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Disproportionate Impact of Named Reactions on Chemical Biology

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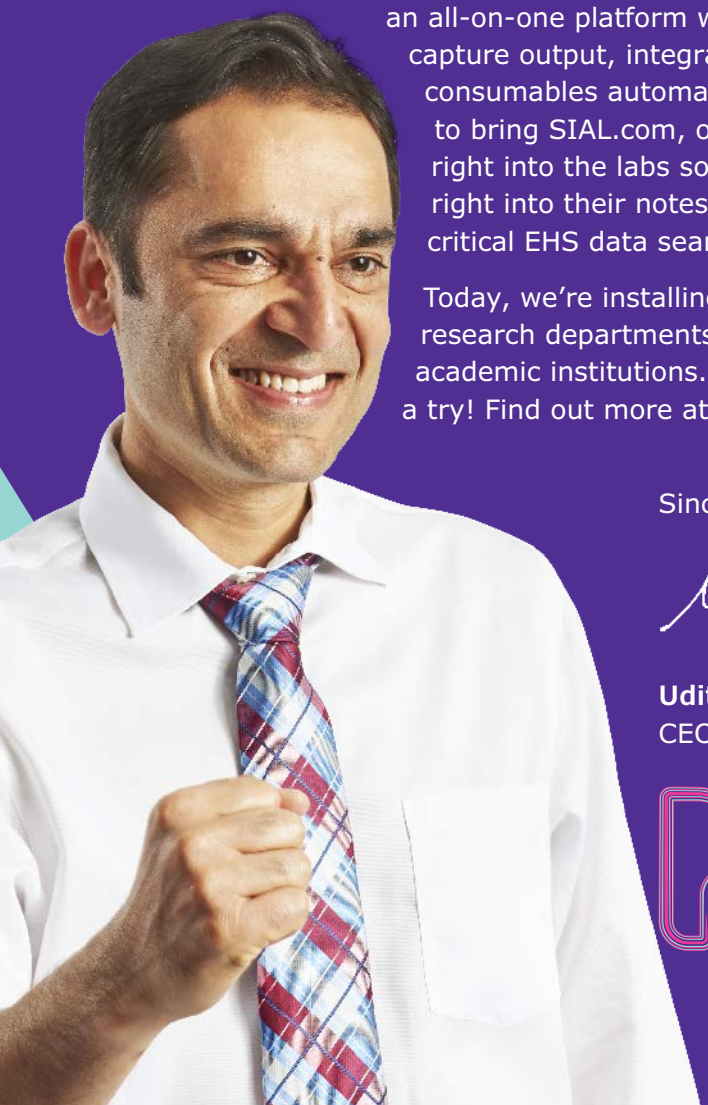

Sincerely yours,

Udit Batra, Ph.D.
CEO, MilliporeSigma

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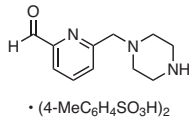
Professor Matthew B. Francis of the College of Chemistry at UC Berkeley kindly suggested that we offer 6-(1-piperazinylmethyl)-2-pyridinecarboxaldehyde bistosylate salt (**808571**). The secondary amine functional group in this reagent readily reacts with commercially available esters of *N*-hydroxysuccinimide to convert nonspecific bioconjugation reagents into labeling agents that can be used in a mild, site-specific, and versatile reaction for selectively modifying protein N-termini without requiring any genetic engineering of the target protein.

MacDonald, J. I.; Munch, H. K.; Moore, T.; Francis, M. B. *Nat. Chem. Biol*. **2015**, *11*, 326.



• $(4\text{-MeC}_6\text{H}_4\text{SO}_3\text{H})_2$

808571
(InChI Key: QDGVEYQPPPITKT-UHFFFAOYSA-N)

808571	6-(1-Piperazinylmethyl)-2-pyridinecarboxaldehyde bistosylate salt	100 mg

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ABOUT OUR COVER

Nymphenburg Palace, Munich (oil on canvas, 68.4 × 119.8 cm) was painted ca. 1761 by the renowned urban landscape painter Bernardo Bellotto (1722–1780). Born and raised in Venice, Italy, Bernardo apprenticed, starting at the age of 13, with his maternal uncle, Antonio Canaletto, another famed Venetian vedutista, or cityscape artist. Bellotto proved to be a very talented student and, early in his career, a faithful imitator of his uncle's painting methods and style. Bellotto spent his early twenties travelling to, and painting, various Italian cities and sites. At age 25, he left Venice permanently, and spent the rest of his life successfully executing commissioned work in the service of royal courts in Saxony and Poland. The result was an impressive, accurate record* of several cities and palaces of central Europe.

Detail from ***Nymphenburg Palace, Munich***. Photo courtesy National Gallery of Art, Washington, DC.

This panoramic view of Nymphenburg Palace, is part of this record and beautifully illustrates Bellotto's exacting style of painting urban landscapes that are not only grand, but also rich in detail about the inhabitants and their daily lives. Even though Bellotto was also an able etcher and draftsman, nevertheless the realism and precision exhibited in this and many of his other compositions are so extraordinary that they beg the question of whether Bellotto's initial sketches for these compositions were produced with the help of a camera obscura, a device well known at the time and already in use by other artists.

This painting is part of the Samuel H. Kress Collection at the National Gallery of Art, Washington, DC.

** Bellotto's cityscapes are so realistic and detailed that they proved invaluable almost 200 years later. To find out more, visit* ***SigmaAldrich.com/acta502***

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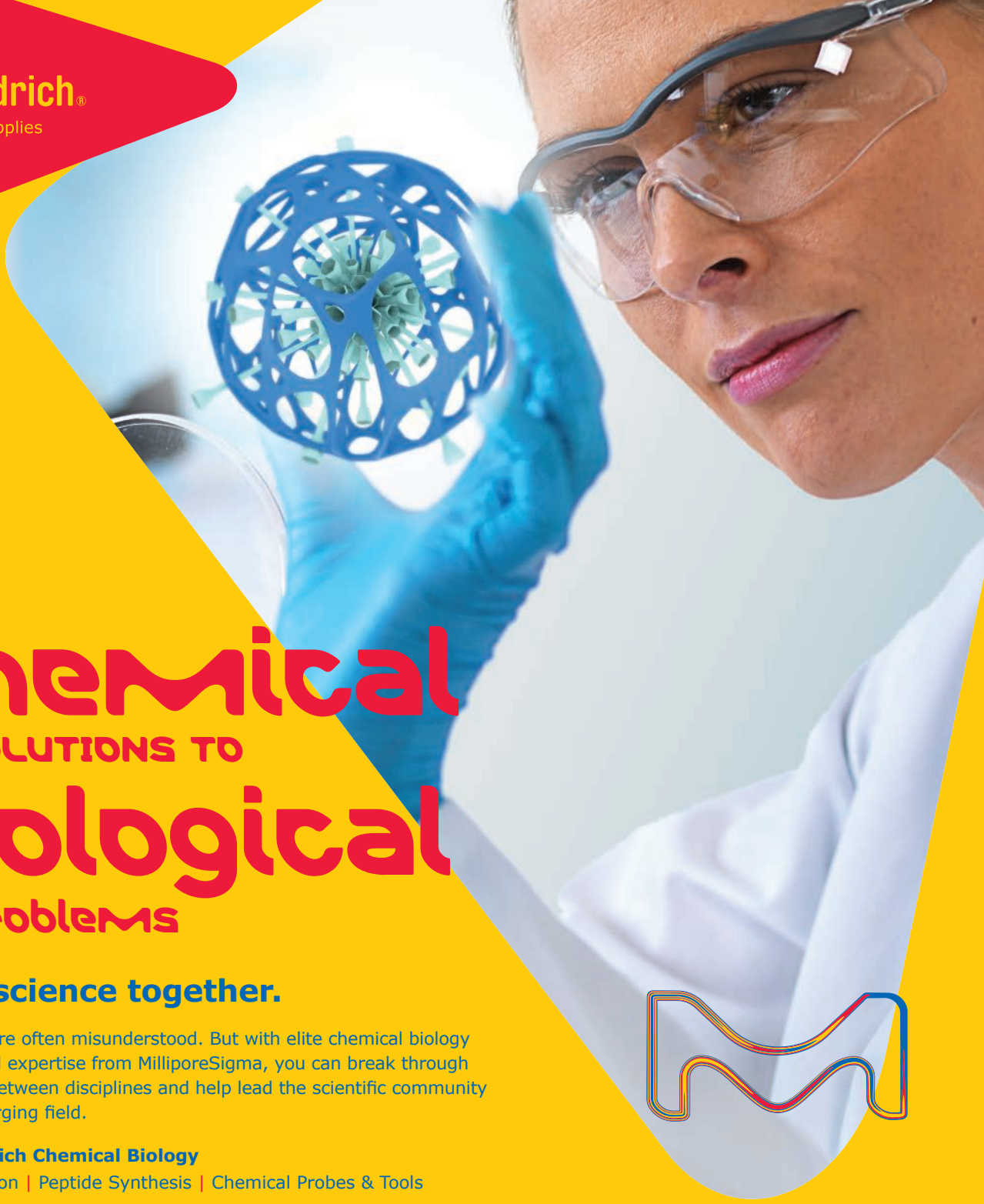

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Disproportionate Impact of Named Reactions on Chemical Biology


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Keywords. chemistry tools; imaging studies; bioconjugation; small molecules; organic chemistry; synthesis.

Abstract. Despite their long, rich history in organic chemistry, named reactions have only recently been applied to chemical biology. This review highlights twelve named reactions that have been applied with great success in chemical biology studies.

Outline

1. Introduction

In 1822, Serullas unintentionally synthesized iodoform and potassium formate from iodine, potassium metal, and ethanol.[1] What he observed was the disproportionation of iodine in the presence of potassium ethoxide generated in situ to yield ethyl hypoiodite. This species rapidly loses hydroiodic acid to generate acetaldehyde, and sequential exhaustive iodination and ethanolysis ultimately yield the observed products. Nearly 50 years later, Lieben conducted the first comprehensive study to determine the scope of this reaction.[1] This work laid the foundation for the industrial production of various haloforms, as well as the development of a robust method to identify methyl ketone functionalities.[2] In recognition of Lieben's contributions, this transformation became known as the Lieben haloform reaction, one of the earliest named reactions in organic chemistry. Other notable examples would soon follow, typically paying homage to the inventor or scientist responsible for advancing the reaction. This reaction naming system has provided chemists at all levels with a convenient frame of reference when discussing a given chemical transformation.

With an ever-growing repertoire of chemical reactions at their disposal, organic chemists set forth to conquer some of the most challenging synthetic targets nature has to offer, doing so with remarkable creativity. Notably, pioneers such as Robert Woodward and E. J. Corey were awarded Nobel Prizes in 1965 and 1990, respectively, for their contributions to the synthesis of complex organic molecules. Other disciplines, including the pharmaceutical and biomedical sciences, have also harnessed the power of synthetic chemistry to access an impressive assortment of molecules. Likewise, synthetic chemistry has allowed scientists working at the interface of chemistry and biology to study complex biological systems with molecular precision through the development of novel chemical tools. However, named reactions merely have served as a means to an end in a majority of these cases. Thus, the aim of this review article is to survey specific named reactions that have been integrated directly into the mode of action of a chemical tool. Since many of these transformations were originally intended for chemical synthesis, careful tuning of reactivity, chemoselectivity, and biocompatibility are essential considerations when developing any tool for chemical biology. As such, we begin by outlining key criteria necessary for successful adaptation of a named reaction for biological applications. Next, we draw attention to recent examples in the literature that feature a named reaction belonging to one of three general categories: (i) pericyclic reactions, (ii) rearrangement

reactions, or (iii) metal-mediated reactions (**Figure 1**). These reactions have been selected because they offer a combination of novelty, unique chemical reactivity, and prospect for future advances. In each instance, we provide a brief historical account leading to the discovery of the reaction and offer insight into the motivation for its development. Furthermore, we discuss the specific challenges each investigator had to overcome and key biological applications. Finally, we conclude by providing a commentary on the outlook for this exciting and rapidly expanding research area.

2. Named Reaction Selection Criteria

Although there is a vast assortment of molecules used in chemical biology investigations, the examples we discuss below are broadly defined as either analyte-specific probes for molecular imaging or selective bioconjugation tools and techniques for biomolecule (e.g., protein) modification.

When developing imaging probes, the primary objective is to detect an analyte of interest in its native biological environment with minimal perturbation, typically within a live cell or animal. One strategy is to employ a named reaction that utilizes the analyte of interest as a key reagent in the transformation. In particular, the reaction must produce a discernable change in the analytical readout such as an enhancement in the fluorescence signal. Design strategies commonly employed to achieve this include chemically masking a key functional group required for emission (e.g., internal charge transfer, ICT) or tuning fluorescence via the photoinduced electron-transfer (PeT) mechanism. The specifics of these design strategies are beyond the scope of this review and have been reviewed elsewhere.[3] On the other hand, the motivations for bioconjugation include tracking, isolating, and augmenting biomolecules. However, any modification should preserve the intrinsic properties of the biomolecule (e.g., stability) since perturbation may result in undesirable consequences such as loss of function.[4] Likewise, it is essential that the reaction conditions be mild to prevent damaging the biomolecule through denaturation processes. Regardless of the application, the contemplated reaction must be (i) biocompatible, (ii) chemoselective or bioorthogonal, and (iii) fast.

A skilled chemist can exclude nearly any undesired component when performing a reaction in vitro. For instance, reagents can be rigorously dried and solvents degassed for moisture- and oxygen-sensitive reactions, respectively. However, this is challenging, if not impossible, in a biological context. As such, any chemical tool or reaction partner must be competent in an aqueous environment. This prerequisite may limit the use of many reactions requiring reagents that are not intrinsically soluble in water or those that are sensitive to hydrolysis. However, as detailed in the following sections, numerous creative approaches can be employed to overcome these constraints.

Chemoselectivity or bioorthogonality is another important criterion, especially for analyte-specific probes, where it is important to unambiguously demonstrate that a signal change is due to detection of the intended target and not from competing species with similar functional groups. Thus, it is highly desirable to begin with a reaction that is intrinsically chemoselective. Incidentally, for a reaction lacking specificity, it is possible to fine-tune its reactivity for a particular application. For example, decreasing the electrophilicity of a tool will favor reactivity with only the most nucleophilic analyte. Similarly, installation of a bulky chemical group can provide steric hindrance to exclude reactivity with larger, off-target molecules. Indeed, many other approaches are available to achieve excellent chemoselectivity. With regard to bioconjugation, careful selection of reactions that utilize abiotic functional groups (e.g., azide and alkyne groups) is important since this decreases the likelihood of cross-reactivity with physiological processes.

Finally, a named reaction should proceed with permissible kinetics at physiological temperatures. Analyte-specific probes typically require a reaction that is sufficiently fast to intercept the target before it is depleted by normal cellular mechanisms. This is often the case with highly reactive metabolites and signaling molecules such as nitroxyl (HNO), which is present only momentarily and at low concentrations.[5] In the same way, since bioconjugation is employed to identify and track rapid and dynamic cellular processes, a large rate constant is also essential.

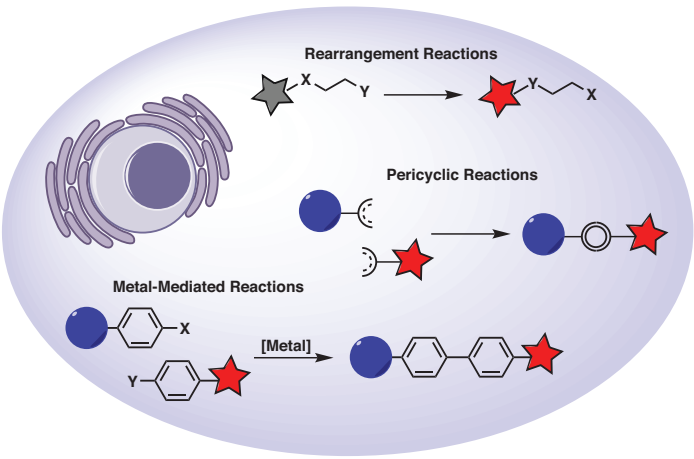


Figure 1. Overview of Reaction Classes Employed in Chemical Tools for Biological Studies.

3. Pericyclic Reactions

Pericyclic reactions are those that proceed through a concerted mechanism and are characterized by a cyclic transition state.[6] These reactions, both unimolecular (e.g., sigmatropic rearrangements) and bimolecular (e.g., intermolecular cycloadditions), are appealing to the synthetic chemist due to their ability to rapidly generate multiple new stereocenters. Nature is also believed to use such reactions to generate complex products, but the prevalence of common reactive partners, such as *s-cis*-dienes, is very low.[7] Thus, pericyclic reactions are attractive to chemical biologists particularly for their potential of affording excellent chemoselectivity and bioorthogonality. In the following subsections, we feature named pericyclic reactions that demonstrate these characteristics in their applications as analyte-specific probes and bioconjugation techniques.

