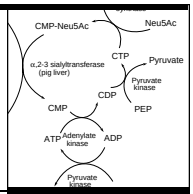
Glycosylation employing bio-systems: from enzymes to whole cells



CHEMICAL SOCIETY REVIEWS

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This contribution will highlight chemoenzymatic approaches to the rather complex task of stereospecific and regiospecific glycosylation. Advantages and problems associated with the application of enzymes from carbohydrate metabolism as organic reagents in aqueous and non-aqueous solvent systems are discussed. One chapter will report on the use of glycosidases in reverse hydrolysis and tranglycosylation reactions. Another section focuses on the syntheses with simple as well as more complex and delicate glycosyltransferases including cofactor regeneration. Further, effective combination of both degrading and synthesising enzyme systems, and finally glycosylations employing cells with direct access to their complete enzyme equipment are treated.

1 Introduction

Within recent years the application of enzymes in synthetic organic chemistry has become a rather well-established technique. In fact, there are now textbooks and laboratory manuals available[1] describing many useful and accessible techniques.

Besides preparation of chiral synthons, the synthesis and modification of carbohydrates by enzymes is one of the most intensely exploited areas of enzyme applications. Whereas some applications of enzymes in sugar chemistry are indeed simple if not trivial several other sophisticated methods put high demands upon a broad knowledge and skills not only in organic chemistry but also in biochemistry, microbiology and immunology.

This review aims to point out some biological aspects and uses of rather complex, sophisticated systems for carbohydrate chemistry.

Glycosylation is considered to be an important method for the structural modification of compounds with useful biological activities. It allows conversion of lipophilic compounds into hydrophilic ones, thus improving their pharmacokinetic properties. Sometimes, by attaching a sugar pharmacodynamic properties are also changed or novel and more effective drug delivery systems (prodrugs) obtained. Enzymatic glycosylation methods in comparison with chemical methods are especially useful in the glycosylation of complex biologically active substances, where generally harsh conditions or use of toxic (heavy metals) catalysts are undesirable. The enzymatic approach is also a good alternative in the chemistry of food additives where the use of synthetic chemistry is sometimes not acceptable.

2 Glycosidases

Glycosidases are cheap enzymes, quite robust to handle, they use cheap donors and show absolute stereoselectivity. The main drawbacks for their use in glycoside synthesis are lower yields and generally low regioselectivity. This, however, can be overcome by the rational choice of appropriate glycosidases with more pronounced regioselectivity and by new sophisticated methods of transglycosylation as, *e.g.* controlled dosing of the reactants and use of co-solvents (Scheme 1).

One of the main advantages 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

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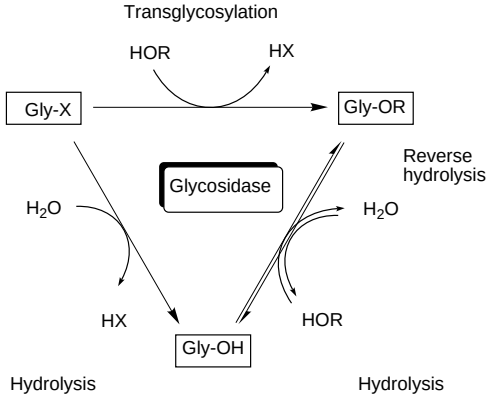
Joachim Thiem

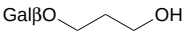


Scheme 1 Transfer reactions catalysed by glycosidases

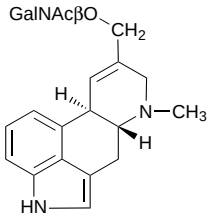
with an inverted anomeric configuration by using glycosyl fluorides or, *e.g.* glycosyl pyridinium ions.

In contrast to glycosyltransferases, glycosidases are able to glycosylate many 'xeno-substrates' with primary or secondary hydroxy groups. Even though the activity of water as acceptor in the reaction mixture is usually one to two orders higher than the activity of the alcohol to be glycosylated, the yields of the glycosides are often much higher than would be expected from simple thermodynamic calculations. This is considered to be caused by the higher affinity of the alcoholic substrates for binding to the 'aglycone site' of the glycosidase compared to water. Speculation about the possible influence of the groups adjacent to the respective hydroxy group is common, and electron rich moieties such as double bonds in the allylic position, conjugated systems of double bonds, aromatic or heteroaromatic systems generally support glycosylation. Aliphatic alcohols are also good acceptors for glycosyl moieties transferred by glycosidases. Amphiphatic properties and the presence of nitrogen in the acceptor molecule usually enhance transglycosylation yields.

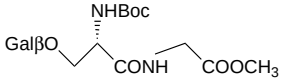
There exists a broad variety of substrates that are glycosylated by glycosidases (Scheme 2)—aliphatic and alicyclic alcohols,[2] phenols, oximes, steroids and terpenes[3] and amino acids—generally, only protected amino acids or peptides can be



Enzyme: β-galactosidase (*E. coli*)
donor: lactose
yield: 42% ref. 2



Enzyme: β-*N*-acetylhexosaminidase (*A. oryzae*)
donor: *p*-nitrophenyl-β-GalNAc
yield: 12% ref. 5



Enzyme: β-galactosidase (*E. coli*)
donor: lactose
yield: 13% ref. 4

Scheme 2 Examples of various glycosides obtained by glycosylations with glycosidases

glycosylated by glycosidases,[4] alkaloids[5] 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, *tert*-butyl alcohol). Concentrations of co-solvents up to 30% are usually well tolerated by most glycosidases. Contrary to, *e.g.* lipases, lowering the water activity by co-solvents usually does not improve yields, the improved solubility of the acceptor and of the donor is the only decisive factor. There are, however, examples of lowering the water activity by, *e.g.* addition of various salts[6] that improves the glycosylation yields.

There are few examples of enzymatic glycosylation of simple alcohols in bi-phasic systems with organic solvents or by polyethyleneglycol-modified glycosidases in organic solvents.[7]

2.1 Enzymatic *versus* chemical glycosylation

The strategy for choice of enzymatic glycosylation *vs.* chemical glycosylation is dictated by several factors.

(1) There is no reliable chemical method for the glycosylation—harsh methods decompose the aglycone, the reaction does not give reasonable yields or it is impracticable. Unwanted anomers are produced by chemical glycosylation, *e.g. en route* to syntheses of α-galactosides or β-mannosides.

(2) Use of heavy metals as catalysts is not acceptable because of their toxicity or high price. This is especially true in the case of products with potential use in the pharmaceutical area or in nutrition.

(3) Enzymatic glycosylation can help to overcome steric hindrance because an unprotected glycosyl residue is introduced that is less bulky than the protected glycosyl donor in the case of chemical reaction.

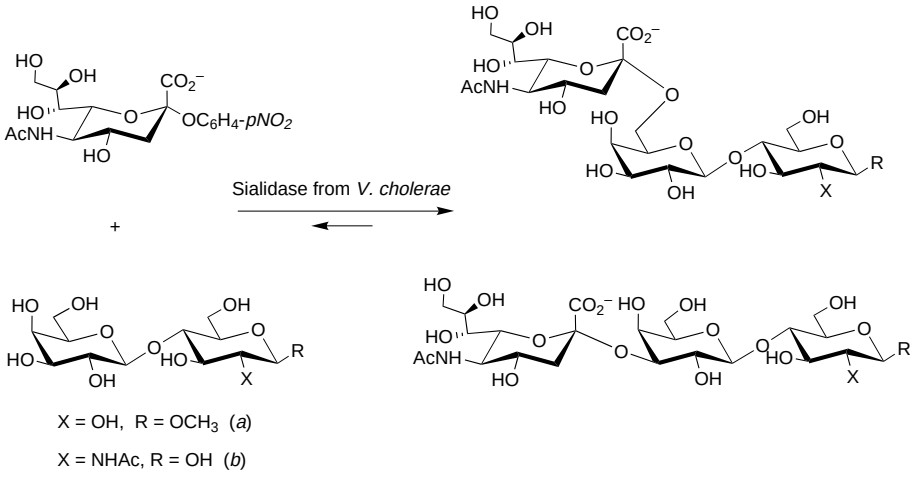
(4) There exists a suitable enzymatic system for effective glycosylation and the substrate is at least partly water-soluble.

