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Review

Recent progress in the field of neoglycoconjugate chemistry

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Abstract

Glycosylation is probably the most complex secondary gene event that affects the vast majority of proteins in nature resulting in the occurrence of a heterogeneous mixture of glycoforms for a single protein. Many functions are exerted by single monosaccharides, well-defined oligosaccharides, or larger glycans present in these glycoproteins. To unravel these functions it is of the utmost importance to prepare well-defined single glycans conjugated to the underlying aglycon. In this review, the most recent developments are described to address the preparation of carbohydrate-amino acid (glycoconjugates). Naturally occurring N- and O-linked glycosylation are described and the preparation of non-natural sugar-amino acid linkages are also included.

Keywords: biomolecular interactions; glycosylation; neoglycopeptides; synthesis.

Introduction

With the sequencing of an ever-increasing number of genomes, and the number of genes encountered, it has become very clear that the primary gene products are mostly unique structural scaffolds and that post-translational modifications (PTMs) of the encoded proteins actually provide the structural diversity required for function. In this context, glycosylation represents the most extended and complex type of PTM, ranging from the attachment of a single monosaccharide [e.g., fucose to Thr61 in human tissue plasminogen activator (1)] to far more complex structural arrangements (e.g., tetrasialylated tetraantennary core-fucosylated complex type N-glycans) in recombinant human erythropoietin (EPO) (2) (Figure 1).

Carbohydrate attachment to the backbone, usually occurring at the protein surface, entails not only modest-to-substantial structure alteration but also often the generation of differently glycosylated variants of a single gene product. Glycosylation has been extensively studied in eukaryotes (3–6) and evidence is growing that in prokaryotes it is also more common than hitherto supposed (7, 8). Glycans have been associated with many biological events such as fertilization (9, 10), cell growth (11), tumor growth/metastasis (12), immune reactions (13, 14), cell communication (15–17), or infections (18, 19). Glycosylation, as opposed to glycation, is an enzymatic process that can occur in the endoplasmic reticulum (ER) as a cotranslational event or in the Golgi apparatus as the newly synthesized protein passes through.

It should be stressed that glycosylation is a contingent cellular process, resulting from non-template-directed, secondary gene events. Different types of glycosylation exist and the two most frequently occurring are N-glycosylation and O-glycosylation (Figure 2). A particular feature of N-glycosylation is that initiation requires a consensus sequon (Asn-Xxx-Thr/Ser; Xxx ≠ Pro). If this motif occurs a preassembled 14-sugar precursor (Figure 2) can be transferred to Asn while the protein is still being synthesized in the ER. Upon completion of protein translation in the ER, the three terminal glucose units are removed by glucosidases and the resulting oligomannose glycan can be trimmed by mannosidases before the glycoprotein is passed onto the Golgi. This trimming is not always quantitative and thus gives rise to structural heterogeneity [ranging from, e.g., Man_3 (20) to Man_9Glc sugars (21)]. In the Golgi several glycosyltransferases are involved in the elongation, and the nature of the resulting glycotope largely depends on the protein migration rate, the availability of donor/acceptor substrate and appropriate enzymes, environmental factors and, last but not least, the preceding enzymatic reaction (22, 23). It is also in the Golgi where the other most abundant type of glycosylation, i.e., O-glycosylation, is initiated. In contrast to N-glycosylation, O-glycosylation builds from the initial addition of an α-linked GalNAc to Ser or Thr and no consensus sequon is known for the initial addition, making this type of glycosylation less predictable. This feature is enhanced through the existence of at least 21 polypeptide-*N*-acetylgalactosaminetransferases (ppGalNAcT-1 to -21) (24) that are encoded by different genes, differ in their amino acid sequences, and catalyze the same reaction. It has be shown that these ppGalNAcTs act in either stand-alone or concerted reaction

