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Enzymatic synthesis of oligosaccharides

Kurt G. I. Nilsson

As the importance of the oligosaccharide moieties of glycoproteins and glycolipids is being increasingly recognized, efforts to synthesize them are expanding. The number of functional groups of carbohydrate monomers and the variety of configurations that oligomers can adopt is greater than with nucleotides/nucleic acids or amino acids/peptides. By reversing the hydrolytic action of glycosidases and by using highly regiospecific glycosyltransferases, enzymatic oligosaccharide synthesis can be performed.

Why is it necessary to synthesize oligosaccharides? The fundamental answer is that we want to explore and exploit for human ends the biological activity of these polymers following synthetic and biosynthetic work on oligopeptides and oligonucleotides. The complex oligosaccharide chains (glycans) of glycoproteins and glycolipids mediate or modulate a variety of biological processes[1–9]. For example, the glycoconjugate oligosaccharides serve as cell surface receptors (e.g. for influenza and other viruses, bacteria, bacterial toxins, blood-group and tumor-specific antibodies, circulating lymphocytes and for a variety of lectins); they are important for intracellular migration and secretion of glycoproteins and for clearance of glycoproteins from circulation by hepatocytes; they are involved in cell adhesion; they serve as modulators of cell growth; and they change during cellular differentiation. Moreover, there are numerous reports on alterations of glycans after malignant transformation[2,4,6–11]. Antibodies against cancer-associated carbohydrate antigens are being used in diagnostic kits (e.g. pancreas, colon cancer)[6] or in immunotherapy (e.g. melanoma patients)[2]. The importance of the carbohydrate portion of serum glycoproteins for their half-lifes in the circulation and their immunogenicity has been recognized[12] and *in-vitro* glycosylation of recombinant proteins has been attempted[13].

Knowledge of the various glycoprotein and glycolipid glycan structures has increased dramatically in the last decade because of the development of permethylation[14] and NMR analysis. It is well known that the combination of different amino acids gives a huge variety of peptides and proteins. But the number of possible combinations of a given number of carbohydrates monomers is much higher because there are many possible linkage sites on each and at each site there is the possibility of different anomeric configuration (α- or β-glycosidic linkages). However, the glycan structures of glycoconjugates are not randomly constructed. On the contrary, they may be divided into families in which structures are similar and contain common oligosaccharide sequences (Table 1). The most common carbohydrate chains in glycoproteins are high-mannose- and complex-type, asparagine-linked (*N*-glycosidic) or serine/threonine-linked (*O*-glycosidic) oligosaccharides. Similarly, glycolipids can be divided into five main structural series. Nevertheless, the diversity of the glycoconjugate oligosaccharides evident from Table 1 allows for biological specificity. Indeed, it has been proposed that 'the specificity of many natural polymers is written in terms of sugar residues and not of amino acids or nucleotides' (Ref. 15). The synthesis of such complexes represents a stiff challenge.

Importantly, however, short fragments of glycan structures (Table 1) are sufficient for biological specificity[1,2,6,11]. These have been used in affinity chromatography, for preparing neoglycoconjugates, for incorporation into liposomes, for immunization, and for characterization of antibodies, glycosidases, glycosyltransferases and lectins. They have also been used in the development of diagnostic kits or targeting of drugs[7–9,16–19]. For example, a sensitive and specific assay for identifica-

Glossary

Aglycon – the non-sugar portion of a glycoside
Bn – Benzyl ($PhCH_2$-)
Bz – Benzoyl
Neu5Ac (NeuAc, Sia) – *N*-Acetylneuraminic acid
Gal – D-Galactopyranose
GlcNAc – 2-acetamido-2-deoxy-D-glucopyranose
GalNAc – 2-acetamido-2-deoxy-D-galactopyranose
Man – D-mannopyranose
Fuc – L-Fucopyranose

Abbreviated nomenclature of oligosaccharides according to IUB–IUPAC recommendations[66].

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Table 1

Glycoconjugates and bioactive glycans

Types of glycan in glycoconjugate	
N-Glycosylproteins	Glycolipids
Oligomannosidic type (high-mannose type)	Globo series
N-Acetyllactosaminic type (complex type)	Lacto series
Hybrid type	Bactofucapentaosyl (IV)Cer
O-Glycosylproteins	Muco series
Mucin type	Gala series
	Ganglio series

Examples of glycan structures	
An *N*-acetyllactosaminic glycan (complex type)	NeuAc(α2–6)Gal(β1–4)GlcNAc(β1–2)Man(α1–3) Man(β1–4)GlcNAc(β1–4)GlcNAc(β1–N)ASn Gal(β1–4)GlcNAc(β1–2)Man(α1–6) Fuc (α1–6) Fuc (α1–3)
A lactoglycolipid glycan	Fuc(α1–2)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc(β1–1)Cer

