Monatshefte für Chemie 133, 541–554 (2002)

Monatshefte für Chemie
Chemical Monthly
© Springer-Verlag 2002
Printed in Austria

The Chameleon of Retaining Glycoside Hydrolases and Retaining Glycosyl Transferases: The Catalytic Nucleophile

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Summary. This paper addresses the existence and role of a catalytic nucleophile in retaining glycoside hydrolases and retaining glycosyl transferases. Although the former has now been established beyond doubt, such is not the case with the latter. We report reliable procedures for the synthesis of various 2-deoxy-2-fluoro glycosyl nucleoside diphosphates, useful donor analogues for the study of the mechanism of action of retaining glycosyl transferases.

Keywords. Glycoside hydrolases; Glycosyl transferases; 2-Deoxy-2-fluoro hexopyranoses.

Introduction

The burgeoning field of glycobiology arose from the recognition that carbohydrates play a pivotal role in an overwhelming number of biological processes [1–6]. Carbohydrates are now known to be of key importance in cell adhesion, infection, differentiation, and regulation as well as in many intercellular communication and signal transduction events [7]. In order to gain a deeper understanding of these events, it was natural for chemists, biochemists, and molecular biologists to probe the mechanism of action of the enzymes that process carbohydrates, mainly the glycoside hydrolases and glycosyl transferases.

This article, therefore, will first address some recent efforts with glycoside hydrolases, and then turn to the more vexed question regarding the mechanism of action of retaining glycosyl transferases.

Results and Discussion

Glycoside hydrolases

The mode of action of glycoside hydrolases, enzymes that catalyze the hydrolytic cleavage of glycosidic bonds in both simple and complex carbohydrate structures, is thoroughly understood thanks to the early and seminal efforts of *Koshland* [8] and *Legler* [9], and more recently by *Sinnott* [10, 11], *Vasella* [12], *Davies* [13, 14],

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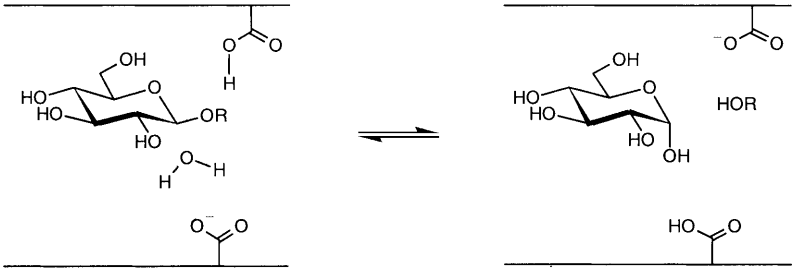
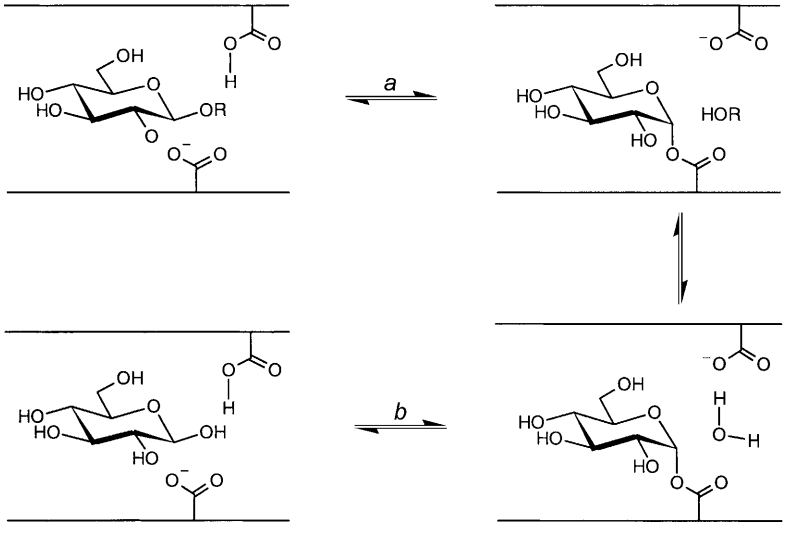
and *Withers* [15–20]. The glycoside hydrolases have been classified into families based on amino acid sequence homology [13, 21, 22], and most of these families more broadly into clans [23]. The members of a family all exhibit the same mechanism of action, whether they be *exo*- or *endo*-hydrolases and whether they act on similar or unrelated substrates. More recently, glycoside hydrolases have also been classified according to the direction from which the glycosidic oxygen is protonatated during hydrolysis (*syn*- or *anti*-protonators) [24].

Glycoside hydrolases catalyze the cleavage of the glycosidic linkage with either inversion or retention of configuration at the anomeric centre; the well established steps in the two separate processes are shown below (for a β-D-glycoside) [8].

Inversion:

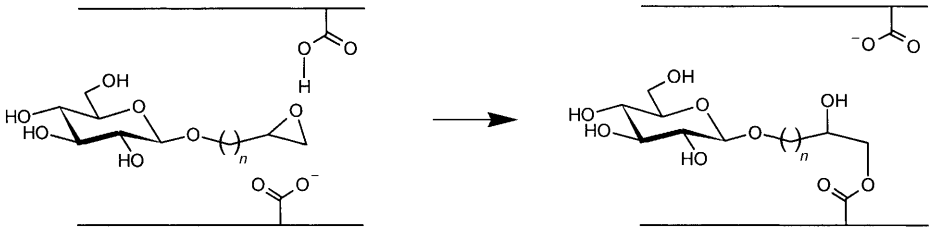


Retention:

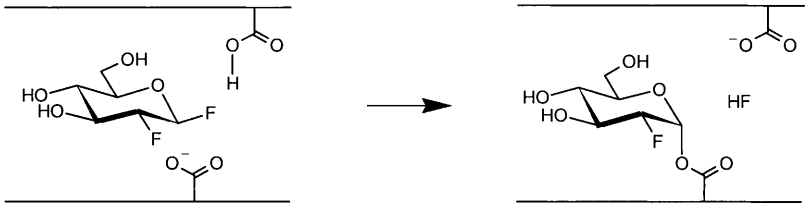


For an inverting glycoside hydrolase, general acid-base catalysis prevails, and there is a need for pK_a modulation of the two relevant carboxylic acid residues. The active site, in order to accommodate the substrate and a molecule of water, is some 10 Å across [13]. The salient points of a retaining glycoside hydrolase are the smaller active site (6 Å), the formation of a true glycosyl-enzyme intermediate through the agency of a catalytic nucleophile (aspartate or glutamate), and the stabilization of the transition states associated with steps *a* and *b* by the hydroxyl group present at C2 [13, 19].

