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FEATURE ARTICLE

Mechanism-based inhibition of carbohydrate-mediated biological recognitions

Pamela Sears† and Chi-Huey Wong

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

Carbohydrates are often associated with specific biological recognition, targeting and signalling processes that play important roles in both normal and disease states. The efforts of many groups have been directed toward the synthesis of complex saccharides and saccharide mimics in the hope of understanding these recognition processes and developing effective agents for their intervention. As compared to research with other classes of biomolecules, however, the pace of major advances in glycobiology and development of carbohydrate-based therapeutics has been relatively slow, due to a combination of factors including the complexity of glycans in natural systems and a lack of facile synthetic techniques and analytical methods available for carbohydrate-related research. This review discusses some of the most recent developments in the field, with particular emphasis on the use of combined chemical and enzymatic approaches for the synthesis of saccharides and mimetics. Some of the highlights include the studies of selectin–carbohydrate and aminoglycoside–RNA interactions, and the synthesis and evaluation of inhibitors of glycoprocessing enzymes.

Although ubiquitous in nature, carbohydrates represent one of the least exploited classes of biomolecules. It is now known that carbohydrates are key elements in various molecular recognition processes. Carbohydrates play a role in infection by a variety of pathogens; they are important for cellular trafficking in acute and chronic inflammation and metastasis; and they play key roles in differentiation, development, regulation and many other intercellular communication and signal transduction events.[1] At the molecular level, carbohydrate-mediated recognition processes are not well understood, however. Although several potential targets for therapeutic intervention have been recognized, the rate of development of saccharide-based pharmaceuticals has been slower than that of the other classes of biomolecules. This is due to a number of factors. First, there are still some extremely difficult technical problems faced by glycobiologists and glycochemists. There is no replication system available for the amplification of minute amounts of carbohydrates to facilitate structure analysis and synthesis, nor is there a machine available for the solid-phase synthesis of oligosaccharides to facilitate the study of their functions. Because cells glycosylate lipids and proteins in a very heterogeneous fashion, it is not feasible to simply grow cells and purify the glycoproteins and glycolipids to homogeneity in large quantity. The heterogeneity of natural glycoconjugates also makes characterization difficult, although recent advances in mass spectral analysis have facilitated structural identification of oligosaccharides with picomoles of material. In addition, the synthesis of free oligosaccharides—not to mention glycoconjugates—in large quantities for research and therapeutic purposes is very difficult and expensive. Secondly, carbohydrates generally possess poor properties for drug development. The affinity of carbohydrates for their protein receptors is almost inevitably weak,[2–6] with dissociation constants in the millimolar range, and carbohydrates are generally orally inactive and sensitive to glycosidases *in vivo*. As a result, carbohydrates may only be used in injectable form for the treatment of acute symptoms.

It is clear, however, that these recognition processes are of fundamental importance in organism development, cell–cell communication, and cell and protein targeting. They are involved in the progression of a variety of diseases, such as invasion and metastasis of tumors. Likewise, many disease states are associated with changes in glycosylation at the cellular level. Pharmaceutical control of such recognition processes may therefore 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

