DEOXY AND DEOXYFLUORO GLYCOSIDES

AS MECHANISTIC PROBES

OF *Escherichia coli (lacZ)* β-GALACTOSIDASE

By

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B.Sc., The University of Victoria, 1988

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Chemistry)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

May 1991

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ABSTRACT

The mechanism of galactoside hydrolysis by *Escherichia coli (lacZ)* β-galactosidase was probed with a series of 2',4' dinitrophenyl deoxy and deoxyfluoro glycopyranosides. A two-step mechanism has been proposed previously for this process:

1) cleavage of the glycosidic bond and formation of a covalent galactosyl-enzyme intermediate ("galactosylation");

2) hydrolysis of the intermediate to give free enzyme and galactose ("degalactyosylation").

A series of deoxy and deoxyfluoro analogs of 2',4'-dinitrophenyl-β-D-galactopyranoside was prepared for this study. The 2-deoxyfluoro derivative was found to be an effective mechanism-based inactivator of *E. coli (lacZ)* β-galactosidase. This compound thus joins a class of 2-deoxy-2-fluoro glycosides which inactivate glycosidases of this type by the accumulation of a stable glycosyl-enzyme intermediate. The active site-directed nature of this inhibitor was shown by protection against inactivation by a competitive ligand and the near 1:1 stoichiometry of dinitrophenolate release with enzyme inactivation. Furthermore, when freed from excess inactivator the 2-deoxy-2-fluorogalactosyl enzyme intermediate turned over slowly in buffer ($t_{1/2}$ = 69 h at 25°C) and exhibited enhanced rates of reactivation in the presence of the acceptors methanol or glucose, providing strong evidence that the intermediate is catalytically competent.

The other deoxy and deoxyfluoro analogs synthesized were substrates for the enzyme although the rates of enzymic hydrolysis were two to four orders of magnitude slower than have been measured for the parent compound. These large rate reductions are thought to result primarily from the loss of important transition state binding interactions due to the substitution of a hydrogen or a fluorine for a hydroxyl at a given position on the galactopyranose ring. These results strongly suggest that much of the catalytic power of

the enzyme is derived from non-covalent interactions between the enzyme active site and the galactopyranose ring of the substrate.

A linear free energy relationship (r = 0.80) was shown to exist between the logarithm of k_{cat}/K_m for the enzyme-catalyzed reaction and the logarithm of the first order rate constant for the spontaneous hydrolysis of the same series of deoxy and deoxyfluoro glycopyranosides. Since the spontaneous process has considerable oxocarbonium ion character at the transition state, these data suggest that the enzymic mechanism involves a similar electron deficient transition state.

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Carbohydrate Structure

βGalPNP	4'-nitrophenyl β-D-galactopyranoside
βGalONP	2'-nitrophenyl β-D-galactopyranoside
βGalDNP	2',4'-dinitrophenyl β-D-galactopyranoside
βGluDNP	2',4'-dinitrophenyl β-D-glucopyranoside
βGalSiPr	isopropylthio-β-D-galactopyranoside
2FβGalF	2-deoxy-2-fluoro-β-D-galactopyranosyl fluoride
2FβGluF	2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride
2FβGluDNP	2',4'-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside
2FβGalDNP	2',4'-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside
3FβGalDNP	2',4'-dinitrophenyl 3-deoxy-3-fluoro-β-D-galactopyranoside
4FβGalDNP	2',4'-dinitrophenyl 4-deoxy-4-fluoro-β-D-galactopyranoside
6FβGalDNP	2',4'-dinitrophenyl 6-deoxy-6-fluoro-β-D-galactopyranoside
2dβGalDNP	2',4'-dinitrophenyl 2-deoxy-β-D-*lyxo*-hexopyranoside
3dβGalDNP	2',4'-dinitrophenyl 3-deoxy-β-D-*xylo*-hexopyranoside
4dβGalDNP	2',4'-dinitrophenyl 4-deoxy-β-D-*xylo*-hexopyranoside
6dβGalDNP	2',4'-dinitrophenyl 6-deoxy-β-D-galactopyranoside

Other Abbreviations

Ac	acetyl
Bz	benzoyl
Ph	phenyl
Ts	*p*-toluenesulphonyl

Physical Constants

k	Boltzmann constant
h	Planck's constant
T	Temperature (K)
R	Gas constant

ACKNOWLEDGEMENT

INTRODUCTION

1. Glycosidases and glycoside hydrolysis

Glycosidases, or glycoside hydrolases, constitute a large family of enzymes which catalyze the hydrolysis of glycosidic linkages by cleavage of the C-O bond between the sugar and the aglycone (R, which may be another sugar or an aryl or alkyl group) as shown in Figure 1.
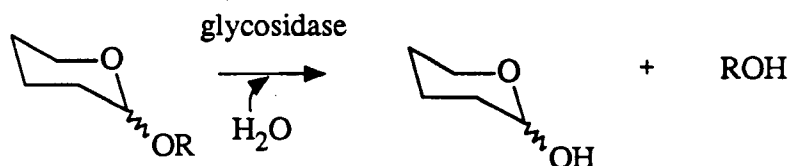
Figure 1. Reaction catalyzed by a glycosidase.

Glycosidases may be subdivided according to:

1) *Anomeric configuration of the substrate.* A β-glycosidase will only catalyze the hydrolysis of β-glycosides while an α-glycosidase is specific for α-glycosides.

2) *Stereochemical outcome of the reaction.* Glycosidases are termed "inverting" or "retaining" depending on the relative anomeric configurations of the substrate hydrolyzed and the product initially released from the enzyme. Thus a "retaining" β-glycosidase will catalyze the hydrolysis of β-glycosides to give β-glycose products.

3) *Glycone specificity*. A given glycosidase usually exhibits maximum activity with a specific type of glycoside, e.g. a galactosidase will be most active against galactosides, in which the C(4) hydroxyl of the sugar is axial, though the hydrolysis of other glycosides may be catalyzed as well (albeit less efficiently).

2. The catalytic mechanism of a "retaining" glycosidase

A general mechanism for "retaining" glycosidases, which are the focus of this study, was proposed by D.E. Koshland in 1953 (shown in Figure 2 for a β-galactosidase).[1] The main features of this mechanism, which are consistent with a large amount of experimental data that has accumulated since that time, are summarized below:

1) The reaction proceeds *via* formation of a covalent glycosyl-enzyme intermediate.

2) Both the formation and breakdown of this intermediate proceed *via* oxocarbonium ion-like transition states.

3) General acid catalysis may assist aglycone departure though this is not essential for all glycosidases with all substrates.

4) Non-covalent interactions between enzyme and substrate are responsible for most of the rate acceleration.

These features will be discussed in some detail below, particularly in relation to the *lac(Z)* β-galactosidase from *Escherichia coli*.

3. *Escherichia coli (lacZ)* β-galactosidase

This large (464 kDa) tetrameric protein, encoded by the *Z* gene of the *lac* operon of *E. coli*, enables the organism to utilize lactose as an energy source. The enzyme exhibits a
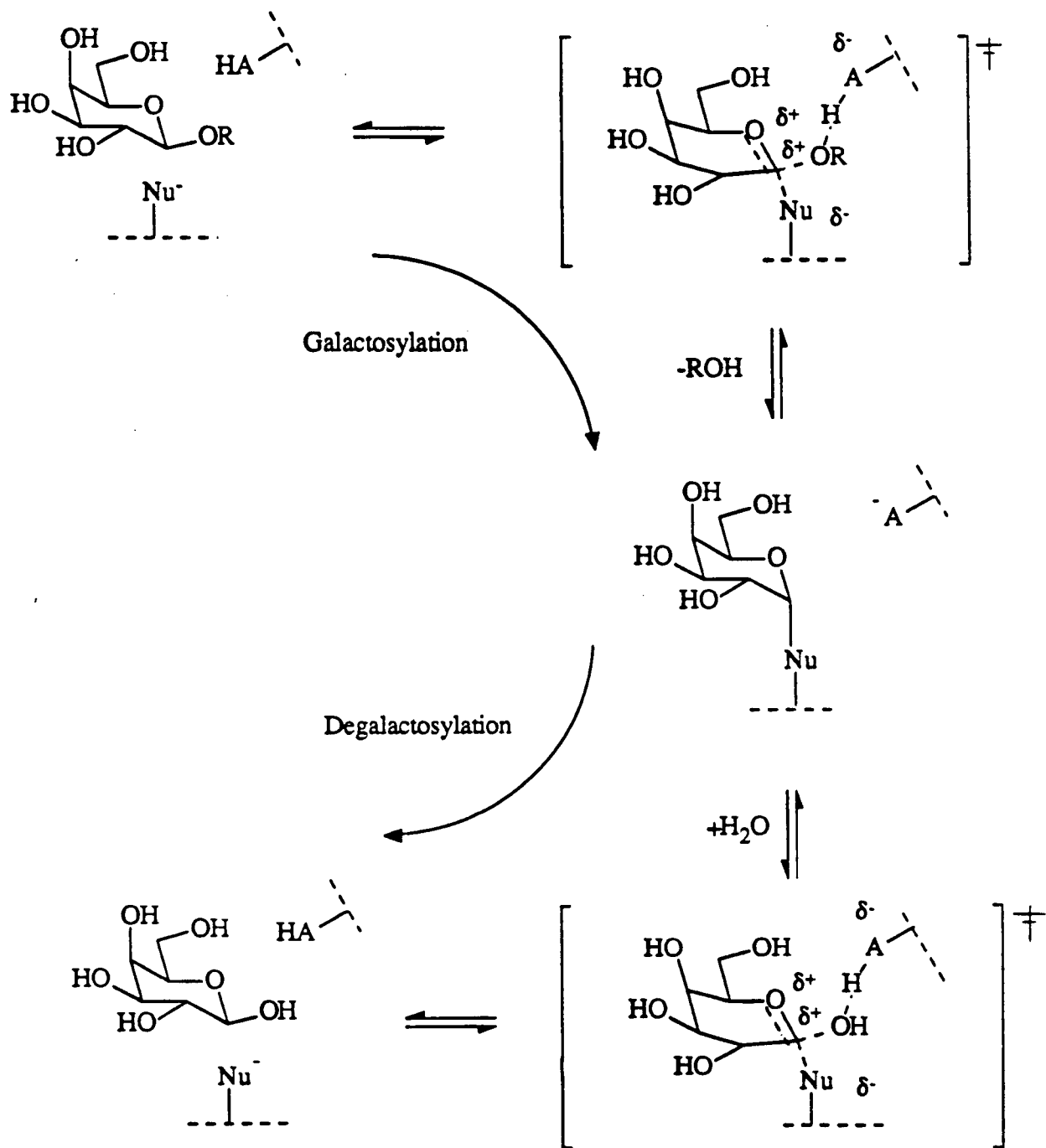
Figure 2. Proposed mechanism for hydrolysis of β-galactosides by a "retaining" β-galactosidase.[1]

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somewhat liberal specificity for the aglycone and consequently may hydrolyze a wide variety of aryl β-galactosides with varying degrees of efficiency. In addition to C-O bonds, C-S, C-F and C-N bonds in appropriate substrates may also be cleaved by the enzyme.[2] The enzyme requires Na^+ and Mg^{2+} for full activity with O-galactosides.[2] Although first crystallized in 1961[3], elucidation of the tertiary structure of the protein by X-ray crystallography has not been achieved. Consequently, a detailed picture of enzyme-substrate interactions in the active site has not yet emerged although a great deal of mechanistic information is available.

3.1 Covalent glycosyl enzyme intermediate

Stokes and Wilson[4] reported a constant partioning ratio between the hydrolysis and methanolysis products formed in the β-galactosidase-catalyzed solvolysis of a series of aryl β-galactosides in mixtures of methanol and buffer (Figure 3 and Table 1).

$$E + S \rightleftharpoons ES \longrightarrow E\text{-}P \begin{array}{c} \xrightarrow{H_2O} P\text{-}OH \\ \xrightarrow{CH_3OH} P\text{-}OCH_3 \end{array}$$
$$\downarrow HX$$
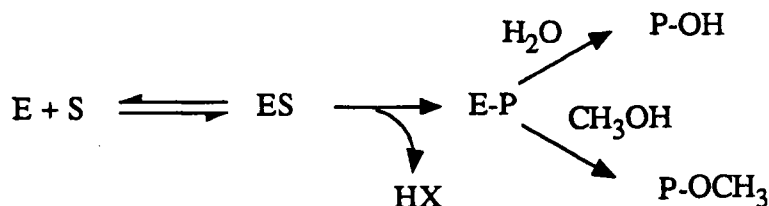
Figure 3. Kinetic model for partitioning of products between water and methanol. S is the substrate, ES the non-covalent Michaelis complex, E-P the glycosyl enzyme intermediate, HX the aglycone, P-OH the hydrolysis product and P-OCH3 the methanolysis product.

Though the rates of enzyme-catalyzed solvolysis vary widely, the constant ratio of products suggests involvement of an enzymic intermediate common to all substrates. However, little information about the nature of the intermediate can be inferred from the data.

Aglycone	Methanolysis/ hydrolysis	V_m (relative)
2'-nitrophenyl	1.97	1.0
3'-chlorophenyl	2.08	0.5
4'-nitrophenyl	1.99	0.2
phenyl	1.94	0.1
4'-bromophenyl	2.02	0.02

Table 1. Partitioning of products between methanol and water in β-galactosidase-catalyzed solvolysis[a]

[a] Data from Stokes, T.M. and Wilson, I.B. (1972) *Biochemistry 11*, 1061 .

The existence of an intimate ion pair, as has been proposed for lysozyme[5], is a possibility. However, given the instability of glycosyl cations (whose lifetime is estimated to be on the order of 10^{-10} seconds in water[6]), it seems unlikely that even a very favourable microenvironment in the active site could allow the leaving group to diffuse out and an acceptor to diffuse in and react before collapse to a covalent species occurred. This suggests that stabilization of the intermediate is occurring covalently *via* a nucleophilic residue on the enzyme. Herrchen and Legler[7] have proposed that a possible candidate for such an enzymic nucleophile is an active site glutamate (Glu-461), identified with the affinity label conduritol C epoxide, which presumably reacts with the enzymic carboxylate group as shown to irreversibly inactivate the enzyme (Figure 4).
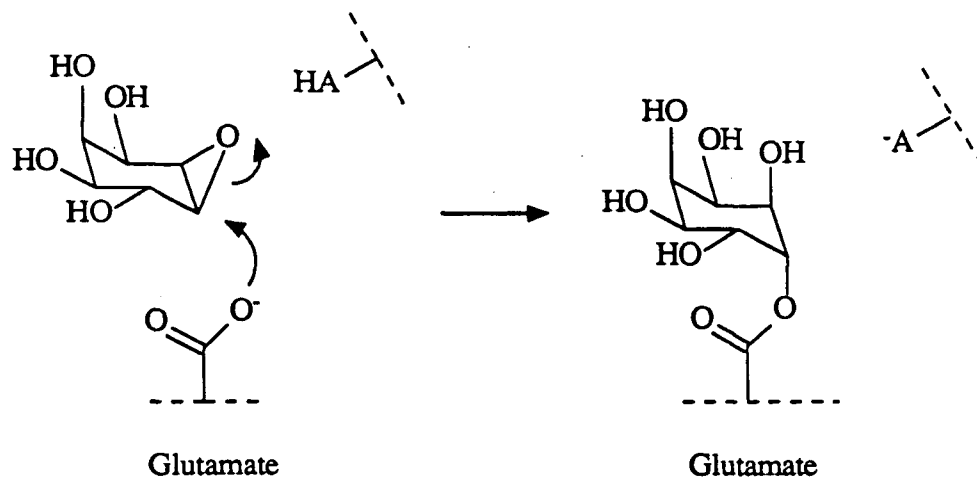
Figure 4. Affinity labeling of an active site glutamate by conduritol C epoxide.

Site-directed mutagenesis studies[8], in which the glutamate was replaced by a glutamine, produced a mutant enzyme which exhibited some 10^{-2} of the activity of the wild type. This residual activity with an uncharged glutamine residue in the mutant enzyme suggests that Glu-461, if it is indeed the enzymic nucleophile, acts not electrostatically but covalently to stabilize the developing positive charge at the anomeric centre.[9]

The covalent nature of a glycosyl-enzyme intermediate in another glycosidase, *Agrobacterium* pABG5 β-glucosidase, has been unequivocally demonstrated.[10] In the reaction of 2',4'-dintrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside with β-glucosidase, the fluorine atom at C(2) of the pyranose ring slows both the glucosylation and deglucosylation steps, while the highly activated leaving group accelerates glucosylation sufficiently to trap the 2-deoxy-2-fluoroglucosyl intermediate and inactivate the enzyme (Figure 5). Evidence for the covalent nature of the intermediate and an α-linkage to the enzyme were obtained by ^{19}F nuclear magnetic resonance spectroscopy. The 2-deoxy-2-fluoroglucosyl enzyme is extremely stable, with a half-life of greater than five hundred hours at 30°C in buffer, but upon incubation with a suitable acceptor the enzyme may be reactivated and a disaccharide product isolated,[11] demonstrating that the intermediate is

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catalytically competent. The identity of the nucleophilic residue, a glutamic acid, was also determined by inactivating the enzyme with a radiolabeled inhibitor, digesting the protein and comparing the sequence of the labeled peptide to the known amino acid sequence of the protein.[11]
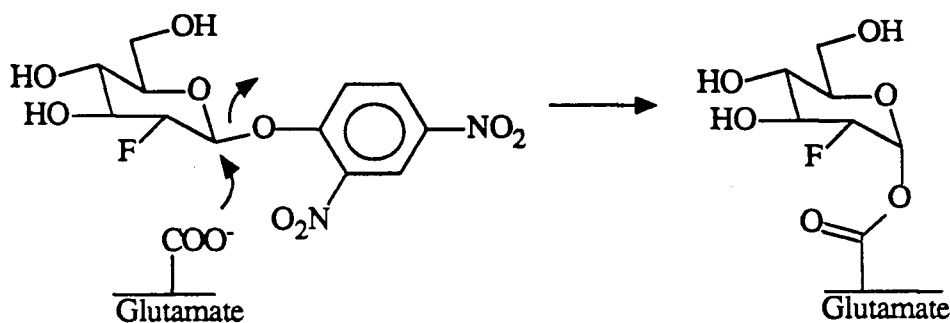
Figure 5. Covalent inactivation of Agrobacterium pABG5 *β-glucosidase by 2',4'-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside.*

3.2 Oxocarbonium ion-like transition states

Much of the evidence for oxocarbonium ion-like transition states associated with both the galactosylation and degalactosylation steps of β-galactosidase-catalyzed hydrolysis is derived from kinetic isotope effect studies. By selection of appropriate aglycones it is possible to obtain substrates for which either the galactosylation step or the degalactosylation step is rate-limiting. The isotope effect on each step may then be determined. The β-galactosidase-catalyzed hydrolysis of C(1)-deuterated β-galactosyl pyridinium salts[12] (Figure 6) exhibited α-secondary deuterium isotope effects of 1.15 to 1.20, indicating substantial sp^2 character at the transition state for formation of the galactosyl-enzyme since galactosylation is rate-determining for these substrates.

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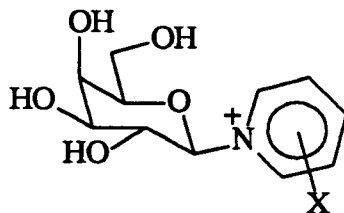
Figure 6. Galactosyl pyridinium salt.

Similar large isotope effects are observed when degalactosylation is rate-determining. In the enzyme-catalyzed hydrolysis of 2',4'-dinitrophenyl β-D-galactopyranoside, an α-deuterium isotope effect of 1.25 was reported[13], suggesting increased sp^2 character at the transition state for hydrolysis of an sp^3-hybridized covalent intermediate.

β-Glycosidases are strongly inhibited by compounds which resemble an oxocarbonium ion in conformation or charge (Figure 7).
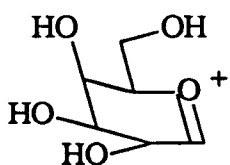
a galactosyl cation
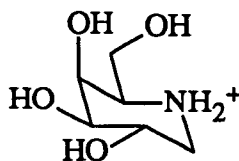
deoxygalactonojirimycin
Ki = 0.0125 mM
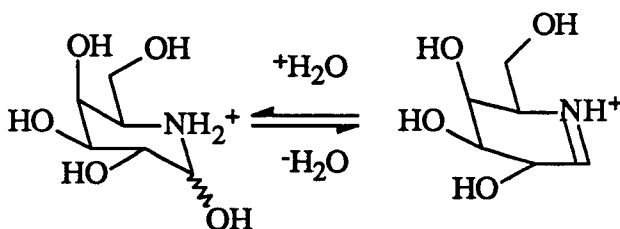
galactonojirimycin
Ki = 0.000045 mM

Figure 7. Comparison of structural and electronic similarity of a galactosyl cation with deoxygalactonojirimycin and galactonojirimycin. The former is isoelectronic with a galactosyl cation; the dehydrated form of the latter is isosteric as well. K_i values shown are for E. coli (lacZ) β-galactosidase.[14]

These transition state analogs bind more tightly to the enzyme than normal, uncharged glycosides that adopt a chair conformation, as would be expected for inhibitors which mimic the positively charged half-chair transition state. The consequent extremely low K_i values for these inhibitors provide supportive evidence for the existence of oxocarbonium ion-like transition states.

3.3 Acid catalysis

The role played by an acid catalyst in the catalytic mechanism of *E. coli (lacZ)* β-galactosidase is presently unclear. However, the efficient hydrolysis of β-galactosyl pyridinium salts (Figure 6), which cannot accept a proton, indicates that acid catalysis is not essential to catalysis by β-galactosidase.[12] However, Mg^{2+}, which binds to the enzyme in the ratio of one Mg^{2+} per protomer[15], contributes significantly to catalysis with some substrates. Removal of Mg^{2+} from the enzyme has little effect on the kinetic parameters of β-galactosyl pyridinium salts while the k_{cat} values of aryl O-galactosides decrease 4- to 150-fold upon removal of Mg^{2+}.[16]

It was originally suggested by Sinnott that Mg^{2+} is required for optimal placement of an acid catalytic group[13], accounting for the decreased rates with O-galactosides and the much smaller effect on β-galactosyl pyridinium salts when Mg^{2+} was removed from the enzyme. A likely candidate for this acid catalytic group is tyrosine-503. Replacement of Tyr-503 by phenylalanine *via* site-directed mutagenesis yields a mutant enzyme with k_{cat} values for nitrophenyl galactosides some 10^{-3} that of the wild type enzyme.[17] Further evidence for a strategically-placed acid catalytic residue has been reported by Lehmann and Schlesselmann.[18] The stereochemistry of the product from hydration of the octenitol in Figure 8 indicates that the substrate was protonated from the β face.
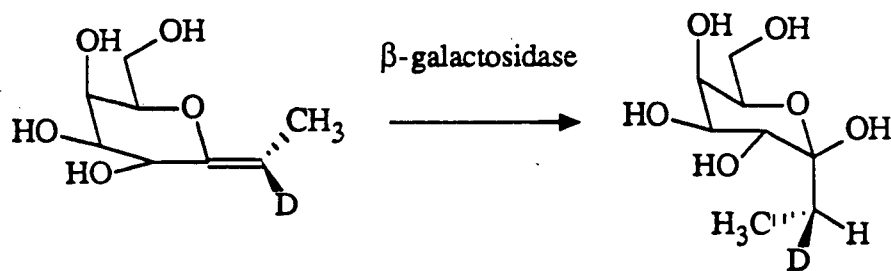
Figure 8. Stereochemistry of E. coli (lacZ) *β-galactosidase-catalyzed hydration of an octenitol derivative.*

Alternatively, it has been more recently proposed (again by Sinnott[9]) that Mg^{2+}, when it can co-ordinate to the aglycone, may act as a simple electrophilic catalyst. The site-directed mutagenesis studies of Edwards et al.[19] show that Glu-461 is important to Mg^{2+} binding and may also be co-ordinated to Mg^{2+}. The results of a previous nuclear magnetic resonance study with Mn^{+2}-enzyme which suggested that a divalent metal ion bound too far from the aglycone to effect electrophilic catalysis now appear to be misleading.[20,9] The stereochemical results of Lehmann and Schlesselman may be explained by addition of a Mg^{2+}-coordinated water. The notion that the enzyme primarily uses electrophilic rather than general acid catalysis to assist aglycone departure is consistent with observations that Mg^{2+} contributes significantly to catalysis even when the aglycone is an acidic phenol which is deprotonated at pH 7. Proton transfer from an acid catalytic residue of higher pK_a, such as the simple phenol of tyrosine, would at best be only partial.

3.4 Non-covalent interactions

Although stabilization of a developing oxocarbonium ion by an enzymic nucleophile and electrophilic or acid catalysis may contribute to the rate accelerations achieved by β-galactosidase, it is unlikely that these factors alone account for the full catalytic power of the enzyme. It is now a well-accepted principle of enzymology that enzymes achieve such

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spectacular rate enhancements by stabilization of the transition states for the steps involved in an enzyme-catalyzed reaction, a concept first suggested by Haldane[21] in 1930 and later elaborated by Pauling.[22] This stabilization of the transition state and consequent decrease in activation energy may be directly attributed to the increased binding energy realized upon tighter binding of enzyme and transition state (see Reference 23 for a detailed discussion). That enzyme complementarity to the transition state rather than the ground state of the substrate leads to an enhancement in rate is illustrated below for a simple enzyme-catalyzed reaction. When the ground state is stabilized by realizing maximum binding energy on binding the substrate (as in Figure 9 A)), the activation energy represented by $\Delta G_c^{\ddagger}$ is increased and the rate is therefore reduced. When the transition state is stabilized by realizing maximum binding energy on binding the transition state (as in Figure 9 B)), $\Delta G_c^{\ddagger}$ is decreased and the rate is enhanced, while the overall activation energy from free enzyme plus free substrate to the transition state (represented by $\Delta G_T^{\ddagger}$) is decreased by ΔG_R. Thus by preferentially stabilizing the transition state through specific binding interactions, an enzyme is able to utilize binding energy for catalysis.

Ligand/protein interactions are the basis for the specificity of enzyme/substrate binding and antigen recognition by immunoglobulins. Such interactions likely take the form of hydrophobic interactions and hydrogen bonding. Given the large number of hydroxyls associated with most carbohydrates, it is likely that hydrogen bonding plays an important role in binding and catalysis by enzymes with carbohydrate substrates. Indeed, the detailed picture of hydrogen bonding interactions between protein and ligand in arabinose binding protein derived from X-ray crystallographic data[24] is an indication of the importance of hydrogen bonding in other carbohydrate-binding proteins.

Elegant work by Fersht[25] has shown that tyrosyl t-RNA synthetase utilizes a complex network of hydrogen bonds to achieve ground state specificity and to stabilize the transition state. Using site-directed mutagenesis, mutant enzymes were prepared in
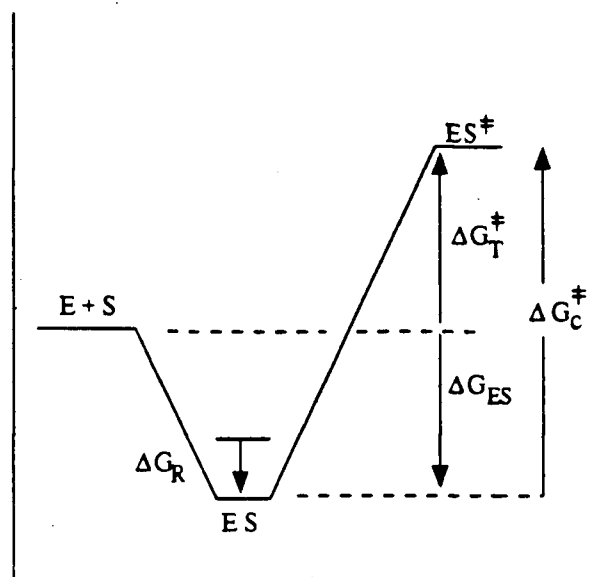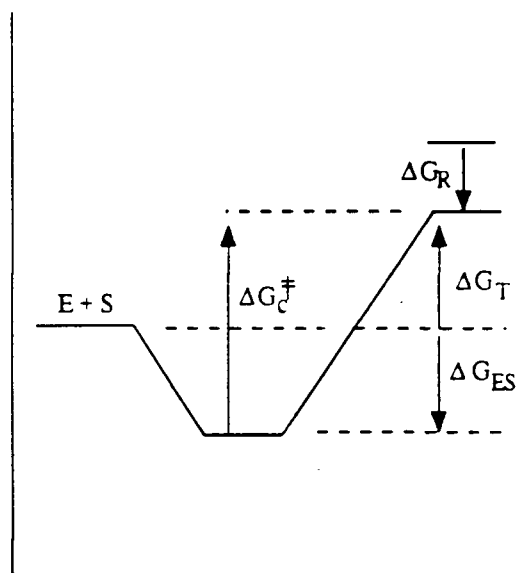
Figure 9. Free energy diagram of an enzymic reaction involving: A) Maximum enzyme-substrate complementarity at the ground state: B) Maximum enzyme-substrate complementarity at the transition state.

which specific enzyme/substrate interactions (identified from the known X-ray crystallographic structure) had been deleted. Comparison of kinetic data for these mutants with that for the wild type enzyme yielded information about the importance of hydrogen bonding at each residue to catalysis. Fersht proposed that such interactions account for virtually all of the rate acceleration provided by this particular enzyme since no other residues which can participate catalytically (e.g. as nucleophiles, acid catalysts etc.) have been identified.