3.1. 2-Aza-Cope Rearrangement

In 1950, Horowitz and Geissman reported that treating α-allylbenzylamine (**1**) with formic acid and formaldehyde at 90–100 °C resulted in the formation of benzaldehyde and trialkylamine **2**, rather than the anticipated α-allylbenzyldimethylamine product (**eq 1**, Part (a)).[8] By excluding formic acid, it was determined that formaldehyde mediates C–N bond cleavage through sequential [3,3]-sigmatropic rearrangement and hydrolysis. Owing to its similarity to the Cope rearrangement, this transformation became known as the 2-aza-Cope rearrangement. However, unlike the Cope rearrangement, which was first reported to proceed at 150–160 °C,[9] the 2-aza-Cope rearrangement can take place under milder conditions (even at room

temperature), especially when accelerated by a positive charge.[10] Because formaldehyde plays an integral role in lowering the activation barrier through iminium ion formation, our group postulated that the 2-aza-Cope rearrangement could be employed to develop a fluorescent probe, **FP1** (eq 1, Part (b)), for visualizing endogenous formaldehyde in living systems.[11] Formaldehyde has attracted significant interest due to its potential role in mediating long-term memory formation and aberrant accumulation in tumors. However, prior to the development of **FP1**, methods of formaldehyde detection involved indirect and invasive protocols, which typically led to the destruction of the biological sample being analyzed. In contrast, formaldehyde readily condenses with the secondary amine of **FP1** under physiological conditions to yield an iminium intermediate. This in turn undergoes a facile 2-aza-Cope rearrangement and hydrolysis to cleave a pendant 4-nitrobenzylamine, which had been installed to quench the fluorescence of the probe. With **FP1** in hand, we successfully imaged formaldehyde non-invasively in multiple cell lines using confocal microscopy and corroborated the results with flow cytometry. At the same time, Chang reported the development of **FAP1**, a spiro-amine-based formaldehyde probe that also utilizes the 2-aza-Cope rearrangement.[12] More recently, Chang developed **FAP488**, which features a modified formaldehyde-selective trigger based on β-elimination of the ketone intermediate.[13] Notably, the Thorpe–Ingold effect was exploited in this example to accelerate the sigmatropic rearrangement.

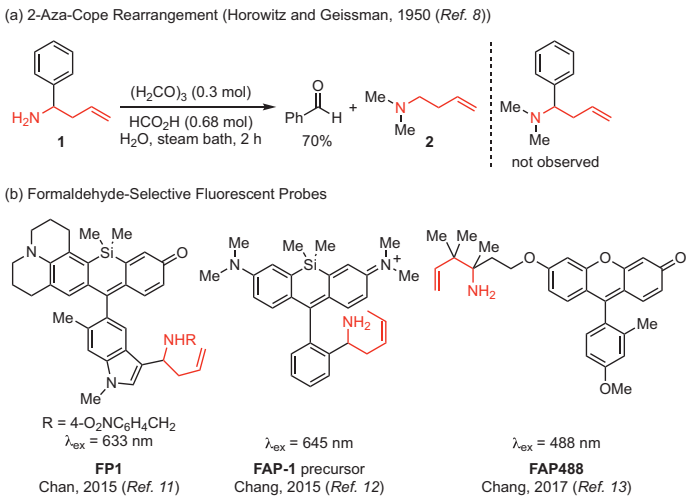
3.2. Alkyne–Azide Huisgen Cycloaddition

Although the alkyne–azide Huisgen cycloaddition is widely recognized today as the gold standard of click chemistry, this was not always the case. In 1910, Dimroth and Fester reported the formation of 1,2,3-triazole products after acetylene and hydrazoic acid or phenyl azide were heated at 100 °C in closed reaction vessels for 70 hours (**Scheme 1**, Part (a)).[14a] The reaction went unappreciated for some 50 years until Huisgen demonstrated its general utility using various 1,3-dipoles and dipolarophiles.[14b] Even then, these studies were performed in organic solvents, and the reaction rates were slow near physiological temperatures.[15] In 2002, Meldal[16] and Fokin and Sharpless[17] independently reported that Cu(I) could accelerate the cycloaddition using Cu(I) salts and Cu(II) salts under reducing conditions, respectively. In both cases, Cu(I) acetylides generated in situ from terminal alkyne precursors could readily react with organic azides at room temperature. Within a year, this reaction was employed for the fluorescent labeling of the functionalized cowpea mosaic virus (CPMV),[18] and for activity-based protein profiling where proteins in the proteasome can be enriched and identified.[19]

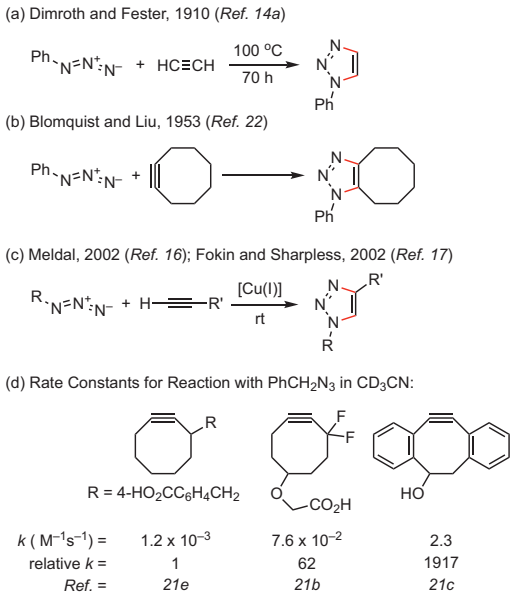
Unfortunately, the use of redox active metal ions such as Cu(I) in biological systems can generate toxic reactive oxygen species through Fenton-like chemistry.[20] To address these concerns, Bertozzi and others developed a series of substituted cyclooctyne reagents (e.g., 3,3-difluorinated cyclooctynes) that were optimized for a Cu(I)-free, strain-promoted variant of the Huisgen cycloaddition reaction.[21] This work was based on an earlier report by Blomquist and Liu that phenyl azide reacts violently with cyclooctyne to afford the corresponding 1,2,3-triazole.[22] Indeed, DIFO (3,3-difluorocyclooctyn-6-oxylacetic acid) and its congeners exhibited rate constants suitable for bioconjugation applications,[21] many of which are highlighted in another review article.[23] Notably, both strain-promoted and Cu(I)-catalyzed methods remain popular in facilitating biological advances that include discovering electrophilic modifications on human proteins,[24] identifying proteins secreted by virulent bacteria,[25] and illuminating the selectivity in the *S*-acylation of proteins.[26]

3.3. Ozonolysis

The reaction between ozone (1,3-dipole) and an alkene (dipolarophile) is another example of a Huisgen 1,3-dipolar cyclcoaddition that has recently been utilized in chemical biology. Harries published a detailed investigation in 1905 on the reactivity of ozone with unsaturated compounds, noting that, under aqueous conditions, the rapidly formed ozonide intermediate decomposes to yield two carbonyl species and hydrogen peroxide (**Scheme 2**).[27] This reactivity has been exploited in water purification processes motivating the development of first-generation probes to quantify ozone levels.[28] Using these probes, ozone was recently shown to be generated in human neutrophils[29]



eq 1



Scheme 1. Huisgen Azide–Alkyne Cycloaddition and Improvements in the Kinetics of Bioconjugation.

and atherosclerotic arteries.[30] However, these early examples reported significant cross-reactivity with singlet oxygen ($^1O_2$).[31] Koide addressed this limitation by developing **3**, a probe which features a homoallyl group appended to a dichlorofluorescein dye platform.[31] Selectivity was achieved because only ozone reacts with the trigger through a pathway first disclosed by Criegee.[32] In particular, a [3 + 2] cycloaddition sets in motion a series of rearrangements that generate two carbonyl species through oxidative cleavage of the alkene precursor. The resulting β-ether aldehyde, **4**, eliminates acrolein and releases compound **5**, which is highly fluorescent. In contrast, reaction of **3** with $^1O_2$ yields an uncleaved, non-fluorescent product, **6**. Using probe **3**, Koide successfully detected ozone in air samples and in BEAS-2B human bronchial epithelial cells.[31]

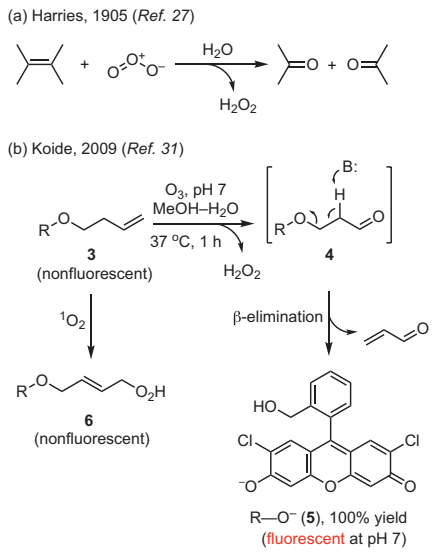
3.4. Diels–Alder Reaction

In 1928, Diels and Alder published the first in a series of articles on the [4 + 2]-cycloaddition reaction between electron-rich dienes and electron-poor dienophiles (**Scheme 3**, Part (a)).[33] This general reaction class would eventually bear their names in recognition of their seminal contributions. A major advance came in 1980 when Breslow disclosed a Diels–Alder cycloaddition that proceeded 700-times faster in water than in a nonpolar hydrocarbon solvent.[34] Owing to its compatibility with aqueous media and the relatively low abundance of *s-cis*-dienes in biological systems, the Diels–Alder reaction became one of the earliest transformations to be applied for bioconjugation. For instance, it was demonstrated that dienes could be site-selectively incorporated into RNA to enable subsequent modification with dienophiles such as maleimide via the Diels–Alder reaction (Scheme 3, Part (b)).[35]

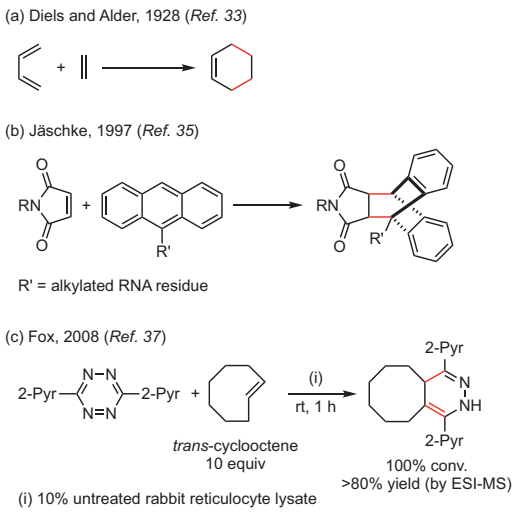
Unfortunately, maleimides are also known to react with thiol nucleophiles such as those found in protein cysteine residues.[36] This limitation prompted Fox to explore the *s*-tetrazine-based, inverse-electron-demand Diels–Alder reaction as a possible alternative (Scheme 3, Part (c)).[37] Electron-poor *s*-tetrazines were found to react with electron-rich or strained alkenes in a [4 + 2]-cycloaddition manner. Subsequently, a rapid retro-[4 + 2]-cycloaddition releases N_2, resulting in the irreversible formation of a dihydropyridazine. In addition to improved bioorthogonality, the rate of this inverse-electron-demand Diels–Alder reaction (minutes)[38] was found to be faster than that of the Diels–Alder reaction (hours).[35,39] The kinetic efficiency in combination with the cell-permeability of the *s*-tetrazine-appended dyes made this an appealing tool for intracellular studies. For instance, when microtubule-bound, *trans*-cyclooctene-modified Taxol® was reacted with tetrazine-BODIPY FL, a robust fluorescence turn-on response could be observed owing to turnover of the fluorescence-quenching monoaryl-*s*-tetrazine moiety.[40b] In addition, the inverse-electron-demand Diels–Alder reaction was employed to couple the functional components of a proteolysis-targeting chimera (PROTAC) in live cells in order to enable degradation of BRD4 and ERK1/2.[41] This was necessary because PROTACs are generally cell-impermeable. Lastly, active-site lysine residues of various protein kinases were modified with a *trans*-cyclooctene carbamate moiety that could be cleaved through an inverse-electron-demand Diels–Alder reaction. Specifically, upon introduction of a tetrazine additive, the dihydropyridazine intermediate that is generated rapidly eliminates to restore enzymatic activity.[42]

4. Rearrangement Reactions

By definition, a rearrangement reaction describes the intramolecular shift from one constitutional isomer to another. This intramolecular event can be initiated by an external stimulus such as an increase in temperature or chemical activation. In the eyes of a chemist, the thermodynamic isomer formed will typically offer new reactivity and/or stability. For the chemical biologist, rearrangement reactions resulting in more stable compounds are ideal for bioconjugation reactions that rely on the formation of new, stable linkers. On the other hand, rearrangement reactions that generate new reactivity are ideal for analyte-specific probes as these can be tailored to directly or indirectly affect an analyzable output. In the following section, we highlight recent examples of both stability-enhancing and reactivity-changing rearrangement reactions.



Scheme 2. Original Ozonolysis Research and Its Application in Ozone-Selective Probes.



Scheme 3. Diels–Alder Cycloaddition and Its Applications in Bioconjugation.

4.1. Smiles Rearrangement

One of the oldest rearrangement reactions was first described by Henriques[43] and Hinsberg,[44] who observed that, following addition of alkaline ferricyanide to bis(2-hydroxynaphth-1-yl) sulfide and bis-(2-hydroxynaphth-1-yl) sulfone, respectively, new rearranged products were generated. However, it was not until 1930 that these reactions, as well as their key Meisenheimer complex intermediates, were fully characterized by Smiles (**Scheme 4**, Part (a)).[45] What Henriques and Hinsberg had observed was an intramolecular S_NAr that would become known as the Smiles rearrangement. In 1958, Burchfield reported that the Smiles rearrangement was occurring when cysteine was reacted with 1-fluoro-2,4-dinitrobenzene.[46] During this reaction, an initial product is formed that has an absorbance maximum in the 340–345 nm range; however, as the reaction proceeds a new product with an absorbance maximum at 360 nm forms. This is consistent with a mechanism in which the thiol initially displaces the fluoride nucleofuge followed by an intramolecular S_NAr with the free amine.

A clever biological application of the sulfur-to-nitrogen Smiles rearrangement was demonstrated in a study initiated to understand the biosynthetic pathway that is responsible for producing mycothiol, a protective thiol of actinobacteria.[47] It was proposed that along this pathway, MshB catalyzes the hydrolytic cleavage of the acetamide in GlcNAc-Ins, a precursor to mycothiol.[48] However, there were no reliable methods to monitor the activity of this enzyme colorimetrically, prompting the development of GlcNAc-SDNP.[47] In particular, in the presence of active MshB, the acetamido group of GlcNAc-SDNP is hydrolyzed to afford primary amine **7**, which is primed for a subsequent Smiles rearrangement. Favoring the aniline product over the sulfide, the Smiles rearrangement generates free thiol **8**, which can then react in vitro with Ellman's Reagent, yielding disulfide **9** and thiol **10**. Free thiol **10** absorbs strongly at 412 nm, allowing reliable colorimetric detection.

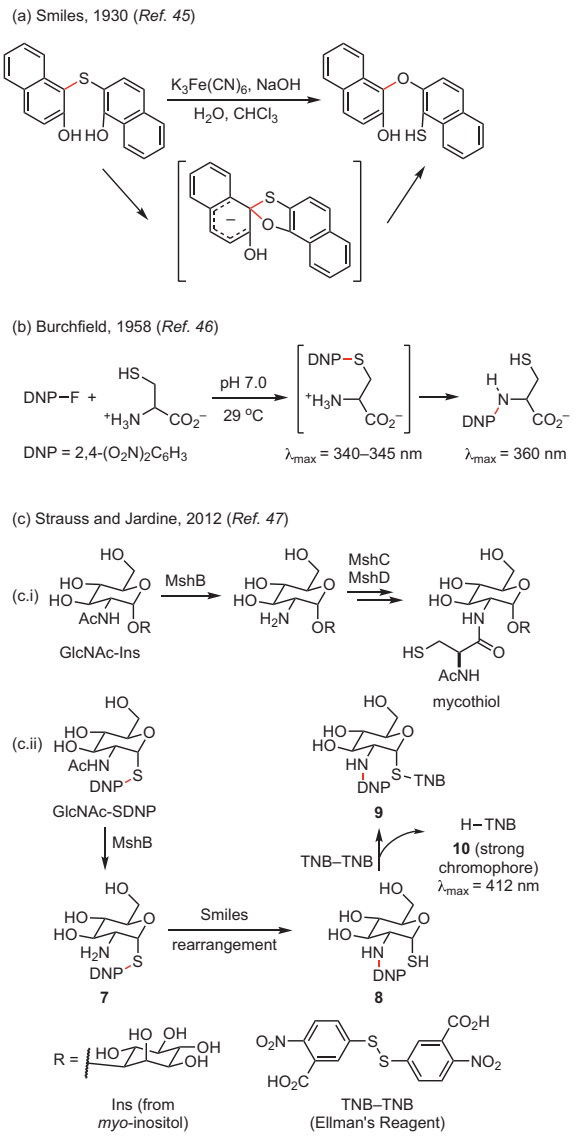
4.2. Pictet–Spengler Reaction

Although numerous approaches exist for installing aldehyde and ketone functional groups into a protein, methods to utilize them for subsequent bioconjugation have suffered from poor chemical stability. For example, condensation with hydrazine and hydroxylamine affords hydrolyzable hydrazone and oxime linkers, respectively.[49] Fukuzawa and Tachibana[50] sought to overcome this through the use of a rearrangement reaction pioneered by Pictet and Spengler[51] in 1911 (**Scheme 5**, Parts (a) and (b)). Specifically, tryptamine **11** condenses with aldehyde moieties to form iminium intermediates that undergo intramolecular nucleophilic attack and rearrangement to afford the stable, fused-ring products **12**. To improve upon the slow kinetics,[52] Bertozzi made two important improvements with **13** that would render the Pictet–Spengler reaction more biocompatible.[49] First, the amine linker was shifted from the C-3 to the C-2 position to reduce steric hindrance at the nucleophilic site; this resulted in a rearrangement-free iso-Pictet–Spengler mechanism to produce **14** (Scheme 5, Part (c)). Additionally, increasing the rate of iminium ion formation through the alpha-effect was implemented by modifying the pendent amino group into an aminooxy functionality. In combination, the rate of the reaction was increased by 3 orders of magnitude. The low pH required for efficient condensation was a final constraint that was addressed through substitution of the aminooxy group with hydrazine.[53]

Of note, the Pictet–Spengler reaction has not been limited to ligation chemistry. Another breakthrough came in the realization by Fukumura that an N-terminus tryptophan could perform a Pictet–Spengler reaction with ^{11}C-labeled formaldehyde, allowing the radiolabeling of short oligopeptides for PET imaging.[54]

4.3. Staudinger–Bertozzi Ligation

In 1919, Staudinger reported that the combination of phenyl azide and triphenylphosphine resulted in a violent reaction (**Scheme 6**, Part (a)).[55] Although it was proposed that a phosphazide intermediate was involved, N_2 evolution was so rapid that only the final iminophosphorane **15** could be isolated. Supplemental water reacted with the electrophilic phosphorus in **15** leading to hydrolysis of the P=N bond to generate an equivalent of phosphine oxide and aniline. On the other hand, reaction of iminophosphorane **15** with anhydrous benzoic acid resulted in the formation of secondary amide **16** (Scheme 6, Part (b)).[56] Requiring five days and elevated temperatures, the reaction of **15** with benzoic acid is significantly slower than the corresponding reaction with water.



