Azides in Carbohydrate Chemistry

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16.1 Introduction

The first azide-containing sugar, a glycosyl azide, was reported in 1930 by Bertho.¹ Since that time various methods have been developed for the introduction of azides at different positions of sugars. A survey of available methods is given in Section 16.2. Until the late 1970s, azides contained in carbohydrate derivatives were simply used as accessible synthons for amines because of their easily performed reduction to amines. Due to their stability against a variety of reaction conditions, azides often can serve as masked amines during the course of carbohydrate synthesis. The development of the diazo transfer reaction facilitated the use of azides also as temporary protecting group for amines. This was extensively applied during the preparation of aminoglycoside derivatives (Section 16.3).

Over the last three decades, azides became an important tool especially for the synthesis of glycopeptides and -proteins. In 1978 Paulsen *et al.* developed the 'azide method' for the preparation of 1,2-*cis*-glycosides of glycosamine derivatives using 2-azido-2-deoxydonors (Section 16.4). This reaction is widely used for the synthesis of *O*-linked glycosyl amino acid building blocks. In *N*-glycoproteins, the glycan chains are attached to the protein via a β -glycosyl amide. Staudinger-type reactions offer a convenient access to such structures and are applied since the 1990s for the synthesis of α - and β -glycosyl amides directly from glycosyl azides (Section 16.5).

An enormous impact on the field of glycobiology during the last decade had the development of two bioorthogonal reactions based on azides: the copper-catalyzed azide-alkyne [3+2] cycloaddition and the Staudinger ligation. Together with the possibility of *in vivo* incorporation of azide and alkyne tags into glycans and proteins, these reactions offer new options for selective labeling and manipulation of biomolecules even within

living cells. Especially the azide-alkyne cycloaddition has been extensively applied for the chemical synthesis of neoglycoconjugates such as glycopeptide and glycoprotein mimics or multivalent glycoclusters (Section 16.6). Metabolic oligosaccharide engineering uses the biosynthetic pathways for the introduction of azide- (and alkyne-)tagged sugar moieties into the glycans of cells that can subsequently be labeled by a detectable probe. This approach is discussed in Section 16.7.

16.2 Synthesis of Azide-Containing Carbohydrates

A common way for the introduction of azides into carbohydrates is the nucleophilic replacement of leaving groups by the azide ion. These reactions can be divided into three groups: substitutions at the anomeric center leading to glycosyl azides, substitutions at primary, and substitutions at secondary carbon atoms.

A widely used method for the preparation of glycosyl azides²⁻⁴ is the conversion of acetylated halogenoses, such as **1**, by treatment with sodium azide based on Bertho's initial work (Scheme 16.1A).¹ While homogeneous one-phase reactions in DMF often require elevated temperatures,⁵ phase-transfer catalysis enables milder conditions.⁶ One limitation of this methodology is the instability of glycosyl halides. Thus, sequential one-pot procedures have been developed that avoid the isolation of glycosyl halides.⁷ An alternative, which circumvents the preparation of glycosyl halides completely, is the direct conversion of glycosyl acetates into the corresponding glycosyl azides using trimethylsilyl azide under Lewis acid catalysis (Scheme 16.1B).⁸ Glycosyl azides with 1,2-*trans*-configuration are easily obtained by the described methods using acyl protecting groups due to their neighboring group participation. Glycosyl azides with 1,2-*cis*-configuration can be prepared from 1,2-*trans*-glycosyl halides in an S_N2-type reaction or from ether-protected glycosyl acetates by treatment with trimethylsilyl azide.²⁻⁴

Scheme 16.1 Preparation of glycosyl azides from (A) peracetylated glycosyl halides under classical homogeneous conditions⁵ and under mild phase-transfer catalysis⁶ and (B) from peracetylated sugars⁹

The introduction of azides at the primary carbon of carbohydrates is conveniently carried out by an S_N2 reaction. The generation of a good leaving group, such as a sulfonate, is often possible in a selective way without need for protection of the secondary hydroxy groups as was shown for GlcNAc derivative **5** (Scheme 16.2).¹⁰ Subsequent substitution with sodium azide usually proceeds at elevated temperatures with good yields.