(5) The substrate is rare or expensive—in the case of enzymatic glycosylation, even if the yields are lower, the unreacted substrate can be recovered near quantitatively.

If there exists a suitable chemical method giving sufficient glycosylation yields of the desired product in the appropriate quality then the use of enzymatic glycosylation would be only *l'art pour l'art*. Nevertheless, even in these cases methodology orientated studies may be of interest with regard to future limits of the chemical processes.

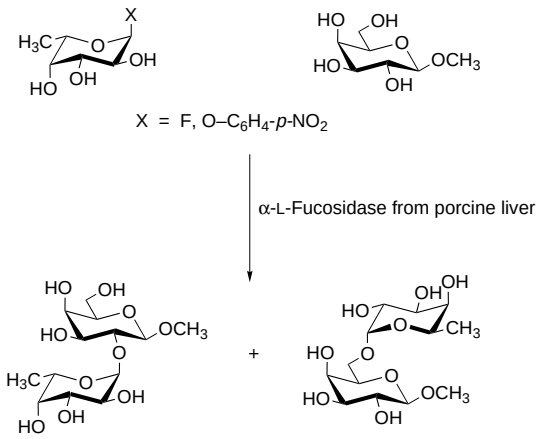
Chemical syntheses of sialyl-glycosides or fucosyl-glycosides often involve considerable problems. The use of glycosyltransferases is limited due to their narrow selectivity and the high price of the enzymes and glycosyl donors—*vide infra*. Therefore, using sialidases and fucosidases represents an interesting alternative.

Sialidases are usually isolated from pathogenic bacteria where they often act as toxins causing desialylation of cell surface glycoconjugates which then causes cell lysis. Sialidase from *Vibrio cholerae* was used for reversed glycosylation of methyl β-galactopyranoside using as a glycosyl donor-free 5-*N*-acetylneuraminic acid (Neu5Ac) affording both the α,2-3- and the α,2-6-linked disaccharides (ratio 1 : 10, ^{1}H NMR) in a mere 1% overall yield.[8] The tranglycosylation concept using as activated donor the α-*p*-nitrophenyl glycoside of Neu5Ac and sialidase immobilized on 'VA-epoxy' as carrier gave considerably higher yields (16%) of α,2-6- and α,2-3-disaccharides in a ratio of 2 : 1. In the case of methyl β-lactoside (*a*) or *N*-acetyllactosamine (*b*) (Scheme 3) as acceptors, a 14–16% yield of trisaccharides was obtained. The α,2-6 isomer, and the α,2-3 isomer, representing the defucosylated sialyl Lewis^X were obtained in a ratio of 2.8 : 1 (Scheme 3).[8] These examples demonstrate well the complementarity of the two 'glycosidase synthesis approaches': reversed glycosylation and transglycosylation.



Scheme 3 Tranglycosylations catalysed by sialidase from *Vibrio cholerae*[8]

One of the few examples of the use of fucosidases for transglycosylation is a chemoenzymatic synthesis of galactosyl fucosides employing porcine α-L-fucosidase[9] giving yields up to 16% (Scheme 4). Here, two types of activated donors were used, *e.g. p*-nitrophenyl α-L-fucoside and α-L-fucosyl fluoride with comparable yield and regioselectivity. Use of other glycosyl fluorides (α-galactosyl fluoride, α-glucosyl fluoride) as activated donors in the enzymatic transglycosylation is an attractive alternative to the nitrophenyl α-glycosides. β-Glycosyl fluorides cannot be used because of their instability.



Scheme 4 Transglycosylation catalysed by α-L-fucosidase from porcine liver[9]

2.2 Regioselectivity of glycosidases

The regioselectivity of glycosidases is rather poor. If more OH groups are available as acceptors, several products are usually encountered in various proportions. Often the regioselectivity of the glycosidases can be partly predicted—*e.g.* in hexopyranoses the affinity of OH groups for the glycosyl transfer generally decreases in the following order: 6-OH ≫ 4-OH ≥ 3-OH > 2-OH. This selectivity also correlates with chemical reactivity of these groups.

The often cited papers of Nilsson[10] show a possible control for regioselectivity by changing the substituent configuration at the anomeric carbon. This case is, however, quite special and subtle changes in structure and conformation in this region could bring about alternative regio-effects. Until now, it has not been demonstrated whether the change of a methyl group in the above case[10] into, *e.g.* an ethyl group would result in the conservation of regioselectivity.

A systematic approach to the regioselectivity problem is, however, the use of glycosidases with their own structural preferences towards a specific position in the carbohydrate pyranose or furanose rings. Provided a broad variety of glycosidases having synthetic properties are available, it is possible to choose the particular glycosidases with appropriate regioselectivity.

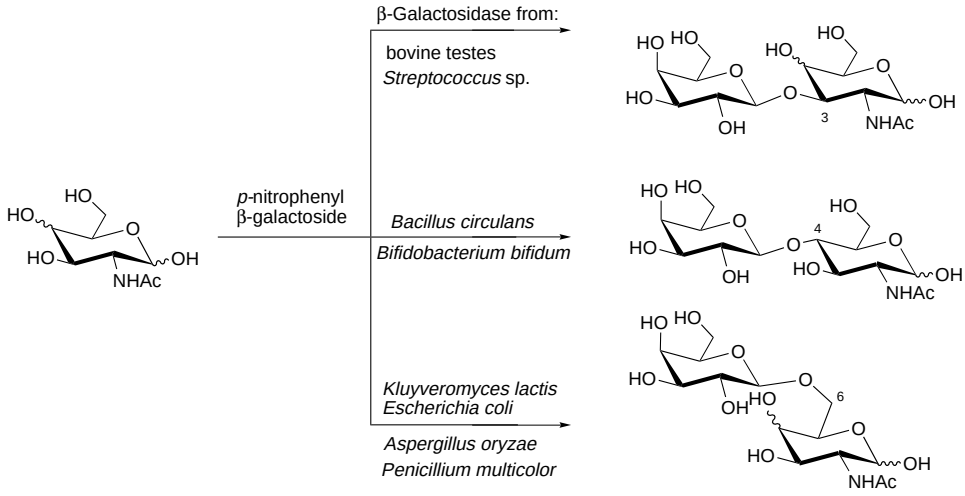
It seems that the regioselectivity of the transglycosylation reactions is analogous to the hydrolysis specificity towards the same type of linkages. This was rather convincingly demonstrated by Ajisaka *et al.*[11] by a kinetic study on the isomers of *N*-acetyl lactosamine and on their sialylation by sialidases. The same authors[11] and many others demonstrated that by rational selection of galactosidases from various sources it is possible to prepare various regioisomers of *N*-acetyllactosamine, *e.g.* the 1→6 isomer using β-galactosidase from *Aspergillus oryzae*, the 1→4 isomer using that from *Bacillus circulans* or *B. bifidum* and the 1→3 isomer using that from *Streptococcus* sp. or from bovine testes[12] (Scheme 5).

Glycosidases also display regioselectivity in a hydrolytic mode that can be well used for preparation of selectively glycosylated substances. β-Glucuronidase from the limpet *Patella vulgata* can selectively cleave glucuronic acid from the aromatic hydroxy group of morphine-3,6-diglucuronide[13] (Scheme 6). This enables the production of morphine-6-glucuronide that has about three times higher analgesic activity than the aglycone.

There is, however, a constant need for novel biocatalysts. Glycosidases with pronounced regioselectivity should be searched mainly among endoglycosidases and screened for the cleavage of a particular glycosidic bond that is to be synthesised. Good substrates for such a screening can be found among natural heteropolysaccharides often bearing the desired glycosyl residue(s).

