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Mechanisms of glycosyl transferases and hydrolases

S.G. Withers*

Protein Engineering Network of Centres of Excellence of Canada and Departments of Chemistry and Biochemistry, The University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z1

Abstract

Glycosidases and glycosyl transferases fall into two major mechanistic classes; those that hydrolyse the glycosidic bond with retention of anomeric configuration and those that do so with inversion. There are, however, two classes of transferases: those that use nucleotide phosphosugars (NP-sugar-dependent) and those that simply transglycosylate between oligosaccharides or polysaccharides (transglycosylases). The latter are mechanistically similar to retaining glycosidases while the mechanisms of NP-sugar-dependent transferases are far from clear.

Retaining glycosidases and the transglycosylases employ a mechanism involving a covalent glycosyl–enzyme intermediate formed and hydrolysed with acid/base catalytic assistance via oxocarbenium ion-like transition states. This intermediate has been trapped on glycosidases in two distinct ways, either by modification of the substrate through fluorination, or of the enzyme through mutation of key residues. A third method has been developed for trapping the intermediate on transglycosylases involving the use of incompetent substrates that allow formation of the intermediate, but prohibit its transfer as a consequence of their acceptor hydroxyl group being removed.

Three-dimensional structures of several of these glycosyl–enzyme complexes, along with those of Michaelis complexes, have been determined through X-ray crystallographic analysis, revealing the identities of important amino acid residues involved in catalysis. In particular they reveal the involvement of the carbonyl oxygen of the catalytic nucleophile in strong hydrogen bonding to the sugar 2-hydroxyl for the β-retainers or in interactions with the ring oxygen for α-retainers. The glucose ring in the −1 (cleavage) site in the intermediates formed on several cellulases and a β-glucosidase adopts a normal $^4C_1$ chair conformation. By contrast the xylose ring at this site in a xylanase is substantially distorted into a $^{2,5}B$ boat conformation, an observation that bears significant stereoelectronic implications. Substantial distortion is also observed in the substrate upon binding to several β-glycosidases, this time to a $^1S_3$ skew boat conformation. Much less distortion is seen in the substrate bound on an α-transglycosylase.

Finally an efficient catalyst for synthesis, but not hydrolysis, of glycosidic bonds has been generated by mutation of the glutamic acid catalytic nucleophile of a β-glucosidase to an alanine. When used with α-glucosyl fluoride as a glycosyl donor, along with a suitable acceptor, oligosaccharides up to five sugars in length have been made with yields of up to 90% on individual steps. These new enzymes have been named Glycosynthases.

Keywords: NP-sugar-dependent; Transglycosylases; Glycosynthases

1. Introduction

In keeping with the wide variety of naturally occurring glycosidic bonds there exists a wide range of different enzymes, glycosidases, whose function is the cleavage of these bonds. Amino acid sequences of well over 1000 different glycoside hydrolases are now available, and these have been arranged into over 60 different families on the basis of sequence similarities (Henrissat & Bairoch, 1996) These sequences are easily accessible through a web-site that gives updated listings of these family members, plus information on the identities of important amino acid residues (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html). Three-dimensional (3D) structures are now available for representatives of at least 28 of these families and these, along with the family relationships, have been reviewed relatively recently (Davies & Henrissat, 1995; Henrissat, Callebaut, Fabrega, Lehn, Mornon & Davies, 1995). Monomer sizes for these enzymes range from approximately 14,000–170,000 Da, the enzymes also varying in their structural composition composition from essentially completely α-helical to almost exclusively β-sheet. This diversity is presumably a consequence both of the diverse nature of their substrates and also of different evolutionary solutions to the problem of constructing an active site capable of hydrolysing a glycosidic bond.

Enzymatic hydrolysis of glycosidic bonds is carried out with one of two stereochemical outcomes; net retention or net inversion of anomeric configuration, thus

* Tel.: +1-604-822-3402; fax: +1-604-822-2847.
E-mail address: withers@chem.ubc.ca (S.G. Withers).

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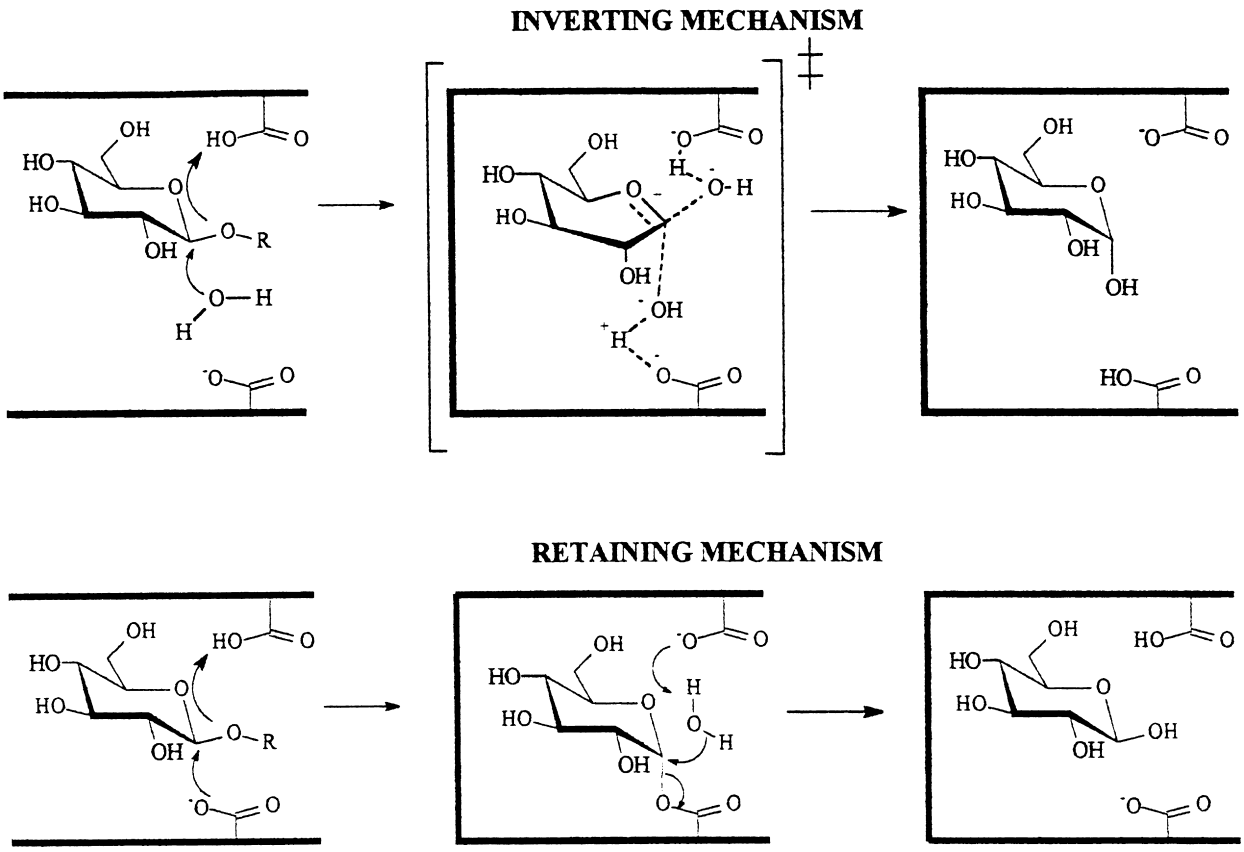


