Mechanistic Studies on α-Glycosidases and α-Glycosyl Transferases

by

Curtis Braun

B.Sc., Simon Fraser University, 1989

A THESIS SUBMITTED IN PARTIAL FULFILMENT 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


In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of CHEMISTRY

The University of British Columbia
Vancouver, Canada

Date Nov 6 / 95
ABSTRACT

The two activities, glucosidase and transferase, of glycogen debranching enzyme are involved in the degradation of glycogen. The glucosidase site was shown to hydrolyze the substrate \( \alpha \)-D-glucosyl fluoride with net inversion of the anomeric configuration. Glycosyl fluorides were shown to be good substrates for the glucosidase and transferase sites, with kinetic parameters \( k_{\text{cat}} = 1104 \text{ min}^{-1} \) and \( K_m = 4.2 \text{ mM} \) for \( \alpha \)-D-glucosyl fluoride with the glucosidase activity, and \( k_{\text{cat}} = 44 \text{ min}^{-1} \) and \( K_m = 11 \text{ mM} \) for \( \alpha \)-maltotriosyl fluoride in the presence of 1.0% glycogen with the transferase activity. 4-Deoxy-\( \alpha \)-maltotriosyl fluoride was found to be an incompetent substrate of the transferase activity as the enzyme would carry out the first step in the double displacement mechanism, glycosylation of the enzyme and release of fluoride ion, but is unable to perform the second step, transfer onto another molecule of itself, because the 4-hydroxy group has been removed. This was shown by the accumulation of a glycosyl-enzyme intermediate as demonstrated by the release of one equivalent of fluoride ion, corresponding to one turnover. Tandem electrospray mass spectrometric (MS/MS) analysis of a proteolytic digest of enzyme reacted with 4-deoxy-\( \alpha \)-maltotriosyl fluoride demonstrated that the trisaccharide was covalently attached to a peptide. Subsequent MS/MS experiments on this peptide, along with sequence alignments permitted the identification of the catalytic nucleophile of the transferase activity as aspartic acid 549.

Human pancreatic \( \alpha \)-amylase is involved in the degradation of starch into simple sugars in the gut. \( \alpha \)-Amylase was shown to have an active site composed of five subsites by the kinetic evaluation and determination of the enzymes “action pattern” with the malto-oligosaccharides, maltotetraose through maltoheptaose, using a novel HPLC method. A Dextropak\textsuperscript{\textregistered} HPLC column from Waters\textsuperscript{\textregistered} was used, which allowed the determination of the stereochemical outcome of the reaction catalyzed by \( \alpha \)-amylase, through identification of the initially formed products. Glycosyl fluorides were also shown
to be good substrates, with $\alpha$-maltotriosyl fluoride being the best ($k_{cat}/K_m = 555 \text{ s}^{-1} \text{ mM}^{-1}$). 2,4,6-Trinitrophenyl 2-deoxy-2,2-difluoro-4-O-$\alpha$-(1,4)-D-glucosyl-$\alpha$-D-arabinohexopyranoside was found to act as an active site-directed, time-dependent inactivator of $\alpha$-amylase ($k_i/K_i = 0.0073 \text{ min}^{-1} \text{mM}^{-1}$), labeling the enzyme stoichiometrically as demonstrated by the release of a single equivalent of 2,4,6-trinitrophenol. The 2,2-difluoro glycosyl-enzyme intermediate was found to be stable, as reactivation of the inactivated enzyme was not observed.

Neither 2-deoxy-2-fluoro-$\alpha$-maltotriosyl fluoride or 2-deoxy-2-fluoro-$\alpha$-maltosyl fluoride were found to act as inactivators of the debranching enzyme or $\alpha$-amylase, respectively, but rather they are both substrates. The apparent inactivation of yeast $\alpha$-glucosidase by 2-deoxy-2-fluoro-$\alpha$-D-glucosyl fluoride (Withers et al. (1988), J. Biol. Chem. 263, 7929) was therefore reevaluated and shown to be due to a contaminant in the analogue while the glycosyl fluoride itself was found to be a substrate ($k_{cat} = 1.6 \text{ s}^{-1}$ and $K_m = 4.8 \text{ mM}$). 2,4,6-Trinitrophenyl 2-deoxy-2,2-difluoro-$\alpha$-D-arabinohexopyranoside was shown to be a mechanism-based inactivator of $\alpha$-glucosidase ($k_i/K_i = 0.25 \text{ min}^{-1} \text{mM}^{-1}$). Electrospray mass spectrometric analyses of proteolytic digests of enzyme inactivated with 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-$\alpha$-D-arabinohexopyranoside and 2-deoxy-2-chloro-2-fluoro-$\alpha$-D-arabinohexopyranosyl chloride confirmed the identification of the catalytic nucleophile as aspartic acid 214.
TABLE OF CONTENTS

ABSTRACT............................................................................................................ ii
TABLE OF CONTENTS.......................................................................................... iv
LIST OF TABLES................................................................................................. ix
LIST OF FIGURES................................................................................................. x
ABBREVIATIONS AND SYMBOLS...................................................................... xiii
ACKNOWLEDGEMENTS...................................................................................... xvi
DEDICATION......................................................................................................... xvii

CHAPTER 1: GENERAL INTRODUCTION....................................................... 1
  1.1 Glycosidases............................................................................................... 1
  1.2 General features of the catalytic mechanism.............................................. 3
    1.2.1. “Inverting” glycosidases................................................................. 3
    1.2.2. “Retaining” glycosidases............................................................... 4
    1.2.3. Acid catalysis.................................................................................. 5
    1.2.4. The active site nucleophile............................................................ 7
    1.2.5. Glycosyl-enzyme intermediate...................................................... 7
    1.2.6. Oxocarbonium ion-like transition states........................................ 10
    1.2.7. The binding energies attributable to noncovalent interactions..... 12
  1.3. Enzyme-catalyzed hydration of glycals.................................................. 13
  1.4. Aims of this thesis.................................................................................... 14
    1.4.1. Research significance and objectives.......................................... 14
    1.4.2. Studies testing substrates for α-glycosidases and
          α-glycosyl transferases................................................................. 15
    1.4.3. Trapping glycosyl-enzyme intermediates.................................... 15

CHAPTER 2: MECHANISTIC STUDIES ON RABBIT MUSCLE
GLYCOGEN DEBRANCING ENZYME.................................................. 17
  2.1. Introduction............................................................................................. 17
    2.1.1. Biochemical role in vivo............................................................... 17
    2.1.2. Assays of the debranching enzyme.............................................. 18
    2.1.3. One or two active sites?............................................................... 19
    2.1.4. General features of the catalytic mechanism................................. 19
    2.1.5. Glyx is part of the α-amylase superfamily.................................... 21
  2.2. The aims of this study............................................................................. 23
    2.2.1. Stereochemical outcome............................................................. 23
    2.2.2. Assay systems............................................................................... 23
    2.2.3. Mechanism-based inhibitors of Glyx........................................... 24
  2.3. Results and discussion........................................................................... 26
    2.3.1. Kinetic evaluation of α-glucosyl fluoride as a substrate of
           the glucosidase activity of Glyx.................................................. 26
    2.3.2. Kinetic evaluation of α-maltotriosyl fluoride as a substrate
           of the transferase activity......................................................... 28
      a. Synthesis of α-maltotriosyl fluoride............................................. 28
b. Substrate evaluation........................................................................................................................................30

2.3.3. \( \alpha \)-Maltosyl fluoride as a substrate of Glyx..................................................................................32

2.3.4. Assessment of the stereochemical outcome of the glucosidase reaction mechanism..................35
   a. \( \alpha \)-Glucosyl fluoride as the substrate........................................................................................................35
   b. Limit dextrin as the substrate.......................................................................................................................36

2.3.5. Further investigation of “inverting” glucosidase..............................................................................38
   a. \( \beta \)-Glucosyl fluoride as the substrate.........................................................................................................38
   b. Investigating the hydrolysis of D-glucal.......................................................................................................41

2.3.6. 2-Deoxy-2-fluoro-\( \alpha \)-maltotriosyl fluoride as a mechanism-based inhibitor of the transferase activity........44
   a. Synthesis of 2-deoxy-2-fluoro-\( \alpha \)-maltotriosyl fluoride........................................................................44
   b. 2-Deoxy-2-fluoro-\( \alpha \)-maltotriosyl fluoride as a mechanism-based inhibitor..............................................45

2.3.7. 4-Deoxy-\( \alpha \)-maltotriosyl fluoride as an incompetent substrate of the transferase activity............47
   a. Synthesis of 4-deoxy-\( \alpha \)-maltotriosyl fluoride..........................................................................................47
   b. Burst experiment.........................................................................................................................................47

2.3.8. Determination of enzymic reaction products....................................................................................50

2.3.9. Identification of the nucleophilic amino acid by electrospray mass spectrometry.........................54

2.3.10. Identification of the sequence of the labeled peptide.......................................................................56

2.3.11. Identification of the catalytic nucleophile.........................................................................................58
   a. Aminolysis experiment...............................................................................................................................59
   b. Sequence comparison.................................................................................................................................60

2.4. Conclusions.............................................................................................................................................60

CHAPTER 3: MECHANISTIC STUDIES ON HUMAN PANCREATIC \( \alpha \)-AMYLSCE.......................................................................................................................63

3.1. Introduction.................................................................................................................................................63

3.1.1. Biochemical role in vivo........................................................................................................................63

3.1.2. HPA is part of the \( \alpha \)-amylase superfamily..............................................................................................64

3.1.3. Assays for HPA......................................................................................................................................65

3.1.4. Inhibitor studies......................................................................................................................................66

3.1.5. General features of the catalytic mechanism........................................................................................68

3.2. Aims of this study.......................................................................................................................................68

3.2.1. Stereochemical outcome.......................................................................................................................68

3.2.2. Oligosaccharides as substrates..............................................................................................................68

3.2.3. Glycosyl fluorides as substrates............................................................................................................69

3.2.4. Design of mechanism-based inhibitors.................................................................................................70

3.3. Results and discussion...............................................................................................................................71

3.3.1. Determination of stereochemical outcome...........................................................................................71
   a. NMR method using \( \alpha \)-maltosyl fluoride as the substrate.................................................................71
   b. NMR method using maltoheptaose as the substrate.............................................................................73
c. HPLC method using maltopentaose as the substrate

3.3.2. Malto-oligosaccharides as substrates for HPA

a. “Action patterns” with malto-oligosaccharides
b. Kinetic evaluation of malto-oligosaccharides

3.3.3. Glycosyl fluorides as substrates for HPA

a. Kinetic evaluation
b. Cloned HPA in baby hamster kidney cell lines

3.3.4. Testing 2-deoxy-2-fluoro-α-maltosyl fluoride as a mechanism-based inhibitor of HPA

3.3.5. Testing 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-4-O-(α-(1,4)-D-glucosyl)-α-D-arabinohexopyranoside as a mechanism-based inhibitor of HPA

a. Synthesis
b. Time-dependent inactivation of HPA
c. Active site protection against inhibition
d. Reactivation experiment
e. Burst experiment

3.4. Conclusions

CHAPTER 4: MECHANISTIC STUDIES ON YEAST α-GLUCOSIDASE

4.1. Introduction

4.2. Aims and goals

4.3. Results and discussion

4.3.1. Reassessment of 2-deoxy-2-fluoro-α-D-glucosyl fluoride as an inactivator of yeast α-glucosidase

a. 2FoGF is a substrate, not a simple mechanism-based inactivator
b. Test for a contaminant

4.3.2. 2FoGF as a substrate for yeast α-glucosidase

4.3.3. 2,4,6-Trinitrophenyl 2-deoxy-2,2-difluoro-α-D-arabinohexopyranoside as a mechanism-based inhibitor

a. Synthesis
b. Time-dependent inactivation of yeast α-glucosidase
c. Protection experiment
d. Contamination experiment
e. Degradation 22FoGTNP by BSA

4.3.4. Identification of the catalytic nucleophile

4.4. Conclusions

CHAPTER 5: MATERIALS AND METHODS

5.1. Organic synthesis

5.1.1. Materials and routine experimental procedures

a. Analytical methods
5.1.2. Routine synthetic procedures
b. Thin-layer chromatography and silica gel column chromatography ........................................... 121
c. Solvents and reagents ......................................................... 122
d. Compounds synthesized and provided by colleagues ........ 122

5.1.3. Synthesis
a. The synthesis of \( \alpha \)-glycosyl fluorides ......................... 125
b. The synthesis of 2-deoxy-2-fluoro-\( \alpha \)-maltotriosyl fluoride .. 126
c. The synthesis of 4-deoxy-\( \alpha \)-maltotriosyl fluoride .................. 127
d. The synthesis of 4-deoxy-\( \alpha \)-maltosyl fluoride .................... 134
e. The synthesis of 2-deoxy-2-fluoro-\( \alpha \)-maltosyl fluoride .............. 141
f. The synthesis of 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-4-O-(\( \alpha \)-(1,4)-D-glucosyl)-\( \alpha \)-D-arabino-hexopyranoside ........... 142
g. The synthesis of 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-\( \alpha \)-D-arabino-hexopyranoside .................. 145

5.2. Enzyme kinetics

5.2.1. Miscellaneous procedures ........................................ 148

5.2.2. Enzymes used in this study and their assays .............. 148
a. Glycogen debranching enzyme ......................................... 148
b. \( \alpha \)-Amylase ................................................................. 149
c. Yeast \( \alpha \)-glucosidase ..................................................... 149

5.2.3. Evaluation of substrates ............................................. 149
a. Determination of \( K_m \) and \( k_{cat} \) for various substrates ........ 149
b. Detailed assay conditions ............................................... 150

5.2.4. Evaluation of reversible inhibitors ............................. 151

5.2.5. Evaluation of irreversible inhibitors ............................ 151
a. Detailed assay conditions ............................................... 151
b. Protection against inactivation ....................................... 152
c. Reactivation of irreversibly inactivated enzymes .............. 153
d. Burst experiments ......................................................... 153
e. Contamination experiments ............................................. 154

5.2.6. Determination of product distribution and kinetic parameters
by HPLC .................................................................. 155
a. HPLC instrument ............................................................. 155
b. Product distribution of the amylase catalyzed malto-oligosaccharide hydrolysis ........................................... 156
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>c. Determination of product stereochemistry by HPLC</td>
<td>156</td>
</tr>
<tr>
<td>5.2.7. Determination of the product stereochemistry by NMR</td>
<td>157</td>
</tr>
<tr>
<td>5.3. Electrospray mass spectrometry</td>
<td>157</td>
</tr>
<tr>
<td>a. Experiments with glycogen debranching enzyme</td>
<td>158</td>
</tr>
<tr>
<td>b. Experiments with yeast α-glucosidase</td>
<td>159</td>
</tr>
<tr>
<td>Appendix I: Supplementary Data</td>
<td>160</td>
</tr>
<tr>
<td>Appendix II: Theory</td>
<td>164</td>
</tr>
<tr>
<td>A-II.1. Enzyme catalysis</td>
<td>164</td>
</tr>
<tr>
<td>A-II.2. Irreversible inhibition of enzyme catalysis</td>
<td>166</td>
</tr>
<tr>
<td>A-II.3. Protection against inactivation: calculations</td>
<td>168</td>
</tr>
<tr>
<td>References</td>
<td>169</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1.1. Reversible inhibition of α-glycosidases by transition state analogues........ 12
Table 2.1. Comparison of substrates of the glucosidase and transferase activities....... 27
Table 3.1. Kinetic evaluation of malto-oligosaccharides as substrates for HPA and porcine pancreatic α-amylase................................................................. 82
Table 3.2. Kinetic evaluation of glycosyl fluorides as substrates for HPA.................. 83
Table 3.3. Active site protection/inactivation experiment...................................... 98
Table 4.1. Kinetic parameters for the reaction between 2FaGF or αGF and yeast α-glucosidase................................................................. 107
LIST OF FIGURES

Figure 1.1. The hydrolysis and glycosyl transfer reactions catalyzed by glycosidases.............................................................. 1
Figure 1.2. Catalysis with overall retention or inversion of anomeric configuration.... 2
Figure 1.3. The proposed mechanism for the hydrolysis of α-glucosides by an “inverting” α-glucosidase.................................................. 3
Figure 1.4. Overall enzymic hydrolysis of β-maltotetraosyl fluoride by β-amylase.... 4
Figure 1.5. The proposed mechanism for the hydrolysis of α-glucosides by a “retaining” α-glucosidase (or α-glucosyl transferase)............... 6
Figure 1.6. The 2-deoxy-2-fluoro glycosyl-enzyme intermediates of Agrobacterium β-glucosidase......................................................... 9
Figure 1.7. Two forms of nojirimycin in aqueous solution.................................................. 11
Figure 1.8. The half-chair conformation and resonance structure of D-glucono-(1,5) lactone........................................................................ 11
Figure 1.9. Hydration of D-glucal by a glucosidase.............................................................. 13
Figure 1.10. The proposed mechanism for the hydration of D-glucal in D2O by “retaining” and “inverting” α-glucosidases........................................... 14
Figure 2.1. The reaction of glycogen debranching enzyme with glycogen phosphorylase limit dextrin.................................................. 18
Figure 2.2. Proposed catalytic mechanism for the transferase activity of the debranching enzyme.................................................. 20
Figure 2.3. Proposed catalytic mechanism for the glucosidase activity of the debranching enzyme.................................................. 21
Figure 2.4. Conserved sequence stretches in the amylase superfamily....................... 22
Figure 2.5. Reaction scheme for the synthesis of α-maltotriosyl fluoride................ 29
Figure 2.6. Determination of the reaction products produced by the transferase activity............................................................................ 31
Figure 2.7. HPLC profiles of the products of the reaction between αG3F and glycogen debranching enzyme.................................................................. 33
Figure 2.8. HPLC profiles of the product distribution from the reaction between α-maltosyl fluoride and Glyx in the absence and presence of deoxynojirimycin................................................................... 35
Figure 2.9. Determination of the stereochemistry of reactions catalyzed by the glucosidase activity of the debranching enzyme by 1H NMR............................................. 37
Figure 2.10. 1H NMR investigation of the action of debranching enzyme on β-D-glucosyl fluoride in the presence of β-cyclodextrin......................... 40
Figure 2.11. 1H NMR investigation of hydration of D-glucal by Glyx......................... 42
Figure 2.12. Reaction scheme for the synthesis of 2-deoxy-2-fluoro-α-maltotriosyl fluoride........................................................................ 45
Figure 2.13. Proposed free energy diagrams for reactions catalyzed by β- and α-glycosidases........................................................................... 46
Figure 2.14. Reaction scheme for the synthesis of 4-deoxy-α-maltotriosyl fluoride.... 48
Figure 2.15. Reaction of 4DαG3F with the debranching enzyme................................... 49
Figure 2.16. HPLC profiles of the products formed from the reaction of 4DaG3F and Glyx in the presence of maltotriose.................................................. 51
Figure 2.17. Mechanism for accumulation of an intermediate of the debranching enzyme with 4DaG3F and turnover via transglycosylation upon addition of maltotriose.................................................. 52
Figure 2.18. Disproportionation of 4-deoxymaltohexaose by debranching enzyme in the presence of excess maltotriose.................................................. 53
Figure 2.19. ESMS/MS experiments of Glyx proteolytic digests.................................................. 55
Figure 2.20. Schematic of ESMS/MS.................................................. 56
Figure 2.21. Tandem MS/MS daughter ion spectrum of the 4DaG3F-labeled active site peptide 1.................................................. 57
Figure 2.22. Tandem MS/MS daughter ion spectrum of the 4DaG3F-labeled active site peptide 2.................................................. 59
Figure 2.23. Identified labeled peptide and the conserved sequence stretches in the amylase superfamily.................................................. 61
Figure 3.1. Hydrolysis of polysaccharides by human pancreatic α-amylase........... 63
Figure 3.2. X-ray crystal structure of HPA.................................................. 65
Figure 3.3. Mode of inhibition of porcine pancreatic α-amylase by acarbose........ 67
Figure 3.4. Mechanism of action of HPA.................................................. 69
Figure 3.5. 1H NMR determination of the stereochemistry of the products in the reaction between αG2F and HPA.................................................. 72
Figure 3.6. 1H NMR determination of the stereochemical outcome in the reaction between maltoheptaose and HPA.................................................. 74
Figure 3.7. HPLC chromatograms of reaction mixtures containing maltopentaose and HPA.................................................. 75
Figure 3.8. HPA catalyzed hydrolysis of maltopentaose.................................................. 77
Figure 3.9. “Action pattern” for HPA-catalyzed hydrolysis of maltotetraose, maltopentaose, maltohexaose, and maltoheptaose.................................................. 78
Figure 3.10. Active site of HPA.................................................. 79
Figure 3.11. Proposed additional subsite in active site.................................................. 80
Figure 3.12. The plot of catalytic efficiency vs. malto-oligosaccharide chain length.................................................. 81
Figure 3.13. HPLC analysis of the products formed in the reaction between αG2F and HPA.................................................. 85
Figure 3.14. HPLC analysis of the products formed in the reaction between 4DaG2F and HPA.................................................. 87
Figure 3.15. HPLC analysis of the products formed in the reaction between αG3F and HPA.................................................. 88
Figure 3.16. Proposed reaction scheme between αG3F and HPA.................................................. 89
Figure 3.17. The Michaelis-Menten plot for the release of fluoride ion from αGF by HPA.................................................. 90
Figure 3.18. HPLC analysis of the products arising from the reaction between αGF and HPA.................................................. 91
Figure 3.19. The Lineweaver-Burk plot for the reaction between 2FaG2F and HPA.................................................. 93
Figure 3.20. Outline of the synthesis of 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-
Figure 3.21. Inactivation of HPA by 22F0¢G2TNP.................................................. 96
Figure 3.22. Inactivation of HPA by 22F0¢G2TNP............................................. 97
Figure 3.23. Inactivation of HPA by 22F0¢G2TNP in the absence and presence of acarbose. Inactivation of HPA by 2,4,6-trinitrophenol........................................... 98
Figure 3.24. Reaction of 22F0¢G2TNP with HPA.............................................. 100
Figure 4.1. The release of fluoride ion from the reaction between 2-deoxy-2-fluoro-α-D-glucosyl fluoride and yeast α-glucosidase.......................................................... 105
Figure 4.2. Inactivation of different concentrations of yeast α-glucosidase in the presence of 2F0¢GF.......................................................... 106
Figure 4.3. Outline of the synthesis of 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-α-D-arabino-hexopyranoside.......................................................... 109
Figure 4.4. Inactivation of yeast α-glucosidase by 22F0¢GTNP.................................. 110
Figure 4.5. Inactivation of yeast α-glucosidase by 22F0¢GTNP.................................. 111
Figure 4.6. Inactivation of yeast α-glucosidase by 22F0¢GTNP without and with pretreatment of yeast α-glucosidase.............................................. 111
Figure 4.7. Inactivation of yeast α-glucosidase by 22F0¢GTNP in the absence and presence of DNJ.......................................................... 113
Figure 4.8. Proposed adduct between BSA and 2,4,6-trinitrophenol....................... 114
Figure 4.9. Partial mass spectra of peptides from a pepsin digest of yeast α-glucosidase inactivated with and without 2-deoxy-2,2-dihalo sugars.............................. 116
Figure 4.10. Daughter ions of the peak at m/z = 943 observed by tandem MS.............. 118
Figure 4.11. Base catalyzed ester hydrolysis of glycosyl-enzyme intermediate.................. 119
Figure 5.1. The Lineweaver-Burk plot for the hydrolysis of αGF by Glyx.............. 160
Figure 5.2. The Lineweaver-Burk plot for the cleavage of αG2F by Glyx .............. 160
Figure 5.3. The Lineweaver-Burk plot for the cleavage of αG3F by Glyx.............. 160
Figure 5.4. The Lineweaver-Burk plot for the hydrolysis of G3 by HPA.................. 161
Figure 5.5. The Lineweaver-Burk plot for the hydrolysis of G4 by HPA .................. 161
Figure 5.6. The Lineweaver-Burk plot for the hydrolysis of G5 by HPA.................. 161
Figure 5.7. The Lineweaver-Burk plot for the hydrolysis of G6 by HPA.................. 161
Figure 5.8. The Lineweaver-Burk plot for the hydrolysis of G7 by HPA.................. 161
Figure 5.9. The Lineweaver-Burk plot for the cleavage of αG2F by HPA.............. 162
Figure 5.10. The Lineweaver-Burk plot for the cleavage of αG2F by BHKHPA........... 162
Figure 5.11. The Lineweaver-Burk plot for the hydrolysis of 4DαG2F by BHKHPA ...... 162
Figure 5.12. The Lineweaver-Burk plot for the cleavage of αG3F by BHKHPA ........... 162
Figure 5.13. The Lineweaver-Burk plot for the hydrolysis of 4DαG3F by BHKHPA ...... 162
Figure 5.14. The Lineweaver-Burk plot for the hydrolysis of αPNPG by Glyx ........... 163
Figure 5.15. The Lineweaver-Burk plot for the cleavage of 2FαGF by Yeast α-glucosidase.......................................................... 163
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>axial</td>
</tr>
<tr>
<td>$A_{\lambda}$</td>
<td>Absorbance at wavelength $\lambda$ (nm)</td>
</tr>
<tr>
<td>Abg</td>
<td>Agrobacterium $\beta$-glucosidase</td>
</tr>
<tr>
<td>$\alpha$GF</td>
<td>$\alpha$-D-glucosyl fluoride</td>
</tr>
<tr>
<td>$\alpha$G2F</td>
<td>$\alpha$-Maltosyl fluoride</td>
</tr>
<tr>
<td>$\alpha$G3F</td>
<td>$\alpha$-Maltotriosyl fluoride</td>
</tr>
<tr>
<td>$\alpha$G6F</td>
<td>$\alpha$-Maltohexaosyl fluoride</td>
</tr>
<tr>
<td>$\alpha$G9F</td>
<td>$\alpha$-Maltononaosyl fluoride</td>
</tr>
<tr>
<td>$\alpha$PNPG2</td>
<td>p-Nitrophenyl $\alpha$-maltoside</td>
</tr>
<tr>
<td>APT</td>
<td>Attached proton test</td>
</tr>
<tr>
<td>Asp</td>
<td>L-Aspartic acid</td>
</tr>
<tr>
<td>A. wentii</td>
<td>Aspergillus wentii</td>
</tr>
<tr>
<td>BHKHPA</td>
<td>Cloned human pancreatic $\alpha$-amylase in baby hamster kidney cell lines</td>
</tr>
<tr>
<td>$\beta$GF</td>
<td>$\beta$-D-glucosyl fluoride</td>
</tr>
<tr>
<td>$\beta$CD</td>
<td>$\beta$-Cyclodextrin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Cloned deoxyribonucleic acid</td>
</tr>
<tr>
<td>2Ce2FaGCl</td>
<td>2-deoxy-2-fluoro-$\alpha$-D-arabinohexopyranosyl chloride</td>
</tr>
<tr>
<td>COSY</td>
<td>Correlation spectroscopy</td>
</tr>
<tr>
<td>4DFaG2F</td>
<td>4-Deoxy-$\alpha$-maltosyl fluoride</td>
</tr>
<tr>
<td>4DFaG3F</td>
<td>4-Deoxy-$\alpha$-maltotriosyl fluoride</td>
</tr>
<tr>
<td>DCI</td>
<td>Desorption chemical ionization</td>
</tr>
<tr>
<td>4D5</td>
<td>4-Deoxymaltopentaose</td>
</tr>
<tr>
<td>4G6</td>
<td>4-Deoxymaltohexaose</td>
</tr>
<tr>
<td>$\alpha$DKIE</td>
<td>$\alpha$-Secondary deuterium isotope effect</td>
</tr>
<tr>
<td>DAST</td>
<td>Diethylaminosulfur trifluoride</td>
</tr>
<tr>
<td>DNJ</td>
<td>Deoxynojirimycin</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E</td>
<td>Enzyme</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>Extinction coefficient</td>
</tr>
<tr>
<td>EC</td>
<td>Enzyme Commission (classification number) of the International Union of Biochemistry</td>
</tr>
<tr>
<td>$E_{\text{mg/mL}}$</td>
<td>The absorbance (per unit pathlength) of a 1 mg/mL solution</td>
</tr>
<tr>
<td>ESMS</td>
<td>Electrospray mass spectrometry</td>
</tr>
<tr>
<td>2FoGF</td>
<td>2-Deoxy-2-fluoro-$\alpha$-D-glucosyl fluoride</td>
</tr>
<tr>
<td>2FoG2F</td>
<td>2-Deoxy-2-fluoro-$\alpha$-D-maltosyl fluoride</td>
</tr>
<tr>
<td>22FoGTNP</td>
<td>2,4,6-Trinitrophenyl 2-deoxy-2,2-difluoro-$\alpha$-D-arabinohexopyranoside</td>
</tr>
<tr>
<td>22FoG2TNP</td>
<td>2,4,6-Trinitrophenyl 2-deoxy-2,2-difluoro-4-O-($\alpha$-(1,4)-D-glucosyl)-$\alpha$-D-arabinohexopyranoside</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>2FoG3F</td>
<td>2-Deoxy-2-fluoro-α-D-maltotriosyl fluoride</td>
</tr>
<tr>
<td>2FβGlcDNP</td>
<td>2,4-Dinitrophenyl 2-deoxy-2-fluoro-β-D-glucoside</td>
</tr>
<tr>
<td>G1</td>
<td>Glucose</td>
</tr>
<tr>
<td>G2</td>
<td>Maltose</td>
</tr>
<tr>
<td>G3</td>
<td>Maltotriose</td>
</tr>
<tr>
<td>G4</td>
<td>Maltotetraose</td>
</tr>
<tr>
<td>G5</td>
<td>Maltopentaose</td>
</tr>
<tr>
<td>G6</td>
<td>Maltohexaose</td>
</tr>
<tr>
<td>G7</td>
<td>Maltoheptaose</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GlcDNP</td>
<td>Glucose 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-glucoside</td>
</tr>
<tr>
<td>G2M</td>
<td>Maltose</td>
</tr>
<tr>
<td>G3M</td>
<td>Maltotriose</td>
</tr>
<tr>
<td>G4M</td>
<td>Maltotetraose</td>
</tr>
<tr>
<td>G5M</td>
<td>Maltopentaose</td>
</tr>
<tr>
<td>G6M</td>
<td>Maltohexaose</td>
</tr>
<tr>
<td>G7M</td>
<td>Maltoheptaose</td>
</tr>
<tr>
<td>Gln</td>
<td>L-Glutamate</td>
</tr>
<tr>
<td>Glu</td>
<td>L-Glutamic acid</td>
</tr>
<tr>
<td>Glyx</td>
<td>Glycogen debranching enzyme</td>
</tr>
<tr>
<td>HF-Pyridine</td>
<td>Hydrogen fluoride/Pyridine (70% HF)</td>
</tr>
<tr>
<td>HPA</td>
<td>Human pancreatic α-amylase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>Imix</td>
<td>Inactivation mixture</td>
</tr>
<tr>
<td>kcat</td>
<td>Catalytic rate constant (turnover number)</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation constant for an enzyme-reversible inhibitor complex</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis constant of a substrate</td>
</tr>
<tr>
<td>kobs</td>
<td>Pseudo first-order rate constant for inactivation</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Liquid chromatography/mass spectrometry</td>
</tr>
<tr>
<td>LRMS</td>
<td>Low resolution mass spectrometry</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionization</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Electrospray tandem mass spectrometry</td>
</tr>
<tr>
<td>NaPi</td>
<td>Sodium phosphate</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PPA</td>
<td>Porcine pancreatic α-amylase</td>
</tr>
<tr>
<td>RF</td>
<td>Range-finding</td>
</tr>
<tr>
<td>TAKA</td>
<td>α-Amylase from <em>Aspergillus oryzae</em></td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion chromatogram</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>TNP</td>
<td>2,4,6-Trinitrophenol</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>U</td>
<td>Units of enzyme activity</td>
</tr>
<tr>
<td>UV-VIS</td>
<td>Ultraviolet-visible</td>
</tr>
<tr>
<td>V_max</td>
<td>Maximum reaction velocity</td>
</tr>
</tbody>
</table>

Amino acid one letter code

<table>
<thead>
<tr>
<th>Letter</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>L-Alanine</td>
</tr>
<tr>
<td>C</td>
<td>L-Cystine</td>
</tr>
</tbody>
</table>
D  L-Aspartic acid
E  L-Glutamic acid
F  L-Phenylalanine
G  L-Glycine
H  L-Histidine
I  L-Isoleucine
K  L-Lysine
L  L-Leucine
M  L-Methionine
N  L-Asparagine
P  L-Proline
Q  L-Glutamine
R  L-Arginine
S  L-Serine
T  L-Threonine
V  L-Valine
W  L-Tryptophan
Y  L-Tyrosine
ACKNOWLEDGEMENTS

I thank my supervisor, Prof. S. G. Withers for the opportunity to work in his laboratory and collaborate with many excellent scientists. I am very grateful for the guidance and vision provided by my supervisor. I would like to thank Dr. Helene Cote for providing me with the cloned α-amylase, and Dr. Thisbe Lindhorst for allowing me to collaborate with her in the synthesis of the 4-deoxy sugars. I also thank Karen Rupitz for her technical assistance. I would also like to thank Prof. S. G. Withers and Natural Sciences and Engineering Research Council for funding throughout my doctoral studies.
DEDICATION

This thesis is dedicated to my parents,

John and Bunny Braun

and to two of the greatest people God has blessed me with,

my wife Wendy and daughter Alicia.
Chapter 1: General Introduction

1.1. Glycosidases

Carbohydrates play essential roles in energy metabolism, cell-cell recognition, and other important biological processes. Glycosyl hydrolysis and glycosyl transfer are important biochemical reactions as they are required for processes such as degradation of starch and utilization of glycogen. Glycosidases are a broad class of enzymes which carry out the hydrolysis reactions by cleaving a glycosidic C-O bond and replacing the leaving group (aglycone) with a water molecule. Glycosyl transferases perform the glycosyl transfer reactions by replacing the aglycone with another sugar molecule (see Figure 1.1).

