested with trypsin and Glu-C, and performed LC-MS/MS analysis looking for the addition of a C_4H_4O adduct on undigested lysines.

We found that both ATP-MA and ADP-MA modify the catalytic Lys 295 (Figure 3.4.1, 3.4.2) as well as the structurally proximal but non-catalytic Lys 423 on the activation loop (Figure 3.4.3). Both labeling sites (Lys 295 and Lys 423) had been previously independently observed in experiments using ATP-biotin.²⁷ We did not observe modifications at non-lysine amino acids under these conditions. Given the absence of supstoichiometric labeling by ADP-MA and ATP-MA by whole protein LC-MS and the similarity of relative MA peptide abundance between ADP-MA and ATP-MA labeled samples, we hypothesized that one site was predominantly labeled and that the second site was likely to be a side product. An alternative hypothesis that remains difficult to rule out is that our Src-MA samples contained a mixed population of singly labeled protein (for example, 50% labeled at Lys 295 and 50% labeled at Lys 423) and that the first labeling event prevents a second labeling event from taking place.

To try to clarify this point, we expressed the Src K295M and K295R mutants and characterized their reactivity with ADP-MA and ATP-MA. We found that K295R was modified by ADP-MA. Unlike K295M, Src K295R-MA crosslinked Srcide (data not shown). We hypothesized that this modification and crosslinking might be mediated through the Arg side

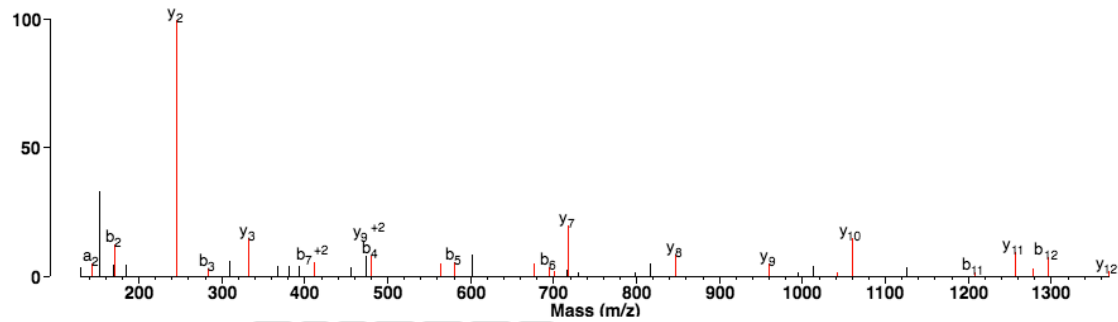
chain. Because this is a non-natural mutation and thus not physiologically relevant, and because we wanted to study protein that could not be modified at this site, we used the K295M mutant for remaining studies as a negative control for electrophile addition to the 295 position in the active site.

We found that K295M labeling was slower than the comparable K295 WT labeling at 50 μ M, that both ADP-MA and ATP-MA could label Src K295M, and that ATP-MA was slightly faster than ADP-MA (Figure 3.4.4). We did not test the K423M mutant, which would be helpful for clarifying these results for future studies.

Because K295M could still be labeled (likely at K423, although we did not evaluate this by LC-MS/MS), albeit at a slower rate than wild-type protein, we cannot rule out the possibility that the dominant site of labeling is K423 or the possibility that both sites are labeled in a mixed population. The K295M mutation could decrease the affinity of Src for ADP-MA and ATP-MA, for example, reducing the rate of MA labeling even if K423-MA was the predominant product. To more fully address this question, the reactivity of ADP-MA and ATP-MA toward the K423M mutant of Src could be tested. Additionally, K295M and K423M and the K295M/K423M double mutant could be incubated with ADP-MA and ATP-MA before digestion and MS/MS to examine the pattern of specific fragment labeling.

We additionally confirmed that ADP-MA is able to modify an unrelated protein kinase, Aurora A, and confirmed labeling of the catalytic Lys as well as two other Lys by trypsinization followed by LC-MS/MS analysis. We found that both ADP-MA and ATP-MA modify Aurora A at Lys 143, the catalytic Lys 162, and Lys 227 (ADP-MA labeled K143MA, K162MA, K227MA correspond to Figures 3.4.5, 3.4.6, and 3.4.7 respectively). (ATP-MA labeled K142MA, K162MA, and K227MA correspond to Figures 3.4.8, 3.4.9, and 3.4.10 respectively).

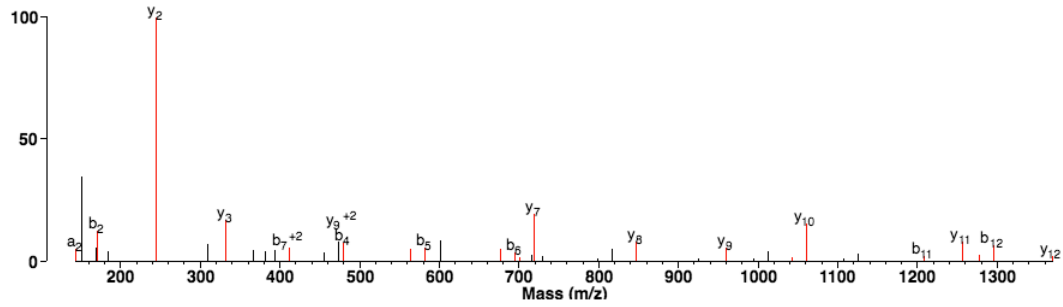
VAIK(MA)TLKPGTMSPE⁺²



b	b ⁺²			y	y ⁺²
---	---	1	V	14	---
171.1128	---	2	A	13	1440.7767 720.8920
284.1969	---	3	I	12	1369.7396 685.3734
480.3180	240.6627	4	K(MA)	11	1256.6555 628.8314
581.3657	291.1865	5	T	10	1060.5343 530.7708
694.4498	347.7285	6	L	9	959.4866 480.2470
822.5448	411.7760	7	K	8	846.4026 423.7049
919.5975	460.3024	8	P	7	718.3076 ---
976.6190	488.8131	9	G	6	621.2549 ---
1077.6667	539.3370	10	T	5	564.2334 ---
1208.7071	604.8572	11	M	4	463.1857 ---
1295.7392	648.3732	12	S	3	332.1452 ---
1392.7919	696.8996	13	P	2	245.1132 ---
---	---	14	E	1	148.0604 ---

Figure 3.4.1. ADP-MA modifies the Src catalytic Lys 295. Detected masses are annotated in red text and +2 masses are annotated with grey highlighting.

VAIK(MA)TLKPGTMSPE+2



b	b+2			y	y+2
---	---	1	V	14	---
171.1128	---	2	A	13	1440.7767 720.8920
284.1969	---	3	I	12	1369.7396 685.3734
480.3180	240.6627	4	K(MA)	11	1256.6555 628.8314
581.3657	291.1865	5	T	10	1060.5343 530.7708
694.4498	347.7285	6	L	9	959.4866 480.2470
822.5448	411.7760	7	K	8	846.4026 423.7049
919.5975	460.3024	8	P	7	718.3076 ---
976.6190	488.8131	9	G	6	621.2549 ---
1077.6667	539.3370	10	T	5	564.2334 ---
1208.7071	604.8572	11	M	4	463.1857 ---
1295.7392	648.3732	12	S	3	332.1452 ---
1392.7919	696.8996	13	P	2	245.1132 ---
---	---	14	E	1	148.0604 ---

Figure 3.4.2. ATP-MA modifies the Src catalytic Lys 295.

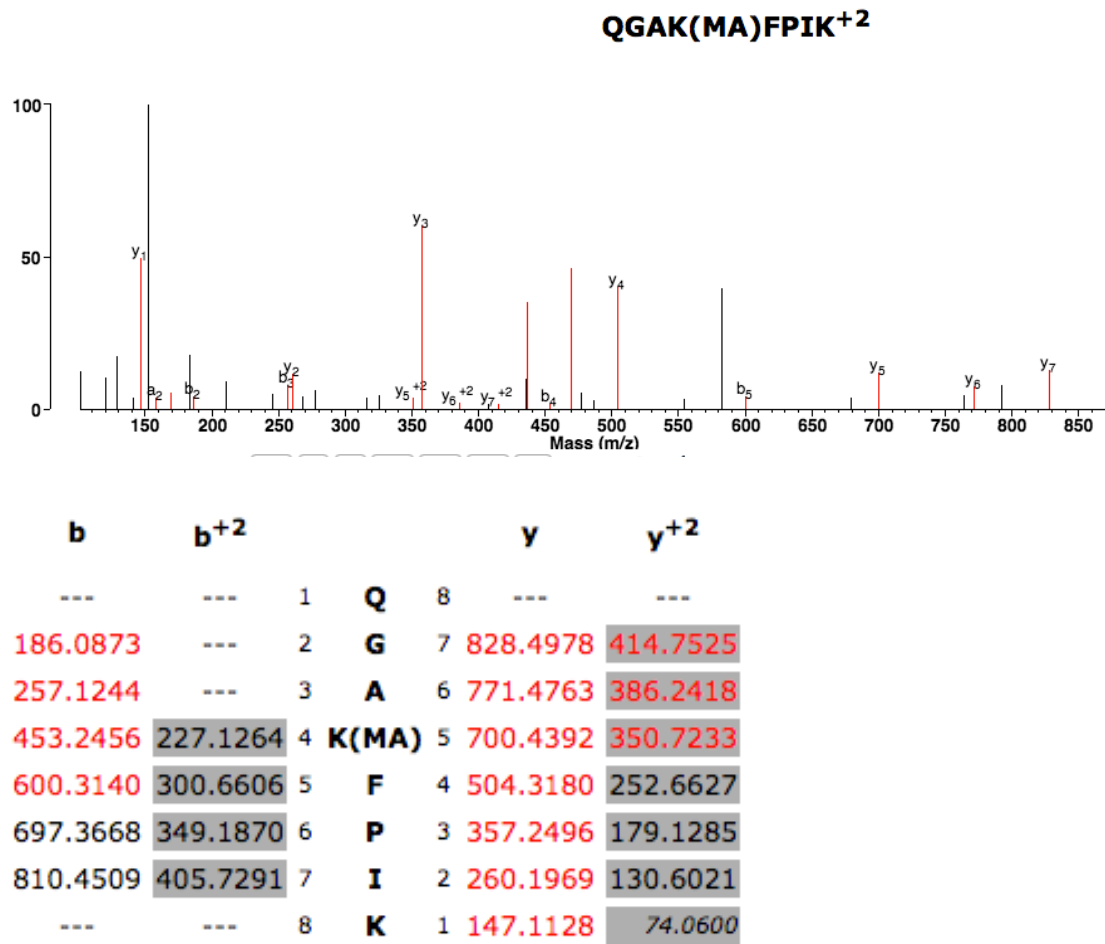


Figure 3.4.3. ATP-MA additionally modifies the Src activation loop Lys 423.

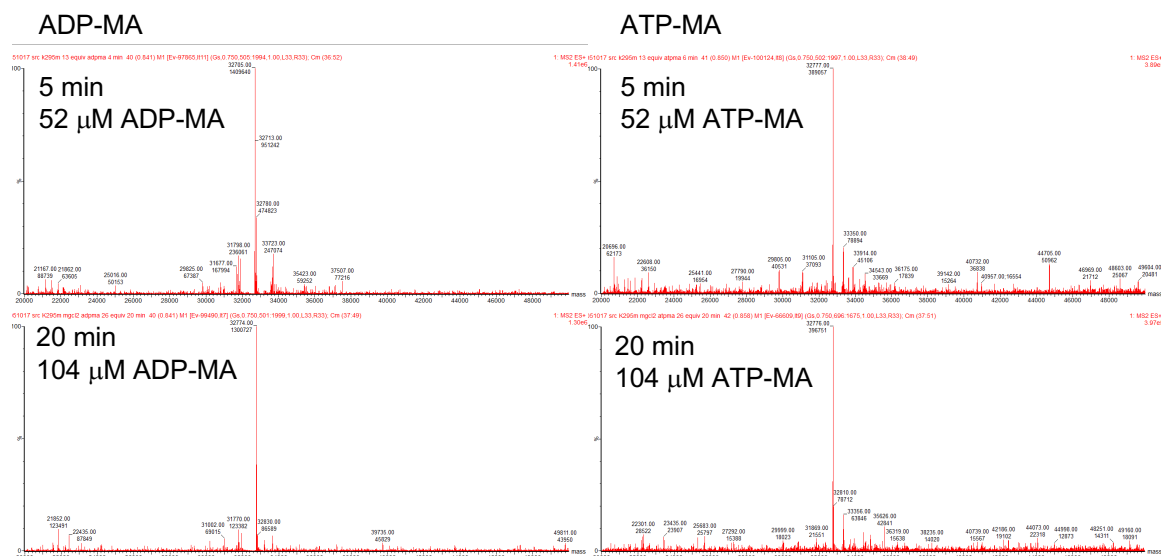


Figure 3.4.4. ADP-MA (left) versus ATP-MA (right) modification of 4 μ M Src K295M at 4 °C. Both probes label Src K295M. At ~5 minutes with 13 equivalents crosslinker, ADP-MA has labeled <30% K295, and ATP-MA has fully labeled K295. At 20 minutes with 26 equivalents crosslinker, both are fully labeled.

