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Recent Advances in Bioorthogonal Reactions

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[§]SCS-DSM Award for best poster presentation in Chemical Biology

Abstract: The ability to selectively perform chemical reactions within living systems has transformed the field of Chemical Biology. These chemoselective processes have had a major scientific impact by enabling studies of cellular processes, producing tools for the ligations of large biomolecules, and advancing our ability to image or track small molecular changes such as posttranslational modifications. As more investigators become involved in the development and application of chemical reactions performed within complex biological settings, there have been a rising number of innovations that create improved tools and resources for understanding nature. This perspective highlights some recent achievements that show how innovation and creativity can provide new opportunities in this exciting and rapidly expanding area of chemical research.

Keywords: Bioorthogonal · Chemical Biology · Chemical Reporter · Chemoselective · Ligation

Rebecca Schäfer received her Bachelor and Master's degrees in chemistry from the Ruprecht-Karls Universität in Heidelberg. During her Master's studies, she performed research in Prof. Nicolas Mézailles's group at the CNRS in Toulouse, followed by an extended stay at the University of California, Berkeley, to conduct her Master thesis with Prof. F. Dean Toste. After her Master's studies, she went to ETH Zürich and is currently pursuing her PhD studies in Chemistry with Prof. Helma Wennemers to develop novel bioorthogonal ligation reactions.

1. Introduction

The identification and validation of targets for various diseases requires an in-depth molecular understanding of the underlying chemical and biological processes. Since early descriptions of live-cell imaging in the early 20th century,[1,2] a multitude of new inventions and discoveries have been developed that enable the study of biomolecules in their native environment ranging from highly efficient optical methods[3,4] to novel fluorophores.[5] Recent advances in the tracking and visualization of proteins include fusions with fluorescent proteins[6] or small peptidic tags [7,8] to further complement these ever-improving optical technologies.

Although proteins comprise the largest fraction of a cell's dry mass, it is estimated that more than half are modified with glycans, lipids, or small metabolites.[9] Thus, methods that enable the selective labeling of both proteins and non-proteinaceous biomolecules provide incredible value for the analysis of cellular downstream processes and further improve the understanding of living systems. Amongst these methods, biocompatible chemical reactions have proven useful to selectively label proteins, nucleic acids, cell surface glycans and lipids, as well as for the controlled ligation of two biomolecules.[10–12] These chemoselective reactions, also coined as *bioorthogonal*,[13] must function reliably under aqueous biological conditions at physiological pH (6.4–8.0) and temperature (approximately 37 °C), as well as in the presence of a high concentration of biomolecules bearing a plethora of potentially competing functional groups.

One specific application for bioorthogonal chemistry is the so-called 'chemical reporter' strategy, which has proven especially useful for *in cellulo* or even *in vivo* tracking of small biomolecules such as cell-surface glycans, lipids or nucleic acids.[12] The chemical reporter approach has two major challenges: first, the genetic or metabolic incorporation of a 'reporter' functional group into the target biomolecule *via* an appropriate chemical precursor; and second, the highly chemoselective reaction of the reporter with an exogenously delivered probe that enables visualization or isolation of the target molecule. To create a successful system, the functional group of the chemical reporter should therefore be both small and biologically inert in order to avoid interference with the natural function of the biomolecule, and the reaction with the probe should be fast and highly selective.[14]

Numerous types of bioorthogonal reactions have been developed to date including dipolar cycloadditions (*e.g.* metal-catalyzed or strain-promoted cycloaddition),[15–19] inverse electron-demand Diels-Alder reactions (tetrazine ligations)[20–22] and polar reactions (*e.g.* the Staudinger ligation[23–26] or imine/oxime/hydrazone ligations[27]). However, there is no single reaction that is ideally suited for every situation,[28–30] and as new biological applications emerge, concurrently, a need for the development of optimal chemical processes arises.

This perspective highlights some recent achievements in bioorthogonal chemistry from the last three years and shows how innovation and creativity can provide new opportunities in this exciting and rapidly expanding area of chemical research.

