Staudinger Ligation

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Staudinger Ligation as a Method for Bioconjugation

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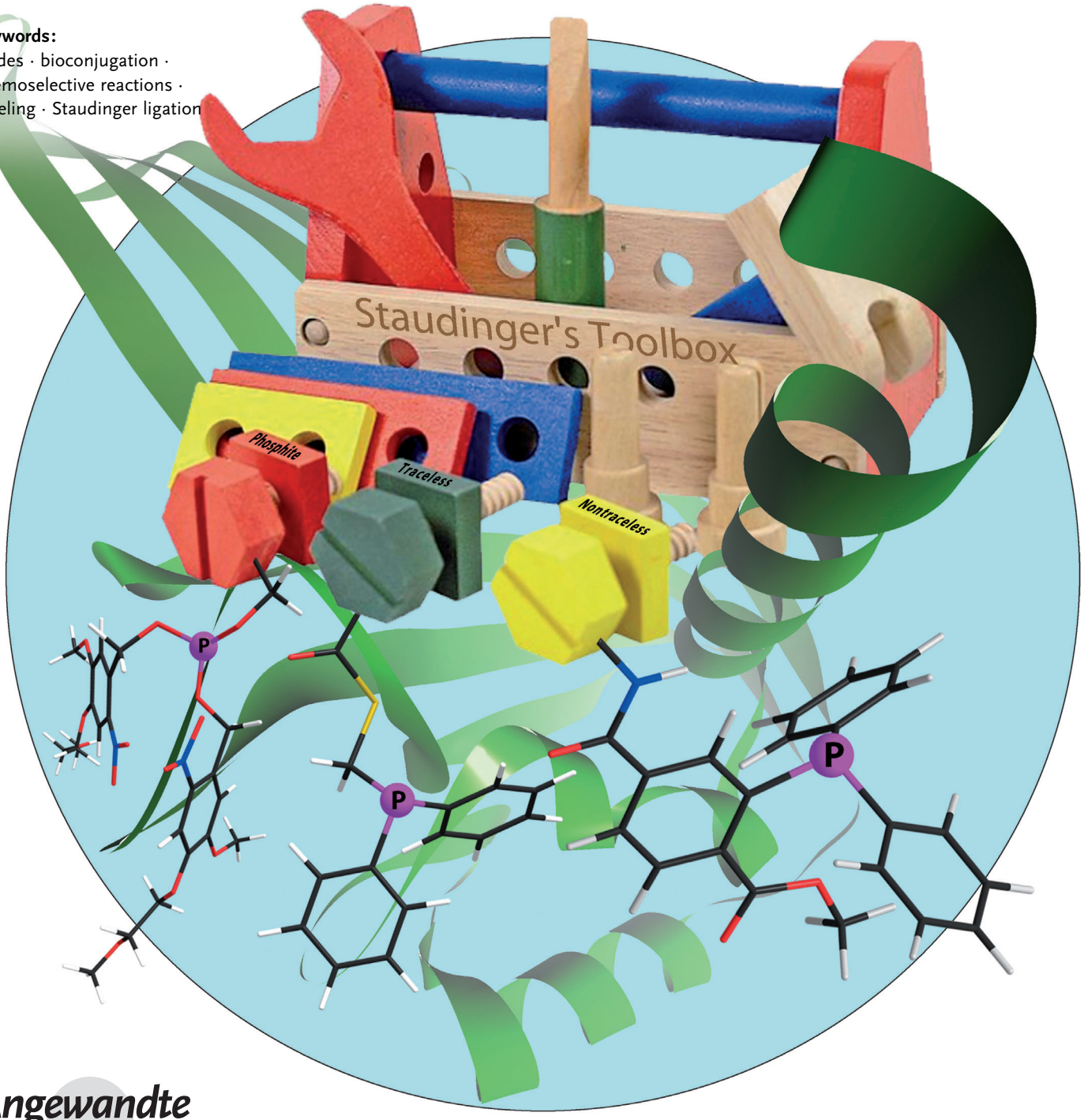

In 1919 the German chemist Hermann Staudinger was the first to describe the reaction between an azide and a phosphine. It was not until recently, however, that Bertozzi and co-workers recognized the potential of this reaction as a method for bioconjugation and transformed it into the so-called Staudinger ligation. The bio-orthogonal character of both the azide and the phosphine functions has resulted in the Staudinger ligation finding numerous applications in various complex biological systems. For example, the Staudinger ligation has been utilized to label glycans, lipids, DNA, and proteins. Moreover, the Staudinger ligation has been used as a synthetic method to construct glycopeptides, microarrays, and functional biopolymers. In the emerging field of bio-orthogonal ligation strategies, the Staudinger ligation has set a high standard to which most of the new techniques are often compared. This Review summarizes recent developments and new applications of the Staudinger ligation.

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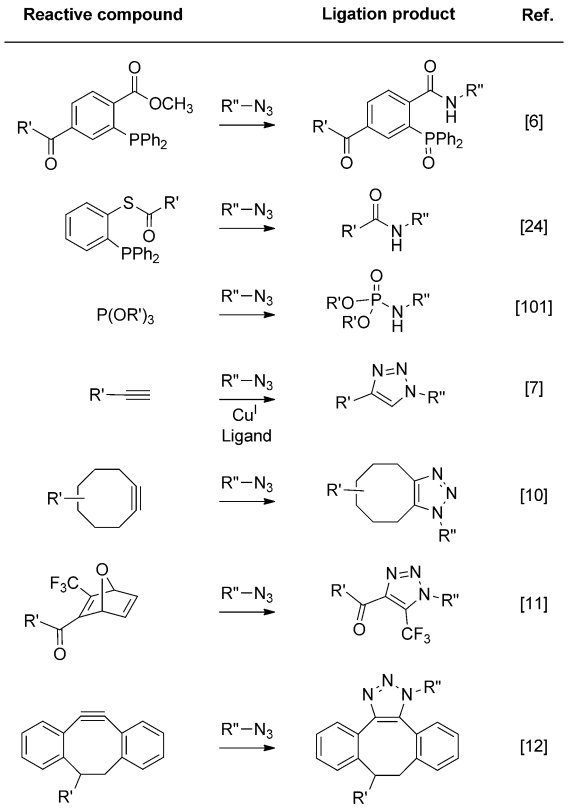
1. Introduction

Several bioconjugation techniques have been developed over the years which enable the covalent linkage of fluorescent probes, affinity tags, or isotope labels to different (bio)molecules. The decoration of various biomolecules (e.g. proteins, glycans, and DNA), cell-surface labeling, and immobilization of proteins under physiological conditions have been achieved by using different coupling strategies. In general, well-established in vitro bioconjugation protocols, such as thiol–maleimide or amine-activated ester coupling reactions, are applied to construct the desired bioconjugates. However, in vivo, a vast variety of accessible competing nucleophiles and electrophiles, which are present on proteins, glycans, and thousands of small organic metabolites, has hampered the use of such techniques in complex biological systems. Several site-specific, bio-orthogonal,[1] conjugation techniques have been developed to overcome the problems encountered with in vivo labeling.[2,3] Site-specific conjugation in a native environment requires unique reactive functional groups which are not present in biological systems. Those unique reactive functionalities—bio-orthogonal chemical reporters—are non-native, nonperturbing chemical handles which can be modified in biological systems only through highly selective reactions.[4] The most frequently applied reporter, used in several bio-orthogonal conjugation techniques, is the azide functionality.[5] Azides are chemically inert, stable under physiological conditions, and extremely rare in biological systems. Not surprisingly, azides have been used in several bioconjugation strategies, including the Staudinger ligation,[6] copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reactions,[7,8] and more recently various strain-promoted cycloaddition reactions with, for example, cyclooctynes,[9,10] oxanorbornadienes,[11] and dibenzocyclooctynes.[12] Whereas azides produce a stable triazole linkage in the cycloaddition reactions (Scheme 1), the reaction of an azide and a phosphine reagent in the Staudinger ligation generates a stable amide bond. The potential of the Staudinger reaction as a chemoselective, bio-orthogonal



Scheme 1. Overview of reactions in which an azide and a complementary reactant are used.

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reaction for utilization in glycobiology was first recognized by Saxon and Bertozzi. In a seminal paper they demonstrated that azide-modified, glycan-containing cell surfaces could efficiently be labeled using a fluorogenic phosphine reagent.[6]

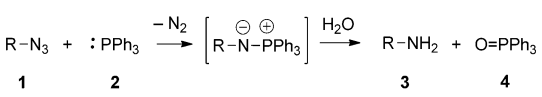
With the rediscovery of the Staudinger reaction as a mild and highly selective method for bioconjugation, Saxon and Bertozzi paved the road for a multitude of applications in chemical biology. As a consequence, this bio-orthogonal type of reaction is often referred to as the Bertozzi–Staudinger ligation.

The progress made with the Staudinger ligation as a chemoselective bioconjugation method was last reviewed by Köhn and Breinbauer in 2004.[13] However, with the rapidly expanding field of chemical biology, the number of new applications and developments concerning the Staudinger ligation has increased considerably. This prompted us to summarize the recent advancements of the Staudinger ligation (from 2004 onwards) in this Review. In addition, the Staudinger ligation is compared to other available bio-orthogonal conjugation techniques and a brief future prospect will be presented.

2. Development of the Staudinger Ligation

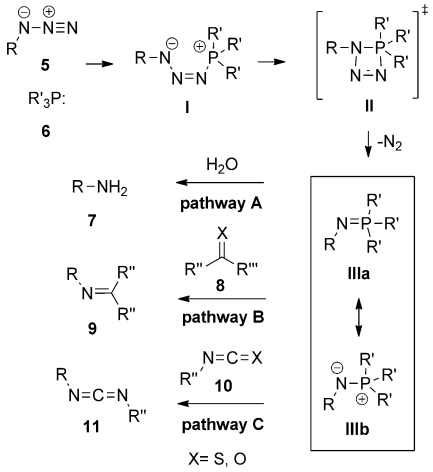
2.1. Staudinger Reaction

In 1919 Staudinger and Meyer documented the reaction occurring between an azide **1** and triphenylphosphine (**2**), which, under the loss of nitrogen, resulted in the formation of an iminophosphorane intermediate.[14] In the presence of water this intermediate is hydrolyzed to produce a primary amine **3** and triphenylphosphine oxide (TPPO, **4**; Scheme 2).



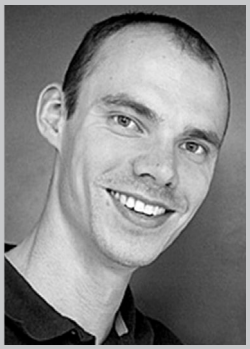
Scheme 2. Classical Staudinger reaction between a phosphine and an azide.

The mechanism of the classical Staudinger reaction has been thoroughly investigated by various experimental[15] as well as computational methods,[16,17] and is known to proceed via several intermediates.[15a,18] The first step involves a nucleophilic attack of the phosphorus atom of **6** onto the azide moiety of **5** to form the phosphazide intermediate **I** (Scheme 3). In a subsequent step, this phosphazide undergoes an intramolecular cyclization to form a four-membered-ring transition state (i.e. intermediate structure **II**) which, upon loss of nitrogen, gives rise to iminophosphorane resonance structures **IIIa** and **IIIb**.



Scheme 3. Mechanism of the Staudinger reaction. Addition of H_2O (as a nucleophile) results in the classical Staudinger reduction (pathway A). The choice of electrophile determines the product outcome in the case of pathways B and C.

The Staudinger reaction can either be a first or second order reaction depending on whether the rate-determining step is the unimolecular decomposition or the bimolecular



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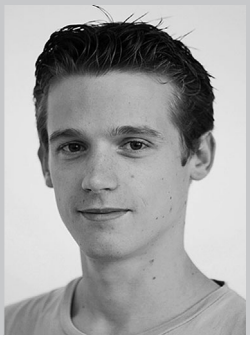
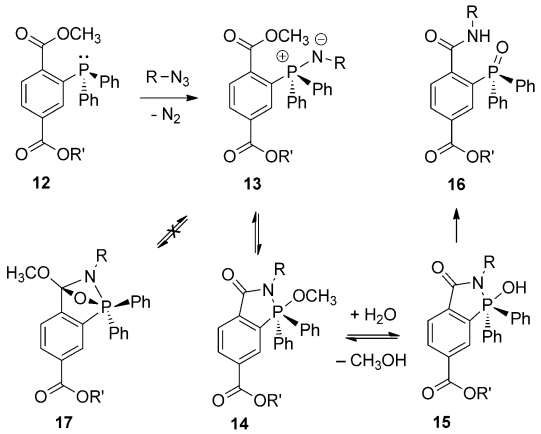


Mark B. van Eldijk received his MSc in molecular life sciences at the Radboud University Nijmegen (The Netherlands) in 2010. As part of the MSc studies he carried out research internships in bio-organic chemistry with Prof. Jan van Hest at the Radboud University Nijmegen and with Prof. David Tirrell at the California Institute of Technology (USA). He is currently a PhD student in the research group of Prof. van Hest working on biopolymer-based stimulus-responsive materials.

