Substituted Aryl Glycosides as Probes of the Mechanism of Spontaneous and Enzyme-Catalyzed Glycoside Hydrolysis.

By

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B.Sc., The University of Alberta, 1988

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY in
THE FACULTY OF GRADUATE STUDIES (Department of Chemistry)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
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Abstract

A series of mono-substituted deoxy and deoxyfluoro 2',4'-dinitrophenyl \( \beta \)-D-glycopyranosides (DNPGlycosides) was synthesized and used to probe the mechanisms of spontaneous and enzyme-catalyzed hydrolysis of \( \beta \)-glycosides. The relative rates of spontaneous hydrolysis of the DNPGlucosides followed the trend 4-deoxy > 3-deoxy > 6-deoxy > parent > 6-deoxy-6-fluoro > 3-deoxy-3-fluoro > 4-deoxy-4-fluoro > 2-deoxy-2-fluoro. These relative rates can be rationalized on the basis of the stabilities of the oxocarbonium ions generated at the transition state, which appear to be principally a function of field effects (as predicted by the Kirkwood-Westheimer hypothesis) exerted by the ring substituents at O5 and C1. Differences in the rates of hydrolysis between different series of hexopyranosides could not be explained simply on the basis of field effects and likely arise from several factors. Based on the large differences in the values of the activation entropy and enthalpy for hydrolysis of the analogously substituted galactosides and glucosides, it is suggested that differences in the hydration structure of these glycosides at the transition state could be contributing to the observed rate differences.

The role of non-covalent interactions between the enzyme active site and the hydroxyl groups of the substrate glycone in the mechanism of Agrobacterium faecalis \( \beta \)-glucosidase, as well as the amount of charge generated at the transition states for the enzymic reaction, were probed using the same DNPGlycosides. Both pre-steady state and steady state kinetic analysis of the hydrolysis of the DNPGlycosides by Agrobacterium \( \beta \)-glucosidase was performed to investigate the effect of the ring substitutions on each of the steps in the enzyme mechanism. Non-covalent enzyme/substrate interactions at the 2, 3 and 6 positions contributed respective binding energies of at least 18, 7 and 3 kJ/mol, to stabilization of the transition state for glycosylation of the enzyme. Binding effects at the transition state for deglycosylation were similar. The interaction at the 4 position is
unique in that it appears to stabilize the transition state for glycosylation to a greater extent than the transition state for glycosylation. Evidence is also presented to support an earlier proposal (Kempton and Withers (1992), *Biochemistry 31*, 9961) that the transition states for glycosylation and deglycosylation of the enzyme may differ in the amount of positive charge generated, with deglycosylation being significantly more oxocarbonium ion like.

The rates of enzymic hydrolysis obtained in this study were then used to investigate the mode of action of castanospermine and 1-deoxynojirimycin as non-covalent inhibitors of *Agrobacterium* β-glucosidase. Dissociation constants ($K_i$) were determined for a series of mono-substituted castanospermines and 1-deoxynojirimycins, and the logarithm of those dissociation constants correlated with the logarithm of the rate constants for the *Agrobacterium* β-glucosidase-catalyzed hydrolysis of the analogously substituted DNPglycosides. In all cases, these parameters correlated poorly, regardless of whether $K_i$ for the inhibitor was compared with $K_d$, $k_{cat}$ or $k_{cat}/K_m$ for the analogous substrates. Based on these data, it is unlikely that the tight binding of castanospermine and 1-deoxynojirimycin to this enzyme is due to their being transition state analogues. Binding of these inhibitors is likely better described as 'fortuitous' wherein the positively charged amines are binding to the anionic active site of an enzyme which has evolved to bind a polyhydroxylated, positively charged transition state.
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List of Abbreviations

Carbohydrate Structure

DNPglucoside 2',4'-Dinitrophenyl β-D-glucopyranoside
2FDNPglucoside 2',4'-Dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside
3FDNPglucoside 2',4'-Dinitrophenyl 3-deoxy-3-fluoro-β-D-glucopyranoside
4FDNPglucoside 2',4'-Dinitrophenyl 4-deoxy-4-fluoro-β-D-glucopyranoside
6FDNPglucoside 2',4'-Dinitrophenyl 6-deoxy-6-fluoro-β-D-glucopyranoside
3dDNPglucoside 2',4'-Dinitrophenyl 3-deoxy-β-D-ribo-hexopyranoside
4dDNPglucoside 2',4'-Dinitrophenyl 4-deoxy-β-D-xylo-hexopyranoside
6dDNPglucoside 2',4'-Dinitrophenyl 6-deoxy-β-D-glucopyranoside
2CIDNPglucoside 2',4'-Dinitrophenyl 2-chloro-2-deoxy-β-D-glucopyranoside
DNPgalactoside 2',4'-Dinitrophenyl β-D-galactopyranoside
4FDNPgalactoside 2',4'-Dinitrophenyl 4-deoxy-4-fluoro-β-D-galactopyranoside
DNPalloside 2',4'-Dinitrophenyl β-D-allopyranoside
3FDNPgalloside 2',4'-Dinitrophenyl 3-deoxy-3-fluoro-β-D-allopyranoside
DNPmannoside 2',4'-Dinitrophenyl β-D-mannopyranoside
2FDNPmannoside 2',4'-Dinitrophenyl 2-deoxy-2-fluoro-β-D-mannopyranoside
PNPglucoside 4'-Nitrophenyl β-D-glucopyranoside
2FPNPglucoside 4'-Nitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside
2NH2PNPglucoside 4'-Nitrophenyl 2-amino-2-deoxy-β-D-glucopyranoside
2dPNPglucoside 4'-Nitrophenyl 2-deoxy-β-D-glucopyranoside
PNPmannoside 4'-Nitrophenyl β-D-mannopyranoside
Other Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>abg</td>
<td>Agrobacterium</td>
</tr>
<tr>
<td>α-DKIE</td>
<td>α-secondary deuterium kinetic isotope effect</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetyl</td>
</tr>
<tr>
<td>Bz</td>
<td>Benzoyl</td>
</tr>
<tr>
<td>LFE</td>
<td>Linear free energy</td>
</tr>
<tr>
<td>LFER</td>
<td>Linear free energy relationship</td>
</tr>
<tr>
<td>ln(khy)</td>
<td>Natural logarithm of the rate constant for hydrolysis</td>
</tr>
<tr>
<td>LSIMS</td>
<td>Liquid Soft Ionization Mass Spectrometry</td>
</tr>
</tbody>
</table>
Acknowledgments

I would like to thank the entire Withers group for making the last four years both challenging and enjoyable. I am especially indebted to my supervisor, Dr. Steve Withers, whose patience with me has at times been extraordinary. I am also grateful for the input of John McCarter, whose contribution as both a friend and source of 'sober second thought' has been indispensable. I would also like to thank Bill Stirtan and Julie Kempton for their help and input and Trevor Andrews and Karen Rupitz for their help with various sections of this project. Finally, I wish to acknowledge the support and encouragement of my wife Gayle, whose patience with me over the last half year has bordered on superhuman.
Chapter 1

Introduction
1.1 General Introduction

Carbohydrates and carbohydrate polymers are ubiquitous in nature. They play a central role in energy metabolism, cell-cell recognition and a variety of other biologically important processes. Their central importance in nature has prompted a great deal of research into the chemistry and biochemistry of sugars. However, many of the fundamental processes involved in the breakdown of carbohydrate polymers are still not well understood. For example, the acid-catalyzed hydrolysis of carbohydrates has been proposed to proceed via protonation of the anomeric oxygen followed by unimolecular decomposition of the glycoside. The cation generated is then attacked by water to produce the hydrolyzed sugar.

![Cyclic carbonium ion mechanism for glycoside hydrolysis.](image)

Though this mechanism is reasonable, hydrolysis has also been proposed to proceed via protonation of the ring oxygen followed by ring opening (1).
Figure 1-2. Acyclic carbonium ion mechanism for glycoside hydrolysis.

Evidence exists to support both proposals (see Chapter 2) which illustrates the fact that a great deal of study is still needed before a definitive explanation can be found. Indeed, different mechanisms may occur under different conditions.

In nature, the hydrolysis of carbohydrate polymers is usually carried out enzymatically. The glycosyl hydrolases are a large and diverse group of enzymes and are involved in a variety of biologically important processes ranging from the breakdown of wood to the post-translational modification of proteins. These enzymes are usually divided into two classes, α- and β-glycosidases, depending on the anomeric configuration of the substrates on which they act. The enzymes are further classified by the initial stereochemistry of the products they release. If the product released has the same anomeric configuration as the substrate, the enzyme is said to be retaining. Conversely, if the product released has the opposite configuration to the substrate the enzyme is termed inverting. Finally, the enzyme is named for the glycone portion of the substrate on which it has the most activity. For example, a glucosidase will have maximal activity on substrates with glucose as the glycone.
Figure 1-3. Reaction catalyzed by a retaining $\beta$-glucosidase.

The work described in this thesis was carried out to investigate the mechanism of spontaneous and enzyme-catalyzed hydrolysis of carbohydrates and the role of the ring hydroxyl groups on the sugar in these reactions. The enzyme investigated in these studies was a retaining $\beta$-glucosidase originally isolated from the bacterium *Agrobacterium faecalis*. The enzyme was first characterized by Han and Srinivasan (2) and further investigated by Day and Withers (3). It has a monomer molecular weight of 50,983 Daltons (4) and is active as a dimer with one active site per monomer. The enzyme is believed to possess a glycone binding site as well as at least two adjacent sites capable of binding carbohydrate moieties, though not as specifically as the glycone site (3). Accordingly, the enzyme shows a moderately lax specificity for the aglycone portion of the substrate, having activity against a variety of aryl and alkyl $\beta$-glucosides. This lack of specificity for the substrate aglycone has been reported in several glycosidases and has been exploited to investigate the mechanism of action of these enzymes (5). In general, glycosidases are far more sensitive to alterations in the glycone portion of the substrate. The *Agrobacterium* $\beta$-glucosidase is interesting in that it shows significant activity against galactosides, mannosides, allosides and xylosides (6).
1.2 Mechanism of β-Glucosidase

The mechanism for retaining glycosidases was first proposed by D.E. Koshland (7) in 1953 and is illustrated for a β-glucosidase (Figure 1-4). After the initial binding step, the anomeric carbon of the glycone is attacked by an enzymic nucleophile leading to the formation of a covalent α-linked glucosyl-enzyme intermediate. This intermediate is then hydrolyzed by the attack of water at Cl yielding the free enzyme and a molecule of β-D-glucose. Both the formation and hydrolysis of the intermediate are believed to proceed via transition states with substantial oxocarbonium ion character. It has also been proposed that an enzyme group acts as a general acid moiety, aiding the departure of the aglycone by protonation of the glycosidic oxygen. Further, the hydrolysis of the intermediate is assisted by general base catalysis, the same group helping to remove a proton from water as it attacks the glucosyl-enzyme intermediate.

1.2.1 The Glycosyl-Enzyme Intermediate

Evidence for the existence of a glycosyl-enzyme intermediate in retaining glycosidases has been steadily accumulating over the last twenty years. One of the first attempts to provide kinetic evidence for the intermediate was carried out by Stokes and Wilson on E. coli (lac-Z) β-galactosidase (8). The enzyme was incubated with a series of aryl-galactosides in buffers containing methanol as a competitive nucleophile. For all of the substrates examined, the ratio of methanolysis to hydrolysis was constant, regardless of the reactivity of the substrate used. It was therefore reasonable to infer that hydrolysis of the substrates tested was proceeding through a common intermediate.
Figure 1-4. Proposed mechanism for a retaining β-glucosidase
Figure 1-5. Partitioning of a galactosyl-enzyme intermediate between methanolysis and hydrolysis.

Studies by Roeser and Legler (9) provided further evidence for the existence of an intermediate in the β-glucosidase from *Aspergillus wentii*. It had been shown previously (10) that glucal (1,5-anhydro-2-deoxy-D-arabino-hex-1-enitol) reacts slowly with β-glucosidase to generate 2-deoxy-D-glucose as the final product. The reaction is believed to proceed via protonation at C2 to generate an oxocarbonium ion, followed by formation of the enzymic intermediate which is subsequently hydrolyzed to release 2-deoxy-D-glucose (Figure 1-6). Using $^{14}$C labeled-glucal the intermediate was shown to accumulate on the enzyme. Subsequent denaturation and sequencing of the labelled enzyme showed the sugar to be attached to an aspartic acid residue.

Figure 1-6. The glycosylation of β-glucosidase with glucal.
A cryoenzymology study by Fink and Good (11) on the hydrolysis of p-nitrophenyl-β-D-glucoside by a retaining β-glucosidase isolated from sweet almonds demonstrated the existence of a 'burst' of phenol release at low temperatures. The release of phenol was shown to be stoichiometric with the amount of enzyme suggesting that, at least under the conditions used (-45 °C in 50% DMSO), the enzymic hydrolysis occurred in two steps; a rapid first step with the formation of an intermediate, followed by a subsequent, slower, hydrolysis step.

The question which remained to be addressed concerned the nature of the glycosyl-enzyme intermediate. Though Koshland's original hypothesis proposed that the intermediate was covalent, it was suggested by Phillips that an ion pair between a negatively charged enzymic group and the glucosyl cation would fulfill the same function (12). Evidence for this proposal comes mainly from the X-ray crystal structure of hen egg white lysozyme into which an N-acetyl glucosamine tetramer was modeled. The structure seems consistent with the enzyme forming an intimate ion pair, principally between Asp-52 and the glycosyl cation. A recent re-evaluation of the lysozyme structure with a trisaccharide N-acetyl muramyl-N-acetyl glucosaminyl-N-acetyl muramic acid bound at the active site also appears to confirm the previous work of Phillips (13). Indeed, if this structure is an accurate representation of the active conformation of the enzyme and if stereoelectronic factors are of major importance, the authors contend that the distance between the enzymic nucleophile and the anomeric centre of the substrate would make formation of a covalent bond impossible. However, there is a great deal of evidence in retaining glycosidases (including lysozyme) which is more consistent with the formation of a covalent glycosyl-enzyme intermediate.

A study on the Agrobacterium β-glucosidase carried out by Withers and Street (14) provided direct evidence for the formation of a covalent α-linked glucosyl-enzyme intermediate. Treatment of the enzyme with 2-deoxy-2-fluoro-β-D-glucosyl fluoride
leads to the complete inactivation of the enzyme by accumulation of the 2-deoxy-2-fluoro-glucosyl-enzyme intermediate. The presence of an electron-withdrawing fluorine adjacent to C1, the atom on which the positive charge is being generated in the enzymic reaction, decreases the rate of formation and hydrolysis of the intermediate. Use of an activated leaving group (fluoride, in this case) selectively increases the rate of formation of the intermediate but has no effect on its breakdown. Indeed, though the enzyme is completely inactivated in seconds, the half life of the 2-deoxy-2-fluoro-glucosyl enzyme intermediate is approximately 500 h (15).

The notable stability of this intermediate allowed its characterization by $^{19}$F nmr. Analysis of the chemical shift of a 2-deoxy-2-fluoro-mannosyl enzyme intermediate showed that the intermediate was indeed $\alpha$-linked. Further, both the 2-deoxy-2-fluoro-mannosyl and -glucosyl-enzyme intermediates were demonstrated to be catalytically competent, suggesting that at least for these glycosides, a covalent intermediate occurs on the usual pathway for their enzymic hydrolysis.

Further kinetic evidence for the covalency of the intermediate comes from $\alpha$-secondary deuterium kinetic isotope effect studies ($\alpha$-DKIEs) on E. coli (lac-Z) $\beta$-
galactosidase by Sinnott and co-workers (16,17). Isotope effects were measured for the hydrolysis of a series of O-aryl galactosides and β-galactosyl pyridinium salts (see below). In the case of aryl galactosides, for which deglycosylation was rate-limiting, α-DKIEs of $k_H/k_D \sim 1.25$ were measured. In the case of the pyridinium salts, glycosylation was rate-limiting and a significant α-secondary deuterium isotope effect ($k_H/k_D = 1.15-1.20$) was again observed. However, isotope effects measured for O-aryl galactosides for which glycosylation was rate-limiting were extremely small ($k_H/k_D = 1.04$).

![Figure 1-8. A deuterio-galactosyl pyridinium salt.](image)

Similar results were obtained with a series of aryl glucosides tested on Agrobacterium β-glucosidase. Substrates for which deglycosylation is rate limiting exhibited isotope effects on $V_{max}$ of $k_H/k_D = 1.11$ while substrates for which the rate limiting step was glycosylation have isotope effects of $k_H/k_D = 1.06$ (6). Similar positive α-DKIEs were reported for hydrolysis of p-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranosyl-1,4-β-D-glucopyranoside by hen egg white lysozyme (18).
Table 1-1. \(\alpha\)-Secondary Deuterium Kinetic Isotope Effects Measured for the Hydrolysis of a Series of Aryl Glucosides by Agrobacterium \(\beta\)-Glucosidase.

<table>
<thead>
<tr>
<th>Aglycone</th>
<th>(pK_a)</th>
<th>(k_H/k_D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2',4'-dinitrophenyl</td>
<td>3.96</td>
<td>1.10 ± 0.02</td>
</tr>
<tr>
<td>4'-nitrophenyl</td>
<td>7.18</td>
<td>1.12 ± 0.02</td>
</tr>
<tr>
<td>3'-nitrophenyl</td>
<td>8.39</td>
<td>1.07 ± 0.02</td>
</tr>
<tr>
<td>4'-bromophenyl</td>
<td>9.34</td>
<td>1.06 ± 0.02</td>
</tr>
<tr>
<td>(\beta)-naphthyl</td>
<td>9.51</td>
<td>1.05 ± 0.02</td>
</tr>
</tbody>
</table>

Data obtained from Kempton and Withers (6).

The observation of a positive \(\alpha\)-secondary deuterium kinetic isotope effect is associated with a change in the hybridization state of the \(\alpha\) carbon from \(sp^3\) to \(sp^2\) as the substrate goes from the ground state to the transition state. The data above are therefore consistent with the transition states for formation and hydrolysis of the intermediate having significant \(SN_1\) character. Further, the intermediate must have more \(sp^3\) character than the two transition states to account for the observation of positive \(\alpha\)-DKIE for both steps. This evidence is more consistent with the proposed covalent glucosyl-enzyme intermediate than the ion pair proposal. Finally, it is estimated that a glucosyl cation in water has stability roughly equal to that of a tertiary alkyl cation (19), giving it a lifetime in water on the order of \(10^{-10}\) sec (20). It seems unlikely that, even within the stabilizing confines of an active site, such an unstable species could exist adjacent to a negative charge without the formation of covalent bond (for a more detailed discussion see Sinnott and Souchard (16)).

Most of the kinetic evidence therefore suggests the intermediate is indeed covalent. However, as was pointed out by Kirsch (21) in his work on lysozyme, these experiments were all conducted on unnatural substrates making it impossible to say
categorically what the mechanism is for the natural substrates. A criticism of the 2-deoxy-2-fluoro glucoside inactivators has been that the 2-deoxy-2-fluoro oxocarbonium ion is much less stable than the fully hydroxylated parent and so forces the formation of a covalent intermediate which is not present with the natural substrate. However, the protonation of glucal by β-glucosidase will produce a 2-deoxy glucosyl oxocarbonium ion which is much more stable than the fully hydroxylated analogue and yet both compounds appear to form covalent glycosyl enzyme intermediates (although the 2-deoxy-glucosyl-enzyme intermediate was trapped only by denaturation of the enzyme). Further, interpretation of the magnitude of α-KDIE measurements in terms of intermediate or transition state structure can be difficult in cases where the kinetic step being examined is not completely rate limiting (as could occur for a multi-step process or in a case where the reaction is partially diffusion controlled). In any case, the bulk of the kinetic evidence for retaining β-glucosidases seems to favor the formation of a covalent intermediate.

1.2.2 Oxocarbonium Ion Transition States

Much of the evidence for the existence of oxocarbonium ion transition states is derived from studies of α-secondary DKIEs and was discussed in the previous section. Aside from isotope effects, the majority of the evidence supporting an oxocarbonium ion transition state is based on studies of competitive inhibitors which resemble the proposed structure of the transition state. The 1,5-glyconic acid lactones and nojirimycins are good examples (Figure 1-9). In the case of the lactones, their enhanced binding to β-glucosidases is believed to be derived from the sp² hybridization of C1 and the polarization of the carbonyl bond which leaves C1 electron deficient with respect to the analogous aldopyranose.
D-glucono-1,5-lactone

1-deoxynojirimycin

Figure 1-9. Transition state analogues for β-glucosidase

In the case of the nojirimycins and 1-deoxynojirimycins, the charge of the ring nitrogen is crucial to their ability to act as inhibitors (22). They are believed to mimic the charged ring oxygen found in the oxocarbonium ion-like transition state for glycoside hydrolysis. The inhibitor approach to transition state analysis is discussed in some detail in Chapter 4 and has been thoroughly explored by Wolfenden (23).

1.2.3 General Acid Catalysis

The mechanism proposed by Koshland for a retaining β-glucosidase suggested that a general acid moiety would aid in the departure of the aglycone by donation of a proton. However, it is generally believed that catalysis by the general acid is not essential for enzymic activity. For example, the hydrolysis of glycosyl pyridinium salts is efficiently catalyzed by several glycosidases (k_{cat}/k_{uncat} = 10^{8}-10^{13}) even though proton donation to these substrates is impossible (16). Work by Legler (24) on hydrolysis of a series of aryl glucosides by β-glucosidase A3 from Aspergillus wentii showed that in cases where glycosylation was rate-limiting, there was little variation in k_{cat} across a large range of leaving group ability. The authors rationalized this as indicating extensive proton donation to the leaving group leading to little negative charge.
development at the transition state, even in the case of relatively acidic phenols such as 2,4-dinitrophenol.

In a solvent isotope effect study on *E. coli* (*lac-Z*) β-galactosidase using a substrate for which the rate limiting step was glycosylation (p-nitrophenyl-β-D-galactopyranoside) it was found that a single proton transfer was involved and a solvent deuterium isotope effect of $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}} = 1.7$ was observed (25). This is consistent with donation of a proton by a general acid moiety during the glycosylation step.

Though it seems clear that the general acid moiety does exist in β-glycosidases the nature of this group remains to be determined. From the X-ray structure of hen egg white lysozyme, a glutamic acid (Glu 35) has been implicated as the acid catalyst in this enzyme since it was found to be in the proper orientation to donate a proton to the leaving group. An affinity labelling study in yeast α-glucosidase (26) implicated a histidine residue as the general acid in that enzyme. In *E. coli* (*lac-Z*) β-galactosidase, it has been suggested that a Mg++ cofactor may be acting as the general acid (or as an electrophilic catalyst) (27) though there is also some evidence to suggest that Tyr 503 (28) may be the acid catalyst in this system. However recently, Gebler et al. (29) have provided evidence that Glu 461 may be the general acid in this enzyme. This ambiguity in the literature points out both the difficulty in determining whether a particular residue is indeed the general acid catalyst and that the amino acid type used in this capacity may vary between systems.

1.3 Non-Covalent Interactions

1.3.1 General Theory

Though nucleophilic and acid/base catalysis may play a substantial role in catalysis with glycosidases, the majority of the catalytic power of these enzymes may be
accounted for by non-covalent interactions between the enzyme and the substrate as was noted by Koshland (7). These interactions must function both to generate substrate specificity and to aid in catalysis. According to Haldane (30) and Pauling (31) these interactions will be maximally effective if they are specific for the transition state structure of the reaction rather than the ground state (see Appendix 1 for a detailed discussion).

To date, the best exploration of the use of non-covalent interactions by an enzyme comes from the elegant work of Fersht on tyrosyl t-RNA synthase (32). Using the X-ray structure of the enzyme as a guide Fersht selectively deleted interactions between the substrate and the enzyme using site-directed mutagenesis to remove the side chain on the protein which was interacting with the substrate. Thorough kinetic analysis of the mutant enzymes showed that protein/substrate hydrogen bonds were being used to select for the proper substrate and to drive catalysis. In many instances, the bonds between the enzyme and the substrate became stronger as the reaction progressed, binding maximally to the transition state of the reaction. Indeed, for this enzyme system it was suggested that the sole source of catalysis was non-covalent interactions since no other catalytic groups (eg. general acid or nucleophilic groups) were identified.

In enzymes which act upon carbohydrates, it seems reasonable that hydrogen bonds between the enzyme and the substrate would play an important role in enzyme/substrate interactions. Such interactions have been identified in the X-ray structures of several carbohydrate-binding proteins and glycosidases (33 and references therein). In cases where the X-ray structure of the enzyme is unavailable (a very common occurrence for β-glycosidases) site-directed mutagenesis cannot be used to probe these interactions systematically since there is no way of determining which interaction has been deleted after the mutation is made. The most reasonable alternative is to resort to the classical method of structure/activity correlation using modified substrates.
1.3.2 Net Hydrogen Bonds

The determination of hydrogen bond strengths between the enzyme and the substrate is complicated by the fact that the measurements must be done in water. We are therefore examining an exchange of hydrogen bonds occurring as the substrate leaves bulk water and binds to the enzyme. For this example, the substrate (S) is arbitrarily shown as the hydrogen bond acceptor and the enzyme (E) as the donor.

\[
E-\overset{\text{H}}{\text{-OH}}_2 + \overset{\text{H}}{\text{OH}}-\overset{\text{A}}{\text{-S}} \rightleftharpoons E-\overset{\text{H}}{\text{-A}}-\overset{\text{S}}{\text{+ \overset{\text{H}}{\text{OH}}-\overset{\text{OH}}{\text{2}}}} \tag{1}
\]

As the substrate binds to the enzyme, it displaces water from the enzyme active site and from itself. Therefore there is no net change in the number of hydrogen bonds in the system. However, the active site of the enzyme has presumably evolved to bind the substrate preferentially and the geometry of the bonds may be more favorable in the ES complex than between the enzyme and water. Further, there is an entropically favorable term associated with the release of enzyme bound water into bulk water which will again promote formation of the enzyme/substrate hydrogen bond.

It can also be shown that there is no net change in the number of hydrogen bonds for the binding of a deoxy substrate:

\[
E-\overset{\text{H}}{\text{-OH}}_2 + \overset{\text{H}}{\text{2O}} + \overset{\text{H}}{\text{-S}} \rightleftharpoons [E-\overset{\text{H}}{\text{H}}-\overset{\text{S}}{\text{]} + \overset{\text{H}}{\text{2O}}-\overset{\text{HOH}}{\text{}}] \tag{2}
\]

However, comparing (1) and (2) it is apparent that when binding of a deoxy and hydroxylated substrate to the enzyme are compared, the binding energy being measured is the net difference in the hydrogen bond strengths between the enzyme and water and
the enzyme and the substrate. For this reason, hydrogen bond strengths measured in this way are considered to be minimum estimates of the actual magnitude of the enzyme/substrate hydrogen bond (34).

There is a reasonable body of evidence which suggests that hydrogen bonds to fluorine bonded to carbon do exist (35) though these interactions are likely weaker than in the case where the oxygen is accepting the hydrogen bond. Indeed, X-ray crystallographic studies have recently shown (36) that a fluorinated galactose molecule is capable of acting as a hydrogen bond acceptor in a carbohydrate binding protein. Therefore, examining the binding of deoxyfluoro sugars to the enzyme, two situations are possible:

\[ E--H--OH_2 + HOH--F--S \leftrightarrow [E--H--F--S] + H_2O--HOH \]  
(3)

or

\[ E--A--H_2O + HOH--F--S \leftrightarrow [E--A F--S] + H_2O--HOH \]  
(4)

In the first case, the enzyme is acting as a hydrogen bond donor and so may interact favorably with the deoxyfluoro substrate. This case is much the same as the fully hydroxylated substrate where there is no net change in the number of hydrogen bonds. In the second case, the enzyme is acting as a hydrogen bond acceptor. Since fluorine cannot possibly donate a hydrogen bond, there is a net loss of a hydrogen bond in the equation (between the enzyme and the substrate). Under these circumstances, the deoxy compound may actually bind more tightly to the enzyme than the analogously substituted deoxyfluoro substrate.

If we wish to specifically probe the nature and magnitude of the enzyme/substrate hydrogen bonds at the glycone site, modifications which are made to the hydroxyls on
the sugar ring must be sterically conservative. Substitutions which are larger than hydroxyl risk generating adverse steric effects, making any meaningful interpretation of the data impossible. For this reason, deoxy and deoxyfluoro substrates are acceptable as probes of these interactions since both hydrogen and fluorine are smaller than the hydroxyl.

![Chemical Structures](image)

**Figure 1-10. Examples of deoxy and deoxy-fluoro glucosides**

<table>
<thead>
<tr>
<th>Group</th>
<th>Bond Length (Å)</th>
<th>Van der Waals Radius (Å)</th>
<th>Total (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-H</td>
<td>1.09</td>
<td>1.20</td>
<td>2.29</td>
</tr>
<tr>
<td>C-F</td>
<td>1.39</td>
<td>1.35</td>
<td>2.74</td>
</tr>
<tr>
<td>C-O(H)</td>
<td>1.43</td>
<td>1.40</td>
<td>2.83</td>
</tr>
<tr>
<td>C-OH</td>
<td>1.43</td>
<td>2.10</td>
<td>3.53</td>
</tr>
</tbody>
</table>

*a* Data taken from (37)

When the hydroxyl group on the sugar ring is replaced by hydrogen, any hydrogen bonding interaction which existed at that position in the enzyme/substrate complex will be deleted. The loss of the interaction will be observed as a change in the observed kinetics for the hydrolysis of the substituted substrate with respect to the fully hydroxylated parent compound. Loss of binding energy at the transition state will manifest itself as a drop in the rate of reaction for the deoxy substrate with respect to the
parent compound. Loss of binding energy at the ground state will result in a higher
dissociation constant (K_d) for the deoxy substrate as compared to the parent compound.

The ratio of the rate constants or dissociation constants between the deoxy
compound and the parent is related to the hydrogen bond strength and provides a
minimum estimate of the strength of the interaction at the position being examined.
These data can be quantitated by the relation

\[ \Delta \Delta G^0 = RT \ln \left( \frac{k_{\text{par}}}{k_{\text{sub}}} \right) \]

where R = 8.314 J/(K mol), T is the temperature in Kelvin, k_{par} is the constant for the
parent compound and k_{sub} the analogous constant for the substituted substrate.
If the same position is now substituted with fluorine, the net hydrogen bonding polarity
at the substituted position can be examined. Fluorine is arguably capable of accepting a
hydrogen bond, but cannot possibly donate one. Therefore, if the enzyme was donating
a hydrogen bond at the substituted position, it may be able to regain some of the binding
energy lost in the deoxy compound, which would manifest itself as an increase in rate
constant for the fluoro compound over the deoxy compound. This approach has been
used to examine the non-covalent interactions in the mechanism of glycogen
phosphorylase (38) phosphoglucomutase (39), \( \beta \)-galactosidase (40) and recently lactase
(41).

Other factors, aside from non-covalent interactions may also affect the observed
rates of hydrolysis of the modified substrates. For example, replacement of a ring
hydroxyl group with fluorine should decrease the rate of hydrolysis of these compounds
with respect to the fully hydroxylated parent glucoside since an electron withdrawing
substituent has been introduced into an electron deficient transition state. Deoxygenation
of the substrate should induce the inverse effect. Another possibility is that substitution
of the ring hydroxyl groups could lead to significant changes in the structure of the
glycoside. Either of these factors could affect the rate of hydrolysis of the substituted
glycosides and must be addressed before the role of enzyme/substrate interactions in the mechanism can be examined. These concerns are addressed in Chapter 2.

1.4 Aims of This Study

In this study we propose to use modified deoxy and deoxyfluoro substrates to probe the role of non-covalent interactions between substrate and enzyme in the mechanism of β-glucosidase from *Agrobacterium faecalis*. A series of mono-substituted deoxy and deoxyfluoro 2',4'-dinitrophenyl-β-D-glycopyranosides was synthesized to probe these interactions. The dinitrophenyl aglycone was chosen to allow the reactions to be easily followed spectrophotometrically and also to provide the best opportunity to examine the pre-steady state kinetics for these substrates (which can only be observed if the glycosylation rate is higher than that of deglycosylation). The deoxy glycosides were used to estimate the apparent hydrogen bond strengths at each position on the sugar ring at the ground state, at the transition state for glycosylation and at the transition state for deglycosylation. By comparing the rate constants for these steps with the deoxy substrates to the same constants for the analogously substituted deoxyfluoro substrate, we may be able to determine the net hydrogen bonding polarity at the substituted position. Once the steady state and pre-steady state data are accumulated for all of the substrates, the role of the interaction at each of the positions around the sugar ring with respect to ground state binding of the substrate or stabilization of the transition state for glycosylation and deglycosylation can be assessed.

The spontaneous hydrolysis rates of these substrates were also examined in order to determine the effect that intrinsic electronic factors have on the rate of hydrolysis and how this may affect the observed enzymic rates. These data will also be helpful in addressing the role that the substituents on the sugar ring play in the spontaneous hydrolysis of glycosides. In this way it should be possible to examine the electronic
nature of the transition states in the enzymic reaction and possibly to probe electronic differences in the transition states for glycosylation and deglycosylation of the enzyme.

Finally, by comparison of the kinetic data accumulated for the substituted DNPglycosides with inhibition constants for similarly substituted inhibitors it is proposed to examine the mode of action of 1-deoxynojirimycin and castanospermine as non-covalent inhibitors of the abg β-glucosidase. Though the tight binding of the amine inhibitors is thought to be due to their resemblance to the oxocarbonium ion transition state there is no evidence available to show this is the case. This study will provide evidence to determine if these compounds are indeed transition state analogues.
Chapter 2

Investigation of the Mechanism of Spontaneous Hydrolysis of Glycosides.
2.1 Introduction

2.1.1 Nature of the Oxocarbonium Ion Generated in the Hydrolysis of Glycosides

The mechanism of glycoside hydrolysis has been intensively studied (1). Based on these studies, two mechanisms have been proposed for the acid-catalyzed hydrolysis of glycosides. According to the first mechanism, the reaction proceeds via a rapid reversible protonation of the exocyclic oxygen of the glycosidic bond, followed by a slow unimolecular decomposition of the protonated glycoside to generate a cyclic oxocarbonium ion. The attack of water on this species yields the hydrolyzed product (Figure 2-1).

![Figure 2-1. Cyclic oxocarbonium ion mechanism for the acid-catalyzed hydrolysis of a glycoside.](image)

In the second mechanism, it is proposed that glycoside hydrolysis proceeds via a rapid reversible protonation of the endocyclic oxygen, followed by a slow cleavage of the O5-C1 bond to generate an acyclic oxocarbonium ion. Recyclization after the attack of water on the acyclic species gives the product (Figure 2-2).
Figure 2-2. Acyclic oxocarbonium ion mechanism for the acid-catalyzed hydrolysis of a glycoside.

Though some evidence supports the acyclic mechanism (42,43), it is generally believed that the predominant pathway for hydrolysis of glycopyranosides proceeds via a cyclic oxocarbonium ion transition state. In an elegant study of isotope effects in the acid-catalyzed hydrolysis of α- and β-methyl glucosides, Bennet and Sinnott (44) have provided the best evidence to date for the cyclic oxocarbonium ion mechanism. Labeling of the ring oxygen with $^{18}$O resulted in a small negative KIE for the acid-catalyzed hydrolysis of methyl glucosides ($k_{O16}/k_{O18} = 0.991 \pm 0.002$ for the β-glucoside). Further, $^{18}$O substitution on the methoxy leaving group generated a positive primary KIE for cleavage of the exocyclic C1-OMe bond ($k_{O16}/k_{O18} = 1.024 \pm 0.001$ for the methyl β-D-glucoside). The observation of a negative isotope effect for hydrolysis of the
18O5 glucoside is inconsistent with the acyclic mechanism and would be expected in the generation of a cyclic oxocarbonium ion. Similarly, the observation of a primary KIE for cleavage of the C1-OMe bond would indicate that cleavage of that bond is rate-limiting, again supporting the cyclic oxocarbonium ion mechanism. Based on the results of this study and others (1) the cyclic mechanism seems likely for the hydrolysis of hexopyranosides.

2.1.2 Stereoelectronic Effects

Perhaps the most controversial aspect of the mechanism of glycoside hydrolysis is the role of stereoelectronic effects. Stereoelectronic theory predicts that the rate of hydrolysis of an α-glycoside should be greater than the rate of hydrolysis of an analogous β-glycoside due to the presence of a lone pair of electrons on the ring oxygen anti-periplanar to the scissile bond in the α-glucoside (45). This lone pair would presumably donate electron density into the $\sigma^*$ orbital of the glycosidic C-O bond and thus assist in cleavage of the glycoside.

![Figure 2-3. Stereoelectronic assistance in the cleavage of an α-glucoside.](image)

It has generally been observed that the hydrolyses of α-glycosides are in fact faster than those of β-glycosides, a finding consistent with predictions based on the anti-periplanar lone pair hypothesis. There are, however, some important exceptions. Capon
(1) noted that the rate of acid-catalyzed hydrolysis of methyl β-glucoside was higher than that of methyl α-glucoside. Similarly, the hydrolysis of β-glucosyl phosphates is faster than the hydrolysis of the α-anomers (46). In many cases, as the steric bulk of the leaving group increases, the rate of hydrolysis for the α-anomers becomes greater than that of the analogous β-glycoside (Table 2-1). Capon therefore suggested that the difference in rates could be rationalized by steric arguments with the hydrolysis rate for the α-glycosides increasing as the steric bulk of the aglycone was increased. Though this does not rule out a contribution from the anti-periplanar lone pair in generating the transition state for glycoside hydrolysis, it does suggest that this contribution may be relatively minor.

