Review

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Staudinger Ligation and Reactions – From Bioorthogonal Labeling to Next-Generation Biopharmaceuticals

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Abstract: In this review, we highlight groundbreaking discoveries and applications of Staudinger reactions in the molecular life sciences, starting from the engineering of the Staudinger ligation as a bioorthogonal reaction until most recent applications in modern bioconjugation methods to generate next-generation biopharmaceuticals. Bioorthogonal reactions refer to a set of chemoselective transformations in biological environments able to take place in presence of naturally occurring functional groups. The Staudinger ligation set a new paradigm of such transformations, resulting in the development of various labeling and bioconjugation strategies for the modification of (bio-)molecules of interest.

1. Introduction

In recent years, researchers in Chemical Biology have developed new bioconjugation methods. They rely on the utilization of chemoselective and bioorthogonal reactions to manipulate and modify biological entities, thus enabling powerful tools to study biological mechanisms and generate promising biopharmaceuticals. Classical organic reactions are an excellent starting point and source of inspiration for expanding this toolbox.

The Staudinger reaction is probably the prime example to illustrate research in this area. In 1919, Meyer and Staudinger reported a reaction between azides and phosphines to form iminophosphoranes eventually resulting in a primary amine and a phosphine oxide in the Staudinger reduction.[1] Staudinger reactions were frequently exploited in organic synthesis, proving useful in several chemical transformations.[2] Several decades later, in 2000, Bertozzi and Saxon[3] unleashed the unprecedented potential of Staudinger transformations in a biological context by introducing the Staudinger ligation, which coined a new paradigm for highly selective transformations: bioorthogonal reactions.[4] Since then, many researchers in this area, including our laboratory, have been inspired by the notion of bioorthogonal reactivity of azides with various P(III)-reagents and engineered a plethora of variations. So far, these efforts have resulted not only in highly selective chemical labeling strategies in living organisms but also in furnishing new bioconjugates applied in pharmaceutical research.[5]

This review underlines the early applications, the strengths and weaknesses as well as the evolution of Staudinger transformations towards bioorthogonal reactions and modern bioconjugation-related applications.

1.1 The Staudinger Reaction

The Staudinger reaction is known to organic chemists primarily for the reaction of organic azides **1** and trialkylphosphines **2** to form an iminophosphorane **5**, which, upon subsequent hydrolysis, generates a primary amine **7** and a phosphine oxide **8** in the so-called Staudinger reduction (Scheme 1). Numerous experimental and computational[6] studies have been devoted to elucidating the mechanism of this unique transformation. The Staudinger reaction is initiated by a nucleophilic attack of the trivalent phosphorus atom to the terminal nitrogen atom of the azide to yield an intermediate phosphazide **3** with retention of the phosphorus center. In a second step, the intermediate phosphazide gets stabilized via a four-membered ring transition state, which upon release of molecular nitrogen gives rise to an iminophosphorane **5**, always in equilibrium with its aza-ylide form **6** (Scheme 1). The kinetics of this two-step process can be second or first order, respectively, depending on whether the formation of the phosphazide or the intramolecular decomposition is the rate-determining step.[2a,7] In the Staudinger reduction, the iminophosphorane will ultimately be hydrolyzed to an amine and phosphine oxide; however, several other pathways are possible. The nucleophilic nitrogen atom in iminophosphoranes can readily react with a variety of electrophiles. One prime example is the reaction between iminophosphoranes and aldehydes or ketones in an aza-Wittig reaction to yield imines.[8]

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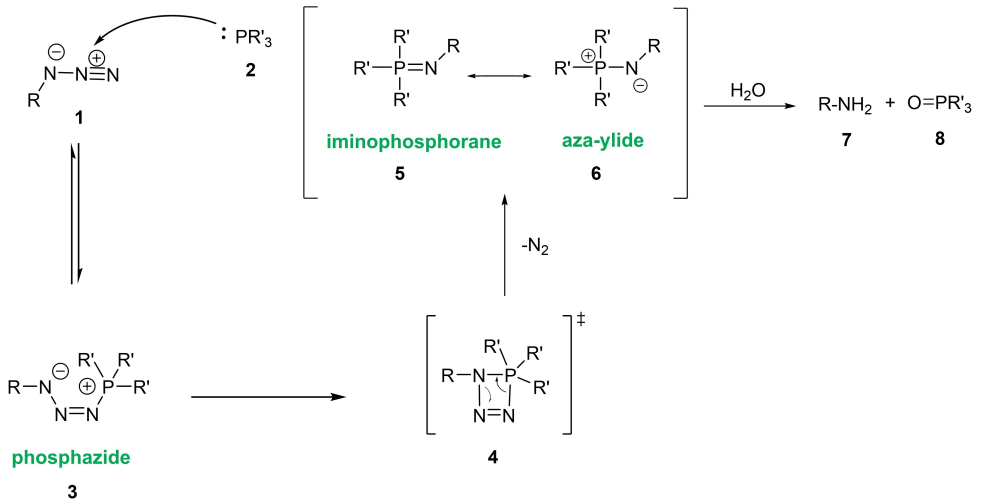

Scheme 1. Mechanism of the Staudinger reaction.

The scope of the Staudinger reaction has been thoroughly explored, and several types of phosphines or other trivalent phosphorus compounds are tolerated in transforming azides.[2,9] Nonetheless, the substituents of the P(III)-derivatives and the azide strongly influence the reaction outcome and rate. For example, while the reaction between a phosphine with strong electron-donating substituents and the azide counterpart to form the phosphazide is irreversible, in the opposite case, the phosphazide can decompose.[2a,10] As a general observation following thorough investigations, electron-poor azides and electron-rich phosphorus compounds can react most efficiently.[2a] Despite the expanded scope of the Staudinger reaction and its countless applications,[11] there are certain drawbacks. The equimolar release of the phosphine oxide, the sensitivity of many phosphorus compounds towards air and moisture, and the low stability of the iminophosphoranes/aza-ylides have limited its widespread use in biological applications.

1.2 Original Development of the Staudinger Ligation

The Staudinger ligation, first reported by Bertozzi in 2000,[3] remains among the most acknowledged bioorthogonal reactions. The ligation uses organic azides and phosphines bearing an electrophilic trap, namely an ester, to yield one single ligated product via an amide bond (Scheme 2a). Mechanistically similar to the Staudinger reduction, a nucleophilic attack of the phosphorus **9** to the terminal nitrogen atom of the azide **10** initiates the reaction. Release of molecular nitrogen gives rise to an iminophosphorane **11**, which again is in equilibrium with its aza-ylide form **12**. The nucleophilic nitrogen atom of the iminophosphorane **11** can be trapped by the proximal

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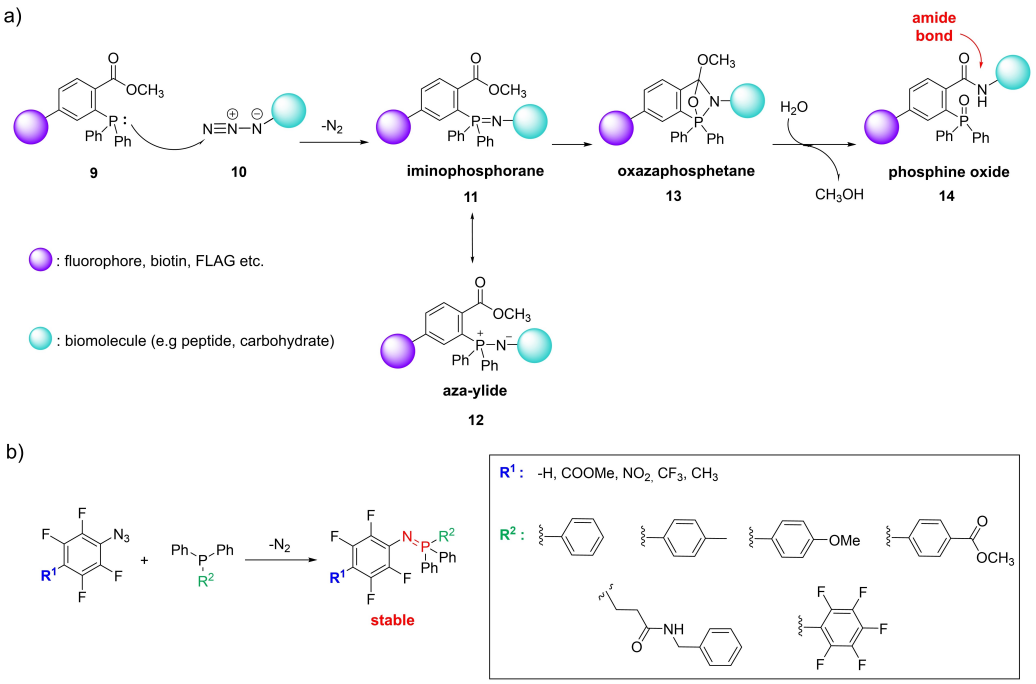
Christian Hackenberger (he/his) studied chemistry in Freiburg, Madison/WI and Aachen. After a postdoctoral stay at MIT, he started his independent career at the Freie Universität Berlin in 2005. In 2012, he became the Leibniz-Humboldt Professor for Chemical Biology at the Leibniz-Research Institute for Molecular Pharmacology and the Humboldt Universität zu Berlin. Throughout his academic life, Christian has been passionate about studying peptide and protein function using bioorthogonal reactions. His laboratory develops next-generation bioconjugation reactions, in particular using Staudinger transformations and phosphorus-electrophiles, to generate protein-based therapeutics against cancer, Alzheimer and viral infections. He is a co-founder of the Munich-based company Tubulis, which engineers better tolerable cancer drugs using technologies from his lab.

