Recent developments in glycoconjugates

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Covering the literature up to January 1999.

1 Introduction

The long history of glycoconjugate synthesis and application has been driven by the ubiquity of glycoconjugates in nature and an overwhelming sense shared by all glycoscientists that their manipulation will, in time, allow fine control of a wealth of biological communication events.[1,2] A detailed description of the broad subject that is glycoconjugate science is beyond the scope of this review and has been well catalogued since the first review on glycolipids in 1937.[3] Even the dissemination of glycoconjugate science has itself been the subject of a review.[4] A dedicated journal, several excellent comprehensive texts[5,6] and a plethora of other reviews have covered many individual aspects of this subject and so it is, instead, the intention of this review to highlight recent developments in the context of other well-established and successful approaches. It is hoped that they might convey a sense of the ingenuity that is being employed in this field and that this will spark yet more exciting work.

It is becoming ever clearer that the very presence of carbohydrate units in naturally occurring structures and their mimetics has a dramatic effect on their physical, chemical and biological properties. Consequently, this review will apply the term *glycosylation* in its broadest sense: as a method that allows the introduction of carbohydrates to structures rather than necessarily as a definition of the formation of the glycosidic bond. Glycoscience is by necessity broad in the range of techniques that it encompasses and it is clear that in this context the oft-applied and somewhat artificial distinction between "chemical" and "biological" techniques is unhelpful. Furthermore, because all such glycoconjugates have potential function there will be no distinction made between synthetic analogues, so-called *neo*glycoconjugates, and those that occur naturally. Indeed, with the advent of an array of techniques adapted from Nature (*e.g.*, see sections 4.3 and 4.4) the distinction between *neo* and natural has become blurred.

Three models for glycoconjugate interactions have been suggested: (i) Carbohydrate Recognition Model – only single oligosaccharide motifs are ligands; (ii) Cluster Model – clusters of many carbohydrate motifs are ligands; (iii) Carbohydrate–Protein Recognition – the binding ligand is both the carbohydrate motif *and* a given region of protein that supports it.[7] It is likely that all play a role and for this reason access to well-defined scaffolds to probe the nature of these models is essential. The elucidation of the mechanism of this binding and its consequences is a dominant primary goal in glycoscience and has driven and continues to drive the synthesis of glycoconjugates. They are the tools of the glycobiology trade.

Typically syntheses of glycoconjugates adopt one of two strategies. The first is the formation of the glycan–aglycone link early, to form, for example, glycosylated building blocks such as glycopeptides or glycosylated dendritic wedges, that may then be assembled. The second is formation of the link late on in the synthesis once the scaffold for its presentation is in place. Given the instability that may be associated with the link and the requirements for protection that need to be considered in the use of glycosylated building blocks, it is clear why the latter has often seemed the most attractive option.

The future of carbohydrate science will be honed by the application of its products; the applications of conjugates are therefore an important backdrop to this review and are the context in which glycoconjugate synthesis should rightly be judged.[8,9] Section 8 therefore provides a brief overview of the extensive applications of such conjugates that are no more limited than the varied utilisation by the body of "Glycocode".

1.1 Inter- and intra-cellular communication and "Glycocode"

Carbohydrate structures are unrivalled in the density of information that they can convey. Precise differences in the nature of the linkages between two residues *e.g.*, 1-2, 1-3, 1-4, 1-6 for two

pyranoses, contrast with the linear nature of proteins and nucleic acids. A comparison of the permutations of hexamer formation illustrates this point well. Whereas, DNA (with a basis set of 4) and amino acids (with a basis set of 20) may construct a biological language for information transfer of 4096 and 6.4×10^7 'words', respectively, carbohydrates have access to greater than 1.05×10^{12} variations.[10] Add to this the additional variety afforded by anomeric stereochemistry, ring size and sub-unit modification (*e.g.*, sulfation, phosphorylation or acylation) and it can be quickly seen that this greater variety of possible combinations gives the language of carbohydrates exquisite eloquence. This language has been christened *glycocode* – a term that well represents the potential level of complex information that carbohydrate structures are able to convey. It should also be noted that this vast number of potential permutations represents a technological barrier and means that no longer can oligosaccharidic structures be made on an iterative basis since there are far too many possible synthetic targets. It is therefore crucial that the design of new carbohydrate-containing structures is guided by the identification of the associated functions of existing structures.

The decipherers of glycocode are typically sugar-binding proteins called lectins which, despite their very shallow binding sites, show a remarkable specificity in their binding of multivalent complex carbohydrate structures.[11] The term lectin, which owes its origin to the Latin word *legere* meaning specific, was first used by Boyd in 1954[12] to describe proteins that show a potent and highly specific ability to bind glycosylated structures. The term has subsequently been redefined to describe carbohydrate-binding proteins that are neither enzymes nor immunoglobulins – although some similarities in modes of binding and some intermediate cases have meant that this distinction is starting to be questioned.

The binding in the so-called carbohydrate recognition domain (CRD) of lectins typically consists of hydrogen bonding from backbone and side chain amide group donors to oxygen lone pair acceptors and from carbohydrate hydroxy group donors to backbone and side chain carbonyls (Fig. 1). Protein-bound calcium ions can also play a role both in coordinating vicinal carbohydrate groups and in 'shawing up' the protein backbone for correct disposition of potential hydrogen-bond donors and acceptors. Moreover, these polar interactions are amplified by van der Waals' interactions between typically aromatic side chains and hydrophobic 'patches' on carbohydrates (Fig. 2). The resulting cradle of non-covalent interactions places a large degree of spatial constraint on which ligands may bind and is part of the source of lectin specificity. Lectins undergo little or no conformational change upon binding and so, in essence, their binding sites are essentially preformed.[13] Scattered around these binding sites are highly ordered, often conserved, water molecules. Since the entropic cost of the presence of these water molecules[14] is largely compensated for by enthalpic gains, these water molecules are important in mediating hydrogen bonds within the binding site and are not "just solvent"; 'water acts as a molecular mortar'.[15]

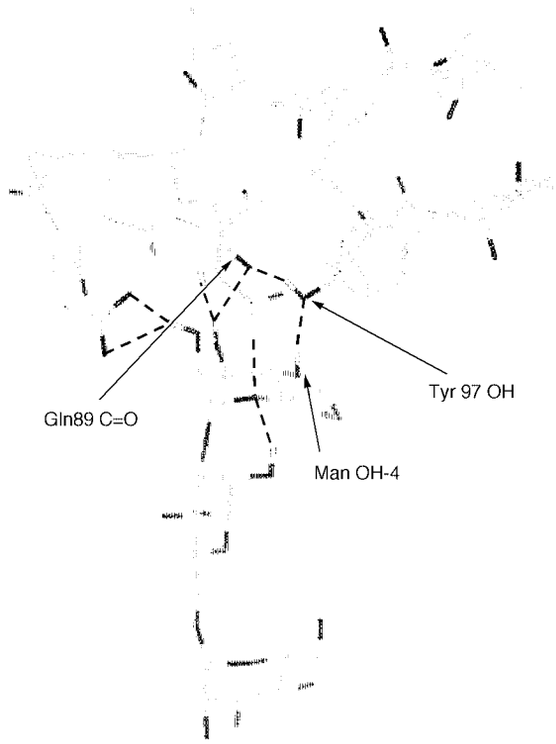


Fig. 1 α-D-Man*p*-(1→6)[α-D-Man*p*-(1→3)]-α-D-Man bound to the snowdrop lectin (*Galanthus Nivalis* Agglutinin, PDB entry ljpc).

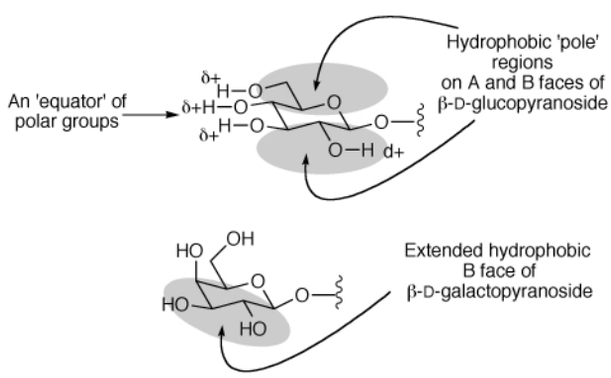


Fig. 2 Hydrophobic and polar 'patches' on carbohydrates.

1.2 The multivalent, oligovalent or "Cluster" effect

The carbohydrate–lectin interaction stands out as an unusually weak and relatively undiscriminating one (K_d in the order of mM for monosaccharides)[16] when compared to others in Nature. This is largely due to the shallow, solvent-exposed nature of the lectin binding sites, which make few direct ligand contacts. The large difference in affinity shown by these shallow sites as compared with deep sites is amply illustrated by the influenza haemagglutinin lectin, which binds sialic acids with an approximately 1000-fold lower affinity than is shown by a neuraminidase found in the same virus.[17] However, when more than one saccharide of the right type and in the right orientation are clustered together there is a rapid increase in both affinity and specificity by the corresponding lectin.[18] This increase is more than would be expected due to the increase in local concentration (statistical effect) alone and has been termed the "cluster" or "multivalent effect".

In a seminal study in 1983, Lee and co-workers highlighted the importance of the "cluster effect" through the synthesis and measurement of the affinity of the hepatic asialoglycoprotein lectin for a range of mono- and multi-antennary β-D-galactosyl terminated structures.[19] The clear importance of multiantennary ligands for binding was demonstrated by the 1×10^6-fold greater avidity of a tetraantennary undecasaccharide than an equivalent monoantennary trisaccharide despite the only 4-fold statistical increase in absolute galactose concentration. Furthermore, whilst internal branch structure had some effect on binding this was shown to be of comparatively minor importance.

The reasons for the cluster effect are yet to be rigorously determined but their implications are profound. Firstly, the steady biological reservoir of soluble monosaccharides are negligible inhibitors of any process that lectins mediate. Secondly, the specificity of this type of binding is exquisitely fine-tuned. It relies not only on the complementarity of the individual binding sites with a particular sugar ligand but also on the relative

arrangement of the binding sites to each other in space and therefore by necessity the corresponding display of each sugar ligand relative to the next. Thirdly, the kinetics of such binding are different to those of monovalent binding and may afford faster "on rates". Fourthly, multipoint attachment is more resistant to shear stresses.

It should be noted that exploitation of the cluster effect does not necessarily rely on mimicking natural multiantennary structures as long as an energetically efficient method for their presentation to binding sites may be found; in this regard polymeric chains that are glycosylated with single residues, for example, may serve as adequate substitutes. In multisite binding, chelation effects are optimized by the presence of just enough scaffold between two ligands to span two sites. Therefore the nature of the scaffold should in theory influence dissociation constant (K_d) values. Any extra structure above that which is needed bears a concomitant entropic cost. Although the limited evidence to date on linkers with different conformational flexibility shows little difference in binding,[20] it is clear that those multivalent scaffolds that are too short to bridge two sites still have higher inhibitory potencies than monovalent ones. This is due to a statistical effect that can be viewed in terms of a high local concentration of ligands that perturbs the dissociation equilibrium.

1.3 Why conjugate?

In Nature multivalent arrays are constructed on the surface of cells either by branched oligosaccharides of glycoproteins which act like sugary hands with each "finger tip" grasping a lectin "bowling ball" or by the sliding together of glycolipids within the lipid bilayers to form carbohydrate-dense, "sticky" patches.[21] There is also a corresponding number of multireceptor display methods, ranging from the oligomerization of single receptor proteins *e.g.*, the asialoglycoprotein receptor in the liver which forms hexamers, through to single proteins which have more than one binding site *e.g.*, mannose-binding snowdrop lectin which has three. Furthermore, in many cellular structures these lectins are themselves multiply expressed in dense collections of membrane protrusions. For example, influenza haemagglutinin is presented by ~500 spikes of lectin trimers.[22] In essence, the arrangement of the clusters of glycans is a second order pattern where the first order pattern is the arrangement of the sugars within the cluster. In this context, Nature ably exploits the tertiary structure of proteins as a scaffold. Yet more complex third order patterns are then in turn the product of the arrangement of these glycoconjugates on cell surfaces. Diverse carbohydrate bonding gives way to diverse oligosaccharide patterns which gives way to diverse cluster pattern which gives way to diverse glycoconjugate patterns. In summary, glycocode is a biological fractal with each layer of structural diversity generating yet another of greater diversity.

There are numerous examples of the important role that the macromolecule that displays a glycan has in determining activity. As early as 1929 it was appreciated that immunological activity toward sugars may be greatly enhanced through conjugation to proteins (for further details see section 8.4).[23] Furthermore, the specificity of such immunological responses to sugars varies greatly with the nature of the protein to which they are conjugated. For example, synthetically prepared glycoproteins bearing the oligosaccharide blood group determinant Lewis-y (Le^y) do not generate antibodies that can react with naturally occurring Le^y bearing structures. This could be due to a number of factors such as different densities of carbohydrate on the conjugate, as well as the influence of the protein or the linker upon conformation or accessibility. However, as yet, the reasons for such striking differences are undefined – an effect is observed and it is one that requires the preparation of glycoconjugates for further investigation.[24] Corresponding differences in lectin binding activities have been well illustrated by the recent preparation and screening of a library of disaccharides on the solid phase by Kahne and co-workers who showed that the order of affinity of these potential ligands for *Bauhinia purpurea* lectin differed greatly on an insoluble resin from when they were separated from the resin and screened in solution.[25]

Conversely there are also many examples of proteins and peptides whose biological activity is enhanced by conjugation to carbohydrates. For example, the activity of the anti-diuretic nonapeptide arginine-Vasopressin is almost doubled through galactosylation.[26] Also, different ribonuclease B glycoforms that were carefully separated using capillary electrophoresis show 4-fold different hydrolysis activities. They also show decreased flexibility and greater protease resistance, possibly through the action of the glycan as a "steric shield" for protease cleavage sites.[27]

The link between the glycan and the molecule to which it is to be conjugated (the spacer arm) can be, within the definition of glycoconjugates, of virtually infinite variety and therefore a detailed discussion of different spacer arms is outside the scope of this review. However certain important structural features should be borne in mind. Several studies have investigated the effect of spacer arm length upon the affinity of some proteins (*e.g.*, selectins[28] and the asialoglycoprotein[29]) for glycoconjugates. The clear consensus result is that an optimal length is required that is long enough to allow accessibility but short enough that the loss of entropy upon binding is not a prohibitive cost in the binding equilibrium.

1.4 The need for homogeneity and pure, well-defined conjugates

Unlike the biosynthesis of proteins and nucleic acids there appears to be no associated mechanism for proof-reading and correcting differently glycosylated biomolecules – the result is mixtures. Therefore, glycoproteins occur naturally in a number of forms (glycoforms)[30] that possess the same peptide backbone, but differ in both the nature and site of glycosylation. The different properties exhibited[27,31] by each component within these microheterogeneous mixtures present regulatory difficulties[32] and problems in determining exact function through structure–activity relationships. It has even been suggested that these naturally occurring mixtures of glycoforms provide a spectrum of activities that can be biased in one direction or another as a means of fine-tuning.[27] Consequently, the few studies that have compared single glycoforms successfully[27] have required abundant sources and extensive chromatographic separation. There is therefore an urgent need for sources of homogeneous glycoconjugates. The development of highly successful small-molecule carbohydrate containing ligands has often involved careful structure–activity relationship (SAR) refinements.[33] If we are to achieve the same successes with large molecule glycoconjugates, then homogeneity must be our first priority.