Table 2-1. First-Order Rate Constants for the Acid-Catalyzed Hydrolysis of Some Glycopyranosides in 2.0 M Hydrochloric Acid

<table>
<thead>
<tr>
<th>Glycopyranoside</th>
<th>k x 10^5 at 60 °C (sec^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl α-D-gluco-</td>
<td>0.708</td>
</tr>
<tr>
<td>Methyl β-D-gluco-</td>
<td>1.26</td>
</tr>
<tr>
<td>Phenyl α-D-gluco-</td>
<td>38.0</td>
</tr>
<tr>
<td>Phenyl β-D-gluco-</td>
<td>9.33</td>
</tr>
<tr>
<td>p-Nitrophenyl α-D-gluco-</td>
<td>25.1</td>
</tr>
<tr>
<td>p-Nitrophenyl β-D-gluco-</td>
<td>2.88</td>
</tr>
<tr>
<td>Methyl α-D-galacto-</td>
<td>3.55</td>
</tr>
<tr>
<td>Methyl β-D-galacto-</td>
<td>5.13</td>
</tr>
<tr>
<td>Ethyl α-D-galacto-</td>
<td>7.58</td>
</tr>
<tr>
<td>Ethyl β-D-galacto-</td>
<td>5.01</td>
</tr>
<tr>
<td>Phenyl α-D-galacto-</td>
<td>128</td>
</tr>
<tr>
<td>Phenyl β-D-galacto-</td>
<td>24.5</td>
</tr>
</tbody>
</table>

a) Data taken from (47). b) Rate constants extrapolated from Arrenhius plots.
Several other inconsistencies in the antiperiplanar lone pair hypothesis have been investigated by Sinnott (48) who contends that stereoelectronic theory is valueless as a tool for the prediction of hydrolysis rates in glycosides and that the entire theory could be reinterpreted in terms of the theory of least motion. Recently, a more moderate hypothesis has been proposed by Kempton and Withers (6) where it was suggested that the requirement for an anti-periplanar lone pair for hydrolysis is predicated on the assumption that these reactions are $S_N$1, and thus lead to a significant amount of oxocarbonium ion character at the transition state. However, it is reasonable to suggest that glycoside hydrolysis can proceed with a significant amount of nucleophilic pre-association in which case the stereoelectronic constraints of the reaction move towards those of an in-line displacement (see 57). Thus predictions based on the requirement for an anti-periplanar lone pair would become less reliable. In any case, general agreement on the role of stereoelectronic effects in the solvolysis of glycosides has not yet been reached. For a thorough discussion of both views on this theory and its application to the hydrolysis of glycosides see (45) and (49).

2.1.3 Transition State Structure

There is general agreement in the literature that a significant amount of oxocarbonium ion character exists at the transition state for the hydrolysis of most glycosides. Most of the evidence for this has been obtained from $\alpha$-DKIE's which provide insight into the hybridization state of the reacting center at the transition state. While there can be problems in interpreting these values (52) they do appear to be reasonable predictors of hybridization states within a series of reactions involving the same chemical types of nucleophiles and leaving groups. $\alpha$-DKIE's have been measured for the acid-catalyzed hydrolysis of 4-methylphenyl and 4-methylumbelliferyl $\beta$-D-glucopyranosides (50) and $\alpha$- and $\beta$-methyl glucoside (44) (Table 2-2). In all cases
moderate $\alpha$-DKIE's effects were observed which is consistent with rehybridization of C1 at the transition state and thus some degree of oxocarbonium ion character.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\alpha$-DKIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Methylphenyl $\beta$-D-glicoside</td>
<td>1.056 +/- 0.012</td>
</tr>
<tr>
<td>4-Methylumbelliferyl $\beta$-D-</td>
<td>1.071 +/- 0.010</td>
</tr>
<tr>
<td>methyl $\alpha$-glucoside</td>
<td>1.137 +/- 0.007</td>
</tr>
<tr>
<td>methyl $\beta$-glucoside</td>
<td>1.073 +/- 0.003</td>
</tr>
</tbody>
</table>

Based on these data and the observation that the $\alpha$-DKIE for acid-catalyzed hydrolysis of $\beta$-glucosides decreased with increasing leaving group ability (data not shown), Van Doorslaer et al. (50) concluded that glycoside hydrolysis proceeded by an A-1 mechanism with $S_N$2 character and further, that the transition state for hydrolysis became more reactant-like with better leaving groups. This hypothesis is somewhat contrary to a study by Sinnott and Jencks (51) which suggested that the transition state for ethanolysis of glycosides became more dissociative with better leaving groups and so would be expected to show larger isotope effects. This disparity emphasizes the fact that the magnitudes of isotope effects are influenced by many factors (see 52) and must be interpreted cautiously.
2.1.4 The Ring Substituents

Relatively little work has been done on examining the influence of the ring substituents on glycoside hydrolysis rates. In general, it is found that the rate of hydrolysis of aldohexopyranosides increases as the number of axial hydroxyl groups on the glycopyranose ring increases. Edward (53) suggested that the factors determining the relative rates of hydrolysis of these glycosides were largely steric. As the reaction proceeds to the transition state, the conformation of the sugar changes from a $4C_1$ chair to a $4H$ half chair due to the formation of the C1-O5 double bond in the oxocarbonium ion. This allows the axial substituents in the carbohydrate ring to move away from each other resulting in relief of steric strain from the 1,3-diaxial interactions present in the ground state chair. Since this effect is greater when these interactions are between a hydroxyl and a hydrogen (rather than two hydrogens) the rate of hydrolysis of glycosides with more axial hydroxyl groups would be expected to be higher.

![Figure 2-4. Half chair transition state proposed for the hydrolysis of a methyl-glucoside.](image)

Although this argument rationalizes the greater rate of hydrolysis of glycosides with more axial hydroxyl groups, it does not address the differences in the rates of hydrolysis for glycosides with the same number of axial hydroxyl groups (for example, the rates of hydrolysis of galactosides are generally found to be higher than the rates of hydrolysis of mannosides).
Polar effects

Polar effects also play an important role in determining the relative rates of hydrolysis of glycosides. Replacing a hydroxyl group on the glucopyranose ring by hydrogen would be expected to result in an increased rate of hydrolysis for the deoxy glucoside since an electron withdrawing substituent has been removed from an electron deficient transition state. Further, the magnitude of this effect might be expected to be related to the distance between the substituent and the anomeric center. While this may be true, several studies have shown that another effect is also at work. In one study, Overend et al. (47) determined the rates of acid-catalyzed hydrolysis of a series of α-methyl glycosides where the ring hydroxyl groups had been mono-substituted for hydrogen. The rates of hydrolysis followed the order 2d>4d>3d>OH. The same order was reported by Mega and Matsushima (54) for the acid-catalyzed hydrolysis of a series of analogously substituted phenyl deoxy-β-D-glucosides where 2d>4d>3d>6d>OH.

A more detailed study of the problem was undertaken by Withers and co-workers (46,55) through examination of rates of acid-catalyzed hydrolysis of a series of α-glucosyl phosphates. The hydrolysis rates for the deoxy compounds in this study again followed the trend observed previously such that 2d>4d>3d>6d>OH. An accompanying study examined the relative rates of hydrolysis of the analogous series of deoxyfluoro glucosyl phosphates and the inverse trend was observed, ie. OH>6F>3F>4F>2F.

The rationale for these results proposed by the authors was based on a combination of inductive effects with a detailed examination of the change in the relative orientation of the dipoles for the C-substituent bonds as the reaction proceeded from the ground state \(^4C_1\) chair to the transition state \(^4H\) half chair. Assuming that an increase in alignment of the dipoles on adjacent positions destabilizes the transition state, it was shown that substitution of hydroxyl by fluorine at the 3 position would have less of an adverse dipole effect than a substitution at the 4 position. Combination of this effect
with the inductive effect based on the distance of the substitution from C1 would yield
the observed order for the hydrolysis rates of the deoxy and deoxyfluoro glucosides
(Figure 2-5).

A recent molecular modeling study by Woods et al. (56) suggests another role for
the ring hydroxyls in glycoside hydrolysis. Assuming that the main factor which
determines the relative rates of hydrolysis of glycopyranosides is the relative stability of
the oxocarbonium ions generated at the transition state, calculation of these stabilities
should allow the relative rates of hydrolysis of these compounds to be predicted. To this
end, the gas phase stabilities of the lyxo, ribo, arabino and xylo oxocarbonium ions were
examined using MM2 87 molecular mechanics calculations. This study indicated that the
relative stabilities of the ions were in part determined by hydrogen bonds between the
ring hydroxyl groups in the half chair transition state. Based on the values determined,
the predicted relative hydrolysis rates of the pentopyranosides would be
arabino>ribo>xylo>lyxosides. If these results are extended to the analogous
aldohexopyranosides, the predicted relative hydrolysis rates would be
galacto>allo>gluco>mannosides. However, the utility of this approach is questionable
since interactions with solvent, leaving group and attacking nucleophile can affect the
structure of the transition state (57) and are not addressed. In addition, the effects of
differences in ground state stabilities are not examined. Indeed, when the relative rates of
hydrolysis of the pentopyranosides are compared it is found that the relative rate of acid-
catalyzed hydrolysis of the methyl glycosides is lyxo>arabino>xyloside (53).
<table>
<thead>
<tr>
<th>Bond Viewed Down</th>
<th>GROUND STATE Conformation of Substrate</th>
<th>&quot;TRANSITION STATE&quot; Conformation of Substrate</th>
<th>TRANSITION STATE Conformation of Oxocarbonium Ion (when different)</th>
<th>CONSEQUENCE in terms of Dipole Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1—O-5</td>
<td><img src="#" alt="Diagram 1" /></td>
<td><img src="#" alt="Diagram 2" /></td>
<td><img src="#" alt="Diagram 3" /></td>
<td>Greater Alignment</td>
</tr>
<tr>
<td>O-5—C-5</td>
<td><img src="#" alt="Diagram 4" /></td>
<td><img src="#" alt="Diagram 5" /></td>
<td><img src="#" alt="Diagram 6" /></td>
<td>Slightly less Alignment</td>
</tr>
<tr>
<td>C-5—C-4</td>
<td><img src="#" alt="Diagram 7" /></td>
<td><img src="#" alt="Diagram 8" /></td>
<td><img src="#" alt="Diagram 9" /></td>
<td>No Change</td>
</tr>
<tr>
<td>C-4—C-3</td>
<td><img src="#" alt="Diagram 10" /></td>
<td><img src="#" alt="Diagram 11" /></td>
<td><img src="#" alt="Diagram 12" /></td>
<td>No Change</td>
</tr>
<tr>
<td>C-3—C-2</td>
<td><img src="#" alt="Diagram 13" /></td>
<td><img src="#" alt="Diagram 14" /></td>
<td><img src="#" alt="Diagram 15" /></td>
<td>Less Alignment</td>
</tr>
<tr>
<td>C-2—C-1</td>
<td><img src="#" alt="Diagram 16" /></td>
<td><img src="#" alt="Diagram 17" /></td>
<td><img src="#" alt="Diagram 18" /></td>
<td>Greater Alignment</td>
</tr>
</tbody>
</table>

Figure 2-5. Relative dipole alignments in ground and transition states. Taken from (46).
2.2 Goals of this Study

In this study, a series of mono-substituted deoxy and deoxyfluoro 2',4'-dinitrophenyl β-D-glycopyranosides was synthesized as a means of examining the role of the ring substituents in glycoside hydrolysis. Both the equatorial and axial epimers of the fluorinated and fully hydroxylated glycosides were synthesized (in part by Dr. Adam Becalski in this laboratory) to explore the dependence of the hydrolysis rates on the configuration of the different centers on the glycone. The choice of deoxy and deoxyfluoro substitutions minimizes steric effects on the reaction rate, both substituents being smaller than hydroxyl. Hydrolysis of a series of deoxy and deoxyfluoro galactosides (synthesized by John McCarter in this laboratory) was also examined.

DNPGlycosides were chosen for these studies because the rate of hydrolysis of these compounds has been shown to be pH independent between pH 2 - 8 (58). Therefore, rate constants determined in the pH independent region will be unaffected by protonation equilibria, providing true first order rate constants for the heterolysis of the glycosidic linkage, free from any complications associated with parallel pathways through neutral and conjugate acid species.

2.3 Synthesis

Synthesis of the deoxy and deoxyfluoro DNPGlycosides used in these studies began with the syntheses of the required protected deoxy and deoxyfluoro glucose derivatives according to published procedures. Generally, these syntheses involve a series of protection steps to isolate one of the hydroxyls on the sugar ring followed by replacement of that hydroxyl with either fluorine or hydrogen. Details of the procedures are provided in Chapter 5.
2.3.1 Synthesis of Per-O-Acetyl-Deoxy-D-Glucopyranoses

Syntheses of two of the deoxy glucosides started from deoxygenated precursors available in this laboratory. The 2',4'-dinitrophenyl 6-deoxy-ß-D-glucopyranoside (6dDNPglucoside) was obtained by deacetylation of 2',4'-dinitrophenyl 2,3,4-tri-O-acetyl-6-deoxy-ß-D-glucopyranoside (previously synthesized by Leise Berven in this laboratory (59)) using HCl/MeOH (60). Synthesis of the 1,2,3,6-tetra-O-acetyl-4-deoxy-D-xylo-hexopyranose was started from methyl 2,3,6-tri-O-benzoyl-4-deoxy-α-D-xylo-hexopyranoside (108). The benzoyl groups were removed in sodium methoxide/methanol (16) and the resulting methyl glucoside hydrolyzed in water in the presence of an acidic resin (Dowex 50W (H+)). The 4-deoxy-D-xylo-hexopyranose obtained was acetylated in a mixture of acetic anhydride/pyridine (109) to give the per-O-acetyl-4-deoxy-D-xylo-hexopyranose.

Synthesis of the 1,2,4,6-tetra-O-acetyl-3-deoxy-D-ribo-hexopyranose from commercially available 1,2:5,6 di-O-isopropylidene glucose utilized a modified Moffatt oxidation (61) to yield the ketone at the 3 position which was stereoselectively reduced with sodium borohydride (62) to give the protected allose (31). Treatment of (31) with sulfuryl chloride gave the protected 3-chloro-3-deoxy-glucofuranose (32). Inversion of configuration of the C3 hydroxyl group was necessary as it had previously been shown that reduction of the axial chloride was not possible (63). Hydrolysis of the isopropylidene protecting groups followed by acetylation of the free sugar yielded 1,2,4,6-tetra-O-acetyl-3-chloro-3-deoxy-D-glucopyranose (33). The per-O-acetylated-3-deoxy-D-ribo-hexopyranose is generated by reduction of the chloride with tributyl tin hydride (64).
Scheme 2-1. Synthesis of per-O-acetyl-3-deoxy-D-ribo-hexopyranose.

2.3.2 Synthesis of Per-O-Acetyl-Deoxyfluoro-D-Glucopyranoses

Syntheses of the deoxyfluoro sugars followed a similar procedure involving isolation of the hydroxyl group to be modified, followed by conversion to the
The exception was the 2-deoxy-2-fluoro-D-glucopyranoside which was synthesized by treatment of tri-O-acetyl-D-glucal with trifluoromethyl hypofluorite yielding the trifluoromethyl per-O-acetyl-2-deoxy-2-fluoro-α-D-glucoside (obtained from Dr. D. Dolphin). Syntheses of the other deoxyfluoro sugars were achieved by treatment of the selectively protected carbohydrate with diethylamino sulfuryl trifluoride (DAST), converting a hydroxyl group to fluorine with inversion of configuration. The use of DAST for the synthesis of fluorinated carbohydrates has been reviewed elsewhere (65,66,67).

**Figure 2-6. Mechanism of the reaction of DAST with a chiral alcohol.**

Synthesis of the 3-deoxy-3-fluoro glucoside began with 1,2,4,6-tetra-O-acetyl-3-deoxy-3-fluoro-β-D-glucopyranose which was available in this laboratory (46). Tetra-O-acetyl-6-deoxy-6-fluoro-D-glucopyranose was obtained in one step by treatment of 1,2,3,4-tetra-O-acetyl-D-glucopyranose (46) with DAST.

Synthesis of the 1,2,3,6-tetra-O-acetyl-4-deoxy-4-fluoro-D-glucopyranose proceeded as follows. Commercially available methyl α-D-galactopyranoside was treated with 3 equivalents of benzoyl chloride in pyridine (68) to yield the selectively
protected galactoside (8). The galactoside was treated with DAST in the presence of a suitable base (2,6-dimethyl pyridine) under anhydrous conditions to generate the deoxyfluoro glucoside (9). Since deprotection of benzyolated DNP glucosides is problematic, the benzoyl protecting groups were replaced by acetate groups. Treatment with sodium methoxide in methanol (16) followed by hydrolysis of the methyl glucoside in water in the presence of an acidic resin (Dowex 50W (H⁺)) yielded the free sugar with an overall yield of 58% for the two steps. The 4-deoxy-4-fluoro-D-glucose produced was purified, then acetylated in a mixture of acetic anhydride and sodium acetate (69) to give the per-O-acetylated carbohydrate (12).

Scheme 2-2. Synthesis of 1,2,3,6-tetra-O-acetyl-4-deoxy-4-fluoro-D-glucopyranose.
Although synthesis of the deoxy and deoxyfluoro glucose derivatives has been thoroughly investigated, synthesis of the deoxy and deoxyfluoro 2',4'-dinitrophenyl glucosides has not been reported. These syntheses are discussed below.

2.3.3 Selective Deacetylation at the Anomeric Center

Several methods are available to selectively deacetylate the C1 hydroxyl group, including treatment of the per-O-acetylated sugar with HBr/acetic acid then hydrolysis (70, 71), treatment with ethanolamine (72), hydrazine acetate (73), tributyl tin methoxide (74) or lipase (75). Of the methods examined in this study the best procedure, especially with the fluorinated compounds, was the use of ethanolamine in ethyl acetate. Yields of the hemiacetal using this procedure were good (70%, 73%, 88% and 88% for the 4-deoxy, 6-deoxy-6-fluoro, 4-deoxy-4-fluoro and 3-deoxy-3-fluoro glucose tri-O-acetates respectively), however long reaction times (typically several days) were required. Attempts to employ a faster method using hydrazine acetate met with mixed success when applied to the deoxyfluoro carbohydrates. Though the mono-deacetylation of penta-O-acetyl-D-glucose could be effected in 15 min with a 90% yield, deacetylation of the fluorinated glucose tetra-O-acetates was much slower and tended to give lower yields (50% for tetra-O-acetyl-3-deoxy-3-fluoro-D-glucose) and mixtures of products. Presumably, these byproducts arose from non-specific reaction with the other acetates on the molecule. Indeed, an attempt to selectively deacetylate 1,3,4,6-tetra-O-acetyl-2-deoxy-2,2-difluoro-D-glucopyranose with hydrazine acetate gave a mixture of products, including the 1,3-di-deacetylated derivative.
2.3.4 Formation and Deprotection of the 2’,4’-Dinitrophenyl β-D-glycosides

After purification, the appropriately protected hemiacetal was treated with 1-fluoro-2,4-dinitrobenzene (FDNB) in the presence of an appropriate base, diazabicyclo[2.2.2]octane (DABCO), to yield the per-O-acetylated-β-D-glucoside (76). Yields for syntheses of the fluorinated glucosides were lower than those obtained for the synthesis of per-O-acetyl DNPglucoside or DNPgalactoside (see Chapter 5 and (76)), typically around 35%. Though the cause of the decreased yields was never investigated, it is possible that the reduced nucleophilicity of the fluorinated hemiacetal compared to the fully hydroxylated compounds may be responsible. Indeed, moderate yields (77% and 64% respectively) were obtained for the DNP coupling step with the more nucleophilic 6-deoxy and 3-deoxy hemiacetals. Further, synthesis of the 2-deoxy-2-fluoro and 3-deoxy-3-fluoro glucosides gave significant amounts of the α-DNPglucosides, with proportionate decreases in the yields of the β-glucosides.

The deprotected glucosides were obtained by removal of the acetate groups in HCl/methanol by the method of Ballardie et al. (60). These reactions proceeded smoothly yielding the unprotected glucosides with little or no breakdown, even with the less stable deoxy glucosides. Since the deprotected DNPglucosides were too unstable for silica gel chromatography, purification of these compounds was accomplished by crystallization.
Scheme 2-3. An example of the synthesis of a DNP glycoside (4FDNP glucoside) from the corresponding per-O-acetylated glucose.

Nmr analyses of the compounds obtained were consistent with the DNP glycosides adopting the expected $^4C_1$ chair conformation. Assignment of the $^1H$ nmr resonances for the glycone protons of the acetylated DNP glucoside showed that coupling constants for adjacent ring protons were approximately 10 Hz. Similar coupling constants were found for adjacent ring protons on the modified glucosides implying the structures of the modified and parent glucoside are comparable. Coupling constants for the deprotected glycosides showed the analogous 10 Hz couplings in the cases where the individual ring protons could be assigned (see Chapter 5). Further, it has previously been demonstrated that 2-deoxy-2-fluoro-β-D-mannopyranosyl fluoride adopts a $^4C_1$ chair despite substitution of 2 hydroxyl groups for fluorine (160), again indicating that substitutions on the glycone ring would not be expected to cause large conformational changes. It is therefore unlikely that the changes in hydrolysis rate observed in this study are related to differences in the conformation of the various glycosides.
2.4 Kinetic Results

Rates of hydrolysis for the DNPglycosides were determined under the same conditions used by Sinnott (58), details of which are found in Chapter 5. Hydrolysis rates were determined in triplicate at three or more temperatures. Rate constants were determined by fitting the series of absorbance values at these time points to a first order equation (77). Activation entropies and enthalpies were determined from Eyring plots of the data and standard errors were placed on the activation parameter based on these plots using GraFit (107).

To minimize possible solute-solute interactions, hydrolyses were carried out on dilute solutions of the glycosides (~10^{-4} M). Further, the rates were measured at three different concentrations of glycoside and found to be independent of the concentration of substrate within error. This would suggest solute-solute interactions have little effect on the observed rates of hydrolysis at these concentrations.

Analysis of these data assumes that hydrolysis of these glycosides occurs via heterolysis of the glycosidic linkage as shown in Scheme 2-5A. However, it was necessary to prove that no other modes of cleavage contributed significantly to the observed rate. Since it was possible that glycoside cleavage occurred by attack of water on C1' of the aryl ring (78,110) rather than at the anomeric centre of the sugar, an experiment was performed in which the hydrolysis of the DNPglucoside at 60 °C in 18O-H2O was carried out under otherwise identical conditions to those used in the kinetic studies. Electron impact mass spectral analysis of the products of hydrolysis showed no incorporation of 18O into the DNP released on hydrolysis of the substrate within the error of the measurement (5%). This result is consistent with hydrolysis of the DNPglycosides proceeding only via attack of water on the sugar ring.
Scheme 2-4. Incorporation of the $^{18}$O label into glucose after attack of water at Cl of glucose. N.B. No $^{18}$O incorporation into DNP will occur if hydrolysis proceeds by this mechanism.

Horton (79) has demonstrated that under basic conditions (0.25 M KOH) the breakdown of α-PNPglucoside proceeds by a series of migrations initiated by the attack of the sugar C2-OH group on the ipso position of the aryl ring to generate the 2-O-p-nitrophenyl-D-glucose. The PNP group then migrates again to the 3 position hydroxyl group and subsequently eliminates yielding a 2,3 unsaturated sugar (Scheme 2-5B). It is unlikely that a significant amount of the phenol release observed in the current experiments was generated by this route for several reasons. Firstly, with the exception of the DNPmannoside, all of the glycosides examined were trans 1,2 glycosides in contrast to α-PNPglucoside where the aryl group and the C2 hydroxyl are cis. This is known to decrease the rate of migration of the aryl group (79). Secondly, the hydrolysis of the DNPglycosides was carried out at pH 6.5 while the migration is believed to be base catalyzed. Thirdly, aliquots taken at several time points during the hydrolysis of DNPglucoside and analyzed by HPLC failed to show the presence of any new UV-active sugar-containing peaks as would be generated by the migration of the DNP group to the
C2 or C3 hydroxyl (though this assumes the subsequent elimination step is rate limiting with respect to the migrations). Interestingly, new UV active peaks were detected in older samples of the DNPmannoside which may suggest that a small degree of migration has taken place with this substrate, in which the aryl group and C2 hydroxyl are cis. 

\(^1\)H nmr analysis of the samples used for kinetics did not reveal the presence of observable

\[
\text{Scheme 2-5. Heterolysis A) and elimination B) mechanisms for the release of DNP during glycoside hydrolysis.}
\]
amounts of these compounds. Also, the release of phenol during the hydrolysis of all the substrates tested followed first order kinetics which is inconsistent with two first order processes with different rates both contributing to the observed release of phenol.

Finally, Sinnott (58) has demonstrated that the 2,3-elimination did not contribute significantly to the release of phenol in the first 10% of the reaction for the hydrolysis of DNPgalactoside. Therefore the rates determined by Sinnott by initial rates analysis should not be altered by migration of the aryl group. The rate constants (4.6 x 10^{-6} sec^{-1} for DNPgalactoside, 1.2x10^{-6} sec^{-1} for DNPglucoside) determined by initial rates analysis were in reasonable agreement with the rate constants determined in this study by following the reaction for several half lives and fitting the release of phenol with time to a first order equation (4.7 x 10^{-6} sec^{-1} for DNPgalactoside and 8.7x10^{-7} sec^{-1} for DNPglucoside) again suggesting that the hydrolysis pathway monitored is that proposed in Scheme 2-5A involving simple heterolysis of the glycosidic bond.

Rate constants and activation parameters determined for the hydrolysis of the DNPglycosides are presented in Table 2-4. All of the rate constants determined for the DNPglycosides at various temperatures and a typical Eyring plot of these data are provided in Appendix 3. The relatively wide range of hydrolysis rates observed with these glycosides made it impossible to obtain reliable data for all compounds at one temperature. The rate constant listed at 37 °C is therefore the value determined by extrapolation or interpolation of the Eyring plot for hydrolysis of each glycoside.

Notably, it was found that repeat determinations of the rate constant from Eyring plots (repeated 3 times for DNPglucoside) were more precise than the activation entropy and enthalpy values derived from the same plots (see Table 2-3) implying that the accuracy of the activation parameters may not be well represented by the statistically calculated errors. For this reason, essentially all the analysis in this thesis is based upon rate data (and therefore ΔG^‡), instead of ΔH^‡ or ΔS^‡; and only large differences in ΔH^‡ or ΔS^‡ will be interpreted.
Data obtained in this study are consistent with previous results from other systems. A plot of the logarithm of the rate constants for hydrolysis of the DNPglucosides versus the logarithm of the analogous rate constants for the substituted \(\alpha\)-glucosyl phosphates showed a strong correlation \((r = 0.997, \text{Figure 2-7})\). Interestingly, a positive slope \((m = 1.1)\) was obtained for this plot indicating a slightly greater sensitivity to ring substitutions for the \(\alpha\)-glucosyl phosphates. Assuming electronic factors dominate in determining the hydrolysis rate, this would indicate a larger amount of positive charge at the transition state for hydrolysis of the glycosyl phosphates. This could be attributed to a number of factors such as the difference in the leaving groups and the stereochemistry at the anomeric center. The latter explanation is particularly attractive since evidence has been found in several systems for hydrolysis of \(\alpha\)-glycosides proceeding via a more dissociative transition state than the analogous \(\beta\)-linked compounds \((80)\).
Figure 2-7. A LFE plot of the rate constants for hydrolysis of the DNPglucosides at 37 °C versus the analogous α-glucosyl phosphates at 25 °C.
Table 2-4. *Kinetic Data for the Spontaneous Hydrolysis of the DNP Glycosides*.^a^  

<table>
<thead>
<tr>
<th>DNP glycoside</th>
<th>Number of temperatures</th>
<th>$\Delta H^\ddagger$ (kJ/mol)</th>
<th>$\Delta S^\ddagger$ (J/mol/K)</th>
<th>Rate constant at 37 °C$^b$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNPGlu</td>
<td>13</td>
<td>117.8 (2.1)</td>
<td>33.8 (0.9)</td>
<td>5.58 x 10$^{-6}$</td>
</tr>
<tr>
<td>2FDNPGlu</td>
<td>4</td>
<td>120.8 (1.5)</td>
<td>13.3 (0.3)</td>
<td>1.45 x 10$^{-7}$</td>
</tr>
<tr>
<td>2ClDNPGlu</td>
<td>4</td>
<td>82.8 (0.1)</td>
<td>-89.3 (6.8)</td>
<td>1.61 x 10$^{-6}$</td>
</tr>
<tr>
<td>3FDNPGlu</td>
<td>8</td>
<td>121.4 (6.2)</td>
<td>29.6 (2.4)</td>
<td>8.23 x 10$^{-7}$</td>
</tr>
<tr>
<td>4FDNPGlu</td>
<td>3</td>
<td>123 (8.2)</td>
<td>27.9 (3.0)</td>
<td>3.74 x 10$^{-7}$</td>
</tr>
<tr>
<td>6FDNPGlu</td>
<td>4</td>
<td>126 (16)</td>
<td>53 (11)</td>
<td>1.99 x 10$^{-6}$</td>
</tr>
<tr>
<td>3dDNPGlu</td>
<td>3</td>
<td>116.7 (1.1)</td>
<td>42.0 (0.6)</td>
<td>2.23 x 10$^{-5}$</td>
</tr>
<tr>
<td>4dDNPGlu</td>
<td>3</td>
<td>109.5 (0.3)</td>
<td>33.0 (0.2)</td>
<td>1.25 x 10$^{-4}$</td>
</tr>
<tr>
<td>6dDNPGlu</td>
<td>4</td>
<td>108.3 (0.7)</td>
<td>16.3 (0.1)</td>
<td>2.60 x 10$^{-5}$</td>
</tr>
<tr>
<td>DNPGal</td>
<td>4</td>
<td>107.0 (1.8)</td>
<td>12.0 (0.3)</td>
<td>2.61 x 10$^{-5}$</td>
</tr>
<tr>
<td>2FDNPGal</td>
<td>4</td>
<td>104.5 (1.7)</td>
<td>-21.2 (0.6)</td>
<td>1.25 x 10$^{-6}$</td>
</tr>
<tr>
<td>3FDNPGal</td>
<td>9</td>
<td>90.7 (4.9)</td>
<td>-48.1 (4.8)</td>
<td>1.04 x 10$^{-5}$</td>
</tr>
<tr>
<td>4FDNPGal</td>
<td>8</td>
<td>107.1 (0.8)</td>
<td>-4.74 (0.06)</td>
<td>3.36 x 10$^{-6}$</td>
</tr>
<tr>
<td>6FDNPGal</td>
<td>4</td>
<td>101.5 (2.3)</td>
<td>-14.1 (0.5)</td>
<td>9.40 x 10$^{-6}$</td>
</tr>
<tr>
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<td>104.3 (3.9)</td>
<td>18.4 (1.1)</td>
<td>1.61 x 10$^{-4}$</td>
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<tr>
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<td>1.18 x 10$^{-5}$</td>
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<tr>
<td>3FDNPgal</td>
<td>4</td>
<td>119.1 (1.4)</td>
<td>23.4 (0.5)</td>
<td>9.51 x 10$^{-7}$</td>
</tr>
<tr>
<td>DNPMan</td>
<td>4</td>
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<td>-18.0 (0.2)</td>
<td>1.09 x 10$^{-6}$</td>
</tr>
<tr>
<td>2FDNPman</td>
<td>4</td>
<td>126.3 (0.7)</td>
<td>18.1 (0.2)</td>
<td>3.05 x 10$^{-8}$</td>
</tr>
</tbody>
</table>

*a* Standard errors for the activation parameters are given in brackets.  

*b* Rates were calculated by extrapolation or interpolation of Eyring plots.
2.5 Discussion

The relative rates of hydrolysis determined in this study for the deoxy and deoxyfluoro DNPglucosides were \(4d>6d>3d>\text{OH}>6F>3F>4F>2F\) which is essentially the same order seen for the substituted \(\alpha\)-glucosyl phosphates. As has been discussed previously in this chapter, this order does not simply reflect the distance of the substitution from C1 and hence cannot be explained simply by the polar effect at this position. However, polar effects likely do play a role in determining the rate of hydrolysis of a glycoside since in all cases deoxygenation of the glucoside increases the rate of hydrolysis with respect to the parent glycoside while fluorination has the inverse effect. In order to examine polar and other effects which may play a role in determining the rate of glycoside hydrolysis, it will be necessary to first consider the structure of the transition state for glycoside hydrolysis, then examine the factors which affect its stability.

2.5.1 Oxocarbonium Ion Characterization

DNPglycosides have been demonstrated to hydrolyze via an \(S_N 1\) mechanism (58) which is believed to lead to generation of an oxocarbonium ion. Oxocarbonium ions are usually represented with the positive charge residing completely at the ring oxygen. This is merely one of the hypothetical resonance forms, the second form having the positive charge residing at C1 (Figure 2-8). Though oxocarbonium ion formation appears to be important in a variety of chemical processes, little information is available about the relative stabilities of these resonance forms and hence the distribution of charge at the transition state is unknown.
Recently, molecular modeling studies have been used to investigate the nature of the oxocarbonium ion. A study by Andrews et al. (86) used \textit{ab initio} calculations (G-316*) to assess the role of oxocarbonium ion formation in the acid-catalyzed breakdown of 2-methoxy tetrahydropyran (Figure 2-9). These studies indicated that lone pair donation from O5 into the no* orbital at C1 was important in attaining the transition state for the reaction and further that cleavage of the C1-O1 bond took place concurrently with formation of the C1-O5 double bond. Therefore, it would appear that at least for this system, the transition state is best described as resembling an oxocarbonium ion rather than a C1 carbonium ion.

Another modeling study carried out by Kajimoto et al. (87) used PM3 in Mopar to calculate the partial charge distribution for the ring carbons and oxygen in α-D-glucose and in a glucosyl oxocarbonium ion. Though the data presented was incomplete (it did not include ring hydroxyl or hydrogen substituents) it implied that the greatest difference in the partial charge between the ground state and the transition state was at
O5 rather than C1. Though this evidence is suggestive, it is important to point out that none of these model studies takes into account the role of solvent, attacking nucleophile or the leaving group in determining the stability of the oxocarbonium ion at the transition state for hydrolysis.

2.5.2 Polar Effects

Polar effects in alkyl systems are composed of inductive and field effects. Inductive effects are caused by through bond polarization of electron density while field effects are due to through space interactions (81). Several studies have convincingly demonstrated that polar effects in alkyl systems in solution are best described as field effects (81,82,83,84,85) and that inductive effects are of relatively minor importance. Several factors influence the magnitude of field effects (see 83). With respect to the present study, the key considerations are the positions at which charge is being created at the transition state for glycoside hydrolysis and the distance of the different ring substituents from that point.

Data obtained from the present study indicate that field effects exerted at both C1 and O5 affect the rates of hydrolysis of the DNPglycosides and several attempts have been made to demonstrate this as follows. Nmr chemical shifts have been used in several studies (88) as an indication of field effects. The correlation presumably arises because electron withdrawing substituents lead to deshielding of the nuclei being examined. The anomeric $^1$H nmr chemical shifts for the DNPglucosides in methanol-D$_4$ were used as a measure of the field effect felt at C1 as a consequence of the ring substitutions. A moderate correlation ($r = 0.74$) was found between the chemical shift and the logarithm of the hydrolysis rate (Figure 2-10) as long as the point for the 2-fluoro compound was excluded. This would indicate that the field effect at C1 has some effect on the relative rates of hydrolysis of the DNPglucosides.
It is more difficult to estimate field effects at O5. The approach we propose is to measure the pKa values of a series of monosubstituted 1-deoxyojirimycins (Figure 2-11), which were obtained from Dr. Dan Getman. Protonation of the ring nitrogen of these compounds generates a positive charge at the same position in the ring as the positive charge at the ring oxygen in an oxocarbonium ion. Therefore, the field effect on the pKa of the amine should be similar to the field effect on the ring oxygen of a glucosyl-oxocarbonium ion. Unfortunately, very few of these compounds were available (none of the di-deoxy compounds were available). However, with the exception of the 2-fluoro compounds, the correlation of this LFE plot is extremely good (r = 0.999). Indeed, even if the 2-fluoro compounds are included, the correlation is still significant (r = 0.95).
Figure 2-11. Protonated 1,3-dideoxy-3-fluoro-nojirimycin.

Table 2-5. pKa Values for the 1-Deoxynojirimycins

<table>
<thead>
<tr>
<th>1-deoxynojirimycin</th>
<th>pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>unsubstituted</td>
<td>6.48</td>
</tr>
<tr>
<td>2-fluoro</td>
<td>5.54</td>
</tr>
<tr>
<td>3-fluoro</td>
<td>5.81</td>
</tr>
<tr>
<td>4-fluoro</td>
<td>5.49</td>
</tr>
</tbody>
</table>

Figure 2-12. A LFE plot of the ln(kdehy) of the DNPglucosides versus pKa of the analogous deoxyfluoro 1-deoxynojirimycins at 37 °C. ○ DNJ; ● 2FDNJ
Therefore, the available evidence suggests that field effects at both C1 and O5 will affect the rates of glycoside hydrolysis and must be addressed. The deviation seen for the 2-fluoro compounds is interesting, and may suggest that the transition state for hydrolysis of the 2 position substituted glycosides may differ significantly from the other glycosides examined (see Section 2.5.5).