In contrast, S_N2 reactions at secondary carbons of the sugar ring system are more complex. The success of such reactions is strongly dependent on the type of sugar (stereochemistry), the position at which the S_N2 reaction is carried out, anomeric configuration, and used protecting groups. Nevertheless, this approach is widely applied for the introduction of azido groups at the ring system. For instance, the mesylate of glucoside **7** was substituted yielding 4-azido galactoside **8** under inversion of configuration (Scheme 16.3).¹¹

Epoxides are also useful precursors for the incorporation of azido groups by nucleophilic attack. According to the Fürst-Plattner rule,¹² ring opening of sugar epoxides by azide ions preferentially leads to the diaxial product. For instance, 2-azido compound **10** is obtained regioselectively by opening of Cerny epoxide **9** with sodium azide (Scheme 16.4).¹³ **10** was further converted into the suitably protected glycosyl donor **11**, which was applied in the synthesis of a heparan sulfate synthon by 1,2-*cis*-glycosylation (cf. Section 16.4).

Azides can also be introduced by radical addition to glycals. The classical azidonitration, developed by Lemieux *et al.* in 1979, is a powerful method for the preparation of

Scheme 16.2 Regioselective introduction of an azido group at the primary carbon of **5** via nucleophilic replacement of a sulfonate intermediate¹⁰ pyr = pyridine

Scheme 16.3 Replacement of a mesylate by an azido group under inversion of configuration at a secondary center of the sugar ring¹¹

Scheme 16.4 Regio- and stereoselective opening of Cerny epoxide **9** leads to 2-azido compound **10**, which can be further converted into glycosyl donor **11**¹³

Scheme 16.5 Azidonitration of galactal **12** leads to an epimeric mixture of the 2-azido-1-nitro-pyranoses **13** and **14** from which glycosyl donor **15** can be prepared directly.¹⁴ CAN = cerium(IV) ammonium nitrate

Scheme 16.6 Typical procedure for the Cu(II)-catalyzed diazo transfer.²⁶ DMAP = 4-(dimethylamino)pyridine

2-azido sugars that is still frequently used (Scheme 16.5).¹⁴ It is especially useful for the synthesis of those 2-azido derivatives, whose corresponding glycosamines lack accessibility from natural sources as in the case of galactosamine. However, while the reaction is highly regioselective, in most cases epimeric mixtures of the 2-azido compounds are formed. The ratio of the epimers strongly depends on the employed glycal substrate.¹⁵ The obtained 1-nitro-pyranoses can easily be converted into glycosyl donors, such as glycosyl halides,¹⁴ trichloroacetimidates,¹⁶ *n*-pentenyl glycosides,¹⁷ or thioglycosides,^{18–20} which are valuable building blocks for the preparation of 1,2-*cis* glycosides of *N*-acetyl-glycosamines (cf. Section 16.4). Similar methods for the synthesis of 2-azido sugars using radical addition to glycals are the azidochlorination²¹ and the azidophenylselenation.^{22,23}

Another possibility for the synthesis of organic azides is the diazo transfer using triflyl azide.²⁴ In contrast to the methods described above, not the entire azido group is incorporated into a molecule but an N_2 moiety is transferred onto an existing amine under retention of configuration. The first diazo transfer onto amino sugars was reported in 1991 by Vasella *et al.*²⁵ They treated different unprotected glycosamines with freshly prepared triflyl azide under basic conditions. After subsequent acetylation, the 2-azido sugars were isolated in good yields. This methodology was further improved by the addition of catalytic amounts of copper sulfate which leads to a much faster and more reliable reaction (Scheme 16.6).^{26,27} Using the diazo transfer, it is possible to employ azides not only as amine synthons but also as temporary protecting groups for amines. This has been applied for example to the synthesis of aminoglycosides (Section 16.3), heparan sulfate fragments,²⁸ heparin fragments,^{29,30} hyaluronan neoglycopolymers,³¹ and *N*-acetyl-neuraminic acid derivatives.³²

16.3 Azides as Protecting Groups during Aminoglycoside Synthesis

Protecting groups commonly employed for masking amino groups include alkyl carbamates such as benzyl-, *tert*-butyl-, and 9-fluorenylmethyl carbamates. If used for the

protection of molecules containing multiple amino groups, however, carbamate protecting groups can seriously complicate the interpretation of NMR spectra. This is due to the occurrence of *E/Z* rotamers that are in slow interconversion leading to multiple sets of signals. The use of azides as protecting groups circumvents this problem. Azides are easily reduced to amines, for example by catalytic hydrogenation or by reaction with thiols or complex hydrides.^{4,33,34} A widely applied method in carbohydrate chemistry is the Staudinger reduction using triaryl- or trialkylphosphines.³⁵ This mild procedure enables the selective reduction of azides in the presence of esters and benzyl ethers which are frequently used as OH-protecting groups. Furthermore, azides can be directly converted into carbamate-protected amines using a variant of the Staudinger reaction (cf. Section 16.5).^{27,36,37}

Aminoglycosides are highly potent, broad-spectrum antibiotics, containing several amino groups presented on an oligosaccharide-like core.^{38–40} Due to the appearance of bacterial strains resistant to these drugs and due to their relatively high toxicity, the synthesis of aminoglycoside derivatives with improved properties is of great interest.⁴¹ Several syntheses of aminoglycoside derivatives using azides as amine protecting groups were reported,^{42,43} for instance the preparation of analogs of neomycin B as shown in Scheme 16.7.^{27,44,45} Starting from commercially available neomycin B (**18**), all six amino groups were converted into azides by diazo transfer. After chemical derivatization of the structure, amines were regenerated by Staudinger reduction.

