Article:
Overkleeft, Herman S, Davies, Gideon J and Williams, Spencer J (2026) Catalyzing Carbohydrate Cleavage:Glycosidases and Their Mechanisms. Chemical Reviews. ISSN: 0009-2665

https://doi.org/10.1021/acs.chemrev.5c00803

Catalyzing Carbohydrate Cleavage: Glycosidases and Their Mechanisms

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Keywords: carbohydrates, glycobiology, enzymes, metabolism

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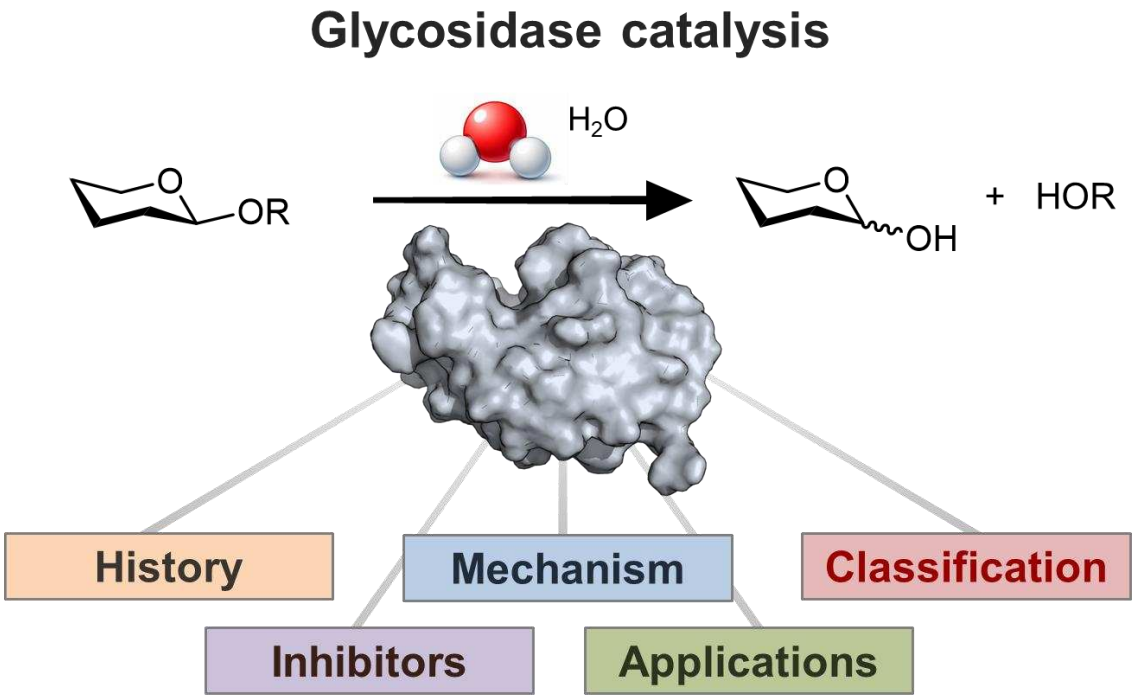

Abstract:

Glycoside hydrolases, “glycosidases”, catalyze carbohydrate catabolism, remodeling, and signaling by accelerating glycosidic-bond cleavage more than 17 orders of magnitude. Distributed across every kingdom of life and grouped into over 180 sequence-defined families, these enzymes exhibit exceptional diversity in fold, mechanism, and physiological function, and many also catalyze trans-glycosylation or phosphorolysis. The classical Koshland paradigms—stereochemical inversion, enzymatic nucleophile-assisted retention, and substrate-assisted retention—are analyzed with emphasis on the conformational itineraries and oxocarbenium ion-like transition states revealed by kinetic isotope effects, linear free-energy relationships, and high-resolution three-dimensional structures. Attention then turns to non-canonical enzymes that employ NAD^+-dependent redox hydrolysis or other cleavage mechanisms. Mechanistic insights have inspired the development of engineered glycosidase-derived catalysts for programmed bond construction, as well as mechanism-based inhibitors, transition-state analogues, and activity-based probes that are driving advances in chemical biology, biotechnology, and drug discovery.

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1. Introduction

Carbohydrates represent the most abundant pool of organic carbon in the biosphere, and their controlled breakdown underpins processes ranging from microbial conversion of plant biomass into biofuels[1, 2] to augmentation of host nutrition by the human gut microbiota.[3, 4] How do living organisms break down the glycosidic bonds found in carbohydrates and other sugar-based biomolecules? The answer mainly lies with glycoside hydrolases (glycosidases), a large group of enzymes that have evolved to catalyze the hydrolysis of these bonds. At neutral pH, the hydrolysis of a glycosidic linkage is exceptionally slow, with a half-life on the order of millions of years.[5] This inherent stability presents a biochemical challenge, yet glycosidases have evolved to solve it in elegant ways. These enzymes can achieve turnover numbers exceeding 1000 s^{-1}. Rate accelerations greater than 10^{17} allow them to overcome the inherent stability of glycosidic linkages and facilitate the timely breakdown of carbohydrates essential for cellular energy production and biosynthesis.[5]

A diverse array of glycosidases has evolved that mirror the structural variety found among the natural glycosides. These enzymes exhibit a finely-tuned selectivity for configuration, ring size, anomeric configuration, and linkage. While it is clear that the structural diversity of glycans far exceeds that of the other natural biopolymers of RNA/DNA and polypeptides,[6, 7] we still do not know the true number of naturally-occurring glycosides and their cognate glycosidases.[8] Formally, glycoside hydrolases catalyze the transfer of a glycosyl group from a substrate glycoside to a water molecule, thus converting an acetal to a hemiacetal. However, many glycosidases (or their homologs) can transfer glycosyl groups to other nucleophiles, such as phosphate or sugar alcohols, thereby acting as glycoside phosphorylases and transglycosylases, respectively. We discourage use of the terms ‘glycosyl hydrolase’ and ‘glycosylase’, which does not reflect the chemical nature of the substrate (a glycoside), and in the latter case is used to describe enzymes that cleave N-glycosidic bonds in nucleic acids.[9] On the other hand, transglycosylase is preferred over transglycosidase, as these enzymes catalyze the transfer of a glycosyl group between a donor and acceptor.[10]

This review traces the advances in the development of our knowledge of how glycosidases recognize their substrates and achieve their remarkable catalytic rates. This knowledge has been foundational in the development of chemical tools for studying glycosidase mechanisms and their biological functions, including the design of glycosidase inhibitors as potential therapeutic agents. We will explain the mechanistic reasons for why these enzymes can exhibit hydrolase, transglycosylase and phosphorylase activities.

Throughout, we will integrate this information with bioinformatic analysis of protein sequence relationships, particularly the Carbohydrate Active Enzyme (CAZy) Database (www.cazy.org),[11] which enables extension of insights from individual enzymes to entire sequence-related families, enhancing our ability to predict the function and mechanism of as-yet uncharacterized enzymes. Finally, we will provide a brief overview of enzymes that cleave glycosides through non-hydrolytic pathways.

1.1 Historical, stereochemical and topological perspectives on glycosidase action

The first enzyme discovered and produced in concentrated form was diastase (from Greek διάστασις, "separation"), which was extracted from malt solution by Anselme Payen and Jean-François Persoz in 1833.[12] Today, we recognize diastases as any of a group of amylases that breakdown starch into maltose. From this precedent arose the naming of enzymes through application of the *-ase* suffix (interestingly, Payen also introduced the *-ose* suffix for naming carbohydrates, commencing with cellulose).

In early studies of glycosidases, it was observed that most enzymes were strictly specific for anomeric stereochemistry, and that glycosidic bond hydrolysis occurred with a unique stereospecific outcome.[13] That is, glycosidases acted only on α- or β-glycosides, with some yielding a hydrolysis product in which the anomeric configuration is inverted relative to the substrate glycoside, while others yielded a hydrolysis product that retained the anomeric stereochemistry of the substrate (**Figure 1a**). The terms *inverting* and *retaining* were introduced to describe these stereochemical outcomes of glycosidase catalysis and are intrinsically connected to the chemical mechanisms that are employed. We now know that there are also non-stereospecific glycosidases that can act on both α- and β-glycosides. These unusual enzymes operate through a distinct chemical mechanism that will be discussed separately.

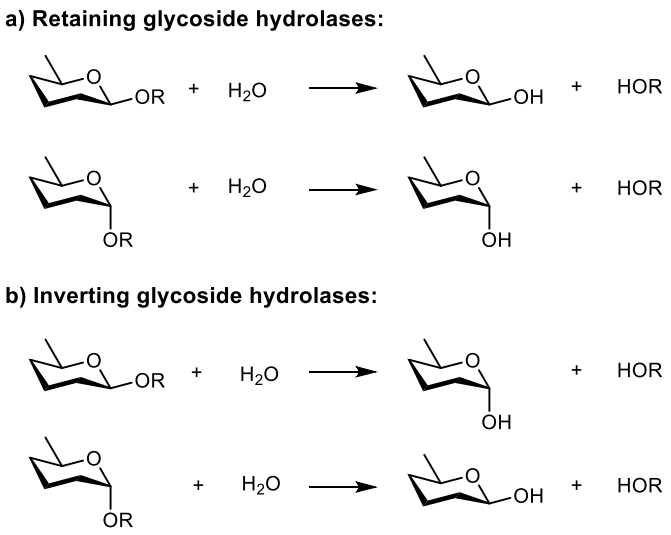


Figure 1. Reactions and stereochemical outcomes catalyzed by (a) retaining glycoside hydrolases, (b) inverting glycoside hydrolases. A C5 substituent has been added to indicate D-sugars.

Glycosidases can be classified as either exo- or endo-glycosidases, depending on where they cleave within a polysaccharide chain (**Figure 2a**). Exo-glycosidases typically cleave one sugar residue at a time, though some can cleave two or three residues, with cleavage usually occurring at the non-reducing end of the substrate. A consistent nomenclature system is crucial for accurately describing where individual sugar residues lie relative to the site of glycosidase cleavage, so as to denote the sugar-binding subsites in the enzyme. One widely adopted system labels sugar residues in an oligo/polysaccharide substrate, along with the enzyme binding sites, from *–n* to *+n*.[14] In this labelling, *–n* designates subsites towards the non-reducing end, while *+n* indicates the direction toward the reducing end (**Figure 2b**). Enzymatic cleavage occurs between the –1 and +1 sugar residues, when bound at the correspondingly numbered subsites in the enzyme.

X-ray crystallography has revealed key structural differences between endo- and exo-glycosidases. For example, exo-glycosidases, such as *Thermotoga maritima* β-glucosidase,[15] have active sites located at the end of a pocket that accommodates the terminus of the substrate chain (**Figure 2c**). This allows for precise cleavage of specific sugar residues. In contrast, endo-glycosidases, like hen egg white lysozyme,[16] feature an active site within a cleft that provides access to internal glycosidic bonds within longer substrates, enabling cleavage within the polysaccharide chain (**Figure 2d**). Some exo-glycosidases, such as *Humicola insolens* cellobiohydrolase II (Cel6A),[17] have active sites that form enclosed, tunnel-like architectures, which promote processivity,[18] the ability to catalyze repeated bond cleavages along a single polymer chain without enzyme dissociation

(**Figure 2e**). While the tunnel topology facilitates efficient, stepwise hydrolysis from chain ends, these enzymes exhibit weak endo-type activity, which likely requires transient opening of the tunnel-forming loops to create a cleft that permits access to internal regions of polysaccharide chains.[19] This structural and stereochemical diversity sets the stage for systematic sequence-based classification.

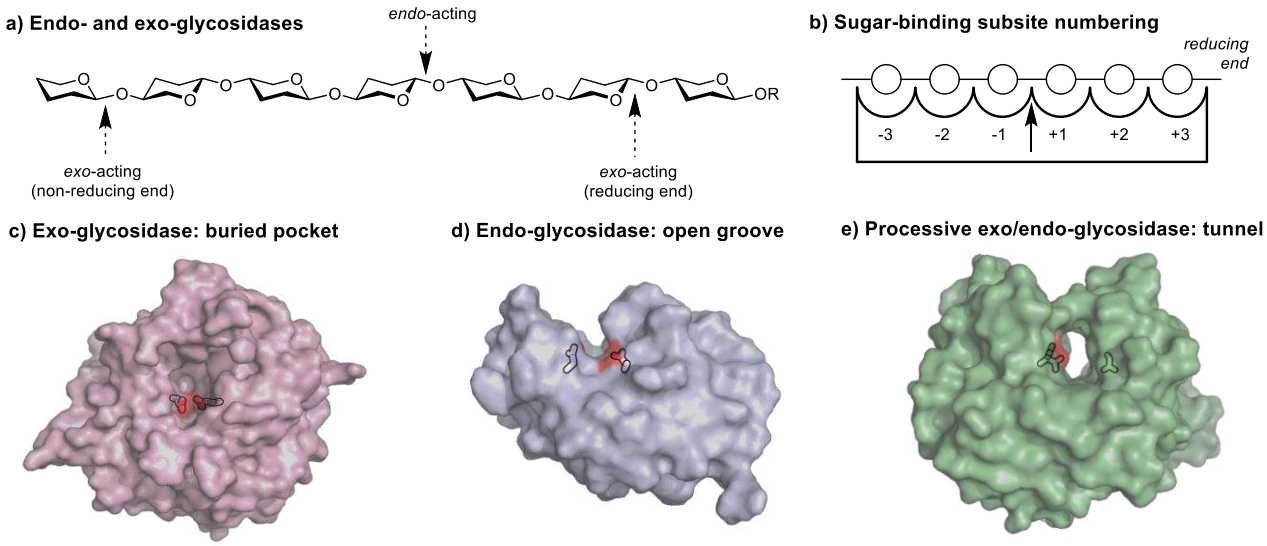


Figure 2. Basic features of glycosidases. (a) The preference for a glycosidase to cleave within (endo) or at the end (exo) of an oligo/polysaccharide chain. Examples of glycosidase topology (b) Glycosidase sugar-binding subsite nomenclature. Surface rendering of 3D structures of endo/exo-glycosidases, with catalytic residues highlighted: (c) *Thermotoga maritima* β-glucosidase, a family GH1exo-glycosidase (PDB 1oin), (d) hen egg white lysozyme (pdb 2vb1), a family GH22 endo-glycosidase, (e) *Humicola insolens* CBH II Cel6A (1bvw), a family GH6 processive exo/endo-glycosidase.

1.2 Sequence-based classification of glycosidases

Millions of glycosidases are encoded across genomes. Making sense of this sequence diversity is essential for functional annotation. The Carbohydrate-Active enZyme Database (CAZy; www.cazy.org), pioneered by Henrissat,[20] addresses this need by clustering catalytic domains into families of closely related amino acid sequences (**Table 1**).[11] Each family is seeded by at least one biochemically characterized enzyme, which serves as a trusted starting point. To expand the family, seed sequences are aligned to identify the amino acid positions that are conserved and therefore likely to be functionally important. From this alignment, a statistical profile termed a hidden Markov model (HMM) is

generated [21], which captures the characteristic sequence features of the family. The HMM is then used to search the GenBank database. Candidate sequences are admitted only after expert inspection, with careful excision of catalytic and ancillary modules in these frequently multi-domain proteins.[11] CAZy currently lists >190 glycosidase families and has grown at roughly five new families per year over the past decade.[11]

Within a family, conserved catalytic motifs allow confident inference of key amino-acid residues and, almost invariably, the stereochemical course of catalysis (retaining versus inverting). Where several functions coexist, finer-grained subfamilies can be delineated to sharpen functional predictions.[22-29] Conversely, families that share a common three-dimensional (3D) fold and superposable catalytic residues—even when their primary sequences are distant—are grouped into “clans,” reflecting the greater evolutionary conservation of structure over sequence.[30, 31]

Despite catalyzing the same fundamental chemical transformation, the hydrolysis of glycosidic bonds, glycosidases exhibit striking diversity in overall three-dimensional architecture. A relatively small number of protein folds nevertheless dominate the glycosidase landscape, most notably the $(\beta/\alpha)_8$ TIM-barrel, β-jelly-roll, lysozyme-like, $(\alpha/\alpha)_{5-7}$ solenoid, β-propeller, and Rossmann-like folds. These architectures recur across multiple CAZy families and, in some cases, across distinct mechanistic classes, reflecting extensive evolutionary re-use of these structural scaffolds. The distribution of folds across glycoside hydrolase families and clans is summarized in **Table 1**.

By grouping enzymes with similar sequences, CAZy enables rapid functional inference for the avalanche of sequences emerging from genomic and metagenomic projects, even though only a minority of entries have been experimentally characterized. The companion resource CAZypedia (www.cazypedia.org)[32] provides a continuously updated, community-curated encyclopedia of CAZy families, clans, and methodologies, further facilitating the translation of sequence space into biochemical knowledge.

Table 1. Glycoside hydrolase (GH) families according to the Carbohydrate Active enZyme (CAZy) classification (www.cazy.org), showing family, clan membership, stereochemical outcome and 3D fold. Data as of 16 Jul 2025. *Multidomain protein, fold cannot be currently assigned. Deleted/reclassified families arise when errors in biochemical characterization are reported, and no longer have any sequence content; family numbers are never reused, to avoid citation confusion.

1.3 General and specific acid/base catalysis

Acid–base catalysis involves reactions in which proton transfer between two species accelerates the transformation. The catalytic efficiency hinges on the intrinsic strengths of the participating acid or base. For non-enzymatic glycoside hydrolysis, the observed rate constant (k_{obs}) can be expressed in the following general form:

$$k_{obs} = k_{SAC}[H_3O^+] + k_{GAC}[HA] + k_{other} \quad (1)$$

where: k_{SAC} is the rate constant of specific acid catalysis,
k_{GAC} is the rate constant for general acid catalysis, and
k_{other} is the rate constant for other, non-acid catalyzed processes

For reactions catalyzed by strong acids the actual species involved is the protonated solvent (for water: H_3O^+). These reactions are termed specific acid catalyzed.[33] In these reactions, a proton is reversibly transferred to a substrate to generate a protonated intermediate, prior to the rate determining step of the reaction. The generalized equation above can be simplified as at low pH where the concentration of $[H_3O^+]$ is high, this will dominate the equation, which then simplifies to a form where the rate of reaction is proportional to the concentration of the protonated solvent:

$$k_{obs} \sim k_{SAC}[H_3O^+] \quad (2)$$

Experimentally, specific acid catalysis can be demonstrated by showing that the observed rate is inversely proportional to pH. In the case of specific acid catalyzed hydrolysis of glycosides, the proton transfer is a rapid equilibrium, and the rate of reaction is proportional to the concentration of the protonated glycoside.

Some reactions are catalyzed by weak acids (HA). Because these acids are too weak to protonate the solvent, HA is directly involved in the reaction itself, and the process is referred to as general acid catalysis. Reaction mechanisms occurring with general acid catalysis involve partial proton transfer from the weak acid to the substrate in the rate determining step. That is, in the transition state there is partial bond breaking from the acid HA and partial bond making to the substrate. Propitious conditions for the detection of general acid catalysis are when the $[H_3O^+]$ is low, but the concentration of HA is high. In practice this occurs close to the pK_a value

of HA. Under these conditions the generalized equation will simplify to a form independent of pH where the observed rate is proportional to the concentration of the weak acid:

$$k_{obs} \sim k_{GAC}[HA] + k_{other} \quad (3)$$

Experimentally, general acid catalysis can be demonstrated by observing an increase in the rate of reaction as the concentration of HA is increased at constant pH.[34]

Moving onto enzyme mechanisms involving acid-base catalysis, it is important to recognize that amino acid side chains, are weak acids and bases: they cannot fully protonate or deprotonate water to generate hydronium or hydroxide. Instead, catalysis proceeds through general acid/base catalysis, in which proton transfer occurs in concert with bond formation or cleavage. In such mechanisms, the transition state is characterized by partial bond order between the amino acid, the substrate, and the proton in transit. General acid/base catalysis enables enzymes to operate efficiently under physiological (near-neutral) pH conditions, where reaction rates scale with enzyme concentration at constant pH.

1.4 Linear free energy relationships

Whereas acid–base catalysis explains the general principles of proton transfer in glycoside hydrolysis, linear free energy relationships (LFERs; also known as linear Gibbs energy relationships) extend this analysis by providing quantitative measures of transition-state structure. By correlating logarithmic changes in reaction rates or binding affinities with systematic modifications to substrate or enzyme structure, LFERs allow one to infer the extent of bond formation, bond cleavage, or charge development in the transition state. More specifically, an LFER is constructed by plotting the logarithm of a rate constant (proportional to activation free energy, *y*-axis) against the logarithm of an equilibrium parameter or substituent constant (proportional to the energy associated with an equilibrium parameter such as an ionization constant, *x*-axis).

In glycosidase chemistry, LFERs are most useful when the enzyme accommodates structural variation at a site of the substrate such as the aglycone or C2 substituent. One commonly applied LFER in enzymology is the Brønsted relationship, in which the logarithm of the catalytic rate constant (k_{cat} or k_{cat}/K_M) is plotted against the pK_a value of the aglycone leaving group:

$$\log k_{cat} = \beta_{lg} \cdot pK_a + c$$

or

$$\log k_{cat}/K_M = \beta_{lg}' \cdot pK_a + c$$

Substrates are usually aryl glycosides, with the leaving groups chosen to span a range of pK_a values. The slope (β_{lg}), typically ranging from 0 to –1, reflects the extent of charge development on the glycosidic oxygen in the transition state, and captures proton transfer from the catalytic general acid. Steep slopes (e.g., $\beta_{lg} \approx -1$) indicate significant bond cleavage and charge build-up, while deviations from linearity, such as downward concavity, signal a switch in the rate-determining step; commonly from glycosylation to deglycosylation in retaining glycosidases. Alternatively, plots of log(k_{cat}/K_M) reflect the free energy difference between the ground state and the transition state of the first irreversible step. One caveat is that if the rate-determining step includes a partially non-chemical process (e.g., conformational change), the correlation may be obscured.

Another LFER tool used to probe electronic and steric influences is the Taft-like relationship, which employs the Taft σ* parameter, an experimental measure of the combined polar and steric effects of a substituent:

$$\log k_{cat}/K_M = \rho \cdot \sigma^* + c$$

This relationship has been applied to glycosidases that rely on neighboring group participation, particularly in studies involving C2-fluorinated analogues of N-acetylated sugars. In these cases, the slope (ρ) can be parsed into steric (δ) and polar (ρ*) contributions, depending on how permissive the enzyme active site is to size or electronic changes. For example, enzymes that strictly discriminate between hydrogen and fluorine may show δ-dominated effects, while those more permissive for steric variation will allow the electronic influence to predominate.

An LFER that allows assessment of transition state mimicry by an inhibitor is the Bartlett relationship,[35] which compares catalytic efficiency (log k_{cat}/K_M) with the binding affinity (log $1/K_I$) of an inhibitor:

$$\log K_M/k_{cat} = \alpha \cdot \log K_I + c$$

This method can be implemented in two distinct ways: (i) by introducing parallel structural changes to both the substrate and inhibitor, or (ii) by creating enzyme variants and measuring how each mutation affects both catalysis and inhibitor binding. A slope (α) of ~1, coupled with a strong correlation ($R^2 \approx 1$), supports the view that the inhibitor closely mimics the transition state, experiencing the same energetic sensitivities as the real transition state does to structural or environmental changes. Lower slopes ($0 < \alpha < 1$) indicate partial mimicry, while $\alpha \approx 0$ suggests a ground-state analogue. Caution is needed, as such analyses assume that binding poses remain conserved across variants, that the catalytic mechanism is unchanged, and that a single rate-limiting step dominates throughout. However, a key limitation of the enzyme variant approach is that catalytic residues cannot be altered, as such mutations inevitably disrupt the mechanism itself.