Examples of short bioactive glycan structures	
Blood group active structures	
Fuc(α1–2)Gal(β1–3)GlcNAc . . .	H structure
GalNAc(α1–3)[Fuc(α1–2)]Gal(β1–3)GlcNAc . . .	A structure
Gal(α1–3)[Fuc(α1–2)]Gal(β1–3)GlcNAc. . .	B structure
Receptors for pathogens	
Gal(α1–4)Gal(β1)–R	P-fimbriated *E. coli*
Tumor-associated antigens	
NeuAc(α2–8)NeuAc(α2–3)Gal(β1–4)Glc(β1)–R	melanoma
NeuAc(α2–3)Gal(β1–3)GlcNAc(β1)–R	various cancers
NeuAc(α2–3)Gal(β1–3)[Fuc(α1–4)]GlcNAc(β1)–R	pancreas, colon cancer
Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1)–R	gastrointestinal adenocarcinoma

Some conversions/applications of various oligosaccharide glycosides	
Allyl glycosides	2-Bromoethyl glycosides
Inhibitors	Neoglycoproteins
Temporary anomeric protection	Affinity supports
Polysaccharide copolymers	Neoglycolipids
Affinity adsorbents	Glycosides for affinity labelling or coupling
Neoglycoproteins	Methyl glycosides
Affinity labels	Inhibition studies
Benzyl glycosides	Temporary anomeric protection
Inhibition studies	*p*-Nitrophenyl glycosides
Temporary anomeric protection	Enzyme substrates
	Inhibition studies
	Coupling to proteins, affinity adsorbents
	Affinity labelling

tion of P-fimbriated *E. coli* (a pathogen characteristic of urinary tract infections) was based on the detection of the disaccharide structure Gal(α1–4) Gal[20,21].

The range of possible applications and the need for well characterized oligosaccharides in sufficient amounts for fundamental studies has made the synthesis of complex carbohydrates a major challenge in synthetic chemistry.

Chemical versus enzymatic synthesis

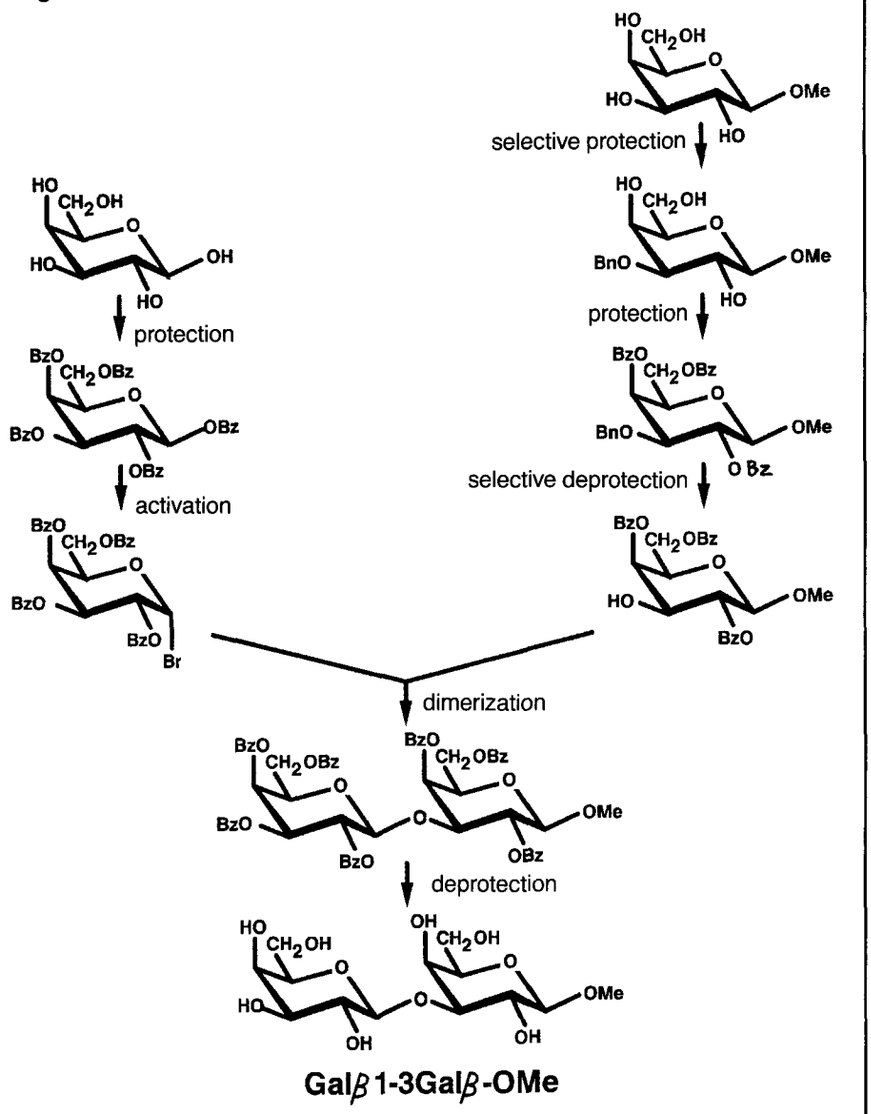
Chemical methods for the synthesis of oligosaccharides are well developed[22–25]. However, carbohydrates contain multiple hydroxyl

groups of similar reactivity and chemical methods are complicated by the many protection and deprotection steps that are necessary for regioselective synthesis (Fig. 1).

