

1-1-2017

# Utilization of alkyne bioconjugations to modulate protein function

Johnathan C. Maza
*College of William and Mary*

Christina A. Howard
*College of William and Mary*

Megha A. Vipani
*College of William and Mary*

Christopher R. Travis
*College of William and Mary*

Douglas D. Young
*College of William and Mary*, dyoung01@wm.edu



## Recommended Citation

Maza, Johnathan C.; Howard, Christina A.; Vipani, Megha A.; Travis, Christopher R.; and Young, Douglas D., Utilization of alkyne bioconjugations to modulate protein function (2017). 10.1016/j.bmcl.2016.11.041

Published in final edited form as:

*Bioorg Med Chem Lett*. 2017 January 01; 27(1): 30–33. doi:10.1016/j.bmcl.2016.11.041.

# Utilization of alkyne bioconjugations to modulate protein function

**Johnathan C. Maza**[1], **Christina A. Howard**[1], **Megha A. Vipani**, **Christopher R. Travis**, and **Douglas D. Young**[*]

Department of Chemistry, College of William & Mary, P.O. Box 8795, Williamsburg, VA 23187, USA

## Abstract

The ability to introduce or modify protein function has widespread application to multiple scientific disciplines. The introduction of unique unnatural amino acids represents an excellent mechanism to incorporate new functionality; however, this approach is limited by ability of the translational machinery to recognize and incorporate the chemical moiety. To overcome this potential limitation, we aimed to exploit the functionality of existing unnatural amino acids to perform bioorthogonal reactions to introduce the desired protein modification, altering its function. Specifically, via the introduction of a terminal alkyne containing unnatural amino acid, we demonstrated chemically programmable protein modification through the Glaser-Hay coupling to other terminal alkynes, altering the function of a protein. In a proof-of-concept experiment, this approach has been utilized to modify the fluorescence spectrum of green fluorescent protein.

### Keywords

Bioconjugations; Green fluorescent protein; Glaser-Hay reaction; Unnatural amino acids

Protein engineering is a powerful tool for the development of new therapeutics, catalysts, and biosensors.[1–8] While many advances in the field have been made, designing novel protein functionality is still a challenge, as it requires an intricate understanding of the subtle interplay between protein structure and function. Current engineering techniques often focus on using selections and screens to optimize or enhance existing protein functionality.[2] While this method has proved useful for optimizing existing function, generating new function where it does not exist is still a hurdle.

Unnatural amino acids (UAAs) represent a unique means to introduce new chemical functionality into proteins. Using the Schultz methodology, UAAs are co-translationally incorporated into a protein's primary sequence, typically in response to a stop codon introduced at the DNA level.[9–11] As such, UAA mutagenesis allows for the site-specific incorporation of a novel chemical functionality into proteins, which can then be exploited

[*]Corresponding author: dyoung01@wm.edu (D.D. Young).

[1]Authors contributed equally to the work.

**A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.11.041

towards the development of novel protein activities. By inserting new chemical reactivity into a protein, UAAs allow for a condensed and facile workflow for engineering new protein functionality without the need to perform tedious and lengthy selections and screens. For example, recent reports from our laboratory and others have site-specifically incorporated fluorescent UAAs into proteins, yielding novel fluorescent properties in a protein in a direct and highly controlled fashion.[12–14]

While UAAs have allowed for the development of unique protein function, the evolved proteins are often limited to a single new function, depending on the UAA incorporated.[2,15] In addition, the functionality is limited to the UAA itself, which suffers from constraints, such as the requisite for an aminoacyl tRNA synthetase capable of recognizing the UAA, the synthetic accessibility of the UAA, and the size of the UAA which may preclude its uptake by a biological system.[10] A more appealing strategy would allow for the generation of a UAA-containing protein ‘‘template” upon which researchers could synthetically introduce different chemical moieties that would in turn lead to altered protein function depending on the moiety employed.