The main (and obvious) difference between an inverting and a retaining hydrolase is the presence of a carboxylic acid residue capable of acting as a catalytic nucleophile. This nucleophile was first detected and, in some instances, identified by the use of affinity labels such as epoxyalkyl glycosides [9] – these molecules somewhat resemble the natural substrate for the enzyme and, additionally, are capable of (sometimes indiscriminate) attachment to a carboxylic residue within the enzyme [25, 26].



Much more impressive as labels of the catalytic nucleophile are the deoxy fluoro sugars recently developed by *Withers*. A 2-deoxy-2-fluoro sugar, suitably activated at the anomeric carbon with an excellent leaving group, is able to label the catalytic nucleophile specifically [27–29].

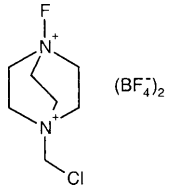


The glycosyl-enzyme intermediate is sufficiently long-lived (owing to the stabilizing effect of the fluorine atom) as to be detected and observed by nuclear magnetic resonance, mass spectrometric, or X-ray crystallographic techniques. Nowhere has this approach been put to better use than in the recent labelling of the catalytic nucleophile of hen egg-white lysozyme, long accepted as the paradigm for retaining glycoside hydrolases and now shown to operate in common with other members of the family [30].

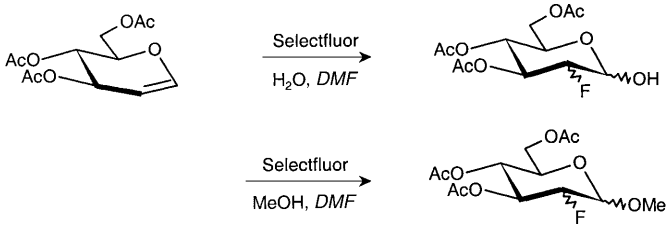
For somewhat related reasons, 5-fluoro sugars, also activated at the anomeric carbon, were found to be effective reagents for the labelling of the catalytic nucleophile [18, 31, 32]. The establishment of a mechanism for retaining glycoside hydrolases has led to effective procedures for glycoside synthesis through the action of transglycosylation and the invention of glycosynthases [33, 34].

We were attracted to the synthesis of 2-deoxy-2-fluoro sugars some years ago, but sorely missed the boat in terms of publication. However, we still feel that some comment is warranted here. Although there are various methods available for the synthesis of 2-deoxy-2-fluoro sugars, none are general, and some involve the use of

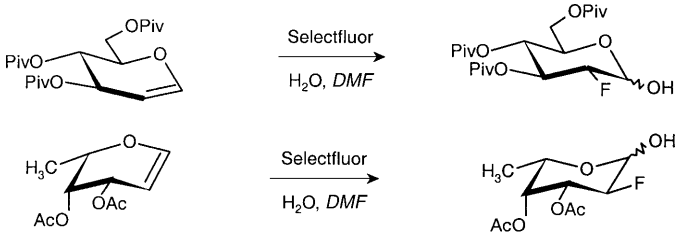
obnoxious reagents such as acetyl hypofluorite or elemental fluorine [35–37]. The whole situation changed with the advent of Selectfluor™, a fluoroammonium salt:



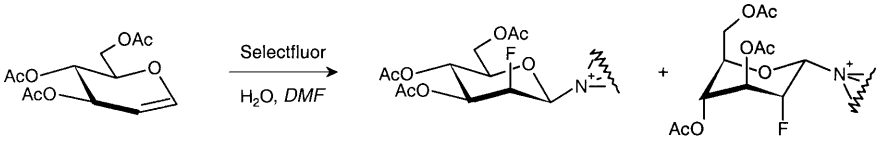
Wong was the first to report the addition of Selectfluor™ to glycals, ultimately to produce mixtures of free sugars and glycosides [38].



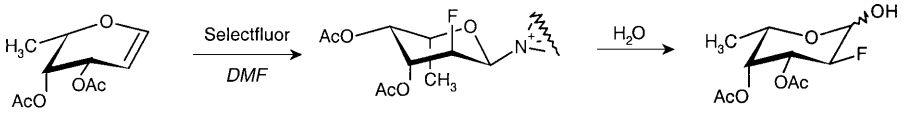
The stereoselectivity of the process can be improved by using bulky protecting groups on the glycal or through the use of a favourable substrate [39, 40].



Most of the mechanistic studies involving the addition of Selectfluor™ to glycals have been conducted by *Dax* [39, 41] and *Wong* [40] and, together with these results, we propose a rationalization for the process. The addition, by necessity, is a concerted process, and with a substrate such as tri-O-acetyl-*D*-glucal, two glycosylammonium salts result [39].



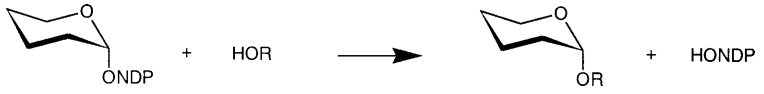
The 2-deoxy-2-fluoro-*D*-mannopyranosyl salt would be present in the $^4C_1$ conformation, and there is some evidence that the corresponding *D*-glucopyranosyl salt is also dominated by a reverse anomeric effect and found in the $^1C_4$ conformation [40]. A subsequent hydrolysis, for example, yields the observed mixtures of free sugars. With glycals such as tri-O-pivaloyl-*D*-glucal and tri-O-acetyl-*D*-galactal, the addition of Selectfluor™ occurs from the less hindered face; di-O-acetyl-*L*-fucal is an ideal substrate in several ways.



