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**Employing Unnatural Amino Acids to Develop Bioconjugations, Multivalent Conjugates, and Protein Modulation Strategies**

Elizabeth King

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Employing Unnatural Amino Acids to Develop Bioconjugations, Multivalent Conjugates, and Protein Modulation Strategies

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Chemistry from The College of William and Mary

by

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EMPLOYING UNNATURAL AMINO ACIDS TO DEVELOP BIOCONJUGATIONS, MULTIVALENT CONJUGATES, AND PROTEIN MODULATION STRATEGIES

Elizabeth Ann King
Wytheville, Virginia

A Thesis Presented at the College of William & Mary in Candidacy for Departmental Honors

Department of Chemistry

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ABSTRACT

Unnatural amino acid technologies have been critical in introducing unique chemical functionality to proteins to allow for specific protein modulation or preparation of well-defined, homogeneous bioconjugates through bioconjugation chemistry. Bioconjugates are tremendously useful in disease treatment, diagnosis, and imaging. Therefore, the development and optimization of novel unnatural amino acids and bioconjugation methods, and their application to protein modulation or bioconjugate formation is a critical field of biochemical investigation. This thesis describes the development of a [2+2+2] cyclotrimerization bioconjugation using a novel unnatural amino acid. Then, several methods of preparing multivalent conjugates, which have a broader range of therapeutic applications than typical bivalent conjugates, are discussed. Finally, efforts to assay and modulate human protein arginine methyltransferase 1 using photoregulation unnatural amino acid technology are described. This thesis aims to expand the bioconjugation chemical toolbox, with high impact medicinal, therapeutic, and material applications.
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CHAPTER 1: INTRODUCTION TO UNNATURAL AMINO ACIDS AND BIOCONJUGATES

Natural and Unnatural Amino Acids

There are twenty naturally occurring amino acids encoded in the human genome that are used in protein synthesis, universally considered canonical code amino acids. An additional two amino acids also naturally found in protein, selenocysteine and pyrrollysine, are not coded for but can be incorporated into proteins via biochemical synthetic mechanisms. Amino acid structure consists of an alpha-carbon attached to a carboxyl group, amino group, hydrogen, and a side chain that serves as the only distinguishing factor between individual amino acids. Amino acids are divided into four classes based on side chain nature: nonpolar, polar uncharged, acidic, or basic (Figure 1.1). The alpha-carbon is usually a stereocenter and occurs naturally most often in the L-configuration. The genetic code for amino acids is degenerate as sixty-four codons encode only twenty amino acids. Three of these codons are naturally occurring stop sequences that signal protein synthesis termination by recruitment of

Figure 1.1. The 20 canonical amino acids. Image adapted from https://socratic.org/questions/which-part-of-an-amino-acid-s-structure-makes-it-unique-from-other-amino-acids
release factors: UAA, UGA, and UAG. The addition of amino acids to a polypeptide chain is
catalyzed naturally by ribosomes and requires anticodon-codon pairing of transfer RNA (tRNA)
to messenger RNA (mRNA) for amino acid delivery (Figure 1.2).1

Noncanonical code exploits the degeneracy of the existing genetic code to include unnatural amino acids (UAAs), defined as amino acids with altered side chains or derivations from normal structure.2-7 Expansion allows enhanced chemical, physical, and biological properties for proteins that contain altered amino acids. Variations can be structural or chemical in purpose and vary in specificity.3,8 Common novel functionalities include metal binding, photo-cross-linking, photocaging, and fluorescence.2 The positive, useful applications of UAA mutagenesis are important in probing structure and function of proteins and in engineering new proteins.6-7 Over one hundred UAAs have been synthesized and incorporated into proteins with various functions, including investigation into existing biological processes and additions to the understanding of protein fundamentals on atomic and molecular levels (Figure 1.3).3-4,8-9

![Figure 1.2. General overview of the process of translation. The ribosome assembles on mRNA and catalyzes the formation of peptide bonds between amino acids in a growing protein chain. The tRNA molecules contain a 3 base pair anticodon that pairs with a corresponding 3 base pair codon on mRNA molecule. Each tRNA is charged with a specific amino acid, which is added to the growing protein chain after proper anticodon-codon pairing. Once a stop codon is reached, there is not tRNA pairing and the ribosome disassembles.](image-url)
Bioconjugates

Chemical biology is an emerging field of present research that integrates chemical synthesis, analysis, and molecules into biological systems to allow for study or manipulability. Bioconjugate chemistry is an important area of chemical biology with broad applications in therapeutics, diagnostics, and materials. Bioconjugates are covalently linked molecules, including at least one biomolecule, such as a protein, carbohydrate, or oligosaccharide. The
biomolecules are typically conjugated to a fluorescent probe, immobilized surface, or small molecule. The partner then provides the bioconjugate with new functionality, such as fluorescence with a probe, target specificity, or inhibition with a small molecule drug.\textsuperscript{11}

Protein bioconjugates are an important class of conjugates that have been used extensively in therapeutic settings\textsuperscript{11-14}. Most notably, these molecules are critical for antibody-drug therapy.\textsuperscript{12-14} The conjugate affords cellular localization using an antibody for specificity to diseased cells with the therapeutic effects of an attached small molecule drug. An advantage to using proteins as the bioactive part of the molecule, rather than just small molecules therapeutics, is that the drug is delivered via protein-protein interactions with a naturally occurring protein.\textsuperscript{13} These interactions between proteins can be more easily modeled and predicted than small molecule drugs, and the specificity of the bioconjugate for its target is much higher.\textsuperscript{14} Therefore, this therapy technique is particularly useful in cancer treatments due to the combination of specificity and potency, alleviating many of the deleterious off-target side effects observed with traditional chemotherapies.\textsuperscript{14-15}

Bioconjugates are formed via bioconjugation reactions, which involve the formation of a stable covalent bond between the two components. These reactions must be biocompatible, meeting several chemical conditions that are necessary in the human body environment.\textsuperscript{16-18} These molecules must have rapid reaction rates at body temperature (37\,°\,C) and pH (\sim 7).\textsuperscript{16} These reactions must also be orthogonal, or chemically unreactive with the wide range of functional groups found on other biomolecules and small organic molecules in the body.\textsuperscript{18} Moreover, this requires minimal use of metal catalysts and organic solvents, making the development and optimization of new biological reactions an important part of the research.\textsuperscript{17}
A major limitation of such reactions is the necessity for novel chemical functionality in the protein. Most commonly, natural protein bioconjugates are formed utilizing the nucleophilic lysine, serine, tyrosine, and cysteine residues. These additions lack site-specificity, as there are many of these residues within a given protein that would react with the conjugation partner. Consequently, this produces a heterogeneous mixture of various products with different sites containing the added molecule (Figure 1.4). Such mixtures are sometimes purifiable, but it is difficult and time-consuming. For example, immunoglobulin G (IgG), which is the most common type of antibody found in the human body and often employed in the preparation of bioconjugates through conjugation at native lysine or cysteine sites, contains 80 lysine residues and 14 cysteine disulfide pairs. Bioconjugation results in a highly heterogeneous product mixture, and the prepared complexes vary significantly in potency, stability, solubility, and kinetics, leading to inconsistent treatment for patients. Reactions of the N- and C- termini have been employed to greatly enhance site selectivity, allowing for only one site in each protein to be modified. However, these sites have limited chemical functionality, might be blocked by post-translational modifications or protein folding, and their modification can interfere with natural protein processing and function in vivo.

One excellent way to prepare protein bioconjugates is to utilize UAA technologies to introduce unique reactivity not found naturally in proteins. This offers a bioorthogonal reactive handle in proteins which can be utilized to prepare well-defined, homogenous, stable protein
There are several known bioconjugations that utilize the unique reactivity of UAAs. One is the copper-catalyzed azide alkyne cycloaddition (CuAAC), or copper click reaction, in which a protein harboring an azide-containing UAA is reacted with an alkyne to generate a bioconjugate (Figure 1.5A). Another is the Staudinger ligation, which forms an amide bond between a protein with an azide-containing UAA and a phoshpine reaction partner, which has been applied to cell membrane modification and in vivo imaging (Figure 1.5B). Additionally, ketone and aldehyde UAA-containing proteins can undergo condensation with alkoxyamines and hydrazines (Figure 1.5C).

Despite these useful strategies, the bioconjugation toolbox is still extremely limited as compared to the total amount of developed chemical reactions. Therefore, it is imperative to investigate incorporation of UAAs with novel functionalities and development of bioconjugations to expand the applicability of these techniques.

**Incorporation Methods for Unnatural Amino Acids**

Incorporation of UAAs into proteins can be achieved through different processes, which can either be chemical or biosynthetic in nature. The most basic chemical approaches involve direct preparation or modification of amino acids. Reagents can react with specific amino acids, in reactions like transamination, resulting in side chains with different properties. Modification
to the thiol group of cysteine, the carboxylate group of aspartate and glutamate, and the N-terminal group are common derivations that occur due to the reactivity of those functional groups. However, this technique is difficult for site-specific modifications in proteins because all amino acids containing these functional groups in the primary structure may react and become modified.

Chemical synthetic approaches, particularly with an established solid-phase, provide a higher level of control over the protein product than biosynthetic methods, a large range of chemical functionality possibilities, and rapid effect and easy automation (Figure 1.6). Total synthesis, or solid-phase peptide synthesis, has become a common practice due to the development

![Diagram of solid-phase peptide synthesis](image)

**Figure 1.6.** Solid-phase peptide synthesis. Each deprotection and coupling cycle could be used to add an UAA, resulting in a synthetic peptide with any number of UAAs.
of solid-phase methods and protecting groups for side chains and the amino group.\textsuperscript{34-35} The C-terminal amino acid is attached to an insoluble resin. Activated amino acids containing a protecting group on the amino group, activating them at the carboxyl group, are added to the N-terminal of the immobilized amino acid. These amino acids are completely and rapidly deprotected to allow the step-by-step addition of more amino acids to the chain.\textsuperscript{33} This technique is useful because unnatural amino acids can be added as many times as desired, significant particularly for noncanonical code because the polypeptide chain could be made entirely of unnatural amino acids. However, due to the number of reactions, diminishing yields, and incomplete reactions, the synthetic peptide is often limited to only contain a maximum of 40 residues, so entire proteins are difficult to synthesize using this technique.\textsuperscript{33,36} Another limitation is low yield of peptide bond formation due to low rate constant, low reactant concentrations, and lack of proteases to aid C-terminal acyl transfer that drives natural protein synthesis.\textsuperscript{33}

The most used chemical approach, semi-synthetic protein ligation, involves chemical combination of two or more protein fragments that can be recombinant or synthetic to make a complete protein.\textsuperscript{33,37} General methodology for protein ligation starts with an initial capture step that combines two peptides using a chemical reaction that is faster than natural intermolecular acyl transfer. This initial step often utilizes reactivity of different functional side chain groups. Acyl transfer is thus made intramolecular, encouraging this reaction to occur more rapidly with higher efficiency. The final step is the chemical or spontaneous replacement of the capture moiety on the amino nitrogen with a hydrogen, resulting in a complete peptide bond.\textsuperscript{33} This technique is advantageous because it can produce peptides that are much longer than the 40-residue limit for total chemical synthesis, and the peptides do not have to be protected prior to ligation.\textsuperscript{33}
technique is also advantageous because it is successful in a variety of different chemical environments: in solution, in aqueous or organic solvent, or on solid support.\textsuperscript{33}

There are many different specific ligation methods, but unnatural amino acids are most commonly introduced to chemical fragments using a native chemical ligation variant, expressed protein ligation (EPL).\textsuperscript{38-39} In native chemical ligation, a thioester peptide and a cysteine-containing peptide are chemically ligated through transthioesterification resulting in a peptide bond, but size constraints exist because the thioesters must be totally chemically synthesized (Figure 1.7).\textsuperscript{33,40} EPL alternatively proposes addition of a peptide to a larger recombinant protein fragment to eliminate this size limit.\textsuperscript{39} The gene of interest is cloned into an intein vector and expressed in E. coli. The cysteine-containing peptide is fused with an intein-chitin binding domain sequence, that contains a defective peptide splicing mechanism that naturally produces thioesters that can then be intercepted for ligation.\textsuperscript{38} The sulfhydril group of cysteine goes through acyl transfer with the amino group to form a thioester with the carboxyl group of the recombinant protein.\textsuperscript{38} A synthetic peptide containing the desired unnatural amino acids is then added in excess with thiophenol to promote transthioesterification, analogous to native peptide ligation.\textsuperscript{38,40} Acyl transfer back to the amino group occurs, resulting in a peptide bond. This method has been successful in UAA incorporation, but limitations include inaccessibility of internal sites that cannot be cleaved and ligated and problems with live cell injection.\textsuperscript{33}

![Native chemical peptide ligation](image)

Biosynthetic methods of genetic incorporation use endogenous cellular machinery to translate the target protein. There are 64 codons total and
61 are “sense” codons that code for amino acids. Unnatural amino acid incorporation can be achieved through reassignment of sense codons to an unnatural amino acid rather than a canonical one under protein expression conditions. These results are achieved by reassigning all codons that encode one specific amino acid, resulting in a residue-specific incorporation (Figure 1.8). Sense codon reassignment is advantageous to biologists in understanding how much change the genetic code can endure and adapt, while also producing proteins with practical functionalities beyond the natural proteome. Useful applications include the incorporation of selenomethionine in place of methionine to advance crystallography or aryl halides that can undergo further palladium-catalyzed coupling reactions.

The first step in this method is auxotroph generation, an expression host that lacks the biosynthesis capabilities for producing one or more of the canonical amino acids. The auxotroph is then transformed with a plasmid that encodes for a specific protein of interest and grown in a medium with all canonical amino acids and necessary antibiotics for the protein.

After several rounds of centrifugation and washing, the expression cells are then placed in medium
containing all canonical amino acids except the one that is the target of reassignment and incubated at expression temperature. Finally, protein expression is induced.\cite{42}

Following auxotroph expression in medium lacking one of the canonical amino acids, reassignment translational activity then relies on the over-expression and activity of aminoacyl-tRNA synthetases (aaRS).\cite{46} Often, aaRS overexpression is not enough, and they must be engineered in two ways. Firstly, the aaRS has a binding pocket that is shape-selective. This must be redesigned to match the shape of the unnatural amino acid at its binding site to allow binding and eventual incorporation. Secondly, the aaRS has a hydrolysis site that edits tRNA that is improperly aminoacylated. This site may also be engineered to allow unnatural amino acids to pass through, thus providing an efficient way to genetically introduce a UAA.\cite{46} However, this entire process excludes one or more canonical amino acids from the protein products, which can be disadvantageous to protein folding and function. This technique, while residue-specific, is not site-specific and will incorporate UAAs at every codon that encodes for the missing canonical amino acid\cite{5}. Amber codon suppression incorporation is the most universal method for UAA site-specific genetic incorporation (Figure 1.9).\cite{47-48} Amber stop codon (UAG) is the least used stop codon and rarely terminates essential gene translation.\cite{3-4,41,49} The UAG triplet normally causes translation to terminate due to release factor 1, but in UAA mutagenesis this triplet can be used to introduce an amino acid using a tRNA at the stop codon. Incorporation at the amber codon requires a tRNA and its related aminoacyl-tRNA synthetase that allows the sequence to recognize the UAA of interest. This machinery must be orthogonal to endogenous machinery to prevent cross
reactions. The tRNA synthetase has minimal interaction with the anticodon of its tRNA, thus mutations to the binding pocket for the UAA are effective.

