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Designing a Reactive Warhead to Bind and Inhibit Pseudomonas aeruginosa's Periplasmic Protein, Inhibitor of Vertebrate Lysozyme

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Designing a Reactive Warhead to Bind and Inhibit *Pseudomonas aeruginosa's*
Periplasmic Protein, Inhibitor of Vertebrate Lysozyme

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Committee Chair   Graduate Program Coordinator

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Committee Member   Department Chair

_____   _____
Committee Member   College Dean

Dedication

For my loving parents, Darren and Traci, who have supported me throughout this

journey.

Abstract

     Pseudomonas aeruginosa is a Gram-negative bacterium commonly found throughout the environment. It is a significant cause of disease and mortality in immunodeficient patients such as those suffering from cystic fibrosis (CF). Due to the emerging antibiotic resistance of *P. aeruginosa*, it is becoming increasingly more challenging to treat an infection by traditional means. Further complicating treatment, *P. aeruginosa* secretes a protein known as Inhibitor of Vertebrate Lysozyme (PaIVY) that binds to and inhibits C-type lysozyme, thus preventing the degradation of the bacterium. A reactive chemical warhead was synthesized from a rhenium(I) tricarbonyl derivative in order to bind to and irreversibly inhibit PaIVY, thereby allowing endogenous host lysozyme to effectively degrade drug-resistant *P. aeruginosa* bacteria. Various biophysical methods, such as fluorescence spectroscopy, NMR, and mass spectrometry are utilized in the characterization of the rhenium(I) tricarbonyl derivative and the binding interaction PaIVY experiences when placed in the presence of the chemical warhead.

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Chapter One

Background and Significance

Cystic fibrosis (CF) is an autosomal recessive genetic disorder that affects about 70,000 people worldwide with 1,000 new cases annually.[1] CF is a disorder of the exocrine glands that results from a mutation in the cystic fibrosis transmembrane conductance regulator protein-coding gene (CFTR). These mutations affect sodium and chloride ion transport at the apical surface level of exocrine gland epithelial cells. In healthy individuals, a thin, salt containing layer of mucus coats the surface of the respiratory tract, trapping debris and pathogens that will be swept out by cilia. For individuals with a mutation in the CFTR protein, the result is too little chloride transport and too much sodium transport. Due to the excess sodium, water is depleted from the secretions creating a thick mucus that accumulates in the lungs allowing for bacterial growth and infection.[2]

Pseudomonas aeruginosa is an opportunistic bacterium widely found throughout the environment in soil and water. For healthy individuals an infection would be mild if established at all. However, for immune compromised individuals, or those suffering from CF, the infection is responsible for a high rate of morbidity and mortality.[3] *P. aeruginosa* is one bacterium that finds home in the thick mucus secretions found in the respiratory tract of patients with CF. The bacterium produces a biofilm which is highly tolerant towards the host immune system and helps enable the pathogen to be up to 1,000 times more tolerant to antibiotics.[4] Further complicating matters, *P. aeruginosa* secrets a

protein known as Inhibitor of Vertebrate Lysozyme (PaIVY) from its periplasm. This protein inhibits C-type lysozyme that would normally compromise the structural integrity of the bacterial cell wall by catalyzing the hydrolysis of the β-1,4 glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) that are the disaccharide building blocks of the peptidoglycan polymers in the bacterial cell wall[5] (**Figure 1**). Eradicating *P. aeruginosa* infection is increasingly more difficult due to its growing antibiotic resistance and production of PaIVY, producing a need for alternative treatment methods.
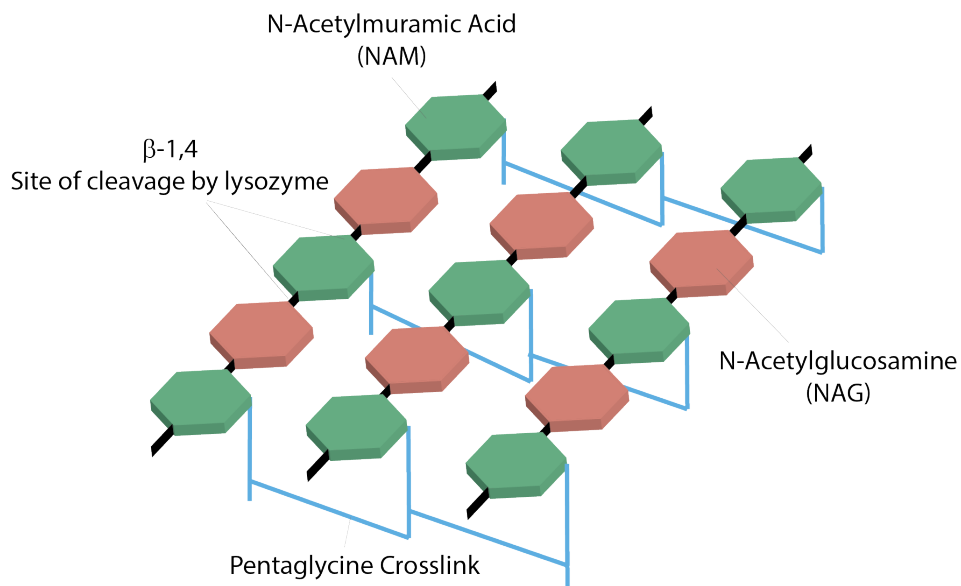
Figure 1. Composition of the bacterial cell wall indicating lysozymes cleavage site; NAG and NAM are represented by the red and green hexagons, respectively, while peptide chains are indicated by the blue lines.

One target for inhibiting PaIVY is an exposed loop with H62 at the center that is involved in the inhibition of lysozyme (**Figure 2**). When PaIVY interacts with lysozyme the loop from PaIVY occupies the active site of lysozyme forming hydrogen bonds with two of the three residues, Aps52 and Glu35, involved in its enzymatic activity.[7]

Transition metals yield promising results for binding amino acids which could block this interaction from occurring.[9]
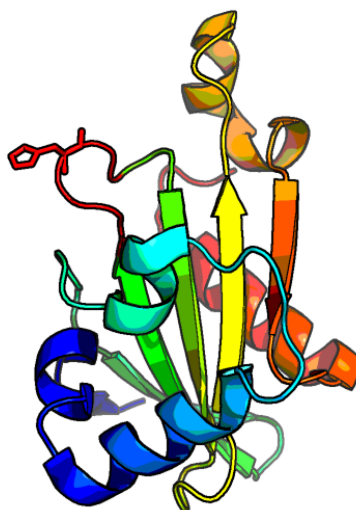
Figure 2. Ribbon structure of PaIVY; shown in red is the exposed loop with H62 indicated by a stick structure on the loop.[8]

Metal complexes have re-emerged as enzyme inhibitors because of their structural diversity, adjustable ligand exchange kinetics, fine-tuned redox activity, and their distinct spectroscopic signatures.[9] These metal complexes are used in biological systems by forming coordination bonds to residues at the protein surface or in the active site. A reactive warhead, or covalent inhibitor, contains a specific group that is designed to react with a target typically an amino acid side chain that is part of the catalytic activity by covalent binding. The covalent binding is much stronger than typically non-covalent interactions such as hydrogen bonds or salt bridges, therefore frequently resulting in an irreversible interaction.[10]

Rhenium cations are increasing in interest due to the potential to be used as a radiological imaging agent or as a therapeutic radiopharmaceutical similar to technetium. There is a further interest in the coordination chemistry of rhenium complexes with biological molecules due to its potential to be used as a bifunctional chelating agent (BFCA) in drug designs.[11] Rhenium(I) tricarbonyl molecules have been identified to bind to nucleic acids, nucleotides, polypeptides, and amino acids. Of particular interest $Re(CO)_3(H_2O)_3^+$ has been identified to form complexes with N-acetyl histidine and histidylhistidine, making it an ideal candidate to design a reactive warhead targeting H62 of PaIVY.[11] Furthermore, diamine rhenium tricarbonyl complexes are favored for their strong luminescence that can be used in characterization.[12] Other techniques such as nuclear magnetic resonance, mass spectrometry, and X-ray crystallography are also often important in characterization and identification of these types of compounds and for monitoring complex formation to biomolecules.[13, 22, 23]

Nuclear magnetic resonance (NMR) has a growing role in the drug discovery process with the ability to identify binding sites, affinities, and ligand poses at the individual amino acid level without necessarily solving the structure of the protein-ligand complex.[13] Heteronuclear single quantum coherence (HSQC) is a 2-dimensional NMR pulse sequence that is frequently used in protein chemistry. Using a ^{15}N-labeled protein, an HSQC provides a fingerprint of the folded protein by showing a peak for each non-proline amino acid in the backbone due to the variation in chemical environment each residue experiences. Once each peak is assigned, HSQC can be used to monitor protein interactions with any unlabeled ligand that is added to the protein solution and afford residue specific information about chemical changes. Chemical shift perturbation arises

from changes in the chemical environment the nucleus experiences from conformational changes induced by a binding event making HSQC a reliable source for identifying if binding of a reactive warhead occurs and at what amino acid residue(s).[14]

Other biophysical methods can be utilized to characterize complex formation including mass spectrometry and fluorescence. There is a wide range of mass spectrometry techniques that can be used for molecule characterization including matrix-assisted laser desorption/ionization (MALDI), liquid chromatography-mass spectrometry (LC-MS), and electrospray ionization mass spectrometry (ESI-MS). ESI-MS is of particular interest for characterization of the warhead alone as well in complex with PaIVY because ESI-MS applications include protein-ligand binding experiments. ESI is a soft ionization technique that converts solution-phase analytes to gas-phase analytes and measures the mass-to-charge ratio (*m/z*).[15] ESI-MS begins by introducing the sample to the ionization source in the mass spectrometer where the solution-phase analytes are converted to gas phase analytes using electrical energy and obtain a positive or negative charge. From here they arrive at the detector based on the m/z ratio. Once the ions are detected the signals are transferred to a computer which displays the information graphically providing a fingerprint for the compound.[16] This information will be used to verify the identity of the synthesized warhead as well as identify where the warhead is interacting with PaIVY.

The use of fluorescence in biological sciences for cellular and molecular imaging has grown rapidly because there is no longer the need for expensive and difficult to handle radioactive tracers while the detection remains highly sensitive. [17] There are many different techniques in which fluorescence can be monitored to reveal valuable

information about a compound such as fluorescence resonance energy transfer (FRET), fluorescence polarization or steady state fluorescence. Steady state fluorescence is the most common type of fluorescence in which the sample is illuminated with a continuous beam of light and the intensity or emission spectrum is recorded.[17] Fluorescence studies can be carried out on many rhenium compounds due to their distinct spectroscopic signatures depending on the different ligands attached and the solvent used. The distinct absorption and emission spectra the rhenium compound exhibits are highly useful in characterization and binding studies. One way fluorescence can be used in binding studies is by determining the rate of the reaction. Most proteins do not exhibit any fluorescence of their own, therefore by attaching a fluorescent molecule the rate at which the two react can be monitored.

Another important technique that can be used in determining where a ligand is binding to a protein is site-directed mutagenesis using polymerase chain reaction (PCR). There are two types of site directed mutagenesis, simple or multiple mutations. A simple mutation method is based on the amplification of double-stranded DNA from the template DNA using complementary oligonucleotides, or primers, carrying the mutation of interest while multiple mutations either obtain the mutation after several rounds of this process or can obtain them simultaneously in one reaction.[18] In a PCR small amounts DNA are amplified or copied using a thermocycler. There are three steps to a PCR reaction: denaturation, where the DNA is heated and denatures becoming single-stranded, annealing where the primers, containing the mutation, bind to flanking regions of the DNA, and extension where DNA polymerase extends the 3' end of each primer along the template strand.[19] There are many commercially available kits for site directed

mutagenesis that make the process simple as well as many useful websites to help design primers. For PaIVY, it was proposed that the H62 loop was the active site that binds to and inhibits lysozyme. Mutating this residue will help indicate if the residue is actually important in lysozyme inhibition as well as help indicate if the warhead is binding to this site.

To determine if lysozyme is still active in the presence of an inhibitor such as PaIVY after site-directed mutagenesis or the addition of a reactive warhead a muramidase assay or a lysozyme activity assay is performed. This commercially available assay utilizes fluorescently labeled cells that indicate how active lysozyme is due to the relationship between the fluorescent signal recorded and activity of lysozyme being proportional.[20] The fluorescent signal increases when lysozyme is properly functioning and lysing the cells. However, because PaIVY inhibits lysozyme the activity of lysozyme decreases and therefore the fluorescent signal also decreases as the concentration of PaIVY increases. This assay plays an important role in determining if the addition of the reactive warhead to PaIVY will eliminate or reduce PaIVY's inhibition activity against lysozyme as well as determine if specific residues of PaIVY are necessary in the inhibition of lysozyme.

Chapter Two

Protein Purification and Mutagenesis

To begin research on the interactions between a reactive warhead and PaIVY

purified protein must be available for use. *Pseudomonas aeruginosa* Inhibitor of

Vertebrate Lysozyme (PaIVY) was purified following a protocol developed by Aaron

Schultz, a previous graduate student, located in appendix two with some modifications.

The original plasmid, PA3902, was purchased from DNASU in a pSpeedET vector with a

six residue His tag containing a TEV cleavage site located at the beginning of the protein

sequence. The plasmid has kanamycin antibiotic resistance.

Following the protocol originally created by Aaron Schultz, the plasmid was

transformed into BL21DE3PLysS single shot cells and onto kanamycin LB agar plates

and left to grow overnight at 37°C. The following day, the transformed cells were used

for an overnight culture. Typically for a 2 L prep, two 100 mL small inoculum flasks

were prepared using LB media or M9 minimal media and left at 37°C overnight. The

following day 50 mL from each flask would be transferred to 1 L flasks containing the

respective media and left to grow to an OD_{600} of 0.6 at 37°C. Once the correct OD was

reached the cells were induced using L-arabinose and left overnight at 37°C. The next

day the cells were spun into a pellet, lysed using a French Press, and centrifuged again

separating the crude lysate from the cell fragments.

Subsequently the purification process consisted of an immobilized metal affinity

chromatography (IMAC) His1 (**Figure 3**), followed by dialysis and TEV cleavage. The

His1 fraction is dialyzed for three hours to remove excess imidazole before TEV is added and then left overnight at room temperature. The following day a His2 (**Figure 4**) is performed and the peak fraction is concentrated down to approximately 2 mL. From this point the concentrated His2 peak is loaded on the size exclusion chromatography (SEC) column which will separate the protein by size (**Figure 5**) and therefore allowing for a pure sample of the protein of interest. In the outlined protocol the SEC is typically run in SEC buffer which consists of 20 mM monobasic potassium phosphate, 20 mM dibasic potassium phosphate and 200 mM potassium chloride at pH 8.0, however a modification using a new buffer from what Aaron previously used was adapted in order to save time on buffer exchange. The new buffer used to run SEC is 20 mM sodium phosphate buffer pH 6.0 or in sterile 18Ω water. Once the SEC was completed the peak at the appropriate place corresponding to the correct molecular size was collected and concentrated to the desired concentration. The concentration of the protein is dictated by the future experiments it will be used for. A typical two-liter preparation PaIVY yielded approximately 1.5 mL at 1.2 mM. After this the protein was aliquoted into smaller amounts, typically 500uL to be used for future experiments or frozen in the -80°C for later use.
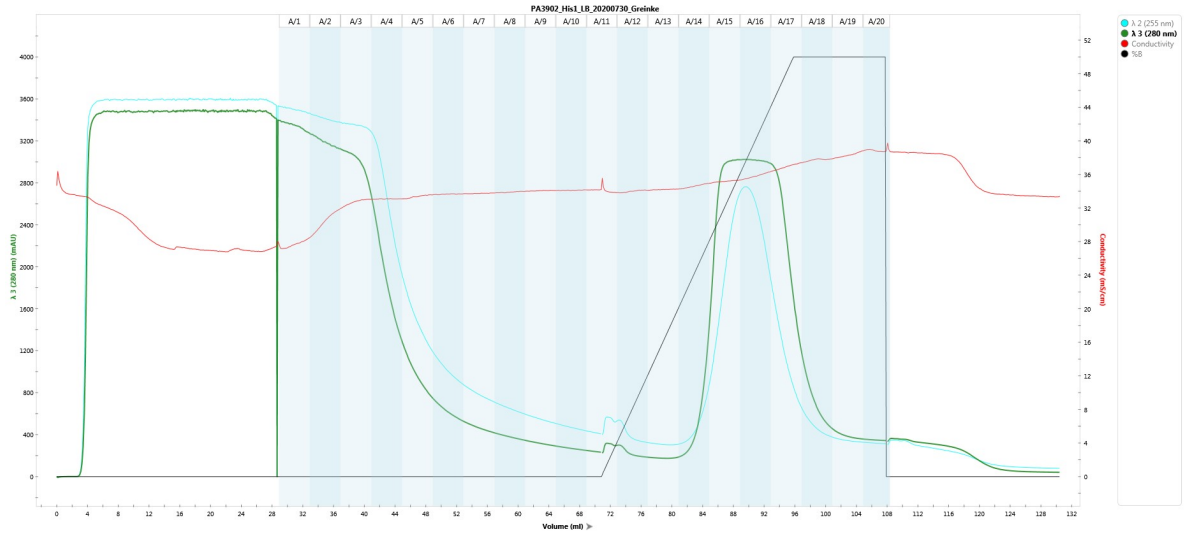
Figure 3. His1 chromatogram of wild type PaIVY purification showing good separation between the 280 nm (green) and 255 nm (teal) wavelength in the elution phase indicating viable protein requiring further purification.
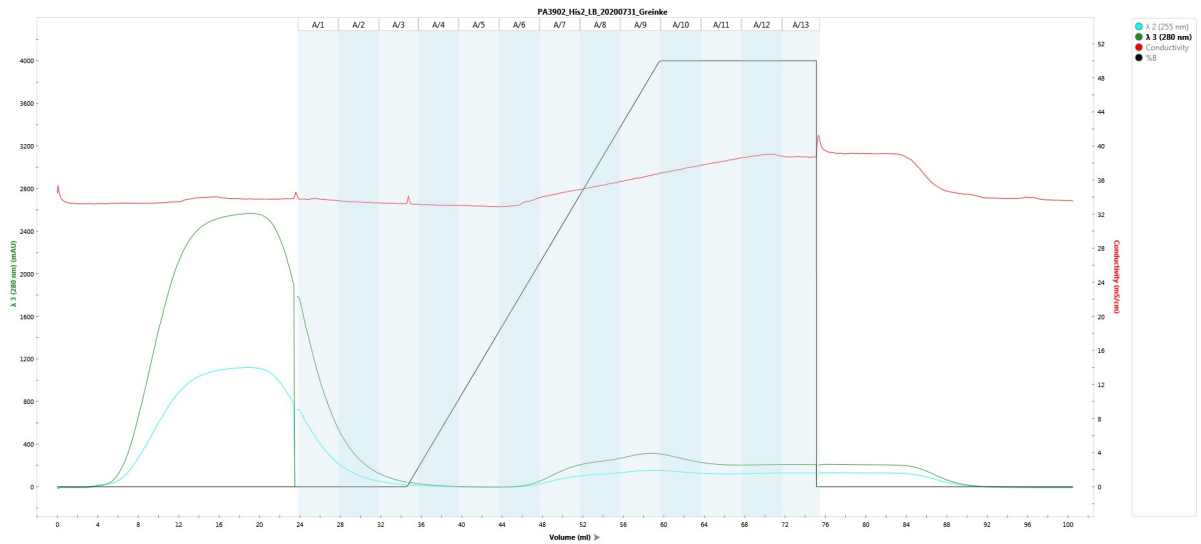
Figure 4. His2 chromatogram of wild type PaIVY purification. After His tag has been removed showing good separation between the 280 nm (green) and 255 nm (teal) wavelength.
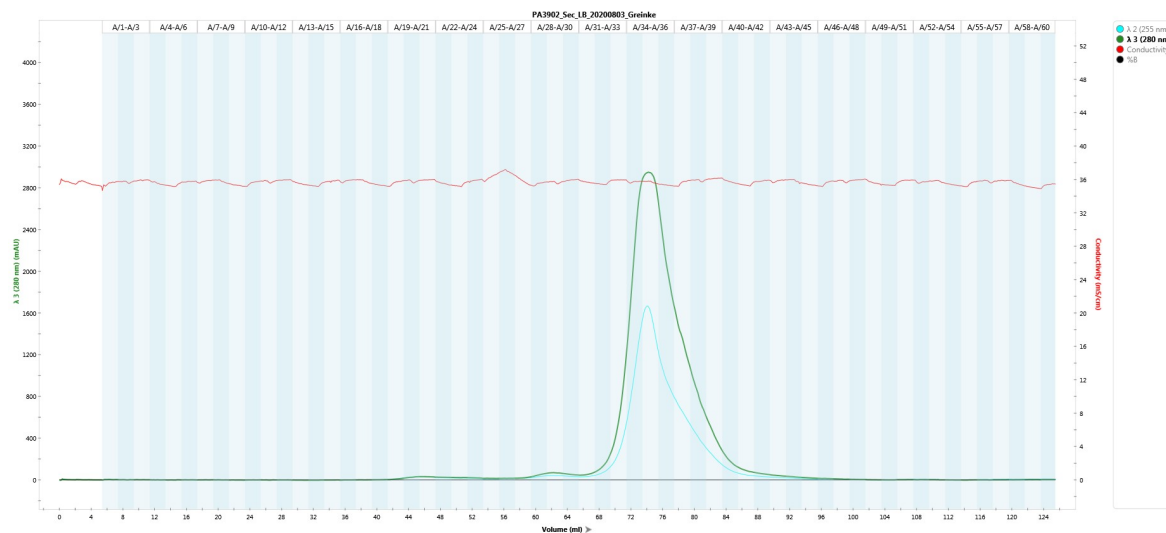
Figure 5. SEC chromatogram of wild type PaIVY purification. A symmetrical peak at the correct molecular weight for the protein of interest indicating pure PaIVY.

Performing site-directed mutagenesis on a protein has the potential to alter the purification protocol due to the potential loss or difference caused in the stabilization of the protein. H62 is the proposed active site of PaIVY making it an important target for site-directed mutagenesis. When the histidine residue is mutated valuable information can be gathered about the significance this residue plays in inhibition of lysozyme as well as its significance in binding to the reactive warhead. Initially site-directed mutagenesis was only intended to be performed for H62, however as data was collected via NMR studies about the warhead binding, further mutagenesis was performed on H20.

Site directed mutagenesis was performed on PaIVY to further understand H62's role in inhibition of lysozyme as well as its importance in warhead reactions. Previously, primers were originally designed by Aaron Schultz to mutate H62 to alanine, although he was never able to successfully obtain H62A before leaving. Site-directed mutagenesis was performed successfully by laboratory technician Sarah Waldron using these primers and following pfuUltra High-Fidelity DNA polymerase AD protocol.[6] Once the correct

mutation was successfully incorporated the DNA was transformed to DH5α cell line and mini-prepped to readily usable plasmid to be used in future experiments. When purifying H62A mutant protein the protocol was followed with the SEC buffer modification. H62A mutant typically did not express as well as wild-type PaIVY, usually only 0.5 mL at 1.2 mM was collected per two-liter prep.

It has been proposed that H62 is the active site of PaIVY in its inhibition of lysozyme and the proposed location that the warhead will bind, therefore mutating this residue should reduce or eliminate lysozyme inhibition, as well as no longer bind to the warhead. However, muramidase assays showed that H62A mutant still inhibits lysozyme and NMR studies showed that there is still chemical shift perturbation when the warhead is reacted with H62A mutant. The chemical shifts seen on the HSQC spectra for both wild-type PaIVY and the H62A mutant point to H20 as the possible alternative binding site. To further investigate this, new primers were designed to mutate H20.

Primers were designed using PrimerX. Once on the website the original plasmid sequence from DNASU of PaIVY was uploaded. Step two was left blank and for step three the primer design protocol drop-down menu was changed to QuickChange site-directed mutagenesis kit by Stratagene. Initially the goal was to mutate His20 to alanine like H62 was, however there were no primers generated for alanine so various other amino acids were tried. On the next page, the plasmid sequence was copied to the box and the mutation was manually changed from CAT coding for histidine to GAT coding for aspartic acid. Once PrimerX generated primers for H20D these primers were copied and pasted into Oligo Calc to identify any hairpins, annealing points, and self-complementary. Aspartic acid was chosen because it only required one nucleotide to be

changed and the primers designed contained one annealing site whereas many others had multiple annealing sites as well as a hairpin formation. Next the forward and reverse primers were ordered from idtDNA.

The primers were initially diluted to 100uM. Then following pfuUltra High-Fidelity DNA polymerase AD protocol, PCR was performed using wild type PaIVY as the template DNA for one PCR reaction. In a separate PCR reaction tube H62A mutant plasmid was used as the template DNA to get the H20D/H62A double mutant PaIVY with no other histidine's in the sequence. Following a DNA gel was run on both sets of mutants and a band was seen indicating that the PCR worked. Next the DNA was transformed using DH5α cells and left to grow overnight on an LB agar kanamycin plate. Colonies from each plate were mini prepped to obtain the plasmid. E.Z.N.A Plasmid DNA Mini Kit II was used. The plasmid was eluted into 40uL sterile deionized water at 219ng/uL and 150ng/uL for H20D and H20D/H62A respectively. The plasmid was then set for sequencing using Genewiz DNA Sequencing Services.

