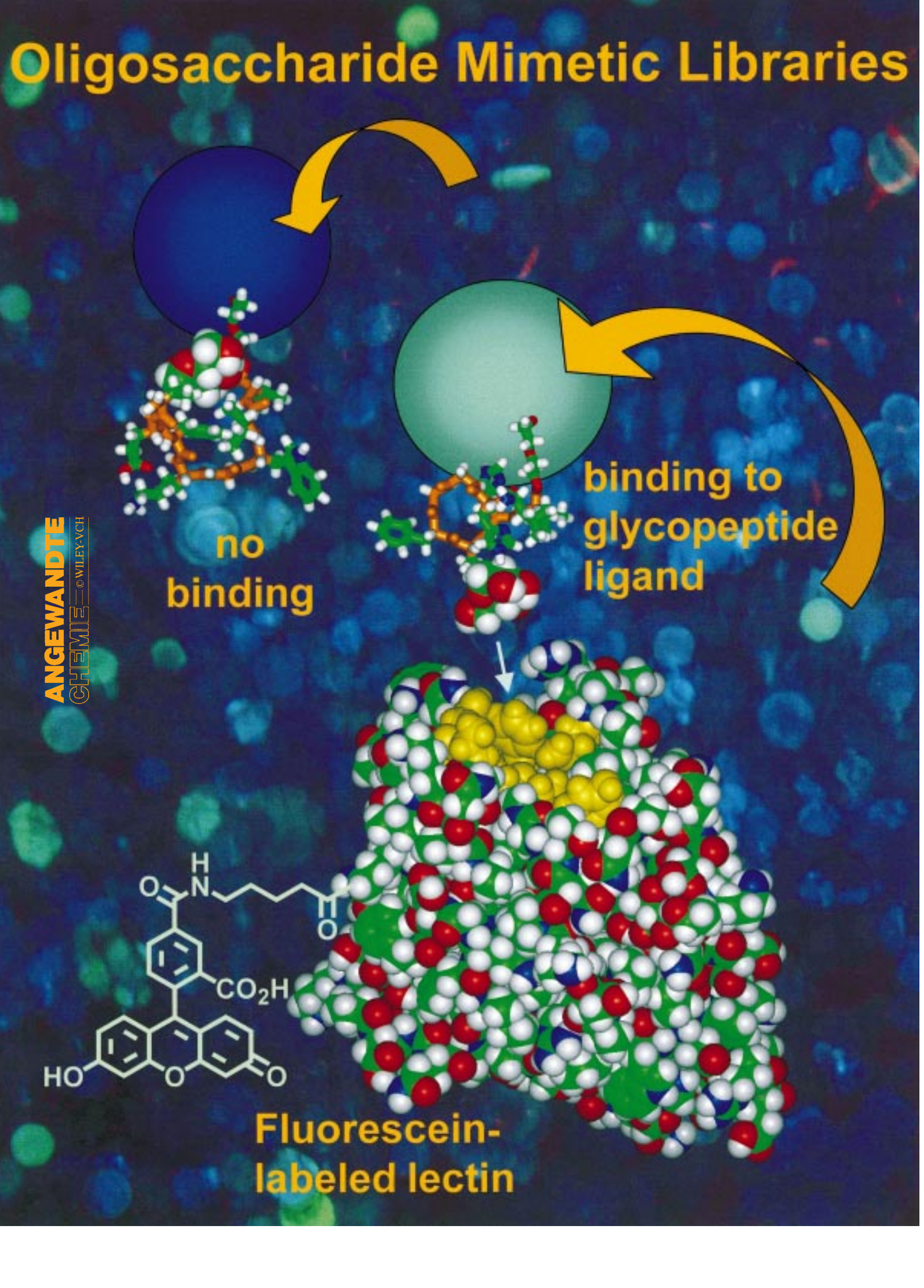
Oligosaccharide Mimetic Libraries



Glycopeptide and Oligosaccharide Libraries

Phaedria M. St. Hilaire and Morten Meldal*

Despite the burgeoning interest in the various biological functions and consequent therapeutic potential of the vast number of oligosaccharides found in nature on glycoproteins and cell surfaces, the development of combinatorial carbohydrate chemistry has not progressed as rapidly as expected. The reason for this imbalance is rooted in the difficulty of oligosaccharide assembly and analysis that renders synthesis a rather cumbersome endeavor. Parallel approaches that generate series of analogous compounds rather than real libraries have therefore typically been used. Since generally low affinity is obtained for interactions between carbohydrate receptors and modified oligosaccharides designed as mimetics of natural carbohydrate ligands, glycopeptides have been explored as alternative mimics. Glycopeptides have been proven in many cases to be superior ligands with higher affinity for a receptor than the natural carbohydrate ligand. High-affinity glycopeptide ligands have been found for several types of receptors including the E-, P-, and L-selectins, toxins, glycohydrolases, bacterial adhesins, and the mannose-6-phosphate receptor. Furthermore, the assembly of glycopeptides is considerably more facile than that of oligosaccharides and the process can be adapted to combinatorial synthesis with either glycosylated amino acid building blocks or by direct glycosylation of peptide templates. The application of the split and combine approach using ladder synthesis has allowed the generation of very large numbers of compounds which could be analyzed and screened for binding of receptors on solid phase. This powerful technique can be used generally for the identification and analysis of the complex interaction between the carbohydrates and their receptors.

Keywords: Combinatorial chemistry · Oligosaccharides · Carbohydrates · Glycopeptides · Receptors

1. Parallel Arrays versus Libraries of Compounds

Combinatorial methodology has been a driving impetus in many areas of research and has gained considerable ground since its introduction at the turn of last decade. This progress, however, has not been reflected in the field of oligosaccharides, primarily due to difficulty in their synthesis and analysis. As an alternative, the readily available glycopeptide libraries have been exploited as potential functional mimics of oligosaccharides. These libraries can be essential tools in the development of carbohydrate-based therapeutics such as tumor vaccines,[1] xenotransplantation aids, and antiinfectives. A few reviews have recently briefly described carbohydrate-based combinatorial libraries and parallel syntheses.[2–9]

Here, we present a comprehensive overview of the many aspects required for the design, synthesis, analysis, and screening of solution and solid-phase carbohydrate-based libraries. In this review, combinatorial libraries have been defined as assemblies of compounds, which are derived through a real combinatorial step leading to exponentially increasing numbers of products relative to the number of reagents used (Figure 1a). This staggering increase in products may be achieved either by mixing reagents to form product mixtures or by introducing mixing of intermediates in solution or compartmentalized on solid phase, for example, by the split and combine approach. Due to the ease with which this process is carried out on solid phase, the potential of solid-phase libraries by far exceeds solution libraries and thus, emphasis is placed on solid-phase libraries in the present review. Combinatorial methods which have only been demonstrated by synthesis of libraries containing a few compounds, but with the potential of generating large numbers of structures are considered. Indeed, the very fact that there are few examples of truly combinatorial oligosaccharide libraries, underscores the great challenge faced by researchers in this field.

Parallel syntheses are also described, particularly in cases where the strategy employed can be extrapolated to create a true library or where interesting biological results have been obtained. It must be noted however, that these are considered to be extensions of conventional synthesis of analogues and

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not libraries since they do not easily give rise to a very large number of compounds, that is, they do not take advantage of combinatorial exponential increase in numbers of products (Figure 1b).

2. Carbohydrate-Binding Proteins

Carbohydrate-binding proteins (CBP's) located in cellular membranes or recirculating in the cytosol or serum are involved in a variety of important biological functions including communication and intercellular adhesion, adhesion of bacteria or viruses, activation of the innate immune system, leukocyte rolling, hepatic clearing of aged serum proteins, and sorting of newly synthesized glycoproteins.[10, 11] Based on their mode of binding, mammalian CBP's have been divided into three major groups: the C-type, the S-type, and the P-type lectins.[12]

The calcium-dependent C-type lectins include the E-, L-, and P-selectins while the galectins are calcium-independent S-type lectins. The latter have a binding site sized for tight interaction with di- or trisaccharides. The former bind their ligands mainly through coordination of two vicinal hydroxyl groups of a single sugar moiety to a bound calcium ion in the carbohydrate recognition domain (CRD) and the surrounding sugars of the oligosaccharide ligand add to the binding specificity through relatively weak additional interactions. Due to the simple nature of this interaction, the specificity of selectin binding is quite broad and can be mimicked by simple monosaccharide-bearing compounds.[13, 14] However, the biological activity of ligands is structure dependent and complicated by a range of factors such as multivalency and clustering.[15, 16]

The calcium-independent receptors of the P-type involved in the clearing and sorting of glycoproteins include the mannose 6-phosphate receptors,[17] the hepatic Gal/GalNAc receptors,[18] and the Man/GlcNAc receptors isolated from liver extracts.[19] These receptors are truly multivalent and bind to the termini of bi-, tri- or tetraantennary N-linked oligosaccharides. Although most known receptors recognize Gal/GalNAc or Man/GlcNAc, there are also receptors, such as the macrophage sialic acid receptor, for the recognition of sialylated complex glycans.[20]

The collectins, mostly involved in the innate immune system,[21] are large surface or serum proteins composed of bundles of structural collagen stalks with trimer heads containing a calcium-dependent CRD's and may be considered C-type by structural similarity. Their binding to simple mannose oligosaccharides is relatively weak and activation of the complement cascade is only effected when the protein interacts multivalently with large polymannans on foreign cell surfaces creating an activation template for complement-processing enzymes.[22] Due to the unspecific nature of this multivalent interaction and the long distance between the sites of interaction (53 Å),[23] multivalent synthetic ligands for these proteins are difficult to design and prepare. However, each CRD may be targeted with high-affinity monovalent ligands.

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Phaedria M. St. Hilaire was born in Dominica in the Eastern Caribbean and received her B.Sc. in Chemistry from the University of South Carolina where she was a Presidential Scholar. As a Fellow of the Integrated Toxicology Program she obtained her Ph.D. in Organic Chemistry from Duke University in 1995 where, under the supervision of Dr. Eric Toone, she characterized the interaction of the Shiga-like toxin with its carbohydrate receptor. An interest in carbohydrate-mediated interactions led to a postdoctoral position at the Carlsberg Laboratory where she has developed methods for the synthesis and analysis of glycopeptide libraries. In 1997, she became a Research Associate and her current research interests include the use of combinatorial libraries to study protein–carbohydrate interactions and to develop novel, specific, protease inhibitors. She also examines fundamental processes related to the solid-phase screening process.