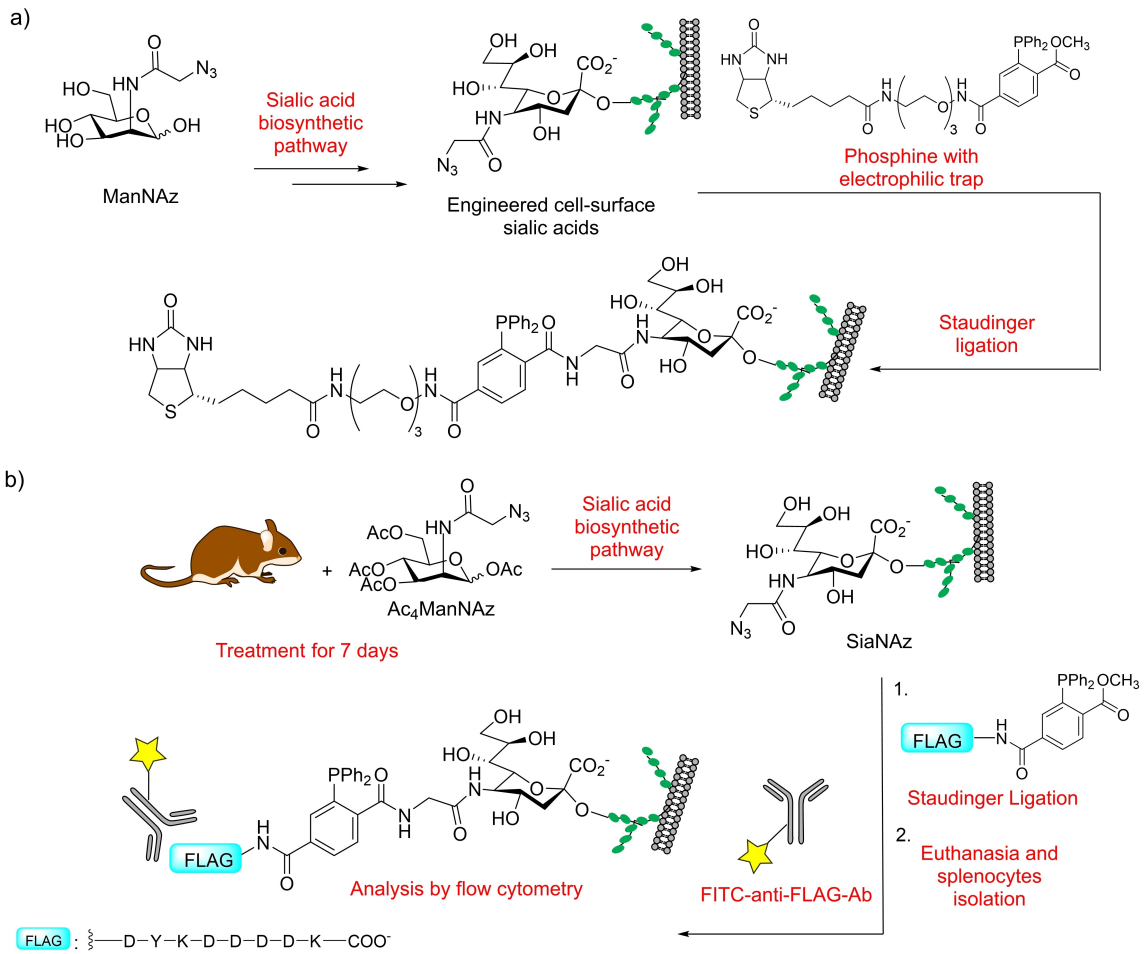


Scheme 2. a) Reaction and mechanism of the original Staudinger ligation; b) principle and scope of the nonhydrolysis Staudinger ligation yielding a stable iminophosphorane.

electrophilic moiety installed initially on the phosphine to form a cyclic oxazaphosphetane intermediate **13**. Finally, hydrolysis of the intermediate takes place to afford the amide-linked ligated product. Owing to the residual phosphine oxide moiety on the end product, the Staudinger ligation – in particular after the development of the traceless variant (see subsection 1.3) – was later termed ''non-traceless''.

Since the first report, many efforts have focused on expanding the scope and investigating the kinetic parameters of this ligation reaction.[12] A wide range of reactants is well accepted; however, both sterics and electronics profoundly affect the reaction rate and product formation. For example, the ester leaving group did not play an important role in the reaction rate; however, the addition of bulky groups led to significant aza-ylide hydrolysis leading to the formation of phosphine oxide and amine instead of the ligated product. In contrast, substituting the phenyl groups directly attached to the phosphorus plays a decisive role in the reaction rate. Similar to the Staudinger reduction, electron-donating substituents on the phosphorus and electron-withdrawing substituents on the azide notably enhanced the reaction rate. This observation guided the development of perfluoroarylated azides as a reaction counterpart in the Staudinger ligation. In this version, which was termed nonhydrolysis Staudinger ligation, the produced iminophosphorane is stable towards hydrolysis (Scheme 2b), and the reaction rates were two orders of magnitude higher than the classical ligation.[13] Lastly, exploration of the solvent effect revealed that the ligation proceeded more rapidly in polar protic solvents, such as H_2O, which suggests its suitability for most biological applications.[3,5a,b,14]

To date, one of the most significant examples of using the Staudinger ligation as a bioconjugation method is Bertozzi's first report, where cell surface glycans were selectively labeled with biotin.[3] More specifically, an azide functionalized sugar, N-azido-acetyl-mannosamine (ManNAz), was delivered to the cell surface of Jurkat cells via the carbohydrate biosynthetic pathway in a process referred to as metabolic oligosaccharide engineering (MOE).[15] Ligation of the azide-containing cell-surface glycans with a biotin functionalized phosphine and subsequent binding with fluorescein-labeled avidin beads lead to fluorescence increase in ManNAz treated cells (Scheme 3a). Similarly, MOE was employed to incorporate *N*-azidoacetylgalactosamine (GalNAz) into mucin-type *O*-linked glycoproteins, which can then be orthogonally labeled via the Staudinger ligation.[4] This selective chemical tag provided a novel approach for the challenging enrichment of *O*-linked glycoproteins in proteomics analyses. A few years later, Bertozzi and coworkers were able to demonstrate the ability to label cell-surface glycans in living organisms without adverse effects in their physiology. Laboratory mice were injected with Ac_4ManNAz for seven consecutive days and subsequently injected with a FLAG-bearing phosphine to perform the Staudinger ligation *in vivo*. The mice were euthanized and their splenocytes were isolated, probed utilizing a fluorescent anti-FLAG antibody and cell-surface SiaNaz was quantified via flow cytometry (Scheme 3b). Only splenocytes from mice treated with Ac_4ManNAz exhibited an increase in fluorescence



Scheme 3. a) The first application of the Staudinger ligation for cell-surface glycan labeling,[3] b) *In vivo* application of Staudinger ligation for quantification of azide-containing cell-surface sialic acid.[16]

compared to untreated mice, indicating the successful implementation of the Staudinger ligation in living animals.[16]

In 2009 Sun *et al.* utilized the Staudinger ligation in a similar manner to Bertozzi to functionalize the surface of liposomes with glycans in aqueous conditions. Notably, the integrity of the lipid bilayer was not influenced offering a versatile approach to modify liposomes with various modalities, to provide functional vesicles.[17] The construction of antibody-label conjugates was also attemted via the Staudinger ligation. In one example van Dongen and colleagues produced azide-modified antibodies to selectively react with radiolabled phosphines for tumor specific imaging and therapy[18] (Scheme 4). Despite the promising *in vitro* results, injection of the radiolabeled agents in mice did not yield effective labeling due to the oxidation of the phosphine probes and the slow kinetics of the Staudinger ligation. In addition to antibodies the Staudinger ligation was employed to label G protein-coupled receptors, transmembrane proteins implicated in various processes.[19] Huber *et al.* made use of amber codon suppression to incorporate azide-containing amino acids in rhodopsin and set out to fluorescently label the protein in a site-specific manner.[20] However, the hydrophobicity of the fluorescein-phosphine label and the slow reaction kinetics hampered the application.

The Staudinger ligation and its broadened scope inaugurated the field of bioorthogonal chemistry and has been a valuable tool in several systems. Since its first report by Bertozzi a wide range of chemical transformations have been developed to successfully probe biology even *in vivo*. Nevertheless, certain drawbacks including the significantly slow reaction kinetics and the residual bulky and hydrophobic phosphine oxide moiety, have limited its widespread use, as highlighted in some of the afore-mentioned examples.

