

Bioorthogonal Chemical Reactions of Alkenes

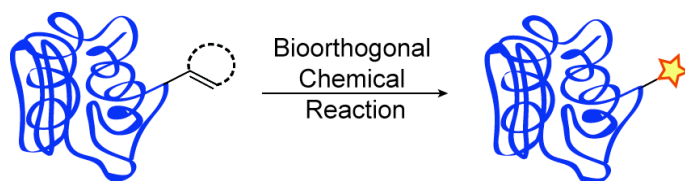
Ellen M. Sletten

Department of Chemistry, University of California, Berkeley, 94720

esletten@berkeley.edu

June 5, 2009

ABSTRACT



A growing area of reaction methodology focuses on the development of chemical transformations with exquisite selectivity, kinetics, and biocompatibility. These transformations are termed “bioorthogonal chemical reactions” and can be used to label biomolecules *in vitro* and *in vivo*. Many of these reactions employ azides or ketone/aldehydes as one of the reagents. However, chemistries involving alkenes are beginning to be utilized for proteins and oligonucleotide modification. Here bioorthogonal chemical reactions involving alkenes are highlighted.

In recent years, chemists have joined forces with biologists to develop new methods to study biomolecules. A key component to many of these approaches is bioorthogonal chemical reactions, or reactions that do not interfere with biology.¹ The functional groups involved in such reactions must react rapidly and selectively with each other yet be inert to the plethora of functionality found within living systems (Figure 1A). Typically, a two-step strategy is employed to gather insightful information regarding a biological process from a bioorthogonal chemical reaction (Figure 1B). In the first step, one of the functional groups involved in the reaction is incorporated into a biomolecule through metabolic uptake of an unnatural substrate. The size of the functional group is essential for the success of this first step, as the perturbation to the metabolite must be small enough that the organism’s biosynthetic machinery accepts the modified metabolite. Upon metabolic incorporation of the unnatural functional group (also referred to as the chemical reporter), the biomolecule can be detected in a second step using a bioorthogonal ligation with the complementary

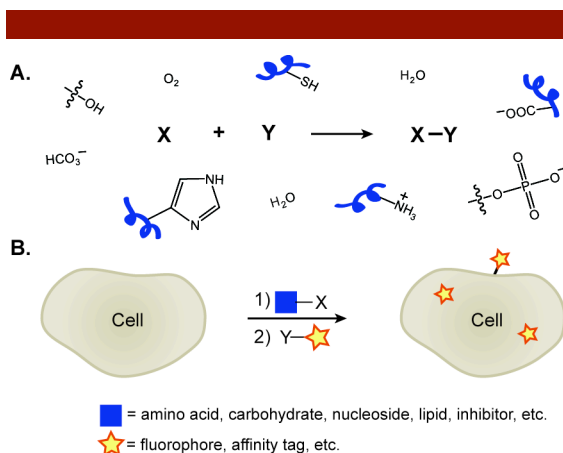


Figure 1. **A.** Generic representation of bioorthogonal chemical reaction between X and Y. **B.** Application of bioorthogonal chemical reactions to living systems. A metabolite is adorned with unnatural functional group X and incorporated into biomolecules via the cell’s biosynthetic machinery. A detection reagent labeled with complementary functional group Y allows for analysis of the labeled biomolecule.

¹ Prescher, J.A.; Bertozzi, C.R. *Nat. Chem. Biol.* **2005**, *1*, 13.