2. Cycloaddition Reactions

2.1 *Metal-catalyzed Cycloadditions*

Cycloaddition reactions are the most commonly used bioorthogonal ligations, and prominent examples include the copper-catalyzed azide-alkyne cycloaddition (CuAAC)[15,16] and the strain-promoted azide-alkyne cycloaddition (SPAAC).[18] Since its discovery, the copper-catalyzed azide–alkyne cycloaddition reaction has found countless applications in many areas of science and has had a significant impact in chemical biology due

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to its effectiveness in aqueous media (Fig. 1a).[17] Limitations in more complex biological settings arise from the restriction of the CuAAC to terminal alkynes, which along with some azides can have cross-reactivity with cellular thiols, but, most importantly, is the cytotoxicity of redox-active Cu(I)-species generated *in situ* during the reaction.[31] Accordingly, most optimization strategies for the CuAAC include the use of copper-stabilizing ligands or variations in the reducing agent that serve to both enhance the rate and reduce cytotoxicity,[32] but by comparison relatively little work has been reported on azide-alkyne cycloadditions catalyzed by alternative metals that are also biocompatible.

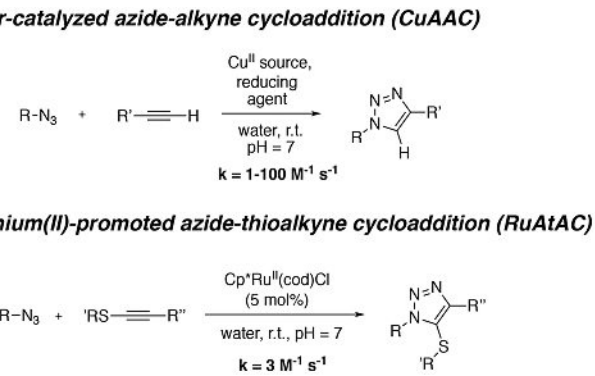

Fig. 1. a) Copper-catalyzed azide-alkyne cycloaddition. b) Ruthenium-promoted azide-thioalkyne cycloaddition.

One example of a well-known metal-catalyzed azide-alkyne cycloaddition reaction re-engineered for biological applications is the ruthenium-catalyzed azide-alkyne click reaction (RuAAC), a synthetic method used to generate 1,5-substituted triazoles.[33,34] Ruthenium catalyzed cycloaddition reactions normally require harsh conditions and is highly sensitive to oxygen or water, precluding its use in bioorthogonal applications. However, recently, Mascareñas and coworkers reported a biocompatible ruthenium(II) catalyzed cycloaddition reaction that functions at room temperature in water (Fig. 1b).[35] Here, they demonstrate that ruthenium catalysts of the type Cp*Ru(cod)Cl can efficiently catalyze the reaction between azides and thio-substituted alkynes at sub-stoichiometric amounts (5 mol% cat) and in good yields. Notably, in contrast to CuAAC, the so-called Ru-promoted azide–thioalkyne cycloaddition (RuAtAC) works without the addition of any external reducing agents and tolerates the replacement of terminal alkynes with internal thio-substituted alkynes. RuAtAC proceeds quickly in aqueous conditions ($k = 3\ M^{-1}\ s^{-1}$ for RuAtAC[35] *vs* $k \sim 1–100\ M^{-1}s^{-1}$ for CuAAC[14,36]) and without any side reactions in complex biological media such as cell lysates.

2.2 *Light-activated Cycloadditions*

Light is a benign and traceless reagent that provides a valuable tool for bioorthogonal chemistry. Irradiation with UV light is frequently used to liberate highly reactive intermediates from stable precursors to enable rapid chemical processes that are otherwise untenable within physiological conditions. As such, light provides temporal control for the chemical activation of a number of chemoselective reactions, and thus enables acute modulation of biomolecules within a distinct time frame.