In the course of these studies, it was observed that the regioselective reduction of a single azide of multiple azide-containing molecules is feasible if only one equivalent of phosphine is used.²⁷ Reduction of neamine derivative **23**, for example, gave mono-amine **24** in a yield of 46 % (Scheme 16.8). Strong evidence was presented that the selectivity is primarily determined by electronic factors with electron-deficient azides being reduced more rapidly and efficiently than electron-rich azides. In compound **23** this is the case for

Scheme 16.7 Synthesis of aminoglycoside derivative **22** using azides as protecting groups for amines. First, the amino groups of **18** were converted into azides by diazo transfer.²⁷ After chemical remodeling of the aminoglycoside (one amino group was replaced by a hydroxy group), the amines were regenerated by Staudinger reduction⁴⁴

Scheme 16.8 Regioselective reduction of tetra-azides **23**²⁷ and **25**.^{46,47} Boc-ON = 2-(tert-butoxycarbonyloxyimino)-2-phenylacetonitrile

the 2'-azide adjacent to the anomeric center. It was shown that the regioselectivity can be predicted on the basis of ^{15}N and, to some extent, ^1H NMR chemical shifts. Consequently, by introduction of electron withdrawing 4-chlorobenzoyl protecting groups in the 5- and 6-position, the selectivity can be tuned in favor for reduction of the 1-azide (**25** $\rightarrow$ **26**).^{46,47}

16.4 Azides as Non-Participating Neighboring Groups in Glycosylations

Although 1,2-*cis* glycosides of 2-amino-2-deoxysugars are less frequently found in natural products compared to their 1,2-*trans* isomers, they are a common motive in important structures. In mucin-type *O*-glycoproteins, e.g. the glycan chains are attached to protein via an α -glycosidic linkage of *N*-acetyl-D-galactosamine to the β -hydroxy group of either serine or threonine, and α -glycosides of 2-acetamido-2-deoxy-D-glucose are found in the glycosaminoglycan heparan sulphate.^{48–51} For the preparation of these 1,2-*cis* glycosides, the commonly employed *N*-acyl protecting groups are not suited because they lead to 1,2-*trans* products **29** via neighboring group participation (Scheme 16.9A).^{15,52,53} In 1978 Paulsen *et al.* showed that 2-azido-2-deoxy-glycosyl halides are suitable donors in 1,2-*cis* glycosylations. This approach preferentially leads to α -glycosides **32** either directly from β -glycosyl halides **30** (Scheme 16.9B)^{54,55} or by *in situ* anomerization⁵⁶ of α -glycosyl halides **33** (Scheme 16.9C).⁵⁷ Since then, the azide method has been widely used^{15,52,58–62} and expanded by use of other glycosyl donors, such as trichloroacetimidates,¹⁶ *n*-pentenyl glycosides,¹⁷ and thioglycosides^{18–20} just to name a few. The required 2-azido-2-deoxy sugars are usually prepared by azidonitration of glycals or by diazo transfer reaction of the corresponding glycosamines as described above. After glycosylation, the azide can

Scheme 16.9 Preparation of *O*-glycosides of 2-amino-2-deoxysugars. (A) Use of *N*-acyl-protected donors **27** results in 1,2-*trans* glycosylation due to neighboring group participation. (B) 1,2-*cis* glycosylation products **32** from β-glycosyl halides **30**^{54,55} or (C) by in situ anom-erization⁵⁶ of α-glycosyl halides **33**⁵⁷

be transformed to the natural acetamido function either in two steps by reduction of the azide and subsequent acetylation or in one step by reductive acetylation using thioacetic acid.^{63,64}

