Glycosidase Mechanisms: Anatomy of a Finely Tuned Catalyst

DAVID L. ZECHEL AND STEPHEN G. WITHERS*
Department of Chemistry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z1

Received June 14, 1999

ABSTRACT

In order to accelerate the hydrolysis of glycosidic bonds by factors approaching 10^{17}-fold, glycosidases have evolved finely tuned active sites optimally configured for transition-state stabilization. Structural analyses of various enzyme complexes representing stable intermediates along the reaction coordinate, in conjunction with detailed mechanistic studies on wild-type and mutant enzymes, have delineated the contributions of nucleophilic and general acid/base catalysis, as well as the roles of noncovalent interactions, to these impressive rate enhancements.

The glycosidic bond, particularly that between two glucose residues, is the most stable of the linkages within naturally occurring biopolymers, with half-lives for spontaneous hydrolysis of cellulose and starch being in the range of 5 million years.[1] Enzymes responsible for the hydrolyses of these materials therefore face a challenging task, yet they accomplish this with rate constants up to 1000 s^{-1}, earning them a reputation as some of the most proficient of catalysts. This Account will focus upon the current view of how this class of enzymes achieves these extraordinary rate enhancements. The review is not intended to be exhaustive: for that the reader is referred to other sources.[2−8] Rather this review will focus primarily upon mechanisms of hydrolysis of β-glycosides, with an emphasis on studies of the *Agrobacterium* sp. β-glucosidase (Abg), the *Cellulomonas fimi* exoglycanase (Cex), and the *Bacillus circulans* xylanase (Bcx). However, most of the principles elaborated should be common to all glycosidases.

There has been a veritable explosion of structural information on glycosidases in recent years both in terms of sequences and in terms of three-dimensional structures. The predicted amino acid sequences of well over 2000 different glycoside hydrolases are now available, and, at last count, these were divided into 76 different families on the basis of sequence similarities, this information being currently available on an excellent Web site (http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html)[9] and having been reviewed recently.[10] Three-dimensional structures have now been determined for representatives of at least 30 of these families, revealing incredible structural diversity despite the fact that all these enzymes catalyze the same reaction, hydrolysis of an acetal. Nonetheless, some families adopt similar folds and on this basis have been assigned to so-called "clans", as also reviewed recently.[4,11] However, many active-site features, such as a pair of carboxyl groups, are retained throughout, as will become apparent.

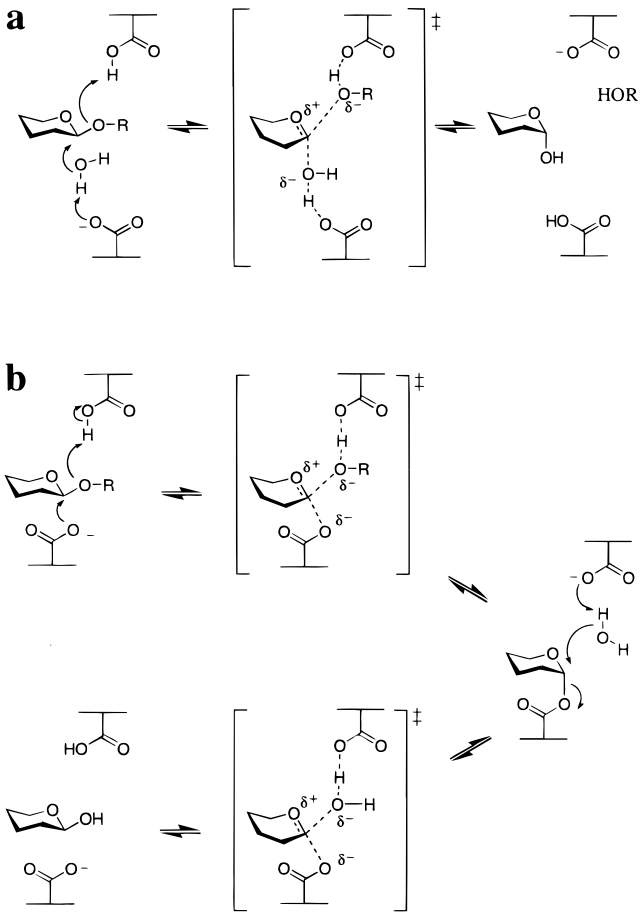

FIGURE 1. General mechanisms for inverting (a) and retaining (b) glycosidases.

Hydrolysis of the glycosidic bond can occur with one of two possible stereochemical outcomes: inversion or retention of anomeric configuration. This observation demands (at least) two different mechanisms (Figure 1). However, all enzymes within a sequence-related family appear to catalyze reactions with the same stereochemical outcome.[12]

The two carboxyl groups in inverting glycosidases serve as general acid and general base catalysts and are suitably placed, some 10.5 Å apart on average,[2,13] to allow the substrate *and* a water molecule to bind between them (Figure 1a). Reaction occurs via a single-displacement

Steve Withers received his B.Sc. (1974) and Ph.D. (1977) degrees in chemistry from the University of Bristol. He then moved to the Biochemistry Department at the University of Alberta as a postdoctoral fellow, where he was able to indulge his passion for whitewater kayaking in the Rocky Mountain rivers. He has since moved to the saltwaters of the West Coast, where he holds the Khorana Chair of Chemistry and Biochemistry at the University of British Columbia.

David Zechel received his B.Sc. from the University of Toronto in 1995. He is currently working toward his Ph.D. degree at the University of British Columbia under the supervision of Steve Withers. His research interests lie in the application of physical and bioorganic chemistry toward the understanding of enzyme mechanisms.

* To whom correspondence should be addressed. E-mail: withers@chem.ubc.ca.

10.1021/ar970172+ CCC: $19.00
Published on Web 11/18/1999

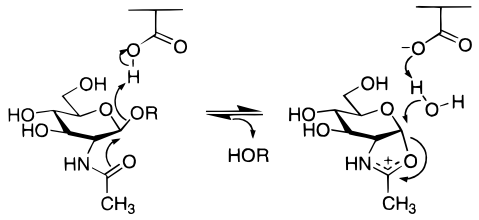

FIGURE 2. General mechanism for retaining β-*N*-acetylhexosaminidases.

