

Developing Novel Bioorthogonal Chemistry Employing Nitron Dipoles

by

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Abstract

The introduction of the concept of Click chemistry by Barry Sharpless has inspired countless chemists to further develop traditional chemical transformations into powerful tools for a number of applications that require extremely fast, efficient, selective and robust reactions. Recently, these concepts have been extended to reactions amenable to complex biological systems allowing researchers to interrogate them with ever greater precision. The field of bioorthogonal chemistry has rapidly been developed to give access to a plethora of robust and powerful tools allowing for the labelling and tracking of biomolecules in living systems. The development of new bioorthogonal reactions displaying faster kinetics and the use of alternative reagents, allows for more complex biological systems to be interrogated, allows for applications requiring multiplex labelling where multiple biomolecules can be simultaneously functionalized through applying orthogonal bioorthogonal reactions, and hopefully allow for the minimal perturbation to biomolecules' native functions. Towards these goals, we have focused efforts towards the development of reactions employing nitron dipoles, which can act as alternative dipoles in a multitude of bioorthogonal cycloaddition reactions. In the context of this thesis, work involving the optimization of the copper-catalyzed alkyne-nitron cycloaddition involving rearrangement (CuANCR), the strain-promoted nitron-cyclooctadiyne cycloaddition, and other exploratory nitron cycloaddition chemistry will be explored.

The optimization of CuANCR, also known as the Kinugasa reaction, follows previous efforts by our research group towards the application of micelles to allow for faster and more efficient reactions under aqueous conditions. Building upon these initial studies, we were

able to further optimize the reaction to apply activated alkynes, propiolamides and propiolic esters, to obtain better product yields under micellar conditions. The development of a fluorescence magnetic bead assay to allow for rapid development of reaction conditions is also presented, thus allowing for rapid screening of biomimetic lipids forming micelles and studying the efficiency of applying amino acids as copper ligands. The applicability of micelle-assisted CuANCR towards bioorthogonal applications was demonstrated by the functionalization of cell membrane-associated peptides. Initial development of dual labelling protocols involving CuANCR, followed by thiol addition to the formed β -lactam products is also presented.

Following CuANCR development, the study of strain-promoted nitron-alkyne cycloadditions (SPANC) between nitrones and cyclic diynes (molecules containing two alkyne moieties, cyclooctadiynes) is presented. These cyclooctadiyne scaffolds allow for the cycloaddition of two separate dipoles, with great rate accelerations observed for the second cycloaddition. Kinetic and computational investigations of this bioorthogonal transformation have allowed for a better understanding of the parameters which influence and accelerate the reaction. Applicability of these SPANC reactions was demonstrated through the synthesis of oligomer materials and through the labelling of bacterial *L. innocua* cells using a sequential protocol of metabolic incorporation of nitron amino acids to cells surfaces, followed by treatment with cyclooctadiyne and finally labelling with a fluorophore azide dipole.

Finally, further development of SPANC chemistry is presented with an analysis of reactions between phenanthridine nitron scaffolds and strained cyclic alkynes. Kinetic evaluation of the reaction showed that these nitrones scaffold show fast reaction kinetics

with second-order rate constants (k_2) in the range of $10^1 \text{ M}^{-1}\text{s}^{-1}$, which while slower than previously examine acyclic nitrones and other small five-membered endocyclic nitrones, represent faster reactions than with analogous azide dipoles. Analysis of the fluorescence properties of the reaction and its fluorogenic potential uncovered a small FRET effect with strained cyclic alkyne fluorophores, which shows the potential for further development of fluorogenic transformations involving nitron dipoles. Initial development of the reactions between nitrones and cyclopropenes and cyclopropenone-caged cyclooctadiyne are also presented, further demonstrating the wide applicability of this versatile class of bioorthogonal dipoles.

Acknowledgements

One does not embark on such a journey without a whole village's support, and this journey has certainly required the help of many. I would firstly like to thank and extend my deepest gratitude to my supervisor, Dr. John Pezacki, for your constant support and mentorship. I truly appreciate the scientific freedom that you have afforded me, in pursuing a variety of projects and collaborations allowing me to explore a breadth of disciplines and techniques that I will carry with me for the rest of my journey. While working in your lab, I was able to scratch that itch for exploring the fascinating interface between chemistry and biology that has kept me motivated throughout the years. These opportunities that I have been given to present my work, participate in worldclass conferences, mentor students in their own scientific careers will have a lasting impact on me. Once again, thank you for the support, mentorship, engaging discussions and the laughs. I will always be willing to drop off a dish and join in on the Pezacki Lab Classic BBQ!

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I owe the biggest debt of gratitude to my wife Karina, whose unconditional love and support has been the biggest contributor to my success. You've held me up when I hit any road bumps and cheered the loudest when I hopped over hurdles. You have always been the best audience to bounce ideas off of and help me see things from a completely different perspective. I sincerely could not have completed this endeavour without you, je t'aime.

Finally to Nora, whom I have yet to meet, but who for the last months has put everything in a new light and helped me focus my efforts. I hope to one day instill that spark of curiosity in you as well and share with you the exciting world of science.

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List of Abbreviations

Au-NP	gold-nanoparticles, 44
BSA	bovine serum albumin, 58
CMC	critical micelle concentration, 56
CTAB	cetyl trimethylammonium bromide, 63
CuAAC	
copper-catalyzed azide-alkyne	
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CuANCR	
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FMO	
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FRET	
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GFP	
green fluorescent protein, 1	
HATU	
hexafluorophosphate azabenzotriazole	
tetramethyl uronium, 69	
HEPES	
4-(2-hydroxyethyl)-1-	
piperazineethanesulfonic acid, 71	
HOMO	
highest occupied molecular orbital, 19	
KDO	
3-deoxy-D-manno-octulosonic acid, 59	
LUMO	
Lowest unoccupied molecular orbital,	
79	
lowest unoccupied molecular orbital,	
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<i>m</i> -CPBA	
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Acetonitrile, 55	
MFN	
multifunctional nanoparticles, 45	
MTO	
methyltrioxorhenium, 35	
NHS	
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NMR	
Nuclear magnetic resonance, 80	
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SPAAC	

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cycloaddition, 18
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nitronc cycloadditions, 57
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TBTA
tris(benzyltriazolymethyl)amine, 57
t-BuOH
tert-butanol, 55
TCO
trans-cyclooctene, 31
TFA
trifluoroacetic acid, 69
TIPS
triisopropyl silane, 69
UHP
urea complexed with hydrogen
peroxide, 35

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Chapter 1 Development of Bioorthogonal Chemistry

1.1. Statement of Introduction Organization

The information presented in the introduction of this thesis and its organization is reflective of review articles in which Didier A. Bilodeau (DAB) has participated in the redaction. The introduction of bioorthogonal chemistry is broadly inspired by the following review article:

Scinto, S. L.; **Bilodeau, D. A.**; Hincapie, R.; Lee, W.; Nguyen, S. S.; Xu, M.; am Ende, C. W.; Finn, M. G.; Lang, K.; Lin, Q.; Pezacki, J. P.; Prescher, J. A.; Robillard, M. S.; Fox, J. M. Bioorthogonal Chemistry. *Nature Reviews Methods Primers* **2021**, *1* (1), 30. <https://doi.org/10.1038/s43586-021-00028-z>.

While the information pertaining to nitrones employed in bioorthogonal transformations is inspired by the following review article:

Bilodeau, D. A.; Margison, K. D.; Serhan, M.; Pezacki, J. P. Bioorthogonal Reactions Utilizing Nitrones as Versatile Dipoles in Cycloaddition Reactions. *Chem. Rev.* **2021**, *121* (12), 6699–6717. <https://doi.org/10.1021/acs.chemrev.0c00832>.

References have been updated with relevant recent developments in the field of bioorthogonal chemistry development where applicable.

1.2. Introduction

The covalent linkage between different biomolecules and the modification of biomolecules with extrinsic functionality in complex cellular environments represent long standing challenges faced by chemical biologists. Broadly speaking, achieving such chemical transformations would allow for a multitude of applications such as cell labelling, biomolecule tracking, drug delivery, and biomaterial synthesis. To achieve these conjugation applications, researchers must employ chemical transformations which are highly chemoselective and which are high yielding, while avoiding any unwanted reactivity with chemical moieties endogenous to the biological systems, such as complex biopolymers (proteins, nucleic acids, glycans, etc.), metabolites, ions, and water. Chemical transformations that demonstrate these specific characteristics can often be classified as bioorthogonal transformations.

Prior to advances in the field of bioorthogonal chemistry, applications requiring the labelling and or tracking of proteins relied on recombinant (also known as fusion proteins) protein expression in order to produce fluorescent proteins and antibodies^{1,2}. These techniques rely on creating fusions between proteins of interest and fluorescent proteins, such as green fluorescent protein (GFP); thus allowing for the visualization of desired proteins and the tracking of complex protein dynamics. Recombinant protein strategies, while having been demonstrated to be widely applicable, do display some significant limitations. Namely, only proteins are amenable to being modified and tracked, other classes of biomolecules such as glycans, lipids and nucleic acids cannot be easily or generally modified, due to recombinant techniques taking advantage of cells' protein synthesis machinery. Furthermore, the fusion of fluorescent proteins to proteins

of interest can greatly disrupt normal protein trafficking and function, due to alterations in overall protein structure. These limitations have therefore spurred the development of bioorthogonal chemistry to enable the study of more complex biomolecules in more complex systems.

Bioorthogonal chemistry was initially developed from the concept of Click chemistry introduced by Sharpless, Kolb and Finn in 2001³. In this seminal review, they identified organic transformations which display excellent atom economy, excellent efficiency, and specificity while forming new carbon-heteroatom and carbon-carbon covalent bonds. Click reactions are defined as chemical reactions which meet several criteria making them “nearly perfect spring loaded” bond forming transformations. These reactions occur under mild aqueous conditions and are orthogonal to many other functional groups which may be present in the reaction milieu, and ideally display high reaction rates owing to high thermodynamic driving forces. Some initially exploited reactions by the Sharpless group consist of nucleophilic opening of strained heterocycles, and various cycloaddition reactions. By taking advantage of chemical moieties not found in biological systems, these click reactions can be adapted to a wide range of applications involving biomolecule functionalization.

These click reactions which are found to be amenable to biological systems can be described as bioorthogonal reactions, a term coined by Bertozzi and coworkers in 2003⁴. The group’s work on employing a modified Staudinger reaction (the reaction between an azide and phosphine) to form amide bonds to achieve cell surface engineering represents the first example of the application of this concept (see Figure 1.1)⁵.

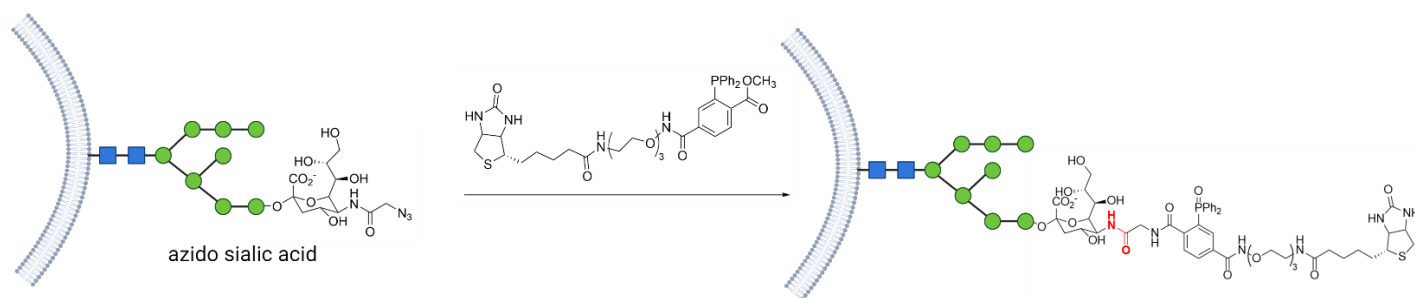


Figure 1.1 Functionalization of cell surface azido sialic acid using a biotinylated phosphine reporter; representing the first example of a bioorthogonal transformation. Adapted from Bertozzi et al.⁵. Created with Biorender.com.

Bioorthogonal reactions are broadly described as any chemical transformation which can occur selectively inside a biological system without interfering with native biochemical reactions. To be termed bioorthogonal, such reactions must: be high yielding (ideally displaying high atom economy); have both orthogonal reacting partners be selective for bond formation between each other (while avoiding side reactivity with native functionality, such as biological nucleophiles and electrophiles); be amenable to physiological aqueous and pH conditions; display fast kinetics to occur under timescales mirroring biological events and to allow for the use of low concentrations of reacting partners; produce stable non-toxic or inert products from non-toxic reacting partners (avoiding the production of by products); and the modification of the biomolecules of interest must minimize any disturbance to its native structure and function⁶. The development of a classical organic chemistry transformation into a bioorthogonal transformation requires the consideration of all these factors, to ensure successful ligation of reacting partners.

This process often begins by identifying chemical transformation in which the reacting partners are abiotic (not canonical to common biological functional groups and chemical motifs)⁷. In early stages of development, factors such as aqueous solvent tolerance

and stability, improvements of reaction rates, proper characterization of products and mechanism determination can be analyzed *in vitro* to ensure that the transition to a complex aqueous biological system is feasible. At this stage of development, the stability of reagents should be examined under conditions which mimic physiological conditions (ex: cell lysate solutions, glutathione solutions, aqueous solutions of relevant biological pH)⁶. The importance of evaluating the reaction rates is paramount to the success of a chemical transformation for bioorthogonal applications. For practical applications, reactions should display second-order reaction rate constants greater than $10^{-4} \text{ M}^{-1}\text{s}^{-1}$ to allow for the ligation to take place under a timescale relevant to biological events, but also to allow for the use of very low concentrations of reacting partners which are necessary if the reaction is to be applied to living systems^{6,7}. Analysis of the reaction mechanism can allow for modifications of reacting partner structures, to improve or tune reaction rates, or in order to allow for new applications. For example, modification to certain reaction mechanisms can be taken advantage of to allow 'click-to-release' strategies⁸. Insights into the reaction mechanism can also allow for the modification of reacting partners structures to alleviate certain limitations to bioorthogonality (improving reagent solubility, limiting cross-reactivity with biological nucleophiles/electrophiles, employing functional group isosteres to improve reactivity, etc.). Once a classical chemical reaction has been modified to be amenable to biological systems, the reaction can be tested in model systems of increasing complexity to demonstrate applicability. Reaction's applicability can be tested starting with simple *in vitro* models involving aqueous or mixed aqueous/organic solvent systems, to solutions containing simple biomolecules or cell lysates, to *in cellulo* models where labelling can occur on cell surfaces or

on target intracellular biomolecules, to finally more complex living organisms (such as zebrafish and mice)⁷.

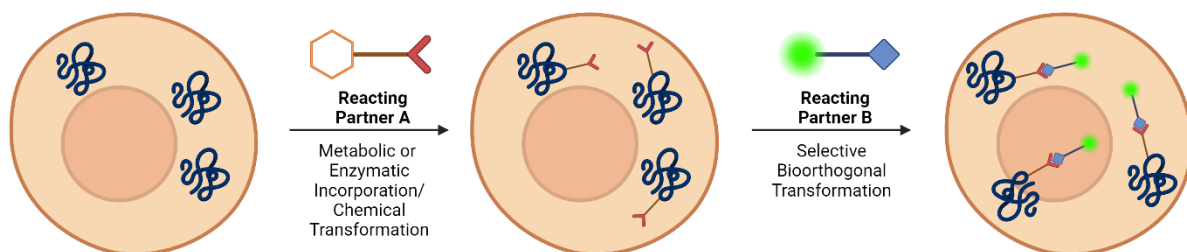


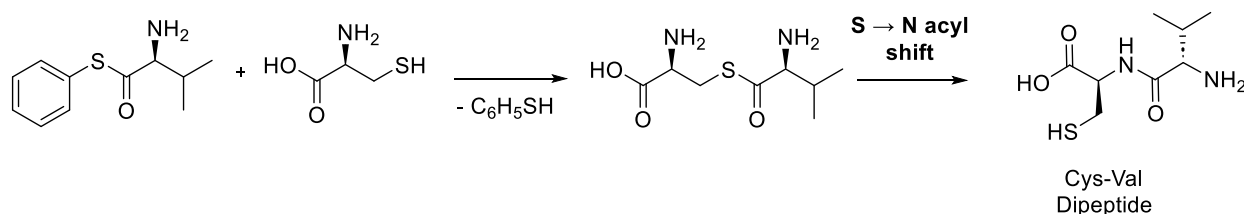
Figure 1.2 General bioorthogonal biomolecule transformation strategy in which a bioorthogonal reagent is incorporated to a biomolecule of interest, and further functionalized by a subsequent bioorthogonal reaction with the second bioorthogonal reacting partner. Created using Biorender.com.

The general strategy (chemical reporter strategy) employed in applying bioorthogonal reactions consists of a two-step approach⁹. Firstly, the first reacting partner is incorporated to the biomolecule of interest, which can be achieved in a number of ways including metabolic or enzymatic engineering by taking advantage of native biochemical processes to incorporate appropriately modified metabolites into the target^{6,9}. Metabolic engineering allows for the modification of a broad range of biomolecules including molecules which are difficult to functionalize by traditional methods, such as lipids, secondary metabolites, and small-molecule cofactors^{10–12}. Other strategies include genetic code expansion (amber codon suppression) to incorporate non-canonical amino acids to site-specifically to engineered protein sequences^{13,14} and biomolecule tagging via reactive substrates (such as metabolites, enzyme inhibitors, ligands, etc.)^{8,15,16}. Once installed, the biomolecule of interest can be reported upon via bioorthogonal ligation with the appropriate reacting partner (reporter molecule) (see Figure 1.2).

The following section will outline the development of some of these organic transformations which have been repurposed for the goals of bioconjugation. These reactions include ligation chemistries such as native chemical ligation, oxime ligation, and the aforementioned Staudinger reaction, as well as cycloaddition chemistry including metal-catalyzed and catalyst-free cycloadditions between alkynes with various dipoles. Other metal-catalyzed reactions such as cross-coupling chemistry and olefin metathesis will also be discussed in the context of their application to bioconjugation. Finally, the development of inverse electron-demand Diels-Alder reactions between strained alkenes and tetrazines, representing a milestone in the development of bioorthogonal chemistry, being the fastest reported applied reaction to date.

1.3. Native Chemical Ligation

The need for the conjugation of chemically synthesized peptides has driven the development of chemoselective reactions forming new covalent linkage between the N-terminus and C-terminus of two peptide fragments. Native chemical ligation, representing the reaction between the C-terminus thioester of one peptide fragment, with a thiol functional group on the N-terminus, consists of one of the most widely applied reactions for this purpose. Wieland and coworkers were the first to report the crucial S→N-acyl shift step in their analysis of the reaction between valine-thioester and cysteine in aqueous buffer (see Scheme 1.1)¹⁷.



Scheme 1.1 Initial demonstration of S→N acyl shift in the ligation between valine thioester and cysteine by Wieland and coworkers¹⁷.

The work of Wieland and coworkers of the reactivity of amino acid thioester reactions led other groups to develop the concepts of chemical ligation and native chemical ligation by taking advantage of the reaction between an ionized thiol residue of one peptide fragment and an appropriate thioester on the second peptide fragment to allow the ligation of unprotected peptide fragments. The development of native chemical ligation was pioneered by the work of Kent, Schnölzer, Dawson, Muir and coworkers^{18–20}. Briefly, after the initial thiol-thioester exchange step between the two peptide fragments, a short-lived thioester intermediate covalently linking the two fragments is formed. This thioester intermediate can then undergo rapid intramolecular S→N acyl shift to form a new native amide bond between the peptides (see Figure 1.3).

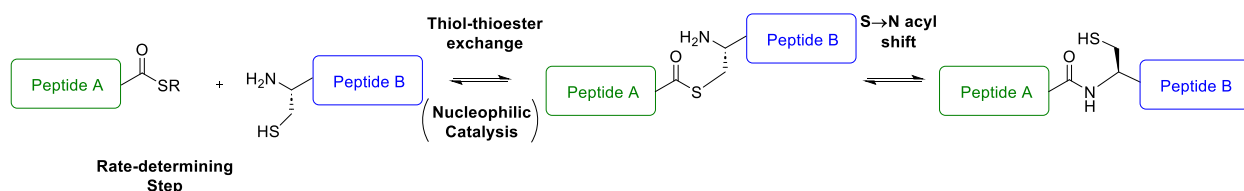


Figure 1.3 Native chemical ligation of peptide fragments for protein synthesis or semi-synthesis.

The rate-determining acyl transfer step can be accelerated by using nucleophilic catalysis. The use of catalysts such as imidazole, or nucleophilic thiols allows for the formation of a more reactive thioester intermediate, facilitating the ligation of peptide

fragments^{20,21}. Variations of native chemical ligation protocols include the use of selenocysteine and selenoesters²², the ligation of expressed proteins to achieve the semi-synthesis of more complex proteins or peptides containing unnatural amino acids²³, the application of thiol-auxiliaries and various methods for the desulfurization of the ligated peptide²⁰.

While native chemical ligation represents a powerful strategy for the synthesis and semi-synthesis of proteins, the transformation is limited to the modification of protein biomolecules, and as such is better suited for *in vitro* applications. Given the need for pH modulation, and potentially the use of small organic catalysts or auxiliaries, native chemical ligation is poorly suited for the rapid conjugation of biomolecules required for *in vivo* and *in cellulo* applications. It can however be classified as a bioorthogonal reaction, as it displays many of the required features such as high atom economy, high chemo- and regioselectivity and displaying fast kinetics under aqueous conditions.

1.4. Oxime and Hydrazone Ligation Chemistry

The condensation reaction between alkoxyamines or hydrazides and ketones/aldehydes to form oximes and hydrazones, respectively, represents another example of a classical chemical transformation that has been adapted to bioorthogonal applications (see Figure 1.4). The oxime condensation reaction has been reported upon as early as 1882, by Meyer and Janny²⁴⁻²⁷. Carbonyl functionalities represent some of the first explored extrinsic moieties for biological ligation applications, due to their relatively low abundance in native biomolecules, inertness towards reaction at neutral pH, and ease of incorporation into biomolecules²⁸⁻³⁰. The first example of oxime ligation applied to a biological ligation consists

of the work of Rideout and coworkers and the *in cellulo* assembly of cytotoxic drug molecules³⁰. These ligations have also been applied to modification of cell surfaces by taking advantage of the metabolic incorporation of keto-carbohydrates into sialic acid residues²⁹. Carbonyl functionality can also be introduced into sialic acids found on cell surfaces via oxidation with periodate³¹ or via the oxidative cleavage of 1,2-diamino alcohols³².

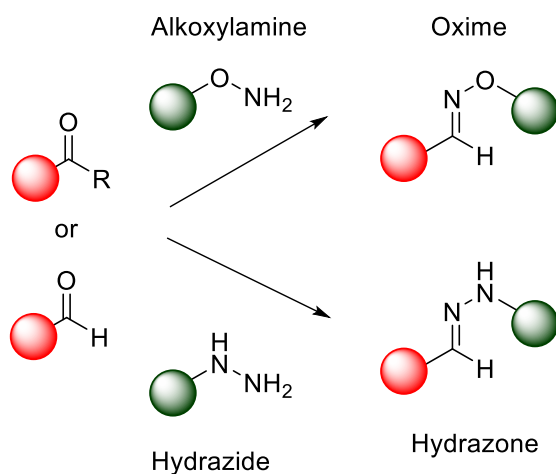
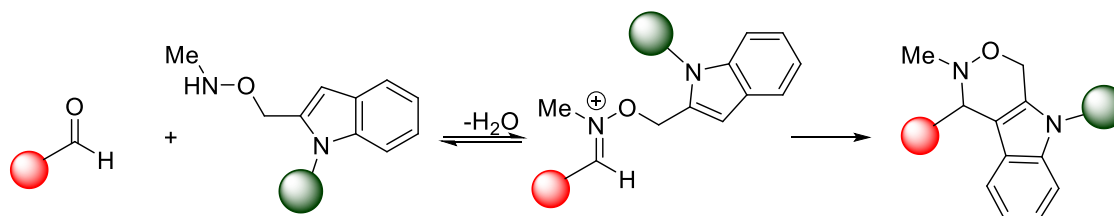


Figure 1.4 Prototypical oxime and hydrazone ligation reactions between carbonyls and alkoxyamines and hydrazides.

Under neutral pH conditions, these reactions display second-order rate constants (k_2) on the order of $0.01 \text{ M}^{-1}\text{s}^{-1}$, which is lower than most commonly applied bioorthogonal reactions^{33,34}. Initial attempts to accelerate these condensations consisted of lowering the pH (pH = 4-6) and the use of aniline as a nucleophilic catalyst, forming a reactive Schiff base³⁵⁻³⁷. Given that the use of low pH conditions is not amenable to many biological contexts, rate accelerations were sought using aniline analogues such as 3,5-diaminobenzoic acid, 2-aminobenzenephosphonic acid and 5-methoxyanthranilic acid. The use of aniline analogues increased the reaction rates up to 40 times, as compared to aniline catalysis^{38,39}. The use of nucleophilic catalysis however limits the potential for intracellular applications which may

require low concentrations of ligation partners. Another variation of this type of condensation consists of the Pictet-Spengler ligation developed by Bertozzi and coworkers (see Scheme 1.2)⁴⁰.



Scheme 1.2 Pictet-Spengler ligation applied to protein chemical modification.

To improve hydrolytic stability, substituted-indolyl nucleophiles are used in condensation reaction with aldehydes. Here the charged oxyiminium intermediate formed can undergo intramolecular C-C bond formation with an indole nucleophile, thus forming a hydrolytically stable oxacarboline product. The group was able to apply this bioconjugation reaction to site-specific modification of glyoxyl-functionalized and formylglycine-functionalized proteins. This variation of the oxime ligation also eliminates the problem of reversibility, by forming a stable C-C bond in the ligated product.

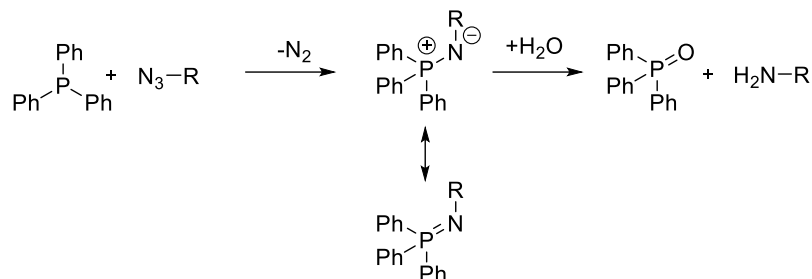
The products of these conventional ligation reactions, oximes and hydrazones, are more stable towards hydrolysis than conventional imine condensation products between carbonyls and amines, due the stabilizing effect offered by the adjacent α -heteroatom to the sp^2 nitrogen, with oximes being more stable than the respective hydrazone counterpart products⁴¹. However, although these products display relative stability towards hydrolysis, these ligation reactions still represent reversible transformation, especially under lower pH conditions which may be present in certain cellular microenvironments. Furthermore, the

chemoselectivity of these transformations is not ideal, given that carbonyls and aldehydes moieties can be found in many intracellular metabolites. While this reversibility can be taken advantage of in certain contexts for the controlled release of desired cargo molecules⁴²⁻⁴⁴, this feature is detrimental to the widespread application of these ligation reactions to most bioorthogonal applications which require permanent covalent linkage, and thus limits their use to mostly *in vitro* applications.

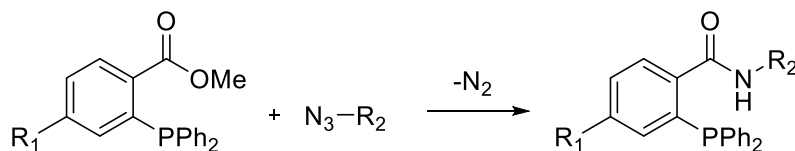
1.5. Staudinger Reaction

The Staudinger reaction, the reaction between organic azides and triaryl phosphines is widely accepted as the first applied truly bioorthogonal reaction^{45,46}. Originally developed for the reduction of aromatic azides, chemical biologists recognized that this historical transformation could be repurposed for bioorthogonal purposes, given that both reaction partners are exogenous to biological systems and the reaction is amenable to aqueous conditions (see Scheme 1.3). Furthermore, the azide is an attractive bioorthogonal reagent, as its small size minimally perturbs the structure and function of the biomolecules it is incorporated into. The Staudinger was first applied to a biological setting for the cell surface engineering by the Bertozzi group in 2000⁵. In order to provide covalent linkage between the two reaction partners, the phosphine reagent was modified to incorporate an ortho methyl ester electrophilic trap to allow for intramolecular amide bond formation with the aza-ylide intermediate formed during the initial nucleophilic attack (see Scheme 1.4). This highly specific ligation chemistry is widely applicable as it has been employed to target most major biomolecules such as for the analysis of proteins^{47,48}, nucleic acids⁴⁹, and lipids^{50,51}. The Staudinger ligation also represents one of the first examples of bioorthogonal

transformations applied to an *in vivo* model for the imaging of glycosylation changes during disease progression in mice⁵².

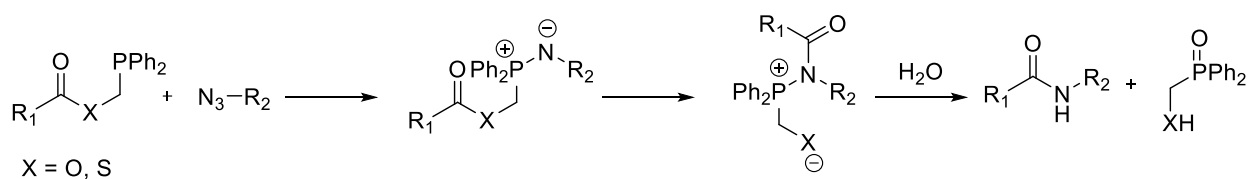


Scheme 1.3 Prototypical Staudinger reaction between an organic azide and triaryl phosphine to yield triaryl phosphine oxide and an amine.



Scheme 1.4 Modified Staudinger reaction with intramolecular methyl ester electrophilic trap for *in vivo* applications

An improvement on initial protocols, the traceless Staudinger ligation was introduced simultaneously by the groups of Bertozzi and Raines (see Scheme 1.5)^{53,54}. The incorporation of a cleavable linker allows for the formation of the native bond, without the phosphine oxide being incorporated into the final product, thus allowing for minimal perturbation of the structure and function of the tagged biomolecule. Other variations of the Staudinger ligation include the use of thioester as an alternative electrophilic trap⁵⁵, the use of alternative phosphine nucleophiles such as phosphonites⁵⁶ and phosphites⁵⁷, and the use of alternative nucleophiles to azides such as cyclopropanones^{58,59} and α,β -unsaturated amides⁶⁰.



Scheme 1.5 Traceless Staudinger ligation for amide bond formation

Although the Staudinger ligation has been shown to be highly specific and applicable to a wide variety of biomolecules and *in vivo* contexts, the reaction suffers from slow kinetics when compared to other bioorthogonal transformations⁶¹. The lower observed second-order reaction rates can require the use of high concentrations of reacting partners for ligation to take place under relevant time scales, often using high concentrations of phosphine reagent. These high concentrations can lead to high background signal observation, especially for fluorescence imaging applications where removal of excess reagent may be difficult (i.e. *in vivo* imaging). Phosphine reagents can also be prone to oxidation, which can limit their incorporation to biomolecules. Nevertheless, the versatility and specificity of the reaction still warrants its use in many bioorthogonal applications.

1.6. Bioorthogonal Alkyne Cycloaddition Chemistry

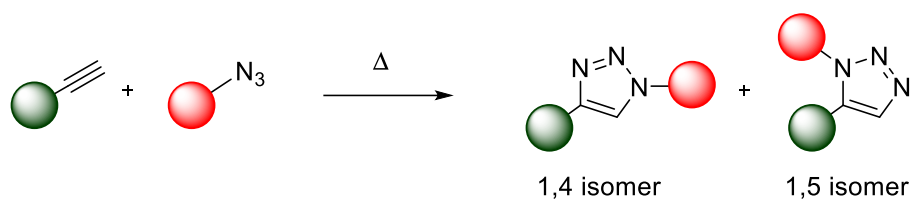
Alkynes represent attractive bioorthogonal reagents as they are rarely found in naturally occurring biomolecules, with some exceptions of terminal alkynes being found in fungi and other rarely occurring secondary metabolites^{62,63}. The small size and relative chemical inertness of alkynes under most biological conditions, also render them good reacting partners in the context of bioorthogonal chemistry. The incorporation of alkynes into biomolecules is also relatively facile through benign coupling reactions or through metabolic incorporation of functionalized metabolites.

In the context of bioorthogonal chemistry, alkynes have found widespread applications for both metal-catalyzed transformations, as well as catalyst free, strain driven cycloaddition chemistry. Amongst these transformations, reactions involving the cycloaddition between azides and alkynes represent some of the most robust and widely applied bioorthogonal transformations; once again driven by both reacting partners being exogenous to most biological systems, being chemically inert towards biological nucleophiles and electrophiles, the rapid kinetics of the reactions and the relative ease of synthesis and incorporation of the reacting partners. The copper-catalyzed azide-alkyne cycloaddition (CuAAC) represents one of the most popular biocompatible transformations, more broadly termed as the prototypical Click reaction, and it has found use in not only the field of chemical biology, but also for polymer and materials chemistry, and for combinatorial chemistry and drug discovery. Metal-free variations of azide alkyne cycloadditions have also been developed to circumvent some of the issues related to the translation of CuAAC to biological systems. These strained-promoted azide-alkyne cycloadditions (SPAAC) take advantage of the driving force of strain-release associated with the cycloaddition of strained cyclic alkynes with various dipoles, most notably azide dipoles.

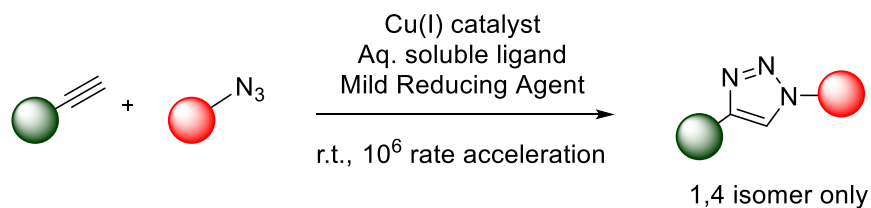
1.6.1. Copper-catalyzed azide-alkyne cycloaddition

The development of CuAAC for bioorthogonal applications and as a Click reaction stems from the prototypical Huisgen 1,3-dipolar cycloaddition between azides and alkynes (see Scheme 1.6)^{64,65}. Uncatalyzed versions of azide-alkyne cycloadditions require high temperatures to occur within reasonable time frames, and therefore occur too slowly to be applicable to biological conditions. A key breakthrough of applying copper(I) catalysis was discovered simultaneously by the groups of Sharpless⁶⁶ and Meldal⁶⁷. The great rate

accelerations obtained by applying metal catalysis and high specificity of the reaction transformed this traditional organic transformation to a widely applicable Click reaction (see Scheme 1.7). As opposed to the uncatalyzed version of the reaction, CuAAC leads to the formation of only the 1,4-disubstituted triazole, a motif that is similar to native *trans*-amide bonds when comparing geometry, and that is extremely stable to cleavage under biological conditions. Other notable improvements to applying CuAAC to a biological context include the use of water-soluble catalyst ligands which stabilize the Cu(I) oxidation state, help accelerate the reaction or allow for the use of lower catalyst loadings and help limit copper-mediated reactive oxygen species formation via copper sequestration^{68,69}. These ligands include copper-binding molecules based on the tris(triazolymethyl)amine scaffold, phenanthroline scaffold and endogenous copper-binding amino acids, such as histidine and proline (see Figure 1.5)⁷⁰⁻⁷³.



Scheme 1.6 Huisgen 1,3-dipolar cycloaddition for the synthesis of 1,2,3-triazoles.



Scheme 1.7 Prototypical Copper(I) catalyzed azide-alkyne cycloaddition (CuAAC)

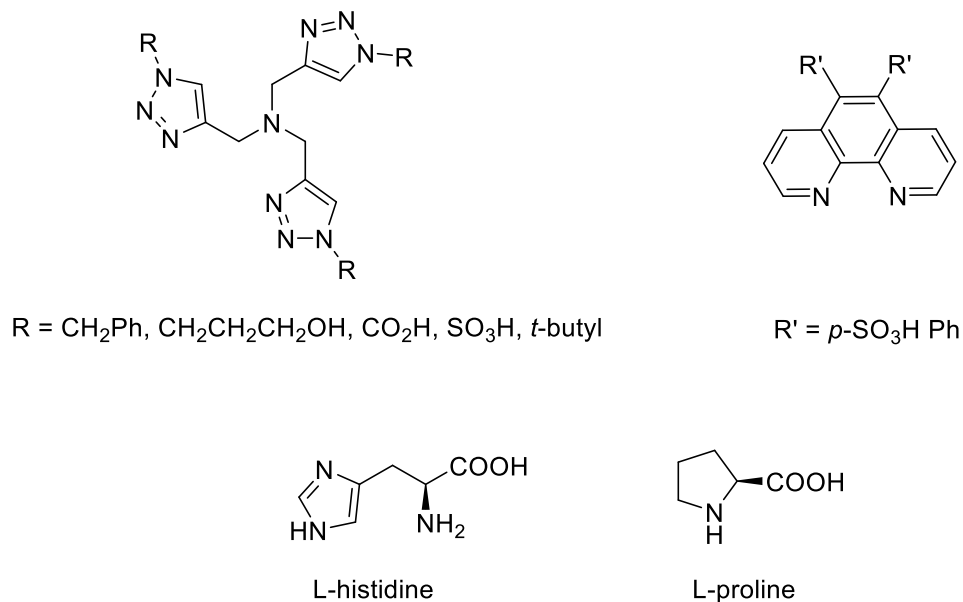


Figure 1.5 Prototypical copper-binding ligands employed in copper-catalyzed azide-alkyne cycloadditions.

The mechanism of CuAAC has been proposed to proceed through a dinuclear copper species, in which a copper acetylide is first formed between the terminal alkyne and the first copper-(I) atom, this is followed by further coordination of the acetylide to a secondary copper-(I) atom which then allows for the coordination of the azide. After ligand displacement takes place, cyclization can take place, followed by final protonation to yield the 1,4-triazole isomer product (see Figure 1.6)⁷⁴.

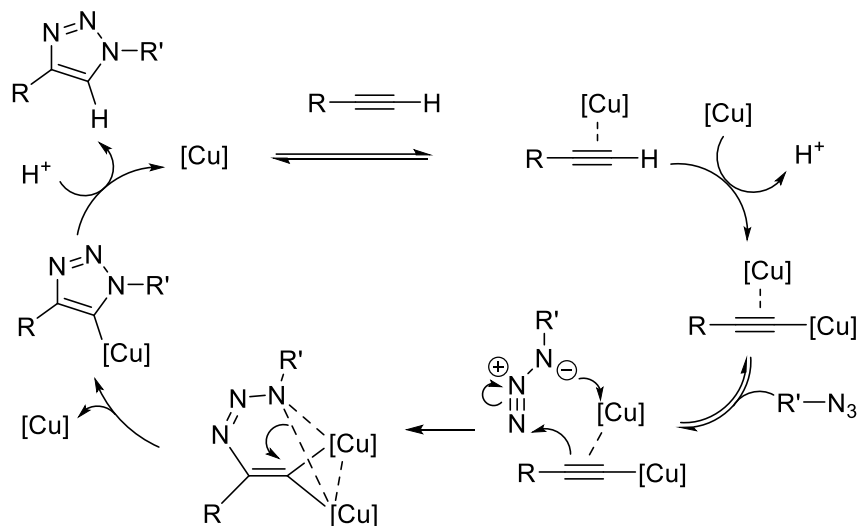
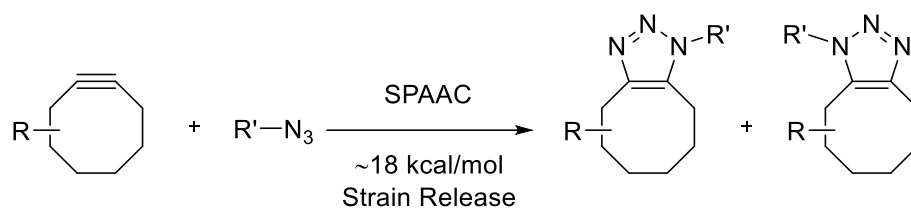


Figure 1.6 Proposed CuAAC catalytic cycle. Adapted from Fokin *et al.* ⁷⁴.

CuAAC has found widespread use in applications such as labelling cell surfaces⁷⁵, for activity-based protein profiling⁷⁶, labelling biomolecules inside cellular complexes and for *in situ* drug synthesis⁷⁷, examples amongst a plethora of other Click chemistry applications^{78,79}. The use of copper catalysis does however present a few limitations for CuAAC bioconjugation. Firstly, the chief limitation is copper toxicity, which can somewhat be addressed with the use of proper ligands. However, given that copper is highly regulated and is endogenously bound to chaperone and other interacting proteins, and as such even with the use of highly binding ligands, metal ion exchange can occur and eventually lead to copper-mediated reactive oxygen species generation⁸⁰. Thiols capable of binding copper are also abundantly present in intracellular environments; these include cysteines and glutathione. Copper binding by thiols can render the catalysts inactive and lead to copper-mediated oxidative toxicity⁷². While these considerations can be avoided in certain contexts, the application of CuAAC to *in vivo* settings remains difficult, and has spurred further research into more appropriate biocompatible copper ligands and metal-free versions of azide-alkyne cycloaddition chemistry.

1.6.2. Strain-Promoted [3+2] Azide-Alkyne Cycloadditions

An alternative to metal-catalysis, alkyne cycloadditions can be accelerated via strain release of cyclic alkynes. The spontaneous reaction between cyclooctyne and phenyl azide was first reported by Krep and Wittig⁸¹. The driving force of the reaction consisting of bond angle deformation that occurs in the transition state from the strained 163° angle of the cyclooctyne reagent. The release of this strain represents approximately 13-18 kcal/mol^{82,83}. The metal-free cycloaddition between alkynes and azides simplifies the translation to bioorthogonal applications, as it avoids the need for optimization of metal catalysts and associated ligands. The strain-promoted [3+2] azide-alkyne cycloaddition (SPAAC) was first applied to a bioorthogonal context by Bertozzi and coworkers (see Scheme 1.8)⁸⁴.



Scheme 1.8 Strain-promoted [3+2] azide-alkyne cycloaddition for bioorthogonal applications.

In this seminal work, the cyclooctyne was functionalized with biotin or fluorophore in order to isolate glycans functionalized with azides which were found on proteins, complex cell lysates and cell surfaces^{84,85}. These initial biological experiments clearly demonstrated that the reaction still proceeded at fast enough rates for efficient labelling, while completely avoiding any toxicity issues associated with copper catalysis. However, two main limitations were quickly evident from initial studies: the poor aqueous solubility of bulkier cycloalkynes and slower reaction rates as compared to metal-catalyzed variants. Various strategies have been employed to improve the characteristics of the cycloalkynes used, as they are more

amenable to chemical modification than their azide reacting partners (see Figure 1.7). Some initial modification of the cyclooctyne (OCT) scaffold include the incorporation of electron-withdrawing groups adjacent to the alkyne in order to lower the lowest unoccupied molecular orbital (LUMO) energy of the alkyne, as the cycloaddition involves the LUMO of the alkyne interacting with the highest occupied molecular orbital (HOMO) of the azide. First-generation cycloaddition with OCT displaying a second-order rate constant of $0.0024 \text{ M}^{-1}\text{s}^{-1}$ for the reaction with benzyl azide. The incorporation of fluorine adjacent to the alkyne led to significant rate constant increases, as seen with MOFO⁸⁵ and DIFO^{86,87}, displaying second-order rate constants of $0.0043 \text{ M}^{-1}\text{s}^{-1}$ and $0.076 \text{ M}^{-1}\text{s}^{-1}$ respectively. Another strategy to increase reaction rates is to destabilize the starting alkyne by increasing ring strain. This can be achieved by the benzannulation of the cyclooctyne core leading to strained alkynes such as DIBO⁸⁸, DBCO^{89,90} and BARAC⁹¹. Again, these modifications led to significant rate constant increases, with BARAC displaying a second-order reaction rate of $0.96 \text{ M}^{-1}\text{s}^{-1}$ with benzyl azide, while the incorporation of the endocyclic amide also allowed for greater aqueous solubility. Increase of ring strain has also been achieved by the formation of bicyclic nonyne structures through cyclopropanation of 1,5-cyclooctadiene, such as with BCN reported by the van Delft group, achieving a second-order rate constant of $0.140 \text{ M}^{-1}\text{s}^{-1}$ with benzyl azide⁹². Smaller, and thus more highly strained, ring sizes have also been reported with the use of thiacycloheptyne (TMTH)⁹³. The increased ring strain and relative smaller steric hindrance allows TMTH to react with benzyl azide with a rate constant of $4.0 \text{ M}^{-1}\text{s}^{-1}$.

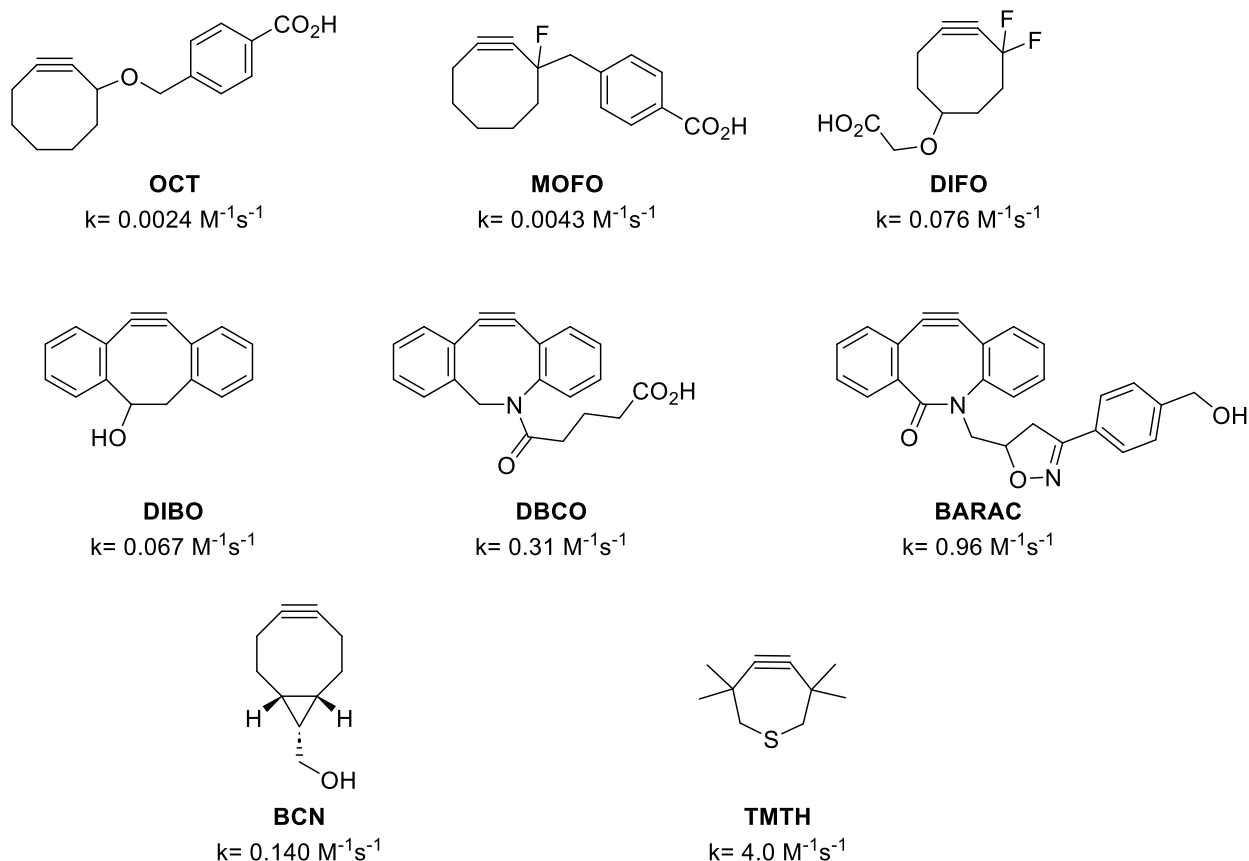


Figure 1.7 Strained cyclooctynes commonly used in strain-promoted cycloaddition reactions with dipoles. Second-order rate constants shown for the reaction between the cycloalkyne and benzyl azide.

An important computational model that has been used to explain reactivity trends in cycloaddition chemistry is the Distortion-Interaction (D/I) or Activation Strain model proposed by the groups of Houk and Bickelhaupt^{94–96}. Briefly this model states that for SPAAC reactions, the differences between the energies required to distort the azide and alkyne to their respective transition state geometry controls the activation energies for the transformation. The activation energy is defined as the sum of the destabilizing distortion energies ($E_d^\ddagger$) and the stabilizing interaction energies ($E_i^\ddagger$)(see Figure 1.8)^{96,97}. While the greatest contributor to the distortion energies in this reaction is the distortion of the azide, more focus has been placed upon analyzing and modifying the alkyne component due to

more facile synthetic manipulations. Using this model, the calculated activation energy for the reaction between acetylene and phenyl azide is 16.2 kcal/mol, whereas the reaction with cyclooctyne is 8.0 kcal/mol, resulting in a 10^6 -fold rate increase. The calculated distortion for cyclooctyne is approximately 3.0 kcal/mol lower than for acetylene, partly resulting in the lower observed activation energy. The D/I model has proven invaluable in predicting reactivity for bioorthogonal reacting partners reacting through cycloaddition chemistry, as well as identifying mutually orthogonal bioorthogonal reaction pairs for multiplexing applications⁹⁸.

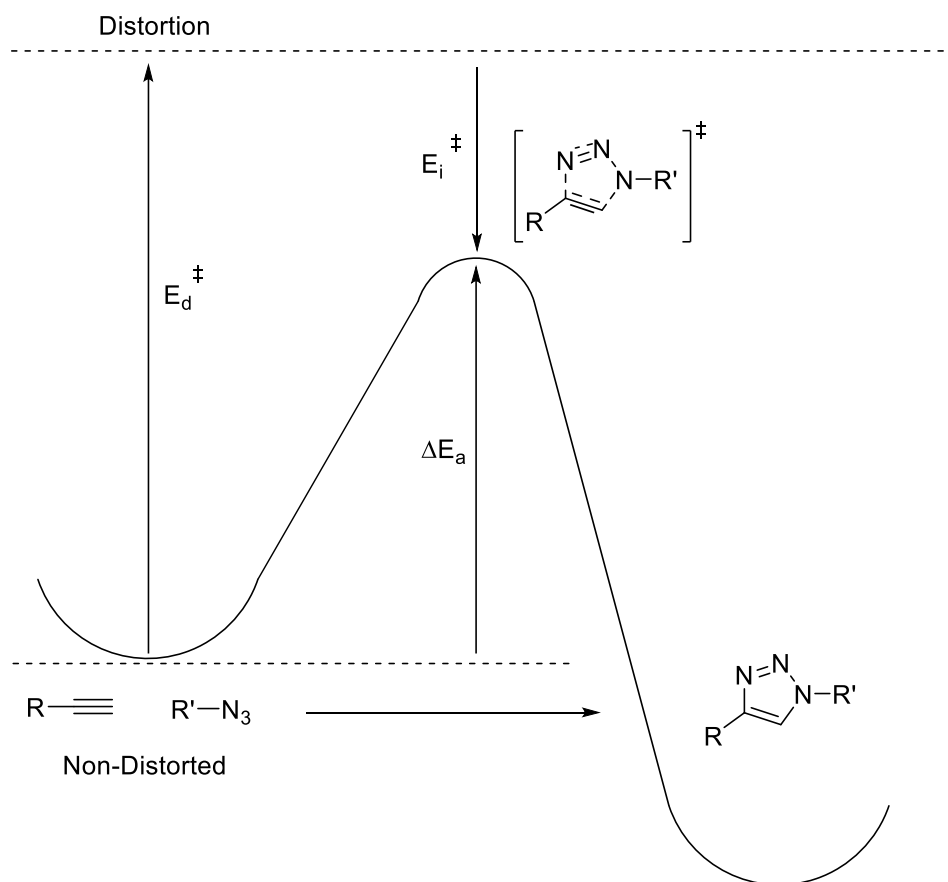
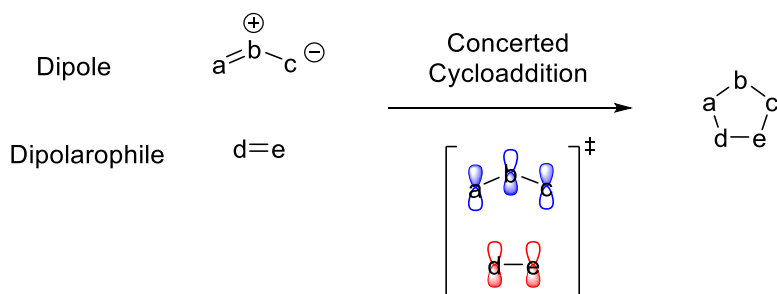


Figure 1.8 Reaction coordinate energy diagram for a model azide-alkyne cycloaddition reaction highlighting the distortion and interaction energies. Adapted from Houk *et al.*⁹⁶

The lack of metal catalyst has allowed SPAAC to be performed in more complex biological systems and for *in vivo* applications^{99,100}. For example, SPAAC was applied to the analysis of glycan formation in zebrafish by the Bertozzi group^{101,102}. By feeding zebrafish with azide-carbohydrate monomers, effective conjugation and visualization after various development timepoints was achieved using DIFO-fluorophores. Similar imaging of glycans was also achieved in the nematodes, *Caenorhabditis elegans*¹⁰³. SPAAC was also shown to be applicable to mammalian animal models by imaging the metabolic incorporation of per-acetylated N-azidoacetylmannosamine into cell surface sialic acids of mouse model animals¹⁰⁴. The incorporation of the azido-sugar was successfully tracked by using various strained functionalized alkynes (incorporating fluorophores or affinity tags such as FLAG-tags). Although these applications do demonstrate that SPAAC can be an invaluable bioorthogonal tool, some of its limitations were also highlighted. Namely, the somewhat low aqueous solubility of the strained alkynes can limit their efficiency, such as with the mouse model study in which it was shown that the DIFO alkynes used showed high affinity for murine serum albumin¹⁰⁴. The slower rate constants offered by strain-promoted cycloadditions also necessitates somewhat longer labelling timeframes which can limit the potential targetable biomolecules and biological processes for *in vivo* applications. Finally, the lower stability of strained alkynes, as opposed to terminal alkynes increases the potential for unwanted side products to form, such as the nucleophilic addition (or radical addition) of endogenous thiols¹⁰⁵. These limitations warrant the development of reactions with alternate dipoles and new strained alkyne scaffolds.

1.6.3. Alkyne 1,3-dipolar cycloaddition with alternative dipoles

To improve upon the reaction kinetics of the SPAAC reaction, the azide reacting partner can be replaced by various other 1,3-dipoles. These alternate dipoles can all potentially react with alkynes via 1,3-dipolar cycloaddition mechanisms, described thoroughly by Huisgen (see Scheme 1.9)⁶⁴. The mechanism of cycloaddition involves 4 π electrons from a 1,3-dipole (ex: azides, nitrones, ozone) and 2 π from a dipolarophile (ex: alkenes and alkynes). The reaction is also classified as a [3+2]-cycloaddition referring to the involved p-orbitals, where the 3 p-orbitals of a dipole can combine with 2 p-orbitals from the dipolarophile in a suprafacial manner to yield a cyclic product in a concerted fashion. These cycloadditions can also be explained in the context of frontier molecular orbital (FMO) theory. In most cases of cycloadditions exploited for bioorthogonal purposes, the LUMO of the dipolarophile interacts with the HOMO of the dipole to undergo concerted bond formation, these cycloadditions are classified by Sustmann as Type-I normal electron demand processes (see Figure 1.9)¹⁰⁶. The interaction can be reversed if the dipole contains strong electron-withdrawing groups such that its HOMO energy is decreased lower than the dipolarophile HOMO, in which case the dominating interaction becomes the dipole's LUMO with the HOMO of the dipolarophile; such transformations are classified as Type-III inverse electron demand processes¹⁰⁶. Type-II processes occur when the HOMO and LUMO energies of the dipole and dipolarophile are of degenerate or very similar energy levels. The reactivity can therefore be modulated by affecting the energy level of these frontier orbitals, either by lowering the energy of the dipolarophile's LUMO or by increasing the energy of the dipole's HOMO (decreasing the HOMO-LUMO energy gap)¹⁰⁷.



Scheme 1.9 Concerted 1,3-dipolar cycloadditions between dipoles and dipolarophiles proposed by Huisgen.

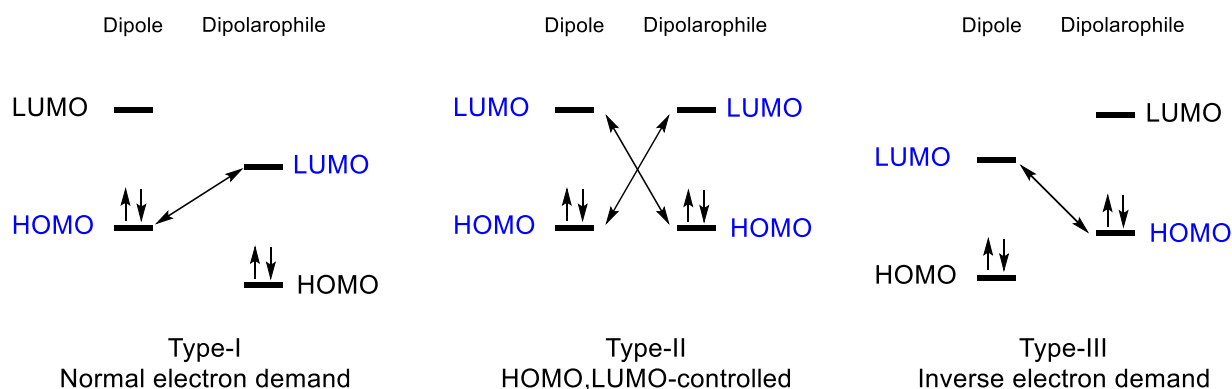


Figure 1.9 Sustmann's Classification for FMO interactions in 1,3-dipolar cycloadditions. Adapted from Domingo *et al.*¹⁰⁷

Some of the successfully applied alternate 1,3-dipoles include nitrile oxides, diazo dipoles, mesoionic compounds such as sydnone, and nitrones (see Figure 1.10). These compounds can offer advantages over the use of azides which allow for different applications, greater tuneability of reactivity, and faster reaction kinetics.

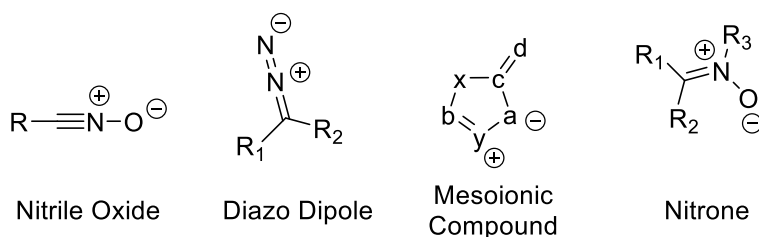
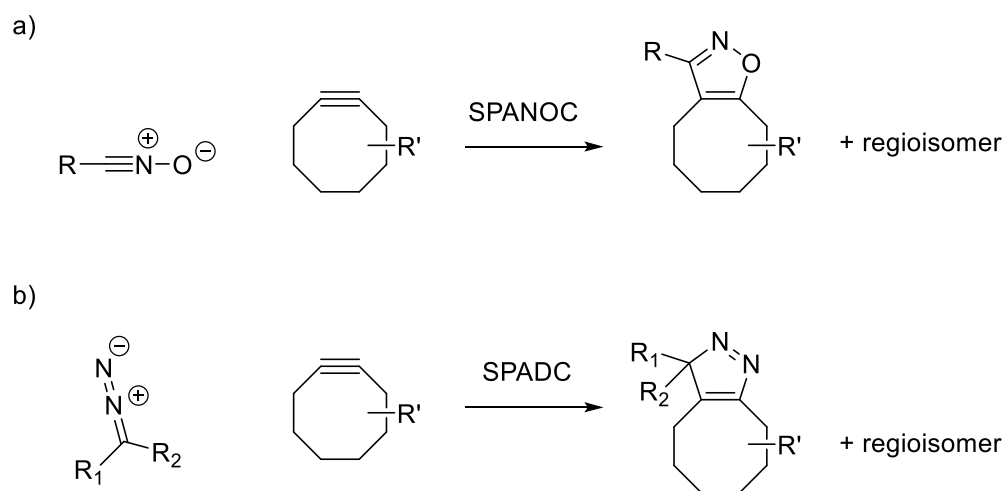


Figure 1.10 Alternative dipoles employed in 1,3-dipolar cycloadditions.

1.6.3.1. Nitrile oxide and Diazo dipoles cycloadditions

Nitrile oxides are highly reactive dipoles that can participate in cycloaddition with a number of potential dipolarophiles. Their great reactivity however does lead to poor stability under physiological conditions which necessitates their *in situ* generation. This can be achieved by oxidizing oximes with mild oxidants such as phenyl iodide bis(trifluoroacetate)¹⁰⁸ or through the deprotonation of arylchlorooximes with mild base¹⁰⁹. Once formed nitrile oxides have been shown to react with dipolarophiles such as norbornene¹⁰⁹ and strained alkynes (strain promoted alkyne-nitrile oxide cycloaddition, SPANOC)^{108,110} for the tagging and analysis of nucleotides, peptides and carbohydrates. SPANOC was shown to have second-order rate constants approximately 10-fold greater than for similar SPAAC transformations¹¹⁰. While these reactions are faster than comparable SPAAC reactions, and the formed isoxazole products are stable, the requirement of *in situ* generation and potential for cross reactivity can limit their application in certain contexts.



Scheme 1.10 Strain-promoted cycloadditions of a) nitrile oxide (SPANOC) and b) diazo dipoles (SPADC).

Diazo dipoles can also be considered as competent alternate dipoles for bioorthogonal cycloaddition reactions¹¹¹. While more highly reactive diazo dipoles could be generated *in situ*, more stable diazoalkynes, and thus more amenable to manipulation, can be accessed through the treatment of azides with activated phosphinoesters¹¹². The rate of reaction between diazo dipoles and strained alkynes can be modulated by careful choice of diazo structure, allowing for reactions occurring faster or more slowly than the analogous azide dipole; offering a range of reaction rate constants differing up to 10³-fold in magnitude. Raines and McGrath were also able to demonstrate that the strain promoted alkyne diazo cycloaddition (SPADC) was accelerated by the use of polar protic solvent, warranting further investigation of this reaction for bioorthogonal applications.

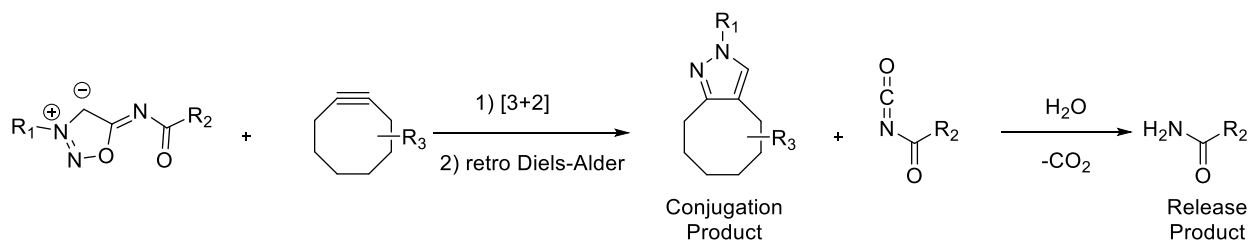
1.6.3.2. Mesoionic dipoles cycloadditions

Mesoionic dipoles consist of a diverse family of cyclic dipoles that can participate in metal-catalyzed or metal-free cycloaddition chemistry; these include sydnones, aza-iminosydnones and iminosydnones (see Figure 1.11)¹¹³. While their cycloadditions with various dipolarophiles have long been studied, for example being studied by Huisgen in his seminal 1,3-dipolar cycloaddition studies¹¹⁴, such mesoionic dipoles were not applied in a bioorthogonal context until 2013 by Taran and coworkers¹¹⁵. They have subsequently been shown to be to react efficiently and quickly with a number of dipolarophiles, including strained alkynes^{116–118}.



Figure 1.11 Selected examples of mesoionic dipoles.

For example, they were applied to the ^{18}F -labelling of antibody conjugates for *in vivo* PET imaging of mice, displaying second-order rate constants of the magnitude of 10^2 - $10^3 \text{ M}^{-1}\text{s}^{-1}$ ¹¹⁷. Another attractive feature of mesoionic dipole chemistry is that certain structures allow for the formation of bicyclic products which can undergo further reaction such as retro Diels-Alder reactions, allowing for click-and-release strategies in which payloads can be released upon initial bioconjugation (see Scheme 1.11)¹¹⁶. Mesoionic dipoles also offer the potential for fluorogenic probes which emit a detectable fluorescent signal upon cycloaddition^{119,120}.



Scheme 1.11 Click-and-release reaction between iminosydnone and strained cyclooctynes. Adapted from Taran and coworkers¹¹⁶.

Over recent years, these mesoionic compounds have emerged as versatile bioorthogonal reagents which display impressive reaction rate constants allowing for *in vivo* applications requiring short time frames and/or low concentrations. As with the development of many bioorthogonal reagent class, further development focussing on

biocompatibility, stability and ease of synthesis should continue to improve the applicability of these ligation reactions.

1.7. Other transition-metal catalyzed ligation reactions

In addition to the widespread use of copper as a transition metal catalyst for bioorthogonal ligation chemistry, other metal catalysts, such as palladium and ruthenium, have been applied in the context of bioconjugation (see Figure 1.12, Figure 1.13)¹²¹. Over recent years, palladium cross-coupling reactions have become some of the most widely applied transformations and has come to display an excellent functional group and aqueous condition tolerance¹²². The fast reaction kinetics, the excellent chemoselectivity of these metal-catalyzed transformations, as well as the use of abiotic transition metals make these cross-coupling and olefin metathesis reactions attractive for bioorthogonality development. The Suzuki-Miyaura cross-coupling was first applied to bioconjugation in 2009 for the modification of peptides bearing a *p*-iodobenzyl cysteine residue with aryl and vinyl-boronic acid derivatives¹²³. A water-soluble Pd-catalyst consisting of the sodium salt of 2-amino-4,6-dihydroxypyrimidine allowed the reaction to take place under mild conditions at 37°C. Another example of homogeneous Pd-catalysis is the Sonogashira cross-coupling reaction between aryl iodides and proteins to which homopropargylglycine has been incorporated; the transformation was also applied to proteins found inside *E. coli* cells¹²⁴. Examples of heterogeneous Pd-catalysis have also been applied for bioorthogonal applications, such as the use of polystyrene microspheres to deliver Pd⁰ into the cytosol of HeLa cells to allow for the *in situ* Suzuki-Miyaura synthesis of fluorophore molecules¹²⁵. It was postulated that the encapsulation of the metal catalyst allows for the minimization of toxicity, while allowing for extended time frame of intracellular catalytic activity. Transition metal catalysis has also

been applied to uncaging applications, to release specific payloads through metal-catalyzed bond cleavage; through Pd-catalyzed deallylation, deallenylation or depropargylation reactions for example¹²⁶.

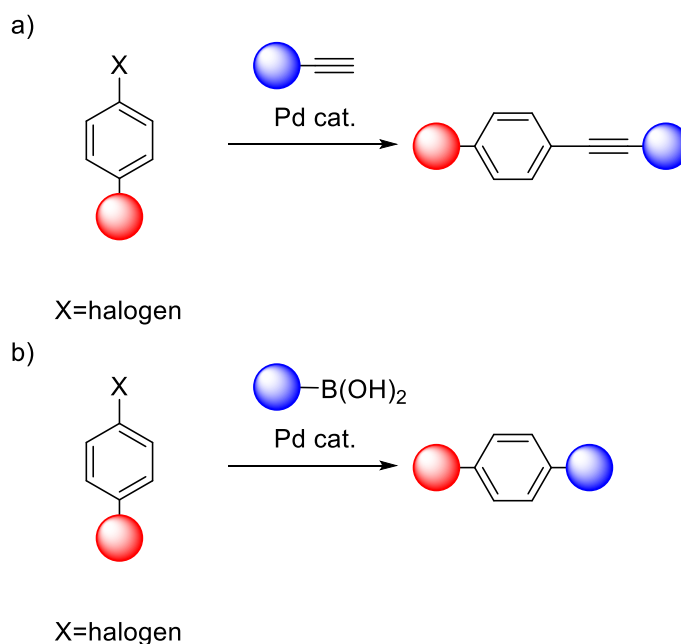


Figure 1.12 Examples of palladium catalyzed cross-coupling reactions applied for bioconjugation; a) Sonogashira cross-coupling, b) Suzuki-Miyaura cross-coupling.

Ruthenium catalyzed olefin metathesis also represents an effective strategy for in vitro bioconjugation applications (see Figure 1.13). Again, the excellent chemoselectivity of the transformation, allows for very specific and efficient bioconjugation to take place. Davis and coworkers were able to apply Hoveyda-Grubbs second generation catalyst to perform bioconjugation of proteins containing alkene containing amino acids¹²⁷. Their initial exploratory results led them to identify S-allylcysteine as a reactive olefin yielding cross metathesis products, while the carbon analogue, homoallylglycine, did not yield any product, most likely due to the instability of ruthenium alkylidenes under physiological conditions. Their investigations also led them to identify Se-allylselenocysteine as a superior reaction

partner, with observed second-order rate constants of $0.3 \text{ M}^{-1}\text{s}^{-1}$ for its reaction with allyl alcohol¹²⁸. Using olefin metathesis, the group was able to modify subtilisin protein from *Bacillus lentus* with various glycosylation and PEGylation functionalities. The reaction did require MgCl_2 and *tert*-butanol as additives to allow for efficient bioconjugation.

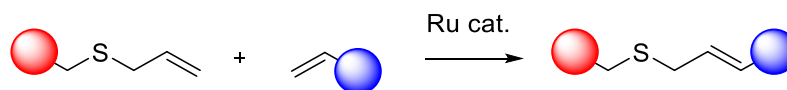


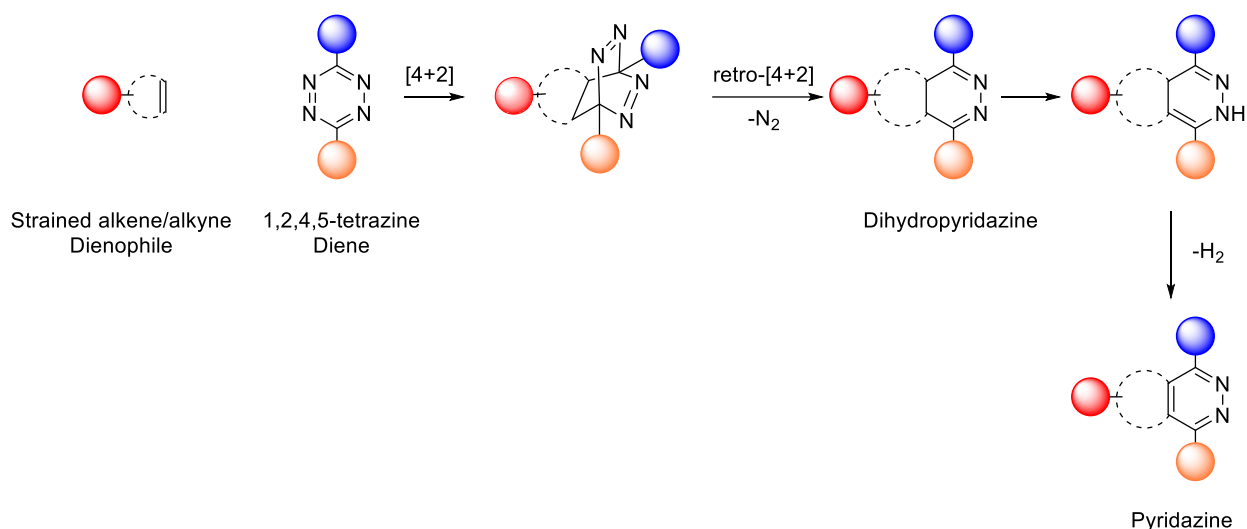
Figure 1.13 Ruthenium catalyzed olefin metathesis applied to bioconjugation of peptides derived with allyl sulfide substrates.

While these transition metal catalyzed transformations have found use in efficient and specific modification of peptides, cell surface labelling and even DNA modification¹²⁹, they suffer from similar limitations to the copper-catalyzed reactions. Namely, their application is mostly limited to *in vitro* and *in cellulo* applications due to mechanisms involving trimolecular species, issues with cell permeability of catalyst species, the potential for sequestration of metal catalysts by non-specific binding to endogenous proteins¹³⁰, and the potential for toxicity to arise in *in vivo* models. Further study to discover new biocompatible catalysts, determine the safety of the reaction and products towards *in vivo* models, and improve reaction rates, is warranted.

1.8. Inverse electron demand Diels-Alder reactions involving tetrazines

The fastest bioorthogonal reactions discovered to date consist of the inverse electron demand Diels-Alder reactions between 1,2,4,5-tetrazines (diene) and dienophiles such as strained alkenes and alkynes. The general mechanism involves a [4+2] cycloaddition, followed by a retro-[4+2] cycloaddition to expel N_2 and afford dihydropyridazine or pyridazine products (see Scheme 1.12). Diels-Alder reactions represent privileged reactions

for the development of bioorthogonal ligation reactions, due to mechanisms involving only two reacting partners, the diene and the dienophile, to apparent rate accelerations observed under aqueous conditions¹³¹, to not necessitating catalysis, and to the formation of stable products. Based on earlier work showing that tetrazines reacted quickly with strained dienophiles¹³², Fox and coworkers first reported the reaction between tetrazines and *trans*-cyclooctene (TCO), with the reaction displaying second-order rate constants up to 2000 M⁻¹s⁻¹, the first bioorthogonal reaction to have k_2 values over 10³ 133. Simultaneously, the Weissleder group also reported the reaction between tetrazines and norbornene, although displaying much slower kinetics than the analogous TCO reaction ($k_2 \approx 2 \text{ M}^{-1}\text{s}^{-1}$)¹³⁴.



Scheme 1.12 Inverse electron-demand Diels-Alder reaction between 1,2,4,5-tetrazine and strained dienophiles.

Since initial reports of this bioorthogonal ligation, many efforts have been undertaken to improve the orthogonality and speed of the reaction, namely focussing on developing alternative dienophiles, improving the stability of the tetrazine reagents, and developing metabolic incorporation methods. Alternative dipoles such as strained alkynes have also been shown to react quite rapidly with tetrazines ($k_2 = 10^2\text{-}10^3 \text{ M}^{-1}\text{s}^{-1}$)^{135,136}. When reagent

size and stability comparative to TCO are considerations, smaller dienophiles such as cyclopropenes¹³⁷, cyclobutenes¹³⁸, isonitriles^{139,140}, and acylazetines¹⁴¹ have also displayed adequate reactivity with tetrazines. Cyclopropenes in particular offer the advantage of being one of the smallest strained dienophiles and allowing for applications necessitating genetic encoding for site-specific incorporation of reporter tags into proteins while still displaying fast kinetics ($k_2=1\cdot 10^4\text{ M}^{-1}\text{s}^{-1}$)¹⁴². Efforts to modulate the reactivity of the tetrazine reacting partners have led to the synthesis of more reactive tetrazines boasting electron-withdrawing groups to improve reaction kinetics (up to 10^6)^{143,144}. If tetrazine stability and size are issues for given applications, alternative 1,2,4-triazine dienes have also been applied to iEDDA reactions¹⁴⁵.

Given the great versatility and fast kinetics that the tetrazine ligation displays, it has found widespread application in the field of chemical biology and beyond. The genetic incorporation of both the tetrazine¹⁴⁶ and TCO¹³⁵ functionalized amino acids has allowed for the detection of low abundance proteins. The high specificity and reaction rates have also allowed for the application of the tetrazine ligation to *in vivo* radiochemistry applications, where the short half lives of the radioisotopes require fast reactivity^{147,148}. Tetrazine-TCO ligations have also been applied to the activation of prodrugs through click to release strategies^{149,150}. Fluorogenic tetrazine-fluorophore conjugates have also been widely applied to live-cell and *in vivo* imaging applications, where the resonance energy transfer from the tetrazine to the conjugated fluorophore diminishes fluorescence. After ligation takes place, the fluorescence intensity is greatly increased resulting in increased signal-to-noise ratios and for the spatiotemporal control of fluorescence¹⁵¹⁻¹⁵⁴. The main limitations of the tetrazine ligation have consisted of the rather bulky size of both reagents (tetrazine and TCO),

and the high reactivity of the reagents which can lead to stability issues (i.e. isomerization of trans-alkenes).

1.9. Nitrones as bioorthogonal reagents

As previously outlined, the Huisgen 1,3-dipolar cycloaddition reaction prototype has proven to be a highly applicable reaction mechanism to bioorthogonal applications. While the cycloadditions of azides with various dipolarophiles have been widely explored, alternative dipoles such as sydnones, nitrile oxides, diazo dipoles and nitrones offer the possibility of adjusting the reactivity through a greater control of stereoelectronics. Nitrones, in particular, offer a wide variety of structural diversity owing to having three sites for functionalization, and also offering the possibility for cyclic structures. Nitrones are allyl-type dipoles in which 4π electrons are distributed in 3 p orbitals which are aligned perpendicularly to the plane of the dipole (see Figure 1.14)¹⁵⁵. They have found applications in the synthesis of numerous pharmacologically and biologically relevant nitrogen-containing molecules such as β -lactams, isoxazolines and isoxazolidines^{156,157}. Nitrones represent privileged dipoles due to their ease of synthesis and purification, general stability, and great structural variety and, to date, they have been applied to a number of bioorthogonal reactions including copper-catalyzed alkyne-nitrone cycloaddition involving rearrangement (CuANCR), strain-promoted alkyne-nitrone cycloaddition (SPANC), and strain promoted nitrone-TCO cycloadditions.

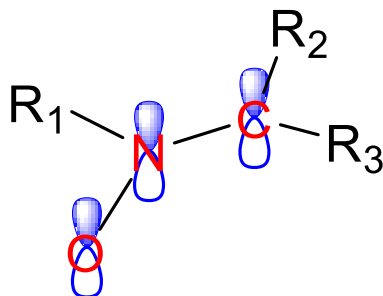
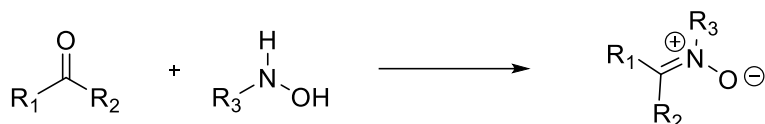


Figure 1.14 General structure of nitrone 1,3 dipoles.

1.9.1. Overview of nitrone synthesis.

Nitrones can be accessed through a wide variety of chemical transformations¹⁵⁶. Perhaps the most widely applicable synthesis of nitrones consists of condensation reactions between *N*-monosubstituted hydroxylamines and carbonyl compounds (aldehydes and ketones) (see Scheme 1.13). Condensation between aldehydes and *N*-alkyl, *N*-alkenyl, *N*-aryl substituted hydroxylamines generates the corresponding nitrone¹⁵⁸; this can be achieved in the presence of a dehydrating agent, such as magnesium sulfate (MgSO_4), in order to perform the reaction under mild room temperature conditions to yield *Z*-isomers. Synthesis and isolation of simple nitrones structures can be achieved by treatment with weak base and filtration through short silica pads, or through recrystallization¹⁵⁹. Without dehydrating reagents, higher temperatures¹⁶⁰, ball-milling¹⁶¹ or microwave irradiation¹⁶² can yield the desired products as well. Given the higher steric requirements of ketone condensation, such reactions require higher temperatures to be productive¹⁶³. Ketonitrones are also accessible via the Cope-type hydroamination of monosubstituted allenes with hydroxylamines¹⁶⁴.



Scheme 1.13 Nitronium synthesis by condensation between hydroxylamine and carbonyls.

Oxidation also serves as a direct and facile route to various nitronium structures; through oxidation of *N,N*-disubstituted hydroxylamines, secondary amines, imines and *N*-alkyl- α -amino acids (see Figure 1.15). The oxidation of *N,N*-disubstituted hydroxylamines is achievable with a variety of stoichiometric oxidizing reagents such as mercuric oxide (HgO), manganese dioxide (MnO₂), sodium hypochlorite (NaOCl), and hypervalent iodine oxidants (*o*-iodoxybenzoic acid, IBX)^{165–168}. Catalytic oxidation of hydroxylamines can also be achieved with a variety of metal catalysts, including palladium¹⁶⁹, gold¹⁷⁰, and rhodium¹⁷¹. Given the great accessibility of secondary amines, their direct oxidation also represents a widely applied nitronium synthesis strategy. The use of hydrogen peroxide in the presence of sodium tungstate (Na₂WO₄•2 H₂O) was first reported by Murahashi and coworkers¹⁷². To improve upon the separation of water-soluble nitroniums, urea complexed with hydrogen peroxide (UHP) can also be used¹⁷³ or other metal-free methods involving *meta*-chloroperoxybenzoic acid (*m*-CPBA)¹⁷⁴. As with the oxidation of hydroxylamines, the oxidation of secondary amines has also been reported for a variety of metal catalysts¹⁵⁶. Similarly to secondary amines, imines are easily accessible through condensation chemistry and are thus attractive starting materials to access nitroniums. Their oxidation with UHP with catalytic methyltrioxorhenium (MTO) provides a straightforward one step method for this synthesis and allows for the synthesis of enantiomerically pure nitroniums from enantiopure imines^{175,176}. The decarboxylative oxidation of *N*-alkyl- α -amino acids provides an efficient method to perform the regioselective synthesis of nitroniums, where the oxidation from the

secondary amine would lead to a mixture of isomers. In addition to the sodium tungstate/H₂O₂, the oxidation of amino acids requires the addition of base (potassium carbonate (K₂CO₃) or phase transfer catalyst)¹⁷⁷.

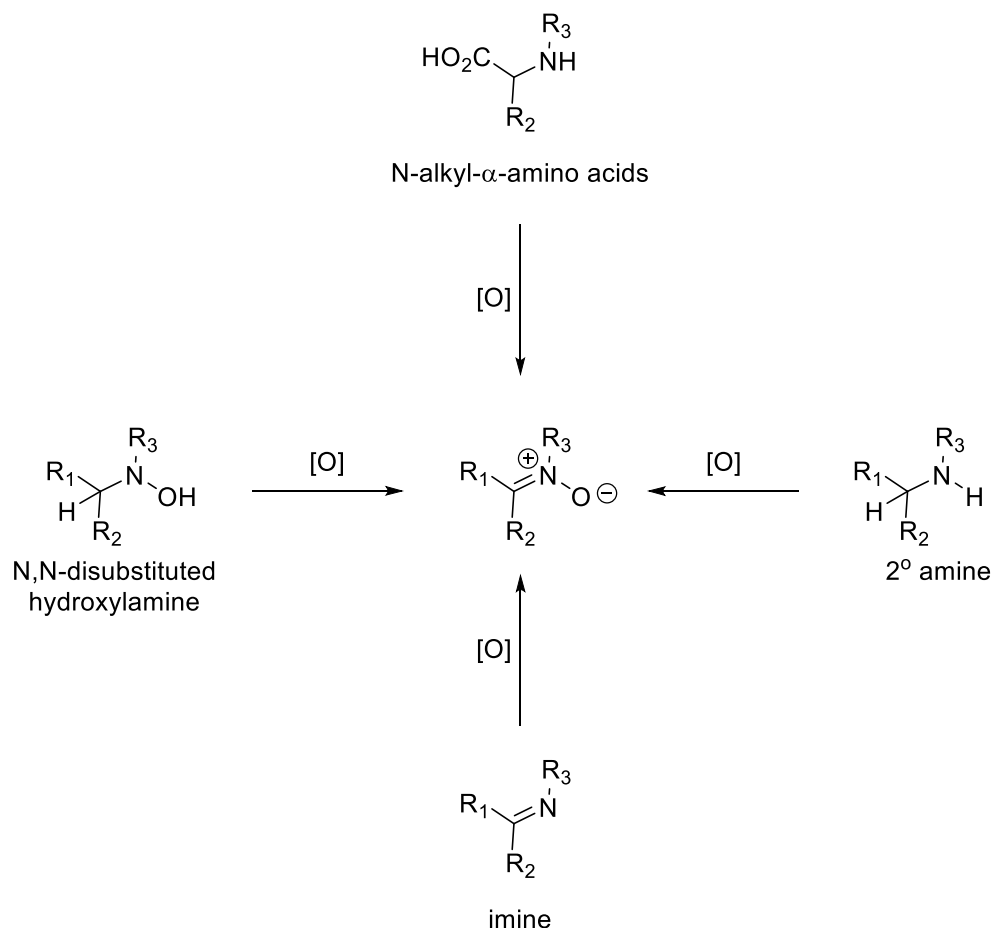
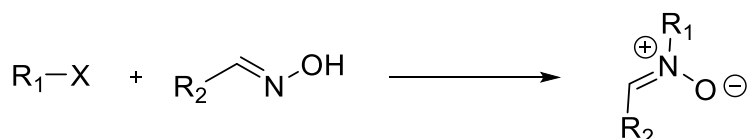


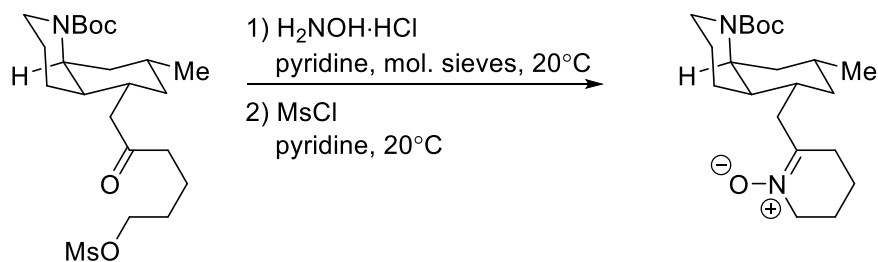
Figure 1.15 Synthesis of nitrones through oxidation.

The reactions between oximes and various electrophiles also provides a useful route to nitrone synthesis (see Scheme 1.14). Chiral nitrones have been prepared from the desilylative cyclization of carbohydrate-derived oximes¹⁷⁸⁻¹⁸¹. Similar intramolecular cyclizations have also been applied to natural product synthesis strategies for the synthesis of lycoposerramine-Z (see Scheme 1.15)¹⁸². Copper catalysis can also be applied to couple

oximes and alkenyl boronic acids to form nitrones^{183,184}. Various nitrones structures including endocyclic nitrones have been accessed through oxime reactions with varied electrophiles such as alkenes^{185–187} and electron-deficient alkenes^{188–190}, alkynes¹⁹¹, allenes¹⁹², and α -keto acids¹⁹³. Endocyclic nitrone structures have been found to be particularly useful for bioorthogonal applications, due to their higher stability to aqueous hydrolysis observed with acyclic nitrone counterparts, and generally faster reactions due to increased strain¹⁹⁴.

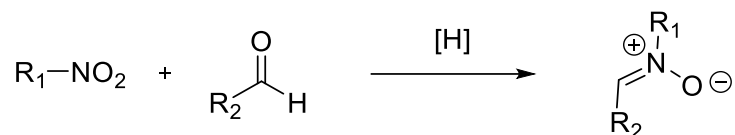


Scheme 1.14 Synthesis of nitrones from oximes.



Scheme 1.15 Synthesis of lycoposerramine-Z. Scheme adapted from Zhang *et al.*¹⁸².