2.5.3 Parameters for Prediction of Polar Effects.

A difficulty in investigating polar effects in glycoside hydrolysis is the need for a suitable indicator of the electron withdrawing ability of a substituent. A variety of \( \sigma \) scales have been developed for use in this type of study and selection of the appropriate scale is important. A scale which was developed based on field effects in alkyl systems is \( \sigma_I \). Thus application of \( \sigma_I \) to examine field effects in the hydrolysis of carbohydrates would seem reasonable. Indeed, \( \sigma_I \) values have recently been used successfully to examine the effect of substitution at the 2 position on the rate of hydrolysis of a series of nicotinamide \( \beta \)-D-arabinosides (90).

Past studies of glycoside hydrolysis have suggested that the relative rates of hydrolysis may not be well predicted on the basis of polar effects (1). For example, a study by Timmell et al. (89) reported that the relative rates of acid-catalyzed hydrolysis of a series of 6 position substituted glucosides could not be rationalized on the basis of polar effects, thus it was suggested that both steric and polar effects may be at work. However, if the data from the Timmell study are plotted against the available \( \sigma_I \) values, a strong correlation is found \((r = 0.97, \text{Figure 2-13})\). Similar results can be obtained plotting the \( \sigma_I \) value for the entire C5 position (i.e. \( \text{CH}_2\text{X} \) for the glucosides and \( \text{H} \) for a xyloside). This implies that the effect of substitution at the 6 position is primarily electronic, and again verifies that \( \sigma_I \) is adequate for examining the role of polar effects in glycoside hydrolysis.
<table>
<thead>
<tr>
<th>Substituent</th>
<th>$\sigma_1$</th>
<th>Substituent</th>
<th>$\sigma_1$</th>
<th>Substituent</th>
<th>$\sigma_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMes$^+$</td>
<td>0.92</td>
<td>COF</td>
<td>0.42</td>
<td>CH$_2$Cl</td>
<td>0.17</td>
</tr>
<tr>
<td>SO$_2$Cl</td>
<td>0.86</td>
<td>I</td>
<td>0.59</td>
<td>NH$_2$NH$_2$</td>
<td>0.15</td>
</tr>
<tr>
<td>SO$_2$F</td>
<td>0.86</td>
<td>OAc</td>
<td>0.59</td>
<td>CH$_2$OH</td>
<td>0.10</td>
</tr>
<tr>
<td>NO$_2$</td>
<td>0.65</td>
<td>OPh</td>
<td>0.38</td>
<td>Ph</td>
<td>0.10</td>
</tr>
<tr>
<td>SOCF$_3$</td>
<td>0.64</td>
<td>CH=CHNO$_2$</td>
<td>0.58</td>
<td>Ph</td>
<td>0.10</td>
</tr>
<tr>
<td>SO$_2$Et</td>
<td>0.63</td>
<td>NO</td>
<td>0.57</td>
<td>NMes$^+$</td>
<td>0.06</td>
</tr>
<tr>
<td>NH$_3^+$</td>
<td>0.60</td>
<td>CHO</td>
<td>0.31</td>
<td>CH=CH$_2$</td>
<td>0.05</td>
</tr>
<tr>
<td>SO$_2$Me</td>
<td>0.59</td>
<td>CO$_2$Et</td>
<td>0.50</td>
<td>CH$_3$NH$_2$</td>
<td>0.00</td>
</tr>
<tr>
<td>SF$_5$</td>
<td>0.57</td>
<td>Ac</td>
<td>0.28</td>
<td>H</td>
<td>0.00</td>
</tr>
<tr>
<td>CN$^-$</td>
<td>0.56</td>
<td>OMe</td>
<td>0.27</td>
<td>Me</td>
<td>0.04</td>
</tr>
<tr>
<td>CNCO</td>
<td>0.55</td>
<td>NHAc</td>
<td>0.26</td>
<td>Et</td>
<td>0.05</td>
</tr>
<tr>
<td>OCF$_3$</td>
<td>0.55</td>
<td>CH$_3$NH$_3^+$</td>
<td>0.25</td>
<td>i-Pr</td>
<td>0.06</td>
</tr>
<tr>
<td>SO$_2$Me</td>
<td>0.50</td>
<td>OH</td>
<td>0.25</td>
<td>t-Bu</td>
<td>0.07</td>
</tr>
<tr>
<td>F</td>
<td>0.50</td>
<td>SH</td>
<td>0.25</td>
<td>B(OH)$_2$</td>
<td>0.08</td>
</tr>
<tr>
<td>SO$_2$NH$_2$</td>
<td>0.46</td>
<td>SO$_3^-$</td>
<td>0.25</td>
<td>SiMes$_3$</td>
<td>0.10</td>
</tr>
<tr>
<td>Cl</td>
<td>0.46</td>
<td>N=NPh</td>
<td>0.25</td>
<td>CH$_3$SiMes$_3$</td>
<td>0.11</td>
</tr>
<tr>
<td>CF$_3$</td>
<td>0.45</td>
<td>SMe</td>
<td>0.23</td>
<td>O$^-$</td>
<td>0.12</td>
</tr>
<tr>
<td>Br</td>
<td>0.44</td>
<td>CH$_2$CN</td>
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<td>0.42</td>
<td>CONH$_2$</td>
<td>0.21</td>
<td>B(OH)$_3^-$</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Data taken from (82).

Figure 2-13. A LFE plot of ln(khy) versus $\sigma_1$ for the acid-catalyzed hydrolysis of a series of 6 position substituted glucosides ($r = 0.97$).
2.5.4 Field Effects in the Hydrolysis of the DNPGlycosides.

If this approach is now applied to the present study, it appears that the relative rates of hydrolysis of the DNPGlucosides are consistent with predictions based on field effects. An indication of the importance of field effects is obtained by investigating the effect of substitution at each sugar position on hydrolysis rate. Hammett plots of the logarithm of the rate of hydrolysis versus $\sigma_I$ for the 3, 4 and 6 position substituted glucosides were all linear ($r = 0.994$ or higher, Figure 2-14) consistent with the relative rates of hydrolysis of the glucosides being dictated largely by electronic effects, in agreement with previous studies (55). Data for the 4 ($r = 0.999$) and 6 position ($r = 0.99$) substituted galactosides were also linear while plots for the 2 position glucosides ($r = 0.96$), 2 position mannosides ($r = 0.98$) and 3 position allosides ($r = 0.95$) showed a somewhat greater degree of scatter. Taken together, these plots provide reasonable evidence that the major factor dictating the relative rates of hydrolysis of the substituted DNPGlycosides is the field effect of the ring substituents on the stability of the oxocarbonium ion.

If this analysis is now extended to examine the relative rates of hydrolysis of the DNPGlucosides substituted at different positions, it appears that these rates are roughly in accord with predictions based on the Kirkwood-Westheimer model (149) for field effects. This model predicts that the relative values of $k$ (the equilibrium or in this study rate constant being studied) for two compounds differing with respect to a single substitution ($x$ and $y$) can be calculated using the formula shown below where $e$ is the charge generated and $\theta$ is the angle between a line joining the point where charge is being generated and the midpoint of the dipole created by the polar substitution (the length of this line being $R$). $\varepsilon$ is the dielectric constant for the space through which the field effect is traveling and $\mu$ is the magnitude of the dipole created by the substitution.
Figure 2-14. LFE plots of \( \ln(\text{khy}) \) for the DNPglucosides versus \( \sigma_f \) for the substituent at the ring position being examined. a) The rate for the 2dDNPglycoside was estimated using the observed relationship between the hydrolysis rate of the DNPglucosides and the \( \alpha \)-glucosyl phosphates (Figure 2-7) and the known hydrolysis rate for the 2-deoxy \( \alpha \)-glucosyl phosphate (55).
\[
\ln(k_x/k_y) = (e/kT) \left[ (\mu \cos\theta/R^2D_E)_x - (\mu \cos\theta/R^2D_E)_y \right]
\]

Figure 2-15. An illustration of determining \( R \) and \( \theta \) for the 6 position with respect to O5.

Since we are comparing substitutions on the same system at one temperature, it is reasonable to assume that \( e \), \( k \), \( T \) and \( D_E \) are all constant and thus will not affect the relative rates. For this analysis, it was assumed that the transition state for glycoside hydrolysis is a \( ^4H \) half chair, and that charge is generated at the transition state at both Cl and O5. Through space distances between O5 or Cl and the substituted centers, along with values for \( \theta \), were calculated for a glucosyl oxocarbonium ion using PCmodel (157). Finally, the relative magnitudes of the dipole for the substituent bond were estimated using the \( \sigma_1 \) value for the substitution. Thus the relative field effect felt at O5 and Cl from a position on the ring may be estimated using the formula

\[
F.E. = e_{C1} [\sigma_1 \cos\theta/(R_{C1})^2] + e_{O5} [\sigma_1 \cos\theta/(R_{O5})^2]
\]

where F.E. is the relative size of the field effect and \( e_{C1} \) and \( e_{O5} \) are the partial charges generated at Cl and O5.

The field effect at O5 and Cl was estimated for each of the ring positions. Values for these parameters were calculated for a number of different charge distributions where
the total amount of charge generated at the transition state (arbitrarily assigned a value of 1) was split between O5 and C1 to differing degrees. Presented in Table 2-7 are the values calculated assuming that 60% of the charge resides on O5 and 40% on C1, which showed the best correspondence with the observed rates of hydrolysis (the observed relative order of the rates of hydrolysis are predicted with 25 to 80% of the charge on O5). The numbers provided in the table represent the sum of all of the field effects due to the substituents on the ring. For example, calculation of the field effect for DNPglucoside requires the field effect at O5 and C1 be calculated for a hydroxyl group at positions 2, 3, 4 and 6, while 2FDNPglucoside would require calculation of the field effect for a fluorine at the 2 position and hydroxyl groups at the 3, 4 and 6 positions and so on. The Kirkwood-Westheimer hypothesis has been applied to a number of different systems, and usually functions best in rigid ring systems (83). It will therefore be difficult to estimate the field effect from the 6 position substitutions since the polar substituent is relatively free to rotate around the C5-C6 bond. For this analysis, the weighted average of the two most populated rotamers for 6-deoxy-6-fluoro α-D-glucose and α-D-glucose in D2O (91) were used, but it is certainly possible that the rotamer populations are different at the transition state.

Given the crude nature of these estimates, the relative field effects parallel the rates of spontaneous hydrolysis to a remarkable degree (Figure 2-16). Thus it would appear that the size of the field effect generated by a substituent on the sugar ring can be estimated using the \( \sigma_1 \) value for the substituent. Further, the relative rates of hydrolysis of the DNPglucosides substituted at the various ring positions correlate well with the magnitude of the field effect at O5 and C1 as predicted by the Kirkwood-Westheimer model. Taken together, these data strongly indicate that the relative rates of hydrolysis of these glucosides are dictated by the relative stabilities of the oxocarbonium ions.
Table 2-7. Estimates of the Relative Sizes of Field Effects
Calculated for the DNPglucosides.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Relative field effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>2FDNPglu</td>
<td>0.1152</td>
</tr>
<tr>
<td>3FDNPglu</td>
<td>0.1046</td>
</tr>
<tr>
<td>4FDNPglu</td>
<td>0.1083</td>
</tr>
<tr>
<td>6FDNPglu</td>
<td>0.0915</td>
</tr>
<tr>
<td>DNPglu</td>
<td>0.0839</td>
</tr>
<tr>
<td>6dDNPglu</td>
<td>0.0764</td>
</tr>
<tr>
<td>3dDNPglu</td>
<td>0.0633</td>
</tr>
<tr>
<td>4dDNPglu</td>
<td>0.0595</td>
</tr>
</tbody>
</table>

Figure 2-16. A LFE plot of the observed rates of hydrolysis for the DNPglucosides versus the predicted size of the field effect at O5 and Cl ($r = 0.98$).
created at the transition state and that these stabilities are principally dependent on electronic factors.

If no other factors are important, it should be possible to predict the relative rates of hydrolysis of all of the DNPglycosides in the manner described for the glucosides. If hydrolysis of the galactosides proceeds via a $^4\text{H}$ half chair, the field effect for the 4 position substituent in the galactosides would be predicted to be smaller than the analogous equatorial substituent due to the increase in $\theta$ on epimerization of the C4 substituent. Thus the faster rate of hydrolysis for the galactosides and similarly for the allosides, with respect to the glucosides would be predicted on the basis of field effects. However, the calculated field effects for the DNPgalactosides only correlated moderately ($r = 0.93$) with the logarithm of the rate constant for hydrolysis. Further, the field effect from C2 in a $^4\text{H}$ half chair should be similar for a mannoside and a glucoside, yet the rates constants for hydrolysis of the mannosides were lower than those found for the analogous glucosides. It is therefore apparent that a detailed application of this theory to the other glycosides will be less successful than it was with the glucosides. It is possible that the structure of the transition state will vary as the configurations of the substituents on the glycone are changed. In one instance, modeling studies have shown that at least two structures of the mannosyl oxocarbonium ion are possible (158) making it difficult to estimate the role of field effects in those systems without detailed knowledge of the transition state structure. Further, the transition state structure may well differ in water solution from the structures calculated from modeling studies in gas phase.

In any case, it seems probable that other factors aside from field effects have a role in determining the rates of hydrolysis, thus accounting for the scatter observed in several of the LFE plots in Figure 2-14. Several factors which may contribute to the observed rate of hydrolysis are discussed below.
### 2.5.5 Differences in the Hydrolysis of the 2 Position Substituted Glycosides.

Several observations from the present study imply that hydrolysis of the 2 position substituted glycosides differs from that of the other glycosides. The 2-deoxy-2-fluoro-glycoside deviated from the trend observed for the other glycosides in both the nmr chemical shift study and 1-deoxynojirimycin pKa study. Further, considerable scatter was seen in the plot of \( \ln(k_{hy}) \) versus \( \sigma_1 \) for the 2 position substituted glucosides. These deviations could arise from several factors.

As the reaction progresses toward the transition state, the position of the substituent at C2 will shift as the half chair is formed. This will change the orientation of the dipole created at this position with respect to C1 and O5 and could account for some of the deviation observed since the field effect at the ground state (assessed by the nmr and pKa studies) would differ from the field effect at the transition state. It is also likely that field effects at the 2 position would be more pronounced than at the other positions since only at C2 is the substitution adjacent to one of the atoms on which positive charge is generated at the transition state. Thus the oxocarbonium ion generated during hydrolysis of the DNP 2-halo-glycosides is likely much less stable and may require a significantly higher degree of nucleophilic pre-association for the transition state to be formed (52). Support for this hypothesis comes from examination of the activation entropy values. The activation entropy, \( \Delta S^\ddagger \), is related to the frequency of productive collisions between reactant molecules (92). If a fairly specific geometry is required for the reaction to take place (i.e. for the transition state to be formed) the number of productive collisions will be low. Thus the overall reaction rate is lowered, as is \( \Delta S^\ddagger \). In this way, \( \Delta S^\ddagger \) provides a measure of the relative 'order' of the reaction when similar compounds are being examined. The \( \Delta S^\ddagger \) values for hydrolysis of the DNP 2-deoxy-2-fluoro, and 2-chloro-2-deoxy glucosides and for hydrolysis of the 2-deoxy-2-fluoro and 2-chloro-2-deoxy-\( \alpha \)-glucosyl phosphates (46) are all lower than \( \Delta S^\ddagger \) values observed for...
the fully hydroxylated parent glycosides. This is consistent with a more pre-associated transition state for hydrolysis of the 2 position substituted glycosides.

Another possibility is that the 2 position hydroxyl group aids in hydrolysis by some type of neighboring group participation. For example, it has been suggested (51) that the 2 position hydroxyl in the parent compound may have a role in positioning the nucleophile (in this case, water) for attack at C1. Therefore replacing the C2 hydroxyl with fluorine could have a dual effect and cause a larger decrease in rate than would be predicted solely from field effects.

2.5.6 Steric Effects

It has been suggested previously that steric effects play a role in determining the relative rates of hydrolysis of glycopyranosides (1). As discussed earlier, Edward (53) has proposed that glycosides with larger axial substituents on the glycone will hydrolyze faster than the analogous glucosides due to release of steric compression present at the ground state. However, the data obtained in this study are not wholly consistent with this proposal. Most notably, the DNPmannoside and 2FDNPmannoside both had lower rates of hydrolysis than the analogous glucosides. Further, while the rate of hydrolysis of the DNPgalactoside is five times higher than the rate of hydrolysis of DNPglucoside, the rate of hydrolysis of the 4FDNPgalactoside is ten times higher than the rate of hydrolysis of the 4FDNPglucoside, despite the fact that fluorine is significantly smaller than a hydroxyl substituent. Therefore, though it is probable that steric factors do play some role in determining the relative rates of hydrolysis of the DNPglycosides, the mechanism by which this is accomplished is unclear and their influence on the rates of hydrolysis of the DNPglycosides remains to be determined.
2.6 Effects Between Series of Glycosides

Field effects appear to provide a reasonable explanation for the relative rates of hydrolysis of the DNPglycosides within a series of compounds (e.g. within the glucosides) but provide only a partial explanation for the changes in the hydrolysis rate seen between series of glycosides (i.e. between the galactosides and the glucosides). To further investigate this problem, kinetic data for the DNPgalactosides and DNPglucosides were compared.

The relative rates of hydrolysis of the DNPgalactosides correlate well with field effects at C1. If the data point for the 2-fluoro compound is again excluded, an excellent correlation ($r = 0.99$) is obtained for a plot of the $\ln(k_{hy})$ versus the chemical shift of H1 in methanol-D$_4$ (Figure 2-17) again confirming the importance of field effects in determining the relative rates of glycoside hydrolysis.

The rates of hydrolysis for the DNPglucosides correlate strongly ($r = 0.98$) with those of the analogous DNPgalactosides when plotted in the form of a LFE plot (Figure 2-18) indicating similarity in the transition states for hydrolysis of these compounds. The slope of this plot ($m = 0.90$) indicates that hydrolysis of the galactosides proceeds via a transition state which is slightly less sensitive to substitution of the ring hydroxyl groups than the glucosides. The simplest explanation is that less positive charge is developed during hydrolysis of the galactosides. Interestingly, the 4dDNPglycoside did not follow the trend for the other glycosides while the 4FDNPgalactoside did. Since the former compound is unique among the galactosides in that it lacks an axial C4 substituent, it might be expected that it would not correlate well.
Figure 2-17. A plot of $\ln(k_{hY})$ at 37°C for the DNPgalactosides versus the $^1$H-nmr chemical shift at H1 for the DNPgalactosides in methanol-$D_4$. □ DNPgals; ● 2FDNPgal

Figure 2-18. A LFE plot of the $\ln(k_{hY})$ for the DNPglucosides versus the DNPgalactosides at 37°C. ○ DNPglys; ● 4dDNPgly
An interesting difference between hydrolysis of the DNPgalactosides and the DNPglucosides is noted on comparison of the values determined for $\Delta H^\ddagger$ and $\Delta S^\ddagger$. Though the rates of hydrolysis of the various galactosides and glucosides differ by only three to ten times (i.e. $\Delta G^\ddagger$ is relatively constant), differences in activation entropies and enthalpies between the two series of compounds are large, with both $\Delta H^\ddagger$ and $\Delta S^\ddagger$ being smaller for the galactosides. Entropy/enthalpy compensations (where the free energy of a reaction remains relatively constant while the entropy and enthalpy vary in the same direction, i.e. both positive or negative) have been noted for a variety of systems such as the hydrolysis of esters (93), ionization of carboxylic acids (94), binding of different ions to a protein (95) and binding of carbohydrates to lectins (96). A rationale proposed for these observations (97) invokes a reorganization of the solvent structure as the particular reaction takes place. For example, if a process involves the generation of charge during the reaction, water molecules may re-orient to stabilize the charge thus decreasing the activation enthalpy. However, the organization of water required for this process to occur is entropically disfavored and will lead to a lower $\Delta S^\ddagger$ for the reaction. Thus reactions of this type would be expected to exhibit entropy/enthalpy compensations. Indeed, solvent reorganizations can be a major determinant of the value of $\Delta S^\ddagger$, especially in cases where the ground state and transition state differ significantly in the amount of charge on the molecule (161).

As has already been discussed, lower $\Delta S^\ddagger$ values can arise as the result of a greater degree of pre-association of the nucleophilic water at the transition state. However, this does not appear to be true for hydrolysis of the DNPgalactosides in comparison to the analogous DNPglucosides. Measurement of the $\alpha$-DKIE's for hydrolysis of DNPgalactoside and DNPglucoside under the usual conditions at 45 °C yielded an isotope effect for hydrolysis of the galactoside ($k_H/k_D = 1.11 \pm 0.01$) which was the same size, or possibly larger than, the isotope effect for hydrolysis of the glucoside ($k_H/k_D = 1.09 \pm 0.02$). Since the size of the isotope effect would be
expected to decrease for the galactosides if hydrolysis was significantly more pre-associative for those compounds, these data suggest that the decrease in $\Delta S^+$ observed for hydrolysis of the galactosides is not due to pre-association, but some other phenomenon.

Since hydrolysis of DNPglycosides proceeds by a predominantly $S_N1$ mechanism, the reaction rates will be largely dependent on the stability of the oxocarbonium ion generated at the transition state. Differences in the rates of hydrolysis of the DNPglucosides and galactosides may therefore be rationalized by postulating that the galactosyl-oxocarbonium ion is more heavily hydrated than a glucosyl-oxocarbonium ion, leading to a more stable transition state by dispersal of the charge. One possibility is that hydration of the ring oxygen at the transition state is more facile for the galactosides. Since O5 appears to possess the majority of the charge, such stabilization could increase the rate of hydrolysis considerably. Indeed, differences in the hydration structure of glucose and galactose have been observed in a number of studies (98,99,100,101,102). Further, it has recently been reported that galactose is more heavily hydrated in solution than glucose (103,104,105,106). Therefore it is not unreasonable to suggest that the transition state hydration structure for the DNPglucosides and galactosides may differ and that increased hydration of the galactosyl cations may be partially responsible for the increase in their rates of hydrolysis with respect to the analogous glucosides.

The evidence derived from this study is consistent with a role for hydration in determining the rate of glycoside hydrolysis. Firstly, the slope of a linear free energy relationship comparing the hydrolysis of the DNPglucosides and galactosides suggests that the galactosides are less sensitive to substitutions on the sugar ring (Figure 2-18). This would be consistent with a greater degree of hydration of the galactosyl oxocarbonium ion where electron donation from the solvent would lead to less charge development on the sugar ring. Secondly, $\Delta S^+$ values for hydrolysis of the galactosides are lower than those observed with the analogous glucosides, a finding consistent with a more 'ordered' hydration structure for the galactosides. Interestingly, though the $\Delta S^+$
values were generally lower for the hydrolysis of the galactosides, values for the galactosides modified at the 4 position (the 4FDNPgalactoside and 4dDNPglycoside) were higher than those observed for the hydrolysis of other galactosides where the oxocarbonium ions generated at the transition state were of comparable stability. For example the 4FDNPgalactoside had a higher $\Delta S^\ddagger$ value than the other fluorinated galactosides. These data imply that the axial hydroxyl at C4 is connected in some manner with the decrease in $\Delta S^\ddagger$ values seen for the galactosides. This would be consistent with that group aiding in hydration of O5 and forming some type of more organized hydration structure.

2.7 Conclusions

The relative rates of hydrolysis observed for the monosubstituted DNPglycosides can largely be rationalized based on field effects related to the distance of the substituent from both O5 and C1, with the assumption that the majority of the charge at the transition state resides on the ring oxygen. Plots of $\sigma_T$ for the various substitutions versus $\ln(k_{hy})$ were generally found to correlate well and provided a good indication of the importance of field effects in determining the relative rates of hydrolysis of the substituted glycosides. The scatter seen in some of these plots could be due to a number of factors including steric and dipolar effects (46).

The reason for the differences in the rates of hydrolysis observed between series of glycosides appears to more complex. Field effects still appear to be important though differences in steric interactions, hydration and dipole alignment could all contribute to varying degrees and the present study provides little information about the relative contributions of these factors to the rates of hydrolysis of glycosides. Another complication arises from the angular dependence of the field effect. The orientation of the dipole created by the polar substituent on the sugar ring will have an effect on the
magnitude of the field effect (83) and without detailed information about the geometry of
the transition state for each glycoside, it is difficult to estimate how these changes will
affect the rate of hydrolysis. For example, estimation of the field effect at C6 is
problematic since rotation of the polar substituent about the C5-C6 bond is possible.
Further, the rotamer populations will vary with the glycoside being examined (91) and
are likely to vary somewhat with the size of the substituent at C6. Indeed, differences in
the structure of the transition state between series of glycosides seem probable, and could
give rise to different field effects in different glycosides. Clearly these factors must be
quantitated if a definitive glycoside hydrolysis mechanism is to be proposed. However,
given the apparent complexity of the problem this will be difficult.
Chapter 3

Investigation of the Mechanism of abg β-Glucosidase
Using Substituted Aryl Glycosides as Probes
3.1 Introduction

From the evidence accumulated in other studies of abg \( \beta \)-glucosidase, it appears that the enzyme functions as a retaining \( \beta \)-glucosidase and generally follows the mechanism proposed for these enzymes by Koshland (7) as discussed in Chapter 1. Assuming only the chemical steps are relevant, a reasonable kinetic scheme for abg \( \beta \)-glucosidase may be written as follows:

\[
E + S \xrightleftharpoons[k_1]{k_{-1}} ES \xrightarrow{k_2} E-S \xrightarrow{k_3} E + P
\]

**Scheme 3-1. A kinetic model for abg \( \beta \)-glucosidase.**

where \( k_1 \) and \( k_{-1} \) are the rate constants for formation and dissociation of the Michaelis complex, \( k_2 \) the rate constant for formation of the glycosyl-enzyme intermediate, and \( k_3 \) the rate constant for hydrolysis of the glycosyl-enzyme intermediate. By the steady state approximation, the formation and breakdown of any intermediate on the reaction pathway occur at the same rate. Therefore

\[
k_2[E\cdot S] = k_3[E-S], \text{ or}
\]

\[
[E-S] = \frac{k_2[E\cdot S]}{k_3} \quad (1)
\]

It will also be true that

\[
k_1[E][S] = k_2[E\cdot S] + k_{-1}[E\cdot S] \quad (2)
\]

If the total amount of enzyme \([E_0]\) is
\[ [E_0] = [E] + [E\cdot S] + [E-S] \] then,
\[ [E] = [E_0] - [E\cdot S] - [E-S] \quad (3) \]

Substituting for \([E]\) in equation 2, gives (4)

\[ k_1[E_0][S] = k_1 ([E\cdot S] + [E-S])[S] + k_2[E\cdot S] + k_{-1}[E\cdot S] \quad (4) \]

Using equation (1) to substitute for \([E-S]\)

\[ k_1[E_0][S] = k_1 ([E\cdot S] + k_2/k_3[E\cdot S])[S] + k_2[E\cdot S] + k_{-1}[E\cdot S] \quad (5) \]

which can be simplified to

\[ k_1[E_0][S] = [E\cdot S] \left( k_1 + k_1k_2/k_3 \right)[S] + (k_{-1} + k_2)[E\cdot S] \quad (6) \]

Since the reaction is assumed to be at steady state and the rate of formation of product is \(d[P]/dt = k_3[E-S]\), it is also true that \(d[P]/dt = k_2[E\cdot S]\) since \(k_2[E\cdot S] = k_3[E-S]\) (equation (1)). Equation 6 can be rearranged to isolate \([E\cdot S]\)

\[ [E\cdot S] = \frac{k_1k_3 [E_0][S]}{k_1k_3 + k_1k_2} \left[ \frac{1}{[S] + \frac{k_{-1} + k_2}{k_1}} + \frac{k_3}{k_2 + k_3} \right] \quad (7) \]

Therefore, the velocity \((v)\) of the reaction is given by
\[ v = \frac{k_2k_3 [E_0][S]}{k_2 + k_3} \]

For such an enzyme system, the parameters \( k_{\text{cat}} \) and \( K_m \) can now be defined as:

\[
k_{\text{cat}} = \frac{k_2k_3}{k_2 + k_3}
\]

\[
K_m = \left( \frac{k_1 + k_2}{k_1} \right) \left( \frac{k_3}{k_2 + k_3} \right)
\]

\[
k_{\text{cat}}/K_m = \frac{k_1k_2}{k_1 + k_2}
\]

The data gained from steady state analysis is somewhat limited since both \( K_m \) and \( k_{\text{cat}} \) consist of several rate constants and thus provide little information about the individual steps in the enzymic reaction. Analysis of the pre-steady state kinetics of the reaction allows the individual steps in the mechanism to be characterized. In these experiments the initial turnover of the Michaelis complex \([E\cdot S]\) to the glycosyl-enzyme intermediate is observed. Since this reaction will only release one mole of aglycone per mole of enzyme, larger amounts of enzyme (rather than catalytic amounts) are required for this type of experiment.
In order to use pre-steady state kinetics to analyze the rate of glycosylation, several conditions must be met. Firstly, formation of the Michaelis complex must be rapid with respect to the first chemical step in the mechanism, otherwise the kinetics observed may be at least partially diffusion-controlled. Secondly, dissociation of the Michaelis complex (k_{-1}) must be rapid with respect to formation of the intermediate (k_2) for pre-steady state kinetics to provide a good estimate of the dissociation constant for the Michaelis complex (K_d). Thirdly, the rate of formation of the intermediate must be greater than the rate of its breakdown. Ideally, glycosylation is much more rapid than deglycosylation so that formation of the intermediate follows first order kinetics (assuming the concentration of substrate is not significantly changed during the reaction). In practice, this is seldom the case and the observed kinetics are usually biphasic (Figure 3-1). The initial release of aglycone follows pseudo first-order kinetics. As the intermediate accumulates, the rate of release of aglycone becomes dependent on the rate of breakdown of the intermediate. This eventually leads to steady state kinetics where the rate of release of aglycone is constant.

The time course for release of aglycone is fit to an equation for a first order reaction followed by a linear steady state phase. The observed rates from the pre-steady state phase obey Michaelis-Menten kinetics and thus can be fit to the standard form of the Michaelis-Menten equation. Since the pre-steady state is unaffected by the rate of
deglycosylation, $k_2$ and $K_d$ (where $K_d = k_{-1}/k_1$) are given directly. Also, as pre-steady state data can only be acquired for substrates which display rate-limiting deglycosylation, it follows that $k_{cat}$ will provide an estimate of the rate constant for deglycosylation, $k_3$ (assuming the rate of product release from the enzyme is rapid with respect to deglycosylation). By combining the data from the steady state and pre-steady state analyses, the individual steps in the reaction can be examined in isolation.

![Graph](image)

**Figure 3-1. A pre-steady state burst for 4FDNPglucoside with abg β-glucosidase at 5 °C (1.07 mM substrate concentration).**

### 3.2 Steady State Kinetic Results

Kinetic data for the steady state hydrolyses were acquired using a conventional UV-visible spectrophotometer to follow the release of aglycone. All of the reactions were carried out at 37 °C in 50 mM sodium phosphate buffer (pH 6.80) in the presence of 0.1% BSA. Substrate concentrations were chosen to bracket $K_m$ whenever possible and the rates were determined using initial rates analysis. Rates of release of aglycone
were found to be linear below 8% substrate depletion. The steady state constants were determined by fitting the data to the Michaelis-Menten equation using weighted regression analysis (107). Standard errors for these constants were calculated by the same fitting program and are given in brackets.

Table 3-1. Steady State Rate Constants for Hydrolysis of DNPGlycosides by abg β-Glucosidase at 37 °C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNPglucoside</td>
<td>0.0218 (0.0012)</td>
<td>130 (3)</td>
<td>5960</td>
</tr>
<tr>
<td>6dDNPglucoside</td>
<td>0.0227 (0.004)</td>
<td>44.8 (0.25)</td>
<td>1974</td>
</tr>
<tr>
<td>4dDNPglucoside</td>
<td>0.170 (0.01)</td>
<td>380 (11)</td>
<td>2260</td>
</tr>
<tr>
<td>3dDNPglucoside</td>
<td>0.029 (0.003)</td>
<td>9.9 (0.42)</td>
<td>342</td>
</tr>
<tr>
<td>6FDNPglucoside</td>
<td>0.0020 (0.0001)</td>
<td>8.68 (0.15)</td>
<td>4340</td>
</tr>
<tr>
<td>4FDNPglucoside</td>
<td>0.020 (0.0016)</td>
<td>9.62 (0.27)</td>
<td>481</td>
</tr>
<tr>
<td>3FDNPglucoside</td>
<td>0.0020 (0.0004)</td>
<td>1.12 (0.05)</td>
<td>560</td>
</tr>
<tr>
<td>2FDNPglucoside$^a$</td>
<td>~2.0x10$^{-7}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNPgalactoside</td>
<td>0.840 (0.10)</td>
<td>175 (8)</td>
<td>208</td>
</tr>
<tr>
<td>4FDNPgalactoside</td>
<td>0.500 (0.034)</td>
<td>137 (4)</td>
<td>271</td>
</tr>
<tr>
<td>DNPalloside</td>
<td>0.0068 (0.0002)</td>
<td>0.100 (0.007)</td>
<td>14.7</td>
</tr>
<tr>
<td>DNPmannoside</td>
<td>0.106 (0.014)</td>
<td>0.126 (0.007)</td>
<td>1.19</td>
</tr>
</tbody>
</table>

$^a$ Data obtained from Street et al. (15)
Table 3-2. Steady State Rate Constants for Hydrolysis of

PNPGlycosides by abg β-Glucosidase at 37 °C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$  (mM)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNPglucoside</td>
<td>0.082</td>
<td>141</td>
<td>1720</td>
</tr>
<tr>
<td>PNPmannoside$^a$</td>
<td>0.020</td>
<td>0.10</td>
<td>5</td>
</tr>
<tr>
<td>2dPNPglucoside$^b$</td>
<td>0.015</td>
<td>0.025</td>
<td>1.67</td>
</tr>
<tr>
<td>PNPgalactoside$^c$</td>
<td>5.0</td>
<td>275</td>
<td>55</td>
</tr>
<tr>
<td>2FPNPglucoside$^d$</td>
<td>$\sim2.0\times10^{-7}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2NH$_2$PNPglucoside</td>
<td>0.056 (0.009)</td>
<td>$6.5\times10^{-4}$ (3.3x10$^{-5}$)</td>
<td>$1.16\times10^{-2}$</td>
</tr>
</tbody>
</table>

a) Data obtained from Day and Withers (3).
b) Data obtained from Karen Rupitz (unpublished results, this lab)
c) Data obtained from Kempton and Withers (6)
d) Data obtained from Street et al. (15)

When the kinetic data obtained for these substrates are plotted using the form of Lineweaver and Burk (111), the resulting plots are linear as would be expected for Michaelis-Menten kinetics, with the exception of the 3FDNPglucoside. With this substrate, the Lineweaver-Burk plot was biphasic, the rate of release of phenol increasing at higher substrate concentrations instead of rendering a limiting (saturating) value. Previous work in this laboratory (6) demonstrated that similar kinetic behavior observed with PNPxyloside arose from transglycosylation at the higher concentrations of substrate, the breakdown of the glycosyl-enzyme intermediate being faster through transfer of the sugar to a second molecule of substrate rather than water. Presumably, the binding energy of the secondary site is used to position the second sugar molecule preferentially for attack on the intermediate, making the sugar a 'better' nucleophile than water. Since transglycosylation requires a second molecule of substrate to bind at the aglycone site, it is usually observed at higher substrate concentrations.
Figure 3-2. A Lineweaver-Burk plot of the hydrolysis of 3FDNPglucoside by the abg β-glucosidase.

Figure 3-3. A schematic model of transglycosylation.

In light of these findings, it is reasonable that transglycosylation could be responsible for the kinetic behavior observed with the 3FDNPglucoside. In order to examine this possibility, the product identity was investigated by HPLC with several DNPglycosides. Aliquots of reaction mixtures containing substrate and abg β-
glucosidase were injected onto an HPLC equipped with a Dextro-Pac column, and eluted with 20% acetonitrile/water. Since authentic standards of the transglycosylation products (the appropriate deoxyfluoro DNPCellobiosides) were not available, the relative mobilities of DNPGlucoside and DNPCellobioside (Figure 3-4) were used to estimate the mobilities expected for the fluorinated glucosides and celllobiosides.

At a 3FDNPglucoside concentration of 0.055 mM, the release of DNP during the reaction was accompanied by accumulation of a product, observed as a new peak with a retention time consistent with that of a DNPCellobioside. These findings were confirmed by liquid SIMS mass spectral analysis of the products as follows. The 3FDNPglucoside reaction was repeated at 1 mM substrate concentration and the putative transglycosylation peak again found. This reaction mixture was evaporated to dryness and the residue acetylated in acetic anhydride/pyridine (109). The solvent was evaporated in vacuo and the resulting residue dissolved in chloroform and washed with water. The compounds present in the organic layer were subjected to liquid SIMS mass spectral analysis, which detected a peak with the predicted mass for the acetylated deoxyfluoro DNPCellobioside (M+1 = 724 g/mol).