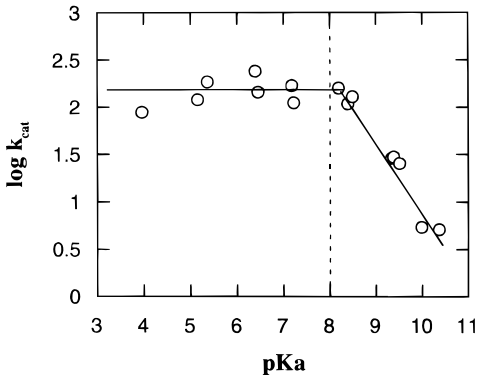

FIGURE 3. Brønsted relationship for a series of aryl β-glucosides with *Agrobacterium* sp. β-glucosidase.

mechanism involving an oxocarbenium ion-like transition state. By contrast, the carboxyl groups in retaining glycosidases are only 5.5 Å apart, consistent with a double-displacement mechanism involving a covalent glycosyl−enzyme intermediate (Figure 1b).[2,4,13] In the first step, one of the carboxyl groups functions as a general acid catalyst, protonating the glycosidic oxygen concomitantly with bond cleavage. The other acts as a nucleophile, forming a covalent glycosyl−enzyme intermediate. In the second step, the side-chain carboxylate deprotonates the incoming water molecule, which attacks at the anomeric center and displaces the sugar. Both steps occur via transition states with substantial oxocarbenium ion character. A variant of this mechanism appears to be followed by some *N*-acetyl β-hexosaminidases (those from families 18 and 20), in which the *N*-acetyl group plays the role of the catalytic nucleophile. Reaction therefore proceeds via an oxazoline intermediate, as shown in Figure 2.[14,15]

Defining Rate-Determining Steps

To fully characterize each mechanistic step, it is necessary to first identify a number of substrates for which different steps are rate-limiting. The aryl glycosides have proved ideal for this purpose with a number of β-glycosidases, early studies being performed on the almond β-glucosidase[16] and *Escherichia coli* β-galactosidase.[17] In the case of Abg, a plot of log k_{cat} values for the hydrolysis of a series of aryl glucoside substrates versus the aglycone pK_a values produces a biphasic, concave-downward Brønsted relationship, indicating a change in rate-determining step as the aglycone leaving group ability increases (Figure 3).[18]

The glycosylation step is rate-limiting for substrates to the right of the break, as indicated by the strong dependence ($\beta_{lg} = -0.7$) in this region. Below the break, the k_{cat} value is independent of the leaving group, indicating that some other step is rate-limiting. This was shown to be the deglycosylation step through nucleophilic competition studies and the observation of "burst" kinetics in the pre-steady state.[18,19]

Noncovalent Interactions

A significant component of catalysis in most enzymes derives from noncovalent enzyme/substrate interactions that are optimized at the transition state. To probe the importance of hydrogen-bonding interactions at each sugar hydroxyl position for each step along the reaction coordinate with Abg, a series of deoxygenated and deoxyfluorinated derivatives of the substrate 2,4-dinitrophenyl β-glucoside were synthesized and subjected to detailed kinetic analysis.[19] Use of the very good 2,4-dinitrophenol leaving group made it probable that the deglycosylation step would be rate-limiting in each case; thus, pre-steady-state and steady-state kinetic parameters for each substrate allowed the apparent contribution of a specific hydroxyl to be evaluated at both the glycosylation and deglycosylation transition states. Key conclusions from this study were that binding interactions at the 3-, 4-, and 6-positions individually contribute 3−10 kJ mol^{-1} to each transition state, whereas contributions in the ground state are much weaker ($\leq$3 kJ mol^{-1}). However, the most interesting interactions are those at the 2-position, which contribute substantially (18−22 kJ mol^{-1}) to stabilization of the glycosylation and deglycosylation transition states. This appears to be a common phenomenon with β-glycosidases,[19] with interactions at this position in some enzymes reaching 45 kJ mol^{-1}.

Transition-State Structure

Substantial oxocarbenium ion character has been shown to be present at the anomeric center in the transition states of the enzyme-catalyzed process, as is also the case for the spontaneous hydrolysis mechanism.[19,20] Perhaps the best such evidence is that derived from measurements of α secondary deuterium kinetic isotope effects (αDKIEs). Such measurements on glycosidases have a long history[6] and are illustrated here in studies with Abg. The αDKIE values measured[18] for two substrates for which deglycosylation was rate-limiting averaged $k_H/k_D = 1.11$, while αDKIE values for the glycosylation step averaged $k_H/k_D = 1.06$. The larger αDKIE values measured for deglycosylation suggest that this step has more oxocarbenium ion character than does glycosylation; thus, it involves a more dissociative transition state. This might be expected since the departing carboxylate is axial and may receive some stereoelectronic assistance from the endocyclic oxygen. The relatively large value of $\beta_{lg} = -0.7$ measured for the glycosylation step clearly indicates substantial bond cleavage at that transition state; thus, the smaller isotope effect must indicate more preassociation by the nucleophile. Indeed, since the leaving group is equatorial (unless distorted, vide infra), this step will require more nucleophilic "push" from the enzymatic nucleophile.

Substrate Distortion

The role of substrate distortion in the mechanism of β-glycosidases is a contentious issue with a long history. First posited for hen egg white lysozyme on the basis of models of bound oligosaccharides, this concept has received support from measurements of subsite affinities, which indicated a negative contribution from the -1 site, and from stereoelectronic arguments, which require such distortion in order to place the lone pair on the endocyclic oxygen antiperiplanar to the scissile bond.[21,22] Structural evidence for such distortion has been ambiguous, possibly because the complexes under investigation in many cases had no sugar filling the $+1$ site. Recently, however, there have been three separate structure determinations of β-glycosidases with uncleaved substrates spanning the active sites, in which large distortions of the pyranoside ring bound in the -1 site into a $^1S_3$ skew boat have been seen.[23−25] Such distortion, which is likely driven by interactions between the enzyme and the substrate in the $+1$ site, can assist bond cleavage in several ways. First, it permits an in-line attack of the enzymic nucleophile at the anomeric center, unencumbered by the repulsive 1,3-diaxial interactions otherwise present when displacing an equatorial leaving group. It also moves the substrate closer to the conformation of the oxocarbenium ion transition state, as well as placing the glycosidic oxygen in an appropriate position for protonation by the general acid catalyst. Finally it is consistent with the dictates of stereoelectronic theory alluded to earlier.