The Schultz method is the classical way to generate orthogonal aaRS and tRNA pairs that will accept UAAs, consisting of different positive and negative selection phases (Figure 1.10). First, a large library of mutant tRNA that contain an amber codon anticodon are transformed into E. coli with an antibiotic resistance gene harboring a TAG codon. This is a positive selection phase as the aaRS should aminoacylate the tRNA and will express the antibiotic resistance protein, conferring increased cellular viability when grown in the presence of that antibiotic. Cells containing aaRS’s that cannot charge the tRNA stop translation at the TAG codon and do not produce the antibiotic resistance protein, leading to lethality and removal from the selection pool. Secondly, a negative phase coexpresses the surviving tRNAs with a heterologous aaRS mutant library of survivors utilizing the cytotoxic barnase gene with 3 TAG codons. These cells are plated
in the absence of the UAA and any aaRS’s that recognized a natural amino acid in the positive selection now read through the TAG codons to produce the toxic barnase gene and die. The synthetase and tRNA pairs that are orthogonal to E. coli expression machinery are the only pairs to survive and are UAA-specific.\textsuperscript{3,51-52}

For site-specific incorporation, the UAA-specific tRNA and aaRS pairs are used to insert an unnatural amino acid at a nonsense codon. An expression plasmid encodes a nonsense mutant and is coexpressed with a corresponding suppressor plasmid containing the orthogonal pair specific to the desired unnatural amino acid.\textsuperscript{49,53} This process led to the development of amber-suppressor plasmid pEVOL, providing an improvement to amber codon mutant development that is not as cumbersome as the multiple phases of the Schultz method.\textsuperscript{51,54} The development system used \textit{glnS} as a constitutive promoter to ensure a basal level of the aaRS.\textsuperscript{55} An inducible promoter, \textit{araBAD}, was added to increase concentration of the aaRS.\textsuperscript{54,56} The tRNA expression was optimized if mutated at several positions and subjected to \textit{proK} promoter control.\textsuperscript{49,57} Ultimately,
one copy of tRNA expression occurs under proK promoter and two copies of synthetase under araBAD and glnS.\textsuperscript{54} This system and other plasmid systems are created to maximize tRNA/aaRS orthogonal air development and UAA expression efficiency, as low yield is common with amber codon suppression.\textsuperscript{54,58}

**Conclusion**

Unnatural amino acid technology is essential in bioconjugate preparation and protein modulation. While unnatural amino acids are incorporated through a variety of biosynthetic and chemical techniques, the Schultz two-plasmid method is employed in our lab due to its simplicity and ability to be applied to a wide variety of proteins with single-residue specificity. Expanding the bioconjugation toolbox with the development of new UAAs and bioorthogonal reactions is necessary to advance this field. This thesis will describe a novel dipropargyl amine UAA and cyclotrimerization bioconjugation, the application and optimization of bioconjugations towards the development of multivalent bioconjugates, and the application of a photoactive UAA in protein function modulation of therapeutically relevant human protein arginine methyltransferase 1.

**References**


CHAPTER 2: GENETIC ENCODING OF A NOVEL DIPROPARGYL AMINE BIOCONJUGATION HANDLE FOR [2+2+2] CYCLOADDITION REACTIONS

Introduction

Bioconjugation reactions, in which a biomacromolecule is covalently linked to a functional partner, are presently widely employed in the fields of medicine, pharmaceuticals, and materials. Protein bioconjugates, particularly those in which an antibody is conjugated to a small-molecule drug, are especially valuable for drug delivery. The attachment of antibody allows for the drug molecule to be targeted to a specific location within the patient, making it more effective in interacting with the target and preventing off-target affects. Ultimately, lower doses can be administered for the same therapeutic effect, leading to reduced side effects for treatments such as chemotherapy for cancer.

Unnatural amino acid (UAA) technology represents a means to utilize bioorthogonal handles to prepare well-defined, homogeneous protein bioconjugate products. These homogeneous conjugates have been shown to be more efficacious than heterogeneous conjugates and to possess several therapeutic advantages. One common bioorthogonal reaction employed to prepare protein bioconjugates is the copper-catalyzed azide alkyne cycloaddition (CuAAC), known as the “copper click” reaction, in which an UAA with either an alkyne or azide group is reacted with an azide-containing or alkyne-containing reaction partner, respectively. More recently we reported the development of a Glaser-Hay bioconjugation reaction involving the coupling of an alkynyl UAA with a soluble terminal alkyne to afford a covalent diyne linkage. However, there are a limited number of bioconjugation reactions available due to the necessity of physiological conditions and lack of cross-reactivity with endogenous biological systems. Thus, it is critical to
develop novel bioconjugation reactions to afford a variety of chemical tools for different applications and expand the scope of bioconjugation reactions.

The [2 + 2 + 2] cyclotrimerization reaction can be catalyzed by a variety of transition metals and relies on three alkyne moieties reacting to yield aromatic compounds. This cycloaddition is widely used and is a key organic methodology to generate a stable, polysubstituted benzene ring. Several [2 + 2 + 2] cycloadditions have been reported in alcohol and water biphasic systems and supercritical water under inert atmosphere. However, to the best of our knowledge, a biological [2 + 2 + 2] cyclotrimerization has not previously been reported, despite the prominence of this reaction in synthetic organic synthesis. Grigg et al. previously applied a rhodium coordination complex to produce various heterocycles from diynes and monoynes. Recently, Wang et al. demonstrated the utilization of a rhodium-catalyzed cyclotrimerization in aqueous conditions at 60°C, illustrating the potential feasibility of the reaction in physiological environments (Figure 2.1A). Consequently, we aimed to adapt these reactions for use on biological macromolecules.

![Figure 2.1](image-url)

**Figure 2.1** Development of aqueous [2+2+2] cycloaddition reactions under mild conditions. A) Wang et al. successfully developed an aqueous [2+2+2] cyclotrimerization reaction at 60 °C. B) To further investigate the feasibility of adapting this reaction to a biological setting, we attempted the same aqueous coupling at room temperature using a biphenyl ligand previously used in the Glaser-Hay bioconjugation, affording a trimer cyclized propargyl ether product in 30% yield.
to afford a new bioconjugation reaction. Ideally, we aimed to contribute a new bioorthogonal reaction to the biological toolbox as a means to assist in disease diagnosis improve drug delivery systems. Herein, we report a novel cyclotrimerization bioconjugation utilizing a newly synthesized and site-specifically incorporated dipropargyl amine UAA. This work has key applications towards the preparation of specific, homogeneous protein bioconjugates.

**Results and Discussion**

**Development of Physiologically Compatible [2+2+2] Cyclotrimerization**

Given the aqueous cycloaddition previously demonstrated, we sought to optimize this reaction at temperatures lower than 60°C using a water soluble carboxylated biphenyl ligand that has previously been demonstrated to provide effective chelation in the Glaser-Hay bioconjugation. As a proof-of-concept experiment, we attempted to dimerize propargyl ether under similar conditions as previously reported (Figure 2.1B). Gratifyingly, when performed at room temperature, product was able to be detected by thin layer chromatography (TLC) and $^1$H NMR (30% yield). Rather than coupling of the propargyl ether to the phenylacetylene, we observed a trimer product of the propargyl ether, which still demonstrated that this rhodium-catalyzed [2+2+2] cycloaddition can occur effectively at room temperature under aqueous conditions.

In order for this reaction to be applicable to a protein context, a dipropargyl functionality must be included into the amino acid component (Figure 2.2A). Thus, a UAA containing the dipropargyl amine functionality based on a substituted phenylalanine backbone, $p$DPrAF was synthesized (Figure 2.2B). Initially, the conditions used for the synthesis of the alkyne containing UAA $p$PrF were attempted with 2 equivalents of propargyl bromide (Figure 2.3A). However, these conditions proved too mild, and addition of only one propargyl alkyne to the amine
functionality was the observed major product. Increased temperature, equivalents of propargyl bromide, and reaction time afforded the desired dipropargyl functionality. Additionally, the use of cesium carbonate as opposed to potassium carbonate increased yield (Figure 2.3B). Though intending for addition of the alkyne-containing propargyl groups to react solely at the amine, we also observed reaction at the unprotected carboxylic acid as well. However, we realized that the standard deprotection protocol would convert the ester back to the desired carboxylic acid while retaining the desired dipropargyl amine functionality. Thus, we chose not to initially protect the carboxylic acid group to reduce the overall number of steps in the synthesis.

Our two-step synthesis afforded the desired product 1 with an overall yield of 47%. The first step, the SN2-like addition of three alkyne-containing propargyl groups to the molecule, resulted in the formation of the intermediate with good yield (48%). The intermediate was then deprotected in a series of acid/base reactions to afford the desired product in excellent yield (98%).

Figure 2.2: Development of the components for a [2+2+2] cycloisomerization bioconjugation. A) Generic [2+2+2] cycloaddition reaction. B) The synthesized pDPnAF UAA (1) to be employed in the bioconjugation. C) Incorporation of 1 into GFP at residue 151 using the pCNF aRS. This polyspecific synthetase has already been demonstrated to incorporate other UAAAs (pPrF and pBrPrF), and only small amounts of protein are detected in the absence of an UAA.
Following the synthesis of 1 (Figure 2.2B), the next requisite for site specific incorporation was the genetic encoding of the new UAA. While this typically requires a double-sieve aminoacyl-tRNA synthetase (aaRS) selection, previous research has demonstrated that several pre-existing aaRS/tRNA pairs confer a degree of polyspecificity towards multiple UAAs. Consequently, we attempted to identify an appropriate aaRS capable of both recognizing 1 and charging it to the appropriate tRNA. We first investigated several synthetases due to either known polyspecificity, or due to their incorporation of structurally similar UAAs. Plasmids encoding both the aaRS and tRNA were co-transformed into BL21(DE3) E. coli with a pET-GFP-TAG-151 plasmid, harboring GFP with a TAG codon at position 151. Following protein expression, GFP mutants were purified using a Ni-NTA resin and analyzed by SDS-PAGE to determine incorporation of 1. Gratifyingly, the promiscuous pCNF aaRS was shown to effectively incorporate 1 into GFP at position 151, in similar yields to its incorporation of pPrF and pBrPrF, two commonly employed alkyne containing UAAs (Figure 2.2C). Successful incorporation was also confirmed through mass
Thus, the previously evolved pCNF aaRS was utilized to express mutant GFP harboring 1 at position 151.

**Development and Optimization of Biological [2+2+2] Cyclotrimerization Conditions**

With 1 successfully incorporated and encoded into a protein, optimization of the [2+2+2] cyclotrimerization in a biological context was investigated. As is the case in other bioconjugations, the amount of transition metal catalyst should be minimized to reduce potential cytotoxicity. Conveniently, the toxicity of rhodium has been demonstrated to be lower than that of other transition metals, including platinum, palladium, cadmium, nickel and chromium. More specifically, in assays examining the impact of transition metals on oxidative damage in epithelial cells, rhodium was demonstrated to be the least cytotoxic of the metals tested. Further, rhodium complexes have also been integrated into proteins to generate stable organometallic proteins.
Thus, it is reasonable to employ rhodium in bioconjugation reactions, provided low concentrations and relatively short reaction times.

To test the viability of this reaction, we sought to couple the pDPrAF-containing GFP with the commercially available AlexaFluor-488 Alkyne (Figure 2.5A). To do this, a rhodium (I) dimer complex, \([\text{Rh(cod)Cl}]_2\) (250 mM in DMSO) was mixed with the biphenyl ligand (500 mM in H2O). Following complex formation, the \(p\text{DPrAF-GFP (pH} = 7.4, \sim 1.0 \text{ g/mL) and AlexaFluor-488}

![Figure 2.5. (2+2+2) cycloaddition bioconjugation. A) Experimental proof-of-concept experiment linking the GFP-containing pDPrAF with a Fluor-488-Alkyne label. Successful cycloaddition reaction yields an isoindole ring linkage. B) SDS-PAGE analysis of the successful bioconjugation. The gels were analyzed first for fluorescence (top gel) and then stained with Coomassie blue to visualize protein (bottom gel). Lane 1: Wild-type GFP protein containing Tyr at residue 151 demonstrating no fluorescent signal after denaturation of the protein. Lane 2: GFP containing 1 at residue 151 that was not subjected to reaction conditions. Lane 3: GFP containing 1 at residue 151 was reacted in the presence of both the Fluor-488-Alkyne and the rhodium system, affording the cycloaddition product and fluorescence due to the covalent linkage of the fluorophore to the protein. Lane 4: GFP containing 1 at residue 151 reacted in the presence of the Fluor-488-Alkyne but no rhodium, demonstrating that non-covalent association of the dye does not occur.](image)
alkyne (1 mM in DMSO) were added. This aqueous reaction proceeded for 12 hours at 4°C. A lower temperature than in the organic test of this aqueous coupling was employed in order to limit protein degradation by rhodium. Gratifyingly, the reaction was successful, as indicated by fluorescence at the appropriate molecular weight by SDS-PAGE analysis (Figure 2.5B). The GFP was denatured prior to the gel analysis, so the fluorescent signal is the result of a direct conjugation between the protein and the fluorophore. Unfortunately, in initial reactions, significant levels of protein degradation were also observed, requiring further optimization of reaction conditions.

It was hypothesized that the observed protein degradation was potentially due to the formation of radicals caused by the presence of the rhodium catalyst. Protein oxidation was previously observed in the Glaser-Hay bioconjugation due to the copper catalyst.\textsuperscript{13-14} In order to mitigate radical formation, catalase and sodium ascorbate were examined as radical scavengers to reduce protein oxidation and minimize protein degradation. The utilization of catalase was previously demonstrated to be successful in the case of the Glaser-Hay reaction.\textsuperscript{15} Moreover, sodium ascorbate has a long history of reducing protein oxidation in bioconjugation reactions, namely in the copper click
reaction and in biological Sonogashira couplings.\textsuperscript{30,31} Both catalase and sodium ascorbate resulted in significantly reduced protein degradation, with sodium ascorbate being most effective, suggesting that damaging radicals are likely formed at some point during the reaction. Overall, we successfully performed a novel bioconjugation reaction between GFP harboring 1 in position 151 and Fluor-488 alkyne through a [2 + 2 + 2] cyclotrimerization.

With the protein oxidation minimized, further optimization of the reaction was attempted, as some degradation was still observed. Initially, a time course was conducted to elucidate the optimal reaction time. Reactions were conducted for durations 0, 2, 4, 8, and 12 hours with all other conditions kept constant (Figure 2.6). It was determined that protein degradation increased fairly linearly with time, and the maximum ratio of coupling to form the bioconjugate product occurred after a reaction time of 2 hours (Figure 2.6). We next investigated the effects of temperature on the reaction, evaluating reaction temperatures of 4°C, 22°C, and 37°C. These reactions were conducted for 2 hours with all other conditions held constant. It was observed that the 22°C reaction afforded the best coupling relative to protein degradation. Reaction at 37°C led to significant protein degradation and...
reduced coupling (Figure 2.7), while reactions at 4°C had similar amounts of degradation with less coupling. Finally, we examined the effect of pH on the reaction. In the original proof-of-concept, GFP harboring 1 in a phosphate-buffer solution with pH 7.4 was employed. Consequently, the cyclotrimerization reaction was conducted for 2 hours at 22 °C on reactions containing protein in solution of pH 6.0, pH 7.4, and pH 8.0. While pH did not appear to have an effect on coupling, it did have minor impact on protein degradation, with the pH 7.4 reaction affording the least protein degradation (Figure 2.8). With the optimized conditions established, the coupling yield was determined via the coupling of the GFP to a SR-Fluor 680-Alkyne ($\varepsilon = 257800 \text{ M}^{-1} \text{ cm}^{-1}$). The unique spectrum of the fluorophore alkyne relative to GFP allowed for quantification of the coupling yield via absorption spectroscopy. Using the absorbance at $\lambda_{\text{max}}$ and extinction coefficients of both the GFP and the alkynyl fluorophore, Beer’s law was employed to demonstrate a 91% coupling yield under similar conditions with rhodium omitted from the reaction. While the coupling efficiency was high, future optimization may be necessary to further increase efficiency and decrease protein degradation. The genetic incorporation of
pDPrAF as a bioconjugation handle is advantageous due to its applicability in a range of bioconjugation reactions.

