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Review

Glycosyl fluorides in enzymatic reactions

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Abstract

Glycosyl fluorides have considerable importance as substrates and inhibitors in enzymatic reactions. Their good combination of stability and reactivity has enabled their use as glycosyl donors with a variety of carbohydrate processing enzymes. Moreover, the installation of fluorine elsewhere on the carbohydrate scaffold commonly modifies the properties of the glycosyl fluoride such that the resultant compounds act as slow substrates or even inhibitors of enzyme action. This review covers the use of glycosyl fluorides as substrates for wild-type and mutant glycosidases and other enzymes that catalyze glycosyl transfer. The use of substituted glycosyl fluorides as inhibitors of enzymes that catalyze glycosyl transfer and as tools for investigation of their mechanism is discussed, including the labeling of active site residues. Synthetic applications in which glycosyl fluorides are used as glycosyl donors in enzymatic transglycosylation reactions for the synthesis of oligo- and polysaccharides are then covered, including the use of mutant glycosidases, the so-called glycosynthases, which are able to catalyze the formation of glycosides without competing hydrolysis. Finally, a short overview of the use of glycosyl fluorides as substrates and inhibitors of phosphorylases and phosphoglucomutase is given.

Keywords: Chemoenzymatic reactions; Glycosidase; Glycosyl transferases; Glycosyl transfer; Transglycosylation; Hehre mechanism; CGTase; Mechanism-based inhibitors; Glycosynthase

Contents

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1. Introduction and scope

Glycosyl fluorides are becoming increasingly important molecules for the study of enzymatic reactions, for use as mechanistic probes and as reagents for enzymatic glycosylations. The interest in these compounds arises not least in that they are relatively stable in aqueous solution; they are also readily accessible synthetically in both anomeric forms. Although originally prepared by the action of anhydrous hydrogen fluoride on acetylated sugars [1], for this conversion the much safer reagent hydrogen fluoride/pyridine may now be substituted [2]. Other methods for the synthesis of glycosyl fluorides include the action of HF/pyridine [2] or diethylaminosulfur trifluoride (DAST) [3] on sugar hemiacetals and the treatment of glycosyl bromides or chlorides with silver fluoride [1] or silver tetrafluoroborate [4]. The action of HF/pyridine usually results in the formation of the thermodynamically more stable anomer whereas the other reagents typically afford the product of inverted anomeric stereochemistry, although the products obtained from the action of silver tetrafluoroborate on glycosyl halides depend on the reaction conditions [4]. The reactivity of glycosyl fluorides in many enzyme systems is particularly important. Being α-fluoro ethers, glycosyl fluorides are relatively reactive compounds, particularly when activated by binding to an enzyme active site. The small size of fluorine, being comparable to that of a hydroxyl group, means that there is little steric demand suffered upon introduction of this atom into a molecule compared to either a proton or a hydroxyl group (Table 1) [5–7]. Additionally, the fluorine atom in a C–F bond is believed to be capable of forming hydrogen bonds with hydrogen bond donors but not hydrogen bond acceptors [6,8]. The naturally occurring isotope of fluorine, ^{19}F, has a spin number of 1/2 and may be readily detected by NMR spectroscopy. This is of particular value as it allows the direct detection and monitoring of fluorine, even when the molecule of interest is interacting with an enzyme. The aim of this review is not to give a detailed and comprehensive review of the literature but rather to provide an overview of the general uses of glycosyl fluorides in the context of enzyme reactions, with specific examples chosen for their illustrative value. The recent review of Dax and co-workers, covering fluorinated carbohydrates deserves special mention as an adjunct to this review [9].

Table 1
Bond lengths and selected van der Waals radii for some elements

Element	Bond length (C–X) (Å)	van der Waals radius (Å)
H	1.09 [5]	1.20 [5]
O	1.43 [5]	1.40 [5]
F	1.37–1.42 [10–15]	1.35 [5]

2. Issues of stability

In enzymatic reactions, glycosyl fluorides are required in a form suitable for an enzyme to act upon, usually as the polyol. Indeed, glycosyl fluorides are the only glycosyl halide stable enough to be deprotected and dissolved in aqueous buffers without appreciable rates of spontaneous hydrolysis. Most deprotected glycosyl fluorides are crystalline compounds that may be stored for reasonable periods. For prolonged storage, glycosyl fluorides are best kept as the per-*O*-acetate that may be deacetylated (with catalytic sodium methoxide in methanol or ammonia in methanol) immediately prior to use. In aqueous solution, most α-D-glycosyl fluorides are stable for periods of weeks or longer, particularly if kept below 0 °C and buffered to minimize auto-decomposition arising from acid-catalyzed solvolysis. However, the β-D anomers are less stable and, for most purposes, solutions should be made up immediately prior to use. β-D-Glucopyranosyl and β-cellobiosyl fluorides are among the most stable β-fluorides; the β-fluorides of

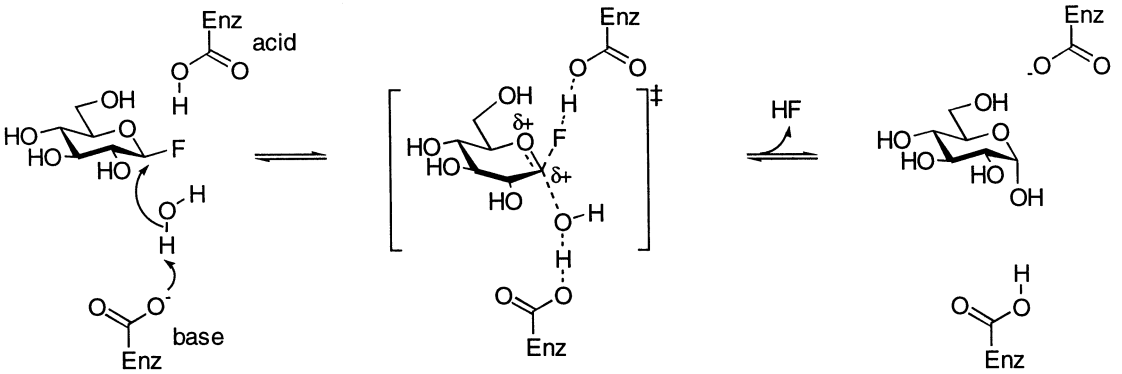


Fig. 1. Mechanism of hydrolysis of β-glucosyl fluoride by an inverting β-glucosidase.

galactose and mannose are somewhat less stable compounds but are still useful substrates provided enzymatic rates of reaction are sufficiently high. For example, measurements made of spontaneous hydrolysis rates of α- and β-D-glucopyranosyl fluoride in 1 M $NaClO_4$ at 50 °C showed that the β-fluoride hydrolyses some 40-fold faster than the α-fluoride [16]. The choice of buffer is also relatively important and, when a new buffer is chosen, it is wise to establish the rate of spontaneous hydrolysis. In particular, strongly acidic and basic buffers are known to accelerate the rate of hydrolysis, as are phosphate and phosphonate buffers [17].

Major exceptions to the stability comments mentioned above are glycosyl fluorides prepared from 2-acetamido-2-deoxy sugars with a 1,2-trans arrangement. These compounds solvolyze relatively rapidly in water or methanol by way of an oxazolinium intermediate [18]. However, 1,2-*trans*-2-acetamido-2-deoxy glycosyl fluorides are stable enough to be used in impure form in enzymatic reactions in aqueous solution provided reaction rates are fast enough to be detected over the background hydrolysis [19]. It has been noted that, in contrast to the stability of α-D-glucopyranosyl fluoride, 5-thio-α-D-glucopyranosyl fluoride undergoes rapid, spontaneous hydrolysis [20]. In addition, these authors are unaware of any simple, deprotected furanosyl fluorides stable in aqueous solution. Another noteworthy point is that certain glycosyl fluorides may adopt unusual conformations that may modify their reactivity and enzymatic recognition. For example, 2,3,4-tri-*O*-acetyl-β-D-xylopyranosyl fluoride is known to prefer a $^1C_4$ conformation in organic solvents [21].

3. Glycosyl fluorides as substrates and mechanistic probes

3.1 Substrates for glycosidases.

A review of the uses of glycosyl fluorides in enzymatic reactions necessarily requires a discussion of the mechanism of action of glycosidases. There exist two classes of glycosidases, those that hydrolyze glycosides with retention of anomeric stereochemistry, and those that operate with inversion of anomeric stereochemistry. Both classes typically perform this task with the aid of two important residues, usually carboxyl groups [22]. Inverting enzymes proceed through a single transition-state with substantial oxacarbenium-ion character, one carboxyl acting as an acid and the other as a base (Fig. 1). These residues are typically spaced some 9–10 Å apart to allow for the interspersing of a water molecule [23]. Retaining glycosidases proceed by way of nucleophilic attack on the anomeric carbon by one carboxyl group (the nucleophile) with the other functioning as an acid (Fig. 2). A glycosyl enzyme intermediate is formed which is hydrolyzed, the other carboxyl group now functioning as a base. The carboxyl groups are spaced approximately 5.5 Å apart to allow for the direct attack on the anomeric carbon by the nucleophile residue. Again, the two transition-states have been shown to possess substantial oxacarbenium-ion character. Glycosidases have been classified into over 80 sequence related families [24]. Within a family,

there is a strict correlation of sequence, structure and the stereochemical outcome of hydrolysis.