With a 28-fold lower concentration of substrate (0.002 mM) the same reaction conditions did not produce the peak assigned to the transglycosylation product to an appreciable extent. This result is consistent with the predicted change in mechanism where the rapid release of DNP at high substrate concentrations is due to transglycosylation, while the less rapid release of DNP at lower concentrations is due to normal hydrolysis of the glycosyl-enzyme intermediate. Examination of the abg β-glucosidase-catalyzed reaction with the DNPGlucoside and 4FDNPglucoside indicated that at saturating substrate concentration (0.4 mM), no transglycosylation was occurring with these substrates confirming that the linear Lineweaver-Burk plots seen for the other substrates in the study represented the normal hydrolysis of the glycosyl-enzyme intermediate.
Figure 3-4. An HPLC trace of DNPglucoside (retention time ~ 11.5 min.) and DNPcellobioside (retention time ~ 13 min.) eluted with 20% acetonitrile/water.

Figure 3-5. HPLC traces of the products from treatment of 3FDNPGlucoside with abg β-glucosidase at A) 0.055 mM and B) 0.002 mM substrate concentration at different reaction times.

Examining the kinetic data obtained, it is clear that substitution of the ring hydroxyl groups changes the Michaelis-Menten parameters for the substituted
DNPglycosides. The $k_{\text{cat}}/K_m$ values measured for the modified glycosides were found to be one to three orders of magnitude smaller than the value for the parent substrate, DNPglucoside. Since $k_{\text{cat}}/K_m$ is the second order rate constant for the first irreversible step in the reaction (in this case, glycosylation), it is apparent that replacement of the ring hydroxyl groups of the DNPglucoside decreases the rate of formation of the glycosyl-enzyme intermediate. A similar decrease in $k_{\text{cat}}$ was also observed in most cases, with the interesting exception of those modified substrates with a hydrogen equatorial at the 4 position (DNPgalactoside, 4FDNPgalactoside and 4dDNPglucoside). In fact, for the 4dDNPglucoside a significant increase in $k_{\text{cat}}$ was observed.

3.2.1 Diffusion Control of the Rate of Glycosylation

Since the $k_{\text{cat}}/K_m$ values observed for the DNPglucosides with abg $\beta$-glucosidase are relatively large, it is conceivable that the rate of glycosylation is diffusion controlled. Work by Dale et al. (112) with sweet almond $\beta$-glucosidase showed that the rate of hydrolysis of DNPglucoside by that enzyme was at least partially diffusion controlled. Some evidence suggested this might also be the case for the abg $\beta$-glucosidase, as follows. A study by Kempton and Withers (6) determined the rates of hydrolysis of a series of aryl $\beta$-D-glucosides by abg $\beta$-glucosidase. When $\ln(k_{\text{cat}}/K_m)$ was plotted against the pKa of the phenol leaving group, a reasonable correlation was found over most of the substrate range. This would be expected since $k_{\text{cat}}/K_m$ is the rate constant for the first irreversible step, that in which aryl-glucose bond cleavage is occurring. However, rate constants for the most activated glucosides, most notably DNPglucoside (see Figure 3-6) fall significantly below this line, suggesting that some other step, most likely the association of the substrate and the enzyme, was becoming rate limiting.
Many examples of diffusion controlled reactions have been reported in enzymic systems (113,114) and a method has been developed by Cerjan and Barnett (115) for investigating diffusion control in these systems through varying the viscosity of the reaction mixture by addition of a viscogen such as sucrose. Theoretically, increasing the viscosity of the solution will only affect those rate constants which are subject to diffusion control. Therefore, $k_{\text{cat}}$ should be unaffected by a change in viscosity, since the substrate will be bound to the enzyme for the chemical steps in the reaction, while $K_m$ will increase for those substrates for which $k_{\text{cat}}/K_m$ is diffusion controlled.

Such an investigation was carried out for abg β-glucosidase by measurement of $k_{\text{cat}}$ and $K_m$ in the presence and absence of 24% sucrose as a viscogen (Table 3-3). These data gave some indication that the rate of glycosylation with DNPglucoside may indeed be diffusion controlled as follows.
Table 3-3. Steady State Kinetic Parameters for the Hydrolysis of Aryl β-D-Glucosides by abg β-Glucosidase in the Presence and Absence of 24% Sucrose.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(k_{\text{cat}}) (sec(^{-1}))</th>
<th>(K_m) (mM)</th>
<th>(k_{\text{cat}}/K_m) (mMsec(^{-1}))</th>
<th>(k_{\text{cat}}/K_m) (mMsec(^{-1})) no sucrose</th>
<th>(k_{\text{cat}}/K_m) no sucrose in sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNPglucoside</td>
<td>198</td>
<td>1.01</td>
<td>196</td>
<td>2171</td>
<td>10.8 (0.1)\textsuperscript{b}</td>
</tr>
<tr>
<td>DNPglucoside</td>
<td>198</td>
<td>0.411</td>
<td>482</td>
<td>6302</td>
<td>13.0 (0.6)\textsuperscript{b}</td>
</tr>
<tr>
<td>p-BrPheglucoside\textsuperscript{c}</td>
<td>-</td>
<td>-</td>
<td>8.7 (0.35)</td>
<td>91.7</td>
<td>10.9 (1)</td>
</tr>
<tr>
<td>6FDPNPglucoside</td>
<td>12.75 (0.05)</td>
<td>0.0264 (0.0004)</td>
<td>483</td>
<td>4417</td>
<td>9.2 (0.6)</td>
</tr>
</tbody>
</table>

a) Standard errors given in brackets.
b) Error given is standard deviation of repeat determinations of viscosity effect.
c) With this substrate, \(k_{\text{cat}}/K_m\) was determined directly from a plot of \(V\) versus [S] at substrate concentrations at least 10 times below \(K_m\).

Large rate reductions were observed for the less activated 4-bromo and 4-nitrophenyl glucosides (for which the rate of glycosylation is likely below the diffusion controlled limit) implying that sucrose is acting as a competitive inhibitor to an appreciable extent in this enzyme and lowering the \(k_{\text{cat}}/K_m\) values. However, since the concentration of sucrose is held constant for all of the determinations, the rate reduction due to inhibition will be approximately the same for all substrates. The larger rate reduction observed for DNPglucoside in sucrose is therefore likely due to a viscosity effect and is indicative of some diffusion control in the value of \(k_{\text{cat}}/K_m\) for the DNPglucoside with abg β-glucosidase. Small increases in \(k_{\text{cat}}\) were observed (10-15%) contrary to what is expected by theory. This is likely due to sucrose acting as a transglycosylation acceptor, thus increasing the observed value of \(k_{\text{cat}}\). Despite this
uncertainty, the viscosity effect study lends support to the previous conclusion, based on the Hammett plot data from the aryl glucosides, both indicating that diffusion is at least partially rate determining in the $k_{cat}/K_m$ value for the DNPglucoside.

Interestingly, no viscosity effect was observed on $k_{cat}/K_m$ for the 6FDNPglucoside, despite the similarity in $k_{cat}/K_m$ for DNPglucoside and 6FDNPglucoside (5960 and 4340 mM$^{-1}$sec$^{-1}$ respectively). This would suggest the reaction with the 6FDNPglucoside is not diffusion controlled. In order to further investigate this, Michaelis-Menten parameters were determined for a series of aryl 6-deoxy-6-fluoro-glucosides (Table 3-4) and a Hammett relationship correlating $\ln(k_{cat}/K_m)$ and aglycone pKa plotted (Figure 3-7). As can be seen, the plot is essentially linear ($\rho = 0.95$) as might be expected if the first irreversible step is indeed bond cleavage. Importantly, however, the value for the 6FDNPglucoside fell essentially on the line. Indeed, omission of this value from the plot improved the fit only marginally ($\rho = 0.95$ to 0.99). This would suggest that $k_{cat}/K_m$ is not significantly diffusion controlled for 6FDNPglucoside. Since this appears to be the case for the fastest of the substituted DNPglucosides, it is unlikely that diffusion control has any effect on the value of $k_{cat}/K_m$ for the other substituted DNPglycosides used in this study since their $k_{cat}/K_m$ values are all lower than that of 6FDNPglucoside.

It is also noteworthy that the slopes of the Hammett plots for the aryl glucosides ($m = 1.8$) and the aryl 6-deoxy-6-fluoro-glucosides ($m = 1.6$) are similar, indicating that the structures of the transition states for the two series of compounds are similar, at least with respect to the degree of negative charge accumulation on the aryl oxygen at the transition state.
Table 3-4. Steady State Kinetic Constants for the Hydrolysis of the
Aryl 6-Deoxy-6-Fluoro-Glucosides by abg β-Glucosidase at 37°C

<table>
<thead>
<tr>
<th>6-Deoxy-6-fluoro-β-D-glucoside</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$k_{cat}/K_m$ (sec$^{-1}$mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNP</td>
<td>0.0020 (0.0001)</td>
<td>8.52 (0.15)</td>
<td>4260</td>
</tr>
<tr>
<td>2-chloro, 4-nitrophenyl</td>
<td>0.0035 (0.001)</td>
<td>12.5 (0.6)</td>
<td>3570</td>
</tr>
<tr>
<td>2-nitrophenyl</td>
<td>0.044 (0.0029)</td>
<td>19.5 (1.2)</td>
<td>443</td>
</tr>
<tr>
<td>PNP</td>
<td>0.359 (0.037)</td>
<td>49.5 (2.5)</td>
<td>138</td>
</tr>
<tr>
<td>phenyl</td>
<td>1.640 (0.380)</td>
<td>0.81 (0.06)</td>
<td>0.494</td>
</tr>
</tbody>
</table>

* a) standard errors given in brackets

Figure 3-7. A Hammett plot of $\ln(k_{cat}/K_m)$ versus leaving group pKa for the hydrolysis of the aryl 6-deoxy-6-fluoro-β-D-glucopyranosides with abg β-glucosidase.
3.3 Pre-Steady State Kinetics

In order to examine how glycoside ring substitutions affect the rates of the individual steps in the αβ-g-glucosidase-catalyzed reaction, pre-steady state kinetic analysis (in which the burst of release of phenol prior to the steady state is examined) is necessary. This allows the glycosylation and deglycosylation steps to be examined separately, with pre-steady state kinetics specifically examining the glycosylation step of the reaction. The pre-steady state data were collected using a stopped flow spectrophotometer, again by following the release of aglycone. Since the size of the observed burst is proportional to the amount of enzyme used, larger quantities of enzyme are required for this type of measurement. Enough enzyme was used to give a burst with a total absorbance change of approximately 0.03A, which was sufficient to give reliable rate determinations under the conditions of the experiment. All reactions were carried out in 50 mM sodium phosphate buffer at pH 6.80. The rate of phenol release for the DNPglycosides was measured at 5 °C while the less activated PNPglycosides were examined at 37 °C. When possible, rate determinations were made at concentrations of substrate which bracketed $K_d$. Values for $k_2$ and $K_d$ were obtained by fitting the observed rates to a weighted version of the Michaelis-Menten equation. The standard errors for all of the kinetic data were obtained in this manner and are given in brackets. Some substrates exhibited no burst phase indicating that a step prior to deglycosylation was rate limiting. No pre-steady state data could be obtained for such compounds.
Table 3-5. Pre-Steady State Kinetic Data for the
DNPGlycosides with αβ-Glucosidase at 5 °C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_d (mM)</th>
<th>k_2 (sec⁻¹)</th>
<th>k_2/K_d (mM⁻¹sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNPglucoside</td>
<td>0.65 (0.022)</td>
<td>1300 (21)</td>
<td>2000</td>
</tr>
<tr>
<td>6dDNPglucoside</td>
<td>2.7 (0.34)</td>
<td>530 (35)</td>
<td>195</td>
</tr>
<tr>
<td>4dDNPglucoside</td>
<td>b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3dDNPglucoside</td>
<td>0.43 (0.04)</td>
<td>33 (1.3)</td>
<td>77</td>
</tr>
<tr>
<td>6FDNPglucoside</td>
<td>5.6 (2.0)</td>
<td>1800 (500)</td>
<td>320</td>
</tr>
<tr>
<td>4FDNPglucoside</td>
<td>2.7 (0.58)</td>
<td>90 (14)</td>
<td>34</td>
</tr>
<tr>
<td>3FDNPglucoside</td>
<td>0.31 (0.04)</td>
<td>81 (5)</td>
<td>266</td>
</tr>
<tr>
<td>2FDNPglucoside</td>
<td>0.34 (0.05)</td>
<td>0.355 (0.02)</td>
<td>1.06</td>
</tr>
<tr>
<td>DNPgalactoside</td>
<td>2.74 (0.37)</td>
<td>22.1 (1.7)</td>
<td>8.1</td>
</tr>
<tr>
<td>4FDNPgalactoside</td>
<td>b</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DNPmannoside</td>
<td>2.65 (0.37)</td>
<td>1.04 (0.10)</td>
<td>0.392</td>
</tr>
</tbody>
</table>

a) K_d and k_2 were estimated from K_m and k_cat determined at 5 °C.
b) These substrates do not show a pre-steady state 'burst'. Therefore, no pre-steady state kinetic data were available.

Table 3-6. Pre-Steady State Kinetic Data for the
PNPGlycosides with αβ-Glucosidase at 37 °C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_d (mM)</th>
<th>k_2 (sec⁻¹)</th>
<th>k_2/K_d (mM⁻¹sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNPglucoside</td>
<td>-</td>
<td>-</td>
<td>3300^a</td>
</tr>
<tr>
<td>PNPmannoside</td>
<td>7.7 (1)</td>
<td>48 (5)</td>
<td>5.5</td>
</tr>
<tr>
<td>2dPNPglucoside</td>
<td>4.4 (1.4)</td>
<td>19 (5)</td>
<td>4.2</td>
</tr>
</tbody>
</table>

a) k_2/K_d was estimated from the slope of a plot of k_observations versus [S]. Individual values of k_2 and K_d could not be determined.
### Table 3-7. Inactivation Rate Constants for the 2-Position-Substituted Glycosides with αβ-Glucosidase at 37°C.

<table>
<thead>
<tr>
<th>Inactivator</th>
<th>$K_i$ (mM)</th>
<th>$k_i$ (sec$^{-1}$)</th>
<th>$k_i/K_i$ (mM$^{-1}$sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2FDNPglucoside</td>
<td>0.25 (0.02)</td>
<td>4.72 (0.22)</td>
<td>19.3</td>
</tr>
<tr>
<td>2FDNPmannoside</td>
<td>1.15 (0.7)</td>
<td>0.50 (0.01)</td>
<td>0.43</td>
</tr>
<tr>
<td>2FPNPglucoside</td>
<td>1.67</td>
<td>2.83x10$^{-4}$</td>
<td>1.65x10$^{-4}$</td>
</tr>
<tr>
<td>2C1DNPglucoside</td>
<td>8.3 (1.5)</td>
<td>0.28 (0.03)</td>
<td>0.035</td>
</tr>
</tbody>
</table>

a) Data obtained from Street et al. (15)

Data obtained by the pre-steady state method appears to be internally consistent with the data obtained from steady state analysis. Values for $k_2/K_d$ for the PNPglycosides were found to be in reasonable agreement with $k_{cat}/K_m$ values obtained from steady state kinetics as would be expected since both parameters pertain to the same step in the reaction. It was noted however, that the $k_2/K_d$ values were generally somewhat higher than the $k_{cat}/K_m$ values obtained by the steady state method.

For the DNPglycosides, a plot of $k_2/K_d$ at 5°C versus $k_{cat}/K_m$ at 37°C would be expected to be linear, in the absence of major differences in the temperature dependences of the reaction with the different glycosides. These parameters were found to correlate well (Figure 3-8, $r = 0.98$) with two exceptions, the DNPglucoside and the 3FDNPglucoside. For the DNPglucoside, the observed deviation likely is a temperature effect since $k_{cat}/K_m$ is at least partially diffusion controlled with DNPglucoside and the temperature effect on association of the substrate with the enzyme is likely to differ from the temperature effect for bond cleavage. This conclusion is supported by the fact that the $k_{cat}/K_m$ value determined for the DNPglucoside at 5°C using the steady state method was in moderate agreement with $k_2/K_d$ (1100 and 2000 mM$^{-1}$sec$^{-1}$ respectively) and was still well above the line seen for the other substrates plotted in Figure 3-8.
Unfortunately, the deviation observed for 3FDNPglucoside could not be similarly analyzed, though a temperature effect again might be a possibility.

Figure 3-8. A plot comparing $k_2/K_d$ at 5 °C and $k_{cat}/K_m$ at 37 °C for the DNPglycosides. ○ DNPglys; □ DNPglu; ■ 3FDNPglu

A possible difficulty with the use of stopped flow techniques for the measurement of very rapid reactions ($k = 1000 \text{ sec}^{-1}$ or higher) is the occurrence of mixing artifacts which can give the appearance of first order kinetics, but do not represent the actual rate constant you wish to measure (A. Fersht, personal communication). The fact that a linear Lineweaver-Burk plot was obtained from the pre-steady state data for DNPglucoside and the similar values obtained for $k_2/K_d$ and $k_{cat}/K_m$ would argue that the pre-steady state data is fairly accurate. Also, tests with DNPgalactoside (a substrate which showed linear pre-steady state kinetics) at short reaction times (10 msec) showed no curvature, above the noise of the experiment, suggesting that mixing of the substrate and the enzyme was complete within the dead time of the instrument. However, pre-steady state data for the
fastest substrates (DNPglucoside and 6FDNPglucoside) must be interpreted with some caution since a large portion of the reaction being observed has taken place within the dead time of the instrument (approx. 1 msec), making it more difficult to determine rate constants for the pre-steady state accurately.

The fact that the rate constant for glycosylation ($k_2$) is so high raises the possibility that $\text{abg} \beta$-glucosidase may be displaying Briggs-Haldane (116) kinetics, especially with the more activated substrates. Briggs-Haldane kinetics apply in cases where the values of $k_2$ and $k_1$ are similar. Under such circumstances, the value for the dissociation constant for the Michaelis complex cannot be obtained from pre-steady state kinetics. Instead, the calculated values for $K_d$ are $K_m$ values for the pre-steady state phase of the reaction, where $K_m = (k_2 + k_1)/k_1$. Given that values for the rate constant for dissociation of the Michaelis complex ($k_1$) were unavailable, it was not possible to determine the relative sizes of $k_2$ and $k_1$ for these substrates. Therefore, the values of $K_d$ determined in this study can only be considered maximum estimates of the true dissociation constant for the Michaelis complex and their application to characterization of ground state binding interactions will be somewhat limited.

Pre-steady state analysis yielded several valuable pieces of information. The observation of saturation behavior for the pre-steady state kinetics of the DNPglucoside implies that glycosylation is at worst only partially diffusion controlled for that substrate, at least at 5 °C (148). Therefore the rate constant obtained for glycosylation is likely a better estimate than originally thought based on the viscosity studies. Secondly, the observation of a pre-steady state burst essentially proves that deglycosylation is rate limiting for the majority of these substrates, as would be expected with the activated DNP leaving group. Interestingly, substrates modified at the 4 position (with the exception of the 4FDNPglucoside) appear to have a different rate limiting step since no pre-steady state burst was observed. This result is discussed in detail in Section 3.7.4.
The changes in the kinetic constants observed in the abg β-glucosidase-catalyzed hydrolysis of the DNPglycosides upon substitution of the glycone hydroxyl groups can be related to changes in the equilibrium (dissociation constants) and activation (rate constants) free energies for the processes being examined. These ΔΔG° or ΔΔG°‡ values are calculated by comparison of the data obtained for the parent substrate (DNPglucoside) to that obtained for the substituted substrates using the expression given below where k_par and k_sub are the kinetic or dissociation constants for the parent and substituted glycosides respectively.

\[ ΔΔG° = RT \ln \left( \frac{k_{\text{par}}}{k_{\text{sub}}} \right) \]

Using the kinetic data obtained by both steady state and pre-steady state analysis, it is possible to calculate the ΔΔG° value for each of the steps in the abg β-glucosidase-catalyzed hydrolysis of these glycosides (Table 3-8). The changes in activation free energy, ΔΔG°‡, derived from k_2, k_{cat}/K_m and k_{cat} and the change in the equilibrium free energy ΔΔG° derived from K_d are labeled accordingly.

It is important to consider the consequences of diffusion control of the rate of glycosylation for the parent DNPglucoside on the values of the ΔΔG°‡. Since k_{cat} is unaffected by the rate of association of the product, ΔΔG°‡ values calculated from that parameter should be unaffected. However, since k_{cat}/K_m and the measured value for k_2 for DNPglucoside are lower than they would otherwise be if bond cleavage were rate limiting, due to diffusion control. Thus ΔΔG°‡ values calculated from those parameters will be smaller than should be the case. However, since diffusion control is significant only for DNPglucoside (section 3.2.1), the calculated ΔΔG°‡ values should be lowered to the same extent for all of the substituted substrates. Thus the relative values of ΔΔG°‡ should still provide the same information about the mechanism of the enzyme.
Table 3-8. Changes in Activation Free Energy and Free Energy of Formation of the Michaelis Complex for the Substituted Aryl Glycosides with abg β-Glucosidase.\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(\Delta G^0(K_d))(^b)</th>
<th>(\Delta G^{0+}(k_2))(^b)</th>
<th>(\Delta G^{0\alpha}(k_{cat}/K_m))(^c)</th>
<th>(\Delta G^{0\alpha}(k_{cat}))(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNPglucoside</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6dDNPglucoside</td>
<td>3.3</td>
<td>2.1</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
<td>4dDNPglucoside</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
<td>-2.8</td>
</tr>
<tr>
<td>3dDNPglucoside</td>
<td>-1.0</td>
<td>8.5</td>
<td>7.4</td>
<td>6.6</td>
</tr>
<tr>
<td>6FDNPglucoside</td>
<td>5.0</td>
<td>-0.8</td>
<td>0.8</td>
<td>7.0</td>
</tr>
<tr>
<td>4FDNPglucoside</td>
<td>3.3</td>
<td>6.2</td>
<td>6.5</td>
<td>6.7</td>
</tr>
<tr>
<td>3FDNPglucoside</td>
<td>-1.7</td>
<td>6.4</td>
<td>6.1</td>
<td>12</td>
</tr>
<tr>
<td>2FDNPglucoside</td>
<td>-1.5</td>
<td>19</td>
<td>15</td>
<td>52</td>
</tr>
<tr>
<td>DNPgalactoside</td>
<td>3.3</td>
<td>9.4</td>
<td>8.6</td>
<td>-0.8</td>
</tr>
<tr>
<td>4FDNPgalactoside</td>
<td>-</td>
<td>-</td>
<td>8.0</td>
<td>-0.1</td>
</tr>
<tr>
<td>DNPgalactoside</td>
<td>-</td>
<td>-</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>DNPmannoside</td>
<td>3.3</td>
<td>17</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>2FDNPmannoside</td>
<td>-</td>
<td>-</td>
<td>25</td>
<td>41</td>
</tr>
<tr>
<td>2dPNPGlucoside(^d)</td>
<td>-</td>
<td>-</td>
<td>18</td>
<td>22</td>
</tr>
</tbody>
</table>

\(^a\) All values are in kJ/mol.
\(^b\) Values calculated from data obtained at 5 °C
\(^c\) Values calculated from data obtained at 37 °C
\(^d\) Values for this compound use PNPglucoside as a parent substrate.

3.5 Discussion

It is clear from the \(\Delta G^0\) values calculated above that substitution of the glycone hydroxyls has a significant effect on each of the stages of the abg β-glucosidase mechanism and further that the magnitude of the effect varies with both the position of
the substituent and the step in the reaction being examined. As has been discussed previously (38), changes in the kinetic parameters observed upon substitution of the various hydroxyl groups on the glycone are a consequence of changes in the electronic nature of the transition state of the reaction and alteration of the non-covalent interactions between the enzyme and the substrate. Though these effects cannot be separated completely, estimates of the relative contribution of these effects can be derived as follows.

3.6 Electronic Effects

Direct examination of the electronic nature of the transition state of an enzymic process is difficult. However, an approach which has been applied successfully (38,40,117) is the use of LFER's to compare enzymic processes with well characterized chemical reactions for which the mechanism is known. In this study, the effect of the ring substituents on the electronic nature of the transition state can be investigated by comparing the rate of enzymic hydrolysis of the DNPglycosides with their rates of spontaneous hydrolysis (Chapter 2). There is a large body of evidence which suggests that the hydrolysis of the DNPglycosides in solution proceeds via a transition state with substantial oxocarbonium ion character. If the enzymic process proceeds via a similar transition state, a correlation should exist between the rates of spontaneous and enzymic hydrolysis for the same substrate. However, any such correlation will necessarily be masked by a background of scatter due to the fact that the rates of enzymic hydrolysis will be affected by differing non-covalent interactions while non-enzymic rates will not.
Figure 3-9. Plots of A) ln($k_{cat}/K_m$) and B) ln($k_{cat}$) versus ln($k_{spon}$)

The plot of ln($k_{cat}$) versus ln($k_{spon}$) shows a very modest correlation (r = 0.73) between the rates of enzymic and spontaneous hydrolysis of the same substrate while the analogous plot with ln($k_{cat}/K_m$) is essentially a scatter plot (r = 0.25). As noted earlier, the scatter in the both plots is likely due to the disruption of binding interactions between the enzyme and the substrate. The larger degree of scatter in the $k_{cat}/K_m$ plot could be due to at least two factors. Firstly, $k_{cat}/K_m$ reflects an overall activation free energy (free enzyme plus free substrate to the bound transition state for the first irreversible step) while $k_{cat}$ examines the free energy difference from a bound intermediate to the bound transition state. Thus differences due to binding interactions will be minimized in the latter parameter. Secondly, these differences in scatter could arise from an inherently greater sensitivity of $k_{cat}$ than $k_{cat}/K_m$ to electronic factors. This could occur if the transition state for the deglycosylation step was more electron deficient than the transition state for glycosylation. The second proposal provides an attractive rationale for several other observations and is consistent with recent work on the nature of the transition state of reactions at Cl of glycosides as described below.

Firstly, Banait and Jencks (57) have recently shown that the transition states for displacements at the anomeric center of glucosides are more pre-associative with charged
nucleophiles than with neutral ones. Accordingly, more $S_N2$ character might be expected in the transition state for attack of the charged enzymic carboxylate on the substrate during the glycosylation step in comparison to that for attack of a neutral water on the glucosyl-enzyme intermediate during deglycosylation.

Secondly, an indication of the degree of oxocarbonium ion character at each transition state comes from measurement of the $\alpha$-DKIE for the hydrolysis of several different substrates. Isotope effects measured on $k_{cat}$ with substrates for which glycosylation was rate limiting were found to be $k_H/k_D = 1.06$ while for substrates for which deglycosylation was rate limiting, isotope effects were approximately $k_H/k_D = 1.11$ (6). An attempt to measure the isotope effect on $k_{cat}/K_m$ for the DNPglucoside estimated $k_H/k_D$ to be between 1.01-1.05 (Karen Rupitz, unpublished results). However, the size of the isotope effect on $k_{cat}/K_m$ for DNPglucoside will be affected by diffusion control of the rate of glycosylation and thus may not be representative of the amount of oxocarbonium ion character for glycosylation with that substrate. Regardless, these measurements are consistent with the notion, that for the aryl glucosides examined, there is a greater degree of oxocarbonium ion character in the transition state for deglycosylation than for glycosylation.

Given that an $\alpha$-DKIE was observed for both glycosylation and deglycosylation, it is reasonable to suggest that both transition states possess some degree of oxocarbonium ion character. Therefore substitutions which remove electron density from the transition state (such as replacement of a hydroxyl on the glycone by fluorine) should slow the rate of reaction. The converse should be true of replacement of a hydroxyl by hydrogen. Furthermore, the more oxocarbonium ion character developed at the transition state, the larger these effects would be. Given that $\Delta AG^0$ values possess an electronic component, it would be expected that $\Delta AG^0$ values calculated for the fluorinated DNPglucosides would be larger for deglycosylation than glycosylation if more $S_N1$ character is generated at the second step in the enzymic reaction. The inverse
should be true for the deoxy glucosides. This appears to be the case for most of the fluorinated DNPglucosides but not for the deoxy glucosides (Table 3-9). However, these results may be rationalized as follows.

It has been previously suggested by Legler (120) that enzyme/substrate binding may be tighter at the transition state for deglycosylation than for glycosylation (at least in *A. wentii* β-glucosidase). Since such an increase in binding would manifest itself as a larger $\Delta \Delta G^\ddagger$ value for deglycosylation for both the deoxy and deoxyfluoro glycosides since in both cases binding has been decreased with respect to the parent DNPglucoside. However, if the second transition state is more $S_N1$ like, the increase in the amount of positive charge at the transition state would be expected to decrease $\Delta \Delta G^{\ddagger}$ for the deoxy substrates but increase $\Delta \Delta G^{\ddagger}$ for the deoxyfluoro compounds. Yet $\Delta \Delta G^{\ddagger}$ values for the deoxy glucosides were the same for glycosylation and deglycosylation. Thus neither an increase in binding nor an increase in positive charge for deglycosylation can alone account for the observed values of $\Delta \Delta G^{\ddagger}$ for the substituted DNPglucosides. However, these data are consistent with an increase in binding and an increase in the amount of positive charge generated at the transition state for deglycosylation. For the deoxy glucosides, the expected decrease in $\Delta \Delta G^{\ddagger}$ due to electronic effects is apparently offset by an increase in $\Delta \Delta G^{\ddagger}$ due to greater loss of binding at the deglycosylation step. Since both electronic and binding effects will increase $\Delta \Delta G^{\ddagger}$ for the deoxyfluoro glucosides a larger increase in $\Delta \Delta G^{\ddagger}$ for deglycosylation is observed as would be expected. Thus, these data would support the notion that more positive charge is generated at the transition state for deglycosylation. Interestingly, this was not true for the 4FDNPglucoside. This may be due to a difference in the nature of the transition state for glycosylation with the 4 position substituted glycosides as will be discussed later (see section 3.7.4).
**Table 3-9. ΔΔG°‡ Values Calculated From $k_{\text{cat}}/K_m$ and $k_{\text{cat}}$ for the Fluorinated Glucosides with $abg$ β-Glucosidase.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ΔΔG°‡glycosylation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ΔΔG°‡deglycosylation&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2FDNPglucoside</td>
<td>14.8</td>
<td>52.3</td>
</tr>
<tr>
<td>3FDNPglucoside</td>
<td>6.1</td>
<td>12.3</td>
</tr>
<tr>
<td>4FDNPglucoside</td>
<td>6.5</td>
<td>6.7</td>
</tr>
<tr>
<td>6FDNPglucoside</td>
<td>0.8</td>
<td>7.0</td>
</tr>
<tr>
<td>3dDNPglucoside</td>
<td>7.4</td>
<td>6.6</td>
</tr>
<tr>
<td>4dDNPglucoside</td>
<td>2.5</td>
<td>c</td>
</tr>
<tr>
<td>6dDNPglucoside</td>
<td>2.9</td>
<td>2.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values calculated from $k_{\text{cat}}/K_m$ are related to differences in the rate of glycosylation of the enzyme.

<sup>b</sup> Values calculated from $k_{\text{cat}}$ are related to differences in the rate of deglycosylation of the enzyme.

<sup>c</sup> A ΔΔG°‡ value for deglycosylation could not be estimated for this substrate.

Similar results were obtained for the $abg$ β-glucosidase-catalyzed hydrolysis of a series of 2 position substituted aryl glycosides. Since the non-covalent interaction at the 2 position for all of these substrates will be somewhat 'crippled', differences in the activation free energies for these compounds will be largely determined by the relative electronic stabilities of the transition states and deleterious steric interactions between the enzyme and the glycosides.

Comparison of the ΔΔG°‡ values for DNPmannoside and 2FDNPmannoside reveals that the rate of glycosylation is essentially unaffected by the electronic nature of the substrate. However, a profound effect can be seen on the rate of deglycosylation. The results are again consistent with more oxocarbonium ion character in the transition state for deglycosylation. The changes in activation free energy for glycosylation also appear to vary with changes in the the leaving group. The ΔΔG°‡ values calculated for the PNPglucosides vary unpredictably in comparison to the analogous DNP substrates.
This could arise from several factors including differing steric effects between the two series and diffusion control of the rate of glycosylation of abg β-glucosidase with the DNPglucoside. A definitive conclusion as to the cause of this effect will require more study.

Table 3-10. Changes in Activation Free Energies Calculated for Substrates Modified at the 2 Position.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\Delta \Delta G^\ddagger (k_{cat}/K_m)$ or $\Delta \Delta G^\ddagger (k_{cat}/K_i)$</th>
<th>$\Delta \Delta G^\ddagger (k_{cat})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2FDNPglucoside</td>
<td>15</td>
<td>52$^a$</td>
</tr>
<tr>
<td>2C1DNPglucoside</td>
<td>31</td>
<td>not available</td>
</tr>
<tr>
<td>DNPmannoside</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>2FDNPmannoside</td>
<td>25</td>
<td>41$^a$</td>
</tr>
<tr>
<td>2FPNPglucoside$^b$</td>
<td>42</td>
<td>52$^a$</td>
</tr>
<tr>
<td>2dPNPglucoside$^c$</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>2NH$_2$PNPglucoside</td>
<td>31</td>
<td>32$^d$</td>
</tr>
<tr>
<td>PNPmannoside$^e$</td>
<td>15</td>
<td>19</td>
</tr>
</tbody>
</table>

a) Calculated from data taken from Street et al. (15).
b) Calculated from data taken from Kempton and Withers (6).
c) Calculated from data obtained from Karen Rupitz (unpublished results)
d) This substrate does not show rate limiting deglycosylation
e) Calculated from data taken from Day and Withers (3)

In summary, the available evidence is consistent with the transition states for both glycosylation and deglycosylation possessing some degree of oxocarbonium ion character, though it would appear that deglycosylation possesses significantly more oxocarbonium ion character. Therefore, based solely on electronic considerations, the deoxy glucosides should have the highest rates of enzymic hydrolysis and conversely, the fluorinated glycosides the lowest. However, given that all the deoxy glucosides possessed lower $k_{cat}/K_m$ values than the parent DNPglucoside, other factors such as
enzyme/substrate binding interactions, must be playing a major role in determining the relative rates of hydrolysis of these glycosides.

### 3.7 Binding Effects

Non-covalent interactions between the substrate and the enzyme are proposed to account for the majority of the rate acceleration seen in glycosidases (7) and are also no doubt involved in substrate specificity. Assuming these interactions take the form of hydrogen bonds between the substrate hydroxyls and the enzyme (an assumption which is well supported by X-ray crystal structures of glycosidases and carbohydrate binding proteins (33,36) ), an analysis of the non-covalent enzyme/substrate interactions can proceed as follows.

Replacement of a glycone hydroxyl group with hydrogen will result in the deletion of any enzyme/substrate hydrogen bonding which is normally present at the substituted position. Thus, in the absence of significant electronic considerations, $\Delta G^0$ or $\Delta G^+/-$ values calculated for each of the steps in the $\beta$-glucosidase-catalyzed reaction with the deoxy glycosides will provide a minimum estimate (see Chapter 1) of the magnitude of the interaction at the substituted position. Since fluorine is arguably capable of accepting a hydrogen bond, but cannot donate one, a deoxyfluoro gluco side may be able to regain some of the binding energy lost with the analogous deoxy substrate, if the enzyme is donating a hydrogen bond at the substituted position and thus may exhibit a higher rate of hydrolysis than the analogous deoxy substrate. Given that electronic considerations would predict that all of the deoxyfluoro compounds should have lower rates of hydrolysis than the analogous deoxy glycosides, it is likely that the enzyme is donating a hydrogen bond at the substituted position in cases where the rate constant for the deoxyfluoro compound is larger than that for the analogous deoxy glucoside. If the deoxyfluoro compound has a lower rate of hydrolysis than the
analogous deoxy glycoside, no conclusion as to the polarity of the interaction at that position is possible since this could arise from electronic or binding effects.

**Examination of Binding Interactions by Position**

### 3.7.1 6 Position

Estimation of the magnitude of the enzyme/substrate interaction at the 6 position is accomplished by comparing the various kinetic constants determined for the 6dDNPglucoside with those values obtained for the parent glucoside. These kinetic constants can then be converted to ΔΔG°‡ values as has been discussed, and these values for the 6dDNPglucoside are presented in Figure 3-10. From pre-steady state analysis a maximum estimate of K_d, the dissociation constant for the Michaelis complex, was obtained for both substrates. The value for 6dDNPglucoside was higher than that for the parent glucoside (K_d = 2.7 and 0.64 mM respectively). Given that these K_d values are maximum estimates of K_d and that it is more likely that the parent glucoside is displaying Briggs-Haldane kinetics (due to the higher value of k_2 for DNPglucoside) it seems likely that the interaction at the 6 position plays some role in formation of the ground state complex.

Examination of differences in the rate constants for glycosylation (k_2) provides the difference in activation free energy required for these two substrates to reach the transition state for glycosylation from the Michaelis complex. In this case, the 6dDNPglucoside has a lower rate of glycosylation than the parent compound (530 and 1300 sec⁻¹ respectively) suggesting that the hydroxyl at the 6 position is also involved in an interaction which stabilizes the transition state for this step. Comparison of the relative values of k_{cat}/K_m provides similar information since k_{cat}/K_m is again a rate constant for the glycosylation step, but is derived from the free energy difference
between the enzyme plus substrate free in solution and the transition state for
glycosylation. Comparison $k_{\text{cat}}/K_m$ for the DNPglucoside and the 6dDNPglucoside
(5690 and 1970 mM$^{-1}$sec$^{-1}$ respectively) again indicates a small decrease in the stability
of the transition state for the deoxy substrate, presumably due to the loss of the
interaction at the 6 position. Examining $k_{\text{cat}}$ for these substrates a similar decrease is
found for deglycosylation as a consequence of deoxygenation (Figure 3-10).