The number of steps increases with the size of the oligosaccharide, so that, while synthesis of a disaccharide may require five to seven steps, a trisaccharide may require more than ten steps. Total yields are often low and large-scale synthesis is not practical. In addition, stereospecific reactions giving the correct anomer (α or β) are often difficult. In particular, in the synthesis of sialic acid-containing oligosaccharides, the desired α-sialoside must be separated from the unnatural β-isomer[9,25–27].

Enzymatic methods are becoming accepted as routine procedures in synthesis of organic compounds[28]. The use of enzymes in synthesis of complex carbohydrates has not been extensively investigated, yet it offers several advantages over chemical methods. A wide variety of regiospecific and often highly regioselective reactions can be catalysed very efficiently without protection of the hydroxyl groups; these take place under mild conditions, often at room temperature and close to neutral pH, and organic solvents and hazardous chemicals or catalysts can be avoided. Immobilization of the enzyme allows reuse while production of the catalyst by fermentation facilitates large-scale synthesis. Two types of enzyme have been used for preparation of complex oligosaccharides, the glycosyltransferases (EC 2.4) and the glycosidases (EC 3.2).

Fig. 1



Typical chemical synthesis of a disaccharide.

Glycosyltransferases and glycosidases as catalysts

The glycosyltransferases[1,2,29] catalyse the stereo- and regiospecific transfer of a monosaccharide from a donor substrate (glycosyl nucleotide) to an acceptor substrate. They are classified by the sugar transferred from donor to acceptor and by the acceptor specificity (Table 2). The regio- and stereospecificity, the high selectivity for the acceptor substrate and the high yields that can be achieved are attractive features of these catalysts.

In most animals glycosyltransferases are present in low concentrations and are bound to intracellular membranes and thus require special methods for purification. However, the literature on purification of these enzymes is extensive and it is relatively easy to purify sufficient amounts for preparative scale synthesis in one to two weeks. Today only a few transferases are commercially available. The cloning of transferase genes will change this: the cloning of the cDNA for β-galactoside-α-2,6-sialyltransferase (EC 2.4.99.1) has been reported[30].

Most, if not all, glycosidases (in this context, strictly *O*-glycosidases) can transfer the glycosyl moiety of a substrate to acceptors other than water and hydrolysis represents merely a special case in which water serves as an acceptor[31,32]. The general reaction can be written:

$$DOR + R'OH \rightleftharpoons DOR' + ROH$$

where DOR symbolizes the glycosyl donor and R'OH the acceptor. Hydrolysis (where R' = H; R = organic group), equilibrium controlled synthesis (R = H; R'OH = sugar) or transglycosidation (kinetically controlled reaction; R = organic group;

Table 2

Reactions catalysed by glycosidases and glycosyltransferases

Examples of glycosyltransferase reactions		Ref.
β-D-*Galactoside(α2–6)sialyltransferase*		
CMP-Neu5Ac + Gal(β1–4)GlcNAc	→ Neu5Ac(α2–6)Gal(β1–4)GlcNAc + CMP	58, 59[a]
β-D-*Galactoside*(α1–2)*fucosyltransferase*		
GDP-Fuc + Gal(β1)–R	→ Fuc(α1–2)Gal(β1)–R + GDP	56[a]
N-*Acetylglucosaminide*(β1–4)*galactosyltransferase*		
UDP-Gal + GlcNAc	→ Gal(β1–4)GlcNAc + UDP	56, 57[a]
UDP–Gal + Fuc(α1–6)GlcNAc(β)–OR	→ Gal(β1–4)[Fuc(α1–6)]GlcNAc(β)–OR	60[a]
CMP–Neu5Ac + Neu5Ac(α2–3)Gal(β1–3)GalNAc(α)–OPh	→ Neu5Ac(α2–3)Gal(β1–3)–[Neu5Ac(α2–6)]GalNAc(β)–OPh	61[a]
CMP–Neu5Ac + Gal(β1–3)GlcNAc(β)–OMe	→ Neu5Ac(α2–3)Gal(β1–3)GlcNAc(β)–OMe	54, 58[a]

[a]Reactions conducted preparatively.

Linkages cleaved by

exoglycosidases

α-*Galactosidase*
Gal(α1)↓–R

β-*Galactosidase*
Gal(β1)↓–R

α-*Mannosidase*
Man(α1)↓–R

β-*Mannosidase*
Man(β1)↓–R

α-*N-Acetylgalactosaminidase*
GalNac(α1)↓–R

β-*N-Acetylhexosaminidase*
GlcNAc(β1)↓–R

α-L-*Fucosidase*
Fuc(α1)↓–R

endoglycosidases

Endo-β-galactosidases

GlcNac(β1–3)Gal(β1↓–4(3))GlcNAc or Glc

Endo-β-N-acetylglucosaminidase

R′ Man(α1–6)
Man(β1–4)GlcNAc(β1↓–4)GlcNAc(β1–N)Asn
R′ Man(α1–3)

R′OH = alcohol, sugar) are, therefore, catalysed by most glycosidases.

