

Itineraries of enzymatically and non-enzymatically catalyzed substitutions at *O*-glycopyranosidic bonds

Wim Nerinckx^{*}, Tom Desmet,[†] and Marc Claeysens

Laboratory for Glycobiology, Department of Biochemistry, Physiology and Microbiology
Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium

E-mail: wim.nerinckx@ugent.be

Abstract

Several crystal structures of glycoside hydrolases in complex with a substrate analog, or of inactive mutants complexed with a substrate, reveal non-ground state carbohydrate conformations within subsite –1 of the active site. These “frozen” local minima as preferred by the enzyme represent pre-transition-state situations along the reaction itinerary. Substantiated by theoretical considerations, this leads to the proposal that substitutions on β -equatorial as well as α -axial D-*O*-glycopyranosidic bonds may always follow an itinerary that is predetermined by the original configuration at the anomeric center, where the formation of a transition state that is similar to a half-chair is always preceded by a conformational change away from the ground state.

Keywords: Hydrolysis, glycoside hydrolase, mechanism, substrate/ligand complex

Abbreviations: TS, Transition State; ALPH, Antiperiplanar Lone Pair Hypothesis; SPLH, Synperiplanar Lone Pair Hypothesis; PMR, Principle of Microscopic Reversibility; PLM, Principle of Least Motion; BEP, Bell-Evans-Polanyi

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^{*} Corresponding author, Tel: (+32) 9-264-5270, Fax: (+32) 9-264-5332

[†] Present address: Laboratory of Industrial Microbiology and Biocatalysis, Department of Biochemical and Microbial Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

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1. Introduction

In Nature, many different carbohydrate monomers, oligomers and polymers play important structural or functional roles in a vast array of biological processes. *O*-glycoside hydrolases (EC 3.2.1.x) as carbohydrate-modifying or -degrading enzymes represent a structurally diverse group, where primary amino acid sequence analysis allows classification into more than 100 different families;¹⁻⁴ many being grouped into one of fourteen fold-related clans.⁵ Most of these enzymes need neither metals nor cofactors, yet achieve k_{cat} values that are in the order of 1–100 s⁻¹, a truly stunning rate enhancement in comparison to k_{uncat} values of 10⁻¹⁵ s⁻¹.⁶ This, together with their stereo- and regioselectivity, and their biological importance, have led to high interest also in their applications, both therapeutic and industrial.⁷⁻¹¹

With almost no exceptions, *O*-glycoside hydrolases possess at least two mechanistically important carboxyl functions at the junction of subsites -1/+1: 1) a semi-lateral *syn*- or *anti*-positioned¹² proton donor close to the glycosidic oxygen; and 2) a nucleophilic carboxylate (retaining hydrolases) or a similar residue assisting solvent nucleophilicity (inverting hydrolases). The general but undetailed catalytic mechanisms of β -D-glycopyranoside hydrolysis

by retaining (double displacement) and inverting (single displacement) enzymes have been proposed by Koshland as early as 1953¹³ and are presented in Figure 1.

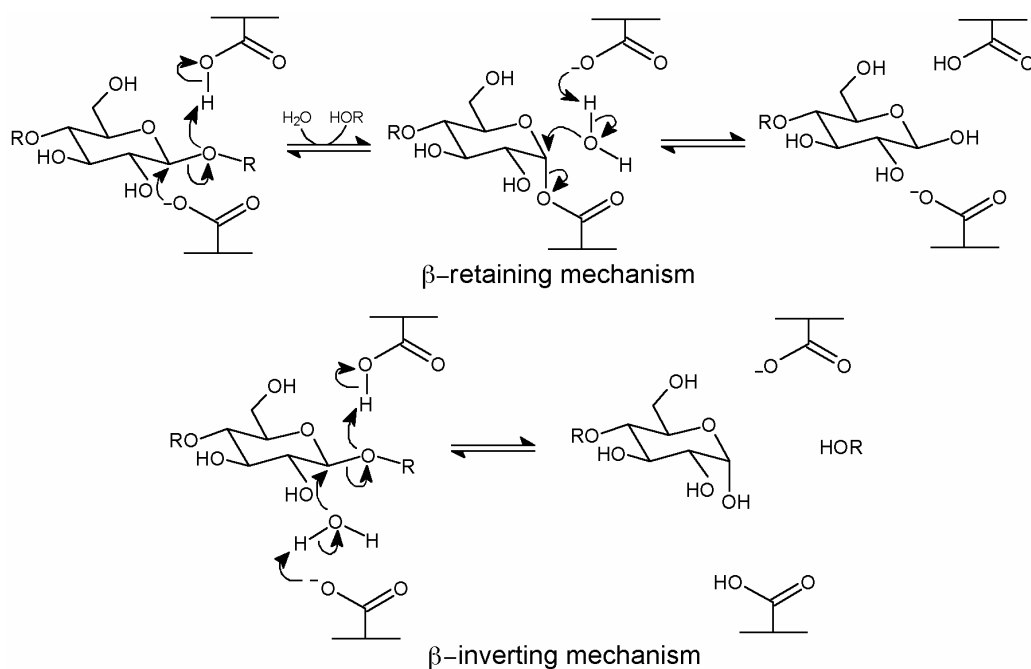


Figure 1. Catalytic mechanisms of β -glycoside hydrolases according to Koshland.¹³

Current understanding of glycosidase mechanisms has been reviewed thoroughly,^{14–17} and recently three new general concepts have been put forward: 1) the *syn/anti* proton donor positioning;¹² 2) the presence of a hydrophobic platform as a common feature within the -1 subsite;¹⁸ and 3) enzymes with *syn*-positioned proton donors perform electrostatic transition state (TS) stabilization (known to be the most important factor for enzymatic catalysis^{19,20}) towards the substrate's ring oxygen by means of the conjugate base of their proton donor, whereas *anti*-protonating enzymes provide a separate electron-rich residue for this purpose.²¹

The considerations in this work pertain to pyranoside glycosidic bond substitutions that operate by a classical exocyclic mechanism on oxyalkyl-type leaving groups, thus not to: 1) the endocyclic mechanism²² which has not yet been observed with glycoside hydrolases; 2) the S_Ni -substitution mechanism that occurs in glycosyl fluoride solvolysis²³ and in-situ anomerisations with good leaving groups,²⁴ and has been proposed to be operative in retaining glycosyltransferases;²⁵ 3) catalysis by GH family 4 (GH4) glycosidases that operate by a redox and elimination mechanism;²⁶ and 4) GH families 18, 20 (Clan K)^{27,28} and 84²⁹ that utilize anchimeric assistance from the substrates' *N*-acetyl group, resulting in a strained oxazoline-type glycosyl intermediate (chitinases/chitobiases). Based on its orientation relative to a reference atom in the Fisher projection (IUPAC nomenclature), an α/β glycosidic bond often – but not always – corresponds with an axial/equatorial substituent orientation in the ground state

conformation. For the rational development of the present discussion, an α -axial or β -equatorial O-glycosidic bond of a 4C_1 ground state D-glycopyranoside will be assumed throughout; the IUPAC-idiosyncratic sialosyl glycosides and some L-glycopyranosides are dealt with in separate paragraphs.

2. Inconsistencies within the current understanding of itineraries for glycopyranosidic bond substitutions

A classical glycopyranosidic bond substitution proceeds via a transient³⁰ oxocarbenium-like TS, with an sp^2 -hybridised geometry at both C1 and O5, allowing a considerable mutual double bond character and resulting in C5–O5–C1–C2 coplanarity. This process does not involve a discrete carbocation in a first-order reaction, but is borderline SN_1 – SN_2 . Recently published primary ${}^{13}C$ ^{31,32} as well as secondary α -deuterium³³ kinetic isotope effects indeed are consistent with SN_2 -type pathways. The four-atom coplanarity at the TS is obtained in four conformational situations: 1) the 4H_3 – and its flanking 4E and E_3 conformers; 2) the 3H_4 – and its flanking 3E and E_4 conformers; 3) the $B_{2,5}$ conformer; and 4) the ${}^{2,5}B$ conformer (Figure 2). The route from the reactant via the transient TS into the product is called the substitution itinerary, which has been lively debated up to now.

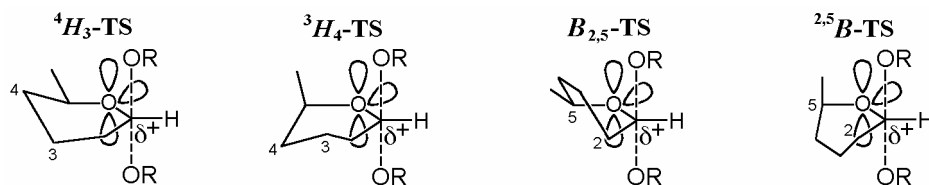


Figure 2. Four TS conformational situations having the C2–C1–O5–C5 four-atom coplanarity.

2.1. The ALPH itinerary for β -glycopyranoside substitutions

According to the Antiperiplanar Lone Pair Hypothesis (ALPH), the substitution of β -equatorial glycopyranosides is preceded by a conformational change away from the ground state chair into a skew conformation where the leaving group has an axial position as well as an antiperiplanar set-up with the ring oxygen's *trans* lone pair, which by hybridization at the TS into a 2pz orbital allows the formation of the partial double bond towards the anomeric center.^{34–36} The reaction then proceeds through an ALPH-compliant β -skew \rightarrow 4H_3 -like TS \rightarrow α - 4C_1 itinerary (Figure 3 from left to right).