Glycosyl fluorides have proven particularly useful as substrates for glycosidases. In contrast to other popular substrates such as aryl glycosides, there are no known examples of glycosidases that cannot process the glycosyl fluoride that corresponds to the substrate. Glycosyl fluorides were first shown to act as substrates for glycosidases with the β-galactosidase of *Escherichia coli* in 1961 [25]. Relatively soon thereafter it was realized that the rapid hydrolysis of glycosyl fluorides by the corresponding glycosidases was a general phenomenon. Typically, glycosyl fluorides are excellent substrates with high k_{cat}/K_m values. High values of k_{cat}/K_m are of particular use for the determination of enzymatic reaction rates with small amounts of enzyme and for the determination of K_i values for tight-binding inhibitors. Moreover, the generality of these compounds as substrates for glycosidases and their high k_{cat}/K_m values is reflected in their common usage to determine the stereochemical outcome of enzyme-catalyzed glycoside hydrolysis [26,27].

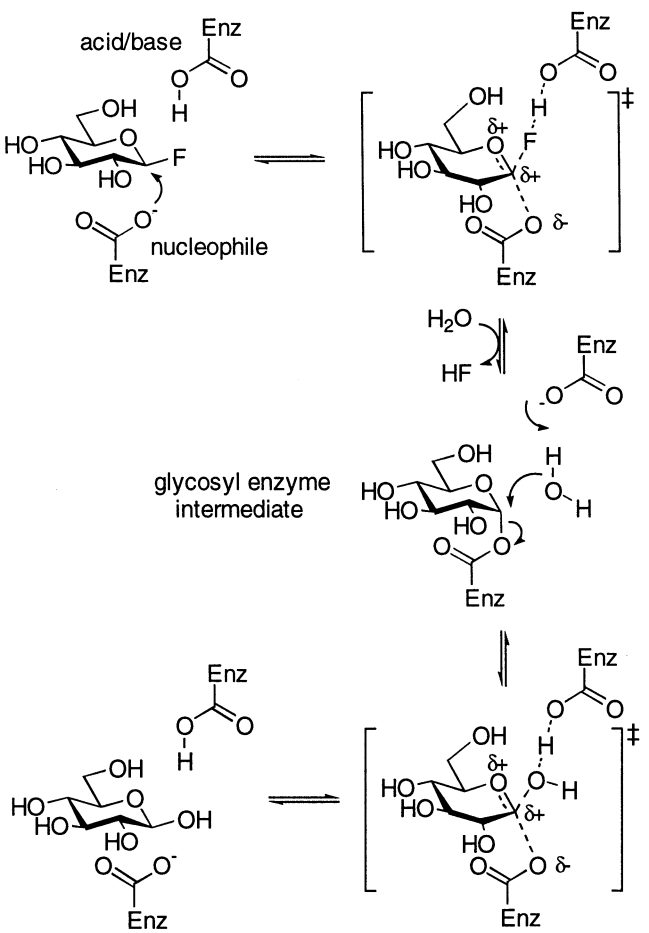


Fig. 2. Mechanism of hydrolysis of β-glucosyl fluoride by a retaining β-glucosidase.

Historically, hydrolysis of glycosyl fluorides was followed by measurement of fluoride ion released with a pH stat [25]. Alternatives to this approach were developed including the use of a coupled assay for the detection of either glucose or galactose released, through use of glucose or galactose oxidase [28]. The availability of fluoride ion-specific electrodes now makes the task of direct measurement of fluoride relatively easy [16]. Alternatively, some workers have used a fluoride specific dye that allows for the direct spectrometric observation of fluoride release [29–31]. Some care must be exercised in the selection of buffers as certain ions such as calcium or manganese may cause precipitation and thus reduction of the observed fluoride concentration. The detection of fluoride with an ion-selective electrode has distinct advantages over the other methods mentioned above due to its generality, simplicity and accuracy.

An illuminating example of the importance of glycosyl fluorides as substrates for glycosidases is that of mammalian pancreatic α-amylase. Aryl α-maltosides have been shown to be rather poor substrates for this enzyme [32], whereas the corresponding α-maltooligosaccharyl fluorides are sensitive and reliable substrates [33].

3.2 Glycosyl fluorides as substrates for glycosidase mutants.

Glycosyl fluorides have evolved into important substrates for analysis of the effects of mutagenesis performed on glycosidases. In particular, the important observations are that the hydrolysis of glycosyl fluorides does not require acid catalysis [34] and that glycosyl fluorides react readily with charged nucleophiles in aqueous solution [35]. These two observations have enabled the assignment of function to amino-acid residues by detailed kinetic studies with mutant enzymes using glycosyl fluorides [36]. This subject has been recently reviewed and only a cursory overview of the approach will be given [36]. In mutant

Table 2
Glycosidases that have been shown to hydrolyze the 'wrong' glycosyl fluoride

Enzyme		Source	Enzyme mechanism
α-Glucosidase	[50]	Sugar beet	Inverting
α-Glucosidase	[50]	Rice seed	Inverting
β-Amylase	[40]	Sweet potato	Inverting
Glucoamylase	[43]	*Rhiziopus niveus*	Inverting
Glucodextranase	[43]	*Arthrobacter globiformis*	Inverting
endo-β-Glucanase A	[51]	*Cellulomonas fimi*	Inverting
β-Xylosidase	[41]	*Bacillus pumilus*	Inverting
Trehalase	[44]	*Candida tropicalis*	Inverting
Trehalase	[44]	Cockchafer	Inverting
Trehalase	[44]	Rat intestinal mucosa	Unknown
Trehalase	[44]	Rabbit kidney	Inverting
Trehalase	[42]	*Trichoderma reesei*	Inverting
Cellobiohydrolase II (Cel6A)	[46]	*Trichoderma reesei*	Inverting
Glucoamylase E400 cysteinesulfinic acid	[45]	*Aspergillus awamori*	Inverting
Glycogen debranching enzyme	[48]	Rabbit muscle	Inverting
α-Glucosidase	[49]	*Aspergillus niger*	Retaining

enzymes, individual amino-acid residues are replaced with alternative residues to modify the properties of the enzyme. To determine whether the mutation has been made at the acid–base residue of either a retaining or inverting glycosidase the relative rates of hydrolysis by the mutant compared to that of the wild-type are determined for a substrate that requires acid catalysis, such as a simple *O*-glycoside, and for a substrate that does not require acid catalysis, typically a glycosyl fluoride. If there exists a large loss of activity towards the substrate requiring acid-catalysis that is not matched with the substrate that does not require acid catalysis then it is likely that this residue provides acid catalysis for glycosyl transfer [37,38]. Further confirmation for this assignment may be gained if substantial rate enhancements are observed with substrates such as glycosyl fluorides when alternative nucleophiles such as azide are included in the reaction mixture and a glycosyl azide of retained stereochemistry is formed. Alternatively, to determine whether the residue mutated is the nucleophile in a retaining glycosidase, two approaches may be used. In one approach, assays with the mutant show no significant activity with activated substrates (typically a dinitrophenyl glycoside or a glycosyl fluoride) until a small nucleophile such as azide or formate is added, when a glycosyl azide product of the opposite anomeric configuration is formed. Thus, the enzyme has been converted from a retaining mechanism to an inverting mechanism. In the other approach, glycosyl fluorides of the 'wrong' anomeric configuration are found to be cleaved by such mutants through a Hehre resynthesis mechanism (vide infra). In this case, there is no hydrolysis of the product owing to the lack of the catalytic nucleophile. This discovery lead to the development of glycosynthases (Section 4.2) [39].

3.3 Enzymatic hydrolysis of 'wrong' glycosyl fluorides.

In 1979 Hehre and co-workers demonstrated that β-amylase, an inverting α-glucan hydrolase, could catalyze the hydrolysis of β-maltosyl fluoride to β-maltose and hydrogen fluoride [40]. This was the first demonstration of a glycosidase catalyzing the hydrolysis of a substrate with the 'wrong' anomeric configuration. Similarly, the inverting β-xylosidase from *Bacillus pumilus* was shown to cleave α-xylopyranosyl fluoride [41]. Detailed analysis has been conducted on the hydrolysis reaction, which has now been observed with many inverting glycosidases and one retaining glycosidase (Table 2).