Bioorthogonal chemistry, which employs reactions that proceed to completion under physiological conditions (pH ~7, 37 °C), offers a unique mechanism to add new chemical functionality to proteins.[16,17] Indeed, a variety of reactions have been developed that can add novel chemistry to living systems. In particular, the cycloaddition between azides and alkynes that is either copper(I) mediated or strain promoted has become a widespread technique to introduce unique chemistry to proteins.[18–21] More recently, our group has developed a bioorthogonal variant of the Glaser-Hay reaction,[22,23] which brings together two terminal alkynes to form a diyne in the presence of copper(I) under physiological conditions. [24–26] The resulting stable diyne linkage has a well-defined linear geometry, and due to the abundance of commercially available terminal alkynes, a variety of chemical moieties can be reacted onto a protein using this technique. As such, we sought to utilize the power of this new chemistry to generate new and different protein function dependent upon the alkyne reaction partner and not the UAA alone.

Specifically, we designed a proof-of-concept experiment to alter the function of green fluorescent protein (GFP) via reaction of different terminal alkynes onto the GFP chromophore. GFP is a 27 kDa protein isolated from *Aequorea victoria* with photochemical properties arising from an internal chromophore composed of Ser65-Tyr66-Gly67.[27–29] New chemical properties afforded by UAA introduction in place of Tyr66 have already been documented to alter GFP’s fluorescence profile.[30] All UAAs incorporated were found to blue-shift the fluorescence profile of GFP, with more highly conjugated UAAs exhibiting a greater degree of spectral shifting. Based on these results, the ability to modulate the conjugation of GFP’s fluorophore using UAA mutagenesis is apparent, and represents a convenient means to rationally design new protein function.[31] However, this mutagenesis approach is limited by the size and complexity of the UAA. An alternative approach involves exploiting the chemical functionality in pre-existing UAAs to serve as functional handles for bioorthogonal reactions, acting as a ‘‘template” for the chemical derivatization of new protein function.

This chemically templated protein function could be achieved via the genetic incorporation of *p*-propargyloxyphenyalanine (*p*PrF, **1**) or *p*-ethynylphenylalanine (*p*EtF, **2**) into residue 66 of GFP (Fig. 1).[32] This provides a terminal alkyne handle for reaction with different chemical moieties via the bioorthogonal Glaser- Hay reaction. The resulting diyne linkage is highly conjugated, and a prime candidate to introduce new photochemical properties into GFP's fluorophore without the need to evolve a new aaRS or express multiple versions of the protein containing different UAAs. Moreover, the *p*EtF (**2**) is directly conjugated with the aromatic ring of the UAA, allowing for a comparison of the conjugation between the different UAAs. We hypothesized that the altered conjugation and chemical properties around the fluorophore would lead to new photophysical properties, demonstrating the utility of a chemically programmable protein engineering strategy. Herein we report our findings on utilizing the Glaser-Hay reaction on GFP's fluorophore to alter its fluorescent properties.

In order to obtain protein possessing an alkynyl moiety, a GFP plasmid harboring a TAG mutation at position 66 was co-transformed with the polyspecific *p*CNF-aaRS/tRNA pair.[33] Conveniently, this aaRS is capable of recognizing both **1** and **2** for expression of an alkyne-containing GFP.[25] As the alkynyl UAA is incorporated into position 66 in GFP's fluorophore, the extended conjugation afforded by the UAA alters the spectral properties of GFP. To assess that *p*PrF-GFP$_{TAG66}$ was successfully produced, spectra for the GFP-variant were compared to the wild type. These spectra exhibited blue-shifts, in agreement with the literature precedent, in the *p*PrF variant relative to the wild type. Incorporation was also confirmed by SDS-PAGE analysis of protein expression in the presence and absence of the UAA (see Supplementary material). A similar expression was performed using the *p*CNF aaRS/tRNA pair and *p*EtF to produce a separate GFP mutant with a bioconjugation handle. Conveniently, due to the modularity of this approach, only a single protein expression is necessary and all functional modification can be achieved synthetically. This is in contrast to previous experiments, which required an individual protein expression for each UAA in order to modify protein function.