1.3 Traceless Staudinger Ligation

The traceless Staudinger ligation was reported shortly after the introduction of the original variant. Bertozzi[21] and Raines[22] published, almost simultaneously, the design and use of phosphines bearing a cleavable electrophilic trap. In these

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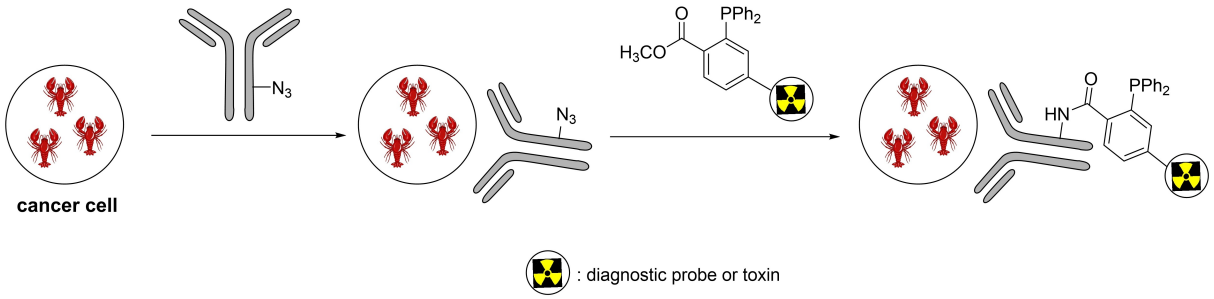


Scheme 4. Schematic overview of the van Dongen approach[18] to apply the Staudinger ligation to create efficacious antibody-label or antibody-toxin conjugates.

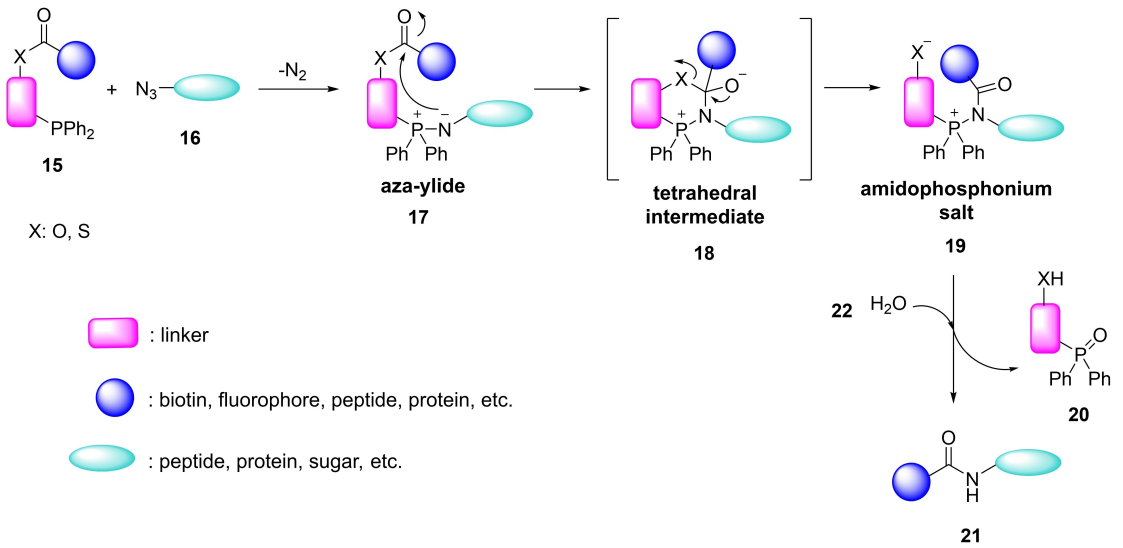
studies, whose findings contrast with the initial Staudinger ligation, the phosphine oxide is released from the target biomolecule upon hydrolysis, granting access to a native amide bond (Scheme 5).

The mechanism of the traceless Staudinger ligation is similar to all other Staudinger transformations. Initially, the trivalent phosphorus atom in phosphines of type **15** nucleophilically attacks an azide **16**, forming an aza-ylide **17**. The nitrogen atom of the aza-ylide intramolecularly attacks the electrophilic ester to afford an amidophosphonium salt **19** via a tetrahedral intermediate **18**.

Raines and coworkers performed numerous studies to verify that the reaction proceeds via an acyl transfer, building on previous literature showing that iminophosphoranes can undergo acyl-transfer reactions.[23] In the last step, hydrolysis of the amidophosphonium salt **19** yields an amide **21** with a loss of phosphine oxide **20**.

The factors affecting the product formation, kinetic processes, and yield of the Staudinger ligation have been thoroughly investigated. In general, the reaction exhibits rate constants of $\sim 10^{-3}\,M^{-1}\,s^{-1}$. Solvent polarity, pH, and the choice of reaction counterparts can significantly affect the ligation efficiency. Table 1.1 summarizes various phosphine reagents utilized for several ligation strategies as well as corresponding solvents and yields of the reactions.

As a general observation, ligations with less sterically hindered amino acids, such as glycine residues, are most efficient, while bulky substituents decrease the yields. To overcome this, Raines reported using phosphinothiol **15h**, which bears two electron-donating substituents, rendering the phosphorus more nucleophilic to enhance the reaction rate.[25] Nonetheless, the added electron density on the phosphorus results in higher sensitivity towards oxidation. Kiessling and coworkers aimed at stabilizing phosphinothiol precursors by borane protection,[27] which can be deprotected under basic conditions to couple glycosylazides and asparagine to obtain an *N*-glycosylated amino acid or in intramolecular reactions for medium-sized lactam synthesis.[32] Shortly after that, the Hackenberger group used acidic deprotection strategies for the chemoselective cyclization of medium-sized peptides.[35] In



Scheme 5. Reaction principle and mechanism of the traceless Staudinger ligation.

Table 1. The phosphines explored in the traceless Staudinger ligation, yields, and solvent conditions reported.

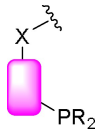
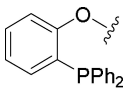
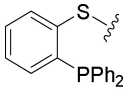
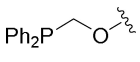
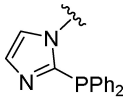
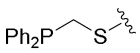
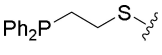
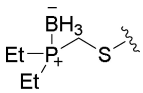
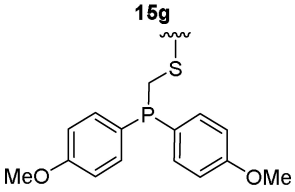
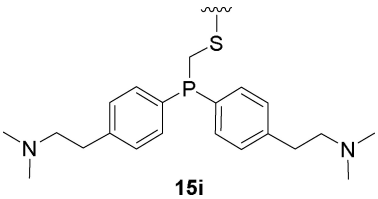
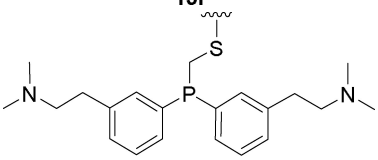
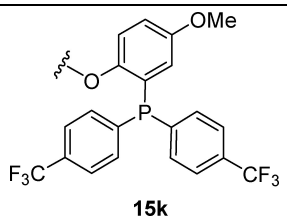
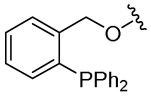
	Coupling	Solvent	Yield (%)	Refs.
 **15a**	Gly–Gly Gly–Phe Ala–Phe Gly–Leu	DMF/H2O DMF/H2O DMF/H2O DMF/H2O	>95 36 32 6	 [21] [24]
 **15b**	Gly–Gly	DMF/H_2O	38	[21–22]
 **15c**	Gly–Gly	DMF/H_2O	11	[21–22]
 **15d**	Gly–Gly	DMF/H_2O	<95	[21]
 **15e**	Gly–Gly Phe–Gly Gly–Ser Ala–Ala	DMF/H_2O DMF/H_2O DMF/H_2O DMF	<95 92 92 36	 [25]
 **15f**	Gly–Gly	DMF/H_2O	39	[26]
 **15g**	Asp–Glycosyl	DMF	54	[27]
 **15h**	Ala–Ala Phe–Ala	DMF 1,4-dioxane	82 84	[25]
 **15i**	Gly–Gly	H_2O	<50	[28]
 **15j**	Gly–Gly	H_2O	<65	[29]

Table 1. continued

	Coupling	Solvent	Yield (%)	Refs.
 **15k**	β,β-dialkylated α,β-dehydroamino acids	H_2O/1,4-dioxane	< 75	[30]
 **15l**	Gly–Gly/Ala/Trp Phe/Leu/Ile/Tyr/Lys Ala–Ala Glu–Gly/Ala	DMF/H_2O/Et_3N DMF/H_2O/Et_3N DMF/H_2O/Et_3N	< 80 78 < 88	[31]

2007, the Raines group designed water-soluble phosphinothiols **15i** and **15j**.[28–29] The dimethylamino substituents appear to contribute to water solubility and minimize aza-ylide protonation. Very recently, Inoue and colleagues designed phosphine **15k** and combined on the same scaffold substituents with electron-donating and electron-withdrawing properties.[30] They further used this phosphine with fine-tuned reactivity to perform Staudinger ligations for the synthesis of the natural product yaku′amide B, achieving sterically-demanding, native β,β-dialkylated α,β-dehydro linkages. Soon after, Kumar *et al.* demonstrated the use of the air-stable phosphine **15l** for successive ligations of simple and sterically hindered amino acids with excellent yields of 78–95 %.[31]

In recent years, the traceless Staudinger ligation has proved a valuable tool for peptide synthesis and macrocyclization. Nevertheless, some disadvantages have limited its use in the applications mentioned above: The slow kinetics might lead to aza-ylide hydrolysis if the acyl transfer does not occur fast enough, thereby not yielding the desired amide bond. Furthermore, the occasional poor solubility and mainly the sensitivity of the phosphines towards oxidation has been the main obstacle to further applications.

