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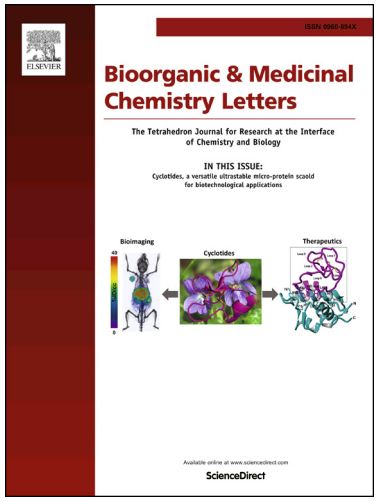

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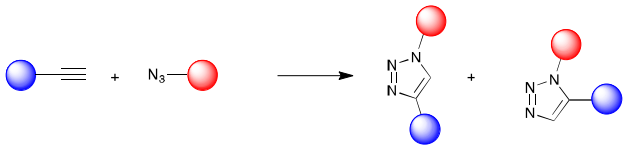
ABSTRACT

The prevalence of 1,3-dipolar cycloadditions of azides and alkynes within both biology and chemistry highlights the utility of these reactions. However, the use of a copper catalyst can be prohibitive to some applications. Consequently, we have optimized a copper-free microwave assisted reaction to alleviate the necessity for the copper catalyst. A small array of triazoles was prepared to examine the scope of this approach, and the methodology was translated to a protein context through the use of unnatural amino acids to demonstrate one of the first microwave-mediated bioconjugations involving a full length protein.

The alkyne/azide 1,3-dipolar cycloaddition reaction has become an indispensible tool for scientists as a mechanism to conjugate an alkyne moiety with an azide (Scheme 1).[1-4] While the general class of pericyclic reactions have proven useful to organic chemists,[5, 6] the robust nature of this particular alkyne/azide "click" reaction in particular has allowed it to become ubiquitous in a plethora of fields such as drug design, sensors, catalysis, materials chemistry, and bioconjugations.[7-14] While this specific cycloaddition was first discovered in 1963, the copper catalyzed variant has expanded its utility via decreasing reaction times and temperatures, while increasing regioselectivity.[3] However, the addition of a copper catalyst has some limitation within biological systems and in some materials applications.[15, 16] These issues have necessitated the development of rapid copper-free conditions, which is typically accomplished via the use of highly strained alkynes, many of which are challenging to synthetically access.[17-19] Alternatively, heterogeneous reactions employing an immobilized catalyst, and non-transition metal catalyzed reactions have been explored.[20, 21] While these methodologies are extremely useful, we became interested in exploring other options for rapid copper-free reaction conditions using unstrained alkynes. Another mechanism to accelerate the reaction has been the application of microwave irradiation, which has been primarily examined under copper-catalyzed conditions.[22-25] Previously, a microwave mediated copper-free cycloaddition toward the preparation of a complex polymer was reported; however, little experimental optimization of the reaction was conducted and yields were significantly low.[26] We aim to significantly expand on this approach, optimizing reaction conditions and expanding its utility towards biological applications.

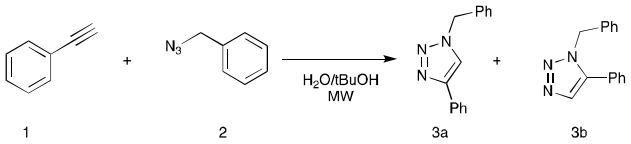


Scheme 1. Standard copper-free 1,3-dipolar cycloaddition

Due to the extensive number of variables associated with microwave irradiation, initial investigations involved the optimization of reaction conditions with a model system.[27] Based on commercial availability of reagents and spectroscopic properties, the reaction of benzyl azide (**2**) and phenylacetylene (**1**) was selected to explore the copper-free microwave mediated 1,3-dipolar cycloaddition of alkynes and azides. The rapid nature of the microwave was expected to facilitate productive reactions in a short amount of time, without necessitating the use of a copper catalyst, and ultimately making the reaction more useful for biological settings or other applications where the use of copper precludes the utilization of the reaction. Using a CEM Discover, various temperatures, microwave powers and microwave settings were explored (Table 1). Standard microwave conditions involve the input of power until a specific temperature is obtained, followed by brief bursts of power to maintain the temperature. Power mode involves the constant input of a microwave power until a set temperature is reached, followed by termination of reaction conditions. Finally, Pulsed Power (SPS) mode involves a power cycling to maintain the temperature within a specific range (δT). A delicate interplay between power input and reactant decomposition was noted, as higher product yields were observed with increased power

