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The physical organic chemistry of glycopyranosyl transfer reactions in solution and enzyme-catalyzed

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Our understanding of the mechanisms of glycopyranosyl transfer that occur in solution, both for the chemical synthesis of complex structures and that for the cleavage of glycosidic bonds has allowed us to design biologically active molecules. Recent efforts on the reactivity of glycopyranosides, which are critical entities in all biological systems, coupled with the advent of modern spectroscopic instrumentation have allowed physical organic chemists to broaden our knowledge of glycosyl transfer reaction transition states, both in solution and for enzyme-catalyzed processes, and of critical high energy intermediates. This review details recent physical organic, kinetic and structural studies that have led to elucidation of several different mechanism for the transfer of glycopyranosyl moieties from various substrates to acceptors, such as water or a sugar hydroxyl group.

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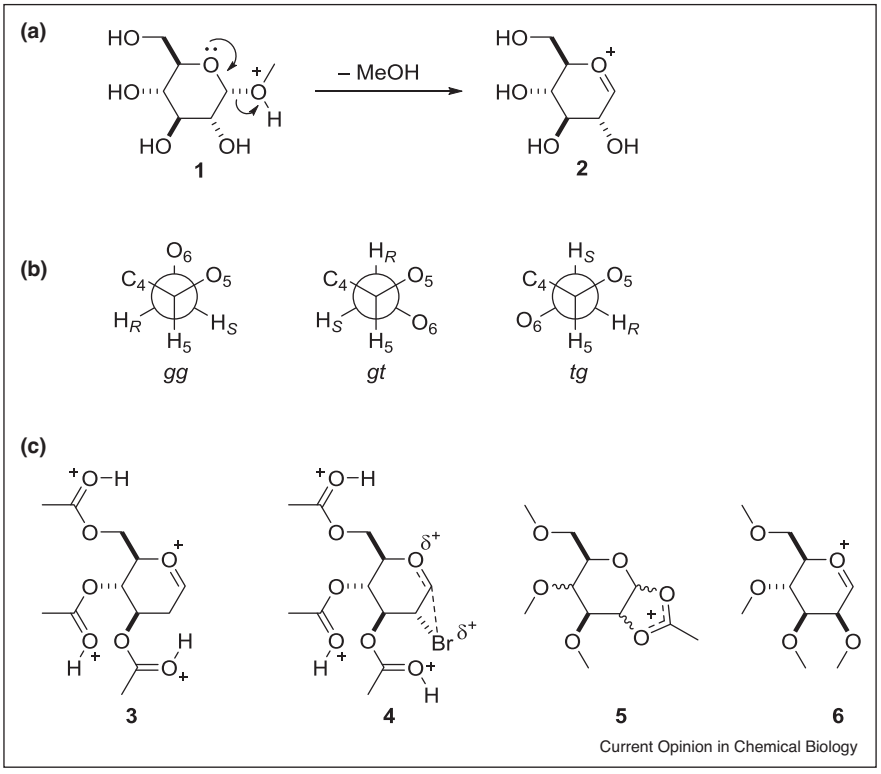
The physical organic chemistry, that is, the study of reaction mechanisms, of carbohydrates has a long and storied history. It is over a century since Hudson, in the early 1900s, performed his seminal studies on the acid-catalyzed and base-catalyzed mutarotation of glucose [1]. Although at that time, Hudson didn't realize that mutarotation, which he noted is catalyzed by numerous different acids and bases [2], is a classic example of general-catalysis [3]. In contrast to the mutarotation of aldoses, where general base-catalysis is more efficient than general acid-catalysis [1,4] most glycosyl transfer reactions in aqueous media are only efficiently catalyzed by the conjugate acid of the solvent (H_3O^+), that is, these reactions are classic examples of specific-acid catalysis. In other words, hydrolysis of *O*-glycopyranosides generally involves a rapid and reversible protonation that is followed by rate-limiting glycosidic bond cleavage. Another facet of glycosyl transfer reactions that is often overlooked is that although chemists represent the glycosidic bond cleavage step occurring with efficient charge delocalization onto the ring oxygen (**1**) to give in this case cation **2** (Figure 1a), this is certainly not always the case [5]. Undoubtedly, imperfect synchronization [6] between glycosidic bond cleavage and charge delocalization is a likely cause, in part, for the high intrinsic barrier of glycopyranosidic bond cleavage in aqueous solution [7,8]. Of note, the classic substitution mechanisms for enzymatic cleavage of glycopyranosides, which exhibit enzyme proficiencies of between 10^{-17} to 10^{-22} M [7], have evolved to exhibit a high degree of synchronization between glycopyranoside bond cleavage and charge delocalization [9–11]. Moreover, these reactions are resplendent with multiple examples of general catalysis, which as a consequence avoids formation of high energy intermediates, such as protonated glycosides, along the enzyme catalyzed pathway.

This review employs the IUPAC recommended nomenclature for reaction mechanism terminology as summarized by Guthrie and Jencks [12], while the conformations of glycosyl units are designated based on IUPAC recommended nomenclature rules [13,14], and the naming of carbohydrates and their simple derivatives adheres to the 1996 IUPAC recommendations [15]. For example, the quintessential cationic intermediate the glucopyranosyl cation is referred to as the glucopyranosylium ion (**2**). In addition, we use the term spontaneous to indicate carbohydrate reactions in solution that are neither catalyzed by acid nor by base.

We have divided this article into four subsections; firstly, we discuss some of the more common physical organic chemistry tools used to study reaction mechanism; secondly, we report on recent structural studies on the conformation of glycopyranosides and glycopyranosylium ions as well as the lifetime of these high energy cationic intermediates; thirdly, we examine glycosyl transfer reactions that occur with positive charge built up at the transition state (TS) on the sugar moiety; and lastly, we detail the corresponding reactions that have anionic TSs. We have not considered articles that predominantly involve theoretical calculations, for example see the review on glycoside hydrolase (GH) mechanisms by Montgomery *et al.* [16], simply to keep this article concise.

Figure 1



(a) Common representation of a glycosidic bond cleavage that occurs with charge delocalization, two events that are often presumed to be synchronous; **(b)** Newman projections along the C5–C6 bond for the three staggered conformations of the C6-hydroxymethyl group. Conformations are labelled as *g* – gauche or *t* – trans with the first and second identifiers referring to the relationship between C6-OH and O5 or C4, respectively; **(c)** Carbohydrate-based cations produced either during the super acid reactions of peracetylated 2-deoxyglucoses (**3** and **4**) or by IR induced desolvation (**5**) or fragmentation (**6**) in the gas phase.