With a *p*PrF-GFP$_{TAG66}$ and *p*EtF-GFP$_{TAG66}$ in hand, we then sought to employ our previously reported bioorthogonal Glaser- Hay reaction to install new and varied chemical functionality into the chromophore of GFP (Fig. 2). To investigate, we performed bioorthogonal Glaser-Hay reactions on the chromophore's alkyne handle to couple terminal alkyne-bearing aliphatic and aromatic compounds with different chemical functionalities. Glaser-Hay reactions were performed by using a working concentration of 500 mM of CuI and TMEDA in the presence of alkyne-UAA bearing GFP$_{TAG66}$ and the cognate alkynyl partner (Scheme 1). Reactions proceeded for 4 h at 4 °C and then were purified via centrifugation with a molecular weight cut-off column. The protein was placed in phosphate-buffered saline solution (pH ~7.2) for analysis using fluorescence spectroscopy. Gratifyingly, our initial attempts to couple the terminal alkynes to the fluorophore were successful. Furthermore, the different characteristics of the installed alkyne moieties successfully shifted the fluorescence profile away from the parental alkynyl-GFP$_{TAG66}$ spectra, each in a unique way. Control reactions in the presence of the alkyne but the absence of either the CuI/TMEDA or the soluble alkyne afforded no spectral shifts,

confirming that the protein modification is indeed due to the coupling reaction. While it might be expected that the requisite for the reaction to occur within the β-barrel of GFP may hinder this reaction from occurring, we hypothesize that the hydrophobic nature of the interior of GFP actually aided in the hydrophobic alkyne localization, thereby facilitating the reaction by increasing the effective concentration. Additionally, SDS-PAGE analysis with Coomassie revealed that the Glaser-Hay reaction only minimally altered protein concentration suggesting only minimal protein degradation (see Supplementary material).

We found that our initial Glaser-Hay reactions on *p*PrF-GFP$_{TAG66}$ had different effects on the fluorescent profile of GFP (Fig. 3A). Reacting 1-hexyne (**3**) on the chromophore caused a general broadening and quasi-red shift of the fluorescent spectra. Reacting propargyl amine (**4**) on the chromophore caused a slight band broadening, as well as potential increase in fluorescence intensity, perhaps due to the increased polarity of the introduced amine group. Interestingly, coupling with an aromatic alkyne resulted in a dramatic red-shift of the fluorescence to above that of wild-type GFP. Both ethynylanisole (**5**) and ethynylaniline (**6**), resulted in excitation spectra maxima above 540 nm, dramatically altering the fluorescence of GFP.

We next sought to explore the effects of reacting terminal alkynes in direct conjugation with the aromatic ring of residue 66. This was feasible with the GFP mutant harboring **2**. Interestingly, this strategy resulted in an even greater blue shift of the *p*EtF-GFP$_{TAG66}$ compared to both the *p*PrF and wild-type variants, likely due to the increased conjugation of the direct attachment of that terminal alkyne on the phenyl ring (see Supplementary material). As a result of this shift, a different excitation wavelength was necessary, as 395 nm was found to no longer excite the *p*EtF-containing chromophore. Based on absorption experiments, we selected 280 nm as the wavelength to excite the *p*EtF-GFP$_{Tag66}$ and all its Glaser- Hay derivatives. In the same fashion as the *p*PrF, the bioorthogonal Glaser-Hay was performed on *p*EtF-GFP$_{TAG66}$ using the same reaction partners. Once again, **3** was found to broaden the fluorescence spectra. Interestingly, **4** had a drastic red-shift relative to the pEtF parent chromophore. We believe this helps validate our initial speculation that the polarity of the amine has a drastic impact on the fluorescent properties of the chromophore, as in this instance the whole system is in direct conjugation. Interestingly, when employing the aromatic alkynes in the fluorophore modulation, a less dramatic effect was observed than with the *p*PrF mutants. Reaction with **5** only slightly red-shifted the spectra; however, **6** had a more significant impact both on the intensity and the red-shifting of the fluorophore. Additionally, attempts to repeat the experiments using the bromo-alkyne derivative of **2** under Cadiot-Chodkiewicz coupling conditions resulted in the identical spectra, but were performed under more mild reaction conditions. These results were expected as the final products of both the Glaser-Hay or Cadiot-Chodkiewicz reactions are identical.[25] This represents a viable alternative reaction to these protein modification approaches.