Acid/Base Catalysis

Cleavage of a glycosidic bond between two sugar residues requires substantial general acid catalytic assistance, as well as general base catalysis to assist the attack of the nucleophilic water molecule, and glycosidases seem to be well evolved to provide such help. Three-dimensional structures generally provide good clues regarding the identities of the acid/base catalysts. Alternatively, candidates identified through sequence alignments can be investigated by detailed kinetic analysis of mutants modified at those conserved residues.[8] However, simple measurement of activities can lead to misassignments.

Replacement of the acid/base catalyst carboxyl group with a methyl group (Ala) or an amide functionality (Asn or Gln) should yield a mutant with kinetic parameters that vary widely with the leaving group ability of the substrate aglycone. Thus, the glycosylation step, as measured through k_{cat}/K_m values or pre-steady-state kinetics, is drastically slowed for substrates such as disaccharides with poor leaving groups that need protonic assistance for departure but is affected relatively little for substrates such as dinitrophenyl glycosides or glycosyl fluorides that need no such assistance. However, the deglycosylation step is slowed equally (typically 200−2000-fold) for all substrates of the same glycone structure, due to removal of general base catalytic assistance. This results in accumulation of the covalent intermediate when reactive substrates are employed,[26−30] as suggested by unusually low substrate

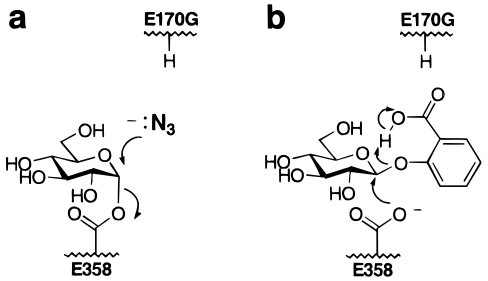

FIGURE 4. Rescue of catalytic activity in the acid/base mutant of *Agrobacterium* sp. β-glucosidase by azide (a) and substrate-assisted protonation (b).

K_m values and "burst" kinetics in the pre-steady state. In some cases, the intermediate can be trapped entirely, and thus isolated, as will be discussed below. The altered dependence on leaving group ability is also reflected in the different slopes of Brønsted plots of $\log(k_{cat}/K_m)$ versus pK_a for hydrolysis of aryl cellobiosides by Cex and its acid catalyst mutant. Removal of the acid catalyst from Cex increases the β_{lg} value for cleavage of aryl cellobiosides from -0.3 in the wild-type enzyme to -1 in the acid catalyst mutant, consistent with the removal of significant proton donation.[28] Interestingly, family 1 myrosinases do not appear to have a general acid/base catalyst, yet they still function effectively.[31] Possibly such enzymes use the substrate's own acid catalyst and compensate for the absence of a general base catalyst by forming more reactive glycosyl−enzymes.

The kinetic characteristics described above are necessary, but not sufficient, indicators of mutation of the acid/base catalyst. Changes in the pH/rate profile should also be observed, ideally involving complete removal of the ionization from the profile,[28,30] though in many cases this can be masked by other proton-transfer events. A more definitive and rapid diagnostic test for having mutated the acid/base catalyst is the observation of a large increase in rate of cleavage of substrates for which deglycosylation is rate-limiting upon addition of nucleophilic anions such as azide, formate, or acetate. This is accompanied by the formation of a new product with retained anomeric configuration (Figure 4a). These effects are due to the anion reacting more rapidly with the glycosyl−enzyme than does water in the absence of general base catalysis. No such effect is seen with wild-type enzyme, charge screening by the base catalyst presumably denying access to the anions in that case. This approach allows relatively facile and certain identification of the acid/base catalyst and appears to be general.[8]

Catalytic activity has also been restored to the acid/base mutant of Abg (Glu170Gly) by the inclusion of a suitably positioned carboxyl group into the substrate.[32] Thus, the k_{cat}/K_m value for cleavage of 2-carboxyphenyl β-glucoside by this mutant is some 10^7-fold greater than that for its 4-substituted isomer, due to intramolecular delivery of a proton, as is shown in Figure 4b.

These results suggest that rigorous positioning of the acid/base catalyst may not be as crucial as that of the catalytic nucleophile, consistent with the differing stereo-

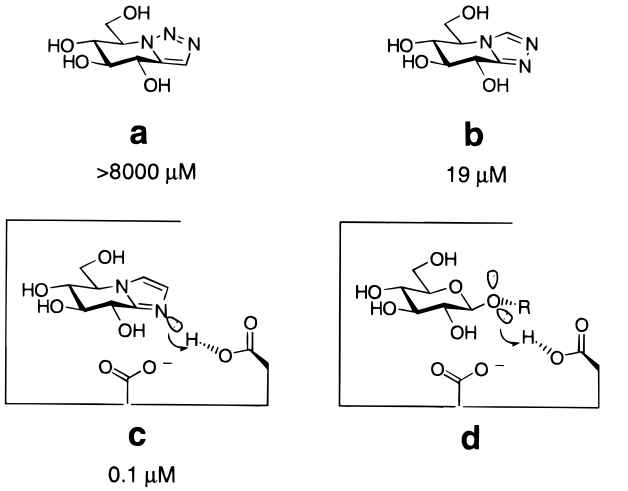

FIGURE 5. Structures of *gluco*-configured triazoles (a and b) and imidazole (c), illustrating "side-on" protonation by the acid/base catalyst (c and d). K_i values for the inhibition of almond β-glucosidase are shown.

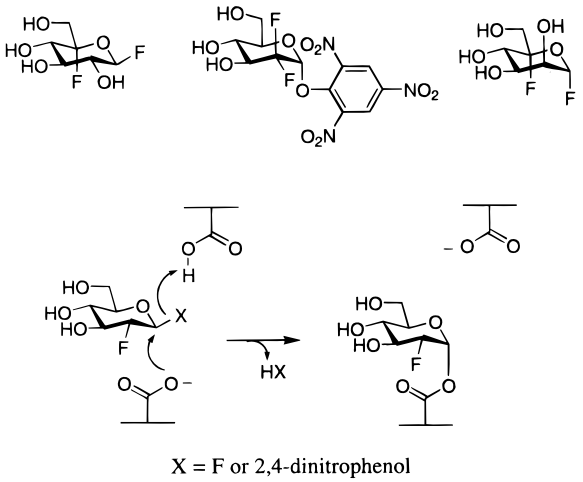

FIGURE 6. Examples of fluorosugars and their mode of mechanism-based inactivation of retaining glycosidases.

