Chapter 13

Enzymes of the Carbohydrate Metabolism and Catabolism for Chemoenzymatic Syntheses of Complex Oligosaccharides

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13.1 Introduction

The chemical access to complex glycostructures can still be rather difficult. Within recent decades the application of enzymes in synthetic organic chemistry became attractive alternatives to classical synthetic routes due to improved and now well-established techniques. In particular, in carbohydrate synthesis the use of very simple but increasingly more complex systems proved to be of great advantage, and today a number of biocatalysts are commercially

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Scheme 13.1 General pathway of chemical and enzymatic glycosylations.

available. Thus, the chemoenzymatic assembly of complex oligosaccharides and glycoconjugates enjoys the application of all enzymes involved in carbohydrate metabolism and catabolism. In comparison to chemical glycosylation, enzymatic glycosylation is of advantage in a number of cases, such as (Scheme 13.1)

- demanding and highly specific chemical procedures for glycosylations;
- challenging protecting group manipulations;
- harsh methods leading to decomposition and generally lower yields;
- wrong anomers formed by chemical glycosylation, which requires tedious separations and results in lower yield;
- employing heavy metal catalysts, which is not acceptable for products with potential use in the pharmaceutical area or in nutrition due to toxicity and high price.

Most prominent is the use of glycosyltransferases in synthesis but also the appropriate application of “glycosylhydrolases” (glycosidases) in the synthetic direction is of considerable interest. This subject has been of growing interest for preparative carbohydrate chemistry within the recent decades and thus has been reviewed several times (Nilsson, 1988; Monsan et al., 1989; Wong and Whitesides, 1994; Wong et al., 1995; Thiem, 1995; Nilsson et al.,

1996; Kren and Thiem, 1997; Vocadlo and Withers, 2000; Shoda, 2001; Ajisaka, 2001; Ajisaka and Yamamoto, 2002; Wong, 2002; Seibel and Buchholz, 2010; Tefsen and van Die, 2013).

13.2 Glycosylation Employing Leloir and Non-Leloir Glycosyltransferases

13.2.1 *Leloir Glycosyltransferases*

In course of the trimming process for glycoprotein biosynthesis in the Golgi apparatus glycosyltransferases (GlyT) perform the regio- and stereoselective formation of higher heterooligosaccharides. Sugar donors employed are the sugar dinucleotides (**1**, Gly-NDP) which are assumed to form an activated intermediate complex **2** with GlyT releasing the dinucleotide leaving group NDP. The complex **2** on treatment with the acceptor substrate **3**, will result in formation of the novel interglycosidic linkage between donor and acceptor substrate to give the product **4** releasing the enzyme GlyT. This transfer is absolutely stereospecific with regard to the anomeric position in the donor entity and similarly regiospecific to the hydroxyl position to be glycosylated in the acceptor unit.

Yields of this transfer process are generally quantitative. However, since most of the rare glycosyltransferases are membrane-bound their use for preparative purposes is not simple but rather demanding. Whereas initially the dogmatic conception in biochemistry required glycosyltransferases to be exclusively specific, there are by now a large number of examples in which GlyT could be used to work with unnatural acceptors and even donors. Another problem in use of GlyT is the requirement of the complex donor structures, which can be difficult to synthesize in convincing yields as discussed by Wagner et al. (2009). A promising novel one-pot reaction in water was recently described by Hindsgaul and coworkers (2012) (Fig. 13.1).

13.2.1.1 β ,1-4-galactosyltransferase

Only a limited number of soluble, non-membrane-bound glycosyltransferases are readily available such as galactosyl transferase (GalT), and therefore the substrate specificity was

GlyT: Glycosyltransferase

NDP-Gly: UDP-Glc, UDP-Gal, UDP-GlcUA, UDP-GlcNAc, UDP-GalNAc, GDP-Man, GDP-Fuc

Figure 13.1 Leloir type glycosyltransferase reactions using sugar dinucleotide donors (NDP-Gly).

extensively studied and often reviewed (Wong and Whitesides, 1994). For these systems, early on the regeneration of the cofactors and diminution of the feedback inhibiting dinucleotide could be convincingly solved. In this original process depicted in Fig. 13.2 glucose (**5**, $R^1=OH$) was phosphorylated by hexokinase to give the 6-phosphate (**6**, $R^1=OH$), and this with phosphoglucomutase led to α -D-glucopyranosyl phosphate (**7**, $R^1=OH$). UDP-glucose-

Figure 13.2 Cofactor regeneration and diminution of feedback inhibition in galactosyltransferase (GalT) reactions.

pyrophosphorylase used UTP to form UDP-glucose (**8**, $R^1=OH$) under release of pyrophosphate (PP_i) which was degraded enzymatically to inorganic phosphate ($2P_i$). Enzymatic oxidation/reduction at C-4 of **8** led to the activated galactose (UDP-galactose, **9**, $R^1=OH$) which as donor substrate suffices to galactosylate the substrate glucose (**10**, $R^2=OH$) or *N*-acetyl glucosamine (**10**, $R^2=NHAc$) with galactosyltransferase to give the disaccharide lactose (**11**, $R^1=R^2=OH$) or *N*-acetyl lactosamine (**11**, $R^1=OH$, $R^2=NHAc$), respectively. The released UDP which is a feedback inhibitor was regenerated to UTP with phosphoenol pyruvate and the corresponding kinase (Fig. 13.2) as shown by Wong et al. (1982). Further studies showed that this GalT reaction could be extended to other, non-physiological acceptor substrates. Instead of GlcNAc (**10**, $R^2=NHAc$) also 6-substituted GlcNAc (Palcic et al., 1987), GlcNAc with varied *N*-substituents (Brockhausen et al., 2005) chitobiose (GlcNAc β 1-4GlcNAc), GlcNAc-asparagin, chitobiose-asparagine, and cellobiose could be employed as acceptor substrates (Thiem and Wiemann, 1990; Streicher et al., 2008).

