Synthesis and Application of Glycopeptide and Glycoprotein Mimetics

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Abstract Glycosylation of proteins is the most complex form of posttranslational modification. Glycan chains of glycoproteins are involved in numerous biological recognition events, such as protein folding, cell–cell communication and adhesion, cell growth and differentiation, as well as bacterial and viral infection. As an alternative to glycoprotein synthesis, approaches to the construction of glycopeptide and glycoprotein mimetics have been developed. These so-called neoglycopeptides and neoglycoproteins, respectively, provide insight on the importance of distinct structural elements on biological activity. Furthermore, they may have improved properties, such as increased stability in the case of S- and C-glycopeptides. This article summarizes recent progress in the field of synthetic glycosylated amino acids, peptides, and proteins with non-natural structural elements with a focus on the carbohydrate–peptide linkage. Linear and convergent approaches are discussed. Application of chemoselective ligation reactions provides access to pure glycoforms of glycoprotein mimetics.

Keywords Glycopeptides · Glycoproteins · Glycobiology · Neoglycoconjugates · Bioconjugation · Chemoselective ligation

Abbreviations

Ac	Acetyl
All	Allyl
Aloc	Allyloxycarbonyl
Azy	Aziridine-2-carboxylic acid
Bn	Benzyl
Boc	<i>tert</i> -Butoxycarbonyl
Bu	Butyl
<i>t</i> -Bu	<i>tert</i> -Butyl
Bz	Benzoyl
CM	Cross-metathesis
COD	Cycloocta-1,5-diene
dba	Dibenzylidene acetone
DBU	1,8-Diazabicyclo[5.4.0] undec-7-ene
DCL	Dynamic combinatorial library
Ddv	1-(4,4-dimethyl-2,6-dioxocyclohexylidene)isovaleryl
DIC	<i>N,N'</i> -Diisopropylcarbodiimide
DIEA	<i>N,N'</i> -Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
DMF	<i>N,N</i> -dimethylformamide
dppf	1,1'-Bis(diphenylphosphanyl)ferrocene
DTT	Dithiothreitol
EDT	Ethane-1,2-dithiol
EPO	Erythropoietin
Et	Ethyl
FITC	Fluorescein isothiocyanate
Fmoc	9-Fluorenylmethoxycarbonyl
HATU	2-(1 <i>H</i> -7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	2-(1 <i>H</i> -Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	1-Hydroxybenzotriazole
KHMDS	Potassium hexamethyl disilazide
ManLev	<i>N</i> -Levulinoylmannosamine
MBHA	4-Methylbenzhydrylamine
Me	Methyl
NaHMDS	Sodium hexamethyl disilazide
NEM	<i>N</i> -Ethylmorpholine
NMM	<i>N</i> -Methylmorpholine
Pfp	Pentafluorophenyl
Ph	Phenyl
Pht	Phthaloyl
Piv	Pivaloyl
PTM	Posttranslational modification
py	Pyridine
PyBOP	Benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate
rt	Room temperature
SBL	Subtilisin <i>Bacillus lentus</i>

SPPS	Solid-phase peptide synthesis
Su	Succinimidyl
TBAHS	Tetra- <i>n</i> -butylammonium hydrogen sulfate
TBS	<i>tert</i> -Butyldimethylsilyl
TFA	Trifluoroacetic acid
Tfa	Trifluoroacetyl
THF	Tetrahydrofuran
TIPS	Triisopropylsilyl
TMS	Trimethylsilyl
Ts	Tosyl
Z	Benzyloxycarbonyl

1

Introduction

The most surprising result of the human genome project probably was the fact that our genome consists of less than 30 000 genes. This low number underlines the importance of posttranslational modifications (PTMs) in order to reach the complexity of higher organisms. Among the PTM events that are known today, glycosylation is the most complex form and requires about 1% of mammalian genes [1]. The functions of the glycan chains of glycoproteins span the complete spectrum from being essentially unimportant to being crucial for the survival of an organism [1–10]. Glycan chains of glycoproteins modulate physicochemical properties of proteins, such as solubility, viscosity, charge, conformation, and dynamics and thereby can bestow stability and resistance to proteolytic degradation and can lead to improved protein efficacy. On the other hand, the glycans provide unique epitopes for molecular recognition that are involved in cell–cell communication, cell growth and differentiation, cancer metastasis, bacterial and viral infection, and they direct protein folding.

Oligosaccharides are attached to proteins mainly via an *N*-glycosidic bond to asparagine (Fig. 1) or an *O*-glycosidic bond to hydroxylated amino acids,

Fig. 1 Common structure of all *N*-glycoproteins: core pentasaccharide attached to the consensus sequence Asn-Xaa-Ser/Thr

Fig. 2 Selected carbohydrate-peptide linkages found in *O*-glycoproteins

such as serine and threonine (Fig. 2) [11–15]. Whereas all *N*-glycoproteins share the same core structure 1, which is derived from a common biosynthetic oligosaccharide precursor, a variety of carbohydrate-peptide linkages are found in *O*-glycoproteins. In the mucins, the GalNAc(α 1-O)Ser/Thr linkage 2 (also called Tn antigen) is found. Another widely occurring *O*-glycosidic bond is the GlcNAc(β 1-O)Ser/Thr linkage 3. Less common *O*-glycosidic linkages include those to hydroxylysine (4) and tyrosine (5, 6).

Since the glycan chains are secondary gene products that are not synthesized in a template-controlled fashion, they usually occur as complex mixtures of glycoforms, making their isolation and analysis a demanding task. Chemists, therefore, have developed synthetic strategies for the preparation of pure glycoforms of proteins required for the evaluation of structure-function relationships [16–26]. The total synthesis of glycoproteins, however, is still an enormous challenge and remains the domain of a few specialized laboratories around the world.

The synthesis of glycopeptide and glycoprotein mimetics, also referred to as neoglycoconjugates [27], represents an alternative strategy to produce single glycoforms for biological studies. The replacement of a natural structure with a non-natural one allows study of the influence of distinct structural elements on biological activity, but has many practical applications as well. Use of chemoselective ligation reactions makes glycoconjugate synthesis accessible to a broader community. Furthermore, *S*- and *C*-glycosidic bonds for example are more stable than the corresponding *O*-glycosides (both chemi-

cally and with respect to enzymatic degradation), which leads to an increased half life of a glycoconjugate within a biological system. Thus, beneficial properties of glycans may be permanently linked to a protein, which is an important aspect for pharmaceutical applications. This article summarizes recent progress in the field of synthetic glycosylated amino acids, peptides, and proteins with non-natural structural elements, with a focus on the carbohydrate–peptide linkage. For a more complete coverage of this wide area, the reader is referred to other excellent reviews [23, 28–38].

2

Strategies for Assembly of Glycopeptide and Glycoprotein Mimetics

Scheme 1 summarizes the main strategies that have been explored in the past for the preparation of glycopeptide and glycoprotein mimetics. Two major approaches can be distinguished: the linear and the convergent assembly. In the linear assembly, carbohydrates are coupled to amino acids **7** to give modified amino acids carrying either mono- (**8**) or oligosaccharides (**10**). These are used as building blocks in solution or (more commonly) solid-phase pep-

Scheme 1 Linear and convergent strategies that have been employed for the synthesis of glycopeptide and glycoprotein mimetics

tide synthesis (SPPS) to provide glycoconjugates **9** and **11**, respectively. Linear approaches are covered in Sect. 3 of the article. In convergent approaches, carbohydrates are coupled to presynthesized peptides or to proteins (**12**) to give **9** and **11**. Depending on the type of chemistry used for carbohydrate attachment, there might be a need for protecting groups at the peptide and carbohydrate, an approach which is confined to synthetic peptides. Conjugation of carbohydrates to full-length proteins is possible via chemoselective ligation to amino acids with a unique reactivity, for example cysteine residues. Combined with site-directed mutagenesis, this approach allows for control of both site of attachment and type of saccharide. Alternatively, genetically engineered proteins containing non-proteinogenic amino acids with a unique chemical reactivity can be employed.