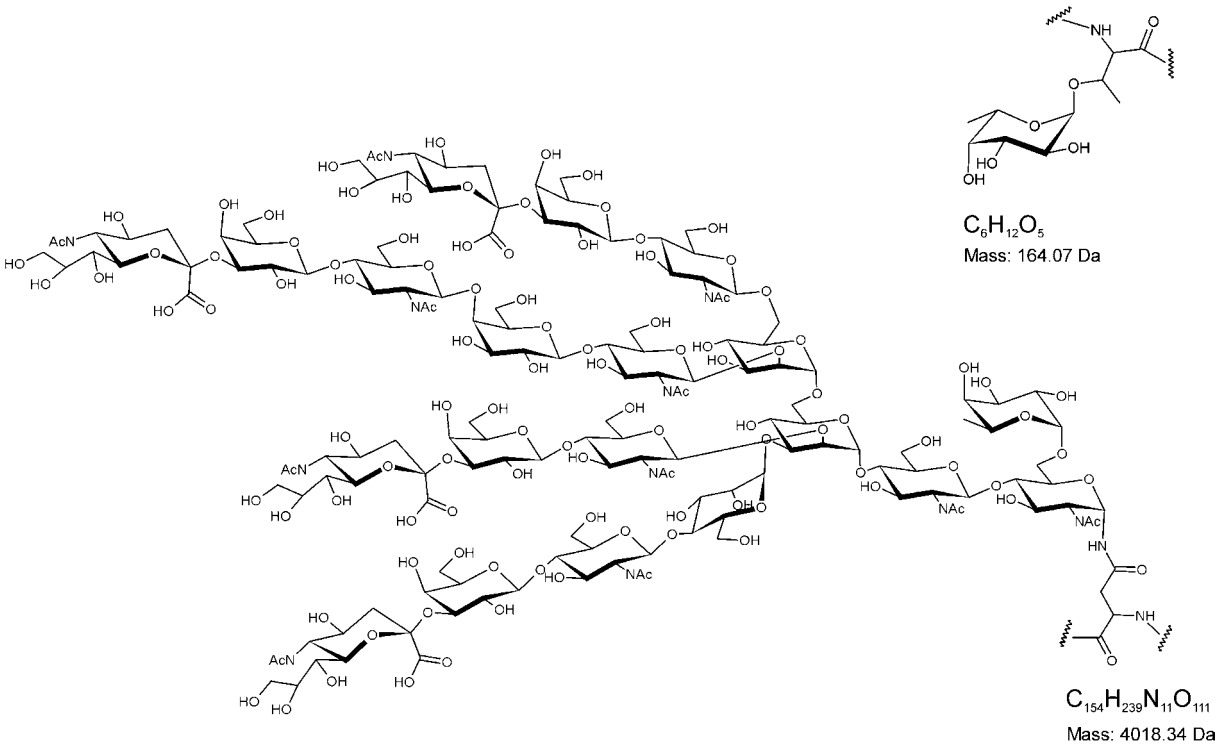

Figure 1 Glycosylation as post-translational modification ranges from a single fucose residue (top) to an *N*-acetyllactosamine repeat containing tetrasialylated tetraantennary core-fucosylated complex type N-glycan (bottom).

modes (25), which suggests that the O-glycosylation is not random at all and that its fine-tuning is not yet understood. Similarly, the other glycosylation steps can be coordinated by several homologous glycosyltransferases, some of which are truly tissue-specific, generating the inherent structural heterogeneity of glycoproteins. The way in which glycans are synthesized, added to the lack of proof-reading mechanisms, has long obscured the understanding of the crucial role played by these entities in the biological phenomena indicated above.

To further unravel the functions that particular glycotopes exert, it is of the utmost importance that chemically well-defined glycans are available, not only as free entities but also as particular glycoconjugates, so that the combined effect of carbohydrate epitope and the underlying structural entity can be evaluated. Much effort has been put into the development of different chemical and enzymatic strategies to generate such glycotope targets in sufficient amounts for structural and functional studies. Unlike DNA, proteins or peptides, where PCR amplification, recombinant DNA technology, and Merrifield solid-phase peptide synthesis (SPPS), respectively, allow for amplification and/or efficient production, carbohydrate synthesis is a technology still under development, relying on non-routine, chemically non-straightforward protocols. Several attempts are being made, particularly by the Seeberger group (26–28), to develop automated synthetic procedures, but despite encouraging results they have not yet reached the universal applicability of their peptide counterpart. In general, glycan synthetic strategies, either linear (glycan incorporated as a preformed glycosyl amino acid building block during peptide synthesis) or convergent (glycosylation carried out on a full-length peptide end product), tend to be as diverse as the structures they target, and to rely on purely chemical, purely enzymatic approaches, or on combinations of both.

In this review, an update of the field is provided in the different areas of glycopeptide and neoglycopeptide synthesis, focusing primarily on chemistries that yield the sugar-amino acid moiety. For simplicity the subject matter is divided between natural and non-natural linkages between the glycan and the non-glycan moieties, and subclassifications are made according to the type of linkage or the chemistry applied during the synthesis. Non-glycosidic linkages between sugars are not included in this review. Further recommended readings in this area can be found elsewhere (28–31).

Natural glycosidic linkages

N-linked glycosylation

Evidently, the optimal mimic of a particular glycoprotein part would be the peptide sequence containing the natural linkage to the peptide-bound monosaccharide and the subsequent glycan epitope. The most frequently occurring glycan types have been addressed by different groups. Thus, Crich et al. (32) employed *N*-benzyloxycarbonyl-L-aspartic cyclic monothioanhydrides and unprotected glycosyl primary amines and *N*-sulfonyl amino acid derivatives in a one-pot synthetic