It is also worth mentioning that some glycal derivatives bearing substituents at C2 are found to be unreactive towards Selectfluor™. Tri-O-acetyl-1,2-dideoxy-2-fluoro-*D*-arabino-hex-1-enopyranose, when treated with Selectfluor™ for several days, failed to produce tri-O-acetyl-2-deoxy-2,2-difluoro-*D*-glucopyranose. A similar lack of reactivity has been observed with tri-O-acetyl-1,2-dideoxy-2-(N,N-diacetylamino)-*D*-arabino-hex-1-enopyranose [42].

Glycosyl transferases

The discussion here will be restricted to those enzymes that catalyze the transfer of a sugar residue from a nucleoside diphosphate to an acceptor molecule, usually another carbohydrate.

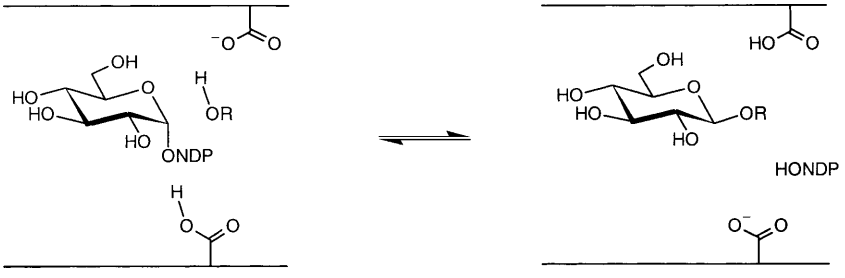


Investigations into the mechanism of action of such glycosyl transferases have sorely lagged behind those of the glycoside hydrolases. The reasons for this lag are numerous, including difficulties in access and supply and the fact that many of the glycosyl transferases are membrane associated – crystallization of the membrane-free enzyme has been, until recently, a difficult affair.

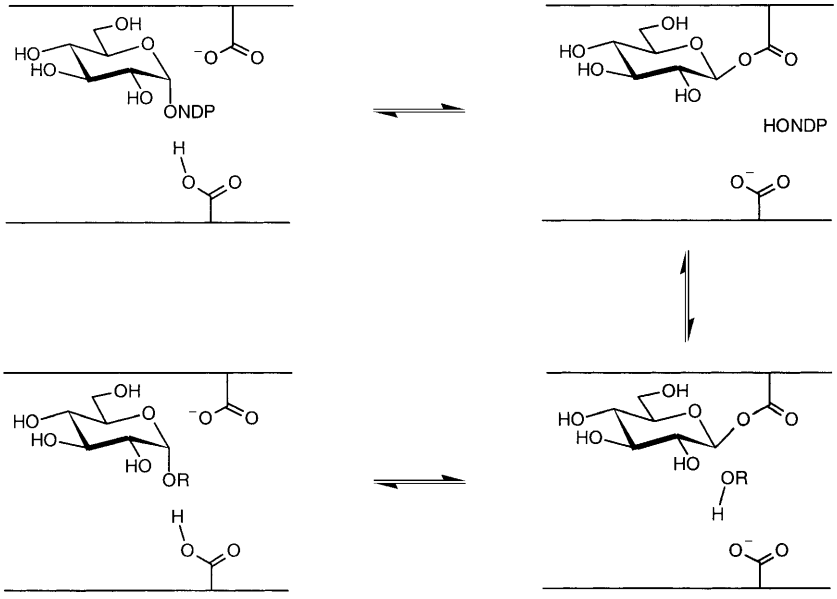
However, in spite of the above problems, glycosyl transferases have been classified into families (along lines similar to those for the glycoside hydrolases), even though the amino acid sequence homology for transferases can be virtually non-existent [43, 44]. The transferases are also classed as inverting or retaining, and this characteristic of the enzyme is easily assessed (in contrast to the glycoside hydrolases) by a simple inspection of the reaction products.

Perhaps one of the follies of carbohydrate chemists and biochemists alike in the late twentieth century will be the belief that the mechanistic principles established for glycoside hydrolases could be applied directly, almost without question, to glycosyl transferases.

Inversion:



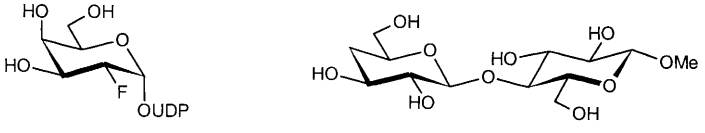
Retention:



The application of such mechanistic principles presents few problems for an inverting glycosyl transferase, with some (limited) experimental evidence to support the case [45–47]. However, a retaining enzyme again requires the involvement of a catalytic nucleophile, generally suggested to be a carboxylic acid (aspartate or glutamate) residue. Although the presence of a carboxylic catalytic nucleophile is suggested on the basis of results from non-specific labelling reagents (carbodiimides) and amino acid sequence alignments (for glycogen synthase) [48], there has never been reported the actual labelling and identification of such a residue for a glycosyl transferase.