Jan C. M. van Hest received his MSc in polymer chemistry in 1991, at the Eindhoven University of Technology, and his PhD in 1996 with Prof. E. W. (Bert) Meijer. As a postdoctoral researcher he investigated the incorporation of artificial amino acids in proteins under the supervision of Prof. David Tirrell, at the university of Massachusetts in Amherst (USA). After working at DSM, in 2000 he was appointed full professor of bio-organic chemistry at the Radboud University Nijmegen. His current interests focus on translating natural structural concepts into bio-inspired materials by using modern organic and polymer chemistry as well as protein engineering.

formation of the phosphazide.[15a] In the presence of water, iminophosphorane **III** will be hydrolyzed to generate an amine **7** and the stable phosphine oxide (Scheme 3, pathway A). This reaction is referred to as the Staudinger reduction and is routinely used in organic synthesis to introduce amines.[15b] Moreover, the highly nucleophilic nitrogen atom of the iminophosphorane readily reacts with a variety of electrophilic compounds to give valuable synthetic intermediates. For example, the aza-Wittig reaction, first observed by Staudinger and Hauser, is a reaction which utilizes iminophosphoranes to produce imines (Scheme 3, pathway B).[19] In this reaction, which displays a high similarity to the Wittig reaction, the iminophosphorane is treated with an aldehyde, ketone, or thioketone **8** to form an imine product **9**. The most important applications of this reaction are found in the synthesis of acyclic and heterocyclic compounds.[20] Furthermore, iminophosphoranes can be used in the synthesis of carbodiimides (**11**; Scheme 3, pathway C) by treating the iminophosphorane with iso-(thio)cyanates (**10**).[21]



Scheme 4. Mechanism of the Staudinger ligation.

2.2. Nontraceless Staudinger Ligation

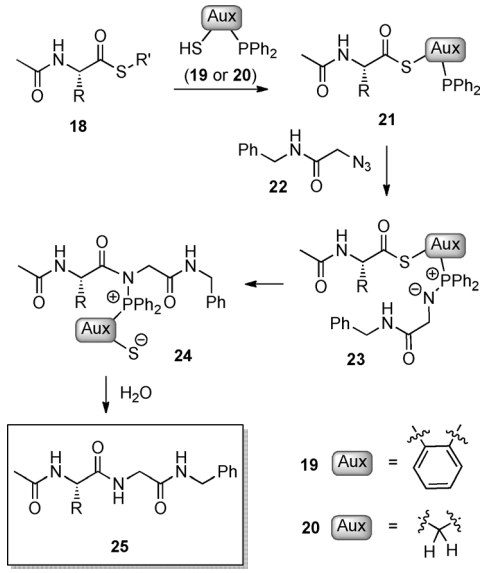
More than 80 years after the discovery of the Staudinger reaction, Saxon and Bertozzi modified this reaction such that it has become a valuable method for bioconjugation.[6] The Staudinger reaction, now termed the Staudinger ligation, was specifically designed for studying the metabolic engineering of cell surfaces. In previous studies, Mahal et al. introduced a chemoselective ligation reaction for cell-surface engineering, by applying the reaction between a ketone and an aminooxy or a hydrazide group.[22] Although this reaction was useful for the labeling of cell surfaces, the use of this reaction for intracellular labeling was limited due to the interference of endogenous metabolites carrying ketone functionalities. This led to the quest for a bio-orthogonal ligation technique with reactive partners which were both abiotic and would form stable adducts under physiological conditions. The Staudinger reaction was envisioned to meet most of the criteria necessary for selective bio-orthogonal ligation. The reaction of a phosphine and an azide proceeds smoothly in water, at room temperature, quantitatively, and—most importantly—both reactants are abiotic bio-orthogonal reporters. Unfortunately, the product of the Staudinger reaction, the aza-ylide, normally undergoes rapid spontaneous hydrolysis to yield a primary amine and the corresponding phosphine oxide. To avoid this hydrolysis a phosphine reagent was designed that carried an intramolecular electrophilic trap to capture the nucleophilic aza-ylide intermediate by an intramolecular cyclization (Scheme 4). This cyclization step produced a stable amide bond (i.e. **16**) rather than the products obtained from aza-ylide hydrolysis. In a preliminary mechanistic study based on ^{31}P NMR spectroscopy, the Staudinger ligation was proposed to proceed via the oxaphosphetane intermediate **17** (Scheme 4).[23] In a recent more elaborate study, however, a different mechanism was reported. Here, intermediate structure **14** was proposed to be involved in the key step (based on ^{1}H NMR as well as 2D ^{1}H and ^{13}C NMR spectroscopy, and X-ray crystallography). It was also shown that the oxygen atom of the phosphine oxide originated from a water molecule.[24]

2.3. Traceless Staudinger Ligation

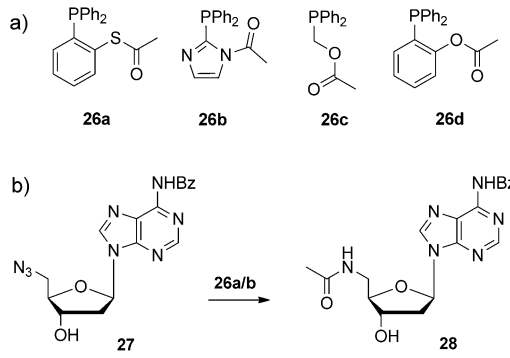
Soon after the publication on the nontraceless Staudinger ligation, Raines and co-workers[25] as well as the Bertozzi research group[26] simultaneously reported the so-called traceless Staudinger ligation. The difference between this variant and the nontraceless Staudinger ligation is the elimination of phosphine oxide from the final product in the hydrolysis step. Raines and co-workers demonstrated that the traceless Staudinger ligation could be used for the conjugation of two peptide fragments as an alternative to native chemical ligation.[25] In their studies, 2-(diphenylphosphino)-benzathiol (**19**) and (diphenylphosphino)methanethiol (**20**) were both used as auxiliaries for the linkage of a thioester and an azide component.[25,27] The first step in this reaction involves the trans-thioesterification of thioester **18** with a phosphinothiol (**19** or **20**). The activated thioester **21** then reacts with azide **22** to form an iminophosphorane **23**. Intramolecular rearrangement of this iminophosphorane leads to the formation of the amidophosphonium salt **24**, which upon hydrolysis gives amide **25** (Scheme 5).[28] Moreover, Raines and co-workers showed that **20**, with its high rate constant and chemoselectivity, was the most efficacious coupling reagent in mediating the Staudinger ligation.

The traceless Staudinger method described by Saxon et al. implemented the use of different acylated phosphine compounds (Scheme 6a) designed to form an amide bond via a cleavable linkage system.[26] A structural prerequisite in these compounds was the presence of two aromatic phosphine substituents to prevent excessive oxidation of the phosphine. In a model reaction, the four acylating phosphine agents (**26a–d**) were treated with azido-nucleoside **27** (Scheme 6b). Compounds **26a** and **26b** both gave the desired Staudinger ligation product **28** in good yields, while compounds **26c** and **26d** only gave the aza-ylide hydrolysis product.

In-depth mechanistic investigations concerning the traceless and nontraceless Staudinger ligation has provided



Scheme 5. Traceless Staudinger ligation by Raines and co-workers using thiol auxiliaries **19** or **20**. Aux = auxiliary.



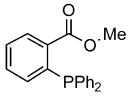
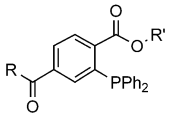
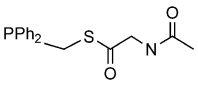
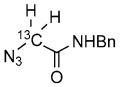
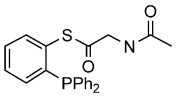
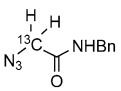
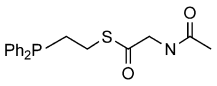
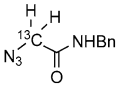
Scheme 6. a) Bertozzi's cleavable linker systems for traceless Staudinger ligation; b) traceless Staudinger ligation of azido-nucleoside **27** with phosphine derivatives **26a** and **26b**. Bz = benzoyl.

significant insight into the reaction kinetics of the different coupling reagents (Table 1). For example, the effects of solvent, azide structure, and phosphine substituents (steric effects) on the reaction kinetics have been probed intensively.[24,28] Moreover, the electronic effects of various phosphine analogues on the rate of the Staudinger ligation have been determined by means of Hammett correlations.[29] The obtained rate constants for the traceless Staudinger ranged from 0.12 to $7.7\times10^{-3}\,\text{M}^{-1}\text{s}^{-1}$ (in DMF/D_2O 6:1), while rate constants between 1.8 and $2.2\times10^{-3}\,\text{M}^{-1}\text{s}^{-1}$ (CD_3CN/D_2O 95:5) were obtained for the nontraceless Staudinger ligation.

3. The Staudinger Ligation as a Labeling Method

In chemical biology, the conjugation of small molecules to biomolecules is one of the most important methods for studying biological systems. The Staudinger ligation, as a conjugation strategy, has been widely applied for the coupling

Table 1: Reaction kinetics of the Staudinger ligation.

Ligation type	Solvent	Azide	k_{obs}[b]	Ref.
reduction				
PPh_3	benzene		10.0	[18]
nontraceless[a]				
	CD_3CN/H_2O(5%)		2.0 ± 0.1	[24]
	CH_3CN/KH_2PO_4 (1:1)		3.8 ± 0.8	[49]
Traceless				
	DMF/D_2O		7.70 ± 0.30	[28]
	DMF/D_2O		1.04 ± 0.05	[28]
	DMF/D_2O		0.65 ± 0.01	[28]

[a] R and R′ as specified in structure **48**. [b] ($\times10^{-3}\,\text{M}^{-1}\,\text{s}^{-1}$).

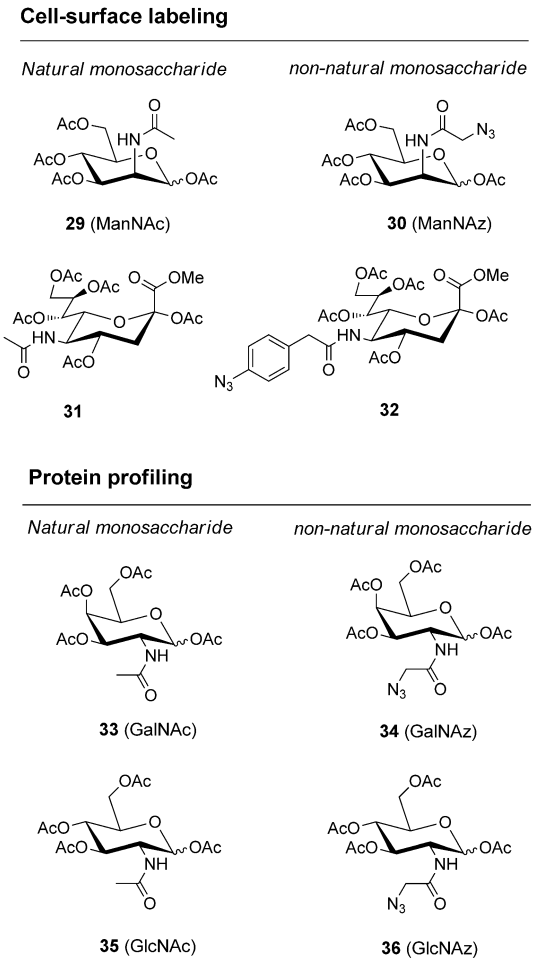
of chemical probes to biomolecules. Implementing the Staudinger ligation as a method for bioconjugation requires the presence of one of the reacting partners (phosphine or azide) in a biomolecule. With respect to incorporation into a biomolecule, the azide is preferred because of its size and stability under physiological conditions. In addition, an azide moiety can easily be incorporated into glycans, proteins, lipids, and DNA either by chemical methods (e.g. diazotransfer) or by biosynthetic pathways. With a wide variety of appropriate azides available, a multitude of applications of the Staudinger ligation can be pursued. In the next sections the use of the Staudinger ligation as a labeling agent for different biomolecules will be discussed. In addition, the use of the Staudinger ligation as a synthetic method in the fields of peptide chemistry and material sciences will be briefly reviewed.