Additionally, a useful general synthetic strategy is the reduction of nitro compounds in the presence of aldehydes (see Scheme 1.16). The reduction can be carried out in a variety of conditions including reduction via zinc metal¹⁹⁵, using ruthenium photoredox catalysis in the presence of weak base¹⁹⁶, and H_2/Pt reductions^{196,197}.

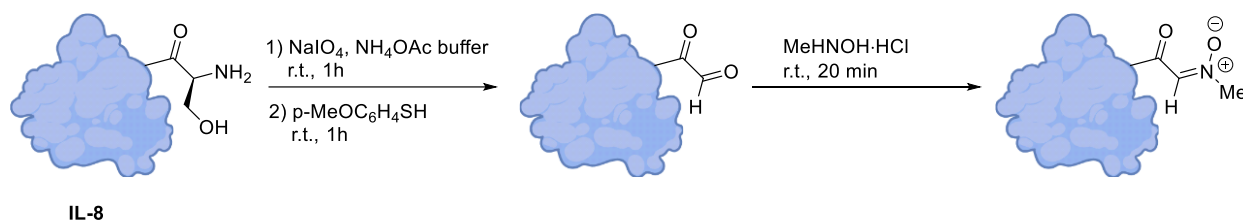


Scheme 1.16 Synthesis of nitrones from the reduction of nitro compounds in the presence of aldehydes.

1.9.2. Nitrones applied to bioorthogonal and biocompatible conjugations

Given their great tuneability, ease of synthesis, and possibility for *in situ* formation, nitrones have found applications as alternative dipoles in bioorthogonal and biocompatible applications. Through taking advantage of Huisgen 1,3-dipolar cycloadditions, chemical biologists have been able to apply these dipoles both metal-catalyzed and metal free cycloadditions with dipolarophiles for the purposes of cell labelling, biomolecule modification, and materials chemistry. Namely nitrones have been applied to strain-promoted alkyne-nitrone cycloadditions (SPANC) and other strained dipolarophiles and to copper-catalyzed alkyne-nitrone cycloaddition involving rearrangement, where they serve as analogues to the widely applied azide dipoles, and in general display faster kinetics. For example, the Van Delft group were able to show that the reaction between α -carboxynitrones and DIBO to have a second-order rate constant of $3.9 \text{ M}^{-1}\text{s}^{-1}$, compared to the analogous reaction with benzyl azide displaying a rate constant of $0.12 \text{ M}^{-1}\text{s}^{-1}$ ¹⁹⁸. Initial investigations by our research group also showed similar trends with cycloaddition reactions with BARAC occurring faster with nitrone dipoles compared to benzyl azide; up to $k_2 = 58.8 \text{ M}^{-1}\text{s}^{-1}$ for nitrones^{199,200}, compared to $0.96 \text{ M}^{-1}\text{s}^{-1}$ for the azide⁹¹. Given the need for the development of rapid bioorthogonal conjugations, allowing low concentrations of reagents to be used, further investigation into nitrone reactivity has emerged in recent years.

The use of nitrones for the development of strain-promoted bioorthogonal chemistry was first reported by McKay and coworkers in 2009, where it was found that a variety of nitron structures reacted more quickly with DIBO than benzyl azide²⁰¹. Almost contemporaneously, SPANC reactions were shown to be applicable for the modification of proteins by the group of van Delft¹⁹⁸. In their investigations, the group was able to achieve small peptide modification via a one-pot three-step protocol. Briefly, the *N*-terminal serine residue of a model hexapeptide was oxidized with sodium periodate to generate an aldehyde, which then allowed for *in situ* nitron formation via condensation with *N*-methylhydroxylamine, which could then undergo SPANC chemistry with DIBO to afford the desired isoxazoline product. They were able to apply this same methodology to the larger protein interleukin-8 (IL-8), a prototypical protein with an *N*-terminal serine, by using SPANC to functionalize it with PEG (polyethylene glycol) (see Scheme 1.17).



Scheme 1.17 Modification of IL-8 via *in situ* nitron formation after oxidation of *N*-terminal serine residues. Figure adapted from Ning *et al.*¹⁹⁸ Scheme created with Biorender.com.

Utilizing a similar strategy of *in situ* nitron formation, the van Delft group were also able to demonstrate the potential for dual functionalization of proteins using SPANC chemistry. By taking advantage of the structural modularity of nitrones, *N*-stearyl hydroxylamine was chosen to form a nitron conjugated to a stearyl functionality. Using this nitron, they were able to demonstrate the dual functionalization of human lactoferrin (hLF) with a chloroquine analogue using SPANC chemistry (via incorporation of

bicyclo[6.1.0]nonyne-trifluoromethyl quinoline (BCN-QN)); thus hLF was modified with stearyl and quinoline functionalities, aiding in its endosomal escape²⁰². In the same study, they were also able to demonstrate the functionalization of enhanced green fluorescent protein (eGFP) using both SPANC and CuAAC chemistry. By selecting *N*-propargyl-hydroxylamine, a terminal alkyne moiety could be introduced to the *N*-terminus of eGFP and thus allow for SPANC chemistry to take place with the nitron moiety, followed by copper catalyzed cycloaddition to the terminal alkyne. After *N*-terminal serine oxidation and nitron formation, eGFP was treated with BCN-biotin, resulting in an isoxazoline product with a terminal alkyne, which then participated in CuAAC chemistry with fluorescein-azide to achieve the dual functionalization of the protein (see Figure 1.16)²⁰².

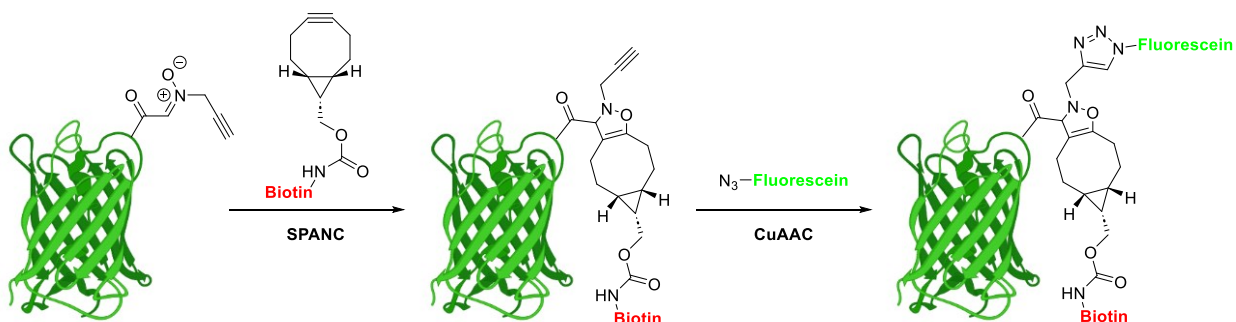
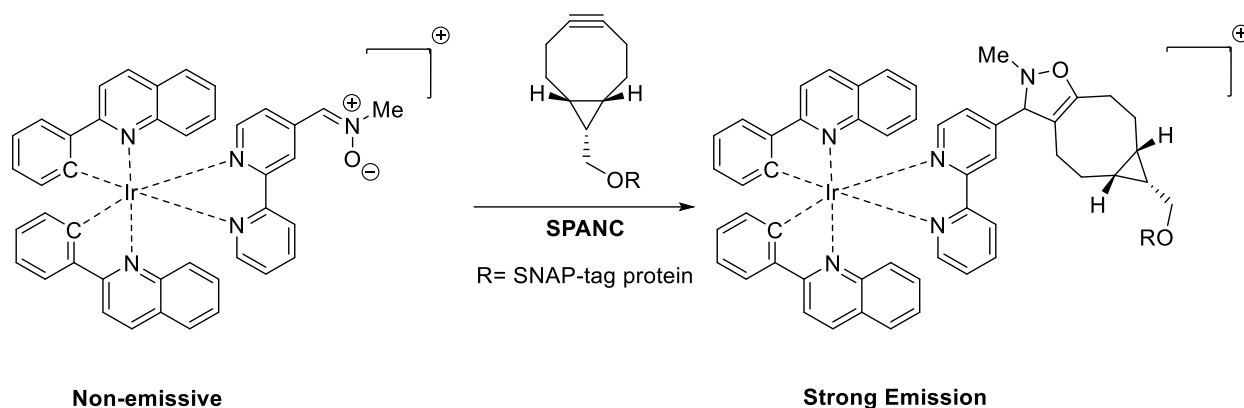


Figure 1.16 Dual functionalization of eGFP via sequential SPANC and CuAAC chemistry. Figure adapted from Temming et al.²⁰² Figure created with Biorender.com.

SPANC chemistry has also been employed to functionalize proteins with phosphorogenic iridium(III) polypyridine nitron complexes^{203,204}. Leung and coworkers were recently able to demonstrate this reactivity to modulate the emission and singlet oxygen, $^1\text{O}_2$, photosensitization of iridium metal nitron complexes with SNAP-tag proteins (DNA repair protein O^6 -alkylguanine-DNA alkyltransferase which reacts specifically with benzylguanine derivatives) functionalized with BCN²⁰⁴. SNAP-tag protein is often fused to

proteins of interest for its self-tagging properties. A reactive cysteine residue can react selectively with benzylguanine residues in order to functionalize the SNAP-tag protein²⁰⁵. In this study, BCN-benzylguanine derivative was used to install strained alkyne functionality to SNAP-tag proteins, which were then incorporated into Chinese hamster ovary-K1 cells (CHO-K1). Upon SPANC reaction, the quenching properties of the nitron (arising from photoisomerization of the C=N bond) are eliminated and the isoxazoline product can emit light and generate $^1\text{O}_2$ (see). Similar ruthenium(II) nitron complexes have also been reported²⁰⁶. Recently, the Kam-Wing Lo group have employed iridium(III) bis-nitron complexes as phosphorogenic reagents for phototheranostic applications, where they were able to demonstrate the synthesis of stapled peptides and the formation of hydrogels²⁰⁷.



Scheme 1.18 SPANC chemistry between iridium metal nitron complex and strained alkynes resulting in turn on emission and $^1\text{O}_2$ photosensitization. Adapted from Lee *et al.*²⁰³

Nitron chemistry has also been employed for the modification of other classes of biomolecules. The radical quenching properties of nitrones (spin traps) have been exploited for pharmacological activity against oxidative-stress mediated diseases²⁰⁸. To try and improve upon the bioactivity of nitrones against oxidative stress, Headley *et al.* incorporated

the endocyclic nitron, 1-pyrroline-N-oxide, to a probe containing two long dodecyl alkyl chains (diC₁₂PO)(see Figure 1.17). This lipophilic derivative of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was able to be incorporated into liposomes containing phosphatidylcholine²⁰⁹. SPANC labelling with DBCO-rhodamine fluorophore was used to confirm the incorporation and localization to intramolecular membranes of the lipid-like nitron in bovine aortic endothelial cells.

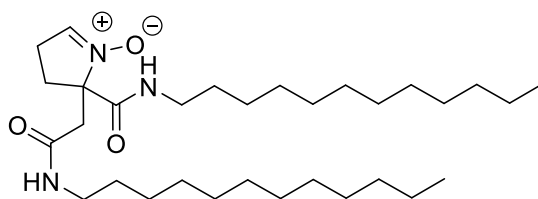


Figure 1.17 Structure of lipid-like nitron (diC₁₂PO) incorporated into phosphatidylcholine liposomes and intracellular membranes. Adapted from Headley *et al.*²⁰⁹

Nitrones cycloadditions have also been applied to the biocompatible modification of nucleic acid polymers and the functionalization of DNA-encoded libraries (DELs). These libraries comprised of small molecules barcoded with unique DNA sequences represent useful tools for rapid affinity-based screening²¹⁰. The Schreiber group employed [3+2]-cycloaddition chemistry of nitrones to expand upon the chemical space and access isoxazoline structures which are rarely found in DELs. In an initial study, the group was able to take advantage of the potential for *in situ* nitron formation from the condensation between hydroxylamines and aldehydes, followed by intramolecular [3+2]-cycloaddition with alkenes (at moderately high temperatures, but accelerated by electron-deficient olefins) to access a variety of isoxazoline products (see Figure 1.18)²¹¹. Nitron cyclization was found to be amenable to many functional groups common to DNA-modification chemistry, including carboxylic acids, amines, and aryl halides. The functionalization of the DNA

barcodes did not impede PCR amplification or lead to DNA degradation. The group was able to expand upon this application by taking advantage of the reaction between nitron dipoles and strained allen²¹². In this study, DNA barcodes were functionalized with triflate piperidine precursors via amide coupling, which could then be converted to reactive strained allen^{es} upon treatment with cesium fluoride (see Figure 1.19). They were able to demonstrate the successful cycloaddition with various nitron structures derived from aliphatic and aromatic aldehydes (under milder room temperature conditions). As with the intramolecular cyclization, the functionalization of the DNA barcodes did not lead to DNA degradation or hampering of DNA amplification.

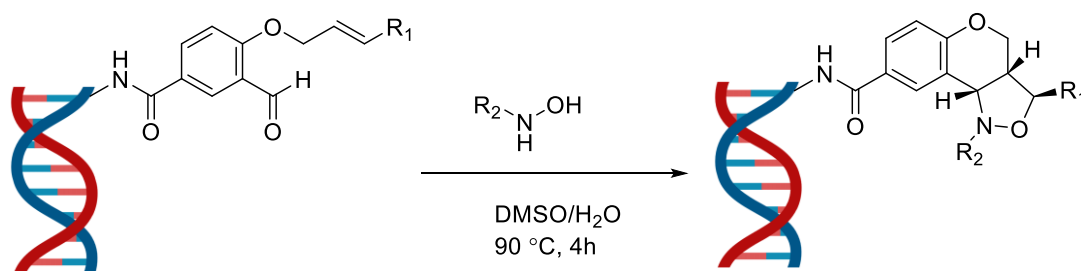


Figure 1.18 Functionalization of DNA barcodes by intramolecular [3+2]-cycloaddition between *in situ* synthesized nitron dipoles and allen^{es}. Adapted from Gerry *et al.*²¹¹ Figure created with Biorender.com.

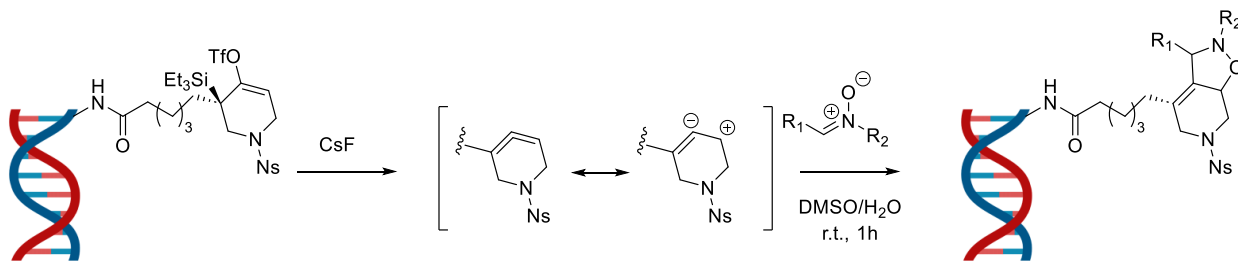


Figure 1.19 Functionalization of DNA barcodes by cycloaddition between nitrones and strained allen^{es} formed *in situ* via fluoride-induced elimination. Adapted from Westphal *et al.*²¹² Figure created with Biorender.com.

Furthermore, nitron chemistry has proven a useful tool for the preparation of functionalized nanoparticles with potential biological applications. The Workentin group has employed various nitron cycloaddition reactions to the synthesis of gold-nanoparticles (Au-NPs). In one study, the group was able to employ maleimide-functionalized Au-NPs in 1,3-dipolar cycloaddition with nitron dipoles to obtain various Au-NP conjugates²¹³. Although the authors note that maleimide hydrolysis and their reactivity towards biological nucleophiles such as amines and thiols, limits the potential for *in vivo* formation of maleimide-nitron conjugates. Expanding upon these initial findings, the Workentin group also pursued the development of Au-NPs functionalized through SPANC chemistry²¹⁴. Nitron Au-NPs were synthesized by treating Au-NPs with nitron-terminated thiol ligands. Taking advantage of the nitron being displayed interfacially on the modified Au-NPs, the nanoparticles could be further functionalized through interfacial-SPANC chemistry (I-SPANC) with strained alkynes. Interestingly, the I-SPANC reaction allowed the Au-NPs to be conjugated to ¹⁸F-radiolabelled prosthetic groups to allow for PET imaging applications. The modified ¹⁸F-Au-NPs maintained their water solubility and investigation of their *in vivo* distribution in model mouse models showed low uptake in background organs; thus demonstrating the potential for SPANC chemistry to be employed to PET labelling studies.

The first example of nitron chemistry being applied to live cell labelling was demonstrated by McKay and coworkers in 2011¹⁹⁴. In this seminal study, human recombinant epidermal growth factor (EGF) protein was functionalized with cyclic pyrroline-*N*-oxide nitron through conjugation with an *N*-hydroxysuccinimide (NHS) ester of the nitron. Human breast cancer cells (MDA-MB-468) overexpressing EGF receptors on the cell surface were then treated with the EGF-nitron conjugate. Successful visualization of

the cell surface was accomplished via SPANC chemistry, where the cells were first treated with DIBO-biotin, and then streptavidin-FITC fluorophore (see Figure 1.20).

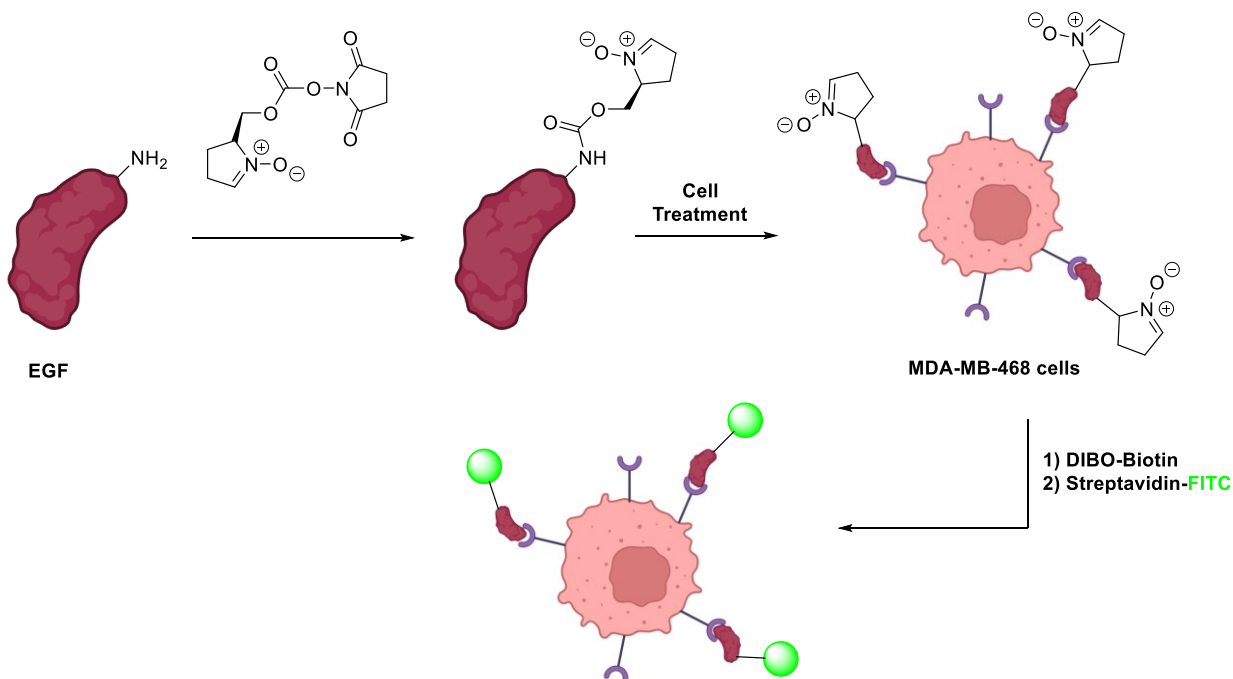
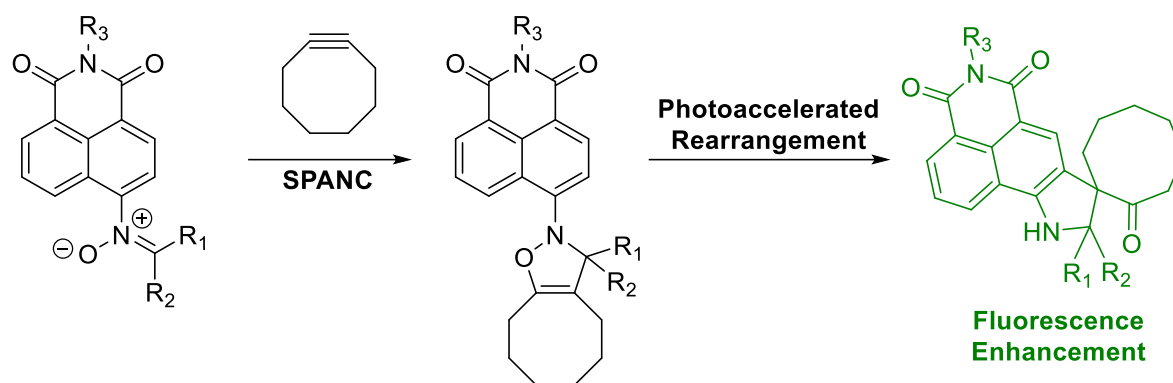


Figure 1.20 Labelling of EGF-EGF receptor interactions on MDA-MB-468 cells via SPANC chemistry between DIBO-biotin and EGF-nitrone. Adapted from McKay *et al.*¹⁹⁴ Figure created with Biorender.com.

Another early example of cell labelling using SPANC consists of work by Colombo and coworkers²¹⁵. The functionalization of single chain variable fragment (scFv) antibodies was accomplished by oxidation of *N*-terminal serine residues to obtain aldehyde functionality which was converted to the nitrone dipole after condensation with hydroxylamine. Multifunctional nanoparticles (MFN) which had been functionalized with strained cyclooctynes (DIBO) could then be conjugated to the nitrone-scFv antibodies through SPANC chemistry. The conjugation allowed the MFNs to be immobilized specifically to target breast cancer cells expressing EGF receptor 2 (HER2).

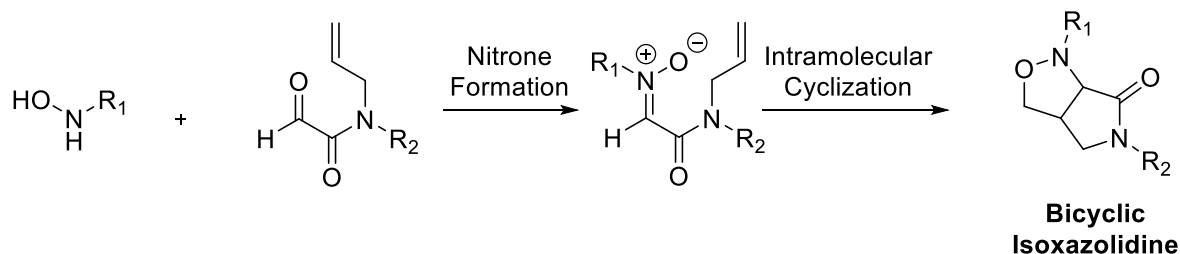
Recently, nitron dipoles have also allowed for fluorescence turn-on probes to be developed. The group of Tian and coworkers were able to synthesize nitron functionalized 1,8-naphthalimide probes which were able to form fluorescent products after photo-accelerated rearrangement of the initial cycloadduct (see Scheme 1.19 Turn-on fluorescence of 4-nitron-1,8-naphthalimide probes after SPANC with strained alkynes followed by rearrangement. Adapted from Teng *et al.*²¹⁶).²¹⁶ The incorporation of the electron-withdrawing nitron moiety to the 1,8-naphthalimide disrupted the internal charge transfer, thus quenching fluorescence emission. Upon cycloaddition with a strained alkyne, the formed isoxazoline can undergo rearrangement restoring the internal charge transfer and leading to great fluorescent emission enhancement; a process which could be accelerated through photoirradiation. The turn-on probe was used to label both proteins (bovine serum albumin (BSA)) and to live-cell imaging. To accomplish cell imaging, HeLa cells were treated with triphenylphosphonium-conjugated BCN and DIBO, which localized to mitochondria. Upon treatment with 1,8-naphthalimide nitron, fluorescence enhancement was observed which coincided with the localization of a commercial mitochondria tracker dye, Mito Tracker.



Scheme 1.19 Turn-on fluorescence of 4-nitrone-1,8-naphthalimide probes after SPANC with strained alkynes followed by rearrangement. Adapted from Teng *et al.*²¹⁶

Intramolecular nitron cycloaddition chemistry has also been recently harnessed by the Nitz group to achieve bioorthogonal conjugation reactions between nitrones and *N*-allylglyoxylamide. Through a cascade, nitron dipoles are formed *in situ* through condensation of the aldehyde of the glyoxylamide, which then undergoes intramolecular cycloaddition to form a bicyclic isoxazolidine product; the reaction was found to be amenable to aqueous conditions and accelerated by aniline/5-methoxyanthranilic acid catalysis (see Scheme 1.19)²¹⁷. In this study, they were able to demonstrate the applicability of this new bioorthogonal transformation through modification of HaloTag7 protein with a 1-chlorohexyl Halotag substrate functionalized with *N*-hydroxylamine. The HaloTag7-hydroxylamine was then conjugated to a glyoxylamide containing fluorescein fluorophore in complex cell lysates. Cell surface labelling of HEK293 cells was also accomplished after the cells were transfected with a plasmid inducing the expression of HaloTag protein to the cell surface. Treatment of the transfected cells with hydroxylamine-HaloTag substrate, followed by glyoxylamide-fluorescein in the presence of 5-methoxyanthranilic acid led to efficient labelling of cell surfaces. The reaction was also shown to be compatible with CuAAC chemistry in multiplex labelling experiments with HaloTag7-hydroxylamine and Z-Domain-

azide model proteins. The authors do state that the challenges of hydroxylamine oxidation, and the necessity of catalysis limit the strategy to *in vitro* applications.



Scheme 1.20 Bioorthogonal conjugation between N-allyl glyoxylamide and *in situ* formed nitrones. Adapted from Lee *et al.*²¹⁷

SPANC chemistry has also been shown to be quite useful in labelling bacterial cell surfaces, due to the ease of incorporation of unnatural metabolites to the cellular membranes, particularly the peptidoglycan layer. Unnatural amino acids bearing nitron side chains have been shown to be readily incorporated into the peptidoglycan of multiple bacteria species, thus allowing the visualization of cell surfaces through SPANC, CuANCR or TCO-nitrone cycloaddition chemistry^{218–221}. Derivatives of D-alanine and D-lysine amino acids with cyclic nitron side chains (CMPO, DMImO) provide stable and relatively small bioorthogonal reacting partners, allowing the labelling of both Gram positive and Gram negative bacteria; with more efficient labelling being observed for Gram positive species such as *Listeria* (*L.*) *innocua* and *L. monocytogenes* (see Figure 1.21). Noncovalent labelling of peptidoglycan was also demonstrated by synthesizing an endocyclic nitron functionalized vancomycin analogue, a pentapeptide glycoprotein antibiotic that binds to D-Ala-D-Ala bonds found in peptidoglycan²¹⁸. The structural diversity and reactivity tuneability of nitrones can also be employed to achieve the multiplex labelling of bacteria²¹⁹. Two different bacteria, *L. innocua* and *L. lactis*, were incubated with two structurally and electronically

different nitron amino acids. *L. innocua* with D-Ala-DMImO nitron with is more electron-poor and *L. lactis* with the more electron rich D-Ala-CMPO nitron. After metabolic incorporation of the nitrones, the bacterial cells could be mixed and treated simultaneously with BCN-CF488 fluorophore and DBCO-TAMRA fluorophore. Given that DMImO nitron displays a greater second order rate constant with BCN, than the analogous reaction between BCN and CMPO, BCN-CF488 could label *L. innocua* exclusively, while DBCO-TAMRA was found to effectively labels both species. This manipulation of nitron reactivity allows for the identification of bacterial species in complex mixtures, with potential applications in the detection of food-borne pathogens.

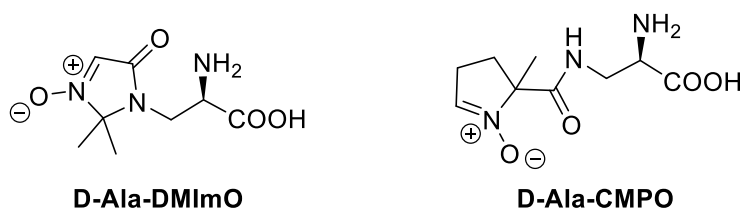


Figure 1.21 Structure of unnatural nitron-functionalized amino acids used for metabolic incorporation into peptidoglycan of bacterial cell surfaces.

1.10. Thesis Outlook

The development of bioorthogonal chemistry has become an increasingly diverse, exciting and impactful field of research. Given its potential to empower scientists with extremely modular and efficient chemical tools, novel applications and more complex applications are continually discovered, allowing for better imaging and labelling of biomolecules, for the synthesis of biomaterials and increasingly more medical applications. The goal of the work presented in this thesis is to further the development of bioorthogonal chemical transformations to improve upon this toolbox of reactions available to biological chemists. While azide dipole chemistry has been thoroughly explored in the context of

bioorthogonal transformations, alternative dipoles, such as nitrones, remain somewhat underexplored comparatively. Given the advantages that nitron dipoles can display over azides, such as greater modularity, tuneability of reactivity and the potential for faster reaction rates, the development of this class of dipoles is highly warranted.

Efforts towards the optimization of the copper-catalyzed alkyne-nitron cycloaddition involving rearrangement (CuANCR) will initially be discussed. Employing micellar conditions, various aspects of successful CuANCR reactions were evaluated, such as micellar composition, ligand nature, alkyne and nitron structure. The use of an alkyne-functionalized magnetic bead for the rapid determination of reaction efficiency/applicability towards bioorthogonal applications will also be discussed. The application of these optimized micellar CuANCR conditions towards the functionalization of membrane soluble peptides will then be explored. Finally, initial development of a dual labelling protocol involving sequential CuANCR and thiol addition (through native chemical ligation type chemistry) will be discussed.

The application of nitron dipoles towards double strain-promoted alkyne-nitron cycloadditions involving dibenzocyclooctadiyne will then be explored. As with studies of similar reactions with azide dipoles, the reactivity with nitrones and cyclic diynes demonstrates an interesting rate acceleration for the second cycloaddition, with efforts to isolate intermediate product of initial cycloaddition being unsuccessful. Mechanistic aspects of the reaction such as reaction kinetics and electronic effects are discussed, through experimental (UV kinetics, Hammett analysis) and computational methods (Distortion/Interaction analysis). The applicability of this dual ligation chemistry is then

demonstrated through the synthesis of oligomers towards potential polymer applications and through the labelling of bacteria via metabolic incorporation of nitronone dipoles.

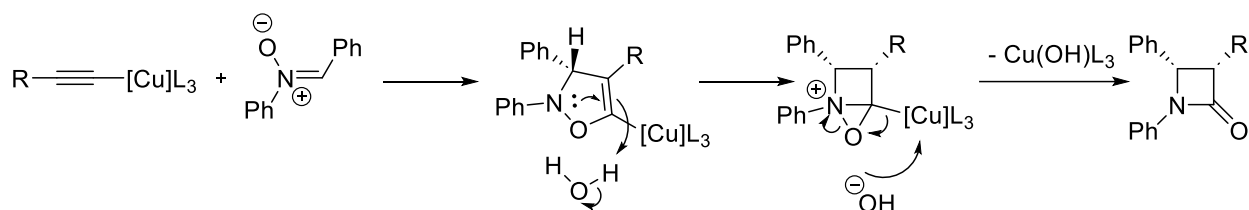
The final sections of this thesis will outline some initial efforts in the development of novel bioorthogonal transformations. These efforts include the further study nitronone dipoles with examples of the reactivity of nitronones with phenanthridine-like structures, and exploration of nitronone-cyclopropene cycloadditions. Initial investigations cycloaddition reactions between cyclopropenone-caged cyclooctadiyne and nitronones will also be disclosed.

Chapter 2 Optimization and Development of Copper-Catalyzed Alkyne-Nitrone Cycloadditions

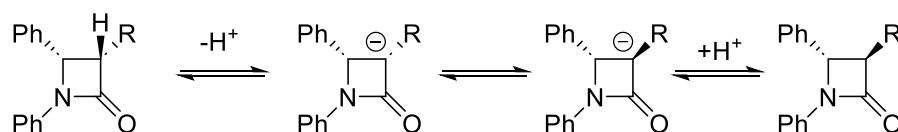
2.1. Introduction

The copper(I)-catalyzed alkyne-nitrone [3+2]-cycloaddition involving rearrangement (CuANCR) remains an underdeveloped reaction in the context of bioorthogonal applications development. The CuANCR reaction is highly analogous to the copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction, which since its initial development as a 'Click' reaction, has found widespread use for bioconjugation and cellular labelling applications^{6,76,222-224}. In contrast to CuAAC, CuANCR offers advantages of ease of synthesis and tuneability of nitrone dipoles, as well as formation of β -lactam products. While offering connectivity between two moieties through β -lactam formation, the conjugation product has the potential to be exploited further either through taking advantage of antibiotic properties of certain β -lactam motifs or through exploiting the electrophilic character of the formed heterocycles for further conjugation chemistry²²⁵⁻²²⁷. The first example of the reaction reported by Kinugasa and Hashimoto involved the Cu(I)-catalyzed cycloaddition of phenylacetylide with various nitrones using anhydrous pyridine as solvent to yield *cis*- β -lactam products in modest (50-60%) yields²²⁸. The first proposed mechanism for the Kinugasa reaction was put forward by Ding and coworkers due to observing mixtures of *cis* and *trans* products while exploring the scope of the reaction²²⁹. Similarly to CuAAC reactions, the reaction is initiated by the formation of copper acetylide under basic conditions. The initial rate-limiting [3+2] cycloaddition can then take place with the nitrone dipole forming a cuprated isoxazoline intermediate, which they proposed rearranged to a strained bicyclic oxaziridium intermediate (see Scheme 2.1). The formation of the favoured *cis*-product was explained by

the isooxazoline intermediate being readily protonated on its less sterically hindered face. Formation of the observed *trans*-products was explained by the isomerization of the β -lactam product under basic reaction conditions (see Scheme 2.2).

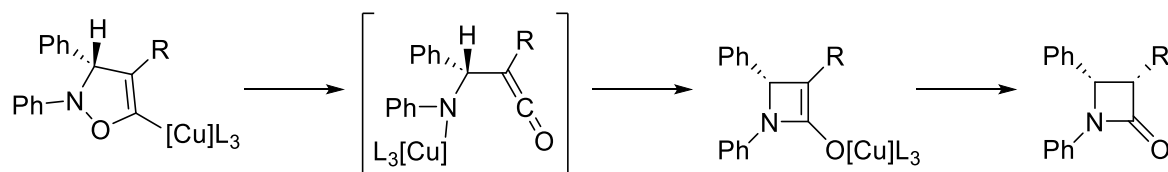


Scheme 2.1 Kinugasa reaction mechanism proposed by Ding and Irwin²²⁹.



Scheme 2.2 Proposed β -lactam product isomerization under basic conditions²²⁹.

Alternate mechanisms have been proposed throughout the years, notably by Tang and coworkers, who proposed that the isooxazoline intermediate undergoes ring opening leading to an intermediate ketene (see Scheme 2.3)²³⁰. Product formation can then proceed through intramolecular cyclization to form an enolate, which can then be protonated. In both proposed mechanisms, the stereochemical configuration at position C-4 is set by the initial cycloaddition reaction between the copper-acetylide and nitronitrone dipole.



Scheme 2.3 Isoxazoline rearrangement in Kinugasa mechanism proposed by Tang et al.²³⁰.

A revised mechanism was more recently reported by Hein and coworkers²³¹. This proposed mechanism is initiated by the formation of the bis-copper-acetylide which was proposed due to the reaction displaying second-order dependence on the catalyst. Rate-limiting [3+2] cycloaddition between the acetylide and nitron yields a key metallocyclic intermediate which then undergoes reductive elimination and protonation forming the isoxazolidine heterocyclic intermediate. The isoxazolidine undergoes a cascade of [3+2]-cycloreversion and [2+2]-cycloaddition to form the β -lactam product (see Figure 2.1). Recent computational studies performed by Himo and Santoro have corroborated the findings of Hein and coworkers' proposed mechanism²³². The calculations considered different mechanistic pathways of β -lactam formation from bisaryl-nitrones and phenylacetylene under copper catalysis with phenanthroline as the ligand and triethylamine as base. The calculations confirmed the initial two step cycloaddition promoted by two copper ions, which is then followed by cycloreversion to generate the imine and ketene intermediates. Rather than the previously proposed [2+2] Staudinger cycloaddition, Himo and Santoro propose that the reaction can proceed through a nucleophilic attack of a ketyl dicopper intermediate on the imine, followed by intramolecular cyclization to yield the product (see Scheme 2.4). In this computational analysis, the authors do state that the energetic differences between different mechanistic possibilities are sensitive to the nature of the

ligand and the base, meaning that these conclusions may not necessarily be extended to all systems, especially when considering the reaction taking place under aqueous conditions.

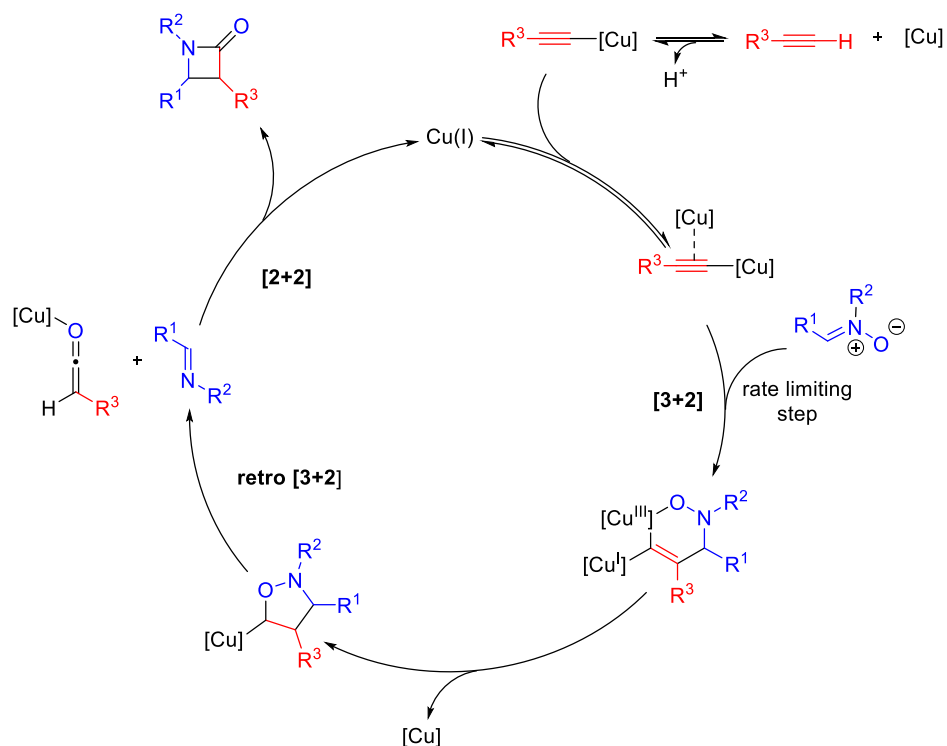
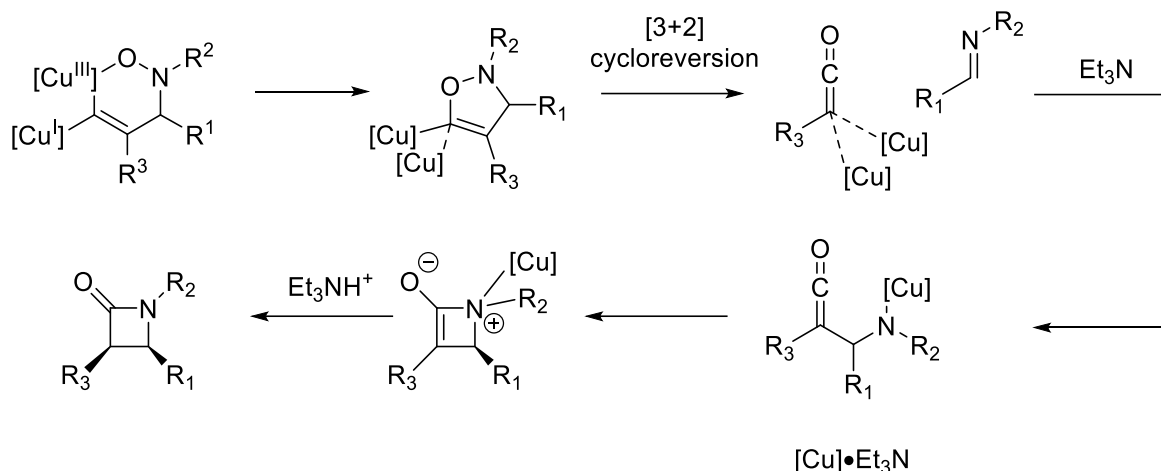


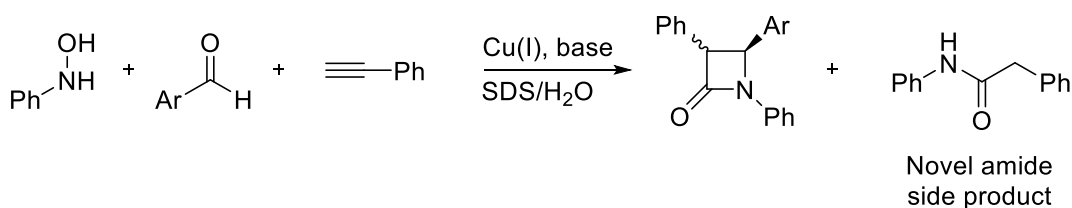
Figure 2.1 Proposed copper(I)-catalyzed alkyne-nitrone [3+2]-cycloaddition involving rearrangement reaction mechanism.^{231,233}



Scheme 2.4 Revised Kinugasa reaction mechanism proposed by Himo and Santoro based on DFT analysis²³².

These inaugural examples and other developments of the CuANCR reaction under organic conditions, as well as the work in developing CuAAC for bioorthogonal applications laid the groundwork for the development of CuANCR reactions amenable to aqueous conditions. The first challenge overcome consisted in applying appropriate Cu(I) catalyst systems to organic/aqueous solvent conditions (referred to as Click conditions), which was achieved by Basak and coworkers with the use of copper (II) sulfate (CuSO₄) and appropriate bioorthogonal reducing agents such as sodium ascorbate in organic/aqueous (1:2) solvent mixtures (DMF:H₂O, MeCN:H₂O, *t*-BuOH:H₂O)²³⁴. These reported conditions however did not result in high β -lactam product yield in fully aqueous solvent. “On water” reaction conditions applying chiral diamine ligands were later reported by Chen and coworkers leading to excellent diastereoselectivity and enantioselectivity (over 99:1 d.r. and 98% *ee*)²³⁵. One of the main hurdles to adapting CuANCR reactions to aqueous conditions has been the poor solubility of the reacting partners, especially when testing unoptimized simple alkyne and nitron structures used in reaction condition development. Such as in the work of Basak and

coworkers, the use of organic co-solvents can lead to successful β -lactam formation, however these solvents are not orthogonal to most biological systems. Our research group therefore hypothesized that the reaction could be promoted in aqueous conditions using surfactant micelles to increase the solubility of reacting partners, and increase their effective local concentration, thus potentially leading to reaction rate acceleration. Over a critical micelle concentration (CMC), surfactants can aggregate to form micelles, creating hydrophobic environments which allow for the solubilization of organic moieties in aqueous environments and allow for their interaction with solutes found in aqueous phase. Furthermore, McKay and coworkers suggested that these micelles could serve as an effective platform for rapidly developing reactions towards bioorthogonal applications, as micelles can serve as biomimetic compartments resembling the complex phospholipid bilayers of cells²³⁶. A similar approach had been employed previously by Chatterjee and coworkers to perform the single-pot synthesis of isoxazolidines from the [3+2]-cycloaddition of α ,N-diaryl nitrones and ethyl acrylate in water at room temperature under micelle-catalyzed conditions²³⁷. Based upon this seminal example, McKay and coworkers performed the multicomponent Kinugasa reactions under micelle conditions in aqueous conditions (see Scheme 2.5)



Scheme 2.5 Surfactant enabled multicomponent Kinugasa reactions in aqueous conditions²³⁶.

The reaction was proposed to proceed through a two-step sequence in which micelle solubilization of the *N*-phenyl hydroxylamine and aryl aldehydes allowed for *in situ* nitron formation, which can then undergo Cu(I)-catalyzed [3+2]-cycloaddition with phenylacetylene to yield diastereomeric mixtures of β -lactam products. The appearance of a novel amide side product (up to 36% yield) was also noted under the reported reaction conditions. Although this amide linked product does not constitute the desired β -lactam linkage, covalent connection between the two reacting partners is maintained, and therefore does not necessarily preclude the reaction from bioorthogonal applications.

In contrast to many other chemical transformations that have been employed for bioorthogonal purposes, both CuAAC and CuANCR face the challenge of necessitating copper catalysis, which can lead to copper-associated cellular toxicity. These toxicity challenges are addressable through the careful selection of ligand and cellular targets^{72,238}. While the initial micelle-assisted multicomponent Kinugasa reactions employed organic amines such as pyridine, bipyridine or tris(benzyltriazolymethyl)amine (TBTA) as the copper ligands; their use is impractical for bioorthogonal applications. The use of natural amino acid ligands, such as L-proline and L-histidine, allows for the copper catalysis to take place without greatly affecting reaction rates, all while greatly reducing toxicity to cells in *in vitro* experiments^{72,238}. Further development of aqueous strain-promoted alkyne-nitron cycloadditions (SPANC) conditions led to the use of endocyclic nitrones which have been shown to be more stable to hydrolysis under bioorthogonal reaction conditions and display fast reaction conditions¹⁹⁴. The problem of nitron hydrolysis to the parent hydroxylamine and aldehyde under aqueous conditions can also be minimized by *in situ* nitron formation and using excess hydroxylamine reagent²³⁹.

CuANCR reactions were first employed for bioorthogonal purposes by Zlatopolskiy and coworkers for use in *in vitro* ^{18}F -radiolabelling of model peptides and proteins²⁴⁰. They postulated that the Kinugasa reaction displayed sufficiently rapid reaction kinetics in order to afford radiolabelled β -lactam functionalized proteins for positron emission tomography (PET) detection and analysis with the ultimate goal of PET detection of bacterial and viral infection. ^{18}F -labelled nitron was synthesized by the acid-catalyzed condensation of phenyl hydroxylamine to *p*- ^{18}F -benzaldehyde which could then undergo cyclization with alkyne-modified proteins and peptides. The group also demonstrated the ^{18}F incorporation using *o*- and *p*-fluorophenyl acetylene. Under mild conditions (CuSO_4 , sodium ascorbate, L-histidine and aqueous MeCN), they achieved the radiolabelling of a propiolyl-functionalized depsipeptide with high radiochemical yield (85%) and the radiolabelling of propiolyl-functionalized bovine serum albumin (BSA) in modest radiochemical yield (32%). These model radiolabelling experiments were completed within a time course of 10-20 minutes, amenable to the short half-life of ^{18}F .

Our research group has also demonstrated the application of CuANR transformations for *in vitro* cellular labelling, such as for the labelling of cell-surface metabolically incorporated modified carbohydrates in both prokaryotic and mammalian cells. It has been demonstrated, that cells readily incorporate functionalized carbohydrates through the synthesis of sialylated glycans³¹. Applying this strategy has allowed for the labelling of Huh-7 human hepatoma cells which were cultured with alkyne-modified mannosamine sugars (see Figure 2.2). After metabolic incorporation, the CuANR reaction could take place with a biotin-

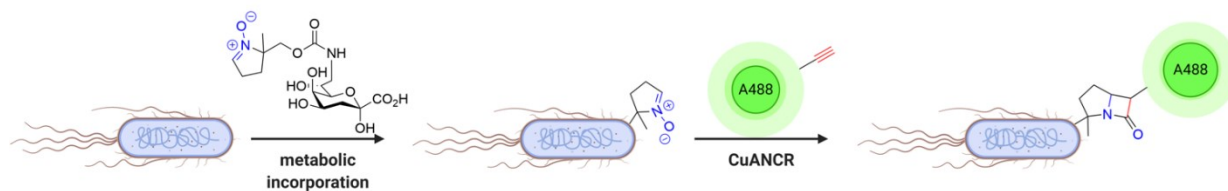


Figure 2.3 Cell surface labelling of *E. coli* cells via the incorporation of KDO derivatives. Legend: A488 = Alexa488. Adapted from Sherratt et al.²⁴¹ and Bilodeau et al.²³³ Created with Biorender.com

The main goals of the study presented in this thesis chapter were to expand upon the development of CuANCR conditions to improve the reaction's applicability to bioorthogonal applications by exploring the scope of applicable nitrones, alkynes and expand upon the various surfactants that can be employed for micelle-assisted β -lactam formation. Its applicability to the functionalization of liposoluble peptides, such as membrane-bound peptides, was also demonstrated by the conjugation of biotin to the E5-TAT peptide. The final section of this chapter will also highlight initial experiments that were performed towards the development of a dual-labelling variation of the Kinugasa transformation, in which the electrophilic properties of the formed β -lactam products would be exploited to perform a second conjugation reaction after the initial conjugation between the nitronium and alkyne reacting partners.

2.2. Author Contributions

The synthesis of nitronium compounds used in the study was performed by Didier Alexandre Bilodeau (**DAB**) with assistance from Kaitlyn Margison (**KM**). **DAB** performed the screen of alkynes in micelle-assisted Kinugasa reactions, including synthesis of propiolamides and propiolic esters. Initial magnetic bead assay ligand screening (Fig. 2) was performed by Dr. Allison Sherratt (**AS**), while further magnetic bead assays screening micelle

composition were performed in conjunction by Dr. Miroslava Strmiskova (**MS**) and **DAB**. Small peptide modifications (Fig. 4) were performed by **DAB**, with assistance from Dr. Noreen Ahmed (**NA**) for peptide selection, western blotting training, and mass spectrometry sample preparation.



Optimized aqueous Kinugasa reactions for bioorthogonal chemistry applications†

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Kinugasa reactions hold potential for bioorthogonal chemistry in that the reagents can be biocompatible. Unlike other bioorthogonal reaction products, β -lactams are potentially reactive, which can be useful for synthesizing new biomaterials. A limiting factor for applications consists of slow reaction rates. Herein, we report an optimized aqueous copper(i)-catalyzed alkyne–nitron cycloaddition involving rearrangement (CuANCR) with rate accelerations made possible by the use of surfactant micelles. We have investigated the factors that accelerate the aqueous CuANCR reaction and demonstrate enhanced modification of a model membrane-associated peptide. We discovered that lipids/surfactants and alkyne structure have a significant impact on the reaction rate, with biological lipids and electron-poor alkynes showing greater reactivity. These new findings have implications for the use of CuANCR for modifying integral membrane proteins as well as live cell labelling and other bioorthogonal applications.

Bioorthogonal chemistry provides invaluable tools to chemical biology research for studying specific biological processes inside cells that are otherwise challenging to probe.^{1–6} A number of bioorthogonal reactions have been developed and are now used as techniques to efficiently, selectively and covalently link two reactive groups.^{7–13} One such reaction is copper(i)-catalyzed azide–alkyne cycloaddition (CuAAC) (Fig. 1a),^{14,15} which has been and continues to be utilized extensively in a myriad of biological labelling applications and is used to perform labelling of cellular targets.^{16–20} Concerns around copper toxicity are addressable with careful choice of copper(i) ligand,²¹ through traceless reagents bearing catalytic copper,²² or development of alternative copper-catalyzed reactions.^{23,24} However, alternatives are still desired to expand the variety of reacting partners and bring additional reactions for use in bioorthogonal applications, allowing for the discovery of more efficiently catalyzed reactions.^{25–27} The classical Kinugasa reaction,²⁸ although notoriously sensitive and challenging, represents a unique prototype reaction for development

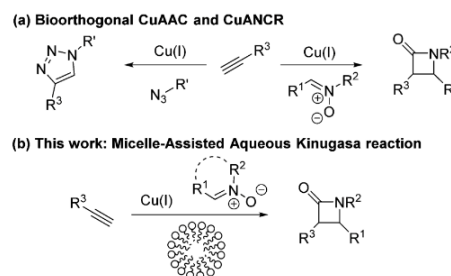


Fig. 1 (a) Comparing Kinugasa/CuANCR with CuAAC bioorthogonal chemistry. (b) Lipid acceleration through micelles.

and optimization for bioorthogonal chemistry applications. Since its initial discovery, the Kinugasa reaction has received considerable recognition as a reaction that produces β -lactams in a variety of conditions.^{28,29} β -Lactams are of significant interest because of their use as antibiotics,^{30,31} and their general properties as electrophiles.³² The Kinugasa reaction has been developed from original reports using pyridine as a base and solvent,^{28,33} to using polar aprotic solvents as well as polar protic/aprotic solvent mixtures,²⁹ and reactions in aqueous conditions,^{34,35} with the potential to be applied to biological systems for bioorthogonal labelling.^{2,3,21,36} Yet, examples of the Kinugasa reaction in aqueous conditions are few, with our group previously reporting one of the first examples.³⁴ Thus, it can be considered a prototype reaction that still needs development and optimization in order to operate as a useful bioorthogonal reaction.

In addition to applications of biological labelling, bioorthogonal chemistry allows for the production and functionalization of nanoparticles and biomaterials.^{37,38} CuAAC chemistry has been used extensively for modification of material surfaces,³⁹ attachment of proteins to materials⁴⁰ and nanomaterial synthesis.⁴¹ The Kinugasa reaction could be applied to similar applications, while producing potentially bioactive β -lactam products directly on biomaterial surfaces. The development of biomaterials with antibacterial properties is vital due to bacterial colonization being a major

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9cc09473c

cause of implant failure.⁴² β -Lactam antibiotic doping has been proposed to produce such materials;⁴³ thus the Kinugasa reaction could allow for direct functionalization of such bioorthogonal implant materials.

Nitrones are excellent tunable bioorthogonal reactants, which have already been demonstrated in strain-promoted alkyne–nitron cycloaddition (SPANC) reactions.^{44,45} We recently discovered a version of the Kinugasa reaction, copper(i)-catalyzed alkyne–nitron cycloaddition involving rearrangement (CuANCR), that can be used as a bioorthogonal reaction for live-cell labelling.⁴⁶ However, reaction rates were slow. Herein, we optimize reaction conditions for the aqueous CuANCR and use surfactant micelles⁴⁷ to accelerate aqueous reaction rates and yields.

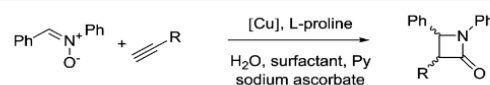
Initially, we sought to optimize the alkyne portion of the reaction. Alkynes have been activated towards [3+2] cycloaddition by conjugation to electron-withdrawing groups, such as esters, through lowering of the energy of their LUMO.⁴⁸ Therefore, other alkynes bearing electron withdrawing groups were tested (Table 1, entries 2–7). The use of propiolamides (Table 1, entries 5 and 6) and propiolic esters (Table S1 (ESI[†]), entry 7) led to increases in observed yield, as did the inclusion of micellar catalysts.³⁰ The scope of acyclic nitrones was also evaluated and the reaction was found to be amendable to a wide range of aryl substitution (see Table S2, ESI[†]). Furthermore, it should be noted that the reported yields of β -lactam products after extraction might not fully represent the total reaction yield, given potential product loss in the extraction process with surfactants/micelles. Additionally, the β -lactam yields do not report on other potential

covalently linked side products which would also lead to successful bioconjugation.³⁶ Thus, percent conversion is thought to be more representative of reaction progression.

Given difficulties in product isolation and quantification due to use of surfactants, the determination of reaction kinetics proves challenging. Therefore, to further evaluate the reaction efficiency and ultimately relative rates, we performed studies utilizing our recently reported alkyne-tagged magnetic bead assay.⁴⁶ The latter represents a simple and convenient approach to assessing reaction progression under biological lipid/micellar catalysis, which otherwise interferes with most conventional spectroscopic approaches. Briefly, we employed a biotin-tagged cyclic nitron (biotin–CMPO) that was reacted with alkyne-functionalized magnetic beads in the presence of CuSO₄ and sodium ascorbate, while varying ligand type and presence of different biomimetic lipids. After magnetic separation, covalently linked biotin conjugated to alkyne-tagged magnetic beads *via* CuANCR, were detected by FITC–streptavidin staining and fluorescence microscopy. An initial comparison between L-histidine and L-proline as bioorthogonal copper ligands led us to choose L-proline to perform further on-bead assays of a variety of lipid micelles, due to higher observed labelling efficiency (Fig. 2 and Fig. S1, ESI[†]) and due to L-proline having been previously shown to be a competent copper ligand.^{49,50}

Next, we sought to evaluate the effects of biomimetic lipids on the efficiency of CuANCR reactions. We have previously reported that CuANCR is an effective approach for cell labelling.⁴⁶ We hypothesized that cell surface components, such as cellular lipids, glycans, glycoproteins and the membrane itself increase the efficiency of the CuANCR labelling reaction, as previously seen by others.⁵¹ To test this hypothesis, we performed a screen of biomimetic lipids with a hydrophilic sugar group or with quaternary ammonium and phosphate groups (Table S3, ESI[†]). We chose an initial lipid concentration of 1.5 mM, corresponding to the highest critical micellar concentration (CMC) amongst all

Table 1 Screen of alkynes in micelle-assisted Kinugasa reactions

		
Entry	Alkyne	Conversion ^a (Isolated yield %)
1		38 (22 ^b)
2		76 (16 ^b)
3		85 (20 ^b)
4		90 (32 ^b)
5		95 (69)
6		81 (64)
7		91 (65)

^a Reactions were conducted in 20 mL Argon degassed H₂O containing 10 mM SDS. Sodium ascorbate (0.2 mmol), pyridine (0.1 mmol), L-proline (0.05 mmol) and CuSO₄ (0.025 mmol) were added successively. Following the addition of alkyne (0.05 mmol), C,N-diphenylnitron (0.05 mmol) was added and stirred for 30 minutes at 25 °C. NMR yields are reported following extraction with 3 × 20 mL EtOAc, using 1,4-dimethoxybenzene as internal standard. ^b Conducted with 3.5 mM CTAB in the absence of L-proline.

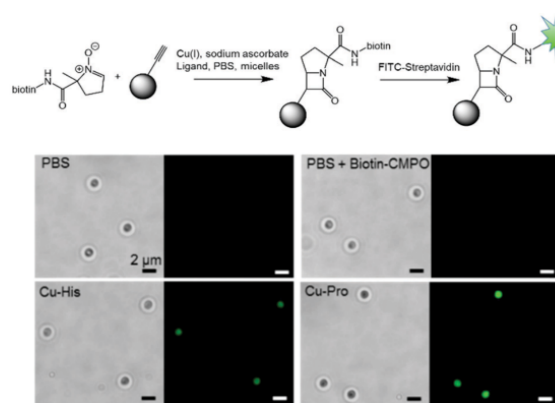


Fig. 2 Effect of ligand on CuANCR labelling of alkyne-tagged magnetic beads. Alkyne beads were incubated for 30 minutes at 37 °C in PBS, PBS with biotin–CMPO or PBS containing 100 μ M CuSO₄, 200 μ M ligand, 2 mM sodium ascorbate and 50 μ M biotin–CMPO. Beads were washed in PBS then stained with FITC–streptavidin prior to imaging. Scale bars = 2 μ m.

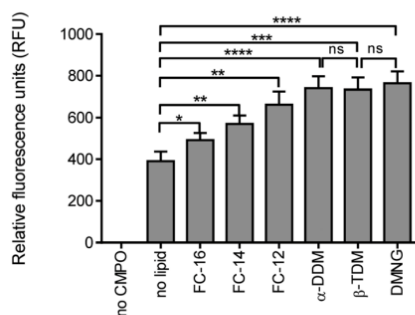


Fig. 3 Lipid screen for micelle-assisted Kinugasa/CuANCR reaction. Alkyne-tagged magnetic bead assay was used. Beads were labelled via CuANCR reaction in 100 μ M CuSO_4 , 2 mM sodium ascorbate, 200 μ M L-proline and 50 μ M biotin-CMPO, in presence of 1.5 mM of indicated lipid. Reaction was carried out in PBS for 30 minutes at 37 $^\circ\text{C}$, after which beads were washed in PBS and incubated with 5 $\mu\text{g mL}^{-1}$ streptavidin-FITC, then washed. Above background fluorescence for at least five beads from two separate field views (10 beads total) was used to determine the average fluorescence of beads for each condition. * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

screened lipids (Table S3, ESI[†]), to ensure that CuANCR is conducted in a micellar environment. The background fluorescence, determined for beads incubated without biotin-CMPO and with Fos-choline 16 (arbitrarily chosen amongst screened surfactant), was subtracted from all samples. All examined lipids contributed to a more efficient CuANCR labelling of alkyne beads (Fig. 3), and maltopyranoside-functionalized lipids increased the efficiency about 2-fold. These results confirmed our hypothesis that biomimetic lipids can significantly accelerate CuANCR reactions.

To further optimize CuANCR reactions for future bioorthogonal applications, we selected an optimal lipid to examine the effects on the rates of reactions. We chose β -TDM, which has the lowest CMC amongst the three maltopyranoside lipids, for further CuANCR condition optimization. We then focused on optimal conditions of this biological lipid for CuANCR, focusing on sub-micellar and micellar conditions. A concentration screen showed that at CMC of β -TDM in water (10 μM), the CuANCR labelling of alkyne beads is roughly as efficient as in 10-fold or 100-fold excess of β -TDM (Fig. S3–S5, ESI[†]). However, in absence of β -TDM and in sub-micellar concentrations of β -TDM, the labelling efficiency drops to or below 50% in comparison, showing the importance of the presence of β -TDM micelles for optimal CuANCR labelling. This suggests that a micellar environment, and not individual lipid species are responsible for catalysis.

Next, we investigated the kinetics of CuANCR labelling in micellar β -TDM, seeking insight in the possible mechanism of micelle catalysis of CuANCR (Fig. S6, ESI[†]). After 5 minutes of CuANCR labelling, there is a significant difference between micelle-assisted CuANCR, and surfactant-free reaction. Under pseudo-first order kinetics with 50 μM biotin-CMPO nitron in 10-fold excess over alkyne, we were able to estimate a $k_{\text{obs-micelle}}/k_{\text{uncat}}$ ratio of ≥ 100 , suggesting that presence of micelles contributes to a faster rate of CuANCR, possibly by solubilizing hydrophobic reactants and effectively increasing their local

concentration, or orienting the reactants for optimal reaction trajectory. We envision membrane and biological lipids to play a similar role for bioorthogonal applications; allowing for greater labelling efficiencies and enhanced reaction rates on cells.

To demonstrate the applicability of micelle-assisted CuANCR chemistry, we performed biotin labelling of a small membrane-associated peptide from the class of cell-penetrating peptides, E5-TAT.^{52,53} Membrane proteins represent an important class of protein acting as membrane receptors, integral structural components and transporters, amongst other functions. Their isolation and functionalization can be challenging requiring hydrophobic environments to maintain proper structure and function. Micellar conditions represent a suitable system in which these membrane proteins can be solubilized and modified.⁵⁴ E5-TAT is a small membrane-associated peptide that contains a fusion between HIV-TAT protein transduction domain (PTD) and a variant of the influenza HA2 domain that inserts into lipid membranes and destabilizes them at low pH.^{52,53} It was chosen as a model peptide to which an alkyne handle could be appended through lysine residues on the cationic TAT sequence (KKRRQRRR). To accomplish labelling, the peptide samples were treated with an *N*-hydroxysuccinimide ester alkyne for 1 hour under micellar conditions (Fig. 4). The alkyne modified peptides were purified by FPLC and modification of expected lysine residues (K26, K27) was confirmed by mass spectrometry analysis (see Fig. S8 and Tables S4, S5, ESI[†]). The modified peptides were then submitted to CuANCR conditions for 1 hour to conjugate a biotin moiety, using biotin-CMPO as the nitron reacting partner. After the two-step labelling protocol, the peptide samples were analyzed by Western blot with results showing a

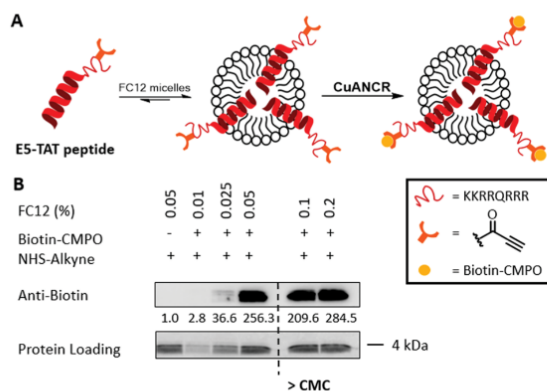


Fig. 4 Biotin conjugation of E5-TAT membrane peptide using CuANCR. (A) Schematic representation depicting the two step biotin conjugation of E5-TAT peptide under micellar assistance. Alkyne functionalization of E5-TAT (20 μM) was carried out using *N*-hydroxysuccinimide ester alkyne (300 μM). Biotin labelling was achieved by treating modified peptide with $\text{CuSO}_4 \cdot \text{H}_2\text{O}$ (20 μM), sodium ascorbate (300 μM), L-proline (40 μM) and biotin-CMPO (300 μM). (B) Top panel represents the Western blot analysis of biotin labelled E5-TAT samples under varying percentages (w/v%) of detergent (FC12) ranging from 0.01–0.2%, CMC = 0.05%. Bottom panel represents the TGX-Stain free blot as a loading control. The presented relative biotin conjugation values consist of densitometry analysis normalized to protein loading.

significant difference in the relative biotin conjugation between the micelle-assisted CuANCR conditions ($\geq$ CMC) and the surfactant-free conditions (>250 fold) (see Fig. S7 (ESI[†]) for non-modified peptide control). These results emphasize the importance of micellar conditions not only for peptide solubility, but as well for achieving robust peptide modification.

In conclusion, we have found that lipids, biological ligands and electron poor alkynes help accelerate and increase the yield of the CuANCR reaction developed from the prototype Kinugasa reaction. The use of micelle catalysis consists of a cost-effective platform on which bioorthogonal reaction development can take place. We have also shown that membrane protein modification is possible through CuANCR chemistry, highlighting the bio-orthogonal nature of the reaction and value for rate acceleration in micellar environments for selective membrane protein modifications. We envision that new findings from optimized CuANCR reactions could be applied in a bioorthogonal manner for *in vitro* labelling to live cell labelling. The development of the CuANCR reaction could also allow for the synthesis of bioactive β -lactam products in biological system *via* membrane lipids, allowing for further functionalization and engineering of cell membranes. Further studies are under way to develop applications of CuANCR and expand upon the potential chemistry of the produced β -lactams.

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Conflicts of interest

There are no conflicts to declare.

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2.4. Development of dual-labelling strategy employing CuANCR reactions

Previous work by our lab has shown CuANCR to be amenable to aqueous conditions and applicable to cell labelling applications. While β -lactams are often exploited for their antibiotic properties, they represent a reactive chemical motif whose electrophilic properties allow for ring opening through nucleophilic addition^{225,226,243}. CuANCR chemistry could thus allow for the covalent linkage of two reacting partners, followed by further modification/labelling via β -lactam ring opening chemistry. This two-stage labelling strategy opens the door to a broad range of bioorthogonal applications, such as multiplex labelling, targeted drug delivery and better temporal control over modification.

In earlier experiments conducted by Dr. Allison Sherratt, the applicability of CuANCR reactions for the labelling of live mammalian cells was demonstrated. Labelling was achieved using alkyne-derivatized D-mannosamine which was metabolically incorporated into cell surface glycoproteins of human hepatoma cells. Given the increased reactivity of propiolamides towards cycloaddition, a metabolic label was synthesized by coupling propiolic acid with D-mannosamine to form D-mannosamine-oxo-propyne (see Figure 2.4). The reactivity and labelling efficiency of this activated alkyne was compared to a similar, but unconjugated alkyne derivative, D-mannosamine-alkyne²⁴¹. D-mannosamine-oxo-propyne, or D-mannosamine-alkyne were added to growth medium of Huh 7.5 cells and was incorporated in cell surface glycoproteins. Labelling of the alkyne metabolic labels on cell surface with biotin-CMPO or biotin-N-phenyl-nitrone via CuANCR showed a higher labelling efficiency when an electron-poor alkyne was used (Figure 2.4, right column). Interestingly,

for both electron-poor and electron-rich alkynes, the use of L-proline ligand led to strikingly better labelling than when L-histidine was used (Figure 2.4(d)).

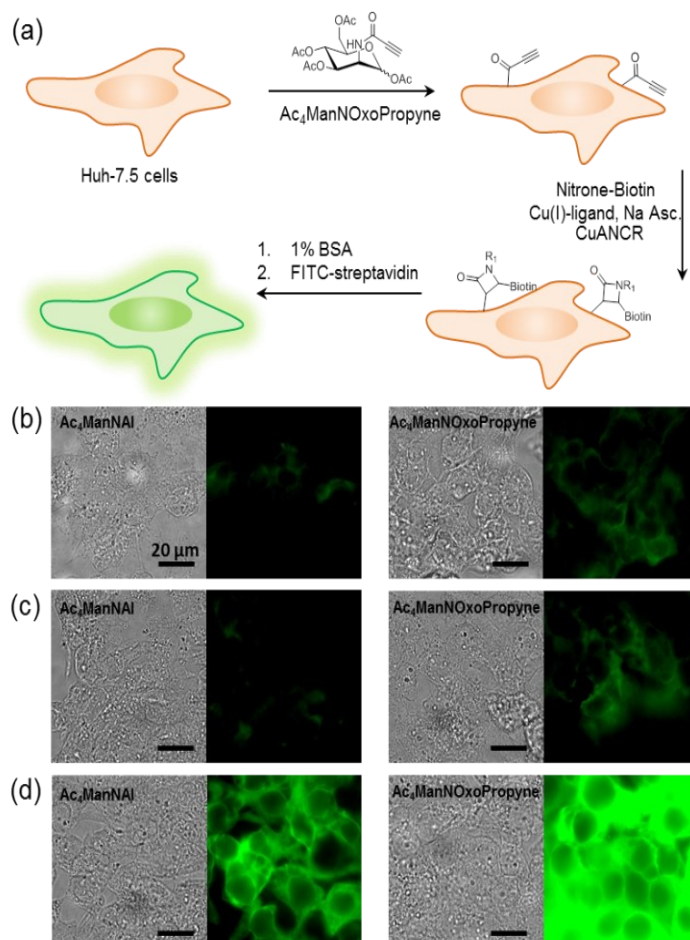
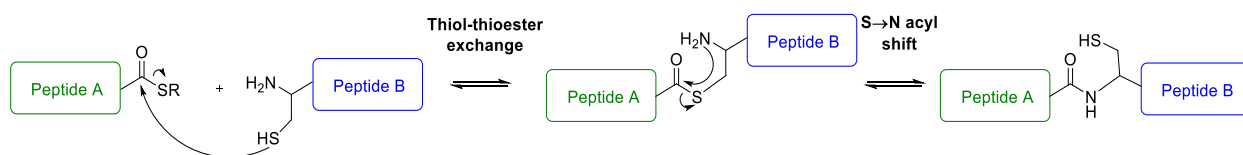
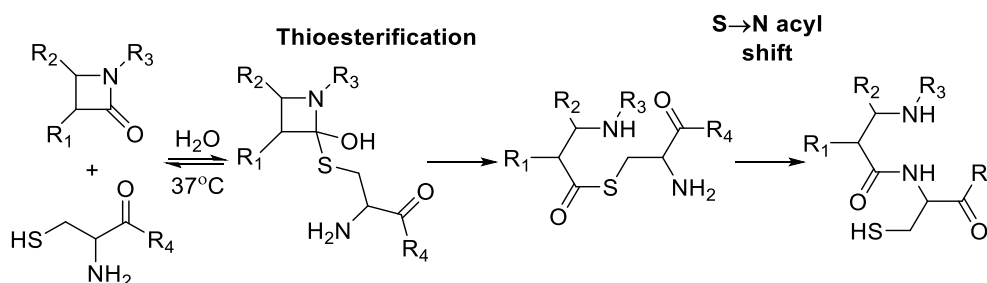


Figure 2.4 . *In situ* labelling of Huh-7.5 human hepatoma cell surfaces by CuANCR. (a) General scheme for metabolic incorporation and fluorescence detection of alkyne-tagged mannosamine. Huh-7.5 cells were cultured in the absence or presence of 10 μ M Ac₄ManNAIkyne or 10 μ M Ac₄ManNOxoPropyne for 72 hours. Cells were then treated for 30 minutes at 37 °C with either (b) 50 μ M Biotin-CMPO in PBS containing 0.1 mM CuSO₄, 0.2 mM L-histidine, 2 mM sodium ascorbate; (c) 50 μ M Biotin-*N*-Phenyl-nitrone in PBS containing 0.1 mM CuSO₄, 0.2 mM L-histidine, 2 mM sodium ascorbate and 100 μ M *N*-phenyl-hydroxylamine; or (d) 50 μ M Biotin-*N*-Phenyl-nitrone in PBS containing 0.1 mM CuSO₄, 0.2 mM L-proline, 2 mM sodium ascorbate and 100 μ M *N*-phenyl-hydroxylamine. Cells were blocked with 1% BSA in PBS, then stained with 5 μ g/ml FITC-streptavidin. All fluorescence images are displayed with identical brightness intensity range, and above background signal obtained for cells cultured in the absence of alkyne-tagged mannosamine. Scale bars = 20 μ m.

To demonstrate the potential for further functionalization of the CuANCR-derived β -lactams, we established reaction conditions for electrophilic ring opening. We postulated that β -lactam ring opening with *N*-terminal cysteine residues should yield intermediates reminiscent of native chemical ligation reactions, ultimately leading to a stable amide bond linkage (see Scheme 2.7)¹⁹. Native chemical ligation represents a widely employed technique in peptide synthesis allowing for the conjugation of two different peptides via a two-step mechanism forming a new amide linkage. The initial step consists of the nucleophilic attack of the *N*-terminal cysteine residue's thiol or thiolate of one peptide fragment to the C-terminal thioester of a second unprotected peptide (see Scheme 2.6)¹⁸. The formed thioester intermediate can then rapidly undergo a rearrangement via an intramolecular S $\rightarrow$ N acyl shift which results in forming the amide linkage between the two peptide fragments. The reaction occurs under mild aqueous conditions at room temperature, making variations of the reaction amenable to bioorthogonal applications.

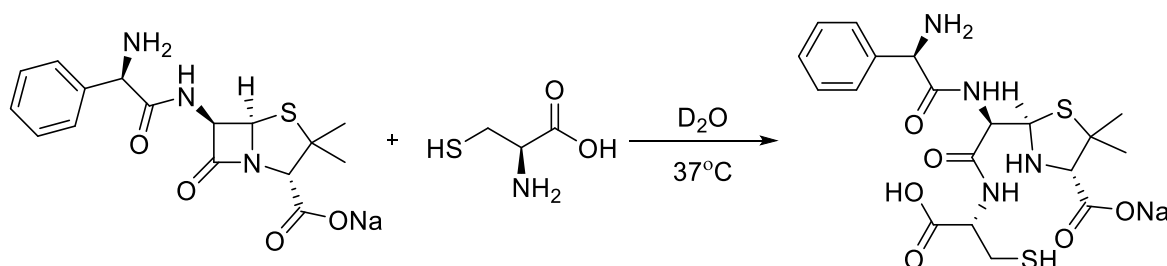


Scheme 2.6 Prototypical Native Chemical Ligation Mechanism



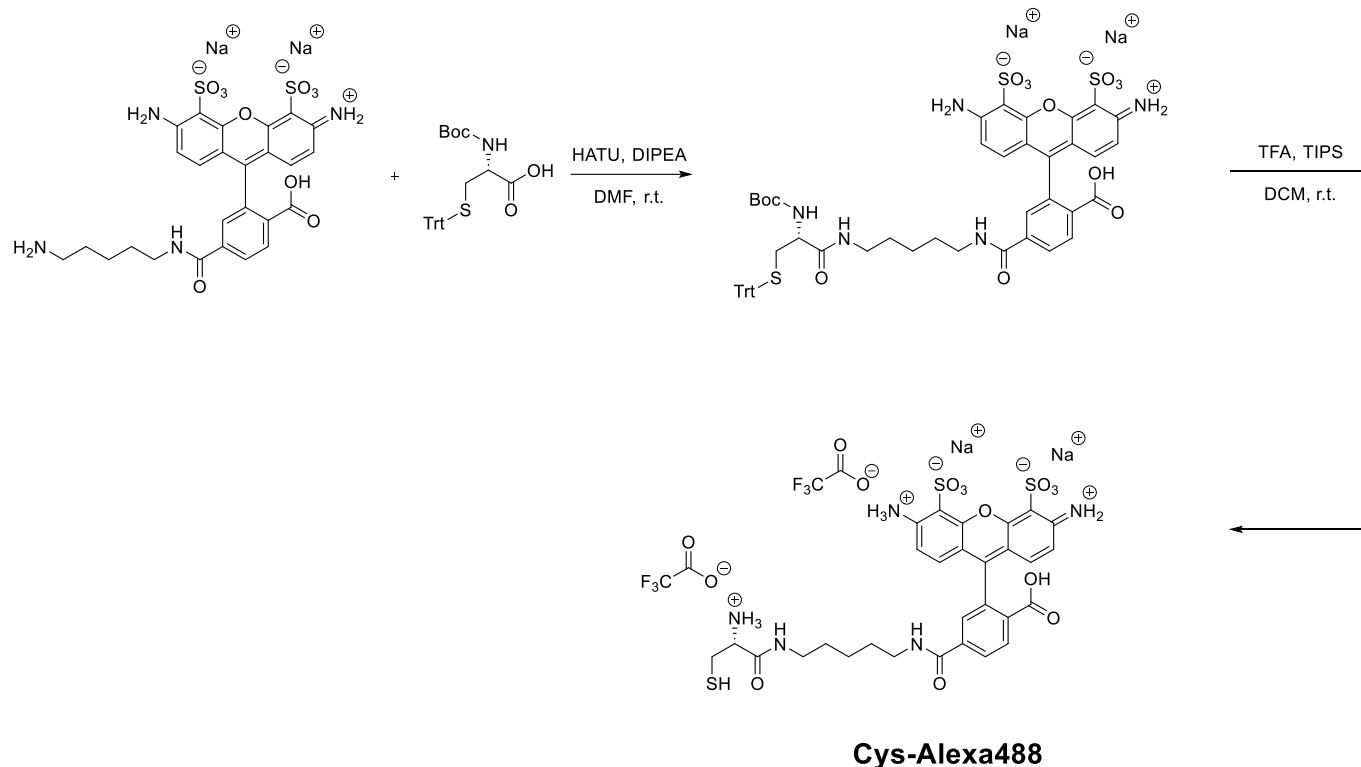
Scheme 2.7 Proposed native chemical ligation via thiol addition to β -lactam products.

To verify that addition of thiols would be feasible to β -lactams, proof of concept experiments were performed with a reactive β -lactam model compound, ampicillin. Ampicillin and cysteine were mixed in a 1:2.5 ratio in D₂O at 37 °C and the reaction was monitored by NMR. The reaction with cysteine (Cys) worked best in aqueous solutions where the β -lactam nitrogen could become protonated to make ring opening facile. Products consistent with ring opening, followed by rearrangement to a stable amide were observed by NMR and the product mass confirmed by UPLC-MS (see Scheme 2.8). The use of aryl thiol catalysts, such as 2-aminothiophenol, 4-aminothiophenol and 4-mercaptophenylacetic acid, was also briefly explored during these initial trial experiments, but were ultimately not pursued due to the noted presence of complex reaction mixtures and side products. Given that the initial nucleophilic addition to ampicillin was successful, we postulated that the reaction could be optimized, and its feasibility demonstrated on less reactive β -lactam products formed after CuANCR reactions. In order to more quickly optimize reaction conditions and mimic conditions more similar to those amenable to bioorthogonal applications, we performed proof-of-concept fluorescence labelling experiments using alkyne functionalized magnetic beads (see Figure 2.5).



Scheme 2.8 Ampicillin β -lactam ring opening via cysteine addition.

We envisioned employing the same micelle-assisted CuANCR reactions to form β -lactam products, while using magnetic core polymer beads to allow for facile separation of reaction products. As with the optimization of aqueous Kinugasa conditions, the use of biomimetic surfactants would allow for the proper solubilization of all reagents, while providing a more similar lipid environment to the one found on complex cell bilayers; with the goal of performing dual labelling on cell surfaces. The use of magnetic beads eliminated the need for the difficult purification of products observed when working under surfactant conditions, thus allowing for a rapid evaluation of various reaction conditions. To establish two stage labelling with CuANCR, followed by β -lactam-derived native chemical ligation, we utilized this alkyne-tagged magnetic bead assay to demonstrate that β -lactams derived from the nitron, CMPO-biotin, could be functionalized with cysteine functionalized with Alexa-488. The assay consisted of performing the CuANCR β -lactam formation between CMPO-biotin and the alkyne covalently linked on magnetic beads. The β -lactam formation was then confirmed by further conjugation to Cyanine3 (Cy3) functionalized streptavidin. Following appropriate washing steps, native chemical ligation step was performed with treatment with synthesized Alexa-488 functionalized cysteine. The cysteine fluorophore was synthesized by the hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU) mediated amide bond formation between the sodium salt of Alexa488 cadaverine and Boc-Cys(Trt)-OH; the crude mixture was purified by HPLC. The purified protected cysteine fluorophore was then treated with trifluoroacetic acid (TFA) and triisopropyl silane (TIPS) for final deprotection to be used in labelling experiments without further purification after solvent evaporation (see Scheme 2.9).



Scheme 2.9 Synthesis of Alexa-488 Cysteine derivative.

Initial attempts at obtaining successful dual labelling showed poor labelling efficiency of the cysteine conjugation step; with no statistical significance shown between the nitron negative control and the fully treated samples. This issue in lower observed fluorescence was resolved by eliminating a 'bead blocking' step using milk and eliminating an ethylenediaminetetraacetic acid (EDTA) washing step after the CuANCR reaction (replaced with additional PBS washing steps). In these initial experiments, biotin conjugation, and thus successful CuANCR chemistry and β -lactam formation, was confirmed by detection of above background Cy3 emission after streptavidin (see Figure 2.6, left PBS results). The lower efficiency of observed Alexa488 emission for the fully treated beads was potentially

explained by the spectral overlap between the emission-excitation spectra of the chosen fluorophores, Alexa488 and Cy3; thus, lowering the observed Alexa488 emission when in proximity to a Cy3-moeity in the same molecule. Furthermore, given that the reactions were initially performed in phosphate-buffered saline (PBS) adjusted to a pH of 7.4, we hypothesized that a greater efficiency of the chemical ligation step could be achieved by increasing the pH of the buffer solution, such that more thiolate would be present in solution, thus favouring nucleophilic attack. Given that desired pH values of approximately 8.0 were beyond the buffer range of PBS, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer was prepared (pH adjusted to 8.1) and used for the cysteine addition step. This change in pH buffer led to a drastic increase in the observed Alexa488 emission (see Figure 2.6, right bars show HEPES results). As compared to 1.52 times greater Alexa488 emission between the negative streptavidin-Cy3 control and the nitron negative control under PBS conditions, the observed Alexa488 emission for the negative streptavidin-cy3 control was 3.75 times greater as compared to the no nitron control under HEPES conditions (see Figure 2.6). This observation seems to indicate that the greater pH of the buffer solution did in fact increase the efficiency of the cysteine chemical ligation step by promoting thiolate formation.

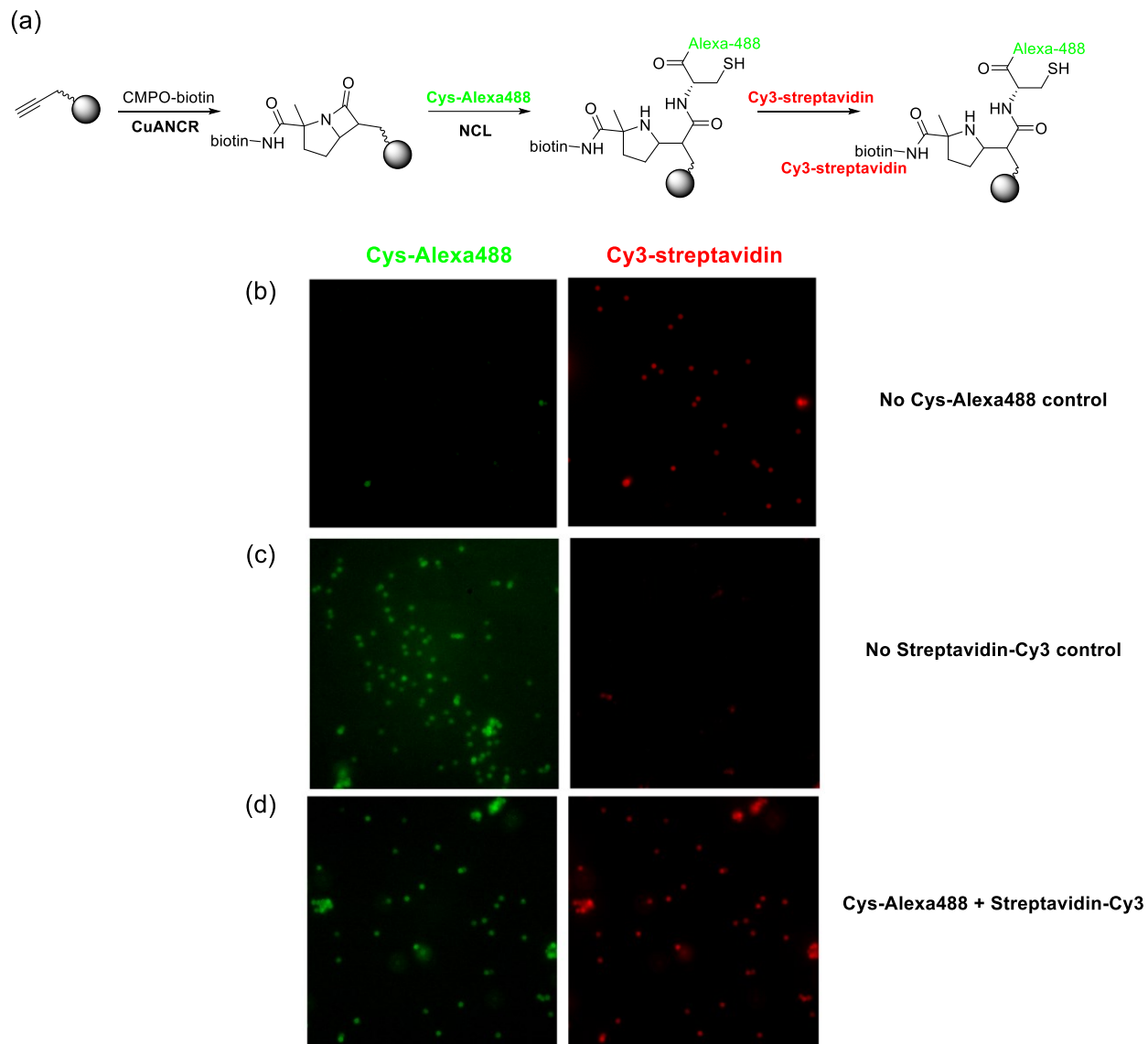


Figure 2.5 Dual labelling using CuANCR and native chemical ligation of alkyne-tagged magnetic beads. (a) General scheme for labelling. Alkyne beads were incubated for 30 minutes at 37 °C in HEPES buffer (pH = 8.0) (b) with biotin-CMPO followed by Cy3-labelled streptavidin; (c) with biotin-CMPO followed by C-terminal Alexa-488-functionalized cysteine; (d) with biotin-CMPO followed by both Cy3-labelled streptavidin and C-terminal Alexa-488-functionalized cysteine showing two stage labelling. Left images show the Alexa488 channel and the images on the right show the Cy3 channel. Legend: NCL = Native Chemical Ligation

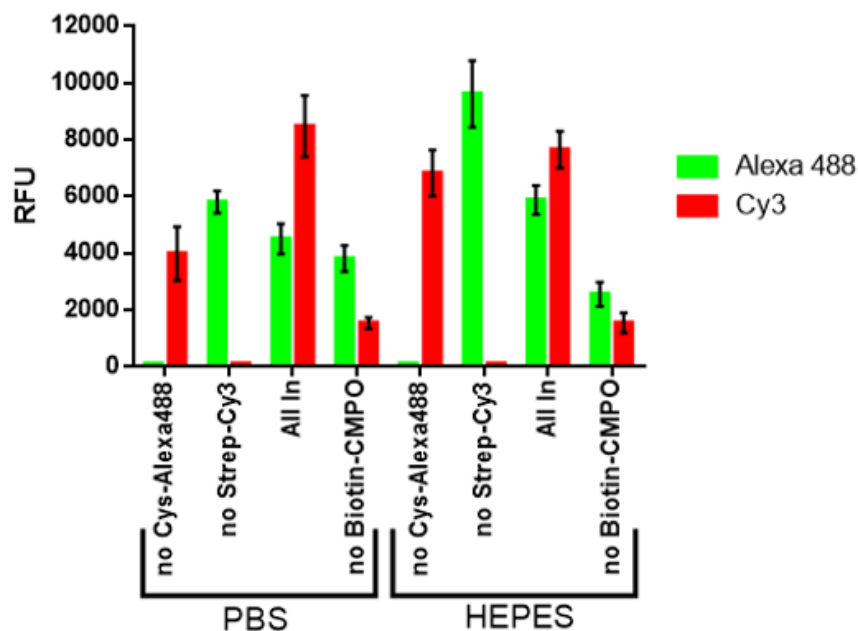


Figure 2.6 Effect of buffer and pH on dual labelling efficiency. Fluorescence signal is reported as RFU relative to the respective non-labelled control background images.