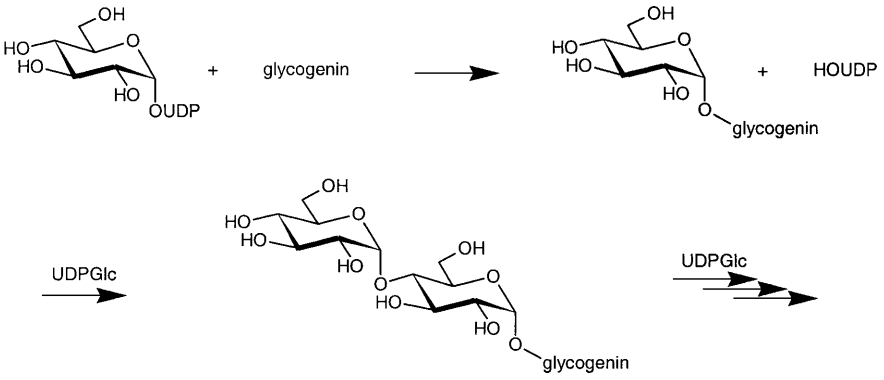
Until recently, no glycosyl transferase had been crystallized, so as to allow mechanistic investigations along the lines of the beautiful piece of work on glycoside hydrolases by *Davies* and *Withers* [49]. However, in the last decade, six glycosyl transferases, all inverting in their action, were crystallized and investigated by X-ray crystallography [50–55]. Several of these enzymes required the presence of a nucleoside diphosphate donor for a successful outcome [51–53]. Unfortunately, no great insights into the enzymes were revealed, particularly when the actual identity of some of the natural substrates remained unknown [52].

The seventh glycosyl transferase to be crystallized, however, was a retaining enzyme [56]. The successful experiment was conducted with a bacterial 1,4-α-galactosyl transferase (family 8), modified by point mutations, and crystallized in the presence of a deactivated donor and modified acceptor:



Surprisingly, the authors could find no evidence for the presence of any catalytic nucleophile. In the absence of a catalytic carboxylic acid (*Asp* and *Glu*) residue, several other contenders were considered, including a glutamine residue and the 6-OH of the acceptor molecule. In some desperation, a chemically unusual S_Ni mechanism was finally proposed [56].

The most striking points for us in this very important paper were the absence of an obvious catalytic nucleophile and the necessity for a metal ion (Mn^{2+}) for successful catalysis. We have been working for some time now on another family 8 glycosyl transferase, glycogenin, a self-glucosylating enzyme that initiates the synthesis of macro-glycogen [57, 58]:



Glycogenin is an α-retaining enzyme, and we have sought to label the catalytic nucleophile by incubation of the enzyme with *UDP*-2-deoxy-2-fluoro-α-*D*-glucose – the results have been, at best, equivocal. However, a notable fact is that glycogenin is also metal ion (Mn^{2+}) dependant [59]. If it were to eventuate that some retaining glycosyl transferases do not employ a nucleophile in their catalytic event, what then is their mechanism of action? For family 8 retaining transferases, one of the few conserved residues (*DXD*) is doubtlessly involved in the binding of the metal ion (Mn^{2+}). This leads us to suggest a general process for the catalytic action of (family 8) retaining glycosyl transferases:

(1) The initial event involves binding of the glycosyl donor into the active site, partly stabilized by an interaction between the pyrophosphate residue and the metal ion (Mn^{2+}).
(2) Next follows the binding of the acceptor, which triggers a change in the shape of the enzyme at the active site, ensuring an anhydrous environment [52, 53].
(3) This change in conformation also allows a hydrogen bond to form between the hydroxyl group of the acceptor and the glycosidic oxygen atom, causing cleavage of the glycosidic linkage and generating an oxacarbenium ion that may be stabilized through non-bonding interactions with adjacent amino acid residues.
(4) The oxacarbenium ion, through the agency of the aforementioned conformational change, is now adjacent to the acceptor and forms the new glycosidic linkage from the appropriate face.

(5) The enzyme undergoes another conformational change, first releasing the new glycoside, followed by the nucleoside diphosphate, thus presenting the enzyme in its native form ready to repeat the cycle.

In the case of the bacterial 1,4-α-galactosyl transferase mentioned previously, the oxacarbenium ion predicted in (3) is likely to be stabilized by a highly conserved glutamine residue (Fig. 1).

We feel that some other points are worth mentioning in the context of the above proposal. With retaining glycoside hydrolases, water is the natural acceptor molecule for the glycosyl-enzyme intermediate; when such hydrolases are used in an unnatural process, *i.e.* glycoside synthesis, the acceptor molecule is generally an alcohol, much less reactive than water and reflected in a slower process. However, the presence of a general acid/base residue in the hydrolase does allow the synthetic process to proceed [34]. If one now translates these observations to retaining glycosyl transferases, hydrolysis of the donor is the unnatural process, and the natural acceptor is an unreactive alcohol. Surely this situation requires a donor far more reactive than a conventional glycosyl-enzyme intermediate, especially when a general acid/base residue appears to be absent. We believe that the new hydrogen bond formed between the acceptor hydroxyl group and the glycosidic oxygen of the donor holds the key.