4. Modified sugars as transition state probes

The complementary approach to that employed by Fersht in probing hydrogen bonding structure at the transition state involves systematic modification of the substrate rather than the enzyme. This more classical approach is appropriate when X-ray crystallographic data is not available, as is the case for *E. coli (lacZ)* β-galactosidase, and should provide essentially the same information. Specifically, systematic structural modifications of the glycopyranose ring within a series of substrates can provide information about: 1) interactions between the glycone and the enzyme at the transition state; and 2) the electronic nature of the transition state.

Changes in the glycone must be sterically conservative to avoid steric clashes in the active site. The substituents in the modified substrates must therefore be no larger than a hydroxyl group. Two substituents which fulfill this criterion are hydrogen and fluorine (Table 2).

Deoxy and deoxyfluoro sugars have been shown to be useful probes of hydrogen bonding interactions between an enzyme and substrate in the work of Street et al. with rabbit muscle glycogen phosphorylase.[27,28] Note that a hydrogen bond consists of two paired functional groups. One group, termed the "donor", is covalently bonded to a

Group	Bond Length (A)	Van der Waals Radius (A)	Total (A)
C-H	1.09	1.20	2.29
C-F	1.39	1.35	2.74
C-O(H)	1.43	1.40	2.83
C-OH	1.43	2.10	3.53

Table 2. Size comparison of C-H, C-F, C-O(H) and C-OH groups.[26]

hydrogen atom which interacts with the lone pair electrons of the "acceptor" atom (which can be F, O or N). Thus a hydroxyl group on an unmodified sugar can both accept and donate a hydrogen bond while the hydrogen of a deoxy sugar cannot participate in any hydrogen bonding interaction. There is evidence from X-ray crystallographic data[29] that fluorine may participate in C-F---H-N hydrogen bonds although the resultant interactions are probably weak since fluorine's lone pair electrons are held tightly by the nucleus. Evidence for C-F---H-O hydrogen bonds in a fluorinated carboxylic acid has also been reported.[30] A deoxyfluoro sugar may therefore be capable of acting as a (weak) hydrogen bond acceptor when an appropriate donor group is available but cannot possibly perform a donor role itself.

All hydrogen bonding interactions between the enzyme and the hydroxyl at a particular position on the normal substrate will be abolished in the corresponding deoxy analog. The enzyme should show a significant loss in binding energy for this analog that will be reflected in increased activation energies for the catalytic steps. The deoxyfluoro analog would be expected to bind poorly if the substrate hydroxyl at that position acted as a hydrogen bond donor to a group on the enzyme. If, on the other hand, the sugar hydroxyl

normally accepts a hydrogen bond from the enzyme then the deoxyfluoro sugar may still bind appreciably. In this way, information about hydrogen bond polarity may be obtained.

Substitution of a hydroxyl by fluorine or hydrogen also has significant effects on the electronic nature of the transition state. The rate of uncatalyzed spontaneous glycoside hydrolysis is decreased by electron-withdrawing substituents on the glycopyranose ring and accelerated by electron-rich groups because of the primarily electronic effects of these substituents on the developing positive charge at the transition state. Similar effects observed for the enzyme-catalyzed hydrolyses would, for example, constitute evidence that the enzymic transition states also have substantial oxocarbonium ion character.

5. Aims of this study

It was proposed to probe the role of non-covalent binding interactions in transition state stabilization by *E. coli (lacZ)* β-galactosidase with a series of deoxy and deoxyfluoro glycopyranosides. In this way, information about the the importance to catalysis of enzyme-substrate interactions at each position on the galactopyranose ring and possibly hydrogen bond polarities may be obtained. The choice of a highly activated aromatic leaving group, 2,4-dinitrophenolate, as the aglycone permits convenient determination of enzymic reaction rates by measuring the rate of aglycone release spectrophotometrically. In addition, an analog incorporating a fluorine at C(2) and a good leaving group as the aglycone may permit trapping of a covalent 2-deoxy-2-fluorogalactosyl enzyme intermediate. The presence of electronegative fluorine adjacent to a developing positive charge at the anomeric centre would be expected to slow both the rates of galactosylation and degalactosylation while a good leaving group can accelerate only the galactosylation step.

The substituent effects of these modified substrates may also provide insight into the electronic nature of the enzymic transition state. Comparison of the kinetic parameters

for the enzymic hydrolysis of these substrates with the spontaneous hydrolysis rates may provide evidence of similarity in electronic structure between the enzymic and non-enzymic transition states. In this way, the contribution of electronic factors to enzymic transition state structure may be investigated.

Chapter II

RESULTS AND DISCUSSION

1. Syntheses of fluorinated analogs of 2',4'-dinitrophenyl β-D-galactopyranoside

All of the monofluorinated analogs of galactose have been synthesized by other workers previously.[31,32,33,34] The intermediates synthesized in this work were characterized by comparison of their ^1H and ^{19}F nmr spectra and/or melting points, where possible, with literature values. The novel deoxyfluoro 2',4'-dinitrophenyl β-D-galactopyranosides were characterized by ^1H and ^{19}F nmr, melting point and elemental analysis. Excluding the characteristic resonances of the dinitrophenyl moiety, the signal farthest downfield in all cases is the anomeric proton. This appears as a doublet, except in the case of the 2-deoxyfluoro compound in which it also shows a 4 Hz coupling to fluorine. The magnitude of $J_{1,2}$ (~ 7.5 Hz) indicates a β-anomeric configuration in all cases. The magnitude of the ^1H-^1H coupling constants observed in the acetylated deoxyfluoro 2',4'-dinitrophenyl β-D-galactopyranosides indicates a *trans*-diaxial relationship between H-2 and H-3, suggesting the galactopyranose ring adopts the expected $^4C_1$ conformation.

1.1 Fluorination at the 2 position

The route employed for the preparation of 2',4'-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside 7 is shown in Figure 10.
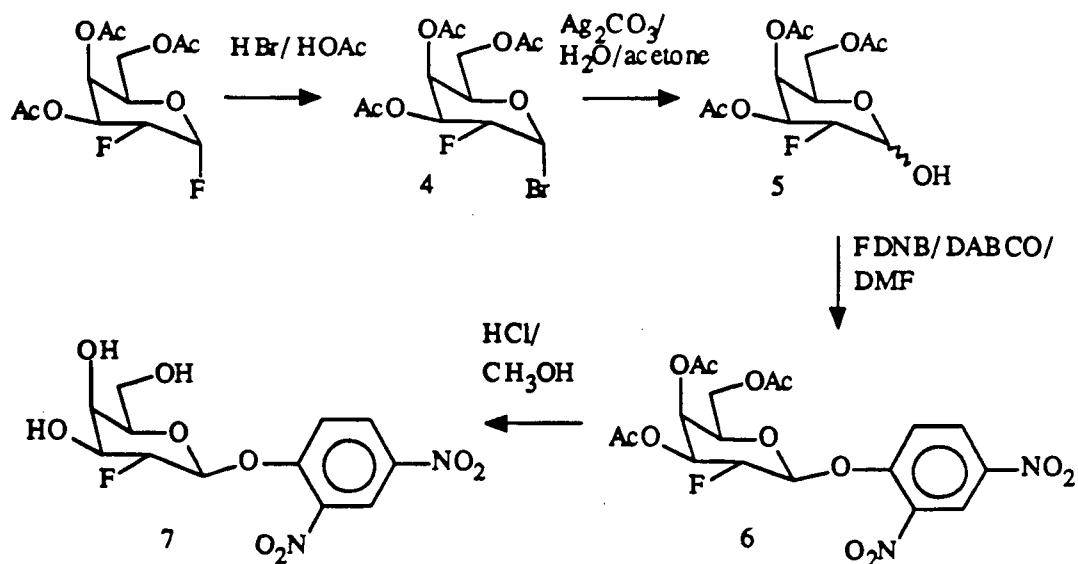
Figure 10. Synthetic scheme for the preparation of 2',4'-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside 7.

1,3,4,6-Tetra-O-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl bromide **4** was prepared in 92% yield from 1,3,4,6-tetra-O-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl fluoride (obtained from Dr. D. Dolphin, Department of Chemistry, U.B.C.) with 45% hydrobromic acid in acetic acid.[35] The bromide **4** was converted to the alcohol **5** in 67% yield by reaction with silver carbonate in a mixture of acetone and water.[36] The galactopyranoside **6** was prepared according to the procedure of van Boom et al.[37] as a 3:1 mixture of β and α anomers by reaction with 1-fluoro-2,4-dinitrobenzene (FDNB) and 1,4 diazabicyclo[2.2.2]octane (DABCO) in N,N-dimethylformamide. The β anomer of **6** was selectively crystallized from ethyl acetate by addition of hexanes and isolated in 42% yield. Subsequent treatment of **6** with hydrogen chloride in methanol[38] gave the deacetylated galactopyranoside **7** in 51% yield.

1.2 Fluorination at the 3 position

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The synthetic route used for the preparation of the 3-deoxyfluoro analogs of galactose and 2',4' dinitrophenyl β-D-galactopyranoside is shown in Figure 11. Oxidation of 1,2:5,6 di-O-isopropylidene-α-D-glucofuranose[39] by acetic anhydride in dimethyl sulphoxide (DMSO) has been reported to give the ulose hydrate **8**.[40] However, in our hands, the major product isolated from this reaction was the 3-thiomethoxy methyl ether **48**

H_3C   O
   X
H_3C   O
                    O
H_3C—S   O   ''O
                        CH_3
                    O   CH_3
          48
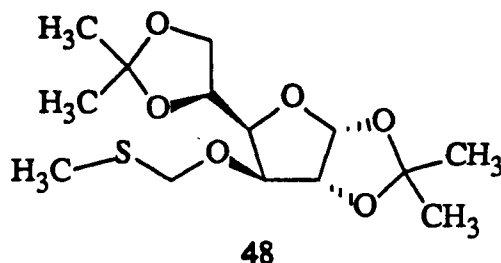
identified by comparison of its ^{1}H nmr spectrum with that reported in the literature.[41] The desired product **8** was instead synthesized in 50% yield by oxidation of 1,2:5,6-di-O-isopropylidene glucofuranose with phosphorus pentoxide in DMSO.[42]

Preparation of the enol acetate **9** was carried out in 47% yield with a mixture of acetic anhydride and pyridine and was subsequently reduced stereoselectively using sodium borohydride in methanol to give the partially protected gulofuranose **10** in 20% yield.[43] This compound was fluorinated with diethylaminosulphur trifluoride (DAST) according to Kovac and Glaudemans[44] to give the protected galactofuranose derivative **11** in 75% yield. The isopropylidene groups were removed by mild acid hydrolysis using Amberlite IR-120(H^{+}) cation exchange resin in aqueous ethanol[32] to give the free deoxyfluoro sugar **12** as a colourless oil in 73% yield. This was acetylated in 97% yield with acetic anhydride and pyridine to give a mixture of the tetra-O-acetates **13** which were selectively deacetylated at the 1 position with 2-aminoethanol[45] for 30 h at room temperature to give a mixture of the tri-O-acetates **14** in 58% yield. The galactopyranoside **15** was prepared in 47% yield by reaction of **14** with FDNB and DABCO[37] giving exclusively the β anomer.

Subsequent treatment of **15** with hydrogen chloride in methanol[38] gave the deacetylated galactopyranoside **16** in 37% yield.
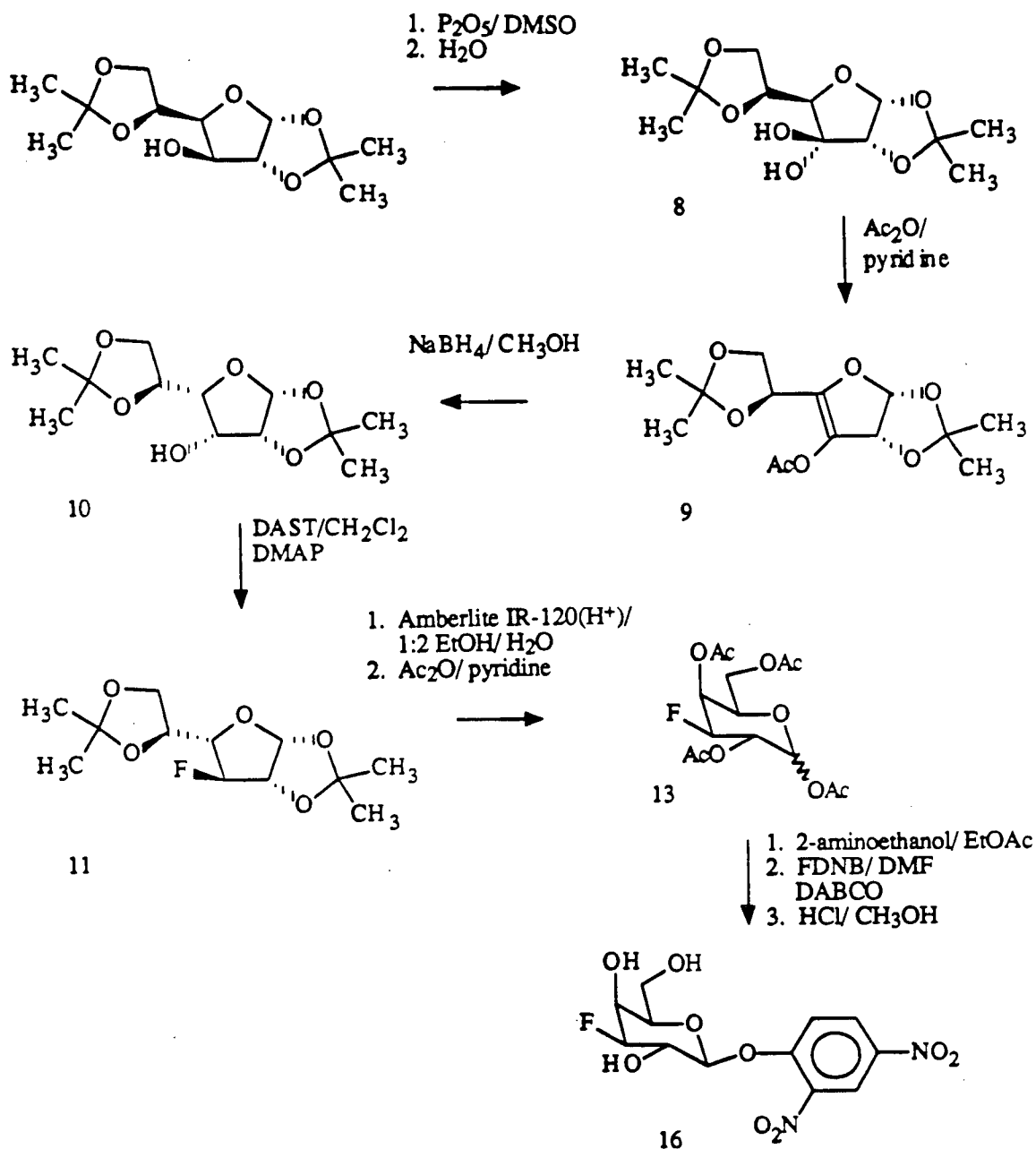
Figure 11. *Synthetic scheme for the preparation of 2',4' dinitrophenyl 3-deoxy-3-fluoro-β-D-galactopyranoside 16.*

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1.3 Fluorination at the 4 position

The deacetylated galactopyranoside **21** was obtained from Dr. A. Becalski, Department of Chemistry, U.B.C. using the procedures indicated in Figure 12. 4-Deoxy-4-fluoro-D-galactopyranose **19** was also synthesized separately in this study.
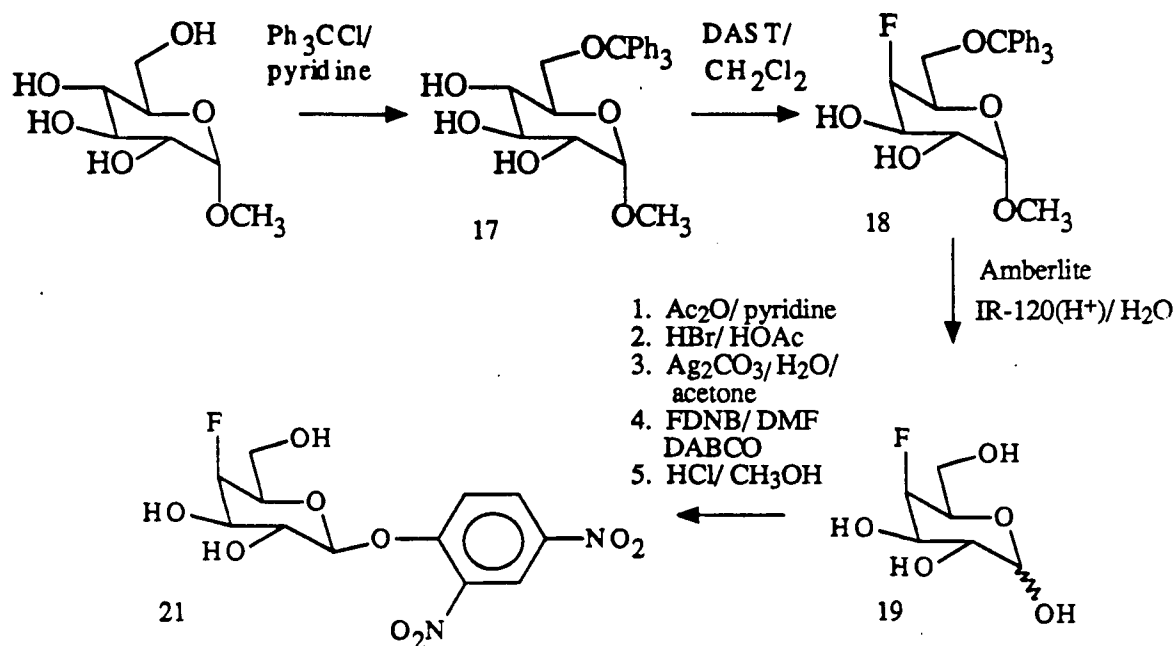
Figure 12 . Synthetic scheme for the preparation of 2',4' dinitrophenyl 4-deoxy-4-fluoro-β-D-galactopyranoside 21.

Methyl α-D-glucopyranoside was treated with triphenylmethyl chloride in pyridine to give the trityl ether **17** in 77% yield. This was fluorinated with DAST[46] to give the 4-deoxyfluoro galactopyranoside **18** in 21% yield and subsequently converted to the free deoxyfluoro sugar **19** in 89% yield by refluxing with Amberlite IR-120(H⁺) cation exchange resin in water for 3 days.

The route employed for the preparation of the 6-deoxyfluoro analogs of galactose and 2',4' dinitrophenyl β-D-galactopyranoside is shown in Figure 13.
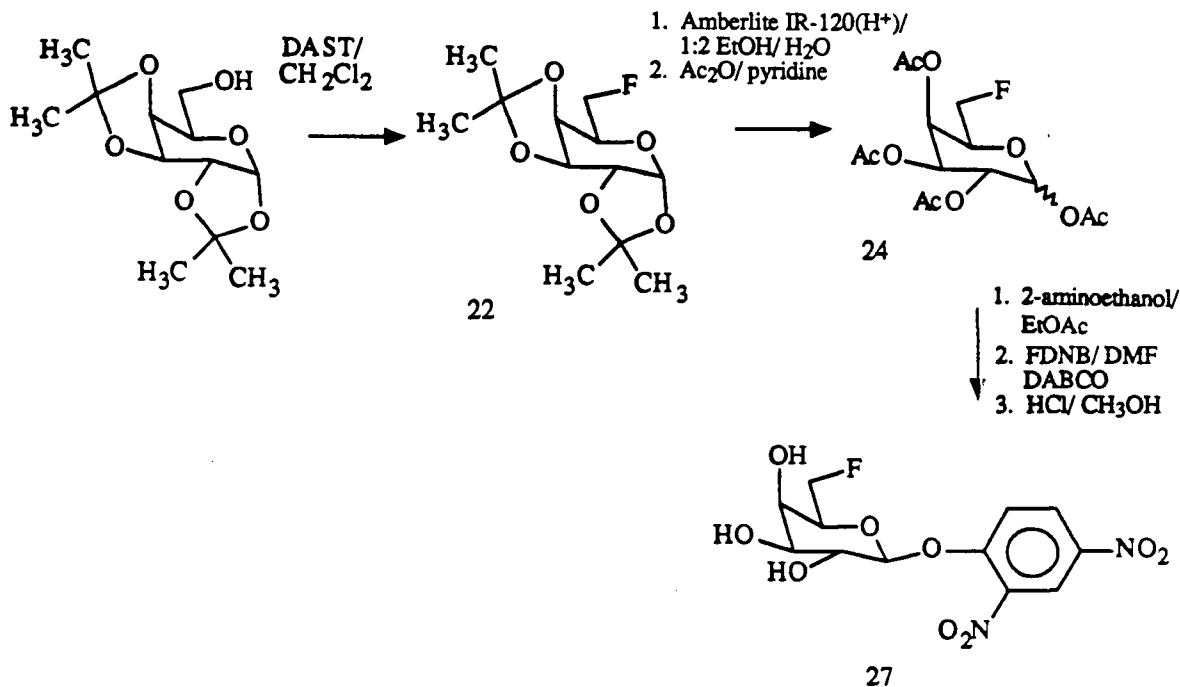
Figure 13. Synthetic scheme for the preparation of 2',4' dinitrophenyl 6-deoxy-6-fluoro β-D-galactopyranoside 27.

1,2:3,4-Di-O-isopropylidene-α-D-galactopyranose may be prepared in one step from D-galactose and acetone in the presence of sulphuric acid.[39] It seemed likely that the 6-deoxyfluoro derivative **22** could be readily prepared by treatment of this compound with DAST in the usual manner. However, initial attempts, upon work-up, produced only a non-fluorinated product or mixtures of this product and the desired 6-deoxyfluoro derivative **22** in low (20 to 25%) yields. This non-fluorinated product was identified as the 6-*chloro*deoxy analog, 1,2:3,4-di-O-isopropylidene-6-chloro-6-deoxy-α-D-galactopyranose **49**, by comparison of its ^{1}H nmr spectrum with that of an authentic

sample prepared by another route.[83] Isolation of this compound was surprising, the only source of chlorine in the reaction being dilute hydrochloric acid used in the work-up. Normal work-up for fluorinations with DAST consists of quenching the reaction with excess methanol at -20°C, allowing the mixture to warm to room temperature and washing with cold, dilute hydrochloric acid to remove 2,4,6-trimethylpyridine or a similiar organic base used in the reaction. When the reaction was quenched with excess methanol by stirring for several hours at room temperature and washed with 1 M aqueous acetic acid, the 6-deoxyfluoro compound **22**, obtained in 40% yield, was the sole isolated product.

Isolation of the chlorodeoxy compound **49** suggests interception of a putative DAST intermediate such as **50** by chloride ion during work-up.
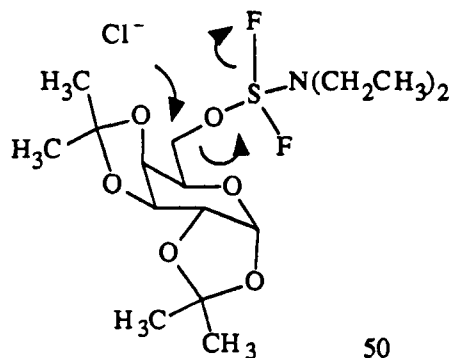
Relatively stable intermediates resulting from reaction of DAST or similar fluorinating reagents with sterically hindered alcohols have been reported in the literature. Tewson and Welch[47] have observed a new ^{19}F nmr signal when DAST was reacted with the hindered alcohol 1,2:5,6-di-O-isopropylidene-α-D-glucofuranose, which they attribute to a DAST intermediate. A fluorinated carbohydrate could not be isolated from this reaction but starting material was recovered upon treatment of the mixture with ethanol, while distillation from pyridine at 80°C yielded an elimination product. Evelyn and Hall[48] have reported that treatment of 1,2:3,4-di-O-isopropylidene-α-D-galactopyranose with the so-called fluoramine reagent, N-(2-chloro-1,1,2-trifluoroethyl)-N,N-diethylamine gave none

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of the expected 6-deoxyfluoro product **22** but that aqueous work-up afforded the chlorofluoroacetate **51**. ^{19}F nmr spectroscopic evidence was presented in support of the following scheme.
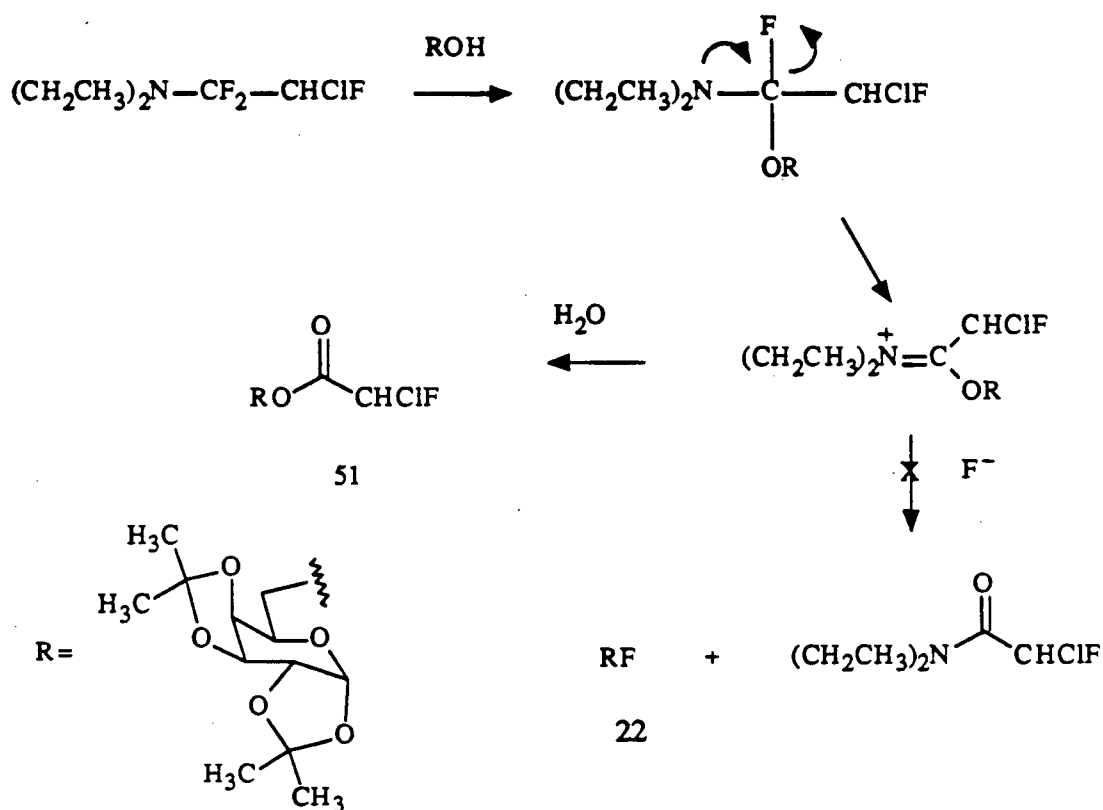
Figure 14. Proposed mechanism for reaction of N-(2-chloro-1,1,2-trifluoroethyl)-N,N-diethylamine with 1,2: 3,4-di-O-isopropylidene-α-D-galactopyranose.[48]

It appears likely that steric hindrance of the 6 position by the axial isopropylidene group at C-4 contributes to the the relative stability of a DAST intermediate such as **50** and accounts for the formation of the 6-chlorodeoxy analog **49** when an aqueous hydrochloric acid work-up is employed in the synthesis of **22**.