Given the success of the dual labelling strategy on magnetic beads and considering successful cell surface labelling demonstrated by experiments performed by Dr. Allison Sherratt (see Figure 2.2), we aimed to apply the dual labelling strategy to mammalian cell surface labelling following metabolic incorporation of bioorthogonal reacting partners. With the help of Dr. Geneviève Desrochers, Huh7 carcinoma cells were cultured in Dulbecco's modified eagle medium (DMEM) and treated for 72 hours with an alkyne-functionalized sugar, D-mannosamine-oxo-propyne (Ac₄ManNOxoPropyne). Following alkyne-sugar treatment, cells were fixed using acetone and pre-treated with cysteine in HEPES for 60 minutes at 37 °C, to limit non-specific binding of the cysteine probe following CuANCR chemistry. Following pre-treatment, the Kinugasa conjugation step was performed using developed conditions (biotin-CMPO nitrene, CuSO₄, sodium ascorbate, L-proline ligand),

followed by a blocking step with 1% BSA. Following β -lactam formation, the cells were incubated for 2 hours at 37 °C with the cysteine-Alexa488 fluorophore in HEPES buffer to perform the native chemical ligation step. Finally, cells were treated with Streptavidin-Cy3 for 30 minutes at room temperature to confirm the presence of the biotin moiety installed by the Kinugasa reaction. 4',6-diamidino-2-phenylindole (DAPI) mounting medium was added, and the cells were analyzed by ratiometric fluorescence microscopy (see Figure 2.7).

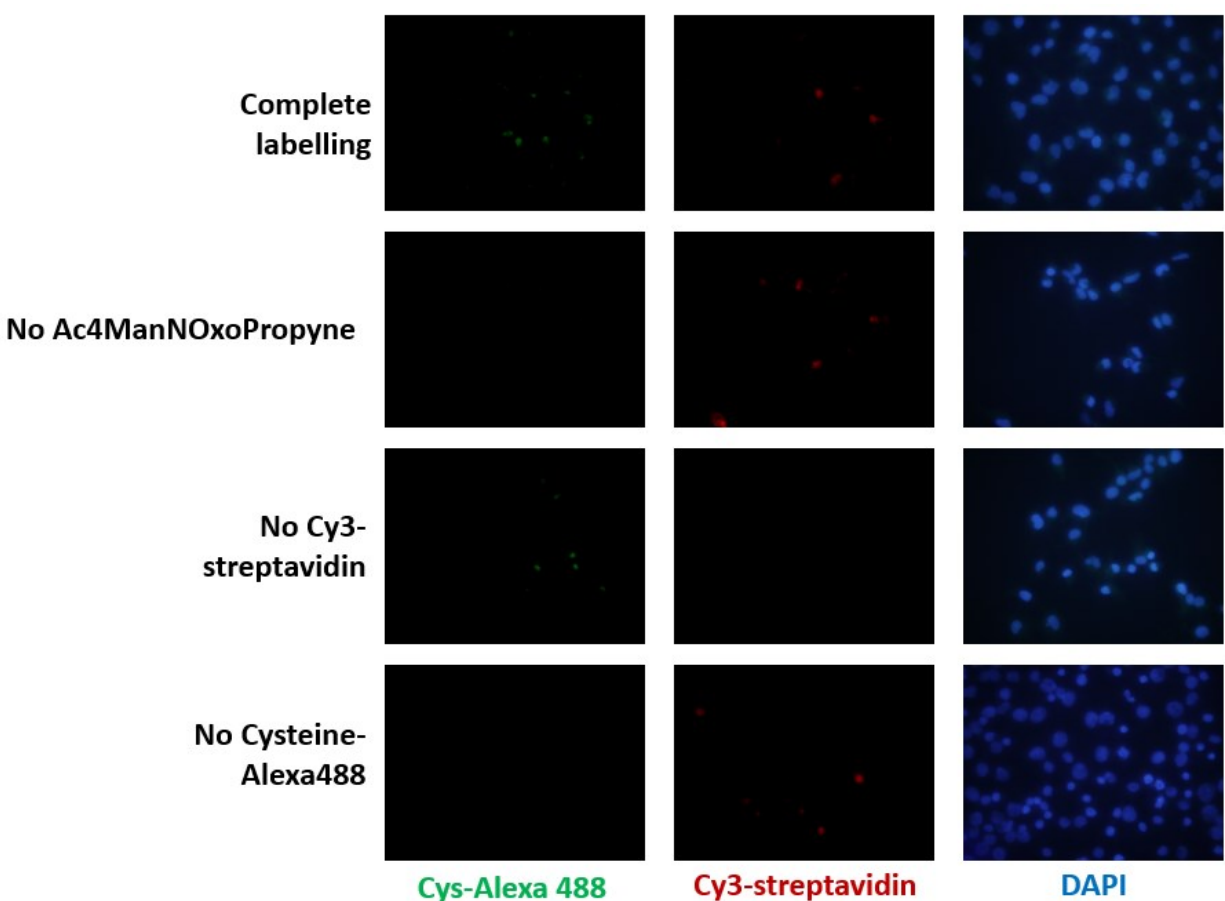


Figure 2.7 *In situ* labelling of Huh7 carcinoma cells by the two step CuANCR conjugation and cysteine native chemical ligation strategy. Left images show the Alexa-488 channel (496 nm), middle images show the Cy3 channel (569 nm), and the right images show the DAPI channel (461 nm).

Interestingly, dual labelling was indeed observed under the developed protocol (see supplementary information, Annex II), however the overall labelling efficiency was found to be rather poor. Overall, the mannosamine alkyne sugar seems to have been poorly metabolically incorporated into the cell surface; with overall low labelling resulting from both biotin-CMPO and Cysteine-Alexa488 conjugation, and issues of cell viability. Nevertheless, these results seem to indicate that there exists a sugar dependant conjugation of the cysteine-Alexa488 fluorophore, as evidenced by the comparison between the alkyne sugar negative control to the Cy3-streptavidin negative control and the fully treated cells. Initial trial experiments also seem to indicate lower viability of cells treated with the alkyne-sugar. In order to remediate these issues, future experiments would aim to incorporate a different functionalized sugar, such as the non-activated alkyne mannosamine sugar, Ac4ManNAlkyne, or a nitron-functionalized sugar and conjugating the nitron to biotin-alkyne. We also envisioned potentially demonstrating the dual labelling strategy on a simpler prokaryotic cell model, such as with the KDO-derivative metabolic incorporation to *E. Coli*; or through incorporation of nitron functionalized unnatural amino acids.

2.5. Summary and Conclusions

The optimization of the Kinugasa reaction under aqueous conditions has shown the to be amenable to a plethora of bioorthogonal applications, such as cell surface labelling and peptide conjugation, thus complimenting widely employed CuAAC transformations. The reaction with nitrones displays faster kinetics, as compared to cycloadditions involving azides, while also allowing for greater structural diversity and stereoelectronic tuneability. The use of lipid micelles for the rapid development of reaction conditions should also prove

useful for adapting novel organic transformations to aqueous conditions, and thus bioorthogonal applications. Micelles provide a simple and cost-effective mimic to cell membranes, and their application in the development of the Kinugasa reaction. While some biomimetic lipids were applied during the presented study, the obtained results warrant further investigation is the use of a wider variety of biomimetic lipids and combinations of lipids, in order to arrive at conditions that more closely resemble the native cellular environment. The use of micelle-assisted CuANCR reactions was also shown to be suitable for the functionalization of lipophilic biomolecules, such as membrane-bound proteins. The use of micelles in such applications allows for both the proper solubilization and maintenance of protein structure, and the acceleration of the conjugation reaction. Furthermore, a wide variety of β -lactam product can be accessed by modifying the nitron structures, allowing both acyclic and cyclic nitrones to be used. While providing covalent connectivity between the two reacting partners, the β -lactam products can be further exploited for their electrophilic properties. Future studies could focus on developing the *in situ* formation of bioactive β -lactams through CuANCR chemistry; potentially allowing for the preparation of biomaterials displaying antibacterial properties, with scaffolds similar to carbapenem antibiotic structures being accessible by careful choice of cyclic nitron structure²²⁷. Future studies should also focus on further developing the dual labelling strategy where further functionalization of conjugated products is achieved by nucleophilic addition to β -lactams. This approach would allow for multiple functionalities to be installed on a single target biomolecule, for example allowing for fluorescence tracking of targeted drug delivery.

Chapter 3 Mechanistic Analysis of Bioorthogonal Double Strain-Promoted Alkyne-Nitrone Cycloadditions Involving Dibenzocyclooctadiyne

3.1. Introduction

Strain-promoted cycloaddition reactions have allowed for a wide variety of bioorthogonal applications to be developed^{6,244,245}. By taking advantage of the energy released by reduction of strain of dipolarophiles upon cycloaddition, conjugation reactions can take place without the need for additional catalysts or high temperatures. The strain-promoted azide-alkyne (SPAAC) was the first example applied *in vivo* and on cells by Bertozzi and coworkers⁸⁴. SPAAC has been found applicable to a great multitude of applications as advances have been made to dipolarophile (strained alkyne) structures and reaction conditions. It has not only been applied in the context of biological studies, but also in the development of new polymer chemistries. The high reaction efficiency, high rates, and mild conditions (aqueous conditions, no catalyst, no initiator) displayed by strain-promoted reactions (also termed Click reactions) make them attractive options for polymerization chemistry. By employing bifunctional monomers containing two strained alkyne or two azide moieties, SPAAC reactions can be leveraged to synthesize new polymer structures²⁴⁶⁻²⁵². Namely, applications which employed monomers with two azide moieties and two dibenzocyclooctyne (DBCO) moieties have found success in the synthesis of polymers and cross-linked gels (see Figure 3.1)²⁴⁶. This work highlighted the ease of preparation of bifunctional azide monomers, but the synthesis of bifunctional strained alkyne monomers was much more difficult to achieve. An alternative to these monomers which are functionalized with two DBCO moieties consist of dibenzo-1,5-cyclooctadiene-3,7-diyne

(diyne, also referred to as Sondheimer diyne) scaffold structures, which contain two strained alkyne functionalities in a single molecule, which are readily accessible and offer the potential for greater structural variety (see Scheme 3.1)^{253,254}.

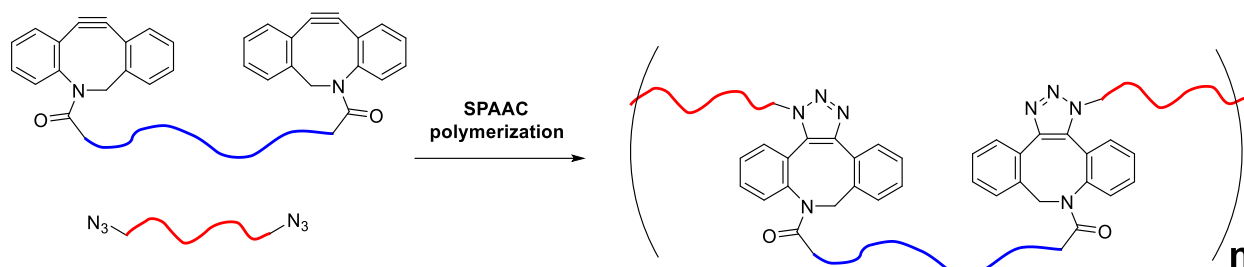
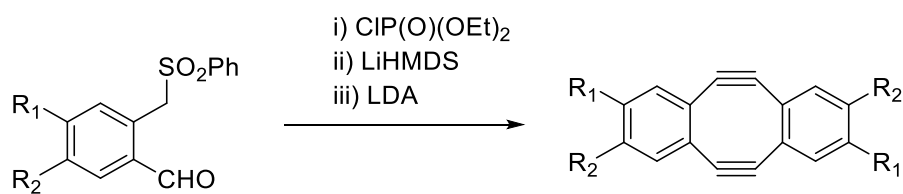


Figure 3.1 Strain-Promoted Azide-Alkyne cycloaddition-mediated step-growth polymerization²⁴⁶.

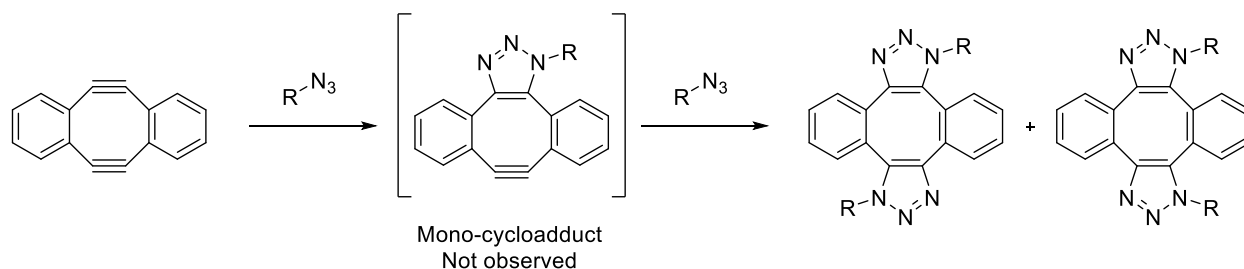


Scheme 3.1 Synthesis of dibenzo-1,5-cyclooctadiene-3,7-diyne scaffolds²⁵³. Legend: $\text{ClP}(\text{O})(\text{OEt})_2$ = Diethyl phosphochloridate, LiHMDS = Lithium bis(trimethylsilyl)amide, LDA = Lithium diisopropylamide.

These cyclooctadiynes have served as strained alkynes in a variety of bioorthogonal applications, such as cross-coupling of biomolecules and peptide stapling^{255,256}. These versatile dipolarophiles were investigated in the context of SPAAC chemistry, where the authors coined the term strain-promoted double-click reaction (SPDC)²⁵⁷. In this study, diyne scaffolds were shown to be amenable to a variety of bioorthogonal applications, acting as an adapter molecule for the conjugation of two azide containing molecules. Dienes were used to label HaloTag-glutathione *S*-transferase (GST) fusion proteins which were functionalized with azide via HaloTag incorporation, to an azide-functionalized fluorophore. The technique was also demonstrated by the three-step labelling of HEK293 cells, by first metabolically

incorporating tetraacetylated *N*-azidoacetyl-D-mannoasmine (Ac₄ManNAz), then treating the cells with diyne and finally achieving cell surface labelling by treatment with an azide-fluorophore. Similarly to other SPAAC applications, the reaction was found to be extremely efficient and display appropriate fast reaction rates. In studying the reaction using simple azide model molecules, the authors were unable to isolate any intermediate yielding from a single cycloaddition reaction (mono-cycloadduct) (see Scheme 3.2), with the reaction with benzyl azide yielding 60% *trans* product and 38% *cis* product. The lack of observed mono-cycloadduct was hypothesized to be due to great rate accelerations for the second cycloaddition step, after initial SPAAC reaction. DFT analysis of the reaction showed that the barriers for both cycloadditions were low, confirming the spontaneous nature of the reaction; with the activation energy of the second cycloaddition being smaller than for the first (+8.8 and +9.5 kcal mol⁻¹ for the *trans* and *cis* products vs +12.4 kcal mol⁻¹ for the mono-cycloadduct). They reported a second order rate constant of $(6.29 \pm 0.05 \text{ SE}) \times 10^{-2} \text{ M}^{-1} \text{ s}^{-1}$ for the first cycloaddition, a comparable rate constant to the reaction between benzyl azide and the monoyne, dibenzocyclooctyne (DIBO). The authors also noted that the Kohn-Sham HOMO and LUMO surfaces of the transition states confirmed the higher reactivity of the mono-cycloadduct, attributed to the highly distorted alkyne bond resulting from steric repulsion of the formed triazole ring. Furthermore, while comparing simultaneous and sequential biological labelling protocols, it was observed that sequential protocols, where the initial azide conjugation takes place with the diyne forming a mono-adduct which is then reacted with a second azide moiety in a subsequent step, allowed for proper labelling without observation of homodimer formation. Therefore, while under conditions employed for kinetic investigation and product characterization, the mono-cycloadduct was not

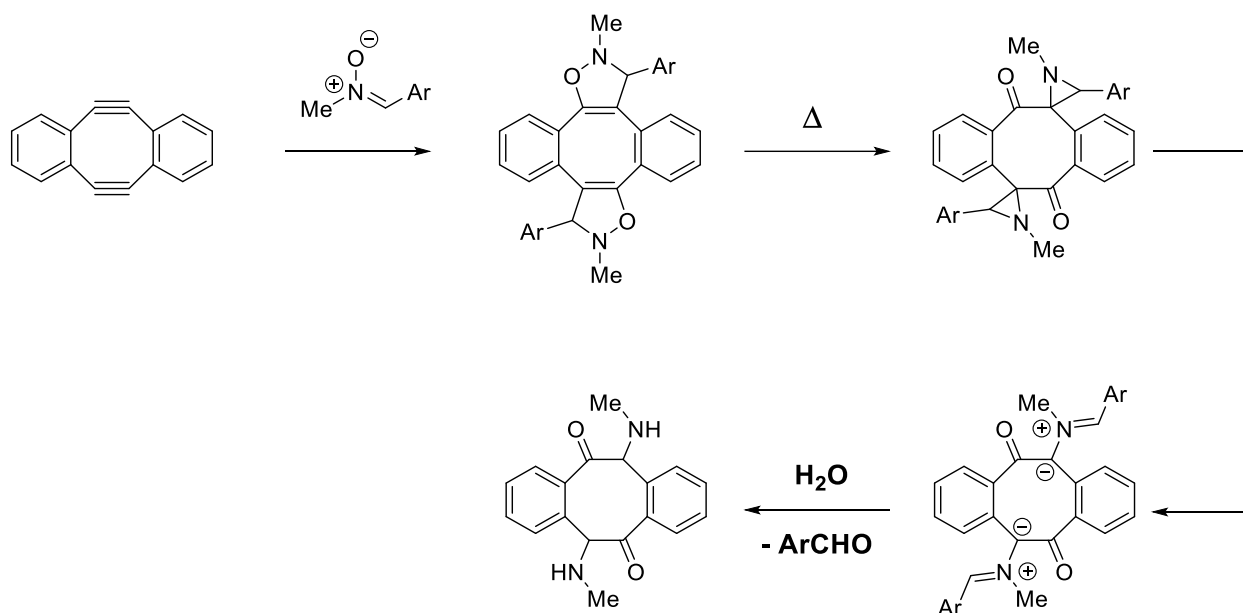
isolated or detected, under biological labelling conditions, the mono-cycloadduct could have a longer lifetime in solution when conjugated to biomolecules, explained due to more dilute concentrations employed and lower motility of large biomolecules.



Scheme 3.2 Azide-Alkyne strain-promoted double-click reaction by Kii *et al*²⁵⁷.

These diyne scaffolds have also been studied in the context of polymer synthesis, where Liu and coworkers exploited the self-accelerating nature of the dual cycloaddition reactions to achieve stoichiometric imbalance-promoted step-growth polymerization of novel high molecular weight ($>10^5$ g/mol) polymer structures using Sondheimer diyne and various non-azide dipoles²⁵⁸. In reactions with diazo dipoles, sydnone dipoles and N-methylnitrone dipoles, higher rate constants were observed for the second cycloaddition reaction, as compared to the rate-determining initial cycloaddition; k_2/k_1 ratios of 180, 246 and 230 respectively and k_2 values in the range of 10^1 M⁻¹s⁻¹ for the second cycloadditions (where k_1 represents the determined second-order rate constant for the first cycloaddition k_2 represents the determined second-order rate constant for the second cycloaddition). Once again, the mono-cycloadducts were not observed, even when monitoring the reaction mixture by ¹H-NMR. These results were of special interest to our research group, as they showed that reactions between nitrone dipoles and diyne displayed faster kinetics than the analogous reaction with azides, with reactions taking place under mild, catalyst-free

conditions. The cycloadducts formed from cycloadditions with sydnone and diazo dipoles showed high molecular weight, good thermal stability, and polymerization was complete in under 1 hour. However, the polymers formed from the reaction with nitron dipoles showed notably lower thermal stability, and a greater propensity for self-degradation as the polymer molecular weight increased. This lack of thermal stability was explained by the N-O bond of the formed isooxazoline heterocycles displaying low stability and undergoing rearrangement reactions to form azo-methine ylide intermediates. Subsequent hydrolysis of the azo-methine ylide then produces an σ -amine-ketone and releases the aldehyde by-product (see Scheme 3.3)^{200,259,260}.



Scheme 3.3 Thermal decomposition of isooxazoline heterocycles formed by strain-promoted double cycloaddition reactions with diynes.

Given that in this study the authors solely employed *C*-aryl-*N*-methyl-nitron dipoles, which have been shown to be less hydrolytically stable than other diaryl acyclic nitrones and cyclic nitrones, we envisioned studying the strain-promoted double-cycloaddition between

Sondheimer diynes and a greater variety of nitron structures to better characterize the reaction kinetics and attempt to optimize the reaction towards bioorthogonal applications. The use of these various nitron structures would also allow us to explore other polymer/oligomer structures, while allowing us to make efforts to isolate and detect the mono-cycloadduct intermediates, as given the observed rate accelerations for the second cycloaddition, stable mono-cycloadducts could prove to be important dipolarophiles in a variety of applications necessitating highly reactive conjugating partners. Furthermore, given that previous studies only focused on unsubstituted diyne scaffolds, we were also interested in exploring the effect of substitution of the diyne structures on the reaction kinetic parameters, as well as the physical properties in relation to bioorthogonal reaction conditions; especially giving the highly hydrophobic properties of the unsubstituted diyne. Lastly, we aimed to demonstrate the advantages of using an adapter like molecule, such as the diyne, in order to achieve cell surface labelling with readily available and/or easily accessible heterologous dipoles, by performing heterologous cycloaddition reactions involving nitrones and azides.

3.2. Author Contributions

Synthesis of the nitrones used in the study was completed by **DAB** with the assistance of Dr. Shadi Sedghi Masoud (**SM**) for compounds **1g** and **1h**. Synthesis of diynes (non-functionalized and tetramethoxy-functionalized) was completed by **DAB**. Kinetic evaluation of the cycloaddition reactions between diynes and nitrones was accomplished by Kaitlyn Margison (**KM**), with assistance from **DAB** for reactions between tetramethoxy diyne (OMe-diyne) and nitrones, as well as for kinetic evaluation of reactions with cyclic nitron **1f** and

diyne. Dr. Jeffrey W. Keillor participated in useful discussions regarding the kinetic evaluation of the cycloaddition reactions, as well as prepared the sequential first-order reaction fitting model (and accompanying spreadsheet) used for data analysis/fitting (see Figure 1). Hammett plot analysis was completed by **KM** with assistance from **DAB** for data interpretation. Distortion/Interaction computational modelling was completed by Dr. Masaya Nakajima (**MN**), with data analysis completed by **DAB** with assistance from **MN**. Oligomerization studies and analysis were completed by **SM** with assistance from **DAB** for experimental design. Metabolic labelling of *L. innocua* was completed by **DAB**.

Mechanistic Analysis of Bioorthogonal Double Strain-Promoted Alkyne–Nitronene Cycloadditions Involving Dibenzocyclooctadiyne

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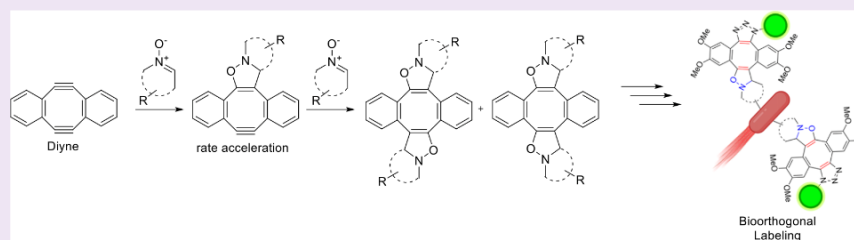
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ABSTRACT: The reactions of nitronenes with cyclooctadiynes were studied to establish the relative rates of sequential reactions and to determine the limits and scope of this bioorthogonal chemistry. We have established the second-order rate constants for the consecutive additions of a variety of nitronenes onto diyne and studied the structure–activity relationships via Hammett plots. Results show that the addition of the second nitronene to the monointermediate occurs significantly faster than the first, with both reactions being faster than analogous reactions with azides. Computational chemistry supports these observations. The rate of second addition increases with electron-deficient nitronenes, as demonstrated by a large ρ value of 2.08, suggesting that the reaction rate can be controlled by nitronene selectivity. To further investigate the kinetic parameters of the reaction, dinitronene monomers containing cyclic and diaryl-nitronenes were designed for use in oligomerization applications. Oligomerization was used as a probe to test the limits of the reactivity and attempt to isolate monocycloaddition products. The oligomer formed from a cyclic nitronene reacts faster, and detailed MALDI mass spectrometry analysis shows that monoaddition products exist only transiently and are not isolatable. These studies inform on the scope and limits of this chemistry in a variety of applications. We successfully demonstrated bacterial cell wall labeling using heterogeneous dual cycloadditions involving nitronene and azide dipoles, where the nitronene was the faster reacting partner on the bacterial cell surface.

Bioorthogonal chemistry has enabled the discovery of a multitude of new tools for the study of living systems at the molecular level.^{1,2} Cycloaddition reactions involving bioorthogonal dipoles and alkyne groups are a cornerstone of bioconjugate chemistry. These reactions can also be useful for purposes outside biological applications. Polymer synthesis employing 1,3-dipolar cycloadditions is well established.^{3–6} Recent reaction developments leading to rate accelerations have allowed for increased versatility.⁷ The strain-promoted azide–alkyne cycloaddition (SPAAC) reaction has been adapted to polymer synthesis, cross-linking bifunctional strained cyclooctyne monomer formed by connecting two dibenzocyclooctynes (DBCOs), and a second monomer functionalized two azides moieties.⁸ In this work, the preparation of diazido monomers was quite facile, but the DBCO monomers proved difficult to synthesize. The sym-dibenzo-1,5-cyclooctadiene-3,7-diyne (diyne) shown in Scheme 1, which can offer an alternative bifunctionalized

strained alkyne monomer, is easily synthesized and offers greater versatility.^{9,10}

Cyclooctadiynes have also recently emerged as a tool for cross-linking, stapling, and creating polymeric materials with distinctive properties.^{11,12} It was applied in SPAAC chemistry, termed the strain-promoted “double-click” (SPDC) reaction. Interestingly, the monointermediate was never isolated nor detected over the course of the reaction, suggesting that the monoyne is significantly more reactive than the unreacted diyne. The difference between the activation energy of the first and second cycloaddition was found to explain these

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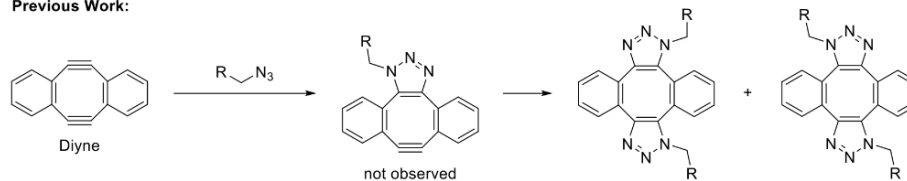
Accepted: September 20, 2023

Published: October 18, 2023



Scheme 1. Strain-Promoted Double-Click Reaction with Cyclooctadiyne and Azide and Nitron Dipoles

Previous Work:



This Work:

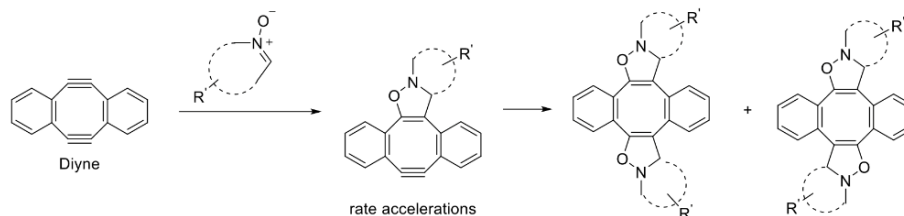
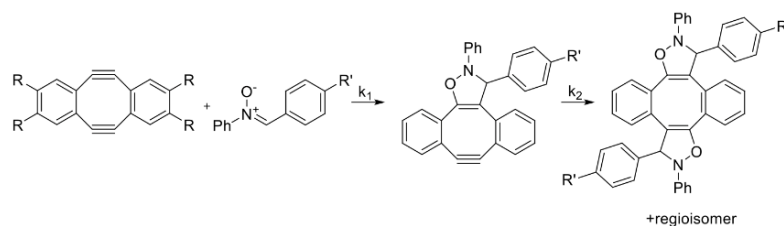


Table 1. Kinetic Evaluation of the Cycloaddition Reactions of Diyne and Acyclic Nitrones



nitron		k_1 ($M^{-1} s^{-1}$)	k_2 ($M^{-1} s^{-1}$)	rate constant ratio ($r = k_2/k_1$)
1a ^a	R'=H	0.17 ± 0.02	0.41 ± 0.09	2.41
1b ^a	R'=OMe	0.09 ± 0.01	0.07 ± 0.04	0.78
1c ^a	R'=F	0.19 ± 0.02	0.47 ± 0.06	2.47
1d ^a	R'=Br	0.17 ± 0.02	0.53 ± 0.10	2.94
1e ^a	R'=CN	0.20 ± 0.01	7.16 ± 0.13	35.8
1a ^b	R'=H	0.36 ± 0.02	1.08 ± 0.11	2.98
1b ^b	R'=OMe	0.14 ± 0.01	0.40 ± 0.06	2.92
1e ^b	R'=CN	0.51 ± 0.05	2.53 ± 0.42	5.02

^aR=H. ^bR=OMe.

observations, with calculated energies of +12.4 and +8.8/9.5 kcal/mol for the first and second (cis/trans adducts) cycloadditions, respectively. A second-order rate constant of $6.29 \pm 0.05 \times 10^{-2} M^{-1} s^{-1}$ was determined for the rate-determining step, the first cycloaddition reaction between the diyne with benzyl azide.¹³ This interesting phenomenon has allowed for interesting applications of the double cycloaddition chemistry in peptide stapling and DNA cross-linking, as examples.

Cycloadditions of cyclooctynes and nitrones have been shown by our group and others to be significantly faster than reactions with azides.^{2,14,15} Therefore, we sought to analyze the reaction of nitrones with cyclooctadiynes (Scheme 1) to determine whether a similar rate acceleration for the second reaction also occurs to gain potential mechanistic insights into reactions employing these bioorthogonal dipolarophiles. Herein we report rate accelerations for nitron cycloadditions with cyclooctadiynes, as well as a similar rate-limiting step to the dual reactions. We examined the scope and mechanism to determine what the limitations are to conducting nitron-

cyclooctadiyne cycloadditions and discovered both mechanistic insights into when the rate-limiting step is altered and structure-activity relationships that give insights into new potential cyclooctyne structures that should have heightened reactivity compared to those previously studied. These mechanistic findings helped to guide us in establishing conditions for successful live cell labeling using dual cycloaddition labeling.

RESULTS AND DISCUSSION

In order to assess initial reaction rates and structure-activity relationships, pseudo-first-order UV-visible kinetic experiments were performed with a series of acyclic nitrones bearing different α -substituted carbon (see Table 1). Although acyclic nitrones are less stable to hydrolysis when compared to cyclic nitrones, they are easily accessed and modified, which allows for rapid structure-activity relationships studies to be conducted.¹⁶ For Hammett analysis, substituted acyclic nitrones are the most straightforward method of determining the sensitivity of the general reactions to substituent effects.

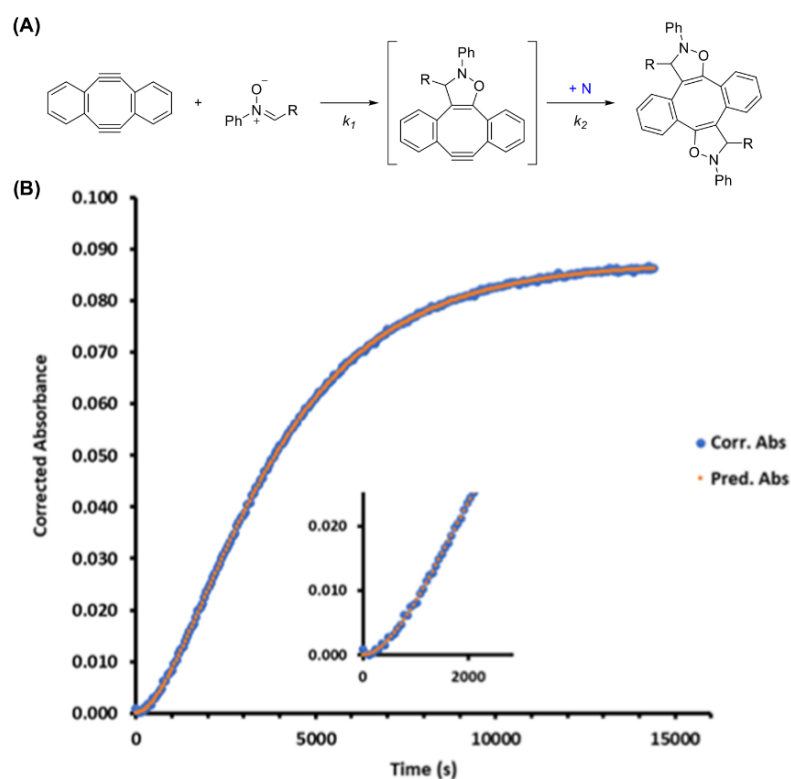


Figure 1. (A) Sequential strain-promoted alkyne–nitrone cycloadditions using acyclic nitrones for reaction kinetics evaluation (N = nitrone). (B) Sequential first-order fitting by the least-squares fitting model of the reaction between 1 mM C-*N*-diphenylnitrone and 25 μ M diyne, with the corrected and predicted absorbance. First 2000 s zoomed in to highlight the lag period.

Monitoring reaction kinetics via NMR was attempted, but due to the complexity of the resulting spectra, UV monitoring allowed for better tracking of both sequential cycloadditions, while product formation was confirmed using mass spectrometry. Kinetic analysis was done using rate laws and explicit equations for sequential pseudo-first-order reactions using nonlinear regression to determine k_1 and k_2 values (Figure 1). Based on studies performed with azide–diyne cycloadditions, it was assumed that the smaller rate constant corresponded to the first addition ($k_1 \leq k_2$).¹³ Using excess nitrone allowed for reactions to reach completion while providing a more accurate fit to the sequential reaction model (Figure 1B). Similar to studies with azides, it was found that the rate constants for the second cycloaddition step were greater than the rate constants for the first cycloaddition. Analyses of products of the reaction were consistent with the assumption that $k_1 \leq k_2$, in that monoaddition products were not observed (*vide infra*).

Detailed analyses of reaction rate constants using a sequential first-order model were performed for a number of substituted nitrones (Table 1 and ESI). The rate constants for the first cycloaddition were found to be comparable to those of other strain-promoted alkyne–nitrone cycloaddition (SPANC) reactions.¹⁷ We observed rate constants that were on par with those observed for cycloadditions between nitrones and 4-dibenzocyclooctynol (DIBO), which has structural and electronic similarities to the diyne.¹⁷ Generally, there is a

trend of increasing rate constants with increasing electron-withdrawing character of the nitrone substituent, with similar rates being observed ($k_1 \approx k_2$) for electron rich substituents, while much greater differences are being observed with electron-withdrawing groups (see Table 1). These trends suggest that the second cycloaddition is more sensitive to substituent effects. The first and second cycloaddition rate constants were also found to be greater when a tetramethoxy-substituted diyne was employed (Table 1). We note that there is a higher HOMO energy associated with the alkoxy-substituted diyne,¹⁸ leading to increased rate constants. Otherwise, for the tetramethoxy-substituted diyne, the same trend of increased rate constants with nitrones bearing electron-withdrawing groups was observed.

To further quantify the sensitivity of the reaction to substituent effects, Hammett plots were constructed, as has been previously performed for other studies of cycloadditions involving nitrones.¹⁹ Hammett plots were constructed for each addition step in the reaction sequence (see Figure 2). Hammett ρ values of 0.28 and 2.08 were observed for the first and second additions, respectively. The ρ value for the first cycloaddition is comparable to ρ values previously observed for cycloaddition between nitrones and biaryl-aza-cyclooctyne (BARAC), a similar strained and electron-deficient alkyne dipolarophile with a high level of electron density conjugation.²⁰

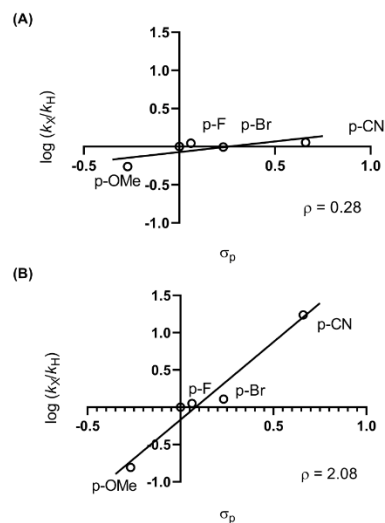


Figure 2. (A) Hammett plot depicting the substituent effects for the first (k_1) cycloaddition between acyclic nitrones and cyclooctadiyne. (B) Hammett plot depicting the substituent effects for the second (k_2) cycloaddition between acyclic nitrones and cyclooctadiyne.

In considering the relative magnitudes of ρ , we note that the observed small ρ values indicate a relatively low sensitivity to substituent electronic effects during the first cycloaddition. There is only a slight acceleration by electron-withdrawing groups. We observed similar values for ρ for the reaction of

nitrones with BARAC.²⁰ In both cases, there is little electron density build up at the nitron α -carbon during the transition state, consistent with a concerted transition-state mechanism. On the other hand, the second nitron cycloaddition to the monosubstituted cyclooctadiyne seems to be much more sensitive to substituent effects by comparison. The ρ value of 2.08 likely represents a nonsynchronous transition state where significant electron density is building up or even a stepwise addition.

In consideration of the differences in sensitivity for the second cycloaddition reaction, we looked to theory as a guide. For the azide–diyne double cycloaddition reactions, Kohn–Sham orbital amplitude plots of transition state structures show that the orbital coefficients of the single cycloaddition monoyne intermediate (LUMO) are localized at the alkyne carbons, possibly explaining the increased electronic sensitivity for the second cycloaddition.¹³ These differences in orbital coefficients are likely to be similar for cycloadditions of nitrones with cyclooctadiynes. Recently, distortion/interaction energies have been highlighted as an important consideration for cycloadditions of cyclooctynes with various dipoles, including nitrones.^{21–23} Given that distortion/interaction energies may play a more important role, we investigated the mechanistic possibilities further by conducting computational studies.

The distortion/interaction (D/I) model, developed simultaneously by the Houk and Bickelhaupt groups, is an invaluable tool to study bioorthogonal cycloaddition reactions and the substituent effects that affect activation barriers.^{21–23} The transition activation barrier energies and distortion/interaction energies were calculated by comparing azide and nitron reactivity as well as for a series of substituted acyclic diaryl-nitrones. Calculations were carried out using M06–2x functionals with the 6-311++G(d,p) basis set (see Table S13

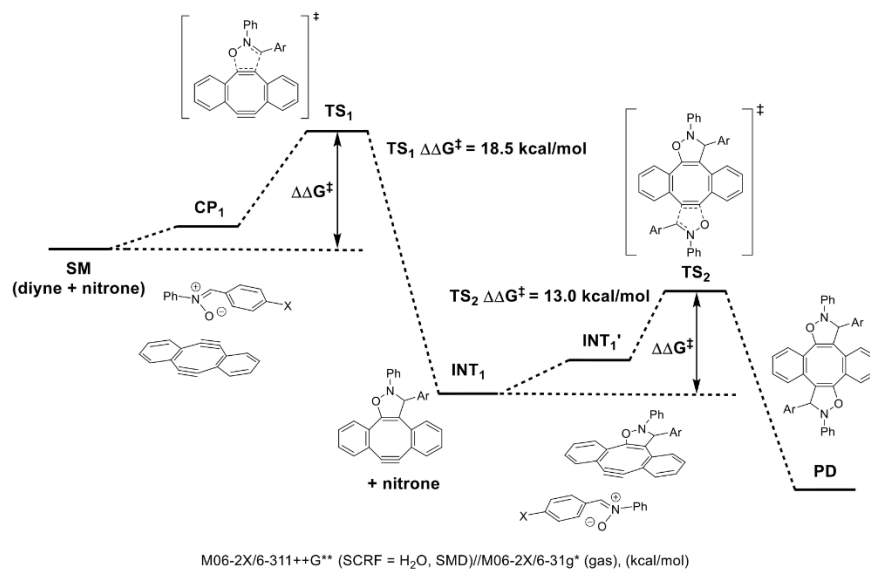
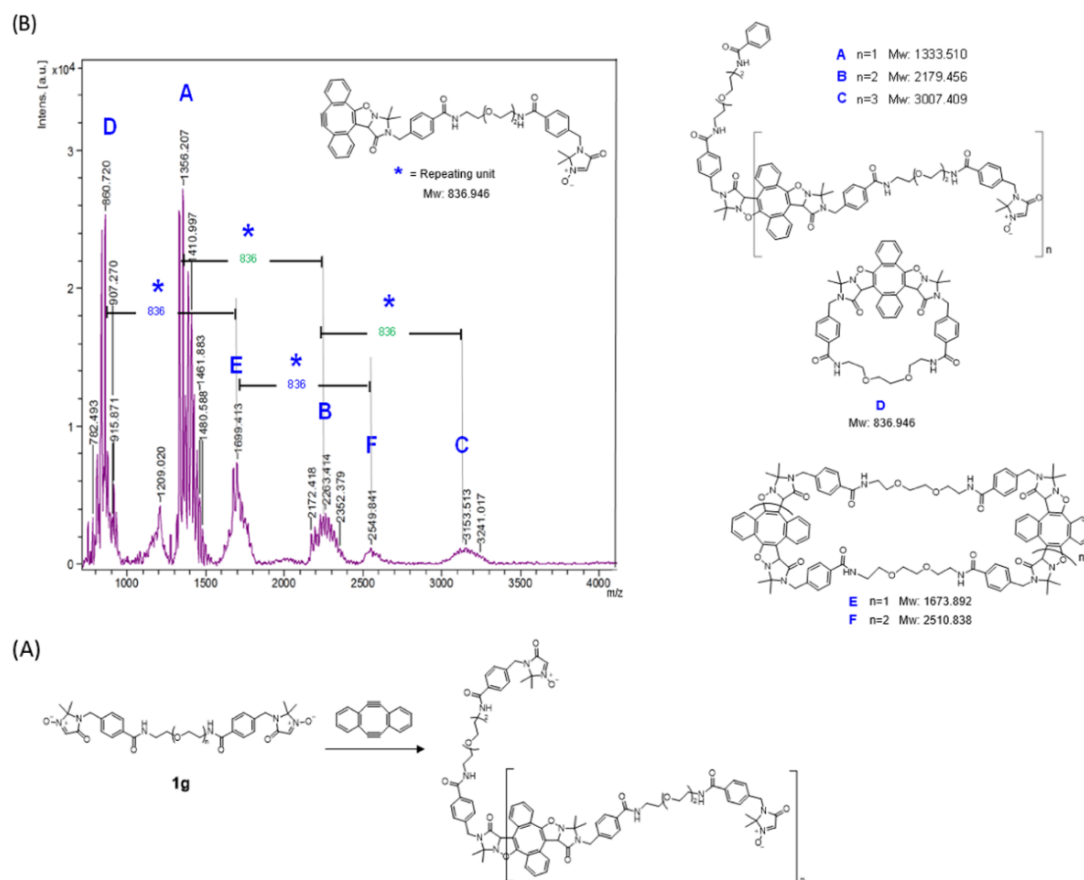
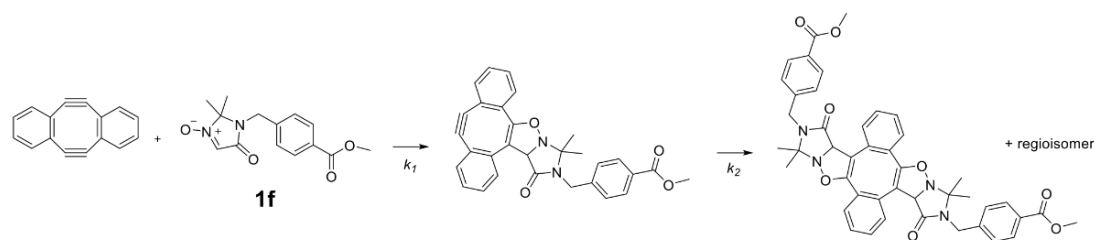


Figure 3. Calculated reaction coordinate diagram for the cycloaddition between nitron dipoles and cyclooctadiyne dipolarophiles. The activation energies are shown for X=H.

Scheme 2. Strain-Promoted Double-Click Reaction of Cyclic Nitron (DMImO) Monomers with Cyclooctadiyne

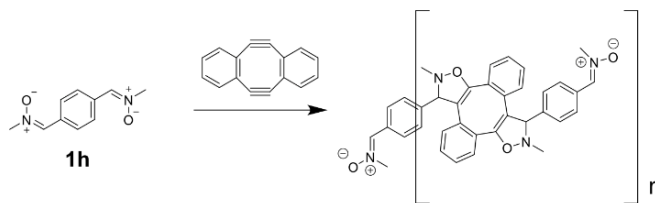
Figure 4. (A) Oligomerization achieved by cycloaddition of di-DMImO (**1g**) and cyclooctadiyne monomers. (B) MALDI mass spectrum of oligomer structures formed by SPANC polymerization of di-DMImO (**1g**) with cyclooctadiyne.

and Figure S15). Similarly to other strain-promoted dipole–alkyne cycloadditions, the activation energies were higher for cycloadditions with azide dipoles than for the comparative nitron dipoles, with the energies calculated for the first transition state (TS1) being larger than for the second transition state (TS2) in all cases, further supporting the observed fast reaction rates for the second cycloaddition (see Figure 3).

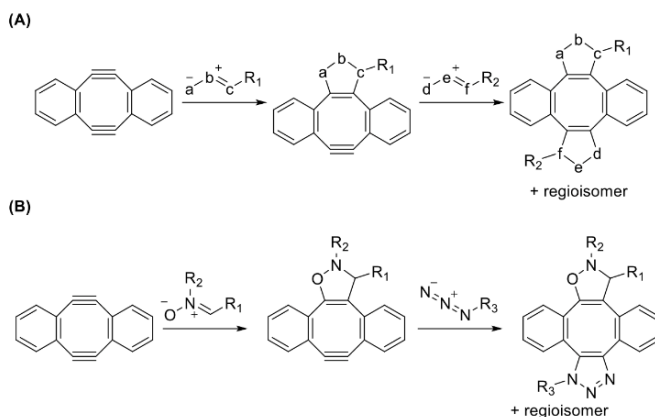
D/I analysis revealed that the dipolarophile (diyne) distortion energy differences between TS1 and TS2 were all

within 1.6 kcal/mol, while the differences in distortion energies for the dipoles were in the range 3.5–7.3 kcal/mol, indicating that the distortion energy of the dipoles is the greatest contributor to the activation barrier. Interaction energies were found to be within 4.8 kcal/mol, with a greater interaction being observed in TS2. A trend of decreasing the activation energy barrier can also be observed for more electron-deficient nitrones. Closer analysis of the transition-state geometries revealed that bond formation distances are 0.1–0.2 Å longer in TS2 and TS1, suggesting greater distortion in TS1 and a later

Scheme 3. Strain-Promoted Double-Click Reaction of Acyclic Dinitrone Monomers with Cyclooctadiyne



Scheme 4. Heterologous Double-Click Reactions with Cyclooctadiyne



transition state (see Figure S15). Moreover, the CCC bond angle of the dipolarophile is found to be distorted to a degree of 3–4° in TS2; however, the distortion energies are almost the same values when comparing TS1 and TS2, meaning that the influence of increased strain in the dipolarophile is negligible to the increase in the activation energy barrier. These findings confirm the observed reaction rate constants and confirm the basis for the relative reactivities and electronic sensitivities of the first and second cycloaddition steps. Computational studies show the importance of employing more electron-deficient nitrones when faster reactions are necessary. Furthermore, taken in combination with the greater hydrolytic stability of cyclic nitrones, these findings help support the use of electron-deficient cyclic nitrones for concrete applications of these cycloaddition reactions.¹⁶

In an attempt to detect the first cycloaddition intermediate for the reactions of nitrones with cyclooctadiyne, we investigated the reaction progression by continuous sampling mass spectroscopy. A TOF mass spectrometer was used, thus allowing the separation of the lower molecular weight (MW) ions (corresponding to starting materials) from the mid-MW ions (monoyne intermediate) and the high-MW ions (double cycloaddition products). C-(4-bromophenyl)-*N*-phenylnitron was chosen as the model nitron due to bromine isotopes facilitating the identification of product ion species and potentially allowing for the identification of the monoyne intermediate. The disappearance of the nitron starting material and the corresponding formation of a double addition product were observed, showing similar changes in peak intensity (see Figure S14). However, the monoyne intermediate was not reliably detected, further confirming that the

second addition of nitron to diyne is significantly faster than the first for electron-poor nitrones.

The scope of the reaction was also further examined by testing more reactive and hydrolytically stable cyclic nitrones in model reactions, cyclic dipoles which have found use in bioorthogonal applications.² For nitron **1f** reacting with cyclooctadiyne according to Scheme 2, we determined k_1 and k_2 to be 0.27 and 33 M⁻¹ s⁻¹, respectively. Given the large rate constant for the second addition, we envisioned employing cyclic nitrones in prototypical SPANC oligomerization reactions to simultaneously determine the efficiency of additions and to apply MALDI mass spectrometry to detect intermediate monoaddition products, in order to provide further mechanistic insight. These experiments were performed to test whether the monoyne intermediates would be detectable under oligomerization conditions.

To perform the oligomerization, dinitrone monomer (**1g**) was synthesized by conjugating a polyethylene glycol (PEG)-based linker to two DMImO nitron moieties. Stock solutions of dinitrone and diyne were prepared and mixed using a ratio of 2:1, respectively. Oligomerization was carried out by removing the solvent under reduced pressure on a rotary evaporator.⁸

MALDI data showed oligomer peaks with repeating units continued to $n = 3$, ionized with Na⁺ (with slight fragmentation) (Figure 4B, peaks A, B, and C). Furthermore, the MALDI spectrum showed peaks that are assigned to as cyclic products (Figure 4B, peaks D, E, and F) formed by intramolecular cyclization and preventing the monomers from forming high molecular weight polymers. The formation of intramolecular cyclic structures which has been previously reported by Liu et al.,⁷ was further confirmed by the addition

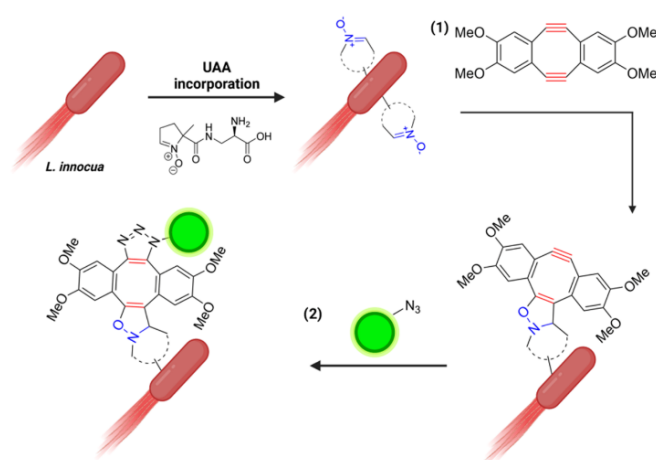


Figure 5. Protocol for metabolic labeling of *L. innocua* with nitron-functionalized UAA, where incorporated UAA is detected by sequential cycloaddition with strained diyne and azide-fluorophore.

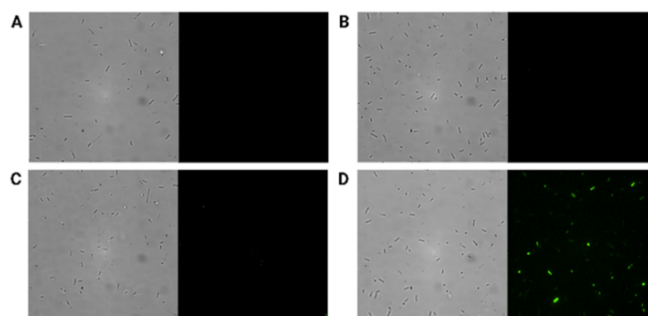


Figure 6. Results showing the incorporation of UAA in *L. innocua*. Bacterial cells were cultured in brain heart infusion (BHI) medium, treated with MeOH control medium or 10 mM of nitron-UAA for 1 h at 30 °C, and washed with PBS, before and after reaction with DMSO control medium or 100 μ M OMe-Diyne for 30 min at 37 °C. Diyne treatment was followed by the addition of DMSO control or 25 μ M N_3 -AlexaFluor488 for 20 min at 37 °C. Brightfield is on the left, fluorescence is on the right. (A) No N_3 -AlexaFluor488 control; (B) no OMe-Diyne control; (C) no nitron-UAA control; and (D) treated sample showing the above background signal.

of an alternative cyclic nitron, benzyl azide, and $\text{Co}_2(\text{CO})_8$, in efforts to trap free monoyne. However, the unaltered MALDI results for oligomers indicate the absence of free alkynes in the reaction mixture and the formation of cyclic structures (see Figures S1 and S2). Oligomerization was also achieved using acyclic dinitrones (see Scheme 3), when chain-growth oligomerization was observed, the free alkyne intermediates were unable to be captured; oligomers arising from acyclic nitrones have also been shown to undergo rapid degradation.⁷ The inability to isolate monocycloaddition intermediates demonstrates that the concentration effects on the rate acceleration for the first and second cycloaddition reactions are similar. This confirms that the predicted reactivity patterns in ideally dilute solutions hold true in nonideal conditions that might mimic biological environments. Encouragingly, these observations also highlight the potential for the generation of highly reactive strained alkyne dipolarophiles *in situ*.

The two strained alkynes of cyclooctadiyne offer the potential to act as an adapter molecule, allowing for the conjugation of two different classes of dipoles for heterologous double-click reactions, allowing for the linking of two dipoles

rather than the archetypical dipole–dipolarophile pairing (Scheme 4). This presents the opportunity for heterologous and modular connectivity that takes advantage of a large swath of commercially available dipoles, such as the abundant option for azides conjugated to various fluorophores or affinity-moieties. The result is a version of dual click that takes advantage of the high reactivity of nitron dipoles as well as the enhanced reactivity of their monoyne cycloadducts for bioconjugation, as indicated by our rate constant data (*vide supra*).

To demonstrate the applicability of the double cycloaddition of diynes with nitrones and to demonstrate its bioorthogonal potential and the adapter properties of diynes in heterologous reactions, we performed bacterial cell labeling with metabolically incorporated nitrones on the cell surface. Specifically, we incorporated nitron-functionalized D-alanine into the peptidoglycan layer of the bacterial cells has been demonstrated to be robust.^{19,24} Briefly, *L. innocua* cells were treated with D-ala-CMPO, washed with phosphate-buffered saline

(PBS), then treated with diyne, further washed with PBS, and finally labeled with azide–functionalized AlexaFluor488 fluorophore (see Figure 5).

The first cycloaddition reaction involved the CMPO-labeled *Listeria* that was then subsequently reacted with a commercially available AlexaFluor488-azide that resulted in robust labeling of the *Listeria innocua* by heterologous dual click chemistry (see Scheme 4). Above background labeling occurred rapidly when using a tetramethoxy-substituted diyne (see Figure 6). Tetramethoxy-substituted diyne was employed to increase water solubility and avoid unwanted background fluorescence due to hydrophobic interactions. Labeling with unsubstituted dibenzocyclooctadiyne also displayed statistically significant cell labeling via heterologous dual click chemistry (see Scheme 4); however, there was a higher observed background fluorescence as compared to the tetramethoxy-substituted diyne experiment (see Figures S19 and S20).

The chemistry on bacterial surfaces demonstrates the capacity for dyes to function as adapter molecules in bioorthogonal applications. These results suggest that a broad range of substrate combinations should be possible with nitrones as the first reactant in heterologous dual click chemistry.

Based on the rate constant data for nitron cycloadditions with the more water-soluble tetramethoxy-substituted diyne, we predicted the fast reactivity of this diyne with nitrones on cell surfaces. We observed strong labeling consistent with this prediction and successful heterologous cycloadditions with the addition of AlexaFluor488-azide. Although isolation of monocycloaddition intermediates has proven elusive *in vitro*, stepwise cycloaddition reactions have been demonstrated for bioorthogonal applications, where the low effective concentration of the initial dipole is sufficiently small to avoid further secondary cycloaddition.²⁵ The spatial distribution of the nitrones on the bacterial cell surface must be sufficient to enable the slower reacting azide to perform the second cycloaddition, as our observations indicate.

CONCLUSIONS

We have demonstrated that nitrones can be applied to the strain-promoted “double-click” reaction with dibenzocyclooctadiyne. As shown for other strain-promoted bioorthogonal reactions, nitrones provide alternative dipoles to azides, which display more favorable reaction kinetics. As observed in previous studies, the rate constants for the second cycloaddition reactions are much greater than for the first cycloaddition, resulting in difficulty observing monoyne intermediates; however, the potential stereoelectronic tunability of nitrones could allow for future study of these intermediates. The further development of the SPDC between nitrones and cyclooctadiyne could enable the synthesis of new polymer materials and be expanded to further bioorthogonal chemistry applications including dual labeling, stapling, and covalent ternary complex formation. Finally, these studies also suggest that new cyclooctynes with structures similar to those of monoaddition products will be faster for bioorthogonal labeling applications. We are currently pursuing these new reactions and related applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscchembio.3c00491>.

Experimental details, materials and methods, compound characterization data, and kinetic and theoretical data (PDF)

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Author Contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript. These authors contributed equally to this work.

Notes

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ABBREVIATIONS

SPDC, strain-promoted “double-click”
UAA, unnatural amino acid

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3.4. Summary and Conclusions

As demonstrated in previous studies for the cycloaddition between nitron dipoles and strained alkynes (SPANC), the reaction between nitrones and dibenzocyclooctadiyne was shown to be suitable for bioorthogonal applications, as the reaction displays fast reaction rates and occurs under mild bioorthogonal conditions. As with the analogous reaction with azide dipoles, rate accelerations were observed for the second cycloaddition reaction, and as such the intermediate product resulting from the first cycloaddition was unable to be detected or isolated. Attempts to observe this monoadduct intermediate were unsuccessful, both by reaction progress monitoring by mass spectrometry, and by preliminary oligomerization studies, where the formation of cyclic oligomers was observed. As with previously studied SPANC reactions, similar trends emerged where the reaction was accelerated by the use of electron-deficient nitrones, and the use of dipolarophiles which display higher HOMO energy, such as the use of tetramethoxy-substituted diyne. These findings were reinforced by analysis of the sequential reactions by Distortion-Interaction computational modelling, where the activation barriers were found to be lower in all cases for the second cycloaddition reactions, and the main contributor to these barriers being the distortion of the dipoles, as compared to dipolarophile distortion energies. Future studies could focus on employing dipoles which necessitate less distortion to achieve transition state conformation to achieve conjugation with higher reaction rates. Furthermore, while we were able to demonstrate the applicability of the diyne scaffolds as adapter molecules allowing for the conjugation of heterologous dipoles, further functionalization of biomolecules via the functionalization of the diyne scaffold itself was not explored. Additional efforts towards the synthesis of unsymmetrical diyne molecules could allow to expand on applications involving

dual labelling of biomolecules. The intercalation of diyne molecules into nucleic acids also constitutes an avenue for future research into these dual-click reactions for the conjugation of azide or nitron modified nucleic acids, with secondary nitron dipoles. Further efforts into the isolation of mono-adducts resulting from a single cycloaddition are also warranted, given the rate accelerations observed for the second cycloaddition. This could be potentially achieved by selective protection of single alkyne moieties of the diyne, for example by using dicobalt octacarbonyl ($\text{Co}_2(\text{CO})_8$)²⁶¹ or cyclopropenone²⁶² alkyne protection. The isolation of these monoadducts could significantly impact the scope of available dipolarophiles for applications requiring extremely fast and efficient reactions.

Chapter 4 Development of Novel Bioorthogonal Transformations involving Nitron Dipoles

4.1. Introduction to additional nitron cycloaddition chemistry

Metal-free strain-promoted cycloaddition reactions have become a cornerstone of bioorthogonal ligation strategies. While the cycloaddition reactions, such as CuAAC and SPAAC, of azide dipoles have been widely studied and applied, alternative dipoles remain somewhat underexplored. Nitrones have thus emerged as viable alternative dipoles due to their ease of synthesis, structural modularity allowing greater control over reactivity and displaying generally faster kinetics than analogous reactions with azides²³³. In addition to the optimization of CuANCR/Kinugasa reactions and investigations of dual strain-promoted cycloadditions with dibenzocyclooctadiynes, nitron dipoles were also investigated in other novel cycloaddition reactions in the context of this thesis. Notably, the reactivity of nitron dipoles based upon a phenanthridine scaffold was investigated with the goal of providing nitron dipoles which maintained the bioorthogonality and reactivity of acyclic diaryl nitrones, while improving stability (aqueous hydrolysis stability) (see Figure 4.1). Given that phenanthridine scaffold molecules offer potential therapeutic benefits, namely antiparasitic properties, and intercalation properties, these novel dipoles could potentially serve as dual therapeutic/bioorthogonal reporter molecules, while displaying fast reaction rates in cycloaddition chemistry with dipolarophiles.

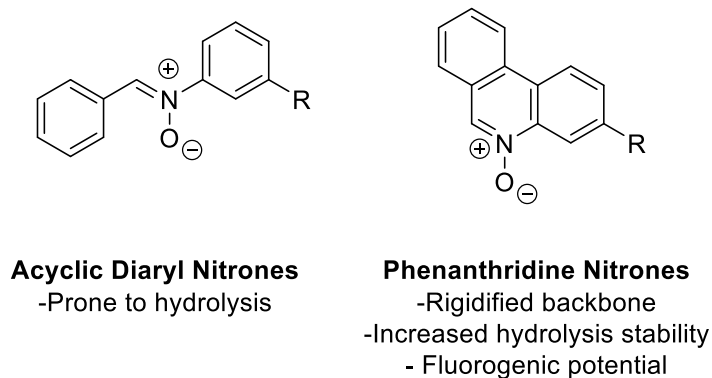


Figure 4.1 Comparison of acyclic diaryl nitron structures to phenanthridine nitron.

In addition to the development of alternative nitron scaffolds, the use of alternative dipolarophiles was also investigated. Although the cycloaddition between nitrones and strained cyclic alkynes proceeds efficiently and rapidly, there are still challenges associated with the use of strained cyclooctynes, namely the bulky size of the alkyne can perturb biomolecule structure, function and intracellular localization, as well as limit the aqueous solubility which can limit the applicability of SPAAC/SPANC reactions. In efforts towards the development of bioorthogonal reactions which minimally perturb biomolecule structure, the cycloaddition reactions between nitron dipoles and cyclopropene dipolarophiles were also investigated. Cyclopropenes represent the smallest strained cyclic alkene structures, and as such have much more limited steric interactions when incorporated into biomolecules or probe molecules^{137,263}. They are also promising dipolarophiles, as they can be selectively incorporated into proteins through genetic code expansion, allowing for excellent control of biomolecule functionalization^{264–266}. The development of cyclopropene bioorthogonal chemistry with alternative reagents would therefore expand upon available tools and further expand the potential for multiplex labelling.

Finally, initial investigations were performed towards developing SPANC chemistry with protected cyclooctadiyne dipolarophiles. Given the successful cycloadditions observed for various nitrone structures and diynes, and the rate accelerations observed for the second cycloaddition reaction, we were interested in applying cyclopropenone protected alkynes for the potential isolation of mono-cycloadducts. Cyclopropenone protected strained alkynes have recently emerged as a method of controlling cycloaddition reactivity through photoirradiation¹³⁶. Upon irradiation with UV light, the reactive strained alkyne can be uncaged and conjugated to appropriate dipoles^{267,268}. Given the successful application of this strategy towards molecules containing a single alkyne (BCN or DBCO derivatives), we envisioned applying the technique to strained diynes and their cycloaddition with nitrone dipoles. Successful isolation of monoadducts resulting from a single cycloaddition, could also lead to the generation of protected strained alkynes displaying enhanced reactivity towards subsequent cycloadditions.

4.2. Author Contributions

The synthesis of nitrones used in the study was completed by Didier A. Bilodeau (**DAB**), with assistance from Mariya Chigrinova (**MC**) in the development of the synthetic route and synthesis of coumarin nitrone (**8**). The analysis of reaction kinetics by UV kinetics was accomplished by **DAB** with assistance from Dr. Miroslava Strmiskova (**MS**). The determination of nitrone toxicity was accomplished by **MS**. The determination of fluorescence spectrum of studied nitrones and control phenanthridine scaffold, as well as the FRET pair analysis was accomplished by **MS** with assistance from **DAB**. DNA intercalation studies were performed by **MS**.



Phenanthridine-based nitrones as substrates for strain-promoted alkyne-nitrone cycloadditions

Miroslava Strmiskova, Didier A. Bilodeau, Mariya Chigrinova, and John Paul Pezacki

Abstract: Over the past decade, bioorthogonal chemistry that facilitates the efficient conjugation of biomolecules has expanded from the copper-catalyzed alkyne-azide cycloadditions to a multitude of diverse reactions, varying additives and reactional partners, and most often offering better alternatives with faster rates and lower toxicity of employed reactants. Among these, the copper-free strain-promoted cycloaddition reactions have been demonstrated to be more promising, offering a reaction without toxic metal catalysts and with faster inherent kinetic rate constants. The strain-promoted alkyne-nitrone cycloadditions are easily tunable from both the (strained) alkyne and nitrone perspective, both compounds giving the opportunity to modulate the rate of reaction by substituting various positions. Previously, acyclic nitrones have been evaluated in the strain-promoted alkyne-nitrone reactions; however, they were notably prone to hydrolysis. Some five-membered ring endocyclic nitrones developed concomitantly offered the advantage of relatively fast kinetics and better resistance to degradation in aqueous conditions and have been successfully used for labelling of biomolecules in living systems. Herein, we have prepared and studied nitrones inspired by the phenanthridine scaffold that efficiently undergo strain-promoted alkyne-nitrone reactions. Phenanthridine nitrones react fast with strained cyclooctynes with large bimolecular rate constants while maintaining bioorthogonality and resistance to hydrolysis.

Key words: phenanthridine, bioorthogonal labelling, nitrone, SPANC.

Résumé : Au cours des dix dernières années, la chimie bioorthogonale facilitant la conjugaison de biomolécules a permis d'étendre les cycloadditions catalysées par le cuivre d'azotures sur des alcynes à une multitude d'autres réactions qui font intervenir différents additifs et diverses paires de réactifs et qui constituent la plupart du temps une option plus avantageuse pour effectuer des réactions plus rapides, utilisant des réactifs moins toxiques. Parmi celles-ci, les cycloadditions sans cuivre accélérées par la tension de cycle se sont révélées les plus prometteuses, car elles procèdent sans catalyseur métallique toxique et présentent des constantes de vitesse cinétique intrinsèques plus élevées. Les cycloadditions accélérées par la tension de cycle de nitrones sur des alcynes sont facilement réglables, tant du point de vue de l'alkyne (par la tension de cycle) que de la nitrone, les deux composés offrant la possibilité de moduler la vitesse de réaction par substitution à diverses positions. Des nitrones acycliques ont déjà été évaluées dans le contexte de réactions accélérées par la tension de cycle entre des alcynes et des nitrones. Cependant, elles étaient remarquablement sensibles à l'hydrolyse. Certaines nitrones endocycliques à cinq membres synthétisées au même moment offraient l'avantage d'une cinétique relativement rapide et une meilleure résistance à la dégradation en conditions aqueuses. Elles s'étaient révélées utiles pour le marquage de biomolécules dans des systèmes vivants. Dans le cadre des présents travaux, nous avons étudié des nitrones inspirées de la structure de la phénanthridine qui réagissent efficacement dans les cycloadditions accélérées par la tension de cycle de nitrones sur des alcynes. Les nitrones dérivées de la phénanthridine réagissent rapidement avec les cyclooctynes, comme en témoignent leurs constantes de vitesse de réaction bimoléculaire élevées, tout en conservant la bioorthogonalité et la résistance à l'hydrolyse. [Traduit par la Rédaction]

Mots-clés : phénanthridine, marquage bioorthogonal, nitrone, SPANC.

Introduction

Studying biological processes in their natural environment has been made possible using bioorthogonal reactions that have little impact on metabolism and homeostasis. The challenging task of selectively targeting one family or one type of biomacromolecule has been tackled by constantly developing the bioorthogonal reaction toolbox.¹ The requirements for a reaction to be considered a good candidate for bioorthogonal chemistry^{2–4} include, but are not limited to, fast reactivity in aqueous biological environment at requisite temperatures, excellent selectivity towards its target in presence of all available biological components, and low toxicity or perturbation to the biological system. As such, the copper-

catalyzed alkyne-azide cycloaddition (CuAAC) has proven itself particularly useful in labelling cell surface and intracellular macromolecules⁵ such as proteins,^{6,7} lipids,^{8–10} nucleic acids,^{11–13} and saccharides.¹⁴ However, the use of toxic copper concentrations has been shown to be incompatible with labelling of living cells. Several ways help to circumvent this, including the use of various ligands that help reduce copper toxicity^{5,15} and, more importantly, development of copper-free reactions using strained alkynes such as the strain-promoted alkyne-azide cycloaddition¹⁶ (SPAAC) or the rapid inverse-electron demand Diels-Alder (IEDDA) tetrazine ligation.^{17,18} The tetrazine ligation was shown to be a valuable tool for PET imaging^{19–22} and radioimaging²³ probe construction, benefiting from fast kinetics, and it is also a useful ligation reaction for chemi-

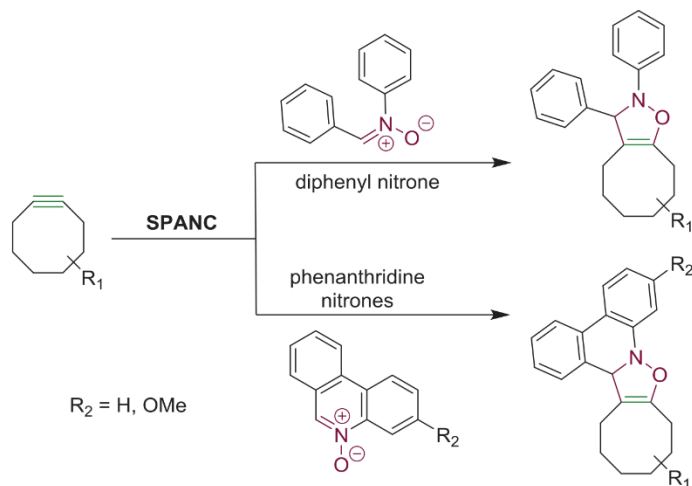
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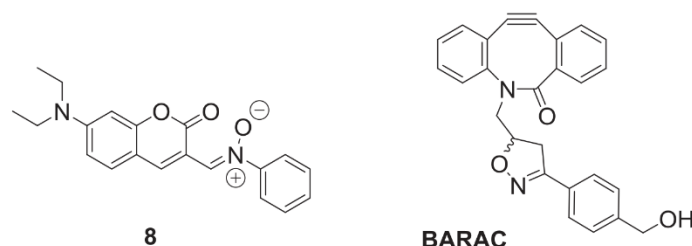
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Scheme 1. Reaction of acyclic diphenyl nitrone and cyclic phenanthridine nitrones with strained alkynes in the strain-promoted alkyne-nitronc cycloaddition. [Colour online.]



Scheme 2. Reference molecules. Coumarin-nitronc **8** (left), based on acyclic phenyl-substituted nitronc, was used as a control in this study. BARAC (right) was used as a model strained alkyne for kinetics measurements.



cal proteomic analysis.¹⁷ As an alternative to azide 1,3-dipoles, nitronc dipoles^{24–26} have been used to enhance reactivity towards cyclooctynes, and their general reactivity has been recently reviewed in detail by Merino et al.²⁷

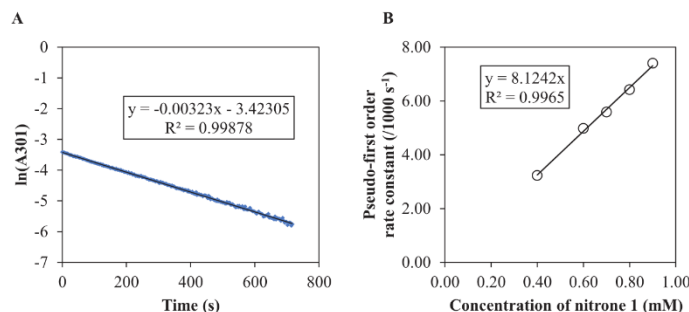
Our group and others have applied the strain-promoted alkyne-nitronc cycloaddition (SPANC) to a variety of living systems, exploring the nitronc-functionalized ligand,²⁸ nitronc-derived unnatural amino acid,^{29,30} antibiotic²⁹ and sugar³¹ incorporation, and also nitronc-functionalized reporters.³² As such, both cyclic and acyclic nitroncs were shown to be broadly useful for labelling living bacteria³⁰ via incorporation of KDO-nitronc or vancomycin-nitronc in bacterial peptidoglycan, as well as for cellular labelling.^{29,33}

We have first investigated the reactivity of acyclic nitroncs by varying substituents on α -C position with the strained dibenzocyclooctyne (DIBO).^{24,26,33,34} We have shown that acyclic nitroncs with electron-withdrawing substituents on α -C position reacted faster than unsubstituted nitroncs or those bearing electron-donating substituents on α -C.²⁴ However, more reactive acyclic nitroncs suffered from poor stability in aqueous conditions (Scheme 1),³³ which are necessary to carry out labelling of biomolecules. Endocyclic nitroncs, on the other hand, are excellent reactants for SPANC due to their stability in aqueous media.²⁸ We have therefore investigated the reactivity of several endocyclic nitroncs with strained alkyne biarylazacyclooctynone (BARAC, Scheme 2), and we reported a series of nitroncs that are highly reactive and resistant to hydrolysis in both acidic and basic con-

ditions,³³ which we demonstrated by using several endocyclic nitroncs for cellular labelling and labelling of living bacteria.^{29,33} Some nitroncs undergo a rearrangement upon SPANC reaction and their reactivity has been described.³⁴ Similarly, a rearrangement of the product can occur in the case of CMPO nitronc used with a terminal alkyne in the copper-catalyzed reaction (CuANCRC).³² Therefore, further reactions based on the nitronc group are warranted with more stable and more reactive nitronc dipoles to be developed. We hypothesized that diaryl nitroncs based on the phenanthridine ring system could lead to increased stability of the resulting nitronc in aqueous conditions while retaining reactivity in strain-promoted cycloaddition owing to its similarity to other diaryl nitroncs (Scheme 1).

Phenanthridines are historically intensively studied biologically active compounds and they have been used in a wide range of applications as the gold-standard DNA- and RNA-intercalating agents. For a long time, the human medical applications of phenanthridines have been avoided due to their potential carcinogenic and mutagenic properties, relevant especially in the case of ethidium bromide derivatives. Recently, the scientific community noted an increased interest in phenanthridines and their alkaloid analogues derivatives due to their antiparasitic properties,³⁵ as recently reviewed by Tumir et al. This could give rise to the possibility of using SPANC chemistry to introduce biologically active compounds to bioconjugates or to construct novel carriers containing the medicinal and biochemical effects of phenanthri-

Fig. 1. Kinetic plots for phenanthridine nitrone **1** reactions with BARAC. (A) Pseudo-first order kinetics of BARAC reaction with 400 μM of **1**. (B) Pseudo-first order rate constants vs nitrone **1** concentration. [Colour online.]



dines. It is also possible that nitrone derivatives will not be as biologically active and serve as bioorthogonal groups.

Here, we report phenanthridine-nitronone derivatives and their kinetic characterization in a SPANC reaction and as promising scaffolds for new bioconjugation reactions. We show that these phenanthridine-nitrones have low toxicity and potential broad applications in cellular labelling, owing to their inherent tunable spectroscopic properties, their stability and compatibility with aqueous conditions.

Results and discussion

To explore the reactivity and stability of phenanthridine-derived nitrones for bioorthogonal SPANC reactions, derivatives first needed to be synthesized. Phenanthridine-derived nitrones **1** and **5** were prepared as previously reported in the literature.³⁶ Briefly, phenanthridine 5-oxide **1** was synthesized in one step from commercially available phenanthridine, using freshly prepared phosphomolybdic acid as catalyst, affording **1** in 73% overall yield. 3-Methoxyphenanthridin-6(5H)-one, a precursor of **5**, was obtained following the procedure reported in the literature (Supplementary Scheme S1).³⁷ Then, the carbonyl of 3-methoxyphenanthridin-6(5H)-one was transformed into (trifluoromethyl)sulfonyl-group using TiF_2O and subsequently removed with palladium acetate to afford 3-methoxyphenanthridine. Finally, **5** was prepared from 3-methoxyphenanthridine in a similar manner as **1**, using phosphomolybdic acid as catalyst, as reported previously.³⁶ All synthetic steps are detailed in the Supplementary data.

Next, to evaluate the reactivity of phenanthridine-derived nitrones, kinetics studies were performed in a SPANC reaction with a model strained alkyne BARAC, as described in the Supplementary data. A typical kinetic profile of nitrone **1** and BARAC under pseudo-first order conditions is shown in Fig. 1, along with a plot of k_{obs} against nitrone concentration. As predicted, the reactivity of both phenanthridine nitrones **1** and **5** (Supplementary Tables S1 and S2; Supplementary Figs. S1 and S2) is diminished in comparison with acyclic phenyl-substituted nitrone **6** (Table 1). This is likely due to the loss of electron-donating effect of the phenyl group of **6** that is now involved in the delocalized electron system of nitrones **1** and **5**. With a k_2 constant approximately 3.5 times higher than the benzyl-substituted acyclic nitrone,³³ the strain offered by the three aromatic six-membered rings gives a notable kinetic advantage to both **1** and **5**. However, when compared with the five-membered ring endocyclic nitrones such as CMPO,³³ the rate constants k_2 of **1** and **5** were about 5 times lower, which is consistent with the fact that five-membered rings provide more strain than six-membered rings. Furthermore, methoxy-substituted phenanthridine-nitronone **5** is slightly more reactive than unsubstituted **1**, probably owing to the increased energy of the HOMO of **5**. Finally, both nitrones **1** and **5** remain

Table 1. Second order rate constants for addition of BARAC on nitrones **1**, **5**, **6**, and **7**.

Nitronone	Structure	k_2 ($\text{M}^{-1}\text{s}^{-1}$)
1		8.1
5		9.7
6		59 ^a
7		0.96 ^b

^aSee MacKenzie et al. 2014.³³

^bSee Jewett et al. 2010.⁴²

about 10 times more reactive in the SPANC reaction than benzylazide in the SPAAC reaction. They are more resistant to hydrolysis than their acyclic counterparts and retain the advantageous position for further development of this scaffold. This establishes nitrones **1** and **5** as useful reactants for bioorthogonal SPANC reactions.

To assess the bioorthogonality of phenanthridine-derived nitrones, toxicity profiles were determined. Certain phenanthridine derivatives are notoriously toxic and even considered to be mutagenic and carcinogenic due to their DNA-intercalating properties. Some phenanthridine derivatives are also effective against human breast cancer cell proliferation.³⁸ Here, we assessed the toxicity of nitrones **1** and **5** using MTT assays. Phenanthridine-nitronone derivatives showed no toxic effect on human hepatoma Huh 7.5 cells (Fig. 2) in the range of concentration commonly used for cellular labelling, likely because of the lack of phenanthridine substituents necessary for intercalation between nucleic acid base pairs.^{39,40} We compared their toxicity with a benchmark compound, coumarin-nitronone **8** (Scheme 2; Fig. 3), for which we determined an IC_{50} of 12.6 μM , owing likely to the intrinsic toxicity of coumarin-derived compounds.⁴¹ The low toxicity in the range of

Fig. 2. Toxicity of phenanthridine nitrones against Huh7.5 human hepatoma cells. Huh7.5 cells were incubated with 0–100 μM of each compound for 24 h, after which a 2.5 mg/mL solution of MTT reagent in PBS was added and cells were incubated for further 3–4 h. Cell viability was determined by measuring absorbance at 562 nm of formed formazan.

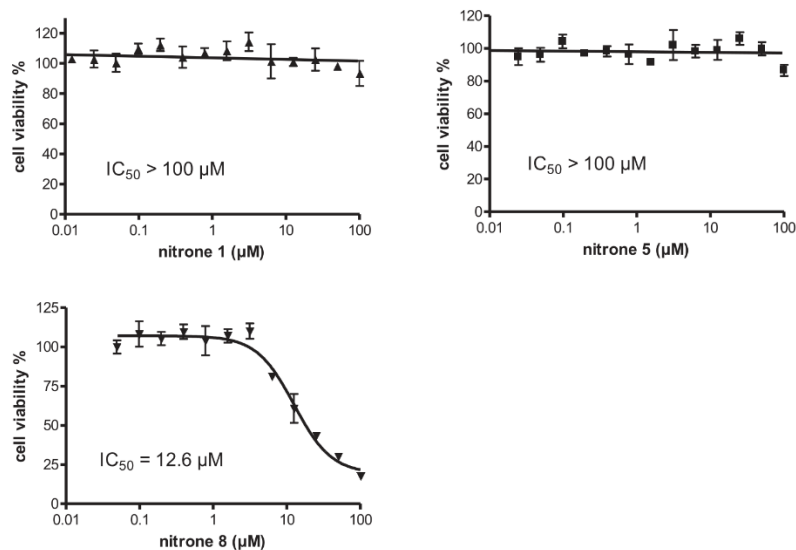


Fig. 3. Fluorescence spectrum of phenanthridine and nitron compounds. Excitation and emission spectra of 2 μM 1, 5, or phenanthridine control and 1 μM 8 (stock in DMSO) have been acquired in PBS at room temperature, using 5 nm increments, on a SpectraMax i3 plate reader (Molecular Devices). Paired excitation and emission spectra have been normalized to the same levels of fluorescence intensity. *Estimated value. Excitation maximum is likely below 280 nm. [Colour online.]

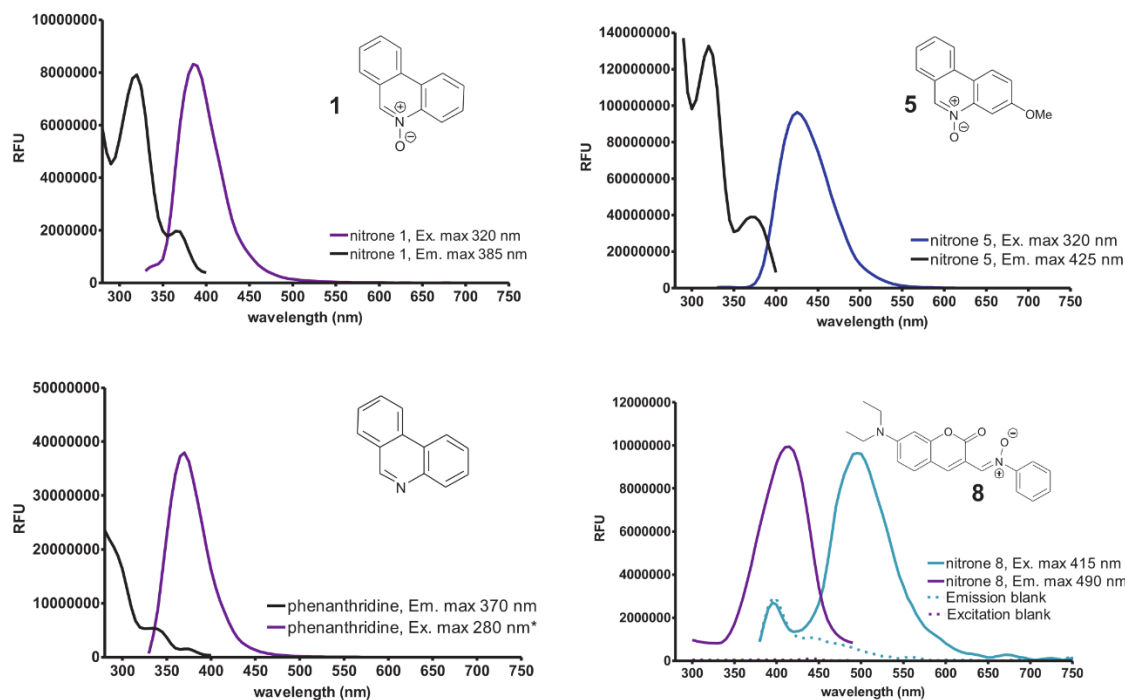
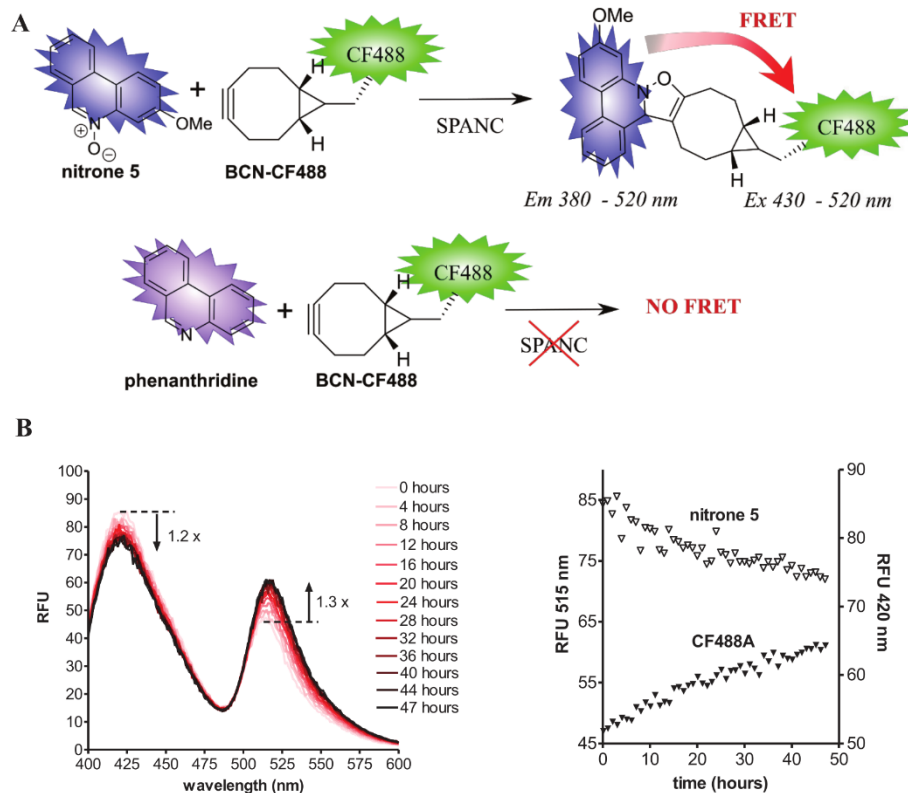


Fig. 4. Nitron 5 as FRET pair with BCN-CF488A. (A) FRET pair design in SPANC reaction. (B) FRET in SPANC reaction of BCN-CF488A (Biotium) and nitron 5. 10 μ M of nitron 5 was combined with 10 μ M BCN-CF488A in PBS and SPANC reaction was followed by FRET between the phenanthridine-nitron 5 donor and the CF488A acceptor upon excitation at 380 nm (left). The reaction was performed under second order conditions to see a comparable increase of acceptor and decrease of donor fluorescence. Fluorescence decrease at 420 nm and increase at 515 nm overtime are shown (right). Fluorescence scans were acquired at 25 $^{\circ}$ C on a Cary-Eclipse Fluorimeter (Varian) equipped with a temperature control unit, using 5 nm slit for both excitation and emission. [Colour online.]



concentration useful for cellular labelling makes phenanthridine nitrones good candidates for further development and use for labelling of biomolecules in living cells.