In conclusion, we have extended our work on the biological Glaser-Hay to utilize the bioorthogonal chemistry to modulate protein function. Using two previously reported alkyne-containing UAAs within the chromophore of GFP (position 66), we have successfully performed the Glaser-Hay reaction on the chromophore of GFP. The resulting diyne linkage alters the fluorescence profile of GFP depending on the moiety attached to the

terminal alkyne. Our future work seeks to extend the reaction to additional aromatic containing alkynes, which we hope will have a greater impact on GFP fluorescence due to the increased conjugation found in aromatic systems. Our findings highlight the potential of bioorthogonal chemistry, particularly diyne-forming chemistries, to modulate protein function without the need for tedious selections and screens.

## Acknowledgments

The authors would like to thank the College of William & Mary and the National Institute of General Medical Sciences of the NIH (R15GM113203). We would also like to acknowledge Dr. Peter Schultz for providing the pEVOL plasmids utilized in the research.

## References

1. Wu A, Senter P. Arming antibodies: prospects and challenges for immunoconjugates. Nat Biotechnol. 2005; 23:1137–1146. [PubMed: 16151407]
2. Brustad EM, Arnold FH. Optimizing non-natural protein function with directed evolution. Curr Opin Chem Biol. 2011; 15:201–210. [PubMed: 21185770]
3. Zhu H, Snyder M. Protein chip technology. Curr Opin Chem Biol. 2003; 7:55–63. [PubMed: 12547427]
4. Tan W, Sabet L, Li Y, et al. Optical protein sensor for detecting cancer markers in saliva. Biosens Bioelectron. 2008; 24:266–271. [PubMed: 18479906]
5. Link AJ, Mock ML, Tirrell DA. Non-canonical amino acids in protein engineering. Curr Opin Biotechnol. 2003; 14:603–609. [PubMed: 14662389]
6. Banghart M, Volgraf M, Trauner D. Engineering light-gated ion channels. Biochemistry. 2006; 45:15129–15141. [PubMed: 17176035]
7. Steen E, Redeker DT, Ta D, Cortens B, Billen W, Guedens P. Adriaensens, protein engineering for directed immobilization. Bioconjug Chem. 2013; 24:1761–1777. [PubMed: 24160176]
8. Presta L. Engineering of therapeutic antibodies to minimize immunogenicity and optimize function. Adv Drug Deliv Rev. 2006; 58:640–656. [PubMed: 16904789]
9. Wang L, Schultz PG. Expanding the genetic code. Angew Chem Int Ed Engl. 2004; 44:34–66. [PubMed: 15599909]
10. Young TS, Schultz PG. Beyond the canonical 20 amino acids: expanding the genetic lexicon. J Biol Chem. 2010; 285:11039–11044. [PubMed: 20147747]
11. Xie J, Schultz PG. A chemical toolkit for proteins – an expanded genetic code. Nat Rev Mol Cell Biol. 2006; 7:775–782. [PubMed: 16926858]
12. Wang J, Xie J, Schultz P. A genetically encoded fluorescent amino acid. J Am Chem Soc. 2006; 128:8738–8739. [PubMed: 16819861]
13. Lee H, Guo J, Lemke E, Dimla R, Schultz P. Genetic incorporation of a small, environmentally sensitive, fluorescent probe into proteins in Saccharomyces cerevisiae. J Am Chem Soc. 2009; 131:12921. [PubMed: 19702307]
14. Lampkowski JS, Uthappa DM, Young DD. Site-specific incorporation of a fluorescent terphenyl unnatural amino acid. Bioorg Med Chem Lett. 2015; 25:5277–5280. [PubMed: 26421994]
15. Liu C, Schultz P, Kornberg R, Raetz C, Rothman J, Thorner J. Adding new chemistries to the genetic code. Annu Rev Biochem. 2010; 79:413–444. [PubMed: 20307192]
16. Sletten E, Bertozzi C. Bioorthogonal chemistry: fishing for selectivity in a sea of functionality. Angew Chem Int Ed. 2009; 48:6974–6998.
17. Maza J, Jacobs T, Uthappa D, Young D. Employing unnatural amino acids in the preparation of bioconjugates (vol. 27, p. 805, 2016). Synlett. 2016; 27:e6–e6.
18. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB. A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective ‘‘ligation” of azides and terminal alkynes. Angew Chem Int Ed Engl. 2002; 41:2596–2599. [PubMed: 12203546]