Whilst the construction of the macromolecule–carbohydrate link is the focus of this review, the importance of a well-defined homogeneous source of glycan should not be understated. The elegant synthesis of oligosaccharides[34,35] must continue hand-in-hand with methods for their conjugation.

This review divides the preparation of glycoconjugates loosely according to scaffold type as this reflects existing similarities in synthetic strategy. However, it is important to note that concentration of the efforts of glycoscience on conjugation to only one type of scaffold or framework for carbohydrate display only serves to create false oppositions between different synthetic camps. Knowles has clearly illustrated the importance of open-mindedness in this respect, through the synthesis of bivalent influenza haemagglutinin inhibitors.[36] Whereas ethylene glycol and piperazine scaffolds displayed poor binding due to too little and too much flexibility, respectively, a glycine scaffold proved well-suited. Clearly, no single framework will

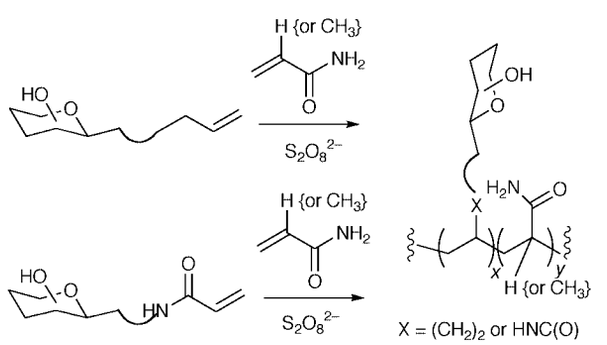
always provide ideal carbohydrate display and this is reflected in the great variety of systems used in the applications highlighted in section 8.

2 Glycopolymer synthesis

2.1 Linear polymers

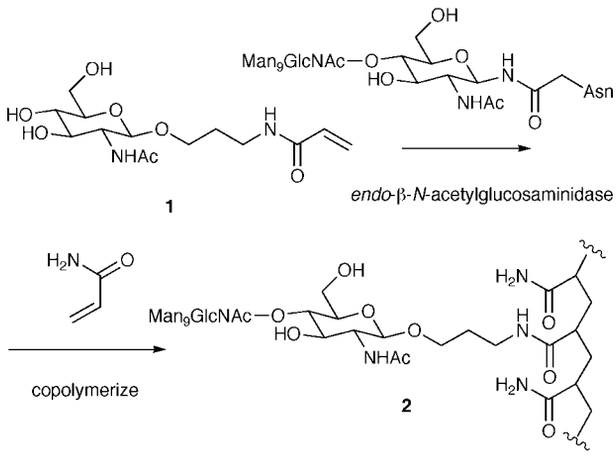
As for all glycoconjugate syntheses, two distinct approaches may be identified in the synthesis of polymers according to the point in the strategy at which the glycan-scaffold bond is made: in this case, pre- or post-polymerization. In this context many standard polymerization techniques have been adopted for the synthesis of pre-polymerization glycosylated polymers by simple extension to glycosylated monomers.[37–39] It is clearly important that in such cases, if unnecessary protection steps are to be avoided, that such methods tolerate the presence of hydroxy groups. Examples of three such methods are the polymerization of acrylamide, styrene and norbornene † monomers.

A large number of spacer-arm linked monomers can be polymerized according to standard polymer techniques (Scheme 1). For example, early glycopolymers were made from allyl glycosides through copolymerization with acrylamide using persulfate as a radical initiator.[40] However, the use of these short spacer arms and the fact that copolymerization with acrylamide favours incorporation of acrylamide and not allyl groups has meant that acrylamide based glycosides have become more popular.[37] An advantage of this technique is that the glycosylated monomers may be prepared by acryloylation of a variety of readily available amine-bearing carbohydrates, *e.g.*, glycosylamines or *p*-aminobenzylglycosides, with acryloyl chloride. Copolymerization of differently functionalized acrylamides allows the introduction of a variety of effector groups along with glycosyl units. For example, D-*N*-acetylgalactosamine (GalNAc) and L-rhamnose (Rha) bearing monomers have been copolymerized with biotin bearing monomers and acrylamide in terpolymerizations.[41] In an elegant and rare example of the use of endoglycosidases in synthesis, the *endo*-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) was used to transglycosylate the *N*-acetylglucosamine monomer **2** with the high mannose core of the *N*-linked glycoprotein soybean agglutinin derived from digestion with the peptidase, pronase (Scheme 2).[42] Subsequent copolymerization with acrylamide gave **2** which was a better inhibitor of mannose binding protein than soybean agglutinin itself.

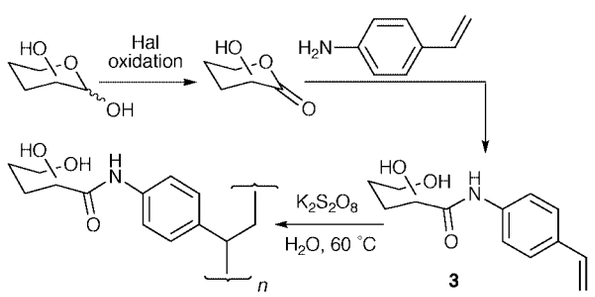


Scheme 1

Standard radical-initiated homopolymerization allows preparation of glycosylated styrenes from *p*-vinylbenzamide *N*-acetyl chitopentaosyl-[43] and maltoheptaosyl-[44] monomers, such as **3**, themselves prepared from sugar lactones (Scheme 3). In a similar approach this method has been extended by reversing the nature of the styrene–sugar link which is instead formed from glycosylamines and *p*-vinylbenzoyl chloride.[45] This has the advantage of not destroying the cyclic nature of the reducing end carbohydrate residue. Recently, further examples of the same type of monomer with more complex glycans have been prepared using glycosidases.[46]



Scheme 2

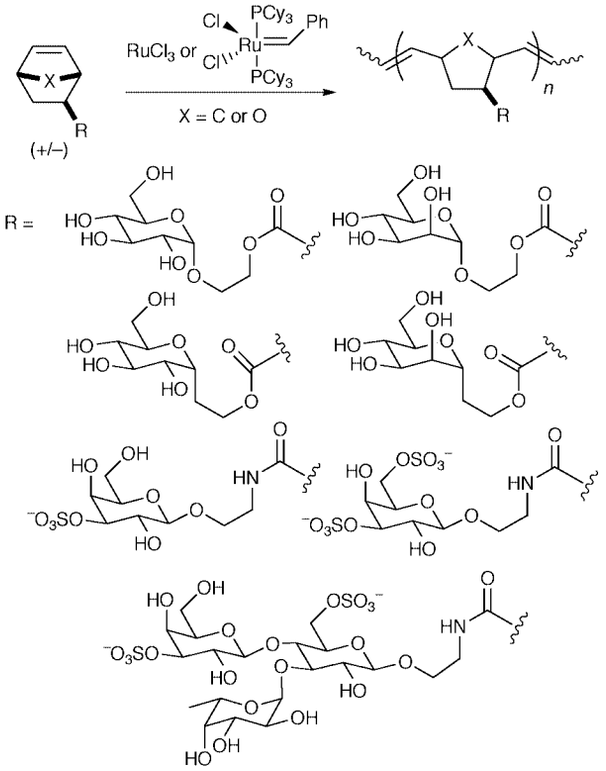


Scheme 3

One disadvantage of standard polymerizations is that often broad mass distributions result and laborious purification will then be required to form homogeneous materials. Ring-opening metathesis polymerization (ROMP) catalyzed by $RuCl_3$ has also been used to create glycopolymers and well defined oligomers (see section 2.3) from glycosylated norbornene monomers bearing one or two pendant D-mannosyl, D-glucosyl residues (Scheme 4).[47,48] As for polyacrylamides, these polymerizations tolerate the presence of polar groups, such as carbohydrate hydroxys and so may be conducted using unprotected sugars in aqueous solution. The use of Grubbs' catalyst in such systems also allows living polymerizations, in which termination does not compete with polymerization. This allows the preparation of polymers with precise degrees of polymerization (DP) and therefore narrow molecular mass dispersities. It should be noted though, that the resulting variety of *cis* and *trans* links (~30–50% *trans*) may lead to an unpredictable local structure. Single 3-sulfo-D-galactosyl and 3,6-disulfo-D-galactosyl monomers, prepared through regioselective sulfation using a temporary borate protection and stannyl ether activation strategy (see section 6.1) have also been used.[49,50] In these latter two cases, emulsion polymerisation was used to overcome difficulties associated with the conventional polymerization technique. Interestingly, higher lectin-binding inhibitory potency was observed for polymers of *C*-glycosides than for *O*-linked glycosides and for those with only one pendant mannosyl or glucosyl residue per repeating unit rather than two.[47] The latter result probably indicates that two saccharides per unit are too close for efficient interaction with binding sites.

Given some of the problems of compatibility and purification associated with polymerization of glycosylated monomers, it is somewhat surprising that post-polymerization glycosylation of already defined polymers has not been more widely adopted. Perhaps this is due to the need for well-defined glycosylation patterns (typically saturative glycosylation) in the context of a perceived lack of methods for high yielding

† The IUPAC name for norbornene is bicyclo[2.2.1]heptane.



Scheme 4

glycosylation of macromolecules. Bovin and co-workers have pioneered a post-polymerization grafting technique that utilizes polyacrylates derived from monomers in which the carboxylate is activated as its *p*-nitrophenyl ester.[51] These polyesters are reacted with ω-aminoalkyl glycosides to glycosylate the polymer; the maximum level of glycosylation being limited by the steric bulk of the carbohydrate structure (*e.g.*, up to 80% for monosaccharides). Any remaining unfunctionalized esters can then be reacted with smaller-weight amines. Once a given polymer background of high homogeneity has been isolated, this method can be used in the preparation of a variety of glycopolymers simply by varying the modifying reagent.

Whitesides and co-workers have compared post- and pre-polymerization glycosylation techniques in a comprehensive study of the formation of sialic acid polymers as shown in Scheme 5. They demonstrated that post-polymerization glycosylation results in glycopolymers that are more effective lectin inhibitors (up to 100-fold in the case of influenza haemagglutinin) – a difference that was attributed to non-random distribution of glycosylated monomers during polymerization that may limit accessibility.[52] The introduction of biotin into these polymers allowed ELISA assays which demonstrated that inhibition of agglutination correlates well with the affinity of the polymers for the lectin.

2.2 Glycoclusters

This term has generally been applied as a catchall category for medium-sized glycoconjugates that are non-linear but which show fewer elements of symmetry than glycodendrimers. Many of the structures described as glycoclusters have been described in the other sections of this review (see, for example, glycopeptoids in section 4.1). However, some uncategorised scaffolding examples remain.

For example, azamacrocycles have been used as a scaffold through the reaction of their amine group with α-D-mannosyl isothiocyanate and show significant inhibition of a lectin used by *E. coli* in binding to erythrocytes.[53] The synthesis of glycosylated calixarenes has also been reported.[54,55]

Recently, β-cyclodextrins bearing one or seven amino groups at their C-6 positions have been functionalized with mono- and di-glycoisothiocyanates. Interestingly, a reverse approach was attempted using glycosylamines and cyclodextrin isothiocyanates but was less successful.[56]

Ley and co-workers have used reactivity tuning methods to assemble selenoglycosides and glycosyl fluorides onto a pentaerythritol-based core. This extension of their powerful one pot glycosidation methodology results in the formation of tetrameric trimannosyl clusters.[57]

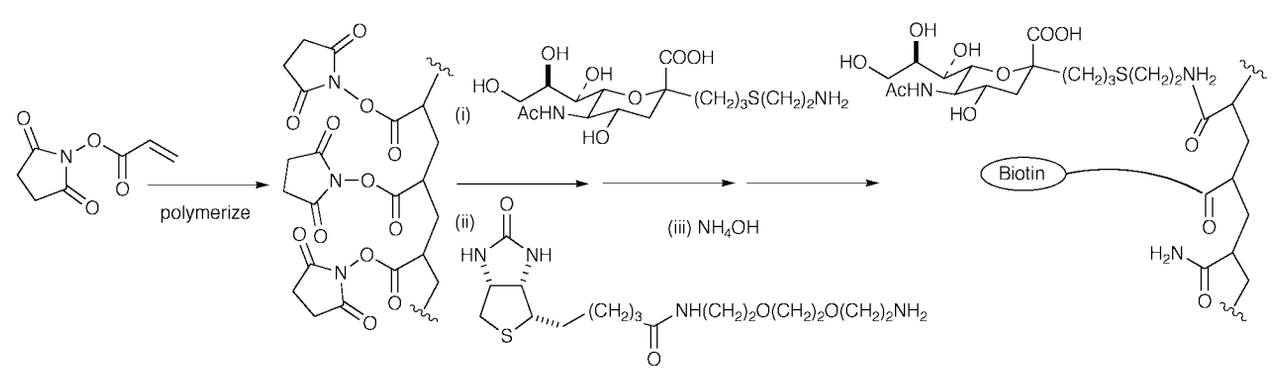
Lee and co-workers have recently described[58] an elegantly simple synthesis (Scheme 6), which does not require chromatography, of a YDD[59] analogue of his original YEE trigalactosyl cluster[60] that has been so successfully used in exploiting the cluster effect. Lysyllysine trigalactosyl clusters, similar in design to Roy's dendrimeric structures (see section 3) have also been described.[29]

2.3 Oligomers

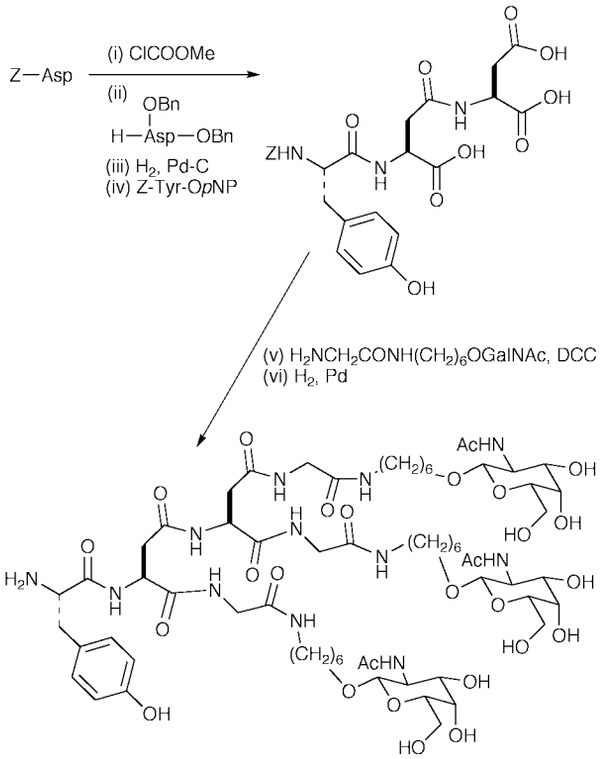
Shorter lengths of linearly repeating glycosylated units (oligomers) often offer advantages in terms of their properties over longer polymers. As well as being more easily prepared in homogeneous form, they may represent minimal structures for efficient binding to proteins that are potentially free of additional entropic costs associated with the binding of longer chains.

Kiessling and co-workers have elegantly exploited the control of the degree of polymerization (DP) that is available in ROMP (see section 2.1) to assemble oligomers of well-defined lengths.[20] This allowed a useful study of the effect of DP upon binding to the mannose-specific lectin concanavalin-A (Con-A). α-D-Mannosyl 10-, 25-, 52- and 143-mers were prepared (Scheme 4) using Grubbs' catalyst. When assessed on the basis of the number of residues present, these showed lectin-binding inhibitory potencies that reached a plateau for DP ~50 or more at levels that are ~2000-fold greater than the corresponding monomer. These results agreed well with modelling that showed that 35-mers are sufficiently long to bridge two of the Con-A CRD's, which are 65 Å apart.

