Synthesis of Complex Carbohydrates and Glycoconjugates: Enzyme-Based and Programmable One-Pot Strategies

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I. Introduction

A. The Biological Significance of Glycoconjugates

Identifying the functional roles of carbohydrates in biological systems has been an area of intense study in recent years. Although carbohydrates were once viewed only as inert molecules protecting the cell-surface, this diverse class of biomolecules is now recognized as playing a significant role in numerous physiological responses (Scheme 1). For example, cell-surface glycoproteins and glycolipids can serve as protein ligands, thereby providing an anchor for intercellular adhesion or a receptor for viral invasion. Alternatively, cell-surface glycans may mask a receptor site, functioning as a "decoy" to evade such recognition events. Carbohydrates are frequently a required component of antibiotic agents that bind to either protein or RNA target structures, and such glycoconjugates therefore have medicinal potential. Furthermore, altered cell-surface glycosylation patterns are diagnostic in certain cancers, and even a mechanism of glycosylation-based signal transduction has been proposed. The functional diversity of glycoconjugates in biosystems is well documented, and several thorough reviews on the subject have appeared in the literature.[1]

B. Hurdles in Obtaining Complex Glycoconjugates for Study

Although the study of carbohydrates and glycoconjugates is an important research area, access to these molecules has been difficult. This stems largely from the fact that no automated system for the synthesis of oligosaccharides exists, as it does for proteins and nucleic acids (Table 1). The central paradigm invoked in biosystems (DNA to RNA to protein) that allows template-driven synthesis and rational biomolecule manipulation unfortunately does not apply to glycoconjugates. Glycan synthesis is not template-driven, but rather occurs post-translationally. Glycan structure can therefore be influenced by a number of factors, including competition of enzymes for the same substrate, enzyme substrate specificity, and

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substrate availability. As such, when glycoproteins are produced in prokaryotic or eukaryotic expression systems, it is not uncommon for them to exhibit glycoform microheterogeneity.[2] It is therefore nearly impossible to obtain homogeneous glycoproteins for study from overexpression systems.

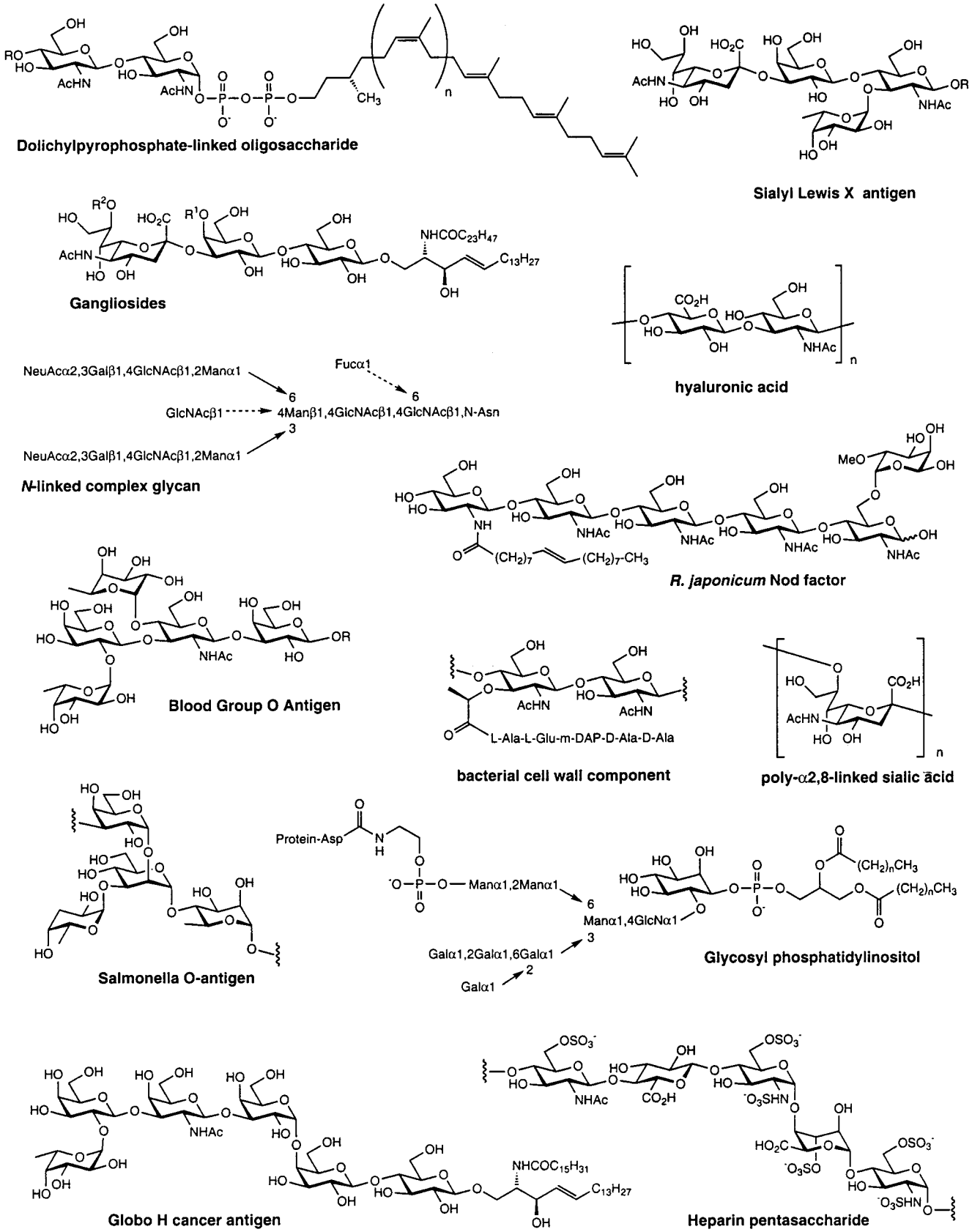
In the biological front, research into carbohydrate structure and function has focused mainly on the characterization of glycan chains isolated from glycoproteins. New glycan sequencing methods have greatly reduced the analytical time commitment in recent years, but these techniques produce only minute quantities of material for further study.[1b] This drawback has attracted the interest of the chemical community, and much effort has been invested in the synthesis of carbohydrate constructs by chemical means. Nonetheless, decades of synthetic research have not yielded robust methods of automated carbohydrate synthesis that are analogous to those available for proteins and nucleic acids. The explanation for this roadblock lies within the intrinsic structures of carbohydrates themselves. For example, the synthesis of an oligopeptide involves the iterative formation of a peptide bond between a single activated carboxylic acid and a free amine. However, in oligosaccharide synthesis, several hydroxyl groups of similar reactivity must be suitably differentiated in order to obtain the desired product with the appropriate regio- and stereoselectivity. Consequently, chemical carbohydrate synthesis often requires laborious protecting group manipulations and long synthetic routes. As could be anticipated, tedious chemical synthesis is not an attractive option to the scientific community as a whole.

C. Tools for the Simplification of Glycoconjugate Synthesis

Efforts to simplify the synthesis of carbohydrates have been of current interest. Toward this goal, enzymes provide an alternative that chemists and biologists alike consider synthetically viable. Glycosyltransferases and glycosidases have become valuable catalysts for constructing stereo- and regiospecific glycosidic linkages in oligosaccharide structures. Enzymes such as proteases and aldolases have found applications in the synthesis of complex glycoconjugates as well. Alternatively, in the chemical arena, quantitation of the chemical reactivity of specific glycosyl donors has resulted in programmable one-pot syntheses that have great potential toward the development of automated oligosaccharide assembly. Both enzymatic and programmable one-pot techniques provide complex carbohydrates without lengthy protection/deprotection strategies and decrease the synthetic time commitment.

At the present time, complex carbohydrate and glycoconjugate synthesis remains much more complicated than that of other biomolecules. However, enzyme-based and one-pot methods have greatly simplified synthetic strategies. This review first describes recent accomplishments in the area of enzyme-based and chemoenzymatic synthesis of complex carbohydrates, glycopeptides, glycoproteins, and glycolipids. In this respect, it does not attempt to

Scheme 1. Biologically Relevant Glycoconjugates



describe every enzyme involved in glycoconjugate biosynthesis, but instead highlights applications of enzymes that have proven useful in preparative-scale syntheses. The remaining section is devoted to one-pot techniques for the chemical synthesis of oligosaccharides. Included in this discussion are studies and observations that had a direct impact on the development of programmable one-pot synthetic methods. Both enzymatic and chemical one-pot synthetic methods are emerging as tools that will aid the advance-

Table 1. General Methods Employed to Obtain Biomolecules

biomolecule	primary methods
proteins	***automated*** peptide synthesis, native peptide ligation, overexpression systems, protease-catalyzed bond formation
nucleic acids	***automated*** nucleic acid synthesis, polymerase chain reaction
carbohydrates	isolation from glycoproteins, chemical synthesis, enzymatic synthesis

ment of glycoscience through simplification of complex glycoconjugate synthesis.

II. Enzyme-Based Glycoconjugate Synthesis

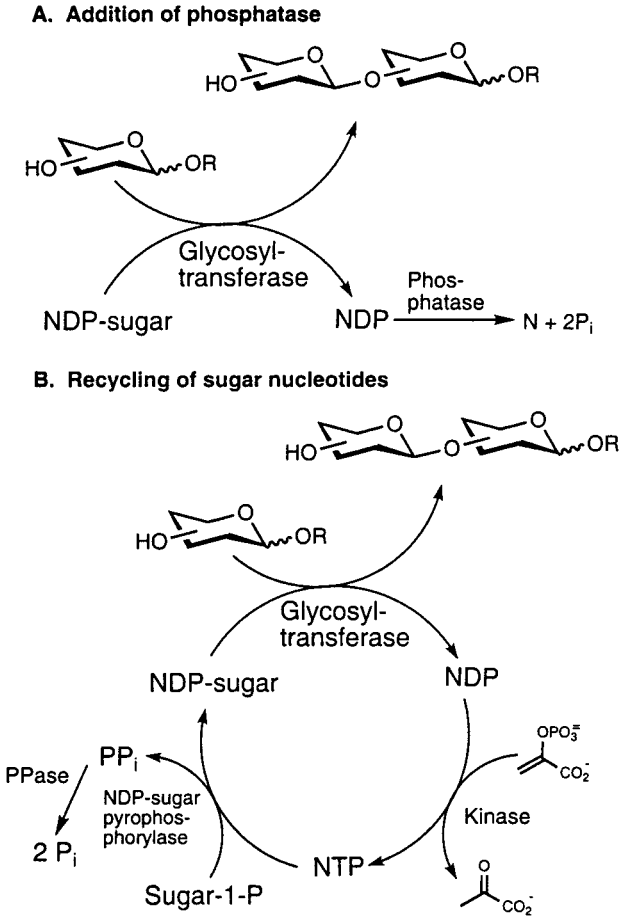
Enzymes have been used in the synthesis of complex glycoconjugates. Glycosyltransferases, exo-glycosidases, and endo-glycosidases are valuable catalysts for the formation of specific glycosidic linkages. Moreover, enzymes such as sulfotransferases, proteases, lipases, and aldolases can be exploited for the synthesis of other distinct structures which are critical to glycoconjugate function. Discussed below are features of the various enzyme classes that have been utilized in the assembly of oligosaccharides and glycoconjugates.

A. Glycosyltransferases

Glycosyltransferases of the Leloir pathway are responsible for the synthesis of most cell-surface glycoforms in mammalian systems.[3] These enzymes transfer a given carbohydrate from the corresponding sugar nucleotide donor substrate to a specific hydroxyl group of the acceptor sugar. A large number of eukaryotic glycosyltransferases have been cloned to date[1c] and, in general, exhibit exquisite linkage and substrate specificity. It is remarkable that such a considerable number of mammalian enzymes have converged on only eight general sugar nucleotides as glycosyl donor substrates. Glucosyl- and galactosyltransferases employ substrates activated with uridine diphosphate as the anomeric leaving group (α-UDP-Glc, α-UDP-GlcNAc, α-UDP-GlcUA, α-UDP-Gal, α-UDP-GalNAc), whereas fucosyl- and mannosyltransferases utilize guanosine diphosphate (β-GDP-Fuc, α-GDP-Man). Sialyltransferases are unique in that the glycosyl donor is activated by cytidine monophosphate (β-CMP-NeuAc). Preparative-scale syntheses of relevant sugar nucleotides have been developed previously, and most are now commercially available.[4] Non-Leloir glycosyltransferases, which utilize sugar phosphates as glycosyl donors, have been applied synthetically in a much more limited fashion and will not be covered further.

As Leloir glycosyltransferases are highly regio- and stereospecific with respect to glycosidic linkage formation and provide products in high yield, these enzymes are often the catalysts of choice for glycoconjugate synthesis. However, glycosyltransferase-based syntheses suffer from two major drawbacks. First of all, the nucleoside diphosphates generated during the reaction are potent glycosyltransferase inhibitors. Furthermore, sugar nucleotide expense can become a burden if large-scale synthesis is required. The feedback inhibition problem can be solved by the addition of a phosphatase into the reaction, which results in breakdown of the NDP product (Scheme 2).[5] However, to circumvent sugar

Scheme 2. General Protocols for Glycosyltransferase-Based Synthesis



nucleotide expense and avoid product inhibition simultaneously, multienzyme recycling systems have been developed.[6] In this case, NDP-sugars are required in only catalytic quantities, as they are generated in situ from inexpensive starting materials. Moreover, the NDPs are recycled to NDP-sugars, thereby avoiding product inhibition.

Glycosyltransferase availability is occasionally considered a third drawback. Although many glycosyltransferases can now be purchased from commercial sources (Table 2), an enzyme specific for every desired glycosidic linkage cannot be obtained at the present time. In some instances, those that are not available commercially can be isolated from tissue sources.[7] The use of glycosyltransferases in carbohydrate synthesis has been reviewed previously.[8]

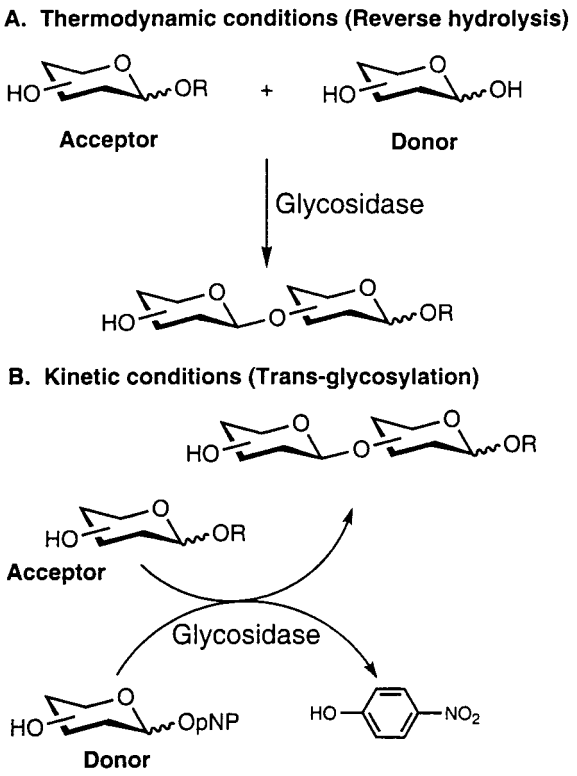
B. exo-Glycosidases

In vivo, exo-glycosidases are responsible for glycan processing reactions that take place during glycoprotein synthesis. The physiological function of these enzymes is the cleavage of glycosidic linkages. However, under controlled conditions, glycosidases can be used to synthesize glycosidic bonds rather than cleavage (Scheme 3). As such, they have been em-

Table 2. Commercially Available Glycosyltransferases

glycosyltransferase	supplier
β1,4-galactosyltransferase	Calbiochem, Fluka, Sigma, Glyko, Worthington
α1,3-galactosyltransferase	Calbiochem
α2,6-sialyltransferase	Calbiochem, Sigma
α2,3-sialyltransferase	Calbiochem
α1,3-fucosyltransferase (III, IV, V, VI, VII)	Calbiochem
α1,2-mannosyltransferase	Calbiochem

Scheme 3. General Protocols for Glycosidase-Based Synthesis



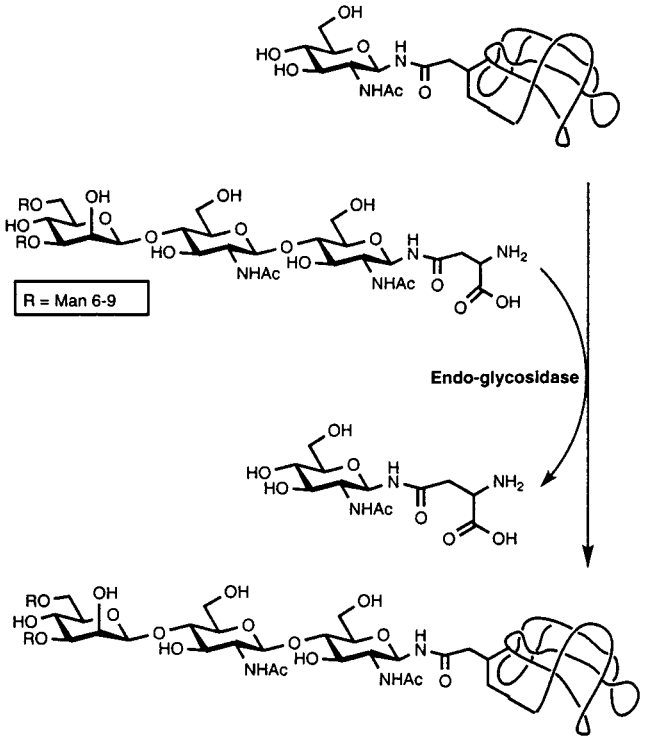
ployed as catalysts in oligosaccharide synthesis.[8a−c,9] In comparison with glycosyltransferases, glycosidases are inexpensive, stable, and readily available. These catalysts require only inexpensive donor substrates, as opposed to expensive nucleotide sugars. Although glycosidases are generally stereospecific, a primary disadvantage is weak regiospecificity, which may result in the formation of multiple products.