Physical organic chemistry tools

Kinetic isotope effects

In order to study reaction mechanisms experimentalists often perturb the reaction, by making subtle changes to one of the following: the reaction conditions, reagent concentrations, or substrate structures, and they note the effect that the perturbation causes to the reaction rate constant. The smallest perturbation commonly used is the incorporation of a heavy isotope into the compound being studied and comparing the rate constants for the unlabelled and labelled substrates. Such experiments give kinetic isotope effects (KIEs). Ideally, multiple KIEs should be measured for all heavy atoms ($^{13/14}$C, ^{15}N, ^{18}O), and any attached protons ($^{2/3}$H), undergoing bond changes at the reaction center. Two typical approaches are used to measure KIEs, and these involve: (i) the separate determination of isotopologue rate constants, and (ii) the competitive determination of rate constant ratios [17,18]. As heavy atom KIEs are quite small (typically between 0.97–1.08) most experiments involve the evaluation of competitive KIEs as such measurements minimize systematic errors [17,19]. A KIE is simply the rate constant ratio of the light and heavy isotopologues under identical conditions (k_{light}/k_{heavy} = KIE). The physical basis of KIEs is that addition of mass (neutrons) to a nucleus lowers the ground state frequencies of vibrations in which the isotopic site is involved (simple harmonic oscillator approximation for a diatomic; Eq. (1), where κ is the bond force constant and μ_{AB} is the reduced mass). Zero-point energy (ZPE; Eq. (2)) differences between isotopologues at the ground state and the corresponding values at the reaction TS result in the observation of a KIE. A simple mnemonic for explaining KIEs is that the heavy isotope prefers sites of stronger bonding, so if at a TS an isotope experiences a reduction in bond strength then the lighter isotopologue reacts faster.

$$\nu = \frac{1}{2\pi}\sqrt{\frac{\kappa}{\mu_{AB}}} \quad \mu_{AB} = \frac{m_A \times m_B}{m_A + m_B} \tag{1}$$

$$ZPE = \frac{1}{2}h\nu \tag{2}$$

An important caveat, which can complicate the analysis of a measured KIE, is that the chemical step of interest is rate-determining for the overall reaction, for example, a common case where this is not true occurs when slow non-chemical steps in enzyme-catalyzed reactions contribute to the measured rate constant.

Linear free energy relationships

Linear free energy relationships (LFERs) correlate two free energy terms, a process that involves a log–log plot of either rate or equilibrium constants for the reaction being probed versus the corresponding values for either a standard equilibrium or a standard reaction. Such correlations are useful tools to probe the finer details of reaction mechanisms.

Common LFERs used in the study of glycosyl transfer reactions include: i) Brønsted plots (correlation between experimental rate constants and the pK_a for the ionization of the conjugate acid of leaving group (slope = β_{lg}) or nucleophile (s = β_{nuc}); ii) Hammett–Taft relationship (s = ρ_I), and iii) Bartlett relationship (transition state analogy).

For instance, systematic changes in the molecular structure of an aryl glycoside result in changes in leaving group ability of the aglycone; the sensitivity of the resulting Brønsted plot [log(k_{obs} versus p$K_{a(ArOH)}$] is the β_{lg} value for the reaction. Specifically, the slope of such a correlation provides insight into certain reaction features. In addition, the shape of the plot can provide information into whether such changes induce a change in either the rate-determining step or in the mechanism itself. Of note, measured β_{lg} values for hydrolyses in solution and those for GH-catalyzed reactions generally range between 0.0 and −1.3. Again caution should be exercised to check whether a non-chemical step factors into the measured rate constants. Hammett-Taft LFERs [20] are used to probe the role of electronic effects for a series of substituents at a single site in a substrate [21]. Here, a more negative sensitivity parameter (ρ_I), for the plot of log(k_{obs} versus σ_I), is consistent with a greater interaction of the substituents with positive charge development at the reaction TS [21].

A Bartlett LFER study relates binding free energy for enzyme inhibitors (y-axis is log K_i) to the TS free energy of the first irreversible step relative to that of free enzyme and substrate (x-axis is log K_m/k_{cat}). Such LFERs are based on TS theory with the associated equilibrium assumptions so that a true transition state analog (TSA) gives a Bartlett plot with a slope of one [22,23].

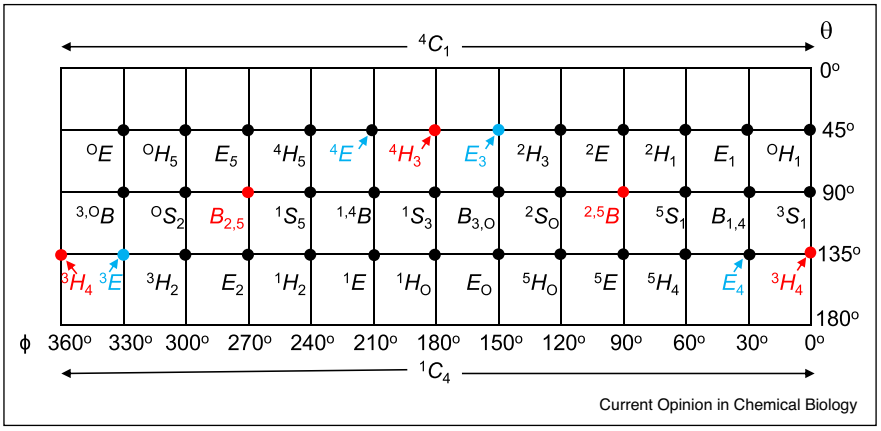
Studies on the ground state glycopyranoside side-chains and glycopyranosylium ions conformations

Remembering that reactivity is determined by the free energy differences between ground state(s) and the transition state(s) for reaction it is important to understand ground state free energies. As noted in a recent study, Amarasekara *et al.* reported the synthesis of bicyclo[4.4.0]decane based carbohydrate mimics, and they analyzed the ^{1}H–^{1}H coupling constants for compounds in which the pseudo-C6OH group is locked in either a: i) gauche–gauche (relative to C4 and O5); ii) gauche–trans; or iii) trans-gauche conformation (Figure 1b) [24]. These authors then used these models for the three staggered conformation of the hydroxymethyl side chain of hexopyranosides to derive limiting $^3J_{H,H}$ coupling constants that are associated with the various C6 side-chain conformers (Figure 1b). As a result these authors could estimate the ground state conformer populations of hexopyranosides [24].

Another important physical organic facet of glycopyranosyl transfers is whether the reaction involves a high energy glycosyl cation, the lifetime (τ) of which is defined as the reciprocal of its pseudo-first-order rate constant for reaction with solvent ($1/k_{SOH}$) [3]. Thus, structural and reactivity studies on such species are particularly pertinent. Glycopyranosylium ion intermediates have traditionally been assumed to be in one of the four low energy conformations in which C2, C1, O5 and C5 are coplanar ($^4H_3$, $^3H_4$, $^{2,5}B$, and $B_{2,5}$; red circles Figure 2) or one of the closely related envelop conformations (E_3, ^3E, E_4, or ^4E; blue circles). Of great importance are the recent advances in spectroscopic techniques that have furnished critical insights into the structures of glycopyranosylium ions in both the gas phase and in a super acid medium (Figure 1c) [25,26••,27••].