A well-suited functional group for light-activated processes is the nitrile imine, which is a highly-reactive moiety that undergoes rapid 1,3-dipolar cycloaddition reactions with various types of linear or strained alkene and alkyne dipolarophiles (Fig. 2).[37] The high instability of nitrile imines prevents their direct use in complex biological settings as they undergo background hydrolysis as well as reactions with biological nucleophiles.[38] Thus, they are instead applied as stable tetrazole precursors that form nitrile imines upon activation by light.[39–41] Recent work by Lin and coworkers demonstrated that the instable nitrile imine moiety can be further shielded against nucleophilic attack through the installation of adjacent bulky groups *ortho* to the nitrile imine.[42] Following extensive screening, it was determined that incorporation of N-Boc pyrrole substituents in both *ortho* positions led to the highest selectivity for cycloadditions over other background reactions if a spirohexene dipolarophile was used. Both the N-substituent as well as its proximity to the nitrile imine appeared to be crucial as a lack of either resulted in decreased selectivity. While nucleophilic attack of thiols was greatly hindered as a result of the increased sterics (predicted to cause >6000-fold rate decrease) the rate of the cycloaddition reaction was also decreased, but by no more than 2-fold. Solution and solid-state analysis suggests that the N-Boc pyrrole substituents form a bowl-shaped shield around the nitrile imine, which has a higher impact on the thiol attack than on the cycloaddition due to the necessary trajectory of the approaching thiol nucleophile. Furthermore, the optimized nitrile imine, after activation, was found to be stable towards hydrolysis for ~100 seconds in phosphate buffer/acetonitrile (1/1), compared to non-substituted analogs, which only last ~8 seconds before hydrolysis. The authors demonstrated the compatibility of the optimized "photo-click reaction" within biological systems through fluorescent labeling of the G-protein coupled glucagon receptor (GCGR)—a member of the class B G-protein-coupled receptor family and a validated drug target for diabetes treatment—in live mammalian cells.[42] GCGR mutants were encoded with a spirohexene amino acid (SphK) and successfully labeled, confirming the applicability of this photo-click reaction in a complex biological environment.[43]

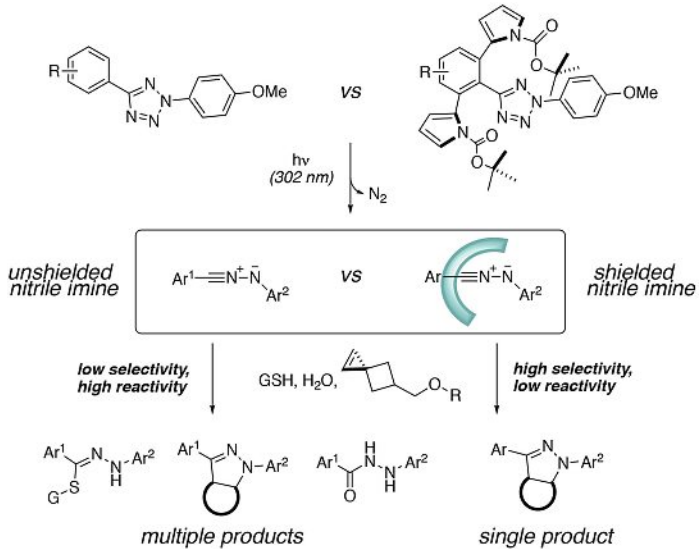

Fig. 2. Light-activation to release nitrile imine and consequent cycloaddition reaction with spirohexene in the presence of competing nucleophiles such as glutathione (GSH) in phosphate buffer/CH_3CN at pH 7.4.

Although light is a valuable reagent for the generation of reactive species, the higher energy UV light often required for these reactions can also create phototoxicity in biological systems. The development of chemoselective reactions that can be initiated with visible-light is therefore important for biomolecule labeling in living organisms. One recent example by Zhang and coworkers reported a visible-light induced [4+2] cycloaddition reaction between electron-rich alkene **1** and 9,10-phenanthrenequinone **2**, forming the fluorescent product phenanthrodioxine **3** (Fig. 3).[44] Although the proposed mechanism suggests the formation of a highly reactive bi-radical intermediate, no side product formation in the presence of biological nucleophiles such as thiols was observed. The reaction relies on the excitation of **2** to

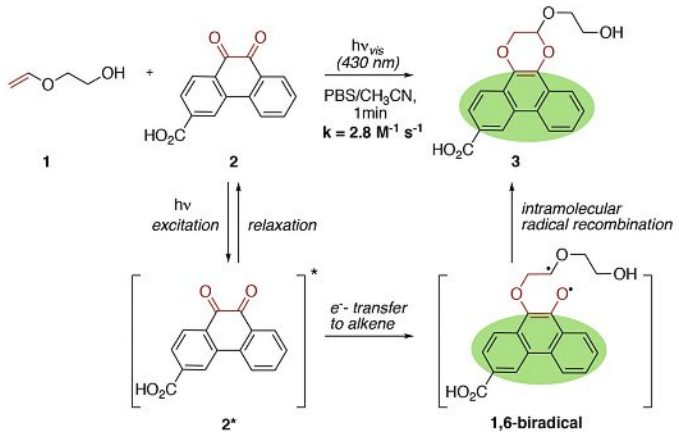

Fig. 3. Visible light promoted [4+2] cycloaddition between electron-rich alkene **1** and 1,9-phenanthrene quinone **2** *via* formation of a 1,6-biradical intermediate, generating a fluorescent dioxin derivative **3**.

