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Review

Glycosidase-catalysed synthesis of alkyl glycosides

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Abstract

Glycosidases catalyse the synthesis of anomerically pure alkyl glycosides in one step. In contrast, chemical synthesis of anomerically pure glycosides is circuitous and expensive. Two methodologies are used in enzymatic glycosylation: thermodynamically controlled reversed hydrolysis and kinetically controlled transglycosylation. The advantages and limitations of both approaches are delineated. Glycosidases exhibit broad specificity with regard to the aglycon: in addition to simple alcohols, hydroxy amino acids, nucleosides, ergot alkaloids and cardiac genins are glycosylated. Non-alcohol acceptors such as oximes and thiols also function as substrates whereas pyranoid glycals act as non-natural donors. Glycosidases exhibit absolute selectivity with regard to the stereochemistry at the anomeric centre and show a high degree of chemoselectivity for different hydroxyl groups, e.g., the order of reactivity is primary > secondary alcohols > phenols; tertiary alcohols are unreactive. Chiral primary alcohols are poorly discriminated, but the enantioselectivity towards a hydroxyl group that is directly attached to a (pro)chiral carbon atom is often high. The synthetic utility of glycosidases would be considerably improved if methods could be found for maintaining their catalytic activity in non-aqueous media.

Keywords: Glycosidase; Transglycosylation; Reversed hydrolysis; Selectivity; Non-natural substrates

1. Introduction

Glycosides are ubiquitous in nature, particularly in plants. Alcohols, phenols and mercaptans exist in plants mainly as their glycosides and are, as such, precursors for aroma compounds. Some glycosides also exhibit antimicrobial activity [1]. Furthermore, glycoside formation can be used to protect a hydroxyl group during the synthesis of a natural flavour, for example, in which for labelling purposes it is necessary to employ a 'natural' method. Acid-catalysed synthesis of alkyl glycosides—Fischer alkylation [2–4]—generally results in a mixture of isomers in which, in the case of glucose or galactose, the α-pyranoside predominates [5]. Chemical methods for the synthesis of anomerically pure glycosides involve protection and deprotection steps and are inherently circuitous [6].

In vivo glycosyl transferases (EC 2.4) mediate the highly regioselective formation of glycosidic bonds. Their use in vitro is, however, prohibitive owing to the requirement for stoichiometric quantities of expensive cofactors, such as uridine diphosphate (UDP). In contrast, glycosidases (EC 3.2.1) catalyse the formation

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of anomerically pure glycosides without the need for a cofactor. In vivo they catalyse the reverse reaction, i.e., glycoside hydrolysis (for an overview of the glycoside hydrolases, see Ref. [7]). In vitro they can be used for glycoside synthesis via reversed hydrolysis [2] (thermodynamic control) or transglycosylation (kinetic control). This is a particularly attractive option for the synthesis of β-glucosides and β-galactosides because their chemical synthesis involves the use of the inefficient Königs–Knorr route [6,8]. Moreover, the required enzymes are readily available and relatively inexpensive. β-Galactosidases, for example, are industrially applied in the hydrolysis of lactose.

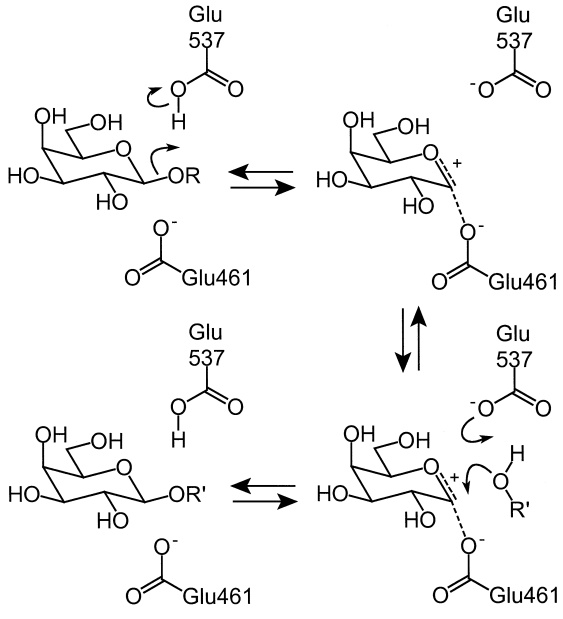
The practical merits of the glycosidase-mediated synthesis of glycosides [3] from non-carbohydrate acceptors are at the focus of this review. Disaccharide and oligosaccharide synthesis will not be discussed because these have been reviewed extensively elsewhere [10,12–14]. The availability and stability of glycosidases, the synthetic methodology and experimental procedures as well as selectivity issues—including enantioselectivity—will be put into perspective.

2. Mechanistic considerations

Glycosidases are subdivided on the basis of whether glycosylation occurs with retention or with inversion of configuration at the anomeric centre. Examples of the former are β-galactosidase, invertase and lysozyme; the inverting glycosidases, such as trehalase and β-amylase, have hardly been used for the synthesis of alkyl glycosides.

The catalytic mechanism of lysozyme, a retaining β-glycosidase, has been the subject of a sustained research effort [15]. The active site contains two glutamic acid residues of which the first one assists in the departure of the leaving (oligo)-saccharide group by proton transfer to the anomeric oxygen atom. An enzyme-bound oxonium ion intermediate [15–18] (Scheme 1) which has been detected by NMR [17], results. The second carboxylate, which is deprotonated in the resting state, stabilises the oxonium ion. In the next step a nucleophile adds to the same face of the glycosyl-enzyme intermediate from which the leaving group was expelled, resulting in net retention of configuration at the anomeric centre. The addition of the nucleophile is assisted by the first carboxylate which in this step reverts to carboxylic acid. Mutagenesis and X-ray structural studies have confirmed that the mechanism of other retaining glycosidases is similar [15,19–21]. The α-glycosidases also operate by a similar mechanism, except that the positions of the carboxylic acid groups are reversed.

As a consequence of the hydrolysis mechanism the anomeric selectivity of glycosidases is absolute. The synthetic utility of the glycosi-



Scheme 1. The mechanism of β-galactosidase from *Escherichia coli* [21].

[2] From an organic synthesis standpoint, the correct name for this reaction would be condensation, i.e., a reaction of two molecules with expulsion of water. However, reversed hydrolysis —which is linguistically equivalent to 'reversed mountain climbing' is firmly entrenched in the glycosidase literature and it will be used for that reason.

[3] For short reviews see Refs. [9–11].

dases stems from this characteristic feature as well as from the wide latitude that the enzyme allows with regard to the nature of the leaving and nucleophilic groups. In the natural, hydrolytic reaction the leaving group is an (oligo)saccharide and the nucleophile (glycosyl acceptor) is water. However, an alcohol or a monosaccharide can also act as glycosyl acceptor. Reversed hydrolysis, the condensation of a monosaccharide and an alcohol in which water is the leaving group (reaction 1), was first reported in 1913 [22,23]. The reaction is thermodynamically controlled, i.e., it proceeds towards an equilibrium. Transglycosylation of a glycoside according to reaction 2, in which the leaving group is often a (mono)saccharide, is an entirely different concept [24]. The reaction can be controlled kinetically, overshooting the equilibrium composition, if the reactant is much more reactive than the product. Water acts as a competing nucleophile and causes parasitic hydrolysis of the reactant.

$$\text{Glycosyl–OH} + \text{R}^1\text{OH} \leftrightarrows \text{Glycosyl–OR}^1 + \text{H}_2\text{O} \quad (1)$$

$$\text{Glycosyl–OR}^1 + \text{R}^2\text{OH} \rightarrow \text{Glycosyl–OR}^2 + \text{R}^1\text{OH} \quad (2)$$

The synthesis of alkyl glycosides, whether enzymatic or otherwise, is generally hampered by the low solubility of carbohydrates in organic media. The use of solvents such, as DMF, DMSO or pyridine, would be self-defeating, considering that the reaction products are intended for use in foods and personal care products. Water is, of course, an excellent solvent for carbohydrates but because water keeps the conversion low in equilibrium syntheses and causes undesirable hydrolysis in transglycosylation, its concentration or, rather, its thermodynamic activity a_w [25] should be kept as low as possible. In the very similar case of the lipase-catalysed esterification of carbohydrates, this problem was solved by the use of an anhydrous medium polarity solvent, such as *tert*-butyl alcohol [26,27]. If necessary, the reaction was performed at an elevated temperature to make up for the low solubility of the carbohydrate [26].

3. Glycosidases: availability and stability

The esterification methodology cited above required the use of a thermostable, low a_w tolerant lipase which was—fortunately—available. However, the commonly employed glycosidases, such as the β-glucosidase from almonds [28,29] and the β-galactosidases from *Aspergillus oryzae* and *E. coli*, seem to need at least some water in the reaction medium in order to remain active. The minimum a_w [4] required by almond β-glucosidase was found to range from 0.4 to 0.5 for C_3-alcohols [28,30] to 0.8 for 1-octanol [29], acetonitrile [31] and *tert*-butyl alcohol [32].

