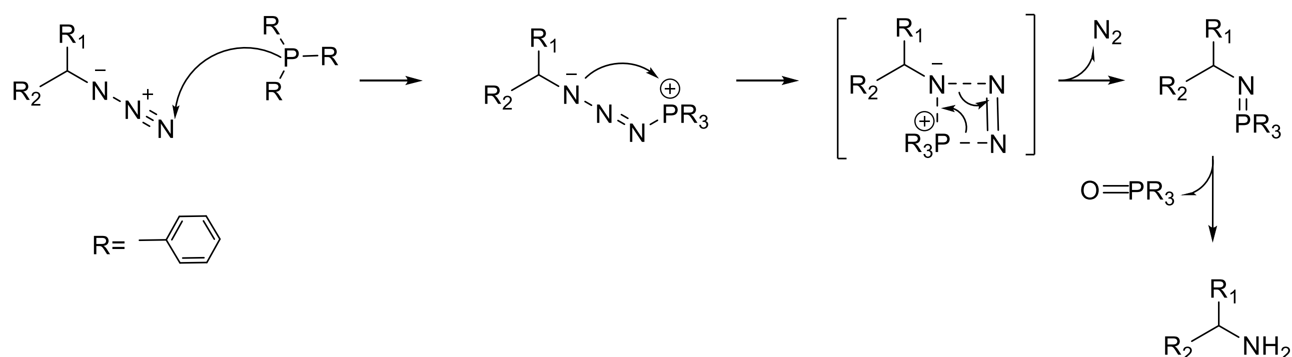
Bioorthogonal labeling of peptides and proteins-two recent examples

Can Araman

Gorlaeus Laboratories, Einsteinweg 55, 2333 CC Leiden, Netherlands email:m.c.araman@lic.leidenuniv.nl

Site-specific labeling of biomolecules *in vivo* is a challenging task. One way to do that is to perform bioorthogonal chemistry in living systems. Bioorthogonal reactions do not interact with other biochemical processes, starting materials are physiologically inert (if desired) and not immunogenic. Thus, this type of chemistry is ideal for labeling of biomolecules, specifically peptides and proteins, *in vivo*. In 2000, Bertozzi and coworkers developed one of the first bioorthogonal reactions, the Staudinger ligation, in order to study interactions of glycans with other biomolecules on the cell surface. [1] This reaction is based on the Staudinger reduction of azides into amines using phosphines (Scheme 1). [2]