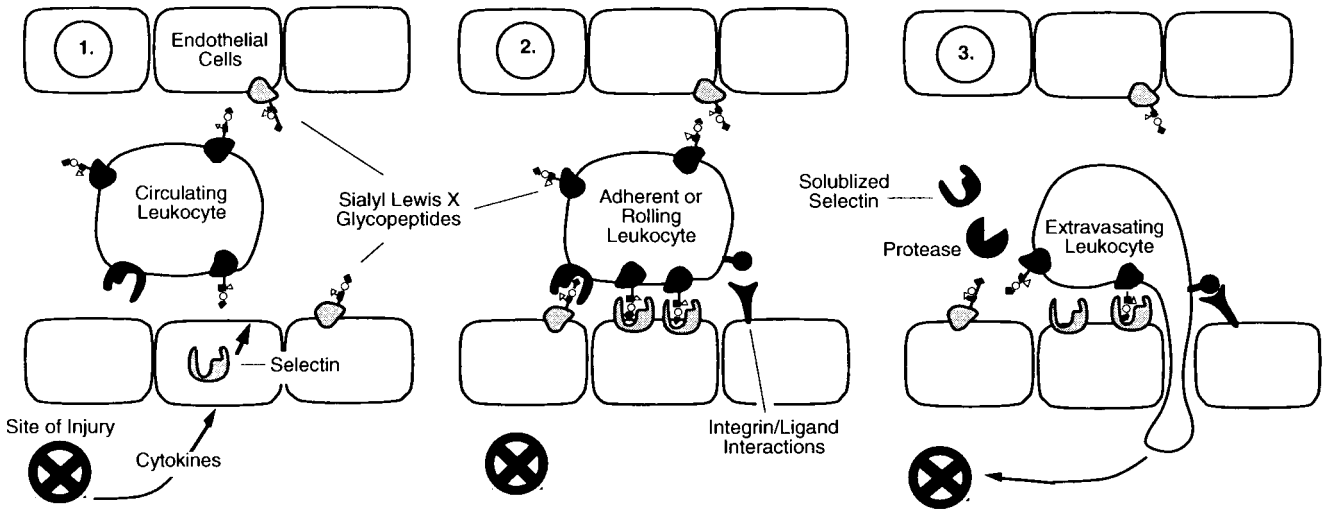


Fig. 1 Sialyl Lewis X-mediated cell adhesion in inflammatory reaction

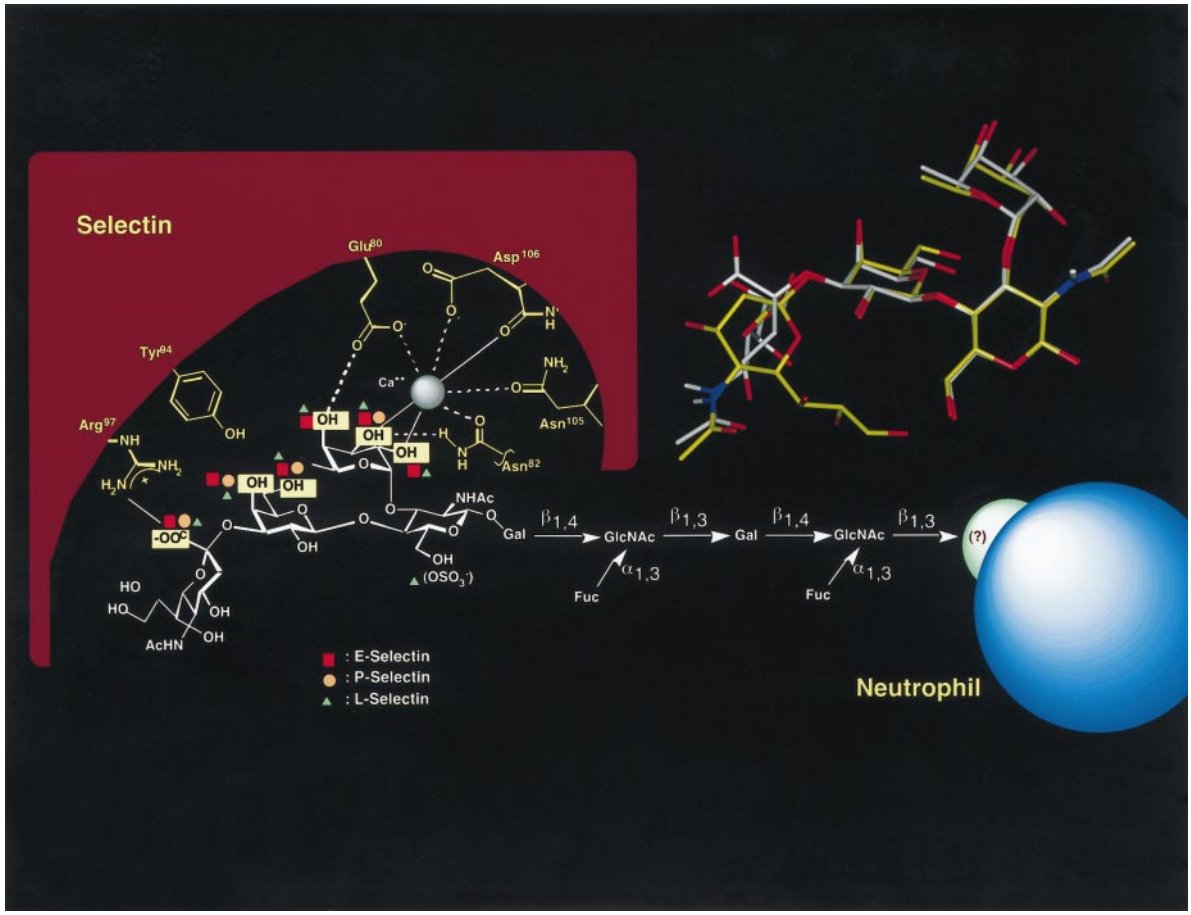


Fig. 2 Overlay of the conformation of sialyl Lewis X bound to E- or P-Selectin (yellow) with that bound to the L-selectin (white), and the functional groups essential for selectin recognition

review focuses on some of the most recent efforts toward the synthesis of complex carbohydrates, carbohydrate mimetics and inhibitors of glycoprocessing enzymes. It concentrates on the development of molecules that inhibit the natural functions of selectins, RNA, glycosidases and glycosyltransferases.

Cellular trafficking *via* selectin–carbohydrate interactions

A new class of carbohydrate-binding glycoproteins called selectins have recently been identified on the surface of specific cell types. These glycoproteins have been named E-,[7] P-[8] and L-selectins[9,10] according to the cell type on which each was initially found (*i.e.* endothelium, platelets and lymphocytes, respectively). The selectins are all homologous and have similar tertiary structures. The *N*-terminal region is made up of a calcium-dependent lectin-like domain and an epidermal growth factor-like (EGF) domain, which are necessary for carbohydrate binding. Following these are a number of modules (~ 60 amino acids) similar to those found in certain complement binding proteins. The exact number of modules depends on the selectin, though their function is, as yet, unknown. The X-ray crystal structure of the lectin and EGF domains of human E-selectin was determined[11] and its amino acid sequence is homologous to another mammalian lectin, the mannose binding protein,[12] for which the structure has also been solved. Carbohydrate ligands that are recognized by the selectins have been identified. E-Selectin recognizes sialyl Lewis X (SLe^x) on the surface of neutrophils.[13–15] P-Selectin also binds sialyl Lewis X on neutrophils or leukocytes with a lower affinity.[16,17] L-Selectin weakly recognizes sialyl Lewis X on endothelial cells but the affinity is higher with a sulfate group on the 6-position of Gal[18,19] or perhaps more likely on the 6-position of the GlcNAc residue.[17,20] Some sulfated Le^x also bind to E- and P-selectin[21] and questions regarding the true physiological ligands for these selectins still exist, particularly since the specificity of the selectins towards these ligands is by no means absolute.

The selectin–carbohydrate interaction is initiated at an early stage of the inflammatory reaction[22,23] or metastasis.[24–26] As illustrated in Fig. 1, when tissue injury occurs, cytokines are released to signal endothelial cells to display P- and then E-selectins to recruit neutrophils to the site of injury. Recruitment is mediated by the adhesion of neutrophils to endothelial cells through the multivalent interaction of SLe^x and P- or E-selectin on the respective cell surfaces, followed by a more tight interaction between integrins on neutrophils and the intercellular adhesion molecule (ICAM-1) on endothelial cells, resulting in the extravasation of neutrophils at the site of injury. Over recruitment of neutrophils can be deleterious, causing damage to normal cells and leading to inflammation. Intervention of this process by partially inhibiting the adhesion step has thus been considered to be a new strategy for creating anti-inflammatory agents, and it is expected that many acute symptoms such as reperfusion injury, stroke, asthma and arthritis may be treated with this approach. In support of this idea, the carbohydrate ligands of these selectins, especially those of E- and P-selectin, have been shown to be potentially useful for the treatment of these acute symptoms.[27,28]

Chemoenzymatic synthesis of oligosaccharides and glycoproteins

The syntheses of SLe^x and related structures[29–34] have played a very important role in defining the structure–function relationship. Studies with these molecules have not only provided confirmation of the function of the ligand but have also unravelled the essential groups involved in ligand recognition (Fig. 2). For SLe^x interaction with E- and L-selectins, it has

been shown that the three hydroxy groups of fucose,[35,36] the 2- and 6-hydroxy groups of galactose[37] and the carboxylate of neuraminic acid[35] are essential for binding and that the GlcNAc residue is not critical.[38] For P-selectin, the essential functional groups are generally the same, but the 2- or 4-hydroxy group of fucose seems not to be critical.[35] These discoveries together with the conformations of SLe^x determined by NMR spectroscopy[39–43] 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 anti-inflammatory agents.