Glycosidases are broadly classified as exoglycosidases, which act on glycosidic linkages at the non-reducing end of saccharide chains (i.e. D = monosaccharide group) or endoglycosidases acting on glycosidic linkages within saccharides. They are further characterized by their specificity for the glycosyl moiety of the donor (i.e. D in the scheme above) and by their stereospecificity (Table 2). A certain degree of aglycon (see Glossary) specificity has been reported[33,34]. However, most glycosidases show a less pronounced selectivity than the glycosyltransferases for the acceptor structure. This means that a given glycosidase can be used for the synthesis of a number of glycosides from a given glycosyl donor by employing different acceptors. The less pronounced regioselectivity can produce purification problems (i.e. a mixture of 1–6, 1–4, 1–3 and 1–2 linkages is often obtained). However, preponderant formation of one linkage usually occurs. Different linkages may be obtained by using different sources of the enzyme.

Glycosidases occur widely in viruses, microorganisms, plants and animal cells and are thus easily available. In addition, there is no need for cofactors as is the case with glycosyltransferases and simple, easily available substrates can be used.

These general properties of glycosidases and glycosyltransferases dictate the type of enzyme used and the synthetic strategy. Glycosidases are interesting for synthesis of shorter oligosaccharides while glycosyltransferases are suitable for synthesis of higher oligosaccharides.

Syntheses involving glycosidases

There are similarities between the principles used in glycosidase-catalysed formation of oligosaccharides and protease-mediated peptide synthesis[35]. Glycosidases and proteases are hydrolytic enzymes and synthesis is carried out either as an equilibrium-controlled or as a kinetically controlled process. The hydrolytic activity of these enzymes can of course also be used for preparation of oligosaccharides or peptides from larger structures.

Equilibrium-controlled synthesis

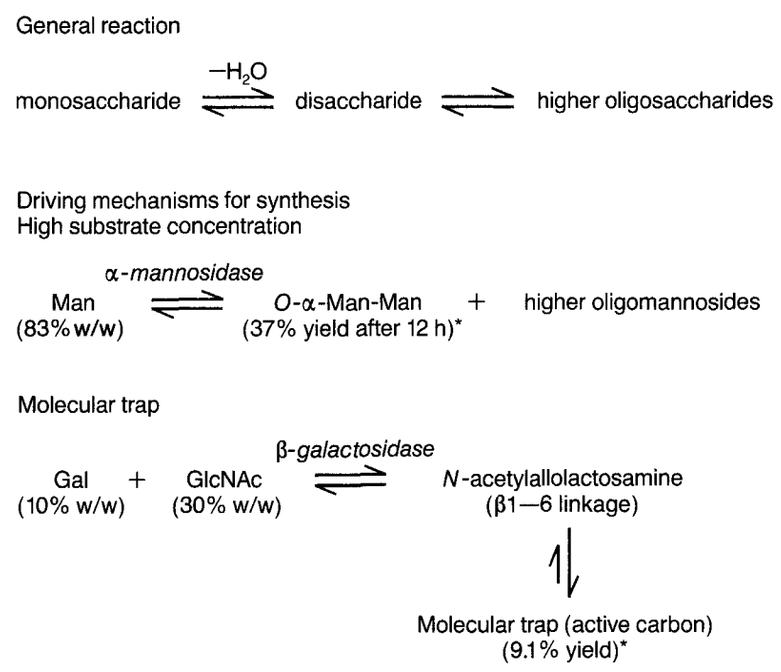
Synthesis of oligosaccharides by reversion of the hydrolytic reaction of glycosidases was demonstrated by Bourquelot and Bridel as early as 1912 (Ref. 36). Since then numerous reports and reviews, mainly describing reactions with glucose or fructose-specific glycosidases have appeared[37,38]. The equilibrium (Fig. 2) favors hydrolysis and the synthetic chemist has to use 'tricks' to increase the oligosaccharide yield.

In protease-mediated peptide synthesis, biphasic systems where hydrophobic products accumulate in the organic phase or products precipitate during the reaction have been used to increase the polymer yield. However, sugars are highly

water-soluble and the above methods have not been applied in glycosidase-catalysed oligosaccharide synthesis. Instead, high concentrations of substrates or molecular traps have been used to obtain reasonable yields.

Oligoglucosides or oligomannosides (mainly 1–6-linked) have been obtained in good yield using a high initial concentration of glucose or mannose (up to 90% w/v) and increased temperature[39,40]. (The high temperature that increased the rate of reaction was possible because high concentrations of sugar stabilize the enzyme activity against heat denaturation.) Wallenfels[41] used a carbon–celite column as a molecular trap to enrich oligosaccharides produced by β-galactosidase. The yield of Gal–GlcNAc isomers (mainly β1–6 linked) produced from galactose and *N*-acetylglucosamine using β-D-galactosidase was also increased from 3% to 15% by circulation of the reaction mixture through an active carbon column[42]. After elution of the products with aqueous ethanol, the column was reused and the total yield of products increased to over 30%, comparable with that obtained by transglycosidation.