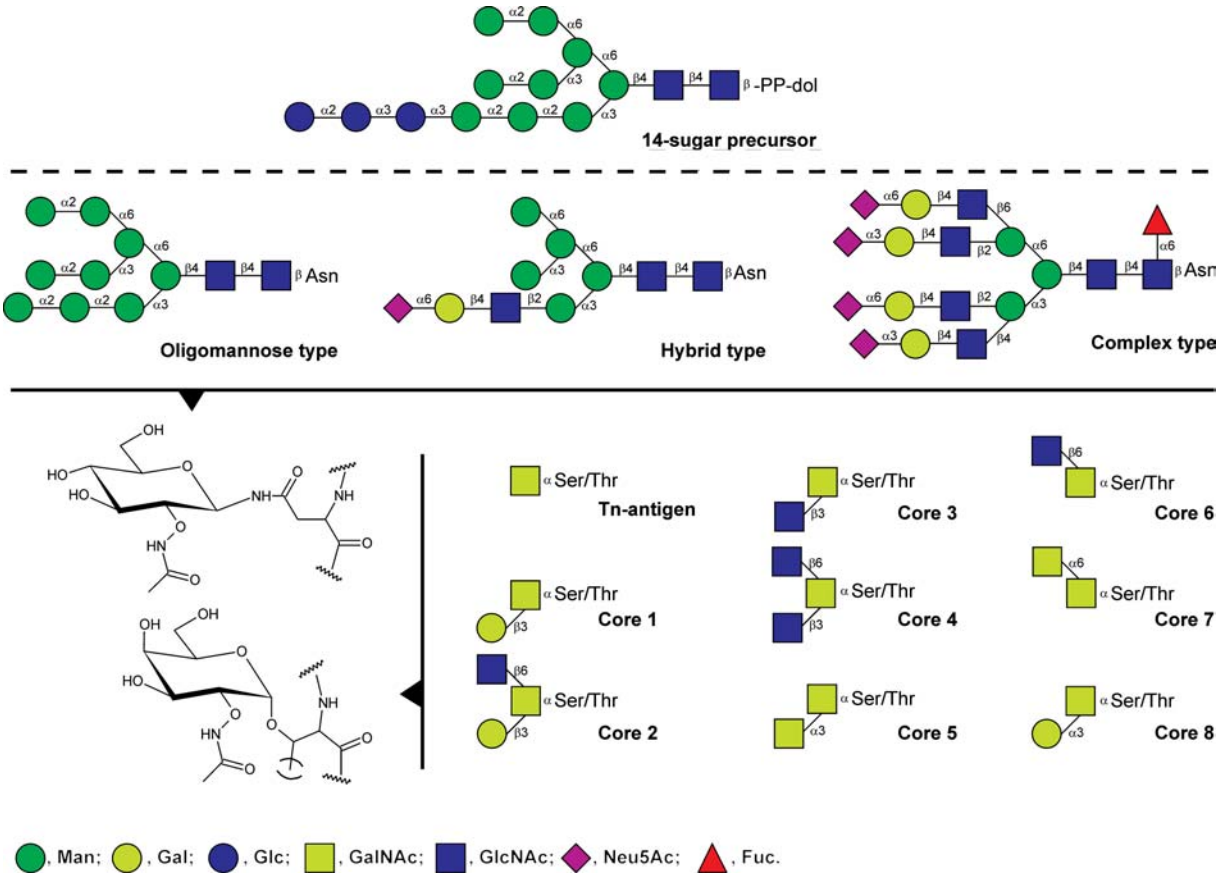

Figure 2 Short-hand notation for the two most frequently occurring types of glycosylation.
The top panel depicts N-type glycans, with the 14-residue dolichol pyrophosphate precursor above the dashed line. Below the dashed line are depicted oligomannose type (left), hybrid type (center), and complex type glycans (right). On the bottom panel on the lower-right, the eight known mucin-type O-glycan core structures are depicted. The symbols are from the Consortium for Functional Glycomics (CFG; http://www.functionalglycomics.org/static/index.shtml).

strategy to generate *N*-glycosyl asparagine derivatives. With the use of sulfonamide-functionalized amino acids the glycan-containing building block could be further elongated to a glycopeptide following a linear strategy. Unfortunately, when the commonly occurring monosaccharide in N-linked glycans, i.e., 2-acetamido-2-deoxyglucose, is a target to be used, the 2-acetamido-2-deoxy-1-glucosamine derivative was found to be a poor nucleophile and limits the yield of this strategy. Also using a glycosylamine but in a convergent strategy, De Bona et al. (33) coupled 6-amino-6-deoxy trehalose to an Aβ pentapeptide in an attempt to produce non-self-aggregating Aβ(1–42) fibrillogenesis inhibitors. In this case the primary hydroxyl functionality was selectively tosylated, then converted to an azido group that upon reduction yielded a primary amine that could be coupled to the side chain carboxyls of Asp/Glu or succinyl-functionalized pentapeptides. In an attempt to combine classical carbohydrate and solid phase peptide synthesis, Swarts et al. (34) generated from glucosamine a fully protected azido-functionalized di- or trisaccharide that was subsequently conjugated to an Asp residue. This element could in turn be incorporated in a conventional SPPS strategy to generate a glycopeptide. The major contribution of this approach resided in the use of a 2-chlorotrityl resin that allowed preservation of acid-labile glycosidic linkages (e.g., the core fucose in N-glycans) upon cleavage from the solid support. Recently, Hu et al. (35) confirmed the utility of this approach in the generation of macrocyclic glycopeptide antibiotics that contained mono- and disaccharides. This synthetic strategy, however, could face limitations when more complex glycans are targeted. Piontek and colleagues worked around this difficulty by making use of unprotected naturally occurring complex type sugars (36). From egg yolk they isolated an Asn-containing biantennary building block of 9–11 monosaccharides that was desialylated and coupled to a functionalized PEGA resin using 1-benzotriazolyloxy-tris(pyrrolidino) phosphonium in the presence of *N,N*-diisopropylethylamine. All free amino and hydroxyl groups were subsequently acetylated to allow elongation of the peptide sequence. Eventually, the glycopeptide and the remaining peptide moiety were put together by native chemical ligation to yield for the first time a synthetic, homogeneously glycosylated glycoprotein of more than 100 amino acids with the expected enzymatic activity (37). Nagorny et al. have embarked on the synthesis of similarly glycosylated glycoproteins building from their expertise in complex glycan synthesis. Their targets include the

β-subunit of follicle stimulating hormone (38) and EPO (39, 40), the latter a glycoprotein of 165 amino acids and four (three N-linked and one O-linked) carbohydrate chains that must be clearly regarded as a major synthetic achievement. Their strategy was based on three fragments (residues 1–28, 29–77, and 78–166), each with one N-glycan and the latter also with the O-glycan at position 126, to be combined by sequential ligation. For glycopeptide generation their strategy relied on the direct attachment of a glycosylamine via Lansbury aspartylation. The outcome of this process is significantly dictated by steric factors, and for the (1–28) fragment glycosylation was only successful (i.e., yields over 50%) when small sugars (disaccharides) were employed. To achieve glycosylation with a dodecasaccharide, smaller peptide sequences and more fragment ligations had to be employed, decreasing the overall yields of the strategy. Despite the fact that the natively occurring glycosylation on EPO [i.e., ultra-complex polysialylated tetraantennary structures (2)] has not been attempted thus far, the Lansbury aspartylation can be employed to generate dense glycoclusters as demonstrated by Krauss et al. (41). No doubt this approach represents a significant step forward in understanding and harnessing the role of single glycoforms. Its future success will largely depend on the availability of C1-amine-functionalized complex type glycans.

