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Published in:
Chemistry : a European Journal

DOI:
[10.1002/chem.201103069](https://doi.org/10.1002/chem.201103069)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Desmet, T., Soetaert, W., Bojarova, P., Kren, V., Dijkhuizen, L., Eastwick-Field, V., Schiller, A., & Křen, V. (2012). Enzymatic Glycosylation of Small Molecules: Challenging Substrates Require Tailored Catalysts. *Chemistry : a European Journal*, 18(35), 10786-10801. <https://doi.org/10.1002/chem.201103069>

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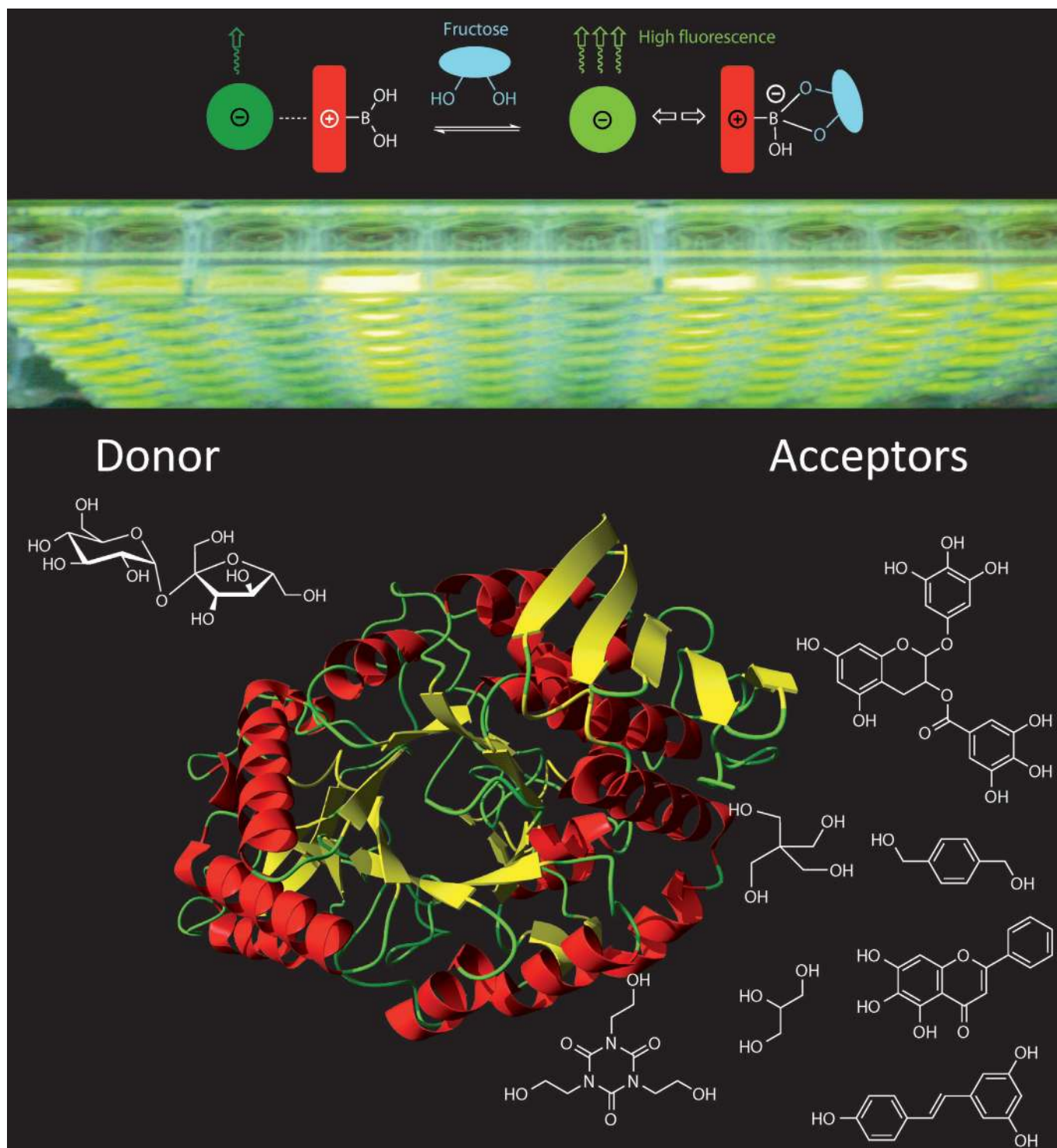
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Enzymatic Glycosylation of Small Molecules: Challenging Substrates Require Tailored Catalysts

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Lubbert Dijkhuizen,^[e] Vanessa Eastwick-Field,^[f] and Alexander Schiller*^[a]



Abstract: Glycosylation can significantly improve the physicochemical and biological properties of small molecules like vitamins, antibiotics, flavors, and fragrances. The chemical synthesis of glycosides is, however, far from trivial and involves multistep routes that generate lots of waste. In this review, biocatalytic alternatives are presented that offer both stricter specificities and higher yields. The advantages and disadvantages of different enzyme classes are discussed and illustrated with a number of

recent examples. Progress in the field of enzyme engineering and screening are expected to result in new applications of biocatalytic glycosylation reactions in various industrial sectors.

Keywords: acceptor specificity • enzyme engineering • glycosylation • glycosyltransferase • high-throughput screening

Introduction

Besides being a source of energy and a structural component of the cell wall, carbohydrates also mediate various recognition processes when attached to proteins or lipids.^[1] Well-known carbohydrate motifs of such glycoconjugates are the cancer epitope Sialyl Lewis X and the AB0 blood group determinants. In addition, glycosylation is an important source of structural diversity of natural products, such as alkaloids, steroids, flavonoids, and antibiotics. Glycosides typically display properties that differ from those of their non-glycosylated aglycons.^[2] A prime example is naringin, a flavanone glycoside that is responsible for the bitter taste of citrus fruits. Removal of the glycon part eliminates the bitter taste, which is one of the main goals of enzymatic treatment of grapefruit juice.^[3] The opposite is true for gly-

cirrhizin, a terpenoid glycoside from sweetwood (*Glycyrrhiza glabra*) that loses most of its sweetness upon hydrolysis.^[4]

Since the transfer of a glycosyl group can influence both the physicochemical and biological properties of an organic molecule, such processes may be used for a wide range of applications. The most obvious advantage of introducing a carbohydrate moiety is the increased solubility of hydrophobic compounds. This is nicely illustrated in flavonoids, the pharmaceutical properties of which can often be efficiently exploited only in the form of their hydrophilic glycosyl derivatives.^[2] Glycosylation may also be used to improve the stability of labile molecules. A famous example is ascorbic acid, a very sensitive vitamin, the long-term storage of which can be drastically extended by glycosylation, resulting in high-value applications in cosmetics and tissue culturing.^[5] Another important application of glycosylation is the reduction of skin irritation caused by hydroquinone, employed in cosmetics for its skin whitening effect.^[6] Glycosides of flavors and fragrances, in turn, can function as controlled release compounds. The α -glucoside of L-menthol, for example, is only slowly hydrolyzed in the mouth, resulting in a prolonged sensation of freshness.^[7] Last but not least, it has been possible to modulate the activity spectrum of glycopeptide antibiotics by varying their carbohydrate moiety, in a process known as “glycorandomization”.^[8,9]

In view of these examples, the development of cheap and efficient glycosylation technologies, useful both in the laboratory and in industry, is highly desirable. In this review, the challenges and recent innovations concerning the glycosylation of small, non-carbohydrate molecules are covered.