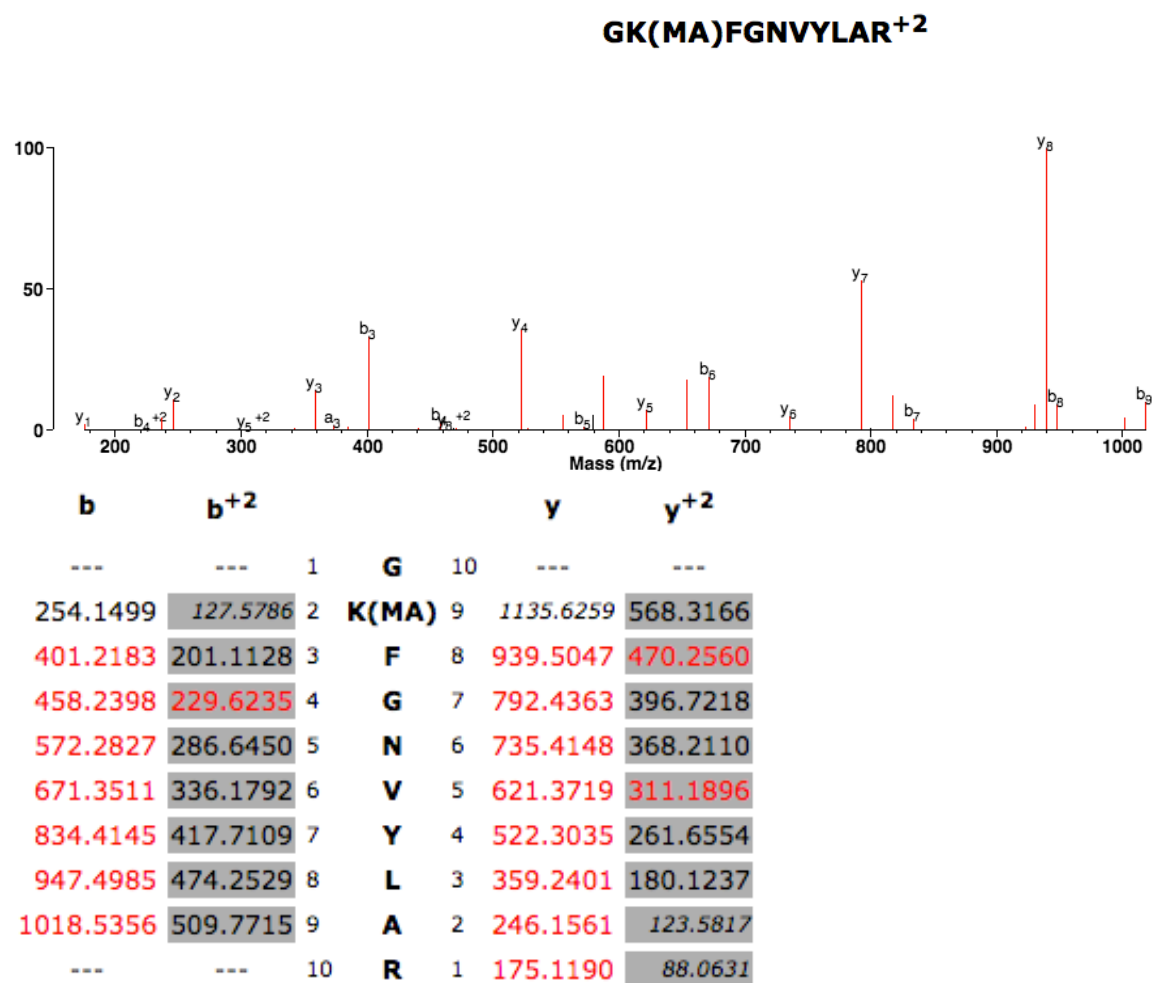


Figure 3.4.5. ADP-MA modifies the Aurora A Lys 143.

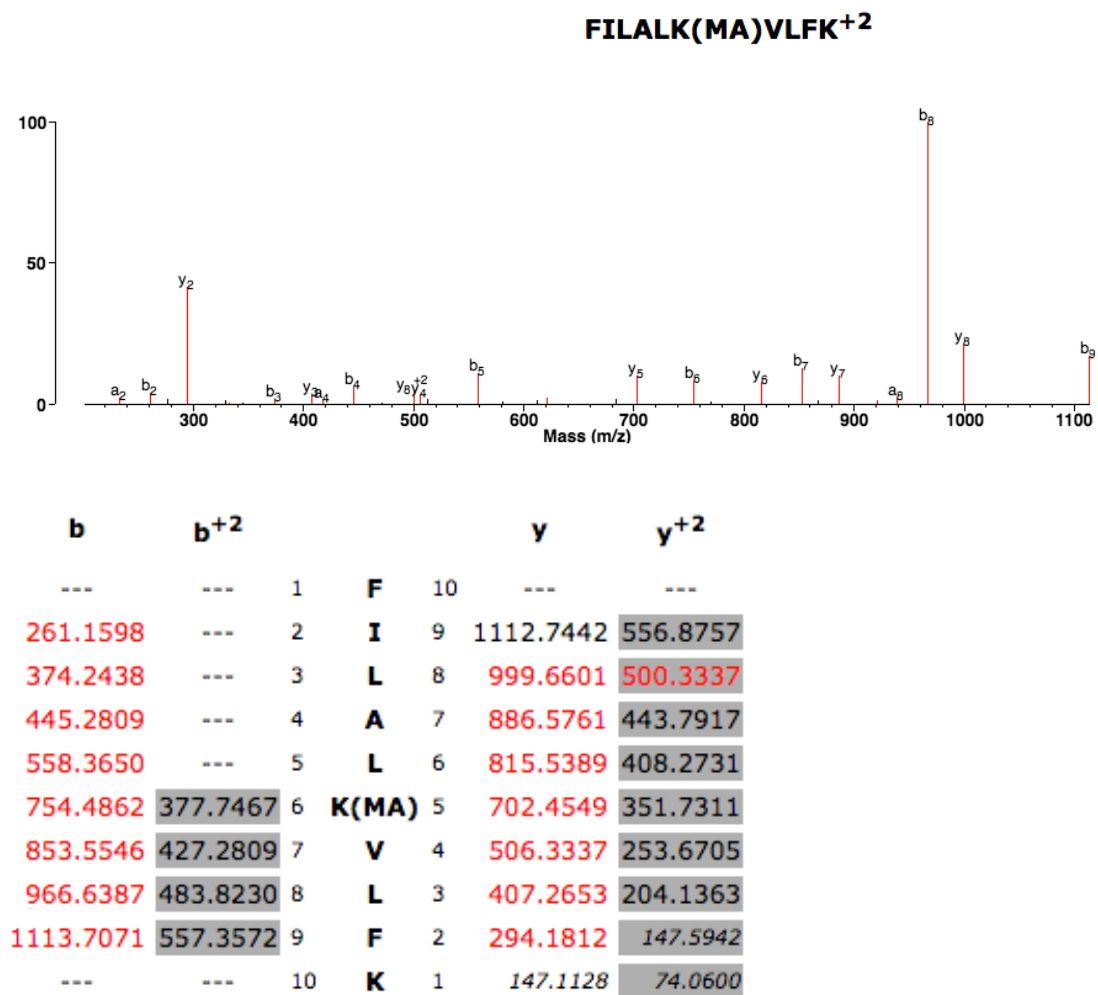


Figure 3.4.6. ADP-MA modifies the Aurora A catalytic Lys 162.

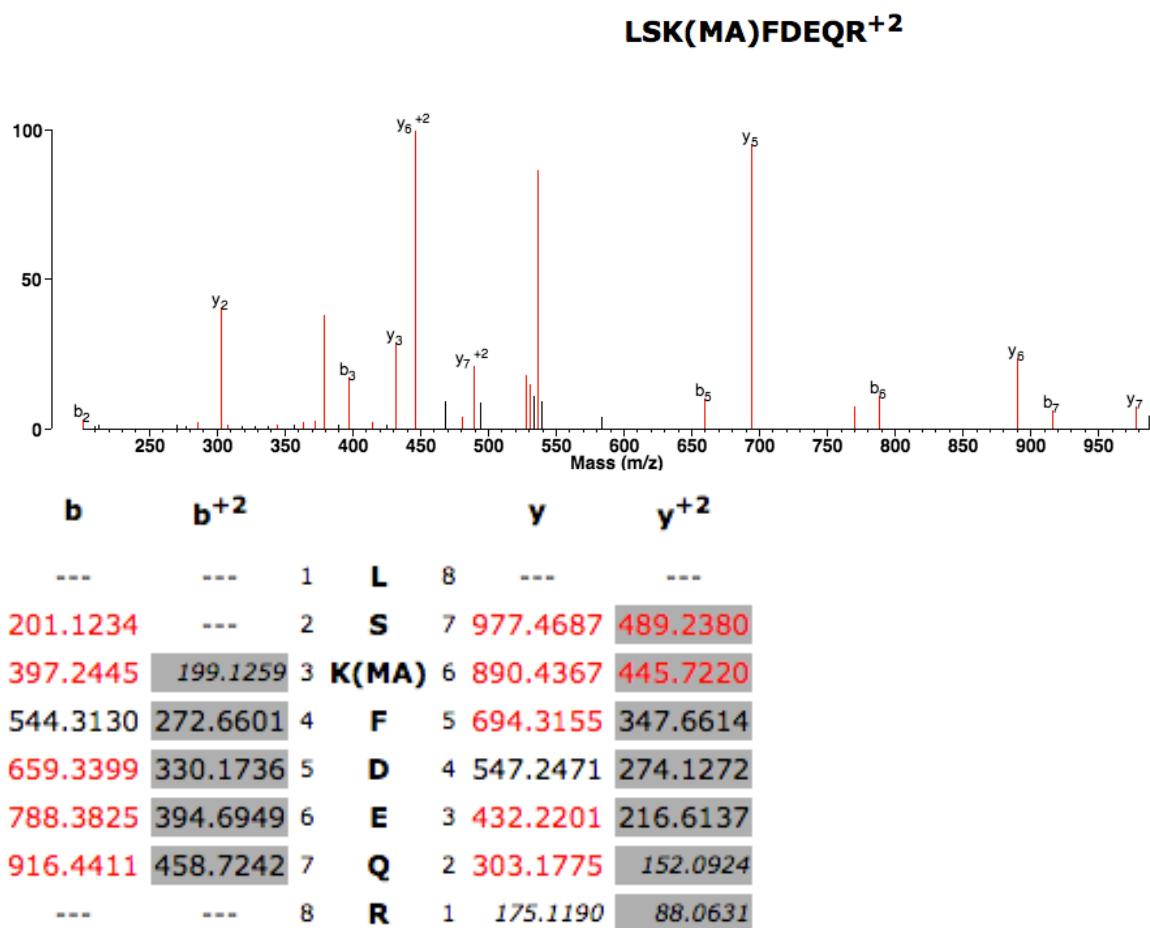
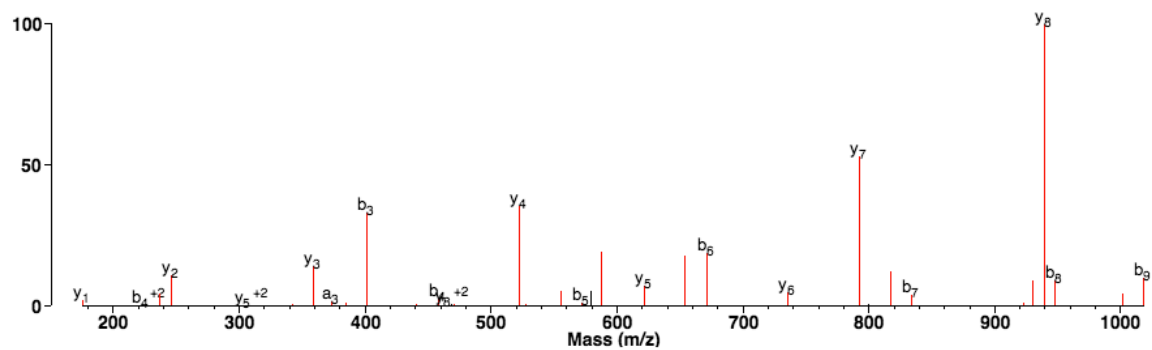


Figure 3.4.7. ADP-MA modifies the Aurora A Lys 227.

GK(MA)FGNVYLAR⁺²



b	b+2			y	y+2
---	---	1	G	10	---
254.1499	127.5786	2	K(MA)	9	568.3166
401.2183	201.1128	3	F	8	939.5047
458.2398	229.6235	4	G	7	792.4363
572.2827	286.6450	5	N	6	735.4148
671.3511	336.1792	6	V	5	621.3719
834.4145	417.7109	7	Y	4	522.3035
947.4985	474.2529	8	L	3	359.2401
1018.5356	509.7715	9	A	2	246.1561
---	---	10	R	1	175.1190

Figure 3.4.8. ATP-MA modifies the Aurora A Lys 143.

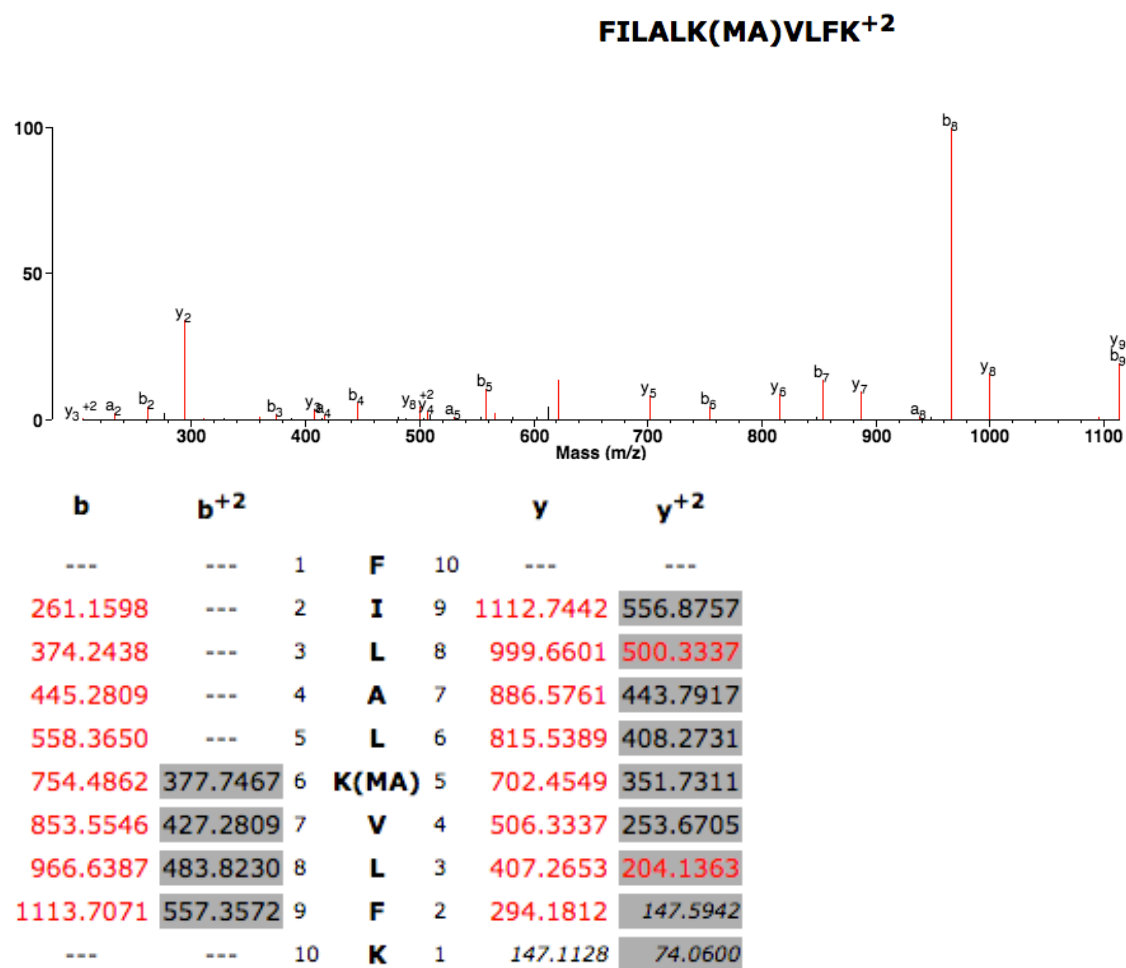


Figure 3.4.9. ATP-MA modifies the Aurora A catalytic Lys 162.

