Bioorganic Chemistry

The Staudinger Ligation—A Gift to Chemical Biology

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Although the reaction between an azide and a phosphane to form an aza-ylide was discovered by Hermann Staudinger more than 80 years ago and has found widespread application in organic synthesis, its potential as a highly chemoselective ligation method for the preparation of bioconjugates has been recognized only recently. As the two reaction partners are bioorthogonal to almost all functionalities that exist in biological systems and react at room temperature in an aqueous environment, the Staudinger ligation has even found application in the complex environment of living cells. Herein we describe the current state of knowledge on this reaction and its application both for the preparation of bioconjugates and as a ligation method in chemical biology.

1. Introduction

In their studies of biological systems molecular biologists and chemists are often faced with the need to link two molecular entities covalently, for example, to link a complex carbohydrate with a peptide or to attach a small molecular probe (such as a fluorescent dye, a radical probe, or an affinity tag) to a biopolymer. As biological systems are both rich in structural complexity and diverse in their functional reactivity, chemoselective ligation reactions have to be developed in which two mutually and uniquely reactive functional groups can be coupled, usually in an aqueous environment under physiological conditions. These uniquely reactive functional groups should be selective for one another and also tolerate a diverse array of other functionalities, thus rendering the use of protecting groups unnecessary and, in the ideal case, allowing its application in the complex environment of a living cell.

Although several bioconjugation techniques are available for the in vitro preparation of bioconjugates substituted with a limited number of functional groups,[1] truly chemoselective ligation reactions are rather limited. Most ligation reactions rely on the reaction of an electrophile with a nucleophile. As biological systems are rich in diverse electrophilic and nucleophilic sites, only a few functional groups are available that exhibit orthogonal reactivity to the functional groups present.[2] Recently, two reactions were introduced in which the azide moiety serves as a reactive functional group, with the following three advantages: 1) the azide moiety is absent in almost all naturally occurring compounds ("bioorthogonal"); 2) despite their high intrinsic reactivity, azides undergo a selective ligation with a very limited number of reaction partners; 3) the azide group is small and can be introduced into biological samples without altering the molecular size significantly. Whereas the "click-chemistry" reaction of Sharpless and co-workers ([3+2] cycloaddition between an azide and a terminal alkyne) requires the presence of a copper catalyst,[3] the Staudinger ligation introduced by Saxon and Bertozzi exploits the smooth reaction between an azide and a phosphane to form a phospha–aza-ylide. This ylide can be trapped by an acyl group with formation of a stable amide bond.[4] Herein we summarize recent applications of this chemoselective ligation method with two bioorthogonal groups.

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2. Staudinger Reaction

In 1919 Staudinger and Meyer reported that azides react smoothly with triaryl phosphanes to form iminophosphoranes (Scheme 1).[5] This imination reaction proceeds under mild conditions, almost quantitatively, and without noticeable formation of any side products. Over the last century detailed mechanistic studies have revealed that the mechanism of this reaction involves several intermediates (Scheme 2).[6] In a

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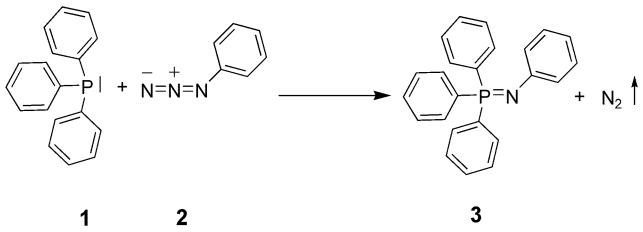
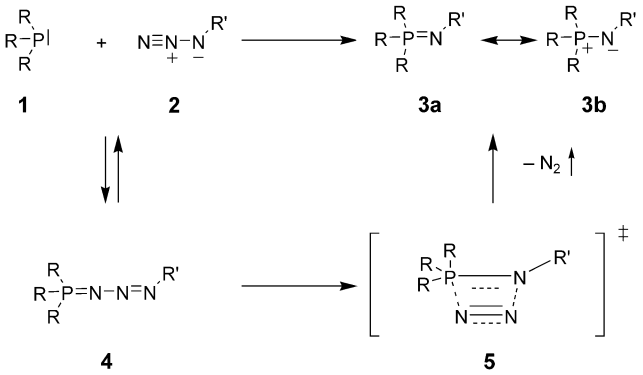


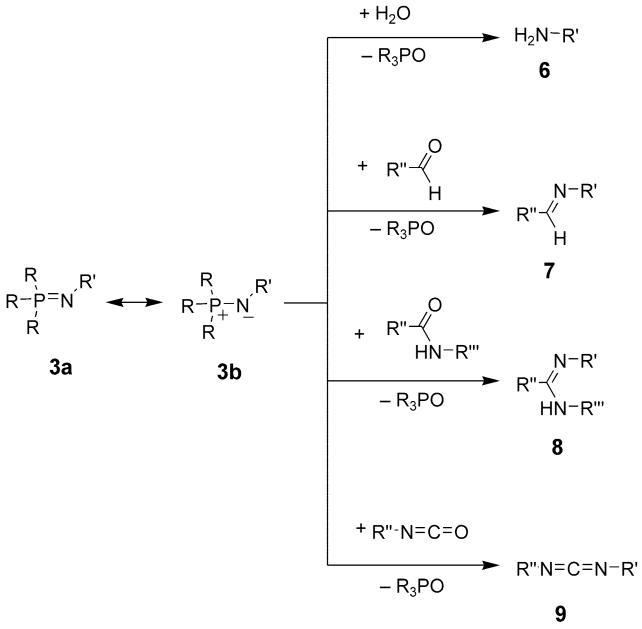
Scheme 1. Staudinger reaction between a phosphane and an azide to form an iminophosphorane.



Scheme 2. Mechanism of the Staudinger reaction.

primary imination reaction **1** and **2** react to form a phosphazide **4**, which, as a rule, decomposes during the reaction with loss of nitrogen. It is interesting that the rate of the formation of **4** is controlled only by the inductive properties of the groups attached to the phosphorus atom and the azide, and that no significant steric influence is observed. Phosphazides **4** are stable at room temperature in organic solvents if substituents are present that delocalize the positive charge on the phosphorus atom and/or provide steric shielding of the phosphorus atom. The subsequent loss of dinitrogen is thought to proceed via the 4-membered-ring transition state **5** with retention of the original configuration at phosphorus, without the participation of either free radicals or nitrenes.

The iminophosphorane **3**, with its highly nucleophilic nitrogen atom, can react with almost any kind of electrophilic reagent, thus resulting in many reactions of significant synthetic importance (Scheme 3).[6,7] If the Staudinger reaction is carried out in an aqueous solvent, the readily formed iminophosphorane **3** is hydrolyzed rapidly to generate the primary amine **6** and phosphane(v) oxide. The so-called Staudinger reduction is a frequently used method for the



Scheme 3. The aza-ylide **3b** can be trapped by a plethora of electrophiles.

smooth reduction of azides to amines. Staudinger himself discovered that **3** can react with aldehydes or ketones to form imines.[8] The synthetic value of the aza-Wittig reaction is reflected by the fact that it has found application in many total syntheses, for example, in syntheses of croomine, quinine, and dendrobine.[7] Less reactive carbonyl electrophiles, such as amides or esters, also undergo reaction with **3**, especially if the electrophilic attack proceeds in an intramolecular fashion. To name one example, the reaction of 2-azidobenzoyl chloride with a suitable amide is an excellent method for the construction of quinazolines (Eguchi protocol).