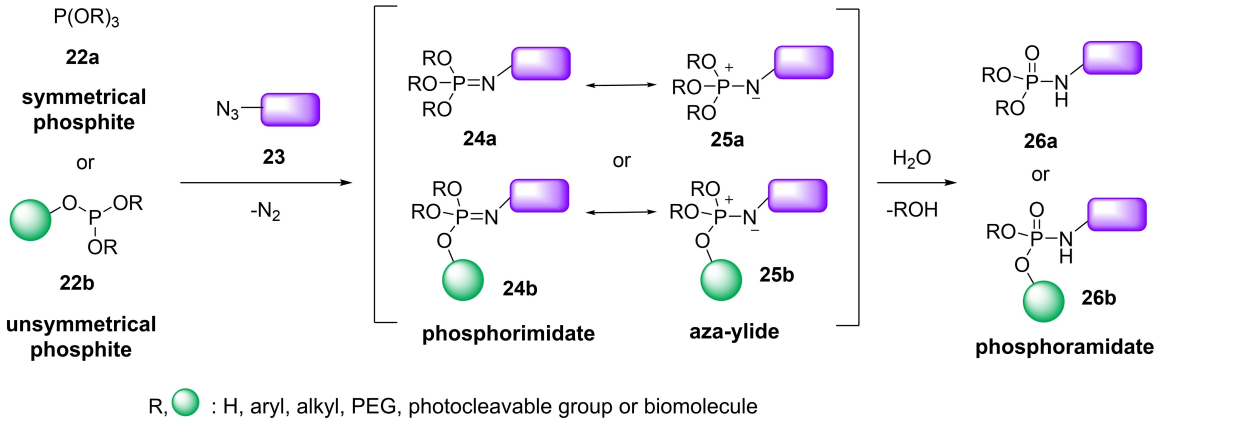
1.4 Staudinger-Phosphite and -Phosphonite Reaction

Chemoselective Staudinger-phosphite and -phosphonite reactions have been introduced by Hackenberger and coworkers[33] and have enabled a plethora of modification strategies for azide-containing biomolecules and polymers. Here, phosphines used in the classical Staudinger reaction are replaced by a phosphite ($P(OR)_3$) or phosphonite ($P(OR)_2R'$). The development of this type of reaction is based on early studies of Kabachnik *et al.*, in which substitution of the alkyl- or aryl-residues of phosphines by alkoxy groups and subsequent hydrolysis did not lead to complete cleavage of the P–N bond in the intermediate imidate, but afforded phosphin-, phosphon,– and phosphoramidates.[34]

In the Staudinger-phosphite reaction, an azide **23** is reacted with a symmetrical or unsymmetrical phosphite **22a** or **22b** via an intermediate phosphorimidate **24a** or **24b**, which is in equilibrium with its aza-ylide form **25a** or **25b**. Hydrolysis of the phosphorimidate leads to the formation of phosphoramidate **26a** or **26b**, in which one of the alkoxy groups of the phosphorus counterpart is released (Scheme 6). The alkoxy group‘s liberation can occur by direct water attack to the phosphorus atom of the aza-ylide or the alkyl group in an arbuzov-type reaction.[35]

For symmetrical phosphites, the release of the alkoxy group leads to one single phosphoramidate. Unsymmetrical phosphites can yield different products depending on the substituents. The Hackenberger group studied the influence of the substituents and found that benzoxy and phenoxy groups are more prone to be released compared to the aliphatic groups and longer chains of aliphatic groups are more retained than the shorter ones.[36]

Significant advantages of applying phosphites in chemoselective Staudinger reactions include their resistance towards oxidation, especially compared to previously used trialkyl phosphines, and their easy synthetic accessibility, given that most of these phosphites can be produced by commercially available phosphoramidite-building blocks that are routinely used in DNA-synthesis. Organic solvents, aqueous media, and cell lysates are well-tolerated conditions for the Staudinger-phosphite reaction.[37] However, phosphites are prone to hydrolyze, mostly at acidic pH, depending on the alkoxy substituents. For example, trimethyl phosphite displayed over 50 % hydrolysis in aqueous solutions of neutral pH, while for a polyethyleneglycol (PEG) -substituted phosphite, less than 20 % hydrolysis was observed under the same conditions.[33a,37–38]

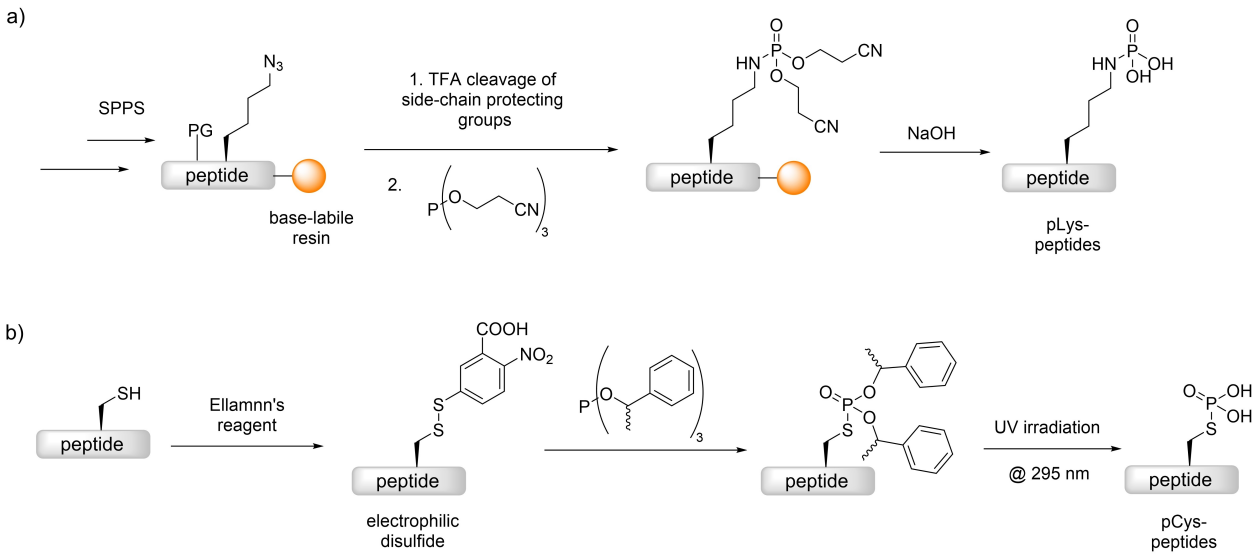


Scheme 6. Mechanism of the Staudinger-phosphite reaction.

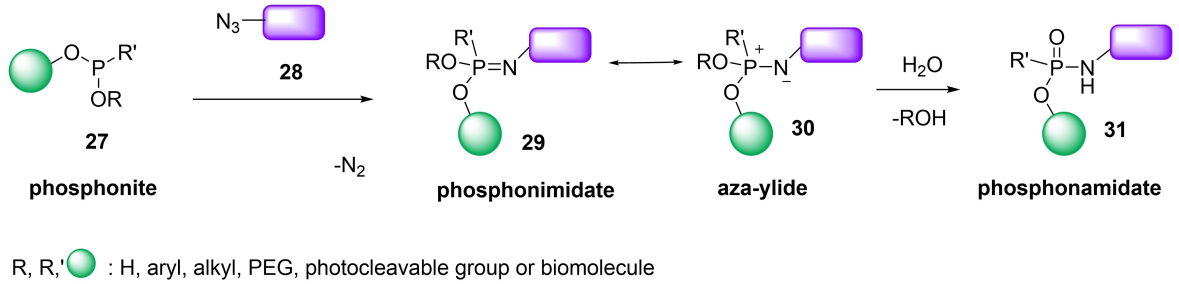
Since its first report, the scope of the Staudinger-phosphite reaction has been significantly expanded. In an initial study, Hackenberger and coworkers used a phosphite with photocleavable groups and an azidophenylalanine-containing protein affording a phosphotyrosine mimic.[33a,38] Access to site-specifically phosphoramidate-linked glycopeptides and lipidated peptides has also been granted via the unsymmetrical Staudinger-phosphite reaction.[39] PEG-substituted phosphites have been employed in reactions with azide-containing peptides and proteins in which the resulting branched PEG-architecture substantially improved the stability of bioconjugates even inside living cells.[38,40] This reaction was further explored to chemically synthesize naturally occurring labile phosphorylations, more specifically, phospholysine (pLys)[41] and phosphocysteine (pCys)[42] peptides (Scheme 7). Taken together with a recent publication where the Staudinger phosphite reaction was used to produce stable, non-hydrolyzable phospholysine mimics,[43] the toolbox to study these rare and poorly understood phosphorylation sites is greatly expanded.[44]

The chemoselective Staudinger-phosphonite reaction, first reported in 2011,[33b] follows the same principle as the Staudinger-phosphite reaction, starting this time with a nucleophilic attack of the trivalent phosphorus of a phosphonite **27** to the azide **28**. Finally, a phosphonamidate **31** is formed via an intermediate phosphonimidate **29** (Scheme 8).