1.5 Non-enzymatic mechanisms of glycoside hydrolysis

Glycosides are acetals and, like their acyclic counterparts, undergo acid-catalyzed hydrolysis through pathways that pass via true or incipient oxocarbenium ion intermediates.[36] In simple acyclic acetals the reaction follows an S_N1 mechanism and produces discrete oxocarbenium ions that are resonance-stabilized by the adjacent oxygen.[37-39] Glycopyranoside hydrolysis proceeds analogously: rapid, reversible protonation of the glycosidic oxygen is accompanied by rate-determining heterolytic C–O bond cleavage.[40] Whether a bona-fide cation forms, or only a highly dissociative transition state, depends on substituents and medium polarity; the lifetime of the glucopyranosyl cation in water is a mere 1–3 ps.[39, 41] Acid-catalyzed cleavage of alkyl furanosides provides a counter-example, proceeding by an S_N2-type ring-opening rather than by furanosyl-cation formation.[42]

Acid hydrolysis of acetals is a case of specific acid catalysis in which hydronium (H_3O^+) is the catalyst and the rate is governed directly by its concentration (i.e., by pH). Although glycosyl cations can be observed under exotic conditions such as the gas phase,[43] superacidic HF/SbF_5 at low temperature,[44] or electrochemical ion pools with weakly nucleophilic counter-ions,[45] those media bear little resemblance to the aqueous realm of enzymes.

More commonly, substitution reactions of glycosides lie on a continuum between the S_N1 and S_N2 extremes. This continuum can be visualized with a two-dimensional More O'Ferrall–Jencks diagram (**Figure 3**), in which C–O bond cleavage (*x*-axis) and C–Nu bond formation (*y*-axis) are tracked simultaneously.[46] An ideal concerted S_N2 reaction follows the diagonal from

lower left to upper right with no stable intermediate. A stepwise S_N1 process first moves along the *x*-axis in the first rate-determining step to the fully dissociated oxocarbenium ion corner and then rapidly ascends the *y*-axis as the nucleophile attacks. Most real reactions trace curved paths between these limits, their exact location reflecting the degree of nucleophilic participation. A carbohydrate example at the concerted end is the reaction of α-glucopyranosyl fluoride with azide, a powerful nucleophile; it proceeds through a highly dissociative 'loose' S_N2-like transition state in which the C–F bond is almost fully broken while the C–N_3 bond is only partly formed.[47]

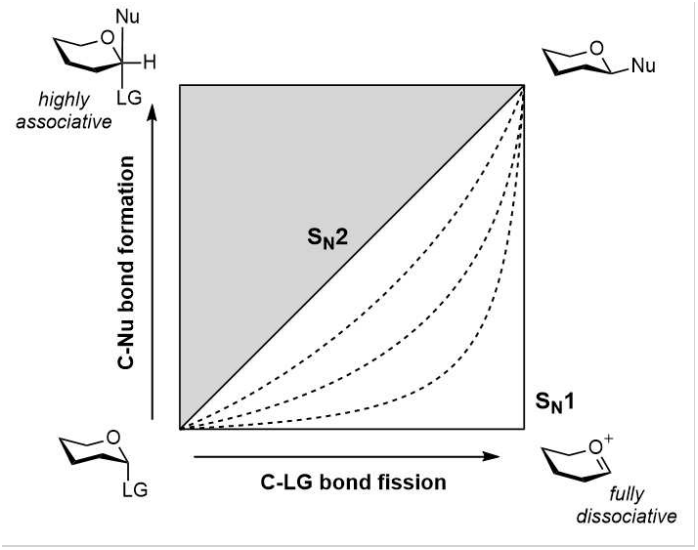


Figure 3. More O'Ferrall–Jencks diagram showing spectrum of pathways for substitution reactions of a glycoside. Trajectories above the diagonal are forbidden for saturated carbon but become accessible at hypervalent or heteroatom centers (e.g., P(V), S(VI)). Adapted with permission from Ref.[48] Copyright 2019 Elsevier Ltd.

1,2-*trans*-Glycosides can also be hydrolyzed under strongly basic conditions through a mechanism involving neighboring-group participation by the C-2 hydroxyl.[49, 50] Deprotonation gives a 2-oxyanion, which in the rate-limiting step attacks C-1 intramolecularly to generate a 1,2-epoxide (glycal epoxide). Subsequent hydroxide attack at C-1 opens the epoxide, and reprotonation furnishes the sugar hemiacetal. The rate is proportional to [OH^-], an example of specific base catalysis.

Such specific acid- or base-driven mechanisms are unavailable to enzymes, which operate near neutral pH where [H_3O^+] and [OH^-] are very low. Under these conditions

glycosides are astonishingly stable—methyl α-D-glucopyranoside has a half-life on the order of 100 million years at room temperature in dilute neutral buffer[5]—because water is a poor nucleophile and an unactivated aglycon is an exceptionally poor leaving group. Yet glycosidases routinely achieve k_{cat} values >10^3 s^{-1}.[5] How do they achieve such prodigious rate accelerations? The next sections examine the catalytic solutions that enzymes have evolved.

2. Glycosidase mechanisms

2.1 The three Koshland glycosidase mechanisms

In a landmark review Koshland outlined a general theory of glycosidase catalysis that explained retaining and inverting stereochemical outcomes, as well as how glycosidases utilize acid and base catalysis at or around neutral pH.[13] Building on the idea that non-enzymatic specific acid-catalyzed acetal hydrolysis involves protonation of the exocyclic acetal oxygen, which converts it into a leaving group, he postulated three distinct glycosidase mechanisms involving general acid catalysis: one that leads to inversion of anomeric configuration and two that lead to retention.

For stereochemically-inverting enzymes, Koshland proposed a one-step mechanism that involves two enzymatic residues in the active site, one acting as general acid while the other acts as general base. He proposed that the general acid residue would protonate the glycosidic oxygen (**Figure 4a**), while at the same time a water molecule attacks the anomeric center as it is deprotonated by the general base residue, displacing the aglycon and furnishing the hydrolysis product with inversion of configuration (axial to equatorial in the example shown).

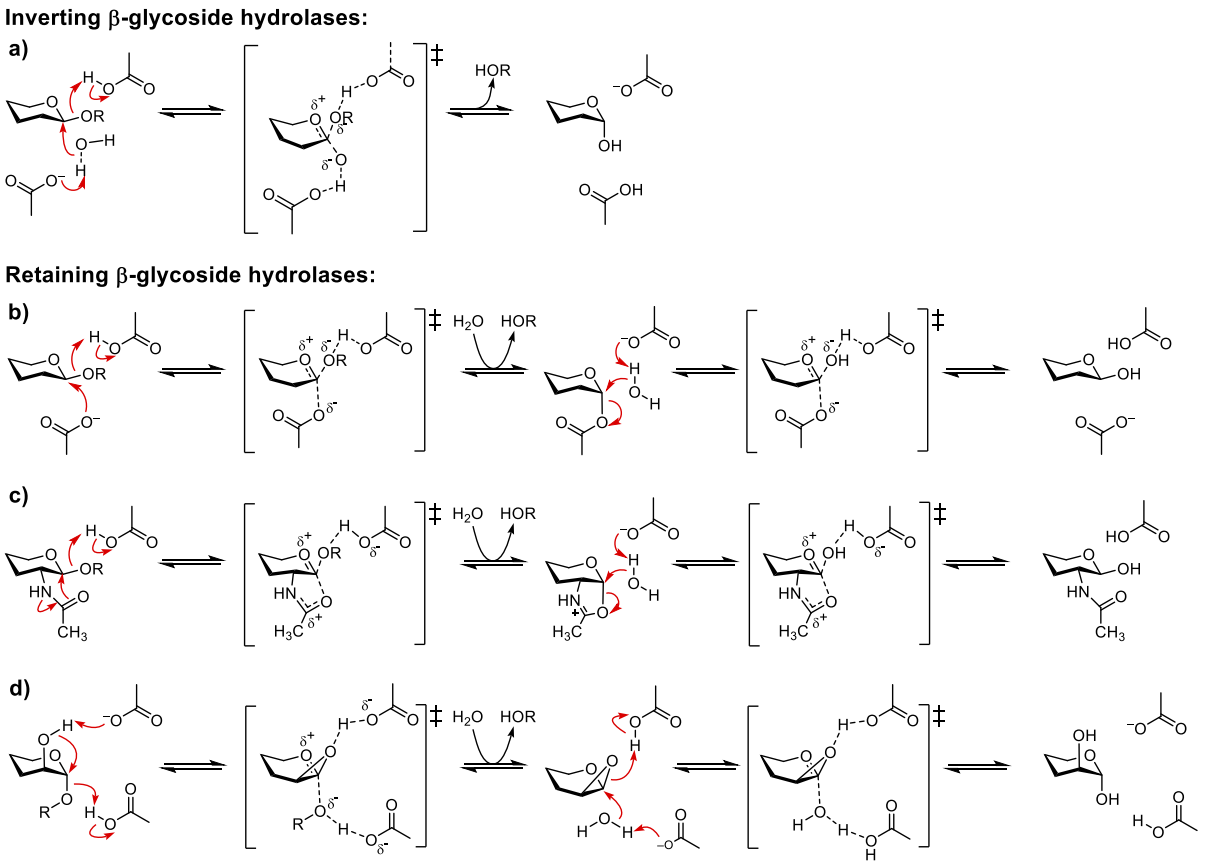


Figure 4. Mechanisms and transition states of glycosidases that proceed through (a) a one-step stereochemically-inverting mechanism, and glycosidases that use two-step stereochemically-retaining mechanisms involving an enzymatic (b) carboxylate residue, (c) a substrate 2-acetamido residue, and (d) a substrate 2-hydroxyl group.

For stereochemically-retaining enzymes, Koshland advanced two different two-step mechanisms. Both involve an enzymatic general acid/base and proceed via an intermediate with inverted configuration. The first retaining mechanism involves an enzymatic nucleophile, which attacks the anomeric carbon, while the general acid simultaneously protonates the glycosidic oxygen, resulting in a substitution reaction with inversion of configuration, and formation of a covalent glycosyl-enzyme intermediate (**Figure 4b**). In the second step, a water molecule attacks the anomeric carbon, while being deprotonated by the now anionic residue that acts as general base, cleaving the glycosyl-enzyme intermediate and forming a sugar hemiacetal with a second inversion of configuration. Overall, this hydrolysis reaction leads to a net retention of configuration.

Koshland's second stereochemically-retaining mechanism invoked a neighboring nucleophile that is part of the substrate, and which attacks the anomeric carbon, while the residue acting as general acid simultaneously protonates the glycosidic oxygen (**Figures 4c,d**). This leads to the formation of a cyclic intermediate that is not covalently bound to the enzyme. In the second step a water molecule attacks the anomeric center of this cyclic intermediate with general base assistance by the other residue. This leads to a second inversion, regenerating the neighboring group, and leading to an overall net retention of anomeric configuration.

At the time of Koshland's writing, the residues involved in the proposed mechanisms were unknown. Today, we know that the general acid and base residues are typically the amino acid side chains of aspartate and/or glutamate, and less commonly histidine. It has been proposed that asparagine or glutamine may also provide acid/base catalysis via their imidic tautomers.[51] The nucleophilic residues are most commonly aspartate or glutamate, and less commonly tyrosine[52] or cysteine.[53] One special case are plant myrosinases, which catalyze the hydrolysis of thioglycosides called glucosinolates, and which lack an enzymatic general acid/base, and instead use ascorbate.[54] Finally, for the neighboring group participation mechanism, these involve a 2-acetamido group leading to an oxazoline (or oxazolinium) cyclic intermediate,[55-57] or a 2-hydroxyl group leading to an epoxide intermediate,[58] and will be discussed below. Other key questions that were unanswered at the time of Koshland's review include:

- What is/are the nature of the transition state(s) in the enzyme-catalyzed reactions?
- What are the conformational changes a substrate glycoside undergoes along the catalysis reaction coordinate?
- Are there other mechanisms for enzymes that catalyze glycoside hydrolysis using "non-hydrolytic" cleavage?

The pursuit of answers to these questions has helped to guide our still growing understanding of glycosidase catalysis, and application of this knowledge has enabled the development of chemical tools to study glycosidases and new applications of glycosidases in synthesis. Remarkably, Koshland's core hypotheses have largely stood the test of time with just a few examples of other mechanisms of hydrolysis, and of other mechanisms for non-hydrolytic cleavage.

2.2 Evidence for covalent glycosyl-enzyme intermediates on retaining glycosidases

Experimental support for covalent glycosyl-enzyme intermediates in Koshland's proposed mechanisms for retaining glycosidases comes chiefly from kinetic isotope-effect (KIE) analysis. Typically, two substrates are synthesized, one bearing an atom at natural abundance (e.g. ^{1}H, ^{12}C, ^{16}O), while the other bears a heavier isotope (e.g. ^{2}H, ^{13}C, ^{18}O) at the center of interest. The KIE is the ratio of the rate constants (or rates at equal concentrations) (k_{light}/k_{heavy} = KIE) measured for the individual isotopic isomers (isotopomers).[59] Because isotopic substitution changes only nuclear mass through varying the number of neutrons, not electronic structure, KIEs probe subtle changes in bond order and geometry between the ground state and the transition state:

- Primary KIEs arise when the isotopically labelled bond is broken in the rate-limiting step. The heavier bond vibrates at a lower frequency (lower zero-point energy) and therefore requires more energy to cleave, giving $k_H > k_D$ and a normal effect (KIE > 1).
- Secondary KIEs report on re-hybridization or inductive changes at a site neighboring that where bonds are broken. Because the bond involving the isotope is not broken during the reaction it can report on changes in vibrational frequencies in the transition state relative to the ground state. In this case effects may be >1 (termed normal KIEs) or <1 (termed inverse KIEs).

For a retaining glycosidase that operates via a covalent intermediate, theory predicts large and normal α-deuterium secondary KIEs for both the glycosylation and deglycosylation steps, reflecting changes from an sp^3-hybridized ground state (a glycoside or a glycosyl-enzyme intermediate) to sp^2-hybridized transition states on *both* sides of the expected covalent glycosyl-enzyme intermediate (**Figure 5**).

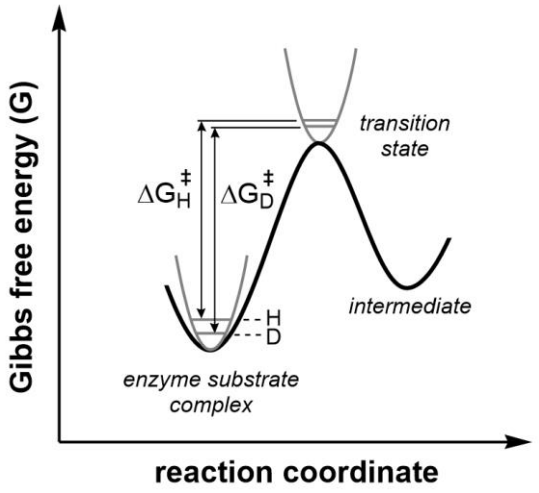


Figure 5. Illustration of a normal α-secondary KIE on the first (glycosylation) step for a deuterium-labelled glycoside cleaved by a retaining glycosidase. The black trace depicts the reaction coordinate; grey curves represent the potential-energy wells of the ground state (left) and transition state (right). In the sp^3 ground state the C–H bond is stiffer (higher force constant) than C–D, giving a larger zero-point-energy gap between isotopomers. At the sp^2-like transition state the C–H/D bond is weakened, the energy gap narrows, and the heavy isotopomer is comparatively stabilized. The net effect is $k_H > k_D$, i.e., a normal secondary KIE (> 1).

To dissect the individual glycosylation and deglycosylation half-reactions experimentally, substrates are required for which each step can be made independently rate-limiting. Aryl glycosides are ideal because the leaving group ability of the aglycone (measured as the pK_a value of the conjugate acid) can be systematically tuned by varying aromatic substituents. Seminal studies by Sinnott and Souchard on *Escherichia coli* β-galactosidase,[60] which built on earlier work by Nath and Rydon on almond β-glucosidase,[61] established the use of linear free energy relationships in the form of Brønsted plots (log k_{cat} versus aglycone pK_a values) to identify the step that limits the rate of turnover. This now classical approach is well illustrated by more recent work on the *Agrobacterium* sp. family GH1 β-glucosidase (Abg), which exhibits a biphasic, concave-downward Brønsted relationship (**Figure 6**).[62] For substrates with poor leaving groups (high pK_a values), a steep slope (β_{lg} = –0.7) demonstrated that glycosylation was rate-limiting, whereas for better leaving groups (low pK_a values), log k_{cat} became independent of aglycone pK_a value ($\beta_{lg} \approx 0$), consistent with deglycosylation as the rate-limiting step. This mechanistic switch was further corroborated by the observation of 'burst' kinetics in the pre-steady state for substrates with leaving groups of low pK_a values.[62, 63]

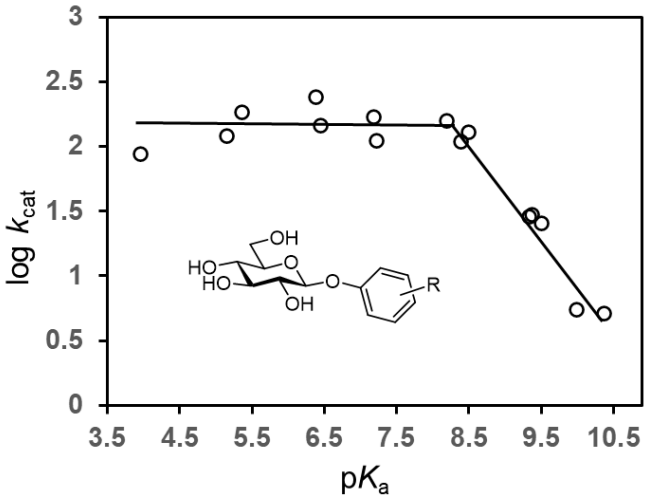


Figure 6. Brønsted plot for a series of aryl β-glycosides with *Agrobacterium* sp. β-glucosidase.[62]

Next, α-deuterium secondary KIEs were measured for Abg using substrates labelled with deuterium at C1 and with the anomeric leaving group chosen to be rate-limiting for the glycosylation or deglycosylation steps, guided by the results of the Brønsted plot.[62] For 4'-bromophenyl glucoside (pK_a 9.34, rate-limiting step: glycosylation) the measured α-deuterium secondary KIE was k_H/k_D = 1.06, and for 2,4-dinitrophenyl glucoside (pK_a 3.96, rate-limiting step: deglycosylation) the value was k_H/k_D = 1.10. These values correspond to normal (i.e. ≥ 1) α-deuterium secondary kinetic isotope effects and thus indicate a change from an sp^3-hybridized ground state to an sp^2-hybridized transition state at the anomeric center for both steps. This data supports the formation of a covalent, tetrahedral (sp^3-hybridized) glycosyl-enzyme intermediate, distinguishing it from mechanisms that involve an oxocarbenium ion intermediate.

Insights into the nature of the transition state have been obtained with retaining and inverting α-glucosidases. α-Deuterium secondary KIEs for the hydrolysis of α-glucopyranosyl fluoride by retaining sugar beet α-glucosidase (k_H/k_D = 1.10) and inverting *Rhizopus niveus* α-glucosidase (k_H/k_D = 1.26) were normal,[64] which are consistent with the formation of oxocarbenium ion-like transition states for both.

As noted above, earlier studies have shown that glycosyl cations in aqueous solution have an extremely short lifetime—so brief that their existence lies at the borderline of being a true intermediate. Given this inherent instability, and the presence of nucleophilic groups positioned near the reaction center within glycosidase active sites, we can infer that any

oxocarbenium ion formed during enzymatic catalysis is, at most, an incipient species that would rapidly convert to an associative species without an energy barrier. Collectively, the three Koshland mechanisms can therefore be considered to involve 'exploded' transition states having substantial oxocarbenium ion character. In such transition states, both leaving group and incoming nucleophile are loosely associated, and the relative degree of participation of each group will depend on the substrate and enzyme, and can be visualized with a Moore O-Ferrall-Jencks diagram through off-diagonal trajectories. In all three cases, in the transition state the leaving group (aglycon) is partially protonated by the general acid. The nucleophile comprises partially deprotonated water for inverting glycosidases, and either the active site enzyme nucleophile or a partially deprotonated neighboring group (acetamide, hydroxyl) for retaining glycosidases that rely upon neighboring group participation.

2.3 Covalent labelling of active site residues with electrophilic sugar derivatives

The use of reactive small molecules as mechanistic probes has been highly successful in elucidating the catalytic mechanisms of glycosidases. In particular, sugar-like reagents that form covalent linkages with the enzyme, commonly termed affinity labelling reagents, have played a formative role in identifying the residues directly involved in catalysis.[65] These include various sugars and sugar-like molecules bearing strategically positioned electrophilic groups have been developed as covalent labelling reagents for glycoside hydrolases, and their application provided some of the earliest experimental evidence for the identities and functional roles of catalytic residues.[66, 67]

An early example is provided by conduritol B epoxide, an electrophilic cyclitol analogue of glucose that undergoes time-dependent inactivation of a variety of α- and β-glucosidases (**Figure 7a**).[68-71] Use of radiolabelled conduritol B epoxide enabled direct identification of covalently modified residues in several enzymes, providing early experimental support for the involvement of nucleophilic carboxylates in catalysis.[70, 72] However, subsequent work revealed that conduritol epoxides may label residues other than the catalytic nucleophile, complicating mechanistic interpretation in some systems.[73] Other important examples of related glycosidase inhibitors include conduritol aziridine[74] and cyclophellitol,[75, 76] which together have laid the foundation for the development of the cyclitol epoxides/aziridines as activity-based reagents for glycosidase profiling, as discussed Section 8.2.