Two general protocols for glycosidase-based synthesis have been reported. When glycosidase-catalyzed reactions are performed under thermodynamic conditions (reverse hydrolysis), products are frequently isolated in low yield. However, improved yields can often be achieved under kinetic conditions (*trans*-glycosylation), usually employing an activated glycosyl donor, organic cosolvent, or donor excess, or using a transglycosidase that prefers transglycosidation to hydrolysis. In general, only simple glycosides can be prepared, and product yields are still greatly decreased when compared to those of glycosyltransferase-catalyzed reactions. In some cases, a glycosidase is available when the corresponding glycosyltransferase is not, thus providing an alternative enzymatic synthetic route.

C. endo-Glycosidases

endo-Glycosidases cleave internal glycosidic linkages in an oligosaccharide chain or glycoconjugate. endo-Glycosidases, like the exo-glycosidases described above, can also catalyze *trans*-glycosylation reactions under appropriate conditions.[10] The enzymes endo-A, endo-M, and endo-F are endo-glycosidases that cleave the bond between the GlcNAc units of the chitobiose disaccharide at the reducing terminus of *N*-linked glycans (Scheme 4). These endo-

Scheme 4. Typical *N*-Linked Glycan endo-Glycosidase *trans*-Glycosylation Reaction



glycosidases require an Asn-linked glycan donor for *trans*-glycosylation and have been employed in the construction of complex glycopeptides and glycoproteins of defined structure in a single transformation. This methodology is currently the most accessible technique to obtain homogeneous glycoproteins, albeit in a relatively low yield. Another endo-glycosidase, ceramide glycanase, has facilitated the synthesis of complex glycolipids.

D. Other Enzymes

Synthetic strategies toward complex glycoconjugates are concerned with linkages other than the glycosidic bond. Glycan modifications, such as the sulfation present on the GlyCAM-1 carbohydrate or the acylation of ganglioside 9-*O*-acetyl-GD_3, have also been investigated by enzymatic methods. The use of proteases as catalysts in peptide-bond formation and lipases for the mild removal of protecting groups have

been extremely beneficial in the area of glycopeptide synthesis. Aldolases have provided access to numerous unnatural carbohydrate derivatives. Furthermore, inteins are applicable to the synthesis of modified glycoproteins. In general, enzyme catalysts have become standard synthetic tools and are increasingly recognized by the chemical community as valuable additions to their arsenal of synthetic methods.

III. Enzymes that Form Glycosidic Linkages

Enzymes that catalyze the formation of glycosidic linkages have been utilized extensively in the construction of complex glycoconjugates. Biologically relevant target structures include the carbohydrate chains of glycoproteins and glycolipids involved in intercellular recognition processes, such as the inflammatory response and cancer cell metastasis. Glycans that have been implicated in viral cell adhesion and organ transplant rejection are also of interest. Much recent effort has focused on the generation of homogeneous glycoproteins. The following section outlines synthetic applications of enzymes that catalyze the formation of glycosidic bonds, and is categorized by the specific mono- or oligosaccharide transferred.

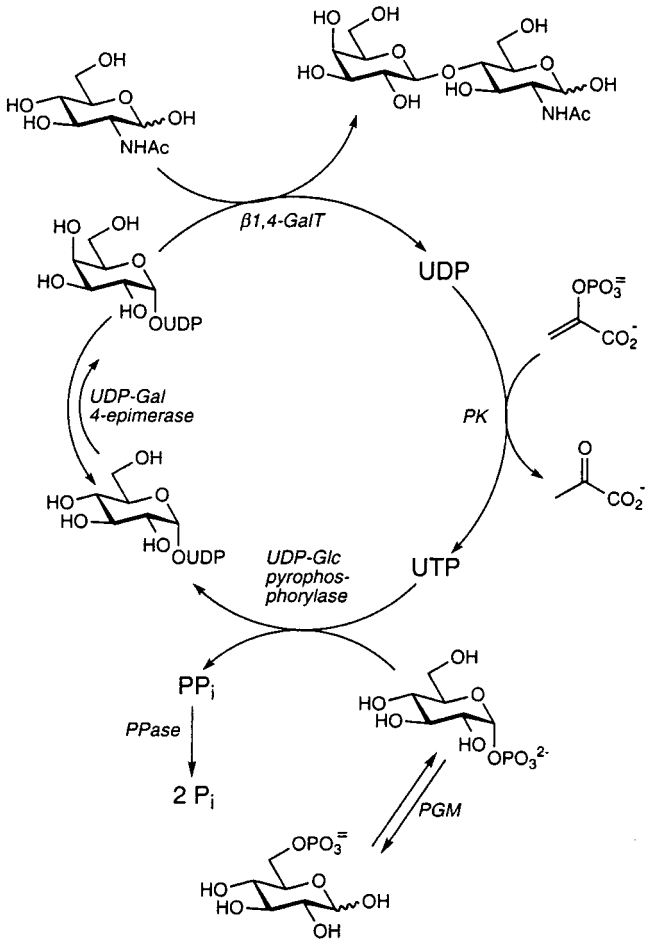
A. Transfer of Monosaccharides

1. Galactose

a. Galactosyltransferases (GalT). Almost two decades ago, Wong and Whitesides published a landmark report of the first multienzyme system for the synthesis of *N*-acetyl-lactosamine (LacNAc) from simple starting materials (Scheme 5).[11] This protocol for the in situ regeneration of UDP-galactose solved the problems of β1,4-GalT product inhibition and procuring expensive sugar nucleotide substrates. Synthesis of LacNAc was accomplished on a multigram scale, a "green" procedure which is now employed in the industrial-scale production of LacNAc. This classic paper also instigated the development of sugar nucleotide recycling systems for syntheses employing various glycosyltransferases. Subsequently, alternative regeneration schemes for UDP-Gal have also been reported,[12] including one in which sucrose synthetase is incorporated into the recycling scheme (Scheme 6).[12a]

β1,4-GalT is one of the most highly characterized and utilized glycosyltransferases for synthesis. Furthermore, β1,4-GalT is the only glycosyltransferase for which a high-resolution X-ray crystal structure has been solved to date.[13] β1,4-GalT has been used for the production of vast numbers of LacNAc analogues,[14] and its substrate specificity has been studied exhaustively.[15] The most unusual result with respect to substrate specificity was the report by Nishida et al. that β1,4-GalT catalyzed the formation of a β,β-1,1-linkage with certain GlcNAc or XylNAc analogues (Scheme 7).[16] These studies revealed a substantial directing effect of the NHAc group within the enzyme active site. β1,4-GalT has been employed for the generation of LacNAc and LacNAc-glycopeptides in

Scheme 5. Recycling of UDP−Gal Employing UDP−Gal 4-Epimerase for the Synthesis of LacNAc[11]

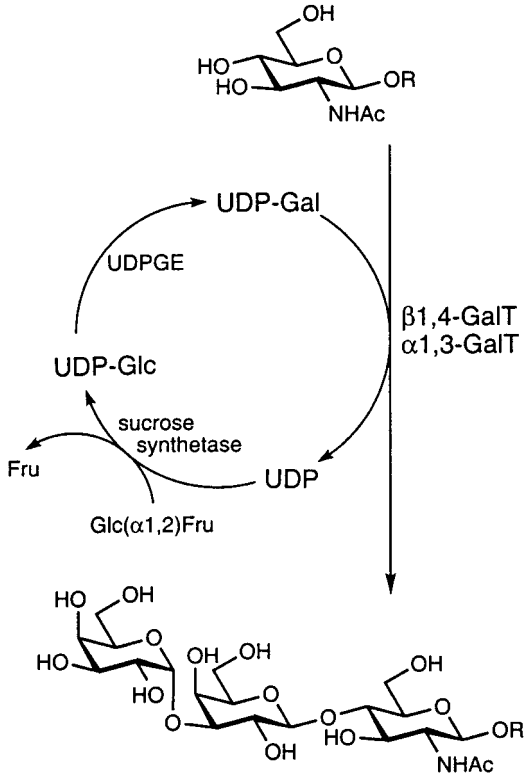


solution[17] and on solid support.[18] Synthesis of mimics of the capsular polysaccharide of *Streptococcus pneumoniae* type 14 have also been undertaken.[19]

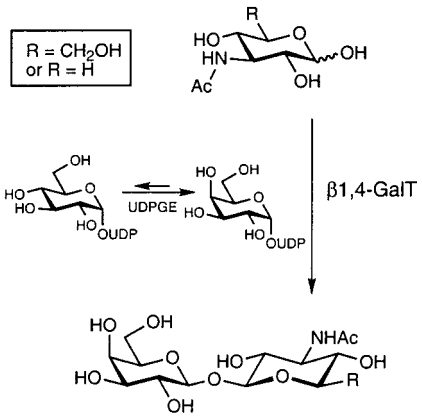
Other than LacNAc-based structures, β1,4-GalT has been utilized for the differentiation between enantiomers of conduritol[20] and to append galactose to various alkaloids for improvement of bioavailability.[21] Transfer of galactose onto cyclodextrin (CD)[22] was performed, based on the premise that recognition of Gal-CD conjugates by galectins will enhance its drug delivery capabilities. Most often, however, β1,4-GalT is employed in conjunction with other glycosyltransferases to provide extremely complex target structures, as will be discussed in later sections.

Although most galactosyltransferase literature focuses on β1,4-GalT, the utility of other galactosyltransferases will emerge as their availability increases. The β1,3-GalT responsible for synthesis of the β1,3 branch of core 2 glycans has recently been studied with regard to substrate specificity,[23] as has β1,4-GalT I which is implicated in poly(LacNAc) synthesis.[24] In an innovative synthetic approach, metabolically engineered bacterial cells were coupled for the production of globotriose (Scheme 8).[25] In this case, *E. coli* was engineered to express the UDP-Gal biosynthetic genes *galT* (Gal-1-P uridyltransferase), *galU* (Glc-1-P uridyltransferase), *galK* (galactokinase), and *ppa* (pyrophosphatase). In addition, *C.*

Scheme 6. Recycling of UDP−Gal Employing Sucrose Synthetase for the Synthesis of the α-Gal Trisaccharide[12h]



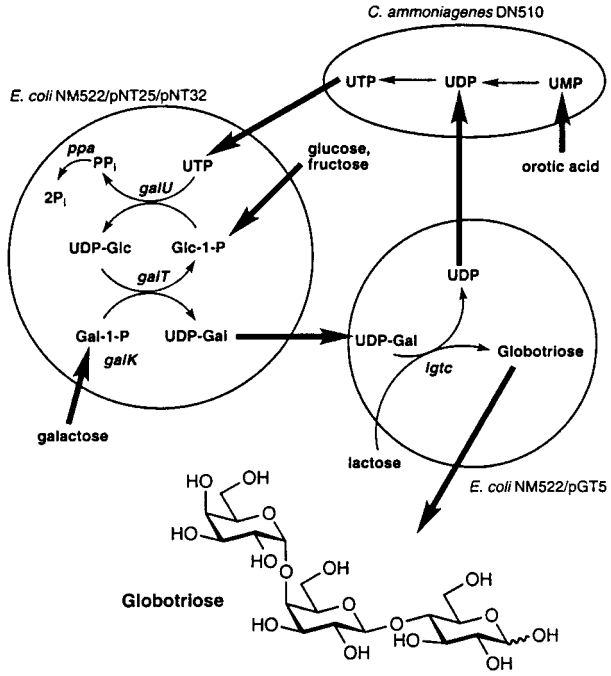
Scheme 7. Formation of Unusual *β,β*,1,1-Linked Disaccharides with *β*1,4-GalT[16]



ammonigenes was engineered to produce UTP from orotic acid. Coupling of these engineered cell lines to *E. coli* expressing *lgtC* (α1,4-GalT) from *N. gonorrhoeae* resulted in the large-scale synthesis of UDP-Gal and globotriose, without side products. Notably, the only additives to the system were the inexpensive substrates galactose, lactose, glucose, fructose, and orotic acid which served as starting materials. A second report citing α1,4-GalT involves the use of galactosyl fluoride as a substrate for the glycosyltransferase in the presence of only catalytic amounts of UDP.[26] If this approach can be scaled up and extended to other glycosyltransferases, it would generally obliterate the problem of sugar nucleotide expense.

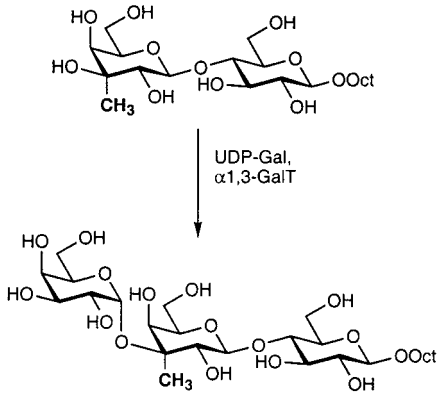
As of late, the retaining galactosyltransferase α1,3-GalT has attracted much attention. This enzyme is

Scheme 8. Large-Scale Production of Globotriose Utilizing Metabolically Engineered Bacterial Cells[25]



responsible for the addition of the nonreducing terminal α-galactose residue of oligosaccharides that cause hyperacute rejection following xenotransplantation.[27] Several studies of α1,3-GalT substrate specificity have been carried out.[28] Interestingly, α1,3-GalT is reportedly capable of galactosyl transfer to an unnatural hindered tertiary hydroxyl of the acceptor sugar (Scheme 9).[29] This hindered transfer of

Scheme 9. α-Galactosyl Transfer to a Tertiary Hydroxyl Acceptor Site[29]

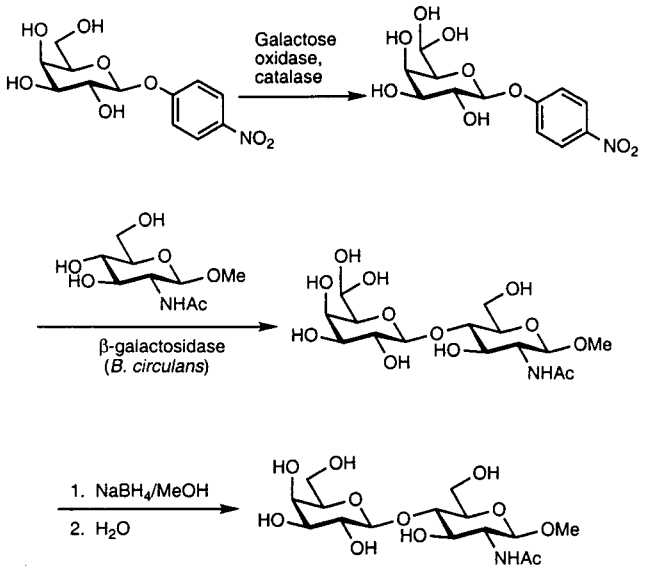


α-galactose was also observed with α1,4-GalT and the human blood group A and B glycosyltransferases (GTA, GTB). The Wang group reported the expression of an α1,3-GalT/UDPGE (UDP-Gal 4-epimerase) fusion enzyme for the facile synthesis of α-galactose linked sugars. With this construct, UDP-Gal can be generated in situ from the relatively inexpensive sugar nucleotide UDP-Glc.[30]

b. Galactosidases. *β*-Galactosidases have also been extensively studied. In parallel with *β*1,4-GalT, this family of enzymes has also been employed in numerous syntheses of the LacNAc disaccharide and

its derivatives.[31] Notably, the tandem use of β-galactosidase from *B. circulans* and galactose oxidase provides LacNAc in nearly double the product yield usually observed in β-galactosidase-catalyzed synthesis (Scheme 10).[32] In this case, when 6-oxo-Gal-

Scheme 10. Tandem Use of Galactose Oxidase and β-galactosidase for the Synthesis of LacNAc Derivatives[32]



pNP is employed as the donor substrate, *trans*-glycosylation is kinetically favored over hydrolysis.

