

Review

Intervention of carbohydrate recognition by proteins and nucleic acids

Pamela Sears and Chi-Huey Wong

Department of Chemistry and the Skaggs Institute of Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037

ABSTRACT Carbohydrates in biological systems are often associated with specific recognition and signaling processes leading to important biological functions and diseases. Considerable efforts have been directed toward understanding and mimicking the recognition processes and developing effective agents to control the processes. The pace of discovery research in glycobiology and development of carbohydrate-based therapeutics, however, has been relatively slow due to the lack of appropriate strategies and methods available for carbohydrate-related research. This review summarizes some of the most recent developments in the field, with particular emphasis on work from our laboratories regarding the use of chemoenzymatic strategies to tackle the carbohydrate recognition problem. Highlights include the study of selectin-carbohydrate and aminoglycoside-RNA interactions and development of agents for the intervention of these recognition processes.

Of the three major classes of biomolecules, carbohydrates perhaps are the least exploited. It has been known that carbohydrates can serve as structural components of natural products, as energy sources, or, more interestingly, as key elements in various molecular recognition processes, including bacterial and viral infections, cell adhesion in inflammation and metastasis, differentiation, development, regulation, and many other intercellular communication and signal transduction events (1). [Representative structures are presented in Fig. 1 (2–16).] The precise mechanism of many carbohydrate-mediated recognition processes are, however, not well understood, and, though potential opportunities exist, the pace of development of carbohydrate-based pharmaceuticals has been slower than that of the other classes of biomolecules. Several reasons may be attributed to this slow pace of development. First, some technical problems in the field are impossible or difficult to solve. There is no PCR equivalent replication system available for the amplification of minute amounts of carbohydrates to facilitate structure analysis and synthesis, and there is no machine available for the solid-phase synthesis of oligosaccharides to facilitate the study of their functions. In addition, synthesis of oligosaccharides in large quantities for therapeutic evaluation and development is in general very difficult and expensive. Second, carbohydrates generally possess undesirable properties for drug development. The affinity of carbohydrates for their protein receptors is relatively weak (17–21), with dissociation constants in the millimolar range, and carbohydrates are generally orally inactive and sensitive to enzymes *in vivo*. As a result, carbohydrates may only be used as injectable form for the treatment of acute symptoms.

Understanding carbohydrate recognition is important, however, as this recognition process often occurs at the early stage of disease development, and intervention of such process may be beneficial. Furthermore, understanding the mechanism of carbohydrate recognition may lead to the development of new concepts and new strategies to tackle the problems of carbohydrate-based drug development. This review highlights the most recent work on the development of practical chemoen-

zymatic methods for the synthesis of complex carbohydrates, carbohydrate mimetics and inhibitors of enzymes associated with carbohydrate biosynthesis and processing (with particular focus on the work from our laboratories regarding the development of inhibitors that target carbohydrate interaction with selectins), RNA, and glycosyltransferases.

Selectins and Their Carbohydrate Ligands in Cell Adhesion

In 1989, a new class of carbohydrate binding glycoproteins called selectins were identified on the surface of certain cells, and subsequently these glycoproteins were designated as E- (22), P- (23) and L-selectin (24, 25), according to the cell type on which the selectin was found (i.e., endothelium, platelets, and lymphocytes, respectively). Each selectin was found to contain an N-terminal calcium-dependent lectin-like domain, an epidermal growth factor repeat, and a number of modules (≈ 60 aa) similar to those found in certain complement binding proteins (26). The x-ray structure of human E-selectin has been determined (27), and its primary sequence is homologous to another structurally known sugar binding protein, the mannose binding protein (28). The carbohydrate ligands that are recognized by these selectins have also been identified. E-selectin recognizes sialyl Lewis X (SLe^x) on the surface of neutrophils (29–31). P-selectin also binds SLe^x on neutrophils or leukocytes with a lower affinity (32, 33). L-selectin weakly recognizes SLe^x on endothelial cells, but the affinity is higher with a sulfate group on the six position of Gal (34, 35) or, perhaps more likely, on the six position of the GlcNAc residue (33, 36). Some sulfated Le^x also bind to E- and P-selectin (37), and questions regarding the real ligands for P- and L-selectin still exist.

The selectin-carbohydrate interaction occurs at the very early stage of inflammatory reaction (38, 39) or metastasis (40–42). For example, as illustrated in Fig. 2, when tissue injury occurs, cytokines are released to signal endothelial cells to synthesize E-selectins to recruit neutrophils to the site of injury. This process is mediated by the adhesion of neutrophils to endothelial cells through the multivalent interaction of SLe^x and E-selectin on the respective cell surface, followed by a more tight interaction between integrins on neutrophils and the intracellular adhesion molecule (ICAM-1) on endothelial cells, which then allows the extravasation of neutrophils to the site of injury. It is not clear how the recognition signal is transduced from outside to inside the cell, though one possibility is through the integrin-FAK-Src pathway (43–45). When too many neutrophils are recruited, however, normal cells will also be damaged, causing inflammation. Control of this process by adequately inhibiting the adhesion step has thus been considered to be a new antiinflammatory strategy, and many acute inflammatory symptoms, such as asthma, myocardial infarction, lung injury, and arthritis, may be treated with this approach. Similarly, new anticancer agents could be developed based on the inhibition of this adhesion process. Indeed, the

Abbreviation: SLe^x, sialyl Lewis X.

