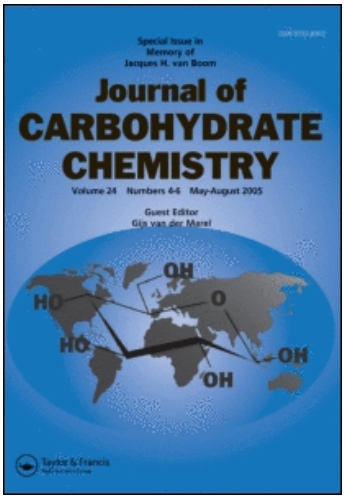

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GLYCOSYLTRANSFERASES IN OLIGOSACCHARIDE SYNTHESIS*

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INTRODUCTION

Recent demonstrations that oligosaccharides play important roles in diverse biological events have resulted in renewed interest in the synthesis of oligosaccharides and their analogs. The availability of such molecules can facilitate studies on carbohydrate-protein recognition and help to elucidate molecular mechanisms of oligosaccharide-mediated biological processes[1,2] that could eventually lead to rationally designed carbohydrate-based therapeutics.[3,4] Despite many advances that have been made over the past decades, the chemical synthesis of oligosaccharides remains a challenge.[5,6] This is attributed to the inherent chemical difficulties presented by this class of molecules. Each monosaccharide carries at least three hydroxyl groups that must be protected and deprotected during synthesis. Also, glycosylation generates a new stereocenter at the anomeric carbon, and there are no general methods for the introduction of all types of glycosidic linkage in a manner that is both stereo-controlled and high yielding. The chemical synthesis of oligosaccharides is therefore very time-consuming and requires specialized expertise. The synthesis of oligosaccharide analogs containing modified sugars is an even more complex task than the preparation of natural structures. Almost twice as many steps are usually required for analog synthesis and the steps are more difficult, since most chemical protocols in the literature have been optimized for natural sugars.

In nature, glycosyltransferase enzymes accomplish the ''daunting'' task of the construction of diverse and complex oligosaccharide.[7–9] These enzymes catalyze

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the transfer of a monosaccharide from a glycosyl donor (usually a sugar nucleotide) to a glycosyl acceptor in a regio- and stereospecific manner (Figure 1). Pioneered by Barker,[10,11] Whitesides[12] and Augé[13] and their colleagues, enzymatic synthesis by means of glycosyltransferases opened up a new venue for the preparation of oligosaccharides, avoiding many of the problems encountered in traditional chemical synthesis. Multistep protection and deprotection sequences are no longer required, since the glycosyl transfer catalyzed by glycosyltransferases is stereo- and regiospecific; thus the configuration of the newly formed anomeric center is absolute. Glycosyltransferases, unlike many catalysts used in organic synthesis, are environmentally benign and operate best in aqueous solution.

Although glycosyltransferases exhibit high specificity for both the sugar nucleotide donor and acceptor, they have been shown to tolerate certain structural changes on both donor and acceptor substrates. An enzymatic approach to unnatural oligosaccharides greatly simplifies the synthetic scheme because the requirement for the chemistry is then reduced to the synthesis of more readily accessible smaller ''primers'' (mono- to trisaccharides), which can be elongated in a regio- and stereospecific manner using glycosyltransferases without additional protection and deprotection steps.

With advances in molecular biology and biotechnology, more than 30 glycosyltransferases from mammalian,[9,14] bacterial,[15–17] and even viral[18] sources have

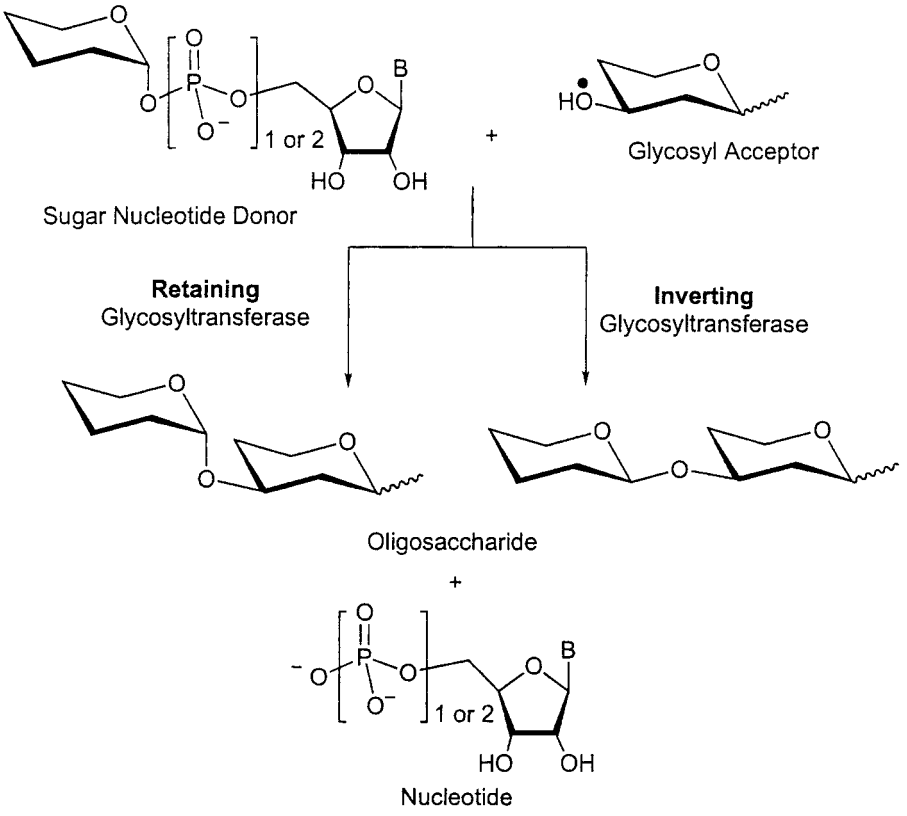

Figure 1. Reactions catalyzed by Leloir glycosyltransferases (B = uracil, guanine, cytosine). The acceptor glycosylation site is highlighted.

been cloned, and many are now readily available in large multiunit quantities. For synthetic applications, the major sugar nucleotide donor substrates for mammalian glycosyltransferases are commercially available, and cost limitations for large-scale synthesis are rapidly being overcome with nucleotide donor recycling systems[12,19] and pathway engineering.[20] Glycosyltransferase-catalyzed synthesis of both natural and unnatural oligosaccharides is an important component of glycochemistry, as evidenced by the numerous comprehensive reviews that have appeared.[21–25]

GLYCOSYLTRANSFERASES

Glycosyltransferases can be divided into the Leloir and non-Leloir types according to the type of glycosyl donors they use.[26] Non-Leloir glycosyltransferases typically use glycosyl phosphates as donors, while Leloir glycosyltransferases utilize sugar nucleotides as donors and transfer the monosaccharide with either retention (retaining enzymes) or inversion (inverting enzymes) of the configuration of the anomeric center (Figure 1). Most of the glycosyltransferases responsible for the biosynthesis of mammalian glycoproteins and glycolipids are Leloir glycosyltransferases. They are typically type II membrane proteins with a short cytoplasmic N-terminal domain, a hydrophobic transmembrane domain, a luminally oriented stem, and a large C-terminal catalytic domain.[27]

With a few exceptions,[28,29] each glycosyltransferase produces a unique glycosidic linkage. According to the ''one enzyme–one linkage'' hypothesis,[30] there are estimated to be hundreds of glycosyltransferases responsible for the production of the diverse and complex oligosaccharide structures found in nature. Glycosyltransferases are classified according to their nucleotide donor sugars, the type of glycosidic linkage formed (α or β), and the specific hydroxyl group in the acceptor to which the monosaccharide is transferred. Mammalian glycosyltransferases use only nine main sugar nucleotides as building blocks to construct complex oligosaccharides. They are activated sugar donors of uridine diphosphate (UDP-Gal, UDP- GlcNAc, UDP-Glc, UDP-GlcNAc, UDP-Xyl, UDP-GlcA), guanosine diphosphate (GDP-Fuc, GDP-Man), and cytidine monophosphate (CMP-sialic acid or CMP-Neu5Ac). All the sugar units have the D configuration except for fucose, which has an L configuration.

This chapter reviews the literature on enzymatic synthesis utilizing Leloir glycosyltransferases, with focus on galactosyltransferases, fucosyltransferases, and sialyltransferases, most of which are commercially available or widely accessible. Also, they are well studied in terms of their donor and acceptor specificity and have been used for the synthesis of both natural oligosaccharides and their analogs. As well, two practical examples for the synthesis of trisaccharide analogs are given.

GALACTOSYLTRANSFERASES

β1,4-Galactosyltransferase

β1,4-Galactosyltransferase (β1,4-GalT, EC 2.4.1.22/38/90) has been commercially available for many years in unit quantities, where a unit is the amount of enzyme that converts one micromole of substrate to product per minute. It is the most

widely studied glycosyltransferase with regard to substrate specificity and use in preparative synthesis and the first mammalian glycosyltransferase for which an X-ray crystal structure was determiend.[31] Historically, β1,4-GalT was also the first enzyme used for the preparation of oligosaccharides on a large scale (>10 g).[12] β1,4-GalT catalyzes the transfer of Gal from UDP-Gal to OH-4 of terminal β-linked GlcNAc to form *N*-acetyllactosamine (LacNAc) (Figure 2). The acceptor specificity shifts to glucose in the presence of the protein modifier α-lactalbumin, giving lactose as the major product.

Since Berliner's pioneering work demonstrating that β1,4-GalT could transfer glucose, 4-deoxy-Gal and arabinose from their corresponding UDP donors,[32] donors with modifications at every OH group have been employed for the synthesis of numerous LacNAc analogs (Figure 3). While the enzyme transfers 2-deoxy-Gal at a rate comparable to that of UDP-Gal,[33] 3-deoxy,[34] 6-deoxy,[35,36] and 6-deoxy-6-fluoro Gal[35,36] are transferred at reduced rates. The enzyme also utilizes UDP-GalNAc, UDP-GalNH_2, and UDP-GlcNH_2 donors[37,38] (Figure 3). Replacement of the oxygen of Gal with sulfur in UDP-5′-thio-Gal is tolerated by β1,4-GalT,[39] including transfer in the presence of α-lactalbumin.[40]

The acceptor specificity of β1,4-GalT is equally relaxed, since numerous modifications in acceptors including the ring oxygen are possible as long as the 4-OH remains available for glycosylation (Figure 3). The 4-OH is a ''key polar group''[41] where replacement or modification yields analogs that no longer bind to the enzyme.