The protected deoxyfluoro sugar **22** was stirred with Amberlite IR-120(H$^+$) cation exchange resin in aqueous ethanol to remove the isopropylidene groups,[32] affording the free sugar **23** which crystallized exclusively as the α anomer in 75% yield. This compound was acetylated with acetic anhydride and pyridine in quantitative yield to give an anomeric mixture of per-O-acetates **24**, then selectively deacetylated at the 1 position with 2-aminoethanol[45] to give a mixture of the tri-O-acetates **25** in 56% yield. The galactopyranoside **26** was prepared by the reaction of **25** with FDNB and DABCO[37] in N,N-dimethylformamide to give the β anomer in 40% yield. This compound was treated with hydrogen chloride in methanol[38] to give the deacetylated galactopyranoside **27** in 70% yield.

2. Syntheses of deoxy analogs of 2',4'-dinitrophenyl β-D-galactopyranoside

Both the 6- and 4-deoxy analogs of 2',4'-dinitrophenyl β-D-galactopyranoside were prepared. The latter compound, 2',4'-dinitrophenyl 4-deoxy-β-D-*xylo*-hexopyranoside **42** was obtained from M. Namchuk, Department of Chemistry, U.B.C. The complete synthesis of the 3-deoxy analog was not achieved. Its immediate precursor 2',4'-dinitrophenyl 2,4,6-tri-O-acetyl-3-deoxy-β-D-*xylo*-hexopyranoside **39** was synthesized but attempts to isolate the deacetylated glycoside after treatment with hydrogen chloride in methanol[38] were unsuccessful.

2.1 Deoxygenation at the 3 position

The preparation of 3-deoxy-*xylo*-hexopyranosides by conventional methods, *i.e.* reductive dehalogenation[49] of the protected halogeno derivative, involves long, multistep syntheses giving the final product in only modest yields. A recent improved synthesis[50] by Lin, Kovac and Glaudemans is reported to give methyl 3-deoxy-β-D-*xylo*-hexopyranoside **52** in 20% overall yield from methyl β-D-galactopyranoside in seven steps. For hexopyranosides, the synthetic problem is complicated by the propensity of 3-deoxy-D-*xylo*-hexose to exist partially in its furanose form (19.5% at equilibrium at 31°C in water[51]).

We proposed an alternative synthesis of methyl 3-deoxy-α-D-*xylo*-hexopyranoside **35** prompted by two observations in the literature: 1) Hedgley et al. have reported that lithium aluminum hydride reduction of methyl 3,4-anhydro-α-D-galactopyranoside **28** afforded methyl 3-deoxy-α-D-*xylo*-hexopyranoside **35** in 76% yield[52]; 2) according to Jones et al., the anhydro compound **28** could be prepared in 74% yield from methyl 3-chloro-3-deoxy-α-D-gulopyranoside **53** in aqueous sodium hydroxide over 3 days at 40°C.[53]

Methyl 2,3,6-tri-O-benzoyl-4-chloro-4-deoxy-α-D-glucopyranoside **54** is available in 55% overall yield in two steps from methyl α-D-galactopyranoside, by selective benzoylation at the 2-, 3- and 6-positions[54] followed by treatment with sulphuryl chloride.[55] The corresponding 4-O-toluenesulphonyl derivative **55**, upon treatment with sodium methoxide in methanol at 0°C for several hours, is reported to give the anhydro compound **28** in 81% yield.[56] It seemed plausible that treatment of the benzoylated 4-chloro-4-deoxy glucopyranoside **54** with base might give the anhydro compound **28** in one step, analogous to the base promoted epoxidation of the 3-chloro-3-deoxy gulopyranoside **53**. Reduction of the anhydro compound **28** as described by Hedgley et al.[52] above would then give the desired 3-deoxy-*xylo*-hexopyranoside **35**.

R7, OR1, R6, R4, R5, R1O, R3, R2, O (structure)

O, OH, HO, O, OCH3 **28**
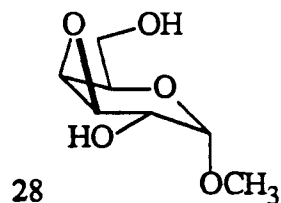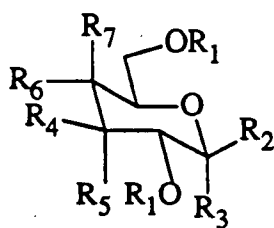
	R_1	R_2	R_3	R_4	R_5	R_6	R_7
29	H	H	OCH_3	OH	H	Cl	H
35	H	H	OCH_3	H	H	H	OH
52	H	OCH_3	H	H	H	H	OH
53	H	H	OCH_3	H	Cl	H	OH
54	Bz	H	OCH_3	OBz	H	Cl	H
55	Bz	H	OCH_3	OBz	H	OTs	H

Bz = benzoyl; Ts = p-toluenesulphonyl

However, neither the aqueous conditions employed by Jones et al.[53] nor sodium methoxide in methanol gave satisfactory yields of the anhydro compound **28** from the debenzoylated 4-chloro-4-deoxy glucopyranoside **29** or the 4-chloro-4-deoxy glucopyranoside **54**, respectively. Room temperature treatment of 4-chloro-4-deoxy glucoside **54** with sodium methoxide in methanol gave only the debenzoylated 4-chloro-4-deoxy glucopyranoside **29** in 87% yield. When 4-chloro-4-deoxy glucopyranoside **54** was heated with sodium methoxide in methanol at 45°C for 60 hours, a ~ 1: 1: 1 mixture of the anhydro compound **28**, the debenzoylated 4-chloro-4-deoxy glucopyranoside **29** and

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the 3,6-anhydrogalactopyranoside **30**, separable by column chromatography, was produced (Figure 15).
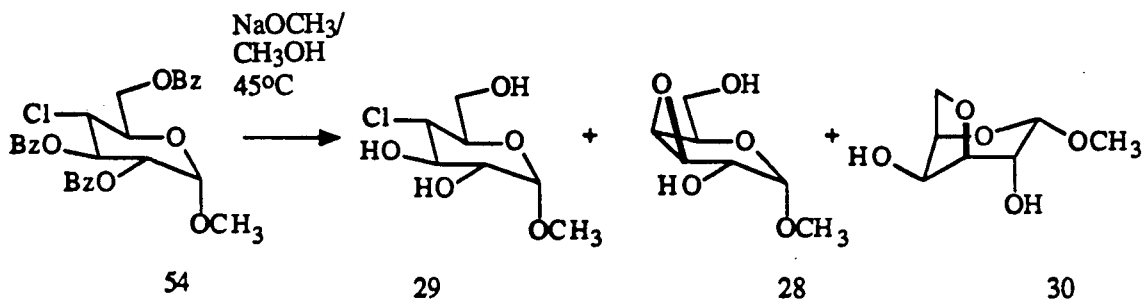
Figure 15. Reaction of methyl 2,3,6-tri-O-benzoyl-4-chloro-4-deoxy-α-D-glucopyranoside 54 with sodium methoxide in methanol at 45°C.

When the 4-chloro-4-deoxy glucopyranoside **54** was refluxed with sodium methoxide in methanol for 18 h, the sole product was the 3,6-anhydro-α-D-galactopyranoside **30**, identified by comparison of its ^{1}H nmr spectrum to literature values[57], obtained in 77% yield. Clearly, it seemed the desired anhydro compound **28** could not be formed from the 4-chloro-4-deoxy glucopyranoside **54** under the conditions examined without irreversible formation of the 3,6-anhydrogalactoside **30** as well.

Formation of a 3,6-anhydro-α-D-galactopyranosyl moiety has been reported[58] upon treatment of 2,3,6,2',3'-penta-O-acetyl-4,1',6'-tri-O-toluenesulphonyl-D-sucrose **56** with refluxing sodium ethoxide in ethanol (Figure 16). Presumably, the 3,4-epoxide ring is formed initially (step 1), followed by migration to the 2,3-position (step 2) which permits closure of the 3,6-anhydro ring (step 3). This behavior is well known[59] in the 2,3-anhydro allopyranoside series.
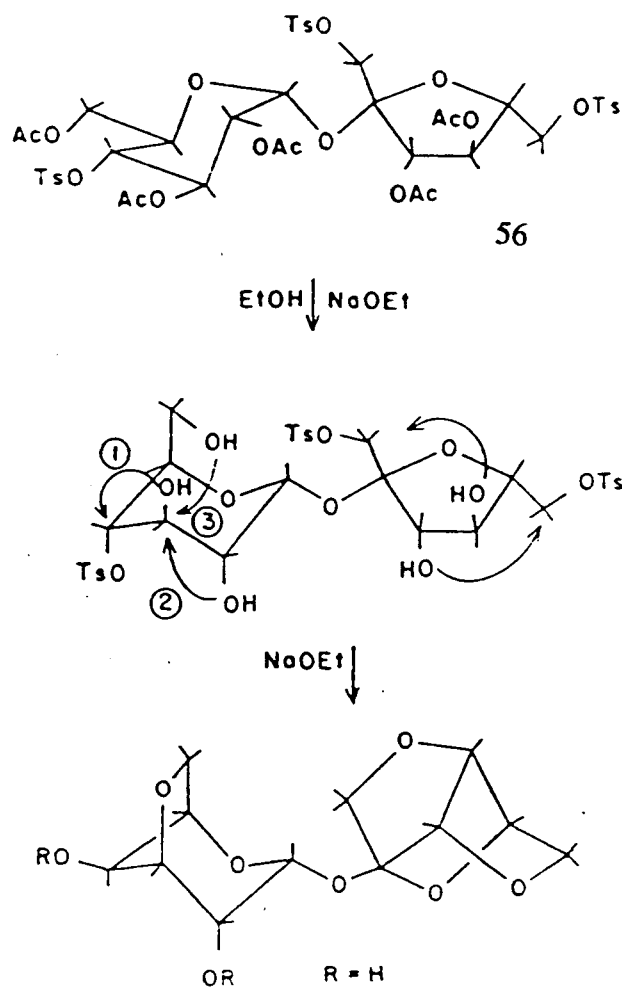
Figure 16. Proposed mechanism for formation of a 3,6-anhydro-α-D-galactopyranosyl structure from a 4-O-toluenesulphonyl glucopyranose moiety.[58]

The low reactivity of the 4-chloro-4-deoxy glucopyranoside **54** compared to the 3-chloro-3-deoxy gulopyranoside **53** and the 4-O-toluenesulphonyl glucopyranoside **55** is not difficult to rationalize. The hydroxyl of the guloside **53** is already well positioned to displace the axial chlorine and the glycopyranose ring does not have to undergo an unfavourable conformational change to position the hydroxyl for an optimal in-line attack. It would be expected, furthermore, that chloride is a much worse leaving group than the *p*-toluenesulphonate group in **55** (Table 3).[60]

Leaving group	k_{rel}
trifluoromethanesulphonate	1.4×10^8
p-toluenesulphonate	3.7×10^4
methanesulphonate	3.0×10^4
I⁻	91
Br⁻	14
Cl⁻	1.0
F⁻	9×10^{-6}

Table 3. Relative solvolysis rates of 1-phenylethyl esters and halides.[a,b]

[a] Data from Noyce, D.S. and Virgilio, J.A. (1972) *J. Org. Chem. 37,* 2643 .
[b] In 80% aqueous ethanol at 75°C.

Iodide is generally assumed to be a better leaving group than chloride and indeed it was found that treatment of methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-iodo-α-D-glucopyranoside **40** with sodium methoxide in 2:1 methanol/ N,N-dimethylformamide at room temperature for 4 days afforded the anhydro compound **28**, identified by comparison of its ^{1}H nmr spectrum to literature values[61], in 66% yield (Figure 17).
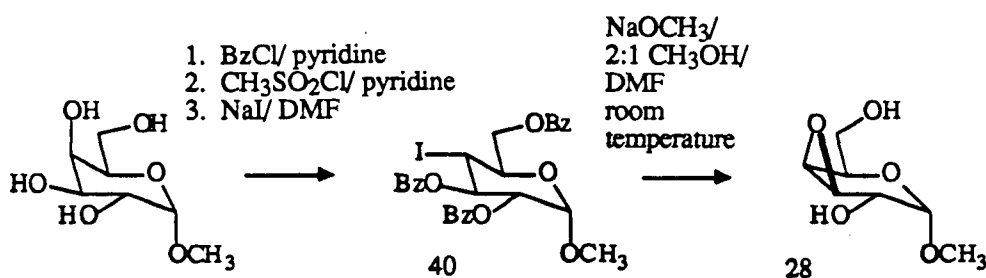
Figure 17. Synthetic scheme for the preparation of methyl 3,4-anhydro-α-D-galactopyranoside 28.

The 4-deoxy-4-iodo glucopyranoside **40** was prepared in 53% yield by iodination of methyl 2,3,6-tri-O-benzoyl-4-O-methanesulphonyl-α-D-galactopyranoside **57** with

sodium iodide in refluxing N,N-dimethylformamide.[62] The methanesulphonate **57** is available in 58% overall yield in two steps from methyl α-D-galactopyranoside by selective benzoylation at the 2-, 3- and 6-positions, followed by reaction with methanesulphonyl chloride in pyridine.[55] Methyl 3-deoxy-α-D-*xylo*-hexopyranoside **35** is thus available in five steps *via* this route in 15% overall yield from methyl α-D-galactopyranoside.

An alternative strategy to block the irreversible formation of the 3,6-anhydro ring was also examined (Figure 18).
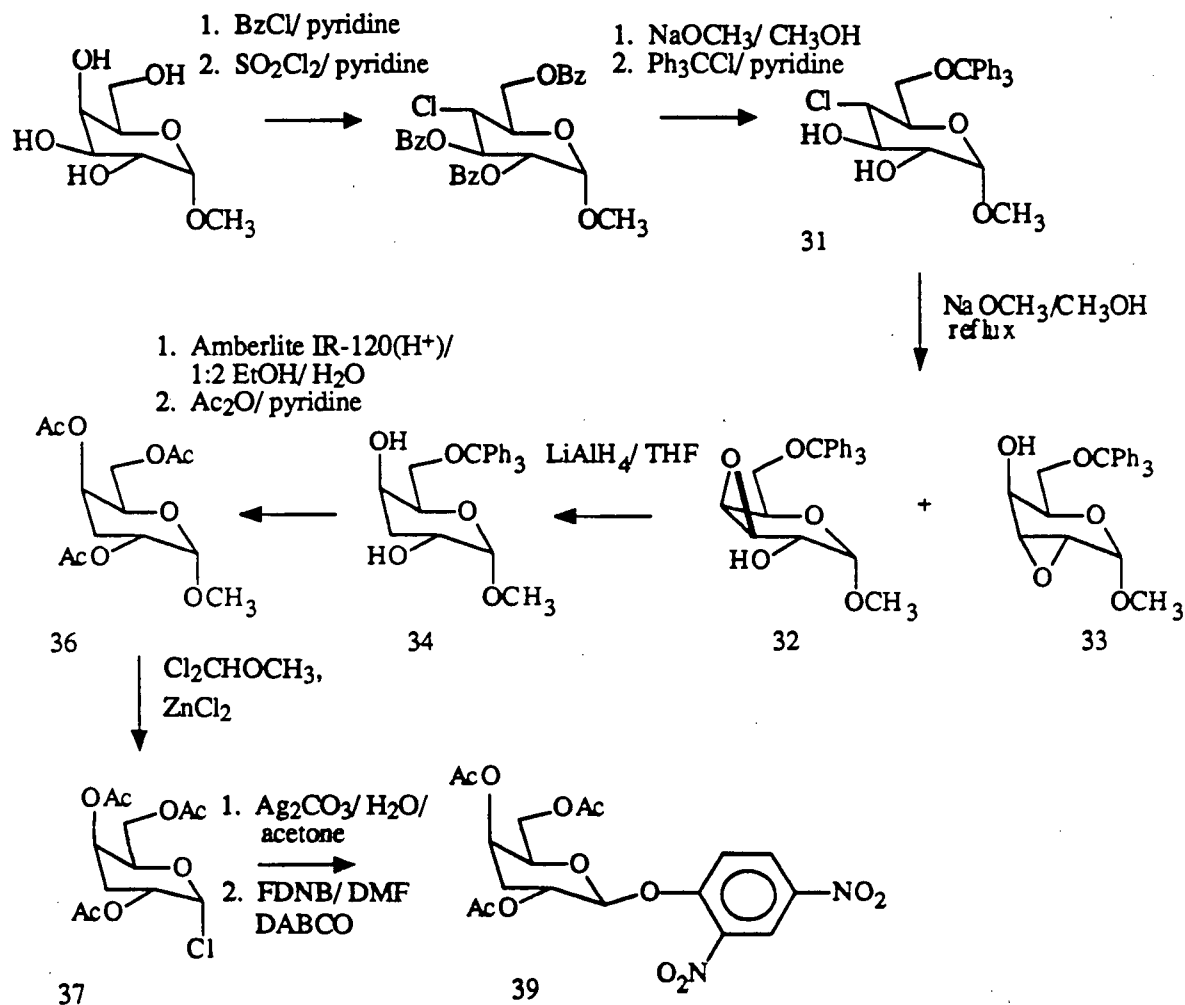
Figure 18. Synthetic scheme for the preparation of 2',4'-dinitrophenyl 2,4,6-tri-O-acetyl-3-deoxy-β-D-xylo-hexopyranoside 39.

The debenzoylated 4-chloro-4-deoxy glucopyranoside **29** was synthesized in 87%

yield from the protected 4-chloro-4-deoxy glucopyranoside **54** as described previously,

then treated with triphenylmethyl chloride in pyridine to give the 6-O-triphenylmethyl

derivative **31** in 76% yield. This compound was refluxed with sodium methoxide in

methanol for 15 h to afford a 1: 1.4 mixture of the 3,4-anhydro galactopyranoside **32** and

the 2,3-anhydro gulopyranoside **33** in a combined yield of 80%. The two isomers were

separated by column chromatography and the pure 3,4-anhydro galactopyranoside **32** was

treated with lithium aluminum hydride in THF to give exclusively the 3-deoxy-*xylo*-

hexopyranoside **34** in 95% yield. The triphenylmethyl group was removed by mild acid

hydrolysis with Amberlite IR-120(H^{+}) cation exchange resin in aqueous ethanol[32] and the

resulting gum acetylated without purification to give the acetylated methyl 3-deoxy-*xylo*-

hexopyranoside **36** in 75% yield from **34**. Primarily because of loss of material as the

unwanted 2,3-anhydroguloside **33**, this route is inefficient, with an overall yield of **36**

from methyl α-D-galactopyranoside of only 9%. Compound **36** was converted to the α-

hexopyranosyl chloride **37** with 1,1-dichloromethyl methyl ether and zinc chloride[63] and

hydrolyzed without purification to the alcohol **38** in 75% yield from **36**.[36] Compound **38**

was converted to the glycopyranoside **39** by reaction with FDNB and DABCO in N,N-

dimethylformamide[37] in 23% yield. Attempted deacetylation of this compound with

hydrogen chloride in methanol[38] gave only hydrolyzed material, but it was anticipated that

the deacetylated 2',4'-dinitrophenyl 3-deoxy-β-D-*xylo*-hexopyranoside would be

extremely labile (see Section 6, p. 60).

2.2 Deoxygenation at the 6 position

The route employed for the preparation of 2',4'-dinitrophenyl 6-deoxy-β-D-

galactopyranoside **46** is shown in Figure 19.
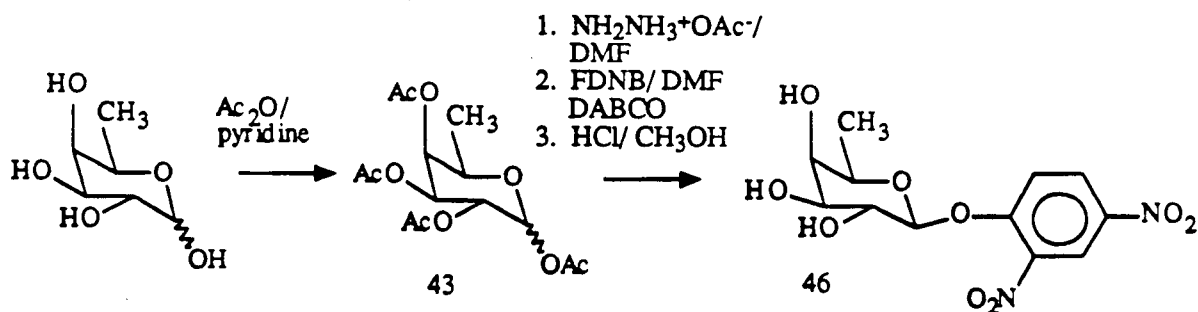
Figure 19. Synthetic scheme for the preparation of 2',4'-dinitrophenyl 6-deoxy-β-D-galactopyranoside 46.

D-Fucose (6-deoxy-D-galactose) was acetylated with acetic anhydride in pyridine to give a mixture of the tetra-O-acetates **43** in quantitative yield. This material was selectively deacetylated at the 1 position with hydrazine acetate in N,N-dimethylformamide[64] in 69% yield and the resulting alcohol **44** converted to the galactopyranoside **45** in 69% yield with FDNB and DABCO in N,N-dimethylformamide.[37] Subsequent treatment with hydrogen chloride in methanol[38] gave the deacetylated galactopyranoside **46** in 25% yield.

3. Inactivation of *E. coli (lacZ)* β-galactosidase with 2-deoxy-2-fluoro galactopyranosides

The general mechanism proposed by Koshland[1] for a "retaining" glycosidase is shown in Figure 2 (see Chapter 1, p. 3). Assuming only the chemical steps are kinetically significant, the mechanism may be depicted as follows.

$$E + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} ES \xrightarrow{k_2} E\text{-}P \xrightarrow{k_3} E + P$$

$$\downarrow HX \qquad \uparrow H_2O$$
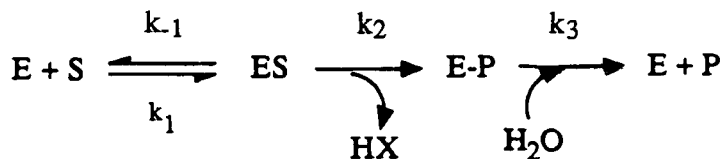
Figure 20. Kinetic model for a "retaining" glycosidase. E is the free enzyme, S the glycoside, HX the aglycone, ES the noncovalent Michaelis complex and E-P the covalent glycosyl enzyme intermediate. k_2 and k_3 are the first order rate constants for glycosylation and deglycosylation, respectively.

Assuming a steady state concentration of ES and E-P is reached during the reaction then

$$k_2[ES] = k_3[E\text{-}P]$$

and

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES]$$

$$= 0$$

The total concentration of enzyme, E_0 is the sum of the concentrations of free enzyme and all enzyme-bound species

$$[E_0] = [E] + [ES] + [E\text{-}P]$$

Therefore, by substituting for [E-P] and rearranging

$$k_1[E_0][S] = (k_{-1} + k_2)[ES] + k_1[ES][S] + \frac{k_1 k_2 [ES][S]}{k_3}$$

$$= (k_{-1} + k_2)[ES] + (k_1 k_3 / k_3 + k_1 k_2 / k_3)[ES][S]$$

Solving for [ES]

$$[ES] = \frac{k_1[E_0][S]}{k_{-1} + k_2 + \frac{k_1(k_2 + k_3)}{k_3}[S]}$$

Since at steady state the rate of production of P, vp is given by

$$\frac{d[P]}{dt} = k_3[E\text{-}P]$$

$$= k_2[ES]$$

then
$$v_P = \frac{\dfrac{k_2k_3}{k_2+k_3}[E_0][S]}{\dfrac{k_3}{k_2+k_3}\dfrac{k_{-1}+k_2}{k_1} + [S]}$$

which follows the standard form of the Michaelis-Menten equation

$$v = \frac{k_{cat}[E_0][S]}{K_m + [S]}$$

Therefore the kinetic parameters for this mechanism are

$$k_{cat} = \frac{k_2k_3}{k_2+k_3}$$

$$K_m = \frac{k_3(k_{-1}+k_2)}{k_1(k_2+k_3)}$$

$$k_{cat}/K_m = \frac{k_1k_2}{k_{-1}+k_2}$$

It can be shown that k_{cat} is the rate constant for the rate-determining step of the reaction and will always be associated with the highest free energy step in the pathway. k_{cat}/K_m will always be the pseudo second-order rate constant for the free enzyme and free substrate proceeding to the transition state of the first irreversible step.[23]

When the aglycone in Figure 20 is a highly activated leaving group (e.g. 2,4-dinitrophenolate), it would be expected that if the rate of glycosylation was increased sufficiently relative to deglycosylation then deglycosylation would become rate determining, i.e. $k_2 \gg k_3$. This has been shown by methanol competition experiments to be the case for the hydrolysis of βGalDNP by *E. coli (lacZ)* β-galactosidase.[65] Assuming

further a rapid, reversible association of enzyme and substrate, i.e. $k_{-1} \gg k_2$, the kinetic parameters k_{cat} and k_{cat}/K_m then become

$$k_{cat} = k_3$$

$$k_{cat}/K_m = \frac{k_1 k_2}{k_{-1}}$$

The Eyring equation relates these rate constants to differences in energy levels among the various species on the reaction pathway.

$$k_{cat} = (kT/h)\exp\{-(G_{EP}^{\ddagger} - G_{EP})/RT\}$$

$$= (kT/h)\exp\{-\Delta G_{EP}^{\ddagger}/RT\}$$

$$k_{cat}/K_m = (kT/h)\exp\{-(G_{ES}^{\ddagger} - G_{E+S})/RT\}$$

$$= (kT/h)\exp\{-\Delta G_T^{\ddagger}/RT\}$$

The free energy diagram for this situation is depicted schematically in Figure 21.
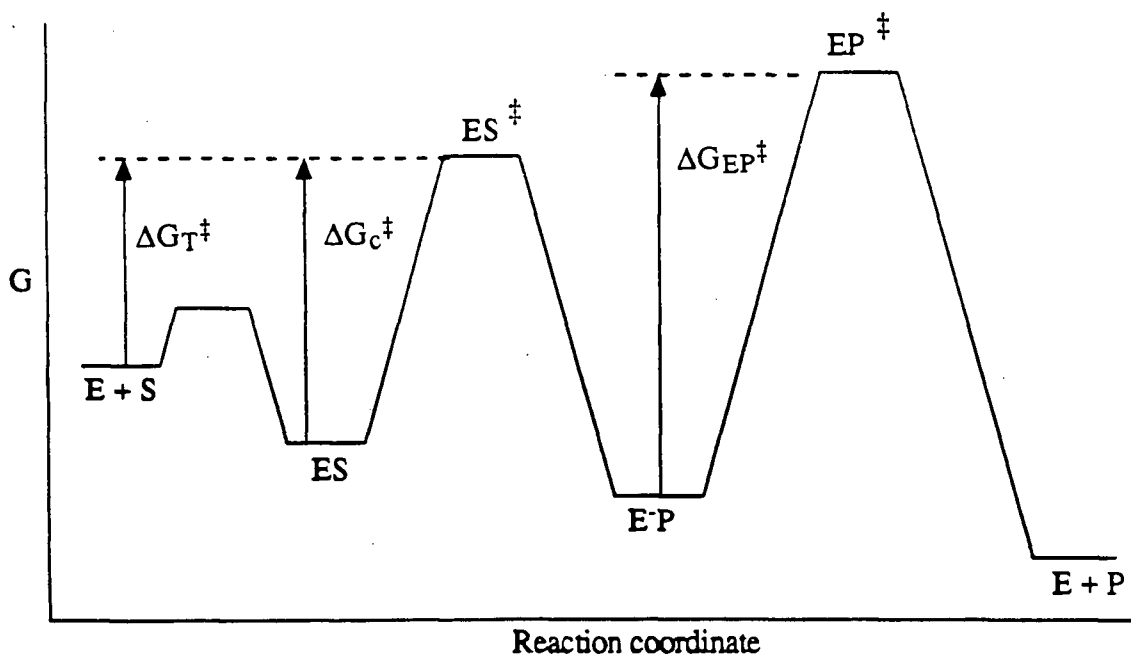
Figure 21. Hypothetical Gibbs free energy diagram for a "retaining" glycosidase showing rate-limiting deglycosylation, i.e. $k_3 \ll k_2$.

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Carrying rate-limiting deglycosylation to its extreme, if k_3 is sufficiently small compared to k_2 such that k_3 approaches zero then the glycosyl enzyme intermediate will accumulate, inactivating the enzyme, and the kinetic model becomes

$$E + I \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} EI \xrightarrow{k_2} E\text{-}P$$
$$\downarrow$$
$$HX$$
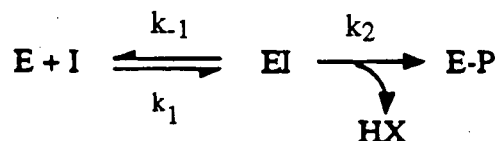
Figure 22. Kinetic model for inactivation via the accumulation of E-P, the glycosyl enzyme intermediate. E is the free enzyme, EI the noncovalent enzyme-inhibitor complex and HX the aglycone.