3.1. Glycan Labeling

Molecular imaging enables visualization of biomolecules in their native environment without substantial perturbation. The in vivo imaging of glycans is particularly interesting since these biopolymers participate in many biological processes, including cell–cell interactions, molecular trafficking, signal transduction, and endocytosis.[30,31] The existing molecular biology techniques that are applied for the profiling and imaging of proteins and DNA cannot be extended to investigate glycans, as they are not directly encoded by the genome. Glycans can be imaged by lectins and antibodies, but

the use of these detection methods in vivo is limited as a result of low affinity.[32] Another method for imaging glycans involves the formation of Schiff bases with either aldehydes or ketones. This method, however, also encounters problems when applied in vivo because of nonspecific binding to endogenous metabolites.[4,22]

A breakthrough in glycan imaging was achieved by Saxon and Bertozzi, who used an azide-modified mannose derivative, that is, *N*-azidoacetylmannosamine (ManNAz, **30**; Scheme 7), as a bio-orthogonal chemical reporter.[6] Incuba-



Scheme 7. Natural monosaccharides and their azide-functionalized analogues which are accepted in the glycan biosynthetic pathways.

tion of Jurkat cells with saccharide **30** resulted in the incorporation of this compound into glycans through the sialic acid biosynthetic pathway (Figure 1).[33] The incorporated ManNAz units were subsequently visualized by applying the Staudinger ligation with a phosphine–biotin probe (**38**, Scheme 8). After treatment of the biotin-labeled glycans with fluorescein-labeled avidin, an increase in the fluorescence of the biotin–avidin complex could be observed.

Other, more bulky analogues of *N*-acetylmannosamine were not, or less efficiently, incorporated. As it turned out, the integration was hampered by the poor transformation of ManNAc derivatives by ManNAc 6-kinase in the biosynthetic

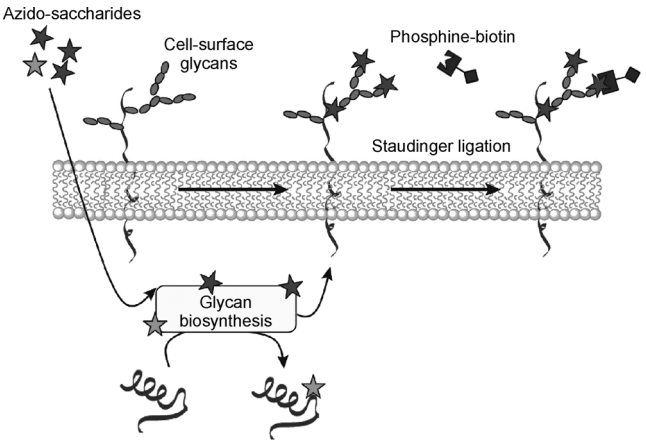
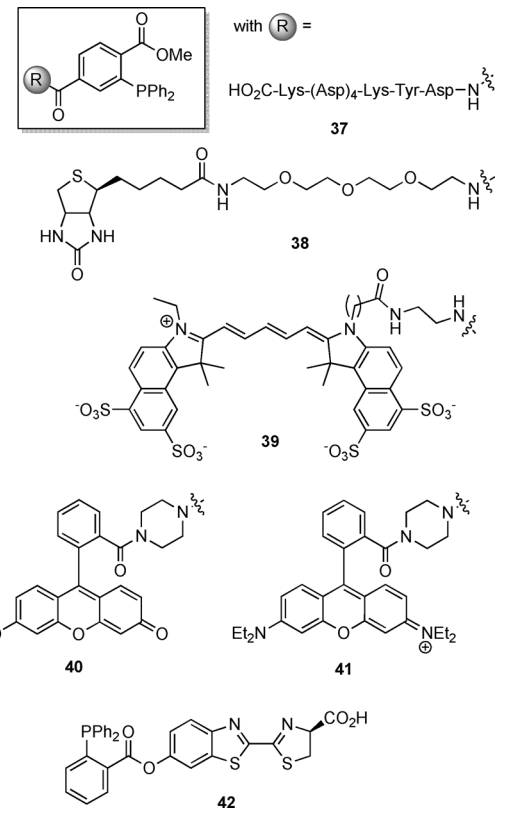


Figure 1. Incorporation of azido-saccharides into glycoconjugates through biosynthetic pathways. Adapted from Ref. [4].



Scheme 8. Various triarylphosphine conjugates: FLAG conjugate (**37**), biotin conjugate (**38**), Cy5.5 conjugate (**39**), fluorescein conjugate (**40**), rhodamine conjugate (**41**), and luciferin conjugate (**42**).

process.[34] In an attempt to get round this specific enzymatic step, sialic acid derivative **32** was introduced, which was found to be delivered efficiently to the cell surface.[35] In a subsequent investigation, Bertozzi and co-workers tested the Staudinger ligation on mice splenocytes. Injection of mice with saccharide **30** gave rise to azide-labeled splenocytes, which in a second step (ex vivo) were subjected to the Staudinger ligation with FLAG compound **37** (Scheme 8). The labeled splenocytes were subsequently detected by flow-

cytometric analysis.[36] Although the FLAG–phosphine conjugate (**37**) and the biotin–phosphine conjugate (**38**) are both suitable probes for either monitoring cell-surface labeling or for the analysis of proteomes, in vivo imaging of glycans cannot be performed with these conjugates. Several fluorescently labeled phosphine probes (**39**–**42**, Scheme 8) were developed to visualize the functioning of glycans in living systems. The Cy5.5–phosphine conjugate (**39**) was shown to be superior over both the fluorescein–phosphine (**40**) and rhodamine–phosphine (**41**) conjugates, as a result of its low background fluorescence. This effect was attributed to the higher charge density, and therefore greater solubility, which allowed more efficient removal of excess phosphine conjugate.[37]

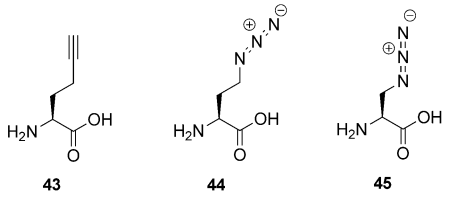
Besides the visualization of glycans by using azidosugars as metabolic labels and phosphine-modified fluorescence imaging probes, the Bertozzi research group recently applied a luciferin–phosphine conjugate **42** for the bioluminescent detection of cell-surface glycans.[38] Upon Staudinger ligation of the luciferin–phosphine conjugate with azido-containing glycans, the liberated luciferin diffuses into the cell where it is converted into the bioluminescent oxyluciferin by luciferase.

3.2. Protein Labeling

A crucial requirement for protein labeling by the Staudinger ligation is the specific protein functionalization with either a phosphine or an azide tag. The azide is preferred as the chemical handle because of the high degree of oxidation of the phosphine moiety in aqueous solutions. The azide can be introduced into a protein chemically, that is, by a diazotransfer reaction[39] or biochemically, either by using genetic engineering for the incorporation of azide-containing amino acids into proteins[40] or by post-translational modifications. The latter concept was used in a combined effort by the research groups of Tirrell and Bertozzi.[41]

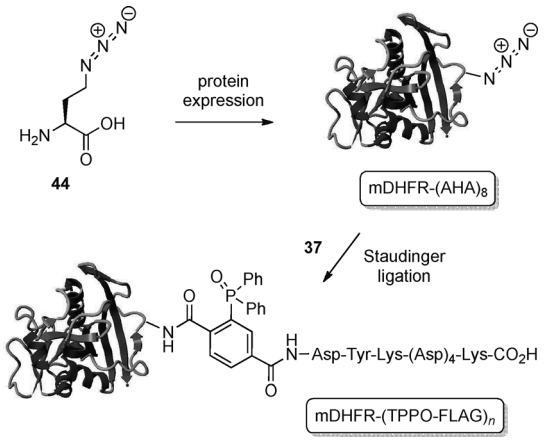
3.2.1. Non-Natural Amino Acids

The introduction of amino acid analogues can be achieved in vitro (i.e. in *E. coli*, yeast, and mammalian cells) by using the cell translational machinery in either a site-specific or a residue-specific manner. This incorporation is most stringently controlled by aminoacyl-tRNA synthetases (aaRS). These enzymes are responsible for the coupling of amino acids to tRNAs. For example, structural analogues of methionine, depicted in Scheme 9, can be incorporated into proteins in a residue-specific manner by the methionyl-tRNA synthetase (MetRS).



Scheme 9. Methionine analogues **43**–**45** used for incorporation by MetRS.

The expression of proteins containing non-natural amino acids, by making use of a methionine-auxotrophic strain of *E. coli*,[42] was elegantly used by the Bertozzi and Tirrell research groups. The in vitro incorporation of azide-containing amino acid analogues of methionine was demonstrated for two azide-functionalized amino acids, that is, azidohomoalanine (**44**, AHA) and azidoalanine (**45**, AAL). These two non-proteinogenic amino acids were first tested computationally as substrates for MetRS. Subsequently, the biosynthesis of murine dihydrofolate reductase (mDHFR) was tested in an *E. coli* methionine auxotroph in methionine-depleted medium, supplemented with the unnatural amino acids **44** or **45**. Results from both assays showed that compound **44** was an outstanding methionine surrogate, as indicated by the 95% replacement of methionine residues by **44**. The incorporation of AHA into mDHFR yielded mDHFR-$(AHA)_8$. The reactivity of the azides in mDHFR-$(AHA)_8$ was investigated in the Staudinger ligation using the triarylphosphine–FLAG conjugate **37** (Scheme 10). The products of the Staudinger ligation were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis with anti-FLAG antibodies. This approach showed that on average five of the eight azides in mDHFR-$(AHA)_8$ had reacted with FLAG–phosphine **37**. The incomplete modification of all eight sites in mDHFR-$(AHA)_8$ was attributed to the reduction of the residual azides.[41] In a complementary experiment, the Staudinger ligation was performed on the crude cell lysate. Also here, the Staudinger ligation could be successfully applied, thus suggesting that this ligation technique could even be used for intracellular labeling applications.

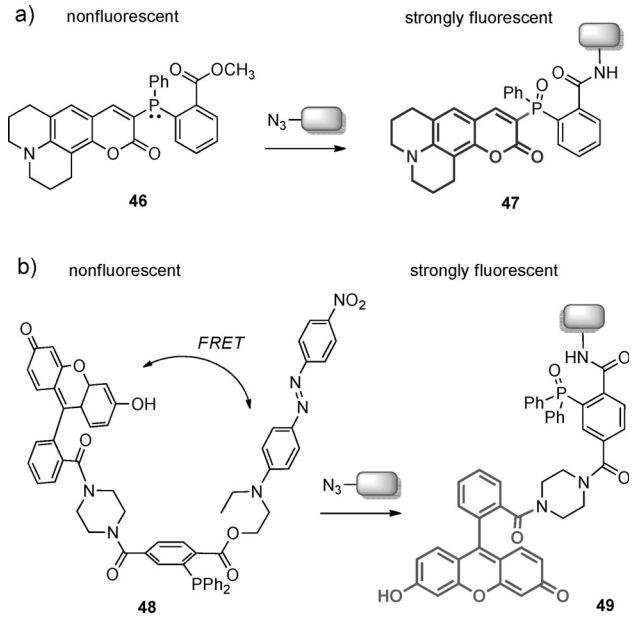


Scheme 10. Incorporation of azidohomoalanine (**44**) into mDHFR to yield mDHFR-$(AHA)_8$, followed by Staudinger ligation with triarylphosphine–FLAG conjugate **37** ($n = \leq 5$).

Site-specific incorporation of azide-containing amino acids can be achieved by using the strategy developed by Schultz and co-workers.[43] This method relies on the construction of a unique codon–tRNA pair and the corresponding aminoacyl-tRNA synthetase. First, an orthogonal tRNA is

generated that is not recognized by any natural aminoacyl synthetases. As a result, only its cognate amino acid is inserted in response to the amber nonsense codon. Next, a synthetase is generated which specifically recognizes this unique tRNA. The substrate specificity of this synthetase is then evolved to recognize only the desired non-natural amino acid, and no endogenous amino acid. This method can be used to efficiently incorporate a large variety of non-natural amino acids into proteins by using *E. coli*, yeast, and mammalian cells.[44] Introducing azide-containing amino acids in this way, followed by Staudinger ligation has been successfully applied by various research groups.[45,46] An elegant example was reported by Yokoyama and co-workers, who used the amber stop codon to introduce *p*-azido-L-phenylalanine. It was then shown that after incorporation of the non-natural amino acid, modification of the azide unit could be accomplished by the Staudinger ligation.[47]

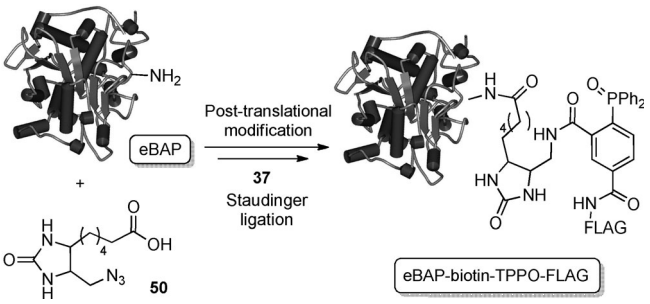
The Staudinger ligation, as described in the examples above, requires a two-step procedure for the visualization of the target compound. This process could be simplified to a single-step method through the design of a Staudinger probe containing a fluorogenic coumarin moiety (**46**).[48] Prior to ligation, the coumarin–phosphine dye is nonfluorescent as a result of quenching of the excited state of the fluorophore by the lone pair of electrons on the phosphorus atom (Scheme 11a). Upon reaction with an azide, the phosphorus atom is oxidized, which results in activation of the fluorescent label (structure **47**). This method was evaluated by ligation of probe **46** with a model azide and subsequently with mDHFR-$(AHA)_8$. Labeling experiments showed that fluorescence was enhanced 60-fold upon oxidation of the phosphine. Non-specific air oxidation of the phosphine, however, appeared to be a disadvantage of this technique. To overcome this problem fluorogenic phosphine reagent **48** was designed, which contains an ester-linked fluorescence resonance energy transfer (FRET) quencher (Scheme 11b). Staudinger ligation of phosphine reagent **48** with azides resulted in cleavage of the ester bond followed by disabling of the quenching.[49] Here, nonspecific oxidation does not interfere with the efficiency of the FRET quenching. The performance of this Staudinger probe was tested by ligation to mDHFR-$(AHA)_8$. The effectiveness of **48** in this labeling experiment triggered the authors to use this fluorescent marker in the staining of an azide-labeled cell surface of living HeLa cells. Moreover, it was shown, through the use of an early apoptosis marker, that **48** was non-cytotoxic and thus can be used in in vivo studies.