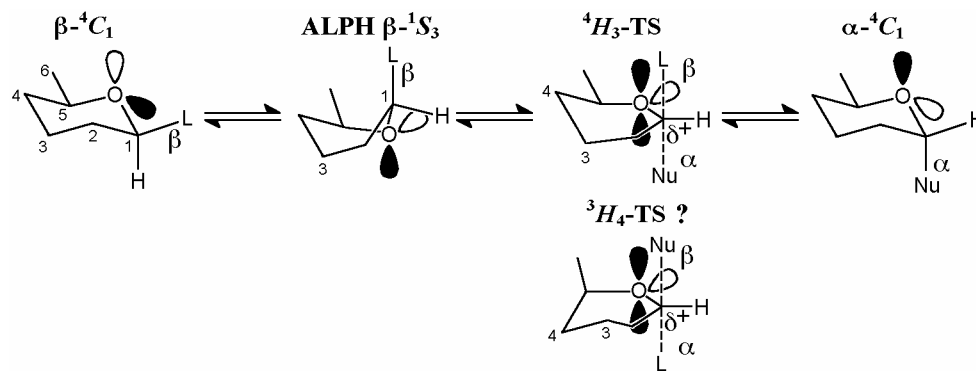


Figure 3. The Deslongchamps-type substitution itinerary for β -D-glycopyranosides. The O5 lone pair that is *trans* to the leaving group is indicated in black. A preceding conformational change into an ALPH-compliant 1S_3 -like pre-TS is followed by a transient 4H_3 -like TS and leads to the α - 4C_1 product. A 3H_4 -like TS does not belong to this itinerary. L = leaving group; Nu = nucleophile.

The relevance of ALPH for glycosidic bond substitutions has been controversial for a long time, but much of the criticism has ceased after a seminal article by P. Deslongchamps.³⁷ Some objections on the general validity of ALPH have since been formulated, e.g. on a conformationally restricted 2-methoxy-1,3-dioxane³⁸ and on cyclic amidinium substrates,³⁹ but they have been re-analyzed in a recent extensive review by S. Chandrasekhar as not to invalidate ALPH.⁴⁰

2.2. The reverse ALPH itinerary for α -glycopyranoside substitutions

In the case of α -axial glycopyranosidic bond substitutions, the leaving group already has an ALPH-compliant set-up when the carbohydrate ring is in the ground state chair conformation. The Principle of Microscopic Reversibility (PMR) has often been invoked to suggest that here the itinerary is the reverse of that for a β -equatorial glycopyranosidic bond substitution, i.e. an ALPH- α - ${}^4C_1 \rightarrow {}^4H_3$ -like TS $\rightarrow \beta$ -skew pathway (Figure 3, from right to left). That “ α -glycosides must hydrolyze via their ground state conformation” has even been explicitly stated by Deslongchamps.³⁴ However, the PMR applies to phenomena occurring within the forward versus reverse path of a single process.⁴¹ In other words, it can be invoked for the reverse path of a given glycosidic bond substitution (e.g. on a given β -glucoside), but it cannot be used to predict the reaction behavior of another glycoside (e.g. a given α -mannoside) that has its own PMR for its reverse path. In fact, this generalizing but subtle principle has often been misapplied for deducing or disproving the mechanism of a reaction, and this has been extensively reviewed.⁴² If the PMR would be a mechanistic axiom for glycopyranosidic bond substitutions, then the substitution of an α -axial glycopyranoside would always progress through the ALPH- α - ${}^4C_1 \rightarrow {}^4H_3$ -like TS $\rightarrow \beta$ -skew pathway, but this would rule out a 3H_4 -like TS, which occurs in the case

of α -inverting GH47 enzymes (see further). Thus, the reverse Deslongchamps pathway – although allowed by the PMR – is not always operative. Later in this work we will propose that conformationally unrestricted α -glycosides may very well prefer an alternative substitution route that has a lower activation energy toward its 3H_4 -like TS (and that has its own PMR), instead of the reverse Deslongchamps pathway with its 4H_3 -like TS.

2.3. Itineraries involving a boat-TS

A possible alternative is a skew \rightarrow boat-TS \rightarrow skew glycopyranoside substitution pathway. This route implies that ALPH is not operative, but instead that the ring oxygen's free electron pair positioned *cis* versus the leaving group participates in the partial double bond towards C1 at the transient boat TS (Synperiplanar Lone Pair Hypothesis, SLPH) (Figure 4).⁴³ Such a substitution is electronically equivalent to a syn-elimination, as has been suggested previously.^{16,17} However, syn-eliminations on a cyclohexane ring are 7000 times slower than anti-eliminations.⁴⁴ On the other hand, synperiplanar effects have recently been shown to be operative in acid hydrolyses of conformationally restricted acetals.^{45,46}

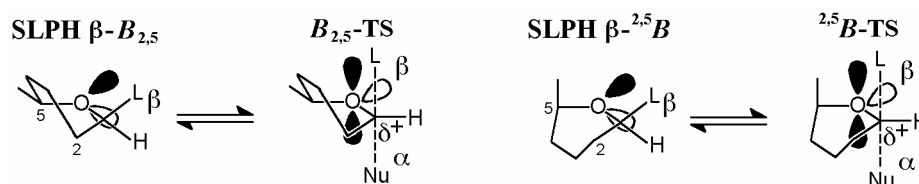


Figure 4. Comparison of the regular $B_{2,5}$ and ${}^{2,5}B$ conformers with the corresponding boat-TS conformations shows a synperiplanar topology of the leaving group versus an O5 lone pair (indicated in black). Only the situations for β -D-glycopyranosides are shown; the topology is analogous for α -D-glycopyranosides. L = leaving group; Nu = nucleophile.

The synthesis of isopropyl and *p*-nitrophenyl α - and β -D-glucopyranosides **1-4** that are locked into the $B_{2,5}$ via a C2–C5 oxymethylene bridge has recently been published (Figure 5).⁴⁷ Their acid-catalyzed hydrolysis is obligatorily SLPH-compliant and proceeds at remarkably similar rates to those of the corresponding unconstrained glucosides,⁴⁸ which indicates that such an itinerary may be used by enzymes as well. However, the $B_{2,5}$ is a relatively high-energy conformation due to an unfavorable C2–C5 flagpole positioning⁴⁹ as well as eclipsed substituents at the C3–C4 bond. The locked-boat compounds do not have a flagpole problem, but the authors caution that at least part of the observed reactivity must derive from a higher ground state energy in these strained bicyclo-[2,2,2] systems. The cellobiose analog **5** (with the $B_{2,5}$ -locked glucoside at the nonreducing end) was also synthesized and was assayed against glycoside hydrolases from 11 different families. It is only a very weak inhibitor for two β -retaining glycanases (from families 3 and 7), and its catalyzed hydrolysis was never observed.⁴⁸ But inspection of the known crystal structures of complexed hexopyranoside hydrolases invariably shows the crucial –1

subsite to be remarkably spacious, so the unnatural oxymethylene bridge should be easily accommodated. Moreover, the C2-hydroxyl group is now replaced by an ether bridge, which removes a possible hydrogen bond interaction with the enzyme. This can only influence K_{ass} and be of relatively minor importance for TS stabilization, which is mainly achieved through electrostatic compensation by the enzyme of the change in charges^{19,20} occurring at the O5 ring oxygen.²¹ Thus, if a $B_{2,5}$ -TS belongs to the substitution itinerary within the tested β -glycoside hydrolases (from families 1, 3, 6, 7 and 18), one should expect the locked-boat disaccharide **5** to be a substrate or a relatively good inhibitor – which is never the case.

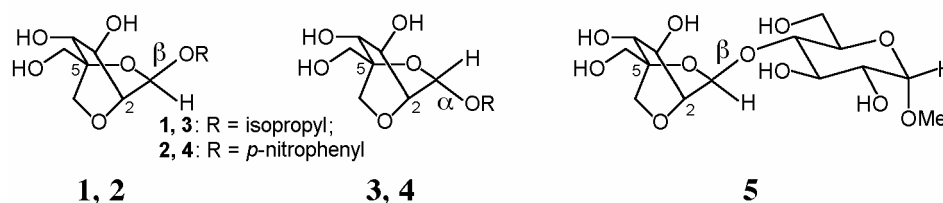


Figure 5. The by Blériot et al.^{47,48} synthesized β - and α - $B_{2,5}$ locked-boat compounds **1-4**, and the locked-boat cellobiose analog **5**.

Boat conformations have nevertheless been proposed as transition states within the mechanism of several glycoside hydrolases on the basis of observed conformers within the liganded complexes, most notably within GH26⁵⁰ and 11.⁵¹ This has been recently reviewed.^{16,17} Further in this work we will show that each of those conformers can just as well be interpreted as indicators of 4H_3 or 3H_4 -like transition states.

2.4. A reinterpretation of kinetic isotope effects on aryl- α -D-glucopyranosides

In an effort to disprove the ALPH hypothesis, Hosie and Sinnott undertook an extensive study on primary and secondary deuterium isotope effects for the hydrolysis of aryl- α -D-glucopyranosides catalyzed by a *S. cerevisiae* retaining α -glucosidase.⁵² They came to the following conclusions:

(1) After formation of the initial enzyme-substrate complex, a kinetically discrete noncovalent event must precede the actual bond-breaking process, which indeed excludes the reverse Deslongchamps-type ALPH- α - ${}^4C_1 \rightarrow {}^4H_3$ -like TS $\rightarrow \beta$ -skew pathway (Figure 3, from right to left), since it comprises a direct ground state \rightarrow TS conversion.