The 2-NHAc group can be replaced with *N*-propanoyl,[42,43] *N*-butanoyl,[42] azido,[38,44] allylcarbamate,[45] and other amide derivatives, including bulky heterocycles, charged groups, and glycuronamides.[46] Acceptors with the 3-OH group deoxygenated,[47,48] alkylated with a methyl or allyl group, or oxidized to the ketone are active as acceptors with reduced reaction rates.[48] The 6-OH group of GlcNAc can be methylated,[43,48] deoxygenated,[47] fucosylated,[43] or replaced with F or SH.[47] GlcNAc bearing α-linked sialic acid at 6-OH is not a substrate; however, if the

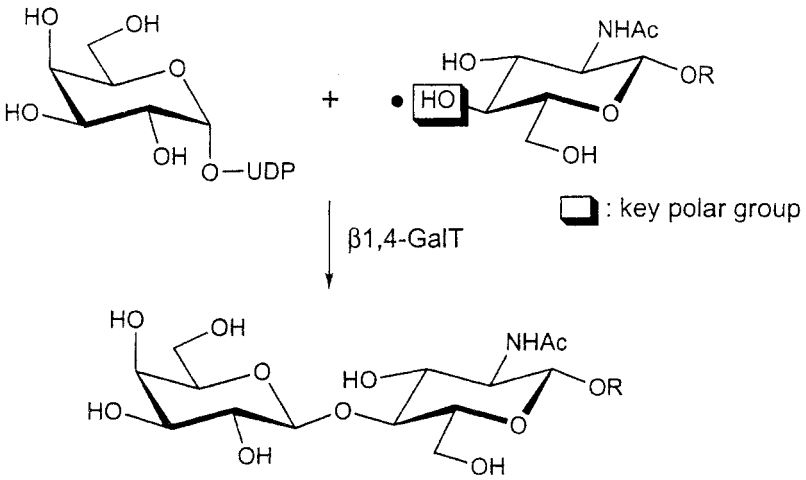

Figure 2. The reaction catalyzed by β1,4-galactosyltransferase.

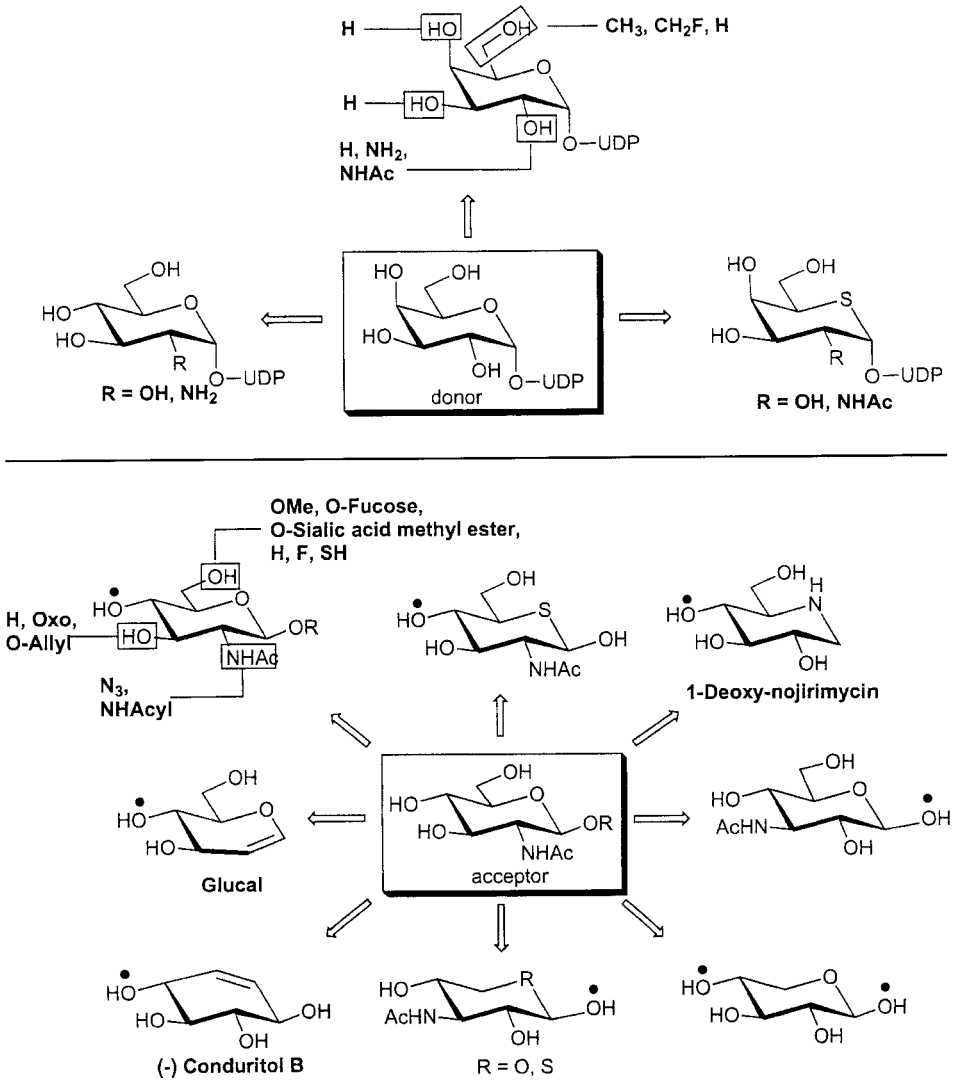

Figure 3. Unnatural donors and acceptors for β1,4-GalT.

carboxylic acid of NeuAc is derivatized with a methyl ester, the resulting compound is an acceptor with 4% the rate of transfer compared to the parent acceptor.[43]

β1,4-GalT will utilize acceptors with the ring oxygen modified, including 5′-thio-Glc and 1-deoxy-nojirimycin.[48] Glucal with a flattened ring[48] and (±)-conduritol B are substrates; the latter mixture can be resolved because only a single galactosylated product (−)-conduritol B is produced.[49] In an exception to the ''one enzyme-one linkage'' hypothesis, the enzyme transfers galactose to the β-anomeric position of 3-acetamido-3-deoxy-D-glucose acceptors giving a $\beta 1 \rightarrow 1$ trehalose type of linkage.[29] This atypical regiochemistry of galactosylation was also reported for *N*-acetyl-gentosamine, *N*-acetyl-5′-thiogentosamine, and xylose acceptors.[50,51] A variety of im-

mobilized acceptors have been employed in solid phase or soluble polymer synthesis,[52–58] and even UDP-GlcNAc is an acceptor substrate, yielding the nucleotide disaccharide UDP-*N*-acetyllactosamine.[59] Reactions at scales of 500 g or more with β1,4-GalT have been reported with in situ donor recycling.[60] Also β1,4-GalT enzymes have been found in bacterial sources, including *Helicobacter pylori* and *Neisseria meningitidis*[61–63] and utilized in 100 g reactions for *N*-acetyllactosamine production through bacterial coupling.[64]

α1,3-Galactosyltransferase

α1,3-Galactosyltransferase (α1,3-GalT, EC 2.4.1.151) has recently attracted much attention because it catalyzes the transfer of Gal from UDP-Gal to 3-OH of the Gal residue in Galβ1 → 4GlcNAc-R to form Galα1 → 3Galβ1 → 4GlcNAc epitopes on glycoproteins and glycolipids (Figure 4).[65–67] This is the major xenoactive antigen that is responsible for hyperacute rejection in xenotransplantation.[68,69] α1,3-Galactosyltransferases from porcine and bovine tissues and recombinant bovine α1,3-galactosyltransferase have all been used for preparative synthesis, the latter for gram-scale reactions.[66,70] Recombinant porcine α1,3-GalT is now commercially available in unit quantities, and a fusion protein comprising UDP-Gal epimerase and α1,3-GalT has been engineered for large-scale synthesis.[71]

α1,3-Galactosyltransferase transfers 3-deoxy-, 4-deoxy-, and 6-deoxy-Gal from the corresponding donor at very low rates (<2%), while UDP-2-deoxy-Gal is a better substrate than UDP-Gal[72] (Figure 5). However, unlike the case of β1,4-GalT, UDP-Glc, GalNAc, GlcNAc, and glucuronic acid are not transferred by α1,3-GalT.[73]

Acceptors other than Galβ1 → 4GlcNAc are utilized by α1,3-GalT, including Galβ1 → 3GlcNAc and Galβ1 → 4Glc,[67,74] and acceptors immobilized on poly-

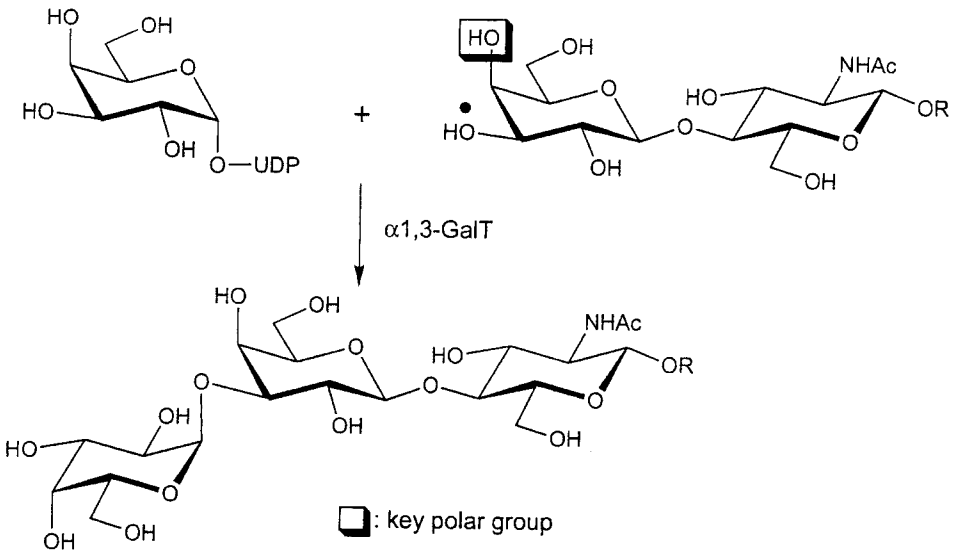

Figure 4. The reaction catalyzed by α1,3-galactosyltransferase.