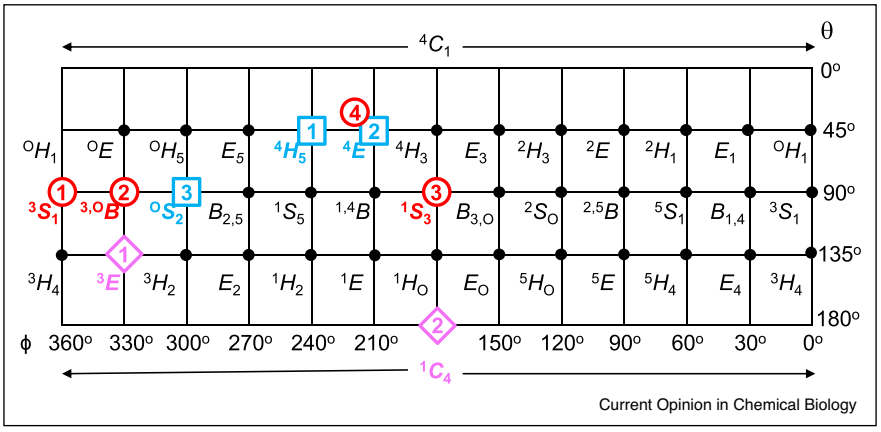
Martin *et al.* reported the production of long-lived glycopyranosyl cations in solution by reacting peracetylated β-glucopyranose or 2-acetamido-2-deoxyglucopyranose in super acid (HF/SbF_5) [25]. These authors observed that the resultant solutions exhibited NMR spectra consistent with neighboring group participation to give fully protonated versions of the known dioxalenium and oxazolinium ions. More importantly, these authors also analyzed the NMR spectra of the cations produced from the super acid reactions of peracetylated 2-deoxy-glucopyranose and 2-bromo-2-deoxy-β-D-glucopyranose, which gave stable ion solutions containing glucopyranosylium ions **3** and **4** (Figure 1c) that the authors concluded were in ^4E and $^4H_5$ conformations, respectively (Figure 3) [25]. These authors then performed two different trapping studies on both of the 2-deoxyglucopyranosylium ions by: i) adding cyclohexane-*d*12 to the super acid media to initiate a deuteride transfer to the anomeric carbon, and ii) pouring the super acid solution into an excess of methanol to generate methyl glycopyranoside products. Even though the starting cations are identical, these two reaction types occur on different pyranosylium ions as deuteride transfer occurs on the fully protonated peracetylated pyranosylium ion, while during the methanol quench two diffusional reactions occur. The

Figure 2



Mercator projection of six-membered ring conformations expected for glycopyranosylium ions, the four conformations shown in red have four adjacent co-planar atoms, while the four labelled in blue are for the four possible envelope conformations.

Figure 3



Mercator projection of six-membered ring conformations of various glycopyranosyl cations: **Red circles** cold ion IR data: peracetylated sugars, **1** – glucose, **2** – mannose, **3** and **4** – galactose: **Pink diamonds** single ion IR data: 2,3,4-tri-*O*-mannosyl, **1** – 6-OMe, **2** – 6-CO_2Me; and **Blue squares** super acid media: 3,4,6-triacetyl sugars: **1** – 2-Br-glucose and 2-OAc-glucose, **2** – 2-deoxyglucose, and **3** – 2-acetamido-2-deoxyglucose.

first being proton transfer from a protonated acetyl group to methanol and the second involves methanol trapping of a pyranosylium ion. In spite of these differences a common pattern of reactivity is displayed; the 2-deoxy cation reacts to give preferentially the α-stereoisomer, while the 2-bromo-2-deoxy cation yield the corresponding β-isomers as the major products, a result consistent with trapping of a bromonium-ion type species [25].

More recently, two related studies that combined the ability of modern mass spectrometers to trap and react single mass separated ions. In these studies following production of glycosyl cations either as separate ions [26••] or enclosed in superfluid helium droplets [27••] absorption of infra-red radiation results in ion fragmentation or helium evaporation, respectively. The mass detection of ions as a function of irradiation frequency generates infrared spectra [26••,27••]. The experimental gas phase IR spectra of these cations were matched to the theoretical modelled IR spectra of possible cationic species in a number of possible conformations in order to allow assignment of ring conformations (Figures 1c and 3). In these studies all ions produced that possessed a participating group at C2 resulted in the formation of cyclic 1,2-dioxalenium ions **5** [26••,27••]. Interestingly, analysis of the IR spectrum of the mannosyl cation **6** without a participating group on C2 (C2-OMe) suggests that the mannopyranosylium ion is, under these conditions, in an envelope conformation (^3E, Figure 2) [26••].

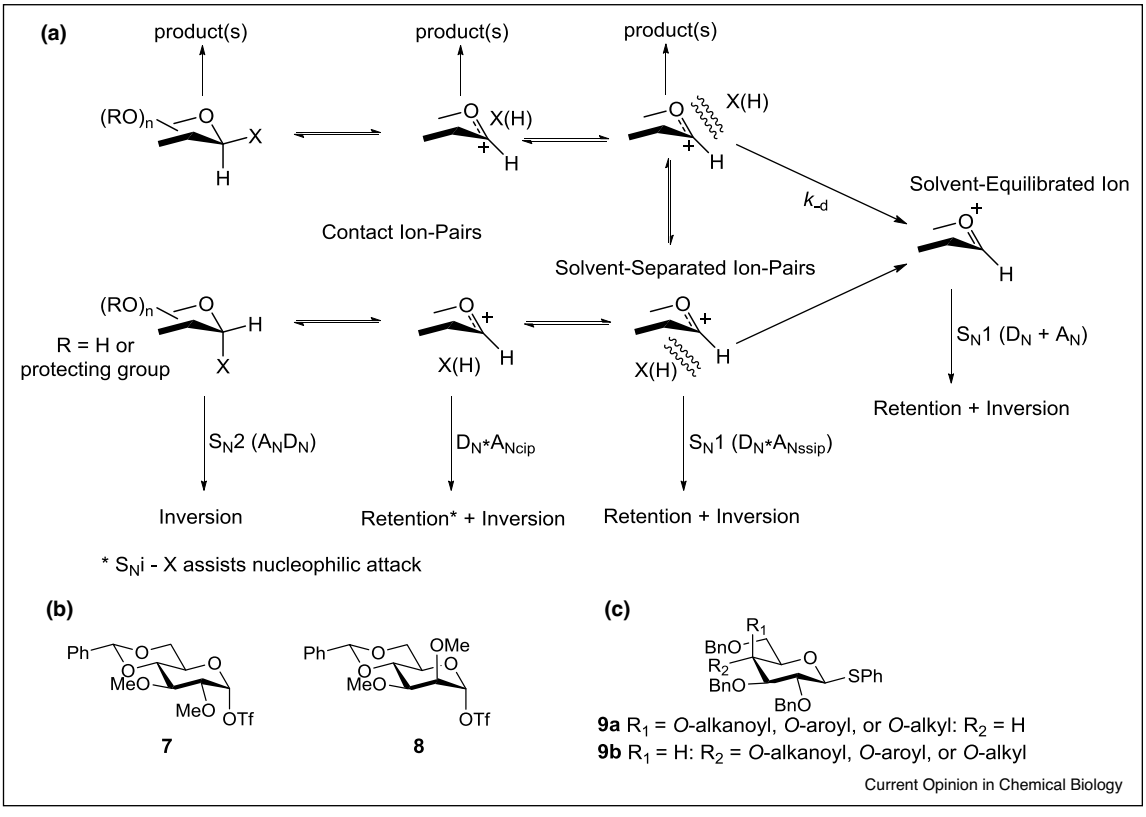
Glycoside reactivity in solution that involve positive charge delocalization on the glycosyl fragment