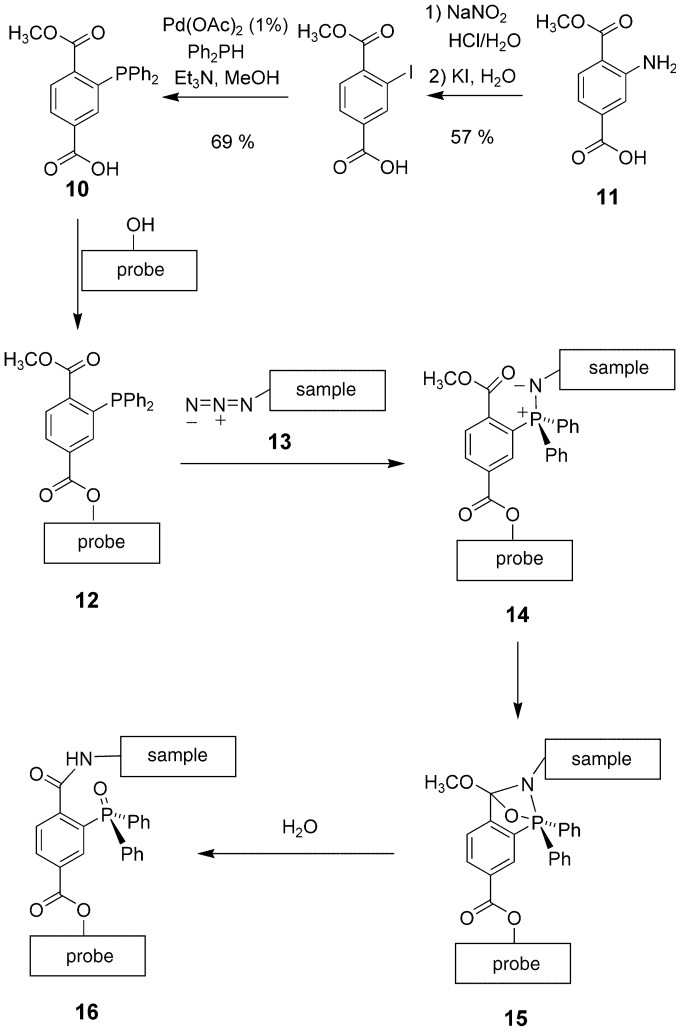
Born in Schärding (Austria) in 1970, Rolf Breinbauer studied chemistry in Vienna and Heidelberg. After his doctoral studies with Prof. M. T. Reetz at the Max-Planck-Institut für Kohlenforschung in Mülheim an der Ruhr (Germany) he went as an Erwin Schrödinger Fellow to Prof. E. N. Jacobsen at Harvard University. Since 2000 he has led a research group at the Max-Planck-Institut für molekulare Physiologie and the Institute of Organic Chemistry of the Universität Dortmund (Germany). His research interests include combinatorial chemistry and chemical biology. (Foto: Jürgen Huhn)

Maja Köhn was born in 1975 in Kiel (Germany), where she also studied chemistry. For her diploma project she carried out research on the derivatization of carbohydrates under the supervision of Prof. T. K. Lindhorst. As a visiting scholar at the CSIC in Seville (Spain) she worked with Dr. J. M. Garcia Fernandez on the synthesis of glycoclusters. Since 2001 she has been a graduate student in the Department of Chemical Biology (Prof. H. Waldmann) at the Max-Planck-Institut für molekulare Physiologie and the Universität Dortmund.

3. Staudinger Ligation

3.1. Nontraceless Staudinger Ligation

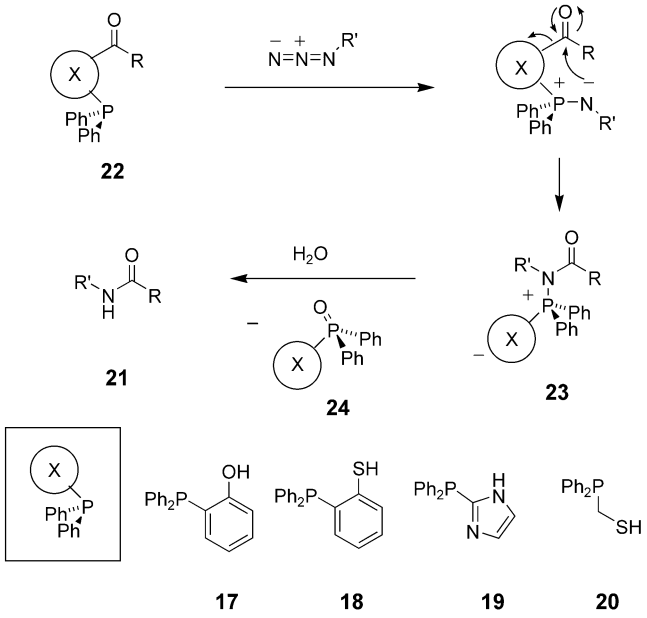
In their pioneering studies on the metabolic engineering of cell surfaces, Bertozzi and co-workers recognized the limitation of the hydrazone ligation method, in which conjugates are formed between a hydrazine probe and a ketone-modified sample.[2] Their search for a milder reaction of two truly bioorthogonal functionalities led them to the Staudinger reaction. As described above, the product of the reaction of an azide with a phosphane, the aza-ylide **3**, undergoes spontaneous hydrolysis to the amine and phosphane oxide in an aqueous environment. Saxon and Bertozzi designed the ligand **10** based on the rationale that an appropriately located electrophilic trap, such as an ester moiety, within the structure of the phosphane, would capture the nucleophilic aza-ylide **14** by intramolecular cyclization (Scheme 4).[4] This process would ultimately produce a stable amide bond before the competing aza-ylide hydrolysis could take place. The ligand **10**, which is not yet commercially available, can be synthesized readily from the aminoterephthalic acid ester **11** by diazotization, followed by iodination and subsequent Pd-catalyzed phosphanylation.[4,9] Standard esterification or amidation methods allow the attachment of the phosphane to the probe to form the conjugate **12**, which undergoes reaction in an aqueous solution with the azide **13**. Mechanistic studies by ^{31}P NMR spectroscopy identified the aza-ylide **14** and the oxaphosphetane **15** as intermediates in the ligation reaction.[10]



Scheme 4. Preparation of the phosphane **10** and its application in the Staudinger ligation.