settings (300W); however, increased irradiation times began to lead to decreased yields. Thus, the SPS setting was found to be optimal as it afforded high power inputs, while reduced times at elevated temperatures. Ultimately, maximizing power to 300W in SPS mode for 20 min afforded a 96% yield and was employed in further reactions. As expected, a mixture of the two regioisomers (**3a** and **3b**) was obtained under all conditions.

Table 1. Optimization of the copper-free microwave assisted 1,3-dipolar cycloaddition.



1 2 3a 3b

Power (W)	Time (min)	Temperature (°C)	MW Setting	Yield (%)
100	10	100	Standard	21.9
100	10	125	Standard	26.0
100	20	125	Standard	31.4
100	10	168	Power	23.0
200	2	168	Power	26.0
300	1	168	Power	49.2
300	2	168	Power	21.9
200	20	168 (δT=15)	SPS	48.1
300	20	168 (δT=15)	SPS	96.2

In order to assess the scope of the reaction, we next examined a variety of alkynes and azides under the optimized reaction conditions. In addition to benzyl azide (**2**), azidoheptane (**4**) and trimethylsilyl azide (**5**) were examined due to commercial availability and chemical functionality. This set of azides was reacted with phenylacetylene (**1**), 1-hexyne (**6**), and propargyl alcohol (**7**) under the previously optimized microwave conditions. When reacting equimolar ratios of the propargyl alcohol and any azide, very little product was recovered <5%, obviating the further optimization of the reaction. We hypothesized that the propargyl alcohol was susceptible to decomposition under the microwave conditions as reactions typically resulted in a very dark crude mixture, which was unobserved with other alkynes. Two remedies were examined to address the low yields, first trityl-protected propargyl alcohol (**9**) and methyl propargylether (**8**) were employed to protect the hydroxyl group, and second, excess of the alkyne was employed to identify if decomposition was an issue. Ultimately, it appears that alkyne decomposition under microwave conditions was the primary factor, as increasing from 1 equivalent to 3 equivalents dramatically increased the yield. While not ideal, eventual translation to bioconjugations typically employ extreme excess of the non-protein partner to drive reactions, so 3 equivalents is relatively minimal. Consequently, the array of triazole products (**3, 10-23**) was re-synthesized using an excess of alkyne (Figure 1). Conveniently, the volatility of the alkyne reactants allowed for easy purification under vacuum or via column chromatography. Overall, the reaction proceeded in moderate to excellent yield (96-47%), with the benzyl azide being the most reactive azide (96-84%), and the azidoheptane exhibiting lower activity (94-46%). This may be a result of some solubility issues, or simply the aliphatic nature of the azide. Trityl protected propargyl alcohol reactions involved the lowest yields, potentially due to decomposition. These yields could be significantly increased via alteration of the protecting group to a methyl substituent. Additionally, reactions performed with the TMS-azide in the microwave resulted in desilylation and yielded the free triazole ring after column chromatography. Control reactions that mimicked the microwave temperature profile without microwave irradiation did afford product, albeit in dramatically lower yield < 20%. Thus, the dramatically increased yields using the optimized microwave conditions demonstrate the utility of this methodology. While these yields are sometimes comparable to previously reported reactions, the combination of decreased reaction times and absence of copper suggest that the methodology may be useful in specific applications.[28]