Chemical versus Enzymatic Glycosylation

Glycosylation reactions by conventional chemical synthesis are used intensively in the field of glycochemistry. Despite the variety of glycosylation protocols developed to date,^[10–25] synthesis of glycosylated compounds largely relies on four non-enzymatic reactions (Scheme 1). One of the first was famously developed by Koenigs and Knorr, in which glycosyl halides, activated with silver salts, are used as glycosyl donors.^[26–28] Glycosyl trichloroacetimidates were later found

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to be very powerful donor substrates with an excellent leaving group.^[29] Alternatively, more stable glycosides, such as thioglycosides and *n*-pentenyl glycosides, can be used when

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Dr. Pavla Bojarová, born in 1978, graduated at Charles University in Prague, Faculty of Sciences, in 2002, and obtained her Ph.D. in biochemistry in 2006. She has participated in several study stays abroad. For her postdoctoral studies she joined Dr. S. J. Williams' group at University of Melbourne, to work on time-dependent inactivation of sulfatases. Since her undergraduate years she has been working in the Laboratory of Biotransformation with Prof. V. Křen. Her main research interests include (chemo-)enzymatic synthesis of complex glycostructures using glycoside hydrolases, and analysis of these enzymes at the molecular level.



Prof. Vladimír Křen, born in 1956 is, currently Head of Laboratory of Biotransformation, and a Head of Department of Biotechnology of Natural Products at the Institute of Microbiology, Academy of Sciences of the Czech Republic. His research interests cover the biotransformation of natural products by enzymes and microorganisms; glycobiology; supramolecular chemistry; flavonoids and antioxidants; secondary metabolites of fungi; and immobilised microbial cells.



activated by electrophilic reagents.^[30] There are, however, two major issues connected with the outcome of these reactions, that is, the regioselectivity and the configuration of the glycosidic linkage. The former can be solved by appropriate protection strategies, whereas the latter is strongly dependent on the neighboring group participation of the C2 substituent. Additionally, solvents and catalysts have an important effect on the anomeric outcome of glycosylation reactions.

The chemical methods suffer from a number of drawbacks: labor-intensive activation and protection procedures, multistep synthetic routes with low overall yields, the use of toxic catalysts and solvents and the amount of waste.^[31] To overcome these limitations, specific enzymes may be used for the synthesis of glycosides. DeRoode et al. have calculated

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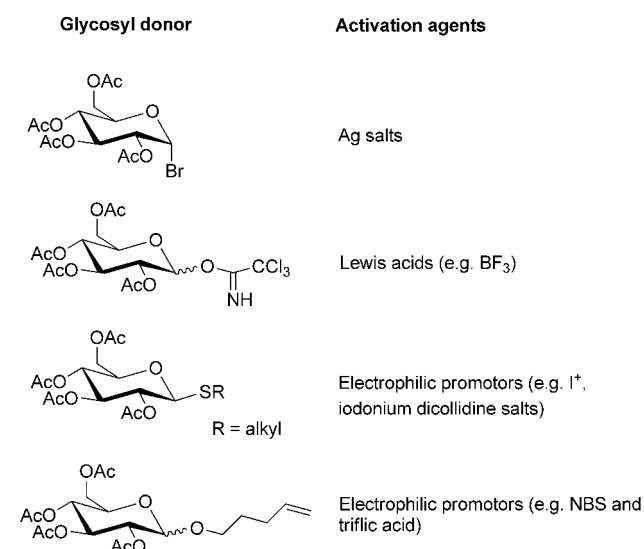


Vanessa Eastwick-Field is founder and Managing Director of Carbosynth Limited, an SME specialising in the development of carbohydrate-based entities for high-value application. She received her Ph.D. from the University of Warwick in 1990 in the electrochemistry of reduced state conducting polymers and subsequently worked in the fine chemical industry. Her career, spanning more than 20 years, has involved in all aspects of managing international SMEs including start-up, acquisition, and divestment.



Alexander Schiller studied chemistry at the University of Munich (LMU) and completed his Ph.D. in 2006 under the supervision of Prof. K. Severin at the École Polytechnique Fédérale de Lausanne (Switzerland). As a postdoc he worked together with Prof. B. Singaram and Dr. R. Wesling (University of California, Santa Cruz) on fluorescent saccharide sensors. At Empa (Switzerland) he was project leader and head of laboratory for stimuli-responsive materials. In 2009 he joined the Friedrich-Schiller University of Jena as a junior professor of the Carl-Zeiss foundation. His present research interests include NO and CO releasing materials and supramolecular analytical chemistry (<http://www.jppm.uni-jena.de>).

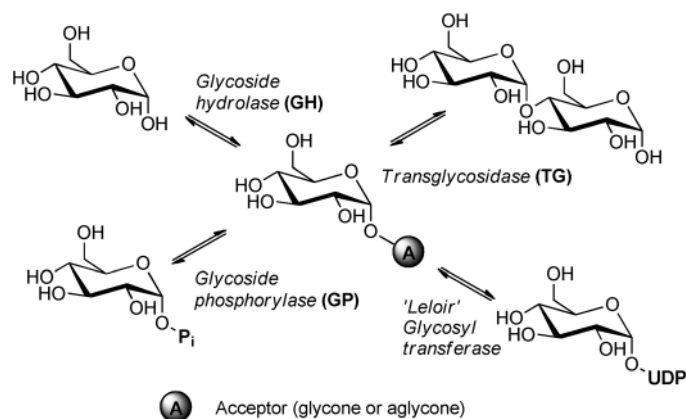




Scheme 1. Prominent glycosyl donors used in chemical synthesis (Ac = Acetyl, NBS = *N*-bromosuccinimide).^[30]

ed that enzymatic glycosylation reactions generate fivefold less waste and have a 15-fold higher space–time yield, a tremendous improvement in eco-efficiency.^[32] Although oligosaccharides, such as isomaltulose, isomalto (IMO), galacto (GOS) and fructo oligosaccharides (FOS), are synthesized industrially with the use of enzymes,^[33–35] this is not yet the case for glycosides. A perspective on the enzymatic glycosylation of small organic molecules will, therefore, be presented in this review.

Several types of carbohydrate-active enzymes (CAZymes) may be used in glycosylation reactions, each with specific characteristics (Scheme 2).^[36,37] Nature's catalysts for glycosylation reactions are known as “Leloir” glycosyl transferases (GT). Although very efficient, these enzymes require expensive nucleotide-activated sugars (e.g., uridine diphosphate glucose) as glycosyl donors, which hampers their application in the laboratory and industry. However, two spe-



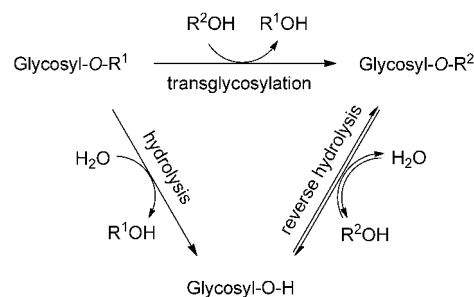
Scheme 2. Glycosylation reactions catalyzed by the various classes of carbohydrate-active enzymes (Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Adapted and reproduced with permission from reference [41]).

cial types of glycosyl transferring enzymes are the proverbial “exception to the rule” and are active with low-cost donors. Glycoside phosphorylases (GP), on the one hand, only require glycosyl phosphates (e.g., glucose-1-phosphate) as donors—compounds that can easily be obtained in large quantities.^[38] Transglycosidases (TG), on the other hand, even employ non-activated carbohydrates (e.g., sucrose) for the transfer of a glycosyl group.^[39] Additionally, glycoside hydrolases (GH) can also be used for synthetic purposes when applied under either kinetic (transglycosylation) or thermodynamic (reverse hydrolysis) control.^[40] In the following sections, the glycosylation reactions catalyzed by GH, GP, and TG will be described in more detail.