Fig. 1. Mechanisms of retaining and inverting glycosidases.

glycosidases are classified as either retaining or inverting. Basic mechanisms for these processes were proposed in 1953 by Koshland (1953), and these have been elaborated upon in the interim both mechanistically, and particularly structurally, as shown in Fig. 1. The inverting glycosidases use a direct displacement mechanism through an oxocarbenium ion-like transition state. Two carboxylic acids at the active site are positioned approximately 11 Å apart (McCarter & Withers, 1994; Wang, Graham, Trimbur, Warren & Withers, 1994; White, Withers, Gilkes & Rose, 1994) such that one provides base catalytic assistance to the attack of water while the other provides acid catalytic assistance to cleavage of the glycosidic bond.

Catalysis by retaining glycosidases and transglycosylases proceeds via a double-displacement mechanism in which a covalent glycosyl–enzyme intermediate is formed and hydrolysed via oxocarbenium ion-like transition states. The active site again contains a pair of carboxylic acids, but in this case they are approximately 5.5 Å apart. Acid/base catalysis is also important for this enzyme class, but in this case is provided by a single carboxyl group at the active site functioning as an acid catalyst for the first (glycosylation) step and as a base catalyst for the second (deglycosylation) step. Background information on these mechanisms, and references to the original publications can be found in the following reviews (Sinnott, 1990; Davies et al., 1998; Withers, 1999; Zechel & Withers, 1999). We have also recently published a review on mutagenesis of glycosidases and glycosyl transferases (Ly & Withers, 1999).

2. Trapping intermediates: retaining β-glycosidases

Our first approaches to the trapping of the glycosyl–enzyme intermediate were carried out on retaining glycosidases and involved modification of the substrate as a means of selectively slowing the second (deglycosylation) step. This was achieved by replacement of the 2-hydroxyl of the substrate with a fluorine, thereby inductively destabilising *both* the positively charged transition states. This substitution also removed a key hydrogen bonding substituent, since interactions with the 2-position hydroxyl have been shown to contribute up to 10 kcal/mol to transition state stabilisation (Wolfenden & Kati, 1991; Namchuk & Withers, 1995; McCarter, Adam & Withers, 1992), thereby also destabilising the transition states. In order to render the intermediate kinetically accessible a reactive leaving group such as dinitrophenolate or fluoride was incorporated into the substrate, thereby accelerating the glycosylation step, but not deglycosylation. These function as shown in Fig. 2, forming an intermediate that is surprisingly stable,

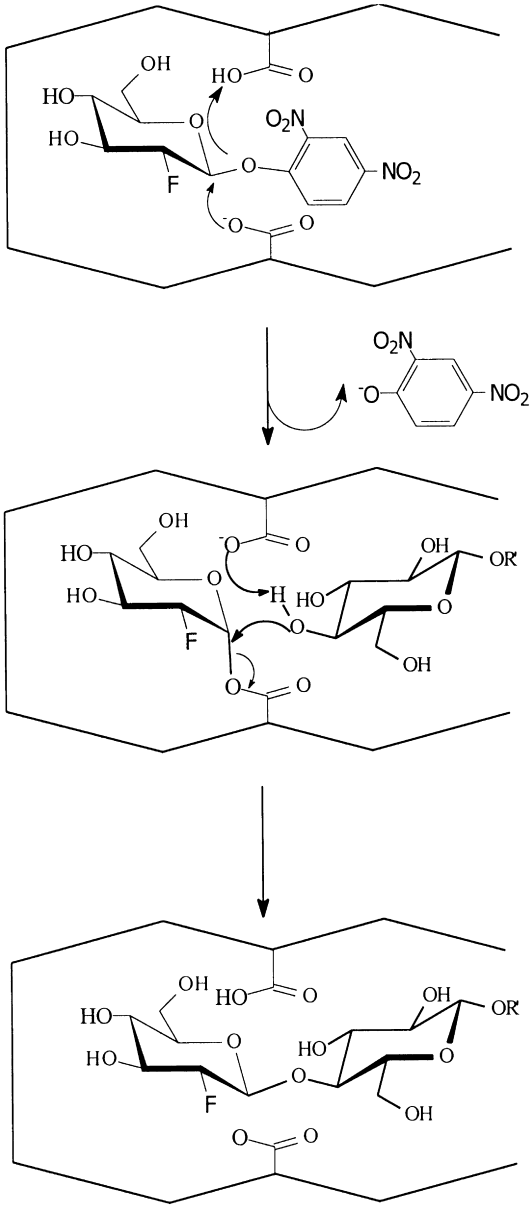
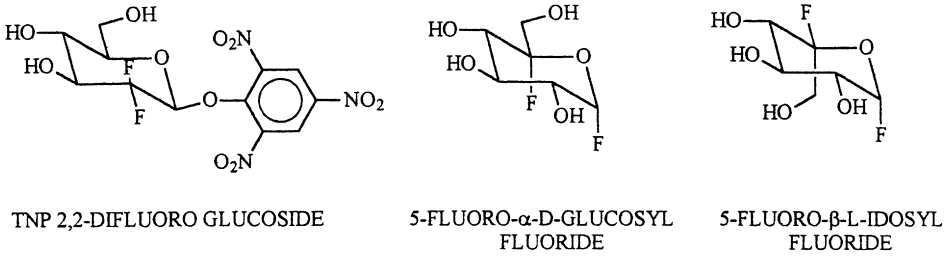


Fig. 2. Inactivation and reactivation with 2-fluoroglycosides.

typical half-lives for hydrolytic turnover being measured in days. However, in the presence of a suitable sugar, reactivation is much faster, occurring by a more efficient transglycosylation reaction (Fig. 2). These fluorosugar reagents have proved extremely valuable for unequivocally identifying the catalytic nucleophiles in retaining glycosidases since they very specifically tag the amino acid of interest. This is achieved via proteolysis of labeled enzyme, then HPLC separation of the resultant mixture using a tandem quadrupole mass spectrometer to analyse the eluate. Particularly valuable has been the use of a neutral loss scan to localise the glycosylated peptide, this technique relying upon the facile fragmentation of the ester bond between the sugar and the peptide. The sequence of the peptide is then determined by further MS/MS analysis, or by Edman degradation of the purified material. In this way the identities of the catalytic nucleophiles in a large number of the families of retaining glycosidases have now been determined (Withers & Aebersold, 1995; McCarter & Withers, 1996; Mackenzie, Davies, Schulein & Withers 1997; McCarter, Burgoyne, Miao, Zhang, Callahan & Withers 1997; Tull, Miao, Withers & Aebersold, 1994). See the following publications and reviews for leading references to this approach (Zechel & Withers, 1999; Withers & Aebersold, 1995; Withers & Street, 1988; Street, Kempton & Withers 1992).