Morten Meldal was born in Denmark in 1954 and is a Senior Scientist heading the Synthesis Group at the Carlsberg Laboratory and also an Adjunct Professor at the Danish Technical University. He directs the combinatorial chemistry research at the SPOCC Center, hosted at the Carlsberg Laboratory. He received the 1990 Danish Chemistry Award, the 1996 NKT Research Award for Chemistry, the 1996 Leonidas Zervas Award, and the 1997 Ellen and Niels Bjerrum Gold Medal in Chemistry. He has over 190 publications and reviews in international journals and has filed 10 patents. His research interests include: solid-phase organic combinatorial chemistry, polymer chemistry, peptide and glycopeptide synthesis, glycobiology and molecular immunology, methodology and automation, and enzymology and enzyme inhibition. He is well-known for the development of novel PEG-based supports suitable for both solid-phase synthesis and screening, and for novel solid-phase assays based on FRET which are used for the determination of enzyme activity and inhibition.

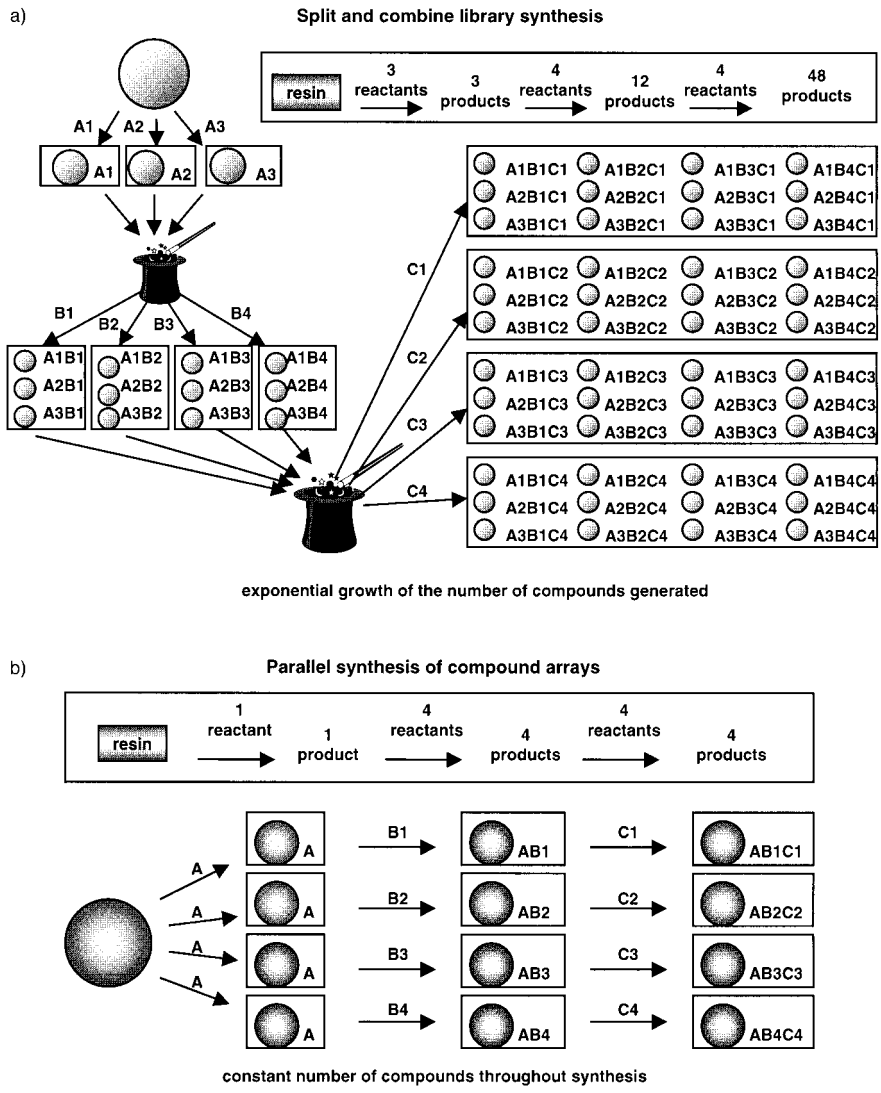


Figure 1. The conceptual difference between the synthesis of a combinatorial library with exponential increase in number of compounds (a) and the syntheses of parallel arrays of discrete compounds (b).

3. The Carbohydrate Ligands

Mammalian glycoproteins consist of 5–90% of glycan structures and these fall in several major groups. There are the N-glycosylations including high-mannose, complex, and hybrid oligosaccharides, and O-glycosylations including the mucins, the blood group determinants, and the proteoglycans (Figure 2).

Glycolipids comprise another group of oligosaccharide-signaling molecules. The high-mannose oligosaccharides are based on 1→2-α-mannosylation of the mannose pentasaccharide core, α-D-Man-(1→3)-{α-D-Man-(1→3)-[α-D-Man-(1→6)-]α-D-Man-(1→6-}β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→N), and these oligosaccharides exist as a mixture of conformational populations around the 1→6 bonds (Figure 3). Any of these conformers may be the active one in contact with a receptor.

The complex antennary glycans are structures obtained by attachment of β-(1→4)-linked lactosamine units to the 2, 4, and 6 positions of terminal mannoses in a α-D-Man-(1→3)-{α-D-Man-(1→6)-}β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→N) core. The terminal galactoses are often sialylated at the 3 or 6 position.

The tetraantennary oligosaccharide shown in Figure 2 has been shown to present an umbrellalike structure as one of the major conformational populations.[24, 25] The distance between terminal sugar residues interacting with receptors may vary from approximately 8–30 Å. In the case of the phosphorylated high-mannose ligands for the mannose 6-phosphate receptor (MPR) the distance between phosphates is about 15 Å.[26]

The mucin oligosaccharides do not appear to have many mammalian receptors. However, bacteria in the gastrointestinal tracts often display receptors that adhere to the mucin-coated epithelia.[27] Recognition of the aberrantly glycosylated forms present in malignant tissue is essential for the rejection of tumors by the immune system.[28–30] The most thoroughly investigated ligands are analogues of the SLe^x antigen[13, 31] involved in leukocyte rolling, adhesion, and shedding by interaction with the E-, P-, or L-selectins for which the optimal ligands and mode of recognition for biological activity are still sought. The fucose residue, one hydroxyl group from the central galactose unit, and the carboxyl group of the sialic acid are essential for the interaction (Figure 4).

The structural investigations of receptors and their carbohydrate ligands indicate that binding of the ligand to the receptor often involves only a few terminal residues. It is therefore anticipated that these receptors will bind to carbohydrate mimetics that present these essential functional groups in the optimal binding orientation. The use of carbohydrate-based combinatorial libraries that are either randomly generated or carefully designed based on knowledge of the receptor and/or ligand will greatly facilitate the discovery of mimetics of complex oligosaccharide ligands.

4. Supports for Solid-Phase Libraries

Parallel arrays as well as libraries of glycopeptides and oligosaccharides can be synthesized in solution or solid phase. The advantages of using solid-phase methods[32] are well documented and detailed descriptions have recently been published.[5, 33]The choice of solid support is crucial and depends on the types of reactions to be carried out as well as the screening methods to be employed. The two most

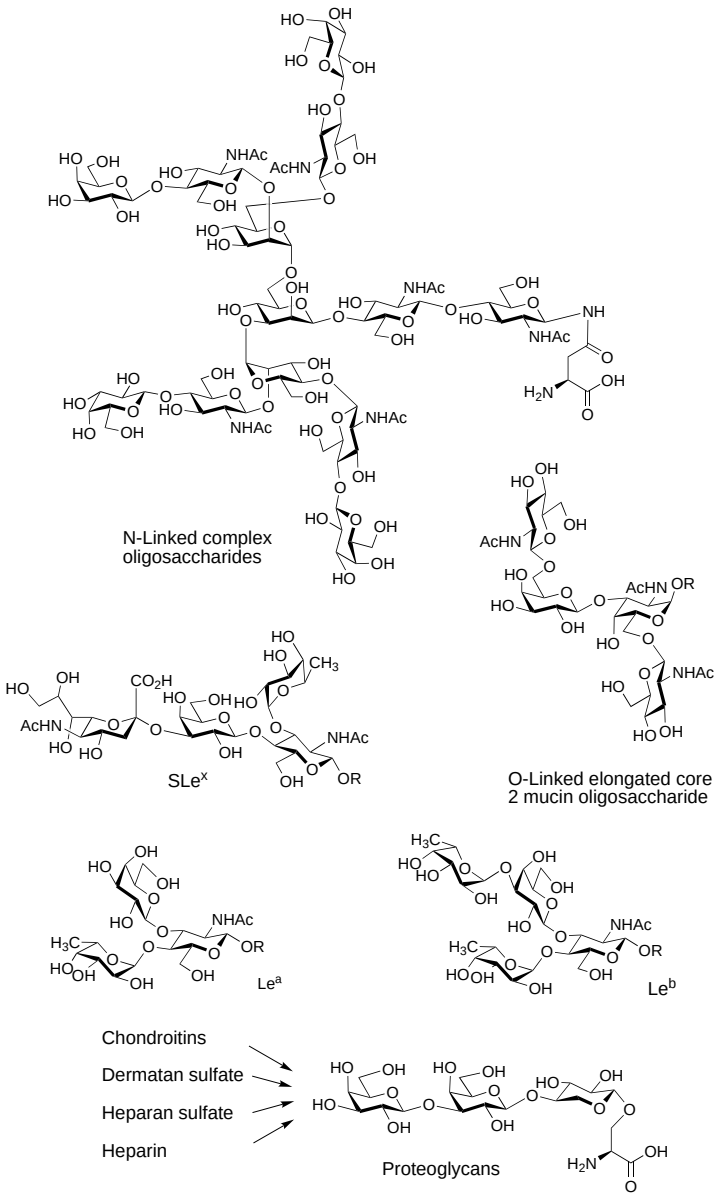


Figure 2. Representative oligosaccharide structures found on mammalian glycoproteins and glycolipids. The complex oligosaccharides may be bi-, tri-, or tetraantennary while the branches may be more or less elongated with 1 →4 linked lactosamine units and they may or may not be sialylated. The SLe^x, Le^a, and Le^b structures represent the different blood group determinants often present on lipids and the elongated core 2 structure is a mucin-type glycosylation. Proteoglycans have a common core to which a variety of linear acidic polysaccharides are attached.

commonly used types of solid supports are polystyrene (PS) based (for example, Merrifield supports[32] including the commonly used Wang resin[34] and TentaGel[35]) or polyethylene glycol (PEG)-based (such as PEGA,[36] POEPOP,[37] POEPS-3,[38] and SPOCC[39]); examples are shown in Figure 5.

Polystyrene resins are comprised of mainly 1–2% divinylbenzene cross-linked backbones with short linkers (Figure 5a). Polystyrene based gels are generally unsuitable for direct on-bead screening, either due to loss of enzyme activity in the polymer or due to their poor swelling in polar media and consequent exclusion of biomolecules from the hydrophobic core of the polymer. This is also true for PS-resins grafted with long PEG chains such as ArgoGel or TentaGel (Figure 5b),[40] although swelling in water is improved and some access to the interior has been achieved.[41, 42] Furthermore, the polystyrene material absorbs light, thus interfering with some fluorescence assays and the hydrophobic core may result in nonspecific protein binding.