For an inverting glycosidase, the hydrolysis reaction is believed to proceed by way of an initial transglycosylation from one glycosyl fluoride molecule to another to form a glyco-

sidic linkage with inversion of anomeric stereochemistry (Fig. 3). The transglycosylation product now has the 'correct' anomeric stereochemistry for the enzyme and is rapidly hydrolyzed, affording the product with inverted stereochemistry; the net result is retention of stereochemistry from the initial glycosyl fluoride. Evidence for this mechanism is the unusual dependence of rate on the concentration of the substrate (being dependent on the square of the concentration) and the increase in rate commonly noted upon addition of alternative acceptor molecules. The mechanism is widely referred to as the Hehre resynthesis–hydrolysis mechanism.

In some cases, the intermediate transglycosylation products have been isolated [41–44]. For example, with the inverting β-xylosidase from *B. pumilus* the reaction of α-xylopyranosyl fluoride in the presence of [^{14}C]xylose was followed and the intermediate, xylobiose, was isolated and shown to contain one xylose residue derived from α-xylopyranosyl fluoride and one residue from the labeled xylose [41]. In a somewhat unusual example, Fierobe and co-workers have reported the preparation of a mutant of the inverting glucoamylase from *Aspergillus awamori* in which the catalytic base, E400 was replaced with cysteine and oxidized to cysteinesulfinic acid [45]. While the wild-type enzyme was unable to catalyze the hydrolysis of β-glucopyranosyl fluoride at detectable rates, the oxidized mutant was able to catalyze the reaction at rates detectable over the rather rapid background hydrolysis. In the case of the enzymatic hydrolysis of α-cellobiosyl fluoride by the inverting β-glycanase, CBHII (Cel6A) from *Trichoderma reesei*, the reaction was originally believed to follow an alternative S_Ni mechanism; ultimately it was shown that this enzyme also follows the Hehre resynthesis–hydrolysis mechanism [46,47]. The hydrolysis of β-glucopyranosyl fluoride has been demonstrated with glycogen debranching enzyme, a mixed α-glucosidase/glycosyl transferase, and is discussed below [48].

The hydrolysis of the 'wrong' glycosyl fluoride by a retaining glycosidase has been demonstrated in only one case, that being for an α-glucosidase from *Aspergillus niger* [49]. Typically, the 'wrong' glycosyl fluorides do not even bind to retaining glycosidases. The mechanism of this hydrolysis has been little investigated other than to determine that the initial product of hydrolysis of β-glucopyranosyl fluoride is α-D-glucose.

Interestingly, the enzymatic hydrolysis of the 'wrong' anomer of a substrate has only been observed for glycosyl fluorides. This is presumably a result of the small size of

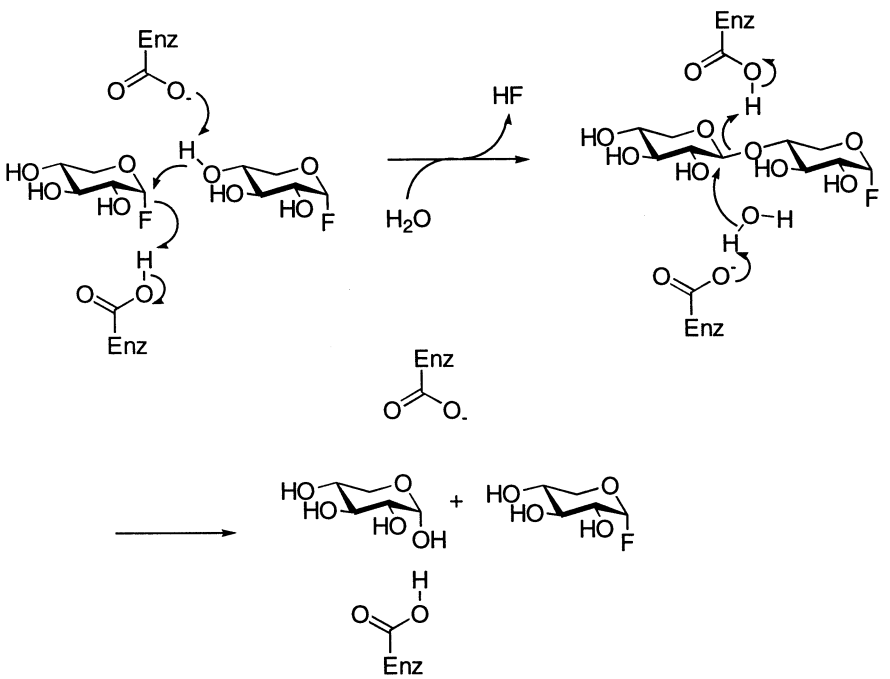
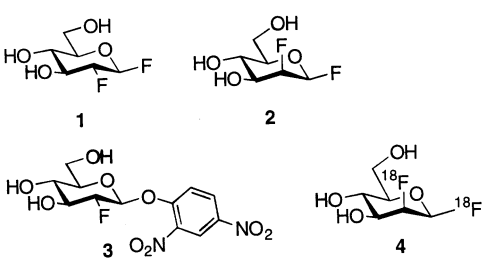


Fig. 3. Hehre resynthesis–hydrolysis mechanism for the hydrolysis of the 'wrong' anomeric fluoride.

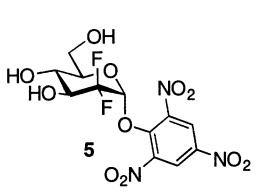
fluorine that allows binding within a space ordinarily evolved to allow only water to bind. The space between the catalytic residues of an inverting glycosidase is sufficient to accommodate a water molecule or a fluorine atom but will not allow even an *O*-methyl group to bind, as evidenced by the absence of methanolysis activity for inverting glycosidases and an inability to cleave the 'wrong' methyl glycosides. In addition, glycosyl fluorides are quite reactive compounds, especially the β anomers. Hehre has suggested that the hydrolysis of the wrong anomer of a glycosyl fluoride by an inverting glycosidase is the result of 'plastic' and 'conserved' phases in glycosidases, wherein protonation occurs by way of flexible, 'plastic' catalytic machinery while hydrolysis occurs by a strictly maintained and rigid trajectory [50]. In response Sinnott has commented that the hydrolysis of these compounds, unexpected based on the implicit stereospecificity of these enzymes, is unsurprising [22]. Glycosyl fluorides have a relatively high intrinsic reactivity which, in the presence of the stereochemically well-defined and highly reactive active-site of an enzyme should be able to participate in reactions of some sort, not least hydrolysis [51].

3.4 Substituted glycosyl fluorides as mechanism-based inhibitors and probes of enzyme mechanism

As indicated earlier, the enzyme catalyzed hydrolysis reaction for retaining glycosidases proceeds by way of a glycosyl enzyme intermediate flanked by oxacarbenium ion-like transition states. A large body of data points to the existence of the glycosyl enzyme intermediate, which has now been observed crystallographically in both α- and β-glycosidases [52–54]. Introduction of fluorine onto the carbohydrate ring will destabilize charge development at O-5 or C-1 at the transition state to an extent that depends upon the proximity of the substitution to these two positions. The replacement of the hydroxyl group at C-2, in particular, with a strongly electronegative fluorine atom destabilizes the two transition-states to such an extent that both the glycosylation and deglycosylation of the enzyme occur at much reduced rates. However, by introduction of a very good aglycone leaving group, the intermediate glycosyl enzyme becomes kinetically accessible, while the hydrolysis of this intermediate remains very slow. Consequently, these compounds act as mechanism-based inhibitors of glycosidases since trapping of the glycosyl enzyme intermediate prohibits binding of normal substrates. Moreover, active-site-directed reversible inhibitors provide protection against inhibition as is to be expected if both inhibitors are competing for the same enzyme active site. Good choices for the anomeric leaving group have been shown to include fluorides and highly reactive aryl groups, in particular 2,4-dinitrophenolate [55,56]. The use of the natural aglycon has also been described in the case of 2-deoxy-2-fluoro-glucotropaeolin (an inhibitor of myrosinase from *Sinapis alba*) [57] and 2′-deoxy-2′-fluoro-β-cellobiose (an inhibitor of Abg from *Agrobacterium* sp.) [58]. 2-Deoxy-2-fluoro-glycosyl fluorides (e.g., **1** and **2**) were originally introduced by Withers and co-workers as a complement to the corresponding 2,4-dinitrophenyl 2-deoxy-2-fluoro-glycosides (e.g., **3**) [56]. Time-dependent inhibition with both of these classes of compounds is usually observed, as expected for their mode of inhibition. However, the stability of the intermediate glycosyl enzyme may vary considerably from one glycosidase to another [56]. The inhibition of β-glucosidase and β-mannosidase by 2-deoxy-2-fluoro-β-glucopyranosyl and mannopyranosyl fluorides, **1** and **2**, has even been demonstrated in vivo [59]. Here, the compounds inactivated their respective glycosidases throughout the rats studied, even crossing the blood/brain barrier and inactivating the glycosidases therein. Radiolabeled 2-deoxy-2-[^{18}F]-fluoro-β-mannopyranosyl fluoride (**4**) has been suggested as a potential imaging probe for glycosidases through the use of positron emission tomography [60].