Beyond just the [2+2+2] cycloaddition, the alkynyl functionalities can also be employed in other reactions. Specifically, the versatility of the UAA 1 was assessed through examining its reactivity in an alkyne/azide 1,3-dipolar cycloaddition ("copper click") bioconjugations, as well as in a Glaser-Hay bioconjugations. For the copper click bioconjugation, the terminal alkyne groups of 1 within GFP were reacted with an azide-containing fluorophore to form a stable triazole ring. For the Glaser-Hay bioconjugation, the terminal alkyne group in 1 in GFP was reacted with an alkyne-containing fluorophore in the presence of a Cu(I)/TMEDA catalyst to generate a linear, stable 1,3-diyn functional group. For each, we also hypothesized that these reactions could occur on both terminal alkynes within the same protein, which would afford a bioconjugate with two fluorophores attached to GFP at position 151. Gratifyingly, SDS-PAGE demonstrated that 1

![Figure 2.9](image.png)

Figure 2.9. Bioconjugation versatility of the pDPrAF UAA. SDS-PAGE analysis shows that pDPrAF UAA 1 is capable of successfully participating in three unique bioconjugation reactions. The gel was analyzed first for fluorescence (top gel) and then stained with Coomassie blue to visualize protein (bottom gel). Lane L: protein ladder. Lane 1: Wild-type GFP protein containing Tyr at residue 151 demonstrating no fluorescent signal after denaturation. Lane 2: GFP containing 1 at residue 151 that was not subjected to any reaction. Lane 3: GFP containing 1 at residue 151 was reacted with Fluor 488-alkyne and the described rhodium system, affording the [2+2+2] cycloaddition bioconjugation product and fluorescence due to the covalent linkage of the fluorophore to the protein. Lane 4: GFP containing 1 at residue 151 reacted with Fluor 488-azide in the copper click reaction, affording the 1,3-dipolar cycloaddition bioconjugation product and fluorescence due to the covalent linkage of the fluorophore to the protein. Lane 5: GFP containing 1 at residue 151 reacted with Fluor 488-alkyne in the Glaser-Hay reaction, affording the linear 1,3-diyn bioconjugation product and fluorescence due to the covalent linkage of the fluorophore to the protein.
incorporated into proteins is capable of being employed in both copper click and Glaser-Hay bioconjugations (Figure 2.9). Analysis by MS indicated the single addition of a fluorophore for both the Glaser-Hay and 1,3-dipolar cycloaddition reactions under these conditions, as well as some double-addition of the fluorophore through the copper click reaction (Figure 2.3). Despite the possibility for two additions due to the presence of two alkyne moieties in the pDPrAF, multiaddition products were not the major products obtained. Further optimization of these reactions could afford conditions more favorable for the secondary addition of the fluorophore. Combined with its use in our newly developed cyclotrimerization bioconjugation, there are at least three distinct bioconjugation reactions in which pDPrAF can participate, making it a valuable amino acid with many potential applications.

**Conclusion**

Overall, the synthesis and incorporation of a novel, dipropargyl amine UAA capable of undergoing both the alkyne/azide 1,3-dipolar cycloaddition and Glaser-Hay bioconjugations was demonstrated. Furthermore, this novel UAA was used in a novel [2+2+2] cyclotrimerization bioconjugation. This bioconjugation affords a polysubstituted benzene ring as part of the conjugate, generating a highly stable covalent linkage between the two reaction partners. While potential further optimization of the reaction is being investigated, this work introduces a new bioconjugation reaction as a biochemical tool with widespread utility in a plethora of applications.

**Experimental**

**General.** pEvol plasmids were obtained from the laboratory of Prof. Peter Schultz. Fluorophores, chemical compounds, and solvents were purchased from Sigma-Aldrich and used without further purification. Reactions were conducted under ambient atmosphere with non-distilled solvents.
NMR data was acquired on a Varian Gemini 400 MHz. All GFP proteins were purified according to manufacturer’s protocols using a Qiagen Ni-NTA Quik Spin Kit.

**Synthesis of p-dipropargylaminophenylalanine (pDPrAF).** p-Aminophenylalanine-OMe (0.50 g, 1 eq, 1.78 mmol) was added to a flame-dried vial. Potassium carbonate (1.23 g, 5 eq, 8.91 mmol) was added, followed by DMF (7 mL). This mixture was stirred at room temperature for 5 minutes. Propargyl bromide (0.78 mL, 5 eq, 8.91 mmol) was then added and the reaction was stirred at 80°C for 96 hours. The reaction was then cooled to room temperature and extracted with DCM and brine. The organic layers were combined, dried with magnesium sulfate, filtered, and excess solvent was removed in vacuo. The reaction was purified via flash chromatography (silica gel, 3:1 hexanes: ethyl acetate) to yield the desired product as a yellow oil (0.33 g, 0.85 mmol, 48% yield).

\[ ^1H \text{NMR (400 MHz, CDCl}_3 \]: } \delta 7.06 (d, J = 9 Hz, 2 H), 6.87 (d, J = 9 Hz, 2 H), 4.96 (d, J = 8 Hz, 1 H), 4.70 (q, J = 18 Hz, 2 H), 4.56 (d, J = 8 Hz, 1 H), 4.08 (s, 4 H), 3.02 (t, J = 8 Hz, 2 H), 2.51 (s, 1 H), 2.24 (s, 2 H), 1.41 (s, 9 H). \]

\[ ^{13}C \text{NMR (400 MHz, CDCl}_3 \]: } \delta 171.4, 155.3, 147.0, 130.3, 126.8, 115.9, 80.1, 79.4, 75.6, 72.9, 54.5, 52.7, 40.6, 37.2, 28.5. \text{ M/Z = 395.3.} }
This product was then dissolved in 1,4-dioxane (2 mL). Then, 1 M lithium hydroxide (2 mL) was added and the reaction was stirred at room temperature for 2 hours. 1,4-dioxane was then removed in vacuo and the resulting water solution was acidified through the addition of 6 M HCl. The reaction was then extracted into ethyl acetate and the organic layer dried with magnesium sulfate.
and filtered. Excess solvent was removed in vacuo to yield a light brown oil. This oil was dissolved in DCM (1.5 mL). Trifluoroacetic acid (TFA, 0.5 mL) was added and the reaction was stirred at room temperature for 1 hour. Excess solvent was removed in vacuo to yield pDPrAF as a brown solid (0.22 g, 0.84 mmol, 98% yield). $^1$H NMR (400 MHz, MeOD): δ 7.18 (d, J = 9 Hz, 2 H), 6.98 (d, J = 9 Hz, 2 H), 4.13 (s, 4 H), 3.07 (dd, J = 9 Hz, 1 H), 2.58 (s, 2 H), 2.23 (s, 2H). $^{13}$C NMR (400 MHz, MeOD): δ 147.6, 129.9, 124.6, 116.0, 78.9, 72.7, 39.8, 35.3. M/Z = 257.1. Overall yield 47.1%.

$^1$H NMR spectrum of deprotected pDPrAF
Expression of pDPraf-containing GFP-151. *Escherichia coli* BL21(DE3) cells were co-transformed with a pET-GFP-TAG-151 plasmid (2.0 μL) and a pEvol-pCNF plasmid (2.0 μL) using an Eppendorf electroporator. Cells were then plated on LB-agar plates supplemented with ampicillin (50 mg/mL) and chloramphenicol (34 mg/mL) and grown at 37°C. After 16 hours, a single colony was used to inoculate LB media (10 mL) supplemented with ampicillin and chloramphenicol. The culture was grown to confluence at 37°C over 16 hours. This culture was then used to begin an expression culture in LB media (20 mL) at OD600 = 0.1, then incubated at 37°C until it reached an OD600 of between 0.7 and 0.8. At this point, mutant protein expression was induced through the addition of 1 M ITPG (20 μL) and 20% arabinose (20 μL), as well as 100 mM pDPraf (200 μL). Induced cells were grown for an additional 16 hours at 30°C, then harvested via centrifugation (10 mins, 5000 rpm). The media was decanted, and the cell pellet was stored in a -80°C freezer for 20 minutes. Mutant GFP was then purified using commercially

13C NMR spectrum of deprotected pDPraf
available Qiagen Ni-NTA Quik Spin Kit according to the manufacturer’s protocol. Protein yield and purity was then assessed via SDS-PAGE and spectrophotometrically via a Nanodrop spectrophotometer. Protein was then transferred into phosphate buffered saline solution (PBS) using 10k MWCO spin columns prior to use in bioconjugation reactions.

**Biological Cyclotrimerization protocol.** To a sterile 1.5 mL Eppendorf tube, the following were added: 5 μL of [Rh(cod)Cl]2 (250 mM in DMSO) and 5 μL of 2,2’-Bipyridine-4,4’-dicarboxylic acid (500 mM in DI H2O). The two solutions were mixed thoroughly by pipetting until a dark red color was achieved. Next, 30 μL of GFP containing pDPrAF at position 151 (GFP/pDPrAF; pH=7.4; ~1.0 mg/mL) and 20 μL of Fluor-488 Alkyne (1 mM in DMSO) were added to the tube. Finally, 5 μL of sodium L-ascorbate (200 mM in DI H2O) was added to the tube. The reaction was incubated at 4°C. After 2 hours, excess reactants were removed via buffer exchange using 10k MWCO spin columns. The reaction was washed with phosphate buffered saline solution (pH 7.4 PBS, 8 x 200 μL) to a final volume of 50 μL. The reaction was analyzed by SDS-PAGE and imaged using a SYPRO Ruby scan to analyze fluorescence. The gel was stained for 3 hours using Coomassie Brilliant Blue, then destained overnight using a methanol solution (60% deionized water, 30% methanol, 10% glacial acetic acid). The gel was then imaged using a Coomassie scan protocol.

**Biological Glaser-Hay protocol.** To a sterile 1.5 mL Eppendorf tube, the following were added: 5 μL of a vigorously shaken CuI solution (500 mM in DI H2O) and 5 μL of tetramethylethylenediamine (500 mM in DI H2O). The two solutions were thoroughly mixed by pipetting. Next, 30 μL of GFP containing pDPrAF at position 151 (GFP151/pDPrAF; pH = 6; ~1.0
mg/mL) and 20 µL of Fluor-488 Alkyne (1 mM in DMSO) were added to the tube. The reaction was incubated at room temperature (22 °C). After 4 hours, excess reactants were removed via buffer exchange using 10k MWCO concentrator columns. The reaction was washed with phosphate buffered saline solution (pH 6 PBS, 8 x 200 µL) to a final volume of 50 µL. The reaction was analyzed by SDS-PAGE as previously described.

Biological copper click protocol. To a sterile 1.5 mL Eppendorf tube, the following were added: 2 µL of CuSO\(_4\) solution (50 mM in DI H\(_2\)O) and 2 µL of TCEP (50 mM in DI H\(_2\)O). The two solutions were thoroughly mixed by pipetting. Next, 20 µL of GFP containing pdPrAF at position 151 (GFP151/pdPrAF; pH = 7.4; ~1.0 mg/mL) and 10 µL of Fluor-488 Azide (1 mM in DMSO) were added to the tube. Finally, 10 µL of TBTA (5 mM in DMSO) was added to the tube. The reaction was incubated at 4 °C. After 16 hours, excess reactants were removed via buffer exchange using MWCO concentrator columns. The reaction was washed with phosphate buffered saline solution (pH 7.4 PBS, 8 x 200 µL) to a final volume of 50 µL. The reaction was analyzed by SDS-PAGE as previously described.

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CHAPTER 3: EMPLOYING UNNATURAL AMINO ACIDS TOWARDS THE DEVELOPMENT OF MULTIVALENT CONJUGATES

Introduction

As previously described, bioconjugates have widespread applications in various fields, including therapeutics, diagnostics, and materials.\(^1,2\) However, each of the bioconjugation reactions previously discussed, as well as nearly all the bioconjugation reactions currently utilized employ only two reaction partners.\(^1-5\) The resulting bivalent bioconjugate product is essentially limited to containing two distinct functionalities. Therefore, the preparation of a multivalent bioconjugate, in which three or more reaction partners (containing at least one biomolecule) are conjugated, could provide a broader range of more powerful and applicable bioconjugates. For instance, an existing antibody-drug conjugate that contains localization and therapeutic functionalities could be reacted with a third fluorescent probe partner. Then, the whole delivery and treatment could be tracked via fluorescence of the same molecule.

Although antibody conjugations with natural amino acids are often nonspecific and result in heterogeneous products, there has been recently reported success in site-specific conjugations of multiple antibodies to a single virus-nanoparticle, which has a variety of drug delivery, imaging, and vaccine applications.\(^6-8\) Most recently, Park et al. (2020) reported site-specific functionalization of human IgG1. A site-specific de-glycosylation of Asn295 made Gln297 enzymatically accessible, followed by a transglutaminase reaction to add an azide functional group to the protein (Figure 3.1).\(^6\)

![Figure 3.1](image-url)
Heterobifunctional ligands were conjugated at multiple sites on the virus nanoparticle, and then the antibody was conjugated at each site using click chemistry to generate a nanoparticle with multiple proteins conjugated to it, resulting in a large complex containing many biomolecules (Figure 3.1). However, while this approach could theoretically be employed to add multiple different biomolecules containing an azide functionality to the virus nanoparticle, such as the antibody and a fluorophore azide, there would be low-yield of a specific desired multivalent product due to cross-reactivity and lack of differential specificity for the two different reaction partners. This lack of specificity is an illustrative example of why residue-specific unique chemical groups are necessary to prevent cross-reaction in multivalent conjugation formation. Additionally, nanoparticles are much bulkier, have limited success on cell entrance, and have evidence of toxicity in human therapeutics, so the development of multiprotein conjugates without the nanoparticle middleman might be more therapeutically relevant.

For bioconjugation reactions involving proteins, unnatural amino acid (UAA) conjugations have already been established as superior to conjugation of a protein’s natural 20 amino acids in the preparation of several bivalent conjugates. Therefore, producing a multivalent conjugate using UAA technology would be favorable to other methods, as UAA conjugation enables a high degree of selectivity over the location of conjugation and avoids production of a heterogeneous product mixture.

Optimization of existing unnatural amino acid technology towards the generation of multivalent conjugates by incorporation of multiple amino acids has already been reported in the literature. Wan et. al (2010) concomitantly incorporated two different UAAs into GFP by simultaneously suppressing both the amber (TAG) and ochre (TAA) stop codons in E. coli. This required the use of two distinct, evolved orthogonal amino-acyl (aaRS)/tRNA pairs. It was
successful on combinations of an azide UAA with multiple partner UAAs containing alkyne, cyclopentane, and tert-butoxy functional groups on their side chains. Xiao et. al (2013) developed this methodology further to incorporate multiple UAAs and generate a trivalent conjugate in mammalian cells. Using simultaneous TAA and TAG codon suppression, the ketone-containing UAA pAcF and azide-containing UAA AzK were incorporated into the heavy chain and light chain of full-length anti-HER2 antibody, respectively. The drug auristatin (nAF) was conjugated to pAcF while a fluorophore was conjugated to AzK, producing a trivalent antibody-drug-fluorophore conjugate (Figure 3.2A). In 2010, Neumann et. al evolved a quadruplet-decoding ribosome and orthogonal aaRS/tRNA pairs in which four base pair sequences encoding blank codons could be recognized and subsequently result in incorporation of UAAs. Using this technology, Neumann genetically encoded an azide and alkyne into the same protein at different sites and successfully linked the groups with an internal Cu(I)-catalyzed cycloaddition reaction (Figure 3.2B). Oh et. al (2014) were able to translationally incorporate five UAAs into GFP using an optimized cell-free protein synthesis system. The synthesis solution containing the plasmid encoding GFP and necessary nucleoside triphosphates was cleared of five canonical amino acids (valine, phenylalanine, arginine, threonine, and tyrosine) and
supplemented with five UAA analogs containing various functional groups to replace them.¹⁵

While these researchers have made advancements towards, and, in the case of Xiao, even generated multivalent conjugates, developing and optimizing cell-free translational systems and suppressing multiple codons with multiple evolved orthogonal synthetases or evolving a novel ribosome are tedious and complicated processes.¹²-¹⁵ If, instead of incorporating multiple UAAs, a single UAA could be incorporated with the potential to undergo two bioconjugation reactions, a multivalent conjugate could be prepared easily in two steps without the need for further genetic manipulation. The result would be a complex with three functional biomolecules conjugated at a single location using well-developed and efficacious methodology.