Glycoside Hydrolases

Glycosidases (*O*-glycoside hydrolases; EC 3.2.1.-) are *in vivo* purely hydrolytic enzymes. Their subclass in the IUBMB system (International Union of Biochemistry and Molecular Biology) comprises over 150 entries. In the CAZy database (Carbohydrate Active Enzymes, <http://www.cazy.org/>) glycosidases are structurally divided into over 130 families.^[42]

Glycosidases split saccharidic chains by transferring the cleaved glycosyl moiety to water as an acceptor substrate (Scheme 3). In laboratory conditions, however, the acceptor



Scheme 3. Synthetic and hydrolytic reactions catalyzed by glycosidases.

molecule may be virtually any structure possessing a hydroxyl group, allowing the formation of a new glycosidic bond, instead of the naturally occurring hydrolysis reaction. Two strategies for such synthetic processes may be applied. First, two reducing sugars react in a thermodynamically controlled condensation process, usually called “reverse hydrolysis”. This approach has been preferentially used for the glycosylation of alcohols.^[43] Besides primary and secondary alcohols, successful glycosylations of sterically hindered tertiary alcohols were accomplished, such as of 2-methylbutan-2-ol, 2-methylpentan-2-ol or *tert*-butyl alcohol.^[44,45] More complex structures are efficiently glycosylated under kinetic control in so-called transglycosylation reactions. In this case, glycoside donors require activation by a good leaving group; this

may be an aromatic structure such as nitrophenyl, methylumbelliferyl, fluorides, azides, and oxazolines. Thereby, glycosyl oxazolines mimic reaction intermediates during the cleavage of hexosamine substrates.^[46] During the cleavage of activated donors, the leaving group is released and the interaction with an acceptor proceeds at the activated anomeric center. Transglycosylations afford higher yields of 20–40% but exceptions of even 80% isolated yield have been reported.^[47] Interestingly, the transglycosylation mode can only be applied with retaining glycosidases (i.e., the glycosidic product has the same anomeric configuration as the substrate entering the hydrolytic reaction). In the last 100 years, glycosidase-catalyzed synthesis has developed from simple glycosylations of alcohols to one-pot multienzyme processes,^[48] tailored enzyme mutants,^[49] and boldly derivatized substrates as building blocks of saccharides with direct medicinal or biotechnological applications.^[50] Glycosidase-assisted synthesis and mechanisms have recently been examined in several reviews.^[51–54]

It is to be emphasized that an important aspect of the industrial application of glycosidases consists in targeted trimming of long, natural polysaccharide chains, thus producing either the desired small active compound directly or embodied in a set of defined derivatives of increasing molecular weight (e.g., di-, tri-, tetrasaccharides, etc.). Thus, a high-mannose oligosaccharide was selectively trimmed by various glycosidases to yield a library of carbohydrate compounds.^[55] Another use is the targeted degradation of plant material, such as of lignocellulose,^[56] arabinoxylan^[57] or lignin-containing polymeric feedstock.^[58]

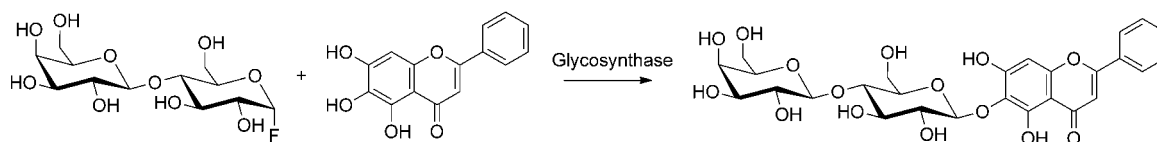
The food, cosmetics, and fine chemicals industries represent the major large-scale applications of glycosidases. Historically, processes involving starch manipulation, milk treatment (sweetness, lactose-free formulas etc.), brewery, and wine industry comprise the typical uses of glycosidases. Other examples include the enzymatic processing of cereal products using cellobiase, *exo*-1,4-glucosidase, mannase, and xylanase.^[59] Another important area is the production of non-digestible galactooligosaccharides (GOS) of prebiotic nature. Here, lactose from various sources is treated with whole cells of *Bifidobacterium bifidum*, which contain cell-bound β -galactosidases.^[60] Improvement and refinement of tea flavor has also been accomplished by using various glycosidases.^[61] Furthermore, through the trimming action of β -galactosidases and β -xylosidases, the antioxidant kaempferol-3-*O*-rutinoside can be prepared from green tea seeds.^[62] Similarly, the derhamnosylated or deglycosylated flavonoid icariin is gained through enzymatic trimming as an anti-ageing, anti-wrinkling, and whitening agent for cosmetic preparations.^[63] Flavonoids, such as hesperidin, naringin or quercetin which are used as anti-oxidants and nutraceuticals, are often glycosylated to increase their water solubility and thus absorbability.^[64]

Glycosidases are characterized by robustness, stability, absolute stereoselectivity, and broad substrate specificity. These properties predestine them to numerous uses, especially when reactive or sensitive substrates are involved that

require mild reaction conditions. Moreover, the resulting products may be usable in medicinal, pharmaceutical, or nutraceutical fields, since they do not generally encounter any harsh reagents in the glycosylation process.^[65,66] Two major issues may need to be overcome when glycosidases are used in the synthetic mode: low regioselectivity leading to mixtures of glycosylated products, and yields that are far from quantitative. Various reaction set-ups have been employed in glycosidase-assisted synthesis to diminish water activity, increase reaction yields, and/or optimize reaction outcome. These include solid-phase synthesis,^[67] reactions in ionic liquids,^[68] inorganic solvents,^[69] microwave irradiation,^[43,70] or various supporting additives, such as salts and cyclodextrins.^[71] Although these attempts have shown promising results, the focus and future of glycosidase catalysis rather lies in ingenious combinations of donor–acceptor–catalyst in aqueous solution. Moreover, such alternative methods are not accepted in the food and cosmetics industries, for which water is the only possible solvent in order to eliminate the potential risks of toxicity.

The most intensively expanding field in glycosidase-catalyzed synthesis is that of enzyme engineering. This relatively young discipline allows construction of glycosidase variants with improved properties, either by site-directed mutagenesis of catalytic residues (well-known glycosynthases) or by randomly introduced mutations through directed evolution.^[72,73] The development of glycosynthase variants from inverting glycosidases, such as the *exo*- β -oligoxylanase from *Bacillus halodurans*^[74,75] and the 1,2- α -L-fucosidase from *Bifidobacterium bifidum*,^[76] represents an important breakthrough because the respective wild-type enzymes are naturally incapable of catalyzing transglycosylation reactions. Another crucial finding is the design of first-generation glycosynthases exercising a substrate-assisted catalytic mechanism. These mutants utilize oxazoline donors mimicking the intermediate of the catalytic process, either by mutating the acid/base residue^[77,78] according to the classical glycosynthase pattern or by mutating the water-stabilizing residue similarly to inverting glycosynthases.^[77]

Recently, two glycosynthases derived from retaining α -L-fucosidases were added to the glycosynthase family.^[79] This is only the third example of α -glycosynthases, after those of a retaining α -glucosidase^[80] and an inverting α -L-fucosidase.^[76] Notably, the α -fucosynthases were able to process β -L-fucosyl azides as glycosyl donors, instead of the generally used fluorides. The concept of glycosyl azides as donors for glycosidases was previously applied with a range of natural glycosidases^[81,82] and also with thioglycoligases.^[83] The thioglycoligase enzymes, together with double mutant thioglycosynthases,^[84] are the first biocatalysts readily synthesizing thioglycosides with good yields, up to 50%. Glycosyl azides are an especially valuable alternative when the respective fluoride donors lack sufficient stability, such as in the case of *N*-acetyl- β -D-hexosaminyl fluorides or β -L-fucosyl fluorides. Their efficient application in glycosynthase-catalyzed synthesis opens a new direction in expanding the repertoire of glycosynthases.