![Figure 1.1](image)

Figure 1.1. The hydrolysis and glycosyl transfer reactions catalyzed by glycosidases.

The aglycone is usually also a sugar in the naturally occurring substrate. However, many enzymes lack specificity for this part of the substrate and as a result other glycosides such as aryl glycosides or glycosyl fluorides will also act as substrates. The naturally occurring substrates for the enzymes studied in this thesis are:
\[ \alpha\text{-Amylase} \quad R = \text{malto-oligosaccharides} \]
\[ \alpha\text{-Glucosidase} \quad R = \text{glucose} \]
\[ \text{Glycogen debranching enzyme} \quad R = \text{glycogen (limit dextrin)} \]

These two classes of enzymes are generally broken down into subclasses based on factors such as substrate specificity, and product stereochemistry. Enzymes are usually very specific towards the anomeric configuration of the substrate. For example, an \( \alpha \)-glycosidase will generally only hydrolyze \( \alpha \)-glycosides. These enzymes also show specificity towards the type of sugar ring (ie. glucose or galactose). Although some enzymes may utilize different sugars as substrates, they are classified according to the substrate for which they have the greatest activity. Lastly, enzymes catalyze the reaction with overall retention ("retaining") or inversion ("inverting") of anomeric configuration (see Figure 1.2 for an example of an \( \alpha \)-glucosidase).

**Figure 1.2.** Catalysis with overall retention or inversion of anomeric configuration.
1.2. General Features Of The Catalytic Mechanism

1.2.1. "Inverting" glycosidases

A general mechanism for "inverting" glycosidases is shown in Figure 1.3. The mechanism is believed to include the following features:

1. Acid catalysis assists in the departure of the aglycone.
2. Base catalysis promotes attack of the nucleophile.
3. Oxocarbonium ion-like transition states may be involved.
4. Various noncovalent interactions increase the rate of the reaction.

Each feature has a similar counterpart in "retaining" glycosidases and will be discussed later (see section 1.2.2).

Many "inverting" glycosidases have been found to have a carboxyl and a carboxylate group in the active site which act as the acid and base catalyst, respectively. The two residues are found to have an average distance of 9.0 Å between the carboxylate oxygens (Wang et al., 1994). This distance allows water to come in and be the nucleophile in the single displacement mechanism.

A general feature of "inverting" enzymes is their ability to not only utilize the appropriate anomer of a glycosyl fluoride as a substrate, but also the "wrong" one. For example the "inverting" β-amylase from sweet potato, which normally cleaves off the

![Figure 1.3. The proposed mechanism for the hydrolysis of α-glucosides by an "inverting" α-glucosidase.](image)
terminal disaccharide from α-(1,4) linked amylose, utilizes both α-maltosyl fluoride and β-maltosyl fluoride as substrates, with the product from both reactions being β-maltose (Hehre et al., 1979). The results with β-maltosyl fluoride were interpreted using the assumption that β-amylase first performs a glycosyl transfer reaction between two substrate molecules to yield a maltotetraoside intermediate (see Figure 1.4). Once the β-maltotetraosyl fluoride intermediate is formed, it now resembles the "natural" substrate and is hydrolyzed by β-amylase to produce β-maltose and β-maltosyl fluoride. Thus in the first step β-amylase functions as a glycosyl transferase and in the second step it carries out the "normal" reaction of a glycosidase.

![Figure 1.4. Overall enzymic hydrolysis of β-maltotetraosyl fluoride by β-amylase. R = α-D-glucose.](image)

1.2.2. "Retaining" glycosidases

In 1953 a general mechanism for "retaining" glycosidases was proposed by Koshland, and was believed to involve a double displacement reaction (Koshland, 1953). A great deal of work since then has further refined the mechanism, but its essential
features are still believed to be valid. The mechanism for a "retaining" α-glycosidase (see Figure 1.5 for an example of an α-glucosidase) is believed to include the following general features:

1. Acid catalysis assists in the departure of the aglycone.
2. The carboxylate group of an Asp or Glu residue of the protein is located near the anomeric center of the substrate on the opposite side of the aglycone.
3. A covalent glycosyl-enzyme intermediate is formed between the carboxylate of the enzyme and C-1 of the sugar.
4. Oxocarbonium ion-like transition states may be involved in both the formation and breakdown of the intermediate.
5. Various noncovalent interactions increase the rate of the reaction.

1.2.3. Acid catalysis

The departure of the aglycone leaving group is believed to be assisted by concerted protonation of the glycoside oxygen by the side chain of an acidic amino acid residue in the active site of the enzyme as the bond is cleaved. In α-amylase from Aspergillus oryzae (TAKA), Glu 230 has been identified as a catalytically important acidic residue as it was found by X-ray crystallography to be positioned to protonate the glycosidic oxygen (Matsuura et al., 1984). Mutagenesis studies of barley α-amylase confirmed the importance of the equivalent residue in this sequence-related enzyme as essentially all activity was lost when Glu 205 (Glu 230 in TAKA amylase) was converted to Gln (Sogaard et al., 1993), presumably because Gln is not able to assist in the departure of the aglycone.
Figure 1.5. The proposed mechanism for the hydrolysis of α-glucosides by a "retaining" α-glucosidase (or α-glucosyl transferase).
The importance of general acid catalysis in the glycosidase reaction mechanism is variable, and in some cases it may not occur at all. For example, it is not possible to protonate glycosyl pyridinium salts, yet the observed rate increases for the hydrolysis of these compounds by β-galactosidase is $10^8$-$10^{13}$ fold compared with the rate of spontaneous hydrolysis (Sinnott & Withers, 1974). Clearly the large rate increases by the enzyme are not due to general acid catalysis in this case.

1.2.4. **The active site nucleophile**

X-ray crystallographic studies of the β-glycosidase lysozyme provided the first evidence for the presence of a carboxylate properly positioned in the active site near the anomeric center of a bound substrate. X-ray diffraction methods have determined the structure of hen egg white lysozyme (Imoto et al., 1972), and a strategically placed carboxylate group was observed (Asp 52). The first evidence of a carboxylate group appropriately located in the active site of an α-glycosidase was demonstrated in TAKA amylase (Matsuura et al., 1984) also from X-ray crystallographic data. Subsequently, the crystal structures of several other related enzymes have been solved (human pancreatic α-amylase (Brayer et al., 1995), cylodextrin glucanotransferase (Klein & Schulz, 1991), *Aspergillus niger* α-amylase (Brady et al., 1991), bacterial α-amylase (Suzuki et al., 1990), porcine pancreatic α-amylase (Qian et al., 1993), and barley α-amylase (Kadziola et al., 1994)) and in all cases the carboxylate group was observed in the active site.

1.2.5. **Glycosyl-enzyme intermediate**

As an alternative to the covalent glycosyl-enzyme intermediate mechanism, it was suggested that a carbonium ion intermediate is formed which is stabilized by the enzyme (Phillips, 1966). X-ray structural studies of hen egg white lysozyme (Blake et al., 1967) suggested that a negatively charged carboxylate (Asp 52) could stabilize a positively charged oxocarbonium ion intermediate, and it was proposed that the lifetime of this
intermediate could be long enough to allow attack of an acceptor molecule (water or another sugar). The lifetime, however, would have to be sufficient for the leaving group to diffuse away and the acceptor diffuse in and react before the ion pair collapsed to form a covalent intermediate.

Further support for the tight ion pair intermediate recently came from the highly refined X-ray crystallographic data from two different α-amylases, barley α-amylase (Kadziola et al., 1994) and porcine pancreatic α-amylase (Qian et al., 1994). The enzymes complexed with the proposed transition state mimic acarbose (see Figure 3.3, p. 67) disfavor the formation of the covalent intermediate as the Asp residue is 3.3 Å away from C-1, some 1.8 Å longer than expected for a covalent bond (Qian et al., 1994). However, the complex that is formed with natural substrates could be different from that with transition state analogues and thus the Asp residue may be close enough to form a covalent bond.

One of the problems with the crystallographic results is that the previously mentioned complexes have been with transition state analogues, not actual substrates. A study with porcine pancreatic α-amylase using $^{13}$C-labeled maltotetraose and cryoenzymology provided evidence for the formation of a covalent bond in a β-glycosyl-enzyme intermediate (Tao et al., 1989). More recently an α-glucosyltransferase from Streptococcus sobrinus has been tagged with radiolabeled fructose in a rapid quench experiment (Mooser et al., 1991). Peptide digests were purified and sequenced and the Asp which corresponds to Asp 206 from TAKA amylase was found to be covalently labeled.

α-Secondary deuterium kinetic isotope effect (αDKIE) studies using E. coli β-galactosidase and a series of arylgalactosides provided additional evidence for the formation of a covalent glycosyl-enzyme intermediate (Sinnott & Souchard, 1973). Substrates for which glycosylation is rate-limiting exhibited $k_H/k_D$ values of 1.15-1.20, and those for which deglycosylation is rate-limiting had $k_H/k_D$ values of 1.20-1.25.
αDKIE values above unity (k_H/k_D > 1) indicate that there is an increase in sp² character at
the anomeric center as the substrate goes from the ground state to the transition state in
the reaction. Conversely, an αDKIE value below unity (k_H/k_D < 1) indicates an increase
in sp³ character. The Koshland mechanism requires both steps to have αDKIE values
greater than 1.0, because there is more sp² character in both transition states than in their
preceeding ground states (see Figure 1.5). Therefore, the fact that αDKIE values
determined experimentally in both steps were greater than 1.0, indicates that a covalent
intermediate is formed.

Inactivation studies of Agrobacterium β-glucosidase (Abg) with an appropriate 2-
deoxy-2-fluoro-β-D-glycoside mechanism-based inhibitor provided strong evidence that
the glycosidase reaction mechanism involves the formation of a covalent glycosyl-
enzyme intermediate. The substitution of an electron-withdrawing fluorine atom at C-2
in the 2-deoxy-2-fluoro glycoside inductively destabilizes both transition states (see
Figure 1.5). This results in a decrease in the rate of formation (k_2) and the rate of
hydrolysis (k_3) of the glycosyl-enzyme intermediate. The presence of a highly reactive
leaving group (2,4-dinitrophenolate or fluoride) will result in an increase in k_2 only. The
net effect (inductive effect and leaving group ability) results in the accumulation of a
glycosyl-enzyme intermediate. The reaction of Abg with 2,4-dinitrophenyl 2-deoxy-2-
fluoro-β-D-glucoside (2FβGlcDNP) resulted in the isolation of a 2-deoxy-2-fluoro

![Figure 1.6. The 2-deoxy-2-fluoro glycosyl-enzyme intermediates of Agrobacterium β-glucosidase.](image)
glucosyl-enzyme intermediate whose half-life was over 500 hours. Thus when the radiolabeled inhibitor \([1^-{\text{3}}H]-2F\beta\text{GlcDNP}\) was mixed with Abg, it was found to be covalently attached to the nucleophile Glu 358 (Withers et al., 1990). Further work involving \(^{19}\text{F} \text{NMR}\) studies of a 2-deoxy-2-fluoro mannosyl-enzyme intermediate of Abg showed that the sugar was covalently attached to the enzyme through an \(\alpha\)-glycosidic linkage (Withers & Street, 1988) (see Figure 1.6).

1.2.6. Oxocarbonium ion-like transition states

A transition state analogue is a reversible inhibitor whose structure resembles the transition state of the enzyme catalyzed reaction. These analogues generally bind more tightly to the enzyme's active site than do normal substrates, which agrees with the hypothesis that the enzyme's active site is designed to stabilize the transition state more so than the ground state (Pauling, 1948). Two features would exist if oxocarbonium ion-like character was present in the transition states of an enzymic reaction: 1) the O-5 and C-1 would share a partial positive charge; and 2) the O-5, C-5, C-1, and C-2 atoms would tend to be coplanar (Sinnott, 1987). Compounds with one or both of these features have been found to be tight-binding, reversible inhibitors of glycosidases. An example of one such compound is nojirimycin, as it potentially contains a positively charged nitrogen atom and is known to exist in two such forms (see Figure 1.7). Each form is believed to act as a glycosidase inhibitor by forming an ion pair with a negatively charged amino acid in the active site (Legler, 1990).

The dissociation constants for binding of 5-amino-5-deoxy-D-glycopyranose (eg. nojirimycin) with several different glycosidases are shown in Table 1.1. As can be seen, all dissociation constants are in the low micromolar to sub-micromolar region; much lower than those for substrates or products. For example, nojirimycin binds \(~10,000\)-fold tighter than the ground state analogue glucose \((K_m = 32 \text{ mM})\) (Nelson et al., 1979) to glycogen debranching enzyme.
Another example of a transition state analogue is D-glucono-(1,5)-lactone, which binds 300-fold better with \( \beta \)-glucosidase (\( A. \) \textit{wentii} \) than does glucose (Legler et al., 1980). Once again, the coplanarity between C-5 and C-2, and the partial positive charge on O-5 in the lactone resembles the transition state in a glycosidase catalyzed reaction (see Figure 1.8).

Further evidence for the presence of oxocarbonium ion-like transition states came from studies using \( \alpha \text{DKIE} \) with \( \beta \)-glycosidases. The results of Kempton and Withers (1992) using a series of aryl glycosides and Abg showed that both the glycosylation and the deglycosylation transition states had oxocarbonium ion-like character (see Figure 1.5). It was also found that the \( \alpha \text{DKIE} \) values were higher for the deglycosylation step \( (k_{H}/k_{D} = 1.11) \) than the glycosylation step \( (k_{H}/k_{D} = 1.06) \), which suggests more oxocarbonium ion-like character in the second transition state.
Table 1.1. Reversible inhibition of α-glycosidases by transition state analogues.

<table>
<thead>
<tr>
<th>Enzyme and source</th>
<th>Inhibitor (5-amino-5-deoxy)</th>
<th>Ki (μM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-D-Glucosidase (Yeast)</td>
<td>-D-glucopyranose</td>
<td>6.3</td>
<td>b</td>
</tr>
<tr>
<td>β-D-Glucosidase (<em>A. wentii</em>)</td>
<td>-D-glucopyranose</td>
<td>0.36(^a)</td>
<td>c</td>
</tr>
<tr>
<td>α-D-Galactosidase (<em>E. coli</em>)</td>
<td>-D-galactopyranose</td>
<td>0.17(^a)</td>
<td>d</td>
</tr>
<tr>
<td>β-D-Galactosidase (<em>E. coli</em>)</td>
<td>-D-galactopyranose</td>
<td>0.045(^a)</td>
<td>d</td>
</tr>
<tr>
<td>α-D-Mannosidase (Almonds)</td>
<td>-D-mannopyranose</td>
<td>21</td>
<td>e</td>
</tr>
<tr>
<td>β-D-Mannosidase (<em>A. wentii</em>)</td>
<td>-D-mannopyranose</td>
<td>7.7</td>
<td>e</td>
</tr>
<tr>
<td>Glycogen debranching enzyme (Rabbit muscle)</td>
<td>-D-glucopyranose</td>
<td>3.9</td>
<td>f</td>
</tr>
</tbody>
</table>

\(a\) slow approach to the inhibition equilibrium; \(^b\) (Hanozet et al., 1981); \(^c\) (Legler et al., 1980); \(^d\) (Legler & Pohl, 1986); \(^e\) (Legler & Julich, 1984); \(^f\) (Nelson et al., 1979)

1.2.7. The binding energies attributable to noncovalent interactions

Pauling, in 1946, first proposed the hypothesis that most of the catalytic power of an enzyme comes from noncovalent interactions between the enzyme and the substrate at the transition state (Pauling, 1946). The importance of noncovalent interactions has been elucidated by replacing the hydroxyl groups of a substrate with a hydrogen or a fluorine atom. Studies with *A. wentii* β-glucosidase revealed that when the C-2 hydroxyl group of glucose is replaced by H, the rate of hydrolysis dropped by a factor of 10\(^6\) (Roeser & Legler, 1981). The C-2 hydroxyl group thus makes a critical interaction with the enzyme that enables transition state stabilization.

Studies with the glycosyl transferase, glycogen phosphorylase also revealed the importance of various hydroxyl groups in noncovalent interactions. For example, when
the C-6 hydroxyl group of glucose-1-phosphate was replaced by H, the rate of transfer onto glycogen decreased 10^4-fold (Street et al., 1989).

1.3. Enzyme-Catalyzed Hydration Of Glycals

Glucal, which has a double bond between C-1 and C-2, is a substrate for glucosidases and is hydrated by the enzyme to produce 2-deoxy-D-glucose (Figure 1.9). Initially, glucal was thought to be a tight binding inhibitor, but the apparent binding was due to the slow deglycosylation and therefore accumulation of a glycosyl-enzyme intermediate. The stereochemistry of the hydration has been investigated with a "retaining" α-glucosidase and the product found to be 2-deoxy-α-D-glucose (Hehre et al., 1977). Unfortunately, experiments with "inverting" α-glucanases were unable to determine the stereochemical outcome because the rate of hydration was much slower than the rate of anomerization (Chiba et al., 1988). The product is probably 2-deoxy-β-D-glucose because this would be the same stereochemical outcome as observed with glycoside hydrolysis by "inverting" α-glucosidases.

![Figure 1.9. Hydration of D-glucal by a glucosidase](image)

1H NMR studies of the hydration reaction in D_2O revealed further information concerning the stereochemistry of protonation at C-2. The results for both "retaining" and "inverting" α-glucosidases are shown in Figure 1.10. The "inverting" α-glucosidase protonates D-glucal from below the plane of the ring (Figure 1.10B); whereas, the "retaining" α-glucosidase from above the plane of the ring (Figure 1.10A). These results
Figure 1.10. The proposed mechanism for the hydration of D-glucal in D₂O by "retaining" (A) and "inverting" (B) α-glucosidases.

demonstrate another fundamental difference in the mechanisms of glycosidases that result in retention and inversion of anomeric configuration.

1.4. Aims Of This Thesis

1.4.1. Research significance and objectives

Substrates and mechanism-based inhibitors are useful in studying enzyme reaction mechanisms. Mechanism-based inhibitors that result in a stabilized glycosyl-enzyme intermediate would aid in the identification of important catalytic residues in the
enzyme active site and thus a better understanding of their specific roles in catalysis would be obtained. These inhibitors may also lead towards the development of, or find applications as, therapeutic drugs. The aim of this thesis was to investigate the mechanism of action of glycogen debranching enzyme and of human pancreatic α-amylase. The approaches include: kinetic studies with oligosaccharide substrates and glycosyl fluorides; and "trapping" of intermediates in catalysis and thereby identification of the nucleophilic residue

1.4.2. Studies testing substrates for α-glycosidases and α-glycosyl transferases.

Glycosidases show considerable specificity for the glycone, but relatively low specificity for the aglycone moiety of the substrate. Leaving groups such as fluorides have been utilized by many α-glycosidases as substrates (Hehre et al., 1973). Based on this finding glycosyl fluorides were synthesized and tested as substrates for α-glycosidases and α-glycosyl transferases, the purpose being to develop artificial substrates which may be used as better assay systems.

Another aim of this study was to do a complete kinetic investigation on human pancreatic α-amylase testing malto-oligosaccharides as substrates. The initial step will be to establish the kinetic mechanism and specificity by determining "action patterns" for degradation of defined oligosaccharide substrates by this enzyme.

1.4.3. Trapping glycosyl-enzyme intermediates.

The aim of this study concerns the identification of the active site nucleophile, and three approaches are proposed. The first approach involves the synthesis of a derivative of a glycosyl fluoride, in which the hydroxyl group at the C-2 position is replaced by a fluorine atom. These analogues should allow for the accumulation of a glycosyl-enzyme intermediate on glycogen debranching enzyme and on human pancreatic α-amylase. The second approach involves the synthesis and testing of a novel
covalent inhibitor, in which the C-2 hydroxyl group and hydrogen are replaced by two fluorine atoms. The aglycone of this glycosyl analogue will be 2,4,6-trinitrophenol, which will make an excellent leaving group, so that the enzyme will be able to carry out the first step in catalysis, forming an intermediate, but this will not hydrolyze. The third approach involves the synthesis of a derivative of maltotriosyl fluoride in which the C-4 hydroxyl group is replaced by hydrogen. 4-Deoxy-α-maltotriosyl fluoride will be investigated as an "incompetent" substrate analogue of the glycosyl transferase activity of glycogen debranching enzyme. Electrospray mass spectrometry will be used to attempt to determine the identity of the amino acid nucleophile in "trapped" glycosyl-enzyme intermediates.
Chapter 2: Mechanistic Studies On Rabbit Muscle Glycogen Debranching Enzyme

2.1. Introduction

2.1.1. Biochemical role in vivo.

Glycogen debranching enzyme (Glyx, amylo-1,6-glucosidase [EC 3.2.1.33] / 4-α-glucanotransferase [EC 2.4.1.25]) plays an important role in carbohydrate metabolism. It works in conjunction with glycogen phosphorylase in the total degradation of glycogen. Glycogen phosphorylase removes glucose units via phosphorolysis from the α-(1,4) chains in glycogen until a branch point is reached. Glyx then removes the α-(1,6) branch points from glycogen phosphorylase limit dextrin by a two step mechanism (see Figure 2.1). The first step requires a glycosyl transferase activity to transfer a maltotriose unit from the four sugar branch to the "main" chain, resulting in an elongated section of α-(1,4) polymer, with a single glucose attached to it via an α-(1,6) linkage. The enzyme then utilizes a glucosidase activity to hydrolyze the glucose stub, thus allowing glycogen phosphorylase to continue to degrade the linearized α-(1,4) polymer. Both the transferase and the glucosidase activities are found on a single polypeptide chain (Brown & Brown, 1966; Nelson et al., 1979) that has recently been determined to have a molecular weight of 177,542 Da (Liu et al., 1993) (rabbit muscle). In humans, defects in the gene (AGL), coding for glycogen debranching enzyme, are responsible for type III glycogen storage disease (Forbes' disease), which is characterized by hepatomegaly, hypoglycemia, short stature, and variable myopathy. The net effect is that glycogen phosphorylase can only degrade glycogen up to the first branch point, with limit dextrin being the end product.
2.1.2. Assays of the debranching enzyme

The two enzyme activities have been classically assayed by their combined action on glycogen phosphorylase limit dextrin (Gillard & Nelson, 1977). The enzyme activities can also be assayed independently; as follows. The glucosidase activity has been assayed by following the reaction in reverse and monitoring the incorporation of radiolabeled D-glucose into glycogen (Nelson & Larner, 1970). The transferase activity has been assayed by monitoring the complexation of amylopectin with iodine, which increases with the length of the chain (Nelson et al., 1970) and more recently by following the disproportionation of oligosaccharides (Tabata & Dohi, 1992).

![Diagram](image)

**Figure 2.1.** The reaction of glycogen debranching enzyme with glycogen phosphorylase limit dextrin (α-(1,6) linkages ‡, and α-(1,4) linkages —).
2.1.3. One or two active sites?

Both the transferase and glucosidase activities are required in order to accomplish the debranching of limit dextrin. However, it was not clear whether the reactions take place at one or two active sites. Initial inhibition studies with 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), which is typically a competitive inhibitor of glucosidases, found it to be a non-competitive inhibitor of Glyx when using limit dextrin as the substrate (combined assay) (Nelson et al., 1969). It was determined that the inhibition was of the "simple linear" dead-end type (Cleland, 1963), which suggests that the enzyme can simultaneously bind the inhibitor and substrate equally well. This was the first evidence for different binding sites for the glucosidase and transferase activities. The result was confirmed when Tris was found to be a competitive inhibitor of Glyx using the glucose-reincorporation assay. More recently, it was found that the transferase activity could be inactivated by a carbodiimide in the presence of an amine, without any appreciable effect upon the glucosidase activity (Liu et al., 1991). This experiment provided the first example of a covalent modification of the transferase site only, clearly distinguishing it from the glucosidase site. These results suggested that the glucosidase activity and transferase activity were due to two separate active sites.

2.1.4. General features of the catalytic mechanism

Glyx catalyses two separate reactions. The first of these is a glycosyl transfer in which an $\alpha$-(1,4) bond is cleaved and a new $\alpha$-(1,4) bond is formed. Clearly this reaction is occurring with retention of anomeric configuration, most likely through a double displacement mechanism as shown in Figure 2.2. The second reaction is that of an $\alpha$-glucosidase in which an $\alpha$-(1,6) glucoside linkage is hydrolyzed. The stereochemical outcome of this reaction was investigated using limit dextrin as substrate and following the reaction by polarimetry (Nelson & Larner, 1970). The conclusion of this study was that the reaction occurred with net retention of configuration, thus again
likely via a double displacement mechanism (Figure 2.3). However, as will become apparent, there was reason to doubt this suggestion.