Comparison of the values of $k_{\text{cat}}/K_m$ for the 6-deoxy and 6-deoxy-6-fluoro
glucoside (1974 and 4340 mM$^{-1}$sec$^{-1}$ respectively) indicates that the rate of
glycosylation of the enzyme is higher for the fluorinated compound, contrary to what
would be expected on purely electronic grounds. This result is consistent with hydrogen
bond donation by the enzyme at the 6 position, a conclusion which is supported by the
analogous values for $k_2/K_d$. However, the values of $K_d$ determined for these compounds
(5.6 and 2.7 mM for the 6-deoxy-6-fluoro and 6-deoxy glucosides respectively) would
indicate that the fluorinated compound is bound weakly in comparison with the deoxy
glucoside on formation of the Michaelis complex. This may simply be due to difficulties
in obtaining a value for $K_d$ for the DNPglycosides or may actually reflect a difference in
the enzyme/substrate interaction as the reaction proceeds from ground state to transition
state. A definitive conclusion is not possible from the data available. Combining data
from each of these analyses, it appears that the enzyme is donating a hydrogen bond at
the 6 position which stabilizes both transition states to about the same degree, but that
this interaction is relatively weak. It is also possible that the 6 position plays a role in
stabilization of the Michaelis complex.
Figure 3-10. Free energy profile comparing the \( \text{abg}\ \beta\text{-glucosidase-catalyzed hydrolysis}\) of the 6dDNPglucoside (----) and the DNPglucoside (---).

It should be noted that the values presented above (with the exception of \( \Delta\Delta G^0_{\text{Kd}} \)) are derived from the steady state parameters \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) to facilitate comparison of the \( \Delta\Delta G^0 \) values for both glycosylation and deglycosylation at the same temperature (37 °C). Mainly for this reason, most of the analysis of binding interactions in this study will be carried using the steady state kinetic data.

3.7.2 4 Position

With the exception of 4FDNPGalactoside, kinetic data for the substrates modified at the 4 position differ markedly from that of the other glycosides tested. Firstly, no pre-steady state burst was observed for these compounds (DNPgalactoside, 4FDNPGalactoside and 4dDNPglucoside) which, coupled with their low \( k_{\text{cat}}/K_m \) values
implies that glycosylation is rate limiting for these glycosides. Interestingly, despite the observed drop in $k_{cat}/K_m$, $k_{cat}$ appears unaffected or may have been slightly increased. Given that enzymes derive specificity from $k_{cat}/K_m$, while $k_{cat}$ is the maximal catalyzed rate the enzyme can achieve, these data suggest that the function of the interaction at the 4 position is mainly regulatory enabling the enzyme to select between galactoside and glucoside substrates.

How then is this selectivity achieved? Since pre-steady state data could not be obtained for these substrates, $\Delta AG^{0+}$ values could not be calculated for the individual steps in the reaction. However, given that glycosylation is rate limiting for these substrates, some degree of selective destabilization of the transition state for glycosylation, but not deglycosylation, must be occurring. Some insight into the mechanism by which $\alpha$-$\beta$-glucosidase destabilizes the transition state for glycosylation for galactosides comes from comparing the glycosylation step for DNPgalactoside and DNPglucoside. The $\alpha$-DKIE on $k_{cat}/K_m$ has been determined for both the DNPglucoside and DNPgalactoside. Recall that the $\alpha$-DKIE for the DNPglucoside was estimated to be between 1 - 5 %. The isotope effect measured for $k_{cat}/K_m$ for the DNPgalactoside was found to be somewhat higher ($k_H/k_D = 1.076 +/- 0.015$) than that seen for the DNPglucoside suggesting that the transition state for glycosylation with the DNPgalactoside is more dissociative than that for the DNPglucoside. However, these isotope effects must be interpreted cautiously since the magnitude of the isotope effect on $k_{cat}/K_m$ for DNPglucoside will be affected by diffusion control of the rate of glycosylation of the enzyme with this substrate.

Similar evidence comes from examining the hydrolysis of 4FDNPglucoside, which was the only substrate altered at the 4 position which still maintained rate limiting deglycosylation. Recall that for the other fluorinated glycosides, $\Delta AG^{0+}$ values calculated for deglycosylation were larger than values calculated for glycosylation, while for the 4FDNPglucoside, $\Delta AG^{0+}$ was about the same for both steps (Table 3-9). This
could arise in two ways. Firstly, if alteration of the interaction at the 4 position does lead to a more dissociative transition state for glycosylation, the electronic and binding effects on the first and the second transition states would become more similar, leading to the equalization of the calculated values of $\Delta \Delta G^{\text{tof}}$ for the two transition states. Alternatively, if the interaction at the 4 position was maximized at the glycosyl-enzyme intermediate, weakening of this interaction would lead to a destabilization of the 4Fglucosyl-enzyme intermediate with respect to the parent glucosyl-enzyme. This would increase the rate of deglycosylation for the fluorinated intermediate with respect to the parent and decrease $\Delta \Delta G^{\text{tof}}$ for the deglycosylation step, again leading to the observed equalization of the $\Delta \Delta G^{\text{tof}}$ for the two transition states.

Given that the available evidence suggests that the transition state for glycosylation of a DNPglucoside is more pre-associated (SN2-like) than that for deglycosylation, one might speculate that the interaction at the 4 position plays some role in forcing pre-association between the enzymic nucleophile (Glu-358) and the substrate. Such an interaction would be expected to be optimized at the glycosyl-enzyme intermediate since at that point Cl and the oxygen of the enzymic carboxylate are bonded and necessarily the optimum distance apart for bond formation. Deletion of this interaction (as seen for the substrates which display rate limiting glycosylation) would lead to a more dissociative transition state for glycosylation and a lower $k_{\text{cat}}/K_m$ value. Unfortunately, in the absence of detailed structural information for abg $\beta$-glucosidase, it is difficult to explore this hypothesis further.

Interestingly, similar kinetic data to that seen for the 4 position substituted glycosides with abg $\beta$-glucosidase have been observed with chymotrypsin, where again $k_{\text{cat}}$ was insensitive to changes in the substrate, while $k_{\text{cat}}/K_m$ differed markedly. Based on these studies, Hedstrom et al. (118) suggested that it might be advantageous for an enzyme which forms a covalent intermediate to have a high turnover number but lower $k_{\text{cat}}/K_m$ value with a non-optimal substrate to enable the enzyme to select for the proper
substrate yet avoid inactivation of the enzyme by accumulation of a stable covalent intermediate. The interaction at the 4 position in the abg β-glucosidase seems to fit this general description. Indeed, since galactose is a common monosaccharide, such an evolutionary development for a β-glucosidase would not be surprising.

Figure 3-11. A possible free energy profile comparing the hydrolysis of DNPglucoside (—) and DNPgalactoside (----) at 5 °C.

3.7.3 3 Position

The interaction at the 3 position plays a significant role in binding to and stabilizing the transition states for both glycosylation and deglycosylation. Comparing the rate constants obtained for 3dDNPglucoside with DNPglucoside (Table 3-1) it is
apparent that at least 7 kJ/mol of binding energy is lost at the transition state for
glycosylation and at least 6 kJ/mol for deglycosylation as a consequence of
deoxygenation (Table 3-8, Figure 3-12). Examining the $k_{cat}/K_m$ values for the 3-deoxy
and 3-deoxy-3-fluoro glucosides (342 and 560 mM$^{-1}$sec$^{-1}$ respectively) it appears the
substrate is accepting a hydrogen bond at the 3 position since the fluorinated glucoside
has a higher value for $k_{cat}/K_m$ than the analogous deoxy glucoside, contrary to
predictions based on electronic effects. This conclusion is supported by the relevant
$k_2/K_d$ values. The $K_d$ values for both the 3-deoxy and 3FDNPglucoside were similar to
the estimated value of $K_d$ for the DNPglucoside. It therefore seems unlikely that the
interaction plays a significant role in ground state binding of the substrate though this
interpretation must again be made cautiously.

Figure 3-12. A free energy profile comparing the hydrolysis of 3dDNPglucoside (----)
and DNPglucoside(—).
3.7.4 2 Position

The enzyme/substrate interaction at the 2 position is the most important in terms of binding energy at the transition state. Comparison of $k_{cat}/K_m$ values for the 2dPNPglucoside and PNPglucoside (Table 3-2, 3-8) indicates that the interaction at the 2 position accounts for at least 18 kJ/mol of binding energy at the transition state for glycosylation. This binding energy would appear to increase at the second transition state by at least 4 kJ/mol as was calculated from the appropriate values for $k_{cat}$.

The role of the 2 position interaction in binding the ground state is difficult to assess. In the case of the 2FDNPglucoside, the substitution appears to have no significant effect on ground state binding since again the $K_d$ value for 2FDNPglucoside is similar to the estimate of $K_d$ for DNPglucoside. However, the PNP 2-deoxy and 2-deoxy-2-fluoro-β-D-glucosides appear to bind poorly in comparison to DNPglucoside. Since a reliable $K_d$ value for the PNPglucoside at 37 °C could not be obtained, it was impossible to determine if the drop in ground state binding for the PNPglucosides was due to real differences in binding at the 2 position or due to the difference in aglycone. The polarity of the hydrogen bond at the 2 position is also difficult to determine since this position is particularly sensitive to the electronic nature of the substituting group. Therefore any effects due to binding could be masked by large electronic effects.

3.8 Refined Mechanism for β-Glucosidase.

In general, an enzyme must satisfy two requirements. It must catalyze the reaction in question and also select the proper substrate on which to act. In the case of the glycosidases, non-covalent interactions between the hydroxyls of the substrate glycone and the enzyme play a crucial role in this process. Applying the data accumulated from this study and others on abg β-glucosidase, it is possible to refine the
Koshland mechanism to reflect this. The roles of the individual non-covalent interactions in the enzyme's mechanism are described below.

Formation of the $\alpha$-linked glucosyl-enzyme intermediate simultaneously utilizes several modes of catalysis. The departure of the leaving group is believed to be aided by donation of a proton from a general acid moiety, though its identity in abg $\beta$-glucosidase is yet to be determined. The interaction at the 4 position may function to stabilize the transition state for glycosylation by aiding in pre-association of the enzymic nucleophile with the substrate. However, the transition state for glycosylation still possesses significant oxocarbonium ion character even though the carboxylate nucleophile of the enzyme is likely to be tightly pre-associated. Generation of this transition state is promoted by the interactions at the 2 and 3 and to a small extent the 6 position which selectively stabilize the transition state structure of the substrate.

The hydrolysis of the glucosyl-enzyme intermediate again proceeds via a transition state with significant oxocarbonium ion character. Interactions at the 2, 3 and 6 positions of the substrate again stabilize the transition state and aid in catalysis. The attack of water is promoted by removal of a proton from the attacking water molecule by a general base. Isotope effect data and analysis of the activation free energies for the fluorinated substrates give some indication that the attacking water is not as tightly pre-associated as the nucleophilic carboxylate, leading to a transition state with substantially greater $S_N1$ character for deglycosylation. The cycle is completed by release of a molecule of $\beta$-D-glucose from the enzyme.
Figure 3-13. Revised mechanism for the abg β-Glucosidase.
3.9 Conclusions

As predicted, non-covalent interactions between the enzyme and the substrate play a crucial role in the catalytic mechanism of the enzyme. These interactions effect catalysis by stabilizing both oxocarbonium ion transition states. Further, formation of the covalent intermediate reduces the entropic cost of the hydrolysis of the glycosides (see Jencks 119). Also, general acid and base catalysis contribute to the formation and hydrolysis of the glycosyl-enzyme intermediate as discussed. Indeed, the glucosidase mechanism is a good example of an enzyme's ability to use several modes of catalysis simultaneously.

A critical consideration in any mechanistic study carried out with modified substrates is the degree to which data from these substrates addresses the mechanism of the enzyme with its natural substrate. The model derived for the abg β-glucosidase is likely a moderately accurate depiction of the mechanism of the enzyme at the glycone site, but completely ignores the role of binding of the aglycone in the enzyme's mechanism. The fact that transglycosylation is seen for some of the substrates tested argues that these interactions play some role in the normal mechanism of the enzyme since transglycosylation is the exact microscopic reverse of hydrolysis of a disaccharide. This can also be seen for the hydrolysis reaction since when the aglycone is another sugar molecule, the leaving group alcohol has a pKa of ~17. However, the $k_{cat}$ value for the hydrolysis of cellobiose by abg β-glucosidase is the same as that of the DNPglucoside (3), implying that deglycosylation is rate limiting in both cases and indicating a significant activation of the glucose leaving group. Indeed, glycosylation is rate limiting for phenyl β-D-glucopyranoside even though the phenyl glucoside should be up to $10^7$ times more reactive than cellobiose, based on the pKa of the leaving group. Clearly the
non-covalent interactions between the aglycone and the enzyme must function in some
manner to accelerate the rate of glycosylation of the cellobiose substrate.

Finally, it is interesting to note the data obtained in this study are similar to those
derived from the study of other β-glycosidases. A common feature of all the β-
glycosidases studied to date is the importance of the interaction at the 2 position in
stabilizing the transition state (Table 3-11). Since the transition states for glycoside
hydrolysis apparently possess some oxocarbonium ion character, the interaction at the 2
position would be ideal for selective binding of the transition state since the orientation
of the hydroxyl at C2 would be expected to change on going from the ground state $^4C_1$
chair to the transition state $^4H$ half chair. Thus it likely that the evolution of a strong
interaction at the 2 position is a consequence of the geometry of the reaction being
catalyzed and may prove to be a general feature of all β-glycosidases. Interestingly, the
2 position interaction appears to less important for α-glycosidases. Clearly more data are
required before any conclusions pertaining to differences in mechanisms of α- and β-
glycosidases is possible and work in this laboratory with α-amylase, glycogen
debranching enzyme and glycogen phosphorylase is currently underway which will aid in
addressing this question.
Table 3-11. Comparison of Changes in Activation Free Energy Calculated From $k_{cat}/K_m$ Using Analogously Substituted Deoxy-Glycoside Substrates$^a$.

<table>
<thead>
<tr>
<th>Modified position</th>
<th>abg β-glu</th>
<th>E. coli (Lac-Z) β-gal$^b$</th>
<th>A. wentii β-glu$^f$</th>
<th>A. oryzae β-glu$^c$</th>
<th>Mammalian Lactase$^d$</th>
<th>Glycogen phosphorlyase$^e$</th>
<th>A. niger glucoamylase$^g$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-deoxy</td>
<td>17.9</td>
<td>&gt; 34</td>
<td>32.1</td>
<td>43-45</td>
<td>h</td>
<td>-</td>
<td>5.2</td>
</tr>
<tr>
<td>3-deoxy</td>
<td>7.4</td>
<td>-</td>
<td>-</td>
<td>34.8</td>
<td>h</td>
<td>26.3</td>
<td>11.3</td>
</tr>
<tr>
<td>4-deoxy</td>
<td>2.5</td>
<td>15.5</td>
<td>21</td>
<td>29.1</td>
<td>5.2</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td>6-deoxy</td>
<td>2.9</td>
<td>18.0</td>
<td>-</td>
<td>16</td>
<td>-4.8</td>
<td>24.7</td>
<td>-1.8</td>
</tr>
</tbody>
</table>

a) Activation free energies in kJ/mol. b) Data from McCarter et al. (40). c) Data from Mega and Matsushima (54). d) Data from Rivera-Sagredo et al. (41). e) Data from Street and Withers (38). f) Data from Roeser and Legler (120). g) Data from Sierks and Svensson (121). h) Rates were too slow to be measured, indicating a large loss of binding energy upon deletion of the interactions at the 2 and 3 positions.
Chapter 4

Investigation of the Mode of Action of Castanospermine and
1-Deoxynojirimycin as Inhibitors of abg β-Glucosidase
4.1 Introduction

The rational design of tight binding non-covalent inhibitors of enzymes is generally based on one of two approaches: the bi-substrate approach or the transition state analogue approach. The bi-substrate model is based on the notion that one of the possible modes of catalysis available is for the enzyme to act as an entropy trap, a concept which has been thoroughly explored by Jencks (122,123). If a particular process is bimolecular, a significant component of the activation free energy is due to the adverse entropic term associated with the two reactants orienting themselves for reaction in solution. It is, therefore, reasonable that an enzyme can catalyze such a process by merely binding the reactants at the active site such that they are well aligned for the reaction to take place. In so doing, the enzyme converts the bimolecular reaction in solution into an essentially unimolecular reaction, thus decreasing the loss of entropy at the transition state and effecting catalysis.

This principle can be exploited in the design of inhibitors. If the enzyme must bind two reactants (A and B) at the active site for the reaction to proceed, there will be a loss in entropy associated with the binding of each of the substrates. If the two substrate molecules are linked in some manner (i.e. A-B) which does not disturb the binding of either A or B, the binding of A-B would be expected to be tighter than that of either A or B individually. This is mainly due to the entropic contribution to binding since binding of either half of A-B will require a similar loss in entropy to the binding of A or B. However, since A-B has already been bound to the enzyme at one site, binding of the other half of the molecule will require little additional entropy loss (for the exceptions to this generalization see 123). Thus the overall ΔG° for binding of the inhibitor A-B should be more favorable. An inhibitor of this type is, in essence, an efficient chelator of the enzyme.
The second approach to the design of non-covalent enzyme inhibitors (and the focus of this Chapter) is the use of transition state analogues. It was originally proposed by Pauling (31) that in order for an enzyme to catalyze a particular reaction, it must bind more strongly to the transition state of that reaction than to the ground state. It can be argued therefore that molecules which mimic the transition state of the reaction should bind more tightly to the enzyme than the analogous substrate molecules. The thermodynamic description of this principle is given in Figure 4-1 (124).

\[
\begin{align*}
E+S & \xrightleftharpoons{K_n} [E+S] \xrightarrow{K_t} E+P \\
ES & \xrightleftharpoons{K_e} [ES] \xrightarrow{K_t} EP
\end{align*}
\]

Figure 4-1. A thermodynamic description of the binding of a substrate to an enzyme at the ground state and the transition state for a reaction.

In this system, \(K_s\) is the dissociation constant for the Michaelis complex and \(K_t\) is the dissociation constant for the enzyme and the substrate at the transition state. From transition state theory, the equilibrium constant between the compound at the ground state and at the transition state is related to the rate constant of the reaction. The equilibrium constants \(K_n\) and \(K_e\) are therefore related to the rate constants for the uncatalyzed and catalyzed reactions respectively. Based on the above thermodynamic description it follows that

\[
\frac{K_n}{K_e} = \frac{K_t}{K_s}
\]
According to this equation, (for a full discussion see 124) the binding of a substrate at the transition state will be tighter than the binding of the substrate at the ground state by an amount proportional to the difference in the rate constants for the catalyzed and uncatalyzed reactions. Since enzymes are known to increase the rates of some reactions up to $10^{14}$ times (124), the potential increase in binding of a transition state analogue in comparison to the binding of a substrate at the ground state could be enormous.

The scheme given above pertains to the relatively common case of an enzyme-catalyzed reaction on a single substrate which proceeds via a single transition state to the product. Wolfenden (125) has pointed out that even in this simple system the design of a true transition state analogue is impossible. For example, at the transition state, bonds are partially formed or broken, a situation which cannot be mimicked with any stable compound. Despite these limitations, transition state analogue inhibitors have been designed for a number of enzymes. Thompson and Bauer (126) have studied the inhibition of serine proteases by a series of peptide aldehydes (Figure 3-2).

These compounds have proven to be potent inhibitors of serine proteases. For example, the inhibitor Ac-pro-ala-pro-ala-CHO binds to elastase with a $K_i$ of 0.0008 mM while the analogous peptide substrate has a $K_m$ of 3.9 mM. The tight binding of these compounds together with the structural similarity between the intermediate formed with the aldehydes and the proposed transition state for peptide hydrolysis made it likely these compounds were transition state analogues. However, tight binding of an inhibitor can arise from a variety of effects and this factor alone does not necessarily qualify a compound as a transition state analogue. Wolfenden (125) has suggested that a true transition state analogue must be a "chemically innocuous" compound so that inhibition is unlikely to arise from non-specific chemical modification of the protein. He also maintained that for inhibitors which are transition state analogues a "mechanistically understandable relationship" should exist between the inhibitors and analogous substrates.
Figure 4-2. A comparison of A) the proposed transition state for deacylation of a serine protease and B) the adduct formed between a serine protease and a peptide aldehyde.

Provided that the first criterion is met, a test for the 'transition state-likeness' of an inhibitor is to examine the relationship between the binding constants for a series of inhibitors and the rates of the enzyme-catalyzed reactions with the analogous series of substrates. If the enzyme is using a specific interaction to aid in catalysis, a change in the structure of the substrate which decreases binding in the enzyme/substrate complex at the transition state will slow the rate of the catalyzed reaction for the modified substrate with respect to an unmodified parent compound. If the analogous modification is carried out on a transition state analogue, binding of the inhibitor should decrease to a similar degree. Therefore, a LFER should exist between the logarithm of the rate constant for the enzyme catalyzed turnover of each of a series of substrates and the logarithm of the binding constant for the analogous transition state analogues.
For the peptide aldehydes, it was demonstrated that for the four enzymes used in the study, a linear relationship existed between \( \log(\frac{k_{cat}}{K_m}) \) for the amide substrates and \( \log(\frac{1}{K_i}) \) for the analogous peptide aldehyde inhibitors. Although these data were far from comprehensive (only two inhibitors and substrates were compared for each enzyme in the study) they did suggest that a plot of the type shown (Figure 4-3) might function as a test of the transition state-likeness of a series of inhibitors.

![Figure 1](image)

**Figure 1:** The similar linear relationship between \( \frac{1}{K_i} \) of a peptide aldehyde and \( \frac{k_{cat}}{K_m} \) of the corresponding peptide amide for four serine proteases. (X) SGP1; (O) SGP3; (+) \( \alpha \)-chymotrypsin; (△) elastase.

Figure 4-3. *Figure taken from (126).*

The best example to date of this type of study comes from work by Bartlett and Marlowe (127) on the use of phosphonamidates as inhibitors of the zinc protease thermolysin. In contrast to the serine proteases, where the peptide is cleaved with formation of a covalent acyl-enzyme intermediate, the zinc proteases catalyze the hydrolysis of peptides *via* direct attack of water on the scissile bond (128). This would lead to generation of a tetrahedral intermediate (*via* a similar transition state) of the type shown in Figure 4-4. Since a phosphonamidate has a similar charge distribution and geometry to the proposed transition state for peptide hydrolysis, it was believed that the tight binding seen for these compounds was due to their 'transition state-likeness'.
Figure 4-4. Comparison of A) the putative transition state for hydrolysis of an amide by a zinc protease to B) the structure of a phosphonamidate.

The data obtained in the Bartlett study demonstrated that for the phosphonamidates, a good correlation exists between the logarithm of the binding constants of these inhibitors and \( \log(\frac{k_{\text{cat}}}{K_m}) \) for the hydrolysis of the analogous substrates. Further, it was demonstrated that for these inhibitors no discernible correlation existed between \( K_m \) and \( K_i \) (Figure 4-5) implying that ground state substrate/enzyme interactions have no significant role in binding of the inhibitors to the enzyme. This relationship fulfills Wolfenden's criterion for a transition state analogue and very strongly indicates that the phosphonamidates are indeed functioning as transition state analogues.
4.2 Glycosidase Inhibitors

Non-covalent glycosidase inhibitors can generally be divided into two classes: the glyconic acid lactones, lactams or related compounds which are sp² hybridized at the anomeric center and the amine inhibitors. A brief outline of the application of these compounds as inhibitors is given below. For a complete discussion of this topic the reader is referred to a recent review by Legler (129).

4.2.1 Lactone and Lactam Inhibitors

A great deal of the work done with this class of inhibitors has been carried out by Leevy (130) and Conchie and Leevy (131). The glyconic acid lactones have been tested
as inhibitors of the number of aldohexopyranosidases. However, a problem with obtaining reliable inhibition constants for this group of inhibitors is isomerization of the 1,5-lactone. Only the 1,5-lactones (Figure 3-6) are believed to possess significant inhibitory activity with these enzymes (129). However, once the 1,5-lactone is dissolved in water, it will begin to isomerize to the thermodynamically more stable 1,4-lactone. Therefore, $K_i$ values determined for these compounds may be mixed binding constants, representing the binding of both forms to the enzyme. Indeed, no reliable data are available for the inhibition of β-galactosidases with the D-galactono-1,5-lactone since this compound can only be generated from the D-galactono-1,4-lactone under acidic conditions in solution and thus the amount of the 1,5-lactone present cannot be known with any certainty (132). These difficulties with most of the other lactones can largely be avoided by carrying out the experiments quickly after the inhibitor solution has been prepared.

![Figure 4-6. Two examples of the lactone class of inhibitors: A) D-glucono-1,5 lactone- and B) D-glucono-1,5-lactam.](image)

The lactones tested have been found to be competitive inhibitors of both α- and β-glucosidases (129). The notable inhibitory activity of these compounds was first rationalized by Reese (133) when it was suggested that the lactone bore a resemblance to the putative oxocarbonium ion transition state for the hydrolysis of a glycoside.
Figure 4-7. A comparison of the structure of A) the transition state for hydrolysis of a β-glucoside and B) D-glucono-1,5-lactone.

The carbon at C1 is sp²-hybridized in both the lactone and the transition state. Further, the dipole of the carbonyl group of the lactone will lead to a larger amount of δ⁺ charge at C1 of the inhibitor than in the analogous aldopyranose providing further similarity with the transition state. Sinnott (16) has proposed that β-glycosidase inhibition by the gluconic acid lactones may be due to reversible formation of a covalent intermediate via attack of the enzymic nucleophile on the lactone carbonyl. Legler (129) suggested this was unlikely as a general rule since gluconolactams still show a significant degree of inhibition with many β-glucosidases, despite being much less likely to form a covalent complex of the type described, due to the decreased susceptibility of amides (in comparison to acids) to nucleophilic attack. However, this could be a possibility in enzymes which are strongly inhibited by lactones, but not by the analogous lactams.

\[ K_i \] values for the lactones are generally found to be 100 - 10,000 times smaller than the \[ K_i \] values for the analogous aldoses (Table 4-1). Inhibition constants for the lactams were generally found to be similar in magnitude to the analogous lactones (129). It is interesting to note that \[ K_i \] values obtained for the lactones with α-glycosidases are often higher than with the β-glycosidases. This may be due to greater structural similarity between a lactone and β-glycoside rather than an α-glycoside (133).
Table 4-1. Inhibition of Glycosidases by Aldono-1,5-lactones and Aldohexoses, Expressed by the Dissociation Constant $K_i$ of the Enzyme Inhibitor Complex$^a$.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_i$ Lactone (mM)</th>
<th>$K_i$ Hexose (mM)</th>
<th>$K_i$ Hexose $/ K_i$ Lactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-D-glucosidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Agrobacterium faecalis</em></td>
<td>0.0017</td>
<td>6.4</td>
<td>3,700</td>
</tr>
<tr>
<td><em>Aspergillus wentii</em></td>
<td>0.0095</td>
<td>2.8</td>
<td>290</td>
</tr>
<tr>
<td>Sweet Almond A</td>
<td>0.20</td>
<td>189</td>
<td>950</td>
</tr>
<tr>
<td>Sweet Almond B</td>
<td>0.036</td>
<td>80</td>
<td>2,200</td>
</tr>
<tr>
<td>Human Liver, cytosolic</td>
<td>0.015</td>
<td>55</td>
<td>~3,700</td>
</tr>
<tr>
<td>α-D-glucosidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit, intestinal sucrase</td>
<td>10</td>
<td>19</td>
<td>1.9</td>
</tr>
<tr>
<td>β-D-Mannosidase</td>
<td>0.017</td>
<td>2.2</td>
<td>130</td>
</tr>
<tr>
<td>(Hen Oviduct)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-D-Mannosidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jack Bean</td>
<td>0.12</td>
<td>22</td>
<td>180</td>
</tr>
<tr>
<td>Rat, epididymis</td>
<td>0.071</td>
<td>12</td>
<td>170</td>
</tr>
<tr>
<td>β-D-Glucuronidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Rat Liver)</td>
<td>0.00017</td>
<td>&gt;0.56$^b$</td>
<td>&gt;3,300</td>
</tr>
<tr>
<td>N-acetyl-β-D-glucosaminidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jack Bean</td>
<td>0.00008</td>
<td>12</td>
<td>15,000</td>
</tr>
<tr>
<td>Human Liver</td>
<td>0.0001</td>
<td>5</td>
<td>50,000</td>
</tr>
<tr>
<td>Slug, <em>Arion rufus</em></td>
<td>0.005</td>
<td>3.6</td>
<td>700</td>
</tr>
<tr>
<td>Lysosome (hen egg)</td>
<td>0.000083</td>
<td>0.01</td>
<td>120</td>
</tr>
</tbody>
</table>

$^a$ Data taken from Legler (129).

$^b$ $K_m$ of substrate PNP-β-D-mannuronic acid
4.2.2 Basic Sugar Inhibitors

It has been reported that for several sugars, replacement of a ring hydroxyl group or the ring oxygen by an amine or nitrogen dramatically increases binding of the sugar to glycosidases. This was demonstrated by Lai and Axelrod (134) when they noted the enhanced binding properties of glycosylamines (1-amino-1-deoxy-D-glycopyranoses) with glycosidases. However, most of the interest in this class of compounds has been focused on the cyclic sugar analogues where the ring oxygen has been replaced by nitrogen. Nojirimycin (135) and 1-deoxynojirimycin (136) were both originally isolated from microbial sources and have been found to be powerful inhibitors of both α- and β-glycosidases.

![Diagram of nojirimycin and anhydronojirimycin compared to the putative oxocarbonium ion transition state for glycoside hydrolysis.]

Figure 4-8. A comparison of A) nojirimycin and anhydronojirimycin and B) 1-deoxynojirimycin with C) the putative oxocarbonium ion transition state for glycoside hydrolysis.

The detailed mode of action of these inhibitors is not known. It has been suggested that these compounds are acting as transition state analogues since after
protonation they possess positive charge in a similar location to that which is generated in an oxocarbonium ion. In the case of nojirimycin, it has been proposed that much of the inhibitor's activity is due to a small percentage of the compound which exists in the dehydrated form shown above (137). However, for many enzymes, this seems unlikely since 1-deoxynojirimycin frequently possesses a similar degree of activity to nojirimycin and cannot undergo dehydration (Table 4-2). It is interesting to note that the enzymes which do show a marked difference in the binding of these compounds bind nojirimycin more tightly and are usually β- as opposed to α-glycosidases. This may suggest that sp² hybridization at C1 for these inhibitors is more important for binding with the β-glucosidases since one difference between nojirimycin and 1-deoxynojirimycin is the anhydro form of nojirimycin. Recall that a similar effect was observed for the binding of D-glucono-1,5-lactones which are also sp² hybridized at C1. Alternatively, the C1 hydroxyl group could be bound more tightly in β-glucosidases, leading to the observed increased binding.

Another theory is that the tight binding of nojirimycins is merely due to formation of an ion pair between the protonated ring amino group and an anionic group in the enzyme active site (perhaps the general acid moiety), rather than any structural or electronic similarity to the transition state. Support for this hypothesis comes from pH studies which measured the $K_i$ of the inhibitor at different pHs with the β-glucosidase from A. wentii. It was found (138) that as the pH was increased, the inhibitor bound more tightly. This supports the hypothesis that it is preferable for the inhibitor to be bound in its neutral form (at least in this enzyme, see 129), then to deprotonate an acidic active site group to form a tight ion pair.
Table 4-2. Inhibition of \( \alpha \)- and \( \beta \)-Glycosidases by Nojirimycin.

1-Deoxynojirimycin or Castanospermine\(^a\).

<table>
<thead>
<tr>
<th>Enzyme and source</th>
<th>5-Amino-5-Deoxy-Hexopyranose ( K_i ) (( \mu )M)</th>
<th>1,5-Dideoxy-1,5-Iminohexitol ( K_i ) (( \mu )M)</th>
<th>Castanospermine ( K_i ) (( \mu )M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )-D-Glucosidases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td>0.01*</td>
<td>0.01*</td>
<td>0.015*</td>
</tr>
<tr>
<td>Yeast</td>
<td>6.3</td>
<td>12.6</td>
<td>&gt; 1500</td>
</tr>
<tr>
<td>Rabbit Intestine (sucrase)</td>
<td>0.13*</td>
<td>0.032*</td>
<td></td>
</tr>
<tr>
<td>Rat Intestine (sucrase)</td>
<td></td>
<td></td>
<td>0.00055*</td>
</tr>
<tr>
<td>( \beta )-D-Glucosidases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Aspergillus wentii}</td>
<td>0.36*</td>
<td>2.7*</td>
<td>0.9*</td>
</tr>
<tr>
<td>Sweet Almonds</td>
<td>0.89*</td>
<td>47</td>
<td>1.5*</td>
</tr>
<tr>
<td>Calf Liver (cytosol)</td>
<td>-</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>Calf Spleen (lysosomes)</td>
<td>4.5</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>\textit{Helix pomatia}</td>
<td>1.1*</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>( \alpha )-D-Galactosidases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coffee Beans</td>
<td>0.0007*</td>
<td>0.0016*</td>
<td></td>
</tr>
<tr>
<td>\textit{E. coli}</td>
<td>0.17*</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>( \beta )-D-Galactosidases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{E. coli}</td>
<td>0.045*</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>\textit{Aspergillus wentii}</td>
<td>0.011*</td>
<td>0.16*</td>
<td></td>
</tr>
<tr>
<td>Pig Brain (galactocerbroside)</td>
<td>1.5</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>( \alpha )-D-Mannosidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jack Bean</td>
<td>1.2*</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Sweet Almonds</td>
<td>21</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>Calf Liver (lysosomes)</td>
<td>4.4</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>( \beta )-D-mannosidases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Aspergillus wentii}</td>
<td>7.7</td>
<td>4,600</td>
<td></td>
</tr>
</tbody>
</table>

* Inhibitor ion showed a slow approach to equilibrium binding value.

\(^a\) Data taken from Legler (129).
It has been observed (129) that in cases where the nojirimycins bind tightly, the onset of inhibition is often slow. Indeed, 15 minutes was required for 1-deoxynojirimycin to fully bind to intestinal sucrase (139). Since association rates for the inhibitor, even in the faster cases, are at least 100 times slower than what is generally observed to be the diffusion controlled limit for association of substrates with enzymes (140), it is clear that another phenomenon is at work. It has been suggested that the inhibitor is initially bound in a low affinity conformation which slowly equilibrates to a tightly bound form. Conversely, it is possible that the change in conformation takes place in the enzyme rather than the inhibitor. In one case Donsimoni et al. (141) monitored a change in the conformation of sweet almond β-glucosidase on binding of D-glucono-1,5-lactone or nojirimycin.

Another example of this class of inhibitors is castanospermine, an indolizine alkaloid originally isolated from *Castanospermium australe* (142). The compound is similar in structure to 1-deoxynojirimycin (Figure 4-9) and presumably has a similar mode of action. However, while castanospermine is an extremely tight binding inhibitor with some enzymes (better than 1-deoxynojirimycin), in others, it has almost no activity (Table 4-2, 129). This is likely due to the rigid, bicyclic structure of the molecule. In cases where castanospermine binds tightly, the molecule is likely being restricted to a preferred conformation for binding (thus offsetting the entropy lost on binding of a more flexible molecule and locking it into the preferred binding mode). In enzymes where castanospermine binds poorly, the structure of the molecule may not be similar to the high affinity conformer of 1-deoxynojirimycin with that enzyme (129). It is also possible that steric effects due to the 5 membered ring may be preventing binding in some cases.
An interesting approach recently used by Tong et al. (143) combines the properties of glucosylamines, nojirimycin and the half chair conformation to develop inhibitors of the type shown in Figure 4-10. The gluco-1-amidine bound tightly to sweet almond β-glucosidase (Kᵢ = 8 µM) in comparison to 1-deoxynojirimycin (Kᵢ = 47 µM). The amidine was also an effective inhibitor of Jack Bean α-mannosidase (Kᵢ = 9 µM).

Although these compounds bind well to glycosidases, their instability in water limits their potential use as inhibitors. Attempts to improve the stability of these compounds have led to the development of several new inhibitors namely the amidrazones (144), amidoximes (144) and nojirimycin tetrazoles (145) (Figure 4-10). These compounds have again proven to be tight binding glycosidase inhibitors and compared with the amidine display greatly enhanced stability in aqueous solutions. Preliminary tests of the tetrazoles carried out in this laboratory (Karen Rupitz, unpublished results) have been promising as these compounds have shown good activity against a number of glycosidases (Table 4-3).
Figure 4-10. An A) amidine, B) amidrazone, C) amidoxime and D) nojirimycin tetrazole.