Scheme 4. The glycosynthase E197S variant of cellulase Cel7B displays activity towards flavonoid acceptors, which are not part of its normal substrate.^[88]

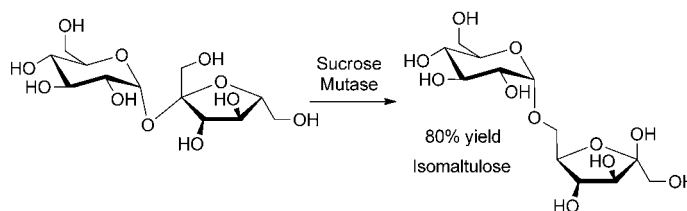
An industrially applicable synthesis using a glycosynthase derived from *Rhodococcus* endoglycoceramidase affords various gangliosides in nearly quantitative yields, on a scale up to 300 mg.^[85] New catalysts with substantially increased catalytic efficiency were also created by directed evolution approach, based on the design of an efficient high-throughput screening method. The validity of this approach was shown for example in thermophilic β -xylosynthase^[86] and β -glycosynthase.^[87] Interestingly, some glycosynthase variants were found to display activity towards acceptors that are not part of their normal substrate. The E197S variant of the cellulase Cel7B from *H. insolens*, for example, is able to efficiently glycosylate flavonoid compounds, with reaction rates that are comparable with those of natural Leloir transferases and with a preparative yield of about 85 % (Scheme 4).^[88]

Transglycosidases

Transglycosidases are basically retaining glycosidases that are able to avoid water as an acceptor substrate during the interconversion of carbohydrate chains.^[36] However, they also display low activity towards non-carbohydrate acceptor substrates, which can be exploited for the synthesis of glycosylated products in vitro. One example is the enzyme cyclodextrin glucanotransferase (CGTase), catalyzing synthesis of cyclodextrins and maltodextrins,^[89] but also a broad range of glycosylation reactions with acceptor substrates, using starch as donor substrate.^[90,91] High-resolution crystal structures of CGTase proteins, and biochemical characteristics of many CGTase mutants, have provided clear insights in the role of specific amino acid residues in the CGTase active site for proper binding of acceptor substrates in glycosylation reactions.^[91,92] A particularly interesting example of a CGTase-catalyzed glycosylation reaction is that of resveratrol, a compound possessing antioxidant, anti-inflammatory, estrogenic, anticancer, cardioprotective, neuroprotective, and immunomodulatory bioactivities.^[93] CGTase of *Thermoanaerobacter* sp. converted resveratrol into α -glucosylated products, reaching 50 % conversion. The water solubilities of these glucosylated derivatives were at least 65-fold higher than that of resveratrol.^[93,94] It is expected that this modification of physicochemical properties (solubility, but also partition coefficient) by glycosylation exerts a positive effect on the bioavailability of these compounds.

Interestingly, several transglycosidases employ sucrose as donor substrate, a very reactive molecule that allows yields to be obtained comparable with those of the nucleotide-activated donors of Leloir transferases.^[95] Although the specific-

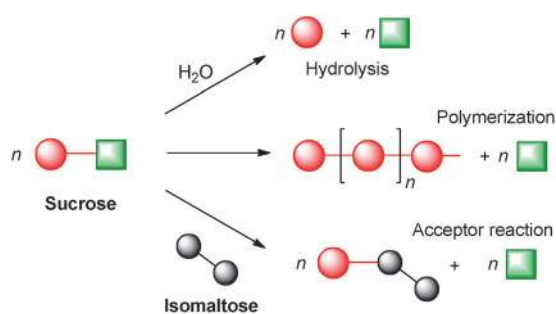
ity of these sucrase-type enzymes is rather limited, the low cost of their glycosyl donor is a major advantage for industrial applications. Transglycosylation reactions with sucrose have already been exploited for the large-scale production of various oligosaccharides. Sucrose mutase, for example, is employed as a whole-cell biocatalyst for the production of isomaltulose, a non-cariogenic and low-glycemic sweetener for use in beverages and food preparations (Scheme 5).^[33,96,97] In turn, fructo-oligosaccharides (FOS)



Scheme 5. The transglycosylation of sucrose (α -1,2-bond) with the enzyme sucrose mutase mainly yields isomaltulose (α -1,6-bond), with 20 % trehalulose (α -1,1-bond) as contaminating product.^[34]

can be produced from sucrose with the help of fructansucrase enzymes.^[98,99] Interestingly, the range of products that can be synthesized with fructansucrases has been extended by the development of sucrose analogues, activated substrates that contain monosaccharides other than glucose and fructose.^[100] In this review, however, the focus will be on glucansucrase enzymes that transfer the glucosyl rather than the fructosyl moiety of sucrose.

Glucansucrases are extracellular enzymes, only reported to occur in lactic acid bacteria, members of the genera *Leuconostoc*, *Streptococcus*, *Lactobacillus*, and *Weissella*.^[101–103] Their major activity is to convert sucrose into α -glucan polysaccharides (Scheme 6), most abundantly with α -1,6-glycosidic linkages (*Leuconostoc* dextransucrase). Additional free hydroxyl-groups, however, exist on a glucose unit, and in more recent years it has become apparent that all other possible glycosidic linkages naturally occur in the α -glucan products of glucansucrase enzymes from various bacteria. Examples are mutan with α -1,3-linkages (*Streptococcus* mutansucrase)^[104] and alternan with alternating α -1,3- and α -1,6-linkages (*Leuconostoc* alternansucrase).^[105] Also a glucansucrase enzyme synthesizing α -1,2-linkages has been reported, again from a *Leuconostoc* strain.^[106] More recently, we have characterized reuteran as a glucan with α -1,4-linkages, always occurring in a mixture with α -1,6-linkages (*Lactobacillus reuteri* reuteransucrase)^[107–109] and a glucansucrase enzyme that cleaves α -1,4-linkages (e.g., in maltodextrins)



Scheme 6. The polymerization, hydrolysis, and acceptor (with isomaltose as example) reactions catalyzed by glucosucrase enzymes, resulting in α -glucan polymer, glucose plus fructose, or oligosaccharide formation, respectively. Glucose, red circle; fructose, green square.

and introduces α -1,6-linkages.^[110–112] These glucosucrase enzymes strongly differ in reaction specificity, introducing different glycosidic linkages in their glucan products. They also differ in the degree and type of branching, and in the molecular mass of their glucan products. The recent identification of glucosucrase enzymes in the genus *Weissella*^[113] suggests that these enzymes occur more widespread in nature, although their distribution at present remains limited to lactic acid bacteria.

Glucosucrase enzymes have been classified in glycoside hydrolase family GH-70 (<http://www.cazy.org>) on the basis of four catalytically important conserved sequence motifs that are similar to those of members of glycoside hydrolase families GH-13 and GH-77, but which occur in a different order. This has led to the proposal that glucosucrases belong to the α -amylase superfamily (or GH-H clan), but have a circularly permuted $(\beta/\alpha)_8$ -barrel as catalytic domain.^[114] However, glucosucrases are much larger enzymes (1600–1800 amino acid residues) than their GH-13 and GH-77 relatives (500–600 amino acids), and contain an N-terminal domain of variable length and unknown function and a C-terminal, putative glucan-binding domain flanking the central catalytic domain.^[101] By analyzing glucosucrase site-directed mutants and hybrid glucosucrase proteins, we have identified active site amino acid residues that determine the specificity of the glycosidic linkage. This has allowed the synthesis of novel α -glucans with strongly varying glycosidic linkage ratios, and even of unnatural glucans with mixtures of α -1,3- and α -1,4- and α -1,6-linkages^[115–117] that have been subjected to a detailed structural characterization.^[118,119]