Lactosylated oligomers may be formed through the quench-



Scheme 5



Scheme 6

ing of free radical polymerizations of acrylamide containing monomers with thiol scavengers such as *tert*-butylmercaptan. This method allows the formation of low molecular weight (DP up to 190) so-called telomers in one step.[61]

2.4 Polyamino acids

Polyamino acids have proved popular as rich homogeneous sources of easily prepared, highly functionalized polymers, for example by the *N*-carboxy anhydride (NCA) method, and have therefore been used frequently as scaffolds for glycosylation. Their use has proved particularly popular in drug delivery systems (see section 8.2) because they are homogeneous biodegradable polymers that are analogues of proteins but with often-lower immunogenicity.

Since they contain the same functional side chains, the formation of glycosylated polyamino acids has adopted a post-polymerization strategy that exploits many of the methods described for glycoprotein synthesis in section 4.2. In particular, the frequent use of poly-L-lysine[62] has meant that methods that functionalize amino groups, such as reductive amination of aldehydes,[63] 2-iminomethoxymethyl thioglycosides[64] or isothiocyanates[65] have proved most popular. More recently, novel pyroglutamate isothiocyanates have also been used for post-polymerization glycosylation of poly-L-lysine.[66] A powerful example of the derivatization of poly-L-lysine (DP = 240) with chloroacetic anhydride has allowed the formation of a fully functionalized soluble sialyl Lewis-x (sLe^x) bearing polymer through sequential reaction with sLe^x, biotin and glycerol derived thiols, in a conceptually similar manner to Bovin's grafting technique (outlined in section 2.1).[67]

Poly-L-glutamic acid has also been used and has been coupled to 8-aminooctyl glycopyranosides using EDCI (*N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide) to activate the side-chain carboxylates.[68] More recently, poly-L-glutamic acid has been used as a scaffold for presenting the glycolipid GM3 in the synthesis of a highly potent inhibitor of influenza haemagglutinin. The lipid moiety of the glycolipid was functionalized with an amino terminus spacer arm before reaction with poly-L-glutamic acid activated as its succinimidyl esters.[69]

3 Glycodendrimer synthesis

As highly branched, high-symmetry molecules dendrimers sit structurally between clusters and polymers. They keep the advantage of being well defined that clusters offer whilst allowing extension of the size of scaffold upon which sugars may be presented up to the levels of polymers by providing a source of branched macromolecules with precise structures through iterative assembly.[70,71]

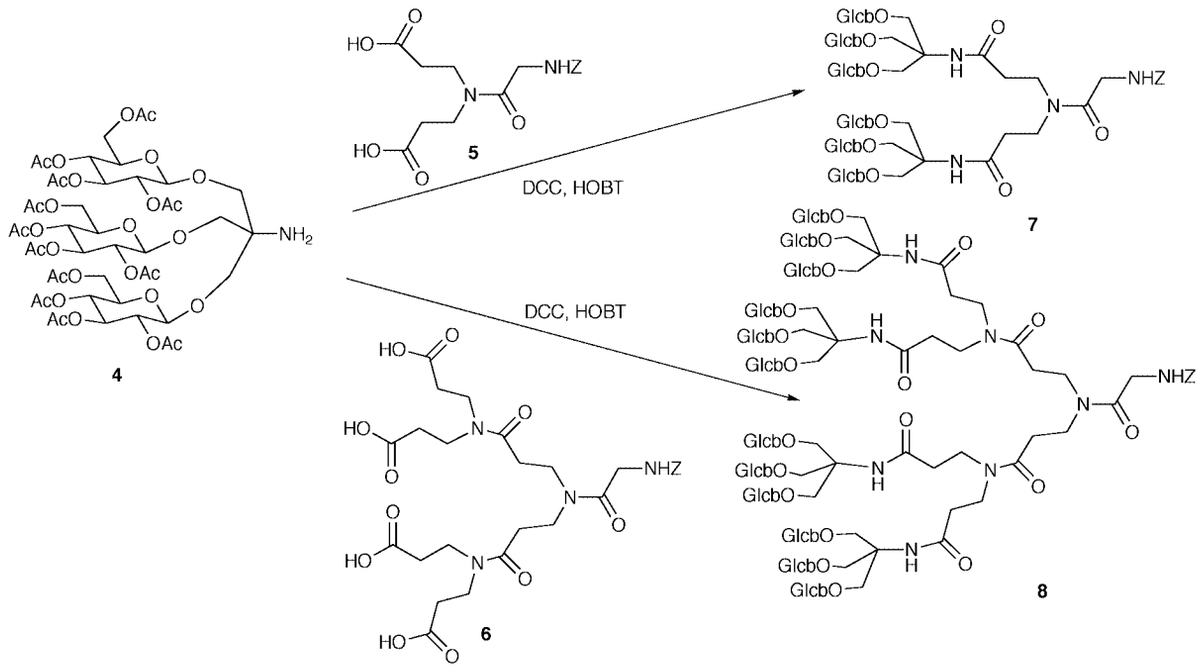
Stoddart has logically set out the scope of glycodendrimer formation as populating any point in a structural spectrum between two conceivable extremes: carbohydrate-coated dendrimers and fully-saccharidic structures in which the carbohydrates also provide the source of branching.[70] Taking advantage of the extensive background of non-carbohydrate dendrimer techniques, the examples described thus far have largely been of the former, sugar-coated variety. To describe the synthetic strategies that have been followed he has applied the terms "convergent" and "divergent" (sometimes called "starburst") to describe the assembly of preglycosylated dendritic wedges onto a branched core and the glycosylation of the termini of preformed dendrimers, respectively. In this sense these terms bear close resemblance to the "linear" and "convergent" strategies described for glycoprotein/peptide synthesis, which introduce carbohydrate moieties before and after scaffold formation, respectively (see section 4).[72]

In an elegant example of the former strategy (Scheme 7), the formation of a tri-β-D-glucosylated TRIS branch **4**, using Koenigs–Knorr chemistry, allowed the formation of 6-mer and 12-mer wedges through DCC-mediated coupling with the di- and tetra-carboxylic acids **5** and **6** in excellent yields and with high purity. Subsequent *N*-deprotection and coupling of **4**, **7** or **8** with a benzene-1,2,5-tricarboxylic acid core allowed the formation of 9-, 18- and 36-mers, respectively.[73,74] It should be noted that this method also allows the potential for the use of asymmetrical wedges with differently glycosylated cores. Two different early glycosylation strategies were employed in the construction of a 36-mer β-D-glucosyl dendrimer; the failure of this method to allow the coupling of a 6-mer wedge onto a hexavalent core in contrast to the success of the coupling of a 12-mer wedge onto a trivalent core illustrates that steric hindrance at the core is a limiting factor in construction.[74] This TRIS branch method has also been extended to α-D-mannosyl dendrimers up to 36-mers.[75]

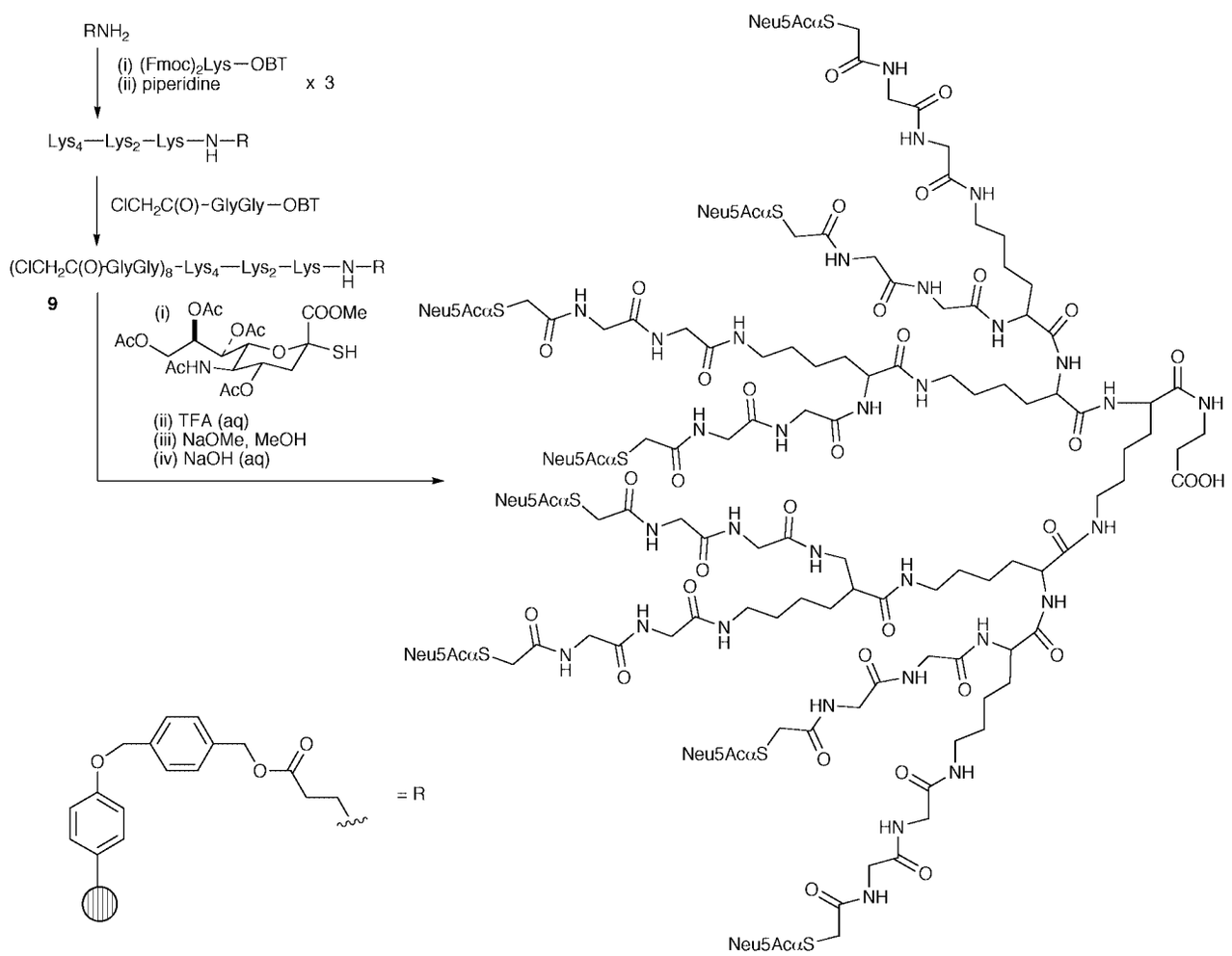
"Divergent" or "late glycosylation" dendrimer syntheses require key high yielding glycosylation techniques and therefore this approach has also largely adapted well-established protein glycosylation techniques (see section 4.2). For example, aldonolactones (to form "sugar balls"),[76] glycosylated serine NCAs (to form "sugar balls II"),[77] glycosyl isothiocyanates[78–81] and *N*-hydroxysuccinimide activated esters[82] have all been used to functionalize the amine termini of dendrimers such as Tomalia's commercially available polyamidoamine (PAMAM) dendrimers with glucosyl-, mannosyl- galactosyl-, cellobiosyl-, and lactosyl- residues.

As was observed in early glycosylation strategies, surface bulk may limit the size and generation levels that are attainable. In this regard, the use of protected glycans is a further limitation on the steric bulk that may be accommodated. Lindhorst and co-workers have overcome this problem by adapting the reaction of unprotected glycoisothiocyanates with amines to functionalize triamino- and PAMAM cores to form α-D-mannosyl trimers and hexamers.[83] Stoddart and co-workers have also coupled deprotected glycosylated TRIS branches to a *N*-hydroxysuccinimide activated tricarboxylic acid core to give a 9-mer.[84]

Roy has pioneered an innovative approach that employs branched poly-L-lysine scaffolds, such as the 8-mer **9**, which may be conveniently synthesized using solid phase Fmoc chemistry (Scheme 8). Functionalization with chloroacetic



Scheme 7



Scheme 8

anhydride allows the subsequent introduction of sialyl-,[85] β-glucosaminyl-, β-lactosyl-, β-lactosaminyl-[86] and α-mannosyl-[87] thiols. The same method may also be applied to gallic acid[88,89] and branched propylamine[90,91] scaffolds. Propylamine scaffolds (2-, 4- and 8-mer) have also been functionalized with *N*-linked α-sialyl residues in a solid phase TBTU (*O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate) mediated amide-coupling approach.[92] Enzymatic methods have

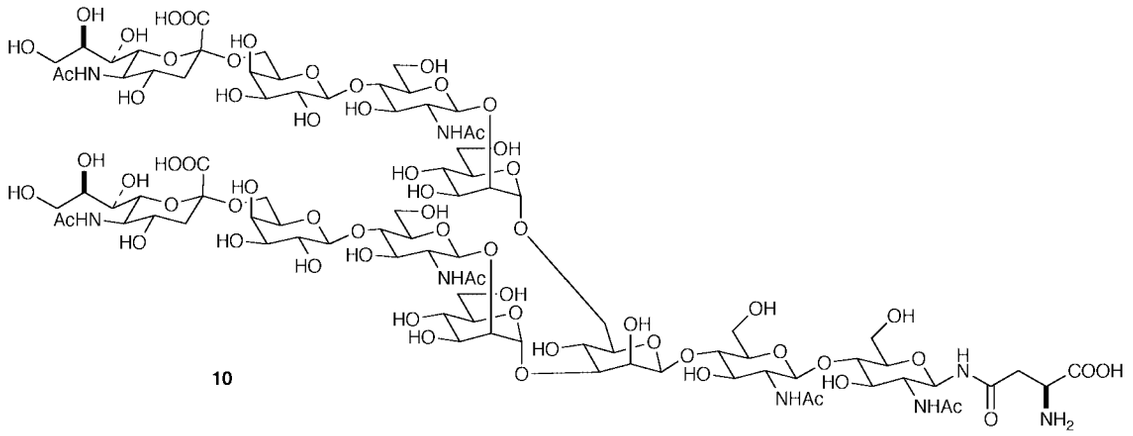


Fig. 3 The synthesis of undecasaccharidic peptide **10** demonstrates that highly glycosylated glycopeptide building blocks are accessible.

also been employed in glycodendrimer synthesis as highlighted by the use of a β(1,4)-galactosyltransferase to modify GlcNAc residues on the surface of 2-, 4- and 8-mers in up to 90% yields.[93] Two further glycosyltransferase mediated steps allowed the formation of some of the most complex glycodendrimer glycan termini yet described: sLe^x-bearing 2-, 4- and 8-mers.[94]

Although no syntheses of glycodendrimers based exclusively on carbohydrate building blocks have yet been reported, the use of 2,5-anhydro-D-mannitol as a C2-symmetric core[95] or D-glucose as a core[96–98] for the synthesis of dendrimers and the recent syntheses of sugar branch point heptasaccharide wedges has highlighted the efficacy of this approach.[99]

The race to build dendrimers has, to date, been driven by a certain desire to go "bigger and better". So it is illuminating that in a number of examples optimal binding constants have been found for medium as opposed to higher generation glycodendrimers. For example, binding of α-D-mannosylated dendrimers to the lectin concanavalin-A was estimated to be optimal between 9- and 18-mers.[75] Indeed, the expected entropic cost involved in the association of larger structures with concomitantly higher degrees of conformational freedom indicates that perhaps such biological signposts should be heeded in future glycodendrimer designs.