The use of more thermostable enzymes might partially solve this problem since, as a rule of thumb, thermostable enzymes more often remain active at low a_w and an elevated reaction temperature could compensate for the low solubility of the reactant. The recent finding [33] that the thermostability of β-galactosidases from mesophilic sources is enhanced at moderate pressure has not yet been exploited for synthesis.

A number of thermostable glycosidases have been identified and characterised in recent years, some of them have already been used in the synthesis of glycosides and one is even commercially available (see Appendix A). The most remarkable one among these newcomers is the β-glucosidase from the hyperthermophilic archeon *Pyrococcus furiosus* [34] which also showed a quite respectable β-galactosidase activity (the heat-resistant β-galactosidase that has been isolated from *P. furiosus* [35] may be the same enzyme). The organism is relatively easy to grow and the β-glucosidase, which is stable for an unprecedented 85 h at 100°C, constitutes

[4] The a_w values have been estimated using the UNIFAC group contribution method, except those that were supplied by the authors.

almost 5% of the cellular protein. The enzyme has also been cloned and overexpressed in *E. coli* [36]. The practical value of thermostable enzymes is illustrated by the synthesis of ethyl galactoside in 5 M (30%, v/v) aqueous ethanol catalysed by the β-glucosidase from *Caldocellulum saccharolyticum* [5] [37] and the β-galactosidase from *Streptococcus thermophilus* [38], whereas the enzymes from mesophilic organisms were rapidly deactivated in this medium [37,39]. At $a_w = 0.9$, this can hardly be due to loss of water, which raises the question of the effects of organic solvents on glycosidases.

Glycosidases often show a lack of activity in organic media, although the effects are generally reversible [40–43]. Almond β-glucosidase, which was stable and active for days in 90% (v/v) aqueous acetonitrile at 40°C [31], [6] or *tert*-butyl alcohol at 50°C [32] ($a_w = 0.8$) seems to be an exception. *Low* (30%, v/v) concentrations of these solvents [42] deactivated the β-glucosidase irreversibly, similar to many other enzymes. It would seem that polar organic solvents at low concentrations deactivate enzymes by inducing structural changes, whereas at high concentrations the enzyme remains frozen in the active conformation due to loss of water. A structural effect of the organic solvent may be apparent with the much-used β-galactosidase from *A. oryzae*, which maintained > 80% of its initial activity for some hours in 20% (v/v) aqueous dioxane at 40°C, but lost 80% activity over 2 h in 20% (v/v) aqueous acetonitrile [44].

Like many enzymes glycosidases are often unstable in biphasic systems. The very stable β-glucosidase from *P. furiosus*, for example, was rapidly ($t_{0.5} = 0.4$ h at 75°C) deactivated in a biphasic system of aqueous 1-(dimethyl-phenylsilyl)-ethanol [45]. Almond β-glucosidase was similarly deactivated in aqueous 1-octanol after 2 days at 60°C [46], although the enzyme was stable almost indefinitely in a biphasic system of 1-octanol and concentrated glucose syrup [47], perhaps due to a stabilising effect of glucose at high concentration.

The efficient use of enzymes in non-aqueous media necessitates their immobilisation on a suitable carrier material, because the use of 'free' protein dispersions is bound to render the major part of the enzyme molecules inaccessible for the reaction medium. Adsorption of the enzyme on an inert material, such as XAD-4 [29,48], enhances the accessibility of the enzyme and it also stabilises almond β-glucosidase against the deactivation by 1-octanol/water mentioned above [29,48]. Almond β-glucosidase on XAD-4 could be reused 3 times with approx. A total of 10% loss of activity per cycle [49]. Gelo-Pujic et al. [50] went one step further by coimpregnating an acidic alumina with β-glucosidase as well as glucose. Due to the combined stabilising effects of the carrier and glucose the resulting catalyst could be used at 80°C. Besides, dispersion of hydrophilic reactants on a polar carrier is known to moderate their incompatibility with organic media due to an increased rate of dissolution [51,52].

We found that β-galactosidase could be adsorbed on polymeric materials such as Duolite A-7 (an anion exchange resin) or Duolite S761 (a neutral resin with methylol functionality). The serious leaching of enzyme from the surface that was observed in aqueous medium was prevented by cross-linking [53] which also stabilised the enzyme. *A. oryzae* β-galactosidase immobilised in this way was active in 100% glycerol, whereas the freely suspended lyophilisate was not (see Table 1) [54]. The *K. fragilis* enzyme required more water to maintain its activity, as is shown in Fig. 1.

Multipoint attachment combines the advantages of adsorption and cross-linking although the carrier material (Eupergit C, a methacrylamide polymer carrying oxirane groups) is expensive. Thus, multipoint attachment of the *P.*

[5] The organism has been reclassified as *Caldicellulosiruptor saccharolyticus*.

[6] In 70% (v/v) aqueous acetonitrile ($a_w = 0.9$) deactivation was rapid ($t_{0.5} = 2$ h) which reconfirms the observation that partial dehydration of an enzyme increases its stability, although it may be detrimental to its activity.

Table 1
Condensation of galactose with glycerol[a]

Enzyme source	Carrier	Cross-link reagent	Yield (%)
A. oryzae	Duolite S-761	1,5-pentanedial	27
A. oryzae	Duolite S-761	dimethyl adipimidate	34
A. oryzae	none (suspended)	none	0

[a]Reaction conditions: 54 mg (0.3 mmol) galactose and 40 U β-galactosidase, 1.1 g (12 mmol) glycerol at 40°C for 18 h.

furiosus enzyme to Eupergit C increased its life time under reaction conditions (vide supra) by an order of magnitude [45]. The entrapment of enzymes in sol–gel glasses emerged quite recently as a promising stabilisation method [55–57] but an attempt to stabilise a β-galactosidase in this way was not successful [58]. Entrapment and multipoint attachment have been combined in the copolymerisation of *A. oryzae* β-galactosidase with a polyisocyanate prepolymer, although the immobilisation yield was rather low [59].

Immobilisation and cross-linking of enzymes are often accompanied by a partial loss of activity which often can be minimised by loading the active site with reactant (lactose in the case of the β-galactosidases [53,54]) or a reactant mimic. One should also keep in mind that many glycosidases need structural ions (typically Mg^{2+} and Na^{+}) for activity [60–62].

Solubilisation of enzymes in organic media by the attachment of non-polar substituents is another approach to rendering them suitable for use in such media. Chemical modification of the β-galactosidase from *A. oryzae* with polyethylene glycol, for example, afforded a conjugate that was soluble in organic media and required less water for its activity than the free enzyme, but the stability was reduced [63]. Chemical modification is rather laborious and, fortunately, it has become evident recently that enzymes can also be solubilised by physical bonding to a modifier. The technique involves the mixing of an aqueous solution of the enzyme with an amphiphile, e.g., a glycolipid, which results in precipitation of the lipid-coated protein [64]. β-galactosidases treated in this way were freely soluble in organic media. In biphasic aqueous–organic reaction systems lipid coated β-galactosidases efficiently catalysed the transfer of a galactosyl group from lactose to non-polar alcohols such as 5-phenylpentanol and 1-octanol without hydrolysis [65], whereas the native enzyme gave only hydrolysis under the same conditions.

3.1. Glycosyl transferases

The enzymes discussed above all operate in the catabolic pathway, as far as their metabolic

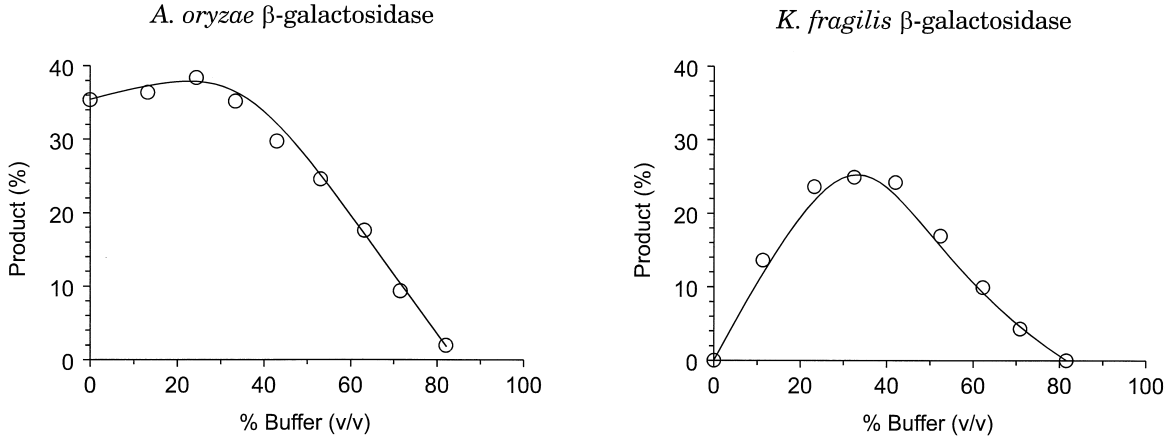


Fig. 1. Condensation of galactose and glycerol catalysed by immobilised β-galactosidase, plot of yield against percent (%) added buffer [54]. Reaction conditions: 0.3 mmol galactose, 12 mmol glycerol, 50% (v/v) buffer pH 6.5, 40 U β-galactosidase, 40°C.

function is known. In the *synthetic* pathway, nature makes use of glycosyl transferases, which selectively effect the transfer of a glycosyl unit from an activated intermediate, such as an UDP derivative. These enzymes are very efficient but have not been much used in vitro, because of the prohibitive cost of the required phosphorylated reagents.