3. Trapping intermediates: retaining α-glycosidases and transglycosylases

Interestingly the 2-fluoro inactivators proved to be relatively ineffective as inactivators of α-glycosidases, serving rather as slow substrates. This necessitated the development of alternative approaches, one of which involved the use of a 2-deoxy-2,2-difluoro glycoside, the idea being that the presence of a second fluorine would ensure that turnover would occur only *much* more slowly. Even more reactive leaving groups are then required in order to allow accumulation of the intermediate, thus trinitrophenyl derivatives such as that shown in Fig. 3 were synthesised and shown to be effective in trapping intermediates on an α-glucosidase as well as on human pancreatic α-amylase (Braun, Brayer & Withers, 1995). Another approach involved substituting the fluorine at the 5-position, close to the endocyclic oxygen, where most of the charge accumulates at the transition state (McCarter & Withers, 1996). Thus compounds such as 5-fluoro-α-D-mannosyl fluoride and 5-fluoro-β-L-gulosyl fluoride were shown to trap an intermediate on jack bean α-mannosidase and were used to identify the catalytic



TNP 2,2-DIFLUORO GLUCOSIDE

5-FLUORO-α-D-GLUCOSYL FLUORIDE

5-FLUORO-β-L-IDOSYL FLUORIDE

Fig. 3. Structures of inactivators.

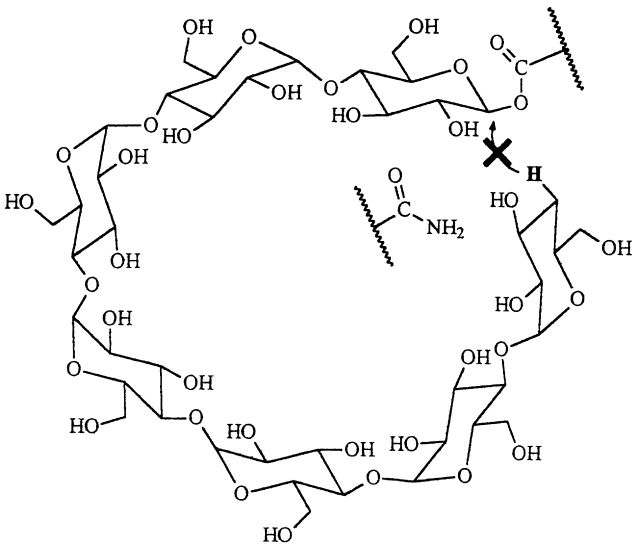


Fig. 4. Trapping the intermediate on CGTase.

nucleophile through proteolysis of the labeled enzyme and LC/MS-MS analysis of the peptide mixture produced (Fig. 3) (Howard, He & Withers, 1998). Similar experiments were performed with 5-fluoro-α-D-glucosyl fluoride and 5-fluoro-β-L-idosyl fluoride on the yeast α-glucosidase (McCarter & Withers, 1996). The compounds of opposite anomeric stereochemistry were shown to be equally effective at trapping intermediates on β-glycosidases.

Another approach to trapping the intermediate was developed for transglycosylases. This was first applied to the transferase activity of glycogen-debranching enzyme (Braun, Lindhorst, Madsen & Withers, 1996) and has since been used with the mechanistically simpler cyclodextrin glucanotransferase (CGTase) (Uitdehaag et al., 1999; Mosi, He, Uitdehaag, Dijkstra & Withers, 1997). The CGTase case will be used to illustrate the approach since this system has also proved amenable to structural analysis. CGTase catalyses the conversion of starch into cyclodextrins through an intramolecular transglycosylation reaction involving a covalent glycosyl–enzyme intermediate. Others had noted earlier (Hehre, Mizokami & Kitahata, 1983; Cottaz, Apparu & Driguez, 1991) that this enzyme could use glycosyl fluorides as alternate substrates, yielding longer malto-oligosaccharyl fluorides and cyclodextrins as products. Reaction was assumed to occur via a glycosyl–enzyme intermediate that undergoes attack by a second bound substrate molecule. We confirmed this interpretation and carried out a kinetic analysis of this process with a series of mutants (Mosi et al., 1997; Mosi, Sham, Uitdehaag, Ruiterkamp, Dijkstra & Withers, 1998). More importantly however, we reasoned that a glycosyl fluoride substrate in which the non-reducing end 'nucleophilic' hydroxyl group had been removed by deoxygenation should be able to carry out the first step in catalysis, formation of the intermediate, but would not undergo the second transfer step since the requisite nucleophilic group was missing. This approach is illustrated in Fig. 4. When tested as a substrate, 4 deoxymaltotriosyl α-fluoride (4DG3αF) was found to react slowly ($k_{cat} = 2\ s^{-1}$) compared to maltotriosyl fluoride ($k_{cat} = 275\ s^{-1}$), but with a much lower K_m value (0.027 versus 2.5 mM), as expected if the intermediate is accumulating. However the turnover was faster than expected, and HPLC analysis revealed that this was due to hydrolysis. In order to slow down this hydrolysis reaction to provide a sufficiently stable intermediate for peptide mapping work and X-ray crystallographic analysis of the trapped intermediate, the experiment was repeated with a mutant of the enzyme modified at the acid/base catalyst (Glu257Gln). The concept here was that the intermediate should still form rapidly since the fluoride leaving group needs no significant acid catalytic assistance. However turnover of this intermediate should be slowed due to the removal of the general base catalyst. This turned out to be the case, the kinetic parameters for cleavage of 4DG3αF being $k_{cat} = 0.6\ s^{-1}$ and $K_m = 0.027$ mM and turnover now being slow enough to permit observation of the intermediate by electrospray ionization mass spectrometry (74,984 versus 74,513 Da for the free enzyme, the difference of 471 being that expected for a 4-deoxymaltotriosyl moiety). Proteolysis using pepsin followed by HPLC/MS-MS analysis in the neutral loss mode allowed the localisation of two peptides (1679 and 1809 Da) which bore the trisaccharide moiety. Purification of these peptides and sequencing via Edman degradation identified these as overlapping peptides containing the residue identified as the catalytic nucleophile, Asp229. This residue is completely conserved within family13 and had previously been suggested to function in this role on the basis of 3D structures and labeling of other hydrolytic family members (Mosi et al., 1997).