O-linked glycosylation

This second most frequently occurring type of glycosylation includes the attachment of Gal to Lys, hydroxy-Pro, and Thr; Glc to Tyr and Thr; GlcNAc to hydroxy-Pro, Ser, and Thr; Ara to hydroxy-Pro; Man to hydroxy-Pro, Thr, and Ser; Fuc to Ser and Thr. This section, however, will focus on the most abundant, so-called mucin-type, glycosylation where a GalNAc is linked to Ser or Thr. This type of glycosylation is produced by many epithelial tissues in vertebrates and serves a variety of functions including protection of the underlying tissue or antifreezing properties by means of T-antigen containing tripeptide repeats [$(Ala\text{-}Ala\text{-}Thr)_n$; n up to 50]. To prepare the latter, Heggemann et al. (42) have converted tri-*O*-acetyl galactal into a 2-azido-1-bromoderivative which by conjugation to Fmoc-Thr and acetamidation of the azido group renders the glycoaminoacid as an α/β epimer mixture in an overall ∼50% yield. This building block can then be incorporated onto any SPPS-generated sequence. In that particular study, the authors were able to produce up to five glycosylated repeats and to demonstrate their effects on the retardation of ice-structuring activity. To generate more complex mucin-type glycosylations, chemoenzymatic approaches (*vide infra*) (43) have been employed on the Tn-containing peptide. Alternatively, the Ser/Thr can be decorated with a core-1 (Gal-β-1,3-GalNAc-α-1-) disaccharide, rather than a monosaccharide, and used as an effective building block in SPPS (44). Baumann et al. used this approach to produce, first, the disaccharide-containing Thr that was subsequently conjugated to a fully protected trichloroacetimidate functionalized tetrasaccharide to produce the sialyl $Lewis^X$ (sLe^X)-containing hexasaccharide amino acid. This building block was subsequently included in the SPPS of the N-terminal 15 amino acid peptide of PSGL-1 (45) with double coupling reactions for the two amino acids following the glycan. Overall, the yield was ∼12–24% but multi-mg amounts could be produced. In a follow-up study, the same group included both enzymatic and chemical tyrosine sulfation (46) to render the true ligand for interaction with P- and E-selectin. Vohra et al. synthesized the same glycan structure on Thr following an alternative approach (47). They also produced the sLe^X-tetrasaccharide and conjugated it ''en bloc'' to the Thr-containing T antigen. To generate the latter, galactosyl trichloroacetimidate was conjugated to a 2-azido-thiogalactosyl acceptor, activated with diphenyl sulfoxide and triflic anhydride in the presence of 2,6-di-t-butyl-4-methylpyridine and threonine to yield the Thr-disaccharide in an overall yield of 61%. A different strategy to produce glycosylated amino acids, reported by Okamoto et al. (48), made use of a sialyl Tn-Thr derivative that was incorporated to a MUC4 sequence by standard SPPS protocols. The disaccharide moiety was itself prepared by coupling of C1-trimethyl- silylethyl (SE)-functionalized 2-azido galactose to a protected Neu5Ac-1-amide-2-phosphite derivative acting as sialyl donor, with catalysis by trimethylsilyl triflate. Conversion of the SE moiety into the trichloroacetamidate and subsequent conjugation to a Ser benzyl ester were very effective (∼70% overall yield) rendering this approach very attractive.

Amino acids other than serine and threonine are also targets for O-glycosylation. In an effort to deliver peptide-based vaccines more efficiently into the cytoplasm of antigen-presenting cells via the mannose-binding receptor, Lee et al. (49) incorporated a mannose residue to Fmoc-hydroxyproline (Hyp) allyl ester using perbenzoylated 1-bromomannose as mannosyl donor. Treatment with $Pd^0(Ph_3P)_4$ yielded the fully protected glycoamino acid ready for SPPS. Biondi et al. (50) described conjugation of per-O-acetylated glucose to both Hyp and Tyr residues in fairly high yields (∼75%).

Glycoconjugate dendrimers

In an attempt to develop tumor-associated carbohydrate antigens with multivalent display and thus enhanced presentation of the underlying peptide aglycon to the immune system, Vichier-Guerre et al. (51) used Koenigs-Knorr condensation, as Heggemann et al. (42), but with an α-1-chloro (instead of 1-bromo) derivative of *N*-acetylgalactosamine and silver carbonate/perchlorate to favor α-anomeric configuration in the end product, which was then incorporated to an Fmoc-protected homoserine acceptor, prepared according to Shiori et al. (52). Other attempts to mimic naturally occurring, dense glycocalyx structures have involved glycoclusters made by coupling trihydroxy amine compounds to trichloracetimidate-functionalized sugars, or trivalent carboxylic acids with aminoethyl-functionalized carbohydrates (53). The Danishefsky group has also made important contributions to this field. In a recent report on the synthesis of a fucosylated GM1 epitope, the pentenyl glycoside of a fully protected hexasaccharide was conjugated to Fmoc-allylglycine benzyl ester by olefin cross-metathesis (OCM) using the Hoveyda-

Grubbs catalyst, yielding after catalytic hydrogenation a five-carbon homolog of the Ser O-glycan (54). They further exploited this approach to build a pentavalent peptide platform displaying the Globo-H, GM2, STn, TF, and Tn carbohydrate epitopes on adjacent amino acids (55). Conjugated to Keyhole Limpet Hemocyanin (KLH) and given to mice, this unique structure elicited antibodies that recognized each of the individual epitopes, thus paving the way for polyvalent carbohydrate vaccines. Jiménez-Barbero et al. (56) parted from a 4,6-silylidene-protected galactal that reacted as dienophile diastereospecifically with a thione-functionalized homoglutamate heterodiene to yield the Gal-β-1,3-Gal-α-1 mimetic which was capable of binding viscumin, a galactose-binding plant lectin. Both synthetic N- and O-linked glycosylation reactions are depicted in Figure 3.