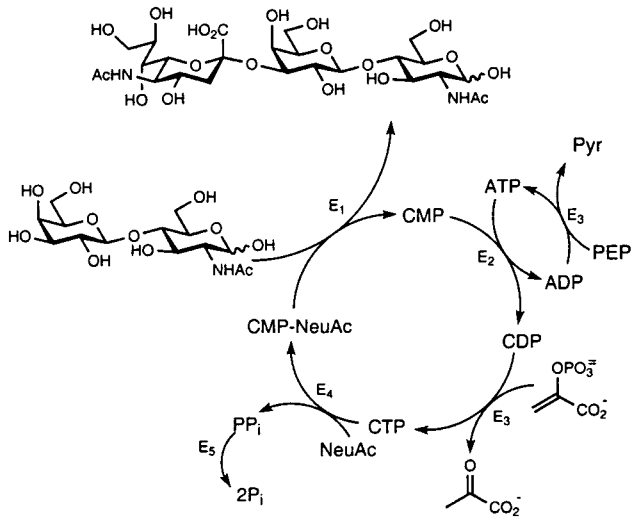
Since β-galactosidases exhibit different regiospecificity depending on the enzyme source, galactosidases from many different sources (*E. coli*, bovine testes, *B. circulans*, *A. oryzae*, *P. multicolor*, *B. bifidum*, *B. singularis*, *S. solfataricus*) are often examined for the formation of a desired linkage.[33] Many β-galactosidases accept galactose[34] or xylose[35] as the acceptor sugar, and Gal-Gal or Gal-Xyl disaccharides have also served as target structures. Transfer of galactose to peptide hydroxyl groups,[36] 1-deoxynojirimycin,[37] simple alcohols,[38] and hydroxycellulose[39] have also been reported. β-Galactosidase has also been utilized as a trimming agent[40] or for the construction of oligomaltose inhibitors of α-amylase hydrolysis.[41] Notably, a lipid-coated β-galactosidase catalyzed glycosylation of long-chain alcohols and protected sugars in organic or biphasic solvent systems, and the reactions proceeded with no observable competing hydrolysis.[42]

The Wang group screened a thermophilic glycosidase library (CLONEZYME) to identify a catalyst that would transfer galactose in a β-linkage to glucosamine, since β1,4-GalT failed to catalyze this transformation.[43] This disaccharide was further elaborated by use of the α1,3-GalT/UDPGE fusion enzyme to provide the α-Gal trisaccharide. α-Galactosidases from several sources (coffee bean, *A. niger*, *A. oryzae*, *P. multicolor*) have been studied with respect to substrate regiospecificity.[44] Galactosidases (α and β) have also been applied as catalysts in the glycosylation of amino acids[45] and cyclodextrin structures.[46]

2. Sialic Acid

a. Sialyltransferases (SiaT). The most greatly utilized sialyltransferases for synthetic purposes are α2,3-SiaT and α2,6-SiaT,[47] although polysialyltransferases are beginning to find synthetic applications. Substrate specificity of α2,3-SiaT[48] and α2,6-SiaT[49] have been studied, in some cases with modified CMP-NeuAc donors.[50] A multienzyme recycling system for CMP-NeuAc[51] that functions with either α2,3-SiaT or α2,6-SiaT has been utilized for large-scale synthesis of the important cell-surface recognition elements 3′- and 6′-sialyl-lactosamine (3′- or 6′-SLN, Scheme 11).[52] The fusion enzyme CMP-NeuAc syn-

Scheme 11. Recycling of CMP−NeuAc[a] for the Synthesis of 3′-SLN[52]



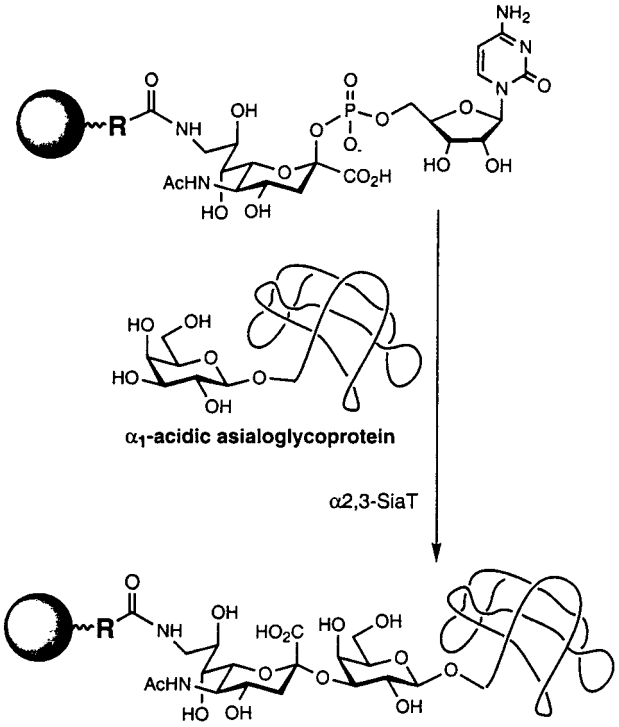
[a] E_1 = α2,3-SiaT, E_2 = nucleoside monophosphate kinase, E_3 = pyruvate kinase, E_4 = CMP−NeuAc synthetase, E_5 = pyrophosphatase.

thetase/α2,3-SiaT has recently been incorporated into this regeneration protocol.[53] This enzyme construct exhibited greater solubility than α2,3-SiaT alone and functioned efficiently at pH 7.5, even though the optimal pH values for α2,3-SiaT (pH 6.0) and CMP-NeuAc synthetase (pH 8.5) differ significantly. Furthermore, several strains of metabolically engineered bacteria have been employed in the large-scale production of CMP-NeuAc and sialylated oligosaccharides.[52b]

Sialyltransferases have been employed to append sialic acid to the terminal galactose residue in an oligosaccharide chain[54] or for the addition of sialic acid to glycolipids[55] or glycoproteins.[56] A mild method to immobilize glycoproteins through reaction of SiaT with solid-support bound CMP-NeuAc has recently been reported (Scheme 12).[57] Analogues of sialic acid, KdN and KDO,[58] are also of biological interest, and a bacterial α2,6-SiaT that is capable of KdN transfer has recently been utilized for the synthesis of KdN trisaccharides.[59]

Polysialyl structures are characteristic features of the capsular polysaccharides of invasive bacteria. The α2,8- and α2,8/2,9-polySiaT's have been characterized with respect to glycolipid substrate specificity,[60] and the resulting glycolipids may be applicable for the induction of CD_1-mediated immune response.[61] Vac-

Scheme 12. Glycoprotein Immobilization through Reaction of α2,3-SiaT with Solid-Support Linked CMP−NeuAc[57]



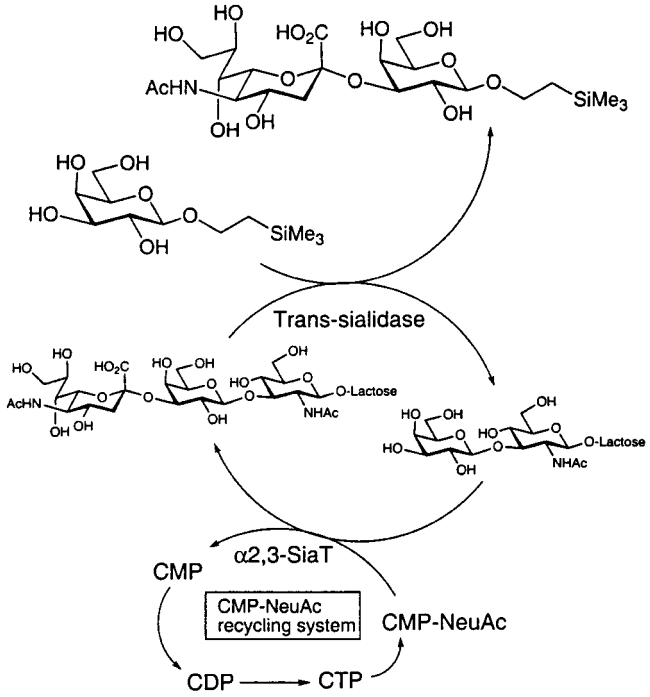
cination against these structures may aid in eradication of pathogenic bacteria from the body through targeting of their carbohydrate coat.

b. Sialidases. Synthesis employing sialidases has not been as economical as that of other glycosidases. For example, reverse hydrolysis using free NeuAc as glycosyl donor with *A. ureafaciens* sialidase gave very low product yields.[62] Although kinetically controlled synthetic conditions with sialidase from *V. cholerae* resulted in higher yields, low regioselectivity was observed.[63] More recently, Ajisaka et al. analyzed sialidases from various sources (*C. perfringens*, *A. ureafaciens*, *V. cholerae*) for synthetic purposes, examining NeuAc-pNP, NeuAc(α2,8)NeuAc, or colominic acid as the glycosyl donor.[33] In contrast to hydrolytic enzymes, *trans*-sialidases catalyze *trans*-glycosylation reactions in vivo and characteristically form very few hydrolysis products when employed synthetically in vitro. The combination of the *trans*-sialidase from *T. cruzi* with an α2,3-SiaT recycling system has been successfully applied to the synthesis of complex sialosides (Scheme 13).[64] *N*-Glycan specificity of this *trans*-sialidase has also been studied.[65] In general, yields were elevated compared with exo-sialidases; however, the donor substrate in this case was also expensive.

3. Fucose

a. Fucosyltransferases (FucT). The L-sugar fucose is an important recognition component of cell-surface glycans. Several human fucosyltransferase isozymes have been characterized, including at least seven α1,3-FucT's. Substrate specificity studies have been carried out on FucT III,[66] FucT VI,[66a,b,67] FucT

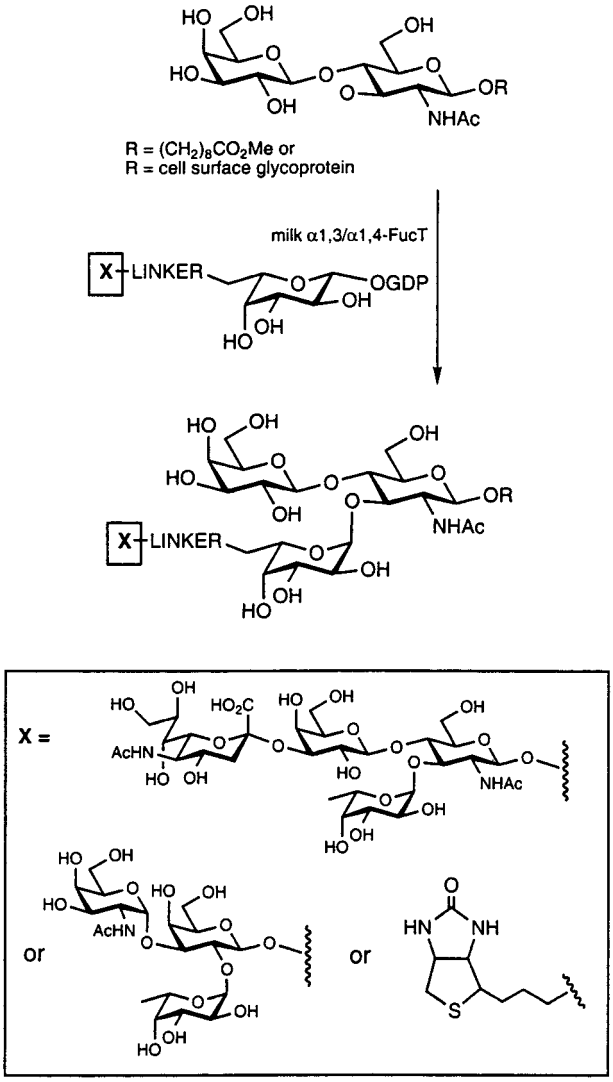
Scheme 13. Combination of α2,3-SiaT and *trans*-Sialidase from *T. cruzi* for the One-Pot Synthesis of Novel Sialosides[64]



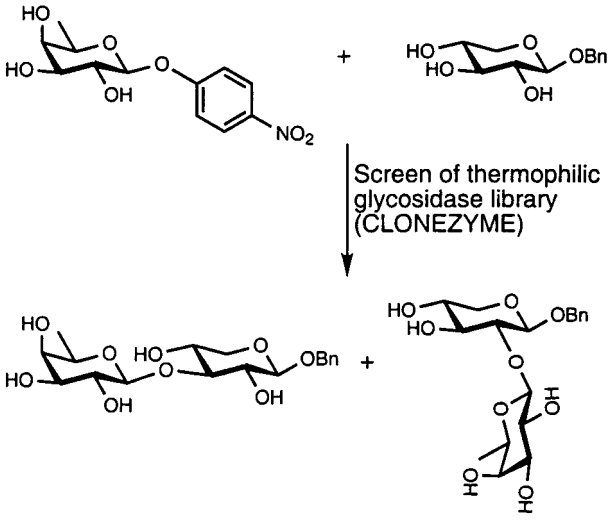
V,[68] and the poly(LacNAc) specificity of FucT IV and FucT VII.[69] Fucosyltransferases have been utilized extensively for the preparation of Lewis x (Le^x) and Lewis a (Le^a) derivatives as well as their sialylated versions.[70,71] As L-fucose is equivalent to 6-deoxy-L-galactose, it is not surprising that L-galactosyltransferase accepts GDP-L-fucose as a substrate.[72] Likewise, α1,3/1,4 human milk FucT can transfer L-galactose from GDP-L-galactose.[73] In fact, the human milk FucT is very permissive with respect to its substrate specificity. This enzyme is capable of fucosyl transfer to tertiary hydroxyl groups on unnatural methylated derivatives of GlcNAc,[74] is active in the presence of sulfation,[75] and even allows extensive modifications to the 6-position of the fucose ring (Scheme 14). Tolerated additions include long alkyl chains,[76] biotinylated linkers,[77] and even linkers terminating in oligosaccharides such as human blood group B[78] and sulfo and sialyl Le^x.[79] This finding has been exploited for the modification of glycoproteins on erythrocytes and CHO cells.

b. Fucosidases. Fucosidases from various sources (*A. niger*, *Corynebacterium* sp., *Amullaria*, porcine liver, *Fussarium oxysporum*, bovine liver and kidney) have been investigated for the ability to transfer fucose to galactose, glucose, and GlcNAc.[80] Those from *A. niger*, *P. multicolor*, and bovine kidney have been found to catalyze the addition of fucose in an α1,3-linkage to GlcNAc under kinetic conditions.[81,31j] *C*-Fucoside affinity chromatography has been employed for the isolation of a porcine liver fucosidase that catalyzes the formation of α1,2- and α1,6-linkages.[82] In contrast, the β-glycosidase from *S. solfataricus* transfers fucose in a β-linkage.[38b] Notably, the Wang group has screened a library of thermophilic glycosidases for a Fuc(β1,2)Xyl-specific enzyme (Scheme 15).[83] Catalyst screening represents

Scheme 14. Utilization of Milk α1,3/α1,4-FucT for the Transfer of Complex Fucosyl Derivatives[77−79]



Scheme 15. Screening of a Thermophilic Glycosidase Library for Discovery of a Fuc(β1,2)Xyl-Specific Enzyme[83]

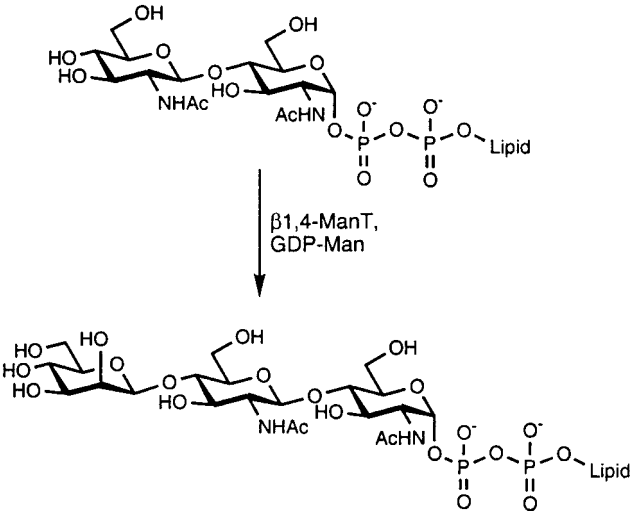


a new strategy in glycosidase-based synthesis. Instead of focusing on the reactions catalyzed by a single glycosidase, several glycosidases are screened for the potential to catalyze formation of a specific linkage.

4. Mannose

a. Mannosyltransferases (ManT). Certain mannosyltransferases have been utilized for synthetic purposes, although very few are readily available. Recombinant β1,4-ManT has been overexpressed and employed in the synthesis of the core dolichol-conjugated trisaccharide of *N*-linked glycans.[84] Various phytanyl and lauryl substitutions of the lipid have also been analyzed (Scheme 16).[85a] β1,4-ManT

Scheme 16. β1,4-Mannosyl Transfer to Lipid-Linked Chitobiose Structures[85]



is especially valuable synthetically as β-mannosyl linkages are exceedingly difficult to form chemically. This was exemplified in the synthesis of the bacterial O antigen of *Salmonella* serogroup E_1.[85b] Furthermore, a recombinant α1,2-ManT has been overexpressed for use in a GDP-mannose recycling system. Several mannosyl conjugates were generated by this strategy, including mannosyl-glycopeptides.[86] This methodology was further extended to the use of whole cells as the source of α1,2-ManT.[87]

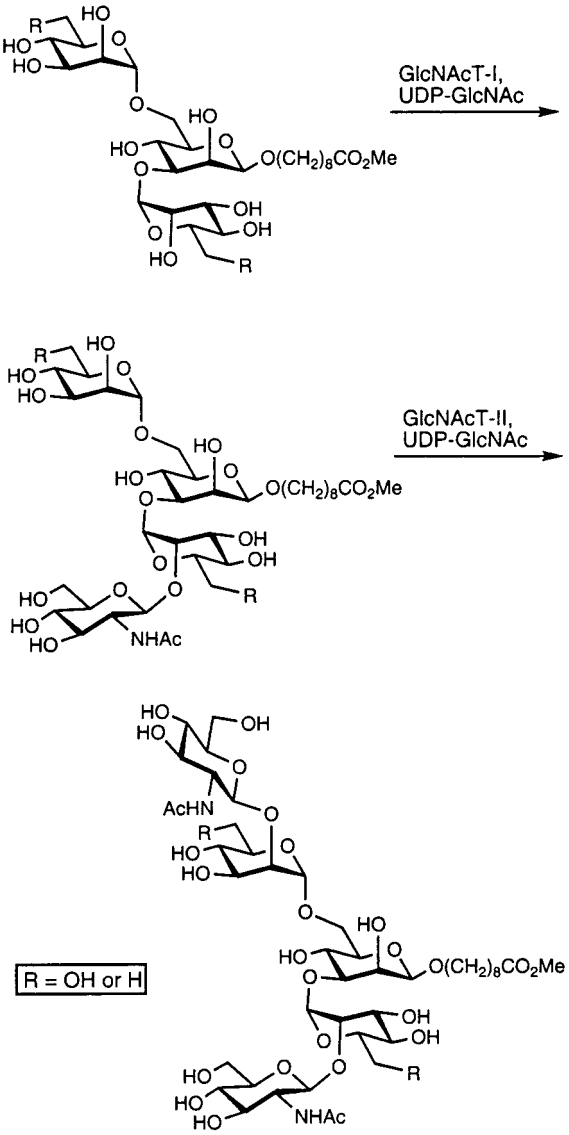
b. Mannosidases. Both α- and β-mannosidases have been applied to synthesis.[38a,44e] The α-mannosidase from *A. niger* characteristically produces mixtures of mannosyl-oligomers.[88] Optimization of separation procedures, however, allowed facile separation of the regioisomeric product compounds. The α-mannosidase from jack bean has been utilized to transfer mannose onto cyclodextrin rings.[89] Notably, the α1,2-mannosidase from *A. phoenicis* was found to produce mannobiose and mannotriose structures with absolute regiospecificity under equilibrium conditions, a rare trait in glycosidases.[90] Furthermore, Thiem and co-workers discovered that β-galactosidase from *A. oryzae* as well as the β-mannosidase from *snail viscera* can catalyze the formation of β-mannosides under kinetic conditions.[91]