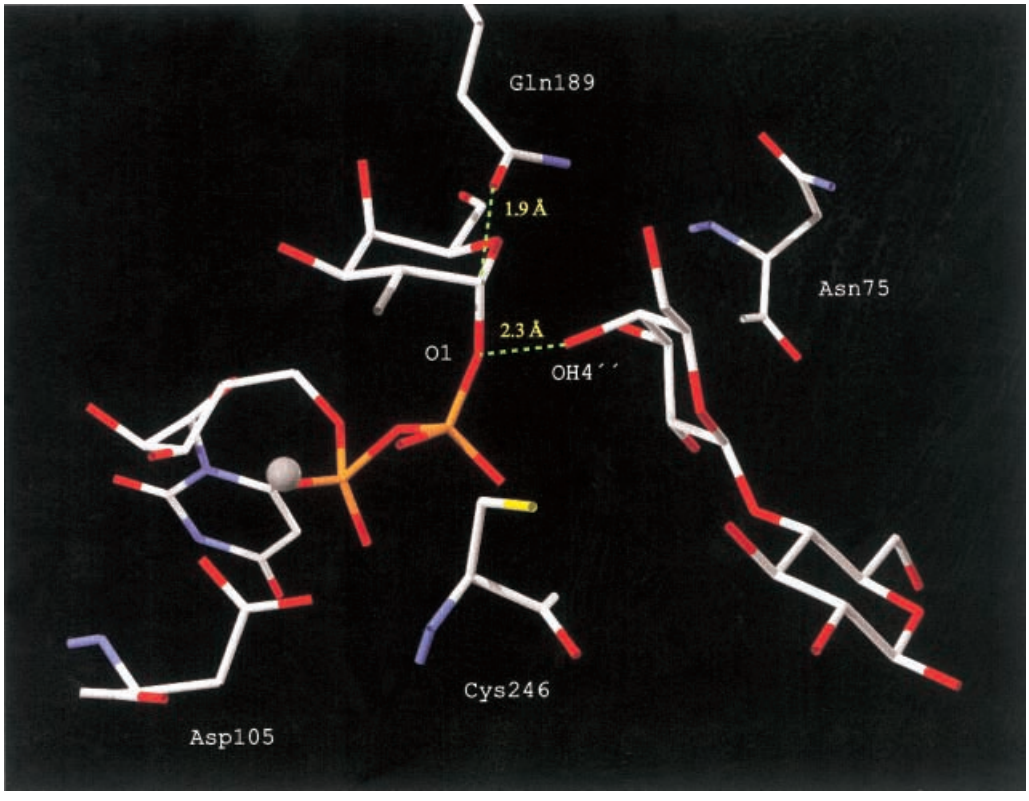


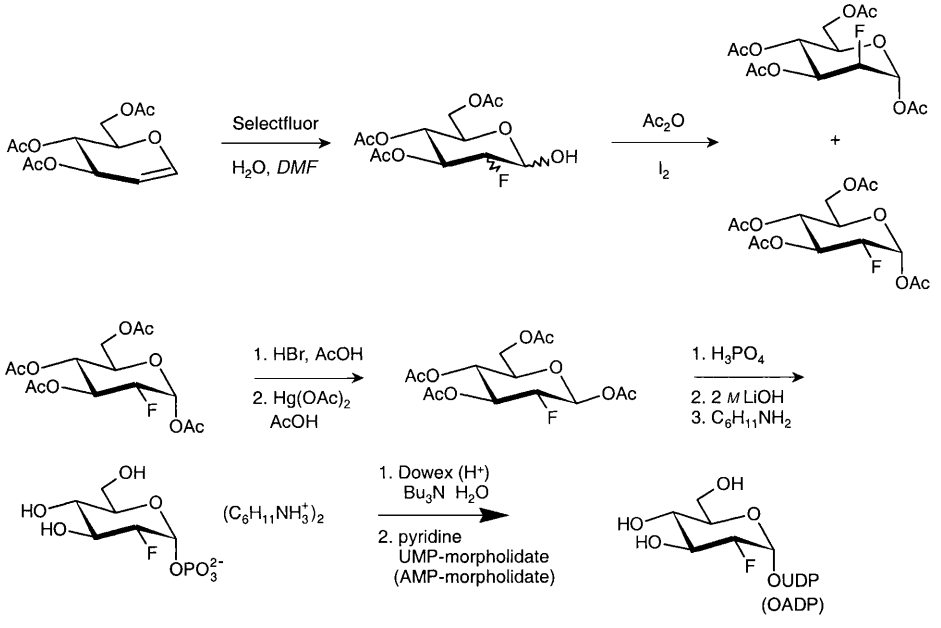
Fig. 1. Depiction of a modified active site arrangement for bacterial 1,4-α-galactosyl transferase (PDB: 1GA8) showing bond distances between C1 and Gln189 (rotated) as well as O1 and OH4″ (inserted)

Syntheses

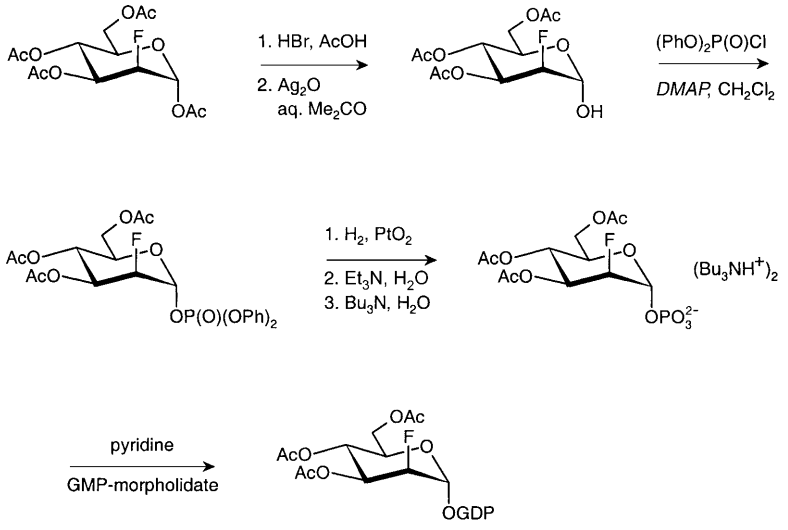
Our final words in this paper are concerned with the synthesis of the glycosyl donors (and related molecules) used in the above investigations: 2-deoxy-2-fluoro glycosyl nucleoside diphosphates.

For the synthesis of *NDP*-2-deoxy-2-fluoro-α-*D*-glucose derivatives, tri-O-acetyl-*D*-glucal (Scheme 1) was treated with Selectfluor^{TM} to give, after selective acetylation and purification, tetra-O-acetyl-2-deoxy-2-fluoro-α-*D*-glucopyranose. Inversion of configuration at the anomeric centre *via* the agency of the bromide gave the β-*D*-anomer, ready for a successful *MacDonald* phosphorylation to provide the α-*D*-glucosyl phosphate isolated as the di(cyclohexylammonium) salt [60]. This salt was then converted into the desired nucleoside derivative (*UDP* or *ADP*), employing a modified morpholidate coupling procedure described by *Wittmann* and *Wong* [61]. It should be noted that this transformation could be conveniently monitored by HPLC, and was found to proceed well using the tri-*n*-butylammonium salt of the α-*D*-glucopyranosyl phosphate.