2.3 Stereoselectivity of glycosidases

Besides regioselectivity of glycosidases one can also observe chiral discrimination displayed by these enzymes. This is a rather common fact observed in many enzymes, *e.g.* lipases, esterases, oxidoreductases and nitrilases—that are able to discriminate between two enantiomers in both the synthetic or the lytic mode of action. There are several examples demonstrating that glycosidases are able to discriminate enantiomers during glycoside synthesis.[14,15] Systematic studies of this problem were undertaken quite recently.[2]



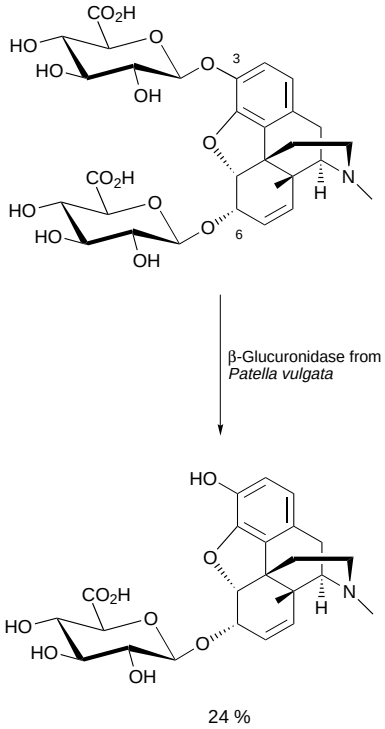
Scheme 5 Regioselectivity of glycosidases from various sources

Boos *et al.*[14] have studied β-D-galactosyl transfer to *sn*-glycerol using a β-D-galactosidase from *E. coli* and demonstrated that only the (2*R*)-glycerol-1-*O*-β-D-galactopyranoside (in addition to smaller amounts of 2-*O*-β-D-galactosyl-glycerol) are formed (Scheme 7). Racemic 2,3-epoxypropanol has also been used as an acceptor.[15] A diastereomeric excess of (2*R*)-2,3-epoxypropyl β-D-galactoside (*R* : *S* = 7 : 3) was obtained with *o*-nitrophenyl β-D-galactoside as donor but no diastereomeric excess could be detected with lactose. This indicates that the stereochemical outcome of the formation—and probably then also of the hydrolysis—of the glycosides is kinetically controlled because both the β-galactosyl donors used have different kinetic properties (K_M, V_{max}).

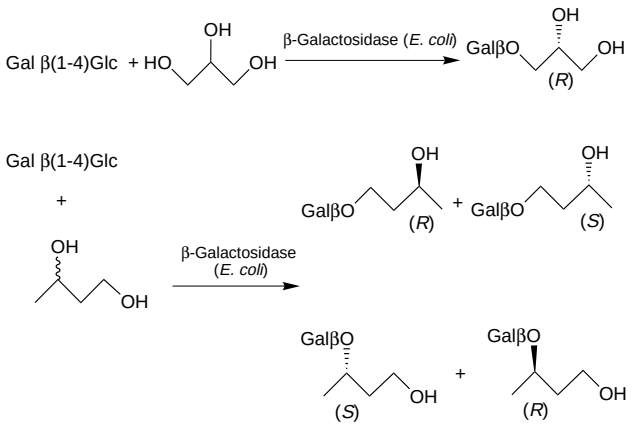
Regio- and stereo-chemical studies have been carried out by Crout *et al.*[2] using lactose as a donor with racemic butan-2-ol and with a variety of diols acting as acceptors. If racemic propane-1,2-diol was used as acceptor all four possible products were formed. The major product contained the galactose attached to the primary OH group of the diol (*R* : *S* = 1.0 : 0.86). A similar selectivity (*R* : *S* = 1 : 0.77) was observed in formation of the minor product by transfer to the secondary hydroxy group. Overall, transfer to the primary hydroxy group was favoured over the secondary by a factor of 1 : 0.35.

Somewhat different results were obtained when racemic butane-1,3-diol was used as acceptor[2] (Scheme 2). In this case there was only a very slight diastereoselectivity in transfer to the primary hydroxy group (*R* : *S* = 1.0 : 0.9), but in the transfer to the secondary hydroxy group there was a marked selectivity in favour of the (*R*)-enantiomer (*R* : *S* = 1 : 0.5). Overall transfer to the primary hydroxy group was favoured over the secondary by a factor of 1 : 0.15. Various results for these diols were interpreted in terms of their possible conformational similarity with glucose and eventual interaction with the hydrophobic 'glucose' binding site of the β-galactosidase (lactase). A number of other examples of glycosidase stereoselectivity have been reviewed recently.[16]

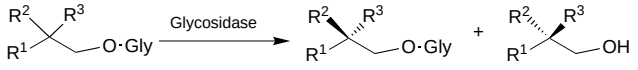
The preceding examples show that some glycosidases are able to distinguish between steric neighbourhoods of the hydroxy group to be glycosylated. It implies the possibility of analogous stereo-discrimination during hydrolysis. We recently demonstrated that β-galactosidases display a significant stereoselectivity during hydrolysis.[16] Chemically synthesised galactopyranosides of several racemic alcohols were subjected to enzymatic hydrolysis by β-galactosidases from different microbial sources, *e.g. E. coli, A. oryzae, Kluyveromyces lactis* and *Bacillus circulans* (Scheme 9). From all β-galactosidases tested one from *E. coli* displayed the most pronounced stereoselectiv-



Scheme 6 Regioselective hydrolytic cleavage of morphine-3,6-diglucuronide by β-glucuronidase from *Patella vulgata*[13]

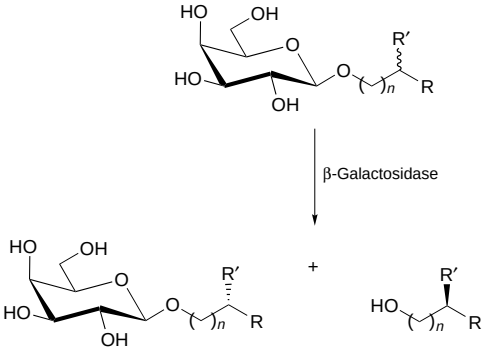


Scheme 7 Stereoselective galactosylation of racemic polyols by β-galactosidase from *E. coli*[2,14]



Scheme 8 Principle of chiral discrimination by glycosidases

ity. The selectivity can be improved by higher reaction temperature and by a short reaction time.



Scheme 9 Chiral discrimination of racemic alcohols by β-galactosidase from *E. coli*[16]

Poor selectivities were observed for the β-galactosides of pentan-2-ol, the 1-β-*O*-galactoside of propane-1,2-diol, and both galactosides of pentane-1,4-diol. On the other hand, β-galactosides of 1,2-*O*-isopropylidene-glycerol, butan-2-ol, and both galactosides of butane-1,3-diol were hydrolysed with good stereoselectivity (Table 1). In comparing these results to

Table 1 Chiral discrimination of synthetic galactosides by β-galactosidase from *E. coli*[a]

β-Galactoside of aglycone	D.e. of synthetic galactoside (%)	Conversion (%)	E.e. of alcohol released (%)
Isopropylidene-glycerol[b]	22 (*S*)	65	11 (*R*)
		40	46 (*R*)
		7	61 (*R*)
Butan-2-ol[c]	20 (*S*)	14	51 (*R*)
		12	60 (*R*)
Pentan-2-ol[c]	11 (*R*)	8	22 (*R*)
Propane-1,2-diol[b]	23 (*R*)	61	15 (*R*)
Butane-1,3-diol[b]	9 (*R*)	22	56 (*S*)
Butane-1,3-diol[b]	23 (*R*)	23	76 (*R*)
Pentane-1,4-diol[b]	0	41	24 (*R*)
Pentane-1,4-diol[b]	8 (*S*)	16	16 (*R*)
		24	16 (*R*)

[a] Concentration of substrates 4 μmol cm^{-3}, reaction temperature 40 °C, enzyme activity 0.8 U cm^{-3}. [b] Incubation time 15 min. [c] Incubation time 30 min.

those obtained by Crout *et al.*[2] it can be concluded that in the case of propane-1,2-diol the extent of chiral discrimination is about the same, however, the preferred absolute configuration appears to be opposite, *e.g.* (*R*) in the transglycosylation and (*S*) in the hydrolytic process. For butan-2-ol and butane-1,3-diol higher selectivities were obtained in the hydrolytic process. Both transgalactosylation of butan-2-ol and the secondary OH group of butane-1,3-diol and hydrolysis of their galactosides showed the same preference for the (*R*)-enantiomer.

In contrast to the observations in transgalactosylation[2] it was observed that in the hydrolytic process[16] the selectivity could not be associated with a similarity with the natural substrate of β-galactosidase, lactose, in particular, to the glucopyranose moiety. The distinguishing effect of β-galactosidase from *E. coli* could be based on hydrophobic interactions. In summary, galactosidase mediated hydrolysis of racemic β-galactopyranosides led to significant enantiomeric enrichments in some of the alcohols released.