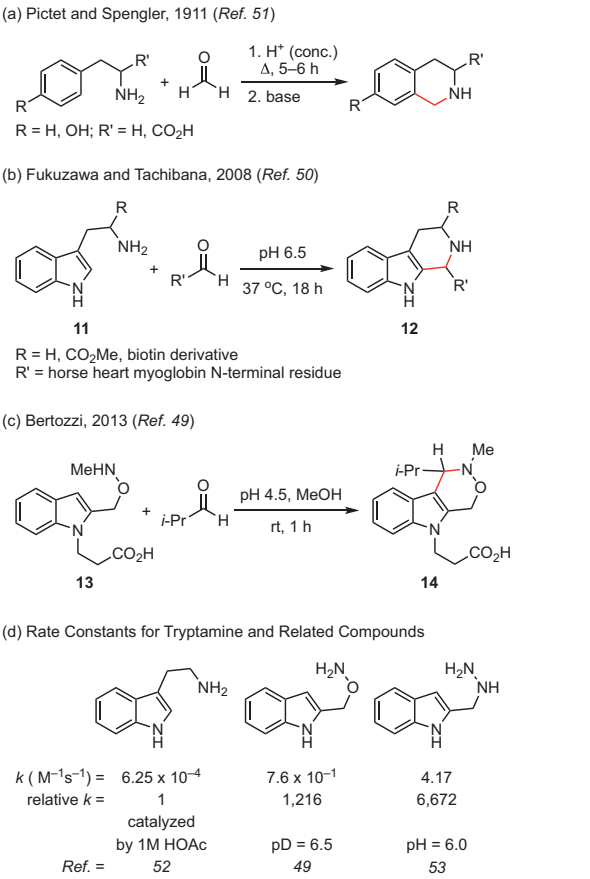
Scheme 4. The Smiles Rearrangement and Developments toward Its Use in Enzymatic Assays.

Bertozzi hypothesized that a carefully designed phosphine with an intramolecular electrophile would capture the iminophosphorane nucleophile before hydrolysis could occur. Indeed, introduction of a methyl ester group at the ortho position of one of the benzene rings in **17** proved sufficient, and, upon reaction with azide, resulted in a stable amide **18** after intramolecular rearrangement (Scheme 6, Part (c)).[57] In its first demonstration, the Staudinger–Bertozzi ligation, as it is now known, enabled the incorporation of a biotin marker only on cells displaying azido sugars on the cell surface.[57] Later, peracetylated *N*-α-azidoacetylmannosamine, an unnatural sugar, was administered to live mice for one week, resulting in glycan modification in the heart, liver, and kidney.[58] Subsequent injection of a FLAG® peptide appended with a reactive phosphine handle enabled the first in vivo application of the Staudinger–Bertozzi ligation. In addition to sugars, successful ligations have been performed on DNA[59] and proteins[60] under aqueous conditions.

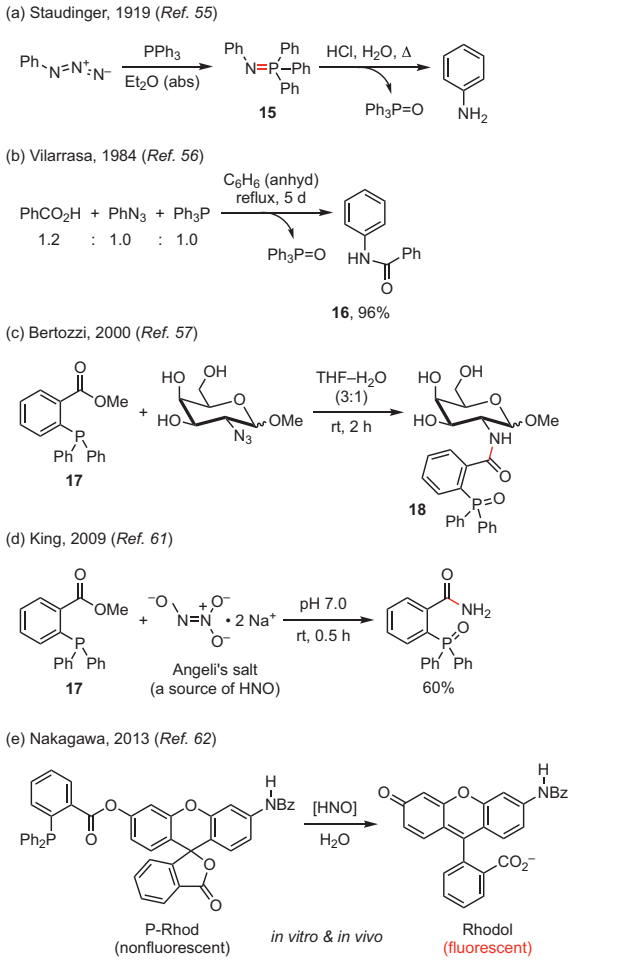
It is worth noting that the Staudinger–Bertozzi ligation does not proceed exclusively with azides. In fact, King demonstrated that HNO, a transient signaling molecule, also reacts with phosphines through a similar mechanism (Scheme 6, Part (d)).[61] Since methods to non-invasively detect HNO are limited, Nakagawa proposed to exploit its reactivity with phosphines to develop imaging probes specific for HNO detection. This led to the development of P-Rhod, a rhodol-based fluorescent probe that was used to image HNO produced upon treatment of A549 cells with Angeli's salt, an HNO donor molecule (Scheme 6, Part (e)).[62] Since this work, a number of other investigators have utilized this trigger to develop a variety of other imaging probes.[63] Unfortunately, the detection of naturally occurring HNO has remained elusive to date, presumably due to inefficient reaction kinetics.

4.4. Baeyer–Villiger Oxidation

In 1899, Baeyer and Villiger were exploring the reactivity of Caro's acid (peroxymonosulfuric acid, $HOS(=O)_2OOH$) with cyclic ketones when they discovered that ring-expanded lactones were being produced (**Scheme 7**, Part (a)).[64] Subsequent studies would establish that other peroxy compounds could also promote this transformation, now known as the Baeyer–Villiger oxidation, in a manner strongly correlated with the p*K*a value of the peroxy compound.[65a] Of note, while oxidation of ketones with H_2O_2 (p*K*a = 11.7)[65b] does occur, the reaction kinetics are generally sluggish compared to Caro's acid (p*K*a = 0.4).[65c] In contrast, 1,2-diketones such as benzil are rapidly oxidized by H_2O_2 in basic aqueous MeOH solutions to afford two benzoic acid products (Scheme 7, Part (b)).[66]

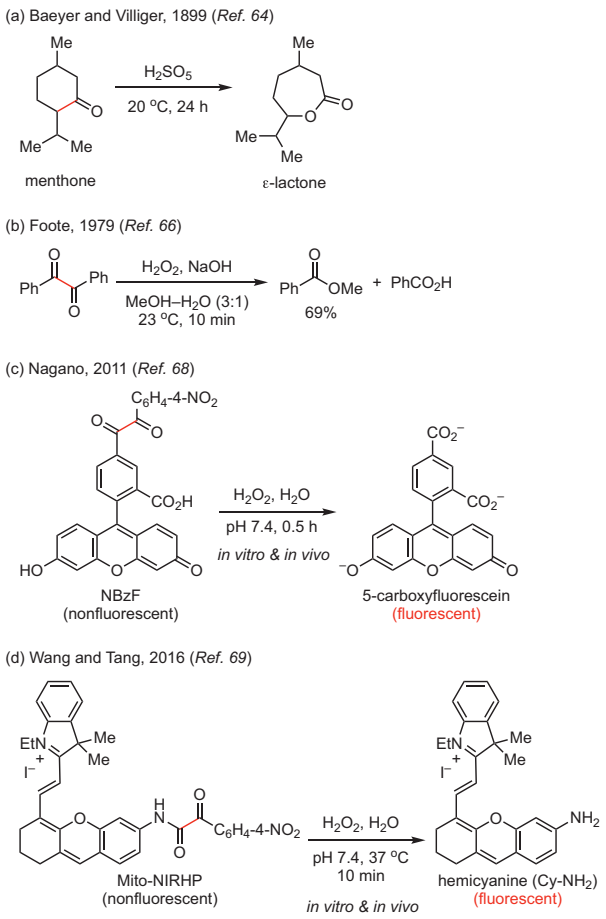


Scheme 5. The Pictet–Spengler Reaction and Its Development for Efficient Bioconjugation.



Scheme 6. The Staudinger Reaction and Subsequent Staudinger–Bertozzi Ligation.

Owing to its involvement in a large variety of physiological processes ranging from the immune response to cell signaling,[67] it is essential to develop chemical tools to detect H_2O_2 within biological samples. In this regard, Nagano designed NBzF, a fluorescein analogue equipped with an electron-deficient 4-nitrobenzil moiety to sense H_2O_2 (Scheme 7, Part (c)).[68] This probe design utilizes the 4-nitrobenzil group to quench the fluorescence of NBzF via the d-PeT (donor-excited photoinduced electron transfer) mechanism. After addition of H_2O_2, the 4-nitrobenzil moiety is oxidized to a hydrolytically unstable anhydride intermediate that spontaneously decomposes to afford 5-carboxyfluorescein, which is accompanied by a dramatic 150-fold turn-on response. The impressive selectivity for H_2O_2 and exquisite sensitivity enabled fluorescence imaging of endogenously produced H_2O_2 in multiple cell lines. In addition to the benzil moiety, the α-ketoamide functionality was recently utilized to develop Mito-NIRHP, a near-infrared, mitochondrially targeted H_2O_2-responsive probe (Scheme 7, Part (d)).[69] The α-ketoamide group functions by masking a key amino functionality crucial for fluorescence. Upon Baeyer–Villiger oxidative cleavage, the latent fluorophore is released with a concomitant increase in fluorescence. Wang and Tang successfully employed Mito-NIRHP to visualize H_2O_2 produced during ischemia-reperfusion injury in live cells and in an animal model.[69]



Scheme 7. The Baeyer–Villiger Oxidation and Its Applications in the Design of H_2O_2-Responsive Fluorescent Probes.

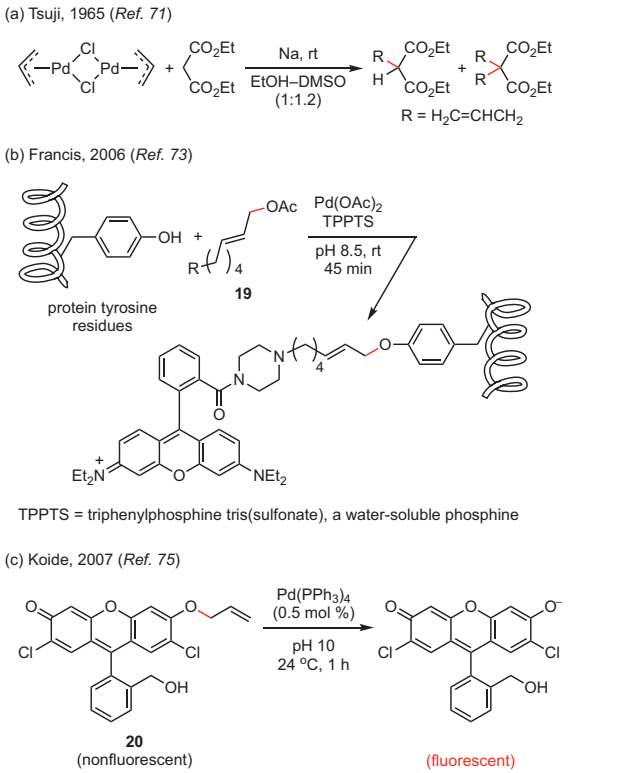
5. Metal-Mediated Reactions

Recently, there has been growing interest in utilizing second and third row transition metals such as ruthenium and palladium to develop chemical biology tools. This represents an exciting avenue for chemical biologists to develop highly bioorthogonal reagents, since these elements are not found naturally in biological systems. If the intrinsic toxicity of such heavy metals can be controlled, the introduction of new metal-mediated reactions with unique reactivities will undoubtedly lead to exciting biological discoveries. In the following subsections, we highlight recent examples involving palladium- and ruthenium-mediated transformations.

5.1. Tsuji–Trost Reaction

In 1965, Tsuji reported the reaction of diethyl malonate carbanion, a soft carbon nucleophile, with a cyclooctadiene-palladium chloride complex.[70] Depending on the workup conditions, either a cyclopropyl product was formed through one-carbon addition to one of the carbon–carbon double bonds, or a mono-substituted, conjugated cyclooctadiene product through double-bond migration. Shortly thereafter, Tsuji published similar chemistry with π-allylpalladium chloride; however, only the allylated product was observed (**Scheme 8**, Part (a)).[71] In this seminal work, pre-forming the π-allylpalladium indicates that the reaction begins with substitution at the metal center followed by reductive elimination and dissociation of the allylated nucleophile to release Pd(0).

Soon after discovery of this reaction, Trost and others made key advances by expanding the substrate scope and enabling a catalytic

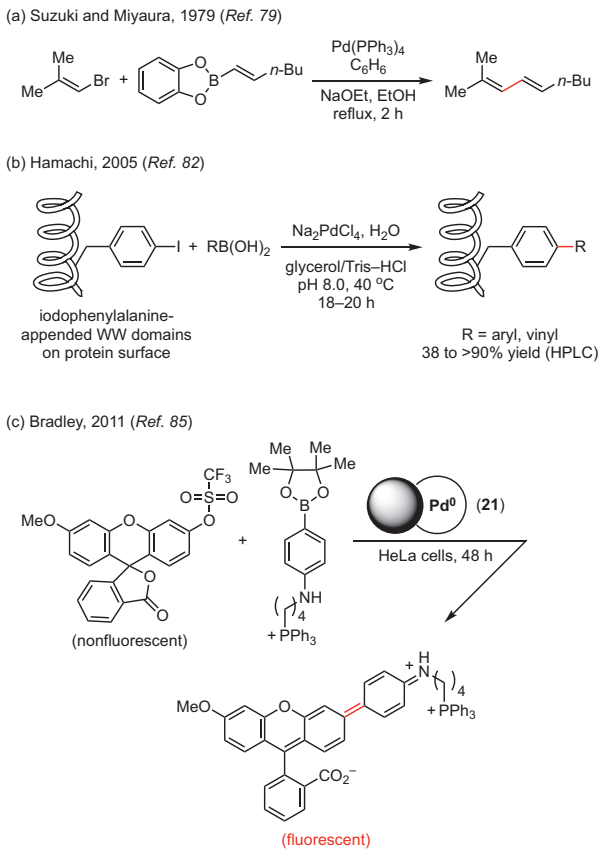


Scheme 8. The Tsuji–Trost Allylation and Its Applications in Bioconjugation and Pd(0) Sensing.

variant.[72] Exploiting the reactivity of phenols, Francis developed a bioconjugation method that is selective for tyrosine residues (Scheme 8, Part (b)).[73] To accomplish this, stoichiometric allyl donor **19**, catalytic $Pd(OAc)_2$, and a water-soluble phosphine (TPPTS) were added to a solution of chymotrypsinogen A, achieving a 50–65% labeling efficiency of the protein. The most impressive aspect of this work was the observation that neither lysine nor cysteine reacted under the labeling conditions. Unfortunately, this chemistry was limited to only ex vivo applications, and no significant improvements in the Tsuji–Trost bioconjugation have been reported since.