It has been found that 2-deoxy-2-fluoro glycosyl fluorides and glycosides are usually more effective inhibitors for β-glycosidases than for α-glycosidases, more commonly acting as slow substrates for which the glycosylation step is rate limiting in the latter cases [61]. This difference is in part a result of the greater stability of the α-glycosyl enzyme intermediate formed on a β-glycosidase than of the β-glycosyl enzyme formed on an α-glycosidase. In one approach to overcome problems associated with the rapid turnover of such intermediates formed on α-glycosidases, Withers and coworkers developed the related 2,2-difluoro glycosides [61]. Here, the second fluorine at C-2 further slowed each step while the reactivity of the leaving group was enhanced further to allow intermediate accumulation by the incorporation of a 2,4,6-trinitrophenyl group. The 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-α-D-glucoside **5** was shown to inactivate yeast α-glucosidase and the corresponding α-maltosyl fluoride **6** was an effective inhibitor of human pancreatic amylase [61].



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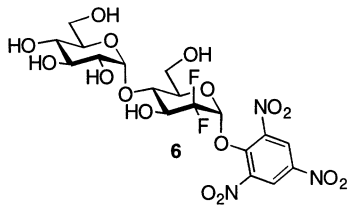

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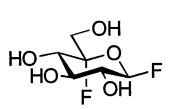
A third class of related compounds is that of the 5-fluoro-glycosyl fluorides, e.g., **7**, which can serve as inactivators or as slow substrates of very low K_m, owing to accumulation of the glycosyl enzyme intermediate. This class of glycosidase inhibitors was introduced by McCarter and Withers [62] and again, the concept here is the introduction of an electron-withdrawing group, this time at the 5-position. The location of the C-5 fluorine directly adjacent to the site of greatest charge development (O-5) [63] should lead to substantial destabilization, especially, since in this case, a hydrogen rather than a hydroxyl has been substituted by a fluorine. Further, recent studies have suggested that the percentage of charge borne on the ring oxygen versus the anomeric carbon is greater for α-glycosidases than for β-glycosidases, possibly explaining why 5-fluoro-glycosyl fluorides function to trap intermediates on both classes of enzyme [23,54,64]. These compounds may be prepared with varying degrees of efficiency from 5-bromo-glycosyl fluorides which may themselves be prepared by photobromination reactions on glycosyl fluorides, a reaction originally developed by Ferrier and Furneaux [65]. 5-Fluoro-glycosyl fluorides may be considered 'double' glycosyl fluorides, as C-5 now also becomes anomeric. This places considerable limitations on the ability to modify the sugar structure once the C-5 fluorine has been installed.

On occasion, during the preparation of 5-fluoro glycosyl fluorides from the corresponding 5-bromides, the compounds epimeric at C-5 may be isolated either as minor products or indeed as the major product. In some cases these derivatives inverted at C-5 with respect to the substrate behave as better inhibitors than the 5-fluoro compounds with the same C-5 stereochemistry as the natural substrate for the enzyme [66]. For example, 5-fluoro-β-L-gulopyranosyl fluoride (**8**) was shown to be a better inactivator of jack bean α-mannosidase than 5-fluoro-α-D-mannopyranosyl fluoride (**9**), the C-5 epimer [67]. Interestingly, 2-deoxy-2-fluoro-α-D-mannopyranosyl fluoride failed to inactivate this enzyme, providing further validation for the use of 5-fluoro-glycosyl fluorides [56].



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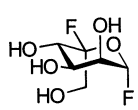

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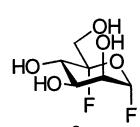

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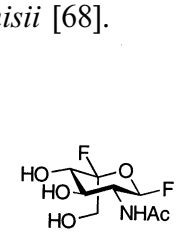
5-Fluoro-glycosyl fluorides are a particularly useful extension of 2-deoxy-2-fluoro glycosyl inhibitors since the modification is conservative, all substituents on the sugar ring being preserved. This has allowed the synthesis of inhibitory molecules that cannot be formed in the 2-fluoro series. A most pertinent example is 2-acetamido-2-deoxy-5-fluoro-α-L-idopyranosyl fluoride (**10**), a compound recently shown to be a mechanism-based inhibitor for the *N*-acetyl-β-D-glucosaminidase from *Vibrio furnisii* [68].



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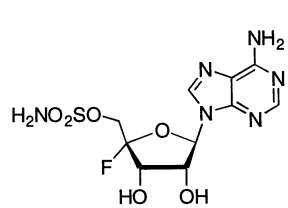

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While all the compounds presented here have been synthetic in origin, the first representative of this class of compounds was the natural product, nucleocidin **11**, a nucleoside antibiotic. This compound was originally isolated from the fermentation broths of *Streptomyces calvus* [7] and contains a rather intriguing 4-fluoro-β-D-ribofuranosyl moiety. Although there exists substantial similarity between this compound and the 5-fluoro sugars discussed above, little is known about the mechanism of action of this antibiotic.

All of these fluoro sugars may be considered highly specific reagents for labeling the catalytic nucleophile of retaining glycosidases. There are no known cases where the labeling occurs in a non-stoichiometric manner nor with residues other than the catalytic nucleophile, unlike many common affinity labels that have been used for glycosidases [69,70]. In fact, the stoichiometric enzyme labeling performed by these compounds has enabled 'active-site titrations'. This technique allows the direct measurement of enzyme concentration by measurement of a 'burst' of either fluoride or 2,4-dinitrophenolate released upon reaction with the enzyme due to the stoichiometric nature of the reaction [31].

Substantial evidence exists for the trapping of such intermediates including the detection and assignment of the anomeric stereochemistry of the glycosyl enzyme formed on the β-glucosidase from *Agrobacterium* sp. and the xylanase from *Cellulomonas fimi* by ^{19}F NMR spectroscopy [52,71]. X-ray crystallographic structure determination of glycosyl enzymes formed from the reaction of 2-deoxy-2-fluoro-glycosides has been used to give insight into the nature of the glycosyl enzyme intermediate and the roles of specific residues in the active site of the enzyme. The first of these trapped intermediates was observed on the retaining *exo*-xylanase Cex from *C. fimi* [52], and they have since been observed on a number of glycosidases as reviewed [23].

2-Deoxy-2-fluoro- and 5-fluoro-glycosyl fluorides and the 2,4-dinitrophenyl 2-deoxy-2-fluoro-glycoside counterparts have proved especially useful reagents for the labeling and identification of the enzymes' catalytic nucleophile [72]. This may be done in a number of ways. Initially this was approached though the use of radiolabeled sugars and differential (high performance liquid chromatography) HPLC mapping of proteolytic digests of the inhibited enzyme compared to an unlabeled sample processed in an identical fashion. The identification of the enzyme nucleophile has now been substantially simplified by the application of electrospray–ionization mass spectrometry (ESIMS). In this approach, the inhibited enzyme is proteolytically digested to provide a mixture of short peptides, one or more of which will be labeled with the inhibitor. The labeled peptide is the sole ester present in the mixture, thus neutral loss scans in a tandem mass spectrometer may be used to locate the labeled peptide within a HPLC chromatogram. Purification of a sample of the labeled peptide and the application of MS/MS to sequence the peptide usually allows the identification of a unique labeled carboxyl residue as the enzyme nucleophile; a result which may be confirmed through Edman degradation. The catalytic nucleophiles of over 20 enzymes have been identified through the use of 2-deoxy-2-fluoro sugars and 5-fluoro-glycosyl fluorides as reviewed [23].

3.5 Kinetic isotope effects and 1-fluoro-glycosyl fluorides as probes of oxacarbenium ion character.

Primary (^{14}C) and secondary kinetic isotope effects have been determined for the enzymatic hydrolysis of α-glucopyranosyl fluoride by several inverting and retaining α-glycosidases [73,74]. These data suggested that the transition-state structure for enzyme catalyzed hydrolysis has considerable oxacarbenium ion character and that the D-glucosyl ring becomes flattened at the transition state as indicated in Fig. 1. Of interest here are detailed kinetic isotope effects that have been measured for the spontaneous hydrolysis of α- and β-glucopyranosyl fluorides [34]. In the case of the α-fluoride, these measurements indicated a transition state with close similarities to that of the enzyme catalyzed reaction. However, in the presence of 2.0 M sodium azide, an S_N2 reaction has been demonstrated [35]. The spontaneous hydrolysis of the β-fluoride, on the other hand, gave kinetic isotope effects indicative of an S_N1-like transition state [34].