Scheme 11. Representation of two fluorogenic phosphine dyes (**46** and **48**) which are activated upon Staudinger ligation. a) Quenching of fluorogenic phosphine dye (**46**) with a lone pair of electrons; b) FRET-based quenching of fluorogenic phosphine dye (**48**).

3.2.2. *Protein-Modifying Enzymes*

Another approach for the site-selective labeling of proteins by the Staudinger ligation is based on post-translational modification using protein-modifying enzymes. Here, an azide-containing tag is covalently attached to a target protein through protein-modifying enzymes which recognize a specific amino acid sequence. Ting and co-workers used the biotin ligase from *P. horikoshii* to site-selectively link the azide-bearing biotin analogue **50** to an endogenous biotin acceptor protein (eBAP). After enzymatic coupling of **50** to the acceptor protein, the Staudinger ligation was performed with the triarylphosphine–FLAG conjugate **37** (Scheme 12). The obtained product was subsequently detected by immunoblotting with anti-FLAG antibodies.[50]



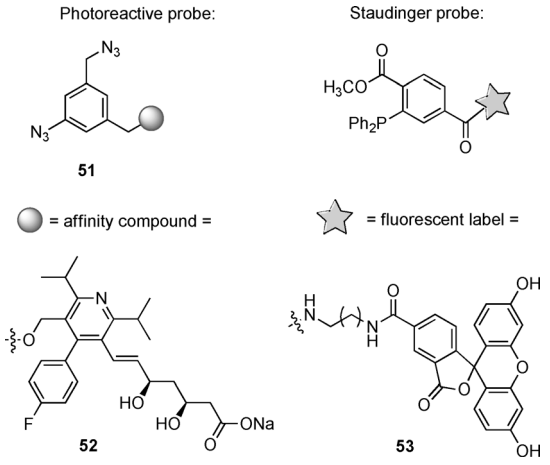
Scheme 12. Post-translational modification of eBAP with azide-bearing biotin analogue **50**, followed by labeling with FLAG–phosphine **37**.

3.2.3. *Activity-Based Protein Profiling*

Mechanism-based probes are critical tools in the evaluation of the enzymatic activities of protein families in complex proteomes and can be used to characterize small-molecule inhibitor species. These probes typically consist of a unique chemical scaffold for binding affinity and a reactive functional group for cross-linking. The reactive functional group can, for example, be an electrophile which reacts with an active-site nucleophile or a photo-cross-linkable group that allows covalent attachment to the target protein upon irradiation with UV light.[51] The degree of labeling directly corresponds to the amount of active protein present in the sample. However, decoration of the inhibitor with a detectable label is

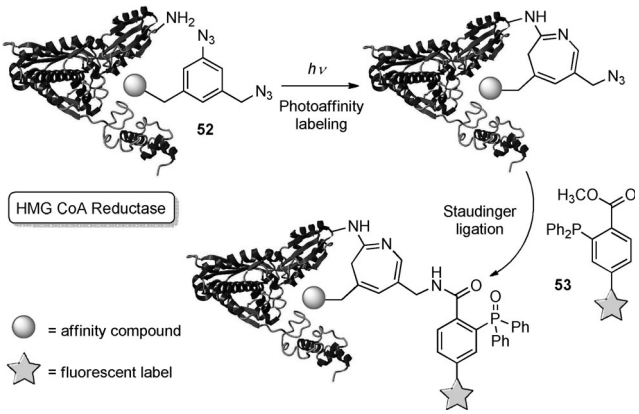
required to quantify the labeling efficiency. Implementing the azide group as a functional handle on an inhibitor allows for the introduction of a detection unit through the Staudinger ligation. Utilization of the appropriate affinity tags allows for the detection of the protein–inhibitor complex. Generally, a biotinylated phosphine or a triarylphosphine–FLAG conjugate is applied as the affinity tag.[51] Azide-modified, covalent inhibitors, in combination with the Staudinger ligation, have been used to identify various enzyme family clusters. These studies have yielded several inhibitors for proteases,[52] cysteine proteases,[53] and glycosidases.[54]

Another type of activity-based protein profiling (ABPP) is photoaffinity labeling (PAL). Here, covalent attachment of a photo-cross-linkable group is used to identify target proteins and elucidate their binding sites. For this purpose photoreactive functional groups are often combined with radio isotopes. Hosoya et al. introduced a new radio-isotope-free approach for PAL. To this end, photoreactive compound **51** was designed with an azido-benzylazide scaffold, which in a later stage could be used for Staudinger ligation with the fluorescein-anchored triarylphosphine derivative **53** (Scheme 13).



Scheme 13. Left: Photoreactive probe **51** equipped with an affinity compound (e.g. cerivastatin derivative **52**); right: Staudinger probe equipped with a fluorescent label (e.g. fluorescein-anchored triarylphosphine **53**).

To prove the concept of PAL, Hosoya et al. synthesized the cerivastatin-functionalized photoreactive probe **52** and used it to explore the binding of cerivastatin to the human 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) as depicted in Scheme 14.[55] Recently, Overkleeft and co-workers demonstrated the efficiency of the Staudinger ligation in a two-step ABPP approach, in which an azide-modified bodipy probe and a phosphine–biotin conjugate (similar to probe **38**) were used.[56] From their experiments they concluded that the efficiency depended on the reactivity of the affinity label towards the target protein and not on the chemoselective ligation employed (i.e. Staudinger ligation). In a recent study, Overkleeft and co-workers



Scheme 14. Concept of photoaffinity labeling. A consecutive Staudinger ligation is applied to introduce a detectable tag.

synthesized trifunctional activity-based protein probes containing an electrophilic trap, a photoreactive group, and a bioorthogonal ligation handle (i.e. Staudinger probe). These probes were subsequently used for the covalent and irreversible modification of proteasomal catalytic sites, followed by photo-cross-linking and visualization of the resulting conjugates through the Staudinger ligation.[57]

Although ABPP is a robust technique, it suffers from the need to introduce a photoreactive group, which might affect the binding properties. In addition, analysis by SDS-PAGE under denaturing conditions might lead to the loss of structural information of a target protein. To overcome these drawbacks Wagner and co-workers devised an alternative method for analyzing a complex proteome under native conditions.[58] In the so-called "noncovalent activity-based protein profiling", a target protein–probe complex is selectively labeled with a fluorophore through the Staudinger ligation. In a subsequent step, the crude lysate is analyzed under nondenaturing conditions, thereby identifying only the target protein (Figure 2). As an example, the authors targeted the GyrB subunit of DNA gyrase by complexing azidonovobiocin to it. In a next step, the complex was labeled using a rhodamine–phosphine tag. The target–probe–label complex (GyrB–Novo–Rhod complex) could then be identified under nondenaturing conditions, thus demonstrating the power of this method.

In an attempt to monitor lymphocyte trafficking Tanaka et al. developed a two-step engineering procedure for the labeling and visualization of glioma cells.[59] Initial studies focused on the tagging of proteins with an azide residue, by making use of the newly developed aza-electrocyclization technique.[60] By applying this method, model protein HSA was treated with aza-electrocyclization precursor **54**. After cyclization, the HSA product was treated with different phosphine reagents (biotin (**38**) or *N*-glycan conjugates (**55** and **56**)) to complete the chemical bioconjugation through the Staudinger ligation (Scheme 15).[59] This method resulted in complete cell death in rat C6 glioma cells, as a result of the high concentration of reactants present. As a result, the authors applied the reversed reaction order, that is, first the Staudinger ligation followed by aza-electrocyclization. This

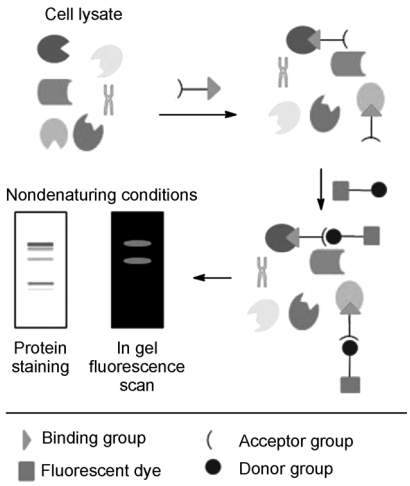
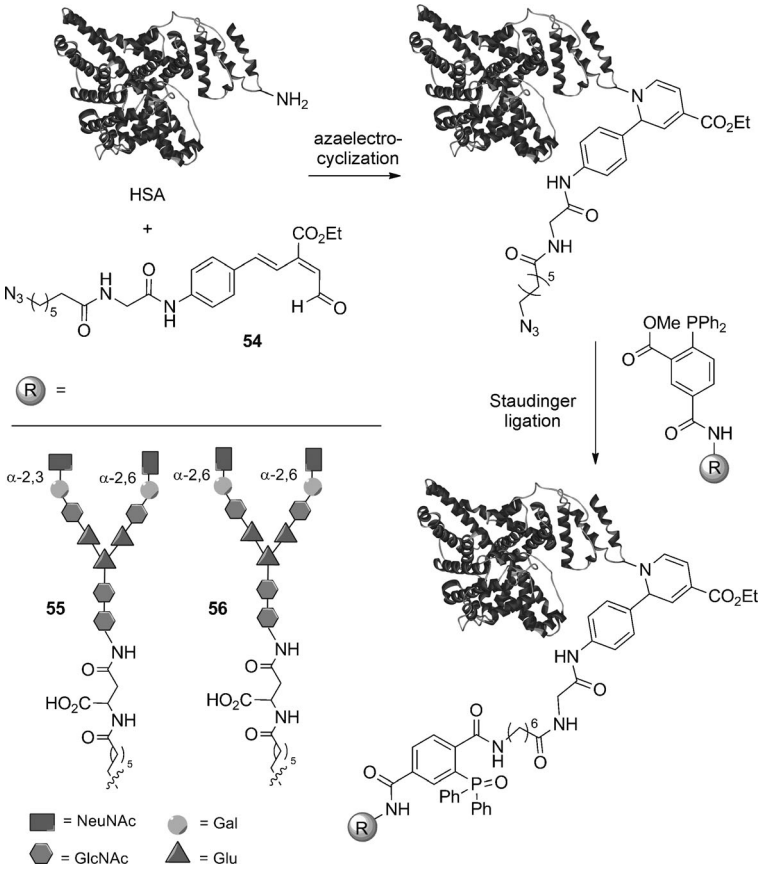


Figure 2. Target identification through noncovalent activity-based protein profiling and nondenaturing analysis. Adapted from Ref. [58].



Scheme 15. Protein modification through a sequential aza-electrocyclization/Staudinger ligation procedure.

procedure resulted in the successful labeling of the cells accompanied by inactivation of the starting materials (aldehyde reduction and phosphine oxidation). Visualization of the labeled glioma cells was achieved by incubation with either rhodamine-labeled avidin (complementary to biotin) or rhodamine-labeled SNA lectin (complementary to *N*-glycans). In a subsequent experiment, lymphocytes were fluorescently labeled and injected into mice bearing DLD-1 human colon carcinoma. Monitoring the trafficking of the lymphocytes showed there to be no accumulation at the tumor site. Surprisingly, co-labeling of the fluorescent lymphocytes with *N*-glycans by aza-electrocyclization was found to give increased localization of the lymphocytes at the tumor region after one week.