5. N-Acetylhexosamines

a. *N*-Acetylhexosaminyltransferases (GlcNAcT, GalNAcT). Since most *N*-acetylhexosaminyltrans-

ferases are not commercially available, studies have been limited. *N*-Acetylglucosaminyltransferases (GlcNAcT) are generally involved in the elaboration of *N*-linked glycans on glycoproteins. As such, many studies have elucidated the specificity of GlcNAcT isozymes (I−IV) for branched trimannosyl core structures,[92] including the evaluation of deoxy substrates (Scheme 17).[93] GlcNAcT I has been employed to

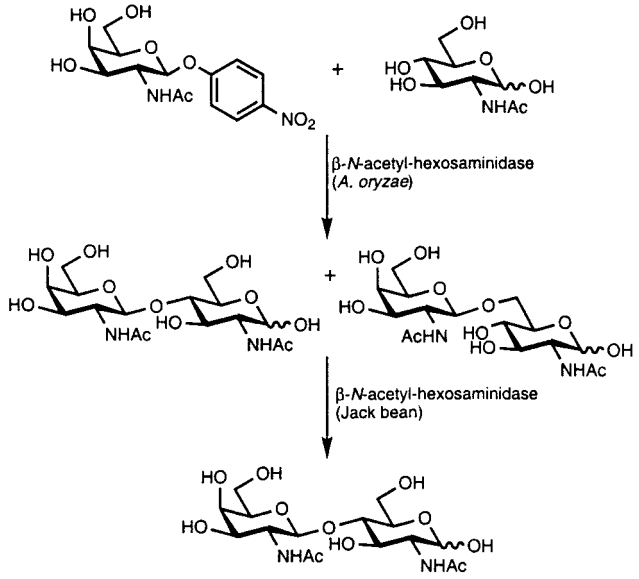
Scheme 17. Specificity of GlcNAcT Isozymes I and II on a Branched Tri-Mannose Core[93]



transfer GlcNAc in a β1,2-linkage to mannose-based trisaccharides.[94] Some modifications to the nucleotide sugar donor are tolerated, as the core 2 GlcNAcT can use *N*-trifluoroacetyl-glucosaminyl-UDP as a substrate.[95] The *N. meningitidis* GlcNAcT can employ either UDP-GlcNAc or UDP-GalNAc as donor substrate, and its acceptor specificity has recently been investigated.[96] A β1,3-GalNAcT has been studied with respect to the role of sulfation on chondroitan sulfate oligomers,[97] and an α1,3-GalNAcT from porcine liver has been utilized for the synthesis of the blood group A trisaccharide.[98] A novel chemoenzymatic synthesis of donor substrate UDP-GalNAc has also recently been published.[99]

b. *N*-Acetylhexosaminidases. As many *N*-acetylhexosaminyltransferases are not commercially available, hexosaminidases offer an alternative synthetic approach.[38a,100] Crout et al. have undertaken many studies on the β-*N*-acetyl-hexosaminidase from *A. oryzae*. This enzyme transfers GlcNAc or GalNAc to monosaccharides under kinetically controlled conditions (Scheme 18).[101] Although mixtures of β1,4- and

Scheme 18. Tandem Use of Two β-*N*-Acetylhexosaminidases for the Synthesis of a Specific Disaccharide Product[101]



β1,6-linked products are generally formed, incubation with a second glycosidase results in hydrolysis of all but the β1,4-linked product. This enzyme has been utilized to construct various chito-oligomers[102] and glycosyl-amino acids,[103] and has been extensively studied with respect to anomeric control.[104] This glycosidase has also been applied in conjunction with β-galactosidase for the construction of trisaccharides.[105] Interestingly, in a study by Murata et al. on β-*N*-acetyl-hexosaminidase from *N. orientalis*, the ratio of product mixture regioisomers was altered when reactions were performed in the presence of α-cyclodextrin.[106]

6. Glucose

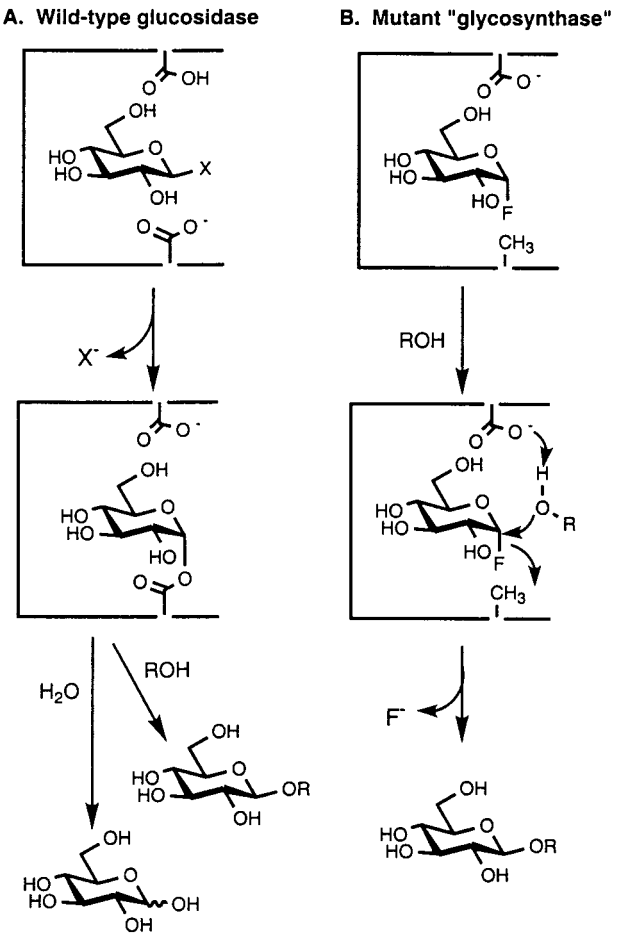
a. Glucosyltransferases (GlcT, GlcUAT). Glucuronosyltransferases have been utilized for the transfer of glucuronic acid to phenolic and steroidal substrates.[107] Similarly, Gygax et al. have reported a recycling system for UDP-GlcUA for the synthesis of GlcUA linked aglycones.[108] In this process, UDP-GlcUA was generated from UDP-Glc, a reaction catalyzed by UDP-Glc dehydrogenase. The required NAD cofactor was regenerated by the action of lactate dehydrogenase on the byproduct NADH. A regeneration system for UDP-Glc in the synthesis of sucrose and trehalose has also been described.[109] Furthermore, β1,4-GalT can reportedly be employed to transfer glucose from UDP-Glc to acceptor sugars.[110] A cyclodextrin glucosyltransferase has been shown

to form linear or branched gluco-oligomers from αCD.[111]

b. Glucosidases. Glucosidases are much more prevalently utilized in synthesis than glucosyltransferases. Several groups have reported the use of glucosidases in the synthesis of gluco-oligomers[112] or the formation of alkyl glucosides.[38,44e,113] Prade et al. reported glucosidase-catalyzed synthesis using various glycosyl donors, such as pNP-ethers, fluorides, and the unlikely glucal.[114] Even a glucosidase-labile protecting group has been published.[115] Remarkably, a solvent-free glucosidase-catalyzed synthesis, involving the use of a solid support and microwave radiation, has been investigated.[116] The reaction rate was greatly increased under these conditions; however, this procedure can be applied only to thermostable glycosidases.

Mutant glucosidases ("glycosynthases", *Agrobacterium* sp. β-glucosidase E358A, *B. lichenifomis* β1,3/1,4-glucanase E134A) have been engineered by two groups (Scheme 19). These enzymes lack a catalytic

Scheme 19. Mechanistic Comparison of Wild-Type Glucosidases and Engineered Mutant "Glyco-Synthases"[117]



nucleophile in the active site and are utilized in conjunction with activated glycosyl donors of the opposite configuration as the desired product. They can synthesize but not hydrolyze the product, thus solving one of the major problems encountered in most glycosidase-based syntheses.[117]

B. Transfer of Oligosaccharides

1. endo-Glycosidases

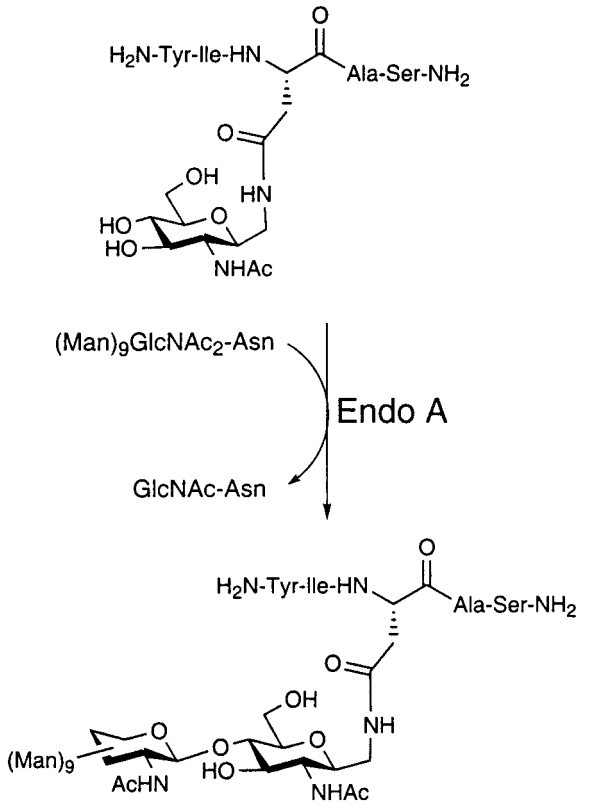
endo-Glycosidases cleave internal glycosidic linkages, generally at a position between two sugars. For example, the endo-glycosidases Endo-A (*A. protophormiae*), Endo-M (*M. hiemalis*), and Endo-F (*F. meningosepticum*) have long been applied to the study of *N*-linked glycan chains of glycoproteins as they cleave the bond between the chitobiose disaccharide. The first reports that endoglycosidases could be employed for synthetic purposes emerged over a decade ago. Endo-F was shown to synthesize *trans*-glycosylation products with high mannose *N*-glycans,[118] and an endo-*N*-acetyl-galactosaminidase from *Diplococcus pneumoniae* was characterized for acceptor specificity.[119] Until recently, the synthetic utility of endo-glycosidases has not been fully realized.

N-Linked glycans are generally categorized as either high-mannose, hybrid, or complex, although some consider poly(LacNAc) linked chains a fourth category. Some endo-glycosidases exhibit stringent substrate specificity, while others accept a broad range of glycan substrates. To date, two of these enzymes have been used largely for synthetic purposes. Endo-A recognizes only high-mannose chains, while Endo-M accepts complex and hybrid glycan chains as well. Endo-A has been examined in terms of acceptor specificity and stability to the addition of organic cosolvent.[120] Furthermore, Endo-A *trans*-glycosylation was utilized to prepare a polyacrylamide conjugate which was subsequently copolymerized to afford a neoglycoconjugate.[121] The synthesis of glycopeptides[122] and unnatural glycopeptide analogues[123] have been undertaken as well. For example, a *C*-linked glycopeptide prepared by Endo-A *trans*-glycosylation was shown to be a potent inhibitor of broad-spectrum glycoamidases (Scheme 20).[124] In this case, the insertion of a single methylene group between the glycan and peptide conferred resistance to *N*-glycanase-based hydrolysis.

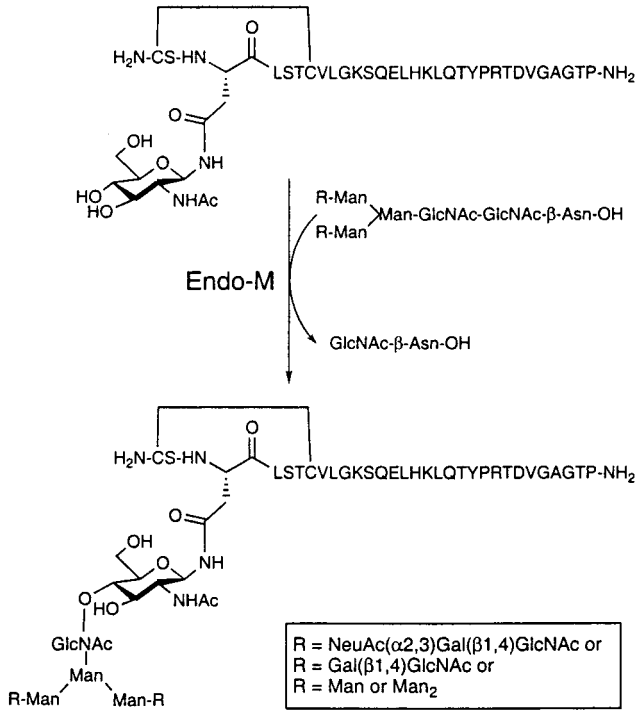
Endo-M, in contrast, has been employed in the synthesis of glycopeptides containing high mannose, complex sialoglycans, and complex asialoglycans (Scheme 21).[125] Glycosylation of calcitonen, which does not naturally contain sugar chains, was accomplished by this method. This allowed structural and functional consequences of glycosylation on biological activity to be assessed. Moreover, Endo-M has been utilized to transfer *N*-glycans onto β-cyclodextrins.[126]

Ceramide glycanase is an endo-glycosidase which cleaves linkages between the carbohydrate and lipid tail of glycolipids. Utilizing this enzyme under *trans*-glycosylation conditions, glycolipid analogues of ganglioside GM_1 and fluorogenic substrates for spectral assay have been prepared.[127] While the oligosaccharyltransferase (OST) responsible for the transfer of dolichol-linked glycans to Asn of a nascent polypeptide chain is not readily available, its substrate specificity has been investigated, albeit to a limited extent.[128]

Scheme 20. Synthesis of a *C*-Linked High-Mannose Glycopeptide by Endo-A *trans*-Glycosylation[124]



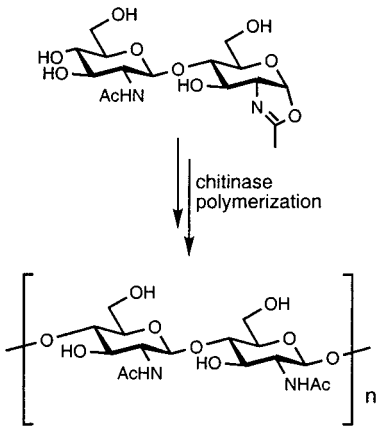
Scheme 21. Synthesis of Glycosylated Derivatives of Calcitonen by Endo-M Transglycosylation[125]



2. Polymerizing Enzymes

Enzymes that hydrolyze glycopolymers in vivo have also been applied to the synthesis of well-defined oligosaccharides in vitro.[129] Chitinase has been utilized to synthesize GlcNAc oligomers from chitobiose.[130] Lysozyme has also been shown to catalyze this transformation on a protected chitotriose.[131] Interestingly, Kobayashi used an oxazoline transition state analogue of the chitin hydrolysis reaction as the monomer substrate for chitinase-catalyzed polymerization (Scheme 22). The premise for this study was

Scheme 22. Utilization of an Oxazoline Transition-State Analog as the Monomer for Chitinase Polymerization[132]



that a distorted glycosyl donor would approximate the hydrolytic transition state structure and would thereby lower the activation barrier for the enzymatic transformation. Glycosylation could then occur at pH values where hydrolysis was unfavorable. In keeping with their theory, chitin polymerization was accomplished at pH 10.6 without the observation of hydrolysis products when employing this oxazoline donor.[132] Cellulase polymerization of lactosyl fluorides and nonglucosidic sugars has also been reported.[133]

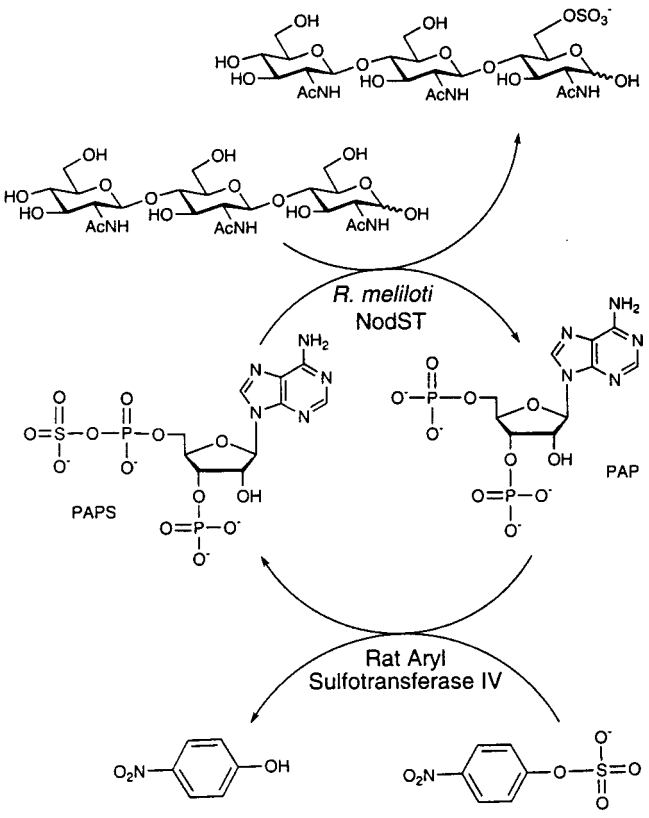
IV. Enzymes that Do Not Form Glycosidic Linkages

Enzymes that are not involved in the formation of glycosidic linkages are also of interest in complex glycoconjugate synthesis. Relevant post-translational modifications of oligosaccharides and glycopeptides often provide a required biological recognition element. Enzymes have also had great utility as protecting group removing agents or in the synthesis of glycopeptides, glycoproteins, glycolipids, and unnatural sugar structures.