electronic demands of the two processes. Thus, increasing or decreasing the length of the side chain of the acid/base catalyst in Bcx (Glu172Ala and Glu172CysCH_2COOH) reduced rate constants for glycosylation only 2−23-fold for aryl xylobiosides and 400-fold for xylan.[29] By contrast, shortening the nucleophile side chain reduced the glycosylation rate for aryl xylobiosides by 1600−5000-fold.[33] Nevertheless, the development of protonation site-specific transition-state analogue inhibitors has elegantly revealed a subtlety in the positioning of the acid/base catalyst.[34] The vastly reduced potency of 1,2,3-triazoles (Figure 5a) relative to 1,2,4-triazoles and imidazoles (Figure 5b and c, respectively) with glycosidases belonging to clan GH-A implies that protonation of the glycosidic oxygen occurs within the plane of the sugar ring rather than from above (Figure 5d). Recent structural analysis of a cellobiose imidazole complex with a cellulase has confirmed the mode of binding.[35]

Exciting insight into the modulation of the pK_a of the acid/base catalyst to suit its role at each step in catalysis was obtained from ^{13}C NMR titrations on Bcx that had been specifically ^{13}C-labeled in the side-chain carboxyl groups of the nucleophile (Glu78) and the acid/base catalyst (Glu172).[36] pK_a values of 4.6 and 6.7 were measured for Glu78 and Glu172, respectively, identical to those deduced from the pH dependence of k_{cat}/K_m. However, when the 2-fluoroxylobiosyl−enzyme intermediate was titrated, it was seen that the pK_a of Glu172 had dropped some 2.5 units, thereby producing the ionization state required for the subsequent deglycosylation step. Such "pK_a cycling" is ascribed to changes in the active-site environment arising from the presence and absence of charge on Glu78 and is most likely a general feature of the mechanisms of retaining glycosidases.

Covalent Intermediate: Trapping

The covalent intermediate shown in Figure 1 is significantly different from the ion-pair generally depicted for the "textbook" glycosidase lysozyme. In reality, the structure of the lysozyme intermediate remains unclear because it has not been possible to make the deglycosylation step rate-limiting. However, the covalent intermediate is now firmly established with a number of other glycosidases. Important evidence has been provided by αDKIEs of $k_H/k_D > 1.0$ measured on the deglycosylation step, which are consistent *only* with a covalent intermediate reacting through an oxocarbenium ion-like transition state.[3] An ion-pair intermediate would demand inverse isotope effects.

Direct trapping of the covalent intermediate has been achieved in several different ways. One approach involves the introduction of a fluorine substituent into the 2- or 5-position of the substrate (Figure 6) to slow the reaction by destabilizing the positive charge that develops at the transition state and removing the important transition-state hydrogen-bonding interactions which develop at the 2-position (for leading references, see refs 37−40). Since such substitutions slow both the glycosylation and deglycosylation steps, a good leaving group, typically fluoride or dinitrophenolate, is generally incorporated into the analogue to ensure that the glycosylation step is faster than the deglycosylation, and thus that the intermediate accumulates.

Inactivation of the enzyme is accompanied by the release of one full equivalent of aglycone per equivalent of enzyme. This has allowed facile active-site titrations to be performed, which have proved invaluable in the characterization of mutant enzymes.[13,41] The formation of a covalent intermediate is now trivially demonstrable by electrospray mass spectrometric analysis, and ^{19}F NMR studies have provided good evidence for the stereochemistry of the linkage.[42,43] The catalytic competence of these intermediates (with typical lifetimes of days) has been evidenced by their accelerated turnover in the presence of acceptor sugars, via the transglycosylation process shown in Figure 7.

The identity of the nucleophilic amino acid residue involved in intermediate formation has been determined in a number of cases by proteolytic cleavage of the labeled

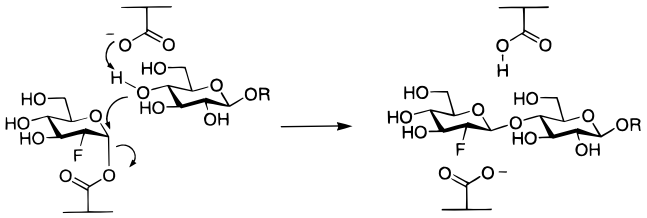

FIGURE 7. Reactivation of glycosidase activity via transglycosylation in the presence of a sugar acceptor.

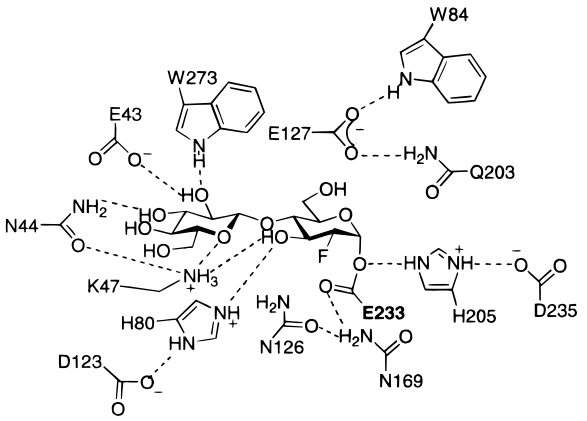

FIGURE 8. Active-site environment of the *Cellulomonas fimi* exoglycanase Cex trapped as a covalent 2-fluorocellobiosyl intermediate.

protein, followed by isolation and sequencing of the glycosylated peptide.[40] This process has been significantly accelerated by the use of LC/MS/MS protocols. Using this or closely related approaches, the active-site nucleophiles of 21 glycosidases have been identified, representing 13 of the 26 different families of retaining glycosidases.[7]

Covalent Intermediate: X-ray Crystallography

Certainly the best insight into the nature of the glycosyl−enzyme intermediate has come from X-ray crystallographic analysis of trapped complexes. Structures of several such complexes have now been solved,[23,31,44−46] the first being that of the 2-deoxy-2-fluorocellobiosyl−enzyme intermediate of Cex[43] (Figure 8).

No significant changes in protein structure occurred upon intermediate formation, and the proximal, covalently linked sugar ring assumed a standard $^4C_1$ chair conformation. Of particular interest were the identities of the amino acid residues that interact with the sugar 2-hydroxyl, and two close contacts with the fluorine were apparent. One of these involved Asn126, located at a distance of 2.8 Å from the fluorine. However, site-directed mutagenesis of this conserved residue indicated[47,48] an interaction worth only about 2.5 kcal mol^{-1}. The other nearby atom is the carbonyl oxygen of the catalytic nucleophile (Glu233) which, despite the fact that its interaction with the fluorine at C-2 must be destabilizing, was only 3.1 Å away, suggesting that an important hydrogen bond might be present when there is a hydroxyl group at the 2-position.