Recently, Dijkhuizen, Dijkstra, and co-workers have elucidated the first high-resolution 3D structure of a glucosucrase protein, GTF180 of *Lactobacillus reuteri* 180.^[120,121] The structure confirms that, compared to GH-13 α -amylases, the catalytic $(\beta/\alpha)_8$ -barrel domain indeed is circularly permuted as was previously proposed.^[114] The active site of the GTF180 protein is very similar to those of GH-13 family members, suggesting that a very similar reaction mechanism is responsible for the cleavage of the α -glycosidic bond of sucrose and the formation of the β -glucosyl–enzyme inter-

mediate. Crystal structures with bound sucrose (donor substrate) and maltose (acceptor substrate), combined with site-directed mutagenesis experiments, showed that GH-70 glucosucrases possess only one active site and have only one nucleophilic residue. These results provide a solid basis for structure-based inhibitor design, and may facilitate the search for unique specific anticancer drugs. These structures with ligands also allowed identification of sugar-binding subsites, targets for mutagenesis to generate new enzymes with improved acceptor substrate reaction specificity and activity.^[121]

Glucosucrase enzymes also catalyze alternative transglycosylation reactions,^[33] transferring a glucosyl moiety to suitable acceptor substrates (acceptor reaction, see Scheme 6). Of industrial relevance is the use of dextransucrase for the production of isomaltoligosaccharides as non-cariogenic and prebiotic components of beverages and food preparations.^[34,96,122] Other successful examples include the synthesis of various oligosaccharides by *Leuconostoc mesenteroides* α -dextranucrase,^[105] and glycosylation of catechols,^[123] salicyl alcohol, phenol and salicin,^[124] flavonoid,^[125] epigallocatechin gallate,^[126] arbutin,^[127] and L-DOPA^[128] by *Leuconostoc mesenteroides* glucosucrase. Seibel et al. evaluated the acceptor substrate specificity of immobilized glycosyltransferase R (GTFR, a glucosucrase) of *Pseudomonas oralis* on substrate microarrays.^[129] GTFR glycosylated a broad range of primary alcohols and amino acid derivatives (peptides), yielding glycoethers and glycosylated amino acids (peptides). Using tosylated monosaccharides as acceptors, GTFR performed a highly efficient synthesis of novel branched thiooligosaccharides.^[130] Glycosylation of non-saccharide molecules is generally hampered by the poor solubility of these compounds in water, and the glucosucrase activity loss in organic solvents. Therefore the activity and stability of glucosucrase enzymes in the presence of water-miscible organic solvents were studied.^[125,131] Only when using such aqueous–organic solvents, *Leuconostoc mesenteroides* glucosucrase enzymes catalyzed glycosylation of the non-water soluble flavonoids, luteolin (44% conversion) and myricetin (49% conversion).^[125] Glycosylation considerably increased the water solubility of these compounds, increasing their potential in pharmacological applications. Also *O*-glucosides of phenolic compounds are more soluble in water than their parent polyphenols, and may find applications in dermatoccosmetic, nutritional and therapeutic compositions.^[132,133] The α -glucoside of caffeic acid provides a very promising example and is of commercial interest as it regulates key factors favoring the anti-photo ageing of human skin. Its industrial production at gram scale and in 75% yield has been reported using a *Leuconostoc* glucosucrase enzyme.^[134]

Unfortunately, glucosucrase productivity in the reaction with non-carbohydrate acceptors generally remains low (due to low affinity, inhibition of activity by solvents used, or by products of the reaction) and will need to be optimized to become economically viable. Furthermore, it is difficult to block water completely as the acceptor substrate, meaning that the yields suffer from a competing hydrolytic reaction

(Scheme 6). Nevertheless, the reported activities are a promising starting point for the engineering of glucansucrase enzyme specificity. To the best of our knowledge, modifying the acceptor substrate specificity of glucansucrase by means of site-directed or random mutagenesis has not yet been performed. The recent elucidation of high-resolution crystal structures of glucansucrase proteins,^[121] and the increased insights in the role of acceptor substrate-binding residues,^[135] provide a firm basis for such engineering approaches in future work. A further drawback is that no thermostable glucansucrase enzymes are yet available, which limits their industrial applications. Therefore, engineering of the enzyme's stability towards high temperatures and the presence of organic solvents (for applications outside food and cosmetics industries, that is, in the chemical and pharmaceutical fields) will have to be performed. The *Lactobacillus reuteri* GTFA enzyme is very promising (temperature optimum 50°C), but does not meet the industrial requirements yet. Alternatively, novel glucansucrase enzymes are screened for in nature as well as in (meta)genomic databases.

Glycoside Phosphorylases

Glycoside phosphorylases (GPs) share characteristics with both glycoside hydrolases and glycosyl transferases.^[38,136] Their physiological role is the degradation of the glycosidic bond in di- and oligosaccharides using inorganic phosphate, which results in the production of a glycosyl phosphate and a saccharide of reduced chain length. Because the phosphate group can be simply transferred from C1 to C6 by a phosphomutase, the product can be metabolized through glycolysis without further activation by a kinase.^[137] The phosphorylytic degradation of saccharides is, therefore, more energy-efficient than their hydrolysis, saving one molecule of ATP. The name “phosphorylase” is a historic anomaly and the more accurate “phosphorolase” is almost never used.

From a functional perspective, phosphorylases are thus very similar to hydrolases, differing only in their use of phosphate instead of water as nucleophile. This difference has, however, an important practical consequence, because the high-energy content of the produced glycosyl phosphate allows the reactions to be reversed and to be used for synthetic purposes *in vitro*. In that respect, GPs resemble Leloir transferases that also employ glycosyl donors activated by a phosphate group, albeit one that is much larger and is substituted with a nucleotide.^[138] Since a glycosyl phosphate is much cheaper than a nucleotide sugar, phosphorylases have received a lot of attention as promising biocatalysts for the production of oligosaccharides and glycosides. The donor glucose-1-phosphate, for example, can be readily produced in large amounts from cheap substrates like starch, sucrose, or trehalose by the action of various phosphorylases.^[139–141]

To lower the cost even further, the glycosyl donor can also be produced *in situ* by means of so-called “phosphorylase coupling”. This strategy is very attractive for the con-

version of a cheap disaccharide, like sucrose, to a more expensive one like cellobiose. Indeed, the latter compound is difficult to obtain in pure form through hydrolysis of cellulose, but can be efficiently produced from sucrose by the combined action of sucrose and cellobiose phosphorylase.^[142] Purification of the product is very simple in that case, as its much lower solubility compared to that of the substrate sucrose and of the intermediate α -glucose-1-phosphate allows cellobiose to be recovered by simple filtration. Phosphorylase coupling has also been proposed as a strategy for the production of trehalose, a non-reducing disaccharide with important applications in the food industry.^[143] Nowadays, however, it is exclusively produced by a two-step process developed by the Hayashibara Company, which involves TG and GH enzymes instead of phosphorylases.^[144] Finally, coupling of sucrose and starch phosphorylase has been implemented for the synthesis of amylose with a defined chain length.^[145] This allowed its use as functionalized biopolymer to replace fossil-based plastics.^[146]

An important disadvantage of GPs compared to Leloir transferases is that their product yields are significantly lower, with equilibrium constants that are close to one.^[38] To circumvent this problem, the use of glycosyl fluorides as alternative, more reactive donor substrates has been evaluated. The synthetic reaction catalyzed by cellobiose phosphorylase was found to become completely irreversible in that case, with the released fluoride being unable to act as nucleophile in the reverse, degradative reaction.^[147] This strategy is reminiscent of the glycosynthase concept in glycosidase chemistry,^[73] but has the additional benefit that the GPs do not need to be mutated and can thus be used as wild-type enzymes with only a tenfold reduction in catalytic efficiency.^[148]