A. Sulfotransferases

Sulfation on either the hydroxyl group of an oligosaccharide or the peptide to which it is attached is an important post-translational modification that has not yet been studied in great detail. However, a few recent reports have advanced the field toward this end. Lin et al. developed a six-enzyme recycling system for the regeneration of PAPS, the universal biological donor of sulfate. This regeneration cycle was employed in the sulfation of chito- and LacNAc-based oligomers.[134] A simpler two-enzyme recycling system which utilizes an arylsulfotransferase for the same purpose has also been described (Scheme 23).[135] The substrate specificity of a GlcNAc 6-sulfotransferase potentially involved in the synthesis of Gly-

Scheme 23. Two-Enzyme PAPS Regeneration System for the Synthesis of Sulfated Chito-Oligomers[135]



CAM-1, an L-selectin ligand, has also been explored.[136] Furthermore, the tyrosylprotein-sulfotransferase responsible for the sulfation of the N-terminus of glycoprotein PSGL-1 has been studied to some extent and is expected to be of great interest in the future.[137] Other sulfotransferases that add sulfate to carbohydrates or peptides have been discovered but largely have not been employed for synthetic purposes.[138]

B. Proteases

Proteases have dual function in the synthesis of glycoconjugates. These enzymes can be utilized for the formation of peptide bonds of glycopeptides or as selective acylating agents. Subtilisin has been a popular choice among enzyme engineers for mutational analysis. The Wells group reported the use of an engineered variant of subtilisin BPN′ (subtiligase) for the synthesis of RNase A with unnatural catalytic residues.[139] Furthermore, the generation of thermostable variants of subtilisin 8397 has led to enzymes with altered stability of broadened substrate specificity.[140] Subtilisin BPN′ has been explored as a catalyst for ligation of unnatural peptide-based structures.[141] The ability to catalyze peptide-bond formation with the glycosylated amino acid occupying different subsites of subtilisin BPN′ was analyzed,[142] and a useful strategy for the synthesis of glycoproteins based on a combination of enzymatic glycopeptide coupling and glycosyltransferase reactions was developed (Scheme 24).[143]

Subtilisin has been utilized both for selective ester hydrolysis[144] and for the esterification of oligosaccharides[145] and glycolipids. It was employed for the acylation of the antigenic 9-*O*-acetyl-GD_3 ganglioside, which is currently a potential cancer vaccine candidate (Scheme 25).[146] The protease papain has been used for deprotection of the C-terminal esters of glycopeptides.[147]

C. Lipases

Lipases have been applied to the selective acylation or hydrolysis of esters at specific positions on a glycoconjugate.[148] This class of enzymes has been particularly important in the synthesis of glycopeptides, as lipase reactions proceed under essentially neutral pH conditions which are compatible with acid- or base-labile glycosidic linkages. Deprotection problems generally associated with traditional protecting groups are thereby overcome. Several lipase-labile esters have been described for glycopeptide synthesis,[149] including heptyl,[150] MEM,[151] PEG,[151] and phenoxyacyl.[152] Butyrylcholine esterase was especially useful for the deprotection of phosphorylated glycopeptides.[153]

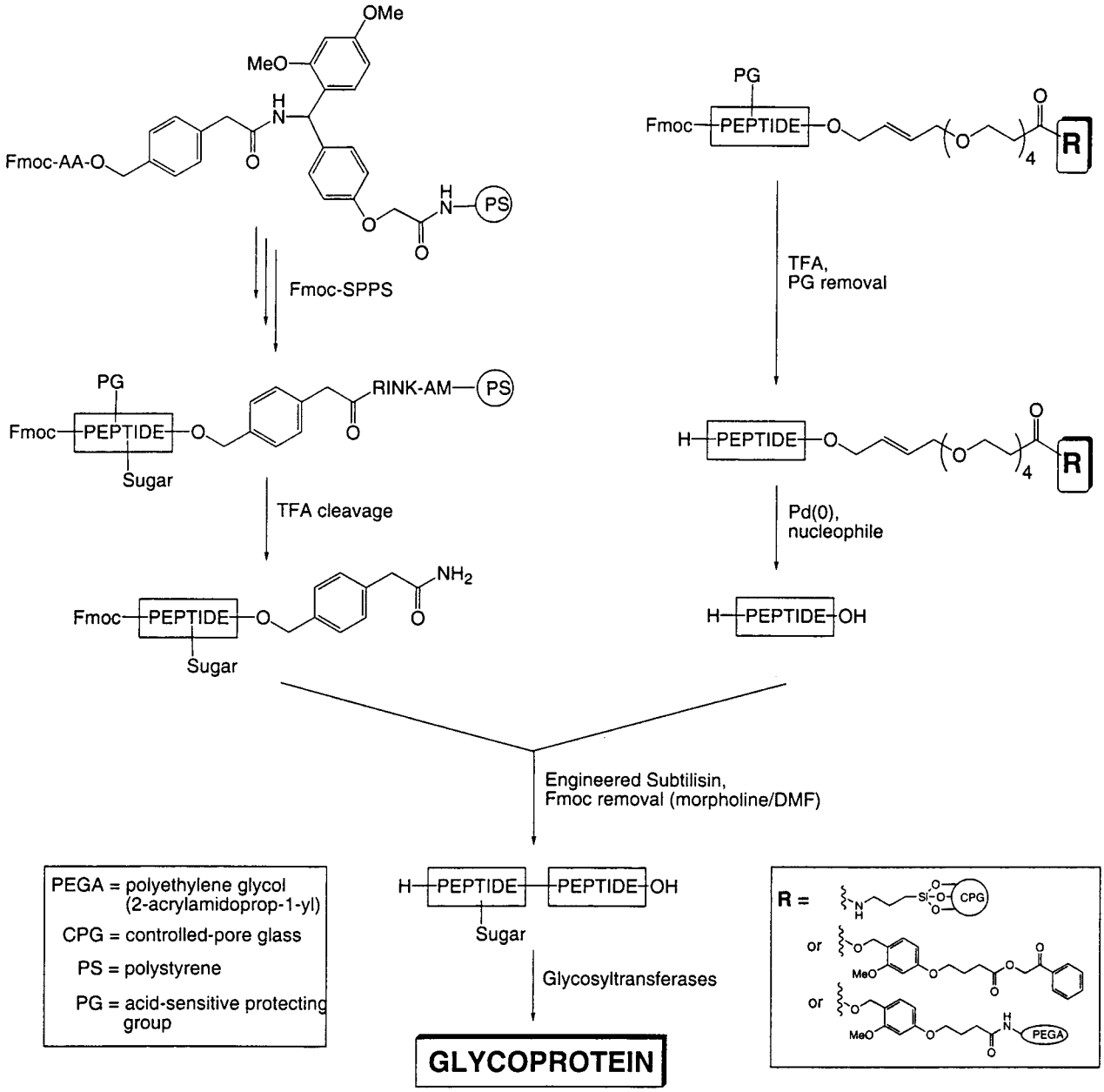
D. Aldolases

Aldolases have proven useful in the formation of monosaccharides and their derivatives, and synthetic applications have been reviewed extensively.[154] DHAP (dihydroxyacetone phosphate)-dependent aldolases have been utilized for the synthesis of carbohydrates containing ^{13}C labels, heteroatoms, and deoxygenated sites.[4b,8f] The condensation of DHAP with pentoses or hexoses affords NeuAc and KDO analogues.[155] Iminocyclitols and L-sugars can also be readily obtained analogously (Scheme 26).

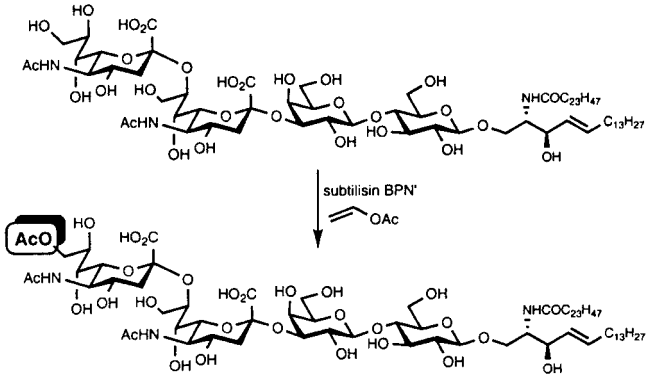
NeuAc aldolase catalyzes the condensation of ManNAc and pyruvate to give NeuAc. As such, the use of NeuAc aldolase has allowed the preparation of various NeuAc derivatives, including aza-sugars.[156] Unlike other aldolase reactions, the NeuAc aldolase reaction is a substrate-control process, i.e. using L-sugars as substrates, L-NeuAc derivatives are obtained. Using the technique of directed evolution, D-KDPG aldolase has been altered to a KDG aldolase which accepts both L- and D-glyceraldehyde as substrate with the same facial selectivity, thus extending the scope of enzymatic aldol addition reactions.[157] Similarly, KDO aldolase is also pyruvate-dependent and has been characterized with regard to substrate specificity.

DERA is an acetaldehyde-dependent aldolase that catalyzes the condensation of two aldehydes. DERA-catalyzed reactions have provided iminocyclitols, among other structures, and the tandem use of DERA and NeuAc aldolase affords deoxy NeuAc derivatives (Scheme 27). Due to the incompatible nature of the two aldolase reactions, however, these enzymes cannot be combined in a one-pot synthesis.[158]

Scheme 24. Combination of Subtilisin and Glycosyltransferases for the Synthesis of Homogeneous Glycoproteins[143]



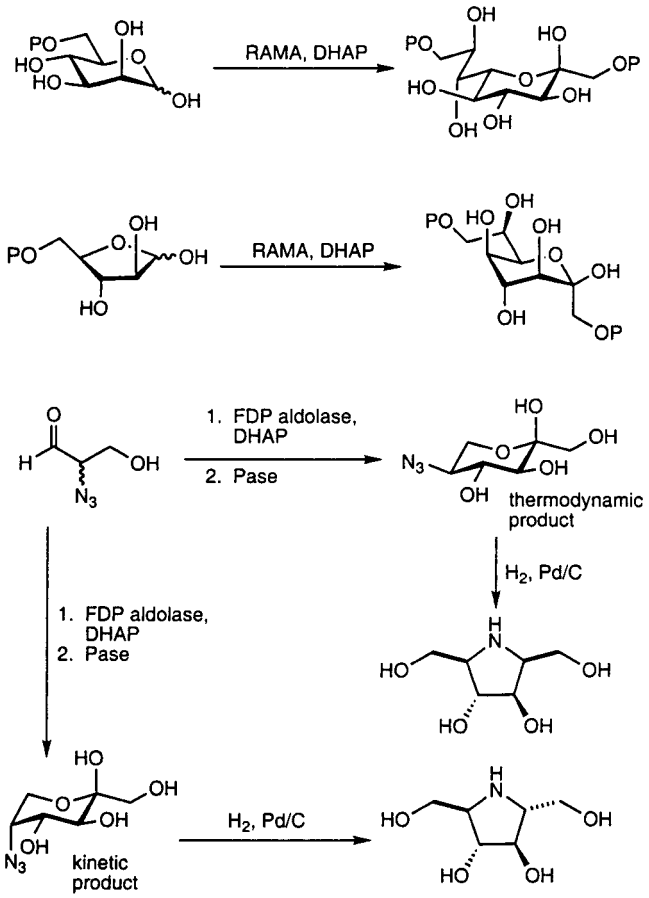
Scheme 25. Subtilisin-Based Acylation for Production of 9-*O*-acetyl-GD$_3$[146]



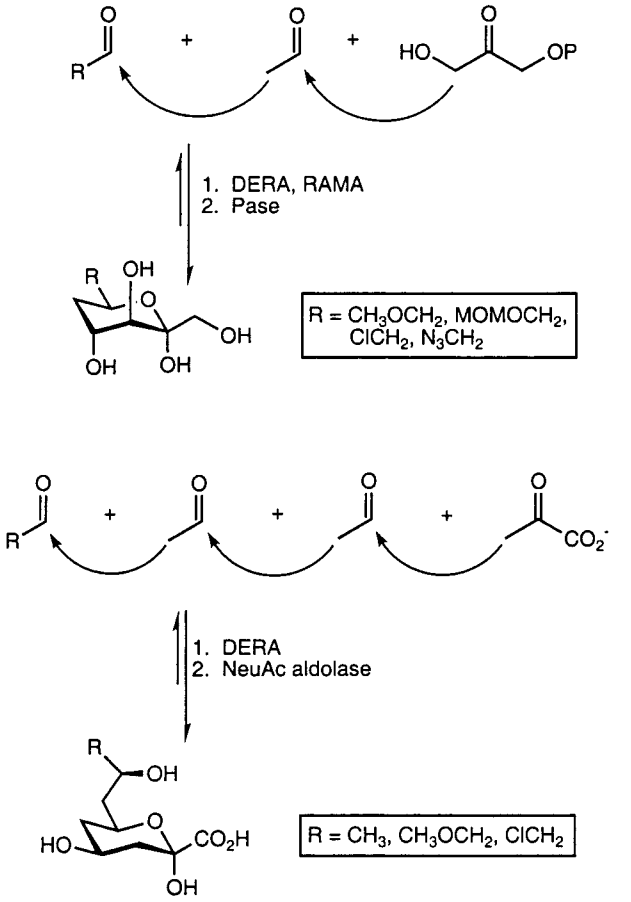
E. Other Enzymes

Other enzymes with interesting activity have found applications in glycoconjugate synthesis as well. A new strategy toward glycoprotein synthesis involves intein-catalyzed peptide ligation, a technique which arose through studies of protein-splicing mechanisms. This method has allowed the preparation of a novel maltose-binding protein with GlcNAc appended to the C-terminus (Scheme 28).[159] This construct was shown to be a substrate for β1,4-GalT and could therefore potentially be further elaborated with other glycosyltransferases to provide complex homogeneous glycoproteins. An alternative strategy employs galactose oxidase as a catalyst in the formation of 6-oxo-galactose structures. The Bertozzi group exploited this enzyme for the synthesis of unnatural glycopeptide conjugates.[160] In this case, 6-oxo-GalNAc-linked peptides were selectively coupled to oxime-functionalized sugars to give novel glycoconjugates that contained the natural GalNAc-α-*O*-Thr linkage.

Scheme 26. Synthesis of Unnatural Carbohydrate Derivatives and Iminocyclitols Employing DHAP-Dependent Aldolases[155]



Scheme 27. Combination of DERA with DHAP- or Pyruvate-Dependent Aldolases for the Synthesis of Deoxy-NeuAc Derivatives[158]



V. Use of Multiple Enzymes for Complex Glycoconjugate Synthesis

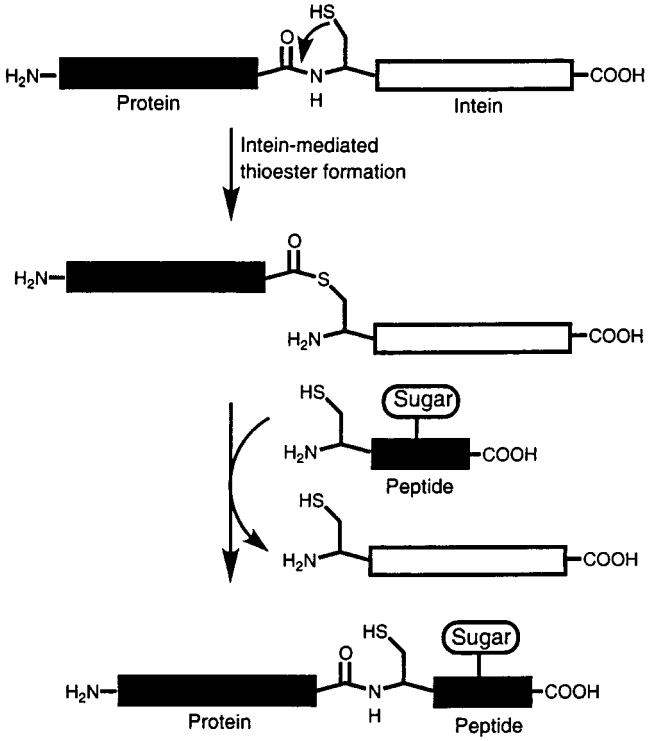
A. Sequential Transfer

1. Multiple Glycosyltransferases

As glycosyltransferases exhibit unique linkage specificity, it has been possible to plan complex syntheses that utilize several of these catalysts in sequence. Of great interest have been sialyl lactosamine (SLN) structures which appear on cell-surface glycolipids and glycoproteins. Utilizing β1,4-GalT and either α2,6-SiaT or α2,3-SiaT in sequence readily provides these epitopes. The SLN trisaccharide and analogues have been enzymatically synthesized in solution,[161] on solid support,[162] attached to N-linked glycopeptides,[163] on glycosphingolipids,[5,164] and even on branched Asn-linked undecasaccharides.[165] Although several studies of glycopeptide structure have been undertaken,[166] it remains difficult to assess the effect of glycosylation on glycoprotein structure. Notably, Miyazaki et al. utilized β1,4-GalT and α2,6-SiaT in sequence to transfer [^{13}C]-Gal and [^{13}C]9-Fluoro-NeuAc to the surface of hen ovalbumin for the purpose of NMR structural analysis (Scheme 29).[167]

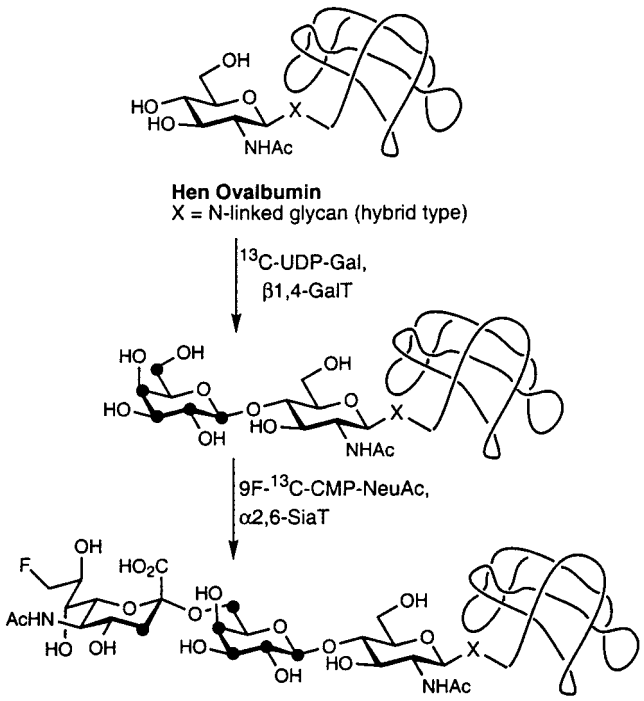
Another carbohydrate structure that has attracted attention is the sialyl Lewis x tetrasaccharide (sLe^x).