PEG-based resins, on the other hand, are cross-linked with long chain PEG macromonomers which also present the amino or hydroxyl functional groups. Therefore, the mechanical and chemical properties of the resins are strongly influenced by the nature of the PEG chains.[33] Additionally, PEG chains are highly miscible with most solvents creating a quasi-homogenous reaction medium. PEG-based resins swell tremendously in aqueous media, thus allowing biomolecules access to the entire bead.[43, 44] These resins are, therefore, suitable for protein ligation,[45] enzymatic reactions, and screening for enzyme activity and inhibition.[42, 46–51]

Since PEG-based polymers confer the advantage of a support that is amenable to both synthesis as well as screening of enzyme activity and protein binding, efforts have been made to generate such polymers with superior properties in both areas. They may be obtained by radical polymerization of long-chain PEG macromonomers to give PEG–polyacrylamide (PEGA, Figure 5c) copolymer[36, 43] or PEG–cross-linked oligostyrene (POEPS-3, Figure 5d).[38] Recently, two novel types of gel supports obtained by anion-catalyzed bulk polymerization of PEG, derivatized with epichlorohydrin (POEPOP, Figure 5e)[37] or by cation-catalyzed bulk polymerization of PEG, derivatized with oxetane (SPOCC, Figure 5f)[39] were introduced. The inert character of the polymers allows the application of harsh organic reactions. The excellent swelling in aqueous buffers has allowed all the above resins to be used in bioassays for enzymes, and PEGA resin was further investigated and showed no nonspecific binding in protein-binding studies.[52]

For the solid-phase synthesis of glycopeptides and oligosaccharides, both polystyrene and PEG-based resins have been successfully used. Experiments that compare the rates of reactions on various resins have revealed that the rate of reaction completely depends on the nature of the reaction itself.[53] Some reactions perform better on hydrophobic resins while others are better on hydrophilic resins. Another issue of particular importance for the synthesis of oligosaccharides on solid phase is the influence of the solid support on the stereochemical outcome of the glycosylation reaction. There are few detailed studies that address this issue, however, preliminary results are contradictory, and some suggest that the anomeric ratio is strongly influenced by the nature of the resin as well as solvent, C-2 group on the donor, and temperature.[54–56] In some cases there seem to be an enhancement of anomeric selectivity on the solid phase.[55] There are presently four examples of successful synthesis of oligosaccharide and glycopeptide libraries in the literature and these have been generated using either TentaGel or a PEG-based resin (PEGA and POEPOP), thus enabling rapid solid-phase screening of the library (see Sections 6.3 and 8.2).

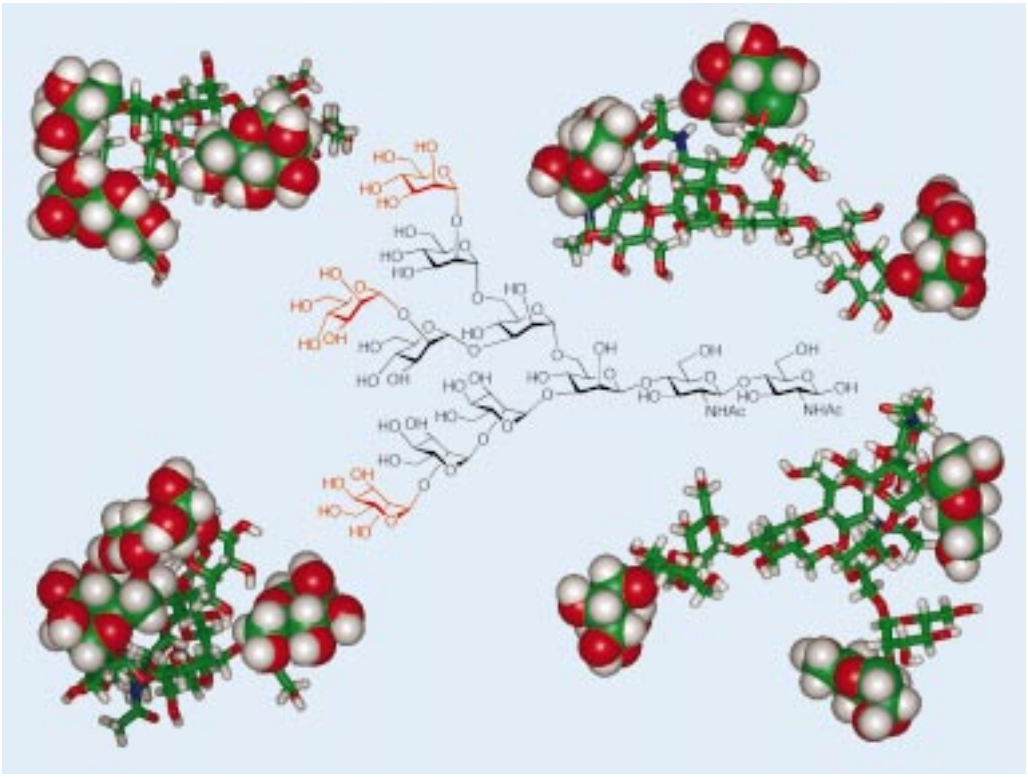


Figure 3. Structural models of four different conformer populations (2 for each 1 →6 link) of a triantennary high-mannose undecasaccharide M9. Terminal mannoses are highlighted as CPK-models.[22]

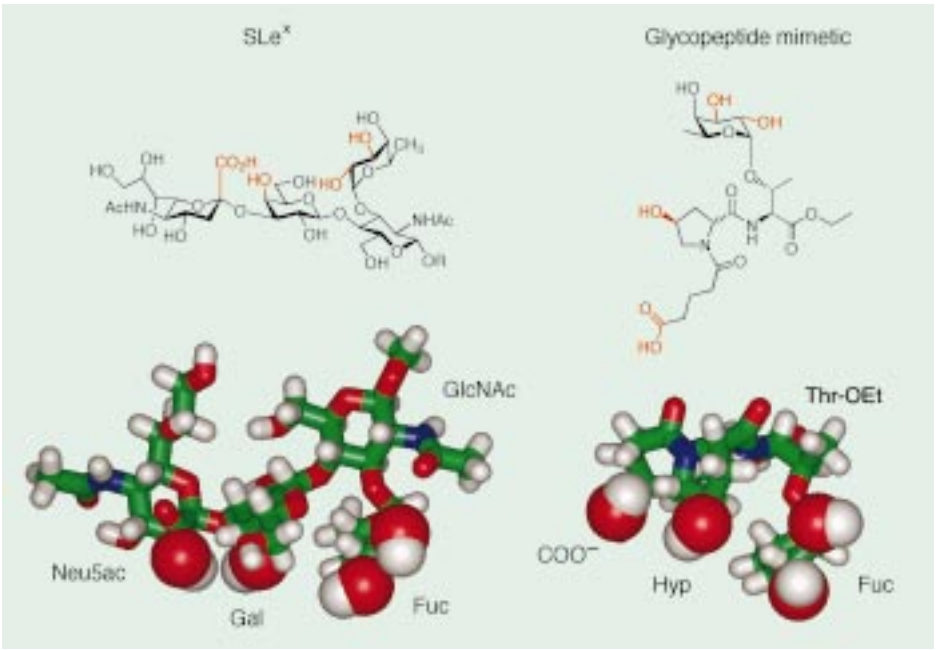


Figure 4. Bound conformation of the SLe^x tetrasaccharide relative to that of an efficient fucosyl glycopeptide mimetic compound.[118] Tightly bound hydroxyl groups are shown as spheres.

5. Analytical Tools for Oligosaccharide and Glycopeptide Libraries

For solution-phase libraries which are composed of mixtures of compounds, the difficulty of analysis escalates with increasing numbers of compounds. Typically, large mixtures of compounds are not analyzed before screening while small ones may be analyzed for reaction completeness using mass spectrometry, HPLC, NMR, or combinations thereof. The identification and analysis of active compounds from these mixtures is painstakingly tedious and complete characterization is only possible after deconvolution procedures and resynthesis of the active compound.[57] For solid-phase libraries, the methods currently developed are discussed below.

5.1. Tagging Techniques in Oligosaccharide Libraries

The number of methods for analyzing non-peptide libraries are increasing and generally fall into two categories: direct methods, usually based on mass spectrometry and NMR spectroscopy, or indirect methods employing encoding—chemical,[58–60] chemoluminescent,[61] or otherwise.[62–64] Many of the methods of chemical encoding are restricted by the additional synthetic effort required and the need to design orthogonal reaction conditions required for the two sets of syntheses. However, chemical coding is the only method that has so far been successfully used for identifying components in an oligosaccharide library.[59] This study utilizes a carbene insertion reaction to attach polyhalogenated aromatic tags that are photochemically released and then analyzed by GC-MS.[60]

5.2. Analysis by Mass Spectrometry

The development of oligosaccharide and glycopeptide libraries obtained by the split and mix method is severely hampered by the lack of concurrent development of general, facile separation and characterization technology. Some headway has been made with chemical coding of the libraries but very few direct methods of analysis exist. One of the promising methods that could be applied to the direct

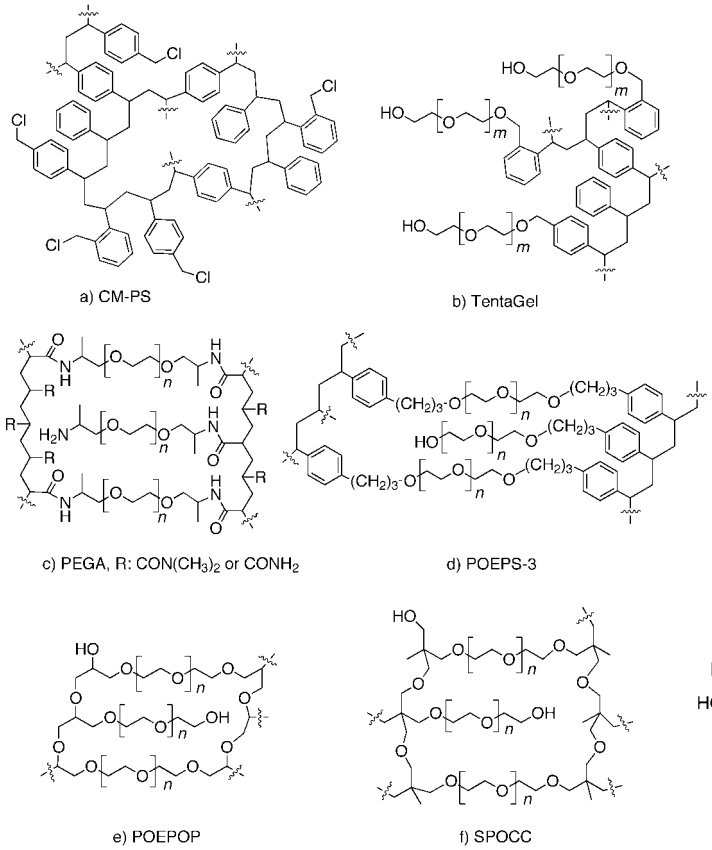


Figure 5. The chemical structure of TentaGel and PEG-based resins. The open structure and inert nature of PEG in biological systems confer ideal properties to the PEG-based resins for bioassays.[33]

characterization of both types of libraries is mass spectrometry, more specifically Matrix Assisted Laser Desorption/Ionisation-Time of Flight mass spectrometry (MALDI-TOF-MS), Electrospray Tandem mass spectrometry (ES-MS-MS), or Fourier Transform-Ion Cyclotron Resonance mass spectrometry (FT-ICR-MS). MALDI-TOF-MS and ES-MS-MS have been used to characterize glycopeptides[65–67] and complex oligosaccharides.[68–72]