**Figure 2.2.** Proposed catalytic mechanism for the transferase activity of the debranching enzyme
2.1.5. Glyx is part of the α-amylase superfamily

Recently it has been concluded, on the basis of sequence alignments, that glycogen debranching enzyme is a member of the α-amylase superfamily (Jespersen et al., 1993). The three dimensional structures of enzymes of this family reveal that the catalytic domain has a (β/α)8-barrel structure. Despite the fact that they are part of a
<table>
<thead>
<tr>
<th></th>
<th>Consensus Sequence 1</th>
<th>Consensus Sequence 2</th>
<th>Consensus Sequence 3</th>
<th>Consensus Sequence 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α-Amylase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>111 GMYLVMVDVANH</td>
<td>201 DGLRIDTVKH</td>
<td>226 YCIAGEVLD</td>
<td>289 LGTFVENHD</td>
</tr>
<tr>
<td>Barley 1</td>
<td>82 GVQAIADIVNH</td>
<td>175 DAVRLDFARG</td>
<td>201 LAVAENWD</td>
<td>283 AATFVDNHD</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>88 GVRITYDVPNFH</td>
<td>181 AGFRVDAAKH</td>
<td>219 YIQSFVID</td>
<td>279 SLVFVDNHD</td>
</tr>
<tr>
<td>Porcine pancreas</td>
<td>90 GVRIVYDAVINH</td>
<td>192 AGFLDASKH</td>
<td>229 FIPQFVID</td>
<td>292 ALVFVDNHD</td>
</tr>
<tr>
<td>Human pancreas</td>
<td>90 GVRIVYDAVINH</td>
<td>192 AGFLDASKH</td>
<td>229 FIPQFVID</td>
<td>292 ALVFVDNHD</td>
</tr>
<tr>
<td><strong>α-Glucosidase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>100 GMKFITDLVINH</td>
<td>170 DGFRIEDTGL</td>
<td>272 MRVGFEVAH</td>
<td>341 ATTYIEH</td>
</tr>
<tr>
<td><strong>α-Glucosyltransferase (GTase-I)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus sobrinus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclodextrinase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus sphaericus</em></td>
<td>232 GMRVLLDAVFNH</td>
<td>321 DGWRDLVANE</td>
<td>351 YILGEIMH</td>
<td>414 SPNLGSHD</td>
</tr>
<tr>
<td>Pullulanase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em></td>
<td>596 GMNVIMDVYNYH</td>
<td>672 DGFRFDMG</td>
<td>702 YPPFEGWD</td>
<td>826 VVNYVSHD</td>
</tr>
<tr>
<td>Isoamylase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas amyloidesmosa</em></td>
<td>298 GIKVYMDVVYNH</td>
<td>369 DGFRFDMIS</td>
<td>412 RILREFTV</td>
<td>499 SINFIDVDH</td>
</tr>
<tr>
<td>Oligo-1,6-glucosidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>92 NHKLMDLVLNHN</td>
<td>194 DGFRMDVINF</td>
<td>251 MTVGEMPG</td>
<td>321 NSLYWNNHD</td>
</tr>
<tr>
<td>Cyclodextrin glucantransferase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus macerans</em></td>
<td>129 NIKVVMDFAPNH</td>
<td>224 DGRFIAVKH</td>
<td>254 FTFGEWFL</td>
<td>321 MVTIFDNDH</td>
</tr>
<tr>
<td>Branching enzyme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>328 GLNVILDWPGH</td>
<td>399 DAVLVDAAS</td>
<td>453 VTMAESEST</td>
<td>517 NFVLPLNDH</td>
</tr>
<tr>
<td>Amylomaltase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus pneumonia</em></td>
<td>218 VAEDSSDMWAND</td>
<td>290 DIVRIDHFRG</td>
<td>328 AAVKBEGL</td>
<td>388 SVMYTGTHD</td>
</tr>
<tr>
<td>Glycogen debranching enzyme</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human muscle</td>
<td>192 NVICITDVYNH</td>
<td>504 QGVRLDNCNS</td>
<td>534 YVVAEFLFT</td>
<td>602 ALFMDITHD</td>
</tr>
<tr>
<td>Rabbit muscle</td>
<td>232 NVLCITDVYNH</td>
<td>544 QGVRLDNCNS</td>
<td>574 YVVAEFLFT</td>
<td>642 ALFMDITHD</td>
</tr>
</tbody>
</table>

Figure 2.4. Conserved sequence stretches in the amylase superfamily
family the sequence similarity between different enzymes is relatively low, and only 8 amino acid residues of the barrel domains have been found to be invariant (Svensson, 1994). A list of the four consensus sequences of the enzymes in this family is shown in Figure 2.4. Based on X-ray crystallographic data, three key carboxylic acid residues, which are completely conserved, are located in the active site (Matsuura et al., 1984). From the crystal structure it was proposed that Glu 230 (TAKA amylase) is the acid catalyst, Asp 206 is the nucleophile, and Asp 297 activates the acceptor water molecule.

2.2. The Aims Of This Study

The aims of this study were three-fold: 1) determination of the stereochemical outcome of the glucosidase reaction; 2) development of a simple, continuous assay system which allows specific, independent monitoring of the two separate activities; and 3) investigation with mechanism-based inhibitors to obtain a more detailed understanding of the reaction mechanism by identifying catalytically important amino acid residues in the active sites.

2.2.1. Stereochemical outcome

Since some doubt existed concerning the stereochemical outcome of the glucosidase activity (see 2.2.3) as determined by monitoring the cleavage of limit dextrin by polarimetric studies, the aim of this study was to use two other techniques, NMR and HPLC, to determine the stereochemistry of the products.

2.2.2. Assay systems

The aim of this study was to synthesize glycosyl fluorides and evaluate them as substrates of Glyx whose turnover could be followed using a fluoride ion selective
electrode. Based on the normal reaction catalyzed by Glyx, α-glucosyl fluoride (1) would be a candidate substrate for the glucosidase activity, and α-maltotriosyl fluoride (2) for the transferase activity. The disaccharide α-maltosyl fluoride (3) would allow testing of the specificity of both sites.

2.2.3. Mechanism-based inhibitors of Glyx

Withers et al. (1988) have shown that 2-deoxy-2-fluoroglycosides rapidly inactivate "retaining" β-glycosidases and act as useful probes for studying the reaction mechanism of these enzymes. Less success, however, was obtained with α-glycosidases. Since yeast α-glucosidase was one of only two α-glycosidases that was inactivated, though only partially, this study was extended to examine the "retaining" α-glucosidase activity of Glyx. 2-Deoxy-2-fluoro-α-glucosyl fluoride (4) was therefore evaluated as a mechanism-based inhibitor of the glucosidase activity of Glyx by collaborators at the University of Alberta and they found that the glycoside did not inactivate the glucosidase activity, but rather functioned as a slow substrate. Presumably the 2-deoxy-2-fluoroglycoside did not inactivate the glucosidase activity either because the fluorine at the C-2 position did not slow the second step in the reaction sufficiently or because this reaction does not occur via a double displacement mechanism. This result was what
initially prompted us to reevaluate the stereochemical outcome of the glucosidase reaction.

By contrast, the transferase is known to be a retaining enzyme thus 2-deoxy-2-fluoro-α-maltotriosyl fluoride (5) was synthesized and tested as a mechanism-based inhibitor of the transferase activity.

Another aim of this study was to design a different approach to the accumulation of the glycosyl-enzyme intermediate in transferases such as glycogen debranching enzyme. This approach is based upon the main difference between glycosidases and glycosyl transferases, which is in the second step of the mechanism. A glycosidase utilizes water as the acceptor, whereas a transferase requires another sugar molecule with a free hydroxyl group to be the acceptor in order for the glycosyl-enzyme intermediate to turn over. Therefore, by making a substrate which is deoxygenated at the 4-position, the transferase activity of the debranching enzyme will be able to perform the first step (glycosylation) but unable to carry out the second step (deglycosylation), and accumulation of an intermediate should occur. 4-Deoxy-α-maltotriosyl fluoride (6) was
thus synthesized and tested for accumulation of an intermediate with glycogen debranching enzyme.

2.3. Results and Discussion

2.3.1. Kinetic evaluation of α-glucosyl fluoride as a substrate of the glucosidase activity of Glyx

Glycosidases have been shown to utilize artificial sugars, such as glycosyl fluorides, as substrates (Hehre et al., 1973; Kitahata et al., 1981; Van Hofsten, 1961). The reactions with glycosyl fluorides can be followed simply by monitoring the release of fluoride ion with an ion-selective electrode in a continuous fashion (Hehre et al., 1979). Since the glucosidase activity of Glyx normally clips off a glucose stub, it seemed reasonable to evaluate α-D-glucosyl fluoride (αGF) as a substrate of the glucosidase activity of Glyx. αGF was synthesized according to a published procedure (Hayashi et al., 1984). Kinetic evaluation was done in collaboration with Professor Madsen's laboratory at the University of Alberta, and αGF was found to be a substrate for the debranching enzyme with the values of $k_{\text{cat}}$ and $K_m$ (see A-II.1 p. 164) shown in
Table 2.1. Comparison of substrates of the glucosidase and transferase activities

<table>
<thead>
<tr>
<th>Substrate + additions</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m$(min$^{-1}$mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-glucosyl fluoride$^a$</td>
<td>4.2 ± 0.4</td>
<td>1104 ± 44</td>
<td>263 ± 36</td>
</tr>
<tr>
<td>$^{[14]C}$ glucose + glycogen$^{a,b}$</td>
<td>32</td>
<td>1505</td>
<td>42</td>
</tr>
<tr>
<td>p-nitrophenyl α-Glucoside$^a$</td>
<td>0.8 ± 0.3</td>
<td>4.7 ± 0.5</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>α-maltotriosyl fluoride$^c$</td>
<td>42 ± 10</td>
<td>31 ± 4</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>α-maltotriosyl fluoride + glycogen (0.1%)$^c$</td>
<td>11 ± 1</td>
<td>27 ± 1</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>α-maltotriosyl fluoride + glycogen (1.0%)$^c$</td>
<td>11 ± 1</td>
<td>44 ± 1</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>α-maltotriosyl fluoride + glycogen (9.8%)$^c$</td>
<td>15 ± 2</td>
<td>80 ± 4</td>
<td>5.3 ± 1.0</td>
</tr>
<tr>
<td>maltopentaose$^{c,d}$</td>
<td>&gt;12</td>
<td>89</td>
<td>7.4</td>
</tr>
</tbody>
</table>

$^a$ glucosidase activity. $^b$ Nelson and Larner (1970) the estimate of their $k_{cat}$ is uncertain. $^c$ transferase activity. $^d$ (Tabata & Dohi, 1992) $k_{cat}$ and $K_m$ values estimated from the double reciprocal plot.

Table 2.1. The overall catalytic efficiency ($k_{cat}/K_m$) for αGF was much greater than that found for the other two substrates to which it was compared. The reasonable Michaelis constant ($K_m$) and relatively high $k_{cat}$ make αGF an ideal substrate for assay of the glucosidase activity. The use of αGF also provides a convenient method for measuring the glucosidase activity without the complication of having to measure radioactivity and have glycogen present, as in the $^{[14]C}$ glucose incorporation assay. Further it is completely free of any dependence on the transferase activity - unlike the $^{[14]C}$ glucose incorporation assay whose rates are increased by subsequent back transfer by the transferase, thereby covering the site.
2.3.2. Kinetic evaluation of α-maltotriosyl fluoride as a substrate of the transferase activity

The normal reaction catalyzed by the transferase activity involves the transfer of a trisaccharide unit from the branch to the main chain (see Figure 2.1). In view of the ability of the glucosidase activity to utilize αGF, and given the ability of other polysaccharide degrading enzymes such as α-amylase to use malto-oligosaccharyl fluorides as substrates (Hehre et al., 1973) it seemed reasonable that α-maltotriosyl fluoride (αG3F) would be a substrate of the transferase activity.

a) Synthesis of α-maltotriosyl fluoride (αG3F)

The method used to synthesize αGF (Hayashi et al., 1984) was initially applied to the synthesis of αG3F, but unfortunately the longer time required (18 h vs. 2 h) resulted in extensive cleavage of the glycosidic linkages. Therefore a modification of the above procedure was used as outlined in Figure 2.5.

Maltotriose was acetylated according to the procedure reported by Wolf from et al. (1949), then the per-O-acetate was selectively deprotected at the anomeric center using hydrazine acetate (Excoffier et al., 1975) to produce deca-O-acetyl maltotriose. The yield of this reaction is quite good for monosaccharides, but appears to be lower with longer oligosaccharides. The reaction did not go to completion even when more hydrazine acetate was added to the reaction mixture. In the second step diethylamino sulfur trifluoride (DAST) was used to obtain the glycosyl fluoride (Posner & Haines, 1985). The product consisted of two diastereomers, β-maltotriosyl fluoride per-O-acetate (major) and α-maltotriosyl fluoride per-O-acetate (minor) as determined by $^{19}$F NMR analysis. The final step required the anomerization of the fluoride
Figure 2.5. Reaction scheme for the synthesis of α-maltotriosyl fluoride (2)

to the thermodynamically more stable α anomer. HF-pyridine was used, but even using the short time required for anomerization (15-30 min) substantial cleavage of the product was observed. The yield seemed to depend upon the condition of the HF-pyridine, as earlier results on small scale reactions were much better as analyzed by TLC. Standard deprotection using sodium methoxide in methanol resulted in the synthesis of αG3F.
b) Substrate evaluation

$\alpha$G3F was evaluated as a substrate for the transferase activity using a fluoride ion selective electrode to monitor the extent of the reaction. The kinetic constants determined are shown in Table 2.1. The maximum concentration of $\alpha$G3F attained (67 mM) was only two times the estimated $K_m$. Nonetheless, the slope of the initial linear portion of the plot yielded a value of $k_{cat}/K_m = 0.7 \text{ min}^{-1}\text{mM}^{-1}$, substantially lower than the value of $k_{cat}/K_m = 7.4 \text{ min}^{-1}\text{mM}^{-1}$ observed for maltopentaose (Tabata & Dohi, 1992). A large part of the reason for the lower catalytic efficiency is most likely that the transferase activity requires a specific polymer to both act as the acceptor and fulfill binding requirements in the ground state. Quite probably another molecule of $\alpha$G3F doesn't fully satisfy these requirements.

To be sure the reaction was occurring at the transferase site, the products formed in the reaction between $\alpha$G3F and Glyx were analyzed by HPLC (Figure 2.6C). Comparison with standard malto-oligosaccharides, Figure 2.6A, reveals that only very small amounts of maltotriose were produced. However, two new peaks of longer retention time, corresponding to longer oligosaccharides, were observed (Figure 2.6C). These presumably correspond to $\alpha$-maltohexaosyl fluoride ($\alpha$G6F) (8.5 min) and $\alpha$-maltononaosyl fluoride ($\alpha$G9F) (11 min). Since no standards of these fluorides were available, the reaction mixture was boiled to hydrolyze the fluoride to the free malto-oligosaccharides for which there are standards. The HPLC profile of this reaction mixture is shown in Figure 2.6B, and clearly reveals the formation of maltotriose (from the excess $\alpha$G3F) and maltohexaose (5.2 min), plus a peak likely corresponding to maltononaose (6.5 min). Therefore reaction is occurring at the transferase site, the products formed being longer malto-oligosaccharyl fluorides (ie. $\alpha$G6F and $\alpha$G9F).
Figure 2.6. Determination of the reaction products produced by the transferase activity

A) HPLC profile of the malto-oligosaccharide standards G2-G7. Chromatographic conditions are provided in Materials and Methods. B) HPLC profile of the hydrolyzed mixture of C). C) HPLC profile of the products of the reaction between αG3F and glycogen debranching enzyme. In B) and C) the peaks marked X at 2 min and 3 min are due to NaPi buffer and glycerin (from Centricon membrane), respectively.
The effects of inclusion of glycogen into the reaction mixture were investigated to see if the binding of \( \alpha \text{G3F} \) could be improved. Table 2.1 shows the Michaelis-Menten parameters determined for reaction in the presence of different concentrations of glycogen. As can be seen, \( K_m \) values are significantly lowered upon addition of glycogen, and \( k_{cat}/K_m \) values raised as glycogen concentrations increase. There are two possible explanations for this behavior. One is that the binding of glycogen results in conformational changes which improve the affinity of the enzyme for the \( \alpha \text{G3F} \) substrate. The other is that glycogen acts as a more effective acceptor of the maltotriosyl moiety than does \( \alpha \text{G3F} \) itself. This second explanation can be verified by HPLC product analysis. Reactions of \( \alpha \text{G3F} \) with Glyx in the presence of three glycogen concentrations (0, 0.2, and 3%) were all followed to the same extent of reaction (30% of total fluoride released). The reactions were stopped by passing the mixtures through a 100,000 molecular weight cutoff membrane, which removes both Glyx and glycogen. If \( \alpha \text{G3F} \) is a better acceptor, then \( \alpha \text{G6F} \) would be the major product; whereas, if glycogen is a better acceptor, transfer would occur onto glycogen, resulting in elongation of the polymer with no production of \( \alpha \text{G6F} \). The results can be seen in Figure 2.7. In the absence of glycogen (Figure 2.7A), relatively large amounts of \( \alpha \text{G6F} \) are formed, but as the glycogen concentration increased to 0.2% (Figure 2.7B) and 3% (Figure 2.7C) the amount of \( \alpha \text{G6F} \) formed decreased substantially. These results confirm that glycogen at higher concentrations is a better acceptor than \( \alpha \text{G3F} \), as evidenced by the decrease in the amount of \( \alpha \text{G6F} \) formed.

### 2.3.3. \( \alpha \)-Maltosyl fluoride as a substrate of Glyx

Assay systems have been developed for the glucosidase and transferase activities using \( \alpha \text{GF} \) and \( \alpha \text{G3F} \), respectively. It was of interest to see how specific these active sites are for the glucosyl and maltotriosyl moieties. The disaccharide derivative \( \alpha \)-
Figure 2.7. HPLC profiles of the products of the reaction between $\alpha$G3F and glycogen debranching enzyme.

In the presence of the different glycogen concentrations: A) 0%; B) 0.2%; and C) 3%. The peaks marked X at 2 min and 3 min are due to NaPi buffer and glycerin (from Centricon membrane), respectively. The additional peak in C) also marked X is due to glucose from the glycogen preparation.
maltosyl fluoride ($\alpha$G2F; synthesized according to a published procedure (Hayashi et al., 1984)) was therefore tested as a substrate of Glyx. The first experiment was to determine what products, if any, would be formed in the reaction with Glyx, and the results can be seen in Figure 2.8A. By integrating the area of each peak it was determined that 80% of the product arose from transglycosylation, of which $\alpha$-maltotetraosyl fluoride was the major component, while 20% was the hydrolysis product maltose. This result therefore differs from those obtained with both $\alpha$GF and $\alpha$G3F, as reaction of the monosaccharide with Glyx resulted only in hydrolysis, while reaction of the trisaccharide resulted only in transglycosylation. Given these differences it was of interest to discover whether $\alpha$G2F bound and reacted at just one or both sites. This was determined by the use of a known competitive inhibitor of the glucosidase activity. Deoxynojirimycin (DNJ) was determined experimentally to bind tightly to the glucosidase site ($K_i = 0.8 \mu M$). Therefore the product distribution was once again determined, but in the presence of 20 mM DNJ. The results of this experiment can be seen in Figure 2.8B. There was essentially no difference in the product distribution even though the glucosidase site should have been totally occupied by DNJ. Therefore the hydrolysis product arose from reaction at the transferase site. It was of interest to note that the transferase activity was capable of functioning as a glycosidase. A possible explanation for this result is that since maltose is not normally used as an acceptor in a disproportionation reaction (Tabata & Dohi, 1992), the disaccharide derivative, $\alpha$G2F, probably does not fulfill the requirements to be a suitable acceptor in the transfer reaction, as well. Therefore, water now competes with $\alpha$G2F as the acceptor and hydrolysis is observed.
Figure 2.8. HPLC profiles of the product distribution from the reaction between \( \alpha \)-maltosyl fluoride and Glyx in the absence (A) and presence (B) of 20 mM deoxynojirimycin.

2.3.4. Assessment of the stereochemical outcome of the glucosidase reaction mechanism

a) \( \alpha \)-Glucosyl fluoride as the substrate

Since \( \alpha \)GF was found to be a good substrate for the glucosidase activity of Glyx, it was used to determine the stereochemical outcome of this reaction. The anomeric stereochemistry of the initially formed reaction products was determined by the use of \( ^1 \)H NMR. Figure 2.9(a) depicts the partial \( ^1 \)H NMR spectra of \( \alpha \)GF and its glucose product formed during the course of the debranching enzyme reaction. Spectrum A shows the anomeric proton region of the spectrum of \( \alpha \)GF in buffer. The large double doublet centered at \( \delta \) 5.66 arises from the anomeric proton, while the broad resonance at \( \delta \) 4.84 is due to water. Spectra B, C, and D were recorded at different times after the addition of Glyx. As can be seen clearly, the decrease in the intensity of the substrate
anomeric proton resonance is accompanied by the rapid appearance of a resonance at $\delta$ 4.60 (doublet, $J = 7.8$ Hz) due to the anomeric proton of $\beta$-D-glucose. Subsequently a resonance appears at $\delta$ 5.20 (doublet, $J = 3.75$ Hz) from $\alpha$-D-glucose that is formed by mutarotation of the initially formed $\beta$-D-glucose. Such mutarotation is complete within 24 hours as shown in spectrum D, when the normal anomeric ratio (64% $\beta$ : 36% $\alpha$) has been established. These data show unequivocally that the initial product of enzymatic hydrolysis of $\alpha$GF is $\beta$-D-glucose, and therefore the glucosidase activity of Glyx is "inverting".

b) Limit dextrin as the substrate

In order to ensure that this result was not a peculiarity associated with the use of the artificial substrate, the experiment was repeated with the natural substrate, limit dextrin (see Figure 2.9(b)). In this case the broad resonance at $\delta$ 5.39 is due to the anomeric protons of the $\alpha$-(1,4) linkages. The resonance due to the $\alpha$-(1,6) linkages is concealed under the water peak. Once again the initial product of enzyme action is seen to be $\beta$-D-glucose, as witnessed by the rapid appearance of the resonance at $\delta$ 4.62 and only subsequent appearance of the resonance due to $\alpha$-D-glucose. It is not at all clear why this result is opposite to that obtained by Nelson and Larner (1970), using polarimetric methods, but the direct observation of the glucose species as it formed suggests confidence in the NMR result. In addition, inversion of anomeric configuration is consistent with the results obtained at the University of Alberta (Liu et al., 1991). 2FaGF was unable to inactivate the glucosidase activity of Glyx, because inverting enzymes have no intermediate to trap (see Figure 1.3).
Figure 2.9. Determination of the stereochemistry of reactions catalyzed by the glucosidase activity of glycogen debranching enzyme by $^1$H NMR.

a) Hydrolysis of $\alpha$-D-glucosyl fluoride (18 mM) by debranching enzyme (0.4 mg) in 100 mM phosphate buffer (0.5 mL), pH 6.8. Spectrum A, time = 0, before enzyme addition; spectrum B, time = 17 min; spectrum C, time = 33 min; spectrum D, time = 24 h. b) Hydrolysis of limit dextrin (28 mg) by debranching enzyme (0.4 mg) in 50 mM phosphate buffer (0.5 mL), pH 6.8. Spectrum A, time = 0, before enzyme addition; spectrum B, time = 15 min; spectrum C, time = 30 min; spectrum D, time = 72 h.
In a separate, related NMR experiment it was shown directly that hydrolysis of the limit dextrin under these conditions probably occurs only through cleavage of $\alpha$-(1,6) linkages. This was achieved by setting up two separate reaction mixtures identical to those employed in the previous experiment, adding enzyme as before, and stopping them at time = 0 and 30 min by placing in a hot water bath (90°C) for 5 min. $^1$H NMR spectra of these samples were then recorded at a high temperature (80°C) in order to move the water peak upfield and away from the resonance due to the anomeric protons of $\alpha$-(1,6) linked residues. Integration of the resonances due to $\alpha$-(1,4) and $\alpha$-(1,6) linked anomeric protons revealed that the number of $\alpha$-(1,6) linked residues dropped significantly during the course of the reaction since the ratio of $\alpha$-(1,4):$\alpha$-(1,6) increased from 8.2:1 (time = 0 min) to 11.4:1 (time = 30 min).

It is extremely interesting, therefore, that the two reactions catalyzed by the enzyme (glycosyl transfer and glycoside hydrolysis) occur with completely different stereochemistry, glycosyl transfer occurring with retention and glycoside hydrolysis with inversion. This therefore provides additional strong evidence for the hypothesis originated by Nelson and his colleagues and summarized in the review by Nelson et al. (1979) that the two activities occur at different catalytic sites, since it is highly unlikely that a single active site would be capable of catalyzing two mechanistically different reactions.

### 2.3.5. Further investigation of "inverting" glucosidase

**a) $\beta$-D-Glucosyl fluoride as the substrate**

As mentioned earlier, "inverting" glycosidases have the ability to utilize the "wrong" anomer, and therefore $\beta$-D-glucosyl fluoride (PGF; 9) was synthesized according to the procedure reported by Micheel and Klemer (1961) and tested with Glyx.

$^1$H NMR was used to follow the reaction of Glyx with PGF. The addition of enzyme to a buffered sample of PGF did not increase the rather large rate of spontaneous...
hydrolysis of this labile compound. The two possible explanations for this result are that either βGF is not a good enough acceptor, or that βGF is simply not utilized as a substrate by Glyx.

Taylor and Whelan (1966) demonstrated that the debranching enzyme will hydrolyze glucose from glucosyl-α-(1,6)-cyclomaltohexaose (Schardinger α-dextrin), and one would therefore predict that the cyclodextrin would act as an acceptor for glucose from βGF. Cyclodextrins would also be the malto-oligosaccharide of choice since there are no hemiacetal anomic protons present, which means the resonance of interest would not be obscured by the acceptor. We chose to use cyclomaltoheptaose (β-cyclodextrin, βCD) instead of cyclomaltohexaose (α-cyclodextrin) because the resonance due to the glycosidic anomic protons of βCD occurs slightly further downfield from the presumed resonance position of the α-(1,6) linkage being sought.

Figure 2.10 shows the anomic proton regions of a series of \( ^1\text{H} \) NMR spectra of an incubation mixture of βGF and the debranching enzyme in the presence of βCD. Spectrum A is that before the addition of enzyme, and the principal peaks are as follows. The large double doublet centered around δ 5.22 is due to the anomic proton of βGF, and the small doublets at 5.20 and 4.62 are due to α-D-glucose and β-D-glucose,
Figure 2.10. $^1$H NMR investigation of the action of debranching enzyme on β-D-glucosyl fluoride in the presence of β-cyclodextrin.

A) β-D-glucosyl fluoride (40 mM) plus βCD (18 mM) in 150 mM phosphate buffer (0.5 mL), pH 6.8, just prior to addition of enzyme. B) 16.5 min after addition of debranching enzyme (0.1 mg). C) 26 min after addition of enzyme. After 30 min a second aliquot of (0.16 mg) of debranching enzyme was added and spectra D and E were collected after a total of 40 and 52 min from the first enzyme addition. Spectrum F was recorded after 24 h.
respectively, which arise from decomposition of βGF. The large resonance at δ 5.06 arises from the anomeric protons of the α-(1,4) linkages of the βCD, while that at 4.72 is due to water. Addition of debranching enzyme brings about the rapid appearance of a new resonance at δ 4.93 (doublet, J = 3.7 Hz), which we assign as the newly formed α-(1,6) linkage between the glucose and the βCD. The coupling constant and chemical shift are consistent with those determined both in this work and previously (Gidley, 1985; Withers, 1990) for the anomeric protons of suitable reference materials such as isomaltose, glycogen, and glycogen limit dextrin. In addition the resonance at δ 4.62 due to β-D-glucose has increased significantly, with relatively little change in the resonance due to α-D-glucose. As the reaction progresses (spectra B-E), there is at first a buildup of the resonance at δ 4.93 due to the intermediate, as well as the resonance due to β-D-glucose, and then the resonance due to the intermediate decreases and finally, after 24 hours (spectrum F), has disappeared completely. At the same time, there is a rapid buildup of the β-D-glucose resonance, but then ultimately this resonance decreases, with a concomitant increase in the resonance due to α-D-glucose such that after 24 hours the expected ratio (64:36) of the two glucose anomers is observed. These data therefore clearly indicate the initial formation of an α-(1,6) linked glucosyl transfer product from βGF and βCD, followed by hydrolysis of this intermediate, again with inversion of anomeric configuration, to give β-D-glucose.

b) Investigating the hydration of D-glucal

As mentioned previously in Chapter 1, glycals are hydrated differently by "retaining" or "inverting" α-glucosidases. Since the glucosidase activity of Glyx results in overall inversion of anomeric configuration, hydration of D-glucal should result in the formation of 2-deoxy-β-glucose with protonation occurring from below (α) the double bond. TLC was used to follow the reaction between D-glucal and Glyx. 2-Deoxyglucose was not produced suggesting that no reaction had taken place. The
Figure 2.11. $^1$H NMR investigation of hydration of D-glucal by Glyx

A) D-Glucal (58 mM) plus β-cyclodextrin (16 mM) in 100 mM phosphate (0.5 mL), pH 6.86 just prior to addition of enzyme. B) 7 days after addition of debranching enzyme (0.8 mg). C) 17 days after enzyme addition.
experiment was repeated in the presence of β-CD, in the hope that this would promote the glucosidase activity. Under these conditions reaction did indeed occur and the product was identified as 2-deoxyglucose.