In synthetic chemistry, one of the most common uses of the Tsuji–Trost reaction is to selectively deprotect O- and N-allylated species under mild conditions.[74] Probes that can selectively visualize palladium are desirable in order to study its toxicity to biological systems, as well as to monitor palladium-based chemotherapeutics. In 2007, Koide utilized the reactivity of the Tsuji–Trost reaction to develop **20**, a fluorescent probe for the detection of Pd(0) through an O-deallylation mechanism (Scheme 8, Part (c)).[75] However, due to the requirements of Pd(0) and high limit of detection, the utility of this and other similar probes were never demonstrated in a biological system.[75,76]

Due to these limitations, subsequent generations of probes began to employ a propargyl protecting group rather than an allyl moiety.[77] In particular, various oxidation states including Pd(0), Pd(II), and Pd(IV) can all mediate the cleavage of the propargyl group to unmask the latent fluorophore. Similar chemistry was used to enable the activation of proteins by addition of palladium to cells, in particular restoring bacterial phosphothreonine lyase activity via depropargylation of a protected active site residue.[78]



Scheme 9. The Suzuki–Miyaura Coupling and Its Applications in Protein Tagging.

5.2. Suzuki–Miyaura Coupling

The Pd-catalyzed coupling of organoboranes with organic halides first introduced by Suzuki and Miyaura is mechanistically similar to other organometallic coupling reactions (**Scheme 9**, Part (a)).[79] However, this coupling, now named after Suzuki and Miyaura, is generally a safer alternative to other reactions such as the Stille coupling, which uses toxic organostannane reagents. In addition to excellent compatibility with water and oxygen, organoboranes and organic halides do not naturally occur in most biological systems. As such, the application of the Suzuki–Miyaura coupling in chemical biology was inevitable.

The first example of a Suzuki–Miyaura coupling that occurred primarily in aqueous solution came in 1990 with the introduction of sulfonated phosphines.[80] This new class of ligands enabled the coupling of aryl and vinyl boronic acids with aryl halides, including an iodonucleoside, albeit at 80 °C. Despite this demonstration of aqueous compatibility, the earliest examples involving unnatural amino acids were performed in organic solvents, also at 80–90 °C.[81] Transitioning to Na_2PdCl_4, a water soluble, phosphine-free catalyst, the Suzuki–Miyaura coupling was successfully performed on a protein under aqueous conditions for the first time in 2005 (Scheme 9, Part (b)).[82] Unfortunately, even when mixed at 40 °C with organic co-solvents, reaction times were still long, taking approximately 18 hours. A 2-aminopyrimidine-based, water-soluble catalyst developed by Davis set a new standard a few years later, reacting in buffered water at 37 °C with yields >90% for couplings of bromo and iodo derivatives of phenylalanine and tyrosine with phenylboronic acid.[83] With this catalyst, synthetic post-translational modifications could be made to surface-expressed *p*-iodophenylalanine (*p*IPhe) in maltose-binding protein. Importantly, the same catalyst could be used in living cells to successfully append either a fluorophore or sugar to *p*IPhe-containing membrane proteins to enable direct visualization of the cell and modification of the glycocalyx, respectively.[84] In these studies, cell impermeability prevented the demonstration of intracellular modifications. To overcome this, Bradley deviated from traditional small ligands in favor of a cell-permeable Pd(0) nanoparticle, **21** (Scheme 9, Part (c)).[85] This stable Pd(0) species successfully catalyzed the coupling of an aryl triflate with an organoborane to produce a fluorescent species within HeLa cells. Future directions include intracellular coupling of biologically relevant partners. Potential catalysts for these new bioconjugations include the aforementioned Pd(0) nanoparticle, as well as the very recently developed artificial Suzukiases.[86]

5.3. Grubbs Reaction (Olefin Metathesis)

The 2005 Nobel Prize was awarded to Chauvin, Grubbs, and Schrock for their pioneering research on olefin metathesis.[87a] In particular, Grubbs was recognized for the development of Ru(II) carbenoid complexes that exhibit good functional-group tolerance.[87b] Utilizing this coupling reaction, unnatural amino acids were some of the first molecules modified for biological studies (**Scheme 10**, Part (a)).[88] Another application was to improve the stability of peptide-based pharmaceuticals by generating rigid, stable linkers within and across secondary peptide structures.[89] Although this peptide chemistry was performed in degassed organic solvents under anhydrous conditions, improvements in catalyst design have allowed for improved reactivity, water solubility, and biocompatibility.[87b,90] With these new catalysts,

aqueous ring-closing metathesis (RCM) and ring-opening metathesis (ROM) polymerizations were readily demonstrated, but robust cross-metathesis (CM) remained elusive. Working with nontraditional CM partners, Davis discovered that *S*-allyl-cysteines, and more so *Se*-allyl-selenocysteines, reacted much more readily than the *O*-allyl species (Scheme 10, Part (b)).[91] This improved reactivity was attributed to the softness of selenium and sulfur compared to oxygen. This softness better facilitates pre-coordination to the ruthenium catalyst and allows CM between proteins and various allyl handles. The future of the Grubbs reaction as a competitive chemical biology tool will depend on continued improvements of the reactivity and stability of the ruthenium catalyst. Additionally, continuing to improve the catalyst-controlled *E*- and *Z*-selectivity is important as the final isomer can effect noticeable changes in the bioactivity of the unnaturally linked peptide.[92]

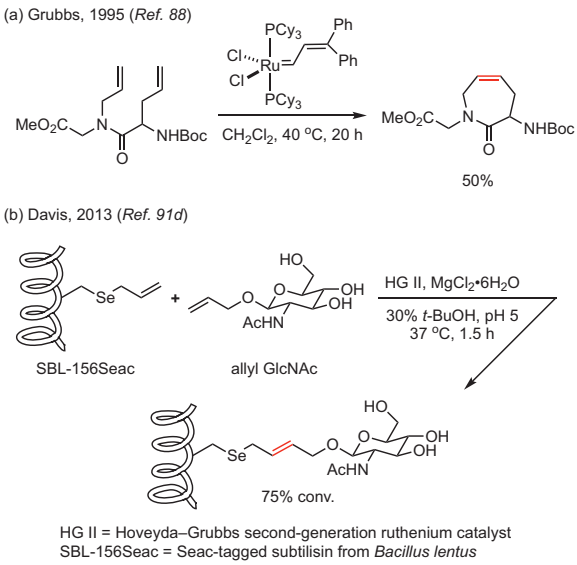
5.4. Oxidative Heck Coupling

In the early 1970s, Mizoroki and Heck independently discovered that mono- or unsubstituted olefins could be coupled to iodobenzene in the presence of base and catalytic $Pd(OAc)_2$.[93] Shortly thereafter, Heck would briefly report the first example of oxidative Heck coupling between methyl acrylate and 1-hexenylboronic acid (**22**), but this required stoichiometric amounts of $Pd(OAc)_2$ (**Scheme 11**, Part (a)).[94] Further work by Uemura enabled the use of catalytic palladium[95] and, almost a decade later, work by Jung made possible the use of O_2 as oxidant[96] and base-free couplings (Scheme 11, Part (b)).[97] With these developments, the oxidative Heck reaction became highly desirable for bioconjugation, namely because it enabled addition of substrates to unfunctionalized olefins. Using the water-soluble catalyst Pd-BIAN, Dekker was the first to successfully perform this bioconjugation in 2014 (Scheme 11, Part (c)).[98] After expressing the R16C mutant of 4-oxalocrotonate tautomerase, in which a single terminal alkene exists, addition of $Pd(OAc)_2$, BIAN, and a fluorescent boronic acid in a 6:1 buffer–DMF solution led to successful fluorescent labeling. Shortly thereafter, Dekker disclosed a fully aqueous variant involving lysine acylation post-translational modification.[99] Notably, the oxidative Heck coupling has been extremely useful for the identification of proteins that have been post-translationally modified by protein methyltransferase.[100] Using the oxidative Heck coupling, a biotin analogue can be appended to proteins allylated by an allyl-SAM (SAM = *S*-adenosyl-L-methionine) analogue for later isolation via an avidin column.

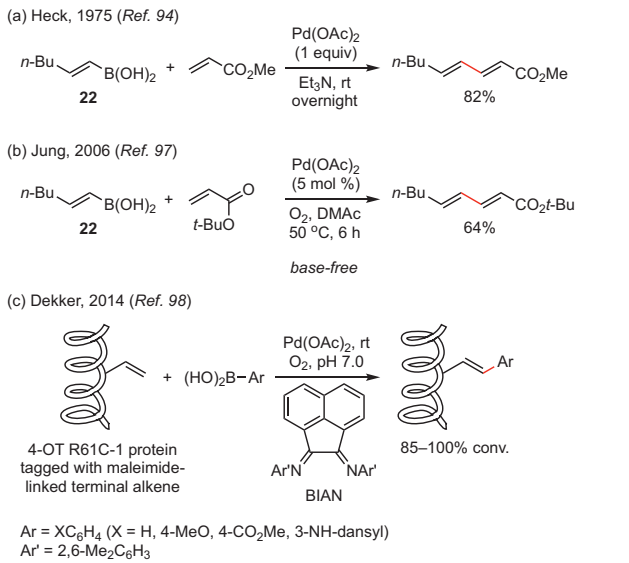
6. Conclusion and Outlook

The adaptation of named reactions for various applications in chemical biology is a rapidly emerging research area. The aim is to develop effective chemical tools with extraordinary chemoselectivity and biocompatibility for deciphering unanswered biological questions, as well as to discover new biological phenomena. Indeed, by utilizing the collective knowledge of countless organic chemists, the process of developing a new chemical tool can be greatly accelerated. However, to do so, one must have a keen eye for identifying reactions that are poised to meet the criteria detailed above in Section 2. For instance, reactions that already operate efficiently in water are favored over those that do not. Similarly, reactions exhibiting kinetics within the desirable range for a specific application will require less elaborate chemical tuning. Moreover, reactions that feature abiotic chemical moieties will suffer less from potential cross-reactivity with endogenous molecules found in the complex biological milieu. Although this is not always possible, researchers new to this area can take comfort that chemical ingenuity can overcome essentially any barrier. For instance, chemical tools that undergo reactions not compatible with aqueous media can be encapsulated within some material such as a conjugated polymer nanoparticle that offers an organic-solvent-like interior. This approach can also accelerate reaction rates by increasing the effective concentration of reactants and limit cross-reactivity by excluding the entry of competing species.

In this review article, we have highlighted select named reactions employed thus far in the development of analyte-specific imaging



Scheme 10. Application of the Grubbs Olefin Metathesis Reaction to Peptide Stabilization and Bioconjugation.



Scheme 11. The Oxidative Heck Coupling and Its Applications in Bioconjugation.

probes, as well as chemical tools and approaches for bioconjugation. However, it is noteworthy to point out that many chemical reactions, named or unnamed, may be appropriate to use. An exciting direction is to employ reactions to selectively cleave a stable chemical bond.[101] This strategy can be exploited to develop reagents (i.e., donors) that can be triggered to release a specific analyte (e.g., metal ion) to perturb normal cellular processes. Likewise, cleavage reactions can be used to deliver drug molecules with precision control. Another area that we anticipate will experience tremendous growth is the application of reversible reactions. Chemical tools based on these transformations could provide investigators a means to study dynamic processes. Lastly, to gain further spatiotemporal control, tools based on photochemically initiated reactions will allow for on-demand responses using light.[102]

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Bioconjugation—A Versatile and Essential Research Tool

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Abstract. Bioconjugation is now firmly established as an important component of successful research in many different fields. The ability to link one molecule to another or to a surface frequently forms the basis for the detection, assay, or targeting and tracking of biomolecules. Basic research, materials science, electronics, clinical diagnostics, and even drug development benefit from the science of bioconjugation.

Outline

1. Introduction

While bioconjugation is now well recognized as an indispensable tool in several areas of research, its methods can often be complicated. Moreover, the choice of the best reagent for a particular conjugation application can be imprecise and time-consuming, especially for someone who is relatively unfamiliar with the methodology. The optimal reagents and conditions for preparing a new bioconjugate can be determined by considering a number of factors, such as a reagent's chemical and physical properties, the functional groups it targets for coupling, its length, its molecular size, its water solubility, its cleavability, and precedents for its application in a given context. While a number of excellent treatments of bioconjugation have been published,[1] this review aims to bring up to date the state of the art, and simplify it enough to make it accessible to the widest audience possible.

2. Crosslinkers

2.1. Homobifunctional Crosslinkers

One of the most fundamental aspects of crosslinker design is whether the reagent is homobifunctional or heterobifunctional. The overwhelming majority of bioconjugate reagents are bifunctional, with two reactive groups usually located at the outer ends of an organic spacer. In a homobifunctional compound, the two reactive groups are identical, whereas in a heterobifunctional compound they are different.

Homobifunctional compounds will react at both ends with the same target functional group, thus forming a covalent crosslink between two molecules using the same type of bond. The most common type of homobifunctional reagent is one that reacts with amines, whereby it links together two different molecules each containing one or more amino groups. This means that proteins containing α-amines as well as lysine ε-amino groups can be conjugated in a single step using a homobifunctional, amine-reactive crosslinker. Some of the more popular homobifunctional, amine-reactive crosslinkers include the bis-imidoester compound dimethyl pimelimidate (DMP), the bis-NHS ester compounds disuccinimidyl tartrate (DST) and disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl) suberate (BS^3), and ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS) (**Figure 1**). When retention of the positive charge on proteins is desired, DMP is reacted with amino groups to form a positively charged amidine bond. The NHS ester and sulfo-NHS ester compounds react with amino groups to form amide bonds, which are more stable than amidine bonds. These reagents have been utilized extensively to capture and study interacting proteins in cells and other biological samples.

Other homobifunctional crosslinkers, with maleimide groups at each end, react with thiol groups to form stable thioether bonds. These compounds can link two biomolecules containing cysteine side-chain thiols, or, in some cases, they can even bridge the gap between two thiols created by disulfide reduction of a protein. Some examples of these reagents include bis(maleimido)ethane (BMOE), dithio-bis(maleimido)ethane (DTME), 1,8-bis(maleimido)diethylene glycol [BM(PEG)$_2$], and 1,11-bis(maleimido)triethylene glycol [BM(PEG)$_3$] (**Figure 2**, Part (a)).

Another type of homobifunctional, thiol-reactive crosslinker is 1,4-bis[3-(2-pyridyldithio)propionamido]butane, which, instead of maleimide groups, has a pyridyldithiol group on each end (Figure 2, Part (b)). This reactive group couples with thiols to form a reversible disulfide linkage, which can be reduced with DTT or another reducing agent to cleave the crosslink after it has formed. In some applications, the ability to reductively cleave the linkage is important for activity of the conjugate. For example, pyridyldithiol coupling has been utilized to form antibody–drug conjugates (ADC) for targeting tumor cells. The resultant dithiol linkage between the antibody and the drug gets cleaved after the bioconjugate is internalized by the cancer cell, thus releasing the toxic drug to kill the tumor.

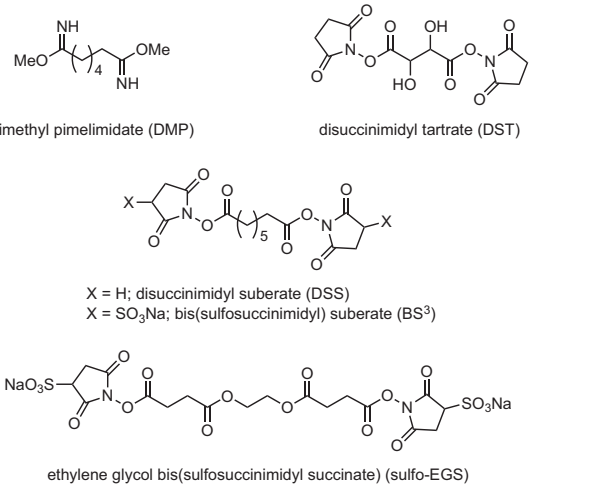


Figure 1. Popular, Amine-Reactive Homobifunctional Crosslinkers.

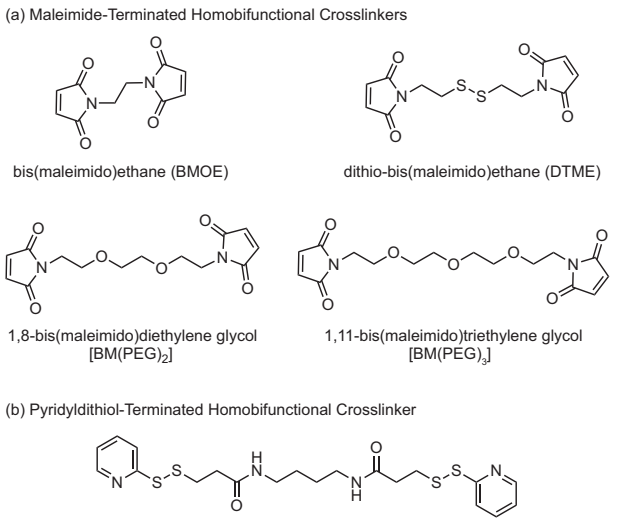


Figure 2. Popular, Thiol-Reactive Homobifunctional Crosslinkers.

The main shortcoming of homobifunctional crosslinkers is that they can easily polymerize proteins or other molecules containing multiple target functional groups. Such multiple coupling reactions with each protein being conjugated can lead the crosslinking molecules to act as bridges for oligomerizing proteins and creating large-molecular-weight complexes. This can be a problem when needing highly defined and reproducible conjugates between two proteins, since it would be difficult to limit such polymerizations simply by adjusting the molar ratios of the reactants or by controlling the reaction time.