Mixed linear and convergent assembly strategies have also been described. Glycopeptide mimetics **9** or **11** synthesized by the linear route have been extended in the glycan part by convergent attachment of an oligosaccharide through chemoselective ligation. Convergent and mixed approaches for assembly of glycopeptide and glycoprotein mimetics are dealt with in Sect. 4. Not shown in Scheme 1 are the assembly of peptide and neoglycopeptide fragments by native chemical ligation [39] and the incorporation of non-proteinogenic glycosylated amino acids into proteins by in vivo suppressor tRNA technology [40–42].

3

Synthesis of Glycopeptide Mimetics by Linear Assembly of Glycosylated Amino Acids with Unnatural Linkages

3.1

O-Glycosides

Several groups have described building blocks for the preparation of glycopeptide mimetics containing O-glycosidic bonds. Based on chemistry developed earlier [43], Nativi et al. prepared α -O-linked glycohomo-glutamates **16** as replacement for naturally occurring α -O-glycopeptides found in mucins (Scheme 2) [44]. β -Ketoesters **13** obtained from aspartic acid and Meldrum's acid were reacted with phthalimidodisulfonyl chloride. Base treatment of **14** gave reactive intermediate **15**, which underwent a hetero Diels–Alder reaction with glycals to give α -O-glycohomo-glutamates **16** as diastereomerically pure isomers. The same approach has been used by Franck et al. for the preparation of N-glycosides [45].

Preparation of the four galactosylated 5-hydroxylysine mimetic building blocks **19–22** was accomplished by Guichard and coworkers (Scheme 3) [46]. The synthesis of the 5-hydroxy amino acid derivative **18** started from hydroxylated piperidinone **17**. Subsequent galactosylation gave **19**. The use of

Scheme 2 Preparation of α-O-glycohomo-glutamates 16 through hetero Diels–Alder reaction with glycals

Scheme 3 Synthesis of building blocks for the preparation of collagen mimetics

pivaloyl-protected galactosyl bromide prevented undesired orthoester formation during the glycosylation step. Building blocks 20–22 were obtained analogously. The galactosylated hydroxylysine mimetics were incorporated into bovine type II collagen (bCII) immunodominant glycopeptides in order to study the fine specificity of bCII-reactive T cells involved in the initiation and/or regulation of collagen-induced arthritis, a mouse model for rheumatoid arthritis [47].

Danishefsky et al. applied β-O-allyl glycosides of mono- and oligosaccharides, including the breast and prostate cancer antigen Globo-H, for a cross-metathesis reaction with Fmoc-L-allylglycine benzyl ester, followed by reduction of the resulting olefins via catalytic hydrogenation, with the concomitant release of the free acid [48]. The obtained non-natural glycosyl amino acids can readily be applied in solid-phase synthesis of potential glycopeptide-based antitumor vaccines.

Peptoids (N-substituted oligoglycines) are peptide mimetics that are characterized by an improved proteolytic stability and high conformational flexibility [49,50]. Roy and coworkers were the first to synthesize glycosylated derivatives of peptoids, which they named glycopeptoids. Several O-linked [51,52] but also N-linked [53,54] and C-linked [52] variants were prepared and used as building blocks for the construction of HIV-1 protease inhibitors [55] and Tn-antigen clusters [56].

Glycopeptide mimetics with a modified peptide backbone in order to reduce biodegradability have been reported by Shin et al. [57]. They synthesized the pentamers **23** and **24** containing O- and N-glycosylated α -aminoxy acids by fragment coupling on solid support (Scheme 4).

Scheme 4 Glycopeptide mimetics with a peptide backbone containing O- and N-glycosylated α -aminoxy acids

3.2

N-Glycosides

In recent years, the copper(I)-catalyzed [3 + 2] cycloaddition of azides and alkynes [58, 59] (often referred to as “click” reaction) has found wide application in the field of bioconjugation [60], including the preparation of glycopeptide mimetics. Depending on whether the azide or the alkyne component is connected to the carbohydrate, *N*-glycosides and *C*-glycosides, respectively, are obtainable. Rutjes and coworkers reacted various glycosyl azides **25** with a series of acetylenic amino acids **26** to obtain triazole-linked glycosyl amino acids **27** and also some peptides (Scheme 5) [61]. With a few exceptions, the yields were generally good to excellent. The triazole linkage appeared to be stable under several acidic and basic conditions (e.g., 1 M HCl in MeOH, 6 h, reflux or 1.25 M aqueous NaOH, 24 h, reflux). Using α - and β -glycosyl acetylenes, several *C*-glycosidically linked triazole-containing glycosyl amino acids were synthesized.

The azide-alkyne cycloaddition was also employed for the preparation of carbohydrate-modified 2(1*H*)-pyrazinones as precursors of glycopep-

Scheme 5 Application of the azide-alkyne cycloaddition for the preparation of triazole-linked glycosyl amino acids

tidomimetics [62] and also for the preparation of glycopeptide and glycoprotein mimetics by convergent strategies (cf. Sect. 4.4).

A different approach towards non-natural *N*-glycosyl amino acids was reported by Klaffke and coworkers [63, 64]. They used disaccharides *N*-glycosidically linked to spacers bearing a second amino function at the other end. This primary amine was linked to the γ -carboxamide function of glutamine within the dipeptide Z-Gln-Gly-OH in a reaction catalyzed by the enzyme transglutaminase. The needed spacer-modified *N*-glycosides were accessible by reaction of the disaccharide (e.g., maltose) with allylamine followed by acetylation and photochemical thioetherification with cysteamine hydrochloride.

Starting from azido-functionalized glucuronic acid **28**, Lindhorst and coworkers prepared glycosyl amino acids with a dendron moiety attached to the sugar giving access to branched glycopeptide mimetics of variable sugar composition (Scheme 6) [65]. Serving as the branching element, amino triester **29** was introduced under standard peptide coupling conditions. Resulting **30**

Scheme 6 Application of a modified Staudinger reaction for the synthesis of dendron-functionalized *N*-glycoside **31**

was converted into the dendron-functionalized *N*-glycoside **31** via a modified Staudinger reaction.

Van Ameijde and Liskamp coupled *N*-linked glyco amino acid building blocks, in which glycosyl amines are connected to the carboxy group of an amino acid via an amide bond, to trivalent cyclotriveratrylene scaffolds with varying spacers [66]. The obtained glycoclusters can be used to study multivalent interactions. Ichikawa et al. explored the use of Steyermark's glucopyranosyl oxazolidinone for anchoring glucosyl moieties onto amines and thiols in aqueous media [67]. The method was applied for the synthesis of a urea-tethered glucosyl lysine conjugate and a corresponding cysteine conjugate.

Thiem and coworkers prepared a *N*⁴-(2-acetamido-2-deoxy- β -D-glucopyranosyl)-L-asparagine analogue in which the side-chain carboxy group of aspartic acid is linked to the 2-amino function of 1,5-anhydro-2-amino-2-deoxyglucitol [68]. The building block was incorporated into a mimic of the V3 loop of the HIV-1 gp 120 envelope glycoprotein by solid-phase peptide synthesis (SPPS).

Inspired by the work of Roy et al. [53, 54], already mentioned in Sect. 3.1, Burger and coworkers reported a preparatively simple synthesis of *N*-linked glycopeptoids starting from iminodiacetic acid using hexafluoroacetone as protecting and activating reagent (Scheme 7) [69]. Dielectrophile **32** reacts with weak nucleophiles such as glycosylamine **33** at the more reactive acid chloride to give the *N*-glycosylated activated glycine derivative **34**. Treatment with sarcosine-*tert*-butylester provided dipeptide **35** under concurrent deprotection of the α -amino group. *N*-Terminal elongation with **36** gave tripeptide **37**.