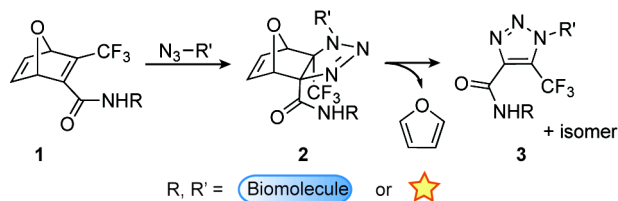
reactive group conjugated to a probe (e.g., fluorophore or affinity tag). Bioorthogonal chemical reactions have successfully been used to study proteins, glycans, lipids, and nucleic acids in living systems.² Originally, ketones or aldehydes were used as chemical reporter groups and detected with amino-oxy or hydrazide reagents.³ Unfortunately, these reactions are not optimal at physiological pH, and due to the presence of intracellular ketone and aldehyde metabolites, are limited to cell-surface labeling. The azide has widely replaced the ketone/aldehyde as a chemical reporter group.⁴ Azides are small, kinetically stable and absent from endogenous metabolites. Azides can be detected through the Staudinger ligation with triarylphosphine reagents,⁵ the copper-catalyzed cycloaddition between terminal alkynes and azides (CuAAC),⁶ or [3 + 2] cycloaddition with strained cyclooctynes.⁷ While the azide is an ideal chemical reporter, the bioorthogonal chemical reactions involving the azide have limitations and their improvement is an ongoing area of research. The Staudinger ligation suffers from slow reaction kinetics and background phosphine oxidation.⁵ CuAAC is a rapid reaction; however, the Cu(I) catalyst is toxic to cells, limiting its applications.⁸ The strain-promoted cycloaddition with fluorinated cyclooctyne reagents is currently the optimal bioorthogonal reaction for the azide, but the cyclooctynes are difficult to synthesize and still exhibit reactivity only one order of magnitude faster than the Staudinger ligation.^{7b}

In order to expand the arsenal of bioorthogonal chemical reactions, multiple groups have recently looked to the alkene. Although alkenes are naturally found in lipids, fatty acids, cofactors and other natural products, most of these new bioorthogonal reactions employ activated alkenes that should be able to react selectively in the presence of endogenous olefins. Many of these reactions have yet to be used extensively in biological systems, but in all cases their bioorthogonality has been demonstrated in model systems.

In a manner similar to the strain-promoted cycloaddition, azides react with strained alkenes. The product of an alkene-azide [3 + 2] cycloaddition is a triazolone, which is significantly less stable than an aromatic triazole and not ideal for applications where an irreversible covalent bond is desired.⁹ Rutjes and coworkers have avoided this problem

by employing an oxanorbornadiene as a dipolarophile. The optimized oxanorbornadiene, **1**, contained an electron-deficient alkene that reacted with azides to produce triazolone **2**, that immediately underwent a retro Diels-Alder reaction to extrude furan and yield the stable, aromatic triazole product **3** (Scheme 1).¹⁰

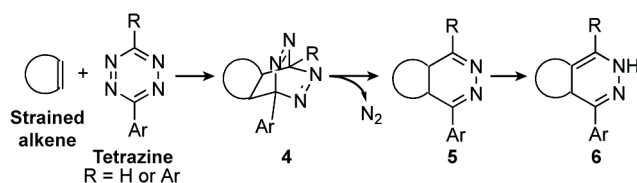
Scheme 1. The cycloaddition of oxanorbornadienes and azides.



Using this metal-free triazole formation, Rutjes and coworkers were able to selectively label an oxanorbornadiene-functionalized protein¹⁰ and an azide-functionalized cyclic peptide.¹¹ The requisite oxanorbornadiene reagents are easy to synthesize; however, the kinetics of this cycloaddition are quite slow (second-order rate constant, $k \sim 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$) and has limited its widespread use.

Strained alkenes have also been used in inverse-electron demand Diels-Alder reactions with tetrazines (Scheme 2). The initial alkene-tetrazine reaction yields cycloadduct **4**, which loses nitrogen to produce dihydropyridazine **5**, which tautomerizes to final ligation product **6**. Recently, three groups independently reported the reaction between aryl tetrazines and olefins activated through ring strain (Scheme 3).

Scheme 2. The inverse electron-demand Diels-Alder reaction of strained alkenes and tetrazines.



Fox and coworkers developed the reaction of *trans*-cyclooctene **7** with bipyridyl tetrazine **8** to yield **9** and its isomers (Scheme 3A).¹² This reaction is the fastest bioorthogonal chemical reaction reported to date ($k \sim 10^3$

² Sletten, E.M.; Bertozzi, C.R. *Angew. Chem. Int. Ed.* **2009**, In press.

³ (a) Cornish, V.W.; Hahn, K.M.; Schultz, P.G. *J. Am. Chem. Soc.* **1996**, *118*, 8150. (b) Mahal, L.K.; Yarema, K.J.; Bertozzi, C.R. *Science* **1997**, *276*, 1125.

⁴ Baskin, J.M.; Bertozzi, C.R. *QSAR Comb. Sci.* **2007**, *26*, 1211.

⁵ Saxon, E.; Bertozzi, C.R. *Science* **200**, 287, 2007.