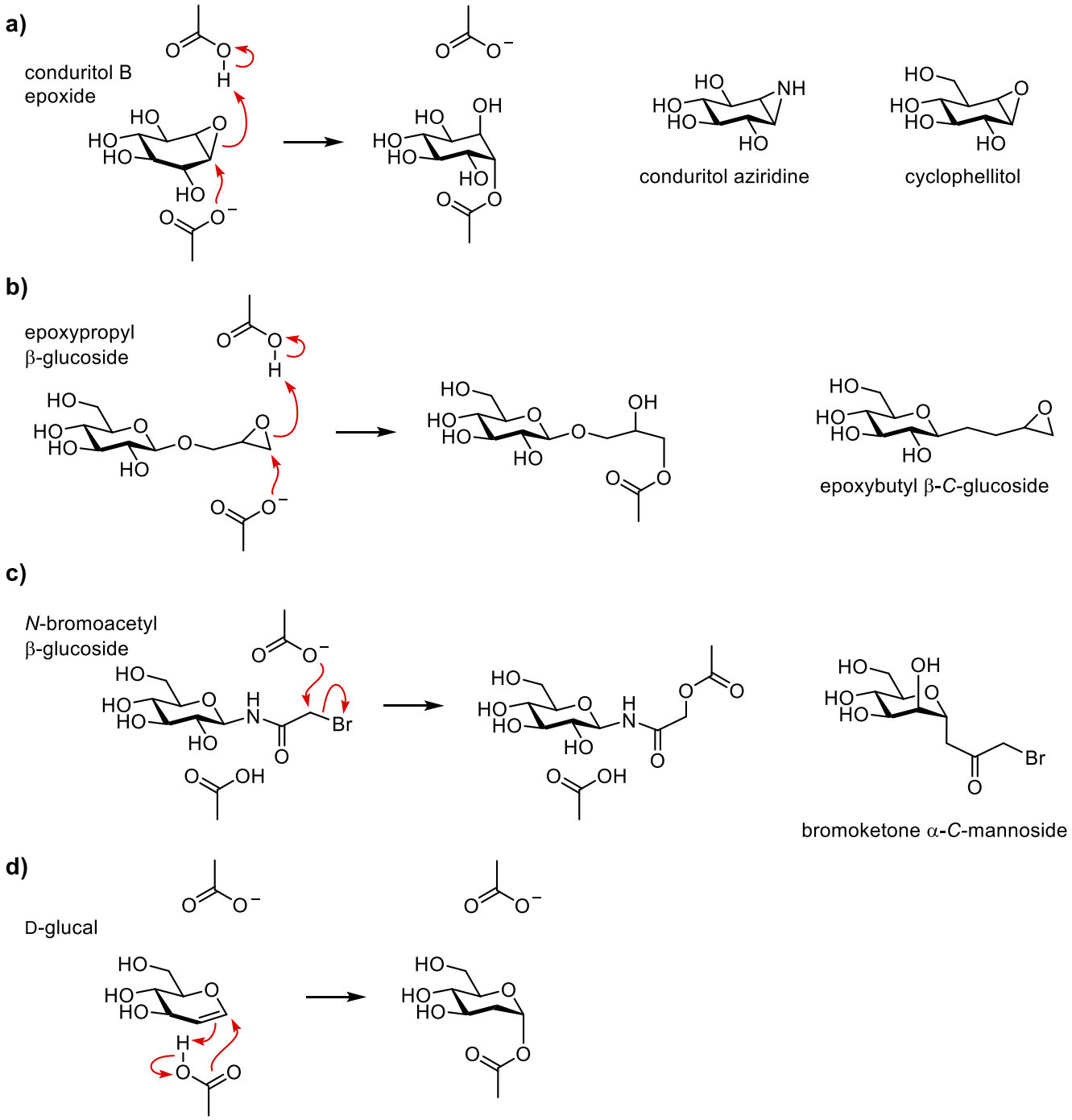


Figure 7. Reaction mechanisms for covalent labelling of active site residues of retaining β-glucosidases with electrophilic sugar derivatives.

Closely related in spirit, and also historically important, are the epoxyalkyl glycosides, in which an epoxide-bearing alkyl chain (most commonly propyl, butyl, or pentyl) is appended to a glycone that matches the substrate preference of the target enzyme (**Figure 7b**). These compounds were originally prepared to study endoglycosidases,[77] and act as time-dependent irreversible inhibitors, forming covalent adducts that can be identified either by mass

spectrometric analysis of proteolytic peptides or, in favorable cases, directly by X-ray crystallography.[65] C-glycoside variants have been developed to reduce the propensity for these compounds to be cleaved by the target glycosidases.[78] Early studies suggested that epoxyalkyl glycosides selectively labelled the catalytic nucleophile of retaining glycosidases. Later investigations, however, demonstrated that covalent modification of the catalytic acid/base residue can also occur, depending on the chain-length of the epoxyalkyl group, a result of the flexibility of the alkyl chain to the epoxide.[79] These findings underscored both the utility and the limitations of this inhibitor class as mechanistic probes.

Another important class of affinity labelling reagents comprises the α-halocarbonyl glycosides, exemplified by *N*-bromoacetyl β-glycosylamines and bromoketone *C*-glycosides (**Figure 7c**).[65] These compounds typically act as time-dependent inactivators and show a preference for modification of the catalytic acid/base residue. *N*-Bromoacetyl β-glycosylamines are easily prepared from sugar hemiacetals in just two steps,[80] and have been used to identify acid/base residues in retaining β-glycosidases,[81-83] although examples of labelling one or more other residues have been reported.[82, 84, 85] Bromoketone *C*-glycosides were developed to target α-glycosidases, circumventing the synthetic challenges associated with *N*-bromoacetyl α-glycosylamines, and similarly favor labelling of the catalytic acid/base.[86, 87]

One final class of historically important affinity labelling reagents are glycals, which are often effective time-dependent inhibitors of classical retaining glycosidases that use an enzymatic carboxylate nucleophile (**Figure 7d**). This behavior arises from a chemical reaction in which the conjugate acid of the catalytic nucleophile protonates the enol ether of the glycal, leading to formation of a covalent 2-deoxyglycosyl–enzyme intermediate.[88, 89] This reactivity has been exploited to identify catalytic nucleophiles by peptide sequencing,[89] and the resulting 2-deoxyglycosyl–enzyme complexes may be sufficiently stable to permit structural characterization by X-ray crystallography.[90] Glycals are ineffective against other classes of retaining glycosidases such as those that use neighboring group participation by a 2-acetamido group[91] or a 2-hydroxy group,[92] or a tyrosine nucleophile,[93] and instead bind as simple competitive inhibitors.

Although the above reagents are now largely superseded by more selective tools, their synthesis, deployment, and analysis were pivotal steps in the historical development of mechanism-based glycosidase inhibitors. As discussed in the following sections, fluorosugars and cyclophellitol epoxides/aziridines ultimately proved superior in terms of mechanistic

specificity and generalizability. By enabling controlled trapping of covalent intermediates with minimal off-pathway reactivity, fluorinated substrates resolved several ambiguities associated with earlier affinity labels and became central to modern structural and kinetic dissection of glycosidase mechanisms.

2.4 2-Deoxy-2-fluoro- and 5-fluoro sugars to identify enzymatic nucleophiles of retaining glycosidases

As discussed earlier, Koshland proposed that hydrolysis by retaining glycosidases proceeds via a covalent glycosyl–enzyme intermediate, which is both formed and hydrolyzed through transition states with substantial oxocarbenium ion character. Withers and co-workers exploited this feature and introduced activated fluorosugars as mechanism-based inactivators of retaining glycosidases (**Figure 8**).[94] Substitution of a fluorine atom adjacent to the anomeric carbon at C2 (by deoxyfluorination, e.g., 2FGlcF and DNP-2FGlc)[95] or at the ring oxygen at C5 (by dehydrofluorination, e.g., 5FGlcF)[96] destabilizes the transition states for both glycosylation and deglycosylation. However, installation of a highly activated anomeric leaving group such as fluoride or 2,4-dinitrophenolate selectively accelerates the first step, rendering glycosylation kinetically accessible while leaving deglycosylation slow. The net result is accumulation of a 2-deoxy-2-fluoro- or 5-fluoro-glycosyl enzyme adduct, through mechanism-based inactivation. Because these inhibitors form covalent linkages to the catalytic nucleophile, they provide a powerful means of nucleophile identification through techniques such as mass spectrometry and/or X-ray crystallography.[65, 94] In the mass spectrometric approach, following inactivation, the covalently-modified enzyme is proteolytically digested to yield short peptides, which are purified by reversed-phase HPLC and analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS), allowing sequencing of the glycosylated peptide and unambiguous assignment of the nucleophilic residue.[97] This approach proved superior to earlier approaches using radiolabeled fluorosugar inhibitors along with Edman sequencing.[98] The precise identification of the catalytic nucleophile of a range of retaining glycosidases belonging to different families supported the development of clans of glycosidases, grouping enzymes with conserved folds and active site residues.[31]

Inactivation by fluorosugars is time-dependent, reflecting the chemistry of covalent modification. The stability of the trapped fluoroglycosyl–enzyme intermediate varies widely across enzymes, with half-lives ranging from milliseconds to days. Hydrolysis ultimately

regenerates enzyme activity with release of the fluorosugar hemiacetal; likewise reactivation can also be achieved by transglycosylation to a suitable sugar acceptor.[97] Consequently, the fluoroglycosyl enzyme can be considered catalytically competent,[98] and fluorosugars behave as slow substrates. Because the release of the anomeric group occurs in strict 1:1 stoichiometry with enzyme inactivation, fluorosugar inactivators can be used as active-site titration reagents.[99] Importantly, these same features underlie their subsequent development as activity-based protein profiling (ABPP) probes, as discussed in Section 8.2.

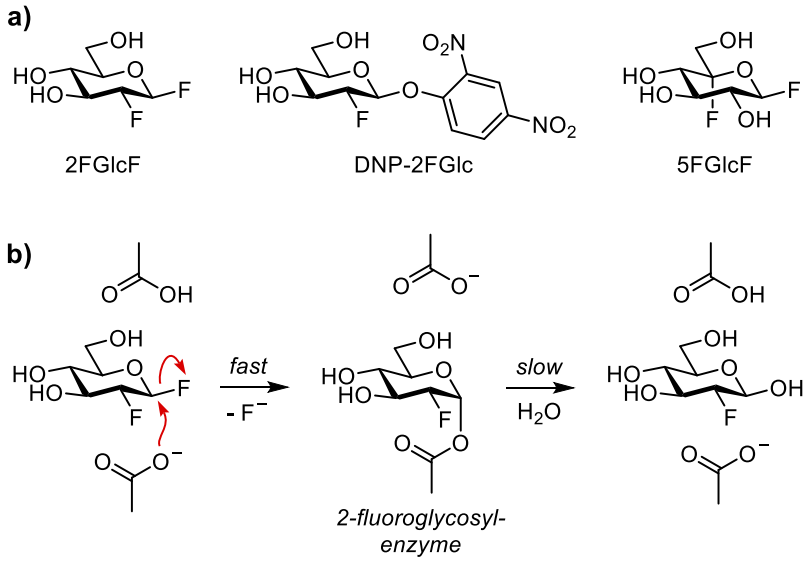


Figure 8. Fluorosugars as mechanism-based inhibitors of retaining glycosidases that use an enzymatic nucleophile. (a) Examples of 2-deoxy-2-fluoro and 5-fluoro sugar inactivators. (b) Mechanism of inactivation of a retaining β-glucosidase by 2-deoxy-2-fluoroglucosyl fluoride.

2.5 Mechanism of HEWL: a retaining glycosidase that uses an enzymatic nucleophile

Hen egg white lysozyme (HEWL) belongs to family GH22 and catalyzes the hydrolysis of (1,4)-β-linkages in peptidoglycan and related oligosaccharides, including chitooligosaccharides composed of repeating *N*-acetyl-D-glucosamine residues. It acts with retention of anomeric stereochemistry, as demonstrated by monitoring the products of glycosyl transfer to methanol.[100, 101] A mechanism involving neighboring group participation by the 2-acetamido group can be ruled out because substrates bearing hydrogen and hydroxyl substitutions at 2-position are hydrolyzed at similar rates to the parent compound bearing a 2-acetamido group.[102, 103] In the 1960s, Phillips and coworkers used X-ray crystallography to determine a series of three-dimensional (3D) structures of HEWL, both alone[104] and in complex

with substrates and inhibitors.[105] The HEWL structure was a watershed accomplishment, being one of the first high-resolution protein 3D structures and the first enzyme 3D structure to be resolved. A notable structure from Phillips' work was the complex of HEWL with chitohexaose, which provided insights into how the enzyme interacted with its substrate.[105] For the protein regions binding the chitobiose units flanking the fissile glycosidic bond (now recognized as the –1 and +1 subsites), Phillips and coworkers observed critical contributions from Glu35 and Asp52. They proposed that Glu35 functioned as the general acid/base catalyst, while Asp52 stabilizes an intermediate carbonium ion.[106] In a later study of a 3D structure of a peptidoglycan-derived trisaccharide bound to HEWL, Strynadka and James argued that Asp52 could not extend far enough to form a covalent intermediate without disrupting the local structure of the enzyme surrounding this residue.[107]

There is a conflict between the intermediates in the retaining glycosidase mechanisms proposed by Phillips for HEWL and by Koshland. Phillips proposed that HEWL operates through a mechanism that involves a non-covalent oxocarbenium ion intermediate.[106] In contrast, application of Koshland's mechanisms invokes a covalent glycosyl-enzyme intermediate, which has been convincingly demonstrated on many retaining glycosidases using approaches such as kinetic isotope effects (KIE) and studies with 2- and 5-fluorosugars, as described above. Consequently, reevaluating HEWL's mechanism using modern methods was essential considering this growing body of evidence. The role of Glu35 as a general acid/base is now widely accepted in HEWL,[108] and the Koshland model of a covalent glycosyl-enzyme intermediate has been firmly established for HEWL through various methods, as discussed below (**Figure 9a**).

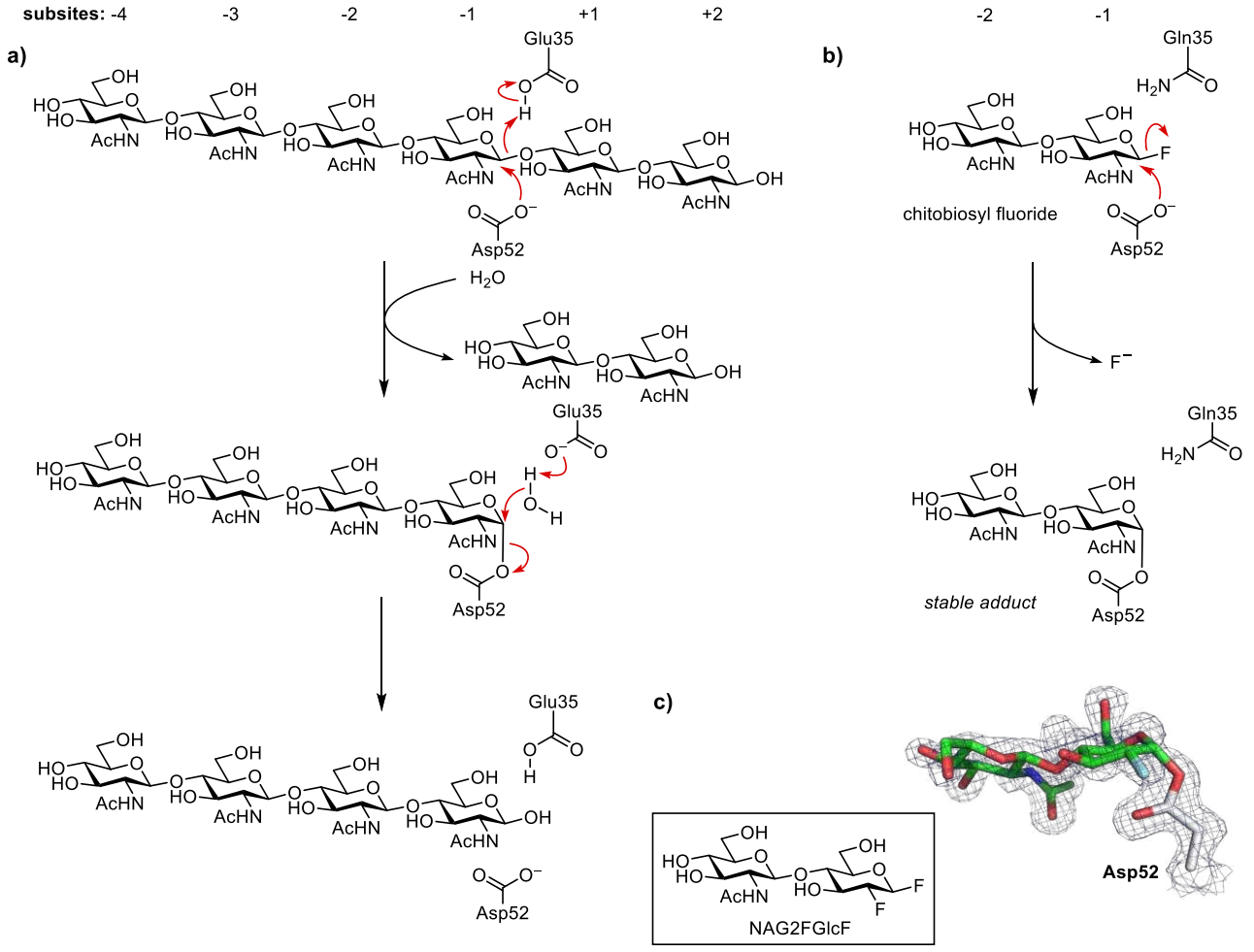


Figure 9. Experimental insights into the retaining mechanism of HEWL. (a) Retaining mechanism of HEWL acting on chitohexaose. (b) Cleavage of β-chitobiosyl fluoride by Glu35Gln HEWL. (c) X-ray structure of the covalent glycosyl enzyme formed by the reaction of NAG2FGlcF (inset) with Glu35Gln HEWL (PDB 1h6m), $2F_o - F_c$ map contoured at 0.4 e/Å^3.

Early insights into the nature of the transition state of HEWL were obtained through KIE measurements using a C1-tritiated substrate, which revealed a large normal k_H/k_T of 1.19, and which was interpreted as evidence for an oxocarbenium ion-like *transition state*.[109] The authors argued that the oxocarbenium ion-like transition state is subsequently converted to a covalent intermediate, which was considered to be a glycosyl-enzyme formed with Asp52 (an alternative oxazoline intermediate involving the substrate *N*-acetyl group was considered, but, as discussed earlier, this is inconsistent with the known substrate tolerance of 2-hydroxylated sugars).[109] More direct evidence in support of a covalent glycosyl-enzyme intermediate in HEWL was late obtained through deliberate perturbation of the system by a combination of site

directed mutagenesis of HEWL and chemical variation of the substrate. Specifically, replacement of Glu35 with a non-acidic glutamine residue (HEWL Glu35Gln) was paired with the use of β-chitobiosyl fluoride, in which fluoride serves as an excellent leaving group (**Figure 9b**).[110] Because β-chitobiosyl fluoride does not require general acid catalysis for cleavage the first displacement step can proceed even in the absence of an acidic Glu35 residue. In contrast, hydrolysis of the resulting intermediate still requires Glu35 to function as a general base. As a consequence, incubation of HEWL Glu35Gln with β-chitobiosyl fluoride led to accumulation of a stable covalent glycosyl–enzyme species, which was directly detected by mass spectrometry and assigned to modification of Asp52.

The lifetime of this intermediate was extended further by replacing the 2-acetamido group of the chitobiose moiety with fluorine. X-ray crystallographic analysis of the complex formed upon reaction with NAG2FGlcF provided unambiguous structural evidence for a covalent glycosyl-enzyme on HEWL (**Figure 9c**).[110] Importantly, this structure demonstrated that Asp52 is able to form a covalent bond to the anomeric centre without requiring any substantial distortion of the enzyme fold.

Additional evidence for covalent glycosyl–enzyme intermediates has been obtained in systems that require little or no chemical or genetic perturbation. In a study of a xyloglucan *endo*-transglycosylase, mechanism-based labelling was achieved using an electronically unactivated, native xyloglucan-derived substrate and a wild-type enzyme, allowing direct observation of a covalent glycosyl–enzyme intermediate without the use of activated leaving groups, fluorinated sugars, or catalytic-residue mutations.[111] This example demonstrates covalent intermediate formation in a physiological transglycosylation system, supporting the relevance of the retaining double-displacement mechanism under native conditions. Related support comes from work on the glycosyltransfer activity of glycogen-debranching enzyme, in which a wild-type enzyme was trapped using 4-deoxy-α-maltotriosyl fluoride.[112] This substrate acts as an incompetent acceptor for self-transglycosylation, leading to accumulation of a covalent glycosyl–enzyme intermediate, which is only released in the presence of a competent acceptor bearing a 4-hydroxyl group. Peptic digestion of the trapped enzyme followed by tandem mass spectrometric analysis of the resulting peptides provided evidence for a covalent linkage to the catalytic nucleophile, Asp549. Together, these examples represent minimally perturbed systems and counter suggestions that covalent intermediates arise only from fluorosugar substitution, highly activated substrates, or enzyme mutagenesis.

2.6 Glycoside hydrolases that use neighboring group participation

Neighboring group participation by a 2-acetamido group was first implicated for some retaining β-hexosaminidases on the basis of: (1) the 3D structure of an inhibitor-enzyme complex of the GH18 chitinase hevamine with allosamidin, a natural product that includes an allosamizoline group that mimics the proposed oxazolinium ion intermediate;[55] (2) the acquisition of the structure of the family GH20 chitobiase from *Serratia marcesans* in which chitobiose was bound, serendipitously unhydrolyzed, across the -1 and +1 subsites, and with the carbonyl of the acetamido group positioned below C1;[56] and (3) potent inhibition of jack bean GH20 exo-β-hexosaminidase by NAG-thiazoline, a chemically-inert molecule that resembles the proposed oxazoline/oxazolinium ion intermediate.[57] Unambiguous evidence for the involvement of the neighboring 2-acetamido group in catalysis has been obtained using substrates that varied in the number of fluorine atoms substituted on the *N*-acetyl group.[113-115] Increasing the number of fluorine atoms decreases the nucleophilicity of the carbonyl oxygen, resulting in a monotonic reduction in the value of k_{cat}/K_M. This can be formally assessed by linear free energy analysis in which the electronic Taft parameter (σ^*) is plotted on the *x*-axis against $\log(k_{cat}/K_M)$ on the *y*-axis. In the case of human β-hexosaminidase from family GH20, this revealed a strongly negative correlation ($\rho = -1$), which was interpreted as suggesting that the carbonyl oxygen acts as a nucleophile, attacking the anomeric center (**Figure 10**).[115]

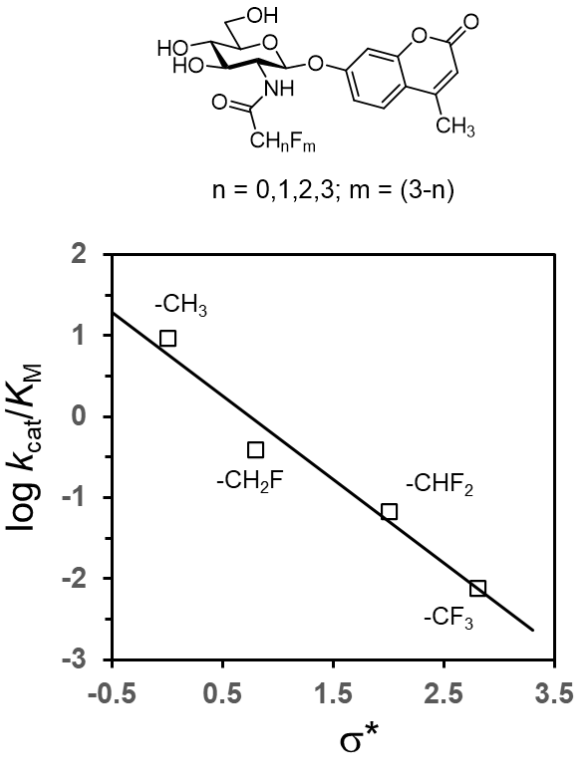


Figure 10. Linear free energy plot of the Taft parameter (σ^*) of the fluoroacetyl substituent of 4-methylumbelliferyl fluoroacetyl-β-D-glucosaminides versus log k_{cat}/K_M for human β-hexosaminidase.[115]

Neighboring group participation by a 2-hydroxyl group was implicated for a retaining endo-α-mannosidase of family GH99 based on a 3D structure of an inhibitor-enzyme complex in which no candidate nucleophile was evident, with minimal ring distortion, and an axial 2-hydroxyl interacting with an adjacent carboxylate residue proposed to act as general base.[116] Direct evidence in support of neighboring group participation was obtained through kinetic isotope effects observed at key reaction sites including C1 and O2.[58] In particular, the $^{16}O/^{18}O$ kinetic isotope effect for the C2-O was 1.052, directly implicating nucleophilic participation by the C2 hydroxyl.[50]

2.7 Geometry of leaving group protonation by general acid residues

Heightman and Vasella,[117] later elaborated by Nerinckx and co-workers,[118] analyzed three-dimensional structures of glycosidases bound to ligands and observed that the general acid/base residue is typically arranged approximately laterally to the plane of the sugar ring.