3 Glycosyltransferases

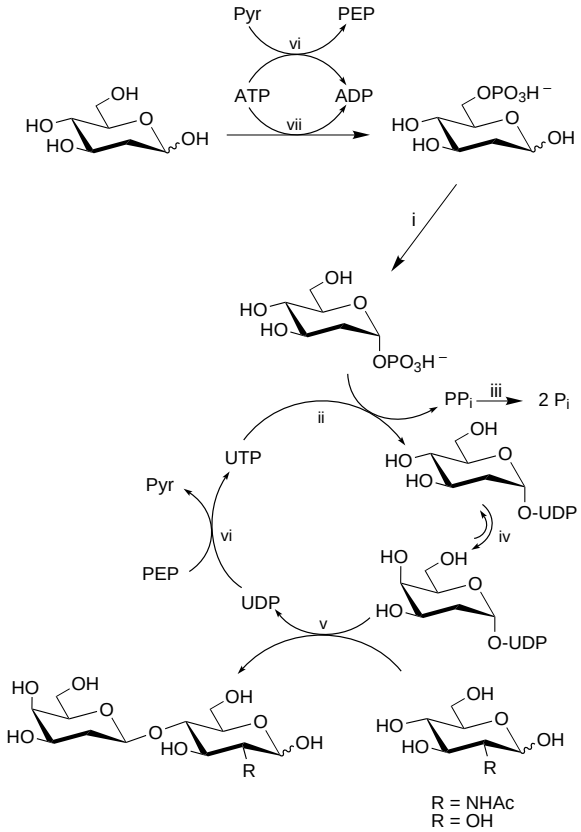
3.1 Leloir-type glycosyltransferases

Glycosyltransferases are more expensive and rarer enzymes. They are also usually more sensitive to environmental conditions, often demanding special buffers or detergents for solubilization. They show, however, better selectivities and give nearly quantitative yields. Many glycosyltransferases have been cloned—for a survey of cloned sialyltransferases see *e.g.* ref. 17 Immobilization of the glycosyltransferases is another effective way to reduce the costs.

Glycosyltransferases are often referred to as being rather stringent towards the distal one to two saccharidic moieties and also very specific to the glycosyl donor (nucleotide). At present, there are, however, numerous examples demonstrating that also glycosyltranferases can be 'persuaded' to work with both unnatural donors and/or acceptors maintaining their main advantages, *i.e.* strict regio- and stereo-selectivity and high yields.

Only a few glycosyltransferases are readily available and among those a considerable number of experiments have been performed with galactosyltransferase (GalT). The substrate specificity of this enzyme has been extensively studied and often reviewed.[1]

The problem of *in situ* UDP-Gal regeneration and GalT feedback inhibition by UDP was solved in 1982 by Whitesides.[18] A recent extension of this approach with regard to donor modification opened up further possibilities for enzymatic approaches (Scheme 10). Hexokinase (**vii**) allowed the forma-

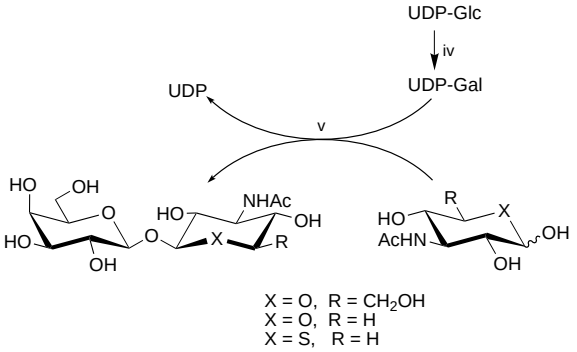


Scheme 10 GalT glycosylation with UDP-2d-Gal as donor (enzymes: **i** phosphoglucomutase; **ii** UDP-glucose-pyrophosphorylase; **iii**, inorganic pyrophosphorylase; **iv**, UDP-galactose-4′-epimerase; **v**, galactosyltransferase (GalT); **vi**, pyruvate kinase; **vii** hexokinase)[19]

tion of 2-deoxy-D-glucose-6-phosphate starting from 2-deoxyglucose (2-d-Glc) and ATP, which in turn could be regenerated from PEP with pyruvate kinase (**vi**). In analogy to ref. 18 the corresponding activated UDP-2-d-Glc and UDP-2-d-Gal could be obtained. Its reaction with GlcNAc or with glucose and GalT alone or in the presence of α-lactalbumin gave 2′-deoxy

analogues of *N*-acetyllactosamine and of lactose in 40 or 25% yields, respectively.[19]

A rather unexpected reaction with GalT (**v**) resulted when unnatural amino sugars were subjected to galactosylation (see Scheme 20 below). The reaction using GalT together with UDP-Gal 4′-epimerase with the 3-amino sugar *N*-acetylkanosamine (X = O, R = CH_2OH) gave a β-galactosylation at the anomeric position in the β-configuration and led to Galβ1-1βGlc3NAc in 22% yield.[20] Analogously, *N*-acetylgentosamine (Xyl3NAc, X = O, R = H) and *N*-acetylthiogentosamine (5SXyl3NAc, X = S, R = H) gave the corresponding trehalose-type linked disaccharide derivatives in good yields.[20] Recently, xylose was shown to be the first ambident substrate for GalT-catalysed galactosylation. Both the β1-4 and the β1-β1 transfer products (Galβ1-4Xyl and Galβ1-1βXyl) could be obtained in a 2 : 1 ratio.[21]



Scheme 11 Frame shifted galactosylation with GalT (**v**)[20,21]

Galactosyltransferases can also be used in glycosylation of non-sugar substances, *e.g.* some natural products. Complex glycosides of ergot alkaloids, required for immunological studies were prepared by use of GalT: for preparation of β-D-galactopyranosyl (1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→*O*)-elymoclavine **2**[5] the extension of 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→*O*)-elymoclavine **1**[5] by the use of bovine β,1-4-galactosyltransferase was chosen (Scheme 12).

For generation of UDP-Gal *in situ*, UDP-Glc and UDP-Gal 4′-epimerase were used. It was found, however, that β,1-4-galactosyltransferase was able to transfer glucose forming in parallel also β-D-glucopyranosyl (1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→*O*)-elymoclavine **3** (Scheme 12). This was later confirmed on a semipreparatory scale using UDP-Glc without the epimerase. The transfer of glucose was confirmed fully by spectral methods.[5] This is the first example of concomitant transfer of glucose and galactose by galactosyltransferase.

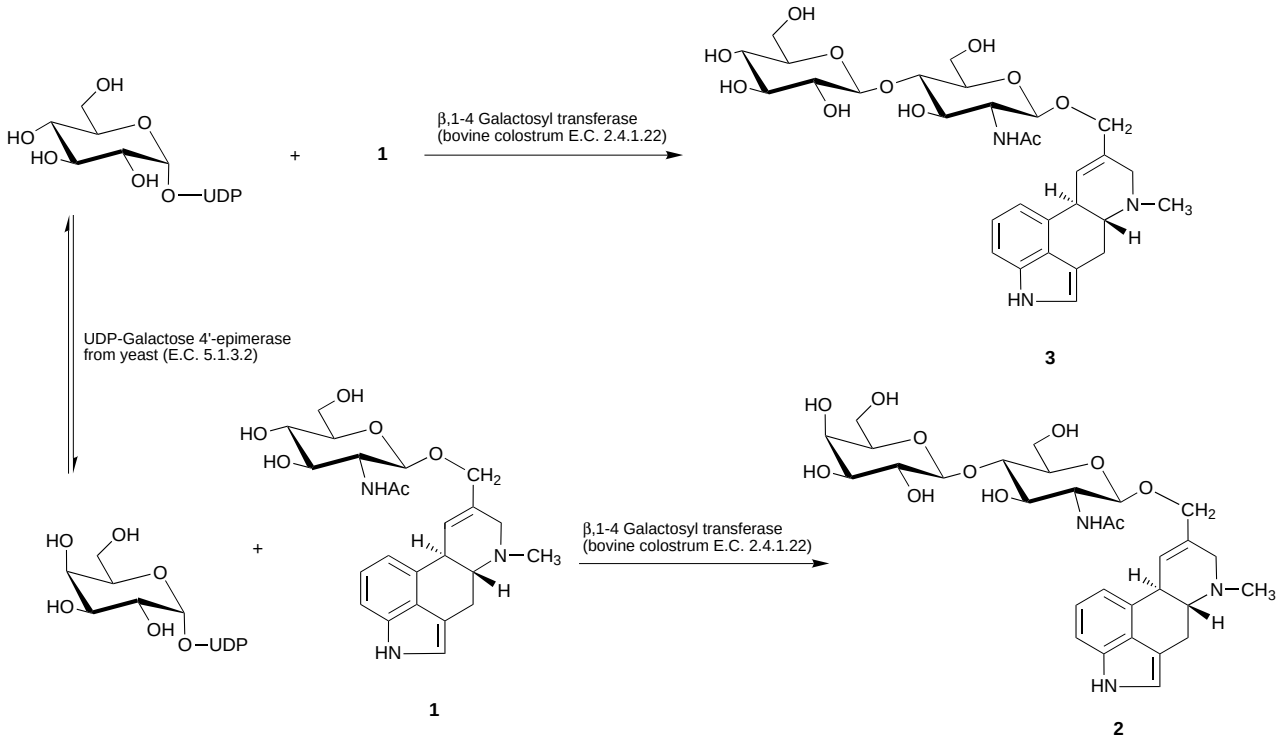
β-Lactosyl elymoclavine was prepared from the respective β-glucoside by use of bovine β,1-4-galactosyltranferase in the presence of α-lactalbumin.[5] Analogously, β-Lac and β-LacNAc derivatives of other ergot alkaloids, *e.g.* 9,10-dihydrolysergene were prepared.[5]