Scheme 7 Synthesis of *N*-linked glycopeptide **37**

azides from glycals [70]. After Staudinger reduction, the obtained glycosylamines were applied in the synthesis of 2-deoxy- β -*N*-glycopeptides.

3.3

S-Glycosides

Replacement of the anomeric oxygen of *O*-glycosides by sulfur to furnish S-glycosides leads to an increased stability towards acid/base and enzymatic hydrolysis. The preparation of S-linked glycopeptides/proteins has been covered by several excellent reviews [23, 32, 38, 71], most notably a very recent one by Pachamuthu and Schmidt [28]. The present article, therefore, will focus on selected examples.

In the past, two main strategies have been employed for the synthesis of S-linked glycosyl amino acids. One uses an anomeric thiolate nucleophile, which is generally configurationally stable, in S_N2 reaction with a halogen-functionalized amino acid, often a 3-bromoalanine derivative. The major side reactions are disulfide formation via air oxidation and β -elimination to the corresponding dehydroalanine followed by Michael addition, which results in an epimeric mixture at the α -carbon of the amino acid product. Alternatively, a thiol-containing amino acid, e.g., cysteine or homocysteine, is glycosylated by reaction with a glycosyl donor. The reaction can be carried out under basic conditions employing a halogenose leading to an S_N2 displacement. This is particularly convenient for equatorial S-glycosides because the halogen atom is generally found in the axial position. Access to axial thioglycosides is given by acid-catalyzed S-glycosylation following an S_N1 -type mechanism.

Knapp and Myers used thiazolines **38**, derived from peracetylated glucosamine and galactosamine, respectively, by treatment with Lawesson's reagent, for the formation of α -GlcNAc and α -GalNAc thiols **39** (Scheme 8a) [72, 73]. Alkylation with 3-iodoalanine derivative **40** in the presence of base provided the S-glycosylated amino acids **41** in high yields without apparent epimerization at the α -carbon of the amino acid.

A similar approach was applied by Zhu and Schmidt for the synthesis of S-glycosylated amino acids and di- and tripeptides **44** (Scheme 8b) [74, 75]. They reacted α -GlcNAc and α -GalNAc thiols **39** as well as β -GlcNAc and β -lactosyl thiols with 3-bromoalanine (**42**) and 4-bromohomoalanine derivatives (**43**) under phase transfer conditions or in a mixture of DMF and water at pH 8.5. Due to the mild conditions, epimerization was not observed and even Fmoc-protected peptides were applicable.

Wong et al. modified the procedure and developed a two-step one-pot reaction with in situ generation of sugar thiolates **46** (Scheme 9) [233]. Glycosyl thioacetates **45** were first S-deacetylated with NaOH in methanol at pH 7.5 followed by reaction with Boc- and Fmoc-protected 3-bromoalanines **47** to get a great variety of S-linked glycosyl amino acids **48**. Two of them were incorporated into a linear and a cyclic analogue of the cyclic cationic decapeptide

Scheme 8 Approaches to S-linked glycosyl amino acids using anomeric thiolate nucleophiles

Scheme 9 A two-step one-pot reaction for the preparation of S-linked glycosyl amino acids 48 generating sugar thiolates 46 in situ

antibiotic tyrocidine A, which is produced in *Bacillus brevis*. The cyclic analogue was shown to have a better antibiotic profile than tyrocidine A.

An efficient solid phase strategy for the synthesis of β -S-glycosyl amino acids 51 was described by Jobron and Hummel (Scheme 10) [76]. Based on an earlier report [77], they used unprotected sugar thiols immobilized via the 6 position to generate sodium thiolates 49 for nucleophilic attachment to different iodine-substituted Fmoc-protected amino acids 50.

For the stereoselective synthesis of S-linked glycosyl amino acids, e.g., 55 and 56, Cohen and Halcomb prepared serine- and *allo*-threonine-derived

Scheme 10 Solid-phase synthesis of β -S-glycosyl amino acids 51

Scheme 11 Application of cyclic sulfamidates **54** (R = H, Me) for the synthesis of S-linked glycosyl amino acids

cyclic sulfamidates **54** (R = H, Me) (Scheme 11) [78,79]. In their approach, the sulfamidates reacted with in situ-formed β -cesium thiolates, such as **53**, to give the unprotected thioglycosides after hydrolysis of the *N*-sulfates. The use of cesium as the counter ion afforded higher yields due to an increased solubility of the thiolate, allowing the application of higher concentrations. The serine-derived sulfamidate was also incorporated into a peptide on a solid support. Addition of a 1-thio sugar afforded a solid-phase bound S-glycodipeptide, which could be extended to a tripeptide using standard peptide synthesis procedures.

Direct base-catalyzed S-glycosylation of cysteine- and homocysteine-containing peptides using protected glycosyl bromides was reported by Schmidt and coworkers [80]. They reacted cysteine-centered tripeptides, which were accessible via chemical ligation, with peracetylated glucosyl and lactosyl bromides under two-phase conditions and observed the formation of S-linked glycopeptides in very good yields. The strategy was also applied for the synthesis of an S-linked glycopeptide analogue derived from human Tamm–Horsfall glycoprotein in the same laboratory [81].

Two types of novel S-neoglycopeptides were published by Schmidt et al. [82]. Glycosylthiomethyl amides were obtained by reaction of glycosylthiomethyl azides with amino acids and peptides containing aspartate and glutamate thio acids, whereas reaction of glycosylthiomethyl bromides with cysteine and homocysteine derivatives afforded the corresponding S-(glycosylthiomethyl) amino acids and peptides.

3.4

C-Glycosides

The existence of C-glycosidic linkages in naturally occurring glycoproteins was first discovered in 1994 in human RNase, in which a mannose residue is connected to C2 of the indole ring of a tryptophan [83]. A few years later, Manabe and Ito established the synthesis of a corresponding building block, which opened the way for the incorporation of ^{13}C or ^{15}N labels [84]. However, even before the discovery of the C-mannosyl-tryptophan linkage, carbon-linked glycosyl amino acids have attracted the interest of synthetic chemists

because of the stability of this linkage. C-Glycosyl glycines for example were reported as early as in the late 1940s [85]. Yet, it took until 1992 to incorporate C-glycosyl amino acids into peptides. Kessler et al. synthesized C-glycosyl alanine derivatives via free radical addition to dehydroalanine derivatives and dehydroalanine-containing peptides [86]. Bertozzi et al. reported on a C-glycosyl analogue of Gal(β 1-O)Ser (i.e., a C-glycosyl homoalanine) and its use for SPPS of an α -helical C-glycopeptide [87]. Since then, C-linked glycopeptides have been extensively studied by many groups as an approach to obtain stable glycopeptide mimetics and several review articles cover this subject [32, 35, 38]. Selected recent publications will be presented.

Nucleophilic attack at the anomeric carbon is a common pathway towards C-glycosides [88, 89]. Following this approach, Kihlberg et al. synthesized the C-linked isostere **62** of β -D-galactosyl hydroxynorvaline (Scheme 12) [90]. The two key steps in this synthesis were the addition of a homoallylic Grignard reagent to per-O-benzylated galactopyranolactone **57** with subsequent reduction of the resulting hemiacetal with triethylsilane and a Wittig reaction of phosphonium salt **59** employing Garner's aldehyde **60**. Building block **62** was then incorporated into a fragment from type II collagen by solid-phase synthesis. Helper T cell hybridomas obtained in a mouse model for rheumatoid arthritis responded to the C-linked glycopeptide when presented by class II MHC molecules although at a higher concentration compared to the natural O-glycosylated hydroxynorvaline.