Two lateral geometries were found, involving protonation of the glycosidic oxygen on the same side (syn) or the opposite side (anti) relative to the endocyclic oxygen (**Figure 11**). Subsequently, an exception to the syn/anti lateral paradigm was identified in Clan O glycosidases (families GH52 and GH116).[119] In these exceptional enzymes, the catalytic acid/base is located above the plane of the pyranose ring. This results in a distinct vertical trajectory of proton delivery, and involves an alternative alignment of the oxygen lone pairs on the glycosidic oxygen.

The lateral arrangement provides an explanation for the strong inhibition of many retaining glycosidases by planar heteroaromatic compounds, such as glycosyltetrazoles and imidazoles (*vide infra*), which rely on in-plane protonation by the general acid residue from the anti orientation.

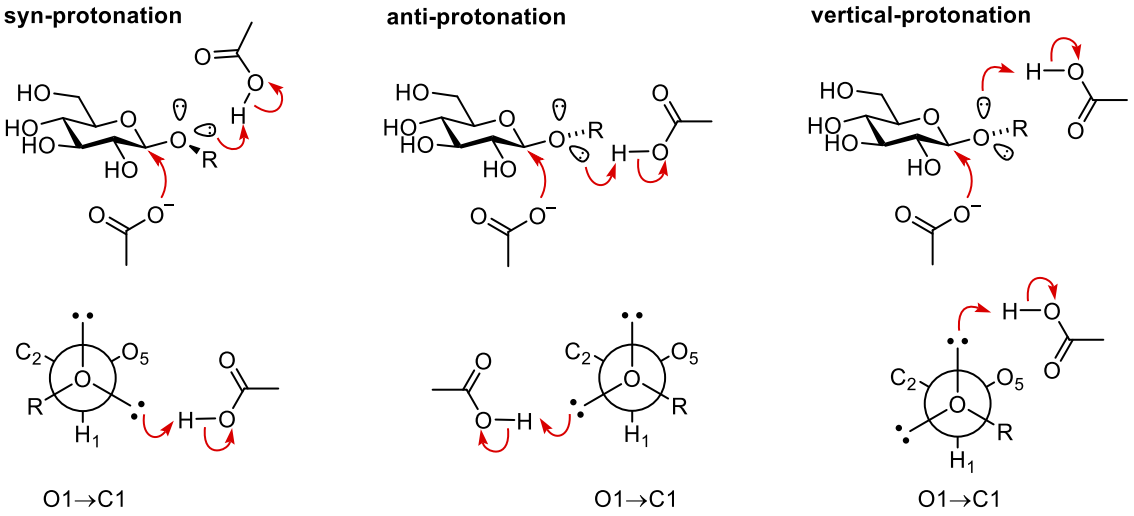


Figure 11. Illustration of general acid protonation geometry for anti, syn, and vertical protonation by glycosidases, illustrated for a β-glucosidase.

3. Conformation during catalysis

3.1 Conformation of glycosidase transition states

The oxocarbenium ion-like transition states for Koshland-type glycosidases described above benefit from stabilization of the developing positive charge on the anomeric carbon through donation of electron density from the ring oxygen, which leads to double bond character of the C1-O5 bond and a near-planar arrangement of the system C5-O-C1-C2.[120] The sugar ring can accommodate this planarity through a continuum of flattened conformations, with

significant species including half-chair (*H*), boat (*B*) and envelope (*E*) conformational isomers (conformers) (**Figures 12, 13**).

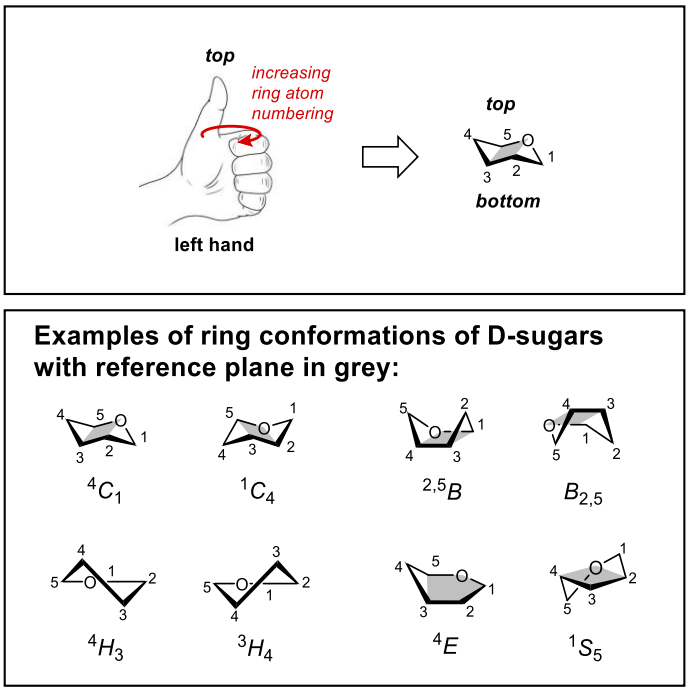


Figure 12. Standard nomenclature for pyranose-ring puckering. Pyranose rings relieve angle strain and steric clash by adopting non-planar cyclohexane-type conformations: *C* (chair), *B* (boat), *H* (half-chair), *S* (skew), and *E* (envelope). For each conformation a reference plane is drawn through ≥ 4 ring atoms; out-of-plane atoms are specified by their ring numbers as a superscript (above the plane) and/or subscript (below the plane) using the left-hand rule (fingers curl with increasing ring numbering). The ring oxygen lowers the symmetry relative to cyclohexane, so the superscript/subscript order matters. In L-sugars the same rule swaps "top" and "bottom," making, for example, α-D-glucopyranose $^4C_1$ the mirror image of α-L-glucopyranose $^1C_4$.

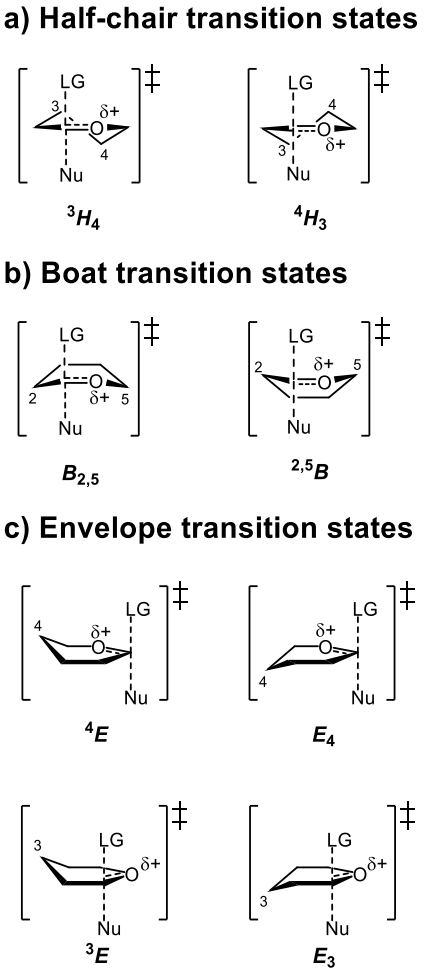


Figure 13. Canonical flattened pyranosyl transfer transition-state conformations.

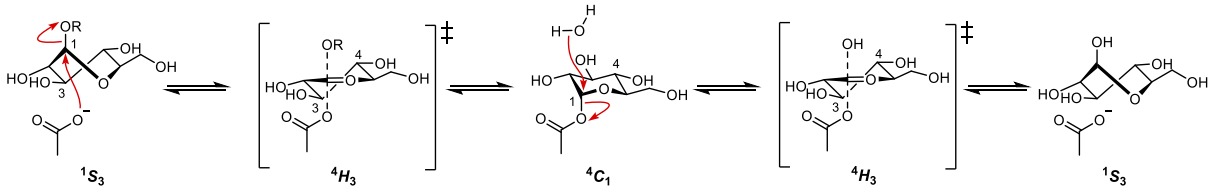
3.2 Conformational changes along the reaction coordinate

A consequence of the glycosidase reaction using an S_N2-like mechanism involving nucleophilic attack (as opposed to an S_N1 mechanism, as proposed by Phillips for HEWL), is that sugar rings will adopt shapes that favor less hindered nucleophilic attack trajectories. That is, glycosidases (and enzymes more generally) bind their substrates in a conformation that predisposes them to react. This can be considered an example of the principle of least nuclear motion, which states that elementary reactions proceeding with the least changes in atomic position and electronic configuration are preferred.[120-122] Thus, glycosidases tend to distort their substrates away from their lowest energy conformation to conformations that promote formation of flattened oxocarbenium ion-like transition states (such as those shown in **Figure 12**).

In the 1990s a series of studies of substrate-bound “Michaelis” (enzyme-substrate) complexes of glycosidases revealed distortion of the substrates to allow in-line nucleophilic

attack, supporting the formation of specific transition-state conformations and conformational itineraries along the reaction coordinate. X-ray structural analysis of such enzyme-substrate complexes was achieved by trapping what are normally reactive complexes using non-hydrolysable substrate analogues,[123] serendipitously [56] or though pH control to alter the ionization state of catalytic residues,[124] (and subsequently using enzyme variants in which the catalytic residues were mutated). **Figure 14a** depicts the reaction mechanism of a canonical retaining β-glucosidase in which the bound substrate adopts the $^{1}S_{3}$-conformation, which is different to the thermodynamically most stable $^{4}C_{1}$ conformation of a β-glucopyranoside in solution. As the first glycosylation step of the retaining mechanism proceeds, the pseudo-axially oriented glycosidic oxygen accepts a proton, the C1-glycosidic oxygen bond weakens, and the anomeric carbon moves down towards the nucleophile, resulting in flattening of the C5-O-C1-C2 system at the transition state, which is in the $^{4}H_{3}$ conformation. Further downwards migration of the anomeric carbon allows bond formation to the enzyme nucleophile, leading to the covalent glycosyl-enzyme with the sugar ring adopting a $^{4}C_{1}$ conformation. The second deglycosylation step of the reaction is the near-microscopic reverse of the first step, but with involvement of a water molecule to hydrolyze the glycosyl-enzyme intermediate and the conformational changes are reversed to form β-glucopyranose. The conformational changes occurring along the catalysis reaction coordinate can be codified into a series of steps termed the conformational itinerary, in this case $^{1}S_{3} \rightarrow [^{4}H_{3}]^{\ddagger} \rightarrow {}^{4}C_{1} \rightarrow [^{4}H_{3}]^{\ddagger} \rightarrow {}^{1}S_{3}$ (**Figure 14b**). As retaining glycosidases use two reaction steps, they will traverse cyclical pathways that revisit the same transition state conformation, while inverting glycosidases will follow a linear pathway with a single transition state.

a) Retaining β-glucoside hydrolase:



b) Classical conformational itineraries

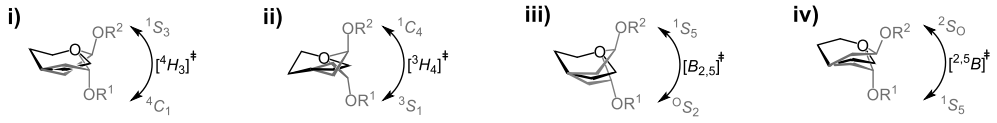


Figure 14. Conformational itineraries of glycosidase mechanisms. (a) Conformational reaction mechanism of a retaining β-glucosidase. (b) Overlay of conformational itineraries of a pyranose ring along the reaction coordinate for various retaining and inverting glycosidases.[125, 126]

Subsequent studies have shown that all four of the major proposed transition-state conformations ($^{4}H_{3}$, $^{3}H_{4}$, $B_{2,5}$, $^{2,5}B$) are used by different glycosidases acting on different substrates, notably with the demonstration that retaining β-mannanases used a $^{1}S_{5} \rightarrow [B_{2,5}]^{\ddagger} \rightarrow {}^{O}S_{2}$ conformational itinerary[127] and followed by many other studies of diverse enzymes (reviewed[125, 126]). Such studies can be combined with computational studies and can be used to inform enzyme inhibitor design (see below).

3.2 Insights into glycosidase conformational itineraries along the reaction coordinate: free energy landscapes

Comprehensive analysis of the conformational preferences of a pyranose ring can be achieved using the computational method of metadynamics.[128] Metadynamics allows systematic and exhaustive sampling of all the possible conformations of a molecule using a reduced set of collective variables, and construction of a conformational free energy landscape showing the relative energy of individual sugar conformations.[129] The resulting free energy landscape can be plotted on the Cremer-Pople pseudosphere[130] or various projections (**Figure 15**). One study used metadynamics to calculate the free energy landscape of methyl α-mannopyranoside, revealing that aside from the global minimum of a $^{4}C_{1}$ conformation, the $B_{2,5}$, $^{O}S_{2}$, $^{3,O}B$, $^{3}S_{1}$, $B_{1,4}$, $^{5}S_{1}$ and $^{2,5}B$ conformations are most stable (**Figure 16a**).[131] Metadynamics was used to calculate the conformational free energy landscape of substrate, α-1,2-mannobiose, constrained within the active site cleft of the inverting *Caulobacter* sp. GH47 α-mannosidase (**Figure 16b**). This revealed that the enzyme restricts the energetically accessible conformations of the reactive -1 mannosyl residue to a different region of the landscape. Structural insight into the conformations of critical points along the reaction coordinate were obtained by X-ray crystallographic analysis of complexes of the α-mannosidase with a non-hydrolysable S-linked methyl α-1,2-mannobioside mimicking the enzyme-substrate complex, mannoimidazole mimicking the transition state complex, and noeuromycin, a mimic of the product complex (**Figure 16c**). Collectively, this work implicated a $^{3,O}B/^{3}S_{1} \rightarrow [^{3}H_{4}]^{\ddagger} \rightarrow {}^{1}C_{4}$ conformational itinerary for this enzyme (**Figure 16d**).

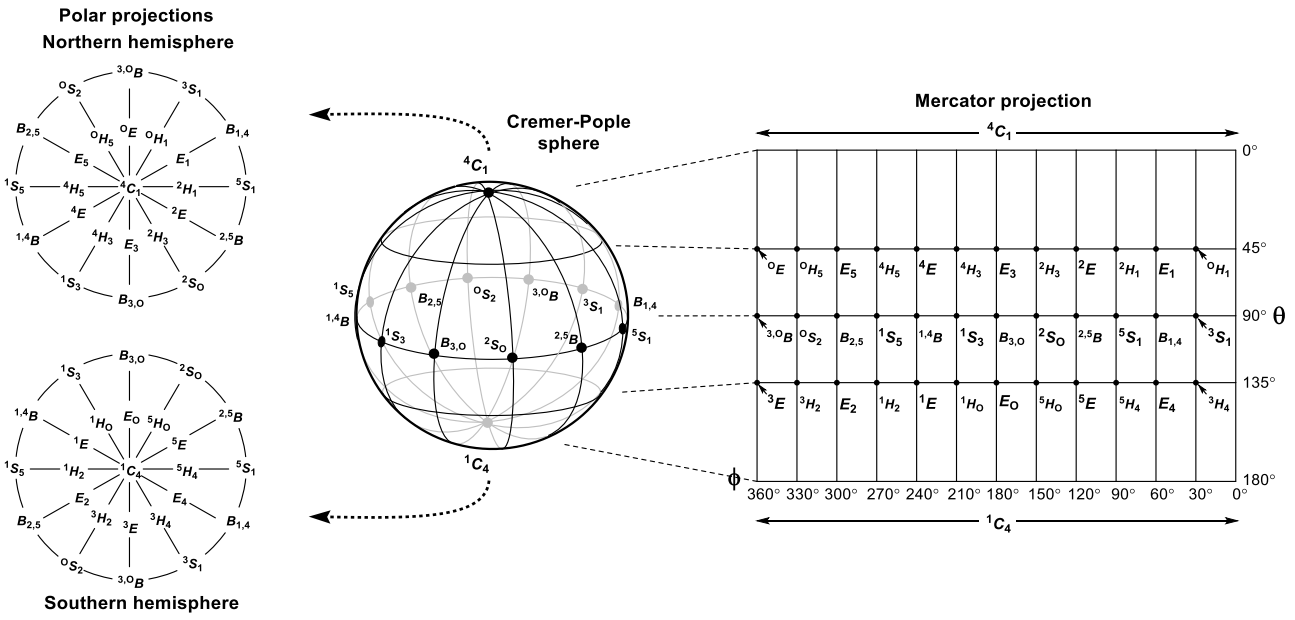


Figure 15. Mapping pyranose “conformational itineraries” with Cremer–Pople ring-puckering coordinates. Any pyranose conformation can be expressed as a point (Q, θ, φ) on a Cremer–Pople pseudosphere, where Q is the total puckering amplitude and θ/φ locate the ring shape on the sphere.[130] Plotting all points on the spherical surface, and converting it to a Mercator or polar projection, yields a continuous landscape on which chair, boat, half-chair, skew, and envelope forms can be visualized. Trajectories traced across this map illustrate the conformational itineraries followed during ring interconversion or along enzyme reaction coordinates.

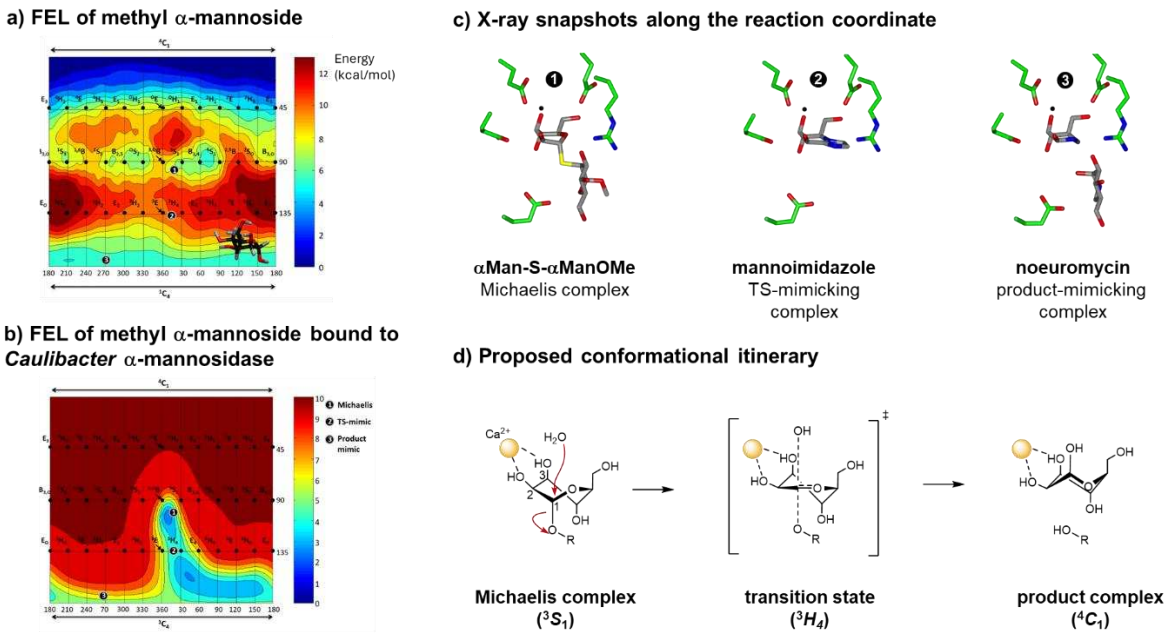


Figure 16. Integrated computational and structural assignment of the conformational itinerary of *Caulobacter strain* K31 GH47 α-mannosidase. (a) Free energy landscapes of methyl α-mannopyranoside (a) 'off-enzyme', and (b) in the active site of α-mannosidase show how the enzyme restricts the energetically accessible conformations on-enzyme to promote substrate pre-activation. (c) X-ray structures of Michaelis complex with a thioglycoside (4AYP), transition state mimicking complex with mannoimidazole (4AYQ), and product mimicking complex with noeuromycin (4AYR), (d) proposed conformational itinerary.

4. Transglycosylation and mutant glycosidases for glycoside synthesis

4.1 Synthesis of glycosidic bonds by transglycosylases

Retaining glycosidases achieve hydrolysis through reaction of water with the glycosyl enzyme intermediate, or with closely related cyclic oxazoline/oxazolinium ions or epoxide intermediates. Retaining glycosidases can be exploited for the synthesis of glycosidic bonds when these intermediates are intercepted by an alternative nucleophile such as a simple alcohol or a sugar.[132, 133] In the case of an alcohol nucleophile, this process converts the substrate glycoside into a new glycoside and thus is referred to as transglycosylation (or less preferably, transglycosidation) (**Figure 17a**). Under these conditions, product formation occurs with retention of anomeric configuration, consistent with the underlying double-displacement mechanism, and can also be used as a test for whether an enzyme is a retaining glycosidase.

It should be noted, however, that at sufficiently high concentrations of free sugars, the net formation of glycosidic bonds can also be observed for both retaining and inverting glycosidases through reversal of the hydrolytic equilibrium, driven by Le Chatelier's principle. In such cases, glycoside formation reflects thermodynamic forcing rather than interception of a covalent intermediate and does not constitute mechanistic transglycosylation.

