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Glycosidases in Carbohydrate Synthesis: When Organic Chemistry Falls Short

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Abstract: Thanks to the stability, good availability, stereoselectivity and broad substrate specificity, oligosaccharide synthesis catalyzed by glycosidases represents an elegant way to complex carbohydrate structures. Two approaches to glycosidase catalysis are presented: i) the use of structurally modified substrates that carry various functional moieties in the molecule, and ii) the design of mutant glycosidases void of hydrolytic activity. Products of glycosidase-catalyzed synthesis are applicable in a range of areas such as immunology, therapy of Alzheimer's or Parkinson's diseases and the synthesis of neoglycoproteins.

Keywords: Carbohydrate · Enzymatic synthesis · Glycosidase · Glycosynthase · Transglycosylation

1. Introduction

Oligosaccharide synthesis is traditionally accomplished by methods of organic chemistry;[1] however, this approach has one serious drawback: a vicious circle of protection-deprotection steps, which often result in a dramatic decline in reaction yields and time-cost efficiency. For instance, the yield of a chemical synthesis of sialyl dimeric Le^x,[2] a complex heptasaccharide, is less than 1%, despite the fact that most of the individual reaction steps are quantitative. In such cases, enzymatic synthesis represents a welcome alternative. Carbohydrate-processing enzymes fall into two classes: glycosyltransferases and glycosidases. The former enzymes, though favored in carbohydrate synthesis due to their absolute selectivity,[3] frequently encounter the problem of limited stability and availability. Another negative point is the high cost of their substrates: nucleotide sugars. In this review, we aim to introduce glycosidases, original hydrolytic enzymes, as potent synthetic tools in many applications.

The first glycosidase-catalyzed synthesis dates back to the end of the 19th century.[4,5] A systematic study of glycosidases as synthetic devices did not happen until the 1950s when β-fructofuranosidases,[6] α-[7] and β-glucosidases[8] as well as α-[9] and β-galactosidases[10] were successfully applied in the preparation of disaccharides. Later on, the first glycosidases were isolated and characterized, such as the pioneer β-*N*-acetylhexosaminidase from a commercial enzyme preparation Takadiastase®.[11] The late 1980s can be called the golden era of glycosidases, connected with the groups of Nilsson,[12] Fujimoto and Ajisaka,[13] Larsson and Mosbach,[14] and others. Nowadays, the emphasis is on highly selective reactions with mutant enzymes or derivatized substrates, not seldom with direct medicinal or biotechnological applications. Fig. 1 shows the development of three main areas concerning the application of glycosidases in carbohydrate chemistry.

2. Fundamentals of Glycosidase Catalysis

Glycosidases (*O*-glycoside hydrolases; EC 3.2.1.-) cleave saccharidic chains *in vivo* by transfering a glycosyl moiety to a water acceptor. Under certain conditions, this strategy can be reversed: other mol-

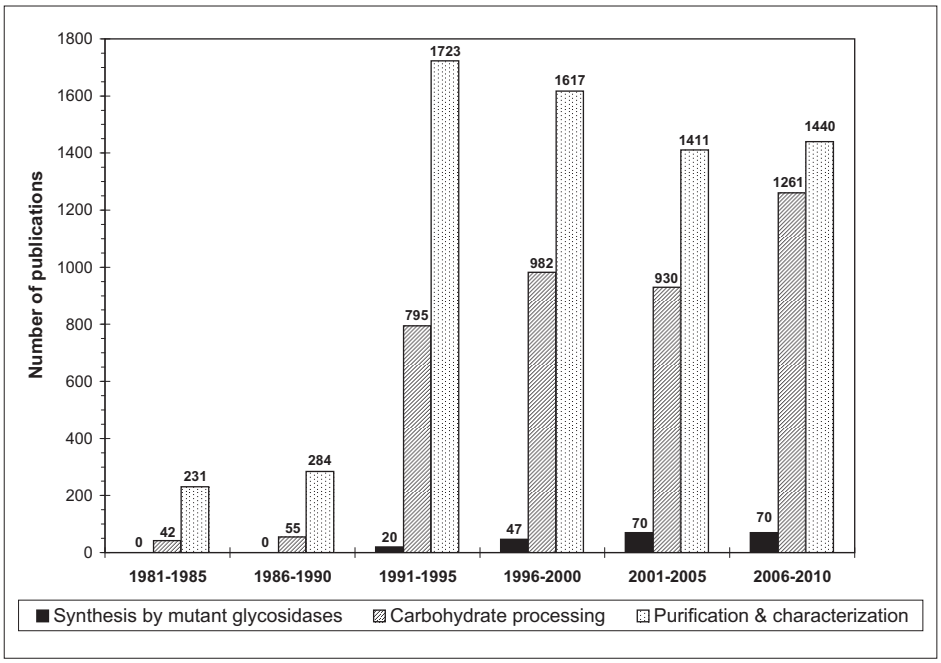

Fig. 1. Use of glycosidases in the period 1981–2010. Publications on three areas of glycosidase application (*i.e.* carbohydrate processing, purification & characterization, and mutant glycosidases in synthesis) were monitored over the given timespan. Source: Web of Science database (*http://isiknowledge.com/*).