Interestingly, the major product from the Selectfluor^{TM} reaction on tri-O-acetyl-*D*-glucal, tetra-O-acetyl-2-deoxy-2-fluoro-α-*D*-mannopyranose (Scheme 1), is inert to the MacDonald phosphorylation procedure – a result of the double deactivation at the anomeric carbon by an axial acetyl group and an adjacent fluorine atom [42]. Instead, tetra-O-acetyl-2-deoxy-2-fluoro-α-*D*-mannopyranose was converted *via* the bromide exclusively to the α-*D*-anomer of the hemiacetal (Scheme 2). Treatment of the hemiacetal with diphenyl chlorophosphate then gave the diphenyl phosphate that was convertible, after catalytic hydrogenolysis, into the desired *GDP*-2-deoxy-2-fluoro-α-*D*-mannopyranose.



Scheme 1



Scheme 2

Conclusions

There is no doubt that two great papers have appeared in the first year of the new century – the first, on the mechanism of action of hen egg-white lysozyme, all but closes the chapter on retaining glycoside hydrolases; the second, on the search for a catalytic nucleophile in a retaining glycosyl transferase, answers some questions and asks of others.

Much work remains to be done, and an upcoming report on the X-ray structure determination of glycogenin [62], another retaining glycosyl transferase of family 8, is sure to continue the debate[1].

Experimental

Experimental details have been given previously [64]. Bio-Gel P-2 Gel (Fine) was purchased from Bio-Rad Laboratories.

Tetra-O-acetyl-2-deoxy-2-fluoro-α-D-glucopyranose and *Tetra-O-acetyl-2-deoxy-2-fluoro-α-D-mannopyranose* ($C_{14}H_{19}FO_9$)

Freshly powdered Selectfluor^{TM} (5.7 g, 16 mmol) was added to a solution of tri-O-acetyl-D-glucal (2.2 g, 8.0 mmol) in H_2O/*DMF* (100 cm^3, 1:1), and the mixture was left to stir at 40° (8 h). The resultant solution was concentrated *in vacuo* and then subjected to a usual workup (EtOAc) to give a colourless syrup. Ac_2O (10 cm^3) and I_2 (300 mg) were then added to this syrup, and the mixture was

[1] During the preparation of this manuscript, a report on a retaining 1,3-α-galactosyl transferase by *Gastinel* and coworkers suggested the detection of a glycosyl-enzyme intermediate [63]; without access to the primary crystallographic data (electron density maps), the claim appears somewhat equivocal.

allowed to stand at room temperature (3 h). The mixture was then concentrated *in vacuo*, subjected to a usual workup (EtOAc), and purified using flash chromatography (30% EtOAc/petroleum ether).

First to elute was the D-gluco derivative (748 mg, 28%); m.p.: 75–77°C (Ref. [65]: 78°C), $[\alpha]_D = +147°$ (Ref. [65]: $+148°$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.01$, 2.15, 2.22, 2.27 (4s, 12H, CH_3), 3.96–4.02 (m, H5,6), 4.23 (dd, $J_{5,6} = 5.1$, $J_{6,6} = 11.5$ Hz, H6), 4.61 (ddd, $J_{2,F} = 47.6$, $J_{1,2} = 4.7$, $J_{2,3} = 9.5$ Hz, H2), 5.02 (t, $J_{3,4} \approx J_{4,5} = 9.6$ Hz, H4), 5.48 (dt, $J_{3,F} = 13.1$ Hz, H3), 6.35 (d, H1) ppm.

Next to elute was the D-manno derivative (1.34 g, 50%); m.p.: 68–69°C (Ref. [35]: 66–68°C), $[\alpha]_D = +65°$ (Ref. [35]: $+60°$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.12$, 2.15, 2.22, 2.27 (4s, 12H, CH_3), 4.13 (ddd, $J_{4,5} = 9.6$, $J_{5,6} = 5.1$, 3.1 Hz, H5), 4.15 (dd, $J_{6,6} = 11.9$ Hz, H6), 6.28 (dd, H6), 4.73 (dt, $J_{2,F} = 48.9$, $J_{1,2} \approx J_{2,3} = 2.1$ Hz, H2), 5.23 (ddd, $J_{3,F} = 23.0$, $J_{3,4} = 9.7$ Hz, H3), 5.41 (dd, H4), 6.23 ($J_{1,F} = 8.5$ Hz, H1) ppm.

Di(cyclohexylammonium) 2-deoxy-2-fluoro-α-D-glucopyranosyl phosphate ($C_{18}H_{38}FN_2O_8P$)

Tetra-O-acetyl-2-deoxy-2-fluoro-β-D-glucopyranose [60] (1.15 g) was stirred with molten anhydrous H_3PO_4 (4.0 g) under vacuum (1 mm Hg) at 60°C overnight. The mixture was allowed to cool slightly (but not enough to allow crystallization) and then treated with *THF* (5 cm^3) to give a dark solution. Aqueous 2 *M* LiOH (80 cm^3) was then added to the solution with cooling, and the mixture was left to stir at room temperature overnight. The mixture was filtered and the filtrate passed through a column of Dowex 50W-X8 (H^+) resin into an excess of cyclohexylamine in H_2O. Concentration of the eluent *in vacuo* and subsequent lyophilization gave di(cyclohexylammonium) 2-deoxy-2-fluoro-α-D-glucopyranosyl phosphate as a pale yellow powder (1.32 g, 87%). $[\alpha]_D = +46°$ (Ref. [60]: $+48.6°$); the ^{1}H NMR spectrum was consistent with that reported [60].