Protein purification was carried out following wild type PaIVY protocol to ensure the same protocol and buffers would work for the mutants. The original protocol worked for both mutants except during the dialysis and TEV cleavage. In the His2 spectrum there is a peak of uncleaved protein in the elution meaning not all of the His tag was cut off from the protein during the dialysis and TEV cleavage. The peak was not significantly large enough to do a second dialysis and TEV cleavage, but there likely needs to be a higher concentration of TEV added to the dialysis in order to cleave all of the protein. At the size exclusion chromatography (SEC) step, both mutants were run in the SEC buffer

initially because they were unlabeled and would not be used for NMR studies. Later the same modification of performing the SEC in other buffers was utilized for NMR studies.

1. Ladder
2. H20D His1
3. H20D His2
4. H20D SEC
5. H20D/H62A His1
6. H20D/H62A His2
7. H20D/H62A SEC (Fraction 14)
8. H20D/H62A SEC (Fractions 16-18)
9. H20D/H62A SEC (Fractions 19-20)
10. H62A 0.43mM
11. H62A + [Re(CO)$_3$(H$_2$O)$_3$]Cl$^+$ (**1**)
12. H62A + [Re(CO)$_3$(Phen)H$_2$O]Cl$^+$ (**3**)
13. Ladder
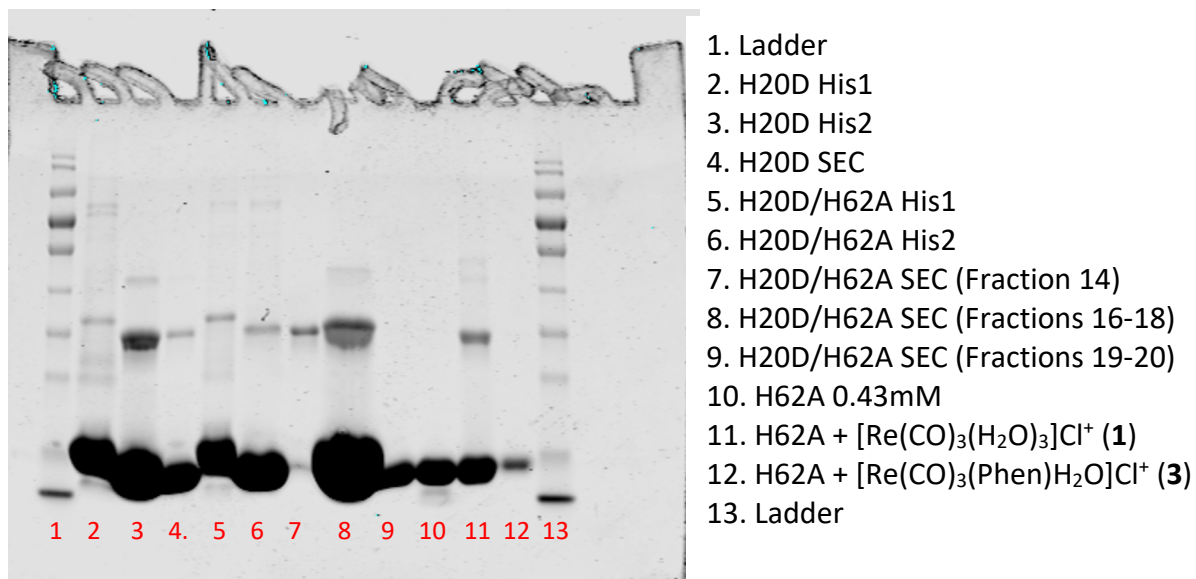
Figure 6. SDS-PAGE gel of H20D PaIVY and H20D/H62A PaVIY

When sequencing arrived back, it was confirmed that the correct mutation was found on both. Moving forward, the next step was to purify the mutant proteins using ^{15}N labeled ammonium chloride in M9 minimal media to use for NMR studies. When preparing samples for NMR, the only purification difference is that the SEC is run in 20 mM sodium phosphate buffer at pH 6.0. to eliminate the need for buffer exchange later.

Chapter Three

Designing a Warhead and NMR Spectrometry

Due to its ability to bind to amino acids and specifically its ability to bind N-acetyl histidine and histidylhistidine residues such as the histidine-62 (H62) residue of PaIVY, the original warhead of focus is a rhenium(I) tricarbonyl complex. $Re(CO)_3(H_2O)_3^+$ (**1**) was found to bind to His15 of Hen Egg White Lysozyme (HEWL)[8] sparking the idea that it would also be an ideal candidate for a reactive warhead targeting the exposed H62 loop on PaIVY. To begin testing this hypothesis $[Re(CO)_3(H_2O)_3]Br^+$ (**1**) was synthesized following the published protocol by Lazarova et al.[24] in which $Re(CO)_5Br^+$ or $Re(CO)_5Cl^+$ was refluxed for several hours with water to produce $[Re(CO)_3(H_2O)_3]Br^+$ (**1**) (**Figure 7**). After the reaction was refluxed to completion, the product containing solution was transferred to a 50 mL falcon tube and placed in the -80°C freezer overnight. The following day the frozen solution was lyophilized for 24 hours. The product was a tan/white, flakey solid that is easily soluble in water. Once $[Re(CO)_3(H_2O)_3]Br^+$ (**1**) was synthesized, a stock solution in water was made at 100 mM concentration which would be used in following experiments.
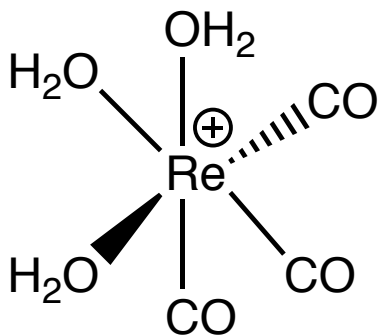
Figure 7. Structure of $Re(CO)_3(H_2O)_3^+$ (**1**)

To test the viability of this warhead (**1**), the stock solution of $[Re(CO)_3(H_2O)_3]Br^+$ (**1**)was combined with ^{15}N labeled PaIVY, purified by adapting a prior student's, Aaron Schultz's, protocol as described in appendix two, to monitor any binding interactions using an HSQC program on the Bruker 400 MHz NMR located at Kennesaw State University. The NMR spectra were all processed using NMRPipe and analyzed with SPARKY.

Several experiments were performed initially to gauge how much of the warhead (**1**) would be necessary and at what time point the reaction between $[Re(CO)_3(H_2O)_3]Br^+$ (**1**) and PaIVY was completed. To test this, 1.25 mM ^{15}N PaIVY was reacted with the warhead (**1**) at 5x molar equivalents and monitored at time zero, 24 hours, 72 hours, and seven days incubation time. At a high concentration of ^{15}N PaIVY and 5x excess warhead (**1**) with a short incubation time of 24 hours, it was found that many of the previously visualized amino acids peaks from the reference ^{15}N HSQC of PaIVY containing no warhead were no longer present after reacting with $[Re(CO)_3(H_2O)_3]Br^+$ (**1**) (**Figure** 8). This result demonstrated some of the limitations of using NMR. One drawback of using NMR is that high molecular weight species are not visible. However, the addition of just the small molecule such as the warhead (**1**) should not cause the molecular weight to

become too large and no longer visible. From this is was speculated that multiple protein molecules were interacting with one warhead (**1**) and creating a dimer or trimer-like complex that would have a much higher apparent molecular weight with broader lineshapes that would make the protein difficult to observe by solution NMR.
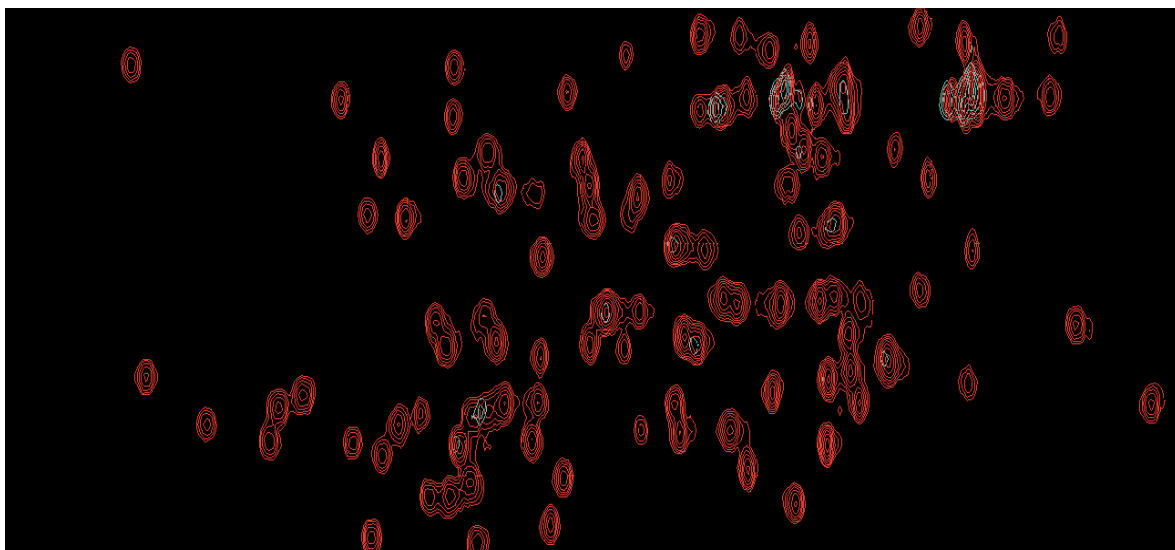
Figure 8. Reference HSQC of ^{15}N PaIVY at 1.23 mM with no warhead(red) overlaid with few residual peaks from 1.25 mM ^{15}N PaIVY reacted with five equivalents of [Re(CO)$_3$(H$_2$O)$_3$]Br$^+$ (**1**) (blue) both in 20 mM sodium phosphate buffer pH 7.4. Due to the disappearance of the peaks, i.e. linebroadening, it is proposed that there is likely high molecular weight species being formed that limit NMR visibility of the compound.

To further investigate this phenomenon, a size exclusion chromatography (SEC) experiment that separates species based on their molecular weight was applied to the NMR sample (**Figure 9**). Interestingly the SEC showed three to four new peaks eluting between 50 and 62 mL whereas purified monomer IVY elutes at 69 to 72 mL. These additional peaks indicate multiple species of higher molecular weight which corresponded to dimers, trimers, and other oligomers of PaIVY that are formed in the presence of the warhead (**1**). This was then confirmed with an SDS-PAGE protein gel (**Figure 10**). In the last four lanes it can be seen that there are two additional bands at higher molecular weight confirming oligomerization. The SEC also showed that at this

high concentration the formation of the trimer-like complex and dimer was favored over the monomer which can be seen by the significantly higher peak on the SEC for the higher molecular weight species.
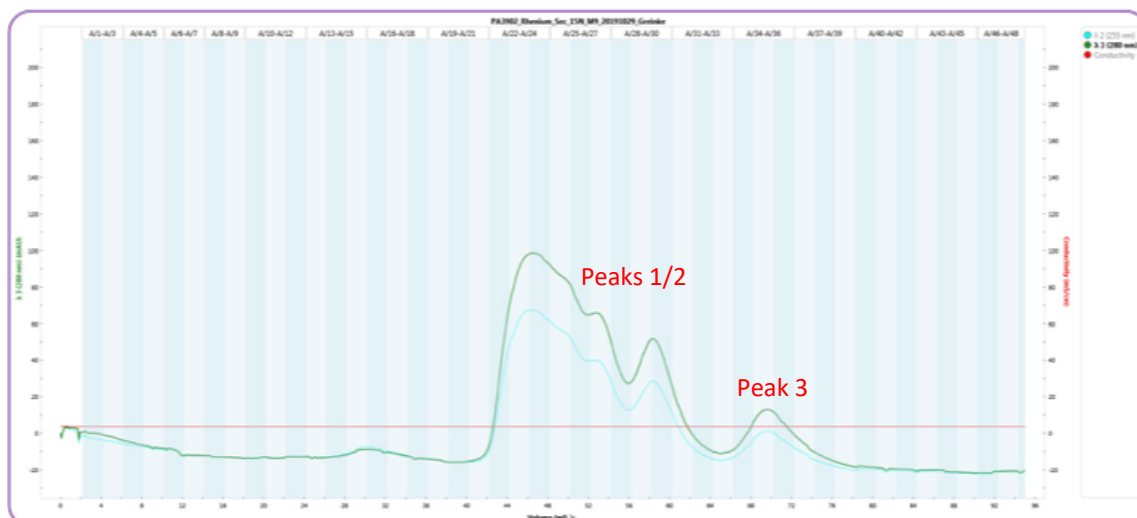
Figure 9. Size exclusion chromatography (SEC) spectrum of 1.25 mM PaIVY reacted with five equivalents of $[Re(CO)_3(H_2O)_3]Br^+$ (**1**) in 20 mM sodium phosphate buffer at pH 7.4. Peaks labeled corresponding to fractions collected and run on SDS-page gel in **Figure 10**. Peaks 1/2 correspond to oligomers of PaIVY. Peak 3 corresponds to the monomer peak of PaIVY.
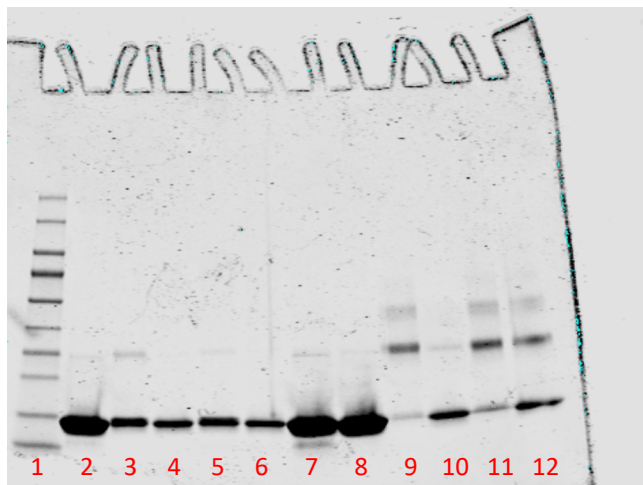
1. Ladder
2. PaIVY heated
3. Peak 1/2 heated
4. Peak 3 heated
5. Peak 1/2 heated, BME
6. Peak 3 heated, BME
7. PaIVY heated, BME
8. PaIVY unheated
9. Peak 1/2 unheated
10. Peak 3 unheated
11. Peak 1/2 unheated, BME
12. All peaks unheated, BME

Figure 10. SDS-PAGE protein gel using various conditions to show PaIVY is present in higher molecular weights than usual indicating one $[Re(CO)_3(H_2O)_3]Br^+$ (**1**) is coordinating to multiple PaIVY molecules. Boiling causes the oligomers to dissociate while they remained in the unheated samples. (Peaks from **Figure 9** SEC).

Due to the formation of these higher molecular weight species the warhead was redesigned to stop the coordination of multiple PaIVY molecules to the rhenium warhead. A rhenium tricarbonyl complex was still the primary focus for a warhead but there needed to only be one site for the complex to coordinate to PaIVYs histidine residue, i.e. only one easily exchangeable water ligand, in order to prevent the formation of dimers and trimers. From this criterion the ideal warhead contains rhenium(I) at the center coordinating to three carbonyls and a bidentate ligand such as phenanthroline or bipyridine that leaves one rhenium coordination site free and able to coordinate to H62 of PaIVY. $Re(CO)_3(Phen)^+$ (**2-4**) became the focus of the new warhead design. **Figure 11** shows a representation of how the warhead (**2-4**) is intended to coordinate to H62 of PaIVY.
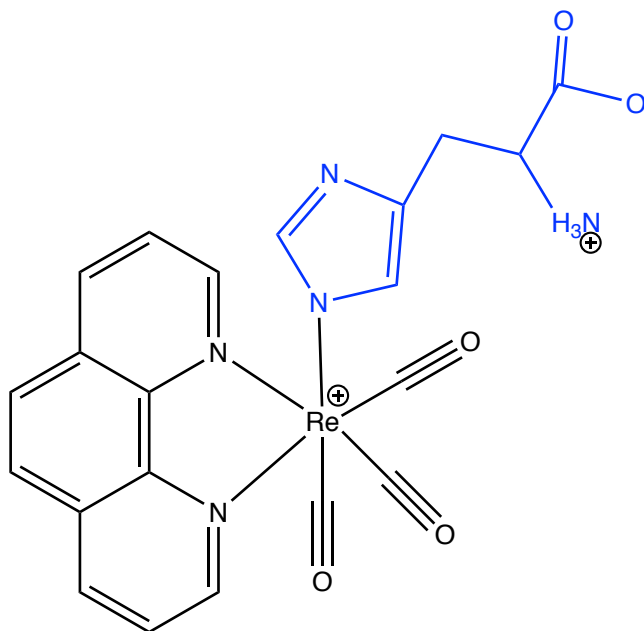
Figure 11. Schematic of $Re(CO)_3(Phen)^+$ (**2-4**), shown in black, coordinating to a histidine, shown in blue.

$Re(CO)_3(Phen)X^+$ was synthesized in two ways. Initially, $[Re(CO)_3(H_2O)_3]X^+$ (**1**) had already been synthesized, so it was utilized in the synthesis for the newly designed

warhead (**2,3**). Previously synthesized $[Re(CO)_3(H_2O)_3]X^+$ (**1**) was mixed with 1,10 phenanthroline in a 1:1.1 molar ratio in a round bottom flask containing acetone at room temperature with stirring to form $[Re(CO)_3(Phen)H_2O]Br^+$ (**2**). The solution immediately turned bright yellow supporting product formation. Next, the mixture was moved to a 50 mL falcon tube and left open to evaporate the acetone over the next few days until a fine yellow powder was left. Another variation of this same warhead is $[Re(CO)_3(Phen)H_2O]Cl^+$ (**3**) in which $Re(CO)_5Cl$ is the starting material instead of $Re(CO)_5Br$. The reaction is carried out in the same way with a difference being the halogen ion that remains in solution as well as the option to carry it out in a H_2O solution containing purified protein in the second step.

A more efficient protocol published by Chakraborty et al.[12] was discovered which required a one-step process. Since this protocol was one-step the remaining H_2O on the warhead from the previous protocol was replaced with a Cl because the starting material is $Re(CO)_5Cl$. The H_2O from the previous protocol and the Cl from the new protocol would be replaced when reacted with PaIVY. A comparison of all the warheads synthesized and their structural differences can be seen in **Figure 12** and **Figure 13**. The synthesis required one molar equivalent of $Re(CO)_5Cl$ to be mixed with 1,10-phenanthroline in chloroform and methanol and then refluxed for 24 hours to create $Re(CO)_3(Phen)Cl^+$ (**4**). The product was then vacuum filtered and left to dry for several days leaving a fine yellow powder.

1. x= Br; Cl
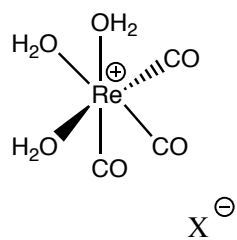
2.
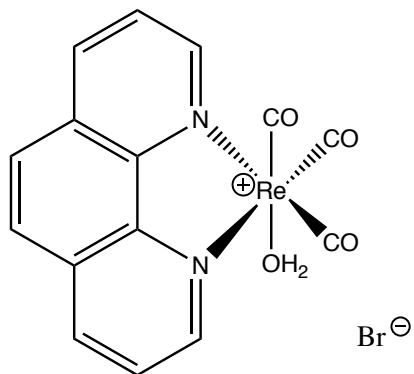
3.
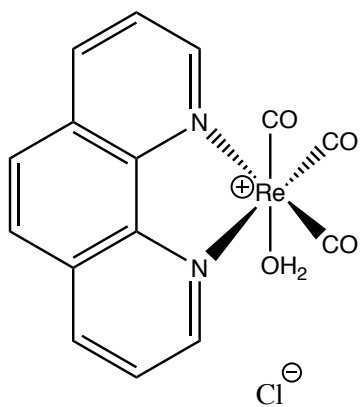
4.
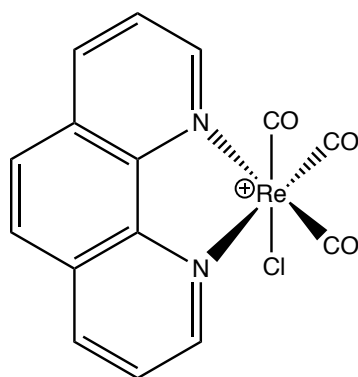
Figure 12. Structures for each warhead (**1-4**).

1.
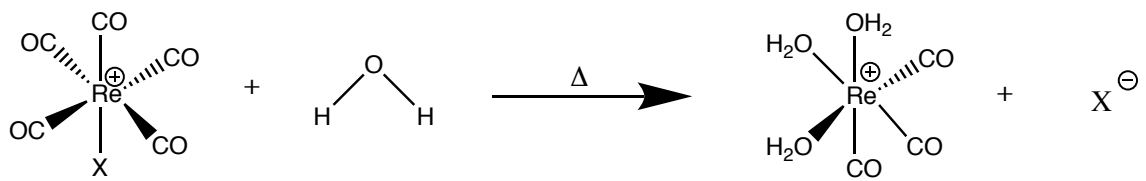
X= Br; Cl

2.

Step 1
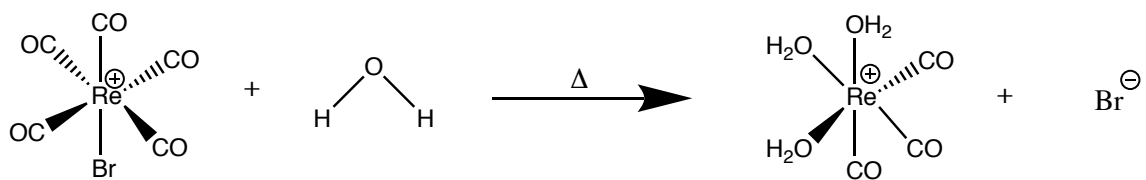
Step 2

3.

Step 1

Step 2

4.
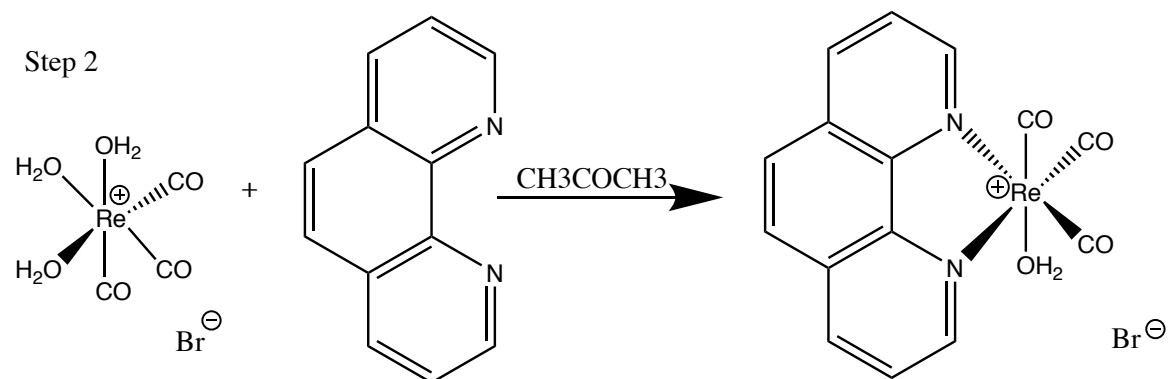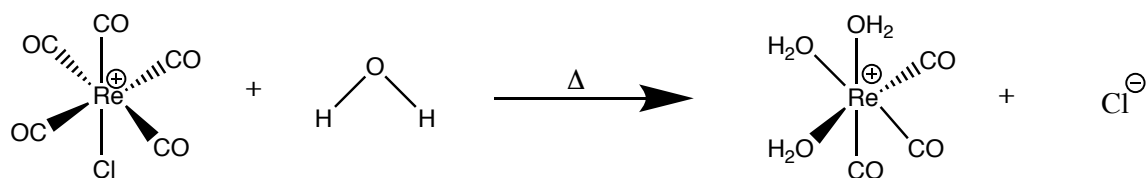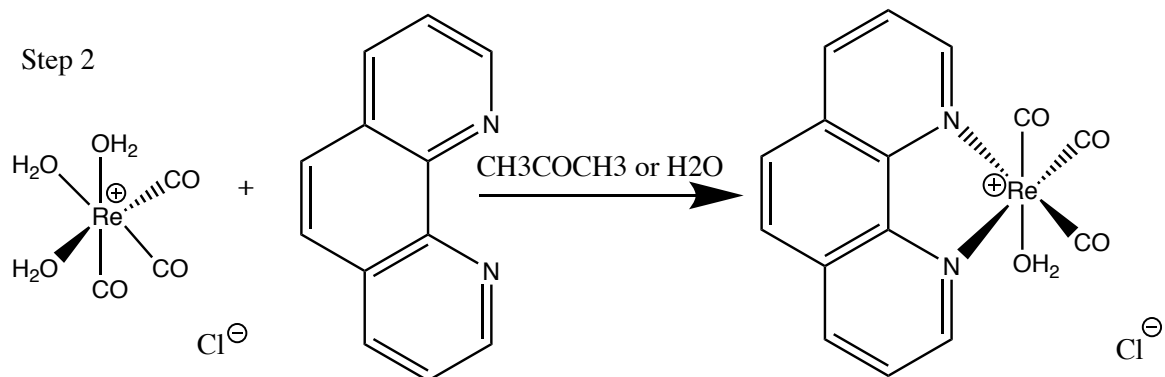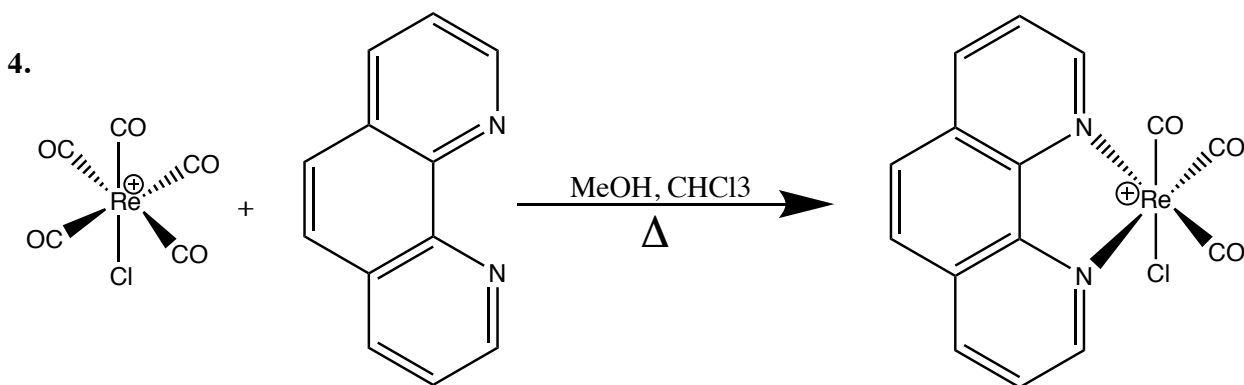
Figure 13. Reaction schemes of each warhead used. **2** and **3** made in the two-step process involving $Re(CO)_3(H_2O)_3^+$ differing only in the halogen ion present. Warhead **4** made in the one-step process.