The analysis of carbohydrates by mass spectrometry presents special challenges in distinguishing between the branch points, isobaric monosaccharides, and the anomeric configuration of the glycosidic bond. Several methods have emerged using MALDI-TOF-MS. In one strategy, the oligosaccharide is treated sequentially with glycosidases, the mass of the resulting fragments recorded and the identity of the original oligosaccharide determined by a process of deduction.[71, 73] Alternatively, the oligosaccharide fragments are generated during the ionization process in several ways: by postsource decay (PSD), delayed extraction with high-energy collision-induced decomposition (CID), in-source fragmentation or tandem MALDI-MS.[68, 69] In all cases, fragmentation occurs across the glycosidic bond in a manner that is dependent in part on the type of linkage (such as 1→6 or 1→3), the anomeric configuration, and the nature of the monosaccharide.[72, 74] Interpretation of PSD spectra can be somewhat difficult and challenging for complex saccharides but should be much simpler if the oligosaccharide libraries are restricted to three to four monosaccharides.

Similarly, the analysis of glycopeptides has been carried out using primarily MALDI-PSD-MS. Two strategies are employed: the glycan portion is first cleaved enzymatically or by base-catalyzed β-elimination[66, 75] and separately characterized by other means (MS or NMR) while the peptide is sequenced using PSD, or the entire glycopeptide is fragmented by PSD and the spectra analyzed.[65, 67] Again, all these spectral analyses are time consuming and not suited for high-throughput screening. The only example to date of a facile, direct method of characterizing glycopeptide libraries generated using the building-block approach is one that combines chemical coding (to determine the glycan portion) with capping steps to generate fragments for the mass analysis (ladder synthesis).[52] An example of the mass spectrum of a bisglycosylated peptide from such a library is shown in Figure 6. The principle of this method and its application in the synthesis of a glycopeptide library are detailed in Section 8.2.

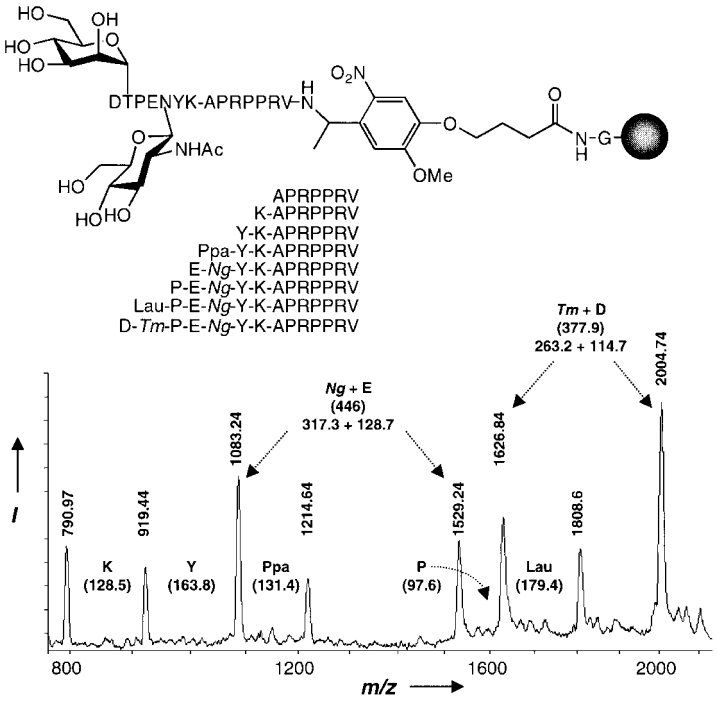


Figure 6. Representative mass spectrum showing the "ladder" of a bisglycosylated glycopeptide obtained from screening of a glycopeptide library. The phenylpropionic acid (Ppa) and lauric acid (Lau) encode for Asn(GlcNAc) and Thr(Man), respectively.[52]

5.3. Structural Analysis by MAS NMR Spectroscopy of Compounds Linked to Single Beads

In the absence of highly-efficient and effective tagging methods, nanoprobe Magic-Angle Spinning (MAS) NMR spectroscopy is the analytical technique which holds most promise for direct identification of single compounds bound to one polymer particle. Furthermore, the completeness of each synthetic step can be assessed in a nondestructive

manner. Gel-phase NMR spectroscopy can give respectable ^{13}C NMR spectra of large amounts of resin bound compounds, but for single beads such analysis is not possible and proton spectra do not show useful resolution. The properties of the polymer have a major influence on the quality of the spectra recorded and, in general, the more mobile and liquidlike the backbone of the polymer, the better the resolution of the ^{1}H NMR spectra. This also holds true for MAS NMR spectroscopy at intermediate spinning rates of 2000–4000 Hz where compounds on the PEG-based resins are much better resolved than on polystyrene or even TentaGel.[76] In fact, solid-phase spectra displaying a resolution indistinguishable from that of solution spectra may be obtained with these resins.[77]

Complete structural elucidation of unknown compounds on single beads remains challenging and most other routine analyses require substantial amounts of resin beads, except in one case where macrobeads of the TentaGel type were used.[78] Typically, analysis has been performed using polystyrene beads and, although generally the 1D ^{1}H MAS NMR spectra showed little or no resolution of coupled resonances, useful 2D MAS NMR spectra could be obtained.[79] Only known reaction products and known compounds on polystyrene or TentaGel have been analyzed so far.[80–83] Recently, the only complete structural elucidation of an unknown peptide of eight residues on a single POEPOP bead (10 nmol) has been achieved.[77] The minimum amount of resin-bound material required for a complete structural elucidation on PEG-based resins at 500 MHz is 2–7 nmol of compound, depending of the complexity of the structure and the resin.[77] The time-consuming nature of NMR structural analysis also demands a more robust (no false positives) and quantitative biological assay in which the rank of the compound activities may be directly quantified in order to limit the number of structural analyses necessary.[84] MAS NMR spectroscopy has been used for the monitoring of solid-phase glycosylation reactions of peptide templates with the aim of analyzing single-bead glycopeptides from consecutive glycosylations of peptide template libraries.[85, 86]

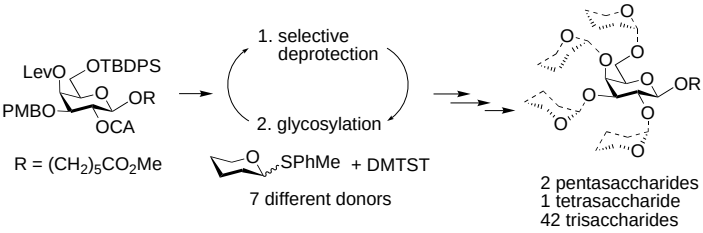
6. Parallel and Library Synthesis of Oligosaccharides

6.1. Introductory Considerations

While tremendous progress has been achieved in the generation of libraries comprised of small molecules, peptides, or nucleotides, the synthesis and characterization of carbohydrate-based libraries has remained elusive. The assembly of carbohydrate libraries is complicated by the chemical complexity of the carbohydrate itself and the reactions involved. A well-planned orthogonal protecting group strategy must be implemented in order to selectively mask and unmask the relevant hydroxyl groups for glycosylation, which must be achieved stereoselectively and in high yields. Three types of glycosyl donors, the thioglycosides[87] the glycosyl sulfoxides,[88] and trichloroacetimidates,[89] have precipitated as being generally effective in coupling reactions providing high yield and reasonable stereoselectivity. Furthermore, the methods offer homogeneous reaction conditions and may therefore be used for solid-phase glycosylation reactions. It must be noted however, that it is still difficult, even in solution, to generate important oligosaccharide linkages such as the β-Man, α-Neu5Ac, and α-Kdo in greater than 50% yield. In solid-phase libraries, the adaptation of glycosylation reactions which perform well in solution to the solid phase is often problematic. These challenges notwithstanding, there have been significant breakthroughs in the generation of oligosaccharide libraries of small to medium size.

6.2. Parallel Synthesis of Oligosaccharide Arrays

The structural diversity of oligosaccharides may be generated through parallel solution synthesis based on a common core entered at the first synthetic step. A recent, elegant solution-phase approach[90] employs four orthogonal protecting groups for the monosaccharide hydroxyl groups and has been used to produce a 45-member array of compounds (42 trisaccharides, one tetrasaccharide, and two pentasaccharides) synthesized by parallel glycosylation of the common acceptor with seven different glycosyl donors (Scheme 1). The key



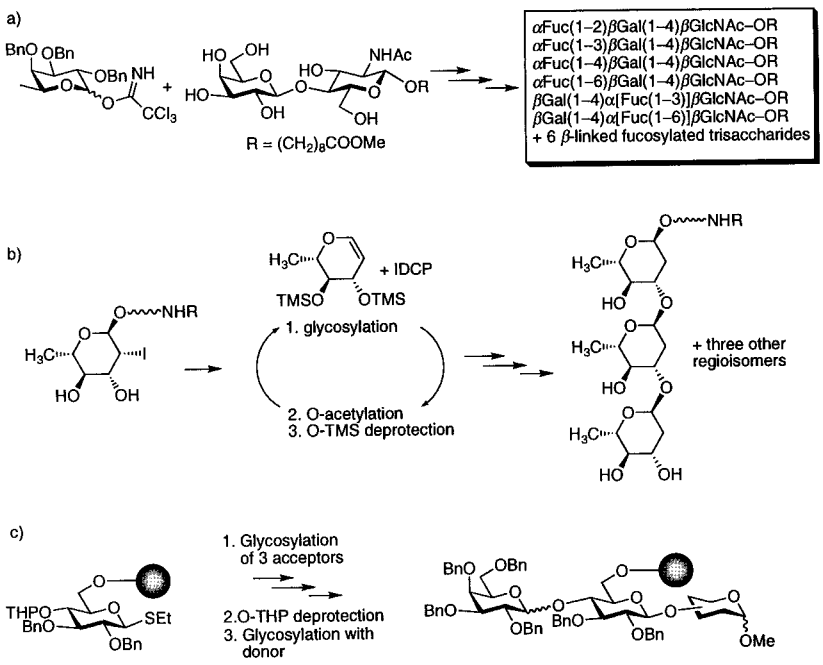
Scheme 1. Solution-phase parallel synthesis of oligosaccharide arrays. Yields of oligosaccharides varied widely, ranging from 5–85% for two glycosylation/deprotection cycles.[90] DMTST = (dimethylthio)methylsulfoniumtrifluoromethanesulfonate, CA = chloroacetyl.

monosaccharide acceptor contained the chloroacetyl (CA), levulinoyl (Lev), *p*-methoxybenzyl (PMBO), and *tert*-butyldiphenylsilyl (TBDPS) protecting groups. The various hydroxyl groups were sequentially unmasked and coupled with thiocresyl donors yielding the products in widely varying yields and stereoselectivity. As the size of the oligosaccharide increased, a decrease in selectivity and yield was observed and overall yields for two deprotection and coupling cycles ranged from 5–85%. Compounds synthesized were completely deprotected and characterized by mass spectrometry and NMR spectroscopy. This strategy as described in solution phase is extremely labor intensive and limited by the requirement of an orthogonal protection scheme.