2* at 430 nm followed by an electron transfer from the excited **2*** to the electron rich olefin **1** to form a 1,6-biradical. The reactive 1,6-biradical intermediate can then recombine intramolecularly to product **3** (Fig. 3). The high chemoselectivity of this reaction was explained by the consideration, that in the absence of an appropriate olefin but in the presence of potential biological nucleophiles, **2*** still relaxes back to the ground state. Furthermore, this radical-mediated [4+2] cycloaddition reaction was shown to be orthogonal towards other commonly used olefins or alkynes. The high selectivity in combination with a fast rate ($k = 2.76\ M^{-1}s^{-1}$ in PBS:CH_3CN (1:1)) for this ligation method enabled biological applications, including site-specific labeling of bovine serum albumin (BSA). In addition, live-cell imaging was performed by attaching an olefin handle (**1**) to the antibody cetuximab, which has a strong binding affinity to extracellular EGFR receptors on cancer cells.[45] As new chemoselective chemistries are developed, orthogonal-bioorthogonal reactions become more and more valuable. Toward this goal, Zhang and coworkers showed that their [4+2] cycloaddition reaction can be reliably used in parallel with other bioorthogonal ligations, including light-induced and strain-promoted cycloadditions.[44]

3. Multifunctionalized Substrates

The Staudinger ligation – a modified version of the Staudinger reaction[46] – is one of the most selective bioorthogonal ligation methods known to date (Fig. 4a).[26] The effectiveness of the ligation arises in part from the exquisite selectivity of the phosphine for organic azides, two abiotic functional groups that are inert towards naturally-occurring functionalities. The Staudinger ligation produces highly stable amide linkages,[23] and traceless examples have been developed which leave behind native amide bonds indistinguishable from those formed by nature.[24,25] The Staudinger ligation proceeds at modest rates ($k \sim 2–8 \times 10^{-3}\ M^{-1}s^{-1}$)[47,48] in comparison to other types of ligations. Electronic modulation of either component can provide faster rates[49] such as by increasing the electrophilicity of the azide or increasing the nucleophilicity of the phosphine, but the improved kinetics generally come at the cost of decreased selectivity as activated azides become substrates for nucleophilic thiols, and aliphatic phosphines can undergo fast oxidation.[50,51] While a number of chemoselective reactions have been investigated for the organic azide,[52] there are relatively few examples of reactions that exploit the unique reactivity of phosphines within a biological context.[53,54]

One example of a fast chemoselective ligation is the reaction of cyclopropenones with phosphines (Fig. 4b).[55] Cyclopropenones are small and biocompatible functional groups, as they have been shown to endure recombinant protein expression *via* genetic code expansion.[55] Cyclopropenones are also chemoselective reagents as they readily react with soft nucleophiles like phosphines.[56] The reaction proceeds *via* a nucleophilic attack of the phosphine to the cyclopropenone, leading to the formation of a ketene-ylide intermediate. This reactive intermediate is intramolecularly trapped by an adjacent nucleophile to form an α,β–unsaturated carbonyl product. The ligation is effective within complex mixtures such as cell lysates, and additionally, Prescher and coworkers showed that this reaction can be used to label a cyclopropenone-functionalized lysosyme.[57] Most notably, the phosphine-mediated cyclopropenone ligation shows higher rate constants compared to the Staudinger ligation with reaction rates of up to $k = 20\ M^{-1}\ s^{-1}$ in benzene.[57] Furthermore, the ligation can be further accelerated by applying cyclopropenethiones instead of cyclopropenones to increase reaction rates by ~300-fold in acetonitrile.[58] Similar to their oxo-analogs, cyclopropenethiones are amenable to *in vitro* protein labeling; however, the cyclopropenethione derivatives are less stable and will require further development for future applications in living-cell experiments. Nevertheless, this innovative approach nicely demonstrates the versatility and the potential for phosphines in biological applications in addition to Staudinger-type reactions.