Once both identities were confirmed, the focus of the warhead became

$Re(CO)_3(Phen)Cl^+$ (**4**) because it was easily produced by the one-step synthesis. To test

the viability of the new warhead (**4**) design, several ^{15}N HSQC NMR experiments were

performed in order to find the ideal incubation time of the reaction to reach completion as

well as how much excess of the warhead (**4**) would be necessary. 1.06 mM purified ^{15}N

PaIVY was reacted with one, three, and five molar equivalents of warhead (**4**). It was

monitored using the 400 MHz NMR located at KSU at time zero, 24 hours, 72 hours,

seven days, and 10 days. Each of the different concentrations showed no changes at time

zero, little to no changes at 24 hours (**Figure 14**), and many slight changes after three

days (**Figure 15**). The ^{15}N HSQC showed some chemical shift perturbation, but it was

not as significant as expected. It was speculated that due to the low solubility of

phenanthroline in water that not enough of the warhead (**4**) was in solution to show a

significant chemical change on the NMR. Unfortunately, there is little to change about

the buffer solution containing PaIVY in order to improve solubility of the warhead (**4**)

due to the protein's need to be in an aqueous environment or risk denaturation. In an

attempt to help improve the solubility of the warhead (**4**), the pH of the phosphate buffer

was reduced from 7.4 to 6. Unfortunately, this did not significantly change the solubility

of the warhead (**4**), so a new technique needed to be adapted in order to achieve the

complex formation between the warhead (**4**) and PaIVY.
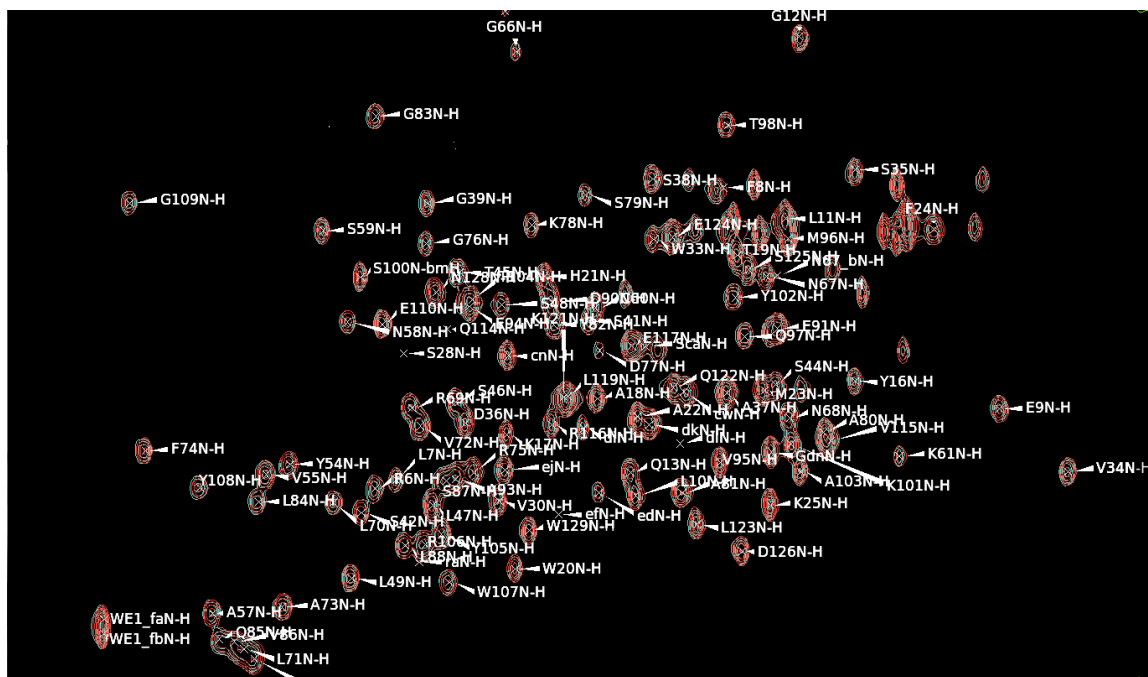
Figure 14. Overlay of the reference ^{15}N HSQC at 1.23 mM (red) and 1.06 mM PaIVY reacted with three equivalents of [Re(CO)$_3$(Phen)H$_2$O]Br$^+$ (**2**)after 24 hours incubation (blue). No chemical shift perturbations noted.
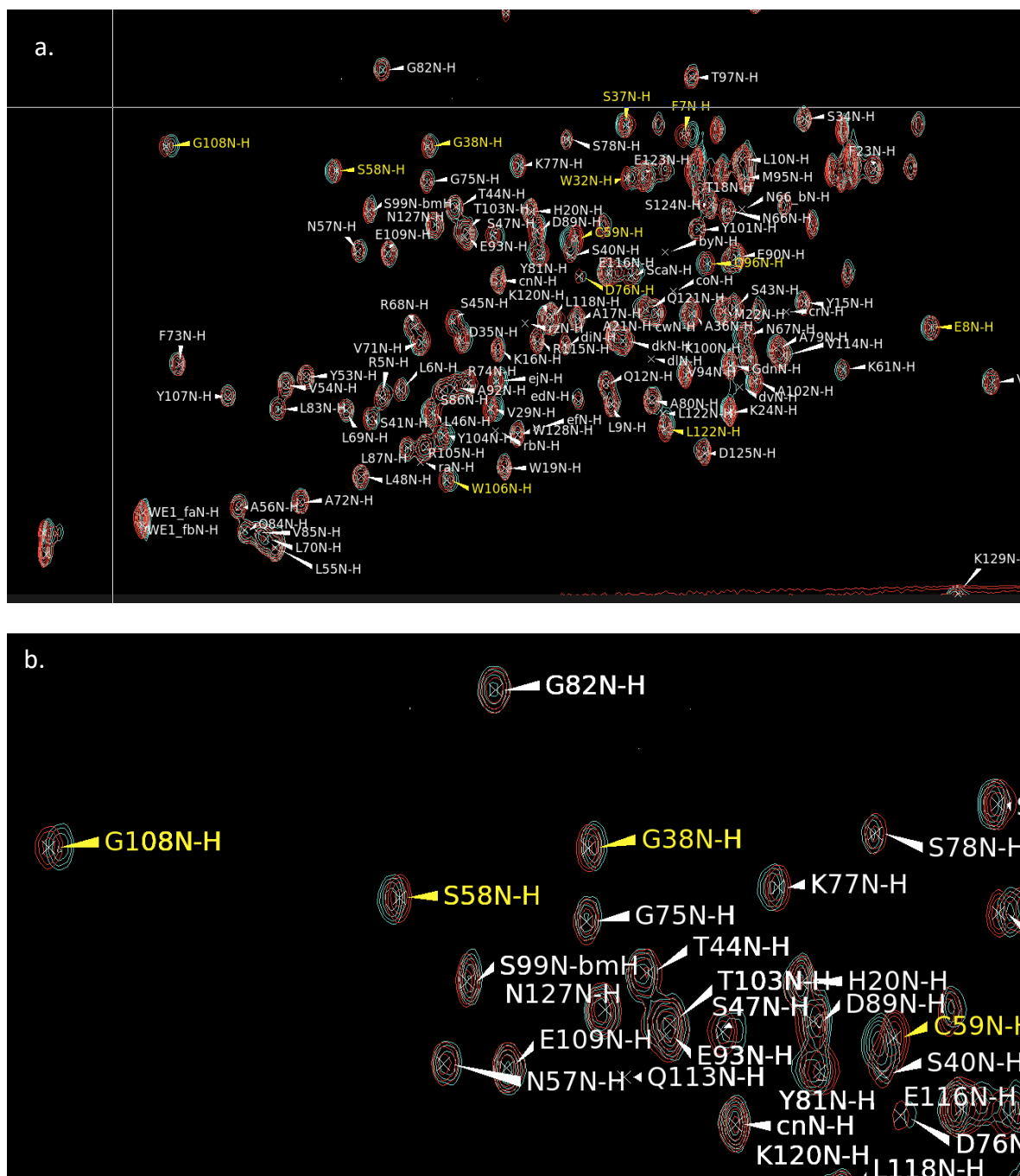
Figure 15. a.) Overlay of the reference ^{15}N HSQC at 1.23 mM (red) and 1.06 mM PaIVY reacted with three equivalents of $[Re(CO)_3(Phen)H_2O]Br^+$ (**2**) after three days incubation (blue). Labels in yellow indicate some of the notable chemical shift perturbations. b.) Zoomed in image from Figure 11 to show the slight chemical shifts noted in yellow.

Due to the solubility issue, it was later decided to do the reaction in two parts.

First allow a two times excess of $[Re(CO)_3(H_2O)_3]X^+$ (**1**) to react with purified PaIVY at

a low concentration, about 0.5 mM, for two days and then add an excess of

phenanthroline into the reaction tube to react for an additional two days. The solution

began turning yellow after the first 24 hours and increased in intensity by the 48 hours

reacting at room temperature. The color after 48 hours can be seen in **Figure 16**. The

reaction was then loaded on the SEC column and the monomer peak was collected

(**Figure 17**), concentrated to 0.5 mM and subsequently an HSQC NMR was performed

using the 600 MHz NMR located at KSU. It can be seen that doing this reaction at a

lower concentration than previously favored the formation of the monomer peak rather

than a trimer or dimer (**Figure 18**). The HSQC spectra showed promising results of

chemical shift perturbation however, many of the peaks were split indicating that not all

of the PaIVY was in complex with the warhead (**3**). Another interesting discovery was

that majority of the amino acid residues that experienced a shift were not located near

H62 as expected but rather near H20 (**Figures 18 and 19**).

Figure 16. Reaction tubes after 1,10-phenanthroline was added to PaIVY reacted with
$[Re(CO)_3(H_2O)_3]X^+$ (**1**) indicating the formation of the warhead (**3**) due to the yellow
color.
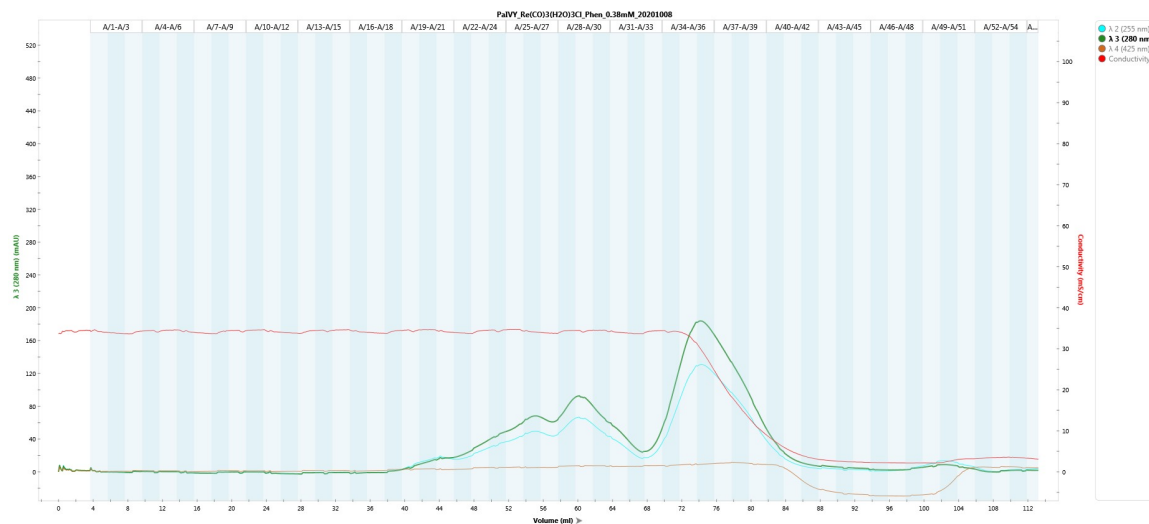
Figure 17. Size exclusion chromatography (SEC) of $[Re(CO)_3(Phen)(H_2O)]Cl^+$ (**3**) reacted with PaIVY
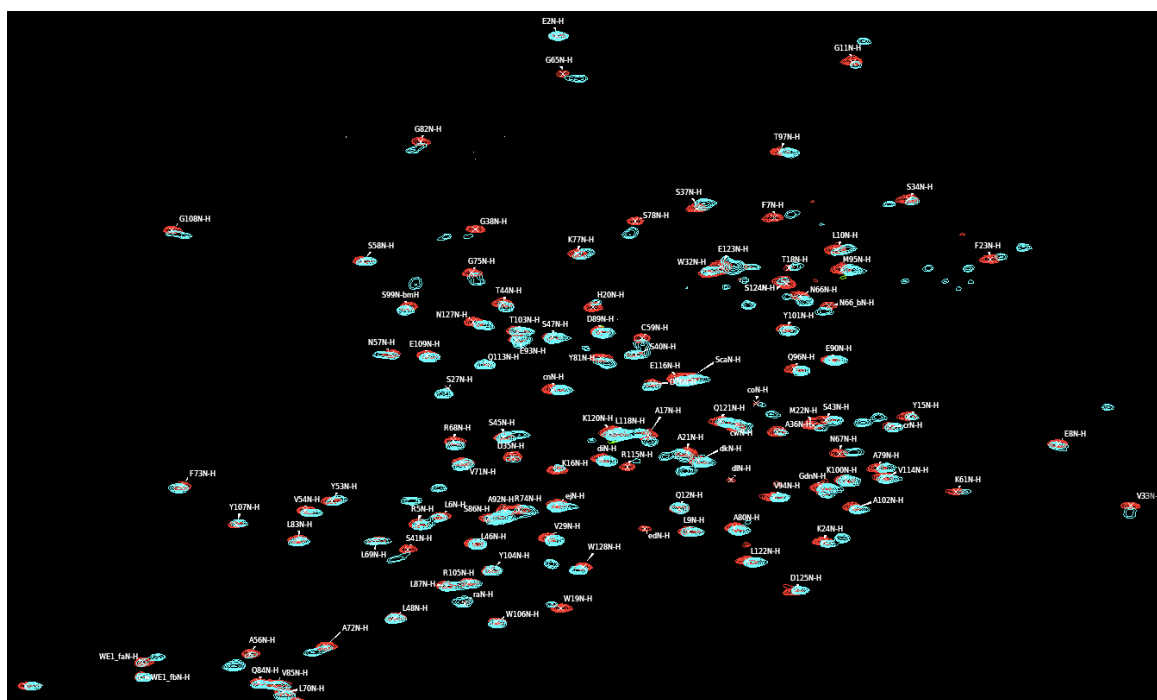
Figure 18. ^{15}N HSQC spectra of monomer peak from SEC of $[Re(CO)_3(Phen)(H_2O)]Cl^+$ (**3**) reacted with PaIVY at 0.3 mM in blue. Reference HSQC in red at 1.06 mM PaIVY after 72 Hr.
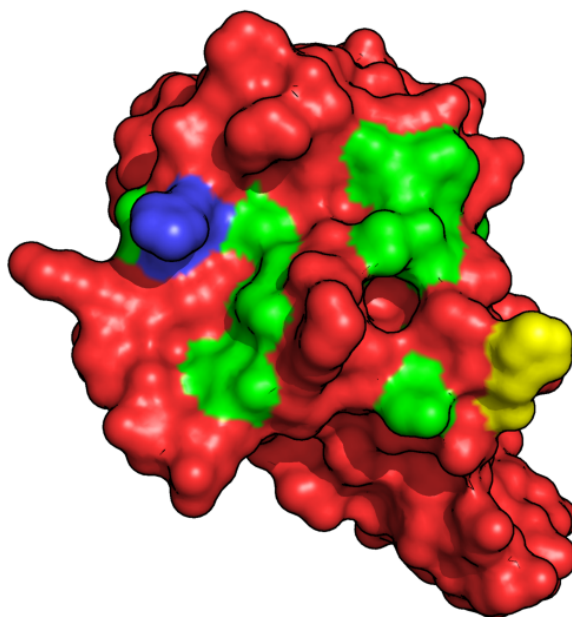
Figure 19. Surface crystal structure of PaIVY.[8] In green are the amino acid residues with the most chemical shift perturbation after reacting with the warhead (**3**). The rest of the amino acid residues are in red. H20 shown in blue. H62 is shown in yellow.

To investigate further that H62 is not the warhead (**3**) binding site site-directed mutagenesis was performed and more NMR studies were done. Using the same technique of reacting purified ^{15}N PaIVY with $[Re(CO)_3(H_2O)_3]X^+$ (**1**) then adding 1,10-phenanthroline and SEC purifying the sample, ^{15}N HSQC was performed at 600 MHz. As it was suspected, the H62A mutant PaIVY still had chemical shift perturbation when it was reacted with the warhead (**3**) (**Figure 20**). This indicated that H62 was not the binding site for the warhead (**3**). Additionally, an SDS-Page gel was run that indicated the H62A mutant was also forming dimer/trimers with PaIVY indicating the warhead (**3**) was reacting with H62A (**Figure 21**). In order to uncover if the warhead (**3**) was binding to H20 instead site-directed mutagenesis was performed for a second time.
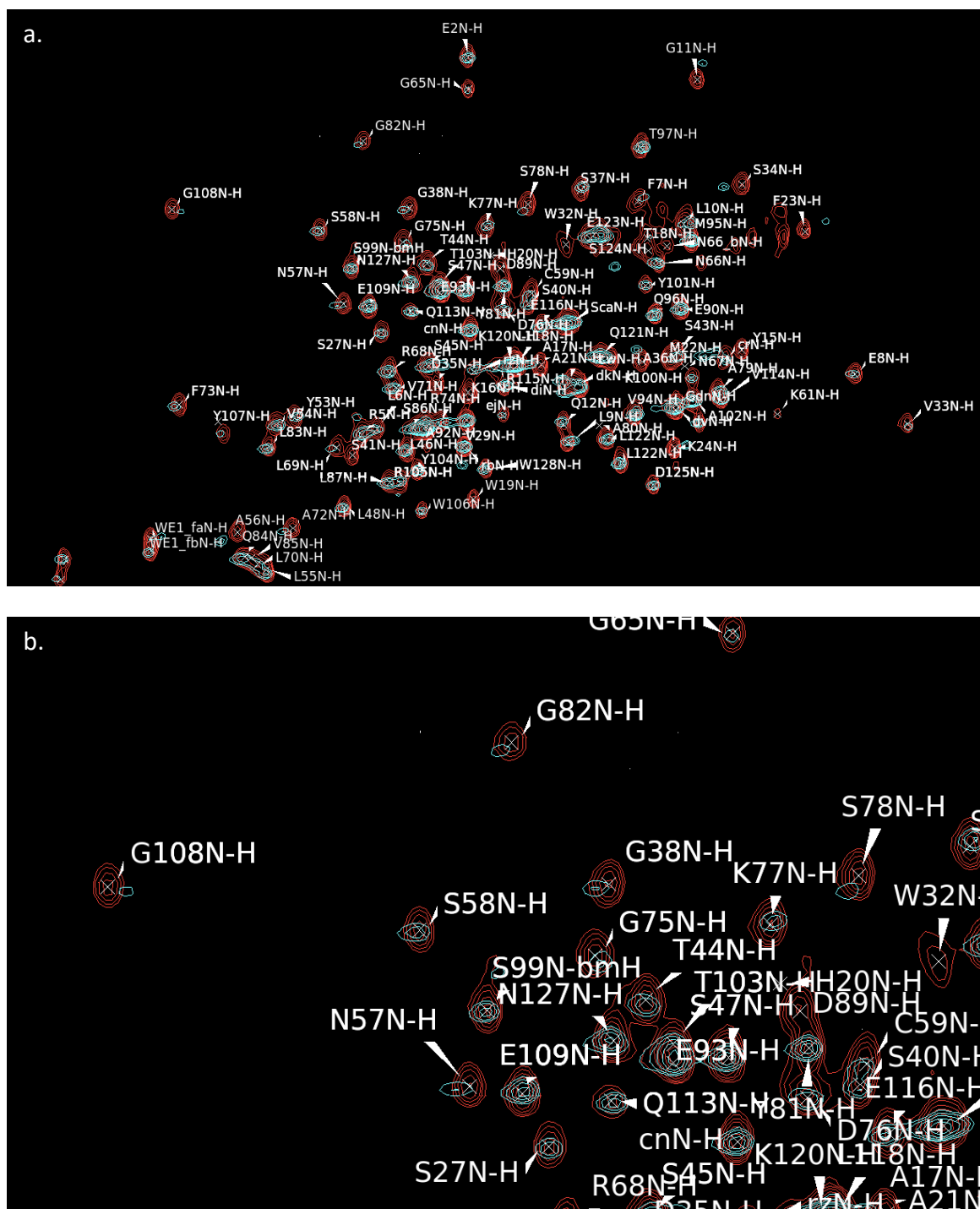
Figure 20. a.) H62A reference in red at 200uM in a 300 mm tube overlaid with H62A reacted with $[Re(CO)_3(Phen)(H_2O)]Cl^+$ (**3**) in blue at 0.5 mM in a 5 mm tube. This indicated the warhead (**3**) is still binding to PaIVY without H62 present. b.) Zoomed in view to see chemical shift perturbation.

1. Ladder
2. PaIVY 0.5mM
3. PaIVY w/ Re(CO)$_3$(H$_2$O)$_3$ 0.38mM
4. PaIVY monomer SEC peak 0.38mM
5. PaIVY dimer SEC peak 0.38mM
6. PaIVY trimer SEC peak 0.38mM
7. PaIVY w/ Re(CO)$_3$(H$_2$O)$_3$ 0.35mM
8. PaIVY w/ Re(CO)$_3$(Phen)Cl$^+$ 0.35mM
9. PaIVY monomer SEC peak 0.35mM
10. H62A PaIVY w/ Re(CO)$_3$(H$_2$O)$_3$ 0.4mM
11. H62A PaIVY w/ Re(CO)$_3$(Phen)Cl$^+$ 0.4mM
12. H62A PaIVY monomer SEC peak 0.4mM
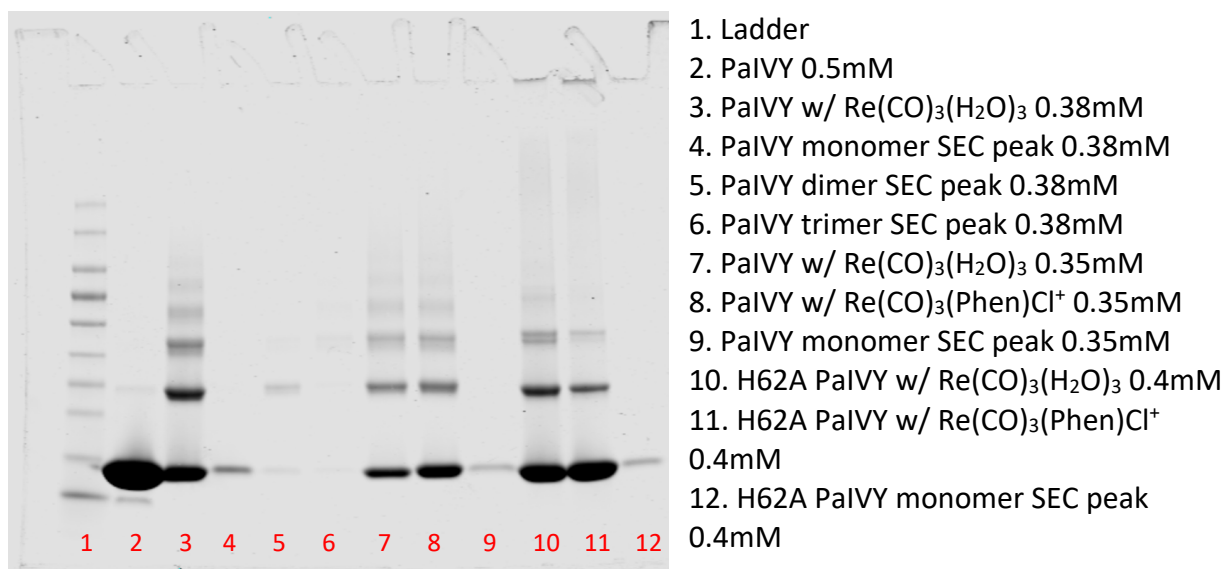
Figure 21. SDS-PAGE gel image of wild type PaIVY and H62A mutant at different stages of soaking with the warhead (**1,3**).