This highly orthogonal protection scheme can be avoided by use of a variety of enzymes with the desired glycosylation specificity. In particular, inversion of the reaction of glycohydrolases has been found to be useful and small arrays of oligosaccharides including the mucin core 1 and 2 structures have been synthesized by this procedure, albeit in low yields.[91] Yields of the products may be improved by the use of thermostable glycosidases at elevated temperatures.[92]

Alternatively, glycosyl transferases may be used for anomeric and regioselective synthesis of glycosidic bonds. Small arrays of nonnatural SLe^x (11 compounds) and SLe^a (16 compounds) analogues were synthesized by sequential incubation of nonnatural *N*-acyl disaccharides with α(2-3) sialyl transferase and either Fucose III or IV transferases.[93]

Solid phase methodology for parallel synthesis of oligosaccharides has also been developed.[55, 56, 88, 94–97] The orthogonal linkers used include those based on the TMSET (trimethylsilylethyl) protective group cleaved with $BF_3 \cdot Et_2O$,[96] the *p*-alkoxybenzyl group cleaved with cerium ammonium nitrate,[95] the hydroxymethylbenzoic acid linker cleaved with dilute base, and photolabile linkers. Acid-labile linkers are sometimes problematic under acidic glycosylation conditions. The glycosylations must be carried out under homogeneous conditions and the ones which have been used are the trichloroacetimidate procedure,[55, 89] the sulfoxide/Tf_2O procedure,[88] pentenylglycoside procedure with iodonium activation (N-iodosuccinimide/triethylsilyl trifluoromethanesulfonate, NIS/TES-OTf),[56] and the glycal procedures using either iodonium intermediates or epoxide[94] with Lewis acid activation. Since these methods are performed on solid phase they would be particular suited for oligosaccharide library synthesis.



Scheme 2. Examples of oligosaccharide library synthesis. a) Solution-phase synthesis with unprotected acceptors.[98] b) Solution-phase synthesis with protected acceptors.[102] c) Solid-phase synthesis.[103] In all cases, libraries were obtained as mixtures of compounds. Ac = acetyl, Bn = benzyl, IDCP = iodonium dicollidine perchlorate, THP = tetrahydropyranyl, TMS = trimethylsilyl.

6.3. Synthesis of Oligosaccharide Libraries

Oligosaccharide libraries have been successfully generated either in solution or on solid phase using primarily chemical synthesis. Solution-phase synthesis of carbohydrate libraries is carried out employing two main strategies: reacting protected glycosyl donors either with unprotected acceptors[98–100] or protected acceptors.[101] The former strategy is designed to eliminate the need for major synthetic efforts required to synthesize the orthogonally protected saccharide building blocks that ensure regioselectivity, thereby reducing the time and cost involved in the synthesis of oligosaccharide libraries. In the seminal work (Scheme 2a),[98] a library of all six possible α-linked trisaccharides was synthesized by coupling of the perbenzylated fucosyl trichloroacetimidate to the unprotected LacNAc acceptor. Key features for the success of this methodology are the incorporation of a UV active grease "tail" in the acceptor to aid separation and detection of the products in the mixture, the use of polar solvents (DMF) for the glycosylation, and the termination of the reaction after 30% completion to prevent multiple glycosylation. Interestingly, the regioisomers were obtained in near-equimolar quantities despite previous evidence suggesting that the different hydroxyl groups have highly differing reactivities toward glycosylation in nonpolar solvents. The mixtures were used to screen for the activity of glycosyl transferases.[100] The main problems of this strategy are separation and identification of the products; despite the use of the hydrophobic tail, separation of the isomers was quite difficult. The scope of the method was limited by difficulty in the identification of the products, possible only after comparison of the mixture NMR spectra with those of the individual products separately synthesized as single compounds.

While glycosylation using unprotected acceptors can be truly random, caution must be exercised in order to prevent multiple glycosylation, which would make deconvolution and separation of the library components even more difficult. Therefore, others have opted to use the approach wherein both donor and acceptor are protected, thus enabling one to drive the reaction to completion without fear of multiple glycosylation. For example, a small library of 20 (including α/β anomers) trisaccharides was generated using the vinyl latent-active strategy.[101] The 3-methyl allyl glycoside with benzyl protecting groups and one acetyl group was used as the common building block and was converted into the glycosyl donor by isomerization to the vinyl glycoside or to the acceptor by removal of the acetyl group. TMSOTf-promoted glycosylation reactions proceeded in greater than 70% yield to give a 1:1 mixture of α/β anomers. This procedure is in fact a combination of parallel synthesis and the split and mix methodology. A common monosaccharide building block with only two different protecting groups was used so very limited diversity was introduced by the split and mix step. At the end of the synthesis of the trisaccharide library, the individual compounds were not separated but analyzed simply on the basis of the trisaccharide mass and monosaccharide composition. Mass spectrometry was also used to analyze the completion of the glycosylation reactions.

In another example utilizing only the split and mix methodology, a small, four-membered library of 2,6-dideoxytrisaccharides was generated also using a common protected glycal building block (Scheme 2b).[102] Thus, the glycal was first converted to the 2-iodoglycoside by iodonium dicollidine perchlorate (IDCP) activation. The protecting groups in the 3 and 4 positions were removed and then simultaneously glycosylated stereoselectively at low temperature with an equimolar amount of donor to yield a mixture of the two α-linked regio-disaccharides in equal amounts. Although reactions were allowed to proceed for as long as possible, surprisingly, there was no mention of the formation of trisaccharides, resulting from glycosylation of both the available positions, or of unreacted starting material. The remaining hydroxyl groups were then acetylated and the TMS groups removed. The new hydroxyl groups were glycosylated as previously to yield a mixture of four 2-iodotrisaccharides which were reduced by treatment with tributyltin hydride. Again, the products were not separated but analyzed as a mixture using mass spectrometry and NMR spectroscopy.

Although most oligosaccharide libraries have been synthesized in solution, there are two examples of solid-phase synthesis of such libraries. In the first example, a small library of 12 different trisaccharides (including α/β anomers) was synthesized on TentaGel resin using the split and mix method with thioethyl glycoside as the donor (Scheme 2c).[103] This library was very limited in its diversity since only one hydroxyl group was used as the acceptor. The starting thioethyl glycoside was attached to the solid support by a succinic ester linker at C6 thus allowing it to be used both as the donor and acceptor in glycosylation reactions.

In a more extensive effort, the split and mix method was used for the synthesis of approximately 1300 di- and trisaccharides as a "one bead–one compound" library (Figure 7).[59] Six suitably protected azide-containing monosaccharides attached to TentaGel were glycosylated with 12 different mono- and disaccharide sulfoxide donors to yield 72 different compounds. Additional diversity was achieved by reduction of the azide and acylation of the oligosaccharide amine with 20 different carboxylic acids. The identity of compounds attached to the beads was established using a haloaromatic tagging technique employing carbene coupling reactions.[60] While this technique provided a way of decoding which carbohydrates were coupled to each other on a bead, the precise nature of the glycosidic linkage could not be conclusively determined. It was assumed, based on previous experience, that the glycosylation reactions were completely stereoselective under the conditions used. The library was screened for active ligands for *Burhinia purpurea* lectin using a solid-phase enzyme-linked assay.

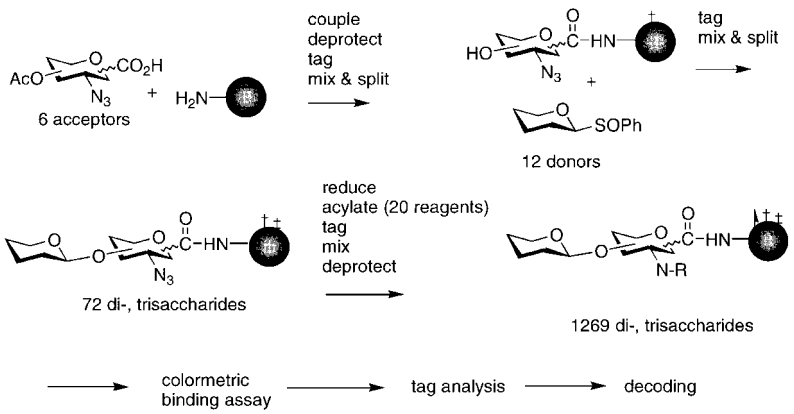


Figure 7. A solid-phase oligosaccharide library synthesized with exponential increase in the number of compounds with each reaction step. The library was screened for active compounds using a solid-phase enzyme-linked assay and the components were identified using chemical coding. The new ligands identified had activity comparable with that of the natural ligand.[59]

Synthesis of oligosaccharide libraries both in solution and on solid phase is by no means trivial. The main impediment to progress in the synthesis of carbohydrate libraries has been the yields and selectivities of the glycosylation reactions and the technology for separation and analysis. Recently, a novel solid-phase approach to S-linked oligosaccharides based on nucleophilic substitution of sugar triflates with thioglycosides was introduced.[104] This approach has not yet been used for libraries, but could alleviate the inherent difficulty in selective glycoside bond formation on solid phase, thereby facilitating the preparation and analysis of split and combine oligosaccharide libraries. In all the examples described above, apart from one instance, the library of oligosaccharide compounds was never separated nor were the individual products precisely identified and characterized. The recent method using orthogonal protecting groups for parallel synthesis in solution (Scheme 1)[105] has great potential for library synthesis on solid phase provided the required analytical methods can be developed. It should also be noted that only in one case of oligosaccharide libraries described[59] has the exponential increase of compound numbers by consecutive reactions been exploited to any significant extent.