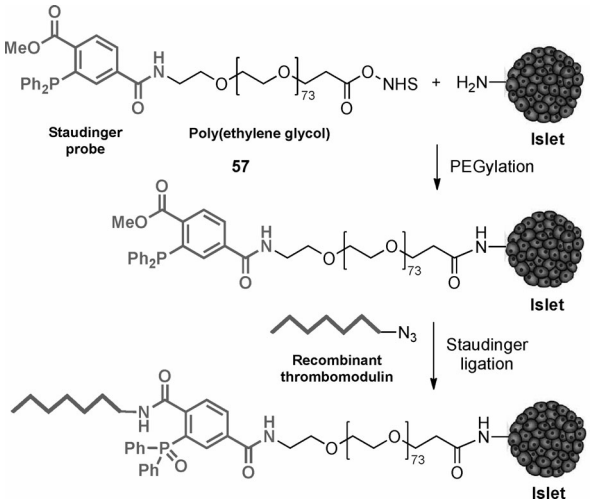
Various excellent examples of whole-cell labeling or chemical modification have recently been reported. Exemplary work has been performed on human pancreatic islets for transplantation into the liver of type 1 diabetes patients. These islets are, however, often targeted by the blood-mediated proteins, thereby resulting in an inflammatory response. A decrease of this immune response can be achieved by reducing the islet-mediated thrombogenicity and increasing the production of activated protein C. To this end, Chaikof and co-workers modified the surface of pancreatic islets, thereby resulting in bioactive islets with reduced thrombogenicity.[61] In a two-step procedure (Scheme 16), recombinantly modified azido-thrombomodulin was conjugated to islet surfaces modified with polyethylene glycol (PEGylated). PEGylation of the islets was performed with bifunctional polyethylene glycol (**57**) consisting of an NHS (NHS = *N*-hydroxysuccinimide) ester and triphenylphosphine probe **12**. Staudinger ligation with azido-thrombomodulin and the PEGylated pancreas islets gave reengineered islets with a reduced immune response.

3.3. Lipid Labeling

Proteolipids are lipid-modified proteins with a wide variety of functions. Lipidation generally occurs co-translationally or post-translationally. *N*-Myristoylation, *S*-palmitoylation, and prenylation are the three major types of lipidation. The attachment of saturated fatty acids onto proteins in eukaryotes facilitates the interaction of a protein with membranes and other proteins. This protein lipidation ultimately influences signaling, subcellular localization, and enzymatic activity. Since lipids are secondary metabolites they cannot be studied through genetically encoded reporters. Similar to the approach applied for the investigation of glycan biosynthesis, bio-orthogonally tagged lipids can be metabolically incorporated. This technique relies on the substrate tolerance of enzymes in the biosynthetic lipidation pathway.

3.3.1. Profiling N-Myristoylation and S-Palmitoylation

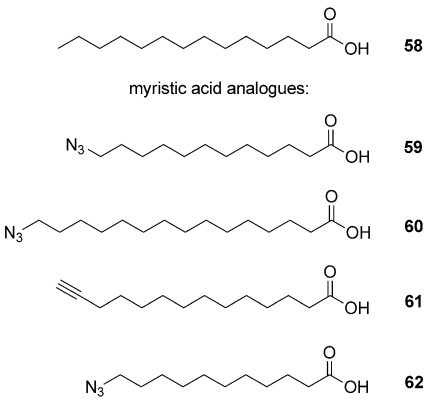
The two major types of protein acylation are the *N*-myristoylation of *N*-terminal glycine residues and the *S*-palmitoylation of cysteine residues. Both modifications play an important role in many biological processes, such as apoptosis, tumorigenesis, and cell differentiation.[62] Radio-



Scheme 16. PEGylation and subsequent Staudinger ligation between recombinant azido-thrombomodulin and a PEG-phosphine linker.[61]

labeled fatty acids have been used to explore these processes, but have encountered the disadvantages of lengthy autoradiographic exposure times and the use of hazardous high-energy ^{125}I-radio isotopes.[63]

In a proof-of-principle experiment Ploegh and co-workers demonstrated that ω-azido fatty acids **59** and **60** (Scheme 17) could efficiently be metabolized by mammalian cells and serve as selective probes for the visualization of *N*-myristoylation and *S*-palmitoylation, respectively.[64] After the in vitro lipidation, the azide was used in a Staudinger ligation with biotinylated phosphine tag **38**. Berthiaume and co-workers subsequently used this method to explore the *S*-palmitoylation of mitochondrial proteins and additionally the *N*-myristoylation of proteins during apoptosis.[65,66]



Scheme 17. ω-Azido fatty acids, which are efficiently metabolized by mammalian cells.

Tate and co-workers extended the scope of this method by developing a general protocol for tagging recombinant proteins. In their approach, a recombinant protein was co-expressed with the myristoyl-CoA protein *N*-myristoyl transferase (NMT) in *E. coli* followed by direct, co-translational labeling. NMT catalyzes the constitutive co-translational *N*-myristoylation, which occurs in all eukaryotic cells, by transferring myristic acid (**58**) from coenzyme A to the N-terminal glycine residue of a protein. Initial experiments demonstrated the tolerance of the NMT from *Candida albicans* towards tetradec-13-ynoic acid (**61**) and ω-azido fatty acid **62**. Tate and co-workers later showed that a recombinant protein with an N-terminal myristoylation motif comprised of the sequence GXXXS (X = any amino acid) could efficiently be acylated with one of the bio-orthogonal reporters **61** or **62** by co-expression with the NMT from *Candida albicans* (Figure 3). No additional synthesis or purification steps were required since the formation of the acyl-CoA thioester and the protein labeling both occurred co-translationally.[67]

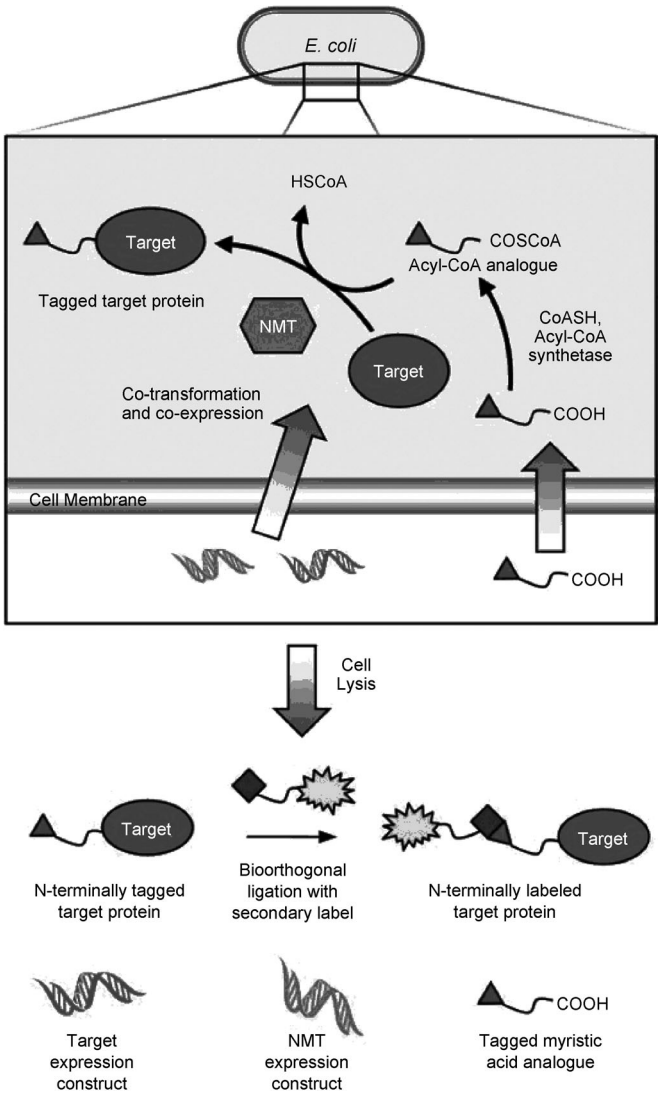
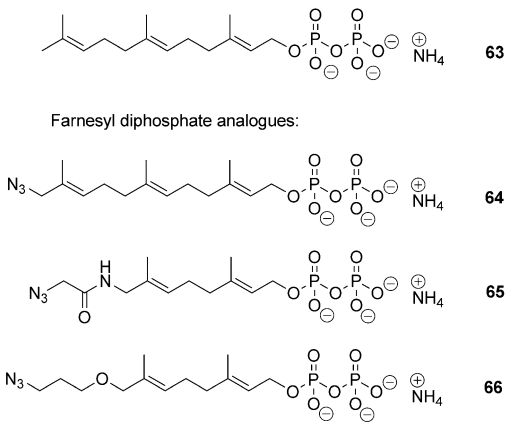


Figure 3. Overview of *E. coli* co-expression system for the *N*-myristoylation of proteins followed by bio-orthogonal Staudinger ligation. Adapted from Ref. [67a].

3.3.2. Profiling Prenylation

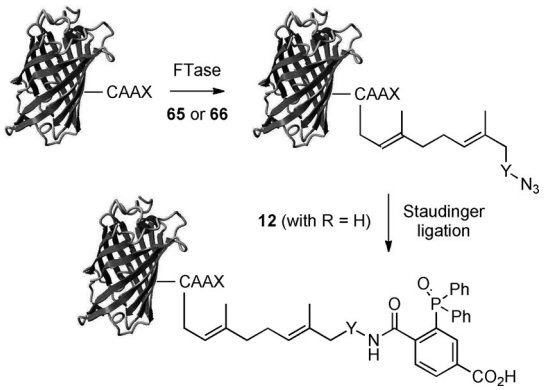
Similar to ω-azido fatty acids, azide-bearing farnesyl analogues have been designed for prenylation, the third major class of lipidation. In 2004, Zhao and co-workers demonstrated the in vitro use of azidofarnesyl diphosphate (**64**, Scheme 18) for the proteomic analysis of farnesylated



Scheme 18. Natural farnesyl diphosphate (**63**) and azidofarnesyl diphosphate derivatives **64**–**66**.

proteins.[68] In the first step, **64** was successfully conjugated to proteins targeted specifically by farnesyltransferase (FTase). This protein-modifying enzyme covalently couples a farnesyl group to the cysteine residue in the CAAX sequence (C is cysteine, A is an aliphatic amino acid, and X can be a variety of amino acids) near the C terminus. After lipidation, the biotinylated Staudinger probe **38** was used to tag the farnesylated proteins for subsequent detection.[68]

Consecutively, Waldmann and co-workers performed an in-depth study on the substrate tolerance of FTase for site-specific protein modification.[69] They showed that several azide-bearing analogues of farnesyl pyrophosphate **64** (Scheme 18) could successfully be conjugated by FTase to the cysteine residue in the CAAX-recognition sequence. After conjugation of an azide-containing farnesyl derivative to a protein, it was subjected to phosphine probe **12** (Scheme 19). The coupling of the two fragments occurred within several hours and was verified by mass spectrometry.



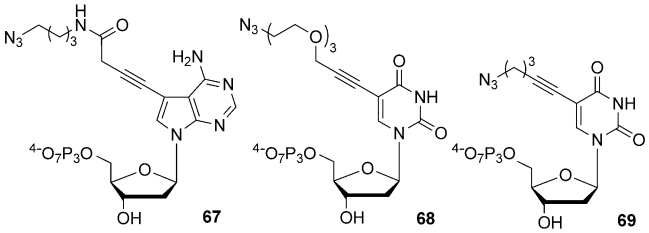
Scheme 19. Prenylation of a protein with the CAAX recognition sequence followed by Staudinger ligation. For linker moieties see **64**–**66** in Scheme 18.

3.4. DNA Labeling

Besides the applications in glycan and protein modification, the Staudinger ligation has also been utilized successfully in the conjugation of small molecules to DNA. The labeling of DNA is of great value to DNA-based molecular diagnostics and in the field of nanotechnology. Methods which are frequently employed in DNA modification are the incorporation of azide-modified nucleotides into the DNA strands, and the incorporation of a chemical reporter by enzymatic modification of the DNA.