A successful approach for the synthesis of *O*-glycopeptides is the assembly of pre-formed, more or less complex glycosyl amino acid building blocks by solid phase peptide synthesis (SPPS).^{60–62,65–67} Based on initial work of Ferrari⁶⁸ and Paulsen,⁶⁹ the azide method is extensively used for the preparation of such glycosyl amino acid building blocks. Especially the synthesis of complex glycosyl amino acids is challenging. Usually, glycosylation is performed with monosaccharides followed by attachment of further sugar residues because glycosylation reactions with oligosaccharide donors and serine or threonine acceptors often proceed with unpredictable stereochemistry. Nevertheless, oligosaccharides have been successfully used in many glycosylations as illustrated by the synthesis of glycosyl threonine building block **38** reported by Danishefsky and coworkers (Scheme 16.10).⁶⁴

16.5 Glycosyl Azides as Precursors for Glycosyl Amides

Beside the *O*-linked glycoproteins, the more prevalent form of glycosylation of proteins is *N*-linked glycosylation.^{48,70,71} *N*-Glycoproteins are characterized by a β-*N*-glycosidic linkage of the terminal *N*-acetylglucosamine of the pentasaccharide core structure to the amide nitrogen of asparagine. The conventional synthetic strategy for the preparation of such glycosyl amides starts from glycosyl amines which are reacted with activated and

Scheme 16.10 Synthesis of glycosyl threonine building block **38** using the azide method.⁶⁴ The 2-azido group is introduced by azidonitration of **34** followed by preparation of donor **36**. Glycosylation using threonine as acceptor leads to 1,2-*cis* glycoside **37**. After conversion of the azide group to an *N*-acetyl group by reductive acetylation, **38** was used as building block in glycopeptide synthesis

suitably protected aspartic acid derivatives to form the amide linkage.^{60–62,65–67} Glycosyl amines are commonly prepared either by reduction of glycosyl azides (cf. Section 16.3) or by amination of unprotected reducing sugars with saturated aqueous ammonium bicarbonate.⁷² Recently, improved variants of the latter procedure employing microwave irradiation^{73,74} and ammonium carbamate,^{75,76} respectively, have been published. Drawbacks of this method are the instability of glycosyl amines and their propensity for dimerization and anomerization. Also, the preparation of α -glycosyl amides is a synthetic challenge.

While the classical Staudinger reaction³⁵ leads to iminophosphoranes which can be hydrolyzed to amines under aqueous conditions (Staudinger reduction, cf. Section 16.3), the addition of acyl donors under dry conditions results in amide formation.^{77,78} This procedure was repeatedly applied for the synthesis of glycosyl amides, thus circumventing the preparation of glycosyl amines. Initially, three-component reactions employing glycosyl azide, activated carboxyl derivative and phosphine were reported (Scheme 16.11). The reaction starts from the β - (**39**) or α -glycoside **45** with the formation of an iminophosphorane (**40** and **42**, respectively), which is then trapped by an acylating agent in the second step. The resulting acylaminophosphonium salt (**43/46**) yields the corresponding glycosyl amide (**44/47**) upon hydrolysis. The intermediate iminophosphorane can undergo anomerization via open-chain form **41** preferring β -configuration. The degree of isomerization is dependent on the efficiency of iminophosphorane trapping by the acylating agent. Differently activated carboxylic acids, such as carboxylic halides,^{79,80} anhydrides,^{80,81} and carbodiimide-activated acids,^{82,83} have been employed as acylating agents. While β -glycosyl amides **44** can be obtained easily from β -glycosyl azides **39**, the stereospecific

Scheme 16.11 Mechanism of the Staudinger reaction with glycosyl azides

Scheme 16.12 Three-component Staudinger-type reaction with β -glycosyl azide **4** stereoselectively leads to the β -glycosyl amides **48**. α -Glycosyl amides can only be obtained from α -glycosyl azide **49** with strong acylating agents to prevent complete anomerization of the intermediate iminophosphorane⁸⁰

synthesis of α -glycosyl amides **47** starting from α -glycosyl azides **45** is only possible with strong acylating agents which trap the intermediate iminophosphorane **42** before anomerization can take place.⁸⁰ Representative examples for the three-component Staudinger reaction are shown in Scheme 16.12. Rarely, the Staudinger reaction with reactive alkylphosphines and free carboxylic acids has been reported.^{84,85} In this case, amide-bond formation is assumed to proceed in a concerted reaction without generation of an iminophosphorane intermediate.