Scheme 12 Synthesis of the C-linked isostere **62** of β -D-galactosyl hydroxynorvaline from galactopyranolactone **57**

On the basis of these results, the same group succeeded in 2003 in the stereoselective synthesis of a C-glycoside analogue of β -D-galactosyl threonine in 14 steps [91]. Recently they reported the synthesis of a C-glycoside analogue of β -D-galactosyl hydroxylysine in 17 steps [92]. An Evans allylation established the configuration at the γ -carbon of hydroxylysine and an asymmetric hydrogenation using Burk's catalyst was applied for the α -amino acid moiety.

Schweizer and coworkers demonstrated that ulosonic acids, which are accessible from the corresponding sugar lactones by reaction with *tert*-butylacetate, can be converted into unnatural *C*-glycosyl amino acids via a one-pot intramolecular Ritter reaction [93]. A diaminobutyric acid-derived building block was used as serine replacement in the solid-phase synthesis of a modified opioid peptide.

The stereoselective synthesis of *C*-glycosyl analogues of phenylalanine was reported by Sinou and coworkers (Scheme 13) [94]. Condensation of hex-2-enopyranoside **63** with an aryl Grignard reagent [95] afforded unsaturated α -*C*-aryl glycoside **64**, which was subjected to a Heck coupling with acrylate **65**. The obtained enamide ester **66** was hydrogenated with a chiral DuPHOS- Rh^+ catalyst and subsequently dihydroxylated to give *C*-mannopyranosyl phenylalanine **68**.

Scheme 13 Synthetic route to *C*-mannopyranosyl phenylalanine **68**

For the gram-scale preparation of the *C*-glucosyl phenylalanine derivative **73**, Augé and coworkers used the Negishi cross-coupling as key reaction (Scheme 14) [96]. High-yield coupling of *p*-iodo-*L*-phenylalanine **70** to silylated glycal **69** gave **71**. Further protecting group manipulations and stereoselective hydroboration of the double bond yielded *C*-glycosylated amino acid **73**, ready for use in SPPS.

Gallagher et al. employed organozinc reagents carrying an aryl iodide function for the reaction with glycals [97]. Cross-coupling of the aryl iodide to an alanyl zinc reagent by Pd(0) catalysis led to *C*-glycosyl analogues of glycosyl tyrosines. A *C*-mannosylated building block was incorporated into a cyclic hexapeptide by solid-phase synthesis.

Through cross-coupling of *C*-linked glycosyl acetylenes with racemic N^α -acetyl-3- and -4-iodophenylalanine methyl esters by Pd(0) catalysis Mel-dal et al. received diastereomeric pairs of *C*-glycosylacetylene phenylalanine

Scheme 14 Application of the Negishi cross-coupling as key step for the synthesis of C-glycosylated phenylalanine 73

building blocks [98]. Separation of the diastereoisomers was achieved by selective enzymatic hydrolysis of the N^α -acetyl group of the L-amino acid by acylase I. After Fmoc protection, the building blocks were used in the solid-phase synthesis of neoglycopeptide analogues of the mouse hemoglobin-derived decapeptide Hb(67–76). Dondoni et al. used this kind of sugar acetylene for the syntheses of ethylene isosteres **76** of N-glycosyl asparagines by coupling of metalated sugar acetylenes **74** with the Garner aldehyde **60** (Scheme 15) [99].

Scheme 15 Synthesis of ethylene isosteres **76** of N-glycosyl asparagines from metalated sugar acetylenes **74**

For the synthesis of C-glycosyl alanine derivatives, Wong and coworkers used an asymmetric Strecker reaction starting from C-glycosyl acetaldehyde derivatives [100]. The stereoselectivity of this reaction was strongly solvent-dependent and was reversed by changing from THF to CH_2Cl_2 . Starting with the same type of C-glycosidically linked aldehyde, Arya and coworkers succeeded in the synthesis of carbon-linked analogues of α - and β -galactosyl-serine glycoconjugates using asymmetric enolate methodology [101]. Methylene-elongated serine and threonine C-glycosyl amino acid building blocks were prepared by Thiem et al. employing regio- and stereo-

selective aziridine ring opening methodology [102]. The needed C-glycosyl aldehyde intermediate was assembled by reaction of peracetylated β -D-galactopyranose with propargyl trimethyl silane in the presence of a Lewis acid, similar to a procedure developed by Bednarski et al. [103]. Wang and Linhardt reported the synthesis of serine-based neuraminic acid C-glycosides by using a neuraminic acid-based glycosyl donor in a samarium diiodide-mediated C-glycosidation reaction [104].

Despite early reports on the use of free radical reactions for the synthesis of C-glycosyl amino acids and peptides [86, 105], this approach is still rarely used in comparison to the above-mentioned nucleophilic approaches. Linker et al. reported a short three-step synthesis of 2-C-branched glyco-amino acids by addition of nitroacetate to various glycals in the presence of ceric(IV) ammonium nitrate and subsequent reduction of the obtained bicyclic isoxazoline *N*-oxides [106]. Caddick and coworkers used tetrafluorophenol-linked acrylate on solid phase for the synthesis of C1- and C6-linked glycopeptide mimetics [107]. The work represents the first C-glycoside synthesis on a solid phase using a free-radical methodology.

Radical allylation of acetobromomannose followed by ozonolysis gave a C-glycosyl aldehyde which was further elaborated into a C-mannosyl amino acid by Toone and coworkers [108]. This building block was incorporated into C-linked glycopeptides that acted as mono- and bivalent ligands for the legume lectin concanavalin A. Binding studies on resin beads and in solution were carried out to investigate the effect of carbohydrate presentation on the carbohydrate-lectin interaction. C-Linked glycopeptides have been used before by the group and evaluated as ligands for the Shiga-like toxin 1 binding subunit [109].

Dondoni et al. used C-glycosyl nitrile oxides and acetylenes for 1,3-dipolar cycloadditions to an alkyne or an azide, respectively, to obtain rigidified heterocycle-tethered C-glycosyl amino acids [110]. The isoxazole- or triazole-tethered C-glucosyl and C-galactosyl alanines are promising building blocks for new glycopeptide mimetics. At the same time, Rutjes and coworkers also reported the synthesis of triazole-linked C-glycosyl amino acids employing 1,3-dipolar cycloadditions (cf. Sect. 3.2) [61].

The cross-metathesis (CM) reaction, being an important method for the preparation of diverse neoglycoconjugates [111], was used by Roy and coworkers for the conversion of β -C-allyl mannoside **77** and (*R/S*)-allyl glycine **78** into C-mannosylated amino acid **79** using Grubbs' catalyst (Scheme 16a) [112]. Earlier, the group had already applied CM in the synthesis of C-glycosylated peptoids [52] which have also been reported by Kessler and coworkers [113]. In 2002 McGarvey et al. used this methodology for the reaction of α -C-allyl glycosides from *N*-acetylglucosamine and lactose with protected allyl glycine derivatives, leading to the corresponding unsaturated C-glycosyl amino acid building blocks [114]. In a convergent approach, the reaction was also applied for the modification of an allyl-

a)

b)

Scheme 16 Applications of cross-metathesis (CM) for the preparation of C-glycosylated amino acids **79** (a) and **83** (b)

glycine-containing tripeptide. Liu and Ben applied CM for the preparation of a series of C-linked antifreeze glycoprotein analogues [115]. Another type of C-linked antifreeze glycoprotein analogue was reported earlier by the same group [116, 117]. Nolen et al. used open-chain sugar precursor **80** for the preparation of C-glycosylated amino acids **83** with a linking carbon chain between the C-glycoside and the amino acid shorter than three carbons (Scheme 16b) [118]. Their CM/cyclization strategy provides a stereoselective entry to C-glucosyl alanines **83** ($n = 0$) and C-glucosyl analogues of glucosyl serines **83** ($n = 1$), without the seriously diminished yields previously observed using allyl or vinyl C-glycosides.