Many naturally occurring enzymes exhibit both glycoside hydrolase and transglycosylase activities. A classic example is *Escherichia coli* LacZ (family GH2) β-galactosidase, which converts lactose (β-Gal-1,4-Glc) to galactose and glucose, and also generates a range of transglycosylation products, including allolactose (β-Gal-1,6-Glc),[134] an inducer of *lac* operon gene expression that acts by binding to and relieving repression by the LacI transcription factor.[135, 136] Because the product of transglycosylation is a glycoside of matching stereochemistry, it will also be a substrate for the enzyme and can itself be subject to a new turnover and hydrolyzed. In some cases, the major or sole product is derived from transglycosylation, such as with GH13 cyclodextrin transglycosylases, which convert linear amylose to cyclic structures termed cyclodextrins;[137] and xyloglucan endo-transglycosylases, which catalyze the cleavage and religation xyloglucan chains in plant cell wall.[111]

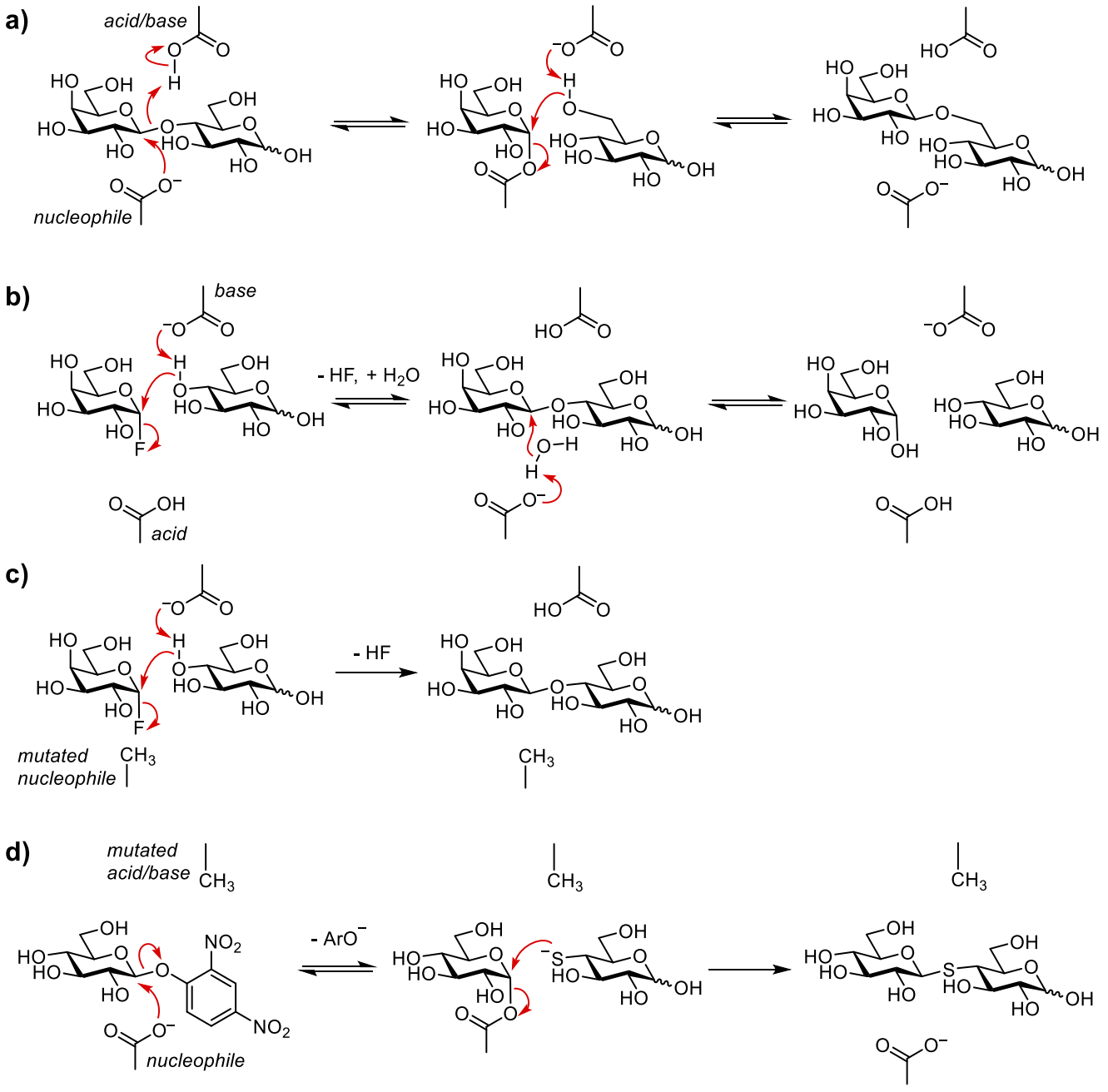


Figure 17. Catalysis of glycosidic bond synthesis by (a) transglycosylation by a retaining glycosidase involving an enzymatic nucleophile; (b) Hehre resynthesis-hydrolysis mechanism by an inverting glycosidase; (c) glycosynthase mechanism for a nucleophile mutant of a retaining glycosidase; (d) thioglycoligase mechanism for acid/base mutant of a retaining glycosidase.

Transglycosylation can be an undesirable side reaction that leads to sugar condensation. For instance, detection of human macrophage chitotriosidase activity with 4-methylumbelliferyl chitobioside is complicated by a futile transglycosylation cycle: the enzyme first generates chitobiose, which then acts as a glycosyl acceptor to form 4-methylumbelliferyl chitotetraoside.[138] Symmetrical cleavage of this product regenerates chitobiose and the original substrate, thereby preventing net release of the fluorophore 4-methylumbelliferone. The result is an apparent inhibition of activity as chitobiose accumulates. This limitation can be circumvented by using 4-methylumbelliferyl 4″-deoxy-chitobioside, which releases 4″-deoxy-chitobiose, a poor glycosyl acceptor incapable of participating in the transglycosylation cycle. This approach was extended to the development of methylumbelliferyl 4-*O*-alkyl-chitobiosides where the alkyl group blocks transglycosylation, and moreover limits cleavage by exo-acting hexosaminidases.[139] Similar issues complicate studies of human pancreatic α-amylase. Methylation at the 4′-position of chromogenic and fluorogenic maltosides blocks transglycosylation, yielding substrates that report exclusively on hydrolytic activity and thereby enable accurate kinetic analysis.[140] In a related approach, a fluorescence-quenched maltopentaoside bearing an indolylethyl group at the anomeric position and a 2-(2-aminoethylamino)-1-naphthalenesulfonate moiety on the non-reducing end sugar was developed.[141] In the intact substrate, fluorescence of the indolyl group is quenched; endo-cleavage by α-amylase separates the fluorophore and quencher, resulting in a fluorescence signal. Importantly, this design not only suppresses transglycosylation but also confers resistance to cleavage by exo-acting glucosidases.

4.2 Glycoside synthesis with endo-β-*N*-acetylglucosaminidases (ENGases) and mutant ENGase synthases

Endo-β-N-acetylglucosaminidases (ENGases) are endoglycosidases that cleave N-linked glycans from glycoproteins by hydrolyzing the β-1,4-glycosidic bond within the conserved *N*,*N*′-diacetylchitobiose core of N-glycans.[133, 142] They are found predominantly in glycoside hydrolase families 85 and 18 and are widely distributed across bacteria, fungi, plants, and animals. Although their primary physiological role is deglycosylation, a subset of ENGases exhibits synthetically valuable transglycosylation activity, enabling the transfer of intact N-glycan oligosaccharides to suitable acceptors. Both hydrolysis and transglycosylation proceed via a substrate-assisted retaining mechanism involving formation of a sugar oxazolinium ion

intermediate through neighboring-group participation of the 2-acetamido group of the GlcNAc residue at the −1 subsite (**Figures 4c, 18a**). In hydrolytic turnover, water adds to this intermediate, whereas in transglycosylation an alternative nucleophile, typically the 4-hydroxyl group of a GlcNAc residue on a peptide or protein acceptor, attacks the oxazolinium ion to form a new β-glycosidic linkage.

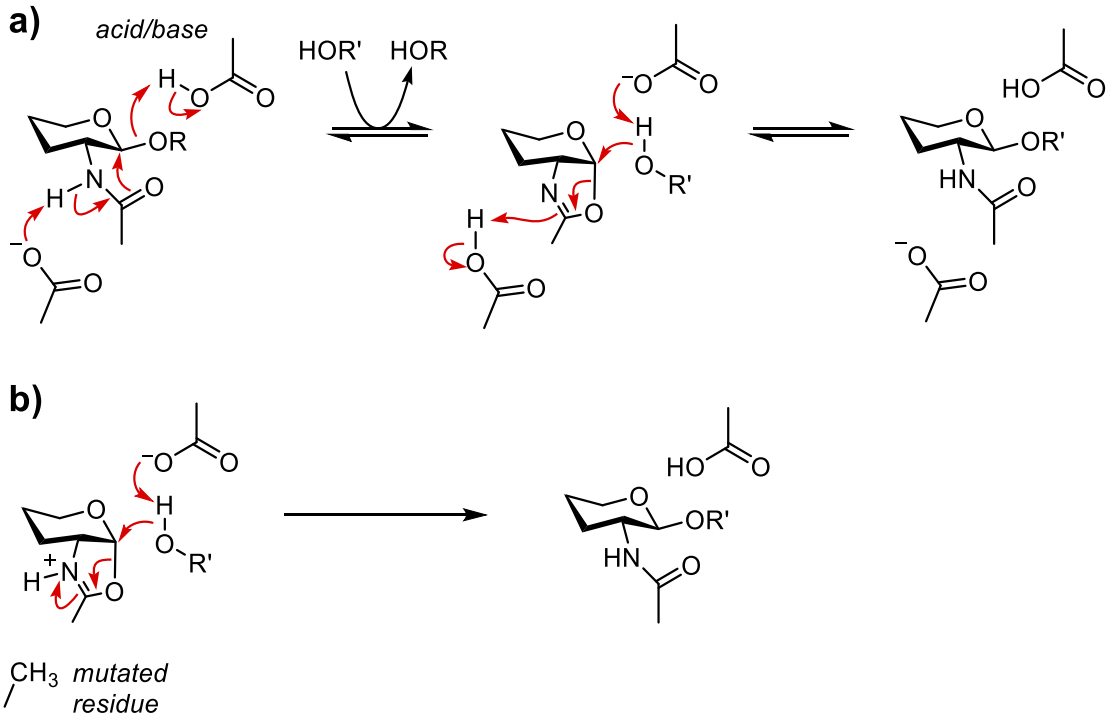


Figure 18. Catalysis of glycosidic bond synthesis by (a) transglycosylation by a retaining hexosaminidase (eg ENGase) involving neighboring group participation; (b) mutant ENG synthase mechanism using an oxazoline glycosyl donor.

A key strategy for exploiting ENGases in synthesis is the use of activated glycan donors in the form of synthetic glycan oxazolines, which can be synthesized directly from complex oligosaccharides without the need for any protecting groups.[143-145] Importantly, wild-type ENGases can often utilize these donors efficiently, even when the glycans are truncated or non-natural in structure. Notably, the resulting truncated glycopeptide products are frequently poor substrates for further hydrolysis, favoring net glycosylation and enabling access to structurally complex N-glycans in high yields.[146] This convergent, en bloc glycan transfer contrasts with glycosyltransferase-based approaches, which assemble glycans in a stepwise, monosaccharide-by-monosaccharide manner, and has established ENGases as valuable

catalysts for the chemoenzymatic synthesis of homogeneous glycoproteins and glycopeptides. For example, Endo A ENGase-catalyzed transglycosylation of a GlcNAc-containing 34-mer peptide derived from HIV-1 gp41 (prepared by automated solid-phase peptide synthesis), using a two-fold excess of a synthetic $Man_3GlcNAc$ oxazoline donor afforded the $Man_3GlcNAc_2$ pentasaccharide in 75% yield (**Figure 19**).[147]

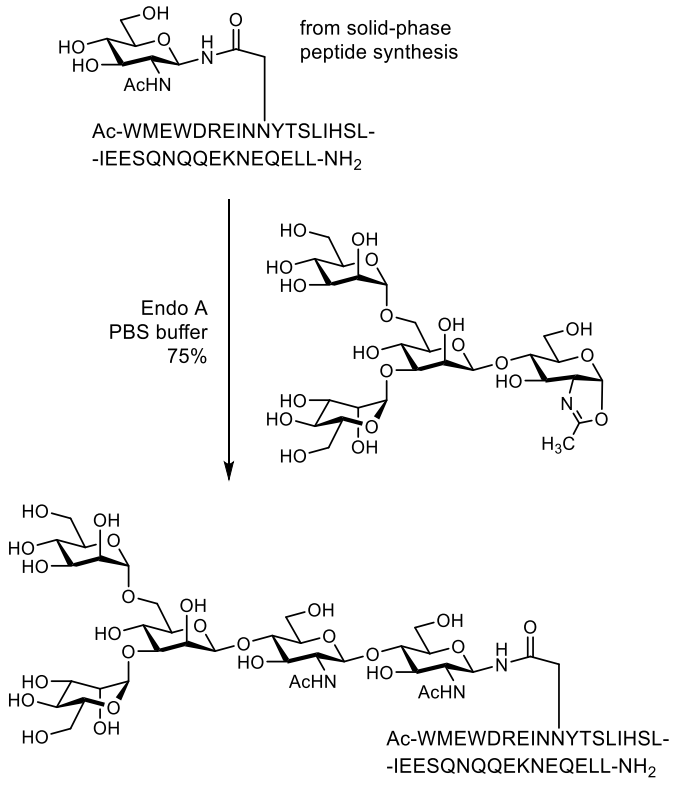


Figure 19. ENGase-catalyzed synthesis of a glycopeptide carrying a core pentasaccharide N-glycan.

Use of wild-type ENGase enzymes for transglycosylation is often compromised by subsequent hydrolysis of the products. This limitation can be overcome through the construction of mutant enzymes in which hydrolytic activity is curtailed.[143-145] In the context of the substrate-assisted mechanism, ENGase synthase mutants have been generated by mutating the catalytic residue responsible for correctly orienting the 2-acetamido group of the −1 GlcNAc, thereby suppressing formation or resolution of the oxazolinium ion intermediate (**Figure 18b**).[148] Alternatively, mutation of the catalytic acid/base residue attenuates hydrolysis while preserving the ability to catalyze glycosyl transfer from activated oxazoline donors.

4.3 Hehre resynthesis-hydrolysis mechanism

Inverting enzymes do not proceed through an intermediate and so cannot be intercepted by a non-water nucleophile. However, Hehre and colleagues demonstrated that inverting glycosidases can often hydrolyze glycosyl fluorides with the 'incorrect' anomeric stereochemistry. For example, *Rhizopus niveus* inverting glucoamylase, which usually cleaves α-linked substrates such as maltooligosaccharides to release β-maltose, is also capable of hydrolyzing β-maltosyl fluoride to give β-maltose.[149] This reaction initiates with transglycosylation from one glycosyl fluoride to another, forming a glycosidic linkage with stereochemistry inverted relative to the original fluoride. Mechanistically, this occurs as the enzyme accommodates the glycosyl fluoride due to the fluorine atom's small size (**Figure 17b**). The reaction yields a glycoside in the 'correct' configuration, which is then rapidly hydrolyzed via a conventional Koshland inverting mechanism. Known as the Hehre resynthesis-hydrolysis mechanism, this process sometimes allows isolation of the intermediate transglycosylation product, as observed with α-xylosyl fluoride and the inverting *Bacillus pumilis* β-xylosidase.[150] The Hehre resynthesis-hydrolysis mechanism has little practical significance as the intermediate glycoside rarely accumulates to useful levels. However, it can be considered a progenitor for the development of engineered enzymes termed glycosynthases.

4.4 Glycosynthases

Glycosynthases are retaining glycosidases that have been mutated to replace the enzymatic nucleophile with a non-nucleophilic residue such as alanine (**Figure 17c**).[151] When the resulting mutant enzyme is incubated with a glycosyl fluoride of the 'wrong' anomeric configuration and a suitable alcohol, it can catalyze the synthesis of a glycosidic bond with a stereochemistry that matches the normal configuration of the enzyme. However, as the glycosynthase lacks an enzymatic nucleophile, the product is not hydrolyzed, and can accumulate to high levels, providing a practical way to make various glycoside products.[133, 152, 153] The glycosynthase concept has been extended to some inverting glycosidases.[154] Glycosynthases derived from endo-glycosidases acting on regular, repeating polysaccharides often suffer from uncontrolled oligomerization or polymerization, since the product itself can serve as a glycosyl acceptor.[155] This issue can be circumvented by masking the acceptor position of the glycosyl fluoride with a removable protecting group such as a tetrahydropyranyl ether,[156] or a galactosyl residue,[152] thereby enabling the controlled, stepwise synthesis of cellooligosaccharides.

4.5 Thioglycoligases

"Thioglycoligases" are retaining glycosidases that have been mutated to replace the acid-base residue with a non-reactive group (**Figure 17d**).[157] These enzymes catalyze the synthesis of thioglycosides from activated donor glycosides such as 2,4-DNP glycosides that do not require acid catalysis. The activated glycoside reacts with the mutant thioglycoligase to form a glycosyl enzyme, but reaction of this species with water in the absence of general base catalysis is slow. Use of a deoxythio sugar as a nucleophile overcomes the recalcitrant reactivity of the glycosyl enzyme, allowing formation of a thioglycoside linkage.

5. Glycoside phosphorylases

Phosphorylases reversibly catalyze the cleavage of glycosidic bonds through substitution with phosphate (phosphorolysis) (**Figure 20a,b**). In contrast to the hydrolysis of glycosides, which is effectively irreversible, phosphorolysis is reversible in practice because the bond energies of the glycoside and the phosphorolysis product are comparable. For example, the equilibrium constant (*K*) for phosphorolysis of cellobiose is 0.3,[158] while that of sucrose is 34.[159, 160] Phosphorolysis can occur with retention or inversion of anomeric configuration. Phosphorylases are *exo*-acting enzymes, and in the phosphorolysis direction leads to the formation of a monosaccharide-1-phosphate (or in rare cases a disaccharide-1-phosphate[161]). In metabolism, phosphorylases may be involved in both the cleavage and synthesis of glycosidic bonds.

Phosphorylases are classified into various glycoside hydrolase (GH13, 65, 94, 130, 149, 161) and glycosyltransferase (e.g. GT4, 35, 91, 108) families based on sequence similarity. The glycoside hydrolase-like glycoside phosphorylases have 3D structures that are similar to glycosidases and generally follow similar mechanisms (**Figure 20c,d**).[162] In some family GH130 mannoside phosphorylases, a proton-relay mechanism has been proposed in which the catalytic aspartate transfers a proton via the substrate 3-hydroxyl, which then relays it to the glycosidic oxygen to promote bond cleavage.[163]

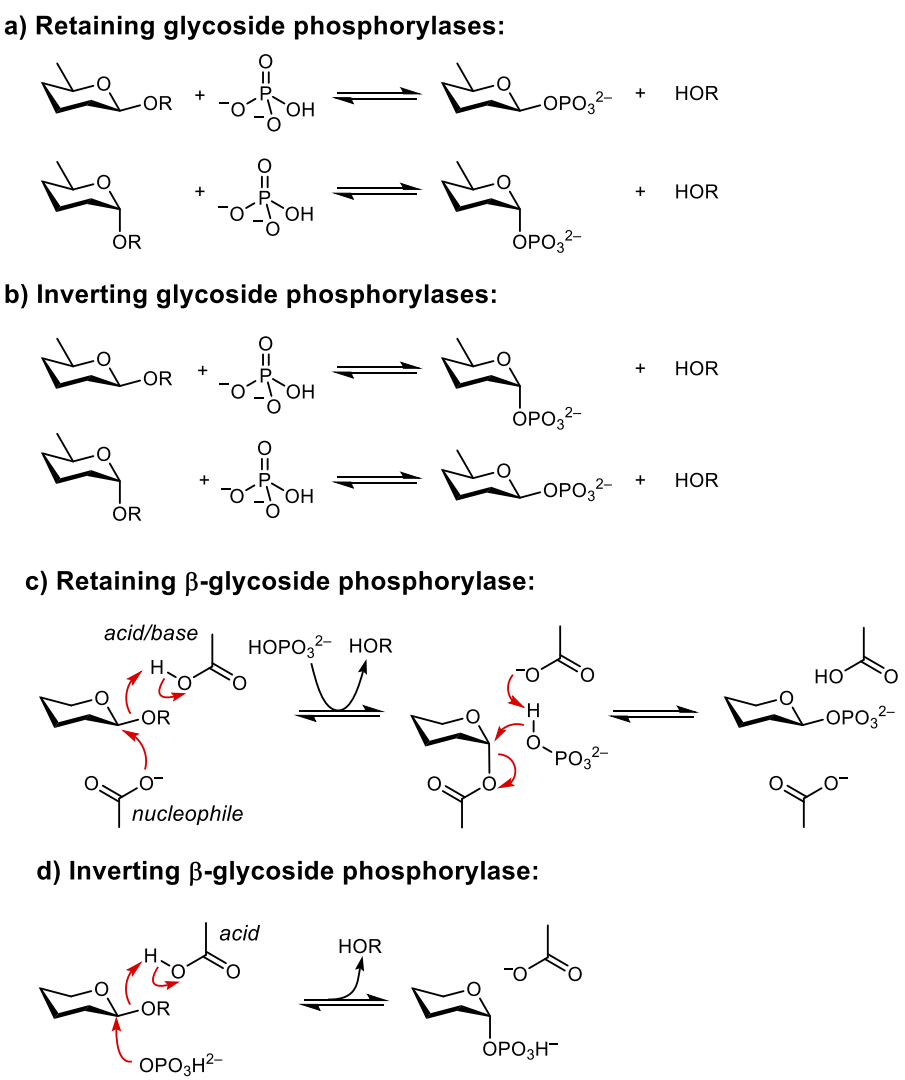


Figure 20. Reactions catalyzed by (a) retaining glycoside phosphorylases, (b) inverting glycoside phosphorylases; a C5 substituent has been added to indicate D-sugars. Catalytic mechanisms for (c) retaining and (d) inverting β-glycoside phosphorylases.

6. Inhibitors of glycosidases

6.1 Overview

Efforts to develop glycosidase inhibitors have drawn heavily on mechanistic insight. Strategies have targeted distinct aspects of catalysis: mimicking features of bound substrates, reproducing elements of the oxocarbenium ion-like transition state, or trapping catalytic intermediates through reactive substrate analogues. Other approaches exploit the enzyme's catalytic machinery, using covalent mechanism-based inhibitors to arrest turnover. Together these

routes have produced a broad arsenal of inhibitors, inspired both by natural discovery and by rational design.

Natural product alkaloids such as nojirimycin, deoxynojirimycin, acarbose, castanospermine, and swainsonine exemplify how sugar-shaped nitrogen heterocycles[164] with substrate-like hydroxylation patterns can bind glycosidases in their -1 subsite by resembling their substrates or transition states. Their discovery has spurred extensive synthetic elaboration to generate probes of enzyme selectivity and as candidate therapeutics. Parallel advances in enzymology and structural biology clarified the structural underpinnings: Koshland's stereochemical paradigms[13] and subsequent crystallographic studies defined catalytic strategies, while Pauling's[165] concept of preferential transition-state stabilization inspired the rational design of iminosugars, azasugars, and sp^2-hybridized glycomimetics.[166] By mimicry of the transition state of glycosidases, such inhibitors allow visualization of this fleeting state and have helped to illuminate catalytic conformational itineraries by protein X-ray crystallography.

Retaining glycosidases that proceed through glycosyl-enzyme intermediates have been fertile targets for intermediate-trapping strategies. Building on early results from Legler and co-workers, [89] as discussed earlier, Withers and co-workers pioneered the use of 2-deoxy-2-fluoroglycosides as slow substrates that accumulate long-lived glycosyl–enzyme adducts. These compounds are valuable chemical tools that enable identification of catalytic nucleophiles, and more recently, activity-based probes (ABP) for profiling of glycosidases. In a complementary vein, mechanism-based covalent inhibition has yielded highly useful probes. The natural products cyclophellitol and conduritol B epoxide inspired the development of cyclitol epoxides and aziridines, which covalently capture catalytic residues and now underpin the most effective ABPs for retaining glycosidases.[167, 168]

Beyond their value in research, several glycosidase inhibitors have translated into the clinic.[169] Acarbose and miglitol are used to manage type 2 diabetes by blocking intestinal α-glucosidases; migalastat serves as a pharmacological chaperone in Fabry disease; and zanamivir, peramivir, and oseltamivir target influenza neuraminidase. Further opportunities lie in therapeutic development for viral infections,[170, 171] Alzheimer's disease,[172] and lysosomal storage disorders.[173]

The following subsections survey major inhibitor classes, beginning with the simplest: non-hydrolysable thioglycosides.