Recently, the synthesis of glycosyl amides has also been achieved employing the traceless two-component Staudinger ligation^{9,86,87} developed in the laboratories of Bertozzi⁸⁸ and Raines^{89,90} (Scheme 16.13). Starting from glycosyl azides **50** and **55**, respectively, the initially formed iminophosphorane **52/57** reacts with an intramolecular (thio)ester group to form the acylaminophosphonium salt **53/58** from which the phosphine moiety is removed by hydrolysis with water. Using benzyl protected α -glycosyl azides such as **50**

Scheme 16.13 Two-component traceless Staudinger ligations using phosphine-derivatized ester **51** (A)⁹ or thioester **56** (B)⁸⁷

and stable phosphine **51** or similar esters in polar aprotic solvents such as DMF, the reaction proceeded stereo conservatively to yield predominantly α -glycosyl amides (Scheme 16.13A).⁹ The use of acetyl protected α -glycosyl azides, however, resulted only in β -glycosyl amides due to isomerization of the less reactive iminophosphorane.

All methods described above have been used for the preparation of the β -glycosyl amide linkage between *N*-acetylglucosamine and the side chain of asparagine in both three-component reactions using free^{84,85} or activated^{82,83} carboxylic acids and two-component reactions as shown in Scheme 16.13.^{9,87} The obtained protected glycosyl amino acids can be used as building blocks in SPPS of *N*-linked glycopeptides.^{91,92} It was also shown that deprotected sugars can be attached to amino acids and whole peptides using the three-component reaction.⁹² Beside Staudinger-type reactions, another route towards the synthesis of glycosyl amides is the reaction of glycosyl azides with thiocarboxylic acids.⁹³

16.6 Synthesis of Glycoconjugates via Azide-Alkyne [3+2] Cycloaddition

Although the azide-alkyne [3+2] cycloaddition⁹⁴ (cf. Chapter 9) is known in carbohydrate chemistry for more than 50 years,⁹⁵ its application for the preparation of glycoconjugates became particularly attractive with the development of the copper(I)-catalyzed variant by Meldal⁹⁶ and Sharpless.⁹⁷ The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC)^{98,99} enables the regioselective formation of 1,4-disubstituted 1,2,3-triazoles under very mild conditions even in a biological context. However, the cellular toxicity of the copper catalyst precludes applications wherein cells must remain viable. Therefore, as an alternative

Scheme 16.14 Coupling of azide-substituted galactoside **60** to alkyne-modified C_{14} hydrocarbon **61** noncovalently bound to the microtiter well surface¹⁰⁸

to CuAAC, strain-promoted azide-alkyne [3+2] cycloadditions have been developed that proceed at room temperature without the need for a catalyst.^{100,101} These reactions are discussed in the next section dealing with metabolic oligosaccharide engineering. Another example of metal-free triazole formation by a tandem [3+2] cycloaddition-retro-Diels-Alder reaction has been developed by van Berkel *et al.* although no carbohydrate-related application was reported.¹⁰²

CuAAC reactions have been extensively applied in carbohydrate chemistry including the synthesis of simple glycoside and oligosaccharide mimetics, glyco-macrocycles, glycoconjugates, glycoclusters, and for the attachment of carbohydrates to surfaces. The field has been thoroughly reviewed^{98,103–107} and, therefore, we will focus on a few selected examples which are of special interest for glycobiology.

One of the first applications of CuAAC in carbohydrate chemistry was – beside the one in the seminal paper of Meldal⁹⁶ – the immobilization of azide-substituted sugars on microtiter plates (Scheme 16.14).¹⁰⁸ The surface-bound sugars such as **62** were screened with various lectins and could be elongated by glycosyltransferase-catalyzed fucosylation. The technique was later on improved by incorporation of a cleavable disulfide bond in the linker allowing mass spectrometric characterization of the carbohydrate array.¹⁰⁹

Neoglycopeptides and -proteins¹¹⁰ differ from naturally occurring structures by replacement of the natural carbohydrate-peptide linkage with a non-natural one. This not only allows studying the influence of distinct structural elements on biological activity, but has many practical applications as well. Use of chemoselective ligation reactions such as

Scheme 16.15 Application of CuAAC for the preparation of triazole-linked glycosyl amino acids **65**¹¹¹

Scheme 16.16 Preparation of tyrocidine derivatives **68** by CuAAC of propargylglycine-containing cyclic peptides **66** and azido-functionalized sugars **67**¹¹³

CuAAC makes glycoconjugates accessible to a broader community. Furthermore, the non-natural linkage often is more stable (both chemically and with respect to enzymatic degradation) which can lead to an increased half life of a glycoconjugate within a biological system. Scheme 16.15 depicts the synthesis of triazole-linked glycosyl amino acids **65** starting from glycosyl azides **63** and different alkyne-containing amino acids **64** which can be used as building blocks in peptide synthesis.^{111,112}