A $^1$H NMR experiment, using the same reaction conditions as above, was performed with the intention of determining the stereochemistry at the anomeric center as well as the direction of protonation upon hydration of D-glucal in D$_2$O. The results are shown in Figure 2.11. Figure 2.11A shows the partial $^1$H NMR spectrum of βCD and D-glucal before the addition of enzyme. The resonance at δ 6.40 results from the enolic anomeric proton, and the multiplet at δ 4.80 is due to the other methine proton at C-2. The doublet at δ 5.06 arises from the anomeric protons of βCD, and the large peak at δ 4.7 is due to water. Spectrum B, run seven days after the addition of enzyme, shows that four new signals are formed. The doublets at δ 5.36 and 4.91 are due to the anomeric proton of 2-deoxy-α- and β-glucose. As has been seen with other "inverting" glycosidases the initial product's anomeric stereochemistry could not be determined because the rate of anomerization was much greater than the rate of production of 2-deoxyglucose. In Spectra 2.11B and C, a double doublet at δ 1.67 ($J$ 11.8 Hz and 2.1 Hz) and a triplet at δ 1.48 ($J$ 10.8 Hz) are also present, due to the proton at C-2 of 2-deoxy-α- and β-glucose, respectively. Based on the coupling constants, and a comparison of the chemical shifts with those in the literature (Chiba et al., 1988), it was possible to determine that these resonances are due to the axial proton and therefore the deuteron must have been added from below the double bond. The results confirm protonation at C-2 occurs from below the plane of the ring as with other inverting α-glycosidases (Chiba et al., 1988).
2.3.6. 2-Deoxy-2-fluoro-α-maltotriosyl fluoride as a mechanism-based inhibitor of the transferase activity

Since the transferase reaction occurs with retention of anomeric configuration, a covalent glycosyl-enzyme intermediate is likely formed during reaction. Based on the finding that αG3F acts as a substrate of Glyx, 2-deoxy-2-fluoro-α-maltotriosyl fluoride (2FαG3F) (5) was synthesized and tested as a mechanism-based inhibitor which might function through the trapping of such an intermediate, as had been seen with β-glycosidases.

a) Synthesis of 2-deoxy-2-fluoro-α-maltotriosyl fluoride

The methods used to synthesize 2FαG3F are outlined in Figure 2.12. Formation of the per-O-acetylated trisaccharyl bromide was accomplished with high yield by treatment of the per-O-acetate with a 40% HBr in acetic acid solution as analyzed by TLC. Reduction of the bromide produced the glycal in only a low yield since three major products were formed: maltotrial per-O-acetate (10), anomerically deprotected maltotriose per-O-acetate, and maltotriose per-O-acetate. The two byproducts result from the presence of water and acetic acid leading to substitution rather than elimination. The per-O-acetylated maltotrial was fluorinated with fluorine (F₂) to afford a mixture (1.1:1) of the 2-deoxy-2-fluoroglycosyl fluorides, 11 and 12, identified by their ¹⁹F NMR spectra. This product ratio is considerably lower than that observed for tri-O-acetyl glucal (3:1) under identical conditions (Satyamurthy et al., 1985), reflecting an influence of the additional sugar residues in the case of addition of the fluorine onto the double bond. The acetylated 2FαG3F was purified by extensive flash chromatography and deacetylated with sodium methoxide in methanol to give 2FαG3F.
b) 2-Deoxy-2-fluoro-α-maltotriosyl fluoride as a mechanism-based inhibitor

Instead of the anticipated inactivation, 2FαG3F turned out to be a slow substrate for Glyx (K_m and k_cat not determined). A rationale for this result was postulated based on the results observed by Kempton and Withers (1992) regarding the amount of positive charge generated at the transition states for both steps in the reaction catalyzed by the β-
As mentioned earlier, the deglycosylation step is believed to have more positive charge than the glycosylation step. The substitution of the electronegative fluorine atom at C-2 therefore results in greater destabilization of the second step than the first, thus leading to the accumulation of the glycosyl-enzyme intermediate. On stereoelectronic grounds it can be argued that the second step for β-glycosidases (α-intermediate → β-product) may be similar to the first step for α-glycosidases (α-reactant → β-intermediate). One may then hypothesize that α-glycosidases may have more positive charge in the glycosylation transition state than in the deglycosylation transition state. If this is the case there would be greater destabilization of the first step and as a result no accumulation of a glycosyl-enzyme intermediate as shown in Figure 2.13. As a consequence, depending upon the extent of destabilization by the fluorine the compound could function as either a competitive inhibitor or as a substrate. The latter was seen to be the case for 2FαG3F (5).
2.3.7. 4-Deoxy-α-maltotriosyl fluoride as an incompetent substrate of the transferase activity

Given the lack of success of the "2-fluoro" approach in accumulating an intermediate, an alternative approach was sought. The approach employed involved the synthesis and use of a 4-deoxy-trisaccharide substrate which can serve as a glycosyl donor, but since its normally nucleophilic 4-hydroxyl is missing (Figure 1.5), not as an acceptor. This should allow trapping of the glycosyl-enzyme intermediate formed at the transferase site. Therefore 4-deoxy-α-maltotriosyl fluoride (4DαG3F) was synthesized and tested with Glyx.

a) Synthesis of 4-Deoxy-α-maltotriosyl fluoride (6)

The synthesis was carried out in collaboration with Dr. Thisbe K. Lindhorst (Lindhorst et al., 1995). The procedure (see Figure 2.14) involved blocking of the 4",6" hydroxyls using a benzylidene protecting group, followed by acetylation. The benzylidene ring was then reductively opened, selectively at the 4-position, using sodium cyanoborohydride. Reaction with triflic anhydride followed by displacement with iodide and then reductive dehalogenation using tributyl tin hydride afforded the 4"-deoxy-6"-O-benzyl-trisaccharide per-O-acetate. The benzyl group was removed using H$_2$ and palladium on carbon as a catalyst. The remaining synthesis involved the methods used for the synthesis of αG3F (see Figure 2.5).

b) Burst experiment

Reaction of 4DαG3F with Glyx was monitored using a fluoride ion selective electrode. As can be seen in Figure 2.15, addition of 41 μM Glyx to an excess (1.8 mM) of 4DαG3F resulted in the relatively rapid initial release of fluoride followed by a slower steady state reaction. The quantity of fluoride released in this initial phase was estimated by extrapolating the linear portion of the plot back to t=0, yielding a value of 43 μM.
Figure 2.14. Reaction scheme for the synthesis of 4-deoxy-α-maltotriosyl fluoride (6)
Figure 2.15. Reaction of 4DαG3F with the debranching enzyme.

(○) Addition of 100 μL debranching enzyme (41 μM) to 150 μL 4DαG3F (1.8 mM) in 100 mM phosphate, 1 mM EDTA buffer, pH 6.90. (●) After 16 min maltotriose (6.5 mg) was added.

fluoride released. This corresponds well with the amount of enzyme present (41 μM) suggesting that this is indeed a true burst of fluoride arising from reaction of one equivalent of 4DαG3F with the enzyme. The second, steady state phase presumably arises from slow hydrolysis of the accumulated intermediate. The experiment was repeated with half the amount of enzyme and produced similar results (data not shown).

Accumulation of an intermediate is possible because no sugar molecule with a free 4-hydroxyl group is present to act as an acceptor. Therefore addition of an appropriate acceptor should result in an increase in the steady state rate and provide
further evidence for the existence of a trapped glycosyl-enzyme intermediate. As shown in Figure 2.15, maltotriose, which has a free 4-hydroxyl group, was added and the rate of fluoride ion release did indeed increase.

Attempts were made to further study the covalently bound glycosyl-enzyme intermediate using electrospray mass spectrometry (ESMS). The technique can determine the difference in mass between the enzyme and that of the glycosyl-enzyme intermediate, which allows one to deduce the identity of the intermediate. Unfortunately, with the LC/MS system used, undigested glycogen debranching enzyme was unable to pass through the liquid chromatography column that was connected to the mass spectrometer. Another technique, Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry was also unable to be used because the mass accuracy obtained for this large enzyme was only ±0.5%, and therefore the error in the mass (±885) is greater than the mass of the label (471).

2.3.8. Determination of the enzymic reaction products

The products of the reaction of 4DaG3F and maltotriose with the debranching enzyme were analyzed by HPLC as shown in Figure 2.16. Two major products were observed each as a mixture of anomers, at 18 and 27 minutes on the HPLC trace. Analysis of these products by MALDI mass spectrometry determined that the product formed first, the doublet at 27 minutes, has a mass of m/z = 997.2 ± 1.0. This agrees closely with the theoretical m/z for 4-deoxymaltohexaose (4dG6+Na⁺ 997.9). The mechanism for the accumulation and turnover of this intermediate is shown in Figure 2.17.

Initially the reaction proceeds as proposed in Figure 2.17, but as is shown in Figure 2.16 another product is also formed. This reaction product, at 18 minutes in the HPLC traces, was determined to be 4-deoxymaltopentaose (4dG5+Na⁺ 835.7) by MALDI mass spectrometry as a peak at m/z 835.3 ± 0.8 was obtained. This product
Figure 2.16. HPLC profiles of the products formed from the reaction of 4DαG3F with Glyx in the presence of maltotriose.

Reaction times after enzyme addition: A) 5.5 min; B) 96 min; C) 186 min; and D) 366 min.
Figure 2.17. Mechanism for accumulation of an intermediate of the debranching enzyme with 4D\(\alpha\)G3F and turnover via transglycosylation upon addition of maltotriose.
Figure 2.18. Disproportionation of 4-deoxymaltohexaose by debranching enzyme in the presence of excess maltotriose.

presumably results from a disproportionation reaction as shown in Figure 2.18; a process which Glyx is known to catalyze (Tabata & Dohi, 1992). Therefore, as 4-deoxymaltohexaose accumulates, the debranching enzyme uses it as a substrate (see Figure 2.18). First the enzyme clips off maltotetraose forming a 4-deoxymaltosyl-enzyme intermediate. Maltotriose then acts as the acceptor resulting in the formation of 4-deoxymaltopentaose. Figure 2.18 reveals that the appearance of 4-deoxymaltopentaose should be accompanied by the appearance of maltotetraose (G4). This is indeed the case, as is seen in Figure 2.16 where G4 appears at 6 min in the HPLC profiles (doublet).

In theory if no acceptor was present then the steady state rate should be zero. But as shown in Figure 2.15, a significant amount of turnover of the intermediate was observed. The product of this steady state reaction was identified to be 4-deoxymaltotriose by HPLC analysis using an authentic standard (data not shown). Therefore, the steady state turnover resulted from the transferase site slowly allowing water to act as the acceptor, resulting in hydrolysis of the intermediate. This result also suggests that a 4-deoxymaltotriosyl-enzyme intermediate is formed.
2.3.9. Identification of the nucleophilic amino acid in Glyx by electrospray mass spectrometry

A recent method that has been used successfully to identify the amino acid involved in the formation of a glycosyl-enzyme intermediate is electrospray tandem mass spectrometry (ESMS) (Tull et al., 1995; Miao et al., 1994; Henderson et al., 1992; and Hunt et al., 1992). Glyx was incubated with 4DαG3F and then digested using the protease pepsin (see Material and Methods for details). The mixture of peptides produced was partially separated by reverse-phase HPLC and the ESMS used as the detector. Scanning the spectrometer in the normal LC/MS mode, the total ion chromatogram (TIC) of the digest displays the large number of peptides in the mixture (Figure 2.19A). In order to determine which peptide had the trisaccharide attached, a second run was performed using the tandem mass spectrometer set up in the neutral loss mode. In this technique the ions are subjected to limited fragmentation by collisions with an inert gas (Ar) in a collision cell located between the two mass analyzers (see Figure 2.20). Since the proposed ester linkage is more labile than a peptide linkage it is anticipated that homolytic cleavage of this bond will preferentially occur. This will result in the loss of a neutral sugar residue of mass 471 Da. The two quadrupoles were therefore first scanned in such a fashion that only ions that differed by mass 471 (singly charged species) could pass through both quadrupoles and be detected, but no peak was observed. Good results were however obtained when the neutral loss was performed searching for the loss of m/z of 235.5, corresponding to the loss of the inhibitor label from a peptide ion in the doubly charged state. Results are shown in Figure 2.19B, along with the results of a similar experiment carried out on a peptic digest of unlabeled enzyme (Figure 2.19C). Comparison of the two spectra shows two distinct peaks which were present in the labeled experiment and not present in the control. These peptides
Figure 2.19. ESMS/MS experiments on Glyx proteolytic digests.

(A) labeled with 4DαG3F, TIC in normal MS mode, (B) labeled with 4DαG3F, TIC in neutral loss mode, (C) unlabeled, in neutral loss mode. (D) Mass spectrum of peptide 1 in panel B, (E) mass spectrum of peptide 2 in panel B.
which were covalently labeled by 4-deoxy-α-maltotriosyl fluoride were determined to have molecular weights of 1692 (± 2) and 2188 (± 2). A search through the amino acid sequence of the debranching enzyme (Liu et al., 1993) using commercially available software revealed that a total of 48 and 45 peptides, respectively, could be derived from Glyx that could satisfy the molecular weights of peptide 1 and peptide 2.

![Schematic of ESMS/MS](image)

**Figure 2.20.** Schematic of ESMS/MS

### 2.3.10. Identification of the sequence of the labeled peptide

Sequence information was obtained without further purification by additional fragmentation of peptide 1 in the daughter ion scan mode. The parent ion of m/z 1081 (doubly charged state) was selected in the first quadrupole and subjected to collision-induced fragmentation in a collision cell in the second quadrupole; then the masses of the daughter ions produced were detected in the third quadrupole. The daughter ions produced are shown in Figure 2.21. The daughter ion at m/z 845 arises from the loss of the 4-deoxymaltotriosyl label. Another daughter ion at m/z 1692 arises from the daughter ion at m/z 845 through the loss of a proton, resulting in the unlabeled peptide in the singly charged state (MH⁺). All the other daughter ions that are at m/z higher than 845 must correspond to singly charged peptides. These fragments arose from further fragmentation of the 845 peptide.
Using a computer program to predict the fragmentation patterns of the 48 possible peptides, only one peptide could have resulted in all of the labeled daughter ions. The sequence of this peptide was:

**VRLDNCHSTPLHVAE**
The complete fragmentation pattern of the labeled peptide can now be explained. The peaks at m/z 1235/1208 are attributed to the loss of the C-terminal tetrapeptide HVAE. The peaks at m/z 1025 and 455 resulted from the C-terminal loss of PLHVAE, and NCHSTPLHVAE, respectively. The other peaks at m/z 736 and 687 resulted from the loss of AE and VAE from the C-terminus, but the peptides are in the doubly charged state.

Peptide 2 (m/z 1329 in the doubly charged state) was also fragmented in the daughter ion scan mode. The daughter ions produced are shown in Figure 2.22. The daughter ion at m/z 1094 arises from the loss of the label. Even though only two other daughter ions were observed, computer generated mass spectra fragmentation patterns of the 45 possible peptides were able to eliminate 40 of the peptides. Therefore only five of the peptides could give rise to the two daughter ions. Since peptide 1 and peptide 2 both contain the catalytic nucleophile, their sequences must overlap. Of the five remaining peptides only one of them overlapped with the sequence of peptide 1. The sequence of this peptide was:

**FQGVRLDNCHSTPLHVAEY**

2.3.11. Identification of the catalytic nucleophile

The catalytic nucleophile has to be one of the amino acids in the two peptides. Within the sequences the only two amino acids which are likely candidates for the nucleophile, based on precedent (Sinnott, 1990; Withers & Aebersold, 1995) and on the facile fragmentation in the mass spectrometer, are Asp 549 and Glu 560.
a) Aminolysis experiment

Covalent attachment of the label to the peptide through an ester linkage was confirmed by aminolysis of the labeled digest. After ammonium hydroxide treatment, the labeled peptide with peaks at m/z 1081.5 (MH$_2^{2+}$), and 721 (MH$_3^{3+}$) was replaced by a new peptide with peaks at m/z 1691.5 (MH$_1^{+}$) and 846 (MH$_2^{2+}$). This result
suggests that aminolysis has removed the label from an acidic residue and therefore the nucleophilic amino acid has been replaced with either an Asn or Gln.

b) Sequence comparison

Further identification of the catalytic nucleophile came from comparison of the amino acid sequences within the α-amylase family. Sequence alignments, as shown in Figure 2.4, reveal that there are four consensus sequences present in the family, with three of the sequences containing the three completely conserved active site carboxylic acids (Svensson, 1994). The peptide that was labeled was found to be partially contained within one of the consensus sequences as shown in Figure 2.22. Asp 549 was found to be one of the three completely conserved active site carboxylates; whereas, Glu 560 was found outside of the consensus sequence and was not conserved in any other members of the α-amylase superfamily. Asp 549 also corresponds to the residue suggested to function as the nucleophile based on the TAKA amylase crystal structure (Matsuura et al., 1984) and to a residue contained in the labeled peptide isolated from the α-glucanotransferase from *Streptococcus sobrinus* (Mooser et al., 1991). Therefore, it is believed that Asp 549 is the catalytic nucleophile in the transferase site of rabbit muscle glycogen debranching enzyme.

### 2.4. Conclusions

The use of αG3F, particularly in the presence of 0.1-1.0% glycogen, provides a convenient, specific assay for the transferase activity of Glyx. Use of this in conjunction with αGF for the glucosidase activity allows specific, independent monitoring of the two separate activities.
<table>
<thead>
<tr>
<th>Enzyme Type</th>
<th>Consensus Sequence 1</th>
<th>Consensus Sequence 2</th>
<th>Consensus Sequence 3</th>
<th>Consensus Sequence 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>α-Amylase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>111 GMYLMVDVVANH</td>
<td>201 DGRLIDTVKH</td>
<td>226 YCIGEVD</td>
<td>289 LGTFVENHD</td>
</tr>
<tr>
<td>Barley 1</td>
<td>82 G0QAIADIVINH</td>
<td>175 DA0RDLFARG</td>
<td>201 LAAEVWD</td>
<td>283 AATFVDNHD</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>88 GVRIVDVVFNH</td>
<td>181 AGFRVDAKH</td>
<td>219 YIVQEVID</td>
<td>279 SLVFVDNHD</td>
</tr>
<tr>
<td>Porcine pancreas</td>
<td>90 GVRIVDAVINH</td>
<td>192 AGFRLDASKH</td>
<td>229 FIFQEVID</td>
<td>292 ALVFVDNHD</td>
</tr>
<tr>
<td>Human pancreas</td>
<td>90 GVRIVDAVINH</td>
<td>192 AGFRLDASKH</td>
<td>229 FIFQEVID</td>
<td>292 ALVFVDNHD</td>
</tr>
<tr>
<td><strong>α-Glucosidase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>100 GMKFI5DLVNH</td>
<td>170 DGR1DSTAGL</td>
<td>272 MRVGEVAH</td>
<td>341 ATTYIENHD</td>
</tr>
<tr>
<td><strong>α-Glucosyltransferase (GTase-I)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus sobrinus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cyclodextrinase</strong></td>
<td></td>
<td>DS1RVDAVD</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus sphaericus</em></td>
<td>232 GMRVMDAVFHN</td>
<td>321 DGWRLDVANE</td>
<td>351 YILGREIMH</td>
<td>414 SFNLLGSHD</td>
</tr>
<tr>
<td><strong>Pullulanase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em></td>
<td>596 GMNVMDVYHN</td>
<td>672 DGFRPDLMGY</td>
<td>702 YFFGEGWD</td>
<td>826 VVNYVSKHD</td>
</tr>
<tr>
<td><strong>Isoamylase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas amyloferosa</em></td>
<td>298 GIKYVMDDVYNH</td>
<td>369 DGFRPDLASV</td>
<td>412 RILREFTV</td>
<td>499 SINFIDVHD</td>
</tr>
<tr>
<td><strong>Oligo-1,6-glucosidase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>92 NHKLMMDLVNH</td>
<td>194 DGFRMDVINF</td>
<td>251 MTVEMPG</td>
<td>321 NSLYWNH</td>
</tr>
<tr>
<td><strong>Cyclodextrin glucanotransferase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus macerans</em></td>
<td>129 NIKVVMDFAPNH</td>
<td>224 DGIRFDDAVKH</td>
<td>254 PTFGEWFL</td>
<td>321 MVTFIDNHD</td>
</tr>
<tr>
<td><strong>Branching enzyme</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>328 GLNVILDWVPGH</td>
<td>399 DALRDVAVS</td>
<td>453 VTMAEST</td>
<td>517 NFVPLNHD</td>
</tr>
<tr>
<td><strong>Amylomaltase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus pneumonia</em></td>
<td>218 VAEDSDDWAND</td>
<td>290 DIVR1DHFRG</td>
<td>328 AAVKEELG</td>
<td>388 SVMYTGTHD</td>
</tr>
<tr>
<td><strong>Glycogen debranching enzyme</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human muscle</td>
<td>192 NVICITDVVYHN</td>
<td>504 QGVRLDNCHS</td>
<td>534 YVVAGLF</td>
<td>602 ALFMDITHD</td>
</tr>
<tr>
<td>Rabbit muscle</td>
<td>232 NVLICITDVVYHN</td>
<td>544 QGVRLDNCHS</td>
<td>574 YVVAGLF</td>
<td>642 ALFMDITHD</td>
</tr>
</tbody>
</table>

Sequence of labeled peptide: VRLDNCSTPLHVAE

Figure 2.23. Identified labeled peptide and the conserved sequence stretches in the amylase superfamily
The glucosidase activity was reexamined and found to be "inverting", since $^1\text{H}$ NMR analysis determined that the initial product in the reaction with $\alpha$GF or limit dextrin was $\beta$-D-glucose. By contrast the transferase activity works with retention of configuration at the anomeric center, and this was also confirmed with small substrates. These results therefore confirm earlier findings that Glyx has two separate active sites. In addition to its action on $\alpha$GF the glucosidase was shown to also utilize the "wrong" anomer, $\beta$GF, ultimately releasing $\beta$-D-glucose when studied in the presence of the acceptor, $\beta$CD. A transient $\alpha$-(1,6) linked glucosyl-CD intermediate was observed by $^1\text{H}$ NMR consistent with the expected mechanism of this reaction (Hehre et al., 1979). Glucal was shown to be hydrated by the glucosidase in the presence of $\beta$CD, protonation occurring from below the double bond. This result is consistent with findings on other inverting $\alpha$-glycosidases.

Addition of 2F$\alpha$G3F to Glyx did not result in the accumulation of a glycosyl-enzyme intermediate. Rather it was found to act as a slow substrate. Addition of 4D$\alpha$G3F did however result in the accumulation of a glycosyl-enzyme intermediate, as demonstrated by the release of one full equivalent of fluoride in a burst phase. Use of electrospray tandem mass spectrometry to analyze proteolytic digests of labeled and unlabeled enzyme, in conjunction with sequence alignments from the amylase superfamily, determined that Asp 549 is the catalytic nucleophile in the transferase site of rabbit muscle glycogen debranching enzyme.
3.1. Introduction

3.1.1. Biochemical role in vivo.

Human pancreatic α-amylase (HPA EC 3.2.1.1.1) plays an important role in carbohydrate metabolism. HPA is an endoglycanase which is involved in the degradation of starch into simple sugars (see Figure 3.1). The digestion of ingested starch occurs in several stages in humans (Semenza, 1987; Truscheit et al., 1981). An initial, partial digestion is provided by the salivary amylase which, to a limited extent, breaks down the polymeric starch to shorter oligomers. Upon reaching the gut the starch is extensively hydrolyzed into oligosaccharides by α-amylase (see Figure 3.1), which is

![Starch hydrolysis diagram](image)

**Figure 3.1.** Hydrolysis of polysaccharides by human pancreatic α-amylase (— α-(1,4) linkages, O = glucose).
produced in the pancreas and excreted into the lumen. This mixture of oligosaccharides, including maltose, maltotriose and a number of $\alpha$-(1,6) linked and $\alpha$-(1,4) oligoglucans then passes through a mucous layer to the brush border membrane, associated with which are a number of $\alpha$-glucosidases whose function is to further degrade the oligosaccharides to glucose, which is then taken up into the blood stream.

HPA is an enzyme of molecular weight 55,798 Da containing some 496 amino acid residues (Nishide et al., 1986). The complete amino acid sequence has been determined by sequencing the cDNA (Nakamura et al., 1984; MacGregor, 1988).

3.1.2. HPA is part of the $\alpha$-amylase superfamily

HPA is a member of the amylase superfamily as discussed in Chapter 2 (see Figure 2.4). Sequence homology among $\alpha$-amylases varies from being quite high (PPA and HPA 84% (Qian et al., 1993)) to essentially nonexistent (Glyx and HPA), but the catalytically important residues are conserved. Information regarding the active site has come from the recent surge in X-ray crystal structures that have been solved in the amylase superfamily. TAKA amylase was originally solved in 1984 (Matsuura et al., 1984). Three dimensional structures of amylases from \textit{Aspergillus niger} (Brady et al., 1991), bacteria (Suzuki et al., 1990), PPA (Qian et al., 1993), barley $\alpha$-amylase (Kadziola et al., 1994), cyclodextrin glucanotransferase (Klein & Schulz, 1991), and HPA (Brayer et al., 1995) have also been recently determined. The active site arrangement in $\alpha$-amylases and other enzymes of family 13, contains the trio of conserved carboxylic acids: Asp 197, Glu 233, and Asp 300 (HPA numbering). The x-ray crystal structure of HPA is shown in Figure 3.2. The catalytic ($\beta/\alpha$)$_8$-barrel domain consists of eight parallel $\beta$-strands surrounded by parallel $\alpha$-helices, with characteristic $\beta_0/\beta_0...$ connectivity. An additional small domain is located in the third $\beta$-$\alpha$ loop and an extra helix is inserted after the 6th $\beta$-strand.
3.1.3. Assays for HPA

Diagnosis of pancreatic disorders is usually carried out by assaying α-amylase levels in serum and urine. Three methods most commonly used in clinical laboratories are the saccharogenic, amyloclastic, and the dye-labeled substrate methods. An example of the saccharogenic method, originally proposed by Somogyi in 1938, is the increase in reducing sugars released by hydrolysis of starch is monitored using a highly alkaline copper reagent, and the amount of copper (I) produced is assayed. The amyloclastic method is a colorimetric method which generally measures the decrease in the iodine-starch complex upon hydrolysis of the starch. These two methods are both convenient and simple but they are either not a continuous or quantitative assay. The third assay, which depends on dye-labeled substrates, originally utilized insoluble dye-coupled starch as a substrate for amylase (Klein et al., 1970). Upon hydrolysis, soluble fragments are released into the supernatant and can be measured spectrophotometrically. A more advanced method of a labeled substrate used soluble substrates such as p-nitrophenyl oligosaccharides (Gillard et al., 1977). This soluble substrate (eg. PNP maltopentaoside) is hydrolyzed by amylase and, upon treatment with an α-glucosidase, releases the
coloured product p-nitrophenol. Even shorter oligosaccharides, such as p-nitrophenyl α-maltoside (αPNPG2), can be used in which amylase directly releases p-nitrophenol. Thus utilization of αPNPG2 as the substrate provides a simple, quantitative, and continuous assay for amylase, however the p-nitrophenyl group is not a very good leaving group and thus results in only slow turnover of this substrate.

3.1.4. Inhibitor studies

Considerable effort in recent years has been expended in a search for inhibitors of starch digestion, with inhibitors of α-amylase being one of the primary targets. One of the most successful inhibitors to date is acarbose (see Figure 3.3), which is a natural product isolated from *Streptomyces* culture supernatant. Animal trials (Truscheit et al., 1981) showed that acarbose has excellent activity in vivo, reducing both post-prandial blood glucose, and serum insulin increases after carbohydrate loads. In healthy human subjects (Clissold & Edwards, 1988), acarbose significantly inhibits post-prandial glucose, insulin and triglyceride responses. Diabetic control is thus improved for patients with non-insulin-dependent diabetes mellitus, but this agent was most successful in insulin-dependent patients. In these cases, significant reductions in post-prandial glucose concentrations were observed, thus reducing daily insulin requirements.

While the general principle of inhibiting carbohydrate metabolism has been established, relatively little work has been performed on the mode of action of acarbose in inhibiting α-amylase, beyond measurement of simple inhibition kinetics. The potency of acarbose was generally believed to be partially due to the half chair conformation of the valienamine ring, which is probably a transition stage analogue. It was recently discovered by X-ray analysis of a complex between acarbose and porcine pancreatic α-amylase (PPA), however, that acarbose was not the compound that was present in the active site (Qian et al., 1994). Amylase clips off a glucose residue from the reducing end of acarbose and attaches this monosaccharide to the non-reducing terminus of another acarbose.
molecule or itself (see Figure 3.3). Transfer of another monosaccharide to the nonreducing end resulted in a pseudopentasaccharide which was found in the crystal structure of PPA.
3.1.5. General features of the catalytic mechanism

HPA is presumed to be a "retaining" glycosidase. Therefore the reaction catalyzed by HPA involves a double displacement as shown in Figure 3.4. The main features of this mechanism include: general acid catalysis; formation of a glycosyl-enzyme intermediate; and general base catalysis (see Chapter 2).

3.2. Aims Of This Study

The aim of this study was to do a detailed kinetic investigation of HPA, which comprised four areas: 1) reevaluate the stereochemical outcome of enzymatic hydrolysis; 2) kinetically evaluate a defined set of oligosaccharides and determine the cleavage pattern for each substrate; 3) kinetically evaluate glycosyl fluorides as substrates; and 4) synthesize and evaluate potential mechanism-based inhibitors of HPA.

3.2.1. Stereochemical outcome

The \( \alpha \)-amylase catalyzed hydrolysis of oligosaccharides is believed to result in retention of anomeric configuration. This result will be reevaluated using two different methods, NMR and HPLC.

3.2.2. Oligosaccharides as substrates

A better understanding of the reaction mechanism is obtained by investigating the products in an enzymic reaction. Thus the next aim of the thesis is to determine the "action patterns" obtained when amylase degrades the defined set of malto-oligosaccharides maltotetraose through maltoheptaose (G4-G7). A novel HPLC assay will be investigated which will enable the determination of cleavage sites without the use of radiolabels. This study will help to identify the number of subsites in the active site of HPA. The determination of the kinetic constants (\( k_{\text{cat}} \) and \( K_{\text{m}} \)) of substrates G3-G7, will be done to determine the specificity of the enzyme.
3.2.3. Glycosyl fluorides as substrates

Artificial substrates, such as glycosyl fluorides, will be kinetically evaluated to further probe the catalytic mechanism. Also, the glycosyl fluorides will be compared with other known substrates such as p-nitrophenyl \( \alpha \)-maltoside and evaluated as assay systems for amylases.
α-Maltosyl fluoride has been shown to be hydrolyzed and transglycosylated by α-amylases (Okada et al., 1979). Various amylases were studied but the human enzyme was not one of them. Therefore the products produced in the reaction between various glycosyl fluorides and HPA will also be investigated. This study will aid in the understanding of the reaction mechanism.