On the other hand, the two most important applications of homobifunctional crosslinkers, and in which they perform very well, are: (i) the capture of interacting proteins by rapidly reacting with them while they are bound together, and (ii) the modification or activation of particles and surfaces. When using homobifunctional reagents to study protein interactions, it is important to limit their concentration so that only those proteins truly interacting are crosslinked and bound together, but not other proteins in the cellular environment nearby. Often, homobifunctional NHS esters are chosen for this purpose, because the interacting proteins usually contain amino groups capable of reacting with the NHS ester, and the reaction rate is fast enough to capture most interactions, except for highly transient ones. This approach has been employed successfully for decades to discover many of the key intracellular protein interactions.[2]

In addition, the use of homobifunctional compounds to modify and activate insoluble matrices such as particles and surfaces is highly valuable and very controllable. In this case, a solid support covered with functional groups (such as amines) can be reacted with an excess of a homobifunctional crosslinker (such as a bis-NHS ester) to create a monolayer of tethers, each of which terminating in a reactive group projecting from the surface and free to be coupled with another molecule, such as a protein. With particles, the use of a large excess of crosslinker during the initial modification reaction prevents particle-to-particle polymerization or undesirable linkages between functional groups on the same particle. In this respect, homobifunctional crosslinkers can function as building blocks to create linker arms and reactive groups on particles and surfaces of all types. After the first modification reaction of a surface or particle, any excess reagent is then simply washed away before using the other reactive end of the attached crosslinker to couple and immobilize a protein or another affinity molecule. The choice of the crosslinker's spacer or cross-bridge also regulates the resultant surface properties of the solid phase after the reaction. For example, a long, hydrophilic spacer (e.g., one containing polyethylene glycol (PEG) units) can provide increased biocompatibility and dramatically reduce nonspecific binding to a particle or surface.

Homobifunctional crosslinkers have also been utilized to create protein–protein conjugates. Some of the very first applications of these crosslinkers were in the formation of antibody–enzyme conjugates for use in immunoassays.[3] Early examples of these reactions used bis-imidoester or bis-aldehyde linkers such as DMP or glutaraldehyde. However, the drawback of forming antibody or protein conjugates with homobifunctional reagents is that each protein typically has more than one group that can react with either end of the crosslinker. Thus, as the reaction progresses, protein oligomers usually form, as multiple linkers on each protein cause polymerization. Very large complexes can result from this reaction, some of which actually precipitate from solution as clumpy or cloudy masses within the solution.[4]

2.2. Heterobifunctional Crosslinkers

Heterobifunctional reagents have major advantages over homobifunctional ones when forming bioconjugates, since one reactive end group would couple with only a specific functional group, while the other reactive end group would react with a different functional group. The discrete nature of this process makes two-step reactions possible, which has the ability to limit undesirable reactions, such as polymerizations during conjugate formation, and thus restrict the size of the resultant complex. In the case of proteins, the first reaction leads to an activated protein intermediate. This activated protein is then mixed with a second protein designed to contain one or more functional groups that were not present on the first protein. Under the right conditions, the remaining reactive, free end group on the crosslinker-modified first protein reacts with the functional targets on the second protein and forms a covalent crosslink or tether between the two proteins, thus creating the final bioconjugate. This is particularly important when the desired result is to create a known molar ratio of the two proteins in the final complex, and do so reproducibly. For instance, in the production of antibody–enzyme conjugates for immunoassays, it is desirable to modify each antibody molecule with multiple enzyme molecules in order to maximize the substrate signal produced by each conjugate. This type of reaction can be carried out repeatedly with a great deal of precision, leading to reproducible detection reagents for the most critical of immunoassay tests, such as in assays designed for clinical diagnostics.[5]

Some of the more popular choices in heterobifunctional crosslinkers include reagents with an amine-reactive NHS ester on one end and a thiol-reactive maleimide group on the other end. Examples include SMCC [4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid *N*-hydroxysuccinimide ester] and sulfo-SMCC [4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt], which have been in use since the 1970s.[6] SMCC is a hydrophobic reagent that contains a cyclohexane ring in its cross-bridge, whereas sulfo-SMCC has a nearly identical structure and reactivity, but also has a sulfonate group on its NHS ester ring (**Figure 3**, Part (a)). This negatively charged sulfonate provides enough polarity to make the compound initially water soluble. The negative charge also permits the labeling of cell surface proteins and avoids penetration of the cell membrane and labeling of intracellular proteins. Other amine-reactive and thiol-reactive heterobifunctionals contain a bromoacetyl, iodoacetyl (e.g., SIAB), or a pyridyldithiol group (e.g., SPDP) instead of a maleimide group (Figure 3, Part (b)).

Another important class of amine-reactive and thiol-reactive crosslinkers is the one incorporating a hydrophilic polyethylene glycol (PEG) based cross-bridge. Such reagents often are extremely water soluble, and can provide increased solubility of the conjugates formed from them. Heterobifunctional PEG-based compounds can be used to impart biocompatibility to surfaces or particles before coupling an antibody or an affinity ligand. Examples of these compounds include maleimide-PEG_{12}-succinimidyl ester (and other PEG chain lengths in this reagent family), dibenzocyclooctyne-PEG_4-maleimide (an azide-reactive and thiol-reactive compound) (Figure 3, Part (c)), and PEG_{12}-SPDP (which is the PEG-based counterpart to the hydrophobic SPDP reagent).

2.3. Functional Groups Targeted

The most reactive functional groups in biomolecules are associated with the heteroatoms N, O, and S, which are nucleophilic due to an unshared pair of electrons and can spontaneously react with the compatible and electrophilic active groups on crosslinkers and modification reagents. In many cases, the nucleophilic functional groups in biomolecules are free and accessible, but in some instances they have to be created to allow reactivity and coupling to take place. There are several specialized reagents available that facilitate the creation of an appropriate functional group for bioconjugation if the desired one is not available. An example of this type of modification reagent is 2-iminothiolane (Traut's reagent; 2-IT),[7] which is a thiolation agent that reacts with an amine to create a free thiol at the end of a short tether. This free thiol can then be utilized for conjugation with thiol-reactive reagents.

Naturally occurring functional groups on biomolecules may consist of any combination of amines, thiols, hydroxyls, carboxylates, aldehydes, organic phosphates, and reactive hydrogens on certain activated carbon atoms. Amines, thiols, and hydroxyls are the main nucleophilic groups, and, under the right conditions, they react directly with the electrophilic reactive groups present on many bioconjugation reagents. In contrast, functional groups consisting of carboxylates, aldehydes, organic phosphates, and reactive hydrogen sites require special activation agents or secondary coupling agents before they will form covalent bonds with another functional group.

2.3.1. Amines

Amines are present in the overwhelming majority of proteins, and, for this reason, they are the number one functional group targeted for bioconjugation. Amines can be found at the N-terminal α-amino group of each polypeptide chain within a protein, provided it is not post-translationally modified and blocked (e.g., through acetylation). Moreover, the most abundant sites for amine conjugation are at lysine side-chain ε-amines that often occur several times within a polypeptide sequence. Large proteins or antibodies can contain dozens of lysine amines on their surfaces, many of which are unmodified and sufficiently accessible within the folded structure of a polypeptide for easy targeting

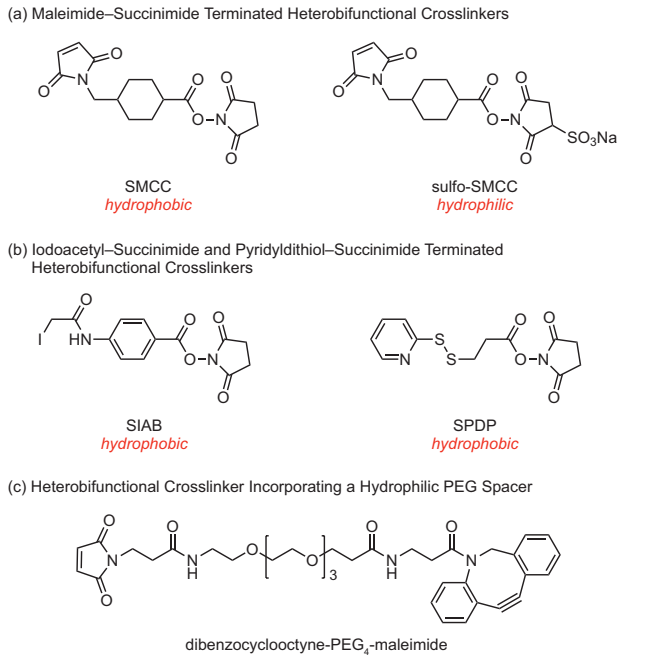


Figure 3. Popular, Amine- and Thiol-Reactive Heterobifunctional Crosslinkers.

and bioconjugation. The products of these conjugation reactions can form very stable amide bonds (e.g., acylation of the amine with an NHS ester or sulfo-NHS ester) or secondary amine bonds (alkylation of the amine), resulting in stable conjugates that are useful in a large variety of applications.

Another important reaction that amines undergo is rapid coupling to an activated phenyl azide group, which is present in many photoreactive crosslinkers. An example of this type of compound is 4-azidophenacyl bromide [4-$N_3C_6H_4C(=O)CH_2Br$], a thiol-reactive and photoreactive reagent that can be used to study protein interactions. Photoactivation of the phenyl azide group with UV light creates an extremely reactive nitrene, which undergoes ring expansion to a dehydroazepine. This reactive group then conjugates with any amine within its reach, forming a secondary amine linkage to a seven-membered ring.[8]

2.3.2. Thiols

Thiols are very good targets for bioconjugation, but since they are often found as disulfide-linked cysteine residues buried within the 3-D structure of a protein, they are not always accessible for conjugation without prior reduction. In some cases, the reduction of a disulfide has to be combined with denaturation conditions in order to unfold and open up the inner structure of the protein. In other cases, the protein subunits may be bonded together through disulfide linkages, and their reduction may result in breakdown of the quaternary structure and loss of activity or, at least, may result in major conformational changes of the protein. Even though disulfide reduction is not always possible without loss of protein structural integrity, antibodies are the one important class of proteins that have been successfully targeted for conjugation by limited reduction of the disulfide linkages without loss of antigen-binding activity.

By employing this strategy, antibody–drug conjugates (ADCs) have been produced after mild reduction of heavy-light chain disulfides to reveal just a few thiols in the structure of the monoclonal antibody cAC10. The free thiols were then successfully conjugated to a cytotoxic drug molecule.[9] In addition, antibodies have been immobilized on solid supports through thiols, and labeled with biotin or fluorescent dyes using thiol reactions.[10–12] The use of disulfide reduction of antibodies as a strategy for bioconjugation has one important benefit in that it keeps any linked proteins or other molecules away from the binding sites at the ends of the Fab regions, and thus does not block them or inhibit the docking of antigens.

2.3.3. Hydroxyls

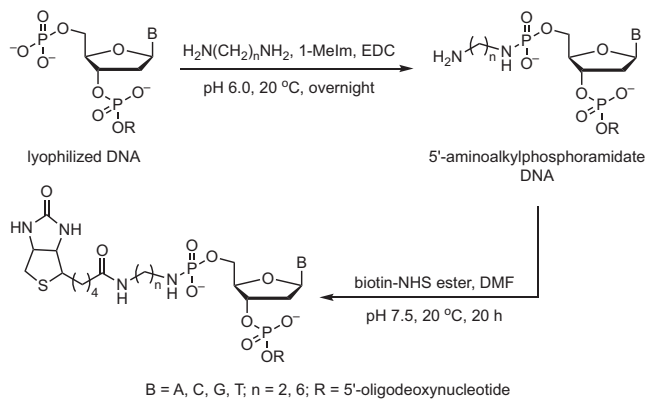
Hydroxyl groups can be found at serine, threonine, and tyrosine residues in proteins as well as on sugars and carbohydrates. They are also present on certain phospholipids and on glycans and glycoproteins, which often decorate cells, proteins, and lipids. Hydroxyl groups are nucleophilic; however, their relative nucleophilicity is less than that of amines and thiols, and thus they are less reactive. In aqueous solution, a hydroxyl group has about the same degree of nucleophilicity as that of the oxygen atom in water, and, since water is in much higher concentration than any OH groups on biomolecules, hydrolysis becomes the predominant reaction, which severely limits the successful conjugation of the hydroxyl group. In addition, if there are any other more nucleophilic groups present in a molecule that contains one or more hydroxyls, then a crosslinker has a much greater chance of reacting with those more reactive functional groups than conjugating to an OH group. An exception to this rule is the case where certain nucleophilic groups in a protein are, in a 3-D structural sense, nearby in sequence, or adjacent, to a hydroxyl-containing residue. If the reaction of the electrophilic crosslinker with such neighboring nucleophiles is transient or reversible—as in the case of an NHS ester reacting with a histidine imidazole group or temporarily hydrogen bonding with an arginine guanidino group—then labeling of a neighboring OH group in serine, threonine, or tyrosine can take place through secondary transfer from the histidine or arginine.[13,14]

Some hydroxyl groups in biomolecules can also be used to create reactive sites for bioconjugation with another molecule. In particular, if there are adjacent hydroxyl groups (1,2-diols), they can be oxidized to form reactive groups. This is especially useful when no other functional group is available for targeting. This approach can also be employed if a hydroxyl group is situated next to an amine, such as at an N-terminal serine residue in peptides, where the α-amino group is next to the serine side-chain hydroxyl and is thus susceptible to oxidation.

Diols are usually found on certain sugars, carbohydrates, glycans, and on the 3' ribose group of RNA. In these cases, one or more reactive aldehyde groups can be generated through periodate oxidation of the diol, which cleaves the carbon–carbon bond between the hydroxyl groups, and oxidizes them to aldehyde groups. Aldehydes created in this manner undergo reductive amination with amines.[15] The aldehydes can also react with hydrazide- or aminooxy-containing crosslinkers to form hydrazone or oxime bonds, respectively (see Section 5).[16–21]

2.3.4. Carboxylates and Organic Phosphates

Carboxylates and organic phosphates are two functional groups very commonly found in biomolecules. Unfortunately, these two groups are not very nucleophilic, and thus do not spontaneously react with the electrophilic reactive groups on crosslinkers or modification reagents. They are also similar in that carbodiimides can activate each to a reactive ester, which can then be utilized to acylate amines on other molecules to form an amide bond (from the carboxylate) or a phosphoramidate linkage (from the organic phosphate). The majority of all conjugation reactions of carboxylates or organic phosphates on biomolecules are accomplished using the water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [EDC, $EtN{=}C{=}N(CH_2)_3NMe_2$] (**Scheme 1**).[22] Moreover, when activating organic phosphate groups with EDC, imidazole is typically added to the reaction to form the intermediate phosphorylimidazolide, which then more effectively couples to amino groups to form phosphoramidate bonds.[22–24]



Scheme 1. Labeling of the 5'-Terminus of Synthetic Oligodeoxyribonucleotides with a Biotinyl Residue. *(Ref. 22)*

2.4. Crosslinker Length

The dimensions or overall linear length of the target molecule before and after conjugation should be considered when choosing a crosslinker or modification reagent for the conjugation reaction. The spacer arm or cross-bridge of the reagent mainly determines the molecular length of the resulting compound. The length of a crosslinking reagent can be determined by use of certain molecular modeling software programs. The crosslinker is initially drawn as linearly as possible using a chemical drawing program and then saved as a three-dimensional model using the protein data bank file format (.pdb) or in a similar format that preserves the coordinates of each atom. Once the saved molecule is opened in a molecular modeling program, it is displayed in three-dimensional space. Its energy is then minimized to orient the structure as realistically as possible based on its bond angles and bond lengths. Many modeling programs then permit the measurement of the distance between any two atoms in the structure in angstroms or nanometers. If the length of a crosslinking reagent is given by the supplier, it usually refers to the approximate maximal molecular distance between the atoms of the crosslinker that are directly linked to the two molecules being conjugated. Although the molecular length of crosslinkers in real-world applications will reflect the vibrations, bending, and folding of such molecules as they undergo molecular dynamic changes in solution,[25] the relative calculated linear lengths of different crosslinkers can be used to estimate distances between crosslinked proteins. Crosslinkers of different sizes thus become molecular rulers for measuring the distances between functional groups in biomolecules. The use of crosslinkers to perform protein structural modeling in this way can lead to the discovery of the conformation of individual proteins, and can determine the orientation of protein complexes formed from multiple interacting proteins.[26] Even the binding domains within proteins have been analyzed using crosslinkers as molecular rulers.[27]

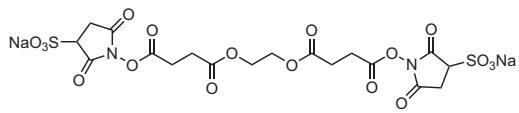
It is possible to eliminate any spacer group between two crosslinked molecules. For instance, a carbodiimide such as EDC is an activating agent that forms an intermediate reactive ester from a carboxylic acid. The reaction of this ester with an amine would result in an amide bond, which is an important step in forming a direct link between two molecules.[28] Short cross-bridges (<5 Å) can be formed from near-neighbor functional groups, or can span very short distances between two biomolecules. Some short-length reagents have even been utilized to covalently crosslink two thiols formed from disulfide reduction. A third arm on this type of linker can enable this bridge to be used to conjugate another molecule to the original site of the disulfide bond within a protein.[29] This type of specialized, short trifunctional crosslinker can help maintain the original conformation of an antibody or protein even after a critical disulfide bond has been broken. It can also create a site of modification away from binding sites or active centers on proteins and enzymes. Crosslinkers of moderate length (5–20 Å) have been employed to produce almost every type of bioconjugate complex imaginable. This includes creating linkages between two proteins to form a defined conjugate, capturing protein–protein interactions, and coupling biomolecules to particles or surfaces. Reagents in this size range offer many options for reactive groups and spacer arms in addition to being hydrophobic or hydrophilic, and cleavable or noncleavable.