The concept of using a biocatalyst that mediates glycoside transfer in vivo for efficient glycosyl transfer in vitro remains very attractive, however. Thus, sucrose phosphorylase (EC 2.4.1.7), which in vivo transfers an α-glucosyl group from sucrose to phosphate, has been employed in the glucosylation of phenolic compounds [66]. Another readily available transglucosidase, cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) has been used in a similar way [67,68].

4. Synthesis of glycosides by reversed hydrolysis

Reversed hydrolysis comprises a reaction of a monosaccharide with a nucleophile such as an alcohol to give the corresponding glycoside and water (reaction 3) until equilibrium is reached (see Fig. 2).

$$\text{Glycosyl–OH} + \text{R}^1\text{OH} \leftrightarrows \text{Glycosyl–OR}^1 + \text{H}_2\text{O} \quad (3)$$

The thermodynamic synthesis of alkyl glucosides has been the subject of a considerable research effort that was mainly driven by the projected application of the products as biosurfactants. In the course of his pioneering work Veibel [69] established the equilibrium constant.

$$K = \frac{C_{\text{GlcOR}^1} C_{\text{H}_2\text{O}}}{C_{\text{Glc}} \cdot C_{\text{R}^1\text{OH}}} \approx 2\text{–}3 \quad (4)$$

for R^1 = methyl to butyl. A very similar value of 1.9 has recently been determined for the C_6 to C_{12} *n*-alkyl alcohols [46]. It is obvious that a low a_w would be desirable to increase the equilibrium yield. A small amount of water is usually added nevertheless, because glycosidases are inactive at low a_w, as has been pointed out above, and to increase the solubility of the carbohydrate. Furthermore, a high concentration of reactant carbohydrate would be desirable to push the equilibrium to the right, but this may give rise to the formation of oligomeric side-products [46]. Reaction temperatures of 40–60°C are routinely used to increase the reaction rate.

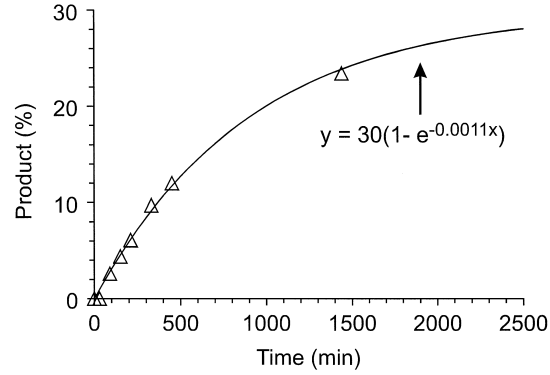


Fig. 2. Time-course of the condensation of galactose and glycerol catalysed by *K. fragilis* β-galactosidase on Duolite A-7 [54]. Reaction conditions: 0.3 mmol galactose, 12 mmol glycerol, 50% (v/v) buffer pH 6.5, 40 U β-galactosidase, 40°C.

This approach has been subjected to numerous variations, resulting in a great number of published procedures. Most of these concern the alkylation of glucose mediated by the β-glucosidase from almonds. The results are not always easy to compare because many authors report data for a single, fixed reaction time so that it is not clear at all whether or not equilibrium has been reached. In most cases the glycosyl accepting alcohol is used as the organic component of the reaction medium, although polar cosolvents have been used in some cases. Because glucose dissolves only sparingly in the usual media it is present as a separate phase, at $a_w < 0.8$ as a solid, otherwise as a (concentrated) solution. Hence, increasing the amount of starting material generally will not increase the yield, although the throughput of the reaction system will increase [29,30,70]. The condensation of

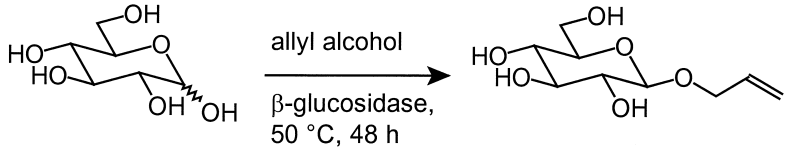


Fig. 3. Synthesis of allyl β-D-glucopyranoside.

glucose and allyl alcohol (Fig. 3) shows how the water concentration should be adjusted carefully to balance enzyme activity against equilibrium yield [7] to optimise the productivity [30] (see Table 2).

Comparable 'minimum water' procedures have been adopted in the glucosylation of, e.g., butanol [71], 1,6-hexanediol [72] as well as medium chain-length alkanols [28] by many workers in this field; for selected results see Table 3. Ljunger et al. [29] found that the reaction time could be reduced and the yield improved if the reaction was started at $a_w = 1$, followed by a gradual removal of water to $a_w = 0.74$.

Gelo-Pujic et al. [50] reported that microwave heating, using a dispersion of glucose and β-glucosidase on acidic alumina at 80°C, increased the glucosylation rate of 1,6-hexanediol by a factor of 6 compared with conventional heating. It would seem that this effect is caused by the local overheating that easily occurs in a microwave field [73]. The yields that were reported on this occasion were rather low and seem to fall short of equilibrium yields [72], either because the reaction time was too short or the catalyst was inactivated under the reaction conditions.

By analogy with the lipase-catalysed esterification of carbohydrates [26] it would seem advantageous to use an inert polar solvent to increase the solubility of the sugar. Acetonitrile, acetone and *tert*-butyl alcohol [31,32,42] containing 10% water ($a_w \sim 0.8$) have been used for this purpose, but the reaction rates and the yields were generally lower than when the acceptor alcohol was used as solvent. Attempts to increase the rate and yield via solubilisation of glucose in benzene as its labile butaneboronic ester [74] were similarly unsuccessful.

As an alternative to the 'minimum water' procedure reactions have also been performed with aqueous glucose at $a_w \sim 1$. Such an approach has the advantage of optimum enzyme activity [29] but its efficiency in terms of equilibrium and yield depends on the extraction of the product into the organic phase (which is also the glycosyl acceptor). Panintrarux et al. [46] have investigated the alkylation of glucose by C_6 to C_{12} alkanols and analysed the results using standard equilibrium theory. Yields increased with increasing volume of the alcohol phase or glucose concentration, as might be expected. Considerable formation of glucose oligomers was also observed, especially at high glucose concentrations. It is worth noting—inter alia—that in the 'minimum water' procedure no glucobiose could be detected, in spite of the presence of a small volume of a saturated aqueous glucose phase [47]. The number of carbon atoms in the alcohol had a major effect on the yield: for each extra carbon atom the equilib-

Table 2
Effect of water on the synthesis of allyl β-D-glucopyranoside[a]

$[H_2O]$ (%, v/v)	Yield (%)	Mole fraction		Thermodynamic activity	
		H_2O	Allyl alcohol	H_2O	Allyl alcohol
20	5	0.49	0.51	0.80	0.54
10	62	0.30	0.70	0.54	0.69
4	56	0.14	0.86	0.24	0.86
2	22	0.07	0.93	0.12	0.93

[a] Reaction conditions: 0.4 mmol D-glucose and 10 mg (6.9 U) almond β-glucosidase in 2 ml of allyl alcohol at 50°C for 48 h. Data have been taken from Ref. [30].

[7] Yields are given in percent (%) of mole product per mole carbohydrate unless stated otherwise.

Table 3
Almond β-glucosidase-catalysed alkylation of glucose using the 'minimum water' procedure

Glycosyl acceptor	Temperature (°C)	Water concentration (%, v/v)	a_w	Yield (%)	References
1-Propanol	60	n.d.	0.4	67	[28]
Allyl alcohol	50	10	0.5	62	[30]
1-Butanol	60	10	0.8	55	[71]
1,6-Hexanediol	50	10	0.8	61	[72]
Benzyl alcohol	50	10	0.9	40	[30]
1-Hexanol	60	n.d.	0.7	36	[28]
1-Octanol	50–60	~3	0.8	15	[47]

rium yield was reduced by about 40%. The yield and productivity of some procedures for β-hexyl and β-octyl glucoside have been compiled in Table 4. The main conclusion is that the 'minimum water' procedure affords the best yield at a comparable productivity (millimole of product per hour per gram enzyme).