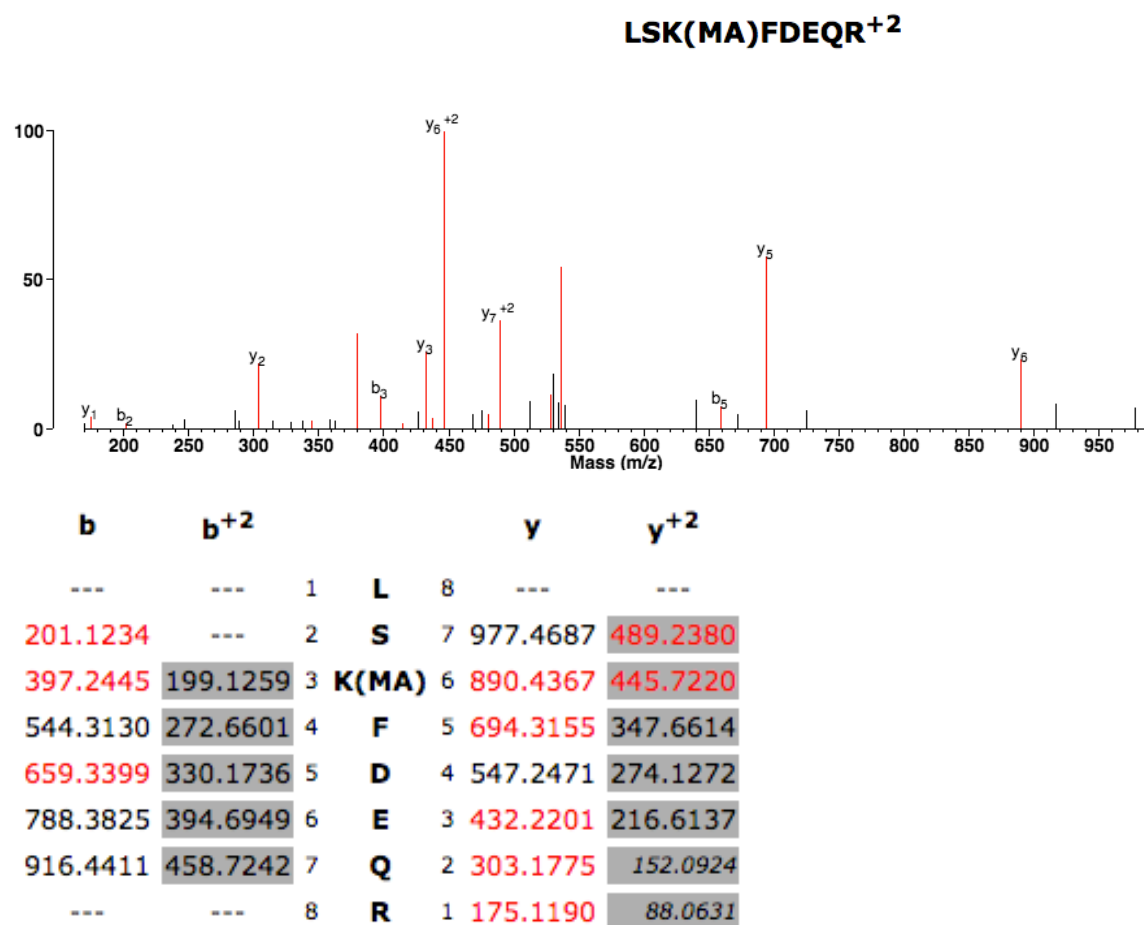


Figure 3.4.10. ATP-MA modifies the Aurora A Lys 227.

Chapter 4.

ADP-MA mediates substrate-selective crosslinking and protein crosslinking *in vitro* and in lysate.

4.1 ADP-MA mediates crosslinking in a kinase-protein substrate system.

Despite the development of many crosslinking probes, to our knowledge the specific crosslinking of kinase to its protein substrate has not previously been demonstrated. Crosslinking to protein rather than peptide is significantly more challenging from a protein engineering perspective. Engineered peptide substrates can be synthesized with a variety of reactive non-native and/or unnatural amino acids in the phosphorylation site and tested in very high concentration relative to protein substrate. The sequence of peptide substrates can exclude reactive amino acid residues that may decorate the surfaces of protein substrates and interfere with crosslinking. Finally, the interaction of kinases with their protein substrates can be regulated by interactions with secondary structures distant from the phosphorylation site or by interaction with scaffolding proteins, necessitating the optimization of interaction conditions.³⁴ Due to these challenges, the development of a tool for kinase-protein crosslinking could assist structural efforts to study the kinase-substrate complex and cell biology efforts to map kinase-substrate signaling networks. To determine if kinase-protein crosslinking was feasible using ADP-MA, we examined the Src substrate Cortactin.

Cortactin is a scaffolding protein that plays a significant role in cellular migration and invasion.^{35,36} Cortactin is a multidomain protein with an N-terminal acidic domain that interacts with Arp2/3³⁷ (NTA), six and a half repeats of a 37 amino acid sequence called Cortactin domains which interact with F-actin, a proline rich domain (PRD), and a Src homology 3 (SH3)

domain which mediates interactions with cytoskeletal proteins including N-WASP, WIP, and others (Figure 4.1.1).^{38,39} The proline-rich domain is phosphorylated by Tyr as well as Ser/Thr kinases.⁴⁰

Phosphorylation positively and negatively regulates Cortactin interactions with its effectors. Cortactin has at least four Src phosphorylation sites towards the C-terminal end of the PRD just before the SH3 domain, Y421, Y466, Y486, and Y489,⁴⁰ and these sites are phosphorylated processively, with Y421 thought to act as a priming phosphorylation for the other sites. When phosphorylated by Src, Cortactin promotes actin polymerization by recruiting Arp2/3 complex proteins to the filament. Cortactin also contains three native Cys residues, two of which are in the Cortactin domains and one of which is in the SH3 domain.

We transiently expressed FLAG-tagged Cortactin mutants in which the native Cys were mutated to Ser (Cyslite) and the phosphorylation sites Y421, Y466, Y486, and Y489 were each mutated to Cys. We found that ADP-MA could mediate Src crosslinking to each of these mutants *in vitro*, observed by western blot (Figure 4.1.1). Importantly, we saw that the crosslinking to the phosphorylation site mutants was more robust than to the native Cys present in wild-type Cortactin, suggesting that substrate specificity is maintained in this system (Figure 4.1.2). We also found that crosslinking could take place in the presence of cellular lysate (Figure 4.1.3).

We expressed FLAG-tagged Cortactin mutants in which the native Cys were mutated to Ser (Cyslite) and the phosphorylation sites Y421, Y466, Y486, and Y489 were each mutated to Cys. ADP-MA mediated Src crosslinking to each of these mutants *in vitro*, observed by western blot (Figure 4.1.2). Crosslinking to the phosphorylation site mutants was more robust than to the native Cys present in wild-type Cortactin, suggesting that substrate specificity is maintained in this system (Figure 4.1.3). We also found that crosslinking could take place in the presence of cellular lysate (Figure 4.1.4). Many of the previous crosslinkers (Figure 1.2.1-1.2.5)

demonstrated reduced or low efficiency in lysate due to off-target interactions with lysate components, so the increased reaction efficiency of our system also represented a significant improvement.

We found that the crosslinking reaction is reversible with heat, which could potentially interfere with detection by western blot (Figure 4.1.5). There is literature precedent for a retro-Michael addition resulting in a reversal of the crosslink with heat.⁴¹ We furthermore had added a competing nucleophile in excess, as is the case when gel electrophoresis samples are boiled in the presence of DTT or BME, and we considered the possibility of competition (Figure 4.1.6). Given these results, in order to best visualize crosslinking by gel shift, all samples made after this discovery were handled without heating and 1 μ L of 1 M BME was added to samples immediately before samples were loaded and run on denaturing gel.

We observed that both ADP-MA and ATP-MA are able to mediate Src crosslinking to protein substrate Cortactin. However, we initially observed that ADP-MA-mediated crosslinking was more efficient than ATP-MA-mediated crosslinking *in vitro* with protein substrates (Figure 4.1.7). In light of our observation that both ADP-MA and ATP-MA modify the same lysine and form the same methacrylamide electrophile, MA, and in the context of kinetics studies demonstrating that Src labeling by ATP-MA is slightly faster than ADP-MA (Figure 2.1.4), we considered several possibilities to explain this observation, ultimately demonstrating that nucleotide or nucleotide-MA binding in the active site affects crosslinking of Src-MA to its protein substrate.

To evaluate whether the presence of nucleotide or nucleotide-MA affects the second step of crosslinking, we first labeled Src with ADP-MA or ATP-MA, then incubated the prelabeled Src-MA/ADP-MA or ATP-MA mixture with Y421C Cortactin in the presence or absence of ADP, ATP, ADP-MA, and ATP-MA. We found that nucleotide and nucleotide-crosslinker

inhibited the crosslinking step, in the following order of most to least inhibition: ATP-MA, ATP, and ADP, with ADP-MA being least inhibitory (4.1.8). We concluded that the less efficient crosslinking by ATP-MA compared to ADP-MA *in vitro* was due to greater inhibition by the ATP generated by ATP-MA reaction with kinase as well as and inhibition by unreacted ATP-MA. Consistent with the hypothesis that nucleotide binding to the active site inhibits crosslinking, EDTA addition to the Src-MA reaction mixture improved crosslinking to Y421C Cortactin (4.1.9). However, we also found that EDTA interferes with the first step of the reaction, the formation of the acrylamide (4.1.10). In summary, these findings suggest that modified Src-MA can bind nucleotide or nucleotide probe and that the substrate-bound protein exhibits a lower crosslinking reaction efficiency to Cys-mutant protein substrate, accounting for our observation that ADP-MA crosslinks more efficiently than ATP-MA *in vitro*.

Subsequently, we were interested to see if nucleotide or nucleotide-MA would affect crosslinking in desalted versus unmodified cellular lysates. Given that cellular lysate contains many nucleotide binding proteins and ATPases, we considered the possibility that ATP-MA and the ATP generated by ATP-MA labeling of Src would be sequestered in other binding sites in cellular lysate. Indeed, we found that ADP-MA and ATP-MA mediate similar levels of crosslinking between Src and Y421C Cortactin in lysate at low and moderate levels, but that ATP-MA, but not ADP-MA, is inhibitory at millimolar concentration (Figure 4.1.11). We additionally found that crosslinking in lysate was not improved or significantly diminished by the addition of EDTA, and that crosslinking was competent in unmodified as well as desalted lysates (Figure 4.1.12). We chose to desalt lysates in further experiments for consistency and because slight improvement in crosslinking was observed in desalted lysate, with the knowledge that this step may not be necessary.

We disproved the alternate hypothesis that Src was capable of using ATP-MA, but not ADP-MA, as a phosphate donor for modification of the wild-type Src phosphorylation sites in Cortactin. We incubated Src with or without wild-type Y-Srctide and added either ATP-MA or ADP-MA before measuring the formation of Src-MA by LC-MS. If Src was capable of using ATP-MA as a substrate, we expected to observe that the formation of Src-MA was inhibited by the competing reaction of MA-phosphorylation of Y-Srctide. Contrary to this hypothesis, the presence of Y-Srctide did not inhibit Src-MA formation for ATP-MA over ADP-MA, and no evidence of the MA-phosphorylated Srctide was detected by LC-MS (Figure 4.1.13). To further disprove this hypothesis, we incubated Src with ADP-MA and ATP-MA *in vitro* until it was fully labeled by LC-MS, and then added the prelabeled Src-MA with ADP-MA or Src-MA with ATP-MA to Y421C Cortactin. If this hypothesis were correct, we would have expected to observe no difference in crosslinking between ADP-MA and ATP-MA prelabeled Src-MA; however, the difference persisted (Figure 4.1.14).

In summary, we observed for the first time that ADP-MA and ATP-MA could mediate specific crosslinking between Src kinase and its protein substrate Cortactin. We found that the crosslinking reaction exhibited preference for introduced Cys at the Src phosphorylation sites over native Cys. We further characterized that formation of the crosslinked product was reversible with heat and that crosslinking was inhibited in the presence of free nucleotide and free ATP-MA but enhanced in the presence of EDTA *in vitro*. Finally, we found that high concentration of ADP-MA and ATP-MA mediate similar levels of Src-Y421C Cortactin crosslinking in cellular lysate.

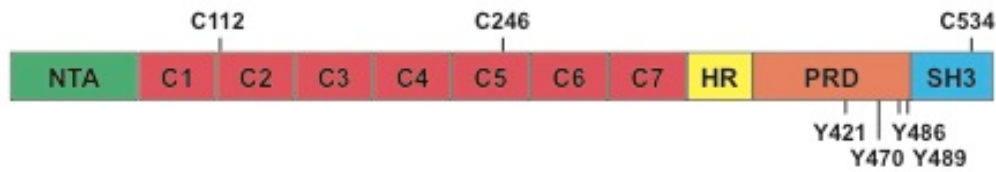


Figure 4.1.1. Cortactin is a monomeric multidomain protein that is phosphorylated by Src towards the end of its proline-rich domain. The N-terminal acidic domain mediates interactions with Arp2/3, C1-C7 are unstructured in solution and mediate interaction with F-actin. The proline-rich domain is phosphorylated by Tyr as well as Ser/Thr kinases. Phosphorylation positively and negatively regulates cortactin interactions with its effectors. The SH3 domain mediates interactions with cytoskeletal proteins including N-WASP, WIP, and others. NTA= N-terminal acidic domain, C1-C7= Cortactin repeats, HR= helical region, PRD= Proline rich domain, SH3= Src homology 3 domain.

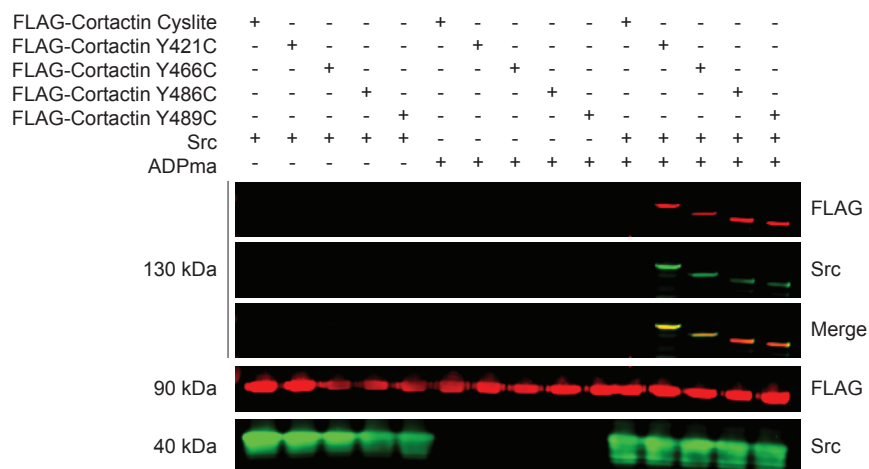


Figure 4.1.2. ADP-MA mediates *in vitro* crosslinking to purified Cortactin phosphorylation site Cys mutants in a Cyslite construct. Conditions for crosslinking were: Src 1.2 μ M, Cortactin 3 μ g (1.8 μ M), ADP-MA 6 μ M, 16 h, 4 $^{\circ}$ C in HEPES 50 mM pH 7.4, NaCl 150 mM, 3XFLAGtide.