4 Glycoprotein synthesis

4.1 Glycopeptide assembly

The need for homogeneous samples (single glycoforms) that was outlined in Section 1.4 has resulted in great effort in the field of *de novo* synthesis of glycoproteins. The linear assembly of glycosylated amino acids, has from the very first examples, such as the use of *N*-acetylglucosaminyl asparagine in the synthesis of a partial sequence of fibroblast interferon,[100] provided well-defined products. Thus, the required carbohydrate structure is attached to an amino acid residue (typically serine and threonine for *O*-linked glycopeptides and asparagine for *N*-linked glycopeptides – an excellent review of methods for the formation of the glycosidic link between peptides and glycans has very recently been published[101] and so will not be addressed here in detail) and used as a building block in strategies that often rely heavily on standard peptide synthesis techniques. That these elegant approaches are still some way off synthesizing peptides of lengths approaching those of proteins is a testament to the inherent difficulties of this approach. Two factors limit the work; firstly the need not only for extensive carbohydrate protection but also amino acid protection regimes; and secondly the acid and base lability of glycosylated amino acid residues. Indeed, this feature of glycoproteins has long been exploited to strip glycans from protein surfaces. The necessary protection and deprotection regimes, the use of solid phase techniques, including the introduction of specific linkers, and coupling methods have all been tailored to be compatible with the presence of carbohydrates. Several excellent reviews[102–107] have already covered these aspects in detail but some recent examples illustrate this strategy well. For example, in an elegant combination of both chemical and glycosyltransferase mediated glycosylation, the synthesis of an undecasaccharide-linked asparagine residue **10** (Fig. 3) has demonstrated that highly glycosylated glycopeptide building blocks are readily accessible.[108]

The first example of the synthesis of a glycophosphopeptide **11** is shown in Scheme 9. The enzyme labile group PhAcOZ was used to protect the *N*-terminus of a serine residue which was glycosylated with GlcNAc. This formed the key building block **12** in the synthesis of **11**. Penicillin acylase allowed the removal of PhAcOZ and the free amine was coupled using carbodiimide to a glycosylated dipeptide, itself prepared from key block **12**. Again, enzyme cleavage allowed the *N*-terminus to be freed before further couplings with peptides and finally a serine residue bearing a protected phosphorylated side-chain. Global deprotection then afforded **11**.[109]

Protease-mediated peptide ligation is as yet not capable of general coupling of glycopeptide blocks due to the often stringent specificities of these enzymes (see section 4.3).[110,111]

A hybrid strategy in which a glycosylated dipeptide was oligomerized up to 12 repeating units has been reported. The tripeptide Z-AAT-Bn was glycosylated with Galβ(1,3)GalNAc, deprotected and then treated with diphenylphosphoryl azide as an activator to form an oligopeptide with significant antifreeze properties.[112]

Block coupling strategies may also be applied to the construction of non-proteolysable (and therefore with potentially higher oral bioavailability) peptide mimics (glycopeptoids), such as the LN(GlcNAc)FKA mimic **13**,[113a] (Fig. 4) which may also show interesting conformational restriction as a result of rotamer formation. Similarly, a linear glycopeptoid mimic of the Tn-antigen (GalNAc-α-Ser/Thr) was prepared using a reiterative TBTU mediated coupling of a single orthogonally protected aminoester building block.[113b] This method has been elegantly extended to the concept of glycopeptoids in which both the interresidue and side chain distance may be varied. Through the incorporation of different aromatic, amine and peptoid spacer units the spatial presentation of *C*-glycosides in these structures may be optimized.[114]

As has been shown above, a linear strategy in glycopeptide synthesis is more usual since direct peptide glycosylation is often unsuccessful, given the variety of functional groups that would be required to protect a peptide of protein length. However, Lansbury and co-workers have pioneered the use of glycosylamines in a convergent approach to glycopeptide synthesis. For example, HBTU (*O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate) mediated coupling of GlcNAc glucosamine with the side chain aspartate carboxylate in the pentapeptide **14** effectively allowed the formation of an Asn

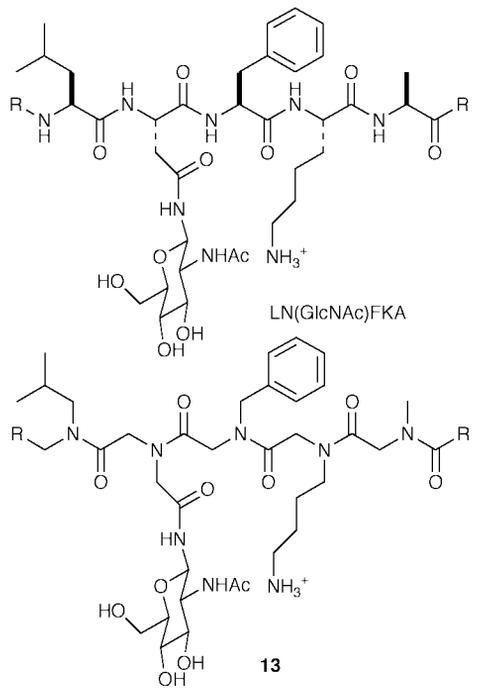
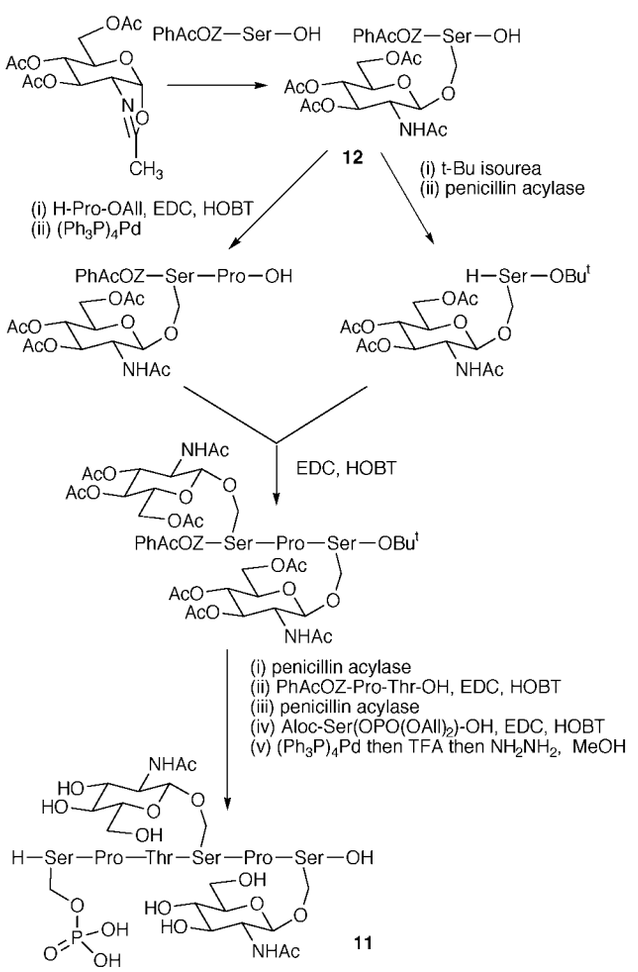
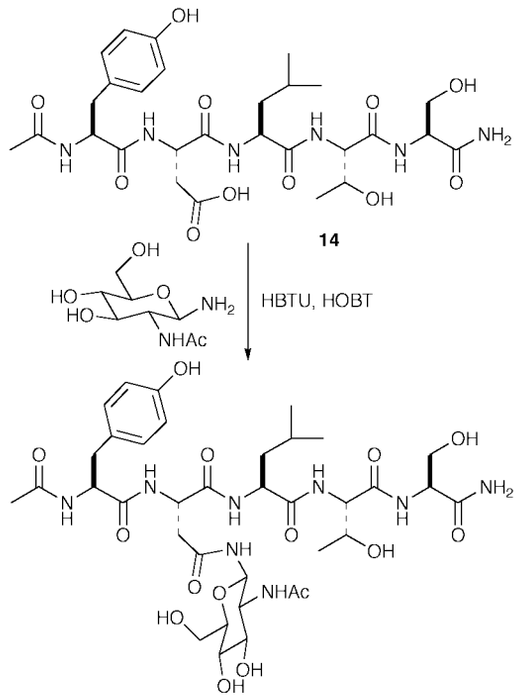


Fig. 4 A comparison of glycopeptoid mimic **13** with glycopeptide LN(GlcNAc)FKA.



Scheme 9

linked *N*-acetylglucoaminyl containing glycopeptide (Scheme 10).[115] This method was successfully extended to peptides containing more complex glycans such as the high-mannose core of *N*-linked glycoproteins, $Man_5(GlcNAc)_2$.[116] Furthermore, this



Scheme 10

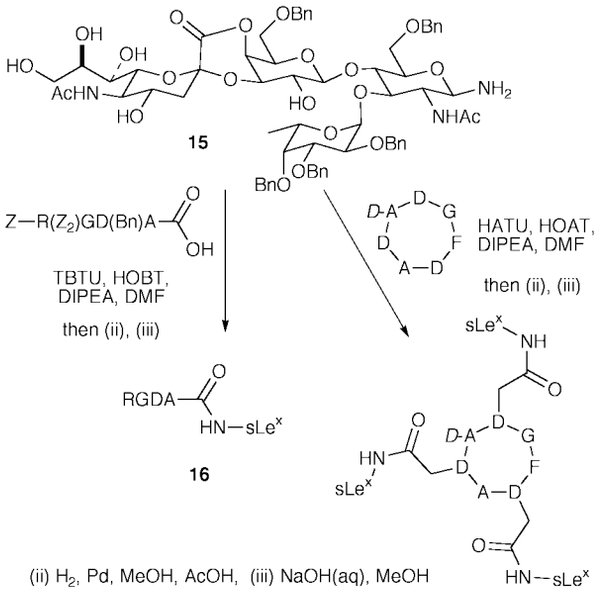
method has recently been extended to encompass solid-phase bound glycosylamines, which are then coupled to side chain carboxylates in pentapeptides before the peptide chain is further extended.[117]

Selective TBTU-mediated coupling of the C-terminus of the tetrapeptide motif RGDA to a partially protected sLe^x glycosylamine **15** allowed the formation of the potent P-selectin inhibitor **16** (Scheme 11).[118] The same sLe^x derivative **15** was also used in a highly convergent HATU-mediated glycosylation strategy in the synthesis of two trivalent cyclic heptapeptides.[119] Danishefsky and co-workers have described the α-glycosylation of the side chains of threonine and serine residues using a trisaccharide phosphite donor, which in turn had been constructed using glycal methodology, before linear assembly into a STSEPV hexapeptide as a powerful example of a mixed linear and convergent strategy.[120]

4.2 Chemical glycoprotein synthesis

Whilst the convergent glycosylation of oligopeptides is successful, it is limited by a lack of suitable functional groups when applied to proteins. For this reason alternative glycoprotein synthesis techniques have been more widely applied.[32,121,122]

The use of 2-iminomethoxymethyl thioglycosides[64] and reductive amination methods[63] are still after 20 years the most frequently used strategies for glycoprotein preparation. The former may be readily prepared by the action of methoxide on cyanomethyl glycosides that may in turn be derived from 1-thioaldoses (Scheme 12a). For the latter, Gray originally modified albumin with lactose through $NaBH_3CN$-mediated reduction[63] (Scheme 12b) although borane may also be used. This method is amenable to other sources of aldehyde functionality such as those generated by ozonolysis of unsaturated spacer arms,[123] through periodate cleavage of diols, or the hydrolysis of acetal-containing spacer arms (Scheme 13).[124] Conjugations through reductive amination are often accompanied by low protein loading levels, that in some cases is due to steric hindrance caused by short spacer arms. In a pragmatic approach to circumventing this problem, a second hydrazide spacer arm (Scheme 13) can be used to extend an existing aldehyde terminated spacer.[125] Reaction of the maleimido terminus of the resulting longer spacer arm with thiols introduced to the



Scheme 11

surface of the protein keyhole limpet haemocyanin (KLH) allowed 5-fold greater loading of the sialyl-GalNAc disaccharide, sTn.

The use of glycosidic aromatic diazonium salts, derived from the corresponding *p*-aminoaryl glycosides, as electrophiles to functionalize proteins was first demonstrated as early as 1929.[23] They modify a wide range of electron rich side chains within protein structures, such as those of aromatic tyrosinyl and tryptophanyl or nucleophilic lysinyl and histidinyl residues (Scheme 12c).[126] Although high levels of functionalization are thus easily accessible, this lack of residue selectivity is often a drawback in the synthesis of well-defined conjugates. *p*-Aminoaryl glycosides may also be elaborated to phenyl-isothiocyanates, which react more selectively with amino groups alone (Scheme 12d).[65] A one-pot, two step preparation of anomeric *p*-nitroanilide(*p*NA) pyroglutamates from unprotected carbohydrates also provides an alternative route to aromatic isothiocyanates.[66] Following glycosylamine formation with the α-amino group of *p*NA glutamic acid, the side chain γ-carboxylic acid readily reacts with the resulting secondary amine to give a pyroglutamate which may then be elaborated (Scheme 12e).

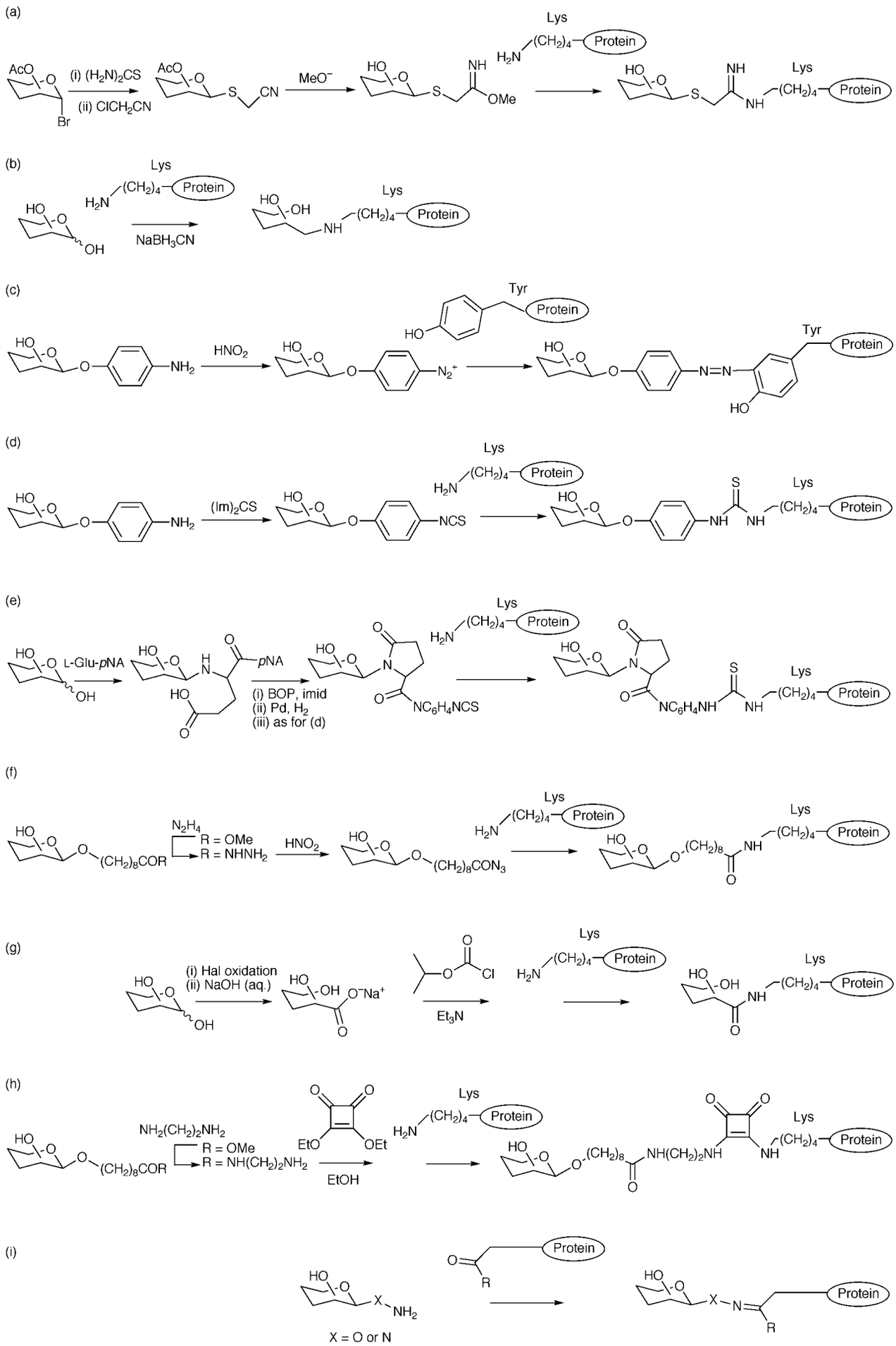
In 1975, Lemieux and co-workers described the use of highly activated acyl azides for the formation of amides from proteinaceous amines and carboxylate ester-terminus spacer arm carbohydrates.[127] The esters were converted to acyl hydrazides before oxidation with nitrous acid to give the corresponding acyl azides (Scheme 12f). Mixed anhydride methods are well tried for the activation of carboxylic acids to form carboxy derivatives and in this way aldonic acids may similarly be coupled to proteinaceous amines (Scheme 12g).[128,129] Similarly, carbodiimide chemistry[130] and the use of *N*-carboxy-anhydrides[131] has also allowed the coupling of aldonates and glycosylated amino acids as sources of glycans bearing carboxylic acids. Hindsgaul has described the use of diethyl squarate for the coupling of carbohydrates bearing amino-terminus spacer arms to amines in BSA (Scheme 12h).[132] 2-Chloroethyl-1-thioglyosides have also been used to indiscriminately alkylate protein amino and hydroxy groups.[133]