Chemoenzymatic synthetic strategies

Strategies based on incorporating preformed glycosyl amino acids as building blocks for SPPS endure the potential drawbacks of low-yield couplings inherent to large molecular entities, and of cleavage of labile glycosidic linkages under the conditions required for peptide deprotection and cleavage from the resin. To address these problems, combined strategies involving enzymatic elaboration of sugar chains on chemically synthesized polypeptides have been devised. Different enzymes allow glycan attachment to a peptide chain in aqueous solution and with no need of protecting groups. These procedures usually start from a monosaccharide-tagged polypeptide, previously made by chemical means (*vide supra*), or from the products of endoglycosidase treatment of natural glycoproteins. Further elongation of this glycan can then be done in two ways. On one hand, glycosyltransferases can be used to extend sugar chains one monosaccharide at a time. Thus, Ueda et al. synthesized glycosylated glucagon-like peptide 1 analogs from a GlcNAc-bearing precursor peptide made by SPPS and subsequently elongated with β-1,4-galactosyltransferase (β-1,4-GalT) and α-2,6-sialyltransferase (α-2,6-SiaT) (57). Similarly, Gutiérrez-Gallego et al. used this strategy to generate the sialyl Lewis X epitope on a core-2 mucin-type glycopeptide (43). Endo-β-*N*-acetylglucosaminidases (ENGases), on the other hand, are advantageous over common glycosyltransferases in that they can attach in a single step an oligosaccharide to a GlcNAc-containing polypeptide. ENGases hydrolyze β-1,4-glycosidic bonds of N-glycans in glycoproteins and transfer the released oligosaccharyl moiety to another glycosyl polypeptide acceptor. The most used ENGases are fungal Endo-M, which can act on the three major types of N-glycans, and bacterial Endo-A, specific toward high mannose type N-glycans. Transglycosylation reactions, however, tend to suffer from low efficiencies (5–20%), and the enzyme requirement for natural N-glycans as substrate donors limits their applicability. To address these problems, Wang explored sugar oxazolines as substrate donors. Di- and tetrasaccharide oxazolines corresponding to N-glycans cores were synthesized and transglycosylated via Endo-A to a GlcNAc-heptapeptide derived from HIV-1 gp120. The reaction was run under mild aqueous conditions and efficiency was improved from 5–20% to 82%, with the resulting glycopeptides proving resistant to hydrolysis (58). Li et al. made a 47-mer peptide with two N-linked pentasaccharides also by this chemoenzymatic approach. The polypeptide, carrying GlcNAc-Asn residues, was first assembled by SPPS and used to transglycosylate N-linked tetrasaccharide oxazolines by means of Endo-A in an excellent 86% yield (59). Zeng et al. studied the structural donor substrate requirements for Endo-A in more detail and established the Man-β-1,4-GlcNAc-oxazoline as the minimum structure recognized by the enzyme. Whereas changes at the C6 did not alter enzyme activity, configuration inversion at the 2′ and/or 4′-hydroxyls of the Man residue caused total loss of activity (60). In another study, Huang et al. showed that native N-linked and triazole-linked GlcNAc were tolerated by Endo-A as acceptor entities (61). Another report by Rising et al. (62) showed excellent (91%) yields for the transglycosylation of Man-α-1,3-Glc-β-1,4-GlcNAc-oxazoline using Endo-M. In the search for new glycoengineering tools, Huang et al. generated and studied several Endo-M and Endo-A mutants to select an enzyme retaining the ability to form glycosidic bonds but devoid of hydrolytic activity. They identified the Endo-M-N175A and Endo-A-N171A mutants as glycosynthases with high transglycosylation rates (around 70% for large N-glycans) and completely non-hydrolytic (63). Matsushita et al. (64) also employed a chemoenzymatic strategy to synthesize a MUC1-related glycoprotein bearing both N- and O-linked glycans. First, per-O/N-acetylated glycosyl amino acid building blocks [GlcNAc-Asn and GalNAc-β-1,6-(Gal-β-1,3-)GalNAc-Thr] were incorporated on short peptides by microwave-assisted SPPS. The more complex step was then achieved through transglycosylation of Neu5Ac-α-2,3-Gal-β-1,4-GlcNAc-β-1,6-Man-α-1,6-(Neu5Ac-α-2,3-Gal-β-1,4-GlcNAc-β-1,6-Man-α-1,3-)Man-β-1,4-GlcNAc to the GlcNAc-Asn residue using Endo-M, whereas the core 2 epitope was elongated to a sialyl hexasaccharide by sequential action of different glycosyltransferases (β-1,4-GalT, α-2,3-SiaT). Finally, complex glycopeptides bearing N and/or O glycans, and LPKTGLR and GG signal sequences, at the *C*- or *N*-termini, respectively, could be assembled employing sortase-A-mediated ligation to yield a complex, multiglycosylated entity.

The last class of enzymes with synthetic applications is oligosaccharyl transferases (OSTs), which transfer oligosaccharide precursor onto Asn residues. One example is the PgIB from *Campylobacter jejuni* used by Glover et al. to glycosylate the peptide acceptor KDFNVSKA with a synthetic disaccharide donor (GalNAc-α-1,3-bacillosamine-pyrophosphate-undecaprenyl) (65).