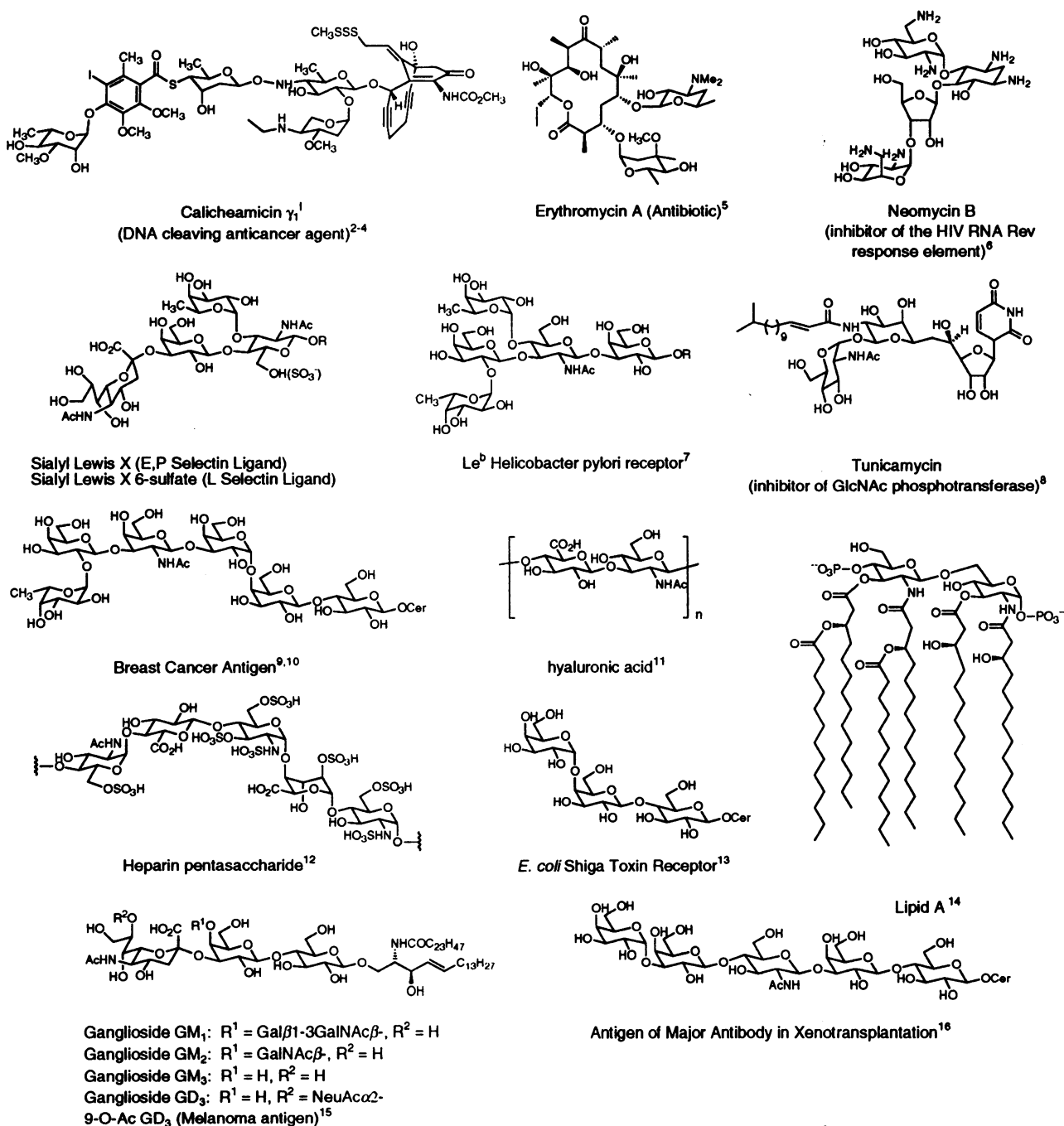


FIG. 1. Representative naturally occurring complex carbohydrates and conjugates with important biological activities.

carbohydrate ligands of these selectins, especially that of E- and P-selectin, have been shown to be potentially useful in the treatment of these acute symptoms (46, 47).