4. Structural studies along the reaction coordinate

Excellent insights into catalysis have come from the determination of the 3D structures of several covalent glycosyl–enzyme intermediates along with structures of enzyme/substrate (Michaelis) complexes in several cases. The 2-deoxy-2-fluorocellobiosyl-enzyme intermediate formed on the exo-xylanase/cellulase Cex from *Cellulomonas fimi* was the first such structure solved, in collaboration with Dr. David Rose (Ontario Cancer Institute, Toronto) (White, Tull, Johns, Withers & Rose, 1996). Interestingly, no significant structural change was observed within the protein upon formation of the glycosyl–enzyme, this enzyme seemingly acting as a relatively rigid cavity preorganised for catalysis. The structure revealed the identities of the amino acid side chains involved in binding and catalysis, including those interacting with the 6-hydroxyl and therefore likely responsible for determining the cellulose/xylan specificity (Fig. 5). Subsequent determination of the structure of the 2-deoxy-2-fluoroxylobiosyl–enzyme revealed that two of the residues that had moved position

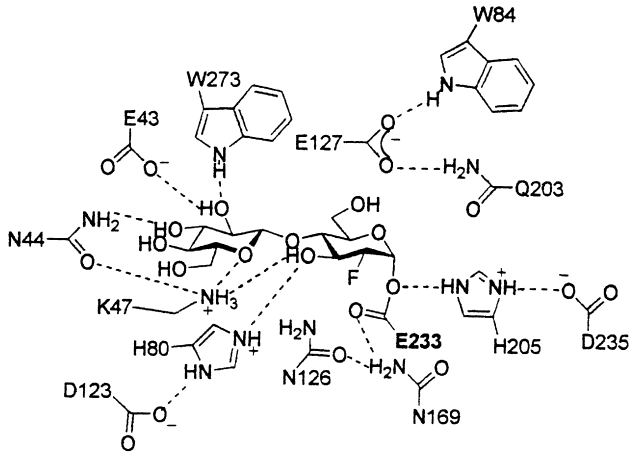


Fig. 5. Schematic of the active site of 2-fluorocellobiosyl-Cex. Adapted from White et al. (1996).

slightly in the fluorocellobiosyl–enzyme complex no longer moved, consistent with the higher specificity for xylan (Notenboom, Birsan, Warren, Withers, & Rose, 1998). However, surprisingly, when mutations at one of these positions were made there was no significant effect upon the cellulose/xylan specificity.

These structures also provided insights into the source of the very strong interactions (>10 kcal/mol) previously shown to be formed with the 2-position of the natural substrate at the transition state (White et al., 1996). The residues closest to the fluorine of the substrate moiety are the highly conserved Asn126 and the carbonyl oxygen of the nucleophile itself. Subsequent mutational studies have shown that interactions with the amide moiety of Asn126 contribute only approximately 2.5 kcal/mol to the stability of the transition state, thus interaction with the charged nucleophile itself seems the most likely source of the majority of this large interaction energy (V. Notenboom, Unpublished Results). In the actual structures determined, the fluorine at the 2-position will not, of course, form a significant hydrogen bond, thus a structure including a 2-hydroxyl is needed to prove that such an interaction could be significant. This required mutating the enzyme instead of modifying the substrate.

Use of a single mutant in which the acid/base catalyst Glu127 was mutated to Ala, in conjunction with a 2,4-dinitrophenyl glycoside substrate with an excellent leaving group, allowed accumulation of the intermediate. However turnover was still too fast for crystallographic analysis. Similar results were obtained with a second mutant in which the histidine residue (His 205) that hydrogen bonds to the catalytic nucleophile, Glu233, was mutated to Asn and Ala. Success was attained through creation of the double mutant His205Asn/Glu233Ala, which relatively rapidly formed a cellobiosyl–enzyme complex when incubated with 2,4-dinitrophenyl cellobioside. Turnover of this intermediate, however, occurred only on a time scale of weeks. The catalytic domain of the Cex double mutant so trapped was then crystallised and its 3D structure solved to 1.8 Å. Some small changes were seen in the positioning of the nucleophile itself and in the hydrogen bonding patterns as a consequence of the mutations, but the overall structure was very similar to that of the wild type enzyme in its 2-deoxy-2-fluorocellobiosyl-enzyme form (Notenboom et al., 1998). Most interestingly though, the carbonyl oxygen of the nucleophile was involved in a short (2.37 Å) hydrogen bond with the sugar 2-hydroxyl. This short distance, and the fact that very strong interactions (>10 kcal/mol) are known to be formed with the 2-hydroxyl at the transition state, suggests that the hydrogen bond formed at this position is a major contributor to transition state stabilisation. Obviously the interaction seen in this structure is not that at the transition state, but being a reactive intermediate it presumably resembles it to some extent. The suggestion is that this interaction is optimised at the transition state both geometrically, as the ring flattens, and electronically, as the positive charge develops at the anomeric centre, acidifying the 2-hydroxyl and thereby increasing its hydrogen bond donor capability, as shown in Fig. 6.

Another interesting glycosyl–enzyme intermediate structure to be solved was that of the 2-deoxy-2-fluoroxylobiosyl–enzyme intermediate formed on the xylanase from *Bacillus circulans* (Sidhu, Withers, Nguyen, McIntosh, Ziser & Brayer, 1999). The interest in this case centres around the fact that the sugar covalently bound to the nucleophile (Glu78) is distorted into a (Davies & Henrissat, 1995; McCarter & Withers, 1994) B conformation, quite unlike the $^4C_1$ conformations seen in all other cases. Such a conformation is mechanistically interesting since it places C-5, O-5, C-1 and C-2 in a plane, just as is required for the generation of double bond character between O-5 and C-1 at the transition state, Fig. 7. Through such distortion, therefore, the enzyme must significantly assist the formation and hydrolysis of this intermediate. Such a conformation is

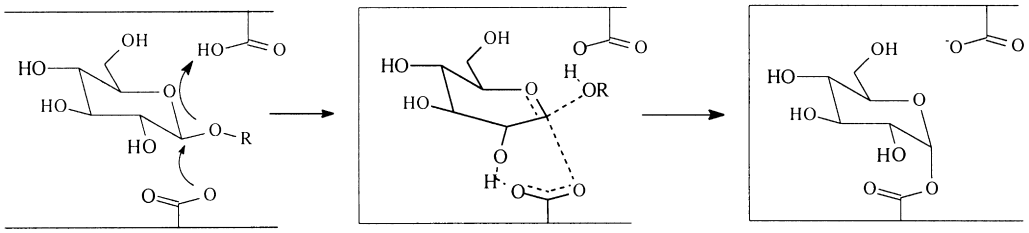


Fig. 6. Hydrogen bonding at the transition state.