3.2.4. Design of mechanism-based inhibitors

2-Deoxy-2-fluoro-glycosyl fluorides have been shown to be mechanism-based inhibitors of glycosidases. In Chapter 2, 2-deoxy-2-fluoro-α-maltotriosyl fluoride was shown only to be a poor substrate for the transferase activity of Glyx. The aim of this study is to determine if 2-deoxy-2-fluoro-α-maltosyl fluoride (22) is utilized as a mechanism-based inhibitor or as a substrate by HPA.

![Diagram of 2-deoxy-2-fluoro-α-maltosyl fluoride](image)

Another aim of this study is to investigate novel mechanism-based inhibitors of α-glycosidases, since inactivation attempts with 2-fluoroglycosides apparently resulted only in limited success. Our initial approach in the design of a mechanism-based inhibitor required that the deglycosylation transition state be more destabilized than with 2-fluoroglycosides since they have been shown to be utilized as substrates (see Chapter 2). The second requirement was that the formation of the glycosyl-enzyme intermediate had to be faster than the breakdown. Addition of a second fluorine atom at C-2 and the
presence of an excellent leaving group, such as trinitrophenol, should result in a reaction in which the glycosylation step is faster than the deglycosylation step, and thus an intermediate should accumulate. Therefore we decided to synthesize 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-\(\alpha\)-maltoside (23) and test it as a mechanism-based inhibitor of HPA.

![Chemical structure of 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-\(\alpha\)-maltoside](image)

3.3. Results and discussion

3.3.1. Determination of stereochemical outcome

a) NMR method using \(\alpha\)-maltosyl fluoride as the substrate

Because \(\alpha\)-maltosyl fluoride (\(\alpha\)G2F) was known to be a substrate of other \(\alpha\)-amylases (Okada et al., 1979), it was utilized to determine the stereochemical outcome of the reaction catalyzed by HPA. The results of this experiment can be seen in Figure 3.5. Figure 3.5A shows the partial \(^1\)H NMR spectrum of \(\alpha\)G2F in buffer before the addition of enzyme. The double doublet centred at \(\delta\) 5.69 is due to the anomeric proton closest to the fluorine. The resonance at \(\delta\) 5.42 is due to the other anomeric proton of \(\alpha\)G2F. The large peak at \(\delta\) 4.73 is due to water. Figure 3.5B is the spectrum 4 min after HPA was added, and the resonance of the anomeric proton at \(\delta\) 5.69 has disappeared and a new resonance at \(\delta\) 5.22 (d, \(J\) 4Hz) has appeared. This resonance is due to the formation of \(\alpha\)-maltose.
Figure 3.5. $^1$H NMR determination of the stereochemistry of the products in the reaction between $\alpha$G2F and HPA

$\alpha$G2F (34 mM) was dissolved in 0.5 mL phosphate buffer (100 mM) pH 6.9. The spectra were acquired after addition of enzyme (70 µg): A) 0 min; B) 4 min; C) 11 min; and D) 24 h.
Spectrum 3.5C (after 11 min) shows the presence of another new resonance at δ 4.65 due to the formation of β-maltose by mutarotation. Spectrum 3.5D, after 24 h, shows the equilibrium mixture of β- and α-maltose in the ratio of 1.6:1.0. Therefore HPA does hydrolyze αG2F with retention of configuration at the anomeric center.

b) NMR method using maltoheptaose as the substrate

In order to ensure the result with αG2F was not associated with the use of an artificial substrate, the experiment was repeated with the "natural" substrate, maltoheptaose (G7; see Figure 3.6). Figure 3.6A shows the partial 1H NMR spectrum of G7 in buffer before the addition of enzyme. The resonance at δ 5.40 is due to the acetal anomeric protons; while the resonances at δ 5.20 and δ 4.65 are due to the α- and β-hemiacetal anomeric protons, respectively. Once again upon addition of HPA the initial product of enzymic reaction is shown to have the α-anomeric configuration, as witnessed by the large increase in the resonance at δ 5.20 with only a small increase in the resonance at δ 4.65. Spectra C and D show the increase in intensity of the resonance at δ 4.65 which results from mutarotation of the initially formed α-anomeric products. Therefore HPA is a "retaining" enzyme. Note that the identification of the products, beyond the stereochemical outcome at the hemiacetal anomeric center, is not possible, because of the similar resonances seen amongst the anomeric protons of malto-oligosaccharides.

c) HPLC method using maltopentaose as the substrate

Another approach to determine the stereochemical outcome of reactions catalyzed by endoglycanases was discovered, which was also capable of identifying the products. A Dextropak® HPLC column from Waters® is able to separate the anomers of glucose oligomers with a degree of polymerization of three or more. Therefore one is able to determine the anomeric configuration of the longer oligosaccharide products. HPLC was thus used to determine the initial product stereochemistry in the reaction between
Figure 3.6. $^1$H NMR determination of the stereochemical outcome in the reaction between maltoheptaose and HPA.

Maltoheptaose (27 mM) was dissolved in 0.5 mL phosphate buffer (100 mM) pH 6.9. The spectra were acquired after addition of enzyme (35 μg): A) 0 min; B) 4 min; C) 12 min; D) 17 min; and E) 2 h.
Figure 3.7 HPLC chromatograms of reaction mixtures containing maltopentaose and HPA.

G5 (0.52 mM) dissolved in 160 µL sodium phosphate (20 mM), sodium chloride (25 mM) buffer pH 6.9 and α-amylase (0.84 µg) were incubated at room temperature for 2 min. (A) A 50 µL aliquot was injected onto the Dextropak® column; (B) a 100 µL sample was placed in boiling water for 2 min and 50 µL of this was injected onto the Dextropak® column.

maltopentaose (G5) and HPA. The results of this experiment are shown in Figure 3.7. Upon addition of HPA two products, maltose and α-maltotriose are produced (Figure 3.7A). Figure 3.7B shows the profile of the same reaction mixture as in Figure 3.7A but the sample was boiled before injection onto the HPLC column. This procedure results in the thermodynamic equilibrium mixture of anomers being formed (1.6:1 β:α). Therefore the results once again demonstrate that the hydrolysis by HPA occurs with retention of
anomeric configuration. In addition, the position of cleavage has been elucidated by identification of the products formed.

3.3.2. Malto-oligosaccharides as substrates for HPA

a) "Action patterns" with malto-oligosaccharides

"Action patterns" involve the determination of initial products formed from the enzymatic hydrolysis of a defined set of oligosaccharides. Malto-oligosaccharides were therefore tested as substrates of HPA, using a method similar to that reported by Beltrame et al. (1987). In the reported procedure, "action patterns" were determined by having one end of the malto-oligosaccharide labeled so that the leaving group (aglycone) and glycone in the reaction could be discerned. We decided to use underivatized malto-oligosaccharides to determine the "action patterns". This approach was feasible because, as mentioned earlier, the Dextropak® HPLC column was able to separate the anomers of malto-oligosaccharides (d.p.>3). Therefore the glycone can be distinguished from the aglycone without having to modify the substrate. An example was already shown in Figure 3.7 in the reaction between G5 and HPA. The cleavage of G5 is shown in Figure 3.8. The reactant exists as the equilibrium mixture of β- and α-anomers with a ratio of 1.6:1, respectively. Since α-maltotriose is formed initially, cleavage must occur two units from the reducing end and therefore the other product maltose must exist as the thermodynamic mixture of anomers. Therefore the glycone (maltotriose) and aglycone (maltose) could be distinguished without prior modification of the substrate. The result also showed that HPA only cleaved at one site as only two products were formed in the reaction. Therefore with G5, only one significant productive binding mode is observed in the reaction with HPA.
Figure 3.8. HPA catalyzed hydrolysis of maltopentaose

The experiment was repeated with the other malto-oligosaccharides and the resulting "action patterns" are shown in Figure 3.9. The arrows indicate cleavage position and the numbers reflect % cleavage observed at each point. In theory these should be proportional to the relative $k_{cat}/K_m$ values for cleavage at each position. The results with $G_4$ show that there are two observed productive binding modes; one where the glycone is a disaccharide and the other where the glycone is a trisaccharide. Of the two binding modes, cleavage occurs preferentially two sugar units from the reducing end, which suggests that maltose makes a better leaving group than glucose for the HPA catalyzed reaction. Two productive binding modes were also observed for cleavage of $G_6$. Since $G_5$ was the only substrate, so far, to be cleaved at only one site, these results suggest there are five subsites which make up the the active site. The results with $G_7$, as would be expected, are more complicated. There are three productive complexes observed, with
only a slight preference for one desired cleavage site. These results are consistent with the five subsite theory since there are three ways in which G7 could occupy all of the subsites.

In the results with substrates G5 and larger, no cleavage is observed less than three sugar units from the non-reducing end and less than two sugar units from the reducing end. The results therefore reveal that the active site is made up of five subsites with three of the subsites making up the glycone site and the other two the aglycone site. The results with the longer oligosaccharides also reveal that, if possible, all subsites will be filled, and only productive complexes which satisfy the above are observed.

\[
\begin{align*}
O-\text{O-} & \text{O-} \text{O-} \text{O-} \text{O} \\
\text{.30} \quad \text{.44} \\
\text{(.15)} \quad \text{(.57)} \\
O-\text{O-} & \text{O-} \text{O-} \text{O} \\
\text{.32} \\
\text{(.32)} \quad \text{.68} \\
\text{(.67)} \\
O-\text{O-} & \text{O-} \text{O} \\
\text{1.00} \\
\text{(.00)} \\
O-\text{O} & \\
\text{.15} \\
\text{(.30)} \\
\text{(.70)}
\end{align*}
\]

Figure 3.9. "Action pattern" for HPA-catalyzed hydrolysis of maltotetraose, maltopentaose, maltohexaose, and maltoheptaose (\textendash \alpha-(1,4) glycosidic linkage; \text{O} reducing sugar, \text{O} nonreducing sugar). Numbers in brackets are for PPA (Robyt & French, 1970).
These results are consistent with those determined for PPA (Robyt & French, 1970) as five subsites were also found. This result is not surprising considering the fact that there is high sequence homology between these two enzymes (84%). Despite this there are still some subtle differences in the "action patterns" particularly for maltoheptaose (see Figure 3.9), which suggests slight differences in the active site of the two enzymes.

![Figure 3.10. Active site of HPA (↓ catalytic nucleophile)](image)

b) Kinetic evaluation of malto-oligosaccharides

Maltotriose (G3) is believed to be cleaved at both glycosidic linkages by HPA as has been demonstrated with PPA using radiolabeled G3 (Robyt & French, 1970), but in our experiment using unmodified malto-oligosaccharides the cleavage position could not be identified and only one $k_{cat}$ and $K_m$ could be determined. G3 had the highest $K_m$ of all the substrates with a value of 10.9 mM (see Table 3.1). G3 also had the lowest $k_{cat}$, approximately 150 times lower than the best substrate maltopentaose.

Maltotetraose (G4) can bind productively in two ways, thus yielding two different sets of kinetic parameters. G4 cleavage to form two moles of G2 has a slightly higher $K_m$ but a significantly higher $k_{cat}$, which results in an overall catalytic efficiency approximately four-fold higher than the cleavage which results in the formation of G3.
and G1. This result correlates well with the cleavage pattern results. Comparing the two catalytic efficiencies \( \frac{k_{\text{cat}}}{K_m} \) it is apparent there is a slight advantage of binding in subsite 5 over subsite 1 (see Figure 3.10). The \( k_{\text{cat}} \) for production of two moles of G2 is higher because amylase prefers to have both of the aglycone subsites filled.

The kinetic results with maltohexaose (G6) were also consistent with the observed "action pattern". The predominant cleavage of G6 into G4 and G2 was observed in the kinetic parameters, since the catalytic efficiency for this reaction was three-fold higher than that for cleavage resulting in the formation of two moles of G3. The difference in catalytic efficiencies was mainly due to the better binding of G6 in the productive complex that resulted in the formation of G4 and G2. Although the difference is not dramatic, the result does suggest that perhaps there is potentially an additional binding subsite in the glycone binding site since in both binding complexes all of the five subsites are filled (see Figure 3.11).

![Proposed additional subsite in active site](image)

**Figure 3.11.** Proposed additional subsite in active site (O - nonreducing sugar; \( O \) - reducing sugar; and — \( \alpha \)-(1,4) linkage)

The cleavage of maltoheptaose (G7) by HPA can occur through three different productive complexes, but kinetic parameters could only be determined for the reactions producing G5 + G2, and G4 + G3 (or G3 + G4). It is not possible to discern between the two ways in which G4 and G3 are formed, because boiling the samples before injection onto the HPLC (denatures HPA), results in the formation of the equilibrium mixture of
G4 and G3 anomers. Therefore only two sets of kinetic parameters can be determined. Summing up the cleavage frequencies for the production of G4 and G3 and comparing this to the formation of G5 and G2, the values were 74% (30% + 44%) and 26%, respectively. Therefore the major products were produced 3-fold more rapidly than the minor products. The kinetic parameters for these two events concurred, since the catalytic efficiency for the formation of G4 and G3 was 2.2-fold higher than that for the formation of G5 and G2. The difference in catalytic efficiencies was due to the two-fold higher $k_{cat}$ for the production of G4 and G3.

The $K_m$ values for all the substrates, except for G3, were between 0.5 and 1.1 mM. This suggests that four of the five subsites are required to be filled in order to obtain optimum binding and further filling of the fifth subsite does not enhance binding. The $k_{cat}$ values increase from G3 to G5 and then decrease slightly with G6. This result demonstrates that even though the fifth subsite does not enhance binding, it does increase the rate of turnover of the substrate. The catalytic efficiency, $k_{cat}/K_m$, follows a better trend than do the individual parameters as the value increases rapidly from G3 to G5 and

![Figure 3.12. The plot of catalytic efficiency vs. malto-oligosaccharide chain length](image)
Table 3.1. Kinetic evaluation of malto-oligosaccharides as substrates for HPA and porcine pancreatic α-amylase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Products</th>
<th>( K_m ) (mM)</th>
<th>( k_{cat} ) (s(^{-1}))</th>
<th>( k_{cat}/K_m ) (s(^{-1})mM(^{-1}))</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPA</td>
<td>maltotriose</td>
<td>G2 + G1</td>
<td>10.9 ± 1.9</td>
<td>2.6 ± 0.2</td>
<td>0.24 ± 0.06</td>
<td>a</td>
</tr>
<tr>
<td>HPA</td>
<td>maltotetraose</td>
<td>G2 + G2</td>
<td>0.86 ± 0.11</td>
<td>100 ± 4</td>
<td>116 ± 19</td>
<td>a</td>
</tr>
<tr>
<td>HPA</td>
<td>maltotetraose</td>
<td>G3 + G1</td>
<td>0.51 ± 0.12</td>
<td>17.4 ± 1.2</td>
<td>27.5 ± 8.4</td>
<td>a</td>
</tr>
<tr>
<td>HPA</td>
<td>maltopentaose</td>
<td>G3 + G2</td>
<td>1.0 ± 0.1</td>
<td>408 ± 17</td>
<td>408 ± 57</td>
<td>a</td>
</tr>
<tr>
<td>HPA</td>
<td>maltohexaose</td>
<td>G4 + G2</td>
<td>0.40 ± 0.09</td>
<td>167 ± 9</td>
<td>418 ± 117</td>
<td>a</td>
</tr>
<tr>
<td>HPA</td>
<td>maltohexaose</td>
<td>G3 + G3</td>
<td>1.1 ± 0.4</td>
<td>150 ± 21</td>
<td>136 ± 68</td>
<td>a</td>
</tr>
<tr>
<td>HPA</td>
<td>maltoheptaose</td>
<td>G4 + G3</td>
<td>0.74 ± 0.11</td>
<td>375 ± 16</td>
<td>507 ± 98</td>
<td>a</td>
</tr>
<tr>
<td>HPA</td>
<td>maltoheptaose</td>
<td>G5 + G2</td>
<td>0.70 ± 0.11</td>
<td>98.1 ± 5.4</td>
<td>140 ± 30</td>
<td>a</td>
</tr>
<tr>
<td>PPA</td>
<td>maltotriose</td>
<td>G2 + G1</td>
<td>0.5</td>
<td>0.13</td>
<td>0.27</td>
<td>b</td>
</tr>
<tr>
<td>PPA</td>
<td>maltotetraose</td>
<td>G2 + G2</td>
<td>0.7</td>
<td>79.1</td>
<td>110</td>
<td>b</td>
</tr>
<tr>
<td>PPA</td>
<td>maltotetraose</td>
<td>G3 + G1</td>
<td>0.7</td>
<td>38.9</td>
<td>54.0</td>
<td>b</td>
</tr>
<tr>
<td>PPA</td>
<td>maltopentaose</td>
<td>G3 + G2</td>
<td>1.08</td>
<td>1360</td>
<td>1260</td>
<td>b</td>
</tr>
<tr>
<td>PPA</td>
<td>maltohexaose</td>
<td>all rxns</td>
<td>0.62</td>
<td>1270</td>
<td>2050</td>
<td>b</td>
</tr>
<tr>
<td>PPA</td>
<td>maltoheptaose</td>
<td>all rxns</td>
<td>1.02</td>
<td>1250</td>
<td>1220</td>
<td>b</td>
</tr>
</tbody>
</table>

\(^a\) This thesis; \(^b\) (Seigner et al., 1987)

then increases slowly from G5 to G7 (see Figure 3.12). This result is consistent with the five subsite hypothesis. It has also been shown that in each case the "action patterns" were in agreement with the determined catalytic efficiencies.

Comparing the kinetic parameters for HPA with PPA (see Table 3.1; (Seigner et al., 1987)) some major differences were observed. The results for G3 showed that the \( k_{cat} \) value with HPA was 20-fold higher than that for PPA, but the \( K_m \) with PPA was
twenty-fold lower than the same reaction catalyzed by HPA. The $K_m$ and $k_{cat}$ results for the hydrolysis of G4 were the same for both HPA and PPA. For G5 through to G7, the $K_m$ values were also very similar but the $k_{cat}$ values for PPA were higher than those obtained for HPA (approximately 3-fold). Therefore with smaller oligosaccharides (G3) the difference between PPA and HPA exists in both the binding ($K_m$) and the turnover ($k_{cat}$), and with larger oligosaccharides (G4-G7) only a slight difference exists in the turnover ($k_{cat}$). The PPA results also suggest that only three of the five subsites were required to be filled in order to obtain tight binding, whereas, with HPA four of the five subsites needed to be filled.

3.3.3. Glycosyl fluorides as substrates for HPA

a) Kinetic evaluation

As mentioned previously, a common assay for HPA involves the cleavage of PNP oligosaccharides. One such sugar which has been utilized as a substrate for HPA is $p$-

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$mM$^{-1}$)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPA</td>
<td>$\alpha$G2F</td>
<td>4.5 ± 0.1</td>
<td>443 ± 5</td>
<td>98 ± 3</td>
<td>a</td>
</tr>
<tr>
<td>HPA</td>
<td>$\alpha$PNPG2</td>
<td>4.2</td>
<td>0.040</td>
<td>0.01</td>
<td>b</td>
</tr>
<tr>
<td>BHKHPA</td>
<td>$\alpha$G2F</td>
<td>3.7 ± 0.4</td>
<td>526 ± 23</td>
<td>142 ± 22</td>
<td>a</td>
</tr>
<tr>
<td>BHKHPA</td>
<td>4D$\alpha$G2F</td>
<td>9.1 ± 1.2</td>
<td>489 ± 27</td>
<td>54 ± 10</td>
<td>a</td>
</tr>
<tr>
<td>BHKHPA</td>
<td>$\alpha$G3F</td>
<td>0.51 ± 0.07</td>
<td>283 ± 12</td>
<td>555 ± 99</td>
<td>a</td>
</tr>
<tr>
<td>BHKHPA</td>
<td>4D$\alpha$G3F</td>
<td>0.49 ± 0.04</td>
<td>303 ± 8</td>
<td>618 ± 67</td>
<td>a</td>
</tr>
<tr>
<td>HPA</td>
<td>2F$\alpha$G2F</td>
<td>4.7 ± 0.9</td>
<td>0.17 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>a</td>
</tr>
</tbody>
</table>

a This thesis; b (Ishikawa et al., 1992)
nitrophenyl $\alpha$-maltoside ($\alpha$PNPG2). However because of the low $k_{\text{cat}}$ value obtained with this substrate, we decided to evaluate $\alpha$-maltosyl fluoride ($\alpha$G2F) as a substrate to see if a better assay system could be developed. The kinetic parameters obtained in the reaction between HPA and the two substrates are presented in Table 3.2. Upon comparison of the results for $\alpha$G2F with those for $\alpha$PNPG2, it is apparent that the glycosyl fluoride has a much higher maximum rate of turnover. This likely reflects the greater leaving group ability of fluoride than p-nitrophenol, suggesting that the rate determining step is the formation of the glycosyl-enzyme.

Cleavage of $\alpha$G2F by amylases other than HPA is known to occur by both hydrolysis and transglycosylation (Okada et al., 1979). The HPA catalyzed cleavage of $\alpha$G2F produced two major products, G2 and G4 (see Figure 3.13). These results are consistent with those determined by Okada et al. (1979). The disaccharide was most likely produced by the hydrolysis of $\alpha$G2F. G4 was produced by first transglycosylation onto another molecule of itself and then subsequent hydrolysis of the fluoride.

3.3.3.b. Cloned HPA in baby hamster kidney cell lines

Isolation of HPA requires a source of human pancreas, and an arrangement with the UBC hospital had provided the enzyme for all the kinetic studies performed up to this point. As can be appreciated, the supply of human pancreas was limited and inconsistent. Work was thus started by others in this group on the cloning of HPA into baby hamster kidney cell lines in collaboration with Professor MacGillivray in the Department of Biochemistry at UBC. The cDNA sequence of the BHKHPA clone produced was identical to the published sequence for HPA (Nishide et al., 1986). However, it was not known if differences in post translational modification might exist and whether they would result in differences in activity. An initial experiment with BHKHPA was therefore performed to determine whether the enzyme was kinetically identical to that obtained from human tissue. $\alpha$G2F was chosen as the most convenient substrate for
Figure 3.13. HPLC analysis of the products formed in the reaction between αG2F and HPA

A) αG2F (3.1 mM) in buffer; B) αG2F (3.1 mM) after addition of HPA (0.18 μg/mL)

The $k_{\text{cat}}$ and $K_m$ values determined are shown in Table 3.2. As can be seen, the cloned and wild type enzymes had very similar kinetic parameters with αG2F. In fact the catalytic efficiency ($k_{\text{cat}}/K_m$) for cleavage of αG2F was slightly higher for BHKHPA than HPA. This result is likely explained by the fact that the activity of the α-amylase obtained from human pancreas depended on several factors, such as the condition of the pancreas and the length of time it had been removed from the body, thus inactive enzyme is probably purified with active enzyme. These factors combined, it is not unreasonable that the enzyme from natural sources had a slightly lower specific
activity than the cloned enzyme. Since the cloned enzyme was shown to be very similar kinetically to the wild type enzyme, herein for all experiments, whether wild type HPA or BHKHPA is used, the enzyme will be referred to as HPA, except where specified.

Since cleavage of αG2F by HPA occurs by hydrolysis and transglycosylation, an interesting substrate would therefore be one in which the hydroxyl group at the 4 position is removed, since it should be unable to undergo transglycosylation. 4-Deoxy-α-maltosyl fluoride (4DαG2F; 24) was thus synthesized and evaluated as a substrate for HPA and the kinetic parameters determined are shown in Table 3.2. The $k_{cat}$ value for 4DαG2F is essentially the same as that for αG2F, suggesting that the rates of hydrolysis and transglycosylation are the same, since 4DαG2F is only cleaved via hydrolysis (see Figure 3.14). This might suggest that the rate determining step for the cleavage of αG2F is formation of the glycosyl-enzyme, since the acceptors, water or another sugar molecule have no effect on the rate of the reaction. The results also show that the 4-position does not make crucial binding interactions at the transition state since the binding is only impaired by a factor of two.

The model developed for the active site of HPA indicates that the glycone site contains three subsites suggesting that αG2F might not be the ideal glycosyl fluoride substrate, since at most it can occupy only two of the subsites. αG3F was thus
Figure 3.14. HPLC analysis of the products formed in the reaction between 4DαG2F and HPA

A) 4DαG2F (2.5 mM) in buffer; B) 4DαG2F (2.5 mM) after addition of HPA (0.084 µg/mL); and C) 4-deoxymaltose in buffer. The column was a Dynamax® amino column (60 Å 8µm, 4.6 mm X 25 cm) from Rainin®, and acetonitrile/water (60/40) was used as the eluent.
I 0.045
0.04
0.035
0.03
0.025
0.05

Time min

Relative Refractive Index

Figure 3.15. HPLC analysis of the products formed in the reaction between αG3F (5.3 mM) and HPA (0.11 μg/mL) using the Dextropak® column.

The product distribution for the trisaccharide analogue, αG3F is shown in Figure 3.15. The results show that the main products formed are αG3, αG6F, αG4, and αG2F. αG3 was formed by the hydrolysis of αG3F and αG6F formed by the transglycosylation of αG3F onto another molecule of itself. The G4 and αG2F are then formed by hydrolysis of αG6F (see Figure 3.16). Based on the large amount of αG3F still present and the fact that the substrate concentration is ten times $K_m$, the hydrolysis of αG6F probably occurred before αG6F could leave the active site. The peaks in the HPLC profile marked with an "X" are unidentified contaminants that are present in the control.

synthesized and evaluated kinetically as a substrate for HPA, and the kinetic constants determined are shown in Table 3.2. The presence of the additional glucose unit reduced the $K_m$ 8-fold compared to αG2F, but interestingly, also reduced the $k_{cat}$ value 2-fold. Even so, the catalytic efficiency ($k_{cat}/K_m$) for αG3F was 4-fold higher than for αG2F, consistent with expectations.
Figure 3.16. Proposed reaction scheme between αG3F and HPA
4-Deoxy-α-maltotriosyl fluoride (4DaG3F) was also evaluated as a substrate (see Table 3.2). Its $K_m$ and $k_{cat}$ values are essentially identical to those of αG3F, again perhaps suggesting that the rate limiting step for both substrates is the formation of the glycosyl-enzyme. As expected the main product formed upon the addition of HPA was 4-deoxymaltotriose (data not shown). Since no difference was observed in the $K_m$ values, this result also shows that no significant ground state interactions are formed with the 4-hydroxyl group of αG3F.

α-D-Glucosyl fluoride (αGF) was also evaluated as a substrate and the results are shown in Figure 3.17. Not only was saturation not observed, but the curve increased exponentially rather than linearly. This suggests that two moles of substrate are required in the rate determining step of the reaction (Hehre et al., 1979). A possible model for this would be an obligate filling of an adjacent site for cleavage of αGF.
In the attempt to kinetically evaluate αGF as a substrate for HPA it was shown that the hydrolysis of glucosyl fluoride does not follow Michaelis-Menten kinetics. The product distribution of this reaction was thus determined to gain a better understanding of how HPA utilizes αGF as a substrate, and the results are shown in Figure 3.18. Upon addition of HPA the three major products formed are G2, G3, and glucose (G). The main product G2 arose from the transglycosylation of αGF onto another molecule of itself followed by hydrolysis of αG2F to form G2. This result is consistent with the kinetic results, that a bimolecular reaction had taken place at the rate limiting step. G3 is probably formed by transglycosylation of αGF onto G2, and G is most likely formed by hydrolysis of G3. With αGF as a substrate HPA most likely acts only as transglycosidase.
3.3.4. Testing 2-deoxy-2-fluoro-α-maltosyl fluoride as a mechanism-based inhibitor of HPA

2-Deoxy-2-fluoro-α-maltosyl fluoride (2FoG2F) was synthesized according to the procedure outlined in Chapter 2 for the synthesis of 2-deoxy-2-fluoro-α-maltotriosyl fluoride, and tested as an inactivator of HPA (not BHKHPA). As seen with Glyx, 2FoG2F was not an inactivator, but rather was used as a slow substrate. Kinetic parameters for its cleavage are shown in Table 3.2. Interestingly, its $K_m$ value is essentially the same as that for αG2F, suggesting that the 2-fluoro group did not impair any ground state binding-interactions. The $k_{cat}$ value, however, was 2600-fold lower than that for α G2F. This decrease in rate was most likely due to inductive destabilization of the oxocarbonium ion-like transition state by the electron withdrawing fluorine atom.

The Lineweaver-Burk plot for the reaction between HPA and 2FoG2F was not linear, but rather biphasic (see Figure 3.19). This biphasic behaviour was not seen with any of the other glycosyl fluorides (see Appendix I), but has been seen with other glycosidases for hydrolysis of substrates whose rate limiting step is deglycosylation. Under these conditions hydrolysis usually predominates at low substrate concentrations, while at high concentrations the aglycone site is occupied and transglycosylation occurs. The break in the line is due to the different rates for the two reactions indicating that the rate-limiting step is deglycosylation. This would suggest that at high concentrations of 2FoG2F the rate of transglycosylation is greater than the rate of hydrolysis, whereas at low concentrations, only hydrolysis is possible.

One definitive result that came out of this work was that 2-deoxy-2-fluoroglycosides act only as slow substrates and not as mechanism-based inactivators of Glyx and HPA. These results thus tend to bring into question the observed inactivation of yeast α-glucosidase by 2FoGF (Withers et al., 1988). The apparent inactivation will be reexamined in Chapter 4 of this thesis.
A novel approach to developing a specific mechanism-based inhibitor of "retaining" α-glycosidases was studied. It was believed that the 2-fluoro substituent does not slow down the deglycosylation step in an α-glycosidase sufficiently in order to accumulate a glycosyl-enzyme intermediate. One solution was to add an additional fluorine to C-2 to further slow the deglycosylation step. This modification will also presumably drastically slow the glycosylation step, so an extremely good leaving group (better than fluorine) was probably going to be required in order for the first step to occur at a useful rate. One very good leaving group would be 2,4,6-trinitrophenol (pK<sub>a</sub> <1), which should be sufficiently reactive to allow the first step in the reaction to take place and thus result in accumulation of a glycosyl-enzyme intermediate. 2,4,6-Trinitrophenyl 2-deoxy-2,2-difluoro-4-O-(α-(1,4)-D-glucosyl)-α-D-arabinohexopyranoside (22FαG2TNP) was thus synthesized and tested as a mechanism-based inhibitor of HPA.