Because some phenanthridines display interesting fluorescence properties, we next sought to study the fluorescence of phenanthridine-based nitrones. Unsubstituted phenanthridine scaffold and a wide range of phenanthridine derivatives are fluorescent and are therefore used as potent markers for DNA, owing to their DNA-intercalating properties.³⁵ Given the high tunability of phenanthridine spectroscopic properties, we measured both the excitation and emission spectra of nitrones **1**, **5**, and **8** (Fig. 3). Although converting nitrogen of the initial phenanthridine to nitron in compound **1** did not cause any significant shift of excitation or emission maxima, nitron **5** emission peak is largely red-shifted, similarly to other phenanthridine derivatives with substituents on C3.³⁹ As spectroscopic properties of phenanthridines are highly tunable, we expect the herein presented nitrones to be first members of a larger groups of analogs with broad range of tunable spectroscopic properties.

We also briefly examined the potential of nitron **1** and **5** to provide fluorogenic reactions. Despite the fact that neither of the two compounds is fluorogenic in reaction with strained alkynes (data not shown), the emission spectrum of **5** is red-shifted enough to be a donor pair for Förster resonance energy transfer

(FRET) to a green fluorophore such as the photostable cyanine-based fluorescent dye CF488A (Fig. 4A). In a SPANC reaction of nitron **5** with the bicyclononyne BCN-CF488A (Biotium, also previously used in SPANC reactions with nitrones³⁰), a time-dependent 1.3-fold increase of CF488A fluorescence at 515 nm upon irradiation of nitron **5** at 380 nm is observed, as well as a similar decrease of nitron fluorescence at 420 nm (Fig. 4B and Supplementary Fig. S3). In absence of BCN, the fluorescence of nitron **5** barely changes over the course of the reaction (48 h). Similarly, in absence of nitron **5** and presence of phenanthridine control (which lacks the reactive nitron moiety), the emission at 515 nm did not change over the course of the reaction, further suggesting that the observed fluorescence increase at 420 nm and decrease at 515 nm is due to FRET of the product of SPANC reaction between nitron **5** and BCN-CF488A (Supplementary Fig. S3). Due to the length and small scale of the reaction, we have not isolated the formed product, which would allow us to further characterize its spectroscopic properties and confirm the nature of the fluorescence change as FRET. However, we can conclude that irrespective of the mechanism, the SPANC reaction with nitron **5** and BCN-CF488A induces spectroscopic changes in the SPANC product, which allow us to easily follow the progress of the reaction (in contrast to simple absorbance or fluorescence measurements which do not change substantially over time). Addi-

tionally, the nitron dipole can be further developed to offer a more efficient energy transfer or simply a higher brightness for direct use as fluorophore-nitron electrophiles for SPANC fluorescent labelling of strained alkyne-tagged biomolecules.

We have also investigated whether the quantum yield of nitrones **1** and **5** changes upon incubation with double-stranded DNA (dsDNA) and observed only a slight decrease of fluorescence intensity (Supplementary Figs. S4 and S5), which suggest, along with the fact that nitrones **1** and **5** lack the necessary components to intercalate DNA, that their spectroscopic properties remain the same, whether in presence or in absence of dsDNA. We also observed a larger decrease of fluorescence intensity after addition of BARAC, likely due to the fact that BARAC has a large absorption band between 220 and 400 nm. Overall, we were able to conclude that addition of dsDNA does not affect nitron spectroscopic properties, which is beneficial for broad applications of phenanthridine-nitrones in cellular and intracellular labelling. Again, this is consistent with the nitrones **1** and **5** being bioorthogonal, rather than bioactive, molecules.

Conclusion

We have designed and synthesized a series of cyclic nitrones based on the phenanthridine scaffold. These were found to be stable to aqueous conditions and showed bimolecular rate constants of $k_2 \sim 10 \text{ M}^{-1} \text{ s}^{-1}$. Phenanthridine-nitrones were then studied in the context of their ability to impart fluorescence onto cyclooctyne reactants. Lack of DNA-intercalating properties and related quantum yield change is in this case beneficial, as the nitron compounds are meant for SPANC labelling of intracellular biomolecules. Furthermore, we demonstrated that phenanthridine-nitron **5** can be used as a FRET donor with suitable fluorophores and has an immense potential for fluorescent microscopy and general bioorthogonal labelling.

Supplementary data

Supplementary data are available with the article through the journal Web site at <http://nrcresearchpress.com/doi/suppl/10.1139/cjc-2018-0253>.

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4.4. Summary and conclusions of phenanthridine nitronc cycloadditions

The study of nitronc dipoles based upon the phenanthridine did demonstrate that they are adequate dipoles for SPANC chemistry, displaying faster kinetics than analogous reactions with benzyl azide ($k_2 \approx 10 \text{ M}^{-1}\text{s}^{-1}$ vs. $0.96 \text{ M}^{-1}\text{s}^{-1}$, respectively). While the observed reaction rates for cycloaddition between BARAC and phenanthridine nitrones were slower than for diaryl acyclic nitrones, their higher stability towards hydrolysis under aqueous conditions makes them an attractive alternative for bioorthogonal application, due to a greater ease of use. The observed Förster resonance energy transfer (FRET) from the conjugation of methoxy-substituted phenanthridine nitronc and an appropriate strained alkyne fluorophore, such as CF488A-BCN, could allow for better tracking of bioorthogonal ligation over time and thus more accurately report on certain biological processes where spatiotemporal analysis is important. The effect observed in the study is rather small, and the synthesis of new nitronc structures which allow for further red-shifting of the nitronc's emission spectrum would be expected to improve upon applications where FRET would be beneficial. The structural variety of the nitrones explored was quite limited in this study, and further synthesis of substituted phenanthridines would allow for a better understanding of stereoelectronic effects and obtaining nitronc dipoles which react more quickly in SPANC reactions.

4.5. Initial exploration of nitronc-cyclopropene cycloadditions

Cyclopropenes represent the smallest class of cyclic strained dienophiles/dipolarophiles, being deemed 'mini-tags'²⁶⁹. This small size reduces the perturbation to the biomolecules/reporter molecules that they are conjugated to, and

therefore should offer a more native-like response/function. They have been used as bioorthogonal reacting partners with tetrazines¹³⁷, nitrile imines²⁷⁰, and tetrazoles²⁷¹. Their reactivity and stability can be modulated by accessing various cyclopropene scaffolds, such as 1,3-substituted²⁶⁹, 3,3-substituted²⁷², spiro[2.3]hex-1-ene²⁷³ and azaspiro[2.3]hex-1-ene²⁷⁴ structures (see Figure 4.2).

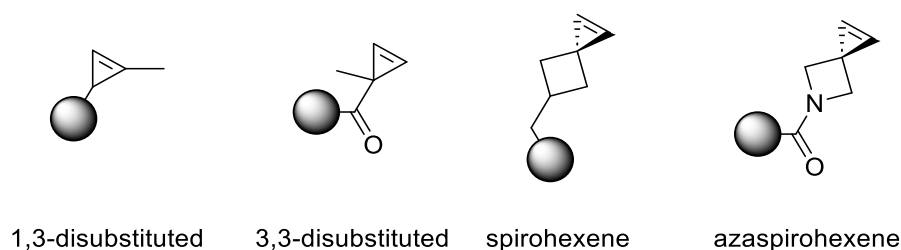
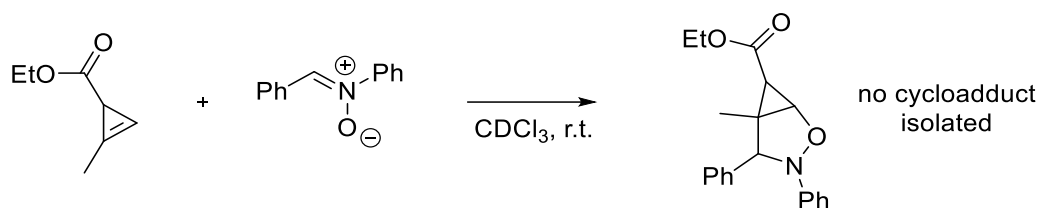


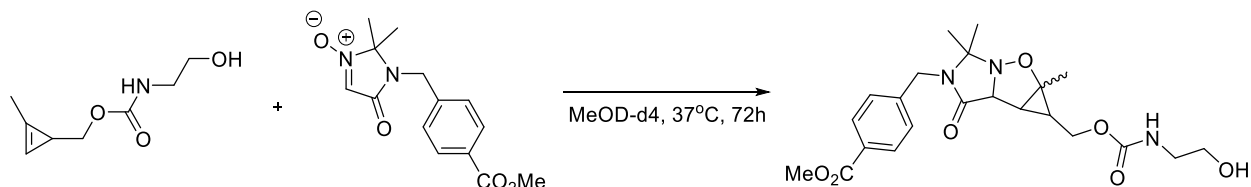
Figure 4.2 Various cyclopropene scaffolds employed as bioorthogonal reagents.

Due to reports upon the successful reaction between diaryl acyclic nitrones and 1,2-diphenylcyclopropenes²⁷⁵, we believed that this type of cycloaddition chemistry could be expanded towards bioorthogonal applications. Furthermore, given the small size of cyclopropene tags and their potential for incorporation through genetic code expansion strategies, we were interested in exploring the potential reactivity of these strained alkenes with nitron dipoles, expanding upon the strain-promoted cycloaddition reactions accessible. Towards this goal, 1,3-disubstituted cyclopropene structures were synthesized according to literature procedures^{276,277}. Initial trial reactions were performed in deuterated solvents to allow for NMR monitoring of reaction progress. While TLC monitoring of initial reactions using acyclic diaryl nitrones showed appearance of new products, successful purification of cycloadducts was unsuccessful (see Scheme 4.1). Given the greater stability of endocyclic nitrones, their reactivity was investigated next. Using increased temperature

(within the limits of bioorthogonal conditions) and extended reaction time, the reaction between 2-methyl-cycloprop-2-enecarboxamide and DMImO nitron in MeOD-d₄ at 37°C (equimolar, ~38 mM) led to the successful isolation of the cycloadduct product through preparatory TLC isolation (see Scheme 4.2). Given the small scale of the reaction and low availability of cyclopropene starting material, overall reaction yield was not determined. Product identity was analyzed through ¹H, ¹³C NMR and exact mass was confirmed via ESI MS analysis.



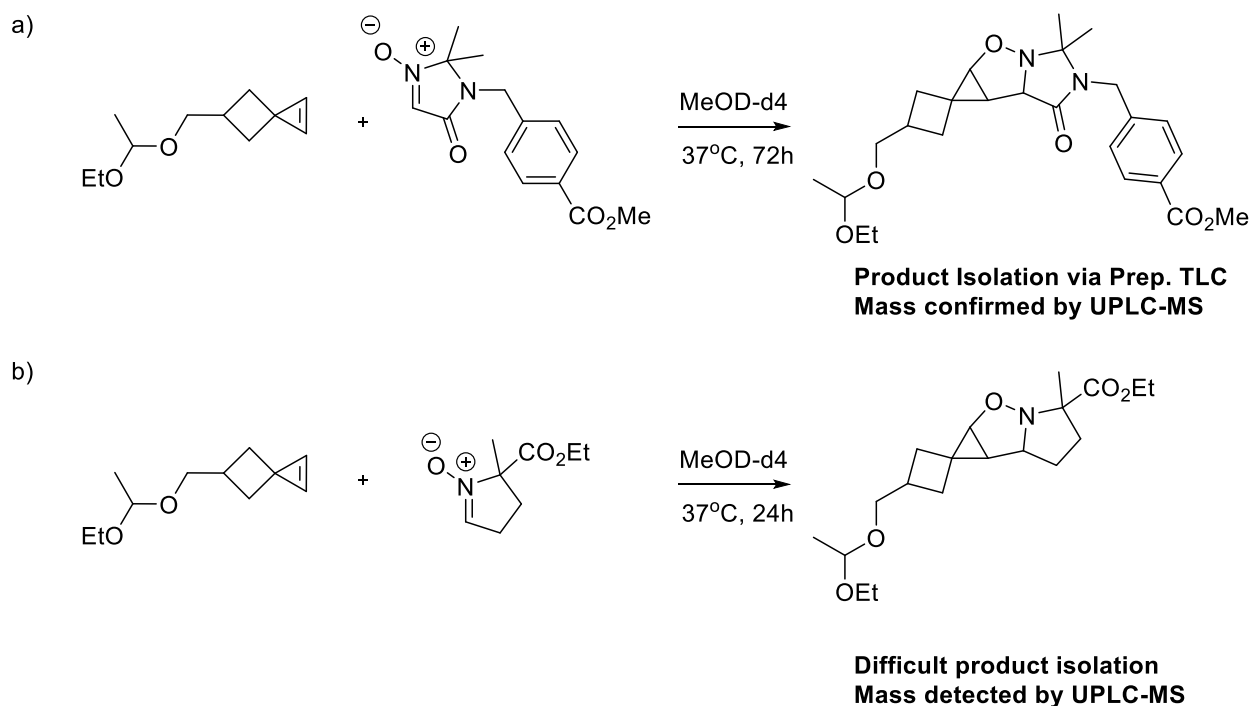
Scheme 4.1 Attempted acyclic nitron-cyclopropene cycloaddition



Scheme 4.2 Cycloaddition between DMImO cyclic nitron and 1,3-disubstituted cyclopropene.

Trial reactions were also attempted with spiro[2.3]hex-1-ene scaffold and endocyclic nitrones (DMImO and EMPO)(see Scheme 4.3). Again, for these cycloaddition reactions with spirocyclic cyclopropenes the reaction with acyclic nitrones led to the generation of new species via TLC monitoring, however no distinct cycloadduct product was retrievable through isolation efforts or UPLC-MS analysis. However, the reaction with endocyclic nitrones, DMImO and EMPO, evidence of cycloadducts was gathered. For the reaction with DMImO, product purification was attempted via preparatory TLC, and the structure of the

isolated adduct was ascertained by NMR and MS analysis (see Annex V); although disappearance of characteristic nitron peaks could be observed after 30 minutes had elapsed, the isolated product shows signs of cycloadduct hydrolysis (appearance of product peaks in the 9.5-10 ppm range) suggesting that the initial cycloadduct is less stable towards hydrolysis and rearrangement. The appearance of cycloadduct with EMPO was only identified by the detection of the appropriate m/z peak in UPLC-MS analysis of a semi-purified product (full product characterization was not accomplished). Preparatory TLC recovery of newly synthesized species led only to mixed product recovery (MS evidence of unreacted EMPO). Higher scale reactions could permit a better analysis and confirmation of formed cycloadducts.



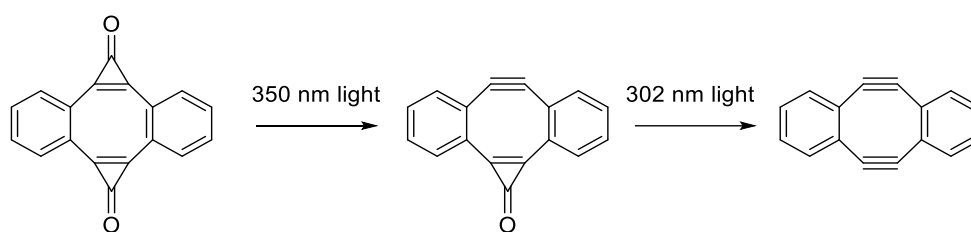
Scheme 4.3 Attempted cycloadditions with protected spiro[2.3]hex-1-ene and endocyclic nitrones a) reaction with DMImO b) reaction with EMPO.

These initial examples of successful cycloaddition between nitrones and cyclopropenes suggest that further reaction development could lead to faster reactions amenable to bioorthogonal applications. The synthesis and evaluation of other cyclopropene scaffolds such as 3,3-disubstituted cyclopropenes and azaspirohexenes would also allow for a better understanding of reaction scope/kinetics. Other small, strained alkenes such as cyclobutenes or azetidines could also be explored and be useful for applicability owing to their greater stability. Similarly, the scope of nitrones studied in these initial experiments was somewhat limited. Studying other nitron dipoles with various electron-withdrawing or electron-donating groups could help accelerate the reaction, in addition to studying solvent effects by moving towards aqueous solvent systems. Computational D/I analysis should also be employed to better guide synthesis efforts. However, given the relatively slower kinetics initially observed, further investigations were suspended.

4.6. Initial development of cyclopropenone-caged diyne and nitron cycloadditions

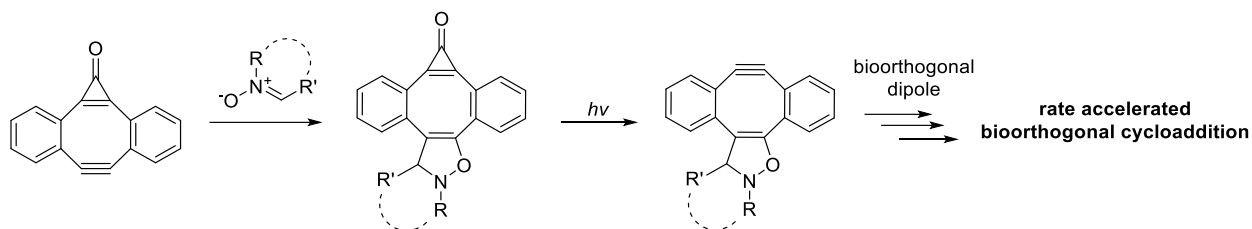
Cyclopropenone-caged strained alkynes serve as a useful tool to modulate and control the reactivity of strained alkynes. The cyclopropenone moiety acts as a protecting group, limiting cycloaddition chemistry and unwanted nucleophilic additions. Upon irradiation with UV light (350-400 nm) and subsequent decarbonylation, the strained alkyne can be uncaged and allowed to react with dipoles for bioorthogonal conjugation (see Scheme 4.4)^{278,279}. This uncaging mechanism allows for a great spatiotemporal control over conjugation chemistry. Recently, dialkyne structures, also referred to as Sondheimer diynes^{257,280}, have emerged as useful tools for bioorthogonal conjugation and polymer synthesis. These diynes can be caged using cyclopropenones to achieve similar modulation of reactivity as with simple strained

alkynes. Interestingly, the uncaging of the alkynes can be accomplished sequentially, by carefully controlling the wavelength of light applied for photoirradiation^{281,282}. This allows for a single alkyne to be uncaged and conjugated to another molecule through cycloaddition chemistry. After initial conjugation, the second alkyne moiety can be uncaged and then conjugated to a second molecule; thus allowing impeccable control over sequential functionalization of biomolecules.



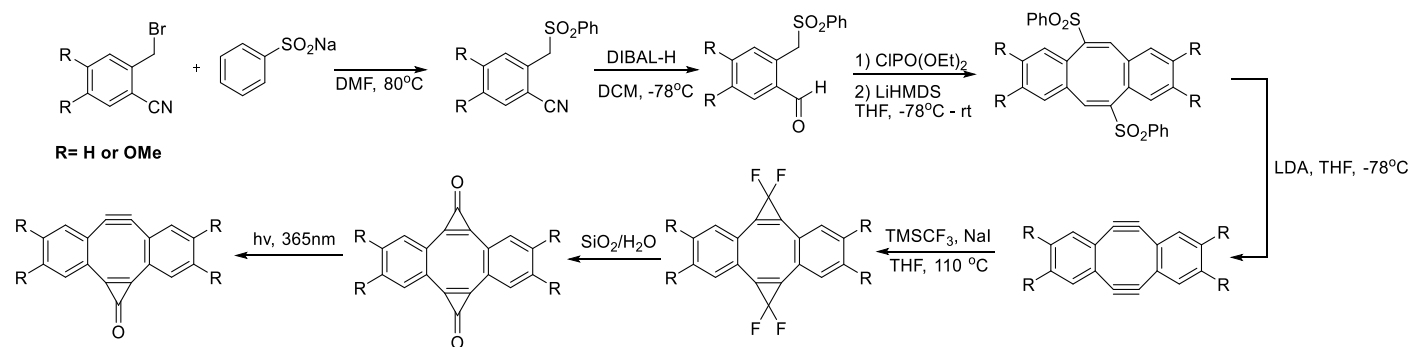
Scheme 4.4 Sequential photochemical decarbonylation of cyclopropenone-caged cyclooctadiyne

Given that this strategy has been successfully applied to azide chemistry by the Popik group²⁸¹, we envisioned being able to apply the strategy towards the development of SPANC chemistry, again taking advantage of the modularity of nitron dipoles. In studying SPANC reactions with unprotected Sondheimer diynes²⁸³, it was observed that the second cycloaddition would occur much faster than the first, and efforts to successfully trap or isolate monoadducts resulting from a single cycloaddition were unsuccessful. We therefore hypothesized that this cyclopropenone-caging strategy could be applied for the stepwise formation of cycloadducts, and thus the isolation of these elusive monocycloadducts. Given the enhanced reactivity of the second cycloaddition, the synthesis and isolation of these cycloadducts could prove an effective route towards strained alkynes with greatly enhanced reaction rates towards cycloaddition chemistry (see Scheme 4.5).

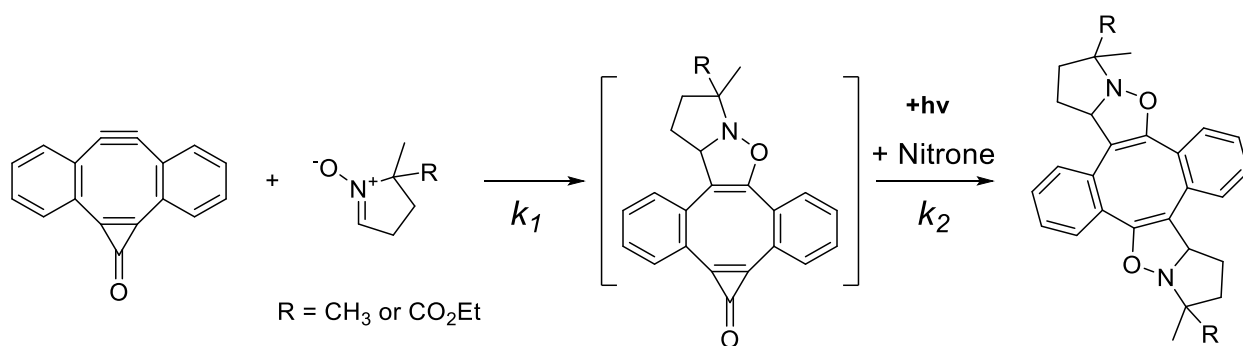


Scheme 4.5 Synthesis of monocycloadducts from nitronene cycloadditions with mono-deprotected diynes

Towards this goal, cyclopropenone-caged diynes were synthesized according to literature procedures (see Scheme 4.6)^{281,282}. Using a pressure vessel was found to greatly increase the yield of cyclopropenone formation. The obtained caged diyne was then photoirradiated with 365 nm light to selectively only deprotect a single alkyne, the reaction completion being monitored using UV spectroscopy. Chromatography then allowed for the recovery of unreacted caged diyne, and separation of fully uncaged diyne. For initial cycloaddition experiments with the mono-protected diyne, endocyclic nitrones were chosen due to their greater stability and greater stability of the formed cycloadducts (see Scheme 4.7).



Scheme 4.6 Synthesis of cyclopropenone-caged diynes.



Scheme 4.7 Proposed SPANC between mono-cyclopropenone-caged diyne and endocyclic nitrones (EMPO, DMPO)

The kinetics of the initial cycloaddition reaction with unsubstituted mono-protected diyne (R=H) were then analyzed via UV spectroscopy under pseudo-first order kinetics using excess nitron. The reaction with DMPO was found to have a second-order rate constant for the first cycloaddition equal to $1.2 \times 10^{-2} \pm 0.6 \text{ M}^{-1}\text{s}^{-1}$, a slightly lower rate constant than for the reaction with unprotected diyne with nitron dipoles and similar to the analogous reaction with azide dipoles (see Figure 4.3)^{281,283}. The reaction was faster with EMPO, displaying a second-order rate constant of $4.4 \times 10^{-2} \pm 0.7 \text{ M}^{-1}\text{s}^{-1}$, a faster reaction most likely owing to the use of a more electron-deficient nitron (see Figure 4.4). Finally, the reaction with DMImO nitron showed a second-order rate constant of $3.6 \times 10^{-2} \pm 0.2 \text{ M}^{-1}\text{s}^{-1}$ (see Figure 4.5). Synthesis of the monoadducts resulting from the cycloaddition of DMPO (R= CH₃) and EMPO (R=CO₂Et) was accomplished in 20:80 CH₂Cl₂:MeOH solvent mixtures at room temperature and isolation of the cycloadducts was accomplished via preparatory TLC.

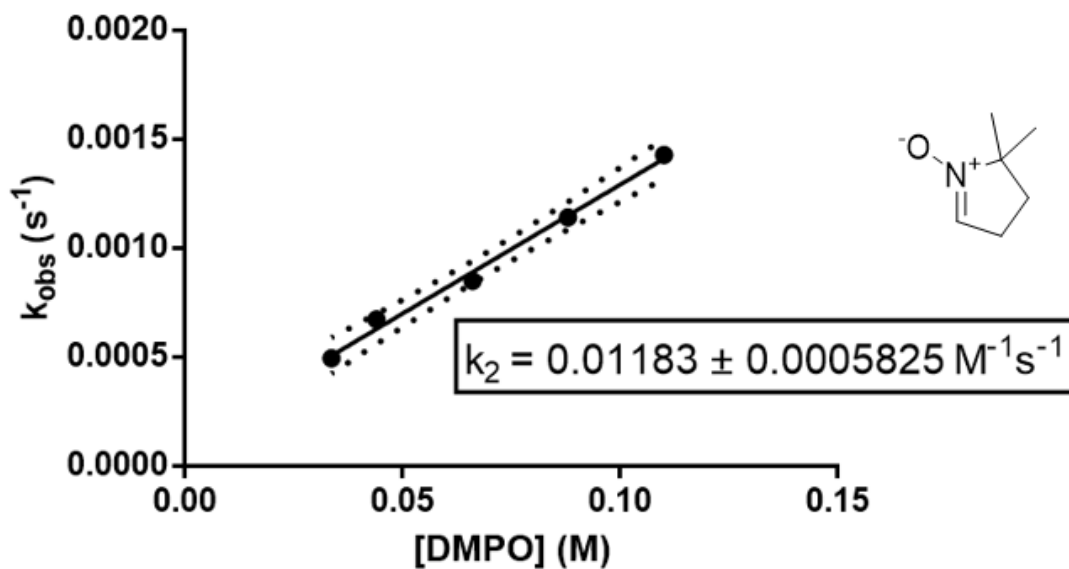


Figure 4.3 Pseudo-first order kinetic analysis of the cycloaddition between DMPO nitron and 2.2 μM of mono-protected diyne

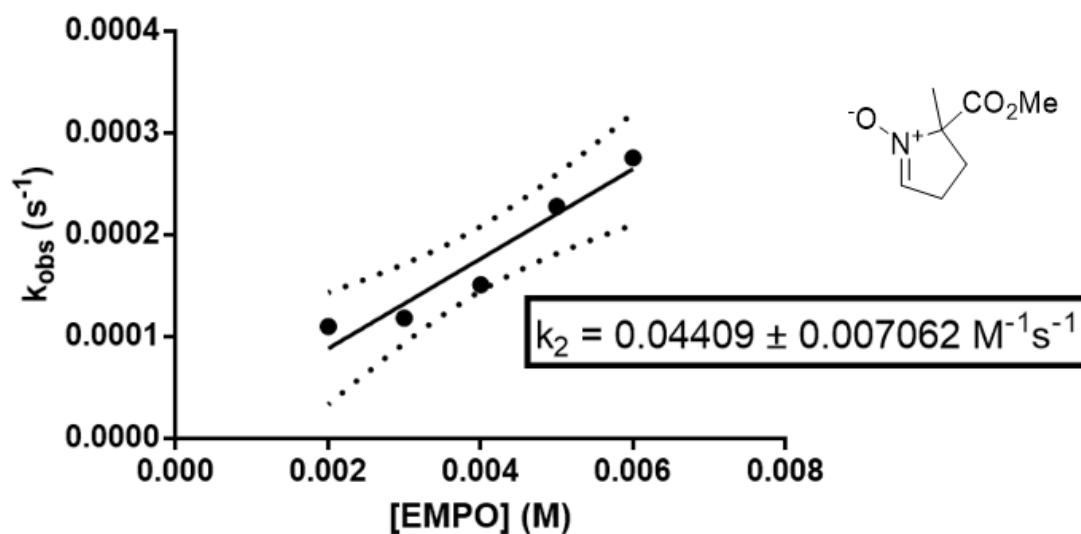


Figure 4.4 Pseudo-first order kinetic analysis of the cycloaddition between EMPO nitron and 100 μM of mono-protected diyne

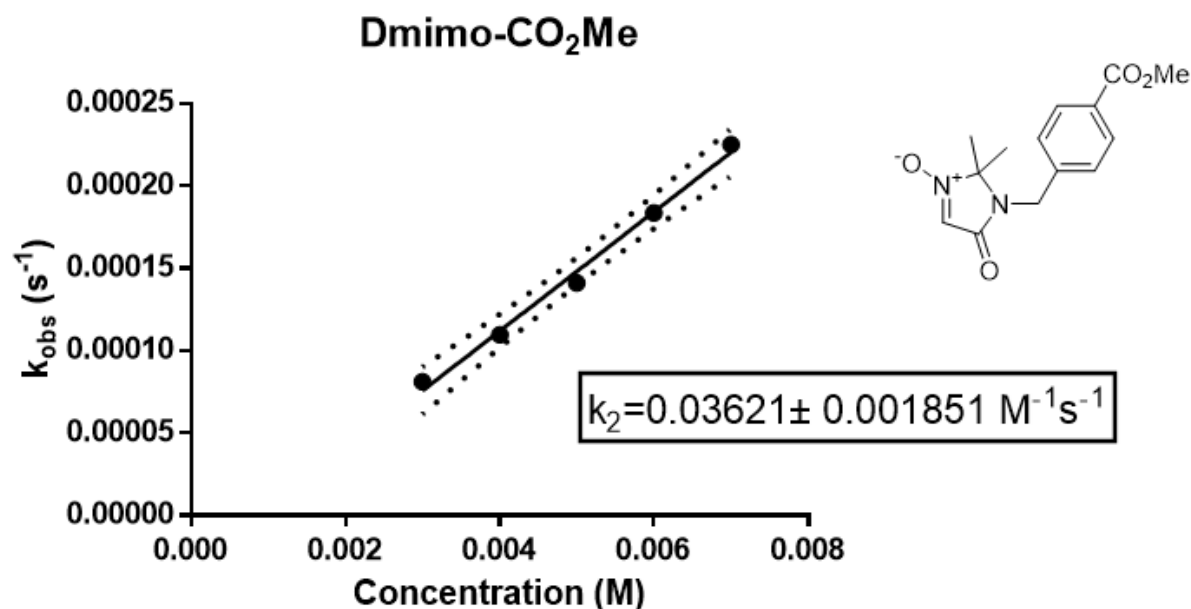


Figure 4.5 Pseudo-first order kinetic analysis of the cycloaddition between DMImO nitrone and 100 μM of mono-protected diyne

Given the initial results of the kinetics experiments, the higher scale synthesis of monoadducts between the monoyne and the cyclic nitrones, CMPO and DMPO, was completed. They were recovered via preparative TLC. The uncaging (using 302 nm light) of the monoadducts was attempted *in situ* with purified monoadduct sample solutions prepared for UV analysis. However, spectral overlap between the nitrone absorbance spectra and the uncaged monoadducts did not permit for easy monitoring of the second cycloaddition progress. The determination of reaction rate constants for the second cycloaddition could perhaps be better determined via NMR kinetics experiments or by synthesizing new nitrone-alkyne cycloadducts with distinctive absorbance peaks allowing for better UV monitoring. Given the low availability of starting materials and obtained monocycloadduct products, NMR kinetics experiments were not performed.

These initial experiments show that SPANC chemistry should be readily applicable to cyclopropenone-caged diynes. The observed reaction rate constants for the first cycloaddition reactions are of similar magnitude to the reactions with azide dipoles with caged-diynes and estimated reaction rates for nitron and unprotected diyne reactions. We were also able to demonstrate the isoxazoline monoadduct products are isolable, although full product characterization needs to be completed, following synthesis scale up. Further study of the reaction kinetics is expected to follow a similar trend of high rate accelerations for the second cycloadditions when compared to initial cycloadditions, with nitron dipoles displaying faster reactions than analogous azide dipoles. Given the success of these initial cycloadditions, further synthesis of functionalized diyne scaffolds, including asymmetrical scaffolds, is warranted, as this would allow for modulation of cycloaddition, as well as facilitate their potential incorporation into biomolecules, in turn allowing their dual functionalization via sequential cycloaddition. The scope of studied nitron was also limited to a few endocyclic nitron structures, but studying the reaction kinetics employing a variety of acyclic diaryl nitrons would allow for a better understanding of the electronic sensitivity of each cycloaddition step. Beyond nitron dipoles, the strategy of sequential uncaging of cyclopropenone-protected diynes opens the potential for the synthesis of a wide variety of mono-cycloadducts with different alternative dipoles, such as nitrile imines, sydnone, nitrile oxides, etc. It is expected that these synthesis efforts will be guided by an initial computational analysis evaluating activation energy barriers for secondary cycloaddition steps.

Chapter 5 Conclusion and Future Directions

5.1 Overview

The further development of bioorthogonal chemistry continually requires the development of new chemical transformations to tailor to a plethora of specific and complex applications. The development of new reactions applicable to biological systems, either *in vitro* or *in vivo* systems, allows for multiplex applications where multiple bioorthogonal transformations can be applied simultaneously to investigate multiple processes or track multiple biomolecules at once. Developing reactions with different reacting partners also allows for modulation of reaction rates better suited to specific applications, better spatiotemporal control of ligation, and greater applicability (through the use of accessible reagents). The work presented in this thesis has outlined recent developments in demonstrating the applicability of nitron dipoles as bioorthogonal reagents in various bioorthogonal ligation reactions; CuANCR and SPANC reactions with a variety of dipolarophiles. Chapter 2 presented the work done in optimizing micelle-assisted CuANCR chemistry, demonstrating the importance of careful reagent choice in obtaining efficient ligation through β -lactam formation. These findings also highlighted the use of micelles, especially employing biomimetic lipids, as a platform that can allow rapid optimization of reaction conditions towards their application to biological systems and their use in the functionalization of membrane associated peptides and proteins. Chapter 3 discussed the further development of SPANC chemistry and specifically the application of cyclooctadiyne scaffolds allowing for rapid bioconjugation between two dipole-containing molecules of interest. The development of nitron cycloadditions with diyne scaffolds holds the potential

for extremely rapid bioconjugation reactions and the synthesis of novel polymer materials with novel properties. Finally, Chapter 4 explored the initial development various strain-promoted reactions with cyclic nitrones. The development of these novel cycloadditions (cyclic phenanthridine nitrone scaffolds with strained alkynes, nitrone/cyclopropene cycloadditions and nitrone/cyclopropenone-caged diynes) highlight the great flexibility and modularity of the nitrone dipoles, and their further development should allow for future multiplex applications. Overall, these findings further highlight the use of nitrones as analogous dipoles to transformations developed with azide dipoles, generally allowing for greater tuneability of reactivity, greater structural variety and generally displaying faster reaction rates. The potential for nitrones to be incorporated to a variety of metabolites, through safe and accessible synthetic methods, and the potential for their *in situ* synthesis also highlights the advantages that they display over azides which have found widespread application in the field of bioorthogonal ligation. While their applicability has been demonstrated in the work presented in this thesis, there still exist many opportunities to further develop nitrone dipoles chemistry and expand upon their role in the bioorthogonal toolbox.

5.2 Perspectives on micelle-assisted CuANCR bioconjugations

One of the main challenges in the development of organic transformations towards their application to biological systems consists of optimization of the reaction for aqueous conditions. The necessity for aqueous conditions can render the optimization and analysis of reaction parameters difficult, as these conditions can limit the use of poorly soluble reagents and render product isolation difficult. The application of micelles and subsequently a fluorescence magnetic bead assay, allowed for the rapid evaluation of parameters affecting

reaction efficiency and provide an accessible platform for the rapid quantification of conjugation efficiency. This platform allowed for the determination of the importance of using electron-deficient propiolic ester and propiolamide alkynes, the potential for using endogenous amino acid copper ligands and the nitrone substrate scope amenable to CuANCR transformation. With optimized reaction parameters in hand, CuANCR could be applied to the functionalization of a membrane-associated peptide, E5-Tat. There exist opportunities to further optimize these methods for the development of more general conditions applicable to a wider variety of other membrane-associated peptides and other biomolecules requiring specific hydrophobic interactions for maintaining proper structure and solubility. Furthermore, the use of micelles in bioorthogonal reaction development has the potential to be applied to other thus underexplored cycloadditions beyond copper-catalyzed transformations, namely a plethora of metal-catalyzed transformations which have thus been underdeveloped owing to poor solubility of reagents. Greater exploration of micelle composition, namely other biomimetic lipids or the use of functionalized lipids (for example ligand containing lipids), is also warranted in order to provide a platform that more closely resembles the native biological environment in which the chemical transformation is to be applied. Finally, CuANCR chemistry provides the noted advantage over CuAAC chemistry that the ligation product consists of β -lactam cycloadducts, as opposed to triazoles. The reactivity of the β -lactam products have to this point been underexplored. Developing on the initial findings presented in this thesis, future work should focus on further developing the potential for secondary ligation through nucleophilic addition to β -lactams. While we were able to demonstrate, using the fluorescence magnetic bead assay, that addition of thiol nucleophiles to β -lactam ligation products, formed through CuANCR, led to the dual

functionalization of beads, more work is required to fully develop this dual ligation strategy. Namely, the scope of added nucleophiles remains underexplored, the conditions for cellular labelling require further optimization, and the scope of applications also needs to be developed beyond simple dual fluorophore functionalization. The optimization of this transformation would present a clear advantage of this strategy over the still widely applied CuAAC reaction. The potential for the *in situ* synthesis of bioactive β -lactam products also remains underexplored, with carbapenem-like scaffolds presenting the simplest potentially attainable scaffold. The synthesis of bioactive products has potential applications for the functionalization of implant materials or cellular membranes with antibiotic properties. While CuANCR chemistry holds great potential for a variety of bioconjugation applications, the limitations common to metal-catalyzed transformations still remain to be completely resolved in order to expand its application beyond *in vitro* applications. Future work towards the development of more biocompatible copper sources and ligands remain challenges to be solved.

5.3 Perspectives on the strain-promoted cycloaddition between Nitrones and Cyclooctadiyne

Cyclooctadiynes consist of interesting dipolarophile scaffolds containing two strained alkynes, allowing for the conjugation of two dipole-containing molecules of interest. The work presented in this thesis allowed for the demonstration of nitrones as competent dipoles for the dual cyclization applications, with evaluation of reaction parameters and kinetics showing that similarly to other strain-promoted cycloadditions involving azide dipoles, nitronc cycloadditions with cyclooctadiynes display faster reaction kinetics and access to a greater structural variety of cycloadducts. An interesting feature of double cycloadditions involving cyclooctadiynes consists of the great rate acceleration that is observed for the

second cycloaddition. Much like previous studies of the cycloaddition involving azide dipoles, we were unable to observe and isolate the products resulting from a single cycloaddition reaction (monocycloadduct). The isolation of these products could lead to the novel strained alkyne scaffolds displaying extremely fast reaction rate constants for other strain-promoted cycloaddition applications, given the rate accelerations determined for the second cycloaddition event. In efforts to observe the monocycloadducts, oligomerization experiments were performed, and while these efforts did not detect the desired adducts, we were able to demonstrate the potential for synthesis of oligomers from the reaction between di-nitrone structures and diynes. These experiments demonstrated the synthesis of cyclic oligomers resulting from intramolecular cyclization, limiting the size of the obtained polymer materials. Therefore, opportunities remain for studying the scope of applied di-nitrones scaffolds towards polymer synthesis and optimization of reaction, in order to obtain higher molecular-weight materials with interesting properties. Furthermore, while the applicability of the dual cycloaddition was demonstrated for the heterologous conjugation between azide and nitronone containing molecules via the labelling of bacterial cellular membranes, applications taking advantage of the diyne scaffold specifically remain underexplored with nitronone dipoles. Namely applications such as peptide stapling, where the potential for the *in situ* generation of nitronone dipoles on aldehyde containing (accessible through serine oxidation for example) peptides, could present an advantage where similar applications where azide incorporation could prove difficult. Finally, future development of the double cycloaddition between nitronones and diynes should focus on expanding the structural variety of diynes employed, allowing functionalization to be introduced not solely through functionalized dipoles, but also the dipolarophile. Exploration of diyne structure would also

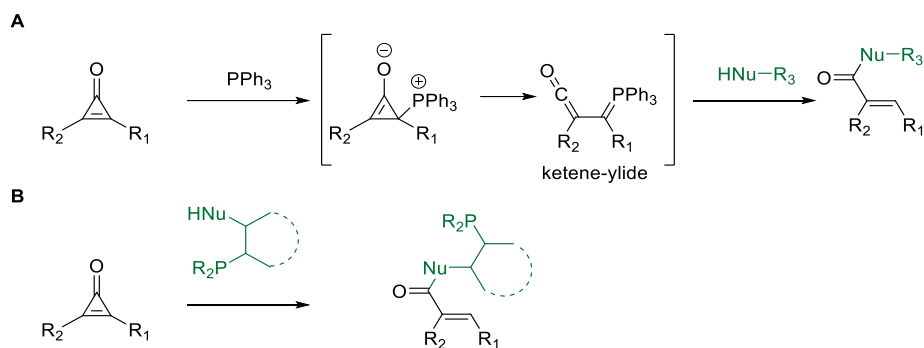
allow for better modulation of reactivity, potentially leading to much faster cycloadditions with nitrones.

5.3 Perspectives on other strain-promoted cycloadditions with Nitrones

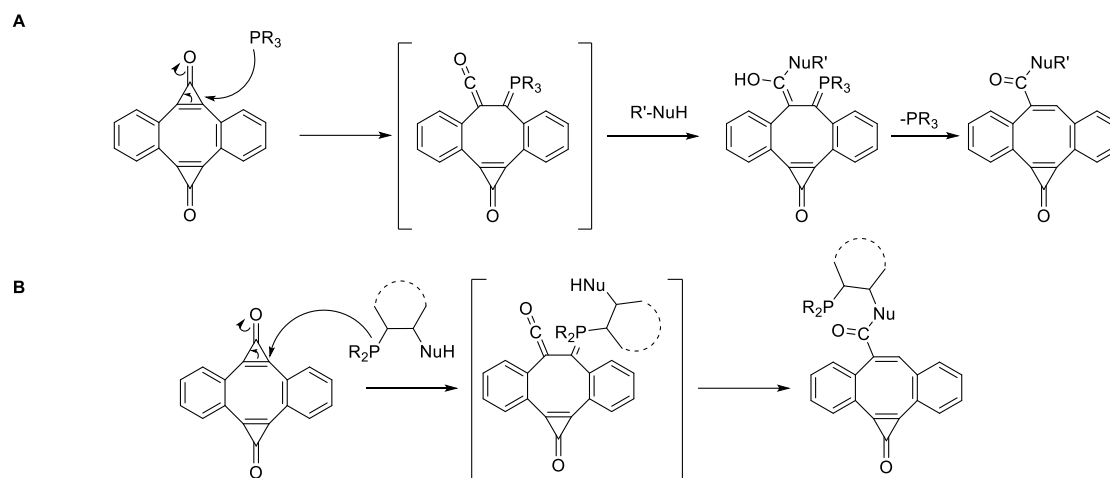
Chapter 4 presented work on the further development of strain-promoted cycloadditions between nitrones and various dipolarophiles. Many of the presented examples consisted of initial exploratory transformations and therefore there remains much work to be accomplished to further develop these ligation reactions and further expand upon the applicability and modularity achievable with nitrone dipoles. The work involving cycloadditions between phenanthridine-like endocyclic nitrones and strained alkynes displayed and examined the reactivity of a novel class of underexplored nitrone dipoles with the potential for being bioactive and also displaying a small FRET effect upon cycloaddition with fluorophore-containing strained alkyne; therefore future work could be done to expand upon this effect which could provide better signal-to-noise ratios where the observed fluorescence signal is modulated only upon successful bioconjugation. The scope of the nitrones structures involved in this study was also quite small, and further functionalization of the phenanthridine-like nitrones could result in obtaining faster cycloadditions more amenable to bioconjugations require low reaction times or low reagent concentration. Secondly, initial experiments involving the cycloaddition between highly strained cyclopropene dipolarophiles and nitrones were presented. While initial efforts did not yield reactions demonstrating very fast reaction kinetics, confirmation of cycloadduct formation does warrant further exploration. The reaction could be analyzed through DFT calculation and D/I computational analysis in order to guide synthesis efforts towards appropriate

cyclopropene scaffolds. Following a better theoretical understanding of the parameters affecting activation barriers, potentially novel nitrones scaffolds could be employed in reactions with cyclopropenes. Given the small size of cyclopropenes, the development of their reaction with nitrones could provide an option for bioconjugations requiring reagents that are minimally perturbing to biomolecule structure and function. The development of this transformation would again expand upon the available bioconjugation reactions and undoubtedly lead to multiplex applications with other bioorthogonal transformations. Finally, exploratory experiments involving cycloadditions between cyclopropenone-caged diynes and nitrones were presented. These cyclopropenone-caged diynes offer the potential for the development of bioconjugations with great spatiotemporal control through selective deprotection of cyclopropenones via UV irradiation. The use of cyclopropenone-caged diynes should also allow for the synthesis of highly-strained caged alkynes, where as observed with the uncaged diynes; the second cycloaddition should display high reaction rates. There remains work to be completed on the full kinetic evaluation of their reactivity with nitrones, expanding upon the scope of nitrones studied, but also fully characterizing the rate constants for the second cycloaddition reactions. This strategy is also not limited to nitron dipoles, and if the synthesis of the highly strained alkynes is demonstrable with nitrones, the scope should be expanded to other dipoles, efforts which should be guided by computational analysis. Beyond cycloaddition chemistry, cyclopropenone-caged diynes could hold the potential for reacting through phosphine-mediated nucleophilic additions. Recently, Prescher and coworkers developed such phosphine-mediated nucleophilic additions to cyclopropenones (see Scheme 5.1)^{59,284,285}. This transformation was shown to be amenable towards biomolecule tracking and crosslinking, as well as being orthogonal to other

bioorthogonal transformations, such as the tetrazine-TCO ligation, allowing for multiplex applications. Given that their work was able to demonstrate the applicability and bioorthogonality of cyclopropanones, a similar strategy should be able to be applied towards cyclopropanone-caged diynes further expanding upon the modularity of their application. Undisclosed initial efforts completed in our laboratory towards the development of these additions to caged-diyne indicate that future work on developing this class of dipolarophiles should focus on exploring this reactivity (see Scheme 5.2).



Scheme 5.1 Phosphine mediated bioorthogonal ligation reactions with cyclopropanones developed by Prescher and coworkers, showing the ketene-ylide intermediate trapped via A) intermolecular or B) intramolecular nucleophilic addition.



Scheme 5.2 Phosphine mediated A) intermolecular or B) intramolecular nucleophilic additions to cyclopropenone-caged Sondheimier diynes (only the first addition is shown).

The development of the phosphine-mediated nucleophilic addition to cyclopropenone-caged diynes would allow for highly modular applications, where for example the diyne can be conjugated to a biomolecule of interest through cycloaddition, and subsequent functionalization could occur via secondary dipole cycloaddition after cyclopropenone deprotection or through phosphine-mediated addition, which would allow for great spatiotemporal control of conjugation. This transformation could also potentially be applied towards protein crosslinking under very mild conditions, where the cyclopropenone-caged diyne is installed on proteins of interest through cycloaddition with preinstalled nitrone or azide dipoles, and protein crosslinking through addition of proximal nucleophiles found on interacting proteins (allowing for the analysis of transient protein interactions) could be triggered by phosphine addition (see Figure 5.1). This strategy employing mild bioorthogonal conditions could provide advantages over traditional protein cross-linking methods where highly electrophilic warheads are used^{286–288}, leading to high

unwanted side reactions or background signal, or where activation of cross-linking moieties through requires high energy UV irradiation²⁸⁹, which can be damaging to some proteins.

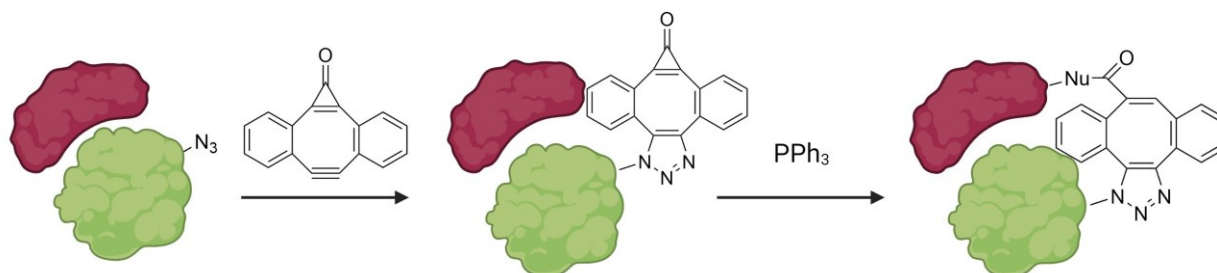


Figure 5.1 Phosphine-mediated crosslinking of azide functionalized protein.

Overall, the further development of nitron cycloadditions with cyclooctadiyne scaffolds, particularly the cyclopropenone-caged variants, holds great potential for both the synthesis of highly reactive strained dipolarophiles, which would benefit many applications where strain-promoted reactivity remains too slow and for new applications attainable through potentially novel phosphine-mediated nucleophilic addition chemistry, thus providing an extremely modular ligation strategy that should be amenable to a great variety of applications.

Other Contributions to Research and Publications

Research Articles

Margison, K. D.; Bilodeau, D. A.; Mahmoudi, F.; Pezacki, J. P. **Cycloadditions of Trans-Cyclooctenes and Nitrones as Tools for Bioorthogonal Labelling.** *ChemBioChem* 2020, 21 (7), 948–951. <https://doi.org/10.1002/cbic.201900627>.

Kaitlyn Margison (KM) and DAB completed the synthesis of nitrones used in this study. Farnaz Mahmoudi with assistance from DAB completed the kinetic evaluation of cyclic nitrones and TCO by NMR. DAB also participated in the redaction of the published manuscript.

Shaw, T. A.; Ablenas, C. J.; Desrochers, G. F.; Powdrill, M. H.; Bilodeau, D. A.; Vincent-Rocan, J.-F.; Niu, M.; Monette, A.; Mouland, A. J.; Beauchemin, A. M.; Pezacki, J. P. **A Bifunctional Nucleoside Probe for the Inhibition of the Human Immunodeficiency Virus-Type 1 Reverse Transcriptase.** *Bioconjugate Chem.* 2020, 31 (5), 1537–1544. <https://doi.org/10.1021/acs.bioconjchem.0c00191>.

In collaboration with Tyler Shaw (TS), DAB completed the synthesis and characterization of probes: Azauracil-azidothymidine (AZT) and Azauracil-azidothymidine triphenylphosphate (AZTTP). Characterization was completed by ^1H , ^{13}C , ^{31}P , HMBC and HSQC NMR and HRMS (ESI+) analysis.

Nakajima, M.; Bilodeau, D. A.; Pezacki, J. P. **Predicting Reactivity for Bioorthogonal Cycloadditions Involving Nitrones.** *RSC Adv.* 2020, 10 (49), 29306–29310. <https://doi.org/10.1039/D0RA05092J>.

DAB completed the redaction of the manuscript from analysis completed by Dr. Nakajima (MN) and Dr. Pezacki (JPP).

Ahmed, N.; Ahmed, N.; Bilodeau, D. A.; Pezacki, J. P. **An Unnatural Enzyme with Endonuclease Activity towards Small Non-Coding RNAs.** *Nature Communications* **2023**, *14* (1), 3777. <https://doi.org/10.1038/s41467-023-39105-0>.

DAB completed the multigram synthesis of racemic and enantiopure unnatural amino acid, 3-(2,2'-bipyridin-5-yl)-L-alanine, for the purposes of incorporation in p19 protein through genetic code expansion techniques.

Review Articles

Scinto, S. L.; Bilodeau, D. A.; Hincapie, R.; Lee, W.; Nguyen, S. S.; Xu, M.; am Ende, C. W.; Finn, M. G.; Lang, K.; Lin, Q.; Pezacki, J. P.; Prescher, J. A.; Robillard, M. S.; Fox, J. M. **Bioorthogonal Chemistry.** *Nature Reviews Methods Primers* **2021**, *1* (1), 30. <https://doi.org/10.1038/s43586-021-00028-z>.

DAB participated in the redaction of sections relating to oxime/hydrazone ligation chemistry and strain-promoted [3+2] bioorthogonal cycloadditions (SPANC), and preparation of related figures.

Bilodeau, D. A.; Margison, K. D.; Serhan, M.; Pezacki, J. P. **Bioorthogonal Reactions Utilizing Nitrones as Versatile Dipoles in Cycloaddition Reactions.** *Chem. Rev.* **2021**, *121* (12), 6699–6717. <https://doi.org/10.1021/acs.chemrev.0c00832>.

DAB participated in the preparation, redaction and editing of this review article; redacting sections pertaining to the introduction, computational Distortion/Interaction analysis, incorporation of bioorthogonal moieties into biomolecules, other dipolarophile-nitrone cycloaddition reactions, and the development of the Kinugasa reaction.

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Supporting Information

Electronic Supplementary Material (ESI) for ChemComm.
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FSupplementary Information (SI)

Optimized Aqueous Kinugasa Reactions for Bioorthogonal Chemistry Applications

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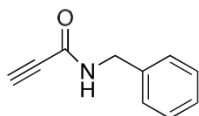
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Materials and Synthetic Methods

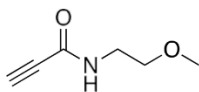
All reagents and solvents were purchased from Sigma-Aldrich, unless otherwise stated, and used without further purification. Deuterated solvents were purchased from Cambridge Isotope laboratories. Thin layer chromatography was performed on SiliCycle Siliplate® silica gel plates (60 Å F254, layer thickness 200µm). Flash chromatography was performed using silica gel (60 Å, particle size 40–63 µm). Fluorescence microscopy was performed with a Nikon Ni-U ratiometric fluorescence microscope equipped with a LED excitation light source and Ultra-sensitive Andor iXon Ultra 897 cooled EMCCD camera. Images were acquired using a Nikon 60x oil dip objective lens and, if indicated, a 2x relay lens. Fluorescence images were obtained under strictly identical conditions of gain and exposure time, on focused beads, typically 2–5 s, and brightfield images were obtained using a 20–50 ms exposure. Images were acquired using Nikon NIS Elements software and processed with ImageJ. All ^1H and ^{13}C NMR spectra were obtained on a Bruker Avance 300 or 400 spectrometer using a frequency of 300 MHz or 400 MHz for ^1H and 100 MHz for ^{13}C and processed using iNMR 4.2.0 software. The following abbreviations were used to designate chemical shift multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved, br = broad signal and J = coupling constants in Hz.

Synthetic Methods

Synthesis of Propiolamides



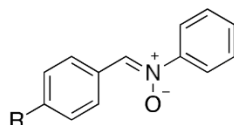
N-Benzylprop-2-ynamide. 0.3 g of propiolic acid was added to 5 mL DMF in a 25 mL round bottom flask equipped with a stir bar. 1.71 g HATU, 0.49 mL benzylamine were added successively to reaction mixture. Lastly, 0.82 mL DIPEA was added and the reaction was let stir for 1 hour at room temperature. Following reaction completion by TLC, the reaction was concentrated in vacuo. Reaction contents were dissolved in 20 mL of ethyl acetate and washed with 10 mL 70% brine 3 times. 60% yield of pure product was recovered following column chromatography using 1:1 EtOAc: Hexanes. ^1H NMR (300 MHz; CDCl_3): δ 7.37–7.30 (m, 5H), 6.10 (s, 1H), 4.50 (d, J = 5.9 Hz, 2H), 2.80 (s, 1H). Spectral data was consistent with previously reported data.¹



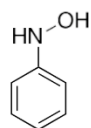
N-(2-methoxyethyl)prop-2-ynamide. Under an Argon atmosphere, propiolic acid (0.3 g, 4.28 mmol) was dissolved in 10 mL of dry DMF, followed by the addition of HATU (1.71 g, 4.50 mmol). 2-Methoxyethylamine (391 µL, 4.50 mmol) was slowly added over 15 minutes, followed by the addition of DIPEA (819 µL). The reaction was allowed to stir at room temperature for 6 hours, after which 10 mL of EtOAc was added. The mixture was extracted three times with 10 mL of a 50% brine solution. The organic layer was recovered, dried over Na_2SO_4 , filtered and concentrated. Pure product (0.24 g) was obtained as a light yellow solid in 44% yield following flash column chromatography using 3:97 MeOH:DCM. ^1H NMR (400 MHz; CDCl_3): δ 6.26 (s, 1H), 3.52–3.46 (m, 4H), 3.37 (s, 3H), 2.79 (s, 1H); ^{13}C NMR (CDCl_3 , 100 MHz) δ = 152.1, 73.2, 70.5, 58.8, 39.5. HRMS: for $\text{C}_6\text{H}_9\text{NO}_2$ ($\text{M}+\text{H}$): calculated: 128.0633; found: 128.0693.

Synthesis N-Phenyl-Nitrone Derivatives

General Nitrone Synthesis A

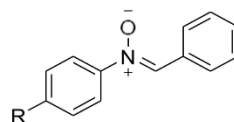


300 mg (2.8 mmol) of phenylhydroxylamine and 3 mmol of *para*-substituted benzaldehyde were added to a dry 25 mL round-bottom flask. 5 mL of dry ethanol was added to the flask and the mixture was stirred for 2 hours at 35 °C. Pure products were obtained following filtration of the precipitated crude product and recrystallization from warm ethanol and hexanes.

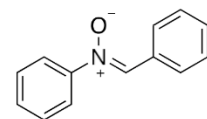


N-Phenylhydroxylamine. A mixture of nitrobenzene (10.8 mL, 0.105 mol), NH_4Cl (6.5 g, 0.12 mol) and degassed H_2O (200 mL) under argon at r.t. was stirred vigorously while zinc dust (15.4 g, 0.21 mol) was added portion wise over 20 minutes. After addition was complete, the reaction mixture was stirred for an additional 20 minutes and was filtered while still warm. The resultant filter cake was washed with hot distilled water (50 mL) and the combined filtrate was saturated with NaCl, and extracted with 3x100 mL of EtOAc. The organic layers were combined, dried over Na_2SO_4 and concentrated under reduced pressure. The crude N-phenyl hydroxylamine was recrystallized from petroleum ether/EtOAc (8.2 g, 72 %), dried thoroughly and stored under an atmosphere of argon at -20 °C. $^1\text{H NMR}$ and MS data are in agreement with that reported previously.² $^1\text{H NMR}$ (300 MHz; CDCl_3): δ 7.32- 7.26 (m, 2H), 7.02-6.97 (m, 3H), 6.77 (dq, J = 2.5, 0.8 Hz, 1H).

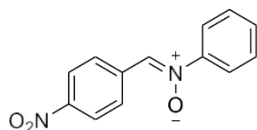
General Nitrone Synthesis B



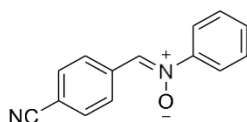
16 mmol of *para*-substituted nitrobenzene and 1.7 g (16 mmol) of benzaldehyde were added to a dry 100 mL round-bottom flask. 29 mL of EtOH and 29 mL of H_2O were added, followed by 0.9 g (18 mmol) of NH_4Cl . The reaction mixture was cooled to 0 °C, and 1.9 g of Zn power (29 mmol) was added over the course of 20 minutes. The reaction was allowed to warm to room temperature and stirred for 16 hours. The reaction was then filtered through Celite, and washed 3 times with 60 mL DCM. The organic layers were combined, dried over Na_2SO_4 , filtered, and concentrated. Pure product was obtained following recrystallization from warm EtOAc and Hexanes.³



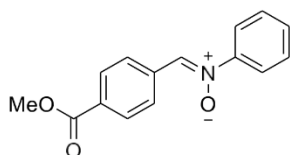
C,N-diphenylnitrone. The nitrone was synthesized according to General Nitrone Synthesis B. The product was obtained in 50% yield. $^1\text{H NMR}$ (300 MHz; CDCl_3): δ 8.42-8.39 (m, 2H), 7.93 (s, 1H), 7.80-7.77 (m, 2H), 7.50-7.48 (m, 6H). Spectral data was consistent with previously reported data.⁴



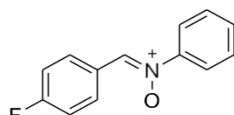
C-(4-nitrophenyl)-N-phenylnitrone. The nitrone was synthesized according to General Nitrone Synthesis A. The product was obtained in 75% yield. $^1\text{H NMR}$ (300 MHz; CDCl_3): δ 8.58-8.53 (m, 2H), 8.34-8.30 (m, 2H), 8.07 (s, 1H), 7.80-7.77 (m, 2H), 7.53 (dd, $J=4.2, 2.5$ Hz, 3H). Spectral data was consistent with previously reported data.⁴



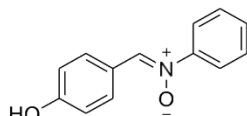
C-(4-cyanophenyl)-N-phenylnitrone. The nitrone was synthesized according to General Nitrone Synthesis A. The product was obtained in 52% yield. $^1\text{H NMR}$ (400 MHz; CDCl_3): δ 8.48 (d, $J=8.4$ Hz, 2H), 8.00 (s, 1H), 7.78-7.74 (m, 4H), 7.52 (t, $J=3.3$ Hz, 3H). Spectral data was consistent with previously reported data.⁴



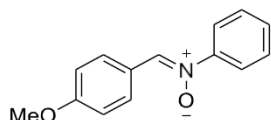
C-(4-methoxycarbonylphenyl)-N-phenylnitrone. The nitrone was synthesized according to General Nitrone Synthesis A. The product was obtained in 63% yield. $^1\text{H NMR}$ (300 MHz; CDCl_3): δ 8.45 (d, $J=8.5$ Hz, 2H), 8.14 (d, $J=8.7$ Hz, 2H), 8.00 (s, 1H), 7.78 (dd, $J=6.9, 2.9$ Hz, 2H), 7.52-7.49 (m, 3H), 3.95 (s, 3H). Spectral data was consistent with previously reported data.⁵



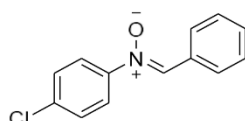
C-(4-fluorophenyl)-N-phenylnitrone. The nitrone was synthesized according to General Nitrone Synthesis A. The product was obtained in 54% yield. $^1\text{H NMR}$ (300 MHz; CDCl_3): δ 8.48-8.43 (m, 2H), 7.91 (s, 1H), 7.79-7.76 (m, 2H), 7.52-7.48 (m, 3H), 7.20-7.15 (m, 2H). Spectral data was consistent with previously reported data.⁴



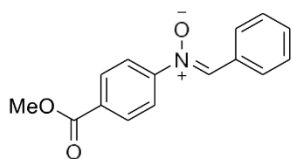
C-(4-hydroxyphenyl)-N-phenylnitrone. The nitrone was synthesized according to General Nitrone Synthesis A. The product was obtained in a 47% yield. $^1\text{H NMR}$ (300 MHz; CD_3OD): δ 8.40-8.35 (m, 2H), 8.27 (s, 1H), 7.84-7.81 (m, 2H), 7.57-7.51 (m, 3H), 6.95-6.90 (m, 2H). Spectral data was consistent with previously reported data.⁵



C-(4-methoxyphenyl)-N-phenylnitrone. The nitron was synthesized according to General Nitron Synthesis A. The product was obtained in 77% yield. $^1\text{H NMR}$ (300 MHz; CDCl_3): δ 8.43-8.39 (m, 2H), 7.86 (s, 1H), 7.80-7.76 (m, 2H), 7.51-7.44 (m, 3H), 7.02-6.99 (m, 2H), 3.89 (s, 3H). Spectral data was consistent with previously reported data.³

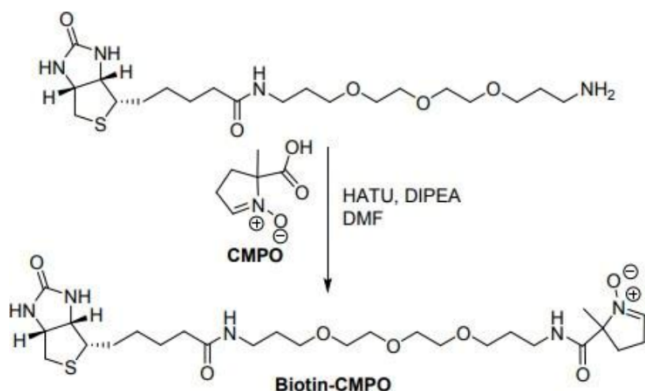


N-(4-chlorophenyl)-a-phenylnitrone. The nitron was synthesized according to General Nitron Synthesis B. The product was obtained in 53% yield. $^1\text{H NMR}$ (300 MHz; CDCl_3): δ 8.40-8.37 (m, 2H), 7.90 (s, 1H), 7.77-7.73 (m, 2H), 7.49 (q, $J=3.3\text{ Hz}$, 4H), 7.45 (t, $J=2.5\text{ Hz}$, 1H). Spectral data was consistent with previously reported data.³



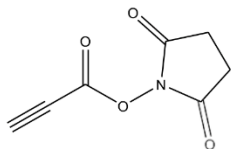
N-(4-methoxycarbonylphenyl)-a-phenylnitrone. The nitron was synthesized according to General Nitron Synthesis B. The product was obtained in 83% yield.⁵ $^1\text{H NMR}$ (300 MHz; CDCl_3): δ 8.43-8.39 (m, 2H), 8.19-8.15 (m, 2H), 7.98 (s, 1H), 7.89-7.85 (m, 2H), 7.52-7.49 (m, 3H), 3.96 (s, 3H). Spectral data was consistent with previously reported data.⁵

Synthesis of Biotin-CMPO



The Biotin-CMPO was synthesized according to previously reported procedures.⁹ Biotin-PEG (91 mg, 0.20 mmol, 1 eq), CMPO (35 mg, 0.24 mmol, 1.2 eq) and HATU (76 mg, 0.20 mmol, 1 eq) were dissolved in DMF (175 μ L). DIPEA (52 μ L, 0.30 mmol, 1.75 eq) was added all at once and the mixture was stirred for 45 minutes. Reaction progress was confirmed by LC-MS. The reaction was concentrated under reduced pressure and stored at -20 °C overnight. The crude was purified using preparatory HPLC with MeCN/H₂O/Formic acid (0.1%) as eluent, running gradient of 10 to 60% acetonitrile over 15 minutes. The product eluted at 7.5-8 minutes and its presence was confirmed by MS; the fractions were pooled and concentrated under reduced pressure. Some starting material was also isolated. The product was obtained as colourless oil (20.1 mg, 0.035 mmol, 17.5 % yield). MS (ESI+) calcd (C₂₆H₄₅N₅O₇S): 572.30 [M+H]⁺, found 572.1; ¹H NMR (400 MHz, MeOD-d₄) δ 7.24 (s, 1H), 4.52 (dd, 1H, J=4.9, 7.7 Hz), 4.33 (dd, 1H, J=4.4, 7.8 Hz), 3.64 (m, 9H), 3.55 (dt, 4H, J=4.7, 6.0, 6.1 Hz), 3.36 (m, 2H), 3.25 (m, 3H), 2.95 (dd, 1H, J=4.9, 12.7 Hz), 2.71 (dd, 3H, J=10.6, 14.1 Hz), 2.22 (t, 3H, J=7.4, 7.4 Hz), 1.79 (ddd, 5H, J=4.0, 6.4, 12.8 Hz), 1.69 (s, 3H), 1.62 (m, 3H), 1.47 (dd, 2H, J=7.5, 15.2 Hz); ¹³C NMR (100 MHz, MeOD-d₄) δ 174.6, 170.8, 141.1, 79.1, 70.1, 69.9, 69.8, 68.6, 68.5, 62.0, 60.2, 55.6, 39.7, 37.0, 36.4, 35.5, 31.0, 29.0, 28.8, 28.4, 28.1, 25.5, 25.0, 21.5. Spectral data was consistent with previously reported data.⁶

Synthesis of N-hydroxysuccinimide ester alkyne

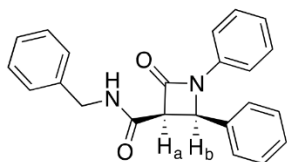


1-[(1-oxo-2-propynyl)oxy]-2,5-pyrrolidinedione. Synthesis was accomplished following a modified procedure.⁷ Pro-piolic acid (500 mg, 7.14 mmol) and N-hydroxysuccinimide (822 mg, 7.14 mmol) were suspended in EtOAc (39 mL) and cooled to 0°C. A solution of *N,N'*-Dicyclohexylcarbodiimide (1.47 g, 7.14 mmol) in EtOAc (13 mL) was added dropwise over the course of 1 hour. The mixture was then stirred at 0°C for 6 hours. The urea byproduct was removed by filtration and the filtrate was concentrated under reduced pressure to approximately 10 mL and then washed twice with brine (5 mL). The organic phase was dried with Na₂SO₄, then concentrated to approximately 1-2 mL. The concentrated organic phase was cooled to -5 °C; 1-2 mL of heptanes was then added and the mixture was

further cooled to -10 °C and the resulting solids were stirred for 2 hours. The solid precipitate was filtered, rinsed with cold heptanes and dried under vacuum to yield the product as a white solid (895 mg, 75% yield). ¹H NMR (300 MHz, CDCl₃): δ 2.87 (s, 4H), 3.31 (s, 1H). Spectral data was consistent with previously reported data.⁷

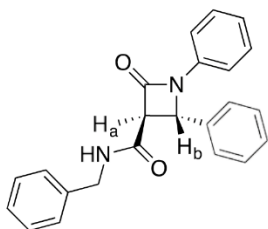
Procedure for isolation of β-lactam products for characterization

The reaction was conducted in 6 mL of argon degassed H₂O. 4 mL of acetonitrile was added to help solubilize organic reagents. L-Proline (36 mg, 0.63 mmol), CuSO₄·5H₂O (39 mg, 0.16 mmol), sodium ascorbate (249 mg, 1.26 mmol) and pyridine (51 μL, 0.63 mmol) were then added successively. Following the addition of N-benzylprop-2-ynamide (50 mg, 0.31 mmol), C,N-diphenylnitrone (62 mg, 0.31 mmol) was added and the mixture was allowed to stir for 1 hour at 25 °C. The reaction was then extracted 3x20 mL of EtOAc. The organic fractions were then dried over Na₂SO₄ and concentrated under reduced pressure. The dried residue was then purified by flash column chromatography using 30% EtOAc in hexanes. The product was recovered as a white solid for analytical and characterization purposes.



N-benzyl-2-oxo-1,4-diphenylazetidine-3-carboxamide, (3S, 4R) -rel-

¹H NMR (400 MHz; CDCl₃): δ 7.38 (d, *J* = 13.6 Hz, 4H), 7.29 (d, *J* = 14.0 Hz, 6H), 7.24 (s, 4H), 7.06 (s, 1H), 6.54 (t, *J* = 0.3 Hz, 1H), 5.41 (d, *J* = 2.6 Hz, 1H), 4.56 (dd, *J* = 14.8, 6.1 Hz, 1H), 4.41 (dd, *J* = 14.8, 5.5 Hz, 1H), 3.86 (d, *J* = 2.6 Hz, 1H); ¹³C NMR (100 MHz; CDCl₃): δ 163.5, 163.1, 137.6, 136.9, 133.9, 129.5, 129.25, 129.21, 129.01, 128.89, 128.82, 128.6, 127.86, 127.81, 127.5, 126.9, 126.2, 124.6, 77.3, 59.4, 58.3, 43.2, 29.9; HRMS (ESI-TOF): for C₂₃H₂₀N₂O₂ (M+Na⁺): calculated: 379.1525; found: 379.1422.



N-benzyl-2-oxo-1,4-diphenylazetidine-3-carboxamide, (3R, 4R) -rel-

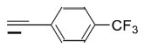
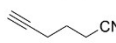
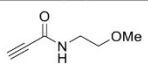
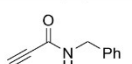
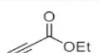
¹H NMR (400 MHz; CDCl₃): δ 7.33 (s, 5H), 7.28 (d, *J* = 7.1 Hz, 4H), 7.25 (s, 3H), 7.08 (d, *J* = 6.9 Hz, 1H), 7.03 (d, *J* = 9.5 Hz, 2H), 6.77 (s, 1H), 5.37 (d, *J* = 6.3 Hz, 1H), 4.52 (d, *J* = 6.2 Hz, 1H), 4.39 (dd, *J* = 14.9, 6.5 Hz, 1H), 4.14 (dd, *J* = 14.8, 5.1 Hz, 1H); ¹³C NMR (101 MHz; CDCl₃): δ 163.5, 163.1, 137.6, 136.9, 133.9, 129.5, 129.25, 129.21, 129.01, 128.89, 128.82, 128.6, 127.86, 127.81, 127.5, 126.9, 126.2, 124.6, 77.3, 59.4, 58.3, 43.2, 29.9; HRMS (ESI-TOF): for C₂₃H₂₀N₂O₂ (M+Na⁺): calculated: 379.1525; found: 379.1422.

In vitro Micelle-Assisted Kinugasa/CuANCR reactions

General Procedure

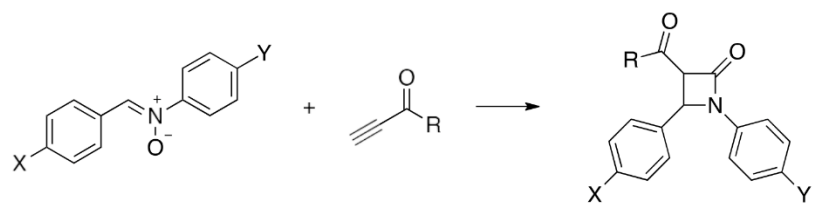
Reactions were conducted in 20 mL of argon degassed H₂O containing 10 mM sodium dodecyl sulfate (58 mg, 0.2 mmol). Sodium ascorbate (40 mg, 0.2 mmol), pyridine (8 μ L, 0.1 mmol), L-proline (6 mg, 0.05 mmol) and CuSO₄ (6 mg, 0.025 mmol) were then added successively. Following the addition of alkyne **1-8** (0.05 mmol), C,N- diphenylnitrone (10 mg, 0.05 mmol) was added and the reaction was stirred for 30 minutes at 25 °C. 3 mL of brine was then added to the mixture, followed by an extraction with 3x20 mL of EtOAc. The organic fractions were then dried over Na₂SO₄ and concentrated under reduced pressure. An internal standard, 1,4-Dimethoxybenzene, was accurately weighed (approximately 0.1 mmol) and added to the dried reaction. NMR yields were obtained by comparing relevant new cis/trans product peaks (β -lactam doublet peaks, 4.5-5.5 ppm range, H_a from the representative product spectra) to the internal standard peak (6.83 ppm, s, 4H). Nitrone conversion was determined by comparing the remaining nitrone peak (8.41 ppm, m, 2H) to the initial amount used (0.05 mmol). The diastereomeric ratio was determined by comparing the calculated NMR yields of both the β -lactam products, while assuming that the minor product was *cis*.

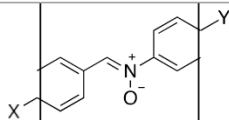
Table S1. Diastereomeric ratios for screen of alkynes used in micelle-assisted Kinugasa reactions^a

Entry	Alkyne	Yield	trans: cis
1		22 ^b	55:45
2		16 ^b	70:30
3		20 ^b	55:45
4		32 ^b	77:23
5		60	80:20
6		64	68:32
7a/b		65/21 ^c	74:26/ 55:45

^aIsolated yield extracted from micellar emulsions. ^bEntries 1, 2, 3 and 4 were conducted in 3.5 mM CTAB (26 mg, 0.07 mmol) instead of SDS and in the absence of L-proline. ^cEntry 7b was conducted in the absence of surfactant.

Table S2. Screen of nitrones used in micelle-assisted Kinugasa Reaction



Entry		A R=OEt (%) yield	B R=NHBN (%) yield	C R=NH(CH ₂) ₂ OCH ₃ (%) yield
1	X=NO ₂ , Y=H	25	38	27
2	X=OCH ₃ , Y=H	53	45	50
3	X=F, Y=H	46	41	56
4	X=OH, Y=H	57	37	54
5	X=CN, Y=H	47	40	44
6	X=CO ₂ CH ₃ , Y=H	30	36	41
7	X=H, Y=CO ₂ CH ₃	59	65	63
8	X=H, Y=Cl	31	55	24

Micelle Assisted Kinugasa/CuANCR Reaction on Alkyne Beads

5 μ L of alkyne-tagged beads (corresponding to 3-5 μ M of reactive alkyne groups, Click Chemistry tools) were washed in PBS prior to use. The reaction was carried out in PBS and consisted of 100 μ M CuSO_4 , 2 mM freshly solubilized sodium ascorbate, 200 μ M L-proline, 50 μ M biotin-CMPO (or vehicle DMSO) to which the indicated amount of surfactant or water solvent was added. The reaction was started by addition of washed beads and was carried out with gentle shaking at 37 $^\circ\text{C}$ for the indicated amount of time. Beads were washed 1x with PBS containing 0.05% Tween20, then 3x with PBS prior addition of 5 $\mu\text{g/mL}$ FITC-streptavidin in PBS. The binding of streptavidin-FITC was carried out at room temperature for 30 minutes in the dark. The beads were washed three more times with PBS and resuspended in PBS containing 5% glycerol. 8 μ L of this solution was applied to a microscopy slide which were imaged using the Nikon Ni-U ratiometric fluorescence microscope equipped with a LED excitation light source and Ultra-sensitive Andor iXon Ultra 897 cooled EMCCD camera. Images were acquired using a Nikon 60x oil dip objective lens and, if indicated, a 2x relay lens. Fluorescence images were obtained under strictly identical conditions of gain and exposure time, on focused beads, typically 2-5 s, and brightfield images were obtained using a 20-50 ms exposure. Images were acquired using Nikon NIS Elements software and processed with ImageJ.

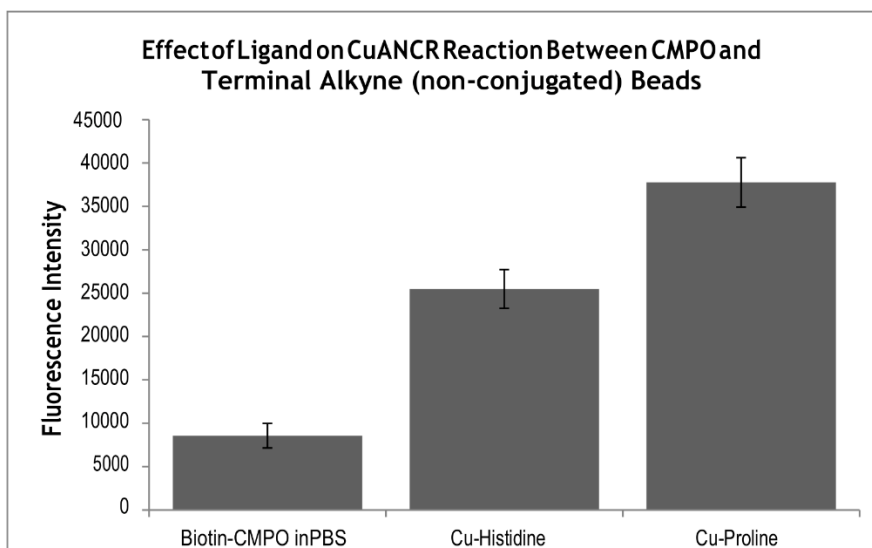


Figure S1. Quantification of Figure 2 CuANCR magnetic beads. Images were set to the same fluorescence intensity levels then converted to 8bit. Integrated density from the same area was then measured for 5 beads per image, which is from different fields of view for each sample

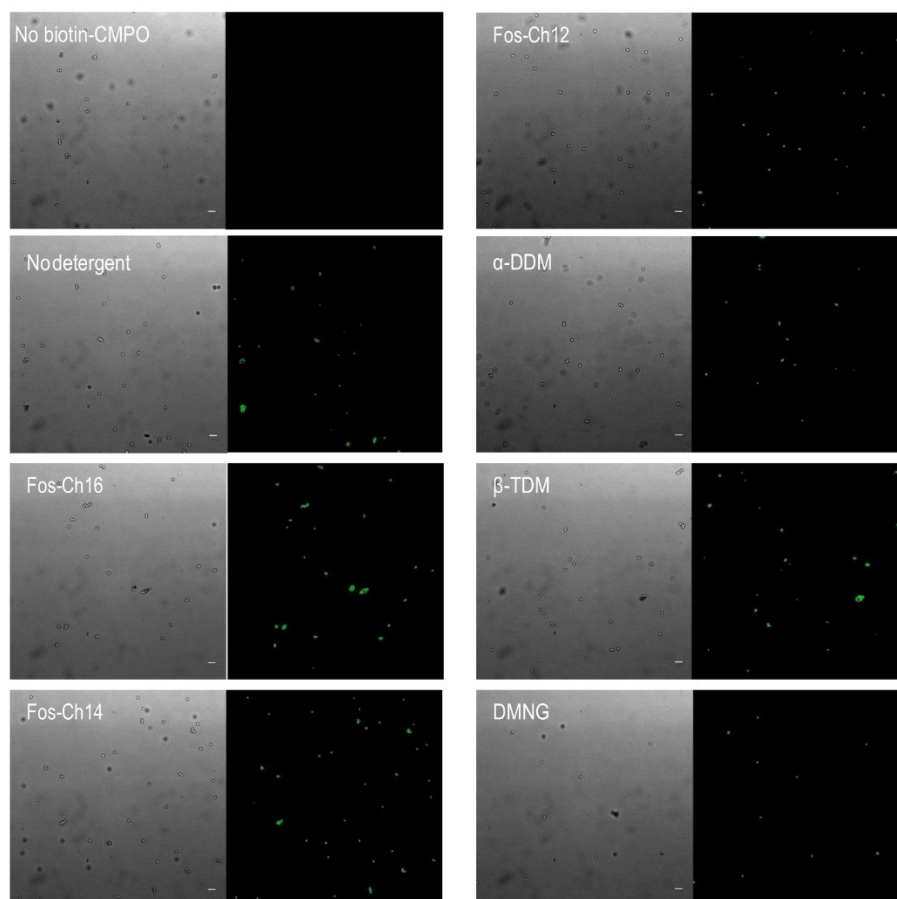
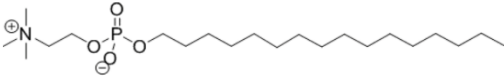
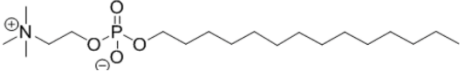
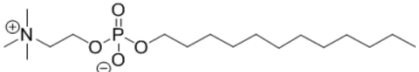
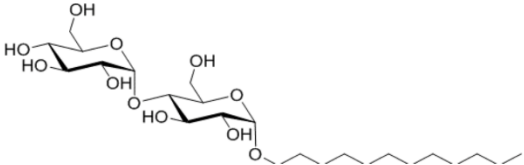
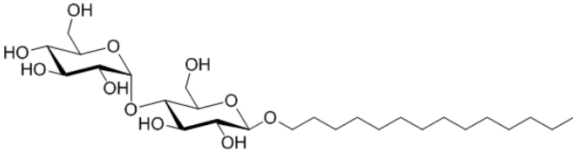
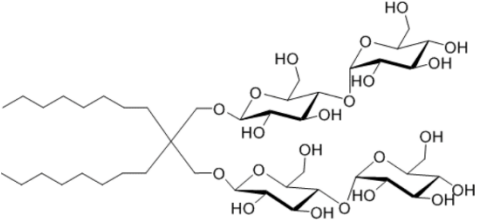


Figure S2. Fluorescence microscopy of micelle-assisted CuANCR with surfactants. Alkyne-tagged beads were labelled with 50 μM biotin-CMPO in presence of 1.5 mM of indicated surfactant (at or above CMC for all detergents) from Table S3. Labelling was carried out for 30 minutes at 37°C after which beads were washed and incubated with 5 $\mu\text{g/mL}$ FITC-streptavidin for another 30 minutes at room temperature in the dark. Images were acquired as indicated in the labelling protocol using a 60x objective with oil dip lens and background fluorescence (sample without biotin-CMPO) was subtracted using software ImageJ. Scale bar indicates 5 μm .

Table S3. Lipids used in screening of Kinugasa reaction on alkyne-tagged beads.

Structures are shown as well as approximate critical micellar concentrations (CMC) in water for each lipid.

Name ^b	Structure	CMC in water ^a
FCH16		~ 0.013 mM
FCH14		~ 0.12 mM
FCH12		~ 1.5 mM
α -DDM		~ 0.152 mM
β -TDM		~ 0.01 mM
DMNG		~ 0.36 mM

^aValues obtained from www.anatrace.com. ^bFCH; Fos-choline, α -DDM; n-Dodecyl- α -D-Maltopyranoside, β -TDM; N-Tetradecyl- β -D-Maltopyranoside, DMNG; Decyl Maltose Neopentyl Glycol.