In other experiments it was shown that the complete circle could be followed starting with 2-deoxy glucose (**5**, $R^1=H$) instead of glucose. Apparently, all the enzymes involved accepted these unnatural substrates, and thus following the circle 2'-deoxy *N*-acetyl lactosamine (**11**, $R^1=H$, $R^2=NHAc$) or 2'-deoxy-lactose (**11**, $R^1=H$, $R^2=OH$) could be obtained in yields of 40% and 25%, respectively (Thiem and Wiemann, 1991).

A novel type with an unprecedented regiodirection of the galactose transfer employing β ,1-4GalT was observed by Nishida et al. (1993) with *N*-acetyl kanosamine and with *D*-xylose (**12**). In addition to the expected β ,1-4-galactosylation to give the disaccharide **13** the symmetrical acceptor molecule xylose is apparently also recognized in reverse orientation. This renders the anomeric β -OH group structurally equivalent to the equatorial 4-OH group and leads to a β 1- β 1- interglycosidic linkage, giving the novel non-reducing disaccharide **14**. Wiemann et al. (1994) could show that recognition of the acceptor substrate in the normal orientation apparently prevails, thus giving Gal β 1-4Xyl (**13**):Gal β 1-1 β Xyl(**14**) in a ratio of 2:1. These experiments were repeated by Hara and Suyama (2000) on a larger scale employing a lactating Holstein cow, and here a ratio of **13**:**14** = 4:1 was observed (Fig. 13.3).

Figure 13.3 Regiodirection of galactose transfer to D-xylose (**12**) and β -L-glucose1-NAc (**15**) / β -L-xylose1-NAc (**16**), respectively, using β ,1-4GalT.

As with some other completely unusual substrates, the *N*-acetylated β -glycosylamines of L-glucose (**15**) and L-xylose (**16**), β -galactosylation could be detected to give the disaccharide derivatives **17** and **18**, respectively, in about 20% yield. It should be noted that the recognition of these acceptor substrates by the enzyme occurred to give the β ,1-3-interglycosidically linked disaccharides **17** and **18** by use of β ,1-4-galactosyltransferase (Fig. 13.3) as demonstrated by Nishida et al. (2000).

In a more recent example Rech et al. (2011) demonstrated a combinatorial one-pot synthesis of poly-LacNAc glycans (**20**, **21**) which play essential roles in carbohydrate-protein interactions and are of interest for, e.g., biomaterial surfaces (Fig. 13.4). By

Figure 13.4 Combinatorial one-pot synthesis of poly-*N*-acetyllactosamine.

employing two glycosyltransferases and uridine-diphospho-glucose 4'-epimerase LacNAc glycans up to six disaccharide (**20**, **21**) units could be obtained for the first time starting from a LacNAc-linker-*t*-Boc (**19**) motive in high yields.

13.2.1.2 α ,1-3/4-fucosyltransferase

The concept of using non-natural donor substrates could be extended to several other glycosyltransferases. Taking as LacNAc acceptor glycoside the β -8-methoxycarbonyloctyl derivative **22** (Gokhale et al., 1990), transfer of the activated modified fucose components GDP-L-Gal (**23**, $R^1 = R^2 = \text{OH}$), GDP-3d-L-Gal (**24**, $R^1 = \text{H}$, $R^2 = \text{OH}$), and GDP-3,6d₂-L-Gal (GDP-3d-L-Fuc, **25**, $R^1 = R^2 = \text{H}$) could be realized by Binch et al. (1998). Employing human milk α ,1-3/4-fucosyltransferase, the corresponding modified Lewis X trisaccharide derivatives **26–28** could be isolated by Stangier et al. (1998) in 84–93% yield (Fig. 13.5). In a similar pathway sialyl Lewis X motives with a number of different fucose derivatives could be obtained by Wu and coworkers (2010).

Human milk oligosaccharides, the gold standard for nourishment of early infants, play a key role in health and development (Bode, 2012; Boehm and Stahl, 2007). Gaining access to these oligosaccharides is of particular interest. Miyazaki et al. (2010) demonstrated the synthesis of the hexasaccharide lacto-*N*-difucohexaose I (**33**), which binds to *Helicobacter pylori*. Starting from lactose (**29**) the core structure, lacto-*N*-tetraose (**30**), was obtained employing β -1,3-GnT (UDP- α -D-GlcNAc) and β -1,3-galactosidase (Gal β -oNP) (Fig. 13.6).

Figure 13.5 Synthesis of modified Lewis x trisaccharides (**26–28**) using non-natural donor substrates (**23–25**) with acceptor β -8-methoxycarbonyloctyl LacNAc (**22**).

Figure 13.6 Synthesis of lacto-*N*-difucohexaose I (33).