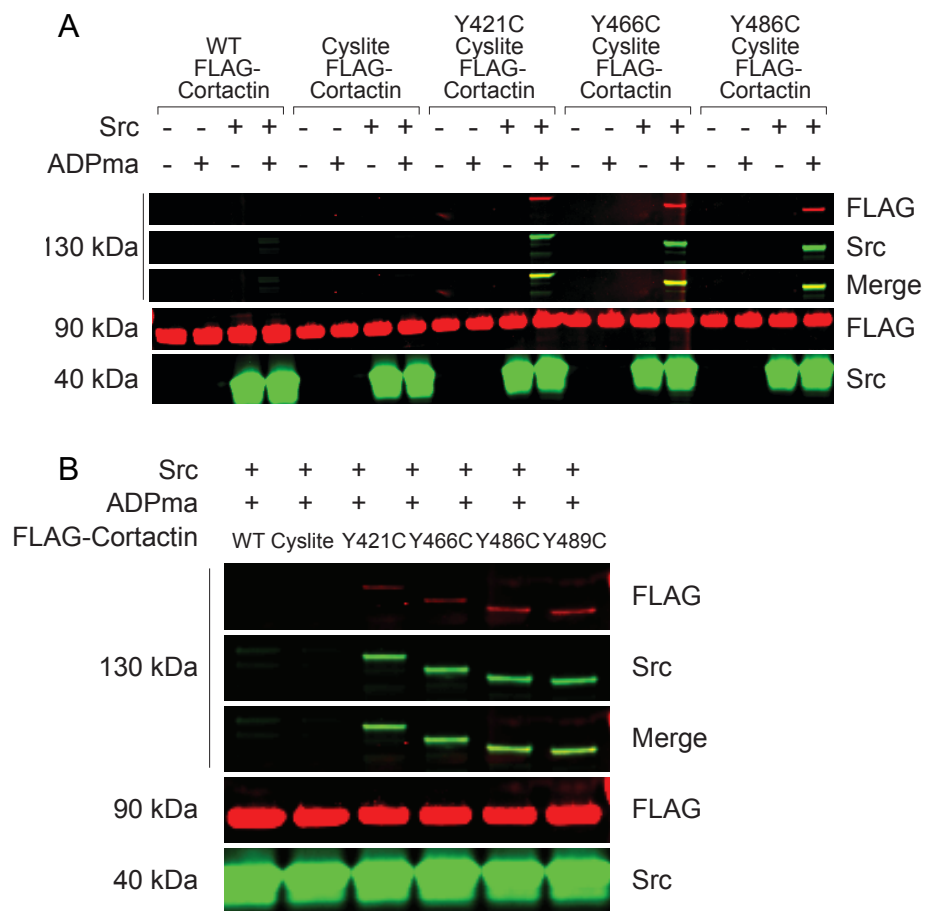


Figure 4.1.3. A. ADP-MA mediates *in vitro* crosslinking to purified Cortactin phosphorylation site Cys mutants preferentially over the three native Cys in Cortactin. B. Crosslinking at different sites migrates differently but reproducibly. Conditions for crosslinking in both A and B were: Src 2 μ M, Cortactin 1.5 μ M, ADP-MA 16 μ M, 16 h, 4 $^{\circ}$ C in HEPES 50 mM pH 7.4, NaCl 150 mM, 3XFLAGtide.

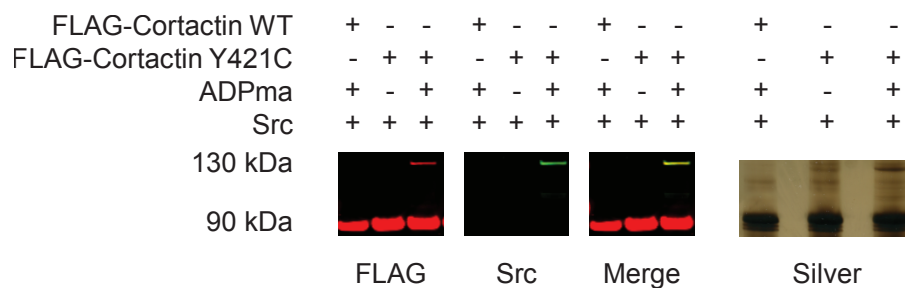


Figure 4.1.4. ADP-MA mediated crosslinking took place in desalted whole cell lysate in which Y421C Cortactin was transiently transfected and 2 μ M Src was supplemented, following by immunoprecipitation for FLAG-Cortactin. Conditions for crosslinking were: Src 2 μ M, ADP-MA 7.5 μ M, 22 h, 4 $^{\circ}$ C in HEPES 25 mM pH 7.4, NaCl 150 mM, MgCl₂ 1 mM.

All lanes have FLAG-Y421C Cortactin, ADPma, Src

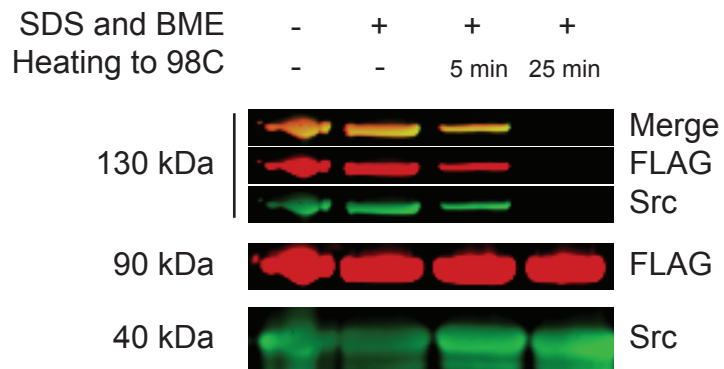


Figure 4.1.5. ADP-MA mediated crosslinking is reversible with prolonged boiling. Conditions for crosslinking were: Src 4.5 μ M, ADP-MA 10 μ M, 16 h, 4 $^{\circ}$ C in HEPES 25 mM pH 7.4, NaCl 150 mM, MgCl₂ 1 mM, BME: 1.43 M. Heating was to 98C for 5 min and 25 min.

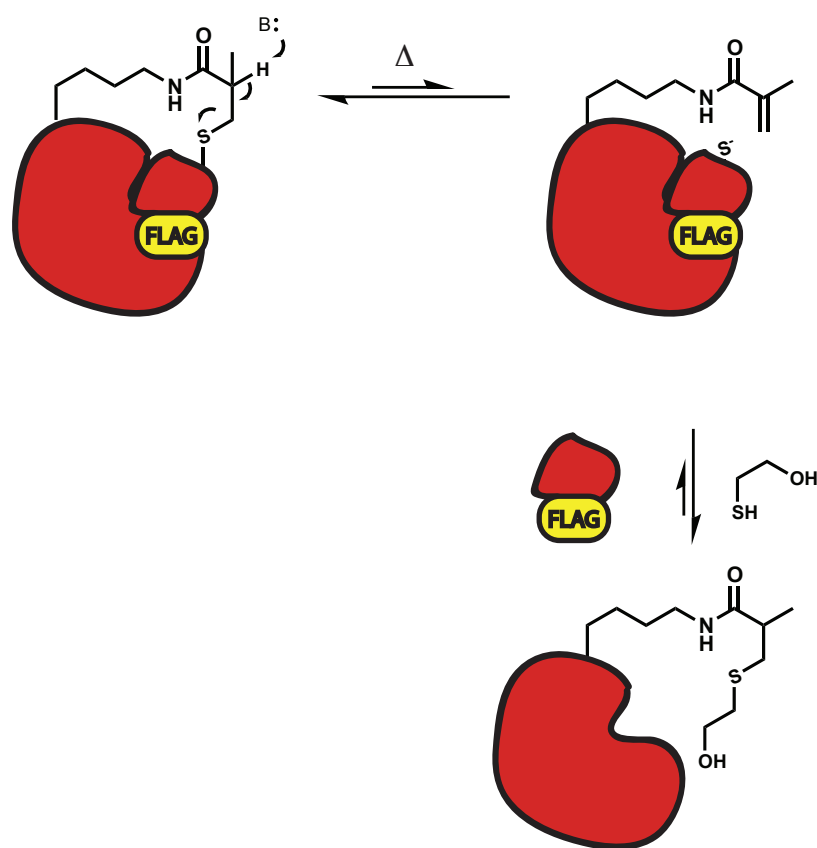


Figure 4.1.6. Proposed mechanism of reversal of ADP-MA mediated crosslinking.

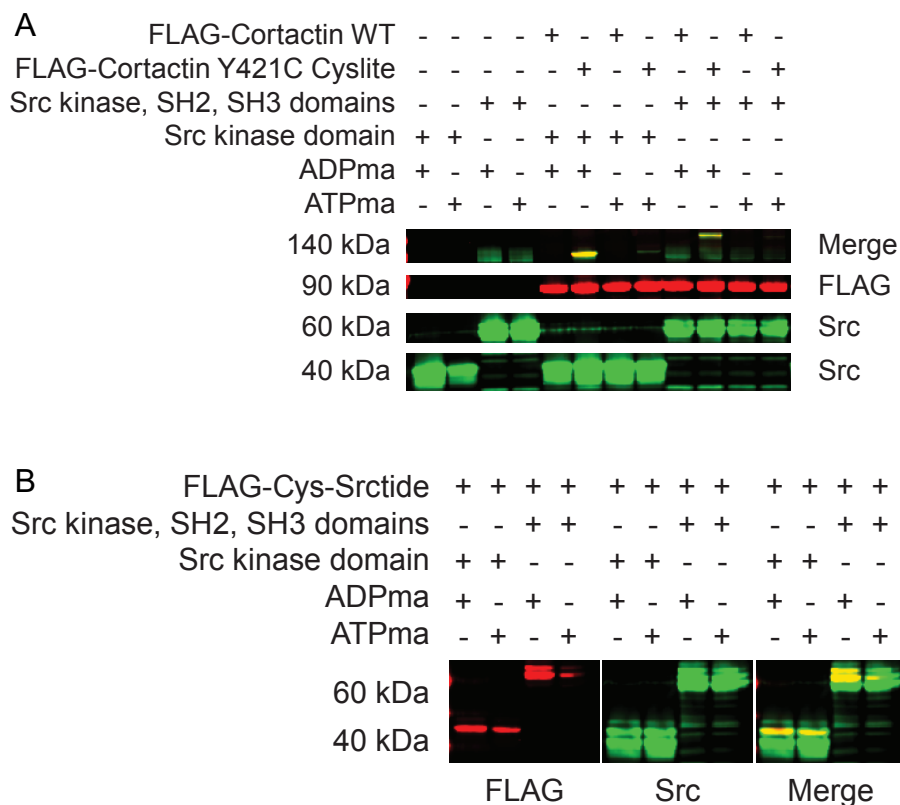


Figure 4.1.7. A. ADP-MA mediates *in vitro* crosslinking between Src and Cortactin phosphorylation site Cys mutants better than ATP-MA with both kinase domain and full length Src kinase. B. ADP-MA and ATP-MA mediate similar levels of *in vitro* crosslinking between Src kinase domain and Srctide. Conditions for crosslinking in both A and B were: Src 3.5 μ M, Cortactin 3.5 μ g/2 μ M (in panel A only), 75 μ M FLAG-Srctide (in panel B only), ADP-MA/ATP-MA 500 μ M, 16 h, 4 $^{\circ}$ C in HEPES 25 mM pH 7.4, NaCl 150 mM, MgCl₂ 1 mM.

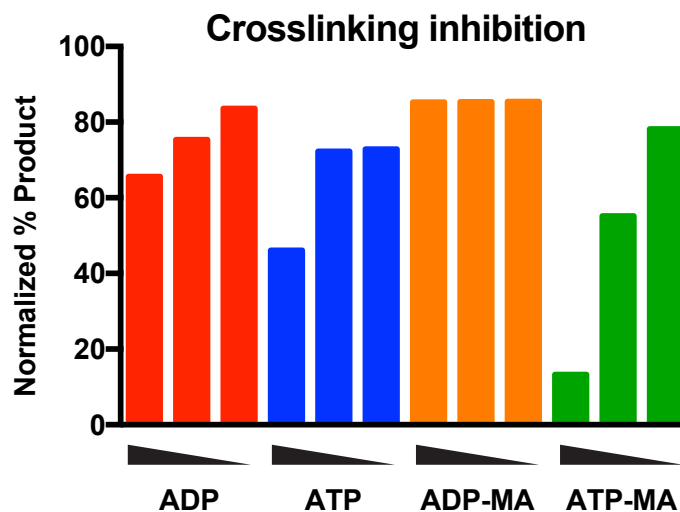
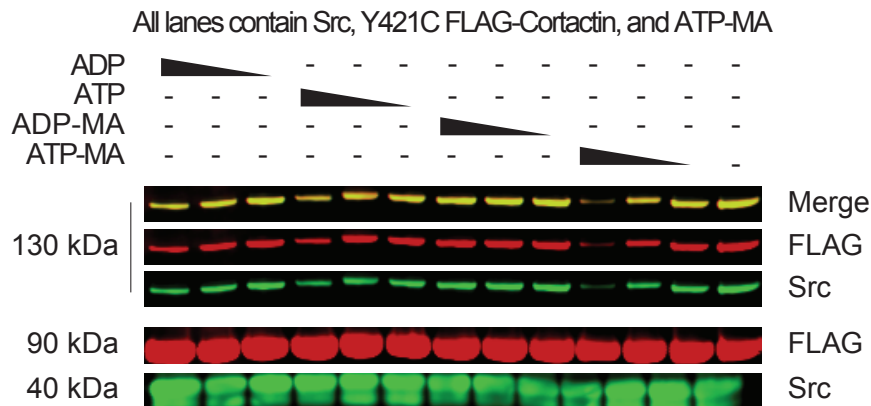


Figure 4.1.8. A. When Src was prelabeled with ADP-MA or ATP-MA and incubated with Y421C Cortactin *in vitro*, ADP, ATP, and ATP-MA interfered with crosslinking. B. ImageJ was used to quantify panel A, and values were normalized to no additive control. Conditions for crosslinking were: pre-labeled: (Src 3 μ M, ADP-MA/ATP-MA ** μ M), Cortactin 1 μ M, 18 h, 4 $^{\circ}$ C in HEPES 25 mM pH 7.4, NaCl 150 mM, MgCl₂ 1 mM, with the addition of a gradient of 2.5 mM, 250 μ M, and 25 μ M ADP-MA, ATP-MA, ADP or ATP.