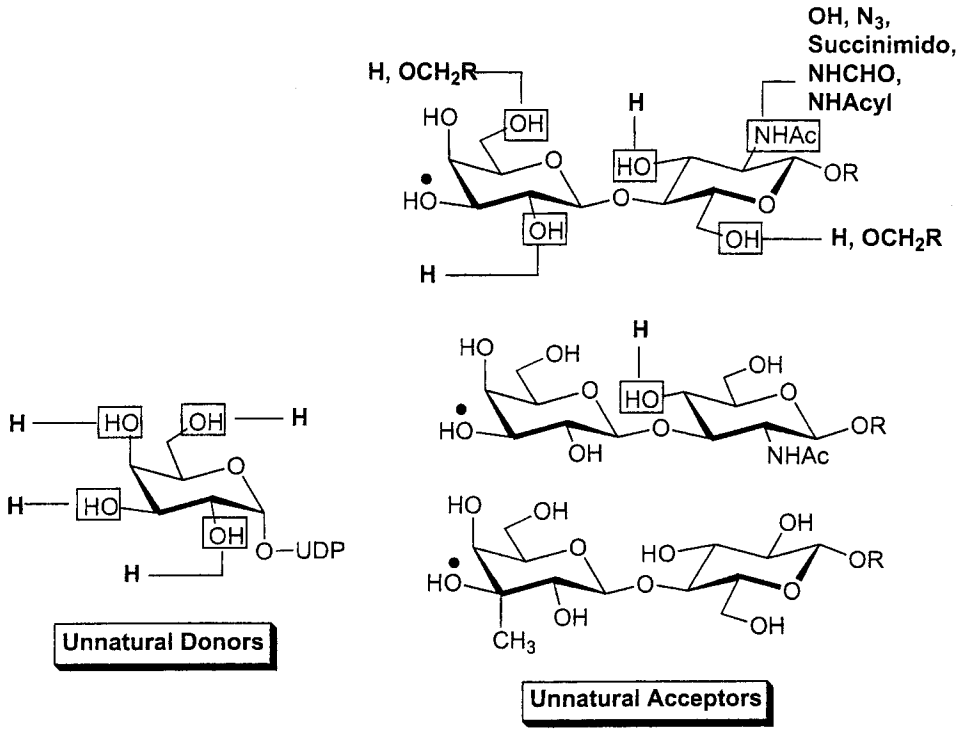

Figure 5. Unnatural donors and acceptors for α1,3-galactosyltransferase.

mers.[71] Modifications on 4-OH of Gal in acceptors give inactive compounds, suggesting that this is a key polar group essential for binding to α1,3-GalT.[67] The *N*-acetyl group in acceptors can be replaced with azido, succinimido, and others with a large number of acyl groups[67,75] (Figure 5). However, replacement of the 2-NHAc with an amino group abolishes activity.[73] As shown in Figure 5, analogs with modifications on 6-OH of GlcNAc, or 2-OH and 6-OH of the terminal Gal residue are substrates, while only deoxygenation of the 3-OH of GlcNAc residue is tolerated.[67] The enzyme can catalyze the transfer of a Gal residue to a very hindered tertiary alcohol acceptor where the carbon-bonded hydrogen at the glycosylation site is replaced with a methyl group, demonstrating that glycosyltransferases can overcome inherent steric limitations to produce oligosaccharide analogs that cannot be easily prepared by chemical methods.[76]

β1,3- and α1,4-Galactosyltransferases

Though not widely available yet, the β1,3-galactosyltransferase that synthesizes Galβ1 → 3GlcNAc (type I) from GlcNAc acceptors has been cloned and expressed.[77,78] Mapping has indicated that *N*-acyl analogs can be utilized as acceptors by the enzyme.[79] α1,4-Galactosyltransferase from *Neisseria meningitidis* transfers Gal from UDP-Gal donors solely to lactose acceptors yielding globotriose.[62,72] The enzyme will transfer arabinose, 2-deoxy-, 4-deoxy-, and 6-deoxy-Gal from modified do-

nors from 2 to 28% of the rate of UDP-Gal; however, 3-deoxy-Gal is not transferred.[72] Large-scale production of UDP-Gal and globotriose (188 g/L) has been achieved by metabolic engineering of bacteria.[20]

Human Blood Group A and B Glycosyltransferases

Human blood group A and B glycosyltransferases (GTA and GTB: EC 2.4.1.40 and 2.4.1.37) are responsible for the biosynthesis of A and B blood group antigens, which are important in cell development, cell differentiation and oncogenesis.[80,81] Both GTA and GTB are retaining enzymes. GTA catalyzes the transfer of GalNAc from UDP-GalNAc to the (O)H antigen (Fucα1 $\rightarrow$ 2Galβ-OR) to give the A antigen GalNAcα1 $\rightarrow$ 3[Fucα1 $\rightarrow$ 2]Galβ-OR. GTB uses the same acceptor but catalyzes the transfer of Gal from UDP-Gal to form the B antigen, Galα1 $\rightarrow$ 3[Fucα1 $\rightarrow$ 2]Galβ-OR (Figure 6).

Chemical mapping studies reveal that OH-4 of the Gal residue is a key polar group for both GTA and GTB, whereas OH-3 of the Gal unit to which the glycosyl residue transfers is not essential for recognition by either enzyme.[82,83] As shown in Figure 7, both enzymes tolerate deoxygenation, substitution, and derivatization of the 6-OH group of the Gal unit.[82] Deoxygenation of any of the hydroxyl groups on the Fuc residue is tolerated by GTA. Methylation of O3′ and O4′ is tolerated by both GTA and GTB. The arabino derivative, where the CH_3 group of the Fuc residue is

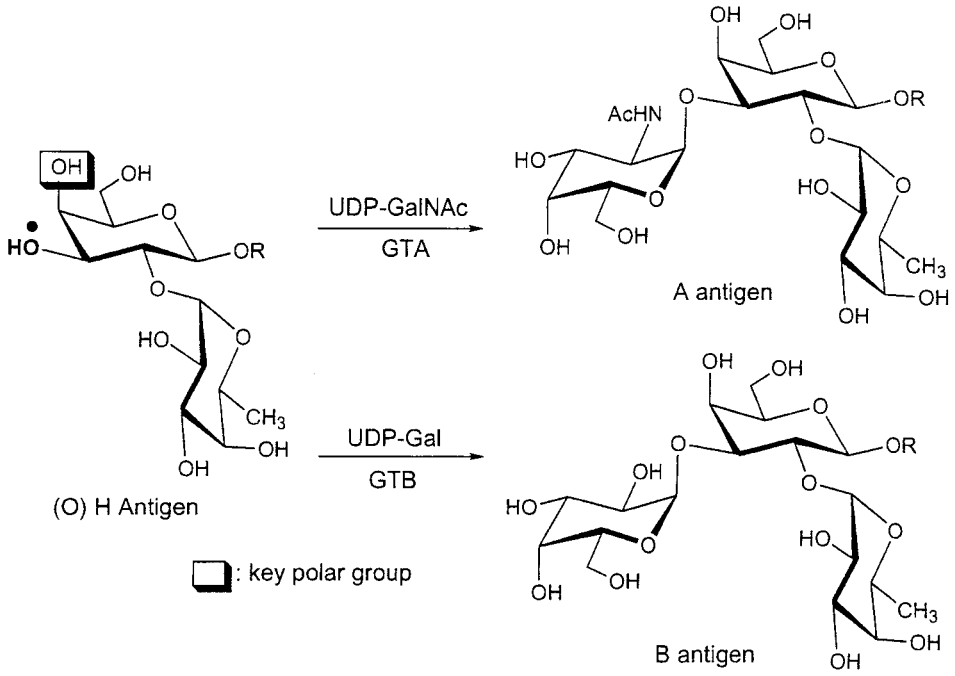

Figure 6. Reactions catalyzed by blood group A and B glycosyltransferases.

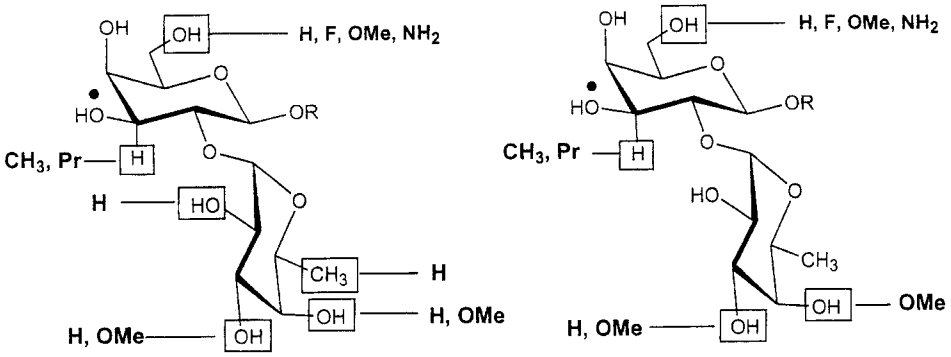

Figure 7. Unnatural acceptors for GTA (left) and GTB (right).

replaced by H, was found to be an acceptor for GTA.[84] Both GTA and GTB, like α1,3-GalT and human milk α1,3/4-FucT, can also act on highly hindered tertiary alcohol acceptors and will even accommodate a large propyl group at the glycosylation site.[76] While crossovers in donor specificity have been reported (i.e., GTA can utilize UDP-Gal and vice versa),[85,86] UDP-Glc is a donor only for GTB and UDP-GlcNAc only for GTA.[87] Donor monosaccharide mapping of GTB shows that modifications to Gal are tolerated at all positions. The rates of transfer relative to UDP-Gal are 2-deoxy-Gal (175%), 3-deoxy-Gal (0.1%), 4-deoxy-Gal (0.2%), 6-deoxy-Gal (18%), and arabinose (0.1%).[72]

FUCOSYLTRANSFERASES

Many antigenic oligosaccharides on the cell surface such as blood group antigens are fucosylated. Along with sialylation, fucosylation by fucosyltransferases is often the last in vivo modification of oligosaccharides. The fucosylated oligosaccharides on cell surfaces are involved in numerous intercellular recognition events.[28,88,89] α1,2-Fucosyltransferases transfer a fucosyl residue from GDP-Fuc to the 2-OH group of β-D-galactosides to form Fucα1 → 2Gal structures, while α1,3/4-fucosyltransferases catalyze the transfer of a fucose residue from GDP-fucose to the 3-OH or 4-OH group of β-D-galactosides in a variety of acceptors to form the blood-group-related antigenic determinants Le^a and Le^x.[28,90–92] α1,3/4-Fucosyltransferases are a multigene family of enzymes with differences in substrate specificities toward Galβ1 → 4GlcNAc, Galβ1 → 3GlcNAc, and NeuAcα2 → 3Galβ1 → 4GlcNAc acceptors; there are differences, as well, with respect to inhibitor sensitivity, pH optima, and tissue distribution. The most common source for the isolation of preparatively useful α1,3/4-fucosyltransferases is human milk.[93] There are two different human milk fucosyltransferases, α1,3/4-fucosyltransferase and α1,3-fucosyltransferase. Five different human α1,3/4-fucosyltransferases have been cloned, FucT III to FucT VII. The in vivo specificities of all five of these human fucosyltransferases expressed in baby hamster kidney (BHK) cells have been elucidated.[94] FucTs IV–VI are now commercially available in 100 mU quantities, sufficient for preparative synthesis.