The concept of the use of non-natural donors has already been extended to other glycosyltransferases. Recently, α,1-3/4 fucosyltransferase from human milk was shown to be able to use as donors GDP-L-Gal, GDP-3d-L-Gal and GDP-3,6d_2-L-Gal the structure of which resembles that of the natural glycosyl donor GDP-L-Fuc. The reactions were accomplished with high yields up to 93% (Scheme 13).[22]

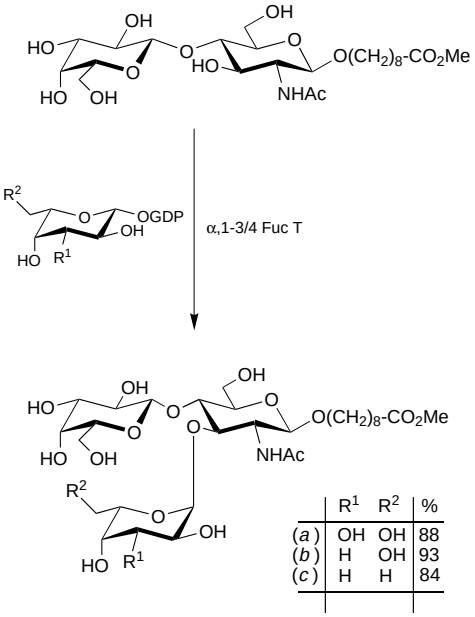
Some other examples of glycosyltransferase use—mostly sialyltransferases—are given in Section 4.

3.2 Non-Leloir glycosyl transferases

A special group of non-Leloir glycosyltransferases are, *e.g.* cyclodextrin glucanotransferases (CGTase). These enzymes are produced by microorganisms and many of them are commercially available. They catalyse cyclodextrination of starch but also a transfer of one or more α-glucosyl units to various acceptors. They can be used for extending glycosides or for α-glucosylation of many compounds, *e.g.* monosaccharides, stevioside, rubuoside, hesperidin, (+) catechin and others. The acceptor specificity of CGTases is rather broad and the enzymes of various origin are also able to glycosylate phenolic OH groups in very good yields. In sugars or glycosides with the D-Glc*p* moiety, the transfer is rather regioselective with

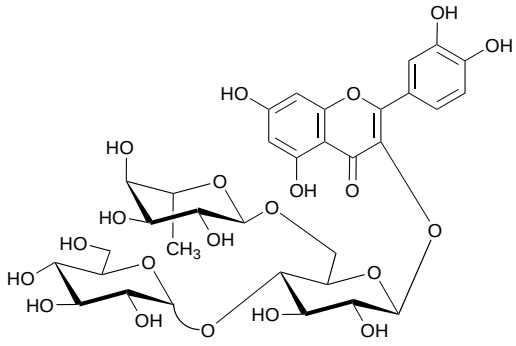


Scheme 12 Concomitant transfer of glucose and galactose by GalT[5]



	R^1	R^2	%
(*a*)	OH	OH	88
(*b*)	H	OH	93
(*c*)	H	H	84

Scheme 13 Glycosylation of lactosamine derivative by α,1-3/4 fucosyltransferase from human milk employing activated donors based on L-galactopyranose; (*a*) GDP-β-L-galactopyranose, (*b*) GDP-β-3-deoxy-L-galactose, (*c*) GDP-β-L-3,6-dideoxy-L-galactose[22]



4^G-α-D-Glucopyranosyl-rutin

Scheme 14 Product of rutin glucosylation by CGTase from *Bacillus stearothermophilus*[23]

preference for 4-OH so that the second Glc unit is always attached by an α(1–4) bond. In other sugars the regioselectivity is, however, rather poor.

CGTase from *Bacillus stearothermophilus* was used for transglucosylation of rutin. The glucosyl unit of rutin was extended by one or more α-glucosyl units and the population of oligoglucosides was trimmed to 4^G-α-D-glucopyranosyl rutin by *Rhizopus* sp. glucoamylase (Scheme 14).[23]

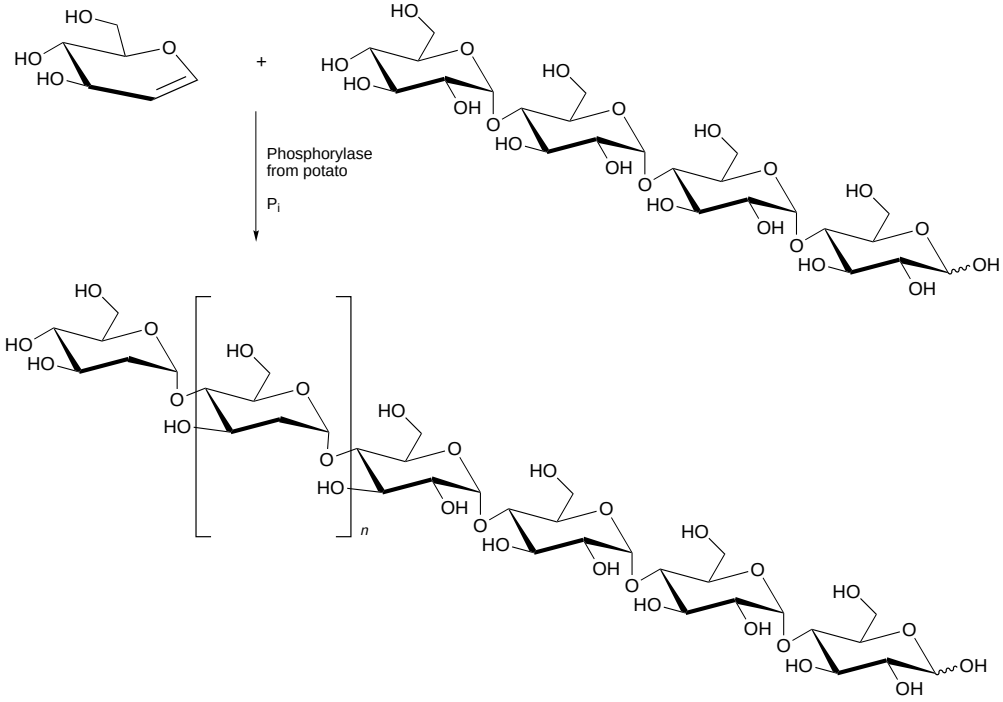
The natural task of another group of non-Leloir glycosyltransferases—phosphorylases—is the phosphorolytic cleavage of a glycosidic bond transferring the glycosyl unit released on the phosphate. Glycosyl transfer can, however, also be accomplished on another acceptor molecule, often with a phenolic OH group. Sucrose phosphorylase is rather complementary—having different regioselectivity—to the CGTases in preparation of α-glucosides of various aglycones, *e.g.* catechins, polyphenols and cyclitols.[24] Some phosphorylases are even able to accept non-natural sugars as glycosyl donors. Potato phosphorylase was able to transfer 2-deoxy-α-glucosyl residues from D-glucal to maltotetraose thus forming 2-deoxy-maltooligosaccharides (Scheme 15).[25]