**Results and Discussion**

**Development of a Biological Thiol-yne Addition**

We first investigated multivalent conjugate preparation using a previously reported organic thiol-yne reaction between an alkyne moiety and thiol reactive group (Figure 3.3A).¹⁶ This is accomplished organically using a catalytic photoinitiator, which produces a reactive chemical species that drives reaction upon irradiation with light. The thiol group adds across a pi bond, producing an alkene from an alkyne, or alkane from an alkene.¹⁶ Assuming the thiol adds across all pi bonds, there could be up to four sites of addition in one 1,3-diyne molecule.

In proof-of-concept experiments under biological conditions, optimization of the thiol

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**Figure 3.3.** A.) Overview of synthesis of a trivalent conjugate using a 1,3-diyne conjugate and a thiol reaction partner. Each different colored orb represents a potential biomolecule or bioconjugate reaction partner (fluorophore, small molecule drug, etc.). The thiol group could theoretically add at either or both alkyne groups. B.) Structure of fluorescent molecule danyl glutathione (dGSH) containing a free thiol group (highlighted in red). C.) Structure of terminal alkyne amino acid ρPrF employed in initial proof of concept thiol-yne additions.
addition was investigated to generate bivalent conjugates. First GFP was expressed with the terminal alkynyl amino acid pPrF (pPrF-GFP) and reacted with a thiol-containing fluorescent molecule called dansyl glutathione (dGSH), either with or without the addition of the photoinitiator DPAP (Figure 3.3B-C). Gratifyingly, pPrF-GFP was fluorescent on SDS-PAGE in the presence of DPAP but not in the absence of DPAP (Figure 3.4C). Additionally, a terminal propargylic alkyne immobilized on a sepharose resin was reacted with the fluorescent dGSH molecule under heterogeneous conditions.

The sepharose resin was concentrated in an Eppendorf tube, washed, and imaged with trans-UV illumination. The resin reaction with DPAP was fluorescent (Figure 3.4A), while the DPAP negative reaction was not fluorescent (Figure 3.4B). Increased time of irradiation and incubation post-irradiation were found to cause significant protein degradation, so time and temperature optimization was necessary to minimize these effects.

1,3-Diyne Bivalent Conjugate Preparation

After confirming thiol-yne addition to terminal alkynyl substrates, we next sought to expand the biological thiol-yne to bivalent conjugates. In order to generate a bivalent starting material, pPrF-GFP was reacted in copper-catalyzed Glaser Hay bioconjugation reactions using previously reported conditions with either a 5 kDa polyethylene glycol (PEG) oligomer containing a terminal alkyne group, a biotin terminal alkyne, an AlexaFluor 488 fluorophore terminal alkyne,
AlexaFluor alkyne and pPrF-GFP conjugation was successful, as visualized by fluorescence at the molecular weight of GFP on SDS-PAGE. The biotin alkyne and pPrF-GFP Glaser Hay reaction was incubated following reaction with a streptavidin-coated resin in an Eppendorf tube, washed to remove unbound molecules, and imaged for fluorescence via trans-UV illumination. Theoretically, due to the incredibly strong preferential binding between streptavidin and biotin, any biotin-containing molecule and its covalently attached conjugation partner would bind the streptavidin resin with high affinity. This did occur and the resin was fluorescent, suggesting successful GFP covalent conjugation to biotin. The DNA and pPrF-GFP Glaser Hay was also successful, as visualized by an approximate 5 kDa molecular weight increase on SDS-PAGE. The reaction was low yielding, likely due to unfavorable steric or charge interactions between the two biomolecules (Figure 3.6A). Nevertheless, three 1,3-diyne bivalent conjugates were successfully generated via Glaser-Hay reactions to next perform thiol-yne addition experiments on: GFP/fluorophore, GFP/biotin, and GFP/DNA.
Preparation of Multivalent Conjugates through Biological Thiol-yne Addition

With the 3 bivalent conjugates generated through Glaser-Hay reactions, the next optimization was the thiol-yne addition to introduce the third conjugation partner. Various thiol conjugation partners were investigated. First, the GFP/fluorophore complex was reacted under thiol-yne biological conditions with a 15-mer DNA oligonucleotide reaction partner containing a 3’-thiol modification. This DNA thiol-yne reaction has been unsuccessful with extensive protein degradation, likely due to radical generation and oxidative damage. Decreased temperature and increased radical scavenger concentration did not result in successful DNA thiol-yne addition. This attempt at multivalent conjugation was abandoned but could be attempted again in the future.

Second, the pPrF-GFP/DNA bivalent complex was subjected to thiol-yne conditions with the fluorophore dGSH. The GFP/DNA conjugate was first concentrated using a size exclusion spin column to remove the GFP monomer, then reacted with dGSH under thiol-yne addition conditions. This appeared successful as visualized by fluorescence at the higher molecular weight on SDS-PAGE, but the negative control lane was also fluorescent (Figure 3.5B). This indicates either dGSH can react without the photoinitiator, or it engages in some form of non-covalent association, possibly with the DNA portion.

![Figure 3.6](image_url)
of the conjugate. These results were not observed in the previous proof of concept dGSH reactions with $p$PrF-GFP or the sepharose resin, so further experimentation is needed to investigate whether this is a consistent result (Figure 3.6A-B).

In a third multiconjugate approach, the GFP/biotin bivalent complex was also subjected to thiol-yne addition conditions with the fluorophore dGSH. After reaction, the product was heated at 98 °C for 10 minutes to denature GFP, eliminating protein fluorescence, and was analyzed via SDS-PAGE. The multivalent complex was fluorescent at the appropriate molecular weight, confirming successful addition of dGSH (Figure 3.7A). The product, now a trivalent conjugate containing GFP, biotin, and dGSH bound at the 1,3-diyne moiety (Figure 3.5), was then immobilized on a streptavidin resin through theoretical interaction with the biotin component of the conjugate. The resin was washed after incubation to remove any unbound reagents and imaged for fluorescence using trans-UV illumination. The DPAP positive reaction on the streptavidin resin was significantly fluorescent, even after the complex had been denatured to remove GFP fluorescence (Figure 3.7B). The DPAP negative control was also fluorescent but significantly less so than the DPAP positive reaction. (Figure 3.7B). This suggests some non-specific or non-covalent interaction in the absence of DPAP, as previously seen in the GFP/DNA thiol-yne addition, while the presence of DPAP causes a significant increase in the addition of dGSH. $p$PrF-GFP and dGSH were separately incubated with streptavidin,
washed, and imaged. Neither of these controls were fluorescent, indicating the resin fluorescence in the thiol-yne addition immobilization was mediated through direct streptavidin-biotin interaction (Figure 3.7B). These results suggest successful thiol-yne addition in the presence of DPAP to generate a multivalent complex consisting of either DNA or biotin, GFP, and dGSH. These conditions must be optimized, and the complexes generated in the absence of photoinitiator must be identified in further experimentation to make it a feasible, selective multivalent bioconjugation strategy.

**1,3-Diyne Bivalent Protein Dimer Preparation for Biological Thiol-yne Addition**

Next, we attempted to expand the scope of this approach by generating 1,3-diyne bivalent conjugates consisting of two proteins that could undergo further conjugation using the developed thiol-yne method. The benefit to this approach is the 1,3-diyne protein dimer (Figure 3.8A) would result in an easily recognizable molecular weight shift on SDS-PAGE, and a subsequent third reaction with a fluorophore would make the dimer conjugate easily visible at the appropriate molecular weight with fluorescence imaging (Figure 3.8B). We attempted 1,3-diyne conjugation between two pPrF-containing proteins using Glaser Hay conditions (Table 3.1, Figure 3.8A). We
also employed the Cadiot-Chodkiewicz reaction, which forms a 1,3-diyn linkage by conjugating a bromoalkyne and a terminal alkyne together. This was attempted biologically by reacting $p$PrF-containing protein with protein harboring the bromoalkyne UAA $p$BrPrF (Table 3.1, Figure 3.8A). All Glaser Hay reactions were catalyzed by CuI and TMEDA and reacted for 4 hours at 4 °C. All Cadiot-Chodkiewicz reactions were catalyzed by CuI and TEA and reacted for 4 hours at 4 °C. Direct dimerization was unsuccessful under all attempted conditions, and only monomeric GFP was observed by SDS-PAGE. This was hypothesized to occur due to unfavorable steric hindrance between the two bulky protein reaction partners or due to electronic repulsion of identical surface potentials between two identical proteins. To remedy this steric interference, linkers of various carbon chain lengths containing alkyne moieties on both ends were investigated to increase the distance between the bulky proteins. This work focused specifically on hexadiyne and dodecadiyne chains (Figure 3.8C). Unfortunately, the only successful dimerization was a Cadiot Chodkiewicz-catalyzed linkage between two $p$BrPrF-GFP molecules and a hexadiyne linker, but this was difficult to replicate and required highly concentrated protein and linker reactants (Figure 3.9).

Consequently, we sought to minimize steric interactions between reaction partners by attempting dimerization using a smaller protein, ubiquitin (Ub). Polyubiquitination of proteins is a common physiological mechanism to signal protein degradation, so therefore, ubiquitin homodimers and heterodimers should be accessible without the potential steric issues observed with GFP. To first test ubiquitin conjugate formation with small biomolecules, ubiquitin was
expressed with pPrF and reacted under Glaser Hay conditions either with a 15-mer DNA-alkyne or 5 kDa PEG-alkyne. The reactions should result in gel shifts of about 5 kDa each, but this was not observed. It was hypothesized that the DNA and PEG-alkynes were preferentially dimerizing with themselves, resulting in unreacted protein monomer. Unfortunately, pBrPrF-Ub also did not dimerize with these molecules under Cadiot-Chodkiewicz conditions, implying more work is needed with highly concentrated substrates or more catalyst to drive reaction favorably towards the desired conjugate.

Next, pPrF-Ub was reacted under Glaser Hay conditions with itself or with pPrF-GFP. This was repeated with pBrPrF-Ub under Cadiot-Chodkiewicz conditions (Table 3.1). The only successful reaction was a direct Glaser-Hay with pPrF-Ub, resulting in the observation of some ubiquitin homodimer (Figure 3.10A).

This bivalent 1,3-diyne product was subsequently employed in an attempted trivalent conjugation utilizing the previously described thiol-yne reaction to add the fluorophore dGSH. Fluorescence was apparent at the molecular weight of the monomeric Ub in the presence of DPAP photoinitiator, but not the molecular weight of the dimer, suggesting that the remaining monomer pPrF-Ub reacted preferentially with little to no dimer reaction (Figure 3.10C). Additionally, the dimer band was absent upon reaction, which we hypothesized was due to...
combination of degradation and dilution (Figure 3.10B). While this reaction also has low reproducibility, further efforts at removing the monomer through size-exclusion chromatography and then reacting concentrated dimer with dGSH are under investigation. Additionally, the direct conjugation of Ub with itself implies linker reactions between monomers of this protein would also be successful. This has not been investigated in current work but is a promising potential alternative in the future to increase conjugation efficiency.

Table 3.1: Glaser Hay (GH) and Cadiot Chodkiewicz (CC) reactions for biomolecule dimerization. Each GH consisted of two pPrF-containing protein reaction partners and was catalyzed by Cul and TMEDA, while each CC consisted of one pPrF-protein partners and one pBrPrF-protein partner and was catalyzed by Cul and TEA. In linker CC reactions, diyne linkers were reacted with only pBrPrF-containing protein. A "-" indicates no dimer formation, and a "+" indicates dimer formation.

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<th>Reaction Partner 2</th>
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**Development of a Biological 1,3-Dipolar Azide-(Bromo)alkyne Cycloaddition**

With several dimerization methods investigated with limited success, we shifted our efforts to a different method of trivalent conjugate preparation beyond the scope of 1,3-diyne linkages. In
order to do so, we began our investigation with the preparation of trisubstituted 1,2,3-triazoles using copper-catalyzed azide-alkyne cycloaddition (“click”) chemistry and an internal alkyne. A variety of methods have been previously reported to synthesize such complexes, most requiring the use of excess metal catalyst or extreme heat, all of which are difficult to achieve in biological systems (Figure 3.11A).\textsuperscript{17-19} However, the cycloaddition of an internal alkyne moiety with an azide moiety is mild and has been catalyzed with a variety of transition metals (Figure 3.11A).\textsuperscript{17} Specifically, terminal haloalkynes reacted with azides and catalyzed by CuI or CuBr can afford a 5-halo-substituted triazole (Figure 3.11B).\textsuperscript{18} The halide group can provide a potential chemical handle for a secondary Sonogashira reaction between an alkyne and an aromatic halide (see p. 58). We sought to translate this reaction to physiological conditions using the bromo-substituted alkynyl amino acid $\rho$BrPrF, which has been used in the previously reported biological Cadot-Chodkiewicz reactions.\textsuperscript{4}
The protected amino acid $p$BrPrF was synthesized in two steps (Figure 3.12). First, $p$PrF was prepared according to the literature protocol, and then dissolved in acetone following purification. The solution was mixed with silver nitrate and N-bromosuccinimide at room temperature for three hours, after which extraction and purification yielded the brominated terminal alkyne in 68% yield. The C- and N-termini were then deprotected according to the literature precedent to give the deprotected amino acid, which was then dissolved in 9:1 water to DMSO solution (100 mM) and solubilized with the addition of NaOH. The amino acid was then incorporated into GFP by co-expression in *E. coli* of the previously described promiscuous $p$CNF-aaRS and tRNA plasmid and the pET-GFP-TAG-151 plasmid. Successful incorporation was confirmed by SDS-PAGE.

Next, the $p$BrPrF-containing GFP was subjected to click reaction conditions derived from previously described tri-substituted 1,2,3-triazole conditions in organic solvents with a Fluor-488 azide reaction partner. Original organic conditions used TCEP as a reducing agent with copper sulfate and TBTA as the catalyst.

**Figure 3.12.** Scheme for the synthesis of the UAA $p$BrPrF. First, the UAA $p$PrF must be synthesized, and then brominated using NBS and silver catalyst. The amino acid is then rapidly deprotected to afford the desired product, with the bromoalkyne group necessary for subsequent trisubstituted triazole formation.

**Figure 3.13.** Click reaction conditions for the conjugation of bromoalkyne-containing GFP and an azide-containing fluorophore. A.) Organic-derived conditions used copper sulfate, TCEP, and TBTA reacted at 4 °C for 16 hours. Optimal biological conditions used copper sulfate, higher concentrations of TBTA and azide fluorophore, and no TCEP reacted at room temperature for an hour.
system at 4 °C overnight (Figure 3.13A). This resulted in successful coupling with low coupling efficiency. As TCEP has been shown to impair protein function and reduce fluorescence of fluorophores, aqueous conditions were optimized based on previously reported click chemistry (Figure 3.13B). The addition of sodium ascorbate as a radical scavenger, aminoguanidine to prevent side chain cross reaction, and the removal of TCEP from the reaction resulted in coupling with higher efficiency, as visualized by fluorescence SDS-PAGE (Figure 3.14B, lane 2). Additionally, the amount of TBTA directly affected coupling efficiency, as more TBTA resulted in a more intense fluorescent signal. The use of more highly concentrated TBTA and AlexaFluor 488 azide partner resulted in a considerably shorter reaction time of one hour (as opposed to 16 hours initially investigated). All reactions were assessed via fluorescence SDS-PAGE imaging and staining with Coomassie blue to visualize protein levels (Figure 3.14A-B).

The scope of this biological bromo-alkyne cycloaddition was next investigated. *E. coli* was co-transformed with plasmids encoding the 8.5 kDa protein ubiquitin and the *p*CNF orthogonal aaRS/tRNA pair. Ubiquitin was expressed with *p*BrPrF, purified, buffer-exchanged into PBS, and then subjected to the optimized click conditions as developed with GFP. Reaction success was confirmed via fluorescence on SDS-PAGE (Figure 3.14B, lane 4). While the proteins harboring *p*BrPrF appeared to have less click conjugation efficiency

![Figure 3.14 A.) Protein staining and B.) fluorescence images of biological click reactions under optimal conditions. Lane 1: pPrF-GFP reacted with AlexaFluor 488 azide. Lane 2: pBrPrF-GFP reacted with AlexaFluor 488 azide. Lane 3: pPrF-Ub reacted with AlexaFluor 488 azide. Lane 4: pBrPrF-Ub reacted with AlexaFluor 488 azide. Fluorescence occurred at 27 kDa in lanes 1-2 and 9 kDa in lanes 3-4.](image-url)
than the same proteins harboring $p$PrF, the difference was not significant enough to cause concern (Figure 3.14B, lanes 1 and 3). Additionally, ubiquitin bands were faint following protein staining, suggesting significant dilution of the starting material, so we concentrated several $p$BrPrF-Ub/fluorophore click reactions prior to further reaction steps (Figure 3.14A). We concluded that production of tri-substituted 1,2,3-triazoles is successful in multiple protein substrates. The next step for the bivalent conjugate containing a bromo-substituted triazole is a Sonogashira coupling with a terminal alkyne reaction partner.