The next step in figuring out if the warhead (**3**) was binding to H20 instead was site-directed mutagenesis to H20D. A single mutant and a double mutant removing both histidine residues in PaIVY was made. Next both of these mutants were reacted with the warhead (**3**) using the same technique and subject to NMR using the 600 MHz located at KSU. The HSQC spectra of the H20D and H20D/H62A mutants do not contain amino acid labels because the spectra were altered when the mutations were made requiring the need for three-dimensional NMR analysis to locate the residues and apply appropriate labeling that was not done. The HSQC spectra of the H20D mutant showed little to no chemical shift perturbation but rather broadening. This is most clearly noticed on residues G38 and S39 (**Figure 22**). These two residues were also affected when wild type PaIVY as well as H62A mutant was reacted with the warhead (**3**). This indicates that [Re(CO)$_3$(Phen)(H$_2$O)]Cl$^+$ (**3**) is still binding to H20D mutant PaIVY but not as widely as previously. One explanation for this is a conformational change may be occurring when the warhead (**3**) binds. To further investigate this the H20D/H62A double mutant was

also subject to a reaction with the warhead (**3**) and an HSQC was performed. This NMR

spectra showed no chemical shift perturbation indicating that the warhead (**3**) was not

binding to any other residue on PaIVY (**Figure 23**). This confirms that there is likely a

conformational change happening when the warhead (**3**) binds that involves both the H20

and H62 residues.
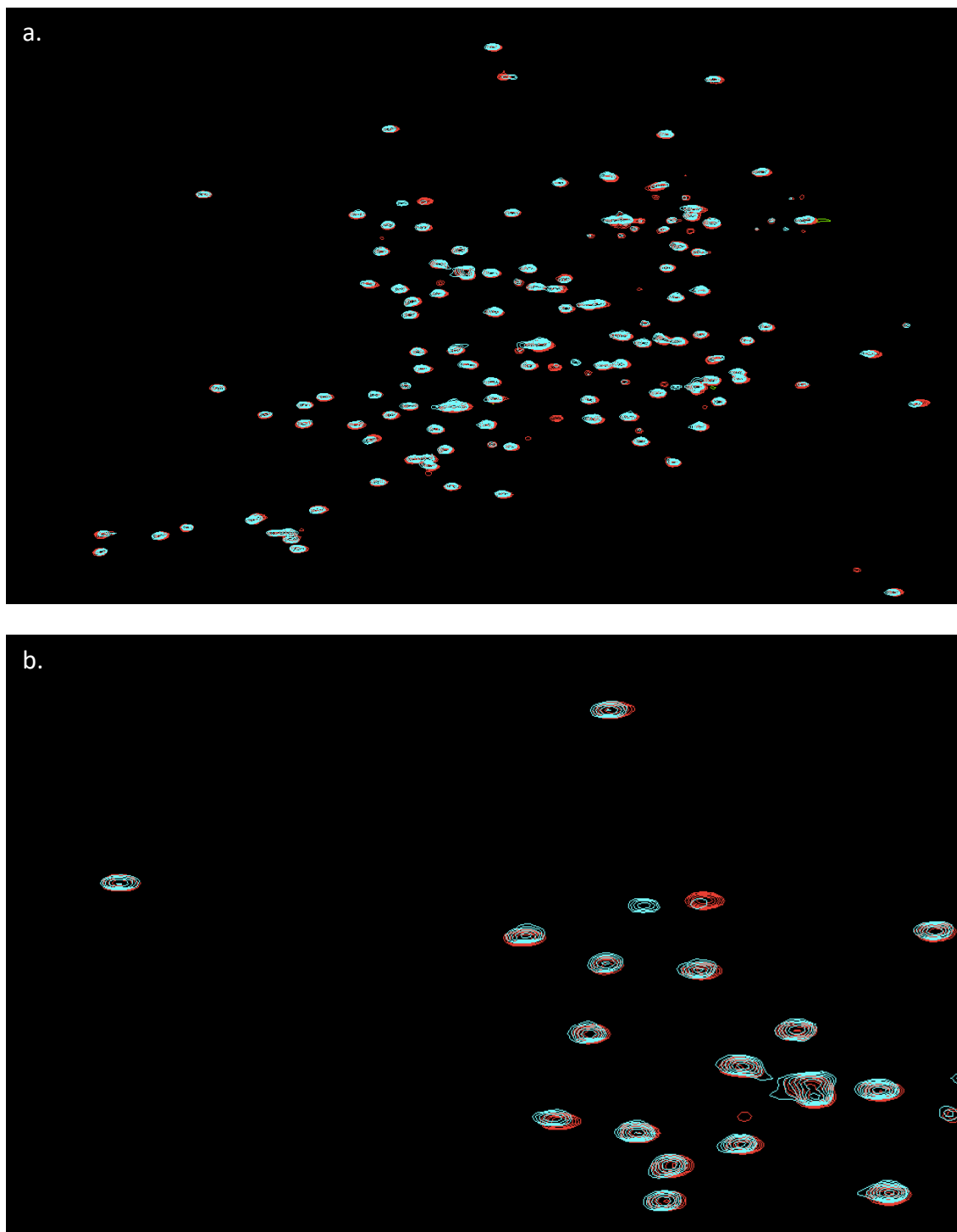
Figure 22. a.) Reference HSQC of H20D at 1.2 mM in red overlaid with H20D reacted with the warhead (**3**) at 0.38 mM in blue. b.) Zoomed in image.
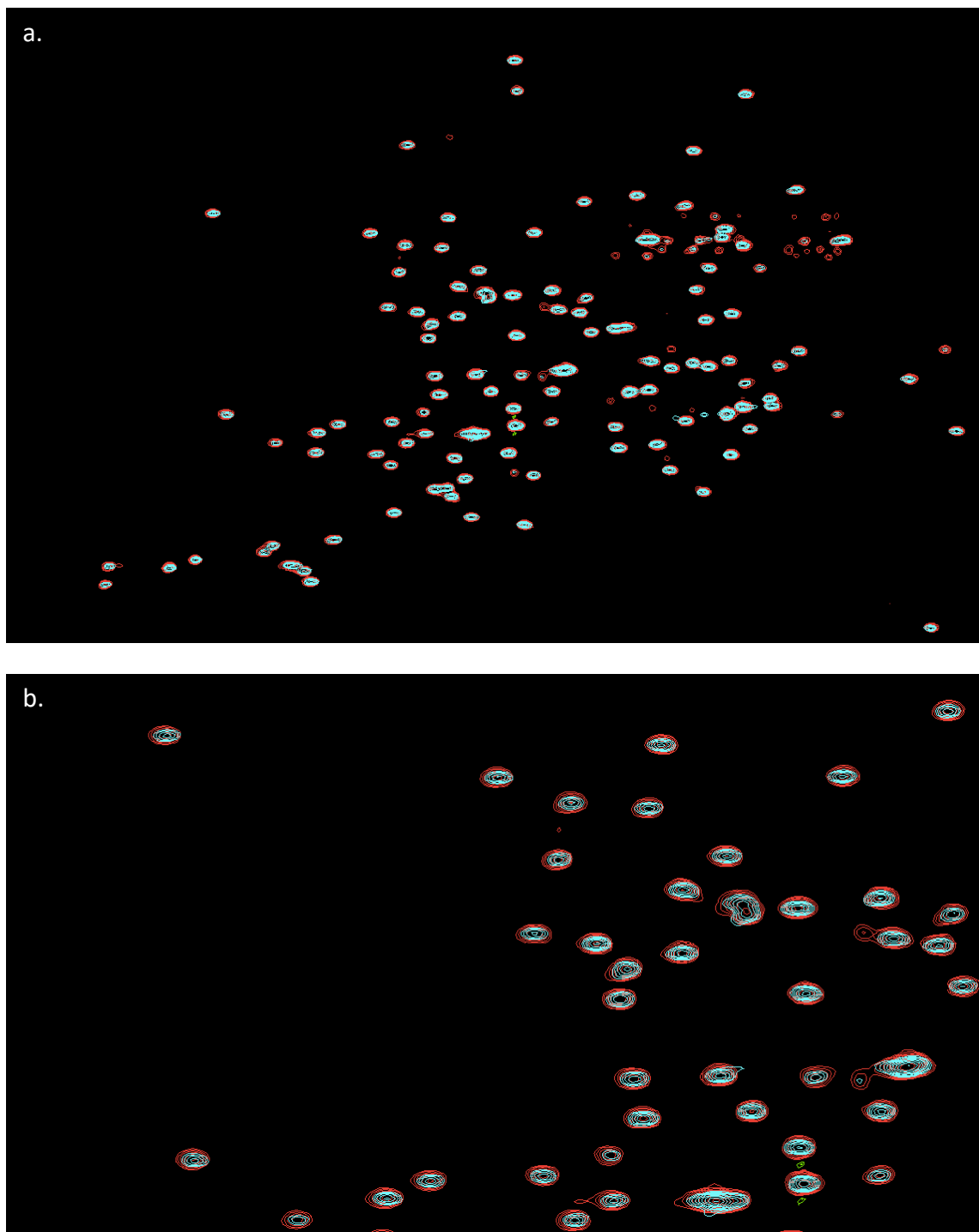
Figure 23. a.) Reference HSQC of H20D/H62A double mutant at 1.4 mM in red overlaid with H20D/H62A reacted with the warhead (**3**) at 0.48 mM in blue. b.) Zoomed in image.

Chapter Four

Muramidase Assay of Wild Type and Mutant PaIVY with and without the

Warhead

A muramidase assay is a lysozyme activity assay in which the ability of lysozyme

to lyse cells is monitored using fluorescence when in the presence of PaIVY. In the assay

lysozyme activity is measured on *Micrococcus lysodeikticus* cell walls which are

fluorescently labeled to allow for quantification of the cells being lysed. The cells are

labeled in such a way that the fluorescence is suppressed when the cells are lysed. The

catalytic activity of lysozyme combats this quenching causing an increase in the

fluorescent signal detected when it is properly functioning. The fluorescent signal

detected is proportional to the activity of lysozyme[20] making this assay extremely useful

in determining if certain residues in the protein sequence are important or necessary for

the inhibition of lysozyme as well as indicate if the addition of a reactive warhead (**3**) will

prevent PaIVY from inhibiting lysozyme. In this assay Hen Egg White Lysozyme

(HEWL) is used. It is expected that when wild type PaIVY is placed in the presence of

lysozyme the cells fluorescence signal will decrease due to PaIVY's ability to inhibit

lysozyme and therefore prevent lysing of the cells. This can be seen in **Figure 24**.

Each muramidase assay was performed using Enzcheck Lysozyme Assay Kit (E-

22013).[20] The DQ cells were prepared following this protocol, however the lysozyme

provided in the kit was not used but rather HEWL purchased from Hampton Research

and was prepared by making a 5 mM stock solution. Each assay was prepared using

Corning 96 well black polystyrene plates with a clear bottom. For each muramidase

performed 25uL of HEWL was used in each well, a varying amount of PaIVY was used

based on the original concentration of the protein, a varying amount 1X reaction buffer to

bring the volume of PaIVY and 1X reaction buffer to 25uL total and lastly each well

contained 50uL of fluorescently labeled DQ cells. The total volume used in each well

was 100uL. The exact amounts used in each well for each reaction can be seen in **Tables**

1 and 2. DQ cells were always added last to ensure the each well reaction started at

approximately the same time. Once each well had the proper amount of each component,

the plate was placed in darkness and left for 30 minutes until the fluorescent signal was

measured using the Synergy H1 fluorescent plate reader. The plate reader was set to an

excitation of 485 nm and emission scan at 530 nm and the background fluorescence was

subtracted using the blank.
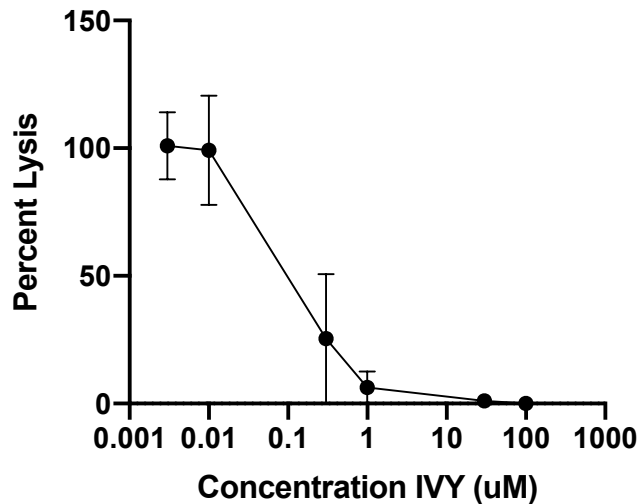
Figure 24. Muramidase assay of wild type PaIVY at various concentrations in the
presence of 100 nM hen egg white lysozyme (HEWL).

The proposed active site of PaIVY's inhibition of lysozyme is the exposed H62 loop, therefore it was hypothesized that when H62 is mutated to H62A there would no longer be a decrease in the fluorescence signal in the assay because lysozyme would be free to effectively lyse the cells. Once the H62 residue of PaIVY was correctly mutated to alanine, a muramidase assay using the mutated protein was performed following **Table 1** concentrations. Interestingly, the H62A mutant behaved almost identical to wild type IVY when in the presence of lysozyme. This is indicated by the fluorescent signal decreasing as the concentration of PaIVY increased (**Figure 25**). The decreasing fluorescence recorded with increasing concentrations of PaIVY in combination with BLI data collected by another graduate student Amani Gaddy, indicated the H62A mutant still binds lysozyme only slightly weaker than wild type IVY disproved the hypothesis that H62 is responsible solely for the inhibition of lysozyme.

Concentration of H62A Mutant PaIVY Vs. Percent Lysis
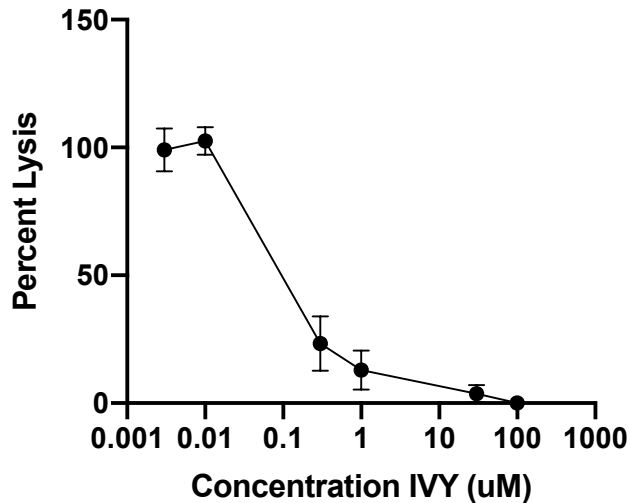
Figure 25. Muramidase assay of H62A mutant PaIVY at various concentrations in the presence of 100 nM hen egg white lysozyme (HEWL).

Table 1. Concentrations of each IVY used in the free protein muramidase assays in **Figures 24, 25, 28, and 29**.

		100 uM IVY	30 uM IVY	1 uM IVY	300 nM IVY	10 nM IVY	3 nM IVY	0 IVY	Blank
H20D	100 nM HEWL	4.8 uL IVY 20.2 uL 1x 50 uL DQ 25 uL HEWL	1.4 uL IVY 23.6 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	25 uL HEWL 25 uL 1x 50 uL DQ	9.5 uL 2.1 mM IVY 40.5 uL 1x 50 uL DQ
H20D/ H62A	100 nM HEWL	5.8 uL IVY 19.2 uL 1x 50 uL DQ 25 uL HEWL	1.8 uL IVY 23.2 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	25 uL HEWL 25 uL 1x 50 uL DQ	11.8 uL 1.7 mM IVY 38.2 uL 1x 50 uL DQ
Wild Type PaIVY	100 nM HEWL	10.5 uL IVY 14.5 uL 1x 50 uL DQ 25 uL HEWL	3.2 uL IVY 21.8 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	25 uL HEWL 25 uL 1x 50 uL DQ	21 uL 0.95 mM IVY 29uL 1x 50 uL DQ
H62A	100 nM HEWL	4.2 uL IVY 20.8 uL 1x 50 uL DQ 25 uL HEWL	2.3 uL IVY 22.7 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	25 uL HEWL 25 uL 1x 50 uL DQ	8.5 uL 2.36 mM IVY 41.5 uL 1x 50 uL DQ

Table 2. Concentrations of each IVY reacted with the warhead (**3**) used in muramidase assays for **Figures 26, 27, 30, and 31**.

		100 uM IVY	30 uM IVY	1 uM IVY	300 nM IVY	10 nM IVY	3 nM IVY	0 IVY	Blank
H20D	100 nM HEWL	12.8 uL IVY 22.2 uL 1x 50 uL DQ 25 uL HEWL	3.8 uL IVY 21.2 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	25 uL HEWL 25 uL 1x 50 uL DQ	25.6 uL 0.78 mM IVY 24.4 uL 1x 50 uL DQ
H20D/ H62A	100 nM HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	25 uL HEWL 25 uL 1x 50 uL DQ	20 uL 1 mM IVY 30 uL 1x 50 uL DQ
Wild- Type PaIVY	100 nM HEWL	25 uL IVY 0 uL 1x 50 uL DQ 25 uL HEWL	7.5 uL IVY 17.5 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	25 uL HEWL 25 uL 1x 50 uL DQ	50 uL 0.4 mM IVY 0 uL 1x 50 uL DQ
H62A	100 nM HEWL	8.3 uL IVY 16.7 uL 1x 50 uL DQ 25 uL HEWL	2.5 uL IVY 22.5 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	10 uL IVY 15 uL 1x 50 uL DQ 25 uL HEWL	3 uL IVY 22 uL 1x 50 uL DQ 25 uL HEWL	25 uL HEWL 25 uL 1x 50 uL DQ	16.6 uL 1.2 mM IVY 33.4 uL 1x 50 uL DQ

Additionally, it was hypothesized that the addition of the warhead (**3**) to PaIVY would block the H62 residue from being able to interact with lysozyme and thus allowing lysozyme to effectively lyse the cells. Muramidase was performed using warhead (**3**)

reacted PaIVY samples reacted using the same technique as previously described for the

NMR experiments of reacting purified PaIVY with $[Re(CO)_3(H_2O)_3]X^+$ (**1**) then adding

phenanthroline and SEC purifying the sample. Curiously, there was not a significant

difference between the free protein in the presence of HEWL as there was with the

warhead (**3**) reacted protein (**Figure 26**). Similarly, the H62A mutant showed no

significant difference between the free protein and the warhead (**3**) reacted protein in the

presence HEWL (**Figure 27**). This indicated for both wild-type PaIVY and H62A mutant

PaIVY that either the warhead (**3**) was not binding to the correct site for inhibition, or

that there was still too much unreacted protein available to inhibit lysozyme.

Figure 26. Muramidase assay of wild type PaIVY at various concentrations in the presence of 100 nM hen egg white lysozyme (HEWL) in blue and wild type PaIVY reacted with an excess of the warhead (**3**) at various concentrations in the presence of 100 nM HEWL.

Concentration of H62A Mutant PaIVY Vs. Percent Lysis

Figure 27. Muramidase assay of H62A mutant PaIVY at various concentrations in the presence of 100 nM hen egg white lysozyme (HEWL) in blue and wild type PaIVY reacted with an excess of the warhead (**3**) at various concentrations in the presence of 100 nm HEWL.

Based off of the NMR studies done previously, it was indicated that H62 was likely not the binding site for the warhead (**3**) but rather H20. Once the H20D and H20D/H62A mutants were successfully expressed, a muramidase assay was done in order to see if these mutations alone or the addition of the warhead (**3**) affected PaIVY's ability to inhibit lysozyme. The muramidase assay showed almost identical curves for both the free H20D and H20D/H62A mutant proteins when in the presence of 100 nM HEWL in which the fluorescent signal diminished as the concentration of PaIVY increased (**Figures 28 and 29**). This indicated that the H20D mutation was not important in the inhibition of lysozyme and that removing both histidine residues from PaIVY also did not affect the inhibition of lysozyme making it all the more pertinent that the warhead (**3**) blocks the interaction between PaIVY and lysozyme.

Concentration of H20D Mutant PaIVY Vs. Percent Lysis

Figure 28. Muramidase assay of H20D mutant PaIVY at various concentrations in the presence of 100 nM hen egg white lysozyme (HEWL).

Concentration of H20D/H62A Mutant PaIVY Vs. Percent Lysis

Figure 29. Muramidase assay of H20D/H62A mutant PaIVY at various concentrations in the presence of 100 nM hen egg white lysozyme (HEWL).

H20D and H20D/H62A PaIVY mutants were reacted with the warhead (**3**) using the same technique as previously described for other experiments and a muramidase assay was performed. For both the H20D and H20D/H62A mutant proteins reacted with

the warhead (**3**), the muramidase assay showed no inhibition. The curve of the warhead

(**3**) reacted proteins followed along in a similar fashion as that of the free protein

(**Figures 30 and 31**). It is to be noted that at a concentration of 0.3uM PaIVY for wild

type and each mutant protein the warhead (**3**) reacted proteins exhibited a higher percent

lysis. This could indicate that the warhead (**3**) is making the reaction slower between

PaIVY and lysozyme, but the interaction is not strong enough to fully prevent PaIVY

from inhibiting lysozyme. When the warhead (**3**) reacted PaIVY is SEC purified the

monomer peak is collected, however because there is not a large discrepancy in

molecular weight of that of free PaIVY and warhead (**3**) attached PaIVY, the peak

collected could contain both free and reacted protein. Therefore, the similar muramidase

curves could also indicate that there is too much free PaIVY that did not react with the