So far, the discussion has remained restricted to the glycosylation of primary alcohols. Secondary alcohols, such as 2-heptanol and 2-octanol are glycosylated slower than their primary counterparts by a factor of 3–5 [75] and the *equilibrium* glycosylation yields are correspondingly reduced [46,76]. Consequently, the glycosylation of secondary alcohols has received only scant attention. However, the method has been successfully applied to the synthesis of β-but-3-en-2-yl glucoside and related compounds [49], which have recently emerged as promising latent–active intermediates in the synthesis of oligosaccharides [77].

The use of reversed hydrolysis has, for quite practical reasons which will be explained later, almost exclusively been applied to glucoside synthesis, although it should work equally well (or badly) with any monosaccharide. The low yield (9%) of methyl β-D-xylopyranoside that has been obtained [78] from methanol and xylose is close to the predicted value [46,69] at the low methanol concentration that was used. It has initially been reported that *A. oryzae* β-galactosidase was not active in the alkylation of galactose [30] but it was later shown [49,72] that yields and rates were only slightly lower than those of almond β-glucosidase in comparable reactions with glucose.

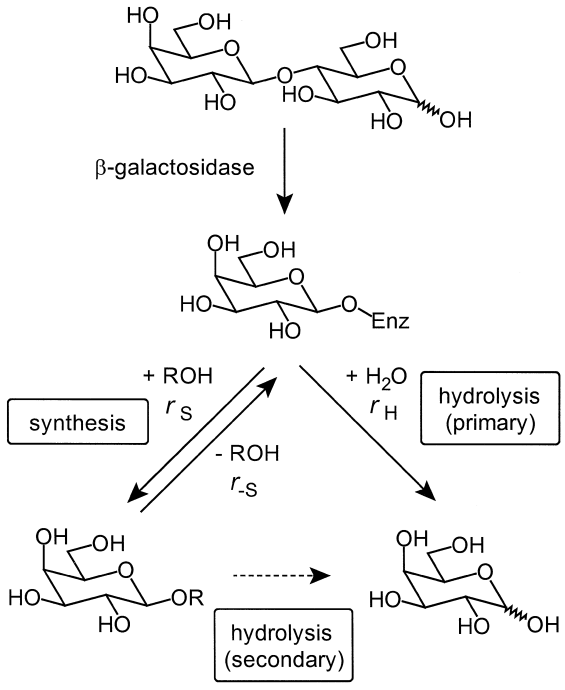
5. Glycoside synthesis by transglycosylation

Reversed hydrolysis, although elegant in its simplicity, is handicapped by the incompatibility of monosaccharides, glycosidases and low-a_w organic media. Transglycosylation according to reaction 5 is an entirely different concept which is based on monopolisation of the catalyst by a reactive glycosyl donor. Because the reaction is controlled kinetically, it becomes possible to

Table 4
Almond β-glucosidase-catalysed reversed hydrolysis of glucose with 1-hexanol and 1-octanol

Reaction medium	a_w	Reactants (mol/mol)	Yield (%)	Time (h)	Productivity (mmol (g enz)$^{-1}$ h^{-1})	References
1-Hexanol/H_2O	1	53	18	48	0.56	[46]
1-Hexanol/H_2O[a]	1	40	20	72	0.7	[48]
1-Octanol/H_2O	1	43	9	48	0.28	[46]
1-Octanol	0.8	32	15	48	0.25	[47]

[a] Enzyme adsorbed on XAD-4.



Scheme 2. Alkylation and competing hydrolysis catalysed by β-galactosidase.

overshoot the equilibrium conversion of reactant into product.

$$\text{Glycosyl–OR}^1 + \text{R}^2\text{OH} \rightarrow \text{Glycosyl–OR}^2 + \text{R}^1\text{OH} \quad (5)$$

As the reactant is consumed the concentration of the product will peak when its rates of synthesis (r_S) and dealkylation (r_{-S}) become equal; at this point kinetic control is lost and the reaction should be stopped before thermodynamic control takes over and the product undergoes enzymatic hydrolysis (see also Scheme 2). Hence, the yield of glycoside is determined by a delicate balance between the rates of donor synthesis and hydrolysis, on the one hand, and product hydrolysis on the other.

The transfer of a glycosyl group from a disaccharide to low-molecular weight alcohols has been extensively investigated as an alternative for reversed hydrolysis. Most studies involved lactose as donor, usually as a 0.1–2 M aqueous solution containing a two- to four-fold molar excess of the acceptor (Scheme 2). Transglycosylation is much faster than reversed hydrolysis and reactions generally take a few hours rather than days.

5.1. Effect of the leaving group

In exerting kinetic control the donor plays a double role:

(i) a fast-reacting donor keeps the reaction times short, which correspondingly decreases the time available for product hydrolysis;

(ii) a donor that binds tightly to the catalyst (low K_m) inhibits product hydrolysis.

Experimental results that illustrate the effect of the leaving group on the galactosylation of glycerol by *A. oryzae* β-galactosidase have been compiled in Table 5 [54]. The nearly quantitative yields obtained with the phenyl galactosides indicate that the reaction is kinetically biased towards synthesis rather than hydrolysis. Consequently, the somewhat lower glycerolysis yields of lactose and lactulose (Galβ(1 → 4)Fru) are caused by product hydrolysis and not by donor hydrolysis.

Activated donors, such as aryl or vinyl [79] glycosides afford superior transglycosylation yields, as exemplified above, but their synthesis via the Königs–Knorr route would make the synthetic use of these intermediates self-defeating. Hence, the recently reported catalytic route towards vinyl glycosides via biocatalytic syn-

Table 5
Glycerolysis of various galactosyl donors catalysed by *A. oryzae* β-galactosidase[a]

Donor	Initial rate (nmol U^{-1} min^{-1})	Product (%)	Time (min)
Galactose	3.5	19	1440
Lactose	85	70	180
Lactulose	172	69	90
β-Gal–OPh	508	100	45
β-Gal–OPh– *p*-NO_2	1330	82	30
β-Gal–OPh– *o*-NO_2	390	99	45

[a]Reaction conditions: galactosyl donor, 0.3 mmol, glycerol, 12 mmol, aqueous buffer pH 4.5 or 6.5, 50% (v/v), galactosidase 30 U, 40°C. The data have been taken from Ref. [54].

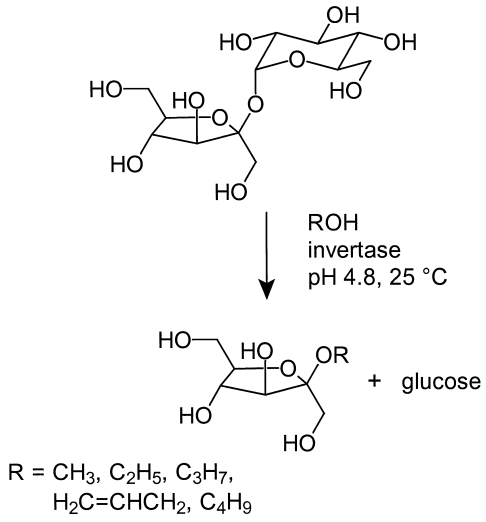


Fig. 4. Alcoholysis of sucrose catalysed by invertase.

thesis of but-3-en-2-yl glycosides [49] followed by a rhodium-catalysed isomerisation step [80], may open new opportunities for increasing the efficiency of enzymatic glycosylation.

5.2. The kinetic synthesis / hydrolysis ratio

The efficacy of transglycosylation depends to a large extent on the kinetic synthesis/hydrolysis ratio r_S/r_H which describes how efficiently the glycosyl-enzyme intermediate is transformed into product (see Scheme 2). Moreover, secondary hydrolysis proceeds via the same intermediate; hence, secondary hydrolysis will be a much more serious problem if r_S/r_H is low. The effect of the medium composition on r_S/r_H of a given reaction is conveniently expressed in thermodynamic parameters:

$$\frac{r_S}{r_H} = S\frac{a_{ROH}}{a_W} \quad (6)$$

in which S is the thermodynamic selectivity parameter which can be determined experimentally.

Because transglycosylation depends on kinetic control, its successful practice requires at least a cursory consideration of reaction kinetics, which is sadly neglected by many authors. The alcoholysis of sucrose [81] (Fig. 4) forms an exception and for that reason it will be discussed in some detail.