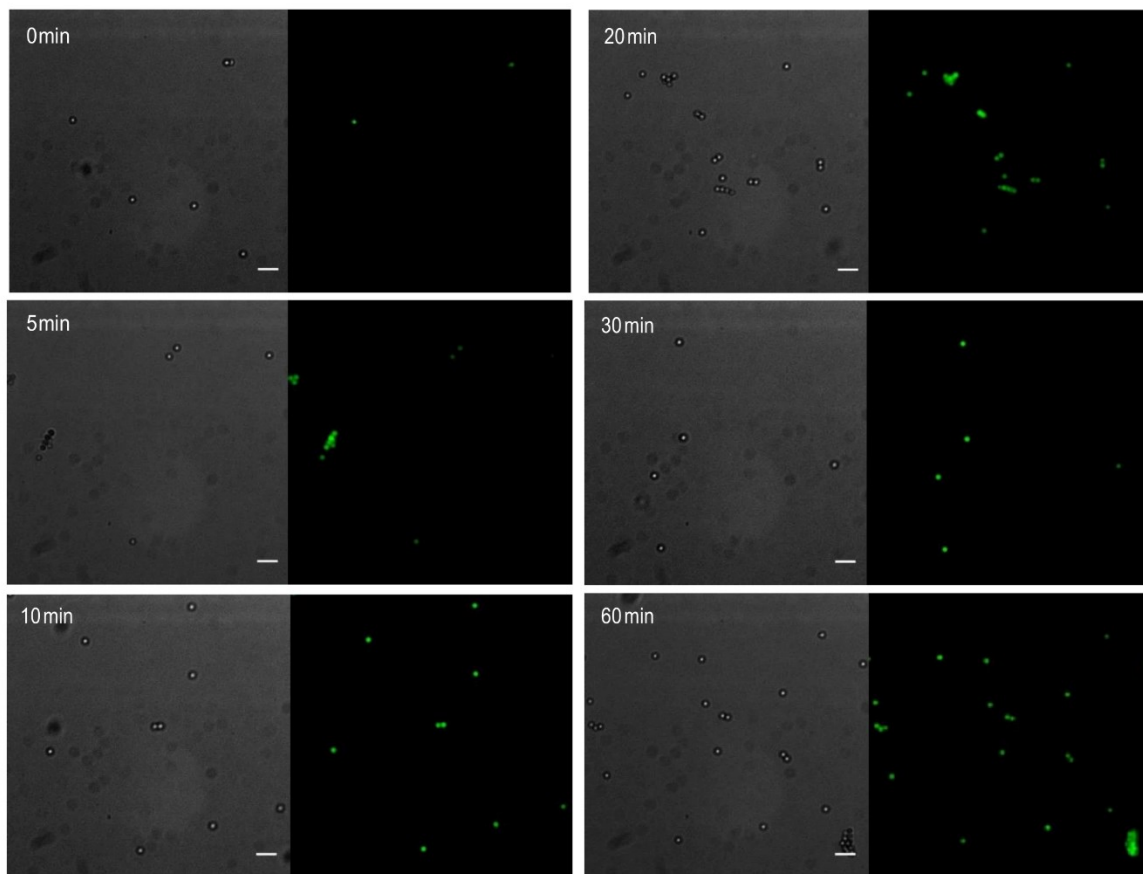


Figure S3. Time course of micelle-assisted CuANCR reaction in presence of β -TDM surfactant. Alkyne-tagged beads were labelled with 50 μ M biotin-CMPO in presence of 150 μ M β -TDM, 100 μ M CuSO_4 , 2 mM sodium ascorbate and 200 μ M L-proline for 0-60 min, as indicated, after which beads were washed with PBS and were then incubated with 5 μ g/mL FITC-streptavidin in the dark. Beads were again washed with 3x PBS and imaged using Nikon Ni-U ratiometric microscope equipped with a 60x objective lens and a 2x relay lens. Average background fluorescence obtained in absence of biotin-CMPO was subtracted from all images. Scale bar indicates 5 μ m.

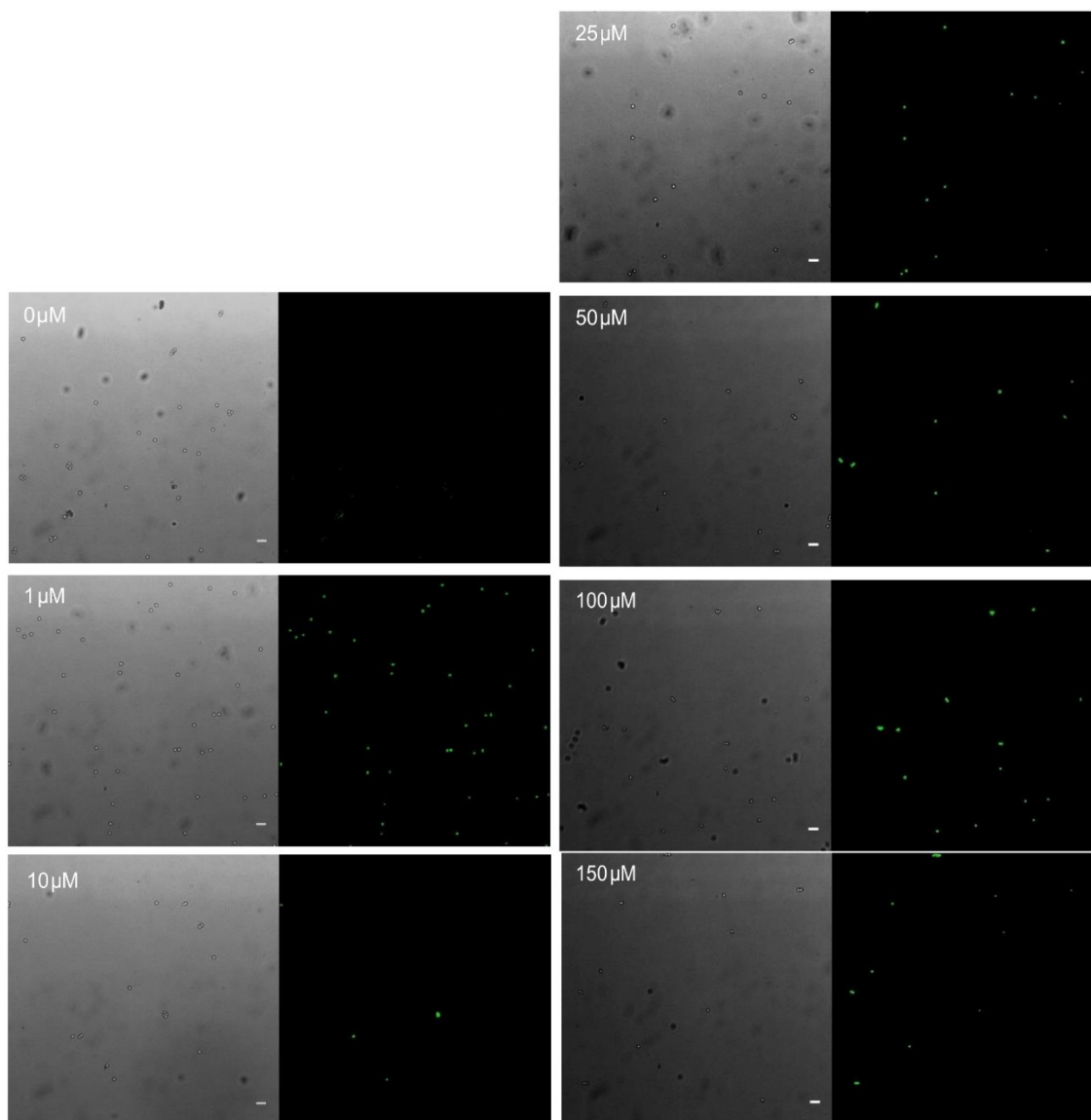


Figure S4. Micelle-dependence of aqueous CuANCR labelling reaction. Labelling of alkyne beads with biotin-CMPO was performed as indicated in the experimental section, in a range of concentrations of β -TDM, both below and above the CMC of β -TDM of 10 μ M (See Table S3). Fluorescence images were acquired using the Nikon ratio-metric microscope Ni-U equipped with an oil-dip 60x objective lens. Background fluorescence was determined as average fluorescence of beads in absence of biotin-CMPO, and was subtracted from all images. Scale bar indicates 5 μ m.

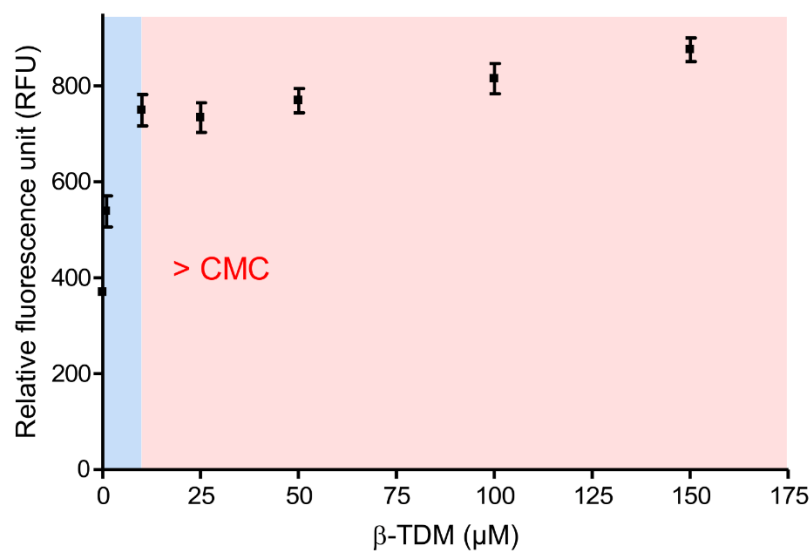


Figure S5. Micelle-dependence of aqueous CuANCR labelling reaction. Labelling of alkyne-tagged beads with 50 μM biotin-CMPO in 100 μM CuSO_4 , 2 mM sodium ascorbate and 200 μM L-proline was performed in a range of concentrations of β -TDM, both below (blue area) and above (light red area) the CMC of β -TDM of 10 μM (See Table S3). Each data point is an average above background fluorescence determined from at least five different beads.

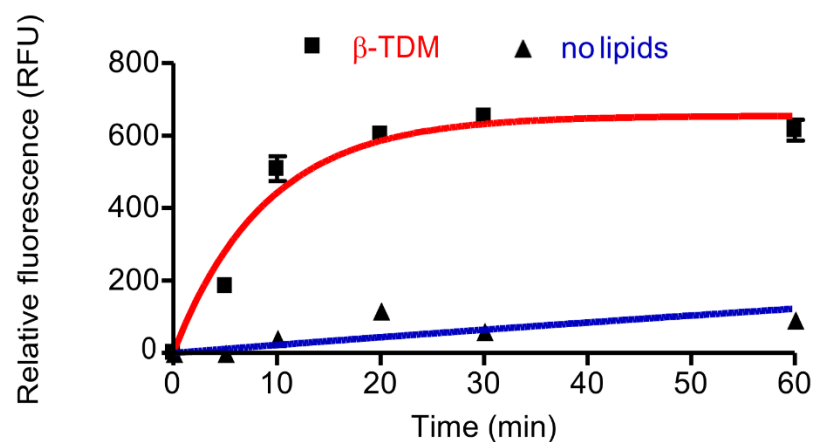


Figure S6. Kinetics of aqueous Kinugasa on alkyne-tagged beads. Alkyne-tagged beads with β -TDM (150 μ M, red curve) and without lipid (0 μ M, blue curve). Alkyne beads (3-5 μ M alkyne groups) were incubated with 50 μ M biotin- CMPO, 100 μ M CuSO_4 , 2 mM sodium ascorbate and 200 μ M L-proline in PBS for the indicated amount of time at 37°C, and then stained with 5 μ g/mL FITC-streptavidin. Beads were washed in PBS before fluorescence imaging. Above background fluorescence of five beads from two independent view fields (10 beads total) was determined using ImageJ software, normalized and plotted against time of labelling (minutes). Data points were fitted to first order kinetics equation in Prism 4. See also Figure S3 for fluorescence imaging data.

E5-TAT peptide modification procedure

Two-Step Biotin Labeling of E5-TAT peptide

E5-TAT peptide (GLFEAIAEFIENGWEGLIEGWYGGRRKRRRR) (GenScript) samples were diluted (20 μ M) in Phosphate-Buffered Saline (PBS) containing varying concentrations of Fos-Choline 12 (FC12) (0.01% to 0.2%, CMC=0.047%). The peptide samples were then treated with 1-[(1-oxo-2-propynyl)oxy]-2,5-pyrrolidinedione (N-hydroxysuccinimide ester alkyne) (300 μ M, DMSO stock) and allowed to stand for 1 hour at room temperature. Sodium ascorbate (300 μ M), L-proline (40 μ M), CuSO₄·5H₂O (20 μ M) and Biotin-CMPO (300 μ M, DMSO stock) were then sequentially added to the samples, which were allowed to sit for an additional hour at room temperature. The samples were then prepared for SDS-PAGE and Western blotting analysis.

Immunoblotting

Labeled E5-TAT peptide (GenScript) samples were loaded and analyzed using SDS-PAGE and western blotting. Samples were run using 12% stain-free polyacrylamide gel electrophoresis (TGX Stain-Free Fastcast Acrylamide kit, Bio-Rad). The proteins were then transferred to a PVDF membrane using the Trans-blot Turbo RTA Transfer Kit (Bio-Rad). Membrane was blocked using Tris-buffered saline with 0.05% Tween-20 (TBS-T) containing 3% W/V Bovine serum albumin (Sigma-Aldrich). Peptides were probed using anti-biotin antibody (1:1000, Invitrogen, MA5-11251) overnight at 4 °C. Blot was washed in TBS-T and probed for one hour at room temperature with HRP-conjugated goat anti-mouse secondary antibody (1:20000, Jackson ImmunoResearch Laboratories, Westgrove, PA). Bands were visualized using Clarity ECL western blotting substrate (Bio-Rad) according to the manufacturer's protocols. Integrated signal was calculated relative to negative control (no Biotin-CMPO) taking into account peptide loading using Image Lab software (Bio-Rad). Figure S7 shows a repeat experiment with the unmodified E5-TAT negative control.

Peptide Modification Analysis

2 mg of E5-TAT peptide dissolved in 1x phosphate-buffered saline (pH 7.5) was treated with 15 equivalents of N-hydroxysuccinimide ester alkyne and left for one hour at room temperature. The peptide sample was then subjected to FPLC purification. The size exclusion chromatography profile was obtained for the alkyne modified E5-TAT using a Superdex 75 size exclusion column (FPLC ÄKTA pure, GE) at a concentration of 1 mg/ml in 1x phosphate-buffered saline (pH 7.5). Absorbance was recorded at 280 nm, with flow rate maintained at 0.8 mL/min (See Figure S8). 10 μ g of purified modified and unmodified peptide were then subjected to desalting using C18 spin columns (ThermoFisher Scientific) according to manufacturer's protocol. Samples were then subjected to mass spectrometry analysis. Proteome Discoverer 2.1 (ThermoFisher Scientific) was used to evaluate the modification of peptide with the N-hydroxysuccinimide ester alkyne. Search engine: SEQUEST-HT implemented in Proteome Discovery was applied for all MS raw files. Search parameters were set to allow for dynamic modification of the N-hydroxysuccinimide ester alkyne (51.995 Da). The peptide-spectrum matches (PSMs) was used to evaluate the alkyne modification of peptides and only peptides for Sequest results of XCorr $\geq$ 2.5 were retained. The obtained PSMs showed modification of lysine residues K26 and K27 (both mono and di-substitution), modifications not found for the unmodified peptide sample.

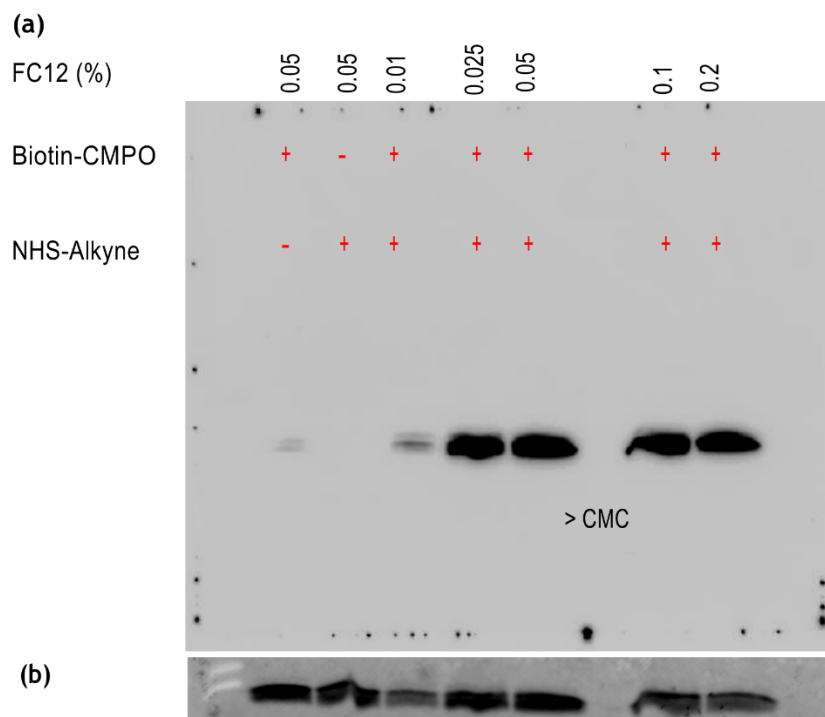


Figure S7. Biotin conjugation of E5-TAT membrane peptide using CuANCR. Alkyne functionalization of E5-TAT (20 μ M) was carried out using N-hydroxysuccinimide ester alkyne (300 μ M). Biotin labelling was achieved by treating modified peptide with $\text{CuSO}_4 \cdot \text{H}_2\text{O}$ (20 μ M), sodium ascorbate (300 μ M), L-proline (40 μ M) and biotin-CMPO (300 μ M). (a) Western blot analysis of biotin labelled E5-TAT samples shown under varying percentages (%W/V) of detergent (FC12) ranging from (0.01-0.2%, CMC=0.05%). (b) TGX Stain-free protein loading control.

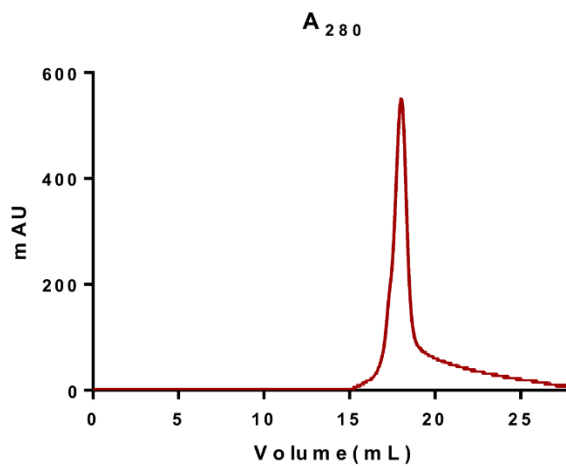


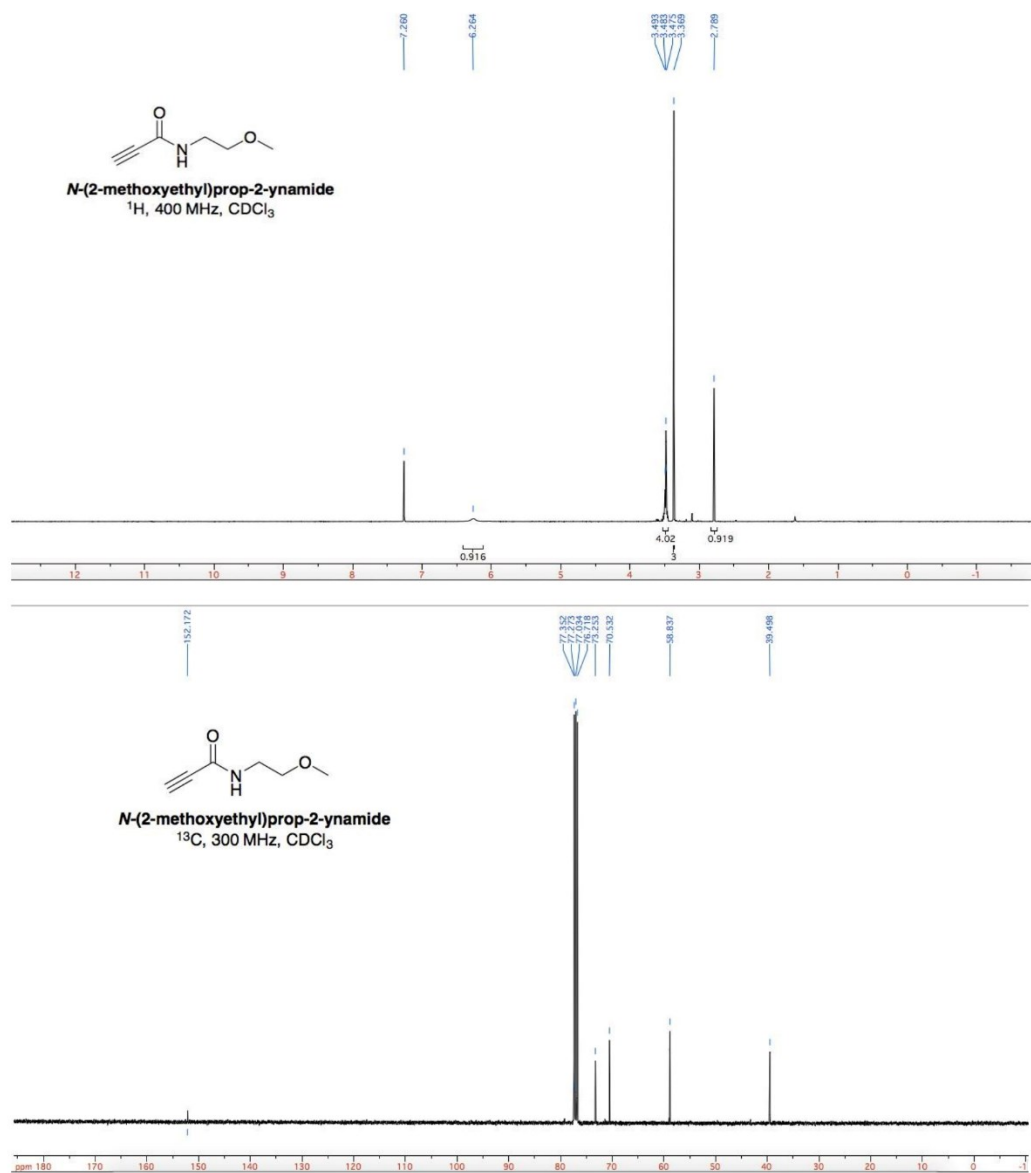
Figure S8. Size exclusion chromatography profile for modified E5-TAT run in 1xPBS, pH7.5 on a Super-dex 75 column(GE).

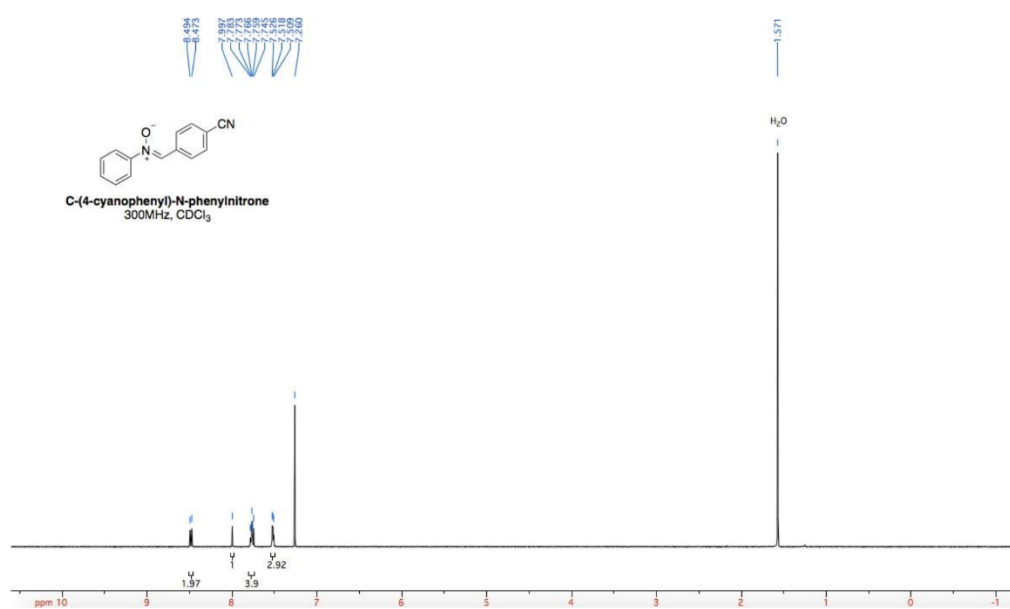
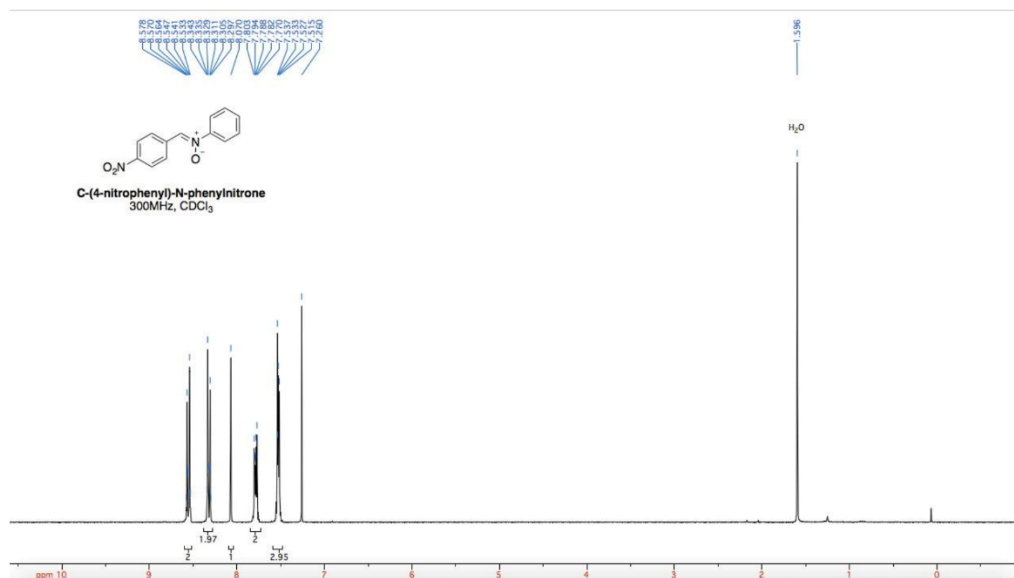
Table S4. Results from SEQUEST-HT implementation in Proteome Discovery for unmodified E5-TAT samples. XCorr values ≥ 2.5 are shown.

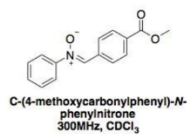
ConfId	SeqIdent	PSM Annot	Modifications	# Protein	# Protein	Master P	Protein A	# Missed	Charge	DataSrc	DataCr	Rank	Search En	ms [Da]	Min [Da]	Delta [Da]	Delta [Da]	Adaptive MS Order	Isolation	Ion Inject	RT [min]	First Scan	Spectrum	Ion Max	XCorr	Area	Area [RT]
High	SeqIdent	Unmodified	(G)GAAAPFENNQ	1	1	peptide	peptide	1	1	1	1	1	96.291	308.10	-1.1164	-0.018	CO/Cella MS2	1	16.464	37.823	2391	1	unmodified	0	1.4360	43.6175	
High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	96.291	308.14	0.0152	0.0047	CO/Cella MS2	1	68.7625	36.715	1482	1	unmodified	0	4.1104		
High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	96.278	308.30	-0.444	-0.004	CO/Cella MS2	1	117.426	57.237	2512	1	unmodified	0	4.0728	100481	
High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	48.141	308.10	-2.2021	-0.014	CO/Cella MS2	1	0.1256	14.234	37.837	1411	1	unmodified	0	1.5617	
High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	96.294	308.12	1.1791	0.0075	CO/Cella MS2	1	94.944	24.585	36189	1448	1	unmodified	0	18.9161	
High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	96.294	308.30	-1.645	-0.004	CO/Cella MS2	1	6.6111	11.7251	37.190	1365	1	unmodified	0	1.9854	
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High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	48.143	308.13	0.3392	0.0034	CO/Cella MS2	1	10.2515	11.3231	35.715	1470	1	unmodified	0	1.8101	
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High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	76.437	308.16	-1.2647	-0.002	CO/Cella MS2	1	6.2652	1.8675	37.041	1436	1	unmodified	0	1.7771	
High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	96.279	308.36	-4.4212	-0.002	CO/Cella MS2	1	4.2072	36.794	1385	1	unmodified	0	1.7464		
High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	48.147	308.10	-1.1261	-0.006	CO/Cella MS2	1	1.6479	13.613	37.857	1424	1	unmodified	0	1.8172	
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High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	58.108	308.10	-2.1408	-0.012	CO/Cella MS2	1	1.9039	17.6375	37.275	2381	1	unmodified	0	1.4691	
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High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	96.281	308.13	4.8849	0.0046	CO/Cella MS2	1	22.8742	2.4711	36.772	1446	1	unmodified	0	1.6154	
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High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	96.293	308.10	-1.1943	-0.018	CO/Cella MS2	1	4.4133	16.441	37.278	1434	1	unmodified	0	1.3741	
High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	76.428	308.11	-2.1432	-0.014	CO/Cella MS2	1	6.3756	6.8325	36.745	1371	1	unmodified	0	1.2748	
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High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	76.424	308.13	0.7465	0.0032	CO/Cella MS2	1	1.07284	15.3486	36.724	1486	1	unmodified	0	1.34275	
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High	SeqIdent	Unmodified	(G)GAAEFENNQ	1	1	peptide	peptide	1	1	1	1	1	76.423	308.11	-0.2814	-0.003	CO/Cella MS2	1	6.37598	1.48514	37.148	1391	1	unmodified	0	1.33845	
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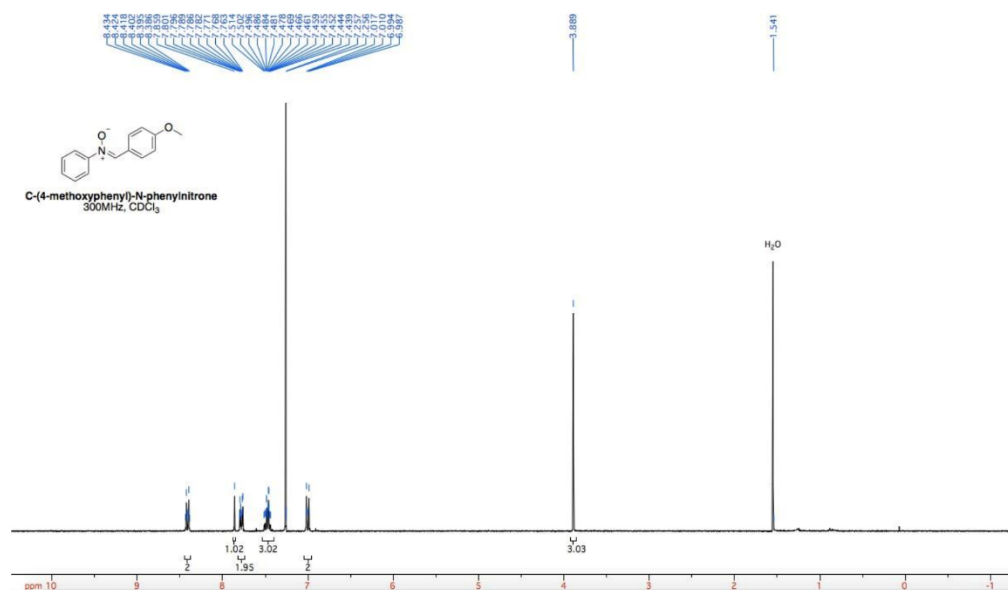
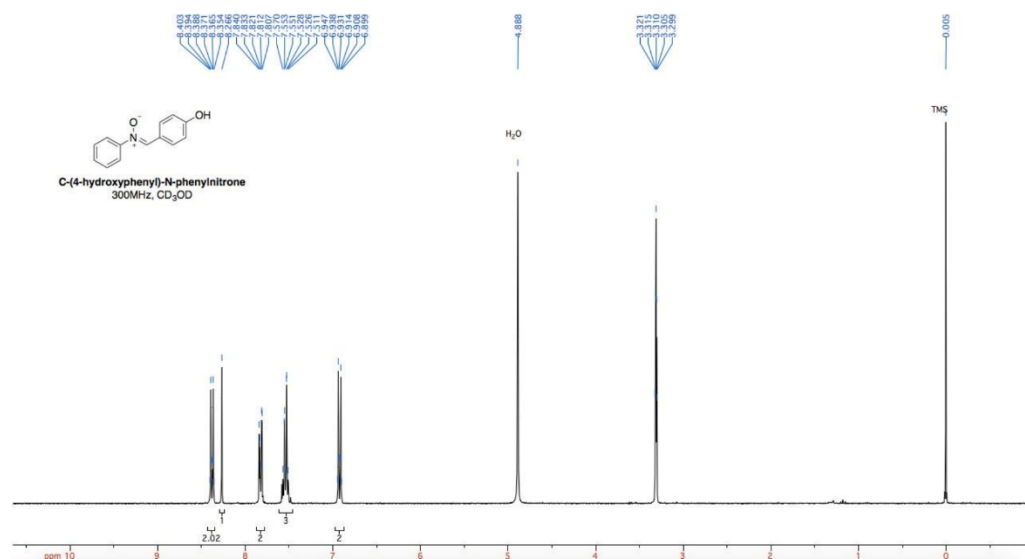
NMR Spectra

Synthesized Activated Alkynes

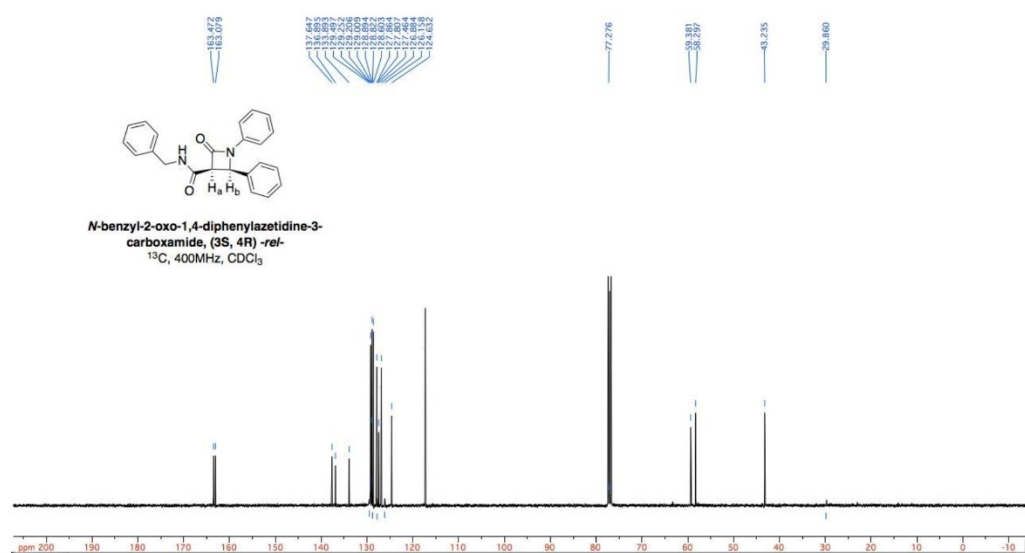


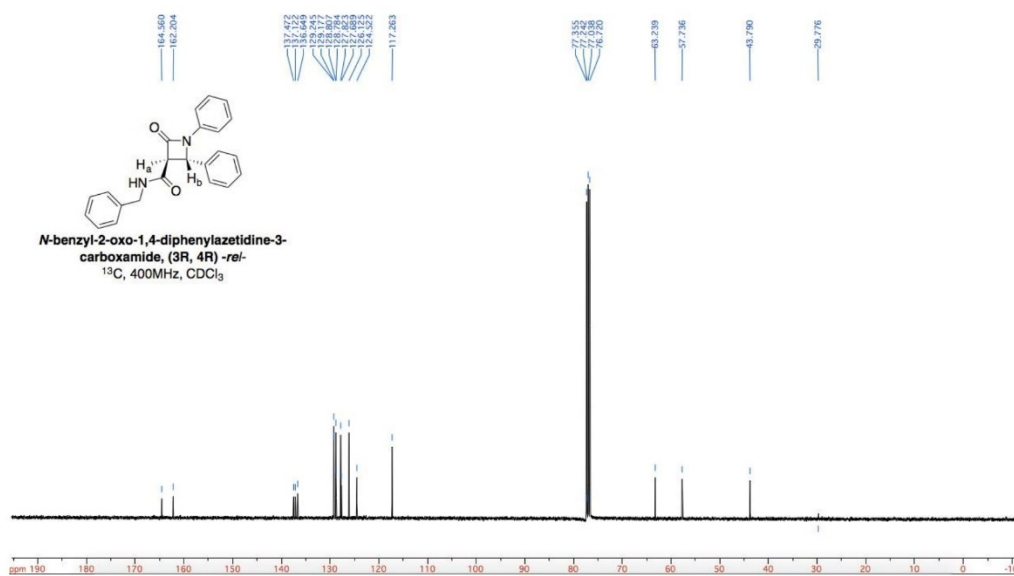
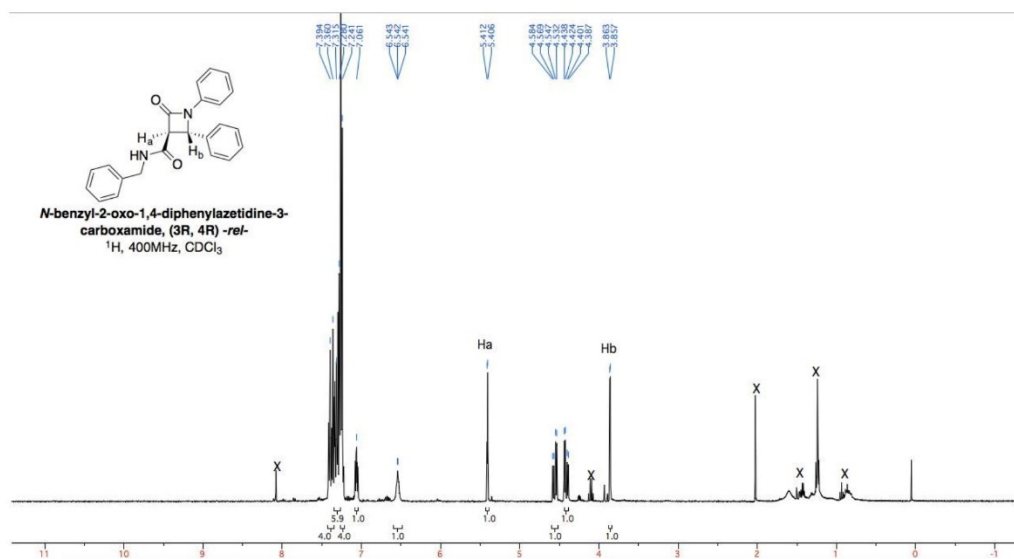




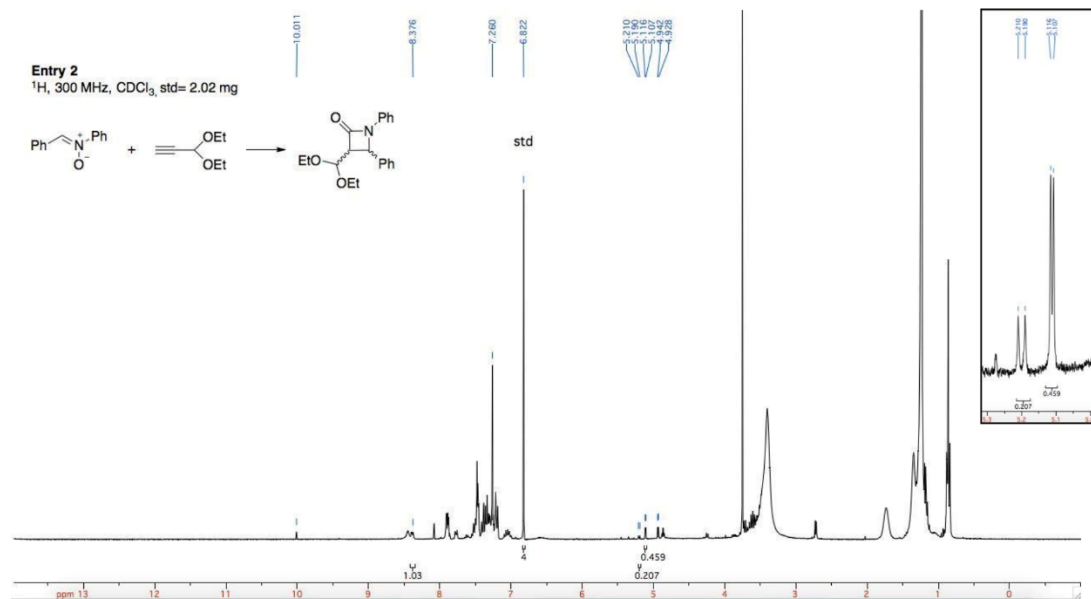
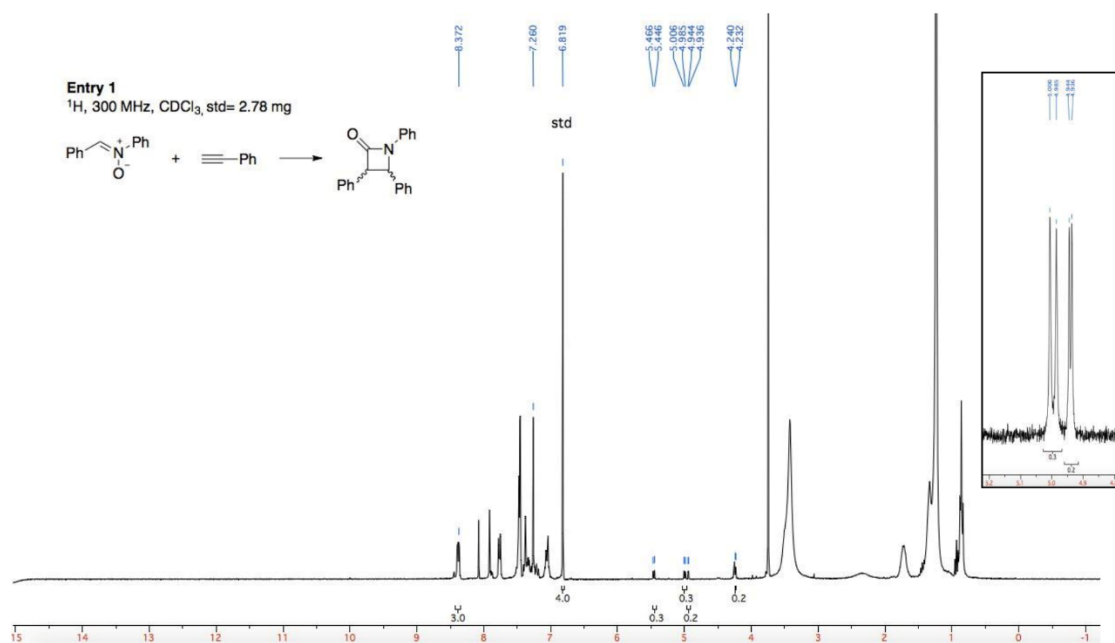


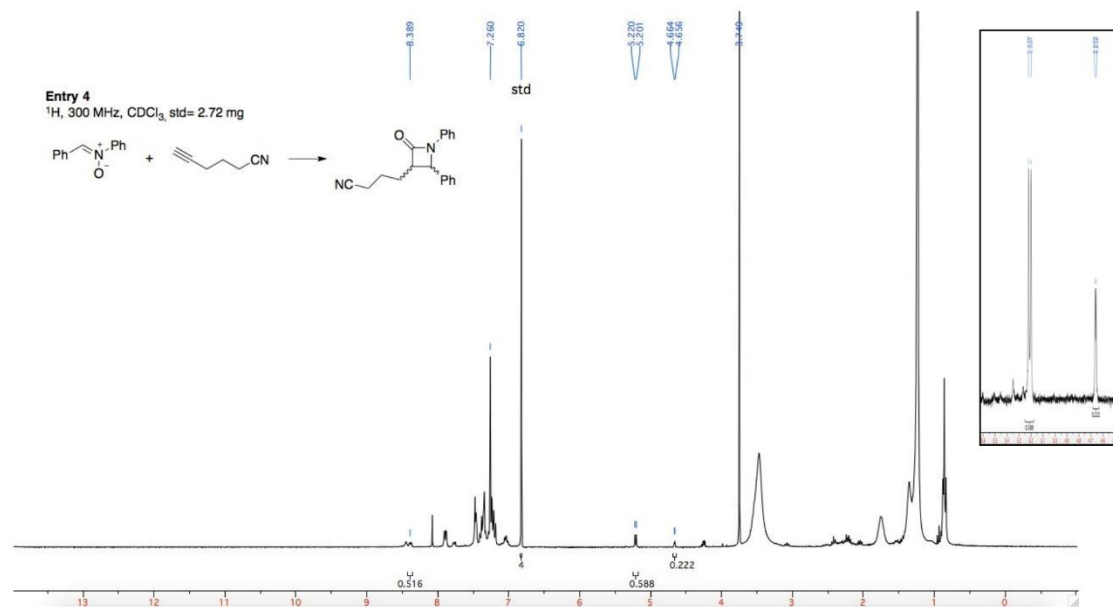
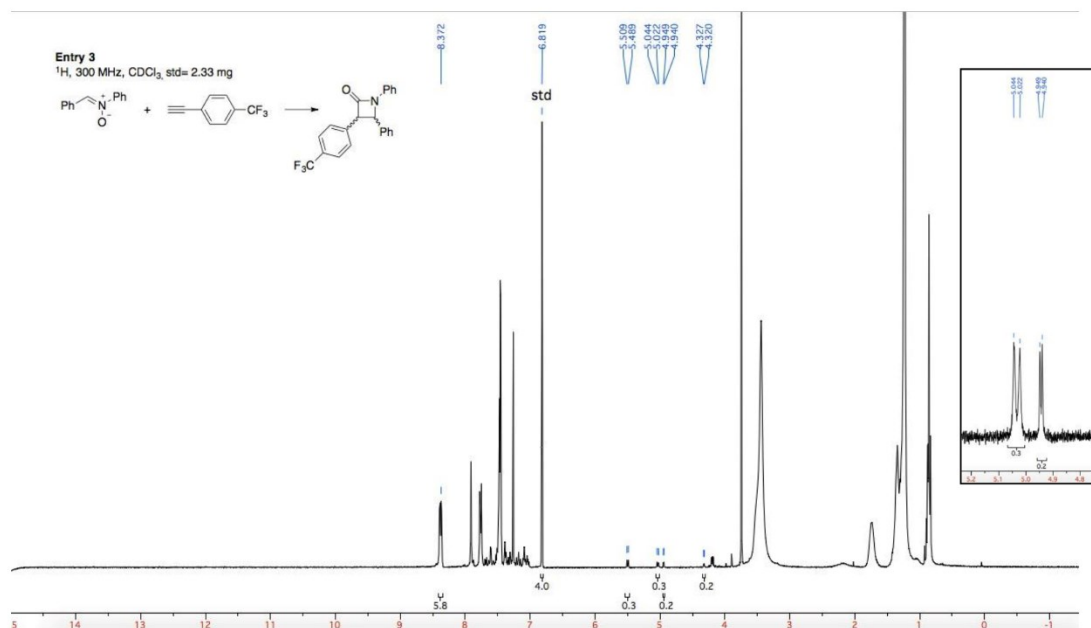


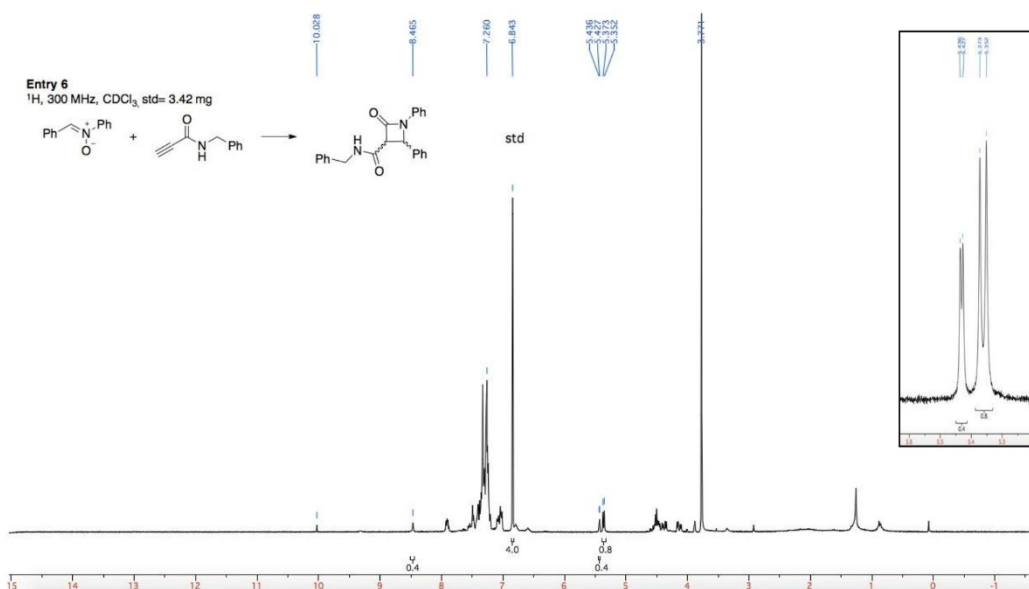
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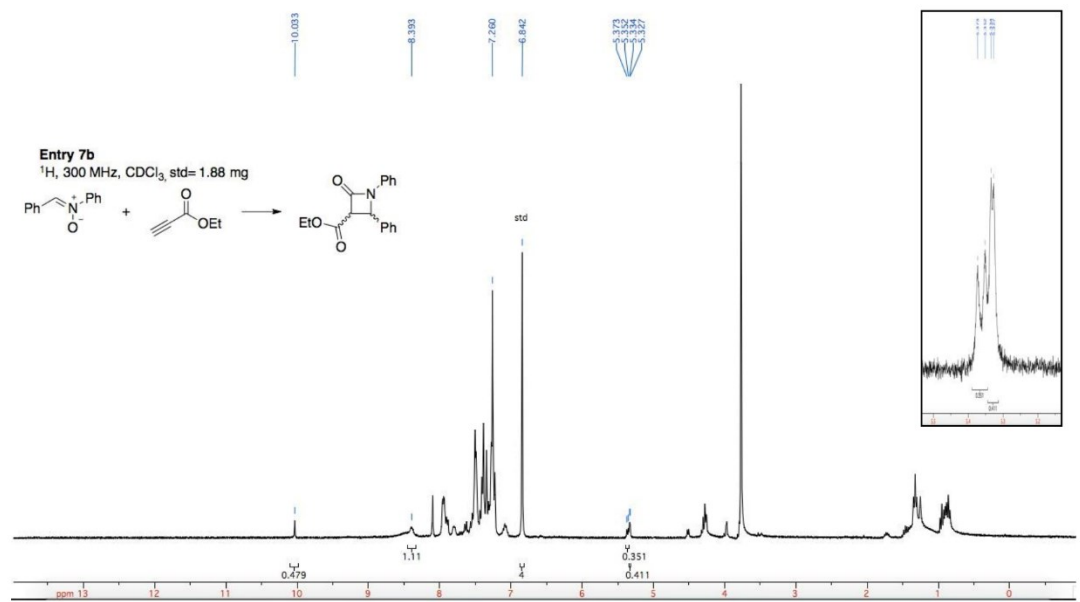
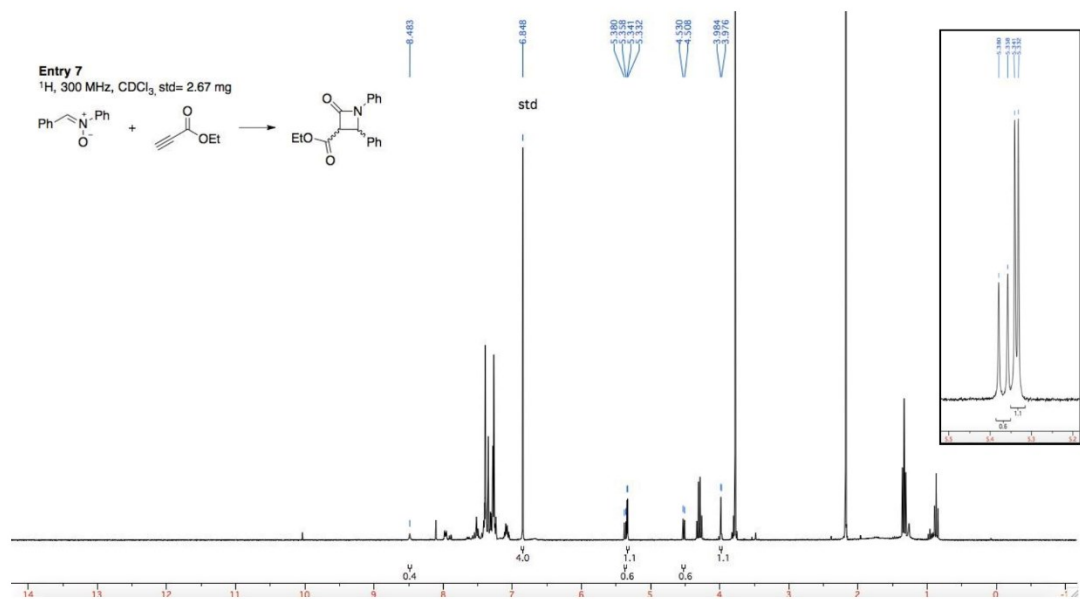


NMR Data for Table 1. Screen of Alkyne Reactivity









NMR Data for Table S2. Screen of Nitron Reactivity

Table S2 yields were calculated based on ^1H NMR Spectra recorded with a frequency of 300MHz. NMR yields are reported using 1,4-Dimethoxybenzene as internal standard.

Table S2. Reaction 1A.

Std= 2.34 mg

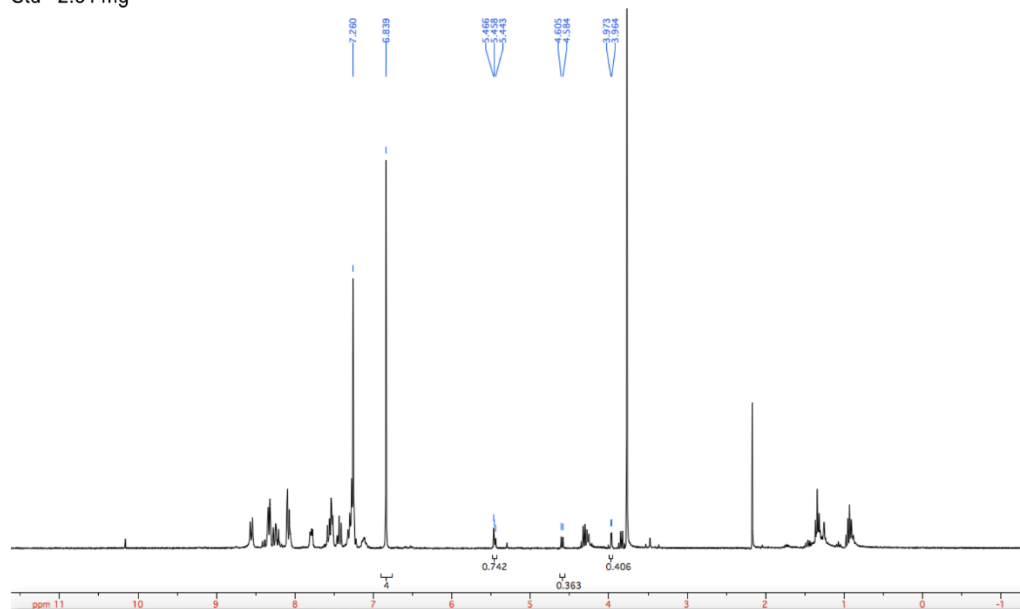


Table S2. Reaction 1B.
Std= 2.32 mg

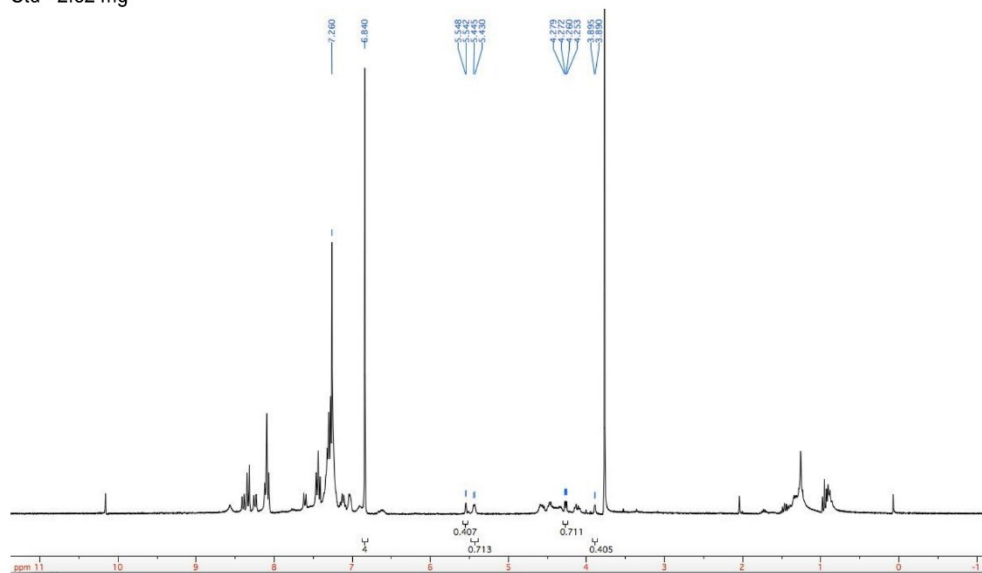


Table S2. Reaction 1C.
Std= 2.50 mg

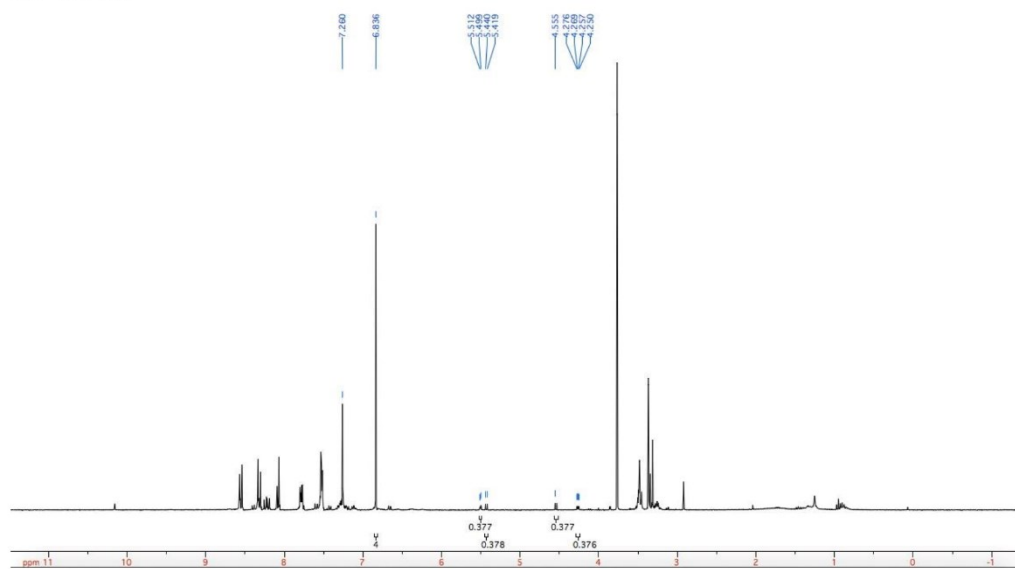


Table S2. Reaction 2A.

Std= 1.76 mg

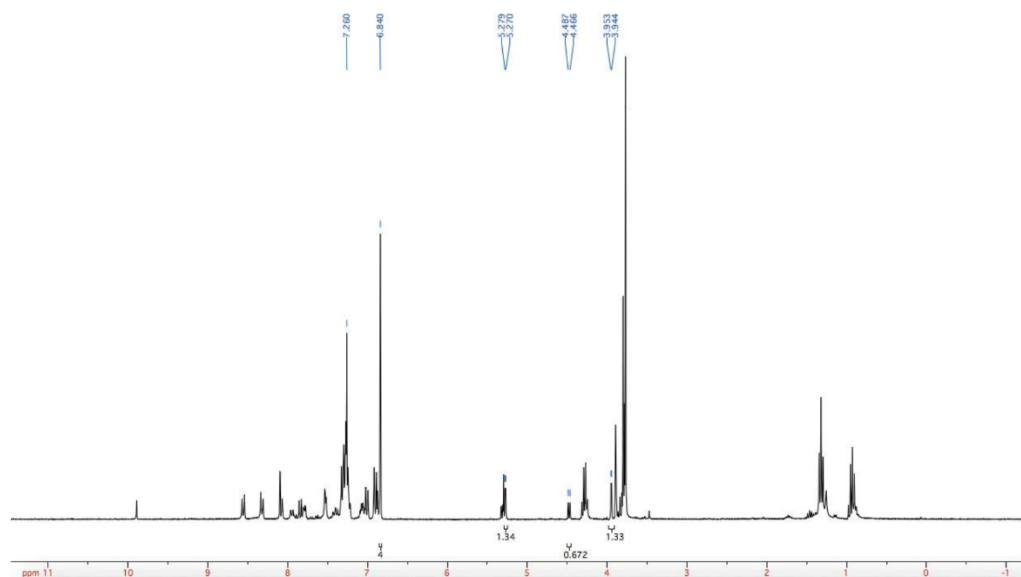


Table S2. Reaction 2B.

Std= 2.90 mg

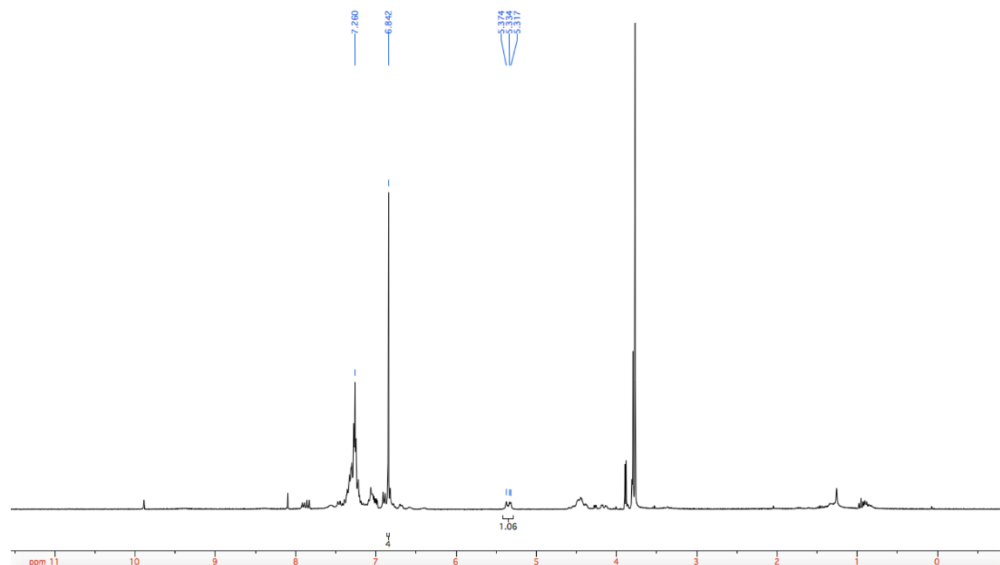


Table S2. Reaction 2C.

Std= 2.47 mg

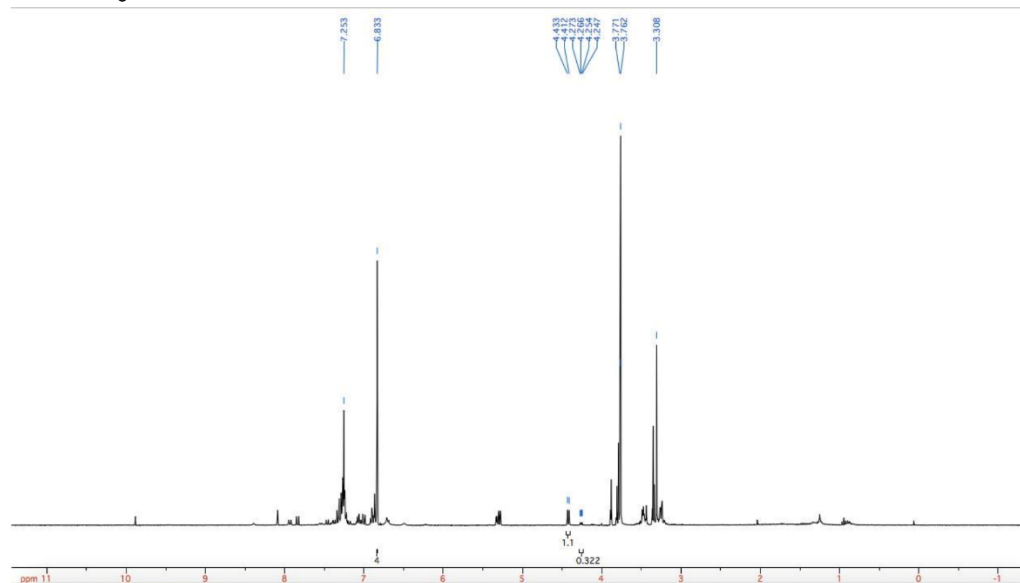


Table S2. Reaction 3B.

Std= 1.81 mg

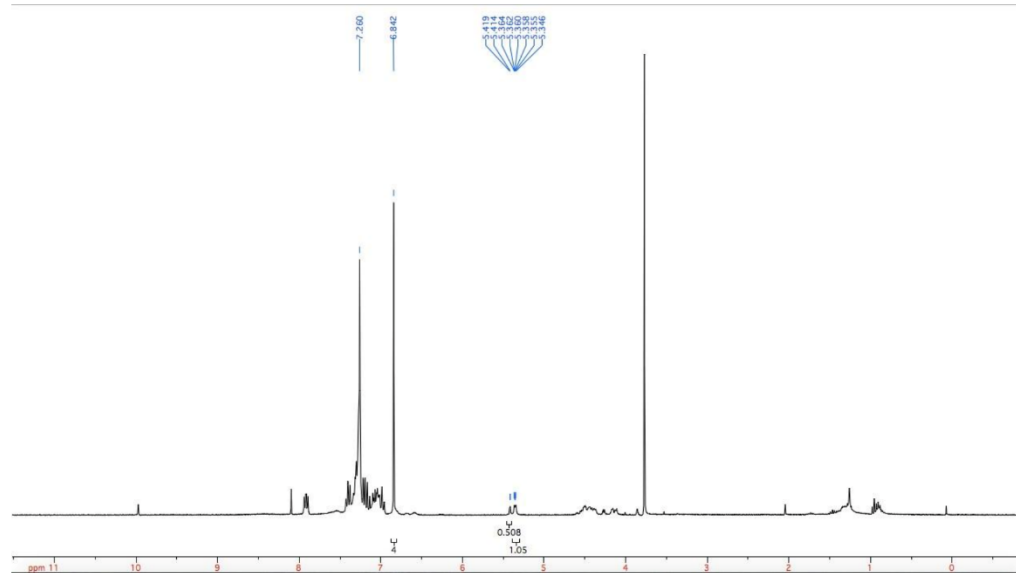


Table S2. Reaction 3C.

Std= 1.52 mg

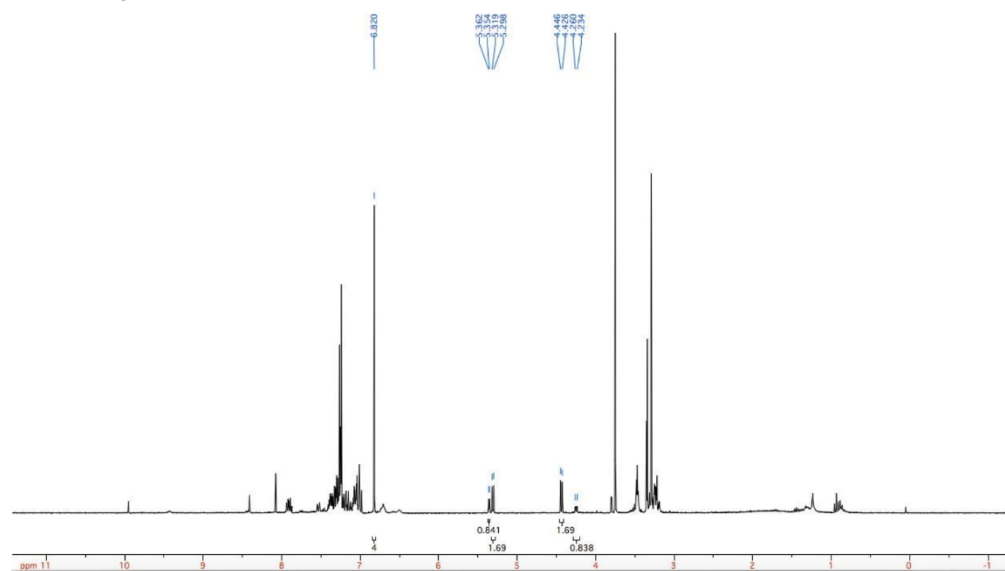


Table S2. Reaction 4A.

Std= 1.90 mg

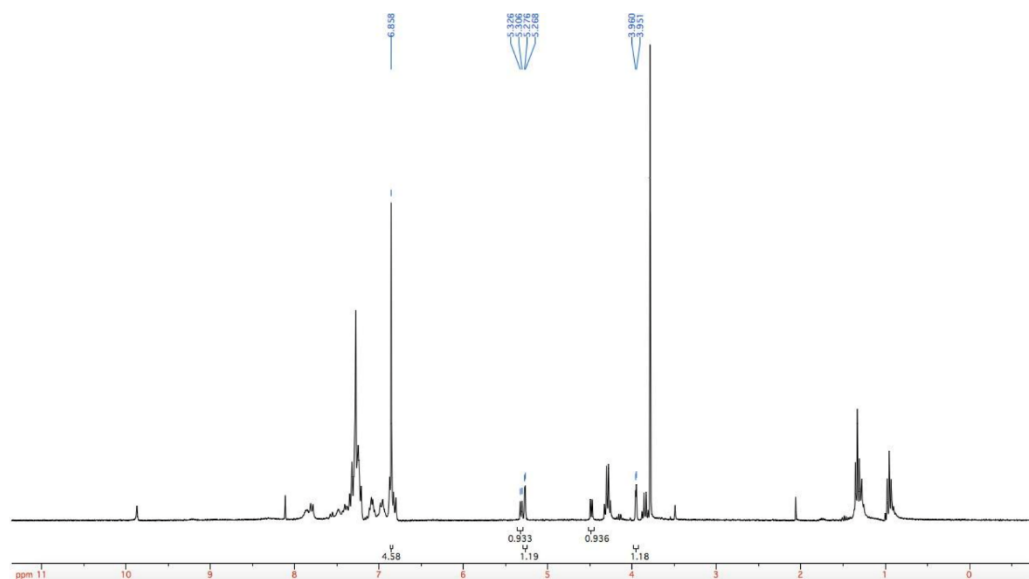


Table S2. Reaction 4B.

Std= 1.92 mg

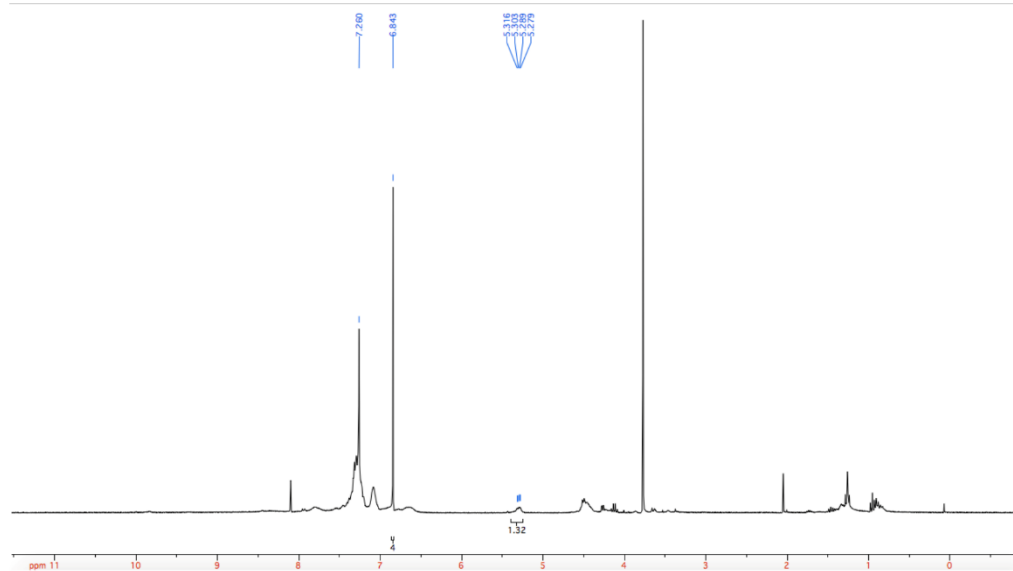


Table S2. Reaction 4C.
Std= 2.78 mg

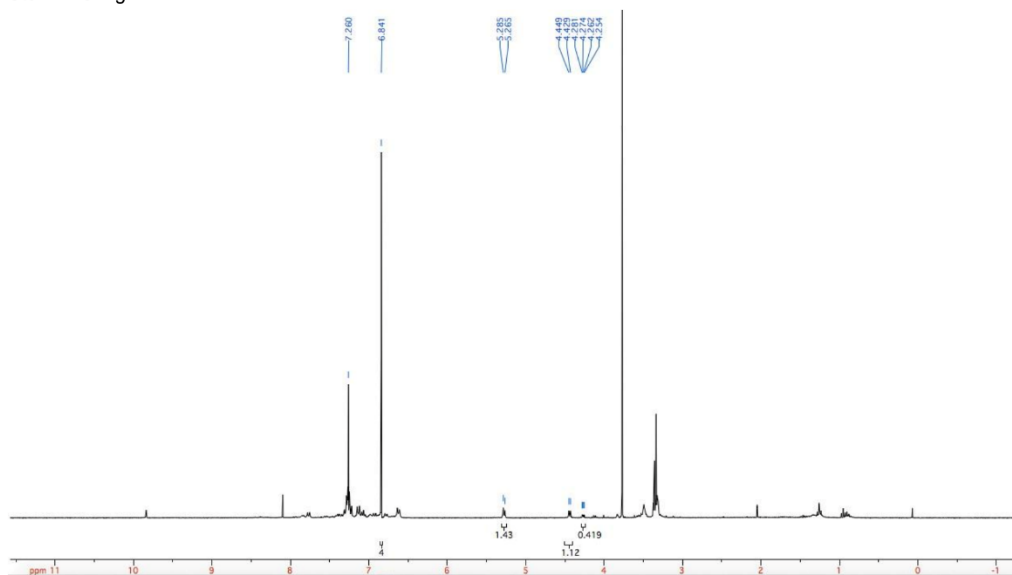


Table S2. Reaction 5A.
Std= 2.14 mg

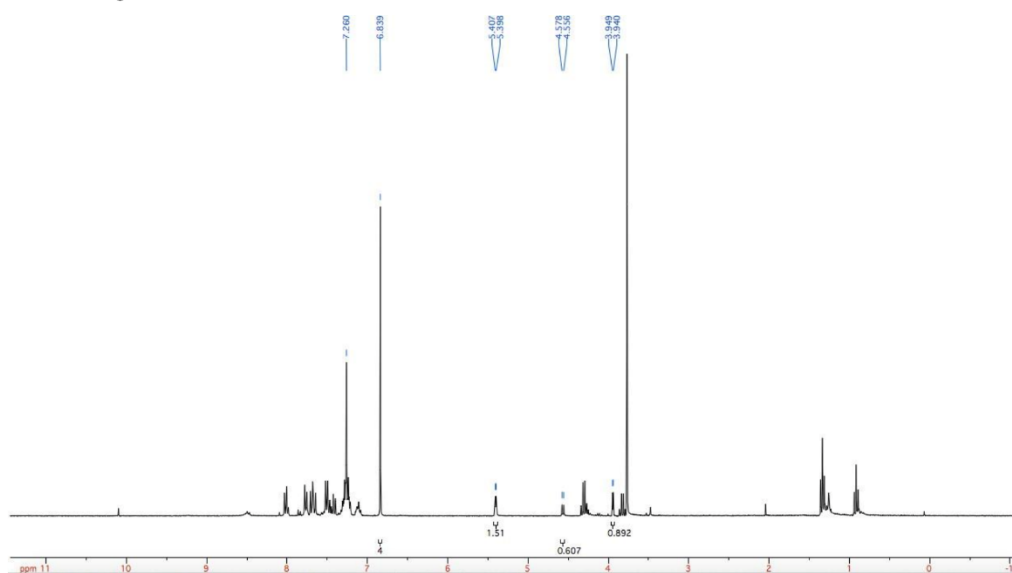


Table S2. Reaction 5B.

Std= 2.23 mg

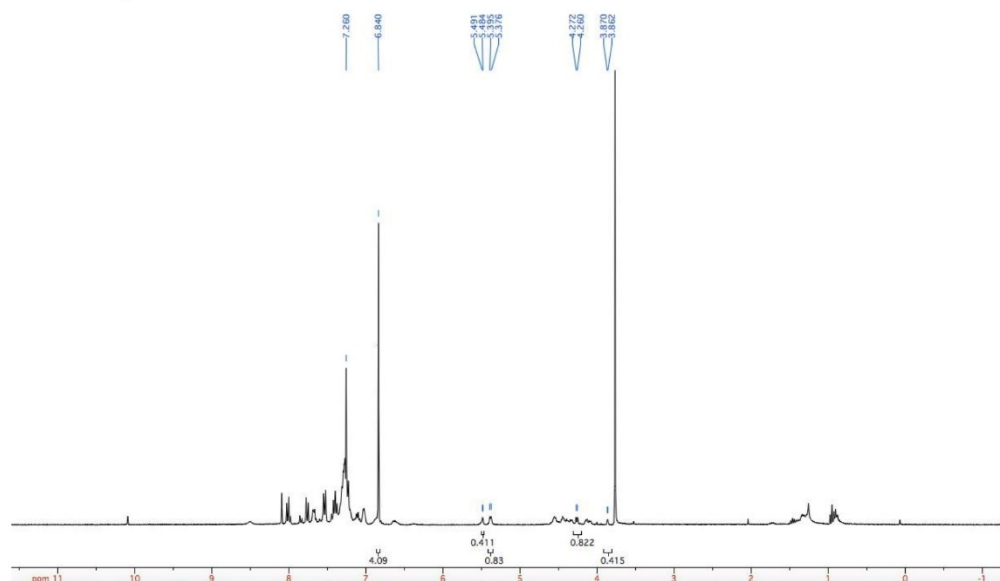


Table S2. Reaction 5C.

Std= 1.84 mg

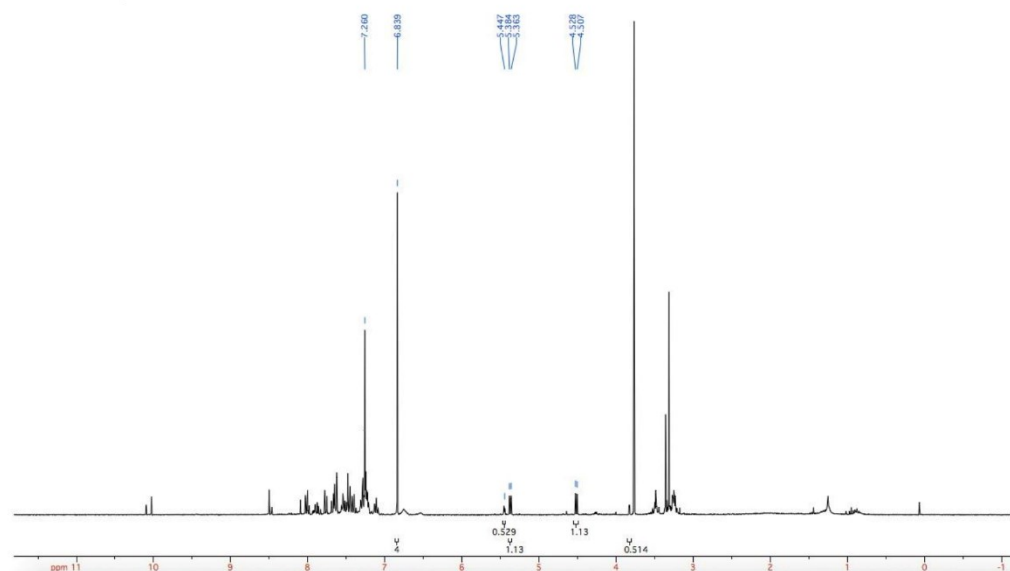


Table S2. Reaction 6A.

Std= 2.61 mg

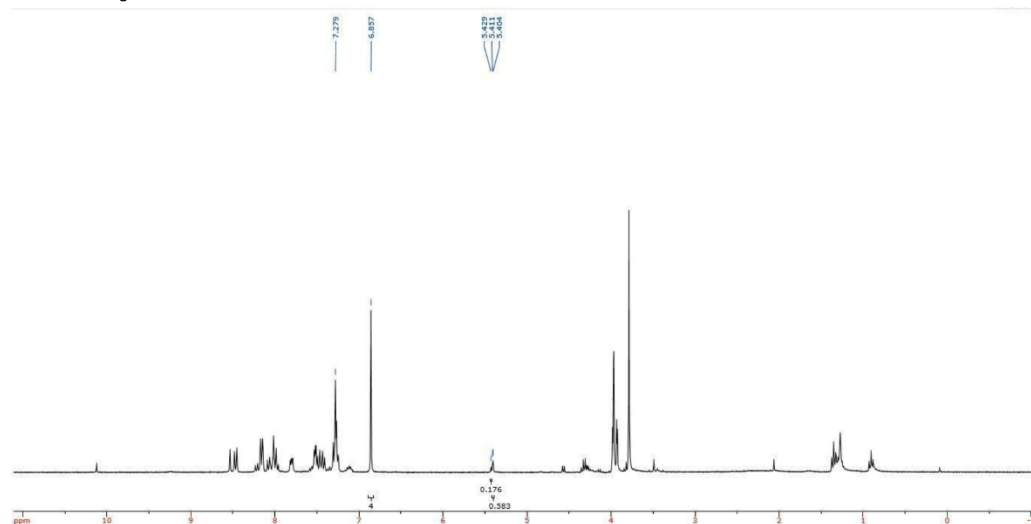


Table S2. Reaction 6B.

Std= 1.41 mg

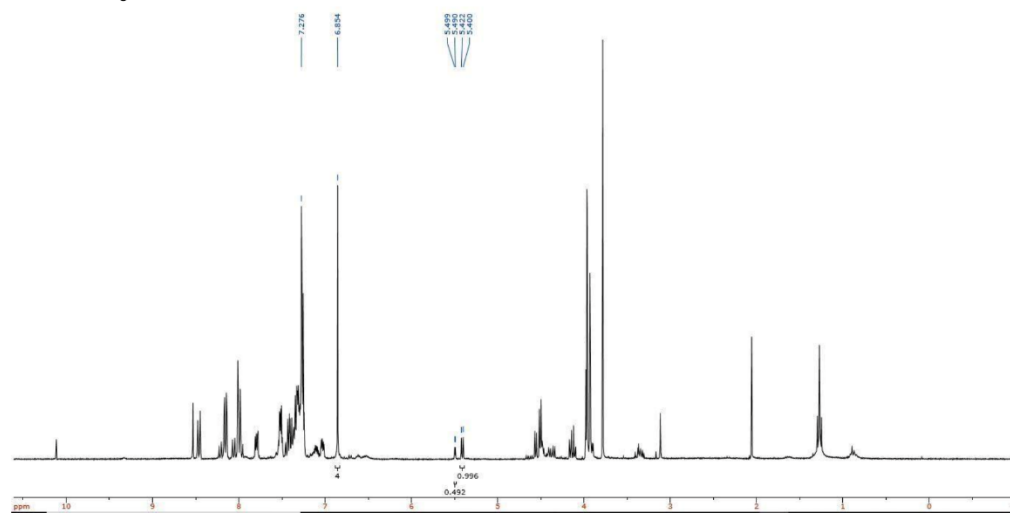


Table S2. Reaction 6C.

Std= 2.96 mg

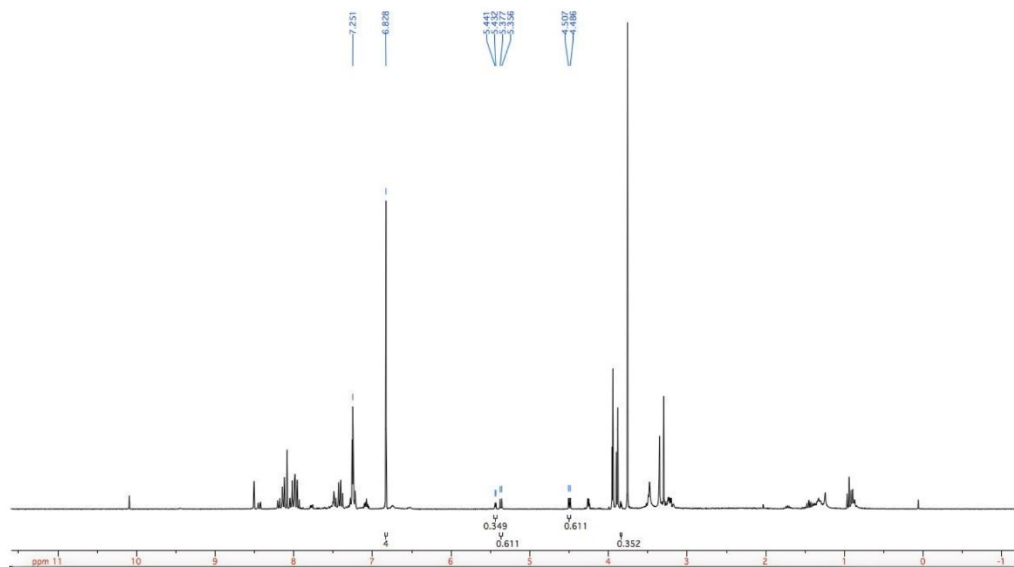


Table S2. Reaction 7A.