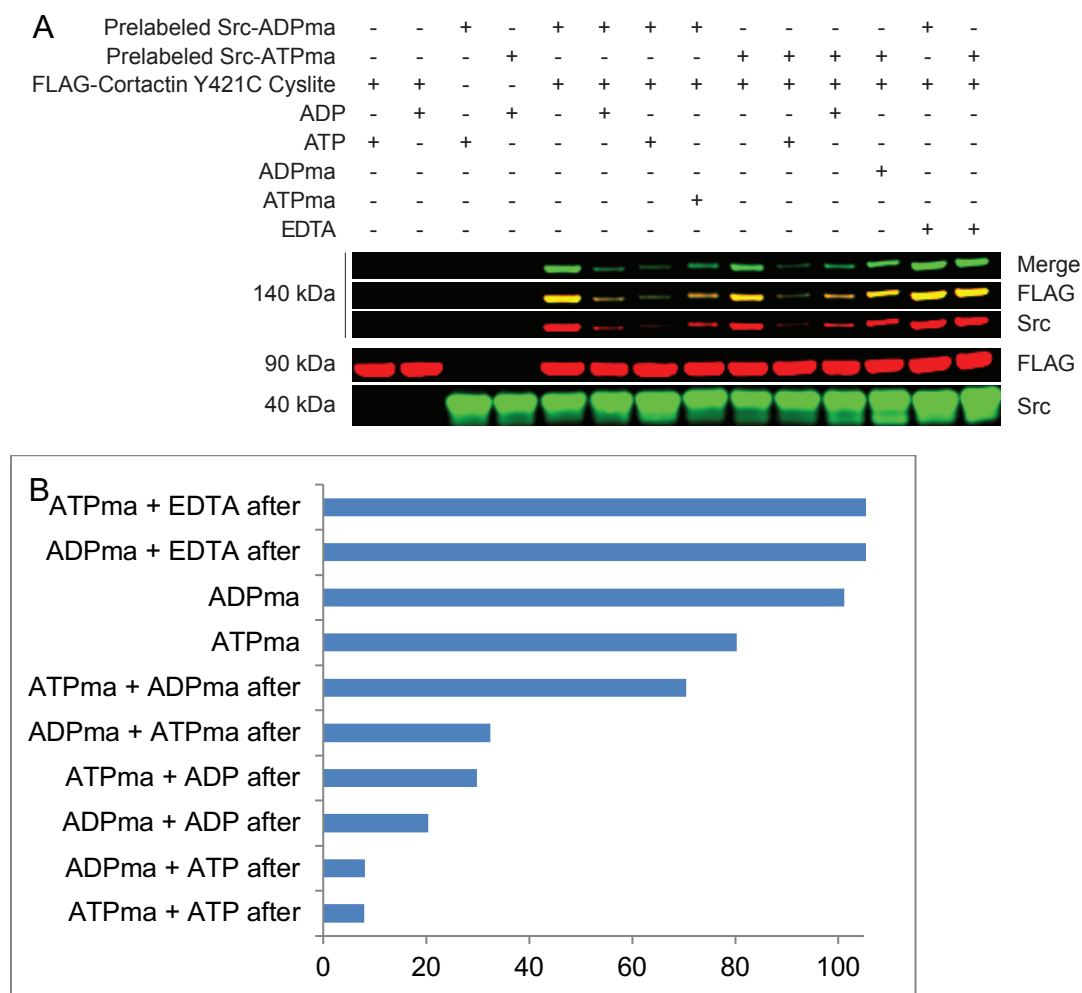


Figure 4.1.9. A. When Src was prelabeled with ADP-MA or ATP-MA and incubated with Y421C Cortactin *in vitro*, EDTA improved prelabeled Src-MA crosslinking. B. ImageJ was used to quantify panel A, and values were normalized to Cortactin loading. Conditions for crosslinking were: prelabeled: (Src 3 μ M, ADP-MA/ATP-MA 50 μ M), Cortactin 1 μ M, 18 h, 4 $^{\circ}$ C in HEPES 25 mM pH 7.4, NaCl 150 mM, MgCl₂ 1 mM, with the addition of 250 μ M ADP-MA or ATP-MA or 5 mM ADP or ATP or 10 mM EDTA.

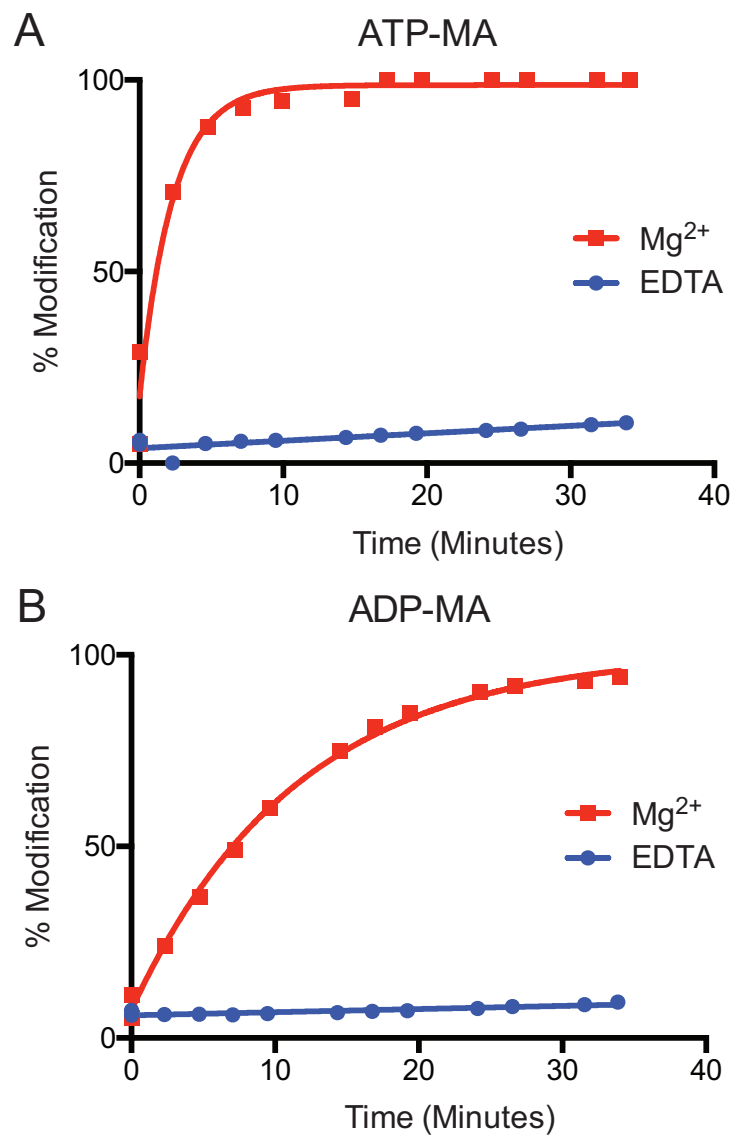


Figure 4.1.10. A. LC-MS analysis of Src-MA formation in the presence of 5 mM Mg²⁺ or 5 mM EDTA with 100 nM Src and 25 μ M (A) ATP-MA and (B) ADP-MA in HEPES buffered saline.

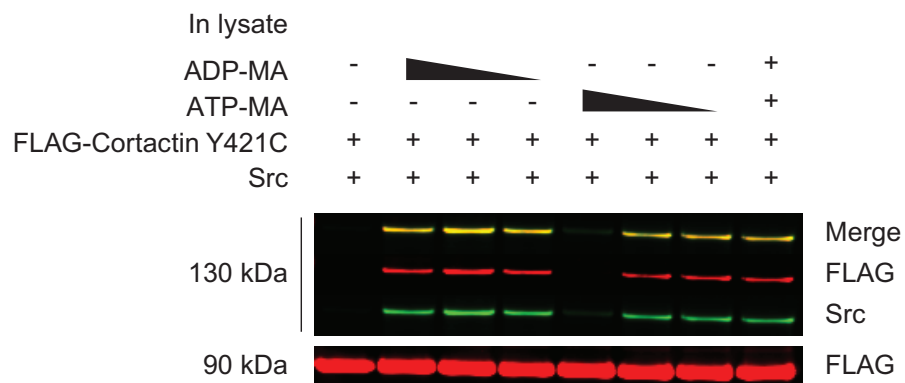


Figure 4.1.11. Crosslinking of 2 μ M Src and transiently transfected FLAG-Cortactin Y421C in lysate mediated by ADP-MA and ATP-MA gradient (1.67 mM, 167 μ M, 16.7 μ M).

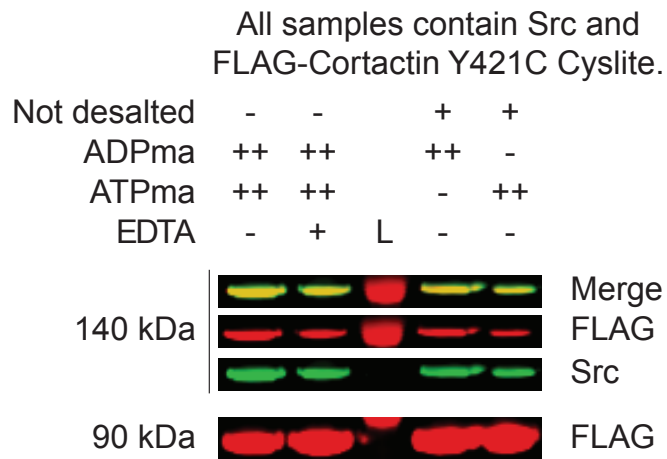


Figure 4.1.12. Src incubated with lysate from 293T cells overexpressing Y421C Cortactin; crosslinking occurs in lysates without desalting. Conditions for crosslinking were: Src 3 μ M, ADP-MA/ATP-MA 15 μ M low, 150 μ M high, 18 h, 4 $^{\circ}$ C in HEPES 25 mM pH 7.4, NaCl 150 mM, MgCl₂ 1 mM. All samples were immunoprecipitated and eluted with 3XFLAGtide and loading dye prior to gel.

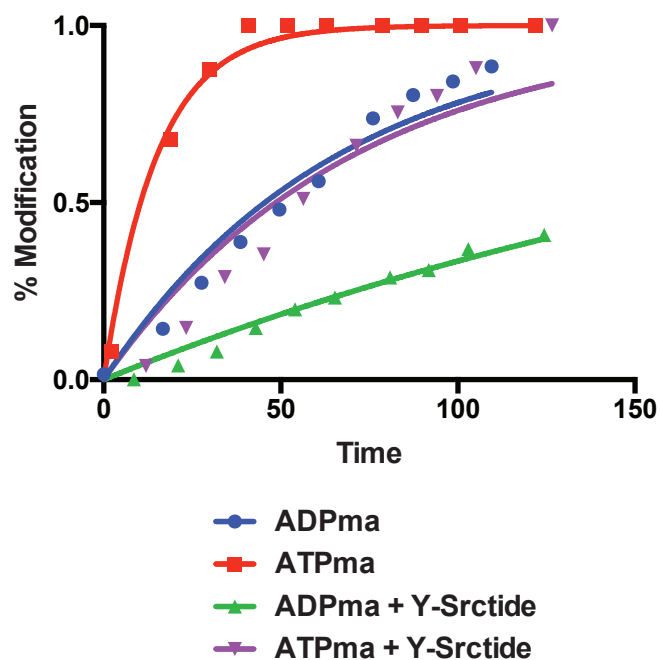


Figure 4.1.13. The rate of both ADP-MA and ATP-MA labeling of Src is reduced in the presence of WT Y-Srctide and ATP-MA labels faster. Reaction was measured unquenched at 4 °C; time elapsed was measured by instrument timestamp. Src was 500 nM, Srctide was 100 μ M, and ADP-MA and ATP-MA were 10 μ M in HEPES 25 mM pH 7.4, NaCl 150 mM, MgCl₂ 1 mM.

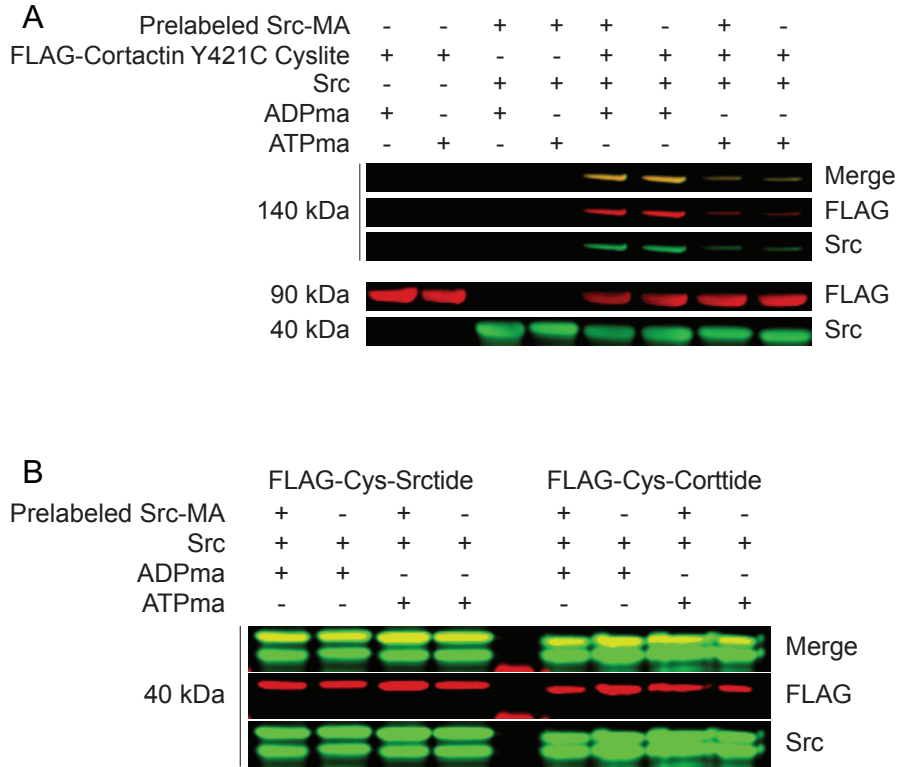


Figure 4.1.14. A. When Src was prelabeled with ADP-MA or ATP-MA and incubated with Y421C Cortactin *in vitro*, ADP-MA mediated more crosslinking than ATP-MA and prelabeling did not significantly improve ATP-MA mediated crosslinking. B. ADP-MA prelabeled and ATP-MA prelabeled Src crosslinked peptides similarly. Conditions for crosslinking in both A and B were: Src 3 μ M, ADP-MA/ATP-MA 50 μ M, Cortactin 12 μ M (in panel A only), FLAG-Srctide or FLAG-Cortide 500 μ M (in panel B only), 17 h, 4 $^{\circ}$ C in HEPES 25 mM pH 7.4, NaCl 150 mM, $MgCl_2$ 1 mM.