Scheme 28. Intein-Catalyzed Synthesis of Homogeneous Glycoproteins[159]



This structure functions as the minimum ligand for the selectin family of glycoproteins. Two general

Scheme 29. Addition of ^{13}C Labeled Carbohydrates to Hen Ovalbumin with β1,4-GalT and α2,6-SiaT[167]

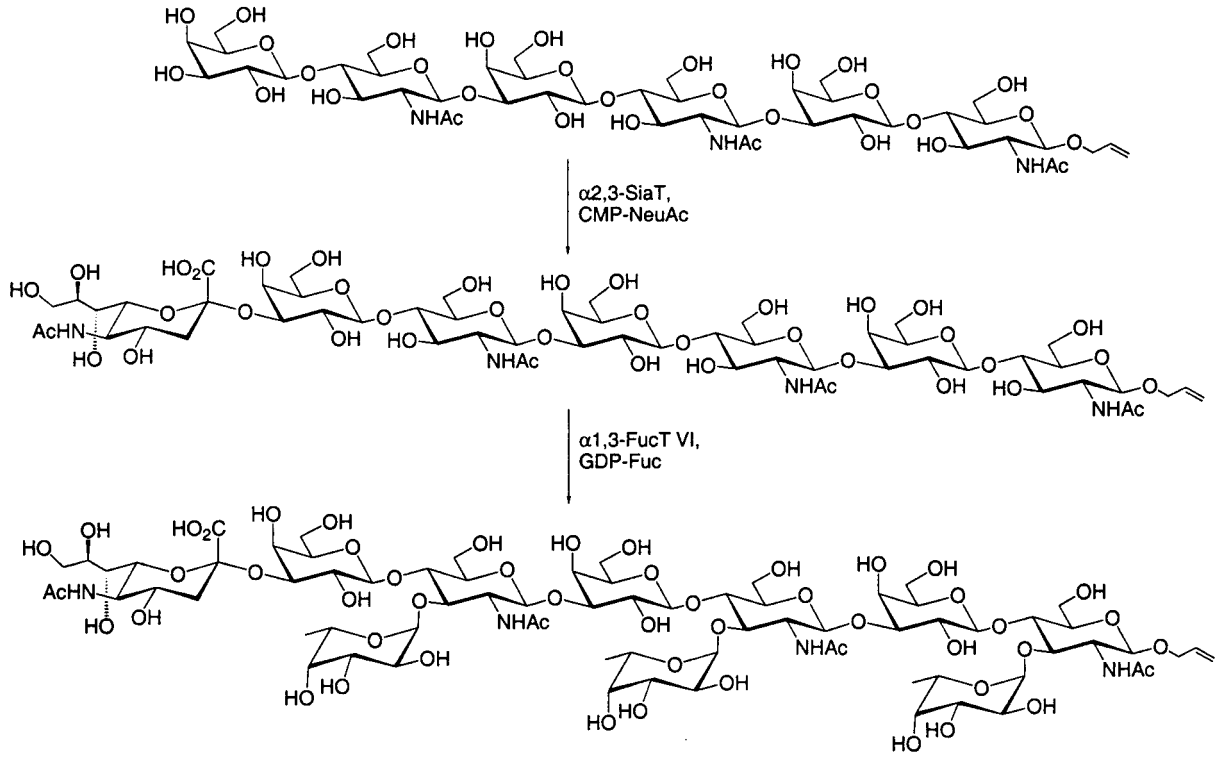


enzymatic strategies have been utilized for the synthesis of this epitope. The first involves applying α2,3-SiaT and α1,3-FucT in sequence to append NeuAc and Fuc to a preformed LacNAc structure. This series has been used in the synthesis of sLe^x dimers,[168] RGD peptide conjugates,[169] Hg-labeled sLe^x,[170] sLe^x on the termini of a trimannose core,[171] and the decasaccharide sialyl−trimeric-Lewis x.[172] The last study is notable in that FucT V and VI were found to have different specificities within the sialyl−tri-LacNAc core (Scheme 30).

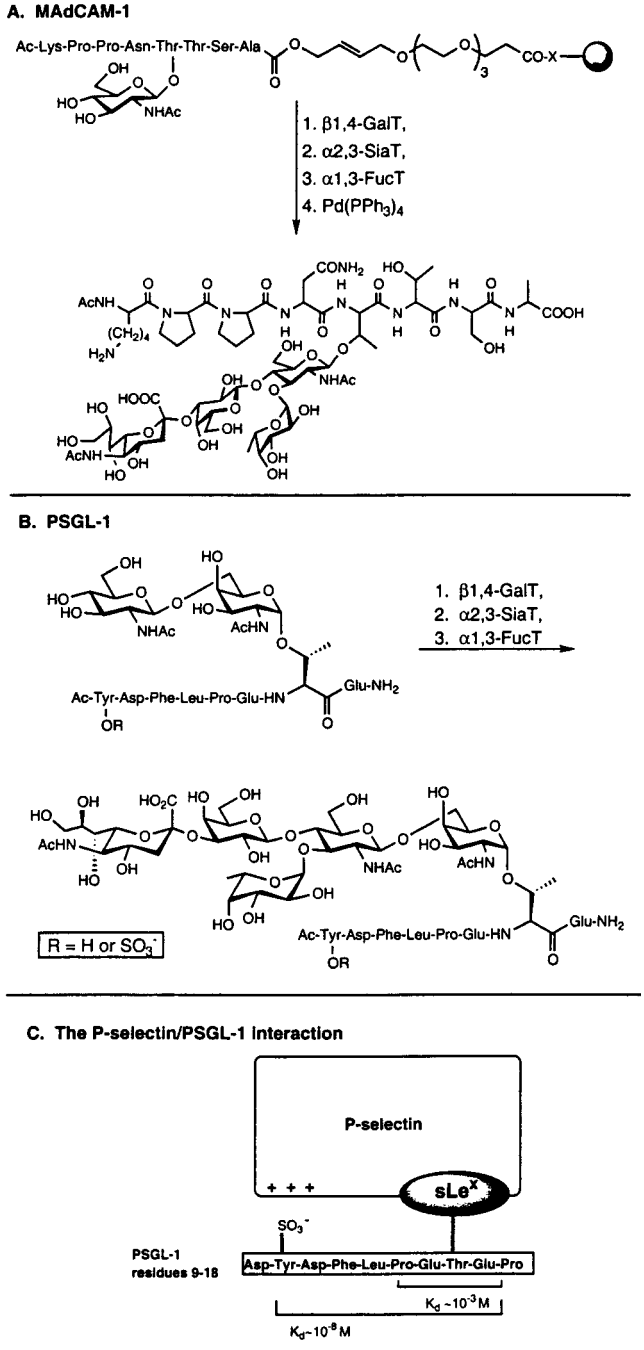
The utilization of β1,4-GalT, α2,3-SiaT, and α1,3-FucT in sequence to form the sLe^x tetrasaccharide from GlcNAc encompasses the second general approach to sLe^x synthesis. This series was first employed for the generation of sLe^x and derivatives with sugar nucleotide recycling systems for each enzyme.[173] This three-enzyme sequence has subsequently been utilized extensively. The synthesis of sLe^x on solid support,[174] in dendritic form,[175] on pseudo-glycopeptides,[176] in fluorescently labeled dimeric form,[177] and in glycoliposomes[178] has been accomplished. The utility of this enzymatic cascade in the synthesis of sLe^x glycopeptides in solid- or solution-phase synthesis[179] has been shown by the synthesis of sLe^x glycopeptides (Scheme 31) from MAdCAM-1[180] and the sulfated sLe^x glycopeptide from PSGL-1.[181] During the synthesis of a PSGL-1 sulfated glycopeptide, it was found that the Tyr-*O*-sulfate group suppresses the activity of β1,4-GalT and α2,3-SiaT involved in the biosynthesis of the *O*-glycan portion of the ligand, suggesting an important role for tyrosine sulfate in regulating the sugar−receptor interaction.

Other related complex structures have also been synthesized using several glycosyltransferases in sequence, including sLe^x on a core 2 glycan[182] and the sLe^x regioisomer sLe^a.[183] A four-enzyme sequence including the β1,6-GlcNAcT (core 2) was used for the synthesis of a branched poly(LacNAc) chain containing four sLe^x tetrasaccharide structures.[184] Alternatively, lacto-*neo*-tetraose has been constructed on a solid phase with β1,3-GlcNAcT and β1,4-GalT.[185] The synthesis of ^{13}C labeled poly(LacNAc) chains[186] and branched poly(LacNAc) have also been reported.[187]

Scheme 30. Utilization of α2,3-SiaT and α1,3-FucT VI for the Synthesis of Sialyl-Trimeric-Lewis x[172]



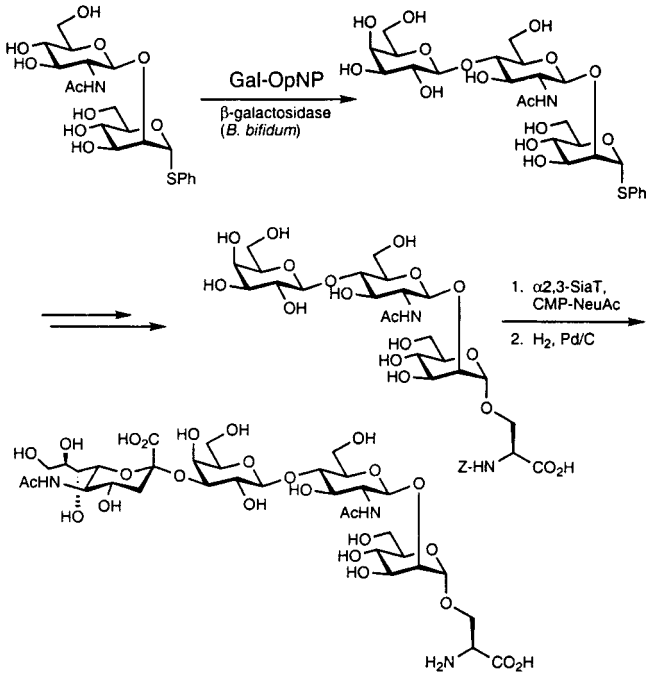
Scheme 31. (a) Solid-phase Chemoenzymatic Synthesis of Glycopeptides from MAdCAM-1.[180] (b) Solution-Phase Chemoenzymatic Synthesis of Sulfated Glycopeptides from PSGL-1.[181] (c) P-Selectin/PSGL-1 Interaction[137]



2. Glycosyltransferases in Combination with Glycosidases

Glycosyltransferases and glycosidases have been employed in synthetic sequence to provide complex target structures. Galactose-terminating oligosaccharides that were prepared with β-galactosidase have further served as substrates for glycosyltransferases,[188] in one case leading to the generation of a neoglycoprotein.[189] In an elegant example, Matsuo et al. employed β-galactosidase to form LacNAc in a β1,2-linkage to a mannosyl-thioglycoside. This construct was then chemically activated for glycosylation to serine and further elaborated with α2,3-SiaT to afford a newly discovered component of α-dystroglycan (Scheme 32).[190] This series exemplifies the efficiency of chemoenzymatic synthetic approaches. *trans*-Sialidase (from *T. cruzi*) has been used in conjunction with β1,4-GalT and α1,3-FucT to provide sLe^x-based structures.[191] In other cases, glycosidases have been used to trim away unnecessary sugars prior to or after glycosyltransferase-catalyzed reactions,[192] thus functioning as protecting group removal agents.

Scheme 32. Employment of a Glycosidase, Glycosyltransferase, and Chemical Glycosylation for the Synthesis of a Component of α-Dystroglycan[190]

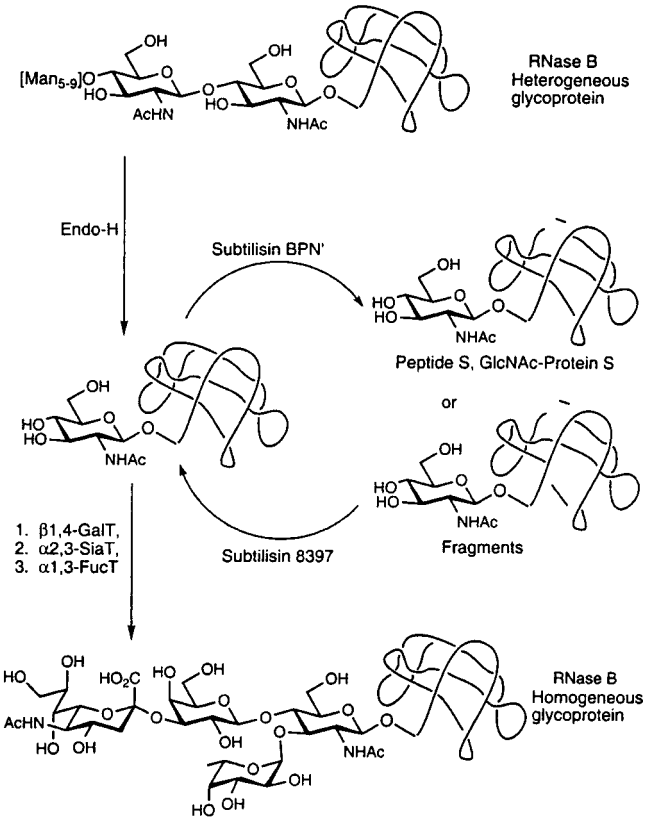


3. Glycosyltransferases in Combination with Other Enzymes

Glycosyltransferase-catalyzed reactions have also been utilized in sequence with proteases and endoglycosidases in recent efforts. The combination of β1,4-GalT and subtilisin has been employed in the preparation of LacNAc-glycopeptides.[193] Subtilisin-catalyzed cleavage and religation of glycopeptide fragments was also explored in the synthesis of a homogeneous RNase B glycoprotein. The natural RNase B *N*-linked glycan was first removed by Endo-H, leaving the reducing terminal GlcNAc on the protein surface. This handle was then elaborated with glycosyltransferases to yield an RNase B glycoprotein with a homogeneous sLe^x glycoform on its surface (Scheme 33).[143]

Ceramide glycanase is another endo-glycosidase that has recently been utilized for the synthesis of glycolipids and derivatives. Yamada et al. developed a synthetic method based on a water-soluble acrylamide polymer. This construct allowed enzymatic transfer of sialic acid to a polymer-linked glycolipid and greatly facilitated purification. *trans*-Glycosylation with ceramide glycanase then released the glycoconjugate from the polymer while simulta-

Scheme 33. Glycoprotein Remodeling with Endo-H, Subtilisin, and Glycosyltransferases[143]



neously affording the glycosyl ceramide structure, GM_3 (Scheme 34).[194]

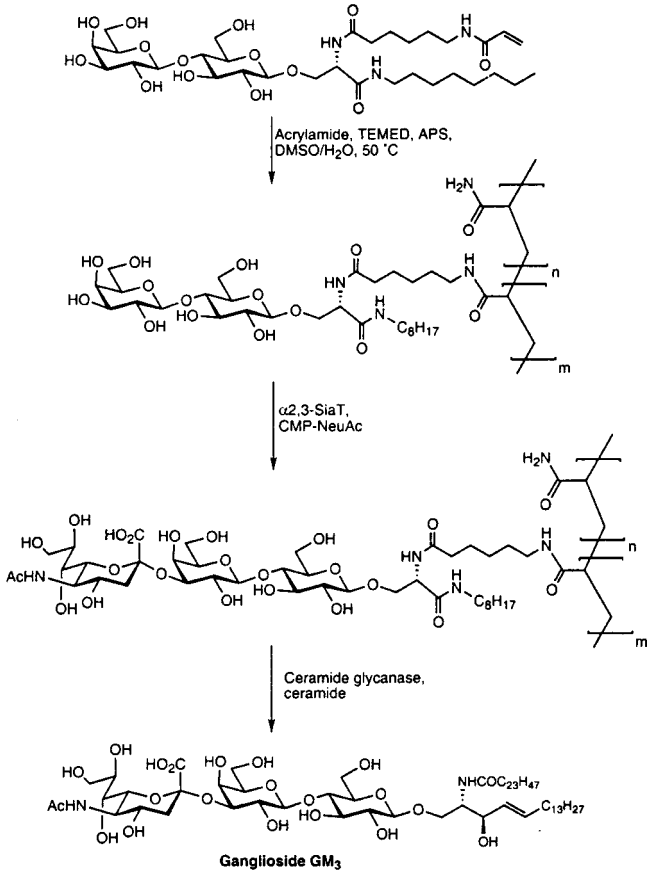
4. Multiple Glycosidases

Reaction protocols which entail the use of glycosidases in sequence have also been reported. The tandem use of *β-N*-acetylhexosaminidase (*A. oryzae*) and *β*-mannosidase (various sources) has provided the core mannose-linked chitobiose trisaccharide of *N*-linked glycans (Scheme 35).[195] α-Mannosidase (*A. niger*), *β-N*-acetyl-hexosaminidase (*B. circulans*), and *β*-galactosidase (*B. circulans*) have been applied in sequence to the synthesis of a trisaccharide common to all complex-type glycans.[196] Furthermore, tandem use of *β*- and α-galactosidases has afforded the α-Gal trisaccharide xenotransplantation epitope.[197]