⁶ (a) Rostovtsev, V.V.; Green, L.G.; Fokin, V.V.; Sharpless, K.B. *Angew. Chem. Int. Ed.* **2002**, *41*, 2596. (b) Tornøe, C.W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057.

⁷ (a) Agard, N.J.; Prescher, J.A.; Bertozzi, C.R. *J. Am. Chem. Soc.* **2004**, *126*, 15046. (b) Baskin, J.M.; Prescher, J.A.; Laughlin, S.T.; Agard, N.J.; Chang, P.V.; Miller, I.A.; Lo, A.; Codelli, J.A.; Bertozzi, C.R. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16793.

⁸ (a) Wolbers, F.; ter Braak, P.; Le Gac, S.; Luttge, R.; Andersson, H.; Vermes, I.; van den Breg, A. *Electrophoresis*, **2006**, *27*, 5073. (b) Sletten, E.M.; Beatty, K.E.; Bertozzi, C.R. Unpublished results.

⁹ Padwa, A. *1,3-Dipolar Cycloaddition Chemistry* Wiley-Interscience, New York, **1984**.

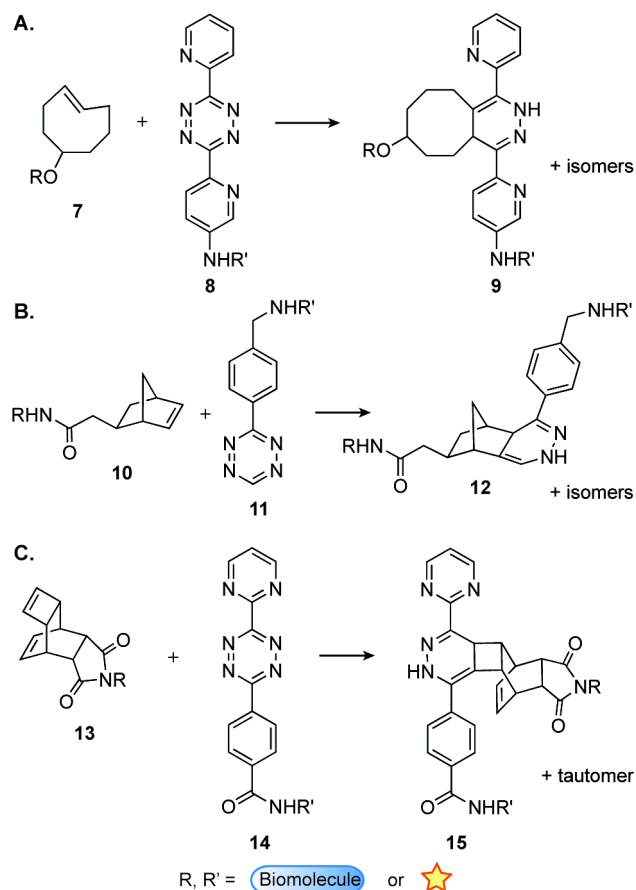
¹⁰ Van Berkel, S.S.; Dirks, A.J.; Debets, M.F.; van Delft, F.L.; Cornelissen, J.J.L.M.; Nolte, R.J.M.; Rutjes, F.P.J.T. *ChemBioChem* **2007**, *8*, 1504.

¹¹ Van Berkel, S.S.; Dirks, A.J.; Meeuwissen, S.A.; Pinggen, D.L.L.; Boerman, O.C.; Laverman, P.; van Delft, F.L.; Cornelissen, J.J.L.M.; Rutjes, F.P.J.T. *ChemBioChem*, **2008**, *9*, 1805.

¹² Blackman, M.L.; Royzen, M.; Fox, J.M. *J. Am. Chem. Soc.* **2008**, *130*, 13518.

$M^{-1}s^{-1}$). In simple biological systems which lack thiols, tetrazine **8** was very effective at selectively modifying *trans*-cyclooctene, as demonstrated by the reaction of *trans*-cyclooctene-functionalized thioredoxin and dipyrindyl tetrazine **8**. However, tetrazine **8**, which had been optimized for reaction rate, displays some background reactivity with amines, thiols, and water, which will hamper its use in complex biological systems.

Scheme 3. Strained alkenes and aryl tetrazines used for modification of biomolecules.