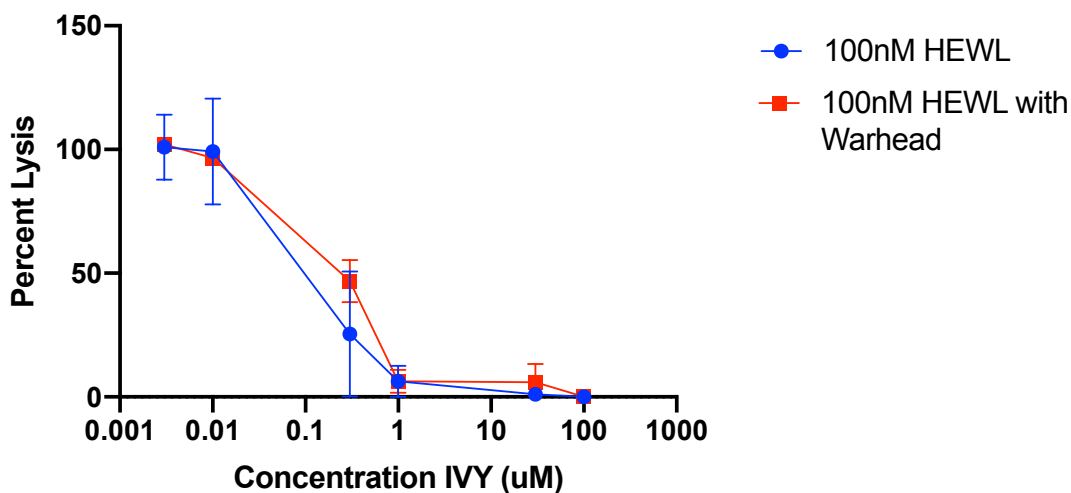warhead (**3**), that is free to interact and inhibit the lysozyme.
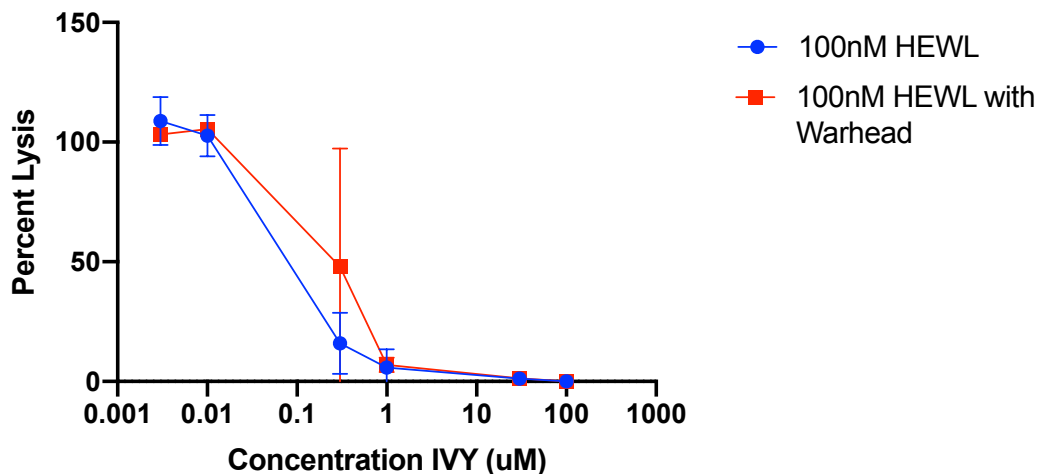
Figure 30. Muramidase assay of H20D mutant PaIVY at various concentrations in the presence of 100 nM hen egg white lysozyme (HEWL) in blue and wild type PaIVY reacted with an excess of the warhead (**3**) at various concentrations in the presence of 100 nm HEWL.
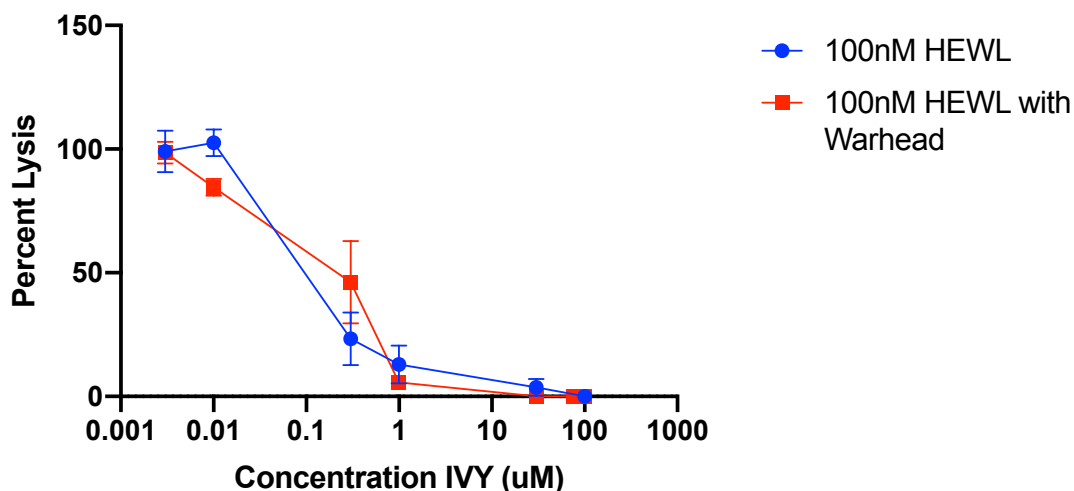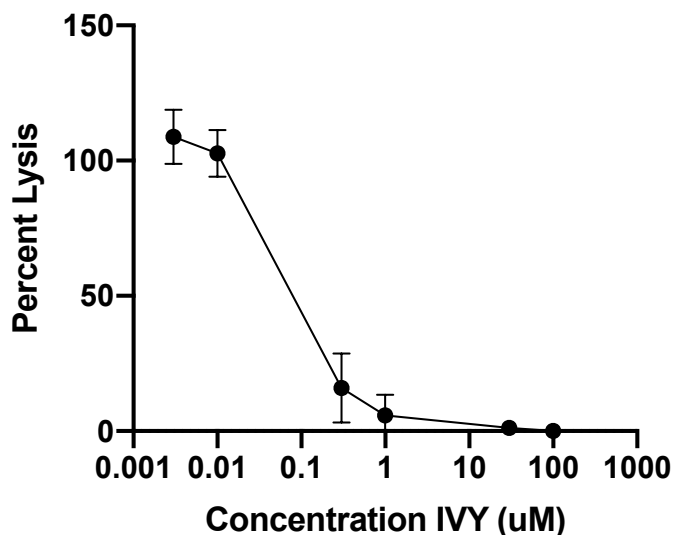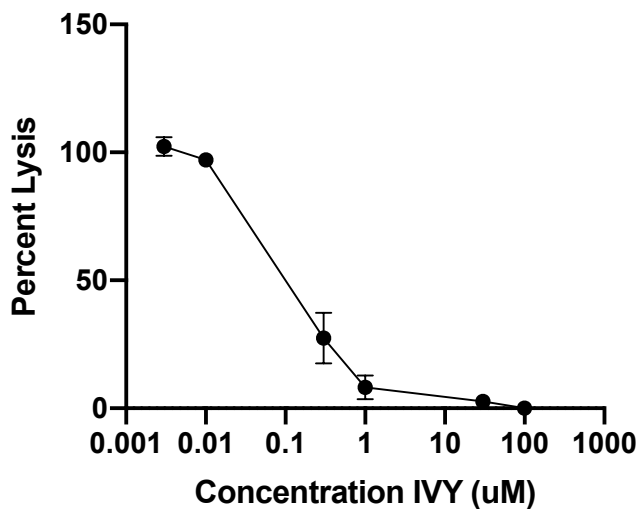
Concentration of H20D/H62A PaIVY Vs. Percent Lysis
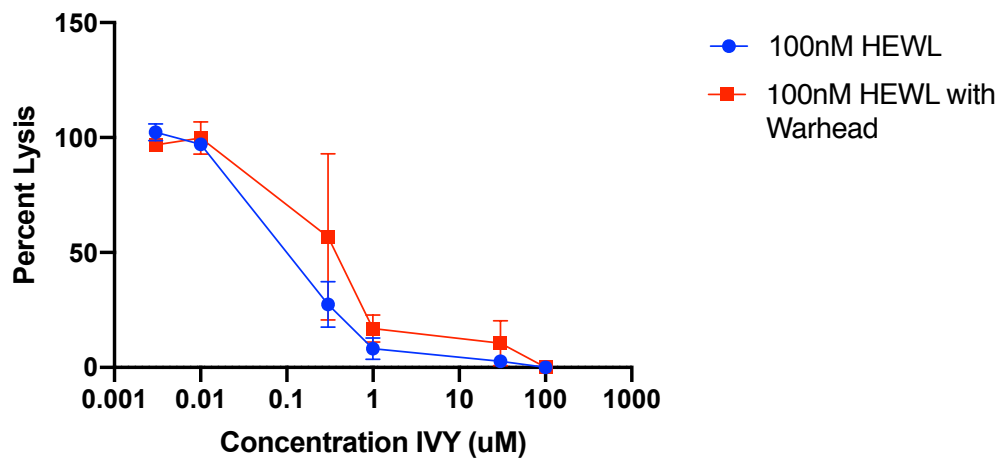
Figure 31. Muramidase assay of H20D/H62A mutant PaIVY at various concentrations in the presence of 100 nM hen egg white lysozyme (HEWL) in blue and wild type PaIVY reacted with an excess of the warhead (**3**) at various concentrations in the presence of 100 nM HEWL.

Chapter Five

Fluorescence and Mass Spectrometry Studies

Fluorescence is a useful tool in characterizing and studying fluorescent molecules. $Re(CO)_3(Phen)Cl^+$ (**4**) is a fluorescent molecule with an excitation at 370 nm and emission at 605 nm according to Chakraborty et al[12]. Because three of the four warheads are fluorescent molecules their fluorescent properties can help indicate whether PaIVY is reacting with the warhead and help determine the rate of the reaction. When the fluorescence was initially being investigated warhead **2** was used for these studies.

$[Re(CO)_3(Phen)H_2O]Br^+$ (**2**), $[Re(CO)_3(Phen)H_2O]Cl^+$ (**3**), and $Re(CO)_3(Phen)Cl^+$ (**4**) each fluoresce, so fluorescence studies were performed to investigate binding to PaIVY. First using Corning black polystyrene 96-well plates with a clear bottom various concentrations of $[Re(CO)_3(Phen)H_2O]Br^+$ (**2**) and PaIVY solutions were monitored over the course of one week as well as individual solutions of just PaIVY and $[Re(CO)_3(Phen)(H_2O)]Br^+$ (**2**) for comparison. Using Biotek Synergy H1 an excitation and emission scan were collected. Initially a fixed excitation was set at 370 nm and emission at 600 nm based off literature values. It was noted that the wavelength corresponding to the excitation and emission may fluctuate based on the solution the compound is in as well as each molecule attached. Later the excitation and emission values were shifted to 370 nm and 520 nm respectively in order to get a more accurate reading based on previous scans. Interestingly the warhead (**2**) complex with PaIVY surpassed the fluorescent signal of just $[Re(CO)_3(Phen)H_2O]Br^+$ (**2**) alone (**Figure 32**).

Being that PaIVY does not fluoresce on its own it was proposed that when the warhead (**2**) is mixed with PaIVY the binding between the two causes more $[Re(CO)_3(Phen)H_2O]Br^+$ (**2**) to be pulled into solution and therefore the fluorescent signal increases. This is plausible because the warhead (**2**) alone is poorly soluble in the 20 mM pH 6 sodium phosphate buffer in which PaIVY is stored. The fluorescence studies also indicated that the complex formation between the warhead (**2**) and PaIVY is time dependent reaching a maximum around day 4 and that the reaction is temperature dependent as well. When the same experiment was conducted at 4°C versus the usual 25°C, the fluorescence did not increase over time indicating that PaIVY was likely not binding the warhead (**2**). The growing fluorescence signal provided a preliminary kinetics curve following second order kinetics (**Figure 33**). All of the fluorescent plate studies were done using $[Re(CO)_3(Phen)H_2O]Br^+$ (**2**) because at the time of the experiments the one-step process to produce $Re(CO)_3(Phen)Cl^+$ (**4**) had not yet been discovered.

0.5 mM

Excitation (au, Ex=370)

Emission (au, Em=520)

Wavelength (nm)

- ◆ 96hr free Re (Ex)
- ▼ 96hr Free Re (Em)
- ■ t0 free Re (Ex)
- ▲ t0 Free Re (Em)
- ● 96hr complex (Ex)
- ■ 96hr complex (Em)
- ▼ T0 complex (Ex)
- ＋ T0 complex (Em)
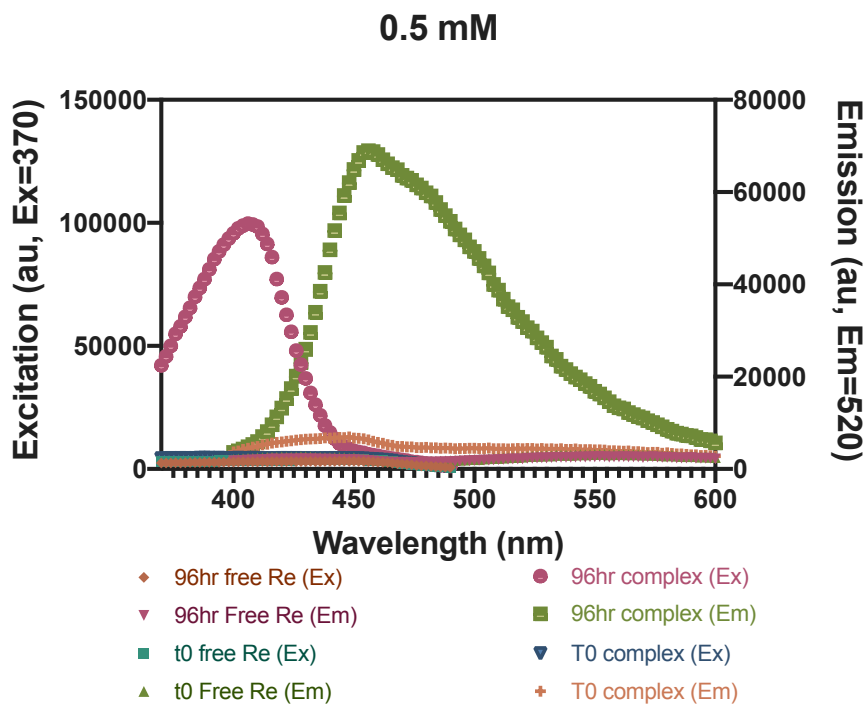
Figure 32. Excitation and emission spectra of free $[Re(CO)_3(Phen)H_2O]Br^+$ (**2**) and in complex with PaIVY at 0.5 mM in 20 mM sodium phosphate buffer at pH 6.

Kinetics

Intensity (au)

Time (hr)

- ● 1mM
- ▲ 0.1mM
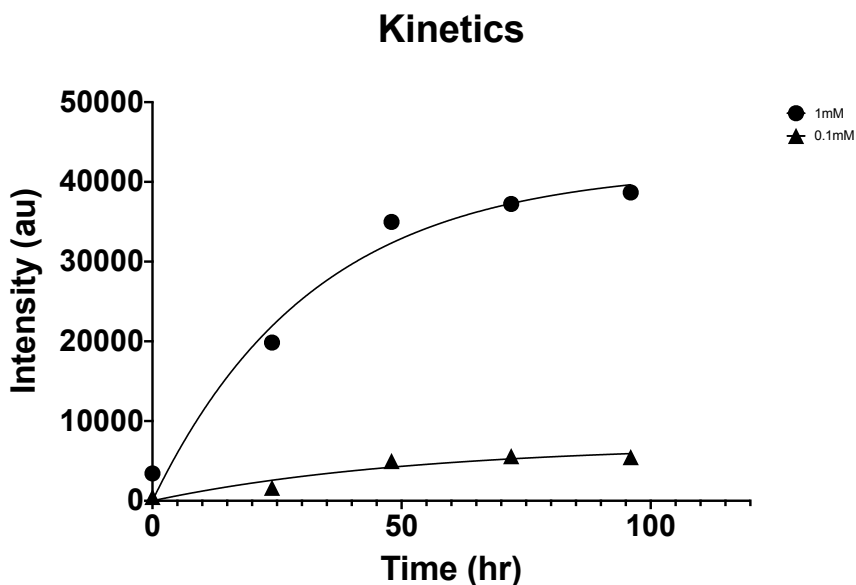
Figure 33. Intensity vs time for 1 mM and 0.1 mM PaIVY and $[Re(CO)_3(Phen)H_2O]Br^+$ (**2**)

Another useful technique to monitor intact proteins and their complexes is

electrospray ionization mass spectroscopy (ESI-MS) because it preserves the protein

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folding and its covalent interactions. The goal of performing ESI-MS was to determine if the warheads were binding to PaIVY. When doing ESI-MS a raw spectrum is provided from which the mass and charge can be calculated . If any of the warheads are binding to PaIVY there will be a shoulder peak on the spectrum that corresponds to the mass of PaIVY plus any adduct. For ESI-MS each sample was prepared following the specifications outlined in **Table 3** and sent to the University of Georgia's Proteomics and Mass Spectrometry facility where the samples were run on the Bruker Esquire 3000 Plus Ion Trap Mass Spectrometer equipped with an ESI ion source.

Table 3. Electrospray Ionization Mass Spectroscopy (ESI-MS) sample specifications.

Sample #	4	5	6	7
Warhead #	**1**	**2**	**4**	-
Sample Makeup	$[Re(CO)_3(H_2O)_3]Br^+$ +PaIVY	$[Re(CO)_3(Phen)(H_2O)]Br^+$ + PaIVY	$Re(CO)_3(Phen)Cl^+$ + PaIVY	PaIVY
MW (complex)	14806 Da	14950 Da	14950 Da	14500 Da
In	water	water	water	water
Molarity	100 uM	100 uM	100 uM	100 uM
Volume	50 uL	50 uL	50 uL	50 uL

In order to extract the valuable information from a raw ESI-MS spectrum, two equations need to be utilized to determine the mass and charge of the peaks present. To determine the mass, Equation 1 is used where M_1 corresponds to the *m/z* value displayed on the spectrum for one ion, M_2 corresponds to the *m/z* value displayed on the spectrum

for the adjacent lower ion, and A corresponds to the mass of the adduct which is typically

a proton but could be a sodium or potassium ion. To determine the charge Equation 2 is

used where z corresponds to the charge present for that peak or cluster of peaks, and all

other variables follow the same as in   **Equation 1**[21].

Equation 1.

$$MW = \frac{(M_1 - A)(M_2 - A)}{M_1 - M_2}$$

Equation 2.

$$z = \frac{M_2 - A}{M_1 - M_2}$$

For the free PaIVY ESI-MS the molecular weight calculation is as follows

corresponding to **Figure 34**. Each calculation was done following this example.

$$MW = \frac{(1038 - 1)(968.9 - 1)}{1038 - 968.9} = \frac{(1037)(967.9)}{69.1} = 14.525kDa$$

$$z = \frac{968.9 - 1}{1038 - 968.9} = 14$$

Once the charges are determined for each cluster of peaks based on the raw data,

this information can be used to calculate the average mass and differences in mass

between shoulder peaks in the same charge cluster. This information indicates if there is

an ion exchange happening at a given charge or if the warhead adduct is forming. The

average masses are listed in **Table 4** for each of the spectra indicating any adduct

formation with its likely identity. For example, in **Figure 34**, it can be seen that there is a

shoulder peak to the right of the +15-charge peak. This peak has a given value of 986.8.

Using this and the charge, the mass is calculated to be 14802 Da with a difference of 270

Da from that of the free protein. This indicates that an adduct is forming on PaIVY

reacted with $[Re(CO)_3(H_2O)_3]Br^+$ (**1**) at a molecular weight of approximately 270 Da.

This was calculated for each of the peaks seen in Figure 38 with an average adduct mass

of 269 Da. The warhead (**1**) adduct should have a mass of approximately 304 Da giving a

difference of 35 Da or 35g. The solution that PaIVY was initially purified in was a 20

mM potassium phosphate buffer. It was desalted into water, but due to the discrepancy of

around 39 Da or 39 g/mol found on each of the spectra it is likely that there was still

leftover potassium in the solution that was being exchanged in each of the samples. The

approximately 35 g/mol discrepancy for the mass of the warhead (**1**) adduct is likely

because there is a potassium ion lost when the warhead (**1**) is interacting with PaIVY.

Table 4. Average calculated masses from raw ESI-MS data in **Figures 34-37**.

	Warhead #	Average Mass of PaIVY	Average Mass of any adduct plus PaIVY	Average Mass of the adduct	Identity of adduct
PaIVY (Figure 37)	-	14532 Da	14571 Da	39 Da	Potassium ion
$[Re(CO)_3(H_2O)_3]Br^+$ plus PaIVY (Figure 38)	**1**	14532 Da	14801 Da	269 Da	$[Re(CO)_3(H_2O)_3]Br^+$ minus potassium ion
$[Re(CO)_3(Phen)H_2O]Br^+$ plus PaIVY (Figure 39)	**2**	14531 Da	14573 Da	42 Da	Potassium ion
$[Re(CO)_3(Phen)]Cl^+$ plus PaIVY (Figure 40)	**4**	14531 Da	14570 Da	39 Da	Potassium ion
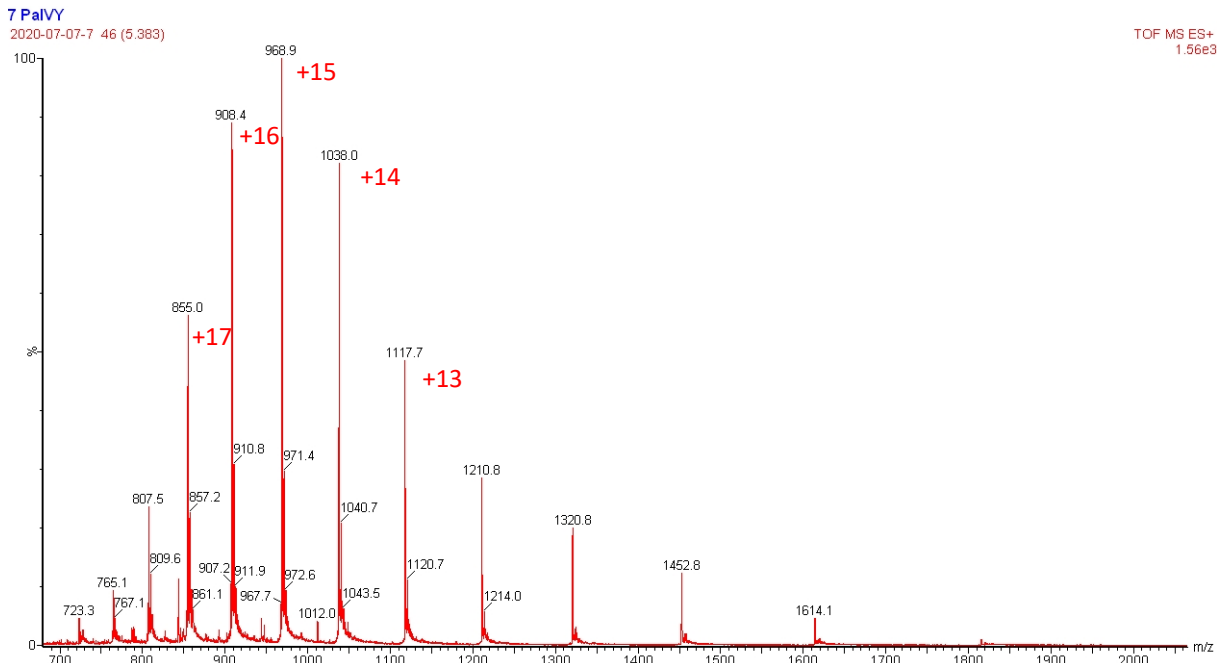
Figure 34. Raw electrospray ionization mass spectrometry data of PaIVY in water at 100uM.

Figure 35. Raw electrospray ionization mass spectrometry data of PaIVY plus [Re(CO)$_3$(H$_2$O)$_3$]Br$^+$ (**1**) in water at 100uM.

Figure 34 shows wild type PaIVY in water at 100uM. The spectrum indicates an average mass of 14532 Da. The most predominate charge for free PaIVY is +15 indicated by the largest relative peak followed by +16 and +14 respectively. There is a small peak for each charge cluster to the right which has an average mass of 14571 Da. It is likely that this small peak is from potassium ion exchange due to leftover potassium from the previous buffer. There are no other adducts forming therefore this spectrum is a good reference to compare the warhead (**1**) reacted ESI-MS spectra such as in **Figure 35**.