Phosphonites are less stable than phosphites towards air oxidation and hydrolysis. Consequently, in initial studies, the Staudinger-phosphonite reaction was performed in dry organic solvents.[33b,45] To perform the Staudinger phosphonite reaction in aqueous systems, Hackenberger and coworkers employed borane-protected phosphonites, which can be deprotected



Scheme 7. a) Synthetic access to phospholysine (pLys) peptides from the solid support via the Staudinger-phosphite reaction, b) Synthesis of phosphocysteine (pCys) peptides using phosphites with electron-deficient disulfides.



Scheme 8. Mechanism of the Staudinger phosphonite reaction.

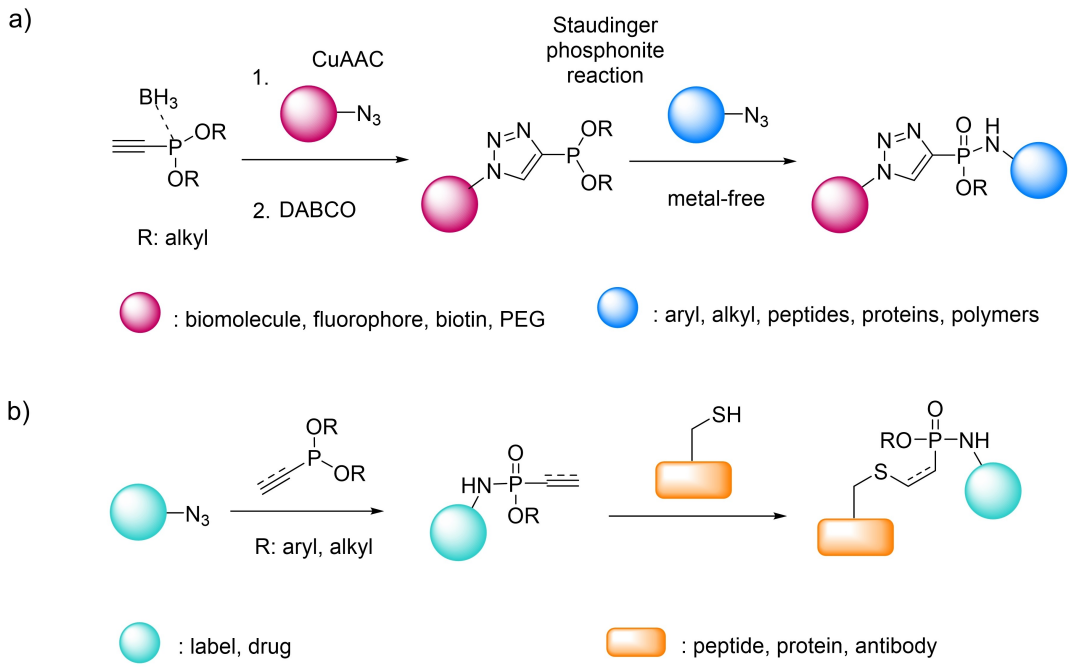
under basic conditions using DABCO.[33b,46] The deprotected phosphonites are then stable for a sufficient time to allow reaction with azides in solution.

Building upon these observations, unsaturated ethynyl-phosphonites were borane-protected and used for modular chemoselective azide-azide couplings by combining copper-catalyzed azide-alkyne cycloaddition (CuAAC) and the Staudinger-phosphonite reaction (Scheme 9a).[46] This method was successfully applied in the embodiment of lactose into azidopolyglycerol, a functionalized polymer used for lectin binding studies.

This transformation laid the basis for discovering a new Cysteine-selective bioconjugation reaction. Upon chemoselective reactions with azides, ethynyl- or vinyl-phosphonites yield electron-deficient phosphonamidates, which show excellent reactivity with thiols and yield various peptide- and protein-conjugates (Scheme 9b).[47] This observation led to the development of the so-called P5-labeling technology, which we cover in detail in the following subsection 1.5.

1.5 P5-labeling: Bioconjugation of Unsaturated P(III)-Reagents

As mentioned in the previous subsection, vinyl- and ethynyl-phosphonamidates bear an electrophilic unsaturated carbon atom, which can be attacked by thiol nucleophiles leading to the formation of stable bioconjugates. In 2019, the Hackenbeger group introduced the use of ethynyl-phosphonamidates for modular Cysteine-selective protein bioconjugations.[47] The method comprises two main transformations. First, an electron-rich ethynyl phosphonite **32** is reacted chemoselectively with an azide, yielding an electron-poor phosphonamidate **33** that undergoes a subsequent



Scheme 9. a) Sequential azide-azide coupling by CuAAC and Staudinger-phosphonite reaction using borane-protected alkyne phosphonites, b) Electron-poor phosphonamidates derived from ethynyl- or vinyl-phosphonites selectively react with cysteines.

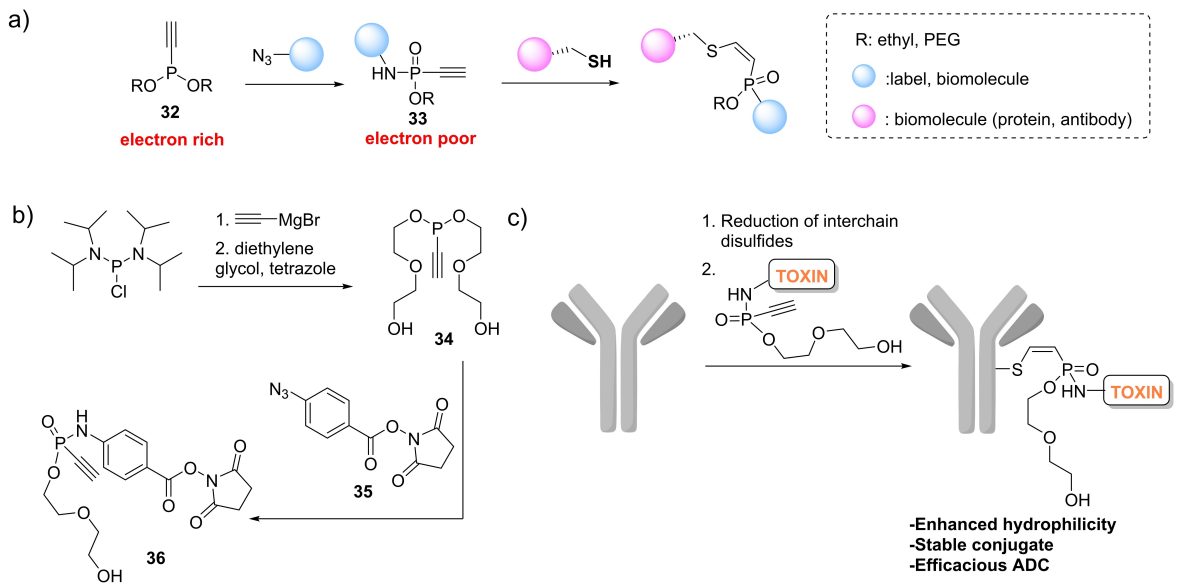
cysteine-selective reaction with proteins and antibodies (Scheme 10a). The facile installation of a Cysteine-selective P(V)-electrophile on complex azide-containing molecules together with the exceptional cysteine selectivity, the tunable reactivity depending on the phosphorus *O*-substituents, and the excellent stability of the final thiol-adducts in biological media render P5-labeling a most valuable and unique bioconjugation tool.

The construction of antibody-drug conjugates (ADCs) containing stable phosphonamidate linkages was the epitome of applications for P5-labeling.[48] The Staudinger-phosphonite reaction performed between phosphonite **34** and an azide bearing an N-hydrosuccinimide (NHS) ester **35** affords a building block **36** for the generation of an ADC with the hydrophobic payloads monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF) (Scheme 10b).[49] Clinically approved ADCs, like Adcetris®, carry maleimide linkers known to undergo thiol exchange reactions in biological fluids, leading to premature release of the toxin.[50] Notably, substituting the maleimide with the phosphonamidate linkage gave rise to significantly more stable ADCs retaining 90% of the toxin in rat serum over the course of 7 days, while in the case of the maleimide-linked Adcetris® only 30% of the payload was retained. A similarly significant finding was that the phosphonamidate-linked ADC exhibited advantageous *in vivo* efficacy at a lower dose (Scheme 10c).