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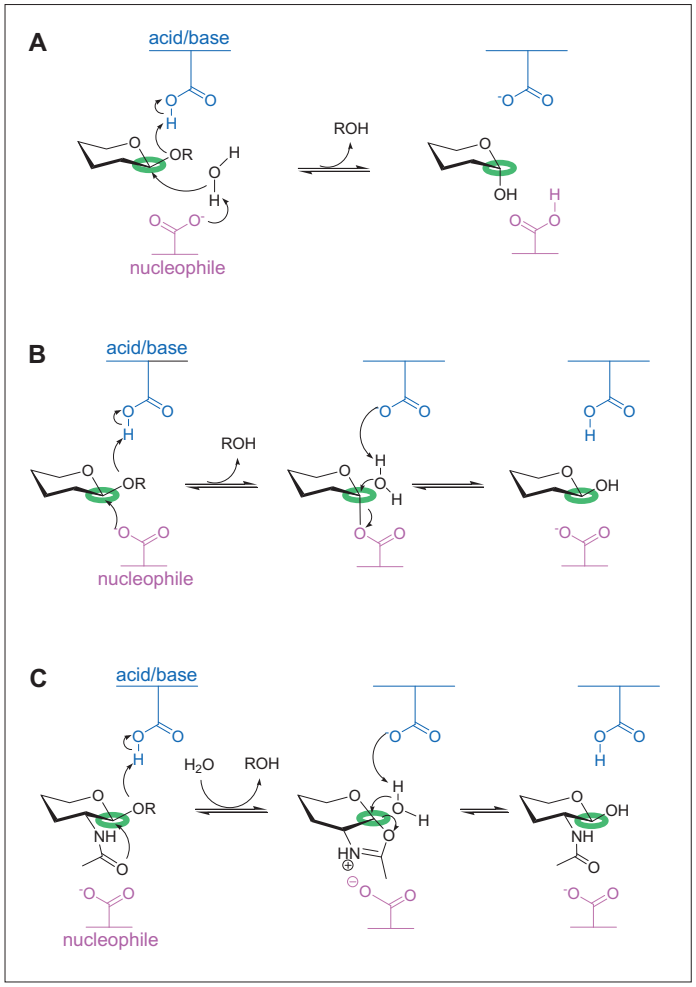


Scheme 1. Hydrolytic mechanisms of wild-type glycosidases. Inverting glycosidases (**A**) act by a single-step, mechanism, with a single inversion at the anomeric centre, shown in a green circle. The catalytic carboxylates are shown in blue (acid-base) and pink (nucleophile). Retaining glycosidases (**B**) act through a double-displacement mechanism (double inversion at the anomeric centre). β-*N*-Acetylhexosaminidases (**C**) utilize a modified retaining mechanism, in which the nucleophilic attack is not performed by the enzyme residue but by the substrate 2-acetamido group, forming an oxazoline intermediate. R = saccharide or alcohol.

ecules possessing an acceptor hydroxyl group are found more attractive than water and a new glycosidic bond is formed. This happens if the activity of water is diminished by, *e.g.* high reactant concentrations,[15] addition of salts,[16] organic solvents or by elimination of the reaction product.[13] If glycosyl donor is a reducing sugar, the synthetic process is under thermodynamic control and is called 'reverse hydrolysis'. In this case, yields are generally below 15%.[17] A more popular reaction system under kinetic control employs glycoside donors activated by a good leaving group, such as *p*-nitrophenyl. This so-called 'transglycosylation' affords higher yields[18] of 20–40%.