Table 4-3. Binding of the Nojirimycin Tetrazoles to Glycosidases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tetrazole Inhibitor</th>
<th>$K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jack Bean α-mannosidase</td>
<td>Manno-</td>
<td>180 +/- 10</td>
</tr>
<tr>
<td>Sweet Almond α-mannosidase</td>
<td>Manno-</td>
<td>700 +/- 80</td>
</tr>
<tr>
<td>Snail β-mannosidase</td>
<td>Manno-</td>
<td>160 +/- 10</td>
</tr>
<tr>
<td>Yeast α-glucosidase</td>
<td>Gluco-</td>
<td>1300 - 5600*</td>
</tr>
<tr>
<td>abg β-glucosidase</td>
<td>Gluco-</td>
<td>1.4 +/- 0.1</td>
</tr>
</tbody>
</table>

* Mixture of competitive and non-competitive inhibition

4.3 Goals of this Study

The goal of this study is to examine the mode of inhibition of abg β-glucosidase by 1-deoxynojirimycin and castanospermine. The roles of the hydroxyl groups around the inhibitor in binding will be examined to determine if changes in these groups affect inhibitor binding. Since the role of these interactions in the hydrolysis of β-glycosides
by abg β-glucosidase has already been studied (Chapter 3), a comparison of the kinetic data available for the enzyme-catalyzed hydrolysis of these compounds with binding data of a series of similarly substituted inhibitors, will aid in determining whether 1-deoxynojirimycin and castanospermine are transition state analogues for abg β-glucosidase.

4.4 Results

The inhibitors used for this study were supplied by other groups through several different collaborations (see Chapter 5). In all cases, the test substrate was PNPglucoside. $K_i$ determinations for both castanospermine and 1-deoxynojirimycin indicated that both these compounds behave as competitive inhibitors (Figure 4-11). Based on these results, it was assumed that all of the modified amine inhibitors tested were also competitive inhibitors.

Figure 4-11. Dixon plots of the $K_i$ determinations for A) 1-deoxynojirimycin and B) castanospermine with abg β-glucosidase.
Approximate $K_i$ values were determined for the other inhibitors using a single substrate concentration and at least six inhibitor concentrations. Values of $K_i$ determined in this manner have generally been found to correspond quite closely (within 50%) in previous studies in this laboratory. Such correspondence was found in this case where $K_i$ values determined in this manner for castanospermine and using a full $K_i$ determination were found to agree within 35% (0.0044 and 0.0028 mM respectively).

For ease of comparison, the ring positions on both the 1-deoxynojirimycins and castanospermines will be referred to using the naming conventions for sugars rather than the conventions usually applied to the naming of alkaloids (Figure 4-12).

![Figure 4-12](image)

*Figure 4-12. Naming conventions used for A) 1-deoxynojirimycin and B) castanospermine.*

It has been noted that for several of the enzymes tested with 1-deoxynojirimycin and castanospermine, binding of the inhibitor to the enzyme is a relatively slow process. No evidence was found for this was found for these inhibitors with abg β-glucosidase when assayed at 37 °C. However, at room temperature (approx. 20 °C), the time course for PNP release had a distinct downward curvature, reaching a steady state after approximately 80 seconds, consistent with slow binding of the inhibitor. Not surprisingly, it appears that the rate of binding of castanospermine is sensitive to
temperature, but, is rapid enough at 37 °C to avoid difficulties in determining the equilibrium $K_i$ values.

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 4-13.** Time courses for hydrolysis of PNPglucoside in the presence of castanospermine at A) room temperature and B) 37 °C. Both reactions were initiated by addition of enzyme to the inhibitor/substrate solution.

It has previously been demonstrated by several groups that the binding of nojirimycins to some enzymes is pH dependent (18,87,129). Since changes in the structure of the inhibitors affect the pKa value of the ring amine (see Chapter 2), it is possible that the differences in the binding of these inhibitors is due to changes in the charge of the amine, rather than changes in the non-covalent interactions between the ring hydroxyl groups and the enzyme. Approximate $K_i$ values determined for 1-deoxynojirimycin with abg β-glucosidase at pH 5.0, 6.8 and 9.0 were found to vary little with pH (0.065, 0.050, and 0.17 mM respectively). Therefore, it is unlikely that the large differences in binding seen in this study can be attributed to a shift in the degree of protonation of the inhibitor.
### Table 4-4. Competitive Inhibition Constants for the Binding of a Series of Monosubstituted 1-Deoxynojirimycins With αβ-glucosidase.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition constant, $K_i$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-deoxynojirimycin</td>
<td>0.012</td>
</tr>
<tr>
<td>2Fdeoxynojirimycin</td>
<td>0.45</td>
</tr>
<tr>
<td>3Fdeoxynojirimycin</td>
<td>0.96</td>
</tr>
<tr>
<td>4Fdeoxynojirimycin</td>
<td>7.5</td>
</tr>
<tr>
<td>mannodeoxynojirimycin</td>
<td>0.21</td>
</tr>
<tr>
<td>galactodeoxynojirimycin</td>
<td>0.23</td>
</tr>
<tr>
<td>xylodeoxynojirimycin</td>
<td>0.029</td>
</tr>
<tr>
<td>L-arabinodeoxynojirimycin</td>
<td>0.77</td>
</tr>
</tbody>
</table>

### Table 4-5. Competitive Inhibition Constants for the Binding of a Series of Monosubstituted Castanospermines With αβ-glucosidase.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibition Constant $K_i$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castanospermine</td>
<td>0.0028</td>
</tr>
<tr>
<td>2Fcastanospermine</td>
<td>5.5</td>
</tr>
<tr>
<td>4Fcastanospermine</td>
<td>1.5</td>
</tr>
<tr>
<td>6Fcastanospermine</td>
<td>~37</td>
</tr>
<tr>
<td>6F epi-castanospermine</td>
<td>1.3</td>
</tr>
<tr>
<td>2-aminocastanospermine</td>
<td>0.33</td>
</tr>
<tr>
<td>allocastanospermine</td>
<td>0.49</td>
</tr>
<tr>
<td>3Fallocastanospermine</td>
<td>&gt;40</td>
</tr>
<tr>
<td>altrocastanospermine</td>
<td>0.20</td>
</tr>
<tr>
<td>mannocastanospermine</td>
<td>2.2</td>
</tr>
</tbody>
</table>
The binding constants determined for 1-deoxynojirimycin and castanospermine are similar in magnitude to those found for other glycosidases (see Table 4-2 and 129). None of the substituted inhibitors bound as tightly as the parent compounds. Castanospermine bound approximately 4 times more tightly than 1-deoxynojirimycin, however, this did not appear to extrapolate into tighter binding for the substituted castanospermines in comparison to the analogous 1-deoxynojirimycins. Indeed, there are no obvious parallels in the effects of the same substitution upon binding in the two series of compounds.

4.5 Discussion

Recall that two criteria have been outlined by Wolfenden (125) for classifying an inhibitor as a transition state analogue. The first criterion maintains that the chemical reactivity of the compounds tested must be low to avoid non-specific modifications of the protein and thus inhibition. Both the castanospermines and the 1-deoxynojirimycins are reasonably chemically inert and non-specific reactions with the enzyme are unlikely to occur. The second criterion maintains that the enzyme-catalyzed reaction with the substrate must vary in a predictable way with respect to the binding of the analogous inhibitor as the structure of these compounds is varied. Examining the LFE plot comparing \( \ln(\frac{k_{cat}}{K_m}) \) for the DNPglycosides and \( \ln(1/K_i) \) for the analogous deoxynojirimycins, it is immediately apparent that no significant correlation exists between these parameters. A similar plot using values for \( k_{cat} \) also shows no correlation. Clearly these compounds cannot be classified as transition state analogues under the operational definition given above. Plotting \( \ln(K_d) \) for the DNPglycosides versus \( \ln(1/K_i) \) for the inhibitors also showed no significant correlation.
Figure 4-14. Relationships between Michaelis-Menten parameters for a series of DNP glycosides and $1/K_i$ values for a series of correspondingly substituted 1-deoxynojirimycins.
Figure 4-15. Relationships between Michaelis-Menten parameters for a series of DNP glycosides and 1/Ki values for a series of correspondingly substituted castanospermines.
The same analysis performed on the castanospermines provided similar results. The LFE plot of ln(k_{cat}/K_{m}) versus ln(1/K_{i}) for the analogously substituted castanospermines was essentially a scatter plot. The same relationship plotted using ln(k_{cat}) also showed no significant correlation. Again, no significant correlation was found for the plot of ln(K_{d}) versus ln(1/K_{i}). Therefore, on the basis of Wolfenden's criterion it must be concluded that neither the 1-deoxynojirimycins nor the castanospermines can be considered to be transition state analogues.

Any interpretation of these data must account for the differing electronic characteristics of the substitutions, which could be affecting the reaction rate, but not inhibitor binding. Clearly substitution of fluorine for hydroxyl on the sugar ring in the DNPglycosides does have an inductively destabilizing effect on the transition state but it seems unlikely that these effects would be large enough to completely overwhelm the effects on binding at the active site (Chapter 3). Indeed, k_{cat}/K_{m} values for the 3F and 6FDNPglycosides are higher than the same values for the analogous deoxy glycosides (probably resulting from fluorine/enzyme interactions stabilizing the transition state), contrary to what would be predicted based on the electronic nature of the transition state. Thus electronic effects cannot be too large.

4.6 Conclusions

The data obtained in this study suggests that neither 1-deoxynojirimycin nor castanospermine are transition state analogues for the reaction catalyzed by abg β-glucosidase. The binding of these inhibitors may be better described as 'fortuitous' wherein these compounds are merely taking advantage of the fact that an enzyme which has evolved to stabilize a positively charged transition state is likely to possess anionic groups in the active site along with a collection of hydrogen bonding groups which normally interact with the sugar in a specific way. Indeed, with the abg β-glucosidase, it
is known that the catalytic nucleophile is a glutamic acid (Glu-358) (146). Further, though the 1-deoxynojirimycins bear some resemblance to the oxocarbonium ion transition state for glycoside hydrolysis, it is difficult to rationalize the tight binding of glucosylamine (1-amino-1-deoxy-D-glucose) in this manner. Yet binding constants for glucosylamines are frequently of the same magnitude as those seen with 1-deoxynojirimycin. Notably, the enhanced binding of the amidrazones and amidoximes to sweet almond β-glucosidase or the binding of castanospermine to abg β-glucosidase could be accounted for by the formation of a single ion pair between the enzyme and the inhibitor (\(\Delta \Delta G_{\text{binding}} = 20\) kJ/mol comparing glucose to castanospermine with abg β-glucosidase (147)). Thus it seems possible that the hydroxyl groups on these inhibitors are simply promoting formation of that ion pair, rather than binding in their transition state conformation.
Chapter 5

Materials and Methods
1 Synthesis

1.1 General Methods and Materials

Melting points (M.p.) were determined on a Laboratory Devices Mel-Temp II melting point apparatus. The melting points provided are uncorrected.

Proton (1H) nuclear magnetic resonance (nmr) spectra were acquired on a 300 MHz Varian XL-300, a 400 MHz Bruker WH-400 or a 200 MHz Bruker AC-200. When possible, tetramethylsilane (TMS) was used an internal reference (δ = 0.00 ppm). The chemical shifts are given on the (δ) scale. Spectra obtained in D2O are referenced externally to 2,2-dimethyl-2-silapentane-5-sulphonate (δ = 0.00 ppm).

19F NMR spectra were acquired on a 200 MHz Bruker AC-200 instrument, with proton decoupling. Chemical shifts are given in the (δ) scale referenced to CFCl3 (δ = 0.00 ppm), though the external reference used was trifluoroacetic acid (δ = 76.53 ppm). Signals upfield of CFCl3 are assigned a positive value.

Micro-analyses were performed by Mr. Peter Borda in the microanalytical laboratory, Department of Chemistry, University of British Columbia, Vancouver, B.C.

The solvents and reagents used were reagent, certified or spectral grade. Dry solvents and reagents were prepared as follows: Methanol was distilled from magnesium methoxide formed in situ by reaction of methanol with magnesium turnings. Acetonitrile (CH3CN) and pyridine were distilled over calcium hydride. Dichloromethane (CH2Cl2) was washed with concentrated sulfuric acid followed by sodium bicarbonate. It was pre-dried over calcium chloride, then distilled over calcium hydride. Dimethyl sulphoxide (DMSO) was distilled over sodium hydroxide under reduced pressure. N,N-dimethyl formamide (DMF) was stirred overnight over MgSO4 then distilled under reduced pressure onto 4 A sieves. Acetyl chloride was refluxed over phosphorus pentachloride.
(PCl₅) then distilled. Tetrahydrofuran (THF) was distilled over sodium metal and benzophenone.

Thin-layer chromatography (tlc) analyses were performed using Merck Kieselgel 60 F-254 analytical plates. Compounds were visualized using UV light or by charring with 10% sulphuric acid in methanol. Amines were detected using a 0.3% solution of ninhydrin in 3.0% acetic acid and n-propyl alcohol. Preparative thin layer chromatography separations were performed on 1 mm thickness Merck GF₂₅₄ Type 60 silica gel plates. Column chromatography was performed using Kieselgel 60 (180-230 mesh) silica gel.

Some of the compounds used in this work were obtained from others: trifluoromethyl 3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-α-D-glucopyranoside was obtained from Dr. D. Dolphin, Department of Chemistry, UBC. The 2-deoxy-2-fluoro, 3-deoxy-3-fluoro, 6-deoxy-6-fluoro, 6-deoxy and unsubstituted 2',4'-dinitrophenyl β-D-galactopyranosides were synthesized by John McCarter. 1,2,4,6-Tetra-O-acetyl-3-deoxy-3-fluoro-β-D-glucopyranose was synthesized by Ian Street. 1,2,3,4-Tetra-O-acetyl-α-D-glucopyranose was synthesized by Dr. Paul Bird. 2',4'-Dinitrophenyl 2-deoxy-2-fluoro-β-D-mannopyranoside, 2',4'-dinitrophenyl 3-deoxy-3-fluoro-β-D-allopyranoside, 2',4'-dinitrophenyl 4-deoxy-4-fluoro-β-D-galactopyranoside and 2',4'-dinitrophenyl β-D-allopyranoside were synthesized by Dr. Adam Becalski. 2',4'-Dinitrophenyl 2,3,4-tri-O-acetyl-6-deoxy-β-D-glucopyranoside was synthesized by Leise Berven. 2',4'-Dinitrophenyl 2-chloro-2-deoxy-β-D-glucopyranoside was synthesized by Eric Lam and 4'-nitrophenyl 2-deoxy-β-D-glucopyranoside by Tim Black. All of the people mentioned were co-workers in this laboratory.

Compounds used in the competitive inhibitor study were all donated through various collaborations. Xylo-1-deoxynojirimycin, D and L-arabino-1-deoxynojirimycin, galacto-1-deoxynojirimycin, castanospermine, 6F and epi-6F-castanospermine, altrocastanospermine, mannocastanospermine, 2Fcastanospermine, 4Fcastanospermine,
3FAltrocastanospermine, 2-amino and allocastanospermines were all supplied by Dr. Richard Furneaux, DSIR, Chemistry section, New Zealand and Dr. M. B. Gravestock, ICI Agrochemical, England. Manno-1-deoxynojirimycin was a gift from Dr. G. Fleet, Department of Chemistry, Oxford University, England. The 2F, 3F and 4Fdeoxynojirimycins were obtained from Dr. Dan Getman at Monsanto, St. Louis.

1.2 General Procedures

1.2.1 Fluorination

An appropriately protected carbohydrate was dissolved in dry dichloromethane in a three neck round bottom flask. In most cases a base was added to the solution, then the solution was cooled to -23 °C (carbon tetrachloride/dry ice bath) and flushed with dry nitrogen. Diethylamino sulphuryl trifluoride (DAST, Aldrich Chemical Co.) was diluted into dichloromethane and added dropwise using a pressure equalized addition funnel over 15 min. The solution was allowed to slowly come to room temperature and stirred until the reaction was complete. The reaction was cooled to 0 °C and quenched by addition of excess methanol. Solvent was evaporated in vacuo and the resulting gum dissolved in chloroform and washed successively with saturated sodium bicarbonate and water, then dried over MgSO₄.

1.2.2 Acetylation

The appropriate carbohydrate was suspended in dry pyridine and cooled to 0 °C. Acetic anhydride was then added to give typically a 1:1 ratio of pyridine and acetic anhydride, then the reaction mixture was allowed to warm up to room temperature and stirred until reaction was complete. The solvent was evaporated in vacuo, the resulting
oil dissolved in chloroform, then washed successively with dilute hydrochloric acid, saturated sodium bicarbonate, and water. The organic layer was dried over MgSO₄ and the solvent evaporated in vacuo.

1.2.3 Selective Deacetylation of Per-O-Acetylated Carbohydrates at the 1-Position

a) With 2-aminoethanol (72)

   The per-O-acetylated carbohydrate was dissolved in ethyl acetate, one equivalent of 2-aminoethanol added and the reaction stirred at room temperature until complete. In some cases more 2-aminoethanol was required to complete the reaction. The solvent was evaporated in vacuo and the resultant oil purified by column chromatography on silica gel using 1:1 EtOAc/hexanes as the elution solvent.

b) With hydrazine acetate (73)

   The per-O-acetylated carbohydrate was dissolved in DMF, 1.1 equivalents of hydrazine acetate added and the solution heated to 50 °C until the hydrazine acetate had dissolved (approx. 3 min). The mixture was allowed to cool to room temperature, stirred until the reaction was complete, then diluted with ethyl acetate and washed several times with brine. The organic layer was dried over MgSO₄ and the solvent evaporated in vacuo. The resulting oil was resuspended in toluene and concentrated in vacuo to an oil several times to remove the remaining DMF.
1.2.4 2',4'-Dinitrophenyl Glycopyranoside Formation (76)

1-Fluoro-2,4-dinitrobenzene (FDNB) was dried in vacuo overnight, then dissolved in dry DMF and placed over 4A sieves for 1 hr. The appropriate hemiacetal and 1,4 diazabicyclo[2.2.2]octane (DABCO) were dissolved in dry DMF and stirred over 4A sieves for several minutes. The FDNB solution was then syringed into the reaction vessel and the solution stirred under nitrogen at room temperature until the reaction was complete. After removal of the sieves by filtration, the solvent was evaporated in vacuo to yield an oil which was dissolved in chloroform and washed successively with saturated sodium bicarbonate, then water and dried over MgSO₄. The solvent was evaporated in vacuo to give an oil which solidified on trituration with ethanol. The solid was filtered and recrystallized from ethanol.

1.2.5 Deacetylation

a) With HCl methanol (60)

The acetylated glycopyranoside was suspended in dry methanol (14 mg/ml), cooled to 0 °C and freshly distilled acetyl chloride added in sufficient quantity to generate a final HCl concentration of 3-5% (w/v). The reaction was then allowed to warm to room temperature, stirred until the solution cleared, then cooled to 4 °C and stirred overnight. After evaporation of the solvent in vacuo, the resulting gum was tritutated with dry diethyl ether (repeated 6-8 times to remove any HCl), then evaporated to dryness. The resulting gum was crystallized and recrystallized from the solvents listed for the individual compounds (vide infra).
b) With sodium methoxide in methanol (69)

The acetylated glycopyranoside was suspended in dry methanol and cooled to 0 °C. Enough sodium metal to generate a final methoxide concentration of 100 mM was then added and the reaction mixture stirred at room temperature until reaction was complete, then quenched using Dowex 50W (H+) resin. The resin was removed by filtration and the filtrate evaporated to dryness in vacuo. The resulting oil was crystallized and recrystallized from the solvents listed for the individual compounds.

1,3 2',4'-Dinitrophenyl-β-D-Glycosides

2,3,4,6-Tetra-O-acetyl-D-glucose (1)

1,2,3,4,6-Penta-O-acetyl-α-D-glucopyranose (69) (250 mg, 0.64 mmol) was dissolved in 10 ml of ethyl acetate and treated with 2-aminoethanol (0.06 g, 0.80 mmol) using the general procedure. The reaction was complete after 18 h and the product was purified in the usual manner to give the hemiacetal as a clear oil (0.169 g, 76%). $^1$H nmr (300 MHz, CDCl$_3$): δ α-anomer 5.54 (t, 1 H, $J_{3,4}$ 9.5, $J_{3,2}$ 9.5 Hz, H(3)), 5.48 (t, 1 H, $J_{1,2}$ 3.2, J$_{1,OH}$, 3.2 Hz, H(1)), 5.10 (t, 1 H, $J_{4,3}$ 9.5, $J_{4,5}$ 9.5 Hz, H(4)), 4.32-4.10 (m, 3 H, H(5)+H(6)), 3.02 (d, 1 H, $J_{OH,1}$ 3.2 Hz, OH), 2.12, 2.10, 2.04, 2.02 (4 x s, 12 H, 4 x OAc). β-anomer 5.27 (t, 1 H, $J_{3,2}$ 8.2, $J_{3,4}$ 8.2 Hz, H(3)), 5.12 (m, 1 H, H(4)), 4.88 (d, 1 H, $J_{1,2}$ 8.2 Hz, H(1)), 4.75 (t, 1 H, $J_{2,1}$ 8.2, $J_{2,3}$ 8.2 Hz, H(2)), 4.32-4.10 (m, 2 H, H(6a+b)), 3.76 (ddd, 1 H, J 8.7, J 4.9, J 2.2 Hz, H(5)).
2',4'-Dinitrophenyl 2,3,4,6-tetra-O-acetyl-\(\beta\)-D-glucopyranoside (2)

The tetra-O-acetate (1) (1.31 g, 3.8 mmol) was dissolved in 14 ml of dry DMF with DABCO (1.44 g, 13 mmol) and stirred over sieves for 15 min. FDNB (0.85 g, 4.6 mmol) was then added according to the general procedure. The reaction was complete after 2 h and was worked up in the usual manner. The product obtained was recrystallized from ethanol to afford the product as a crystalline solid (1.06g, 55%). M.p. 171-172°C (lit. 76°C 174-175°C); \(^1\)H nmr (400 MHz, CDCl\(_3\)): \(\delta\) 8.71 (d, 1 H, \(J_{3',5'}\) 3 Hz, H(3')), 8.42 (dd, 1 H, \(J_{5',6'}\), 9 , \(J_{5',2'}\) 3 Hz, H(5')), 7.48 (d, 1 H, \(J_{6',5'}\) 9 Hz, H(6')), 5.48-5.38 (m, 3 H), 5.30 (t, 1 H, J 10 Hz),, 4.28-4.22 (m, 2 H, H(6)), 3.98-3.92 (m, 1 H, H(5)), 2.11, 2.09, 2.07, 2.06 (4 x s, 12 H, 4 x OAc).

2',4'-Dinitrophenyl \(\beta\)-D-glucopyranoside (3)

The tetra-O-acetate (2) (1.0 g, 1.9 mmol) was deacetylated using the general deacetylation procedure in HCl/methanol. The product was isolated as a gum, crystallized and recrystallized from a mixture of methanol, acetone and dichloromethane (0.30 g, 45%). M.p. 100-103°C (lit. 60°C 99-101°C); \(^1\)H nmr (200 MHz, CD\(_3\)OD): \(\delta\) 8.76 (d, 1 H, \(J_{3',5'}\) 3 Hz, H(3')), 8.51 (dd, 1 H, \(J_{5',6'}\) 10, \(J_{5',2'}\) 3 Hz, H(5')), 7.67 (d, 1 H, \(J_{6',5'}\) 10 Hz, H(6')), 5.27 (d, 1 H, \(J_{1,2}\) 7.8 Hz, H(1)), 3.91 (dd, 1 H, \(J_{6a,6b}\) 12, \(J_{6a,5}\) 2.0 Hz, H(6a)), 3.70 (dd, 1 H, \(J_{6b,6a}\) 12, \(J_{6b,5}\) 5.6 Hz, H(6b)), 3.62-3.34 (m, 4 H, H(2)+H(3)+H(4)+H(5)). Anal. calc. for C\(_{12}\)H\(_{14}\)O\(_{10}\)N\(_2\) + 0.7 acetone + 0.5 H\(_2\)O; C, 42.77%; H, 4.89%; N, 7.08%; Found: C, 42.90%; H, 4.85%; N, 6.94%.
1,2,3,4,-Tetra-O-acetyl-6-deoxy-6-fluoro-β-D-glucopyranose (4)

1,2,3,4,-Tetra-O-acetyl-β-D-glucopyranose, (0.25 g, 0.71 mmol) was dissolved in 20 ml of CH₂Cl₂. The solution was treated with 2,4,6-trimethyl pyridine (0.17 ml, 1.5 mmol) and DAST (0.19 ml, 1.5 mmol) according to the general fluorination procedure. The product was isolated in the usual manner and purified by column chromatography on silica gel (10% EtOAc/CH₂Cl₂), then crystallized from methanol and isolated as a white powder (0.10 g, 40%). M.p. 123-125 °C (lit. 125-126 °C); ¹H nmr (300 MHz, CDCl₃): δ 5.73 (d, 1 H, J₁,₂ 7.6 Hz, H(1)), 5.27 (t, 1 H, J₃,₂ 9.6, J₃,₄ 9.6 Hz, H(3)), 5.20-5.10 (m, 2 H, H(4)+H(2)), 4.49 (ddd, 1 H, J₆a,F 47.0, J₆a,₆b 10.6, J₆a,₅ 2.4 Hz, H(6a)), 4.45 (ddd, 1 H, J₆b,F 47.0, J₆b,₆a 10.6, J₆b,₅ 4.1 Hz, H(6b)), 4.10 (dddd, 1 H, J₅,F 22.1, J₅,₄ 8.0, J₅,₆b 4.1, J₅,₆a 2.4 Hz, H(5)), 2.11, 2.06, 2.04, 2.01 (4 x s, 12 H, 4 x OAc).

2,3,4,-Tri-O-acetyl-6-deoxy-6-fluoro-D-glucopyranose (5)

The tetra-O-acetate (4) (0.050 g, 0.14 mmol) was treated with ethanolamine (11 μl, 0.18 mmol) in 3 ml of ethyl acetate according to the usual selective deacetylation procedure. The reaction was stirred at room temperature for 48 h, purified as usual and the product isolated as a clear oil (32 mg, 73%). ¹H nmr (300 MHz, CDCl₃): δ α-anomer 5.57 (t, 1 H, J₄,₃ 9.1, J₄,₅ 9.1 Hz, H(4)), 5.50 (t, 1 H, J₁,₂ 3.2 J₁,OH 3.2 Hz, H(1)), 5.07 (t, 1 H, J₃,₄ 9.1, J₃,₅ 9.1 Hz, H(3)), 4.90 (dd, 1 H, J₂,₃ 9.1, J₂,₃ 3.4 Hz, H(2)), 4.47 (dd, 2 H, J₆,F 4.6, J₆,₅ 3.2 Hz, H(6)), 4.18 (ddt, 1 H, J₅,F 23, J₅,₄ 9.1, J₅,₆ 3.2 Hz, H(5)), 3.30 (d, 1 H, J₀H,₁ 3.2 Hz, H(OH)), 2.10, 2.07, 2.05 (3 x s, 9 H, OAc). β-anomer 5.57 (t, 1 H, J₄,₃ 9.1, J₄,₅ 9.1 Hz, H(4)), 5.29 (t, 1 H, J₃,₂ 9.1, J₃,₄ 9.1 Hz, H(3)), 5.08 (t, 1 H, J₄,₃ 9.1, J₄,₅ 9.1 Hz, H(4)), 4.88 (d, 1 H, J₁,₂ 9.1 Hz, H(1)), 4.77
2',4'-Dinitrophenyl 2,3,4-tri-O-acetyl-6-deoxy-6-fluoro-β-D-glucopyranoside (6)

The hemiacetal (5) (0.260 g, 0.84 mmol) was dissolved in 10 ml of dry DMF and treated with FDNB (0.20 g, 1.2 mmol) and DABCO (0.33 g, 1.2 mmol) using the usual procedure. The reaction mixture was filtered to remove the sieves, then evaporated to dryness in vacuo and worked up in the usual manner. The product was obtained as a clear oil which solidified on trituration in ethanol and was crystallized from ethanol to give a white, crystalline solid (0.165 g, 41%). M.p. 187-189 °C; $^1$H nmr (300 MHz, CDCl₃): δ 8.72 (d, 1 H, $J_{3',5'}$ 3 Hz, H(3')), 8.45 (dd, 1 H, $J_{5',2'}$ 3, $J_{5',6'}$ 9 Hz, H(5')), 7.51 (d, 1 H, $J_{6',5'}$ 9 Hz, H(6')), 5.40-5.10 (m, 4 H, H(1)+H(2)+H(3)+H(4')), 4.50 (dd, 2 H, $J_{6,F}$ 46.5, $J_{6,F}$ 4.2 Hz, H(6')), 4.09-3.95 (m, 1 H, H(5')), 2.13, 2.10, 2.06 (3 x s, 9 H, 3 x OAc). $^{19}$F nmr (188.3 MHz, D₂O): δ -230.30. Anal. calc. for C₁₈H₁₉O₁₂N₂F: C, 45.58%; H, 4.04%; N, 5.91%; Found: C, 45.31%; H, 4.20%; N, 6.00%.

2',4'-Dinitrophenyl 6-deoxy-6-fluoro-β-D-glucopyranoside (7)

The acetylated glucoside (6) (0.120 g, 0.25 mmol) was deprotected using the general HCl/methanol procedure. The reaction was stirred for 4 h at room temperature until clear then stirred 24 h at 4 °C. In this case another 10 h of stirring at room temperature was required for complete reaction. Work up by the usual procedure yielded the product as a slightly yellow gum which solidified after trituration in a mixture of diethyl ether and hexanes. The product was recrystallized from a mixture of methanol, diethyl ether and hexanes to yield the product as white crystals (0.035 g, 40%). M.p.
Methyl 2,3,6-tri-O-benzoyl-α-D-galactopyranoside (8)

Methyl α-D-galactopyranoside (10 g, 51 mmol) was dissolved in 100 ml of dry pyridine and cooled to 0 °C. Benzoyl chloride (20.6 ml, 177 mmol) was added dropwise with stirring after which the reaction was allowed to slowly warm to room temperature, stirred overnight, then allowed to stand for 3 days at room temperature. The reaction was cooled to 0 °C and treated with 300 ml of ice cold saturated sodium bicarbonate and stirred until evolution of CO₂ had ceased. The solution was then extracted with CHCl₃, and the organic layer washed with water then dried over MgSO₄. The solvent was evaporated in vacuo to yield a clear oil which was crystallized from methanol to yield the product as white crystals (8.5 g, 33%). M.p. 133-135 °C (lit. 68-132-136 °C); ¹H nmr (300 MHz, CDCl₃): δ 8.08-7.97 (m, 6 H, phenyl (H)), 7.62-7.34 (m, 9 H, phenyl (H)), 5.77 (dd, 1 H, J₃,₂ 10.3, J₃,₄ 2.5 Hz, H(3)), 5.69 (dd, 1 H, J₂,₃ 10.3, J₂,₁ 3.6 Hz, H(2)), 5.22 (d, 1 H, J₁,₂ 3.6 Hz, H(1)), 4.69 (dd, 1 H, J₆a,₆b 11.4, J₆a,₅ 6.2 Hz, H(6a)), 4.57 (dd, 1 H, J₆b,₆a 11.4, J₆b,₅ 6.8 Hz, H(6b)), 4.42 (s, 1 H, H(4)), 4.36 (dd, 1 H, J₅,₆b 6.8, J₅,₆a 6.2 Hz, H(5)), 3.47 (s, 3 H, OCH₃).

Methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-fluoro-α-D-glucopyranoside (9)

The tri-O-benzoate (8) (7 g, 13.8 mmol) was dissolved in 10 ml of dry CH₂Cl₂ and treated with 4-dimethylaminopyridine (3.43 g, 28.2 mmol). The solution was
cooled to -23 °C and treated with DAST (3.6 ml, 32 mmol) dissolved in 10 ml of dry CH₂Cl₂ using the usual procedure. The reaction was stirred overnight, quenched and the solvent was evaporated in vacuo to yield a black oil. The oil was re-dissolved in a minimum amount of CH₂Cl₂ and excess methanol added. Upon cooling the product crystallized and was isolated as off white crystals (3.22 g, 46%) which were recrystallized from ethanol to give the product as colorless crystals. M.p. 137-138.5 °C (lit. 138-139 °C); ¹H nmr (300 MHz, CDCl₃): δ 8.13-7.96 (m, 6 H, phenyl (H)), 7.63-7.35 (m, 9 H, phenyl (H)), 6.11 (dt, 1 H, J₃,F 15, J₃,5 10, J₃,2 10 Hz, H(3)), 5.22 (dd, 1 H, J₂,3 10, J₂,1 3 Hz, H(2)), 5.18 (d, 1 H, J₁,2 3 Hz, H(1)), 4.78 (dt, 1 H, J₄,F 52, J₄,3 10, J₄,5 10 Hz, H(4)), 4.66 (m, 1 H, H(5)), 3.48 (s, 3 H, OCH₃).

*Methyl 4-deoxy-4-fluoro-α-D-glucopyranoside (10)*

The per-O-benzoylated glucoside (11) (2.2 g, 4.31 mmol) was suspended in 10 ml of dry methanol and treated with 34 mg of sodium metal using the usual methoxide deprotection procedure. The reaction was complete after 18 h at which time it was quenched by addition of Dowex 50W (H⁺) resin, filtered and the solvent evaporated in vacuo to yield the product as a colorless oil which was carried directly to the next step without further treatment.

*4-deoxy-4-fluoro-D-glucose (11)*

The methyl glycoside (10) was dissolved in 75 ml of water and treated with 40 ml of Dowex 50W (H⁺) resin. The reaction was stirred at reflux for 28 h, then filtered to remove the resin. The solvent was evaporated in vacuo to yield the product as a clear oil (0.455 g, 58% from 9). The product can be isolated as clear crystals by dissolving the oil in a minimum of methanol, cooling to 0 °C and addition of diethyl ether. This was
usually not done if the compound was being used for further synthesis. M.p. 177-180 °C (lit.\textsuperscript{46} 184-186 °C); \textsuperscript{1}H nmr (300 MHz, D\textsubscript{2}O): δ α-anomer 5.20 (t, 1 H, J\textsubscript{1,2} 3.1 Hz, H(1)), 4.30 (dt, 1 H, J\textsubscript{4,F} 50.6, J\textsubscript{4,5} 9.4, J\textsubscript{4,5} 9.4 Hz, H(4)), 4.04-3.62 (m, 4 H, H(3)+H(5)+H(6)), 3.54 (dd, 1 H, J\textsubscript{2,3} 9.6, J\textsubscript{2,1} 3.1 Hz, H(2)). β-anomer 4.66 (d, 1 H, J\textsubscript{1,2} 8.4 Hz, H(1)), 4.30 (dt, 1 H, J\textsubscript{4,F} 50.6, J\textsubscript{4,3} 9.4, J\textsubscript{4,5} 9.4 Hz, H(4)), 4.04-3.62 (m, 4 H, H(3)+H(5)+H(6)), 3.26 (dd, 1 H, J\textsubscript{2,3} 9.4, J\textsubscript{2,3} 8.4 Hz, H(2)).

\textit{1,2,3,6-Tetra-O-acetyl-4-deoxy-4-fluoro-D-glucopyranose (12)}

The non-crystalline sugar (11) (300 mg, 1.6 mmol) was treated with acetic anhydride (15 ml) and sodium acetate (300 mg), then stirred at 110 °C until the reaction was complete (45 min). The reaction mixture was cooled, then slowly poured into ice cold saturated sodium bicarbonate, extracted with CHCl\textsubscript{3} and the solvent was removed \textit{in vacuo} to give the product as a clear oil (225 mg, 72%). The β-anomer can be selectively crystallized from diethyl ether and pentanes (M.p. 123-126 °C (lit.\textsuperscript{46} 126-128 °C)), however for synthetic purposes the α- and β-anomers of the per-O-acetylated glycopyranoses were carried to the next step in the synthesis without further purification.

\textit{2,3,6-Tri-O-acetyl-4-deoxy-4-fluoro-D-glucopyranose (13)}

The per-O-acetylated glucoside (12) (20 mg, 0.058 mmol) was dissolved in 2 ml of ethyl acetate and treated with ethanolamine (4 μl, 0.086 mmol) using the usual procedure. The reaction was complete after 48 h. The product was worked up as usual and isolated as a clear oil (17 mg, 88%). \textsuperscript{1}H nmr (300 MHz, CDCl\textsubscript{3}): δ α-anomer 5.73 (m, 1 H, H(3)), 5.42 (d, 1 H, J\textsubscript{1,2} 3.3 Hz, H(1)), 4.84 (dd, 1 H, J\textsubscript{2,3} 10.2, J\textsubscript{2,1} 3.3 Hz, H(2)), 4.47 (dt, 1 H, J\textsubscript{4,F} 50.4, J\textsubscript{4,3} 9.3, J\textsubscript{4,5} 9.3 Hz, H(4)), 4.46-4.22 (m, 3 H, H(3)).
3.80 (m, 1 H, H(5)), 3.41 (d, 1 H, J_{OH,1} 3.3 Hz, H(OH)), 2.13, 2.11, 2.10 (3 x s, 3 H, 3 x OAc).