Chemoenzymatic Approaches to the Synthesis of Complex Carbohydrates, Glycopeptides, and Glycoproteins

The chemistry developed for the synthesis of SLe^x and related structures (48–53) has played a very important role in the study of structure–function relationship. It has not only confirmed the function of the ligand but also unraveled the essential groups of ligand involved in recognition. In the case of E- and L-selectin–SLe^x interaction, it has been shown that the three hydroxy groups of fucose (54, 55), the 2- and 6-hydroxyl groups of galactose (56) and the carboxylate of neuraminic acid (54)

are essential for binding, and that the GlcNAc residue is not critical (57). For P-selectin, the essential functional groups are generally the same except that the 2- or 4-hydroxyl group of fucose seems not to be critical (54). These discoveries together with the conformation of SLe^x determined by NMR (58–61) provide a basis for the design of new structures to mimic the active conformation of SLe^x, which may lead to the discovery of new and better antiinflammatory agents.

The conformation of SLe^x in solution (58) is different from that bound to E-selectin (61), especially the orientation of the neuraminic acid residue (Fig. 3). The energy difference of these two conformations is estimated to be ≈ 1.5 kcal/mol (62), suggesting that it is possible to design a conformationally constrained SLe^x mimetic to improve the inhibition potency.

With regard to the synthesis of SLe^x, enzymatic methods have been developed for the large-scale process (58). The use

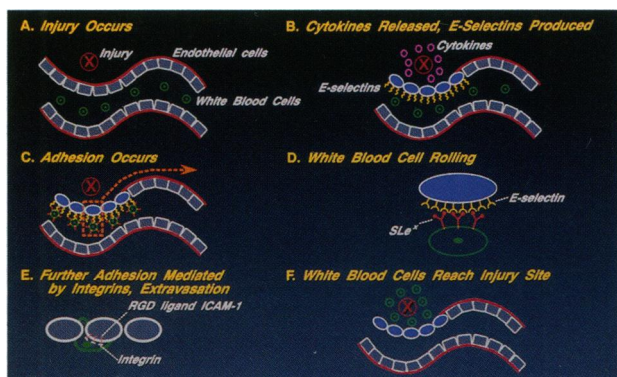


FIG. 2. SLe^x -mediated cell adhesion in inflammatory reaction.

of glycosyltransferases coupled with regeneration of sugar nucleotide substrates, first illustrated in the synthesis of *N*-acetyl lactosamine (63), has proven to be useful for large-scale processing, and SLe^x has been prepared on kilogram scales based on this strategy (Fig. 4). This multienzyme system not only eliminates the problem of product inhibition caused by the released nucleoside phosphate but also reduces the cost of the expensive sugar nucleotide. This method has been extended to the synthesis of hyaluronic acid (64), and it is believed that any oligosaccharide in mammalian system can be prepared in large quantities based on this methodology as methods for the

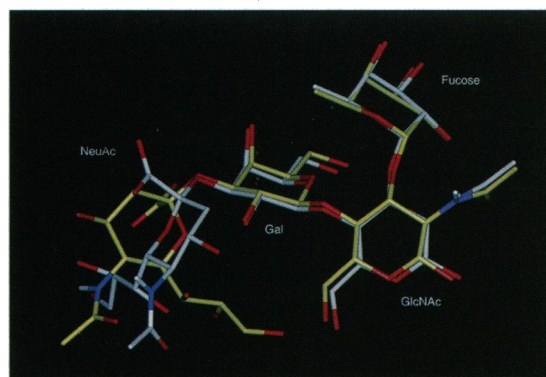


FIG. 3. Overlay of the solution conformation of SLe^x and that bound to the receptor E-selectin, determined by NMR.

regeneration of all sugar nucleotides have been developed (65). The development of new expression systems [especially using yeast (66), baculovirus (58), and *Aspergillus* expressing systems (67)] for the preparation of glycosyltransferases has made possible the large-scale enzymatic synthesis of oligosaccharides.

Enzymatic sulfation coupled with regeneration of 3'-phosphoadenine 5'-phosphosulfate (PAPS) has also been developed for the large-scale synthesis of sulfated oligosaccharides (68). *N*-Acetyl-lactosamine-6-sulfate, for example, has been