This model predicts a rapid time-dependent inactivation of the enzyme. If [I] is much greater than $[E_0]$ and thus [I] is assumed to be essentially constant during the reaction, then pseudo first-order kinetics with respect to enzyme concentration will be observed. In Michaelis-Menten form, the equation for this process is

$$v_i = \frac{k_i[E_0][I]}{K_i + [I]} \qquad (1)$$

where k_i is the rate constant for inactivation and K_i is an apparent dissociation constant for all forms of enzyme-bound inactivator. As [I] is assumed to be constant, Equation (1) can be rewritten as

$$v_i = k_{obs}[E_0]$$

where
$$k_{obs} = \frac{k_i[I]}{K_i + [I]} \qquad (2)$$

The rate of inactivation, v_i is equal to

$$\frac{-d[E_o]}{dt} = k_{obs}[E_o]$$

Thus
$$\ln[E_o] = -k_{obs}t$$

From inspection of Figure 21, it can be seen that k_i is equal to the rate of glycosylation, k_2. Thus, in Eyring form

$$k_i = k_2 = (kT/h)\exp\{-\Delta G_c^{\ddagger}/RT\}$$

with
$$K_i = \frac{k_{-1}}{k_1}$$

since the association of enzyme and inhibitor is assumed to be rapid and reversible, i.e. $k_{-1} \gg k_2$,

then
$$k_i/K_i = \frac{k_1k_2}{k_{-1}} = (kT/h)\exp\{-\Delta G_T^{\ddagger}/RT\}$$

Both k_i and k_i/K_i are associated with the energy barriers for glycosylation, but k_i reflects the height of the energy barrier from the Michaelis complex to the transition state for glycosylation while k_i/K_i reflects the barrier height from free enzyme and free inactivator to the glycosylation transition state. Note that in this scheme, k_i/K_i is analogous to k_{cat}/K_m in terms of the individual rate and equilibrium constants for each step.

Recently it has been shown[66] that incubation of *E. coli (lacZ)* β-galactosidase with 2-deoxy-2-fluoro-β-D-galactopyranosyl fluoride at 37°C led to a rapid, time-dependent inactivation of the enzyme. Protection against inactivation by a competitive ligand, isopropylthio-β-D-galactopyranoside, indicated that inhibition was occuring at the enzyme active site. It was proposed that substitution of the highly electronegative fluorine at C(2) of the glycopyranose ring destabilizes the oxocarbonium ion-like transition states involved in *both* the glycosylation and deglycosylation steps, while acceleration of glycosylation by

deoxy-2-fluoro-glycosyl enzyme intermediate (Figure 5, p. 7). Similar behavior was observed when *Agrobacterium* pABG5 β-glucosidase was incubated with various aryl 2-deoxy-2-fluoro glucosides.[67]

Prompted by these results, *E. coli (lacZ)* β-galactosidase was tested for similar time-dependent inactivation by 2',4'-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside at 25°C. Enzyme was incubated at 25°C in the presence of inactivator. Aliquots were removed at intervals and assayed for residual activity by dilution into a saturating concentration of βPNPGal. This prevents further inactivation by diluting the inactivator with a large excess of a competing ligand. The observed pseudo first-order rate constants for the inactivation process, k_{obs}, at each inhibitor concentration were determined by plotting the logarithm of the fraction of remaing active enzyme against time. Values for K_i and k_i were calculated by fitting the rate constants so determined to Equation (2) using a weighted non-linear regression program.

As expected, incubation of β-galactosidase with 2FβGalDNP resulted in inactivation of the enzyme according to pseudo first-order kinetics. The half-life of inactivation at saturating concentrations of inactivator was estimated to be 63 seconds. This result is consistent with the kinetic model discussed earlier in which deglycosylation is slow compared to glycosylation and the E-P complex rapidly accumulates (Figure 22). The results are shown in Figure 23. Values for k_i and K_i were found to be 0.011 s^{-1} and 0.89 mM, respectively.

The active site-directed nature of the inactivator was shown by protection against inactivation by βGalSiPr, a competitive ligand ($K_i = 0.085$ mM[71]) which binds reversibly at the active site (Figure 24).

A)

B)
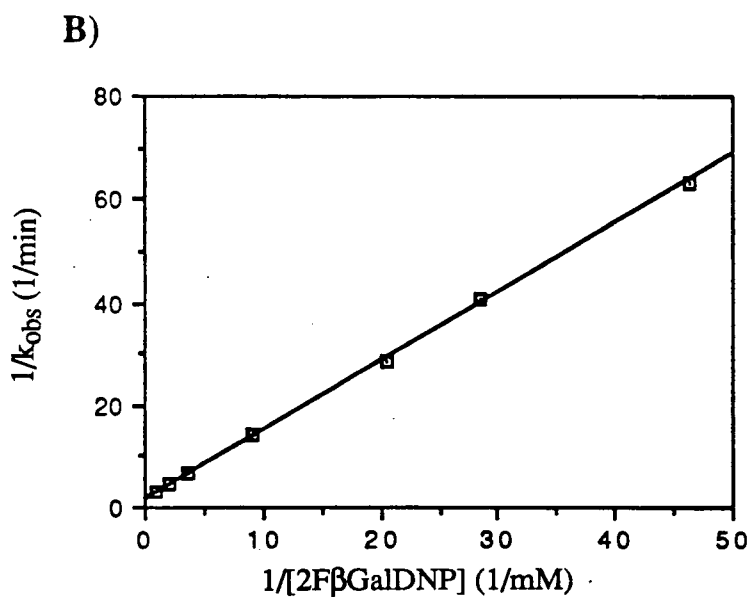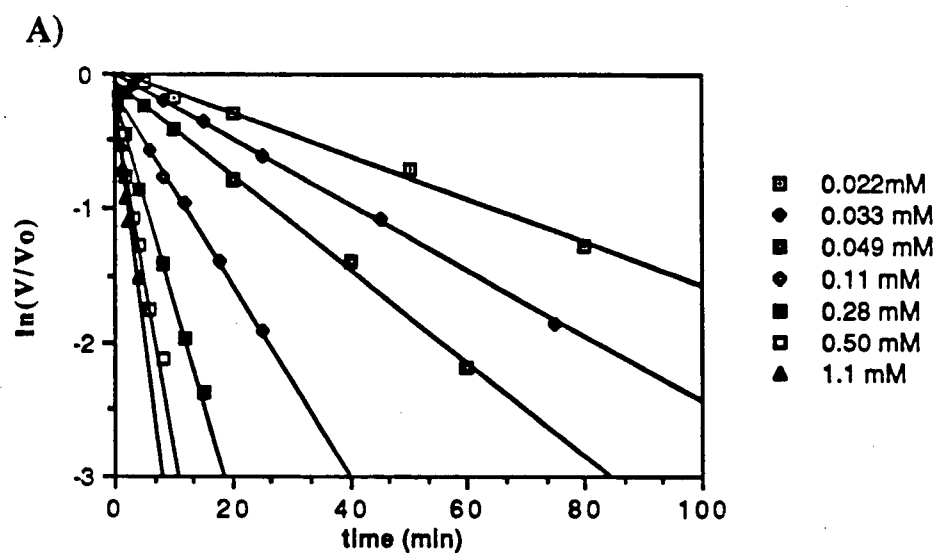
Figure 23. Inactivation of E. coli (lacZ) *β-galactosidase by 2FβGalDNP.*

A) *A semilogarithmic plot of residual activity vs. time at 25°C, pH 7.00 in 50 mM sodium phosphate buffer containing 1 mM Mg^{2+}, 0.1% BSA. Inhibitor concentrations are as indicated.* **B)** *Double reciprocal plot of the following values obtained for k_{obs} at their respective inhibitor concentrations: 0.016, 0.024, 0.035, 0.071, 0.15, 0.25 and 0.35 min^{-1}.*
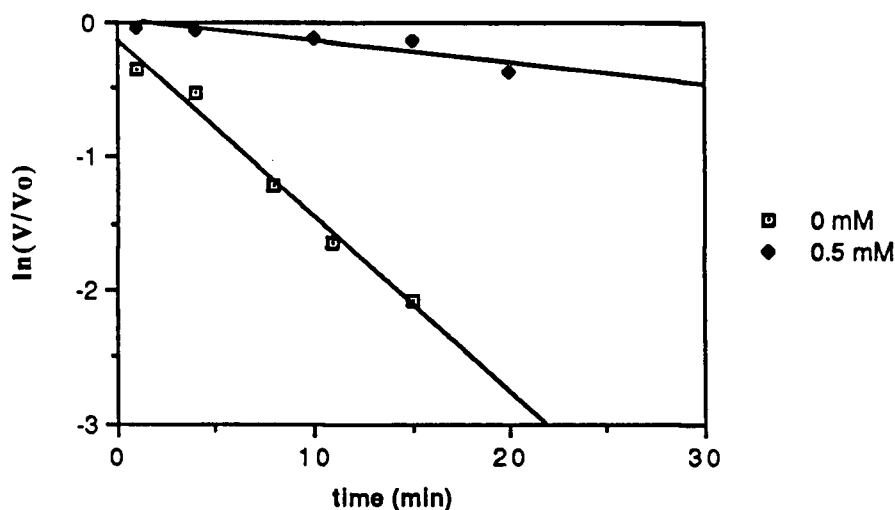
Figure 24. Protection against inactivation by βGalSiPr. Inactivation at a single fixed concentration of 2FβGalDNP (0.32 mM) with the indicated concentrations of βGalSiPr: 0 mM (k_{obs} = 0.13 min^{-1}), 0.50 mM (k_{obs} =0.016 min^{-1}).

4. Turnover of the E-P complex: hydrolysis and transglycosylation

Inactivation of glycosidases by trapping of a 2-deoxy-2-fluoroglycosyl enzyme intermediate represents the first step in turnover of an exceedingly slow substrate. When freed from excess inhibitor, the inactivated glycosyl enzyme would be expected to slowly hydrolyze, releasing a 2-deoxy-2-fluoro sugar and the free enzyme. The return of activity with liberation of free enzyme could then be assayed with a normal substrate.

Water is not the only molecule that can act as an acceptor for the 2-deoxy-2-fluorogalactosyl moiety released upon turnover of the galactosyl enzyme. The transglycosylase activity of *E. coli (lacZ)* β-galactosidase is well known.[68,69] When the natural substrate of the enzyme, lactose (a β(1-4)-linked disaccharide), is hydrolyzed, approximately 50% of the lactose consumed is converted by an intramolecular

trangalactolysis to allolactose, a β(1-6)-linked disaccharide.[68] Allolactose is the natural inducer of the *lac* operon and its synthesis in this manner thus has a specific physiological role (Figure 25).
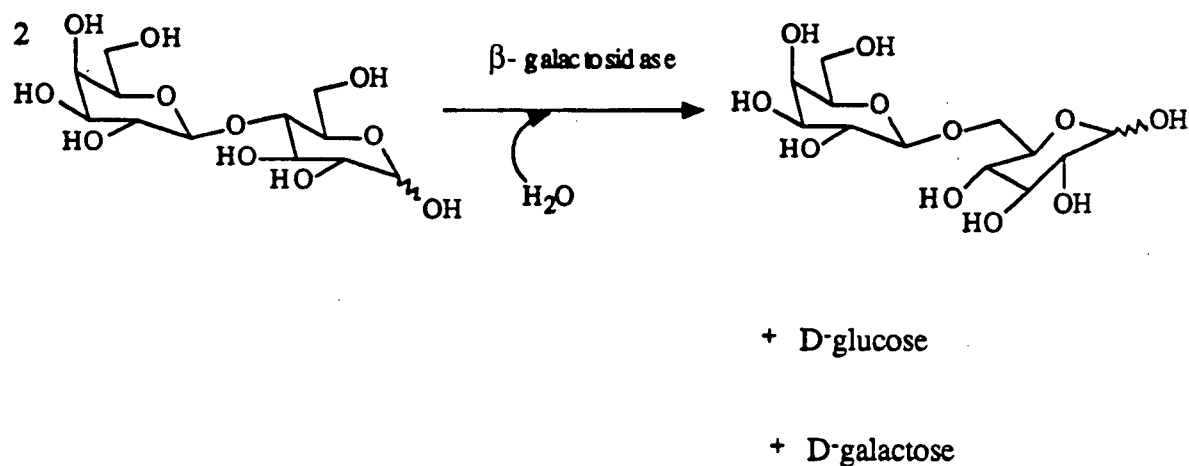
Figure 25. Formation of allolactose by E. coli lac(Z) *β-galactosidase via transglycosylation*

The efficiency of this reaction implies the presence of a second discrete "glucose" binding site adjacent to the active site which provides a high local concentration of a glycosyl acceptor enabling the transgalactolysis reaction to compete effectively with hydrolysis. Indeed, such a site might be expected since the natural substrate has a sugar "aglycone". Such multiple binding sites have been identified in hen egg white lysozyme by X-ray crystallography.[70] However, unlike lysozyme or other glycohydrolases which have evolved to bind and hydrolyze oligosaccharides, the secondary binding site of *E. coli lac(Z)* β-galactosidase is apparently quite small since tri- or tetra-saccharides have no effect on the kinetic parameters of βGalONP hydrolysis by the enzyme,[71] indicating that these compounds are unable to interact with the active site.

The enzyme catalyzes intermolecular transglycosylations to a wide variety of monosaccharides and aliphatic alcohols when these compounds are present during the enzyme-catalyzed hydrolysis of βGalONP.[69] It has been shown that compounds which could form transfer products were uncompetitive effectors of the enzyme, binding preferentially to the glycosyl enzyme rather than free enzyme. For example, Deschavanne et al.[71] found that glucose binds relatively weakly to the free enzyme with a K_i greater than 600 mM while a dissociation constant (= k_{-s}/k_s in Figure 26 below) for glucose binding to the glycosyl enzyme has been determined by Huber et al. to be 17 mM.[69]

A kinetic model for turnover of the isolated 2-deoxy-2-fluorogalactosyl enzyme in the presence of ligands which may act as transglycosylation acceptors is shown below.
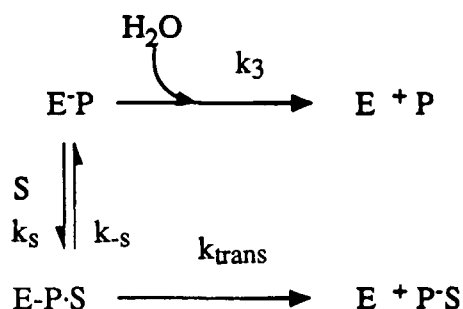
Figure 26. A kinetic model for turnover of the E-P complex via transglycosylation. EP and
k_3 are as defined previously, S is the ligand, E-P·S is the glycosyl enzyme-ligand
complex, k_{trans} is the rate constant for transglycosylation and
$K_{trans} = (k_{-s} + k_{trans})/k_s$.

The rate of decomposition of E-P is given by

$$\frac{-d[E\text{-}P]}{dt} = (k'_3 + k'_{trans})[E\text{-}P]$$

where $k'_{trans} = \dfrac{k_{trans}[S]}{K_{trans} + [S]}$ and $k'_3 = k_3[H_2O]$

Note that K_{trans} is equivalent to a true dissociation constant (= k_{-s}/k_s) for binding of S to the glycosyl enzyme if k_{trans} is slow compared to a rapid and reversible association of S

and E-P, i.e. $k_{trans} \ll k_{-1}$. When [S] is large compared to [E-P], the reaction will be pseudo first-order with respect to [E-P] and the observed rate constant, k_{obs}, will be equal to $k'_3 + k'_{trans}$. The resulting time-dependent increase in activity can be related to the full activity of the sample by

$$[E] = [E_0](1 - \exp\{-k_{obs}t\})$$

Inactivated β-galactosidase was produced by adding a fifty-fold excess of 2FβGalDNP to ~ 0.1 mg of enzyme in 1.6 mL buffer and incubating at 25°C for several hours to ensure complete inactivation. The inactivated galactosyl enzyme was freed of excess inactivator by centrifugation of the enzyme sample in a Millipore Ultrafree-PF filter unit fitted with a polysulphone membrane (30 000 molecular weight limit), diluted with buffer and assayed for activity at 25°C by diluting small aliquots into βGalPNP at saturating concentration. The results are shown below (Figure 27).

A)

B)
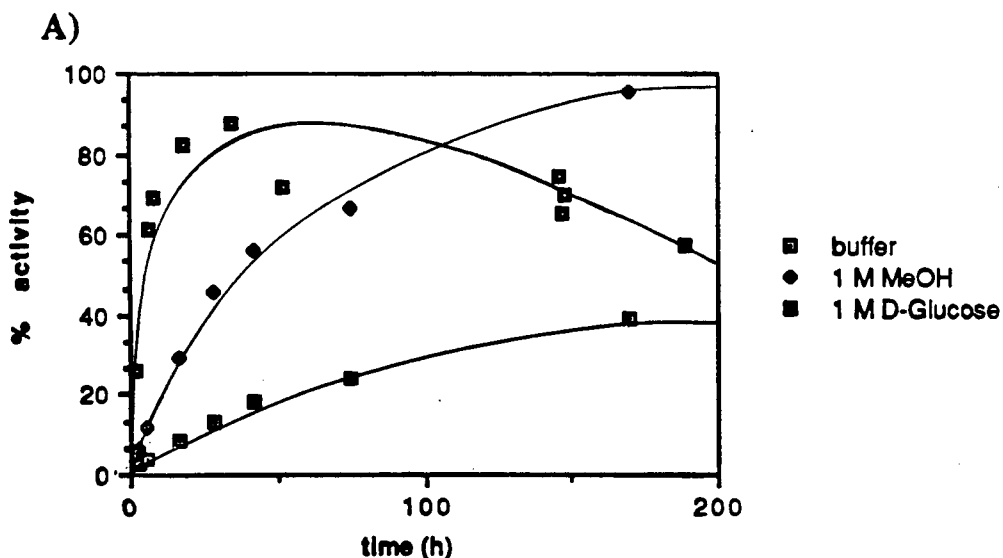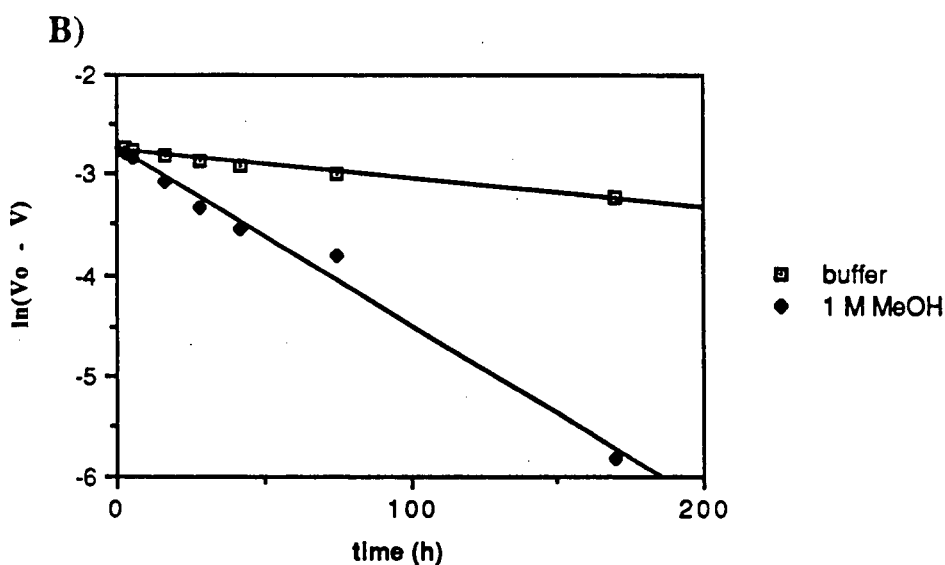
Figure 27. Turnover of the 2-deoxy-2-fluoro galactosyl enzyme in the presence of various ligands. A) Plot of activity vs. time for reactivations in buffer, 1 M methanol and 1 M D-glucose, respectively B) Semilogarithmic plot of activity vs. time for reactivations in buffer and 1 M methanol, respectively. All reactivations were performed in 50 mM sodium phosphate, 1 mM Mg^{2+}, pH 7.00 at 25°C.

Return of activity for the 2-deoxy-2-fluorogalactosyl enzyme in the presence of buffer alone or 1 M methanol followed a single exponential time course (Figure 27 **B**)). The half-life of the 2-deoxy-2-fluorogalactosyl enzyme in buffer at 25°C was estimated to be 69 hours. Control samples with *non-inactivated* enzyme to account for denaturation in buffer or 1 M methanol over the time course of the experiment showed negligible loss of activity. It can be seen from Figure 27 **A**) that the reactivation of the galactosyl enzyme in the presence of 1 M D-glucose does not follow simple pseudo first-order kinetics. The k_{obs} value was estimated from the initial exponential increase in activity during the first 30 hours. Though it appears that some inactivation process is superimposed upon the reactivation curve, controls with *non-inactivated* enzyme in the presence of 1 M glucose showed only a very slight decrease in activity over ~ 75 hours. A speculative rationale that may account for the shape of the observed curve is that the 2-deoxy-2-fluorogalactosyl

enzyme is more susceptible to denaturation in the presence of a high concentration of glucose than the free enzyme.

acceptor	k_{obs} (s^{-1})	relative rate	half-life (h)
H$_2$O	2.8 x 10^{-6}	1	69
1 M methanol	5.2 x 10^{-6}	1.9	37
1 M D-glucose	~ 5 x 10^{-5} *	~ 20	~ 3.5

Table 4. Turnover of the 2-deoxy-2-fluorogalactosyl enzyme in the presence of various ligands.

* Calculated from initial rate of reactivation based on return of activity in first 30 h.

From inspection of Figure 27 A) and Table 4 it is clear that both methanol and D-glucose can act as efficient transglycosylation acceptors, reactivating the enzyme at rates faster than in buffer alone. D-Glucose has been shown to bind relatively strongly to the galactosyl enzyme and to give transfer products when present during the enzyme-catalyzed hydrolysis of βGalONP.[69] Sinnott and Viratelle found that the rate of solvolysis of βGalDNP at saturating concentration by *E. coli lac(Z)* β-galactosidase increased approximately two-fold in the presence of 1 M methanol.[65] This rate acceleration in the presence of a better nucleophile than water was interpreted as evidence that degalactosylation of the enzyme was rate-determining for this substrate. These observations are wholly consistent with the reactivation data obtained in this study, which provides good evidence for a catalytically competent 2-deoxy-2-fluorogalactosyl enzyme intermediate that has not perturbed the normal mechanism other than by slowing the rates of glycosylation and deglycosylation.

Although K_{trans} or k_{trans} values for methanol or glucose were not determined in this study, accelerated transglycosylation rates with these compounds compared to hydrolysis

are evident. These rate increases probably arise from several factors: 1) greater nucleophilicity of the alcohol or sugar hydroxyl relative to water; 2) favourable binding of the acceptor by the "glucose" subsite to orient the nucleophile for attack and; 3) utilization of the binding energy from binding of the acceptor at the "glucose" subsite to stabilize the transition state for degalactosylation. The greater nucleophilicity of methanol, which is unlikely to bind strongly to the "glucose" site, probably accounts for the modest increase in turnover of the glycosyl enzyme compared to hydrolysis. Glucose itself, however, is more likely to utilize specific binding contacts at the secondary site to stabilize the transition state for the cleavage of the glycosyl-enzyme bond. Support for this notion is provided by comparison of k_{cat} values for the enzymic hydrolysis of βGalPNP (163 s^{-1}), for which glycosylation is though to be rate-limiting, with enzymic hydrolyses of lactose (66 s^{-1}) and allolactose (102 s^{-1}).[72] Though the leaving group ability (in terms of pK_a) of glucose differs from that of 4-nitrophenol by many orders of magnitude, the rates of enzymic hydrolyses of the disaccharides are only 40 to 60% lower than βGalPNP.

Note that k_{obs} for the turnover of the 2-deoxy-2-fluoro galactosyl enzyme in buffer alone with water acting as a glycosyl acceptor represents a k_{cat} value for 2FβGalDNP since $k_{obs} = k'_3 = k_3[H_2O]$ and $k_{cat} = k_2 k'_3/(k_2 + k'_3) = 2.8 \times 10^{-6}$ s^{-1}. Recall that for inactivation of glycosidases by 2-deoxy-2-fluoro glycosides, k_2 is equivalent to the k_i value, which was determined earlier. Comparing the k_{cat} value of 2FβGalDNP above with the k_{cat} of the unsubstituted 2',4'-dinitrophenyl galactopyranoside (1100 s^{-1}; see Table 6, p. 56) provides a measure of the decrease in deglycosylation rate caused by placement of the fluorine atom at C(2) since deglycosylation is rate-limiting for both compounds. The k_{cat} of the 2-deoxy-2-fluoro galactopyranoside is thus reduced approximately 10^8 -fold. This vast decrease in deglycosylation rate probably arises from two factors. First, inductive destabilization of an oxocarbonium ion-like transition state by the electron withdrawing effect of fluorine. Second, poorer binding of the 2-deoxy-2-fluorogalactosyl

enzyme intermediate at the transition state, possibly by disruption of specific non-covalent interactions (see Chapter 1, Section 3.4).

A transition state with substantial positive charge would be expected to be inductively destabilized by the electron withdrawing effect of an adjacent fluorine. It has been shown that the rates of spontaneous hydrolysis of 2',4'-dinitrophenyl glycopyranosides are independent of pH between pH 2 and 8 and that the hydrolyses proceed *via* oxocarbonium ion-like transition states.[73] Fluorine substitution in the pyranose ring at C(2) results in a 20-fold decrease in the spontaneous hydrolysis rate of 2FβGalDNP at 25°C relative to the parent compound (Table 7, p. 61). Clearly, the rate reduction arising from purely inductive destabilization of an electron-deficient transition state in the non-enzymic reaction cannot alone account for the 10^8-fold decrease in the enzymic rate. Some other factor, probably loss of specific binding involving the C(2) hydroxyl at the transition state must also contribute to the observed rate reduction. Note however that the transition states for the enzymic and spontaneous reactions may differ considerably in terms of charge development and hence the magnitude of the electronic effects of substitution on the different transition state structures may vary.

Additional evidence that this rate reduction arising from substitution at the 2 position does not simply involve inductive destabilization of electron deficient transition states alone is provided by studies of the enzymic hydrolysis of 1-deoxyglycerol-1-yl-2-deoxy-β-*lyxo*-hexopyranoside **57** and its *galacto* analog **56** by Brockhaus et al.[74] (Table 5). It can be seen that deletion of the 2-hydroxyl reduces the rate of β-galactosidase-catalyzed hydrolysis of **57** some 10^4-fold compared to **56**. Note that on purely electronic grounds, the 2-deoxy compound **57** would be expected to be hydrolyzed *faster* than the galactopyranoside **56** with its C(2) hydroxyl intact. That this reduction is wholly due to poor *ground state* binding is unlikely by comparison of the K_i values for the corresponding hexitols **59** and **58**. These results underline the importance of the C(2) hydroxyl to catalysis by the enzyme.
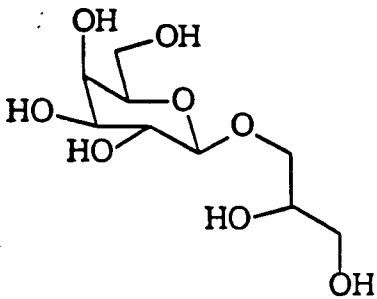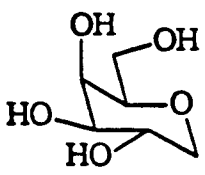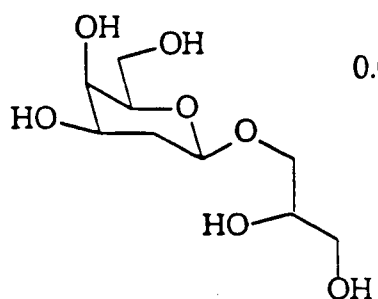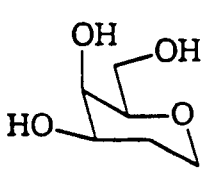
Substrate	Relative rate of enzymic hydrolysis ($\mu mol L^{-1} min^{-1}$)	Inhibitor	K_i (mM)
56	100	58	7.8
57	0.0074	59	69.6

*Table 5. Effect of deoxygenation at the 2 position on the hydrolysis of 1-deoxyglycerol-1-yl-β-D-galactopyranoside 56 by E. coli (lacZ) β-galactosidase.**

* Data from M. Brockhaus et al. (1979) *Carb Res. 69*, 264 .