Figure 3.19. The Lineweaver-Burk plot for the reaction between 2FαG2F and HPA
a) Synthesis

Starting from maltose per-O-acetate the synthesis was similar to the synthesis of 2FαG2F up to the synthesis of maltal per-O-acetate. The overall scheme used to synthesize 22FαG2TNP is outlined in Figure 3.20. The glycal was fluorinated with acetylhypofluorite using the large scale procedure (see Material and Methods) to synthesize the 2-deoxy-2-fluoro disaccharide per-O-acetate. Unfortunately, some F₂ was also present, and added across the glycal double bond resulting in a mixture of four disaccharide products: 2-deoxy-2-fluoro-α-"gluco" fluoride and per-O-acetate; and 2-deoxy-2-fluoro-β-"manno" fluoride and per-O-acetate. Separation of the product mixture was too difficult at this stage and so the mixture was used in the next reaction without further purification. This next step was formation of the bromide using HBr/HOAc, which initially caused problems because instead of taking 45 min (OAc at C-2), the reaction required 2 days, presumably because of the inductive effect of the fluorine substituent. A competing reaction was brominolysis of the glycosidic linkage resulting in considerable degradation to monosaccharides. The next step in the reaction scheme was elimination of HBr with triethylamine and once again the presence of the fluorine at C-2 resulted in a longer reaction time. The 2-fluoro "manno" compound did not undergo the elimination reaction using this procedure, presumably because of the cis arrangement between H-2 and the bromine at C-1. At this stage the mixture could finally be partially purified (95%) before proceeding to the next step. Fluorination of 2-fluoromaltal per-O-acetate required similar reaction conditions to those used for fluorination of maltal per-O-acetate, but in this case addition from the top or bottom face by acetylhypofluorite was of no concern, because the product from both additions was the 2,2-difluoro compound. The reaction mixture was purified at this stage. An initial plan was to attempt to make the α-bromide, but all efforts to afford the bromide were unsuccessful. Therefore we decided to selectively deprotect the anomeric center using hydrazine acetate, which once again took 2 days at elevated temperatures compared with 45 min at room temperature.
Figure 3.20. Outline of the synthesis of 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-4-O-\(\alpha\)-(1,4)-D-glucosyl-\(\alpha\)-D-arabinopyranoside (23)

for the unmodified glycoside. Even though the starting material for this reaction was a mixture of the \(\alpha\)- and \(\beta\)-O-acetates, the only product isolated was the \(\alpha\)-OH product. A side reaction, however, was the deprotection of the 3-position, probably because the two fluorines at C-2 helped to stabilize the formation of the alkoxide ion. Attempts were made to tosylate or mesylate the anomeric center as a means of introducing a reactive
leaving group. However, these were unsuccessful. This product was finally reacted with fluorotrinitrobenzene, which itself was synthesized according to a published procedure (Shaw & Seaton, 1961). The reaction took ten days to go to completion and form the α-trinitrophenyl product. Deprotection using HCl/MeOH at 4°C afforded 23 in 2 days. A major side reaction during deprotection was the formation of the free 2-deoxy-2,2-difluorodisaccharide and methyl picrate, via attack of methanol at C'-1 of the trinitrophenyl moiety via a nucleophilic aromatic substitution.

b) Time dependent-inactivation of HPA

22FaG2TNP was evaluated as mechanism-based inhibitor of HPA. As can be seen in Figure 3.21 time-dependent inactivation was observed. Unfortunately, only a maximum inhibitor concentration of 11 mM could be obtained due to insolubility, yet the $K_i$ was estimated to be approximately 90 mM. Therefore, saturation of the enzyme's

![Figure 3.21. Inactivation of HPA by 22FaG2TNP](image)

Semilogarithmic plot of residual activity vs time at the indicated inactivator concentrations: (◊) 7.5 mM; (▼) 5.6 mM; (●) 3.7 mM; (■) 2.5 mM; (○) 1.9 mM.
active site by the inhibitor could not be achieved. From the slope of the line of the
double reciprocal plot a $k_i/K_i$ of 0.0073 min$^{-1}$mM$^{-1}$ was obtained for the inactivation of
HPA by 22FaG2TNP (see Figure 3.22).

c) Active site protection against inhibition

A known competitive inhibitor of amylases, acarbose, was used to test if the
inhibition was due to an event occurring at the active site. The results of this experiment
are shown in Figure 3.23. As can be seen, the presence of acarbose protected the enzyme
against inactivation by 22FaG2TNP, indicating that inhibition is indeed a result of
reaction at the active site.

![Figure 3.22. Inactivation of HPA by 22FaG2TNP](image)

Replot of first-order rate constants from Figure 3.15.
Figure 3.23. Inactivation of HPA by 22FαG2TNP (5.6 mM) in the (○) absence and presence of (○) acarbose (65 μM). Inactivation of HPA by (□) 2,4,6-trinitrophenol (7.2 mM).

Also tested in this experiment was the concern that inhibition might be caused by 2,4,6-trinitrophenol (TNP) released. The observed results, at a TNP concentration higher than would be possible in the actual experiment, showed essentially no time-dependent inhibition. Therefore the inactivation was not due to the release of TNP by enzymatic and spontaneous processes. The results of these experiments are summarized in Table 3.3.

<table>
<thead>
<tr>
<th>Inactivation mix</th>
<th>Inactivation rate (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPA + 22FαG2TNP</td>
<td>0.0436</td>
</tr>
<tr>
<td>HPA + Acarbose + 22FαG2TNP</td>
<td>0.0084</td>
</tr>
<tr>
<td>HPA + 2,4,6-trinitrophenol</td>
<td>0.0018</td>
</tr>
</tbody>
</table>
d) Reactivation experiment

The excess inactivator was removed using a Centricon 30 kDa molecular weight cutoff membrane, and a reactivation experiment was attempted in the presence and absence of 50 mM maltose. Aliquots were assayed periodically over 30 days and compared with a control. The control over that time lost essentially no activity, and the inactivated enzyme did not recover any activity.

e) Burst experiment

If 22FαG2TNP is truly a mechanism-based inhibitor of HPA then the reaction should involve a single enzymatic event and one mole of TNP should be released per mole of enzyme upon formation of a glycosyl-enzyme intermediate. Reaction of 22FαG2TNP with a relatively large amount of HPA was monitored continuously using a spectrophotometer to follow the release of TNP. As can be seen in Figure 3.24, the time course for the release of TNP was biphasic. The first phase involved a relatively rapid initial release of TNP, followed by a slower second phase, which was a steady state turnover of the substrate. The linear portions of both phases were extrapolated back to time zero and a burst magnitude of 26 μM was obtained. This result corresponds well with the amount of HPA present (23 μM). The experiment was repeated with twice the amount of enzyme and the burst was also found to be increased two-fold (data not shown). Therefore, the results show that inactivation of the enzyme is accompanied by the release of a single equivalent of aglycone.
The steady state turnover of the inhibitor was found to be quite rapid. This release of TNP was due to either turnover of the inactivated enzyme or a spontaneous process with free inhibitor. However, on the basis of the reactivation experiment where no reactivation was seen even after 30 days, it seemed quite unlikely that the turnover was due to an enzymatic process. A reaction mixture was therefore set up which was identical to the burst experiment, except that no enzyme was added. The result was a release of TNP at a rate equivalent to the steady state rate, which demonstrates that the steady state phase was due to a non-enzymatic spontaneous hydrolysis process.
3.4. Conclusions

$^1$H NMR analysis of reaction mixtures containing $\alpha$G2F or G7 and HPA reconfirmed that HPA cleaves its substrates with net retention of anomeric configuration. A novel method using a Dextropak® HPLC column was developed which allows determination of the stereochemistry of the initial products formed by an endoglycanase through identification of the products. This method was used to determine the "action patterns" for the hydrolysis of the malto-oligosaccharides, G4 to G7. The "action pattern" results reveal that there are five subsites which make up the active site. Three subsites make up the glycone site and two subsites make up the aglycone site. Kinetic evaluation of these oligosaccharides confirmed these results.

Both glycosyl fluorides, $\alpha$G2F and $\alpha$G3F, were found to be excellent substrates for HPA. Product distribution studies revealed that turnover of these substrates involved both hydrolysis and transglycosylation reactions. $k_{cat}$ values for the 4-deoxy glycosyl fluorides were determined to be identical to those for 4-hydroxy glycosyl fluorides, thus suggesting that the rate limiting step with these substrates is the formation of the glycosyl-enzyme intermediate. HPA also utilized $\alpha$GF as a substrate, but the reaction did not follow Michaelis-Menten kinetics.

Reaction of 2F$\alpha$G2F with HPA did not result in inactivation via the accumulation of a glycosyl-enzyme intermediate. Rather it was found to act as a slow substrate with a $K_m$ value very similar to that determined for $\alpha$G2F, suggesting that fluorine substitution did not disrupt any ground state binding-interactions. A break in the Lineweaver-Burk plot was observed suggesting that deglycosylation was the rate-limiting step for this substrate.

22FaG2TNP was found to act as a time-dependent inactivator of HPA. Protection studies using the competitive inhibitor acarbose revealed that the inactivation was active site-directed. Further reaction was shown to result in the release of one full equivalent of TNP per equivalent of enzyme in a burst phase, as expected for such a
mechanism-based inactivator which functions via the accumulation of a glycosyl-enzyme intermediate. Attempts were made to reactivate the enzyme but even after 30 days no recovery in activity was observed, indicating that an extremely stable intermediate had been formed.
Chapter 4: Mechanistic Studies On Yeast α-Glucosidase

4.1. Introduction

2-Deoxy-2-fluoro-β-glycosyl fluorides have been shown to be mechanism-based inactivators of "retaining" β-glycosidases (Withers et al., 1988). 2-Deoxy-2-fluoro-α-glycosyl fluorides from the same study, however, only partially inactivated two of the five α-glycosidases tested. Further 2-deoxy-2-fluoro-α-glycosyl fluoride studies on HPA (Chapter 3) and Glyx (Chapter 2) reveal that these compounds are utilized only as slow substrates. These results, in conjunction with the observation of incomplete inactivation of the two α-glycosidases, bring into question whether 2-deoxy-2-fluoro-α-glycosyl fluorides act as mechanism-based inactivators or just as substrates of "retaining" α-glycosidases.

4.2. Aims and goals

The initial goal of this study is to reinvestigate 2-deoxy-2-fluoro-α-glucosyl fluoride (2FαGF) as a mechanism-based inactivator of yeast α-glucosidase. If inactivation is not observed then 2FαGF will be evaluated as a substrate for yeast α-glucosidase.

Another goal of this study is to develop a novel mechanism-based inactivator of α-glycosidases. In a similar series of experiments to those performed in Chapter 3, 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-α-D-arabino-hexopyranoside (22FαGTNP; 44) will be tested as a mechanism-based inactivator of yeast α-glucosidase.

A final goal of this study is to identify the catalytic nucleophile of yeast α-glucosidase that is responsible for formation of the glycosyl enzyme intermediate. This will be accomplished using two inactivators of yeast α-glucosidase: 22FαGTNP, and 2-deoxy-2-chloro-2-fluoro-α-D-arabino-hexopyranosyl chloride (2Cl2FαGCl; 45).
4.3. Results and discussion

4.3.1. Reassessment of 2-deoxy-2-fluoro-α-D-glucosyl fluoride as an inactivator of yeast α-glucosidase

a) 2-Deoxy-2-fluoro-α-glycosyl fluoride is a substrate, not a simple mechanism-based inactivator.

Reaction of 2-deoxy-2-fluoro-α-glycosyl fluoride (2FoGF) with yeast α-glucosidase was monitored using a selective fluoride ion electrode. As is clear from Figure 4.1 α-glucosidase is able to release several equivalents of fluoride ion from the inhibitor 2FoGF, indicating that 2FoGF is acting as a substrate, not as a simple mechanism-based inactivator of yeast α-glucosidase.

b) Test for a contaminant

An attempt was made to see if the time-dependent inactivation observed previously was due to the presence of a contaminant. An inactivation experiment was devised in which the concentration of enzyme was varied rather than the potential inhibitor 2FoGF. If a very low level contaminant is responsible for the inactivation of α-glucosidase, then the amount of contaminant can be titrated by simply increasing the amount of enzyme until
time-dependent inactivation is no longer observed. As well as assaying for residual activity, the inactivation mixture will be assayed for total fluoride released to determine how much, if any, 2FaGF was still present. The goal of this experiment was to find a concentration of enzyme at which inactivation did not occur yet 2FαGF was still present in excess in the inactivation mixture. If these conditions are satisfied then the inactivation of α-glucosidase must be due to a low level contaminant and not be a property of 2FαGF itself. The results of this experiment can be seen in Figure 4.2. In the presence of excess 2FαGF (10 mM), the extent of inactivation was different at the two highest enzyme concentrations. For example the 17 μM enzyme sample was only 17% inactivated, whereas, the 4.3 μM sample was 65% inactivated. In both cases excess 2FαGF was still present in the inactivation mixtures, even though no further enzyme inactivation was observed. Indeed the enzyme continued to hydrolyze the remaining 2FαGF. These results

Figure 4.1. The release of fluoride ion from the reaction between 2-deoxy-2-fluoro-α-D-glucosyl fluoride (3.5 mM) and yeast α-glucosidase (0.22 μM).
Figure 4.2. Inactivation of different concentrations of yeast α-glucosidase in the presence of 2FαGF

The initial concentration of 2FαGF in all inactivation mixtures was 10 mM. The following concentrations of enzyme were present in the inactivation mixtures: 0.26 μM (○); 1.3 μM (▼); 4.3 μM (□); and 17.1 μM (■). Appropriate dilutions of the inactivation mixtures were made so that the same amount of enzyme was present in all of the reaction cells.

suggest that the inactivation of α-glucosidase could not due to 2FαGF, but rather be due to a low level contaminant.

At the two highest enzyme concentrations, 4.3 and 17.1 μM, the amounts of enzyme inactivated were 2.8 and 2.9 μM, respectively. Assuming that inactivation is a stoichiometric process this result suggests there is contaminant in the 2FαGF preparation at the 0.03% level {\((0.003 \text{ mM} / 10 \text{ mM}) \times 100\)}.

Preliminary studies into the identification of the contaminant suggest that a possible candidate is trifluoromethyl 2-deoxy-2-fluoro-α-D-glucopyranoside (46). This compound was a byproduct in the synthesis of the 2FαGF. It could possibly act as a suicide inhibitor because enzymatic
hydrolysis would release the very unstable product trifluoromethanol, which would decompose rapidly, releasing carbonyl fluoride which could react with any nucleophilic amino acid side chain on the enzyme. Further studies are required to unequivocally identify the contaminant.

![Image](46)

4.3.2. 2FaGF as a substrate for yeast α-glucosidase

Since 2FaGF was shown not to be a mechanism-based inactivator of α-glucosidase, it was therefore evaluated as a substrate for the enzyme, and the kinetic parameters determined are shown in Table 4.1. Its $K_m$ value is 5-fold higher than that for αGF, suggesting that the 2-fluoro group somewhat impairs ground state binding-interactions. The $k_{cat}$ value for 2FaGF was only 16-fold lower than for αGF possibly suggesting that the 2-fluoro group inductively destabilizes the oxocarbonium ion-like transition state only to a small extent. However, these results are similar to the those

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$mM$^{-1}$)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>2FaGF</td>
<td>4.8 ± 0.8</td>
<td>1.6 ± 0.1</td>
<td>0.33 ± 0.08</td>
<td>a</td>
</tr>
<tr>
<td>αGF</td>
<td>0.93</td>
<td>24.8</td>
<td>26.7</td>
<td>b</td>
</tr>
</tbody>
</table>

*a This thesis; b (Konstantinidis & Sinnott, 1991)
obtained for α-amylase with the disaccharide analogues, where the 2-fluoro sugar was also shown to be a slow substrate (see Chapter 3).

4.3.3. 2,4,6-Trinitrophenyl 2-deoxy-2,2-difluoro-α-D-arabinopyranoside as a mechanism-based inhibitor

a) Synthesis

The method used to synthesize 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-α-D-arabinopyranoside (22FoGTNP; 44) was similar to that described in Chapter 3 for the synthesis of the disaccharide analogue. The synthetic route is outlined in Figure 4.3.

b) Time-dependent inactivation of yeast α-glucosidase

22FoGTNP was evaluated as a mechanism-based inactivator of α-glucosidase. As can be seen in Figure 4.4 time-dependent inactivation was observed. Unfortunately, the residual activity of the later time points deviated away from the single exponential decay curve. One possible explanation for this result is that perhaps the concentration of inhibitor is decreasing during the inactivation experiment as a result of hydrolysis. This would then result in the observed lower rate of inactivation. The rate of spontaneous hydrolysis was checked and found to be quite high ($k_{\text{spontaneous}} = 0.0047 \text{ min}^{-1}$ at 37°C), but this alone could not account for the decrease in inactivation rate. Another control experiment was set up containing 0.1% Bovine Serum Albumin (BSA, which was required in the inactivation experiment to stabilize the α-glucosidase) plus 22FoGTNP in buffer. Under these conditions the rate of degradation was five times higher than that of spontaneous hydrolysis. At this rate the concentration of 22FoGTNP would decrease significantly during the inactivation experiment. The amount of BSA in the inactivation
Figure 4.3. Outline of the synthesis of 2-deoxy-2,2-difluoro-α-D-arabino-hexopyranoside (44)
mixture was thus decreased to attempt to minimize the breakdown of the inhibitor. A BSA concentration of 0.0005%, which was the lowest concentration possible since yeast α-glucosidase supplied by Sigma contains approximately 25% BSA, was tried first. At this level the α-glucosidase control activity was found to decrease significantly in one hour, thus more BSA was required. The next concentration of BSA tried was 0.005%, and stability tests confirmed that α-glucosidase was 100% active even after 1 h. The inactivation experiment was therefore repeated, and even under these conditions the longer time points (> 10 min) still fell off the single exponential decay curve somewhat. Therefore the solution to this problem was to take inactivation data points only up to 2-3
Figure 4.5. Inactivation of yeast $\alpha$-glucosidase by 22F$\alpha$GTNP

Semilogarithmic plot of the residual activity vs. time at $[\text{BSA}] = 0.005\%$ and the indicated inactivator concentrations: $(\Diamond) \ 3.2 \ \text{mM}; (\nabla) \ 2.7 \ \text{mM}; (\square) \ 2.2 \ \text{mM}; (\blacksquare) \ 1.6 \ \text{mM}; (\bullet) \ 1.1 \ \text{mM}$.

Figure 4.6. Inactivation of yeast $\alpha$-glucosidase by 22F$\alpha$GTNP without ($\Diamond$) and with ($\nabla$) pretreatment (see Section 4.3.3 (d)) of yeast $\alpha$-glucosidase
half-lives (< 10 min). The time-dependent inactivation results are shown in Figure 4.5. Since the plot of rate of inactivation vs. inhibitor concentration (see Figure 4.6) only shows slight curvature, saturation was not achieved, thus the individual kinetic parameters could not be determined. From the slope of the line a $k_i/K_i$ value of $0.25 \text{ min}^{-1}\text{mM}^{-1}$ was obtained.

c) Protection experiment

A competitive inhibitor of $\alpha$-glucosidase, DNJ, was used to protect the enzyme from inactivation. This experiment determines if the inactivation is active site-directed. The results of the experiment are shown in Figure 4.7. As can be seen, the presence of DNJ protected the enzyme against inactivation by 22FoGTNP, which suggests that the inactivation of $\alpha$-glucosidase was due to an event taking place at the enzyme's active site. To further confirm this result, the degree of protection is predictable based on the $K_i$ (12.6 $\mu$M) (Hanozet et al., 1981) for DNJ with yeast $\alpha$-glucosidase. Inserting this value into the modified competitive inhibition equation (see Appendix II.3), a rate of inactivation of $0.079 \text{ min}^{-1}$ should have been observed, which compares favorably with the experimentally determined value of $0.073 \text{ min}^{-1}$. Therefore this result confirms that inactivation of $\alpha$-glucosidase by 22FoGTNP is active site directed.
d) Contamination experiment

In order to determine whether inactivation was due to 22FaGTNP or to a contaminant, the following experiment was carried out. The enzyme was incubated with a 20-fold excess of 22FaGTNP for 30 min. The mixture was then passed through a Centricon® 30,000 Da molecular weight cutoff membrane to remove the inactivated α-glucosidase. The inhibitor was then treated with fresh α-glucosidase to see if inactivation was once again observed. If inactivation was observed at a rate comparable to that obtained by the inhibitor without pretreatment with α-glucosidase then the results would suggest that inactivation was due to the inhibitor and not a contaminant. Time-dependent inactivation was once again observed; however, the rate of inactivation was lower than the expected value by a factor of two (see Figure 4.6). This result can be explained by using the earlier finding that the inhibitor is susceptible to relatively rapid breakdown in the presence of BSA. An approximate value for the non-enzymatic breakdown was estimated
to be 40%, which resulted in the two-fold reduction in the rate of inactivation. Therefore the results suggest that inactivation of $\alpha$-glucosidase is due to 22F$\alpha$GTNP.

e) Degradation of 22F$\alpha$GTNP by BSA

Sanger’s reagent (1-fluoro-2,4-dinitrobenzene) is known to label proteins typically at lysine side chains. The 22F$\alpha$GTNP inhibitor may also be reactive in a similar way, derivatizing the side chain amino group of lysines as shown in Figure 4.8. This is particularly likely with BSA which is known to have a high surface concentration of lysine residues. The result was qualitatively confirmed by TLC analysis in hexane/EtOAc (1:1) since the main breakdown product in the presence of BSA was not trinitrophenol, but rather a highly polar compound that did not move off the baseline, suggesting that the trinitrophenyl ring is attached to the polar BSA. In addition comparison of UV/VIS spectra of samples degraded in buffer and in BSA suggest that the product formed in the presence of BSA is different from that formed in buffer alone, because it has its maximum absorbance at 346 nm; different from that of TNP (356 nm).

![Figure 4.8. Proposed adduct between BSA and 2,4,6-trinitrophenol](image)

4.3.4. Identification of the catalytic nucleophile

The initial approach used to identify the catalytic nucleophile was similar to the method used in Chapter 2 for Glyx. In the first experiment, we used tandem mass spectrometry to look for the loss of the neutral label from a peptide within the peptic
digested 22FaGTNP inactivated α-glucosidase. A loss of m/z 183 was expected, however, no such neutral loss was observed. A neutral loss of the label from a doubly (91.5) or triply charged (61) peptide was also not seen. Thus it was not known whether the label was still covalently attached and just did not fragment under normal neutral loss conditions or whether the label degraded during proteolytic digestion. Several experiments were performed in which the digestion times were reduced (eg. 30 min compared with overnight) to minimize the exposure of the label to pH 2 conditions, and even then no neutral loss was observed. Therefore, there was no concrete evidence to prove if the label was still present or not.

At the time of this study another inactivator of α-glucosidase, 2Cl2FaGCl (45, p. 101) was being investigated by John McCarter and Wei Yeung in Withers' laboratory. 2Cl2FaGCl was determined to be a mechanism-based inactivator of α-glucosidase and thus perhaps could be used as a handle in the identification of the catalytic nucleophile. We first attempted to look for the neutral loss of this 2-chloro-2-fluoro sugar label (m/z 199), presumably present in the digest of α-glucosidase inactivated with 2Cl2FaGCl. Once again loss of the label was not observed under normal neutral loss conditions.

At this point we decided to carefully inspect the peptides formed in the three digests: the control, non-inactivated enzyme, the 22FaGTNP-inactivated sample, and the 2Cl2FaGCl-inactivated sample. This would allow us to attempt to determine which peptide contains the labels by looking for predicted mass differences. The mass of the peptide containing the 2Cl2FGCl label will have a m/z 16 higher than that containing the 22FaGTNP label. Searching through the mass spectra of the three samples, there was only a single peptide for which a difference of 16 m/z was observed between the two labeled peptide digests and for which both peaks were not present in the control. The results are shown in Figure 4.9. Figure 4.9A displays the masses of the peptides present in the control between m/z 700-1000 m/z in the fraction that elutes off the column between 18-18.5 min. Figure 4.9B shows the same region, but contains peptides from the 2Cl2FaGCl-inactivated sample. The
Figure 4.9 Partial mass spectra of peptides from a pepsin digest of yeast α-glucosidase inactivated with and without 2-deoxy-2,2-dihalo sugars

A solution (80 μL) of α-glucosidase (1.0 mg/mL), dithiothreitol (2 mM) and pepsin (0.1 mg/mL) in phosphate buffer (50 mM) pH 2 was incubated at 25°C for 30 min. Samples (10 μL) injected onto LC/ESMS were peptic digests of: A) control, B) α-glucosidase inactivated with 2C12FaGCl (3.3 mM), and C) α-glucosidase inactivated with 22Fa GTNP (1.4 mM). Peptides shown eluted from the LC column between 18-18.5 min.
GCl-inactivated sample. A prominent peak at m/z 943.5 is present which is not in the control. Figure 4.9C shows the peaks present in the 22FoGTNP inactivated sample. A new peak at m/z 927.5, which is 16 lower than the 943.5 peak seen in B, is detected but again it is not observed in the control. Therefore, we conclude that the peak at m/z 927.5 contains the 2,2-difluoro sugar label and the peak at 943.5 contains the 2-chloro-2-fluoro sugar label. Further, the active site peptide which is covalently labeled by the sugars must have a mass of 745. Such a peptide is seen in all three samples. A search through the amino acid sequence of yeast α-glucosidase for peptides with mass 745 ± 1 determined that there are 6 candidate peptides with the following sequences:

- **EPKWW** (10-14)
- **PKWWP** (11-15)
- **PNNWKS** (151-156)
- **RQVDLN** (180-185)
- **RIDTAGL** (212-218)
- **GQEIGQI** (388-394)

In order to further identify which of these 6 peptides is the one containing the catalytic nucleophile, tandem MSMS experiments were performed on the 943.5 and 927.5 peptides. The results for fragmentation of the 943.5 peptide are shown in Figure 4.10. Two peaks are observed with the main peptide ion being the unfragmented 943 peak and the other a small peak with a m/z of 745. This small peak resulted from the loss of the 2Cl2FGCl label giving the parent peptide. Thus, it appears that the label which results from the treatment of α-glucosidase with 2Cl2FoGCl can be fragmented but only to a limited extent and much higher collision gas thickness is required when compared with other acyl ester linkages (550 CGT vs. 350 CGT). This fragmentation of the label was not
A solution (80 μL) of α-glucosidase (1.0 mg/mL), dithiothreitol (2 mM) and pepsin (0.1 mg/mL) in phosphate buffer (50 mM) pH 2 was incubated at 25°C for 30 min then injected (40 μL) onto LC/ESMS. CGT = 550 (X 10^{12} molecules/cm^2).

observed in the 22FGTNP samples, which may suggest that the transition state for homolytic cleavage of the 2,2-difluoro compound is more destabilized than with the 2-chloro-2-fluoro analogue.

Attempts were made to further digest the labeled peptides, 927 and 943, with trypsin. Unfortunately, digestion with trypsin requires the pH to be maintained between 7.5 and 8.5, and under these conditions both peaks disappeared, even without the addition of the protease presumably due to the hydrolysis shown in Figure 4.11. Even though no positive results came from the trypsin digest experiments, the disappearance of essentially
Figure 4.11. Base catalyzed ester hydrolysis of glycosyl-enzyme intermediate

only those two peaks, 928 and 943, does lend support to the idea that the labels were covalently attached, but hydrolyzed at the higher pH values. A possible reason for the greater lability of these ester linkages is that the two halogens at C-2 may help to inductively stabilize the formation of the negative charge on the exocyclic oxygen and thus increase the rate of the reaction (see Figure 4.11).

At this time the labeling of yeast α-glucosidase by another inactivator was also being investigated by John McCarter. In that case, labeled peptides could be fragmented under normal neutral loss conditions. Subsequent fragmentation of the peptide backbone of the labeled peptide in this way allowed the identification of the following active site peptide:

```
R I D T A G L
```

This sequence is indeed one of the 6 candidate sequences we identified with the other two inactivators.
The susceptibility of the labeled peptide to aminolysis (see Chapter 2) also showed that the label must have been attached through an ester linkage, and thus aspartic acid 214 is the catalytic nucleophile.

4.4. Conclusions

2FaGF was found not to be a mechanism-based inactivator of yeast \(\alpha\)-glucosidase as originally proposed, but rather it acts as a slow substrate. 22Fa\(\alpha\)GTNP, however, was found to be a mechanism-based inactivator of \(\alpha\)-glucosidase and a \(k_i/K_i\) value of 0.25 min\(^{-1}\)mM\(^{-1}\) was determined for this process. Comparative LC/MS analysis of proteolytic digests of enzyme inactivated with 22Fa\(\alpha\)GTNP and 2C12Fa\(\alpha\)GCl, in conjunction with tandem MS experiments on these digests and on those involving another inactivator identified aspartic acid 214 as the catalytic nucleophile in yeast \(\alpha\)-glucosidase.
Chapter 5: Materials and Methods

5.1. Organic Synthesis

5.1.1. Materials and routine experimental procedures

a) Analytical methods

Melting points (mp) were determined on a Laboratory Devices Mel-Temp II melting-point apparatus. Melting points were uncorrected.