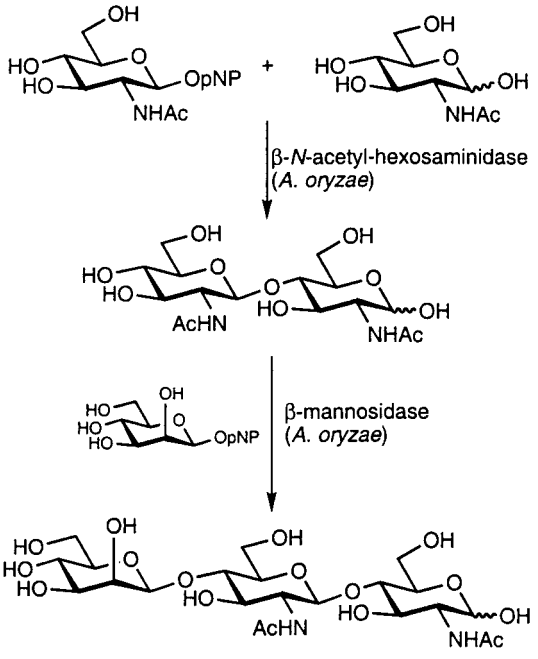
B. One-Pot Multienzyme Synthesis

A large majority of enzymes function optimally near neutral pH in aqueous solution. In many cases, conditions for one-pot glycoconjugate synthesis involving several enzymes can therefore often be employed. Recently, a four-enzyme one-pot synthesis of 5-deoxy-5-ethyl-xylulose has been reported (Scheme 36).[198] Although the enzymes employed had a range of pH optima, enzyme activities were controlled by variation of pH over the course of the reaction. Glycosyltransferase-based systems in which the regeneration of sugar nucleotides is accomplished have been reported for the synthesis of 6′-SLN (Scheme 37),[199] the α-Gal epitope,[12h,200] and a hyaluronic acid polymer.[201] A one-pot synthesis of Le^x using *β*1,4-GalT and α1,3-FucT has also been achieved.[202] Gly-

Scheme 34. Enzymatic Glycolipid Synthesis on a Water-Soluble Polymer, Culminating in Ceramide Glycanase-Catalyzed *trans*-Glycosylation[194]

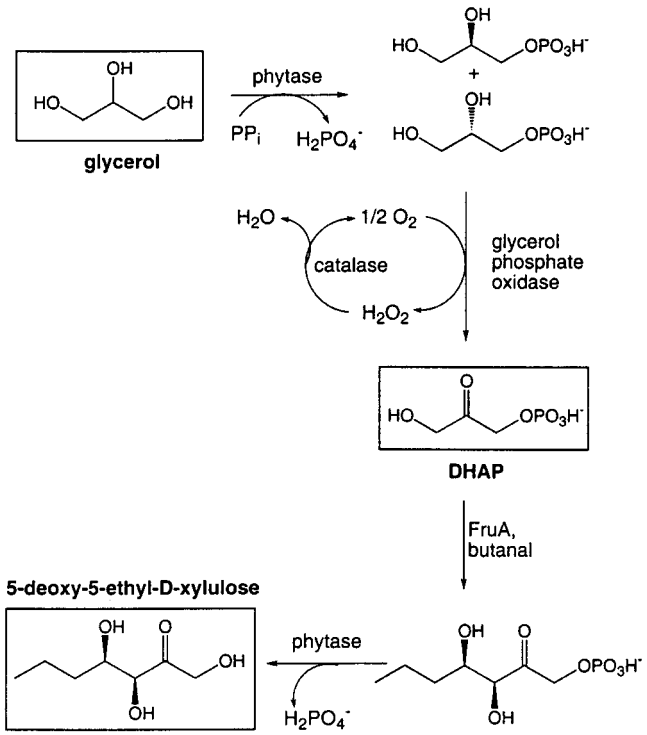


Scheme 35. Use of Multiple Glycosidases in the Synthesis of an *N*-Linked Glycan Trisaccharide[195]

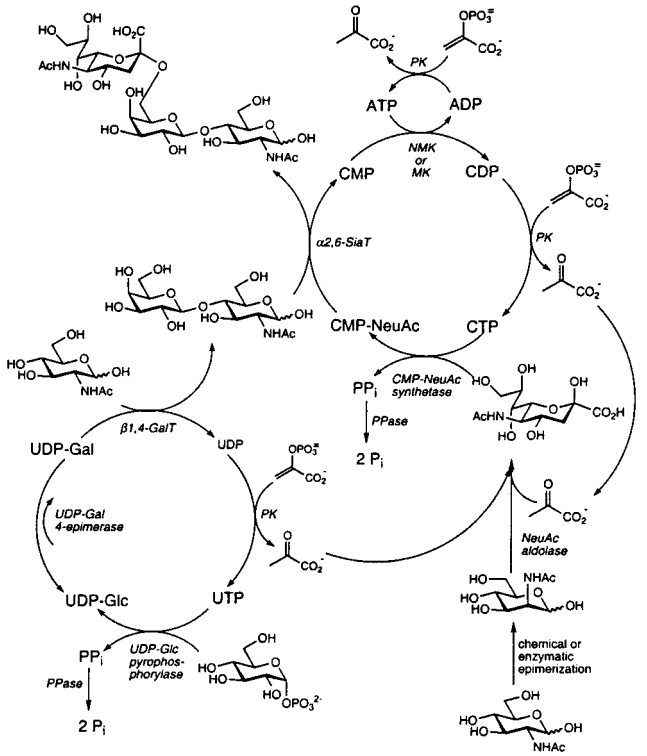


cosidases and glycosyltransferases have also been utilized together in one-pot syntheses. In this case, the drawback of thermodynamic glycosidase reactions is overcome, as the glycosyltransferase removes the product and drives the glycosidase equilibrium in the forward direction. This strategy has been used

Scheme 36. Four-Enzyme, One-Pot Synthesis of 5-Deoxy-5-ethyl-D-xylulose[198]



Scheme 37. Combination of UDP−Gal and CMP−NeuAc Recycling Systems for the One-Pot Synthesis of 6′-SLN from GlcNAc[199]

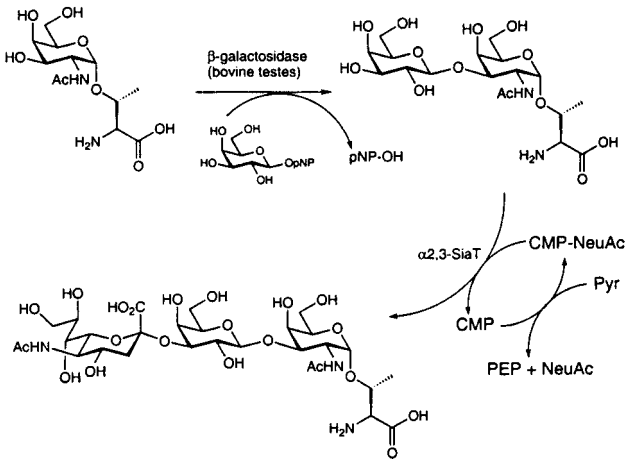


in the synthesis of core 2-trisaccharide,[203] the sialyl-TF antigen (Scheme 38),[204] and 6′-SLN.[205]

VI. Programmable One-Pot Oligosaccharide Synthesis

It is readily apparent that the use of enzymes has greatly simplified glycoconjugate synthesis. In certain

Scheme 38. Combination of β-Galactosidase and α2,3-SiaT for the One-Pot Synthesis of the Sialyl-TF Antigen[204]

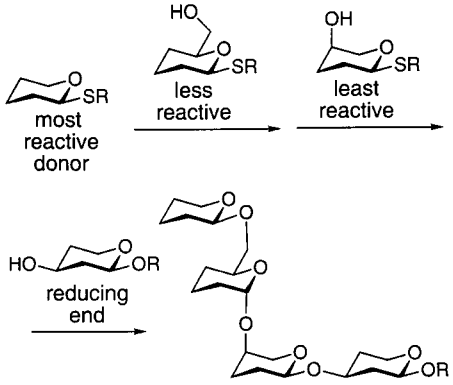


cases, complex target structures can be assembled in one pot. However, enzymes that catalyze every specific desired linkage in a carbohydrate chain are not currently available. Furthermore, since enzymatic reaction conditions are not always compatible with one another, not all enzymatic reactions are adaptable to one-pot synthesis. Enzymes may also be unpredictable when unnatural structures are the desired substrates. As such, chemical and chemoenzymatic carbohydrate synthesis remains a valuable pursuit. Moreover, if a general reaction condition for chemical glycosylation can be identified, chemical synthesis may be more generally amenable to one-pot strategies. The goal of the recently reported programmable one-pot synthesis of oligosaccharides is to establish a protocol for the accurate construction of glycosidic bonds with specificity that mirrors that of enzyme-catalyzed glycosylation reactions.

A. One-Pot Synthetic Techniques

Chemical methods for one-pot syntheses of oligosaccharides have been explored by numerous research groups. Through various strategies, the one-pot syntheses pursued involve several glycosyl donors selected to react in a specific order, thus resulting in a single oligosaccharide product (Scheme 39). The end result of these efforts has been the development of programmable one-pot synthesis, the nearest precur-

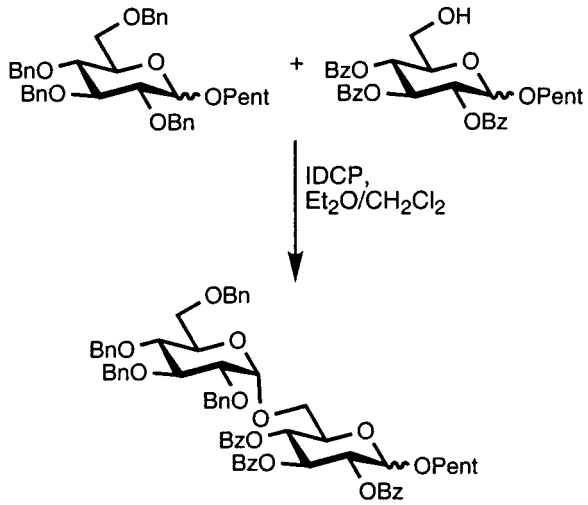
Scheme 39. Design of One-Pot Sequential Oligosaccharide Synthetic Strategies



sor to automated oligosaccharide synthesis that exists today. The following synopsis relates advancements and observations that had a direct impact on the development of this method and is not intended to give exhaustive coverage of the field.

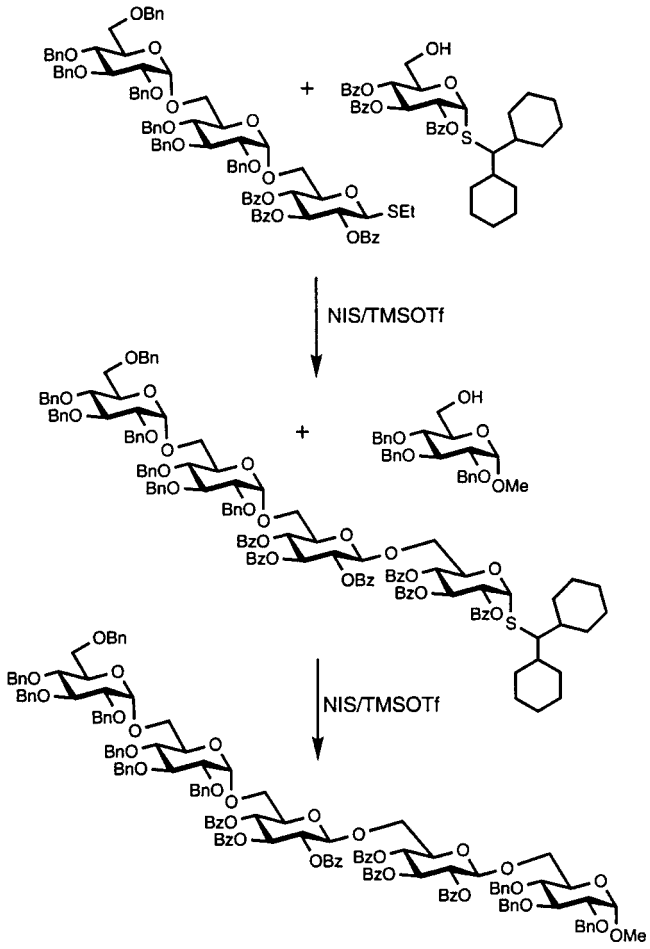
The ability to control glycosyl donor reactivity by careful selection of hydroxyl protecting groups is one of the underlying principles of programmable one-pot oligosaccharide synthesis. The viability of this concept was first documented in the 1970s by Paulsen with the recognition that glycosyl halides bearing 2-*O*-ester protecting groups were much less easily hydrolyzed than their 2-*O*-ether counterparts.[206] Fraser-Reid subsequently described the ability to arm or disarm *n*-pentenyl glycosyl donors by manipulation of the 2-position hydroxyl protecting group.[207] As such, sugars bearing 2-*O*-ether protecting groups ("armed") were shown to react preferentially over those bearing 2-*O*-ester groups ("disarmed"). The "armed−disarmed" strategy was employed in the synthesis of specific disaccharide products in the presence of two potential glycosyl donors (Scheme 40). The Boons group, in turn, reported a complementary strategy in which reactivity of thioglycoside donors was manipulated through the steric bulk of the anomeric thioether substituent.[208] This strategy arose through the reasoning that control of glycoside reactivity solely by the 2-position protecting group was risky, as it is often a major determinant of the stereochemical outcome of a glycosylation reaction. Steric modulation of thioglycoside donor reactivity was then employed in the multistep synthesis of pentasaccharides without protecting-group manipulations (Scheme 41).

Scheme 40. Fraser-Reid's Armed−Disarmed Strategy in the Synthesis of a Disaccharide[207]



In 1993, the first true one-pot chemical glycosylation reactions were achieved. The initial report by Kahne et al. involved sequential activation of phenyl sulfoxide and thioglycoside glycosyl donors, leading to the one-pot synthesis of the trisaccharide of ciclamycin 0 (Scheme 42).[209] Takahashi et al. then described the application of various glycosyl donors in sequence (halides, thioglycosides, trichloroacetimidates) for selective glycosylation reactions.[210] This group has found that selective activation of different glycosyl donors can be employed in one-pot syntheses by varying the activating agent, as exemplified by the synthesis of a hexaglucoside (Scheme 43). Chenault et al. also reported the concept of selective glycosyl donor activation in the one-pot synthesis of a trisaccharide.[211]

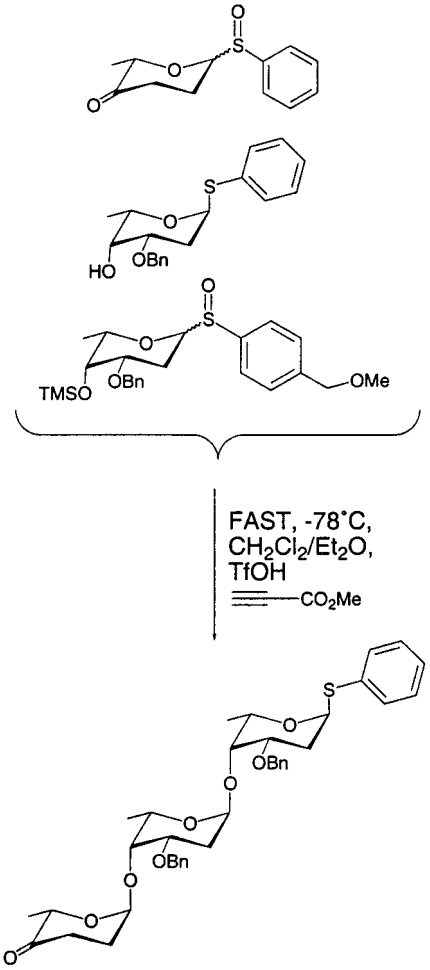
Scheme 41. Steric Modulation of Thioglycoside Reactivity As Reported by the Boons Group[208]



The Ley group then initiated studies that would have profound implications in the one-pot synthesis of oligosaccharides.[212] Insightful advances include the observation that a "semi-disarmed" thioglycoside glycosyl donor state could be accomplished using diacetal protecting groups, giving a intermediate level of reactivity control by Fraser-Reid's standards. Moreover, investigation of thio- and selenoglycosides revealed different reactivity profiles in the presence of the same activator, and these orthogonal glycosyl donors could further be induced to react in a controlled fashion in one-pot reactions (Scheme 44). The major contribution of the Ley group, however, was the notion of quantifying these various observations, which is discussed below.

Rather than one-pot synthesis, the Wong group originally focused on a multistep glycosylation strategy employing an orthogonally protected carbohydrate core structure.[213] Selective deprotection and glycosylation could be accomplished in an iterative fashion to give a library of compounds (Scheme 45). However, as isolation of the product after every step was required, generation of this modest-sized library

Scheme 42. Kahne's One-Pot Synthesis of the Trisaccharide from Ciclamycin 0[209]



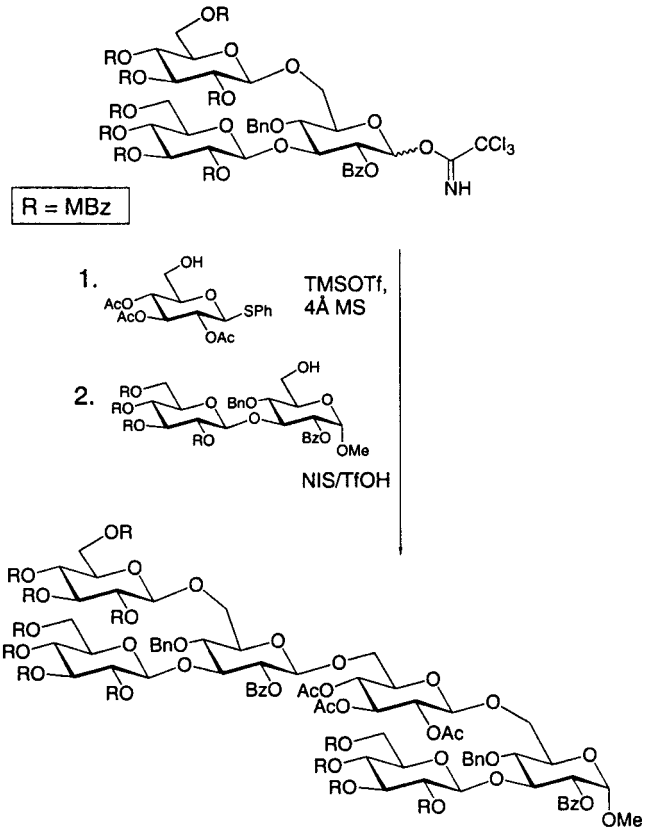
became tedious. Eventually, one-pot methods were pursued as a practical and more efficient alternative.