Having discussed some of the significant recent advances in ground state and high energy cationic species we turn to reactivity in glycosyl transfers. For this section, we use a modification of the Winstein ion-pair mechanism (Figure 4a) for reactions that involve positive charge build up on the glycosyl fragment [28]. The lifetime of the archetypal glucopyranosylium ion in water ($1/k_{H2O}$) has been estimated to be $\sim 1.0–2.5 \times 10^{-12}$ s [29,30]. Such a lifetime, in water, is too short to allow diffusional separation ($k_{-d} \sim 10^{-10}\ s^{-1}$; Figure 4a) [31] of the leaving group from the cation so as to give solvent-equilibration ions. Thus, the aqueous reactions of such ions, and by extension those in less polar media, occur via ion-pairs. To date, the only report that is consistent with the formation of a solvent-equilibrated glycopyranosylium ion is for the solvolysis of α-D-glucopyranosyl fluoride in the ionizing, but weakly nucleophilic solvent, hexafluoroisopropanol [32]. Unfortunately, the lifetime of the glucopyranosylium ion (**2**) has not yet been determined in this solvent. We note that the barrier to interconversion of the two half-chair conformers in cyclohexene is around 8.4–12.1 kcal mol^{-1} [33,34], and although the barrier for pyranosylium ion conformational interconversion has not be determined, the large free energy barrier for cyclohexene suggests that once formed glycopyranosylium ion-pairs will react before becoming conformationally equilibrated.

Synthetic reactions

It is well recognized that the identity of protecting groups on the glycosyl donor affects reaction rates; with electronegative esters being labelled as 'disarming' while 'arming' ethers result in faster coupling reactions [35]. It should be remembered that for typical S_N1-type reactions that the rate constant is generally determined by the first ionization step, while the subsequent product forming steps are not related to the reaction rate constant. That is, the ultimate goal of stereospecific glycosylation reactions remains difficult to achieve due to: i) the need for activated leaving groups, which easily form ion-pair intermediates, ii) the low nucleophilicity of alcohols that reduces the occurrence of S_N2-type reactions, and iii) the loss of selectivity that can occur at the solvent-separated ion-pair stage via ion-pair rearrangements (Figure 4a) [35].

Figure 4



(a) General mechanistic scheme for the reactions of glycopyranosyl derivatives, where reactions can occur either by an S_N2 reaction or by way of glycopyranosylium ion-pairs; **(b)** reactive intermediates formed by triflic anhydride activation of glycopyranosyl sulfoxides (**7** and **8**); **(c)** Various protected sugars (**9**) used in a study on ground state effects.

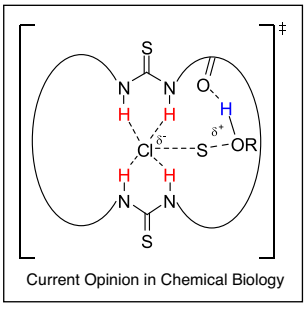
The pioneering ^{13}C-KIE studies on the coupling reactions of α-D-glycopyranosyl triflates by Huang et al. laid the foundation for a more detailed mechanistic understanding of glycosylations [36]. In this paper they concluded that the reactions of **7** and **8** (Figure 4b), reactive intermediates formed by triflic anhydride activation of glycosyl sulfoxides, to form the inverted β-glycosides occur via 'exploded' S_N2 reactions likely on the contact ion-pair (Figure 4a) [36]. While these authors concluded that the minor α-glucoside and α-mannoside products were formed in these reactions via a S_N2-like TS and a S_N1-type reaction, respectively. These minor pathways likely both involve β-triflyl ion-pairs that are formed by facile ion-pair rearrangements (Figure 4a) [36]. Of note, such processes lower the anomeric selectivity of these reactions. As a result, an ongoing and active area of research involves attempts to modulate the conformational flexibility of glycosyl donors to facilitate reactions via a TS ensemble that gives pyranosylium ion-pair intermediates with a predilection for diastereoselective nucleophilic attack.

Recently, Dharuman *et al.* explored whether protecting groups that alter the conformational preferences of the C6-CH_2O– side chain exert a similar or larger effects at the TSs that control reaction rates and those that result in product formation, which are not necessarily the same TS [37•]. These authors investigated the influence of the C4 axial or equatorial protecting group in phenyl 2,3,6-tri-*O*-benzyl-1-thio-β-D-galactopyranosides and glucopyranosides (**9a**,**b**; Figure 4c), respectively, on the side chain distribution of the three staggered conformations (Figure 1b). This study allowed the authors to characterize the influence of electronic and steric perturbations on the ground state side chain populations as a critical first step towards an understanding of whether such effects, of relative side chain conformational free energies, are maintained, accentuated or even diminished at the TS ensemble for ionization on route to glycopyranosylium ion-pairs [37•]. Recently, Hansen *et al.* reported a joint synthetic and computational study on the reactivity of glycopyranosylium ions [38]. This interesting study reports on the computational analysis, using a polarizable continuum model for solvation in CH_2Cl_2, of the conformational energies of a large number of glycopyranosylium ion intermediates. The authors used their generated energy maps to analyze which conformations are preferentially attacked from either the top or the bottom face [38]. The authors then assumed that 'The relative population of all conformational states can be calculated, . . . , utilizing the Boltzmann equation'. The Boltzmann distributions of cation conformations and face selectivities were used to calculate expected anomeric product distributions. Although the authors report good agreement between their calculations and experiment we suggest that the rate constant for the interconversion of conformations with an 8 kcal mol^{-1} barrier (see cyclohexene [33,34]) is $\sim 2 \times 10^6$ s^{-1} at 0 °C, a rate constant that does not allow conformational equilibration of pyranosylium ion-type intermediates, which have very short lifetimes [29,30].