Non-natural glycosidic linkages

The term neoglycopeptide was coined to define a new class of glycopeptides characterized by having a non-native sugar-peptide linkage formed between two highly reactive functional groups normally not present in natural glycoproteins. In addition to facilitating glycopeptides synthesis, the non-

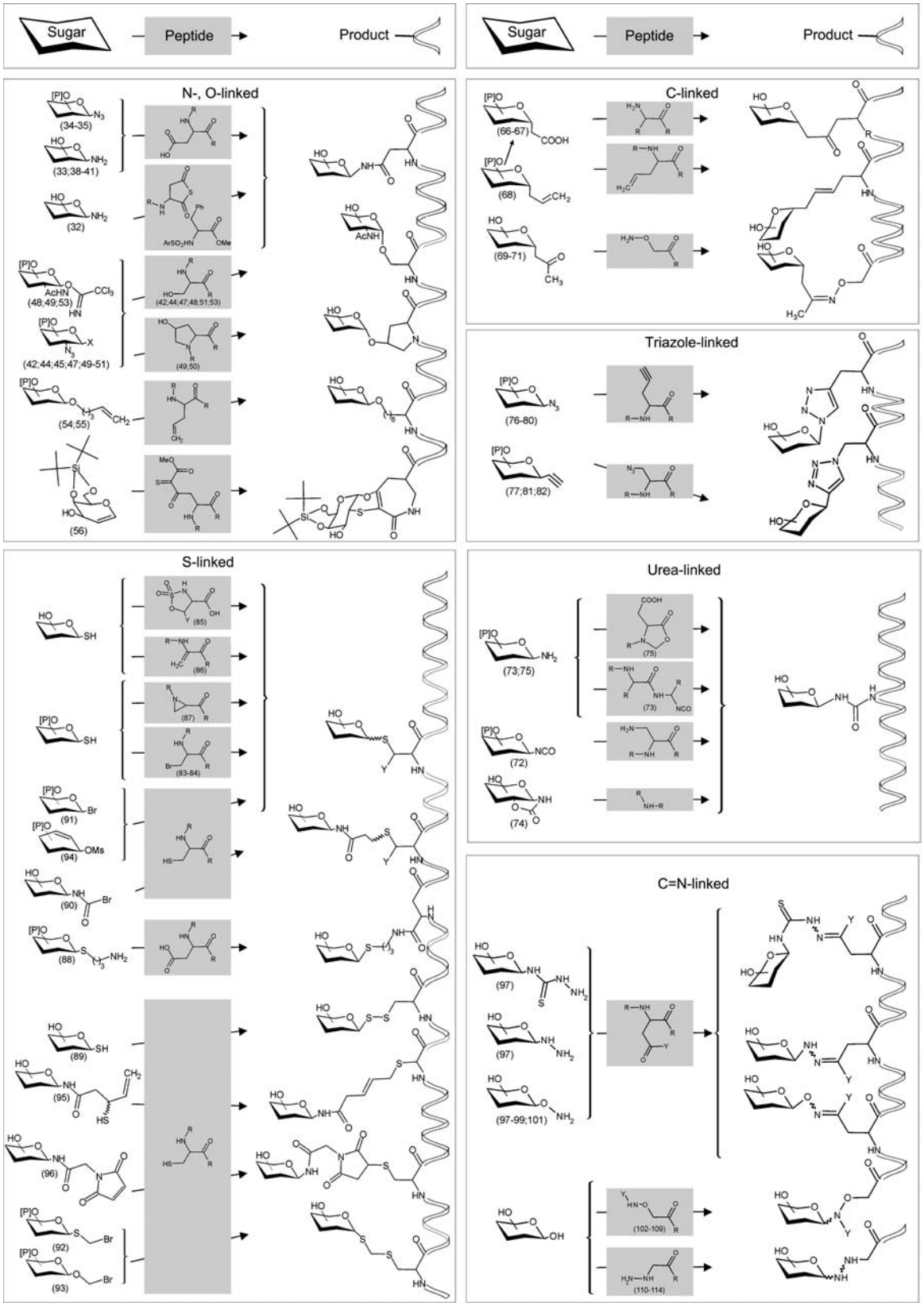

Figure 3 Synthetic chemistries for natural and non-natural glycoconjugate linkages. Chemistries are grouped according to the sugar-peptide linkage; top drawings indicate the direction of the reactions. P, protecting groups for sugar hydroxyls; R, protecting groups in the case of amino acids or remainder of the sequence in the case of peptides; X, bromine, chlorine, or thiophenyl; Y, proton or methyl. Numbers between parentheses correspond to references.

native sugar-peptide linkage can impart stability toward proteases or other adverse conditions, e.g., pH, temperature.

Among the several types of non-natural linkages between sugar and peptide, C-linked glycopeptides have been successfully synthesized and tested under different conditions (Figure 3). Thus, C-fucosyl-acetic acid building blocks were incorporated to a dendrimer library by SPPS and tested as inhibitors of biofilm formation by the pathogen *Pseudomonas aeruginosa* (66, 67). OCM was employed to incorporate C-allyl glycosides into neoglycopeptides by either linear or convergent strategies (68). Another route to C-linked glycopeptides, chemoselective ligation, has the advantage of being done in aqueous media and not requiring auxiliary coupling agents nor protecting groups on either peptide or saccharide. By this approach, C-glycopyranosyl-ketones derived from allyl-glycosides were conjugated to a peptide containing an aminooxy group on the N-terminus or the side chain to give an anticancer vaccine successfully tested against mouse mammary carcinoma (69–71).

Urea-linked glycopeptides, another class of neoglycopeptides, are more water-soluble and enzyme-stable than natural glycopeptides and thus serve as ideal analogs of peptide drugs or enzyme inhibitors. Urea-tethered glycopeptides have been prepared either in a linear strategy, using glycosyl amino acids formed by reaction between *N*-acetyl-D-glucosaminyl isocyanate and amino acid derivates (72), or in a convergent strategy by conjugation of glycosyl amines and peptidyl isocyanates in solution (73). Another approach to urea-linked glycosylated amino acids relied on the reaction between glucopyranosyl oxazolidinone and Lys ε-amino group (74), or vice versa, between glycosyl amines and Fmoc-protected Asp/Glu-5-oxazolidone (75) (Figure 3).