19. Wang Q, Chan TR, Hilgraf R, Fokin VV, Sharpless KB, Finn MG. Bioconjugation by copper(I)-catalyzed azide-alkyne [3+2] cycloaddition. J Am Chem Soc. 2003; 125:3192–3193. [PubMed: 12630856]
20. Agard NJ, Prescher JA, Bertozzi CR. A strain-promoted [3+2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. J Am Chem Soc. 2004; 126:15046–15047. [PubMed: 15547999]
21. Baskin JM, Prescher JA, Laughlin ST, et al. Copper-free click chemistry for dynamic in vivo imaging. Proc Natl Acad Sci USA. 2007; 104:16793–16797. [PubMed: 17942682]
22. Glaser C. Ber Dtsch Chem Ges. 1896; 2:422–424.
23. Shi W, Lei A. 1,3-Diyne chemistry: synthesis and derivations. Tetrahedron Lett. 2014; 55:2763–2772.
24. Maza JC, McKenna JR, Raliski BK, Freedman MT, Young DD. Synthesis and incorporation of unnatural amino acids to probe and optimize protein bioconjugations. Bioconjug Chem. 2015
25. Maza JC, Nimmo ZM, Young DD. Expanding the scope of alkyne-mediated bioconjugations utilizing unnatural amino acids. Chem Commun. 2016; 52:88–91.
26. Lampkowski JS, Villa JK, Young TS, Young DD. Development and optimization of Glaser-Hay bioconjugations. Angew Chem Int Ed Engl. 2015
27. Pakhomov A, Martynov V. GFP family: structural insights into spectral tuning. Chem Biol. 2008; 15:755–764. [PubMed: 18721746]
28. Ormö M, Cubitt AB, Kallio K, Gross LA, Tsien RY, Remington SJ. Crystal structure of the Aequorea victoria green fluorescent protein. Science. 1996; 273:1392–1395. [PubMed: 8703075]
29. Craggs T. Green fluorescent protein: structure, folding and chromophore maturation. Chem Soc Rev. 2009; 38:2865–2875. [PubMed: 19771333]
30. Young D, Jockush S, Turro N, Schultz P. Synthetase polyspecificity as a tool to modulate protein function. Bioorg Med Chem Lett. 2011; 21:7502–7504. [PubMed: 22041062]
31. Maza J, Villa J, Landino L, Young D. Utilizing unnatural amino acids to illustrate protein structure-function relationships: an experiment designed for an undergraduate biochemistry laboratory. J Chem Edu. 2016; 93:767–771.
32. Deiters A, Schultz PG. In vivo incorporation of an alkyne into proteins in Escherichia coli. Bioorg Med Chem Lett. 2005; 15:1521–1524. [PubMed: 15713420]
33. Young D, Young T, Jahnz M, Ahmad I, Spraggon G, Schultz P. An evolved aminoacyl-tRNA synthetase with atypical polysubstrate specificity. Biochemistry. 2011; 50:1894–1900. [PubMed: 21280675]