3.4.1. Non-Natural Nucleic Acids

The utilization of chemical reporters in the selective labeling of DNA requires the functionalization of nucleic acids with a bio-orthogonal functional handle. Both acetylene- and azide-containing analogues of thymidine have been developed and successfully applied to generate uniformly labeled DNA. Recognizing the potential of using modified nucleic acid building blocks, Weisbrod and Marx synthesized azide-containing nucleic acids for the modification of DNA by means of the Staudinger ligation. Hence, the azido-2′-deoxyadenosine-5′-*O*-triphosphate (dATP) analogue **67** was synthesized and subsequently enzymatically incorporated by the DNA polymerase from *Pyrococcus woesei* (*Pwo*).[70] In addition, two other azide-modified 2′-deoxythymidine-5′-*O*-triphosphate (dTTP) analogues (**68** and **69**, Scheme 20) were



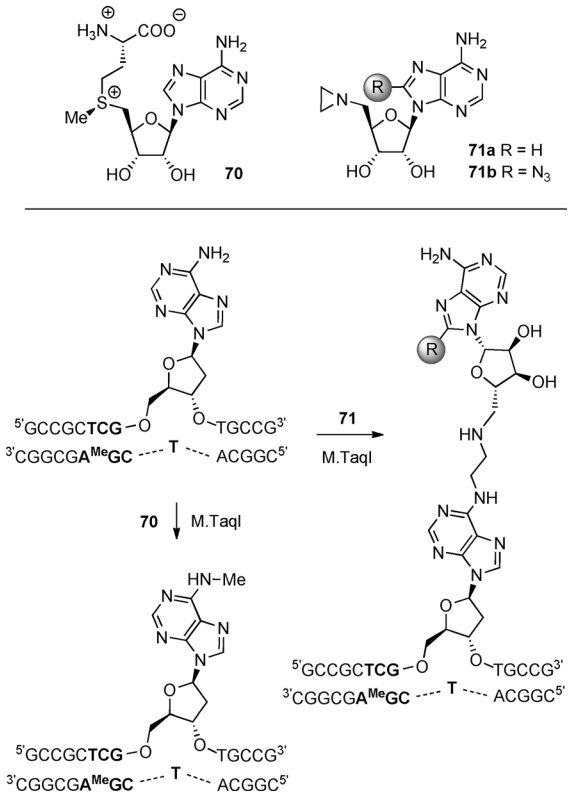
Scheme 20. Structure of azido-dATP analogue **67** and azido-dTTP analogues **68** and **69**.

designed, which were also accepted by the DNA polymerase. After incorporation of these azide-modified building blocks, the Staudinger ligation was successfully applied to the DNA fragments.[71]

3.4.2. DNA-Modifying Enzymes

DNA-modifying enzymes have been used on several occasions for the selective labeling of DNA. The activity of these enzymes, for example, *S*-adenosyl-L-methionine-dependent methyltransferase, was found to be affected by the

change of cofactor.[71] In nature, the methyl group of cofactor *S*-adenosyl-L-methionine (**70**) is transferred to the exocyclic amino group of 2′-deoxyadenosine within the double-stranded 5′-TCGA-3’ DNA sequence (Scheme 21). Such a



Scheme 21. Methyltransferase (M.TaqI) catalyzes the methylation of DNA at a recognition site and can be used as an azidonucleosidyl transferase in combination with cofactor **71b**. Bold letters indicate the M.Taq1 recognition sequence.

transfer reaction also occurred when *N*-adenosylaziridine (**71a**) was used instead of **70**, as was demonstrated by Comstock and Rajski.[72]

After transformation of cofactor **71a** into azide-containing compound **71b**, DNA could elegantly be labeled with azides (Scheme 21). After the site-specific labeling of the DNA, it could be biotinylated through the Staudinger ligation and accordingly analyzed.[73] In the follow-up study, Comstock and Rajski expanded this method to a methyltransferase-directed DNA strand scission upon Staudinger ligation with a copper-complexing phosphine ligand.[74]

By following a facile cellular RNA-detection strategy, Franzini and Kool designed a probe suitable for templated fluorescence activation.[75] The so-called “quenched Staudinger-triggered α-azidoether release” (Q-STAR) probes are fluorophore-containing DNA probes containing a quencher unit, which is attached through an α-azidoether linker (Figure 4). Reduction of the azide moiety with triphenylphosphine releases the quencher from the DNA strand, thereby resulting in the reinstatement of the fluorescence. As a result of the favorable kinetics and high bio-orthogonality of

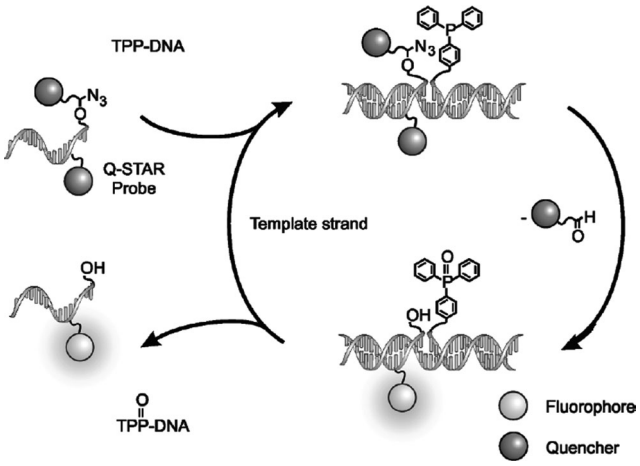


Figure 4. Principle of the quenched Staudinger-triggered α-azidoether release (Q-STAR) process. Reproduced from Ref. [75].

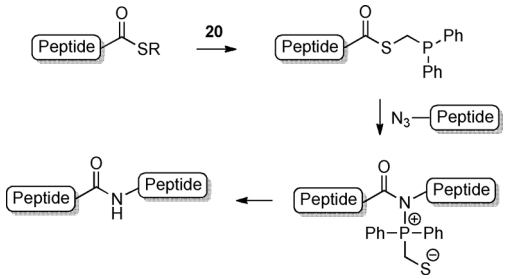
the templated Staudinger reduction, this method could successfully be applied in the sequence-selective detection of nucleic acids both in vitro and directly in prokaryotic cells.

4. The Staudinger Ligation as a Synthetic Method

4.1. Peptide and Cyclic Peptide Conjugates

An excellent example of a highly selective reaction for the assembly of polypeptide structures is native chemical ligation, first discovered by Wieland et al.[76] and further developed by Kent and co-workers.[77] This chemoselective reaction allows the coupling of two unprotected peptide fragments under physiological conditions to produce a single product in high yield. Not surprisingly, native chemical ligation is one of the most frequently utilized methods for the ligation of peptide fragments to produce synthetic proteins.[2,78] The basic principle of this ligation technique is the reaction between a C-terminal thioester of one peptide fragment and an N-terminal cysteine residue of another peptide fragment. A limitation of this reaction is the requirement of a cysteine residue at the ligation junction. In addition, free cysteine residues are relatively uncommon in proteins, where they are mainly found in the form of disulfide bridges. Moreover, modern peptide synthesis is limited to peptides containing up to 50 residues, thus excluding the complete chemical synthesis of a protein.[25,27] Raines and co-workers recognized the potential of the Staudinger ligation to form peptide bonds from an azide and a thioester, thus enabling the synthesis of long peptide fragments (Scheme 22).

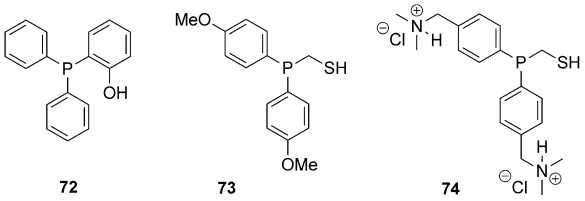
The principle of polypeptide synthesis using the traceless Staudinger ligation was elegantly demonstrated in the coupling of various peptide fragments.[25,27] Further synthetic efforts resulted in the complete build-up of the RNase A protein by a combination of expressed protein ligation (EPL) and the traceless Staudinger ligation. In the first step, two peptide fragments, obtained by solid-phase peptide synthesis, were coupled through the Staudinger ligation. The obtained



Scheme 22. Traceless Staudinger ligation for peptide conjugation.

product was subsequently ligated through native chemical ligation to a peptide expressed in *E. coli*.[79]

The traceless Staudinger ligation for peptide ligation, as proposed initially, only proceeded in high yield for the formation of a dipeptide with a glycine residue at the junction by using phosphine **20**. Liskamp and co-workers devised a route that utilized *ortho*-2-(diphenylphosphine)phenol (**72**, Scheme 23) instead of phosphine **20** to form esters instead of

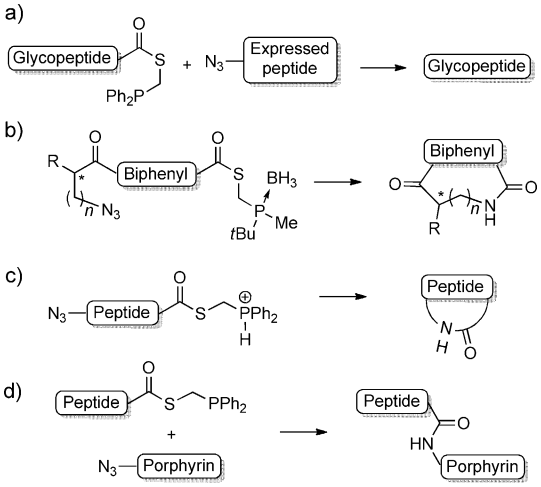


Scheme 23. Staudinger probes applied for the traceless Staudinger ligation of peptides.

thioesters, thereby eliminating these disadvantages.[80] They demonstrated the traceless Staudinger ligation-mediated synthesis of tetra- and pentapeptides. However, the use of phosphine **72** for peptide ligation still suffered from low yields. Later, Raines and co-workers showed the thioester phosphine compatibility with non-glycyl amino acid residues by properly tuning the electron density on the phosphorus atom, thereby resulting in the *p*-methoxy-substituted diphenyl phosphinomethanethiol **73**.[81] The Staudinger ligation with phosphine **73** gave the desired product with high chemoselectivity, that is, minimal nonspecific aminolysis, and high yield (>80%). Moreover, water-soluble phosphonium salt **74** (Scheme 23) was developed by the Raines research group to enable ligation under physiological conditions. The compatibility of **74** was elegantly demonstrated by its use in expressed protein ligation.

It was shown that the phosphinothioester could be installed at the C terminus of bovine pancreatic ribonuclease (RNase A).[82] Recent investigations demonstrated that the introduction of cationic moieties on phosphine **72** or variation of the cationic group on phosphinothiol **74** significantly improved the ability of these phosphine probes to mediate the traceless Staudinger ligation in water.[83,84]

In recent years the traceless Staudinger ligation has found various applications in the field of peptide science.[3] For example, Liu et al. demonstrated the convergent coupling of glycopeptides to recombinantly expressed peptide segments by constructing glycopolypeptides (Scheme 24a).[85] Another elegant example of peptide ligation is the intramolecular



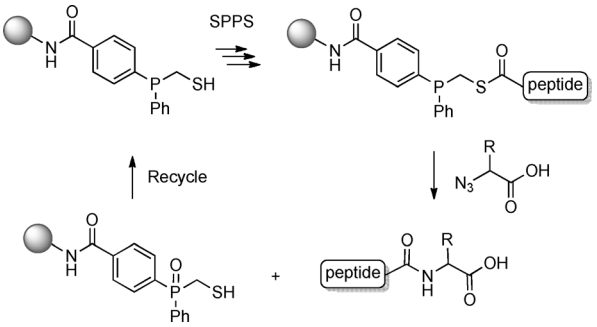
Scheme 24. The traceless Staudinger ligation applied in the construction of various peptide conjugates.

Staudinger cyclization introduced by van Maarseveen and coworkers.[86] They devised a strategy in which an air-stable borane-protected phosphinothiol auxiliary was used to generate biaryl-containing macrolactams (Scheme 24b). This method was subsequently used by Kleineweischede et al., who showed that the Staudinger-based peptide ligation could be performed in the presence and absence of protecting groups on the peptide side chain by making use of masked phosphinothioesters.[87] The authors obtained cyclic peptides in good yields under mild reaction conditions by using either protonated or borane-protected phosphinothiol (Scheme 24c). The application of either the Staudinger ligation or the copper(I)-catalyzed azide–alkyne reaction to azide-functionalized porphyrins and peptide thioesters yielded various peptide–porphyrin conjugates in good yields (Scheme 24d).[88]

To circumvent the troublesome purification often encountered after peptide synthesis using the Staudinger ligation, Kim et al. developed a solid-phase Staudinger ligation strategy for the preparation of small peptides (Scheme 25).[89] The Staudinger ligation could successfully be performed with a modified HiCore resin. After regeneration, the solid support could be reused up to five times without a loss of performance.