6.2 Non-hydrolysable thioglycosides

Replacing the glycosidic oxygen with sulfur yields thioglycosides, which are resistant to enzymatic cleavage because sulfur is less basic than oxygen. They function as stable substrate analogues, revealing how enzymes bind glycosides without undergoing turnover.[174] Thioglycosides are valuable probes in crystallographic studies for capturing bound substrate conformations. For example, sulfur-linked cellopentaosides bound to *Fusarium oxysporum* GH5 endoglucosidase I revealed a $^{1}S_{3}$ conformation, providing insight into substrate distortion during catalysis (**Figure 21**).[123] Despite their generally weak potency as inhibitors, thioglycosides remain useful mechanistic tools.

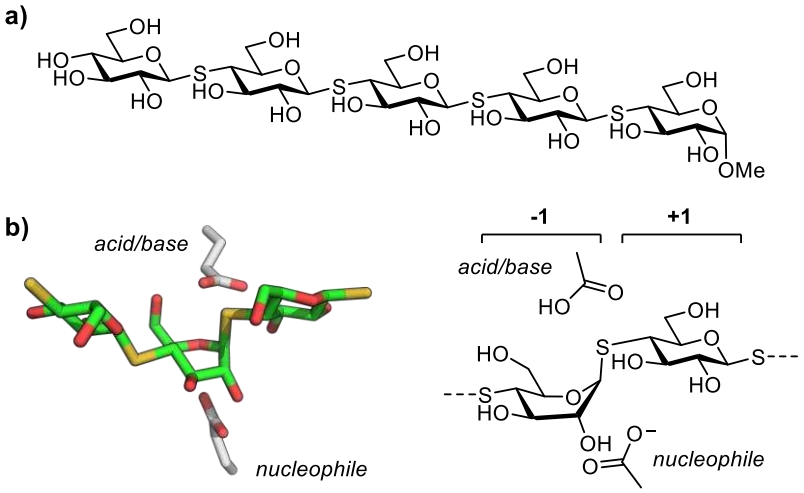


Figure 21. Thioglycosides as non-hydrolysable substrate mimics. (a) structures of an S-linked cellopentaoside. (b) 3D X-ray structure of S-linked cellopentaoside bound in the active center of *Fusarium oxysporum* endoglucosidase I (PDB 1ovw); acid/base Glu202, nucleophile Glu197.

6.3 Iminosugars and azasugars

Various sugar-shaped nitrogen heterocycles have been developed as potent glycosidase inhibitors. Two important classes are iminosugars (where nitrogen replaces the ring oxygen) and azasugars (where nitrogen replaces the anomeric carbon). The parent compounds are deoxynojirimycin[175, 176] and isofagomine,[177] respectively (**Figure 22a**). Interactions of these compounds in the active site of a glycosidase typically involve an ionic interaction between the protonated inhibitor and a deprotonated active site residue (either the nucleophile or acid/base). In particular, for retaining glycosidases with an enzyme nucleophile, protonated isofagomine-type inhibitors form an ion pair with the nucleophile.[178, 179] Deoxynojirimycin and isofagomine can be considered mimics of the two resonance forms of a glycosyl cation (**Figure 22b**) and

arguably achieve inhibition through transition state mimicry. Comparison of the structures of the oxocarbenium ion-like transition states of the standard Koshland mechanisms with the protonated forms of deoxynojirimycin and isofagomine reveals that they exhibit mimicry of the partial development of charge at O5 and C1. However, they do not mimic the flattened structures of the transition states.

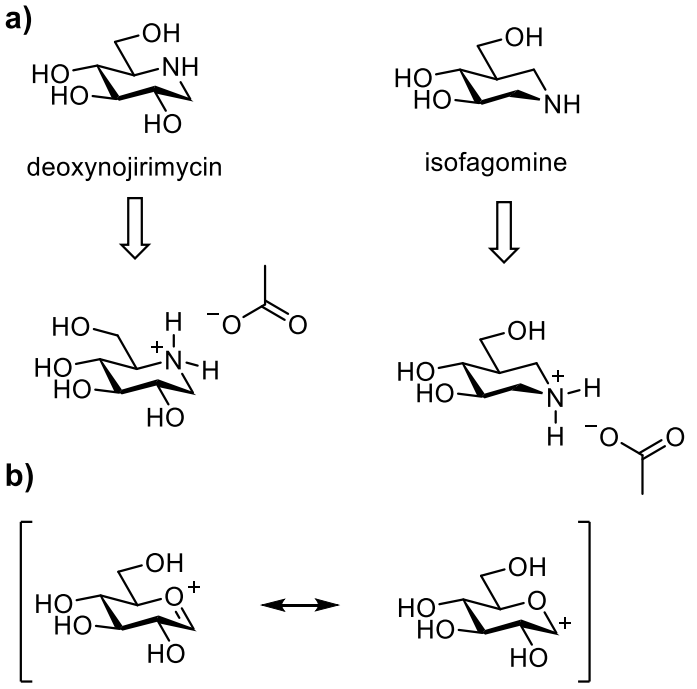


Figure 22. Iminosugar and azasugar inhibitors of glycosidases. (a) Structures of deoxynojirimycin and isofagomine, showing how their protonated forms can interact with enzymatic carboxylate residues. (b) Comparison of the resonance forms of a glycosyl cation.

Analogues of deoxynojirimycin and isofagomine varied in stereochemistry and other functionality are usually effective inhibitors of the corresponding glycosidases. For example, various drugs based on these inhibitor scaffolds include miglitol (inhibitor of intestinal α-glucosidase),[180] forodesine (immunicillin-H, inhibitor of purine nucleoside phosphorylase),[181] and migalastat (chaperone for lysosomal α-galactosidase)[182, 183] (**Figure 23a**). *N*-Butyldeoxynojirimycin (zavesca) is an inhibitor of a glycosyltransferase, glucosylceramide synthase.[184, 185] Other representatives of this class of molecules are the hemiaminals noeuromycin,[186] which is configurationally fluid at C1 and C2 and acts as an inhibitor of both mannosidases and glucosidases, and nojirimycin,[187] an inhibitor of α- and β-glucosidases. The

natural product siastatin is an *N*-acetyl aminal that inhibits sialidases, β-glucuronidases, and *N*-acetylglucosaminidases.[188] In the case of β-glucuronidases, however, siastatin itself is not the true inhibitor. Instead, it undergoes a sequence of chemical transformations that generate several inhibitory products, among them galacturonic noeuromycin.[189] Various natural products glycosidase inhibitors can be considered ring annulated analogues of iminosugars. These include the mannosidase inhibitors swainsonine[190] and kifunensine,[191-193] and the glucosidase inhibitors castanospermine[194] and australine[195] (**Figure 23b**).

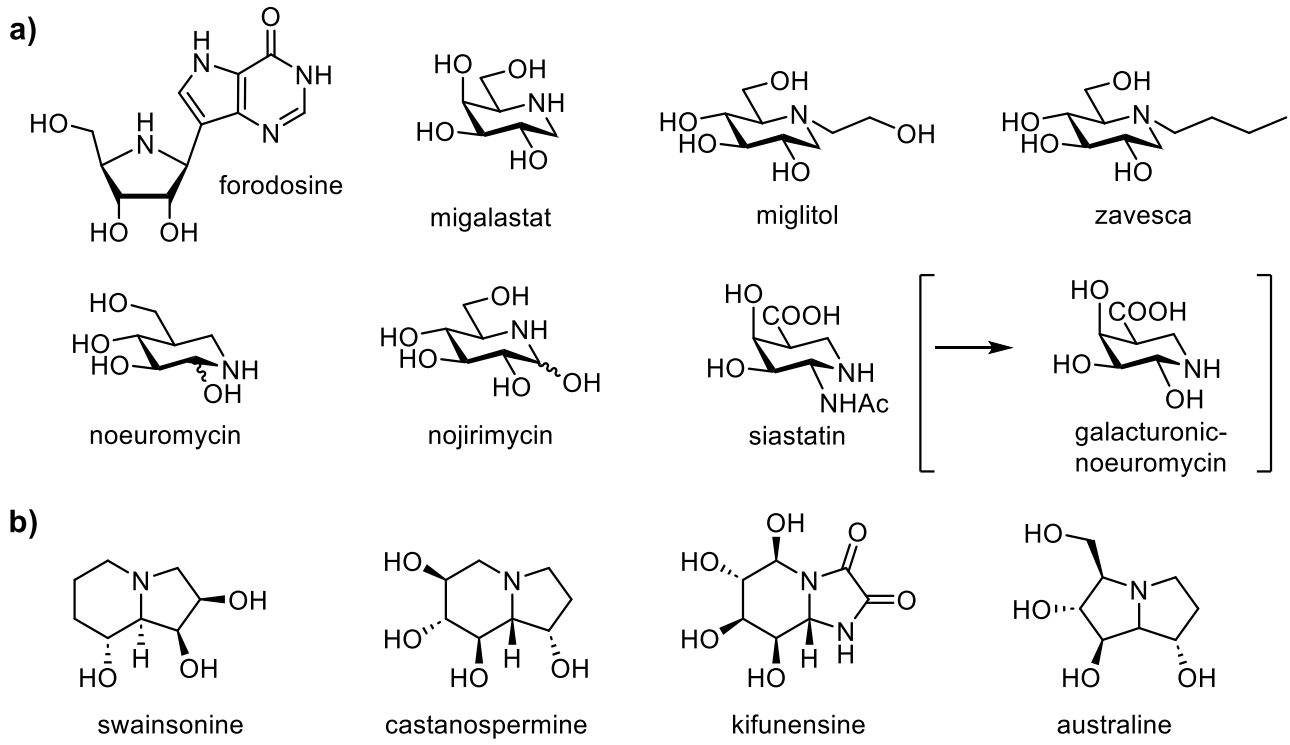


Figure 23. Additional iminosugar and azasugar inhibitors of glycosidases. (a) Structures of various analogues of deoxynojirimycin and isofagomine. (b) Indolizidine (1-azabicyclo[4.3.0]nonane) and pyrrolizidine (1-azabicyclo[3.3.0]octane) alkaloid natural products.

Mimicry of the flattened structure of the transition state is achieved in various glycomimetics that contain an sp^2-hybridized 'anomeric' carbon. The earliest representative of this class is gluconolactone,[196] which is a moderately strong inhibitor of various glycosidases. More potent examples include lactone oximes,[197] lactam oximes,[198] and bicyclic glucotetrazole,[199, 200] glucoimidazole[201] and gluco-1*H*-imidazole[202] (**Figure 24a**). Nagstatin is a natural product representative of this class, and is an inhibitor of *N*-acetyl-β-glucosaminidase.[188]

These compounds achieve effective inhibition when the lone pair on the 'anomeric' nitrogen is directed towards an appropriately located general acid residue in the active site of a glycosidase, a phenomenon achieved when the general acid is located anti to the C1-O5 bond of the sugar (**Figure 24b**).[117, 203] Glycosidases with the general acid located syn to the C1-O5 bond are typically poorly inhibited by these compounds. Glycals have been suggested as possible transition state mimics,[204] however, as discussed earlier, these are typically substrates for retaining glycosidases, and turned over to the hydrated 2-deoxy sugars.[149, 205]

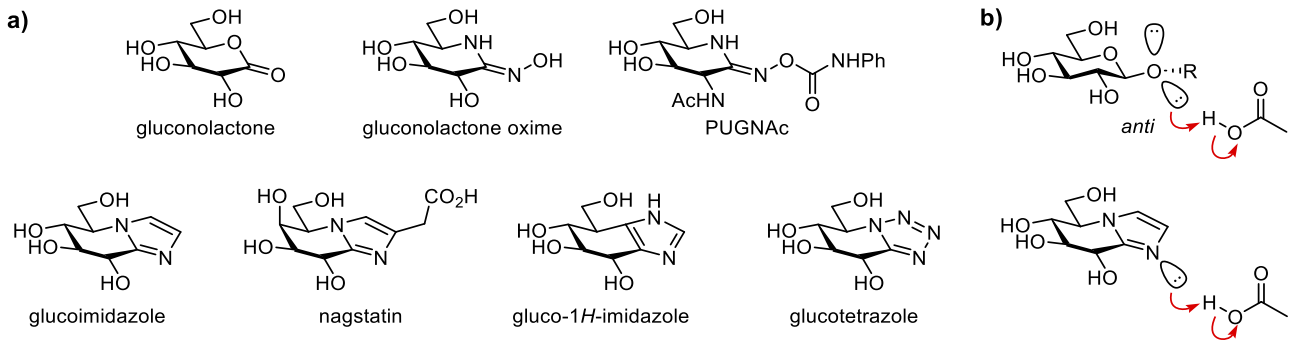


Figure 24. Glycomimetic glycosidase inhibitors containing an *sp*^{2}-hybridized 'anomeric' carbon. (a) Structures of various inhibitors directed at glucosidases. (b) In plane 'lateral' protonation by the acidic residue of an anti-protonating glycosidase.

6.4 Aminocyclitols

Aminocyclitols are carbohydrate mimics derived from cyclitol scaffolds in which one or more hydroxyl groups are replaced by an amino group. Their resemblance to monosaccharides and their ability to adopt flattened, transition state–like conformations can confer glycosidase inhibition. Examples include acarbose (an inhibitor of intestinal α-glucosidase used to treat type 1 diabetes),[206] mannostatin A (an inhibitor of Golgi mannosidase II),[207, 208] and validamycin[209, 210] and trehazolin[211] (inhibitors of trehalase used for control of rice blight) (**Figure 25a**). Other aminocyclitol glycosidase inhibitors include the influenza drugs oseltamivir (a prodrug)[212] and peramivir,[213] which target viral sialidase (and were derived from the neuraminidase glycal-based inhibitors DANA and zanamivir[93, 214]) (**Figure 25b**).

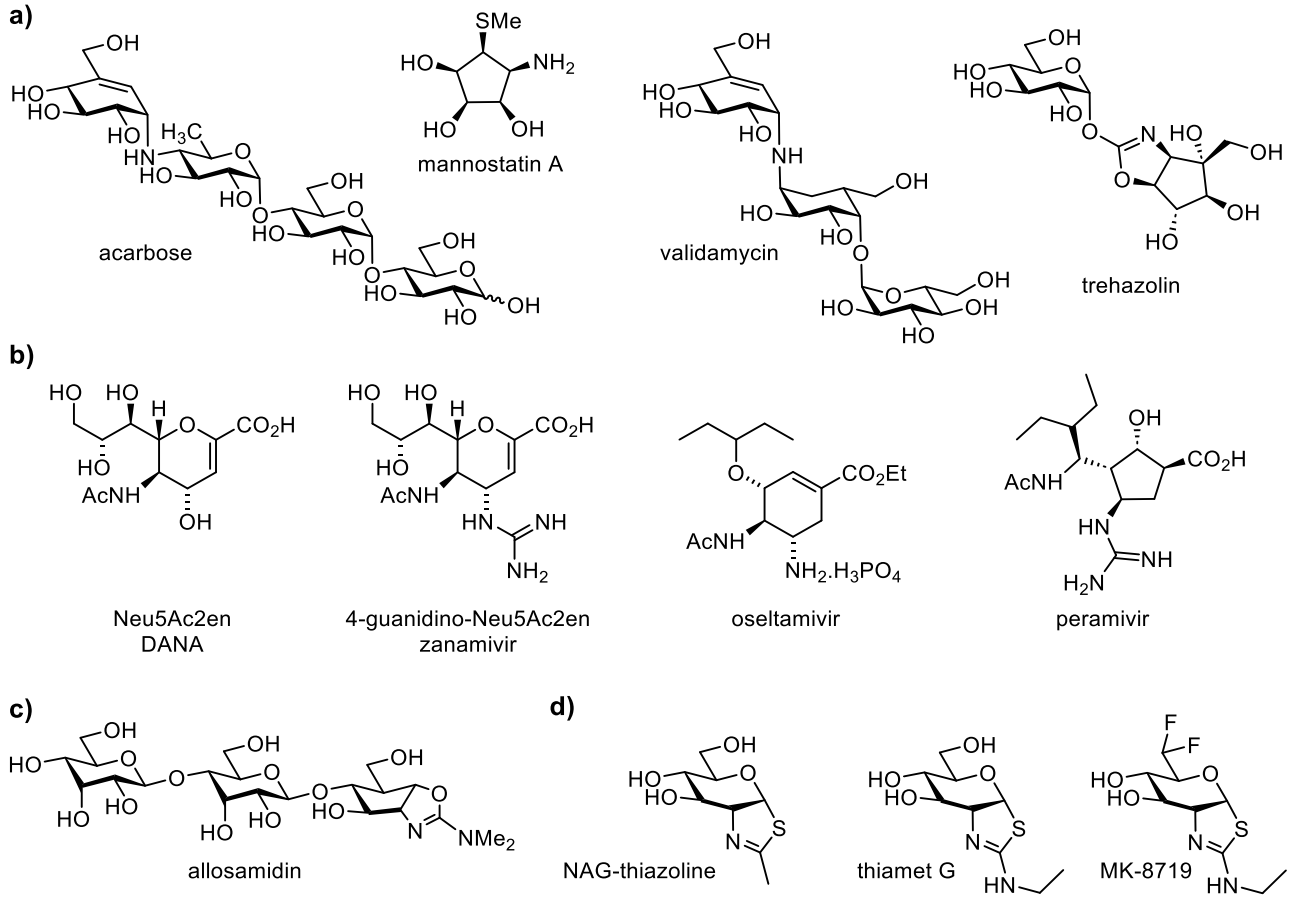


Figure 25. Aminocyclitol, glycal, oxazoline, and thiazoline inhibitors of glycosidases. (a) Acarbose, validamycin and mannostatin A, inhibitors of intestinal α-glucosidase, trehalase, and α-mannosidases respectively. (b) Drugs that target influenza virus sialidases. (c) Allosamidin, an inhibitor of chitinases that use neighboring group participation. (d) Various inhibitors of hexosaminidases based on mimicry of the oxazoline/oxazolinium ion intermediate in the neighboring group participation mechanism.

6.5 NAG-thiazoline and analogues

Retaining hexosaminidases that use substrate-assisted catalysis proceed via an oxazoline/oxazolinium ion intermediate. The natural product allosamidin is a potent inhibitor of chitinases that use this mechanism, and contains an allosamizoline ring that mimics the intermediate (**Figure 25c**).[55] Replacement of oxygen in an *N*-acetylglucosamine derived oxazoline with sulfur provides NAG-thiazoline, which is a stable species that is not turned over by the enzyme (**Figure 25d**).[57] NAG-thiazoline is a potent inhibitor of these hexosaminidases, and can be used to study the 3D X-ray structures of glycosidase-NAG-thiazoline complexes to

explore how the true oxazoline intermediate is bound. NAG-thiazoline and related molecules are typically potent inhibitors of hexosaminidases that use substrate-assisted catalysis, which may be because of mimicry of the intermediate, but may also be because the slightly greater bond distance of a C-S bond versus a C-O bond provides mimicry of the elongated partial bonds at the transition state. Two significant analogues of NAG-thiazoline have been developed that are highly potent and selective inhibitors of human GH84 O-GlcNAcase, namely thiamet G[215] and the Alzheimer's drug candidate MK-8719.[216]

7. Transition state analogy of glycosidase inhibitors

One way to rationalize the potency of many glycosidase inhibitors is to show that they reproduce the fleeting transition state of glycoside cleavage, an idea that can be traced to Pauling's prediction that enzymes bind their transition state with exceptional affinity.[217] The transition state of classical Koshland glycosidases resembles an oxocarbenium ion with trigonal hybridization at the anomeric carbon, substantial positive charge delocalized onto the endocyclic oxygen, partial bonds to the leaving group and incoming nucleophile, and a flattened ring conformation. Effective inhibitors that emulate these features of the transition state should bind tightly by recapitulating the interactions that stabilize the transition state.

Linear free energy relationships (LFERs) developed by Bartlett[35] provide a method for evaluating whether an inhibitor truly mimics the transition state of an enzyme-catalyzed reaction. The approach can be applied in various ways to assess whether changes to an enzyme, substrate, or inhibitor affect binding affinity and catalytic rates proportionally, allowing for a systematic comparison between inhibitors and the transition state.

Mosi *et al*. studied the glycosidase inhibitor acarbose using an approach involving a series of active-site mutants of the family GH13 cyclodextrin glycosyltransferase, and yielded good correlations of log K_I values measured with acarbose against log K_M/k_{cat} values measured for a monosaccharide substrate, which was assumed to be a suitable test for the -1 subsite of the enzyme where the distorted valienamine residue lies.[218] The mutant enzyme approach was also applied to *Cellulomonas fimi* family GH11 xylanase and is discussed in more detail to illustrate the active-site mutant approach. Residues that hydrogen-bond directly to the –1 xylose or lie in the second shell were replaced individually (**Figure 26a**), and for each variant the k_{cat}/K_M value for 2-nitrophenyl β-xylobioside (chosen because the glycosylation step is rate-limiting) K_I values for a xylobiose-derived imidazole (with a flattened, sp^2 anomeric center) and a xylobiose-

derived isofagomine (saturated piperidine ring that carries an endocyclic ammonium cation) were measured. The resultant Bartlett plot of log (k_{cat}/K_M) against log K_I for the imidazole gave a line of fit with R^2 = 0.93 (**Figure 26b**), showing that each mutation weakened catalysis and inhibitor binding to almost identical extents, implying that the imidazole replicates both the charge build-up and the planar geometry of the enzymatic TS. By contrast, the isofagomine plot furnished a noticeably poorer fit (R^2 = 0.65, **Figure 26c**). The cationic nitrogen evidently captures the electrostatic component of binding, but presumably the puckered piperidine ring cannot emulate the flattened $^2H_3/B_{2,5}$ TS conformation of the enzyme.

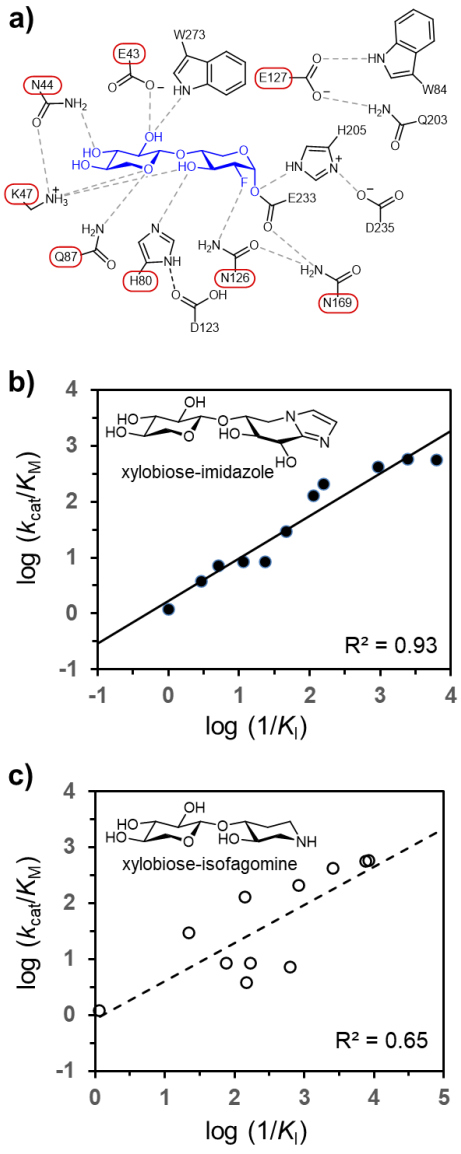


Figure 26. Linear free energy analysis of transition state mimicry for *Cellulomonas fimi* xylanase. a) Cartoon showing 2-deoxy-2-fluoro-xylobiosyl-enzyme with residues that interact

with the sugar or reside in the second shell. Red circles indicate residues that were mutated. Bartlett plot correlating log(k_{cat}/K_M) and log ($1/K_I$) for b) xylobiose-derived imidazole, and c) xylobiose-derived isofagomine.