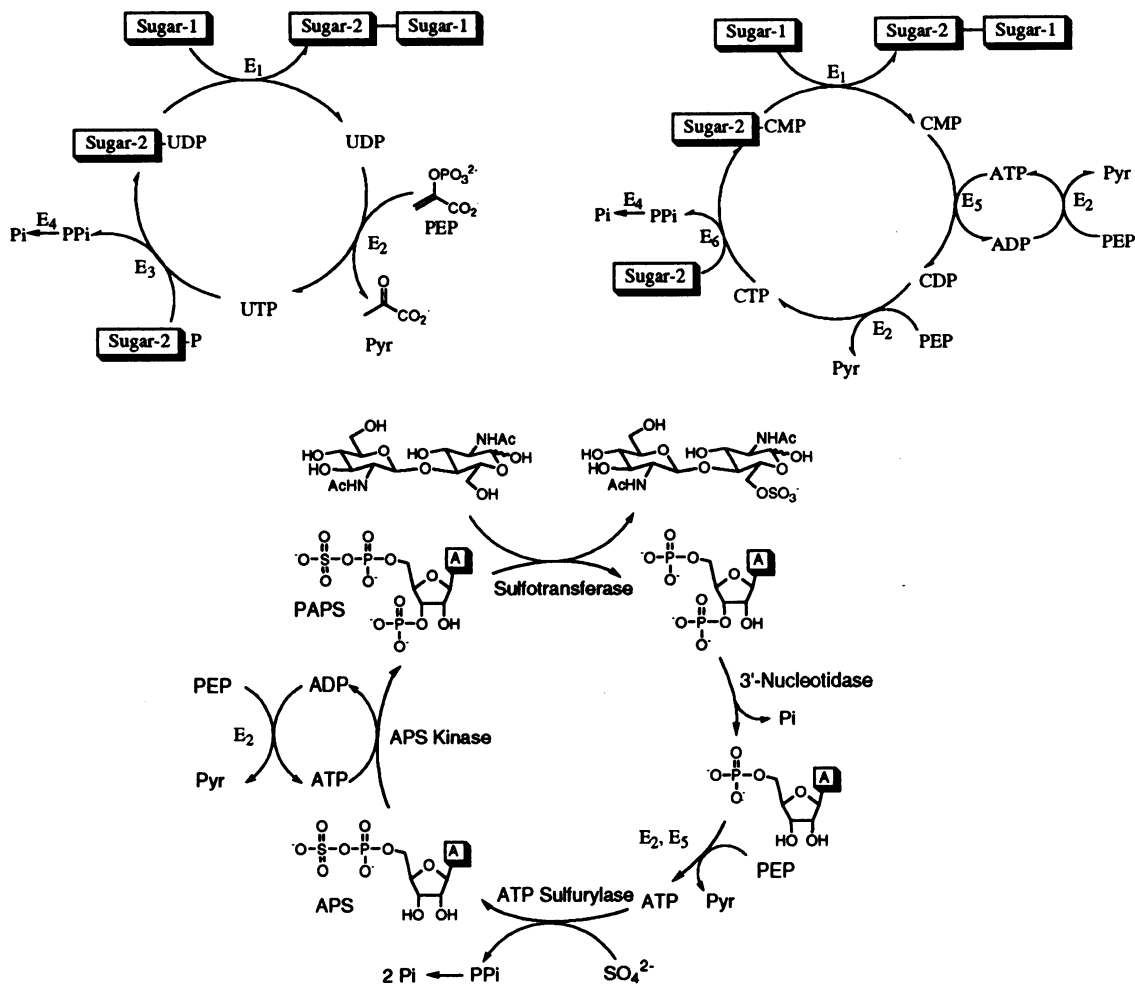


FIG. 4. Multiple enzyme systems used in the large-scale synthesis of oligosaccharides and their sulfates. E₁, glycosyltransferase; E₂, pyruvate kinase; E₃, sugar nucleotide pyrophosphorylase; E₄, pyrophosphatase; E₅, myokinase; E₆, sugar nucleotide synthase; A, adenine; APS, adenosyl phosphosulfate; PEP, phosphoenol pyruvate; and Pyr, pyruvate.

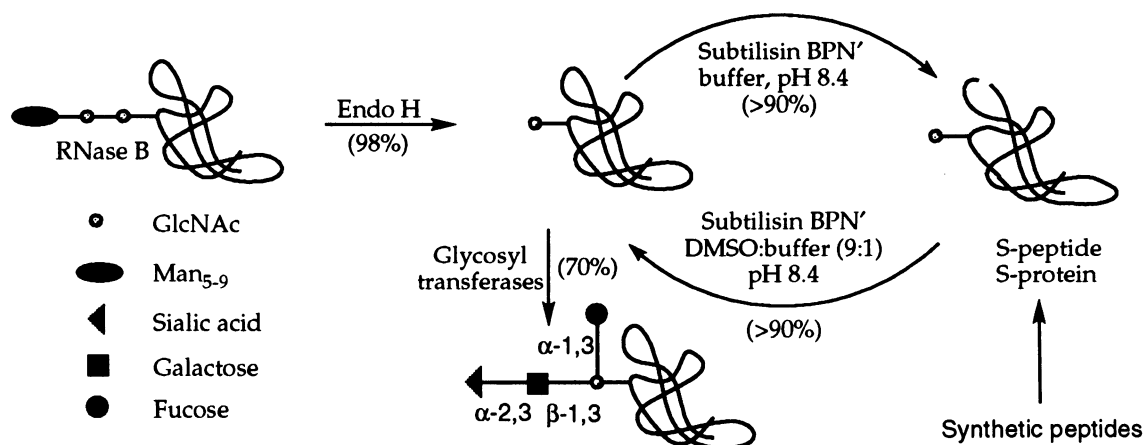


FIG. 5. Enzymatic synthesis of ribonuclease glycoproteins (RNase B glycoforms) through carbohydrate remodeling and glycopeptide coupling.