Tri-O-acetyl-2-deoxy-2-fluoro-α-D-mannopyranose ($C_{12}H_{17}FO_8$)

HBr in AcOH (2 cm^3, 30% w/w) was added to tetra-O-acetyl-2-deoxy-2-fluoro-α-D-mannopyranose (1.80 g, 5.4 mmol), and the mixture was left to stir at room temperature (4 h). Usual workup (EtOAc) gave the α-D-mannosyl bromide as a colourless oil (2.1 g) which was used without further purification.

Ag_2CO_3 (1.14 g, 4.13 mmol) was added to a solution of the above bromide (1.4 g, 3.8 mmol) in aqueous acetone (20 cm^3, 1:10), and the mixture left to stir (2 h). The mixture was then filtered through a plug of silica and concentrated *in vacuo*. Usual workup (EtOAc) of the residue followed by flash chromatography (40% EtOAc/petroleum ether) gave the title hemiacetal as a colourless oil (1.15 g, 98%). The spectroscopic data (^{1}H and ^{13}C NMR) were consistent with those reported [39].

Diphenyl (tri-O-acetyl-2-deoxy-2-fluoro-α-D-mannopyranosyl) phosphate ($C_{24}H_{26}FO_{11}P$)

A solution of tri-O-acetyl-2-deoxy-2-fluoro-α-D-mannopyranose (0.76 g, 2.5 mmol) and *DMAP* (0.55 g) in CH_2Cl_2 (20 cm^3) was cooled to −10°C. Diphenyl chlorophosphate (1.0 cm^3, 4.8 mmol) was added dropwise, and the solution was allowed to stir (4 h) under gradual warming to 10°C. The solution was diluted with CH_2Cl_2 (20 cm^3) and washed sequentially with cold H_2O, 0.5 *M* HCl, and saturated $NaHCO_3$ solution, dried ($MgSO_4$), and concentrated *in vacuo*. Flash chromatography (20% EtOAc/petroleum ether) of the residue gave the title phosphate as a colourless oil (1.01 g, 76%).

$[\alpha]_D = +46°$; found: C 53.5, H 4.7; calcd.: C 53.3, H 4.8; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.95$, 2.00, 2.09 (3s, 9H, CH_3), 3.88 (dd, $J_{5,6} = 1.4$, $J_{6,6} = 12.5$ Hz, H6), 3.98–4.04 (m, H5), 4.13 (dd, $J_{5,6} = 4.2$ Hz, H6), 4.74 (dddd, $J_{2,F} = 48.9$, $J_{1,2} \approx J_{2,3} = 2.2$, $J_{2,P} = 0.5$ Hz, H2), 5.25 (ddd, $J_{3,F} = 27.2$, $J_{3,4} = 10.3$ Hz, H3), 5.37 (ddd, $J_{4,F} = 1.2$, $J_{4,5} = 10.2$ Hz, H4), 5.99 (ddd, $J_{1,F}$, $J_{1,P} = 7.1$, 8.5 Hz, H1), 7.15–7.39 (m, 10H, Ph) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 20.47$, 20.55 (3C, CH_3), 61.19 (C6),

64.59, 70.67 (C4,5), 68.75 (d, $J_{3,F} = 17$ Hz, C3), 85.98 (dd, $J_{2,F} = 182$, $J_{2,P} = 12$ Hz, C2), 95.19 (dd, $J_{1,F}$, $J_{1,P} = 6.0$, 32 Hz, Cl), 119.90–150.11 (12C, Ph) ppm.

2-Deoxy-2-fluoro-α-D-mannopyranosyl-bis(tributylammonium) phosphate ($C_{30}H_{66}FN_2O_8P$)

PtO_2 (40 mg) was added to a solution of diphenyl 3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-α-D-mannosyl phosphate (400 mg) in MeOH (15 cm^3), and the mixture was stirred vigorously under an atmosphere of H_2 at room temperature (2 d). The mixture was filtered, diluted with H_2O (2 cm^3) and Et_3N (2 cm^3), and the solution was left to stand at 4°C overnight. The mixture was concentrated *in vacuo*, taken up in H_2O (5 cm^3), and passed through a column of Dowex 50 W-X8 (H^+) resin into a vigorously stirred mixture of H_2O and excess tributylamine. Concentration *in vacuo* and subsequent lyophilization gave the title phosphate as a white powder (416 mg).

^{1}H NMR (300 MHz, D_2O): $\delta = 0.72$ (t, 18H, CH_3), 1.10–1.21, 1.35–1.51 (2m, 24H, CH_2), 2.85–2.98 (m, 12H, CH_2N), 3.48–3.69 (m, 4H, H4,5,6), 3.76 (ddd, $J_{3,F} = 30.8$, $J_{2,3} = 2.5$, $J_{3,4} = 9.6$ Hz, H3), 4.59 (bddd, $J_{2,F} = 48.7$, $J_{1,2} = 2.4$ Hz, H2), 5.38 (ddd, $J_{1,P}$, $J_{1,F} = 6.0$, 8.1 Hz, H1) ppm; ^{13}C NMR (75.5 MHz, D_2O): $\delta = 24.05$ (6C, CH_3), 30.52, 36.66 (12C, CH_2), 63.89 (6C, CH_2N), 71.57 (C6), 77.62, 84.66 (C4,5), 80.40 (d, $J_{3,F} = 17.3$ Hz, C3), 100.97 (dd, $J_{2,F} = 174.4$, $J_{2,P} = 9.2$ Hz, C2), 104.18 (dd, $J_{1,F} = 4.5$, $J_{1,P} = 30.6$ Hz, C1) ppm.

Uridine 5′-(trihydrogendiphosphate), P′-(2-deoxy-2-fluoro-α-D-glucopyranosyl) ester ($C_{15}H_{23}FN_2O_{16}P_2$)

Di(cyclohexylammonium) 2-deoxy-2-fluoro-α-D-glucopyranosyl phosphate (370 mg, 803 μmol) was dissolved in H_2O (5 cm^3) and passed through a column of Dowex 50 W-X8 (H^+) resin into a vigorously stirred mixture of H_2O and excess tributylamine. The eluent was then concentrated *in vacuo*, coevaporated with MeOH (2 × 10 cm^3), and dried under high vacuum (2 d) to give the *bis*(tributylammonium) salt (410 mg).