The conformation of SLe^x in solution[39] is different from that bound to E- and P-selectin,[42] especially in the orientation of the neuraminic acid residue, though similar to that bound to L-selectin.[43] The conformational and structure–function relationship studies suggest it should be possible to design small molecules that are constrained to resemble the active form of SLe^x and thus improve the inhibition potency (Fig. 2).

Regarding the preparation of SLe^x, enzymatic techniques have been developed for its large-scale synthesis,[39] and the use of glycosyltransferases coupled with regeneration of sugar nucleotide substrates, first illustrated in the synthesis of *N*-acetyl lactosamine,[44] has proven to be useful for the large-scale process. SLe^x has been prepared on kilogram scales based on this strategy. This multienzyme system not only eliminates the problem of product inhibition caused by the released nucleoside (di)phosphates, but also reduces the cost of the expensive sugar nucleotide. This method has been extended to the synthesis of hyaluronic acid,[45] and we believe that, once the appropriate glycosyltransferases are available, it should be possible to produce any biologically known oligosaccharide in large quantities based on this methodology, since methods for the regeneration of all sugar nucleotides have been developed.[46] To date, a wide variety of glycosyltransferases have been cloned. An up-to-date listing can be found in our recent review.[47] Many of the enzymes are inactive when produced in bacterial expression systems, but the development of new high-level eukaryotic expression systems (especially using yeast,[48] baculovirus,[39] and *Aspergillus*[49]) for the preparation of glycosyltransferases has made possible the large-scale enzymatic synthesis of oligosaccharides.

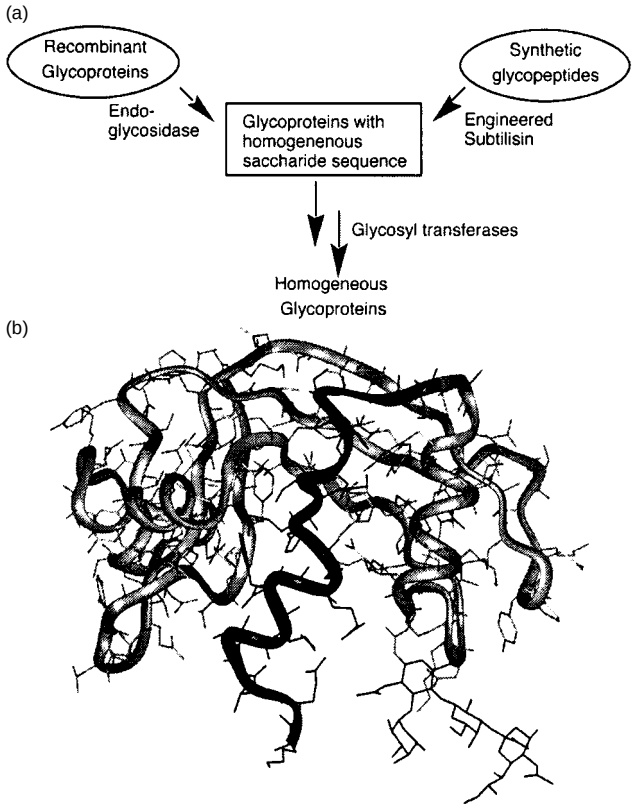


Fig. 3 (a) Strategies for enzymatic synthesis of glycoproteins containing well-defined oligosaccharides; (b) sialyl Lewis X-Ribonuclease A glycoform

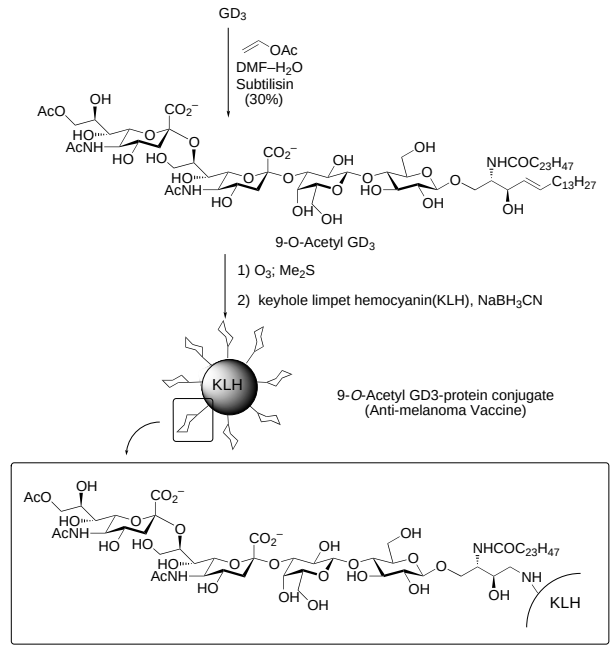
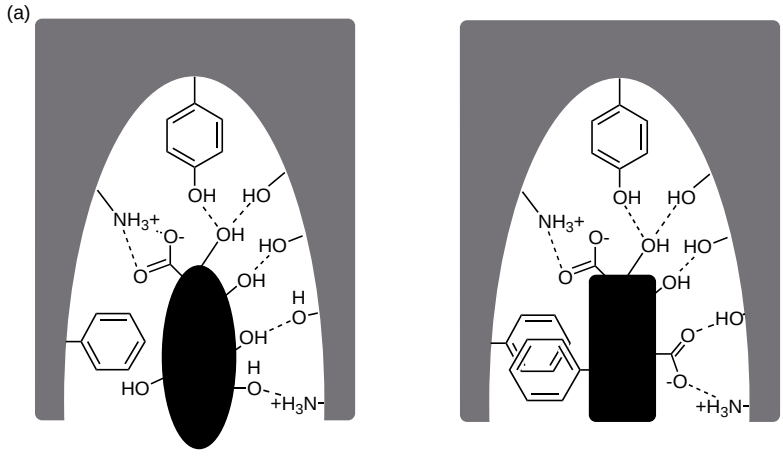


Fig. 4 Enzymatic synthesis of the melanoma antigen 9-*O*-acetyl-GD_3 for the preparation of vaccines

Sulfation of saccharides, too, can be accomplished enzymatically, and can be coupled with regeneration of PAPS for the large-scale synthesis of oligosaccharide sulfates.[50] *N*-Acetyllactosamine-6-sulfate, for example, has been prepared from *N*-acetyllactosamine, and this disaccharide can be easily converted to SLe^x-6-sulfate with commercially available α-1,3-fucosyltransferase and α-2,3-sialyltransferase.