Extremely long spacer arms, spanning distances >20 Å in length and sometimes even >100 Å, allow for greater distances between crosslinked molecules. Included in this set of bioconjugation reagents are some of the discrete and polymeric polyethylene glycol (PEG) containing compounds, which can have repeating ethylene oxide units in their spacer arms of even longer than PEG_{24}. A long linker arm also creates a longer span between the point of modification on a protein and the detection molecule or affinity tag that is attached to the other end of the linker. Furthermore, this often provides greater access to other binding molecules, as in the case of streptavidin binding to a long, PEG-based biotin tag on an antibody. When the conjugation reaction is carried out in aqueous medium, it is crucial to use hydrophilic spacers, because long hydrophobic cross-bridges would only get buried in hydrophobic pockets in proteins or cause unacceptable nonspecific binding or aggregation in conjugates. By contrast, long PEG-based crosslinkers and modification reagents maintain extreme hydrophilicity and very low nonspecific binding. In fact, modification with long PEG-based reagents actually improves the hydrophilicity and dramatically decreases the tendency of the conjugate to aggregate, as compared to the corresponding antibodies or proteins before being modified.[30]

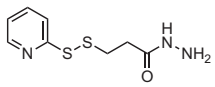
2.5. Cleavable vs Noncleavable Crosslinkers

If interacting biomolecules that have been captured by crosslinking subsequently need to be isolated and analyzed, it is important for the spacer arm of the crosslinker to be cleavable (**Figure 4**). For example, a purified protein is modified with a heterobifunctional crosslinker through its amine-reactive end, while the other end of the linker contains a photoreactive group that is capable of covalently linking to any nearby proteins after being exposed to UV light. The modified protein containing the photoreactive group is mixed with a biological sample to fish out any potential binding partners. After an incubation period, the sample is photolyzed with UV light and the photoreactive end of the modifying linker is then able to crosslink to any unknown interacting proteins within molecular reach. The interaction complex is then isolated using an immobilized antibody against the initial bait protein or through other known affinity interactions. After purification, the unknown interacting proteins can then be isolated away from the bait protein for individual analysis. It makes it easier to determine what proteins have bound to the bait protein if the photo-crosslinks can be reversed, and the unknown proteins can be obtained in purified form away from any covalent conjugates.[31]

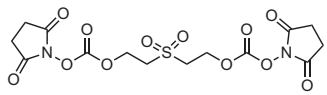
Another important application of cleavable linkers is in the use of label transfer reagents for the study of interacting proteins. In this instance, a trifunctional crosslinker is utilized that contains a spontaneously reactive (i.e., an NHS ester) component on one arm, a photoreactive group on a second arm, and a label, that is either detectable (e.g., fluorescent dye) or has an affinity tag (e.g., biotin) at its end, on the third arm. The linker



ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS)
(ester groups cleavable with H_2NOH)



3-(2-pyridyldithio)propanoyl hydrazide (PDPH)
(disulfide cleavable with DTT, 2-MEA, BME, or TCEP)



bis[2-(*N*-succinimidyloxycarbonyloxy)ethyl] sulfone (BSOCOES)
(sulfone cleavable with TRIS at pH 11)

Figure 4. Crosslinkers with Cleavable Spacer Arms.

to the spontaneously thermoreactive group is also designed to have a chemically cleavable site within its cross-bridge to permit subsequent release of the bait protein. The bait protein is first labeled with the trifunctional reagent through the thermoreactive group and then allowed to interact with proteins in a sample. After an incubation period, the sample is exposed to UV light to crosslink any interacting proteins. If the trifunctional crosslinker has a biotin handle, then the entire complex can be purified using an immobilized streptavidin support. Once the non-interacting components are washed off the support, the cleavable linker is broken and the bait protein is removed. What is left on the support is any of the interacting proteins that were captured during the photoreaction process. After the cleavage reaction has taken place, the arm containing the label is effectively transferred to the interacting proteins from the initially labeled bait protein. This is the reason these crosslinkers are called label transfer reagents.

2.5.1. Cleavable Disulfides

Crosslinkers and modification reagents containing a disulfide group within their spacer arms are some the most common cleavable linkers, and allow for the easy reversal of a conjugate through disulfide reduction. An example of this type of cleavable crosslinker is 3-(2-pyridyldithio)propionyl hydrazide (PDPH), which contains a thiol-reactive pyridyldithiol group at one end and an aldehyde-reactive hydrazide group at the other end. The pyridyldithiol group couples with thiols in proteins or other molecules to form a disulfide bond. Incubation of the conjugate with a disulfide reducing agent such as dithiothreitol (DTT), 2-mercaptoethylamine (2-MEA), β-mercaptoethanol (BME), or tris-carboxyethyl phosphine (TCEP) can be utilized to cleave the disulfide crosslink.[32]

2.5.2. Cleavable Esters

Some crosslinkers contain relatively stable ester bonds within their cross-bridges, which can be cleaved only with a strong base such as hydroxylamine (H_2NOH). For instance, sulfo-EGS (see Figures 1 and 4) contains two esters within its spacer formed by esterification of the carboxylates of succinic acid with the hydroxyls of ethylene glycol. Both ester bonds are completely cleaved with 0.1 M hydroxylamine, when the incubation is carried out at 37 °C and pH 8.5 for at least 3 hours.[33]

2.5.3. Cleavable Sulfones

An example of this type of cleavable reagent is bis[2-(*N*-succinimidyloxycarbonyloxy)ethyl] sulfone (BSOCOES), which is a homobifunctional compound with a central sulfone group surrounded at both ends by amine-reactive NHS carbonate groups. After BSOCOES crosslinks proteins in a sample—perhaps to capture interacting proteins—and the conjugates isolated from the rest of the proteins, then any interacting proteins can be purified from the bait protein by cleavage of the sulfone (0.1 M TRIS, pH 11, 37° C, 2 h).[34] In certain circumstances, denaturing conditions can also be used to disrupt any strong protein interactions and permit isolation of the interacting proteins.

2.6. Hydrophobic vs Hydrophilic Crosslinkers

Many of the early reagents for crosslinking and modification employed aliphatic or aromatic moieties, which imparted a generally hydrophobic nature to the reagents. Even with the advent of sulfo-NHS esters, which possess a strong negative charge and water solubility, their underlying base chemical structure remains quite hydrophobic. In some applications, reagent hydrophobicity can be an advantage, especially when an application involves a needed penetration of cell membranes. Hydrophobic reagents without any strongly polar groups will be able to quickly pass through membranes and crosslink or label internal cell proteins. However, those otherwise hydrophobic compounds that contain one or more negatively charged sulfo-NHS groups will still be restricted to reacting with the proteins on the outer membrane surfaces of cells due to their negative charge. The ability to switch between cell surface labeling and internal cellular labeling just by choosing charged or uncharged reagents is one benefit of using hydrophobic crosslinkers. For instance, the homobifunctional compound DSS has two uncharged NHS esters, is very hydrophobic, and, therefore, can crosslink interacting proteins within cells. By contrast, the similar compound BS^3 has two negatively charged sulfo-NHS esters and will be restricted to labeling outer membrane proteins. The two compounds are otherwise identical in their cross-bridge structure and reactivity.

In recent years, relatively short and discrete polyethylene glycol (PEG) spacers have resulted in new crosslinking and modification reagents that are extremely water soluble without the use of additional charged groups. The advantages of using such reagents are many: They can enhance the performance of a bioconjugate by increasing sensitivity in assays, enhancing signal-to-noise ratios, decreasing nonspecific binding, lowering off-target effects, and improving the stability of conjugates by decreasing aggregation. For example, using non-PEG hydrophobic reagents in the modification of antibodies often results in aggregation or precipitation in aqueous solution with potential loss of antigen binding activity.[35] Using PEG-based reagents instead doesn't cause aggregation or precipitation of antibodies or proteins, and dramatically increases the overall hydrophilicity and water solubility of antibodies and proteins modified by them. The use of hydrophilic bioconjugation reagents also results in greater biocompatibility. In contrast to conjugates or proteins crosslinked with hydrophobic reagents, those modified with PEG-based reagents will result in lower off-target effects in vivo, and will significantly reduce backgrounds in assays.[36]

Microparticles or nanoparticles that have a tendency to clump and aggregate in aqueous solution can be modified with PEG-based linkers to eliminate particle interactions and prevent aggregation. Creating a lawn or monolayer of PEG modifications on a particle or surface also can reduce nonspecific binding of biomolecules during assay or capture of target molecules. PEG-based reagents can nearly eliminate background due to nonspecific binding events and enhance signal in assays, because of less fouling of nonspecifically bound proteins at the surfaces.[37]

3. Conjugation of Amino Groups with Reactive Groups

Only a few of the many types of reactive esters that have been developed for use in bioconjugation have become reagents of choice for forming amide bonds with amine-containing molecules. The most popular of these active esters facilitate reactions that can easily occur in either organic solvents or in aqueous media at or near physiological pH. In the majority of such cases, proteins and other sensitive biomolecules can be quickly modified with reactive esters without loss of activity.

3.1. *N*-Hydroxysuccinimide (NHS) Esters

The electron-withdrawing ability of the two carbonyl oxygens in the *N*-hydroxysuccinimide ring makes the active ester—formed from NHS and a carboxylate—unstable enough to be readily displaced (primarily in an S_N2-type mechanism) upon attack by a primary amine nucleophile on the biomolecule to be crosslinked. An example of this reaction is illustrated for laminin β1 and laminin γ1 (**Scheme 2**).[13] The best amine nucleophile for the reaction with an NHS ester is one that

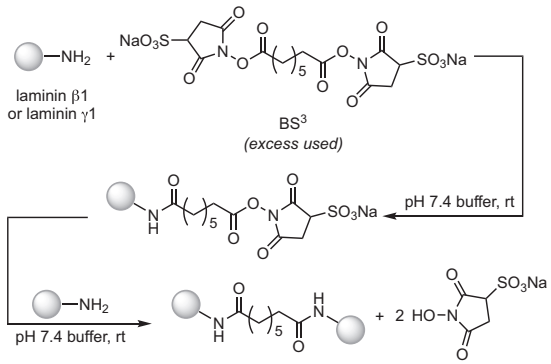
has its unshared pair of electrons fully exposed for interaction with the partial positive charge on the carbonyl group of the ester. Moreover, a primary amine that is in an unprotonated state in aqueous solution will make the best nucleophile for participation in the substitution reaction, and thus the most favorable conditions for conjugation are at pH values slightly above (and, optimally for many, at least one pH unit above) the pK_a of the amine groups being targeted for modification.

This dependence of reactivity on the protonation state of an amine-containing compound in solution, or of an amine group as part of a complex protein, is not as absolute as it might appear at first. For instance, lysine dissolved in an aqueous solution has a pK_a for its α-amine of approximately 8.9 and for its side-chain ε-amine of about 10.5. These pK_a values might lead one to believe that an NHS ester would not efficiently react with the α-amine of lysine until the pH exceeds pH 8.9, and with the ε-amine until the pH is above 10.5. In reality, the crosslinking reactions do occur over a broad pH range, even at pH values much lower than the pK_a of the amine. The pK_a values for lysine represent the pH values at which exactly 50% of the α- or ε-amine groups are protonated. Since the protonated and unprotonated forms are in equilibrium, even at pH units above or below the pK_a, there will still be enough protonated or unprotonated form present for the reaction to take place. Complicating matters further, lysine amines in proteins can have different pK_a values than they do when lysine is in solution. This is due to the intricate folded structure of protein chains that can cause micro-environmental changes to the protonation state or pK_a of an ionizable amino acid residue. For this reason, the pH titration curve of a protein is often considerably more complex than might be suggested by the titration curve of each independent amino acid residue. This is particularly the case when two ionizable residues can interact in the three-dimensional structure of a protein, perhaps forming salt bridges. Such interacting residues may have protonation states at a given pH far different than their solution-phase pK_a values would indicate.[38]

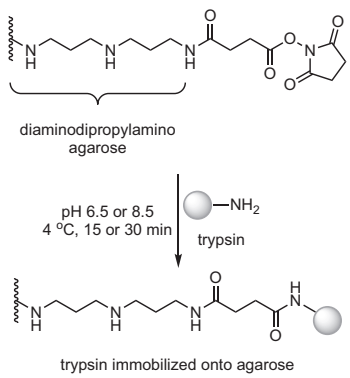
Isom et al. have reported that the ε-amine of lysine can undergo large shifts in its pK_a value, depending on where the side chain is located within the 3-D structure of staphylococcal nuclease.[39] After creating 25 mutants of the nuclease with lysine at different positions, the authors found pK_a values ranging from 5.3 to 10.4. They also discovered that, when the lysine residue was somewhat buried and therefore around more hydrophobic regions in the polypeptide chains, its pK_a was significantly depressed. This means that reactions with NHS esters may occur with high efficiency at physiological pH values, even if lysine in solution displays a much more alkaline pK_a. Since the population of protonated, [BH^+], and unprotonated, [B], lysine ε-amine groups is always in equilibrium at a given pH, the existence of even a small percentage of unprotonated amine will cause reaction with NHS esters and result in amide bond formation, as well as a shift in the [B]/[BH^+] equilibrium toward generating more [B] and more reaction, even if the pH is well below the pK_a of the amine. For these reasons, the reactivity of amino groups in proteins toward acylation with NHS esters occurs over a much broader pH range and at far more acidic values than their individual pK_a values might imply. In practice, reactions can be carried out with high efficiency and in good yields with ε-amino groups down to about pH 7, and with N-terminal α-amino groups down to about pH 5–6.

The amino group reactivity within proteins is also dependent upon *where* in the 3-D structure it resides. Leavell et al. probed the NHS ester reactivity toward primary amines in ubiquitin using mass spectrometry.[40] They found that the N-terminal amine on methionine, along with several other lysine ε-amines, were highly accessible and highly reactive, while several other amines were moderately reactive. However, they also discovered that there was a group of lysine ε-amines that were not reactive, and they correlated these residues to certain locations within the crystal structure of the protein that were involved with strong hydrogen-bonding interactions with neighboring amino acids. The results showed that the number of crosslinks observed in ubiquitin and their locations clearly followed the relative reactivity of the amines within the protein molecule. These observations are similar to the findings of Gibson and co-workers that certain lysine ε-amines in bovine cytochrome c were highly reactive in forming intraprotein crosslinks, while others were not reactive at all.[41]

An early study by Cuatrecasas and Parikh on the use of NHS ester reactive groups for the immobilization of proteins onto agarose chromatography supports revealed that proteins with aliphatic or aromatic amino groups could be immobilized through stable amide bonds over a pH range of 6–9 (**eq 1**).[42] The authors found that the rate of the crosslinking reaction over a given time frame and its yield were greater at pH 8.6 than at pH 6.3, but that the stability of the NHS ester groups toward hydrolysis was much greater at the lower pH. In addition, when the concentration of the amine-containing ligand was increased at both the low and high pH conditions, the lower pH reaction resulted in greater coupling yield than the higher pH reaction. The major reason for this result was a consequence of the rate of NHS ester hydrolysis in different pH environments.



Scheme 2. Example of the Crosslinking Reaction of *N*-Hydroxysuccinimide (NHS) Esters with Amines. *(Ref. 13)*



eq 1 *(Ref. 42)*

3.1.1. Hydrolysis of NHS Esters

The main competing reaction with the acylation of amines with NHS esters in aqueous medium is hydrolysis of the ester with water. Even though the oxygen in water is less nucleophilic than the nitrogen atom in the amine, water is typically far more abundant in concentration than the amine-containing molecule targeted for conjugation. These two competing reactions, crosslinking and hydrolysis, occur at varying rates at every pH value in aqueous solution, and it is finding the optimal balance between them that results in the best yield of amide bond formation. Hydrolysis of an NHS ester can occur at any pH, but the reaction rate generally increases as the environment becomes more alkaline. After only 40 min in aqueous solution at pH 8.6, over 80% of the NHS ester of agarose is hydrolyzed, whereas at pH 6.3 only about 23% is hydrolyzed over the same period (**Figure 5**).[42]

The stability of NHS esters toward hydrolysis in aqueous solution also depends upon the type of bioconjugation reagent containing the ester group. Both the overall hydrophobicity of the reagent and the relative hydrophobicity of the part of the reagent immediately adjacent to the ester can influence its hydrolysis rate. Thus, an NHS ester that is a part of an aliphatic, water-insoluble crosslinker often has a half-life in aqueous solution that is much longer than that of a similar reagent formed using water-soluble components. Even for extremely water-soluble crosslinkers, a hydrophobic moiety adjacent to the active ester group can significantly extend the latter's half-life. In this regard, PEG-based NHS esters are more hydrolytically stable when there is a longer aliphatic component immediately next to the ester but before the hydrophilic PEG chain. The presence of a single methylene group between the PEG chain and the carbonyl group of the NHS ester results in a highly reactive reagent, with a hydrolysis half-life at pH 8 and 25 °C of only $t_{1/2}$ = 0.75 min (hydrolyzes so fast it is almost unusable in aqueous solutions). Introducing one additional methylene group into the bridge between the PEG chain and the ester decreases the hydrolysis rate and increases the half-life to $t_{1/2}$ = 16.5 min, while inserting three methylene groups into the bridge decreases the hydrolysis rate further and increases the half-life to $t_{1/2}$ = 23 min (**Figure 6**).[43]

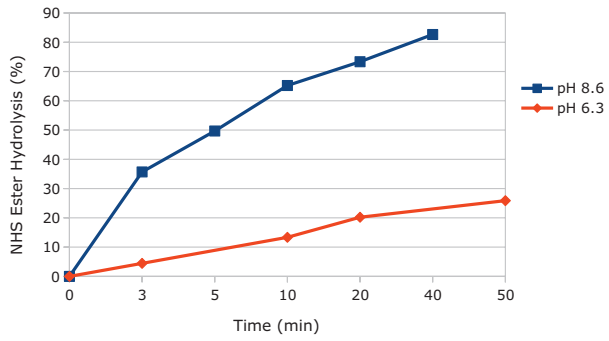
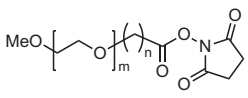


Figure 5. NHS Ester Hydrolysis at Different pH Values. *(Ref. 42)*



Abbr.	n	$t_{½}$, min
SCM-PEG	1	0.75
SPA-PEG	2	16.5
SBA-PEG	3	23

NHS ester hydrolysis half-life at pH 8 and 25 °C.