Fig. 2



*Equilibrium-controlled synthesis by glycosidases. a: High enzyme concentration and/or temperature is used to speed up the reaction. b: Synthesis can be favored by high substrate concentration and the use of molecular traps such as active carbon. Driving the reaction by partitioning the product into an organic phase or precipitating it is not practical for highly water-soluble oligosaccharides. *(The yields are based on HPLC measurements of unpurified reaction mixtures and are much lower after purification.)*

Kinetically controlled synthesis (transglycosidations)

The transglycosidation reaction was reported in 1935 by Rabate[43]. In this approach a glycoside (e.g. an oligosaccharide like lactose or a glycoside with an aliphatic or aromatic aglycon) is used as glycosyl donor[31,32]. Rapid accumulation of the product in a higher concentration than the equilibrium concentration can be achieved by using an efficient donor substrate. Thus, a much lower amount of enzyme can be used and the yield of product from the kinetically controlled reaction will be higher than that of an equilibrium system where no 'tricks' are used. However, the donor glycoside is consumed during the reaction and its reuse is not possible. Hydrolysis competes and the maximum yield depends on the rate of product formation relative to the rate of hydrolysis (Fig. 3a and b). An efficient acceptor used in a high concentration will favor transglycosidation. As the donor glycoside is consumed while product is formed, the rate of hydrolysis of the transglycosidation product eventually becomes larger than the rate of synthesis. Secondary hydrolysis of product will then decrease the yield to that of equilibrium controlled process. The reaction should, therefore, be followed (e.g. by HPLC) and stopped at the point of maximum yield. If the donor has a high reactivity relative to the product and an excess of acceptor is used, the reaction can be prolonged until the donor is almost consumed[48] (Fig. 3c and d).

Glycosides with aromatic aglycons (e.g. nitrophenyl glycosides) are often used as donors because of their high reactivity. Nitrophenol released during the reaction enables the amount of consumed donor to be estimated spectrophotometrically. Most of these donors are available commercially or are easy to prepare. Cheap oligosaccharides (lactose, raffinose) have also been used successfully as donors.

Yield and regioselectivity

Although there are close similarities with synthesis using proteases, there are also important differences. Both peptide and glycoside synthesis involve condensation between an activated donor acting as electrophile and an acceptor group acting as nucleophile. Hydroxyl is less nucleophilic than NH_2 so that glycoside bond formation will be less favored than, for instance, peptide bond formation. In addition the multiple hydroxyl groups exacerbate problems of regioselectivity. This put high demands on the catalyst in glycoside synthesis. Most glycosidases show a low selectivity for the acceptor structure. As a result, yields and regioselectivity are often

lower in glycosidase-catalysed synthesis than in protease-catalysed synthesis.

The yield of protease-catalysed peptide synthesis increases considerably when the water concentration is lowered by the addition of organic cosolvent. This facilitates, for instance, synthesis of D-amino acid-containing peptides[49]. However, preliminary studies with glycosidases show the reverse: yield is decreased from 32% to 21% upon addition of 30% v/v organic cosolvent[50]. The effect is most pronounced (decreased to 14% with 30% v/v solvent addition) with a more hydrophobic acceptor glycoside, indicating a hydrophobic interaction between the acceptor binding site of the enzyme and the acceptor glycoside (the regioselectivity was very little affected by addition of the cosolvent). Furthermore, the cosolvent decreased the enzyme's catalytic activity. As mentioned already, the hydrophilicity of sugars means they do not tend to precipitate or accumulate in the organic solvents and only further studies will show if organic solvents are of any benefit in glycosidase-catalysed oligosaccharide formation.