Std= 1.47 mg

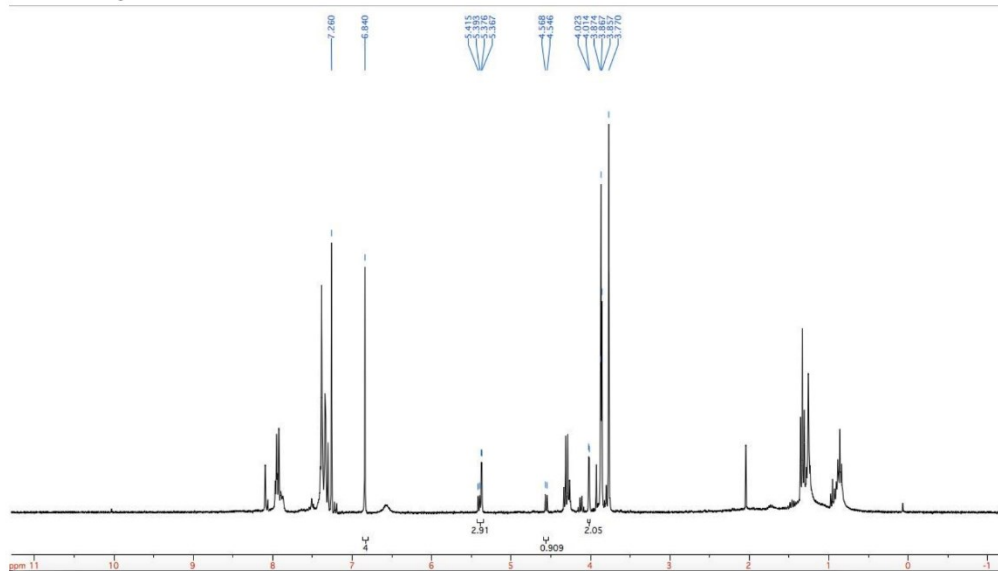


Table S2. Reaction 7B.
Std= 3.34 mg

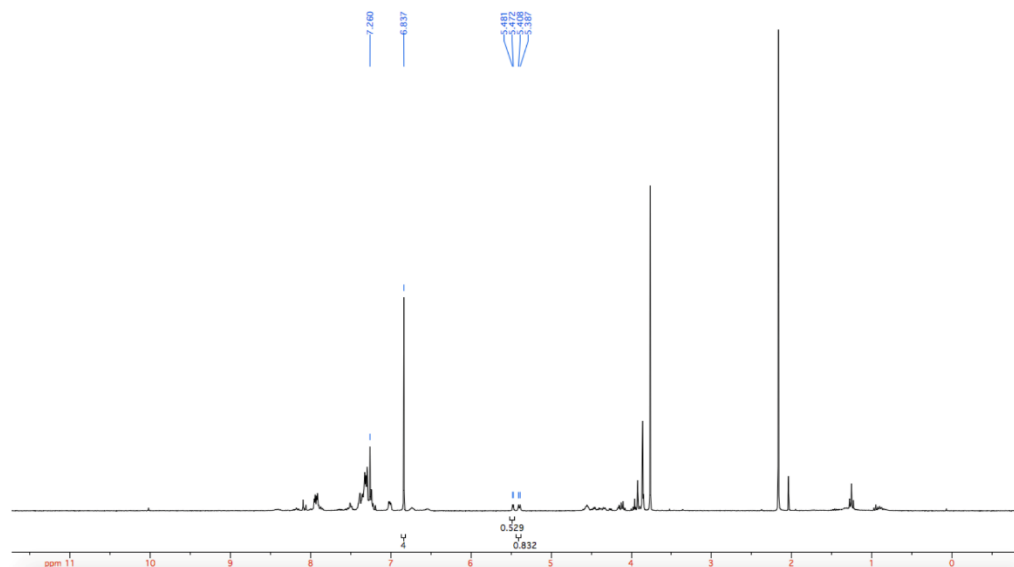


Table S2. Reaction 7C.
Std= 2.42 mg

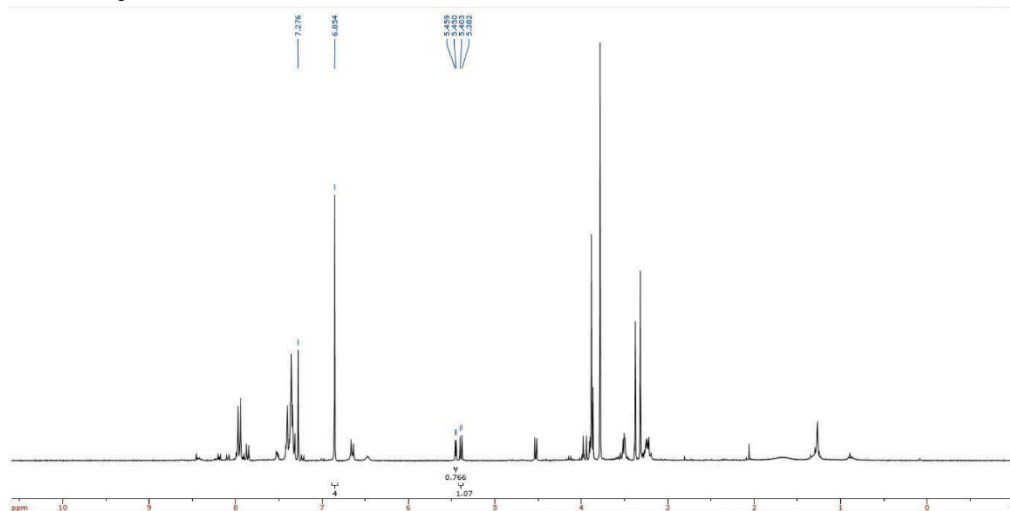


Table S2. Reaction 8A.

Std= 3.02 mg

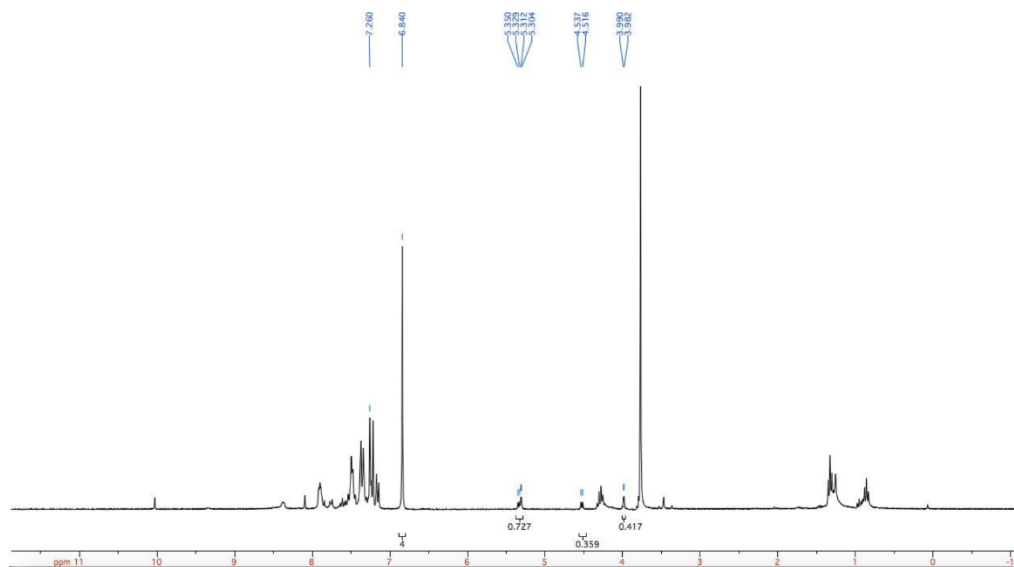
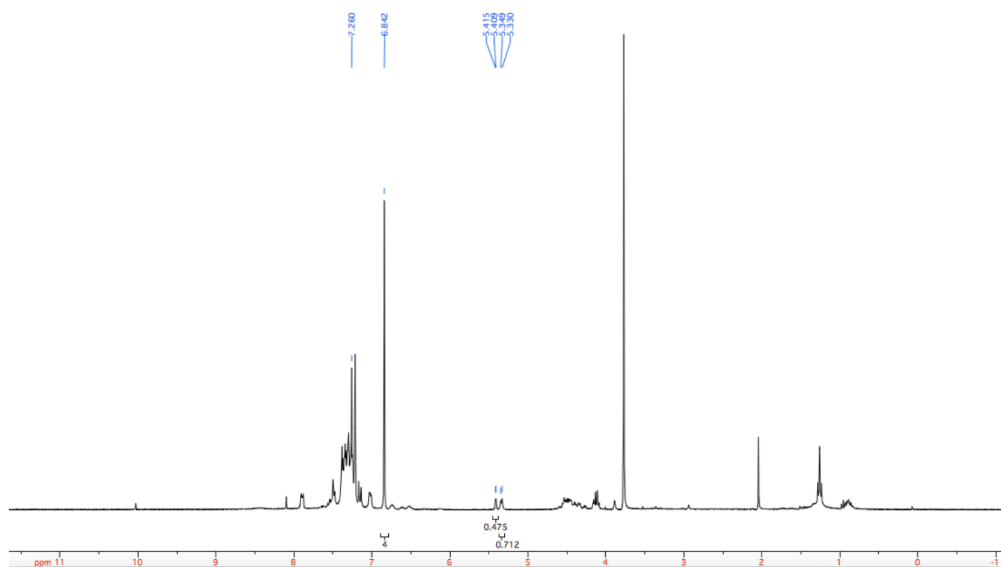
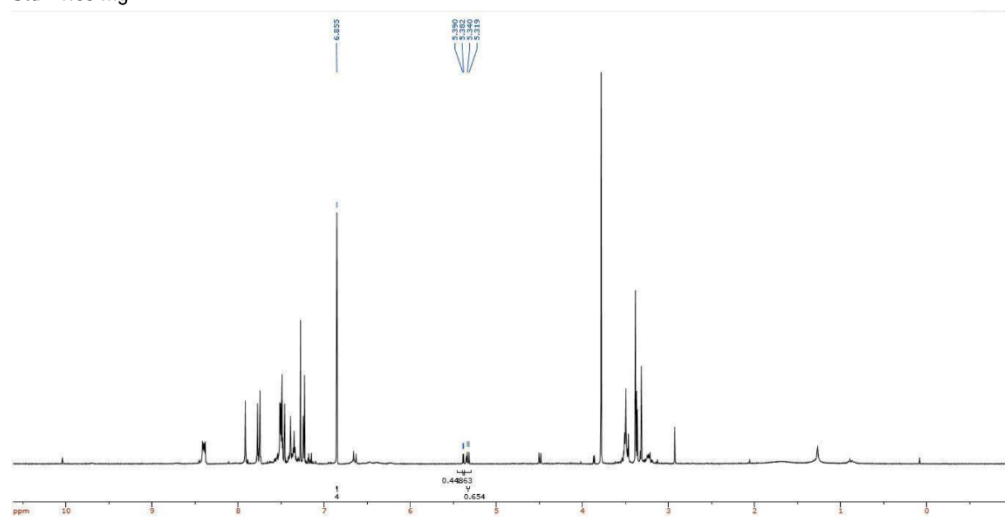


Table S2. Reaction 8B.

Std= 3.23 mg



Std= 1.69 mg

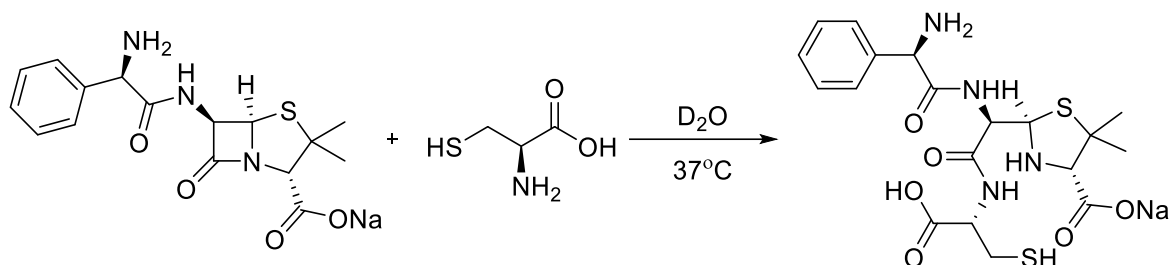


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Annex II – Development of dual-labelling strategy employing CuANCR reactions Supporting Information

Nucleophilic ampicillin β -lactam ring opening with N-terminal cysteine



Ampicillin sodium salt (10 mg, 0.027 mmol) was dissolved in 1 mL of D₂O. L-cysteine (16.36 mg, 0.135 mmol) was then added and the mixture was heated to 37 °C in an oil bath. The mixture was analyzed by NMR after 2 hours. The mixture was then analyzed on a Waters Acquity H-class UPLC, Xevo TQD (70:30 H₂O:MeCN with 0.1% formic acid, 0.1 mL/min, ES+). Tentative crude mixture ¹H NMR (300 MHz, D₂O) δ 7.40 (s, 5H), 5.11 (d, J = 1.4 Hz, 1H), 4.89 (d, J = 6.6 Hz, 1H), 4.33 (dd, J = 6.5, 1.2 Hz, 1H), 4.25 (t, J = 5.1 Hz, 1H), 3.85 (ddd, J = 5.5, 4.1, 1.2 Hz, 4H), 3.06 – 2.97 (m, 2H), 2.90 (dd, J = 4.4, 1.2 Hz, 3H), 2.87 – 2.75 (m, 3H), 1.16 (s, 3H), 1.00 (s, 3H). **MS (ESI-TQD):** for C₁₉H₂₆N₄O₆S₂ (M+H): calculated: 471.14; found: 471.05; (M+Na): calculated: 493.12; found: 493.08.

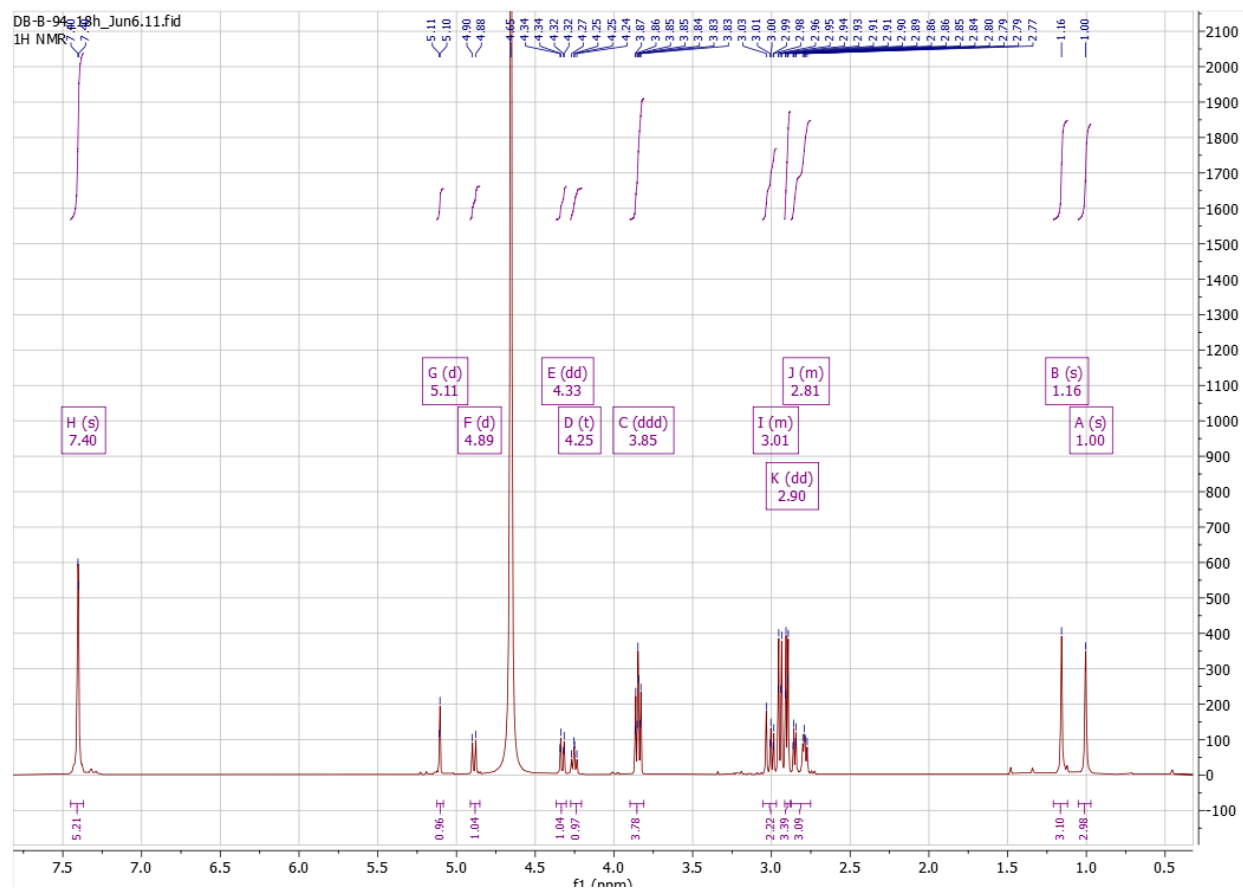


Figure S1. ^1H NMR spectra of crude reaction mixture containing 1 equivalent of ampicillin and 3 equivalents of L-cysteine after 18 hours.

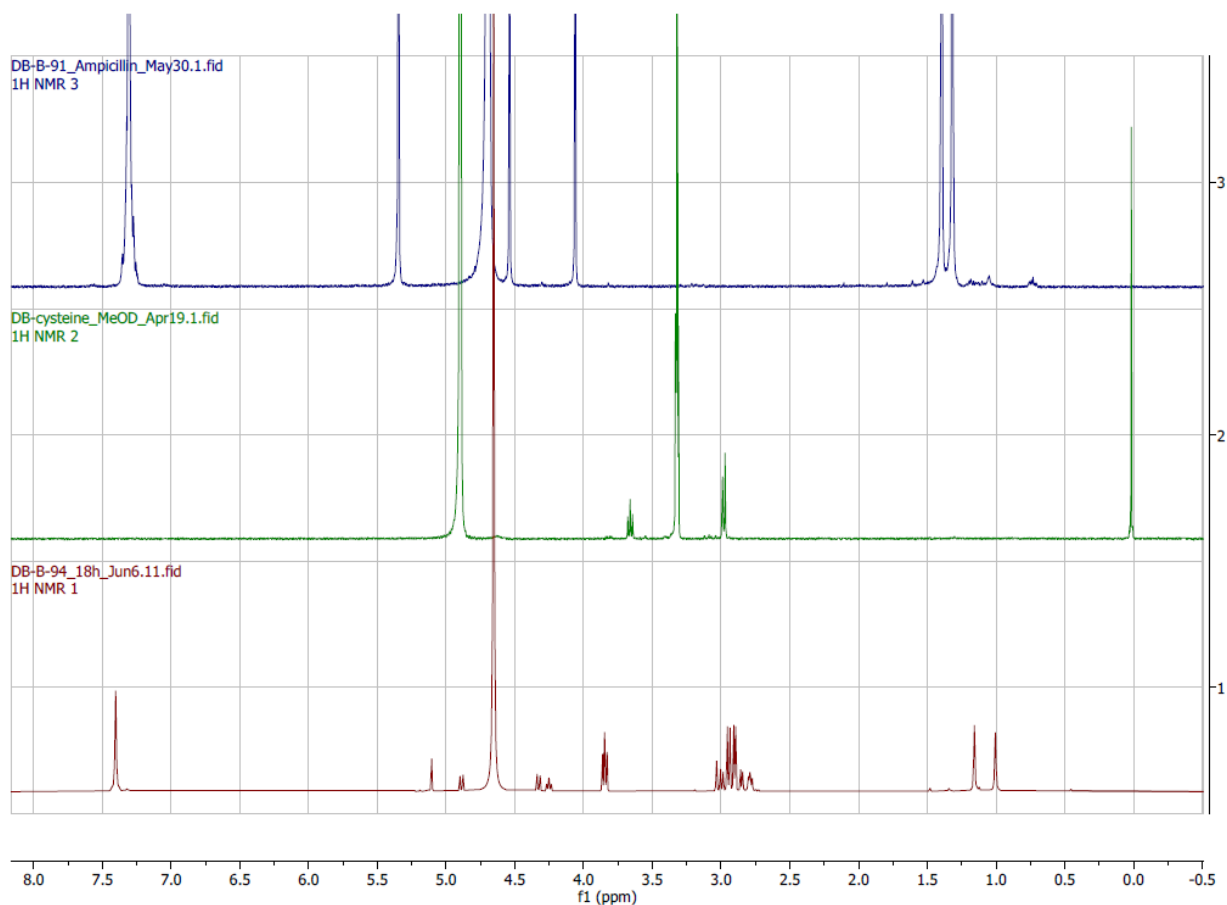


Figure S2. Overlapped ¹H NMR analysis of ampicillin ring-opening by L-cysteine. Top panel shows ¹H NMR of ampicillin in D₂O, middle panel shows ¹H NMR of L-cysteine in MeOD-d₄, bottom panel shows the crude reaction mixture after 18 hours.

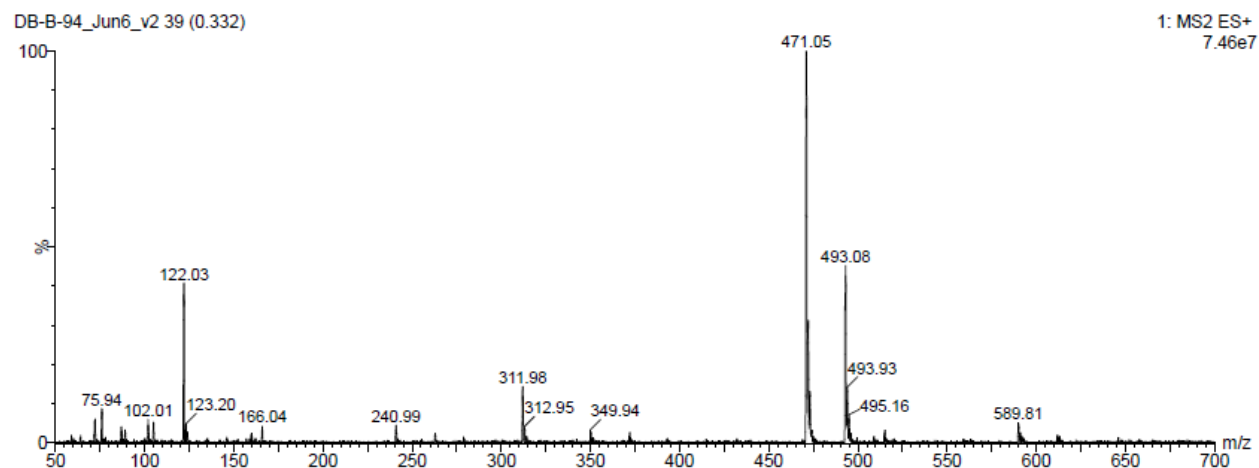
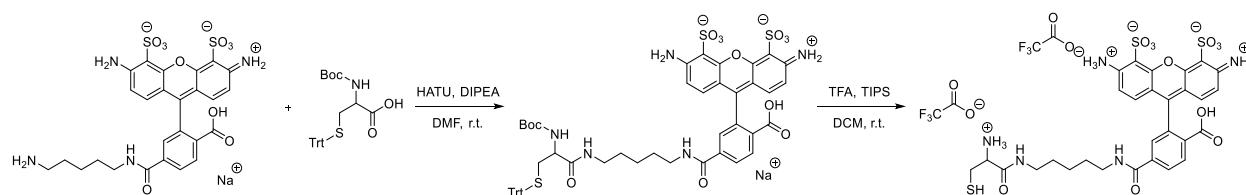


Figure S3. Mass spectrum of ampicillin-cysteine conjugation product.

Dual labelling with Micelle Assisted Kinugasa/CuANCR Reaction and Cysteine β -lactam ring opening on Alkyne Beads

5 μ L of alkyne-tagged beads (corresponding to 3-5 μ M of reactive alkyne groups (Click Chemistry Tools) were pretreated with 100 μ M L-cysteine and then washed 3x in PBS prior to use. The CuANCR reaction was carried out in PBS (pH = 7.4) and consisted of 100 μ M CuSO₄, 2 mM freshly solubilized sodium ascorbate, 200 μ M L-Proline, 150 μ M β -TDM surfactant and 50 μ M biotin-CMPO (or vehicle DMSO) to obtain a total of 500 μ L reaction volume. The reaction was started by addition of washed beads and was carried out with gentle shaking at 37 °C for 30 minutes. Beads were washed 1x with PBS containing 0.05% Tween20, then 3x with PBS. The native ligation reaction was then carried out by first solubilizing the beads in HEPES buffer (pH = 8.1) adding 50 μ M Cysteine-Alexa488 conjugate, followed by gentle shaking at 37 °C for 2 hours, taking care to protect the reaction from light. Beads were then washed 3x in PBS. The binding of streptavidin-Cy3 was carried out by adding 50 μ M at room temperature for 30 minutes in the dark. The beads were then washed 3x with PBS and resuspended in PBS containing 5% glycerol. 8 μ L of this solution was applied to a microscopy slide which were imaged using the Nikon Ni-U ratiometric fluorescence microscope equipped with a LED excitation light source and Ultra-sensitive Andor iXon Ultra 897 cooled EMCCD camera. Images were acquired using a Nikon 60x oil dip objective lens and, if indicated, a 2x relay lens. Fluorescence images were obtained under strictly identical conditions of gain and exposure time, on focused beads, typically 2-5 s, and brightfield images were obtained using a 20-50 ms exposure. Images were acquired using Nikon NIS Elements software and processed with ImageJ. To determine relative fluorescence intensity, densitometry analysis was accomplished using ImageJ software. Background signal from the corresponding fluorophore (no Alexa-488 or no Cy3) control was subtracted from the analyzed image. A uniform 9x9 pixel circle was used to delineate individual beads and measure their fluorescence intensity (Relative Fluorescence Units, RFU).

Synthesis of Cysteine-Alexa488 conjugate



Alexa Fluor 488 cadaverine (sodium salt) (1 mg, 1.6 μ M, 1 eq.) was dissolved in 100 μ L of anhydrous DMF and put under argon. To the mixture was added HATU (2 mg, 5.2 μ M, 3.2 eq.), followed by Boc-Cys(Trt)-OH (2 mg, 4.3 μ M, 2.7 eq.) and finally *N,N*-diisopropylethylamine (2 μ L, 2.7 μ M, 1.7 eq.) was added. The mixture was then stirred at room temperature, in the dark, for 18 hours. 950 μ L of MeOH was then added to the reaction vial and purified via preparative HPLC using gradient method with 5-95% MeCN over 15 minutes. Product eluted at ~0.5-1.5 minutes and its presence was confirmed by MS (on a Waters Acquity H-class UPLC-Xevo TQD, 70:30 H₂O:MeCN with 0.1% formic acid, 0.1 mL/min, ES⁺); the fractions

were pooled and concentrated under reduced pressure via lyophilization. **MS (ESI-TQD):** for $C_{53}H_{52}N_5O_{13}S_3^-$ (M+2H): calculated: 1064.29; found: 1064.62.

Following coupling and purification, the protected Cys-Alexa488 conjugate was dissolved in 500 μ L of a solution of DCM:Trifluoroacetic acid (TFA):Triisopropylsilane (TIPS) (50:47:3), under argon and covered from light. The mixture was allowed to stir at room temperature for 3 hours. Excess solvent was evaporated via flowing argon gas. The dry residue (1.57 mg) was then dissolved in DMSO and stored at -20 $^{\circ}$ C; the compound was used without further purification as the TFA salt. The deprotected product was subjected to UPLC-MS analysis (Waters Acquity H-class UPLC-Xevo TQD, 70:30 H_2O :MeCN with 0.1% formic acid, 0.1 mL/min, ES+). **MS (ESI-TQD):** for $C_{29}H_{30}N_5O_{11}S_3$ (M+2H): calculated: 722.13; found: 722.21.

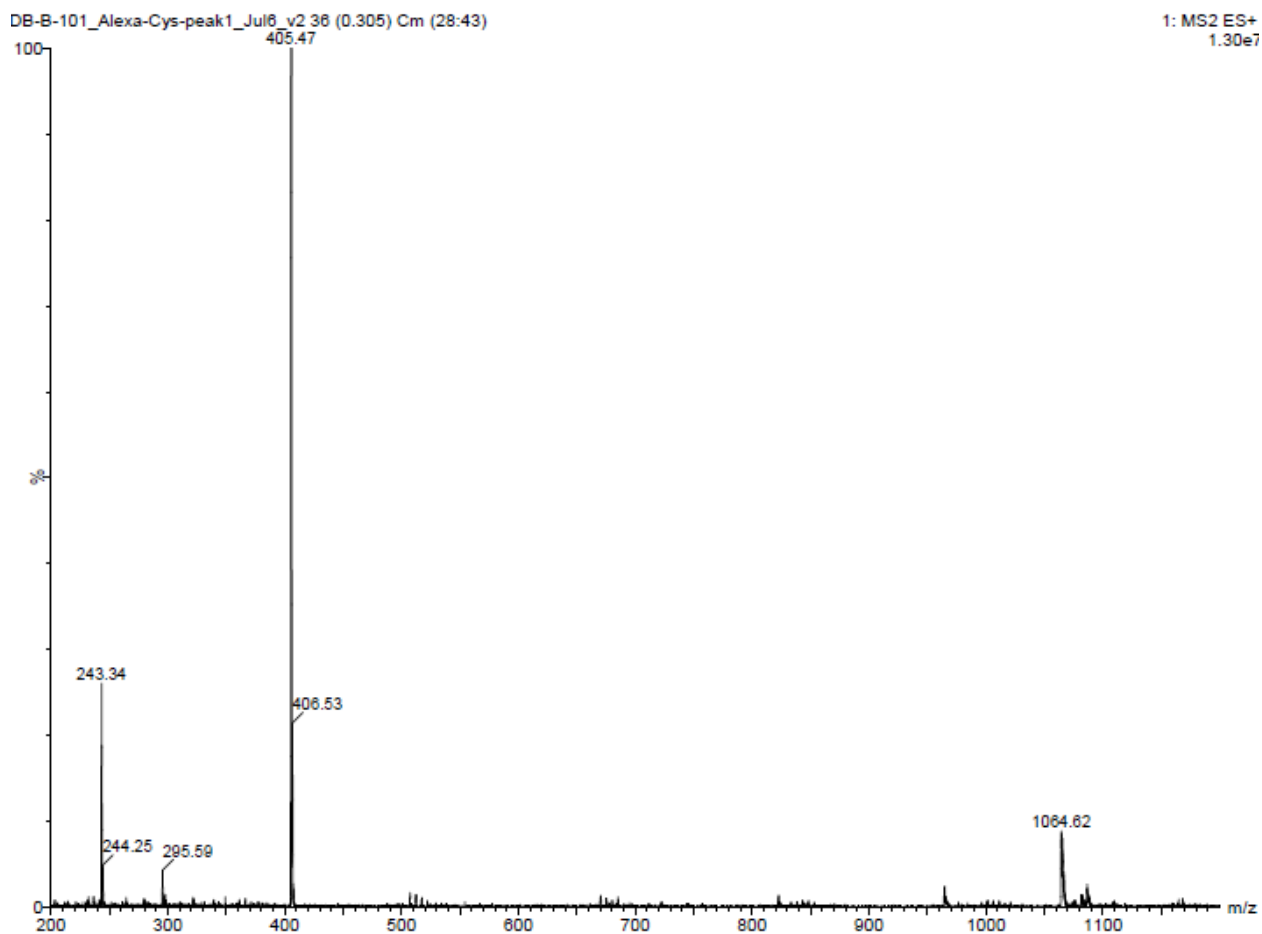


Figure S4. Mass spectrum of protected Cysteine-Alexa488 conjugation intermediate product.

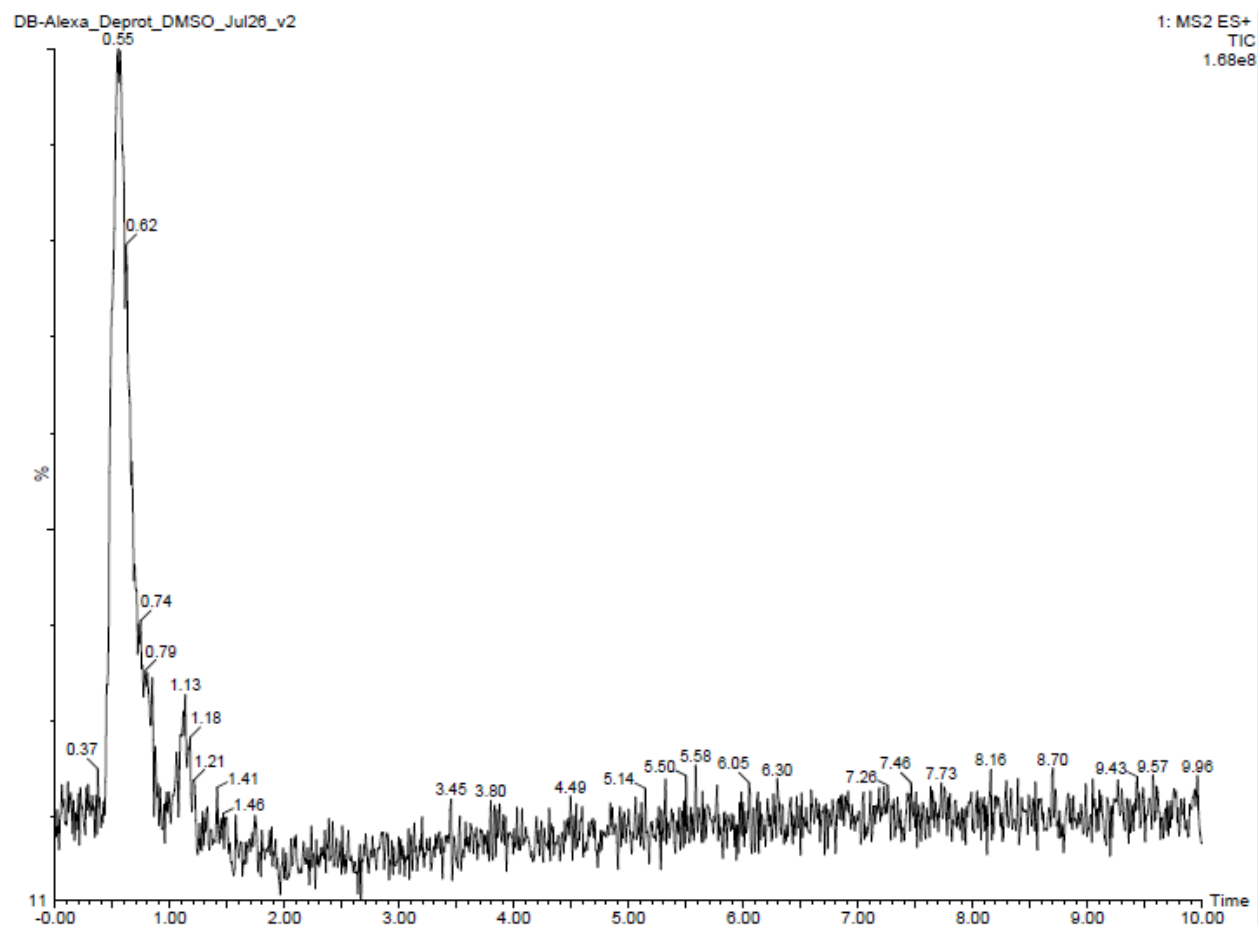


Figure S5. Total ion chromatogram of Cysteine-Alexa488 conjugation product.

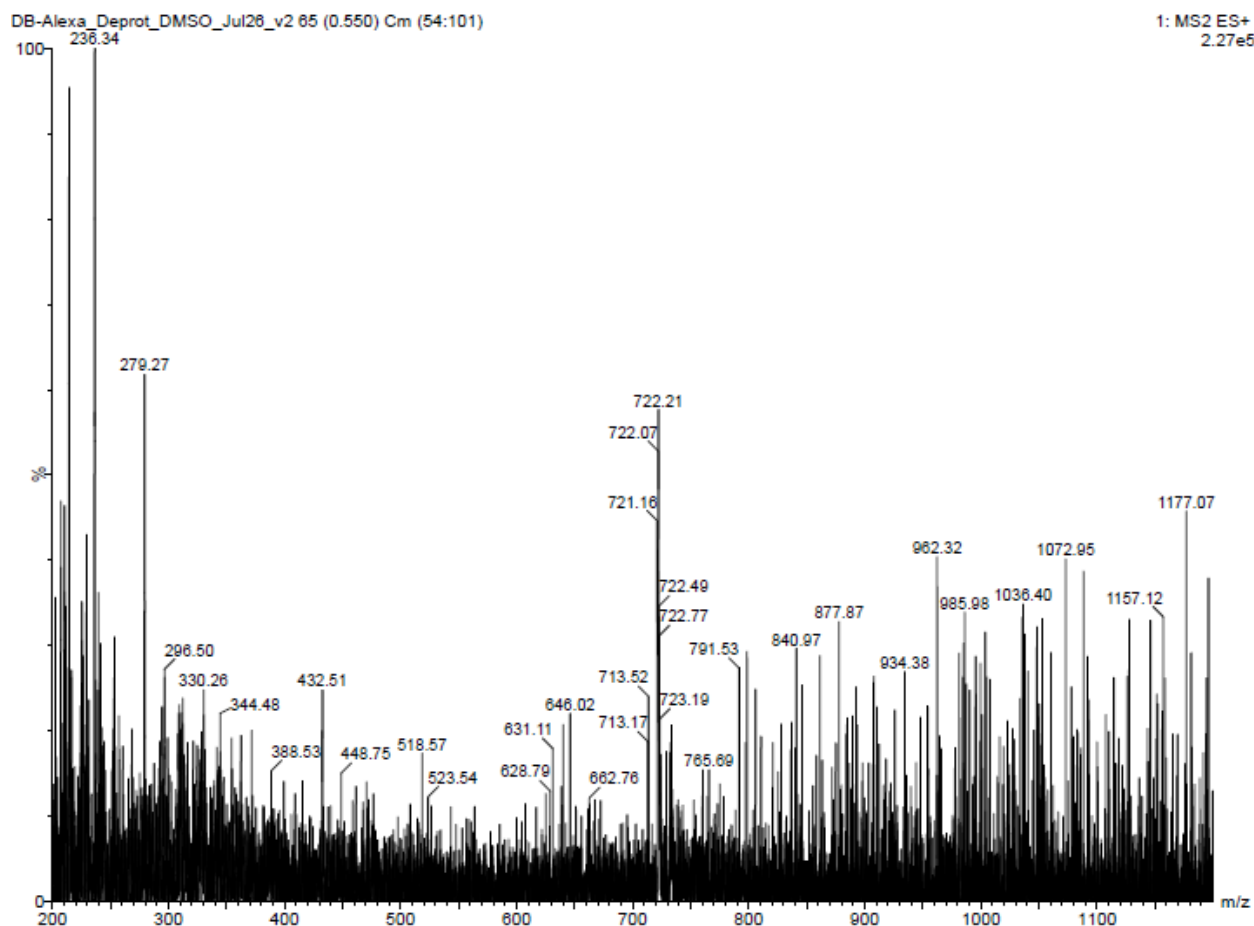


Figure S6. Mass spectrum of Alexa488-cysteine conjugation product.

Dual labelling of HuH7 carcinoma cells with two-step Kinugasa/CuANCR reaction and cysteine β -lactam ring opening

Huh 7 carcinoma cells were seeded in 8-chamber microscopy slides (10000 cells per slide) and cultured in DMEM with 10% FBS and 1 % NEAA. The cells were treated with 50 μ M Ac4Man-N-OxoPropyne or vehicle control for 72 hours. After treatment, cells were 3 times with PBS and then treated for 10 minutes using acetone to fix the cells, and further washed 3 times with PBS. Cells were then treated with 1 mM cysteine in HEPES buffer (pH = 8.1) for 1 hour at 37 °C and washed 3 times with PBS. The CuANCR labelling was then achieved by treating the cells for 30 minutes at 37 °C with 50 μ M Biotin-CMPO nitron, 0.1 mM CuSO₄, 0.2 mM L-proline, and 2 mM sodium ascorbate (200 μ L total volume in PBS). After treatment and washing 3 times with HEPES buffer, the cells were blocked using 1% BSA for 20 minutes at room temperature. The cysteine native chemical ligation was then undertaken by adding Cysteine-Alexa488 in HEPES buffer and sodium ascorbate (2 mM) and incubating the cells for 2 hours at 37 °C and washing the cells 3 times with PBS. After cysteine conjugation, the cells were treated with Cy3-streptavidin (50 μ M) and incubated for 30 minutes at room temperature in the dark and washed 3 times with PBS. Finally, DAPI mount solution was

added to the cells and a coverslip was added. Cells were visualized using the Nikon Ni-U ratiometric fluorescence microscope equipped with a LED excitation light source and Ultra-sensitive Andor iXon Ultra 897 cooled EMCCD camera. Images were acquired using a Nikon 60x oil dip objective lens and 2x relay lens. Fluorescence images were obtained under strictly identical conditions of gain and exposure time, on focused cells, typically 2-5 s, and brightfield images were obtained using a 20-50 ms exposure. Images were acquired using Nikon NIS Elements software and processed with ImageJ.

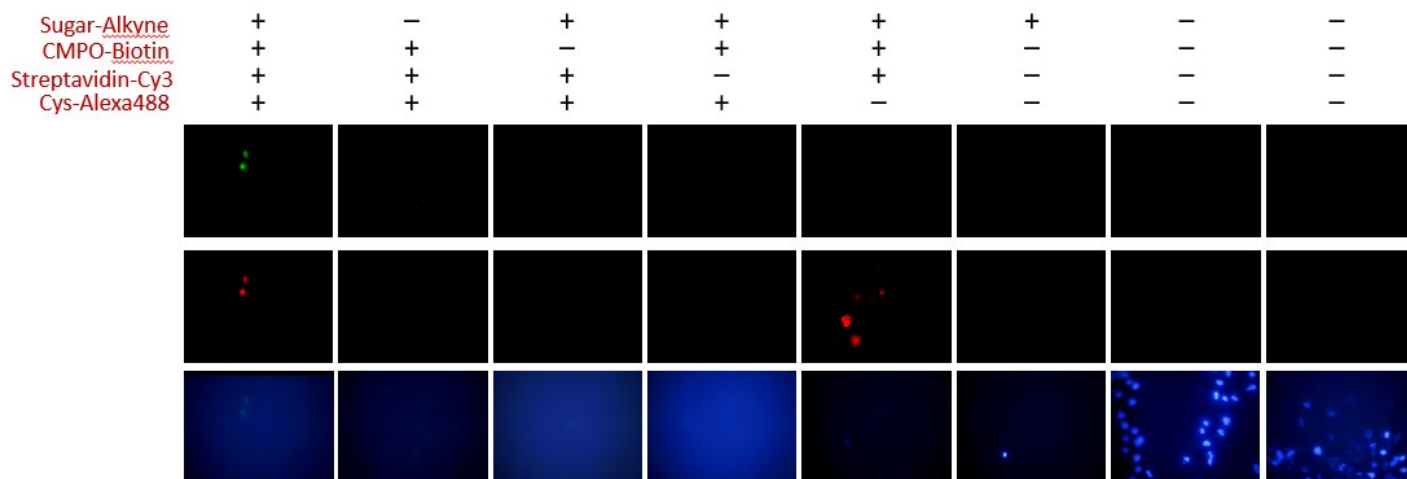


Figure S7. Initial trial of *in situ* labelling of Huh7 carcinoma cells by the two step CuANCR conjugation and cysteine native chemical ligation strategy. Top row images show the Alexa-488 channel (496 nm), middle row images show the Cy3 channel (569 nm), and the bottom row images show the DAPI channel (461 nm).

Annex III - ACS Chemical Biology, 2023, 18, 2430–2438. Supporting Information

Mechanistic analysis of bioorthogonal double strain-promoted alkyne-nitrone cycloadditions involving dibenzocyclooctadiyne.

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Supplementary Information

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Materials and methods

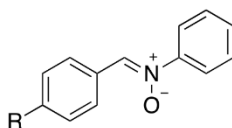
All commercially available reagents were purchased from Alfa Aesar and Sigma Aldrich, and were used without further purification. All deuterated solvents were purchased from Cambridge Isotopes and were used as received, except for deuterated chloroform that was neutralized by passing through a pad of basic alumina (such treated CDCl_3 will be signaled below by an asterisk, i.e. ' CDCl_3^* '). Flash chromatography was performed using silica gel (60 A, particle size 40-63 μm).

Mass Spectroscopy was performed on Waters Synapt High Definition Mass Spectrophotometer with positive electrospray ionization (ESI^+). MALDI-TOF mass spectra were recorded on an Autoflex III Smart Beam MALDI-TOF mass spectrometer (BRUKER) equipped with a Nd-YAG UV 355 nm laser. The acceleration voltage was 20 kV utilizing LP (Linear Positive) acquisition method.

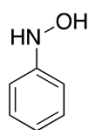
Kinetics of chemical reactions are measured with UV-Vis spectroscopy (Agilent Technologies, Cary 60). All ^1H and ^{13}C NMR spectra were obtained using 300 MHz, and 400 MHz Bruker NMR spectrometers and processed using MestReNova iNMR 4.2.0 software. The units of the NMR spectra peaks are ppm and referenced against the residual solvent peak. Multiplicity of peaks recorded as abbreviated form: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved, br = broad and J = coupling constants in Hz.

Synthesis

General Nitrone Synthesis

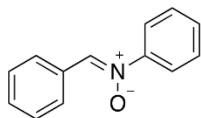


300 mg (2.8 mmol) of phenylhydroxylamine and 3 mmol of *para*-substituted benzaldehyde were added to a dry 25 mL round-bottom flask. 5 mL of dry ethanol was added to the flask and the mixture was stirred for 2 hours at 35 °C. Pure products were obtained following filtration of the precipitated crude product and recrystallization from warm ethanol and hexanes.



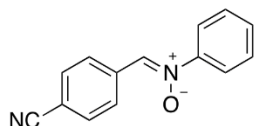
N-Phenylhydroxylamine. A mixture of nitrobenzene (10.8 mL, 0.105 mol), NH₄Cl (6.5 g, 0.12 mol) and degassed H₂O (200 mL) under argon at r.t. was stirred vigorously while zinc dust (15.4 g, 0.21 mol) was added portion wise over 20 min. After addition was complete, the reaction mixture was stirred for an additional 20 minutes, and was filtered while still warm. The resultant filter cake was washed with hot distilled water (50 mL) and the combined filtrate was saturated with NaCl and extracted with 3x100 mL of EtOAc. The organic layers were combined, dried over Na₂SO₄ and concentrated under reduced pressure. The crude N-phenyl hydroxylamine was recrystallized from petroleum ether / ethyl acetate (8.2 g, 72 %), dried thoroughly and stored under an atmosphere of argon at -20 °C. ¹H NMR and MS data are in agreement with that reported previously.¹

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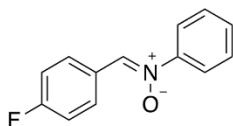
C, N-diphenylnitrone. The nitron was synthesized according to General

Nitrone Synthesis A. The product was obtained in 50% yield. **¹H NMR** (300 MHz; CDCl₃): δ 8.42-8.39 (m, 2H), 7.93 (s, 1H), 7.80-7.77 (m, 2H), 7.50-7.48 (m, 6H). Spectral data was consistent with previously reported data.²



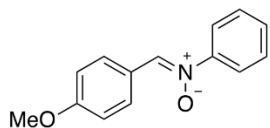
C-(4-cyanophenyl)-N-phenylnitrone. The nitron was synthesized

according to General Nitrone Synthesis A. The product was obtained in 52% yield. **¹H NMR** (400 MHz; CDCl₃): δ 8.48 (d, *J* = 8.4 Hz, 2H), 8.00 (s, 1H), 7.78-7.74 (m, 4H), 7.52 (t, *J* = 3.3 Hz, 3H). Spectral data was consistent with previously reported data.²

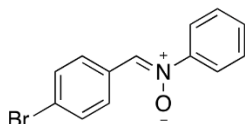


C-(4-fluorophenyl)-N-phenylnitrone. The nitron was synthesized

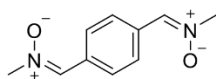
according General Nitrone Synthesis A. The product was obtained in 54% yield. **¹H NMR** (300 MHz; CDCl₃): δ 8.48-8.43 (m, 2H), 7.91 (s, 1H), 7.79-7.76 (m, 2H), 7.52-7.48 (m, 3H), 7.20-7.15 (m, 2H). Spectral data was consistent with previously reported data.²



C-(4-methoxyphenyl)-N-phenylnitrone. The nitrone was synthesized according to General Nitrone Synthesis A. The product was obtained in 77% yield. **¹H NMR** (300 MHz; CDCl₃): δ 8.43-8.39 (m, 2H), 7.86 (s, 1H), 7.80-7.76 (m, 2H), 7.51-7.44 (m, 3H), 7.02-6.99 (m, 2H), 3.89 (s, 3H). Spectral data was consistent with previously reported data.²

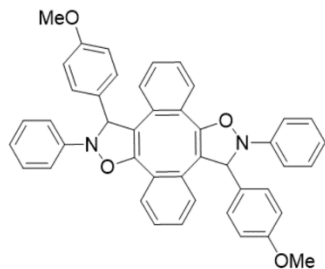


C-(4-bromophenyl)-N-phenylnitrone. The nitrone was synthesized according General Nitrone Synthesis A. **¹H-NMR** (400 MHz; CDCl₃): δ 8.30 (d, *J* = 8.5 Hz, 2H), 7.91 (s, 1H), 7.77 (d, *J* = 9.8 Hz, 2H), 7.62 (d, *J* = 8.6 Hz, 2H), 7.50 (dd, *J* = 5.1, 1.9 Hz, 3H). Spectral data was consistent with previously reported data.³



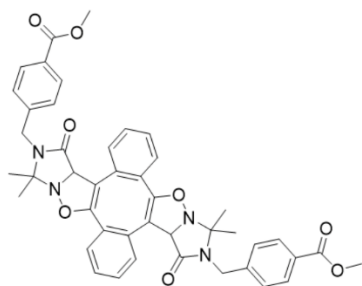
Compound 1h, N-Methyl(4-[[methyl(oxido)

iminio]methyl]phenyl)methylideneamine N-oxide⁵ Terephthalaldehyde (32 mg, 0.238 mmol) was added to a solution of N-methylhydroxylamine hydrochloride (50 mg, 0.598 mmol) in CH₂Cl₂ (1.2 ml). NaHCO₃ (60 g, 0.714 mmol) was added and the mixture refluxed for 2 h. The product was crashed out by hexane, filtered and collected. **¹H-NMR** (300 MHz; CDCl₃): δ 8.24 (4H, s), 7.40 (2H, s), 3.89 (6H, s). Spectral data was consistent with previously reported data.⁵



Compound 2b C-(4-methoxyphenyl)-*N*-phenylnitrone (2.1

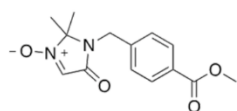
equiv.) and Diyne (1 equiv.) were added to a flask and placed under Argon. Acetonitrile (6 mM) was added and the reaction was stirred for 30 min. at room temperature, concentrated under reduced pressure and the product was quickly purified by short column chromatography on neutralized silica (10:1 to 5:1/Hexane:Ethylacetate) in the form of isomers mixture. **¹H NMR** (400 MHz, CDCl₃^{*}) δ 7.78-7.74 (m, 2H), 7.71-7.68 (m, 2H), 7.42-7.34 (m, 4H), 7.16-7.12 (m, 4H), 7.11-7.08 (m, 4H), 7.08-7.03 (m, 4H), 6.94-6.86 (m, 4H), 6.84-6.80 (m, 2H), 6.67 (s, 1H), 6.63 (s, 1H), 3.89 (s, 3H), 3.88 (s, 3H); **¹³C NMR** (100 MHz, CDCl₃) δ 160.48, 147.25, 145.97, 140.73, 140.02, 134.05, 133.75, 131.29, 129.86, 129.60, 129.14, 128.60, 128.18, 127.94, 127.82, 122.11, 119.95, 118.86, 114.33, 98.56, 98.12, 60.55, 55.52; **HRMS (ESI+)** calcd (C₄₄H₃₄N₂O₄Na): 655.2597, [M+Na]⁺ found 655.2617.



Compound 2f DMImO (2.1 equiv.) and Diyne (1 equiv.)

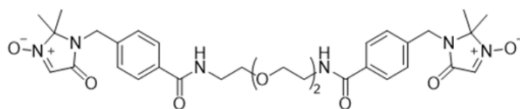
were added to a flask and placed under Argon. Acetonitrile (6 mM) was added and the

reaction was stirred for 30 min. at room temperature, concentrated under reduced pressure and the product was quickly purified by short column chromatography on neutralized silica (Chloroform). **¹H NMR** (500 MHz, CD₃CN) δ 7.90 – 7.88 (m, 4H), 7.54-7.52 (m, 2H), 7.47-7.42 (m, 4H), 7.32-7.29 (m, 2H), 7.23-7.20 (m, 4H), 5.29 (s, 2H), 4.73 (d, *J* = 16.6 Hz, 2H), 4.31 (d, *J* = 16.6 Hz, 2H), 3.82 (s, 6H), 1.38 (s, 6H), 1.35 (s, 6H). **¹³C NMR** (125 MHz, CD₃CN) δ 171.58, 167.45, 151.60, 144.38, 133.83, 131.54, 131.29, 130.76, 130.45, 128.45, 128.08, 118.26, 85.06, 72.95, 52.47, 43.60, 26.63, 22.83. **HRMS (ESI+)** calcd (C₄₄H₄₀N₄O₈Na): 775.2744, [M+Na]⁺ found 775.2739.



Methyl 4-[(2,2-dimethyl-3-oxido-5-oxo-2,5-dihydro-1H-imidazol-1-yl)methyl]benzoate (DMImO). Synthesized

according to a modified version of previously reported literature.⁴ The product was obtained in 26.4% yield over 5 steps. **¹H NMR** (400 MHz, CDCl₃) δ 8.04 – 7.94 (m, 2H), 7.42 – 7.34 (m, 2H), 7.09 (s, 1H), 4.69 (s, 2H), 3.90 (s, 3H), 1.47 (s, 6H); **¹³C NMR** (100 MHz, CDCl₃) δ 166.52, 162.27, 141.76, 130.20, 130.08, 127.89, 123.49, 90.48, 52.23, 43.36, 25.06; **HRMS (EI)** calcd for (C₁₄H₁₆N₂O₄): 276.11101, [M] found 276.10923.



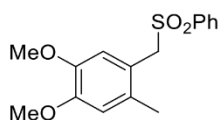
Compound 1g DMImO (200 mg, 0.724

mmol) was dissolved in 2.5 % aqueous sodium hydroxide (7 ml) and the reaction was stirred for 30 min. at 60 °C. The residue was neutralized by resin (AmberChrom, 50WX4 hydrogen form, Sigma-Aldrich), filtered through a Celite pad and concentrated. The crude was purified

by column chromatography (100:2/Chloroform:Methanol) to give Methyl 4-[(2,2-dimethyl-3-oxido-5-oxo-2,5-dihydro-1H-imidazol-1-yl)methyl]benzoic acid. To the solution of DMImO-carboxylic acid in dichloromethane (50 mg, 0.19 mmol) at 0 °C was added 4-Dimethylaminopyridine (11.6 mg, 0.095 mmol, 0.5 equiv.), EDCI (72.8 mg, 0.38 mmol, 2 equiv.) and 1,2-bis(2-aminoethyl)ethane (14 μ l, 0.096 mmol, 0.5 equiv.) respectively and the reaction was stirred overnight. The reaction was diluted with distilled water and extracted with dichloromethane. The organic phase was dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The crude was purified by column chromatography (100:2/DCM:MeOH) to give **1g** (49 mg, 0.077 mmol, 40%). **¹H NMR** (300 MHz, CDCl₃) δ 7.76 (d, J=6 Hz, 4H), 7.39 (d, J=9 Hz, 4H), 7.09 (s, 2H), 6.70-6.63 (m, 2H), 4.69 (s, 4H), 3.69-3.59 (m, 12H), 1.49 (s, 12H); **¹³C NMR** (100 MHz, CDCl₃) δ 166.9, 162.3, 140.3, 134.3, 128.1, 127.6, 123.5, 90.5, 70.2, 69.7, 43.3, 39.8, 25.1 ppm; **HRMS (ESI+)** calcd for C₃₂H₄₀N₆NaO₈ 659.2805, found 659.2825.

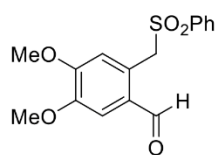
Synthesis of OMe substituted Diyne

The OMe-substitued diyne was synthesized according to previously reported literature procedures.⁶



α -benzenesulfonyl-4,5-dimethoxy-*o*-xylene. In a dry rbf, a suspension of 3,4-dimethoxytoluene (2.25g, 15.0 mmol) and paraformaldehyde (1.35g, 22.5 mmol) in 25 mL of dry CCl₄ was cooled to 0°C, under N₂. A solution of HBr/AcOH (33%, 6 mL) was added dropwise and the mixture was stirred for 4h at 0°C. The mixture was then poured into H₂O (50 mL). The organic layer was then separated and washed with 5% NaHCO₃ and

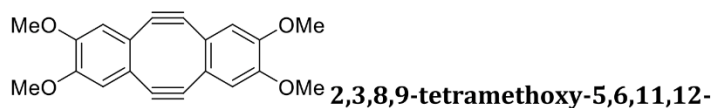
dried with Na₂SO₄. Evaporation of solvent *in vacuo* yielded a crude white solid, which was used in the next step without further purification. The crude product was then dissolved in DMF (25 mL) and benzenesulfinic acid sodium salt dihydrate (3.6g, 18.0 mmol) was added to the mixture. The mixture was then heated to 80°C overnight. After letting the mixture cool to room temperature, H₂O (50 mL) and EtOAc (50 mL) were added and extracted a total of 3 times with EtOAc. The organic layers were separated, dried with Na₂SO₄ and the solvent evaporated *in vacuo*. The crude product was purified via f.c.c. (EtOAc:Hexanes, 1:4) to yield 4.13g (13.5 mmol, 90% yield) of white powder. ¹H NMR (300 MHz, CDCl₃) δ 2.05 (s, 3H), 3.65 (s, 3H), 3.84 (s, 3H), 4.31 (s, 2H), 6.41 (s, 1H), 6.61 (s, 1H), 7.47 (t, J = 7.3 Hz, 2H), 7.62 (t, J = 7.3 Hz, 1H), 7.66 (d, J = 8.3 Hz, 2H). Spectral data was consistent with previously published data.⁶



4,5-Dimethoxy-2-((phenylsulfonyl)methyl)benzaldehyde. Under N₂,

to a suspension of α-benzenesulfonyl-4,5-dimethoxy-*o*-xylene (1.67 g, 5.4 mmol) in CCl₄ (50 mL) were added NBS (1.01 g, 5.7 mmol) and AIBN (89.2 mg, 0.54 mmol) at 90 °C. The resulting mixture was heated at 100 °C for 6 hours. After cooling to room temperature, the reaction was worked up with CH₂Cl₂/NH₄Cl(aq.), the combined organic layers were dried with Na₂SO₄ and evaporated. The crude residue was used for the next step without further purification. To this crude material were added CaCO₃ (5.43 g, 54.3 mmol), 1,2-dimethoxyethane (18 mL), and H₂O (18 mL). The resulting mixture was heated to 120°C overnight under N₂. After cooling to room temperature, the mixture was neutralized with

dilute (1M) HCl, followed by extraction with CH₂Cl₂/H₂O (3 times). The organic layers were dried and evaporated *in vacuo*, and the crude product was used for the next step without further purification. This crude product was dissolved in CH₂Cl₂ (18 mL) and MnO₂ (4.72g, 54.3 mmol) was added. The resulting mixture was heated at 50°C overnight under N₂. After cooling to room temperature, the mixture was filtered and the solvent was evaporated *in vacuo*. The residue was purified by f.c.c. (EtOAc:Hexanes, 1:1) to give the product (1.26 g, 72.4% yield) as a white solid. ¹H NMR (300 MHz, CDCl₃) δ 3.90 (s, 3H), 3.95 (s, 3H), 4.91 (s, 2H), 6.79 (s, 1H), 7.24 (s, 1H), 7.48 (t, J = 7.6 Hz, 2H), 7.62 (t, J = 7.3 Hz, 1H), 7.68 (d, J = 7.3 Hz, 2H), 9.78 (s, 1H). Spectral data was consistent with previously published data.⁶



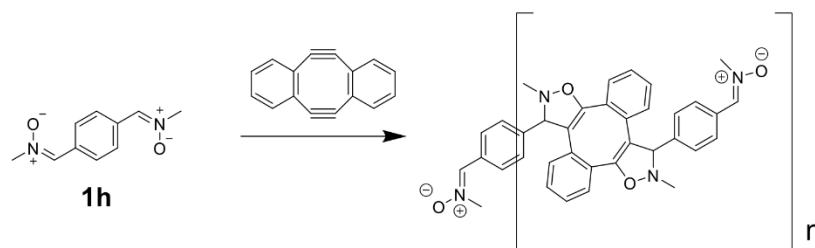
tetradehydrobenzo[a,e]cyclooctyne. A flame dried round bottom flask was loaded with 4,5-Dimethoxy-2-((phenylsulfonyl)methyl)benzaldehyde (630 mg, 1.97 mmol), Diethyl chlorophosphate (0.34 mL, 2.4 mmol), and dry THF (40 mL). A solution of LiHMDS (1.0 M in THF, 4.0 mL, 4.0 mmol) was added dropwise at -78°C. The mixture was stirred at -78°C for 30 minutes and then allowed to warm to room temperature over 1.5 h. The resulting mixture was cooled to -78°C again, and a solution of LDA (1.0 M in THF/hexanes, 10.0 mL, 10.0 mmol) was slowly added dropwise. The resulting mixture was stirred at -78°C for 2 h. After the elapsed reaction time, saturated aqueous NH₄Cl was slowly added to the mixture to quench the mixture. H₂O and EtOAc were then added and the mixture was extracted 3 times with EtOAc. The organic layers were collected, dried over Na₂SO₄, and evaporated *in vacuo*. The crude residue was subjected to f.c.c. (CH₂Cl₂) to give the OMe-

Diyne (160.2 mg, 50% yield) as a bright yellow powder. **¹H NMR** (300 MHz, CDCl₃) δ 3.77 (s, 12H), 6.26 (s, 4H); Spectral data was consistent with previously published data.⁶

Polymer Analysis

General Procedure for Synthesis of Polymers

The stock solutions of di-nitrone and Diyne (2 mM in dichloromethane) were prepared and mixed in a 2:1 ratio (1 ml dinitrone and 0.5 ml diyne), respectively. Polymerization was carried out by removing the solvent under reduced pressure.



Scheme S1. Polymer structure formed by the SPANC polymerization of monomer **1h** with diyne.

MALDI analysis

The polymer sample were dissolved in CH₂Cl₂, 1μl of mixture (sample and matrix) was deposited on a stainless steel plate for analysis by MALDI. The spectra were acquired using DCTB matrix.

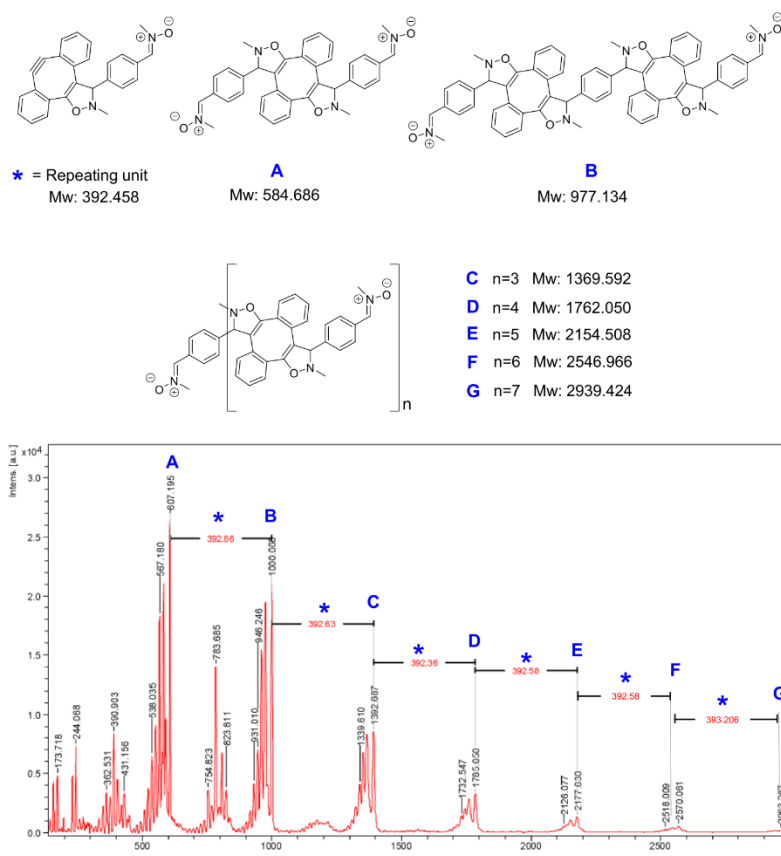


Figure S1. MALDI mass spectrum of polymer structure formed by SPANC polymerization of monomer **1h** with diene from scheme S1.

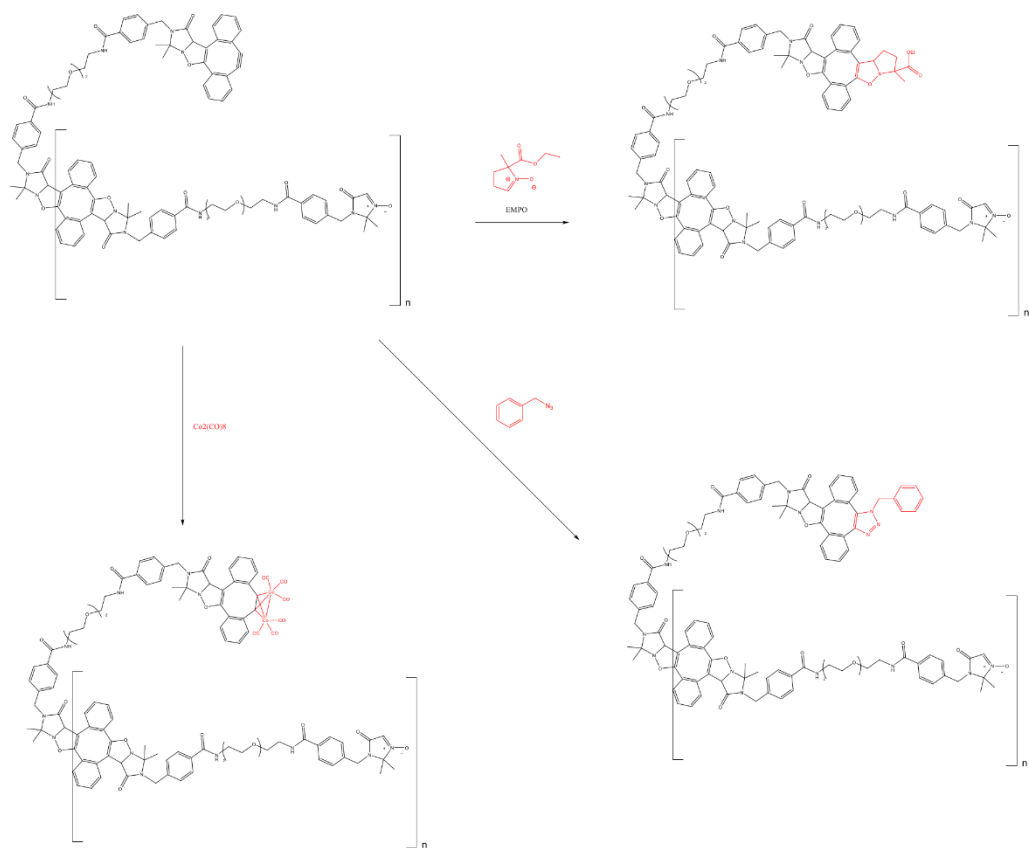
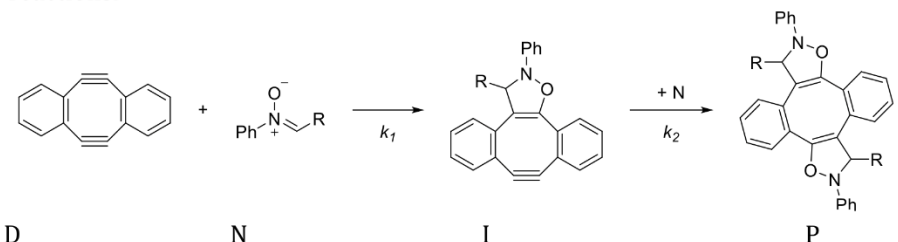


Figure S2. Predicted polymer structures after addition EMPO, benzyl azide and dicobalt octacarbonyl to confirm the cyclic structures. These structures were not observed in MALDI analysis which indicates the formation of cyclic structures arising from intramolecular cyclization and absence of free alkyne in the reaction mixture.

Kinetic experiments

Predicted reaction kinetics of SPDC between nitrones and diyne

Table S1. Rate laws and corresponding explicit equations used to determine kinetics of the SPDC reactions.

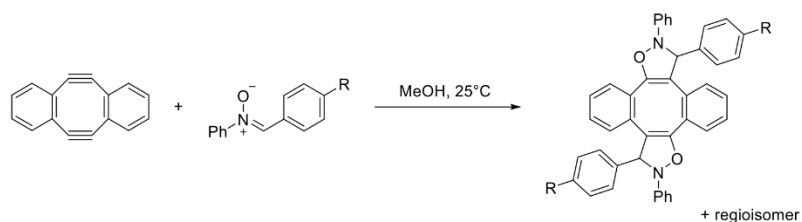
 D N I P	
Rate law	Explicit Equation
$d[D]/dt = -k_1[D]$	$[D] = [D]_0 e^{-k_1 t}$
$d[N]/dt \approx 0$, where $N \gg D$	$[N] = [N]_0 - [I] - 2[P]$
$d[I]/dt = k_1'[D] - k_2'[I]$	$[I] = -k_1'[D]_0 / (k_2' - k_1') e^{(k_1' t - k_2' t)}$
$d[P]/dt = k_2'[I]$	$[P] = [D]_0 - [D] - [I]$

Kinetics Studies and Methodology

The rate measurements of cycloadditions of diyne with various nitrones were performed using UV-visible spectroscopy in methanol at 25 ± 0.1 °C in duplicate. Reactions were done under pseudo-first order conditions with the nitron in 20 - 100-fold excess of the diyne and were monitored by following the increase of the absorbance at the characteristic wavelengths specified in **Table S2**. Note that due to spectral overlap and difficulties

observing individual reactant peaks, the absorbance changes used for kinetic analyses are assumed to be those associated with the formation of the double-addition product.

Table S2. Reaction conditions and absorption wavelengths used to perform UV-Visible kinetic studies of cycloadditions between diyne and nitrones.



Nitrone		Product
		Wavelength λ (nm)
1a	R= H	380
1b	R= p-OCH ₃	402
1c	R= p-F	384
1d	R= p-Br	384
1e	R= p-CN	402

Stock solutions of diyne and nitrone were prepared as 5 mM stocks in screw cap vials in OptimaGrade Methanol and stored at -20 °C. Diyne was then diluted down to 1 mM in methanol and covered with aluminum foil. The cuvettes used were 1.5 mL Quartz screw cap (10mm pathlength). Before preparation of each reaction, UV-vis cuvettes were cleaned with acetone 4 times and let dry for 20 minutes. Cuvettes were wiped with Kimwipes prior to reading to remove any residue on the outer surface.

Prior to reactions, the UV-vis spectrophotometer was blanked with a 1 mL of methanol. Next, reactions were set up with addition of methanol and nitrone (**Table S3**), followed by quick addition of diyne, mixing of the reaction by inverting the cuvette and immediate insertion for wavelength reading. In order to choose a wavelength for product monitoring, the following spectra (200 nm to 800 nm) were obtained over the entire reaction period:

- Solvent only as baseline
- Diyne (25 μM)
- Nitron (1000- 2500 μM)
- Reaction mixture containing 25 μM Diyne, 1000-2500 μM Nitron and remaining amount of methanol according to the volumes shown in **Table S3**

Table S3. Volume of stock solutions used for the pseudo-first order kinetics of acyclic nitrones with diyne.

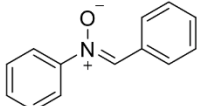
Reaction	Vol. of 1 mM Diyne stock (μL)	Vol. of 5mM nitron stock (μL)	Vol. of methanol (μL)	Total Volume of reaction (μL)
25 μM Diyne 500 μM Nitron	25	100	875	1000
25 μM Diyne 1000 μM Nitron	25	200	775	1000
25 μM Diyne 1500 μM Nitron	25	300	675	1000
25 μM Diyne 2000 μM Nitron	25	400	575	1000
25 μM Diyne 2500 μM Nitron	25	500	475	1000

Once reaction was complete, indicated by a plateau at the indicted wavelength, the highest point of the peak was chosen as the wavelength to use for kinetic calculations. Using a least square fitting model of consecutive first-order reactions, a line of best fit based off predicted absorbance over time in seconds was used to determine k_1' and k_2' . An example of this fitting is shown in **Figure 1** of the reaction between 1000 μM C-*N*-diphenylnitrone with 25 μM Diyne.

General procedure for the double cycloaddition kinetic studies.

A calculated amount of stock solution of nitrone (excess reagent) required to achieve the desired final concentration was diluted in a calculated amount of additional methanol in a cuvette. A calculated amount of stock solution of diyne required to achieve the desired final concentration was rapidly mixed with the thermally equilibrated solution and immediately inserted into the UV-vis spectrophotometer. Absorption was measured at pre-set time intervals of 2 min until it reached a plateau at the wavelength chosen as shown in **Table S2**.

Table S4. Observed rate constants for the consecutive pseudo-first-order kinetic studies of cycloaddition reactions of nitron **1a** with diyne, correlated to tested concentrations of nitron in methanol.

Nitron	Concentration of Nitron (10^{-3} M)	k_1' (10^{-4} s $^{-1}$)	k_2' (10^{-4} s $^{-1}$)
	1.00	3.44 ± 0.02	8.52 ± 0.74
	1.25	4.05 ± 0.45	9.04 ± 0.03
	1.50	4.37 ± 0.15	10.4 ± 0.92
	2.00	5.18 ± 0.08	12.5 ± 1.95

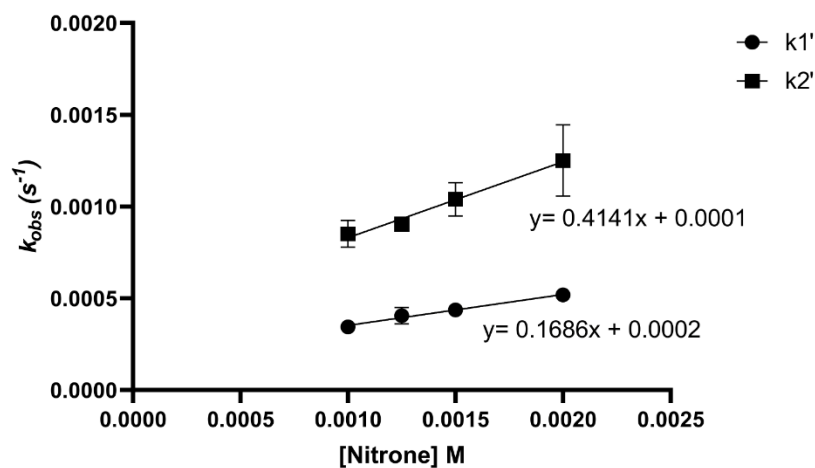
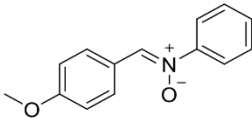


Figure S3. Pseudo-first order rate constants (k_1 and k_2) of nitron **1a** with diyne cycloadditions. Determined by plotting the k_{obs} (s $^{-1}$) of each cycloaddition against concentration of nitron used (M). $R^2(k_1') = 0.92$, $R^2(k_2') = 0.77$

Table S5. Observed rate constants for the sequential-pseudo first-order kinetic studies of cycloaddition reactions of nitrone **1b** C-(4-methoxyphenyl)-*N*-phenylnitronone with diyne, correlated to various concentrations of nitronone in methanol.

Nitronone	Concentration of Nitronone (10^{-3} M)	k_1' (10^{-4} s $^{-1}$)	k_2' (10^{-4} s $^{-1}$)
	1.00	1.06 ± 0.02	9.76 ± 0.45
	1.25	1.48 ± 0.12	9.87 ± 0.58
	1.50	1.49 ± 0.20	9.96 ± 0.69
	2.00	2.05 ± 0.06	10.4 ± 0.37

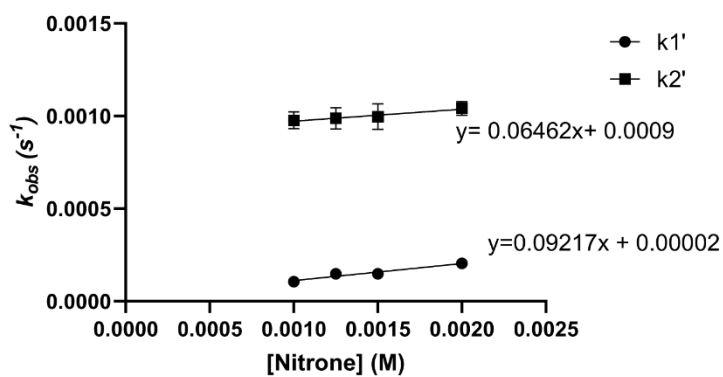
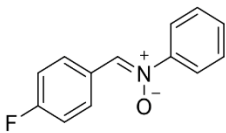


Figure S4. Pseudo first-order rate constants of the first (k_1) and second (k_2) cycloadditions of **1b** C-(4-methoxyphenyl)-*N*-phenylnitronone with diyne plotted against the various concentrations of nitronone (M). $R^2(k_1') = 0.89$, $R^2(k_2') = 0.28$

Table S6. Observed rate constants for the sequential-pseudo first-order kinetic studies of cycloaddition reactions of nitrone **1c** C-(4-fluorophenyl)-*N*-phenylnitron with diyne, correlated to various concentrations of nitron in methanol.

Nitron	Concentration of Nitron (10 ⁻³ M)	k_1' (10 ⁻⁴ s ⁻¹)	k_2' (10 ⁻³ s ⁻¹)
	0.50	1.76 ± 0.18	0.96 ± 0.10
	1.00	3.30 ± 0.21	1.02 ± 0.29
	1.50	3.97 ± 0.22	1.24 ± 0.05
	2.00	4.66 ± 0.25	1.64 ± 0.01

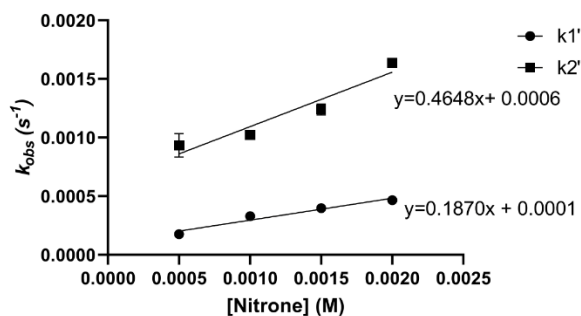
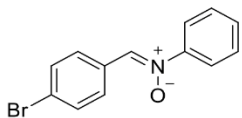


Figure S5. Pseudo first-order rate constants of the first (k_1) and second (k_2) cycloadditions of **1c** C-(4-fluorophenyl)-*N*-phenylnitron with diyne plotted against the various concentrations of nitron (M). $R^2(k_1') = 0.93$, $R^2(k_2') = 0.90$

Table S7. Observed rate constants for the sequential-pseudo first-order kinetic studies of cycloaddition reactions of nitron **1d** C-(4-bromophenyl)-*N*-phenylnitron with diyne, correlated to various concentrations of nitron in methanol.

Nitron	Concentration of Nitron (10 ⁻³ M)	<i>k</i> ₁ ' (10 ⁻⁴ s ⁻¹)	<i>k</i> ₂ ' (10 ⁻³ s ⁻¹)
	0.50	4.32 ± 0.06	1.14 ± 0.07
	1.00	5.24 ± 0.29	1.09 ± 0.00
	1.50	5.41 ± 0.09	1.42 ± 0.08
	2.00	6.12 ± 0.03	1.61 ± 0.08

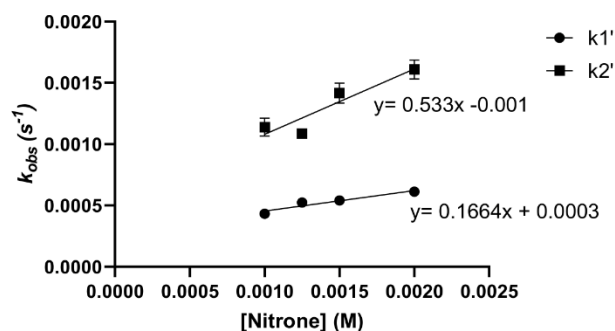
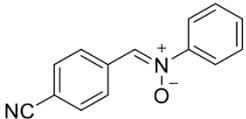


Figure S6. Pseudo first-order rate constants of the first (*k*₁) and second (*k*₂) cycloadditions of **1d** C-(4-bromophenyl)-*N*-phenylnitron with diyne plotted against the various concentrations of nitron (M). *R*² (*k*₁') = 0.89, *R*² (*k*₂') = 0.82.

Table S8. Observed rate constants for the sequential-pseudo first-order kinetic studies of cycloaddition reactions of nitrone **1e** C-(4-cyanophenyl)-*N*-phenylnitron with diyne, correlated to various concentrations of nitron in methanol.

Nitron	Concentration of Nitron (10 ⁻³ M)	k_1' (10 ⁻⁴ s ⁻¹)	k_2' (10 ⁻³ s ⁻¹)
	0.50	4.35 ± 0.05	2.11 ± 0.10
	1.00	5.05 ± 0.06	3.20 ± 0.13
	1.50	5.40 ± 0.06	5.46 ± 0.46
	2.00	5.83 ± 0.11	7.99 ± 0.00

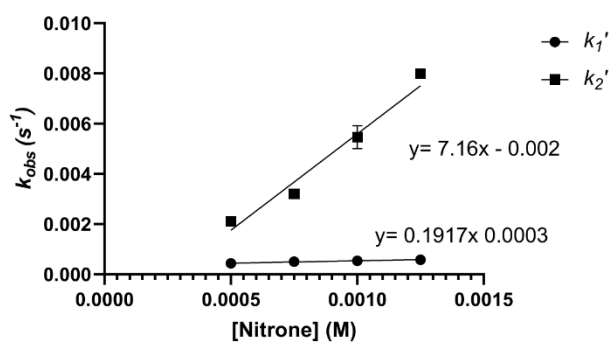
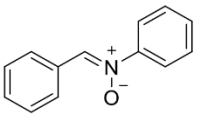


Figure S7. Pseudo first-order rate constants of the first (k_1) and second (k_2) cycloadditions of **1e** C-(4-cyanophenyl)-*N*-phenylnitron with diyne plotted against the various concentrations of nitron (M). $R^2(k_1') = 0.97$, $R^2(k_2') = 0.96$.

Table S9. Observed rate constants for the sequential-pseudo first-order kinetic studies of cycloaddition reactions of nitrone **1a** C-(phenyl)-*N*-phenylnitronone with tetramethoxy-diyne, correlated to various concentrations of nitrone in methanol.

Nitronone	Concentration of Nitronone (10^{-3}M)	k_1' (10^{-4} s^{-1})	k_2' (10^{-3} s^{-1})
	1.00	4.25 ± 0.14	3.83 ± 0.17
	1.25	5.27 ± 0.06	4.24 ± 0.14
	1.50	6.24 ± 0.22	4.60 ± 0.18
	2.00	7.89 ± 0.16	4.93 ± 0.17

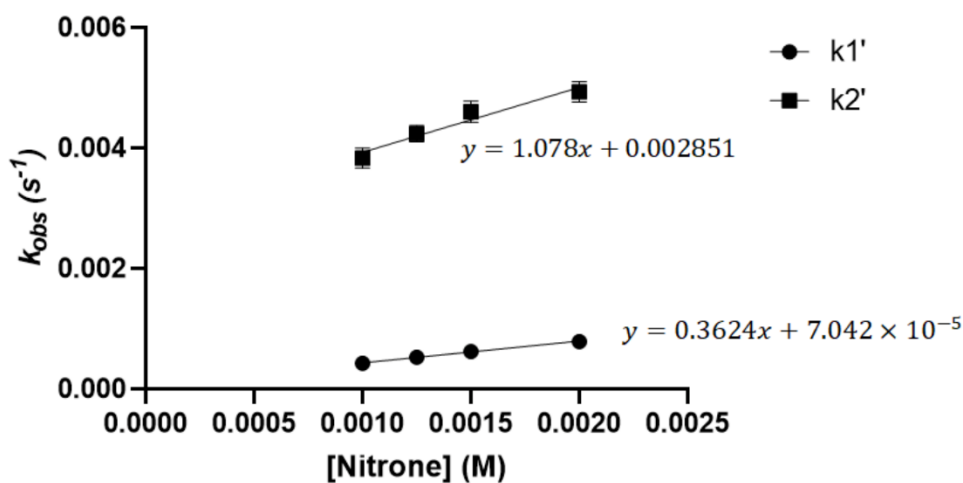
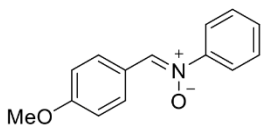


Figure S8. Pseudo first-order rate constants of the first (k_1) and second (k_2) cycloadditions of **1a** C-(phenyl)-*N*-phenylnitronone with tetramethoxy-diyne plotted against the various concentrations of nitronone (M). $R^2(k_1') = 0.99$, $R^2(k_2') = 0.87$.

Table S10. Observed rate constants for the sequential-pseudo first-order kinetic studies of cycloaddition reactions of nitrone **1b** C-(4-methoxyphenyl)-*N*-phenylnitron with tetramethoxy-diyne, correlated to various concentrations of nitron in methanol.

Nitron	Concentration of Nitron (10 ⁻³ M)	k_1' (10 ⁻⁴ s ⁻¹)	k_2' (10 ⁻² s ⁻¹)
	1.00	1.43 ± 0.01	1.75 ± 0.01
	1.25	1.78 ± 0.06	1.76 ± 0.01
	1.50	2.16 ± 0.07	1.77 ± 0.01
	2.00	2.79 ± 0.31	1.79 ± 0.01

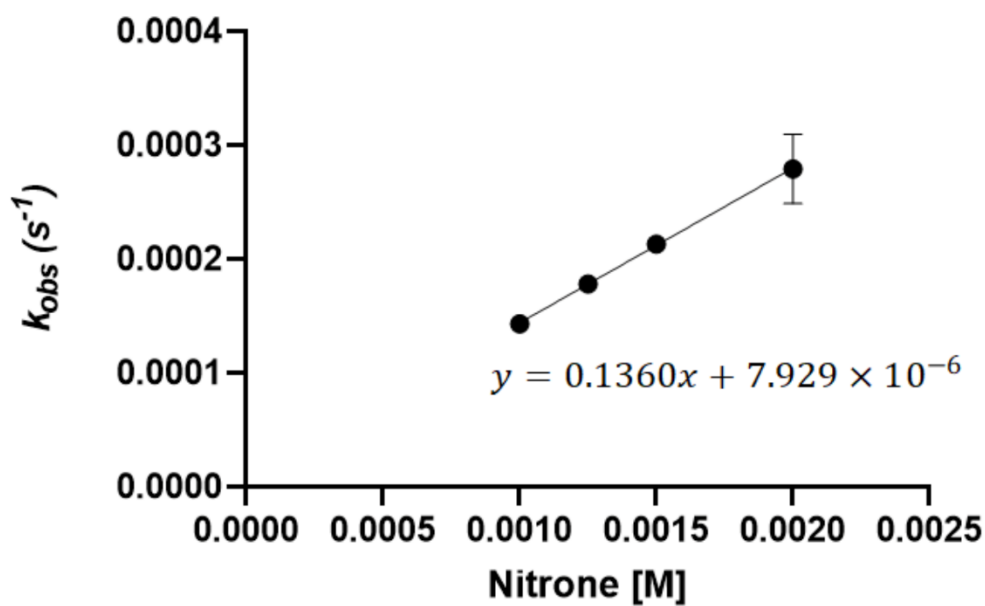


Figure S9. Pseudo first-order rate constants of the first (k_1) cycloaddition of **1b** C-(4-methoxyphenyl)-*N*-phenylnitron with tetramethoxy-diyne plotted against the various concentrations of nitron (M). $R^2 (k_1') = 0.94$.