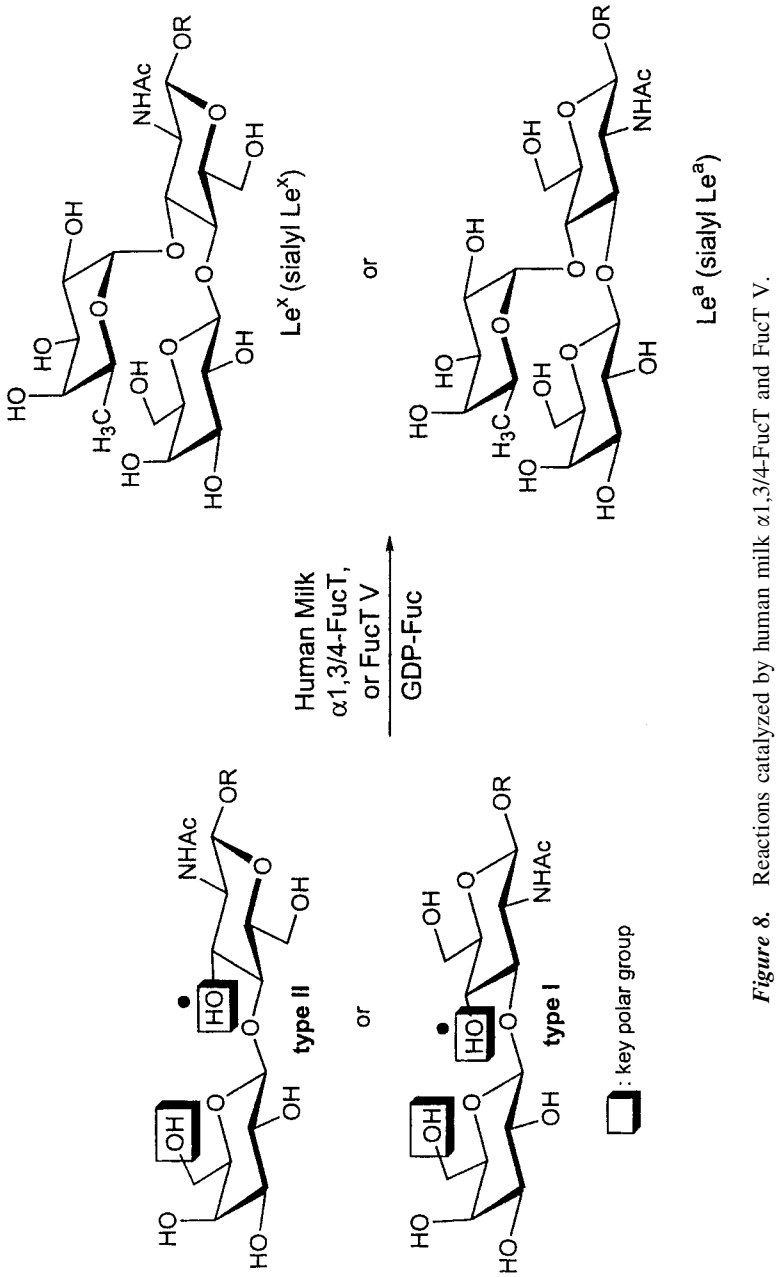

Figure 8. Reactions catalyzed by human milk α1,3/4-FucT and FucT V.

Human Milk α1,3/4-Fucosyltransferase

Human milk α1,3/4-FucT (EC 2.4.1.65) is another exception to the ''one enzyme-linkage'' hypothesis. This enzyme catalyzes the transfer of fucose to both type I and type II acceptors to produce Le^a and Le^x structures, respectively (Figure 8). The enzyme was found to tolerate several structural changes on the donor substrate. The enzyme utilizes GDP-3-deoxy-fucose, GDP-arabinose, and GDP-L-galactose donors in transfers to Galβ1 → 3GlcNAcβ-O-$(CH_2)_8$COOMe as an acceptor (Figure 9).[95] In 1998 a group used GDP-3-deoxy-Fuc and GDP-L-Gal in the preparative synthesis of Le^x analogs, with Galβ1 → 4GlcNAcβ-O$(CH_2)_8$COOMe as an acceptor.[96] The enzyme was also shown to tolerate the addition of a propyl group at 6-OH of L-Gal in GDP-L-Gal. Even larger substitutents can be introduced at C6 of the Fuc residue in GDP-Fuc, including tethered blood group B trisaccharide.[97] Virtually any group can be attached to the C6 position of Fuc, as was demonstrated by the transfer of modified fucose residues containing biotin and blood group A trisaccharide (Figure 9).[98]

Human milk α1,3/4-FucT uses both Galβ1 → 3GlcNAc (type I) and Galβ1 → 4GlcNAc (type II) acceptors. As shown in Figures 8 and 9, modifications are tolerated at every hydroxyl group in the sugar rings except for two key polar groups, 6-OH of the Gal and 3-OH or 4-OH of the GlcNAc residue to which fucose is transferred.[99] The 2-NHAc group of GlcNAc residue in NeuAcα2 → 3Galβ1 → 3GlcNAcβ-O$(CH_2)_8$-COOMe or NeuAcα2 → 3Galβ1 → 4GlcNAcβ-O$(CH_2)_8$-COOMe can be replaced with azido, amino, or propionamido groups.[100] Thiolinked *N*-acetyllactosamine, in which the interglycosidic oxygen is replaced by sulfur, is also a good acceptor for the enzyme.[101] Ether- and imino-linked octyl *N*-acetyl-5*a*′-carba-β-lactosamides were also found to be acceptors for human milk α1,3/4-FucT.[102] As with α1,3-GalT and blood group B galactosyltransferase, α1,3/4-FucT can tolerate the introduction of a large methyl group directly at the site of fucosylation. It transfers fucose to the hindered tertiary alcohol acceptor 3-*C*-methyl-*N*-acetyllactosamine to produce a carbon-branched Le^x analog.[103]

Fucosyltransferase V

FucT V is similar to human milk α1,3/4-FucT because it will utilize both type I and type II acceptors (Figure 8). This important industrial enzyme has been used for the large production of sialyl $Lewis^x$. FucT V also has similar acceptor specificity as human milk α1,3/4-Fuc, requiring 6-OH of Gal and 3-OH or 4-OH of GlcNAc for substrate binding.[99] Galβ1 → 4Glucal, Galβ1 → 4(-5-S)-Glc, 3-sulfo-Galβ1 → 4Glc-4GlcNAc, and Galβ1 → 4-(6-sulfo)-GlcNAc are acceptors for FucT V.[44,104]

Fucosyltransferases III and VI

FucT III transfers fucose onto 4-OH of GlcNAc residues of Galβ1 → 3GlcNAc or NeuAcα2 → 3Galβ1 → 3GlcNAc to form Le^a or sialyl Le^a, respectively (Figure 10). FucT VI uses Galβ1 → 4GlcNAc or NeuAcα2 → 3Galβ1 → 4GlcNAc to form Le^x or sialyl Le^x products (Figure 10). Despite their close sequence homologies, they exhibit

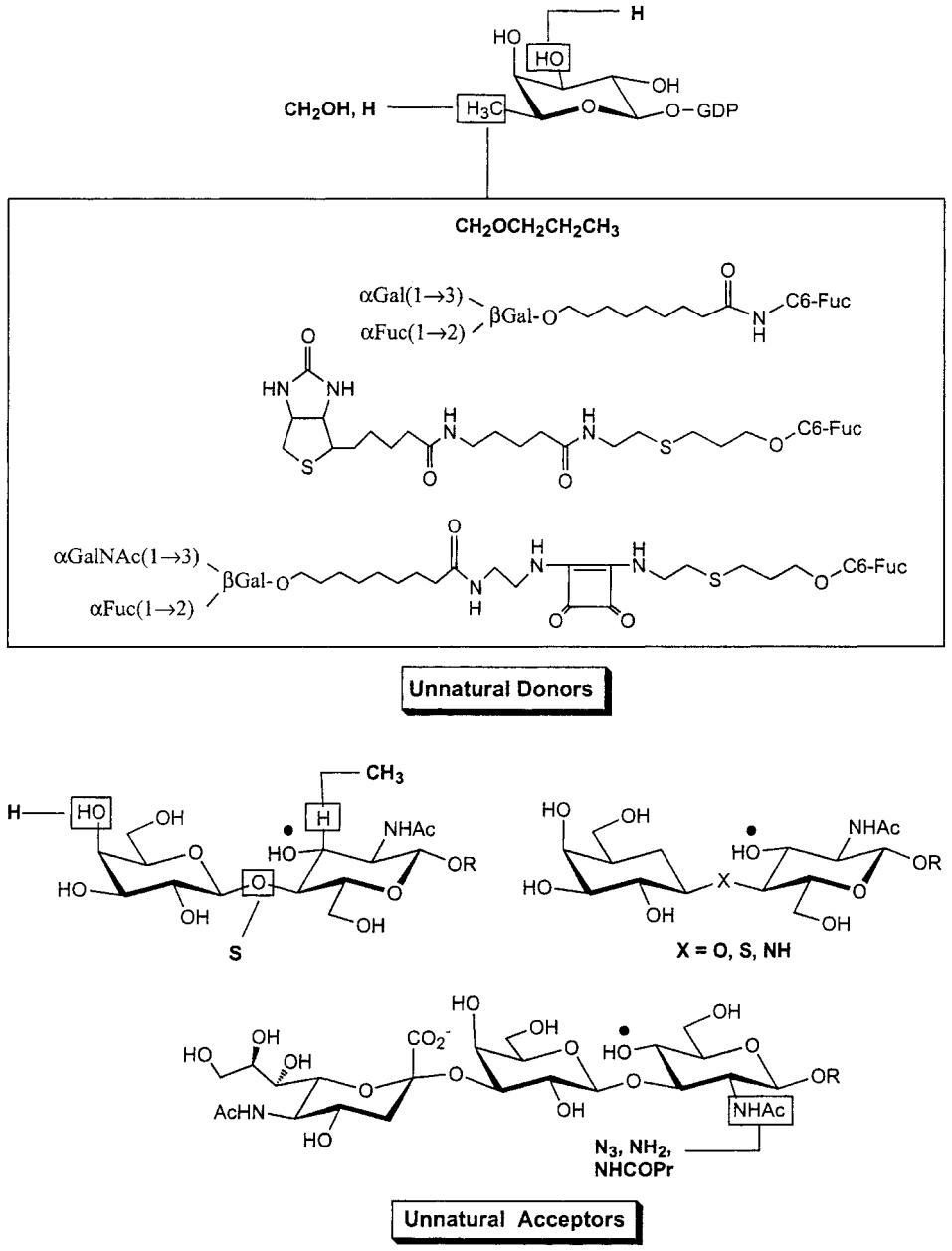

Figure 9. Unnatural donors and acceptors for human milk α1,3/4-FucT.

differences in their recognition of unnatural donors.[105] FucT III transfers L-Gal, arabinose, L-Glc, 2-amino-2-deoxy-fucose, and 2-fluoro-2-deoxy-fucose (Figure 11). FucT VI does not tolerate modifications on the 2-OH group of the fucose but will transfer L-Gal and arabinose from their corresponding donors[105] (Figure 12). Both enzymes