Selective $\alpha 1\text{-3''}$ - and $\alpha 1\text{-2''''}$ -fucosylation of this core structure employing FUT1 ($\text{GDP}\beta\text{-L-Fuc}$) and FUT3 ($\text{GDP}\beta\text{-L-Fuc}$) gave lacto-*N*-difucohexaose I (33) in a small number of steps not achievable by a chemical approach.

13.2.1.3 $\alpha,2\text{-3-}$ and $\alpha,2\text{-6-}$ sialyltransferases

Since the classical glycosylation for *N*-acetyl-neuraminic acid was always more demanding than that of normal sugar derivatives, and although some convincing solution were proposed (Okamoto and Goto, 1990; De Ninno, 1991) regio- and stereospecific enzymatic procedures giving preparative yields would be of particular interest. Early on the synthesis of the activated *N*-acetylneuraminic acid (CMP-Neu5Ac) was solved (Kean and Roseman, 1966; Haverkamp et al., 1979).

Then by use of $\alpha,2\text{-6-}$ sialyltransferase from bovine colostrum (Paulson et al., 1977) the sialylation of various *N*-acetyl-lactosamine derivatives and glycosides **34** gave the $\alpha,2\text{-6-}$ sialylated trisaccharides **36** in good yields (Thiem and Treder, 1986; Sabesan and Paulson,

Figure 13.7 Sialylation of various *N*-acetyl-lactosamines (**34**) to give α ,2-3- (**35**) and α ,2-6- (**36**) trisaccharides with cofactor regeneration.

1986; Unverzagt et al., 1990; Ichikawa et al., 1991; Stangier et al., 1993). The α ,2,6-sialyltransferase of *P. damsela* (Pd2,6ST) was found to even glycosylate internal galactose residues in poly-LacNAc structures (Paulson et al., 2013).

Further work in this direction could employ the corresponding α ,2-3-sialyltransferase, and by cofactor regeneration as depicted in Fig. 13.7 also trisaccharide compounds of the structure Neu5Ac α ,2-3Gal β 1-4GlcNAc β 1-OR (**35**) could be obtained by Ichikawa et al. 1992 (Fig. 13.7). In 2011 the group of Sohng employed the α ,2,3-SiaT from *Pasteurella multocida*, which has a broad substrate specificity, for the decoration of the vancomycin carbohydrate moiety. However, recently the α ,2-3-sialyltransferase of *Photobacterium* sp. JTISH-224 was found to even recognize cyclitols with a 3-OH/ 4-OH galactose like structure (Yamamoto et al., 2010). A comprehensive review, covering a number of sialyltransferases is available from Yu and Withers (2015).

13.2.2 Non-Leloir Glycosyltransferases

There are several transferases in Nature that are dependent on complex NDP-leaving groups but can rather make use of simpler glycosyl derivatives such as phosphates as donor structures.

Figure 13.8 CGT reactions employing glucopyranosyl fluoride (37) as unnatural donor leads to α - and β -cyclodextrins and maltooligomers.

13.2.2.1 Cyclodextrin- α ,1-4-glucosyltransferase (CGT)

Cyclodextrin- α ,1-4-glucosyltransferase (CGT) from *Klebsiella pneumonia* or from *Bacillus macerans* proved to be versatile enzymes and useful in preparative chemistry. Taking amylose as substrate, employment of CGT will result in a mixture of 17% α -, 19% β -, and 6% γ -cyclodextrins (Bender and Komiyama, 1978). However, according to Treder et al. (1986) with α -D-glucopyranosyl fluoride (37) as unnatural donor substrate only α -cyclodextrin (38, 30%), β -cyclodextrin (39, 38%), no γ -cyclodextrins, and maltooligomers (40, $n = 0-8$, 32%) were obtained (Fig. 13.8). Recently Pennec et al. (2014) studied *Thermoanaerobacterium sp.* cyclodextrin glucosyltransferases with aryl glucopyranosides or furanosides to obtain mainly α -Glc-(1,3)-Araf or Galf linked structures. Utilization of microwave irradiation showed a positive effect on the reaction outcome

13.2.2.2 Potato phosphorylase

The natural task of another group of non-Leloir glycosyltransferases, the phosphorylases, is the phosphorolytic cleavage of glycosidic

Figure 13.9 Elongation of maltotetraose (**42**) with glucal **41** as donor using non-Leloir type potato phosphorylase to give oligo-2-deoxy-maltooligomers (**43**).

bonds transferring the glycosyl unit onto the phosphate. By employing potato phosphorylase also D-glucal (**41**) could be used as donor structure (Klein et al., 1982), and with maltotetraose (**42**) as primer molecule by excess of phosphate 2-deoxy α-D-glucopyranosyl phosphate could be obtained in 65% yield. Evers et al. (1984) demonstrated that with lower amounts of phosphate elongation of the primer with 2-deoxy-α-D-glucopyranosyl units were observed. After 4–6 h penta-, hexa-, and heptasaccharides carrying one to three 2-deoxy-α-D-glucopyranosyl units attached to the primer at the non-reducing end could be isolated. After 24 h the resulting product was **43** with $n = 10$ –19 equivalent to maltotetraose elongated by 11 to 20 2d-α-D-Glc units (Fig. 13.9).