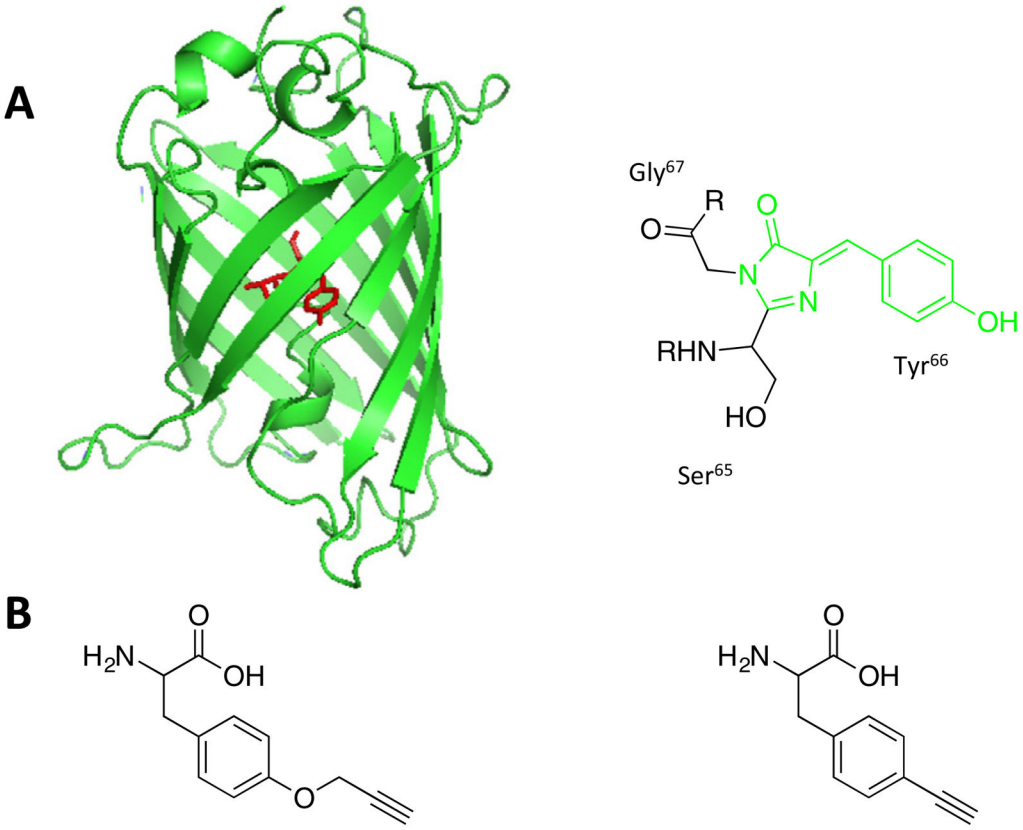



**Fig. 1.**

Modification of GFP function via UAA mutagenesis. (A) GFP β-barrel (green) with the chromophore (red) and chromophore structure. (B) Unnatural amino acids employed as bioconjugation handles.