Lin and Walsh applied CuAAC for the attachment of 21 different azido-functionalized monosaccharides **67** to 13 derivatives **66** of the cyclic decapeptide tyrocidine containing one to three propargylglycine residues at positions 3–8 (Scheme 16.16).¹¹³ Head-to-tail cyclization of the peptides was accomplished using a thioesterase domain from tyrocidine synthetase. Antibacterial and hemolytic assays showed that the two best glycopeptide mimetics had a 6-fold better therapeutic index than the natural tyrocidine. CuAAC has further been used to attach carbohydrates to whole virus particles^{114,115} and DNA.¹¹⁶

More challenging is the modification of bacterially expressed proteins by site-specific attachment of carbohydrates. Crucial step is the introduction of a chemical tag, which can be chemoselectively modified, into the protein. It has been shown that alkyne- and azido-modified amino acids, such as 2-amino-5-hexynoic acid (homopropargylglycine, Hpg),¹¹⁷ 4-azidohomoalanine (Aha),^{118,119} and with less efficiency also 3-azidoalanine, 5-azidonorvaline, and 6-azidonorleucine,¹²⁰ act as methionine surrogates that are acti-

vated by the methionyl-tRNA synthetase of *E. coli* and replace methionine in proteins expressed in methionine-depleted bacterial cultures. This, together with other methods for the incorporation of non-canonical amino acids into proteins,^{121–123} offers the possibility to use azide-alkyne cycloaddition (and also Staudinger ligation^{124–126}) not only for protein labeling within cells or on cell surfaces^{119,120} but also for the preparation of neoglycoproteins.

Davis and coworkers expanded the diversity of chemical protein modification by a combination of this CuAAC-based labeling and disulfide bond formation via genetically engineered cysteine (Cys) residues.¹²⁷ Aha and Hpg, respectively, were introduced into engineered proteins by the auxotrophy-based residue-specific method. Subsequent CuAAC reactions with alkyne- and azide-substituted carbohydrates, respectively, resulted in homogeneous protein glycoconjugates. As second modification reaction, conjugation of Cys residues with substituted methanethiosulfonates was chosen. Applying these two orthogonal protein modification reactions, derivatives of the LacZ reporter enzyme carrying the tetrasaccharide sialyl Lewis X and a sulfotyrosine mimic were created that allowed detection of mammalian brain inflammation.

Recently, Merkel *et al.* reported efficient N-terminal glycoconjugation of proteins by the N-end rule.¹²⁸ Bulky amino acids at the second and third sequence position of the barnase inhibitor barstar efficiently prevent excision of N-terminal methionine analogue Aha introduced by the auxotrophy-based residue-specific method. The created azide tag at the protein's N-terminus was subsequently conjugated to propargyl glycosides of *N*-acetylglucosamine and *N,N'*-diacetylchitobiose, respectively, by CuAAC. The obtained glycoprotein mimetics show binding affinity to the lectin wheat germ agglutinin whereas the natural activity of barstar is conserved.

Lectins are carbohydrate-binding proteins other than immunoglobulins without enzymatic activity towards the recognized sugars.^{129–131} Carbohydrate-lectin interactions are involved in numerous intra- and intercellular events during development, inflammation, immune response and cancer metastasis.^{132–136} Multivalency appears to play an important role in lectin-mediated interactions,^{137–140} and many lectins are found to recognize individual carbohydrate epitopes only with low affinity. Preparation of carbohydrate clusters, therefore, is a common strategy to obtain high-affinity lectin ligands.^{141–144} Because of its robustness, CuAAC is excellently suited for the simultaneous attachment of several carbohydrate epitopes to a scaffold. Initially, Santoyo-González and coworkers prepared different multivalent mannose clusters starting from propargyl mannosides and azide-containing scaffolds.¹⁴⁵ This strategy as well as the opposite approach based on azide-containing carbohydrates and alkyne-bearing scaffolds have been used intensively for the preparation of glycoclusters.^{98,103–107} Glycosyl azides are easily produced, however, the direct attachment of a triazole to the sugar may interfere with the recognition of the carbohydrate by the protein and, therefore, linkers of varying length have been introduced between the sugar and the triazole moiety. It would be far beyond the scope of this chapter to mention all applications. Exemplarily, the asymmetrical, bifunctional dendrimer **69** containing 16 mannose units and two coumarin chromophores¹⁴⁶ and poly(methacrylate)-based glycopolymer **70**¹⁴⁷ are depicted in Scheme 16.17.