The sub-subclass of glycosidases comprises 154 entries in the IUBMB enzyme nomenclature system (International Union of Biochemistry and Molecular Biology, status from July 14, 2010). Another source of information on the variety of glycosidases is the CAZy database (Carbohydrate Active Enzymes, *http://www.cazy.org/*), founded by Henrissat at the onset of the 1990s.[19] This classification system is based on amino acid sequences and divides glycosidases into over 100 families.[20]

As a result of a glycosidase-induced cleavage,[21] the new glycosidic bond either retains the same anomeric configuration (retaining enzymes) or inverts it to form the other anomer (inverting enzymes). Inverting glycosidases (Scheme 1A) act by a one-step, single-displacement mechanism with acid/base assistance (normally by active-site Glu and Asp). Retaining enzymes (Scheme 1B), which often exhibit synthetic potential, act *via* a double-displacement mechanism involving a covalent glycosyl-enzyme intermediate. In the first step (glycosylation), the nucleophilic carboxylate displaces the aglycon to form the glycosyl-enzyme intermediate, which is facilitated by acid-catalyzed departure of the leaving group mediated by the other residue. In the second step (deglycosylation), the glycosyl enzyme is hydrolyzed by an incoming nucleophile, with the other residue now acting as a base catalyst that activates the nucleophile for the attack. Glycosidases of families 18, 20, 25, 56, 84, and 85 process substrates with a 2-acetamido moiety. This moiety acts as an intramolecular nucleophile and instead a covalent glycosyl enzyme, an oxazolinium ion is the reaction intermediate (Scheme 1C). Other mechanistic modifications have been reviewed.[20,21] Mutant glycosidases utilize somewhat altered reaction mechanisms, which are reported on below.

3. Tailored Substrates – Building Blocks of Target Saccharides

The sturdy, easy-to-handle and readily available glycosidases are a potent tool for a green one-step synthesis of oligosaccharides, provided that the problem of reaction regioselectivity is overcome. This may be resolved by a careful selection of enzyme source coupled with an ingenious design of both glycosyl acceptors and donors.

3.1 *Glycosylation of Complex or Sensitive Compounds*

Attachment of a sugar unit(s) to an aglycone may increase its stability or/and water solubility, which is especially important for pharmaceutical preparations. A classical example is the galactosylation of chlorphenisin and chloramphenicol antibiotics by *Aspergillus oryzae* β-galactosidase.[22] Chemical glycosylation of ergot alkaloids has not been reported so far, probably due to the complexity and sensitivity of these compounds.[23,24] Alkaloid glycosides obtained by glycosidase-catalyzed glycosylation are expected to be good prodrugs with improved pharmacokinetic properties (*e.g.* glucopyranosides easily passing the hemato-encephalitic barrier). Alcohols, including L-serine,[25] are common substrates for enzymatic glycosylations.[17] Secondary alcohols are glycosylated 3–5 times more slowly than the respective primary counterparts[26] and tertiary alcohols are even poorer substrates.[27,28] Glycosidases can also be applied to create difficult-to-prepare glycosidic linkages, such as β1,4-mannosidic bond (*endo*-β-mannosidase from *Lilium longiflorum*)[29] or Gal-α1,4 linkage (α-galactosidase from *Bifidobacterium breve*).[30]

3.2 *Functionalized Substrates Exploit Enzyme Natural Potential*

Thanks to their undemanding substrate specificity, many glycosidases are able to utilize specifically functionalized substrates as illustrated in Fig. 2. Glycosyl donors like nitrophenyl glycosides suffer from several flaws, such as limited solubility (especially in combination with other hydrophobic groups in the molecule), a notable ratio of autocondensation products (nitrophenyl disaccharides) in the reaction mixture, and a difficult purification of the released nitrophenol. Therefore, glycosyl donors with other leaving groups have been proposed such as 3-nitro- (**1**) and 5-nitro-2-pyridyl glycosides,[31] vinyl glycosides (**2**),[32] 1-*O*-acetyl glycosides (**3**)[33] and oxazoline derivatives (*e.g.* **4**).[34] The latter structures mimic reaction intermediates in the mechanism of substrate-assisted catalysis[21] (Scheme 1C).