To circumvent the problems associated with S_N1-type reactions, Jacobsen *et al.* are developing macrocyclic catalysts to facilitate cooperative S_N2-type mechanisms so as to give stereoselective syntheses of β-linked disaccharides and tri-saccharides from α-D-glycopyranosyl chloride donors [39]. As part of their mechanistic studies this group developed a new method for the determination of competitive ^{13}C-KIEs, and they measured four carbohydrate ring ^{13}C-KIEs for the reaction of 2,3,4,6-tetra-*O*-methyl-α-D-galactopyranosyl chloride with benzyl alcohol in the presence of their macrocyclic bis-thiourea catalyst (proposed generic TS structure is shown in Figure 5) [40•]. These authors argued, based on the measured C1, C2, C4 and C5 ^{13}C-KIEs, in conjunction with theoretical modelling, that a cooperative mechanism occurs in which simultaneous activation of both nucleofuge (Cl^-) departure and nucleophilic attack occurs via H-bonds between the reagents and the catalyst [40•]. Good levels of stereoselectivity were obtained and the authors measured intermolecular [41] C1-deuterium KIEs for the formation of the major ($k_H/k_D = 1.145$; β-glycoside, S_N2-like) and minor ($k_H/k_D = 1.247$; α-glycoside, S_N1 via ion-pairs) products [39]. Clearly, dissimilar TSs must be responsible for the differences in the experimental KIE values. We suggest that additional measurements of ^{2}H-KIEs for the exchangeable hydrogen atoms as a fraction of deuteration, a so-called proton inventory [42], will provide information on the acceptor (blue) and leaving group (red) activation effects that are proposed to occur during these catalyzed glycosylation reactions (Figure 5).

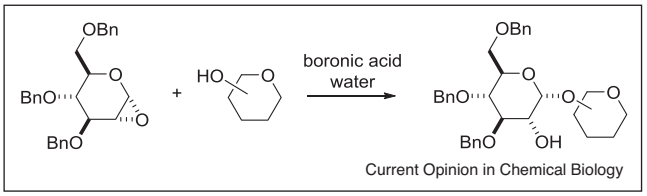
Tanaka *et al.* reported a separate type of glycosylation reaction, which occurs with retention of configuration, for opening of 1,2-anhydrosugar donors (oxiranes) by unprotected sugar acceptors in the presence of boronic acids and water to give 1,2-*cis*-stereoselective glycopyranosides

Figure 5



Proposed transition state structure for the reaction of α-D-glycopyranosyl chlorides (S–Cl) with and alcohol accepter (ROH) catalyzed by macrocyclic bis-thioureas.

Figure 6



Generic reaction for the coupling with retention of configuration of protected 1,2-anhydrosugars and unprotected acceptor in the presence of boronic acids and water.

(Figure 6) [43]. Specifically, the authors propose that these reactions occur via an S_Ni-type mechanism in which glycoside bond formation commences before the termination of the epoxide ring opening. In other words, they argue that the reaction occurs via a highly dissociative, yet concerted, S_Ni mechanism. The principal mechanistic underpinnings for this proposal are a measured anomeric ^{13}C KIE that, within experimental error, is equal to 1.00, and a low α-SDKIE of 1.055. The authors used these values to constrain a theoretical TS model for the proposed concerted S_Ni mechanism [43]. However, we assume that for this reaction the calculated potential energy of the modelled TS, relative to the boronic ester ground state, is smaller than the activation free energy, since the reaction should exhibit a large negative entropy of activation. Of note, even assuming this, the calculated potential energy of the modelled TS is too large for the reaction to be complete within the experimental window of 20 hours at 0 °C [43]. As a result, we invoke Occam's razor and suggest that the reaction is more likely a classical two-step S_Ni reaction with the first step determining the reaction rate, followed by the product determining TS. Further mechanistic studies will be required to confirm or refute this suggestion.

Solvolytic reactions

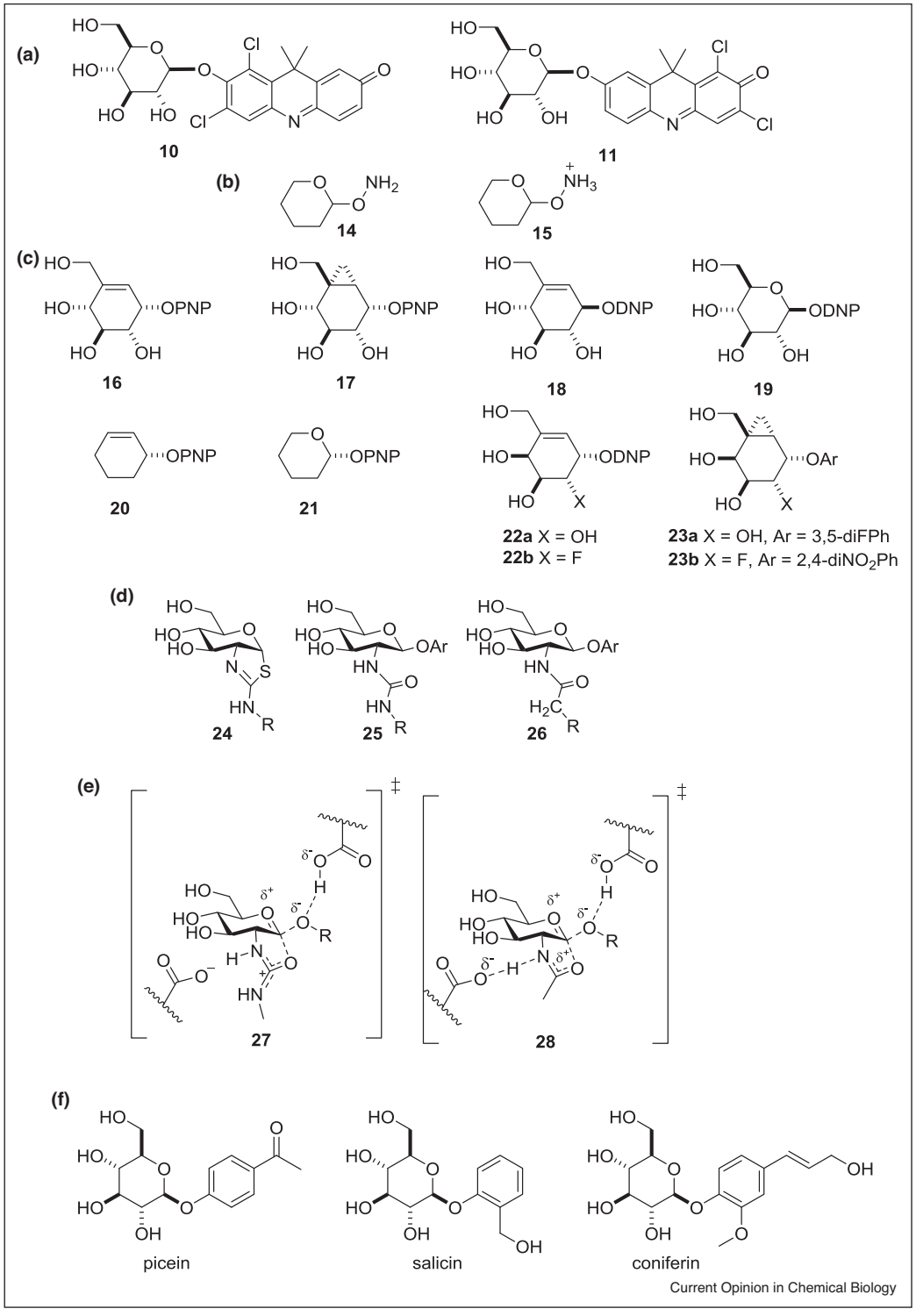
It has been recognized for a long time that the spontaneous hydrolysis reactions of acetals, including tetrahydropyranosyl and glycopyranosyl, are characterized by large negative β_{lg} values (–1.2 to –1.3) [44,45] and that these values are consistent with late TSs. Therefore, the recent report detailing large reactivity differences for a pair of isomeric β-D-glucopyranosides (**10** and **11**) that produce the same anion (**12**) following glycosidic C–O bond cleavage is perhaps surprising [46]. In this instance a reported rate difference of 10^5 was measured for the spontaneous hydrolyses of **10** and **11** (Figure 7a, with the 2′,6′-dichloro isomer (**10**) being the more reactive [46]. The authors concluded that caution should be used when construction Brønsted-type plots using the pK_a value, of the leaving group's conjugate acid, as a measure of leaving group ability. In other words, the use of a macroscopic thermodynamic quantity (K_a) is invalid for situations in which more than one site of protonation (or deprotonation) exists within a molecule. It can be shown that the macroscopic dissociation constant K_a (Scheme 1a) is related to the two microscopic equilibrium constants (a and b) by the expression $1/K_a = 1/a + 1/b$, while the tautomeric equilibrium constant K_T is given by a/b [47]. This thermodynamic cycle requires that the minor component of a pair of equilibrating tautomers, which produce a common anion, has the proton on the more acidic site (**13**) [47].