Another type of linkage found in neoglycopeptides is the triazole ring, formed via copper(I)-mediated 1,3-dipolar cycloaddition (Huisgen cyclization, also known as click chemistry) between an alkyne and an azide group (Figure 3). This approach is of particular interest because of the orthogonality of azide and alkyne with other functional groups, and the compatibility with aqueous medium. Several neoglycopeptides have been made from glycosyl azides and alkyne-containing peptides. The alkyne group is usually incorporated to the peptide as a non-natural amino acid (e.g., propargylglycine) (76–78); alternatively, the alkyne functionality has been generated by reaction of a Cys residue with 2-bromoacetyl propargylamide (79), or of a Lys with N-succinimidyl-4-pentynoate (80). Optimized conditions were devised for the reaction between GalNAc-azide and propargyl-containing unprotected peptides; subsequently, two of the resulting triazole-linked GalNAc-neoglycopeptides were joined by native chemical ligation, demonstrating the compatibility between the two chemistries (76). In combination with chemoenzymatic methods, click chemistry has allowed to make carbohydrate-modified cyclic peptides that could find use as antibiotics. Thus, Lin and Walsh used a linear peptide containing propargylglycine that was first cyclized by tyrocidine synthetase and afterwards coupled to 21 different azido monosaccharides (78). To synthesize more complex neoglycopeptides, an azido ''handle'' displaying either STn antigen or clustered Tn was synthesized and conjugated to an alkynyl 10-mer polypeptide. Best yields were obtained when the Cu-catalyzed Huisgen cyclization was carried out in phosphate buffered saline at pH 7.2 (80). Click chemistry can also be performed with inverted functionalities (acetylenic glycosides and azide-containing amino acids), reportedly with slightly lower yields over the original type (77). Nevertheless, GM2-derived anticancer vaccines were efficiently synthesized by ligation between a propargylated GM2 and azide-containing peptide (81) with rates improved from hours to minutes by the use of microwave irradiation (82).

Another category of chemoselective ligation is characterized by the reaction between thiol groups and a variety of electrophiles. As depicted in Figure 3, S-linked glycopeptides have been synthesized mainly by two different strategies. In the first approach, thiohexoses have been reacted with peptides bearing electrophilic moieties such as cyclic sulfamidates, dehydroalanine (Dha), aziridine or halogenated residues (83, 84). For instance, S-linked glycopeptides were made by reaction of Ser or Thr-derived sulfamidates and various unprotected 1-thio sugars, either in solution or on solid support (85). It should be noted that this strategy is limited to the incorporation of mono-, not di- or polysaccharides, at exclusively the N-terminus of the peptide, as sulfamidates do not stand the basic conditions of Fmoc deprotection, and glycosidic linkages are labile to the protic or Lewis acids required to remove the N-sulfate. Another type of chemoselective ligation, the coupling of thiohexoses and Dha-containing peptides was used by Galonic et al. to conjugate four different tumor-associated carbohydrate antigens (Tn, T, STn, and 2,6-ST) to tripeptides. The reaction generated a pair of diastereoisomers at the newly formed Cys α-carbon, but the configuration of the anomeric center was fully retained (86). The same group later used an aziridine 2-carboxylic acid (Azy)-containing peptide and explored conditions for its conjugation with α-SH-GalNAc (87). Halogenated amino acid residues such as β-bromoalanine also allow conjugation of thiosugars, generating S-linked glycosyl amino acids for solid phase or solution-based strategies (83, 84). Thiohexoses have also been incorporated to proteinogenic amino acid residues such as Asp (88) or Cys (89). Several reports have also appeared of the reverse mode of S-linked glycopeptides synthesis, involving thiol-containing peptides and sugars modified at the anomeric carbon as bromides or thiomethyl bromides (90–93), mesylates (94), thiomethylazides (90–92), allylic thiols (95), or maleimides (96).

The last group of chemoselective reactions relies on C=N linkages, such as oximes, hydrazones, and thiosemicarbazones, mimicking the natural structural motifs of N- and O-linked glycopeptides (Figure 3). One approach consists of functionalizing the reducing end of the sugar moiety as a nucleophile (aminooxy, hydrazine, thiosemicarbazide) and reacting it with a carbonyl group in a synthetic peptide (97). In this way, aminooxy carbohydrates have been prepared by reaction of *N*-hydroxysuccimide with glycosyl chlorides (98) or *N*-hydroxyphthalimide with fluoride-activated sugars (99). The electrophilic carbonyl on the peptide is generated either by ozonolysis of dehydroleucine (98) or by periodate oxi-