Michael and coworkers employed aminomethyl-C-glycosides in a photochemical approach towards unnatural C-glycosyl asparagine derivatives [119, 120]. The products could be obtained by phototransamidation of the aminomethyl-C-glycosides with photoreactive asparagine derivatives under neutral conditions. Conformationally constrained C-glycosyl α - and β -amino acids via Diels–Alder reaction have been reported by Jayakanthan and Vankar [121].

4

Convergent Approaches to Glycopeptide and Glycoprotein Mimetics

Compared to linear approaches, the convergent synthesis of glycopeptide and glycoprotein mimetics offers greater flexibility with respect to the sugars at-

tached to a peptide. Thus, the preparation of several well-defined glycoforms of the same peptide/protein becomes possible. The approach is also of interest for combinatorial glycopeptide synthesis. If chemoselective ligation reactions are employed for the sugar attachment, it is possible to modify unprotected peptides and even whole proteins.

4.1

Formation of C = N Bonds

In 1996 Mutter et al. reported oxime formation as a means to chemoselectively couple reducing oligosaccharides to aminoxy-functionalized peptides [122]. Since then, oxime formation and related C = N bond formations have been widely used to link carbohydrates and peptides with either the carbohydrate or the peptide acting as the carbonyl component. The possibility of using water as solvent and the high chemoselectivity of the reaction, making the use of protecting groups superfluous, are essential advantages of this methodology, which has also been applied in analytical chemistry [123]. However, one has to keep in mind that the reaction is potentially reversible.

Mimetics of *O*-glycopeptides with native sugar-peptide linkages were obtained by Bertozzi and coworkers [124]. A synthetic *O*-glycopeptide with a single *N*-acetylgalactosamine residue was oxidized by galactose oxidase and the obtained GalNAc C-6 aldehyde was reacted with several aminoxy-substituted carbohydrates. Later Bertozzi et al. applied aminoxy-labeled carbohydrates for a chemoselective ligation with (2*S*)-aminolevulinic acid-containing peptides prepared by SPPS to obtain glycopeptide mimetic **84** (Fig. 3) [125]. Similarly, hydrazide- and thiosemicarbazide-modified sugars were used to prepare the corresponding hydrazones and thiosemicarbazones, respectively. Wong and Schultz and their coworkers incorporated *p*-acetyl-L-phenylalanine into the Z domain of staphylococcal protein A by a genetic approach in response to the amber nonsense codon [126] according to a method established earlier [127, 128]. Aminoxy-functionalized mono- and oligosaccharides were ligated and in part further elongated by glycosyltransferase-catalyzed glycosylations. The relatively low mass of the obtained glycoprotein mimetic **85** (approx. 8 kD) facilitated high-accuracy

Fig. 3 Glycopeptide and glycoprotein mimetics obtained by oxime ligation

mass spectrometry and thus a good control of the selective attachment of the unprotected aminooxy-modified saccharides.

In a series of papers, the Dumy group explored cyclic peptides with regioselectively addressable side-chain amino groups for the tetravalent presentation of mono- and disaccharides [129–132]. Recently, they reported the synthesis of a cyclic peptide template with two separated addressable domains (Scheme 17) [131]. Four Boc-protected lysine residues were used for the tetravalent attachment of carbohydrates. Two lysines orthogonally protected by Aloc groups served for attachment of molecular probes. Thus, **86** was first treated with a Pd(0) catalyst and phenylsilane to remove the Aloc groups, followed by attachment of a biotin and a fluorescein residue, respectively. TFA treatment and coupling of pentafluorophenyl (Pfp) ester **87** under basic conditions gave tetra keto-substituted peptides **88** and **89**, re-

Scheme 17 Application of the cyclic peptide template **86** with two separated addressable domains for the preparation of labeled neoglycopeptides **91** and **92**

Nicotra and coworkers applied oxime ligation to the covalent attachment of the C-glycoside analogue **94** of the Tn epitope to T cell epitope peptides, such as **93** modified with two aminooxy acetic acid residues (Scheme 18) [136–139]. The obtained neoglycopeptide **95**, bearing two B-epitopes and one T-epitope, could be presented to and recognized by the

Scheme 18 Ligation of C-glycosyl ketone **94** to aminooxy-substituted T cell epitope peptide **93**

T cell receptor with higher efficiency than related neoglycopeptides having only one B-epitope.

A number of papers describe the use of aminooxy-modified peptides for the preparation of glycopeptide mimetics by chemoselective ligation to unmodified reducing mono- and oligosaccharides. The advantage of this approach is that oligosaccharides isolated from natural sources (e.g., from chemical or enzymatic degradation of glycoconjugates) can directly be used for conjugation to a peptide. Imperiali et al. used this approach to obtain several glycopeptide mimetics containing single GlcNAc residues as inhibitors of oligosaccharyl transferase [140, 141]. Besides aminooxy-modified peptides, peptides with aspartic acid hydrazide were also employed. A problem associated with oxime and acyl hydrazone formation is the fact that the ligation products can exist in two forms, cyclic and acyclic, and each of them as several diastereoisomers (Scheme 19). It has been reported that the acyclic form dominates in the case of ligations with alkoxyamines (Scheme 19a) [142, 143] whereas the cyclic form is preferred with hydrazides (Scheme 19b) [143–147]. The product ratio, however, is dependent on the carbohydrate. In order to fix the sugar in a defined acyclic form, subsequent reduction with, e.g., sodium cyanoborohydride to give the corresponding hydroxylamine and hydrazide, respectively, may be carried out. This process, however, generates new nucleophilic centers (which might have additional utility [148]) and is no longer a sheer ligation reaction.

Scheme 19 Oxime (a) and acyl hydrazone (b) formation leads to mixtures of acyclic and cyclic ligation products

As an alternative strategy, the use of *N,O*-disubstituted hydroxylamines has been described [149]. In contrast to oxime formation, the cyclic nature of the saccharide reducing unit is completely preserved. The reaction also proceeds with high stereoselectivity to give under thermodynamic control the β or α pyranose forms depending on the sugar. Carrasco et al. followed this approach and prepared differently protected amino acids with

a methyl aminoxy group and a methoxyamino group, respectively, in their side chain [150–152]. SPPS following the Boc strategy gave unprotected peptides **101–104** that were chemoselectively glycosylated to generate neoglycopeptides **105–107** (Scheme 20). The ligation yields were in the range of 75% and more. Glycosylation with the sterically hindered threonine derivate **104**, however, failed. The same strategy was successfully applied by Rocchi et al. for the synthesis of opioid deltorphin I analogues [153].

Scheme 20 Peptides containing *N,O*-disubstituted hydroxylamines and their use in chemoselective ligation reactions with unprotected glucose

Aminoxy- and methylaminoxy-substituted homoalanines were incorporated into T4 phage lysozyme and streptavidin by Nishimura and coworkers [154] employing the frameshift suppression strategy on the basis of the four-base codon/anticodon system [155]. Chemoselective coupling between the methylaminoxy-containing mutant streptavidin and unmodified mono-, di-, and trisaccharides allowed for the combinatorial synthesis of several glycoprotein mimetics.

4.2

Formation of C – N Bonds

Arya and coworkers developed automated, multistep, solid-phase strategies for the parallel synthesis of libraries of carbon-linked glycopeptide mimetics, such as **116–118** (Scheme 21) [156–158]. Starting from solid-phase bound peptide **112**, a C-glycosyl aldehyde **113** was attached by reductive amination followed by amide bond formation using C-glycosyl carboxylic acid derivatives **115** to result in neoglycopeptides **116**. Using this approach, a variety of α - or β -carbon linked carbohydrates can be incorporated either at the N-terminus or at internal nitrogens of short peptides in a highly flex-

Scheme 21 Strategy for the combinatorial synthesis of carbon-linked glycopeptide mimetics **116**. Structures **117** and **118** may be obtained analogously

ible manner. The glycopeptide mimetics were used as probes for studying carbohydrate–protein interactions as well as protein folding and trafficking.