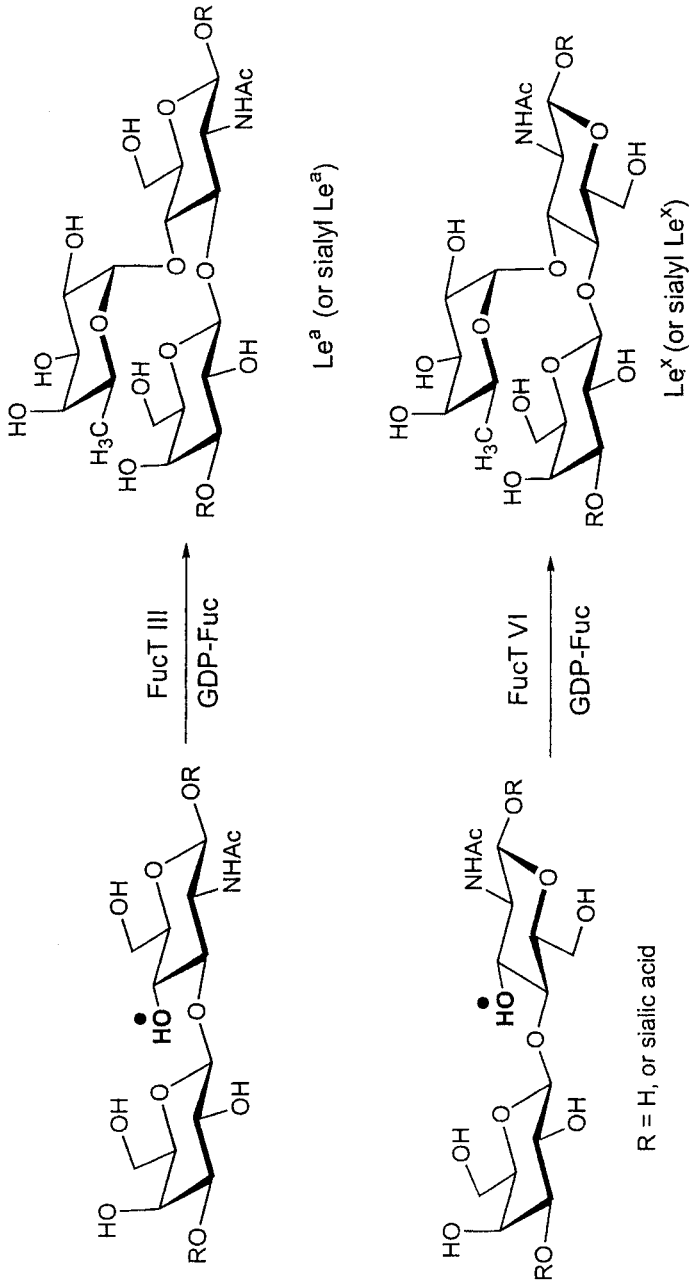

Figure 10. Reactions catalyzed by FucT III and FucT VI.

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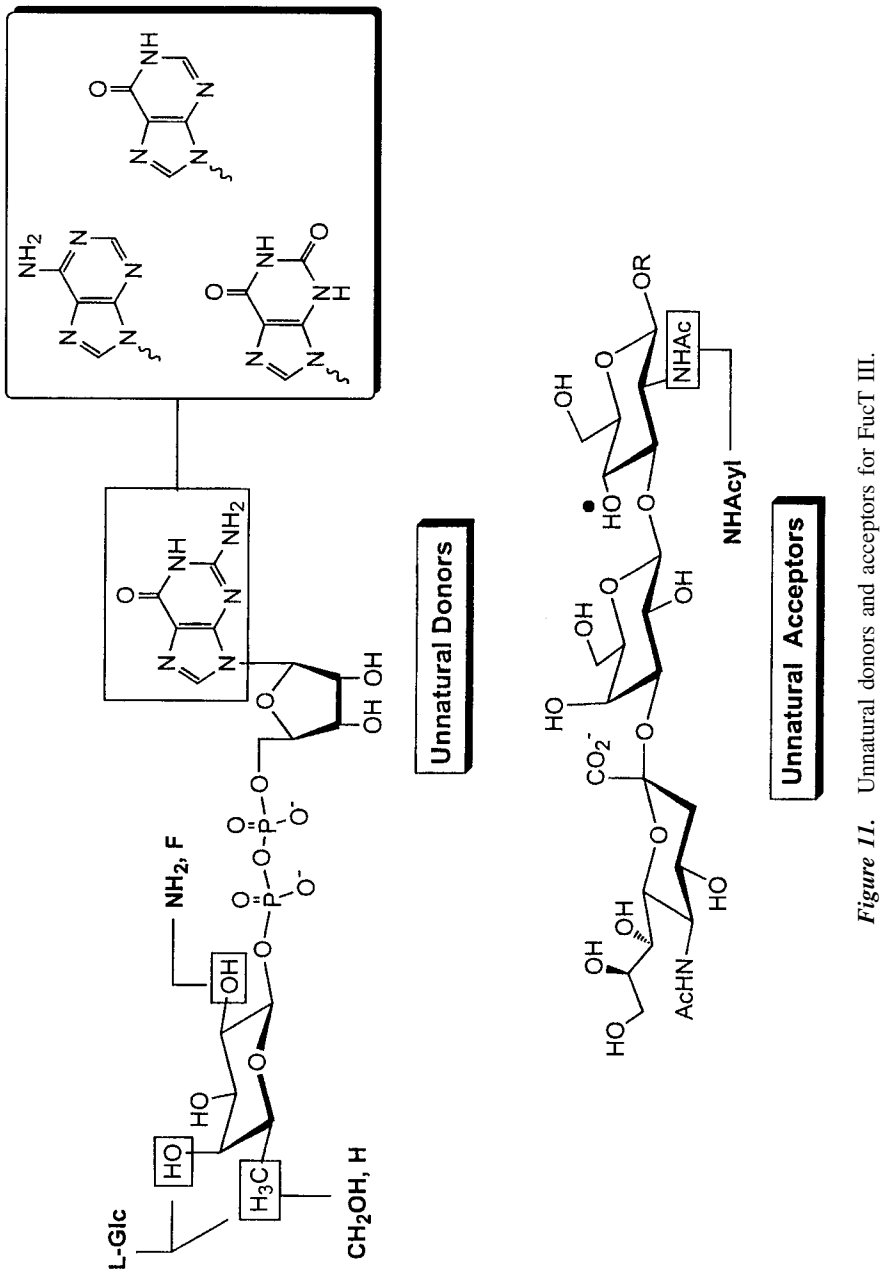

Figure 11. Unnatural donors and acceptors for FucT III.

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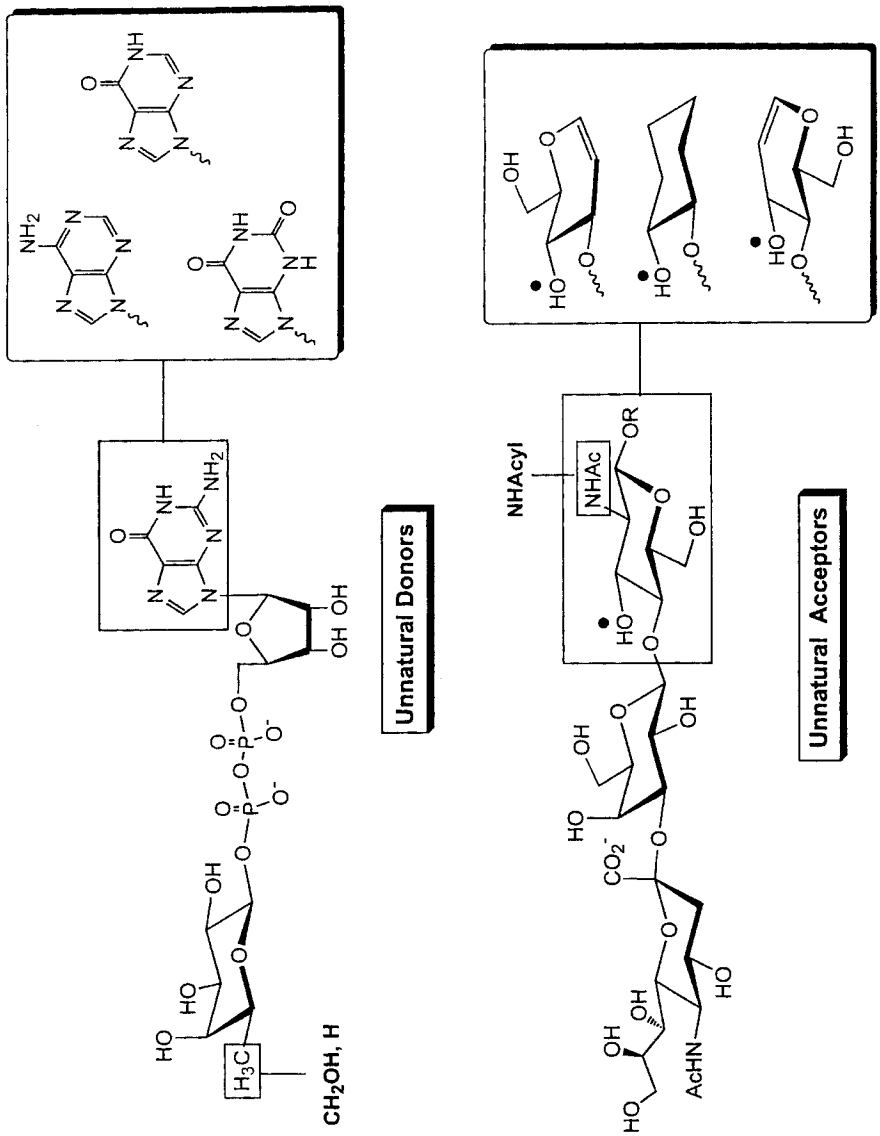

Figure 12. Unnatural donors and acceptors for FucT VI.

accept replacements and modifications of the *N*-acetyl group of the GlcNAc unit.[106–108] FucT VI has been shown to tolerate replacement of the GlcNAc unit with glucal and cyclohexane diol.[109] FucT III and FucT VI have further been used to construct libraries of sialyl Le^a or sialyl Le^x derivatives using both unnatural donors and unnatural acceptors.[110–112]

FucT III and FucT VI have been probed with unnatural donors with a modified purine base. They can tolerate exchange of the guanine by other purine bases such as adenine.[113] These unnatural donors proved to be preparatively efficient in the enzymatic synthesis of Le^a or Le^x, suggesting that natural sugar nucleotide donors can be replaced with inexpensive ones to lower the cost of enzymatic synthesis.