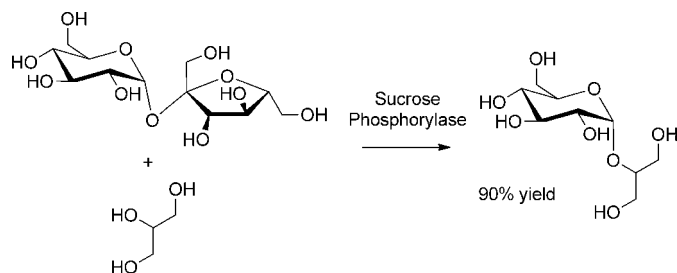
Another disadvantage of GPs is that their substrate specificity is rather limited. To date, only about a dozen of these enzymes have been reported and their donor specificity is largely restricted to glucose-1-phosphate, in either the α - or β -configuration.^[36] The only known exceptions are chitobiose phosphorylase and the phosphorylases from GH-112 that use α -*N*-acetylglucosamine-1-phosphate and α -galactose-1-phosphate, respectively, as glycosyl donors.^[149,150] However, we have found that the donor specificity of phosphorylases can be expanded towards other glycosyl phosphates by means of enzyme engineering. Indeed, directed evolution of the cellobiose phosphorylase from *Cellulomonas uda* has resulted in a number of enzyme variants that also display activity towards α -galactose-1-phosphate.^[151,152] These enzymes are best described as lactose phosphorylases (LPs), a specificity that has not yet been observed in nature.

The acceptor specificity of phosphorylases is typically somewhat more relaxed than their donor specificity, and comprises a range of mono-, di-, and oligosaccharides. Cellobiose phosphorylase, for example, shows a loose specificity at the C2- and C5-positions of the acceptor substrate, resulting in activity towards glucosamine, mannose, xylose, isomaltose, and gentiobiose.^[136] In contrast, a strict specificity is observed at the anomeric position, which must carry a free

hydroxyl group in the β -configuration.^[148] We have found, however, that this strict requirement can be alleviated by means of enzyme engineering.^[153,154] In this way, enzyme variants could be created for the production of various alkyl or aryl β -cellobiosides, which have applications as detergents or as ligands for cellulases.

Non-carbohydrate acceptors have also been reported for GP enzymes. In that respect, sucrose phosphorylase (SP) probably is the most interesting biocatalyst. This enzyme has been found to display activity towards aliphatic, aromatic, and sugar alcohols; ascorbic and kojic acid; furanones; and catechins.^[155] Even a carboxyl group can be used as a point of attachment, resulting in the synthesis of an ester instead of an ether bond. The latter reactions proceed more efficiently at low pH, which probably means that a carboxyl group can only be accepted in protonated form. Under those conditions, the acyl α -glucosides of formic, acetic, benzoic, and caffeic acid could be produced with the relatively stable SP enzymes from *Bifidobacterium longum*^[156] and *Streptococcus mutans*.^[157,158] Interestingly, spontaneous migration of the acyl group from the C1- to the C2-position was observed, generating a mixture of compounds in the final product. Nevertheless, an overall yield of about 80% could be obtained, at least when high concentrations (ca. 40% w/v) of donor were applied.

Recently, the first commercial application of SP for the production of glycosides has been implemented by the German company Bitop AG, based on a process developed by the group of Nidetzky at TU Graz.^[159] They have shown that the SP from *Leuconostoc mesenteroides* is able to glycosylate glycerol with exceptional efficiency and regioselectivity (Scheme 7).^[160] By careful optimization of the reaction



Scheme 7. The enzymatic production of glucosyl glycerol with the enzyme sucrose phosphorylase proceeds with near-quantitative yields.^[160]

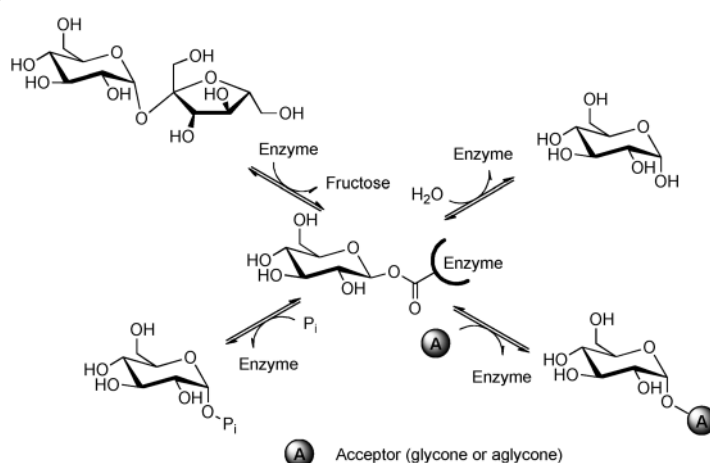
conditions, the competing hydrolytic reaction could be completely suppressed, resulting in near quantitative yields. Furthermore, the glucosyl moiety is exclusively attached to the C2-OH, whereas the glycosylation of glycerol by cyclodextrin glucosyltransferase (CGTase) also involves the C1-OH.^[161] The product is now commercially available under the trade name Glycoin and is used as moisturizing agent in cosmetic formulations.

Despite its interesting acceptor specificity, the large-scale application of sucrose phosphorylase has been hampered by

the low thermostability of the available enzymes. Indeed, this specificity has not yet been discovered in thermophilic organisms, in contrast to most other types of phosphorylases.^[155] Consequently, SP is typically inactivated in a matter of minutes at a process temperature of 60°C. It was recently shown, however, that the enzyme from *Bifidobacterium adolescentis* is the exception to the rule, as it stays active for several hours under these conditions.^[162] Its sequence thus presents the most promising template for the engineering of the stability and specificity of sucrose phosphorylases for industrial applications. To that end, the recently developed high-throughput screening system for SP based on the use of constitutive promoters will be an indispensable tool.^[163]

To increase the operational stability of *B. adolescentis* SP at elevated temperatures, several immobilized enzyme formulations have been developed. Covalent attachment of SP to a Sepabeads enzyme carrier was found to increase the optimal temperature for activity by 7°C,^[162] while immobilization of SP in the form of a cross-linked enzyme aggregate (CLEA) increases the optimum by an impressive 17°C.^[164] More importantly, the latter preparation remained fully active for more than one week at 60°C, during which it could be recycled at least ten times for use in repetitive substrate conversions. This easy and cheap procedure should result in a cost-efficient exploitation of various glycosylation reactions catalyzed by SP at the industrial scale.

Because sucrose phosphorylase follows a double displacement mechanism, it can also be applied as a transglycosidase without the participation of (glycosyl) phosphate (Scheme 8). In this case, a glucosyl group is transferred directly from sucrose to an acceptor substrate, similar to the reaction catalyzed by glucansucrases. Sucrose is not only a cheaper donor substrate than glucose-1-phosphate, but is also significantly more reactive. Optimizing the specificity of SP towards non-carbohydrate acceptors should thus result in very powerful biocatalysts for the glycosylation of specific target molecules. To achieve that goal, it will be imperative



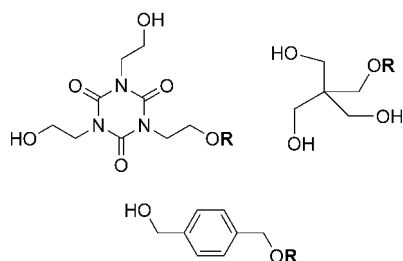
Scheme 8. The different reactions catalyzed by sucrose phosphorylase. A covalent glucosyl–enzyme intermediate is formed, from which the glucosyl moiety can be transferred to phosphate (phosphorolysis), water (hydrolysis), or an alternative acceptor (glycosylation).

to increase the ratio of transfer over hydrolysis, both of which are minor side-reactions in the wild-type enzyme, to minimize the degradation of the glycosylated product.