Tailford *et al.* applied LFER analysis to active-site variants of a family GH2 *Bacteroides thetaiotaomicron* β-mannosidase using a panel of mannosidase inhibitors.[219] The plot of log K_M/k_{cat} versus log K_I for substituted mannoimidazoles showed strong correlations with slopes close to unity, consistent with effective transition state mimicry. X-ray crystallography revealed that these inhibitors bound in a $B_{2,5}$ conformation, and together the results support the view that the enzymatic transition state also adopts this conformation.

Ermert *et al.* used an approach to study Bartlett LFERs in which the structure of the inhibitor was varied along with equivalent changes to the substrate. They found that glucotetrazole and mannotetrazole inhibitors displayed a linear relationship between logarithmic plots of inhibition (K_I value) and enzyme efficiency (log k_{cat}/K_M) for the corresponding 4-nitrophenyl glycosides pNP-β-D-Glc and pNP-β-D-Man across a range of enzymes, suggesting effective transition state mimicry (**Figure 27a**).[200] Shidmoossavee *et al.* measured kinetic parameters for a range of 4-nitrophenyl sialosides and inhibition constants for the corresponding analogues of zanamivir and found poor correlation of either log K_M/k_{cat} or log ($K_M \times k_{uncat}$)/k_{cat} and log K_I, while observing a better correlation of log K_M and log K_I with slope 0.8, which was interpreted to suggest that zanamivir mimics the Michaelis complex (**Figure 27b**).[220] Whitworth and co-workers explored inhibition of wildtype family GH84 human O-GlcNAcase using a series of NAG-thiazoline inhibitors and corresponding 4-methylumbelliferyl glycoside substrates sharing systematic variation at the 2-acyl position (**Figure 27c**).[221] A plot of log K_I versus log K_M/k_{cat} exhibited a strong correlation ($r^2 = 0.98$) with a slope of 0.97 ± 0.06. On this basis, the authors proposed that the transition state mimicry exhibited by NAG-thiazolines may arise, at least in part, from the extended C–S bond length of the thiazoline ring (1.86 Å), compared to the shorter C−O bond length in the corresponding oxazoline (1.45 Å). The thiazoline thus better reflects the partial bond order (0.26) between the nucleophilic carbonyl oxygen and the anomeric center expected at the transition state of the neighboring group participation mechanism. Similar results were observed for the related NAG-aminothiazolines.[222]

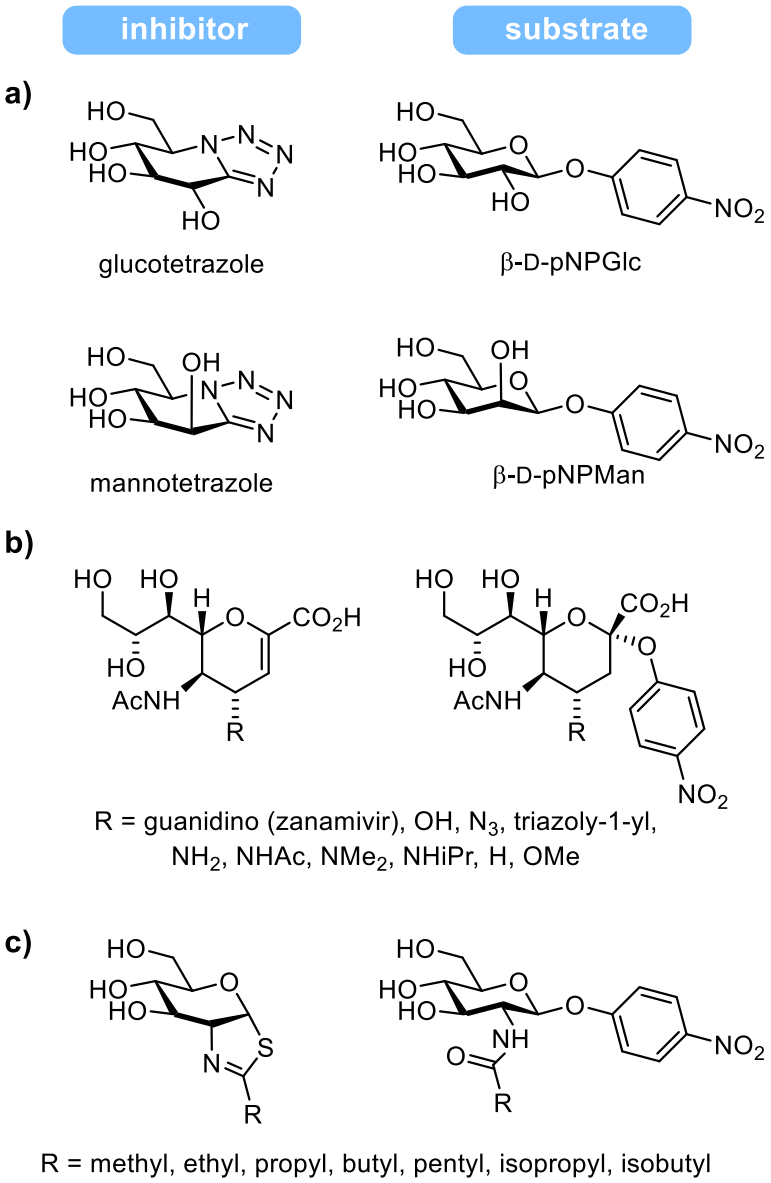


Figure 27. Parallel substrate and inhibitor variation for the study of transition state mimicry using Bartlett linear free energy analysis.

8. Cyclitol epoxides/aziridines as inhibitors of retaining glycosidases

8.1 Mechanism-based inhibition by cyclitol epoxides/aziridines

Legler reported that the sugar-mimicking cyclitol, conduritol B epoxide (CBE), irreversibly inhibits retaining α- and β-glucosidases that proceed through a glycosyl enzyme intermediate (**Figure 28a**).[223, 224] CBE is pseudo-symmetric and serves as an analogue of both α- and β-glucopyranosides (**Figure 28b,c**). Nucleophilic ring opening, assisted by the general acid/base residue, yields an ester-linked enzyme-inhibitor adduct, which is stable over time. Evidence that

both α-glucosidases and β-glucosidases target the 'anomeric' carbon of CBE was obtained by treatment of the ester-linked adducts with hydroxylamine. (+)-*Chiro*-inositol was obtained from CBE-inactivated *Aspergillus wentii* retaining β-glucosidase,[223] and *scyllo*-inositol from CBE-inactivated rabbit intestine sucrase-isomaltase (a retaining α-glucosidase).[225]

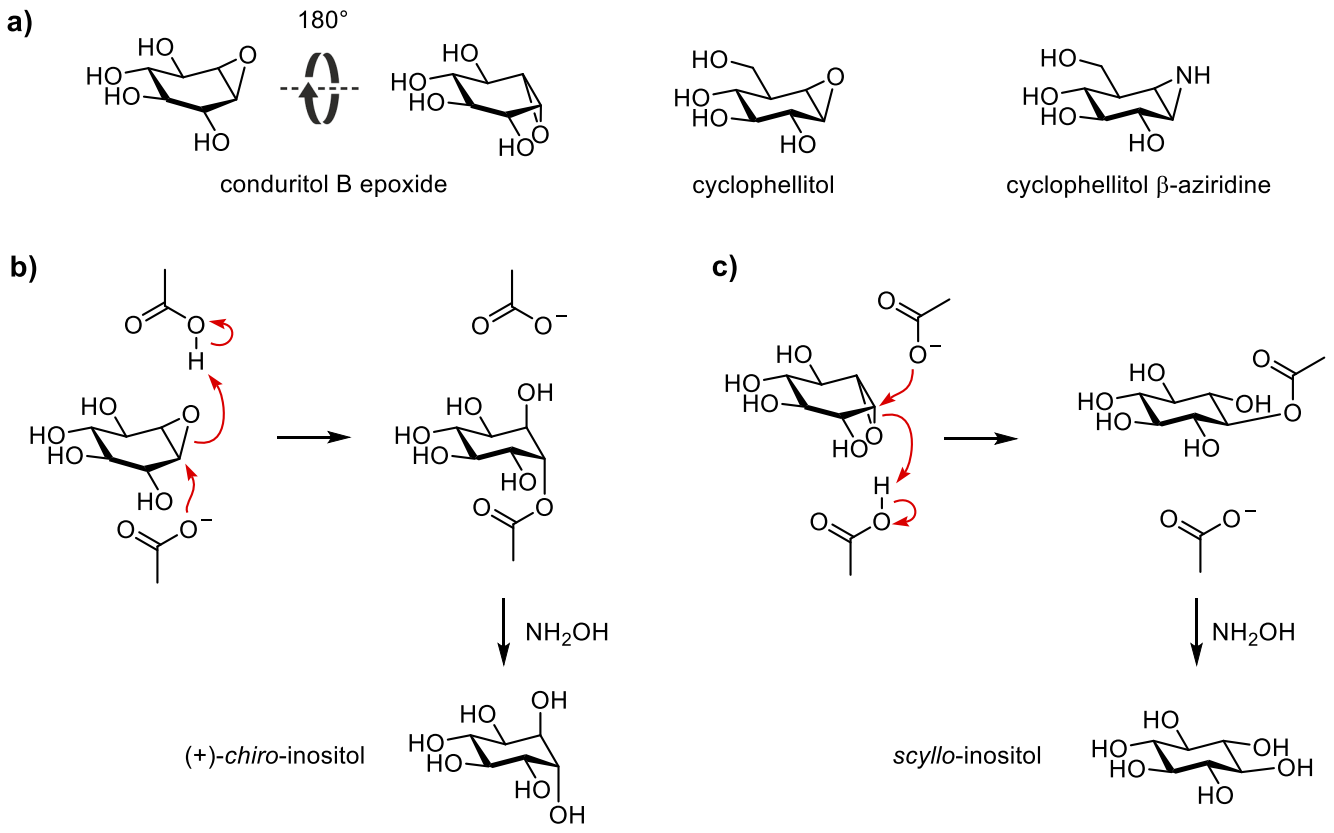


Figure 28. Cyclitol epoxides and aziridines as mechanism-based inhibitors of retaining glycosidases. (a) Structures of conduritol B epoxide and cyclophellitol epoxide and aziridine. Inhibition of (b) β-glucosidase and (c) α-glucosidase by conduritol B epoxide.

Caron and Withers identified conduritol B aziridine as a potent inactivator of retaining α- and β-glucosidases.[74] They hypothesized that breaking the pseudosymmetry of conduritol B epoxide and its aziridine analogue by converting one of the two hydroxyl groups adjacent to the epoxide/aziridine ring into a hydroxymethyl group would produce more selective and potent mechanism-based inactivators for retaining α- and β-glucosidases, respectively. Shortly thereafter, the natural product cyclophellitol was discovered, which was shown to be a highly potent and selective retaining β-glucosidase inactivator (**Figure 28a**).[75, 76] In solution,

cyclophellitol adopts a $^4H_3$ conformation, matching the retaining β-glucosidase transition state conformation. The 3D X-ray structure of the complex of *Thermotoga maritima* retaining GH1 β-glucosidase inactivated by cyclophellitol revealed the adduct bound in the $^4C_1$ conformation, thereby tracing the reaction pathway of substrate processing in these enzymes.[226] Configurational isosteres of cyclophellitol and cyclophellitol aziridine have been developed that are inactivators of a range of retaining glycosidases.[167, 227, 228]

8.2 Activity-based retaining glycosidase profiling

Activity-based probes (ABPs) represent a transformative approach for the study of glycosidases, enabling the direct detection of enzyme activity across various biological contexts. Unlike transcriptomics and proteomics, which infer potential activity through gene or protein abundance, ABPs reveal active enzymes, providing insights into functional enzyme populations and complementing other omics approaches.

By introducing a reporter functionality, inhibitors that covalently and irreversibly bind to target enzymes under denaturing conditions can be transformed into ABPs. Both fluorosugar and cyclitol epoxides/aziridines have been developed into such probes. Vocadlo and Bertozzi created an ABP for *E. coli* LacZ GH2 β-galactosidase, using 2,6-trideoxy-1,2-difluoro-6-azido-β-galactoside, which modifies the active site nucleophile, Glu537 (**Figure 29a**).[229] The covalently labeled enzyme was visualized through bioorthogonal labeling in a Cu(I)-catalyzed azide-alkyne cycloaddition reaction to install a FLAG epitope tag, and then western blot analysis. Hekmat *et al.* developed a biotinylated 2-deoxy-2-fluoro-xylobioside inactivator with a cleavable linker attached at the non-reducing end (**Figure 29b**).[230] This ABP targeted endoglycosidases and was used to label, isolate, and identify a novel retaining GH10 β-1,4-glycanase in *Cellulomonas fimi*.

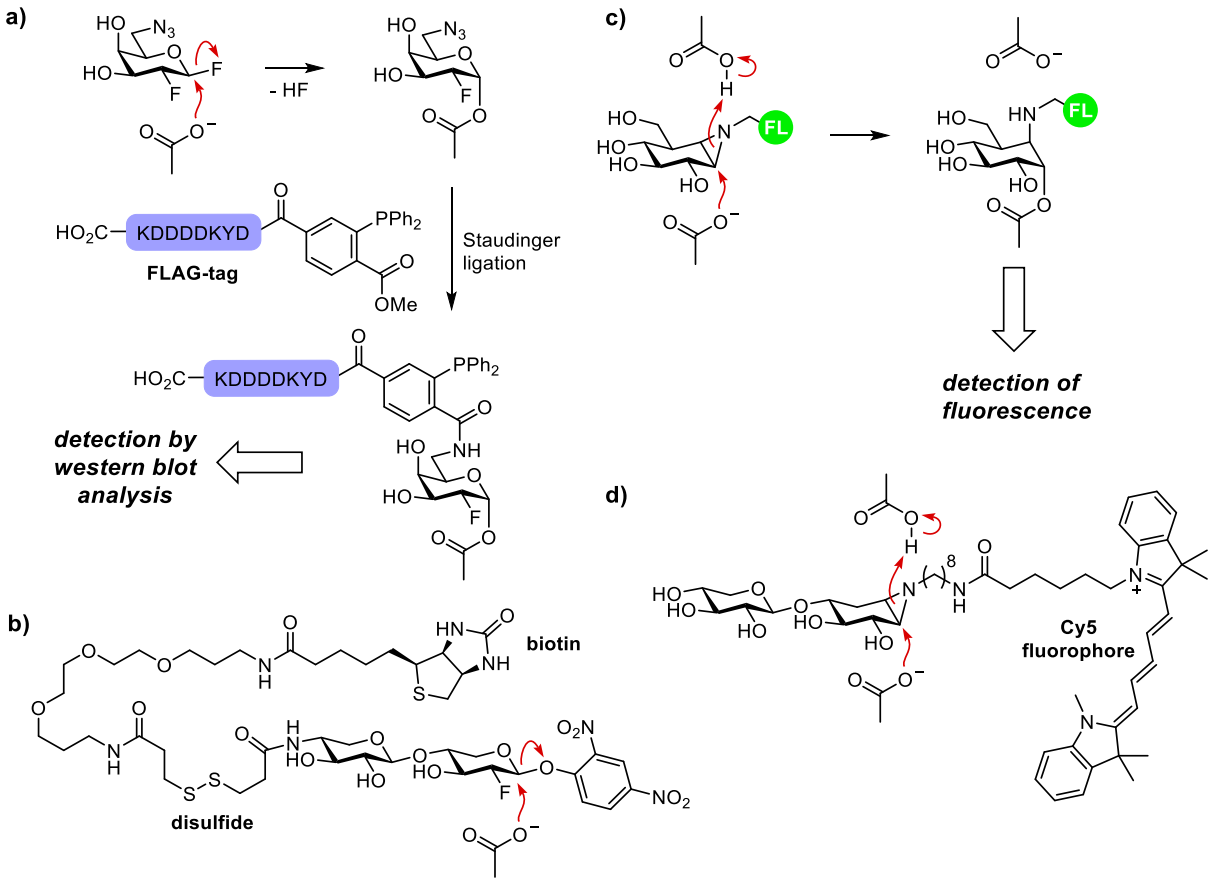


Figure 29. Activity based profiling of retaining glycosidases. (a) Stepwise inhibition of LacZ β-galactosidase with an azide modified 2-deoxy-2-fluorosugar inactivator, and subsequent labelling with an epitope tag for detection by western blot analysis. (b) One-step labelling of a galactosidase with a fluorophore-modified cyclitol aziridine for fluorescence analysis. (c) Example of a biotinylated 2-deoxy-2-fluorosugar inactivator with a cleavage disulfide-containing linker targeting endo-xylosidases. (d) Example of a fluorophore-labelled cyclitol aziridine for ABP analysis of endo-xylosidases.

Cyclophellitol aziridine modified at the aziridine nitrogen was applied to tag and detect three human retaining β-glucosidases—the glucocerebrosidases GBA1, GBA2, and GBA3 (**Figure 29c**).[231] The modification at nitrogen of cyclitol aziridines enables selective targeting of both endo- and exo-glycosidases by the same inactivator framework. For example, a fluorescent Cy5-tagged xylobiosyl aziridine was employed to examine the temperature sensitivity of endo-1,4-xylanases (**Figure 29d**).[232] Collectively, these two mechanism-based inactivator classes offer a powerful platform for the identification and characterization of a diverse range of retaining exo- and endoglycosidases.

9. Glycomimetic unsaturated cyclitol ethers

9.1 Glycosyl cations versus allylic cations

The Bennett and Withers groups independently synthesized glycomimetic carbocycles in which a sugar-mimicking cyclohexenitol ether contains a double bond allylic to the ether oxygen (**Figure 30**).[233, 234] These "valienol" analogues are cleaved both non-enzymatically and by glycosidases in a manner analogous to the corresponding glycosides; however, they progress through an allylic-cation-like, rather than oxocarbenium ion-like, transition state.

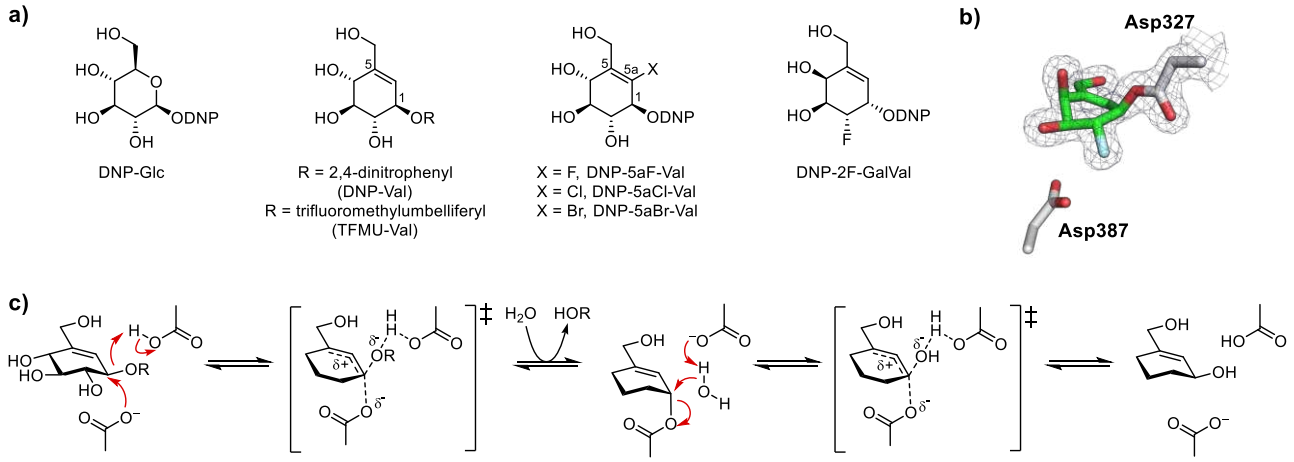


Figure 30. Unsaturated cyclitol ethers as substrates and mechanism-based inhibitors of retaining glycosidases that use an enzymatic nucleophile. (a) Examples of cyclohexenitol ether slow substrates, and 5a-halo and 2-deoxy-2-fluoro inactivators. (b) X-ray structure of the covalent adduct formed by the reaction of DNP-2F-GalVal with *Thermotoga maritima* α-galactosidase (PDB 6gwg), $2F_o - F_c$ map contoured at 2 σ. (c) Catalytic mechanism for hydrolysis of a valieniol substrate by a retaining β-glucosidase.

At pH 6.5 (so, within the pH-independent region), 2,4-dinitrophenyl β-valienol hydrolyzes with rate constants and activation parameters essentially identical to those of 2,4-dinitrophenyl β-glucoside (**Table 2**).[234] The α-deuterium KIE for DNP-1-²H-valienol (k_H/k_D = 1.11 ± 0.01) likewise pointed to an allylic carbocation-like transition state, mirroring the value obtained for DNP-1-²H-Glc (k_H/k_D = 1.09 ± 0.02). Non-enzymatic hydrolysis yielded a mixture of allylic alcohols arising from direct attack of water at C1 and allylic rearrangement with attack at C5, each produced in both α- and β-anomeric forms.

Table 2. Rate constants and activation parameters for hydrolysis of DNP valienol and glucoside at 37 °C.

	DNP-Val	**DNP-Glc**
k (s^{-1})	4.91×10^{-6}	7.41×10^{-6}
$\Delta G^{\ddagger}$ (kJ mol^{-1})	108	106
ΔH (kJ mol^{-1})	112	111
ΔS (J mol^{-1} K^{-1})	13.1	14.4

Glycosidases cleave valienol ethers stereospecifically, faithfully reproducing the stereochemical outcome observed with true glycosides.[234] For example, hydrolysis of 4-trifluoromethylumbelliferyl-β-valienol by the retaining *Agrobacterium* sp. β-glucosidase furnished exclusively the β-configured allylic alcohol, with no C5 substitution as seen under non-enzymatic conditions. The secondary α-deuterium KIE for DNP-1-^{2}H-valienol (k_H/k_D = 1.15 ± 0.03) indicates appreciable allylic-cation character in the enzymatic transition state and closely matches that for DNP-1-^{2}H-Glc (k_H/k_D = 1.10 ± 0.02) with the same enzyme. Overall, these data are consistent with these glycomimetic valienol glycosides undergoing a two-step hydrolysis mechanism that mirrors that for glycosides, via a valienyl-enzyme intermediate (**Figure 30c**). Covalent valienyl-enzyme adducts can be detected by mass spectrometry upon incubation of DNP-Val with Abg[234] or a maltotriose-like pseudotrisaccharide with *Aspergillus oryzae* amylase.[235] Because the reaction of the valienol ethers with a retaining glycosidase occurs in a 1:1 fashion, with the corresponding release of the anomeric group, this compound has been developed as an active site titration reagent for amylases.[235]

Collectively, these data underscore a close mechanistic relationship between prenyl and glycosyl group-transfer reactions. However, despite these mechanistic parallels, valienol ethers are markedly poorer substrates than their glycoside counterparts: across a panel of β-glucosidases, k_{cat}(Glc)/k_{cat}(Val) ranges from 24 to 6000, a difference that is unsurprising given the exquisite evolutionary tuning of glycosidases to stabilise sugar oxocarbenium ion-like transition states.