This possibility was probed by the trapping and structural solution of a covalent glycosyl−enzyme complex in Cex involving a true cellobiosyl moiety.[45] This was achieved

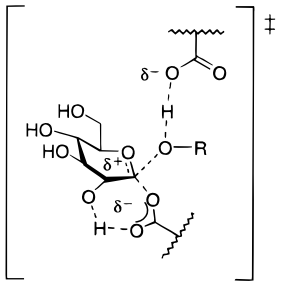

FIGURE 9. Transition-state optimization of a strong hydrogen bond between the 2-hydroxyl and the nucleophile of a β-retaining glucosidase.

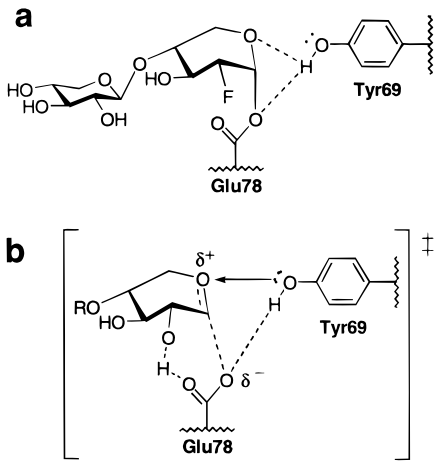

FIGURE 10. Active-site structure of *Bacillus circulans* xylanase trapped as a covalent 2-fluoroxylobiosyl intermediate (a) and implications for the transition state (b).

by deleting both the acid/base catalyst (Glu127Ala) and a histidine (His205Asn) that modulates the position and ionization state of the nucleophile, and reacting this double mutant with 2,4-dinitrophenyl β-cellobioside. No interaction was observed between the sugar 2-hydroxyl and Asn126 (4.4 Å) in this structure, while that with the carbonyl oxygen of the nucleophile, Glu233, had become unusually short (2.4 Å), strongly suggesting that the very strong interaction at the 2-hydroxyl deduced from kinetic studies is, indeed, a hydrogen bond with Glu233. The interaction observed would become shorter and stronger at the transition state through flattening of the sugar ring *and* approach of the carboxylate. In addition, positive charge developed at the anomeric center must transiently increase the acidity of the 2-hydroxyl, making it a better hydrogen bond donor, as shown in Figure 9. This may be a general phenomenon for retaining glycosidases with appropriately configured 2-hydroxyls.

A surprise was obtained when the three-dimensional structure of the 2-deoxy-2-fluoroxylobiosyl-enzyme intermediate formed on Bcx was determined.[44] While the distal xylose moiety adopts a normal $^4C_1$ conformation, the proximal xylosyl moiety, through which the sugar is linked to the enzyme via an ester bond, is distorted into a $^{2,5}B$ boat conformation, as shown in Figure 10a. In contrast, the corresponding 2-deoxy-2-fluoroxylobiosyl−enzyme intermediate in Cex (which possesses dual xylan/cellulose specificity) accommodates both xylose rings in undistorted

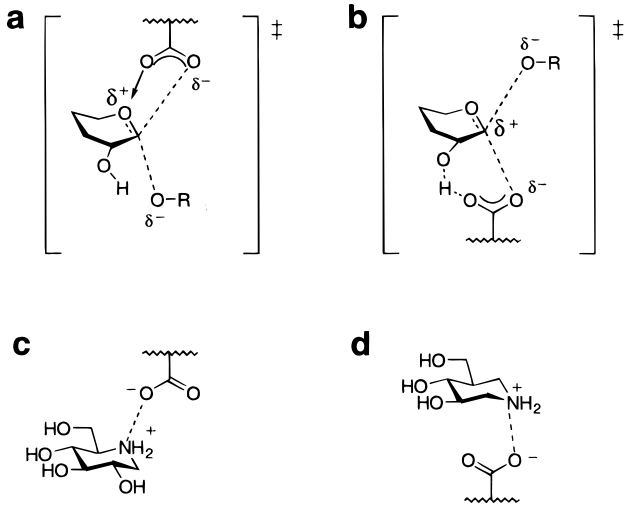

FIGURE 11. Comparison of the transition states for α- (a) and β- (b) retaining glucosidases and the corresponding potent transition state-analogue inhibitors deoxynojirimycin (c) and isofagomine (d).

$^4C_1$ chair conformations.[46] Such distortion has not been seen previously in a glycosyl−enzyme intermediate but, as proposed earlier[49] carries significant mechanistic implications. In such a conformation, C-5, O-5, C-1, and C-2 are held in a planar array ideally set up for stabilization of oxocarbenium ion character at the transition state. Bcx may therefore react through a boat transition state rather than a half-chair!

Another intriguing interaction is a bifurcated hydrogen bond formed by the Tyr69 hydroxyl group to the "ether" oxygen of Glu78 and the sugar ring oxygen (Figure 10a). Upon bond cleavage and positive charge development at O-5, the symmetry of this arrangement will be broken, and a hydrogen bond will develop to the partially negatively charged Glu78 oxygen. Simultaneously, a stabilizing electrostatic or dipolar interaction could develop with the ring oxygen, as shown in Figure 10b. This extended network of interactions from the 2-hydroxyl around to the ring oxygen could serve to disperse charge and stabilize the flattened conformation.