A conceptually similar system was analyzed by Kirby *et al.* who were interested in the tautomeric equilibrium constant (K_T; Scheme 1b) for the interconversion of ammonium oxide and hydroxylamine [48]. Kirby *et al.* realized that knowledge of the macroscopic K_a for protonation of hydroxylamine meant that to calculate K_T these authors needed an estimate for one of the two microscopic acidity constants (a or b). To this end, Kirby and co-workers made the *O*-tetrahydropyranosyl hydroxylamine **14**, and measured the second-order rate constant for its acid-catalyzed cleavage, a process that involves formation of tetrahydropyranosylium ions, which was assumed to occur from the *N*-protonated form **15** (Figure 7b) [48]. Kirby *et al.* then used their experimental rate constant with the known β_{lg} value [44] for the hydrolysis of tetrahydropyranosyl derivatives to estimate the acidic constant b and thus obtain an estimate for the equilibrium constant K_T [48]. Therefore, if we assume that a β_{lg} value of –1.3 [45] is operative for the spontaneous hydrolysis of glycosides **10** and **11**, then we can calculate, based on the known reactivity differences, that the microscopic pK_a values for a and b (Scheme 1a) are ∼4 units apart, a value that is close to the difference in measured pK_a values for phenol (9.81) and 2,6-dichlorophenol (6.78) [49]. We therefore conclude that the reactivity difference between **10** and **11** is not abnormally large.

Lately, the use of carbasugars, especially cyclohexenyl and cyclopropylmethyl analogs (such as **16** and **17**), have garnered interest as new classes of mechanistic probes for GHs (Figure 7c) [50•,51•,52•]. Such systems react via carbocationic intermediates (S_N1-like) or cationic TSs (S_N2-like) in which the electron pair that stabilizes charge development originates from either a double bond (**16**) or a strained C–C sigma bond (**17**), thus mimicking the reactions of carbohydrates where the ring oxygen (n_p–lone pair) performs this task. Moreover, for the case of such allylic systems the six-membered ring conformations of the resultant cation, which in its lowest energy conformations must have five adjacent co-planar atoms (^3E or E_3; Figure 2), closely mimics the half-chair conformations of glycopyranosylium ions ($^4H_3$ or $^3H_4$) with four sequential co-planar atoms.

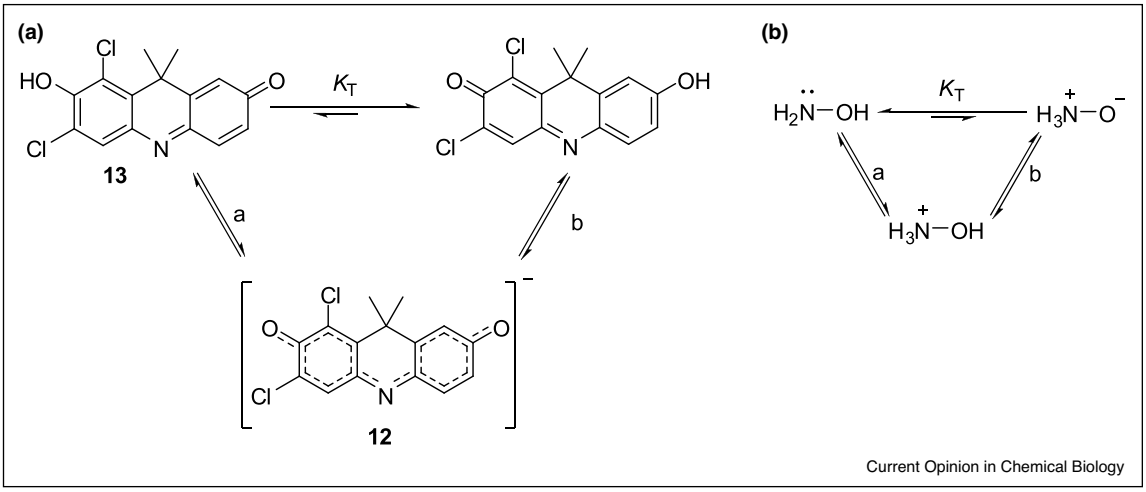
In a similar fashion to the S_N1-like spontaneous reactions of activated glycopyranosides, which give rise to products of both anomeric configuration, the hydrolysis of

Figure 7



(a) Glucopyranosides (**10** and **11**) that yield the same anion following glycosidic bond cleavage; **(b)** *O*-Tetrahydropyranosyl hydroxyamine (**14**) and its *N*-protonated form; **(c)** carbasugar analogues and associated acetals (**16**–**23**) used to study charge delocalization during C–O bond cleavage reactions; **(d)** Inhibitors (**24**) and substrates (**25** and **26**) used to study the mechanism of *O*-GlcNAc hydrolase; **(e)** Proposed transition states (**27** and **28**) for the *O*-GlcNAc hydrolase-catalyzed hydrolysis of **25** and **26**, respectively, **(f)** Structures of three natural product aryl β-D-glucopyranosides.