2',4'-Dinitrophenyl 2,3,6-tri-O-acetyl-4-deoxy-4-fluoro-β-D-glucopyranoside (14)

The hemiacetal (13) (173 mg, 0.56 mmol) was dissolved in 7 ml of dry DMF and treated with DABCO (0.22 g, 2.0 mmol) and FDNB (0.13 g, 0.7 mmol) (dissolved in 3 ml of DMF) using the general procedure. The reaction was stirred for 3 h, then worked up as usual, the product being isolated as white crystals after trituration of the resulting oil in ethanol and recrystallization from the same solvent (85 mg, 32%). M.p. 161-163 °C (lit. 171-173 °C); \(^1\)H nmr (300 MHz, CDCl\(_3\)): \(\delta\) 8.71 (d, 1 H, J_{3',5'} 3.3 Hz, H(3')), 8.44 (dd, 1 H, J_{5',6'} 9.0, J_{5',3'} 3.3 Hz, H(5')), 7.47 (d, 1 H, J_{6',5'} 9.0 Hz, H(6')), 5.43 (dt, 1 H, J_{3}F 15.8, J_{3}2 9.0, J_{3}4 9.0 Hz, H(3)), 5.35-5.25 (m, 2 H, H(1)+H(2)), 4.68 (dt, 1 H, J_{4,F} 50.1, J_{4,3} 9.0, J_{4,5} 9.0 Hz, H(4)), 4.55 (m, 1 H, H(6a)), 4.28 (ddd, 1 H, J_{6b,6a} 12.7, J_{6b,5} 5.5, J_{6b,F} 1.6 Hz, H(6b)), 4.06-3.97 (m, 1 H, H(5)), 2.16, 2.08 (2 x s, 9 H, 3 x OAc). \(^19\)F nmr (188.3 MHz, D\(_2\)O): \(\delta\) -199.54. Anal. calc. for C\(_{18}\)H\(_{19}\)N\(_2\)O\(_{12}\)F: C, 45.58; H, 4.13; N, 5.91. Found: C, 45.43; H, 4.13; N, 5.78.

2',4'-Dinitrophenyl 4-deoxy-4-fluoro-β-D-glucopyranoside (15)

The per-O-acetylated glucoside (14) (80 mg, 0.17 mmol) was suspended in 5 ml of dry methanol and treated with acetyl chloride (0.4 ml, 0.5 mmol) as outlined in the general deacetylation procedure. The reaction was complete after being stirred 23 h and the usual workup yielded the product as a clear gum which was crystallized from a mixture of acetone, dichloromethane and hexanes to give the product as a white powder (16 mg, 27%). M.p. 108-111°C (dec); \(^1\)H nmr (300 MHz, CD\(_3\)OD): \(\delta\) 8.72 (d, 1 H, J_{3',5'} 3.3 Hz, H(3')), 8.48 (dd, 1 H, J_{5',6'} 9.0, J_{5',3'} 3.3 Hz, H(5')), 7.62 (d, 1 H, J_{6',5'} 3.3 Hz, H(5')).
9.0 Hz, H(6'), 5.30 (d, 1 H, J₁₂ 7.5 Hz, H(1)), 4.37 (dt, 1 H, J₄,₂F 51.4, J₄,₃ 8.3, J₄,₅
8.3 Hz, H(4)), 3.95-3.60 (m, 4 H, H(2)+H(3)+H(6)). Anal. calc. for C₁₂H₁₃O₉N₂F +
2H₂O: C, 37.50; H, 4.45; N, 7.29. Found: C, 37.56; H, 4.17; N, 7.08.

2,4,6-Tri-O-acetyl-3-deoxy-3-fluoro-D-glucopyranose (16)

1,2,4,6-Tetra-O-acetyl-3-deoxy-3-fluoro-β-D-glucopyranose (46) (22 mg, 0.063
mmol) was dissolved in 2 ml of ethyl acetate and treated with ethanolamine (4 µl, 0.066
mmol) using the usual procedure. The reaction was complete after 24 h and was worked
up in the usual manner to yield the product as a clear oil (19 mg, 88%). ¹H nmr (300
MHz, CDCl₃): δ α-anomer 5.50 (br s, 1 H, H(1)), 5.23 (m, 1 H, H(2)), 5.04-4.92 (m,
1.5 H, H(4)+1/2 H(3)), 4.81 (t, 0.5 H, J₃,₂ 8.7, J₃,₄ 8.7 Hz, 1/2 H(3)), 4.26-4.08 (m, 3
H, H(5)+H(6)), 2.18, 2.14, 2.10 (3 x s, 9 H, 3 x OAc).

2',4'-Dinitropheryl 2,4,6-tri-O-acetyl-3-deoxy-3-fluoro-β-D-glucopyranoside (17)

The hemiacetal (16) (178 mg, 0.57 mmol) was dissolved in 7 ml of dry DMF and
treated with DABCO (22 mg, 2.0 mmol) and FDNB (13 mg, 0.7 mmol) dissolved in 3 ml
of dry DMF as described in the general procedure. The reaction was worked up as usual
to give the product as a yellowish oil which solidified on trituration in ethanol. The
product was re-crystallized from the same solvent to yield white crystals (82 mg, 30%).
M.p. 150-153 °C; ¹H nmr (300 MHz, CDCl₃): δ 8.72 (d, 1 H, J₃',₅' 3.3 Hz, H(3')),
8.48 (dd, 1 H, J₅',₆' 9.0, J₅',₃' 3.3 Hz, H(5')) , 7.62 (d, 1 H, J₆',₅' 9.0 Hz, H(6')) , 5.51
(ddd, 1 H, J₂₂,₃ 13.0, J₂₂,₃ 9.2, J₂₂,₁ 7.8 Hz, H(2)), 5.34 (ddd, 1 H, J₄₃,₁ 12.3, J₄₃,₅ 9.7,
J₄₃,₅ 9.2 Hz, H(4)), 5.20 (d, 1 H, J₁₂,₂ 7.8 Hz, H(1)), 4.69 (dt, 1 H, J₃₃,₁ 52.0, J₃₃,₂ 9.2,
J₃₃,₅ 9.2 Hz, H(3)), 4.28 (d, 2 H, J₆₅,₂ 4.0 Hz, H(6)), 3.86 (m, 1 H, H(5)), 2.20, 2.15,
2.11 (3 x s, 9 H, 3 x OAc). $^{19}$F nmr (188.3 MHz, D$_2$O): $\delta$ -196.66. Anal. calc. for C$_{18}$H$_{19}$N$_2$O$_{12}$F: C, 45.58; H, 4.04; N, 5.91. Found: C, 45.36; H, 4.04; N, 6.09.

2',4'-Dinitrophenyl 3-deoxy-3-fluoro-\(\alpha\)-D-glucopyranoside (18)

The tri-O-acetate (17) (90 mg, 0.19 mmol) was suspended in 7 ml of dry methanol and treated with acetyl chloride (0.5 ml, 0.63 mmol) as described in the general procedure. The reaction was complete after being stirred overnight. The product was obtained as a clear oil which crystallized upon trituration in a mixture of CH$_2$Cl$_2$ and hexanes (33 mg, 50%) and was recrystallized from a mixture of methanol, dichloromethane and hexanes. M.p. 147-149 °C; $^1$H nmr (300 MHz, CD$_3$OD): $\delta$ 8.72 (d, 1 H, J$_{3',5'}$, 3.3 Hz, H(3')), 8.48 (dd, 1 H, J$_{5',6'}$, 9.0, J$_{5',3'}$, 3.3 Hz, H(5')), 7.62 (d, 1 H, J$_{6',5'}$, 9.0 Hz, H(6')), 5.33 (d, 1 H, J$_{1,2}$, 7.8 Hz, H(1)), 4.40 (dt, 1 H, J$_{3,F}$, 53.0, J$_{3,2}$, 9.0, J$_{3,4}$, 9.0 Hz, H(3)), 4.10-3.50 (m, 5 H, H(2)+H(4)+H(5)+H(6)). Anal. calc. for C$_{12}$H$_{13}$O$_9$N$_2$F + 0.75 H$_2$O: C, 39.84; H, 4.04; N, 7.74. Found: C, 39.54; H, 3.79; N, 7.77.

3,4,6-Tri-O-acetyl-2-deoxy-2-fluoro-\(\alpha\)-D-glucopyranosyl bromide (19)

Trifluoromethyl 3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-\(\alpha\)-D-glucopyranoside (151) (2.0 g, 5.3 mmol) was dissolved in 20 ml of 48% HBr/acetic acid and stirred for 6 days at room temperature. The reaction was then poured into ice water, stirred for 5 min, extracted with CHCl$_3$ and the organic layer washed extensively with cold saturated sodium bicarbonate and water and then dried over MgSO$_4$. The solvent was evaporated in vacuo to yield the product as a clear oil which was carried to the next step without further purification or characterization (1.4 g, 71%).
3,4,6-Tri-O-acetyl-2-deoxy-2-fluoro-D-glucose (20)

The bromide (19) (1.4 g, 3.8 mmol) was dissolved in 6 ml of acetone and 4 drops of water and was treated with Ag₂CO₃ (0.85 g, 3.1 mmol) at 0 °C. The reaction was complete after 45 min. The reaction mixture was diluted with EtOAc, then filtered through Celite to remove the insoluble silver salts. The solvent was evaporated in vacuo to yield the product as a clear oil which was carried to the next step in the synthesis without further purification or characterization.

2',4'-Dinitrophenyl 3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-0-D-glucopyranoside (21)

The hemiacetal (20) (~1.1 g, 3.8 mmol) was dissolved in 10 ml of dry DMF and treated with DABCO (0.15 g, 13.3 mmol) and with FDNB (0.9 g, 4.9 mmol) dissolved in 5 ml of dry DMF according to the usual glycoside formation procedure. The reaction was stirred at room temperature for 1.5 h and worked up in the usual manner to give the product as a slightly yellow oil (0.99 g, 53% from the trifluoromethyl glucoside). The product was approximately 80% the β-glycoside by 1H nmr and the α- and β-glucosides were almost indistinguishable on tlc. The α-anomer of the glucoside could be selectively crystallized by dissolving the mixture in a minimum volume of boiling EtOAc and allowing the solution to cool slowly to room temperature. This was filtered and excess petroleum ether added to the filtrate to precipitate the remaining compound as a white solid which was about 95% the β-anomer. The mixture was further purified by preparative thin layer chromatography (3:2 EtOAc/petroleum ether). The silica gel containing the β-anomer was extracted with EtOAc to yield the product as a clear oil after the solvent was evaporated in vacuo. The product was then crystallized from ethyl acetate/petroleum ether and recrystallized from ethanol to give the β-glucoside as white crystals. M.p. 150-152 °C (lit.59 145-147 °C ); 1H nmr (300 MHz, CDCl₃): δ 8.77 (d,
1 H, J3',5' 3.3 Hz, H(3') ), 8.45 (dd, 1 H, J5',6' 9.0, J5',3' 3.3 Hz, H(5') ), 7.41 (d, 1 H, J6',5' 9.0 Hz, H(6') ), 5.50-5.39 (m, 2 H, H(1)+H(3) ), 5.15 (t, 1 H, J4,3 9.5, J4,5 9.5 Hz, H(4) ), 4.28 (dd, 1 H, J6a,6b 12.2, J6a,5 5.4 Hz, H(6a) ), 4.20 (dd, 1 H, J6b,6a 12.2, J6b,5 2.5 Hz, H(6b) ), 3.99 (ddd, 1 H, J5,4 9.5, J5,6a 5.4, J5,6b 2.5 Hz, H(5) ), 2.14, 2.08 (2 x s, 9 H, 3 x OAc).

$^{19}$F nmr (188.3 MHz, D$_2$O): δ -198.98. Anal. calc. for C$_{18}$H$_{19}$N$_2$O$_{12}$F: C, 45.58; H, 4.04; N, 5.91. Found: C, 45.70; H, 4.03; N, 5.89.

**2',4'-Dinitrophenyl 2-deoxy-2-fluoro-β-D-glucopyranoside (22)**

The tri-O-acetate (21) (70 mg, 0.15 mmol) was suspended in 15 ml of dry methanol and treated with 1 ml (1.26 mmol) of acetyl chloride according to the general deacetylation procedure. The reaction was stirred for 24 h at 4 °C at which time it was worked up in the usual manner to yield the product as a clear gum which was crystallized from a mixture of acetone, dichloromethane and hexanes to yield the product as a white solid (50 mg, 45%). M.p. 104-106 °C (lit. 6 123-126 °C); $^1$H nmr (300 MHz, CD$_3$OD): δ 8.73 (d, 1 H, J3',5' 3.3 Hz, H(3') ), 8.29 (dd, 1 H, J5',6' 9.0, J5',3' 3.3 Hz, H(5') ), 7.57 (d, 1 H, J6',5' 9.0 Hz, H(6') ), 5.57 (dd, 1 H, J1,2 7.8, J1,3' 4.2 Hz, H(1) ), 4.34 (ddd, 1 H, J2,3 51.8, J2,3 9.0, J2,1 7.8 Hz, H(2) ), 3.93 (dd, 1 H, J6a,6b 12.2, J6a,5 2 Hz, H(6a) ), 3.82-3.67 (m, 2 H, H(3)+H(6b) ), 3.66-3.59 (m, 1 H, H(5) ), 3.46 (t, 1 H, J4,3 9.5, J4,5 9.5 Hz, H(4) ). Anal. calc. for C$_{18}$H$_{19}$N$_2$O$_{12}$F + 0.6 acetone + 0.4 mol H$_2$O: C, 42.46; H, 4.49; N, 7.12. Found: C, 42.57; H, 4.49; N, 6.85.

**2',4'-Dinitrophenyl 6-deoxy-β-D-glucopyranoside (23)**

2',4'-Dinitrophenyl 2,3,4-tri-O-acetyl-6-deoxy-β-D-glucopyranoside (59) (150 mg, 0.33 mmol) was suspended in 10 ml of dry methanol and treated with acetyl chloride (0.79 ml, 1.0 mmol) according to the usual procedure. The reaction was stirred at room
temperature for 3 h and at 4 °C for an additional 44 h. The reaction was worked up as usual to give the product as a clear oil which was crystallized from a mixture of acetone, dichloromethane and hexanes to yield the product as a white crystals (70 mg, 65%).

M.p. 113-116 °C; $^1$H nmr (300 MHz, Acetone-D$_6$): $\delta$ 8.73 (d, 1 H, J$^{3',5'}$ 3.3 Hz, H(3')) , 8.50 (dd, 1 H, J$^{5',6'}$ 9.0, J$^{5',3'}$ 3.3 Hz, H(5')) , 7.57 (d, 1 H, J$^{6',5'}$ 9.0 Hz, H(6')) , 5.40 (d, 1 H, J$^{1,2}$ 7.1 Hz, H(1)) , 3.80-3.65 (m, 1 H, H(5)) , 3.65-3.47 (m, 2 H, H(2)+H(3)) , 3.19 (t, 1 H, J$^{4,3}$ 8.9, J$^{4,5}$ 8.9 Hz, H(4)) , 1.30 (d, 3 H, J$^{6,5}$ 7 Hz, H(6)).

Anal. calc. for C$_{12}$H$_{11}$O$_9$N$_2$ + 1 H$_2$O: C, 41.38; H, 4.63; N, 8.04. Found: C, 41.46; H, 4.63; N, 7.70.

*Methyl 4-deoxy-\(\alpha\)-D-xylo-hexopyranoside* (24)

Methyl 2,3,6-tri-O-benzoyl-4-deoxy-\(\alpha\)-D-xylo-hexopyranoside (108) (1.2 g, 2.2 mmol) was suspended in 8 ml of dry methanol, cooled to 0 °C and treated with sodium metal (18 mg, 0.78 mmol). After the sodium had dissolved the reaction was allowed to slowly warm to room temperature, stirred a further 19 h then quenched by addition of Dowex 50W (H$^+$) resin, filtered and the solvent evaporated *in vacuo* to yield the product as a clear oil. It was carried to the next step without further purification or characterization.

*4-Deoxy-D-xylo-hexopyranose* (25)

The methyl glucoside (24) was dissolved in 40 ml of water and 20 ml of Dowex 50W (H$^+$) resin added. The reaction mixture was stirred at reflux for 1.5 h, filtered, the resin was washed repeatedly with methanol and combined filtrates concentrated *in vacuo* to give the product as an oil. The product was used in the next step of the synthesis without further purification. $^1$H nmr (300 MHz, CD$_3$OD): $\delta$ 5.17 (d, 0.4 H, J$^{1,2}$ 4.5
Hz, H(1α), 4.43 (d, 0.6 H, J1,2 7.5 Hz, H(1β)), 4.10-3.84 (m, 1 H, H(3)), 3.70-3.28
(m, 3.4 H, H(2α)+H(5)+H(6)), 3.05 (t, 0.6 H, J2,1 7.5, J2,3 7.5 Hz, H(2β)), 1.92 (dd,
1 H, J4e,4a 14, H4e,3 5 Hz, H(4e)), 1.38 (dd, 1 H, J4a,4e 14, J4a,3 10 Hz, H(4a)).

1,2,3,6-Tetra-O-acetyl-4-deoxy-D-xylo-hexopyranose (26)

The product (25) was treated with 1.8 ml of acetic anhydride in 2.5 ml of dry
pyridine according to the general acetylation procedure. After 24 h the solvent was
evaporated in vacuo, the product redissolved in CHCl3, washed successively with
saturated sodium bicarbonate and water, then dried over MgSO4. The solvent was
evaporated in vacuo to yield an anomeric mixture of products as a clear oil (0.56 g, 72%
from the methyl tri-O-benzoyl glycoside). 1H nmr (300 MHz, CDCl3): δ 6.35 (d, 0.4
H, J1,2 3.5 Hz, H(1α)), 5.61 (d, 0.6 H, J1,2 8.0 Hz, H(1β)), 5.30 (m, 0.4 H,
H(3α)), 5.10-5.00 (m, 1.6 H, H(2)+H(3β)), 4.30-4.10 (m, 2.4 H, H(5α)+H(6)), 3.98-
3.88 (m, 0.6 H, H(5β)), 2.20-2.02 (m, 13 H, 4 x OAc+H(4e)) 1.78-1.60 (m, 1 H,
H(4a)).

2,3,6-Tri-O-acetyl-4-deoxy-D-xylo-hexopyranose (27)

The per-O-acetate (26) (0.56 g, 1.7 mmol) was dissolved in 10 ml of ethyl acetate
and treated with ethanolamine (200 μl, 3.3mmol) according to the general procedure. An
additional 200 μl of ethanolamine was required for complete reaction after about 4 days
at room temperature. The reaction was worked up in the usual manner to give the
product as a clear oil (0.34 g, 70 %) (mixture of α- and β-anomers). 1H nmr (300 MHz,
CDCl3): δ α-anomer 5.46 (br t, 1 H, J1,2 4, J1,OH 4 Hz, H(1)), 5.37 (ddd, 1 H, J3,4a
12, J3,2 10.8, J3,4e 5.7 Hz, H(3)), 4.89 (dd, 1 H, J2,3 10.8, J2,1 3.6 Hz, H(2)), 4.40-
4.30 (m, 1 H, H(5)), 4.20-4.07 (m, 2 H, H(6)), 2.30-2.05 (m, 10 H, 3 x OAc+H(4e)), 1.68-1.53 (m, 1 H, H(4a)).

2',4'-Dinitrophenyl 2,3,6-tri-O-acetyl-4-deoxy-β-D-xylo-hexopyranoside (28)

The hemiacetal (27) (0.338 g, 0.74 mmol) was dissolved in 10 ml of dry DMF and treated with DABCO (440 mg, 3.90 mmol) and FDNB (0.26 g, 1.55 mmol) using the usual procedure for glycoside formation. The reaction was stirred at room temperature for 40 min, worked up in the usual way and the product isolated as an oil which solidified on trituration in ethanol. The product was recrystallized from ethyl acetate/petroleum ether (214 mg, 40%). M.p. 166-170 °C (lit. 180-181 °C); 1H nmr (300 MHz, CDCl3): δ 8.70 (d, 1 H, J3',5' 3.3 Hz, H(3')) , 8.42 (dd, 1 H, J5',6' 9.0, J5',3' 3.3 Hz, H(5')) , 7.48 (d, 1 H, J6',5' 9.0 Hz, H(6')) , 5.28 (t, 1 H, J2,1 8.5, J2,3 8.5 Hz, H(2)) , 5.21 (d, 1 H, J1,2 8.5 Hz, H(1)) , 5.15-5.05 (m, 1 H, H(3)) , 4.22 (d, 2 H, J6,5 5.5 Hz, H(6)) , 4.09-3.98 (m, 9 H, 3 x OAc), 1.79 (dt, 1 H, J4a,4e 13, J4a,3 11, J4a,5 11 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.

2',4'-Dinitrophenyl 4-deoxy-β-D-xylo-hexopyranoside (29)

The tri-O-acetate (28) (90 mg, 0.20 mmol) was suspended in 6 ml of dry methanol, cooled to 0 °C and treated with acetyl chloride (0.45 ml, 0.57 mmol) using the usual procedure. The reaction was stirred until clear at 0 °C then overnight at 4 °C and worked up in the usual way to give the product as a clear oil which was crystallized from dichloromethane, diethyl ether and petroleum ether (25 mg, 43%). M.p. 76-79 °C. 1H nmr (400 MHz, Acetone-D6): δ 8.70 (d, 1 H, J3',5' 3 Hz, H(3')) , 8.50 (dd, 1 H, J5',6' 9, J5',3' 3 Hz, H(5')) , 7.45 (d, 1 H, J6',5' 9 Hz, H(6')) , 5.30 (d, 1 H, J1,2 8 Hz, H(1)),
4.10-3.00 (m, 5 H, H(2)+H(3)+H(5)+H(6a,6b) ), 2.00 (ddd, 1 H, J4e,4a13, J4e,3 6 Hz, J4e,5 3 Hz, H(4e) ), 1.55 (dt, 1 H, J4a,4e13, J4a,3 11, J4a,5 11 Hz, H(4a) ). Anal. calc.

C12H14O9N2 + 2H2O; C, 39.35; H, 4.95; N, 7.65; Found: C, 39.68; H, 4.78; N, 5.88.

1,2:5,6-Di-O-isopropylidene-α-D-glucofuran-3-ulose (30) (61)

To freshly distilled DMSO (20 ml) cooled in an ice bath was added phosphorus pentoxide (2 g, 14.1 mmol) then the solution allowed to warm to room temperature and 1,2:5,6-di-O-isopropylidene-α-D-glucofuranose (Aldrich Chemical Co.) (2.0 g, 7.7 mmol) added and stirred overnight under nitrogen. The reaction was diluted in CHCl3, washed with saturated sodium bicarbonate, water, then dried over MgSO4. The solvent was evaporated in vacuo to yield the product as a yellow oil which solidified on addition of petroleum ether (1.0 g, 50%). The product was carried directly to the next step in the synthesis.

1,2:5,6-Di-O-isopropylidene-α-D-allofuranose (31) (62)

The 3-keto compound (30) (1 g, 3.85 mmol) was suspended in 15 ml of 98% ethanol, cooled to 0 °C then treated with sodium borohydride (120 mg, 3.2 mmol). The solution was stirred for 10 min at 0 °C then at room temperature for 1 h. The solvent was then evaporated in vacuo to give a clear syrup which was redissolved in 1:2 water/CHCl3. The aqueous layer was extracted twice with CHCl3 and the combined extracts washed with water and the solvent evaporated in vacuo to give an oil which was crystallized from benzene and petroleum ether to give (30) as clear crystals (380 mg, 40%). M.p. 76-78 °C (lit. 62 77-78 °C). The product was carried to the next step in the synthesis without further purification or characterization.
1,2:5,6-Di-O-isopropylidene-3-chloro-3-deoxy-α-D-glucofuranose (32) (108)

The protected allose (31) (1 g, 3.8 mmol) was dissolved in 10 ml of dry pyridine, cooled to 0 °C and treated dropwise with SO$_2$Cl$_2$ (0.5 ml, 5 mmol). The solution was allowed to slowly warm to room temperature, stirred overnight, then quenched by addition of water and the solvent evaporated in vacuo to give a black tar which was re-dissolved in CH$_2$Cl$_2$, washed repeatedly with water, then dried over MgSO$_4$. The product was purified by column chromatography (9:1 CH$_2$Cl$_2$/EtOAc) and isolated as a clear semi-solid (0.5 g, 47%). $^1$H nmr (300 MHz, CDCl$_3$): δ 5.83 (d, 1 H, J$_{1,2}$ 3.5 Hz, H(1)), 4.71 (d, 1 H, J$_{2,1}$ 3.5 Hz, H(2)), 4.40 (d, 1 H, J$_{3,4}$ 3.0 Hz, H(3)), 4.33 (m, 1 H, H(5)), 4.15 (dd, 1 H, J$_{4,5}$ 8.5, J$_{4,3}$ 3.0 Hz, H(4)), 4.12 (dd, 1 H, J$_{6a,6b}$ 8.9, J$_{6a,5}$ 8.5 Hz, H(6a)), 4.06 (dd, 1 H, J$_{6b,6a}$ 8.9, J$_{6b,5}$ 4.5 Hz, H(6b)), 1.50, 1.41, 1.35, 1.31 (4 x s, 12 H, 4 x CH$_3$)

1,2,4,6-Tetra-O-acetyl-3-chloro-3-deoxy-D-glucopyranose (33)

The chloro sugar (32) (0.5 g, 1.8 mmol) was dissolved in 20 ml of ethanol and 40 ml of water, then treated with 4 ml of Amberlite IR-120 (H$^+$) resin and heated to 60 °C for 24 h. The resin was removed by filtration, washed thoroughly with methanol and the solvent evaporated in vacuo to give 3-chloro-3-deoxy-D-glucopyranose as a clear oil. The oil was dried overnight in vacuo, dissolved in 5 ml of dry pyridine plus 2 ml of acetic anhydride and stirred overnight at room temperature. The solvent was evaporated in vacuo and the resulting oil redissolved in CHCl$_3$, washed with dilute HCl and water then dried over MgSO$_4$. The product was further purified by column chromatography (1:1 EtOAc/hexanes) and was isolated as a colorless semi-solid which was found to be a mixture of α- and β-anomers (0.343 g, 52%). $^1$H nmr (300 MHz, CDCl$_3$): δ 6.30 (d, 0.5 H, J$_{1,2}$ 4 Hz, H(1α)), 5.68 (d, 0.5 H, J$_{1,2}$ 8.5 Hz, H(1β)), 5.40-5.12 (m, 2 H, α+β
H(2), H(4), 4.32-4.06 (m, 3.5 H, α+β H(4), H(6), H(5α)), 3.85 (m, 0.5 H, H(5β)), 2.19-2.06 (multiple s, 12 H, OAc).

1,2,4,6-Tetra-O-acetyl-3-deoxy-D-ribo-hexopyranose (34) (64)

The protected chloro sugar (33) (0.34 g, 0.93 mmol) was dissolved in 5 ml of dry toluene containing AIBN (1 mg). Dry nitrogen was bubbled through the solution for 20 min. Tributyltin hydride (0.4 ml, 1.5 mmol) was added, the reaction stirred for 30 min at room temperature and a further 24 h at 80 °C. The solvent was evaporated in vacuo and the resulting oil thickened by addition of pentanes. The liquid was poured off and the remaining gum purified on a silica gel column (ethyl acetate) to give the product as a clear oil containing a mixture of the α- and β-anomers (0.264 g, 86%). 1H nmr (300 MHz, CDCl3): δ 6.21 (d, 0.5 H, J1,2 4.0 Hz, H(1α)), 5.70 (d, 0.5 H, J1,2 8 Hz, H(1β)), 5.05-4.80 (m, 2 H, H(2)+H(4)), 4.30-4.20 (m, 1 H, H(6a)), 4.17-4.08 (m, 1 H, H(6b)), 4.04-3.95 (m, 0.5 H, H(5β)), 3.90-3.82 (m, 0.5 H, H(5α)), 2.62+2.37 (dt, 0.5 H, J3,3a 12, J3,2 4.8, J3,4 4.8 Hz, H(3eα+β)), 2.20-1.90 (m, 13 H, 4 x OAc+H(3a)).

2,4,6-Tri-O-acetyl-3-deoxy-D-ribo-hexopyranose (35)

The tetra-O-acetate (34) (264 mg, 0.79 mmol) was dissolved in 2 ml of 45% HBr/AcOH and stirred at room temperature for 90 min. The solution was poured into 40 ml of ice water, stirred for 5 min, then extracted with CH2Cl2. The organic layer was then washed successively with ice cold saturated sodium bicarbonate and water concentrated in vacuo and the resulting oil was dissolved in 5 ml of acetone containing a few drops of water and in the absence of light treated with 0.8 g (2.9 mmol) of Ag2CO3. The reaction was stirred at 0 °C for 90 min then filtered through a pad of Celite and the solvent evaporated in vacuo to yield the product as a clear oil. The product was further
purified using column chromatography (1:1 EtOAc/Hexanes) (0.126 g, 55%) then carried
to the next step without further characterization.

$2',4'-\text{Dinitrophenyl 2,4,6-tri-O-acetyl-3-deoxy-\textbeta-D-ribo-hexopyranoside (36)}$

The hemiacetal (35) (0.31 g, 1.1 mmol) was treated with of DABCO (420 mg,
3.74 mmol) and FDNB (250 mg, 1.3 mmol) in the usual manner. The reaction was
stirred for 2 h at room temperature and worked up as usual to give the product as a
yellowish solid which was recrystallized from ethanol to give the product as white
crystals (0.31 g, 64%). M.p. 146-148 °C; $^1$H nmr (400 MHz, CDCl$_3$): $\delta$ 8.72 (d, 1 H,
J$_3$;$5$ 3.3 Hz, H(3$'$)), 8.44 (dd, 1 H, J$_5$;$6$' 9.0, J$_5$;3$'$ 3.3 Hz, H(5$'$)), 7.50 (d, 1 H, J$_6$;$5$' 9.0 Hz,
H(6$'$)), 5.32 (d, 1 H, J$_1$;2 6.7 Hz, H(1$'$)), 5.14 (ddd, 1 H, J$_2$;3a 9.5, J$_2$;1 6.7,
J$_2$;3e 4.9 Hz, H(2$'$)), 4.96 (ddd, 1 H, J$_4$;3a 9.5, J$_4$;3e 4.9, J$_4$;5 3.9 Hz, H(4$'$)), 4.28 (dd,
1 H, J$_6$a;6b 12.2, J$_6$;5 3.5 Hz, H(6a$'$)), 4.20 (dd, 1 H, J$_6$b;6a 12.2, J$_6$b;5 6.0 Hz, H(6b$'$)),
(ddd, 1 H, J$_5$;6b 6.0, J$_5$;4 3.9, J$_5$;6 3.5 Hz, H(5$'$)), 2.68 (dt, 1 H, J$_3$;e;3a 13.2, J$_3$;e;2 4.9,
J$_3$;e;4 4.9 Hz, H(3e$'$)), 2.12, 2.09, 2.06 (3 x s, 9 H, 3 x OAc), 1.84 (dt, 1 H, J$_3$a;3e 13.2,
J$_3$a;2 9.5, J$_3$a;4 9.5 Hz, H(3a$'$)). Anal. calc. for C$_{18}$H$_{20}$O$_{12}$N$_2$: C, 47.37; H, 4.42; N,
6.14. Found: C, 47.36; H, 4.48; N, 6.11.

$2',4'-\text{Dinitrophenyl 3-deoxy-\textbeta-D-ribo-hexopyranoside (37)}$

The tri-O-acetate (36) (100 mg, 0.22 mmol) was suspended in 7 ml of dry
methanol, cooled to 0 °C, treated with acetyl chloride (0.53 ml, 0.67 mmol) then stirred
at 4 °C overnight. The reaction was worked up in the usual manner to yield the product
as a clear gum which was crystallized from a mixture of acetone, diethyl ether and
petroleum ether (26 mg, 38%). M.p. 100-104 °C; $^1$H nmr (400 MHz, Acetone-D$_6$): $\delta$
8.70 (d, 1 H, J3',5' 3 Hz, H(3')), 8.50 (dd, 1 H, J5',6' 9, J5',3' 3 Hz, H(5')), 7.45 (d, 1 H, J6',5' 9 Hz, H(6')), 5.34 (d, 1 H, J1,2 7 Hz, H(1)), 3.95-3.46 (m, 5 H, H(2)+H(4)+H(5)+H(6)), 2.48-2.38 (m, 1 H, H(3e)), 1.71 (q, 1 H, J3a,3e 11, J3a,2 11, J3a,4 11 Hz, H(3a)). Anal. calc. for C_{12}H_{14}O_{9}N_{2} + 1H_{2}O + 0.6 acetone; C, 43.26; H, 5.16; N, 7.31; Found: C, 43.34; H, 5.16; N, 7.31.

2,3,4,6-Tetra-O-acetyl-D-mannopyranose (38)

1,2,3,4,6-penta-O-acetyl-D-mannopyranose (109) (1.15 g, 2.83 mmol) was dissolved in 25 ml of DMF and treated with hydrazine acetate (0.4 ml, 4.3 mmol) by the usual method. The reaction was worked up in the usual way and the product isolated as a clear oil, which was further purified on a silica gel column (1:1 EtOAc/hexane) and again isolated as a clear oil. It was carried directly to the next step in the synthesis. The isolated alcohol appeared to be almost entirely the α-anomer by nmr. \textsuperscript{1}H nmr (200 MHz, CDCl\textsubscript{3}): \delta 5.75 (d, 1 H, J1,2 2.0 Hz, H(1)), 5.00-4.80 (m, 3 H, H(2)+H(3)+H(4)), 3.95 (dd, 1 H, J6a,6b 12.5, J6,5 5.5 Hz, H(6)), 3.83-3.66 (m, 2 H, H(5)+H(6b)).

2',4'-Dinitrophenyl 2,3,4,6-tetra-O-acetyl-β-D-mannopyranoside (39)

The hemiacetal (38) (0.9 g, 2.6 mmol) was dissolved in 10 ml of dry DMF and treated with DABCO (1.2 g, 10.7 mmol) and FDNB (0.54 g, 2.8 mmol) dissolved in 5 ml of DMF. The reaction was stirred for 1 hour at room temperature and worked up in the usual way. The isolated oil was a mixture containing mostly the α-mannoside and was therefore run down a silica gel column (1:1 EtOAc/hexanes) three times to obtain the pure β-anomer. The compound was crystallized from acetone/petroleum ether. Yield of β-anomer from hemiacetal (80 mg, 6%). M.p. 140-142 °C; \textsuperscript{1}H nmr (400 MHz, CDCl\textsubscript{3}):
δ 8.77 (d, 1 H, J3',5' 3.3 Hz, H(3') ), 8.43 (dd, 1 H, J5',6' 9.0, J5',3' 3.3 Hz, H(5') ), 7.40 (d, 1 H, J6',5' 9.0 Hz, H(6') ), 5.73 (dd, 1 H, J2,3 4.0, J2,1 1.8 Hz, H(2) ), 5.57 (d, 1 H, J1,2 1.8 Hz, H(1) ), 5.31 (t, 1 H, J4,3 8.4, J4,5 8.4 Hz, H(4) ), 5.29 (dd, 1 H, J3,4 8.4, J3,2 4.0 Hz, H(3) ), 4.34 (dd, 1 H, J6a,6b 12.0, J6,5 6.8 Hz, H(6a) ), 4.28 (dd, 1 H, J6b,6a 12.0, J6b,5 4.0 Hz, H(6b) ), 4.00-3.94 (m, 1 H, H(5) ), 2.35, 2.13, 2.10, 2.06 (s 4 x, 12 H, 4 x OAc). Anal. calc. for C20H22N2O14: C, 46.70; H, 4.50; N, 5.43. Found: C, 46.38; H, 4.46; N, 5.43.

2',4'-Dinitrophenyl β-D-mannopyranoside (40)

The per-O-acetylated mannoside (39) (120 mg, 0.23 mmol) was suspended in 10 ml of dry methanol, cooled to 0 °C, then treated with 0.75 ml (10.5 mmol) of acetyl chloride. The reaction was stirred at room temperature until the solution was clear, then overnight at 4 °C and worked up as usual to give a clear oil which was crystallized from methanol and diethyl ether to give the product as a white powder (38 mg, 47%) M.p. 96-98 °C; 1H nmr (400 MHz, CD3OD): δ 8.73 (d, 1 H, J3',5' 3 Hz, H(3') ), 8.47 (dd, 1 H, J5',6' 9.2, J5',3' 2.8 Hz, H(5') ), 7.64 (d, 1 H, J6',5' 9.2 Hz, H(6') ), 5.50 (d, 1 H, J1,2 1.2 Hz, H(1) ), 4.13 (d, 1 H, J2,3 3.2 Hz, H(2) ), 3.93 (dd, 1 H, J3,4 9.2, J3,2 3.2 Hz, H(3) ), 3.53-3.47 (m, 3 H, H(4)+H(6) ), 3.16-3.13 (m, 1 H, H(5) ). Anal. calc. for C12H14N2O10: C, 41.63; H, 4.075; N, 8.09. Found: C, 37.45; H, 4.37; N, 6.22

1.4 Other Aryl-β-D-Glycosides
3,4,6-Tri-O-acetyl-2-ammonium-2-deoxy-α-D-glucopyranosyl bromide hydrobromide (41)

Glucosamine hydrochloride (Aldrich Chemical Co.) (10 g, 46.6 mmol) was placed in a 3 neck flask and acetyl bromide (25 g, 0.20 mol) added using the procedure of Wolf from (152) and heated with stirring to 60 °C. The HBr produced during the reaction was removed using a water aspirator drawing gases from the reaction over a series of 8 tubes alternating between CaCl$_2$ and NaOH as an adsorbant. The reaction was run until the product turned red and solid (approximately 2 h). The crude mixture was then dissolved in ethanol-free chloroform, filtered and the product crystallized by addition of diethyl ether to the filtrate and cooling. The product is isolated by filtration as reddish white crystals (10.1 g, 49%). M.p. 147-148 °C. (lit. 152 149-150 °C); $^1$H nmr (200 MHz, CDC$_3$): δ 8.63 (broad s, 3 H, NH$_3$), 7.02 (d, 1 H, J$_{1,2}$ 3.8 Hz, H(1)), 5.42-5.20 (t, 2 H, J 9.8 Hz, H(3)+H(4)), 4.40-4.21 (m, 2 H, H(2)+H(6a)), 4.12 (d, 1 H, J$_{6b,6a}$ 11Hz, H(6b)), 4.00-3.80 (m, 1 H, H(5)), 2.21, 2.10, 2.06 (3 x s, 9 H, 3 x OAc).

p-Nitrophenyl 3,4,6-tri-O-acetyl-2-ammonium-2-deoxy-β-D-glucopyranoside hydrochloride (42).