(2) At the transient TS, the C2-H bond must be near-perpendicular to the planar oxocarbenium-type system. This was interpreted as to be only compatible with the occurrence of the ${}^{2,5}B$ -TS, in spite of its severe C2-H to C5-hydroxymethyl flagpole interaction as well as the 1,3-syn-diaxial positioning of the C5-hydroxymethyl group versus the incoming nucleophile. Although a passage through the 3H_4 -TS was not considered as an alternative possibility, their experimental results perfectly agree with this: the preceding discrete non-covalent event would then be a conformational change of the ground state α - 4C_1 into an ALPH-compliant α - 3S_1 conformation,

whereas a near-perpendicular C2–H bond versus the C1–O5 bond is present in the 3S_1 pre-TS as well as in the structurally nearby 3H_4 -TS conformation (Figure 6).

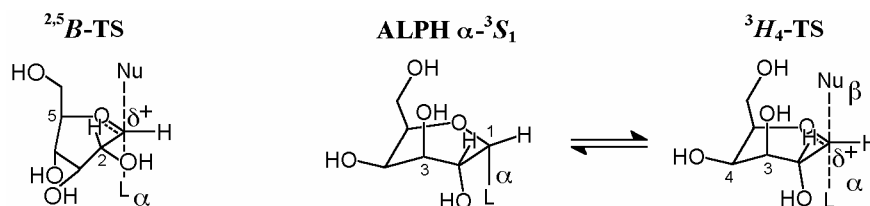


Figure 6. A ${}^{2,5}B$ -TS of an α -D-glycopyranoside has a near-perpendicular positioning of the C2-H bond versus the C1–O5 bond, but the same relative positioning occurs in the 3S_1 pre-TS and its overall-similar 3H_4 -TS.

3. The α -inverting GH47 enzymes use an alternative ALPH-compliant substitution itinerary comprising a 3H_4 -like TS

Several all- α - 4C_1 oligosaccharide Michaelis complexes have been observed in structures of α -glycoside hydrolases, e.g. the GH13 *Bacillus circulans* cyclodextrin glycosyltransferase inactive mutant in complex with maltonaose (pdb entry 1cxk),⁵³ and these appear to indicate that a reverse Deslongchamps-type ALPH- α - ${}^4C_1 \rightarrow {}^4H_3$ -like TS $\rightarrow \beta$ -skew pathway is operative. Deduction of mechanism itineraries based on complexed enzyme structures should however be conducted cautiously, since these will always contain a local energy minimum, which not necessarily represents the productive substrate conformation. This is *a fortiori* so for the 4C_1 ground state, which by its own nature will have a tendency to be a local minimum within the –1 subsite of glycoside hydrolases.

A recently published crystal structure of the human GH47 Class 1 α -1,2 mannosidase in complex with a thiodisaccharide substrate analog (pdb entry 1x9d) reveals the α - 3S_1 conformation in the –1 subsite which, taking into account the close shape similarity, suggests a 3H_4 -like TS.⁵⁴ Furthermore, a detailed docking study on the analogous *Saccharomyces cerevisiae* enzyme with its disaccharide substrate in different glycon conformations indicates that the binding pathway passes through the α - 3S_1 just before reaching the TS, which is likely the 3E .⁵⁵ Clearly, this enzyme family uses an itinerary towards the TS that does not proceed via a direct substitution on the ALPH-compliant α - 4C_1 , but that involves a prior conformational reorganization into the α - 3S_1 , which also is ALPH-compliant. The authors of the 3D structure have realized that the initial conformation of the end-product very likely is the β - 1C_4 inverted chair, since when the Principle of Least Motion (PLM)⁵⁶ is applied to the conversion of the 3S_1 into the 3H_4 (or into the closely similar 3E), only a slight atomic displacement further in the same overall direction leads to a collapse into the 1C_4 . Corroborating this,⁵⁴ kifunensine as well as 1-deoxymannojirimycin, both very potent aza-inhibitors of GH47 enzymes, adopt the 1C_4 within

the -1 subsite of the human α -1,2 mannosidase, even though the 4C_1 is the ground state of 1-deoxymannojirimycin.⁵⁷

In summary, the conformational itinerary for the GH47-catalyzed hydrolysis of the α -1,2-D-mannosidic bond apparently proceeds through an alternative ALPH-compliant route (Figure 7): ground state α - 4C_1 \rightarrow enter the skew-boat pseudorotational series and then adopt an ALPH-compliant conformation \rightarrow α - 3S_1 (or closely similar; now the substitution can start) \rightarrow 3H_4 -TS (or closely similar such as the 3E) \rightarrow β - 1C_4 (end of substitution). Only thereafter will the product return to the ground state β - 4C_1 .

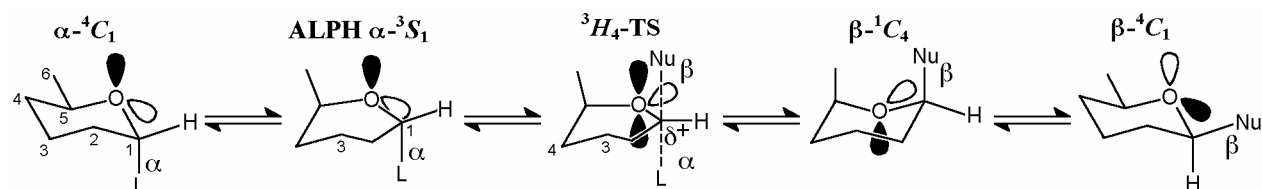


Figure 7. The alternative ALPH-type substitution itinerary for α -D-glycopyranosides. The O5 lone pair that is *trans* to the leaving group is indicated in black. A preceding conformational change into an ALPH-compliant 3S_1 -like pre-TS is followed by a transient 3H_4 -like TS and initially leads to the β - 1C_4 product. The latter converts to the ground state β - 4C_1 in an independent process. L = leaving group; Nu = nucleophile.

4. Towards a mechanistic simplification of *O*-glycopyranoside substitution itineraries

One could argue that a direct substitution on a ground state 4C_1 α -D-mannopyranoside is severely disfavored because of steric hindrance from the axial C2-hydroxyl group towards the incoming nucleophile, and therefore the alternative ALPH trajectory (Figure 7) is exceptionally preferred over the reverse Deslongchamps-type α - 4C_1 \rightarrow 4H_3 -like TS \rightarrow β -skew pathway (Figure 3, from right to left). However, the itinerary used by GH47 passes the same 3S_1 pre-TS and subsequent 3H_4 -TS conformations as those that can be deduced for aryl- α -D-glucopyranosides when re-interpreting the isotope effect data from Hosie and Sinnott⁵² (Figure 6), which is even more remarkable since glucopyranosides have no steric clash between their ground state equatorial C2-OH and the axial nucleophile. This hints to the intriguing possibility that many, if not all, classical α -glycopyranosidic bond substitutions may follow the alternative ALPH-compliant α -skew \rightarrow 3H_4 -like TS \rightarrow β - 1C_4 itinerary (Figure 7). And concomitantly, since a substitution itinerary that passes through a SLPH-compliant boat TS is unlikely for conformationally unrestricted compounds (as outlined above), all classical β -glycopyranosidic bond substitutions may follow the Deslongchamps-type ALPH- β -skew \rightarrow 4H_3 -like TS \rightarrow α - 4C_1 itinerary (Figure 3). The α - and β -*O*-glycopyranoside substitution itineraries may then have a remarkable mechanistic similarity: they may both be always preceded by a conformational change into an ALPH-compliant skew conformation, pass through a half-chair TS, and end in a chair conformation.

And glycoside hydrolases that operate with the classical exocyclic mechanism might also exclusively use these two itineraries, and this suggests a reassessment of the currently known complexed structures, which is the further focus of this work. But first the most important question has to be addressed: could there be an intrinsic advantage for these two itineraries?

5. Basic principles in organic chemistry yield arguments for two inherently preferred substitution itineraries: Deslongchamps-type for β -glycopyranosides, alternative ALPH-type for α -glycopyranosides

The above question can only be addressed by focusing on the TS, since it is the passage through the lowest-energy TS that will dictate the preferred itinerary and its necessary conformational changes, not the other way round (S. Chandrasekhar, personal communication). So, why may a skew \rightarrow half-chair TS \rightarrow chair substitution be the pathway with the lowest activation energy towards the TS, for both classical β - and α -glycopyranoside substitutions?