Figure 6. Effect of the Increasingly Hydrophobic Environment Adjacent to the NHS Ester Group on the Hydrolysis Rate of the Latter. *(Ref. 43)*

For many reactions in aqueous buffers, an NHS ester containing compound will react with an amine with good yield and reasonable efficiency at a pH range of 7.0–7.5. While raising the pH to more alkaline conditions will accelerate the reaction, it would also greatly accelerate the hydrolysis process and possibly negate any advantage that the higher pH condition may create. However, for some hydrophobic NHS ester compounds, such as large fluorescent dyes, the labeling of protein amines at a pH around neutral would result in unacceptably slow reactivity and poor coupling yields. Even most fluorescent dyes with sulfonates or other hydrophilic groups attached to them to make them more water soluble are best reacted at a pH of about 8.5 to obtain the best modification efficiency on antibodies and other proteins.

3.1.2. NHS Ester Reactivity toward Amino Acids in Proteins

The reactivity of NHS esters toward amino acids in proteins is often thought to be solely directed toward the N-terminal α-amines as well as the ε-amino groups of lysine residues. However, NHS esters have also been reported to react with other amino acid side-chain groups. In one study, Zenobi and co-workers carried out epitope mapping on the bovine prion protein to locate the exact positions of coupling when using homobifunctional NHS ester crosslinkers.[44] The authors found that, when crosslinking a monoclonal antibody–prion protein complex with disuccinimidyl suberate (DSS) or disuccinimidyl glutarate (DSG), both serine and tyrosine hydroxyl groups were sites of crosslinks in addition to the primary amino groups. In another report, laminin β1 and laminin γ1 N-terminal recombinant constructs were studied by crosslinking using BS^3 and BS^2G [bis(sulfosuccinimidyl) glutarate]. Kalkhof and Sinz found that up to 20% of the linkages formed were with hydroxyl-containing residues.[13] Mass spectrometric analysis of the modified peptides showed that 12.5% of these linkages to hydroxyl groups were with serine, 4.3% with tyrosine, and 3% with threonine residues.

A systematic study of DSS reactivity utilized synthetic peptides to determine the nucleophilic residues most likely to be modified.[14] The peptides were protected at their N-terminal to prevent α-amines from reacting, and each contained one or two reactive residues in their sequence. These residues were derived from the hydrophilic amino acids lysine (ε-amino), serine (primary OH), threonine (secondary OH), tyrosine (aromatic OH), arginine (guanidino group), and histidine (imidazole group). For peptides containing only a single amino acid that could potentially couple with an NHS ester (Lys, Arg, His, Tyr, Thr, or Ser), conjugation occurred only to the peptide containing a lysine ε-amino group.[14] None of the other amino acids could couple when they occurred alone within the peptide sequence. The authors assumed that histidine likely reacted with the NHS ester, but that its rapid hydrolysis prevented them from observing the labeled product. However, when they placed two potentially reactive amino acids close together in the peptide sequence, then amino acids other than lysine were observed to couple. For instance, the presence of a His close to a Ser or Tyr residue resulted in the labeling of the OH group on these residues. The likely path for this reaction is through initial formation of an acyl imidazole with the His side-chain nitrogen, and, since such

an intermediate is highly reactive, the acyl group migrates to the neighboring hydroxyl group of the Ser or Tyr residue, before the acyl imidazolide is hydrolyzed by water. When Ser or Tyr appeared alone in the peptide sequence without a neighboring His, no modification was observed, because of the competing NHS ester hydrolysis, which takes place before the NHS ester has a chance to react with the hydroxyl groups. It is worth noting that, in the complex folded structure of a protein, His does not have to be close in sequence to a hydroxyl-containing amino acid, just close enough in the 3-D structure of the folded protein to allow acyl transfer.

Other groups have reported similar modifications to Ser, Thr, or Tyr in the course of biotinylating proteins with NHS ester biotinylation reagents.[45–50] For example, several NHS ester containing biotinylation reagents were tested for their amino acid reactivity in the decapeptide [D-Lys-6-labeled] gonadotropin-releasing hormone.[50] While the majority of biotinylated peptide was modified once at the D-Lys-6 side-chain ε-amino group, a significant amount was di-biotinylated and occurred through amine reaction with D-Lys-6 and O-acylation at Ser-4 or Tyr-5. The researchers also found a novel conjugation event with the Arg-8 guanidino group, which is typically unreactive toward NHS esters, because the guanidino group is nearly always protonated in aqueous solution and therefore not nucleophilic.

The effect of neighboring arginine on the ability to acylate Ser, Thr, or Tyr hydroxyl groups in proteins or peptides with NHS esters DSS or BS^3 has also been investigated.[51] Arginine may affect such reactions, most likely due to its ability to stabilize structural intermediates through charge interactions or hydrogen bonding. This study found that unblocked Arg residues next to the hydroxyl-containing amino acids caused an increase in the modification of the hydroxyl groups by the NHS ester, while peptides with blocked guanidino groups did not have their hydroxyl residues modified except in very small amounts. The proposed mechanism for the reaction has the crosslinker first reacting with an amino group using one end of the homobifunctional compound. The carbonyl group of the NHS ester at the other end of the linker then forms a hydrogen bond with the guanidino group of Arg. This association traps the crosslinker immediately next to the hydroxyl-containing amino acid, and allows it to esterify the OH group.

Since NHS ester crosslinkers and modification reagents are capable of reacting with a number of protein functional groups, the exact location of each coupling reaction within a peptide or complex protein may not be entirely predictable just through knowledge of its sequence, because hydrogen bonding and complex polypeptide chain folding can bring residues together and enhance or inhibit their reactivity. Therefore, if bioactivity is compromised after reaction with an NHS ester reagent, it may not be solely due to the modification of α-amines or lysine ε-amines, but could also be due to O-acylation at hydroxyl-containing amino acids or even, in rare cases, modification at exposed cysteine thiols or arginine guanidino groups. Mass spectrometric analysis may ultimately be the best method for determining which amino acid residues end up being modified with NHS ester reagents.

3.2. Sulfo-NHS Esters

Sulfo-NHS esters have nearly identical properties to NHS esters, but are particularly useful in aqueous solution. The first two members of this class of crosslinkers were synthesized by Staros.[52] The sulfonic acid group is deprotonated under all pH conditions useful for coupling with amines, and its resulting strongly polar character often will be enough to drive an entire, otherwise hydrophobic, crosslinker into solution in aqueous buffers. For example, the hydrophobic NHS ester DSS must first be solubilized in a water-miscible organic solvent such as DMF, DMSO, or DMAC before adding it to an aqueous reaction medium, whereas the structurally very similar, but hydrophilic sulfo-NHS ester BS^3 can be added directly to the aqueous medium. When a hydrophobic NHS ester crosslinker is predissolved in organic solvent and an aliquot added to an aqueous reaction medium, a cloudy micro-precipitate typically ensues. In contrast, with a sulfo-NHS ester—even when predissolved in an organic solvent to prevent prior hydrolysis of the ester—a micro-precipitate is not formed and, thus, the crosslinker has much better access to water-soluble biomolecules in the still-homogeneous reaction medium. Similarly, the water solubility of sulfo-SMCC results in a more efficient conjugation reaction—as compared to that of the hydrophobic SMCC—with a higher yield of amine modification during the initial amide-bond-forming step of the two-step conjugation process. It is worth mentioning that a sulfo-NHS ester containing compound may display initial water solubility due to the presence of the sulfonate group, but, once that leaving group is lost in the amine-modification step, the rest of the crosslinker (depending on its structure) may no longer possess its beneficial solubilizing effects.

Crosslinkers or modification agents containing a sulfo-NHS ester group are generally membrane impermeable and, thus, their reactions with amines would be restricted to the outer membrane proteins on the exterior cell surface. This can be a great advantage when studying protein interactions on cell surfaces through crosslinking, or when the isolation of membrane proteins using immobilized streptavidin is desired. However, when labeling a cell surface, a sulfo-NHS ester present on a PEG-based crosslinker or modification reagent may not always be cell impermeable despite the negative charge on the sulfonate. This is because the ethylene oxide units are both water soluble and organic soluble and, thus, may have a tendency to penetrate cell-membrane structures even though the reagent displays extreme hydrophilicity and water solubility. For this reason, the concentration of the reagent in the cell suspension may have to be adjusted downward to minimize or prevent intracellular labeling.

The reactions of NHS esters (or sulfo-NHS esters) with biomolecules are typically carried out in aqueous buffers containing perhaps salts and a number of other additives. Since buffers and additives are usually present in much higher concentrations than the proteins or biomolecules being modified, it is important that they not contain substances that compete with the protein amines for the ester of the crosslinker. Such interfering substances include nitrogenous buffers (e.g., TRIS and imidazole), and amino acids in buffers, such as glycine, which is often added to control pH. They also include any salt additive containing an ammonium ion such as ammonium sulfate—which is commonly used in fractionating proteins from cells and tissues or for stabilizing proteins—ammonium bicarbonate, ammonium citrate, ammonium acetate, and any other salt containing the NH_4^+ ion. Moreover, other functional groups that can react with NHS esters and lead to interfering side reactions are: (i) Azides, which are often found as preservatives in solutions. (ii) Urea, which is often used to extract proteins from cells and tissues or to re-solubilize proteins from inclusion bodies. Urea can break down into unstable carbamic acid, which then decomposes into CO_2 and ammonia. (iii) DTT or other types of thiol-containing reducing agent, which are commonly used to promote protein stability or prevent oxidation. All of these small-molecule interfering substances can be removed from extracts or solutions by simple dialysis or size-exclusion chromatography.

Another category of potentially interfering substances is other proteins or blocking agents that contain amine groups or other nucleophiles. Antibodies in particular are often supplied with BSA or some other protein-blocking agent, or even as crude antisera, all

of which may improve their stability in storage and prevent their aggregation, but will also cause them to compete with NHS ester reactions. However, antibodies can be purified away from other proteins by affinity chromatography on immobilized protein A or protein G, or by using an immobilized antigen.

3.3. Fluorophenyl Esters

Esters derived from fluorinated phenols are growing in popularity for conjugation applications.[53–58] These reactive esters are derived primarily from three different types of phenol: pentafluorophenyl (PFP), tetrafluorophenyl (TFP), and sulfo-tetrafluorophenyl (STP). PFP esters and TFP esters are uncharged and hydrophobic and much more so than NHS esters (**Figure 7**). For this reason, they often are more hydrolytically stable in aqueous buffers than NHS esters, and typically give higher yields of amide bonds than the corresponding NHS esters at a given reaction pH. An STP ester has greater hydrophilicity than its PFP and TFP counterparts, but less so than its sulfo-NHS ester analogue.

PFP esters and TFP esters are perhaps the most commonly used fluorophenyl esters in bioconjugation and in organic synthesis, and, similarly to NHS esters, they undergo nucleophilic substitution at a slightly basic pH. Fluorophenyl esters have been utilized extensively as amine-reactive groups for linking fluorescent dyes to antibodies to make probes for cells and tissues,[53] for coupling bifunctional metal chelates to antibodies to make radiolabeled probes for imaging,[54–56] for the conjugation of drugs to monoclonal antibodies to make ADCs,[57] and for immobilizing molecules onto surfaces.[58]

The presence of a TFP ester group on a PEG chain, which is extremely hydrophilic, provides increased stability toward hydrolysis of the ester group than when an NHS ester is present. As with their NHS ester counterparts, TFP esters can undergo several side reactions, including hydrolysis, reaction with OH groups in proteins and other biological molecules, and formation of inactive cyclic NHS derivatives when the TFP ester is located at the end of a succinic acid spacer (**Scheme 3**).[57] Medley and co-workers undertook a detailed study of the rate of TFP ester hydrolysis over a pH range of 3 to 9 and a temperature range of 10 to 30 °C.[57] The authors reported that the TFP ester was most stable at pH 5–7, was also least reactive toward amines in this pH range, and that the reaction rate increased with increasing temperature over all pH conditions studied. Thus, the coupling reactions of TFP esters with amines are best carried out at ambient temperatures and pH conditions that are slightly alkaline (pH 7–8.5).

3.4. EDC (EDAC)

A carbodiimide is considered a zero-length crosslinker, because it creates a direct link between a carboxylic acid and an amine without an intervening spacer.[28,59] The water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC or EDAC) is one of the most commonly used linking agents for the immobilization of proteins, antibodies, and other amine-containing molecules onto carboxylated microparticles, nanoparticles, or other nanomaterials,[60–62] and for the coupling of affinity ligands onto surfaces.[63] Because EDC is hygroscopic and susceptible to hydrolysis, it should be kept dry until needed. Gilles et al. examined the rate of hydrolysis of EDC in aqueous solution at various pH values, and found that the half-life of EDC ($t_{1/2}$) decreases with a decrease in pH: 37 h (pH 7), 20 h (pH 6), and 3.9 h (pH 5).[64]

For a conjugation reaction in an aqueous medium, EDC initially reacts with a carboxylate group to form an intermediate *O*-acylisourea. This is another type of active ester that can react with an amine to form an amide bond.[65] The *O*-acylisourea intermediate can also undergo a number of side reactions, such as hydrolysis back to the unreactive carboxylate and an inactive isourea,[28] reaction with a neighboring carboxylate group to form an anhydride, or an irreversible rearrangement into an inactive *N*-acylisourea, which may cause nonspecific binding in some applications. The adverse effects of these side reactions can be mitigated by employing EDC/NHS or EDC/sulfo-NHS combinations. When a conjugation reaction is carried out in the presence of EDC and NHS or sulfo-NHS, the intermediate *O*-acylisourea would then react with the OH group of these molecules and form a more hydrolytically stable intermediate NHS or sulfo-NHS ester. An example of the power of this approach was demonstrated in the coupling of radiolabeled glycine to keyhole limpet (*Megathura crenulata*) hemocyanin (KLH) or to bovine serum albumin as a model system for the linking of peptide haptens to carrier proteins (**Scheme 4**).[66] The reaction yield (defined as the percent of total glycine added that was precipitated with the protein, after correcting for background) increased from 0.9% with EDC alone to 21% using EDC/sulfo-NHS (for hemocyanin) and from 1.9% to 38% (for bovine serum albumin). The coupling of peptides containing a free α-amine and lysine ε-amine to carrier proteins can become so efficient with the use of the EDC/sulfo-NHS system that nearly 100% of the peptide hapten can become conjugated to the carrier protein. In addition to improving the yields of such coupling reactions, the negatively charged sulfo-NHS can create a negative-charge repulsion between particles during their activation, thus inhibiting particle aggregation during the coupling reaction. This is extremely important with particles that have a base substrate possessing highly adsorptive or hydrophobic properties.

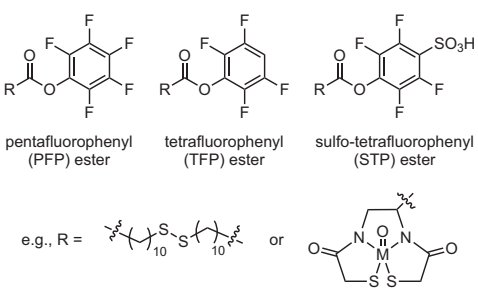
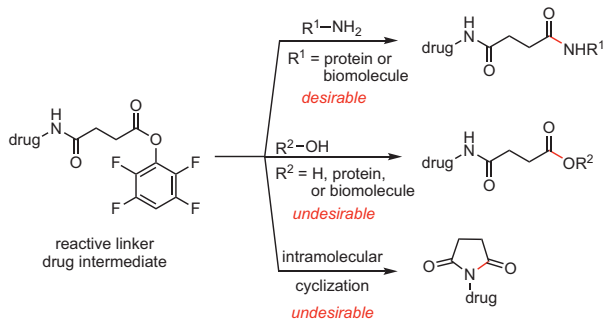


Figure 7. Popular, Reactive Fluorophenyl Esters.