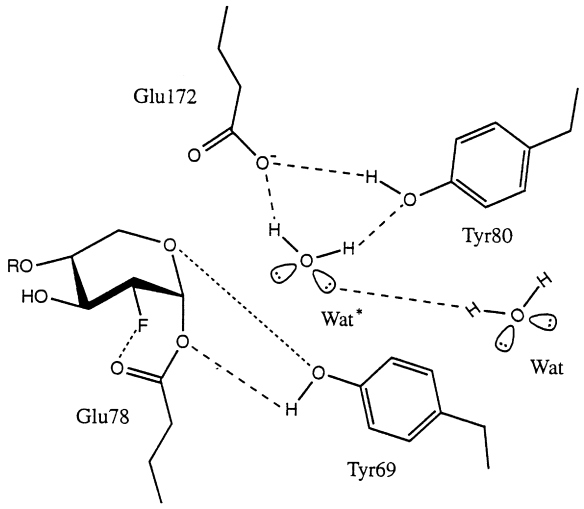


Fig. 7. The 2-deoxy-2-fluoroxylobiosyl-enzyme intermediate formed on the *Bacillus circulans* xylanase. Adapted from Sidhu et al. (1999).

much more readily accessible for *xylo* sugars than for *gluco* sugars since they do not have the bulky hydroxymethyl substituent at C-5 that would be forced axial for a *gluco* sugar, thereby resulting in significant strain due to "bowsprit" interactions within the sugar, and, in this case, substantial steric clashes with the enzyme. Other interesting observations in this complex include the close approach of the fluorine at C-2 to the carbonyl oxygen of the nucleophile, and an ordered water molecule held in place for nucleophilic attack at the sugar anomeric centre through hydrogen bonding interactions with the acid/base catalyst (Glu172) and the conserved Tyr80. In addition there is a surprisingly close approach (2.95 Å) of the hydroxyl group of another conserved residue (Tyr69) to the sugar ring oxygen. Tyr 69 also comes close to the 'ether' oxygen of Glu78 in its ester linkage with the sugar, thus may be involved in a bifurcated hydrogen bond between the two oxygen atoms. Mutation of Tyr69 to Phe leads to complete loss of enzyme activity (Wakarchuk, Campbell, Sung, Davoodi & Yaguchi, 1994), indicating a very important role for this hydroxyl group. It seems probable that as the glycosidic bond in this intermediate cleaves, with generation of negative charge on Glu78 and positive charge on the sugar ring oxygen, the symmetry of this H-bonding situation is broken and a full H-bond develops from Glu78 to Tyr69, while a stabilising electrostatic or dipolar interaction develops between the sugar O-5 ($\delta +$) and the hydroxyl group of Tyr69 ($\delta -$).

Structural studies, in collaboration with Dr. Gideon Davies on the endoglucanase Cel5A from *Humicola insolens* have provided "snapshots" of this enzyme at every stable state along the reaction coordinate diagram: free enzyme, Michaelis complex, covalent intermediate and product complex (Davies et al., 1998) (Fig. 8). The native enzyme, whose pH optimum is around 7.0, was crystallised at pH 4.5, and the structure solved to 0.95 Å resolution. Using the very slow substrate 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-cellobioside in conjunction with the sub-optimal pH, the structure of the Michaelis complex was solved, revealing substantial distortion of the sugar bound in the -1 site to a $^{1}S_{3}$ skew boat. This conformation places the leaving group dinitrophenyl moiety in a pseudo-axial orientation, consistent with the dictates of stereoelectronic theory, and should serve to facilitate the formation of the planar transition state structure. It also removes the barrier to attack of the nucleophile at the anomeric centre arising from 1,3-diaxial interactions present in the $^{4}C_{1}$ conformation. The structure of the covalent intermediate was solved by reacting the enzyme with 2,4-dinitrophenyl 2-deoxy-2-fluoro β-cellobioside (and also the cellotrioside) at pH 7, then lowering the pH to 4.5 and crystallizing the enzyme in that form. The sugar intermediate was clearly seen in an undistorted $^{4}C_{1}$ conformation, much as had been seen with Cex.

A final example to be discussed is that of a family 13 transglycosylase, the CGTase from *Bacillus* sp., for which both Michaelis and covalent glycosyl–enzyme complexes have been solved by Uitdehaag et al. (1999) in collaboration with the group of Dr. Bauke Dijkstra, Fig. 9. The structure of the Michaelis complex was solved by soaking oligosaccharides into crystals of an essentially inactive double mutant (D229N/ E257Q) in which both the nucleophile and the acid/base catalyst, respectively, have been mutated. This structure revealed a nonasaccharide bound across the active site and occupying subsites -7 to $+2$. The sugar bound in the -1 site appears to be slightly distorted with a flattening of the C2–C1–O5–C5 torsion angle towards the planarity expected at the transition state. This distortion appears to be caused, at least in part, by residues Asp328, which bridges O2 and O3 of the -1 site sugar, and His140, which forms a buried hydrogen bond with O6 of this same sugar residue.

The structure of the covalent glycosyl–enzyme intermediate was solved by soaking into the crystal the incompetent substrate analogue 4-deoxy-maltotriosyl fluoride described earlier. Crystallographic analysis of the complex was hindered at first by the fact that CGTase is crystallised in the presence of high concentrations of maltose, which would act as an acceptor for the 4-deoxymaltotriosyl–enzyme trapped, thereby reactivating the enzyme. Exchanging the maltose in the crystals with 4-deoxymaltose prior to addition of 4DG3αF solved this problem and collection of data under these conditions led to the determination of the structure of the intermediate; the first such structure to be determined for an α-retaining enzyme. Of particular note is the fact that the β-linked sugar in the -1 site is bound in a normal $^{4}C_{1}$ chair conformation. This absence of any distortion is again contrary to what might be expected on the basis of stereoelectronic theory. Also of particular interest is the finding that the carbonyl oxygen of the nucleophile (Asp229) is located only 2.7 Å from the sugar ring oxygen. This must be a destabilising interaction since there is no proton to be shared between the two atoms. However, at the transition states flanking this intermediate a strongly stabilising electrostatic interaction may well develop