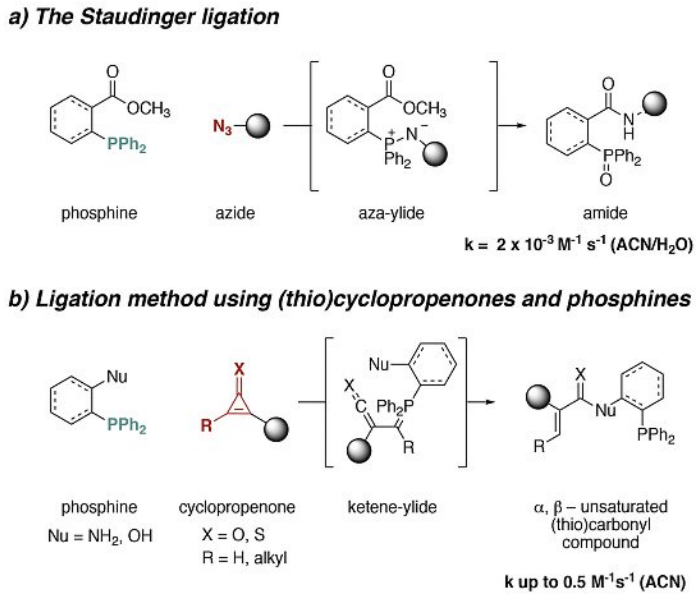

Fig. 4. a) Staudinger ligation using phosphines and azides. b) Ligation method between phosphines and cyclopropenones.

Some of the earliest known chemoselective ligations such as oxime and hydrazone forming reactions are based on dynamic covalent chemistry, yet these reactions have only found limited applications in live cells and organisms.[59] This limitation is partially a result of their relatively slow reaction kinetics, but is perhaps ultimately caused by the issue of their reversibility under aqueous conditions, particularly in acidic media.[60] As another widely used reversible reaction, boronic acids readily react with vicinal diol-containing molecules – such as those found in many biologically occurring saccharides – to form boronic esters.[61] However, boronic ester formation, despite being a fast process, shows reversibility under aqueous conditions and thus demonstrates promising but limited applicability within biological applications where stable covalent linkages are desired, such as in live-cell imaging.

In recent years, several synergistic systems have been developed that combine two or more reversible reactions to obtain stable ligation products and enable their biological applicability.[62] Most recently, Hall and coworkers demonstrated that boronic ester formation can be reliably used for living-cell experiments when combined intramolecularly with a thiosemicarbazone forming reaction

(Fig. 5).[63] The bifunctional system **5** contains a pinene-derived nopoldiol (blue) and a semithiocarbazide functional group (green) in order to react irreversibly and rapidly ($k = 9\ M^{-1}s^{-1}$) with a second bifunctional molecule **4** that bears both a ketone (green) and a phenylboronic acid (blue). The acetyl group improves both the ligation rate and the stability of the resulting semicarbazone (**6**, green) due to the formation of a dative B–>N bond.[63] Previously, the formation of these dative bonds have been shown to enhance the stability of boronic esters.[64–67] Additionally, the Hall group showed that this system remains effective in a complex biological context by introducing the boronic acid into the extracellular domain of beta-2-adrenergic receptors in HEK293T cells through a enzyme-mediated SNAP-tag approach[68] and reacting it with a fluorophore-bearing semithiocarbazide-nopoldiol reagent. This technique demonstrates how intrinsically reversible reactions, once considered not suitable for bioconjugation applications, can create effective and specific ligation products.

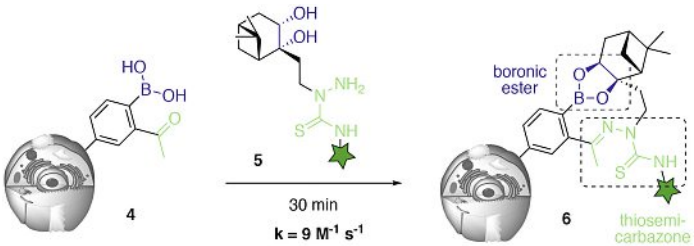

Fig. 5. Biocompatible boronic ester click reaction based on the synergistic effect of thiosemicarbazone formation (**6,** green) and boronic ester reaction (**6,** blue) tethered to each other.

4. Novel Reactivity for Bioorthogonal Chemistry

New chemoselective methodologies often arise from reactions that utilize reagents traditionally considered too unstable for biological conditions. The diazo group – when placed alpha to a carbonyl group and thus stabilized through resonance – is one such example of a reactive functional group that can effectively undergo chemoselective reactions in biological systems, including 1,3-dipolar cycloadditions, esterifications, and metal-carbene mediated alkylations.[69] However, the closely related diazonium compound – also known as a diazonium salt – is generally thought to undergo substitution with nucleophiles or hydrolysis too rapidly to be considered possibly chemoselective.