Scheme 1



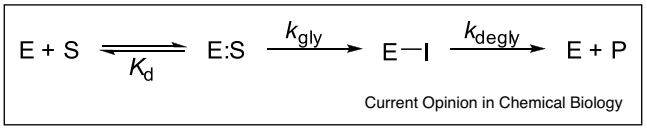
Equilibria constants for the ionization of equilibrating tautomers that form a single ion: Panel **(a)** equilibria pertinent to the formation anion **12**; Panel **(b)** equilibria pertinent to the formation hydroxylammonium cation.

DNP-Val (**18**) gave three products: the pseudo α-anomers and β-anomers as well that resulting from water attack at the tertiary center of the allylic cation intermediate [50•]. Also, consistent with the reactions of **18** occurring via an S_N1 mechanism is the measured α-SDKIE ($k_H/k_D = 1.11$), a value consistent with the TS involving rehybridization ($sp^3 \rightarrow sp^2$) at the pseudo-anomeric center. Interestingly, the two dinitrophenyl compounds (**18** and **19**) undergo spontaneous hydrolyses with nearly identical rate constants [50•]. In contrast, the 4-nitrophenyl unsubstituted analogs (**20** and **21**) display markedly different first-order rate constants for their spontaneous hydrolyses with **21** reacting around three orders of magnitude faster [52•]. These striking reactivity differences between the carbohydrate-based systems (**18**/**19**) and their parent compounds (**20**/**21**) are likely caused by the electron-withdrawing hydroxyl groups retarding hydrolysis of glucosides more than they do for the cyclohexene-based compounds (Figure 7c). A hypothesis that can be tested readily with a Hammett-Taft LFER study similar to that performed by Namchuk *et al.* for glycopyranoside hydrolyses [21].

Enzyme-catalyzed reactions and inhibition

Notably, the cyclohexenyl carbasugars **16** and **18** appear to possess reactivity with the corresponding retaining glycoside hydrolases that is opposite to, and therefore complementary with, the 2-deoxy-2-fluoroglucopyranosides [50•,52•]. That is, 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside is a covalent inhibitor of β-glucosidases ($k_{gly} > k_{degly}$, Scheme 2) and the carbasugar is a slow substrate ($k_{degly} > k_{gly}$) [50•], while the opposite holds true for the pseudo α-anomers; the carbasugar is a covalent inhibitor ($k_{gly} > k_{degly}$)[52•] and the 2-deoxy-2-fluoroglycoside is a slow

Scheme 2



Kinetic scheme for reactions catalyzed by glycoside hydrolases, k_{gly} and k_{degly} are the first-order rate constants for formation and hydrolysis of the covalent enzyme intermediate.

substrate ($k_{degly} > k_{gly}$) [53]. Interestingly, both pseudo anomeric cyclohexenyl sugar analog react with retention of configuration for their GH-catalyzed hydrolyses via covalent enzyme bound intermediates, as would be expected for compounds that utilize the catalytic machinery of retaining GHs [50•,51•]. In other words, bond formation, during the catalytic cycle, to give the covalent enzyme-intermediate occurs with an inversion of stereochemistry as does the subsequent hydrolysis of the intermediate [51•]. Another mechanistic feature of the enzyme-catalyzed reactions of **22** and **23** is that substitution of the 2-hydroxyl group for a fluorine atom has a much more profound rate reducing effect for the bicyclic inhibitors (**23**). A result that is consistent with positive charge delocalization being more advanced at the allylic cation-like TS, while charge is more concentrated on the pseudo-anomeric center at the TS for covalent labelling by the bicyclic inhibitors (**23**). Structures of the intermediates formed from the reactions of **22b** and **23b** have been solved and both are, as expected, β-carbasugars covalently linked to the enzymatic nucleophile [51•,54]. Further mechanistic studies, such as Brønsted LFER experiments, are needed for more in-depth enzymatic TS characterization for covalent inhibition by these carbasugars.

Another area of current interest is the investigation of whether tight-binding competitive inhibitors are TSA inhibitors. For example, in order to characterize whether a series of tight-binding competitive aminothiazoline (**24**) inhibitors of O-GlcNAc hydrolase (OGA), the enzyme that removes *O*-GlcNAc from proteins, are TSAs Cekic *et al.* performed two Bartlett LFER experiments (Figure 7d) [55••]. Specifically, they measured K_i values for a series of inhibitors (**24**) and k_{cat}/K_m values for two series of substrates (**25** and **26**), and they calculated the slopes of the correlations between $\log(K_i)$ and $\log(K_m/k_{cat})$. These authors concluded that aminothiazoline (**24**) are TSA inhibitors of the OGA-catalyzed hydrolysis of 2-deoxy-2-urea-β-D-glucopyranosides (**25**), a deduction based on a good Bartlett correlation that exhibited a slope of approximately unity. Unexpectedly, when these authors performed a similar analysis using the catalytic efficiencies (CEs; k_{cat}/K_m) of 2-acetamido-2-deoxy-β-D-glucopyranosides (**26**) the Bartlett-type plot displayed a slope of 2.3 ± 0.3. A result that is consistent with the OGA-catalyzed TS (**27**), which leads to an aminooxazolinium ion intermediate, supporting more positive charge on the carbohydrate fragment than the corresponding TS (**28**) that gives rise to the oxazoline intermediate (Figure 7d). In other words, the combined LFERs support an OGA catalytic mechanism that involves general-base catalysis by the proximal carboxylate (**28**) rather than the alternative, which has been proposed, where this residue simply performs an electrostatic stabilization role during the formation of the enzyme-bound oxazolinium ion intermediate.

In a noteworthy study, Kötzler *et al.* made, semi-synthetically using a natural peptide ligation strategy, two enzymes that incorporated site-specifically either a single monofluoro or a difluoroglutamic acid as the nucleophilic residue in a circular permutant variant of a retaining *endo*-β-xylosidase [56••]. Unfortunately, both fluorine containing enzymes exhibited K_m values that are far above the solubility limits for two of the seven substrates tested so that only minimal values for k_{cat} can be calculated. Of note, these minimal k_{cat} values are larger than, or similar to, the measured values for the other five substrates. As a result, we only consider the reported CEs (k_{cat}/K_m) for these enzymes. Notably, the difluoroglutamate and fluoroglutamate enzymes display very similar CEs (k_{cat}/K_m), at their pH optimum, for their catalyzed-hydrolyses of aryl xylobioside substrates. That is, the ratio of CEs varies between 0.45 and 2.67 over the seven substrate panel that covers over 3.5-orders of magnitude in leaving group ability (pK_a of their conjugate acids span the range 4.2–7.8). These authors performed a series of Brønsted LFERs (slopes are β_{nuc}) based on the pK_a of the nucleophile (obtained from the pH-rate profiles for the three enzymes) and the logarithm of the CE (k_{cat}/K_m). Notably, for two of the leaving groups (6,8-difluoro-4-methylumbelliferone; $pK_a = 4.7$ and 4-methylumbelliferone; $pK_a = 7.8$) the derived β_{nuc} values are close to zero while for all other leaving groups (pK_a values of the conjugate acids range from 4.2 to 7.2) exhibit β_{nuc} values that are of a similar magnitude. We therefore conclude that nucleophilic participation at the enzymatic TS for glycosylation by these three enzymes is dependent on the aglycone structure, but not in a predictable fashion. This groundbreaking study on an exceedingly complex system has paved the way forward for more detailed mechanistic studies, perhaps on compounds that more closely resemble the natural substrate and by use of physical organic tools that perturb the system to a smaller degree, such as KIEs.