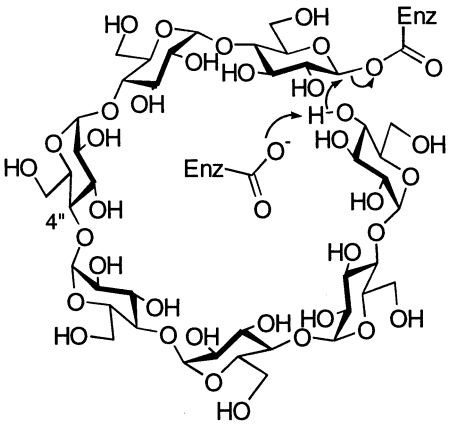
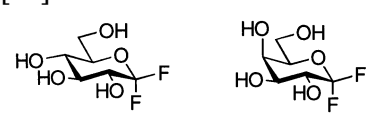


Fig. 4. Glycosyl enzyme intermediate formed on cyclodextrin glycosyl transferase showing mechanism of formation of cyclodextrin product.

1-Fluoro-glycosyl fluorides are an interesting class of sugars originally introduced by Praly and Descotes [75] and first employed as probes of glycosidase mechanism by Konstantinidis and Sinnott [16]. Surprisingly, these compounds do not act as inhibitors of retaining glycosidases but rather as slow substrates for both retaining and inverting α- and β-glycosidases [16]. For example, with the inverting amyloglucosidase II from *A. niger*, hydrolysis of 1-fluoro-D-glucopyranosyl fluoride **12** occurs according to Michaelis–Menten kinetics with a k_{cat} value 2700-fold lower than α-glucopyranosyl fluoride, while with the retaining β-glucosidase from *Aspergillus wentii* the turnover was 240-fold slower than that of β-glucopyranosyl fluoride [16]. These relatively small rate reductions compared to those seen for 5-fluoroglycosyl fluorides are likely a result of the fact that the location of a fluorine substituent directly on a carbenium ion can actually stabilize, rather than destabilize it owing to back-bonding from the fluorine p-orbitals. Such effects have been observed in the addition of trifluoroacetic acid to 2-fluoropropene [76].



12 **13**

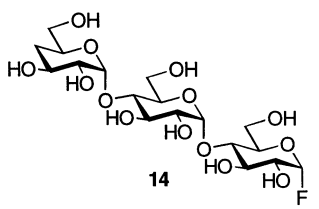
1-Fluoro-glycosyl fluorides have been employed as probes of the extent of charge development at the first transition-state of glycoside hydrolysis, thereby complementing the use of isotopically labeled substrates in the measurement of α-kinetic isotope effects. Specifically, the ratio of the second order rate constants (k_{cat}/K_m values) for the hydrolysis of a glycosyl fluoride and the corresponding 1-fluoroglycosyl fluoride, may be used to determine the degree of charge formation at the transition-state [77]. Such a method has been used with 1-fluoro-D-galactopyranosyl fluoride **13** to track changes in transition state structure during the experimental evolution of the catalytically feeble β-galactosidase ebg from *E. coli* [78].

3.6 Glycogen debranching enzyme and cyclodextrin glycosyl transferase.

Cyclodextrin glycosyl transferases (CGTases) degrade starch into cyclodextrins or short linear oligosaccharides. In particular, the transfer occurs with net retention of anomeric stereochemistry suggesting a two-step, double displacement mechanism analogous to that by which retaining glycosidases are believed to catalyze transglycosylations, namely via an intermediate glycosyl enzyme (Fig. 4). α-Glucopyranosyl, α-maltosyl and α-maltotriosyl fluorides have been shown to be effective substrates for the CGTases from *Bacillus circulans*, *Bacillus megaterium* and *Bacillus macerans* [20,38,79,80]. In the case of the CGTase from *B. circulans*, while both α-maltosyl and α-glucopyranosyl fluorides have greater K_m values than α-maltotriosyl fluoride, their k_{cat} values are of the same order indicating that once the substrate has bound to the enzyme, there is no advantage to the use of longer substrates [38]. The principal reaction product obtained in all these cases is β-cyclodextrin accompanied by small amounts of maltooligosaccharides and other products arising from disproportionation reactions.

Another approach used for the accumulation of usually transient intermediates has been demonstrated with cyclodextrin glycosyl transferase. By use of 4″-deoxy-α-maltotriosyl fluoride (**14**), a compound with a good leaving group at C-1, the glycosyl enzyme should form readily. However, the absence of a hydroxyl group at C-4″ should prevent the transglycosylation of the glycosyl enzyme onto a second molecule of substrate. Interestingly, **14** acts as a slow substrate for the enzyme, as the

intermediate glycosyl enzyme hydrolyzes slowly, but at a sufficient rate to prohibit significant accumulation, a mechanism possibly evolved by the enzyme to prevent inactivation by modified maltooligosaccharides [38]. A solution to the problem of trapping this intermediate was developed involving site-directed mutagenesis of the putative acid–base residue from Glu257 to Gln which caused a significant reduction in the rate of the second hydrolytic step while the first glycosylation step remained accessible due to the high reactivity of the glycosyl fluoride. Subsequent proteolytic digestion followed by a sequencing protocol as described above allowed identification of the catalytic nucleophile [38] which was subsequently confirmed by determination of the three-dimensional X-ray crystal structure [54,81].



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Glycogen debranching enzyme catalyzes the removal of α-(1→6)-linked maltotetraosyl branch points in limit dextrin (glycogen degraded extensively by glycogen phosphorylase alone) through two main steps, that of glycosyl transfer of a maltotriosyl unit from the (1→6) branch to the main chain, and then hydrolysis of the single remaining α-(1→6)-glucosyl group. The enzyme has been shown to have two distinct sets of catalytic machinery, one for transferase action and one for hydrolytic action. Braun and Withers have described an assay for the transferase activity of this enzyme simply using α-maltotriosyl fluoride [48]. It was shown that the action of glycogen debranching enzyme on α-maltosyl fluoride resulted in the formation of maltohexaosyl and maltononaosyl fluoride, thereby confirming binding at the transferase site. The catalytic nucleophile in this transferase site was then identified by a protocol similar to that discussed above for CGTase using 4″-deoxy-α-maltotriosyl fluoride (**14**) as a slow substrate [82].

Interestingly, α-glucopyranosyl fluoride functions as an excellent substrate for the α-glucosidase machinery of the enzyme [48]. Unexpectedly, 2-deoxy-2-fluoro-α-D-glucopyranosyl fluoride functioned as a slow substrate for the enzyme, rather than a mechanism-based inhibitor, forcing a re-evaluation of the enzyme's hydrolytic mechanism, which was then shown to occur by way of inversion [82]. Perhaps unsurprisingly, the enzyme was then shown to be capable of transferring β-glucopyranosyl fluoride to β-cyclodextrin to form an α-(1→6) linkage. This observation is exactly analogous to that discussed above for the Hehre resynthesis–hydrolysis mechanism.

4. Glycosyl fluorides as donors for enzymatic glycosylation

4.1 Transglycosylation with glycosidases.

Carbohydrate structures have an undeniable importance in biology, including the carbohydrate motifs that decorate glycoproteins, the common glycone portions of a variety of antibiotics and the intricate carbohydrate assemblies that are required for signaling processes and as highly discriminating receptors. In most cases, the specificity and activity of these compounds rely on the carbohydrate portions of the molecule [83,84]. In addition, there exists a great amount of interest in the synthesis of specific oligosaccharide structures. Conventional chemical synthetic approaches are, in many cases, inadequate to provide substantial quantities of oligosaccharides in a timely fashion owing to the complexities involved in the selective protection and subsequent manipulations of the various monosaccharide donors and acceptors. Ultimately, in order to provide useful quantities of materials, organic chemistry relies on a good combination of yield and brevity that in many cases must be sought by a combination of chemical and enzymatic techniques [85].