α- vs β-Retaining Glycosidases

A similar mechanism appears to exist in α-retaining glycosidases, but of course with the complementary stereochemical itinerary involving a β-linked intermediate. Good evidence for this has come from kinetic studies,[6] from the trapping of intermediates, and from the three-dimensional structure of one such intermediate.[50] Several lines of evidence, however, point toward a subtle difference in the oxocarbenium ion character of the transition structures formed on α- and β-glycosidases. On β-glycosidases, the *syn* interaction of the nucleophile carboxyl oxygens with the anomeric center and the 2-hydroxyl will tend to favor a greater share of the positive charge on the anomeric carbon (Figure 11b**)**. This interaction is not possible for an α-glucosidase, where instead a *syn* interaction of the nucleophile carboxyl oxygens with the anomeric center and the endocyclic oxygen is observed in the

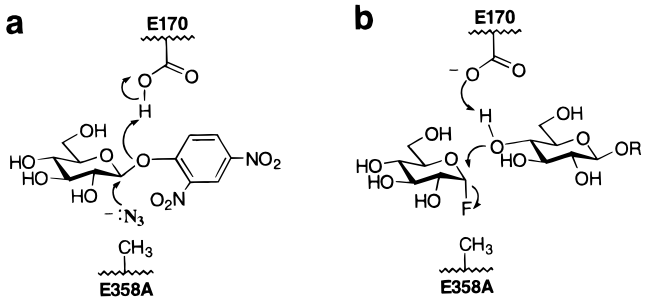

FIGURE 12. Nucleophile mutant of *Agrobacterium* sp. β-glucosidase (Glu358Ala) acting as an "inverting" glycosidase (a) and a "glycosynthase" (b).

intermediate.[50] This interaction will favor positive charge development on the endocyclic oxygen (Figure 11a).

Experimental support for this hypothesis is drawn from observations with two classes of inhibitors, as follows. The efficacy of the 2-fluorosugar inhibitors with β-glycosidases, but not with α-glycosidases, could be due to the fact that only with the β-glycosidases, wherein the charge is localized to a greater extent on the adjacent anomeric carbon, is there sufficient inductive destabilization to affect rates significantly. By contrast, the 5-fluoro inhibitors function with both α- and β-glycosidases, consistent with the proximity of the fluorine to the developing charge on the endocyclic oxygen. The specificities of the reversible azasugar inhibitors of the deoxynojirimycin and isofagomine classes could be explained in this way too. As has been pointed out previously,[34,51,52] the deoxynojirimycins (Figure 11c), which apparently mimic charge developed on the ring oxygen, are potent α-glycosidase inhibitors but very modest inhibitors of β-glycosidases. By contrast, the isofagomines (Figure 11d), which arguably mimic charge development on the anomeric carbon, have the complementary inhibitory profile.

Changing Mechanisms through Mutagenesis

The similarity of the transition states for inverting and retaining glycosidases has made it possible to change mechanisms by changing the relationships of the active-site carboxyl groups. The normally inverting bacteriophage T4 lysozyme has been converted into a retaining enzyme by introduction of a nucleophilic residue (Glu or His) into a position within the active site that is normally occupied by a water nucleophile.[53−55] The opposite change has been carried out on Abg by mutating the nucleophile to alanine (Glu358Ala). The resultant mutant was hydrolytically inactive.[13] However, when presented with an alternate nucleophile such as azide or formate needing no general base catalytic assistance, the reaction proceeded relatively efficiently, yielding the substitution product of inverted configuration (Figure 12a). Consistent with the inverting mechanism now followed,[56] this β-glucosidase mutant was able to cleave α-glucosyl fluoride, forming a disaccharide product, α-cellobiosyl fluoride. This led to the concept of "glycosynthases", mutant glycosidases that can synthesize oligosaccharides but do not degrade them.[57] As shown in Figure 12b, reaction of the mutant with α-glucosyl fluoride

in the presence of a suitable β-linked "acceptor" sugar resulted in efficient synthesis of oligosaccharides in yields of up to 92%. This approach has since been adopted by others[58] and has considerable potential for large-scale enzymatic oligosaccharide synthesis.

Conclusions

The picture that emerges of the active site of a retaining β-glycosidase is of a largely structurally static, but electronically dynamic, cavity containing a highly evolved array of functional groups. It is the substrate that is choreographed through a series of conformational changes within this largely static framework, and in this way problems of rate-limiting protein conformational change can presumably be avoided. Of critical importance are the stabilizing effects applied to the oxocarbenium ion transition states, such as flattening of the sugar ring through a half-chair or ^{2,5}B boat conformation, which appear to be favored by a strong hydrogen bond between the sugar 2-hydroxyl and the nucleophile, and electrostatic interactions with the sugar endocyclic oxygen. Finally, it is also overwhelmingly clear that, for the majority of retaining β-glycosidases examined thus far, these transition states bracket a *covalent intermediate*, and not an ion-pair intermediate as traditionally shown in biochemistry textbooks.

We thank our many co-workers and collaborators for their contributions over the years. We also thank the Natural Sciences and Engineering Research Council of Canada, the Protein Engineering Network of Centres of Excellence of Canada, the British Columbia Science Council, and the Killam foundation for financial support.