13.2.2.3 β,1-3-Galactosyl-N-acetylhexosamine Phosphorylase

Derensy-Dron et al. (1999) used this phosphorylase from *Bifidobacterium bifidum* 20082 to catalyze reversibly the phosphorolytic cleavage of Galβ1-3GlcNAc-terminated oligosaccharides. Employing α-D-galactopyranosyl phosphate (**45**) as donor and α-glycosides of N-acetyl-glucosamine (**44**) as acceptors this enzyme was used by Farkas et al. (2000) to synthesize a series of Galβ1-3GlcNAc disaccharide glycosides (**46**) in 30–60% yield (Fig. 13.10). A series of Galβ1-3GlcNAc and Galβ1-3Gal motives could be obtained with the phosphorylase from *Bifidobacterium infantis* (BiGalHexNAcP) in one-pot synthesis with a galactokinase in a similar manner (Yu et al., 2015).

Figure 13.10 Synthesis of Gal β 1-3GlcNAc disaccharides (**46**) using phosphate donor (**43**) and *N*-acetyl- α -D-glucosamine (**44**).

13.3 Glycosylation Employing Glycohydrolases and Glycosynthases

13.3.1 *Exo-Glycosidases*

According to Kornfeld and Kornfeld (1985) generally exo-glycosidases, e.g., involved in the trimming processes of glycoprotein biosynthesis, are used by Nature to cleave the terminal non-reducing saccharide unit of an oligosaccharide (Gly-OR, **48**). Whereas they show a high specificity for that unit, they are less specific concerning the interglycosidic linkage, and the aglycon moiety R may even vary widely. The mechanism is understood that an activated intermediate is formed, and subsequently with water hydrolysis will occur. The process can be inverted and then reverse hydrolysis may be used to synthesize lower oligosaccharides. This is a thermodynamically controlled reaction which is favored, e.g., by increase of concentration of the starting materials (Gly-OH, **49** and ROH) or enhanced temperature.

In addition glycosidases may be employed for transglycosylation, which requires an activated glycosyl donor component (Gly-X, **47**) carrying a good anomeric leaving group. In this case the product formation is kinetically controlled. Therefore, in order to minimize the competing hydrolytic cleavage before equilibrium is reached the reactions must be quenched at the maximum of product formation. Generally, yields in transglycosylation are considerably higher than in reverse hydrolysis, and therefore the former is largely preferred for preparative purposes (Fig. 13.11).

Figure 13.11 Various modes for synthetic use of glycosylhydrolases (glycosidases) by transglycosylation and reverse hydrolysis.

Glycosidases are broadly available and comparatively inexpensive enzymes, and they need also cheap donor substrates. They are quite robust to handle and show absolute stereoselectivity in substrate recognition. The main drawbacks for use in glycoside synthesis are generally low yields and little regioselectivity. This can be partly controlled by choice of the appropriate glycosidase with more pronounced regioselectivity, or in transglycosylations by dosing

of reactants and use of co-solvents. Another eminent advantage of using glycosidases in glycosylation is their good stereoselectivity. These enzymes are considered to be “retaining enzymes.” There are, however, a few exceptions of inverting glycosidases, which lead to products with an inverted anomeric configuration by using glycosyl fluorides or, e.g., glycosyl pyridinium ion derivatives.

A broad variety of substrates were efficiently glycosylated by glycosidases such as aliphatic and alicyclic alcohols, (Crout et al., 1990) phenols, oximes, steroids and terpenes (Ooi et al., 1985), amino acids (Attal et al., 1992), alkaloids (Kren, 1997), and many other substances. The substrate to be glycosylated by a glycosidase should be at least partly soluble in water. The solubility can be enhanced by addition of water-miscible solvents, e.g., acetonitrile, dimethylformamide, dimethylsulfoxide, dioxane, or *tert*-butyl alcohol. Concentrations of co-solvents up to 30% are usually well tolerated by most glycosidases.

In general glycosidases show rather poor regioselectivities, and with respect to their original biofunction this is expected. Nevertheless, many of them exhibit preferential cleavage of distinct glycosidic linkages and thus are useful as well for the corresponding transglycosylation. In acceptor substrates with more than one hydroxyl group the formation of several glycosylated isomers will be encountered. Often the regioselectivity can be estimated, e.g., in hexopyranose acceptor substrates the affinity of hydroxyl groups is roughly 6-OH >> 4-OH ~ 3-OH > 2-OH resembling the chemical reactivity. Today glycosidases are wide spread in industrial large scale applications as, e.g., in starch manipulation or milk treatment (lactose-free) and many other processes.

13.3.1.1 β ,1-3-Galactosidases

Due to the availability and their easy handling β -galactosidases have become a preferred choices in preparative enzymatic glycoside synthesis often in positive competition to classical synthetic protocols. *En route* to modified sialylated Thomsen-Friedenreich antigen components the core disaccharide motif Gal β 1-3GalNAc needed to be easily accessible and thus β ,1-3-galactosidase from bovine testis (Hedbys et al., 1989; Johansson et al., 1991) was employed. A considerable number of α -glycosides of *N*-acetyl-

Figure 13.12 Synthesis of β ,1-3-linked Gal-GalNAc (**52**) by transglycosylation using *p*-nitrophenyl β -galactopyranoside (**50**).

galactosamine GalNAc **51** could be transgalactosylated using *p*-nitrophenyl β -galactopyranoside (**50**) to give the desired β ,1-3-linked Gal-GalNAc disaccharide derivatives **52** in 15–40% yield (Kren and Thiem, 1995; Gambert and Thiem, 1997; Gambert et al., 1997). In more recent studies modifications could be employed to furnish yields up to 70% (Kröger and Thiem, 2005; Kröger et al., 2006). If β -galactosidases of *Xanthomonas manihotis* (Xm) (Fujimoto, 1997) or recombinant *Bacillus circulans* (bgaC-gene, Bc) (Fujimoto et al., 1998) were employed for the same reaction in addition to **52** the corresponding regioisomeric Gal β 1-6GalNAc derivative was formed [ratio 10:1 (with Xm) and 3:2 (with Bc)]. By in situ hydrolytic cleavage of the latter with β -galactosidase (*E. coli*) (Hedbys et al., 1989) the overall yields were 15–20% (with Xm) and about 30% (with Bc), respectively according to Kröger and Thiem (2005), and Kröger et al. (2006) (Fig. 13.12).