A novel high temperature 'baking' method has been described for the modification of lyophilized proteins with reducing oligosaccharides.[134] Remarkably, despite being heated with the carbohydrate in air at 95–120 °C for up to 40 minutes both trypsin and an IgG antibody survived with little loss of biological activity. The results of tryptic digests and conjugate hydrolyses suggest that the mechanism of conjugation involves an Amadori rearrangement with protein lysines which destroys the integrity of the reducing end residue.

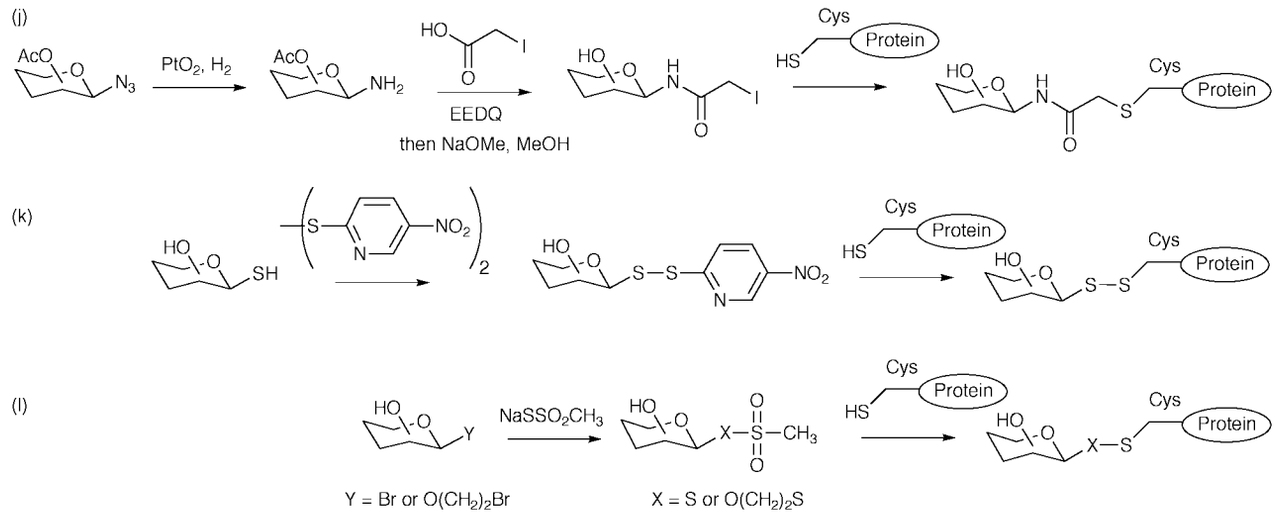
In an attempt to increase the selectivity and predictability of protein glycosylation, various novel approaches have been described all of which exploit the chemoselectivities of different enzymatic and traditional methods. As Scheme 12i illustrates, Bertozzi has exploited the selectivity of galactose oxidase to introduce an aldehyde tag to the C-6 of a GalNAc residue in the antimicrobial 19-residue peptide drosocin.[135] (This strategy has been christened chemoselective ligation and its application to a wide range of bioconjugates has recently been reviewed.[136]) This tag was then selectivity reacted with aminooxy glycosides to introduce further saccharides *via* the formation of an imine, in a manner previously demonstrated for the conjugation of spacer-arm hydrazides with cell surface aldehydes.[137] That this non-native glycopeptide shows comparable biological activity illustrates that certain unnatural linkages can in certain circumstances be tolerated. Unfortunately, this approach still requires the linear construction of an initial glycopeptide and so suffers from the same disadvantages of protection and lability outlined in section 4.1. However, it does hold the advantage that its application may be based on other methods for the introduction of a ketone tag (see section 4.4). A similar chemoselective ligation approach has been applied to glycopeptide synthesis. Both α-amino and lysine ε-amino groups were derivatized with aminooxyacetyl groups before reaction of the free amine introduced with reducing sugars. The use of orthogonal *N*-protection also allowed regioselective glycosylation.[138]

In a sense, these strategies still duck the most overriding issue – the formation of the sugar–protein link – by relying on the presence of a single glycan in the protein structure as a tag for reaction. Similarly, although Wong and co-workers have elegantly constructed an unnaturally glycosylated RNase-B through a series of protease and glycosyltransferase catalyzed ligations the method still requires that a protein-*N*-glycan link be present from the start (see section 4.3). As these methods alter one glycan structure for another they are therefore better described as glycoprotein remodelling (GPR) and they afford the glycoscientist no choice over the site of glycosylation. Several methods have been proposed that tackle this central issue. Among the first approaches was that of Flitsch and co-workers, who reacted the α-iodoacetamide of *N*-acetyl-D-glucosamine with oxidized bovine serum albumin (BSA) to modify the single free cysteine present (Scheme 12j).[139] Later this method was applied by Dwek to introduce chitotriose and a heptasaccharide stripped from the surface of horseradish peroxidase to BSA.[140] Boons has used the dithiopyridyl methodology to make disulfide linked BSA-*N*-acetyl-D-glucosamine constructs (Scheme 12k).[141]

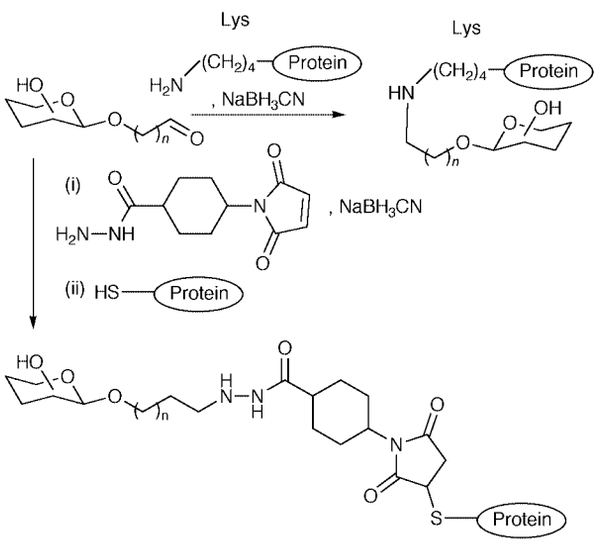
For full control of glycosylation both choice of site and glycan is needed. Recently, a combined site-directed mutagenesis and chemical modification approach has addressed this problem for the first time.[142] The strategy involves the introduction of cysteine as a chemoselective tag at preselected positions and then reaction of its thiol residue with a variety of glycomethanethiosulfonate reagents (Scheme 12l). Methanethiosulfonate reagents react specifically and quantitatively with thiols[143] and allow the controlled formation of neutral disulfide linkages. Four sites at different locations in the representative serine protease subtilisin *Bacillus lentus*, which were both externally and internally disposed, were selected for mutation to cysteine. This controlled glycosylation also allowed the SARs of glycosylation upon enzyme catalysis to be studied in a precise way for the first time. Whilst the above methods are elegant, the selective formation of the naturally occurring *N*- and *O*-anomeric linkages must surely be the goal of future chemical developments.



Scheme 12



Scheme 12 (*Contd.*)



Scheme 13

4.3 Enzymatic glycoprotein synthesis

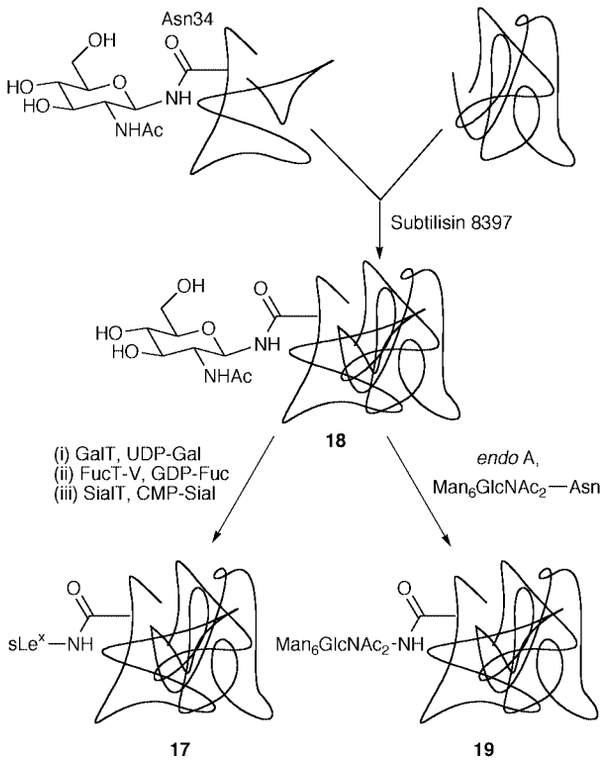
The two distinct methods outlined in the previous two sections are equally available to enzyme-catalyzed techniques. For example, subtilisins have been elegantly used to catalyze the synthesis of glycopeptides,[110,111] in spite of the fact that the natural specificity of these enzymes has limited these peptide ligations to those in which the glycosylated residues are typically at least one residue distant (P_2,P_3... or P_2',P_3'...) from the amide bond formed. For example, while ligation of Z-Gly-OBz with H-Gly-Ser(Ac_3GlcNAcβ)-NH_2 was successful, no yield of product was obtained with H-Ser(Ac_3GlcNAcβ)-NH_2. In a rare use of enzymatic glycosylation on the solid phase, the sLe^x-Asn-Phe dipeptide was synthesized using aminopropyl silica as a support and subsequently cleaved from a glycine linker by the peptidase chymotrypsin.[144] In this context, it is interesting to note that the ability of glycosyltransferases to modify glycans on glycopeptides is greatly influenced by the peptide backbone and the sites that are glycosylated within it.[145] For example, prolines, negatively charged residues and the presence of disaccharides all inhibited the glycosylation of an adjacent GlcNAc-Thr residue.

Convergent enzyme-catalyzed techniques have proved more adaptable than linear ones. One of the very first examples of the use of glycosyltransferases in glycoprotein synthesis was demonstrated by Paulson and co-workers who used a sialyltransferase and CMP-*N*-Ac-neuraminic acid to restore 95% of the sialic acids to a fully desialylated protein.[146] The use of enzymes in glycoproteins synthesis has recently culminated in the elegant synthesis of a single unnatural glycoform of ribonuclease B (RNase-B) **17** using a series of protease and glycosyltransferase catalyzed reactions (Scheme 14).[147] Takegawa and co-workers have also applied their endoglycosidase mediated transglycosylation method (see section 2.1) to the same partially deglycosylated RNase-B **18** in the synthesis of the $Man_6GlcNAc_2$ glycoprotein **19**.[148] Interestingly, Nature employs a similar mechanism in the case of *Trypanosoma cruzi*, the protozoon that causes Chagas' disease. This parasite does not synthesize sialic acid, but instead expresses a transsialidase that catalyzes the transfer of sialic acid from glycoconjugates found in the host to its own surface proteins. The resulting sialylated glycoproteins are then bound by host sialic acid binding receptors thereby allowing cellular invasion.[149] Enzymatic approaches have even been used to directly modify cell surface proteins.[150] The use of a milk fucosyltransferase that displays a broad substrate specificity allowed modification of glycans with fucosyl residues bearing a range of substituents at C-6.

An attractive approach to enzymatic glycoprotein synthesis is to exploit the biosynthetic mechanism for the formation of the sugar–protein link in *N*-linked glycoproteins. The enzyme responsible, oligosaccharyltransferase, transfers a high mannose core oligosaccharide from a fatty acid pyrophosphate carrier to the side chain amide of an asparagine (Asn) residue in the consensus sequence Asn-X-Thr/Ser of the nascent glycoprotein. The use of this enzyme in isolated form in glycoconjugate synthesis has however met with only modest success. Whilst transfer of carbohydrates to a 17-residue peptide containing an unusual Asn-Asn-Thr-Ser sequence was possible, direct glycan transfer to RNase-A failed.[151] Thus, such site specific glycosylation of proteins still remains an elusive goal and as a result there is still no general enzymatic method for the synthesis of homogeneous glycoproteins. However, through the use of enzymes the unification of both the strategies outlined chemically in sections 4.1 and 4.2 can be imagined: enzyme catalyzed peptide ligation to construct a (glyco)-protein scaffold, perhaps bearing single glycan tags, before convergent glycosylation by oligosaccharyltransferase or glycosyltransferases.