Reactions with negatively charged transition states

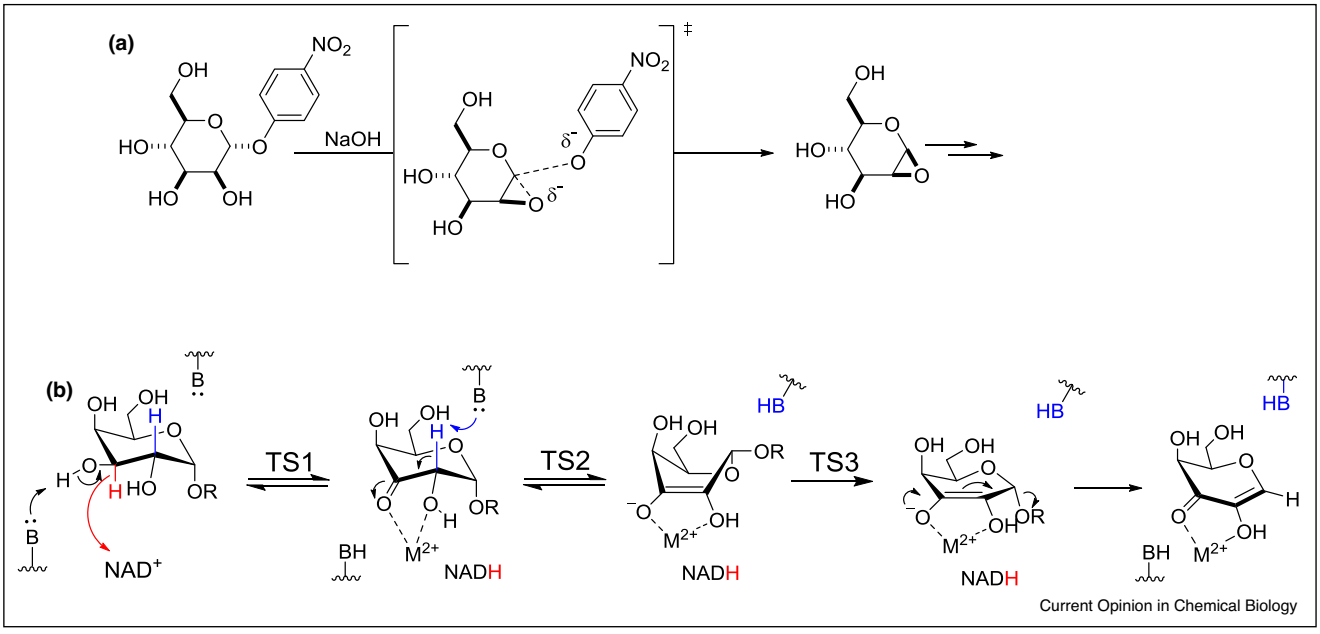
Reactions in solution

It has been known for over 100 years that aryl glycopyranosides in which the aglycone is *trans* to the C2-hydroxyl group, for example, the natural products picein, salicin, and coniferin (Figure 7f), undergo base-promoted solvolyses [57], which occurs at an accelerated rate relative to the corresponding *cis*-glycosides [58–60]. A recent detailed KIE study, in conjunction with theoretical modelling, showed that the specific-base promoted reaction of 4-nitrophenyl α-D-mannopyranoside to give a 1,2-anhydro-β-D-mannopyranose intermediate occurs via a concerted, late, TS with the nucleophilic attack of the C2-oxyanion occurring simultaneously with 4-nitrophenoxide departure (Figure 8a) [61]. Interestingly, a similar mechanism has been proposed for GH99 endo-α-mannosidases that involves general catalysis, thus avoiding oxyanion formation within the active site [62]. We note that the first synthesis of unprotected 1,2-anhydro-monosaccharides has been reported [63], although no mechanistic studies have been undertaken on these reactive intermediates.

Enzyme-catalyzed reactions

A glycoside hydrolase mechanism that involves anionic TSs during the hydrolytic cycle involves the oxidation of C3 to give a ketone intermediate and this is followed by abstraction of the C2-H with aglycone departure to give a glycal intermediate (Figure 3) [64]. To probe this mechanism in more detail, Sannikova *et al.* used a remote labelling strategy, in conjunction with ^{19}F NMR spectroscopy, to measure a series of competitive hydride and proton transfer KIEs on an α-galactosidase-catalyzed hydrolyses of a panel of fluoroaryl α-D-galactopyranoside [65•]. These authors noted that the magnitudes of both the C3 and C2 ^{2}H-KIEs varied with both the leaving group, even though the leaving group had only a marginal effect on the reaction rate constant (k_{cat}/K_m), and the identity of the metal ion that is necessary for enzymatic activity. Such observations are consistent with three sequential steps in the catalytic cycle being partially rate limiting. That is, these authors concluded that these

Figure 8



(a) Rate-limiting formation of a transient oxirane intermediate in the specific-base promoted reactions of 4-nitrophenyl α-D-mannopyranoside; **(b)** Current mechanism for the first half of the catalytic cycle for GH4 enzymes. The three transition states that partially limit the overall reaction rate constants are: TS1 hydride transfer, TS2 proton abstraction, and TS3 conformational change.

elementary steps are; hydride transfer to the onboard NAD^+, proton abstraction from C2, and a non-chemical step that is likely a conformational change that precedes aglycone departure (Figure 8b) [65•].

Future prospects

The continuing discoveries of new glycoside hydrolase families and the large number of glycoside hydrolases from other GH families that hydrolyze the same glycosidic linkage, we would argue, necessitates that mechanistic studies on these enzymes involve the use of natural substrates or natural substrate analogs for kinetic characterization. In this way more relevant, to the natural system, mechanistic deductions will emerge. Moreover, we look forward to the adoption of the remote ^{19}F-label protocol for the measurement of heavy-atom KIEs on GHs as the ^{19}F nucleus, which has a natural abundance of 100%, possesses the best NMR characteristics (sensitivity and spectral dispersion) for such important mechanistic experiments. With regard to synthetic carbohydrate chemistry, an area that is in need of attention is the determination of rate constants for the interconversion of various glycosyl cationic ion-pairs in conjunction with those for nucleophilic trapping of these intermediates. Such experiments will enhance our knowledge of how to perturb the various reaction pathways to provide the desired product. Finally, we also note that new spectroscopic methods, the routine generation of protein variants, and the ever increasing power of modern computational chemistry means that more precise structural and mechanistic investigations will allow future researcher a better comprehension of the intricacies of complex biological systems.

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Conflict of interest statement

Nothing declared.

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