Further acceptor modifications in such galactosylations were studied. Thus, 2-deoxy-galactose and *N*-acetyl-galactosamine structures were recognized by all these galactosidases and gave the corresponding disaccharides as shown by Kröger and Thiem (2005) in 20–70% yield. With *N*-acetyl-chitobiose and -chitotriose β ,1-3- and β ,1-4-galactosylations (ratio 1:1) were reported by Gambert et al. (2000). Treatment of food saccharides such as sucrose, lactose, isomalt (isomaltitol), isomaltulose, isomelizitose, raffinose, and 1-kestose with lactose as donor substrate and β -galactosidase (bovine testes) furnished terminally β ,1-3-galactosylated-tri- to tetrasaccharides in 10–15% yield. The reactions could be performed on 30–40 gram-scale and gave the products in about 20% yield (Schröder et al., 2004).

Modifications of donor substrates were also of interest. Generally, aromatic β -galactopyranosides were employed with good yields and easy UV-survey of the reaction due to the phenolic leaving group. Among them *o*- and *p*-nitrophenylgalactopyranosides represent standard donors, and also methylumbelliferyl as well as resorufinyl β -D-galactopyranosides were reported to be good donors. Often their solubility in aqueous-organic solvent mixtures causes problems. In a recent study by Kröger and Thiem (2007) sixteen aromatic and heteroaromatic β -galactopyranosides of type **50** were prepared. Their β ,1-3-galactosylation potential for allyl *N*-acetyl- α -D-galactopyranoside were studied employing bovine testis galactosidase to give the corresponding disaccharides in 10–75% yield. The solubility was also tackled in experiments studying various galactosides; however, *p*NP- β -D-Galp was superior to lactose and that again much better than both lactulose and lactitol in donor substrate efficiency (about 30:10:2:1) (Schröder et al., 2004).

A multienzyme system consisting of sequential actions of both a glycosidase and a glycosyltransferase coupled with in situ cofactor regeneration could be elaborated in the formation of the sialyl Thomsen-Friedenreich (T-antigen) epitope **55**. Initially with *p*NPGal (**50**) as donor GalNAc (**53**) could be galactosylated employing β ,1-3 galactosidase (bovine testis) to give the disaccharide Gal β ,1-3GalNAc (**52**). Since this is the immediate substrate for the α ,2-3-sialyltransferase (pig liver) the equilibrium of the transglycosylation is shifted to the product side. Irreversibly then the desired trisaccharide Neu5Ac α ,2-3Gal β 1-3GalNAc (**55**) could be obtained in good isolated overall yields employing CMP-Neu5Ac (**54**) regenerated as depicted in Fig. 13.13. As one of the problem to be solved the pH optima of the three most important enzymes α ,2-3-sialyltransferase (pH 7.5), CMP-Neu5Ac synthase (pH 9.0), and α ,1-3-galactosidase (pH 4.3) had to be harmonized (Kren and Thiem, 1995; Gambert and Thiem, 1997).

13.3.1.2 β ,1–4-Galactosidases

Early on, there were reports by Sakai et al. (1992) on the preparative use of β -galactosidase from *B. circulans* for the synthesis of β ,1-4-galactosylated components. This enzyme is

Figure 13.13 Combined use of glycosidase and glycosyltransferase in synthesis of Neu5Ac α ,2-3Gal β 1-3GalNAc (**55**) with cofactor regeneration.

commercially available under the brand name “Biolacta®” (Lactase, EC 3.2.1.23). Biolacta is a quite stable enzyme ($t_{1/2} = 226$ h at 22°C and pH 7.0), which allows for easy transfer reaction between pH 5.0–9.5 and up to 55°C (Daiwa Kasei). Its activity for transglycosylation in aqueous solvent mixtures was determined, and it showed considerable tolerance to several organic solvents. The activity in 30% aqueous organic solvent mixtures at pH 7.0 and 30°C after one hour were measured and gave: DMSO (85%), acetone (70%), DMF (67%), dioxane (59%), and acetonitrile (12%) (Priya and Loganathan, 1999). Studies toward the transglycosylation synthesis of *N*-acetyl-lactosamine and derivatives were of particular interest and performed by several groups (Vic et al., 1996; Takayama et al., 1996; Farkas and Thiem, 1999). As demonstrated pNP-galactopyranoside (**58**, X=OpNP) was superior to lactose (**57**, X=4-O-Glc), and with α - as well as β -allyl *N*-acetyl-glucosaminide (**56**) the *N*-acetylglucosaminides **58** could be obtained by Farkas and Thiem (1999) in up to 60% yield in acetonitrile/aqueous phosphate buffer. Correspondingly, with the phenyl β -thio-galactopyranosides (**59**) the appropriate Gal β 1-4Gal disaccharide **60** could be synthesized in 50–70% yield (Fig. 13.14).