Glycosyl fluorides are widely used with glycosynthases (Scheme 3; C–F bond

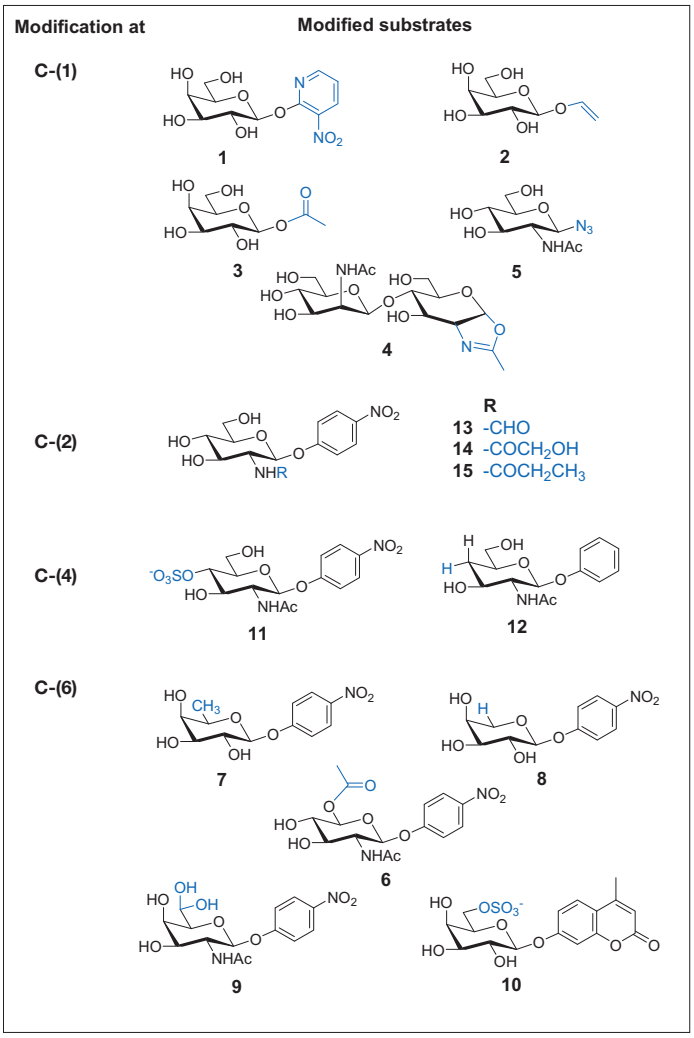


Fig. 2. Overview of modified substrates used in transglycosylation reactions catalyzed by glycosidases.

cleaved).[35] Although their stability in H_2O is limited, they are very efficient due to the small size of fluorine, its easy detection by ^{19}F NMR, and its high electron-withdrawing effect. Glycosyl azides (*e.g.* **5**)[36] (C–N bond cleaved) are good alternative substrates especially for *N*-acetyl-D-hexosamines, the fluorides of which are unstable. They have also been used with β-galactosidases, β-glucosidases, α-mannosidases[37] and thioglycoligases.[38]

Protecting the primary hydroxyl of the acceptor polyol as an acetate, *e.g.*[39] enhances the reaction regioselectivity; thus, the synthesis is likely to be directed to C(4) (*gluco*-substrates) or C(3) positions (*galacto*-substrates). A large number of modifications at C(6), including substitutions by, *e.g.* aldehyde, methyl, carbene, carbyne or fluorine, have been studied including for β-galactosylations by Wong,[40] MacManus,[41] and Hušáková (*e.g.* **6**).[39c] Weingarten and Thiem[42] also developed this topic by presenting nine C(6) modified glycosides (*e.g.* **7**, **8**) as potential substrates for the β-galactosidase from *Bacillus circulans*. An elegant reaction sequence starting from *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-*galacto*-hexodialdo-1,5-pyranoside (**9**) yielded immunoactive oligosaccharides in one pot (Scheme 2).[43] After enzymatic glycosylation, the aldehyde moiety is oxidized chemically to a carboxyl, the carrier of the immunomodulation potential. These structures are strong ligands of the activation receptors of human natural killer cells that are potentially applicable in the treatment of cancer.[43] The originated *N*-acetyl-β-D-galactosaminuronates are all the more interesting because such structures are quite difficult to prepare chemically[44] and no specific transferases for *N*-acetyl-β-D-hexosaminuronates have been reported up to date.

Another widely studied substrate modification is sulfation. Sulfated oligosaccharides are vital in many natural processes, such as cell-cell adhesion, homing of lymphocytes, binding of bacteria, and hormone regulation. The pioneer experiments were performed with the β-galactosidases from *B. circulans* and *E. coli* and 4-methylumbelliferyl 6-sulfo-β-D-galactopyranoside donor (**10**).[41] Glycosylations with 6-*O*-sulfo-*N*-acetyl-D-glucosaminyl moiety was demonstrated in several recent works.[45] Loft and Williams[46] published challenging syntheses of regioselectively sulfated *p*-nitrophenyl 2-acetomido-2-deoxy-β-D-glucopyranosides (6-*O*-sulfate, 4-*O*-sulfate **11**, and 3-*O*-sulfate) and their cleavage by sulfatases and fungal β-*N*-acetylhexosaminidases.