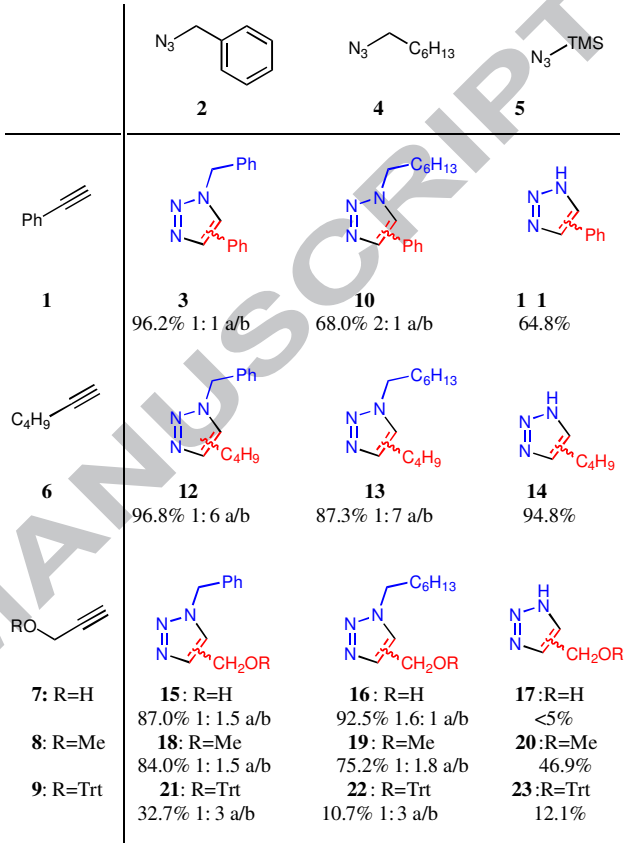


Figure 1. Triazole array prepared to assess the scope of the microwave assisted copper-free 1,3-dipolar cycloaddtion. Reactions were performed with 3 eq. alkyne, using SPS mode 300W, 20 min.

In order to further apply the microwave assisted copper-free 1,3-dipolar cycloadditions, we next investigated its use in a biological context. This is especially relevant due to the propensity of copper to generate radicals that degrade proteins, and due to the general cytotoxicity of copper.[15] To accomplish this aim, an alkynyl unnatural amino acid was incorporated using the Schultz amber suppresion technology into green fluorescent protein (GFP).[29-34] GFP was selected due to its nascent fluorescent properties and well-documented use as a reporter protein. The *p*-propargyloxyphenylalanine (**24**) was expressed at residue 151 of GFP, which is located within the rigid β-barrel of the protein (Figure 2).[13, 35, 36] With the alkyne-containing protein in hand, we next investigated the ability to translate our previously optimized reaction to a biological context. The mutant GFP was reacted with Alexafluor-488 azide to generate a fluorescent bioconjugate that could be analyzed via SDS-PAGE. Not surprisingly, when subjected to microwave irradiation in the standard CEM Discover under a variety of conditions (including the previously optimized SPS conditions), protein degradation was observed at high temperatures, providing no observable conjugated product (data not shown).

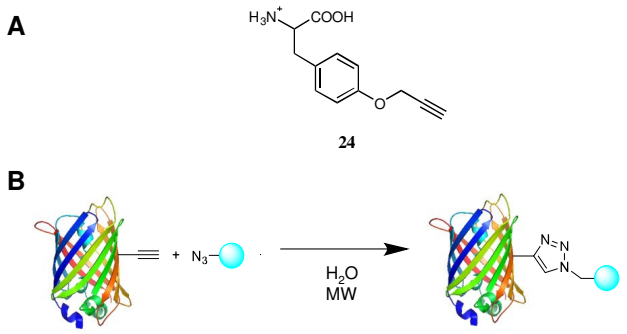


Figure 2. Incorporation and cycloaddition using an unnatural amino acid. A) Structure of *p*-propargyloxyphenylalanine incorporated into GFP. B) Proposed 1,3-dipolar cycloadditon reaction between the mutant GFP and an azide-containing fluorophore in the microwave.

In order to prevent protein degradation, the reaction was translated to a CEM Coolmate system, which utilizes a jacketed reaction vessel to allow microwave transparent cooling fluid to be flowed through to significantly reduce reaction temperatures while still affording microwave irradiation.[37] The SPS setting previously employed is not feasible under Coolmate conditions, and thus modulation of microwave power was the most logical variable to examine. Reactions containing the mutant protein and azide fluorophore were conducted at microwave powers of 100 W, 200 W, and 300 W. The coolant was pre-chilled to -50 °C and used to cool the reaction to -30 °C prior to microwave irradiation, and the reaction was then irradiated until the temperature reached 40 °C.