3.2. Traceless Staudinger Ligation

Although the reaction detailed above works well in a biological environment, a modification in which an amide bond is formed between the two coupling partners to give a product without a triaryl phosphane oxide moiety appears even more attractive. Shortly after their first report, Bertozzi and co-workers[11] and—in a parallel effort—Raines and co-workers[12] reported a traceless Staudinger ligation, in which the phosphane oxide moiety is cleaved during the hydrolysis step (Scheme 5).



Scheme 5. Traceless Staudinger ligation.

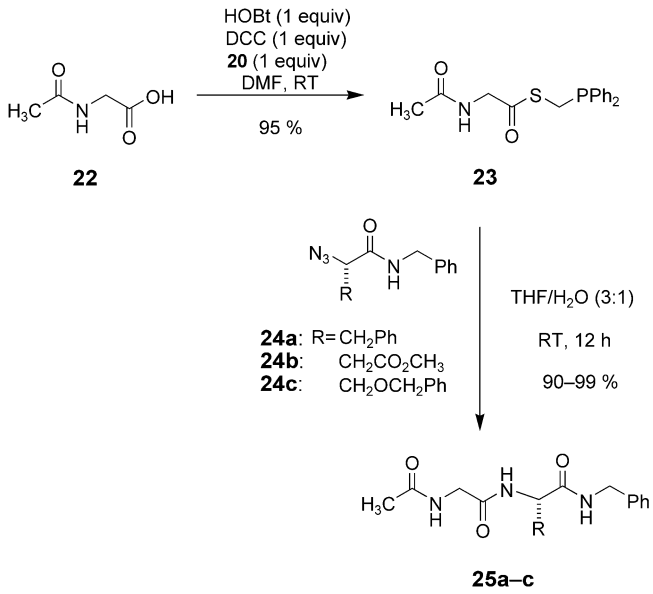
In this reaction, the phosphanes **17**–**20** are first acylated and then treated with the azide. The nucleophilic nitrogen atom of the aza-ylide then attacks the carbonyl group to cleave the linkage with the phosphonium species. Hydrolysis of the rearranged product **23** produces the amide **21** and liberates the phosphane oxide **24**. Among the phosphanes tested, 2-diphenylphosphanylphenol (**17**; readily prepared by the Pd-catalyzed reaction of 2-iodophenol with diphenylphosphane)[11] and diphenylphosphanylmethanethiol (**20**; now commercially available) exhibit the best reactivity profiles and have found widespread application, as will be detailed below. Because of its alkyl substituent, **20** is readily

oxidized in air and is best handled as an adduct with BH_3, from which it can be liberated by treatment with a powerful amine nucleophile, such as dabco (1,4-diazabicyclo[2.2.2]octane).

4. Applications

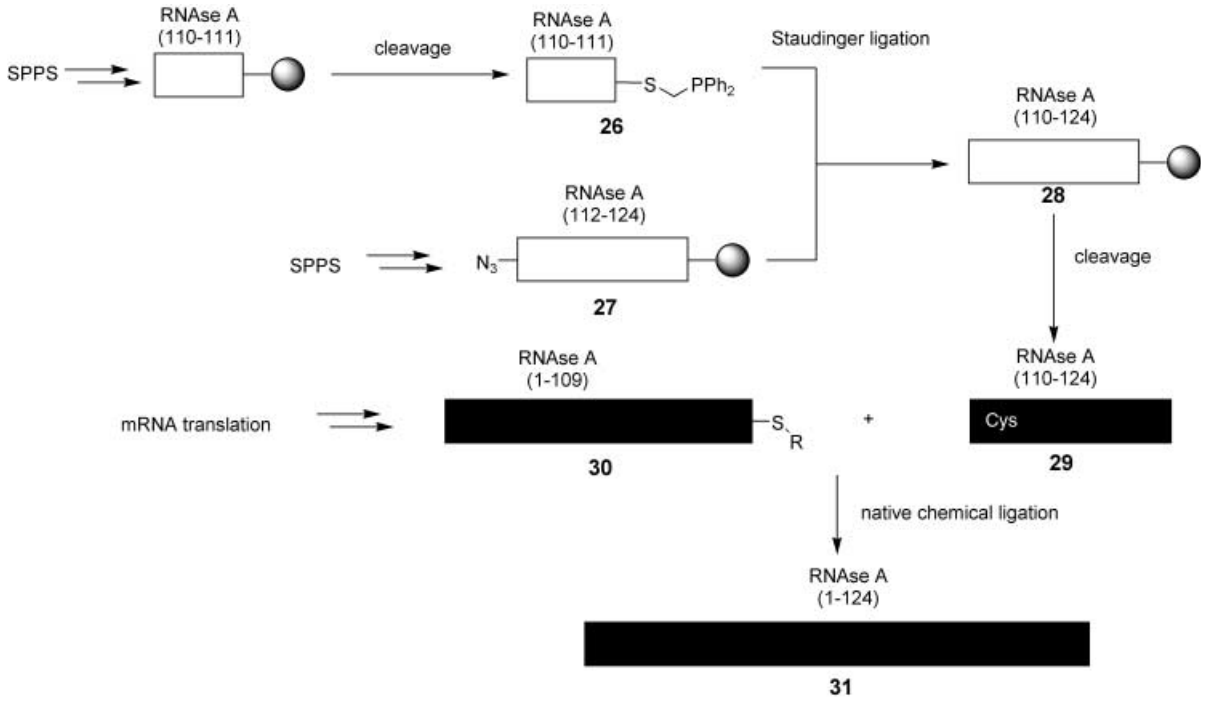
4.1. Peptide Ligation

The total synthesis of proteins requires chemoselective ligation methods in which shorter peptide fragments (which can be synthesized by solid-phase peptide synthesis) are assembled to give the final protein.[13] The most common ligation method, the native chemical ligation of Wieland et al. and Kent and co-workers, requires the presence of a Cys residue at the N terminus of the junction site.[14] Raines and co-workers have proposed the Staudinger ligation as a peptide ligation method which does not depend on the side chain present.[12b] In a proof-of-concept experiment they converted *N*-acetylglycine (**22**) into the thioester **23**, which reacted smoothly in aqueous THF with the protected azido amino acids **24a–c** to give the dipeptides **25a–c** in very good yields and without any detectable epimerization (Scheme 6). The required azido amino acids can be prepared quite simply from the corresponding amino acids by the diazo transfer procedure of Roberts and Wong.[15,16]



Scheme 6. Peptide formation through a traceless Staudinger ligation by Raines and co-workers.