Farkas et al. (2003) formed potential novel food additives by β ,1-4-galactosylation of natural oligosaccharides. Compared to the results in β ,1-3-galactosylation (Schröder et al., 2004) sucrose and 1-kestose were cleanly β ,1-4 galactosylated at the position of the incorporated glucopyranose residue to give the corresponding

Figure 13.14 Preparative transglycosylation using β ,1-4-galactosidase of *B. circulans*.

trisaccharide ("lactosucrose") and tetrasaccharide, respectively, in 20–30% yield. In contrast, both isomelizitose (**61**) and raffinose (**62**) gave rise to the formation of two isomeric tetrasaccharides. For isomelizitose (**61**) both available glucopyranose units were β ,1-4-galactosylated to give **63** and **64** (ratio 1:1, 21%), and for raffinose (**62**) the β ,1-4-galactosylation of the glucose residues gave **66**, and that of the terminal galactopyranose ring led to **65** (ratio 3:4, 38%) according to Farkas et al. (2003) (Fig. 13.15).

Modifications of gluco-, galacto-, *N*-acetyl-gluco-, and *N*-acetyl-galacto-configured acceptor structures have been extensively studied. Generally, in the *gluco*- the equatorial- and in the *galacto*-components the axial 4-hydroxy groups are galactosylated. With regard to the anomeric function, there were apparently no restrictions because

Figure 13.15 Tetra- and pentasaccharides of lactosucrose, raffinose and isomelizitose formed by β ,1-4-galactosylation.

alkyl- and aryl(thio)glycosides as well as *N*-acyl-glycosylamines could be employed (Priya and Loganathan, 1999; Vic et al., 1996; Takayama et al., 1996; Usui et al., 1996; Usui et al., 1993; Herrmann et al., 1993; Fang et al., 1998; Komba and Ito, 2002). Interestingly, galactosylation of inositols could be also effected. Treatment of 1D-chiroinositol as well as of 1D-pinitol under standard conditions lead to the corresponding galactosylated species, respectively, in 45% yield. Apparently, these acceptors structures seem to resemble galactopyranose acceptors (Hart et al., 2001).

Further work by Weingarten and Thiem (2004) and by Neumann et al. (2007) checked donor modifications in *B. circulans*-catalyzed galactosylations of allyl α -*N*-acetyl-hexosaminides carrying a number of different functions at C-5 position.

13.3.1.3 α -Sialidases

Neuraminic acid (Neu5Ac) is the major constituent in a variety of glycoconjugates such as glycoproteins, gangliosides, milk oligosaccharides and oligosaccharides occurring in higher animals, viruses, bacteria, protozoa, and pathogenic fungi. The crucial role of sialylation in regulation of cellular and molecular recognition in biological systems associated with the variation in the glycosylation pattern of cell surface glycoconjugates lead to particular interest for the synthesis of the predominantly terminally sialylated components. In addition to many studies using the difficult to access and handle sialyltransferases there are some reports employing sialidases (Thiem and Sauerbrei, 1991; Ajisaka et al., 1994; Makimura et al., 1998). A comprehensive study revealed the synthetic potential of various sialidase in the preparation of sialylated Thomsen-Friedenreich antigen structures (Schmidt et al., 2000). Treatment of Neu5Ac β NP (**67**) with the α -threonine disaccharide glycoside **68** and several sialidases gave both the regioisomeric α ,2-6- (**69**) and the α ,2-3-linked trisaccharide derivatives (**70**) in varying amounts. Application of sialidases from *Vibrio cholerae* and *Chlostridium perfringens* gave virtually exclusively (>99%) the α ,2-6-amino acid trisaccharide **69**. On the other hand sialidase from *Salmonella typhimurium* (86–99%) and Newcastle disease virus sialidase (>99%) showed almost exclusively the formation of the α ,2-3-sialylated structure **70**. Generally, the yields ranged from about 10%

Figure 13.16 Sialidase reactions of Neu5AcpNP (**67**) with the α -threonin glycoside **68** yielding both regioisomeric α ,2-6- (**69**) and α ,2-3-linked trisaccharides (**70**).

to 25%, which required subsequent selective hydrolytic glycosidase cleavages of the non-desired side product and separations (Fig. 13.16).

An extension of this method could be shown by employing MPEG polymer-supported acceptor glycoside structures. Again these could be sialylated with **67** in solution-phase giving about 15–25% yield. Whereas the sialylated polymer-bound components did not undergo glycosidic cleavage, the excess donor substrates did on subsequent treatment with β -galactosidase (*E. coli*) and β -*N*-acetylhexosaminidase (*A. oryzae*), and thus a facile separation and purification approach could be established (Schmidt and Thiem, 2000).