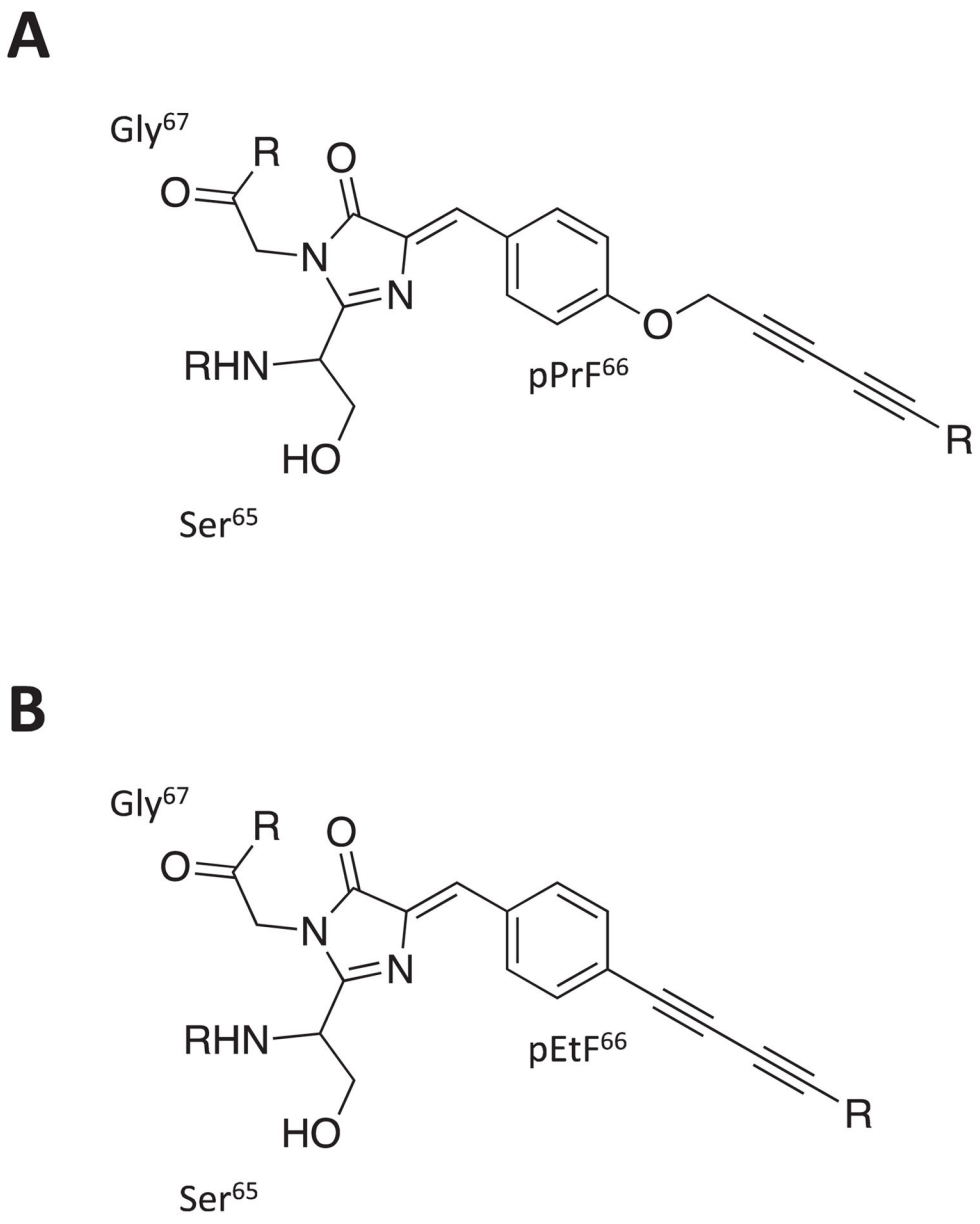



**Fig. 2.**

Structures of the fluorophores generated when performing a Glaser-Hay reaction with the two UAAs. (A) Product from the coupling of a terminal alkyne to a GFP mutant containing **1**. (B) Product from the coupling of a terminal alkyne to a GFP mutant containing **2**.