**Development of a Biological Sonogashira Coupling Reaction**

In order to investigate biological Sonogashira multivalent reactions, physiological, aqueous conditions were derived from previously reported biological organopalladium reactions in the literature. Initially, the bivalent Sonogashira reaction alone required optimization with our protein systems before attempting the preparation of trivalent conjugates. As a proof-of-concept, we used the same expression system in *E. coli* as above to express GFP151 with the halogenated amino acid 4-iodophenylalanine ($p$IF). The $p$IF-GFP protein was reacted with Fluor-488 alkyne in the presence of palladium and copper catalysts. Reactions were allowed to progress 2 hours, 4 hours, 16 hours, and 24 hours at room temperature, after which they were washed to remove metal catalyst and visualized via SDS-PAGE (Figure 3.15A-B). There was

![Figure 3.15 A) Protein staining and B) fluorescent images of time course Sonogashira reactions with $p$IF-GFP and AlexaFluor 488 alkyne. Lane L: reference protein ladder. Lane 1: 2 hour Sonogashira. Lane 2: 4 hour Sonogashira. Lane 3: 16 hour Sonogashira. Lane 4: 24 hour Sonogashira. Fluorescence indicates successful coupling between protein and fluorophore and occurred at MW of 27 kDa.](image-url)
significant product conversion to fluorophore-conjugated GFP at 24 hours as opposed to no product conversion at other shorter timepoints, indicating the biological Sonogashira coupling required approximately a day for product formation to occur (Figure 3.15B).

With moderately optimized conditions in hand, the biological Sonogashira was attempted on the bivalent bromo-substituted click products (Figure 3.16). Initially, the GFP/fluorophore bivalent conjugate was reacted with a 15-mer single-stranded DNA oligonucleotide containing a 5’-hexynyl modification. After 24 hours, the resulting product no longer exhibited fluorescence and had significant protein degradation, which we hypothesized was due to extensive oxidative damage from radical species. Addition of the radical scavenger sodium ascorbate and decreased temperature did not result in decreased degradation. When the GFP/fluorophore complex was reacted with a 5 kDa PEG-alkyne, protein degradation was not as severe but no gel shift was observed, which we hypothesized was due to unfavorable interactions between the bulky PEG oligomer and protein complex. However, future work in which the amount of metal catalyst is increased, and reaction time is decreased, might be successful with these substrates considering how little protein degradation was observed in the proof-of-concept experiment.

**Figure 3.16** Overview of multivalent conjugation strategy. First, the bromoalkyne-containing protein (GFP or Ub) is reacted with the fluorophore azide under biological click conditions. Then, the bromo-substituted triazole is reacted with a terminal alkyne reaction partner (DNA, PEG, fluorophore, biotin, etc.) catalyzed by palladium and copper for 24 hours at room temperature to yield the trivalent conjugate with the alkyne substituted at the bromo position.
Biological Sonogashira Coupling Towards a Biotin-Containing Trivalent Conjugate

In a search for a smaller third conjugation partner, we reacted the GFP/fluorophore bivalent conjugate with a biotin-alkyne under Sonogashira conditions (Figure 3.16). Following reaction, the products were washed and analyzed via SDS-PAGE. The products were then incubated with a streptavidin-coated resin, washed again, and imaged immediately by trans-UV illumination to observe fluorescence. If the resin was fluorescent, it was transferred to the center of a 96-well plate and fluorescence was quantified by excitation at 485/20 nm and a scan for emission at 528/20 nm. Fluorescence levels were normalized to an empty streptavidin resin control.

Initial reactions between GFP/fluorophore click product and biotin alkyne exhibited no fluorescence on the resin but were still fluorescent on protein gel, indicating no successful biotin addition. Surprisingly, when the GFP/fluorophore starting material was buffer exchanged from PBS (pH=7.4) to TAPs (0.2 M, pH=7.4) successful conjugation occurred, resulting in visible fluorescence on SDS-PAGE (Figure 3.17, Lane 2) and visible fluorescence on the streptavidin resin as observed in an Eppendorf tube via trans-UV illumination (Figure 3.18A), which was later quantified at 528/20 nm (Table 3.2). Furthermore, when the Ub/fluorophore conjugate was reacted under the same conditions with biotin-alkyne, fluorescence was visible on SDS-PAGE and the resin, and was subsequently quantified. (Figure 3.17, Lane 3; Table 3.2).
Based on the resin fluorescence measurements, all attempted conjugations were low yield (Table 3.2). To try to improve conjugation, temperature was increased to 37 °C, but this only resulted in more protein degradation (Figure 3.17, Lane 1). Next, the concentration of biotin-alkyne was increased tenfold and reacted with the GFP/fluorophore click product, resulting in significantly increased fluorescence measurements on the resin (11x greater), while the gel fluorescence remained the same (Table 3.2). However, the increase in fluorescence was so great that this experiment needs to be repeated to ensure consistency in results. We further investigated

**Figure 3.18** Analysis of biotinylated Sonogashira products bound to streptavidin resin beads. A.) Left tube contains the resin with bound GFP/fluorophore/alkyne conjugate and is faintly fluorescent. The right tube contains resin incubated with just the GFP/fluorophore conjugate lacking biotin addition and is not fluorescent. B.) Merged microscopic image of streptavidin beads incubated with pBrPrF-GFP + biotin alkyn Sonogashira reaction. The lack of fluorescence confirms Sonogashira conditions do not allow coupling between unreacted pBrPrF-GFP and the terminal alkyn. C.) Merged microscopic image of streptavidin beads incubated with denatured GFP/fluorophore/alkyne conjugate. Fluorescence would only be present due to fluorophore. D.) Fluorescent microscopic image of streptavidin beads incubated with Ub/fluorophore/alkyne conjugate.
streptavidin immobilization by imaging the resin beads via fluorescence microscopy (Figure 3.18B-D). Fluorescence was seen coating the beads in the successful GFP/fluorophore/biotin Sonogashira but not in the positive control, which was GFP/fluorophore incubated with streptavidin in the absence of biotin-alkyne. This signifies that the trivalent conjugates do indeed exist; albeit in low yields. These promising results suggest that with further optimization this could be a valid approach to multivalent conjugate formation.

We next sought to confirm the fluorescence was not due to competitive reaction of unreacted \( pBrPrF-GFP \) with the biotin alkyne under Sonogashira conditions to form a bivalent GFP/biotin conjugate. First, we reacted \( pBrPrF-GFP \) with AlexaFluor 488alkyne under Sonogashira conditions and observed no fluorescence after 24 hours. This was confirmed via fluorescence microscopy, which showed the beads not coated in fluorescence (Figure 3.18B).

**Table 3.2**: fluorescence (at 528/20 nm) of 96-well plates containing streptavidin-bound Sonogashira reactions with varying reaction partners and biotin-alkyne concentrations

<table>
<thead>
<tr>
<th>Reaction Partner 1</th>
<th>Reaction Partner 2</th>
<th>Concentration of alkyne</th>
<th>Fluorescence @ 528/20 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP/fluorophore</td>
<td>DNA alkyne</td>
<td>1 mM</td>
<td>0</td>
</tr>
<tr>
<td>GFP/fluorophore</td>
<td>PEG alkyne</td>
<td>1 mM</td>
<td>0</td>
</tr>
<tr>
<td>Ub/fluorophore</td>
<td>DNA alkyne</td>
<td>1 mM</td>
<td>0</td>
</tr>
<tr>
<td>GFP/fluorophore</td>
<td>Biotin alkyne</td>
<td>1 mM</td>
<td>2,963</td>
</tr>
<tr>
<td>Ub/fluorophore</td>
<td>Biotin alkyne</td>
<td>1 mM</td>
<td>1,304</td>
</tr>
<tr>
<td>GFP/fluorophore</td>
<td>Biotin alkyne</td>
<td>10 mM</td>
<td>33,291</td>
</tr>
<tr>
<td>GFP</td>
<td>Biotin alkyne</td>
<td>1 mM</td>
<td>0</td>
</tr>
</tbody>
</table>

Additionally, we conjugated \( pPrF-GFP \) and biotin-alkyne through a Glaser Hay, immobilized GFP on the resin, and imaged for fluorescence with the same excitation and emission wavelengths. The GFP had no fluorescence as compared to empty streptavidin control, confirming GFP without the attached fluorophore would show little to no signal at the emission wavelength of 528/20 nm (Table 3.3). The GFP/fluorophore/biotin trivalent conjugate was denatured at 98 °C.
for 10 minutes prior to resin immobilization and then microscopically imaged (3.18B). Fluorescence was still observed coating the beads despite denaturation, confirming the fluorescence on the beads originates from fluorophore and not GFP because denatured GFP is not fluorescent (Figure 3.16C). Furthermore, Ub/fluorophore/biotin conjugate was also significantly fluorescent on the streptavidin beads, as confirmed via microscopy (Figure 3.18D). Ubiquitin is not naturally fluorescent, so the fluorescence on the beads would be a direct result of trivalent conjugation. Given the low reaction efficiency for all conjugates, particularly in the ubiquitin conjugation, further optimization of these conditions are necessary. However, the tenfold increase of biotin alkyne reactant concentration appears promising and must be tested with Ub/fluorophore substrate to see if the same trend in fluorescence increase is observed.

**Preparation of Bromo-substituted Triazole Protein Dimers**

Finally, we again attempted to dimerize protein, this time using the optimal copper-catalyzed cycloaddition conditions. We tested a variety of protein combinations (Table 3.3). Click reactions using two unique protein partners, pAzF-Ub and pBrPrF - GFP, and the other combination, to form protein heterodimers were unsuccessful. Homodimerization of GFP or Ub was also unsuccessful. Given our previous justification for the use of linkers, we synthesized dibrominated versions of the diyne linkers that were used in Glaser Hay and Cadiot Chodkiewicz reactions using AgNO₃ and NBS in a similar fashion to the preparation of pBrPrF from pPrF
(Figure 3.19A). The linkers were dissolved (100 µM in DMSO) and added to click reactions containing only pAzF- GFP or pAzF-Ub. GFP dimerization using this approach was unsuccessful. Ub dimerization was initially unsuccessful. When a more concentrated pAzF-Ub solution was reacted with the same concentration of dibromo hexadiyne, it appeared to produce a gel shift, with one band below the 10kDa reference band and one above (Figure 3.19B). The suspected Ub/Ub dimer contained the bromo-substituted triazole, so it was subsequently reacted under Sonogashira conditions with excess AlexaFluor 488 alkyne. Initial results appear promising, as there are two fluorescent bands visible on the gel, suggesting there is some trivalent conjugate composed of a Ub/Ub dimer and conjugated fluorophore at the higher molecular weight (Figure 3.19C). Additionally, it appears that the Sonogashira conditions may catalyze a click reaction between the unreacted pAzF-Ub and the fluorophore alkyne to cause fluorescence at the lower monomer weight. This must be confirmed in repeated experiments to ensure its reproducibility and must be analyzed by mass spec to ensure the proper molecular weight. Additionally, the dibromo dodecadiyne linker was not tested, but would presumably display similar results to the dibromo hexadiyne.

![Figure 3.19](image-url)
Table 3.3: Click reactions employed towards the development of a protein dimer. Linkers, if used, are specified below. A “-” represents a reaction that did not result in a visible gel shift upon analysis, while a “+” did result in a gel shift. All clicks used optimal biological conditions. Entry 9 is not a protein dimer but was tested as a conjugation between less bulky reaction partners.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Reaction Partner 1</th>
<th>Reaction Partner 2</th>
<th>Linker</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pAzF-GFP</td>
<td>pBrPrF-GFP</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>pAzF-GFP</td>
<td>pAzF-GFP</td>
<td>Dibromo hexadiyne</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>pAzF-GFP</td>
<td>pAzF-GFP</td>
<td>Dibromo dodecadiyne</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>pAzF-GFP</td>
<td>pBrPrF-Ub</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>pAzF-GFP</td>
<td>pAzF-Ub</td>
<td>Dibromo hexadiyne</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>pAzF-Ub</td>
<td>pBrPrF-GFP</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>pAzF-Ub</td>
<td>pBrPrF-Ub</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>pAzF-Ub</td>
<td>pAzF-Ub</td>
<td>Dibromo hexadiyne</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>GFP/pBrPrF</td>
<td>PEG azide</td>
<td>None</td>
<td>+</td>
</tr>
</tbody>
</table>

Finally, to alleviate unfavorable protein-protein interactions, pBrPrF-GFP was subjected to click reaction conditions with the addition of a 5 kDa PEG azide partner rather than a bulkier Ub or GFP protein partner (Table 3.3). The GFP/PEG click reaction was successful, as confirmed by a 5 kDa shift on SDS-PAGE (Figure 3.20A). The product contained the bromo-substituted triazole group and was subjected to Sonogashira conditions with AlexaFluor 488 alkyne. The experiment initially did not fluoresce at the GFP monomer weight (27 kDa) or the GFP/PEG dimer weight (33 kDa). Metal catalysts were increased by 1.5 equivalents and the reaction was repeated. The GFP/PEG/fluorophore complex was faintly fluorescent at the desired molecular weight (Figure 3.20B) but it did not correlate well with the amount
of protein observed when Coomassie stained (Figure 3.20A), indicating a low yielding conjugation. Therefore, this illustrates another example of multivalent conjugation utilizing the biological Sonogashira reaction. This will be expanded upon in the future to increase yield by increasing catalyst and fluorophore equivalents, attempting dimerization with \( p\text{BrPrF-Ub} \), and concentrating the protein/PEG dimer prior to Sonogashira reaction.

**Conclusion**

Multivalent bioconjugates, particularly with a protein as one of the conjugate partners, have the potential to be even more powerful than their bivalent relatives. Utilizing a site-specific UAA within a protein with subsequent bioconjugation reactions would be the simplest, quickest, and most efficient way to prepare such complexes. Therefore, several methods have been described and investigated with varying degrees of success.

A biological thiol-yne reaction that adds a thiol-containing molecule across a pi bond in the presence of a photoinitiator was employed. This was successful in proof of concept experiments using a fluorescent thiol molecule (dGSH), and later also successfully resulted in fluorescence to the 1,3-diyn conjugates of GFP/DNA and GFP/biotin, although further work exploring the photoinitiator and degradation is necessary. Protein dimers were also attempted as to be employed as 1,3-diyn bivalent conjugates for further conjugation. However, these were incredibly difficult to achieve, even with the use of carbon chain diyne and dibromo diyne linkers. GFP was ultimately dimerized under Cadiot-Chodkiewicz conditions with a hexadiyne linker and ubiquitin was dimerized under Glaser Hay conditions without linker, but these both were inconsistent in further experimentation, and the ubiquitin dimer biological thiol-yne reaction with dGSH was only fluorescent at the monomer weight. Although protein dimers may be successful with increased catalyst and starting material concentration, or with removal of the monomer prior
to thiol-yne addition, the most promising starting material for biological thiol-yne reactions are protein/small molecule conjugates.