$^1$H Nuclear magnetic resonance (NMR) spectra were recorded on the following instruments: a Brüker AC-200 (200 MHz), a Varian XL-300 (300 MHz), a Brüker WH-400 (400 MHz), or a Varian Unity 500 (500 MHz). Spectra were referenced externally to 2,2-dimethyl-2-silapentane-5-sulfonate ($\delta = 0.00$ ppm). All chemical shifts were reported using the $\delta$ scale. $^{19}$F NMR spectra were recorded with proton coupling on a Bruker AC-200 (188 MHz) instrument. Chemical shifts were reported using the $\delta$ scale referenced to CFCl$_3$ ($\delta = 0.00$ ppm), although the external reference used was CF$_3$COOH. Signals upfield of CFCl$_3$ were assigned negative values. Where required the interpretation was supported by COSY or APT NMR experiments.

Desorption chemical ionization (DCI) mass spectra were recorded on a Delsi Nermag R10-10C mass spectrometer using NH$_3$ as the chemical ionization gas. Electrospray mass spectra were recorded on a Sciex API III mass spectrometer.

Microanalyses were performed by Mr. Peter Borda in the Microanalytical Laboratory, Department of Chemistry, at the University of British Columbia.

b) Thin-layer chromatography and silica gel column chromatography.

Thin-layer chromatography (TLC) was performed using analytical plates (silica gel 60 F$_{254}$, Merck). Compounds were visualized under UV light or after charring with 10% H$_2$SO$_4$ in methanol or ammonium molybdate-H$_2$SO$_4$ solution in methanol.
Column chromatography was performed using Kieselgel 60 (230-400 mesh) silica gel from Merck or Iatrobeads purchased from Iatron Laboratories, Inc. (Tokyo, Japan).

c) Solvents and reagents

Solvents and reagents were either reagent, certified, or spectral grade. Dry solvents and reagents were prepared as described as below. Dichloromethane was first washed with concentrated sulfuric acid followed by water and sodium bicarbonate, predried over calcium chloride, and finally distilled over calcium chloride. Diethyl ether was distilled over sodium metal and benzophenone. Methanol was distilled from magnesium methoxide (formed by reaction of methanol with magnesium turnings in the presence of a catalytic amount of iodine). Pyridine was predried for several days by standing over pellets of NaOH, and then distilled over calcium hydride. Acetyl chloride was refluxed over phosphorus pentachloride then distilled. Dimethylformamide (DMF) was stirred overnight over magnesium sulfate, then distilled under reduced pressure over 4-Å molecular sieves.

d) Compounds synthesized and provided by colleagues.

The compounds not synthesized in the thesis were: 3,4,6-tri-O-acetyl-2-deoxy-2-fluoro-α-glucosyl fluoride, which was generously provided by Dr. David Dolphin, Department of Chemistry, UBC; 2-deoxy-2-chloro-2-fluoro-α-D-arabinofuranosyl fluoride which was synthesized by Wei Yeung and John McCarter in Dr. Withers' laboratory.

5.1.2. Routine synthetic procedures

a) Acetylation procedures

The glycoside was dissolved in dry pyridine (0°C) and acetic anhydride was added (ratio typically 1:1), then the reaction mixture was allowed to warm up to room
temperature or elevated temperatures (50°C) and stirred until the reaction was complete. The excess anhydride was quenched by the addition of solid ice. After an aqueous workup the reaction mixture was purified by column chromatography.

b) Deacetylation with sodium methoxide in methanol

The following procedure was adapted from the work of Zemplen & Pacsu (1929). A catalytic amount of sodium methoxide, freshly prepared by adding sodium to dry methanol (2.3 mg/ml), was added to the acetylated glycoside dissolved in methanol. The reaction mixture was stirred at room temperature until the reaction was complete. The excess base was neutralized by adding Dowex 50W resin in the H⁺ form. The resin was filtered and the mixture was purified by column chromatography.

c) Deacetylation with ammonia-saturated methanol

The following work was adapted from the work of Fritz et al. (1983). The acetylated glycoside dissolved in cold (0°C), dry methanol, was saturated with dry ammonia. The mixture was left at 0°C until the reaction was complete, and then the solvent was evaporated in vacuo. The resulting residue was purified by column chromatography.

d) Deacetylation with HCl-methanol

The glycoside was suspended in dry methanol, cooled to 0°C and freshly distilled acetyl chloride was added to generate a final HCl concentration of 4% (w/v). The suspension was allowed to stir at 4°C until the reaction was complete. After evaporation of the solvent in vacuo, the gum was triturated with dry diethyl ether (6 times to remove excess HCl), then evaporated to dryness. The product was purified by column chromatography.
e) Selective deacetylation with hydrazine acetate

The per-O-acetylated glycoside was selectively deprotected at the anomeric center using hydrazine acetate according to the procedure of Excoffier et al. (1975). The sugar was dissolved in DMF, 1.2-2 equivalents of hydrazine acetate added and the reaction mixture heated to 50°C until the hydrazine acetate dissolved. The mixture was either cooled to room temperature or left at 50°C until the reaction was complete, then diluted with ethyl acetate and washed several times with brine. The organic layer was dried over MgSO₄ and evaporated in vacuo, and purified by column chromatography.

f) Fluorination of selectively deprotected glycosides

The glycoside was dissolved in dry dichloromethane and the solution was cooled to -23°C and flushed with dry nitrogen. Diethylaminosulphur trifluoride (DAST) was diluted in dichloromethane and added slowly dropwise. The solution was allowed to warm to room temperature and stirred until the reaction was complete. The reaction was cooled to 0°C and quenched by the addition of methanol. The solvent was removed and an aqueous work up followed by column chromatography was required to purify the compound.

g) Fluorination of glycals

Small scale procedure:

The glycal (~0.1 mmol) was dissolved in CFCl₃ and cooled in a dry ice acetone bath at -78°C. A mixture of F₂ diluted with Ne was bubbled through the solution at 150 mL/min. This reaction was repeated until all of the starting material was consumed and the reaction progress was followed by TLC and NMR analysis. The CFCl₃ was removed and column chromatography was required to purify the compound.
Large scale procedure:

\[ \text{F}_2 (5\%, 3 \times 40 \text{ psi}) \] was bubbled into a slurry of NaOAc (1 g) and glacial acetic acid (3 mL) in CFCl\(_3\) (30 mL) at -78°C. A solution of glycal peracetate (1.5 mmol) in CFCl\(_3\) (10 mL) was then added to the slurry containing acetylhypofluorite \((\text{in situ})\). The cooling bath was removed and the reaction was allowed to take place. The reaction progress was followed by NMR analysis. The CFCl\(_3\) was removed and an aqueous workup followed by column chromatography resulted in purification of the compound.

5.1.3. Syntheses

a) The synthesis of \(\alpha\)-glycosyl fluorides

\(\alpha\)-Glucosyl fluoride (1) and \(\alpha\)-maltosyl fluoride (3) were synthesized according to the procedure of Hayashi et al. (1984). \(\alpha\)-Maltosyl fluoride was found to be identical to that reported by Genghof et al. (1978). \(\beta\)-Glucosyl fluoride was synthesized according to the procedure of Helferich & Gootz (1929).

2,3,4,6,2',3',6',2'',3'',4'',6''-Undeca-O-acetyl-maltotriose \((7)\).—Maltotriose peracetate (0.75 g, 0.78 mmol) was dissolved in DMF (4 mL) and reacted with hydrazine acetate (0.10 mg, 1.09 mmol) at 50°C for 25 min. EtOAc was then added and the solution was washed with water, saturated (satd) NaHCO\(_3\) solution, and water, and dried (MgSO\(_4\)). Evaporation \textit{in vacuo} followed by flash chromatography (2:1 EtOAc-Hexanes) yielded the hemiacetal \(7\) (0.41 g, 57%).

2,3,4,6,2',3',6',2'',3'',4'',6''-Undeca-O-acetyl-\(\alpha\)-maltotriosyl fluoride \((8)\).—\(7\) (0.147 g, 0.16 mmol) was dissolved in dry CH\(_2\)Cl\(_2\) (3 mL) and fluorinated using DAST (24 mL, 0.20 mmol) by the general procedure. Flash chromatography (2:1 EtOAc-Hexanes) afforded mainly the \(\beta\)-fluoride; \(^{19}\)F NMR (CDCl\(_3\)): \(\delta\) -132.1 (\(\beta\)-F), -149.2 (\(\alpha\)-F). To the syrup (0.11 g, 0.12 mmol) was added HF-pyridine (0.10 mL, 3.5 mmol) at -78°C in a plastic vial and the reaction was allowed to stir under a blanket of N\(_2\) for 15 min, while the cooling bath was removed. Ether was added and washed with water, saturated
aqueous NaHCO₃ solution, and water, and dried (MgSO₄). Evaporation in vacuo followed by flash chromatography (ether) yielded 8 (77 mg, 70%) as a colourless gum; $^{19}$F NMR (CDCl₃): $\delta$ -149.2 (dd, $J_{F1,1}$ 53.2, $J_{F1,2}$ 3.7 Hz, F-1).

$\alpha$-Maltotriosyl fluoride (2).—8 (1.5 g, 1.59 mmol) was deprotected with NaOMe according to the general procedure. Flash chromatography (5:2:1 EtOAc-MeOH-water) afforded pure 2 (0.6 g, 74%) as a colourless gum; $^1$H NMR (D₂O): $\delta$ 5.67 (dd, 1 H, $J_{1,F1}$ 53.5, $J_{1,2}$ 2.7, H-1), 5.39 (d, 1 H, $J_{1',2'}$ 3.9 Hz, H-1'), 5.36 (d, 1 H, $J_{1''}$ 3.8 Hz, H-1''); $^{19}$F NMR (D₂O): $\delta$ -150.4 (dd, $J_{F1,1}$ 53.6, $J_{F1,2}$ 26.3 Hz, F-1). Anal. calcd. for C₁₈H₃₁FO₁₅. H₂O: C 41.22, H 6.34. Found: C 41.12, H 6.52.

b) The synthesis of 2-deoxy-2-fluoro-$\alpha$-maltotriosyl fluoride (5)

3,6,2',3',6',2'',3'',4'',6''-Nona-O-acetyl-maltotriol (10).—To a solution of maltotriose peracetate (1.36 g, 1.43 mmol) in glacial acetic acid (10 mL) was added 45% HBr in AcOH (2.2 mL) and the mixture stirred under N₂ for 1 h at 0°C. The mixture was dissolved in cold CHCl₃, washed successively with water, saturated aqueous NaHCO₃ solution, and water, and dried (MgSO₄). Evaporation of solvent in vacuo gave a gum which was triturated in cold petroleum ether to form a white powder. This was dissolved in 1:1 water-AcOH (15 mL), activated zinc (3.3 g, 50.5 mmol) added, and the mixture stirred overnight at 0°C. The mixture was dissolved in cold CHCl₃ and washed successively with water, saturated aqueous NaHCO₃ solution, and water, and dried (MgSO₄). Flash chromatography (1:2 petroleum ether- EtOAc) yielded pure 10 (0.51 g, 42%) as a colourless gum; $^1$H NMR (CDCl₃): $\delta$ 6.44 (dd, 1 H, $J_{1,2}$ 6.0, $J_{1,3}$ 1.0 Hz, H-1), and 2.15-1.95 (9 s, 9 OAc); HRMS calcd. for C₃₆H₅₂NO₂: (M+NH₄⁺), 866.2930; found: (M+NH₄⁺), 866.2881.

2-Deoxy-2-fluoro-$\alpha$-maltotriosyl fluoride (5).—A solution of 10 (344 mg, 0.405 mmol) in CFCl₃ was treated with F₂ at -78°C according to the small scale procedure to give a 1.1:1 mixture of the 2-deoxy-2-fluoro maltotriose 11 and $\beta$-addition product 12,
identified by their $^{19}$F NMR spectra. Flash chromatography (2:1:1 EtOAc-petroleum ether-CHCl$_3$) yielded 11 (133 mg, 37%) as a colourless gum; $^1$H NMR (CDCl$_3$): $\delta$ 5.68 (dd, 1 H, $J_{1,F1}$ 52.7, $J_{1,2}$ 2.6 Hz, H-1), and 2.15-1.95 (9 s, 9 OAc); $^{19}$F NMR (CDCl$_3$): $\delta$ -151.2 (d, $J_{F1,F2}$ 18.8 Hz, F-1), -205.3 (d, $J_{F2,F1}$ 18.8 Hz, F-2). To a solution of 11 (106 mg, 0.12 mmol) in dry MeOH (10 mL) was added NaOMe (3 mM, 1.76 mmol) and the reaction was stirred for 1 h under N$_2$. Dowex Ag-50W X2 ($H^+$) resin was added to neutralize the solution and the solvent was evaporated in vacuo. Flash chromatography (5:2:1 EtOAc-EtOH-water) yielded pure 5 (30 mg, 49%) as a colourless gum; $^1$H NMR (D$_2$O): $\delta$ 5.85 (dd, 1 H, $J_{F1,F2}$ 53.5, $J_{1,2}$ 2.3 Hz, H-1); $^{19}$F NMR (D$_2$O): $\delta$ -150.3 (d, $J_{F1,F2}$ 19.8 Hz, F-1), -204.6 (d, $J_{F2,F1}$ 19.8 Hz, F-2). Anal. Calcd. for C$_{18}$H$_{30}$F$_2$O$_4$H$_2$O: C 41.06; H, 6.13. Found: C, 40.80; H, 6.16.

c) The synthesis of 4-deoxy-α-maltotriosyl fluoride (6)

$4'$-O-(4,6-O-Benzylidene-α-D-glucopyranosyl)-maltose (13).—Maltotriose (5.00 g, 9.91 mmol) was dissolved in DMF (100 mL), then half of the volume distilled off in order to remove traces of water. p-Toluene sulfonic acid (~20 mg) and benzaldehyde dimethylacetal (1.6 mL, 10.65 mmol) were added and the reaction mixture was kept rotating for 8 h at 60-70°C under a slight vacuum, to remove the MeOH formed (TLC in 7:2:1 EtOAc-MeOH-water). The mixture was then neutralized with a basic ion exchange resin (DOWEX-1, OH$^-$ form), filtered, evaporated and purified by column chromatography (7:2:1 EtOAc-MeOH-water) to yield pure 13 (1.9 g, 3.2 mmol, 32%) as a colourless syrup; the compound was characterized after acetylation.

$1,2,3,6,2',3',6'$-Hepta-O-acetyl-4'$-O-(2,3-di-O-acetyl-4,6-O-benzylidene-α-D-glucopyranosyl)-maltose (14).—The hemiacetal 13 (1.90 g, 3.21 mmol) was dissolved in pyridine (50 mL) and acetylated with acetic anhydride (20 mL) overnight at 50°C. CH$_2$Cl$_2$ (70 ml) was added, the mixture poured into saturated aqueous NaHCO$_3$ solution and then the aqueous phase was extracted three times with CH$_2$Cl$_2$ and the combined
organic phases washed with water. Coevaporation with toluene yielded 14 (3.10 g, 3.19 mmol, 99.8%) as an almost colourless syrup which was not further purified; $^1$H NMR (CDCl$_3$, 200 MHz), selected data $\alpha$- and $\beta$-anomer only: $\delta$ 7.38-7.28 (m, 5 H, aromatic), 6.18 (d, $J_{1,2}$ 4.0 Hz, H-1$\alpha$), 5.70 (d, $J_{1,2}$ 8.0 Hz, H-1$\beta$), 5.50-5.10 (m, 6 H, H-1', H-1", H-3, H-3', H-3", PhCH); DCIMS calcd for C$_{43}$H$_{54}$O$_{25}$: (M+NH$_4^+$), 984.19; found: (M+NH$_4^+$), 984.

$^{1,2,3,6,2',3',6'}$-Hepta-O-acetyl-4-O-(2,3-di-O-acetyl-6-O-benzyl-$\alpha$-D-glucopyranosyl)-maltose (15).—Compound 14 (2.90 g, 2.98 mmol) was dissolved in dry THF (50 mL) and Na(CN)BH$_3$ (2.00 g, 31.8 mmol) added. Under a constant flow of nitrogen a saturated solution of HCl gas in dry ether was added until the gas development ceased and the pH of the solution remained acidic. After 30 min of stirring reaction was complete (TLC in 2:1 EtOAc-hexanes). After concentration of the reaction mixture to ~10 mL, CH$_2$Cl$_2$ (100 mL) was added and the mixture washed with saturated aqueous NaHCO$_3$ solution. The aqueous phase was extracted three times with CH$_2$Cl$_2$ and the combined organic phases were washed with water, dried over MgSO$_4$, evaporated and purified by column chromatography (2:1 EtOAc-hexanes) yielding 15 as a colourless syrup (2.40 g, 2.46 mmol, 83%); $^1$H NMR (CDCl$_3$, 200 MHz), $\beta$-anomer only: $\delta$ 7.35 (m, 5 H, aromatic), 5.72 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 5.40-5.10 (m, 5 H, H-1', H-1", H-3, H-3', H-3"), 4.95 (dd, 1 H, $J_{1,2}$ 8.0, $J_{2,3}$ 9.0 Hz, H-2), 4.77 (dd, 1 H, $J_{1',2'}$ 4.0, $J_{2',3'}$ 10.0 Hz, H-2"), 4.70 (dd, 1 H, $J_{1',2'}$ 4.0, $J_{2',3'}$ 10.0 Hz, H-2"), 4.56 (s, 2 H, PhCH$_2$), 2.70 (s, 1 H, 4"-OH), 2.12-1.90 (9 s, 27 H, 9 OAc); DCIMS calcd for C$_{43}$H$_{56}$O$_{25}$: (M++NH$_3$), 989.93; found: (M++NH$_3$), 990.

$^{1,2,3,6,2',3',6'}$-Hepta-O-acetyl-4'-O-(2,3-di-O-acetyl-6-O-benzyl-4-deoxy-4-ido-$\alpha$-D-galactopyranosyl)-maltose (16).—Compound 15 (2.40 g, 2.46 mmol) was dissolved in dry CH$_2$Cl$_2$ (50 mL) and pyridine (10 mL) and cooled to 0°C under a constant flow of nitrogen. Triflic anhydride (2.0 mL, 11.89 mmol) was slowly added and stirring was continued at room temperature until the reaction was complete (20 min) according to
TLC analysis (2:1 EtOAc-hexanes). Water was added and the aqueous phase was extracted two times with CH$_2$Cl$_2$, then the combined organic phases were washed with water and co-evaporated with acetonitrile. The resulting syrup was dissolved in DMF (50 mL) and stirred with NaI (2.50 g, 16.69 mmol) overnight. The 4''-iodo compound could not be distinguished from the 4''-triflate by TLC analysis. After addition of CH$_2$Cl$_2$ (50 mL) the reaction mixture was poured into water and the aqueous phase was extracted twice with CH$_2$Cl$_2$. The combined organic phases were dried (MgSO$_4$), evaporated, and purified by flash column chromatography (1:1 EtOAc-hexanes) to yield 16 (1.90 g, 1.75 mmol, 71%) as a white foam; $^1$H NMR (CDCl$_3$, 500 MHz), $\beta$-anomer only: $\delta$ 7.37-7.25 (m, 5 H, aromatic), 5.69 (d, 1 H, $J_{1',2''}$ 8.1 Hz, H-1), 5.32 (d, 1 H, $J_{1'',2''}$ 4.3 Hz, H-1''), 5.32 (d, 1 H, $J_{2',3'}$ 10.3 Hz, H-3'), 5.26 (dd, 1 H, $J_{2,3}$ 8.9, $J_{3,4}$ 9.1 Hz, H-3), 5.21 (d, 1 H, $J_{1,2'}$ 3.9 Hz, H-1'), 5.16 (dd, 1 H, $J_{1'',2''}$ 4.3, $J_{2'',3''}$ 10.7 Hz, H-2''), 4.93 (dd, 1 H, $J_{1,2}$ 8.1, $J_{2,3}$ 8.9 Hz, H-2), 4.75 (bd, 1 H, $J_{3'',4''}$ 3.3, $J_{4'',5''}$<1 Hz, H-4''), 4.67 (dd, 1 H, $J_{1',2'}$ 3.9, $J_{2',3'}$ 10.3 Hz, H-2'), 4.49 (m, 3 H, PhCH$_2$, H-6a'), 4.41 (m, 2 H, H-3', H-6a), 4.25 (dd, 1 H, $J_{5,6_b}$ 4.3, $J_{6_a,6_b}$ 12.3 Hz, H-6b), 4.09 (dd, 1 H, $J_{5',6'_b}$ 2.1, $J_{6'_a,6'_b}$ 12.2 Hz, H-6'b), 3.96 (dd, 1 H, $J_{3,4}$ 9.1, $J_{4,5}$ 9.2 Hz, H-4), 3.87 (m, 2 H, H-4', H-5'), 3.82 (m, 1 H, H-5), 3.52 (m, 1 H, H-5''), 3.37 (m, 2 H, H-6'a, H-6'b), 2.12, 2.06, 2.05, 2.04, 2.00, 1.98 (6 s, 18 H, 6 OAc), 1.97 (s, 9 H, 3 OAc) ppm; DCIMS calcd for C$_{43}$H$_{55}$O$_{24}$: (M+NH$_4^+$), 1100.83; found: (M+NH$_4^+$), 1101.

1,2,3,6,2',3',6'-Hepta-O-acetyl-4-O-(2,3-di-O-acetyl-6-O-benzyl-4-deoxy-α-D-xylo-hexopyranosyl)-maltose (17).—A solution of 16 (1.90 g, 1.75 mmol) in benzene (10 mL) was added to a hot solution of tributyl tinhydride (2.60 mL, 9.66 mmol) and a catalytic amount of AIBN (2,2'-azobisisobutyro nitrile) in benzene (20 mL). The reaction mixture was refluxed for 45 min when a small amount of starting material could still be detected by TLC (2:1 EtOAc-hexanes) while a slightly slower moving byproduct had started to form in addition to the desired product. Heating was stopped after 50 min, then the reaction mixture was evaporated, dissolved in acetonitrile and washed several
times with hexanes. After evaporation of the solvent the product was purified over a silica gel column which had been packed with hexanes and was eluted with 1:1 EtOAc-hexanes, yielding 17 as a colourless syrup (1.58 g, 1.65 mmol, 94%); $^1$H NMR (CDCl$_3$, 500 MHz), $\beta$-anomer only: $\delta$ 5.72 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1), 5.38 (d, 1 H, $J_{1',2'}$ 3.8 Hz, H-1'), 5.36 (dd, 1 H, $J_{2,3'}$ 10.3, $J_{3',4'}$ 9.3 Hz, H-3'), 5.27 (t, 1 H, $J_{2,3}$ 8.9, $J_{3,4}$ 8.9 Hz, H-3), 5.24 (d, 1 H, $J_{1',2'}$ 4.1 Hz, H-1'), 5.16 (ddd, 1 H, $J_{3''4''}$ 4.9 Hz, H-3''), 4.94 (dd, 1 H, $J_{1,2}$ 8.1, $J_{2,3}$ 8.9 Hz, H-2), 4.79 (dd, 1 H, $J_{1',2'}$ 3.8, $J_{2',3'}$ 10.4 Hz, H-2'), 4.71 (dd, 1 H, $J_{1',2'}$ 4.1, $J_{2',3'}$ 10.3 Hz, H-2'), 4.54 (d, 1 H, PhCH), 4.52 (d, 1 H, PhCH), 4.45-4.11 (m, 4 H, H-6a, H-6b, H-6'a, H-6'b), 4.00-3.81 (m, 5 H, H-4, H-4', H-5, H-5', H-5''), 3.44 (m, H-6''a, H-6''b), 2.12 (ddd, 1 H, H-4''e), 2.13, 2.08, 2.04, 2.03, 2.00, 1.98, 1.97 (7 s, 21 H, OAc), 1.99 (s, 6 H, 2 OAc), 1.70 (ddd, 1 H, H-4''a); DCIMS calcd for C$_{43}$H$_{56}$O$_{24}$: (M+NH$_4^+$), 974.93; found: 975.

$\text{1,2,3,6,2',3',6'-Hepta-O-acetyl-4'-O-(2,3-di-O-acetyl-4-deoxy-\alpha-D-xylo-hexopyranosyl)-maltose (18).}$—To a solution of 17 (1.58 g, 1.65 mmol) in EtOAc (10 mL) and EtOH (50 mL) Pd-catalyst (10% on charcoal, 100 mg) was added and the mixture was hydrogenated. After the reaction was complete (TLC: 2:1 EtOAc-hexanes) the reaction mixture was filtered through a Celite bed, concentrated by evaporation, and purified by column chromatography (2:1 EtOAc-hexanes) to yield 18 (1.35 g, 1.56 mmol, 94%) as a colourless glass; the compound was characterized after acetylation; DCIMS calcd for C$_{36}$H$_{50}$O$_{24}$: (M+NH$_4^+$), 884.81; found: (M+NH$_4^+$), 885.

$\text{1,2,3,6,2',3',6'-Hepta-O-acetyl-4'-O-(2,3,6-tri-O-acetyl-4-deoxy-\alpha-D-xylo-hexopyranosyl)-maltose (19).}$—

a) A solution of 18 (1.30 g, 1.50 mmol) in pyridine (10 mL) was stirred with acetic anhydride (0.5 mL) overnight at room temperature, CH$_2$Cl$_2$ was added and the mixture was poured into water and extracted with CH$_2$Cl$_2$. The combined organic phases were washed with water and were co-evaporated with toluene. Evaporation from ether/hexanes gave 18 (1.35 g, 1.48 mmol, 99%) as a white foam.
b) Compound 19 could also be obtained directly from 17 by catalytic hydrogenation and subsequent acetylation. The iodide 16 (240 mg, 0.22 mmol) was dissolved in EtOH (20 mL), one drop of NEt₃ was added and the mixture was hydrogenated with Pd (10% on charcoal, 100 mg) for 3 days. TLC analysis (2:1 EtOAc-hexanes) showed that the 4"-iodo substituent was reduced first. After the reaction was complete, the mixture was filtered, evaporated and acetylated using pyridine (10 mL) with acetic anhydride (1 mL) overnight. After addition of CH₂Cl₂ (10 mL) the reaction mixture was poured into saturated aqueous NaHCO₃, the aqueous phase extracted twice with CH₂Cl₂ and the combined organic phases washed with water and co-evaporated with toluene. The resulting syrup was purified by column chromatography (1:1 EtOAc-hexanes) to yield 19 (180 mg, 0.198 mmol, 90%) as a colourless syrup; ¹H NMR (CDCl₃, 500 MHz), β-anomer only: δ 5.37 (d, 1 H, J₁",₂" 3.9, H-1"'), 5.36 (dd, 1 H, H-3'), 5.35 (d, 1 H, J₁,₂ 8.0 Hz, H-1), 5.27 (dd, 1 H, J₂,₃ 8.9, J₃,₄ 8.8 Hz, H-3), 5.23 (d, 1 H, J₁",₂" 4.1 Hz, H-1"'), 5.15 (ddd, 1 H, J₃",₄" 4.9 Hz, H-3"), 4.93 (dd, 1 H, J₂,₃ 8.9 Hz, H-2), 4.78 (dd, 1 H, J₁",₂" 3.9, J₂",₃" 10.4 Hz, H-2"'), 4.71 (dd, 1 H, J₁",₂" 4.1, J₂",₃" 10.3 Hz, H-2"'), 2.20-1.83 (m, 31 H, 10 OAc, H-4"e), 1.58 (ddd, 1 H, H-4"a); DCIMS calcd for C₃₈H₅₂O₂₅: (M+NH₄⁴⁺), 926.84; found: (M+NH₄⁴⁺), 927.

4'-O-(4-Deoxy-α-D-xylo-hexopyranosyl)-maltose.—The per-O-acetate 19 (40 mg, 44.01 mmol) was dissolved in dry MeOH (10 mL) and stirred with a catalytic amount of solid sodium methoxide overnight. After neutralization with ion exchange resin (BIO-RAD, AG 50W-X 12, 50-100 mesh, H⁺-form), the reaction mixture was filtered and evaporated to yield a colourless syrup (20 mg, 40.95 mmol, 93%); ¹H NMR (D₂O, 400 MHz): δ 5.36 (2 d, 2 H, H-1', H-1"'), 5.19 (d, J₁,₂ 3.7 Hz, H-1α), 4.62 (d, J₁,₂ 7.9 Hz, H-1β), 3.47 (dd, J₁,₂ 3.9, J₂,₃ 9.8 Hz, H-2α), 3.24 (dd, J₁,₂ 7.9, J₂,₃ 9.4 Hz, H-2β), 1.96 (ddd, 1 H, H-4"e), 1.42 (ddd, 1 H, H-4"a).

2,3,6,2',3',6'-Hexa-O-acetyl-4'-O-(2,3,6-tri-O-acetyl-4-deoxy-α-D-xylo-hexopyranosyl)-maltose (20).—The per-O-acetate 19 (1.25 g, 1.38 mmol) was dissolved
in DMF (10 mL) and reacted with hydrazine acetate (250 mg, 2.71 mmol) at 50°C for 25 min. EtOAc (20 mL) was then added and the solution was washed with water. The aqueous phase was extracted twice with EtOAc, the combined organic phases washed with water then concentrated by evaporation and purified by flash chromatography (2:1 EtOAc-hexanes) to yield the hemiacetal 20 (1.12 g, 1.29 mmol, 94%).