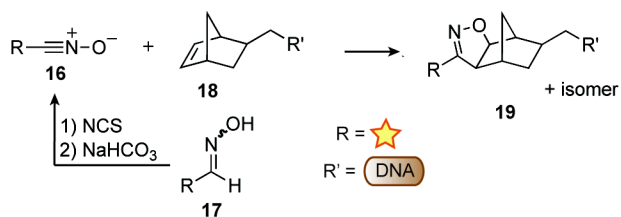
Hilderbrand and coworkers developed an alkene-tetrazine ligation and used it to label an antibody on live cells. The Hilderbrand version of the tetrazine ligation (Scheme 3B) involves the reaction of norbornene **10** and mono-aryl tetrazine **11** to yield ligation product **12** (and isomers).¹³ This reaction is slower ($k \sim 1 M^{-1}s^{-1}$) than the reaction reported by Fox and coworkers but still faster than the strain-promoted cycloaddition ($k \sim 10^{-1} M^{-1}s^{-1}$). The bioorthogonality of this reaction was tested by the conjugation of norbornene and rhodamine to a monoclonal antibody. The doubly labeled antibody was introduced to cells expressing the corresponding antigen. After allowing time for clearing of excess antibody, the cells were

incubated with tetrazine **11** linked to a near IR dye (VT680). The cells were imaged and robust colocalization between the rhodamine and VT680 was observed, indicating that the tetrazine ligation is selective for norbornene on live cells.

The third tetrazine ligation was developed by Pipkorn and coworkers as a method to ligate a peptide to the drug candidate TMZ.¹⁴ This ligation involved the Diels-Alder reaction of cyclobutene **13** and biaryltetrazine **14** to form product **15** and its tautomer (Scheme 3C). The kinetics and selectivity of the ligation reaction were not analyzed in this report. However, the reaction is compatible with aqueous solution containing amines and disulfides.¹⁴

The final bioorthogonal reaction involving strained alkenes developed to date is the 1,3-dipolar cycloaddition between nitrile oxide **16**, which is generated in two steps from oxime **17**, and norbornene **18** to yield *exo*-1,5 isoxazoline product **19** and the corresponding *exo*-1,4 isoxazoline isomer. Carell and coworkers have applied this reaction to the modification of norbornene-functionalized oligonucleotides.¹⁵ While this reaction is useful for the modification of DNA either on the solid phase or in solution, it is rather sluggish and has yet to be tested in complex environments.

Scheme 4. The cycloaddition of nitrile oxides and norbornene used for DNA modification.



Light can also be used to activate a 1,3-dipolar cycloaddition involving alkenes. Lin and coworkers have developed “photoclick chemistry,” which involves the cycloaddition of alkenes and nitrile imines, which are generated *in situ* by UV irradiation of tetrazoles. The first report of this bioorthogonal reaction involved the cycloaddition of nitrile imine **20**, generated from irradiation of tetrazole **21** with 290 nm light, with electron-deficient alkene **23** to yield pyrazoline **24** (Scheme 5).¹⁶ The formation of dipole **20** is fast ($k \sim 10^{-1} s^{-1}$), as is the cycloaddition ($k \sim 10^1 M^{-1}s^{-1}$). Since the initial report, the aryl groups on the tetrazole have been optimized so that lower energy light (365 nm), which is less harmful to biological systems, can be used to generate the nitrile

¹³ Devaraj, N.K.; Weissleder, R.; Hilderbrand, S.A. *Bioconjugate Chem.* **2008**, *19*, 2297.

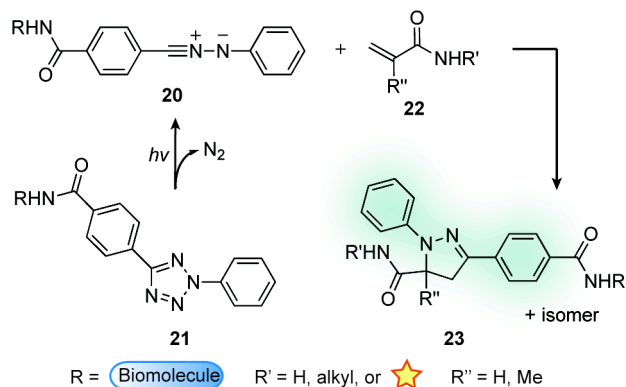
¹⁴ Pipkorn, R.; Waldeck, W.; Didinger, B.; Koch, M.; Mueller, G.; Wiessler, M.; Braun, K. *J. Pept. Sci.* **2009**, *15*, 235.