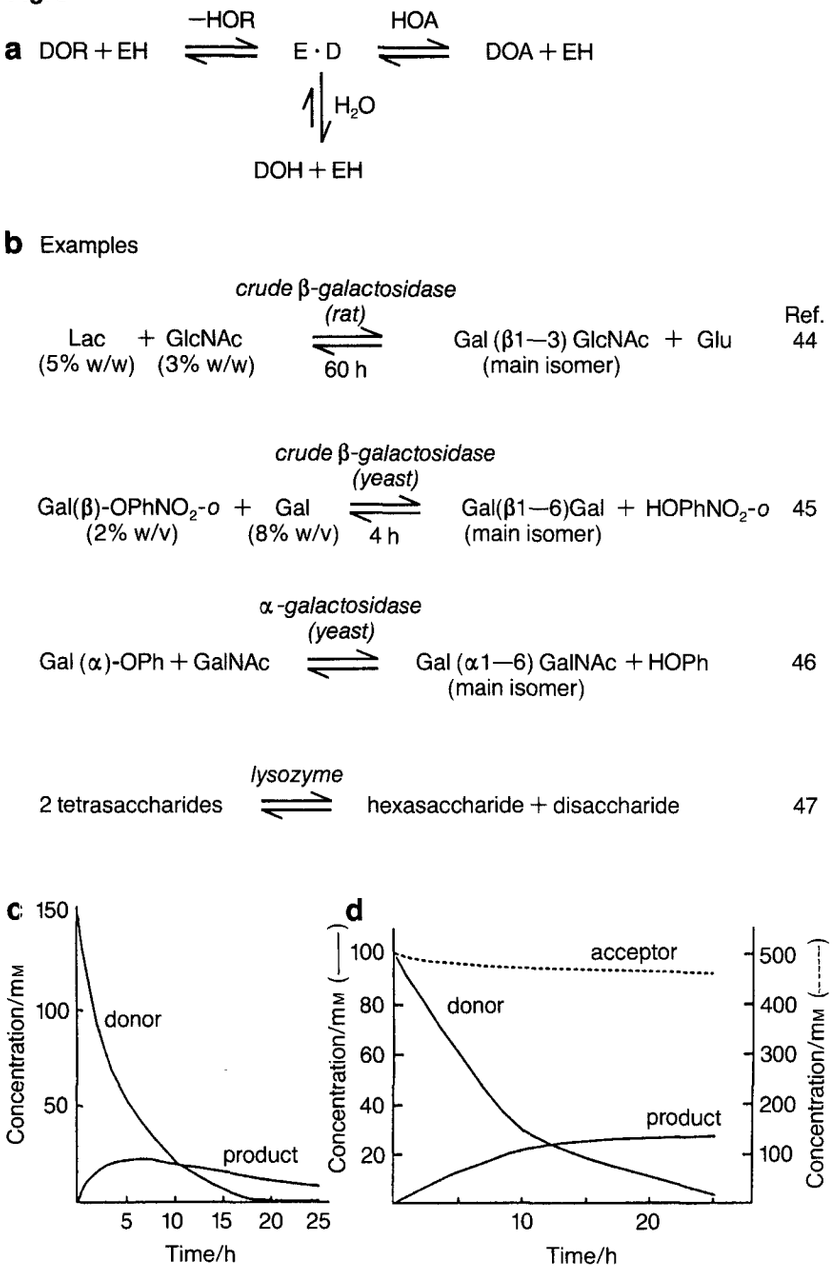
Pressure and the temperature may, however, effect the yield. In one study, the yield of disaccharide increased from 33% to 47% when the reaction temperature was decreased from 50°C to 4°C (Ref. 48). The various 'tricks' used in equilibrium-controlled synthesis (molecular trap etc.) may increase the yield of transglycosidation.

The wider use of glycosidases for synthesis of complex carbohydrates has been hampered not by the 20–50% yields but by the frequently occuring formation of 1–6-linked products; most of the interesting saccharides contain linkages where the secondary hydroxyl groups are involved (i.e. 1–2, 1–3 or 1–4 linkages). More extensive screening may provide a glycosidase that synthesizes the desired linkage. Thus, β-D-galactosidase from *E. coli* catalyses mainly the formation of β1–6-linked Gal–GlcNAc, while a *Lactobacillus* β-galactosidase[51] and β-galactosidase from rat mammary gland[44] almost exclusively synthesize Gal(β1–4)GlcNAc and Gal(β-1–3)GlcNAc, respectively. It is also possible to manipulate the reaction's regioselectivity[52].

Changing the regioselectivity of glycosidase-catalysed synthesis

It has been found that both the structure of the aglycon (R in

Fig. 3



Kinetically controlled synthesis of oligosaccharides by glycosidases – transglycosidation. a: The process is facilitated by efficient glycoside donors (DOR), efficient glycosyl acceptors (HOA) and high concentrations of the acceptor. The yield of the reaction is determined by the relative rates of formation of the product and hydrolysis. b: Examples of transglycosidation reactions. c and d: Concentration profiles during transglycosidation when (c) the glycosyl donor also acts as acceptor, or (d) a high concentration of a separate acceptor prolongs product formation.

Table 3) and the anomeric configuration of the acceptor glycoside may have a pronounced influence on the regioselectivity of disaccharide formation[52]. In this way one glycosidase can be used for the preponderant formation of different linkages. In addition this approach provides a way of synthesizing common glycosides suitable for various applications (Table 1). For example methyl, 2-bromoethyl, allyl, benzyl and trimethylsilyl oligosaccharide glycosides have been conveniently prepared[53] and the use of other aglycons like amino acid or peptide derivatives is also possible. The acceptors can be prepared *in situ* and thus some structures can be synthesized in a 'one-pot' procedure from a simple oligosaccharide and an alcohol[53]. Various glycosides of Gal(β1–3)Gal (a structural unit of several glycolipids) were obtained in a one-pot reaction from lactose and the respective alcohol (allyl alcohol, benzyl alcohol, methanol, etc.) using β-galactosidase. In addition, the monogalactosides were obtained in high yields. In these reactions the concentration of the alcohol had to be low (10–2%, V/V) again indicating the hydrophobic character of the acceptor binding sites. Purification of the product glycosides (by column chromatography) is usually straightforward since the glycosidations are stereospecific and no anomerization of the acceptor glycoside occurs. Other glycosidases (e.g. fucosidases and hexosaminidases; Ref. 54 and unpublished) can also be used in this approach. Most of the disaccharide structures in glycoconjugates can be synthesized using the proper acceptor glycoside. Some trisaccharide glycosides [e.g. Man(α1–2)Man(α1–2)-Man(α)–OMe and Gal(α1–3)Gal-(β1–4)GlcNAc(β)–OEt] have also been prepared[52,55]. The use of endoglycosidases should be beneficial for synthesis of higher oligosaccharide glycosides.