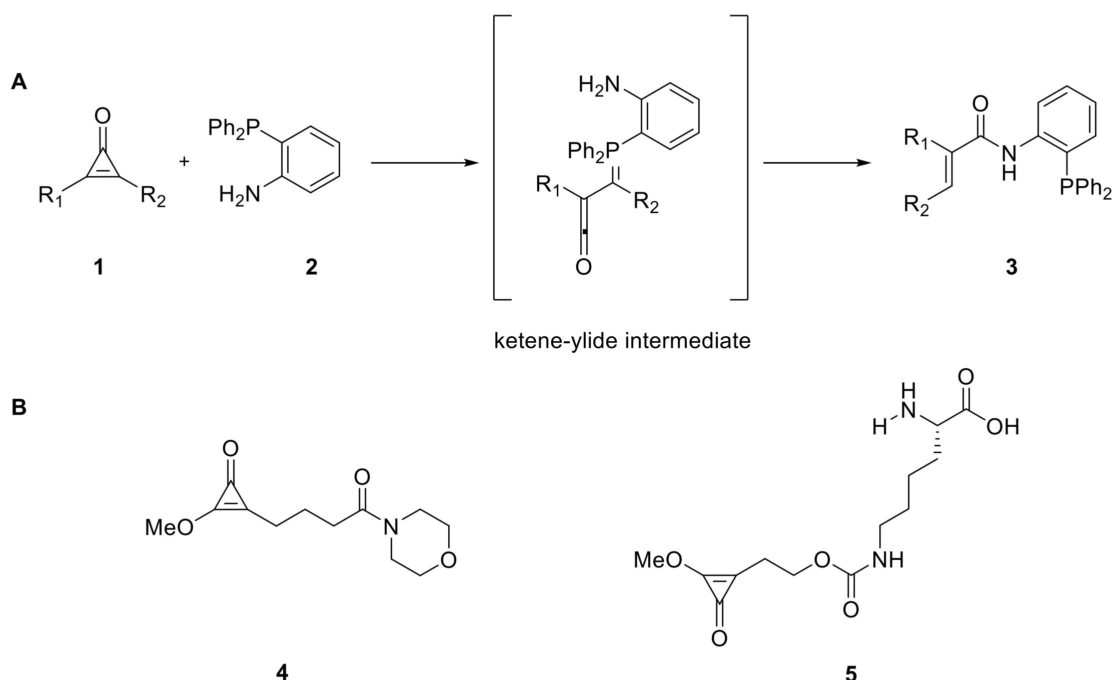


Scheme 1. Staudinger ligation

An electrophilic methoxy ester was introduced into the triphenyl core structure of the phosphine, which prevents the aza-ylide from hydrolyzing into an amine, leading instead to a nucleophilic attack of the nitrogen on the carbonyl moiety giving rise to an amide bond upon hydrolysis. The discovery of the traceless Staudinger ligation allowed conjugation of a variety of handles into biomolecules *in vitro* and *in vivo*.[3, 4] Nevertheless, phosphines are reducing agents that can be oxidized *in vivo* yielding undesired phosphine oxide byproducts. This explains ongoing efforts to improve the conditions of Staudinger-like chemistries *in vivo*. For example, the strain promoted azide alkyne cycloaddition (SPAAC) as well as the inverse electron demand Diels-Alder ligation (IEDDA) have been shown to be valuable alternatives to Staudinger-type ligations *in cellulo*. This commentary will give an overview of two recently published articles describing new bioorthogonal peptide/protein labeling reactions, cyclopropenone ligation (Row *et al. J. Am. Chem. Soc.* **2017**, 139, 7370–7375) [5] and sulfonyl based reversible amine conjugation (Cowell et al. *ChemBioChem* **2017**, 18, 1688 – 1691) [6] respectively.

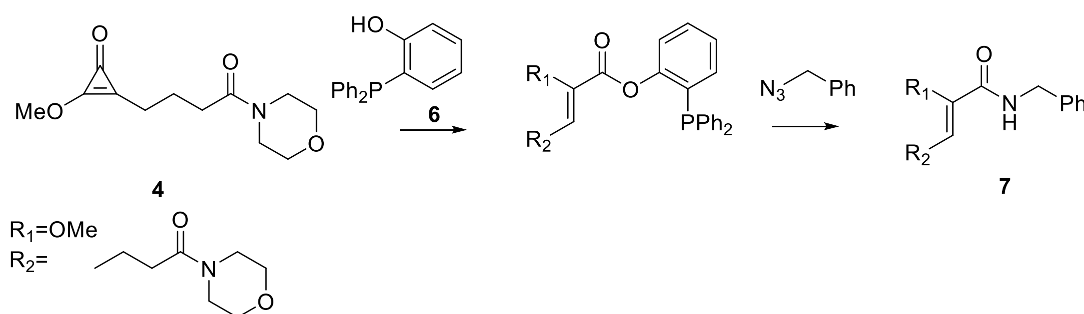
CYCLOPROPENONE LIGATION

As described previously, bioorthogonal chemistries suffer from formation of byproducts in living systems. Jennifer Prescher's lab is specialized in the improvement and optimization of distinct bioorthogonal chemistries. Among many others, one method seems to be very promising, namely the cyclopropenone conjugation. Already in 2015, Prescher and coworkers reported the synthesis of a new type of bioorthogonal reporter, mono-alkylated cyclopropenone compounds (Scheme 2A, **1**). [7] In this previous work, the researchers trapped and identified the ketene-ylide intermediate with a nucleophile. Specifically, they used a triarylphosphine nucleophile where one of the aryl moieties was an aniline (Scheme 2A, **2**). The reaction can be summarized as a Michael-type nucleophilic addition of the aniline derivative to the carbonyl moiety of a ketene-ylide intermediate giving rise to an α,β-unsaturated amide (Scheme 2A, **3**). However, the monoalkylated cyclopropenones (Figure 1, R_1=H) were susceptible to react with cysteine or other thiols in biological systems above pH 7.0, making them unsuitable for *in vivo* utilization. Thus, Prescher and coworkers recently presented the second generation of cyclopropenone compounds, which are described below.



Scheme 2. Cyclopropenone ligation mechanism

The researchers synthesized a variety of dialkylated cyclopropenones with enhanced stability towards biological thiols. 2-Methyl-3-(4-morpholino-4-oxobutyl)cycloprop-2-en-1-one (Scheme 2B, **4**) was found to be the most stable compound, yielding no byproduct when reacted with cysteine *in vitro*. Moreover, the authors successfully synthesized a non-canonical amino acid, N^{ε}-((2-(2-methyl-3-oxocycloprop-1-en-1-yl)ethoxy)carbonyl)-L-lysine (Scheme 2B, **5**), and incorporated it into an *E.coli* strain expressing GFP via amber codon suppression, a common method for site-specific incorporation of unnatural amino acids. [8] Even though the incorporation efficiency was low, the newly formed GFP-cyclopropenone (GFP-Cpo) could be ligated to a triarylphosphine-biotin conjugate and characterized in the cell lysate, whilst the wild type GFP could not. Furthermore, an attempt was made to carry out sequential labeling reactions by combining the cyclopropenone and the traceless Staudinger ligation reactions. Using in a first step compound **4** and a hydroxyaryl phosphine **6**, shown to enhance cyclopropenone ligation reactivity via H-bonding, and performing a traceless Staudinger ligation subsequently, the researchers were able to demonstrate the first *in vitro* sequential cyclopropenone- traceless Staudinger ligation, **7** (Scheme 3).