Wang and coworkers established a general method for rapid generation and screening of a combinatorial C-glycopeptide library in the one-bead one-compound format [159]. An α -C-galactosyl carboxylic acid derivative **115** was activated as a pentafluorophenyl ester and coupled to the N-terminus of the peptides under standard solid-phase conditions. Partial termination during peptide synthesis (ladder synthesis [160, 161]) allowed for mass spectrometric identification of lead structures bound to single beads. Several glycopeptide sequences mimicking the Gal(α -1,3)Gal epitope in binding to human natural anti-Gal antibodies were identified.

Wittmann and coworkers reported the convergent solid-phase synthesis of multivalent cyclic neoglycopeptides to study the influence of carbohydrate presentation on the binding affinity to lectins (Scheme 22) [162, 163]. Starting from library **119** of linear nonapeptides, side-chain cyclization was achieved by Pd(0)-catalyzed removal of the Alloc and allyl groups followed by HBTU/HOBt activation. Hydrazine treatment liberated Ddv-protected (Ddv = 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)isovaleryl) side-chain amino groups of the cyclic peptides. For the attachment of carbohydrates a new linker based on the Alloc protecting group has been developed.

Scheme 22 Preparation of library **121** of 19440 conformationally restricted glycoclusters for the spatial screening of multivalent lectin ligands

Thus, treatment with activated carbonate **120** led to urethane formation in virtually quantitative yields. The obtained combinatorial one-bead one-compound library **121** of 19440 conformationally restricted, spatially diverse glycoclusters has been screened for binding to wheat germ agglutinin (WGA) by a solid-phase enzyme-immuno lectin-binding assay. Several tetra-, penta-, and hexavalent high-affinity ligands were identified by single-bead Edman degradation after palladium-catalyzed detachment of the sugars according to a strategy described earlier [164]. It was concluded that the spatial presentation of the sugars on the cyclopeptide scaffolds is responsible for the high affinity to WGA.

Cyclic peptides as scaffolds for the attachment of carbohydrates have also been reported by Nishimura et al. [165]. Aminohexyl-spacer modified disaccharides were coupled to glutamine side chains by a reaction catalyzed by transglutaminase according to a method described by Klaffke et al. [63] and further elongated by an α -2,3-(*N*)-sialyltransferase-catalyzed glycosylation. The obtained trivalent glycopeptide mimetics displayed different binding affinity to influenza hemagglutinin depending on the peptide conformation. For the best ligand, a tridentate glycopeptide-protein complex was suggested, based on computer calculations.

Finn and coworkers described the use of whole icosahedral cowpea mosaic virus particles as multivalent carbohydrate display platforms [166]. When treated with α -D-mannopyranosyl-phenylisothiocyanate, up to 120 mannose

residues were attached to lysine residues on the virus surface by thiourea formation. Aggregation of labeled virus with the plant lectin concanavalin A was studied by UV/VIS spectroscopy, transmission electron microscopy, and fluorescence resonance energy transfer. The nature of this multivalent interaction was shown to depend upon the density and position of functionalization of the virus. Modification of adenovirus (AV) lysine residues by 2-imino-2-methoxyethyl-1-thioglycosides was used by Davis and coworkers to modulate the ability of AV to function as gene delivery vector [167]. It was possible to “switch off” AV transfection by the normal receptor pathway and “switch on” a specific sugar-mediated pathway. The same imidate chemistry which had originally been developed by Lee and coworkers for the preparation of neoglycoproteins [168] was applied by the Davis group to generate glycosylated forms of rhamnosidase for use in a bipartite drug-delivery system called lectin-directed enzyme-activated prodrug therapy (LEAPT) [169].

4.3

Sulfur as Nucleophile

4.3.1

S-Alkylation

The first report on the use of the reaction of glycosyl iodoacetamides with cysteine residues for the synthesis of N-linked glycoprotein mimetics came from the Flitsch group [170] and was later followed by a report on the modification of unprotected synthetic peptides containing a single cysteine residue in solution [171]. Dahmén and Kihlberg et al. coupled 2-bromoethyl β -glycosides of galabiose [Gal(α 1–4)Gal], globotriose [Gal(α 1–4)Gal(β 1–4)Glc], and 3'-sialyllactose [Neu5Ac(α 2–3)Gal(β 1–4)Glc] to cysteine and homocysteine residues in T cell-stimulating peptides by cesium carbonate promoted alkylation in yields of 52–95% [172]. Neither β -elimination nor epimerization of the neoglycopeptide stereocenters occurred. More recently, the iodoacetamide approach was applied by Flitsch and coworkers for the semi-synthesis of homogeneous glycoforms of human erythropoietin (EPO) [173]. Several mutants in which N-glycosylation site asparagines had been changed to cysteines were reacted with GlcNAc iodoacetamides in yields of about 50%. This was possible even in the presence of additional cysteine residues involved in disulfide bridges. Purification was readily carried out by lectin affinity chromatography.

Glycosyl iodoacetamides may also be applied to solid-phase glycopeptide synthesis. This allows for selective modification of certain cysteine residues in peptides with several cysteines employing orthogonal protection. Macmillan and Flitsch and coworkers applied the 4-sulfamylbutyryl-AM (safety-catch) resin [174] to obtain neoglycopeptide thioesters derived from the first 28 amino acids of the glycoprotein hormone EPO (Scheme 23) [175]. Peptide 122

Scheme 23 Application of glycosyl iodoacetamide 123 to the solid phase synthesis of glycopeptide mimetic 125

was prepared by Fmoc SPPS and selectively deprotected by reduction with dithiothreitol (DTT). The free thiol was then alkylated with iodoacetamide 123 to give glycopeptide mimetic 124. The glycopeptide was released from the resin with benzyl mercaptan in the presence of catalytic NaSPh after activation with iodoacetonitrile. Deprotection with concentrated TFA gave glycopeptide thioester 125. Later it was shown by Macmillan and Arham [176] that glycopeptide thioesters obtained by this approach are suitable for the synthesis of glycoproteins by native chemical ligation [177]. In the same year, a complex-type disialyl-undecasaccharide was appended to a cysteine residue of an undecapeptide using a bromoacetamidyl oligosaccharide [178]. It was shown that the obtained glycopeptide analogue was resistant to enzymatic digestion by peptide:N-glycosidase F (PNGase-F).

Bertozzi and coworkers adapted Flitsch's haloacetamide method to convergently ligate bromoacetamides of Gal and GlcNAc to a C-3 thiol of a GalNAc(α 1-O)Thr-containing peptide with natural carbohydrate-peptide linkage [179]. The obtained thioether-linked structures can be regarded as mimetics of "core 1" and "core 3" structures of mucin-type glycoproteins. Later the work was extended to the synthesis of thioether-linked analogues of the mucin-related 2,3-sialyl-TF and MECA-79 antigens [180] as well as to the synthesis of N-linked glycopeptide mimetics that replace the glycosidic linkages extending from the core pentasaccharide with thioethers

Scheme 24 Application of the haloacetamide ligation for the convergent assembly of neoglycopeptide **128** containing an undecasaccharide mimetic

(Scheme 24) [181]. A protected pentasaccharide-Asn analogue containing two thiol residues was incorporated into CD52 (a glycopeptide expressed on human lymphocytes and sperm cells) by Fmoc SPPS to give, after deprotection, **126**. Undecasaccharide mimetic **128** was then readily generated by alkylation of **126** with bromoacetamido trisaccharide **127**.

The application of 1-thiosugars for the preparation of S-glycosylated amino acid building blocks has already been discussed in Sect. 3.3. The approach, however, can also be carried out in a convergent manner. To this end, Zhu and Schmidt used O-acetyl protected 1-thio derivatives of GalNAc, GlcNAc, and lactose and attached them to β -bromoalanine- or γ -bromo-homoalanine-containing di- to hexapeptides to give S-linked neoglycopeptides [75]. The reaction selectively gave α - and β -S-glycosides and even worked well with O-unprotected 1-thiosugars. Glycosylthiomethyl bromides and azides have also been applied in a convergent approach [82].