4.4 Molecular and cell biological techniques

In vivo methods, that alter the natural machinery of glycosylation, offer promising opportunities[152] but, as yet, still lead to heterogeneous products.[153] The task is made difficult by the daunting array of biosynthetic glycosylation products and thus



Scheme 14

the corresponding array of pathways that need to be controlled or adapted. By expressing a particular glycosyltransferase or glycosidase in one organism different glycosylation patterns may arise to those found by expression of the same enzyme in another. In this way glycosylation patterns may be guided in a particular direction. For example, mutant cell lines in which extra glycosyltransferase expressing genes have been introduced may be used to enhance the presence of particular sugars in glycan structures. The addition of a sialyltransferase to a Chinese hamster ovary (CHO) cell line resulted in the increased "misglycosylation" of *N*-linked glycoproteins to give glycans bearing α(2-6)Gal- instead of α(2-3)Gal-linked sialic acid terminated residues.[154]

The inhibition of enzymes involved in the biosynthesis of glycoproteins offers an alternative way of controlling their structure. For example, the glycosyltransferase inhibitor tunicamycin inhibits the synthesis of the lipid-linked pyrophosphate oligosaccharide precursor that is used as a glycosyl donor in the formation of *N*-linked glycoproteins.[155] The resulting lack of donor prevents formation of Asn-linked glycans and results in only *O*-glycosylated proteins. Less drastic inhibition of later trimming steps that are mediated by glycosidases can be used to create smaller than natural ranges of Asn-linked glycoforms rather than none at all. For example, the use of glucosidase inhibitor *N*-butyl deoxynojirimycin resulted in a reduction in the number of glycoforms of the HIV surface protein gp120 that were produced from more than 100 to 3.[2]

The prospects are also good for the glycosylation of larger biomolecular complexes by taking advantage of the often-relaxed specificities of biosynthetic pathways. Indeed, the use of an unnatural *N*-levulinoylmannosamine as a precursor simply by feeding it to cells has allowed the introduction of a unique ketone tag into sialic acid residues found at cell surfaces[156] which then allowed the selective introduction of further glycans through reaction with aminooxy and hydrazide-functionalized carbohydrates to form imines and hydrazones according to the manner described in section 4.2 (Scheme 12i).[157] This strategy is similar to one previously applied to aldehydes introduced chemically to cell surfaces.[137] In a similar manner, neural cell surfaces have been also engineered by introducing an unnatural *N*-propanoylneuraminic acid precursor.[158]

5 Glycolipids

More than 300 naturally occurring glycolipids have been isolated. Unlike glycoproteins they are structurally diverse in their core carbohydrates and are typically limited to oligosaccharides containing less than five residues.[159] Where syntheses differ is in the scale of the target. Unlike glycoproteins the smaller size of glycolipids has allowed a number of total syntheses to be completed.[160–162] However, their complexity has still driven the synthesis of simpler mimetics in an attempt to identify the minimal structural features required for biological activity.

Models of glycolipids require only that they are amphiphilic, that is they have a lipophilic moiety conjugated to a glycan and so consequently may be as simple as long-chain alkyl glycosides. Many of the techniques developed in glycoprotein synthesis (section 4.2) have also been adopted in this field. For example, reductive amination of sorbose allowed the introduction of an amine group into the carbohydrate which was subsequently *N*-acylated with fatty acid chains.[163] Alternatively, naturally occurring oligosaccharides may be stripped from proteins by hydrazinolysis and then reductively aminated by phosphatidylethanolamine to form unnatural "double-tailed" glycoceramide-like structures.[164] In an adaptation of this method, Wong and co-workers stripped the oligosaccharide from the bioactive triterpene glycoconjugate Julibroside I using allyl alcohol. Ozonolysis, reaction with decylamine and then decanoylation yielded an alternative glycolipid conjugate for the study of Julibroside's antitumour properties.[165] Large scale access to allyl spacer sialyl(α2,8)sialosides has also allowed the formation of linear GD3 and branched GO1b ganglioside mimics that are constructed through reductive amination.[166]

The synthesis of glycolipids has unsurprisingly concentrated on traditional glycoside synthesis methods for the formation of the glycan–lipid bond (indeed, many aspects of oligosaccharide formation methodology have been optimized in the pursuit of glycolipid synthesis). For example, L-glucosylation of sphingosine using Koenigs–Knorr chemistry allowed the preparation of a non-labile L-glucocerebroside for the study of lipid accumulation that is found in Gaucher disease.[167]

Schmidt's azidosphingosine glycosylation procedure has been widely applied. Glycosylation of the hydroxy group of a sphingosine in which the amine group is masked as an azide using trichloroacetimidates, gives ready access to glycosphingolipids and through *N*-acylation with fatty acids to various glycoceramides.[168] This versatile method has recently culminated in the synthesis of the nonasaccharide glycoceramide KH-1.[169] This target has also been constructed by Danishefsky and co-workers using the glycal methodology.[170]

Glycosylphospholipids have also been constructed using trichloroacetimidates as glycosyl donors (Scheme 15);[171] the lability of the resulting glycosidic phosphate ester bond (phosphate linked glycolipids also serve as glycosyl donors to certain classes of glycosyltransferases, *e.g.*, oligosaccharyltransferase, in which the lipid phosphate acts as the leaving group) provides a convincing reason for their lack of occurrence in Nature, which instead utilizes the more stable carbocyclic pseudosugar inositol at this position. The structure of these GPI-anchors,[172] so called because they are lipid-bearing protein attachments which sit in lipid bilayers of cell membranes, was first elucidated by Ferguson and co-workers in 1988.[173] They contain a phosphatidylethanolamine link between the protein and the C-6 hydroxy of a non-reducing end carbohydrate and the reducing end of the glycan is linked to ceramides *via* an inositol phosphate link. Ogawa and co-workers have provided pioneering examples of glycolipid syntheses. One such example is the landmark synthesis of the GPI anchor of *T. brucei* **20** (Fig. 5)

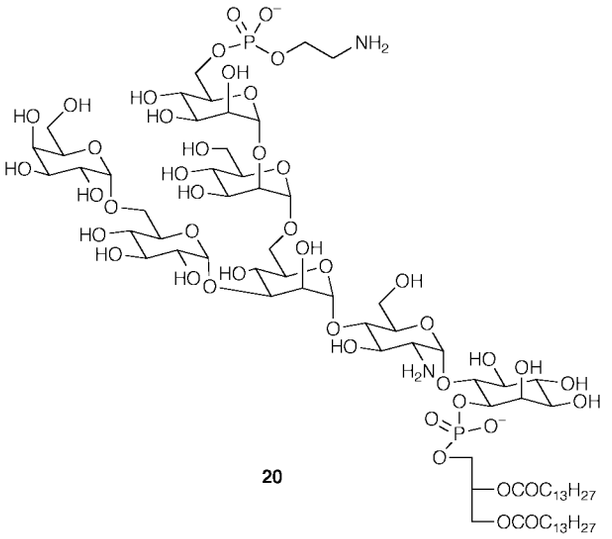
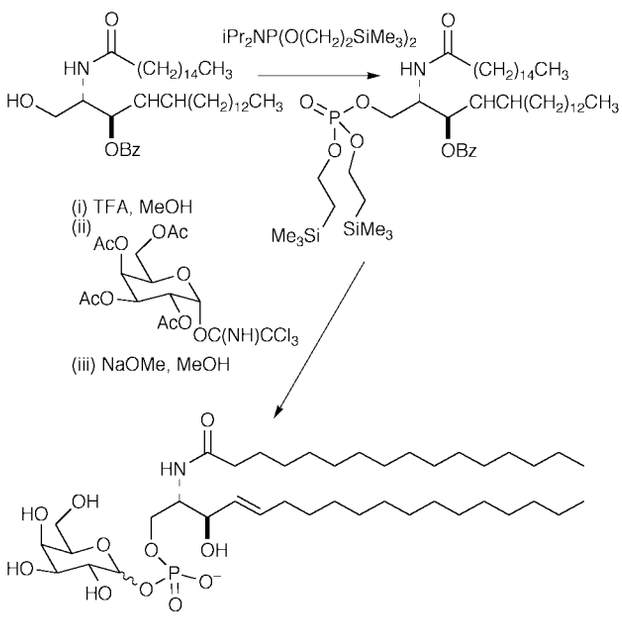


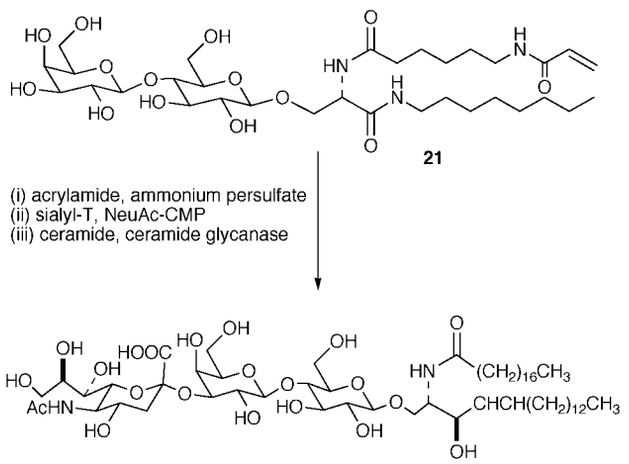
Fig. 5 GPI anchor of *T. brucei.*



Scheme 15

that utilized intermediates containing two H-phosphonates which were later oxidized using iodine to the corresponding phosphodiesters.[174]

Combined chemical and enzyme-mediated strategies can also be employed. For example, the oligosaccharide portion of ganglioside GM3, one of the most widespread glycolipids, was prepared from 2-*O*-pivaloyllactose using a sialyltransferase mediated sialylation that gave a better yield than for either lactose or lactosamine as a substrate. This was then coupled to ceramide using trichloroacetimidate chemistry before deprotection to give GM3.[175] GM3 may also be prepared through a versatile polymerization and enzyme-mediated method (Scheme 16). Acrylamide containing lactolipid **21** was copolymerized with acrylamide using ammonium persulfate as an initiator. The resulting water-soluble polymer was then sialylated with sialyltransferase. GM3 was released through transglycosidation using ceramide and ceramide glycanase in a remarkable 58% overall yield.[176] Increased concentrations of the lactone form of GM3 are associated with melanoma cells. A physiologically stable analogue containing a CH_2O– at C-1 of the sialic acid residue rather than the naturally-occurring COO– has been synthesized.[177] Reduction of the lactone using a Red-Al derivative followed by hydrogenolysis of an *O*,*S*-



Scheme 16

acetal removed the C=O functionality. The azidosphingosine lipid conjugation method then completed the synthesis.

An exciting example of a novel polymeric glycolipid structure has been constructed as shown in Scheme 17. Taking advantage of its propensity for self-assembly the monomer **22** was polymerized using UV light to give what can be thought of as a polymeric glycoliposome.[178]

Magnusson and co-workers have synthesized a large number of derivatives of the globo series of glycolipids to map the interactions with lectins in the pathogens *E. coli* and *S. suis*.[179] This work provides a valuable guide for the design of anti-adhesives (see section 8.3 for other examples). Some drawbacks in the therapeutic application of glycolipids exist. Their effectiveness depends intimately on the micellar concentrations that may be achieved and which are hard to control predictably. Furthermore, their application *in vivo* is limited by their non-specific adsorption onto the surfaces of cells, which leads to rapid decreases in the doses delivered.

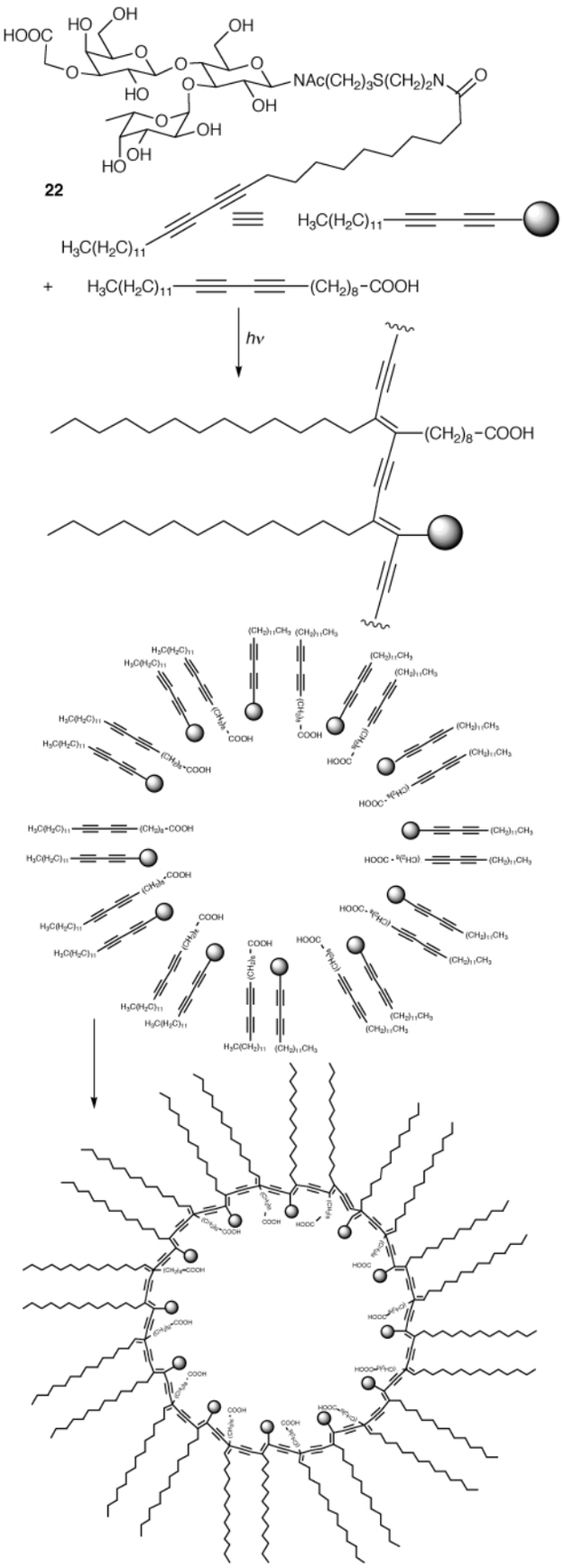
6 Post-glycosylation modifications

6.1 Sulfation

Sulfated oligosaccharides are present in many naturally occurring glycoprotein ligands. Indeed the importance of sulfation in carbohydrate–protein interactions has been emphasized by the discovery that GlyCAM-1, a glycoprotein ligand of L-selectin which is important in leukocyte adhesion, contains two crucially sulfated 6-*O*-Gal and 6-*O*-GlcNac moieties.[180] It should be noted that some procedures for stripping glycans from proteins might also cause sulfate loss.[181]

For the synthesis of sulfated glycans, as might be expected, simple activation of sulfuric acid with DCC leads, as for other non-selective sulfation methods, predominantly to *O*-6 sulfation.[182] Flitsch and co-workers first described the use of stannylene acetals for regioselective sulfation,[183,184] a method that was later combined with temporary boronate protection by Vasella and co-workers.[185] These are now generally accepted methods and have been used, for example, in the selective sulfation of the trisaccharides Le^a [186,187] and Le^x.[49,187,188] Such methods have even allowed syntheses of all possible regioisomeric sulfates of certain carbohydrates.[189] Flitsch and co-workers have also recently introduced the use of trifluoroethyl ester as a sulfate protecting group, which can be introduced by trifluorodiazoethane and cleaved with butoxide.[190] Ring-opening of cyclic sulfates with carboxylic acids allows regiospecific syntheses of acylated and sulfated carbohydrates.[191]

Crout and co-workers have elegantly exploited the regioselectivity of esterase-catalyzed hydrolysis and acetylation reactions to produce a number of regioselectively sulfated carbohydrates.[192] Hydrolysis of peracetylated carbohydrates selectively freed O-6 for sulfation. Alternatively, hydrolysis then



Scheme 17

acid catalyzed acetate migration gave selective access to O-4 for sulfation.