prepared from *N*-acetylglucosamine (Fig. 4), and this disaccharide can be easily converted to SLe^x-6-sulfate as the necessary sialyltransferase and fucosyltransferase for use in the reactions are known (36, 69).

Glycosyltransferases can also be applied to solid-phase synthesis (70, 71) and coupled with other enzymatic reactions to expand the scope of their synthetic application. A glycosidase-catalyzed synthesis of disaccharides, for example, can be coupled *in situ* with a glycosyltransferase reaction to improve the overall yield (72). A monoglycosylated peptide can be linked with another peptide in aqueous solution catalyzed by an engineered subtilisin via aminolysis, followed by further elongation of the sugar chain catalyzed by glycosyltransferases (73, 74). Several engineered thermostable thiosubtilisins have proven to be quite useful for glycopeptide synthesis, and the mechanisms of increased aminolysis to hydrolysis and stabilization have been elucidated (74, 75). A recent new development with the use of subtilisin is in the synthesis of new ribonuclease glycoforms. As illustrated in Fig. 5, the S-peptide and monoglycosylated S-protein, prepared by subtilisin cleavage, can be religated in the presence of dimethyl sulfoxide/water (9:1 vol/vol, pH 8), and the sugar chain can be elongated with glycosyltransferases (76). Other engineered subtilisins useful for peptide ligation have been developed by Wells and colleagues (77) for the total synthesis of ribonuclease A and analogs. The enzymatic synthesis of glycoproteins is useful, as

currently there is no method available for the preparation of homogenous glycoproteins. The strategy illustrated in Fig. 5 may be useful in this regard. The glycoproteins with heterogeneous distribution of carbohydrate composition prepared by recombinant DNA may be remodeled to a homogeneous species via enzymatic removal of the heterogeneous saccharide units, followed by addition of new sugars with glycosyltransferases. Alternatively, enzymatic ligation of glycopeptides followed by incorporation of additional sugars is another useful strategy. The enzymatic method for glycopeptide synthesis is complementary to the solution- and solid-phase chemical approaches (78–80) but may be more suitable for the synthesis of large glycopeptides.

Enzymatic modification of the sugar moiety of a glycoconjugate is another useful route to biologically important glycoconjugates. For example, the melanoma antigen 9-*O*-acetyl-ganglioside GD₃ can be prepared from GD₃ via subtilisin-catalyzed acetylation in dimethylformamide using vinyl acetate (81), or may be prepared from ganglioside GM₃ via GD₃ synthase (82)-catalyzed transfer of 9-*O*-acetylneuraminic acid from its CMP-derivative, which is in turn prepared enzymatically (83) as shown in Fig. 6.

Rational Design and Synthesis of Carbohydrate Mimetics: A Case Study of SLe^x

As described previously, complex carbohydrates may not be ideal candidates for drug development, and so the development of carbohydrate mimetics, which are simpler, more stable, more active than the parent structure, and perhaps more orally active, has become an interesting subject for research. In the case of the SLe^x-selectin interaction, the structure–function relationship study and conformational analysis have led to the rational development of SLe^x mimetics, which may be comparable to or even better than the natural ligand as inhibitors of selectins. Several groups have been actively engaged in this effort, and several SLe^x mimetics developed (see Fig. 7 and relative activity; refs. 63 and 84–92) have been shown to have affinities for E-selectin increased from the millimolar range for SLe^x (58, 93) to the micromolar range for the mimetics. It is notable that the design of C-linked glycosides as SLe^x mimetics was based on the fundamental work of Kishi (94) on the synthesis and conformational analysis of C-linked saccharides, and the hydroxylation method used in the synthesis of noncarbohydrate component is based on the Sharpless and colleagues (95) hydroxylation and on enzymatic aldol reactions developed in our laboratories (90, 91).