B. Quantitation of Glycosyl Donor Reactivity

The majority of one-pot synthetic techniques undertaken to date have been largely qualitative. However, the strategies pursued by the Ley and Wong groups are of note for their quantitative nature. In these cases, methods for the estimation of glycosyl donor reactivity for various sugar constructs were necessary. The Ley group was the first to assign relative reactivity values (RRV) to glycosyl donors.[214] In this case, the RRV values were determined by NMR. These RRVs describe the product ratio when two glycosyl donors compete for a single acceptor and were initially developed to rationalize the results of one-pot syntheses utilizing fully protected mannose and rhamnose donors. These values could in turn be utilized to predict the product outcome in a one-pot synthesis where multiple glycosyl donors were present and could also aid in the selection of donor sugars.

Subsequently, the Wong group took an alternative route to determining glycosyl donor reactivity.[215] Competition experiments performed by HPLC were used to assign RRV's to various thioglycoside-activated glycosyl donors and donor−acceptors (i.e., a thioglycoside with one hydroxyl exposed). In contrast to Ley's studies, these assignments were made

Scheme 43. Takahashi's One-Pot Synthesis of a Hexaglucoside[210]

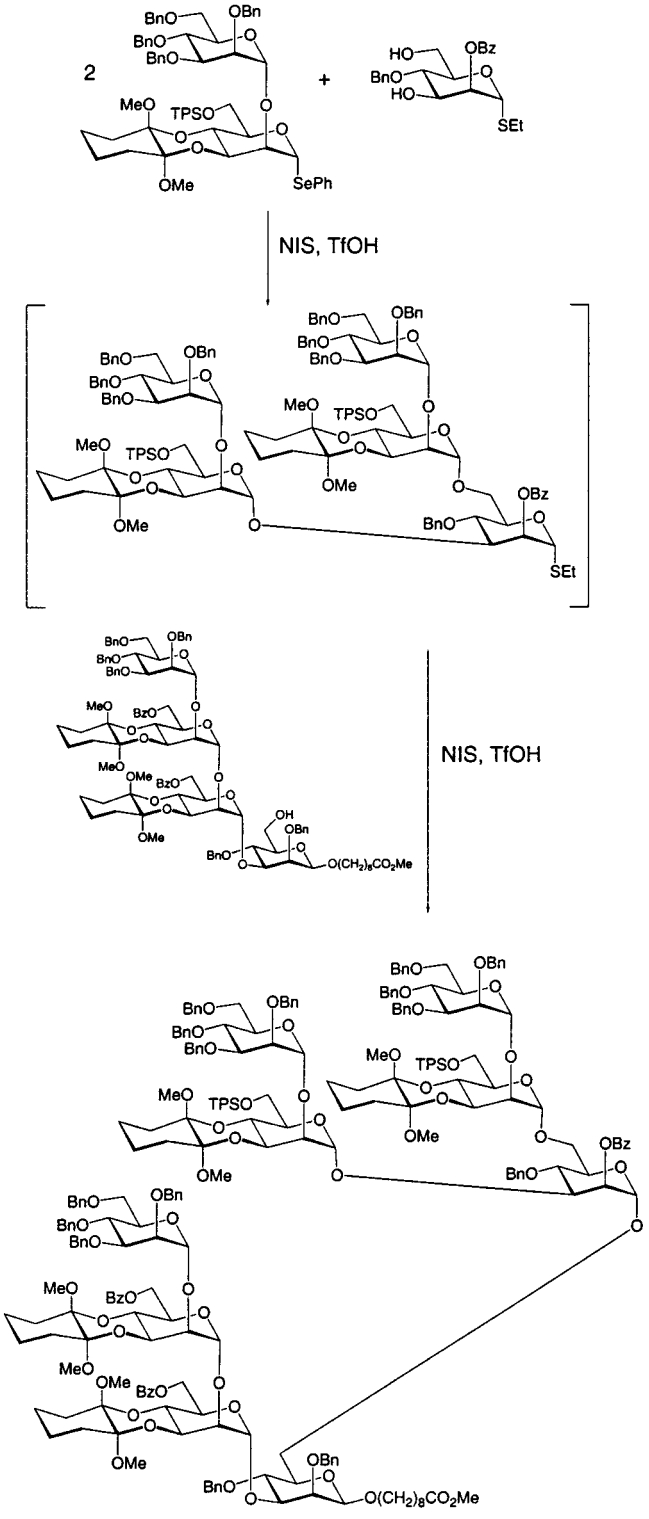


for several carbohydrates (glucose, GlcNAc, galactose, GalNAc, fucose, and mannose), including the fully protected thioglycosides and those containing one free hydroxyl group. The donors and donor−acceptors evaluated contained the *p*-methylthiophenyl leaving group and various protecting group patterns that allowed trends in reactivity to be identified within a given series of carbohydrates. The building blocks prepared by the Wong group and the RRV values are listed in Scheme 46. Several trends in glycosyl donor reactivity were identified by this analysis, and are as follows. (1) Pyranosides show reactivities that differ as a function of sugar. (2) ^{1}H NMR is predictive of reactivity within a series. (3) The reactivity of aminosugars can be tuned by choice of *N*-protecting group. (4) A general trend in protecting group effects exists. (5) The position of the pyranoside that most greatly affects the reactivity varies from carbohydrate to carbohydrate. (6) The magnitude of any effect is attenuated by its position on the pyranoside.

C. Programmable One-Pot Synthesis

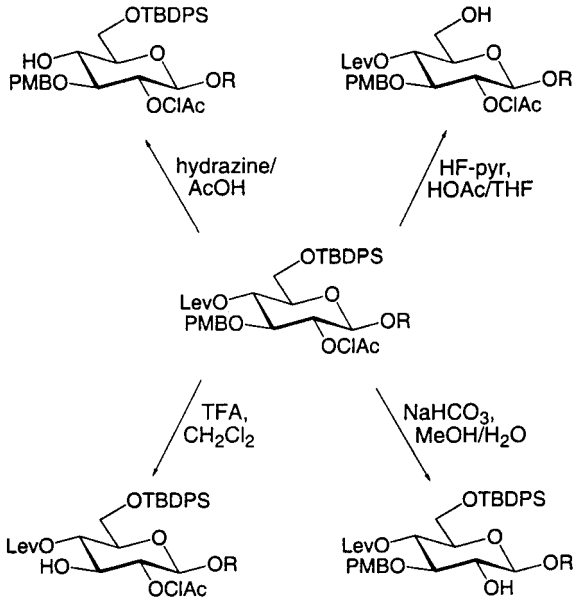
The reactivity data collected was tabulated and employed to create both a database and a computer program (Optimer) to aid in one-pot synthetic design (Scheme 47).[215] Optimer is composed of a reactivity database and a search engine that contains information such as the name of the residue, the position of unprotected hydroxyl groups, and whether the 2′-substituent directs glycosylation in an α- or *β*-linkage. After the user selects a structure of interest, the program lists the 100 best combinations of reagents for its preparation and the predicted yield. To prove

Scheme 44. Ley's Orthogonal Glycosyl Donors for the One-Pot Synthesis of Complex Mannosides[212]



its usefulness, Optimer was initially employed in the design of one-pot syntheses of five different oligosaccharides. When the building blocks that Optimer had selected were combined sequentially in solution, the predicted products were isolated. Thus, this program can be used for the design and preparation of linear or branched (Scheme 48) oligosaccharides in a controlled fashion. More recently, the construction of a small oligosaccharide library has been accomplished by these methods.[216]

Scheme 45. Wong's Orthogonal Deprotection Strategy for Generation of a Library[213]

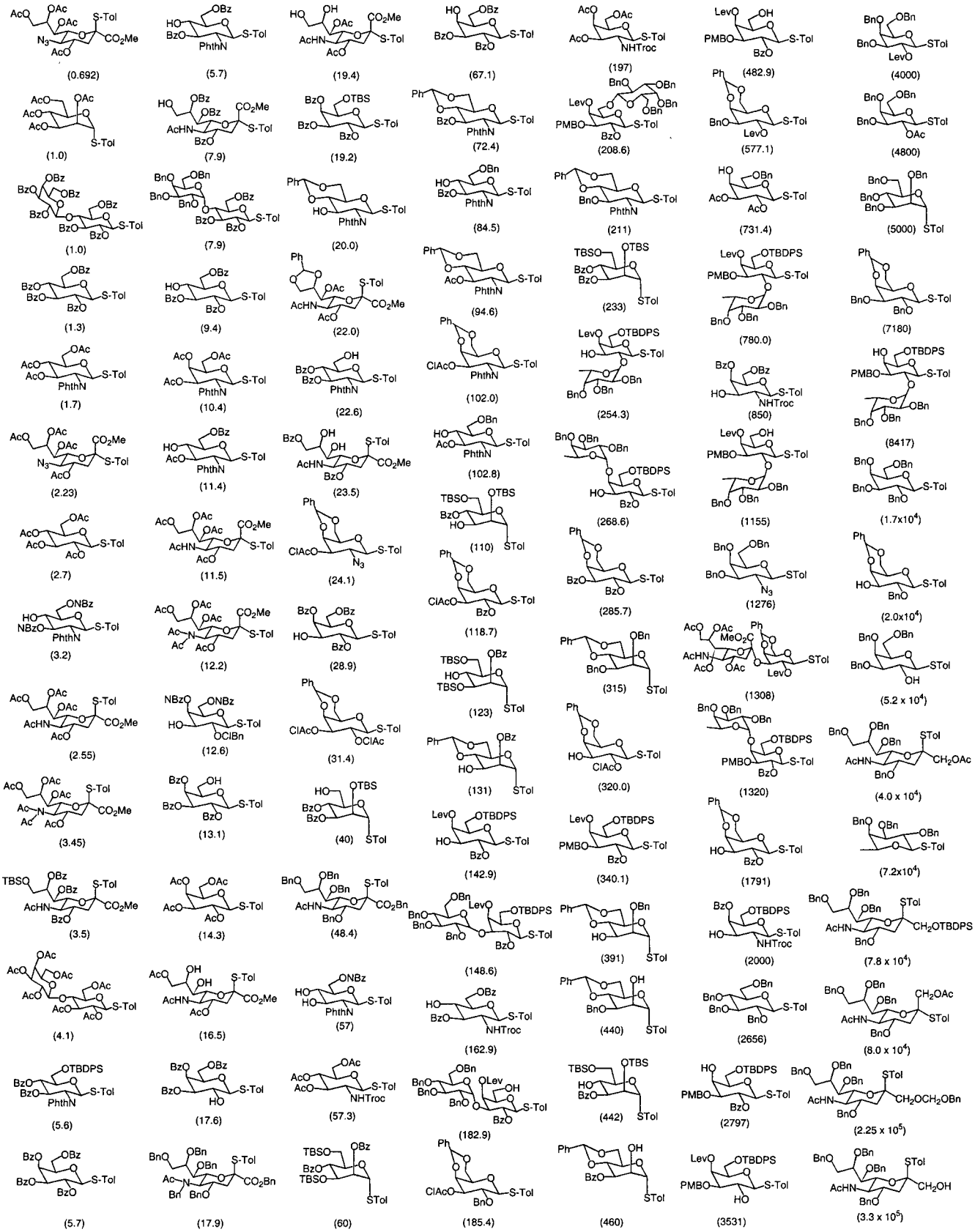


To date, the characterization of reactivity parameters for six monosaccharides has been undertaken. Future plans include the generation of RRVs for other sugars, including complex carbohydrates such as sialic acid. Though the current methodology allows limited options for branched oligosaccharide synthesis, the one-pot procedure will be developed toward this end. The determination of reactivity data for selenoglycosides (as used by Ley) will also be pursued. This glycosyl donor can be activated in the same fashion as the thioglycosides already employed and may provide access to structures that are low yielding at present. Finally, alternative activation strategies will be investigated. Although the current strategy utilizes NIS as activator, favorable results have also been obtained with DMTST. All in all, the end goal is to generate the largest database possible so that any desired oligosaccharide structure can be formed in a single one-pot reaction designed by Optimer. At the present time, programmable one-pot oligosaccharide synthesis is the closest analogue to automated synthesis that exists. With continuing development, it will provide a valuable tool for biologists and chemists alike in the study of carbohydrate function.

VII. Future Directions

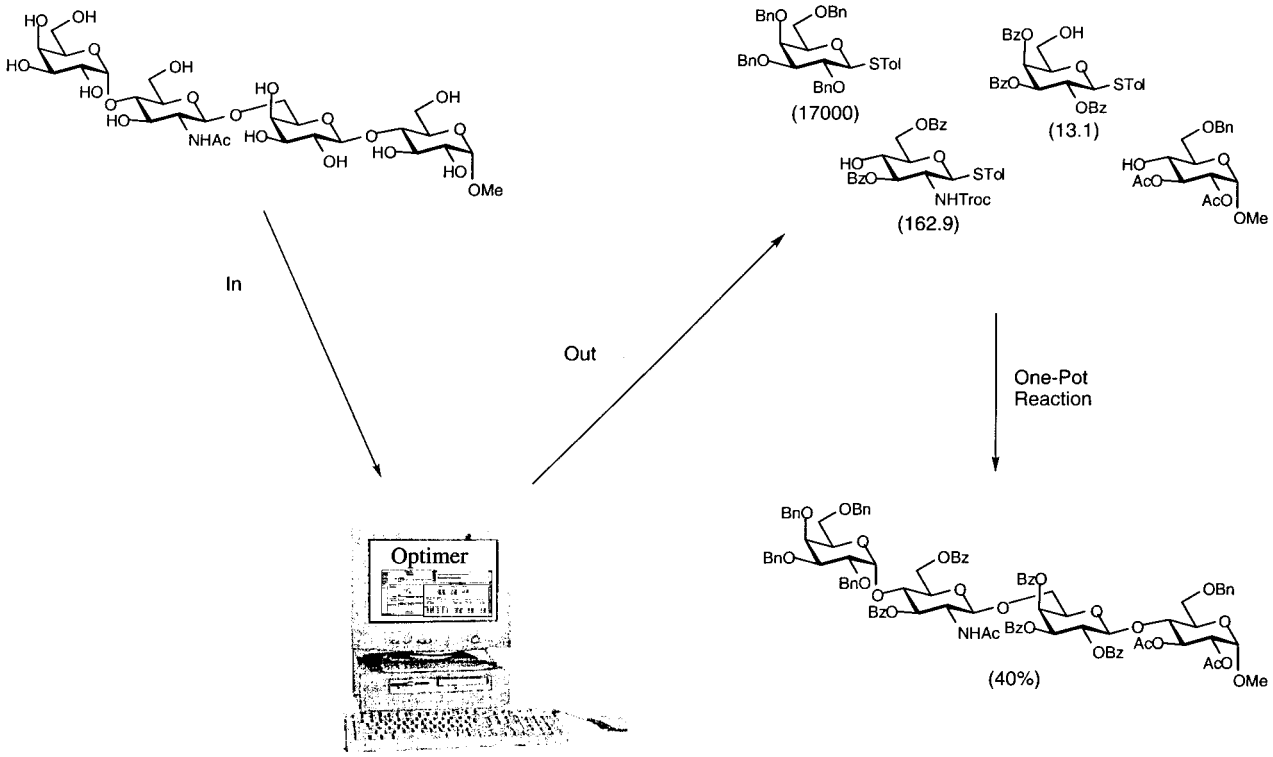
This review has described recent advances in enzymatic and chemical methods for the facile generation of complex oligosaccharides and glycoconjugates. While much synthetic progress has been accomplished in the past, research opportunities in this area remain abundant. In the enzymatic arena, increased commercial availability of glycosyltransferases and decreased sugar nucleotide cost would promote further investigations of glycosyltransferases as catalysts in glycosidic bond formation. Synthetic methods toward regiospecifically esterified, sulfated, or phosphorylated glycoconjugates are also of interest in the study of post-translational modification func-

Scheme 46. Table of Glycosyl Donor and Donor−Acceptor Reactivity As Determined by HPLC[215]

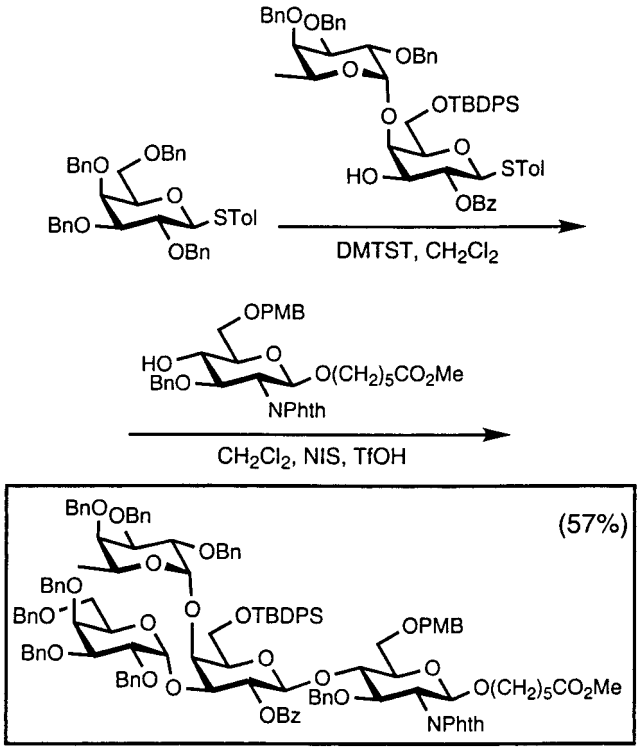


tion. Access to homogeneous glycoproteins is also necessary to truly understand the functional and structural consequences of glycosylation on a protein backbone. Although substantial advances in enzymatic and chemoenzymatic syntheses have been made, access to all desired glycoconjugates remains a daunting challenge. In the chemical front, advances in chemical one-pot synthetic methods have yielded a database of glycosyl donor reactivities and a computer program to aid in the synthesis of oligosac-

Scheme 47. Optimer-Programmed Synthesis of a Linear Oligosaccharide[215]



Scheme 48. Optimer-Designed Synthesis of a Branched Tetrasaccharide[215]



charides. This methodology shows great promise toward the development of automated carbohydrate synthesis and will be a method applicable to the rapid synthesis of complex oligosaccharides. In general, glycosylation remains one of the most poorly understood posttranslational modifications, although general themes in glycoconjugate function are slowly emerging. Further progress in this field relies on the availability of material for study, and the methods described herein represent the state-of-the-art in facile glycoconjugate synthesis to date.

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