The glucosyl bromide (41) (0.5 g, 1.1 mmol) was dissolved in 10 ml of acetone and treated with p-nitrophenol (0.8 g, 5.8 mmol) and 2 M NaOH (2 ml), then stirred for 1.5 h at room temperature. The reaction mixture was then diluted with chloroform, the organic layer washed with 2 M NaOH and the solvent was evaporated in vacuo to yield a crude oil which was dissolved in excess diethyl ether and filtered. The product was precipitated from the filtrate by bubbling HCl gas through the solution, filtered and crystallized from ethanol. The compound was extremely hygroscopic. (0.10 g, 20%). M.p. >210 (dec). $^1$H nmr (200 MHz, DMSO-D$_6$): δ 8.98 (broad s, ~1.5 H, NH$_3$), 8.30,
7.40 (d, 4 H, J2,3 9 Hz, (aryl H) ), 5.81 (d, 1 H, J1,2 8 Hz, H(1) ), 5.40, 4.98 (t, 2 H, J 9.8 Hz, H(3)+H(4) ), 3.90-4.15 (m, >3 H, H(2)+H(6)+water) ), 3.90-3.68 (m, 1 H, H(5) ), 2.10, 2.06, 2.01 (3 x s, 9 H, 3 x OAc). Anal. calc. for C18H23N2O10Cl + 1/2H2O: C, 45.82; H, 5.13; N, 5.94. Found: C, 46.04; H, 5.10; N, 5.86.

p-Nitrophenyl 2-ammonium-2-deoxy-β-D-glucopyranoside hydrochloride (43)

The acetylated glucoside (42) was deprotected using the HCl/methanol procedure with the following modifications. The glucoside (150 mg, 0.33 mmol) was dissolved in 20 ml of dry methanol, cooled to 0 °C and treated with acetyl chloride (0.3 ml, 4.2 mmol). The reaction mixture was stirred at room temperature for 48 h, then 4 h at 40 °C and worked up using the usual procedure to yield the product as an oil. The product solidified after several evaporations in vacuo with diethyl ether and drying in vacuo for several days. The compound was further purified on a silica gel column (7:2:1 ethyl acetate/ethanol/water) and isolated as a flaky white solid (73 mg, 68%). M.p. >210°C(dec): 1H nmr (200 MHz, D2O): δ 8.20, 7.22 (d, 4 H, J2,3 9.0 Hz, H(2')+H(3')+H(4')+H(5') ), 5.57 (d, 1 H, J1,2 9.0 Hz, H(1) ), 3.96-3.35 (m, 6 H, H(2)+H(3)+H(4)+H(5)+H(6) ). Anal. Calc. for C12H17N2O6Cl + 0.6 CH3CH2OH: C, 45.51; H, 5.91; N, 8.15. Found: C, 45.20; H, 5.63; N, 7.72.

Phenyl 6-deoxy-6-fluoro-β-D-glucopyranoside (44).

Phenyl β-D-glucopyranoside (6) (200 mg, 0.8 mmol) was dissolved in approximately 5 ml of dry CH2Cl2, cooled to -23 °C then treated with DAST (0.72 ml, 4 mmol) using the procedure of Card and Reddy (66). The solution was slowly warmed to room temperature, stirred for 45 min, again cooled to -23 °C and quenched by addition of excess methanol. After evaporation of the solvent in vacuo, the resulting syrup was
purified on a silica gel column (5% MeOH/EtOAc) and the product crystallized from ethyl acetate and petroleum ether and isolated as a white powder (70 mg, 40%). Mp. 135-137 °C. (lit.66 143-145 °C); 1H nmr (300 MHz, CD3OD): δ 7.30 (m, 2 H, H(2') +H(6') ), 7.14-6.99 (m, 3 H, H(3')+H(4')+H(5') ), 4.91 (d, 1 H, J1,2 7.5 Hz, H(1) ), 4.69 (ddd, 1 H, J6a,F 48, J6a,6b 10, J6a,5 2 Hz, H(6a) ), 4.65 (ddd, 1 H, J6b,F 48 Hz, J6b,6a 10 Hz, J6b,5 6 Hz, H(6b) ), 3.73-3.40 (m, 4 H, H(2)+H(3)+H(4)+H(5) ). 19F nmr (188.3 MHz, D2O): δ -235.52. Anal. calc. for C12H15O5F + 0.75 H2O: C, 53.04; H, 6.12. Found: C, 52.78; H, 5.93.

*p-Nitrophenyl 6-deoxy-6-fluoro-β-D-glucopyranoside* (45)

p-Nitrophenyl β-D-glucopyranoside (400 mg, 1.3 mmol) was dissolved in 10 ml of dry CH2Cl2, cooled to -23 °C and treated with DAST (0.8 ml, 1.3 mmol). The solution was slowly warmed to room temperature, stirred for 35 min then worked up in the same manner as (44) and the product isolated as white crystals (35 mg, 9%). M.p. 178-181 °C. (lit.66 186-189 °C); 1H nmr (300 MHz, Acetone-D6): δ 8.23 + 7.27 (d, 4 H, J2;3' 8 Hz, aryl (H) ), 5.27 (d, 1 H, J1,2 8 Hz, H(1) ), 4.88-4.51 (m, 2 H, H(6) ), 3.86 (dddd, 1 H, JH,F 25, J5,4 10, J5,6a 5, J5,6b 2 Hz, H(5) ), 3.65-3.46 (m, 3 H, H(2)+H(3)+H(4) ). 19F nmr (188.3 MHz, D2O): δ -235.60. Anal. calc. for C12H14NO7F + 0.5 H2O: C, 46.15; H, 4.84; N, 4.49. Found: C, 45.83; H, 4.87; N, 4.18.

*o-Nitrophenyl 6-deoxy-6-fluoro-β-D-glucopyranoside* (46)

o-Nitrophenyl β-D-glucopyranoside (Aldrich Chemical Co.) (70 mg, 0.23 mmol) was suspended in 5 ml of dry CH2Cl2, cooled to -23 °C and treated with DAST (0.090 ml, 0.74 mmol). The reaction was slowly warmed to room temperature, stirred for 35
min, cooled again to -23 °C and quenched by addition of excess methanol. The solvent was evaporated in vacuo and the resulting oil purified by column chromatography (5% MeOH/EtOAc). The product was isolated as a clear oil (12 mg, 17%). $^{1}$H nmr (200 MHz, Acetone-D$_6$): δ 7.76 (d, 1 H, J$_3'$,4' 9 Hz, H(3') ), 7.56 (t, 1 H, J$_4'$,3' 9, J$_4'$,5' 9 Hz, H(4') ), 7.40 (d, 1 H, J$_5'$,5' 9 Hz, H(5')), 7.14 (t, 1 H, J$_5'$,4' 9 Hz, J$_5'$,6' 9, H(5')), 5.13 (d, 1 H, J$_1$,2 7 Hz, H(1) ), 4.56 (ddd, 1 H, J$_6$a,F 47, J$_6$a,6b 9, J$_6$a,5 2 Hz, H(6a) ), 4.53 (ddd, 1 H, J$_6$b,F 47, J$_6$b,6a 9, J$_6$b,5 5 Hz, H(6b) ), 3.80-3.55 (m, 1 H, H(5) ), 3.52-3.30 (m, 3 H, H(2)+H(3)+H(4) ) $^{19}$F nmr (188.3 MHz, D$_2$O): δ -235.17.

2'-Chloro-4'-nitrophenyl 6-deoxy-6-fluoro-β-D-glucopyranoside (47)

2'-Chloro-4'-nitrophenyl β-D-glucopyranoside (153) (50 mg, 0.15 mmol) was suspended in 5 ml of dry CH$_2$Cl$_2$, cooled to -23 °C and treated with DAST (0.070 ml, 0.58 mmol). The reaction was worked up in the exact same manner as (45) to yield the product as a clear oil which was crystallized from EtOAc/petroleum ether (15 mg, 30%). M.p. 172-175 °C; $^{1}$H nmr (200 MHz, Acetone-D$_6$): δ 8.32 (s, 1 H, H(6') ), 8.21 (d, 1 H, J$_4'$,5' 10 Hz, H(4') ), 7.59 (d, 1 H, J$_5'$,4' 10 Hz, H(5') ), 5.39 (d, 1 H, J$_1$,2 7.8 Hz, H(1) ), 4.71 (ddd, 1 H, J$_6$a,F 48, J$_6$a,6b 10, J$_6$a,5 2 Hz, H(6a) ), 4.63 (ddd, 1 H, J$_6$b,F 47, J$_6$b,6a 10, J$_6$b,5 5 Hz, H(6b) ), 3.99-3.75 (m, 1 H, H(5) ), 3.70-3.44 (m, 3 H, H(2)+H(3)+H(4) ) $^{19}$F nmr (188.3 MHz, D$_2$O): δ -235.64. Anal. calc. for C$_{12}$H$_{14}$NO$_8$CIF + 0.2 H$_2$O: C, 42.23; H, 4.00; N, 4.10. Found: C, 42.29; H, 4.29; N, 3.90.

2,3,4,6-Tetra-O-acetyl-D-galactono-1,5-lactone (48) (154)

2,3,4,6-Tetra-O-acetyl-D-galactopyranose (73) (180 mg, 0.5 mmol) was treated with a mixture of acetic anhydride (0.4 ml) in DMSO (0.6 ml) (which had been pre-
stirred together at room temperature for 4 h) and stirred at room temperature for 36 h. The reaction mixture was then poured into ice water, stirred for 1 h, the solution frozen, lyophilized, and the resulting oil dissolved in CHCl₃ and washed with water. The solvent was evaporated in vacuo to yield an oil which was purified by column chromatography (1:1 EtOAc/Hexanes) giving the product as a clear oil (73 mg, 41%).

\[ \text{1H nmr (200 MHz, CDCl₃): } \delta 5.55 \text{ (d, } 1 \text{ H, J}_{2,3} 7 \text{ Hz, H(2) }), 5.37 \text{ (t, } 1 \text{ H, J}_{3,2} 7, J_{3,4} 7 \text{ Hz, H(3) }), 5.25 \text{ (ddd, } 1 \text{ H, J}_{5,6b} 6.2, J_{5,6a} 5.8, J_{5,4} 3 \text{ Hz, H(5) }), 4.54 \text{ (dd, } 1 \text{ H, J}_{4,3} 7, J_{4,5} 3 \text{ Hz, H(4) }), 4.29 \text{ (dd, } 1 \text{ H, J}_{6a,6b} 11.8, J_{6a,5} 5.8 \text{ Hz, H(6a) }) \]

\(2',4'-\text{Dinitrophenyl } \{1-^{2}H\} \text{ 2,3,4,6-Tetra-O-acetyl-}\beta\text{-D-galactopyranose (49)} (155)\)

The lactone (48) (250 mg, 0.72 mmol) was dissolved in dry THF (12 ml), cooled to 0 °C and treated with NaBD₄ (33 mg, 0.79 mmol) suspended in D₂O (0.15 ml). The reaction was stirred for 2 h, quenched by addition of AG 1-X8 (H⁺) resin, filtered and the solvent evaporated in vacuo to give the product as an oil which was purified by column chromatography (1:1 EtOAc/Hexanes) to yield the product as a clear oil containing both the α- and β- anomers of the hemiacetal (0.123 mg, 49%).

\[ \text{1H nmr (400 MHz, CDCl₃): } \delta \text{ β-anomer } 5.48 \text{ (dd, } 1 \text{ H, J}_{4,3} 3.3, J_{4,5} 1.2 \text{ Hz, H(4) }), 5.42 \text{ (dd, } 1 \text{ H, J}_{3,2} 10.8, J_{3,4} 3.3 \text{ Hz, H(3) }), 5.16 \text{ (d, } 1 \text{ H, J}_{2,3} 10.8 \text{ Hz, H(2) }), 4.75 \text{ (dt, } 1 \text{ H, J}_{5,6a} 6.6, J_{5,6b} 6.6, J_{5,4} 1.2 \text{ Hz, H(5) }), 4.17-4.08 \text{ (m, } 2 \text{ H, H(6) }) \]

\(2',4'-\text{Dinitrophenyl } \{1-^{2}H\} \text{ 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (50)}\)

The hemiacetal (49) (120 mg, 0.34 mmol) was treated with FDNB (90 mg, 0.5 mmol) and DABCO (170 mg, 1.5 mmol) in the usual way, stirred at room temperature
for 2 h and worked up to yield a solid which was recrystallized from ethanol to give the product as slightly yellow crystals (86 mg, 49%). M.p. 174-176 °C \( ^1H \) nmr (400 MHz, CDCl\(_3\) \( \delta \) 8.72 (d, 1 H, \( J_{3',5'} \) 3 Hz, H(3')), 8.45 (dd, 1 H, \( J_{5',6'} \) 9, \( J_{5',3'} \) 3 Hz, H(5')), 7.50 (d, 1 H, \( J_{6',5'} \) 9 Hz, H(6')), 5.60 (d, 1 H, \( J_{2,3} \) 11 Hz, H(2)), 5.50 (d, 1 H, \( J_{4,3} \) 3.5 Hz, H(4)), 5.14 (dd, 1 H, \( J_{2,3} \) 11, \( J_{3,4} \) 3.5 Hz, H(3)), 4.30-4.10 (m, 3 H, H(5)+H(6)), 2.20, 2.15, 2.10, 2.05 (4 x s, 12 H, 4 x OAc). Anal. calc. for C\(_{20}H_{21}N_2O_{14}D\): C, 46.61; H, 4.11; N, 5.44. Found: C, 46.53; H, 4.29; N, 5.33.

2',4'-Dinitrophenyl \{1-\( ^2H \}\} \( \beta \)-D-galactopyranoside (51)

The protected galactoside (50) (66 mg, 0.13 mmol) was suspended in dry methanol (5 ml) and treated in the usual way with acetyl chloride (0.35 ml, 0.44 mmol), then stirred at 4 °C overnight to give the product as a clear oil which crystallized on addition of diethyl ether. The resulting solid was recrystallized from acetone/diethyl ether to give the product as white crystals (25 mg, 56%). Mp. 132-135 °C\(_{\text{dec}}\) \( ^1H \) nmr (200 MHz, CD\(_3\)OD): \( \delta \) 8.70 (d, 1 H, \( J_{3',5'} \) 3 Hz, H(3')), 8.48 (dd, 1 H, \( J_{5',6'} \) 9, \( J_{5',3'} \) 3 Hz, H(5')), 7.65 (d, 1 H, \( J_{6',5'} \) 9 Hz, H(6')), 4.20-3.20 (m, 6 H, H(2)+H(3)+H(4)+H(5)+H(6')). Anal. calc. for C\(_{12}H_{13}N_2O_{10}D + 0.8 H_2O + 1.5 CH_3OH: C, 39.57; H, 4.82; N, 6.84. Found: C, 39.84; H, 4.57; N, 6.50.

2 Spontaneous Hydrolysis Kinetics

2.1 General Kinetic Experiments

The procedure used to determine the rates of spontaneous hydrolysis of the 2',4'-dinitrophenyl glycosides is essentially that used by Sinnott (58) for the determination of the rates of hydrolysis of a series of DNPgalactosides. All rates were determined at pH
6.50 in 25 mM sodium phosphate buffer and 0.400 M KCl. Glycoside concentrations were approximately 0.15 mM. All solutions were prepared with Nano-pure quality water. Hydrolysis rates were determined at three or more temperatures with each glycoside. The temperature was regulated by a Neslab RTE-210 circulating bath. Temperatures were recorded from the available digital readout and were checked using an external thermometer and found to be accurate within +/- 0.2 °C. Temperature during the experiment did not fluctuate more than +/- 0.2 °C. Each rate was determined by fitting the A390 of the solution at specific times to a first order equation. All rates were fit to a first order equation using the work station on an Applied Photophysics MV 17 stopped flow spectrophotometer using the available fitting routine (based on a robust implementation of the Marquart algorithm, P. R. Bevington (77)). Typically seven points were used to determine the rates and the reactions were allowed to proceed to at least 3 half lives. The rates at each temperature were measured three times and averaged. As a check of the first order fits obtained, several rate constants were also determined by plotting ln((A_\infty - A_t)/A_\infty) vs time. The rates obtained were essentially identical to those found using the fitting routine.

Thermodynamic constants for the substrates were obtained from Eyring plots (156) of the logarithm of the averaged value for the rate constant determined at each temperature divided by temperature versus the reciprocal of the absolute temperature. These data were analyzed by linear regression using GraFit (107) which provided the slope and intercept for the line obtained and errors from these values. Since the Eyring equation is \( \ln(k/T) = \ln(k/h) - \Delta H^\ddagger /RT + \Delta S^\ddagger /R \) values for \( \Delta H^\ddagger \) can be calculated from the slope of the line and values for \( \Delta S^\ddagger \) from the y intercept. The rate constants provided were calculated from the equations defining the lines obtained for Eyring plots of the data.
2.1.1 Procedure for High Temperature Hydrolysis Rates

To determine the rates of hydrolysis of the DNPglycosides at temperatures higher than 50 °C the following procedure was employed. A solution of the glycoside was prepared as described above, then solutions of one half and one quarter the original concentration were prepared by dilution of the original stock with buffer. Approximately 1 ml aliquots of the solutions were pipeted into 1 ml Wheaton vials. The vials were flushed with nitrogen, sealed using a bunsen burner, then immersed in a water bath at the required temperature and allowed to equilibrate for 5 min before the initial reading was taken. The samples were removed at different times and immediately cooled in an ice bath then frozen. The solutions were later thawed and the A390 for each of the vials measured at room temperature using a Phillips PU 8000 UV-visible spectrophotometer equipped with a sipper cell. The increase in absorbance with time was then fit to a first order equation in the manner described.

2.1.2 Procedure for Low Temperature Hydrolysis Rates

To determine hydrolysis rates below 50 °C the solutions are prepared in the same manner as described above. The solutions were placed in quartz cuvettes fitted with Teflon plugs and sealed with Parafilm. The cells were then placed in a Perkin Elmer λ II spectrophotometer equipped with a thermostatted cell block and temperature probe. The absorbances were automatically recorded on an Epson Equity I+ computer then fit to a first order equation as previously described.
2.2 Secondary α-DKIE Measurements

The isotope effects for spontaneous hydrolysis of glycosides were all carried out at 45 ± 0.1 °C using the same procedure applied to determining the low temperature hydrolysis rates. Rate constants were determined at least six times for both the deuterated and protiated compounds. Isotope effects were determined from the ratio of consecutive measurements of the rates for the protiated and deuterated compounds and errors provided as the standard deviation in the isotope effects determined.

2.3 pKa Determinations for the 1-Deoxynojirimycins

All pH determinations were carried out using a Radiometer PHM 82 pH meter equipped with an Orion 8103 Ross pH electrode. Dilute solutions of the 1-deoxynojirimycins (6 mM) were prepared in Nano-pure water and placed in a cell equipped with a circulating bath. The amine solution (100 μl) was equilibrated to 37 °C, then acidified by addition of 2-3 μl of HCl(aq). The solution was then back-titrated by addition of 1 μl aliquots of 0.75 M NaOH(aq) using an Eppendorf pipet. The pH after each addition was recorded and fit to a pH titration curve using GraFit (107).

3 Enzyme Kinetics

3.1 General Procedures

The abg β-glucosidase used for these studies was obtained from E. coli paste provided by Dr. Don Trimbur, Department of Microbiology, UBC. The enzyme was purified by the method of Kempton and Withers (6). Enzyme concentrations were determined by their absorbance at 280 nm (ε = 2.184 mg ml⁻¹cm⁻¹). All absorbance
measurements were made on a PU-8800 UV-Visible spectrometer equipped with a circulating water bath. Extinction coefficients for all of the aglycones used in this study were obtained from Kempton and Withers (6).

3.1.1 Steady State Rate Determinations

Steady state rate constants for the hydrolysis of the aryl glycosides by abg β-glucosidase were determined at 37 °C in 50 mM sodium phosphate buffer pH 6.80 in the presence of 0.1% BSA. All buffers were prepared with doubly deionized water. The concentration of enzyme used with each substrate is provided in Appendix 2. Substrate concentrations were determined by measuring the absorbance of the intact DNP-glycoside at 255 nm (ε = 10.7 cm⁻¹mM⁻¹) or by total hydrolysis of the glycoside and determination of the final concentration of phenol released. All rate measurements were made in 1 cm quartz cells. Solutions of substrate and BSA were allowed to equilibrate to 37 °C and the reactions initiated by addition of enzyme. Rates were determined by initial rates analysis following the release of phenol with time which was found to be linear below 8% substrate depletion. Substrate concentrations were chosen to be approximately 0.15 to 7 times K_m. The value of K_m was initially estimated using three or four substrate concentrations then several other concentrations would be chosen based on this estimate. Values for k_cat and K_m were determined by weighted fitting of the observed rate data to the Michaelis-Menten equation using GraFit (107).

3.1.2 Pre-steady State Rate Determinations

All of the stopped flow measurements for this work were carried out using an Applied Photophysics MV 17 microvolume stopped flow spectrophotometer equipped with a Grant constant temperature bath. The data was accumulated using the stopped
flow work station and fit using a robust implementation of the Marquardt algorithm, P. R. Bevington (77). The stop volume was set at 100 µl and the reaction monitored by following the release of dinitrophenol at 360 nm (ε = 14.0 mM⁻¹cm⁻¹).

The concentration of enzyme used in each reaction was chosen such that a burst with a total absorbance change of ΔA₃₆₀ = 0.03 was obtained in each case. For the dinitrophenyl substrates, the reactions were carried out at 5 °C in 50 mM sodium phosphate buffer at pH 6.80. Rates were determined at 5 or more concentrations of substrate which were chosen to bracket Kᵦ for that substrate whenever possible. The rate was repeated four times at each concentration of substrate and the traces averaged using the averaging facility on the work station. The rate of glycosylation (k₂) was determined by fitting the averaged trace to a first order equation or to an equation which fits a pre-steady state followed by a steady state, as was appropriate for the substrate. Kᵦ and k₂ were determined by fitting the observed rate constants to the Michaelis-Menten equation by a weighted non-linear regression, using the GraFit (107) program. Standard error values for these parameters were provided by the same program.

Rates for substrates having a p-nitrophenyl leaving group were determined in the same manner as the 2,4-dinitrophenyl substrates with the exception that the reaction was followed at 400 nm (ε = 7.18 mM⁻¹cm⁻¹) and carried out at 37 °C. The concentration of enzyme was also adjusted accordingly to provide a burst of ΔA = 0.03.

3.2 Viscosity Effects Measurements

Measurements of k₅₃/Kₐ₅ for these experiments were carried out by determining k₅₃ and Kₐ₅ in the manner described above. Measurements were done in pairs where k₅₃ and Kₐ₅ determinations for each substrate were carried out in the presence and absence of sucrose (24% w/v).
3.3 HPLC Procedures for Product Characterization

Hydrolysis of the DNPglycosides was carried out as described for steady state analysis. All analyses were carried out on a Waters HPLC equipped with a UK6 manual injector and Waters 441 UV detector. Aliquots (30 µl) of the reactions were injected onto an analytical Dextro-Pak column and detected by their absorbance at 280 nm. Enzyme was removed from these solutions by a pre-column of Iatro beads (water insoluble silica (Iatron Laboratories)). Products were identified by their retention time in comparison with standards, using the Baseline 710 operating system.

3.4 Secondary α-DKIE Measurements on $V_{\text{max}}/K_m$

Values for $V_{\text{max}}/K_m$ were determined by the method of Waley (159). One ml solutions of enzyme and 0.1% BSA in 1 ml plastic cuvettes were placed in a UV-Visible spectrophotometer equipped with a circulating bath and allowed to equilibrate to 37 °C. The reaction was initiated by injection of 10 µl of a concentrated stock solution of either the protio or deuterio substrate. The substrate concentration was chosen such that the final substrate concentration in the assay cell was at least 15 times below $K_m$. The release of phenol was monitored at 400 nm and the time course was fit to a first order equation using Grafit (107) to yield a pseudo-first order rate constant. These rate constants were determined in pairs of the protiated and deuterated substrates and the isotope effects calculated from the ratio of the rates of hydrolysis. These determinations were repeated at least 10 times and the isotope effect reported was the average of the isotope effects determined. Errors provided are the standard deviation of the average isotope effect value.
4 Competitive Inhibition Experiments

4.1 General Procedure

All inhibition experiments were carried out at pH 6.80 in 50 mM sodium phosphate buffer in the presence of 0.1% BSA at 37 °C using PNPglucoside as a substrate. All assays were carried out in 1 ml plastic cuvettes on a Perkin Elmer λ II UV-Visible spectrophotometer equipped with a circulating bath. Estimates of the $K_i$ values for the inhibitors were determined as follows. An accurate $V_{max}$ value was determined with PNPglucoside for each enzyme sample used as described earlier. Cuvettes were then prepared containing PNPglucoside (0.20 mM), 0.1% BSA and the inhibitor being tested. Each inhibitor was tested at six or more concentrations. The cells were equilibrated at 37 °C and the reaction initiated by injection of enzyme, rates being determined by initial rates analysis of the release of p-nitrophenol with time. $K_i$ values were determined by calculating the equation for the line obtained from a plot of the inverse of the rate of release of phenol versus inhibitor concentration, then determining the inhibitor concentration where $1/velocity$ equals the $1/V_{max}$ value determined for PNPglucoside.

Accurate $K_i$ values were determined in the same manner but, at several substrate concentrations. Apparent $K_m$ values for PNPglucoside at each concentration of inhibitor were calculated and plotted against inhibitor concentration to provide $K_i$. 
Appendix 1, Theory of Binding Energy in Catalysis

Enzymes are proteins which function as catalysts. As such, they are governed by the same principle which applies to all catalysts. The only manner in which the rate of a reaction can be increased is by lowering the energy required for the transition state of that reaction to be reached. That being said, there are several ways in which a catalyst can accomplish this goal; acid/base catalysis, electrostatic catalysis and reduction of the entropy of reaction being a few (for a comprehensive review see 150).

In analyzing the degree to which a particular enzyme catalyzes a reaction, it is important to first define a standard for comparison. Selection of this standard reaction is somewhat arbitrary, but should reflect the particular effect being examined in the study. Since the products of the reaction has no effect on the kinetics, they have been omitted from the diagram for simplicity.

![Reaction Coordinate Diagram](image)

*Figure A1-1. Reaction coordinate diagram for the standard reaction.*
The term $\Delta G_u^+$ defines the activation free energy for the standard, uncatalyzed reaction.

One way in which an enzyme can catalyze a reaction is by the use of non-covalent interactions between the enzyme and the substrate which specifically stabilize the transition state of the reaction. The free energy of these interactions is termed the binding energy. The term arises from the theory of Haldane (30) and Pauling (31) which suggests that the way in which enzymes effect catalysis is to preferentially bind to and stabilize the transition state of the reaction. It should be noted that any interactions which stabilize the enzyme/substrate ground state complex are inhibitory in nature and will therefore lessen the degree of catalysis the enzyme can provide.

This statement is best illustrated by adding an enzyme to the free energy diagram for the standard reaction.

Figure A1-2 Reaction coordinate diagram comparing an enzyme-catalyzed and uncatalyzed reaction.
The terms used in the diagram are defined as follows:

\[ \Delta G_{ct}^{\dagger} \] is the free energy difference between the transition state for the catalyzed reaction and the Michaelis complex.

\[ \Delta G_{t}^{\dagger} \] is the free energy difference from the ground state for the substrate plus enzyme free in solution to the transition state for the enzyme catalyzed reaction.

\[ \Delta G_{B}^{0}gs \] is the free energy released when the enzyme binds the ground state structure of the substrate, and is algebraically positive.

\[ \Delta G_{B}^{0}ts \] is the free energy released when the enzyme binds the transition state structure of the substrate, and is algebraically positive.

For the simple enzyme shown above, the free energy differences are directly related to the commonly determined enzymic constants, \( k_{cat} \), \( K_m \) and \( k_{cat}/K_m \) where \( K_m \) is in this case a simple dissociation constant for the formation of the Michaelis complex. The free energy for formation of the complex is \( \Delta G_{B}^{0}gs \); therefore using the Gibbs equation at equilibrium:

\[ \Delta G_{B}^{0}gs = -RT\ln(K_m) \]

The free energy difference between the Michaelis complex and the transition state is \( \Delta G_{ct}^{\dagger} \) and can be related to \( k_{cat} \) by use of the Gibbs equation such that:

\[ \Delta G_{ct}^{\dagger} = RT\ln(kT/h) - RT\ln(k_{cat}) \]
Finally, $k_{\text{cat}}/K_m$, can be related to $\Delta G_t^+$ in the same manner.

$$\Delta G_t^+ = RT\ln(kT/h) - RT\ln(k_{\text{cat}}/K_m)$$

Using these definitions, the enzymic constants can also be related to the standard un catalyzed reaction. From the above energy diagram it is apparent that:

$$\Delta G_{ct}^+ = \Delta G_u^+ + X \text{ where } X = \{\Delta G^0_{Bgs} - \Delta G_{Bts}\}$$

Similarly it can be shown that:

$$\Delta G_t^+ = \Delta G_{ct}^+ - \Delta G^0_{Bgs}$$

or

$$\Delta G_t^+ = \Delta G_u^+ + \Delta G^0_{Bgs} - \Delta G_{Bts} - \Delta G^0_{Bgs}$$

due to

$$\Delta G_t^+ = \Delta G_u^+ - \Delta G_{Bts}$$

These equations are related to $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ as has already been illustrated.

Since $\Delta G_{ct}^+$ is related to $k_{\text{cat}}$, it is apparent that the size of $k_{\text{cat}}$ is a function of the difference in the enzyme's binding of the ground state and the transition state of the substrate. An enzyme where the transition state is bound more tightly than the ground state (i.e. $X < 0$) is the only instance where $k_{\text{cat}}$ will be larger than the rate constant for the uncatalyzed process. Also, since $\Delta G_t^+ = \Delta G_u^+ - \Delta G_{Bts}$, the value of $k_{\text{cat}}/K_m$ with
respect to the uncatalyzed process is only a function of the enzyme's ability to stabilize the transition state of the reaction.

The conclusion therefore is that if an enzyme has a finite amount of binding energy available, selective binding of the transition state is the most efficient way to effect catalysis. Binding of the ground state will only decrease the amount of catalytic power the enzyme has available. This can be illustrated by examining two theoretical extremes for the application of the binding energy.

\[ \Delta G_t \]

\[ \Delta G_u \]

\[ \Delta G_{ct} \]

\[ \Delta G_{Bgs} \]

Figure A1-3. A free energy diagram comparing an uncatalyzed reaction with the same reaction in the presence of a protein which binds specifically to the ground state structure of the substrate.

If a protein functions only to bind the ground state conformation of the substrate, all the binding energy is expended in formation of the Michaelis complex. Under these
circumstances the most rapid mode of reaction for a substrate molecule is via the uncatalyzed route. Binding of the substrate by the protein will actually be anti-catalytic since the free energy barrier for the protein bound process is larger than $\Delta G_u^\dagger$.

![Free Energy Diagram](image)

Figure A1-4. A free energy diagram comparing the uncatalyzed reaction with the reaction in the presence of a protein which only binds the transition state structure of the substrate.

If the same amount of binding energy is now used to bind the transition state, the largest possible rate acceleration will be achieved. In this case $k_{cat}$ is only a function of $\Delta G_u^\dagger$ and $\Delta G_{Bts}$ thus the rate acceleration in this instance is not limited by ground state interactions as was shown for the previous examples.
Appendix 2. Plots of Kinetic and Inhibition Data.

Steady State Lineweaver-Burk Plots

Figure A2-1. Hydrolysis of DNPglucoside.  
Enzyme Concentration = 3.2x10^-5 mg/ml

Figure A2-2. Hydrolysis of DNPmannoside.  
Enzyme Concentration = 0.0573 mg/ml

Figure A2-3. Hydrolysis of DNPalloside.  
Enzyme Concentration = 0.0114 mg/ml

Figure A2-4. Hydrolysis of DNPgalactoside.  
Enzyme Concentration = 1.03x10^-4 mg/ml

Figure A2-5. Hydrolysis of 4FDNPgalactoside.  
Enzyme Concentration = 1.03x10^-4 mg/ml

Figure A2-6. Hydrolysis of 3FDNPGlucoside.  
Enzyme Concentration = 3.45x10^-4 mg/ml
Figure A2-7. Hydrolysis of 4FDNP glucoside.
Enzyme Concentration = $8.87 \times 10^{-5}$ mg/ml

Figure A2-8. Hydrolysis of 6FDNP glucoside.
Enzyme Concentration = $2.73 \times 10^{-5}$ mg/ml

Figure A2-9. Hydrolysis of 6FPhe glucoside.
Enzyme Concentration = 0.0114 mg/ml

Figure A2-10. Hydrolysis of 6FPNP glucoside.
Enzyme Concentration = $2.05 \times 10^{-4}$ mg/ml

Figure A2-11. Hydrolysis of 6FONP glucoside.
Enzyme Concentration = $9.94 \times 10^{-4}$ mg/ml

Figure A2-12. Hydrolysis of 6F-2'Cl,4'NO₂ Phe glucoside
Enzyme Concentration = $2.49 \times 10^{-4}$ mg/ml
Figure A2-13. Hydrolysis of 2-amino-2-deoxy-PNPglucoside. 
Enzyme Concentration = 0.366 mg/ml

Figure A2-14. Inactivation with 2FDNPglucoside
Enzyme concentration = 0.175 mg/ml

Figure A2-15. Inactivation with 2C1DNPglucoside
Enzyme concentration = 0.26 mg/ml

Figure A2-16. Inactivation with 2FDNPmannoside
Enzyme concentration = 0.142 mg/ml
Pre-Steady State Lineweaver-Burk Plots

Figure A2-17. Glycosylation with DNPglucoside
Enzyme concentration = 0.303 mg/ml

Figure A2-18. Glycosylation with 2FDNPglucoside
Enzyme concentration = 0.152 mg/ml

Figure A2-19. Glycosylation with 3FDNPglucoside
Enzyme concentration = 0.152 mg/ml

Figure A2-20. Glycosylation with 4FDNPglucoside
Enzyme concentration = 0.152 mg/ml
Figure A2-21. Glycosylation with 6FDNPglucoside  
Enzyme concentration = 0.152 mg/ml

Figure A2-22. Glycosylation with 3dDNPglucoside  
Enzyme concentration = 0.152 mg/ml

Figure A2-23. Glycosylation with 6dDNPglucoside  
Enzyme concentration = 0.152 mg/ml

Figure A2-24. Glycosylation with DNPMannoside  
Enzyme concentration = 0.152 mg/ml
Figure A2-25. Glycosylation with PNP glucoside
Enzyme concentration = 0.201 mg/ml

Figure A2-26. Glycosylation with 2dPNP glucoside
Enzyme concentration = 0.263 mg/ml
**Dixon Plots for the Castanospermines**

*Figure A2-27. A Dixon plot for 2F castanospermine.*

*Figure A2-28. A Dixon plot for 4F castanospermine.*

*Figure A2-29. A Dixon plot of 6F-epi-castanospermine.*
Figure A2-30. A Dixon plot for castanospermine.

Figure A2-31. A Dixon plot for mannocastanospermine.

Figure A2-32. A Dixon plot for 2-aminocastanospermine.

Figure A2-33. A Dixon plot for allocastanospermine.

Figure A2-34. A Dixon plot for alirocastanospermine.
Dixon Plots for the 1-Deoxynojirimycins

Figure A2-35. A Dixon plot for 2Fdeoxynojirimycin.

Figure A2-36. A Dixon plot for 3Fdeoxynojirimycin.

Figure A2-37. A Dixon plot for 4Fdeoxynojirimycin.

Figure A2-38. A Dixon plot for mannodeoxynojirimycin.
Figure A2-39. A Dixon plot for galactodeoxynojirimycin.

Figure A2-40. A Dixon plot for xylodeoxynojirimycin.

Figure A2-41. A Dixon plot for L-arabinodeoxynojirimycin.
### Appendix 3.

**Rate Constants for Spontaneous Hydrolysis of the DNPglycosides.**

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### DNPgalactosides

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### DNPgalactosides and DNPmannosides

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Figure A3-1. An example of a typical Eyring plot for the DNP glycosides. This plot is of the hydrolysis of DNP glucoside.
References


63 S. G. Withers, personal communication.


70 S. G. Withers, personal communication.


Org. Chem. 30, 2312.


80 An example is that α-DKIE measured for hydrolysis of α-glycosides are often higher than with the analogous β-linked compound (see 44). Also, stereoselectivity for product formation is higher for ethanolysis of β- as opposed to α-glucosides (51). Also, see (1).


   And references therein.


153 Obtained from Mark Claisen, Universiteit Gent, Belgium.