Aziridine-2-carboxylic acid (Azy)-containing peptides represent alternative electrophilic peptides for conjugation with thiols. Van der Donk and Gin and coworker prepared aziridine-containing peptide **132** starting from

Scheme 25 Conjugation of 1-thio-disaccharide **133** to solid phase-bound Azy-containing peptide **132** leads to S-glycopeptide **134**

Fmoc-Azy-OH **129** and solid phase bound alanine **130** (Scheme 25) [182]. Attempts to extend **131** by sequential coupling of single Fmoc-amino acids were unsuccessful, presumably due to intramolecular aziridine N-deacylation by the liberated amine upon Fmoc removal at the [Azy + 2] position (numbering according to the direction of the synthesis). This side reaction could be avoided by employing a dipeptide building block for coupling of the residues in the positions [Azy + 2] and [Azy + 3] leading to **132**. Addition of 1-thio-disaccharide **133** and deprotection gave S-glycopeptide **134** containing the thio analogue of the sialyl Tn (STn) antigen. Similarly, other 1-thiosugars were conjugated. In all cases, base-promoted aziridine ring-opening occurred with high regioselectivity delivering only small amounts of the corresponding β^2 -amino acid derivative.

4.3.2

Conjugate Addition to Michael Acceptors

The conjugate addition of thiols to maleimido moieties [183] has been widely used in bioconjugate chemistry [184]. Hansen and coworkers were the first to apply this reaction to the preparation of neoglycopeptides [185]. A reducing disaccharide was reacted with S-trityl-2-mercaptoethylamine and stabilized in the cyclic form by N-acetylation. After detritylation, the thiol-functionalized carbohydrate was coupled to maleimido-functionalized pep-

tides to result in neoglycopeptides with a flexible linker between carbohydrate and peptide moieties.

Shin et al. synthesized maleimido-modified sugars for the chemoselective reaction with cysteine-containing peptides and proteins (Scheme 26) [186, 187]. Since deacetylation of **135** and similar sugars under mild basic conditions was not possible neither before nor after thiol ligation due to imide opening, TBS-protected sugars, such as **136**, were employed giving access to **137**. Alternatively, unprotected maleimido-modified sugars were obtained by a two-step procedure in 54–95% as shown for the conversion of **138** into **139**. These sugars were successfully attached to glutathione (γ -Glu-Cys-Gly), the synthetic Fas peptide **140**, and to bovine serum albumin possessing a single reduced cysteine at position 58. Later, Shin et al. used thiol-maleimide ligation also for the fabrication of carbohydrate microarrays [188, 189].

Scheme 26 Application of maleimido-modified sugars for the chemoselective ligation with cysteine-containing peptides

Van der Donk and coworkers used dehydroalanine-containing peptides for the chemoselective conjugate addition of glycosyl thiolates [190, 191]. As the dehydroalanine precursor, *Se*-phenylselenocysteine was introduced via SPPS. Oxidative elimination of this precursor was effected by sodium periodate addition and provided the dehydropolypeptide. Subsequent ligation with different α -glycosyl thiolates resulted in thio isosteres of the tumor-associated carbohydrate antigens Tn, T, STn, and 2,6-ST in high yields. The process proceeded with complete retention of the α -anomeric configuration. No stereoselectivity, however, was observed at the newly formed cysteine stereocenter. The

incorporation of selenocysteine into peptides is not only of interest as a precursor for chemoselective modification of peptides but also for the preparation of larger peptides and proteins by selenocysteine-mediated native and expressed protein ligation [192].

4.3.3

Disulfide Bond Formation

The modification of cysteine residues within proteins combined with site-directed mutagenesis offers a strategy for site-selective incorporation of glycans into proteins. Beside the alkylation reactions discussed in Sects. 4.3.1 and 4.3.2, disulfide bond formation has been explored by several groups towards this goal. Boons et al. used 5-nitropyridine-2-sulfonyl activated thioglycosides for the preparation of disulfide-linked BSA-GlcNAc conjugates [193]. More recently, they reported the direct coupling of 1-thiosugars (thioaldoses) with the thiol group of free cysteine-containing peptides and proteins due to difficulties encountered in synthesizing nitropyridinesulfonyl thioglycosides of oligosaccharides [194]. A number of thioaldoses derived from several oligosaccharides including the core pentasaccharide of *N*-glycoproteins were synthesized. Incubation of 1–2 equivalents of 1-thio-chitobiose with cysteine-containing glutathione and BSA resulted in the site-specific formation of the disulfide-linked neoglycoconjugates together with the homodisulfides of glutathione and BSA in statistical amounts. The same technology was applied to the “glycosylation” of an aglycosylated human IgG1-Fc antibody fragment, engineered to have Cys-297 in place of glycan-linked Asn [195]. Mass spectrometry analysis verified site-selective and complete saccharide incorporation without affecting other Fc protein disulfides. The neoglycoproteins displayed enhanced inhibition of superoxide production by γ -interferon-stimulated U937 cells relative to the aglycosylated N297C protein, where increased glycan size correlated positively with increased inhibition.

Davis et al. reported a whole series of carbohydrate derivatives suitable for chemoselective, cysteine-specific synthesis of neoglycoproteins (Scheme 27) [196–202]. Methanthiosulfonates bound to carbohydrates either via a linker (142) or directly (143) (glyco-MTS derivatives) react specifically with thiols of cysteine-containing proteins 146 and allow the specific synthesis of disulfide-linked glycoproteins 147 in high yields [196–198]. This was demonstrated by site-selective conjugation of glyco-MTS derivatives to cysteine mutants of the serine protease subtilisin *Bacillus lentus* (SBL), which does not naturally contain cysteine. A library of 48 glycosylated forms of SBL as single, pure species has been prepared and detailed glycan structure-hydrolytic activity relationships have been determined. The efficiency of the approach of disulfide bond formation using glyco-MTS derivatives was later improved by using glycosyl phenylthiosulfonates (glyco-PTS) 144 [199]. In most cases these reagents gave superior yields of neoglycoproteins 147.

Scheme 27 Glyco-MTS (142, 143), glyco-PTS (144), and glycosyl selenenylsulfide (145) derivatives developed for the synthesis of neoglycoproteins 147 and glycodendriproteins, such as 148

The glyco-MTS strategy has also been used for the attachment of different bi- and tetravalent glycodendrimers to the Ser156Cys mutant of SBL to give so-called glycodendriproteins, such as 148 [200, 201]. These galactose-presenting synthetic glycoprotein mimetics turned out to be nanomolar inhibitors of the co-aggregation of the pathogen *Actinomyces naeslundii* with co-pathogen *Streptococcus oralis*. It was shown that both the multivalent presentation of galactose and the protein degrading activity of this SBL neoglycoconjugate are essential for optimal inhibition.

Recently, Davis and coworkers described selenenylsulfide-mediated glycoconjugation as a new strategy for the synthesis of disulfide-linked glycoproteins 147 ($\text{X} = \text{S}$) [202]. Two alternative routes were studied. Either a 1-thio mono- or oligosaccharide was converted into the glycosyl selenenylsulfide 145 and then reacted with a cysteine-containing protein 146 or the protein was first converted into the corresponding (phenylselenenyl)sulfide by treatment with phenylselenenyl bromide and then reacted with 1-thio mono- or oligosaccharides. With this approach it was possible to prepare neoglycoproteins from several model proteins modified with mono- and oligosaccharides of up to seven saccharide units.