Invertase, a β-fructofuranoside-specific glycosidase, hydrolyses sucrose into fructose and glucose via a double inversion on the fructose moiety. The binding constant K_m of sucrose is 38 mM, but because sucrose is also a weak inhibitor ($K_i = 1.2$ M) the hydrolysis rate peaks at 0.2 M sucrose [82]; alcohols, acetone and dioxane were also found to act as weak competitive inhibitors [43,81]. In the presence of lower alcohols the corresponding alkyl β-D-fructofuranosides were synthesised. Some results, together with the initial r_S/r_H and S calculated from the experimental data, are summarised in Table 6. From the initial r_S/r_H the expected

Table 6
Invertase-catalysed alcoholysis of sucrose[a]

ROH	Concentration (%, v/v)	Initial r_S/r_H	a_{ROH}	a_w	S	Fructoside yield	
						η (%)	Found
CH_3OH	40	3.20	0.32	0.81	8.1	76	40
C_2H_5OH	10	0.23	0.17	0.97	1.3	19	9
	30	0.39	0.36	0.92	1.0	28	9
	50	0.60	0.44	0.89	1.2	37	13
	70	0.35	0.54	0.81	0.5	26	10
n-C_3H_7OH	55	0.43	0.52	0.95	0.8	30	10
$CH_2=CHCH_2OH$	10	0.69	0.30	0.98	2.2		n.d.
	75	1.40	0.49	0.87	2.5	58	16
n-C_4H_9OH	50	0.36	0.65	1.00	0.6	26	8

[a]Reaction conditions: sucrose 0.44 M (MeOH, EtOH, PrOH) 0.3 M (other), pH 4.8, 25°C.

Table 7
Alcoholysis of disaccharides

Enzyme type	Source	Donor	Acceptor	r_S/r_H	a_{ROH}	a_w	S	Yield		References
								η (%)	exp	
β-galactosidase	*A. oryzae*	lactose	C_2H_5OH (2 M)	1.3	0.2	0.97	6	56	33	[37]
β-galactosidase	*C. saccharolyticum*	lactose	C_2H_5OH (2 M)	0.6	0.2	0.97	3	37	43	[37]
β-xylosidase	*A. niger*	xylobiose	C_2H_5OH (25%)	>40	0.3	0.94	>100	98	84	[83]
β-xylosidase	*A. niger*	xylobiose	*i*-C_3H_7OH (25%)	13	0.5	0.97	25	92	90	[83]
β-xylosidase	*T. reesei*	Xylβ–OCH_3	C_2H_5OH (2 M)	6	0.2	0.97	30	86	52	[78]
β-xylosidase	*T. reesei*	Xylβ–OCH_3	*n*-C_3H_7OH (2 M)	3	0.4	0.97	7	75	53	[78]
Cellulase	*A. niger*	mannobiose	C_2H_5OH (25%)	1.8	0.3	0.94	5	64	52	[84]

yield, η, in the absence of secondary hydrolysis has been extrapolated according to Eq. (7). By comparing η with the experimental peak yield of alkylation product the role of secondary hydrolysis can be assessed.

$$\eta = \frac{r_S/r_H}{r_S/r_H + 1} \quad (7)$$

The results in Table 6 make it clear that the thermodynamic selectivity parameter *S*, which expresses the alkylation efficiency, declines with increasing alcohol chain length, with allyl alcohol as an interesting exception. It would seem attractive to boost r_S/r_H by using the highest acceptor concentration compatible with enzyme activity but the example of ethanol, which shows a drop in *S* at high ethanol concentrations, shows that this tactic may become counterproductive. This result implies that at high ethanol concentrations ethanolysis is inhibited more than hydrolysis, but the mechanism is obscure. An extra complication that should be reckoned with is a reduction of r_S/r_H at high sucrose concentrations that has been reported for the reaction with allyl alcohol [81]. Secondary hydrolysis is prominent in these reactions as shown by the large difference between the predicted and observed yields.

5.3. The efficiency of glycosyl transfer

Quite a number of publications on enzyme-catalysed glycosidation of alcohols have appeared in recent years, but only in a few cases could the initial value of r_S/r_H be calculated from the experimental data; these are compiled in Table 7. An interesting case is presented by the two β-galactosidases from *A. oryzae* and *C. saccharolyticum* [37]. The former enzyme is a more efficient alkylation catalyst as judged by *S*, yet the *C. saccharolyticum* enzyme gave the highest yield because it hardly suffered from secondary hydrolysis [8]. The β-xyloside from *A. niger* is a remarkably efficient glycosyl transfer catalyst that gave high ($>80\%$) yields of alkyl xylosides [83] with primary as well as secondary alcohols from methanol up to the butanols [9]. The β-xylosidase from *T. reesei* [78], in contrast, was less efficient and it also gave more secondary hydrolysis [10]. A crude cellulase preparation from *A. niger* acted as a mannosidase with moderate r_S/r_H [84], but secondary hydrolysis was slow [85].

The effect of the acceptor chainlength was investigated with the latter system. The optimum conversion to mannoside declined severely with increasing carbon number, from 81%

[8] The experimental yield is higher than the prediction on the basis of r_{syn}/r_{hydr}, which is probably due to erroneous data. The experimental yield of 43% indicates a r_S/r_H of 0.8 rather than 0.6, but this does not change the reasoning.

[9] The reported, unprecedented formation of *tert*-butyl xyloside might actually be the isobutyl xyloside formed from a low amount of 2-methylpropanol that often is present in commercial *tert*-butyl alcohol.

[10] β-methyl xyloside was used as donor in this case but the leaving group has no effect on the reaction of the enzyme-bound glycoside with the nucleophile.

(methanol) to 5% (1-heptanol) [85]. The relationship between acceptor structure and reactivity has not attracted much further interest. It would seem that the plateau yield is influenced by the polarity of the acceptor rather than by its size, because diols tend to give yields comparable with mono-alcohols with half the number of carbon atoms [39], but the data are too fragmentary to draw any firm conclusion.

5.4. Concentration dependent effects

In a number of cases inhibition (or inactivation) of the catalyst by lower alcohols has been observed. *A. oryzae* β-galactosidase, for example, was inhibited by ethanol at > 4 M [37] and by allyl alcohol at moderate concentrations.

Surprisingly, the same catalyst performed well with propargyl alcohol [86]. The *K. lactis* enzyme was also inhibited by allyl alcohol [39], unlike the β-galactosidases from *E. coli* [87] and *S. thermophilus* [88]. With the latter enzyme a decrease of the synthesis/hydrolysis ratio upon increasing the allyl alcohol concentration from 2 to 4 M can be deduced from the progress curves; at still higher concentrations the reaction rapidly stagnated due to inhibition. A similar pattern can be observed in the fluoroethanolysis of lactose catalysed by *S. thermophilus* β-galactosidase [38].

The efficiency of the galactosyl transfer from lactose to ethanol or 2-fluorethanol by the β-galactosidases from *A. oryzae* and *S. thermophilus* also depended on the donor concentration. An increase in lactose concentration resulted in a lower yield of galactoside [38] analogous to the negative effect of increasing donor concentration on the kinetic synthesis/hydrolysis ratio observed with invertase [81].

5.5. Experimental procedures

Transglycosylation reactions have generally been performed in homogeneous aqueous media that contain an excess of the acceptor alcohol in concentrations up to 20% (v/v) [86]. Lower concentrations and/or cosolvents are used with less polar acceptors. An excess of *donor* is often used to glycosylate expensive acceptors efficiently, although such a procedure requires the separation of the product from a large amount of saccharidic material.

Alternative procedures are few. The coimpregnation of alumina with glycosidase and donor deserves to be mentioned [50], but because the technique has so far been used only for the alcoholysis of β-phenyl glucoside, its synthetic significance cannot be assessed yet. The use of β-galactosidase that has been rendered soluble in organic media [65] appears to have potential. The reactions have been carried out in a two-phase system with an aqueous lactose phase and an organic phase that contains the biocatalyst and the acceptor. Glycosylation yields with acceptor alcohols containing up to 8 carbon atoms are in the range of 50–70%. Even β-*p*-nitrophenyl galactoside could be prepared in 35% yield. The reactions proceed quite slowly, however, which may be due to the low (typically 10 mM) reactant concentrations used. Consequently, the productivity of the catalyst is low (0.4 mmol (g enz)$^{-1}$ h^{-1}) and is in the range observed for reversed hydrolysis.

6. Comparison of reversed hydrolysis with transglycosylation

6.1. Reaction rate and yield

Examples of straightforward comparison of reversed hydrolysis and transglycosylation are scarce, for reasons that will be explained below. The compilation in Table 8 shows that disaccharides react much faster than monosaccharides, which seems to represent a general pattern [78]. For example, the *equilibrium* yield of the condensation of galactose and glycerol (which would require 2 days reaction time, see Fig. 2) is less than half the yield of the glycerolysis of lactulose after 1.5 h.