References

(1) Wolfenden, R.; Lu, X.; Young, G. Spontaneous hydrolysis of glycosides. *J. Am. Chem. Soc.* **1998**, *120*, 6814−6815.
(2) McCarter, J.; Withers, S. G. Mechanisms of enzymatic glycoside hydrolysis. *Curr. Opin. Struct. Biol.* **1994**, *4*, 885−892.
(3) Sinnott, M. L. Catalytic mechanisms of enzymic glycosyl transfer. *Chem. Rev.* **1990**, *90*, 1171−1202.
(4) Davies, G.; Henrissat, B. Structures and mechanisms of glycosyl hydrolases. *Structure* **1995**, *3*, 853−859.
(5) Legler, G. Glycoside hydrolases: mechanistic information from studies with reversible and irreversible inhibitors. *Adv. Carbohydr. Chem. Biochem.* **1990**, *48*, 319−385.
(6) Davies, G.; Sinnott, M. L.; Withers, S. G. Glycosyl transfer. In *Comprehensive Biological Catalysis*; Sinnott, M. L., Ed.; Academic Press: 1998; Vol. 1, pp 119−208.
(7) Zechel, D. L.; Withers, S. G. Mechanisms of Glycosyl Transfer. In *Comprehensive Natural Products Chemistry*; Poulter, C. D., Ed.; Elsevier: New York, 1999; Vol. 5, pp 279−314.
(8) Ly, H.; Withers, S. G. Mutagenesis of Glycosidases. *Annu. Rev. Biochem.* **1999**, 487−522.
(9) Henrissat, B.; Bairoch, A. Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* **1996**, *316*, 695−696.
(10) Henrissat, B.; Davies, G. Structural and sequence-based classification of glycoside hydrolases. *Curr. Opin. Struct. Biol.* **1997**, *7*, 637−644.
(11) White, A.; Rose, D. R. Mechanism of catalysis by retaining β-glycosyl hydrolases. *Curr. Opin. Struct. Biol.* **1997**, *7*, 645−651.
(12) Gebler, J.; Gilkes, N. R.; Claeyssens, M.; Wilson, D. B.; Beguin, P.; Wakarchuk, W.; Kilburn, D. G.; Miller, R. C., Jr.; Warren, R. A. J.; Withers, S. G. Stereoselective hydrolysis catalysed by related β-1,4-glucanases and β-1,4-xylanases. *J. Biol. Chem.* **1992**, *267*, 12259−12262.
(13) Wang, Q.; Graham, R. W.; Trimbur, D.; Warren, R. A. J.; Withers, S. G. Changing enzymatic reaction mechanisms by mutagenesis: conversion of a retaining glucosidase to an inverting enzyme. *J. Am. Chem. Soc.* **1994**, *116*, 11594−11595.
(14) Knapp, S.; Vocadlo, D.; Gao, Z.; Kirk, B.; Lou, J.; Withers, S. G. NAG-Thiazoline, an N-acetyl-β-hexosaminidase inhibitor that implicates acetamido participation. *J. Am. Chem. Soc.* **1996**, *118*, 6804−6805.
(15) Terwisscha van Scheltinga, A. C.; Armad, S.; Kalk, K. H.; Isogai, A.; Henrissat, B.; Dijkstra, B. Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and x-ray structure of a complex with allosamidin: evidence for substrate assisted catalysis. *Biochemistry* **1995**, *34*, 15619−15623.
(16) Nath, R. L.; Rydon, H. N. The influence of structure on the hydrolysis of substituted phenyl β-D-glucosides by emulsin. *Biochem. J.* **1954**, *57*, 1−10.
(17) Sinnott, M. L.; Souchard, I. J. L. The mechanism of action of β-galactosidase. *Biochem. J.* **1973**, *133*, 89−98.
(18) Kempton, J. B.; Withers, S. G. Mechanism of *Agrobacterium* β-glucosidase: kinetic studies. *Biochemistry* **1992**, *31*, 9961−9969.
(19) Namchuk, M. N.; Withers, S. G. Mechanism of *Agrobacterium* β-glucosidase: kinetic analysis of the role of non-covalent enzyme−substrate interactions. *Biochemistry* **1995**, *34*, 16194−16202.
(20) McCarter, J.; Adam, M.; Withers, S. G. Binding energy and catalysis: fluorinated and deoxygenated glycosides as mechanistic probes of *Escherichia coli* (lac Z) β-galactosidase. *Biochem. J.* **1992**, *286*, 721−727.
(21) Imoto, T.; Johnson, L.; North, A.; Phillips, D.; Rupley, J. *The Enzymes*; Academic Press: New York, 1972.
(22) Deslongchamps, P. *Stereoelectronic Effects in Organic Chemistry*; Pergamon Press: New York, 1983.
(23) Davies, G. J.; Mackenzie, L. F.; Varrot, A.; Dauter, M.; Brzozowski, A. M.; Schulein, M.; Withers, S. G. Snapshots along an enzymatic reaction coordinate: analysis of a retaining β-glycoside hydrolase. *Biochemistry* **1998**, *37*, 11707−11713.
(24) Sulzenbacher, G.; Driguez, H.; Henrissat, B.; Schulein, M.; Davies, G. J. Structure of the *Fusarium oxysporum* endoglucanase I with a nonhydrolyzable substrate analogue: substrate distortion gives rise to the preferred axial orientation for the leaving group. *Biochemistry* **1996**, *35*, 15280−15287.
(25) Tews, I.; Perrakis, A.; Oppenheim, A.; Dauter, Z.; Wilson, K. S.; Vorgias, C. E. Bacterial chitobiase structure provides insight into catalytic mechanism and the basis of Tay-Sachs disease. *Nat. Struct. Biol.* **1996**, *3*, 638−648.
(26) Richard, J. P.; Huber, R. E.; Heo, C.; Amyes, T. L.; Lin, S. Structure−reactivity relationships for β-galactosidase (*Escherichia coli*, lac z). 4. Mechanism for reaction of nucleophiles with the galactosyl−enzyme intermediates of E461G and E461Q β-galactosidases. *Biochemistry* **1996**, *35*, 12387−12401.
(27) MacLeod, A. M.; Lindhorst, T.; Withers, S. G.; Warren, R. A. The acid/base catalyst in the exoglucanase/xylanase from *Cellulomonas fimi* is glutamic acid 127: evidence from detailed kinetic studies of mutants. *Biochemistry* **1994**, *33*, 6371−6376.
(28) Macleod, A. M.; Tull, D.; Rupitz, K.; Warren, R. A. J.; Withers, S. G. Mechanistic consequences of mutation of active site carboxylates in a retaining β-1,4-glycanase from *Cellulomonas fimi*. *Biochemistry* **1996**, *35*, 13165−13172.
(29) Lawson, S. L.; Wakarchuk, W. W.; Withers, S. G. Positioning the acid/base catalyst in a glycosidase: studies with *Bacillus circulans* xylanase. *Biochemistry* **1997**, *36*, 2257−2265.
(30) Wang, Q.; Trimbur, D.; Graham, R.; Warren, R. A.; Withers, S. G. Identification of the acid/base catalyst in *Agrobacterium faecalis* β-glucosidase by kinetic analysis of mutants. *Biochemistry* **1995**, *34*, 14554−14562.
(31) Burmeister, W. P.; Cottaz, S.; Driguez, H.; Iori, R.; Palmieri, S.; Henrissat, B. The crystal structures of *Sinapis alba* myrosinase and a covalent glycosyl−enzyme intermediate provide insights into the substrate recognition and active-site machinery of an S-glycosidase. *Structure* **1997**, *5*, 663−675.
(32) Wang, Q.; Withers, S. G. Substrate-assisted catalysis in glycosidases. *J. Am. Chem. Soc.* **1995**, *117*, 10137−10138.
(33) Lawson, S. L.; Wakarchuk, W. W.; Withers, S. G. Effects of both shortening and lengthening the active site nucleophile of *Bacillus circulans* xylanase on catalytic activity. *Biochemistry* **1996**, *35*, 10110−10118.
(34) Heightman, T. D.; Vasella, A. T. Recent insights into inhibition, structure, and mechanism of configuration-retaining glycosidases. *Angew. Chem., Int. Ed.* **1999**, *38*, 750−770.
(35) Varrot, A.; Schulein, M.; Pipelier, M.; Vasella, A. T.; Davies, G. J. Lateral protonation of a glycosidase inhibitor. Structure of the *Bacillus agaradhaerens* Cel5A in complex with a cellobiose-derived imidazole at 0.97 Å resolution. *J. Am. Chem. Soc.* **1999**, *121*, 2621−2622.