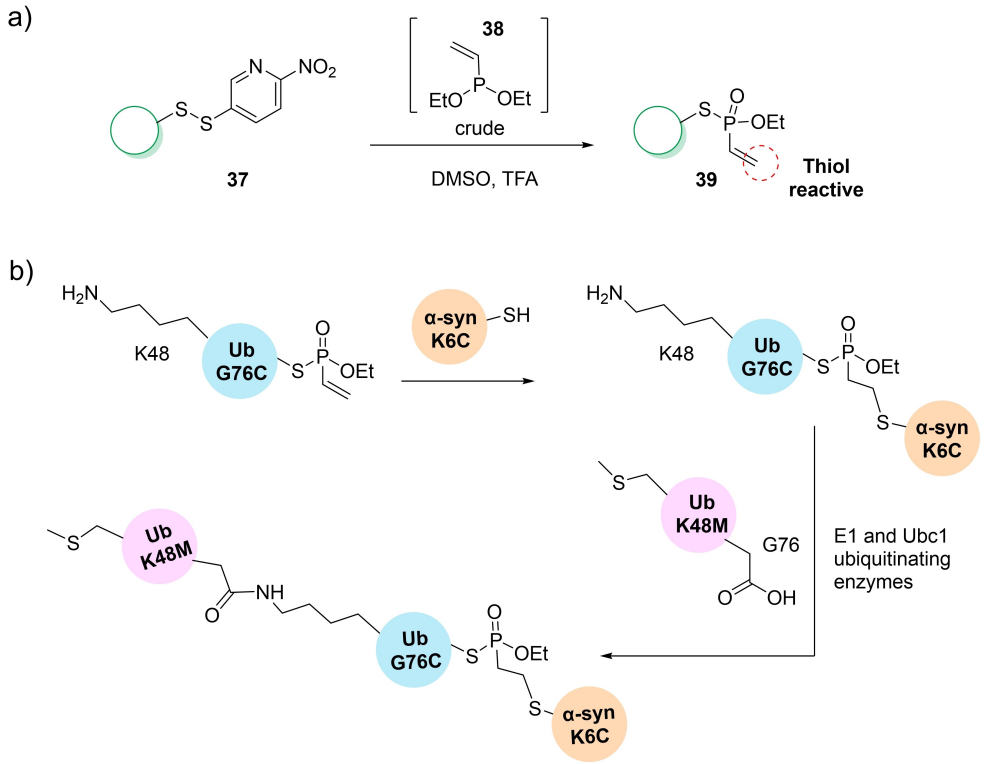
A few months later, the reaction between vinyl-phosphonites and azides was applied to generate peptide macrocycles enabling access to pharmacologically relevant stapled peptides.[51] The asset of this technique lies in the capacity to further modulate the macrocycle's properties by just installing different functional moieties through alteration of the *O*-substituents of the phosphonite.

These results encouraged Hackenberger and coworkers to investigate the applicability of other classes of P(V)-electrophiles in bioconjugation. In 2019, the use of vinylphosphonothiolates was reported for the thiol-thiol conjugation of biomolecules.[52] In a similar principle of converting an electron-rich phosphonite to an electron-deficient phosphonamidate via the Staudinger-phosphonite reaction, a nucleophilic thiol is transformed into an electrophilic vinylphosphonothiolate that can subsequently capture another thiol group. In more detail, a thiol-containing (bio-)molecule is first turned into an electrophilic mixed disulfide **37** with the addition of 2,2′-dithiobis(5-nitropyridine), which is reacted *in situ* with diethylvinylphosphonite **38** under acidic conditions to yield a vinylphosphonothiolate **39** in less than a minute (Scheme 11a). This method was successfully applied in antibody labeling and site-specific conjugation of proteins containing a unique cysteine residue. In a model application, ubiquitin-ubiquitin and ubiquitin-α-synuclein constructs with a non-hydrolyzable vinylphosphonothiolate linkage were synthesized and recognized as substrates for further enzymatic ubiquitination (Scheme 11b).

Hackenberger and colleagues further expanded the repertoire of P(V)-based electrophiles by designing novel diethynyl-phosphinates for bioconjugation reactions.[53] These molecules allow for a facile double thiol modification since they bear two electrophilic positions. Such compounds are easily accessible, starting from diethyl phosphoramidous dichloride **40** performing a Grignard reaction with ethynyl magnesium bromide. Tetrazole-mediated exchange of the diethylamino

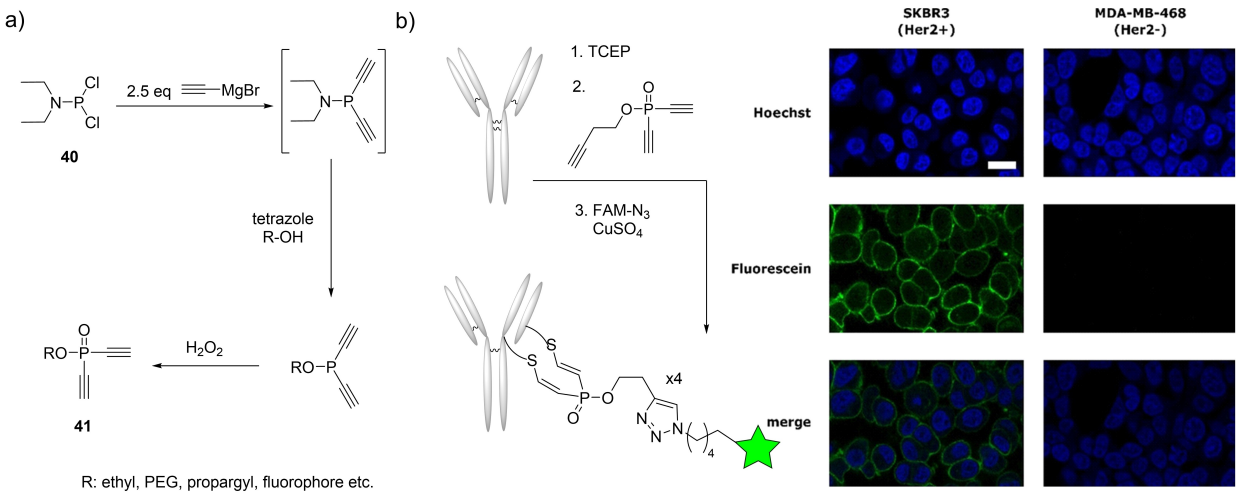


Scheme 10. a) Staudinger-phosphonite reaction between an electron-rich ethynyl-phosphonite and an azide yields an electron-deficient phosphonamidate for selective thiol bioconjugation; b) reaction scheme for the synthesis of a P5-module for subsequent antibody-toxin conjugation, c) generation of stable and efficacious ADCs bearing the P5-linkage technology.

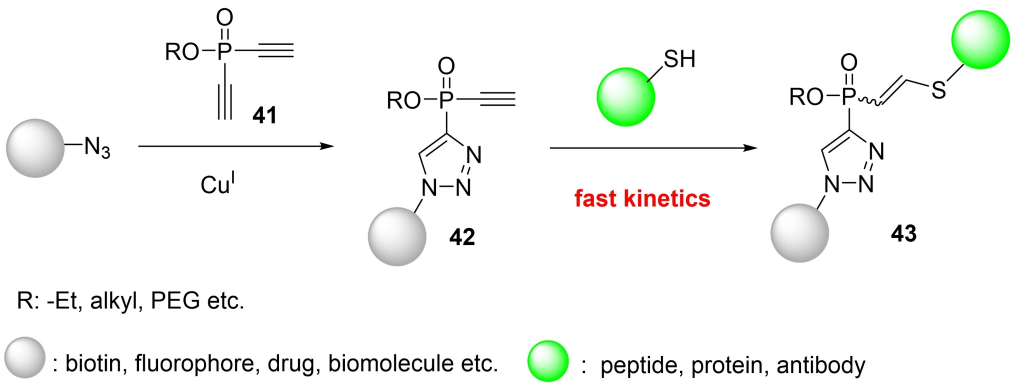


Scheme 11. a) One pot-synthesis of vinylphosphonothiolate electrophiles, b) Ubiquitin- α-synuclein conjugate via a stable phosphonothiolate linkage is recognized as a substrate for subsequent enzymatic ubiquitination.

substituent with the desired alkoxy group and oxidation with hydrogen peroxide in the last step affords the final diethynylphosphinate **41** (Scheme 12a). As for phosphonamidates and phosphonothiolates, diethynyl phosphinates exhibit excellent thiol selectivity and stability of the double-thiol adducts under physiological conditions, allowing their application in several bioconjugation reactions. Apart from protein-protein or peptide-protein conjugation, one prime example is the implementation of diethynyl phosphinates in antibody rebridging. Reduction of the four interchain disulfides and reaction with diethynyl phosphinates leads to the rebridged half-antibody and the full construct with a precise antibody-to-cargo ratio of



Scheme 12. a) Synthetic route to obtain diethynyl phosphinate electrophiles; b) Rebridging of trastuzumab via a fluorescein-diethynyl phosphinate affords a conjugate with an antibody-to-fluorophore ratio of 4. The antibody still retains activity after rebridging.



Scheme 13. Synthesis of ethynyl triazolyl phosphinates via diethynyl phosphinates and subsequent thiol addition exhibiting superior kinetics than previously reported P(V) electrophiles.

four. The latter was demonstrated with a fluorescein-functionalized diehtynyl phosphinate Trastuzumab to additionally prove with fluorescence microscopy that the antibody's function was not compromised upon rebridging.[53] Trastuzumab is a recombinant monoclonal antibody that binds to the extracellular domain of Her2, a receptor overexpressed in breast cancer patients.[54] The functionalized Trastuzumab only stained cell membrane on Her2 positive cells, while no fluorescence signal was observed on the Her2 negative cell line, verifying the antibody's integrity (Scheme 12b).