For most D-glycopyranosides, the 4C_1 is the ground state and the 1C_4 is the second-lowest energy conformation,⁵⁸ since only in a chair do all ring substituents occupy staggered positions. Then a combination of the Bell-Evans-Polanyi (BEP) principle⁵⁹⁻⁶³ with the Hammond postulate⁶⁴⁻⁶⁶ suggests an inherent lowest TS pathway. This is shown in the simplified overall energy profile in Figure 8, idealized for a β -glycopyranoside substitution with an approximately 50/50 equilibrium of reactants and products (the analysis is analogous for reactions with an equilibrium more to the right of the reaction coordinate). Since this is a comparison of two possible itineraries for a carbon substitution on the same molecule (thus with the same reacting substituents), one expects a normal Hammond behavior for the respective TS positioning on the reaction coordinate.⁶³

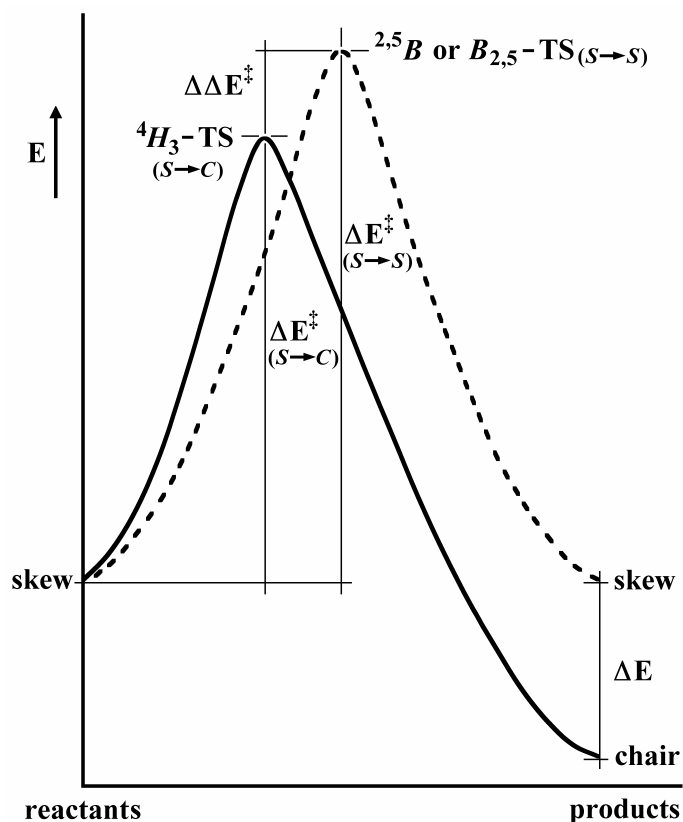


Figure 8. Simplified overall energy profile illustrating Hammond and BEP considerations for a β -glycopyranosidic bond substitution. Full line: skew \rightarrow half-chair TS \rightarrow chair itinerary; dashed line: skew \rightarrow boat TS \rightarrow skew itinerary.

If a β -glycopyranoside substitution would proceed via a SLPH-compliant β -skew \rightarrow boat TS \rightarrow α -skew itinerary, the energy profile would be roughly symmetrical (dashed line), since skews are often of comparable steric energy. In contrast, an ALPH-compliant β -skew \rightarrow half-chair TS \rightarrow α -chair itinerary (full line), ending in the ground state conformation, which is of considerably lower energy than any skew (up to 6 kcal/mol depending on the sugar type⁵⁸), would result in a TS that is more reactant-like due to the Hammond postulate (the TS shifts to the left in the reaction coordinate). But more importantly, the BEP principle predicts that the energy of activation $\Delta E_{(S \rightarrow C)}^\ddagger$ for a skew \rightarrow half-chair TS \rightarrow chair itinerary should be lower compared to $\Delta E_{(S \rightarrow S)}^\ddagger$ in the related skew \rightarrow boat TS \rightarrow skew itinerary.

The energetic advantage $\Delta \Delta E^\ddagger$ from a BEP-Hammond effect cannot be more than a few kcal/mol. However, this should be compared to the relative disadvantage of a boat TS with its fully eclipsed bond and flagpole interaction, and belonging to a SPLH-compliant itinerary that has as yet only been confirmed with conformationally-locked and torsionally-strained ring systems.⁴⁵⁻⁴⁸ The dual effect (advantage half-chair TS/disadvantage boat TS) may then become substantial in comparison to the ± 30 kcal/mol estimate for the enthalpy of activation of spontaneous glycoside hydrolysis.⁶

A reverse-ALPH chair \rightarrow half-chair TS \rightarrow skew pathway (Figure 8, full line from right to left) would result in an activation barrier that is increased by the energy difference ΔE between a skew and a chair conformation. For many, if not all, *O*-glycopyranosides this may be large enough so that an alternative skew \rightarrow half-chair TS \rightarrow chair itinerary is preferred over a reverse-type pathway.

The energy profile for a glycopyranosidic bond substitution is however not simply two-dimensional as in Figure 8, but is a multidimensional landscape, wherein the actual TS is the lowest saddle point within a high-energy mountainous ridge-barrier that represents all possible TS conformations, including many that are energetically implausible. The above considerations then indicate that the lowest-energy saddle point will always be a half-chair TS. In its vicinity will be a minor energy well representing the local minimum energy conformer that precedes the TS; the Hammond postulate indicates that this conformer also structurally resembles the TS. This would be a skew-boat that is ALPH-compliant, and such conformation can be trapped in glycoside hydrolases with e.g. Driguez' thiooligosaccharide analogs.⁶⁷ One does not expect that a pre-TS conformation is a strict boat, since this contains an unfavorable flagpole interaction as well as Pitzer tensions from eclipsed substituents. The BEP principle predicts that the newly formed product should fall into the deepest nearby energy minimum right after the half-chair TS. For the substitution of β -D-glycopyranosides into their α -products, this end-conformation often is the α -⁴C₁ ground state, overall resulting in the ALPH- β -skew \rightarrow ⁴H₃-like TS \rightarrow α -⁴C₁ itinerary (Figure 3) in full accordance with Deslongchamps' theory.³⁷

In the case of α -D-glycopyranosides substituting into their β -products, the analogous lowest-energy route in Figure 8 represents the alternative ALPH-type α -skew \rightarrow ³H₄-like TS \rightarrow β -¹C₄ itinerary (Figure 7), thus initially ending in the β -inverted chair (as in GH47). But some β -skews are of comparable energy – and sometimes even of slightly lower energy – than the β -¹C₄,⁵⁸ often mainly due to the presence in the latter of an unfavorable syn-diaxial interaction from the β -C1-glycosidic bond versus the D-C5-hydroxymethyl group. However, *and this holds for substitutions on α - as well as β -glycopyranosides*, syn-diaxial interactions are partly relieved in a C3–C4 half-chair TS. Moreover, at the stage of the transient TS, the bonds to the leaving and incoming groups are semi-formed and therefore much longer, hence syn-diaxial interactions towards the C1 substituents are not yet present. And, the newly formed product at the start of the energy descent, very shortly after the TS, still resembles it (Hammond postulate). So, neither the TS, nor the species shortly thereafter endure the steric repulsions that will be encountered at the end of the substitution process. Hence, the downwards energetic pathway will be in the direction of a chair anyway, since this is the *initial steepest descent* that provides maximal initial ring strain release, irrespective of whether the end result will be a ⁴C₁ or ¹C₄. Any lower energy conformation may then only be reached by crossing another barrier – but by then the actual substitution is already over. This suggests that substitutions of α -D-*O*-glycopyranosides into their β -products may very well, if not always, proceed via the alternative ALPH-type α -skew \rightarrow ³H₄-like TS \rightarrow β -¹C₄ itinerary, and only thereafter will the product return into the β -⁴C₁ ground state.

6. Demarcation of preferred substitution itineraries within the glycopyranoside conformational space

In Figure 9, a map is presented that is a mechanism-based adaptation of Dowd's Mercator projection for the conformational space of aldopyranosides,⁵⁸ which itself is an adaptation of the Cremer-Pople puckering sphere for six-membered rings.⁶⁸ Herein, the pseudorotational series of the subsequent ideal skew and boat conformations is spread over the equator, all having a Θ orientational puckering of 90° with the Φ orientational puckering varying between 0° and 360° . The ideal 1C_4 and 4C_1 conformations reside respectively at the North Pole ($\Theta = 180^\circ$) and at the South Pole ($\Theta = 0^\circ$), whereas the two pseudorotational series of the subsequent ideal half-chair and envelope conformations are near the Tropic of Cancer and Tropic of Capricorn, respectively. At the skew-boat pseudorotational series and at both chair conformations, the value above the conformational descriptors represents the ideal torsional angle between the glycosidic bond of an α -glycopyranoside versus the *trans*-positioned free electron pair of the ring oxygen, and the value below represents the same for a β -glycopyranoside. It is obvious that the ALPH requirement is fulfilled when this value reaches $150\text{--}180^\circ$. Such Mercator projection (Figure 9) should be preferred over the much used^{15,17} conformational wheel, which is in essence a simplified azimuthal projection of the Cremer-Pople sphere. In order to cover the complete conformational space, one needs in fact two azimuthal projections, i.e. one from each Pole. Moreover, these do not take into account the relative positioning of envelopes within the conformational series near both tropics.

Within the general setting of the Mercator projection, a classical β - as well as α -D-glycopyranosidic bond substitution are both suggested to be preceded by a conformational change, away from the ground state 4C_1 at the South Pole ($\Theta = 0^\circ$), into an ALPH-compliant conformation belonging to the skew-boat pseudorotational series at the Equator ($\Theta = 90^\circ$). From there, the PLM indicates that the reaction itinerary should follow a geodesic along the Cremer-Pople sphere, whereas the BEP principle indicates a route in the direction of a chair conformation; thus only a small variation in Φ should be involved in the process. The ultimate substitution is a fast process³⁰ that passes through the lowest saddle point in the high-energy mountain ridges of TS conformation possibilities near the Tropic of Capricorn or Tropic of Cancer and ends in a chair at $\Theta = 0^\circ$ or 180° for either a β - or an α -original glycosidic bond configuration.