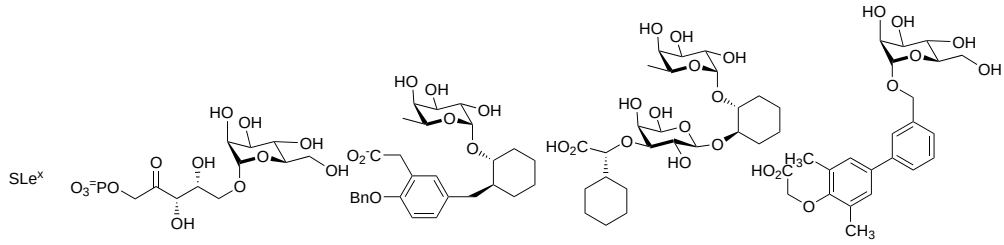
Glycosyltransferases can be applied to solid-phase synthesis[51,52] and coupled with other enzymatic reactions to expand the scope of their synthetic application. As an alternative approach, the normally hydrolytic glycosidases can be forced into the synthetic direction by addition of large amounts of acceptor, or by sequestering the product. A glycosidase-catalyzed synthesis of disaccharides, for example, can be coupled *in situ* with a glycosyltransferase reaction to improve the overall yield.[53] Complex glycopeptides and glycoproteins, while not easily accessible by normal solid-phase synthesis, can be synthesized chemoenzymatically as well. A (short) monoglycosylated peptide ester may be ligated to another peptide in aqueous solution *via* subtilisin-catalyzed aminolysis, and the resulting glycopeptide may then be further elaborated with glycosyltransferases.[54–56] 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.[54,57,58] Recently, subtilisin has been applied to the synthesis of new ribonuclease glycoforms, as illustrated in Fig. 3.[55] Other engineered subtilisins useful for peptide ligation have been developed by Wells and co-workers for the total synthesis of ribonuclease A and its analogs.[59] 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. 3 may be useful in this regard. Glycoproteins produced by fermentation, which are inevitably heterogeneous in their carbohydrate composition, may be remodelled to a homogeneous species *via* enzymatic (endoglycosidase) removal of the heterogeneous saccharide units, followed by addition of new sugars with glycosyltransferases. The enzymatic method for glycopeptide synthesis is complementary to the solution- and solid-phase chemical approaches,[60–62] but may be more suitable for the synthesis of large glycopeptides.



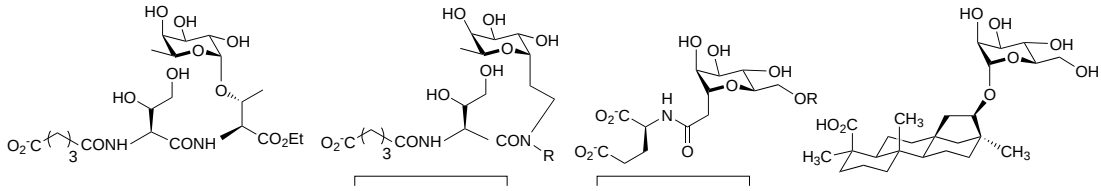
$\Delta G°$ = -4 to -6 kcal mol^{-1}
K_D = ~10^{-3} – 10^{-5} M

$\Delta G°$ = -6 to -10 kcal mol^{-1}
K_D = ~10^{-5} – 10^{-8} M

(b)



E-Selectin	0.7 mM	100 μM	0.63 mM	80 μM	50 μM
P-Selectin	8 mM	0.6 μM	—	—	—
L-Selectin	4 mM	95 μM	—	—	—



			R=$CH_2CONH(CH_2)_{13}CH_3$	R=H	R=$(CH_2)_{15}CH_3$	R=H
E-Selectin	0.5 mM	37 μM	0.3 mM	53 μM	0.1 mM	>3 mM
P-Selectin	1 mM	7 μM	4 mM	2.2 μM	>3 mM	80 μM
L-Selectin	5 mM	190 mM	>5 mM	7.6 μM	2.7 mM	>3 mM

Fig. 5 (a) Strategy for the development of carbohydrate mimetics. (b) Representative sialyl Lewis X (SLe^x) mimetics and their relative activities against selectins as compared to SLe^x. (For a review on SLe^x mimetics, see Simanek *et al.*[100]).

Enzymatic elaboration can be extended to lipids, as well. The ganglioside GM_3 may be converted to GD_3 with α-2,8-sialyltransferase (GD_3 synthase), and the melanoma antigen 9-*O*-acetyl-GD_3 can be prepared from GD_3 *via* subtilisin-catalyzed acetylation in DMF using vinyl acetate[63] (Fig. 4).

Rational design and synthesis of sialyl Lewis X mimetics

As mentioned previously, complex carbohydrates may not be ideal candidates for drug development, and so the development of carbohydrate mimetics which contain additional recognition groups (hydrophobic or charged) and are simpler, more stable, more active than the parent structure, and perhaps orally active has become an interesting subject for research [see Fig. 5(a) for an overall strategy]. 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. 5(b) and relative activity][64–72] have been shown to have IC_{50} values for the selectins decreased from the millimolar range for SLe^x to the low micromolar range for the mimetics.