Wong and co-workers have described a useful multienzyme system that provides sulfotransferase-mediated regioselective sulfation of oligosaccharides as well as coenzyme regeneration.[193] The use of sulfatases in a combined chemical regioselective sulfation and enzymatic regioselective desulfation strategy has also been described for the synthesis of sulfated galacto- and lacto-sides.[194]

6.2 Phosphorylation

Phosphorylation is a crucial element in signal transduction and many of the most basic sugar-processing biosynthetic pathways utilize 6-*O*-phospho glycans. The use of phosphorylating enzymes (kinases) for the formation of phosphorylated carbohydrates is therefore attractive but is hampered by the need for recycling the nucleotide triphosphate coenzyme that is the source of the phospho group. This may be achieved using a cheap source of phosphate, such as phosphoenol pyruvate which in the presence of nucleotide diphosphate and pyruvate kinase forms nucleotide triphosphate and pyruvate.[195]

For polyphosphorylation chemical methods provide a less selective but more reactive source. For example, deprotonation of free hydroxy groups with LDA followed by treatment with tetrabenzylpyrophosphate is an effective method[196] that has been exploited in inisitol phosphate syntheses.[197] As alternatives the H-phosphonates can be formed using, for example, salicylchlorophosphite,[198] or phosphites can be generated using a diethylaminodioxaphosphepane[199] which can then be oxidized under mild conditions to the corresponding phosphates.

In the preparation of glycopeptides containing mannose-6-phosphate, phosphorylation with bis(2,2,2-trichloroethyl)-phosphorochloridate proved a more successful choice than the introduction of allyl protected phosphate. After glycosylation and peptide coupling steps, the trichloroethyl groups were removed by catalytic hydrogenation.[200]

6.3 Pyruvate acetals

Pyruvate acetals are frequent structural elements of bacterial polysaccharides[201] and consequently a number of methods for their specific introduction into carbohydrates have been developed. The use of ethyl pyruvate in direct acid catalyzed ketal formation typically affords low yields of diastereomeric mixtures.[202] More successful and specific methods include the oxidation of (3,4-dimethoxyphenyl)methyl acetals with ruthenium tetraoxide[203] and the SO_2Cl_2–triflic acid catalyzed transacetalation using pyruvate dithioacetals.[204]

6.4 Acetylation

9-*O*-Acetyl sialic acid is an example of an important glycocode motif present in naturally occurring glycoconjugates. Furthermore it is an antigen found in humans almost exclusively on the surface of cancerous cells. Although the attempted selective chemical synthesis of this saccharide has failed, protease catalyzed regioselective esterification using vinyl acetate as an acetyl donor was successful both for sialic acid and the sialylated glycolipid GD3.[205]

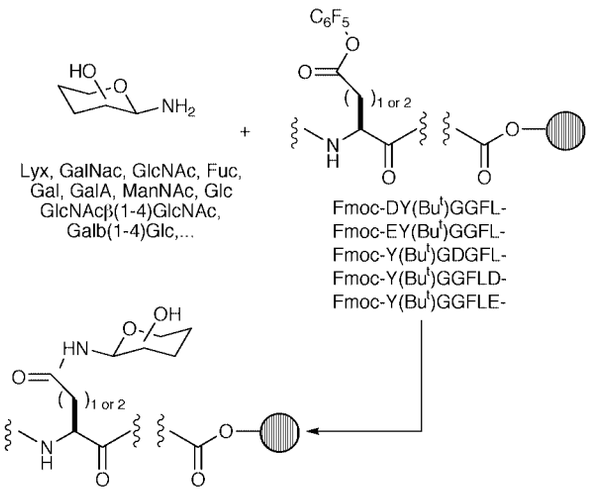
7 Glycoconjugate libraries

Combinatorial or parallel synthesis methods as a means to generating libraries of potentially biologically potent compounds are a valuable and powerful additional tool in chemistry. However associated problems of reactivity, regioselectivity and stereoselectivity have particularly hampered the development of carbohydrate containing libraries.[206,207]

Linear glycopeptide libraries have been constructed along lines which essentially adapt standard solid phase peptide synthesis but with application to glycosylated amino acids as building blocks. For example, the position of the Tn antigen (GalNAc-Thr/Ser) residue in a decapeptide was systematically varied to give rise to a small isomeric library for probing the most compatible site for glycosylation in terms of immune response.[208] Once identified, the nature of the glycan and pep-

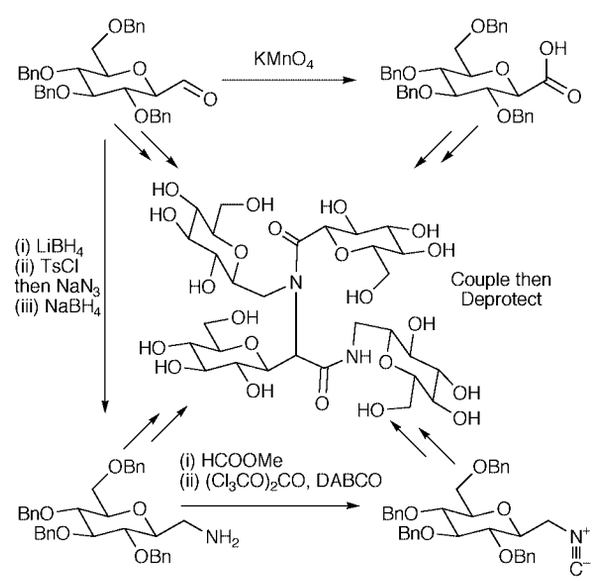
tide at this position were varied generating a second generation library of 12.

Vetter and co-workers have adapted the concept of Lansbury's convergent glycopeptide synthesis approach (see section 4.1) to construct a small library on a polystyrene resin. A total of 18 unprotected glycosylamines were coupled with 5 different hexapeptides containing aspartate and glutamate residues whose carboxy side chains were activated as their corresponding pentafluorophenyl esters (Scheme 18).[209] Recently, a small library of tentagel bound glycosylamines, have been used in a rare example of 'on-bead' lectin screening.[210]



Scheme 18

Ugi four-component condensation of C-glycoside aldehydes and/or C-glycoside carboxylic acids with various isocyanates and Rink resin-based amines has allowed a powerful solid phase approach to a 192-compound library of potential sLe^x glycopeptoid mimics.[211] More recently the same condensation concept has been applied to the construction of a small library of glycoclusters in which all four components are carbohydrate derived (Scheme 19).[212]



Scheme 19

As yet, and despite its widespread and elegant application in other areas of glycoconjugate synthesis, there are no examples of the use of biocatalysis in the preparation of glycoconjugate libraries.

8 Applications and uses

The functions and versatility of glycosylated biomolecules are, as a consequence of their ability to transmit sophisticated information, incredibly broad and the potential applications of glycoconjugates are therefore correspondingly wide. For example, lectins have been implicated in physiological processes ranging from receptor mediated endocytosis, glycoprotein quality-control, the interaction and subsequent invasion of pathogens, and the triggering of effects that lead to the release of biomodulators. A thorough understanding of these processes is essential to their successful exploitation in pharmaceutical therapies that either block lectin-mediated processes through inhibition or that exploit this binding to target designed glycoconjugates to lectin-expressing cells. Indeed, the recent failure of a number of carbohydrate-based drugs may be attributed to a poor understanding of their supposed mechanism of action rather than due to any inherent flaws associated with carbohydrate therapeutics.[213]

Uses and applications have largely reflected the discovery and understanding of various types of lectins, which may be divided according to the ligands that they bind. In this regard, three sub-types have dominated glycoconjugate application: the Gal/GalNAc binders, such as the hepatic asialoglycoprotein,[214] the Man binders, such as the macrophage mannose-binding protein[215] and the sialic acid binders, which are carried by a number of infective agents, such as influenza virus and *H. pylori*, that exploit the proliferation of sialic acids found on human glycan termini. It is interesting to note that in certain systems glucosylation may transfer similar degrees of binding affinity as galactosylation in the targeting of galactose specific lectins.[216] Whilst this may appear puzzling at first inspection it should be noted that only very few naturally occurring glycoproteins *e.g.*, collagen, bear glucosyl residues and it is therefore not an element of glycocode that typically needs to be discriminated. Exceptions to these generalizations are antibodies, which by their very nature bind variable ligand types (see section 8.4 for exploitation of glycoconjugates in this area). Moreover, some interactions rely on far more complex ligands. An example is the tetrasaccharide sLe^x ligand of selectin binding proteins (see section 8.6).[217]

8.1 Probing protein activity, function and mechanism

In addition to their critical role in communication events, glycosylated proteins have long been known to have greater resistance to thermolysis and proteolysis.[122] A much less widely explored aspect is that they also display altered catalytic properties.[218–220] For example, carbohydrate–protease conjugates, which show greater stabilities at high temperatures[221] and in organic solvents,[222,223] also catalyze high yielding peptide syntheses.[224] Furthermore, glycosylation of active site binding pockets of a serine protease with a variety of different mono- and di-saccharides[142] resulted in up to 8-fold greater activity levels than those shown by the corresponding native enzyme.[225] They also allowed the synthesis of dipeptides that were not possible using the unglycosylated catalyst. RNase A which has been mono- and di-glycosylated by EDC-mediated coupling of D-glucosamine to carboxylates at Asp 53 and Glu 49 displays lower catalytic activity but greatly enhanced thermal stability.[226]

An interesting aspect of *N*-linked protein glycosylation is its role in the "quality control" of proteins that are found during translation. Without correct glycosylation many proteins fail properly to fold. This suggests a novel role for added glycans as indicators of correct protein structure. In *N*-linked glycoprotein biosynthesis a 14-residue oligosaccharide core is added as a first step and then trimmed down to size. It has been suggested that, if nascent protein fails to fold properly, these glycans are incorrectly displayed and cannot be processed in these trimming steps, leading to rejection and degradation. Therefore, these apparently superfluous trimming steps may not simply be a means to glycan structure but steps along a "quality controlled" protein production line.[227] It has also been suggested that gly-

cans aid the folding and transport of proteins by protecting them from proteolysis.[228]

In aqueous solution a turn is induced in a SYSPTSPSYS segment of the C-terminal domain of RNA polymerase II when the threonine side chain is *N*-acetyl-D-glucosaminylated, whereas the corresponding non-glycosylated peptide adopts a randomly-coiled structure.[229] This striking difference in structure in aqueous solution is a clear indication of the importance of glycosylation, even by a single saccharide, upon local peptidic structure and opens the door to a host of such crucial structural subtleties in glycosylated proteins. Moreover, this result tallies well with recent suggestions that reversible glycosylation of this site might act as a regulatory mechanism for the control of transcription akin to phosphorylation.[230]

Glycoproteins which act as antifreeze in the serum of deep-sea fish allow them to survive at temperatures as low as −2 °C. Their ability to lower the freezing point is not proportional to the concentration and is not accompanied by altered melting points. This non-colligative effect is thought to arise from a mechanism that inhibits ice nucleation and crystal growth. Oligomeric glycopeptide analogues of such proteins have been prepared and show significant antifreeze properties.[112]

8.2 Drug delivery and targeting

The specificity of the asialoglycoprotein Gal/GalNac specific receptor[214] has been widely exploited for liver targeting and in many cases has been the model for targeted drug delivery. Covalent attachment of drugs through various degradable linkers to lactosaminylated and galactosylated human serum albumin has allowed the targeted delivery of antiinflammatory agents, such as Naproxen,[231,232] and antivirals, such as arabanoside-AMP,[233] which are absorbed into hepatic cells through receptor-mediated endocytosis.[234] This process, which is an internalization of receptors such as lectins, is employed by all cells and so is potentially unlimited in its targeting potential. Interestingly, mannosylated HSA (human serum albumin) is also absorbed but gives rise to a markedly altered drug distribution within the liver.[231] An extensive study of the degree of galactosylation of a variety of proteins has revealed a direct correlation with the pharmcokinetics of liver uptake; but revealed little amplification past densities of 0.001 galactosyl residues per Å^2 of protein surface area.[235] Furthermore, the immunogenicity of such neoglycoproteins is low if prepared with a high degree of homogeneity.[231]

Positively-charged polymers greatly enhance the lifetime in the blood of oligonucleotides through complexation with the negatively-charged phosphate backbone and reduce their particular size, thereby affording them protection against serum nucleases. This protection may be utilized to protect oligonucleotides during delivery to target organs. This in turn allows the production of genes encoding biologically important proteins to cells that may express them; this is gene therapy. Gene therapy drug delivery systems based on glycosylated poly-L-lysine systems can present significant advantages over the formation of protein–polylysine conjugates, in which the protein acts as a targeting ligand, as these typically form useless insoluble aggregates during preparation. It has been shown that lactosylation and galactosylation of poly-L-lysine increases both the targeting and expression levels of a given plasmid to human hepatoma cells but decreases the stability of the electrostatic plasmid–poly-L-lysine complex by reducing the level of the charge.[236,237] Similarly, mannosylated, but not galactosylated, poly-L-lysine introduces plasmids into macrophages.[238] Glycopeptide–poly-L-lysine conjugates have also been used in the specific delivery to the liver of genes expressing luciferase as a reporter enzyme.[239]

A number of amphiphilic glycosylated bile acid-derived steroids have been demonstrated as effective enhancers of the transport of polar molecules across cellular membranes.[240] Although no obvious structure–transport activity relationship was observed and so the precise mode of action is uncertain, the implications for the enhancement of the delivery of hydrophilic molecules without cellular disruption are profound.

8.3 Infection or pathogenesis control

Proteins on pathogens, termed adhesins, mediate infection and colonization of a host by binding to ligands on host cell surfaces.[241] These adhesins are typically lectins. The field of glycoconjugate anti-adhesives has consequently been one of intense activity and the design of antiinfluenza and antiulcer compounds provide good illustrations. The key step in infection by influenza is the binding of cellular α-sialic acid residues with viral haemagglutinin (HA) lectins.[22] A number of sialic acid modified glycopolymers[52,242] and glycodendrimers[85] have been synthesized as potential antiinfectives that inhibit this binding. In line with expected clustering effects, inhibitory potencies increase with the glycosylation valency, although the ability of such polymers to prevent the binding of a polyclonal antibody of influenza virus suggests that they also act by limiting the steric access of the viral particle to the blood cell (an effect known in polymer aggregation as steric stabilization).[52,243] The use of the method shown in Scheme 5 to form a polymer without biotin resulted in the formation of a powerful HA inhibitor ($K_i = 1$ nM based on sugar concentration).[244] Interestingly, a synergy is observed in the inhibition of HA when inhibitors of the neuraminidase in influenza are also added. The displacement of the glycoconjugate HA inhibitor from neuraminidase active sites is suggested as a mechanism that increases steric stabilization.[243] Only recently has a more potent inhibitor, based on a glycolipid presenting poly-L-glutamic acid scaffold, been synthesized.[69] A sequential galactosyltransferase and sialyltransferase mediated synthesis of various heptasaccharides allowed variation of the attachment sites on a D-galactose as a bisecting branch to prepare sialic acid presenting probes of influenza HA binding.[245] Although not strictly within the definition of glycoconjugates used in this review, this work valuably established an optimal interbinding site distance of ~9 Å.

Helicobacter pylori are the bacteria that cause gastric ulcers. They attach themselves to gut cells by binding to extracellular sialylated glycoproteins. This adhesion has been effectively inhibited, as part of an antiulceritic strategy, by albumin glycosylated with 3′-sialyllactosyl residues.[246] It is interesting to think that the array of sialylated conjugates in human milk might be serving the same anti-adhesive function as the synthetic glycoconjugates described in this section and once again highlights the need to study Nature's tactics as models for our own.