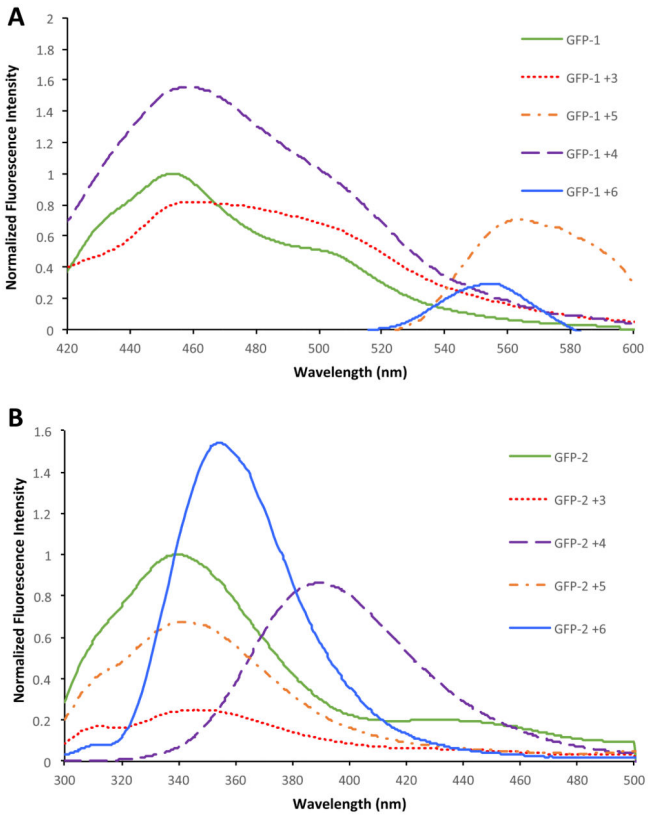
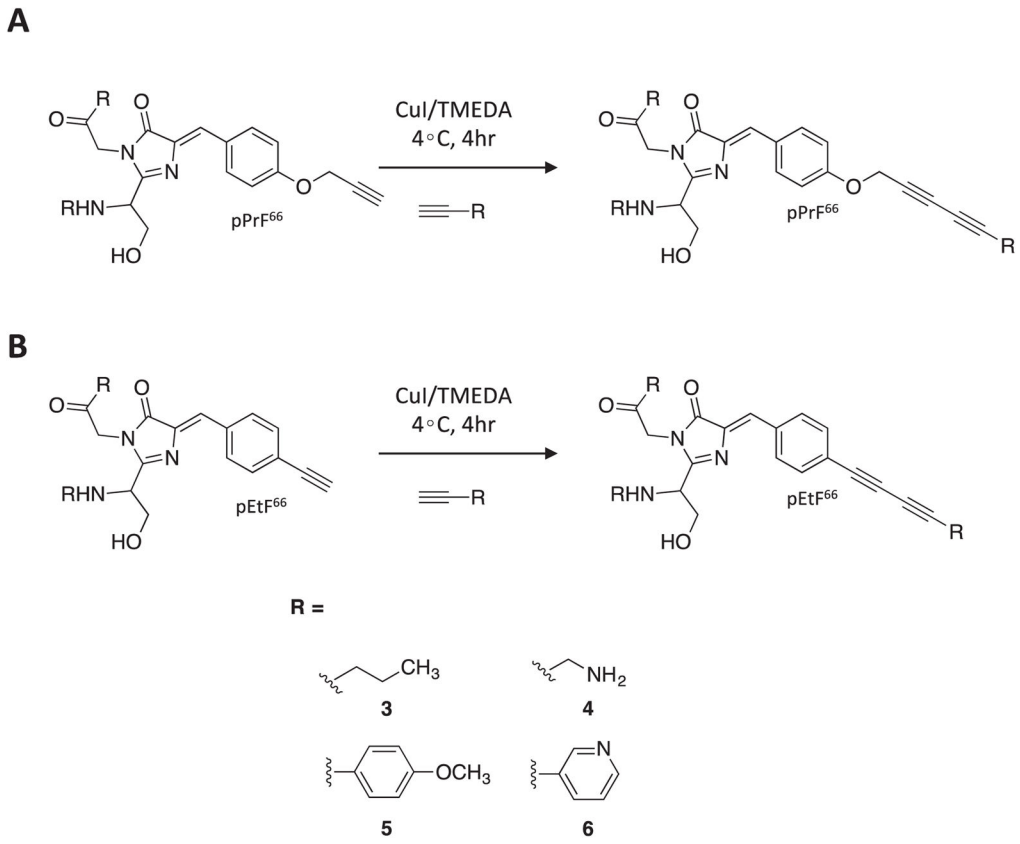



**Fig. 3.**

Fluorescent profile obtained after reacting various alkyne containing partners with pPrf or pEtF-containing $GFP_{TAG66}$. (A) Fluorescent spectra for Glaser-Hay modified pPrF-GFP chromophore. (B) Fluorescent spectra for Glaser-Hay modified pEtF-GFP chromophore.




**Scheme 1.**
Glaser-Hay bioconjugations to modify the GFP fluorophore.