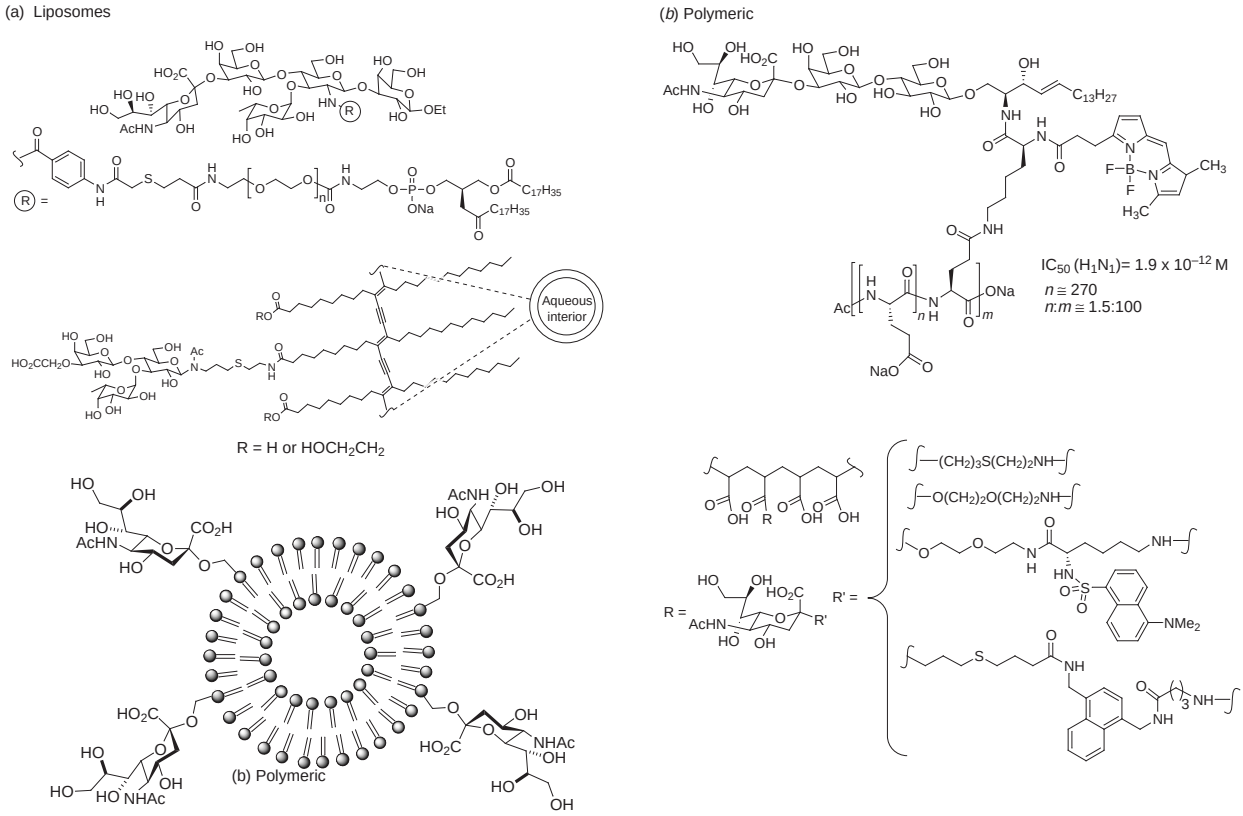


Fig. 6 Representative carbohydrate-based multivalent inhibitors of selectins and influenza haemagglutinin, both (a) liposome-based and (b) polymer-based

Polyvalent inhibitors of receptor–ligand interaction

Cell-surface receptor–ligand interactions, such as selectin–ligand interaction, are often multivalent, and so the inhibitor prepared in a multivalent form is expected to increase the 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- and P-selectin.[73,74] One should be careful, however, in extrapolating *in vitro* binding constants to *in vivo* activities. Polyvalent structures composed of ligands that are expected to show moderate non-specific binding in their monomeric form may show not only an increase in the strength of specific binding, but an increase in the strength of non-specific binding as well. For example, a polymer composed of repeating units of a ligand with a single cationic group will bind strongly to a polyanionic species, even though the monomer binds the polyanionic species only weakly. This is the principle of ion exchange

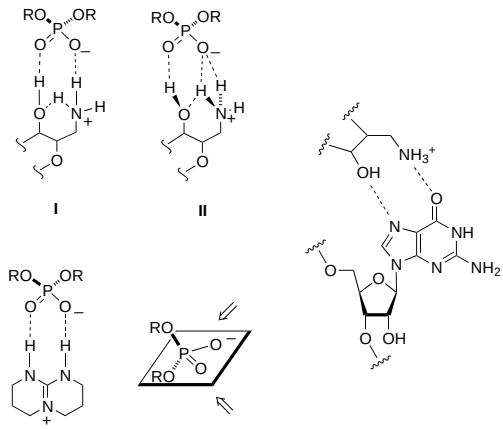


Fig. 7 Interactions of hydroxamic acids with nucleic acids, showing the binding to the phosphodiester backbone as compared to a guanidino group, and binding to the Hoogsten face of guanine

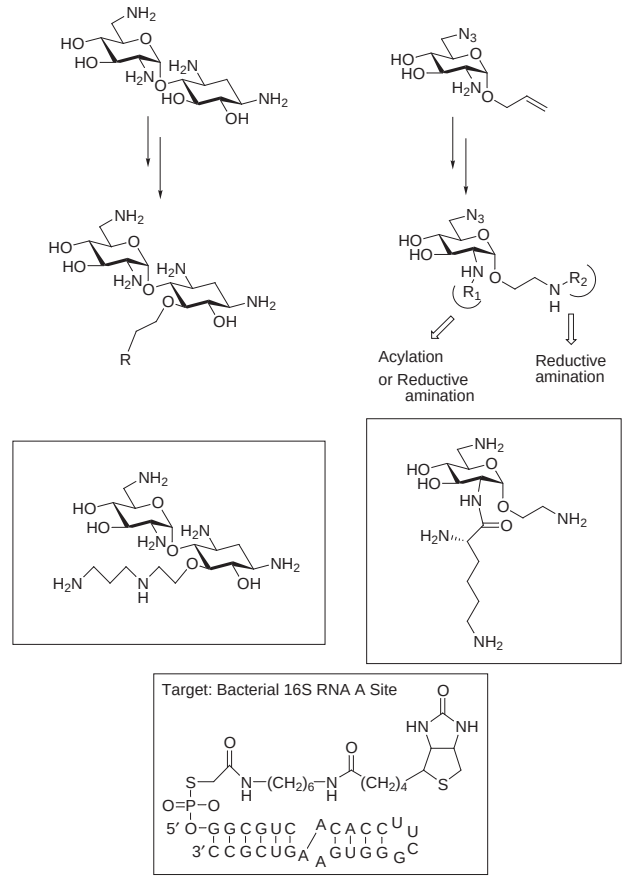


Fig. 8 Library approach for discovering new aminoglycosides with antibiotic activity. Synthetic route to 2,6-dideoxy-2,6-diaminoglucose and neamine-based libraries.