Since cell-surface receptor–ligand interactions, such as selectin–ligand interaction, are often multivalent, the inhibitor prepared in a multivalent form is expected to increase the

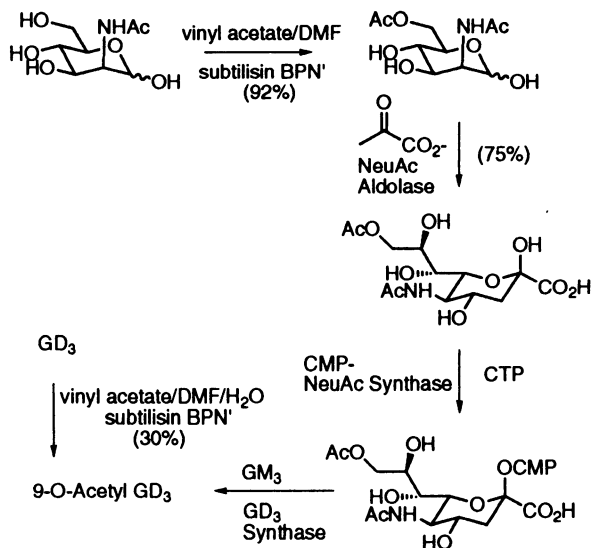


FIG. 6. Enzymatic synthesis of the melanoma antigen 9-*O*-acetyl-ganglioside GD₃.

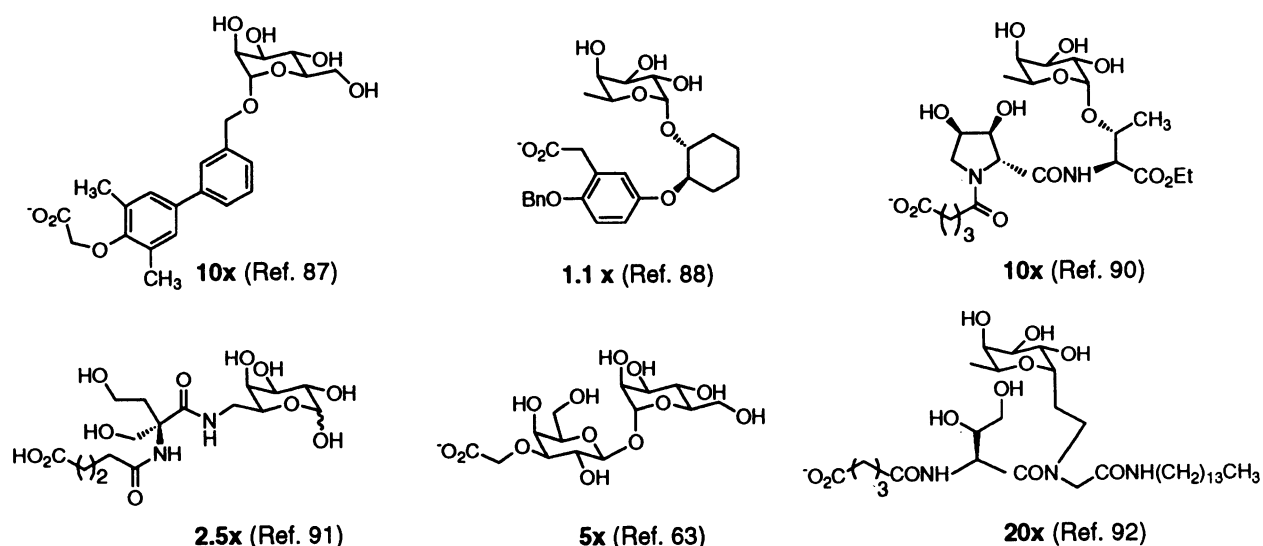


FIG. 7. SLe^x mimetics and their relative activities against E-selectin as compared with SLe^x. (For example, 20× indicates that the mimetic has an affinity for E-selectin that is 20-fold greater than SLe^x.)

inhibition potency. Indeed, both polymeric and liposome-like SLe^x derivatives have been shown to be much more active (by a factor of ≈ 70 –5000, depending on the structure and formulation) than the monomeric species as inhibitors of E-selectin (57, 96, 97). The mimetics perhaps can be converted to the multivalent form to increase the activity.

Combinatorial Approach to the Discovery of Carbohydrate Mimetics: A Case Study of Neomycin B

The combinatorial approach has also been used in finding peptides that bind to E-selectin (98). In cases where the carbohydrate–receptor interaction is not well understood, the aforementioned rational design of carbohydrate mimetics becomes difficult, and the combinatorial synthesis approach is perhaps the most effective way of finding lead inhibitors. For example, oligonucleotides have been found as inhibitors of the HIV Rev protein and the Rev response element (99). The aminoglycoside antibiotic neomycin B has recently been found as inhibitor of Rev response element (100), but the inhibition has not been well studied with regard to the origin of the selectivity. This is true with respect to the interaction of many other aminoglycoside antibiotics with certain sequences of RNA, including ribozymes (101). However, one interesting common feature of these antibiotics is that most of them contain a trans-1,3-hydroxylamine or 1,3-diamine motif. Our recent model study also indicates that phosphodiester complex the gluco-type 1,3-hydroxyamine more strongly than a bicyclic guanidine. This finding has led us to

use a combinatorial approach to rapidly synthesize a library of neomycin B mimetics containing the neamine core and screen this library for new compounds that bind to Rev response element (ref. 102; Fig. 8). As a result, several neomycin B mimetics have been found to be more active than neomycin B itself. The combinatorial synthesis is facilitated by the use of the polyethylene glycol ether as the carrier. The product can be easily isolated by precipitation with ether, so that chromatography is not necessary (103). Perhaps other libraries can be prepared similarly and used to screen for new leads.