Table 3

Changing the regioselectivity of glycosylase-catalysed synthesis. The aglycon moiety (R) and the anomeric configuration (α or β) of the acceptor glycoside ($HOAR_2$) influence the regiospecifity of disaccharide formation from a given glycosyl donor (DOR_1). The disacharide glycosides produced occur widely in natural glycoconjugates.

$$EH + DOR_1 \overset{-HOR_1}{\rightleftharpoons} E{\cdot}D \overset{+\ HOAR_2}{\rightleftharpoons} DOAR_2 + EH$$

$$E{\cdot}D \uparrow\downarrow H_2O \quad DOH + EH$$

Enzyme	Glycosyl donor	Glycosyl acceptor	Main glycosides formed
α-Galactosidase			
	Gal(α)–$OPhNO_2$-*p*	Gal(α)–OMe	Gal(α1–3)Gal(α)–OMe
	Gal(α)–$OPhNO_2$-*p*	Gal(β)–OMe	Gal(α1–6)Gal(β)–OMe
	Gal(α)–$OPhNO_2$-*p*	Gal(α)–$OPhNO_2$-*p*	Gal(α1–3)Gal(α)–$OPhNO_2$-*p*
	Gal(α)–$OPhNO_2$-*o*	Gal(α)–$OPhNO_2$-*o*	Gal(α1–2)Gal(α)–$OPhNO_2$-*o*
β-Galactosidase			
	Gal(β)–$OPhNO_2$-*o*	Gal(α)–OMe	Gal(β1–6)Gal(α)–OMe[a]
	Gal(β)–$OPhNO_2$-*o*	Gal(β)–OMe	Gal(β1–3)Gal(β)–OMe[a]
	Gal(β)–$OPhNO_2$-*o*	GlcNAc(β)–OMe	Gal(β1–3)GlcNAc(β)–OMe[b] Gal(β1–4)GlcNAc(β)–OMe
	Gal(β)–$OPhNO_2$-*o*	GlcNAc(β)–$OEtSiMe_3$	Gal(β1–3)GlcNAc(β)–$OEtSiMe_3$[b]
α-Mannosidase			
	Man(α)–$OPhNO_2$-*p*	Man(α)–OMe	Man(α1–2)Man(α)–OMe
	Man(α)–$OPhNO_2$–*p*	Man(α)–$OPhNO_2$-*p*	Man(α1–2)Man(α)–$OPhNO_2$-*p*
α-Fucosidase			
	Fuc(α)–$OPhNO_2$-*p*	Gal(α)–OMe	Fuc(α1–3)Gal(α)–OMe
	Fuc(α)–$OPhNO_2$-*p*	Gal(β)–OMe	Fuc(α1–6)Gal(β)–OMe

[a] *E. coli* enzyme
[b] Bovine testis enzyme

Synthesis with glycosyltransferases

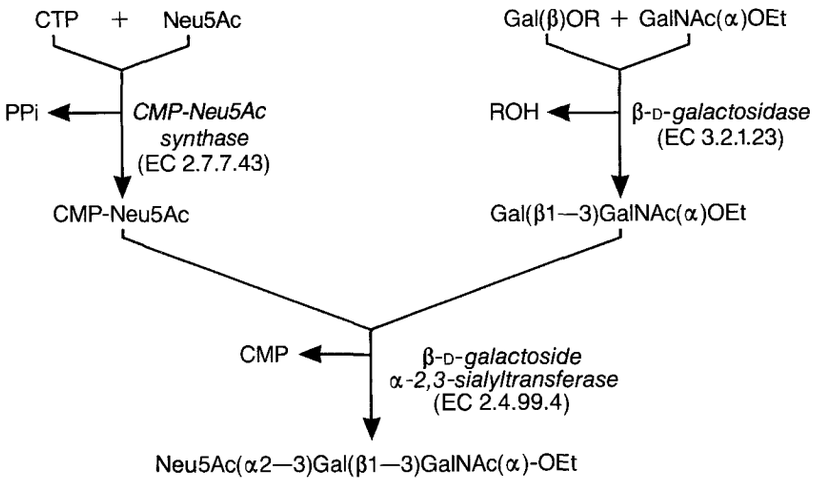
Unlike glycosidases, glycosyltransferases naturally catalyse the quantitative, regio- and stereospecific synthesis of oligosaccharides. A pure glycosyltransferase will catalyse the formation of just one linkage (Table 2) and purification of the product is usually straightforward. Synthesis problems are caused only by the availability of the enzyme and the donor, although this is not a small difficulty. The high acceptor specificity often extends beyond the non-reducing terminal sugar so that, for instance, transfer to monosaccharides or their glycosides is often less efficient than transfer to disaccharides or higher oligosaccharides. Although it is possible to prepare disaccharides with transferases[56,57], the use of glycosidases is preferable for synthesis of shorter oligosaccharides because of their simple reaction systems.