In an impressive effort demonstrating the power of methods involving orthogonal chemical ligation, Raines and co-workers completed the total synthesis of ribonuclease A (**31**, RNAse A, 124 amino acids) by linking three fragments (Scheme 7).[17] RNAse A(110–111) was synthesized as the C-terminal phosphanyl thioester FmocCys(Trt)Glu(O*t*Bu)-SCH_2PPh_2 (**26**) by using a Kenner-type safety-catch linker. RNAse A(112–124) was synthesized as the N-terminal azide $N_3CH_2C(O)$Asn(Trt)ProTyr(*t*Bu)ValProValHis(Trt)PheAsp-(O*t*Bu)AlaSer(*t*Bu)Val (**27**) by a standard Fmoc strategy on a PEGA resin. The two peptide fragments were connected on the resin by a Staudinger ligation. After cleavage from the resin and deprotection of the acid-labile protecting groups, RNAse A(110–124) (**29**) was isolated in 61 % yield. The N-terminal Cys residue of this fragment allowed its attachment



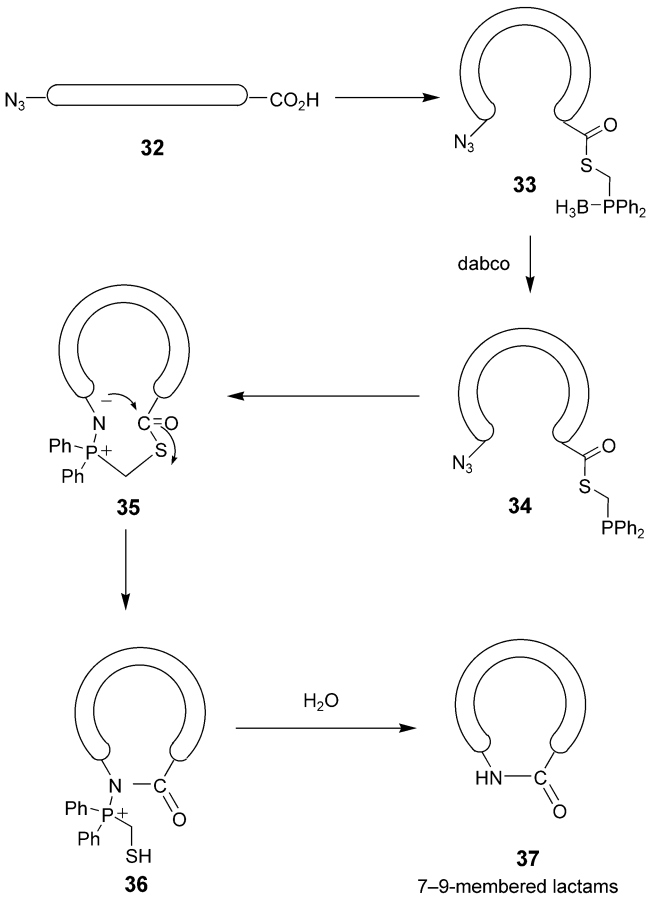
Scheme 7. Total synthesis of RNAse A with three different chemical ligation methods (Raines and co-workers).

by native chemical ligation to the RNAse A(1–109) thioester **30** produced biosynthetically. The resulting protein **31** proved after folding to be fully functional RNAse A.

This impressive result should not detract from the fact that the full scope and limitations of the Staudinger ligation in peptide chemistry have not yet been fully explored. Future efforts will be aimed at the ligation of more-complex peptide fragments, which should answer the question of whether or not side-chain protection is required.

Liskamp and co-workers have begun to address this issue by studying the ligation of peptide fragments in which no glycine residue is present at the junction site.[18] Starting from N-terminal α-azido peptides and C-terminal-peptide ortho-(diphenylphosphanyl)phenyl esters (readily prepared from the phosphane **17**) they prepared tetra- and pentapeptides in 6–36% yield under non-aqueous conditions. The coupling of an unprotected peptide containing a Lys residue not only delivered the desired product but also the product of non-specific aminolysis caused by nucleophilic attack of the ε-amino group of the Lys residue. Kinetic studies revealed that in all cases the formation of the aza-ylide intermediate proceeded very fast, thus suggesting that the slow formation of the ligation product might be a result of the increased steric congestion in the intermediates or the presence of amino acid side chains in the peptide fragments.

An interesting application of this amide-bond-forming reaction was disclosed by Maarseveen and co-workers, who present a solution to the problem of ring-closure to form medium-sized lactams.[19] The reaction sequence is outlined in Scheme 8. The acyclic azido carboxylic acid **32** is converted into the borane-protected phosphanyl thioester **33**. Upon deprotection with dabco the phosphorus atom regains its ability to act as a nucleophile. Nucleophilic attack at the azide occurs with formation of the aza-ylide **35**, and electrophilic trapping of the ylide by the thioester then reduces the ring size by three atoms. In this reaction the enthalpy gain associated with amide formation compensates for the large increase in steric strain accompanying the formation of medium-sized rings. Hydrolysis removes the phosphane auxiliary and furnishes the 7–9-membered lactams **37** in yields that are typically higher than those of traditional lactamization methods.

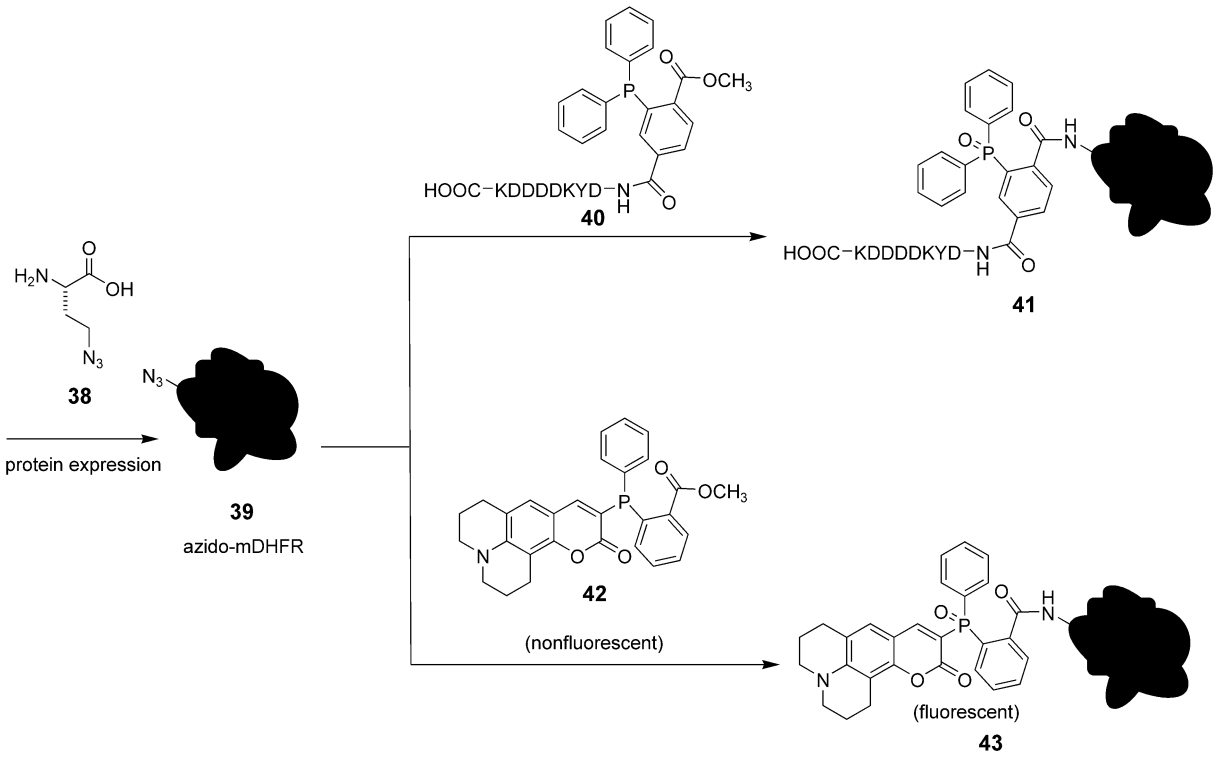


Scheme 8. A general strategy for the formation of 7–9-membered lactams.