A biological copper-catalyzed azide-alkyne cycloaddition to afford a bromo-substituted triazole, along with a subsequent biological Sonogashira reaction with a terminal alkyne and the triazole were developed and employed towards multivalent conjugate formation. The most successful of these was the addition of a biotin alkyne to a protein/fluorophore triazole complex, which resulted in fluorescent streptavidin beads and was demonstrated with both ubiquitin and GFP. There has been preliminary success in the generation of a ubiquitin dimer using a dibrominated diyne linker and the cycloaddition protocol, followed by fluorescence upon Sonogashira reaction with a fluorophore alkyne. GFP was also dimerized with a small PEG oligomer and has shown low yield success in the same subsequent Sonogashira. Overall, this method of preparation of multivalent conjugates has been successful on several biomolecules and reaction partners and is the most promising investigated to date. These successes have, unfortunately, all been relatively low yield. However, optimization of catalyst, reactant concentration, temperature, and buffer could all lead to a high yield preparation of conjugates linked at one unnatural amino acid site. This could be applied in the development of antibody-drug-probe conjugates for specific delivery and tracking of drugs in a variety of disease treatments.

**Experimental**

**General.** General solvents and reagents, including the AlexaFluor 488 Alkyne and Azide, were obtained from Sigma Aldrich, Acros Organics, or Alfa Aesar and used without further purification. Plasmids were provided by the laboratory of Dr. Peter Schultz at The Scripps Research Institute,
as well as by the laboratory of Dr. Ashton Cropp at Virginia Commonwealth University. The dGSH molecule was provided by the laboratory of Dr. Lisa Landino at William & Mary. Reactions were conducted under ambient atmosphere with solvents directly from the manufacturer without further purification. All proteins were purified according to manufacturer’s protocols using a Qiagen Ni-NTA Quik Spin Kit. Unless otherwise indicated, all solutions were prepared in deionized water (pH ~ 7). SDS-PAGE gels were imaged on a Bio-Rad Molecular Imager (Gel Doc XR+). All NMR spectra were acquired on an Agilent Technologies 400 MHz NMR spectrometer.

Synthesis of brominated linkers. 1,5-Hexadiyne (0.10 g., 1.28 mmol) was added to vial containing AgNO3 (0.04 g, 0.26 mmol) and N-bromosuccinamid (NBS, 0.50 g, 2.81 mmol) in acetone (10 mL). The mixture was allowed to stir for 2 hours at room temperature, after which it was diluted with hexanes (20 mL) and the resulting crystals were filtered. The filtrate was then evaporated under reduced pressure and filtered through a silica plug using hexanes as eluent, then concentrated in vacuo to afford the desired product as a brown solid (0.140 g., 0.593 mmol, 46% yield). H NMR (400 MHz, CDCl3): 2.43 (t, 4 H) ppm.

1,11-Dodecadiyne (0.200 g, 1.23 mmol) was added to vial containing AgNO3 (0.043 mg, 0.26 mmol) and N-bromosuccinamid (NBS, 0.48 g, 2.70 mmol) in acetone (10 mL). The mixture was allowed to stir for 2 hours at room temperature, after which it was diluted with hexanes (20 mL) and the resulting crystals were filtered. The filtrate was evaporated under reduced pressure and filtered through a silica plug using hexanes as an eluent, then concentrated in vacuo to afford the desired product as a brown oil (0.210 g, 0.656 mmol, 53% yield). H NMR (400 MHz, CDCl3): 2.48 (t, 4 H), 1.23-1.49 (m, 12 H) ppm.
Synthesis of \( p \)-propargyloxyphenylalanine (\( p \)PrF). Boc-Tyrosine-OMe (0.500 mg, 1.69 mmol) was added to a flame-dried vial. Cesium carbonate (0.826 mg, 2.54 mmol) was then added, followed by dry DMF (10 mL). This mixture was stirred at 60\(^\circ\)C for 30 minutes. 3-bromopentyne (513 \( \mu \)L, 6.77 mmol) and catalytic potassium iodide were added to the vial. The reaction was stirred overnight at 60\(^\circ\)C, then cooled to room temperature, filtered, and washed with brine (20 mL) and extracted with methylene chloride (20 mL x 3). The organic layers were combined and back extracted with brine (20 mL x 3). The organic layer was dried with magnesium sulfate, filtered, and concentrated in vacuo. The resulting oil was purified via flash chromatography (3:1 hexanes/ethyl acetate) and concentrated to yield the desired product as a white crystalline solid (0.464 mg., 83% yield). \(^1\)H NMR (400 MHz, CDCl\(3\)): \( \delta \) 7.02 (d, 2 H), 6.82 (d, 2 H), 4.95 (d, 1 H), 4.53 (d, 2 H), 4.27 (d, 1 H), 3.71 (s, 3 H), 3.02 (m, 2 H), 2.39 (t, 1 H), 1.41 (s, 9 H). \(^13\)C NMR (400 MHz, CDCl\(3\)): \( \delta \) 172.4, 157.9, 130.3, 127.9, 114.5, 83.5, 79.9, 68.8, 66.0, 54.5, 52.2, 37.4, 28.3, 28.2, 21.1, 15.1

Hydrolysis of methyl ester and subsequent acidification, a 1:1 LiOH/Dioxane solution (3 mL) was added to protected \( p \)PrF (0.464 g., 0.73 mmol) on ice and stirred for 2 hours at room temperature. The dioxane was then removed in vacuo, the aqueous solution was cooled on ice, and 6 M HCl was added dropwise to the solution until a pH of 4 was achieved. The reaction was extracted and washed with water and EtOAc, and the organic layer was dried over MgSO\(4\) and concentrated in vacuo to afford a yellow oil. To remove the tert-butoxy protecting group, the oil was resuspended in 50\% TFA solution (2 mL TFA/2 mL DCM) on ice and allowed to warm to room temperature for 1 hour. The solvent was then removed in vacuo, and the product was obtained as a brown solid (0.301 g., 98% yield). \(^1\)H NMR (400 MHz, CD\(3\)OD): \( \delta \) 7.19 (d, 2 H), 6.93 (d, 2 H), 4.91 (s, 1 H),
4.14 (m, 1 H), 3.02 (m, 2 H), 2.54 (t, 1 H). $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 168.4, 155.7, 128.6, 125.4, 113.5, 73.4, 54.4, 52.3, 45.4, 25.5.

Synthesis of p-bromopropargyloxyphenylalanine (pBrPrF).

Protected $p$-Propargyloxyphenylalanine (pPrF, 0.37 g., 1.11 mmol) was synthesized according to the literature, and dissolved in acetone (10 mL). This solution was then transferred to a vial containing AgNO$_3$ (0.019 g., 0.110 mmol) and N-bromosuccinamide (0.21 g., 1.22 mmol). The mixture was allowed to stir for 3 hours at room temperature, after which it was diluted with hexanes and the resulting crystals were filtered off. The filtrate was then evaporated under reduced pressure and purified via flash chromatography (5:1 hexanes/ethyl acetate) yielding the desired product as a white crystal (0.30 g., 90% yield). $^1$H NMR (400 MHz, CD$_3$OD): $\delta$ 7.19 (d, 2 H), 6.93 (d, 2 H),
4.91 (s, 1 H), 4.14 (m, 1 H), 3.02 (m, 2 H), 2.54 (s, 1 H). \(^{13}\)C NMR (400 MHz, CDCl3): \(\delta\) 168.4, 155.7, 128.6, 125.4, 113.5, 73.4, 54.4, 52.3, 45.4, 25.5.

Hydrolysis of methyl ester and subsequent acidification, a 1:1 LiOH/Dioxane solution (3 mL) was added to protected \(p\)BrPrF (0.30 g., 0.73 mmol) on ice and stirred for 2 hours at room temperature. The dioxane was then removed in vacuo, the aqueous solution was cooled on ice, and 6 M HCl was added dropwise to the solution until a pH of 4 was achieved. The reaction was extracted and washed with water and EtOAc, and the organic layer was dried over MgSO4 and concentrated in vacuo to afford a yellow oil. To remove the tert-butoxy protecting group, the oil was resuspended in 50% TFA solution (2 mL TFA/2 mL DCM) on ice and allowed to warm to room temperature.
for 1 hour. The solvent was then removed in vacuo, and the product was obtained as a brown solid (0.13 g., 58% yield). $^1$H NMR (400 MHz, CD$_3$OD): $\delta$ 7.19 (d, 2 H), 6.93 (d, 2 H), 4.91 (s, 1 H), 4.14 (m, 1 H), 3.02 (m, 2 H). $^{13}$C NMR (400 MHz, CDCl$_3$): $\delta$ 168.4, 155.7, 128.6, 125.4, 113.5, 73.4, 54.4, 52.3, 45.4, 25.5. MS: calcd for C$_{12}$H$_{13}$BrNO$_3$: 299.14, found 298.1 and 300.1.

$^1$H NMR of $p$BrPrF

General Procedure for Biological Thiol-yne Reaction$^{16}$

The solution was incubated at room temperature for 2 hours to ensure any oxidized dGSH or fluorophore was sufficiently reduced. To another Eppendorf tube, the following were added to make a Tris-HCl reaction buffer: 6 μL of 1 M Tris-HCl (pH=6.8), 10 μL of 10 mM TCEP in H$_2$O, 10 μL of 10% SDS, and 24 μL of H$_2$O. To the Tris-HCl buffer was added 6.25 μL of 2,2-dimethoxy-2-phenylacetophenone (DPAP). To control reactions, 6.25 μL of H$_2$O was added instead of DPAP. In another Eppendorf tube, 5 μL of the DPAP/Tris-HCl buffer solution was
added to 20 μL of GFP151/pPrF, GFP-Biotin, or GFP-DNA and the resulting solution was incubated at room temperature for 10 minutes. Next, 20 μL of the dGSH thiol or thiol fluorophore substrate solution was added, and the resulting solution was incubated at room temperature for another 10 minutes. The reaction was irradiated at 365 nm for 5 minutes and then incubated at room temperature for 5 minutes. The reaction was heated to 98°C for 10 minutes to denature proteins and then analyzed via SDS-PAGE.

**General Procedure for Biological Glaser-Hay Coupling.** To a sterile 1.5 mL Eppendorf tube, the following were added: 5 μL of a vigorously shaken solution of freshly prepared CuI (500 mM in H₂O) and 5 μL of bidentate nitrogenous ligand (TMEDA or BIPY, 500 mM in H₂O). The two solutions were thoroughly mixed by pipetting and sat for 10 minutes prior to next step. Next, reaction partners 1 and 2 were added to the mixture and mixed by pipetting. Reaction partners consisted of pPrF-GFP or pPrF-Ub (20 μL, ~1 mg/mL in PBS), alkyne DNA oligonucleotide (20 μL, 1 mM in H₂O), alkyne PEG (20 μL, 1 mM in DMSO). In reactions involving linkers, 5 μL of 10 mM linker dissolved in DMSO was added. The reaction was incubated at 4 °C for 4 hours, unless otherwise stated. The reaction was stopped by removing excess reactants via buffer exchange using Spin-X UF concentrator columns and the products were concentrated to a volume of 50 μL and subsequently analyzed via SDS-PAGE.

**General Procedure for Biological Cadiot-Chodkiewicz Coupling.** To a sterile 1.5 mL Eppendorf tube, the following were added: 5 μL of a vigorously shaken solution of CuI (500 mM in H₂O) and 5 μL of TEA (500 mM in H₂O). The two solutions were thoroughly mixed by pipetting. Next, reaction partners 1 and 2 were added and mixed by pipetting. Reaction partners consisted of GFP
or Ub containing alkyne or bromoalkyne UAAs (20 μL, ~1 mg/mL in PBS), alkyne DNA oligonucleotide (20 μL, 1 mM in H₂O), or alkyne PEG (20 μL, 1 mM in DMSO). In reactions involving linkers, 5 μL of 100 μM linker dissolved in DMSO was added. The reaction was incubated at 4°C for 4 hours. The reaction was stopped by removing excess reactants via buffer exchange using Spin-X UF concentrator columns and concentrated to a volume of 50 μL, then subsequently analyzed via SDS-PAGE.

**Initial Conditions for Biological 1,3-Dipolar Azide-alkyne Cycloaddition.** To a sterile 1.5 mL Eppendorf tube, the following were added: 2 μL of Cu(SO₄)₂ (50 mM in H₂O), 2 μL of TCEP (50 mM in H₂O). Next, reaction partners 1 and 2 were added to the mixture and mixed by pipetting. Reaction partners consisted of GFP151 containing either azide or bromoalkyne UAA (20 μL in PBS), Ub48/pAzF (15 μL in PBS), or AlexaFluor 488 Azide (10 μL, 1 mM in DMSO). Lastly, 10 μL of TBTA (5 μM in DMSO) was added, followed by 20 μL PBS. In reactions involving linkers, 5 μL of 100 μM linker dissolved in DMSO was added. The reaction was incubated at 4°C for 16 hours. The reaction was stopped either by performing SDS-PAGE immediately or by removing excess reactants via buffer exchange using Spin-X UF concentrator columns, 73 and then subsequently analyzing the purified products by SDS-PAGE.

**Optimal General Procedure for Biological 1,3-Dipolar Azide-(Bromo)alkyne Cycloaddition.** To a sterile 1.5 mL Eppendorf tube, the following were added: Bromoalkyne-containing biomolecule (25 μL in PBS) and PBS buffer (pH 7, 392.5 μL) Next, AlexaFluor 488 Azide was added (50 μL, 1 mM in DMSO) and mixed. Then, 7.5 μL of a premixed catalyst solution of CuSO₄ (3 μL, 50 mM in H₂O), TBTA (15 μL, 50 mM in DMSO), and H₂O (7.5 μL) was added and mixed.
thoroughly. Next, aminoguanidine (25 μL, 100 mM in H₂O) was added. Lastly, a fresh solution of 100 mM Na ascorbate (11 mg/mL of H₂O) was prepared and added (25 μL). The tube was closed, mixed by inverting several times, and incubated at room temperature for 1 hour. The reaction was stopped by removing excess reactants via buffer exchange with PBS (pH 7) using Spin-X UF concentrator columns and concentrated to a volume of 50 μL, then subsequently analyzing by SDS-PAGE.

General Procedure for Biological Sonogashira Coupling Reaction

To a sterile 1.5 μL Eppendorf tube, the following were added and mixed: 3 μL of palladium (II) acetate (Pd (OAc)₂, 20 mM in H₂O) and 3 μL of triphenylphosphine-3,3′,3′′-trisulfonic acid trisodium salt (TPPTS, 100 mM in H₂O). Then, 2 μL of copper (II) triflate (CuOTf, 12 mM in H₂O) was added. Next, 30 μL of first reaction partner, either bromotriazole-containing dimer of GFP151/pBrPrF with AlexaFluor 488 Azide, pAzF-Ub, or pAzF-GFP, or linked bromotriazole-containing dimers of pAzF-GFP or pAzF-Ub with dibromo diyne linkers, was added. In proof-of-concept, 30 μL of pIF-GFP was added. Next, 20 μL of the third reaction partner, consisting of either DNA alkyne (1 mM in DMSO), biotin alkyne (1 mM in DMSO or 10 mM in DMSO), or AlexaFluor 488 alkyne (1 mM in DMSO) was added. Finally, 5 μL of Na ascorbate (100 mM in H₂O) and 5 μL of 0.2 TAPs buffer (pH 7) were added and mixed. Initially time and temperature were varied according to table. Following optimization, all Sonogashira reactions were reacted at room temperature for 24 hours. After reaction, excess reactants were removed via buffer exchange using 10k MWCO concentrator columns and were concentrated to final volumes of 30 μL. The products were heated to 98°C for 10 minutes and analyzed by SDS-PAGE for fluorescence, then
stained with Coomassie blue for 30 minutes and destained with an alcohol mixture (6:3:1 H₂O: MeOH: acetic acid) overnight, then analyzed.

**General Procedure for Streptavidin Resin Immobilization**

To a sterile 1.5 mL Eppendorf tube was added 50 μL of Streptavidin Agarose Resin (max binding: 28 μg biotin/mL of resin). The tube was centrifuged (13200 rpm) for 2 minutes and the supernatant was removed. Then, 50 μL of PBS (pH7) was added, centrifuged (13200 rpm) for 1 minutes and excess supernatant was discarded. This wash step was repeated 3 times. Then, 20 μL of the biotin-containing Sonogashira reaction product was added to the tube and incubated at room temperature with slight shaking (250 rpm) for one hour. In denaturation reactions, the Sonogashira reaction product was heated to 98°C for 10 minutes, then transferred to the resin tube. Control resins were incubated either with only PBS (pH7) or with pPrF-GFP conjugated to AlexaFluor 488 alkyne. Following immobilization, resin was washed with PBS (50 μL) 8 times and then transferred to a 96-well plate for analysis. A BioTek Synergy HT microplate reader was used to measure fluorescence in the plate with excitation at 485/20 nm and emission scan at 528/20 nm. Microscopic imaging was performed using a Bio-Rad ZOE Fluorescent Cell Imaging System and fluorescence was measured on the green channel (excitation of 480/17 nm and emission of 517/23 nm).