One excellent approach to the regioselective and stereoselective synthesis of the glycosidic bond involves the use of retaining glycosidases in transglycosylation reactions [86–91]. In this approach, hydrolytic enzymes are run in reverse by use of activated glycosyl donors in

the presence of acceptor alcohols. Thus, hydrolysis of a donor sugar with a retaining glycosidase according to the established mechanism proceeds by way of a glycosyl enzyme. Normal hydrolysis occurs when this intermediate reacts with water. If the glycosyl enzyme is intercepted with an alcohol such as a sugar, then the result will be glycosyl transfer or transglycosylation to afford a new glycosidic linkage with net retention of stereochemistry (Fig. 5). Obviously, techniques that reduce the rate of deleterious hydrolysis should increase the yield of the desired transglycosylation reaction. Thus, reduction of the effective concentration of water, running the transglycosylation reactions at high concentrations and the addition of large excesses of the acceptor molecule have all been shown to improve yields. Transglycosylation products are kinetically controlled so careful monitoring of the reaction is necessary to ensure maximum yields. Many glycosyl donors have been used for transglycosylation reactions, the most successful and common being aryl glycosides, oligosaccharides such as lactose, and glycosyl fluorides. One major consideration in a transglycosylation reaction is that the activated donor substrate must function as a significantly better glycosyl donor than does the product to allow the transglycosylation product to accumulate. An equivalent statement is that the donor substrate must have a greater value of k_{cat}/K_m than the product. Typically, nitrophenyl glycosides and glycosyl fluorides have among the greatest values of k_{cat}/K_m and are consequently the best donors.

Glycosyl fluorides have an advantage over aryl glycosides and oligosaccharide donors related to their use as substrates, namely that they appear to be universally processed by all glycosidases. Indeed, the formation of transglycosylation products was noted in some of the earliest studies using glycosyl fluorides as substrates [28,30]. Moreover, in the case of a glycosyl fluoride donor, the major byproduct of a transglycosylation reaction is fluoride ion, which is easy to separate from the transglycosylation product. Unfortunately, the yields of transglycosylation reactions rarely exceed 60% and are more typically on the order of 10% or less. Nevertheless, transglycosylation reactions have been widely used to deliver many complex products.

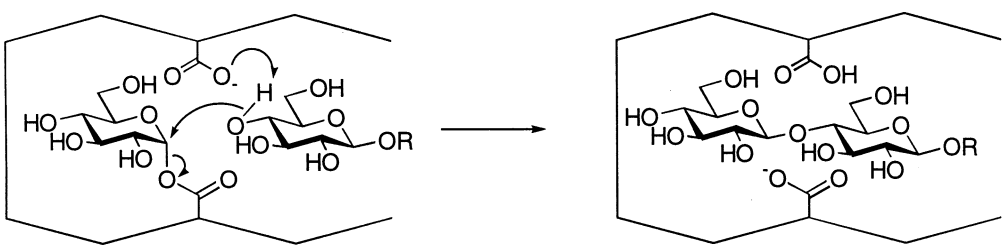
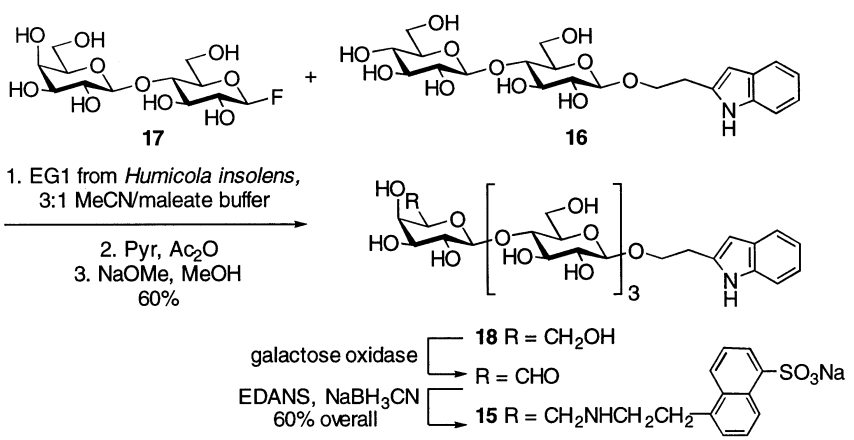
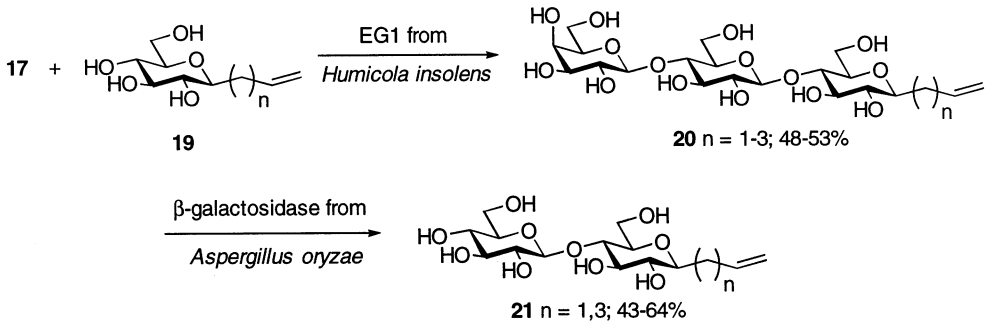


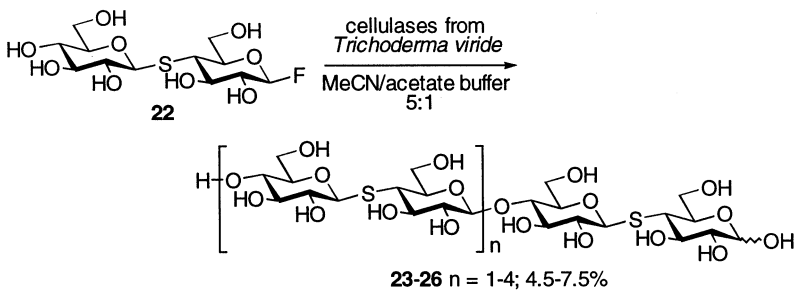
Fig. 5. Mechanism of transglycosylation with a retaining β-glycosidase.



Scheme 1. Ref. [99].



Scheme 2. Ref. [104].



Scheme 3. Ref. [105].

The synthesis of oligosaccharides by the use of glycosyl fluorides as donors in enzymatic transglycosylation reactions has mostly focussed on the use of β-glycosidases [92–97], however, a few examples of the use of α-glycosidases have been reported [42,98].

The general approach is illustrated in Scheme 1 for the preparation of the fluorogenic substrate **15** for cellulases [99]. Enzymatic transglycosylation of the indolethyl β-cellobioside **16** with β-lactosyl fluoride **17** under the agency of endoglucanase 1 from *Humicola insolens* afforded the tetrasaccharide **18** in an excellent yield of 60%. The use of a 3:1 mixture of acetonitrile to buffer illustrates the use of co-solvents to improve yield. Unwanted oligomerisation was prevented by the use of a glycosyl donor with an axial hydroxyl at C-4′, which was sufficiently substrate-like to be transferred by the enzyme but was unable to act as an acceptor. Additional processing involved the selective enzymatic oxidation of C-6 of the terminal galactopyranosyl residue of **18** with a galactose oxidase from *Dactylium dendroides* and reductive amination with 5-(2-aminoethylamino)-1-naphthalenesulfonate to afford **15**. A similar approach was used earlier for the synthesis of a fluorogenic substrate for amylase [100].

A creative approach to the synthesis of β-(1→4)-glucopyranosyl linkages was developed by Shoda and co-workers [101–103]. Here, use of β-lactosyl fluoride as a glycosyl donor with an endoglucanase allows for the selective transfer of a single lactosyl unit. As above, repeated transfer of the donor is prevented by the axial substituent at C-4′. Isolation of the condensation product and, subsequently, treatment with a β-galactosidase cleaves only the unwanted galactopyranosyl unit from the adduct, the net result being the transfer of a single glucopyranosyl unit to the original acceptor. An interesting application of this approach was that used by Fairweather and co-workers to address problems associated with the chemical synthesis of some epoxyalkyl cellobiosyl *C*-glycosyl compounds (Scheme 2) [104]. Thus, the *C*-glycosyl derivatives **19** were shown to act as excellent acceptors for β-lactosyl fluoride **17** using EG1 from *H. insolens*, affording the trisaccharides **20** in yields of up to 53%. Treatment of the trisaccharides **20** with a β-galactosidase from *Aspergillus oryzae* afforded the cellobiosyl

C-glycosyl compounds **21**. These were subsequently transformed into epoxides, putative inhibitors of cellobiohydrolases, the *C*-glycosyl linkage being included to preclude adventitious enzymatic hydrolysis.

A rather useful enzymatic transglycosylation was reported by Moreau and Driguez (Scheme 3) [105]. Oligomerisation of the *S*-linked cellobiosyl fluoride **22** with a mixture of cellulases from *Trichoderma viride* provided a mixture of oligomers of alternating *O* and *S* linkages from which the tetra-, hexa-, octa- and decasaccharides **23**–**26** were isolated in 4.5, 7.5, 5.7 and 5.0% yields, respectively. These compounds have potential to act as substrate-like inhibitors of cellulases. These hemithiocellodextrins are of particular interest as they are all water-soluble whereas cellodextrins beyond about cellooctaose are insoluble in water.