4.2 Selectivity for substrate over non-substrate peptides and phosphorylation site Cys over native Cys is retained.

Substrate selectivity, or selectivity of the methacrylamide labeled kinase to crosslink its substrate preferentially over other Cys containing proteins, is essential for our probe to covalently stabilize the correct kinase in the kinase-substrate complex. It was previously demonstrated that ATP-MA-mediated crosslinking between Src and its substrate peptide is preferred over peptides lacking its consensus motif.¹⁶ We performed a kinase assay to demonstrate that Src tide peptide was a Src substrate, but AK Tide and CK2 tide peptides (peptide sequences listed in Figure 2.2.1) were not Src substrates, and found that ADP-MA mediates crosslinking preferentially to the Cys-substrate peptide Cys-Src tide (Figure 4.2.1). In conjunction with the finding that Src preferentially crosslinks its four Cortactin phosphorylation site mutants over the three native Cys residues in wild-type Cortactin (Figure 4.1.3), we concluded that Src-MA crosslinking to Cys exhibits selectivity for Cys located in Src phosphorylation sequences.

A

	K_m
Srctide	$9.6 \pm 0.6 \times 10^{-6}$
AKTide	Not detected
CK2tide	Not detected

B

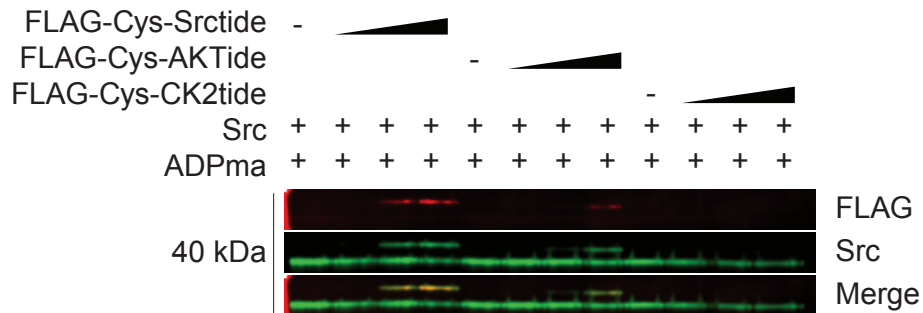


Figure 4.2.1. A. Kinase assay with Tyr containing Src substrate and non-substrate peptide sequences. B. ADP-MA mediates crosslinking preferentially for Src substrate peptide. Panel A values were determined by P^{32} dot blot assay in triplicate with 5 nM Src, a time gradient of 1, 3, 5, 10, 30, and 60 minutes, and a Srctide concentration gradient of 0, 6.25, 12.5, 25, 50, 100, 200, 400 μ M peptide. Conditions for panel B were: Src: 2 μ M, peptide 0, 5, 25, 125 μ M, ADP-MA 7.5 μ M, for 3 h at 4 $^{\circ}$ C in HEPES 25 mM pH 7.4, NaCl 150 mM, $MgCl_2$ 1 mM.

4.3 Using unbiased detection by MS/MS to evaluate crosslinking by ADP-MA and other crosslinkers in cellular lysate.

Having demonstrated that ADP-MA and ATP-MA mediate Src- Cys-Cortactin crosslinking in lysate by western blot, we considered whether our crosslinker could facilitate the covalent capture of kinase-substrate complexes for enrichment by immunoprecipitation followed by tandem mass spectrometry. This pipeline would be followed and optimized using the model kinase and substrate pair Src and Cortactin, and the same pipeline could eventually be followed to facilitate the identification of endogenously expressed kinases crosslinked to orphan substrate phosphorylation sites.

We first transiently transfected HEK 293T cells with FLAG-tagged WT and Y421C Cyslite Cortactin. This overexpression system was used to make desalted cellular lysates containing Cortactin, the substrate of interest. In our initial experiment, we supplemented cellular lysates made from cells overexpressing substrate with 2 μ M bacterially expressed Src kinase domain before incubating in the presence or absence of ADP-MA. We then immunoprecipitated FLAG-tagged substrate and substrate-kinase complexes and eluted before visualizing by silver stain and western blot (Figure 4.3.1). In the Y421C + ADP-MA sample only, we observed the formation of a new band at 130 kDa, the sum of the observed shifts for Src kinase domain (40 kDa) and FLAG-Cortactin (90 kDa). This band at 130 kDa was labeled by both anti-FLAG antibody and anti-Src antibody. By silver stain we were also able to visualize a new band at 130 kDa. We performed in-gel digest and MS/MS on the 130 kDa region of all three samples and found enrichment for Src in the Y421C + ADP-MA sample, but no enrichment in Y421C without probe samples (Figure 4.3.2). We considered this to be a proof of principle that enrichment for kinase could be observed by ADP-MA mediated crosslinking,

immunoprecipitation, and LC-MS/MS. We also tried to perform on-bead digest rather than in-gel digest on crosslinking samples, but when we performed on-bead digest, we found that Src was present in all samples, and that levels of crosslinked Src were not higher than background (Figure 4.3.3). In these experiments we observed free Src by western blot at 40 kDa in samples immunoprecipitated for FLAG-Cortactin, suggesting that at high levels of enriched kinase, unbound kinase was also immunoprecipitated either through interaction with the substrate or through nonspecific interaction with the solid support, suggesting that for very abundant kinases, further enrichment through in-gel digest and gel shift was necessary. We thought that this finding would be less likely to occur at the endogenous expression levels of less abundant kinases.

From these results, we looked to evaluate whether ADP-MA could crosslink, enrich, and enable for subsequent identification of endogenous levels of kinase from lysate. We performed crosslinking in desalted lysates without supplementing with kinase, followed by affinity purification, on-bead digest and MS/MS. Using a data dependent acquisition protocol and three biological replicates, we did not observe Src kinase, nor did we observe significant differences in other kinase enrichment between ADP-MA and no probe controls (Figure 4.3.4). Using a targeted method on the same samples, we did not observe Src in control or probe samples but did observe Src in positive control samples (data not shown).

To verify the first step of the reaction was taking place in lysate, we used an alkyne containing covalent kinase occupancy probe targeting the catalytic Lys, XO44.⁴² Competition was used to demonstrate that ADP-MA was modifying the catalytic Lys of kinases under the conditions used in LC-MS/MS. HEK293T lysate was incubated with ADP-MA, ADP as a control for reversible inhibition, or KL094 (an XO44 analog without the reactive alkyne) as a positive control before incubation with XO44 followed by Cu (I) catalyzed click addition of TAMRA-azide. Overnight pre-incubation of lysate with ADP-MA at 15 μ M or KL094 at 15 μ M

was able to compete with 2 μ M XO44 for labeling of the catalytic Lys (Figure 4.3.5). These results suggest that the first step of the crosslinking reaction was taking place in lysate under the in-lysate crosslinking conditions.

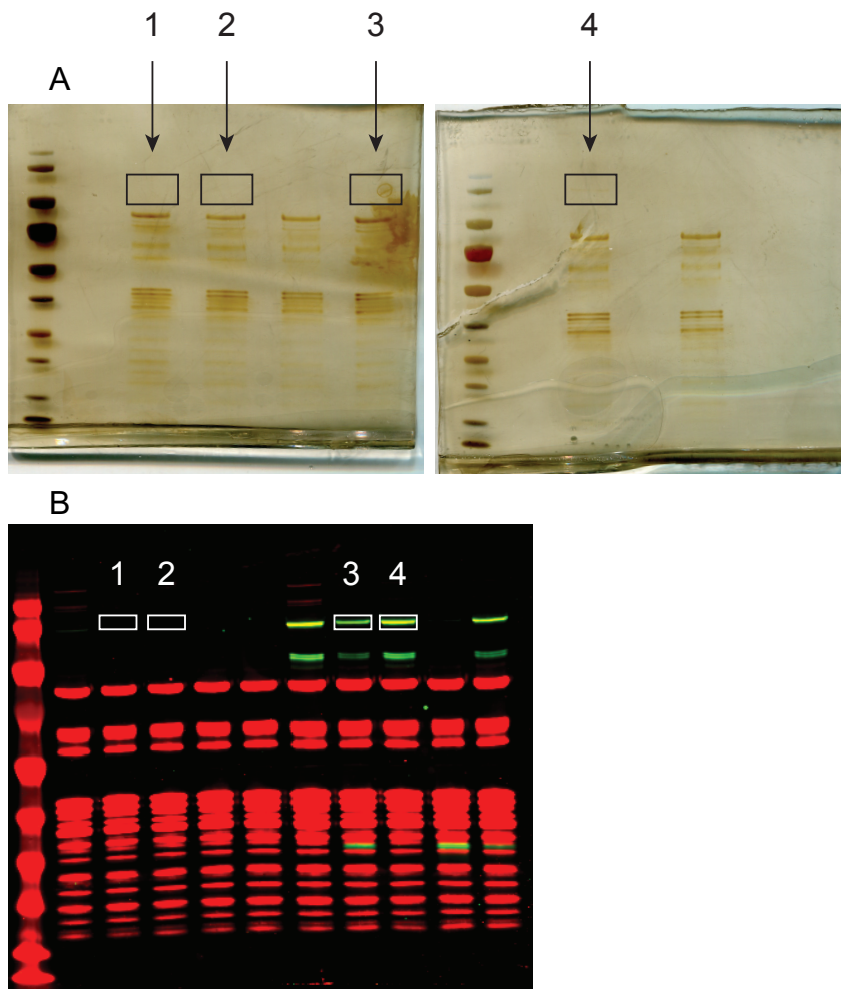


Figure 4.3.1. A. Silver stain of in-gel digest samples. Samples 1 and 2 are lysate from Y421C Cortactin transfected cells incubated with $0.65\ \mu\text{M}$ Src with no ADP-MA negative controls. Samples 3 and 4 are lysate from Y421C Cortactin transfected cells incubated with $0.65\ \mu\text{M}$ Src with $5\ \mu\text{M}$ ADP-MA. All samples were immunoprecipitated with $100\ \mu\text{L}$ M2-FLAG bead slurry and eluted with 3X Flagtide before gel and in-gel digest. Black boxes indicate the region of the gel that was excised for in-gel digest. B. Western blot of the samples from panel A. White boxes correspond to the black boxes in panel A.

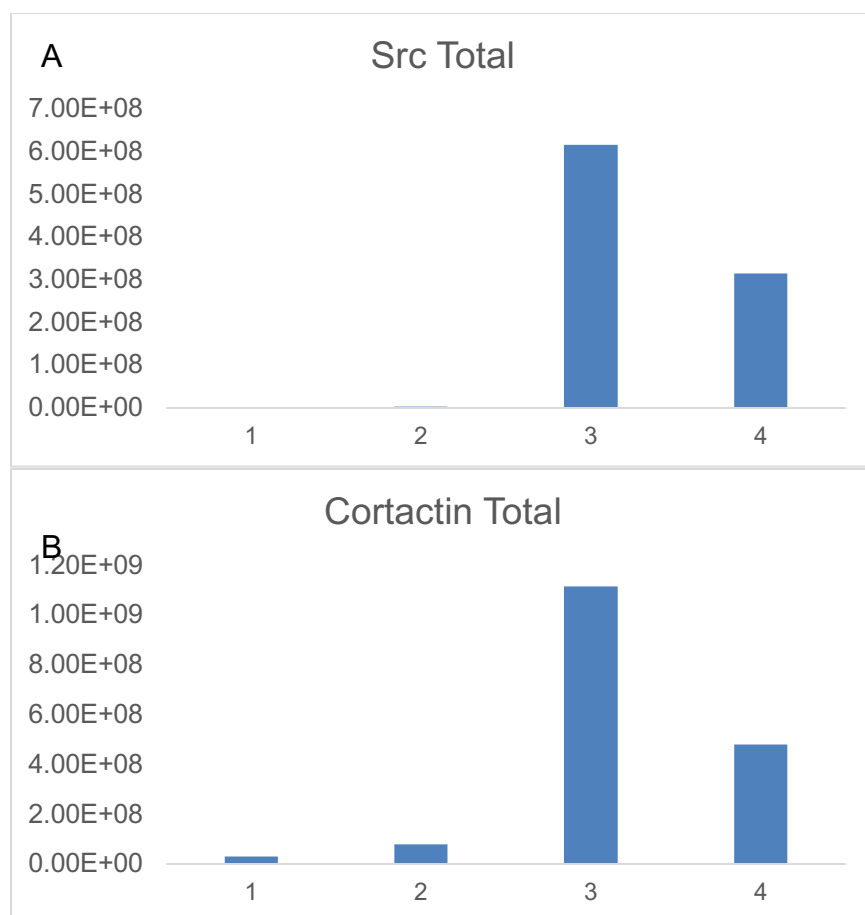


Figure 4.3.2. Area under the curve for all peptides corresponding to Src (Panel A) and Cortactin (Panel B) identified in the LC-MS/MS experiment in Figure 4.3.1. Samples 1 and 2 are lysate from Y421C Cortactin transfected cells incubated with 0.65 μ M Src with no ADP-MA negative controls. Samples 3 and 4 are lysate from Y421C Cortactin transfected cells incubated with 0.65 μ M Src with 5 μ M ADP-MA.

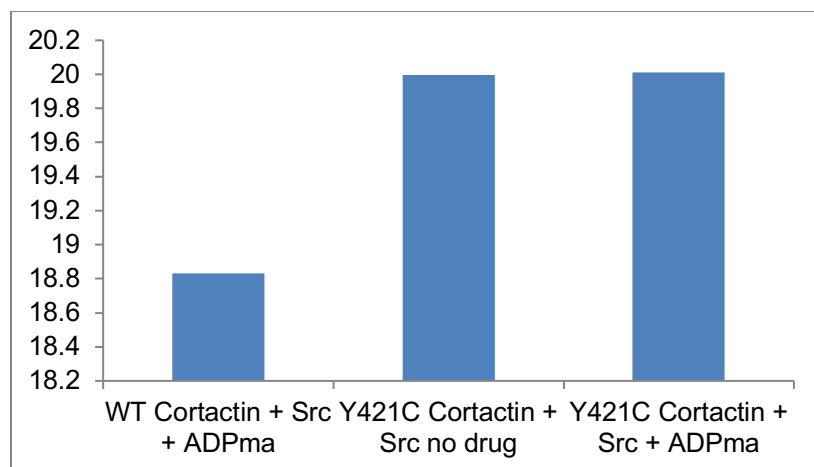


Figure 4.3.3. Area under the curve for all peptides corresponding to Src identified from on-bead digest of in-lysate crosslinking samples with supplemented kinase. Note that units are arbitrary. Conditions for crosslinking were: Src 1 μ M, ADP-MA 15 μ M, Cortactin lysate from 4 10 cm dishes per sample, 18 h, 4 $^{\circ}$ C in HEPES 25 mM pH 7.4, NaCl 150 mM, MgCl₂ 5 mM.