Scheme 3. Sequential cyclopropenone-traceless Staudinger ligation

In my opinion, this method will open up new possibilities in live cell imaging and labeling, once optimized for mammalian cell systems.

SULFONYL BASED REVERSIBLE AMINE CONJUGATION (REVAMINE)

In their recent ChemBioChem article, Gray and colleagues describe the reversible conjugation of functional molecules/warheads to peptides and proteins with a pH-sensitive linker. Hereby, they made use of the fact that the succinimidoyl esters of 2-alkylethylsulfonyl compounds are very prone to hydrolysis at pH > 8.0 but undergo fast aminolysis reactions at physiological pH as described four decades ago. [9] The authors present the first successful conjugation and subsequent cleavage reaction using functionalized 2-alkylethylsulfonyl linkers (Figure 1, **8**) under biocompatible conditions with two peptides ($CD31_{665–674}$: HNDDVRNHAM and the HA antigen peptide: YPYDVPDYA) and a protein, recombinant BSA, *in vitro*. Furthermore, *in cellulo* experiments were done on cell surface biomolecules harboring primary amines (proteins, sugars etc.).

In addition, the researchers used a second generation linker (Figure 1, **9**) and performed proteomics analysis of cell wall associated proteins in *E.coli*. Upon incubation with the 2nd generation RevAmine linker **9**, cells were lysed and incubated with avidin-loaded beads in order to capture biotinylated proteins. Then, the samples were treated with a buffer at pH 9.0 resulting in traceless cleavage of the linker. Subsequent trypsin cleavage with LC-MS^2 enabled an adequate proteomics analysis and characterization of labeled proteins in *E.coli*.

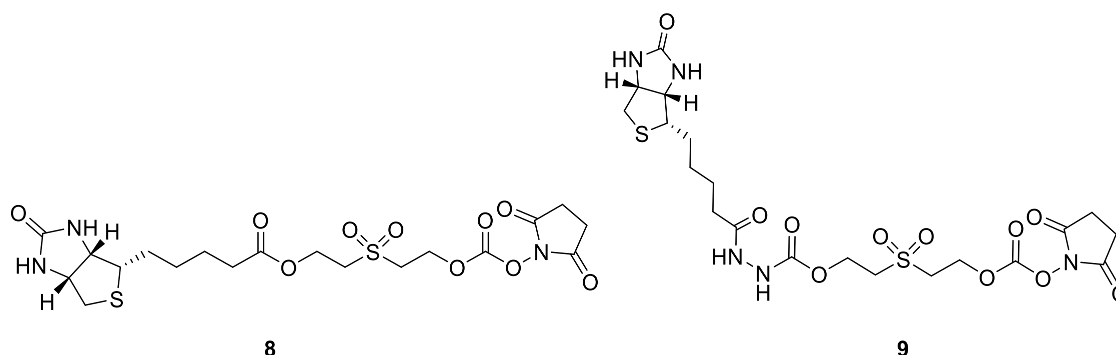


Figure 1. Revamine linkers; 8: first generation, 9: second generation

The chemistry described herein is not complicated and can be applied in the majority of peptide/protein chemistry laboratories. Nevertheless, crucial questions such as "is the linker prone to cleavage *in cellulo* and/or *in vivo*?" as well as "how can one prevent the formation of vinyl-sulfone-derived byproducts during the cleavage reaction?" are not addressed. To conclude, the method described in this publication is based on the pH-controlled cleavage of a sulfonyl-linker and is suitable for *in vitro* use. Nevertheless, there is a chance that the scope of this conjugation method can be expanded if the researchers continue to work on its application *in cellulo* and *in vivo*.

To summarize, I have given an overview of two recent biorthogonal protein/peptide labeling strategies. The first article is an expansion of a method published 2 years ago by Prescher and coworkers, who now have been able to eliminate the susceptibility of cyclopropenone derivatives towards nucleophilic addition reactions with sulfhydryl groups. Furthermore, they demonstrated that a non-canonical amino acid carrying a Cpo can be site-specifically introduced to a protein and successfully conjugated to other handles (e.g. biotin) under physiological conditions. Gray and colleagues, on the other hand, reported the utilization of the pH-driven cleavage of a reversible sulfonyl-linker for peptide and protein labeling *in vitro*. Once optimized for mammalian systems, both methods might have an impact on monitoring of cellular events.

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