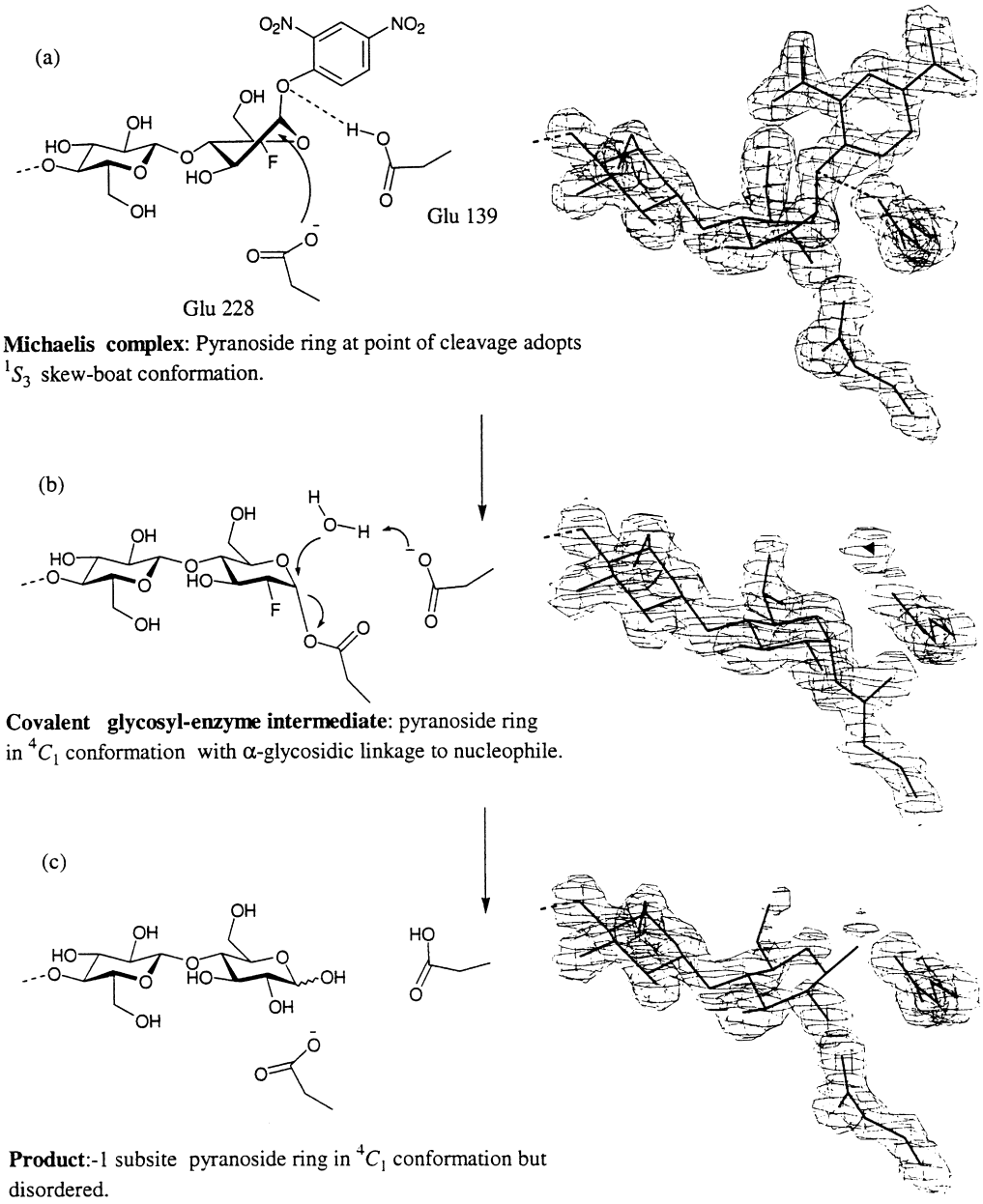


Fig. 8. Snapshots along the reaction coordinate of Cel5A. Structures of the Michaelis complex, the glycosyl-enzyme and the product complex. Adapted from Davies et al. (1998).

between the partially positive ($\delta +$) sugar ring oxygen and the partially negative ($\delta -$) 'carbonyl' oxygen of the nucleophile (Asp229). This interaction, which is reminiscent of that between the sugar ring oxygen and Tyr69 in the xylanase described earlier, may contribute very substantially to catalysis.

In summary, therefore, these structural studies of bound species have provided another level of understanding of how catalysis is effected by glycosidases and transglycosylases. The theme that emerges is one in which the enzyme provides a pre-organised cavity in which the substrate can optimally undergo bonding rearrangement. Very little change occurs in the enzyme structure, though the substrate can be orchestrated through substantial conformational changes. Through a series of precisely placed interacting groups the enzyme disperses charge at the transition state, both through a hydrogen bonding network and through electrostatic interactions. The carbonyl oxygen of the nucleophile appears to play a particularly important role, interacting with the 2-hydroxyl of retaining β-glucosidases and with the ring oxygen of the retaining α-glycosyl transferases and, by inference of α-glycosidases. This is consistent with the reality that substantial changes in electron density must occur at this centre between the ground and transition states, thus the enzyme has evolved to optimally capitalise upon these changes.