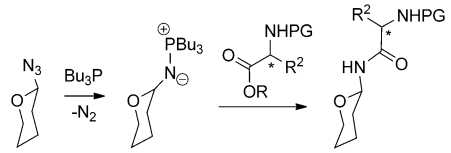
4.2. Glycopeptide Conjugates

The undisputed importance of glycopeptides and glycoproteins in biological systems challenged chemists to develop efficient methods for the coupling of glycosyl moieties to amino acids. Here, the traceless Staudinger ligation also proved to be a useful method for constructing glycoamino acids (see below) and conjugates of carbohydrate haptens to



Scheme 25. Solid-phase peptide synthesis (SPPS) based on the Staudinger ligation.

immunogenic protein carriers.[90] Davis and co-workers introduced the three-component Staudinger ligation for the preparation of *N*-linked glycoamino acids (Scheme 26).[91]

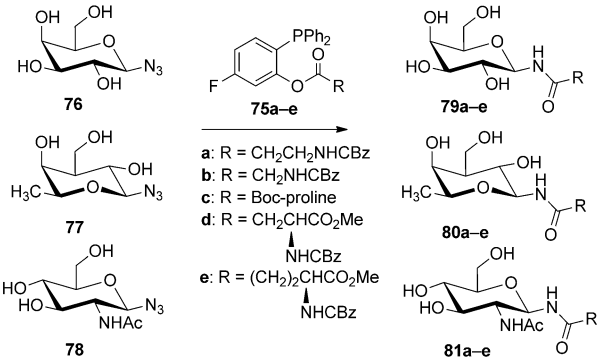


Scheme 26. Three-component Staudinger ligation in the synthesis of glycoamino acids. PG = protecting group.

This method could be applied to a range of carbohydrates and was compatible with different peptide fragments, thus allowing the build-up of biologically relevant N-linked glycopeptides. However, the use of trialkyl phosphine compounds (e.g. PBu_3) has been shown to possibly mediate a contraction reaction, in which a single sulfur atom is lost from a disulfide glycoprotein to give the corresponding S-linked glycoprotein.[92] Nonetheless, the research groups of Green and Davis applied the three component Staudinger ligation in a combined effort to prepare carbohydrate-decorated single-walled carbon nanotubes (glycol-SWNTs).[93] As a consequence of the difficulties associated with the detection of these functionalized nanostructures, the authors used iodine-tagged carbohydrates to demonstrate the successful functionalization of the SWNTs.

Recently, Lindhorst and co-workers applied this three-component Staudinger ligation to access amide-linked glycomimetics such as *N*-mannosyloxyethyl- and *N*-glycosyloctanamide amino acids as well as trivalent glycocluster amino acids.[94] The three-component Staudinger ligation was also successfully applied by Burés et al. for the synthesis of various dipeptides.[95] Deviating from the Davis protocol, this particular method makes use of catalytic amounts of PhSeSePh for the activation of the carboxylic acid.

Bernardi and co-workers investigated the ligation of amino acids to unprotected α- and β-glycosylazides by using fluorinated phosphine esters **75a–e** (Scheme 27).[96] First, 2-(diphenylphosphino)-5-fluorophenol was coupled to the carboxylic acid moiety of an α- or β-amino acid. In a second step, the Staudinger ligation was performed with unprotected α- and β-glycosylazides **76–78** to afford glycosyl amino acids (i.e. **79a–e**, **80a–e**, and **81a–e**) in good yields. This method works particularly well for unprotected β-azides of the *gluco*, *galacto*, and *fuco* series, while α-glucosylazides and modified GluNAc perform significantly less.

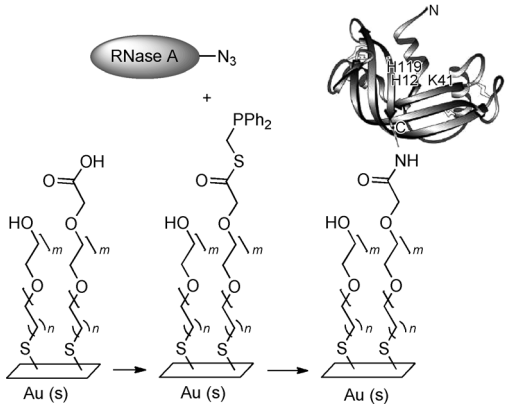


Scheme 27. Three-component Staudinger ligation in the synthesis of glycoamino acids. Boc = *tert*-butoxycarbonyl.

4.3. Surface Modifications

The growing interest in the site-specific immobilization of peptides and proteins on surfaces is clearly demonstrated by the increasing number of publications that have appeared in recent years.[97] Not surprisingly, the Staudinger ligation has played a pivotal role in the covalent attachment of biomolecules to surfaces.[98] The first examples of surface modification using the Staudinger ligation were simultaneously reported by Raines and co-workers[99] and the research groups of Breinbauer, Niemeyer, and Waldmann.[100]

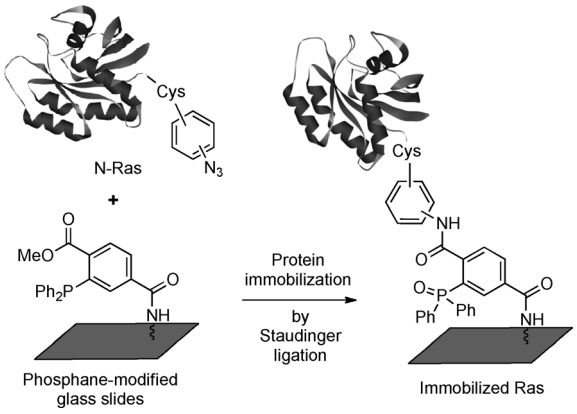
Both examples demonstrated the power of the traceless Staudinger ligation in the immobilization of peptides and proteins on glass surfaces. More recently, Raines and co-workers demonstrated the site-specific immobilization of RNase A on phosphinothioester-containing gold surfaces (Scheme 28).[101] The covalent attachment was verified by an



Scheme 28. Site-specific immobilization of azido-modified RNase A on a phosphino-thioester-coated gold surface. Modified from Ref. [101].

anti-RNase A rabbit primary antibody immunoassay and ellipsometric measurements.

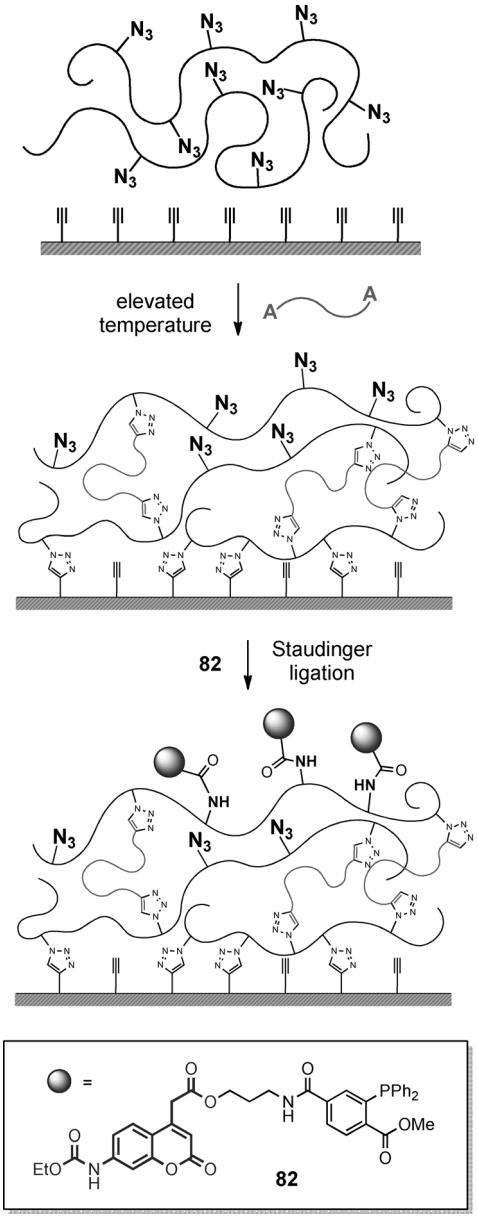
In a combined effort, the research groups of Waldmann and Goody performed site-specific immobilization of an azide-tagged Ras protein on a phosphine-functionalized glass surface.[102] The modified Ras protein was obtained by expressed protein ligation, whereas the phosphine moiety was introduced by the coupling of peptides to glass slides coated with a poly(amidoamine) (PAMAM) dendrimer. The Staudinger ligation was subsequently applied to immobilize the Ras protein onto the glass surface in a site-specific manner (Scheme 29). Combining the strategy of post-translational



Scheme 29. Site-specific immobilization of Ras protein on a phosphine-modified surface. Adapted from Ref. [102].

protein modification and phosphine-modified surface immobilization, Gauchet et al. demonstrated the regio- and chemoselective immobilization of proteins on glass surfaces.[103] Based on the farnesyltransferase assay of Zhao and co-workers,[68] an azidofarnesyl derivative was post-translationally incorporated to obtain azido-GFP and azido-GST (GFP = green fluorescent protein, GST = glutathione S-transferase), while phosphine-derivatized glass slides for Staudinger ligations were prepared according to the procedure described by Raines and co-workers.[99]

More recently, van Hest and co-workers described an approach for the copper-free construction of azide-containing surfaces for the immobilization of functional molecules.[104] Thermal cross-linking of azide-containing polymers and appropriate cross-linkers led to coatings that were suitable for further modification. In a consecutive step, the reactivity of the coatings towards different fluorescently labeled probes was tested (including several reactive compounds depicted in Scheme 1). The reactivity of the Staudinger probe **82** (Scheme 30) towards surface modification was found to be modest compared to the dibenzocyclooctyne and oxanorbornadiene counterparts.

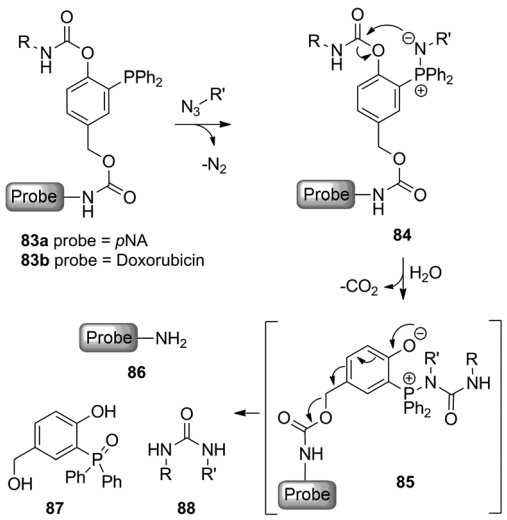


Scheme 30. Synthesis of functional, azide-containing, coatings. The azide content was determined by fluorescently labeled Staudinger probe **82**.

4.4. Biomedical Applications

An interesting biomedical application of the Staudinger ligation was reported by Florent and co-workers, who explored the use of the Staudinger ligation as a trigger for drug release.[105] For this purpose they developed phosphine probes **83** with a modified electrophilic trap that can act as a potential prodrug release system. The release of 4-nitroaniline (*p*NA, **86a**) was studied in a model reaction, thereby demonstrating the efficiency of the release mechanism. In a subsequent study, the release of the anticancer agent doxorubicin was monitored (Scheme 31). Treatment of the prodrug **83b** with an azide led to the formation of aza-ylide **84**. An intramolecular rearrangement afforded phenol anion intermediate **85**, which led to a 1,6-quinone methide rearrangement that released the active substance (doxorubicin, **86b**).

In another biomedical related example, Zhang et al., demonstrated the efficient and chemoselective surface func-



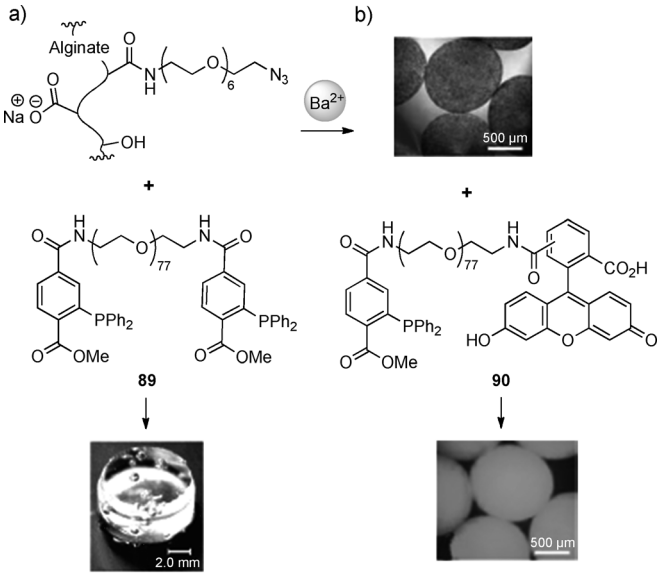
Scheme 31. Drug release triggered by Staudinger ligation.

tionalization of liposomes by using the Staudinger ligation.[106] A phosphine-labeled liposome surface was treated with azidolactose under biocompatible reaction conditions, thereby generating a glycoliposome suitable for lectin binding. The Staudinger ligation was found to be an attractive alternative to all of the current protocols for the surface functionalization of liposomes. Related to the functionalization of nanosized liposomes is the modification of viral coat proteins by means of the Staudinger ligation.[45] Site-specific incorporation of the non-natural amino acid *p*-azidophenylalanine into the coat protein of the Z domain of a phage system could be utilized in the Staudinger ligation for reaction with fluorescein-tethered phosphines. The ligation occurred with high efficiency, without affecting the phage system. Moreover, the build-up of a nonviral gene-delivery system has been pursued by making use of the Staudinger ligation.[107] To this end, the formation of discrete nanoparticles by complexation of a DNA nucleotide with a cationic polyamidoamine was studied. For improved cell uptake, the azide-containing cationic polymer was treated with a phosphine-containing RGD peptide both before and after formation of the particles. Complexation after formation was found to be superior and gave more stable particles.