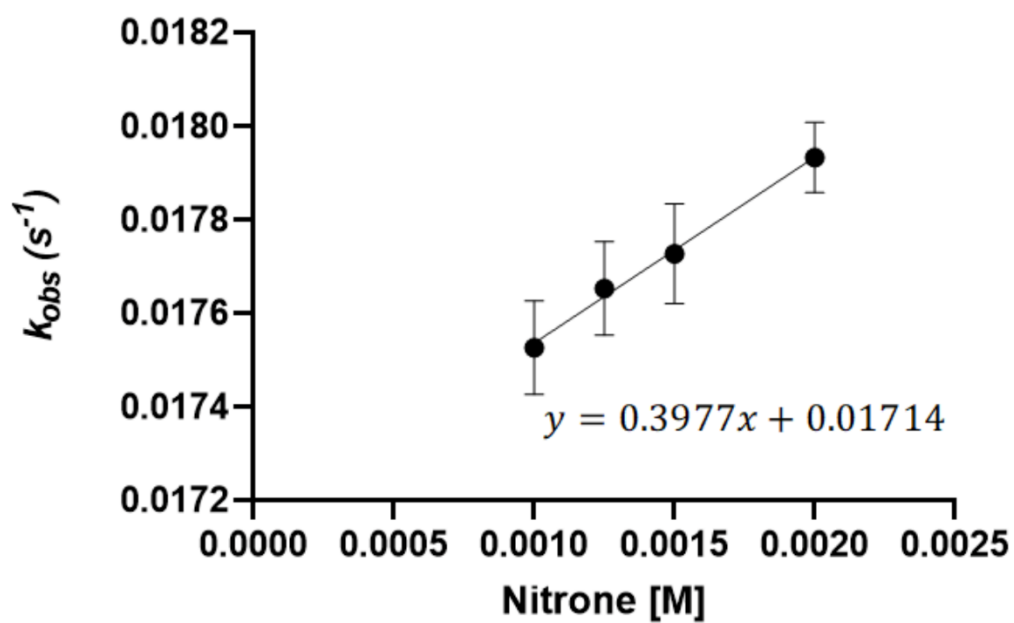
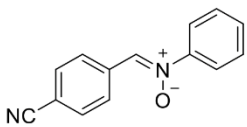


Figure S10. Pseudo first-order rate constants of the second (k_2) cycloaddition of **1b** C-(4-methoxyphenyl)-*N*-phenylnitron with tetramethoxy-diyne plotted against the various concentrations of nitron (M). $R^2 (k_1') = 0.77$.

Table S10. Observed rate constants for the sequential-pseudo first-order kinetic studies of cycloaddition reactions of nitrone **1e** C-(4-cyanophenyl)-*N*-phenylnitron with tetramethoxy-diyne, correlated to various concentrations of nitron in methanol.

Nitron	Concentration of Nitron (10 ⁻³ M)	k_1' (10 ⁻³ s ⁻¹)	k_2' (10 ⁻³ s ⁻¹)
	1.00	3.03 ± 0.06	3.12 ± 0.03
	1.25	3.21 ± 0.08	3.78 ± 0.16
	1.50	3.32 ± 0.02	4.47 ± 0.04
	2.00	3.55 ± 0.10	5.66 ± 0.39

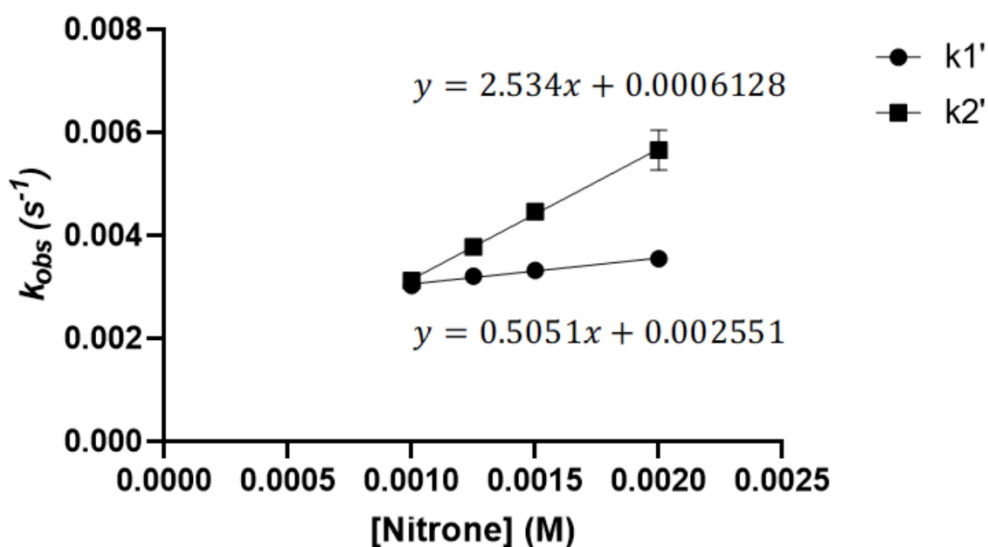


Figure S11. Pseudo first-order rate constants of the first (k_1) and second (k_2) cycloadditions of **1e** C-(4-cyanophenyl)-*N*-phenylnitron with tetramethoxy-diyne plotted against the various concentrations of nitron (M). $R^2(k_1') = 0.90$, $R^2(k_2') = 0.97$.

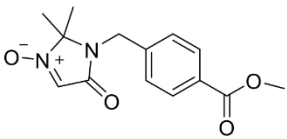
Table S10. Hammett plot data for the first addition (k_1) of the reaction of acyclic nitrones with diyne

Nitrone	Sub. Constant σ_p	k_1 ($M^{-1}s^{-1}$)	$k_1(1x)/$ $k_1(1a)$	$\log(k_1(1x)/$ $k_1(1a))$
$R_2=p\text{-OMeC}_6\text{H}_4$	-0.27	0.09	0.55	-0.26
$R_2= C_6H_5$	0	0.17	1.00	0.00
$R_2= p\text{-FC}_6\text{H}_4$	0.06	0.19	1.13	0.05
$R_2= p\text{-BrC}_6\text{H}_4$	0.23	0.17	0.98	-0.01
$R_2= p\text{-CNC}_6\text{H}_4$	0.66	0.19	1.14	0.06

Table S11. Hammett plot data for the second addition (k_2) of the reaction of acyclic nitrones with diyne.

Nitrone	Sub. Constant σ_p	k_2 ($M^{-1}s^{-1}$)	$k_2(1x)/$ $k_2(1a)$	$\log(k_2(1x)/$ $k_2(1a))$
$R_2=p\text{-OMeC}_6\text{H}_4$	-0.27	0.07	0.16	-0.81
$R_2= C_6H_5$	0	0.41	1	0
$R_2= p\text{-FC}_6\text{H}_4$	0.06	0.47	1.12	0.05
$R_2= p\text{-BrC}_6\text{H}_4$	0.23	0.53	1.28	0.11
$R_2= p\text{-CNC}_6\text{H}_4$	0.66	7.16	17.30	1.24

Table S12. Observed rate constants for the sequential-pseudo first-order kinetic studies of cycloaddition reactions of nitrone **1g** with diyne, correlated to various concentrations of nitrone in methanol.

Nitrone	Concentration of Nitrone (10 ⁻³ M)	k_1' (10 ⁻⁴ s ⁻¹)	k_2' (10 ⁻² s ⁻¹)
	0.50	1.26 ±	2.66 ±
		0.03	1.01
	1.00	2.45 ±	4.19 ±
		0.02	1.23
	1.50	3.68 ±	5.66 ±
		0.05	0.64
	2.00	5.42 ±	7.69 ±
		0.22	0.49

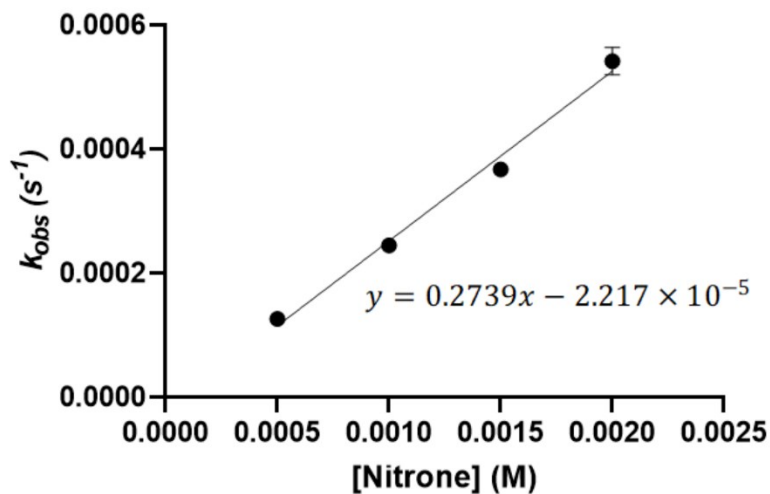


Figure S12. Observed rate constant (k_1) for the pseudo first-order kinetic studies of cycloaddition reaction of DMImO (**1g**) with diyne, correlated to various concentrations of nitron in methanol. $R^2 (k_1') = 0.99$.

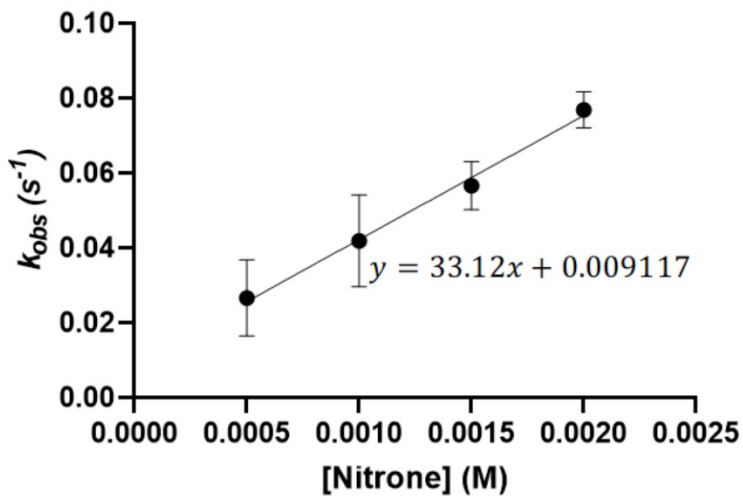


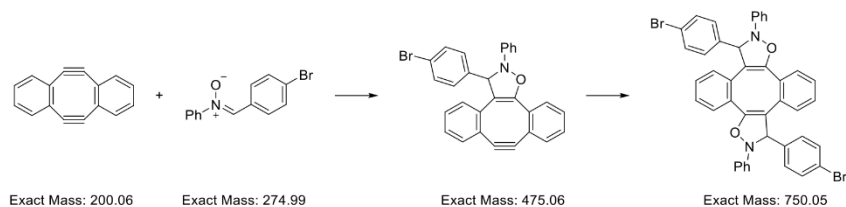
Figure S13. Observed rate constant (k_2) for the pseudo first-order kinetic studies of cycloaddition reaction of DMImO (**1g**) with diyne, correlated to various concentrations of nitron in methanol. $R^2 (k_1') = 0.86$.

Mass spectroscopy studies

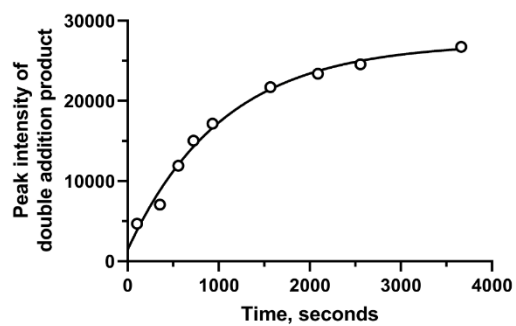
General procedure for the double cycloaddition mass spectroscopy studies.

Both nitron and diyne were pre-dissolved in 5.0 mL of acetonitrile ($\sim 200\ \mu\text{M}$) and mixed at equimolar concentrations of $100\ \mu\text{M}$ at room temperature in a vial, then the reaction was taken up with a 5 mL syringe and continuously injected via syringe pump directly into the Synapt Mass Spectrometer at a rate of $50\ \mu\text{L}/\text{min}$ for about 60-70 minutes. A summary of 50 scans of peak intensity were taken of the masses of both the product and the nitron at time points over the course of the reaction and plotted as a function of time (s). Data is of one trial.

Data analysis of mass spectroscopy studies



a)



b)

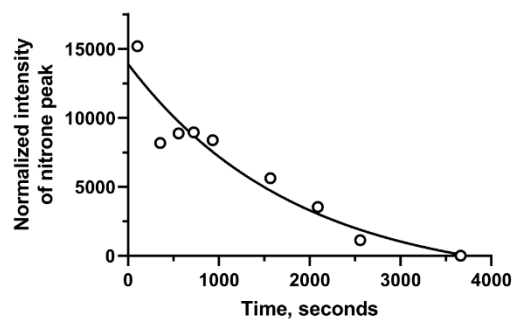
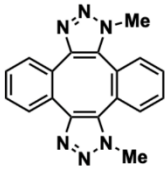


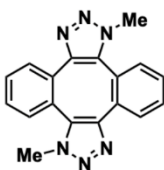
Figure S14. Graph of the peak intensities (50 scans) representing a) product (M+H), = 750, 751 (M+Na)⁺ = 775, 777 and b) nitron peak (M+H)⁺ = 276, 278, (M+Na)⁺ = 298, 300 over the course of the reaction in (s).

Computational Analysis

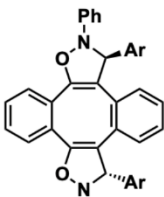
Table S13. Distortion-Interaction analysis of cycloaddition reactions between azide and nitrono dipoles



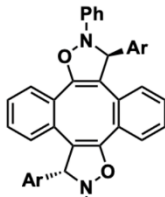
cis



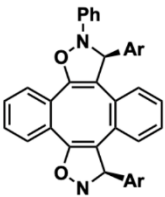
trans



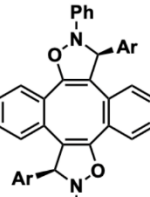
A



B



C



D

Energy Profile of the Cycloaddition (G)												D/I analysis (E)															
dipoles		ΔG						ΔΔG‡				ΔΔE‡				TS1								TS2			
		CP1	TS1	INT1	INT1'	TS2	PD	TS1	TS2	TS1	TS2	dis_nit	dis_yne	dis_total	int	dis_nit	dis_yne	dis_total	int								
MeN3	cis	6.7	24.7	-60.0	-52.3	-39.0	-137.1	24.7	20.9	11.7	7.4	16.9	3.4	20.3	-8.6	13.4	1.9	15.3	-7.9								
	trans				-52.0	-39.3	-137.2		20.7		7.3					13.0	1.9	14.9	-7.6								
PhN3	cis	1.8	24.7	-56.6	-53.0	-37.4	-129.7	24.7	19.2	12.2	4.7	19.5	3.6	23.1	-10.9	14.8	2.2	17.0	-12.3								
	trans				-53.6	-36.5	-130.6		20.2		7.5					14.2	2.2	16.4	-8.9								
X = H	A				-21.8	-15.0	-77.3		13.2		-2.5					5.2	4.8	10.1	-12.6								
	B				-21.7	-15.3	-75.0		13.0		-3.1					4.9	4.6	9.6	-12.7								
	C	1.0	18.5	-28.3	-22.2	-11.7	-72.7	18.5	16.5	5.3	0.2	11.9	4.2	16.1	-10.8	7.7	5.4	13.1	-12.8								
	D				-23.3	-14.7	-76.9		13.5		-2.3					5.3	4.6	10.0	-12.3								
X = OMe	A				-22.8	-13.5	-74.2		13.9		-2.3					5.1	5.8	10.9	-13.1								
	B				-20.1	-14.7	-74.8		12.7		-3.8					4.8	5.0	9.8	-13.6								
	C	0.4	18.8	-27.4	-26.3	-10.7	-70.5	18.8	16.7	5.7	0.5	12.1	4.2	16.3	-10.6	7.8	5.3	13.1	-12.5								
	D				-21.7	-13.5	-74.9		13.9		-1.9					5.5	4.6	10.1	-12.0								
X = F	A				-28.2	-16.2	-77.3		11.7		-3.4					5.1	4.5	9.6	-13.0								
	B				-31.9	-15.6	-77.2		12.3		-2.6					5.3	4.5	9.8	-12.5								
	C	0.5	18.4	-27.9	-22.2	-11.9	-72.6	18.4	16.0	5.3	0.3	12.1	4.2	16.2	-10.9	7.9	5.4	13.2	-12.9								
	D				-32.2	-14.8	-76.9		13.1		-2.2					5.5	4.6	10.2	-12.4								
X = Br	A				-20.0	-12.8	-77.3		15.4		-1.2					5.4	5.7	11.2	-12.4								
	B				-19.5	-13.1	-72.8		15.1		-0.5					5.7	5.3	11.0	-11.5								
	C	-1.1	18.0	-28.2	-22.8	-12.6	-75.5	18.0	15.5	5.0	0.1	11.9	4.1	16.0	-11.0	7.8	5.3	13.1	-13.0								
	D				-32.4	-15.4	-77.2		12.8		-2.4					5.4	4.6	10.0	-12.4								
X = CN	A				-30.6	-17.2	-79.9		11.6		-5.3					6.6	3.2	9.8	-15.0								
	B				-31.4	-15.7	-78.7		13.2		-2.4					5.8	5.0	10.8	-13.2								
	C	-1.9	17.6	-28.9	-23.6	-13.3	-76.5	17.6	15.6	4.6	0.0	11.7	4.1	15.8	-11.2	7.7	5.5	13.3	-13.2								
	D				-33.7	-16.2	-78.1		12.7		-2.5					5.4	4.8	10.3	-12.8								

M06-2X/6-311++G** (SCRF = H2O, SMD) //M06-2X/6-31g* (gas), (kcal/mol)

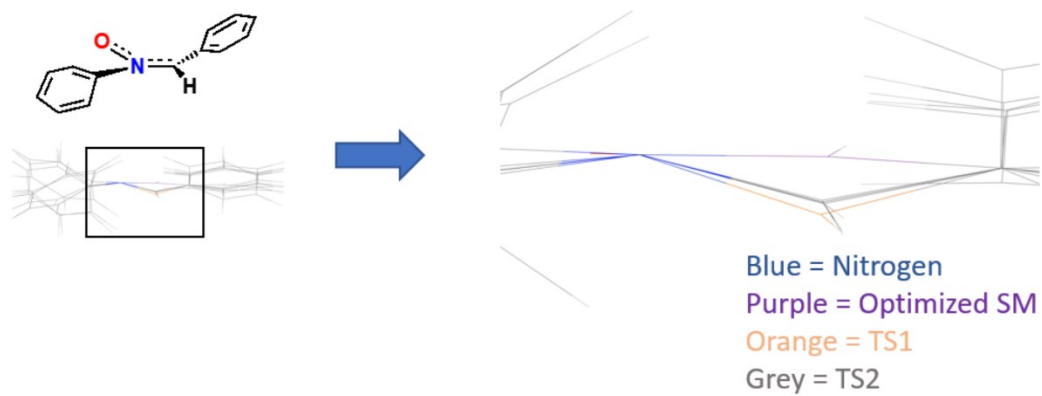
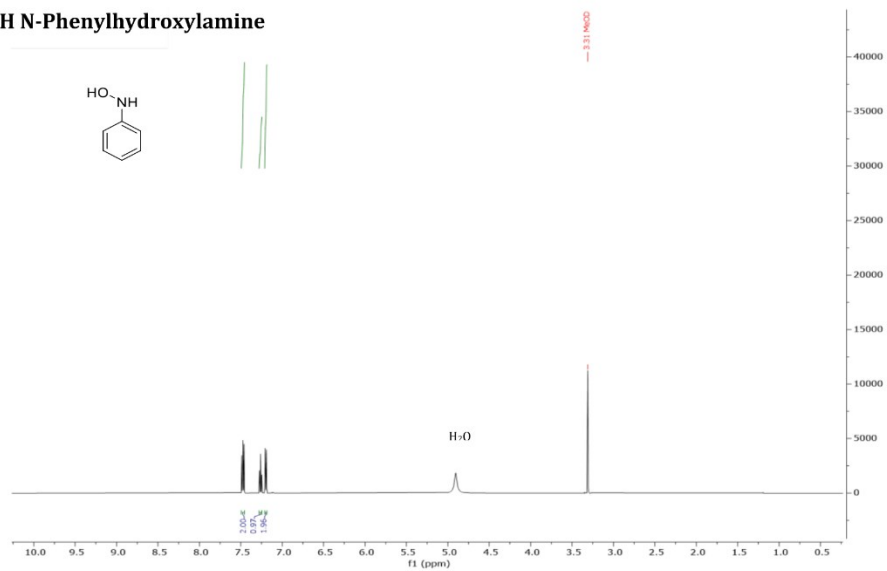


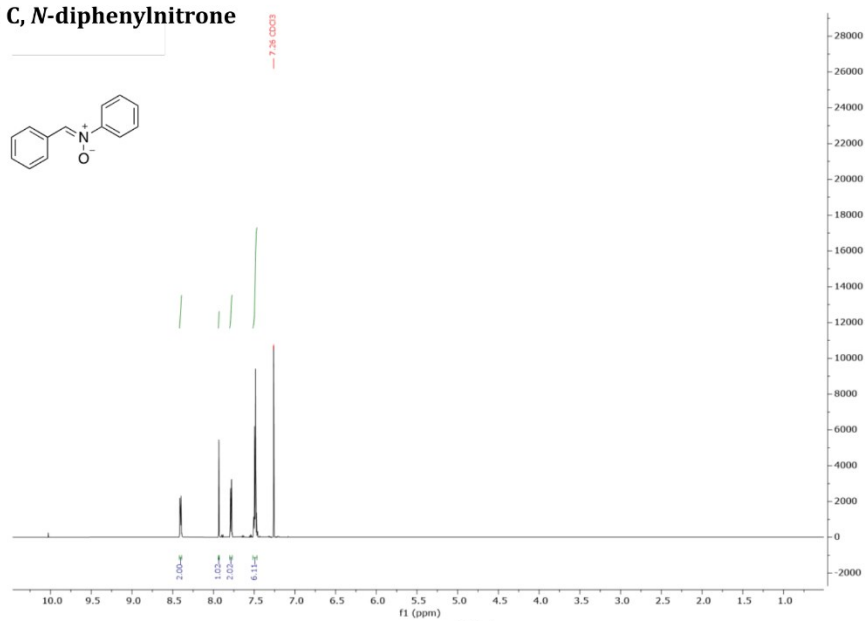
Figure S15. Calculated distortion of diphenyl nitrene during transition states, TS1 and TS2.

NMR Spectra

^1H N-Phenylhydroxylamine

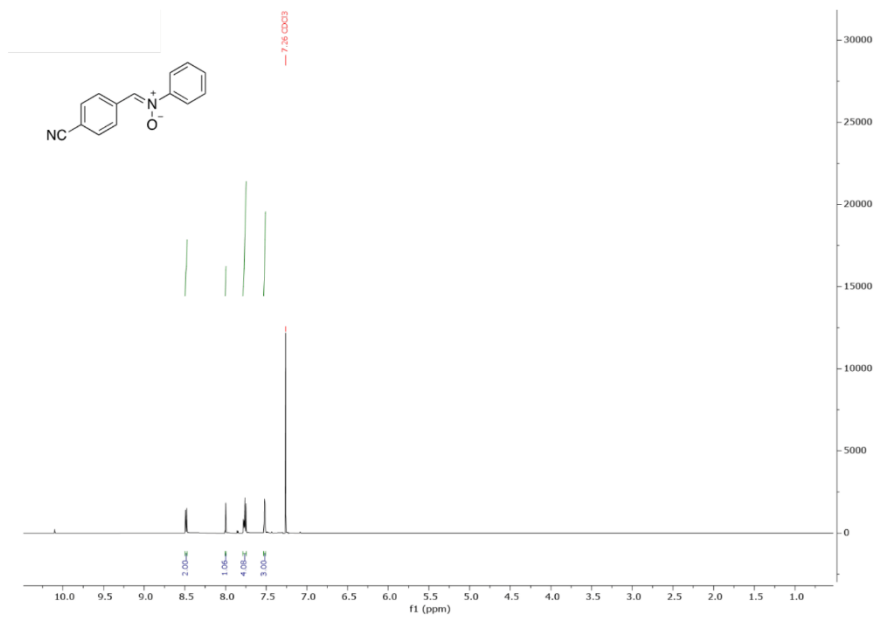


^1H C, N-diphenylnitron

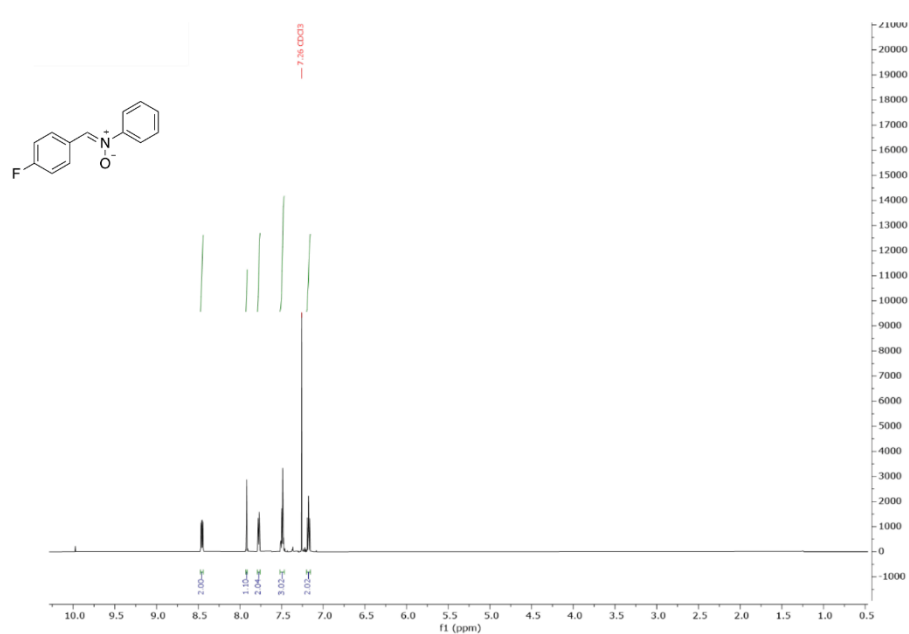


S34

¹H C-(4-cyanophenyl)-N-phenylnitrone

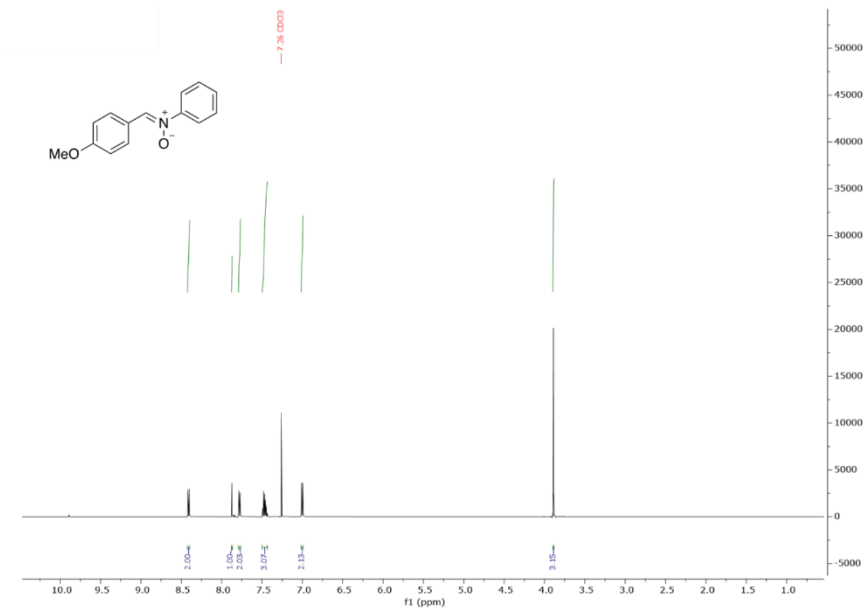


¹H C-(4-fluorophenyl)-N-phenylnitrone

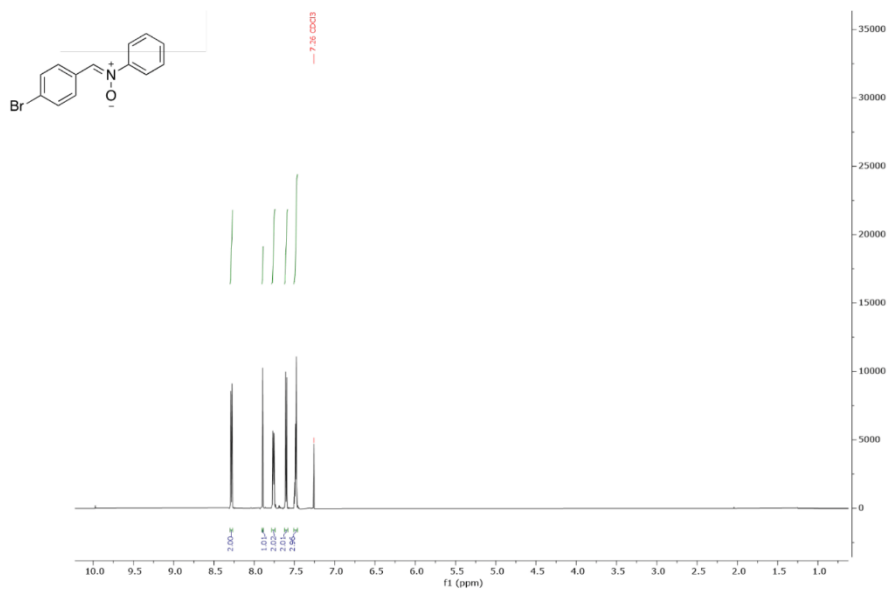


S35

¹H C-(4-methoxyphenyl)-N-phenylnitron

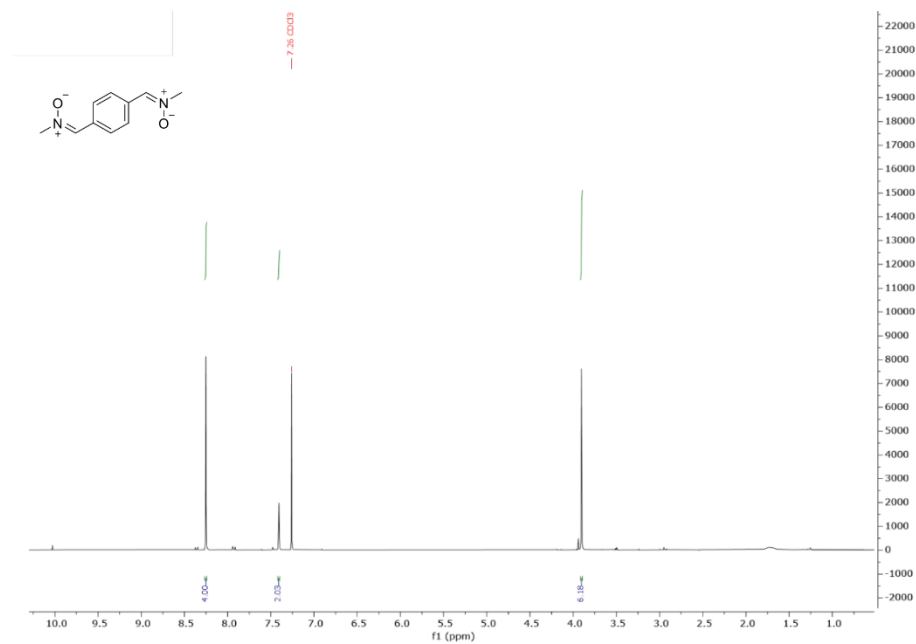


¹H C-(4-bromophenyl)-N-phenylnitron

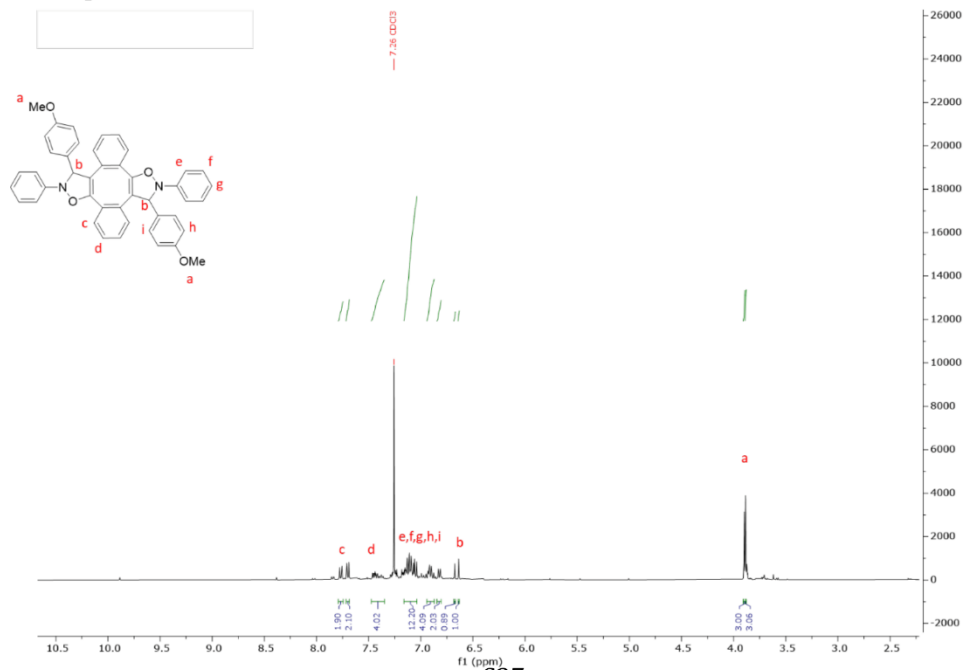


S36

¹H Compound 1h

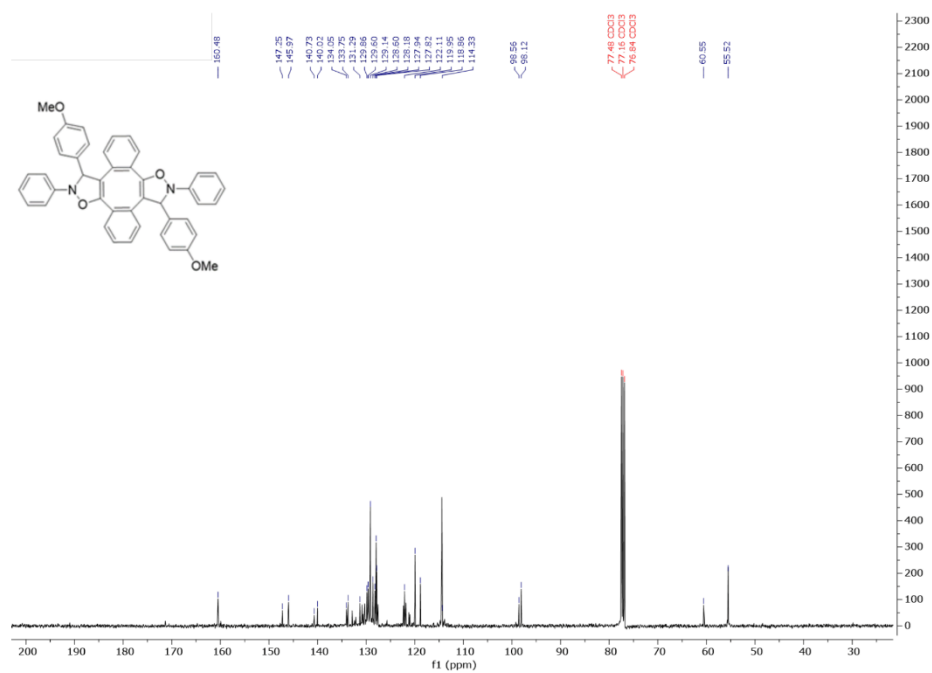


¹H Compound 2b

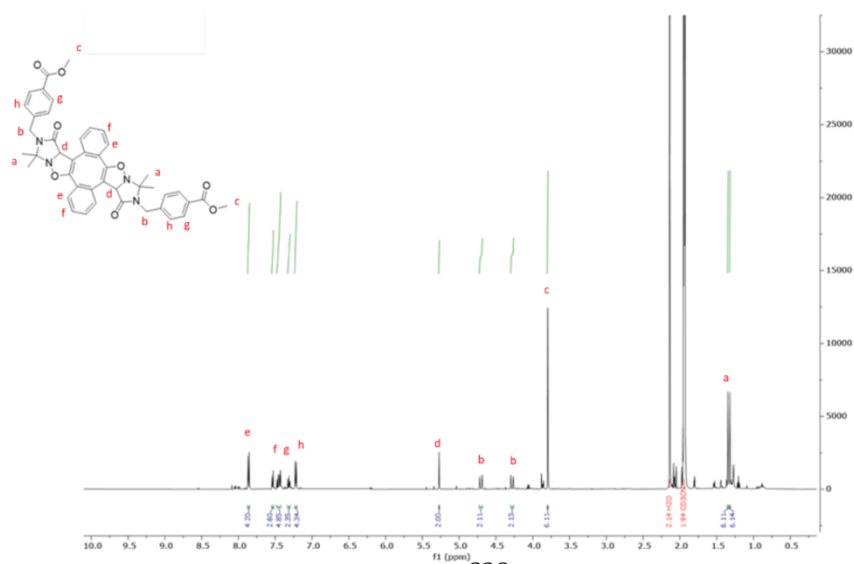


S37

¹³C Compound 2b

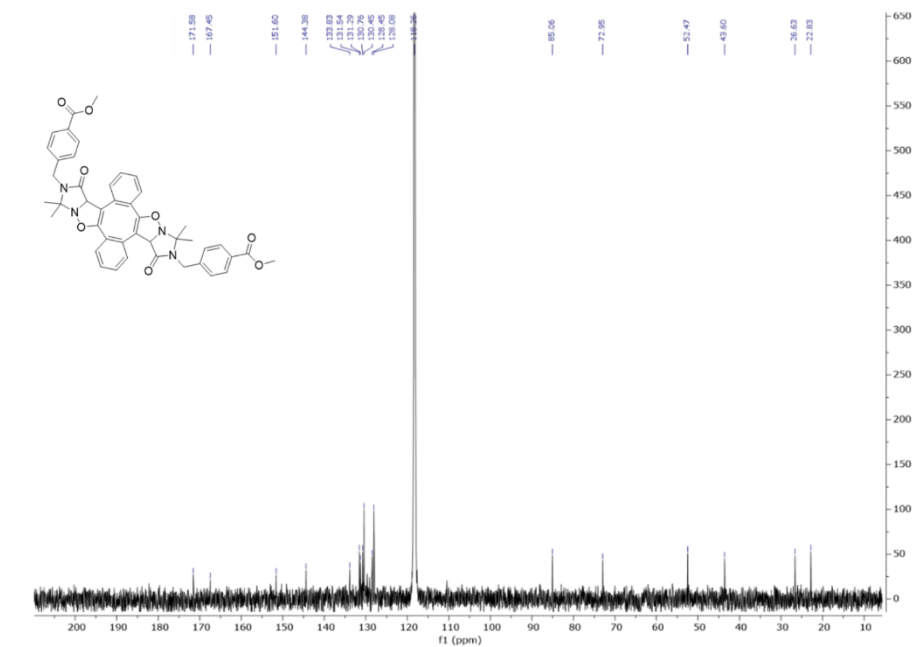


¹H Compound 2f

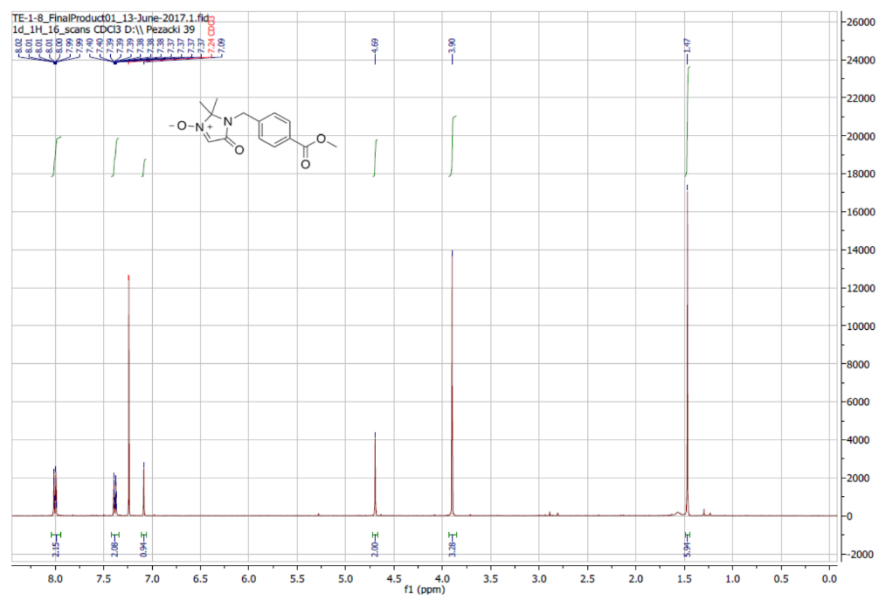


S38

¹³C Compound 2f

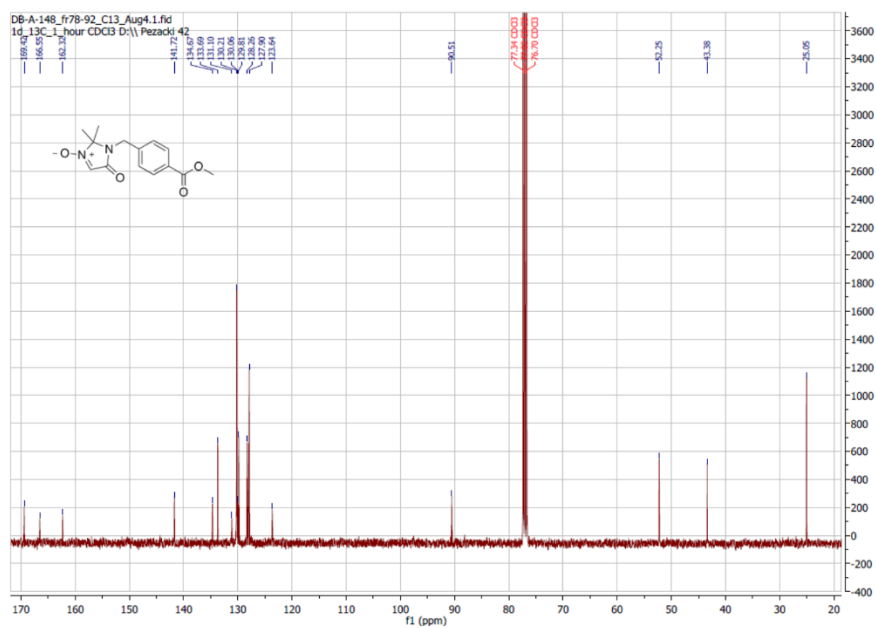


¹H DMImO

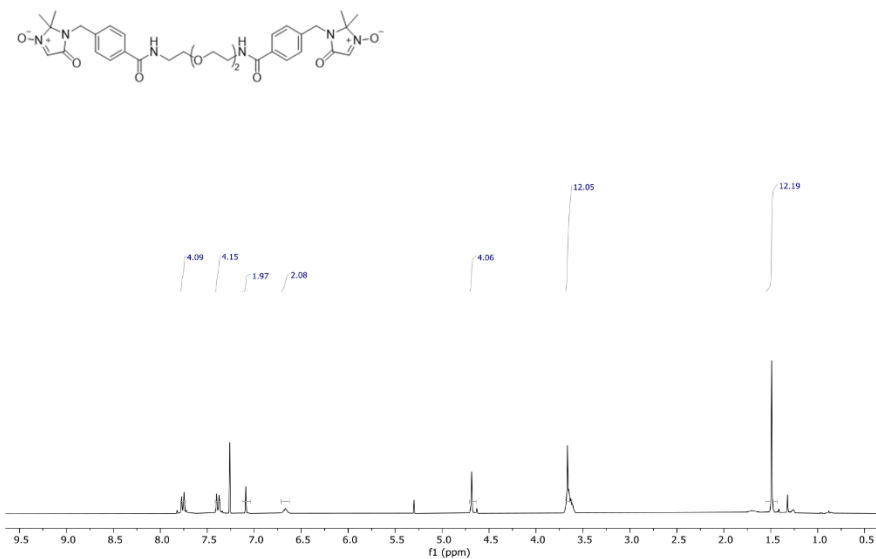


S39

¹³C DMImO

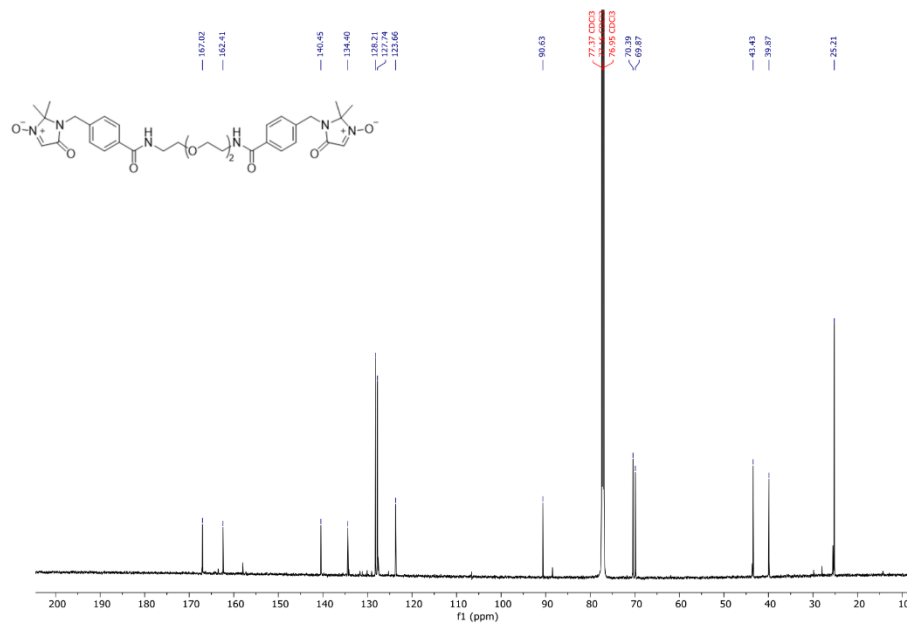


¹H Compound 1g

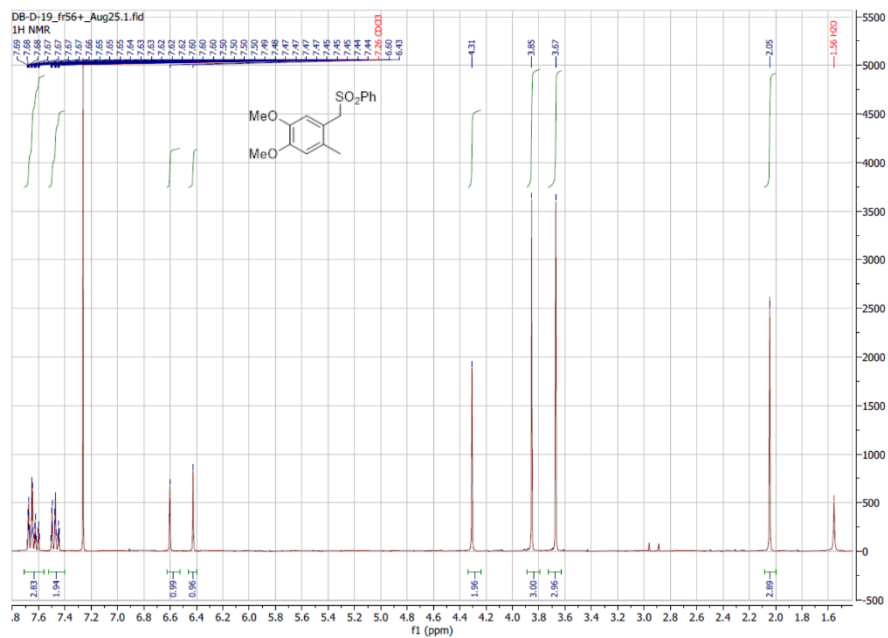


S40

¹³C Compound 1g

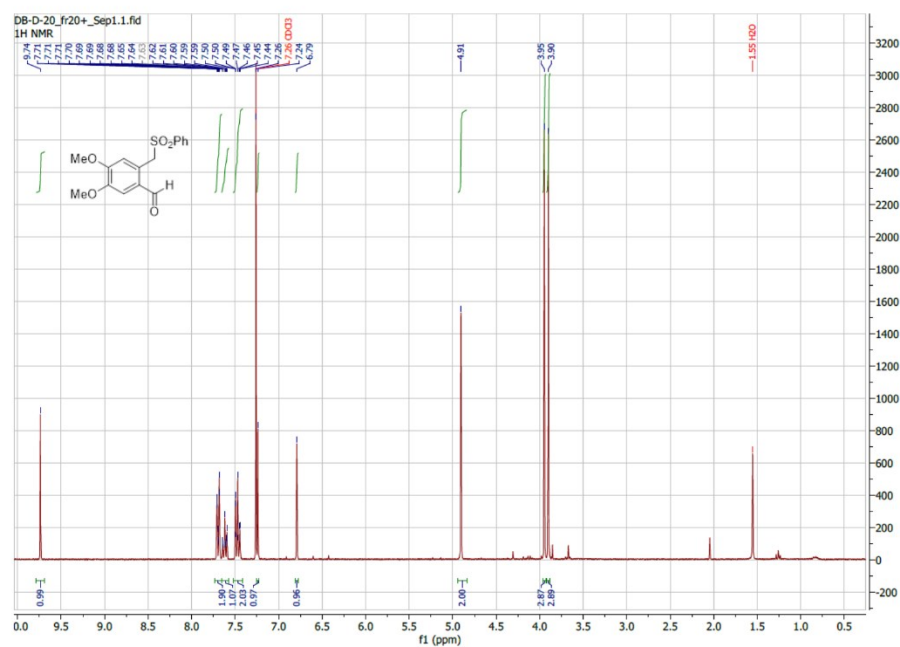


¹H α-benzenesulfonyl-4,5-dimethoxy-*o*-xylene.

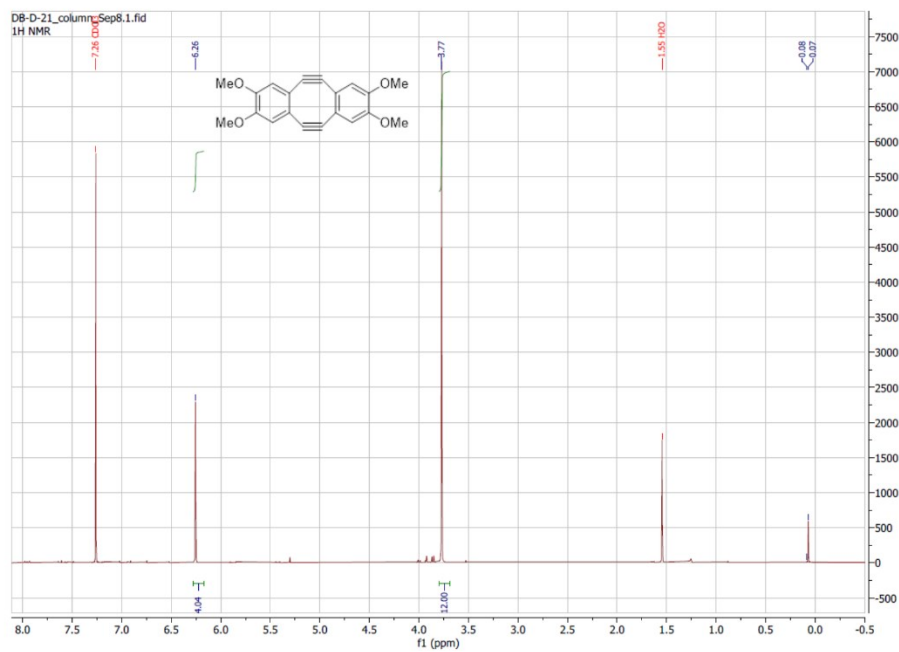


S41

¹H 4,5-Dimethoxy-2-((phenylsulfonyl)methyl)benzaldehyde.



¹H 2,3,8,9-tetramethoxy-5,6,11,12-tetrahydridibenzo[a,e]cyclooctyne.



Mass Spectroscopy Spectra

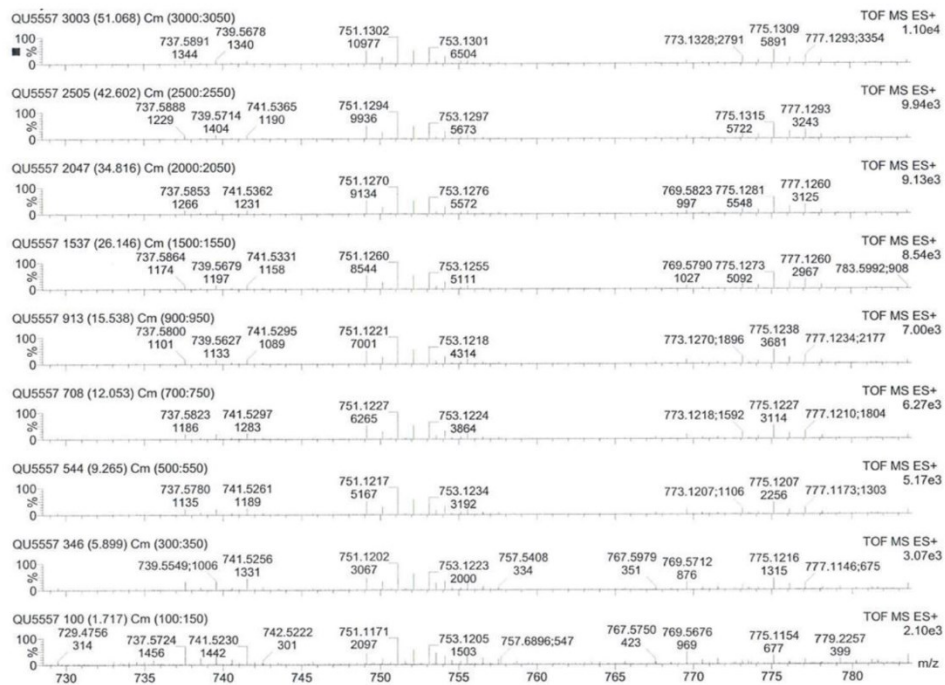


Figure S16. MS analysis of reaction of 100 μ M diyne 100 μ M C-(4-bromophenyl)-N-phenylnitrone over 60 minutes, using Waters Synapt High-Definition Mass Spectrophotometer Q-TOF (ESI+ mode, direct injection). Intensities of peak region where the di-product ions are found. **MS (ESI+)** calcd ($C_{42}H_{28}Br_2N_2O_2$): 750.05. Found ($M+H$)⁺: 751.13, 753.13 and ($M+Na$)⁺: 775.12, 777.13

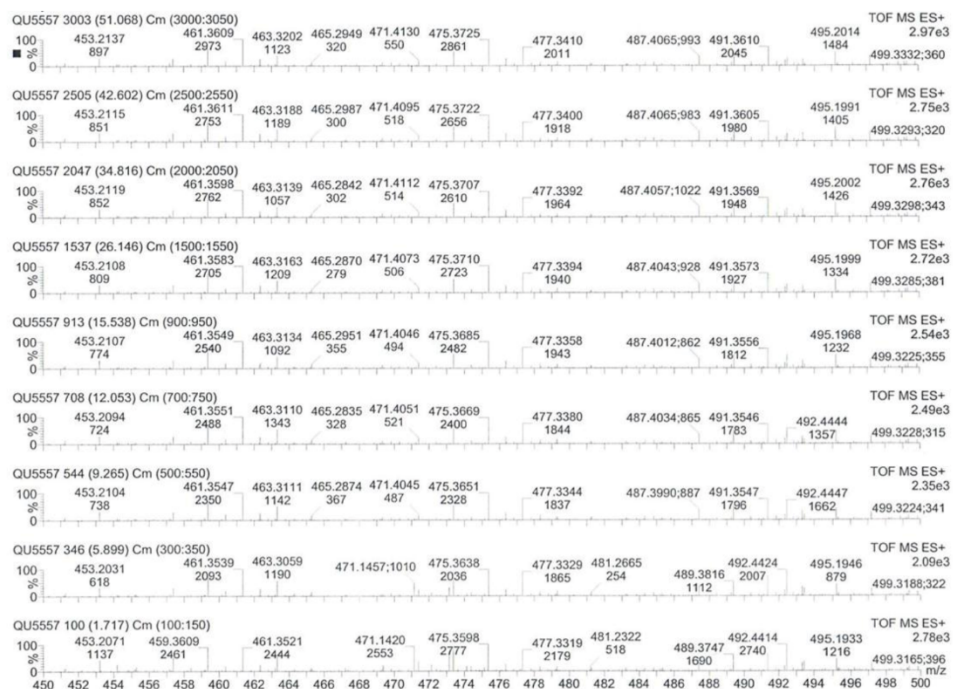


Figure S17. MS analysis of reaction of 100 μM diyne 100 μM C-(4-bromophenyl)-N-phenylnitrone over 60 minutes, using Waters Synapt High-Definition Mass Spectrophotometer Q-TOF (ESI+ mode, direct injection). Intensities of peak region where the mono-intermediate ions would be found. **MS (ESI+)** calcd (C₃₀H₂₁BrNO): 475.06

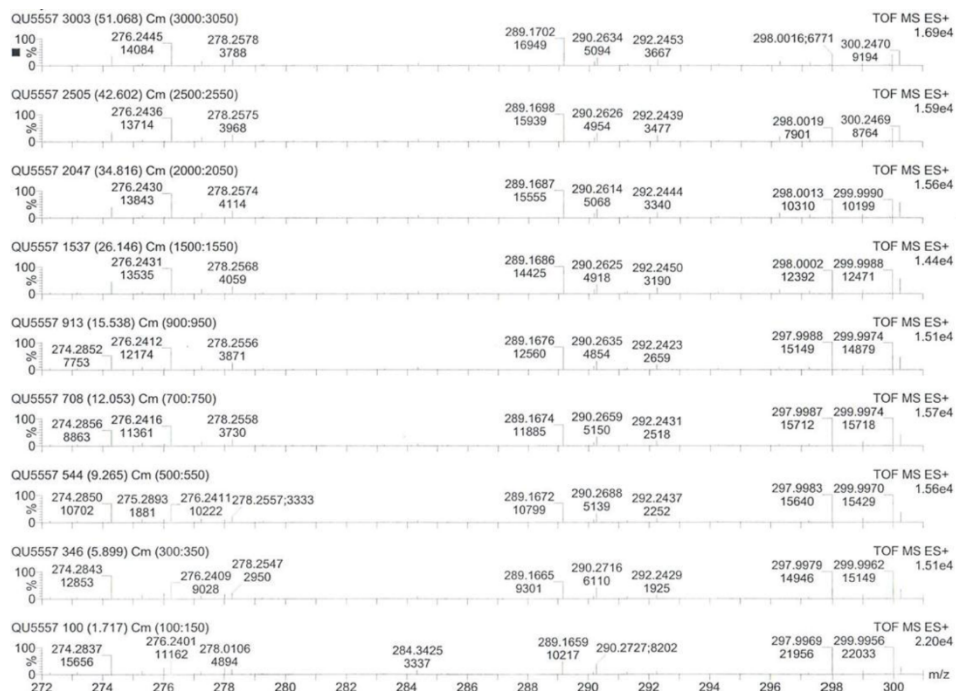


Figure S18. MS analysis of reaction of 100μM diyne 100 μM C-(4-bromophenyl)-N-phenylnitrone over 60 minutes, using Waters Synapt High Definition Mass Spectrophotometer Q-TOF (ESI+ mode, direct injection). Intensities of peak region where the C-(*p*-bromophenyl)-N-phenylnitrone ions are found. **MS (ESI+)** calcd (C₁₃H₁₀BrNO): 274.99. Found (M+H)⁺: 276.24, 278.25 and (M+Na)⁺: 298.00, 300.00

Metabolic Labelling

General metabolic labeling of bacterial cells via nitronene/azide diyne cycloadditions

Listeria innocua were cultured in BHI medium until mid-log phase growth, and then treated with 1.2 μ L of MeOH control, or 10 mM of D-alanine-CMPO (nitronene) UAA for 1h at 30 °C. Cells were then washed with PBS and then treated with 6.25 μ L of DMSO control, or 100 μ M cyclobenzooctadiyne for 30 minutes at 37 °C. After centrifugation and washing with PBS, cells were finally treated with 1.7 μ L DMSO control or 25 μ M N₃-AlexaFluor488 for 20 minutes. Cells were then imaged by fluorescence microscopy.

Fluorescence microscopy

All microscopy images were acquired using a Nikon Ni-U Ratiometric Fluorescence Microscope with Dual Excitation Sources; equipped with a 60x objective lens and a 2x relay lens; above background fluorescence (defined as fluorescence of the no N₃-AlexaFluor488 control sample) is shown. Quantification/densitometry of fluorescence was accomplished using ImageJ software. Statistical analysis was accomplished with GraphPad Prism, applying a Welch's t-test, with samples treated with either diyne showing statistically significant differences in fluorescence intensities comparing the "No UAA" and "Treated" samples ($p < 0.0001$).

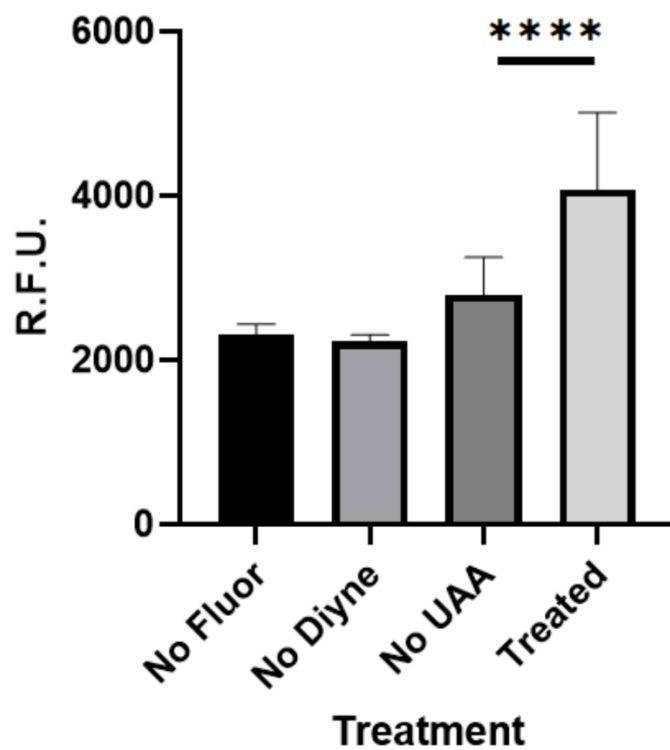


Figure S19. Quantification of fluorescence for *L. innocua* samples treated with OMe-Diyne.

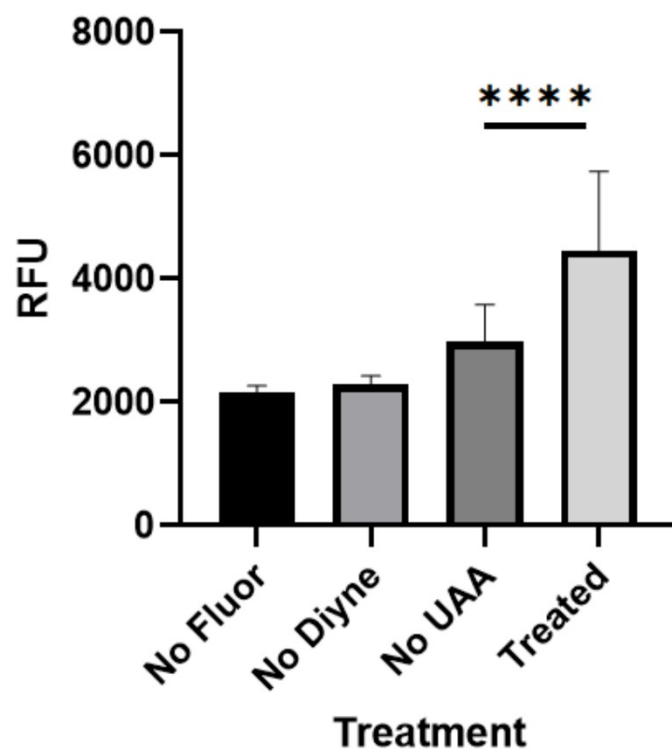


Figure S20. Quantification of fluorescence for *L. innocua* samples treated with unsubstituted Diyne

Computational Methods

All calculations were performed with Gaussian 16 program. Structure optimizations were carried out at 298.15 K, using the M06-2X functional with an ultrafine grid and the 6-31G(d) basis set in gas phase. The energy was corrected by the single point calculation with M06-2X/6-311++G(d, p) in water using the solvation model based on density (SMD). Harmonic vibrational frequencies were computed at the same level of theory of geometry optimization to confirm no imaginary vibration was observed for the optimized structure, and only one imaginary vibration was observed for the transition state. The intrinsic reaction coordinate (IRC) method was used to track minimum energy paths from transition structures to the corresponding local minima.

Cartesian Coordinates and Energies

diyne_opt.log	C	0.000000	1.312702	0.606801
Energy (E) = -614.122710892 Hartree	C	0.000000	1.312702	-0.606801
Energy (E) of 6-311++G** in H2O = -	C	0.000000	-0.694186	4.333267
614.278924083 Hartree	C	0.000000	-1.400026	3.126978
Enthalpy (H) = -613.927919 Hartree	C	0.000000	-0.716488	1.918593
Gibbs free energy (G) = -613.975717	C	0.000000	0.716488	1.918593
Hartree	C	0.000000	1.400026	3.126978
Charge = 0, Spin = 1	C	0.000000	0.694186	4.333267
C 0.000000 -1.312702 0.606801	H	0.000000	-1.238479	5.272044
C -0.000000 -1.312702 -0.606801	H	0.000000	-2.484918	3.123078

S49

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Annex IV - Canadian Journal of Chemistry, 2019, 91(1), 1-6. Supporting Information

Supplementary Material

Phenanthridine-based nitrones as substrates for strain-promoted alkyne-nitrone cycloadditions

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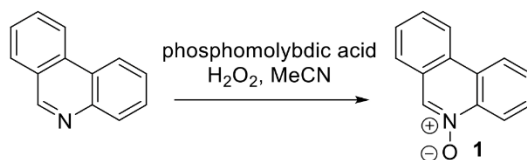
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Materials and Methods

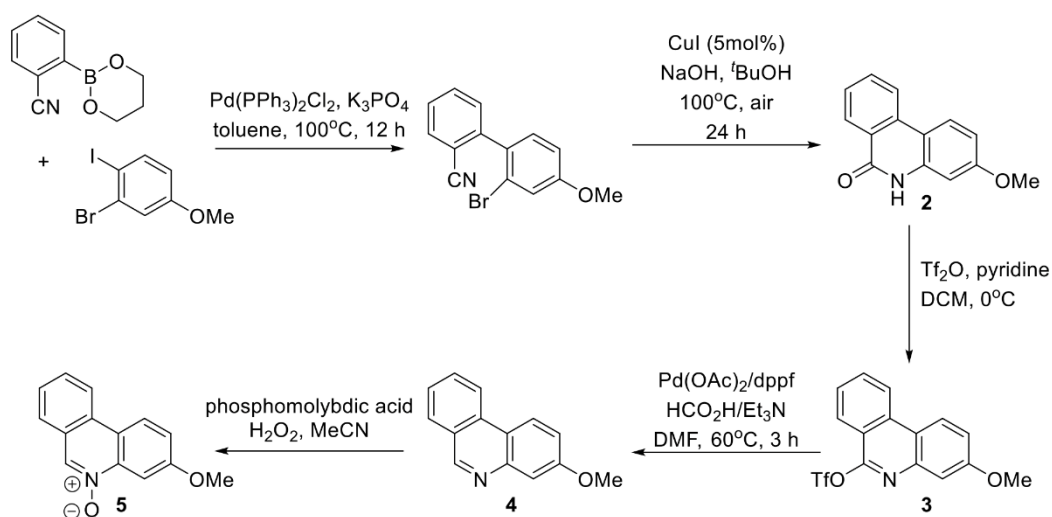
All reagents and solvents were purchased from Sigma-Aldrich, unless otherwise stated, and used without further purification. Deuterated solvents were purchased from Cambridge Isotope laboratories. Thin layer chromatography was performed on Analtech Uniplat® silica gel plates (60 Å F254, layer thickness 250 µm). Flash chromatography was performed using silica gel (60 Å, particle size 40–63 µm). LC-MS/MS spectra were obtained using Waters Alliance 2795 liquid chromatograph quipped with Waters 996 PDA diode array detector and connected to Micromass ZQ2000 mass spectrometer equipped with pneumatically assisted electrospray ionization source, operating in both positive and negative mode. Samples were run with gradient elution of acetonitrile/water/0.1% formic acid on the Waters SunFire C18 (2.1 x 100 mm, 3.5 µm) column with flow rate of 0.2 mL/min. Preparatory HPLC was performed on a Waters Delta Prep 4000 equipped with Waters 996 PDA diode array detector and column Waters SunFire C18 (19 x 100 mm, 5 µm) and Waters fraction collector. Column effluents were monitored at wavelength specified in respective procedures. Mobile phase was acetonitrile/water/0.1% formic acid, degassed with helium sparge at flow rate of 17 mL/min. Aliquots were injected onto column via a Pheodyne 7125 injector fitted with a 5 mL loop. All ¹H and ¹³C NMR spectra were obtained on a Bruker-DRX-400 spectrometer using a frequency of 400 MHz for ¹H and 100 MHz for ¹³C and processed using Bruker TOPSPIN 2.1 software. The following abbreviations were used to designate chemical shift multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet or unresolved, br = broad signal and *J* = coupling constants in Hz. Chemical shifts are reported in ppm as δ referenced to residual solvent and coupling constants (*J*) are reported in Hz.

Synthesis of Phenanthridine-based nitrones

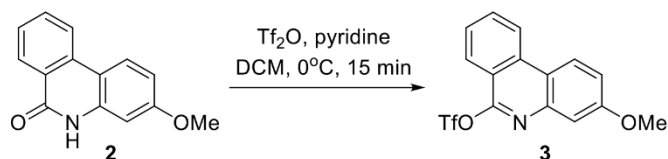


Phenanthridine 5-oxide (1) was synthesized following procedure previously reported in the literature.¹ Accordingly, phenanthridine (50 mg, 0.28 mmol, 1 eq) was dissolved in acetonitrile (200 μ L) and hydrogen peroxide (83 μ L, 0.84 mmol, 3 eq, 30% in water) was added dropwise, followed by phosphomolybdic acid (25 μ L of freshly prepared 0.2 M solution in EtOH, 0.005 mmol, 1.8 mol%), also dropwise. The reaction was allowed to proceed at 50°C overnight. The mixture was cooled to room temperature, diluted with saturated ammonium chloride and extracted with dichloromethane twice. The organic extracts were combined, dried over Na₂SO₄ and concentrated under reduced pressure. The crude mixture was purified by column chromatography (0-20% MeOH gradient in 3:7/Hx:EtOAc) to yield **1** as pale beige powder (40 mg, 0.21 mmol, 73%, R_f = 0.16 in 100% EtOAc). **HRMS (ESI+)** calcd for **1** (C₁₃H₉NO): 195.0684, [M+H]⁺ found 196.0757; **¹H-NMR** of **1** (400 MHz, CDCl₃): δ 8.88 (ddd, J =1.5, 7.8 Hz, 1H), 8.82 (s, 1 H), 8.49 (d, J =7.6 Hz, 1 H), 8.41 (s, 1 H), 7.73 (m, 4 H), 7.60 (t, J =7.4 Hz, 1 H); **¹³C-NMR** of **1** (100 MHz, CDCl₃): δ 139.3, 134.5, 129.5, 129.4, 129.3, 128.8, 126.6, 126.5, 126.0, 122.7, 122.0, 120.6.

3-methoxyphenanthridin-6(5H)-one (2) was synthesized following the procedure reported in the literature (Scheme S1).²

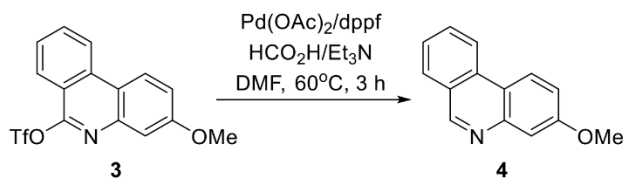


Scheme S1. Synthesis of 3-methoxyphenanthridine 5-oxide.



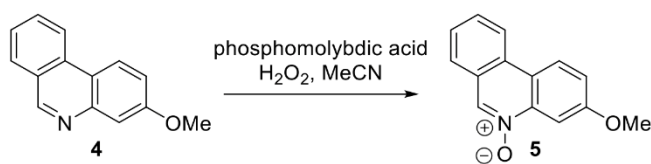
3-Methoxy-, 6-(trifluoromethyl)sulfonyl-, phenanthridin-6(5H)-one (3). **2** (1.01 g, 4.49 mmol, 1 eq) was dissolved in dichloromethane and brought to 0°C in an ice bath. Pyridine (0.56 mL, 6.96 mmol, 1.5 eq) and Tf₂O (1.16 mL, 6.90 mmol, 1.5 eq) were slowly injected into the solution of **2** keeping the temperature ~0°C. The reaction mixture was stirred at 0°C for 15 minutes. The reaction

was diluted with dichloromethane and extracted with saturated NaHCO_3 . The organic layer was dried with Na_2SO_4 , filtered and concentrated under reduced pressure. The crude mixture was purified by column chromatography (1:1/Hx:EtOAc) to yield **3** as pale yellow solid (1.38 g, 3.87 mmol, 86%, $R_f = 0.86$ in 1:1/Hx:EtOAc). **MS (ESI+)** calcd for **3** ($\text{C}_{15}\text{H}_{10}\text{F}_3\text{NO}_4\text{S}$): 357.03 $[\text{M}+\text{H}]^+$, found 358.1; **$^1\text{H-NMR}$** of **3** (400 MHz, CDCl_3): δ 8.50 (d, $J=8.4$ Hz, 1 H), 8.40 (d, $J=9.1$ Hz, 1 H), 8.17 (dd, $J=0.5, 8.3$ Hz, 1 H), 7.91 (ddd, $J=1.3, 7.1, 8.4$ Hz, 1 H), 7.69 (ddd, $J=0.9, 7.2, 8.1$ Hz, 1 H), 7.45 (d, $J=2.7$ Hz, 1 H), 7.33 (dd, $J=2.7, 9.1$ Hz, 1 H), 3.98 (s, 3H); **$^{13}\text{C-NMR}$** of **3** (100 MHz, CDCl_3): δ 160.9, 152.1, 142.9, 136.5, 132.5, 127.4, 124.1, 123.4, 122.0, 120.3, 119.0, 118.4, 117.3, 117.1, 113.9, 109.5, 55.7.



3-Methoxyphenanthridine (4). The reaction was conducted under Argon atmosphere. The reaction flask was charged with **3** (1.38 g, 3.87 mmol, 1 eq), Pd(OAc)_2 (44.8 mg, 0.20 mmol, 5.4 mol%) and dppf (277.2 mg, 0.50 mmol, 13 mol%), followed by 39 mL DMF. The mixture was stirred at room temperature under Argon for ~20 minutes. Formic acid (0.53 mL, 14.1 mmol, 3.6 eq) and Et_3N (1.63 mL, 11.6 mmol, 3 eq) were injected into the reaction mixture. The reaction was then stirred at 60°C for 3 hours. The crude reaction mixture was cooled to room temperature, diluted with saturated NaHCO_3 and extracted with dichloromethane. The organic layer was dried with Na_2SO_4 , filtered and concentrated under reduced pressure. The crude solid was pre-adsorbed to silica gel and purified by column chromatography (1:1/Hx:EtOAc) to yield **4** as white solid (780

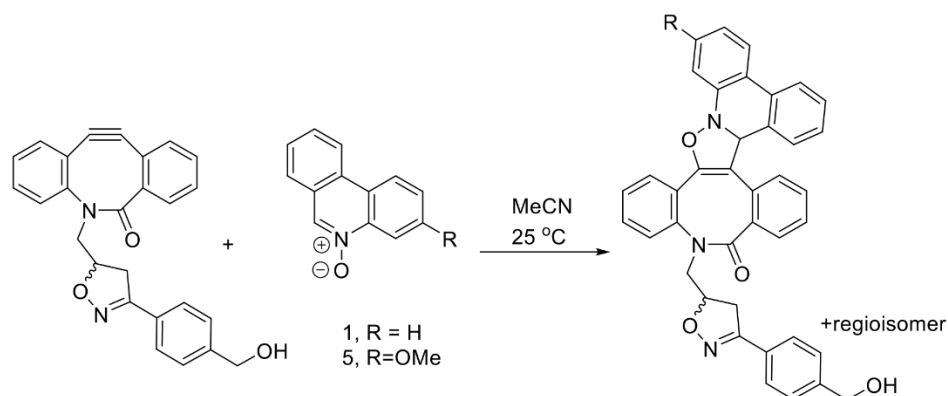
mg, 3.73 mmol, 96%, $R_f = 0.31$ in 1:1/Hx:EtOAc). **MS** (ESI+) calcd for **4** ($C_{14}H_{11}NO$): 209.08 $[M+H]^+$, found 210.2; **1H -NMR** of **4** (400 MHz, $CDCl_3$): δ 9.06 (s, 1 H), 8.16 (t, $J=9.0$ Hz, 2 H), 7.75 (d, $J=7.9$ Hz, 1 H), 7.55 (t, $J=7.6$ Hz, 1 H), 7.45 (s, 1 H), 7.39 (d, $J=7.3$ Hz, 1 H), 7.12 (t, $J=7.3$ Hz, 1 H), 3.83 (s, 3 H); **^{13}C -NMR** of **4** (100 MHz, $CDCl_3$): δ 159.9, 153.8, 145.9, 132.5, 130.8, 128.5, 126.2, 125.3, 123.2, 121.1, 117.9, 117.8, 109.8, 55.4.



3-Methoxyphenanthridine 5-oxide (5). **4** (103 mg, 0.49 mmol, 1 eq) was dissolved in acetonitrile (400 μL) and hydrogen peroxide (150 μL , 1.47 mmol, 3 eq, 30% in water) was added dropwise, followed by phosphomolybdic acid (50 μL of freshly prepared 0.2 M solution in EtOH, 0.01 mmol, 2 mol%), also dropwise. Upon heating, the starting material dissolved completely and the reaction was stirred at 65°C overnight. The mixture was cooled to room temperature, diluted with saturated ammonium chloride and extracted with dichloromethane twice. The organic extracts were combined, washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The mixture was purified by preparatory HPLC with MeCN/ H_2O /FA (0.1%) as eluent, using gradient of MeCN 30-33% over 6 minutes. Starting material **4** eluted at ~3-4 minutes, product **5** eluted at ~5-5.4 minutes. The identities were confirmed by MS and fractions were pooled accordingly; MeCN was removed under reduced pressure. The product was extracted from the aqueous solution with dichloromethane, the organic layer dried over Na_2SO_4 and concentrated under reduced pressure to yield **5** as pale yellow solid (29 mg, 0.129 mmol, 26%, $R_f = 0.47$ in 1:1/Hx:EtOAc).

HRMS (ESI+) calcd for **5** (C₁₄H₁₁NO₂): 225.0790, [M+H]⁺ found 226.0865; **¹H-NMR** of **5** (400 MHz, CDCl₃): δ 8.87 (s, 1 H), 8.41 (d, *J*=9.1 Hz, 1 H), 8.35 (d, *J*=8.3 Hz, 1 H), 8.30 (d, *J*=0.9 Hz, 1 H), 7.72 (d, *J*=8.0 Hz, 1 H), 7.67 (t, *J*=7.5 Hz, 1 H), 7.57 (t, *J*=7.5 Hz, 1 H), 7.38 (dd, *J*=1.4, 9.0 Hz, 1 H), 4.01 (s, 3 H); **¹³C-NMR** of **5** (100 MHz, CDCl₃): δ 161.1, 140.7, 135.3, 129.7, 127.7, 127.1, 126.2, 125.3, 124.2, 121.4, 120.4, 120.4, 100.7, 56.0.

Kinetic Studies of SPANC with Phenanthridine-based Nitrones



The rate measurements of cycloadditions of BARAC with various nitrones were performed using UV-visible spectroscopy in aqueous (0.1% water) acetonitrile at 25 ± 0.1 °C. Reactions were done under pseudo-first order conditions with nitron in 40-100 fold excess and were monitored by following the decay of the absorbance at 301 nm for **1**, 308 nm for **5**. At least five trials were done for each cycloaddition reaction, changing the concentration of the excess reagent by at least 10%.

General procedure for the cycloadditions kinetic studies

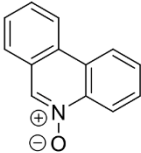
A calculated amount of stock solution of excess reagent (nitron) required to achieve the desired final concentration was diluted in a calculated amount of additional acetonitrile in a cuvette, and the solution was thermally equilibrated to 25 °C. A calculated amount of stock solution of the reacting partner required to achieve the desired final concentration was rapidly mixed with the thermally equilibrated solution and immediately inserted into the UV-vis spectrophotometer. Absorption was measured at preset time intervals of 3 seconds at the wavelength chosen. The time

taken for the physical manipulations was taken into account automatically. Measurements were stopped once no absorption decrease was observed for a substantial amount of time to give a statistically accurate final absorbance value. Due to complexity of NMR spectra of the mixture of regio- and diastereomeric products in each reaction, mass spectrometry was used to confirm the formation of products.

Data analysis

The data analysis was identical in all kinetic experiments. The final absorption (average of at least 15 final recorded values) was subtracted from each measured absorption value to obtain a relative absorption. Natural logarithms of relative absorption values were plotted against time (in seconds). Only the linear portion of the regression was used to fit a linear trend line, the negative slope of which represented the observed rate constant (k_{obs}) for the given experiment at the given concentration of the excess reagent (nitron). The observed rate constants were then plotted against the corresponding excess reagent (nitron) concentrations (Figures S1, S2). The slope of the linear trend line fitted to the data points represents the second-order rate constant (k_2) for each of the cycloadditions.

Table S1. Observed rate constants for the kinetic studies of cycloadditions of BARAC with **1** correlated to the various concentrations of **1** in aqueous (0.1% water) acetonitrile at $25 \pm 0.1^\circ\text{C}$.

Nitrone	Structure	Concentration of nitrone	k_{obs} (s^{-1})
		($\times 10^{-3}$ M)	
1		1.00	8.46×10^{-3}
		0.90	7.40×10^{-3}
		0.80	6.42×10^{-3}
		0.70	5.59×10^{-3}
		0.60	4.98×10^{-3}
		0.40	3.23×10^{-3}

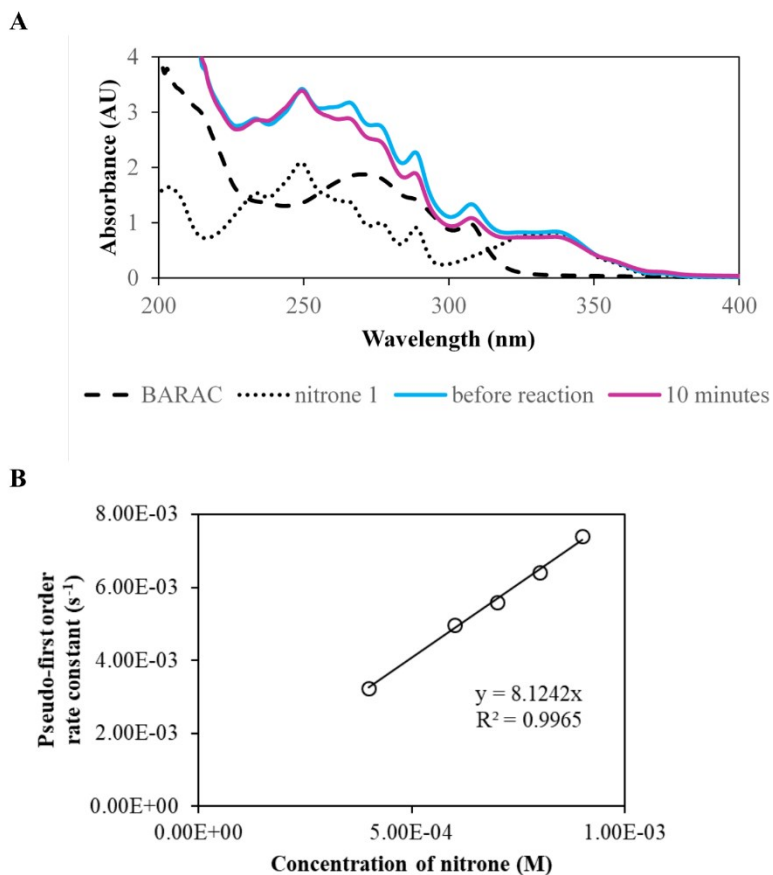
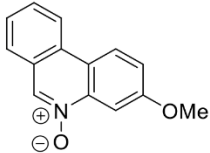


Figure S1. (A) Absorbance spectra of SPANC reactants: BARAC (*dashed line*), nitrone **1** (*dotted line*), and spectra of progressing SPANC reaction at the beginning of the reaction (*cyan*) and after 10 minutes (*magenta*). (B) Pseudo first-order rate constants of the cycloadditions of **1** with BARAC plotted against the various concentrations of the nitrone. All reactions were done in aqueous (0.1% water) acetonitrile at $25 \pm 0.1^\circ\text{C}$.

Table S2. Observed rate constants for the kinetic studies of cycloadditions of BARAC with **5** correlated to the various concentrations of **5** in aqueous (0.1% water) acetonitrile at $25 \pm 0.1^\circ\text{C}$.

Nitrone	Structure	Concentration of nitrone ($\times 10^{-3}$ M)	k_{obs} (s^{-1})
5		0.40	3.74×10^{-3}
		0.36	3.50×10^{-3}
		0.32	3.16×10^{-3}
		0.28	2.73×10^{-3}
		0.24	2.31×10^{-3}
		0.20	1.82×10^{-3}
		0.16	1.52×10^{-3}

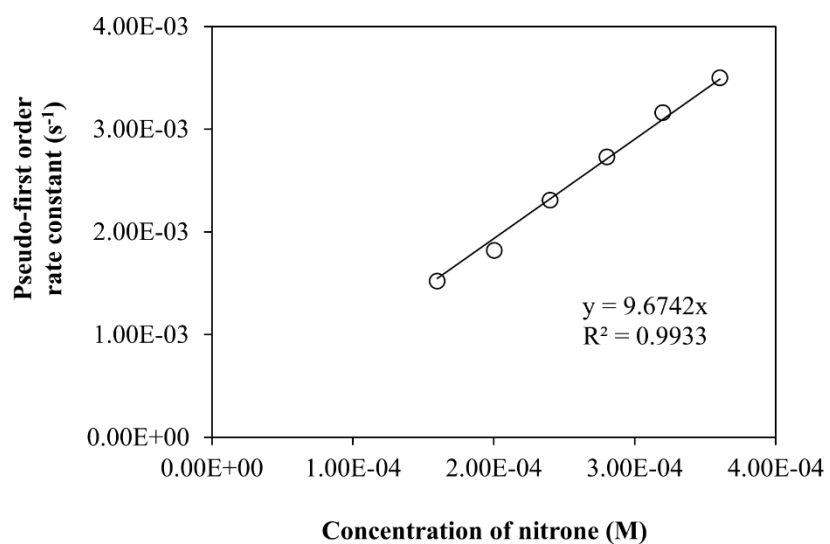


Figure S2. Pseudo first-order rate constants of the cycloadditions of **5** with BARAC plotted against the various concentrations of the nitrone. All reactions were done in aqueous (0.1% water) acetonitrile at $25 \pm 0.1^\circ\text{C}$.