5. Dinitrophenolate burst and active site titration

Accurate determinations of the concentration of enzyme in a sample often rely on spectrophotometric methods which depend on knowledge of the extinction coefficient of the enzyme at a particular wavelength and will often overestimate the enzyme concentration

if the sample is not homogenous. However, with some enzymes, the concentration of active enzyme may be determined by measuring an initial burst of product. Such a burst will occur when the first step in a two-step process releases one product while the second step is slow, allowing an enzyme-bound intermediate to accumulate. One mole of enzyme will initially react with one mole of substrate to form one mole of the first product and one mole of the enzyme-bound intermediate. If this intermediate turns over very slowly, then the concentration of active enzyme may be determined from the amount of the first product released. This is, of course, the kinetic scheme for inactivation of β-galactosidase by 2FβGalDNP (see Figure 22).

Such pre-steady state, burst phase kinetics have been observed in β-glycosidases previously. Mg^{2+}-free *E. coli (lacZ)* β-galactosidase at 4°C showed a burst with βGalDNP[75] and a burst observed with inactivation of pABG5 β-glucosidase by 2FβGluDNP was used to quantitate the amount of enzyme present.[55] In a similar experiment described below, the concentration of *E. coli (lacZ)* β-galactosidase in a sample was determined upon inactivation by 2FβGalDNP.

The enzyme concentration in a dialyzed, homogenous sample was determined by absorbance at 280 nm ($\varepsilon = 2.1$ mg^{-1} mL cm^{-1}).[76] A fifty-fold excess of 2FβGalDNP (0.41 mM) was added to the enzyme sample and the release of 2,4-dinitrophenol was followed at 400 nm for approximately 45 minutes at which time the change in absorbance showed a slight linear increase with time (Figure 28). The initial exponential rate of 2,4-dinitrophenol release ($k_{obs} = 0.19$ min^{-1}) is within 20% of the calculated rate of inactivation at this concentration of 2FβGalDNP ($k_{obs} = 0.23$ min^{-1}; see Figure 23 A), p. 40) confirming, within experimental error, that the inactivation and release of dinitrophenolate are associated with the same event. The concentration of phenol released in the initial burst was determined by back-extrapolation of the linear portion of the curve. A plot of protein concentration (from absorbance at 280 nm) *vs.* 2,4-dinitrophenol concentration is linear (r

= 0.9990) with a slope of 0.9, indicating, within experimental error, approximately one mole of 2,4-dinitrophenol released per mole of enzyme (Figure 29).
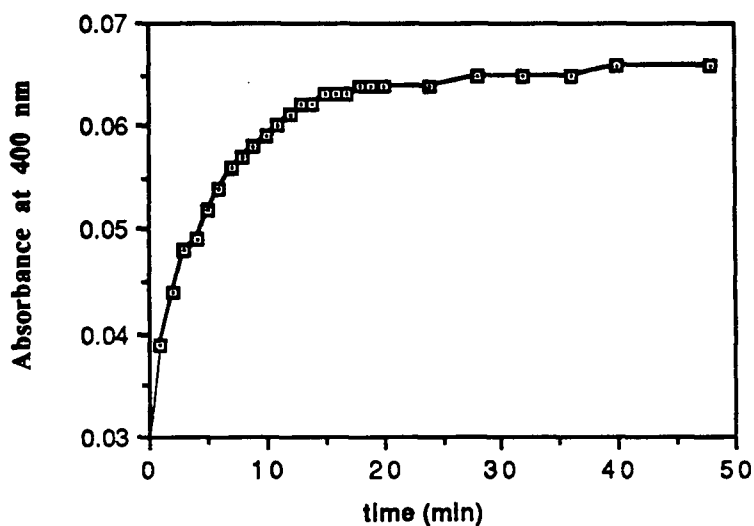
Figure 28. 2,4-Dinitrophenolate burst observed upon incubation of E. coli (lacZ) *β-galactosidase with 0.41 mM 2FβGalDNP in 50 mM sodium phosphate, 1 mM Mg^{2+}, pH 7.00 at 25°C.*
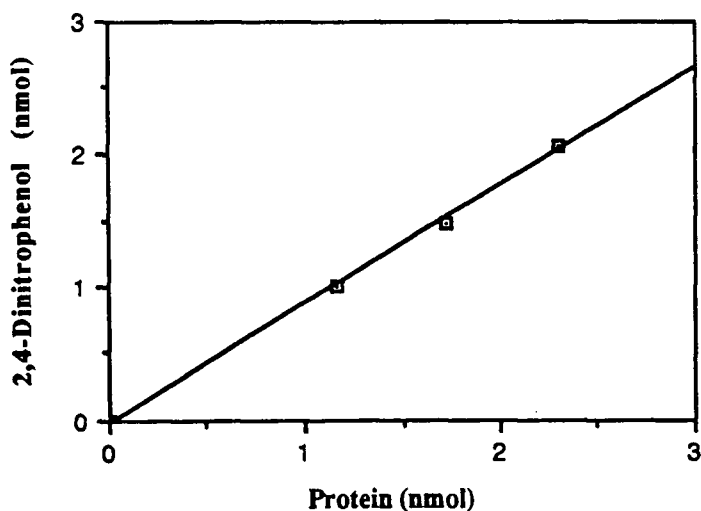
Figure 29. Stoichiometry of 2,4-dinitrophenol released from β-galactosidase incubated with 2FβGalDNP.

51

This near 1:1 stoichiometry is further evidence of the active site-directed nature of the inhibitor. Burst phase kinetics from 2FβGalDNP are readily observable at room temperature though the relatively slow rate of inactivation (and hence 2,4-dinitrophenolate release) and the significant rate of spontaneous hydrolysis of the galactoside are experimental drawbacks to the routine application of this method to active site titration of the enzyme.

6. Modified sugars as transition state probes

Recall that non-covalent interactions between enzyme and substrate at the transition state are proposed to be primarily responsible for the rate acceleration of an enzyme-catalyzed reaction.[23] In glycosyl transferases, these interactions are likely to be primarily due to hydrogen bonding between appropriate groups in the enzyme active site and the hydroxyl groups of the glycone. Hence deoxygenation (substitution of OH for H) at a particular position on the glycopyranose ring should result in deletion of any hydrogen bonding at that position between the glycone and the enzyme at the transition state. Substitution by fluorine, which can arguably accept a hydrogen bond but cannot possibly donate one, may still bind appreciably if the glycone normally accepts a hydrogen bond from the enzyme at that position. Deoxygenation or fluorination of the glycopyranose ring can also affect the rate of glycoside hydrolysis through purely electronic effects. Deoxygenation will tend to stabilize the developing oxocarbonium ion, increasing the rate of hydrolysis, while fluorination will have the reverse effect. Thus analysis of the kinetics of hydrolysis of a series of substrates with these modifications can provide information about both the electronic nature of the enzymic transition state and non-covalent interactions between the glycone and the enzyme active site (see Chapter 1, Section 4).

Hydrogen bonding energetics in water. Evaluation of the contribution of hydrogen bonding to the binding energy between substrate and enzyme is complicated by the fact that such binding involves an *exchange* of hydrogen bonds between substrate plus water and substrate plus enzyme, i.e.

(3)

$$E\text{-}H\text{---}OH_2 + HO\text{-}H\text{---}A\text{-}S \rightleftharpoons [E\text{-}H\text{---}A\text{-}S] + HOH\text{---}OH_2$$
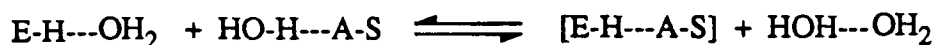
where the enzyme donates a hydrogen bond to an acceptor, A on the substrate, S. The hydrogen bonding roles of the enzyme and substrate could, of course, be the reverse of that shown. There is no change in the number of hydrogen bonds upon binding (though bond geometries may differ considerably) but there is an increase in entropy associated with the release of an enzyme-bound water molecule into bulk water which gives rise to the favourable binding energy of the resulting hydrogen bond in the enzyme-bound complex.[23]

Deletion of the hydrogen bonding group from the substrate will probably not result in loss of the full hydrogen bonding energy because no hydrogen bond could exist between water and the substrate before binding, i.e.

(4)

$$E\text{-}H\text{---}OH_2 + HOH + S \rightleftharpoons [E\text{-}H \; S] + HOH\text{---}OH_2$$
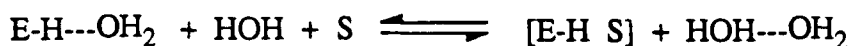
Any free energy difference between the two systems represented by Equations (3) and (4) will be equal to the *difference* in hydrogen bond strengths between enzyme plus substrate and enzyme plus water. A hydrogen bond between a substrate and an enzyme that has evolved to specifically bind the substrate might well be expected to be stronger than that between a protein residue and a free water molecule in solution.

While deoxygenation results in deletion of any hydrogen bond between the enzyme and substrate at that position, substitution of a hydroxyl by a fluorine atom results in two possible scenarios. In Equation (5), the enzyme donates a hydrogen bond which the

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fluorine may accept, while in Equation (6) the enzyme accepts a hydrogen bond and therefore cannot interact favourably with the fluorine.

$$(5)$$
$$\text{E-H} + \text{HO-H---F-S} \rightleftharpoons \text{[E-H---F-S]} + \text{HO-H---OH}_2$$

$$\text{E-A---H-OH} + \text{HO-H---F-S} \rightleftharpoons \text{[E-A} \quad \text{F-S]} + \text{HO-H---OH}_2 \qquad (6)$$
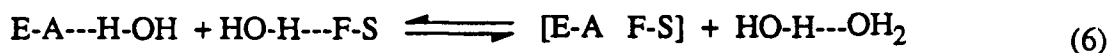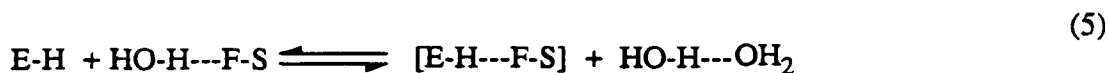
In Equation (6), as the fluorine on the substrate may still act as a hydrogen bond acceptor with water in solution, a net loss of hydrogen bonds results. In this case, the deoxy analog may bind more tightly than the deoxyfluoro substrate.

Kinetic parameters of modified 2',4'-dinitrophenyl glycopyranosides. The series of deoxy and deoxyfluoro 2',4'-dinitrophenyl glycosides synthesized for this study were all substrates for *E. coli (lacZ)* β-galactosidase with the exception of 2FβGalDNP which undergoes degalactosylation sufficiently slowly to be an excellent inactivator (see Sections 3 and 4). Rates of hydrolysis were determined spectrophotometrically by measuring the rate of release of 2,4-dinitrophenol. The data so obtained were fitted to the Michaelis-Menten equation using a weighted least squares non-linear regression program and the kinetic constants k_{cat} and K_m thus determined (Table 6). The data are presented graphically according to the method of Lineweaver and Burk[77] for convenient visual inspection only. These plots were not used to determine k_{cat} and K_m values due to the non-linear error span (Figures 30 to 36).

The enzyme concentrations of the diluted stocks used for each determination are shown in the legends of Figures 30 to 36. Because of low solubility in buffer or high background absorbance, the kinetic parameters of two substrates (6FβGalDNP and 4FβGalDNP, respectively) were determined at substrate concentrations ranging from approximately one-tenth the reported K_m to only slightly above the reported K_m. Other substrates (4dβGalDNP and βGluDNP) bound sufficiently poorly that plots of rate *vs.* [S]

were linear over the substrate concentration range observed. In these cases, approximate values of k_{cat}/K_m were estimated from the Michaelis-Menten equation with $[S] << K_m$

$$v = \frac{k_{cat}[E_0][S]}{K_m}$$

Kinetic parameters of the remaining substrates were generally determined using substrate concentrations bracketing K_m, ranging from one-seventh to seven times K_m.

2',4'-dinitro-phenyl glyco-pyranoside [a]	K_m (mM)	k_{cat} [b] (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)	$\Delta\Delta G^{o\ddagger}$ [f] (kcal mol^{-1})
βGalDNP	0.14 +/- 0.01	1100	7900	---
6dβGalDNP	4.2 +/- 0.5 [c]	23	5.4	4.3
4dβGalDNP	---	---	16 [d]	3.7
3dβGalDNP	n.d.[e]	n.d.[e]	n.d.[e]	
2dβGalDNP	n.d. [e]	n.d.[e]	n.d.[e]	
6FβGalDNP	0.61 +/- 0.06	5.5	9.0	4.0
4FβGalDNP	2.9 +/- 0.3	2.2	0.76	5.5
3FβGalDNP	1.2 +/- 0.1	0.63	0.53	5.7
2FβGalDNP	K_i = 0.89 +/- 0.02	k_i = 0.011 k_{cat} = 2.8 x 10^{-6}	k_i/K_i = 0.012	7.9
βGluDNP	---	---	0.13 [d]	

Table 6. Kinetic parameters of 2',4'-dinitrophenyl β-D-glycopyranosides with E. coli *(lacZ) β-galactosidase.*

[a] All measurements were made in 50 mM sodium phosphate buffer, 1 mM Mg^{2+}, pH 7.00 at 25°C.

[b] k_{cat} values are reported relative to βGalPNP (k_{cat} = 156 s^{-1}) in accordance with Sinnott and Souchard[13] and are +/- approximately 10%.

[c] The maximal concentration of 6dβGalDNP in buffer is approximately 40% of the reported K_m.

[d] Saturation of enzyme not possible due to very poor binding of substrate, i.e. [S] << K_m.

[e] Not determined.

[f] $\Delta\Delta G^{o\ddagger}$ = - RTln{$(k_{cat}/K_m)_2$/ $(k_{cat}/K_m)_1$} where $(k_{cat}/K_m)_2$ is the value for the analog and $(k_{cat}/K_m)_1$ is the value for βGalDNP.
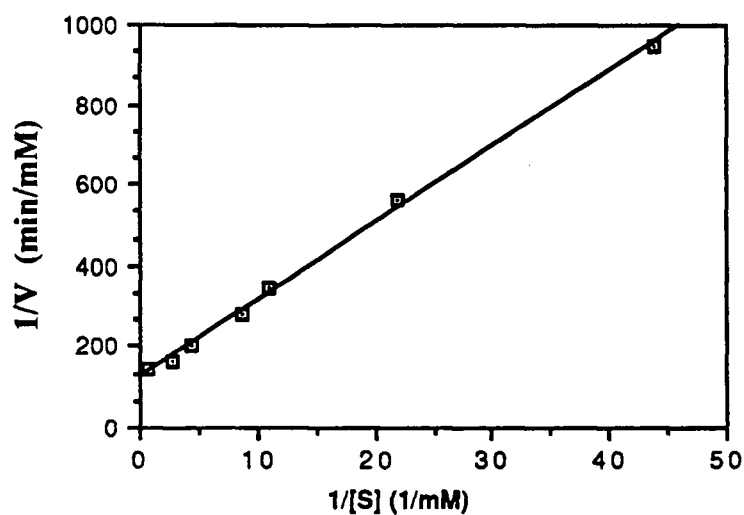
Figure 30. Hydrolysis of 2',4'-dinitrophenyl β-D-galactopyranoside.
Enzyme concentration = 2.24 x 10⁻³ mg mL⁻¹.
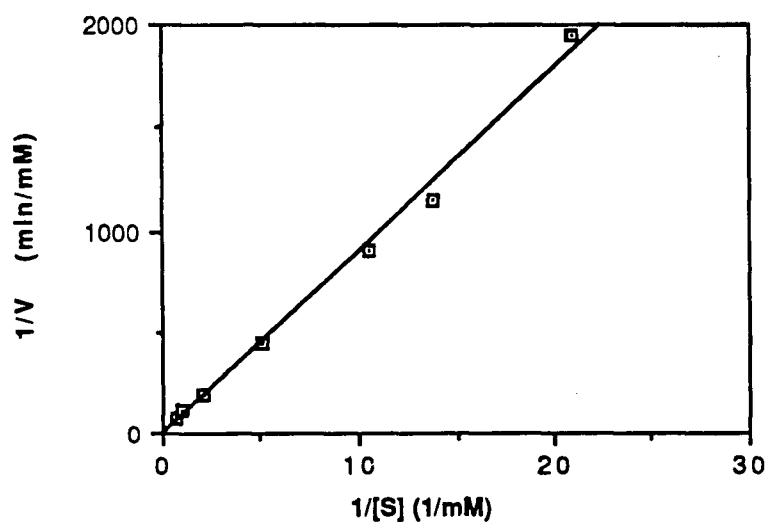
Figure 31. Hydrolysis of 2',4'-dinitrophenyl 6-deoxy-β-D-galactopyranoside.
Enzyme concentration = 1.79 mg mL⁻¹.
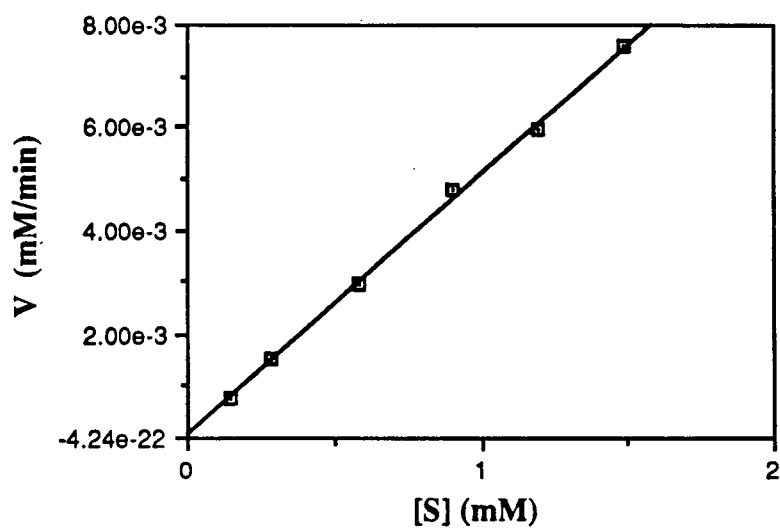
Figure 32. Hydrolysis of 2',4'-dinitrophenyl 4-deoxy-β-D-xylo-hexopyranoside.
Enzyme concentration = 6.78 x 10⁻¹ mg mL⁻¹.
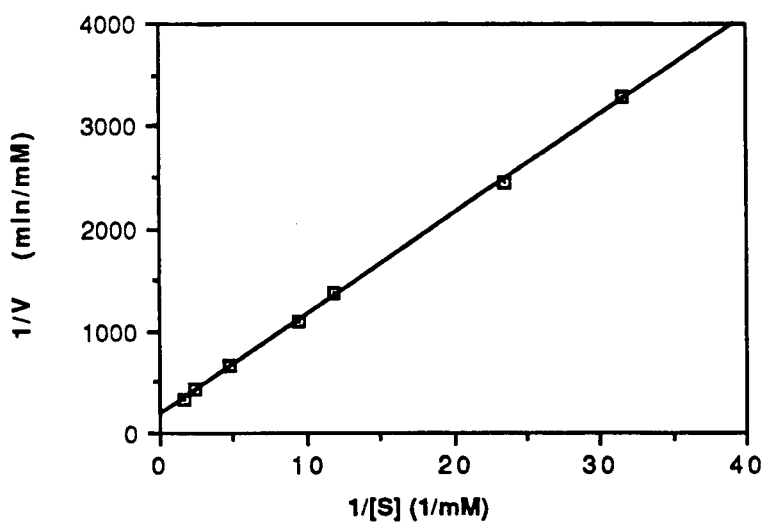
Figure 33. Hydrolysis of 2',4'-dinitrophenyl 6-deoxy-6-fluoro-β-D-galactopyranoside.
Enzyme concentration = 9.10 x 10⁻¹ mg mL⁻¹.
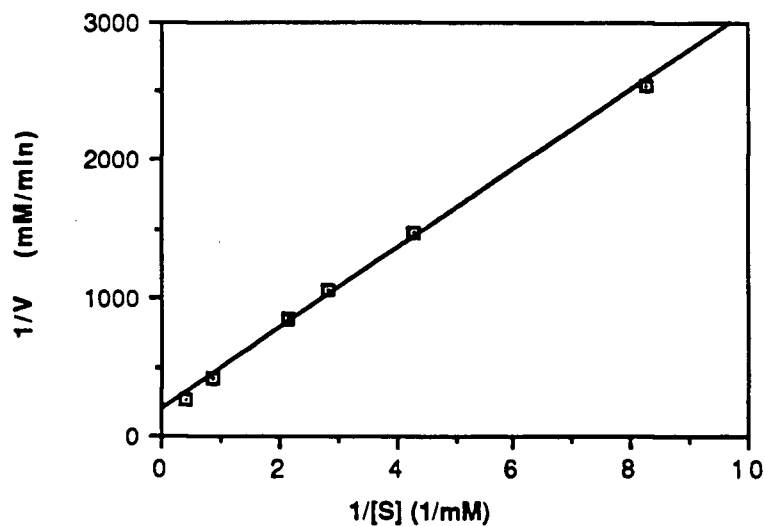
Figure 34. Hydrolysis of 2',4'-dinitrophenyl 4-deoxy-4-fluoro-β-D-galactopyranoside. Enzyme concentration = 1.56 mg mL⁻¹.
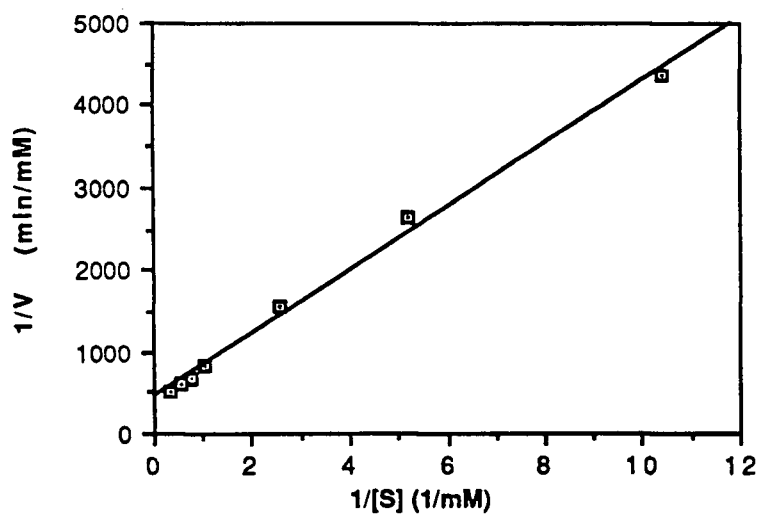
Figure 35. Hydrolysis of 2',4'-dinitrophenyl 3-deoxy-3-fluoro-β-D-galactopyranoside. Enzyme concentration = 7.40 x10⁻¹ mg mL⁻¹.
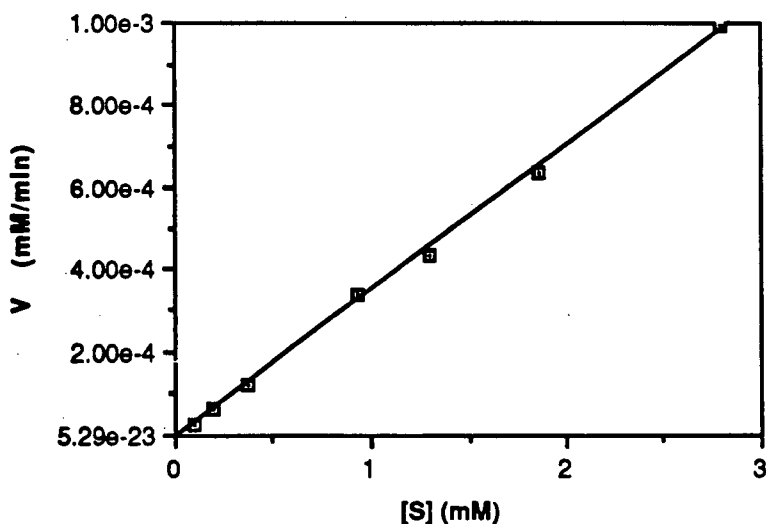
Figure 36. Hydrolysis of 2',4'-dinitrophenyl β-D-glucopyranoside.
Enzyme concentration = 5.70 mg mL^{-1}.

Intrinsic electronic and other effects on glycoside hydrolysis. The double displacement mechanism proposed for the hydrolysis of aryl glycosides by glycosidases (see Figure 2, p. 3) involves transition states with considerable oxocarbonium ion character. On the basis of inductive effects alone, substitution of electronegative fluorine for a hydroxyl on the glycopyranose ring should destabilize these electron-deficient transition states while deoxygenation should have the reverse effect. The effect of such substitutions on the spontaneous, non-enzymic rates of hydrolysis, which have also been shown to proceed *via* oxocarbonium ion-like transition states[73], were determined in order to interpret the effects on enzymic rates. The following spontaneous hydrolysis data were obtained from M. Namchuk, another worker in this laboratory (Table 7).

2',4'-dinitrophenyl glycopyranoside	k(hydrolysis) x 10^{-6} at 25°C (s^{-1})
βGalDNP	4.63
6dβGalDNP	29.3
4dβGalDNP	21.8
6FβGalDNP	1.72
4FβGalDNP	0.662
3FβGalDNP	1.68
2FβGalDNP	0.235
βGluDNP	0.870

Table 7. Spontaneous hydrolysis of a series of deoxy and deoxyfluoro 2',4'-dinitrophenyl β-D-glycopyranosides.[a, b]

[a] Data obtained from M. Namchuk.
[b] Hydrolyses were performed in 25 mM sodium phosphate buffer, 0.8 M KCl, pH 6.5 at various temperatures ranging from 15°C to 85°C., depending on the rate of hydrolysis of the glycoside. Measurements were made at three temperatures for each glycoside and the value of k(hydrolysis) at 25°C determined by extrapolation or interpolation from the resulting Arrhenius plots.

It is clear from the above data that the fluorinated derivatives are all hydrolyzed more slowly than the parent galactopyranoside, while the deoxy analogs are hydrolyzed faster. This is consistent with involvement of an oxocarbonium ion-like transition state which is inductively stabilized or destabilized depending on the electronic nature of the substituent. However, with the deoxyfluoro compounds, the magnitude of the rate constant does not simply reflect the distance of the fluorine atom from the anomeric centre since the observed order is 6-fluoro > 3-fluoro > 4-fluoro > 2-fluoro. This is the same order that was observed by Street et al. in the acid-catalyzed hydrolysis of a series of monofluorinated α-D-glucopyranosyl phosphates,[78] which also hydrolyze with C-O bond cleavage *via* oxocarbonium ion-like transition states between pH 2 and 5. It was proposed

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by these authors that a combination of inductive and dipolar effects, electronic in nature, accounted for the observed order of hydrolysis rates, in particular the larger rate constant for the 3-deoxyfluoro derivative than for the 4-deoxyfluoro derivative. The reverse order was predicted for the deoxy analogs, i.e. 2-deoxy > 4-deoxy > 3-deoxy > 6-deoxy, and was in fact observed with the α-D-glucopyranosyl phosphate derivatives.[79] However, the factors determining the effect of substitution on the rates of hydrolysis of these and similar systems involving glycopyranosyl cation-like transition states have not yet been elucidated unambiguously.

The same order is not observed for the deoxy galactopyranosides synthesized in this study. However, it has been shown[80] that axial substituents which are better accomodated for steric reasons in the more planar half-chair conformation of the oxocarbonium ion transition state than the chair conformation of the ground state (Figure 37) will result in an increase in hydrolysis rate. Thus βGalDNP hydrolyzes some
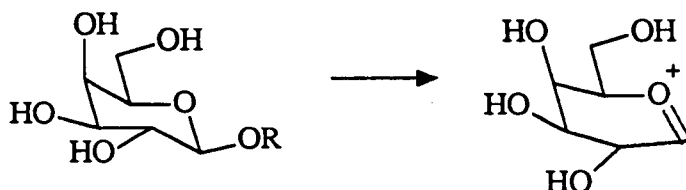
Figure 37. Chair to half-chair transition in the hydrolysis of a β-D-galactopyranoside via an oxocarbonium ion intermediate.

five-fold faster than the corresponding *gluco* analog. This is sufficient to affect the order since the 4-deoxy derivative, which lacks an axial substituent at C(4), would be expected to hydrolyze more slowly relative to the other deoxy galactopyranosides. The predicted order

of hydrolysis rates will then be 2-deoxy > 3-deoxy > 6-deoxy > 4-deoxy > parent. This order is observed for the limited available deoxy analogs and the failure to isolate the 3-deoxy derivative is in itself an indication of its evident instability.