8.4 Vaccines and immunotherapy

The use of complex oligosaccharides as haptens for the induction of antibodies has a rich history dating back to the discovery in 1929 that oligosaccharides may be rendered immunogenic through their attachment to proteins.[23,247] As early as 1930 their use as a strategy to combat pneumococci was described.[248] However, until the 1970's this work was limited by the often-minute amounts of oligosaccharides available from natural sources. In a seminal series of four papers in 1975[127] Lemieux and co-workers completed the total synthesis of the Lewis-a (Le^a) trisaccharide using newly developed glycosidation techniques and conjugated it to BSA using the acyl azide method (see section 4.2) which he developed for the purpose. This hapten successfully produced antibodies that agglutinated Le^a red blood cells. In a recent triumphant example of the marriage of complex oligosaccharide synthesis and conjugation Pozsgay has constructed human serum albumin conjugates that contain from one to four repeating immunogenic tetrasaccharide units in an attempt to probe the level of saccharidic hapten presentation that is required to develop what may

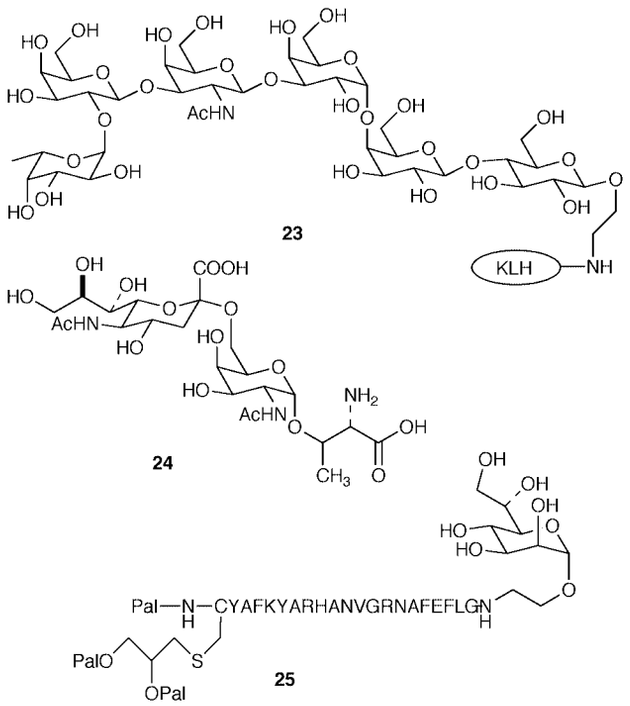


Fig. 6 Glyconjugate vaccines.

become the first example of a vaccine against *Shigella dysenteriae*.[249]

As an approach to a potential anticancer vaccine, Danishefsky has used reductive amination to construct the hexasaccharide-keyhole limpet haemocyanin (KLH) protein conjugate **23** (Fig. 6), at an approximate ratio of sugar–protein of 1 : 150. The hexasaccharide moiety, termed globo H, which was constructed using the glycal methodology, was originally isolated from a glycoceramide associated with breast cancer. The synthetic globo–KLH conjugate was successfully used to induce high anti-globo H antibody titers and induced cell lysis, in the presence of human complement, at levels approaching those of monoclonal antibodies raised against cancerous cells.[250] Other potential anticancer vaccines have been reported based on other tumour-associated epitopes,[251] such as the sialyl-Tn motif **24**; which as a KLH-conjugate has shown promisingly higher survival rates in clinical trials.[252]

Typically, a small array of different proteins, such as diptheria toxin, tetanus toxin and BSA, are used as vaccine carriers due to their well characterized immunogenicity. However, their repeated use is associated with a degree of vaccination tolerance. To circumvent this problem Boons and co-workers have adopted an alternative approach to vaccine synthesis through the incorporation of shorter, more specific peptide sequences.[253] The simple tricomponent vaccine **25** (Fig. 6), which was constructed using conventional solid phase methodology and EDC mediated coupling, has been suggested as a minimal meningitis vaccine structure. It contains an L-glycero-D-manno heptoside and a short peptide sequence associated with *Neisseria meningitidis*. The palmitoylated cysteine terminus allows aggregation of **25** into liposomes for recognition by the immune system.

T-cells mediate intercellular immune responses and are particularly useful as they allow even infants under the age of two to be effectively vaccinated. Whilst oligosaccharides alone do not typically elicit T-cell responses, the discovery[254] that glycopeptides, has allowed immune responses to sugars to be probed systematically and recent developments in the synthesis of more complex glycans bound to peptide and glycoproteins in a site-specific manner (see section 4.1 and 4.2) should allow this to be probed in ever greater detail in the future.

Glycosylation can also, in contrast, lead to reduced immunogenicity. For example, the glycosylation with a heptasaccharide

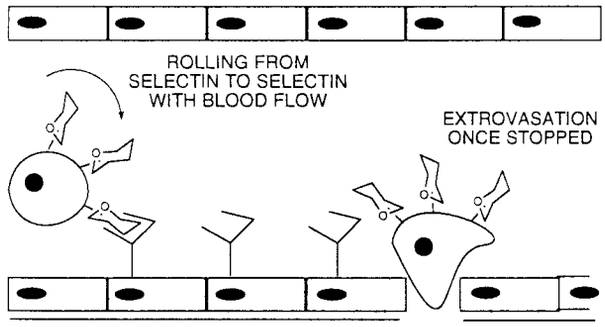


Fig. 7 Schematic representation of leukocyte rolling during the inflammatory response.

of a normally T-cell reactive peptide from the influenza virus led to a reduced response by T-cells and may have implications for the evasion of the immune system shown by some pathogens.[255]

8.5 Avoiding immunogenicity in xenotransplantation

In the field of xenotransplantation a major problem is caused by antibody mediated rejection. The primary response is the expression of anti-carbohydrate antibodies and in particular against any carbohydrate with a α-D-Gal*p*-(1→3)Gal terminus.[256] In fact, this antigen is so commonly encountered that, on average, anti-galactose antibodies are the most abundant natural antibody in humans representing 1–2% of total IgG and 3–8% of total IgM in humans. A successful strategy that has been employed so far has been to treat the transplant recipients serum with conjugates bearing this so-called α-Gal epitope in an attempt to "wash-out" the anti-α-Gal antibodies.[257] To this end the Wang group has used recombinant galactosyltransferase[258,259] to prepare α-Gal epitope containing structures. Transgenic expression of a galactosidase to cleave the α-Gal epitope on cell surfaces and a fucosyltransferase to modify it has also been described. This elegant *in vivo* approach successfully resulted in a 30-fold drop in surface α-Gal epitope concentration as well as a 10-fold drop in immune response to pig cells.[260]

8.6 Antiinflammatory strategies

During the inflammatory response, damage in tissues surrounding a blood vessel causes the influx of signalling molecules. These initiate the rapid expression of carbohydrate-binding proteins, selectins, on the inner surface of blood vessels (**e**ndothelium – hence **E**-selectin) and on **p**latelets (**P**-selectin), which bind complex carbohydrates with high avidity in a specific manner. This binding causes white blood cells (**l**eukocytes) to adhere to the vessel walls, which complete a two-way binding process by expressing **L**-selectins. In the flow of blood, the leukocytes roll towards the site of damage where they pass through to the surrounding tissue (Fig. 7). Whilst this is a well-controlled process in healthy individuals, in excess it is a cause of septic shock, arthritis, asthma and myocardial infarction.[217]

The precise identity of the physiological selectin carbohydrate ligands is not known, however the tetrasaccharide sialyl-Lewis × (sLe^x) is weakly bound by all types and serves as a useful benchmark. Several copies of this motif are present in glycoproteins, such as GlyCAM-1 and PSGL-1, that are more tightly bound ligands and present in high concentrations on the surface of neutrophils. Other potent ligands such as sialyl-di-(Lewis x) have also been identified.[261] It should be noted that the difficulty of correctly assessing binding is complicated by the recent discovery that simple acidic ion exchange resins also bind to selectins.[262]

The three selectin types (L-, E- and P-) show structural

homology. Selectivity in the inhibition of these different types may allow control of their similar but non-identical functions. Kiessling and co-workers have explored this aspect of inhibition with their ROMP-prepared polymers to great effect.[49,50] 3-Sulfo and 3,6-disulfo D-galactose and 3′,6-disulfo Le^x trisaccharide[263] containing polymers were chosen to mimic the charge distribution in ligands such as GlyCAM-1. The 3,6-disulfo polymer typically showed more potent inhibition of P-selectin than sLe^x with an up to 100-fold selectivity preference for P- over L-selectin. Interestingly, some of these polymers not only acted as inhibitors but also promoted a dose-dependent L-selectin shedding mechanism.[263,264] This recruitment of, as yet unidentified, shedding peptidases has broad implications for control of extracellular lectin presentation.

The RGD-sLe^x conjugate **16** (Scheme 11) is a more potent P-selectin inhibitor (IC_{50} 26 μM) than many clustered or multiantennary sLe^x conjugates and suggests that protein-conjugation is as important, if not more so, to avidity than multivalency.[118] Glycosylated liposome-like polymers bearing a sLe^x mimic (Scheme 17) display nanomolar inhibitory constants.[178]

8.7 Other therapeutic strategies

The approaches used to target drugs described in section 8.2 may also be used to target physiologically beneficial enzymes. For example, the mannosylation of enzymes has allowed the targeting of enzymes to particular diseased cells. Replacement β-glucocerebrosidase, an enzyme which is lacking in Gaucher disease,[265] and the beneficial anti-oxidant effects of superoxide dismutase (SOD)[266] have both been directed to macrophages. SOD has also been conjugated with sodium hyaluronate, a polymer of the dimeric motif GlcNAc-glucuronic acid. This combined the ability of SOD to catalyze superoxide anion decomposition with the hydroxyl radical scavenging capacity of hyaluronate in a potentially dual-action antiinflammatory.[267]

Adhesive lectin-mediated processes have also been implicated in tumour metastasis and this work has led to clinical trials that successfully reduced liver invasion by blocking hepatic lectins.[241]

A testis Gal specific receptor[268] and selectin-like interactions[269] have been identified, and may indicate a role for carbohydrate–protein interactions, in sperm-egg recognition with concomitant implications for the use of glycoconjugates in contraception or fertility treatments.

8.8 Analytical techniques and other applications

Analytical techniques also rely on glycoconjugates. For example, deposition of a mannose specific lectin and mannosylated enzymes layer-by-layer on a platinum surface allowed the preparation of a sensitive active enzyme electrode.[270]

Enhanced staining techniques that employ biotinylated glycopolymers and glycoproteins (glycohistopathology) have revealed different tumour types with a degree of specificity and sensitivity that is comparable to that of antibodies. They have also provided a further level of tumour subtyping according to their relative levels of expression of different types of carbohydrate binding sites.[271] It should be noted that simply by providing evidence for the presence of specific binding sites the use of these conjugates in lectin detection also demonstrates their potential pharmaceutical use in the targeting of lectin structures.

An exciting area of research with great potential is tissue engineering, which in principle will allow the design and growth of artificial organs by correct moulding and arrangement of cell types. The role of receptor–ligand interactions is crucial in correctly designing such systems and for this reason the use of glycoconjugates, largely glycopolymers, to culture *e.g.*, hepatocytes, is at the forefront of this technology.[272]

9 Electronic information resources

As a consequence of the vast output of results that glycoscience creates, rapid analysis of this data is becoming ever more important and aspects of glycoscience on the internet have recently been reviewed.[273] The following are highlighted sources of valuable information that relates to glycoconjugates:

The Complex Carbohydrate Structure Database (CCSD or CarbBank) [http://bssv01.lancs.ac.uk/gig/carbbank.htm] acts as the oligosaccharide equivalent to GenBank. It allows access to information about structure, author *etc.* and the compilation of records that may be submitted to the CCSD. A guide to its use has recently been published.[274,275] Sugabase [http://www.boc.chem.ruu.nl/sugabase/databases.html] is a carbohydrate-NMR database that combines CCSD Data with proton and carbon chemical shift values and allows searches for both carbohydrate structures and/or NMR data as well as literature references.

Five excellent sources of glycoscience related information are The Glycoscience Network (TGN) [http://www.vei.co.uk/TGN/], The Carbohydrate Research Foundation [http://www.zestec.nl/crf/], CarbHyd [http://www.public.iastate.edu/~pedro/carbhyd/carbhyd.html], Glycoforum [http://www.glycoforum.gr.jp/] and Forum: Carbohydrates Coming of Age (FCCA) [http://www.gak.co.jp/FCCA]. The latter includes Glycoword [http://www.gak.co.jp/FCCA/glycoword/wordE.html] which contains a number of informative articles intended to act as a quick reference to key terms and SugarMap an elution co-ordinate database that supports a three-dimensional (3-D) sugar mapping technique that utilizes carbohydrate elution positions on three HPLC columns.

The University of Alberta Glycobiology Group [http://glyco2.chem.ualberta.ca/MISC/storage/glycosyl_top.html] have made available an excellent and comprehensive searchable list of over 700 different types of glycosidic bonds and linkages between carbohydrates extracted from the literature of 1994 by Hindsgaul and Barresi.[276]

The Complex Carbohydrate Research Centre at (CCRC) at the University of Georgia is the home of CarbNet [http://www.ccrc.uga.edu/web/ccrcnet/], a neural network-based pattern recognition search engine that uses submitted GC profiles and NMR spectra to identify a limited number of oligosaccharide structures.

O-GLYCBASE[277] [http://www.cbs.dtu.dk/databases/OGLYCBASE] is a database of over 170 glycoproteins with experimentally verified O-linked glycosylation sites, compiled from protein sequence databases and the literature. It contains information about the glycan, the sequence, literature references and is http-linked to other databases. The information in this database has also provided the basis for a predictive neural network that may be applied to predictions of mucin type GalNAc *O*-glycosylation sites in mammalian proteins and is available at [http://www.cbs.dtu.dk/services/NetOGlyc/].

10 Future directions

Carbohydrates have been used successfully as non-proteolysable scaffolds in the design of peptidomimetics.[278,279] The use of D-glucose as a rigid three-point scaffold of secondary structure has also allowed the creation of 16 libraries each of 48 different motifs on a tentagel-based resin.[280] Very recently, carbohydrate oligomers have been synthesized which form turns and elements of folding structure even for very small numbers of residues.[281,282] The unusually high levels of secondary structure that exist in such structures allows one to envisage a new generation of designer conjugates in which carbohydrates provide not only the surface ligands but an extremely well-defined and predictable backbone as well.

Glycosylation of enzymes may allow the development of novel synthetic catalysts. The ability of glycosylation methods[142] to glycosylate the binding pockets of synthetically useful enzymes also creates opportunities to broaden substrate

specificity. For instance, such an array of hydrogen bonding hydroxy groups may enhance the specificity of peptidases towards hydrogen bonding substrates such as glycosylated amino acids and overcome some of the short comings of existing enzyme-catalyzed glycopeptide formation.[110,111]

Finally, in order that the application of glycoconjugates in therapeutic strategies becomes more widespread certain features must be addressed. Any of the glycoprotein structures described above represent potential sources of immunogenicity and hence much attention has been paid to the development of biocompatible polymers as alternatives. By building on the advances of glycoprotein applications, certain large (>100 kDa) biocompatible polymers, may offer all the advantages of glycoproteins but with reduced immunogenicity.[283] Unfortunately many glycoconjugates are also poorly characterized and so future efforts must also focus on homogeneity as a goal. In this context, carbohydrate science is no longer a question of chemistry *or* biology – it is now both. Collaborations between those making glycoconjugates and those applying them will no longer be viewed as multi- or inter-disciplinary – they are part of a new unified discipline intent on making exciting, well-defined molecules with enormous potential for the treatment of disease. A recent commentary expressed the view that "there are Nobel prizes awaiting those who can interpret the language of the carbohydrates in biological communication" [284] – perhaps more importantly this interpretation will at last allow truly specific therapies in the business of making people's lives better.

This review has scratched the surface of glycoconjugate science. The opportunities for glycoscientists to make yet further progress in all of these areas and more are enormous.

11 Notes and references

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