Recently, Hackenberger *et al.* published the discovery of ethynyl triazolyl phosphinates (ETPs) for modular bioconjugation reactions.[55] Essentially, azide-containing molecules can undergo CuAAC-reactions with a diethynyl phosphinate **41** in aqueous buffer to yield an ethynyl triazolyl phosphinate **42** (Scheme 13). The resulting electrophile shows a remarkably higher reactivity towards cysteine, about tenfold compared to the previously reported diethynyl phosphinates and phosphonamidate electrophiles. The triazole-induced faster kinetics were predicted by density functional theory calculations, which previously were also applied to predict the kinetics of thiol addition to other P(V)-electrophiles.[56] Thiol selectivity of the ethynyl triazolyl phosphinates was probed in proteome-wide experiments, in which the intrinsic selectivity of cysteines was verified.[55] The capacity of such electrophiles to be employed in the formation of bioconjugates - such as protein-protein conjugates - in fully aqueous systems, together with the fast access to labeled antibodies and ADCs, further prove the outstanding potential of ETP-reagents in even challenging bioconjugation schemes.

2. Summary & Future Objective

Owing to Bertozzi's seminal report of the bioorthogonal Staudinger ligation about twenty years ago, scientists in the field of Chemical Biology have strived to implement the Staudinger reaction and its variants in a plethora of bioconjugation reactions. Tuning the reactivity, air stability, and water solubility of the phosphine reagents has enabled even *in vivo* applications. To broaden the potential of Staudinger ligation, phosphites and phosphonites were employed in chemoselective reactions with azides to furnish new Cysteine-selective P(V) electrophiles, enabling the synthesis of numerous bioconjugates. Building on this, new P5-labeling was implemented as a singularly valuable bioconjugation platform displaying superior reactivity and facile installation on biologically relevant molecules to generate functional protein conjugates and next-generation biopharmaceuticals.

Combining synthetic chemistry and biology in the engineering of highly selective labeling reactions has resulted in answers to long sought biological questions of high importance. On this note, the Staudinger ligation has led to unprecedented opportunities for researchers in the chemical and molecular life sciences and laid the foundation of bioorthogonal reactions, which have been recognized not only by this year's Wolf Award but also a couple of months later with the Nobel Prize in 2022 to Carolyn Bertozzi.

Currently, we are witnessing how researchers further advance this field by addressing limitations and discover new applications. We anticipate the development of novel reactions of even higher specificity and in vivo compatibility, which will enable bioconjugation chemistries to be widely applied in the clinics to battle currently untreatable pathologies.

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References

[1] H. Staudinger, J. Meyer, *Helv. Chim. Acta* **1919**, *2*, 635–646.
[2] a) Y. G. Gololobov, L. F. Kasukhin, V. S. Petrenko, *Phosphorus Sulfur Relat. Elem.* **1987**, *30*, 393–396; b) Y. G. Gololobov, I. N. Zhmurova, L. F. Kasukhin, *Tetrahedron* **1981**, *37*, 437–472.
[3] E. Saxon, C. R. Bertozzi, *Science* **2000**, *287*, 2007–2010.
[4] H. C. Hang, C. Yu, D. L. Kato, C. R. Bertozzi, *Proc. Natl. Acad. Sci. U.S.A* **2003**, *100*, 14846-14851.
[5] a) C. Bednarek, I. Wehl, N. Jung, U. Schepers, S. Bräse, *Chem. Rev.* **2020**, *120*, 4301–4354; b) T. K. Heiss, R. S. Dorn, J. A. Prescher, *Chem. Rev.* **2021**, *121*, 6802–6849; c) M. Köhn, R. Breinbauer, *Angew. Chem., Int. Ed.* **2004**, *43*, 3106–3116.
[6] W. Q. Tian, Y. A. Wang, *J. Chem. Theory Comput.* **2005**, *1*, 353–362.
[7] a) J. E. Leffler, R. D. Temple, *J. Am. Chem. Soc.* **1967**, *89*, 5235–5246; b) C. Widauer, H. Grützmacher, I. Shevchenko, V. Gramlich, *Eur. J. Inorg. Chem.* **1999**, 1659–1664.
[8] P. I. Molina, M. J. Vilaplana, *Synthesis* **1994**, *12*, 1197–1218.
[9] Y. G. Gololobov, L. F. Kasukhin, V. S. Petrenko, *Phosphorus Sulfur Silicon Relat. Elem.* **1987**, *30*, 393–396..
[10] H. Goldwhite, P. Gysegem, S. Schow, C. Swyke, *J. Chem. Soc. Dalton Trans.* **1975**, 12–15.
[11] a) M. Fottner, A.-D. Brunner, V. Bittl, D. Horn-Ghetko, A. Jussupow, V. R. I. Kaila, A. Bremm, K. Lang, *Nat. Chem. Biol.* **2019**, *15*, 276–284; b) M. Fottner, M. Weyh, S. Gaussmann, D. Schwarz, M. Sattler, K. Lang, *Nat. Commun.* **2021**, *12*, 6515; c) J. Luo, Q. Liu, K. Morihiro, A. Deiters, *Nat. Chem.* **2016**, *8*, 1027–1034; d) Z. A. Wang, Y. Zeng, Y. Kurra, X. Wang, J. M. Tharp, E. C. Vatansever, W. W. Hsu, S. Dai, X. Fang, W. R. Liu, *Angew. Chem. Int. Ed.* **2017**, *56*, 212–216; *Angew. Chem.* **2017**, *129*, 218–222; e) J. S. Wesalo, J. Luo, K. Morihiro, J. Liu, A. Deiters, *ChemBioChem* **2020**, *21*, 141–148.
[12] a) F. L. Lin, H. M. Hoyt, H. van Halbeek, R. G. Bergman, C. R. Bertozzi, *J. Am. Chem. Soc.* **2005**, *127*, 2686–2695; b) E. Saxon, S. J. Luchansky, H. C. Hang, C. Yu, S. C. Lee, C. R. Bertozzi, *J. Am. Chem. Soc.* **2002**, *124*, 14893–14902.
[13] a) L. Cheng, X. Kang, D. Wang, Y. Gao, L. Yi, Z. Xi, *Org. Biomol. Chem.* **2019**, *17*, 5675–5679; b) M. Sundhoro, S. Jeon, J. Park, O. Ramström, M. Yan, *Angew. Chem. Int. Ed.* **2017**, *56*, 12117–12121; *Angew. Chem.* **2017**, *129*, 12285–12289.
[14] O. Nieto-García, M. B. Jaffee, M. Mühlberg, C. P. R. Hackenberger, *Chemoselective and Bioorthogonal Ligation Reactions: Concepts and Applications* **2017**, *1*, 97–115.
[15] a) S. Hinderlich, R. Tauber, C. R. Bertozzi, C. P. R. Hackenberger, *ChemBioChem* **2017**, *18*, 1141–1145; b) P. R. Wratil, R. Horstkorte, W. Reutter, *Angew. Chem. Int. Ed.* **2016**, *55*, 9482–9512; *Angew. Chem.* **2016**, *128*, 9632–9665.
[16] J. A. Prescher, D. H. Dube, C. R. Bertozzi, *Nature* **2004**, *430*, 873–877.
[17] H. Zhang, Y. Ma, X.-L. Sun, *Chem. Commun.* **2009**, 3032–3034.
[18] J. Vugts, A. Vervoort, M. Stigter-van Walsum, G. W. M. Visser, M. S. Robillard, R. M. Versteegen, R. C. M. Vulders, J. D. M. Herscheid, G. A. M. S. van Dongen, *Bioconjugate Chem.* **2011**, *22*, 2072–2081.
[19] W. I. Weis, B. K. Kobilka, *Ann. Rev. Biochem.* **2018**, *87*, 897–919.
[20] H. Tian, S. Naganathan, M. A. Kazmi, T. W. Schwartz, T. P. Sakmar, T. Huber, *ChemBioChem* **2014**, *15*, 1820–1829.
[21] E. Saxon, J. I. Armstrong, C. R. Bertozzi, *Org. Lett.* **2000**, *2*, 2141–2143.
[22] B. L. Nilsson, L. L. Kiessling, R. T. Raines, *Org. Lett.* **2000**, *2*, 1939–1941.
[23] a) M. B. Soellner, B. L. Nilsson, R. T. Raines, *J. Am. Chem. Soc.* **2006**, *128*, 8820–8828; b) A. Tam, R. T. Raines, *Bioorg. Med. Chem.* **2009**, *17*, 1055–1063.
[24] a) X. Ariza, F. Urpí, C. Viladomat, J. Vilarrasa, *Tetrahedron Lett.* **1998**, *39*, 9101–9102; b) I. Bosch, P. Romea, F. Urpí, J. Vilarrasa, *Tetrahedron Lett.* **1993**, *34*, 4671–4674; c) I. Bosch, F. Urpí, J. Vilarrasa, *Chem. Commun.* **1995**, 91–92; d) J. Garcia, F. Urpí, J. Vilarrasa, *Tetrahedron Lett.* **1984**, *25*, 4841–4844.
[25] R. Merkx, D. T. S. Rijkers, J. Kemmink, R. M. J. Liskamp, *Tetrahedron Lett.* **2003**, *44*, 4515–4518.
[26] a) B. L. Nilsson, L. L. Kiessling, R. T. Raines, *Org. Lett.* **2001**, *3*, 9–12; b) M. B. Soellner, B. L. Nilsson, R. T. Raines, *J. Org. Chem.* **2002**, *67*, 4993–4996; c) M. B. Soellner, A. Tam, R. T. Raines, *J. Org. Chem.* **2006**, *71*, 9824–9830.
[27] A. Tam, R. T. Raines, *Methods in Enzymology, Vol. 462* (Eds.: T. W. Muir, J. N. Abelson), Academic Press **2009**, pp. 25–44.
[28] Y. He, R. J. Hinklin, J. Chang, L. L. Kiessling, *Org. Lett.* **2004**, *6*, 4479–4482.
[29] A. Tam, M. B. Soellner, R. T. Raines, *J. Am. Chem. Soc.* **2007**, *129*, 11421–11430.
[30] H. Itoh, K. Miura, K. Kamiya, T. Yamashita, M. Inoue, *Angew. Chem. Int. Ed.* **2020**, *59*, 4564–4571; *Angew. Chem.* **2020**, *132*, 4594–4601.
[31] K. Bajaj, G. G. Pillai, R. Sakhuja, D. Kumar, *J. Org. Chem.* **2020**, *85*, 12147–12159.
[32] a) R. Kleineweischede, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2008**, *47*, 5984–5988; *Angew. Chem.* **2008**, *120*, 6073–6077; b) M. Mühlberg, D. Jaradat, R. Kleineweischede, I. Papp, D. Dechtrirat, S. Muth, M. Broncel, C. P. R. Hackenberger, *Bioorg. Med. Chem.* **2010**, *18*, 3679–3686; c) O. David, W. J. Meester, H. Bieräugel, H. E. Schoemaker, H. Hiemstra, J. H. van Maarseveen, *Angew. Chem. Int. Ed.* **2003**, *42*, 4373–4375; *Angew. Chem.* **2003**, *115*, 4509–4511.
[33] a) R. Serwa, I. Wilkening, G. Del Signore, M. Mühlberg, I. Claußnitzer, C. Weise, M. Gerrits, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2009**, *48*, 8234–8239; *Angew. Chem.* **2009**, *121*, 8382–8387; b) M. R. J. Vallée, P. Majkut, I. Wilkening, C. Weise, G. Müller, C. P. R. Hackenberger, *Org. Lett.* **2011**, *13*, 5440–5443.
[34] a) M. I. A. G. Kabachnik, *Bull. Russ. Acad. Sci. Phys.* **1956**, *5* (7), 809–816; b) T. A. M. Mastryukova, N. V. Odinets, I. L. Petrovskii, P. V. Kabachnik, *Zh. Obshch. Khim.* **1988**, *58*, 1967–1973.
[35] A. E. Arbuzov, *J. Russ. Phys. Chem. A* **1906**, *38*, 687.
[36] V. Böhrsch, R. Serwa, P. Majkut, E. Krause, C. P. R. Hackenberger, *Chem. Commun.* **2010**, *46*, 3176–3178.
[37] M. R. J. Vallée, P. Majkut, D. Krause, M. Gerrits, C. P. R. Hackenberger, *Eur. J. Chem.* **2015**, *21*, 970–974.
[38] R. Serwa, P. Majkut, B. Horstmann, J.-M. Swiecicki, M. Gerrits, E. Krause, C. P. R. Hackenberger, *Chem. Sci.* **2010**, *1*, 596–602.
[39] a) D. S. M. M. Jaradat, H. Hamouda, C. P. R Hackenberger, *Eur. J. Org. Chem.* **2010**, 5004–5009; b) N. Nischan, M. A. Kasper, T. Mathew, C. P. R. Hackenberger, *Org. Biomol. Chem.* **2016**, *14*, 7500–7508.
[40] a) E. Hoffmann, K. Streichert, N. Nischan, C. Seitz, T. Brunner, S. Schwagerus, C. P. Hackenberger, M. Rubini, *Mol. BioSyst.* **2016**, *12*, 1750–1755; b) N. Nischan, A. Chakrabarti, R. A. Serwa, P. H. M. Bovee-Geurts, R. Brock, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2013**, *52*, 11920–11924; *Angew. Chem.* **2013**, *125*, 12138–12142.
[41] a) J. Bertran-Vicente, M. Schümann, P. Schmieder, E. Krause, C. P. R. Hackenberger, *Org. Biomol. Chem.* **2015**, *13*, 6839–6843; b) J. Bertran-Vicente, R. A. Serwa, M. Schümann, P.