Table 8
Comparison of transglycosylation and reversed hydrolysis

Enzyme	Acceptor	Donor and yield (%)				Productivity ratio		References
		Transglycosylation		Reversed hydrolysis		Initial	Over-all	
Almond β-glucosidase	$HO(CH_2)_3CONHC_4H_9$	cellobiose	45	glucose	11	59	13	[76]
Almond β-glucosidase	$CH_3CH(OH)CH_2COOC_2H_5$	cellobiose	10	glucose	2	29	13	[76]
A. oryzae β-galactosidase	glycerol	lactose	70	galactose	19	58	24	[54]
K. fragilis β-galactosidase	glycerol	lactose	69	galactose	23	17	9	[54]

When comparing both techniques one should not put too much emphasis on yield alone, because transglycosylation liberates at least 1 mol of monosaccharide per mol of product which also must be separated somehow from the desired reaction product.

6.2. Side-reactions

The transglycosylation of lactose results in the liberation of an equivalent of glucose, which subsequently is slowly alkylated either because the substrate specificity of the β-galactosidases is not absolute or because of contaminating activity. Consequently, the reaction products will often contain a few percent of the corresponding β-glucoside. Lactulose, in contrast, does not suffer from this problem because its by-product fructose hardly reacts in reversed hydrolysis.

6.3. Product / waste ratio and space–time yield

The generation of non-recyclable waste is increasingly perceived as one of the major problems confronting the fine-chemical industry. The *E* factor [89,90] (kilogram waste per kilogram product) is a suitable yardstick to compare the performance of various glycosylation procedures in this respect (see Table 9). The Königs–Knorr procedure, two examples of which have been included for comparison, is, not surprisingly, at the high end as a waste generator. In the case of the classical Königs–Knorr procedure [91,92] nearly half the waste consists of acetic acid, but the modified procedure for β-but-3-en-2-yl glucoside [93] generates ca. 5 g of mercuric salts for each gram of product. The enzymatic procedures perform rather better; moreover, the waste mainly con-

Table 9
Waste/product ratios and space–time yields of some procedures

Starting compound	Procedure	Product	*E* factor[a] (kg waste per kg product)	Space–time yield ($g\ l^{-1}\ d^{-1}$)	References
Galactose	Königs–Knorr	Galβ–OPh–2-NO_2	10	26	[91,92]
Lactose	transglycosylation[b]	Galβ–OPh–4-NO_2	16	0.07	[65]
Glucose	reversed hydrolysis[c]	Glcβ–$O(CH_2)_6OH$	4	640	[50]
Glucose	reversed hydrolysis	Glcβ–$O(CH_2)_6OH$	0.5	5.6	[72]
Lactose	transglycosylation	Galβ–$O(CH_2)_6OH$	3	2380	[39]
Glucose	Königs–Knorr	Glcβ–$OCH(CH_3)CH{=}CH_2$	20	36	[93]
Glucose	reversed hydrolysis	Glcβ–$OCH(CH_3)CH{=}CH_2$	2	10	[49]
Glucose	reversed hydrolysis	Glcβ–$OCH_2CH{=}CH_2$	0.7	21	[30]
Lactose	transglycosylation	Galβ–$OCH_2CH{=}CH_2$	4	580	[88]
Sucrose	transglycosylation	Fru^{f}β–$OCH_2CH{=}CH_2$	25	380	[81]

[a] Excess side-chain, donor and volatile solvents, which are easily recyclable, are not counted as waste.
[b] Enzyme–lipid complex.
[c] Enzyme and reactant coimpregnated on a silica carrier.

sists of mono- and disaccharides. The alcoholysis of sucrose lags in this respect, which is undoubtedly due to the strain that is inherent in the glycosidic bond of ketoses. Fermentative digestion by an invertase-deficient yeast is, inter alia, a neat way to separate the alkyl fructoside from the mass of saccharidic material [94].

Space–time yield, which influences the capital costs, is another yardstick to compare procedures. An industrial process for a synthetic intermediate would require a space–time yield of at least 100 g l^{-1} d^{-1} to be economically viable. Even a synthetic procedure for laboratory use should perform better than 1 g l^{-1} d^{-1} or multigram synthesis would become unpractical. Transglycosylation generally performs very well in this respect whereas the space–time yield of reversed hydrolysis is very much lower, excepting the procedure that makes use of coimpregnated enzyme and glucose on alumina [50].

6.4. Cost-price considerations

It is significant that almost all examples of reversed hydrolysis (Section 4) concern glucose whereas lactose predominates in transglycosylation. Synthesis of β-alkyl glucosides, particularly if aimed at application in surfactants, necessarily starts with glucose because other glucosyl donors would be too expensive for such applications. By the same reasoning, β-alkyl galactosides are preferably synthesised from lactose which is much cheaper than galactose and, hence, by transglycosylation.

7. Acceptor selectivity issues

It has already been noted that secondary alcohols are glycosylated slower than primary ones by a factor of 3–5. Equilibrium yields [46,75] are reduced by a similar factor, indicating that the steric strain that destabilises the *sec*-alkyl glycosides is already fully developed in the transition state. Phenols are reactive leaving groups and, hence, weak acceptors that are inefficiently glycosylated by glycosidases [95] (vide infra). The reaction could be accomplished, however, by bovine liver β-glucuronidase (EC 3.2.1.31) [95].

Glucosyl transferases, such as sucrose phosphorylase [66] and cyclodextrin glucanotransferase [67,68], do not perform a hydrolytic reaction as their natural function. These enzymes are quite remarkable in transferring an α-glucosyl group from sucrose or maltodextrin, respectively, to phenolic acceptors with some efficiency. Sucrose phosphorylase even glucosylated phenol faster than benzyl alcohol by a factor of 6 [66].

7.1. Regioselectivity

In line with the preference for primary alcohols noted above, 1,2-propanediol was preferentially glycosylated at the primary hydroxyl group with about 70% selectivity by a number of β-glycosidases [50,96–98]. With 1,2-butanediol, in which the secondary hydroxyl function is more hindered, the selectivity increased to 87% [96]. A similar preference for the primary hydroxyl group is often apparent when hexoses or their derivatives are used as acceptor.

The low reactivity of glycosidases towards phenol has already been mentioned. Accordingly, hydroxybenzylalcohols were exclusively glycosylated at the benzylic position by the β-glucosidases from almonds [99] or *Sulfolobus solfataricus* [98]. Sucrose phosphorylase, which shows a preference for the phenolic hydroxyl group in these reactants [66], is exceptional in this respect.

7.2. Enantioselectivity

In discussing enantioselection towards the acceptor it should be remembered that the carbohydrate moiety is chiral and that, consequently, the glycosylation products are diastereomers and may differ in stability. In the kinetic resolution of chiral alcohols glycosidases generally perform much worse than, e.g., lipases [100]. An early report on the resolution of 1,2-di-*O*-iso-

Table 10
Kinetic resolution of secondary alcohols

Preferred enantiomer	Donor	Enzyme source	*de* (%)	References
(*R*)-**1**	lactose	*E. coli*	33	[96]
(*S*)-**1**	Galβ–OPh	*Sul. solfataricus*	95	[98]
(*R*)-**2**	Galβ–OPh–*o*-NO_2	*E. coli*	64	[107]
(*R*)-**3**	Galβ–OPh–*o*-NO_2	*E. coli*	80	[107]
(*R*)-**4**	Galβ–OPh–*o*-NO_2	*E. coli*	98	[107]
(1*R*,2*S*)-**5**	lactose	*E. coli*	96	[105]
(1*R*,2*S*)-**5**	Galβ–OPh	*E. coli*	89	[105]
(1*S*,2*R*)-**5**	Galβ–OPh	*A. oryzae*	70	[105]
(1*R*,2*S*)-**6**	lactose	*E. coli*	80	[106]
(1*R*,2*S*)-**7**	lactose	*E. coli*	75	[105]
(1*R*,2*S*)-**7**	Galβ–OPh	*E. coli*	90	[105]
(1*S*,2*R*)-**7**	Galβ–OPh	*A. oryzae*	38	[105]
(1*S*,4*R*)-**8**	Galβ–OPh	*E. coli*	50	[105]

propylideneglycerol (solketal) [101,102] proved to be erroneous [96,103] and it became clear that the enantioselectivity of glycosidases towards primary hydroxyl groups, such as those in 1,2-propanediol [96,98], glycerol [104] or glycidol [103] is negligible.

Somewhat better results were obtained in the resolution of secondary alcohols (Table 10, see Fig. 5 for glycon structures and Fig. 6 for the structures of the acceptors). These data should be interpreted with some caution, however, because the donor conversion at which *de* has been measured ranges from about 10% to 20% [105] to full conversion [98]. A decrease in *de* in the course of the reaction has been observed [105,106], which was ascribed [105] to the faster hydrolysis of the preferentially formed diastereomer [107]; this is probably a general phenomenon. In reversed hydrolysis, which is equilibrium-driven, efficient enantioselection is not to be expected, as indeed proved to be the case [49]. Conversely, an activated donor would be expected to favour enantioselection because it prevents secondary hydrolysis. This may explain why galactosyl transfer from β-phenyl galactoside to the prochiral diol **7** was more enantioselective than from lactose [107].