Scheme 3. Typical Desirable and Undesirable Reactions of Reactive Tetrafluorophenyl (TFP) Esters. *(Ref. 57)*

4. Conjugation of Thiols with Reactive Groups

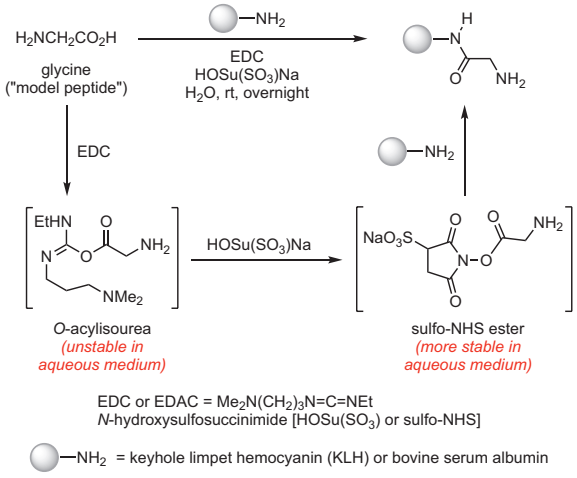
4.1. Maleimides

Reactive maleimide groups are the most popular choice for conjugation with thiols, leading to thioether bonds, which are reasonably stable linkages that cannot be reductively cleaved. However, the reaction of maleimides with thiols or amines is pH dependent. When the pH is around 7, coupling of maleimides with thiols is about 1000 times faster than their coupling with amines, which results in excellent chemoselectivity toward cysteine thiols in proteins.[67] At a much higher pH, the maleimide group becomes more reactive toward amines and thus loses its chemoselectivity.[68] The principal side reaction of maleimides in aqueous solution is hydrolysis of the ring imide to an open maleamic acid form consisting of an amide and a terminal carboxylate group with consequent loss of thiol coupling ability to the internal double bond. The rate of this hydrolysis increases with increasing pH and is dependent on the nature of other nearby structural elements present in the crosslinker. One of the most stable crosslinkers in this regard is SMCC (see Figure 3). The maleimide end of SMCC (and sulfo-SMCC) is unusually stable due to the presence of an adjacent cyclohexane ring, which probably limits access by water to the imide bond and, thus, water's ability to hydrolyze it. A study of the coupling reaction rate, as well as the hydrolysis rate, of the model maleimide, *N*-ethylmaleimide (NEM), with glutathione as a model peptide reports that the thiol coupling reaction takes place very rapidly and goes to maximal yield in only 3 minutes at pH 7 or 1 min at pH 8 (**Scheme 5**).[69] The study also found that the hydrolysis rates were much slower and similarly increased with an increase in pH. It is worth noting that hydrolysis of the maleimide group can also occur after the coupling of a thiol has taken place, and leads to two possible succinamic acid thioether isomers, depending on which side of the ring opens up relative to the position of the thioether. These isomers may result in different activities of a conjugate when used in detection applications or for in vivo therapeutic and diagnostic purposes. Moreover, ring opening after thioether bond formation yields a new, negatively charged carboxylate in the cross-bridge, which may result in new electrostatic interactions with biomolecules and unexpected nonspecific binding or off-target effects. To reduce this potential heterogeneity around the thioether bond, methods have been developed to purposely hydrolyze the imide after thiol coupling.[70] After such treatment, the thioether bond region will not undergo any further chemical changes. This strategy was applied by Lyon et al. toward stabilizing antibody–drug conjugates by creating self-hydrolyzing maleimides.[71] Maleimides in antibody–drug conjugates (ADCs) can be unstable in plasma or in circulation in vivo due to elimination of the maleimide–thiol bond through a retro-Michael process. This reaction happens if the cross-bridge is held in close proximity to a lysine amino group such as within the pocket of serum albumin. The retro-Michael reaction results in the amino group of lysine displacing the thioether linkage to the succinimide ring, and thus breaking apart the conjugate. The self-hydrolyzing maleimide was created by incorporating a basic amino group adjacent to the maleimide through the use of diaminopropionic acid.[71] ADC stabilization through accelerated hydrolysis of maleimide bonds was also accomplished through the use of *N*-arylmaleimides, such as *N*-phenyl and *N*-fluorophenyl, which were found by Christie et al. to have an inherently rapid hydrolysis rate relative to their *N*-alkyl counterparts.[72]

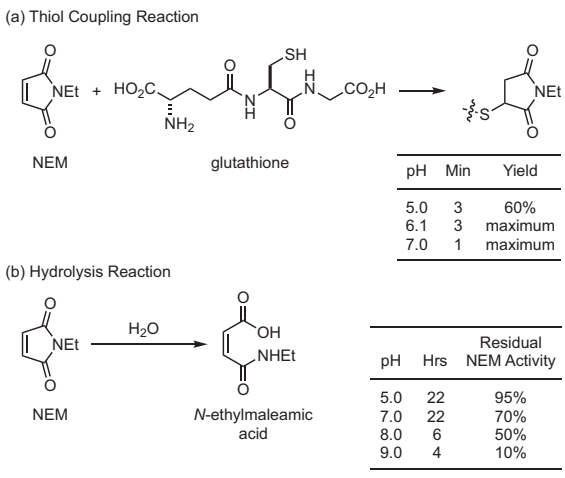
Other undesirable reactions that interfere in the coupling of thiols to maleimides involve thiol-containing reducing agents such as DTT, BME, and 2-MEA, as well as tris(2-carboxyethyl)phosphine (TCEP), a reagent that contains no thiol groups.[73,74] Glutathione (GSH)—present in cells in millimolar concentrations[75] that are orders of magnitude greater than the concentrations of most thiols in proteins from cell lysis or tissue extraction—can compete with solubilized proteins for coupling with maleimides, and should be removed by dialysis or size exclusion chromatography prior to attempting the coupling reaction. Finally, thiol coupling reactions with maleimides should not be attempted in the presence of any group VI oxometallates, such as molybdate and chromate, since these catalyze the rapid hydrolysis of the maleimide ring imide bond and inactivate the double bond toward subsequent coupling with thiols.[70]

4.2. Cyanoethynyls (3-Arylpropiolonitriles; APNs)

A new class of thiol-specific conjugation reagents consisting of a thiol-reactive 3-arylpropiolonitrile (APN) group, with good stability in aqueous media, has recently been described.[76,77] The APN group



Scheme 4. Dramatic Effect of Adding *N*-Hydroxysulfosuccinimide on the Yield of the Coupling of Glycine to Hemocyanin with EDC (Refer to the Discussion on Page 52). *(Ref. 66)*



pH	Min	Yield
5.0	3	60%
6.1	3	maximum
7.0	1	maximum

pH	Hrs	Residual NEM Activity
5.0	22	95%
7.0	22	70%
8.0	6	50%
9.0	4	10%

Scheme 5. The Coupling Rates of *N*-Ethylmaleimide (NEM) with Glutathione at Different pH Values, and the Hydrolysis Rates of NEM at the Same pH Values. *(Ref. 69)*

allows the targeted coupling of thiols in biomolecules, and results in stable thioether linkages without the risk of subsequent side reactions that can occur with maleimides. This mode of coupling is illustrated by the use of the heterobifunctional crosslinker CBTF to prepare antibody conjugates that are remarkably stable in plasma (**Scheme 6**).[77] First, the STP end of CBTF reacts with amine-containing molecules or proteins to provide intermediates containing the thiol-reactive APN group, which then undergoes coupling to a thiol group in another molecule or protein to provide the desired conjugate. This inherent stability of APN-based reagents and derived conjugates holds promise for better performance in many biological applications than the maleimide-based counterparts.

4.3. Iodo- and Bromoacetyls

Another type of thiol-reactive group is haloacetyl, in particular bromo- and iodoacetyls, which can form stable thioether linkages. While this substitution reaction can take place with a number of nucleophilic groups in a pH dependent way, it is most specific for sulfhydryl groups in proteins when carried out at a slightly alkaline pH.[78,79] Haloacetyl compounds are very hydrolytically stable in aqueous environments under normal conditions. However, iodine can be lost due to light-catalyzed degradation or exposure to a reducing agent. Therefore, reagents containing haloacetyl groups should be handled in subdued light and reducing agents carefully removed before attempting the reaction with thiols. SIAB is an example of a heterobifunctional crosslinker containing an amine-reactive NHS ester at one end and an iodoacetyl group at the other, separated by a short *para*-aminobenzoate spacer (see Figure 3). Sulfo-SIAB is a sulfonate analogue of SIAB with similar reactivity, but is water soluble and cell membrane impermeable.

4.4. Pyridyldithiols

The pyridyldithiol group has been used for decades to form reversible conjugate linkages to sulfhydryl groups in proteins or other molecules.[80,81] This takes place through a disulfide interchange reaction, whereby a free cysteine SH group displaces the pyridine-2-thiol leaving group and forms a new mixed disulfide with the crosslinking or modification reagent. The new disulfide bond can then be cleaved, allowing the rupture of conjugate crosslinks in vitro or in vivo. One advantage of this approach is providing intracellular cleavability for biologics. For example, the pyridyldithiol group could be used to form an antibody–drug conjugate (ADC) for tumor targeting. After docking on the cell surface and internalization, the disulfide bond between the antibody and the drug is then cleaved in the reductive environment within the cell, releasing the toxic drug to kill the tumor. **Figure 8** shows some of the more popular pyridyldithiol-containing reagents, both hydrophobic and hydrophilic.

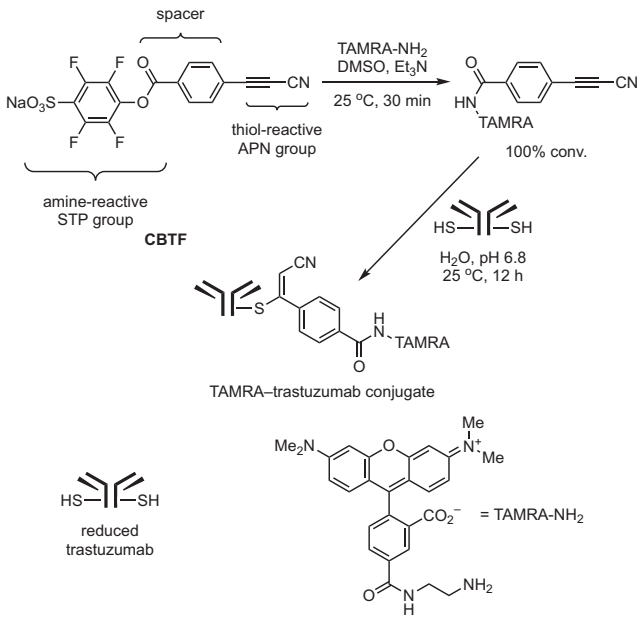
5. Conjugation of Carbonyl Groups

Aldehydes, derived from carbohydrates in biomolecules either by ring-opening of the carbohydrate or by oxidation of diols, may be specifically conjugated using hydrazide, hydrazine, or aminooxy functional groups, leading to the spontaneous formation of hydrazone or oxime linkages. Another route to conjugations using these reactive groups is to pair crosslinkers containing a hydrazide, hydrazine, or aminooxy group with crosslinkers containing an aldehyde. The opposite end of each of these crosslinkers can contain another reactive group that can be used to modify biomolecules so that one biomolecule is derivatized to contain an aldehyde and the other one is derivatized to contain a hydrazide, hydrazine, or aminooxy group. Mixing these two modified molecules together then spontaneously forms the desired bioconjugate through hydrazone or oxime linkages.

Since most biological macromolecules do not normally contain aldehydes or ketones—or hydrazide, hydrazine, or aminooxy groups—crosslinkers containing these reactive groups can find each other in complex solutions and conjugate together with excellent efficiency. Hence these complementary groups can be considered bioorthogonal. Hydrazide or aminooxy reagents can also target ketone groups in unnatural amino acids or sugars., as in the cell-surface labeling of glycoproteins and glycolipids.[82,83] The study of protein glycoengineering has been enabled by use of aminooxy- and aldehyde-based reagents,[84] and glycoconjugates can be analyzed in western blots using hydrazide-based probing.[85]

5.1. Hydrazides and Hydrazines

The hydrazone linkage, formed from the reaction of hydrazide with an aldehyde or ketone in aqueous medium, is much more stable than the corresponding Schiff base formed between an aldehyde and an amine. Nevertheless, hydrazone linkages may still hydrolyze and leach



Scheme 6. Application of Thiol-Reactive 3-Arylpropiolonitriles (APNs) to the Preparation of Remarkably Stable Antibody Conjugates. *(Ref. 77)*

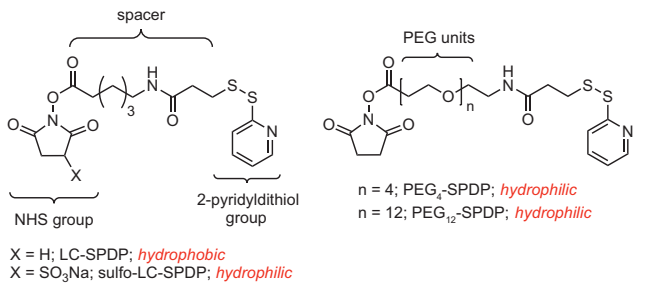


Figure 8. Popular Pyridyldithiol Crosslinking Agents.

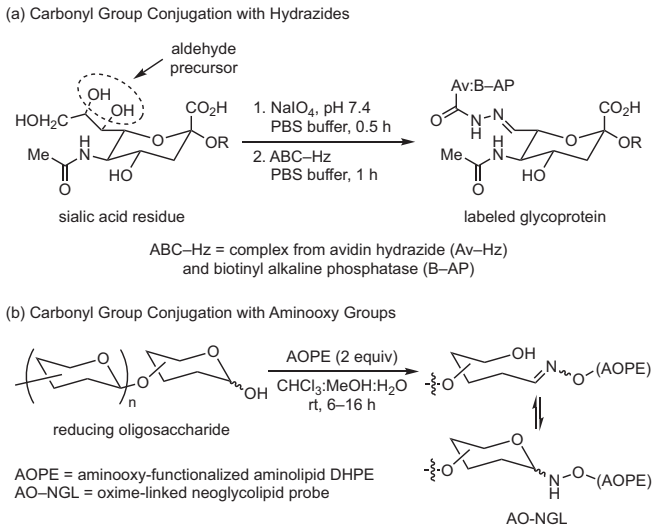
off conjugated molecules over time. To stabilize the linkage further, it may be converted into a hydrolytically stable *N*-alkylhydrazino bond by reduction with sodium cyanoborohydride. Some examples of hydrazide crosslinking reagents include the homobifunctional adipic acid dihydrazide [$(H_2NNHC(=O)CH_2CH_2)_2$] and the shorter and water-soluble carbohydrazide [$(H_2NNH)_2C=O$].

A bis-hydrazide reagent can modify a protein at points of glycosylation on the protein after aldehyde groups are generated by mild periodate oxidation of the carbohydrate groups on the protein. A bis-hydrazide reagent can also react with carboxylate groups on a protein in the presence of a carbodiimide such as EDC. Hydrazide-modified avidin prepared in this manner has been employed to detect glycoproteins on cell surfaces after periodate oxidation (**Scheme 7**, Part (a)).[86–88] Adipic acid dihydrazide has been utilized to create hydrazide functionalities on surfaces, particles, or polymers that contain aldehyde groups, as in the preparation of hydrazide-activated supports for the coupling of aldehyde-containing affinity ligands.[89,90]

5.2. Aminooxy Groups

The aminooxy or alkoxyamine ($RONH_2$) group exhibits a reactivity similar to that of hydrazides in their reactions with ketones or aldehydes, leading to the formation of oximes. However, aminooxy groups react more rapidly with ketones than do amines or hydrazides, allowing ketones in biomolecules to be effectively conjugated. Moreover, the oxime bond is far more stable in aqueous solution than the corresponding Schiff base or hydrazone, does not undergo hydrolysis under normal conditions, and rarely requires reduction (as the hydrazone linkage does in some cases) to be stable.

Aminooxy groups on crosslinking or modification reagents can be used to specifically link to aldehyde groups on other reagents or macromolecules (Scheme 7, Part (b)).[21,91–93] This bioorthogonal reaction can be highly chemoselective even in the presence of other proteins and biomolecules, provided keto-group-bearing small-molecule metabolites such as pyruvate are removed from biological samples by dialysis or size exclusion chromatography prior to the aminooxy reaction. Once the aldehyde group becomes available—through periodate oxidation of glycosylated proteins or through cleavage of glycans from glycoproteins revealing their reducing ends—it can be targeted for coupling away from active sites or binding regions in proteins or antibodies. The reaction of an aminooxy group with aldehydes or ketones can be catalyzed by arylamines, such as aniline.[18–20,94] When added in excess, aniline would condense rapidly with the aldehyde or ketone. The intermediate imine formed would then undergo protonation at moderately acidic pH more rapidly than the oxygen of the aldehyde or ketone. Thus, it is this protonated Schiff base that reacts with the aminooxy group to form the final oxime linkage.[95]



Scheme 7. Carbonyl Group Conjugation with (a) Hydrazides and (b) Aminooxy Groups. *(Ref. 21,88)*

6. References

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Greg T. Hermanson is President of Greg T. Hermanson, Inc., a bioscience consulting company, and the Chief Technology Officer and Principal at Aurora Microarray Solutions, Inc. Greg has over three decades of experience in the development of life science products for research and diagnostics, with broad expertise in protein chemistry, assay development, immobilization, and bioconjugation. He is the author of two bestselling textbooks: *Bioconjugate Techniques*, now in its third edition, is an extensive manual on the methods of bioconjugation, and *Immobilized Affinity Ligand Techniques*, which is a manual on the covalent attachment of affinity ligands to solid supports and affinity chromatography. Greg's significant impact on the bioscience fields is attested to by nearly 27,500 citations to his publications and patents, with one of his publications being in the top 50 cited references of all time.

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