Recently, however, Chatterjee and coworkers demonstrated with their chemoselective rapid azo-coupling reaction (CRACR) that unstable reagents like aryl diazonium compounds can indeed be applied for highly selective bioorthogonal ligations when used in combination with a suitable reaction partner.[71] Building upon the original report by the Francis group that performed azo-couplings with tyrosine residues (**7**) present on protein surfaces to generate reactive intermediates for functionalization (Fig. 6a),[70] Chatterjee and coworkers developed a direct azo-coupling ligation, which instead employs a non-canonical hydroxy-substituted tryptophan (**8**) amino acid (Fig. 6b).[71] Due to the higher electron density of the indole core, **8** shows a high rate enhancement in the reaction with aryl diazonium ions relative to the coupling with tyrosine. Thus, even in the presence of tyrosine residues the reaction between hydroxy-substituted tryptophan (**8**) and various aryl diazonium derivatives (**9**, **10**, **11**) proceeds with high selectivity and a rate constant of up to $k = 63000\ M^{-1}\ s^{-1}$. Further, the similarity to naturally occurring tryptophan also ensured that **8** can be site-specifically incorporated into a protein of interest in both *E. coli* and eukaryotic cells, and these proteins could be selectively functionalized *via* azo-couplings with readily available fluorescein-based diazoniums. 5-Hydroxytryptophan (**8**) is also naturally generated at low levels *in vivo* as a metabolic precursor to the neurotransmitters serotonin and melatonin, such that this approach could also be used in biosensor applications. As the diazonium salts still lack the intrinsic stability of other chemoselective probes, temporal control of the reaction is possible as the diazonium ions can be generated *in situ* by light irradiation of aryl-trizabutadiene derivatives and thus protected derivatives can be implemented if necessary. Furthermore, Chatterjee showed that their CRACR ligation is not only fast and selective, but that the method could be 'unclicked' for protein catch-and-release applications[71] as the azo-linkages can be easily cleaved with biocompatible dithionite.[70]

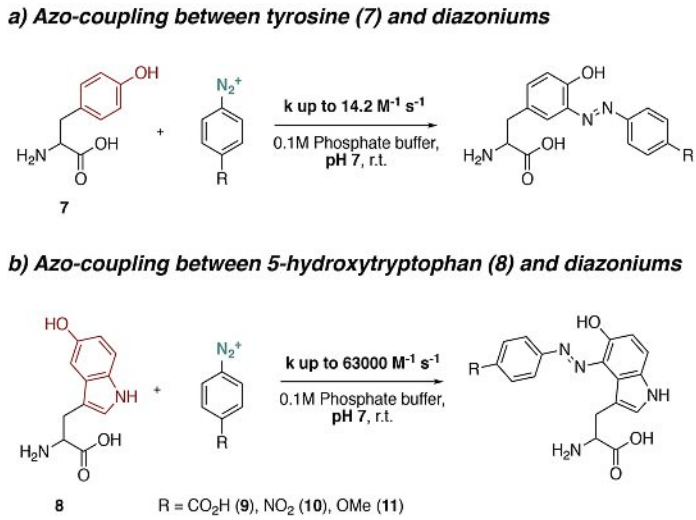

Fig. 6. a) Azo-coupling between tyrosine (**7**) and diazoniums b) Azo-coupling between modified tryptophan (5-hydroxy-tryptophan, **8**) and diazonium ions in phosphate buffer at pH 7 and r.t.

5. Conclusion and Outlook

The substantial number of new reactions developed for biological applications over the last twenty years demonstrate the ever-growing interest in this exciting area of chemical research. Recent years have shown that there is still room for optimization of even the most established chemoselective reactions. While not trivial, these examples illuminate some guiding principles for different ways to continue to improve the field of bioorthogonal chemistry. They demonstrate that when creatively approached and properly optimized, a large spectrum of different chemistries can be used to successfully perform chemoselective reactions in a cellular environment. Over the past century, organic chemists have developed a huge toolbox of chemical transformations to solve the problems faced in the laboratory, however, only a relatively small number of these techniques have seen implementation in the biological realm. Open-mindedness towards yet unexplored organic reactions can encourage the development of new bioorthogonal chemistries, and this expanded chemical toolbox will further empower chemists and biologists to transition these approaches into valuable biological applications.

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