resins, after all. One should therefore begin with a molecule that has good specificity for the target of interest. In the future, perhaps the SLe^x mimetics described above, which already have better affinities for the selectins than SLe^x itself, can be converted to a multivalent form to increase the activity and specificity.

Previous work on the inhibition of the influenza virus haemagglutinin interactions with the host cell-surface sialoglycoproteins has also demonstrated the effectiveness of polyvalent inhibitors, and highly effective inhibitors with IC_{50} values ranging from 10^{-11}–10^{-12} M have been developed.[75,76] Some representative carbohydrate-based polyvalent systems are shown in Fig. 6.

Combinatorial approaches for discovering carbohydrate mimetics

The combinatorial approach has also been used in finding peptides that bind to E-selectin.[77] In many cases, the carbohydrate–receptor interaction is not well understood, so the aforementioned rational design of carbohydrate mimetics becomes difficult. In such cases, the combinatorial synthesis approach is perhaps the most effective way of finding lead inhibitors. Alternatively, the combinatorial approach can be useful for lead optimization. For example, the aminoglycoside antibiotic Neomycin B has recently been found as inhibitor of the Rev response element (RRE),[78] 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.[79] However, one interesting common feature of these antibiotics is that most of them contain a *trans*-1,3-hydroxylamine or *trans*-1,3-diamine motif. Our recent model study also indicates that phosphodiesters complex the gluco-type 1,3-hydroxylamine more strongly than a bicyclic guanidine, and may also interact with the Hoogsten face of guanine[80] (see Fig. 7). This finding has led us to use a combinatorial approach to rapidly synthesize a library of

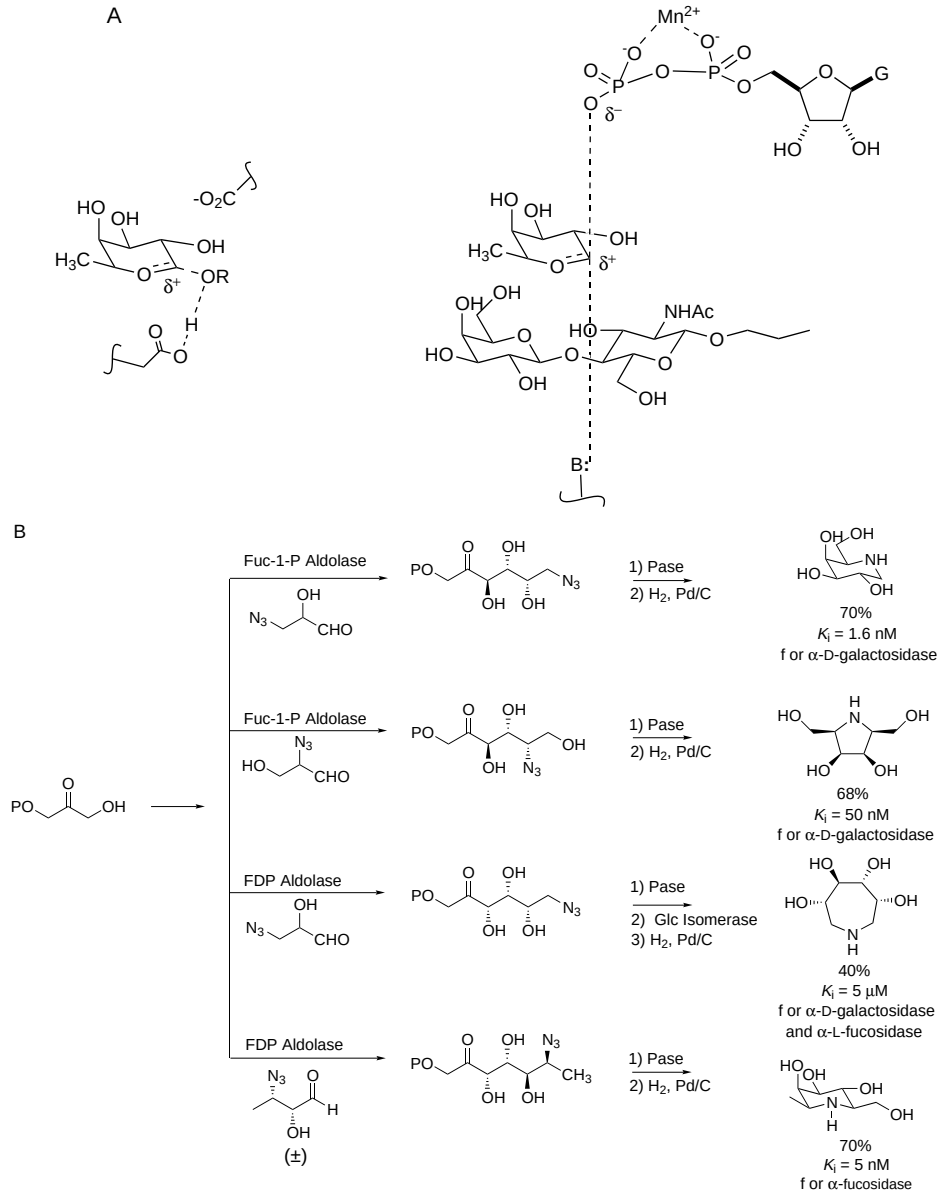
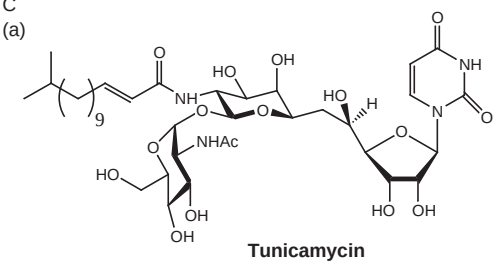