(36) McIntosh, L. P.; Hand, G.; Johnson, P. E.; Joshi, M.; Korner, M.; Plesniak, L. A.; Ziser, L.; Wakarchuk, W. W.; Withers, S. G. The pKa of the general acid/base group of a glycosidase cycles during catalysis: A ^{13}C-NMR study of *Bacillus circulans* xylanase. *Biochemistry* **1996**, *35*, 9958−9966.
(37) McCarter, J. D.; Yeung, W.; Chow, J.; Dolphin, D.; Withers, S. G. 2-Deoxy-2-fluoro disaccharides: incorporating aglycone specificity into mechanism-based glycosidase inhibitors. *J. Am. Chem. Soc.* **1997**, *119*, 5792−5797.
(38) McCarter, J. D.; Withers, S. G. 5-Fluoro glycosides: a new class of mechanism-based inhibitors of both α-and β-glucosidases. *J. Am. Chem. Soc.* **1996**, *118*, 241−242.
(39) Withers, S. G.; Rupitz, K.; Street, I. P. 2-Deoxy-2-fluoro-D-glycosyl fluorides. A new class of specific mechanism-based glycosidase inhibitors. *J. Biol. Chem.* **1988**, *263*, 7929−7932.
(40) Withers, S. G.; Aebersold, R. Approaches to labeling and identification of active site residues in glycosidases. *Protein Sci.* **1995**, *4*, 361−372.
(41) Street, I. P.; Kempton, J. B.; Withers, S. G. Inactivation of a β-glucosidase through the accumulation of a stable 2-deoxy-2-fluoro-α-D-glucopyranosyl−enzyme intermediate: a detailed investigation. *Biochemistry* **1992**, *31*, 9970−9978.
(42) Withers, S. G.; Street, I. P. Identification of a covalent α-D-glucopyranosyl enzyme intermediate formed on a β-glucosidase. *J. Am. Chem. Soc.* **1988**, *110*, 8551−8553.
(43) White, A.; Tull, D.; Johns, K.; Gilkes, N. R.; Withers, S. G.; Rose, D. R. Crystallographic observation of a covalent catalytic intermediate on a β-glycosidase. *Nat. Struct. Biol.* **1996**, *3*, 149−154.
(44) Sidhu, G.; Withers, S. G.; Nguyen, N. T.; McIntosh, L. P.; Ziser, L.; Brayer, G. D. Sugar ring distortion in the glycosyl−enzyme intermediate of a family G/11 xylanase. *Biochemistry* **1999**, *38*, 5346−5354.
(45) Notenboom, V.; Birsan, C.; Nitz, M.; Rose, D. R.; Warren, R. A. J.; Withers, S. G. Active site mutations lead to covalent intermediate accumulation in *Cellulomonas fimi* β-1,4-glycosidase Cex: insights into transition state stabilization. *Nat. Struct. Biol.* **1998**, *5*, 812−818.
(46) Notenboom, V.; Birsan, C.; Warren, R. A. J.; Withers, S. G.; Rose, D. R. Exploring the cellulose/xylan specificity of the β-1,4-glycanase Cex from *Cellulomonas fimi* through crystallography and mutation. *Biochemistry* **1998**, *37*, 4751−4758.
(47) Roberge, M.; Dupont, C.; Morosoli, R.; Shareck, F.; Kluepfel, D. Asparagine 127 of xylanase A from *Streptomyces lividans*, a key residue in glycosyl hydrolases of superfamily 4/7. *Protein Eng.* **1997**, *10*, 399−403.
(48) Notenboom, V., personal communication, 1999.
(49) Hosie, L.; Sinnot, M. L. Effects of deuterium substitution α and β to the reaction centre, ^{18}O substitution in the leaving group, and aglycone acidity on hydrolyses of aryl glucosides and glucosyl pyridinium ions by yeast α-glucosidase. *Biochem. J.* **1985**, *226*, 437−446.
(50) Uitdehaag, J. C. M.; Mosi, R.; Kalk, K. H.; van der Veen, B. A.; Dijkhuisen, L.; Withers, S. G.; Dijkstra, B. W. X-ray structures along the reaction pathway of cyclodextrin glycosyltransferase elucidate catalysis in the α-amylase family. *Nat. Struct. Biol.* **1999**, *6*, 432−436.
(51) Bols, M. 1-Aza sugars, apparent transition state analogues of equatorial glycoside formation/cleavage. *Acc. Chem. Res.* **1998**, *31*, 1−8.
(52) Ichikawa, Y.; Igarashi, Y.; Ichikawa, M.; Suhara, Y. 1-N-Iminosugars: Potent and selective inhibitors of β-glycosidases. *J. Am. Chem. Soc.* **1998**, *120*, 3007−3018.
(53) Kuroki, R.; Weaver, L.; Matthews, B. W. A covalent enzyme−substrate intermediate with saccharide distortion in a mutant T4 lysozyme. *Science* **1993**, *262*, 2030−2033.
(54) Kuroki, R.; Weaver, L. H.; Matthews, B. W. Structure-based design of a lysozyme with altered catalytic activity. *Nat. Struct. Biol.* **1995**, *2*, 1007−1011.
(55) Kuroki, R.; Weaver, L. H.; Matthews, B. W. Structural basis of the conversion of the T4 lysozyme into a transglycosidase by reengineering the active site. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8949−8954.
(56) Kasumi, T.; Tsumuraya, Y.; Brewer, C. F.; Kersters-Hilderson, H.; Claeyssens, M.; Hehre, E. J. Catalytic versatility of *Bacillus pumilus* β-xylosidase: glycosyl transfer and hydrolysis promoted with α- and β-D-xylosyl fluoride. *Biochemistry* **1987**, *26*, 3010−3016.
(57) Mackenzie, L. F.; Wang, Q.; Warren, R. A. J.; Withers, S. G. Glycosynthases: mutant glycosidases for oligosaccharide synthesis. *J. Am. Chem. Soc.* **1998**, *120*, 5583−5584.
(58) Malet, C.; Planas, A. From β-glucanase to β-glucansynthase: glycosyl transfer to α-glycosyl fluorides catalyzed by a mutant endoglucanase lacking its catalytic nucleophile. *FEBS Lett.* **1998**, *440*, 208−212.

AR970172+