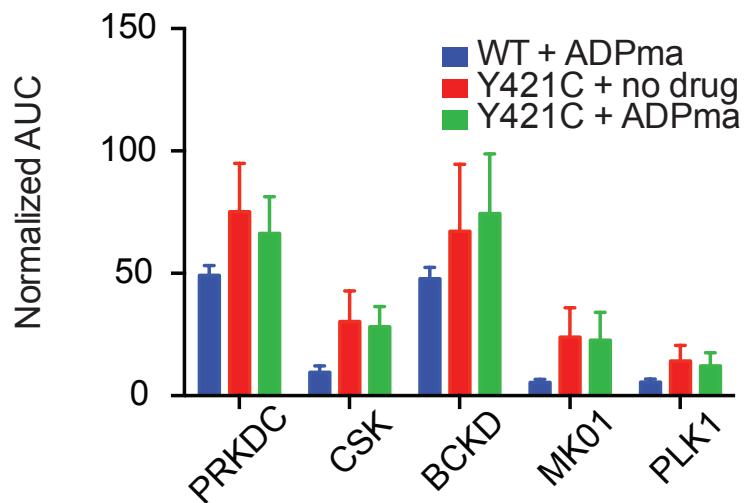


Figure 4.3.4. Kinases detected in crosslinking samples with no supplemented kinase. No significant difference was observed between samples in three biological replicates.

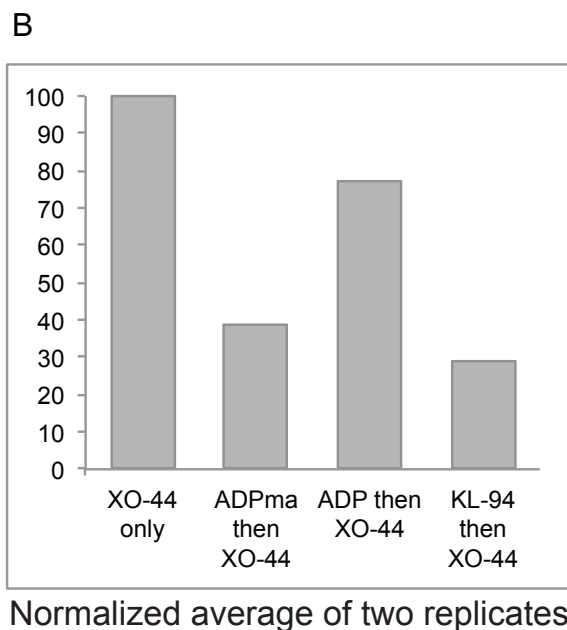
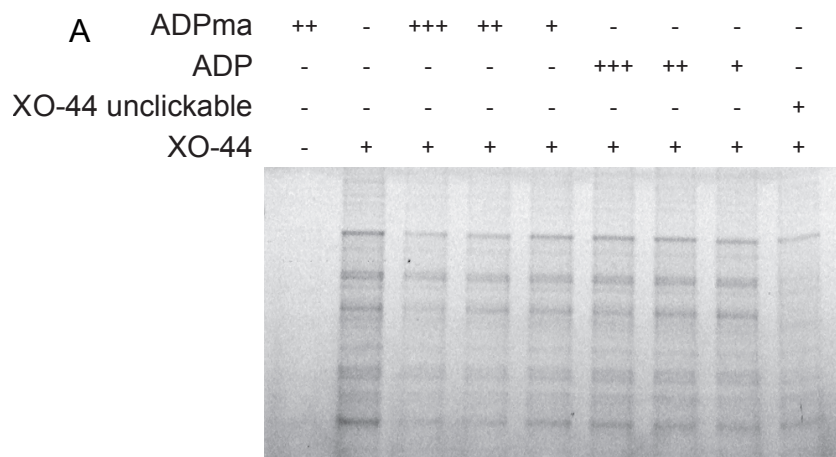


Figure 4.3.5. HEK293T lysate was incubated with ADP-MA or ADP before incubation with XO44, an alkyne containing covalent kinase occupancy probe targeting the catalytic Lys. Overnight pre-incubation with ADP-MA at 15 μ M or KL094 (an unclickable XO44 analog without the alkyne) at 15 μ M was able to compete with 2 μ M XO44 for the catalytic Lys. A. XO44 occupancy was measured by Cu (I) catalyzed click addition of TAMRA-azide and fluorescent imaging on a Typhoon instrument (Typhoon FLA 9500, GE Healthcare). B. Normalized quantification of fluorescence intensity.

4.4 Methods

Cell culture

HEK293T cells (CRL-3216, ATCC) were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Axenia BioLogix) without antibiotics. Cells were newly purchased and the first three passages were frozen for subsequent use. Cells were grown in a 37 °C incubator humidified and maintained at 5% CO₂ and passaged every third day at a 1:14 seeding density. Cells doubled roughly every 24 hours. Transient transfection was performed with 30 µL Lipofectamine LTX (Thermo Fisher) and 15 µg of maxiprep DNA in 1 mL Opti-Mem (Invitrogen) per 10 cm dish of cells at 50% confluence. Cells were incubated for 48 hours without removal of transfection reagent after transfection before harvest by pipetting.

Preparation of cell lysates

HEK293T cells were transfected with WT or phosphorylation site mutant substrate as described above. Media was removed and cells were pooled and suspended in PBS and collected by centrifugation at 500 g at 4 °C for 5 minutes. Cells were washed with 50 mL PBS 3 times total. Pellets were suspended in lysis buffer containing 1% Triton-X-100, HEPES 25 mM pH 7.4, NaCl 150 mM, MgCl₂ 5 mM (MHBS) supplemented with Roche protease inhibitor cocktail, EDTA free (5056489001, Roche), at a ratio of 0.6 mL lysis buffer to 4 10 cm plates of cells. Suspended cells were lightly vortexed and incubated on ice for 20 minutes before centrifugation at 20817 rcf at 4 °C for 10 minutes in 1.5 mL Eppendorf tubes in an Eppendorf 5417R tabletop centrifuge. Supernatant was removed and applied to a 2 mL 7 kDa MWCO Zeba spin desalting column (89890, Thermo Fisher Scientific) that had been previously washed 4 times with 1 mL HEPES 25 mM pH 7.4, NaCl 150 mM, MgCl₂ 5 mM buffer and spun at 1000 g for 2 min each

wash. Spin desalting column with sample was spun for 2 min at 1000 g in a fresh 15 mL Falcon tube and desalted lysate was collected for immediate use.

Immunoprecipitation of FLAG-tagged substrate

For each sample, 40 μ L of anti-FLAG M2 magnetic bead slurry (M8823, Sigma-Aldrich) was washed with 1 mL MHBS in a 2 mL low-retention siliconized tube (02-681-321, Fisher Scientific). Lysate containing crosslinked or uncrosslinked FLAG-tagged substrate was applied to the washed beads and rotated at 4 °C for 1 hour. Supernatant was removed and each sample was rotated on a tube rotator for 5 minutes at 4 °C with 1 mL of MHBS (25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$) + 1% Triton X-100 (T9284, Sigma-Aldrich). Supernatant was removed and wash was repeated twice total. Samples were then washed twice with 1 mL of MHBS for 5 minutes at 4 °C per wash, transferring to a fresh low bind tube each time. Samples were then washed twice with 1 mL of 20 mM Tris pH 8 and 2 mM $CaCl_2$ buffer for 5 minutes at 4 °C per wash, changing to fresh low-bind tubes each time. Beads were frozen at -80C for on-bead digest or sample was eluted with 3X Flagtide for 1 h at 4 °C and BCA assay (Rapid Gold BCA Assay Kit, A53225, Life Technologies) was performed before *in vitro* crosslinking, in-gel digest and/or western blot.

Src-Cortactin crosslinking *in vitro*

Src kinase domain (*Gallus gallus*, residues 251-533) was expressed in BL21 cells as previously described.⁴³ FLAG-tagged WT, Cyslite, and Cys phosphorylation site mutant Cortactin were overexpressed and purified from HEK293T cells with Lipofectamine LTX transient transfection and M2-FLAG-beads as described above. In a typical experiment, purified Cortactin was incubated with the addition of 2 μ M bacterially expressed Src before the addition of 16 μ M

ADP-MA, for 16 h at 4 °C in HEPES 50 mM pH 7.4, NaCl 150 mM in a total volume of 15-20 μ L. Individual experiments may differ slightly in concentrations and volume, and specific conditions are listed under each figure.

Src-Cortactin crosslinking in lysate

FLAG-tagged WT, Cyslite, and Cys phosphorylation site mutant Cortactin were transiently transfected in HEK293T cells. Cells were lysed in lysis buffer (1% Triton-X-100, HEPES 25 mM pH 7.4, NaCl 150 mM, $MgCl_2$ 1 mM supplemented with Roche protease inhibitor cocktail, EDTA free (5056489001, Roche)) and desalted with Zeba spin desalting column as described above. 2 μ M bacterially expressed Src and/or 15 μ M ADP-MA was added to lysate and incubated for 16 h at 4 °C. Crosslinked and uncrosslinked FLAG-substrate was immunoprecipitated with magnetic-FLAG beads as described above and eluted with 3XFLAGtide for 30 min at 4 °C for analysis.

Preparation of ADP-MA and ATP-MA labeled Src for LC-MS/MS analysis

At 4 °C, to each sample of 7 μ g of kinase (Src and Aurora A) in 20 μ L of HEPES 25 mM pH 7.4, NaCl 150 mM, ADP-MA or ATP-MA was added to a final concentration of 15 equivalents. Samples were incubated for 4h at 4 °C and frozen overnight. Samples were then thawed and denatured with the addition of loading buffer and run on a 4-12% gradient gel for 1 hour at 180 volts with MOPS buffer (0.02 M, pH 7). Gel was removed and washed with 50 mL distilled water for 1 minute 3 times and stained for 10 minutes with SafeStain Coomassie dye (LC6065, Thermo Scientific). Bands corresponding to kinase were cut out for in-gel tryptic digest, described below.

Src samples were additionally dried in a GeneVac instrument (SP Scientific) for 1 hour and rehydrated with 0.75 μ L 1 mg/ mL Glu-C (11420399001, Sigma-Aldrich) and incubated at 37 °C for 4 h. Trypsin-Glu-C double digest samples were repurified with C18 zip tip (EMD Millipore) before LC-MS/MS analysis.

In-gel tryptic digest

Gel pieces were diced and placed in siliconized tubes. Gel pieces were washed twice with 100 μ L 25 mM ammonium bicarbonate (ABC, 09830-500G, Sigma-Aldrich): 50% acetonitrile solution for 10 minutes with agitation. 100 μ L 5 mM DTT (DTT10, Goldbio) solution was added to gel pieces and tubes were heated to 56 °C for 30 minutes. Samples were cooled and 100 μ L 2 mM freshly made iodoacetamide (I6125-5G, Sigma-Aldrich) solution was added and samples were incubated in the dark at room temperature for 30 minutes. Supernatant was discarded and gel pieces were washed twice with 100 μ L 25 mM ammonium bicarbonate (ABC): 50% acetonitrile solution for 10 minutes with agitation. Gel pieces were dried completely in a GeneVac instrument (SP Scientific) and 50 μ L of 5 ng/ μ L trypsin (V5113, Promega) was added. Gel pieces were rehydrated for 15 minutes at room temperature and 25 μ L 25 mM ammonium bicarbonate solution was added and digestion was incubated at 37 °C overnight. Gel pieces were extracted three times with 50 μ L 2% formic acid (A117-50, Fisher Scientific), 25 mM ammonium bicarbonate (ABC): 50% acetonitrile solution with agitation for 20 minutes and samples were dried fully and zip tip purified or further digested with Glu-C as above.

On-bead tryptic digest

In a low-retention siliconized tube, beads were suspended in 9 μ L of 20 mM Tris-HCl pH 8.0. A volume of 0.4 μ L 100 mM DTT was added and beads were incubated at room temperature for 30

minutes with agitation. 0.6 μ L of 100 mM freshly made iodoacetamide solution was added to each sample and incubated at room temperature in the dark for 10 minutes with agitation. 500 ng of trypsin was added and beads were incubated at 37 °C with rotation overnight. Sample was spun down and magnetized and supernatant was transferred to a fresh tube. 500 ng fresh trypsin in 5 μ L of 20 mM Tris-HCl pH 8.0 buffer was added and incubated for 3 hours at 37 °C with agitation. Formic acid was added to a final concentration of 2% and zip tip purification using large OMIX C18 tips (A57003100, Agilent) was performed.

Zip tip purification

100 μ L of 2% formic acid/98% acetonitrile was passed through an OMIX C18 tip and expelled. Tip was washed 3 times with 50 μ L of 2% formic acid/49% acetonitrile/49% water, then 3 times with 50 μ L of 2% formic acid/98% water. Sample was passed through the tip 10 times. Tip was washed 3 times with 50 μ L of 2% formic acid/98% water, then eluted 3 times with 50 μ L of 2% formic acid/49% acetonitrile/49% water. Eluate was dried in a GeneVac to complete dryness.

LC-MS/MS analysis

Zip tip-purified peptides were analyzed on a NanoAcquity Ultra Performance UPLC (Waters) system connected to an Orbitrap Velos (Thermo Fisher Scientific) for fragment labeling and in-gel digest or an Orbitrap Fusion Lumos Tribrid (Thermo Fisher Scientific) for endogenous kinase detection. An EasySpray C18 column (ES800, Thermo Fisher Scientific) was used to resolve peptides using a 90 minute gradient of 0.1% formic acid in water-acetonitrile and HCD fragmentation unless otherwise specified. Samples were indexed against the database SwissProt.2016.9.6.random.concat with linear tail fit used to calculate expectation value.

LC-MS/MS analysis of ADP-MA and ATP-MA labeled Src

On the Orbitrap Velos (Thermo Scientific), data dependent acquisition mode was used to switch between MS and MS/MS and the top 6 precursor ions with a charge state of 2+ or higher were fragmented by HCD. Peak lists were searched on Protein Prospector using a mass tolerance of 20 ppm for precursor masses and ± 0.6 Da for fragment masses with cysteine carbamidomethylation as a fixed modification and missed cleavage and C₄H₄O modification of Lys as a variable modification. The digestion was specified as Trypsin + Glu-C with 3 maximum missed cleavages allowed. The UniProt protein identifiers for Src and Cortactin were specified (P00523 and Q14247 respectively).