9.2 Halogenated cyclohexenitol mechanism-based inhibition of retaining glycosidases

Glycomimetic unsaturated cyclitol ethers with effective leaving groups are slow substrates for retaining glycosidases due to the accumulation of a cyclitol-enzyme intermediate, formed via a mechanism analogous to glycoside cleavage. Bennet and co-workers reported the synthesis of a DNP 2-deoxy-2-fluoro-galacto-valienol compound, where the DNP group accelerates formation of the covalent enzyme intermediate, while the fluorine substituent slows its hydrolysis.[236] This compound acted as a time-dependent inactivator of the retaining α-galactosidase from *Thermotoga maritima*, with the covalent adduct stable enough for 3D structure determination by X-ray crystallography (**Figure 30b**). Using isotope-labeled analogues and experimental KIEs, the transition state for inhibition was modeled, revealing charge development at the C5-allyl center and a reaction proceeding through an 'exploded' S_N2 transition state without a discrete enzyme-bound cationic intermediate.[237]

In another approach, Danby and co-workers employed halogenation at the vinylic C5a carbon to stabilize the covalent enzyme intermediate.[238] The C5a-F compound was the fastest enzyme inactivator for several retaining β-glucosidases, but its covalent adduct was highly labile and rapidly hydrolyzed. Conversely, the C5a-Br compound was the slowest inactivator but formed a highly stable, persistent covalent adduct.

10. Beyond Koshland: Non-classical glycosidases and lyases

Koshland proposed three general mechanisms for the glycosidases. While these mechanisms describe the action of most glycosidases, Nature has evolved enzymes that utilize other mechanistic approaches to achieve glycoside hydrolysis or elimination processes.

10.1 NAD^+-dependent glycosidases

Several glycosidase families (GH families 4, 109, 177, 179 and 188)[239-244] use a cryptic redox addition/elimination mechanism involving an NAD^+ cofactor for glycoside hydrolysis (**Figure 31a**). This mechanism involves four steps: oxidation of a glycoside at the 3-hydroxyl to give a 3-keto glycoside and NADH; rate limiting elimination of the glycoside by deprotonation at C2 to give a 3-keto-2-hydroxyglucal; conjugate addition of water to give a 3-keto sugar; and finally, reduction of the 3-keto sugar to give the product sugar and NAD^+.[240] This entire process is catalytic in NAD^+, and sometimes enzymes that use this pathway can act on both α- and β-glycosides. Nature has evolved variants of this process that involve multiple enzymes to achieve the individual steps, namely glycoside 3-dehydrogenase, 3-keto-glycoside lyase, and

3-keto-2-hydroxy-glucal hydratase; these enzymes can be extraordinarily promiscuous in their substrate breadth.[245-248]

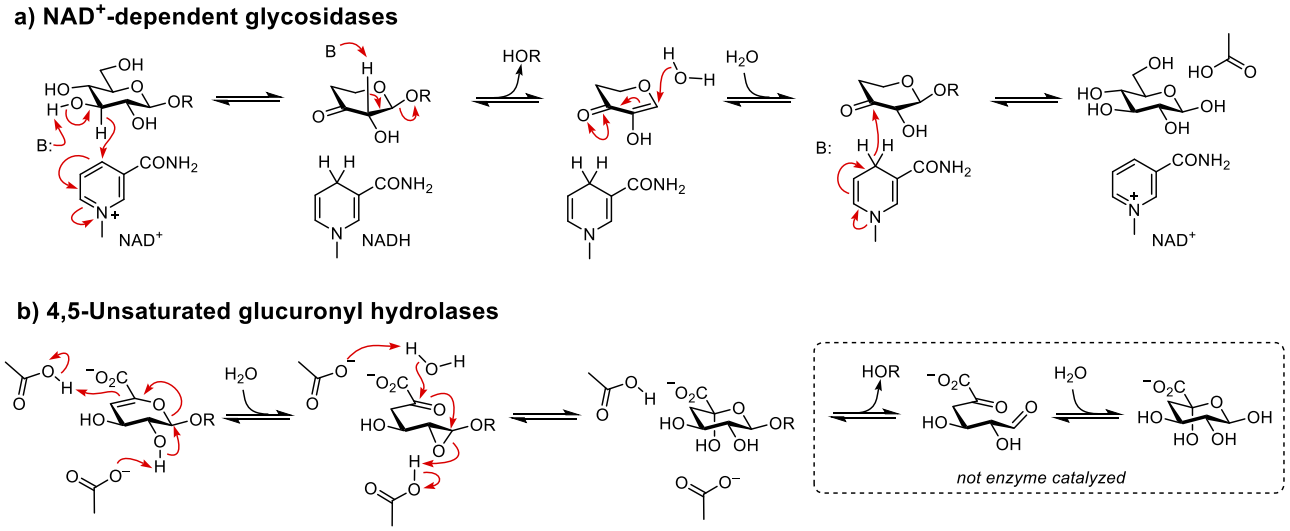


Figure 31. Proposed mechanisms of non-classical glycosidases. (a) NAD^+-dependent glycosidases. Some enzymes of this type are dependent on divalent metal ions. (b) 4,5-Unsaturated glucuronyl hydrolases. There are several proposed mechanisms for this class of enzyme. The one shown involves enzyme catalysis of hydration of the enol ether, with elimination of the aglycon-derived alcohol occurring without enzyme catalysis.

10.2 4,5-Unsaturated β-glucuronide hydrolases

4,5-Unsaturated β-glucuronides are products of hexuronate lyase enzymes that cleave glycosidic bonds of glucuronate-containing polysaccharides through a non-hydrolytic elimination mechanism (**Figure 31b**), and can also be formed through the action of uronate 4,5-dehydratases.[249, 250] The 4,5-unsaturated β-glucuronides can be cleaved through an unconventional hydrolysis mechanism catalyzed by 4,5-unsaturated β-glucuronide hydrolases (GH families 88, 105).[251, 252] These enzymes catalyze a multistep reaction that remains only partially understood. One proposed mechanism that is consistent with kinetic isotope effects involves: protonation at C4 triggering cleavage of the endocyclic C1-O5 bond, with neighboring group participation by OH-2 and formation of a 1,2-epoxide intermediate; recyclization by addition of O5 to C1, with ring-opening of the epoxide to give a C5 hemiacetal.[253] It is proposed that the C5-hemiacetal can undergo elimination, expelling the aglycon derived alcohol and

giving the acyclic 4-deoxy-5-keto-glucuronic acid derived alcohol, which exists in equilibrium with the cyclic pyranose form.

11. Cleavage of glycosidic bonds by lyases and oxygenases

There are several classes of enzymes that catalyze cleavage of glycosides through non-hydrolytic processes. These are not the focus of this review so are only discussed briefly for context. They are not covered in detail.

11.1 Hexuronate lyases

Hexuronate lyases (also known as uronic acid lyases) act on 1,4-linked polysaccharides containing hexuronate groups (**Figure 32a**).[254] These enzymes catalyze the cleavage of the bond between C4 and the glycosidic oxygen through an elimination reaction.[255] Pectin lyases use a divalent metal ion (usually Ca^{2+} or Mn^{2+}) to balance charge on the carboxylate, while most other lyases appear to use a metal-independent mechanism.[254] Deprotonation at C5 by a general base (usually tyrosine, histidine, lysine or arginine) supports an E1cb mechanism where the glycosyloxy group is a leaving group, most likely with general acid assistance.[256, 257] [258] Hexuronate lyases have been classified into Polysaccharide Lyase families as part of the CAZy database.[259]

11.2 Glucan lyases

Glucan lyases act on α-1,4-glucans to cleave individual 1,5-anhydrofructose groups from the non-reducing end (**Figure 32b**).[260] These enzymes belong to family GH31 and use the same catalytic residues as for retaining glycosidases in this family, but for different roles. The first step is identical to that of a retaining Koshland mechanism involving an enzymic nucleophile, namely nucleophilic substitution by the enzymic nucleophile with general acid assistance by a second carboxyl residue. However, the resulting glycosyl enzyme undergoes an elimination reaction involving deprotonation at C2. The 2-hydroxyglucal product tautomerizes to 1,5-anhydrofructose.

11.3 (Lytic) polysaccharide monooxygenases (LPMO/PMOs)

LMPO/PMOs are copper-containing metalloenzymes that catalyze the hydroxylation of 1,4-linked polysaccharides at either C1 or C4 to give the corresponding hemiacetals (**Figure**

32c).[261] These enzymes use a single copper ion and oxygen (and/or hydrogen peroxide) for C-H bond oxygenation. The resulting hemiacetals are unstable species that eliminate, cleaving the glycoside, and forming the corresponding carbonyl compound. Representative substrates include cellulose, xylan, starch and chitin. LMPOs have been classified into Auxiliary Activity families as part of the CAZy database.[262]

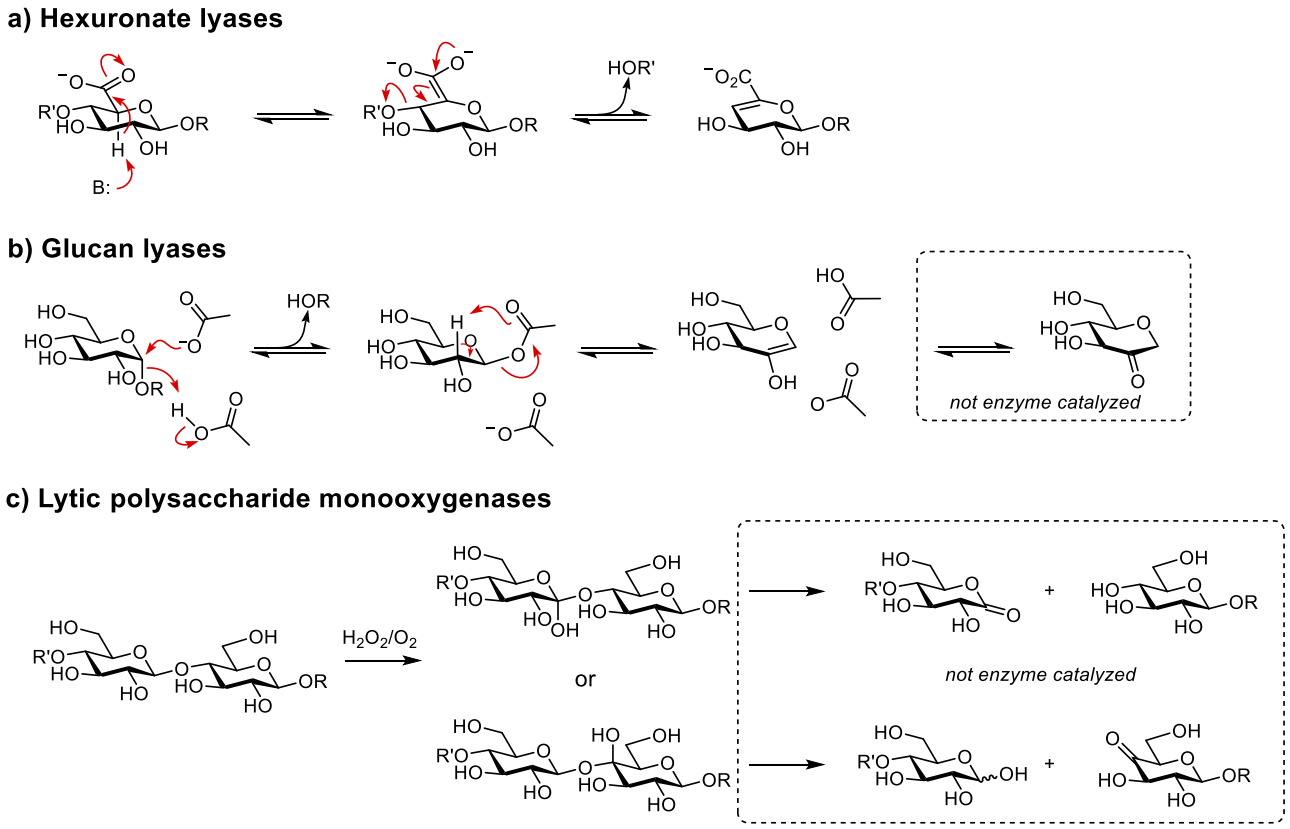


Figure 32. Enzymatic cleavage of glycosidic bonds by non-hydrolytic enzymes. (a) hexuronate lyases; (b) glucan lyases; (c) lytic polysaccharide monooxygenases.

12. Future perspectives

Beyond their roles as biological catalysts and drug targets, glycosidases are increasingly being deployed directly as biomedical and biotechnological tools, exploiting their exceptional stereochemical and linkage specificity. These applications are rooted in decades of mechanistic and structural insight and increasingly benefit from enzyme engineering to enhance stability, selectivity, and performance in non-native contexts.

One prominent medical application is the enzymatic conversion of blood group antigens on red blood cells to generate universal donor cells by editing cell-surface glycan epitopes. Highly selective glycosidases are used to remove terminal antigenic sugars from A and B type

red blood cells, producing the H antigen, and converting to an O type “universal” donor red blood cell.[263-265] Current efforts focus on discovery of suitable enzymes and using site-directed mutagenesis to enhance catalytic efficiency, stability, and activity on intact cell surfaces, improving scalability and clinical feasibility.[241, 266]

Enzyme replacement therapies using glycosidase enzymes are a cornerstone of treatment for several lysosomal storage diseases.[267] In this approach recombinant enzymes are delivered and restore missing enzymatic activity in congenital disorders of glycosylation. Several recombinant glycosidases are now approved drugs,[268] and there is strong interest in improving these enzymes through mutagenesis to address suboptimal pharmacology includingpoor lysosomal targeting, limited catalytic efficiency in vivo, and immunogenicity.[269]

In biopharmaceutical engineering, endo-acting glycosidases, particularly endo-β-N-acetylglucosaminidases, have emerged as useful tools for controlled remodeling of N-glycans on antibodies and other therapeutic glycoproteins.[142, 270] Engineered ENGase variants with attenuated hydrolytic activity and enhanced transglycosylation capability enable the production of homogeneous antibody glycoforms with tuned Fc-effector functions.[271]

Conjugate vaccines are a cornerstone of antibacterial immunization and consist of bacteria-specific capsular polysaccharides covalently linked to antigenic carrier proteins.[272] Conventionally, these polysaccharides are isolated from bacterial cultures and chemically depolymerized prior to conjugation.[273] However, chemical depolymerization can damage chemically labile substituents and typically yields heterogeneous mixtures of fragments. Controlled enzymatic depolymerization, particularly using phage-derived glycosidases, enables the generation of well-defined oligo- or polysaccharide fragments while preserving sensitive functional groups and reducing structural heterogeneity, thereby offering improved control over vaccine composition. [274]

Another emerging application for glycosidases is in cancer glycocalyx engineering.[275] Antibody-glycosidase conjugates can be used to overcome immune evasion by cancer hypersialylation.[276] A HER-2 targeted antibody fused to a sialidase enhanced natural killer cell killing and antibody-dependent cell-mediated cytotoxicity by desialylation of the antibody ligand and of ligands for inhibitory ‘checkpoint’ siglecs.[276, 277]

Finally, glycosidases are useful in glycoproteomics workflows.[278] Enzymatic removal of glycans, or reduction of glycan heterogeneity, concentrates analytical signal into a smaller number of peptide species, thereby enhancing detection and site assignment of glycosylation

events. Sequential treatment with exoglycosidases of known specificity can be used to identify linkages and specific sugar residues.[279] The endo-α-N-acetylgalactosaminidase PNGase F is widely used to cleave intact N-glycans, converting the modified asparagine to aspartic acid, while Endo F and Endo H leave a single GlcNAc residue on the modified asparagine.[280] OglyZOR, an endo-α-N-acetylgalactosaminidase, hydrolyses core 1 and, to some extent, core 3 O-glycans from native glycoproteins and is typically used in combination with an exo-sialidase following desialylation.[280]

Glycosidase research has developed dramatically over the past 70+ years and remains an area with significant potential for discovery and new applications. While the Koshland mechanisms explain many glycosidase activities, the past two decades have revealed non-classical glycosidases that operate via alternative mechanisms. Notably, NAD^+-dependent glycosidases that leverage cryptic redox processes may be far more prevalent than currently recognized. This hypothesis is supported by the vast size of the short-chain dehydrogenase/reductase (SDR) superfamily, to which these enzymes belong, encompassing millions of sequences with unknown functions. However, because this mechanism requires the enzymatic base and the NAD^+ cofactor to act from opposite faces of the enzyme, it imposes a *trans*-diequatorial relationship between the C2 and C3 hydroxyl groups of the sugar ring and is therefore limited to glycans that contain this motif.

The search for new glycosidases with novel substrate specificities and mechanisms is increasingly driven by sophisticated ‘genomic enzymology’ approaches. Two notable strategies include the Enzyme Function Initiative toolset[281] and the study of polysaccharide utilization loci—clusters of physically linked genes that coordinate the degradation of specific glycans.[282] In addition, viruses[283, 284] and archaea[285] represent vast reservoirs of unannotated genomic data with immense potential for discovery. The current pace of new glycoside hydrolase family creation, estimated at around five new families per year, underscores the considerable diversity of glycosidases yet to be explored.

Beyond classical glycosidases, non-hydrolytic glycoside-cleaving enzymes—such as lyases, oxygenases, and phosphorylases—play crucial roles in polysaccharide breakdown. These enzymes frequently collaborate with glycosidases and metabolic enzymes to facilitate the complete depolymerization and catabolism of complex polysaccharides. Consequently, the discovery of novel glycoside-cleaving enzymes may facilitate the discovery of new metabolic

pathways for polysaccharide degradation, and thus advance applications in biofuel production and biomass processing.

Looking ahead, the application of machine learning and artificial intelligence has the potential to revolutionize glycosidase research. These tools can analyze the vast sequence data now available, enabling the identification of misannotated GH family members that may possess distinct substrate preferences or catalytic mechanisms. Additionally, AI-driven approaches can sift through large volumes of dark genomic matter to generate testable hypotheses, integrating functional, sequence, contextual, and structural data to predict new enzyme functions and mechanisms.

Advancements in computational methods are paving the way for the de novo design of entirely novel, highly active enzymes, potentially with unprecedented folds, capable of hydrolyzing a wide range of glycoside substrates. Recent studies have demonstrated some promising results,[286] however, they still face significant challenges. For instance, these methods require the generation and screening of vast numbers of candidates, and even the most successful designed enzymes remain orders of magnitude less efficient than their naturally evolved counterparts.

All enzyme catalysts, whether natural or designed, must adhere to the fundamental principles of chemistry. Consequently, any successful artificial enzymes will need to operate within the constraints of logical catalytic mechanisms. As such, it is likely that any new, designed catalysts will align with the mechanistic frameworks outlined in this review, reinforcing the relevance of established enzymatic paradigms while opening new avenues for enzyme engineering and industrial applications.

As our understanding of glycosidase mechanism has grown, there is an opportunity to use this knowledge to design specific inhibitors for individual glycosidases. This could lead to more precise tools for studying glycobiology and potentially new therapeutic agents with fewer side effects.[287] One potentially fruitful approach is to continue the development of transition state analogues that more closely mimic the charge distribution and shape of the flattened oxocarbenium ion-like structure. This could involve exploring new chemical scaffolds or modifications to existing inhibitor classes. Advanced computational methods coupled with experimental techniques could provide deeper insights into the conformational changes that occur during the catalytic cycle. This could inform the design of inhibitors specific to certain conformational itineraries and help explain substrate specificity. Perhaps the most promising

opportunity for impact in the field of glycosidases is through the development of drugs that targeting these enzymes. This could include drugs that inhibit glycosidases but also those that enhance their activity and stability, such as for treatment of congenital disorders of glycosylation that involve mutations in glycosidases leading to loss of activity.

An alternative approach for glycosidase inhibitor discovery is through the development of peptidic inhibitors through high-throughput panning methods such as phage-display[288] or RNA display.[289] A promising computational approach involves deep learning using RoseTTAFold diffusion[290] in tandem with Alphafold for structure prediction.[291, 292]

Building on the success of glycosynthases and thioglycoligases for the synthesis of glycosidic bonds, there is potential to apply these approaches to grow a palette of engineered enzymes that can be used for construction of glycosidic bonds to order. The tolerance of many glycosidases for substrate variation provides the potential to incorporate unnatural functionality to create new carbohydrate-based molecules and materials. To date, the major class of glycosidases that have been engineered for the synthesis of glycosidic bonds derive from retaining enzymes, including both those that proceed through a glycosyl enzyme or a non-covalent cyclic intermediate. There has been limited exploitation of inverting glycosidases for synthetic applications, and no reports have described the use of cryptic redox glycosidases for synthetic applications, even though the 2-hydroxyglycal intermediate could, in theory at least, be intercepted by a nucleophile other than water.

Expanding the diversity and tuning the selectivity of activity-based probes (ABPs) could significantly enhance the use of ABPs in profiling of glycosidase activity in complex biological systems, potentially unlocking new diagnostic tools and therapeutic targets. Currently, most ABPs are designed to target retaining glycosidases that operate via a glycosyl-enzyme intermediate. However, developing strategies to probe glycosidases that utilize alternative catalytic mechanisms would greatly broaden the scope and applicability of ABPs.

Furthermore, glycosidase-targeting ABPs are typically highly selective for individual enzymes, limiting their utility in broader applications. The development of broad-spectrum ABPs that can target classes of enzyme, echoing those developed for serine proteases,[293] would be a major advance. Such probes, with reduced specificity would provide the capacity to monitor multiple glycosidases, and could facilitate rapid, large-scale profiling of glycosidase expression and inhibition.

Notes

The authors declare no competing interests.

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Acknowledgements

The authors thank past and present collaborators, mentors, students, and friends for their patience and generosity in teaching us, over the course of our careers, the intricacies of glycosidase catalysis. We thank in particular Harry Brumer, Harry J. Gilbert, Bernard Henrissat, Carme Rovira, Michael Sinnott, Robert V. Stick, Nicolas Terrapon, Andrea Vasella, David J. Vocadlo, and Stephen G. Withers, among many others who have inspired us, shaped our thinking, and contributed to this field. We thank the Australian Research Council

for generous funding (DP240100126, DP250100819). GJD thanks the Royal Society for the Ken Murray Research Professorship. G.J.D. and H.S.O. acknowledge support from the European Research Council (ERC-2020-SyG 951231 Carbocentre).

Abbreviations

CAZy	Carbohydrate Active enZyme database
CBH	cellobiohydrolase
3D	three-dimensional
DNA	deoxyribonucleic acid
EC	Enzyme Commission
ENGase	
GH	glycoside hydrolase
HMM	hidden Markov model
KIE	kinetic isotope effect
LFER	linear free energy relationship
LPMO	lytic polysaccharide monooxygenase
NAD^+	nicotinamide adenine dinucleotide
$NADP^{+`}$	nicotinamide adenine dinucleotide phosphate
NAG	*N*-acetyl-D-glucosamine
PMO	polysaccharide monooxygenase
PNP	p-nitrophenyl
PUGNAc	O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenylcarbamate
TS	transition state

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