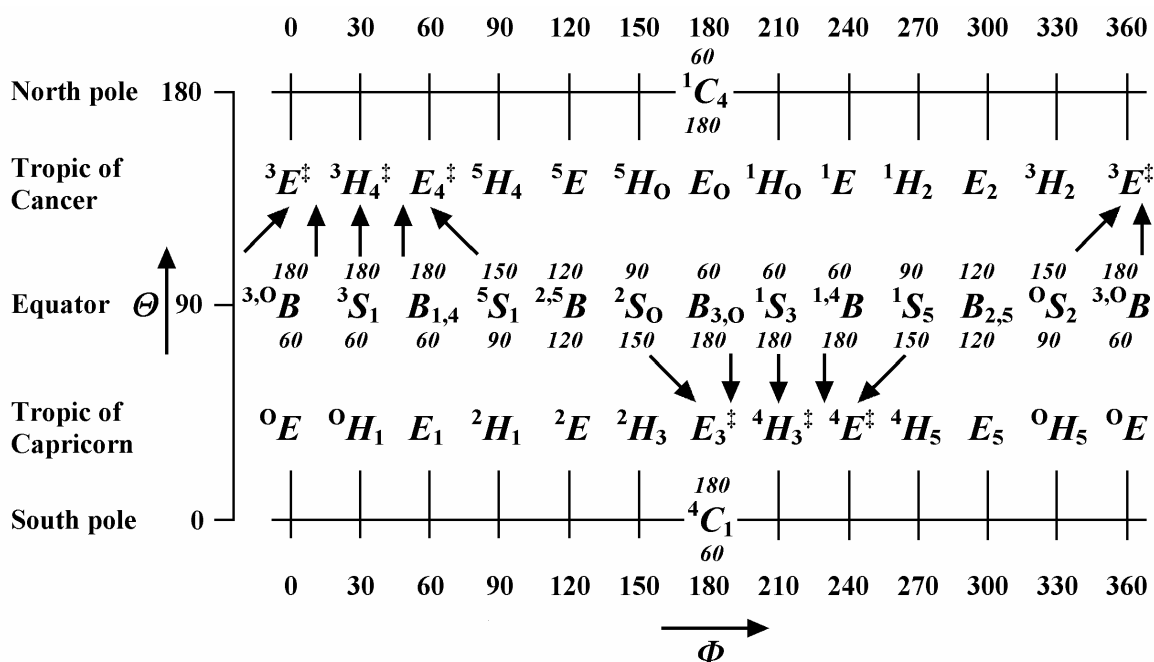


Figure 9. Mercator projection map of the Cremer-Pople puckering sphere (adapted from Dowd⁵⁸), showing the locations of characteristic conformers. This is a mechanism-based adaptation for D-glycopyranosides. Pre-TS conformations at $\Theta = 90^\circ$ are ALPH-compliant, thus with a torsional angle between the glycosidic bond versus the *trans*-oxygen free electron pair of at least 150° , indicated above (for α -D-glycopyranosides) or below (for β -D-glycopyranosides) the conformational descriptors. Preferred transition states are near a pre-TS (PLM) and are indicated with the Cross of Lorraine. The arrows indicate alternative ALPH-type (left) and Deslongchamps-type (center) substitution itineraries.

Consequently, this results in the following two preferred general itineraries:

(1) For β -D-glycopyranosides: Deslongchamps-type. These comply with the ALPH requirement when $\Theta = 90^\circ$ at $\Phi = 150^\circ$ to 270° , comprising the 2S_0 , 1S_3 and 1S_5 skews as possible pre-TS local minima (boat conformations are seldom local minima⁵⁸). TS candidates should have a nearby value of Φ , and should be coplanar at C5–O5–C1–C2, which is only the case with the E_3 , 4H_3 and 4E conformations (indicated with the Cross of Lorraine in Figure 9) at the $\Phi = 180^\circ$ to 240° range. The reaction itinerary is then a fast passage from one of the ALPH-skews over the lowest saddle point in the high-energy mountains near the Tropic of Capricorn into the α - 4C_1 , thus with $\Theta = 90 \rightarrow 0^\circ$ and with minimal variation of Φ . Note that the opposite conformations with Φ between 180° and 240° that are near the Tropic of Cancer do not have coplanarity at C5–O5–C1–C2, and therefore impose an insurmountable energy barrier ridge for a $\Theta = 90 \rightarrow 180^\circ$ itinerary towards the α - 1C_4 .

(2) For α -D-glycopyranosides: Alternative ALPH-type. These comply with the ALPH requirement when $\Theta = 90^\circ$ at $\Phi = 330^\circ$ to 90° , comprising the 0S_2 , 3S_1 and 5S_1 skews as possible

pre-TS local minima. TS candidates are then only the C5–O5–C1–C2 coplanar 3E , 3H_4 and E_4 conformations (indicated with the Cross of Lorraine in Figure 9) at the $\Phi = 0^\circ$ to 60° range. The reaction itinerary now passes over the Tropic of Cancer into the β - 1C_4 , thus with $\Theta = 90^\circ \rightarrow 180^\circ$ and again with minimal variation of Φ . Note that in this case the opposite conformations with Φ between 0° and 60° that are near the Tropic of Capricorn do not have coplanarity at C5–O5–C1–C2, and therefore impose an insurmountable energy barrier ridge for a $\Theta = 90^\circ \rightarrow 0^\circ$ itinerary directly towards the ground state β - 4C_1 . The latter will eventually be reached via an independent process, long after the substitution has occurred.

In Figure 10, these two general itineraries are shown together with the respective Θ and Φ values in relation to the shape of the conformers. If one does not take the ring substituents into account, both ultimate skew \rightarrow TS \rightarrow chair substitutions are in essence ring-plane-mirrored situations of each other. Each is subjected to relatively small atomic movements, which is in full accordance with the PLM. Indeed, the positions at C2–C3–C4–C5 vary only minimally, whereas large repositionings have occurred before the actual substitution, i.e. at the respective conformational change into an ALPH-compliant pre-TS skew.

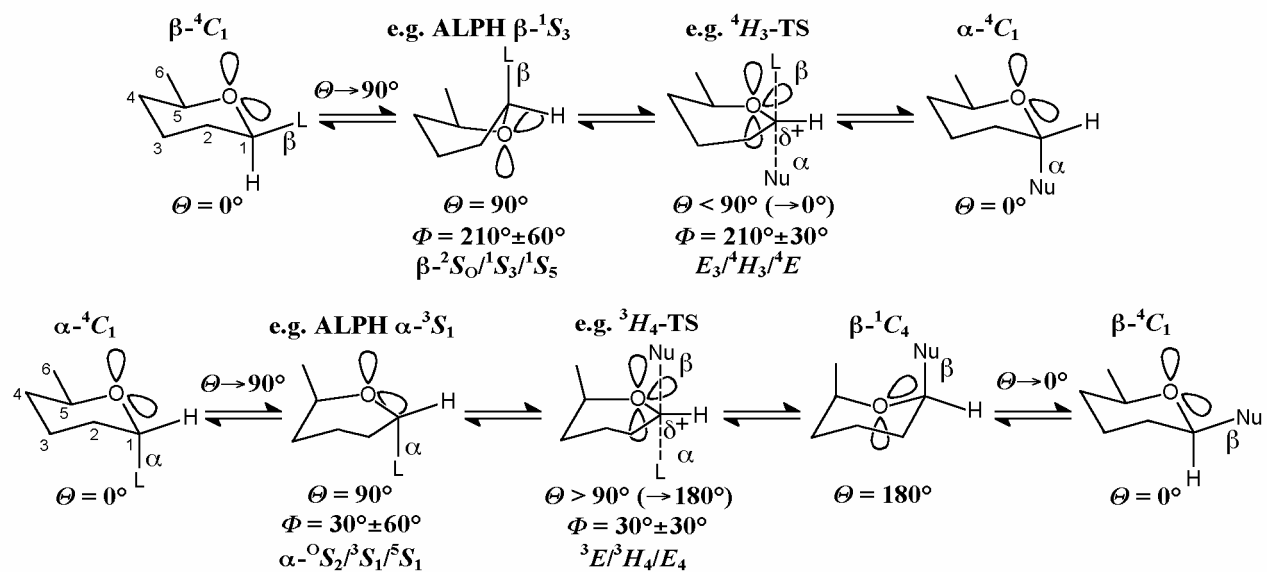


Figure 10. Comparison of preferred β -D-*O*-glycopyranoside Deslongchamps-type (top) and α -D-*O*-glycopyranoside alternative ALPH-type (bottom) substitution itineraries, with indication of the Θ and Φ orientational puckering parameters of the respective conformers within the conformational space. L = leaving group; Nu = nucleophile.

7. Several glycopyranoside hydrolase structures reveal ligands in a pre-TS skew conformation

Since enzymes have evolved to preferentially stabilize transition states,^{69,70} one may assume that the active site of an enzyme has also evolved to allow the conformational changes that the substrate has to undergo in order to be able to reach the TS. In the case of glycopyranoside hydrolases, the possible substitution itineraries contain non-ground state local minimum conformations that belong to the pseudorotational series and have a chance of being trapped by the enzyme, e.g. when using substrate-analogous inhibitors.

Table 1 is a compilation of known skew- or boat-complexed glycoside hydrolase structures, for which the carbohydrate moiety in subsite –1 does not contain a double bond (as e.g. in acarbose) since this results in a ground state ligand having an imposed half-chair conformation, whereas the focus is on non-ground state local minima that are preferred by the enzyme. It is reasonable to assume that these represent pre-TS situations, since an enzyme would prefer to bind a high-energy conformer that is en route to the TS, as a means to decrease the activation barrier by avoiding the low-energy ground state. In the further case-by-case analysis, we will show that even those glycosyl–enzyme intermediates that have been observed as (skew-) boat conformers can as well be interpreted as pre-TS local minima.