Figure 35 shows wild type PaIVY reacted with $[Re(CO)_3(H_2O)_3]Br^+$ (**1**). The predominate charge is +15 followed by +16 and +14, the same as free PaIVY along with the average molecular weight remaining consistent. There is a shoulder peak on each of the charge clusters that is about half the size of the main peak for its respective charge. This peak is PaIVY plus the adduct with an average mass of 14801 Da. In this case the peak corresponds to $[Re(CO)_3(H_2O)_3]Br^+$ (**1**) attached to PaIVY indicating that this warhead (**1**) is binding to PaIVY. There is a slight mass discrepancy of 35 Da that is likely due to the missing potassium ion. Further to the right of this peak there is a slightly shorter peak belonging to the same charge cluster that has an average mass of 14839 Da making the adduct approximately 307 Da or 307g/mol which corresponds closely to that of the molecular weight of the warhead (**1**).
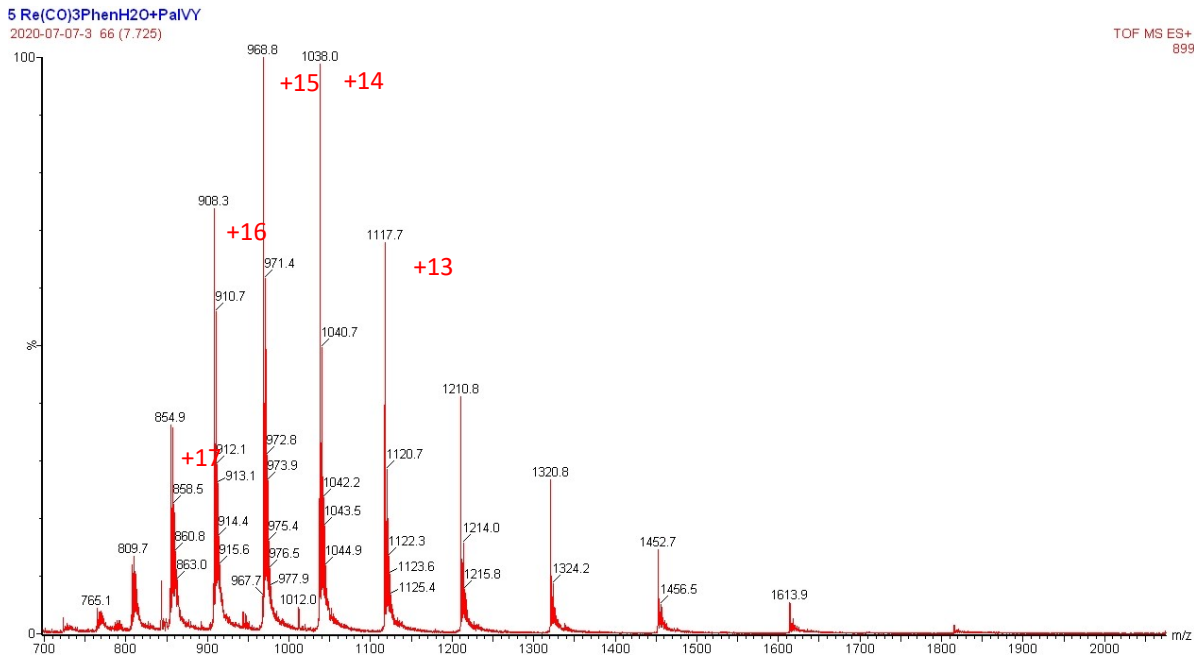
Figure 36. Raw electrospray ionization mass spectrometry data of PaIVY plus [Re(CO)$_3$(Phen)H$_2$O]Br$^+$ in water at 100uM.
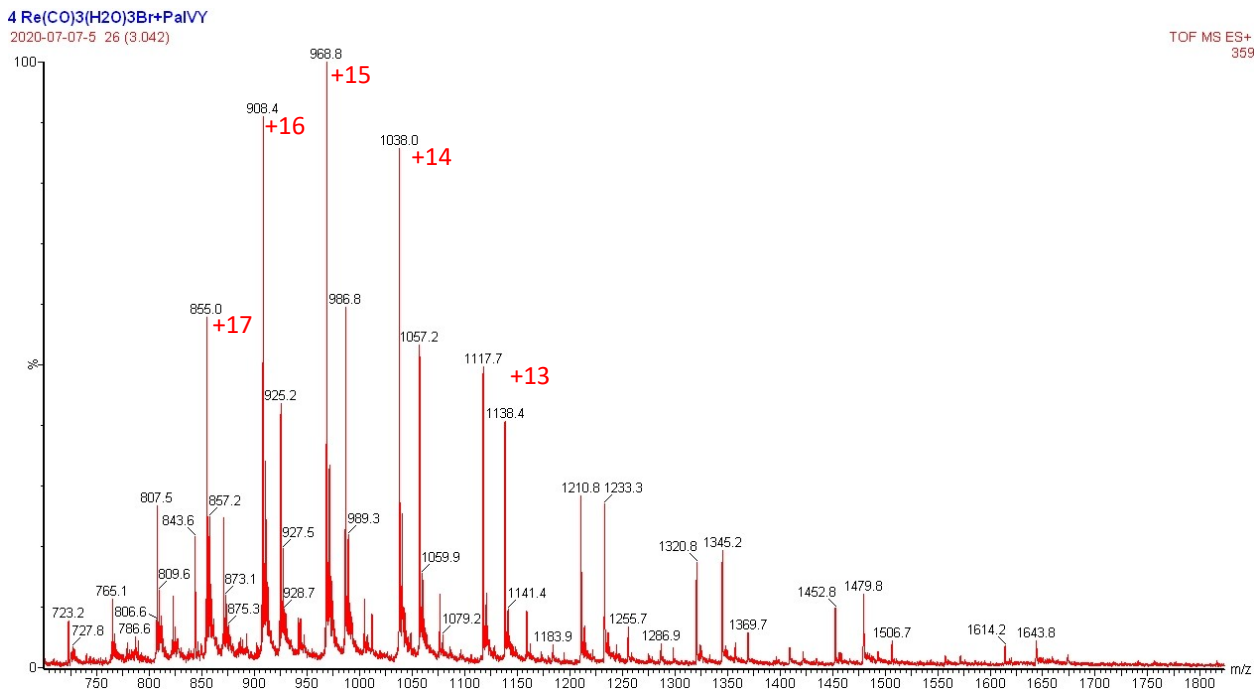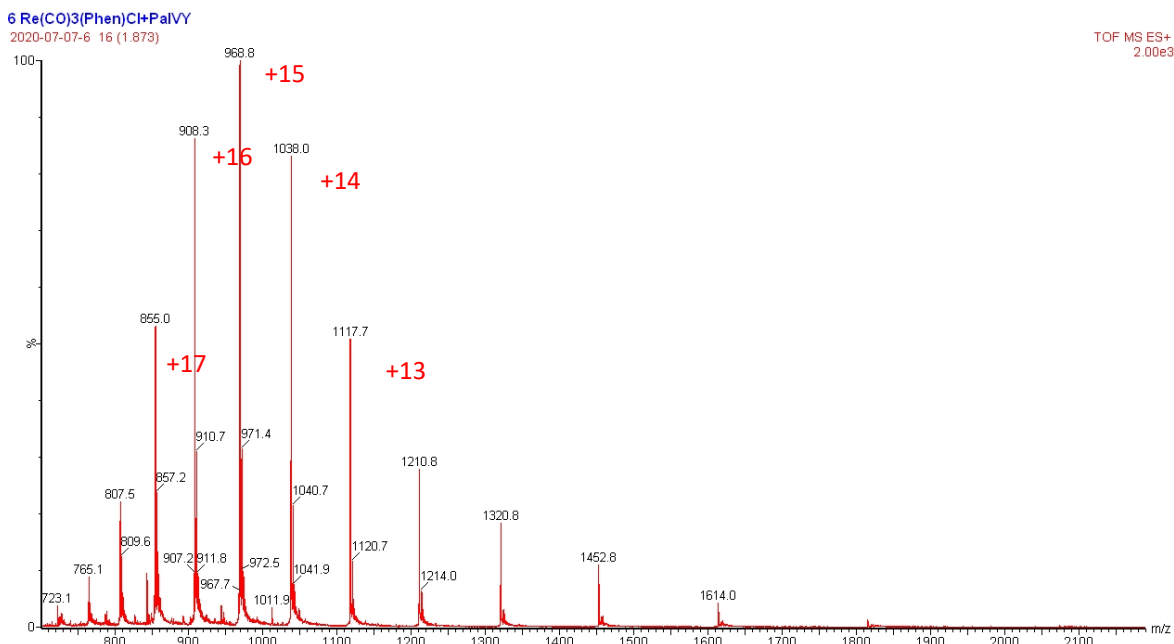

Figure 37. Raw electrospray ionization mass spectrometry data of PaIVY plus [Re(CO)$_3$(Phen)]Cl$^+$ in water at 100uM.

Figure 36 shows PaIVY reacted with [Re(CO)$_3$(Phen)H$_2$O]Br$^+$ (**2**) in water at

100uM. Based on the spectrum the average mass of PaIVY is negligibly lower than the

previous two spectra at 14.531kDa. The predominate charge is 15+ followed closely by +14 and +16 which differs from the spectrum of the free protein. This spectrum does have a lot of relatively small shoulder peaks, however none of them are predominant relative to that of the main peak in the charge cluster or correspond remotely close to the molecular weight of the warhead (**2**). This indicates that the warhead (**2**) is not binding. The failure for PaIVY to bind to this warhead (**2**) is likely due to the warhead (**2**) not being very soluble in water. Similarly, for **Figure 37**, it can be concluded that the warhead (**4**) is also not binding to PaIVY likely for the same reason due to the lack of any shoulder peaks that correspond to the molecular weight of the warhead (**4**). This spectrum has the same average mass of 14.531kDa, with the most predominate charge being +15 followed by +16 and +14. The spectrum is almost identical to that of the free protein supporting the fact that the warhead (**4**) is not bound to PaIVY. There is only one small shoulder peak on this spectrum similar to that of free PaIVY which corresponds to a potassium ion exchange.

Based off the ESI-MS data, PaIVY is only readily reacting with $[Re(CO)_3(H_2O)_3]Br^+$ (**1**) and not the fluorescent warheads (**2-4**) containing phenanthroline. This data verifies that $[Re(CO)_3(H_2O)_3]Br^+$ (**1**) is reacting with PaIVY which was indicated by previous NMR experiments. However, this data did not indicate any dimer/trimer complexes forming, but this could be due to the molecular weight of those species being outside of the set parameters on the mass spectrometer. This data also supports the theory that $Re(CO)_3(Phen)Cl^+$ (**4**) and $[Re(CO)_3(Phen)H_2O]Br^+$ (**2**) are not soluble enough in water to react with PaIVY. This information is valuable in that it helped lead to the decision to react PaIVY with readily water soluble

$[Re(CO)_3(H_2O)_3]Br^+$ (**1**) first and then add phenanthroline to synthesize the desired

warhead (**3**) PaIVY complex.

Chapter Six

Conclusion and Future Work

P. aeruginosa is a common threat to patients suffering from cystic fibrosis. These patients typically have a reduced lifespan and often die from respiratory failure caused by chronic bacterial lung infection with *P. aeruginosa* being a common contributor. It is often difficult to fight off a *P. aeruginosa* infection due to its antibiotic resistant biofilm production which may also be exacerbated by the secretion of a periplasmic protein, Inhibitor of Vertebrate Lysozyme, named after its inhibition of lysozyme PaIVY In order to inhibit the inhibitor a reactive warhead was designed and tested to see if mutating specific residues or adding the reactive warhead would ultimately allow lysozyme to effectively degrade the bacterium. Unfortunately based upon the data collected, mutating specific residues and the addition of the reactive warhead (**3**) did not have an effect on the inhibition PaIVY exhibits towards lysozyme suggesting that current models for how IVY inhibit lysozymes[7] may need to be reexamined.

Due to the potential impact inhibiting PaIVY could have, it has been worked on by multiple individuals. A previous graduate student designed the protein purification process that served as a basis for protein purification during this project, however modifications were made to increase efficiency in this new work. This protocol consists of an immobilized metal affinity chromatography nickel column His1, followed by a dialysis with TEV cleavage to remove the His tag. Next the protein is run over the nickel column a second time to separate the His tag from the protein. Subsequently the collected

fraction is concentrated and run over a size exclusion chromatography column to ensure purity of the correct protein. The only modification frequently used to his protocol was the use of a different buffer during the SEC to eliminate the need for a buffer exchange later. This modification is important because it allows for less time between protein purification and having the final concentrated protein available for experiments. This is especially important when reacting the protein with the any of the warheads as eventually proteins degrade so the less time spent concentrating the more time there is for experiments to be conducted with less of a chance of protein degradation. Primers were designed to mutate the histidine 62 residue of PaIVY to alanine, and PCR was completed for further investigation of this residue. This made it possible to observe the importance of the H62 residue of PaIVY in the inhibition of lysozyme using a muramidase assay as well as the importance of this residue when a reactive warhead is reacted with PaIVY.

The initial warhead designed was $[Re(CO)_3(H_2O)_3]Br^+$ (**1**), which was easily water soluble. This compound did bind to PaIVY, but due to the formation of oligomers its binding location was unable to be successfully seen on an HSQC NMR spectrum. From here the warhead was redesigned to contain 1,10-phenanthroline (**2-4**) to block PaIVY from forming these oligomeric complexes with the warhead. Both $[Re(CO)_3(Phen)H_2O]Br^+$ (**2**) and $Re(CO)_3(Phen)Cl^+$ (**4**) were poorly water soluble making it difficult to get an HSQC NMR spectrum or do any further experiments. In order to combat this obstacle, the easily water soluble $[Re(CO)_3(H_2O)_3]Br^+$ (**1**) was reacted with PaIVY and then 1,10-phenanthroline was added. In order to ensure only the monomer PaIVY in complex with the reactive warhead (**3**) was used for further

experiments a size exclusion chromatography column was utilized. This became the primary technique to form the reactive warhead (**3**) in complex with PaIVY.

Once the design of the reactive warhead and optimization of the complex formation was achieved for the warhead (**3**), it became evident that this warhead was not binding to the proposed active site, H62. This was seen on the wild type ^{15}N PaIVY HSQC NMR spectrum that when the protein was in complex with the warhead (**3**), majority of the residues that experienced chemical shift perturbation were located near H20. This was also confirmed on the H62A mutant ^{15}N PaIVY HSQC NMR spectrum that there were still chemical shifts once the H62 residue was no longer available for binding. From this point site-directed mutagenesis was performed a second time to mutate the histidine 20 residue to aspartic acid. The H20D mutant NMR spectra indicated that H20 was experiencing little chemical shift perturbation but rather linebroadening when reacted with the warhead warhead (**3**). This was clearly noticed in residues G38 and S39 which were also residues that experienced chemical shift perturbation in the wild type PaIVY spectrum as well as the H62A mutant spectrum. This data indicated that H20 alone was not the binding site for the warhead (**3**). It was proposed that perhaps a conformational change was occurring when binding occurs. The H20D/H62A double mutant NMR spectra confirms the theory that a conformational change is likely occurring because in this spectrum there is no chemical shift perturbation. It was also noted from this spectrum that some free protein did not react with the warhead (**3**). This is seen by peak splitting on the spectrum as well as on both wild type and mutant ^{15}N PaIVY HSQC spectra.

Once it was identified where warhead (**3**) was likely binding, muramidase assays were performed for both wild type and all mutant PaIVYs with and without the addition of the warhead (**3**) in order to identify if these specific residues or the addition of this warhead had an effect on the inhibition of lysozyme. It was expected that wild type PaIVY in the presence of lysozyme, specifically HEWL, would cause the fluorescent signal to decrease at higher concentrations of PaIVY because the cells were not being lysed. The muramidase assay confirmed this trend. However, the same trend was seen for all mutant IVYs including H62A. This indicated that H62 may not be the only important residue in the inhibition of lysozyme and that the residues neighboring H62 on the exposed loop may also play a more important role in the inhibition than was previously suggested by the crystal structure of IVY bound to HEWL.[7] The muramidase assays with the reactive warhead (**3**) also followed the same trend in which the fluorescence decreased at higher concentrations of PaIVY for all mutants and wild type indicating that warhead (**3**) was not binding to the correct site to block inhibition of lysozyme. However, it is possible that there is still too much free protein that is unreacted with the warhead (**3**) that was able to inhibit lysozyme. This hypothesis is supported by the fact that there were split peaks on the NMR spectra that showed not all of the protein was in complex with PaIVY, wild type or mutant. From this information it is difficult to conclude whether or not warhead (**3**) really has an effect on the inhibition of PaIVY there is no way to separate the free protein from the reacted protein. Resolving this ambiguity is important for future studies of this system.

In the future there is still work that could be done to further understand where this warhead (**3**) is binding as well as how the inhibition of lysozyme if affected when the

warhead (**3**) is reacted with PaIVY. As suggested previously H62 may not be the only important residue in the inhibition of lysozyme. In order to fully understand the inhibition PaIVY exhibits towards lysozyme the residues neighboring His62, proline and aspartic acid, should be mutated individually as well in conjunction with each other. This would help indicate if all are responsible for the inhibition of lysozyme together. From here the previously done experiments should be repeated with these mutants to get a better understanding of the interaction with lysozyme as well as the effect the warhead (**3**) might have when these residues are mutated.

Other mass spectrometry techniques would also be useful in identifying where the warhead (**3**) is binding precisely to PaIVY such as a hydrogen-deuterium exchange mass spectrometry (HDX-MS) experiment in conjunction with tandem mass spectrometry (MS-MS) experiment. In an HDX-MS experiment the binding site and interaction is revealed by monitoring the exchange rates between hydrogen and deuterium of the amide proton of the protein. Hydrogen atoms not involved in the protein-warhead interaction will exchange at a much more rapid pace than those involved in the interaction thus indicate where binding is occurring.[22] These experiments should be done in order to provide valuable information indicating where the warhead (**3**) is binding and what residues are involved which has yet to be obtained concretely from the research thus far. Doing these experiments with the H62A, H20D, and H20D/H62A double mutant would verify that this warhead has some promiscuity when binding between the H20 and H62 residues which can be addressed by experiments outlined further below.

Additionally, it would be beneficial to do fluorescence polarization studies on PaIVY in complex with the warhead (**3**). Fluorescence anisotropy/polarization is a widely

used technique in which polarized light is sent through a sample and the degree to which the light is polarized is inversely proportional to the rate of molecular motion. A small ligand will have rapid free rotation and therefore emit largely depolarized light while a much larger molecule will have a limited rotation and therefore emit largely polarized light.[23] When the light reaches the detector the degree of polarization can indicate if a ligand, or in this case the warhead (**3**) is bound and what percentage is bound to the protein. This technique has many advantages such as not requiring any separation of bound and free ligand, being nonradioactive, carrying out in solution phase, and requiring relatively low volumes of sample making it an ideal technique to be used in order to determine what percentage of protein is bound to the warhead (**3**).[23] This experiment should be done because it would indicate what percent of the warhead is bound to PaIVY. One of the issues discussed previously is that after collecting the monomer peak from the SEC of warhead (**3**) reacted PaIVY is that it is unknown how much free protein is still in solution left to react and inhibit lysozyme. Knowing how much free protein is left would give a good indication of how reactive this warhead (**3**) truly is in an aqueous environment.

Furthermore, some of the previous work done with PaIVY by Aaron Schultz was finding various drug fragments that bind to PaIVY. It would be beneficial to be able to link these fragments with the warhead (**3**). Using the warhead (**3**) design outlined here, in the future variations of phenanthroline or bipyridine complexes, such as dihydroxy phenanthroline, can be used to help link previously identified drug fragments to the reactive warhead (**3**) as well as help increase the solubility of the molecule, which was one of the challenges in this project. Linking the warhead to previously identified drug

fragments would increase the rate of the reaction by increasing the binding affinity and specificity for the drug molecule towards PaIVY. There are many histidine containing molecules found in the cellular environment. By linking these fragments, the warhead will have a high specificity and be directed towards the target His62 residue rather than H20 or any other histidine containing molecules. It would be beneficial to choose a strong binding drug fragments near the H62 residue to link to the warhead in order to increase the likelihood that the warhead would bind to the H62 residue instead of the H20 residue and therefore result in an irreversible inhibition of PaIVY. Linking the warhead to different drug fragments or multiple would give a better understanding of binding and the required residues for inhibition of lysozyme by PaIVY.

Lastly to expand on this research even further, an artificial sputum closely resembling that of the sputum found in the lung environment of Cystic Fibrosis patients should be used to understand how this warhead (**3**) would behave in a more realistic environment. The artificial septum would contain many other cellular components found in the lung environment that may have histidine residues. By using a realistic environment, it would prove that once a full drug-molecule is created by linking the drug fragments and the warhead (**3**) that there is high specificity of the complex towards His62 of PaIVY. Using this medium would also give a better understanding of how the warhead complex would behave in an imperfect environment rather than a filtered aqueous buffer that was used in the research done thus far.

References

1. Rafeeq, M.; Murad, H. Cystic brosis: current therapeutic targets and future approaches. **2017**.

2. Scott, A. Cystic Fibrosis. *Radiol. Technol.* **2013**, *84*, 493-518.

3. Høiby, N.; Frederiksen, B.; Pressler, T. Eradication of early Pseudomonas aeruginosa infection. *Journal of Cystic Fibrosis* **2005**, *4*, 49-54.

4. Soren, O.; Rineh, A.; Silva, D. G.; Cai, Y.; Howlin, R. P.; Allan, R. N.; Feelisch, M.; Davies, J. C.; Connett, G. J.; Faust, S. N.; Kelso, M. J.; Webb, J. S. Cephalosporin nitric oxide-donor prodrug DEA-C3D disperses biofilms formed by clinical cystic fibrosis isolates of Pseudomonas aeruginosa. *jac* **2019**, dkz378.

5. Leysen, S.; Vanderkelen, L.; Van Asten, K.; Vanheuverzwijn, S.; Theuwis, V.; Michiels, C. W.; Strelkov, S. V. Structural characterization of the PliG lysozyme inhibitor family. *Journal of Structural Biology* **2012**, *180*, 235-242.

6. PfuUltra High-Fidelity DNA Polymerase. *https://www.agilent.com/cs/library/usermanuals/public/600380.pdf*(accessed October 2, 2020).

7. Abergel, C.; Monchois, V.; Byrne, D.; Chenivesse, S.; Lembo, F.; Lazzaroni, J.; Claverie, J. Structure and Evolution of the Ivy Protein Family, Unexpected Lysozyme Inhibitors in Gram-Negative Bacteria. *Proceedings of the National Academy of Sciences of the United States of America* **2007***, 104*, 6394-6399.

8. Joint Center for Structural Genomics, (JCSG) RCSB PDB 4PS6. *https://www.rcsb.org/structure/4PS6* (accessed June 15, 2020).

9. Meggers, E. Targeting proteins with metal complexes. *Chemical Communications* **2009**, 11.

10. Lonsdale, R.; Ward, R. A. Structure-based design of targeted covalent inhibitors. *Chemical Society reviews* **2018***, 47*, 3816-3830.

11. Binkley, S. L.; Leeper, T. C.; Rowlett, R. S.; Herrick, R. S.; Ziegler, C. J. Re(CO)3(H2O)3+ binding to lysozyme: structure and reactivity. *Metallomics* **2011***, 3*, 909.

12. Chakraborty, I.; Jimenez, J.; Sameera, W. M. C.; Kato, M.; Mascharak, P. K. Luminescent Re(I) Carbonyl Complexes as Trackable PhotoCORMs for CO delivery to Cellular Targets. *Inorganic Chemistry* **2017***, 56*, 2863-2873.

13. Ziarek, J. J.; Peterson, F. C.; Lytle, B. L.; Volkman, B. F. Binding site identification and structure determination of protein-ligand complexes by NMR a semiautomated approach. *Methods in enzymology* **2011***, 493*, 241-275.

14. Matte, A.; Kozlov, G.; Trempe, J.; Currie, M. A.; Burk, D.; Jia, Z.; Gehring, K.; Ekiel, I.; Berghuis, A. M.; Cygler, M. Preparation and characterization of bacterial

protein complexes for structural analysis. *Advances in protein chemistry and structural biology* **2009***, 76*, 1.

15. Konermann, L.; Ahadi, E.; Rodriguez, A. D.; Vahidi, S. Unraveling the Mechanism of Electrospray Ionization. *Analytical Chemistry* **2013***, 85*, 2-9.

10. Ho, C. S.; Lam, C. W. K.; Chan, M. H. M.; Cheung, R. C. K.; Law, L. K.; Lit, L. C. W.; Ng, K. F.; Suen, M. W. M.; Tai, H. L. Electrospray ionisation mass spectrometry: principles and clinical applications. *The Clinical biochemist. Reviews* **2003***, 24*, 3-12.

17. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*. **2006**.

18. Fabiola Castorena-Torres,,Katia Peñuelas-Urquides,Mario Bermúdez de León. Site-Directed Mutagenesis by Polymerase Chain Reaction. In *Site-Directed Mutagenesis by Polymerase Chain Reaction Reaction.* **2017**; pp 159-171.

19. PCR Basics. *https://www.thermofisher.com/us/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/pcr-education/pcr-reagents-enzymes/pcr-basics.html* (accessed February 7, 2021).

20. EnChek Lysozyme Assay Kit. *https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2Fmp22013.pdf&title=RW56Q2hlayBMeXNvenltZSB Bc3NheSBLaXQ=* (accessed February 7, 2021).

21. Chemistry LibreTexts ESI-MS
    Data. *https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_*
    Modules_(Analytical_Chemistry)/Analytical_Sciences_Digital_Library/Active_Lear
    ning/In_Class_Activities/Biological_Mass_Spectrometry%3A_Proteomics/Instructor
    's_Manual/Section_2%3A_Electrospray/Section_2B._ESI-MS_Data#mjx-eqn-
    1 (accessed March 1, 2021).

22. shii, K.; Noda, M.; Uchiyama, S. Mass spectrometric analysis of protein–ligand
    interactions. *Biophysics and Physicobiology* **2016***, 13*, 87-95.

23. Moerke, N. J. Fluorescence Polarization (FP) Assays for Monitoring Peptide-Protein
    or Nucleic Acid-Protein Binding. *Current Protocols in Chemical Biology* **2009***, 1*, 1-
    15.

24. Lazarova, N.; James, S.; Babich, J.; Zubieta, J. A convenient synthesis, chemical
    characterization and reactivity of [Re(CO)3(H2O)3]Br: the crystal and molecular
    structure of [Re(CO)3(CH3CN)2Br]. *Inorganic Chemistry Communications* **2004***, 7*,
    1023-1026.