4.2. Synthesis of Bioconjugates

At the heart of chemical biology is the preparation of bioconjugates in which a reporter group (dye, spin label, affinity tag, recognition motif) is attached to a biological sample or biopolymer.[1]

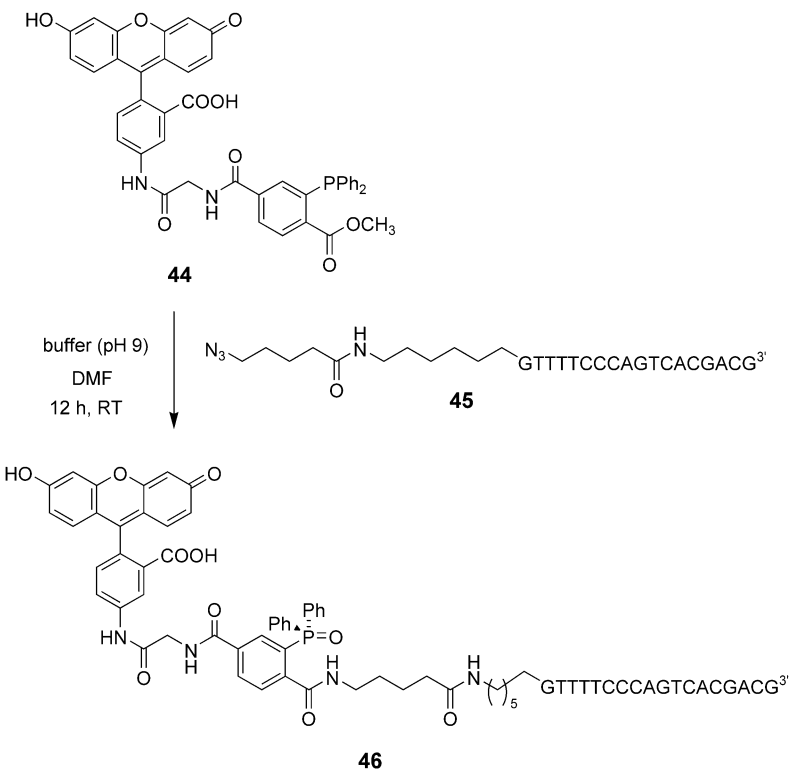
The research groups of Tirrell and Bertozzi have described a generally applicable strategy for the incorporation of azides into recombinant proteins for subsequent chemoselective modification by the Staudinger ligation.[20] Azidohomoalanine (**38**) is activated by the methionyl-tRNA synthetase (MetRS) of *Escherichia coli* and replaces methionine in proteins expressed in methionine-depleted bacterial cultures. In a proof-of-concept experiment murine dihydrofolate reductase (mDHFR) was expressed in an *E. coli* methionine auxotroph in a medium supplemented with azidohomoalanine (**38**). Amino acid analysis of the purified protein **39** indicated 95 ± 2% replacement of methionine. The purified protein was subjected to Staudinger ligation with two different probes (Scheme 9). Azido-mDHFR (**39**) was treated with the phosphane **40** (250 μM) containing the FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) in a buffer at 47 °C for 6 h. The labeled protein **41** was characterized either by incubation with a FITC anti-FLAG antibody (FITC = fluorescein isothiocyanate) or by tryptic digestion and MALDI-TOF analysis. Although the Staudinger ligation took place at several sites, exhaustive modification of all eight possible sites was not observed. Reduction of the azide of the azidohomoalanine residue had probably already taken place to a certain extent. Interestingly and importantly, the labeling process also functions with a crude cell lysate. To avoid a two-step labeling procedure, which would impose limitations on the use of real biological samples, Bertozzi and co-workers designed the fluorogenic coumarin phosphane dye **42**, which itself is nonfluorescent but is activated by the Staudinger ligation with azides.[21] The treatment of **42** with azido-mDHFR (**39**) resulted in the specific labeling of the azido protein. Unlike in the previous experiment with the FLAG conjugate, the labeled protein **43** could be observed directly, without the need for Western blotting, washing, or secondary labeling steps.



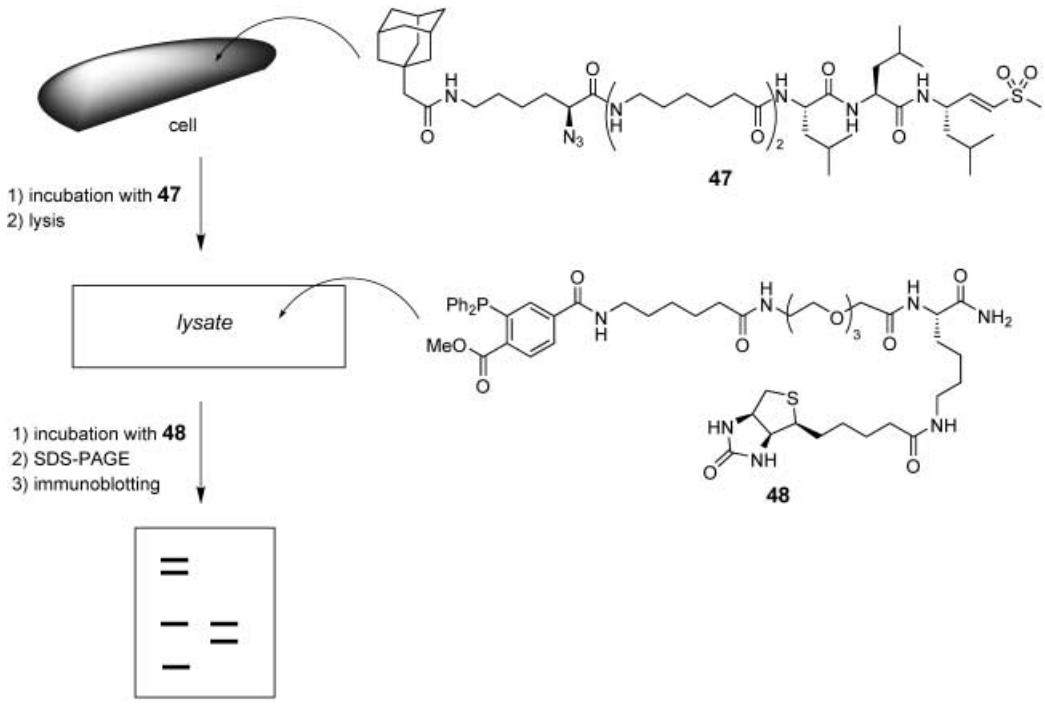
Scheme 9. Recombinant expression of azide-bearing proteins and their labeling with the Staudinger probes **40** and **42.**

Ju and co-workers have demonstrated the site-specific labeling of DNA by using the Staudinger ligation.[22] An oligonucleotide **45** modified at its 5′ end with an azido group underwent selective reaction with the fluorescein-modified phosphane (Fam) **44** under aqueous conditions to produce the Fam-labeled oligonucleotide **46** in approximately 90% yield (Scheme 10). The fluorescent oligonucleotide **46** was then used as a primer in a Sanger dideoxy sequencing reaction to produce fluorescent DNA extension fragments, which were analyzed with a fluorescence electrophoresis DNA sequencer.