The formation of disulfides under conditions allowing thiol/disulfide exchange can be used for the preparation of dynamic combinatorial libraries (DCLs) [203–206]. First applications of DCLs in carbohydrate chemistry were reported by Sasaki et al. [207] and Lehn et al. [208, 209]. Sando and Aoyama and coworker prepared DCLs of glycopeptide mimetics based on disulfide-linked sugar–peptide coupling [210]. A mixture of 1-thiogalactose and two peptides containing two cysteine residues each afforded in the presence of DTT a disulfide library of at least ten sugar–peptide conjugates and cyclic peptides. Preliminary surface plasmon resonance experiments showed that the library contained active divalent galactoside species capable of cross-linking soluble peanut lectin (PNL) to PNL immobilized on the sensor-chip surface.

A similar approach was applied by Davis et al. [211]. They used their selenenylsulfide-mediated glycoconjugation approach for the preparation of several disulfide-linked glycopeptide mimetics containing glucose and GlcNAc and observed the composition of the library under conditions allowing thiol/disulfide exchange. Upon addition of wheat germ agglutinin, a GlcNAc-binding plant lectin, an enrichment of GlcNAc-containing glycopeptides was observed.

4.4

Azide-Alkyne [3 + 2] Cycloaddition

The copper(I)-catalyzed azide-alkyne cycloaddition [58, 59] represents a mild method for the regioselective preparation of 1,4-disubstituted 1,2,3-triazoles even in a biological environment and is now widely used in bioconjugate chemistry [60]. In their seminal paper, Meldal et al. applied this reaction to the synthesis of unnaturally linked glycopeptides [58]. 2-Azidothiogalactoside **150** was reacted in the presence of two equivalents of copper(I) iodide with solid-phase bound peptides substituted with a terminal alkyne group either at the peptide backbone or at the amino-acid side chain, as shown for propargylglycine derivative **149** (Scheme 28). The reactions gen-

Scheme 28 Copper(I)-catalyzed azide-alkyne cycloaddition for the solid-phase synthesis of triazole-linked glycopeptide mimetics

erally worked with > 95% conversion and > 95% purity (78% purity in the case of 151).

Lin and Walsh applied the copper(I)-catalyzed azide-alkyne cycloaddition for the attachment of 21 different azido-functionalized monosaccharides to 13 derivatives of the cyclic decapeptide tyrocidine with one to three propargyl-glycine residues incorporated at positions 3–8 [212]. 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 sixfold better therapeutic index than the natural tyrocidine.

It has been shown that alkyne- and azido-modified amino acids, such as 2-amino-5-hexynoic acid [213], azidohomoalanine [214,215], and with less efficiency also azidoalanine, azidonorvaline, and azidonorleucine [216], act as methionine surrogates that are activated by the methionyl-tRNA syn-

Scheme 29 Application of the copper(I)-catalyzed azide-alkyne cycloaddition for the preparation of glycoviruses

thetase 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 [41, 217], offers the possibility to use azide-alkyne cycloaddition (and also Staudinger ligation [218–220]) not only for protein labeling within cells or on cell surfaces [215, 216] but also for the preparation of glycoprotein mimetics.

Based on work carried out earlier by the same group [221], Finn and co-workers succeeded in the attachment of more than 100 copies of the two complex tetrasaccharides **152** and **153** to the cowpea mosaic virus (CPMV) (Scheme 29) [222]. To allow for ready quantification of the conjugation, the sugar azides **152a** and **153a** were first converted into fluorescein-labeled compounds **152b** and **153b** by azide-alkyne cycloaddition to the corresponding fluorescein dialkyne. In a second step, dye alkyne derivatives **152b** and **153b** were grafted to CPMV azide **156** to give **157** with loadings of 115 and 105 per virion, respectively. Likewise, attachment of sugar azides **152a** and **153a** to alkyne-modified CPMV **154** under similar conditions gave glycoviruses **155**, assuming the same reactivity as with the fluorescein-tagged sugars. The unfortunate tendency of copper ions to accelerate the hydrolytic cleavage of peptide bonds was suppressed by the use of an excess of the sulfonated copper ligand bathophenanthroline **158** to restrict accessibility of the metal center.

4.5

Metabolic Oligosaccharide Engineering and Subsequent Bioorthogonal Ligation

Metabolic oligosaccharide engineering offers the possibility of using the cell's biosynthetic machinery to introduce carbohydrates with unnatural structural elements into the glycan chains of glycoproteins in cell culture and in animals. Reutter and coworkers have demonstrated that mannosamine derivatives, in which the *N*-acetyl group of ManNAc was substituted with for example *N*-propanoyl, *N*-butanoyl, or *N*-pentanoyl groups, can be taken up by cells and are converted into the corresponding sialosides. These are incorporated into sialoglycoconjugates, replacing in a cell-type specific manner 10–85% of normal sialic acids [223–225]. Application of this approach to different biological systems revealed important and unexpected functions of the *N*-acyl side chain of sialic acids. Sialic acids modified in such a way led, for example, to markedly altered binding and/or infection of different primate polyoma viruses that depend on cell surface sialic acids for entry. Both inhibition (over 95%) and enhancement (up to sevenfold) of virus binding and infection were observed, depending on the modification [224].

The promiscuity of the sialic acid biosynthetic pathway has been exploited by Bertozzi et al. to introduce ketone [226] and azide groups [218] into cell surface sialoglycoconjugates. Due to their unique chemical reactivity, these groups can be used for attachment of chemical probes through bioorthogonal

coupling reactions. The versatility of this technology was demonstrated by an example of selective drug delivery [226]. Several human cell lines were treated with *N*-levulinoylmannosamine (ManLev). Ketone-containing cell surface glycans were conjugated to biotinamidocaproyl hydrazide through the formation of an acyl hydrazone to provide a tag for subsequent detection with fluorescein-labeled avidin or for delivery of a ricin A chain–avidin conjugate. Ricin toxin A chain is a potent inhibitor of protein synthesis. At certain concentrations, the ricin A chain–avidin conjugate showed selective toxicity for cells treated with ManLev.

Later, Bertozzi et al. developed an improved bioorthogonal ligation reaction based on a modified Staudinger reaction [218, 219]. This so-called Staudinger ligation allows a selective reaction between azides and modified triphenylphosphines containing an intramolecular electrophilic trap leading to formation of an amide bond (Scheme 30). The azide and phosphine are both abiotic and essentially unreactive toward cellular biomolecules. Since *N*-azidoacetylmannosamine (ManNAz) **159** is metabolized by cells and incorporated into surface glycoconjugates **160**, the Staudinger ligation can be used with labeled triarylphosphines **161** to produce stable cell-surface adducts **162**. Recently, Bertozzi et al. could show that the Staudinger ligation can also be executed in living animals [227]. The process of cell surface engineering offers a variety of applications including the generation of artificial cellular receptors for drug targeting, viral gene delivery, and diagnostics, the improvement of glycoprotein properties, and the attachment of labels to study the behavior of glycoproteins in vivo [225, 228, 229]. The metabolic labeling approach can also be used for proteomic analysis of mucin-type O-linked glycoproteins [230] and O-GlcNAc-modified proteins [231, 232].

Scheme 30 Cell surface engineering by the Staudinger ligation

5 Conclusion

The biological importance of glycans and the need for pure glycoforms of glycoproteins for biological studies and pharmaceutical applications have been the major driving forces toward the development of strategies for the preparation of these structures. The synthesis of glycopeptide and glycoprotein mimetics allows for structure–activity relationship studies but has many practical applications as well. Replacement of labile *N*- and *O*-glycosidic bonds by more stable *C*- and *S*-glycosides generates compounds with longer half-lives and simplifies their preparation by linear assembly strategies due to the increased stability of the corresponding building blocks. Convergent approaches using chemoselective ligation reactions provide access to homogeneous glycoprotein mimetics that are likely to impact our understanding of how specific glycoforms mediate physiological processes. Despite the progress made in the field, many challenges remain, e.g., the development of methods for the controlled introduction of multiple (different) glycans into proteins.

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