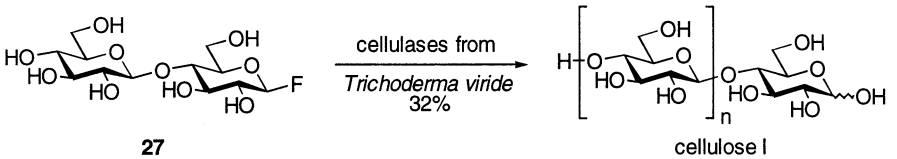
Kobayashi and Shoda have described a number of approaches to the synthesis of carbohydrate polymers, most notably cellulose (Scheme 4) [90,106]. Treatment of a solution of β-cellobiosyl fluoride **27** with a partially purified cellulase preparation from *T. viride* in a 2:1 mixture of acetonitrile and buffer afforded a white powder. This material was shown to exhibit an electron diffraction pattern characteristic of impure cellulose I, a crystalline form of cellulose with a parallel arrangement of linear β-(1→4)-glucan chains [107]. This approach was also used earlier to prepare synthetic cellulose II (consisting of antiparallel, linear β-(1→4)-glucan chains) [106], synthetic xylan [108], and later a novel hybrid polymer having a β-Glc*p*-(1→4)-β-Xyl*p*-(1→4) repeating unit [109].

Glycosidases capable of catalyzing transglycosylation reactions are almost always retaining glycosidases. However, as discussed above, the Hehre resynthesis–hydrolysis reaction may be considered a transglycosylation reaction and on occasion intermediate products have been detected and isolated [41–44].

4.2 Glycosynthases.

Glycosynthases are mutant enzymes formed from retaining glycosidases that are capable of catalyzing glycosyl transfer from glycosyl fluorides to acceptor alcohols without hydrolysis of the product [110]. These enzymes possess an unreactive group, an alanine residue, in place of the crucial, nucleophilic carboxyl residue required for hydrolysis by the wild-type enzyme. Such enzymes now function, in part, like inverting glycosidases, synthesizing oligosaccharides from the 'wrong' glycosyl fluoride in a Hehre resynthesis mechanism, but being incapable of the hydrolysis of this product since they lack the crucial catalytic nucleophile. Consequently, these enzymes can catalyze the direct transfer of a glycosyl residue from a glycosyl fluoride to an acceptor alcohol without the undesired deleterious hydrolysis of product typical in the use of wild-type hydrolases in transglycosylation (Fig. 6). Notably, transglycosylation yields may approach quantitative.

Two glycosynthases have been described thus far. The first of these was the Glu358Ala mutant of the exo-acting β-glucosidase Abg from *Agrobacterium* sp. [110]. This mutant



Scheme 4. Ref. [107].

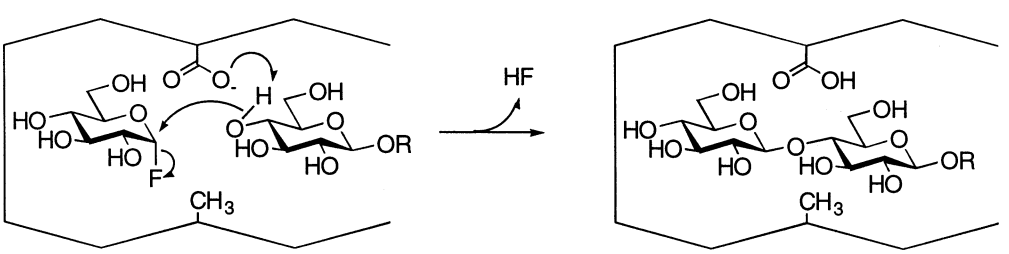
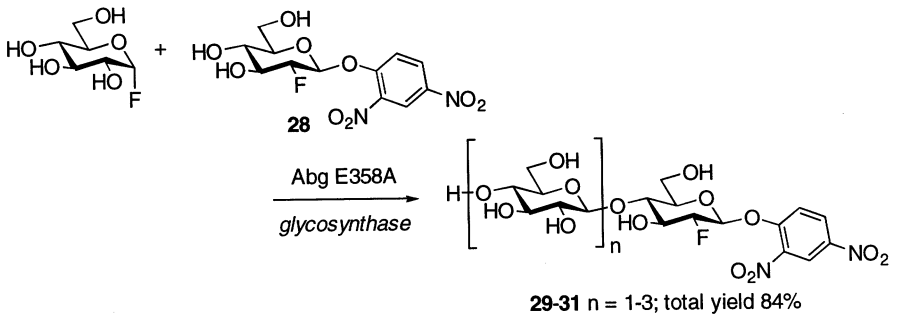
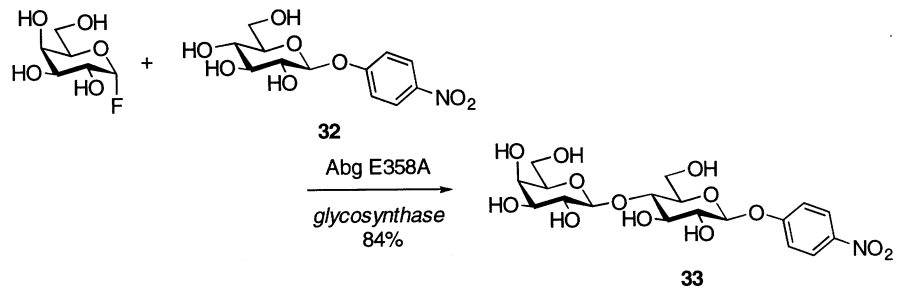


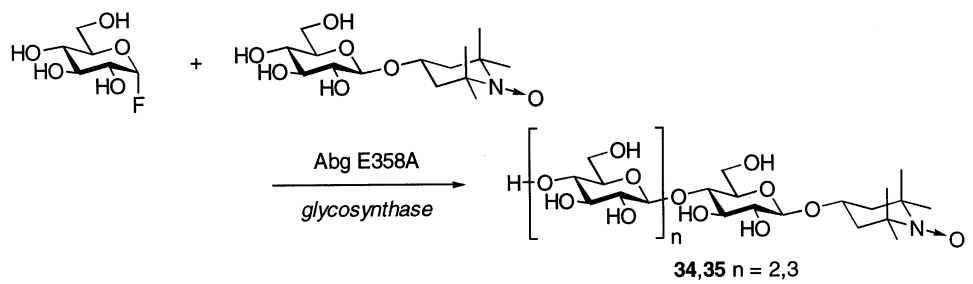
Fig. 6. Mechanism of transglycosylation with a glycosynthase.



Scheme 5. Ref. [110].



Scheme 6. Ref. [110].



Scheme 7. Ref. [111].

enzyme was shown to be capable of catalyzing the reaction of 2,4-dinitrophenyl β-D-glucopyranoside with charged nucleophiles such as azide and formate with inversion of configuration [39]. This glycosynthase will catalyze the transfer of the sugar moiety from α-glucopyranosyl fluoride and α-galactopyranosyl fluoride to suitable acceptor sugars. In the case of α-glucopyranosyl fluoride, the product itself functions as an acceptor, thus a mixture of longer oligosaccharides is obtained in an excellent overall yield. On the other hand, transglycosylation reactions with α-galactopyranosyl fluoride afford a single product, the axial hydroxyl at C-4 being unable to accept an additional glycosyl group.

Glycosynthases have been shown to be particularly useful for transglycosylation reactions with molecules that could function as either substrates or inhibitors for the wild-type enzyme. For example, treatment of 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucoside (**28**) with α-glucopyranosyl fluoride and the Glu358Ala Abg glycosynthase afforded a mixture of the di-, tri- and tetrasaccharides **29**–**31** in a combined yield of 84% (Scheme 5) [110]. Using a conventional glycosidase for this reaction would have been impossible, as the monosaccharide acceptor would rapidly inactivate the enzyme. By contrast, a single galactopyranosyl transfer from α-galactopyranosyl fluoride to 4-nitrophenyl β-D-glucoside (**32**), yielding the lactoside **33**, was possible in 84% yield with this enzyme (Scheme 6) [110]. Again, this reaction would have been impossible with a wild-type glycosidase, as the

monosaccharide acceptor would have functioned as a substrate for the enzyme.

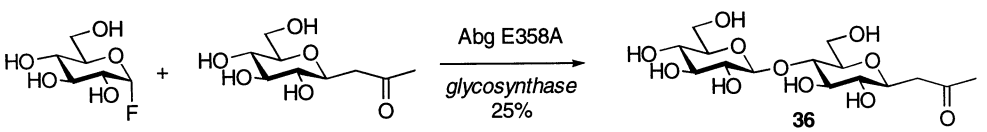
Further use has been made of this mutant to prepare a pair of spin-labeled cellodextrins **34** and **35** for the study of relative binding orientations on cellulose binding domains (Scheme 7) [111], and for the preparation of the cellobiosyl *C*-glycosyl derivative **36** which was elaborated into a mechanism based inhibitor of β-glucosidases (Scheme 8) [112].