dation of Ser (100). Oxime ligation between these aminooxy and carbonyl components has allowed the synthesis of both linear and multivalent neoglycopeptides. Although usually carried out in solution, Renaudet and Dumy described a combined oxime ligation/solid phase approach to RAFT-derived neoglycopeptides that allowed recovery of the excess of aminooxy carbohydrate (101). These examples aside, the absence of either aldehydes or ketone groups on the side chains of natural amino acids has caused this type of chemoselective ligations to be usually performed in the reverse mode, i.e., with the aminooxy group on the peptide, either at the *N*-terminus (102) or on the side chain (103). One application of this methodology was simply analytical, as it was found that ligation of complex glycans to basic aminooxy peptides enhanced the sensitivity of their mass spectrometric detection compared to underivatized versions (102). Several neoglycopeptides prepared by this approach have been used as glycoprobes to characterize carbohydrate-lectin interactions. Thus, photoreactive glycoprobes bearing a biotin moiety were synthesized to detect lectins by chemiluminescence (104). Other biotinylated glycoprobes with two post-translational modifications (glycosylation and sulfation) were prepared by two orthogonal chemoselective ligations to study different glycosyl ligands of P-selectin (103). To study carbohydrate-lectin interactions without the requirement of labeling or high sample amounts, an oxime-linked glycopeptide was synthesized and used as immobilized ligand in surface plasmon resonance (SPR) studies (105). In this type of oxime-linked neoglycopeptides the monosaccharide closed to the peptide scaffold is expected to exist in a variety of structural forms (cylic α/β and acylic E/Z isomers), the latter (i.e., open forms) not recognizable by lectins. To overcome this problem, a variation of chemoselective ligation making use of N′-methyl derivatives of the aminooxy peptides, which ensure a closed conformation in the sugar close to the peptide backbone, has been developed. Peptides bearing this N′-methyl-aminooxy at the N-terminus (106) or side chain (106–108) were synthesized and conjugated with several different monosaccharides. A comparative study on the functionality of both types of neoglycopeptides (with versus without N′-methylation) in carbohydrate-lectin interaction studies by SPR was done; both nuclear magnetic resonance and mass spectrometry data demonstrated that for the non-methylated aminooxy function a rather unfavorable 70:30 distribution of open versus closed structures existed. SPR interaction studies with several lectins showed higher responses of the N′-methylated glycopeptides, indicative of a single binding event and in contrast with the behavior of non-methylated analogs (109). Hydrazide is another nucleophile employed to conjugate carbohydrates to peptides, with a strong α-effect that preserves a cyclic conformation of the sugar. Oligosaccharyl transferase inhibitors have been synthesized by this ligation strategy, as well as by oxime chemistry (110). In a comparison with their native-like counterparts, neoglycopeptides showed equal or better binding affinity to the enzyme (111). Hydrazine-linked glycopeptides were also used as probes in photoaffinity labeling studies (112). Possibly owing to their lower stability (compared with oxime-linked) at pH 4–6 (113), hydrazide-derived neoglycopeptides have found their main use as an enrichment handle and signal-enhancing method in N-glycan mass spectrometric analysis (114).

Outlook

The recognition that carbohydrates, despite their structural heterogeneity and non-template driven synthesis, are crucial for many biological processes has brought about considerable efforts both to analyze and describe naturally occurring glycosylation on proteins and to synthesize these glycans, both with and without aglycon, in chemically well-defined form. This, in turn, has made possible the unraveling of additional biological mechanisms where glycans play a crucial part (115–119). Recent examples include the study of the sialylation of Von Willebrand factor (VWF) (115). It was demonstrated that platelet tethering is induced by multimerization of VWF, in turn regulated in plasma by ADAMTS13. Desialylation of VWF enhanced susceptibility to ADAMTS13 proteolysis (but protected against proteolysis by serine and cysteine proteases) indicating a potential role of the absolute quantity of sialic acid on VWF in the platelet tethering. In a study on how cancer cells bind to vascular surfaces and extravasate into target organs Barthel et al. (116) described the upregulation of 3 fucosyltransferases (FT3, 6, and 7) that mediate the synthesis of α-1,3 linked fucose, in liver-metastatic PCa cells. Several membrane proteins were shown to contain the E-selectin binding determinants that subsequently bound firmly to bone marrow endothelium demonstrating that the single addition of a fucose residue dictates the fate of migrating cancer cells. A final example relates to the common flu where host glycans (principally sialic acid, SA) play an important role in the anchoring of viral hemagglutinin (HA) glycoprotein (119). Partial deglycosylation of HA increased binding to SA that was accompanied by a reduced specificity. Furthermore, the authors showed that removal of structurally irrelevant HA glycans elicited better antibody response with higher binding affinities and improved neutralization activity paving the way for next generation vaccines.

Being aware of the importance of glycosylation in nature, enormous efforts have been brought about to synthesize neoglycoconjugates mimicking the natural glycosylation but with improved biological properties (increased stability and solubility), what makes them more suitable for drug development. Thus, Cipolla et al. synthesized and tested a non-hydrolyzable vaccine against Tn-expressing carcinomas by substituting typical O-glycosidic linkage with a non-hydrolyzable C-linkage (71). Other groups centered the focus of their attention in improving the solubility in aqueous media and bioavailability and synthesized urea-linked analogs as potent enzyme inhibitors and peptide drugs analogs (73). Other stable neoglycopeptides, such as S-linked or triazole-linked glycoconjugates, were prepared and tested as analogs of the natural antibiotic tyrocidine. Whereas S-linked analogs achieved a two-fold improvement of the therapeutic index (84) in comparison with the natural component, similar tri-

azole-linked analogs showed more than six-fold increase in therapeutic index (78). Triazole-linked analogs were also considered as an alternative approach to obtain GM2 glycoconjugate vaccines, avoiding the limitations derived from the traditional hemisynthetic strategy (81). Other neoglycopeptides, e.g., S-linked glycoconjugates containing tumor-associated carbohydrate antigens, were synthesized and considered also as potential drugs in cancer vaccine therapies (86). However, well-characterized structural entities do not always lead to straightforward findings. An example at hand is the use by Joyce et al. of mono-, di-, and trivalent $Man_9GlcNAc_2$ containing cyclopeptides by Lansburry aspartylation (120). These glycopeptides had been designed to serve as HIV-1 vaccines based on the specificity of the human 2G12 mAb. Although high titers of carbohydrate-specific antisera capable of competing with 2G12 were generated, they could not neutralize viral isolates nor bind to the glycopeptide immunogen, suggesting that nature's fine-tuning of biomolecular recognition events is still not completely within our grasp. In this respect, future endeavors will certainly have to take a closer look to the non-carbohydrate part of the glycoprobes, as recent examples have shown that the specificity of the molecular communication resides in an adequate combination of carbohydrate and peptide structural information (121).

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