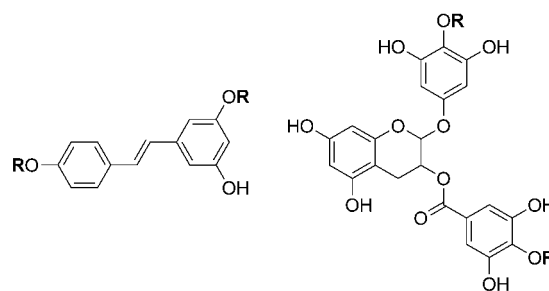
Challenging Acceptors

Generally, most molecules bearing a hydroxyl group can be glycosylated using one of the many chemical protocols. Limitations arise when the hydroxyl group is unreactive, sterically hindered, leads to unwanted reactions such as isomerization and elimination, or when other hydroxyl groups of similar reactivity are present in the molecule. Enzymatic methods can overcome many of these obstacles, although they can suffer from other limitations, in particular the poor solubility of the acceptor substrate in water and/or in polar solvents. A number of dogmas exist about the inability of enzymes to attack some types of substrates, such as tertiary alcohols, thiols, phenols, branched polyols, hydroxyamino acids, and so forth. However, with the expanding knowledge of enzymatic reactions, most of these dogmas have fallen. In the following, we illustrate glycosylation reactions of “difficult” acceptors to demonstrate the utility and flexibility of biocatalysis as alternative to chemical synthesis.

Sterically hindered hydroxyl groups (typically tertiary alcohols) are often unreactive, also in chemical synthesis.^[165] For reactions with hydrolases (including glycosidases), *tert*-butanol has been advocated as a suitable, inert, water-miscible co-solvent to bring hardly soluble substrates into aqueous solutions.^[43] However, a few studies have shown that *tert*-butanol and other tertiary alcohols (e.g., 2-methylbutan-2-ol) can be enzymatically glycosylated in quite good yields.^[45,166] In turn, substrates carrying more than one accessible hydroxyl group (primary alcoholic) are typically glycosylated at a single site by glycosidases, with subsequent glycosylations leading just to an extension of the first glycosidic moiety.^[167] This phenomenon is useful for the selective monoglycosylation of polyhydroxylated compounds without the need for protection chemistry (Scheme 9).^[168] In contrast, glycosylation at multiple sites has been reported for transglycosidases and glycoside phosphorylases. Indeed, cyclodextrin glucanotransferase (CGTase) and sucrose phosphorylase (SP) can be used for the synthesis of the diglycoside of resveratrol and epigallocatechin gallate, respectively



Scheme 9. Selective monoglycosylation of polyhydroxylated compounds without the need for protection chemistry by glycosidases (**R** = glycosides).^[168]

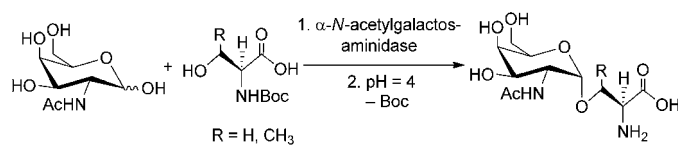


Scheme 10. Synthesis of diglycosides (indicated by “**R**” of resveratrol (left) and epigallocatechin gallate (right) using cyclodextrin glucanotransferase (CGTase) and sucrose phosphorylase (SP).^[93,169]

(Scheme 10).^[93,169] Alternatively, diglycosylation of a divalent acceptor, {5-[(allyloxy)methyl]-1,3-phenylene}dimethanol, has been achieved by the sequential action of two glycosidases, that is, β -galactosidase from bovine liver (also accepting β -Glc), followed by β -galactosidase from *E. coli*.^[170]

A unique example of enzymatic glycosylation has been reported for the unusual acceptor oxime, affording reasonable yields (15–31%) of its glycoside with the β -galactosidase from *A. oryzae*.^[171] Another type of difficult substrate for enzymes is thiols, which are more nucleophilic than alcohols. Although thiols can act as protein poison causing reductive inactivation, glycosylation of 1,3-dithiopropene has been achieved with almond β -glucosidase.^[172] A second report on the glycosylation of thiols by almond β -glucosidase even describes the production of both *O*- and *S*-mercaptoethyl glucosides from mercaptoethanol and glucose, although no spectral characterization was provided.^[173] Many other glycosidases, however, were shown to be inactive towards thioalcohols.^[174] A modern approach for the enzymatic preparation of thioglycosides consists in the use of mutant glycosidases, known as thioglycoligases and thioglycosynthases.^[175] Rather surprisingly, proteinogenic hydroxyl amino acids have been found to be difficult substrates for enzymatic glycosylation.^[176] In contrast, *N*-Boc-protected hydroxylated amino-acids can be easily glycosylated; the Boc group can be removed in situ by acidification of the reaction mixture to pH 4 (Scheme 11). This trick has enabled the synthesis of Tn antigen (GalNAc- α -O-Ser/Thr) with α -*N*-acetylgalactosaminidase.^[177]

Phenolic hydroxyl groups are usually not accepted by GH enzymes, but can be glycosylated by TG or GP enzymes, as illustrated by the above examples of resveratrol and epigallocatechin gallate. In fact, CGTase as well as SP have a remarkably broad acceptor specificity that includes various



Scheme 11. Glycosylation of hydroxylated amino acids.^[177]

phenolic substrates.^[91,155] In contrast, substrates that have both an alcoholic and phenolic OH are typically only glycosylated at the alcoholic group by glycosidases, as exemplified by the glycosylation of kojic acid^[178] or pyridoxine.^[179] Similarly, carboxyl groups are generally not accepted by GHs, but have been used by GPs for the production of glycosyl esters. With SP, for example, the α -glucosides of acetic, benzoic and caffeic acid could be obtained at low pH values.^[156–158]

Finally, some acceptors have so far resisted the glycosylation attempts by various enzyme classes. This is the case for geminal hydroxyl groups, which are stable typically in chloral hydrate or dihydroxymalonic acid. A similar situation occurs when glycosylating the anomeric hydroxyl group of a saccharide acceptor, which is actually the hemiacetal of a geminal diol at C1.^[180,181] In this respect, it is interesting to note that the enzyme will only act on one of the two anomers, although the α - and β -configurations are in equilibrium.

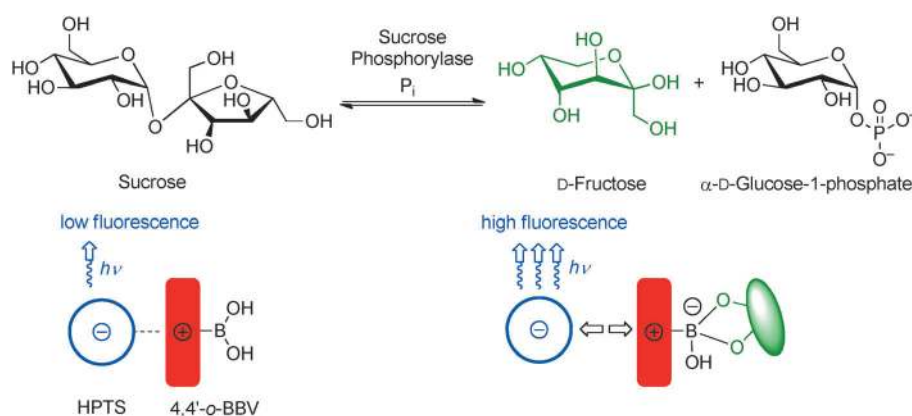
Screening for Improved Biocatalysts

In general, one of the most crucial aspects for the industrial application of enzymes is their operational stability. Indeed, biocatalysts need to be sufficiently robust to withstand the harsh conditions of industrial processes. For glycosylation reactions in particular, enzymes are required that are both tolerant to high temperatures and to the presence of organic (co-)solvents. Nearly all carbohydrate conversions in industry are performed at 55–60°C, mainly to avoid microbial contamination. Organic solvents, in turn, are needed to solubilize the hydrophobic compounds that are used as glycosyl acceptors. Fortunately, the stability of enzymes at high temperatures and in solvents usually coincide, and can thus be improved simultaneously.^[182] Besides stability, the specificity of the biocatalysts also requires engineering, as most of the enzymes described here have a strong preference for carbohydrate acceptors.