The observed pre-TS conformers allow us to infer the subsequent TS conformations for the respective glycosylation, deglycosylation or hydrolysis, and these are indicated in Table 1. For the first entry, the density at subsite –1 of the 4-nitrophenyl-1-thio- β -glucopyranoside Michaelis complex with the β -retaining GH1 maize 1,4-glucosidase (pdb entry 1e1f) was interpreted as indicative of the β -^{1,4}B but with a problematic sp² geometry at C1;⁷¹ the observed C6–C5–(O5)–C1–O1 W-angle of –15° instead suggests the presence of the β -²S₀ and is as such included.

8. On β -glycoside hydrolases

The glycosylation step of retaining β -glycoside hydrolases yields a covalent α -glycosyl–enzyme intermediate, which is subsequently hydrolyzed in the deglycosylation step (Figure 1). Several structures of such glycosyl–enzyme intermediates have been solved, many of them as Withers-type 2-deoxy-2-fluoro derivatives.⁷² With notable exceptions, which will be discussed in the following sections, many show a ground state α -⁴C₁ and therefore ALPH-compliant glycon conformation. This may suggest that a reverse Deslongchamps-type α -⁴C₁ \rightarrow ⁴H₃-like TS \rightarrow β -skew deglycosylation pathway is operative, but might just as well be interpreted as the end situation of a Deslongchamps-type β -skew \rightarrow ⁴H₃-like TS \rightarrow α -⁴C₁ glycosylation itinerary (Figure 3).

As noted earlier, one should be very cautious when deducing an enzyme's reaction itinerary solely based on a ground state conformation in a complexed enzyme structure. Using the PMR for comparing a glycosylation to a deglycosylation is again inappropriate since these

are two separate reaction steps; in other words, the PMR does not make it obligatory for the deglycosylation step to follow a reverse-like itinerary of the one that formed the glycosyl enzyme intermediate. This even accounts for a glycosylation versus a subsequent transglycosylation: although here the return reaction for the glycosylation appears to be the same as a subsequent transglycosylation, the PMR only indicates that a reverse Deslongchamps-type return reaction (or transglycosylation) is possible, but it does not exclude the possibility that a lower-energy alternative ALPH-type itinerary that has its own PMR is operative.

Table 1. A compilation of observed pre-TS skews/boats in Michaelis complexes or in deglycosylation intermediates of glycoside hydrolase structures, together with the inferred TS conformation

Fam.	Mechanism	Organism	Enzyme	PDB	Ligand	Michaelis	TS	Deglycos. TS
1/A	anti β ret.	<i>Z. mays</i>	1,4-glucosidase	1e1f	Glu-S-PNP	β - ² S ₀	<i>E</i> ₃	
5/A	anti β ret.	<i>B. agaradhaerens</i>	1,4-endoglucanase	4a3h	Glc-Glc2F-DNP	β - ¹ S ₃	⁴ H ₃	
6	syn β inv.	<i>H. jecorina</i>	cellobiohydrolase	1qk2	Glc ₂ -S-Glc ₂	β - ² S ₀	<i>E</i> ₃	
6	syn β inv.	<i>H. insolens</i>	cellobiohydrolase	1oc5	Glc ₂ -S-Glc ₂ -OMe	β - ² S ₀	<i>E</i> ₃	
6	syn β inv.	<i>H. insolens</i>	cellobiohydrolase	1ocn	Glc-isofagomine	β - ^{2,5} B/ ² S ₀	⁴ E	
7/B	syn β ret.	<i>F. oxysporum</i>	1,4-endoglucanase	1ovw	thio-Glc ₅	β - ¹ S ₃	⁴ H ₃	
8/M	anti β inv.	<i>C. thermocellum</i>	1,4-exoglucanase	1kwf	cellohexaose	β - ^{2,5} B/ ² S ₀	<i>E</i> ₃	
11/C	syn β ret.	<i>B. agaradhaerens</i>	xylanase	1h4h	xylotriose	β - ^{2,5} B/ ² S ₀	<i>E</i> ₃	
11/C	syn β ret.	<i>B. agaradhaerens</i>	xylanase	1qh6	Xyl ₂ 2F-glycenz.			α - ^{2,5} B/ ⁵ S ₁ <i>E</i> ₄
11/C	syn β ret.	<i>B. circulans</i>	xylanase	1bv v	Xyl ₂ 2F-glycenz.			α - ^{2,5} B/ ⁵ S ₁ <i>E</i> ₄
11/C	syn β ret.	<i>B. circulans</i>	xylanase	1c5i	Xyl ₂ 2F-glycenz.			α - ^{2,5} B/ ⁵ S ₁ <i>E</i> ₄
12/C	syn β ret.	<i>H. grisea</i>	1,4-glucanase	1w2u	cellopentaose	β - ¹ S ₃	⁴ H ₃	
13/H	anti α ret.	<i>S. scrofa</i>	α -amylase	1ppi	modified acarbose	α - ^{2,5} B	<i>E</i> ₄	
26/A	anti β ret.	<i>C. japonicus</i>	mannanase	1gvy	(Man) ₃ 2F-DNP	β - ¹ S ₅	⁴ E	
26/A	anti β ret.	<i>C. japonicus</i>	mannanase	1gw1	(Man) ₃ 2F-glycenz.			α - ⁰ S ₂ ³ E
29	syn α ret.	<i>T. maritima</i>	α -L-fucosidase	1hl9	Fuc2F-glycenz.			β - ³ S ₁ ³ H ₄
31	anti α ret.	<i>E. coli</i>	α -xylosidase	1xsk	Xyl5F-glycenz.			β - ¹ S ₃ ⁴ H ₃
33/E	anti α ret.	<i>T. cruzi</i>	sialidase	1s0i	sialyl-lactose	α -B _{2,5}	<i>E</i> ₅	
38	anti α ret.	<i>D. melanogaster</i>	α -mannosidase	1qwn	Gul5F-glycenz.			β - ¹ S ₅ ⁴ E
38	anti α ret.	<i>D. melanogaster</i>	α -mannosidase	1r33	5S-Man-NH ₂			α - ^{1,4} B/ ¹ S ₅ ⁴ E
38	anti α ret.	<i>D. melanogaster</i>	α -mannosidase	1r34	5S-Man-amidine			α - ^{1,4} B/ ¹ S ₅ ⁴ E
47	syn α inv.	<i>H. sapiens</i>	α -1,2-mannosidase	1x9d	Man-S-(1,2)-Man	α - ³ S ₁	³ H ₄	
56	anti β ret.	<i>A. mellifera</i>	hyaluronidase	1fcv	(hyaluron.) ₄	β - ¹ S ₃	⁴ H ₃	

8.1. GH26 (Clan A)

With the β -retaining GH26 mannanase from *Pseudomonas cellulosa* (Man26A), the crystal structure of the proton donor mutant in Michaelis complex with 2,4-dinitrophenyl 2-deoxy-2-fluoro- β -mannotrioxide (pdb entry 1gvy) shows the glycon in the -1 subsite clearly in an ALPH-compliant β - 1S_5 , whereas that in the corresponding 2-deoxy-2-fluoro-mannotrioxyl-enzyme intermediate (pdb entry 1gw1) adopts an ALPH-compliant α - 0S_2 .⁵⁰ Since these two conformations flank the $B_{2,5}$ within the skew-boat pseudorotational series, it was suggested that the TS for formation of this glycosyl-enzyme intermediate has a $B_{2,5}$ conformation. However, as argued above, a $B_{2,5}$ as TS in a mannopyranoside is not very likely because of an unfavorable C2-C5 flagpole positioning as well as eclipsed substituents at the C3-C4 bond, and because it should react according to the SLPH instead of ALPH. On the other hand, both observed β - 1S_5 and α - 0S_2 conformations are each fully consistent with the proposed general ALPH-skew \rightarrow half-chair-like TS \rightarrow chair pathways as indicated in Figures 9 and 10, even though the glycosyl-enzyme intermediate contains an acetal-ester bond:

(1) The glycosylation step of GH26. The observed β - 1S_5 at $\Phi = 270^\circ$ in the Michaelis complex is the pre-TS local minimum for a Deslongchamps-type β - $^1S_5 \rightarrow$ 4E -TS \rightarrow α - 4C_1 glycosylation itinerary (Figure 3). The enzyme apparently favors a 1S_5 β -mannopyranoside that has an equatorial C2-OH, providing the least steric hindrance for the axial nucleophilic attack on the anomeric center. A reaction itinerary starting from a β - 1S_5 with the least atomic motions (minimal $\Delta\Phi$) then indicates the passage through the 4E -TS ($\Phi = 240^\circ$) and a subsequent fast descent into the α - 4C_1 energy minimum ($\Theta = 90 \rightarrow 0^\circ$). The latter is, however, not the starting conformation for the subsequent deglycosylation step.

(2) The deglycosylation step of GH26. The observed α - 0S_2 at $\Phi = 330^\circ$ of the glycosyl-enzyme intermediate has arisen from a conformational change away from the α - 4C_1 , which was the end of the glycosylation step, back into the skew-boat pseudorotational series. Here the enzyme has favored the local-minimum 0S_2 α -mannopyranosyl ester with again an equatorial C2-OH, which does not hinder the axial attack on C1 of the incoming water nucleophile. This then suggests that an alternative ALPH-type α - 0S_2 skew \rightarrow 3H_4 -like TS \rightarrow β - 1C_4 deglycosylation itinerary is operative (Figure 7). However, the D-mannopyranoside α - 0S_2 conformation has a 1,3-syn-diaxial interaction between C3-OH and the incoming nucleophile that is partly relieved in the nearby 3S_1 conformation at $\Phi = 30^\circ$, making this a better pre-TS candidate. And starting from a pre-TS α - 0S_2 or α - 3S_1 , the PLM (thus with minimal $\Delta\Phi$) suggests a passage through the 3E ($\Phi = 0^\circ$) or 3H_4 ($\Phi = 30^\circ$) TS, followed by a fast descent into the nearby β - 1C_4 energy minimum ($\Theta = 90 \rightarrow 180^\circ$). The return to the ground state β - 4C_1 is then a subsequent independent process.