4 Multienzyme glycosylation approaches

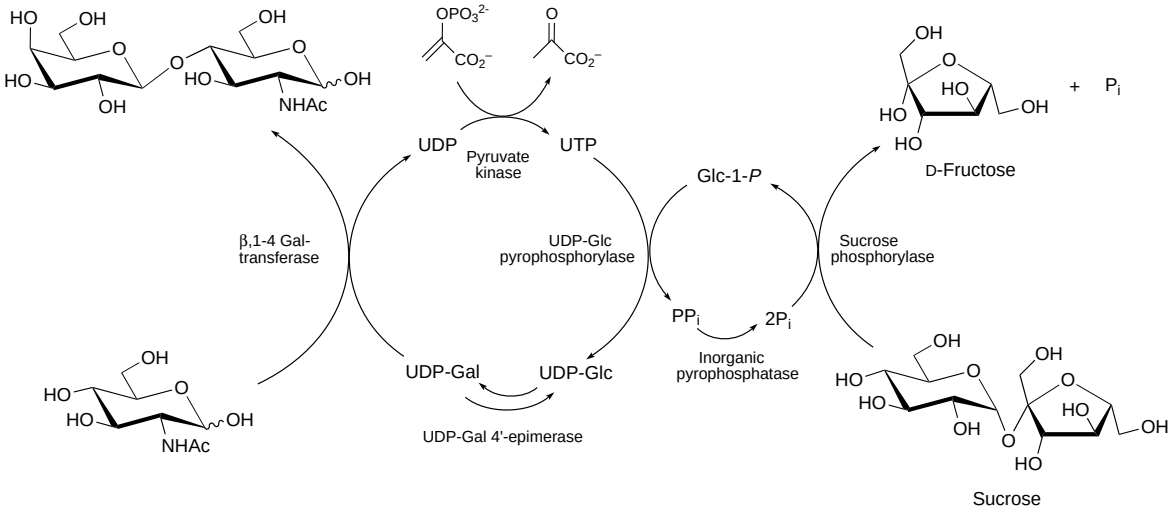
The first basic approaches using the multienzyme system in glycosylation were mostly targeted to regeneration of the activated glycosyl donors (kinases, phosphorylation sequences) or the generation of glycosyl donors *in situ*.

5-*N*-Acetylneuraminic acid is still a rather expensive starting material and therefore its generation *in situ* from *N*-acetylmannosamine with neuraminic acid aldolase was developed. This procedure can be integrated well with further enzymatic sequences generating the CMP-Neu5Ac required for enzymatic sialylation.[26]

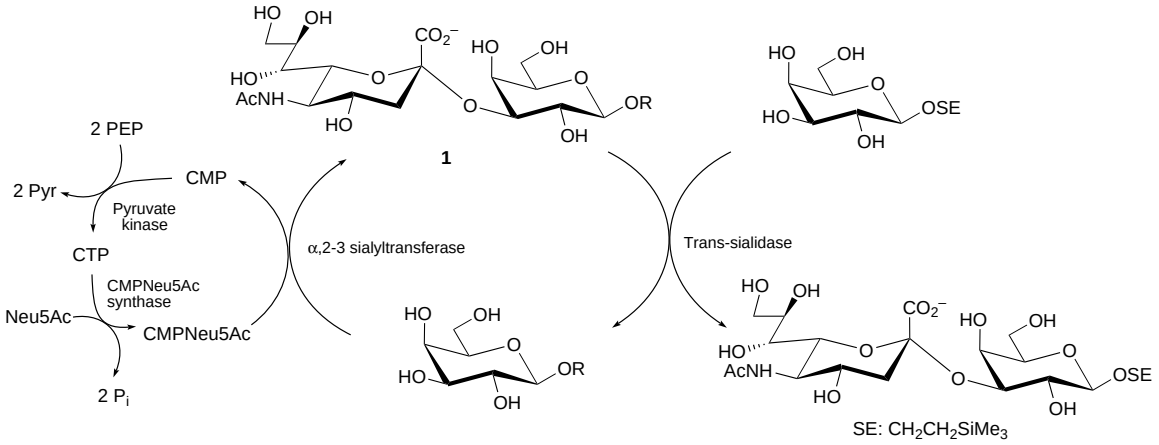
Generation of dinucleoside glycosyl donors, *e.g.* UDP-Gal was developed by Whitesides *et al.*[18] for *N*-acetyllactosamine synthesis in 1982. This reaction sequence started from glucose-



Scheme 15 Phosphorolytic formation of modified maltooligosaccharides containing 2-deoxy-α-D-arabino-hexopyranosyl residues[25]



Scheme 16 Galactosyltransferase reaction combined with sucrose phosphorylase reaction[27]



Scheme 17 Multienzyme system for synthesis of α,2-3 sialosaccharides using α,2-3-sialyltransferase from *Trypanosoma cruzi*[28]

1-phosphate, and recently this approach was improved by Ichikawa *et al*.[27] Starting from sucrose, glucose-1-phosphate could be favourably generated with sucrose phosphorylase (Scheme 16). One of the main advantages of this method is the removal of part of the potential ultimate inhibitor—inorganic phosphate. All enzymes used in this multienzyme system are now commercially available.

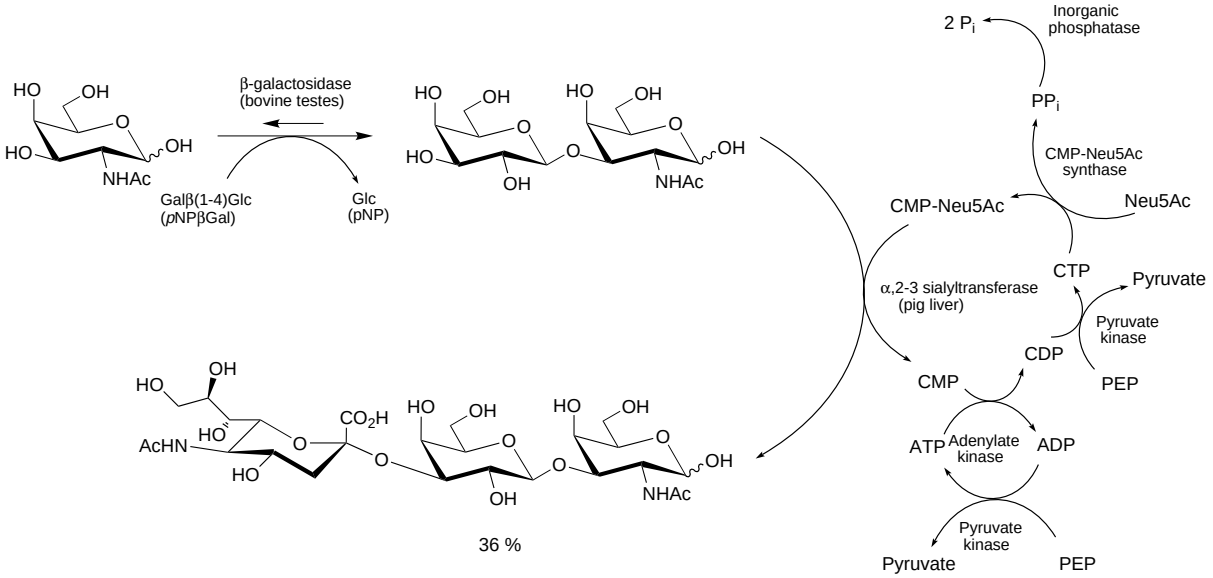
Recently, more sophisticated approaches have emerged, *i.e*. sequential use of glycosyltransferases including cofactor regeneration or even sequential use of glycosidases and glycosyltransferases. These approaches sometimes simulate real enzymatic sequences working *in vivo.*

Multienzyme systems were applied mainly in preparation of complex sialooligosaccharides. Sialic acid is a common structure in glycoconjugates and sialylated structures are involved in a variety of biological processes. Besides their role in secretion, immunogenicity, circulation half-life of glycoproteins, and cellular recognition phenomena they are identified as crucial structural elements of some antigenic determinants which have been identified as tumour markers.