Fig. 9 (A) Postulated transition-state structure of α-fucosidase and human α-1,3-fucosyltransferase and a designed synergistic inhibitor complex; (B) aldolase reactions for production of iminocyclitol inhibitors; (following pages) (C) representative inhibitors of glycosidases and glycosyltransferases: a, Heifetz, *et al.*;[101] b, Palcic, *et al.*;[99] c, Murray, *et al.*;[94] d, Lu, *et al.*;[97] e, Hashimoto, *et al.*;[98] f, Schmidt and Frische;[102] g, Tropea, *et al.*;[103] h, Wong, *et al.*;[89] i, Elbein, *et al.*;[104] j, Pan, *et al.*;[105] k, Dorling, *et al.*;[106] l, Cottaz, *et al.*;[107] m, Tsuji, *et al.*;[108] n, Asano, *et al.*;[109] o, Dong, *et al.*;[110] p, Bernotas, *et al.*;[111] q, Wong, *et al.*;[112] r, Moris-Varas, *et al.*;[95] s, Jeong, *et al.*;[90] t, Ichikawa and Igarashi;[92] u, Schedler, *et al.*;[113] v, Takayama, *et al.*;[114] w, Knapp, *et al.*;[115] x, Sollis, *et al.*;[116] y, Kim, *et al.*[117]

C

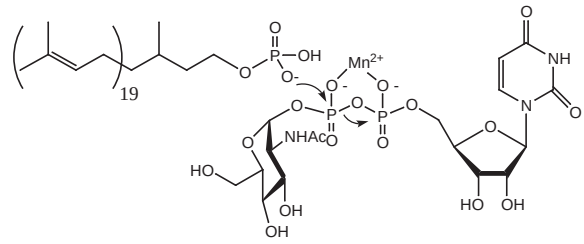
(a)



Tunicamycin

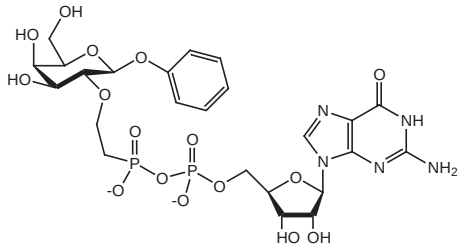
UDP-GlcNAc:Dolichol phosphate GlcNAc Phosphotransferase

IC_{50} = 7 nM



GlcNAc phosphotransferase reaction

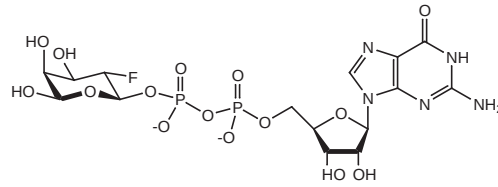
(b)



α-1,2-Fucosyltransferase

K_i = 2–16 μM

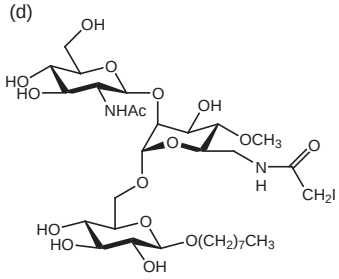
(c)



α-1,3-Fucosyltransferase

K_i = 4 μM

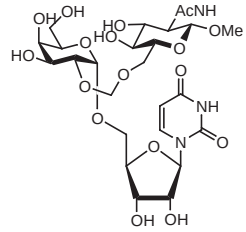
(d)



N-Acetylglucosaminyltransferase V

K_i = 21 μM

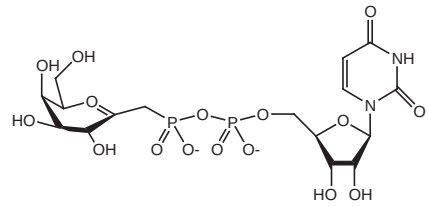
(e)



β-1,4-Galactosyltransferase

K_i = 1.35 μM

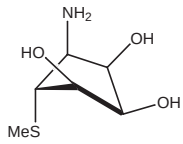
(f)



β-1,4-Galactosyltransferase

K_i = 62 μM

(g)

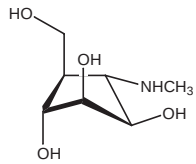


Mannostatin A

α-Mannosidase I I

IC_{50} = 10–15 nM

(h)

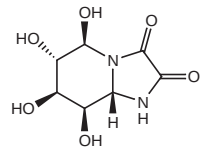


α-D-Mannosidase

(Jack Bean)

IC_{50} = 62 nM

(i)



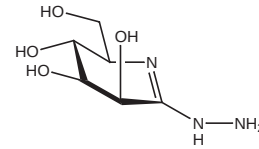
Kifunensine

α-Mannosidase I

(Mung Bean)

IC_{50} = 20-50 nM

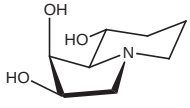
(j)



Bovine a-mannosidase I

IC_{50} = 4 μM

(k)

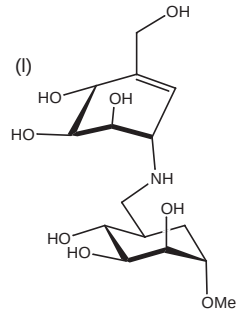


Swainsonine

Lysosomal mannosidase

IC_{50} = 100 nM

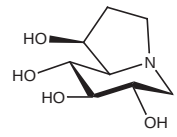
(l)



α-D-Mannosidase

K_i = 30 μM[I]

(m)

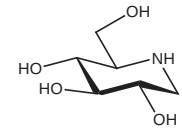


Castanospermine

α-Glucosidase I

IC_{50} = 1.2 μM

(n)



1-deoxynojirimycin

α-Glucosidase II

(rat liver)