Binding effects. Comparison of the data in Tables 6 and 7 reveals that the decreases in enzymic rate observed upon replacement of a hydroxyl by a fluorine in the deoxyfluoro galactopyranosides (10^3 to 10^8-fold) are significantly greater than the decreases in the rate of spontaneous hydrolysis of the same compounds (3 to 20-fold), indicating that some factor which only affects the enzymic rate contributes to the bulk of the observed rate reductions. As in the case of the 2-deoxyfluoro derivative (discussed in Section 4), this factor is probably related to the loss of specific transition state binding interactions. The importance of such binding interactions to catalysis is perhaps best probed by examining the data for the deoxy glycopyranosides. Purely on the basis of electronic effects, the deoxy substrates would be expected to undergo enzymic hydrolysis faster than the parent galactopyranoside, while in fact they are hydrolyzed more *slowly* by the enzyme. Recall that k_{cat}/K_m represents the change in free energy from free enzyme and free substrate to the transition state for the first irreversible step (Figure 21, p. 36). Thus the effect of modifications on a substrate that alter the stability of this transition state will be reflected in changes in k_{cat}/K_m. Neglecting for the moment other factors which may affect the structure of this transition state, comparison of k_{cat}/K_m for the parent compound with that for the modified substrates gives a measure of the energetic contribution to the stability of the transition state due to enzyme-substrate interactions at that position. These values are expressed as Gibbs free energies ($\Delta\Delta G^{o\ddagger}$) in Table 6 (see Reference 23, p. 350). However, as noted above, the intrinsic electronic effects of the substituents affect transition state stability and also contribute to the value of $\Delta\Delta G^{o\ddagger}$. With the deoxy analogs, these electronic effects will only serve to accelerate the reaction, thus the observed rate decreases

are likely due solely to transition state binding interactions and the $\Delta\Delta G^{o\ddagger}$ values are

minimum estimates of the loss of apparent binding energy resulting from deletion of the

hydroxyl at that position. It has been proposed in a similar study with glycogen

phosphorylase[28], that such values are a measure of the *difference* in hydrogen bond

strengths between enzyme plus substrate at the transition state (of the first irreversible step)

and the unbound substrate plus water. The apparent binding energies are approximately 4

kcal mol^{-1} at the 4 and 6 positions. These values are consistent with estimates by Fersht[23]

that a strong hydrogen bond between one neutral and one charged group may contribute 3

kcal mol^{-1} or more to the overall binding free energy. Thus, assuming a deleted hydrogen

bond is responsible for the bulk of the loss of binding energy, these results may suggest

one strong, or several weak, hydrogen bonds at each of the 4- and 6-positions at the

transition state. Unfortunately, no data is available for the 2- or 3-deoxy derivatives.

Because the $\Delta\Delta G^{o\ddagger}$ values for the deoxyfluoro substrates cannot be simply interpreted in

terms of hydrogen bonding, it is not possible to assign hydrogen bond polarities at each

position.

It is noteworthy that the *gluco* analog, βGluDNP, which differs from the

corresponding galactopyranoside only in the orientation of its C(4) hydroxyl, is hydrolyzed

by the enzyme some 10^4-fold slower than βGalDNP. In contrast, the 4-deoxyfluoro

analog, in which fluorine has been substituted for hydroxyl while retaining the *galacto*

configuration, suffers a smaller reduction in rate even with a more unfavourable intrinsic

electronic factor. It is likely that the great reduction in the rate of βGluDNP hydrolysis is

due not simply to a loss of favourable hydrogen bonding interactions alone but to a severe

steric clash in the active site as a hydroxyl group is thrust into a space normally occupied by

a hydrogen atom.

Electronic effects. It is thought that the effect of substitution of hydrogen or fluorine for hydroxyl on the spontaneous hydrolysis rates of 2',4'-dinitrophenyl galactopyranosides is primarily electronic in nature.[73,78,79] Fluorine substitution decreases the hydrolysis rate while deoxygenation leads to a marked increase. If the enzymic reaction proceeds through a transition state similar to the non-enzymic process and the electronic effect of the substitutions was the only factor affecting the free energy of the transition state, then a similar relationship between hydrolysis rate and substituent should also be seen with the enzymic reaction. This linear free energy relationship may be expressed as

$$k_A = Ck_B{}^\beta$$

where k_A and k_B are the rate constants for the two reactions, A and B, while C and β are constants. From the Eyring equation

$$k_A = (kT/h)\exp\{-\Delta G^{\ddagger}{}_A/RT\}$$

and $$k_B = (kT/h)\exp\{-\Delta G^{\ddagger}{}_B/RT\}$$

where $\Delta G^{\ddagger}$ is the activation energy for reaction of A or B. Thus plotting the logarithm of the rate constants for the two processes against each other provides information about the similarity of the transition states involved. The correlation coefficient (r) reflects the degree of certainty in the relationship, while the slope (β) of the line is a measure of the similarity of the two transition states in terms of charge development or some other parameter.

However, we have already seen evidence for the importance of binding effects in catalysis by *E. coli (lacZ)* β-galactosidase. In addition to the electronic effects of deoxygenation and fluorine substitution on transition state stability, these modifications are also likely to perturb enzyme-substrate binding at the transition state. Thus the electronic

effects on transition state stability will be superimposed upon perturbations of transition state energy due to altered binding interactions. A linear free energy relationship considering electronic factors alone may then be masked by binding effects.

Street et al.[28] found a reasonable linear free energy correlation (r = 0.9) between $\log(V_{max})$ of the enzyme-catalyzed hydrolysis of a series of deoxy and deoxyfluoro α-D-glucopyranosyl phosphates and $\log(k)$ of the acid-catalyzed hydrolysis of the same compounds. Since the acid-catalyzed reaction is known to proceed *via* an oxocarbonium ion-like transition state, these results provided good evidence that the enzymic process involves a similar transition state at the rate-limiting step of the reaction. A plot of $\log(V_{max}/K_m)$ *vs.* $\log(k)$ for the same compounds was essentially a scatter plot. This scatter was interpreted as being due to a greater contribution from binding interactions in this plot, since V_{max}/K_m represents the overall activation free energy from *free* enzyme and *free* substrate to the *enzyme-bound* transition state of the first irreversible step, while k_{cat} represents the energy difference between an *enzyme-bound* intermediate and the *enzyme-bound* transition state of the rate-determining step (see Figure 21, p. 36). Thus in the latter case, some of the contribution of the binding energy is in effect cancelled.

A similar treatment was applied to the data in this study and the results are shown in Figure 38. The plot of $\log(k_{cat}/K_m)$ *vs.* $\log(k_{hydrolysis})$ shows a correlation (r = 0.80), indicating that the electronic nature of the enzymic and non-enzymic transition states is similar. Since the spontaneous process has been shown to proceed *via* oxocarbonium ion-like transition states[73], these data are consistent with an enzymic mechanism involving a similar transition state at the galactosylation step. Recall that k_{cat}/K_m is associated with the energy barrier between free enzyme and free substrate and the transition state of the first irreversible step (presumably galactosylation).
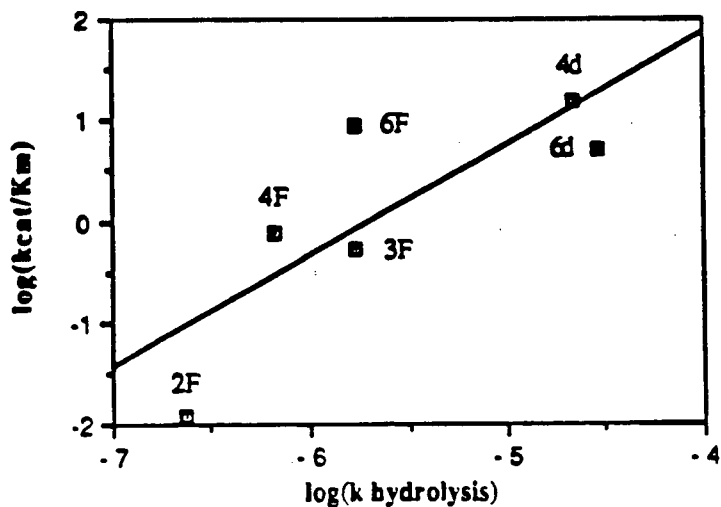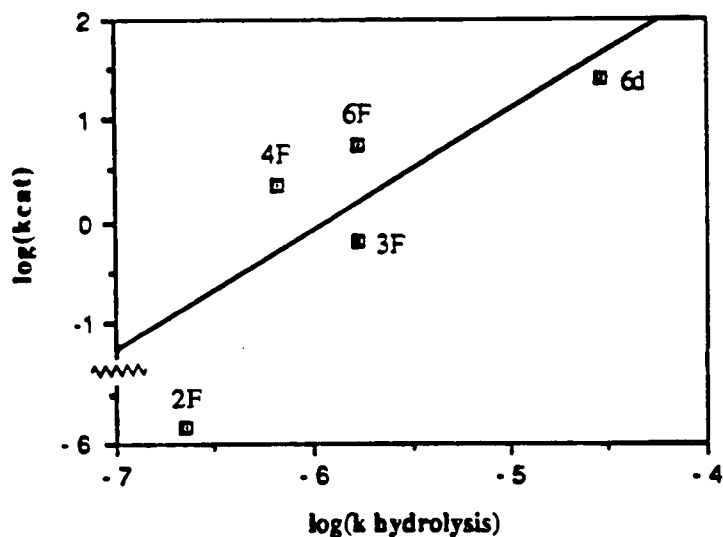
Figure 38. Linear free energy relationships between kinetic parameters for the β-galactosidase-catalyzed reaction and the spontaneous hydrolysis of a series of deoxy and deoxyfluoro 2',4'-dinitrophenyl glycopyranosides. A) Plot of log(k_cat/K_m) vs. the logarithm of the spontaneous hydrolysis rate constant; B) plot of log(k_cat) vs. the logarithm of the spontaneous hydrolysis rate constant.

That a correlation is observed between $\log(k_{cat}/K_m)$ and $\log(k_{hydrolysis})$ in Figure 38 A), even with interferences from binding effects, might be interpreted as suggesting that binding effects are relatively unimportant to stabilization of this transition state. However, this is unlikely in light of the vast decreases in rate observed even with the deoxy substrates. The very fact that the correlation is *not* perfect suggests the presence of transition state binding interactions not present in the non-enzymic reaction.

Note that scatter due to binding effects will occur *if the magnitude and polarity of the contributions to transition state stabilization due to binding at each position are significantly different from each other*. If, for example, hydrogen bonding interactions at each position contributed approximately the same stabilization to the transition state then a linear free energy correlation reflecting electronic effects might still be observed among the deoxy substrates. However, the ability of the deoxyfluoro substrates to accept hydrogen bonds from appropriately positioned donor residues and consequent possible rate increases for these substrates may introduce considerable scatter in a correlation reflecting electronic effects alone. Nevertheless, an efficient enzyme would be expected to utilize potential hydrogen bonding interactions at *all* positions as fully as possible in order to stabilize the transition state. Specific changes in the geometry of the substrate as the transition state is approached, however, impose certain restrictions on the relative importance of binding interactions at a given position. Thus, in the chair to half-chair transition of a developing oxocarbonium ion (Figure 37), interactions at C(2), where a considerable amount of restructuring is occuring at the transition state, would be expected to be more important. However, in a highly efficient enzyme, these differences would likely be minimized because contributions at other positions would be utilized to their fullest potential.

The plot of $\log(k_{cat})$ *vs.* $\log(k_{hydrolysis})$ in Figure 38 B) shows a similar correlation ($r = 0.78$), if the point for the 2-deoxyfluoro deriviative is excluded. Recall that k_{cat} is associated with the activation energy for the rate determining step (degalactosylation for the parent galactopyranoside and the turnover of the 2-deoxy-2-fluorogalactosyl enzyme). The

essential difference between the two plots arises from the very slow turnover of the 2-deoxy-2-fluorogalactosyl enzyme, reflected by a very large decrease in k_{cat}. This may be accounted for by the loss of strong transition state binding interactions with the C(2) hydroxyl which are more important at the transition state for degalactosylation than at the transition state for galactosylation, or perhaps more likely, that the transition state for degalactosylation has more oxocarbonium ion character and is inductively destabilized to a greater extent by the adjacent fluorine atom.

Excluding the data for the 2-deoxyfluoro derivative from both plots, the slopes are 0.60 for the $\log(k_{cat}/K_m)$ plot in Figure 38 A) and 0.73 for the $\log(k_{cat})$ plot in Figure 38 B). This suggests that both the transition state associated with galactosylation and the transition state associated with degalactosylation have significantly less oxocarbonium ion character than the transition state for spontaneous hydrolysis. This is consistent with participation of an enzymic nucleophile which can pre-asssociate with a developing oxocarbonium ion-like species. A greater degree of oxocarbonium ion character at the transition state for degalactosylation as suggested above may be accounted for by preassociation of the nucleophile to a greater extent at the galactosylation transition state. This is reasonable since the nucleophile in the galactosylation step, a potentially charged carboxylate, can readily interact with the developing positive charge at the anomeric centre. In the degalactosylation transition state, the nucleophile is a neutral water and hence is likely to preassociate to a lesser extent with a developing oxocarbonium ion-like species. This interpretation is supported by the α-deuterium kinetic isotope effect data of Sinnott and Souchard[13] as follows. Aryl galactosides with activated leaving groups (e.g. 2,4-dinitrophenolate and 3,5-dinitrophenolate), where degalactosylation is rate limiting (as shown by methanol competition experiments[65]), exhibited larger kinetic isotope effects than galactosides with less activated leaving groups (e.g. 4-nitrophenolate), where galactosylation is thought to be the rate determining step.[81]

7. Conclusions

2FβGalDNP was found to be an effective mechanism-based inactivator of *E. coli (lacZ)* β-galactosidase. This compound thus joins a class of 2-deoxy-2-fluoro glycosides which inactivate "retaining" glycosidases by the accumulation of a stable glycosyl-enzyme intermediate. The active site-directed nature of 2FβGalDNP was shown by protection against time-dependent inactivation by a competitive ligand and the near 1:1 stoichiometry of dinitrophenolate release with enzyme inactivation. Furthermore, when freed from excess inactivator the 2-deoxy-2-fluorogalactosyl enzyme intermediate turned over slowly in buffer and exhibited enhanced rates of reactivation in the presence of the acceptors methanol or glucose, providing strong evidence that the intermediate is catalytically competent.

A series of deoxy and deoxyfluoro analogs of βGalDNP was prepared for this study. All of the compounds, with the exception of 2FβGalDNP which undergoes degalactosylation sufficiently slowly to be an excellent inactivator, were substrates for *E. coli (lacZ)* β-galactosidase although the rates of enzymic hydrolysis were two to four orders of magnitude slower than have been measured for βGalDNP. These large rate reductions are thought to result primarily from the loss of important transition state binding interactions due to the substitution of a hydrogen or a fluorine for a hydroxyl at a given position. These results strongly suggest that much of the catalytic power of the enzyme is derived from non-covalent interactions between the enzyme active site and the galactopyranose ring of the substrate.

A linear free energy relationship was shown to exist between the logarithm of k_{cat}/K_m for the enzyme-catalyzed reaction and the logarithm of the first order rate constant for the spontaneous hydrolysis of the same series of deoxy and deoxyfluoro glycopyranosides, $k_{hydrolysis}$. Since the spontaneous process has considerable oxocarbonium ion character at the transition state, this suggests that the enzymic transition

state associated with k_{cat}/K_m (presumably representing the galactosylation step) is also electron deficient and is affected by the electronic effects of the substituents in a manner similar to that observed for the spontaneous process. A similar correlation is observed in a plot of the logarithm of k_{cat} *vs.* the logarithm of $k_{hydrolysis}$, if the point for the 2-deoxyfluoro derivative is excluded (k_{cat} represents the degalactosylation step for the parent compound, the turnover of the 2-deoxy-2-fluorogalactosyl enzyme and presumably represents degalactosylation for the other glycosides as well). While it is possible that galactosylation has become rate determining for some substrates, this seems unlikely since both the fastest and slowest substrates (the parent compound and the 2-deoxyfluoro derivative, respectively) exhibit rate-limiting degalactosylation. The very small value of k_{cat} for the 2-deoxyfluoro derivative is significant, and when interpreted in light of the linear free energy relationships discussed above, may suggest that some binding or electronic factor associated with substition of fluorine at C(2) destabilizes the transition state for degalactosylation to a greater extent than the transition state for galactosylation.

Chapter III

MATERIALS AND METHODS

1. <u>Synthesis</u>

1.1 <u>General methods and materials</u>

Melting points (m.p.) were determined on a Laboratory Devices Mel-temp II melting point apparatus, and are uncorrected.

^{1}H-nuclear magnetic resonance (nmr) spectra were recorded on 300 MHz Varian XL-300 or Bruker 400 MHz WH-400 instruments. Chemical shifts are given in the delta (δ) scale. With samples in CDCl$_3$ or CD$_3$OD, tetramethylsilane (TMS) was used as an internal reference (δ = 0.00 ppm). Samples in D$_2$O are referenced to external 2,2-dimethyl-2-silapentane-5-sulphonate (δ = 0.00 ppm).

^{19}F nmr spectra were recorded on a 200 MHz Bruker AC-200 instrument and are proton decoupled. Chemical shifts are given in the delta (δ) scale with reference to CFCl$_3$ (δ = 0.00 ppm). Signals upfield of this resonance are assigned a positive value. External trifluoroacetic acid (δ = 76.53 ppm) was used as reference.

Micro-analyses were performed by Mr. P. Borda, Microanalytical laboratory, University of British Columbia, Vancouver, B.C.

Solvents and reagents used were either reagent grade, certified or spectral grade. Dry solvents or reagents where indicated were prepared as follows: Methanol was distilled from magnesium methoxide prepared *in situ* by reaction of methanol with magnesium turnings. Acetonitrile, dichloromethane and pyridine were distilled from calcium hydride. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl. Dimethyl sulphoxide (DMSO) was distilled from sodium hydroxide under reduced pressure. N,N-

dimethyl formamide (DMF) was pre-dried over calcium sulphate then distilled from 4 Å molecular sieves. Acetyl chloride was dried by refluxing several hours over phosphorus pentachloride, followed by distillation.

Thin-layer chromatography (tlc) separations were performed using Merck Kieselgel 60 F-254 analytical plates. Compounds were visualized (where possible) under UV light or by charring with 10% sulphuric acid in methanol. Column chromatography was performed according to the method of Clarke-Still et al.[82], using silica gel columns of Kieselgel 60 (180-230 mesh). The following solvent systems were commonly employed: Solvent **A**: 1:1 ethyl acetate/ hexanes; Solvent **B**: 5% methanol/ ethyl acetate; Solvent **C**: 7:2:1 ethyl acetate/ ethanol/ water.

Several compounds or precursors in this work were obtained from others: From Dr. D. Dolphin; 3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl fluoride. From Dr. A. Becalski; 2',4'-dinitrophenyl 4-deoxy-4-fluoro-β-D-galactopyranoside. From M. Namchuk; 2',4'-dinitrophenyl β-D-glucopyranoside and 2',4'-dinitrophenyl 4-deoxy-β-D-xylo-hexopyranoside. From I. Street; methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-chloro-α-D-glucopyranoside and methyl 2,3,6-tri-O-benzoyl-4-O-methanesulphonyl-α-D-galactopyranoside.

1.2 General procedures

1.2.1 Fluorination

To a 3-necked round bottom flask fitted with a pressure equalized addition funnel was added the suitably protected carbohydrate derivative and the appropriate base dissolved in dry dichloromethane. The apparatus was flushed with dry nitrogen and cooled to - 20°C (carbon tetrachloride/ dry ice bath). Diethylaminosulphur trifluoride (DAST, Aldrich Chemical Co.) was then slowly added over a period of 15 min. After the addition was

complete, the mixture was allowed to warm slowly to ambient temperature. Upon completion, the reaction mixture was cooled to -20°C and quenched with addition of excess methanol. Solvent was removed *in vacuo* , the resulting gum was dissolved in dichloromethane, washed with a saturated sodium bicarbonate solution, dried (MgSO$_4$) and purified by column chromatography.

1.2.2 Acetylation

Acetic anhydride was added dropwise to an ice-cooled solution of pyridine and the carbohydrate derivative. The mixture was stirred until the reaction was complete by tlc (Solvent A) then cooled (0°C), stirred with excess methanol for 1 h and the solvent evaporated. Removal of pyridine was facilitated by several co-evaporations with toluene. The resulting oil was then washed successively with 5% hydrochloric acid, saturated sodium bicarbonate and water and dried (MgSO$_4$) or purified by column chromatography.

1.2.3 Selective deacetylation at the 1 position

a) *with 2-aminoethanol*[45]

The appropriate peracetylated carbohydrate derivative was dissolved in ethyl acetate, 2-aminoethanol was added and the resulting mixture stirred at ambient temperature. Upon completion of reaction, as indicated by tlc (Solvent A), solvent was removed *in vacuo*, the residual oil was dissolved in chloroform, washed once with water, dried (MgSO$_4$) and purified by column chromatography.

b) *with hydrazine acetate*[64]

A suspension of the appropriate peracetylated carbohydrate derivative and hydrazine acetate was heated in DMF at 50°C for ~ 3 minutes to dissolve the starting material. The mixture was then stirred ~ 20 minutes at ambient temperature. Upon completion of reaction, as indicated by tlc (Solvent A), the mixture was diluted with ethyl acetate, washed with a saturated brine solution and dried (MgSO4). DMF was removed by several co-evaporations with toluene and the residual oil was purified by column chromatography.

1.2.4 2',4'-Dinitrophenyl glycopyranoside formation[37]

The appropriate 1-deacetylated glycopyranose derivative and 1,4 diazabicyclo[2.2.2]octane (DABCO) were dissolved separately in DMF and stirred over 4 A molecular sieves for 2 to 3 h. The DABCO solution was added to the carbohydrate derivative under nitrogen and 1-fluoro-2,4-dinitrobenzene (FDNB, dried over P2O5 for 3 h) was added. The mixture was stirred for 2 h at ambient temperature, filtered to remove sieves and concentrated *in vacuo* to an orange syrup. This was dissolved in dichloromethane, washed with saturated sodium bicarbonate solution and water and dried (MgSO4). Removal of solvent gave a brownish yellow gum which yielded a solid upon trituration with ethanol. Subsequent recrystallization from ethanol or ethyl acetate/ hexanes gave white or faintly yellow crystals of the acetylated glycopyranoside.

1.2.5 Deacetylation with HCl/ methanol[38]

The acetylated glycopyranoside was suspended in dry methanol (14 mg/mL), cooled to 0°C under nitrogen and sufficient freshly distilled acetyl chloride added to give a final HCl concentration of 3-5% (w/v). The reaction mixture was stirred ~ 18 to 36 h at 4°C until the reaction was complete by tlc (Solvent B) then the solvent was evaporated *in vacuo*, and the product was washed several times with anhydrous diethyl ether to remove

residual acid. The resulting gum was dissolved in dry methanol or acetone, then triturated with diethyl ether or diethyl ether plus hexanes to induce crystallization.

1.3 2',4'-Dinitrophenyl glycopyranosides

2',4'-Dinitrophenyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (2)

1,2,3,4,6-Penta-O-acetyl-D-galactopyranose (1.00 g, 2.60 mmol) was selectively deacetylated at the 1 position with 2-aminoethanol (0.23 mL, 3.8 mmol) according to the general procedure by stirring in ethyl acetate (75 mL) at room temperature for 60 h. Column chromatography (Solvent A) afforded an anomeric mixture of tetra-O-acetates **1** as a faintly yellow foam (0.82 g, 2.4 mmol, 60%). This material (0.54 g, 1.6 mmol) was converted to the galactopyranoside **2** according to the general procedure by reaction with DABCO (0.60 g, 5.5 mmol) and FDNB (0.34 g, 1.8 mmol). The resulting solid was recrystallized from ethanol to afford a white crystalline powder **2** (0.50 g, 0.97 mmol, 63%). M.p. 174-175°C (lit.[38] m.p. 174-176°C); ^{1}H nmr (300 MHz, CDCl$_3$): δ 8.72 (d, 1 H, $J_{3',5'}$ 3 Hz, H(3')), 8.45 (dd, 1 H, $J_{5',6'}$ 10, $J_{3',5'}$ 3 Hz, H(5')), 7.50 (d, 1 H, $J_{5',6'}$ 10 Hz, H(6')), 5.60 (dd, 1 H, $J_{1,2}$ 8, $J_{2,3}$ 11 Hz, H(2)), 5.50 (d, 1 H, $J_{3,4}$ 3.5 Hz, H(4)), 5.23 (d, 1 H, $J_{1,2}$ 8 Hz, H(1)), 5.14 (dd, 1 H, $J_{2,3}$ 11, $J_{3,4}$ 3.5 Hz, H(3)), 4.3-4.1 (m, 3 H, H(5), H(6, 6')), 2.20, 2.15, 2.10, 2.05 (4 x s, 12 H, 4 x OAc). Anal. calc. for C$_{20}$H$_{22}$O$_{14}$N$_2$; C, 46.70; H, 4.31; N, 5.45%; Found: C, 46.77; H, 4.40; N, 5.33%.

2',4'-Dinitrophenyl β-D-galactopyranoside (3)

The tetra-O-acetate **2** (300 mg, 0.585 mmol) was deacetylated according to the general deacetylation procedure for 18 h. The product crystallized upon trituration with

diethyl ether and was recrystallized twice from acetone/ diethyl ether to give white powdery crystals of **3** (64 mg, 0.185 mmol, 32%). M.p. 134-135°C (dec) (lit.[38] m.p. 161-163°C); ^{1}H nmr (400 MHz, CD$_3$OD): δ 8.70 (d, 1 H, J$_{3',5'}$ 3 Hz, H(3')), 8.48 (dd, 1 H, J$_{5',6'}$ 10, J$_{3',5'}$ 3 Hz, H(5')), 7.65 (d, 1 H, J$_{5',6'}$ 10 Hz, H(6')), 5.22 (d, 1 H, J$_{1,2}$ 8 Hz, H(1)), 4.2-3.2 (m, 6 H, H(2-6,6')). Anal. calc. for C$_{12}$H$_{14}$O$_{10}$N$_2$+0.5CH$_3$OH+H$_2$O; C, 39.48; H, 4.77; N, 7.37%; Found: C, 39.15; H, 4.39; N, 7.05%.

3,4,6-Tri-O-acetyl-2-deoxy-2-fluoro-α-D-galactopyranosyl bromide (4)

2-Deoxy-2-fluoro-α-D-galactopyranosyl fluoride (1.0 g, 3.2 mmol) was dissolved in 45% w/v hydrogen bromide in glacial acetic acid (10 mL), 5 drops of acetic anhydride was added and the reaction mixture was stirred at room temperature for 4 h. The mixture was dissolved in dichloromethane, washed with saturated sodium bicarbonate solution and water, and dried (MgSO$_4$). Evaporation of solvent gave **4** as a white semi-solid mass which was stored under hexanes at 0°C (1.10 g, 2.97 mmol, 92%). [1]H nmr (300 MHz, CDCl$_3$): δ 6.62 (d, 1 H, J$_{1,2}$ 4 Hz, H(1)), 5.55 (t, 1 H, J$_{4,F}$ 3, J$_{3,4}$ 3 Hz, H(4)), 5.49 (dt, 1 H, J$_{3,4}$ 3, J$_{2,3}$ 10, J$_{3,F}$ 10 Hz, H(3)), 4.77 (ddd, 1 H, J$_{1,2}$ 4, J$_{2,3}$ 10, J$_{2,F}$ 51 Hz, H(2)), 4.53 (t, 1 H, J$_{5,6'}$ 6.5, J$_{5,6}$ 7 Hz, H(5)), 4.16 (AB multiplet, 2 H, J$_{6,6'}$ 11.5, J$_{5,6'}$ 6.5, J$_{5,6}$ 7 Hz, H(6,6')), 2.16, 2.07, 2.06 (3 x s, 9 H, 3 x OAc). [19]F nmr (CDCl$_3$): δ 195.52.