SIALYLTRANSFERASES

Sialyltransferases (SialTs) catalyze the transfer of *N*-acetyl-neuraminic acid from CMP-Neu5Ac to acceptors at or near the nonreducing terminus of oligosaccharide chains of glycoproteins or glycolipids.[114–116] Cell surface sialic acid residues are important as antigenic determinants, as ligands for cellular adhesion, and as receptors for the binding of viruses and bacteria.[117] Sialic acids are usually found in terminal positions linked through an α-glycosidic linkage. The stereoselective synthesis of α-sialosides remains a challenge, since the glycosides have a kinetically and thermodynamically unfavorable equatorial orientation and there is no participating group at C3 to direct the stereochemistry in their synthesis.[118] Enzymatic sialylation therefore offers an attractive alternative to prepare α-sialosides in an efficient and stereocontrolled manner. To date, 16 different mammalian sialyltransferases, several bacterial sialyltransferases, and a viral sialyltransferase have been identified. They are α2,3-, α2,6-, and α2,8-sialyltransferases. A systematic classification for sialyltransferases has been proposed on the basis of the regiochemistry of the sialylated reaction product and the acceptor substrate preference.[116] Two different recombinant α2,3-sialyltransferases and an α2,6-sialyltransferase from rat liver are now commercially available in sufficient quantities (100 mU) for use in preparative synthesis.

α2,3-Sialyltransferase and α2,6-Sialyltransferase

α2,3-SialT (α2,3-SialT, EC 2.4.99.6, ST3Gal III) from rat liver transfers a sialic acid unit from CMP-sialic acid to 3-OH of the terminal Gal residue in Galβ1 → 4GlcNAc or Galβ1 → 3GlcNAc sequences (Figure 13) while α2,6-SialT (α2,6-SialT, EC 2.4.99.1) transfers the sialic acid to 6-OH of the terminal Gal residue in Galβ1 → 4GlcNAc (Figure 14).[115,116]

The finding that CMP-sialic acid synthetase can accept many sialic acid analogs has facilitated the production of donor analogs for sialyltransferase specificity studies.[119–121] Both α2,3-SialT and α2,6-SialT tolerate substitutions at C9 of CMP-sialic acid (Figures 15 and 16).[120,122,123] The 9-OH group can be replaced with fluoro, azido, amino, acetamido, hexanoylamido, benzamido, and fluorescent labels. 9-*O*-Acetylsialic acid is also transferred. Donors with the 5-NHAc of sialic acid replaced with OH, $NHC(O)CH_2OH$, or NHCbz (Cbz = benzyloxycarbonyl) are also utilized by

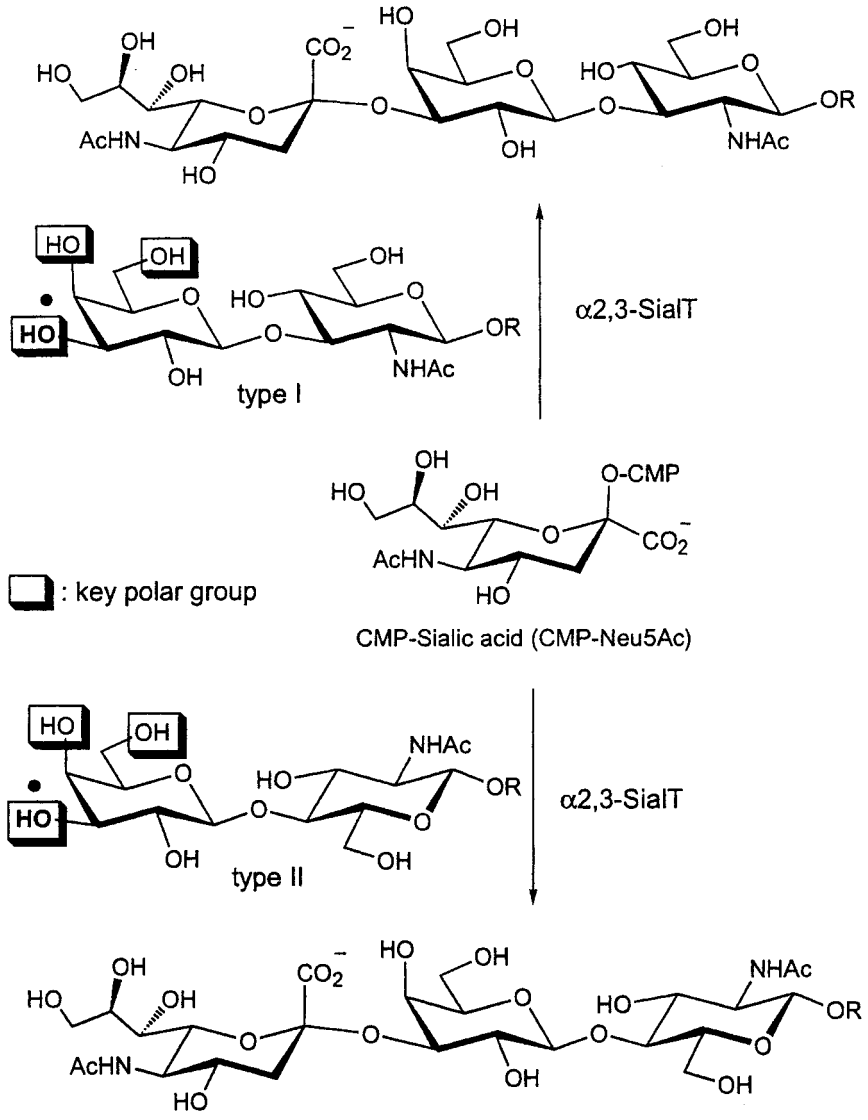

Figure 13. Reactions catalyzed by α2,3-SialT.

α2,3-SialT.[124] CMP-4-deoxysialic acid was found to be a donor substrate for α2,6-SialT.[125]

Chemical mapping studies of acceptor binding have indicated that 6-OH of Gal and 2-NHAc are required for recognition by rat liver α2,6-SialT, while rat liver α2,3-SialT requires an intact 3,4,6-triol system on the Gal residue.[126] Analogs of Galβ1 → 4GlcNAcβ1 → 2Manα-OOct, where the 3-OH or 4-OH group of the Gal residue is deoxygenated or substituted with a fluoro atom, were found to be active as substrates for α2,6-SialT (Figure 16).[127] The 4″-*O*-methyl derivative of this trisaccharide is an ac-

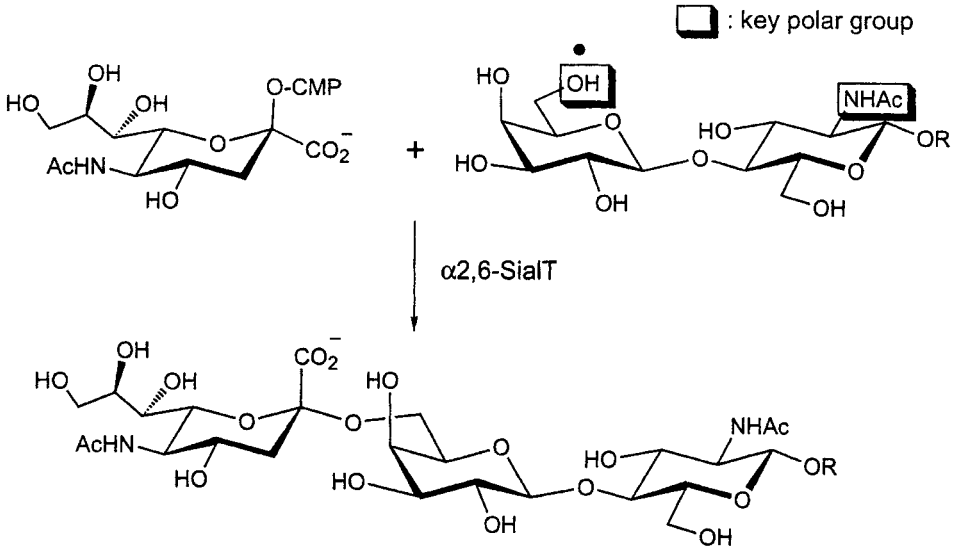

Figure 14. Reaction catalyzed by α2,6-SialT.

ceptor for α2,3-SialT.[127] A variety of substitutions on the *N*-acetyl group of either type I or type II acceptors are tolerated by α2,3-SialT.[128,129] α2,3-SialT also transfers sialic acid to lactal, lactose, and 2-*O*-pivaloyl lactose acceptors (Figure 15).[44] α2,3-SialTs from *N. meningitidis*[17,130] and myxoma virus[18] and an α2,6-SialT from *Photobacterium damsela*[16] have acceptor specificities somewhat different from those of their mammalian counterparts, allowing for broader synthetic applications. Large-scale production of CMP-NeuAc and sialylated oligosaccharides has been achieved by bacterial coupling.[131]

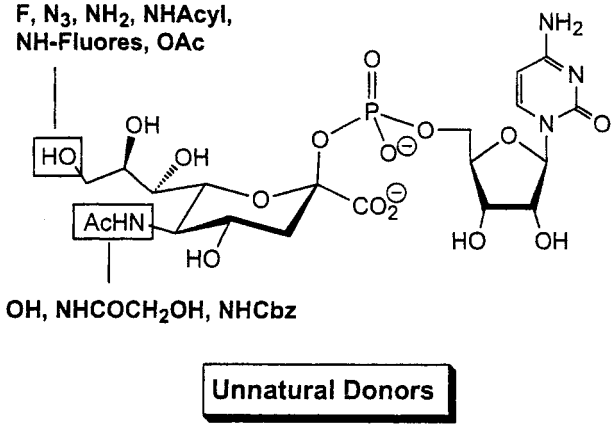

Figure 15. Unnatural donors and acceptors for α2,3-SialT.