The glycosylation of 1,2-propanediol (**1**) was accompanied by approx. 30% reaction at the secondary hydroxyl group. The *E. coli* β-galactosidase showed a slight preference for the (*R*)-enantiomer [96] whereas the *Sul. solfatari-*

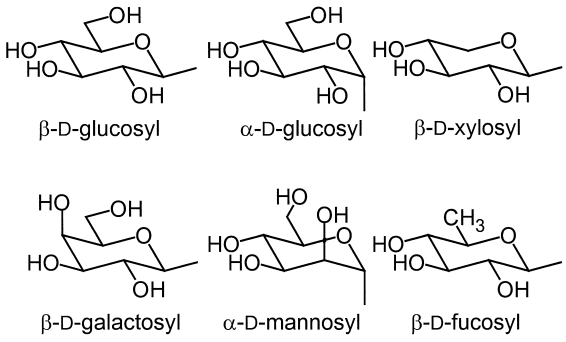


Fig. 5. Monosaccharide structures.

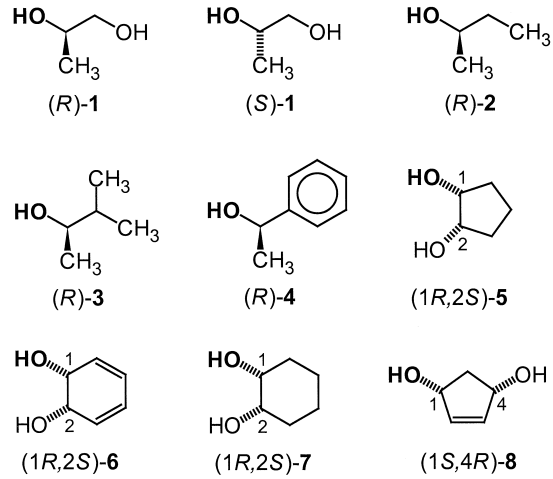


Fig. 6. Chiral and prochiral glycosyl acceptors. The preferentially glycosylated hydroxyl group is in bold.

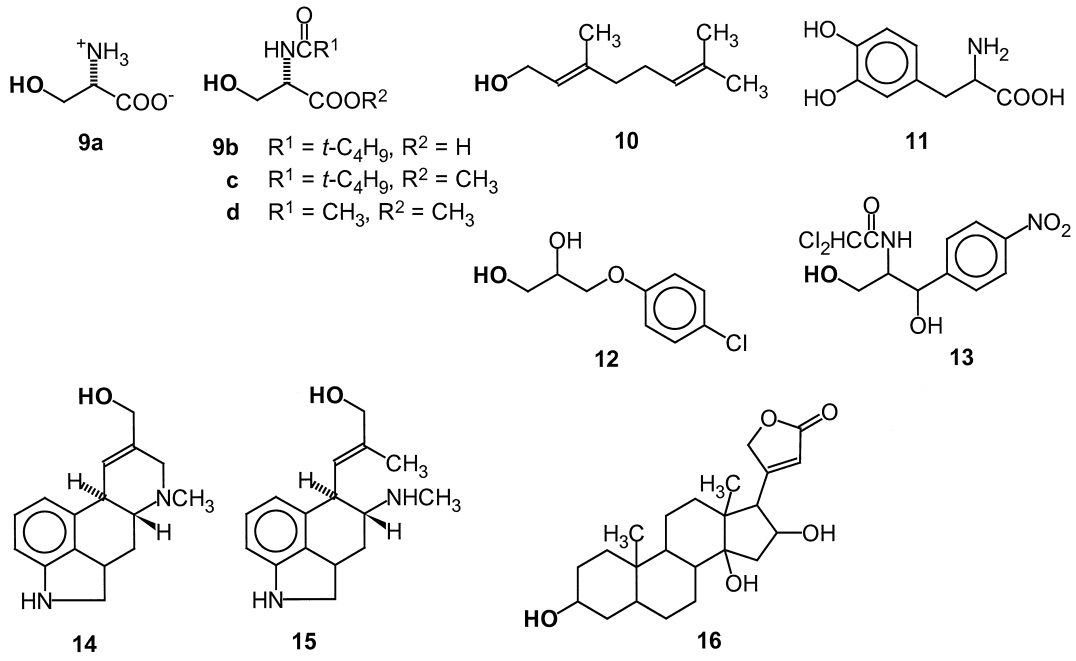


Fig. 7. Complex alcohols that act as glycosyl acceptor. The preferentially glycosylated hydroxyl group is in bold.

cus enzyme was highly selective for (*S*)-**1** [98]. Because the glycosylation of the secondary hydroxyl group plays only a minor role this result is of limited synthetic value. The effect of the side-chain size is illustrated by the increasing enantiopreference of the *E. coli* enzyme for (*R*)-**2**–(*R*)-**4** [107]. This enzyme also converted the prochiral *cis*-diols **5**–**7** with high *de*, in contrast with *A. oryzae* β-galactosidase [105,106].

8. Glycosylation of complex alcohols and non-alcohol acceptors

8.1. Complex alcohols

Transglycosylation is the method of choice for the glycosylation of complex alcohols and carbohydrates. The donor—which may be a disaccharide, but the more reactive aryl glycosides are frequently used—is, in contrast with the previous examples, often used in excess to obtain a high conversion of the expensive acceptor. A prominent theme in this field is the enzymatic glycosylation of carbohydrates, the complex alcohols par excellance, which has been developed into a tool for the synthesis of complex oligosaccharides. Because this subject has been extensively reviewed [10,12–14] it falls outside the scope of this paper. Nucleosides, their close relatives, have been galactosylated by *p*-nitrophenyl β-galactoside in conjunction with *A. oryzae* β-galactosidase, but the yields were low (3–7%) [108].

The enzymatic glycosylation of L-serine (**9a**) and its derivatives **9b**–**d** (see Fig. 7) has attracted considerable attention in recent years because the reaction products are useful intermediates in the synthesis of glycoproteins. These are of interest due to their action as specific receptor structures for proteins and cells in vivo. The procedures and reaction conditions vary widely, which makes the results difficult to compare. Yields [11] are generally mediocre (10–15%) in reversed hydrolysis as well as in transglycosylation. The former procedure has success-

[11] Yields are given in percent (%) of mole product per mole acceptor in this section unless stated otherwise.

fully been used with mannose in conjunction with **9a** [109] as well as **9b** [110]; the somewhat higher yield of 19% that was reported in the latter case is probably due to the very high mannose concentration used. Transgalactosylation from β-2-nitrophenyl galactoside to **9a** was mediated by *A. oryzae* β-galactosidase [111], but this enzyme was inefficient in the galactosylation of **9b** [110] which may be due to steric hindering by the *tert*-butyloxycarbonyl group. The galactosylation of **9c** was accomplished, however, with *E. coli* β-galactosidase [112]. The beneficial effect of excess donor in preventing secondary hydrolysis is illustrated by the almond β-glucosidase mediated glucosyl transfer from β-2-nitrophenyl glucoside to **9d**: the yield nearly doubled from 13 to 25% upon an increase of the excess of donor from three-fold to six-fold [113].

Glycosylation is, in principle, an attractive biocompatible protection technique for labile bioactive compounds such as acid- or oxidation-sensitive fragrances and pharmaceuticals. Besides, glycosylation renders non-polar compounds more soluble in water and may increase the bioavailability of pharmaceuticals. The monoterpene alcohol geraniol (**10**) and its close structural relatives nerol and citronellol were transformed into their β-glucosides [114] or β-galactosides [115] by glycosidase catalysis. Unattractively low ($\leq 4\%$) yields were obtained, which is probably due to the highly non-polar character of the acceptors.

The oxidation-sensitive catecholamines such as, e.g., dopa (**11**) were transformed into their more stable α-glucosyl derivatives by transglucosylation [67]. Cyclodextrin glucanotransferase performed this reaction with a reasonable efficiency ($\sim 35\%$ yield of two positional isomers), which is doubtlessly due to the known [67,68] preference of the catalyst for phenol acceptors.

The antibiotics chlorphenisin and chloramphenicol (**12** and **13**, respectively) were transformed into their glycoconjugates by transgalactosylation from lactose [44] in mixed water–organic media. In spite of the four-fold excess of donor that was used the yields were rather low ($< 12\%$), probably because the reaction ceased before the donor had been consumed. This may be due to product inhibition as is indicated by the K_m values of the corresponding hydrolysis reactions (18 mM for lactose [116] and 1.2 and 2.7 mM for the galactosyl derivatives of **12** and **13** [117], respectively).