Following irradiation, the protein was denatured and analysed by SDS-PAGE. Due to the covalent modification of the protein with a fluorophore, successful reactions were expected to yield a fluorescent product even after denaturation of the protein fluorophore. Reaction success was determined by first examining the fluorescence of the gel, and then staining the gel with coomassie blue to ascertain the presence of protein. Reactions at 300 W displayed significant protein degradation, while reactions at 100 W did not exhibit significant fluorophore coupling. However, reactions at 200 W displayed significant coupling without protein degradation. While other types of bioconjugations have been performed in the microwave, we believe this to be one of the first reported microwave-mediated bioconjugations involving full length proteins.[38] In order to further optimize the coupling, the reaction was subjected to 2 pulses of microwave irradiation at 200 W prior to purification (Figure 3). Based on both absorption spectroscopy of the conjugates and densiometry measurements of the gels the coupling yields are ~85%. Additionally, control irradiations in the absence of the fluorophore were performed, demonstrating that GFP was still fluorescent after irradiation, signifying that it was not denatured as a result of microwave irradiation at low temperature (see supplementary information). This is an important consideration when considering the utility of these reactions within the context of the microwave. Reactions under identical temperature profiles to the microwave yielded no observable conjugate. It is important to note that GFP is a relatively hearty protein and the presence of copper does not necessarily lead to degradation; however, many other less stable proteins may require the copper-free conditions, or the eventual application of the protein in a biological setting may necessitate the absence of copper to prevent cytotoxicity. Attempts to translate these Coolmate conditions to the previously optimized small molecule reactions resulted in some coupling but only around 20% for many of the reaction partners. We hypothesize this is due to the significantly higher reagent concentrations compared to the protein system, coupled with substantially shorter reaction times. Thus, optimal conditions for the microwave-mediated click reaction are dependent on the nature of the reactants (proteins vs. small molecules).

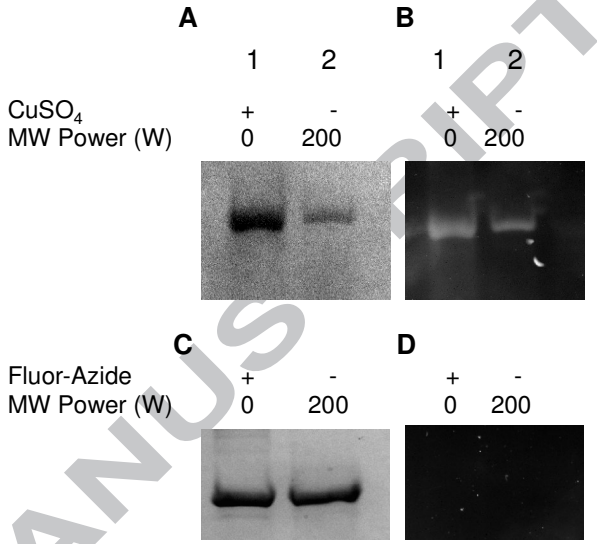


Figure 3. Microwave mediated 1,3-dipolar cycloadditions on GFP. A) SDS-PAGE Coomassie stain indicating the presence of GFP both with a control reaction not utilizing the microwave (Lane 1) and under microwave conditions in the absence of copper (Lane 2) . B) SDS-PAGE fluorescence image, demonstrating the effective coupling between the protein and the azide fluorophore to generate a triazole linkage. The difference in protein concentration is potentially due to some ninor degradation under microwave conditions. C) Copper-free control reactions in the absence of either microwave irradiation or the fluorescently labelled reaction partner. While GFP is present and non-degraded in both reactions when stained with Coomassie blue. D) Fluorescence imaging indicates that no bioconjugation occurs under the two control conditions as no fluorescence is observed

In conclusion, we have demonstrated that it is feasible to conduct alkyne/azide 1,3-dipolar cycloadditions in the microwave without the requisite of a copper catalyst. This has far-reaching applications within the realms of both biology and materials science where copper may be prohibitive to specific reactions. The microwave-mediated reaction was optimized to afford high yields of triazole products and further applied to a protein context via the utilization of unnatural amino acids. Overall, we believe this to be one of the first reported protein bioconjugations utilizing microwave irradiation. Moreover, the methodology developed facilitates an extremely rapid method to obtain bioconjugates and in the absence of potentially cytotoxic copper.

Acknowledgments

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