Chemistry-based approaches in functional proteomics have been developed in which synthetic compounds that modify a selected subset of proteins covalently and irreversibly are used. Irreversible protease inhibitors have been used in the profiling of serine proteases and cysteine proteases.[23] Overkleeft and co-workers designed the azide-containing inhibitor **47**, which inhibits all catalytically active β subunits of both the constitutive and the interferon-γ-inducible immunoproteasome.[24] EL-4 cells were incubated with **47** overnight. After lysis the cell extract was incubated with the biotinylated phosphane probe **48** (Scheme 11). After separation by SDS-PAGE and immunoblotting the different subunits of the proteasome could be visualized. In this experiment in vivo labeling proved more effective than the corresponding procedure carried out in vitro.



Scheme 10. Preparation of a fluorescent-dye-labeled DNA oligonucleotide.



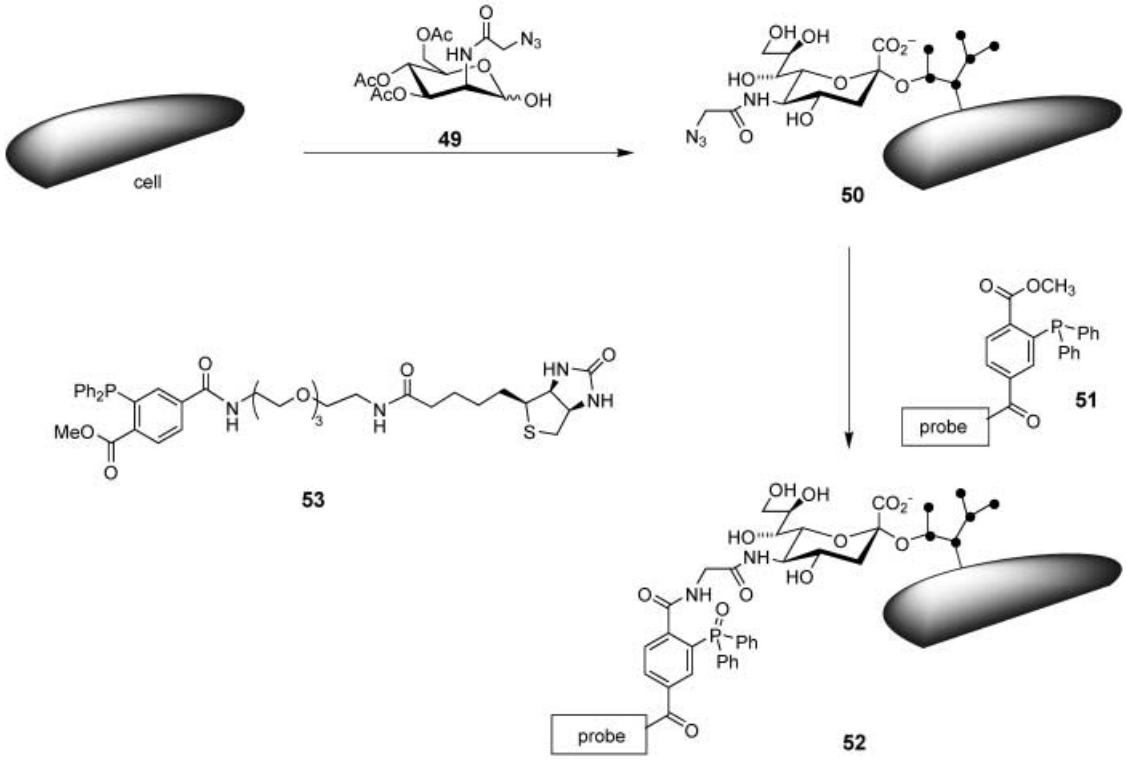
Scheme 11. Detection of active proteasomes by using an irreversible inhibitor probe, which is derivatized through a Staudinger ligation.

4.3. Metabolic Cell Engineering

The interactions that take place on the surface of a cell are of critical importance to the cell cycle and to the communication of cells within complex tissues, including cell–cell adhesion and virus–cell interactions. Carbohydrates have been recognized as central players in these recognition events. Although targeted disruption of glycosylation genes has provided significant information about the function and diversity of carbohydrates, this approach has several limitations, such as embryonic lethality and the upregulation of compensating pathways.[9] Therefore, there is tremendous interest in chemical methods that modulate cell-surface molecules so that their function in the context of intercellular communication can be probed. The research group of Bertozzi has applied a technique in which non-natural biosynthetic precursors are converted into non-natural cell-surface polysaccarides with altered biological functions.[4,9,10] Of particular interest for the application of the Staudinger reaction is the observation that mammalian cells incubated with peracetylated azidoacetylmannosamine (Ac_4ManNAz, **49**) take up this substrate and process it in their own sialic acid biosynthesis pathway to produce azidoacetylsialic acid (SiaNAz), which is incorporated instead of sialic acid into cell-surface glycoconjugates (Scheme 12). The cell-surface azide groups react with a phosphane–probe conjugate **51** (e.g. the biotinylated phosphane **53** or the FLAG phosphane **40**) in a Staudinger ligation. Thus, the probe is attached covalently to the surface glycoprotein, which makes the use of flow cytometry possible for quantitative measurements. In comparison experiments with their own cell-surface ketone–hydrazine reaction the Bertozzi research group could demonstrate that the cell-surface Staudinger reaction was superior in several aspects: a) after cell metabolism to the sialic acid derivative, the Staudinger ligation led to a twofold increase in fluorescence relative to the ketone–hydrazine reaction; b) in contrast to a ketone, an azide has a unique reactivity in a cellular context as a result of its abiotic nature; c) the yield of the Staudinger ligation appears to be pH-independent over a wide range of pH values (5–8.5), whereas the hydrazine–ketone reaction is slow at pH 7.0 and has optimal reactivity at pH 6.5, an acidic environment that causes partial cell death. Control experiments revealed that neither azide reduction by endogenous monothiols (such as glutathione) nor the reduction of disulfides on the cell surface by the phosphane probe takes place. Thus, no side reactions that could obscure results occur.