Although organic azides are stable against most reaction conditions, compounds containing multiple azide residues (like multivalent scaffolds) are potentially explosive. Therefore, several one-pot procedures to generate azides *in situ* followed by CuAAC have

Scheme 16.17 (A) Asymmetrical, bifunctional dendrimer **69** containing 16 mannose units and two coumarin chromophores¹⁴⁶ and (B) poly(methacrylate)-based glycopolymer **70**¹⁴⁷ prepared by CuAAC and used for lectin binding studies with concanavalin A

been reported.^{148–154} While the azides in most of these procedures are introduced by a nucleophilic substitution of a leaving group in allyl, benzyl, glycosyl, or similar position,^{148–152} aliphatic¹⁵⁴ and aromatic¹⁵³ amino groups may also serve as precursors. We reported, for example a one-pot procedure for diazo transfer and subsequent CuAAC which allows the preparation of multivalent structures starting from commercially avail-

Scheme 16.18 Sequential one-pot procedure for diazo transfer and CuAAC.¹⁵⁴ First, diamine **71** is transformed to the corresponding diazide by Cu(II)-catalyzed diazo transfer. After completion, Cu(I) required for subsequent CuAAC with **72** is generated by addition of reducing agent Na ascorbate. MW = microwave; TBTA = tris(benzyltriazolylmethyl)amine

able amine scaffolds without need for isolation of the azide-containing intermediates.¹⁵⁴ As an example, divalent glycoconjugate **73** was synthesized from diamine **71** and propargyl glycoside **72** as shown in Scheme 16.18.

Azides can also undergo [3+2] cycloaddition reactions with nitriles giving access to 1,5-disubstituted tetrazoles. Intermolecular reactions, however, require electron deficient nitriles and very forcing conditions to occur with sufficiently high reaction rates.^{155–157} High yields have been reported for the reaction of sulfonyl and acyl cyanides with unhindered aliphatic azides by neat, thermal fusion.^{158,159} Intramolecular [3+2] cycloaddition reactions of organic azides to nitriles occur more readily.^{160–164} Still, they require high reaction temperatures and yields are with few exceptions¹⁶⁵ not satisfactory. When precisely positioned on a rigid carbohydrate scaffold, however, azides can undergo cycloaddition reactions with nitriles under exceptionally mild conditions. Thus, 3-azido-1,2-*O*-cyanoethylidene-3-deoxy-allopyranose was shown to form a tetrazole embedded in a bridged tetracyclic ring system even at room temperature.¹⁶⁶

16.7 Metabolic Oligosaccharide Engineering

Glycosylation of proteins is an important co- and posttranslational event that has been estimated to occur on more than 50% of eukaryotic proteins.¹⁶⁷ The glycan chains of cell-surface glycoproteins are involved in numerous recognition processes such as cell adhesion and attachment of bacteria or viruses. Inside cells, glycans direct protein trafficking and they modulate structure and activity of proteins.^{132–136} Hence, in vivo monitoring of glycosylation processes is of utmost interest.¹⁶⁸ While fluorescent fusion proteins and other genetically encoded tags provide a means for labeling specific proteins in live cells, analogous techniques are not available for secondary gene products including glycans. Metabolic oligosaccharide engineering offers the possibility to introduce carbohydrates with unnatural structural elements into the glycans without genetic manipulation making use of the cell's biosynthetic machinery.¹⁶⁹ If suitable chemical reporter groups are introduced, subsequent addition of an exogenously delivered detectable probe allows for tagging of the glycans by a chemoselective ligation reaction. Examples for such reporter groups include ketones^{170,171} and thiols.¹⁷² However, the azido group is much more

Scheme 16.19 Metabolic oligosaccharide engineering: peracetylated ManNAz **74** is taken up by mammalian cells and converted into an azide-containing sialic acid derivative which is incorporated into sialic acid-bearing glycans **75**. In the next step, a detectable probe **76** can be attached to **75** via Staudinger ligation^{124,173}

suited for this approach because azides can take part in two important bioorthogonal ligation reactions, Staudinger ligation¹²⁴ (cf. Section 16.5) and azide-alkyne [3+2] cycloaddition (cf. Section 16.6 and Chapter 9).