Considerable effort has been devoted to the glycosylation of ergot alkaloids [118], many of which are pharmacologically active. The galactosylation of elymoclavine (**14**) by Galβ–OPh–*p*-NO_2 (*A. oryzae* β-galactosidase) which yielded 37% of the desired product [118,119] is a representative example. α-Mannosylation of **14** was accomplished by reversed hydrolysis (α-mannosidase from jack bean) in the surprisingly high yield of 18%, whereas the equilibrium yield of the related chanoclavine (**15**) was only 11%. The relative inefficiency of reversed hydrolysis is underscored by comparing this latter result with the mannosyl transfer from Manα–OPh–*p*-NO_2 to **15**, which gave 28% product at more than 60 times the reaction rate under otherwise identical reaction conditions [120].

Glycosylation of cardiac genins such as **16** would seem an attractive way to increase their stability towards acids and bases, but the galactosylation yield—Galβ–OPh and *A. oryzae* β-galactosidase—was only $\leq 3\%$ [121]. This unattractive result underscores the problems of glycosylating a secondary alcohol.

8.2. Non-alcohol acceptors

Examples of non-alcohol acceptors in glycosidase catalysis are rare when compared, once more, with lipases [100]. Galactosyl transfer from *o*-nitrophenyl β-D-galactoside or lactose to number of oximes was mediated by *A. oryzae* β-galactosidase in yields ranging from 15% to 30% with respect to glucose [122], which is remarkable when considering that in lipase catalysis oximes are regarded as sluggish acceptors.

Thiols acted as glycosyl acceptors towards almond β-glucosidase in reversed hydrolysis as well as in transglucosylation [32,123], whereas *A. oryzae* β-galactosidase was inactive. Condensation of glucose with 2-mercaptoethanol mediated by almond β-glucosidase gave a product mixture (65% yield with respect to the donor) in which the product of *S*-glucosylation predominated [123]. The course of the reaction was unusual in that the yield of β-2-mercaptoethyl glucoside passed through a maximum; the suggestion [123] that this product overshoots the equilibrium composition for kinetic reasons must be erroneous, because the reaction is thermodynamically controlled. Diglucosylation to an undetected product would seem a more likely explanation for the observed result.

9. Donor selectivity and non-carbohydrate donors

9.1. Carbohydrate selectivity

The anomeric selectivity of glycosidases is inherent in their structure and, hence, it is absolute. The glycosyl selectivity is often more relaxed and it is sometimes possible to exploit this characteristic feature synthetically. Bovine liver β-galactosidase has not seen much synthetic use, but it is conspicuous by its wide substrate tolerance, which encompasses β-D-xylopyranosides, β-D-fucopyranosides and α-D-arabinopyranosides [124,125]. The thermostable β-glycosidases from *P. furiosus* [34], *Sul. solfataricus* [126] and *C. saccharolyticum* [127] likewise have a very broad substrate specificity (see Table 11).

Some crude enzyme preparations have a relaxed substrate specificity, but it is not clear whether this is a characteristic of the main active component, or that it is caused by contaminating activity. The β-D-xylosidase from *T. reesei* (Novo SP431), which also hydrolyses β-D-glucosides and α-L-arabinofuranosides [78] is a good example, as is the mannosidase capacity of a number of crude cellulase preparations [84]. The activity of almond β-glucosidase in the synthesis of β-allyl galactoside [30,31] has been ascribed to contaminating activity by later authors [49].

9.2. Non-carbohydrate donors

The inhibitory action of pyranoid glycals (1,5-anhydro-2-deoxyhex-1-enitols) on glycosi-

Table 11
Substrate specificity of some thermostable glycosidases

Glycosidase source	Reactant	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m		References
				$mM^{-1}\ s^{-1}$	Percent (%)	
Sul. solfataricus	D-Glcβ–OPh–*p*-NO_2	0.5	542	1084	100	[126]
	D-Galβ–OPh–*p*-NO_2	4.7	1020	217	20	
	D-Fucβ–OPh–*p*-NO_2	1.0	772	772	71	
	cellobiose	30	1333	44	4	
	laminaribiose[a]	1.0	908	908	84	
P. furiosus	D-Glcβ–OPh–*p*-NO_2				100	[34]
	D-Galβ–OPh–*p*-NO_2				34	
	cellobiose				52	
	laminaribiose[a]				7	
C. saccharolyticum	D-Glcβ–OPh–*p*-NO_2	0.5	334	658	100	[127]
	D-Galβ–OPh–*p*-NO_2	1.9	460	237	36	
	D-Fucβ–OPh–*p*-NO_2	0.24	296	1249	190	
	cellobiose	18.2	396	22	3	
	laminaribiose[a]	3.0	964	323	49	

[a] Gluβ(1 → 3)Glu.

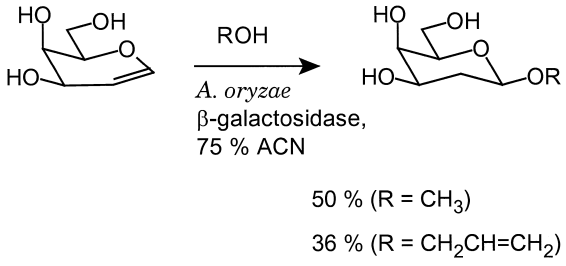


Fig. 8. The glycosidase-mediated alkylation of D-galactal.

dases has been known since the late 1960s [128]. The resulting enzyme-bound 1-anhydroglycosyl intermediate reacts slowly with water [129,130] or alcohols [131], in the latter case to give the corresponding β-alkyl 2-deoxyglycoside (see Fig. 8).

10. Conclusions and future prospects

The glycosidases catalyse the synthesis of anomerically pure alkyl glycosides in one step with a broad specificity for the aglycon. The reactivity of glycosidases towards hydroxyl groups decreases in the order primary > secondary alcohols > phenols; tertiary alcohols are unreactive. Chiral primary alcohols are poorly discriminated, but the enantioselectivity towards a hydroxyl group that is directly attached to a (pro)chiral carbon atom is often high.

In spite of the potential advantages the synthetic utility of glycosidase catalysis is limited owing to the low conversions that are generally observed. The latter are due to the presence of water which is necessary to maintain the enzyme's activity but also causes substrate and product hydrolysis as serious side reactions. Summarising, in order to have real synthetic utility a breakthrough is needed with regard to the operational stability of glycosidases at low water activities.

It is worth emphasising, however, that most of the reported studies of glycosidases have involved the use of the enzyme in a free (non-immobilised) form. By analogy with other enzyme types one would expect that immobilisation, e.g., in the form of cross-linked enzyme crystals, would render glycosidases more tolerant to low water activities in organic solvents. Similarly, protein engineering could be used to generate more stable enzymes, and possibly improve the diastereo- and enantioselectivity. Alternatively, glycosidases from extremophiles may exhibit the required tolerance to low water activities.

Working at low water activity introduces the problem of the poor solubility of carbohydrates in most organic media. This problem has been solved for the lipase-catalysed synthesis of carbohydrate esters, and there is little doubt that a similar approach will be successful with glycosidases, once low a_w-tolerant enzymes become available.

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Table 12
Thermal deactivation of some β-glycosidases

Source	Type	$t_{0.5}$ (h)	°C	References
E. coli	β-galactosidase	0.04	50	[132]
		0.75	40	[133]
		1.5	55	[33]
A. oryzae	β-galactosidase	0.24	50	[116]
		0.12	55	[33]
		72	50	[135]
		833	50	[136]
C. saccharolyticum	β-glucosidase	15	80	[127]
Sul. solfataricus	β-glucosidase	> 24	75	[126]
		3	85	[34]
P. furiosus	β-glucosidase	85	100	[34]

Appendix A. Thermostability of glycosidases

Because the thermostability of glycosidases is an important issue, some literature data on the thermal deactivation of a number of β-glycosidases have been compiled in Table 12. These are, in some cases, conspicuous by their wide divergence. The discrepancy in the reported thermostability date for *E. coli* β-galactosidase, which varies by a factor of 75 [33,132,133], is probably caused by differences in purification and buffers used in the deactivation measurements. Addition of 1% caseinate, for example, improved the half-life of the purified enzyme by two orders of magnitude [134]. Besides, the enzyme is dimeric and purification may cause partial dissociation of its subunits.

Stabilisation by contaminating protein may also contribute to divergence in half-life times reported for *A. oryzae* β-galactosidase [33,116, 135,136]. Isozymes produced by different strains of *A. oryzae* may also be involved: isozymes with half-lives of 1 h at 50 and 64°C, respectively, have been isolated from two *A. oryzae* mutant strains [137]. To complicate matters further, a recent study indicated that the commercial preparation contains two isozymes with different thermostability [138].

The β-glucosidases from *C. saccharolyticum* [127] and *Sul. solfataricus* [34,126] are stable for hours at 80°C, which is a very considerable improvement. The most stable glycosidase isolated so far is the β-glucosidase from *P. furiosus* [34].

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