Terminally substituted sialyl glycosides can either be prepared by sialyltransferases or by sialidases—*vide supra*.

In a quite prominent place among the sialidases used in sialoglycoside synthesis are the sialidases from the blood parasite *Trypanosoma cruzi*, which causes Chagas disease. This enzyme, named trans-sialidase (TS), catalyses the transfer of sialic acid from host glycoconjugates to acceptor molecules of the parasite plasma membrane. There is evidence that sialic acid receptors on the surface of *T. cruzi* mediate the initial stages of trypanosome invasion of the host cells. TS from *T. cruzi* has the unique property of catalysing the reversible transfer of Neu5Ac from a donor substrate of the sequence Neu5Acα2→3βGal-*O*-R to virtually any galactoside acceptor βGal-*O*-R[2] to yield as new product Neu5Acα2-3Gal-*O*-R[2]. Its use for synthetic purposes was, however, limited by the fact that the desired product is produced at the expense of another sialoside used as the donor substrate. This problem and also the shift of equilibrium in favour of the desired sialoside was solved by coupling this reaction to a complex system generating a donor of Neu5Ac for TS. This multienzyme system consists of a catalytic CMP-Neu5Ac regeneration cycle and α,2-3 sialyltransferase[28] (Scheme 17) and it allows for the enzymatic preparation of virtually any terminal Neu5Acα2-3Gal sequence without limitation by acceptor specificity (as with sialyltransferases) or low yield and poor regioselectivity (as with sialidases).

A multienzyme system consisting of sequential action of glycosidase and glycosyltransferase coupled with *in situ* regeneration of sugar nucleosides was demonstrated in the production of sialyl Thomsen–Friedenreich (*T*-antigen) epitope[12] (Scheme 18). The concept is based on the pronounced selectivity of β-galactosidase from bovine testes for the β,1-3 glycosidic bond formation. Although small amounts of other regiomers are formed, only the β,1-3 isomer is accepted by α,2-3 sialyltransferase. The product is no longer a substrate for galactosidase and this shifts the equilibrium of the trans-galactosylation reaction towards the desired product and allows the synthesis of the trisaccharide in an irreversible manner. In



Scheme 18 Multienzyme system for synthesis of *T*-antigen epitope[12]

this multienzyme reaction the problem of bringing rather distant pH optima of sialyltransferase, the cofactor regenerating system (pH 7.5–9.0) and β-galactosidase (pH 4.3) into harmony was faced. This was achieved by rational manipulation of reaction conditions and the use of activated substrates. Thus, it could be demonstrated that the enzymes with rather distant pH optima could be employed, when carefully rationalised and optimised. This concept opens new perspectives for the syntheses of glycosides having up to three or four glycosyl units in one-pot reactions.

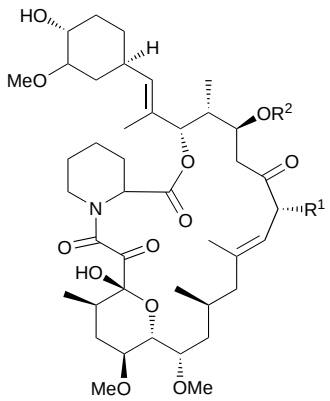
The systems described above are rather complicated, but they help to avoid multistep reactions and laborious purification procedures of the intermediates. They also considerably lower the overall costs mostly due to the integrated cofactor regeneration *in situ*. When this concept is combined with the use of immobilised enzymes or *e.g.* membrane reactors[26] they can be used for effective production of some rare complex oligosaccarides.

5 Glycosylation by whole living cells

The above described multienzyme systems tend to simulate the situation in the living cell where much more complicated multistep reactions take place. Use of living cells combines the advantages of the multienzyme approaches, *e.g.* high selectivity and cofactor regeneration and helps to avoid expensive and laborious isolation of the respective enzymes. For this purpose microorganisms, both prokaryotic and eukaryotic, and plant cell cultures are often used. Mostly, glycosyltransferases are responsible for the glycosyl transfer in these systems.

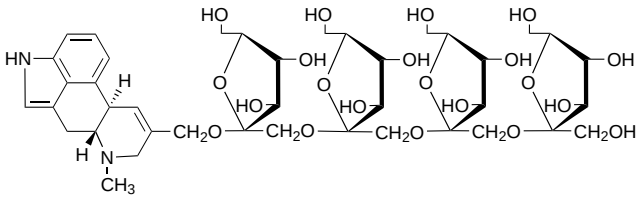
There exist few examples, in which living microbial cells were used for glycosylations. An example of the glycosylation of a rather complicated molecule by resting cells of *Bacillus subtilis* is a preparation of the 24-*O*-β-glucopyranoside of the immunosuppressive drugs FK 506 and of immunomycin[29] (Scheme 19). A strict regioselectivity of the enzyme can be noted because glycosylation at only one of the three available OH groups occurs. The selectivity is also documented by the fact that the glycosylating strain was identified after screening of approximately 1000 strains.

The above microbial glycosylation was catalysed by glycosyltransferase. There are also whole microbial cell biosystems possessing glycosidases with transglycosylation activity. These enzymes are usually bound to cells quite often in the periplasmatic space. Well documented cases are, *e.g.* fructosylations. The growing culture of *Claviceps purpurea* is able to glycosylate ergot alkaloids, either introduced or produced *per*



1 R^1 = $CH_2CH{=}CH_2$, R^2 = H
2 R^1 = CH_2CH_3, R^2 = H
3 R^1 = $CH_2CH{=}CH_2$, R^2 = β-Glc
4 R^1 = CH_2CH_3, R^2 = β-Glc

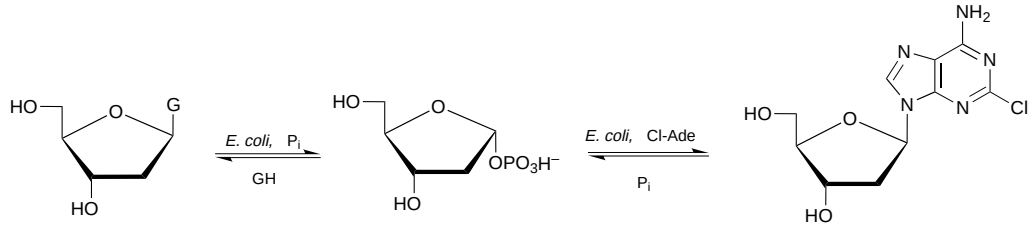
Scheme 19 Structure of FK 506 **1** (R^1 = CH_2-CH=CH_2, R^2 = H), immunomycin **2** (R^1 = CH_2- CH_3, R^2 = H) and their 24-β-glucopyranosyl derivatives **3**, **4** (R^2 = βGlc) produced by *Bacillus subtilis* ATCC 55060[29]



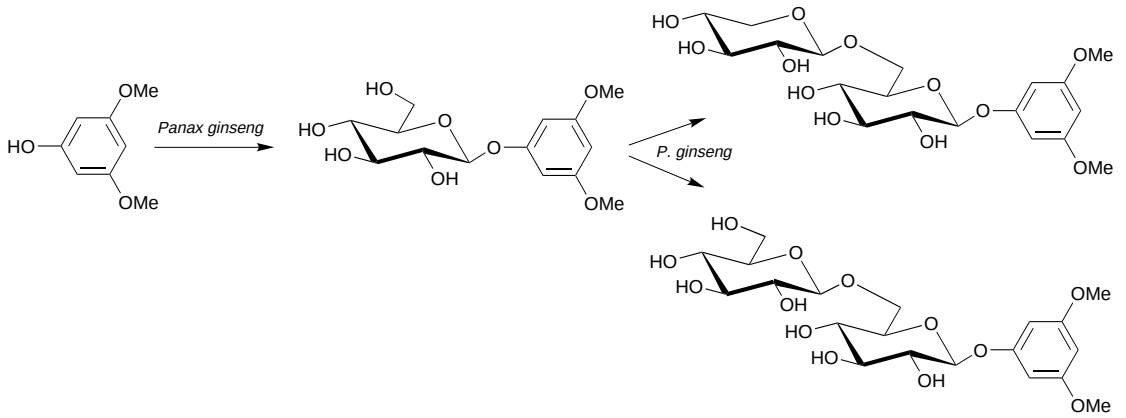
Scheme 20 Tetrafructoside of elymoclavine produced by transfructosylation activity of *Claviceps purpurea*[5]

se, forming alkaloid-oligofructofuranosides.[5] Sucrose served as a donor for this transfructosylation reaction. Glucose was utilised by the fungus for its growth and the fructose released was transferred onto the aglycone. Various fructosides having up to four fructosyl units were isolated (Scheme 20).