7. Glycopeptides as Oligosaccharide Mimetics

In the quest for carbohydrate-based therapeutics, the synthesis of large numbers of diverse oligosaccharides is an expensive, synthetically challenging and time-consuming process. Whereas the use of oligosaccharide libraries would save time during synthetic operations and screening, the expense and the difficulties of the synthesis and analysis of active compounds from the library would still be problematic. Furthermore, it has been shown that small modifications of the natural oligosaccharide ligand rarely confer any increase in binding efficacy.[106–108] This has been attributed to the enthalpy–entropy compensation often found for analogous structures in the interaction with their receptors in aqueous media.[109–111] The free energy of binding of a receptor with any ligand of similar structure has been correlated to the preorganization of water molecules in the media close to the binding site.[112] If this is the case, only ligand structures, which allow significantly different interactions with both the receptor and the aqueous environment, are expected to have affinities significantly different from that of the natural ligand.

When glycopeptides were first introduced as mimetics of oligosaccharides,[113] it was envisaged that the saccharide moiety would provide the specificity of the binding by directing the ligand to the oligosaccharide binding site, while the peptide would function as a scaffold for optimal orientation of the glycan portion. However, since peptide ligands generally bind with high affinity to peptide receptors, it is also expected that the glycopeptide could furthermore interact favorably with the carbohydrate-binding receptor through the peptide scaffold, thus leading to increased binding affinity.[26] The source of this enhanced affinity of peptides may lie in their ability to perform an induced fit because the relatively limited flexibility around peptide bonds leads to fast on-rates for the binding. In the peptide architecture, this flexibility is obtained with the minimum entropic penalty. Conversely, the carbohydrates generally display conformational assemblies located closely around one global energy-minimum conformation (or a multiple of two for each 1 →6 linkage included, see Figure 3). In contrast to glycopeptides, highly flexible neoglycoconjugates[114] do not show high affinity due to the large entropic penalty.[115, 116]

The principle of glycopeptides mimicking oligosaccharides was consolidated through binding studies with an array of phosphorylated glycopeptides and the divalent mannose 6-phosphate receptor.[26] The structural similarity between the oligosaccharide and the most active glycopeptide ligand was supported by molecular dynamics (Figure 8).

Similar results were later obtained with glycopeptides designed for binding to the selectins.[14] A glycopeptide mimic of the SLe^x tetrasaccharide containing fucose on a peptide scaffold had a greater than tenfold increased binding affinity for E-selectin.[117] There was no further significant increase in affinity when the ligands were immobilized in a polymeric multivalent arrangement in liposomes.[118] In another example, a high-affinity divalent adhesin ligand which contained αGal(1 →4)αGal linked by peptide bonds to an aromatic nucleus was prepared for *Streptococcus suis*. The assay with structurally similar tetravalent ligands showed no significant increase in binding indicating the interaction to be truly divalent.[27] Glycopeptide mimics of galactose or GlcNac containing oligosaccharides afforded inhibitors ($K_d = 1.7$ μM) of galactosidase.[119] and GlcNAc-transferase inhibitors,[120] respectively. In another approach, glycopeptide-like azasugar inhibitors were prepared; however, reduction of inhibitory activity was found when compared with the parent azasugar without the peptide moiety.[121]

Glycopeptides are excellent mimics of the complex oligosaccharides and may be utilized in a library format to identify high-affinity ligands.[122] The ease with which glycopeptides are synthesized using preactivated amino acids and glycosylated amino acid building blocks can, by careful assembly of a library, ensure the generation of a single compound in each bead. For the preparation of glycosyl amino acid building blocks, the glycosylation of Fmoc – amino acid – OPfp esters or free Fmoc – amino acids have proved a general and versatile method useful for the preparation of complex compounds for direct incorporation into the glycopeptide libraries (Fmoc = fluoren-9-ylmethoxycarbonyl, Pfp = penta-

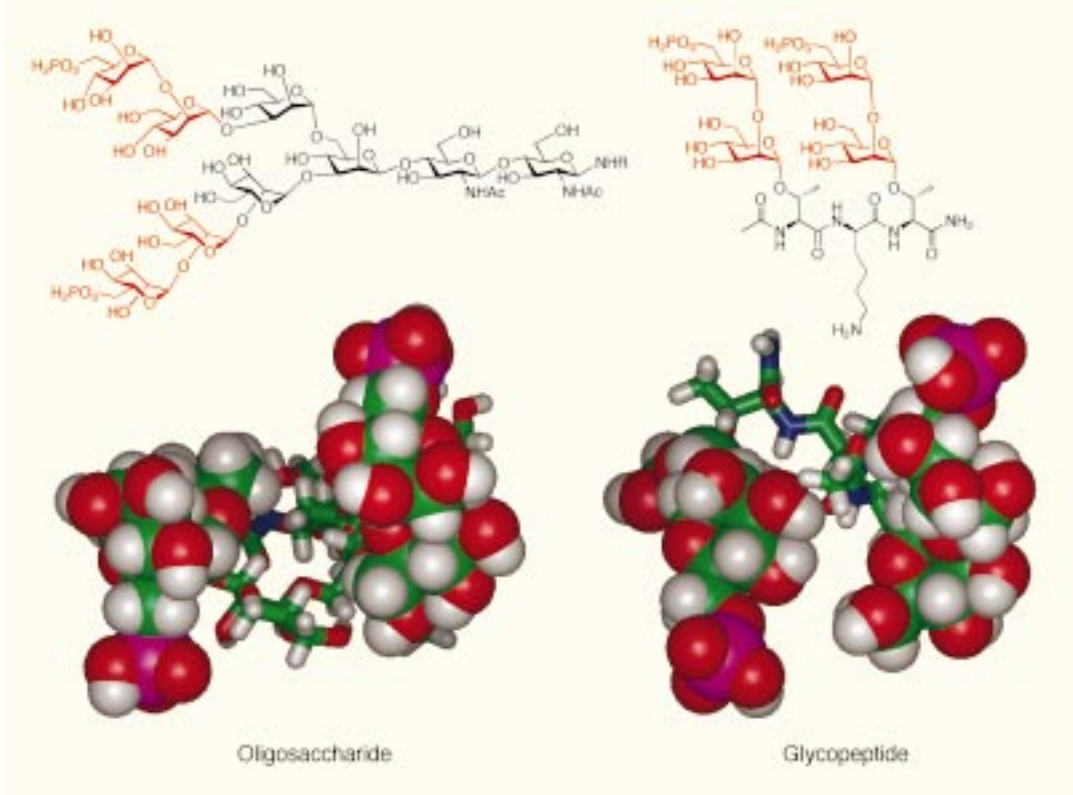


Figure 8. Models of a phosphorylated high-mannose M7 and a glycopeptide mimic which show similar conformations. They are seen from the point of interaction with the mannose-6-phosphate receptor. Disaccharide phosphates are emphasized as spheres.[26]

fluorophenyl).[123–127] Many other strategies have also been successfully employed for glycopeptide synthesis. However, these all require further manipulations of the glycosylated building blocks before use in peptide synthesis.[128] In addition to the relative ease of synthesis, facile characterization of active compounds makes libraries of glycopeptides very attractive alternatives to oligosaccharide libraries.

8. Parallel and Library Synthesis of Glycopeptides

8.1. Parallel Synthesis of Glycopeptide Arrays

When considerable a priori structural knowledge of a protein carbohydrate interaction is available, it is possible to synthesize a range of active glycopeptide analogues by biased design using a parallel synthetic approach. The requisite knowledge is the nature of the dominant sugars involved in the interaction and their spatial orientation in the receptor interaction. With such information available from X-ray crystal data or from transfer NOE NMR spectroscopic experiments, a range of high-affinity ligands of the glycopeptide type have been developed for several receptors by parallel synthesis.

Twenty bidentate glycopeptide ligands for the mannose 6-phosphate receptor (MPR) were synthesized by parallel synthesis and high-affinity ligands with approximately 20-fold increased affinity for the receptor were obtained.[26] The array of analogues contained glycosylated linear tri- to pentapeptides and cyclic hexa- to octapeptides. The glycans were phosphorylated 1→2 and 1→6 linked mannodisaccharides and phosphorylated mannosides. Glycopeptides were synthesized by solid-phase multiple-column peptide synthesis (MCPS)[129, 130] using Fmoc–amino acid–OPfp esters and PEGA resin. The glycosylated building blocks were protected as acetates or benzoates and the phosphate with trichloroethyl groups. Deprotected glycopeptides were obtained in high yield and purity. Assays of the compounds demonstrated that a minimum of two disaccharides on a scaffold was required for the specific interaction with the receptor and clearly showed the necessity to have sufficient but not excessive flexibility in the scaffold. Two 6-P-α-Man-(1→2)-α-Man disaccharides on a linear tripeptide had the highest affinities for the receptor. Attempts to increase the affinity through cyclization of the peptide were unsuccessful.[131]

An array of 120 galactose-containing compounds (30 mixtures of four diastereomers) was prepared in parallel by base-catalyzed Michael addition of β-D-$(C_{12}H_{25}CO)_4$Gal-SAc to four different unsaturated ketones and an α-chloro ketone, followed by reductive amination with six amino acids. The thirty products were purified by solid phase extraction and tested as inhibitors of galactosidase. Galactose provided the specificity for the enzyme, while the affinity was obtained through interaction with the aglyconic scaffold. In this way, inhibitors of the enzyme activity ($K_d = 1.7\ \mu M$) were obtained.[119]

An array of glycopeptide ligands, which bind to MHC class II E^k and interact with the T-cell receptor (Figure 9a) in which the glycan part was varied, were synthesized by MCPS.[132, 133]