Azide derivatization of monosaccharides represents a subtle structural change that is accepted by several metabolic pathways. Thus, azide derivatives of *N*-acetylmannosamine (i.e. *N*-(azidoacetyl)mannosamine, ManNAz), *N*-acetylgalactosamine (i.e. *N*-(azidoacetyl)galactosamine, GalNAz), *N*-acetylglucosamine (i.e. *N*-(azidoacetyl)glucosamine, GlcNAz), and *L*-fucose (i.e. 6-azido-*L*-fucose) have been explored.^{168,169} Initially, ManNAz was employed to tag sialylated cell surface glycans of mammalian cells *in vitro* (Scheme 16.19).^{124,173} Cells are grown in the presence of peracetylated ManNAz **74** which can be taken up by the cells more easily than ManNAz. After de-*O*-acetylation by cellular esterases, resulting ManNAz is metabolized similarly to its native counterpart *N*-acetylmannosamine and integrated into cellular glycans. Finally, the azide-labeled glycans are reacted with a detectable probe by Staudinger ligation. GalNAz can be metabolically introduced at the core position of mucin-type *O*-linked glycoproteins.¹⁷⁴ Thus, a selective labeling of mucin-type glycoproteins is possible. Both, the metabolic labeling of sialylated glycans with ManNAz¹⁷⁵ and labeling of mucin-type *O*-glycoproteins with GalNAz¹⁷⁶ can be carried out *in vivo*. Analogously, GlcNAz has been used for the labeling of *O*-GlcNAc glycosylated proteins.¹⁷⁷ Recently, cells were labeled simultaneously with an azide- and a ketone-containing sugar.¹⁷⁸ Using orthogonal ligation reactions, glycans bearing these sugar residues can be visualized in parallel on the same cells.

In the cases mentioned so far, fluorescence labeling has been achieved by a two-step procedure. First, a biotin label¹²⁴ or FLAG tag (octapeptide Asp-Tyr-Lys-Asp-Asp-Asp-Lys)^{173,174,177} is covalently attached to the azide-containing glycan by Staudinger ligation at high concentration. In a second step, a fluorescently labeled receptor (avidin and anti-FLAG antibody, respectively) is added at lower concentration. To avoid the problem of high background fluorescence caused by the application of fluorescent dyes,

Scheme 16.20 Generation of fluorescent triazole **80** by CuAAC of fluorogenic ethynyl-naphthalimide **78** and azide-labeled glycoproteins **79** applicable for intracellular localization of fucosylated glycoconjugates¹⁷⁹

Wong and coworkers developed a one-step labeling method based on CuAAC ligation using fluorogenic dyes (Scheme 16.20).¹⁷⁹ 6-Azido-L-fucose was applied for tagging of fucosylated proteins by metabolic oligosaccharide engineering. Reaction of alkyne-substituted naphthalimide **78** and azide-modified glycoprotein **79** results in formation of fluorescent triazole **80**. Since **78** is not fluorescent, it can be applied at high concentrations without producing a background signal. The method was used for cell surface glycoprotein analysis and intracellular localization of fucosylated glycoconjugates by using fluorescence microscopy.

Other examples for the application of CuAAC for labeling and visualization of glycoproteins in cells have been published by the research groups of Bertozzi¹⁸⁰ and Wong.¹⁸¹ The main advantage of CuAAC over Staudinger ligation is its much faster reaction kinetics. However, the use of CuAAC for applications *in vivo* is limited due to the cellular toxicity of copper ions. This led to the development of copper-free variants of this cycloaddition. Based on observations made by Wittig who described the exothermic cycloaddition of cyclooctyne with phenyl azide,¹⁸² Bertozzi and coworkers reported the copper-free, strain-promoted cycloaddition between azides and substituted cyclooctyne **81** for covalent modification of biomolecules in living systems (Scheme 16.21).¹⁰⁰ The reaction rates were lower than those of CuAAC but comparable to those of Staudinger ligation.¹⁸³ The validity of the approach was demonstrated by functionalization of modified Jurkat cells with a biotin derivative of **81**.¹⁰⁰ Reaction rates of the strain-promoted azide-alkyne cycloaddition could be dramatically improved by introduction of electron-withdrawing fluorine substituents in α position of the triple bond (Scheme 16.21, **82–84**) with the difluorinated cyclooctyne (DIFO) derivatives **83** and **84** possessing comparable kinetics to those of CuAAC.^{183–185} Similar reaction rates were observed with dibenzocyclooctyne derivative **85**.¹⁰¹ These reactions are not regioselective but proceed chemoselectively within minutes on live cells with no apparent toxicity.^{101,184,185} Latest application of DIFO derivative **83** is the *in vivo* imaging of membrane-associated glycans in live

Scheme 16.21 Cyclooctyne derivatives for use in copper-free, strain-promoted azide-alkyne [3+2] cycloadditions designed by Bertozzi (**81**,¹⁰⁰ **82**,¹⁸³ **83**,¹⁸⁴ **84**¹⁸⁵) and Boons (**85**¹⁰¹). The second-generation difluorinated derivative **84** is easier to synthesize than **83**

developing zebrafish.¹⁸⁶ Using two derivatives **83** with different fluorophores attached, it was possible to perform a spatiotemporal analysis of glycan expression and trafficking.

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