Spectroscopic Properties of Nitrones 1 and 5

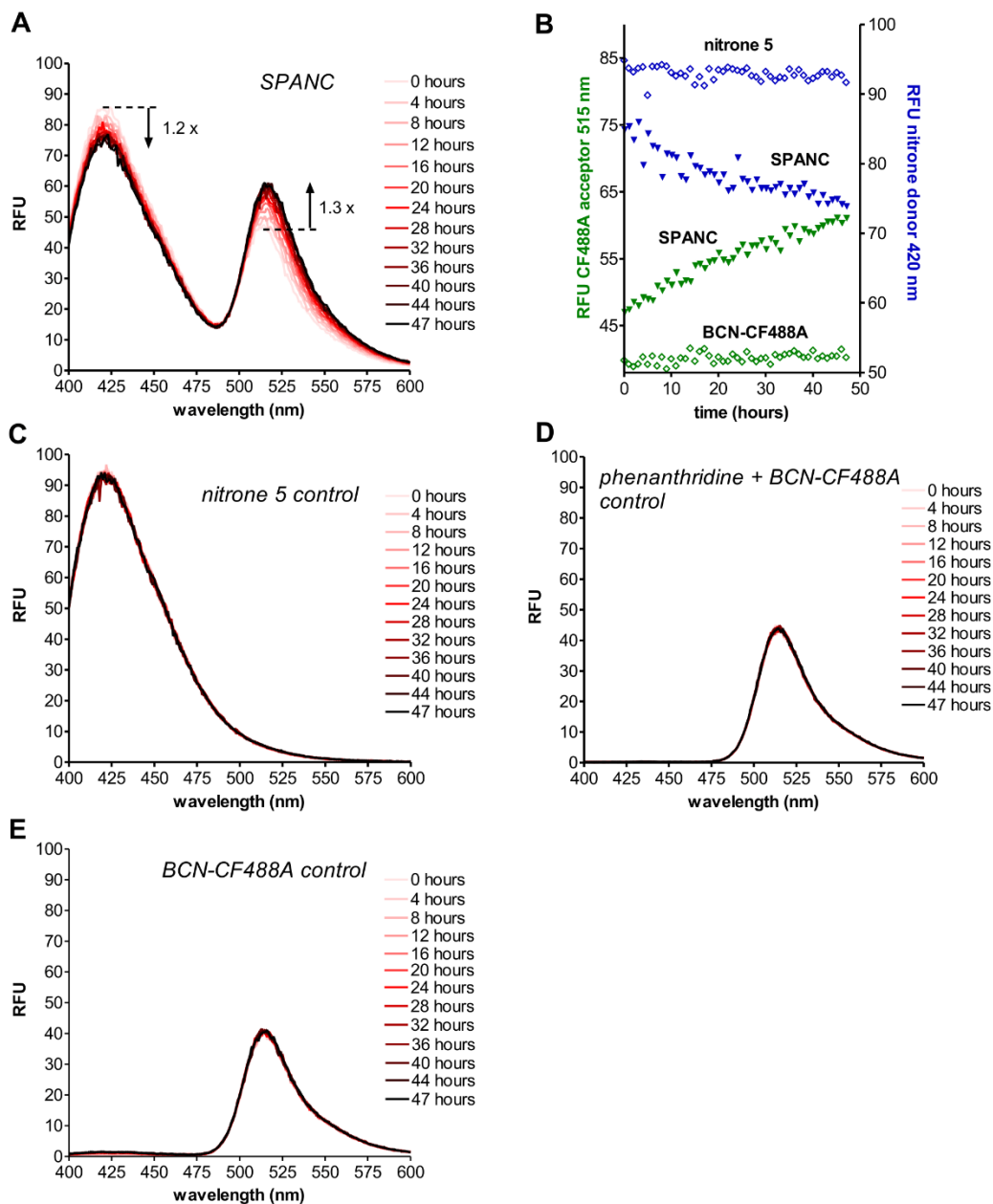


Figure S3. SPANC reaction of nitrone **5** and BCN-CF488A followed by FRET. 10 μ M of nitrone **5** was combined with 10 μ M BCN-CF488A and SPANC reaction was followed by FRET between the phenanthridine-nitrone donor and the CF488A acceptor upon excitation at 380 nm. Reaction was performed under second order conditions where both reactants were used in equimolar concentrations. Fluorescence scans were acquired in PBS at 25°C with excitation at 380 nm, using 5 nm slit for both excitation and emission, on a Cary-Eclipse fluorimeter (Varian) equipped with a Cary-Eclipse cooling unit. Panel A: SPANC kinetics followed over 48 hours. Panel B: Fluorescence increase of CF488A at 515 nm (green) and decrease of nitrone **5** at 420 nm (blue) overtime, consistent with FRET transfer. Panel C: Control reaction performed in absence of BCN-CF488A. Panel D: Control reaction performed between phenanthridine (lacking nitrone electrophile) and BCN-CF488A. Panel E: control reaction performed in absence of nitrone **5**.

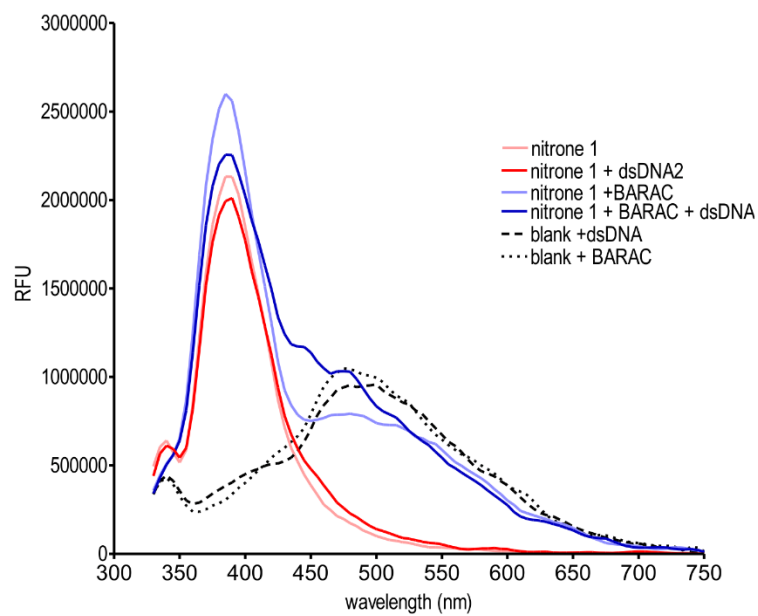


Figure S4. Emission spectrum of **1** in presence of dsDNA (30-mer). Spectra were acquired at room temperature upon excitation at 305 nm of samples containing 2 μ M of nitrone **1**, 20 μ M BARAC and 40 μ M of dsDNA 30-mer in PBS. Reaction of **1** with BARAC was allowed to progress for 4 hours before spectra acquisition.

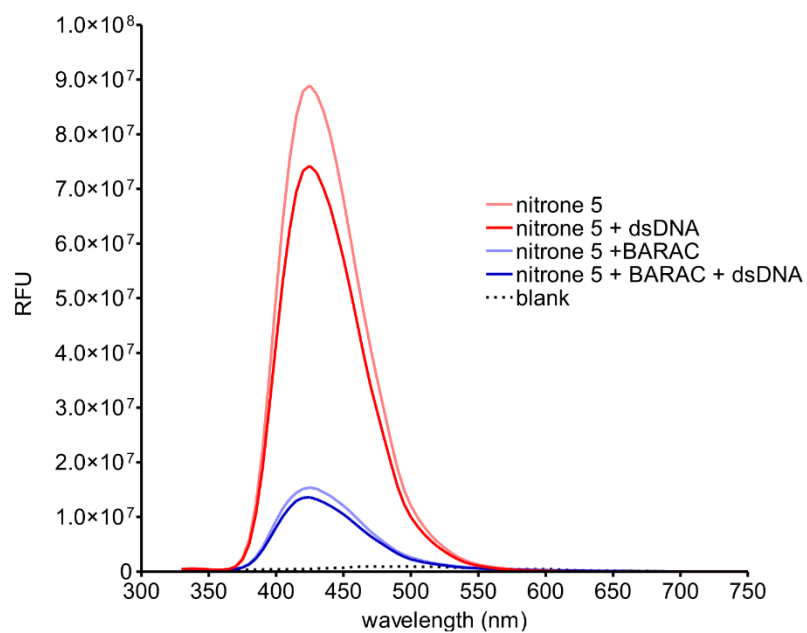
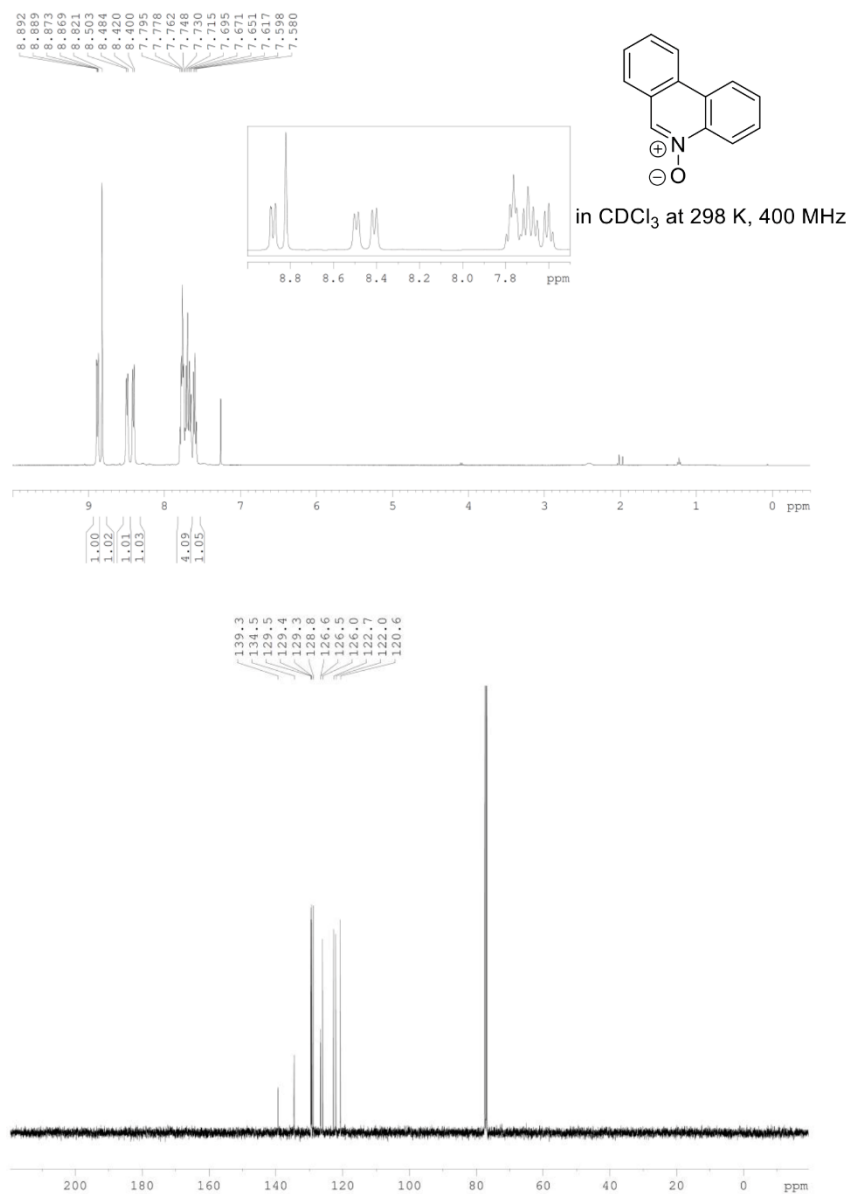


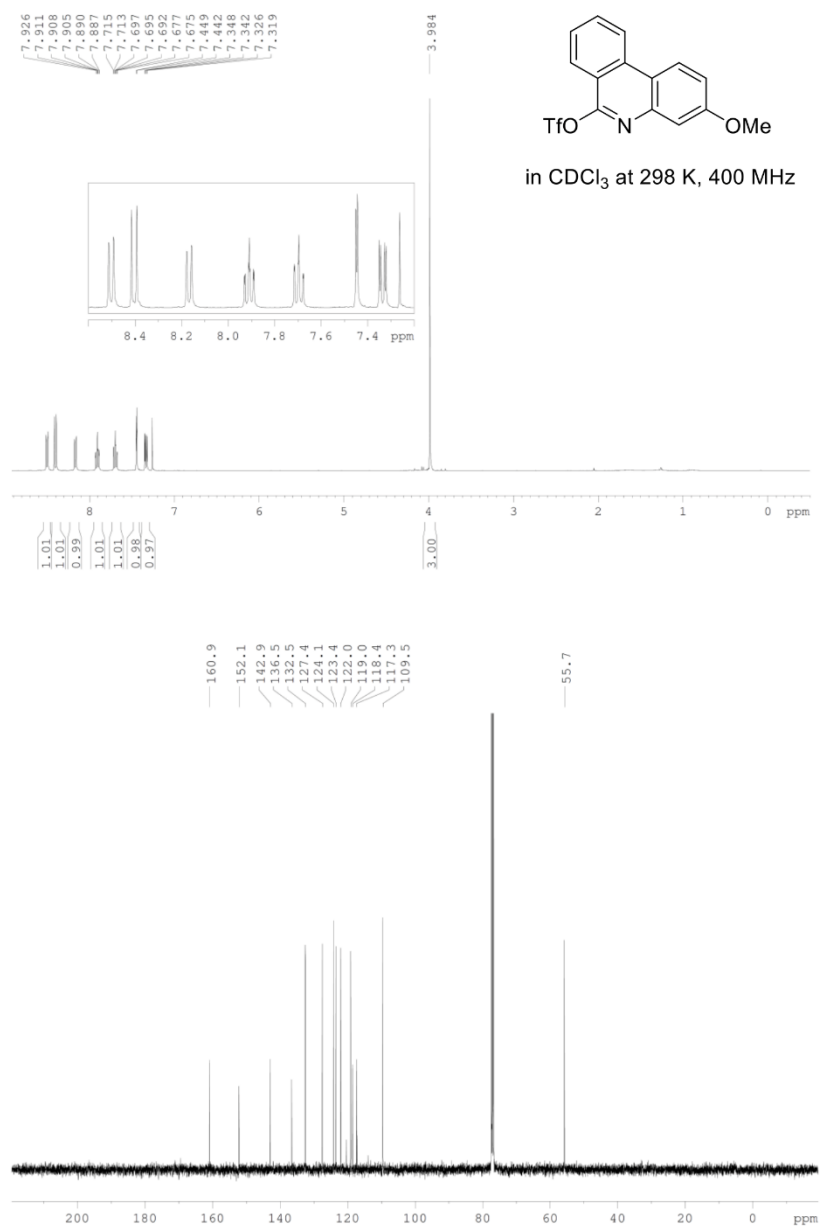
Figure S5. Emission spectrum of **5** in presence of dsDNA (30-mer). Spectra were acquired at room temperature upon excitation at 305 nm of samples containing 2 μ M of nitron **5**, 20 μ M BARAC and 40 μ M of dsDNA 30-mer in PBS. Reaction of **5** with BARAC was allowed to progress for 4 hours before spectra acquisition.

NMR Spectra

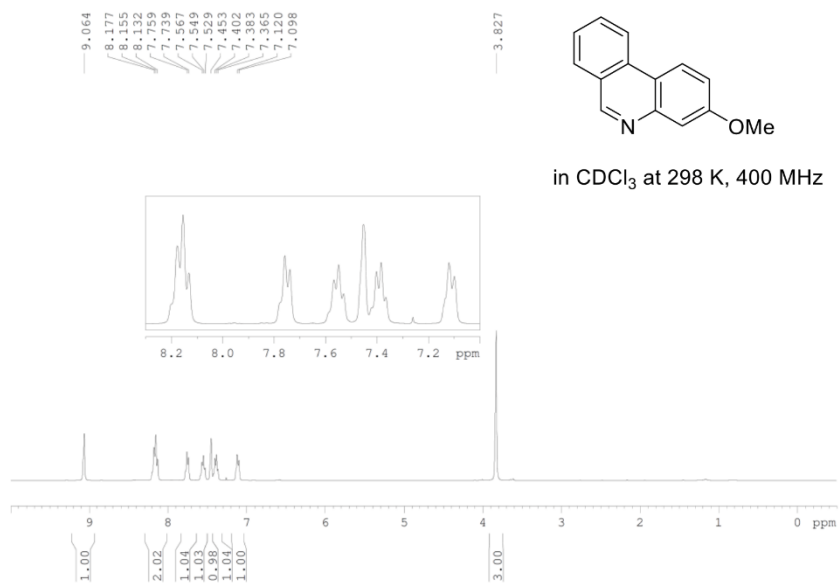
Phenanthridine 5-oxide (1). ^1H and ^{13}C NMR

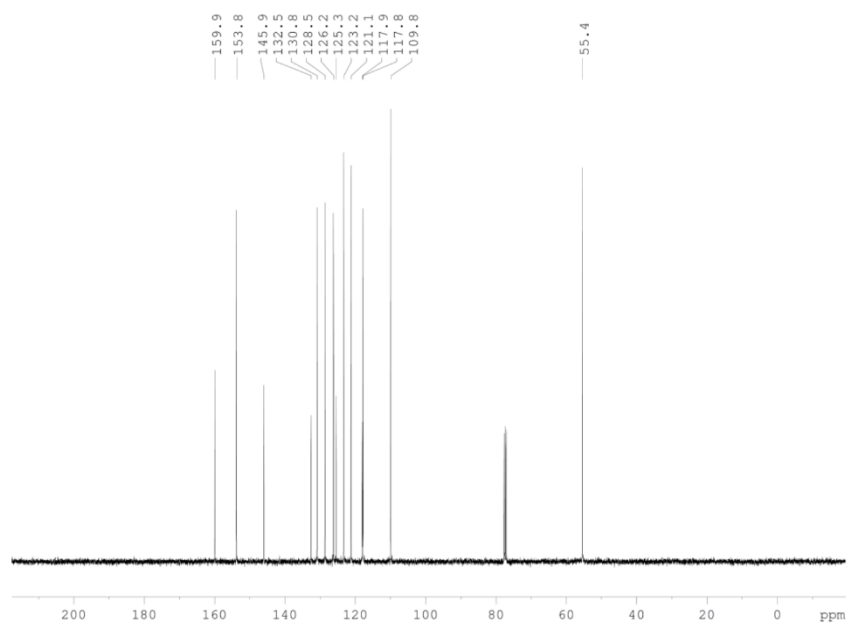


3-Methoxy-, 6-(trifluoromethyl)sulfonyl-, phenanthridin-6(5H)-one (3). ^1H and ^{13}C NMR

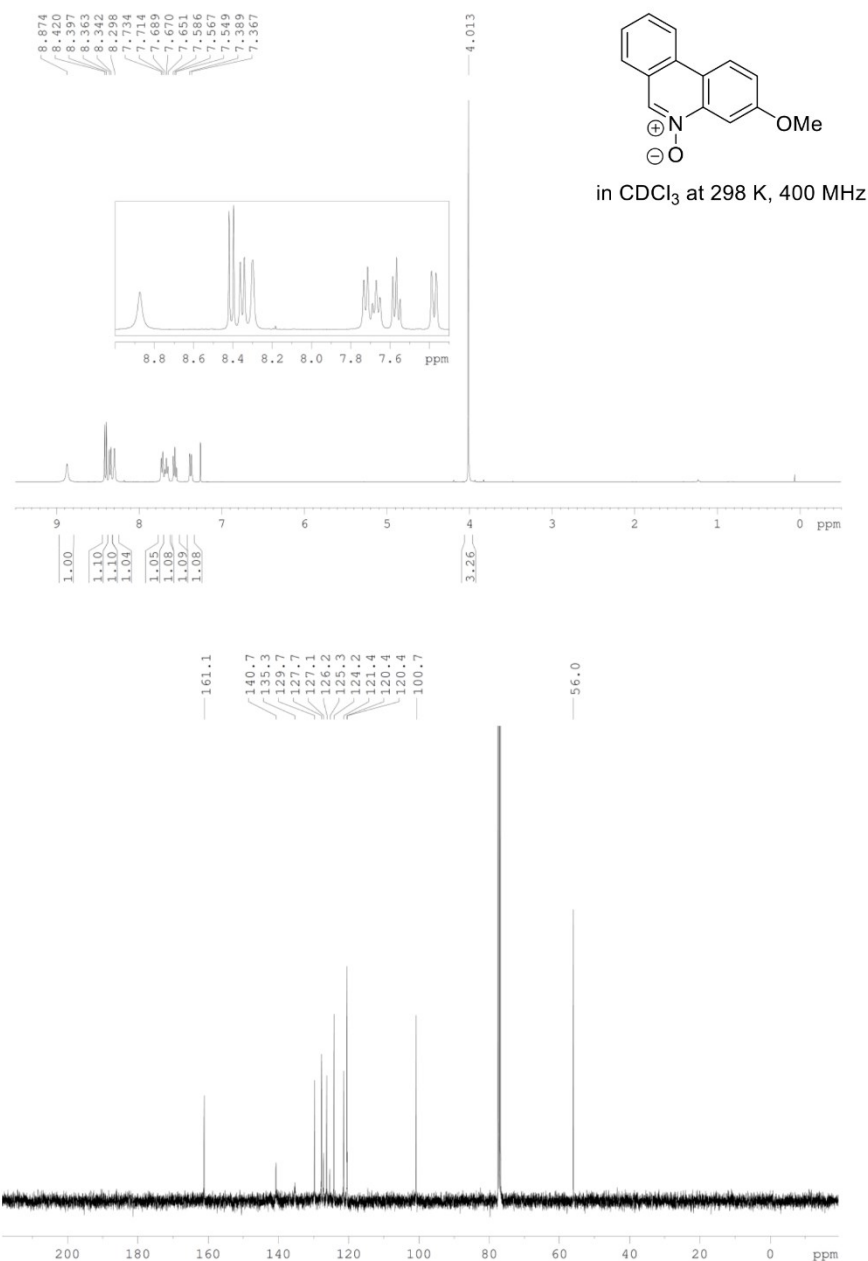


3-Methoxyphenanthridine (4). ^1H and ^{13}C NMR





3-Methoxyphenanthridine 5-oxide (5). ^1H and ^{13}C NMR



References

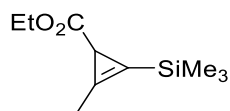
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- (2) Chen, Y.-F.; Wu, Y.-S.; Jhan, Y.-H.; Hsieh, J.-C. *Org. Chem. Front.* **2014**, *1*, 253.

Annex V – Initial exploration of nitron-cyclopropene cycloadditions

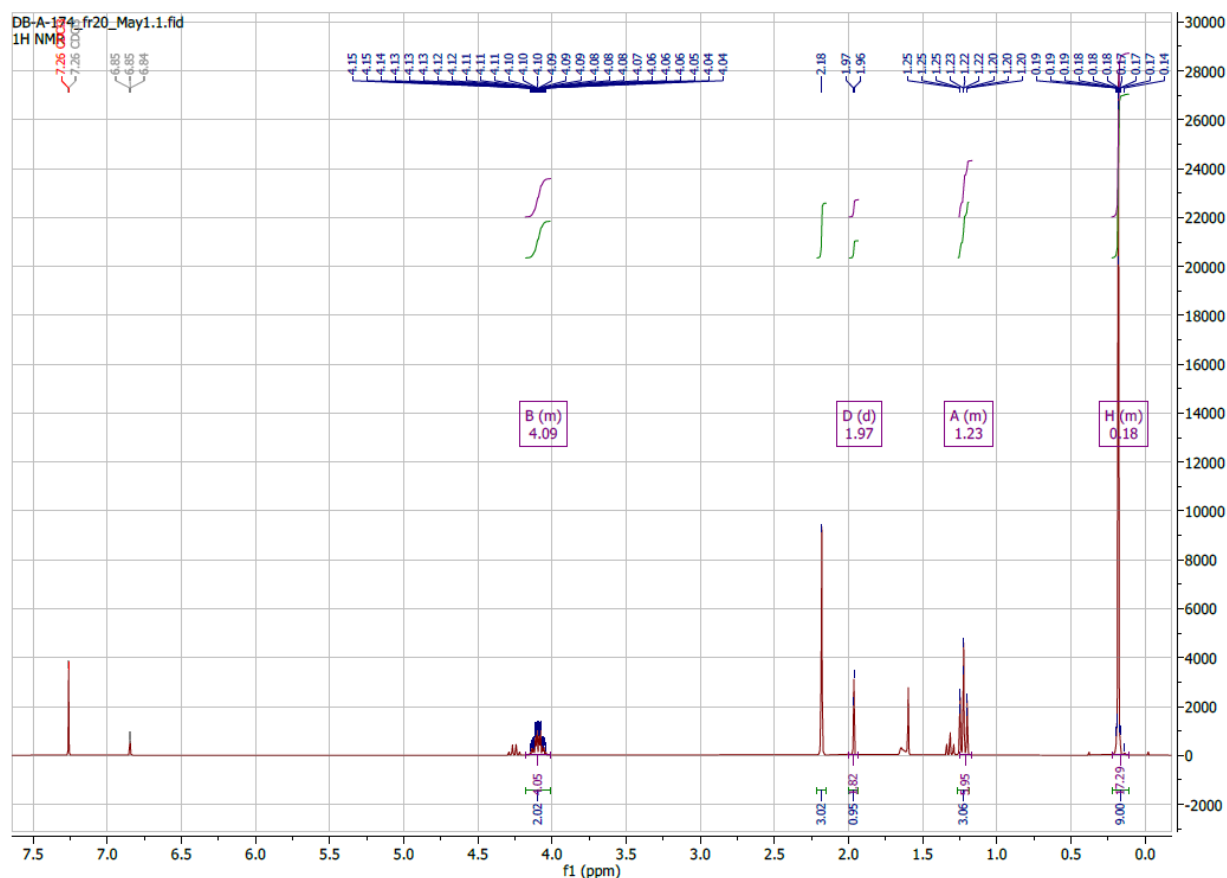
Supporting Information

Synthesis of strained cyclopropenes

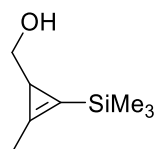
2-methyl-3-(trimethylsilyl)-cycloprop-2-ene-1-carboxylic ethyl ester



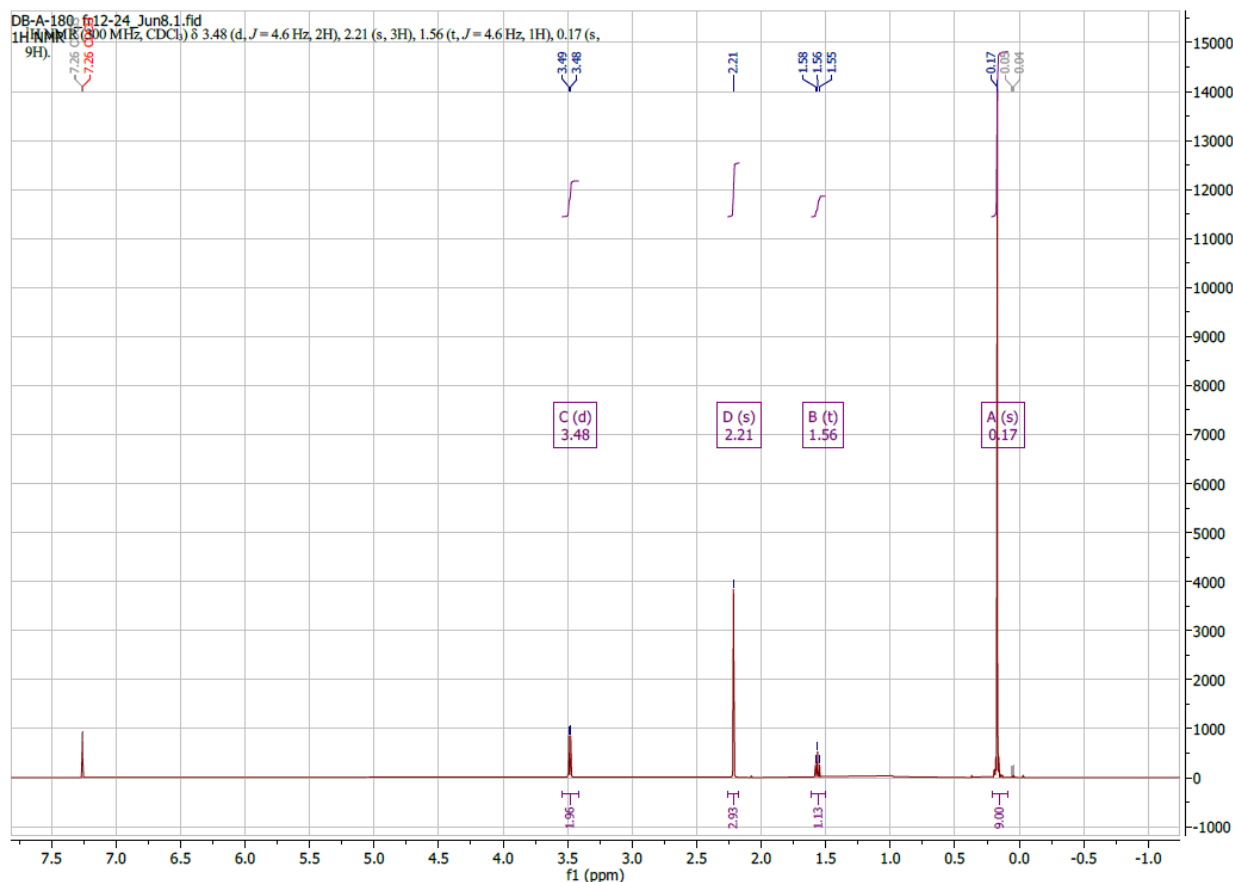
The TMS-cyclopropene precursor was synthesized according to literature procedures²⁹⁰. To a flame-dried round-bottomed flask, under argon atmosphere, was added $\text{Rh}_2(\text{OAc})_4$ (20 mg, 0.045 mmol, 0.02 eq.), followed by trimethylsilyl acetylene (437.1 mg, 4.45 mmol, 2 eq.). Using a syringe, 200 μL of 13% by weight in CH_2Cl_2 of ethyl diazoacetate solution (250 mg, 2.23 mmol, 1 eq.) was slowly added over a period of 20 minutes. The mixture was allowed to stir at room temperature for an additional 40 minutes. The reaction was then concentrated under reduced pressure and purified by column chromatography on silica gel (gradient, 10 $\rightarrow$ 30% CH_2Cl_2 in hexanes). 209 mg of clear colourless oil was recovered (47.3% yield). ^1H spectra corresponded to reported spectra. ^1H NMR (300 MHz, CDCl_3) δ 4.18 – 4.01 (m, 4H), 2.18 (s, 3H), 1.97 (s, 1H), 1.25 – 1.17 (m, 3H), 0.22 – 0.11 (m, 9H).



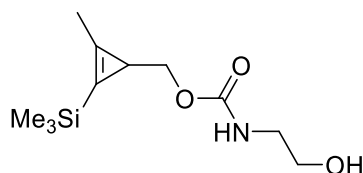
(2-methyl-3-(trimethylsilyl)cycloprop-2-en-1-yl)methanol



The hydroxyl-cyclopropene precursor was synthesized according to literature procedures²⁷⁶. In a dry round-bottomed flask, under an argon atmosphere, was added 2-methyl-3-(trimethylsilyl)-cycloprop-2-ene-1-carboxylic ethyl ester (473.77 mg, 2.52 mmol, 1 eq.) and dry THF. The solution was stirred using a magnetic stir bar and cooled down to 0 °C. DIBAL-H (1M in THF) (6.25 mL, 6.31 mmol, 2.5 eq.) was added over the course of 15 minutes. After stirring for 3 hours, the reaction was carefully quenched with distilled H₂O. The mixture was filtered and the filtrate was concentrated under reduced pressure. The crude oil was purified by column chromatography on silica gel (20% EtOAc:Hexanes). 88.5 mg of clear oil product was recovered (23.7 % yield). ¹H spectra corresponded to reported spectra²⁷⁶. ¹H NMR (300 MHz, CDCl₃) δ 3.48 (d, J = 4.6 Hz, 2H), 2.21 (s, 3H), 1.56 (t, J = 4.6 Hz, 1H), 0.17 (s, 9H).

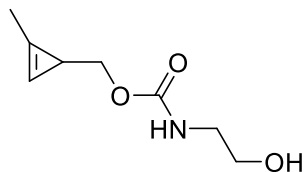


2-methyl-3-(trimethylsilyl)cycloprop-2-enecarboxamide

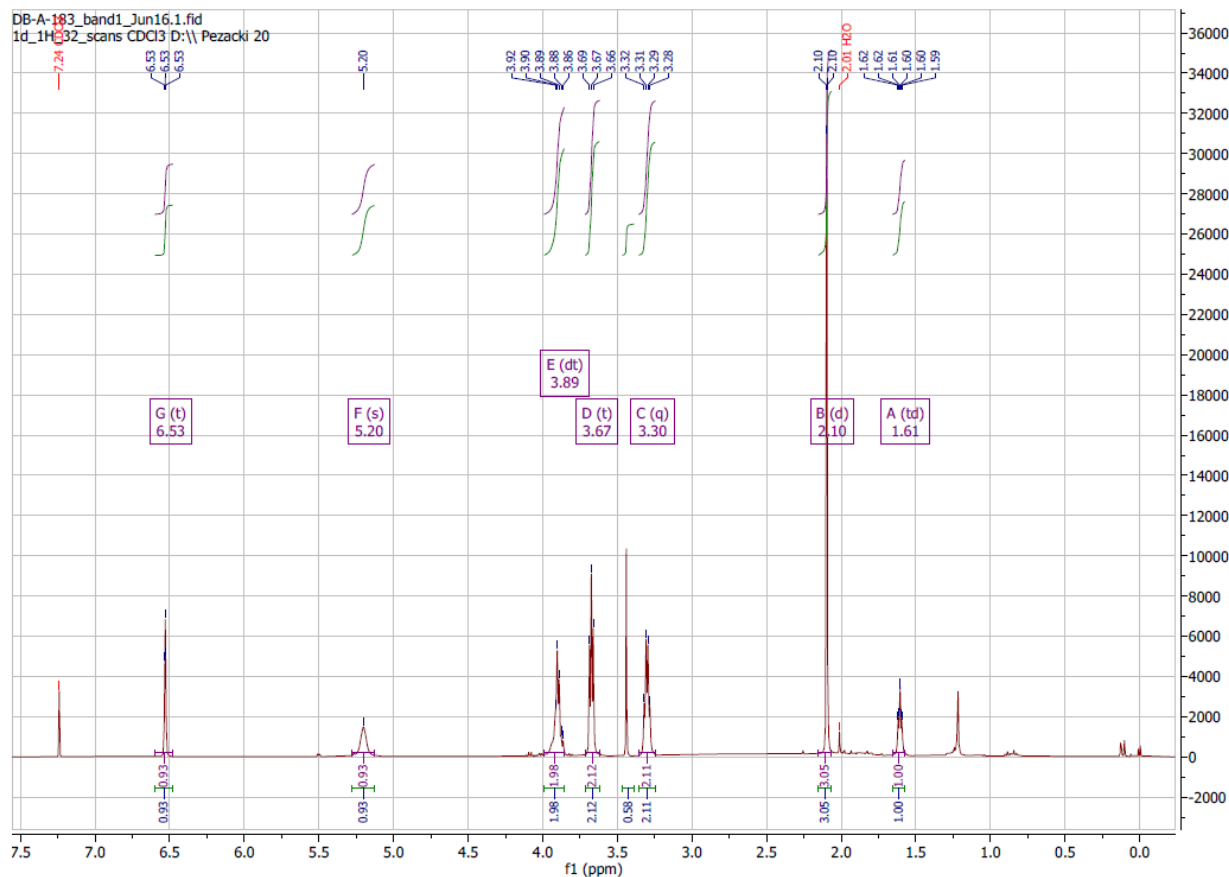


The TMS protected cyclopropene was synthesized according to literature procedures²⁷⁶. To a stirred solution of (2-methyl-3-(trimethylsilyl)cycloprop-2-en-1-yl)methanol (88.5 mg, 0.57 mmol, 1.0 eq.) in dry THF (3.8 mL), under argon atmosphere, was added carbonyldiimidazole (CDI, 111.9 mg, 0.69 mmol, 1.2 eq.). The mixture was stirred at room temperature for 3 hours. After the elapsed time, ethanolamine was slowly added (42.1 mg, 0.69 mmol, 1.2 eq.). The resulting mixture was stirred for 18 hours, after which it was concentrated under reduced pressure. The crude mixture was purified using preparative TLC (Hexanes:EtOAc, 2:1) to yield 79.6 mg of crude product as a clear oil (57.4% yield) and used in the following without further purification. ¹H NMR spectra corresponded to reported spectra²⁷⁶.

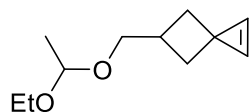
2-methyl-cycloprop-2-enecarboxamide



2-methyl-3-(trimethylsilyl)cycloprop-2-enecarboxamide (79.6 mg crude mixture) was dissolved in dry THF (3.0 mL) in a dry round-bottomed flask, under argon atmosphere. A solution of tetra-*n*-butylammonium fluoride (TBAF, 1M in THF)(0.5 mL, 0.5 mmol, 1.0 eq.) was then carefully added. The resulting mixture was stirred at room temperature for 18 hours, and then concentrated under reduced pressure. The resulting crude product was purified by preparative TLC (Hexanes;EtOAc, 1:1) to afford 20 mg of clear oil product. ¹H NMR spectra corresponded to reported spectra²⁷⁶. ¹H NMR (400 MHz, CDCl₃) δ 6.53 (t, *J* = 1.4 Hz, 1H), 5.20 (s, 1H), 3.89 (dt, *J* = 10.9, 5.1 Hz, 2H), 3.67 (t, *J* = 5.1 Hz, 2H), 3.30 (q, *J* = 5.4 Hz, 2H), 2.10 (d, *J* = 1.2 Hz, 3H), 1.61 (td, *J* = 5.1, 1.6 Hz, 1H).



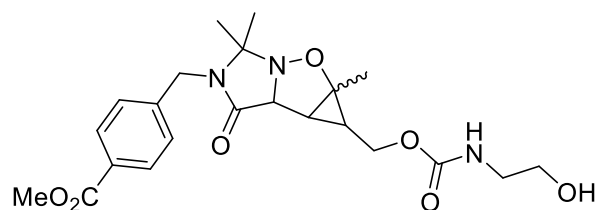
(±)-5-((1-Ethoxyethoxy)methyl)spiro[2.3]hex-1-ene



The spiro-hexene cyclopropene was synthesized according to literature procedures¹, and completed by other members of our research group, Kaitlyn Morrill and Isaac Kuk. Obtained ¹H NMR matched reported data seen here¹: ¹H NMR (400 MHz, CDCl₃) δ 7.43 (d, J = 1.0 Hz, 1H), 7.39 (d, J = 1.0 Hz, 1H), 4.73 (q, J = 5.5 Hz, 1H), 3.72-3.62 (m, 2H), 3.54-3.47 (m, 2H), 2.48-2.39 S37 (m, 1H), 2.26-2.22 (m, 2H), 1.88-1.83 (m, 2H), 1.32 (d, J = 5.0 Hz, 3H), 1.22 (t, J = 7.0 Hz, 3H)

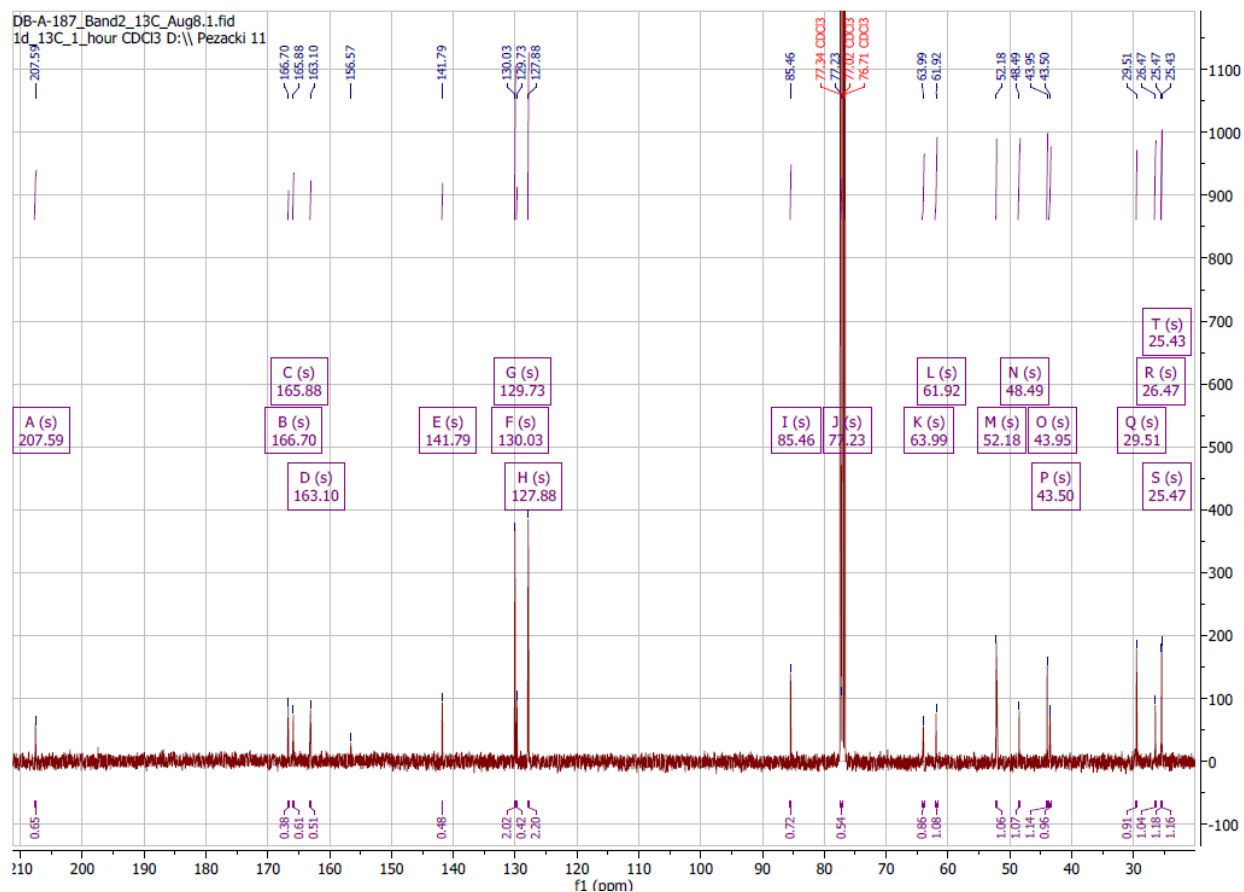
Synthesis of cyclopropene:nitrone cycloadducts

DMimO : 2-methyl-cycloprop-2-enecarboxamide cycloadduct

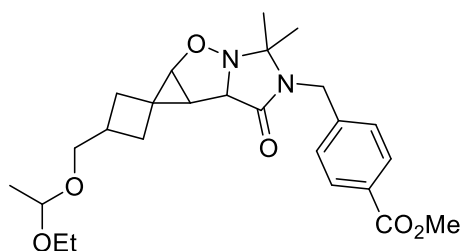


In a dry NMR tube, DMImO nitron (7.25 mg, 0.029 mmol, 1.0 eq.) was dissolved in MeOD- d_4 (750 μ L). 2-methyl-cycloprop-2-enecarboxamide (5 mg, 0.029 mmol, 1.0 eq.) was then added to the tube and placed under an argon atmosphere and sealed. The mixture was then heated to 37 $^{\circ}$ C and stirred overnight (18 h). After the reaction was deemed completed by observing the disappearance of cyclopropene alkene peak by NMR, the crude mixture was directly purified by preparative TLC (MeOH:DCM, 7:93). Given silica gel contamination, and small recovered mass, an accurate yield was not determined. ^1H NMR (400 MHz, CDCl_3) δ 8.02 – 7.95 (m, 2H), 7.38 – 7.32 (m, 2H), 5.22 (s, 1H), 4.63 (s, 2H), 4.42 – 4.27 (m, 2H), 3.90 (s, 3H), 3.78 – 3.67 (m, 2H), 3.47 (d, J = 6.1 Hz, 1H), 3.33 (dd, J = 10.0, 4.4 Hz, 2H), 3.07 (dd, J = 17.2, 8.1 Hz, 1H), 2.75 (dd, J = 17.2, 6.0 Hz, 1H), 2.28 (s, 3H), 1.24 (s, 3H), 1.22 (s, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 207.59, 166.70, 165.88, 163.10, 141.79, 130.03, 129.73, 127.88, 156.57

85.46, 77.23, 63.99, 61.92, 52.18, 48.49, 43.95, 43.50, 29.51, 26.47, 25.47, 25.43. HRMS (ESI) Calculated Exact mass (M): 447.20. Found(M+Na): 470.2437.

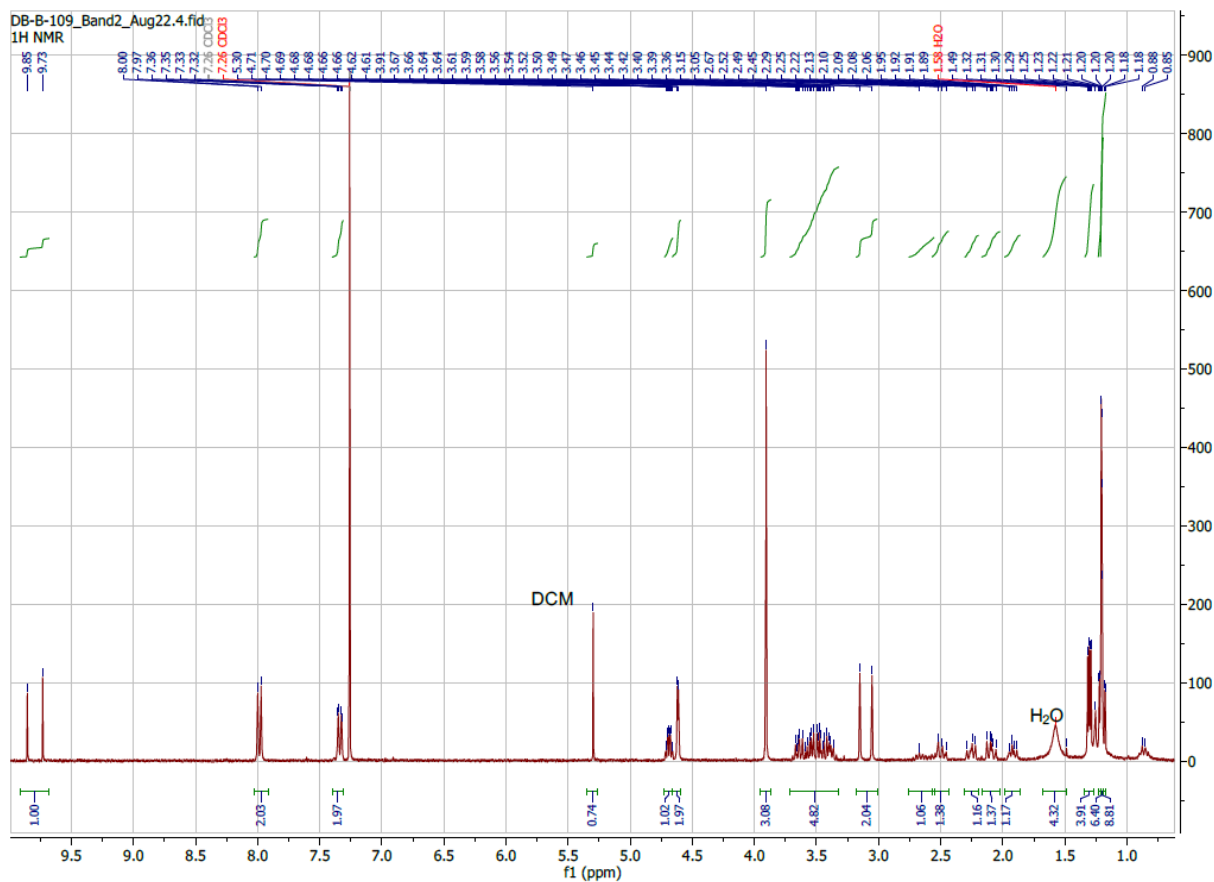


DMImO:(±)-5-((1-Ethoxyethoxy)methyl)spiro[2.3]hex-1-ene cycloadduct



In a dry NMR tube, DMImO nitron (7.4 mg, 0.027 mmol, 1.0 eq.) was dissolved in MeOD-d₄ (500 μ L). 2-methyl-cycloprop-2-enecarboxamide (5 mg, 0.027 mmol, 1.0 eq.) was then added to the tube and placed under an argon atmosphere and sealed. The mixture was then heated to 37 °C and stirred overnight (18 h). After the reaction was deemed completed by observing the disappearance of cyclopropene alkene peak by NMR, the crude mixture was directly purified by preparative TLC (MeOH:DCM, 2:98). Given silica gel contamination, and small recovered mass, an accurate yield was not determined. Observation of ¹H NMR signals around 9.5-10 ppm suggest aldehyde functionality in the recovered compound, and not the initial cycloadduct product. ¹H NMR (300 MHz, CDCl₃) δ 9.85 (s, 1H), 9.73 (s, 1H), 7.99 (d, *J* =

8.1 Hz, 2H), 7.41 – 7.30 (m, 2H), 4.72 – 4.65 (m, 1H), 4.62 (d, $J = 2.7$ Hz, 2H), 3.91 (s, 3H), 3.69 – 3.32 (m, 4H), 3.15 (s, 1H), 3.05 (s, 1H), 2.68 (d, $J = 7.4$ Hz, 1H), 2.55 – 2.42 (m, 1H), 2.32 – 2.19 (m, 1H), 2.16 – 2.03 (m, 1H), 1.92 (dd, $J = 11.0, 6.8$ Hz, 1H), 1.30 (dd, $J = 5.3, 2.8$ Hz, 4H), 1.23 – 1.17 (m, 8H). UPLC-MS (ESI) Calculated Exact mass (M): 458.2417. Found(M+H): 459.55.



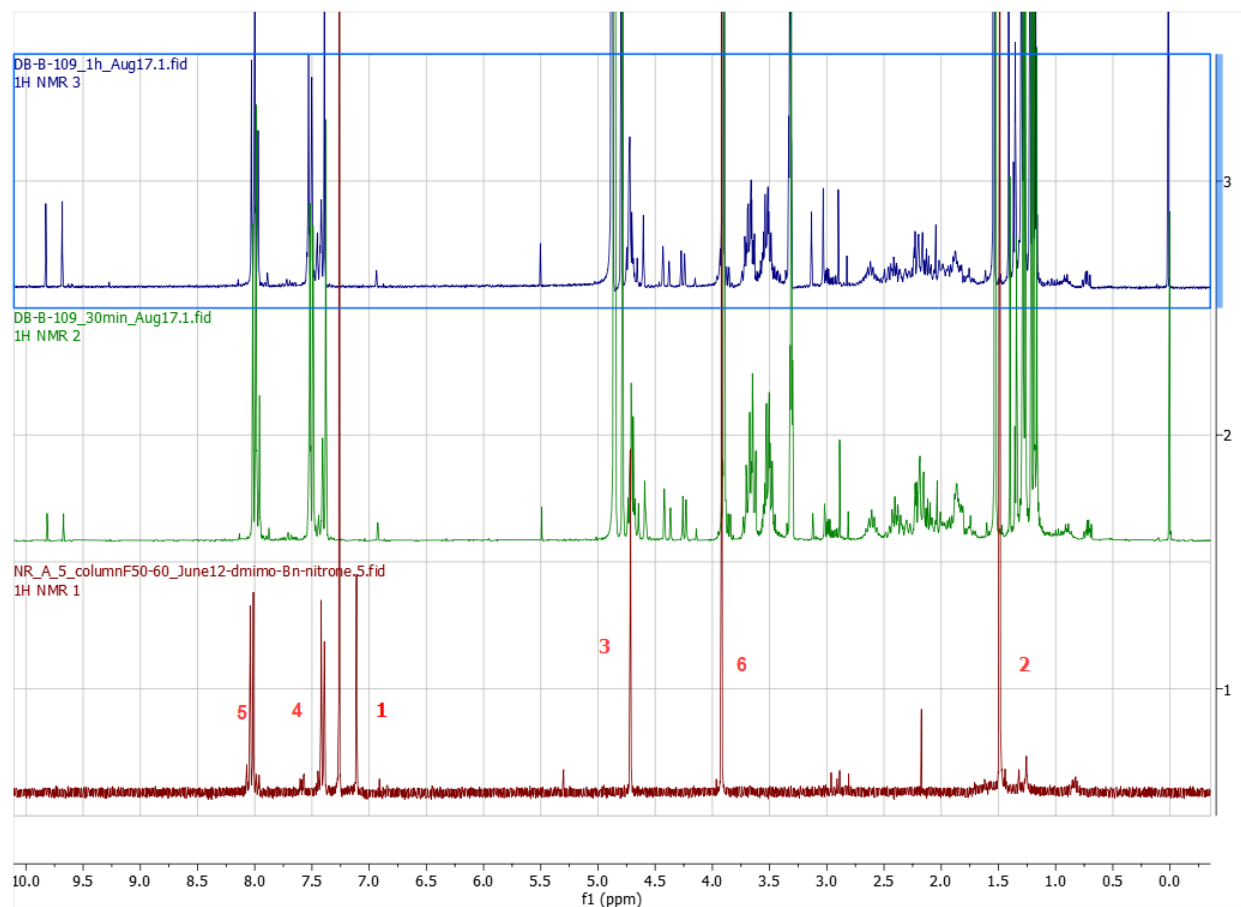
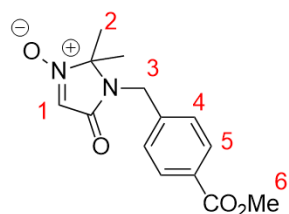


Figure S8. ^1H NMR overlay of reaction between DMImO nitronium and ($\pm$)-5-((1-Ethoxyethoxy)methyl)spiro[2.3]hex-1-ene. Bottom panel shows ^1H NMR spectrum of DMImO nitronium; middle panel shows reaction progress after 30 minutes; top panel shows reaction progress after 1 hour.

References

- (1) Yu, Z.; Lin, Q. Design of Spiro[2.3]Hex-1-Ene, a Genetically Encodable Double-Strained Alkene for Superfast Photoclick Chemistry. *J. Am. Chem. Soc.* 2014, 136 (11), 4153–4156. <https://doi.org/10.1021/ja5012542>.

Annex VI - Initial development of cyclopropenone-caged diyne and nitronc cycloadditions Supporting Information

Cyclopropenone-Caged Diyne Nitronc Cycloaddition Kinetics Studies and Methodology

The rate measurements of cycloadditions of diyne with various nitrones were performed using UV-visible spectroscopy (Agilent Technologies, Cary 60) in methanol at 25 ± 0.1 °C in triplicate. Reactions were done under pseudo-first order conditions with the nitronc in 15 - 60-fold excess of the cyclopropenone-caged monoyne and were monitored by following the decrease of the absorbance at the characteristic wavelength, 354 nm. The absorbance changes used for kinetic analyses are assumed to be those associated with the formation of cycloadduct product, as monitoring of solutions of each substrate in isolation do not show absorbance spectra changes over the reaction time course. Nitronc and cyclopropenone-caged monoyne stocks were prepared fresh for each trial reaction (in triplicate) using a mixture of DCM:MeOH (1:1). Added solvent volume was adjusted according to the initially prepared substrate stock solutions to obtain the desired final concentrations.

A calculated amount of stock solution of nitronc (excess reagent) required to achieve the desired final concentration was diluted in a calculated amount of additional DCM:MeOH in a dry cuvette. A calculated amount of stock solution of diyne required to achieve the desired final concentration was rapidly mixed with the thermally equilibrated solution and immediately inserted into the UV-vis spectrophotometer. Absorption was measured at pre-set time intervals of ~6 seconds until it reached a plateau at the wavelength chosen. The decrease in absorbance was then fit to a first-order exponential decay model (One phase decay) using Graphpad Prism 8.4.3 (absorbance vs time (s)). Second-order rate constants could then be determined by performing linear regression on a graph of observed first-order rate constants, k' , vs concentration of nitronc (M).

Table S1 Preparation of reaction mixtures used for the pseudo-first order kinetics of DMPO with cyclopropenone-caged monoyne.

Reaction	Concentration Monoyne (μM)	Concentration Nitron (μM)	Vol. of DCM:MeOH (1:1) (μL)	Total Volume of reaction (μL)
15x	2.2	33	909.6	1000
20x	2.2	44	828.6	1000
30x	2.2	66	715.7	1000
40x	2.2	88	790.6	1000
50x	2.2	110	757	1000

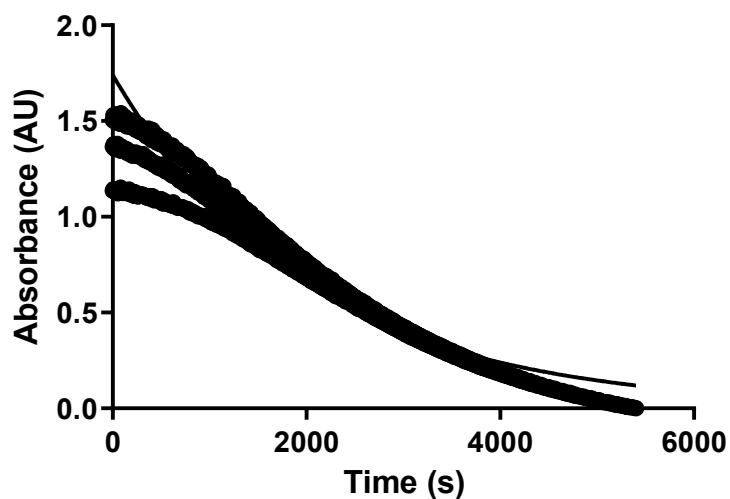


Figure S9. Absorbance (354 nm) of cycloaddition reaction between DMPO (15X) and cyclopropenone-caged monoyne (1X)

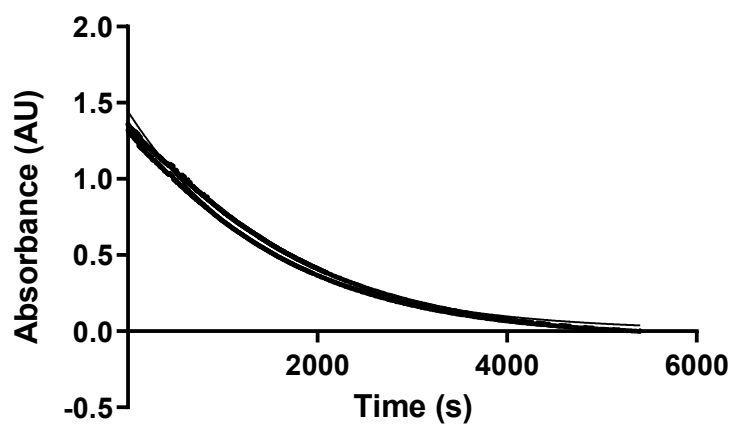


Figure S9. Absorbance (354 nm) of cycloaddition reaction between DMPO (20X) and cyclopropenone-caged monoyne (1X)

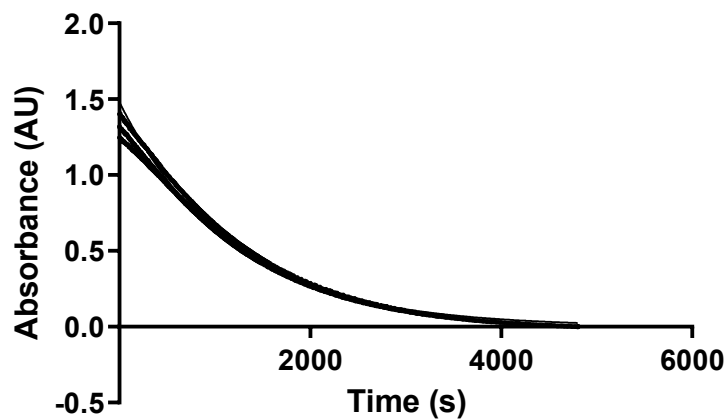


Figure S10. Absorbance (354 nm) of cycloaddition reaction between DMPO (30X) and cyclopropenone-caged monoyne (1X)

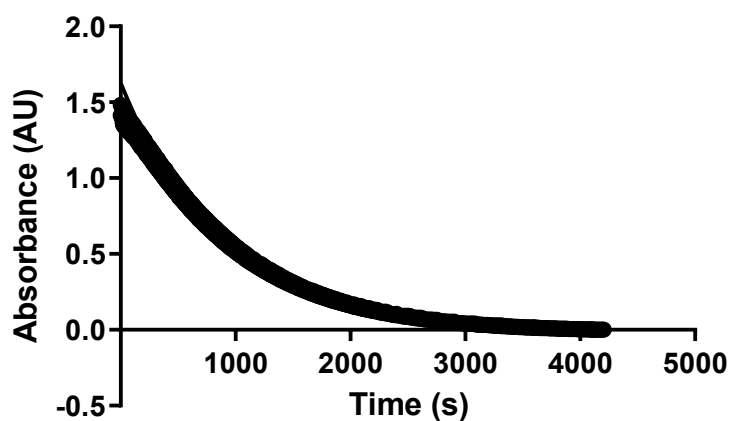


Figure S11. Absorbance (354 nm) of cycloaddition reaction between DMPPO (40X) and cyclopropenone-caged monoyne (1X)

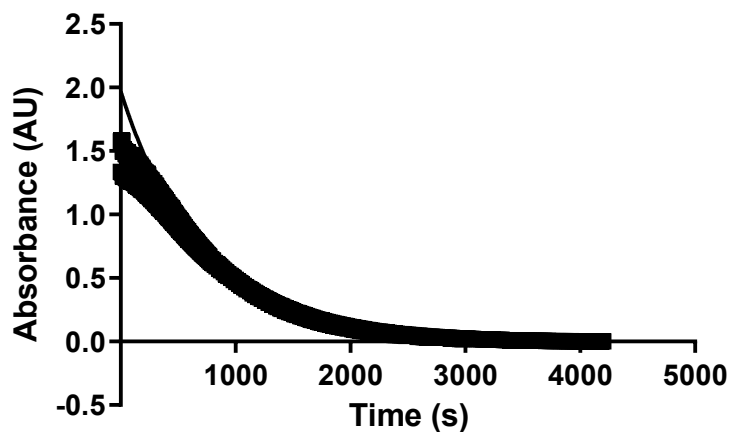
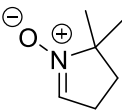


Figure S12. Absorbance (354 nm) of cycloaddition reaction between DMPPO (50X) and cyclopropenone-caged monoyne (1X)

Table S2. Observed first-order rate constants for the cycloaddition reaction between DMPPO and cyclopropenone-caged monoyne

Nitrone	Concentration (M)	k' (s^{-1})
	0.033601	0.0004958
	0.043987	0.0006742
	0.066023	0.0008515
	0.087996	0.001143
	0.110002	0.001429

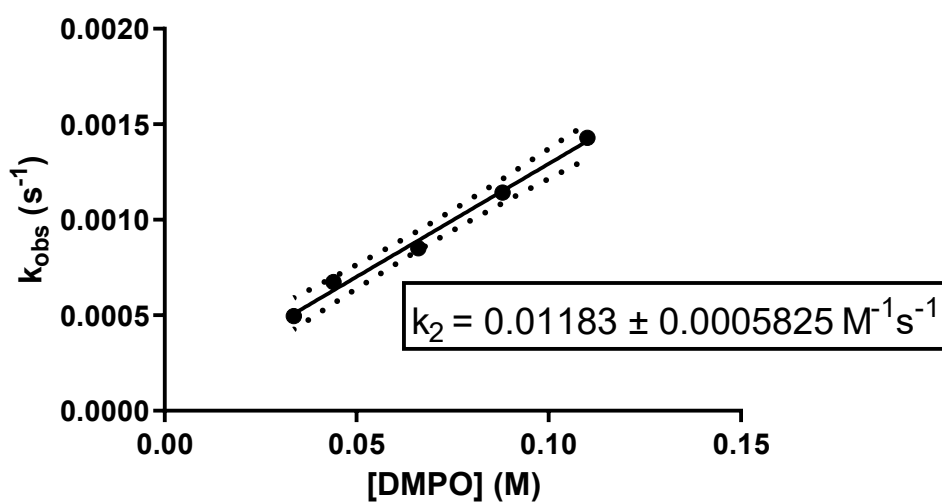


Figure S12. Pseudo-first order kinetic analysis of the cycloaddition between DMPO nitron and 2.2 μM cyclopropenone-caged monoyne. $R^2=0.9928$ (dotted line represents the 95% confidence interval)

Table S3. Volume of stock solutions used for the pseudo-first order kinetics of EMPO with cyclopropenone-caged monoyne.

Reaction	Concentration Monoyne (μM)	Concentration Nitron (μM)	Vol. of DCM:MeOH (1:1) (μL)	Total Volume of reaction (μL)
20x	100	2000	948.6	1000
30x	100	3000	935.7	1000
40x	100	4000	918.1	1000
50x	100	5000	900.4	1000
60x	100	6000	882.8	1000

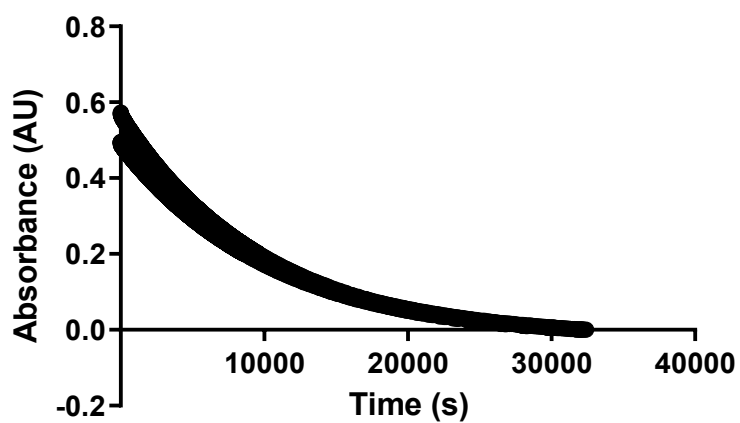


Figure S13. Absorbance (354 nm) of cycloaddition reaction between EMPO (20X) and cyclopropanone-caged monoyne (1X)

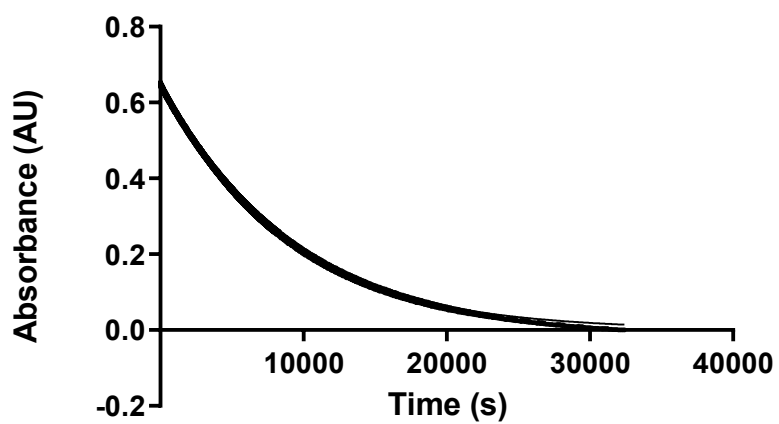


Figure S14. Absorbance (354 nm) of cycloaddition reaction between EMPO (30X) and cyclopropanone-caged monoyne (1X)

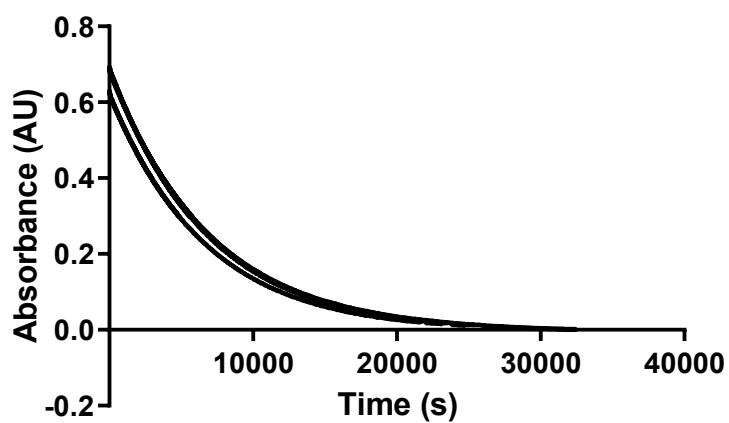


Figure S15. Absorbance (354 nm) of cycloaddition reaction between EMPO (40X) and cyclopropenone-caged monoyne (1X)

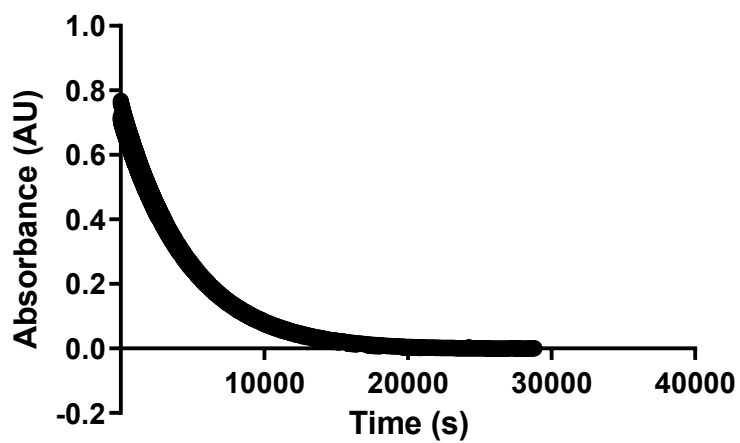


Figure S16. Absorbance (354 nm) of cycloaddition reaction between EMPO (50X) and cyclopropenone-caged monoyne (1X)

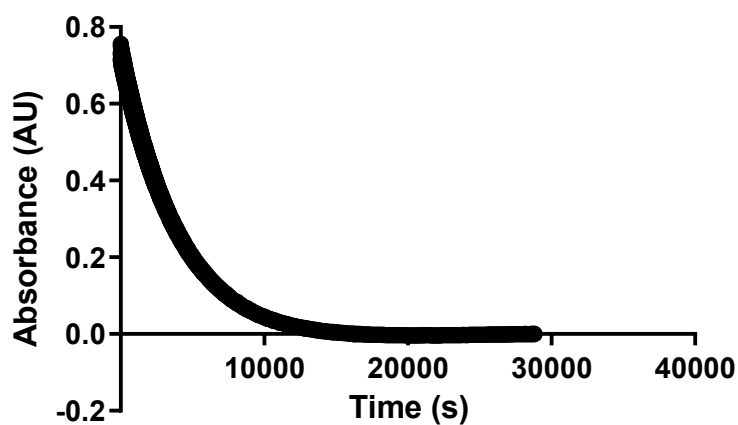
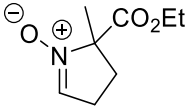


Figure S17. Absorbance (354 nm) of cycloaddition reaction between EMPO (60X) and cyclopropenone-caged monoyne (1X)

Table S4. Observed first-order rate constants for the cycloaddition reaction between DMPO and cyclopropenone-caged monoyne

Nitrone	Concentration (M)	k' (s ⁻¹)
	0.002	0.0001101843
	0.003	0.0001184493
	0.004	0.0001511334
	0.005	0.0002281
	0.006	0.0002758

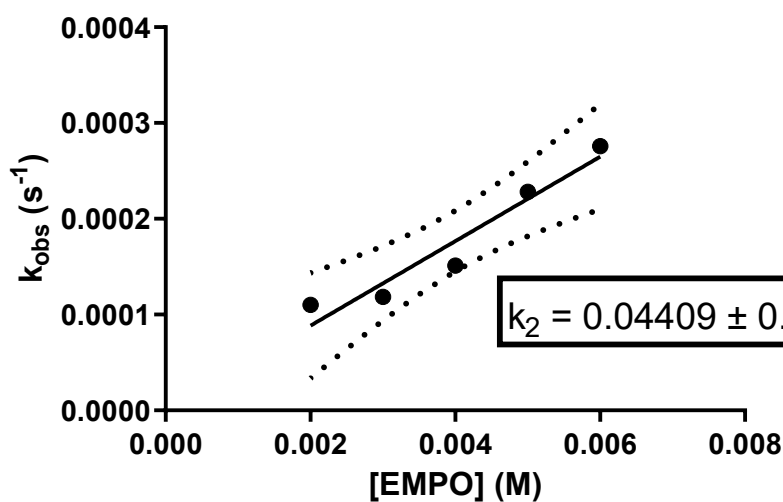


Figure S18. Pseudo-first order kinetic analysis of the cycloaddition between EMPO nitron and 100 μ M cyclopropenone-caged monoyne. $R^2=0.9285$ (dotted line represents the 95% confidence interval)

Table S5. Volume of stock solutions used for the pseudo-first order kinetics of DMImO with cyclopropenone-caged monoyne.

Reaction	Concentration Monoyne (μ M)	Concentration Nitron (μ M)	Vol. of DCM:MeOH (1:1) (μ L)	Total Volume of reaction (μ L)
30x	100	3000	956.5	1000
40x	100	4000	945.6	1000
50x	100	5000	934.8	1000
60x	100	6000	923.9	1000
70x	100	7000	863.5	1000

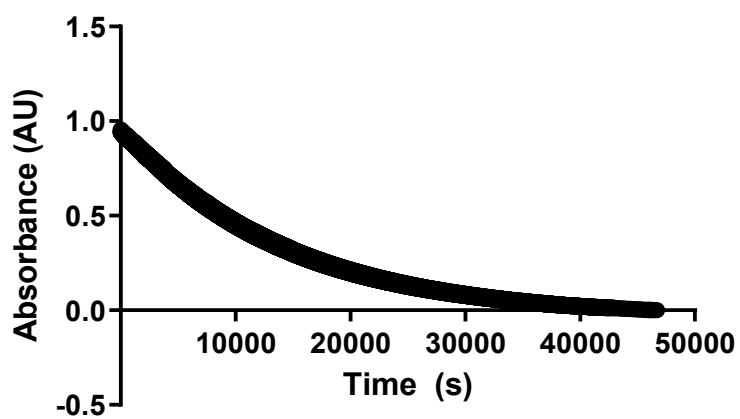


Figure S19. Absorbance (354 nm) of cycloaddition reaction between DMImO (30X) and cyclopropenone-caged monoyne (1X)

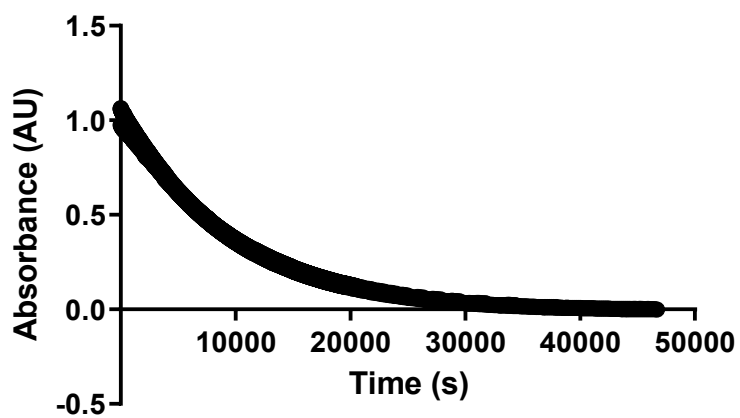


Figure S20. Absorbance (354 nm) of cycloaddition reaction between DMImO (40X) and cyclopropenone-caged monoyne (1X)

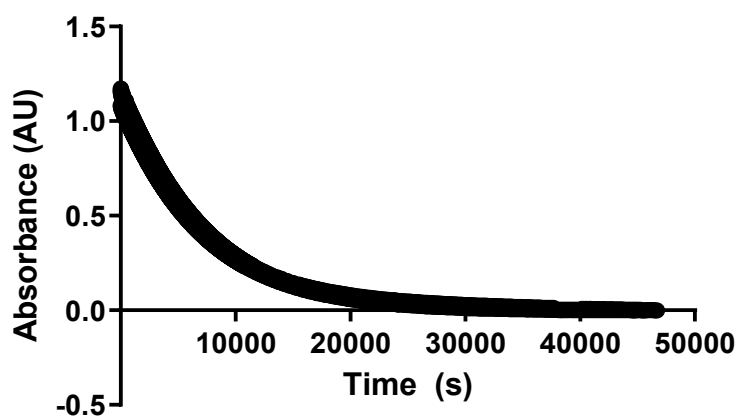


Figure S21. Absorbance (354 nm) of cycloaddition reaction between DMImO (50X) and cyclopropenone-caged monoyne (1X)

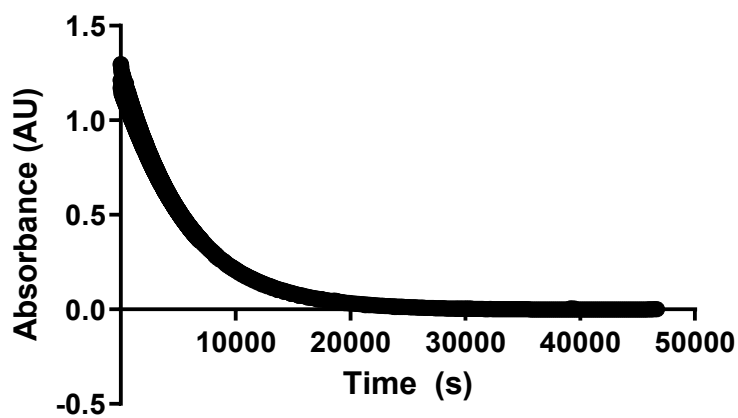


Figure S22. Absorbance (354 nm) of cycloaddition reaction between DMImO (60X) and cyclopropenone-caged monoyne (1X)

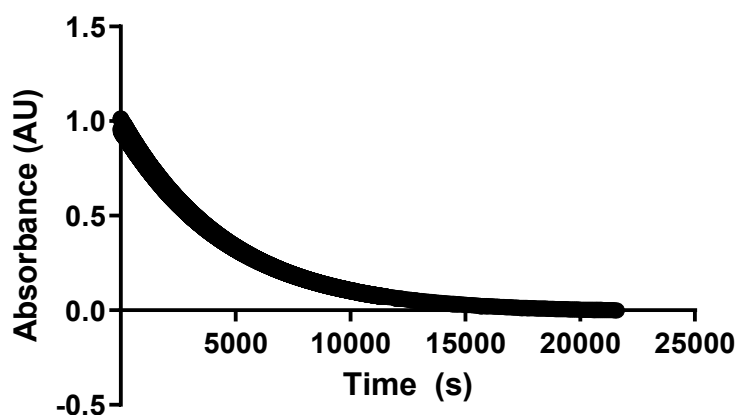
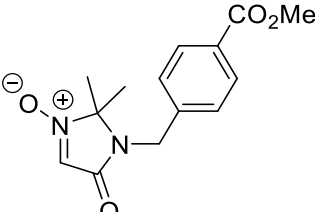


Figure S23. Absorbance (354 nm) of cycloaddition reaction between DMImO (70X) and cyclopropenone-caged monoyne (1X)

Table S6. Observed first-order rate constants for the cycloaddition reaction between DMPO and cyclopropenone-caged monoyne

Nitrone	Concentration (M)	k' (s^{-1})
	0.003	0.00008107
	0.004	0.0001097
	0.005	0.0001412
	0.006	0.0001837
	0.007	0.0002251

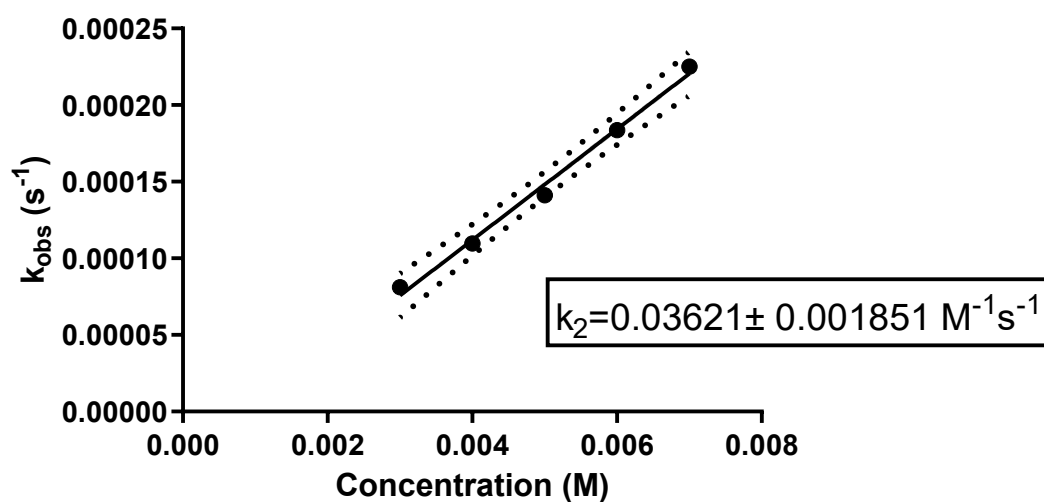
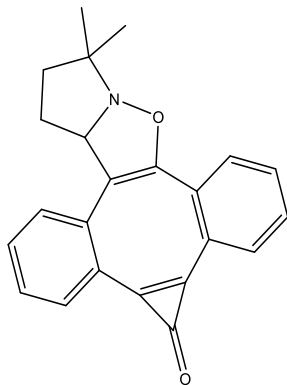


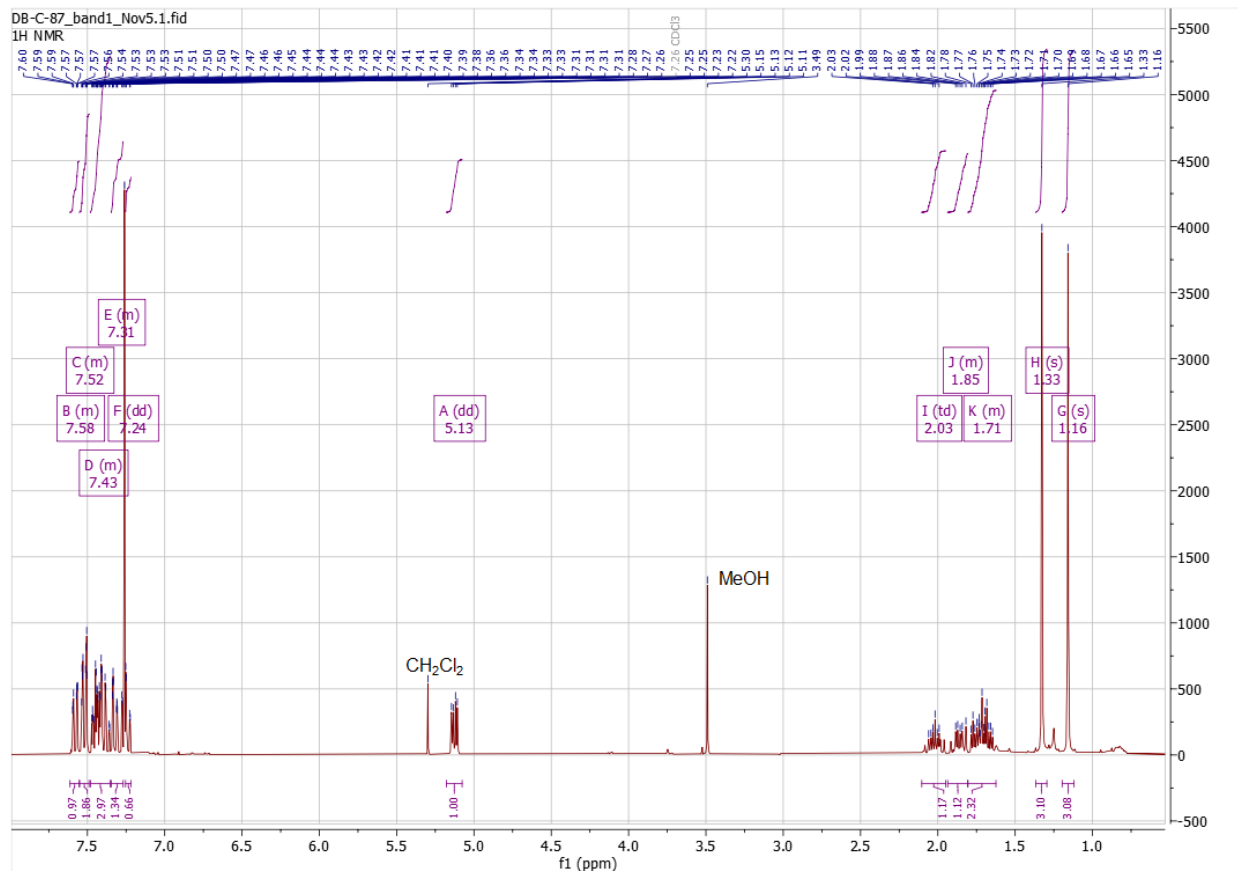
Figure S24. Pseudo-first order kinetic analysis of the cycloaddition between DMImO nitrone and 100 μ M cyclopropenone-caged monoyne. $R^2=0.9922$ (dotted line represents the 95% confidence interval)

Synthesis of Nitrone:Monoyne Cycloadducts

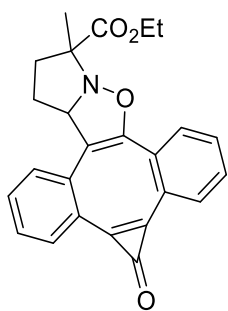
DMPO:Mono-caged cyclooctadiyne (6,7-Dehydrodibenzo[a,e]cyclopropa[c][8]annulen-1-one) cycloadduct



In a dry NMR tube, DMImO nitrone (4.96 mg, 0.044 mmol, 2.0 eq.) was dissolved in MeOD- d_4 (1.6 mL). A solution of monoyne (6,7-Dehydrodibenzo[a,e]cyclopropa[c][8]annulen-1-one) (5 mg, 0.022 mmol, 1.0 eq.) in $CDCl_3$ (0.4 mL) was then added to the tube and placed under an argon atmosphere and sealed. The mixture was left sealed at room temperature for 18 h. After the reaction was deemed completed by observing the disappearance of cyclopropene alkene peak by NMR, the crude mixture was directly purified by preparative TLC (MeOH:DCM, 1:99). 5.3 mg (71% yield) of yellow oil product was collected. 1H NMR (300 MHz, $CDCl_3$) δ 7.61 – 7.55 (m, 1H), 7.55 – 7.49 (m, 2H), 7.48 – 7.35 (m, 3H), 7.34 – 7.27 (m, 1H), 7.24 (dd, J = 7.5, 1.3 Hz, 1H), 5.13 (dd, J = 7.9, 3.5 Hz, 1H), 2.03 (td, J = 8.0, 4.1 Hz, 1H), 1.94 – 1.81 (m, 1H), 1.81 – 1.62 (m, 2H), 1.33 (s, 3H), 1.16 (s, 3H).



EMPO:Mono-caged cyclooctadiyne (6,7-Dehydrodibenzo[a,e]cyclopropa[c][8]annulen-1-one) cycloadduct



In a dry NMR tube, EMPO nitronc (7.53 mg, 0.044 mmol, 2.0 eq.) was dissolved in MeOD-d₄ (1.6 mL). A solution of monoyne (6,7-Dehydrodibenzo[a,e]cyclopropa[c][8]annulen-1-one) (5 mg, 0.022 mmol, 1.0 eq.) in CDCl₃ (0.4 mL) was then added to the tube and placed under an argon atmosphere and sealed. The mixture was left sealed at room temperature for 18 h. After the reaction was deemed completed by observing the disappearance of cyclopropene alkene peak by NMR, the crude mixture was directly purified by preparative TLC (MeOH:DCM, 1:99). The product was recovered as a yellow oil product; product yield was not

determined. ^1H NMR (300 MHz, CDCl_3) δ 7.71 – 7.27 (m, 8H), 5.32 (dd, $J = 7.1, 3.5$ Hz, 1H), 4.22 (q, $J = 7.2$ Hz, 2H), 2.40 – 2.24 (m, 1H), 1.99 – 1.82 (m, 2H), 1.79 – 1.65 (m, 1H), 1.55 (s, 3H), 1.29 (td, $J = 7.1, 0.5$ Hz, 3H).