4.5. Polymer-Based Staudinger Ligation

Besides the numerous applications of the Staudinger ligation in chemical biology, an increasing number of publications report the use of this conjugation strategy with biomacromolecular materials. Recently, the Staudinger ligation was elegantly applied in the cross-linking and functionalization of alginate. This biocompatible hydrogel-forming material has been used as a cell-stabilizing matrix and as a biosensor coating.

Alginate is able to form hydrogels upon treatment with multivalent cations (e.g. Ba^{2+} or Ca^{2+}). Gattás-Asfura and Stabler demonstrated that the modification of alginate with an azide-functionalized ethylene glycol oligomer, followed by cross-linking with phosphine-containing PEG cross-linker **89**, also resulted in the formation of stable hydrogels (Scheme 32a).[108] In addition, the authors showed that azide-modified alginate was still able to form hydrogels upon treatment with Ba^{2+} ions and could be efficiently visualized upon Staudinger ligation with a carboxyfluorescein-labeled phosphine tag **90** (Scheme 32b).

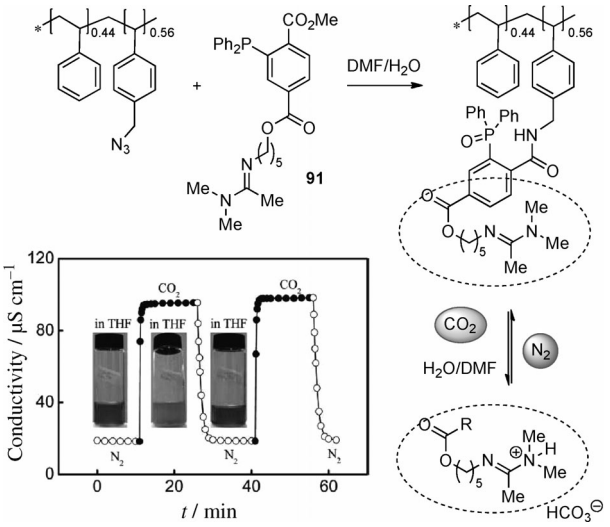


Scheme 32. a) Formation of a hydrogel from azido-alginate by Staudinger-mediated PEG cross-linking. b) Formation of a hydrogel using Ba^{2+} ions followed by fluorescent labeling. Reproduced from Ref. [108] with permission.

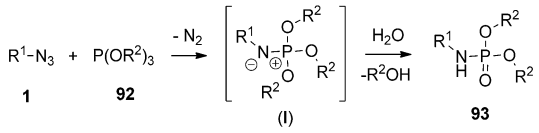
Another interesting application in the field of polymer science was reported by Zhou et al., who created a neutral-charged-neutral switchable polymer, based on the formation of amidinium cations upon treatment of an aqueous amidine-containing polymer solution with CO_2 gas (Scheme 33).[109] To this end, an amidine motif was attached to a PAMS-*co*-PS (PAMS = poly(*p*-azidomethylstryrene), PS = polystyrene) copolymer by making use of the Staudinger ligation. Of the different methods applied for the incorporation of the amidine function onto the copolymer, the Staudinger ligation with phosphine **91** was found to be the most suitable. The obtained functional polymer could be reversibly switched between a neutral and a charged state in solution by alternatively bubbling through either CO_2 or N_2 gas (insert Scheme 33).

5. Novel Synthetic Concepts

Recently, Hackenberger and co-workers introduced the Staudinger-phosphite reaction for the chemoselective modification of proteins.[110] This reaction, first described by Kabachnik and Gilyarov,[111] is based on a modified Staudinger reaction, in which a phosphoramidate **93** is formed in a two-step process between a phosphite **92** and an azide **1** (Scheme 34). Having demonstrated the concept of the



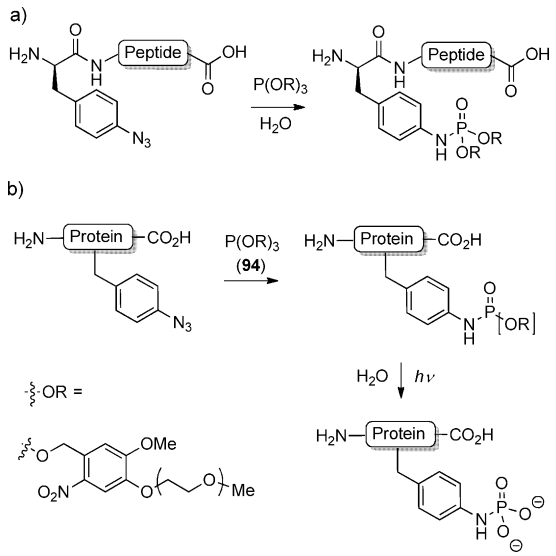
Scheme 33. Synthesis and application of switchable polymers upon treatment with CO_2 or N_2. Reproduced from Ref. [109] with permission.



Scheme 34. Staudinger-phosphite reaction between a phosphite and an azide.

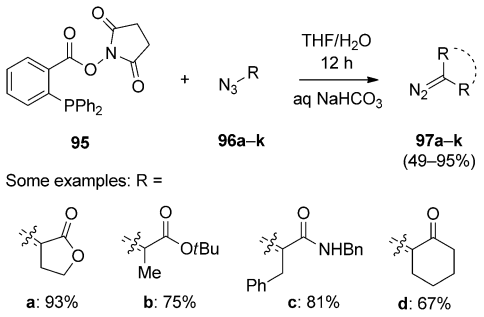
Staudinger-phosphite reaction, a chemoselective modification of azide-containing peptides and proteins was pursued (Scheme 35a,b).

In one example (Scheme 35b), the authors demonstrated the applicability of the Staudinger-phosphite reaction in the selective phosphorylation of model protein SecB. The use of photocleavable phosphite **94** enabled quantitative phosphorylation to be achieved upon irradiation of the protein, which resulted in the in situ hydrolysis of the phosphoramidate. This concept was further explored by using unsymmetrical phosphites.[46] Although the synthesis of unsymmetrical phosphite reagents was accomplished successfully, their application gave mixtures of phosphoramidites. Nevertheless, the biotinylation of a model azido-protein was pursued, which resulted in sufficient transformation into the desired product, as was shown by Western blot analysis. More recently, Hackenberger and co-workers demonstrated the efficient PEGylation of an azide-containing protein.[112] By using symmetrical PEG phosphites, the Staudinger-phosphite reaction could be successfully conducted on *E. coli* lysate. Moreover, the same research group applied the Staudinger-phosphite method to the solid-phase synthesis of phosphoramidate-containing peptides.[113] This method was extended towards the synthesis of phosphoramidate-linked glycopeptides.[114] The strategy involved a two-step approach in which serine-containing peptides were first converted into phosphite peptides by phosphitylation with phosphoramidites. In a second step the phosphite peptides were treated with glycosylazides, thereby generating phosphoramidate-linked glycopeptides. However, cleavage from the solid support with trifluoroacetic acid was accompanied by the formation of several by-products, including phosphotriesters and dehydroalanine residues.



Scheme 35. Applications of the Staudinger-phosphite reaction.

Moving away from the Staudinger ligation, Myers and Raines demonstrated the applicability of Staudinger-type phosphines to produce diazo compounds upon reaction with azides.[115] The importance of the diazo compounds in organic synthesis is demonstrated by the variety of reactions in which this type of compounds is being used, for example, cyclopropanations, ylid formation, and acid-catalyzed cyclization reactions. Myers and Raines found that the use of phosphine-*N*-hydroxysuccinimidyl ester **95**, a highly reactive acylating agent, promoted the formations of acyltriazene compounds upon treatment with various azides (**96a–k**). In a subsequent de-imidogenation reaction, the corresponding diazo compounds (**97a–k**) could be obtained in excellent yields (Scheme 36). This protocol is expected to have a significant impact in the field of synthetic organic chemistry as it produces diazo compounds under mild reaction conditions starting from readily available starting materials.



Scheme 36. Phosphine-mediated conversion of azides into diazo compounds.

6. Conclusions and Outlook

Bio-orthogonal conjugation techniques are of general interest for both biologists and chemists. This relatively new field in chemistry is expanding rapidly, and has found widespread application in the field of systems biology. Moreover, new conjugation strategies allow for the modification of complex biological targets which could not be investigated with previously existing techniques.[116] The Staudinger ligation was introduced as one of the first reactions to meet the criteria of bio-orthogonal ligation chemistry.[6] Consequently, the Staudinger ligation has proven itself particularly useful in the labeling of biomolecules in their native environment. Besides use as an efficient labeling agent, the Staudinger ligation has demonstrated to be a proficient method for surface modification and the construction of polypeptides, glycopeptides, and glycoproteins.

Although the traceless Staudinger reaction has received significant attention over the last years, it is not as frequently applied as its nontraceless counterpart due to the problems encountered with its orthogonality with various nucleophiles. These selectivity issues were addressed by Liskamp and coworkers, who demonstrated that nonspecific aminolysis takes place with unprotected peptides in the case of pentapeptide formation.[80] Moreover, steric hindrance at the ligation junction (as in nonglycyl coupling reactions) significantly diminishes the yield of the ligation. Finally, two structural drawbacks of the traceless Staudinger probe can be distinguished. Firstly the electron-rich phosphine is prone to undergo oxidation,[117] and secondly the less-stabile thioesters are not always synthetically easy to access.

Besides the Staudinger ligation, other strategies for selective and controlled conjugation have recently been developed. In 2005 the copper-catalyzed 1,3-dipolar cycloaddition between an azide and an alkyne was introduced as a novel bioconjugation method. The development and applications of this copper-catalyzed cycloaddition reaction has been reviewed on several occasions.[8,118] Both unique chemical handles (azides and alkynes) can readily be introduced in vivo and can still be used for conjugation purposes after cell lysis. In many of the above described applications of the Staudinger ligation, the conjugation was performed on the cell lysate and could, therefore, also be replaced by the copper-catalyzed azide–alkyne cycloaddition (CuAAC). The main advantages of CuAAC over the Staudinger ligation are the increased reaction rate and the possibility of using both the azide and the alkyne as chemical reporters. This reaction is, however, mainly effective for the in vitro conjugation of biomolecules, since copper is toxic to living systems.

To circumvent the use of copper, strain-promoted cycloaddition reactions have been developed as a new type of bio-orthogonal conjugation technique. These cycloaddition reactions require the use of an azide and a strained cyclic counterpart (e.g. oxanorbornadienes, cyclooctynes, and dibenzocyclooctynes).[5a] Several different types of cyclooctynes have been designed with various properties. Most promising are the difluorinated cyclooctynes (DIFO),[10c,d] the bicyclononyne (BCN),[119] dibenzocyclooctynols,[12] and the azadibenzocyclooctynes (DIBAC).[120] Besides the strain-promoted azide–alkyne cycloaddition (SPAAC), the cycloaddition of tetrazines and *trans*-cyclooctenes, which has been used to label live cancer cells, holds great potential.[121] The aforementioned cycloaddition reactions may be more favorable than the Staudinger ligation as a result of their higher reaction rates. Various reviews focusing on the development and the applications of these and other bioorthogonal approaches have recently appeared.[122]

Even though some of these emerging strategies have superior properties, the Staudinger ligation remains an interesting bioconjugation method and, because of its well-established position, is still frequently applied in the field of chemical biology. This well-established position is demonstrated by the commercial availability of the triarylphosphine–FLAG conjugate. Moreover, the Staudinger ligation sets a high standard to which most of the new bioconjugation techniques are often compared.[10b] This was illustrated by a recent head-to-head comparison of the nontraceless Staudinger probe and different cyclooctynes for the in vivo labeling of azide-containing splenocytes. Despite the inherent lower reactivity of the Staudinger probe towards azides, compared to the cyclooctynes, it was demonstrated to be the most robust labeling strategy.[123] The ideal bio-orthogonal conjugation method thus depends on the experimental set-up. When a well-established in vivo protocol is desired, the Staudinger ligation is a highly suitable method. Conversely, the strain-promoted cycloaddition reaction may be a more reliable technique for rapid ex vivo labeling of cells.[10c]

For all bio-orthogonal reactions it should be remembered that the native situation is modified by the introduction of a small, physiologically stable, non-native chemical reporter. This subtle change to the native environment may influence the structure and thereby the activity, localization, and stability of a biomolecule. Therefore, the quest for other bio-orthogonal and biomimetic chemical reporters that do not rely on the azide group as a chemical handle remains a continuous challenge.

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