Fluoroglycosides[35] inhibit glycosidases. Consequently, these compounds serve as mechanism-based indicators for active-site labeling in particular 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glycoside,[47] 2-deoxy-2-fluoro-β-D-glycosyl fluoride,[48] and 5-fluoro-D-glycosyl fluorides.[49]

Glycosidases exhibit quite a high tolerance to modifications of the C(4) position (*e.g.* **12**). For β-*N*-acetylhexosaminidases (GH family 20), the cleavage of both *gluco*- and *galacto*-configurations, though at different ratios,[50] is a typical feature.[28,51,52]

C(2) hydroxyl and its modifications have a special importance for enzymes using the substrate-assisted hydrolytic mechanism, such as β-*N*-acetylhexosaminidases.[21c] The acetamido group is crucial for stabilization of the oxazoline reaction intermediate and therefore, it cannot be much altered without a substantial decline in enzyme activity. Nevertheless, changes to shorter or longer acyls or substitution by a hydroxyl (**13**–**15**) are tolerated, contrary to highly electronegative or charged moieties.[53] Substrates modified at the C(2) position have recently been applied with transsialidases[54] as well as α-L-arabinofuranosidases.[55]

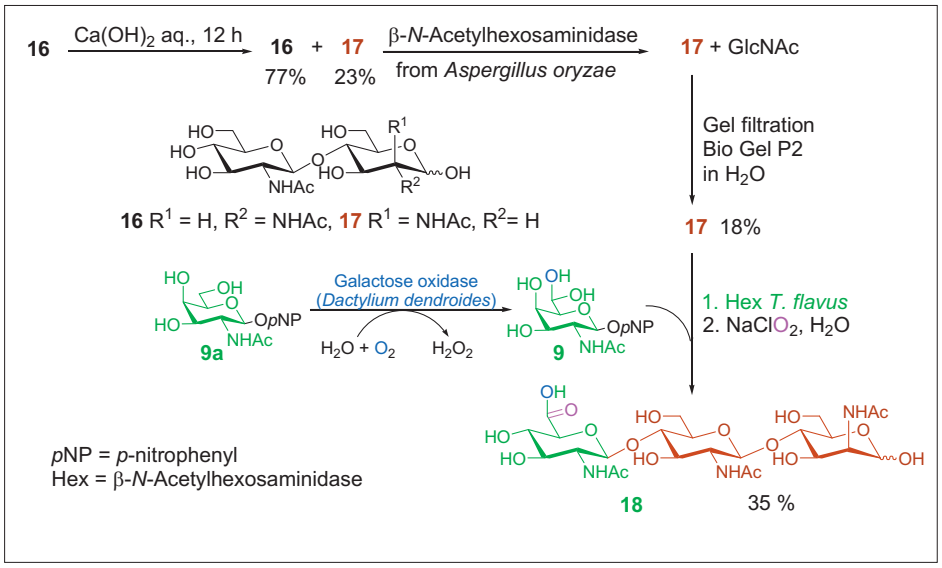
4. Mutant Glycosidases – High-power Synthetic Engines

4.1 *Glycosynthases*

Glycosynthases, site-directed mutants of glycosidases, were invented in 1998[56] and their appearance smashed all existing rules on the role of glycosidases in carbohydrate synthesis. Ingeniously simple, if the active-site catalytic nucleophile (Asp or Glu) is mutated to a non-nucleophilic residue like Ala, the resulting mutant becomes hydrolytically inactive but it can transfer a suitably activated sugar donor (*e.g.* a glycosyl fluoride) onto acceptors with nearly quantitative yields (>80%) (Scheme 3). A typical acceptor is an aryl glycoside that binds well to the active site but cannot be cleaved by itself.[35,57]

The first glycosynthase originated from the β-glucosidase from *Agrobacterium* sp., which is a remarkable catalyst even in wild type.[58] It served as a model enzyme for optimization of glycosynthetic abilities. Three mutations were introduced into this enzyme: Glu358Ala,[59] Glu358Ser,[60] and Glu358Gly.[61] In this order, an enhancement of catalytic activity was observed in the mutants as manifested by higher yields, increased reaction rate, and a broader choice of acceptors (however, this order may not be as strict as shown in ref. [62]).