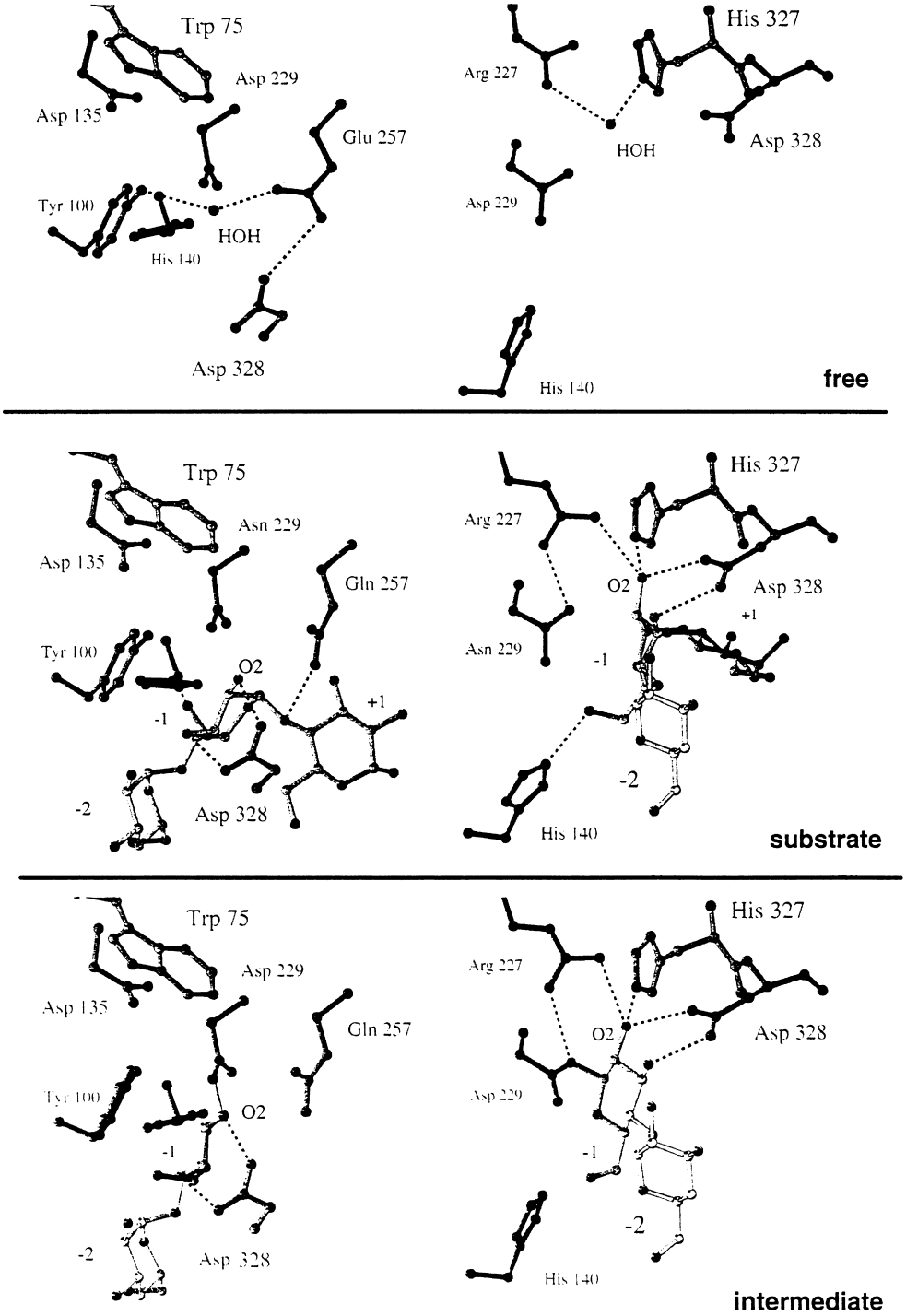


Fig. 9. Snapshots along the reaction coordinate of CGTase. Structures of the Michaelis complex, the glycosyl-enzyme and the product complex. Adapted from Uitdehaag et al. (1999).

5. Glycosynthases: mutant glycosidases for oligosaccharide synthesis

These and other mechanistic insights have been put to use in the generation of a new class of mutant enzyme for the synthesis of oligosaccharides. Despite considerable interest in the use of oligosaccharides as possible therapeutics (Sears & Wong, 1996; Simon, 1996; Zopf & Roth, 1996) progress has been slow due, in part, to the difficulties attendant in the large scale synthesis of such molecules. Attention has therefore turned to the use of enzymes, with Nature's own catalysts, the glycosyl transferases, providing an obvious

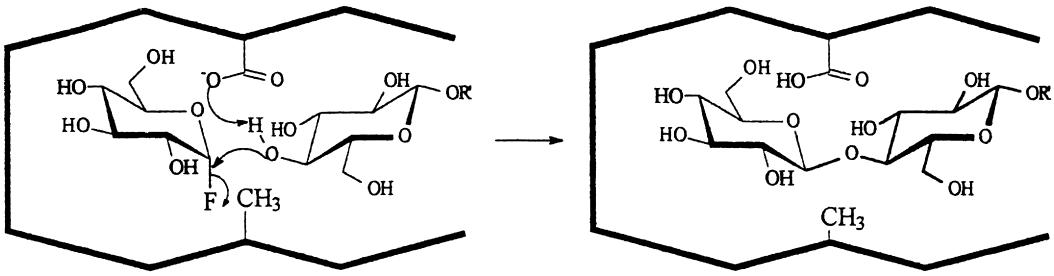


Fig. 10. Mechanism of glycoside synthesis with a Glycosynthase. Adapted from Mackenzie et al. (1998).

approach (Wong, Halcomb, Ichikawa & Kajimoto, 1995; Thiem, 1995; Ichikawa, Look & Wong, 1992a; Ichikawa et al., 1992b). The high cost of the nucleotide diphosphosugars is still a problem with this approach, but one that is slowly being overcome as new strains of bacteria that overproduce these reagents are developed.

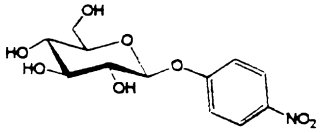
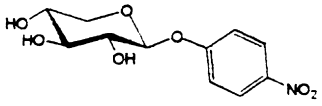
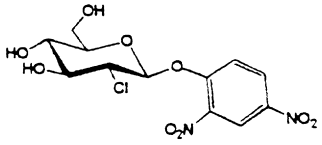
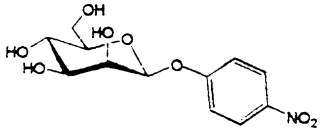
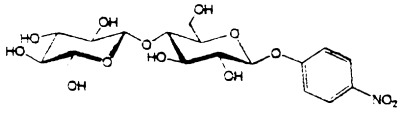
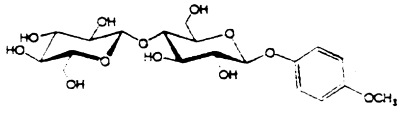
Glycosidases run 'in reverse' are another alternative; indeed one that has been pursued for over 85 years (Thiem, 1995; Crout & Vic, 1998; Watt, Lowden & Flitsch, 1997; Bourquelot & Bridel, 1912; Nilsson, 1988). The equilibrium approach involves mixing very high concentrations of the sugars of interest in the presence of enzyme, and allowing the reaction to approach equilibrium; typically with very low yields and complex product mixtures. The more useful kinetic approach involves the use of a reactive glycosyl donor to generate a steady state concentration of the glycosyl–enzyme intermediate, which can be intercepted with a second sugar acceptor molecule rather than water. This approach tends to yield more specific products, and better yields, but these yields are still typically rather low unless some method is used to limit product hydrolysis. Unfortunately most of these methods are specific to the product in question, involving, for example, selective extraction or crystallisation or the use of some coupled assay system to continuously remove product. Our approach to this problem was to apply the methods of protein engineering to generate a mutant that could carry out the desired glycosyl transfer reaction, but which could not hydrolyse these products once formed (Mackenzie, Wang, Warren & Withers, 1998).