**References**


CHAPTER 4: PHOTOREGREULATION OF HUMAN PROTEIN ARGININE METHYLTRANSFERASE 1

Introduction

Protein Methyltransferases and Human PRMT1

Post-translational modifications greatly expand the capabilities of enzymes depending on the chemical properties or conformation changes caused by the added groups.\(^1\) Methylation, which is the addition of a methyl group, is a common post-translational modification found ubiquitously in the body, seen in the diverse range of effects this change has both on cellular and systems level processes.\(^1\)\(^3\) Protein methyltransferases catalyze the methylation of proteins at positively charged amino acids lysine and arginine by transferring a methyl group from the cofactor S-adenosyl-L-methionine (Figure 4.1).\(^2\)\(^3\)

Therefore, methyltransferases can be divided based on which amino acid they methylate: histone lysine methyltransferases (HKMT) and protein/histone arginine methyltransferases (PRMT).\(^1\)\(^5\)

Histones, which are the proteins that wrap DNA tightly in the cell and are enriched in lysine and arginine residues to allow for strong ionic interactions with the negatively charged backbone of DNA, are commonly methylated by methyltransferases.\(^3\)\(^4\) Histone methylation is the most common form of epigenetic modification of chromatin, primarily determining which sections of the genome are transcriptionally active (euchromatin) or inactive (heterochromatin) (Figure 4.2).\(^5\)
Chromatin methylation patterns not only affect gene expression, mitosis, genome stability, and DNA repair mechanisms, but are also relevant in disease pathogenesis. Specifically, cancer has been significantly associated with abhorrent methylation of histones and DNA, and small molecule drug development is presently interested in investigating modulation of histone methyltransferases for potential therapeutics or chemical probe development.

The PRMT family of methyltransferases is responsible for a regulation of a variety of cellular mechanisms such as protein localization, cell fate, and cell signaling. Currently, there are nine known PRMTs in humans which are all highly conserved, particularly within their catalytic domains. They rely on S-adenosyl-L-methionine (SAM) substrate as the methyl donor and all add a methyl group to the terminal amine in the arginine guanidinium side chain. Ultimately, the methylated amino acid retains its positive charge but has reduced hydrogen bond donor sites and altered steric effects.

PRMTs can be split into groups based on distinct alterations they make to arginine’s guanidinium group: monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), or symmetric dimethylarginine (SDMA) (Figure 4.3). The different locations and amounts of methyl groups allows for specificity of subsequent binding proteins that either activate or repress transcription. For example, asymmetric dimethylation of histone 4 at arginine 3 results in
transcriptional activation, whereas symmetric dimethylation at the same site results in transcriptional repression. Most PRMTs in the body, including PRMT1, are classified as Type I enzymes that catalyze production of ADMA, as opposed to Type II enzymes that catalyze SDMA (Figure 4.3). These are particularly important to understand in disease pathology because free ADMA, which is a result of protein hydrolysis, has been associated with endothelial function. Specifically, ADMA inhibits nitric oxide intracellular production through inhibition of nitric oxide synthetase (NOS) and cationic amino acid transporters responsible for delivery of arginine to NOS. Associated diseases include hypertension, chronic kidney disease, and cardiovascular disease, so regulation of Type I PRMT activity might be critical in therapeutic treatment for these diseases.
PRMT1 accounts for over 85% of all PRMT activity, although activity and specificity of all PRMTs remains to be well documented. It is detectible in all tissues, found in all eukaryotes, and closely related with neuronal development. Structurally, the most common splice variant of the PRMT1 protein monomer has 353 amino acids and four core parts: the N-terminal domain, the SAM binding domain, the unique PRMT family β barrel domain, and the dimerization arm (Figure 4.4A). PRMT homodimerization is necessary for SAM binding and enzymatic methylase activity, although active oligomers of extended PRMT1 polymers formed through disulfide bonds have been observed biologically.

![Figure 4.4](image)

**Figure 4.4.** Structure of human PRMT1 and its active site. A.) PRMT1 is biologically active as a homodimer. Each monomer contains four core domains: the N-terminus (blue), the dimerization arm (red), the conserved β-barrel structure (green), and the SAM (or AdoHcy) binding domain (orange). B.) The active site hairpin loop is depicted, including the substrate arginine residue (highlighted in green), and the key active site glutamate residues E144 and E153 that interact with arginine through ionic and hydrogen bonding. The SAM (AdoHcy) substrate fits in the bottom of the active site pocket to contribute the methyl group.

Substrates of PRMT1 include histones, as well as various peptides and transcription factors like STAT1 and FOXO1. The binding site is likely to be acidic to have strong ionic interactions with basic arginine, but such sites are ill-defined by electron density studies. The identification of three potential binding sites suggests specificity for different substrates or for different orientations of the same substrate, but this has not been clearly elucidated. Regardless,
the active site clearly requires the target arginine buried deeply between the SAM binding and β barrel domains and requires a hairpin loop of residues that includes two necessary glutamates (E144 and E153) (Figure 4.4B). \[13\]

**Photochemical Control of Proteins**

In nature, proteins are regulated by complex and precise mechanisms. Exogenous control of protein function is a desirable and powerful tool for studying the mechanism, function, and regulation of proteins. \[14-18\]

An ideal external stimulus for these mechanisms is light because it is easily tunable and highly defined through wavelength and intensity. It can also be toggled on and off precisely with high spatiotemporal resolution. \[14\] A common mechanism of regulation are light-removable protecting groups, called “caging groups”. These are usually aromatic ring compounds that are installed on small molecule effectors or even directly on amino acids in the protein of interest. The caging group is placed based on active site and native conformation such that one group is enough to inhibit protein activity. \[14\] The ortho-nitrobenzyl moiety is a well-defined such group that is

![Diagram of photochemical control of proteins](image_url)

**Figure 4.5.** Methods of photochemical modulation of protein function. A.) A photochemically caged small molecule effector of an active protein is first introduced. Upon UV irradiation the caging group is removed, and the effector inactivates the protein. B.) A protein is photochemically caged to inactivate protein function. Upon UV irradiation, the caging group is removed and the protein regains active function.

![Ortho-nitrobenzyl group](image_url)

**Figure 4.6.** The photochemical caging ortho-nitrobenzyl group is substituted at the free hydroxyl group of the amino acid tyrosine to form a photocaging unnatural amino acid (ONBY). After irradiation at 365 nm, the caging group undergoes radical cleavage, yielding a substituted benzaldehyde and the original tyrosine residue on the peptide chain.
employed in proteins as a caged tyrosine or lysine amino acid (ONBY or ONBK). Brief irradiation with UV light affords photolytic cleavage of the nitrobenzyl group to produce nitroso-aldehyde and the original amino acid (Figure 4.6). These have been used in cells to study DNA and RNA, kinases, ion channels, and other enzymes. 

Traditionally, non-specific reactions of surface amino acids with photoliable reagents have been used to prepare caged proteins. However, a more efficient way to site-specifically incorporate a photocaged amino acid is through UAA technology. As mentioned previously, an orthogonal aaRS/tRNA pair is needed to introduce the amino acid, and several pairs have been evolved to recognize and incorporate photoreactive UAAs, including ONBY. Replacement in this manner of the free hydroxyl group in tyrosine, which can be key to protein function due to hydrogen bond interactions, can result in successful photocaging with completely inhibited protein function. This has been well-established in GFP at residue Tyr66, in which substitution with ONBY causes a quench of fluorescence that is restored post-irradiation (Figure 4.7A-B). Additionally, Tyr151 modification does not affect fluorescence, so it is a site-selective mechanism.

**Figure 4.7.** Photocaging of GFP through selective residues. A.) Scheme for the photocaging of GFP to inhibit fluorescent activity using ONBY, which is removed to return fluorescence when irradiated at 365 nm. B.) GFP WT is always fluorescent regardless of irradiation. GFP expressed in the absence of UAA is never fluorescent, GFP harboring ONBY at residue Tyr66 is not fluorescent initially but regains fluorescence through time during irradiation, and GFP harboring ONBY at residue Tyr155 is always fluorescent regardless of irradiation.
(Figure 4.7B). This has also been effective in regulating Cre recombinase with ONBY mutation at key catalytic residue Tyr324 but ineffective at other tyrosine residues.\textsuperscript{20}

A similar mechanism may be used to control PRMT1 activity. Residue Tyr291 was determined as important for protein activity because phosphorylation at this site limited enzymatic activity (Figure 4.8A).\textsuperscript{9} Therefore, we sought to utilize synthesized and site-specifically incorporated ONBY at Tyr291 to spatially and temporally control PRMT1 activity without the need for small molecule effectors.

**Results and Discussion**

In order to prepare the caged PRMT1, first ONBY was synthesized according to previously reported one-step synthetic protocol under light-free conditions, and the N- and C- termini were deprotected according to previously reported protocol in a total yield of 39\% (Figure 4.9).\textsuperscript{15} Then, the ONBY was dissolved in water and DMSO (9:1, 100 mM) and

---

**Figure 4.8.** The regulation of PRMT1 using site-specific residues. A.) Residue Tyr291 in PRMT1 has been shown as necessary for substrate interaction and protein function, which is abrogated by phosphorylation at that site.\textsuperscript{9} B.) Proposed photocaging of PRMT1 to inhibit methyltransferase activity. The incorporation of ONBY at residue 291 would result in no substrate interaction. When the ONBY UAA is decaged using UV light, the protein will return to normal function (adapted from 9).

**Figure 4.9.** Synthesis of ONBY.\textsuperscript{15} First, protected tyrosine is reacted with 2-nitrobenzyl bromide and cesium carbonate to afford protected ONBY (42\% yield). Then, the N- and C-termini are deprotected in two steps to give ONBY (90\% yield). Thus, ONBY is obtained in three total steps with an overall yield of 39\%.
solubilized by addition of NaOH. Site-specific incorporation of the UAA was then attempted using a previously evolved orthogonal aaRs/tRNA pair plasmid (pEvol-ONBY), which was co-expressed in E. coli with a PRMT1 protein plasmid (pET-PRMT1-TAG291), harboring the TAG codon at residue 291. Previous work in our lab suggesting toxicity of the nitrobenzyl group towards E. coli necessitated using only 40% of the normally administered volume of the UAA solution. PRMT1 wild type was expressed as a control comparison using a pET-PRMT1 plasmid transformed into E. coli. Gratifyingly, mutant protein expression was confirmed in the presence of UAA but not in the absence of UAA by SDS-PAGE (Figure 4.10). Mutant and wild type protein was purified to a concentration of about 0.500 mg/mL for analysis of activity. Importantly, all steps of expression, harvest, and purification were conducted in light-free conditions, and all steps of purification were conducted on ice to minimize protein degradation.

**PRMT WT and ONBY-PRMT Relative Activity Assay**

Following successful incorporation of ONBY, PRMT1 mutant and wild-type proteins were assessed for relative activity using a GBiosciences SAM510: Methyltransferase Assay. In this assay, as histone H4 is methylated, SAM is converted to S-adenosyl-L-homocysteine (SAH), triggering a cascade that results in color change that can be quantitated via absorbance measurements (Figure 4.11). This assay accounts for PRMT1’s slow turnover rate by measuring
product formation rather than reactant depletion. An additional enzyme in the mix called AdoHcy Nucleosidase converts SAH into S-ribosyl-L-homocysteine to remove feedback inhibition of SAH on PRMT1. The byproduct of this conversion, adenine, is deaminated by Adenine Deaminase into hypoxanthine, which is rapidly oxidated into urate and hydrogen peroxide.

The rate of hydrogen peroxide formation is measured by changes in absorbance at 510 nm in response to the added colorimetric reagents and can roughly be used to measure PRMT1 activity because hydrogen peroxide byproduct is indirectly produced through the methyltransferase activity.

First, we sought to use this assay to confirm photocaging control of PRMT1 activity at residue Tyr291. To do so, absorbance measurements of PRMT WT, ONBY-PRMT, and positive and negative controls were taken at 510 nm every minute for one hour. Following an hour, the entire plate was irradiated with UV light at 365 nm for 10 minutes to decage ONBY. Then, absorbance measurements were taken every minute for an additional 20 minutes. Another trial in which samples were all irradiated (365 nm, 10 min) prior to addition of the enzyme master mix.
was run and absorbance was measured every minute for an hour. All samples were run in triplicates and standard deviation was calculated as the measure of data variation.

The absorbance measurements of adenine were converted to PRMT1 activity. First, absorbance was graphed over time and the slope of the line of best fit was calculated. The average slope of triplicates was normalized by subtracting the slope of the negative control. The rate of A/min was obtained, then Beer-Lambert law was used to convert to M/min. The extinction coefficient for adenine is 13.4 mM$^{-1}$ cm$^{-1}$ and the path length of the wells was 0.577 cm., and the reagent dilution factor of (0.115 mL/0.014 mL) was accounted for to get final activity in mM/min. The specific activity of PRMT1 was obtained by dividing the activity by the concentration of the sample. This was performed for all trials.

Assay runs in which significant time had passed between protein expression and assay performance demonstrated no significant difference in ONBY-PRMT activity between non-irradiated and irradiated protein, suggesting protein decaging occurs either as a result of time or accidental light exposure during storage (Figure 4.12B). However, trials on fresh protein produced preliminary results in which ONBY-PRMT activity was significantly higher following UV
irradiation, despite large standard deviation between triplicate measurements (Figure 4.12A). This suggested that protein activity was inhibited pre-irradiation and restored post-irradiation after decaging of ONBY. Additional trials to confirm these results resulted in similar activity trends for mutant PRMT, but the wells that contained PRMT WT had significantly less activity as compared to irradiated ONBY-PRMT (Figure 4.12). This could be due to time-related protein degradation or PRMT WT could have been inefficiently expressed or purified in earlier steps, resulting in highly contaminated WT samples. Regardless, the results from freshly expressed caged protein when normalized to negative and positive controls demonstrated the expected trends with enough significance to attempt further assay experimentation.

**PRMT WT and ONBY-PRMT Kinetic Activity Assay**

Next, we sought to use the SAM methyltransferase assay to track absorbance changes, and therefore PRMT1 kinetic activity, through time. We ran samples of irradiated and non-irradiated PRMT1 WT and irradiated and non-irradiated ONBY-PRMT on the same assay plate, measuring absorbance every 51 seconds for a total of 45 minutes (all conditions in table 4.1). Each sample was run in triplicate including the positive control, negative control of PRMT WT and buffer with no added histone, and negative control of just buffer and histone. Absorbance was normalized to the background and plotted as a function of time, and endpoint absorbance was also evaluated with calculated standard deviation as a measure of data variation.

In initial kinetic experiments, we irradiated the samples at 365 nm for 15 minutes, let them rest for 10 minutes, and then ran the assay. These trials resulted in preliminary data with highly inconsistent results between irradiated and non-irradiated wild-type samples. The irradiated PRMT WT exhibited no activity at all, while the non-irradiated PRMT WT had activity closely matching the irradiated ONBY-PRMT. Irradiated ONBY-PRMT had significantly higher absorbance than
the non-irradiated ONBY-PRMT, which remained at baseline with no activity for the entirety of
the assay (Figure 4.13A). At the endpoint of the assay, the absorbance of irradiated ONBY-PRMT
and non-irradiated WT did not significantly differ but were both significantly higher than non-
irradiated ONBY-PRMT. As mentioned previously, the irradiated WT had similar endpoint
absorbance to the non-irradiated ONBY-PRMT (Figure 4.13B). These results are consistent with
the hypothesis that photocaging of Tyr291 inhibits protein function, and function is restored upon
removal of the caging group. Although the ONBY-PRMT data presented such promising results
with little deviation between triplicate samples, we chose to further investigate this assay due to
the significantly varied PRMT WT activity. We hypothesized that WT was not degraded during
irradiation because the ONBY-PRMT protein was irradiated for the same length of time and
exhibited significant activity. Different expressions of PRMT WT had been used for the irradiated
and non-irradiated samples, so we concluded that the WT sample used for the irradiation sample
group had either degraded in storage or was significantly contaminated by other biomolecules.