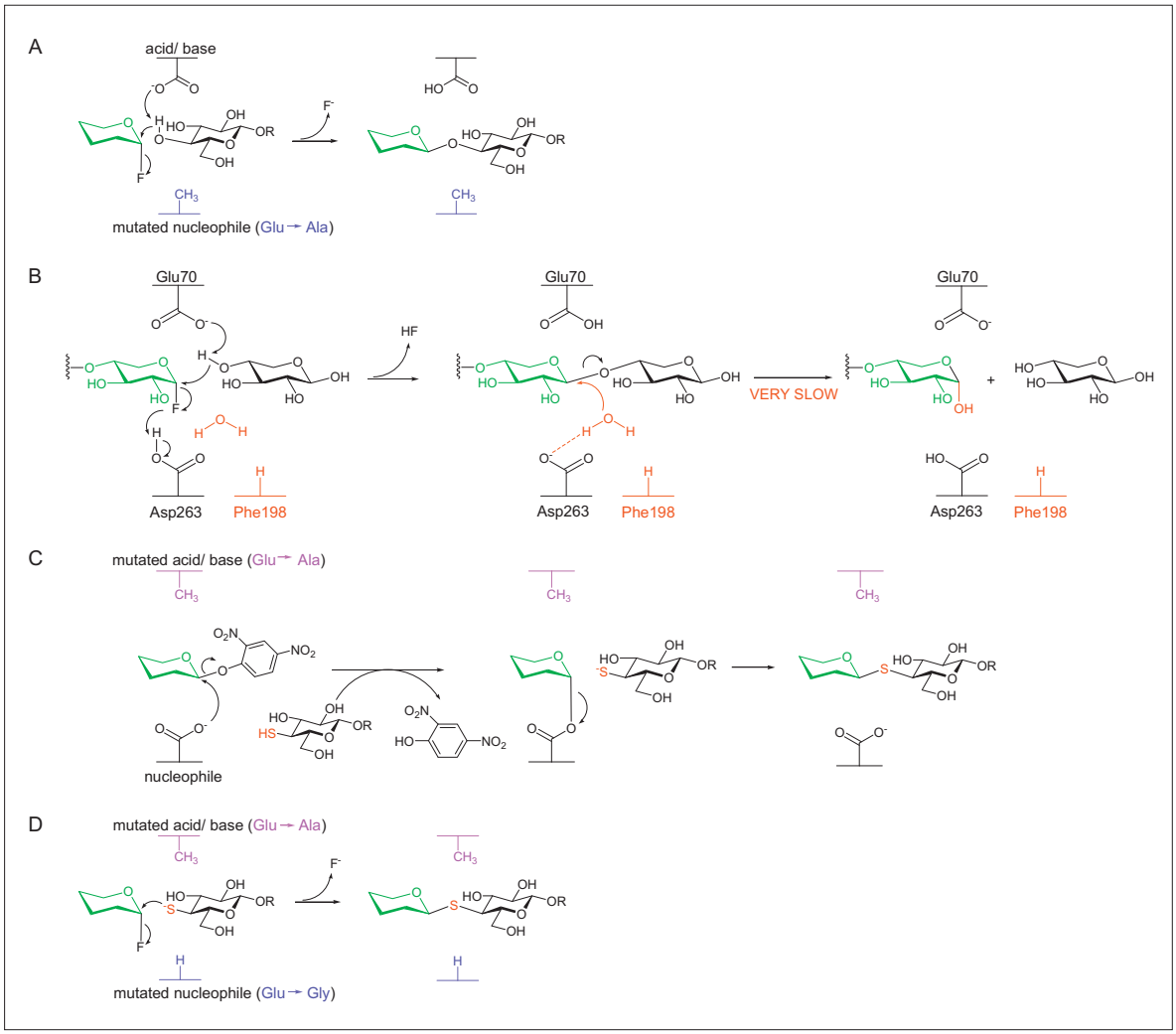
Recently *endo*-glycosynthase,[63] α-glycosynthases,[64] and thermophilic gly-



Scheme 2. Synthesis of an immunoactive trisaccharide using a glycosidase.[43b] β-D-Glc*p*NAc-(1→4)-D-Glc*p*NAc (**16**) is partially epimerized to β-D-Glc*p*NAc-(1→4)-D-Man*p*NAc (**17**). After selective hydrolysis of the reaction mixture by the β-*N*-acetylhexosaminidase from *Aspergillus oryzae*, the desired disaccharide β-D-Glc*p*NAc-(1→4)-D-Man*p*NAc (**17**) is purified by gel filtration. **17** is used as an acceptor in a transglycosylation reaction catalyzed by the β-*N*-acetylhexosaminidase from *Talaromyces flavus* with *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-*galacto*-hexodialdo-1,5-pyranoside (**9**) donor. Subsequent chemical oxidation of the aldehyde group yielded β-D-Gal*p*NAcA-(1→4)-β-D-Glc*p*NAc-(1→4)-D-Man*p*NAc (**18**; 35%). Activated donor aldehyde **9** is prepared by selective enzymatic oxidation of alcohol **9a** (galactose oxidase from *Dactylium dendroides*).