K_i = 4.6 μM

Fig. 9 continued

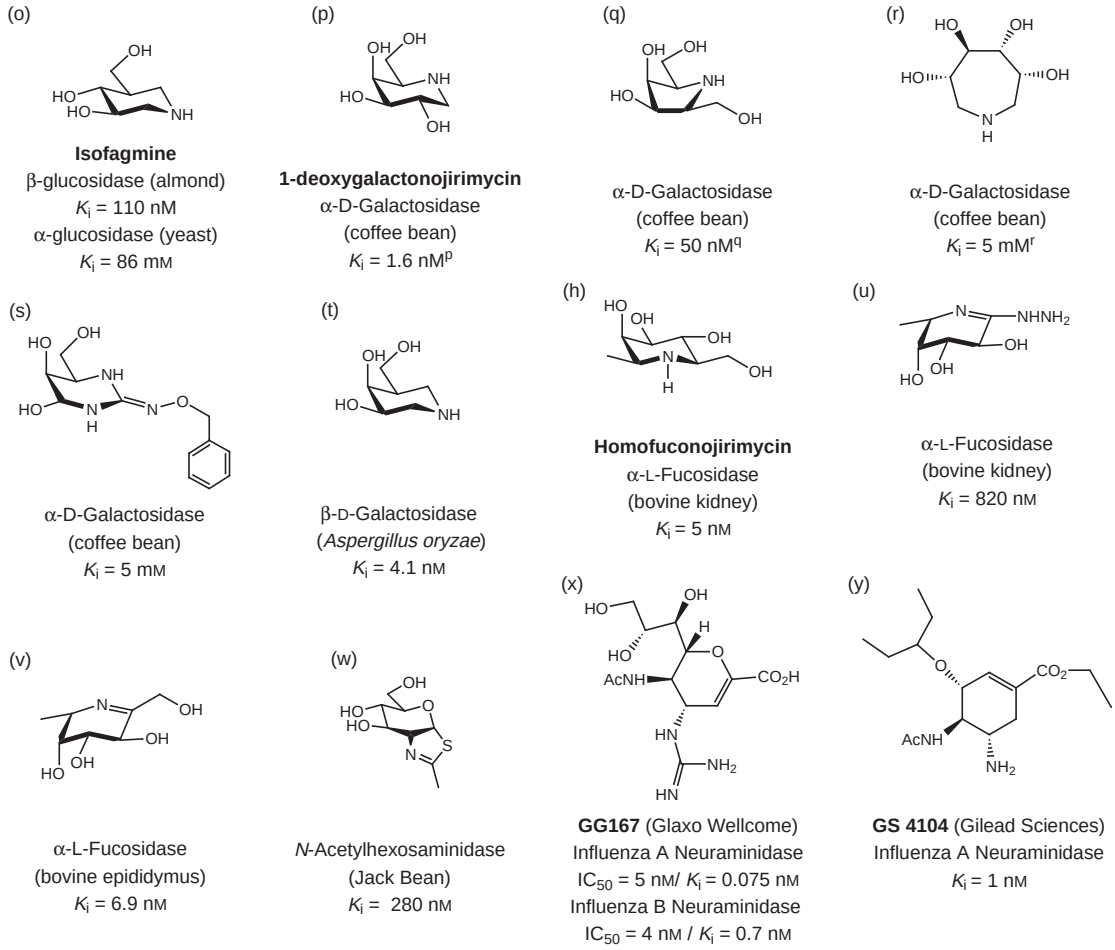


Fig. 9 continued

aminoglycoside mimetics containing either the neamine core or the 2,6-dideoxy-2,6-diaminoglucose (Fig. 8) and screen the library for new compounds that bind to RRE[81] or other RNA sequences, especially the bacterial 16S rRNA A site, a useful target for preventing bacterial infection.[82] As a result, several Neomycin B mimetics have been found to be more active than Neomycin B itself.[81] 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.[83] Both rational and library approaches have also been used to develop new aminoglycoside antibiotic mimetics to target the bacterial 16S rRNA A site and some potent new antibiotics have been discovered.[82]

Inhibition of glycosidases and glycosyltransferases

Another strategy for the intervention of carbohydrate recognition processes is to inhibit the enzymes associated with oligosaccharide biosynthesis. 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[84–86] and means for inhibition of these enzymes have been developed.[87–92] 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 sp^2 character developed at the anomeric center [Fig. 9(A)].[49,93,94] Based on this mechanistic rationale, many transition-state analog inhibitors of glycosidases, especially iminocyclitols, have been developed [Fig. 9(B),(C)]. The five-, six- and seven-membered iminocyclitols have been synthesized based on the sequence of aldolase reaction and Pd-mediated reductive amination.[89,95] These nitrogen-containing heterocycles have also been used as key components in the synthesis of glycosyltransferase inhibitors.[89] In addition, both bisubstrate and trisubstrate analogs and fluorinated sugar nucleotides have been developed as glycosyltransferase inhibitors.[94,96–99]

Conclusions 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 signalling 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. As the principles of carbohydrate recognition become well understood, carbohydrate mimetics will be developed to overcome some of the undesirable properties of parent structures, and 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. Finally, the multivalency strategy can then be utilized to further increase the activity and to control *in vivo* the function of carbohydrates.

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Chi-Huey Wong received his BS and MS degrees from the National Taiwan University and a PhD in Chemistry from the Massachusetts Institute of Technology under George M. Whitesides in 1982. After a year of postdoctoral research at Harvard University, he taught at Texas A&M University for six years, after which he moved to The Scripps Research Institute where he became the Ernest W. Hahn Professor of Chemistry in 1989. He is also head of the Frontier Research Program on Glycotechnology at RIKEN, Japan. His research interests are in the fields of bioorganic chemistry and biocatalysis.

Pamela Sears received her BS and MS degrees from University of California at Berkeley, and her PhD in Macromolecular Structure and Chemistry from the Scripps Research Institute in 1996 with Chi-Huey Wong. After a year of postdoctoral research, she became an Assistant Professor of Chemistry in 1997 at the Scripps Research Institute. Her research interests include enzymatic synthesis of biomolecules and evolution of enzymes for saccharide biosynthesis.

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† E-mail: psears@scripps.edu

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8/00838H