A special case of glycosylation by microorganisms is a preparation of *N*-2′-deoxy-β-ribosides of heterocyclic bases. The chemical synthesis of 2′-deoxy-β-ribosides suffers, compared to the preparation of β-ribosides, often from poor enantioselectivity, giving anomeric mixtures of deoxy-ribosides. The enzyme responsible for these reactions—nucleoside



Scheme 21 Synthesis of 2-chloro-2′-deoxyadenosine by *E. coli* BMT-1D/1A cells selected for high purine nucleoside phosphorylase[30]



Scheme 22 Glycosylation of 3,5-dimethoxyphenol by *Panax ginseng* plant cell culture[32]

2′-deoxyribosyl transferase—ensures full stereoselectivity and regioselectivity. A preparation of the potent antitumour drug 2-chloro-2′-deoxyadenosine was accomplished on a preparative scale by using glutaraldehyde-crosslinked whole cells of a selected strain of *Escherichia coli*[30] with the glycosyl donor deoxy-guanosine and 10 mM inorganic phosphate necessary for a good yield (65%) (Scheme 21).

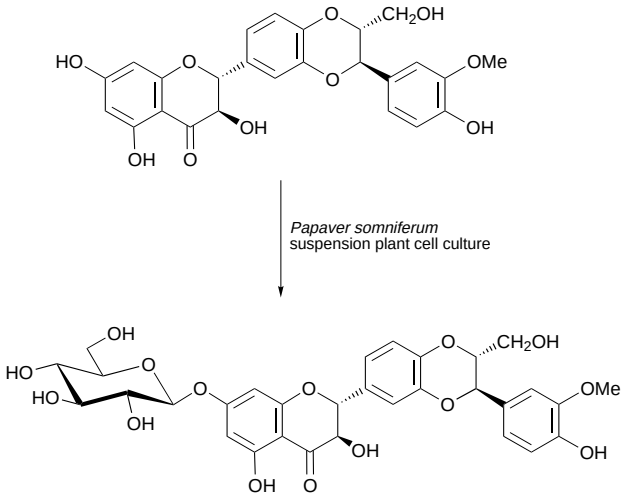
Whereas glycosylations by growing microoorganisms are rather scarce, plant cell cultures have often been used mostly for β-glucosylation. Plant glycosyl-transferases are often specific for various flavonoids. Some of them have somehow 'wobbling' specificity and therefore they can be used for glycosylation of, *e.g.* phenols, steroids, flavonoids, cardenolides and stevioles. These systems can be well used for glycosylation of phenolic hydroxy groups and also for the preparation of glycosyl esters in deprotected form.

The main disadvantages are longer reaction times (days) and sometimes complicated handling of the biosystems, but the main advantages are the glycosylation of barely accessible phenolic hydroxy groups and carboxy groups.

For glycosylations by plant cells, plant homogenates, tissue slices or even whole plants (*e.g.* seedlings) can be used. However, the most suitable are suspension plant cell cultures. In the last two decades their application has been developed so that they can be used without special prerequisites.

Salicylic acid can be β-glucosylated by a cell suspension culture of *Mallotus japonicus*. The yield can be increased up to 1.1 g l^{-1} by continuous feeding of the substrate and the process can be scaled up (5 l fermenter). It was demonstrated that this glycoside exhibited quicker and longer lasting analgesic effects than the aglycone alone.[31] The root culture of *Panax ginseng* has very good glycosylation activity towards aromatic phenols and carboxylic acid and was found to glucosylate, *e.g.* 3,5-dimethoxyphenol, methyl salicylate, *p*-hydroxyacetophenone and coniferyl alcohol, forming in addition to β-D-glucosides, β-D-gentiobiosides and β-D-primeverosides (Scheme 22).[32]

Flavonoid and flavolignan glycosides can often be found in plant tissues and respective glycosylation systems (usually UDP-Glc dependent glycosyltransferases) can be used for glycosylation of various flavonoid compounds. Glycosylating systems with quite pronounced regioselectivity can often be found. Selective glycosylation of silybin (an effective hepatoprotective compound with low water solubility) was accomplished in nearly quantitative yield by suspension cell culture of *Papaver somniferum*. The resulting compound was many times more water soluble than the parent compound and therefore its bioavailability greatly increased[33] (Scheme 23).



Scheme 23 Glucosylation of silybin by *Papaver somniferum* var. *setigerum* plant cell culture[33]

The chemical preparation of glycosyl esters in their deblocked form is sometimes a rather complicated task due to their lability during deblocking procedures. Plant cell cultures facilitate their preparation under very mild conditions in a single step. A good example is the preparation of the potential antitumour agent 6-*O*-butyryl-D-glucose by the cell suspension culture of *Nicotiana plumbaginifolia*.[34] Using plant cultures anomeric glycosyl esters can also be prepared.

Using plant tissue slices or homogenates is now rather obsolete but sometimes good results can be obtained with

highly metabolically active whole young plants, seedlings or with germinating seeds. 5′-*O*-(β-D-Glucopyranosyl) pyridoxine and 4′-*O*-(β-D-glucopyranosyl) pyridoxine were formed by germinating seeds of wheat, barley and rice cultured on a pyridoxine containing medium; the ratio was 1 : 1 but germinating soy bean seeds formed only 5′-*O*-(β-D-glucopyranosyl) pyridoxine.[35]

Preparation of β-glucuronides of various xenobiotics is a good task to be accomplished by bio-systems. β-Glucuronides are important for the catabolism and pharmacokinetic study of most drugs. Moreover, some glucuronides have different pharmacodynamic properties, compared to the original drugs. In man, morphine is glucuronidated at position 6 and the resulting 6-β-glucuronide of morphine has a three times higher analgesic effect than the aglycone[36] (contrary to the morphine-3-β-glucuronide—see also Scheme 6).

β-Glucuronides could be prepared by the use of a subcellular microsomal fraction from beef liver containing glucuronyl transferase. The extremely expensive UDP-glucuronic acid serves as a glucuronic acid donor in this case. Regeneration of this substrate can be accomplished well by the intact organism—*vide infra*—or by, *e.g.* using whole perfused liver. This, rather sophisticated, technique recently became commonly used for liver physiology or drug metabolism studies[37] and its further application in glycobiology is expected.

There is probably a single example of glycosylation by using whole living organisms, *e.g.* preparative glucuronylation of various xenobiotics. Animals—usually rabbits—are fed or injected with the substance to be glucuronylated. The respective glucuronide is isolated from the urine. Fishman[38] used this method for preparation of β-glucuronide of phenolphthalein used as a chromogenic substrate for β-glucuronidase determination. Rabbits were injected with sodium phenolphthalein phosphate and the phenolphthalein glucuronide was isolated as the cinchonidine derivative. This method has certain drawbacks, *e.g.* possible degradation of substrate or unwanted conjugations, sulfonation, and also ethical problems.

6 Conclusions

Synthetic applications of enzymes effectively supplement and/or complement established carbohydrate synthetic methods. Individual applications should be carefully adopted and rationalised. Enzymatic methods should always be used in a way complementary to synthetic steps.

Glycosidases have become widely used in more subtle ways due to pronounced knowledge of their regio- and stereoselectivity. This allows use of these cheaper and more robust enzymes in applications where previously only glycosyltransferases or laborious synthetic sequences could be employed. Glycosyltransferases, formerly believed to be rather stringent towards acceptor and donor, can now be used in more flexible ways with structurally diverse analogues. Multienzyme approaches combine both the advantages of glycosidases and glycosyltransferases and they enable one-pot syntheses of complex trisaccharides or even tetrasaccharides. Whole-cell systems can selectively glycosylate often very complex substrates that would be easily available neither by synthetic nor by enzymatic methods. These methods demand, however, broad screening of suitable systems but the eventual results are often rewarding.

7 Acknowledgements

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