2',4'-Dinitrophenyl 3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-β-D-galactopyranoside (6)

The bromide **4** (0.58 g, 1.57 mmol) was dissolved in acetone (5 mL) containing 4 drops of water and freshly prepared silver carbonate (0.37 g, 1.34 mmol) at 0°C in the dark. The reaction mixture was stirred for 1 h at 0°C then 24 h at room temperature. The suspension was filtered, the precipitated solids washed well with acetone and the filtrate

evaporated to give a yellow oil. This was purified by column chromatography (Solvent A) to give an anomeric mixture of **5** (0.32 g, 1.04 mmol, 67%), as a colourless oil. [19]F nmr (CDCl$_3$): δ 207.38, 207.86. This material (0.30 g, 0.97 mmol) was converted to the galactopyranoside **6** according to the general procedure by reaction with DABCO (0.38 g, 3.30 mmol) and FDNB (0.22 g, 1.18 mmol). [1]H nmr indicated the resulting yellow syrup was a 3:1 mixture of β and α anomers. This syrup was dissolved in ethyl acetate and hexanes were added to selectively crystallize the β anomer. This material was recrystallized from ethyl acetate/ hexanes to afford white needles of **6** (0.19 g, 0.40 mmol, 42%). M.p. 160-161°C; ^{1}H nmr (300 MHz, CDCl$_3$): δ 8.80 (d, 1 H, $J_{3',5'}$ 3 Hz, H(3')), 8.45 (dd, 1 H, $J_{5',6'}$ 10, $J_{3',5'}$ 3 Hz, H(5')), 7.42 (d, 1 H, $J_{5',6'}$ 10 Hz, H(6')), 5.54 (t, 1 H, $J_{4,F}$ 3, $J_{3,4}$ 3 Hz, H(4)), 5.39 (dd, 1 H, $J_{1,2}$ 7.5, $J_{1,F}$ 4 Hz, H(1)), 5.25 (dt, 1 H, $J_{3,4}$ 3, $J_{2,3}$ 10, $J_{3,F}$ 10 Hz, H(3)), 4.90 (ddd, 1H, $J_{1,2}$ 7.5, $J_{2,3}$ 9, $J_{2,F}$ 52 Hz, H(2)), 4.18 (m, 3 H, H(5), H(6,6')), 2.20, 2.12, 2.10 (3 x s, 9 H, 3 x OAc). [19]F nmr (CDCl$_3$): δ 206.74. Anal. calc. for C$_{18}$H$_{19}$O$_{12}$N$_2$F; C, 45.58; H, 4.04; N, 5.91%; Found: C, 45.71; H, 4.10; N, 5.85%.

2',4'-Dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside (7)

The tri-O-acetate **6** (80 mg, 0.169 mmol) was deacetylated according to the general deacetylation procedure for 33 h. The product was recrystallized from methanol/ diethyl ether/ hexanes to give white crystals of **7** (30 mg, 0.086 mol, 51%). M.p. 174-175°C; ^{1}H nmr (400 MHz, CD$_3$OD): δ 8.73 (d, 1 H, $J_{3',5'}$ 3 Hz, H(3')), 8.48 (dd, 1 H, $J_{3',5'}$ 3, $J_{5',6'}$ 10 Hz, H(5')), 7.67 (d, 1 H, $J_{5'6'}$ 10 Hz, H(6')), 5.50 (dd, 1 H, $J_{1,2}$ 7.5, $J_{1,F}$ 4 Hz, H(1)), 4.65 (ddd, $J_{1,2}$ 7.5, $J_{2,3}$ 9, $J_{2,F}$ 52 Hz, H(2)), 4.0-3.7 (m, 5 H, H(3-6,6')). [19]F nmr (D$_2$O): δ 216.32. Anal. calc. for C$_{12}$H$_{13}$O$_9$N$_2$F; C, 41.39; H, 3.76; N, 8.04%; Found: C, 41.41; H, 3.80; N, 7.98%

1,2:5,6-Di-O-isopropylidene-α-D-ribo-hexofuranos-3-ulose hydrate (8)

1,2:5,6-Di-O-isopropylidene-α-D-glucofuranose[39] (10 g, 38.4 mmol) was dissolved in DMSO (100 mL) then phosphorus pentoxide (10 g, 70.4 mmol) was added slowly with ice-bath cooling. After stirring 60 h at room temperature, the mixture was diluted with dichloromethane (100 mL) and washed successively with water, saturated sodium bicarbonate solution and water, then dried ($MgSO_4$). The resulting syrup was chromatographed (Solvent A) to afford faintly yellow crystals of **8** (5.0 g, 18.1 mmol, 50%). M.p. 98-99°C (lit.[40] m.p. 109-111°C); ^{1}H nmr (300 MHz, $CDCl_3$): δ 6.15 (d, 1 H, $J_{1,2}$ 4 Hz, H(1)), 4.42-4.3 (m, 3 H, H(2), H(4), H(5)), 4.10-4.00 (m, 2 H, H(6,6')), 1.47 (s, 3 H, CH_3), 1.45 (s, 3 H, CH_3), 1.34 (s, 6 H, 2 x CH_3).

3-O-Acetyl-1,2:5,6-di-O-isopropylidene-α-D-erythro-hex-3-enofuranose (9)

To a solution of the ulose hydrate **8** (5.0 g, 18.1 mmol) in dry pyridine (50 mL) was added acetic anhydride (25 mL, 210 mmol). The flask was sealed and heated to 75°C for 16 h. After evaporation of the pyridine *in vacuo*, the resulting syrup was dissolved in dichloromethane, washed with cold 0.5 N hydrochloric acid, saturated sodium bicarbonate solution and water, charcoal-treated and dried ($MgSO_4$). After evaporation of solvent the gummy residue was crystallized from methanol/ water to give white crystals of **9** (2.53 g, 8.43 mmol, 47%). M.p. 60°C (lit.[42] m.p. 62°C); ^{1}H nmr (300 MHz, $CDCl_3$): δ 6.06 (d, 1 H, $J_{1,2}$ 5.5 Hz, H(1)), 5.41 (d, 1 H, $J_{1,2}$ 5.5 Hz, H(2)), 4.71 (t, 1 H, $J_{5,6}$ 7, $J_{5,6'}$ 7 Hz, H(5)), 4.09 (m, 2 H, H(6, 6')), 2.21 (s, 3 H, OAc), 1.55, 1.48, 1.45, 1.38 (4 x s,12 H, 4 x CH_3).

1,2:5,6-Di-O-isopropylidene-α-D-gulofuranose (10)

To an ice-cooled solution of enol acetate **9** (2.50 g, 8.33 mmol) in dry methanol (60 mL) was added quickly sodium borohydride (1.25 g, 33.0 mol). The mixture was stirred at 0°C for 30 min then at room temperature for 30 min before quenching with water. Solvent was evaporated *in vacuo* then the residue was dissolved in chloroform, washed once with water and dried (MgSO4). Column chromatography (Solvent A) afforded **10** as a white solid (0.44 g, 1.69 mmol, 20%). M.p. 103°C (lit.[43] m.p. 105-106°C); ^{1}H nmr (300 MHz, CDCl3): δ 5.79 (d, 1 H, $J_{1,2}$ 3.5 Hz, H(1)), 4.67 (dd, 1 H, $J_{1,2}$ 3.5, $J_{2,3}$ 6 Hz, H(2)), 4.48 (ddd, 1 H, $J_{2,3}$ 6, $J_{3,4}$ 6, $J_{3,OH}$ 7 Hz, H(3)), 4.24 (m, 2 H, H(4), H(5)), 3.80 (AB multiplet, 2 H, $J_{5,6}$ 6, $J_{5,6'}$ 7, $J_{6,6'}$ 9 Hz, H(6,6')), 2.68 (d, 1 H, $J_{3,OH}$ 7 Hz, 3-OH), 1.63, 1.47, 1.43, 1.39 (4 x s, 12 H, 4 x CH3).

1,2:5,6-Di-O-isopropylidene-3-deoxy-3-fluoro-α-D-galactofuranose (11)

The partially protected gulofuranose **10** (0.44 g, 1.69 mmol) was treated with DAST (0.60 g, 3.72 mmol) and 4-(dimethylamino)pyridine (0.41 g, 3.38 mmol) according to the general fluorination procedure and stirred at room temperature for 18 h. Column chromatography (10% ethyl acetate/ hexanes) gave white crystals of **11** (0.33 g, 1.26 mmol, 75%). M.p. 46-47°C; ^{1}H nmr (400 MHz, CDCl3): δ 5.90 (d, 1 H, $J_{1,2}$ 4 Hz, H(1)), 4.82 (ddd, 1 H, $J_{2,3}$ 0.5, $J_{3,4}$ 3, $J_{3,F}$ 52 Hz, H(3)), 4.72 (ddd, 1 H, $J_{2,3}$ 0.5, $J_{1,2}$ 4, $J_{2,F}$ 15 Hz, H(2)), 4.34 (m, 1 H, H(5)), 4.08 (m, 2 H, H(4), H(6)), 3.80 (dd, 1

H, $J_{5,6'}$ 6.5, $J_{6,6'}$ 8 Hz, H(6')), 1.52, 1.43, 1.35, 1.33 (4 x s, 12 H, CH$_3$). ^{19}F nmr (CDCl$_3$): δ 187.79.

3-Deoxy-3-fluoro-D-galactopyranose (12)

The protected galactofuranose derivative **11** (0.33 g, 1.26 mmol) was suspended in 1:2 ethanol/ water and stirred with Amberlite IR-120(H$^+$) cation exchange resin for 6 h at 60°C. The resin was removed by filtration and the residual gum purified by column chromatography (Solvent C) to afford an anomeric mixture of the free deoxyfluoro sugar **12** (0.17 g, 0.93 mmol, 73%) as a colourless oil. ^{19}F nmr (D$_2$O): δ 199.14, 203.25.

1,2,4,6-Tetra-O-acetyl-3-deoxy-3-fluoro-D-galactopyranose (13)

The deoxyfluoro sugar **12** (0.16 g, 0.90 mmol) was dissolved in a cold (-20°C) stirred solution of dry pyridine (10 mL) to which acetic anhydride (4.0 mL, 42 mmol) was added dropwise according to the general acetylation procedure. The mixture was stirred for 16 h at room temperature then worked up in the normal manner to afford a colourless oil of **13** (0.30 g, 0.86 mmol, 97%), as an anomeric mixture. ^{1}H nmr (300 MHz, CDCl$_3$): δ 6.41 (t, 1 H, $J_{1\alpha,2\alpha}$ 4, $J_{1\alpha,F}$ 5 Hz, H(1α)), 5.64 (d, 1 H, $J_{1\beta,2\beta}$ 8 Hz, H(1β)), 5.63 (m, 2 H, H(4αβ)), 5.39 (m, 2 H, H(2αβ)), 4.86 (ddd, 1 H, $J_{2\alpha,3\alpha}$ 10, $J_{3\alpha,4\alpha}$ 4, $J_{3\alpha,F}$ 48 Hz, H(3α)), 4.70 (ddd, 1 H, $J_{2\beta,3\beta}$ 10, $J_{3\beta,F}$ 48 Hz, H(3β)), 4.20-3.95 (m, 6 H, H(5αβ), H(6,6'αβ)), 2.18-2.05 (8 x s, 32 H, 8 x OAc). ^{19}F nmr (CDCl$_3$): δ 200.75 (β), 204.30 (α).

2',4'-Dinitrophenyl 2,4,6-tri-O-acetyl-3-deoxy-3-fluoro-β-D-galactopyranoside (15)

The tetra-O-acetate **13** (0.30 g, 0.86 mmol) was selectively deacetylated at the 1 position with 2-aminoethanol (0.90 mL, 1.46 mmol) according to the general procedure by stirring in ethyl acetate (10 mL) for 30 h at room temperature. Column chromatography (Solvent A) afforded an anomeric mixture of tri-O-acetates **14** as a colourless syrup (0.15 g, 0.49 mmol, 58%). ^{19}F nmr (CDCl$_3$): δ 200.45, 205.33. This material (0.15 g, 0.49 mmol) was converted to the galactopyranoside **15** according to the general procedure by reaction with DABCO (0.19 g, 1.65 mmol) and FDNB (0.11 g, 0.59 mmol). The resulting solid was recrystallized from ethanol to give a white crystalline powder (109 mg, 0.23 mmol, 47%). M.p. 187-188°C; ^{1}H nmr (300 MHz, CDCl$_3$): δ 8.70 (d, 1 H, $J_{3',5'}$ 3 Hz, H(3')), 8.42 (dd, 1 H, $J_{3',5'}$ 3, $J_{5',6'}$ 10 Hz, H(5')), 7.50 (d, 1 H, $J_{5',6'}$ 10 Hz, H(6')), 5.68 (m, 2 H, H(2), H(4)), 5.18 (d, 1 H, $J_{1,2}$ 7.5 Hz, H(1)), 4.77 (ddd, 1 H, $J_{2,3}$ 9, $J_{3,4}$ 5, $J_{3,F}$ 48 Hz, H(3)), 4.25 (m, 2 H, H(6, 6'), 4.10 (m, 1 H, H(5)), 2.25, 2.20, 2.10 (3 x s, 9 H, 3 x OAc). ^{19}F nmr (CDCl$_3$): δ 201.00. Anal. calc. for C$_{18}$H$_{19}$O$_{12}$N$_2$F; C, 45.58; H, 4.04; N, 5.91%; Found: C, 45.48; H, 4.03; N, 5.97%.

2',4'-Dinitrophenyl 3-deoxy-3-fluoro-β-D-galactopyranoside (16)

The tri-O-acetate **15** (100 mg, 0.21 mmol) was deacetylated according to the general deacetylation procedure for 19 h. The product was recrystallized from methanol/ diethyl ether/ hexanes to give white crystals of **16** (27 mg, 0.078 mmol, 37%). M.p. 162-163°C; ^{1}H nmr (400 MHz, CD$_3$OD): δ 8.73 (d, 1 H, $J_{3',5'}$ 3 Hz, H(3')), 8.45 (dd, 1 H, $J_{3',5'}$ 3, $J_{5',6'}$ 10 Hz, H(5')), 7.63 (d, 1 H, $J_{5',6'}$ 10 Hz, H(6')), 5.25 (d, 1 H, $J_{1,2}$ 7.5 Hz, H(1)), 4.48 (ddd, $J_{2,3}$ 3, $J_{3,4}$ 9, $J_{3,F}$ 48 Hz, H(3)), 4.2-4.1 (m, 2 H, H(2), H(4)), 3.85-3.65 (m, 3 H, H(5), H(6,6')). ^{19}F nmr (D$_2$O): δ 199.51. Anal. calc. for

$C_{12}H_{13}O_9N_2F+0.5H_2O$; C, 40.34; H, 3.95; N, 7.84%; Found: C, 40.24; H, 4.04; N, 7.64%.

Methyl 6-O-triphenylmethyl-α-D-glucopyranoside (17)

To a suspension of methyl α-D-glucopyranoside (3.0 g, 15.5 mmol) in dry pyridine (15 mL) was added triphenylmethyl chloride (4.9 g, 17 mmol). The mixture was stirred at 40°C for 20 h. Pyridine was removed by several co-evaporations with toluene and the residue was dissolved in chloroform, washed with water and dried (MgSO4). The resulting solid was recrystallized from ethanol and dried thoroughly (under vacuum at 75°C) to give colourless needles of **17** (5.21 g, 11.9 mmol, 77%). M.p. 150-152°C; ^{1}H nmr (300 MHz, CDCl3): δ 7.60-7.20 (m, 15 H, 3 x phenyl), 4.79 (d, 1 H, $J_{1,2}$ 4.5 Hz, H(1)), 3.70-3.40 (m, 6 H, H(2-6,6')), 3.45 (s, 3 H, OMe).

Methyl 6-O-triphenylmethyl-4-deoxy-4-fluoro-α-D-galactopyranoside (18)

A suspension of the protected glucopyranoside **17** (2.00 g, 4.57 mmol) was treated with DAST (3.3 g, 21 mmol) in 25 mL dry dichloromethane according to the general fluorination procedure. After 3 days at room temperature, the reaction was quenched and the product purified by column chromatography (ethyl acetate) to afford colourless needles of **18** (0.42 g, 0.96 mmol, 21%). M.p. 158-159°C; ^{1}H nmr (300 MHz, CDCl3): δ 7.60-7.20 (m, 15 H, 3 x phenyl), 4.93 (dd, 1 H, $J_{3,4}$ 2.5, $J_{4,F}$ 49 Hz, H(4)), 4.81 (d, 1 H, $J_{1,2}$ 4.5 Hz, H(1)), 3.90-3.30 (m, 5 H, H(2), H(3), H(5), H(6,6')), 3.42 (s, 3 H, OMe). [19]F nmr (CDCl3): δ 221.66.

4-Deoxy-4-fluoro-D-galactopyranose (19)

The protected deoxyfluoro galactopyranoside **18** (0.40 g, 0.91 mmol) was stirred with Amberlite IR-120(H$^+$) cation exchange resin (4 mL) and water (40 mL) at reflux for 3 days. The mixture was filtered hot to remove resin and the bulk of the triphenylmethanol and the precipitated solids were washed with water. The filtrate was washed with dichloromethane and the aqueous portion evaporated to afford a gum which was purified by column chromatography (Solvent **C**) to give an anomeric mixture of the free deoxyfluoro sugar **19** as a colourless oil (0.15 g, 0.80 mmol, 89%). ^{19}F nmr (D$_2$O): δ 217.48, 220.04.

2',4'-Dinitrophenyl 4-deoxy-4-fluoro-β-D-galactopyranoside (21)

The above compound was obtained from Dr. A. Becalski, Department of Chemistry, U.B.C. Its tri-O-acetate **20**, had m.p. 243-245°C; ^{1}H nmr (400 MHz, CDCl$_3$): δ 8.68 (d, 1 H, J$_{3',5'}$ 3 Hz, H(3')), 8.40 (dd, 1 H, J$_{5',6'}$ 10, J$_{3',5'}$ 3 Hz, H(5')), 7.52 (d, 1 H, J$_{5',6'}$ 10 Hz, H(6')), 5.63 (dd, 1 H, J$_{2,3}$ 10, J$_{1,2}$ 8 Hz, H(2)), 5.23 (d, 1 H, J$_{1,2}$ 8 Hz, H(1)), 5.05 (ddd, 1 H, J$_{3,4}$ 2.5, J$_{2,3}$ 10, J$_{3,F}$ 26.5 Hz, H(3)), 4.95 (dd, 1 H, J$_{3,4}$ 2.5, J$_{4,F}$ 49 Hz, H(4)), 4.36 (AB multiplet, 1 H, J$_{5,6}$ 6, J$_{5,6'}$ 7, J$_{6,6'}$ 12 Hz, H(6,6')), 4.06 (dt, 1 H, J$_{5,6}$ 6, J$_{5,6'}$ 7, J$_{5,F}$ 25 Hz, H(5)), 2.14, 2.12, 2.11 (3 x s, 9 H, 3 x OAc). ^{19}F nmr (CDCl$_3$): δ 216.80. Anal. calc. for C$_{18}$H$_{19}$O$_{12}$N$_2$F; C, 45.57; H, 4.04; N, 5.90%; Found: C, 45.68; H, 3.99; N, 5.94%. The deacetylated galactoside **21** had m.p. 127°C (dec); ^{1}H nmr (400 MHz, CD$_3$OD): δ 8.73 (d, 1 H, J$_{3',5'}$ 3 Hz, H(3')), 8.48 (dd, 1 H, J$_{5',6'}$ 10, J$_{3',5'}$ 3 Hz, H(5')), 7.66 (d, 1 H, J$_{5',6'}$ 10 Hz, H(6')), 5.28 (d, 1 H, J$_{1,2}$ 7.5 Hz, H(1)), 4.82 (dd, 1 H, J$_{3,4}$ 3, J$_{4,F}$ 50 Hz, H(4)), 4.00-3.10 (m, 5 H, H(2), H(3), H(5), H(6,6')). ^{19}F nmr (D$_2$O): δ 217.62. Anal. calc. for C$_{12}$H$_{13}$O$_9$N$_2$F; C, 41.39; H, 3.76; N, 8.04%; Found: C, 41.21; H, 3.93; N, 7.94%.

1,2:3,4-Di-O-isopropylidene-6-deoxy-6-fluoro-α-D-galactopyranose (22)

Thoroughly dried 1,2:3,4-di-O-isopropylidene-α-D-galactopyranose[39] (2.20 g, 8.45 mmol) was treated with DAST (2.40 mL, 18.2 mmol) and 2,4,6-trimethylpyridine (2.20 mL, 16.7 mmol) in dry dichloromethane (30 mL) according to the general fluorination procedure and stirred at room temperature for 24 h. The reaction was quenched by stirring with excess methanol at room temperature for 2 h and washed with 1 M aqueous acetic acid. Column chromatography (10% ethyl acetate/ hexanes) afforded **22** as a colourless oil (0.87 g, 3.30 mmol, 40%). ^{1}H nmr (300 MHz, CDCl$_3$): δ 5.55 (d, 1 H, $J_{1,2}$ 5 Hz, H(1)), 4.63 (dd, 1 H, $J_{2,3}$ 3, $J_{3,4}$ 8 Hz, H(3)), 4.56 (dm, 2 H, $J_{6/6',F}$ ~ 47 Hz, H(6,6')), 4.35 (dd, 1 H, $J_{2,3}$ 3, $J_{1,2}$ 5 Hz, H(2)), 4.27 (dd, 1 H, $J_{3,4}$ 8, $J_{4,5}$ 4 Hz, H(4)), 4.08 (m, 1 H, H(5)), 1.55 (s, 3 H, CH$_3$), 1.47 (s, 3 H, CH$_3$), 1.35 (s, 6 H, 2 x CH$_3$). ^{19}F nmr (CDCl$_3$): δ 231.67.

1,2:3,4-Di-O-isopropylidene-6-chloro-6-deoxy-α-D-galactopyranose (49)

A solution of 1,2:3,4-di-O-isopropylidene-α-D-galactopyranose[39] (0.70 g, 2.69 mmol) and triphenylphosphine (0.70 g, 2.67 mmol) in carbon tetrachloride (5 mL) was refluxed for 12 h. The mixture was cooled, the precipitated solids removed by filtration and the resulting clear solution was evaporated to a syrup which was purified by column chromatography (10% ethyl acetate/ hexanes) to give **49** (0.31 g, 1.11 mmol, 41%). ^{1}H nmr (400 MHz, CDCl$_3$): δ 5.54 (d, 1 H, $J_{1,2}$ 4 Hz, H(1)), 4.65 (dd, 1 H, $J_{2,3}$ 3, $J_{3,4}$ 8 Hz, H(3)), 4.35 (m, 2 H, H(2), H(4)), 3.95 (m, 1 H, H(5)), 3.65 (AB multiplet, 2 H, $J_{5,6}$ 8, $J_{5,6'}$ 6, $J_{6,6'}$ 12 Hz, H(6,6')), 1.55, 1.45, 1.37, 1.33 (4 x s, 12 H, 4 x CH$_3$).

6-Deoxy-6-fluoro-α-D-galactopyranose (23)

The protected deoxyfluoro sugar **22** (0.71 g, 2.70 mmol) was dissolved in 1:2 ethanol/ water (100 mL) and stirred at 65°C with Amberlite IR-120(H$^+$) cation exchange resin (8 mL). The reaction was complete by tlc (Solvent C) after 15 h. The resin was removed by filtration and the gummy residue was crystallized from ethanol/ diethyl ether to afford white crystals of the free sugar **23** (0.37 g, 2.0 mmol, 75%), which crystallized exclusively as the α anomer. M.p. 159-160°C (lit.[34] m.p. 160°C); ^{1}H nmr (300 MHz, D$_2$O): δ 5.25 (d, 1 H, J$_{1\alpha,2\alpha}$ 4 Hz, H(1)), 4.80-3.40 (m, 6 H, H(2-6,6')). ^{19}F nmr (D$_2$O): δ 229.63.

1,2,3,4-Tetra-O-acetyl-6-deoxy-6-fluoro-D-galactopyranose (24)

The deoxyfluoro sugar **23** (0.33 g, 1.80 mmol) was treated with acetic anhydride (0.85 mL, 9.0 mmol) in pyridine (20 mL) according to the general acetylation procedure and purified by column chromatography (30: 70 ethyl acetate/ hexanes) to afford **24** (0.64 g, 1.80 mmol, 100%) as a colourless oil. ^{1}H nmr (400 MHz, CDCl$_3$): δ 6.35 (d, 1 H, J$_{1\alpha,2\alpha}$ 4.5 Hz, H(1α)), 5.77 (d, 1 H, J$_{1\beta,2\beta}$ 8 Hz, H(1β)), 5.65-5.10 (m, 8 H, H(2-5αβ)), 4.70-4.30 (m, 4 H, H(6,6'αβ)), 4.25 (m, 1 H, H(5α)), 4.12 (m, 1 H, H(5β)), 2.09-2.01 (8 x s, 32 H, 8 x OAc). ^{19}F nmr (CDCl$_3$): δ 232.37 (β), 233.69 (α).

2',4'-Dinitrophenyl 2,3,4-tri-O-acetyl-6-deoxy-6-fluoro-β-D-galactopyranoside (26)

The tetra-O-acetate **24** (0.65 g, 1.9 mmol) was selectively deacetylated at the 1 position with 2-aminoethanol (0.17 mL, 2.9 mmol) according to the general procedure by stirring in ethyl acetate (50 mL) for 60 h at room temperature. Column chromatography (Solvent A) gave an anomeric mixture of tri-O-acetates **25** as a colourless syrup (0.32 g, 1.1 mmol, 56%). ^{19}F nmr (CDCl$_3$): δ 231.54, 231.66. This material (0.32 g, 1.0 mmol) was converted to the galactopyranoside **26** according to the general procedure by reaction

with DABCO (0.40 g, 3.5 mmol) and FDNB (0.23 g, 1.3 mmol). The resulting solid was recrystallized from ethyl acetate/ hexanes to give white crystals (196 mg, 0.41 mmol, 40%). M.p. 181-182°C; ^{1}H nmr (300 MHz, CDCl$_3$): δ 8.70 (d, 1 H, J$_{3',5'}$ 3 Hz, H(3')), 8.45 (dd, 1 H, J$_{5',6'}$ 10, J$_{3',5'}$ 3 Hz, H(5')), 7.55 (d, 1 H, J$_{5',6'}$ 10 Hz, H(6')), 5.63 (dd, 1 H, J$_{2,3}$ 10, J$_{1,2}$ 8 Hz, H(2)), 5.52 (d, 1 H, J$_{3,4}$ 4 Hz, H(4)), 5.30 (d, 1 H, J$_{1,2}$ 8 Hz, H(1)), 5.15 (dd, 1 H, J$_{2,3}$ 10, J$_{3,4}$ 4 Hz, H(3)), 4.55 (dm, 2 H, J$_{6/6',F}$ 48 Hz, H(6,6')), 4.22 (m, 1 H, H(5)), 2.20, 2.15, 2.05 (3 x s, 9 H, 3 x OAc). ^{19}F nmr (CDCl$_3$): δ 230.08. Anal. calc. for C$_{18}$H$_{19}$N$_2$F; C, 45.58; H, 4.04; N, 5.91%; Found: C, 45.38; H, 4.16; N, 5.75%.

2',4'-Dinitrophenyl 6-deoxy-6-fluoro-β-D-galactopyranoside (27)

The tri-O-acetate **26** (100 mg, 0.210 mmol) was deacetylated according to the general deacetylation procedure for 18 h. The product was recrystallized from acetone/ diethyl ether to give white powdery crystals of **27** (51 mg, 0.15 mmol, 70%). M.p. 180-181°C; ^{1}H nmr (400 MHz, CD$_3$OD): δ 8.73 (d, 1 H, J$_{3',5'}$ 3 Hz, H(3')), 8.50 (dd, 1 H, J$_{3',5'}$ 3, J$_{5',6'}$ 10 Hz, H(5')), 7.65 (d, 1 H, J$_{5',6'}$ 10 Hz, H(6')), 5.25 (d, 1 H, J$_{1,2}$ 7.5 Hz, H(1)), 4.60 (dm, 2 H, J$_{6/6',F}$ 48 Hz, H(6,6')), 4.12 (m, 1 H, H(5)), 3.95-3.85 (m, 2 H, H(2), H(4)), 3.63 (dd, 1 H, J$_{2,3}$ 10, J$_{3,4}$ 4 Hz, H(3)). ^{19}F nmr (D$_2$O): δ 230.37. Anal. calc. for C$_{12}$H$_{13}$O$_9$N$_2$F+H$_2$O; C, 39.35; H, 4.13; N, 7.65%; Found: C, 39.15; H, 4.22; N, 7.30%.