It appears at first sight unlikely that such large conformational changes within the -1 subsite are still possible with a molecule that is covalently attached, and/or that an inverted chair can be temporarily accommodated within this crucial subsite. However, as indicated above, the known crystal structures of complexed hexopyranoside hydrolases always show a remarkably

spacious -1 subsite, especially at the region where the C5-hydroxymethyl group of the substrate resides.

8.2. GH11 (Clan C)

A crystal structure of the β -retaining GH11 xylanase from *Bacillus circulans* reveals the covalent 2-deoxy-2-fluoro-xylobiosyl-enzyme intermediate in the α - $^{2,5}B$ that is slightly distorted towards the 5S_1 (pdb entry 1bvj).⁷³ The same glycosyl-enzyme intermediate on the GH11 *Bacillus agaradhaerens* xylanase (pdb entry 1h4g) even apparently shows a strict α - $^{2,5}B$ in spite of the C3-C4 eclipsed positioning and the C2-C5 flagpole, whereas the xyloetraose Michaelis complex with the nucleophile G94A mutant (pdb entry 1h4h) rather seems to adopt a conformation that is between the β - $^{2,5}B$ and 5S_1 for the xylosyl moiety within subsite -1 .⁵¹ This has led these authors to suggest a start- $^4C_1 \rightarrow ^2H_3 \rightarrow ^2S_0 \rightarrow ^{2,5}B$ -TS xylosylation itinerary.

Such pathway would be different from the reaction itinerary within the GH12 glucanases where the $^{2,5}B$ -TS of their glucoside glycon would place the C5-hydroxymethyl group in axial position, thereby imposing a severe flagpole interaction with the C2-hydrogen atom; however, both families belonging to the same GH Clan C (thus having a common ancestor⁵) could tentatively be expected to operate with similar itineraries. But the GH12 *Humicola grisea* endoglucanase cellopentaose Michaelis complex (pdb entry 1w2u) reveals the ALPH- β - 1S_3 within the -1 subsite⁷⁴ whereas the *Streptomyces lividans* endoglucanase's 2-deoxy-2-fluoro-celotriosyl-enzyme intermediate (pdb entry 2nlr) clearly shows its glycon in the α - 4C_1 ,⁷⁵ both in accordance with respectively the pre-TS and the end-situation of a Deslongchamps-type β - $^1S_3 \rightarrow ^4H_3$ -TS $\rightarrow \alpha$ - 4C_1 glycosylation itinerary (Figure 3).

The $^{2,5}B$ is at $\Phi = 120^\circ$ in the $\Theta = 90^\circ$ skew-boat pseudorotational series, flanking the 5S_1 and 2S_0 at $\Phi = 90^\circ$ and 150° , respectively (Figure 9). In the absence of a C5-hydroxymethyl group, interconversion between these skews is relatively unhindered, and as such a $^{2,5}B$ D-xylopyranoside may even become a local minimum within an enzyme, as apparently is observed in GH11. As outlined above, the two ALPH pathways (Figures 9 and 10) may very well be operative, instead of an itinerary passing through a SLPH-compliant $^{2,5}B$ -TS. Both again start with a prior conformational change from the 4C_1 into the skew-boat pseudorotational series:

(1) The glycosylation step of GH11. Slight atomic movements of the observed β - $^{2,5}B$ in the Michaelis complex can easily yield the ALPH-compliant β - 2S_0 at $\Phi = 150^\circ$, enabling a Deslongchamps-type β - 2S_0 skew $\rightarrow E_3$ -TS $\rightarrow \alpha$ - 4C_1 glycosylation itinerary ($\Theta = 90 \rightarrow 0^\circ$ with minimal $\Delta\Phi$). The β - 2S_0 is now preferred since this has an equatorial C2-OH yielding the least steric hindrance for the incoming nucleophile towards C1 (the above β -mannopyranoside uses the β - 1S_5 for the same reason). The end conformation is then the α - 4C_1 , as observed in the GH12 *S. lividans* glycosyl-enzyme intermediate,⁷⁵ but that is not the productive conformation for the deglycosylation step.

(2) The deglycosylation step of GH11. A re-entry into the skew-boat pseudorotational series has occurred ($\Phi = 0 \rightarrow 90^\circ$). Slight atomic movements of the apparently observed α - $^{2,5}B$

glycosyl–enzyme intermediate can easily yield the ALPH-compliant α - 5S_1 , now enabling an alternative ALPH-type α - 5S_1 skew $\rightarrow E_4$ -TS $\rightarrow \beta$ - 1C_4 deglycosylation itinerary ($\Theta = 90 \rightarrow 180^\circ$ with minimal $\Delta\Phi$). The α - 5S_1 is now preferred since this has an equatorial C2-OH, yielding the least steric hindrance for the incoming water nucleophile towards C1. And the return to the ground state β - 4C_1 then occurs in a subsequent independent process.

With a half-chair-like TS in both itineraries, the orthogonal ring oxygen lone pair (forming a partial double bond towards C1) can also interact with the phenolic oxygen of a GH11 invariant tyrosine; this interaction has been proposed to be essential for electrostatic TS stabilisation.^{21,51,73} And proximity of the C2 hydroxyl group towards the nucleophile is also obtained; such interaction may be important in different β -glucoside hydrolases.^{16,76} The apparent strict xylan preference by GH11 enzymes may then not be achieved through an exotic reaction itinerary but through a simple steric incompatibility towards cellulose. Indeed, especially the +1 subsite appears to be too narrow to accept a glucose entity, whereas it has been noticed in the original references^{51,73} that a conserved leucine or valine in the –1 subsite is in Van der Waals contact with the C5-methylene of the substrate, blocking the accommodation of a C5-hydroxymethyl group.

8.3. Other β -glycoside hydrolases

The GH7 *Fusarium oxysporum* retaining 1,4-endoglucanase (Cel7B) in Michaelis complex with a non-hydrolysable thiocellopentaose (pdb entry 1ovw)⁷⁷ reveals the β - 3S_1 in subsite –1, indicative of a Deslongchamps-type β - $^3S_1 \rightarrow ^4H_3$ TS $\rightarrow \alpha$ - 4C_1 glycosylation itinerary (Figure 3). Although many structures of β -retaining glycoside hydrolases with trapped glycosyl–enzyme intermediates apparently exhibit an α - 4C_1 glycon, some are reported with ligands having imprecise bond lengths and/or angles. These are the GH2 *Escherichia coli* galactosidase (pdb entry 1jz0),⁷⁸ the GH3 *Hordeum vulgare* exo-1,3-1,4-glucanase (pdb entry 1iew)⁷⁹ and the GH22 *Gallus gallus* lysozyme (pdb entry 1h6m).⁸⁰ Their glycon's C6–C5–(O5)–C1–O1 W-angles are respectively 90° , 85° and 102° , which is too deviant from the corresponding 120° in an ideal α - 4C_1 , but suggests the partial presence of a skew conformation as pre-TS local minimum for an alternative ALPH-type α -skew $\rightarrow ^3H_4$ -like TS $\rightarrow \beta$ - 1C_4 deglycosylation (Figure 7).

With the β -inverting GH6 cellobiohydrolases (Cel6A) from *Hypocrea jecorina* and *Humicola insolens*, the β - 2S_0 is observed in Michaelis complexes with non-hydrolysable thiooligosaccharides (pdb entries 1qk2 and 1oc5, respectively),^{81,82} whereas the 3D structure of *H. insolens* Cel6A in complex with a gluco-isofagomine cellobioside analog (pdb entry 1ocn) shows an isofagomine conformation that is between the $^2,^5B$ and the 2S_0 , which has again led the authors to suggest a β - $^2S_0 \rightarrow ^2,^5B$ -TS hydrolysis itinerary.⁸³ And the proton donor mutant of the β -inverting GH8 (Clan M) *Clostridium thermocellum* endoglucanase CelA in Michaelis complex with cellopentaose (pdb entry 1kwf) again reveals a conformation in subsite –1 that is between a β - $^2,^5B$ and β - 2S_0 .⁸⁴ All these (skew-)boat conformations can be interpreted as belonging to a Deslongchamps-type β - 2S_0 skew $\rightarrow E_3$ -TS $\rightarrow \alpha$ - 4C_1 hydrolysis itinerary (Figure 3).

9. On α -glycoside hydrolases

The formation of the glycosyl–enzyme intermediate by α -retaining glycoside hydrolases, as well as the direct hydrolysis catalyzed by α -inverting glycoside hydrolases, are both proposed to proceed through itineraries that start with a conformational change into the skew-boat pseudorotational series, followed by an alternative ALPH-type α -skew \rightarrow 3H_4 -like TS \rightarrow β - 1C_4 substitution (Figure 7). This can be supported by kinetic isotope effects on the enzymatic hydrolysis of aryl- α -D-glucopyranosides, as noted earlier. For α -inverting enzymes, this initially yields the inverted chair conformation for the liberated glycoside, and a return to the ground state will be an independent process.