Schmieder, E. Krause, C. P. R. Hackenberger, *J. Am. Chem. Soc.* **2014**, *136*, 13622–13628.
[42] J. Bertran-Vicente, M. Penkert, O. Nieto-Garcia, J.-M. Jeckelmann, P. Schmieder, E. Krause, C. P. R. Hackenberger, *Nat. Commun.* **2016**, *7*, 12703.
[43] A. Hauser, E. Poulou, F. Müller, P. Schmieder, C. P. R. Hackenberger, *Eur. J. Chem.* **2021**, *27*, 2326–2331.
[44] A. Hauser, M. Penkert, C. P. R. Hackenberger, *Acc. Chem. Res.* **2017**, *50*, 1883–1893.
[45] I. Wilkening, G. del Signore, W. Ahlbrecht, C. P. R. Hackenberger, *Synthesis-Stuttgart* **2011**, *17*, 2709–2720.
[46] M. R. J. Vallée, L. M. Artner, J. Dernedde, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2013**, *52*, 9504–9508; *Angew. Chem.* **2013**, *125*, 9682–9686.
[47] M.-A. Kasper, M. Glanz, A. Stengl, M. Penkert, S. Klenk, T. Sauer, D. Schumacher, J. Helma, E. Krause, M. C. Cardoso, H. Leonhardt, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2019**, *58*, 11625–11630; *Angew. Chem.* **2019**, *131*, 11751–11756.
[48] M.-A. Kasper, A. Stengl, P. Ochtrop, M. Gerlach, T. Stoschek, D. Schumacher, J. Helma, M. Penkert, E. Krause, H. Leonhardt, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2019**, *58*, 11631–11636; *Angew. Chem.* **2019**, *131*, 11757–11762.
[49] a) I. Hollander, A. Kunz, P. R. Hamann, *Bioconjugate Chem.* **2008**, *19*, 358–361; b) C. D. Medley, J. Kay, Y. Li, J. Gruenhagen, P. Yehl, N. P. Chetwyn, *Anal. Chim. Acta* **2014**, *850*, 92–96.
[50] a) A. Beck, L. Goetsch, C. Dumontet, N. Corvaïa, *Nat. Rev. Drug Discovery* **2017**, *16*, 315–337; b) B. Q. Shen, K. Xu, L. Liu, H. Raab, S. Bhakta, M. Kenrick, K. L. Parsons-Reponte, J. Tien, S. F. Yu, E. Mai, D. Li, J. Tibbitts, J. Baudys, O. M. Saad, S. J. Scales, P. J. McDonald, P. E. Hass, C. Eigenbrot, T. Nguyen, W. A. Solis, R. N. Fuji, K. M. Flagella, D. Patel, S. D. Spencer, L. A. Khawli, A. Ebens, W. L. Wong, R. Vandlen, S. Kaur, M. X. Sliwkowski, R. H. Scheller, P. Polakis, J. R. Junutula, *Nat. Biotechnol.* **2012**, *30*, 184–189.
[51] M.-A. Kasper, M. Glanz, A. Oder, P. Schmieder, J. P. von Kries, C. P. R. Hackenberger, *Chem. Sci.* **2019**, *10*, 6322–6329.
[52] A. L. Baumann, S. Schwagerus, K. Broi, K. Kemnitz-Hassanin, C. E. Stieger, N. Trieloff, P. Schmieder, C. P. R. Hackenberger, *J. Am. Chem. Soc.* **2020**, *142*, 9544–9552.
[53] C. E. Stieger, L. Franz, F. Körlin, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2021**, *60*, 15359–15364.
[54] D. Gajria, S. Chandarlapaty, *Expert Rev. Anticancer Ther.* **2011**, *11*, 263–275.
[55] C. E. Stieger, Y. Park, M. A. R. de Geus, D. Kim, C. Huhn, J. S. Slenczka, P. Ochtrop, J. M. Müchler, R. Süssmuth, J. Broichhagen, M.-H. Baik, C. Hackenberger, *Angew. Chem. Int. Ed.* n/a.
[56] Y. Park, A. L. Baumann, H. Moon, S. Byrne, M.-A. Kasper, S. Hwang, H. Sun, M.-H. Baik, C. P. R. Hackenberger, *Chem. Sci.* **2021**, *12*, 8141–8148.

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