Obtaining novel biocatalysts with improved properties can be achieved by two complementary approaches: either by searching in previously unexplored natural habitats, or by the *in vitro* creation of enzyme variants. In the former approach, the development of metagenomic tools has dramatically increased the natural diversity that can be accessed by bypassing the need to isolate and cultivate individual microbial species.^[183–185] In the latter approach, directed evolution has been shown to be an extremely powerful algorithm that mimics natural evolution

through random mutagenesis and subsequent selection/screening of the resulting enzyme libraries.^[186–189] In any case, high-throughput screening typically is the bottleneck for the identification of improved enzymes in natural as well as mutant libraries. Indeed, a fast and reliable assay is required to accurately measure the activity of thousands of candidate enzymes in a reasonable time span.^[190] Although several assays are available for the detection of GH, TG, and GP activities, they all have specific disadvantages. For sucrose phosphorylase, for example, glycosylation reactions can be detected by measuring either the release of inorganic phosphate from glucose-1-phosphate as donor^[154] or the release of fructose from sucrose as donor,^[162] with the latter assay also being available for the analysis of TG enzymes (Scheme 8). Unfortunately, these procedures are destructive and can therefore only be used for end-point measurements.^[191] Evidently, a continuous assay would be much more valuable as it increases both the accuracy and the throughput of the screening. Although chromo- and fluorogenic substrates are typically well accepted by GHs, these have not yet been reported for TGs or GPs. Furthermore, identifying enzyme variants with high activity on chromogenic substrates can result in a loss of activity on the natural donor, as famously stated in the first law of directed evolution: “you get what you screen for”.

Supramolecular tandem enzyme assays have been recently introduced by the group of Nau.^[192–197] These assays are based on the molecular recognition of unlabeled substrates or products by artificial supramolecular macrocyclic receptors. However, these enzyme assays have been developed for amino acid decarboxylases and not for carbohydrate modification reactions. In contrast, a supramolecular real-time fluorescent assay has been introduced for sucrose phosphorylase and phosphoglucomutase by Schiller in collaboration with the Singaram group.^[198] In the case of SP, this non-destructive assay makes use of a selective carbohydrate sensing system that detects the unlabeled enzymatic product fructose (Scheme 12). It is perfectly suited for the screening



Scheme 12. Enzyme assay for sucrose phosphorylase with selective detection of the unlabeled product fructose by *N,N'*-bis-(benzyl-2-boronic acid)-4,4'-bipyridinium dibromide (4,4'-*o*-BBV). Adapted and reprinted from reference [198] with permission from Elsevier.

of glycosylation reactions with sucrose as donor (e.g., sucrose phosphorylase as well as glucansucrase), because fructose is always released as product (Scheme 6 and 8). The sensing system is composed of commercially available 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) as the reporter unit and *N,N'*-bis-(benzyl-2-boronic acid)-4,4'-bipyridinium dibromide (4,4'-*o*-BBV) as selective receptor for fructose. Sucrose and glucose-1-phosphate do not show any significant binding affinity to 4,4'-*o*-BBV.^[198,199] The saccharide-sensing system was originally introduced by Singaram and Wessling for continuous-glucose sensing. Progress in this endeavor is documented in a series of publications detailing the chemistry of the system and the development of sensors based on immobilization of the sensing components in hydrogels.^[199–208] The sensor will be commercialized for continuous glucose monitoring in intensive care units.^[209] In collaboration with the laboratory of Schiller^[41,210] the sensing system was used to go beyond continuous glucose sensing,^[210] such as multivariate analysis,^[199,202] hydrogels in multiwell plates,^[211] and enzyme assays.^[198]

The proposed signaling mechanism involves ground-state complex formation between the anionic fluorescent dye and the cationic receptor (a boronic acid based bipyridinium salt) that facilitates electron transfer from the dye to the bipyridinium salt. This results in a static quenching of the fluorescence intensity of the dye. When a reducing saccharide is added to the ground-state complex, the boronic acids are converted into anionic boronate esters, partially neutralizing the net charge of the cationic viologen. This reduces the quenching efficacy and increases the fluorescence intensity. The change in fluorescence can be converted into product concentration, allowing initial reaction velocities and Michaelis–Menten kinetics to be calculated. The assay can be carried out in multiwell plate formats, making it suitable for high-throughput screening.

In contrast to a standard indicator displacement assay (IDA), formulated by Anslyn et al.,^[212,213] the indicator in the Singaram system is displaced by the analyte from a so-called “allosteric interaction”.^[41,210] This means that the analyte does not compete at the same binding site with the indicator. It binds at another site (allos stereos *Greek* “other object”), thereby inducing a decrease in the affinity of the indicator for the receptor. This new type of assay can be called an “allosteric indicator displacement assay” (AIDA). Recently, the group of Schiller combined fluorescence correlation spectroscopy with an AIDA saccharide probe to detect fructose at a nanomolar dye concentration. Digital analysis revealed a complementary implication/notimplication logic function.^[214]

It is important to note that the AIDA enzyme assay was also used recently by the group of Seibel for sucrose isomerases. An isomaltulose synthase was redesigned to an isomaltulose synthase by site-directed mutagenesis. The enzyme assay gave important information as to how much of the substrate sucrose was hydrolyzed to glucose and fructose in the side reaction.^[215]

Conclusions and Perspectives

To circumvent the problems associated with the chemical synthesis of glycosides, several classes of carbohydrate-active enzymes can be recruited, but all of them have their own advantages and disadvantages. On the one hand, glycoside hydrolases and “Leloir” glycosyl transferases offer a very wide range of specificities, but their use is hampered by low yields and the high price of the glycosyl donors, respectively. On the other hand, transglycosidases and glycoside phosphorylases comprise fewer specificities but are active with low-cost donor substrates that generate moderate to good yields. In any case, a biocatalysts' productivity may be further improved by enzyme engineering to increase the commercial potential of their glycosylation reactions. In that respect, the recent elucidation of a high-resolution 3D structure of the GTF180 glucansucrase^[120,121] represents a major breakthrough, since it will allow the use of more rational engineering strategies.

A prospective approach to the development of new glycosylation reactions catalyzed by GH, TG, and GP enzymes is to select specificities that transfer a glycosyl group from cheap and readily available donor substrates (e.g., sucrose) to a range of acceptor compounds. Here, the glycosylation of small organic molecules, like flavonoids, alkaloids, and steroids, offers a yet-to-be-explored reservoir of applications. For that goal, new enzymes with high activity and stability could probably be identified in either natural environments or mutant libraries, using the novel fluorescent probes as tools for high-throughput screening. Collaboration between academia and industry would then allow the evaluation of the economic potential of selected reactions in pilot-scale processes. In that way, several new glycosides may become commercially available for application in the food, feed, chemical, cosmetic, and pharmaceutical industries.

Acknowledgements

All authors thank the EC for financial support through the FP7-project “Novosides” (grant agreement nr. KBBE-4-265854; <http://www.novosides.eu/>). A.S. thanks the Carl-Zeiss foundation for a Junior Professor fellowship and the Fonds der chemischen Industrie (FCI). L.D. acknowledges financial support from the Carbohydrate Competence Center (<http://www.cccresearch.nl>). T.D. and W.S. thank the Multidisciplinary Research Partnership “Ghent Bio-Economy” for support.

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Published online: August 8, 2012