For α -retaining enzymes, the alternative ALPH-type glycosylation step initially results in formation of a β -axial glycosyl–enzyme intermediate with the 1C_4 inverted chair conformation. A return to the ground state β - 4C_1 may occur, but this results in a β -equatorial anomeric substituent that cannot be a productive species for hydrolysis in the deglycosylation step. A direct substitution on the 1C_4 conformer would be via the reverse of an alternative ALPH-type itinerary (Figure 7, from right to left), leading initially to a product with a local minimum skew conformation. But a re-entry into the pseudorotational series, followed by a Deslongchamps-type β -skew \rightarrow 4H_3 -like TS \rightarrow α - 4C_1 deglycosylation pathway (Figure 3), leads directly to the ground state product and thus is expected to be the preferred deglycosylation itinerary.

As with the β -glycoside hydrolase structures and with the same caution for interpretation, many liganded α -glycoside hydrolase structures show ground state chair conformers within the –1 subsite. But the observed non-ground state conformers may very well represent pre-TS situations that are indicators for the reaction itineraries that are operative.

9.1. α -Retaining glycoside hydrolases

The GH38 *Drosophila melanogaster* Golgi α -mannosidase II's 5-fluoro-gulosyl–enzyme intermediate (pdb entry 1qwn) reveals the pre-TS ALPH- β - 1S_5 ($\Phi = 270^\circ$) that has an equatorial C2-OH.⁸⁵ This was again interpreted as indicative of a SPLH-compliant $B_{2,5}$ -TS, but can just as well be interpreted as indicative of a Deslongchamps-type β - ${}^1S_5 \rightarrow$ 4E -TS \rightarrow α - 4C_1 pathway for the deglycosylation (Figure 3). The same enzyme in complex with 5-thio-D-mannopyranosyl amine and with the corresponding benzyl amidinium bromide (pdb entries 1r33 and 1r34, respectively) in both cases also reveal the ${}^{1,4}B$ ($\Phi = 240^\circ$) that appears to be slightly distorted towards the 1S_5 .⁸⁶ These α -substituted amino compounds, however, show an equatorial anomeric substituent and therefore do not mimic a productive pre-TS Michaelis complex, which is expected to have an axial ALPH- α -skew conformation with Φ between 330° and 90° . Such conformation is probably disfavored due to a steric clash of the ligand's large sulfur atom with the nearby Arg228 residue, and the enzyme may then have favored a non-ground state conformation that reflects the pre-TS of the deglycosylation step.

The GH31 *E. coli* α -xylosidase 5-fluoro-xylosyl–enzyme intermediate (pdb entry 1xsk) adopts the deglycosylation pre-TS ALPH- 1S_3 ($\Phi = 210^\circ$) with a half-axial/half-equatorial C2-OH,⁸⁷ which suggests the occurrence of a 4H_3 -TS in a Deslongchamps-type itinerary (Figure 3). The IUPAC nomenclature for L-glycopyranosides *inverts the numbering* at conformational descriptors, but the orientational puckering parameters Θ and Φ are unaffected. So, the ALPH- β - 3S_1 (now also at $\Phi = 210^\circ$) is observed in the 2-deoxy-2-fluoro-L-fucosyl–enzyme intermediate of the GH29 *Thermotoga maritima* α -L-fucosidase (pdb entry 1hl9)⁸⁸ as pre-TS conformation for a Deslongchamps-type deglycosylation, suggesting the 3H_4 -TS (also at $\Phi = 210^\circ$).

9.2. Clan E sialidases-neuraminidases

The α -retaining GH families 33, 34 and 83 comprise the sialidases-neuraminidases, for which a triple idiosyncrasy in the IUPAC nomenclature complicates the picture: the ring atom numbering is shifted by one unit, the scissile bond is alpha although it is equatorial in the ground state, and the latter is the inverted chair 2C_5 . Staying with the IUPAC nomenclature, the ALPH-compliant itineraries are (Figures 9 and 10):

(1) The glycosylation step: Alternative ALPH-type. From the ground state α - 2C_5 ($\Theta = 180^\circ$) a conformational change into the skew-boat pseudorotational series, followed by ALPH- α - 4S_2 (or very similar, at $\Theta = 90^\circ$ and $\Phi = 330^\circ$ to 90°) \rightarrow 4H_5 -TS (or very similar, at $\Phi = 0^\circ$ to 60°) \rightarrow β - 2C_5 sialosyl–enzyme intermediate ($\Theta = 90 \rightarrow 180^\circ$).

(2) The deglycosylation step: Deslongchamps-type. A return to the skew-boat pseudorotational series followed by ALPH- β - 2S_4 (or very similar, at $\Theta = 90^\circ$ and $\Phi = 150^\circ$ to 270°) \rightarrow 5H_4 -TS (or very similar, at $\Phi = 180^\circ$ to 240°) \rightarrow α - 5C_2 ($\Theta = 90 \rightarrow 0^\circ$).

In the GH33 *Trypanosoma cruzi* trans-sialidase Michaelis complex (pdb entry 1s0i),⁸⁹ the conformation of the α -sialosyl moiety appears to be a strict ALPH- α - $B_{2,5}$ and indicates a subsequent E_5 -TS (both at $\Phi = 60^\circ$) into the β - 2C_5 as itinerary for the glycosylation. With the aldopyranoside numbering this coincides with the α - $B_{1,4}$ followed by the E_4 -TS into the β - 1C_4 , thus similar to the alternative ALPH-type pathway for a classical α -axial glycopyranosidic bond substitution. The same sialidase's 2-fluoro-sialosyl–enzyme intermediate with the Tyr342 nucleophile (pdb entry 1s0k)⁸⁹ shows the β - 2C_5 , apparently in accordance with the end of the glycosylation step.

9.3. GH90 Tailspike protein

In GH90 α -inverting *Salmonella* phage P-22 tailspike protein complexes with *O*-antigen fragments (pdb entries 1tyx, 1tyu and 1yw), the terminal L-rhamnose atom densities at subsite –1 have been interpreted as being non-ALPH α - 5S_1 conformations.⁹⁰ However, these are not –1/+1 subsite-spanning product complexes for which one expects to find the lower energy ground state 1C_4 conformations. An α -L-rhamnopyranose should have a Θ - Φ puckering map that is highly similar to that of an α -D-mannopyranose, but the corresponding 1S_5 at $\Theta = 90^\circ$ and $\Phi = 270^\circ$ is

not a local minimum (the ALPH- α - 0S_2 is, at $\Phi = 330^\circ$) and has an estimated steric burden of 7 kcal/mol versus the ground state α - 4C_1 at $\Theta = 0^\circ$.⁵⁸ A docking study on this enzyme also indicates that the terminal L-rhamnose ground state α - 1C_4 should largely be preferred over the α - 5S_1 . The same study *inter alia* suggests that the assigned L-rhamnose C2-hydroxyl oxygen could have actually been the oxygen from a nearby water molecule; the opposite assignment indeed would fit the more realistic α - 1C_4 conformation.⁹¹ The predicted itinerary (Figures 9 and 10 but with L-glycopyranoside nomenclature: *inverted numbering* at the conformational descriptors) is then an alternative ALPH-type α - 2S_0 ($\Phi = 330^\circ$; C2-OH equatorial) $\rightarrow E_3$ -TS ($\Phi = 0^\circ$) $\rightarrow \beta$ - 4C_1 ($\Theta = 180^\circ$) glycoside hydrolysis pathway.

10. Conclusions

From a recapitulation of current inconsistencies within the literature, as well as considerations of basic principles in organic chemistry, it appears that pyranoside glycosidic bond substitutions that operate by a classical exocyclic mechanism on oxyalkyl-type leaving groups proceed via two inherently preferred itineraries: 1) β -equatorial D-glycopyranosides are expected to follow a Deslongchamps-type β -skew \rightarrow 4H_3 -like TS \rightarrow α - 4C_1 itinerary (Figure 3); and 2) α -axial D-glycopyranosides may very well not react by a direct substitution on the ground state in a reverse Deslongchamps pathway, but instead follow an alternative ALPH-type α -skew \rightarrow 3H_4 -like TS \rightarrow β - 1C_4 itinerary, with an independent return to the ground state β - 4C_1 occurring thereafter (Figure 7). Both substitutions are in essence ring-plane mirrored situations of each other, in which the route to the transient TS is preceded by a conformational change into an ALPH-compliant conformation belonging to the skew-boat pseudorotational series. The route demarcations within the conformational space are presented in Figures 9 and 10.

A reassessment of the currently known 3D structures of glycoside hydrolases that are ligand-complexed with non-ground state carbohydrate conformers indicates that these respective itineraries are operative in enzymes as well. Even deglycosylations or transglycosylations of glycosyl-enzyme intermediates appear to proceed in this way, and not through a reverse-type itinerary of the preceding glycosylation step. Arguments are given against the possible occurrence of a boat TS within a SLPH-compliant itinerary, whereas the alternative ALPH-pathway (Figure 7) can be supported by experimental evidence when revisiting results from a study⁵² on kinetic isotope effects observed with enzymatic hydrolyses of aryl α -D-glucosides (Figure 6).

The route demarcations suggest a general mechanistic strategy for protection of the glycosyl-enzyme intermediate by transglycosidases: these may lock their intermediate in a nonproductive conformation for as long as the aglycon subsites are still occupied by water molecules solely, but once these are properly replaced by the carbohydrate-nucleophile, a minor amino acid residue reorientation (induced fit) may favor the productive pre-TS conformation. This explains the equatorial anomeric positioning of the β - 4C_1 4-deoxymaltotriosyl-enzyme

intermediate in the α -retaining GH13 *B. circulans* cyclodextrin glycosyltransferase (pdb entry 1cxl).⁵³

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