cosynthase[65] mutants with altered substrate specificity have been obtained. An example is the xylosynthase derived from *Agrobacterium* sp. β-glucosidase.[66] Tolborg *et al.*[67] reported on the first synthesis using a glycosynthase on solid phase, namely on poly(ethylene glycol) polyacrylamide copolymer. Quantitative glucosylations were catalyzed by Glu358Ser and Glu358Gly β-glucosidase from *Agrobacterium* sp. A great variety of glycosyl acceptors were tested with the hyperthermophilic glycosynthase from *Sulfolobus solfataricus*.[68] The potential of glycosynthases was demonstrated in a number of challenging syntheses, such as the preparation of glycosphingolipids, possibly useful in the therapy of Parkinson's and Alzheimer's diseases,[69] or the glycosylation of flavonoids with immunomodulation effect.[70] A number of alcohols can be glucuronylated by a glucuronyl synthase derived from Glu504Gly β-glucuronidase from *E. coli*.[71]

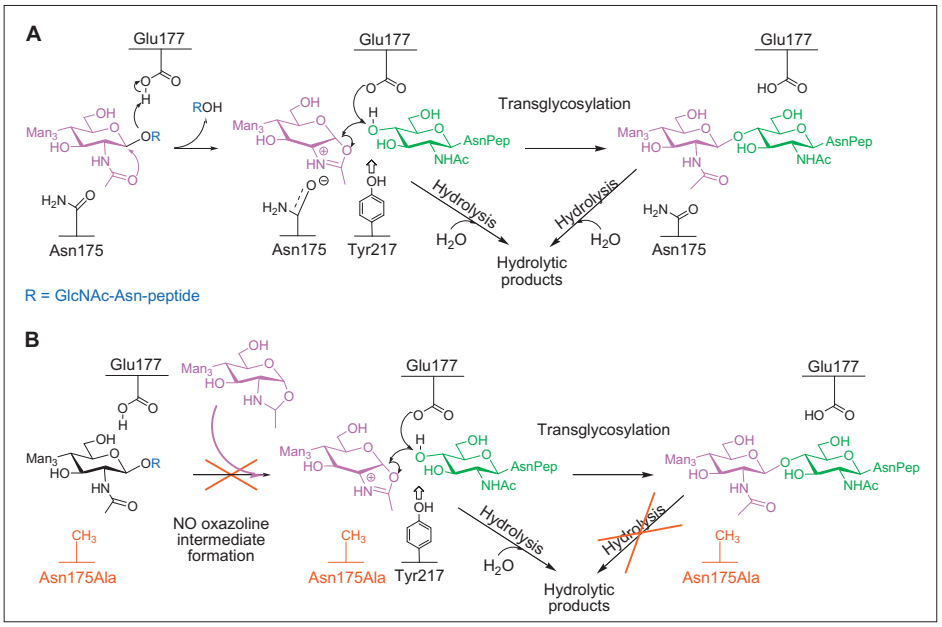
A novel concept in glycosynthase technology was presented by Honda *et al.* – they prepared the first glycosynthase from the reducing end xylose-releasing *exo*-oligoxylanase from *Bacillus halodurans*, which is an inverting glycosidase. Two active-site



Scheme 3. Reaction mechanisms of mutant glycosidases. Glycosynthase (**A**), in which the catalytic nucleophile Glu was mutated to Ala, uses fluoride of the opposite anomeric configuration as a donor. The only inverting glycosynthase, Tyr198Phe xylanase from *Bacillus halodurans* (**B**), can glycosylate using a fluoride donor with negligible side hydrolysis due to the mutation of water-stabilizing Tyr198 to Phe. Thioglycoligase (**C**), in which the catalytic acid-base Glu was mutated to Ala, uses reactive 2,4-dinitrophenyl glycoside donors and nucleophilic thiosugar acceptors. Double mutant thioglycosynthase (**D**), with the catalytic acid-base Glu mutated to Ala and the catalytic nucleophile Glu mutated to Gly, uses inverted glycosyl fluoride donors and thiosugar acceptors. R = aglycon, *e.g. p*-nitrophenyl.

mutations were studied, Asp263Cys[72] (catalytic base residue), and Tyr198Phe.[73] By mutating the general base Asp263 to Cys, the hydrolytic activity was considerably diminished but so was the F^- releasing activity. By mutating the water-stabilizing residue Tyr198 to Phe, the F^- releasing activity was slightly increased compared to the wild-type whereas the hydrolyzing activity was drastically reduced. Thus, Tyr198Phe is a better inverting glycosynthase than the mutant of the base residue. (Scheme 3).