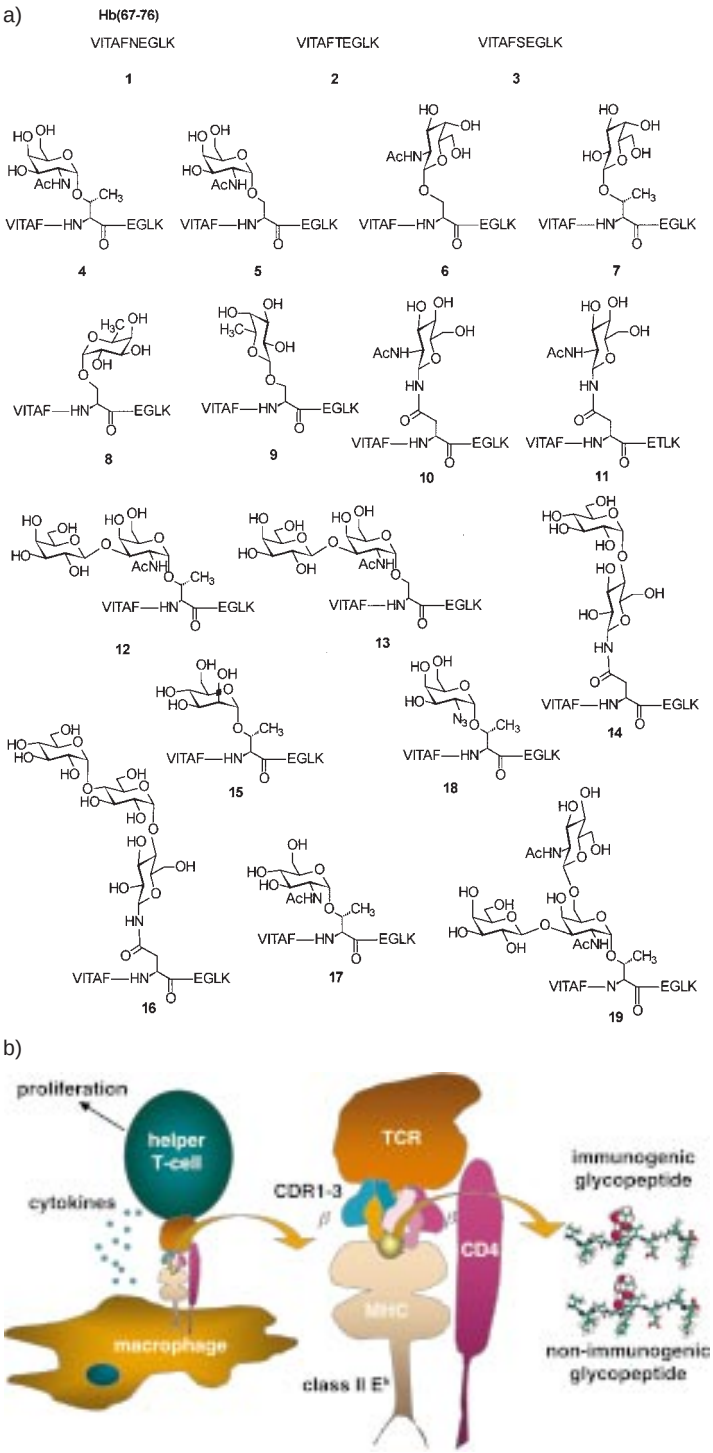


Figure 9. a) An array of glycopeptides for the study of glycan specificity of the MHC/TCR recognition. When mice were injected with the glycopeptides and the proliferation index of T-cells isolated from lymph nodes was measured, only the Tn and T antigens were strong immunogens.[28] b) Glycopeptide/MHC/TCR-interaction. The minor modification of substitution of α-GalNAc with α-GlcNAc, namely change of an axial 4-hydroxyl group to an equatorial one (as illustrated in the two glycopeptides), completely abolished immunogenicity.[28]

The glycosylated building blocks were synthesized by glycosylation of Fmoc–amino acid–OPfp esters and these were directly used for coupling reactions. The glycans included the mucin Tn (α-D-GalNAc) antigen core 1–4 structures, as well

as a large array of mucin-unrelated saccharides. Using the panel of glycopeptides shown in Figure 9a, it was found that mainly the T and Tn antigens were recognized and they were considerably more immunogenic (yielding much higher T-cell titers in mice) than other glycans (Figure 9b).[28] The results indicated that there is a repertoire of TCR's specifically directed towards tumor-associated antigens and elongation with GlcNAc (such as cores 2 and 4) gave nonimmunogenic compounds.

Parallel synthesis of 62 different fucosylated tripeptides resulted in two ligands with submicromolar affinity for the P-selectin, however, the desired activity for the E-selectin was not observed.[134] For the E-selectin selectivity it was necessary to incorporate a hydroxyl group, which mimics the 4-hydroxyl group of the central Gal in SLe^x, in addition to a Fuc-residue, and a carboxylate, to obtain ligands with greater than 10-fold increased activity over that of the SLe^x tetrasaccharide.[117] One of the best ligands was obtained from Thr(α-Fuc)-OEt, which was first N-acylated with a hydroxyamino acid and then elongated with a diacid to furnish the acid mimic of the sialic acid carboxylate (Figure 10). This approach was further developed as a solid-phase method where the molecule was linked to a solid support through the invariable fucosyl moiety.[135]

In an elegant and different approach, an array of C-linked glycopeptide-like mimics of SLe^x were synthesized in parallel by an Ugi four-component reaction.[136] Reaction of different anomeric two or three carbon extended C-glycosyl aldehydes or acids with resin-bound amines, isonitriles, and other acids or aldehydes yielded an array of C-linked analogues. The method is easy to perform in good yield on solid phase. However, this strategy can only be used for a mixed library and not for a one bead–one compound library. It is derived through a multicomponent reaction where all components are introduced in a single step and deconvolution strategies such as positional scanning or iterative synthesis are required for identification of the components of the library. On the other hand, deconvolution is feasible with fast screening because the products are generated rapidly.

8.2. Preparation and Analysis of Solid-Phase Glycopeptide Libraries

While parallel synthesis of arrays of glycopeptides is readily achieved by implementation of the building-block approach (Scheme 3a),[125] glycopeptide library synthesis in a combinatorial manner by the split and mix method has yet to prove

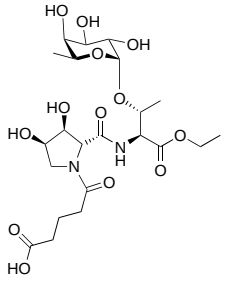
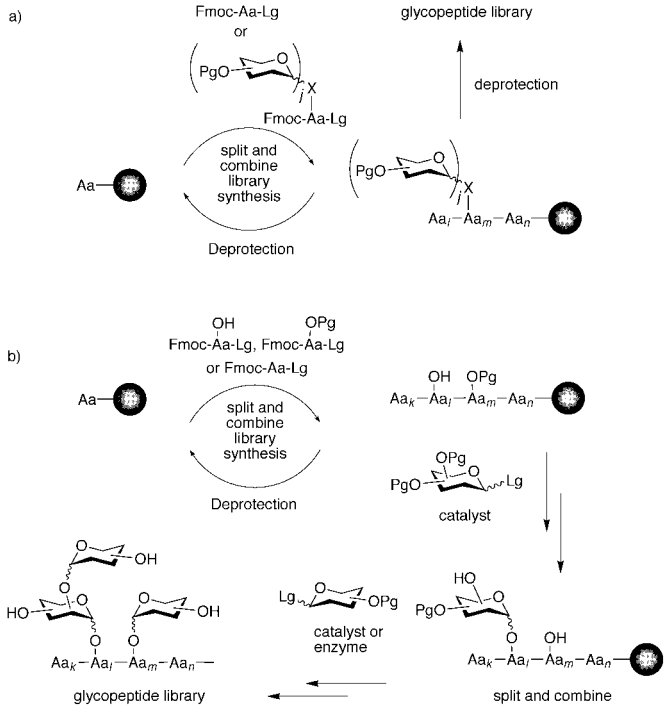


Figure 10. A ligand with high affinity for E-selectin. The receptor interacts with the 2- and 3-hydroxyl groups of Fuc, probably the hydroxyl groups of the dihydroxyproline ring, and the carboxylate moiety. A similar high-affinity ligand is compared with the SLe^x structure in Figure 4.[118]



Scheme 3. Strategies for glycopeptide library synthesis. a) The building block approach (X = O, C, S, N). b) Chemical or enzymatic glycosylation of peptide or glycopeptide. While enzymes have not yet been used in the solid-phase synthesis of glycopeptide libraries, several resin-bound glycopeptides have been glycosylated enzymatically.[44, 148] Lg = leaving group, Pg = protecting group.

routine. The difficulty lies in the structural analysis of the vast number of compounds generated in picomolar quantities on a single bead. Whereas peptides on beads can be conveniently analyzed by solid-phase ladder sequencing[137] or Edman degradation, neither of these methods are suitable for glycopeptides because of the instability of the glycosidic bond under the acidic and basic conditions employed.

An early report described a pentaglycopeptide library containing three randomized positions and an Asn(GlcNAc) building block fixed at position 4.[138] However the library members were not characterized and screening results were not described. Presently, only one example of a combinatorially generated glycopeptide library suitable for screening and structural analysis has been reported.[52] Generation of a 300 000 member library and analysis of its components was made possible in part by the development of a technique based on mass spectrometry for identification of the sequence of individual glycopeptides in the library. In this method, the synthetic history of the glycopeptide is captured on the beads by capping a small percentage of the growing oligomer chain in each synthetic step.[139] Thus, a series of related fragments is generated on the bead rather than a single compound (Figure 6). The difficulty arises when this technique is applied for a glycopeptide library in which the amino nucleophiles present significant reactivity differences. To account for amine reactivity differences, an in situ capping method based on Fmoc amino acids mixed with 10% of the Boc analogue was developed (Boc = *tert*-butoxycarbonyl).[122] The glycosylated Fmoc–amino acid–OPfp esters were encoded by capping

with selected carboxylic acid – OPfp esters. The library was linked to the solid support by a photolabile linker (Figure 6) which facilitated the immediate MALDI-TOF mass spectrometry analysis of compounds released from the resin beads by 20 minutes of irradiation with an Hg lamp. The purity of the library was assessed by MALDI-TOF mass spectrometry through collection and analysis of a few beads. Most of the beads afforded spectra of the ladders, which could easily be deciphered using mass-difference assignment software.

In a departure from the building-block approach, glycopeptide libraries were also be obtained by glycosylation of a preattached glycan or the hydroxyl group of an amino acid side chain from a peptide library (Scheme 3b). In preliminary studies,[55, 140] good yields of (approximately 35 % of the initial resin loading) of glycopeptides containing di- and trisaccharides were obtained using 5 – 8 equivalents of the perbenzoylated glycosyl trichloroacetimidate donor to glycosylate a known glycopeptide. Glycosylation was attempted on four resins: Polyhipe, TentaGel, PEGA1900, and Macrosorb-SPR250 but was successful only on PEGA and Polyhipe.

Early attempts of direct solid-phase glycosylation of a longer peptide were not very successful although the presence of glycopeptide product could be demonstrated.[128, 141] Direct glycosylation of the amino acid side chain hydroxyl groups was partially successful albeit in low yield.[55, 142] The failure of polar resins such as PEGA may be due to the many primary amides in the backbone, which interfere with the carbocation intermediate. Peptide amide bonds seem to show less interference. However, since polar resins are required for solid-phase bioassays, new types of polar resin containing only ether bonds were developed for the solid-phase glycosylation of peptides. Quantitative glycosylation of a known peptide was achieved on a PEG-based resin (POEPOP) containing only ether bonds[37] using 5 – 8 equivalents of the peracetylated or benzoylated trichloroacetimidate. In a one bead – one compound approach, two resin-bound peptides bearing protected and unprotected hydroxyl groups were first glycosylated with galactose and then with fucose after deprotection of the second hydroxyl group, affording a small library of four glycopeptides. The glycopeptides were cleaved off, separated, and characterized by mass spectrometry.