The original Abg Glu358Ala glycosynthase has since been improved by substituting the nucleophile with serine [113]. A marked improvement in rates of transglycosylation was observed that resulted in improved yields of condensation products and reduced reaction times. Thus, poor acceptors for the Abg Glu358Ala glycosynthase, for example 4-nitrophenyl *N*-acetyl-β-glucosaminide, are successfully galactosylated by the Glu358Ser mutant, allowing the efficient synthesis of 4-nitrophenyl β-LacNAc.

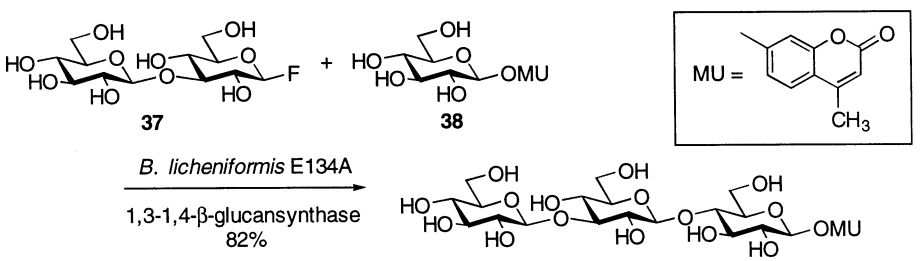
Another glycosynthase based on an identical strategy has been prepared from an endo-acting retaining (1→3)-(1→4)-β-glucanase from *Bacillus licheniformis* [114]. The nucleophile residue of the wild-type enzyme was identified and the Glu134Ala mutant prepared. This mutant enzyme was shown to catalyze the transfer of α-laminaribiosyl fluoride **37** to mono- and disaccharide acceptors, e.g., **38**, in excellent yield (Scheme 9). In this case the analysis of products was facilitated by the use of the wild-type enzyme which is highly specific for hydrolytic cleavage of the β-(1→4) linkage in a β-Glc*p*-(1→3)-β-Glc*p*-(1→4)-Glc structure.

4.3 Glycosyl transferases.

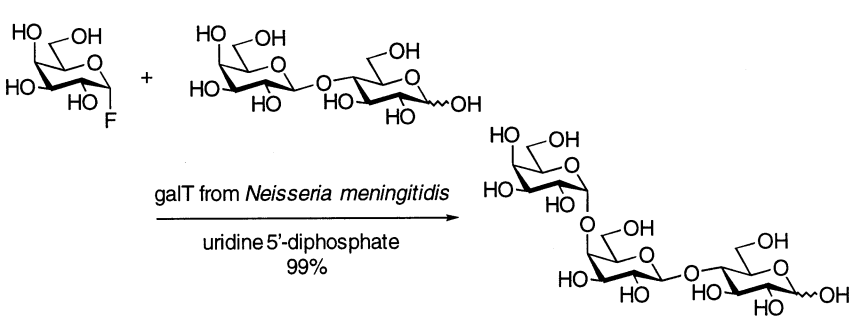
An exciting recent development is the demonstration by Lougheed and co-workers that α-galactopyranosyl fluoride may act as a substrate, albeit slow, for the α-galactosyltransferase from *Neisseria meningitidis* [115]. Here, α-galactopyranosyl fluoride in the presence of 1 mM uridine 5′-diphosphate (UDP) was shown to act as a substitute for the natural substrate, uridine-5′-diphospho-α-D-galactose. In the absence of UDP, no transfer



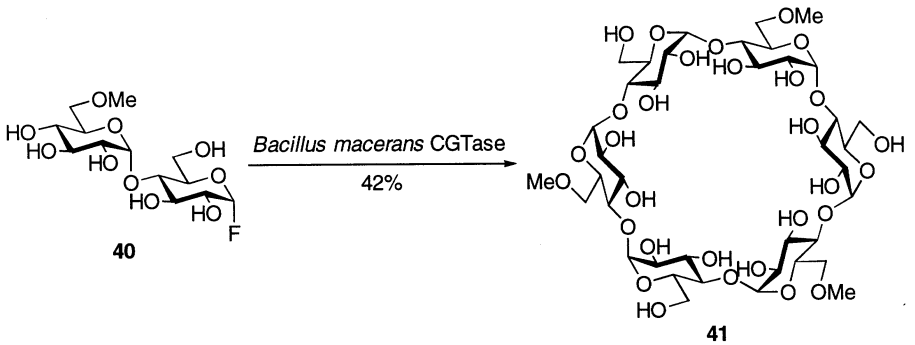
Scheme 8. Ref. [112].



Scheme 9. Ref. [114].



Scheme 10. Ref. [115].



Scheme 11. Refs. [20,116].

was seen. Under these conditions, small-scale reactions provided a quantitative yield of the trisaccharide **39** from reaction of lactose and α-galactopyranosyl fluoride (Scheme 10). Further, in the absence of a suitable acceptor, the transferase catalyzed the formation of uridine-5′-diphospho-α-D-galactose from a mixture of α-galactopyranosyl fluoride and uridine 5′-diphosphate. This result should provide impetus for further investigation into the use of alternative substrates for glycosyl transferases to overcome the difficulties associated with the preparation of large quantities of the natural substrates, sugar nucleotides and with the product inhibition arising from high concentrations of UDP.

CGTases catalyze the transfer of maltooligosaccharyl groups from starch to form cyclodextrins and oligosaccharides and, as noted earlier, these enzymes can use glycosyl fluorides as substrates. Further, these enzymes have been shown to catalyze the transfer of modified glycosyl fluorides to acceptor sugars [80]. For example, treatment of 6′-*O*-methyl-α-maltosyl fluoride (**40**) with the CGTase from *B. macerans* afforded the tri-*O*-methyl-α-cyclodextrin **41** in 42% yield (Scheme 11) [20,116]. The CGTase from *B. circulans* has been shown to catalyze the condensation of *S*-linked α-maltosyl fluoride to afford both cyclic and linear oligosaccharides whereas the *C*-linked α-maltosyl fluorides afforded only linear oligosaccharides [117,118].

5. Other enzymes

α-D-Glucopyranosyl fluoride has been shown to act as a substrate for sucrose phosphorylase from *Pseudomonas saccharophilia* [119]. This compound is an effective substrate for this enzyme, having a 3-fold greater k_{cat} than the natural substrate, sucrose. α-D-Glucopyranosyl fluoride has also been shown to act as a slow substrate for potato and *E. coli* maltodextrin phosphorylases in the presence of oligosaccharide primers [120]. Similarly, α-D-glucopyranosyl fluoride was also shown to act as a slow substrate for the retaining trehalose phosphorylase from *Schizophyllum commune* [121], resulting in the formation of α-glucopyranose-1-phosphate

Glycosyl fluorides have been shown to function as competitive inhibitors for a number of other enzymes such as phosphoglucomutase and glycogen phosphorylase [122]. Thus, α-glucopyranosyl fluoride-6-phosphate was shown to be a good inhibitor of phosphoglucomutase from rabbit muscle ($K_i = 41$ μM) [123] and the enzyme inhibitor complex was investigated by ^{19}F NMR spectroscopy [124]. Interestingly, very large downfield chemical shift changes ($\Delta\delta = -14$ and -19 ppm) were observed upon binding of either 6-deoxy-6-fluoro-α-glucose-1-phosphate or α-glucopyranosyl fluoride-6-phosphate, leading to the suggestion that these two fluorine substituents may be bound in the same portion of the active site. This result, in conjunction with others, strongly supported a mechanism involving substrate rotation in the active site rather than an enzymatic conformational change. A range of α- and β-glycopyranosyl fluorides have been shown to be moderate inhibitors in the millimolar and sub-millimolar range for glycogen phosphorylase from rabbit muscle, binding in the glucose binding site [120,122,125,126].

6. Conclusions

From an early start as an enzymatic curiosity, glycosyl fluorides have evolved into remarkably useful tools for probing and exploiting enzymatic glycosyl transfer reactions. Their high reactivity yet reasonable stability, coupled with the small steric demand of the fluorine substituent endows them with considerable flexibility as mechanistic probes, particularly of mutant enzymes. When coupled with further fluorine substitution on the sugar ring they can acquire the ability to function as reagents for trapping glycosidase reaction intermediates, thereby allowing structural access to this key component of the enzymatic reaction coordinate. However, despite their ease of synthesis, especially using reagents such as hydrogen fluoride/pyridine, they have not been as widely exploited by the biochemical community, as perhaps they should. Commercial availability of these sugars, if only in protected form, would solve the supply problem and broaden their application.

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