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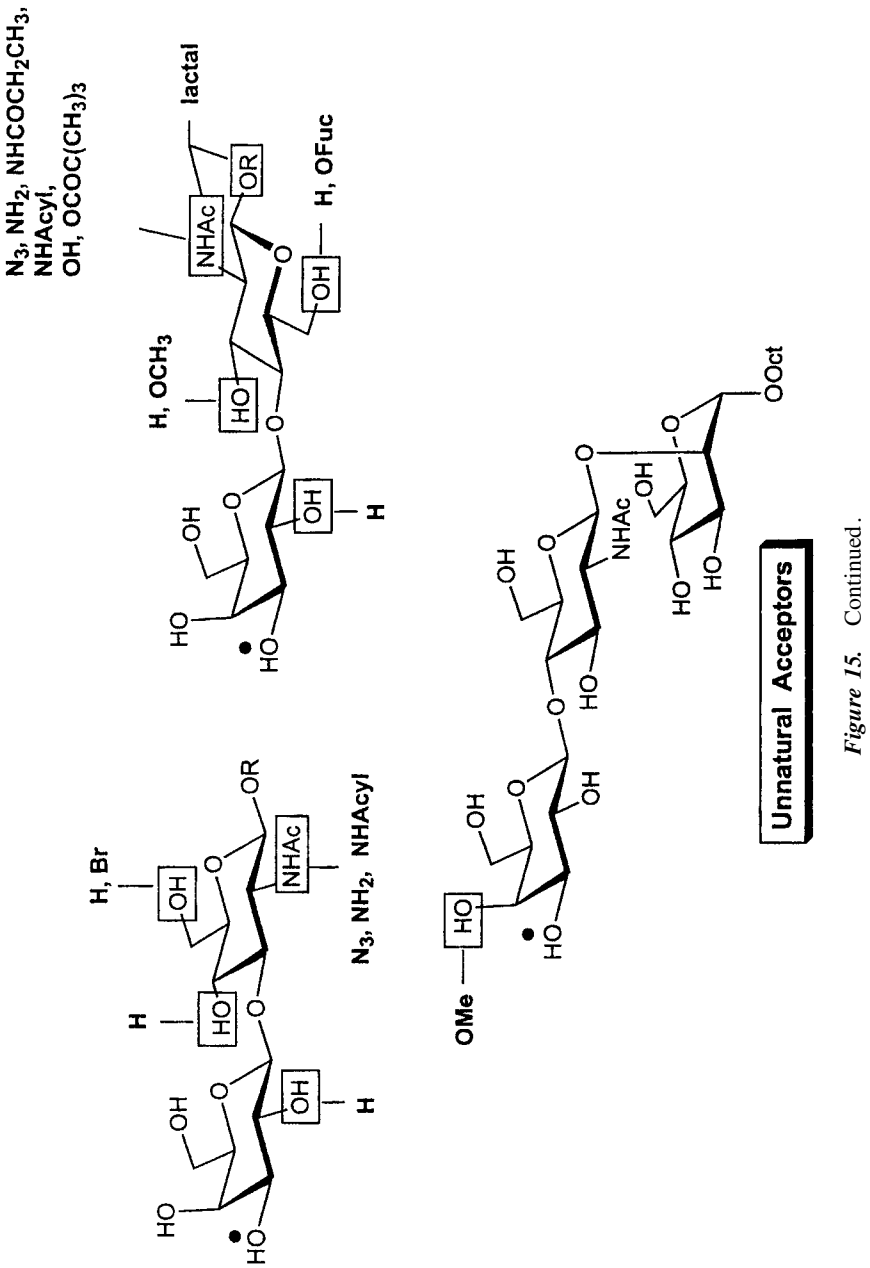

Figure 15. Continued.

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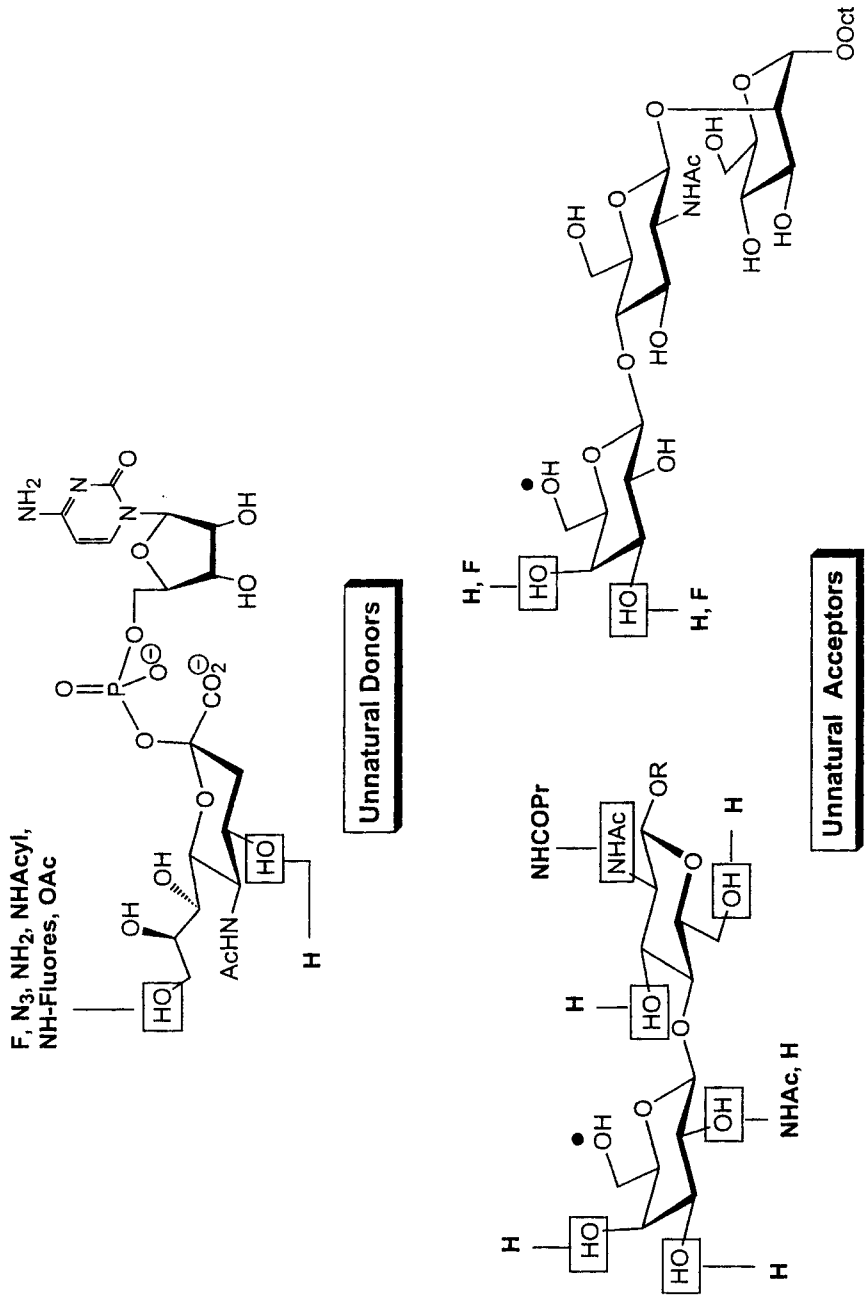

Figure 16. Unnatural donors and acceptors for α2,6-SialT.

PRACTICAL BIOCATALYSIS WITH GLYCOSYLTRANSFERASES

There are no fundamental differences between chemical catalysts and biological enzyme catalysts. Enzymes accelerate the rates of chemical reaction without being changed in the overall transformation; they reduce the activation energies of chemical reactions. The only practical differences between chemical and enzyme catalysts are that enzymes are generally sensitive to temperature, pH, and shear.

The most critical parameter for using an enzyme in synthesis is defining the quantity or activity of enzyme that will be added to ensure that the reaction goes to completion. This amount should first be determined in a defined standard kinetic assay and then with the substrate that will be employed in the reaction. The amount of enzyme activity is defined by units (U), where one U of enzyme activity is the amount producing 1 μmol of product per minute. Substrate and product inhibition are frequently observed in enzyme catalysis; therefore it is desirable to carry out a full kinetic analysis by varying substrate concentrations and determining the V_{max} (maximal rate of reaction at saturating substrate) and K_M for the substrate. This analysis will show whether substrate inhibition occurs at high concentrations that would reduce the reaction rate. As well, by estimating the rate of reaction at a given substrate concentration, the time required for completion can be estimated. A standard assay can also be used to check the viability of the enzyme catalyst throughout the reaction by removing a small aliquot from the reaction mixture and assaying for remaining activity in a standard assay.

Water is the usual reaction medium for enzymes; however, organic solvents or cyclodextrins can be added to increase the solubility of poorly soluble compounds. While multienzyme donor recycling systems can be used for in situ donor production,[12,19] the following examples utilize stoichiometric donors.

Glycosyltransferase Assays

After the glycosyltransferase of interest has been purchased or isolated, it is desirable to verify the activity of the enzyme with a standard substrate and the compound of interest under the conditions of the preparative synthesis. Methods for assaying glycosyltransferase activity are summarized in Table 1, and a suitable method should be selected based on the characteristics of the substrate and the required sensitivity of the assay. Kinetic studies to obtain K_M and V_{max} values for the substrate and optimization of reaction conditions, including buffer and pH, should also be carried out.

If the compound of interest has a very low reaction rate ($<1\%$ of the activity of a known standard substrate), further evaluations with concentrated enzyme are recommended. There are examples of traces of reactive substrates remaining at these low levels in inactive compounds. Since 0.1% conversion of a reactant to product can easily be detected in radiochemical glycosyltransferase assays, confirmation that the low rate of conversion is not due to the presence of contaminants is essential. This level of contamination in modified synthetic substrates is sometimes difficult to detect even by high-field NMR spectroscopy. Therefore before the actual preparative synthesis is performed, 10% conversion of substrate to product should be verified by assay to ensure that the low rate of reaction is not due to traces of substrate in a largely inactive compound.

Table 1. Assays for Glycosyltransferases

Acceptor	Donor	Additives	Method	Ref.[a]
1 Glycosides	Radioactive sugar nucleotides		C18 Sep-Pak	1
2 Glycosides	Radioactive sugar nucleotides		HPLC	2
3 Glycosides	Radioactive sugar nucleotides		Ion exchange column chromatography	3
4 Glycosides	Radioactive sugar nucleotides		Thin-layer chromatography	4
5 Glycoprotein	Radioactive sugar nucleotides		Affinity chromatography	5
6 Glycoprotein	Radioactive sugar nucleotides		Particle exclusion assay	6
7 Long alkyl glycosides	Radioactive sugar nucleotides		Organic solvent extraction	7
8 Glycosides	Radioactive sugar nucleotides		GC and mass spectometry	8
9 Glycoprotein	Radioactive sugar nucleotides		Filtration with membranes	9
10 Glycosides with high molecular weights	Radioactive or labeled sugar nucleotides		Size-exclusion column chromatography	10
11 Glycosides-conjugates	Sugar nucleotides	Antibody (or antiserum, lectin)	ELISA	11
12 Glycosides	Sugar nucleotides	Coupling enzymes (NADH)	Coupled enzyme assays	12
13 Glycosides	Sugar nucleotides	Fluorochrome	Microtiter-based fluorescence assay	13
14 Labeled glycosides	Sugar nucleotides		HPLC	14
15 Labeled glycosides	Sugar nucleotides		Capillary electrophoresis	15
16 Biotin-labeled glycan	Sugar nucleotides	HRP-avidin	Affinity chromatography	16
17 Glycosides	Sugar nucleotides		Mass spectrometry HPLC	17
18 Radioactive acceptor	Sugar nucleotides		HPLC, size exclusion column chromatography	18

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Enzyme and Substrate Concentrations

The activity obtained in an assay allows an estimation of the amount of enzyme that will be required to completely convert substrate to product in a reasonable reaction time (e.g., 6–12 h). Reaction rates will decrease as the substrate is consumed and its concentration drops. Therefore reaction times to achieve quantitative conversions are always three to ten times longer than those calculated, and even longer for substrates with high K_M values. Preparative synthesis should start with substrates at kinetically saturated concentrations, about five times their K_M values. In the case of substrates with a severe solubility problem, the addition of organic solvents to the buffer may aid dissolution.[46] Plastic containers (polypropylene falcon tubes, microcentrifuge tubes, or scintillation vials) are preferred to glass for glycosyltransferase reactions, since these enzymes are inactivated on glass surfaces. The addition of bovine serum albumin (BSA: $\sim$1 mg/mL) or glycerol ($\leq$50%) to glycosyltransferase reaction mixtures can stabilize the enzymes during long reactions. Reactions can be carried out at ambient temperature or at 37°C. In stoichiometric reactions, nucleotides are produced after the monosaccharide is transferred from the donor to the acceptor saccharide. Nucleotides are potent inhibitors of glycosyltransferases; however, they can be hydrolyzed to their corresponding weakly inhibitory nucleosides by adding alkaline phosphatase to the reaction mixtures.