13.3.2 *Trans*-Sialidase

The pathogen responsible for Chagas disease is *Trypanosoma cruzi*. This upon infection expresses a *trans*-sialidase (TSia) that catalyzes transfer of terminal Neu5Ac of the host's sialoglycoconjugates onto its own surface mucins and thus masks its own epitopes to prevent degradation by the host's natural killer cells (Schenkman et al., 1991; Pontes de Carvalho et al., 1993; Schenkman et al., 1994). Based on its primary structure TSia belongs to the glycosidase family 33 which also includes multiple bacterial sialidases (Henrissat, 1998). Kinetic and crystallographic studies of the closely related TSia from *T. brucei* provided information on the binding pocket and the transfer mechanism (Buschiazzi et al., 2000, 2002; Watts et al., 2003). The easy handling of TSia and the excellent stereoselectivity

Figure 13.17 Neu5Ac transfer by *trans*-sialidase (TSia) from *T. cruzi*.

in transferring Neu5Ac from various donors in α ,2-3-linkages to galactose-terminated oligosaccharides was recognized and synthetically used (Sabesan and Paulson, 1986; Vandekerckhove et al., 1992; Turnbull et al., 2002; Agusti et al., 2005; Mendoza et al., 2006). Some recent extensions of this approach allowed to synthesize an array of sialylated structures associated with T-antigen determinant components as well as Neu5Ac-terminated oligosaccharide motifs for binding of the myelin-associated glycoprotein (MAG) (Kröger et al., 2006; Neubacher and Thiem, 2005; Neubacher et al., 2005). Treatment of various lactose and *N*-acetyllactosamine structures **72** (R¹=SPh, R²=N₃; R¹=R²=OH; R¹=OAll, R²=OH; R¹=OMe, R²=NHAc) with *p*-nitrophenyl sialylate **67** and *trans*-sialidase gave the corresponding trisaccharides **73** in 30–60% yield. Correspondingly, a β ,1-3-linked isolactosamine precursor could be reacted with muraminyl sialylate **70** to give the trisaccharide in 30% yield (Neubacher et al., 2006) (Fig. 13.17).

Further transformations were done with the β ,1-3-galactobiose structure **74**. Here Gal β 1-3-GalNAc α Thr and Gal β 1-3-GalNAc α Ser (**74**, R³=Thr or Ser, R⁴=NHAc, R⁵=OH) could be used as acceptor structures, and also 2-deoxy (R⁴=H) and 2-azido (R⁴=N₃) were α ,2-3-sialylated with TSia and **67** or **71** as donors to give the modified trisaccharides **75** in 30–50% yield (Kröger et al., 2006; Neubacher et al., 2005). In the derivatives **72** the terminal galactose unit which

directly accepts the sialyl residue, could be modified. 2-Deoxy-galactose-(**74**, $R^6=H$, $R^7=CH_2OH$) and also the D-fucose-(**74**, $R^6=OH$, $R^7=CH_3$) and the L-arabinose-(**74**, $R^6=OH$, $R^7=H$) terminated disaccharides were nicely sialylated to give the corresponding derivatives **75** (30–50% yield) (Kröger et al., 2006) (Fig. 13.17).

In recent studies modified donor substrates were obtained from *p*NPNeu5Ac **67** by selective oxidative cleavage. Dependent on the ratio **67**: oxidant the octose (**78**, *p*NPOct5Ac) or the heptose (**80**, *p*NPHe5Ac) donor substrates were obtained in high yields. Surprisingly, their transfer onto methyl β-lactoside (**76**) under TSia catalysis did not show any problems; in fact, the yields of the octose (**79**) or heptose analog of neuraminic acid carrying trisaccharides (**81**) were identical to the original derivative **77**, that is 55–65% yield. Apparently, *trans*-sialidase will easily recognize these modified structures. In contrast, STD-NMR studies showed that only the original trisaccharide **77** is a well binding substrate for MAG and the analogous structures **79** and **81** are not (Neubacher et al., 2006) (Fig. 13.18).

Employing a nine step synthesis starting with the α-glycosyl chloride of the peracetylated methyl ester of Neu5Ac a number of other modified, yet *N*-acylated analogs of the above donor structure **67** could be prepared and studied in transfer reaction with *trans*-sialidase (Schroven et al., 2007). Their transfer onto the

Figure 13.18 Systematic donor variation in TSia glycosylations to obtain trisaccharides with enhanced binding affinity to myelin associated glycoproteins (MAG).

Figure 13.19 Modified *N*-acetylated analogues in TSia reactions.

allolactoside Gal β 1-6Glc α OMe (**82**) as a good acceptor structure with **67**, the *N*-propionyl- (**84**) or the *N*-glycolyl donors (**86**) could be realized to give the trisaccharides **83** (87%), **85** (32%), and **87** (60%) (Fig. 13.19). In contrast, *N*-acyl groups larger than propionyl were not accepted, and the same applied to other 5-nitrogen-substituted derivatives of Neu5Ac as well as for KDN, the 5-hydroxy analog of Neu5Ac (Scheppokat et al., 2010). The recent progress of *Trypanosoma cruzi* trans-sialidase as synthetic tool and biological target has been reviewed by Meinke and Thiem (2012).

Recently (Cheng et al., 2010) for the first time α 2,6-trans-sialidase activity was discovered in a previously reported recombinant truncated α 2,6-sialyltransferase from *Photobacterium damsela* (Δ 15Pd2,6ST).