Mechanism-Based Inhibition of Glycosidases and Glycosyltransferases

In addition to the use of carbohydrate ligands and carbohydrate mimetics as inhibitors of the carbohydrate receptor, inhibition of the enzymes associated with carbohydrate biosynthesis is another effective strategy. Both glycosidases and glycosyltransferases are important enzymes involved in the processing and synthesis of oligosaccharides and are therefore obvious targets for intervention. The mechanisms of glycosidases have been well studied (104–106), and means for inhibition of these enzymes have been developed (107–112). Glycosyltransferase reactions are thought to proceed through transition states similar to those of the glycosidase reactions, which are believed to proceed through a half-chair transition state with a substantial sp² character developed at the anomeric center (67, 113). Based on this mechanistic rationale,

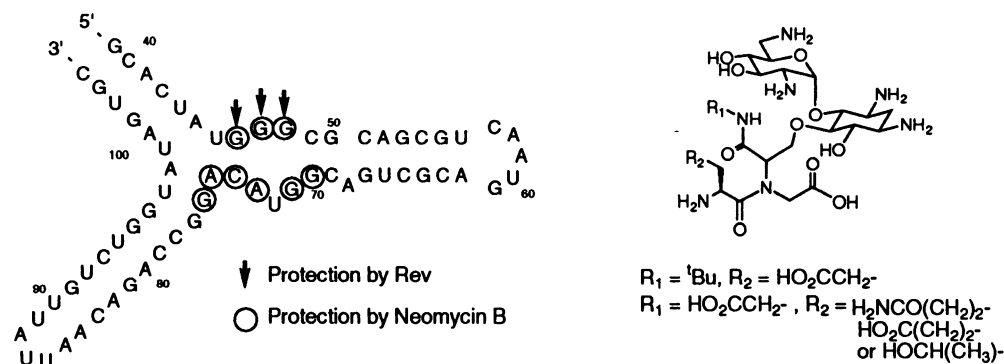


FIG. 8. HIV Rev response element and neomycin B mimetics.