The best method for completely eliminating activity in a retaining glycosidase through a single mutation involves mutation of the catalytic nucleophile to a non-nucleophilic residue such as alanine, carefully prepared mutants having maximal rates at least 10^5 times lower than the wild type enzyme (Wang et al., 1994). CD measurements, and even X-ray crystallographic analyses in several cases (Brayer, Rose & Withers, Unpublished results) have revealed that such mutants fold correctly, thus have an active site capable of binding both the glycone and aglycone moieties. This active site should be optimised for stabilization of the oxocarbenium ion-like transition state, although of course it lacks the negative charge from the carboxylate. It also contains an acid/base residue which, due to the absence of a charged nucleophile, is likely in the deprotonated form, therefore is optimally set up as a base catalyst (McIntosh et al., 1996). The mutant enzyme is therefore set up to carry out the transglycosylation step, but of course has not formed a reactive α-glycosyl–enzyme intermediate so cannot do so. However, if an α-glycosyl fluoride is used as glycosyl donor, chemically mimicking the reactive α-glycosyl–enzyme intermediate, then transfer can be effected to a suitable acceptor, as shown in Fig. 10. The small fluorine substituent is nicely accommodated within the pocket created by the mutation: indeed α-glycosyl fluorides do *not* bind to the wild type enzyme due to steric conflicts with the glutamate nucleophile (Mackenzie et al., 1998).

The β-glucosidase/galactosidase from *Agrobacterium sp.* (Abg), a Family 1 β-glycosidase with broad specificity that has been studied extensively in our group (Namchuk & Withers, 1995; Withers & Street, 1988; Street et al., 1992; Kempton & Withers, 1992) served as the test-bed for this approach, reaction being performed in high buffer concentrations (typically150 mM sodium phosphate, or ammonium bicarbonate) in order to neutralise the HF released stoicheometrically. As an example, on the 2 ml scale, the Glu358Ala mutant of Abg was incubated with α-galactosyl fluoride (40 mM) as donor and *p*-nitrophenyl β-cellobioside (30 mM) as acceptor in the presence of AbgGlu358Ala (0.01 mg) in 150 mM ammonium bicarbonate buffer. Reaction was complete within 8 h and was worked up by ultrafiltration to remove the enzyme (which can be re-used). Lyophilisation, then HPLC purification, yielded 34 mg (92% isolated yield) of Gal-β-1,4-Glu-β-1,4-Glu-β-*p*-nitrophenyl.

As is shown in Table 1, the mutant is capable of transferring galactose residues to a range of different acceptors, the products formed being themselves poor acceptors for the enzyme, thus reaction terminates after a single transfer. α-Glucosyl fluoride also acts as an excellent donor, but since the products are also excellent acceptors a series of longer oligomers is produced. The size of the oligosaccharide product can be controlled to some extent through the number of equivalents of donor employed, as shown in Table 2. A specific example, which illustrates the power of this approach, involves the synthesis of some 2-deoxy-2-fluorocello-oligosaccharide glycosides as potential cellulase inactivators. Incubation of AbgGlu358Ala with

Table 1
Glycosynthase-catalyzed transglycosylations using α-galactosyl fluoride as donor. Taken from Mackenzie et al. (1998)

#	Acceptor	Products (% yield) β-1,4 linked(unless otherwise stated)
Disaccharide		
1		84
2		81 (β-1,3 linked)
3		64
4		66
Trisaccharide		
5		92
6		88

2,4-dinitrophenyl 2-deoxy-2-fluoro β-glucoside plus 1.5 equivalents of α-glucosyl fluoride yielded an intentional mixture of 2,4-dinitrophenyl 2-deoxy-2-fluoro β-cellobioside and cellotrioside (84% total yield) that was readily resolved by HPLC. This yield is considerably better than any we had been able to achieve using classical synthetic approaches. In addition, it is noteworthy that this reaction could not be carried out by standard glycosidase transglycosylation since the 2-fluorosugar would have inactivated the enzyme, a process that is not possible with the mutant.

This new approach has now been reproduced in several other laboratories with different enzymes (Malet & Planas, 1998; H. Driguez, personal communication; M. Moracci, personal communication), thus appears to have considerable potential. Our current efforts are focussing upon the extension of this approach to a range of glycosidases in order to generate catalysts for the synthesis of a range of glycosides, particularly those of pharmaceutical interest.

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Table 2
Glycosynthase-catalyzed transglycosylations using α-glucosyl fluoride as donor. Taken from Mackenzie et al. (1998)

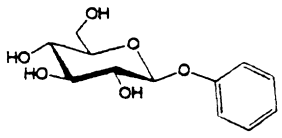
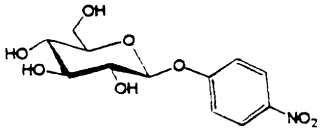
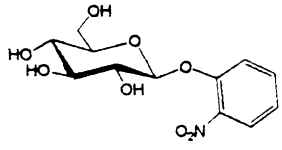
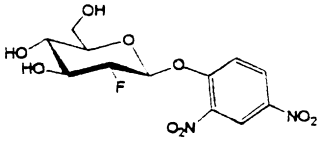
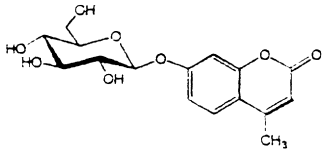
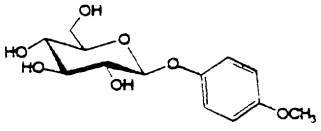
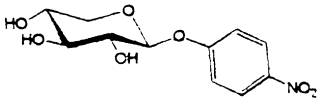
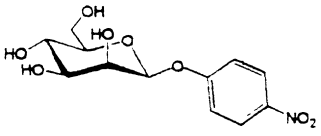
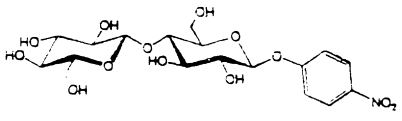
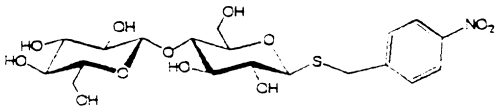
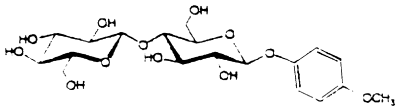
#	Acceptor	Products (% yield) β-1,4 linked (%)			
		Disaccharide	Trisaccharide	Tetrasaccharide	Total yield[a]
7		48	34	–	82
8		38	24	10	72
9		41	29	6	76
10		38	42	4	84
11a		–	75*	8%*	83*
11b			23*	54*	77*
12a		44	29	–	73
12b		12*	66*	8*	86*
13		12	51	3	66 (β-1,3 linked)
14		31	42	6	79
15a			79	13	92

Table 2 (*continued*)

#	Acceptor	Products (% yield) β-1,4 linked (%)			
		Disaccharide	Trisaccharide	Tetrasaccharide	Total yield[a]
15b			8*	64*	72*
16			64	21	85
17			59	12	71

[a] Reactions performed with 1–1.4 equivalents or (*) with 2.2–3.0 equivalents of glycosyl donor.

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