XO44 chase experiment

A T175 flask of HEK 293Ts at 90% confluence, approximately 20×10^6 cells, was harvested by removing media and resuspending in 50 mL ice-cold PBS and centrifuged at 500 g for 5 minutes at 4 °C. Cells were washed with 50 mL ice-cold PBS with centrifugation at 500 g for 5 minutes at 4 °C twice and snap frozen. Cells were lysed in 0.6 mL lysis buffer (1% Triton-X-100, HEPES 25 mM pH 7.4, NaCl 150 mM, MgCl₂ 1 mM supplemented with Roche protease inhibitor cocktail, EDTA free (5056489001, Roche)) with gentle vortexing and centrifuged at 20817 rcf at 4 °C for 10 minutes in a 2 mL Eppendorf tubes in an Eppendorf 5417R tabletop centrifuge. Supernatant was desalted with Zeba spin desalting column as described above. Lysate was diluted for BCA as follows: 1/8X, 1/1X6, 1/32X. All dilutions were measured in triplicate by BCA assay (Rapid Gold BCA Assay Kit, A53225, Life Technologies) and the values were multiplied by the dilution factor and averaged to determine lysate concentration. 100 μ L lysate in 15 μ L final volume was used per sample. Samples were incubated overnight at 4 °C with ADP-MA, ADP, or KL094 at 15 μ M, or with an equivalent volume of water for no probe control

sample. After overnight incubation 2 μ M XO44 was added and samples were warmed to room temperature and incubated for 10 minutes. A stock solution of click reaction reagents was made. Per sample, the click reagent stock solution consisted as follows: 20% SDS, 0.625 μ L. 5 mM TAMRA-N₃, 0.125 μ L. 50 mM TCEP, 0.25 μ L. 1.7 mM TBTA, 0.625 μ L. 50 mM CuSO₄, 0.25 μ L. DMSO 0.25 μ L, with a total of 2.125 μ L per 10 μ L of sample. 10 μ L of each sample was removed to a fresh PCR strip tube well and 2.125 μ L click reaction stock solution was added to each sample. Reaction was incubated for 1 h at room temperature in the dark. 4X LDS sample buffer (NP0007, Thermo Fisher Scientific) was added to all samples and samples were run on a 4-12% gradient gel with MOPS buffer for 1 h at 200 V. The gel was removed and washed once for 1 minute with 10 mL 10% methanol Towbin transfer buffer (25 mM Tris pH 8, 10% methanol) and rinsed three times with 10 mL water before imaging on a Typhoon (Typhoon FLA 9500, GE Healthcare) instrument.

Chapter 5.

Bioorthogonal substrate based protein profiling crosslinkers.

5.1 Bioorthogonal ADP scaffolded crosslinkers.

Bioorthogonal chemistry offers many potential advantages for our goal of chemically stabilizing the kinase-substrate interaction for the unbiased identification of kinases that phosphorylate a site of interest.^{44,45} Chief among these advantages is that bioorthogonal reactions are stable in aqueous environments and do not react with native cellular functional groups, reducing background and off target interactions. Additionally, this field boasts a large and growing toolkit of chemical compounds and reaction pairs of varying affinity dependence and efficiency. Over 20 bioorthogonal reaction pairs have been validated in complex biological environments.⁴⁵ The majority of these pairs can be classified as nucleophilic addition to an electrophile or cycloaddition reactions. Briefly, a few of the best validated bioorthogonal reaction pairs include the Staudinger ligation between organic azides and triaryl phosphines, Cu(I) catalyzed azide-alkyne cycloaddition, and strain-promoted azide-alkyne cycloaddition (SPAAC).⁴⁶⁻⁵⁰

Bioorthogonal chemistry has been successfully used for the labeling of important biological molecules that are otherwise difficult to selectively target. For example, the Staudinger ligation was originally used to target cell surface glycans, which are decorated with many groups of similar reactivity and therefore difficult to target selectively without the introduction of orthogonal functional groups.⁴⁷ Bioorthogonal chemistry has furthermore been applied successfully to *in vivo* ABPP approaches, for example to assess enzyme function in cancer.^{51,52}

However, significant obstacles to this avenue of exploration exist. Many bio-orthogonal reaction pairs are bulky or require bulky cofactors and do not resemble native amino acid side chains; therefore, maintaining substrate specificity may be a challenge in these systems. Furthermore, incorporating these pairs into the kinase-substrate system remains challenging. Unlike mutation to Cys, a canonical amino acid, mutation of the phosphorylation site to an unnatural amino acid residue requires a method for modification or expression, which can require significant manipulation of biological samples. In this chapter we discuss a Cu(I) catalyzed click chemistry approach, the synthesis and characterization of an ADP-pentyne crosslinker probe, and our efforts to incorporate azide into the substrate phosphorylation site.

We chose to first investigate a Cu(I) catalyzed alkyne-azide cycloaddition approach due to the small size of the components, straightforward synthesis, and relative ease of incorporating azide into the substrate phosphorylation site in the form of azidophenylalanine. We thought that the relatively small size of the alkyne and azide would be least disruptive to the kinase-substrate interaction interface, relative to a strained cyclooctyne. An additional consideration was that our primary model system, Src kinase, is a tyrosine kinase. We felt that introducing azidophenylalanine, with its similar bulk and hydrophobicity to tyrosine, could be potentially advantageous for maintaining substrate specificity. For our purposes of crosslinking in lysate, Cu(I) toxicity was not considered to be a liability; however, for *in vivo* studies this toxicity would be a significant obstacle.

Using the mixed anhydride approach described in Chapter 2 and below, we made an ADP-pentyne probe for incorporation of alkyne in the kinase active site. We considered several approaches to incorporating azide in the substrate phosphorylation site. We purchased peptides containing azidophenylalanine (PheN₃), tried to label Cys mutant substrate with electrophile-azide compounds and used an unnatural amino acid incorporation system based on amber stop

codon suppression developed by Dr. Lei Wang.^{53,54} Our attempts to label Cys with thiol-reactive benzyl azides were unsuccessful. We synthesized and tested 3-azidobenzyl iodide, 3-azidobenzyl bromide and 3-azidobenzyl mesylate among others, but formation of the desired product was minimal by LC-MS. For this reason, we focused on the commercially available peptides and unnatural amino acid incorporation.

We found that Src was labeled by ADP-pentyne, but that the reaction gave multiple products and was difficult to resolve by LC-MS. The subsequent reaction with PheN₃-Src tide could not be resolved by LC-MS. However, we observed Cu(I) catalyzed crosslinking by western blot (Figure 5.1.1). We found that the addition of a Cu(I) catalyst was required for this reaction to take place; after 18 hours we did not observe crosslinking in the absence of Cu(I) catalyst.

We additionally found that besides crosslinking Src tide, Src-pentyne was able to crosslink the peptides AurAtide and SRPK tide (peptide sequences in Figure 3.2.1), neither of which possess a Src phosphorylation motif, in the presence of Cu(I), suggesting that greater affinity dependence or lower concentrations of protein and substrate may be needed (Figure 5.1.2). Slight preference for substrate peptide was observed in this experiment although all three peptides tested (Src tide, AurAtide, and SRPK tide) were crosslinked. We also saw multiple crosslinking events, shown by laddering, which may account for the difficulty in resolving LC-MS spectra. In contrast, the cysteine reactive ADP-MA mediated crosslinking system described in Chapter 3 displayed clear substrate specificity, crosslinking Src to Src tide preferentially over AKT tide and CK2 tide. However, one caveat of this comparison is that different peptides were tested (with ADP-MA, AKT tide and CK2 tide; with ADP-pentyne, AurAtide and SRPK tide). Further experiments using similar peptide sequences are warranted in order to ensure that observed differences are due to the crosslinker rather than the peptides.

Finally, we used an Amber stop codon suppression system developed by Dr. Lei Wang to incorporate Y421PheN₃ into our mammalian Cortactin expression system.⁵⁴ In this system, a plasmid containing a FN₃-specific tRNA is cotransfected along with plasmid containing a rare stop codon (TAG) at the desired site of expression and cellular growth media is supplemented with the free amino acid (F-3075, Bachem). We found that expression with a C-terminal tag is needed to separate product, Y421PheN₃ containing Cortactin, from the major contaminant, Cortactin that is prematurely truncated at the amber stop site at residue 421.

In closing, a bio-orthogonal chemical approach offers many new advantages and disadvantages relative to the cysteine-methacrylamide system described in Chapter 4. The cysteine-methacrylamide approach successfully crosslinked kinase-substrate complexes *in vitro* and in lysate in a substrate-specific, affinity dependent manner. However, this approach was unable to crosslink kinase-substrate complexes above the detection threshold of LC-MS/MS when only endogenous levels of kinase were present. Attempts to optimize this reaction pair by increasing the reactivity of the thiol-reactive electrophile were thwarted by the intrinsic reactivity of cysteines present in the kinase itself. The reactivity of a bio-orthogonal chemical pair could potentially be ‘tuned’ to maximize crosslinking yield without unwanted reactivity with native effectors, which would address this principal limitation of the cysteine-methacrylamide approach. However, the bulk, hydrophobicity, and high reactivity of many alkyne-azide reaction pairs suggest that preserving substrate specificity may pose a challenge in this system. We hope that future work will consider this exciting approach to this problem.

Synthesis of azidoacetic acid⁵⁵

Bromoacetic acid (0.5 g, 3.6 mmol) was dissolved in 15 mL water with sodium azide (0.4679 g, 2 equiv) and stirred at room temperature for 48 hours. Reaction mixture was acidified with 0.1 M

aqueous HCl solution to pH 2 and extracted with 5 mL of diethyl ether 3 times. The organic layer was dried over MgSO₄ and solvent was removed *in vacuo*. The crude mixture was kept at or below room temperature during concentration in consideration of the azide moiety. The crude mixture was carried forward without characterization.

Synthesis of pent-4-ynoyl 2,2-dimethylpropanoate

4-Pentynoic acid (250 mg, 2.55 mmol) and triethylamine (0.7104 mL, 2 equiv) was dissolved in 5 mL acetonitrile at room temperature. 2,2-dimethylpropanoyl chloride (0.3136 mL, 1 equiv) was added dropwise and reaction was stirred and monitored by TLC. After 4 hours solvent was removed *in vacuo* and residue was dissolved in ethyl acetate and loaded onto silica. Flash chromatography with a 5 g prepacked 40-60 micron silica column (693873236, Teledyne Isco) was performed using a hexanes: ethyl acetate gradient and fractions containing product were pooled. Product was a clear oil (0.462 g, 99% yield).

Synthesis of ADP-pentyne

In a dry scintillation vial, ADP-TEA (1.00 g, 2.536 mmol) was suspended in a mixture of 8 mL of dry 6:1:1 DMSO: DMF: Dioxane. To this mixture dry triethylamine (1.41 mL, 4 equiv) was added. 4-Pentynoyl 2,2-dimethylpropanoate (0.462 g, 1 equiv) was added dropwise with stirring. The reaction mixture was stirred for 72 h at room temperature and was then concentrated under reduced pressure. Purification by MCI gel column was performed as described in Chapter 3, but the product was not fully characterized.

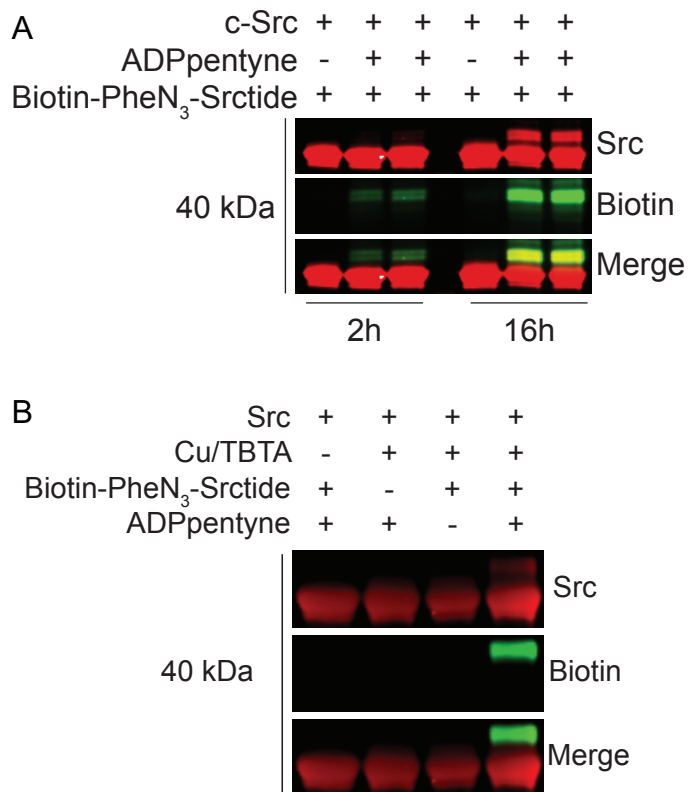


Figure 5.1.1. A. Western blot visualization of Cu(I) catalyzed ADP-pentyne mediated Src-Biotin-PheN₃-Src tide crosslinking. B. Cu(I) is required for crosslinking to take place. Crosslinking conditions in both panels: 8 μ M Src was incubated with 50 μ M peptide for 2-16 h at 4 °C. Buffer components: HEPES 50 mM pH 7.4, NaCl 150 mM, CuSO₄ 1 mM, TCEP 0.5 mM, TBTA 0.085 mM, DMSO 2%.

1. Src + Biotin-PheN₃-Src tide
 2. Src + Biotin-PheN₃-AurAtide
 3. Src + Biotin-PheN₃-SRPK tide
- ADP-pentyne, Cu(I) in all samples

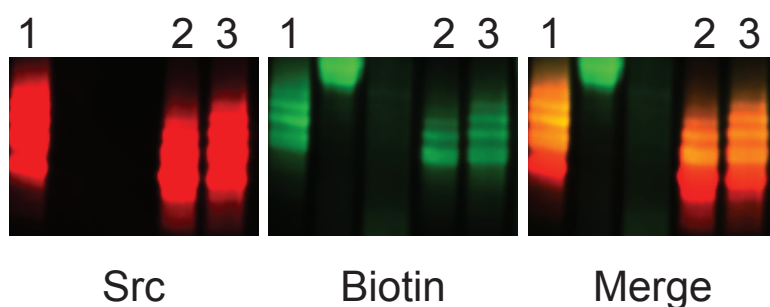


Figure 5.1.2. Western blot visualization of Cu(I) catalyzed ADP-pentyne mediated crosslinking of Src to substrate peptide Biotin-PheN₃-Src tide as well as non substrate peptides AurAtide and SRPK tide. Laddering probably due to multiple crosslinking events was also observed. There appeared to be a preference for crosslinking to Src tide over SRPK tide or AurAtide, but all three peptides were crosslinked. Crosslinking conditions: 2 μ M Src was incubated with 400 μ M ADP-pentyne and 500 μ M peptide for 16 h at 4 °C. Buffer components: HEPES 50 mM pH 7.4, NaCl 150 mM, CuSO₄ 1 mM, TCEP 0.5 mM, TBTA 0.085 mM, DMSO 2%.

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