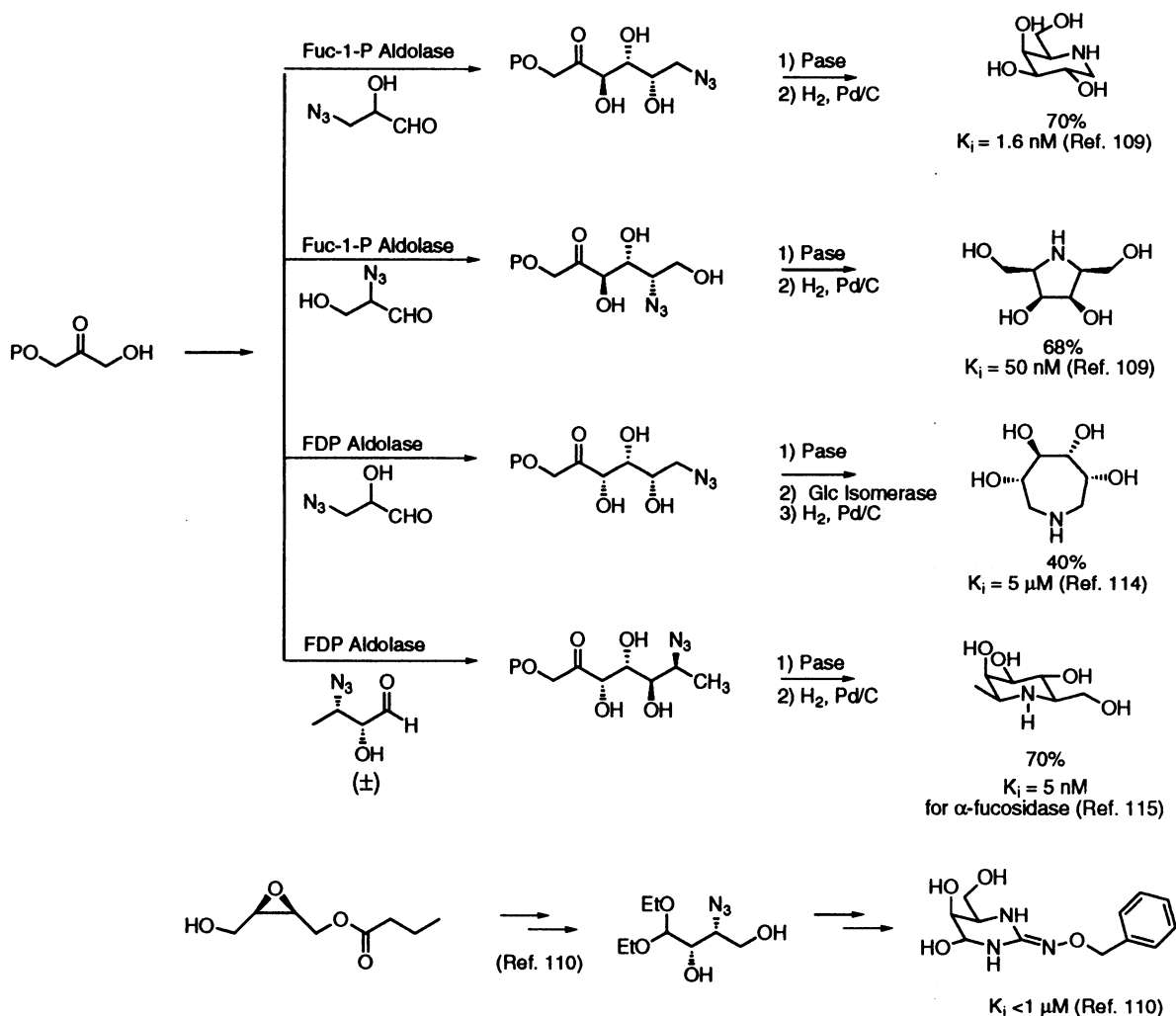


FIG. 9. Representative chemoenzymatic synthesis of five-, six- and seven-membered heterocycles as inhibitors of α -galactosidase and α -fucosidase, as indicated. Fuc, fucose; P, phosphate; Pase, phosphatase; FDP, fructose-1, 6-diphosphate; and Glc, glucose.

many transition-state analog inhibitors of glycosidases, especially iminocyclitols, have been developed. The five-, six-, and seven-membered iminocyclitols have been synthesized based on the sequence of aldolase reaction and Pd-mediated reductive amination (refs. 109 and 114; Fig. 9). These nitrogen-containing heterocycles have been used as key components in the synthesis of glycosyltransferase inhibitors. An inhibitor of α -1,3-fucosyltransferase, for example, has been prepared by the attachment of the *N*-acetylglucosamine moiety to an iminocyclitol-type α -fucosidase inhibitor. In the presence of GDP,

the azatrisaccharide acts as a strong inhibitor of the enzyme (the concentration that causes 50% inhibition is $\approx 31 \mu\text{M}$; Fig. 10; ref. 115). Given the relatively weak binding (K_m in the millimolar range) of the transferase to its substrate, the azatrisaccharide represents a very good inhibitor, and the synergistic inhibition strategy points toward a new direction for the development of better inhibitors. Another effective strategy is the development of bisubstrate analogs as inhibitors of glycosyltransferases (116).

Conclusion and Future Prospects

Molecular recognition of carbohydrates and related structures in biological systems represents a new frontier of research. Many of these recognition events occur at the very early stage of disease development and other signaling processes, and new strategies and techniques are needed to study the recognition events in detail. Chemistry will continue to play a key role in uncovering the molecular mechanism of carbohydrate recognition and in development of novel structures to control the recognition process and combat disease. The combination of chemical and enzymatic methods (117) for the synthesis of complex carbohydrates and glycoconjugates will continue to support research both in the discovery stage and in process development, as illustrated in the representative case studies presented in this article. As the principles of carbohydrate recognition become well understood, carbohydrate mimetics

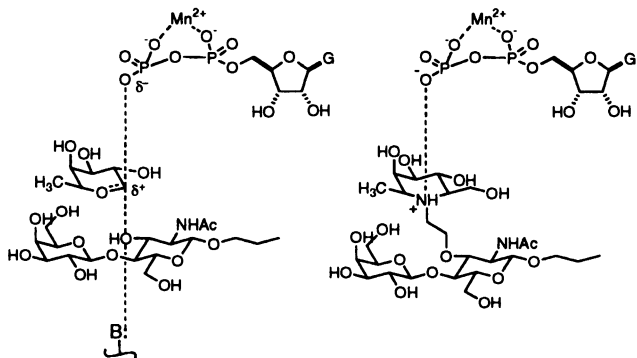


FIG. 10. Postulated transition-state structure of human α -1,3-fucosyltransferase and a designed synergistic inhibitor complex.

will be developed to overcome some of the undesirable properties of parent structures. Perhaps additional groups (such as hydrophobic groups) complementary to the receptor can be further incorporated to the mimetics to enhance binding and to improve the activity, and the multivalency strategy can then be used to further increase the activity and to control *in vivo* the function of carbohydrates.

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