¹⁵ Gutsmidl, K.; Wirges, C.T.; Ehmke, V.; Carell, T. *Org. Lett.* **2009**, ASAP.

¹⁶ Song, W.; Wang, Y.; Qu, J.; Madden, M.M.; Lin, Q. *Angew. Chem. Int. Ed.* **2008**, *47*, 2832.

imine.¹⁷ The additional advantage of the alkene-tetrazole reaction is that the resulting pyrazoline cycloadducts are fluorescent, facilitating direct detection of the reaction within a biological system.

Scheme 5. The photoactivated 1,3-dipolar cycloaddition between nitrile imines, generated from biaryl tetrazoles, and alkenes.



Since photoclick chemistry does not require a large, strained alkene, it has greater utility for studying biomolecules in their native environments. Unnatural alkenes have been incorporated into proteins using a variety of unnatural amino acid metabolites.¹⁸ Lin and coworkers have used *O*-allyl tyrosine (**24**) to incorporate olefins into a Z-domain protein in *E. coli* using Peter Schultz's amber stop codon methodology.^{18b,19} The *E. coli* were then subjected to tetrazole **25** and 302 nm light. Microscopy of the *E. coli* showed that the reaction proceeded selectively in this complex environment. The olefin of *O*-allyl tyrosine was less activated than the

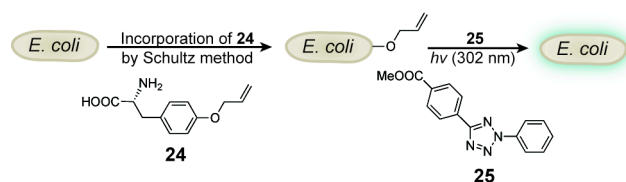


Figure 2. The application of photoclick chemistry to a living system.

originally reported alkene, resulting in a reaction that was 75 times slower.¹⁹ The decreased reaction kinetics necessitated an overnight reaction, which hampers the ability of photoclick chemistry to detect rapid biological

processes. Nevertheless, with optimization, photoclick chemistry has the potential to be used in a variety of biological systems.

Within the last decade, bioorthogonal reactions have become essential tools for chemical biologists. The impact that these chemistries can have on deciphering biological problems has been recognized, and chemists have taken an active role in expanding the repertoire of reactions that can be performed under physiological conditions. Recently, strained alkenes have been utilized as dienophiles and dipolarophiles in bioorthogonal chemical reactions. Many of these new reactions are faster than existing transformations. To date, the main limitation to the majority of the reactions described above is that the strained alkenes are rather large, preventing their metabolic incorporation. The only bioorthogonal reaction for the alkene that has successfully been used to detect a biomolecule that was modified using an organism's biosynthetic machinery was photoclick chemistry. While photoclick chemistry with unactivated olefins is slow, further optimization of the tetrazole or metabolic incorporation of an electron deficient alkene could increase the reaction kinetics.

The reactions discussed herein exemplify progress for the development of new bioorthogonal chemical reactions in two important areas: pericyclic reactions and alternative forms of energy. All the transformations described are cycloadditions which are poised for use in biological systems. Pericyclic reactions are advantageous because they undergo rate enhancement in aqueous solution²⁰ and their concerted mechanisms leave little room for interruption by biological nucleophiles and electrophiles. Photoclick chemistry is an important development because it represents the use of light, as opposed to heat or exogenous reagents, to create an activated reagent *in situ*. It is likely that photoclick chemistry will prompt the exploitation of light and other biocompatible modes of energy in the development of new bioorthogonal reactions.

¹⁷ Wang, Y.; Hu, W.H.; Song, W.; Lim, R.K.V.; Lin, Q. *Org. Lett.* **2008**, *10*, 3725.

¹⁸ (a) Link, A.J.; Mock, M.L.; Tirrell, D.A. *Curr. Opin. Biotechnol.* **2003**, *14*, 603. (b) Wang, L.; Schultz, P.G. *Angew. Chem. Int. Ed.* **2005**, *44*, 34.

¹⁹ Song, W.; Wang, Y.; Qu, J.; Lin, Q. *J. Am. Chem. Soc.* **2008**, *130*, 9654.

²⁰ Breslow, R. *Acc. Chem. Res.* **1991**, *24*, 159.