13.3.3 Endo-Glycosidases

In oligosaccharides or glycoconjugates internal glycosidic linkages are cleaved by endo-glycosidases, and under kinetic control their transglycosylation activity is favored. Some of them could be employed in preparative approaches (Yamamoto et al., 1997; Ashida et al., 2001; Akaike et al., 2004), and recently the commercially available cellulose from *Trichoderma reesei* was shown to transfer entire lactose or lactosamine units employing the corresponding *p*-nitrophenyl β -glycosides **88** or **90**. With aliphatic alcohols as well as lower di- and triols yields of **89** and **83** in the range of 1–20% were obtained. However, with mannose or glucose LacNAc β pNP

Figure 13.20 Transfer of *p*-nitrophenyl β -lactose or *p*-nitrophenyl β -lactosamine (**88** or **90**) to aliphatic alcohols and lower di- and triols.

(**90**) gave the corresponding β ,1-4-linked trisaccharides **91** in 13% and 9% yield, respectively (Totani et al., 2001) (Fig. 13.20).

Endo- β -*N*-acetylglucosaminidase from *Mucor hiemalis* (endo-M) shows transglycosylation activity with a number of different substrates. It could be used to transfer high-mannose, hybrid-type, as well as complex-type oligosaccharides and thus glycopeptides became available (Thayer and Wong, 2007). Employing this enzyme for the transfer of a complex-type oligosaccharide chain in **93** from the GlcNAc-CD52 (**92**) gave the GlcNAc peptide **94** and the complex-type CD52 target structure **95** (Li et al., 2005) (Fig. 13.21). However, the transglycosylation activity of endo- β -*N*-acetylglucosaminidases has been recently reviewed and discussed by Wang (2011).

Figure 13.21 Endo- β -N-acetylglucosaminidase transglycosylation activity towards GlcNAc peptide (**94**) and complex-type CD52 (**95**).

13.3.4 Glycosynthases

A particular improvement in using glycosidases for preparative oligosaccharide synthesis came up with the introduction of glycosynthases by Mackenzie et al. 1998. As elaborated for retaining glycosidases the problem of product hydrolysis in transglycosylation could be solved by bio-engineering of suitable mutants. Thus, the active-site catalytic nucleophile was removed and replaced by a non-nucleophilic amino acid side chain. Hydrolysis and transglycosylation for a wild-type retaining β -glycosidase leads to hydrolysis and transglycosylation products. It is generally accepted that the donor substrate reacts with the nucleophilic amino acid to give a glycosyl-enzyme intermediate. This on treatment with water involving a proton transfer to the acid-base amino acid and subsequent release of the nucleophilic amino acid yields the hydrolysis product (see 12.3.1, Fig. 12.11). In transglycosylation an acceptor substrate is transformed correspondingly to give the disaccharide product. Mutant glycosidases fold apparently in the same way as the wild-type enzymes, however, devoid of a nucleophilic site they cannot form the above intermediate, and thus hydrolysis does not occur. However, if a donor substrate with opposite anomeric configuration will be offered such as a glycosyl fluoride with a small leaving group the mutant glycosidase, the glycosynthase, will recognize it as an analogue of the covalent glycosyl-enzyme intermediate. Thus, employing the same transglycosylation mechanism as for the wild-type enzyme the acceptor sugar is transferred to give again a disaccharide. Since these mutant enzymes cannot display hydrolytic activity the yields are generally excellent.

Within recent years a considerable number of glycosynthases have been described both from exo- as well as from endo-glycosidase and the subject was also reviewed several times (Thayer et al., 2007; Jakeman and Withers, 2002; Williams and Withers 2002; Perugino et al., 2004, 2005; Cobucci-Ponzano and Moracci, 2012). In the original work a well-known β -glucosidase Abg from *Agrobacterium* sp. was studied. Mutation of the nucleophilic amino acid E358 from glutamate to alanine could be achieved. Condensation of α -glucopyranosyl fluoride (**97**) with several acceptor glucopyranosides such as the *o*-nitrophenyl

Figure 13.22 Endo-glycosynthase reaction using *o*-nitrophenyl β -D-glucopyranoside or 2-deoxy-2-fluoro- β -D-glucopyranoside (**96**) yielding di- to tetrasaccharides (**98**).

β -glucopyranoside **96** (X=OH) or the 2,4-dinitrophenyl 2-deoxy-2-fluoro- β -D-glucopyranoside (**96**, X=F) gave transglycosylation yields of di- to tetrasaccharides **98** (X=OH, and X=F) respectively, in 76–84% overall yield (Mackenzie et al. 1998) (Fig. 12.22).

A further quite impressive example was reported by collaboration of Spanish-French scientists. In a tandem-type glycosylation using the aforementioned endo-glycosynthases, the α -cellobiosyl fluoride with a 4'-tetrahydropyran protection **99** was transglycosylated to the acceptor disaccharide α -laminaribiosyl fluoride **100** employing the mutant cellulase to give the tetrasaccharide **101**. Its further use as donor structure with the mutant 1,3-1,4- β -glucanase and the acceptor cellobioside **102** gave the β ,1,4- and β ,1-3-linked hexasaccharide glucan **103** stereoselectively and in excellent yield (Fajes et al., 2001) (Fig. 12.23).

Figure 13.23 Tandem-type endo-glycosynthase and glucanase reactions towards hexasaccharide (**95**).

In a recent example the glycosynthase TnG-E338A, which was developed from *Thermus non-proteolyticus* β -glycosidase, has been used for generating an oligosaccharide library (Wei et al., 2013). The broad substrate utility allowed construction of a small library of oligo- and steroidal glycosides, which could be of particular interest for e.g. research in glycotherapeutics. Furthermore, the scope of glycosynthases could be extended towards combinatorial chemistry.

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