Table 4.1: All conditions initially tested in the SAM510 Methyltransferase Assay. A “--” denotes no addition of the
corresponding component. Each condition was run in triplicate and all samples were run on one 96-well plate.
Irradiation was conducted prior to assay performance. All proteins were used at a concentration of ~0.5 mg/mL.

<table>
<thead>
<tr>
<th>Conditions Tested</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone H4</td>
<td>1 µL</td>
<td>1 µL</td>
<td>1 µL</td>
<td>1 µL</td>
<td>--</td>
<td>1 µL</td>
<td>--</td>
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<tr>
<td>PRMT1 WT</td>
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<td>10 µL</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>10 µL</td>
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<tr>
<td>ONBY-PRMT1</td>
<td>--</td>
<td>--</td>
<td>10 µL</td>
<td>10 µL</td>
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<td>--</td>
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<td>--</td>
<td>--</td>
<td>5 µL</td>
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</tr>
<tr>
<td>Buffer</td>
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<td>4 µL</td>
<td>4 µL</td>
<td>10 µL</td>
<td>14 µL</td>
<td>5 µL</td>
</tr>
<tr>
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<td>365 nm 15 min.</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
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</table>
We conducted the assay again on fresh protein that was expressed using newly purified plasmid DNA to ensure no contamination in ONBY-PRMT or PRMT WT samples. The protein was purified the day of experimentation. In the interest of minimizing irradiation time, we irradiated the new samples at 305 nm for 5 minutes, rested them on ice for 5 minutes, then performed the assay under the same conditions as the previous experiment (Table 4.2).

Table 4.2: All conditions tested in the second attempt SAM510 Methyltransferase Assay. A “--” denotes no addition of the corresponding component. Each condition was run in triplicate and all samples were run on one 96-well plate. Irradiation was conducted prior to assay performance. All proteins were used at a concentration of ~0.65 mg/mL.

<table>
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<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td>Histone H4</td>
<td>1 µL</td>
<td>1 µL</td>
<td>1 µL</td>
<td>1 µL</td>
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</tr>
<tr>
<td>PRMT1 WT</td>
<td>10 µL</td>
<td>10 µL</td>
<td>--</td>
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<td>10 µL</td>
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<tr>
<td>ONBY-PRMT1</td>
<td>--</td>
<td>--</td>
<td>10 µL</td>
<td>10 µL</td>
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<td>--</td>
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</tr>
<tr>
<td>Positive Control</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>5 µL</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Buffer</td>
<td>4 µL</td>
<td>4 µL</td>
<td>4 µL</td>
<td>4 µL</td>
<td>10 µL</td>
<td>14 µL</td>
<td>5 µL</td>
</tr>
<tr>
<td>Irradiation</td>
<td>305 nm</td>
<td>None</td>
<td>305 nm</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

Following data analysis, several trends were observed. First, the irradiated and non-irradiated PRMT WT samples had no significant differences between them, suggesting that the new irradiation conditions do not cause WT protein degradation and both samples contained the desired WT activity. Second, the irradiated ONBY-PRMT samples returned to WT activity starting immediately and closely matched the WT curves through time. Third, the non-irradiated ONBY-PRMT samples remained close to zero absorbance throughout the experiment, with a slight increase at the end that could indicate the beginning of decaging (Figure 4.14A). Additionally, the endpoint absorption was analyzed taking standard deviation into account, and both WT samples
**Figure 4.13.** PRMT methyltransferase assay data. Each sample’s data points are an average of the triplicate runs and error bars represent standard deviation. A “−” in the figure legend denotes no irradiation, while a “+” denotes irradiation at 365 nm for 15 min prior to assay. A.) PRMT assay background-normalized absorbance at 510 nm through time for each of the four sample groups. PRMT WT non-irradiated and ONBY-PRMT irradiated have similar curves and are significantly higher in absorbance than the PRMT WT irradiated and ONBY-PRMT non-irradiated samples. B.) Normalized endpoint (44–15 min) absorbance at 510 nm for each of the sample groups. PRMT WT non-irradiated and ONBY-PRMT irradiated do not significantly differ from each other but have significantly higher absorbance than PRMT WT irradiated and ONBY-PRMT non-irradiated.
Figure 4.14. PRMT methyltransferase assay data for second attempt. Each sample’s data points are an average of the triplicate runs and error bars represent standard deviation. A “-” in the figure legend denotes no irradiation, while a “+” denotes irradiation at 305 nm for 5 min prior to assay. A) PRMT assay background-normalized absorbance at 510 nm through time for each of the four sample groups. Non-irradiated and irradiated PRMT WT and irradiated ONBY-PRMT samples have similar curves and are significantly higher in absorbance than the non-irradiated ONBY-PRMT samples. B) Normalized endpoint (44.15 min) absorbance at 510 nm for each of the sample groups. Non-irradiated and irradiated PRMT WT did not significantly differ from each other or from irradiated ONBY-PRMT, and all 3 groups had significantly higher absorbance than non-irradiated ONBY-PRMT.
and the irradiated mutant samples had significantly higher absorbance than non-irradiated mutant at the end of the experiment, suggesting a sustained significant lack of activity in the ONBY caged protein (Figure 4.14B). Overall, the PRMT WT samples and irradiated ONBY-PRMT all exhibited significantly increased histone methylation activity than the non-irradiated ONBY-PRMT, suggesting photocaging modulation of PRMT activity. More analysis is necessary to convert absorbance to PRMT1 biological activity using Beer-Lambert law, which could reveal trends in protein kinetics and will give a more accurate measurement of specific methyltransferase activity.

**Conclusion**

The use of UAAs allows for site-specific incorporation, which adds a sophisticated level of control in dictating protein function. PRMTs can act on arginines within a wide range of protein types, indicating their critical function in homeostatic maintenance, but their regulation has been difficult to precisely achieve. Highly specific and tunable exogenous control of human PRMT1 could be important in modulating epigenetic factors and post-translational modifications that result in changes in gene expression, particularly with the application to control histone methylation in disease states like cancer.

In this study, the photochemical caging of PRMT1 was attempted and achieved by incorporating the unnatural amino acid ONBY at key residue Tyr291. Methyltransferase activity of the caged mutant was analyzed based on absorption as compared to PRMT1 wild-type protein. Despite inconsistencies in initial results with the wild-type protein, all assay results, whether investigating individual time points or the complete assay through time, have shown irradiated (decaged) ONBY-PRMT to have significantly higher methyltransferase activity than non-irradiated. Additionally, non-irradiated ONBY-PRMT activity remains close to nil throughout
time except for assays conducted on old protein samples, which is likely caused by accidental
decaging through time or light exposure. Most recent assay results conducted on both WT and
mutant PRMT immediately following expression and purification showed consistent activity
throughout time for irradiated WT, non-irradiated WT, and irradiated ONBY-PRMT, while non-
irradiated ONBY-PRMT had little to no activity.

All these results point towards successful inhibition of PRMT1 activity through the site-
specific incorporation of one photocaging amino acid. This eliminates the need for small molecule
effectors or complex protein-protein interactions. This inhibition is easily toggled with UV
irradiation, conferring a higher degree of temporal specificity and tunability to this inhibition
technique as compared to typical PRMT inhibition methods. On top of these benefits, PRMT1
can be modulated spatially potentially even within the same organism. For example, tissues that
require the methyltransferase activity would have active PRMT1 upon UV irradiation while tissues
with overactive or abhorrent methyltransferase activity would have no PRMT1 activity. However,
more investigation into temperature and time stability of the caged PRMT1, as well as analysis of
specific PRMT activity, protein concentration effects, and optimization of irradiation conditions,
is necessary and will be completed in future work. While these results are preliminary and in no
way indicate potential in vivo success, it is a promising method of photo modulation that can be
extended to other proteins, as well, for future application in therapeutics and diagnostics.

Experimental

General. pEvol plasmids were obtained from the laboratory of Prof. Peter Schultz. Fluorophores,
chemical compounds, and solvents were purchased from Sigma-Aldrich and used without further
purification. Reactions were conducted under ambient atmosphere with non-distilled solvents.
NMR data was acquired on a Varian Gemini 400 MHz instrument. All PRMT proteins were purified according to manufacturer’s protocols using a Qiagen Ni-NTA Quik Spin Kit.

**Synthesis of O-(2-Nitrobenzyl)-L-tyrosine.** A solution of Boc-Tyrosine-OMe (1.00 g, 1 eq, 3.35 mmol) in DMF (10 mL) was prepared in a foil-covered, flame-dried vial. To this solution, cesium carbonate (3.27 g, 3 eq, 10.0 mmol) was added and the reaction stirred at room temperature for 10 minutes. Then, 2-nitrobenzylbromide (0.60g, 1.5 eq, 5.04 mmol) was added, and the reaction was stirred overnight at room temperature, then filtered into a foil-covered round-bottom flask. The reaction was then extracted using DCM and water (3 x 20 mL each) and the organic layer was dried with MgSO4. Solvent was removed *in vacuo*. The crude product was purified on a foil-covered silica gel column using hexanes:EtOAc (3:1). Pure fractions were combined and concentrated to afford the desired protected product as a brown oil (0.613 g, 1.43 mmol, 42% yield). H NMR (400 MHz; CDCl3): δ 8.176 (d, 1H), 7.862 (d, 1H), 7.675 (t, 1H), 7.484 (t, 1H), 7.072 (d, 2H), 6.898 (d, 2H), 5.438 (s, 2H), 4.174 (m, 1H), 3.644 (s, 1H), 3.204 (m, 1H), 2.045 (s, 9H), 1.988 (s, 1H), 1.401 (s, 3H), 1.153 (m, 1H).

Hydrolysis of methyl ester and subsequent acidification, a 1:1 LiOH/Dioxane solution (3 mL) was added to the product on ice and stirred for 2 hours at room temperature. The dioxane was then removed in vacuo, the aqueous solution was cooled on ice, and 6 M HCl was added dropwise to the solution until a pH of 4 was achieved. The reaction was extracted and washed with water and EtOAc, and the organic layer was dried over MgSO4 and concentrated in vacuo. To remove the tert-butyloxy protecting group, the yellow oil was dissolved in 50% TFA solution (2 mL TFA/2 mL DCM) on ice and allowed to warm to room temperature for 1 hour. The solvent was then
removed in vacuo, and the product was obtained as white solid (408 mg, 90%).  H NMR (400 MHz; d-MeOH): δ 8.062 (d, J=3325, 1H), 7.775 (d, J=3110, 1H), 7.675 (t, J=3070, 1H), 7.518 (t, J=3009, 1H), 7.210 (d, J=2885, 2H), 6.934 (d, J=1775, 2H), 5.287 (s, J=2116, 2H), 4.174 (m, J=1670, 1H), 3.644 (s, J=1458, 1H), 3.204 (m, J=1282, 1H), 1.988 (s, J=785, 1H), 1.153 (m, J=454, 1H).

Expression of PRMT WT and ONBY-PRMT1. *Escherichia coli* BL21(DE3) cells were either co-transformed with a pET-PRMT1-TAG-291 plasmid (2.0 μL) and pEVOL-ONBY plasmid (2.0 μL), or only a pET-PRMT1 plasmid, using an Eppendorf electroporator. Cells were then plated on LB-agar plates supplemented with kanamycin (10 mg/mL) and chloramphenicol (34 mg/mL), or kanamycin only (10 mg/mL) for growth, and incubated at 37°C. After 16 hours, a single colony from each plate was selected and used to inoculate 2XYT media (10 mL) supplemented with kanamycin and chloramphenicol, or kanamycin only. The culture was grown to confluence at 37°C over 16 hours. These cultures were used to begin expression cultures in 2XYT media (100 mL) at OD600 = 0.1, then incubated at 37 °C until they reached an OD600 of between 0.7 and 0.8. At this point, PRMT1-TAG-291 cultures were covered completely in aluminum foil and cells were
induced with 1 M IPTG (100 µL), 20% arabinose (100 µL) and 100 mM ONBY (400 µL), while PRMT1 WT cultures were left uncovered and cells were induced only with 1 M IPTG (100 µL). Induced cells were grown for an additional 16 hours at 37°C, then harvested via centrifugation (10 min, 5,000 rpm). The media was decanted, and the cell pellet was stored in a -80°C freezer for 20 minutes. PRMT WT protein and ONBY-PRMT mutant protein were then purified using a commercially available Ni-NTA Quik Spin Kit according to manufacturer’s protocol. Protein yield and purity was assessed by SDS-PAGE and spectrophotometrically using a Nanodrop spectrophotometer. Successful expression was confirmed via SDS-PAGE gels stained by Coomassie Blue for protein band visualization.

**PRMT1 Initial Assay.** SAM510 Methyltransferase Assay provided by GBiosciences was used according to manufacturer’s protocol to determine the relative activity of the PRMT1 mutants and wild type either pre- or post-irradiation. A BioTek Synergy HT microplate reader was used to measure the absorbance at 510 nm. Readings were taken every minute for an hour. Either before or after initial reading, the plate was irradiated at 365 nm for 10 min to decage the ONBY group. Absorbance rates were converted to enzyme activity by finding the slope of the change in absorbance, subtracting the negative control slope, and using Beer-Lambert law and adenine’s molar absorptivity to calculate activity. PRMT1 protein samples were run in triplicate. Substrate for PRMT1 methylation was human recombinant histone 4 (1 mg/ML) from New England Biolabs. Negative controls were made by excluding histone or PRMT1 protein. Positive control was provided in the kit and diluted (1:10) (5 µL of positive control in 45 µL of assay buffer). 14 µL of PRMT1 WT or ONBY protein was added to each well (or 14 µL of buffer for the negative control). H4 substrate (1 µL) was then added to give a total volume of 15 µL/ well. For the positive control,
5 μL was diluted into 10 μL of buffer. Following sample preparation, the SAM master mix was prepared by adding 3.3 mL of buffer, 100 μL SAM (lyophilized SAM dissolved in 20mM HCl) and 200 μL of the kit’s enzyme mix. Immediately before the start of the assay, 100 μL of the master mix was added to each well and the plate was placed in the plate reader to immediately begin measuring absorbance. Absorbance measurements were taken every minute for an hour at 37°C. After an hour, the plate was irradiated with UV light (365 nm) for 10 min to decage ONBY. Absorbance measurements were then taken every minute for an additional 20 min. A second trial was then run in which samples were irradiated at 365 nm for 10 min before the addition of the enzyme master mix. The absorbance was then measured every minute for an hour. PRMT1 activity was then calculated and assay was repeated to verify results.

**PRMT1 Kinetics Assay.** SAM510: Methyltransferase Assay was used according to the manufacturer's protocol and 12 different conditions were tested (see Table 4.1 and Table 4.2). Irradiation conditions consisted of subjecting samples to UV light (365 nm or 305 nm) for either 15 minutes or 5 minutes, respectively. Following irradiation, samples were returned to ice for 5-15 minutes and plated for the assay in wells B2-G4 and B7-G9 of a 96-well plate. Differences in activity between PRMT1 WT with and without irradiation were compared to differences in activity between ONBY-PRMT1 both with and without irradiation. This allowed for significant differences only seen between conditions 3 and 4 to be attributed to successful decaging of the UAA via irradiation. Condition 5 served as a positive control for enzyme activity, and condition 6 allowed for measurement of background activity. Each condition was repeated in triplicate and 100 μL of freshly prepared SAM Master Mix was added to each well. Immediately following the addition of the SAM Master Mix, absorbance measurements were taken at an OD510 every 51
seconds for 45 minutes. The data was plotted as a function of absorbance vs. time for each condition. The slope of the best-fit line was used as a rough measure to compare enzymatic activity between the tested conditions. Additionally, for each condition, the change in absorbance between the initial and final absorbance readings was compared in order to compare absorbance changes between conditions.

References