Umekawa and coworkers[74] prepared the first glycosynthase from a glycosidase exercising substrate-assisted catalytic mechanism. In the *endo*-β-*N*-acetylglucosaminidase from *Mucor hiemalis*, the Asn175 residue, critical for stabilizing the oxazoline reaction intermediate, was mutated to Ala. The resulting mutant is unable to hydrolyze any activated substrates but it can quantitatively transfer oxazoline donors (Scheme 4).[73] Other attempts have previously been made to suppress hydrolytic activity by mutating other residues than catalytic nucleophile, with variable results. A noteworthy example is the introduction of transsialidase activity into hydrolyzing-only *Trypanosoma rangeli* sialidase by six site-directed mutations, selected by comparison to *Trypanosoma cruzi* transsialidase.[75]



Scheme 4. Reaction mechanism of a glycosynthase derived from the *Mucor hiemalis endo*-β-*N*-acetylhexosaminidase.[74] In the wild-type enzyme (**A**), Glu177 activates the substrate glycosidic bond and Asn175 assists the orientation of the donor 2-acetamido group for the nucleophilic attack, leading to the formation of the oxazolinium ion intermediate (violet). This structure is then hydrolyzed to form a new glycosidic bond with the incoming acceptor (green). The mutation of Asn175 to Ala (red) (**B**) disables the formation of the oxazoline intermediate but the mutant can glycosylate using sugar oxazoline donors (violet).

4.2 *Thioglycoligases and Thioglycosynthases*

The substitution of the acid/base catalytic residue by another amino acid, mainly Ala and Gln, changes a retaining glycosidase into a thioglycoligase[76] (Scheme 3). These enzymes process activated donors, such as dinitrophenyl glycosides, with thiol acceptors. Thiosugars are strong nucleophiles that do not need activation by the acid/base catalytic residue to form the glycosidic linkage. Therefore, they are optimum acceptors for thioglycoligases, in which this residue is substituted, contrary to wild-type enzymes. The pioneer thioglycoligases, the Glu171Ala β-glucosidase from *Agrobacterium sp.* (β-thioglucoligase) and the Glu429Ala β-mannosidase Man2A from *Cellulomonas fimi* (β-thiomannoligase)[76] glycosylate thiosugars with up to 80% yields. An improved thioglycoligase was designed by Müllegger *et al.* by saturation mutagenesis; the Glu170Gln β-glucosidase from *Agrobacterium sp.* used β-D-glucopyranosyl azide as a glycosyl donor.[38] The same research group constructed the thermostable β-thioglucuronoligase from *Thermotoga maritima*[62] and used the thioglycoligase from *Agrobacterium* sp. for thioglycosylation of glycoproteins.[77] Kim *et al.*[78] created a library of thioglycosides, potential chaperones of lysosomal glycosidases, under catalysis by the β-thiogalactoligase from *Xanthomonas manihotis*. The thioglycoligase concept was further extended to thioglycosynthases, double mutants of retaining glycosidases. These enzymes lack both the catalytic nucleophile and the acid/base residue and they require a combination of a glycosyl fluoride donor and thiol acceptors[79] (Scheme 3).[57]

Thioglycoligases and thioglycosynthases are the only catalysts that can reliably synthesize thioglycosides – and these exclusively as β-anomers. Although some glycosidases, *e.g.* *O*-GlcNAcase (β-*N*-acetylglucosaminidase),[80] can efficiently cleave thioglycosides, there are no reports on the enzymatic synthesis of thiosugars. If so, they encompass solely simple thiols, such as thiopropane[81] or 2-mercaptoethanol.[82] Interestingly, the 6-phospho-β-glucosidase from *Thermotoga maritima* (GH 4, *http://www.cazy.org/*) can naturally cleave thioglycosides with a rate similar to that of corresponding *O*-glycosides.[83] This is probably because it uses a redox-elimination-addition catalytic mechanism that employs anionic transition states[21b] and not the classical retaining mechanism as described in Section 2. Thus, members of GH family 4 could become alternatives to thioglycoligases and thioglycosynthases in the preparation of thiosugars.

4.3 *Transglycosidases*

Through directed evolution[84] a library of randomly created mutant proteins is (automatically) screened for the required property. The best variants may then be further mutated and repeatedly screened to accumulate positive mutations. Contrary to glycosynthases, transglycosidases created by random mutagenesis process classical glycosyl donors (aryl glycosides) and they are still able to synthesize self-condensation products. For instance, the introduction of a double-mutation into the β-glycosidase from *Thermus thermophilus* resulted in a transglycosidase with a significantly improved transglycosylation performance.[85] In *E. coli* β-galactosidase, six random mutations induced a completely new β-D-fucosidase activity.[86]

5. Conclusion

The increasing need for complex glycostructures has stimulated great advances in glycosidase research, including new activities of glycosynthases as well as the use of ingeniously modified glyosidase substrates. Glycosidase catalysis is a simple, adaptable an green alternative.

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