Reaction Monitoring

Thin-layer chromatography (TLC) is a convenient means of monitoring the progress of reactions throughout the preparative synthesis. Both donor and acceptor can be monitored by using Silica Gel 60-F254 plates. After aqueous enzymatic reactions have been spotted, the plates should be completely dried under high vacuum. Then the plates are developed with appropriate solvent mixtures. For UDP-Gal donor, $CHCl_3$/MeOH/H_2O/AcOH (4:5:2:1) will differentiate between the donor and its reaction products UDP, UMP, and uridine. Both UV and spray reagents [anisaldehyde/H_2SO_4/EtOH (1:1:18) with heating] can be used for detection. If the presence of nucleotides is observed during the reaction, the alkaline phosphatase is probably not active. In this case, the addition of more alkaline phosphatase or adjustment of the pH with base will be required. Sugar nucleotides also can degrade in reactions, especially in the presence of Mn^{2+} ions. This requires the addition of sugar nucleotide donors to drive reactions to completion. Aliquots of reaction mixtures can also be analyzed by high-performance liquid chromatography (HPLC), matrix-assisted laser desorption ionization (MALDI) mass spectrometry, or NMR spectroscopy to monitor the reaction progress. At the end of the reaction it is often difficult to establish that all the acceptor has been completely converted to product. An aliquot of the reaction mixture can be removed and used in place of an acceptor substrate solution in a standard radiochemical assay. Additional glycosyltransferase and radiolabeled donor must be added. If no activity (radiolabeled product) is detected, the acceptor has been depleted from the reaction mixture.

The activity of glycosyltransferases can also be monitored throughout the reaction course by removing a small aliquot from the reaction, followed by dilution and assay of

activity by one of the standard assays with defined substrates in Table 1. It is desirable to monitor the pH of the reaction closely, especially when the concentration of the substrate is relatively high compared to molarity of the buffer. The pH of glycosyltransferase reactions drops as reactions proceed as a result of the production of phosphoric acid by alkaline phosphatase. Acidic pH values can inactivate glycosyltransferases and substrates, and decrease the efficiency of alkaline phosphatase.

Product Isolation

For hydrophobic products like glycolipids or other aliphatic aglycones, C_{18} reversed phase supports can be used for their isolation from enzyme reactions. In milligramscale synthesis, C_{18} Sep-Pak cartridges from Waters perform well. The resin or cartridge should be preconditioned before use by washing with MeOH, then equilibrated with water before aqueous enzymatic reaction samples are loaded. Organic solvents such as glycerol or DMSO in enzymatic reactions prevent substrates from binding to the C_{18} resin. In these cases, a dilution with water to less than 1% organic solvent in the enzymatic samples prior to loading on columns is recommended.

Ion exchange chromatography can be used for the isolation of sialylated products and oligosaccharides with amine groups. Size-exclusion chromatography serves well for the isolation of reducing sugars or glycoconjugates with high molecular weights. Adsorption on charcoal is another practical method for the purification of oligosaccharides. A recent publication focuses on the separation of oligosaccharides.[132]

Example 1: Synthesis of α-D-Gal*p*(1→3)-β-D-Gal*p*(1→4)-β-D-Glc*p*-O-$(CH_2)_8CO_2Me$

Recombinant α1,3-GalT was assayed by standard radiochemical assay. In this assay, acceptor (β-D-Gal*p*(1→4)-β-D-Glc*p*-O-$(CH_2)_8CO_2Me$, 47 nmol), donor (UDP-Gal, 10 nmol), and radioactive donor (UDP-[^{3}H]Gal, about 70,000 dpm) were incubated with enzyme in assay buffer (100 mM sodium cacodylate, 20 mM $MnCl_2$, 0.1 mg/mL BSA, pH 6.5) in a final volume of 20 μL for 10 min at 37°C. The reaction mixture was diluted with water to 200 μL and loaded onto a reversed-phase SepPak C_{18} cartridge that had been preequilibrated with MeOH (10 mL) and then with water (10 mL). After loading, the cartridge was washed with water to remove buffer components and unreacted donor until the counts in the eluent were background (about 50 mL). Radiolabeled trisaccharide product was eluted with MeOH (3.5 mL). The MeOH eluates were quantitated for radioactivity by liquid scintillation counting after addition of EcoLite (+) scintillation cocktail (from ICN, 10 mL). The enzyme activity of the stock solution was 4.4 U/mL.

The components of a 4.5 mL mixture of acceptor (β-D-Gal*p*(1→4)-β-D-Glc*p*-O-$(CH_2)_8CO_2Me$, 122 mg, 238 μmol, 50 mM in the reaction), UDP-Gal (dipotassium salt, 168 mg, 262 μmol, 55 mM in the reaction), α1,3-GalT (1 mL, 4.4 U/mL), and alkaline phosphatase (Boehringer Mannheim, 23.8 μL, 1 U/μL) were combined in a 15 mL plastic falcon tube. The buffer was 124 mM MOPS, pH 7.5, containing 2 mM $MnCl_2$ and 1 mg/mL BSA. The mixture was incubated at room temperature with gentle rotation.

After 24 h, the pH of the solution was 6.5. TLC ($CHCl_3$/MeOH/H_2O, 65:35:8) showed that almost all the starting disaccharide had been converted to trisaccharide product. Unreacted, excess UDP-Gal and uridine were also observed by TLC ($CHCl_3$/MeOH/H_2O/AcOH, 4:5:2:1). When an aliquot of the reaction mixture (6 μL, 1/30 diluted enzymatic reaction mixture) was assayed by the standard assay described above, the MeOH eluent gave 13,000 dpm, indicating that α1,3-GalT in the reaction mixture was still active. The same assay in the absence of additional acceptor with 6 μL (undiluted) of the reaction mixture gave 430 dpm in the methanol eluent, indicating that a trace of unreacted disaccharide remained.

After 48 h, the pH of the reaction was 6.5. The standard radiochemical assay for activity mixture gave 12,000 dpm in the MeOH eluent, showing minimal loss of enzyme activity in the reaction mixture. Assay in the absence of added acceptor solution gave 211 dpm, close to background 100–200 dpm, indicating that all the disaccharide had been converted to trisaccharide product.

The reaction mixture was centrifuged ($1450 \times g$), and the resultant clear supernatant was loaded onto a C_{18} column (5 g, 125 Å) that had been pretreated with MeOH and conditioned with water (30 mL) prior to the loading of the centrifuged sample. The column was washed with water (100 mL) then 1% acetonitrile (70 mL). The desired trisaccharide product was eluted with MeOH (HPLC grade, 120 mL). This eluate was concentrated under reduced pressure, and the product was dissolved in water (10 mL). This solution was passed through a Millex-GV filter (Millipore) to remove any residues of C_{18} resin. The filtrate was lyophilized to yield product as a fluffy white powder (133 mg, 196 mmol, 82%). NMR spectroscopy confirmed that the conversion was complete.

Example 2: Synthesis of α-D-Gal*p*(1→3)-β-D-Gal*p*(1→4)-α-D-Glc*p*-O-$(CH_2)_8CO_2Me$

Based on the standard radiochemical assay described above, an α-lactose derivative (β-D-Gal*p*(1 → 4)-α-D-Glc*p*-O-$(CH_2)_8CO_2Me$) has a relative rate of transfer by α1,3-GalT that is 0.03% compared to that of the corresponding β-lactoside (β-D-Gal*p*(1 → 4)-β-D-Glc*p*-O-$(CH_2)_8CO_2Me$). Therefore, α1,3-GalT has 1 mU of activity for the α-lactoside compared to 3.3 U of activity for the β-lactoside. Expression cloning of α1,3-GalT[66,71] provides sufficient quantities for conversion of substrates with such low activities. For the following reaction, standard enzymatic activity was calculated for the α-lactoside. A mixture of acceptor (β-D-Gal*p*(1 → 4)-α-D-Glc*p*-O-$(CH_2)_8CO_2Me$, 20 mg, 39 μmol), UDP-Gal (dipostassium salt, 30 mg, 47 μmol), α(1 → 3) GalT (152 mU) in 3.8 mL of 190 mM MOPS buffer, pH 7.5 containing 3.8 mM DTT, 1 M sodium chloride, $MnCl_2$ solution (1 M, 98 μL), BSA solution (100 mg/mL, 40 μL), and alkaline phosphatase (1 U/μL, 20 μL) was incubated in a 15 mL plastic falcon tube at room temperature with gentle rotation. After 4 days, the enzyme was still active when assayed with an aliquot of the enzymatic reaction and the β-lactoside as an acceptor. After 5 days, TLC was performed ($CHCl_3$/MeOH/H_2O, 65:35:8) and showed that all the starting disaccharide had been converted to trisaccharide. The reaction mixture was loaded onto a preconditioned Sep-Pak reversed phase cartridge. The cartridge was washed with water (100 mL) and 1% acetonitrile (20 mL); then trisaccharide product was eluted in 20% acetonitrile (100 mL). This

eluate was concentrated, and the residue was loaded on a 75 mL Iatrobead column (Iatron Laboratories) in $CHCl_3$/MeOH/H_2O (65:35:5). Chromatography was performed under gravity, with collection of 400 drop fractions. The fractions containing the desired products (nos. 21–36) were collected and concentrated to dryness under reduced pressure. The dried sample was loaded on a preconditioned Sep-Pak cartridge, and the cartridge was washed with water (20 mL) to remove Iatrobeads. Product was eluted with MeOH (20 mL) and this eluate was concentrated. Water (10 mL) was added to the residue and this solution was passed through a Millex-GV filter to remove residues of C_{18} resin. The filtrate was lyophilized to yield product as a fluffy white powder (18 mg, 26 μmol, 66%).

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