The problem of enzyme availability can be addressed by immobilizing glycosyltransferases, permitting their reuse[57,62]. Some of the expensive nucleotide-sugars substrates (e.g. UDP-Gal, CMP-Neu5Ac) can be conveniently prepared with enzymes[57,59,62]. Immobilized multienzyme systems containing transferase and synthase (for regeneration of the nucleotide-sugar during synthesis) have also been devised[57] and there are some promising results using glycosyltransferases for solid-phase synthesis of oligosaccharides[63].

Combining glycosidases and glycosyltransferases

To synthesize larger oligosaccharides, the more available glycosidases can be used to produce shorter fragments and glycosyltransferases used to catalyse the final steps when demands on regiospecificity are higher. For example, an abundant sialylated trisaccharide structure was obtained by using β-D-galactosidase and β-D-galactoside α-2,3-sialyltransferase in sequence[54] (Fig. 4). The chemical synthesis of

Fig. 4



Sequential use of glycosidase and glycosyltransferase in the synthesis of an oligosaccharide.

this structure involves more than ten steps[27]. Neu5Ac(α2–3)Gal(β1–3)GlcNAc, which was prepared with the same enzyme[54], is the central part of sialylated Le^a, a well-characterized tumor-associated antigen[6]. The spacer glycosides of these structures suitable for covalent attachment to proteins or affinity adsorbents can be obtained. The less abundant sialyltransferase can be reused after immobilization to tresylchloride-activated agarose[62].

The high acceptor specificity of the glycosyltransferase means the enzyme will select the correct disaccharide isomer. Therefore the glycosidase-synthesized product does not need to be completely purified. Indeed, the use of coimmobilized transferase and glycosidase may allow the one-pot preparation of the trisaccharides above and similar structures. The transferase then acts like a molecular trap minimizing secondary hydrolysis of the glycosidase-synthesized product.

In-vitro glycosylation of proteins (natural or recombinant) has been attempted using glycosyltransferases. For example, after endoglycosidase H was used to hydrolyse the high-mannose chains of proteins produced in yeast, glycosyltransferases (Gal-transferase and Sia-transferase) were used to glycosylate the remaining asparagine-linked GlcNAc residues[13]. However, further improvements are required.

Combined chemical and enzymatic synthesis

Chemical and enzymatic methods have been combined in various ways to prepare oligosaccharides. Sialylated oligosaccharides have been prepared using chemical methods to synthesize precursor disaccharides and sialyltransferases to assemble the final product (Table 2).

Chemically synthesized monosaccharide glycosides (e.g. nitrophenyl glycosides) are routinely used as substrates in glycosidase-catalysed reactions. Conversely, glycosidases have been used for synthesis of oligosaccharides suitable as precursors in organic synthesis[52–54]. For example, Gal(α1–3)Gal(α)–OMe synthesized with α-galactosidase is a suitable precursor for organic synthesis of the blood group B determinant. Furthermore, glycosidase–catalysed hydrolysis of polysaccharides, such as the production of Gal(α1–4)Gal(β)–OMe from polygalacturonic acid[64], may give material useful in organic synthesis. In addition, oligosaccharide glycosides with various noncarbohydrate aglycons (e.g. allyl, benzyl, nitrophenyl) can be prepared using glycosidase and glycosyltransferases, allowing a number of chemical modifications to be carried out subsequently (Tables 1 and 2). Furthermore, organic synthesis is used for preparation of sugar analogs and derivatives. Such compounds can be used as acceptors in glycosidase-catalysed reactions and in this way useful inhibitors or intermediates for further reactions are obtained. For instance, α-mannosidase has been used to synthesize Man(α1–6)-2,3-*O*-isopropylidene-Man(α)–OMe using methyl 2,3-*O*-isopropylidene-α-D-mannopyranoside as acceptor (unpublished).

The future

Even though, for many purposes, small oligosaccharides have appropriate biological activity, it can be anticipated that enzymatic methods for synthesizing higher oligomers will be developed. Instrumental in such processes will be efforts to clone the genes coding for glycosyltransferases which will ultimately increase the availability of the enzymes. For the glycosidases where availability is not the main problem, the use of site directed mutagenesis could yield enzymes with modified activity, of particular interest being the development of enzymes with modified acceptor selectivity.

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