Methyl 4-chloro-4-deoxy-6-O-triphenylmethyl-α-D-glucopyranoside (31)

Methyl 2,3,6-tri-O-benzoyl-4-chloro-4-deoxy-α-D-glucopyranoside **54**[54,55] (3.0 g, 5.76 mmol) was debenzoylated in 0.13 M sodium methoxide/ methanol (10 mL) for 20 h at room temperature, neutralized with Amberlite IR-120(H$^+$) cation exchange resin and

filtered to afford the crude debenzoylated compound **29** as a white solid. This was dissolved in dry pyridine (12 mL) and triphenylmethyl chloride (1.66 g, 1.95 mmol) added. The mixture was stirred at 40°C for 65 h then pyridine was removed by successive co-evaporations with toluene and the residue was dissolved in dichloromethane, washed with water and dried (MgSO₄). Column chromatography (2.5% methanol/ ethyl acetate) gave **31** as a white foam (1.73 g, 3.81 mmol, 66% from **54**). ^{1}H nmr (300 MHz, CDCl₃): δ 7.65-7.20 (m, 15 H, 3 x phenyl), 4.90 (d, 1 H, $J_{1,2}$ 4 Hz, H(1)), 3.95-3.80 (m, 3 H, H(3-5)), 3.65 (dt, 1 H, $J_{1,2}$ 4, $J_{2,3}$ 7.5, $J_{2,OH}$ 7.5 Hz, H(2)), 3.50 (m, 4 H, H(6), OMe), 3.29 (dd, 1 H, $J_{5,6'}$ 6, $J_{6,6'}$ 9 Hz, H(6')), 2.70 (br s, 1 H, 3-OH), 2.25 (d, 1 H, $J_{2,OH}$ 7.5 Hz, 2-OH).

Methyl 3,4-anhydro-6-O-triphenylmethyl-α-D-galactopyranoside (32) and methyl 2,3-anhydro-6-O-triphenylmethyl-α-D-gulopyranoside (33)

The methyl glucopyranoside **31** (0.10 g, 0.22 mmol) was dissolved in dry methanol (5 mL) containing sodium methoxide (6 mg, 0.26 mmol) and heated at reflux for 15 h. Solvent was evaporated *in vacuo* and the residue was chromatographed (Solvent A) to afford **32** (30 mg, 0.067 mmol, 33%, R_f 0.48) and **33** (43 mg, 0.096 mmol, 47%, R_f 0.38), both as white foams. ^{1}H nmr (400 MHz, CDCl₃) **32**: δ 7.50-7.20 (m, 15 H, 3 x phenyl), 4.60 (d, 1 H, $J_{1,2}$ 5 Hz, H(1)), 3.98 (t, 1 H, $J_{5,6}$ 5, $J_{5,6'}$ 5 Hz, H(5)), 3.78 (dd, 1 H, $J_{1,2}$ 5, $J_{2,OH}$ 10 Hz, H(2)), 3.42 (s, 3 H, OMe), 3.35 (m, 3 H, H(6,6'), H(4)), 3.22 (d, 1 H, $J_{3,4}$ 4.5 Hz, H(3)), 2.48 (d, 1 H, 2-OH). ^{1}H nmr (400 MHz, CDCl₃) **33**: δ 7.50-7.20 (m, 15 H, 3 x phenyl), 5.00 (d, 1 H, $J_{1,2}$ 4 Hz, H(1)), 4.10-4.00 (m, 2 H, H(4), H(5)), 3.52 (s, 3H, OMe), 3.43-3.22 (m, 4 H, H(2), H(3), H(6,6')), 2.40 (d, 1 H, 4-OH).

Methyl 6-O-triphenylmethyl-3-deoxy-α-D-xylo-hexopyranoside (34)

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The 3,4-anhydro galactopyranoside 32 (0.41 g, 0.98 mmol) was dissolved in dry THF (25 mL), cooled to 0°C and lithium aluminum hydride (0.15 g, 4.0 mmol) added in one portion. The mixture was stirred under nitrogen at 0°C for 15 min then at room temperature for 5 h. The reaction was quenched with water, extracted with diethyl ether and dried (MgSO₄). Evaporation of solvent *in vacuo* afforded a chromatographically homogenous (Solvent A) white foam 34 (0.39 g, 0.93 mmol, 95%). ^{1}H nmr (400 MHz, CDCl₃): δ 7.50-7.20 (m, 15 H, 3 x phenyl), 4.72 (d, 1 H, $J_{1,2}$ 4 Hz, H(1)), 4.10-3.95 (m, 2 H, H(2), H(4)), 3.79 (t, 1 H, $J_{5,6}$ 5, $J_{5,6'}$ 5 Hz, H(5)), 3.45 (s, 3 H, OMe), 3.40-3.00 (m, 2 H, H(6,6')), 2.08 (dt, 1 H, $J_{3e,4}$ 4, $J_{2,3e}$ 4, $J_{3e,3a}$ 12.5 Hz, H(3e)), 1.75 (dt, 1 H, $J_{3a,4}$ 3, $J_{2,3a}$ 12, $J_{3e,3a}$ 12.5 Hz, H(3a)).

Methyl 2,4,6-tri-O-acetyl-3-deoxy-α-D-xylo-*hexopyranoside (36)*

The tritylated methyl glycopyranoside 34 (0.36 g, 0.86 mmol) was stirred in 1:2 ethanol/ water (25 mL) at room temperature for 6 h with Amberlite IR-120(H⁺) cation exchange resin (10 mL). The mixture was filtered to remove the resin and precipitated triphenylmethanol and the solvent evaporated *in vacuo* to give methyl 3-deoxy-α-D-*xylo*-hexopyranoside 35 as a yellow gum which was acetylated according to the general procedure without purification and chromatographed (Solvent A) to afford 36 as a colourless oil (0.19 g, 0.65 mmol, 75% from 34). ^{1}H nmr (400 MHz, CDCl₃): δ 5.13 (m, 1 H, H(4)), 5.08 (ddd, 1 H, $J_{1,2}$ 4, $J_{2,3e}$ 5, $J_{2,3a}$ 12 Hz, H(2)), 4.90 (d, 1 H, $J_{1,2}$ 4 Hz, H(1)), 4.15-4.05 (m, 3 H, H(5), H(6,6')), 3.45 (s, 3 H, OMe), 2.20-2.10 (m, 2 H, H(3e,3a)), 2.15, 2.10, 2.08 (3 x s, 9 H, 3 x OAc).

2,4,6-Tri-O-acetyl-3-deoxy-D-xylo-*hexopyranose (38)*

The acetylated methyl glycopyranoside **36** (0.19 g, 0.65 mmol) was dissolved in 1,1-dichloromethyl methyl ether (3 mL) and stirred with a catalytic amount of freshly fused zinc chloride under nitrogen at 65°C. After 3 h, the excess ether was removed *in vacuo* and the residue was dissolved in dichloromethane, washed with saturated sodium bicarbonate solution and dried (MgSO$_4$). Evaporation of solvent afforded 2,4,6-tri-O-acetyl-3-deoxy-α-D-*xylo*-hexopyranosyl chloride **37** as a pale yellow oil which was used without further purification. ^{1}H nmr (400 MHz, CDCl$_3$): δ 6.32 (d, 1 H, J$_{1,2}$ 4 Hz, H(1)), 5.25-5.15 (m, 2 H, H(2), H(4)), 4.35 (m, 1 H, H(5)), 4.17 (AB multiplet, 2 H, J$_{5,6}$ 5.5, J$_{5,6'}$ 7, J$_{6,6'}$ 11 Hz, H(6,6')), 2.15, 2.09, 2.07 (3 x s, 9 H, 3 x OAc). To the above oil, dissolved in acetone (3 mL) and 2 drops water, was added freshly prepared silver carbonate (0.15 g, 0.55 mmol) at 0°C in the dark. The mixture was stirred for 24 h at room temperature then the suspension was filtered, and the precipitated solids washed thoroughly with acetone. Column chromatography (Solvent A) gave **38** as a syrupy mixture of anomers (0.14 g, 0.48 mmol, 75% from **36**). ^{1}H nmr (400 MHz, CDCl$_3$): δ 5.43 (d, 1 H, J$_{1\alpha,2\alpha}$ 3 Hz, H(1α)), 5.18 (m, 2 H, H(4αβ)), 5.15-4.95 (m, 2 H, H(1β), H(2α)), 4.90 (m, 1 H, H(2β)), 4.68 (br d, 1 H, β-OH), 4.35 (m, 1 H, H(5α)), 4.20-4.00 (m, 4 H, H(6,6'αβ)), 3.92 (m, 1 H, H(5β)), 2.32 (dm, 1 H, J$_{3e,3a}$ 13 Hz, H(3eβ)), 2.20-2.00 (m, 19 H, 6 x OAc, H(3eα)), 2.00-1.80 (m, 2 H, H(3aαβ)).

2',4'-Dinitrophenyl 2,4,6-tri-O-acetyl-3-deoxy-β-D-xylo-hexopyranoside (39)

The tri-O-acetate **38** (0.14 g, 0.48 mmol) was converted to the glycopyranoside **39** according to the general procedure by reaction with DABCO (0.18 g, 1.6 mmol) and FDNB (0.11 g, 0.57 mmol). The resulting solid was chromatographed (Solvent A) and recrystallized from ethyl acetate/ hexanes to give yellowish crystals (49 mg, 0.11 mmol, 23%). M.p. 116-119°C; ^{1}H nmr (400 MHz, CDCl$_3$): δ 8.65 (d, 1 H, J$_{3',5'}$ 3 Hz, H(3')), 8.35 (dd, 1 H, J$_{5',6'}$ 10, J$_{3',5'}$ 3 Hz, H(5')), 7.42 (d, 1 H, J$_{5',6'}$ 10 Hz, H(6')), 5.30-5.20

(m, 2 H, H(1), H(2)), 5.15 (m, 1 H, H(4)), 4.20-3.95 (m, 3 H, H(5), H(6,6')), 2.43 (dt, 1 H, $J_{2,3e}$ 4, $J_{3e,4}$ 4, $J_{3e,3a}$ 15 Hz, H(3e)), 2.09, 2.08, 1.98 (3 x s, 9 H, 3 x OAc), 1.84 (ddd, 1 H, $J_{3a,4}$ 3, $J_{2,3a}$ 10, $J_{3a,3e}$ 15 Hz, H(3a)). Anal. calc. for $C_{18}H_{20}O_{12}N_2$; C, 47.38; H, 4.42; N, 6.14%; Found: C, 47.38; H, 4.79; N, 5.57%.

Methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-iodo-α-D-glucopyranoside (40)

Sodium iodide (0.38 g, 2.50 mmol) was added to a solution of methyl 2,3,6-tri-O-benzoyl-4-O-methanesulphonyl-α-D-galactopyranoside 57[55] (0.50 g, 0.85 mol) in dry DMF (10 mL). The mixture was refluxed for 3 h then washed with water, charcoal-treated and dried ($MgSO_4$). Column chromatography (20% ethyl acetate/ hexanes) afforded 40 as a white foam (0.28 g, 0.45 mmol, 53%). [1]H nmr (300 MHz, $CDCl_3$): 8.30-7.31 (m, 15 H, 3 x OBz), 6.08 (t, 1 H, $J_{2,3}$ 10, $J_{3,4}$ 10 Hz, H(3)), 5.22 (m, 2 H, H(1), H(2)), 4.75 (AB multiplet, 2 H, $J_{5,6}$ 4, $J_{5,6'}$ 6, $J_{6,6'}$ 13 Hz, H(6,6')), 4.30 (t, 1 H, $J_{3,4}$ 10, $J_{4,5}$ 10 Hz, H(4)), 3.50 (s, 3 H, OMe). [1]H nmr showed minor resonances, presumably arising from the presence of the *galacto* isomer, but the desired *gluco* compound was favoured by a ratio of greater than 10 to 1.

Methyl 3,4-anhydro-α-D-galactopyranoside (28)

A solution of the iodo glucopyranoside 40 (48 mg, 0.078 mmol) in 2:1 methanol/ DMF (3.5 mL) containing sodium methoxide (2.3 mg, 0.10 mmol) was stirred at room temperature for 4 days. Tlc (9:1 dichloromethane/ methanol) showed ~ 75% methyl 4-deoxy-4-iodo-α-D-glucopyranoside and ~ 25% 28 after 18 h. The reaction was worked up when methyl 3,6-anhydro-α-D-galactopyranoside 30 began to appear after 90 to 100 h at room temperature. Solvent was evaporated and the residue was chromatographed (Solvent B) to afford 28 as a gummy solid (9 mg, 0.051 mmol, 66%). [1]H nmr (300

MHz, D$_2$O): δ 4.70 (d, 1 H, J$_{1,2}$ 4 Hz, H(1)), 4.11 (m, 1 H, H(5)), 3.85 (m, 2 H, H(2),

H(6)), 3.72 (dd, 1 H, J$_{5,6'}$ 7, J$_{6,6'}$ 12 Hz, H(6')), 3.45-3.40 (m, 4 H, H(4), OMe), 3.31

(d, 1 H, J$_{3,4}$ 4.5 Hz, H(3)).

2',4'-Dinitrophenyl 4-deoxy-β-D-xylo-hexopyranoside (42)

The above compound was obtained from M. Namchuk, Department of Chemistry,

U.B.C. Its tri-O-acetate **41** had m.p. 169-170°C; ^{1}H nmr (300 MHz, CDCl$_3$): δ 8.70 (d,

1 H, J$_{3',5'}$ 3 Hz, H(3')), 8.40 (dd, 1 H, J$_{3',5'}$ 3, J$_{5',6'}$ 10 Hz, H(5')), 7.49 (d, 1 H,

J$_{5',6}$ 10 Hz, H(6')), 5.30-5.05 (m, 3 H, H(1-3)), 4.20 (m, 2 H, H(6,6')), 4.05 (m, 1 H,

H(5)), 2.25 (ddd, J$_{4e,5}$ 3, J$_{3,4e}$ 6, J$_{4e,4a}$ 13 Hz, H(4e)), 2.15, 2.11, 2.10 (3 x s, 9 H, 3

x OAc), 1.80 (q, 1 H, J$_{4a,5}$ 13, J$_{3,4a}$ 13, J$_{4e,4a}$ 13 Hz, H(4a)). Anal. calc. for

C$_{18}$H$_{20}$O$_{12}$N$_2$; C, 47.37; H, 4.42; N, 6.14%; Found: C, 47.53; H, 4.58; N, 6.07%. The

deacetylated glycoside **42** had m.p. 76-79°C; ^{1}H nmr (300 MHz, C$_3$D$_6$O): δ 8.70 (d, 1 H,

J$_{3',5'}$ 3 Hz, H(3')), 8.50 (dd, 1 H, J$_{3',5'}$ 3, J$_{5',6'}$ 10 Hz, H(5')), 7.45 (d, 1 H, J$_{5',6'}$ 10 Hz,

H(6')), 5.30 (d, 1 H, J$_{1,2}$ 8 Hz, H(1)), 4.10-3.00 (m, 5 H, H(2), H(3), H(5), H(6,6')),

2.00 (ddd, 1 H, J$_{4e,5}$ 3, J$_{3,4e}$ 6, J$_{4e,4a}$ 13 Hz, H(4e)), 1.55 (q, 1 H, J$_{3,4a}$ 13, J$_{4a,5}$ 13,

J$_{4a,4e}$ 13 Hz, H(4a)). Anal. calc. for C$_{12}$H$_{14}$O$_9$N$_2$+2H$_2$O; C, 39.35; H, 4.95; N, 7.65%;

Found: C, 39.68; H, 4.78; N, 5.88%.

2,3,4,-Tri-O-acetyl-6-deoxy-D-galactopyranose (44)

D-Fucose, 6-deoxy-D-galactose (0.655 g, 3.99 mmol) was acetylated with acetic

anhydride (20 mL, 210 mmol) in pyridine (50 mL) for 18 h according to the general

procedure to afford an anomeric mixture of tetra-O-acetates **43** (1.32 g, 3.99 mmol, 100%)

as a pale yellow oil. This material (1.30 g, 3.91 mmol) was selectively deacetylated at the

1 position with hydrazine acetate (0.44 g, 4.80 mmol) according to the general procedure to

afford, after chromatography (Solvent A), a white solid **44** (0.79 g, 2.72 mmol, 69%). M.p. 112-113°C; ^{1}H nmr (300 MHz, CDCl$_3$): δ 5.49 (t, 1 H, $J_{1\alpha,2\alpha}$ 4, $J_{1\alpha,OH}$ ~4 Hz, H(1α)), 5.40 (dd, 1 H, $J_{2\alpha,3\alpha}$ 11, $J_{3\alpha,4\alpha}$ 4 Hz, H(3α)), 5.32 (m, 1 H, H(4α)), 5.28 (m, 1 H, H(4β)), 5.18 (dd, 1 H, $J_{2\alpha,3\alpha}$ 11, $J_{3\alpha,4\alpha}$ 4 Hz, H(2α)), 5.08 (m, 1 H, H(2β), H(3β)), 4.65 (dd, 1 H, $J_{1\beta,2\beta}$ 8, $J_{1\beta,OH}$ 10 Hz, H(1β)), 4.42 (q, 1 H, $J_{5\alpha,6\alpha}$ 6 Hz, H(5α)), 3.85 (q, 1 H, $J_{5\beta,6\beta}$ 6 Hz, H(5β)), 3.50 (d, 1 H, $J_{1\beta,OH}$ 10 Hz, β-OH), 2.85 (br s, 1 H, α-OH), 2.18, 2.12, 2.02 (6 x s, 18 H, 6 x OAc), 1.23 (d, 3 H, $J_{5\alpha,6\alpha}$ 6 Hz, H(6α)), 1.15 (d, 3 H, $J_{5\beta,6\beta}$ 6 Hz, H(6β)).

2',4'-Dinitrophenyl 2,3,4-tri-O-acetyl-6-deoxy-β-D-galactopyranoside (45)

The tri-O-acetate **44** (0.79 g, 2.72 mmol) was converted to the galactopyranoside **45** according to the general procedure by reaction with DABCO (1.04 g, 9.25 mmol) and FDNB (0.61 g, 3.27 mmol). The resulting solid was recrystallized from ethyl acetate/ hexanes to afford white crystals (0.86 g, 1.89 mmol, 69%). M.p. 166-167°C (lit.[38] m.p. 169-172°C); ^{1}H nmr (300 MHz, CDCl$_3$): δ 8.70 (d, 1 H, $J_{3',5'}$ 3 Hz, H(3')), 8.43 (dd, 1 H, $J_{3',5'}$ 3, $J_{5',6'}$ 10 Hz, H(5')), 7.49 (d, 1 H, $J_{5',6'}$ 10 Hz, H(6')), 5.58 (dd, 1 H, $J_{1,2}$ 7.5, $J_{2,3}$ 9 Hz, H(2)), 5.35 (m, 1 H, H(4)), 5.22 (d, 1 H, $J_{1,2}$ 7.5 Hz, H(1)), 5.12 (dd, 1 H, $J_{2,3}$ 9, $J_{3,4}$ 4.5 Hz, H(3)), 4.10 (m, 1 H, H(5)), 2.22, 2.15, 2.05 (3 x s, 9 H, 3 x OAc), 1.32 (d, 3 H, $J_{5,6}$ 6 Hz, H(6)). Anal. calc. for $C_{18}H_{20}O_{12}N_2$: C, 47.37; H, 4.42; N, 6.14%; Found: C, 47.31; H, 4.52; N, 6.00%.

2',4'-Dinitrophenyl 6-deoxy-β-D-galactopyranoside (46)

The tri-O-acetate **45** (175 mg, 0.38 mmol) was deacetylated according to the general deacetylation procedure for 20 h. The product was recrystallized from methanol/ diethyl ether/ hexanes to give faintly yellow crystals of **46** (31 mg, 0.094 mmol, 25%).

M.p. 115-118°C (dec) (lit.[38] m.p. 135-136°C); ^{1}H nmr (400 MHz, CD$_3$OD): δ 8.72 (d, 1 H, $J_{3',5'}$ 3 Hz, H(3')), 8.47 (dd, 1 H, $J_{3',5'}$ 3, $J_{5',6'}$ 10 Hz, H(5')), 7.63 (d, 1 H, $J_{5',6'}$ 10 Hz, H(6')), 5.19 (d, 1 H, $J_{1,2}$ 8 Hz, H(1)), 3.93 (q, 1 H, $J_{5,6}$ 6 Hz, H(5)), 3.82 (dd, 1 H, $J_{1,2}$ 8, $J_{2,3}$ 9 Hz, H(2)), 3.70 (d, 1 H, $J_{3,4}$ 3 Hz, H(4)), 3.59 (dd, 1 H, $J_{2,3}$ 9, $J_{3,4}$ 3 Hz, H(3)), 1.30 (d, 3 H, $J_{5,6}$ 6 Hz, H(6)). Anal. calc. for $C_{12}H_{14}O_9N_2+2H_2O$; C, 39.35; H, 4.95; N, 7.65%; Found: C, 38.96; H, 5.06; N, 5.41%.

2. Enzymology

2.1 General procedures

Escherichia coli (lacZ) β-galactosidase (β-D-galactoside galactohydrolase; EC 3.2.1.23) was purchased as a suspension in 1.7 M ammonium sulphate from the Sigma Chemical Co. All absorbance measurements were made on a Pye Unicam PU-8800 UV-Visible spectrophotometer equipped with a circulating water bath. Matched quartz cells were used throughout except for the determination of K_i and k_i for 2FβGalDNP, for which plastic cells were used. All cells used in this work had a path length of 1 cm.

2.2 Extinction coefficients

Enzymatic hydrolyses of 2',4'-dinitrophenyl glycopyranosides were followed at 400 nm, the wavelength of maximal absorbance (λmax) of 2,4-dinitrophenol. The intact glycosides do not absorb at this wavelength so the rate of change in absorbance (ΔA_{400}/min) is proportional to the rate of 2,4-dinitrophenol release. The extinction coefficient (ε) of 2,4-dinitrophenol at 400 nm at 25°C in phosphate buffer (pH 7.00) was determined in the following manner. A stock solution of accurately known (by weight) phenol concentration was prepared. This stock solution was diluted with buffer to give

absorbance values of 0.2 to 1.0. Accurate absorbance values at 400 nm were recorded, and the extinction coefficient determined from Beer's Law:

$$\varepsilon = A/bc$$

where A is absorbance, b is cell path length and c is the molar concentration of the solution. The extinction coefficient $(10^{-3} \varepsilon)$ of 2,4-dinitrophenol at 400 nm was determined to be 11.1 $M^{-1} cm^{-1}$ at 25°C in 50 mM sodium phosphate buffer at pH 7.00.

2',4'-Dinitrophenyl glycopyranoside concentrations were determined from their absorbance at 255 nm $(10^{-3} \varepsilon = 10.7 M^{-1} cm^{-1}$ for βGluDNP at 37°C in 50 mM sodium phosphate buffer at pH 6.8). The extinction coefficient of the glucoside was determined in the following manner by J. Kempton and M. Namchuk, other workers in this laboratory. A solution of βGluDNP of known absorbance at 255 nm was incubated with pABG5 β-glucosidase until hydrolysis was complete. Since the initial concentration of glucoside is equal to the concentration of phenol released, $\varepsilon_{glucoside}$ could be calculated from the final absorbance at 400 nm. Extinction coefficients for the deoxy and deoxyfluoro glycosides were assumed to be the same as that of the unsubstituted glucoside.

2.3 Determination of K_m and k_{cat} for hydrolyses of 2',4'-dinitrophenyl β-D glycopyranosides by *E. coli (lacZ)* β-galactosidase

All kinetic measurements were performed at 25°C in 50 mM sodium phosphate buffer (pH 7.00) containing 1 mM Mg^{2+} (added as $MgCl_2 \cdot 6H_2O$) and 0.1% bovine serum albumin (BSA). Enzyme concentrations and reaction times were chosen so that less than 10% of the total substrate was hydrolyzed to ensure linear kinetics. The enzyme dilution used for each substrate was assayed for activity with βGalPNP at saturating concentration

(~ 0.3 mM). All k_{cat} values are standardized with respect to a value for βGalPNP of 156 s^{-1}, in accordance with Sinnott and Souchard.[13]

Rates of enzymic hydrolysis at each substrate concentration were determined as follows. Substrate stock, BSA and buffer were mixed in a quartz spectrophotometer cell to give the desired substrate concentration. The cell was thermally equilibrated at 25°C, and an appropriate dilution of β-galactosidase (10 μL) was added to the cell, which was mixed several times by inversion. The rate of hydrolysis was determined spectrophotometrically at 400 nm. Values of K_m and k_{cat} were determined by measuring rates at six to eight substrate concentrations bracketing K_m and ranging from, if possible, 7 times to 0.15 times K_m. The rate of spontaneous glycoside hydrolysis in each cell was subtracted from the observed rate.

A slightly different procedure was employed for the deoxy substrates because of their high rates of spontaneous hydrolysis. Thermally pre-equilibrated buffer was added to a weighed quantity of the deoxy galactoside, the stock concentration was determined by absorbance at 255 nm and appropriate volumes added to thermally pre-equilibrated cells containing buffer and BSA. Enzyme was added immediately and the rates determined as above.

The data are presented as double reciprocal plots according to the method of Lineweaver and Burk[77], but this method was not used for calculating kinetic parameters due to the inaccuracy introduced by the non-linear error span. The values of K_m and k_{cat} were calculated from a weighted non-linear regression program, *Curvefitter*, written for an Apple IIe computer by I. Street. Some of the glycosides were sufficiently insoluble in buffer (or bound so poorly) that the concentrations of saturated solutions were far below K_m. In these instances, approximate values for k_{cat}/K_m were determined from plots of v vs. [S] using the Michaelis-Menten equation with [S] $\ll K_m$

$$v = \frac{k_{cat}[E_0][S]}{K_m}$$

where $[E_o]$ is the total enzyme concentration and $[S]$ is the concentration of substrate.

2.4 Determination of K_i and k_i for 2',4'-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside by time-dependent inactivation

Solutions containing several concentrations of 2FβGalDNP and 0.1% BSA were incubated at 25°C. β-Galactosidase was added to each inactivation mixture, and aliquots were removed at intervals until the enzyme was ~ 90% inactivated. Residual activity at each time interval was determined by diluting aliquots (10 μL) of the inactivation mixture into 1.6 mL of βGalPNP at saturating concentration (~ 0.3 mM), and measuring the rate of release of 4-nitrophenolate, which is directly proportional to the amount of remaining active enzyme. The rate constant at each inactivator concentration, k_{obs}, was determined by plotting the logarithm of the fraction of remaining active enzyme against time. K_i and k_i were found by fitting the rate constants so determined to

$$k_{obs} = \frac{k_i[I]}{K_i + [I]}$$

using the *Curvefitter* weighted regression program, described previously.

2.5 Determination of β-galactosidase concentration by absorbance at 280 nm and 2,4-dinitrophenolate burst

Several concentrations of freshly dialyzed β-galactosidase in 50 mM phosphate buffer (pH 7.00) containing 1 mM Mg^{2+} were incubated in quartz cells at 25°C in the spectrophotometer. Absorbances at 280 nm were recorded and the concentration of protein

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determined from Beer's Law ($\varepsilon_{280} = 2.1$ cm^{-1} mg^{-1} mL[76]). A solution of 2FβGalDNP to give a final inhibitor concentration of ~ 0.4 mM (approximately one-half K_i and fifty times the estimated enzyme concentration) was added to the cell, which was mixed several times by inversion. The change in absorbance at 400 nm over ~ 45 minutes was recorded. After corrections had been made for the added volume, the initial absorbance and the spontaneous hydrolysis of the galactoside during the course of the experiment, the enzyme concentration could be calculated according to

$$\text{concentration of enzyme (mol L}^{-1}) = \frac{\Delta A_{400} \times \text{Sample Volume (}\mu L)}{1.11 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}}$$

since the moles of enzyme present is equal to the moles of 2,4-dinitrophenolate released.

2.6 Reactivation of covalently inactivated β-galactosidase

Inactivated enzyme (Section 2.5) was centrifuged (3 x 30 min, 5500 rpm) in a Millipore Ultrafree-PF filter unit fitted with a polysulphone membrane (30 000 molecular weight limit) using a Sorvall RC-5B centrifuge with an SS-34 rotor to free it of excess, non-covalently bound inhibitor. The sample was reduced to a volume of approximately 50 μL, washed with approximately 2 mL of buffer after each centrifugation and finally diluted with 2.5 mL of buffer. The end point activity was calculated from the ratio of the absorbances at 280 nm before and after centrifugation multiplied by the initial activity (prior to inactivation). Samples of the inactivated enzyme were then incubated in 50 mM phosphate buffer (pH 7.00) containing 1 mM Mg^{2+} at 25°C in the presence of any desired transglycosylation acceptors. Reactivation was monitored by the removal of aliquots at

appropriate time intervals and assaying for activity by addition to 2.5 mL of βGalPNP at saturating concentration (~ 0.3 mM). The observed rate constant, k_{obs}, for each reactivation process was determined by fitting the data to a single exponential function using an Archimedes 410/1 computer with an Applied Photophysics kinetic workstation. The return of activity can be related to the full activity of the enzyme sample by

$$[E] = [E_0](1 - \exp\{-k_{obs}t\})$$

Control samples with *non-inactivated* enzyme were assayed for activity with βGalPNP to account for denaturation during the time course of the experiment with the incubation conditions used.

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