By using known inhibitors of glycosylation the type of glycoprotein that hosts SiaNAz on the cell surface was identified. Incubation with tunicamycin (an inhibitor that blocks the *N*-linked glycosylation of proteins entirely) led to a steep decrease in cell-surface azide expression relative to that observed in control experiments, thus indicating that the metabolic product derived from Ac_4ManNAz (**49**) is resident within *N*-linked oligosaccharides. An incubation experiment with deoxymannojirimycin (a mannosidase I inhibitor that leads to truncated glycans which lack terminal residues) indicated that the cell-surface azides are present in the terminal sugars of *N*-linked glycans, such as sialic acid.

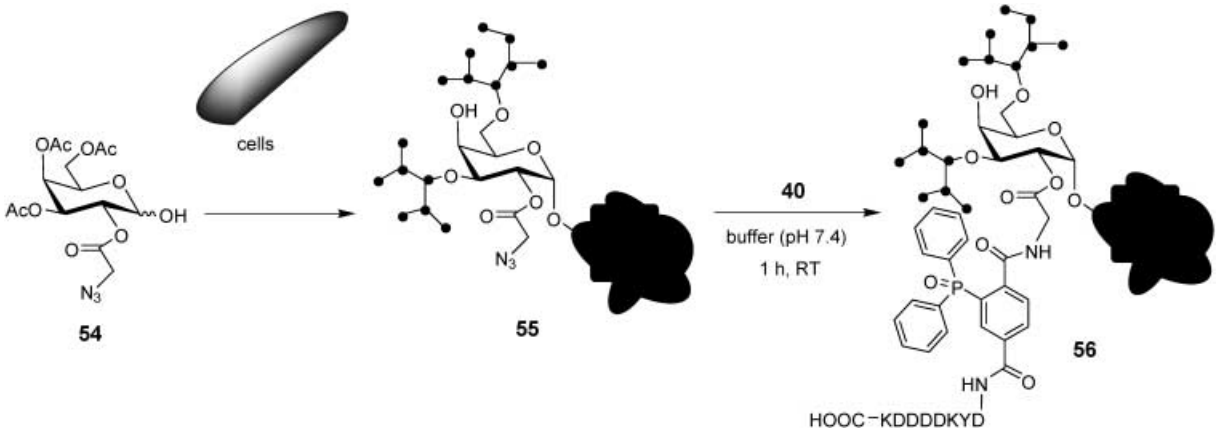
O-Linked glycosylation (at Ser or Thr residues) is the second major type of protein glycosylation. The predominant form of *O*-linked glycosylation is the mucin-type, which is characterized by an initial *N*-acetylgalactosamine (GalNAc) residue α-linked to the hydroxy groups of Thr or Ser side chains. Bertozzi and co-workers have reported a strategy for labeling such mucin-type *O*-linked glycoproteins with a



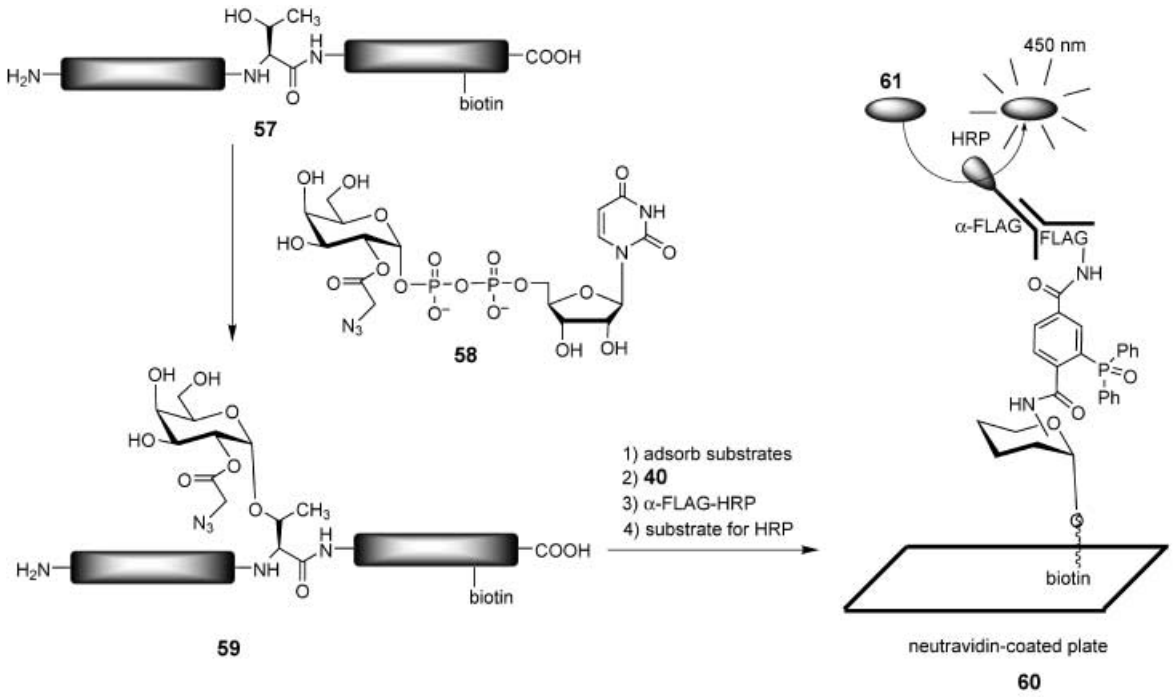
Scheme 12. Metabolic cell-surface engineering with azide-modified glycoproteins prepared biosynthetically.

bioorthogonal azide tag. In this approach they exploit the one common feature of these glycoproteins: the conserved GalNAc residues.[25] The strategy involves feeding CHO (Chinese hamster ovary) cells the peracetylated GalNac analogue **54** (tetraacetyl-*N*-azidoacetylgalactosamine, Ac_4-GalNAz), which is incorporated metabolically into mucin-type *O*-linked glycoproteins **55**, which then react with the probe **40** in a Staudinger ligation to produce labeled glycoproteins **56** (Scheme 13). This method is sensitive enough that glycoproteins expressed at endogenous levels in mammalian cells could be detected in complex cell lysates.

One challenge in glycobiology is the identification of the substrates and functions of the glycosyltransferases that build glycans within the secretory compartments. The polypeptide *N*-α-acetylgalactosaminyltransferases (ppGalNAcTs, a family with approximately 24 isoforms) play a critical role in the biosynthesis of mucin-type *O*-linked glycoproteins by attaching the initial GalNAc unit to Ser or Thr residues of the polypeptide scaffold. In this process UDP-*N*-acetylgalactosamine (UDP-GalNAc; UDP = uridine diphosphate) serves as the glycosyl donor. Bertozzi and co-workers have described an "azido-ELISA" assay (ELISA = enzyme-linked immunosorbent assay) in which a biotinylated protein substrate **57** is glycosylated by ppGalNAcTs with UDP-GalNAz (**58**) as the glycosyl donor (Scheme 14).[26] The reaction mixture is adsorbed onto a 96-well plate coated with neutravidin, to which the FLAG–phosphane **40** and an anti-FLAG antibody chimera with horse radish peroxidase (HRP) are added. The



Scheme 13. Metabolic approach to the labeling of mucin-type *O*-linked glycoproteins.