While solid phase glycosylation is undoubtedly a feasible alternative for the generation of truly random glycopeptide libraries with diversity in both the peptide and glycan portions, analysis of such libraries will present quite a challenge. One possibility is the use of fragmentation of the compounds by mass spectrometry or, alternatively, MAS NMR as discussed in Section 5.

9. Screening of Oligosaccharide and Glycopeptide Libraries

An important requirement for the successful application of the combinatorial library approach to the drug discovery process, is the ability to utilize the library in high-throughput screening (HTS) procedures. HTS screening of oligosaccharide and glycopeptide libraries is dependent on the mode of library synthesis and can be achieved by screening mixtures of compounds in solution, discrete compounds in solution, or discrete resin-bound compounds. Screening mixtures of compounds is nontrivial and the various methodologies that can be used for this purpose have been recently reviewed.[57] One method that has been used for screening an oligosaccharide "library" is based on NMR transfer nuclear Overhauser effects (tr-NOE's).[143, 144] In one demonstration, the *Aleuria aurantia* agglutinin was bound to α-L-Fuc(1 $\rightarrow$6)-β-D-GlcNAc-OMe in the presence of 5 or 14 other nonbinding oligosaccharides.[143] This methodology is limited by the number of compounds in the mixture that can be screened simultaneously, the difficulty in detecting low or very high-affinity ligands and the time required for analysis of spectra.

In another approach, the mixture of 12 trisaccharides obtained by a solution-phase random glycosylation strategy (Section 6.3) was used to screen for the activity of fucosyl transferases by measurement of incorporated radio-labeled GDP-Fuc.[98] This transfer was successfully detected even on acceptors that made up 5 % of the mixtures. Recently, a library of 640 carbohydrate triazine derivatives obtained by the reaction between *p*-aminophenyl pyranoside with cyanuric chloride was rapidly screened as inhibitors of the proteases Factor Xa and plasmin.[145] The synthesis of the libraries as parallel arrays of discrete compounds in 96-well plates facilitated the high-throughput screening of protease activity in the same plates using spectrophotometric measurement of the *p*-nitroaniline derivative released during substrate hydrolysis. No carbohydrate-based inhibitors were found.

As discussed earlier in this review, the split and mix methodology facilitates the rapid production of large number of compounds. Traditionally, the compounds were subsequently cleaved then screened and analyzed in solution. More recently, the development of solid supports that are compatible with both organic and aqueous media has allowed screening of the library to take place on the solid support itself. Active ligands are detected using immunostaining or colorimetric methods or, more directly, by use of fluorescent-labelled receptors. For example, carbohydrate ligands bound to TentaGel resin were screened in parallel arrays using digoxigenin-labelled *Maackia amurensis* agglutinin (a sialic acid specific lectin).[146] Beads binding fluorescent-labeled lectin were detected using flow cytometry.[146] The solid-phase screening of the 1300-compound carbohydrate library (Section 6.3) was carried out on the entire library utilizing a colorimetric assay.[59] Beads were incubated with biotin-labeled *Burhinia purpurea* lectin and bound lectin was detected by secondary incubation with alkaline phosphatase conjugated streptavidin. The screening yielded several non-natural oligosaccharide ligands for the lectin but, surprisingly, the known natural ligand that should have been present in the library was not detected. The reason for the lack of detection is either that binding of the lectin to the ligand was somehow hindered or this particular oligosaccharide was never synthesized. When using tagging methods, the failure of a synthetic step is not detected whereas analysis of the resin-bound compounds using methods such as MS or NMR readily indicates any problems in the synthesis. In subsequent

solution-phase assays, the natural ligand and the mimics showed comparable binding affinity.

Glycopeptide libraries have also been screened in solid-phase assays using soluble receptors. For example, the library generated by ladder synthesis on PEGA resin (Section 8.2) was incubated with the fluorescent-labeled lectin from *Lathyrus odoratus* (Figure 11 a). The most fluorescent beads were collected and ligands identified by MALDI-TOF mass spectrometry. The most active compounds were glycopeptides which contained only mannose (T(α-D-Man)ALKPTHV, LHGGFT(α-D-Man)HV, T(α-D-Man)EHKGSKV, GT(α-D-Man)-FPGLAV, and T(α-D-Man)-LFKGFHV) and these displayed up to a 25-fold increase in fluorescence compared to lectin binding with resin-bound mannose. Binding of the lectin to resin-bound mannose was inhibited by the active glycopeptides synthesized (Figure 11 b) which suggests that the glycopeptides and the natural carbohydrate ligand bind the same or closely related binding sites of the lectin.

Parallel arrays of resin-bound glycopeptides and resin-bound glycopeptide libraries have also been used in high-throughput screening of whole cells. A GlcNAc containing pentapeptide library attached to polystyrene resin was incubated with erythrocytes and adherence was observed but not further investigated.[138] More recently, Tn-antigen containing glycopeptide dendrimers bound to TentaGel resin were used in rosetting tests and showed positive reaction with anti-Tn antibodies and Tn+ erythrocytes.[147] Immunization of animals with one of the most active glycopeptide dendrimers led to an amazing increase in the level of anti-Tn.

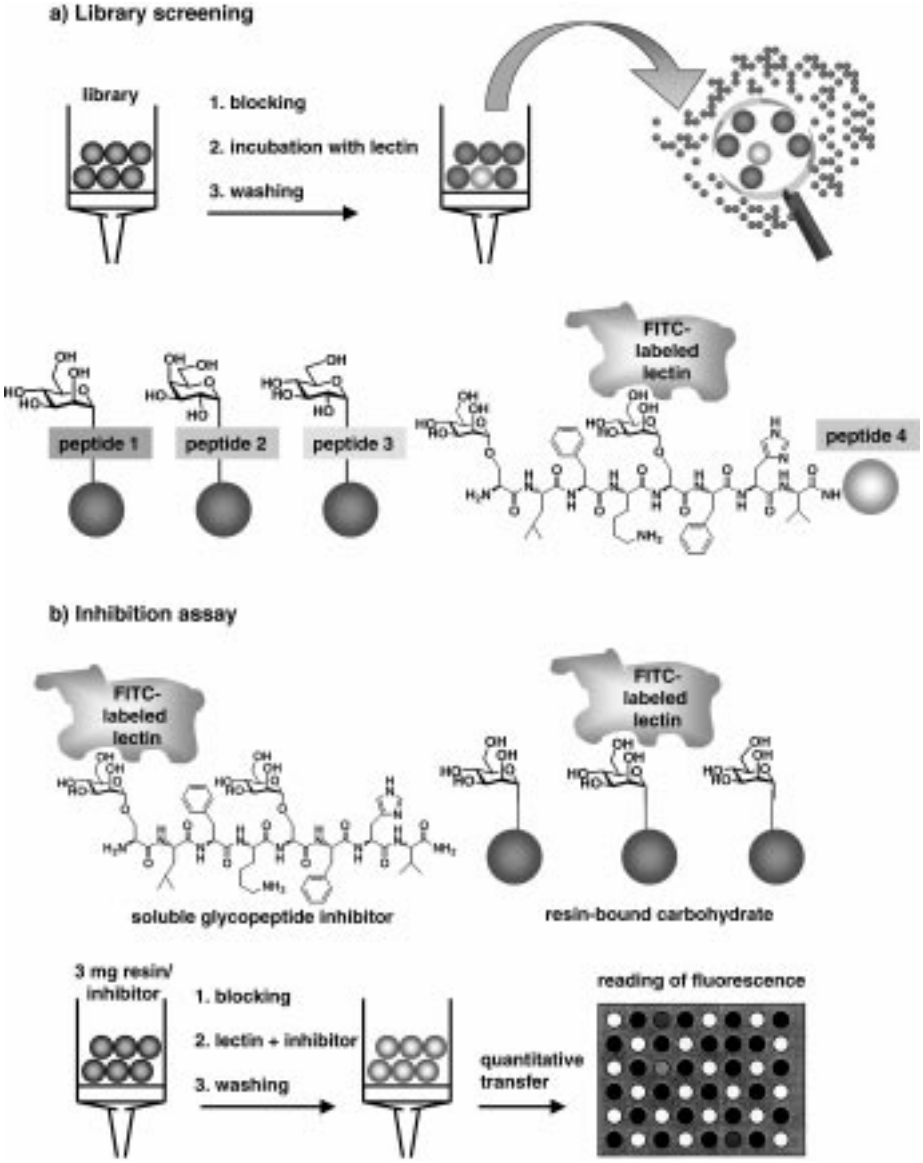


Figure 11. a) Screening of a glycopeptide library using a fluorescent-labeled lectin and ligands bound to PEGA-beads. The active compounds are analysed by mass spectrometry. b) FITC-labeled lectin binding to resin-bound mannose could be inhibited by soluble glycopeptides obtained from a library screen. Percentage inhibition was quantified by recording lectin fluorescence. Only every second well of the microtiter plate was used and nonfluorescent beads indicated good inhibitors.[52] FITC = fluorescein isothiocyanate

10. Conclusions

It is clear that protein–carbohydrate interactions are essential in numerous biological processes and that the development of carbohydrate mimetics that interfere with these processes would provide a powerful methodology for both modulation and amelioration of specific biological activity. In recent years, novel methodologies have been presented for the synthesis of oligosaccharide and glycopeptide libraries that should provide rapid access to such carbohydrate mimics. The technology for the identification of active oligosaccharide or glycopeptide ligands isolated from solid phase libraries for these carbohydrate-binding proteins has also been developed to a certain extent. In spite of its conceptual appeal however, there are major limitations that hamper the progress of carbohydrate-based library synthesis, characterization, and screening. These limitations are related in part to the lack of proper methods for miniaturization and analysis of compounds on solid phase. Additionally, the effect of the solid support on the outcome of the screening results needs to be thoroughly investigated. Although the synthesis and analysis of oligosaccharide libraries remains challenging, alternative use of larger solution-phase libraries requires difficult deconvolution synthesis, which is not practical with oligosaccharide synthesis due to its complexity and lengthy nature. Alternatively, glyco-

peptides, in which the peptide portion confers additional favorable binding affinity and which may easily be formed on solid phase and are less difficult to prepare in high purity, hold a lot of promise. Currently, the most versatile method for glycopeptide library synthesis utilizes an in situ capping procedure with a mixture of Boc and Fmoc amino acids while glycosylated amino acids are separately encoded by capping with carboxylic acids. The analysis of structures is facilitated by direct photolytic release from PEG-based supports for MALDI-TOF-MS analysis. Application of this technique yields high affinity ligands for carbohydrate-binding proteins.

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