Appendix One
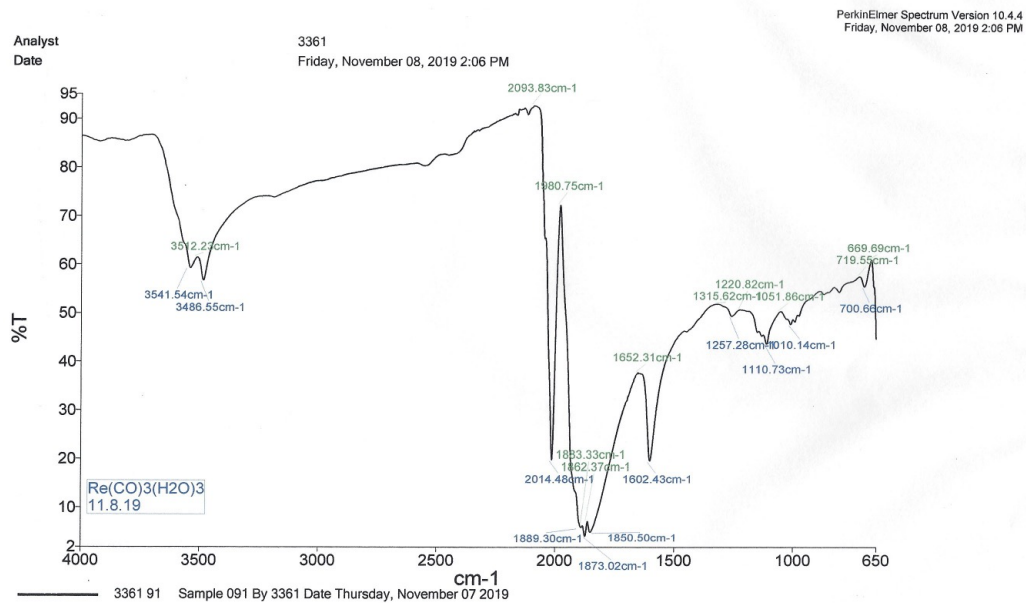
Figure A1.1. IR $[Re(CO)_3(H_2O)_3]Br^+$ (**1**)
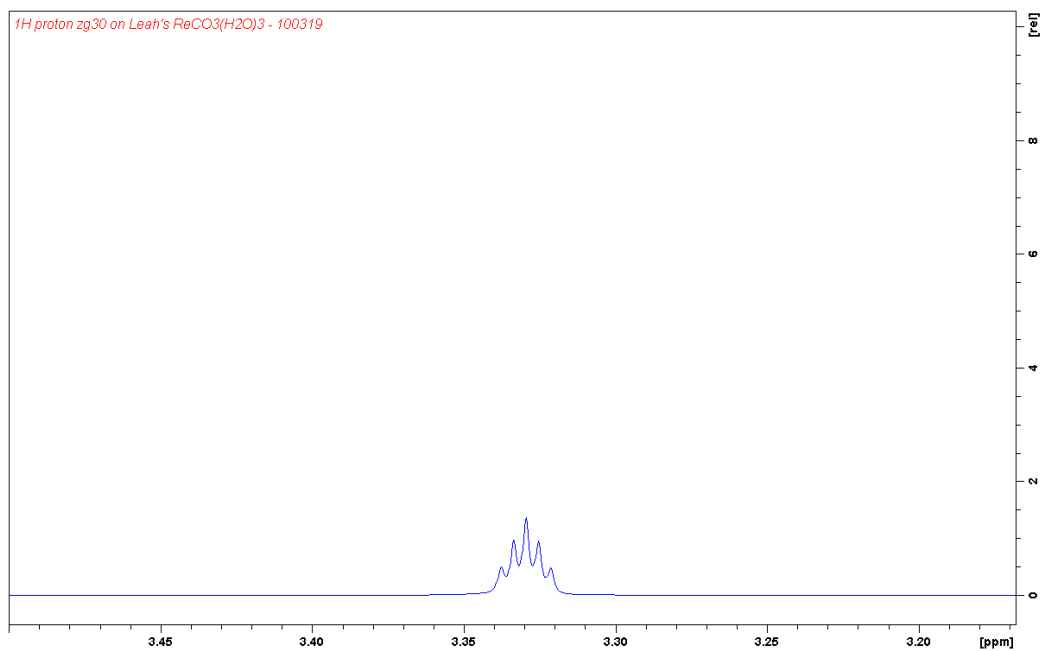
Figure A1.2. 1H NMR $[Re(CO)_3(H_2O)_3]Br^+$ (**1**)
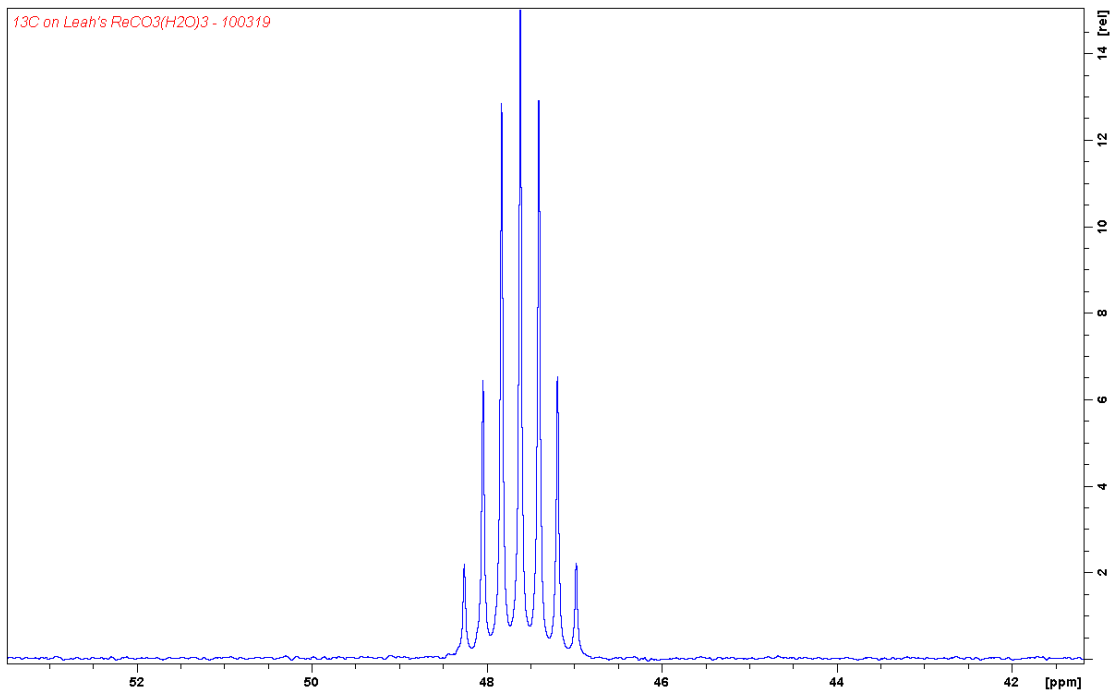
Figure A1.3. 13C NMR $[Re(CO)_3(H_2O)_3]Br^+$ (**1**)
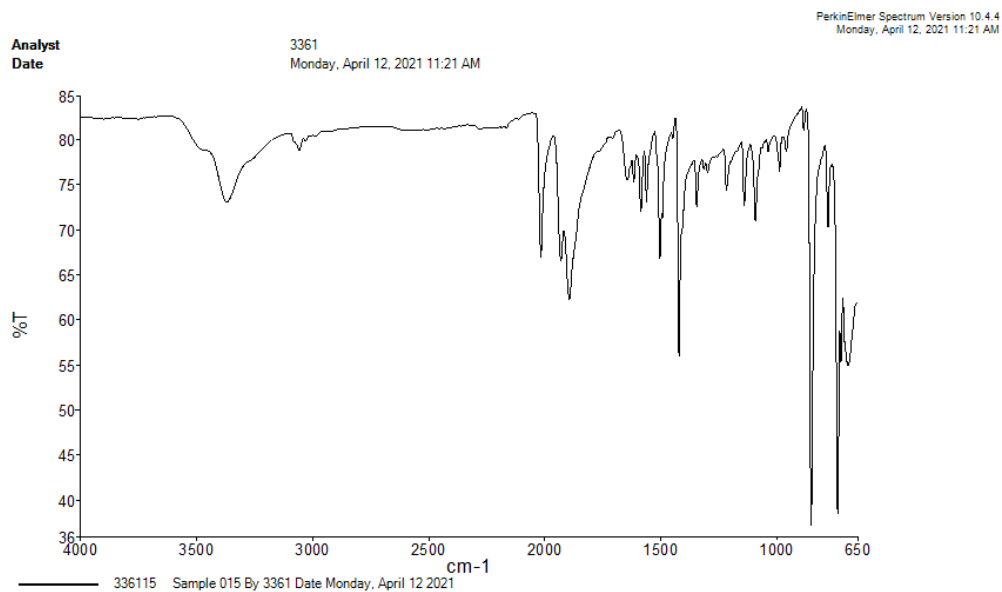
Figure A1.4. IR $[Re(CO)_3(Phen)H_2O]Br^+$ (**2**)

1H proton zg30 on ReCO3PhenH2OBr1 4.13.21

a.

8   6   4   2   [ppm]

[rel]

15

10

5

0

1H proton zg30 on ReCO3PhenH2OBr1 4.13.21

b.

9.2   9.0   8.8   8.6   8.4   [ppm]

[rel]

15

10

5

0

68
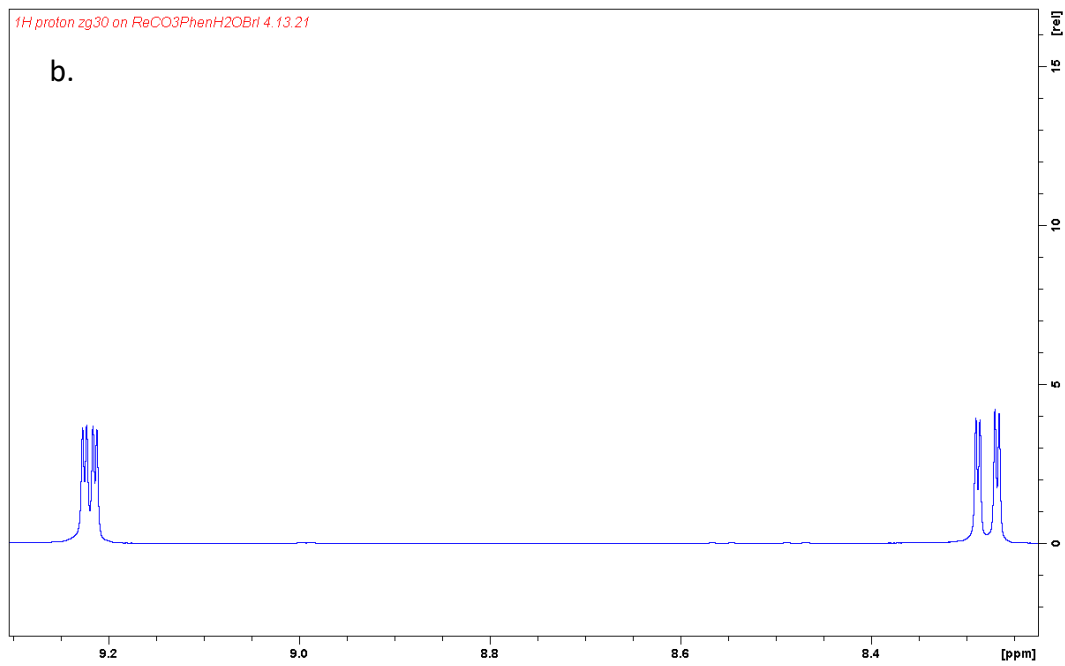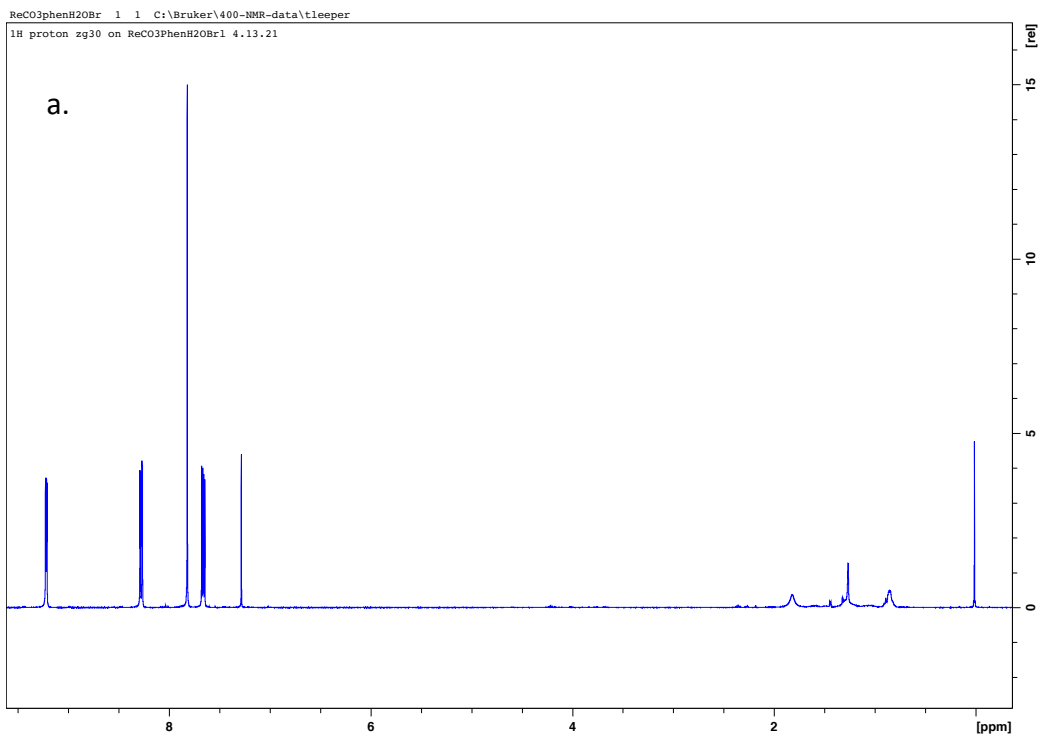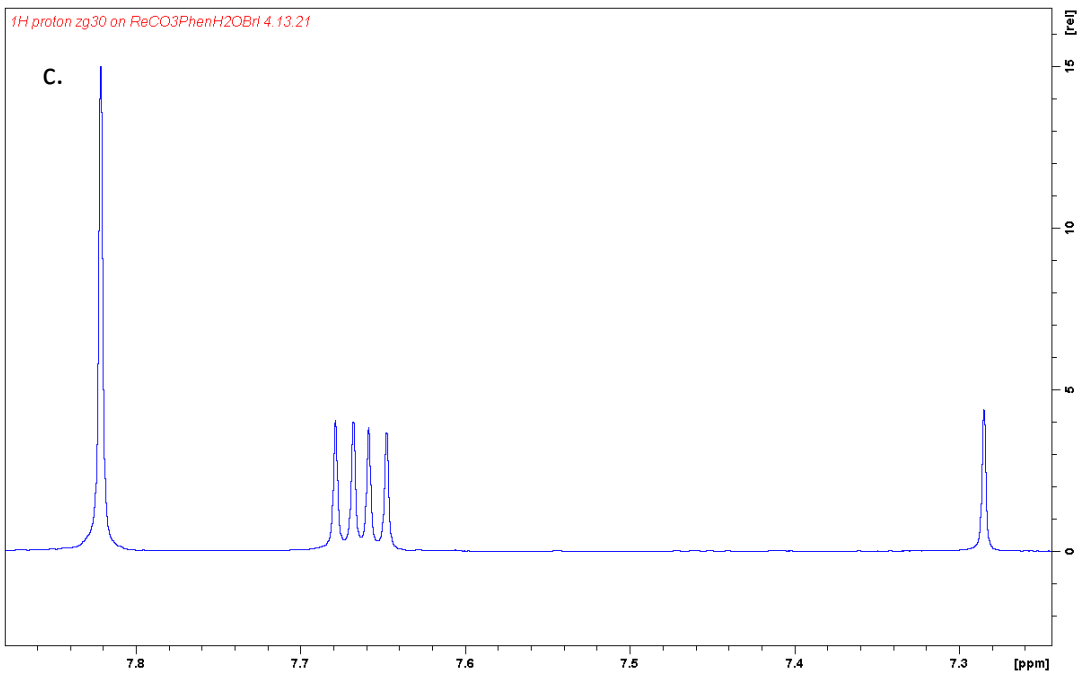
Figure A1.5. a.) 1H NMR [Re(CO)$_3$(Phen)H$_2$O]Br$^+$ (**2**) full spectra b.) Zoomed in downfield. C.) Zoomed in downfield
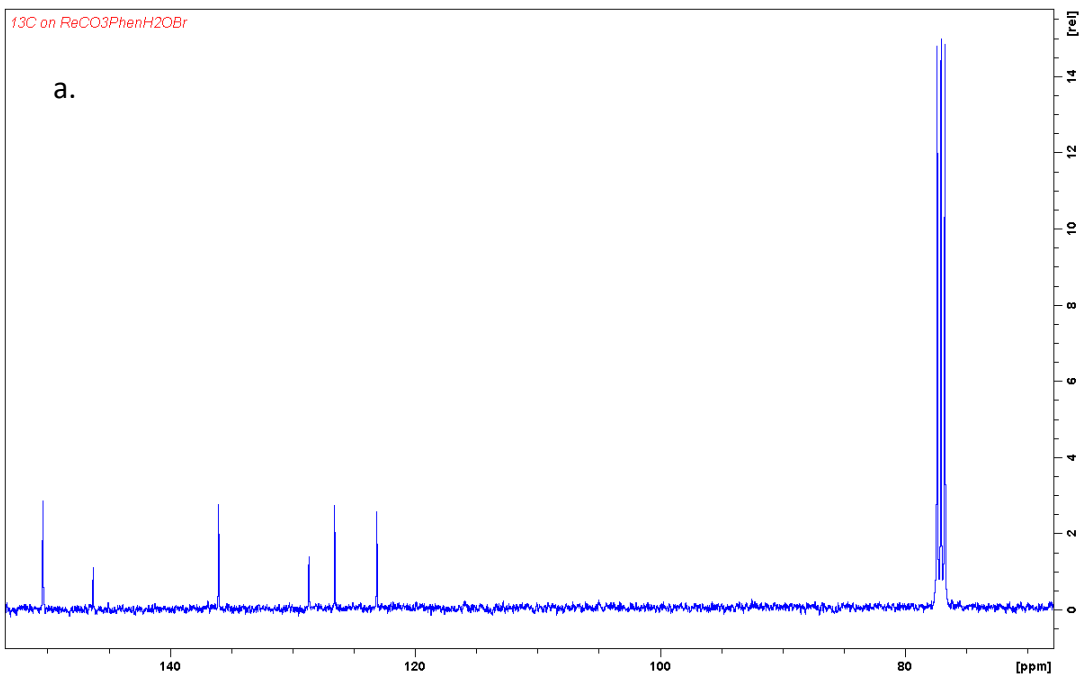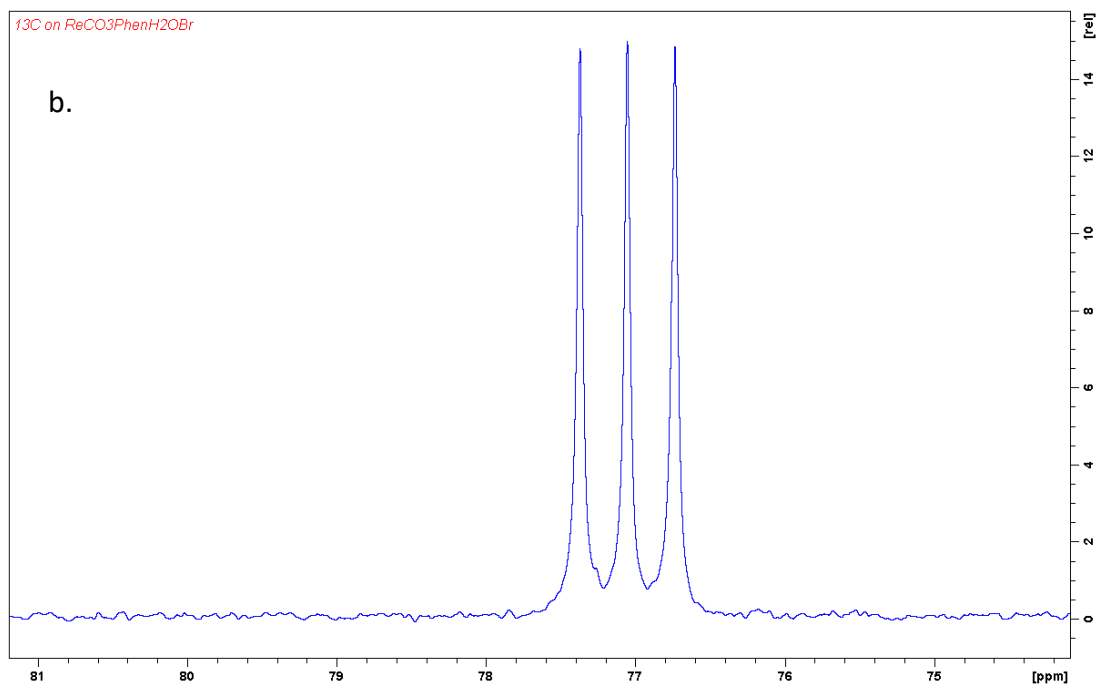
Figure A1.6. a.) 13C NMR $[Re(CO)_3(Phen)H_2O]Br^+$ (**2**) full spectra b.) Zoomed in upfield
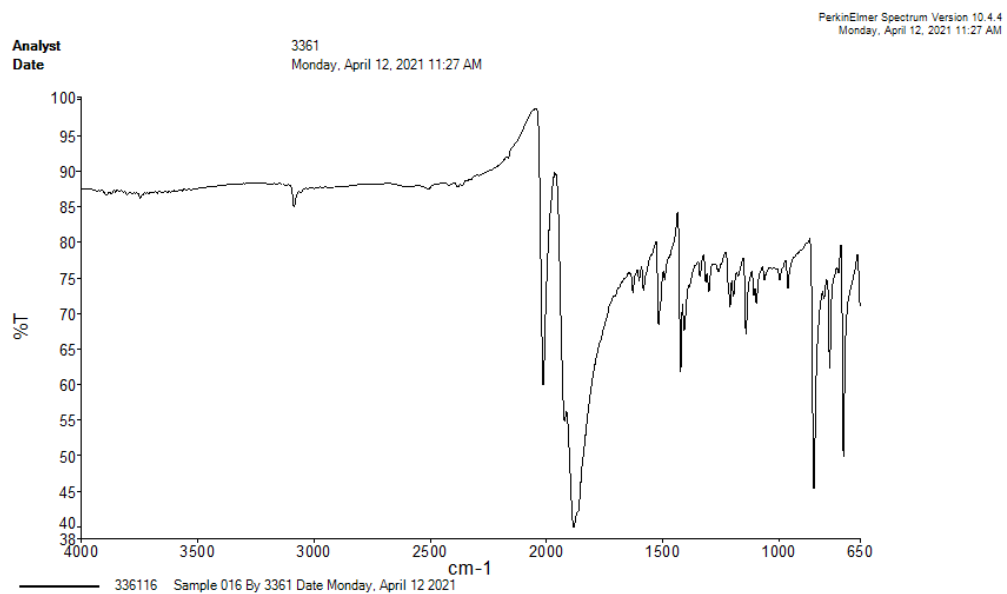
Figure A1.7. IR [Re(CO)$_3$(Phen)]Cl$^+$ (**4**)
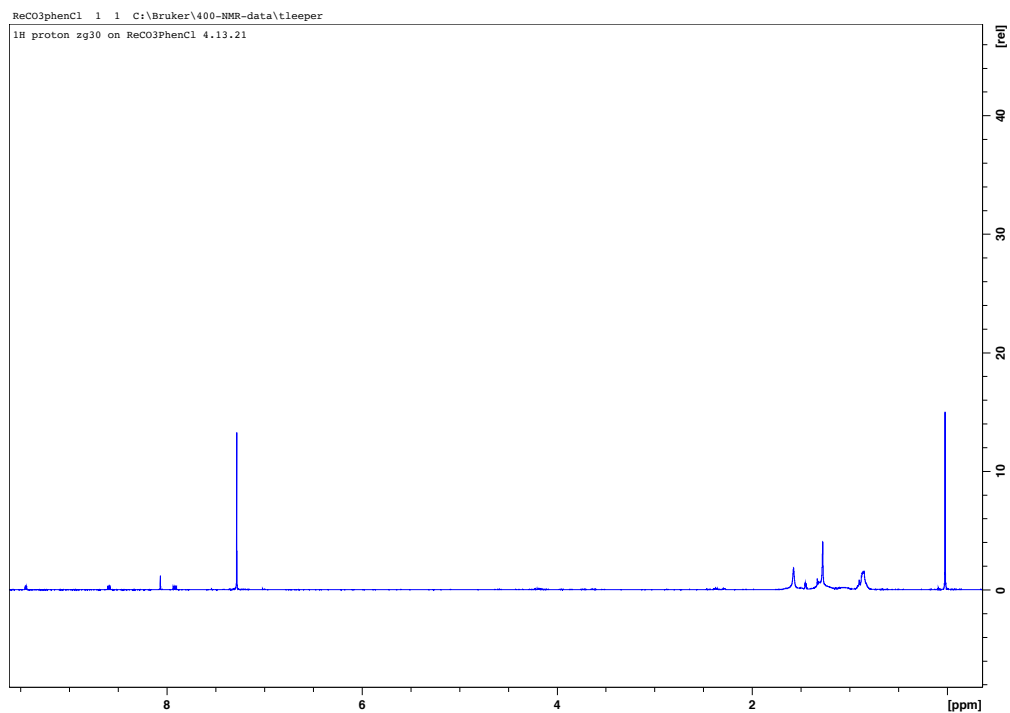
Figure A1.8. 1H NMR [Re(CO)$_3$(Phen)]Cl$^+$ (**4**)
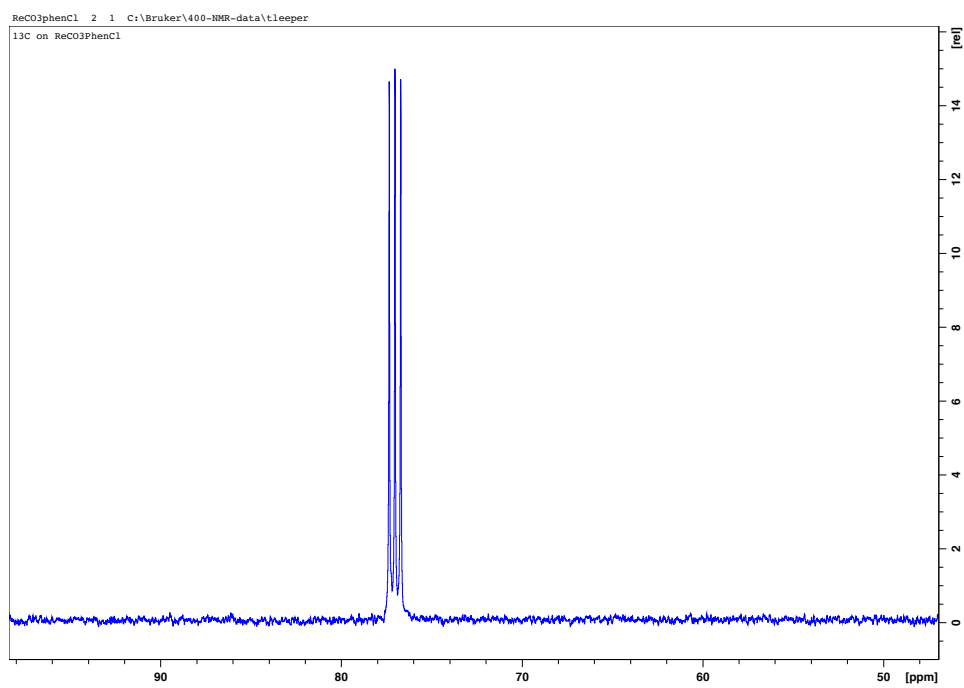
Figure A1.9. 13C NMR [Re(CO)$_3$(Phen)]Cl$^+$ (**4**)

Appendix Two

PaIVY Protocols

Aaron Schultz

Vectorless Host: *E. coli* BL21DE3PlysS

Plasmid: Pa3902 in PSpeedET vector

Promoter: Pbad-Forward

Antibiotic resistance: Kanamycin

Induction: 0.1% (w/v) L-(+)-Arabinose @ OD$_{600}$ 0.4-0.6

Extinction Coefficient: 31065

Molecular Weight: 14.5 kDa

pI: 5.69

Buffer Conditions: 40 mM KPhos, 200 mM KCl pH 8.0

Room temp stable

Withstands freeze-thaw cycles

Withstands de-salting into water

Transformation

<u>Materials:</u>

Bucket with ice

 SOC media

Plasmid

 BL21DE3PLysS single shot cells

2 kanamycin LB/Agar plates

 1 ampicillin LB/agar plate

<u>Method</u>

1. Place BL21DE3PLysS single shot cells ampule and plasmid in ice bucket to thaw

2. Place SOC media on benchtop from refrigerator to warm up

3. Place all three LB/Agar plates in incubator at 37°C

4. Once cells and plasmid are thawed, pipet 80 ng plasmid **<u>directly into cells</u>** and

   put cells back on ice for 30 minutes.