^{1}H NMR (300 MHz, D_2O): $\delta = 0.72$ (t, 18H, CH_3), 1.07–1.16, 1.25–1.37 (2m, 24H, CH_2), 2.89–2.93 (m, 12H, CH_2N), 3.29 (t, $J_{3,4} \approx J_{4,5} = 9.9$ Hz, H4), 3.56 (dd, $J_{5,6} = 4.8$, $J_{6,6} = 12.3$ Hz, H6), 3.63–3.69 (m, H5,6), 3.80 (dt, $J_{2,3} = 9.6$, $J_{3,F} = 13.1$ Hz, H3), 4.21 (dddd, $J_{1,2}$, $J_{1,P} = 2.3$, 3.7, $J_{2,F} = 49.4$ Hz, H2), 6.13 (dd, $J_{1,P} = 6.6$ Hz, H1) ppm; ^{13}C NMR (125.8 MHz, D_2O): $\delta = 15.14$ (6C, CH_3), 21.61, 27.51 (12C, CH_2), 54.96 (6C, CH_2N), 62.52 (C6), 71.15 (d, $J_{4,F} = 8.3$ Hz, C4), 73.37 (d, $J_{3,F} = 17.3$ Hz, C3), 74.78 (C5), 91.89 (dd, $J_{2,P} = 6.8$, $J_{2,F} = 187$ Hz, C2), 94.25 (dd, $J_{1,P} = 4.6$, $J_{1,F} = 22.3$ Hz, C1) ppm.

The above *bis*(tributylammonium) salt (410 mg) and 4-morpholine-N,N′-dicyclohexylcarboxamidinium uridine 5′-monophosphomorpholidate (662 mg, 964 μmol) were coevaporated with dry pyridine (3 × 2 cm^3). 1*H*-Tetrazole (112 mg, 1.61 mmol) and dry pyridine (4 cm^3) were added, and the solution was then concentrated to half of its original volume and left to stir at room temperature (2 d). The solution was then diluted with H_2O (30 cm^3), washed with Et_2O, and concentrated *in vacuo*. The residue was purified on a Bio-Gel P-2 column (2.5 × 95 cm) eluting with 250 m*M* NH_4HCO_3 solution and lyophilized to give the title compound as a white powder (320 mg, 68%).

^{1}H NMR (500 MHz, D_2O): $\delta = 3.34$ (t, $J_{3'',4''} \approx J_{4'',5''} = 9.8$ Hz, H4″), 3.59 (dd, $J_{5'',6''} = 4.5$, $J_{6'',6''} = 12.5$ Hz, H6″), 3.67 (dd, $J_{5'',6'} = 2.2$ Hz, H6″), 3.73 (ddd, H5″), 3.85 (dt, $J_{2'',3''} = 9.7$, $J_{3'',F} = 12.8$ Hz, H3″), 3.97–4.10, 4.16–4.22, 4.27–4.32 (3m, 6H, H2′,3′,4′,5′,2″), 5.59 (dd, $J_{1'',2''} = 3.5$, $J_{1'',F} = 7.3$ Hz, H1″), 5.77–5.82 (m, H5,1′), 7.78 (d, $J_{5,6} = 8.1$ Hz, H6) ppm.

Adenosine 5′-(trihydrogendiphosphate), P′-(2-deoxy-2-fluoro-α-D-glucopyranosyl) ester ($C_{16}H_{24}FN_5O_{14}P_2$)

Following the above procedure, di(cyclohexylammonium) 2-deoxy-2-fluoro-α-D-glucopyranosyl phosphate (285 mg, 618 μmol) was treated with 4-morpholine-N,N′-dicyclohexylcarboxamidinium

adenosine 5′-monophosphomorpholidate (527 mg, 742 μmol) and 1*H*-tetrazole (88 mg, 1.2 mmol) in dry pyridine (2 cm^3) to give the title compound as a hygroscopic white solid (257 mg, 71%).

^{1}H NMR (300 MHz, D_2O): $\delta = 3.35$ (dd, $J_{3'',4''} = 11.1$, $J_{4'',5''} = 10.9$ Hz, H4″), 3.56–3.78 (m, 3H, H5″,6″), 3.87 (ddd, $J_{2'',3''} = 11.0$, $J_{3'',F} = 50.9$ Hz, H3″), 4.06–4.41 (m, 6H, H2′,3′,4′,5′,2″), 5.51 (dd, $J_{1'',2''} = 4.1$, $J_{1'',F} = 7.2$ Hz, H1″), 5.80 (d, $J_{1',2'} = 4.5$ Hz, H1′), 7.86, 8.00 (2s, H2,8) ppm.

Guanosine 5′-(trihydrogendiphosphate), P′-(2-deoxy-2-fluoro-α-D-mannopyranosyl) ester ($C_{16}H_{24}FN_5O_{15}P_2$)

Following the previous procedure, *bis*(tributylammonium) 2-deoxy-2-fluoro-α-D-mannopyranosyl phosphate (320 mg, 694 μmol) was treated with 4-morpholine-N,N′-dicyclohexylcarboxamidinium guanosine 5′-monophosphomorpholidate (605 mg, 833 μmol) and 1*H*-tetrazole (98 mg, 1.4 mmol) in dry pyridine (2 cm^3) to give the title compound as a white powder (281 mg, 65%). Spectroscopic data (^{1}H and ^{13}C NMR) were consistent with those reported [66].

Acknowledgments

The authors would like to thank the Australian Research Council for financial assistance, as well as Air Products and Chemicals, Inc. for a kind donation of Selectfluor™ and financial assistance.

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Received October 16, 2001. Accepted November 7, 2001