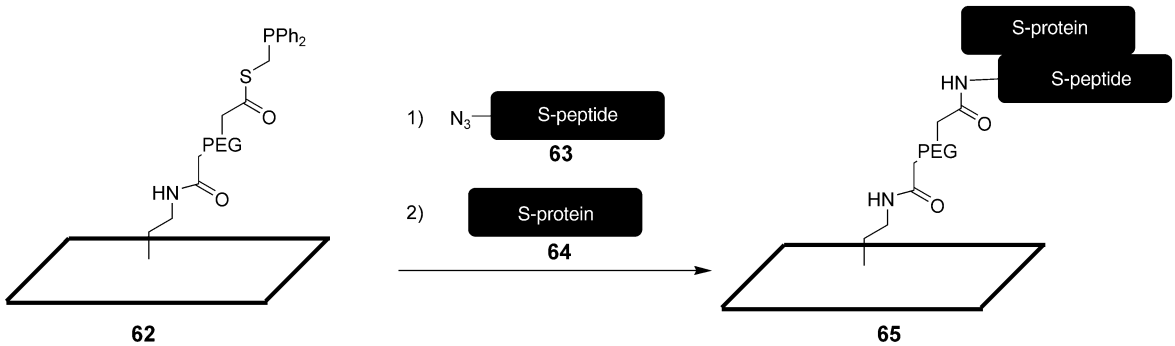
Scheme 14. "Azido-ELISA" allows profiling of the activity of glycosyltransferases.

process is quantified based on measurements of the conversion of the HRP substrate **61** at 450 nm. This platform can be used to profile the substrate specificity of an entire enzyme family.

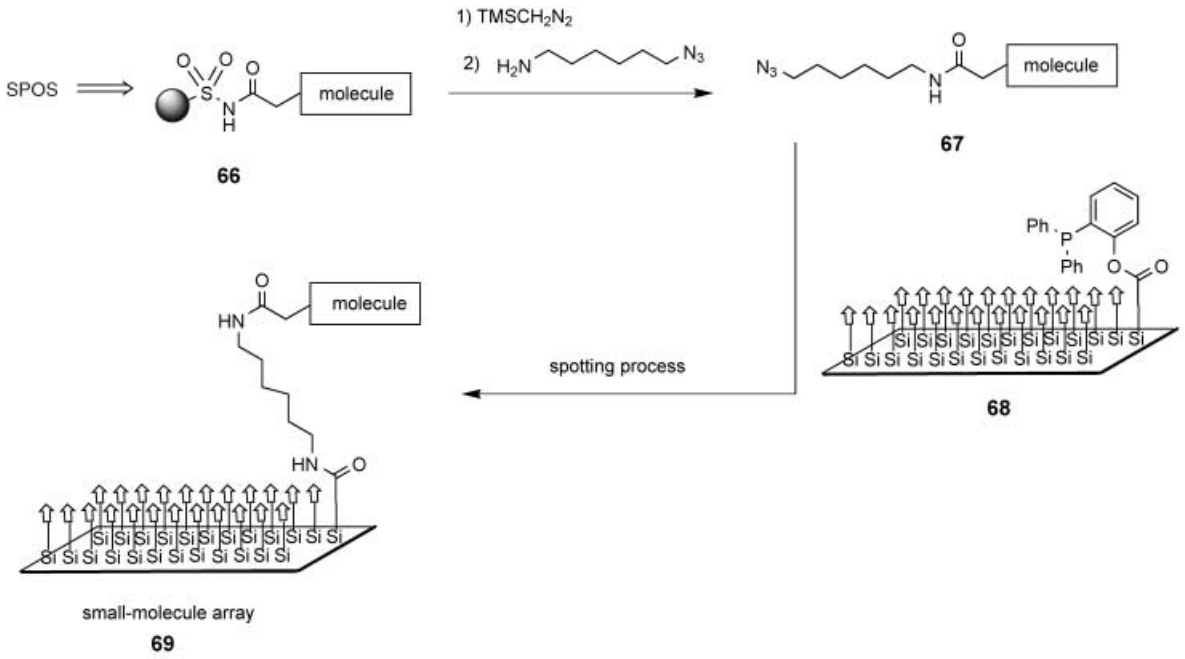
4.4. Preparation of Arrays

The unique chemoselectivity of the Staudinger ligation has stimulated its application as an immobilization technique for the preparation of protein and small-molecule arrays. Raines and co-workers modified an aminopropylsilane-functionalized glass slide with a bifunctional polyethyleneglycol (PEG) spacer and finally attached the phosphane **20**.[27] The azide-functionalized N terminus of the S-peptide **63** (residues 1–15 of RNAse A) is ligated covalently to the slide **62** derivatized with a phosphanyl thioester. Incubation with the S-protein **64** (residues 21–124 of RNAse A) leads to the formation of an adduct **65**, which can be assayed based on ribonucleolytic activity and immunostaining (Scheme 15). Thus, it was shown in a proof-of-concept experiment that the traceless Staudinger ligation can be exploited for the preparation of protein arrays.

In a collaborative effort, the research groups of Breinbauer, Niemeyer, and Waldmann used the phosphane **17** for the traceless immobilization of small molecules, which can be prepared by combinatorial solid-phase synthesis by using the Kenner safety-catch linker **66**.[28] The azide functionalities are introduced upon cleavage from the solid phase. Staudinger ligation of the azide-modified compounds **67** with the phosphane-derivatized dendrimer coating on the slide **68** resulted in the covalent immobilization of the molecules in the sample. The small-molecule arrays **69** prepared in this way were screened for binding with fluorescence-labeled proteins (Scheme 16). The Staudinger ligation provides the advantage of chemoselectivity over many other ligation methods for the



Scheme 15. Site-specific immobilization of proteins through the Staudinger ligation.



Scheme 16. Preparation of a library of azide-terminated small molecules and their immobilization on phosphane-decorated glass slides for the preparation of small-molecule arrays. SPOS = solid-phase organic synthesis.

preparation of small-molecule arrays, thus tolerating a wider range of functional groups that might be necessary for efficient ligand–target binding.

5. Summary and Outlook

Although the Staudinger ligation is just three years old, many examples, as described above, give testimony to its high practical value in research in chemistry and biology. In particular, the phosphane reagent **10** introduced by Bertozzi and co-workers has proven its practicability not only in model experiments but also in actual biological studies. Although it can not be used in a traceless ligation, its stability against oxygen and superior functional-group tolerance makes it the reagent of choice for most applications. At present the Staudinger ligation is probably the mildest and most chemoselective ligation reaction. It should therefore be the first reaction considered for the preparation of bioconjugates or when chemistry has to be carried out in the complex environment of a living cell.

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