5. Heat-shock for 42 seconds at 42°C

6. Place back on ice to rest for 5 minutes

7. Add 0.95mL SOC media to cells. Place in 37°C shaker @ 260 rpm for 1 hour

8. Pipet 100 µL and 200 µL cells onto kanamycin plate and 200 µl onto ampicillin
   plate, spread onto plate evenly

9. Incubate at 37° overnight

10. Parafilm and store in refrigerator. Shelf life ~ 2 weeks

Small Inoculum

<u>Materials</u>

Two (2) 250 mL Erlenmeyer flasks (autoclaved) with 100 mL sterile LB or M9 in each flask

Kanamycin stock

Transformation plate

Bunsen burner

Wire loop

<u>Method</u>

1. Pull transformation plates and set up loop assembly and Bunsen burner
2. Sterilize loop until red hot, allow to cool.
3. Loop 2-3 isolated colonies and stir into flask. Repeat for second flask
4. Add 100 μL kanamycin stock to each flask.
5. Cover top of flask with foil and place in shaker at 37ºC overnight

Large Inoculum

Materials

Two (2) autoclaved 2600 mL fernbach flasks with 1000mL sterile LB or M9 media
2000 mL flask with 500 mL media may be used as a substitute.
Flasks from small inoculum
Spectrophotometer
Plastic cuvettes
Four (4) 0.5 g Arabinose single shots
Kanamycin stock solution

Method

1. Turn on spectrophotometer
2. Spike 1000 mL media with 1000µL kanamycin stock solution if applicable
3. Reserve 1 mL of media as a blank.
4. Pull small inoculum from the shaker, pour contents into fernbach flasks. Cover and shake to homogenize
5. Pipet 1 mL into cuvette, record OD_{600}. Induction OD_{600}=0.4-0.6 mAU
   a. If OD_{600} is below 0.4, shake at 37°C for 20-minute intervals, taking OD_{600} readings at each interval until absorbance is 0.4-0.6.
   b. If OD_{600} is within range, move to step 6.
6. Reserve 4-6 mL non-induced mixture and place in 15 mL falcon tube
7. Induce with L-(+)-arabinose to 0.1%(w/v)
   0.1% (w/v) is 1 g arabinose per 1000 mL media
8. Label flask and tape 15 mL non-induced control mixture to the fernbach flask
9. Place in shaker at 37°C overnight

Large Inoculum Centrifuge Protocol

Materials

Sorvall RC6 plus super centrifuge

F10-6X500Y Rotor from cold room

500 mL centrifuge bottles with screw caps (6 for 2 L prep)

Cultures from large inoculum

Method

1. Turn on centrifuge and place rotor inside unit, screwing lid onto rotor, then securing top screw into centrifuge.
2. Quick-temp the centrifuge by setting rotor on screen. Start spin then immediately stop. This kickstarts the compressor.
3. Set parameters to 8000 RPM at 15 minutes, 4°C
4. Gather two 1 mL samples, induced and uninduced control, in eppendorf tubes
5. Pour large inoculum culture into centrifuge bottles. Volume should be around 2/3 height of bottle.
6. Balance filled bottles against each other using Harvard balance.
7. Place balanced bottles across from one another to balance rotor
8. Secure lid to rotor
9. Secure rotor to centrifuge
10. Confirm rotor and spin settings
11. Start and step back

<u>WARNING! ROTOR IS SUSCEPTIBLE TO FAILURE DURING ACCELERATION AND DECELERATION</u>

12. Pour off clear supernatant and store pellets in -20°C freezer
13. Bleach supernatant and culture bottles for 20 minutes
14. Bleach work area and balance

Buffers

Materials

1 L or 2 L graduated cylinder

2.5 L beaker with stir bar

Dibasic potassium phosphate

Monobasic potassium phosphate

Potassium chloride

Imidazole

Hydrochloric acid

Potassium hydroxide

Digital analytical balance

Stir plate

pH meter

Vacuum filter apparatus with appropriate filter paper

Methods

1. Place ~500 mL 18 MΩ deionized water in beaker with stir bar. Place beaker on stir plate
2. Weigh ingredients using analytical balance
3. Dissolve ingredients into water
4. Adjust pH to 8.0 using pH meter and appropriate acid or base
5. Pour into appropriate graduated cylinder and bring up to volume using 18 MΩ DI H_2O
6. Vacuum filter and degas buffer.
7. Store Buffer A, Buffer B, and SEC buffer in clean 1L glass screw cap bottle
8. Dialysis buffer may be stored in a 2.5 L Nalgene beaker covered with plastic wrap
9. Apply appropriate label with name, date, product name, contents and pH.
Example: AS 7/1/19 PaIVY Buffer A 40 mM KPhos 200 mM KCl, 20 mM imidazole pH 8
10. Store in deli-style refrigerator

Buffer Contents

Buffer Name	Running Buffer "Buffer A"	Elution Buffer "Buffer B"	SEC Buffer	Dialysis Buffer
Volume	1 Liter	1 Liter	1 Liter	2 Liters
pH	8.0	8.0	8.0	8.0
Dibasic Potassium Phosphate K_2HPO_4 (Anhydrous)	20 mM 3.846 g	20 mM 3.846 g	20 mM 3.846 g	20 mM 6.972 g
Monobasic Potassium Phosphate KH_2PO_4 (Anhydrous)	20 mM 2.722 g	20 mM 2.722 g	20 mM 2.722 g	20 mM 5.444 g
Potassium Chloride KCl	200 mM 14.910 g	200 mM 14.910 g	200 mM 14.910 g	200 mM 29.820 g
Imidazole	20 mM 1.362 g	400 mM 27.232 g	Do not add	Do not add

Homogenization

<u>Materials</u>

500 mL centrifuge bottles with pellets from large inoculum centrifugation

20 mL buffer A

60 mL homogenizer with plastic plunger

Ice bucket with ice

<u>Method</u>

1. Pour 20 mL buffer A into one centrifuge bottle with pellet. Vortex until pellet has completely loosened into the buffer.
2. Pour off contents of bottle into next centrifuge bottle with pellet.
3. Repeat steps 1-2 until all pellets have been loosened
4. Pour contents into homogenizer and place in ice
5. Use plunger with an up/down and twisting motion to homogenize cells into buffer A. Product should be a uniform, creamy, light tan solution

French Press

CAUTION! VERY DANGEROUS. USES A COMBINATION OF HIGH PRESSURE FROM AN OPEN HYDRAULIC PRESS AND 10 kg STEEL CELL. SEVERE INJURY MAY OCCUR WITH IMPROPER USE.

<u>Materials</u>

open hydraulic french press

french press cell with T-bar plunger

homogenized cell culture

500 mL beaker with ice

Bucket with ice

<u>Method</u>

1. Ensure new french press machine is set to "down" and "pause". Turn on machine
2. Lubricate rubber on plunger with petroleum jelly and assemble cell body with T-bar plunger to "MAX FILL" line
3. Place upside down onto cell stand
4. Assemble base of cell with outlet nozzle and flow restrictor screw **tightened**
5. Pour homogenized sample into cell body. Make sure to leave about 3 cm of room for the cell base.
6. Place cell base onto cell body and invert to right side up.
7. Place assembled cell into press with the T-bar above the T bar plunger notches. Cell should be positioned with the plastic button on the cell body in line with the steel screw rod, outlet nozzle facing forward, and flow restrictor screw easily accessed to the left.
8. Secure cell in place using steel cross bar with screw assembly.
9. Attach outlet nozzle hose

10. Set to "LOW" and "RUN". Allow pressure to stabilize (~1750 PSI). Make sure cell has not moved from its restraints and that the T-bar has made flush contact with the top plate. You should not see any flow through the outlet hose.

11. Set to "HIGH". Allow pressure to stabilize. You should not see flow through the outlet hose.

12. Using the small mallet, tap the flow restrictor screw in a counter-clockwise fashion until a slow flow is seen in the outlet hose.

13. Once flow has reached the end of the outlet hose, regulate the flow to 1-2 drops per second into a small centrifuge bottle. Contents in the hose should look like a dark tan liquid with cream liquid on top.

14. Run until the plunger reaches "STOP". Set machine to "PAUSE"

15. Set machine to "DOWN" and "RUN".

16. Repeat steps 3-15 two times.

Crude Lysate Centrifugation

Materials

Sorvall RC6 plus super centrifuge

F21S 8X50Y centrifuge rotor from cold room

Crude lysate from french press

Two (2) 30 mL centrifuge bottles

Method

1. Turn on centrifuge
2. Input run conditions 18000 RPM, 45 Min, 4°C
3. Install rotor fastening lid first by tightening bottom screw, then top screw
4. Select rotor
5. Quick start compressor by starting and immediately stopping run
6. Balance samples using Harvard balance
7. Place samples in centrifuge directly across from one another (i.e. slots 1 and 5)
8. Fasten bottom screw and top screw to rotor
9. Confirm run conditions and rotor selection
10. Start and step back.

WARNING! ROTOR IS SUSCEPTIBLE TO FAILURE DURING ACCELERATION AND DECELERATION

11. After run is complete immediately pour off supernatant into a 50 mL falcon tube.
12. Take a 100μL sample of the supernatant placing it in a 1.5mL epi tube. Label *CRUDE*
13. Inspect pellet. The outside edge of the pellet should be glassy and transparent. This represents cell membrane fragments. As you inspect closer to the center, the

color should be tan with a dark bullseye representing heavier bacterial cell fragments.

CromLab Program Basics for NGC Liquid Chromatography System
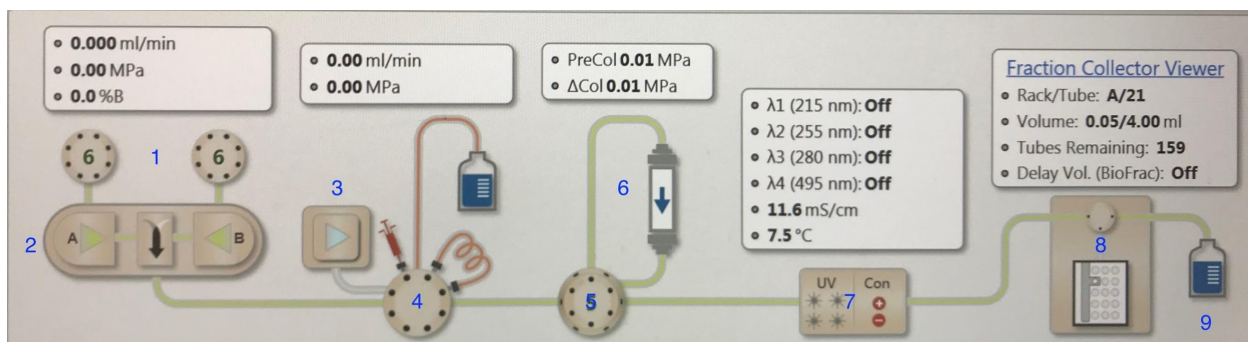
1. System pump buffer selection icon for pump A and pump B
   a. Allows to change between buffers using valve
2. System pump operation icon
   a. Allows pump A and B operation. Isocratic or gradient.
3. Sample pump operation icon
   a. load and/or inject sample
4. Sample pump flow selection icon
   a. Changes selection to loop or direct column inject for sample pump and system pump
5. Column selection icon
   a. changes flow to selected column
6. Column
7. UV/Connectivity icon
   a. toggle lamps on/off, zero absorbance readings
8. Fraction collection icon
   a. set collection tube parameters- type/volume/amount
9. Waste icon

1st Ni-column (His-1) Purification by Immobilized Metal Affinity Chromatography (IMAC)

The purpose of this purification step is to separate our protein from the rest of the bacterial proteins by leveraging the utility of the 6 histidine tag. When the crude lysate flows across the nickel column, the nickel binds PaIVY which contains a his tag. Low imidazole concentration running buffer that flows over the column in the next step helps kick off weakly binding proteins. High concentration imidazole in an elution buffer knocks PaIVY off of the nickel column. There are a few important cautionary steps whenever purifying protein on the NGC. First, make sure you do not introduce any air into the system.Introducing air into a column can dry out the column matrix and destroy the column. Second, make sure flow is within operating range for the column you are using. Using a flow rate too high for a column will compress the matrix and destroy the column. Third, ensure that the fraction collector is turned on and is connected to the NGC before loading your protein. If the fraction collector is not communicating with the NGC, the entire system must be restarted. Finally, once the protein is loaded, do not zero out the absorbance. This will skew your results.

Materials

NGC liquid chromatography system running ChromLab program
PaIVY buffer A (see page 8) placed in buffer selector 3A
PaIVY buffer B (see page 8) placed in buffer selector 3B
Centrifuged crude lysate
three (3) 50 mL falcon tubes labelled 1) buffer A 2) flow through 3) peak fractions

Methods

1. Equilibrate the Ni-column.
   a. Set column selection icon to slot 1
   b. Run DI water (system pump buffer selector slot 2) at 5 mL/min for 25 mL
   c. Run buffer B (system pump buffer selector slot 3 100%B) @ 5mL/min 25 mL
   d. Run buffer A (system pump buffer selector slot 3 0%B) @5mL/min 25 mL, zeroing out the absorbance under UV/Conn icon within 5 mL of completion
   e. set column selection icon to bypass
   f. set sample pump flow selection to "Sample pump direct inject, system pump waste." line on computer diagram should be light blue from the sample pump through the column and into waste.
   g. using sample pump, direct inject 10 mL buffer A to clear out sample pump.
   h. turn column selection icon back to position 1
   i. clear out run.
2. Load the crude lysate
   a. take the empty 50 mL falcon tube labelled "flow through" and place in plastic waste jug with lines in the falcon. The tube should not fall through into the bottom. it should sit on the lid of the waste collector.
   b. determine volume of centrifuged lysate and set injection volume of sample pump.
   c. place falcon tube into clamp by sample pump. Insert brown sample loading tube to the bottom of the falcon.
   d. load sample using sample pump at 5 mL/min. stop pump just before all sample is gone. you should see a large plateau on the chromatogram.
   e. add approximately 10 mL buffer A at 5 mL/min to residual crude lysate and run sample pump for <10 mL to ensure injection of all crude lysate.
   f. remove "flow through" falcon tube from the waste container

g. switch sample pump flow selection to "Manual inject loop, system pump to column." Flow line on computer diagram should show a red injection loop and a green line running from the system pump through the column and to waste.

3. Set up fraction collection
    a. ensure fraction collector is on, has clean racks in it, and is connected to the system.
    b. click on the blue fraction collector viewer icon and press both "remove empty racks" and "remove all"
    c. close out window
    d. double click on fraction collector to open window. Rack type should be F1 (12-13 mm X 100mm tubes) Start collection from A1 and end at B90. Fraction size should be 4 mL.

4. Achieve adequate separation.
    a. Set system pump to 5 mL/min, 0% B, for 25 mL. Start pump
    b. turn on fraction collection by double clicking fraction collector and pressing "collect." you are now collecting fractions.
    c. absorbance line should be near or at baseline after 25 mL run. if not, run 10 mL at a time until the baseline of A280 is less than 50 mAU

5. Elute PaIVY from the column.
    a. Under the system pump, choose gradient, initial %B 0%, final %B 100%, 25 mL then click the check box to run final B% for an additional 15 mL.
    b. Start flow. you should start to see a peak around 30%B
    c. at 100%B, you will notice that A280 flattens out around 300 mAU. To establish this is absorbance from the high concentration imidazole, run 15 mL 0%B and watch the absorbance line and the connectivity line. the line shapes should be very similar. A280 should drop down close to zero.

6. Finishing up

a. Stop collection

b. press "reset arm"

c. collect fractions from peak in 50 mL falcon labelled "peak fraction"

d. add 1:1000 EDTA to peak fraction falcon.

    i. if you collected 16 mL sample, add 16μL EDTA

e. save run under style PaIVY_(media used)_(labelling if applicable)_His1_Yearmonthday_(last name)

    i. PaIVY_M9_15N_His1_20190708_Schultz

    ii. PaIVY_LB_His1_20190708_Schultz

f. close out run and open up analysis

g. deselect every line trace except A280, %B, and connectivity

h. Create run report under File

i. save in a folder created on desktop.

Dialysis and TEV cleavage

The purpose of this step is to remove excess imidazole from the peak fractions to allow cleavage of the TEV cleavage site ENLYFQG to separate the 6-his tag from PaIVY

<u>Materials</u>

Pooled peak fractions containing 0.5 mM (1:1000 dilution) Disodium EDTA from 0.5 M stock
TEV
3500 MW Snakeskin® dialysis tubing with green tube clamps
2L dialysis buffer (see page 8)

<u>Methods</u>

1. Soak a strip of 3500 MW Snakeskin® dialysis tubing in dialysis buffer to hydrate dialysis membrane
2. Fold the first end of the dialysis tubing in a "greek key" motif by making one large fold, then folding the large fold in half. clamp the folded membrane with a green clip.
3. Using a disposable pipet, transfer the pooled fraction containing EDTA into the dialysis tubing. Clamp the second end of the tubing using the same folding technique. make sure there is an air bubble in the dialysis tubing, allowing the tubing to float in the dialysis buffer.
4. Place dialysis tubing in dialysis buffer for at least 3 hours to reduce the imidazole concentration in the pooled fractions.
5. After dialysis, transfer the dialyzed protein into a clean 50 mL falcon tube.
6. add TEV at a ratio of 1 part TEV to 50 parts protein. (eg. if you have 50 mg PaIVY, add 1 mg of TEV)
7. set on benchtop overnight

2nd Ni-column (His-2) Purification by Immobilized Metal Affinity Chromatography (IMAC)

The purpose of this purification step is to further purify your protein by separating the cleaved PaIVY protein from the TEV, N-terminal his-tag, and any non-specific binding proteins that co-eluted with PaIVY during the first nickel column. We use a cleave and clear strategy to remove the his tag from our protein. By running our protein over a nickel column a second time, the flow through will contain PaIVY and the His tag along with TEV will come off of the column with the elution fraction.

<u>Materials</u>

NGC liquid chromatography system running ChromLab program

PaIVY buffer A (see page 8) placed in buffer selector 3A

PaIVY buffer B (see page 8) placed in buffer selector 3B

Dialyzed PaIVY with TEV

Two (2) 50 mL falcon tubes labelled 1) buffer A 2) His 2 Peak Fraction

<u>Methods</u>

1. Equilibrate the Ni-column.
    - j. a.Set column selection icon to slot 1
    - k. Run DI water (system pump buffer selector slot 2) at 5 mL/min for 25 mL
    - l. Run buffer B (system pump buffer selector slot 3 100%B) @ 5mL/min 25 mL
    - m. Run buffer A (system pump buffer selector slot 3 0%B) @5mL/min 25 mL, zeroing out the absorbance under UV/Conn icon within 5 mL of completion
    - n. set column selection icon to bypass

o. set sample pump flow selection to "Sample pump direct inject, system pump waste." line on computer diagram should be light blue from the sample pump through the column and into waste.

p. using sample pump, direct inject 10 mL buffer A to clear out sample pump.

q. turn column selection icon back to position 1

r. clear out run.

7. Load the PaIVY and TEV sample

a. take the empty 50 mL falcon tube labelled "His 2 Peak Fraction" and place in plastic waste jug with lines in the falcon. The tube should not fall through into the bottom. it should sit on the lid of the waste collector.

b. determine volume and set injection volume of sample pump.

c. place falcon tube into clamp by sample pump. Insert brown sample loading tube to the bottom of the falcon.

d. load sample using sample pump at 5 mL/min. stop pump just before all sample is gone. you should see a large plateau on the chromatogram.

e. add approximately 10 mL buffer A at 5 mL/min to residual protein and run sample pump for <10 mL to ensure injection of all protein.

f. remove "His 2 peak fraction" falcon tube from the waste container. this is where your protein is.

g. switch sample pump flow selection to "Manual inject loop, system pump to column." Flow line on computer diagram should show a red injection loop and a green line running from the system pump through the column and to waste.

8. Set up fraction collection

a. ensure fraction collector is on, has clean racks in it, and is connected to the system.

b. click on the blue fraction collector viewer icon and press both "remove empty racks" and "remove all"

c. close out window

d. double click on fraction collector to open window. Rack type should be

F1 (12-13 mm X 100mm tubes) Start collection from A1 and end at B90. Fraction size should be 4 mL.

9. Achieve adequate separation.
   a. Set system pump to 5 mL/min, 0% B, for 25 mL. Start pump
   b. turn on fraction collection by double clicking fraction collector and pressing "collect." you are now collecting fractions.
   c. absorbance line should be near or at baseline after 25 mL run. if not, run 10 mL at a time until the baseline of A280 is less than 100 mAU

10. Elute His tag and TEV from the column.
    a. Under the system pump, choose gradient, initial %B 0%, final %B 100%, 25 mL then click the check box to run final B% for an additional 15 mL.
    b. Start flow. you should start to see a peak around 30%B
    c. at 100%B, you will notice that A280 flattens out around 300 mAU. To establish this is absorbance from the high concentration imidazole, run 15 mL 0%B and watch the absorbance line and the connectivity line. the line shapes should be very similar. A280 should drop down close to zero.

11. Finishing up
    a. Stop collection
    b. press "reset arm"
    c. save run under style PaIVY_(media used)_(labelling if applicable)_His1_Yearmonthday_(last name)
       i. PaIVY_M9_15N_His2_20190708_Schultz
       ii. PaIVY_LB_His2_20190708_Schultz
    d. close out run and open up analysis
    e. deselect every line trace except A280, %B, and connectivity
    f. Create run report under File
    g. save in a folder created on desktop.

Size Exclusion Chromatography (SEC) purification

The purpose of this step is to ensure that the protein collected from the previous purification steps is pure, and is the molecular weight expected for PaIVY. This column is much more fragile than the IMAC columns. Maximum flow through these columns is 0.5-0.7 mL/min. minimum volume required to equilibrate column is 120 mL so plan accordingly. If the column is stored in ethanol, it will require 120 mL of water, then 120 mL of SEC buffer to equilibrate, taking about 6-8 hours.

Materials

NGC liquid chromatography system running ChromLab program
PaIVY SEC buffer (see page 8) placed in buffer selector 6
His-2 purified peak fraction concentrated to under 5 mL.
one (1) 50 mL falcon tube labeled SEC purified PaIVY

Methods

1. Ensure SEC column (column selector 5) is equilibrated to PaIVY SEC buffer (buffer selector 6) and baseline is zeroed.
2. direct inject the His-2 purified, concentrated PaIVY onto the SEC column at 0.5 mL/min
3. Change the sample pump flow selector to "system pump to column, sample pump direct inject"
4. Set system pump for 0%B 120 mL at 0.5 mL/min and start the pump.
5. Immediately start fraction collection (4 mL)
6. PaIVY will come off the column around 80 mL.
7. Pool the peak fractions into the 50 mL falcon tube labeled SEC purified PaIVY and store in deli-style refrigerator.
8. Save run.