2,3,6,2',3',6'-Hexa-O-acetyl-4'-O-(2,3,6-tri-O-acetyl-4-deoxy-\(\alpha\)-D-xylo-hexopyranosyl)-\(\alpha\)- and \(\beta\)-D-maltosyl fluoride (21 and 22).—The hemiacetal 20 (130 mg, 0.143 mmol) was dissolved in dry CH\(_2\)Cl\(_2\) (5 mL) and DAST (0.05 mL, 0.378 mmol) was added at 0°C. After stirring for 30 min at room temperature reaction was complete (TLC in 2:1 EtOAc-hexanes). The mixture was quenched with MeOH, concentrated by evaporation and purified by column chromatography (2:3 EtOAc-hexanes). The maltotriosyl fluorides (21 and 22) were obtained as an amorphous white solid, (anomeric mixture with \(\beta/\alpha \sim 8 : 1\) by \(^1\)H NMR, 120 mg, 0.139 mmol, 97%); \(^1\)H NMR (CDCl\(_3\), 400 MHz), selected data: \(\delta \) 5.63 (dd, \(J_{1,F} 52.8, J_{1,2} 2.8 \) Hz, H-1\(\alpha\)), 5.57 (dd, \(J_{2,3} 40.0, J_{3,4} 9.2 \) Hz, H-3\(\alpha\)), 5.16 (dd, \(J_{1,F} 51.6, J_{1,2} 4.9 \) Hz, H-1\(\beta\)), 5.25 (d, \(J_{1,2'} 4.0 \) Hz, H-1'), 5.15 (ddd, H-3\(\alpha\)), 4.92 (dd, \(J_{1,2} 4.9, J_{2,3} 8.4, J_{2,F} 6.2 \) Hz, H-2\(\beta\)), 4.79 (dd, \(J_{1,2'} 4.0, J_{2',3'} 10.2 \) Hz, H-2\(\beta\)), 2.14, 2.11, 2.08, 2.07, 2.03, 2.01, 1.99, 1.98, 1.97 (9 s, 9 \(\beta\)-OAc and H-4\(\alpha\)B), 1.60 (q, H-4\(\alpha\)B); \(^{19}\)F NMR (CDCl\(_3\), 188 MHz): \(\delta -131.99 \) (B), -149.20 (\(\alpha\)); the compound was further characterized after anomerization.

2,3,6,2',3',6'-Hexa-O-acetyl-4'-O-(2,3,6-tri-O-acetyl-4-deoxy-\(\alpha\)-D-xylo-hexopyranosyl)-\(\alpha\)-D-maltosyl fluoride (21).—To the \(\beta\)-fluoride 20 (600 mg, 0.691 mmol) in a plastic vial was added 2 mL of HF-pyridine. This was kept at -70°C for 10 min and at 0°C for another 50 min, then the reaction mixture was diluted with CH\(_2\)Cl\(_2\) and quenched with saturated aqueous NaHCO\(_3\). The aqueous phase was extracted twice with CH\(_2\)Cl\(_2\), the combined organic phases were washed with water then concentrated by evaporation and investigated by \(^{19}\)F NMR-spectroscopy. TLC analysis (2:1 EtOAc-
hexanes) showed the formation of four products with \( R_f \) values of 0.68, 0.53, 0.50, and 0.25, that at 0.53 corresponding to the main product. Column chromatography (2:1 EtOAc-hexanes) yielded 69 mg (0.24 mmol) of the least polar material; 2,3,6-tri-O-acetyl-4-deoxy-xylo-hexopyranosyl-\( \alpha \)-D-fluoride (crystalline, \(^{19}\)F NMR: \( \delta \) -148.64 ppm), 20 mg of the most polar, which arises from hydrolysis and 510 mg of a mixture of the two middle spots. This product mixture was acetylated overnight in pyridine (10 mL) and acetic anhydride (0.5 mL), washed with \( \text{CH}_2\text{Cl}_2 \), washed with water and the aqueous phase extracted twice with \( \text{CH}_2\text{Cl}_2 \). The combined organic phases were washed with water, co-evaporated with acetonitrile and purified by column chromatography (2:3 EtOAc-hexanes, later 1:1) yielding 360 mg of 21 (0.41 mmol, 60\%) as a white amorphous solid; \(^1\)H NMR (CDCl\(_3\), 500 MHz): \( \delta \) 5.63 (dd, 1 H, \( J_{1,F} \) 53.6, \( J_{1,2} \) 2.6 Hz, H-1), 5.53 (dd, 1 H, \( J_{2,3} \) 10.0, \( J_{3,4} \) 9.2 Hz, H-3), 5.37 (m, 2 H, \( J_{1''},J_{2''} \) 4.0 Hz, H-1”, H-3”), 5.28 (d, 1 H, \( J_{1',1''} \) 10.0 Hz, H-1”), 5.16 (ddd, 1 H, H-3”), 4.81 (ddd, 1 H, \( J_{2,F} \) 23.8, \( J_{1,2} \) 2.6, \( J_{2,3} \) 10.0 Hz, H-2), 4.79 (dd, 1 H, \( J_{1'',1''} \) 4.0, \( J_{2'',3''} \) 10.4 Hz, H-2”), 2.12, 2.08, 2.06, 2.05, 2.01, 1.99, 1.98 (8 s, 28 H, 9 OAc and H-4”e), 1.59 (q, 1 H, H-4”a); \(^{19}\)F NMR (CDCl\(_3\), 188 MHz): \( \delta \) -149.22; DCIMS calcd for C\(_{36}\)H\(_{49}\)FO\(_2\):: (M+NH\(_4^+\)), 886.73; found: (M+NH\(_4^+\)), 887.

4’-O-(4-Deoxy-\( \alpha \)-D-xylo-hexopyranosyl)-\( \alpha \)-maltosyl fluoride (6).—A solution of 21 (170 mg, 0.195 mmol) in dry MeOH (20 mL) was stirred with a catalytic amount of solid sodium methoxide at room temperature overnight. After 17h TLC analysis (7:2:1 EtOAc-MeOH-water) revealed a single polar product. The reaction mixture was neutralized with ion exchange resin (Bio-Rad, AG 50W-X 12, 50-100 mesh, H\(^+\)-form), filtered and evaporated. Freeze drying yielded 6 (86 mg, 90\%) as an amorphous white solid; \(^1\)H NMR (D\(_2\)O, 400 MHz): \( \delta \) 5.75 (dd, 1 H, \( J_{1,2} \) 2.8, \( J_{1,F} \) 53.6 Hz, H-1), 5.47-5.45 (2d, 2 H, \( J_{1',2'} \) 4.0, \( J_{1''},J_{2''} \) 4.0 Hz, H-1”, H-1”), 3.57 (dd, 1 H, \( J_{1',2'} \) 4.0, \( J_{2',3'} \) 9.8 Hz, H-2”), 2.04 (ddd, 1 H, \( J_{3'',4',4''} \) 4.8, \( J_{4'',c',d'} \) 12.6, \( J_{4'',c'',d''} \) 2.0 Hz, H-4”e), 1.50 (q, 1 H, \( J_{3'',4',4''} \) ~J\(_{4'',a',4''} \) ~J\(_{4'',a',5''} \) ~12.5 Hz, H-4”a); \(^{19}\)F NMR (D\(_2\)O, 188 MHz): \( \delta \) -150.35.
NMR (D$_2$O, 188 MHz, coupled): $\delta$ -150.40 (dd, $J_{1,F}$ 54.2, $J_{2,F}$ 26.7 Hz). Anal. Calcd. for C$_{18}$H$_{31}$O$_{14}$F$\cdot$2H$_2$O: C, 41.06; H, 6.70. Found: C, 41.25; H, 6.61.

d) The synthesis of 4-deoxy-$\alpha$-maltosyl fluoride (24)

1,2,3,6,2',3'-Hexa-O-acetyl-4',6'-O-benzylidene-maltose (28).—Maltose (40.00 g, 111.01 mmol) was dissolved in dry DMF (100 mL), benzaldehyde dimethylacetal (18 mL, 119.92 mmol) and p-toluenesulfonic acid (~100 mg) were added, and the mixture was rotated at 60-70°C on the rotary evaporator under a weak vacuum to remove MeOH that formed. Reaction was continued for 6 h, with occasional replenishment of DMF. TLC analysis (7:2:1 EtOAc-MeOH-water) showed one major and several other minor products. The reaction mixture was neutralized with a basic ion-exchange resin (Dowex-1, OH$^-$ form), filtered and co-evaporated with toluene. The remaining syrup was subjected to water-EtOAc extraction, the organic phase being extracted twice with water, and the combined aqueous phases then washed three times with EtOAc. This procedure removes the more nonpolar byproducts from the aqueous phase, thus facilitating the purification procedure. The aqueous phase was concentrated by evaporation and purified by flash chromatography (8:1:1 EtOAc-MeOH-water). The 4',6'-O-benzylidene compound (20.40 g, 47.40 mmol, 43%) obtained was then dissolved in pyridine (300 mL) and acetylated with acetic anhydride (200 mL). After the acetylation reaction was complete, the reaction mixture was diluted with CH$_2$Cl$_2$ and neutralized with saturated aqueous NaHCO$_3$. The aqueous phase was extracted three times with CH$_2$Cl$_2$ and the organic phases were washed twice with water. Coevaporation with toluene yielded 28 (31.8 g, 46.6 mmol, 42% from maltose) as an almost colourless syrup; $^1$H NMR (CDCl$_3$, 200 MHz), the $\beta$-anomer only: $\delta$ 7.45-7.30 (m, 5 H, aromatic), 5.72 (d, 1 H, $J_{1,2}$ 8.3 Hz, H-1), 5.46 (s, 1 H, PhCH), 5.44 (dd, 1 H, $J_{2,3}$ 10.3, $J_{3,4}$ 9.8 Hz, H-3'), 5.34 (d, 1 H, $J_{1',2'}$ 4.2 Hz, H-1'), 5.29 (dd, 1 H, $J_{2,3}$ 9.6, $J_{3,4}$ 8.6 Hz, H-3), 4.96 (dd, 1 H, $J_{1,2}$ 8.3, $J_{2,3}$ 9.6 Hz, H-2), 4.86 (dd, 1 H, $J_{1',2'}$ 4.2, $J_{2',3'}$ 10.3 Hz, H-2'), 4.48 (dd, 1 H, $J_{5',6'a}$ 2.3,
$J_{6'a,6'b}$ 12.5 Hz, H-6'a), 4.30-4.17 (m, 2 H, H-6'b, H-6a), 4.03 (dd, 1 H, $J_{3,4}$ 8.6, $J_{4,5}$ 9.6 Hz, H-4), 3.89-3.55 (m, 4 H, H-6b, H-5, H-5', H-4'), 2.10, 2.09, 2.05, 2.03, 2.01, 2.00 (6 s, 18 H, 6 OAc); DCIMS calcd for $C_{31}H_{38}O_{17}$: (M + NH$_4^+$), 700.66; found: (M + NH$_4^+$), 701.

1,2,3,6,2',3'-Hexa-O-acetyl-6'-O-benzyl-maltose (29).—To a mixture of 28 (18.70 g, 27.39 mmol) in dry THF (200 mL), Na(CN)BH$_3$ (15.00 g, 238.70 mmol) was added. The mixture was stirred at room temperature, and a saturated solution of HCl gas in dry ether was added in small portions until gas development ceased and the reaction mixture remained acidic. The reaction was finished after another 30 min of stirring (TLC in 1:1 EtOAc-hexanes). The reaction mixture was concentrated by evaporation to ~20 mL, CH$_2$Cl$_2$ (200 mL) was added, and the mixture neutralized with saturated aqueous NaHCO$_3$. The aqueous phase was extracted three times with CH$_2$Cl$_2$, and the combined organic phases were washed with water, dried (MgSO$_4$), filtered and evaporated. Column chromatography (1:1 EtOAc-hexanes) yielded 29 (18.20 g, 26.58 mmol, 97%) as a white foam; $^1$H NMR (CDCl$_3$, 500 MHz), the $\beta$-anomer only: $\delta$ 7.38-7.28 (m, 5 H, aromatic), 5.71 (d, 1 H, $J_{1,2}$ 8.3 Hz, H-1), 5.34 (d, 1 H, $J_{1',2}$ 3.9 Hz, H-1'), 5.25 (dd, 1 H, $J_{2,3}$ 9.0, $J_{3,4}$ 9.1 Hz, H-3), 5.18 (dd, 1 H, $J_{2',3'}$ 10.4, $J_{3',4'}$ 9.2 Hz, H-3'), 4.94 (dd, 1 H, $J_{1,1'}$ 8.3, $J_{2,2'}$ 3.9, $J_{2',2'}$ 10.4 Hz, H-2'), 4.57, 4.55 (2 d, 2 H, PhC#), 4.44 (dd, 1 H, $J_{5,6a}$ 1.8, $J_{6a,6b}$ 12.3 Hz, H-6a), 4.16 (dd, 1 H, $J_{5,6b}$ 4.3, $J_{6a,6b}$ 12.3 Hz, H-6b), 4.01 (t, 1 H, $J_{3,4}$ 9.2, $J_{4,5}$ 9.2 Hz, H-4), 3.79 (m, 1 H, H-4'), 3.72 (m, 3 H, H-6'a, H-5, H-5'), 3.60 (dd, 1 H, $J_{5,6b}$ 3.3 $J_{6'a,6'b}$ 10.3 Hz, H-6'b), 2.73 (s, 1 H, 4'-OH), 2.08, 2.06, 2.03, 2.01, 1.99, 1.98 (6 s, 18 H, 6 OAc); DCIMS calcd for $C_{31}H_{40}O_{17}$: (M + NH$_4^+$), 702.68; found: (M + NH$_4^+$), 703.

1,2,3,6-Tetra-O-acetyl-4-O-(2,3-di-O-acetyl-6-O-benzyl-4-deoxy-4-iodo-$\alpha$-D-galactosyl)-glucose (30).—A solution of 29 (3.00 g, 4.38 mmol) in dry CH$_2$Cl$_2$ (30 mL) and dry pyridine (10 mL) was cooled to -20°C under a nitrogen atmosphere and triflic anhydride (2.00 mL, 11.88 mmol) slowly added through a septum. The reaction mixture
was allowed to warm to room temperature and was stirred until the reaction was complete (TLC in 1:1 EtOAc-hexanes). Occasionally, in addition to the main product, a slightly slower moving byproduct, which was not further characterized, was detected by TLC in minor amounts. When the reaction was complete, the volume of the reaction mixture was doubled with CH$_2$Cl$_2$, and the solution was poured into saturated aqueous NaHCO$_3$. The aqueous phase was extracted twice with CH$_2$Cl$_2$ and the combined organic phases were washed with water. The organic phase was coevaporated with toluene or acetonitrile at low temperature. The resulting syrup was dissolved in DMF (50 mL), treated with NaI (3.00 g, 20.01 mmol), and stirred at room temperature overnight. After addition of CH$_2$Cl$_2$ (50 mL), the solution was poured into water, the aqueous phase extracted twice with CH$_2$Cl$_2$, and the combined organic phases were washed twice with water. The organic phase was concentrated by evaporation and purified by flash chromatography (4:6 EtOAc-hexanes). The reaction yielded 30 (2.74 g, 3.45 mmol, 79%) as a colourless foam; $^1$H NMR (CDCl$_3$, 500 MHz), the β-anomer only: δ 7.38-7.26 (m, 5 H, aromatic), 5.70 (d, 1 H, J$_{1,2}$ 8.1 Hz, H-1), 5.33 (d, 1 H, J$_{1,2}$ 4.2 Hz, H-1'), 5.23 (dd, 1 H, J$_{2,3}$ 9.2, J$_{3,4}$ 8.8 Hz, H-3), 5.18 (dd, 1 H, J$_{1,2}$ 4.2, J$_{2,3}$ 10.7 Hz, H-2'), 4.94 (dd, 1 H, J$_{1,2}$ 8.1, J$_{2,3}$ 9.2 Hz, H-2), 4.74 (d, 1 H, J$_{3',4'}$ 4.1, J$_{4',5'}$<1 Hz, H-4'), 4.51 (m, 3 H, PhCH$_2$, H-6a), 4.42 (dd, 1 H, J$_{2',3'}$ 10.7, J$_{3',4'}$ 4.1 Hz, H-3'), 4.14 (dd, 1 H, J$_{5,6b}$ 5.1, J$_{6a,6b}$ 12.2 Hz, H-6b), 3.97 (dd, 1 H, J$_{3,4}$ 8.8, J$_{4,5}$ 9.6 Hz, H-4), 3.78 (ddd, 1 H, J$_{4,5}$ 9.6, J$_{5,6a}$ 2.6, J$_{5,6b}$ 5.1 Hz, H-5), 3.53 (m, 1 H, J$_{4',5'}$<1, J$_{5',6a}$ 3.5, J$_{5',6b}$ 6.0 Hz, H-5'), 3.41 (m, 2 H, H-6'a, H-6'b), 2.08, 2.07, 2.04, 2.03, 2.00, 1.98 (6 s, 18 H, 6 OAc); $^{13}$C NMR (CDCl$_3$, 50 MHz): δ 170.62, 170.30, 170.14, 169.67, 169.61, 168.80 (6 s, 6 CH$_3$CO), 137.46, 128.44, 127.85 (aromatic C), 96.24 (C-1), 91.18 (C-1'), 75.33, 73.69, 73.22, 72.96, 72.41, 70.98, 70.03, 67.95, 67.62 (C-2, C-3, C-4, C-5, C-6, C-2', C-3', C-5', C-6'), 62.73 (PhCH$_2$), 36.70 (C-4'), 20.91, 20.82, 20.75, 20.60, 20.55 (6 CH$_3$CO); DCIMS calcd for C$_{31}$H$_{39}$I$_{10}$O$_{16}$: (M$^+$ + NH$_3$), 811.57; found: (M$^+$ + NH$_3$), 812.
1,2,3,6,2',3'-Hexa-O-acetyl-6'-0-benzyl-4'-deoxy-maltose (31).—A solution of 30 (650 mg, 0.818 mmol) in benzene (10 mL) was added to a hot mixture of tributyltin hydride (1.00 mL, 3.72 mmol) and a catalytic amount of AIBN in benzene (40 mL) and the reaction mixture heated under reflux. TLC analysis (1:1 EtOAc-hexanes) showed the formation of a new spot which was slightly slower moving than the starting material. The reaction was stopped as soon as all starting material had disappeared (0.5-4 h), concentrated by evaporation, dissolved in acetonitrile and then washed with hexanes three times. The acetonitrile phase was concentrated and purified by flash column chromatography (elution first with hexanes then with 1:1 EtOAc-hexanes), yielding 31 (500 mg, 0.75 mmol, 91%) as a colourless syrup; \(^1\)H NMR (CDCl\(_3\), 500 MHz), the \(\beta\)-anomer only: \(\delta\) 7.37-7.25 (m, 5 H, aromatic), 5.70 (d, 1 H, \(J_{1,2}\) 8.1 Hz, H-1), 5.38 (d, 1 H, \(J_{1',2'}\) 4.1 Hz, H-1'), 5.25 (dd, 1 H, \(J_{2,3}\) 9.2, \(J_{3,4}\) 9.1 Hz, H-3), 5.15 (ddd, 1 H, \(J_{2,3'}\) 10.6, \(J_{3',4'a}\) 5.0, \(J_{3',4}\) 11.3 Hz, H-3'), 4.94 (dd, 1 H, \(J_{1,2}\) 8.1, \(J_{2,3}\) 9.2 Hz, H-2), 4.79 (dd, 1 H, \(J_{1',2'}\) 4.1, \(J_{2',3'}\) 10.6 Hz, H-2'), 4.54, 4.52 (2 d, 2 H, PhC\(\beta\)), 4.43 (dd, 1 H, \(J_{5,6a}\) 2.3, \(J_{6a,6b}\) 12.3 Hz, H-6a), 4.17 (dd, 1 H, \(J_{5,6b}\) 4.7, \(J_{6a,6b}\) 12.3 Hz, H-6b), 4.02 (dd, 1 H, \(J_{3,4}\) 9.1, \(J_{4,5}\) 9.5 Hz, H-4), 3.95 (m, 1 H, H-5'), 3.77 (ddd, 1 H, \(J_{4,5}\) 9.5, \(J_{5,6a}\) 2.3, \(J_{5,6b}\) 4.7 Hz, H-5), 3.42 (m, 2 H, H-6'a, H-6'b), 2.11 (ddd, 1 H, \(J_{3',4'e}\) 5.0, \(J_{4'e,5}\) 2.2, \(J_{4'e,4'a}\) 12.8 Hz, H-4'e), 2.07, 2.03, 2.01, 1.99, (4 s, 12 H, 4 OAc), 1.98 (s, 6 H, 2 OAc), 1.68 (q, 1 H, H-4a') ppm; DCIMS caleld for C\(_{31}\)H\(_{40}\)O\(_{16}\); (M + NH\(_4^+\)), 686.68; found: (M + NH\(_4^+\)), 687.

1,2,3,6,2',3'-Hexa-O-acetyl-4'-deoxy-maltose (32).—To a solution of 31 (400 mg, 0.598 mmol) in EtOH (30 mL) a catalytic amount of Pd/C (10%) catalyst was added and the reaction mixture was hydrogenated until reaction was complete (TLC in 1:1 EtOAc-hexanes). The mixture was filtered, evaporated, and purified over a short column (2:1 EtOAc-hexanes) to yield 32 (340 mg, 0.587 mmol, 98%) as a colourless syrup; \(^1\)H NMR (CDCl\(_3\), 400 MHz): \(\delta\) 6.22 (d, \(J_{1,2}\) 3.6 Hz, H-1\(\alpha\)), 5.72 (d, \(J_{1,2}\) 8.2 Hz, H-1\(\beta\)), 5.49 (dd, \(J_{2,3}\) 10.2, \(J_{3,4}\) 8.4 Hz, H-3\(\alpha\)), 5.39 (d, \(J_{1,2'}\) 4.0 Hz, H-1\(\alpha\')), 5.36 (d, \(J_{1,2'}\) 4.0 Hz, H-1\(\beta\)),
5.26 (dd, J_{2,3} 9.4, J_{3,4} 8.8 Hz, H-3β), 5.19 (m, H-3′α), 5.16 (ddd, H-3′β), 4.95 (dd, J_{1,2} 8.2, J_{2,3} 9.4 Hz, H-2β), 4.94 (dd, J_{1,2} 3.6, J_{2,3} 10.2 Hz, H-2α), 4.78 (dd, J_{1,′2′} 4.0, J_{2,3′} 10.6 Hz, H-2′α), 4.77 (dd, J_{1′,2′} 4.0, J_{2′,3′} 10.4 Hz, H-2′β), 4.44 (dd, J_{5,6b} 2.2, J_{6a,6b} 12.3 Hz, H-6bβ), 4.42 (dd, H-6bα), 4.20 (dd, H-6aα), 4.19 (dd, J_{5,6a} 4.5, J_{6a,6b} 12.3 Hz, H-6aβ), 4.04 (m, H-5α), 4.01 (dd, J_{3,4} 8.4, J_{4,5} 10.0 Hz, H-4α), 4.00 (dd, J_{3,4} 8.8 J_{4,5} 9.8 Hz, H-4β), 3.87 (m, H-5′α), 3.86 (m, H-5′β), 3.79 (ddd, J_{4,5} 9.8, J_{5,6a} 4.5, J_{5,6b} 2.2 Hz, H-5β), 3.60 (dd, J_{5′,6′a} 3.6, J_{6′a,6′b} 12.0 Hz, H-6′bβ), 3.59 (dd, H-6′bα), 3.52 (dd, H-6′α), 3.51 (dd, J_{5′,6′a} 5.8, J_{6′a,6′b} 12.0 Hz, H-6′aβ), 2.11, 2.08, 2.04, 2.02, 2.01, 2.00 (6 s, 18 H, 6 OAcβ), 1.57 (ddd, H-4′α), 1.56 (ddd, H-4′β). The compound was further characterized after acetylation.

1,2,3,6,2′,3′,6′-Hepta-O-acetyl-4′-deoxy-maltose (33).—A solution of 32 (870 mg, 1.50 mmol) in pyridine (50 mL) was acetylated with acetic anhydride (5 mL) at room temperature overnight. CH$_2$Cl$_2$ (50 ml) was added, and the mixture poured into water, extracted twice with CH$_2$Cl$_2$ then the combined organic phases washed with water. Co-evaporation with acetonitrile yielded 31 (920 mg, 1.48 mmol, 99%) as a white foam; $^{1}$H NMR (CDCl$_3$, 500 MHz), the β-anomer only: δ 5.72 (d, 1 H, J_{1,2} 8.1 Hz, H-1), 5.35 (d, 1 H, J_{1,2} 4.1 Hz, H-1′), 5.25 (dd, 1 H, J_{2,3} 8.9, J_{3,4} 9.1 Hz, H-3), 5.15 (ddd, 1 H, H-3′), 4.95 (dd, 1 H, J_{1,2} 8.1, J_{2,3} 8.9 Hz, H-2), 4.79 (dd, 1 H, J_{1′,2′} 4.1, J_{2′,3′} 10.5 Hz, H-2′), 4.42 (dd, 1 H, J_{5,6a} 2.2, J_{6a,6b} 12.2 Hz, H-6a), 4.18 (dd, 1 H, J_{5,6b} 5.0, J_{6a,6b} 12.2 Hz, H-6b), 4.11-3.95 (m, 4 H, H-4, H-5′, H-6′a, H-6′b), 3.89 (ddd, 1 H, J_{4,5} 9.6, J_{5,6a} 2.2, J_{5,6b} 5.0 Hz, H-5), 2.13 (ddd, 1 H, J_{3′,4′e} 4.6, J_{4′e,5′} 1.8, J_{4′e,4′a} 12.6 Hz, H-4′e), 2.09, 2.04, 2.00, 1.99, 1.98 (5 s, 15 H, 5 OAc), 2.07 (s, 6 H, 2 OAc), 1.59 (q, 1 H, H-4′a); DCIMS calcd for C$_{26}$H$_{36}$O$_{17}$: (M$^+$ + NH$_3$), 637.59; found: (M$^+$ + NH$_3$), 638.

4′-Deoxy-maltose.—A solution of the per-O-acetate 33 (50 mg, 80.57 mmol) in dry MeOH (10 mL) was treated with a catalytic amount of solid sodium methoxide at room temperature. The mixture was stirred until the deprotection reaction was complete (TLC in 7:2:1 EtOAc-MeOH-water), then neutralized with an acidic ion exchange resin (BIO-
RAD, AG 50W-X 12, 50-100 mesh, H\(^+\)-form), filtered and the solvent removed in vacuo to yield 25 mg (76.61 mmol, 95%) of a colourless syrup. \(^1\)H NMR (D\(_2\)O, 400 MHz): \(\delta\) 5.37 (d, 1 H, \(J_{1,2}\) 3.9 Hz, H-1'), 5.19 (d, \(J_{1,2}\) 3.9 Hz, H-1\(\alpha\)), 4.61 (d, \(J_{1,2}\) 8.0 Hz, H-1\(\beta\)), 3.47 (dd, \(J_{1,2}\) 3.9, \(J_{2,3}\) 9.8 Hz, H-2\(\alpha\), H-2'), 3.24 (dd, \(J_{1,2}\) 8.0, \(J_{2,3}\) 9.4 Hz, H-2\(\beta\)), 1.96 (dd, 1 H, H-4'e), 1.41 (q, 1 H, H-4'a); DCIMS calcd for C\(_{12}\)H\(_{22}\)O\(_{10}\): (M + NH\(_4\))\(^+\), 344.63; found: (M + NH\(_4\))\(^+\), 345.

2,3,6,2',3',6'-Hexa-O-acetyl-4'-deoxy-maltose (34).—A solution of the per-O-acetate 33 (310 mg, 0.50 mmol) in DMF (4 mL) was reacted with hydrazine acetate (100 mg, 1.08 mmol) at 50°C. The reaction was carefully monitored by TLC (1:1 EtOAc-hexanes). After 30 min all starting material had disappeared. EtOAc (10 mL) and water (10 mL) were added and the aqueous phase was extracted twice with EtOAc. The combined EtOAc-phases were washed with water, evaporated and purified by flash chromatography (1:1 EtOAc-hexanes, later 2:1) to yield 34 (200 mg, 0.345 mmol, 69%) as a colourless syrup. Analysis of the anomeric protons the \(^1\)H NMR spectrum (CDCl\(_3\), 200 MHz) indicated selective deprotection of the anomeric center. Detailed assignment was not possible. The reducing sugar 34 was further characterized after the next step.

2,3,6,2',3',6'-Hexa-O-acetyl-4'-deoxy-\(\alpha\)- and \(\beta\)-maltosyl fluoride (35 and 36).—To a solution of 34 (180 mg, 0.311 mmol) in dry CH\(_2\)Cl\(_2\) (10 mL), DAST (0.05 mL, 0.378 mmol) was added at 0°C. The mixture was stirred at room temperature for 30 min until the reaction was complete: TLC (1:1 EtOAc-hexanes). The reaction mixture was quenched with water, extracted twice with CH\(_2\)Cl\(_2\), and the combined organic phases were washed once with water. After evaporation of the solvent flash chromatography (1:1 EtOAc-hexanes) yielded the glycosyl fluorides (35 and 36) (175 mg, 0.301 mmol, 97%) as a colourless syrup which mainly comprised the \(\beta\)-anomer (36) (\(\beta/\alpha\) ~ 9:1 by \(^1\)H-NMR); \(^1\)H NMR (CDCl\(_3\), 500 MHz), the \(\beta\)-anomer only: \(\delta\) 5.38 (dd, 1 H, \(J_{1,2}\) 52.6, \(J_{1,2}\) 5.3 Hz, H-1'), 5.36 (d, 1 H, \(J_{1,2}\) 4.1 Hz, H-1'), 5.18 (ddd, 1 H, \(J_{2,3}\) 10.4, \(J_{3,4}'\) 4.7, \(J_{3,4}'\) 11.1 Hz, H-3'), 5.12 (dd, 1 H, \(J_{2,3}\) 6.7, \(J_{3,4}\) 7.5 Hz, H-3), 4.92 (ddd, 1 H, \(J_{1,2}\) 5.3,
$J_{2,3}$ 6.7, $J_{2,F}$ 9.2 Hz, H-2), 4.78 (dd, 1 H, $J_1',2'$ 4.1, $J_{2',3'}$ 10.4 Hz, H-2'), 4.52 (dd, 1 H, $J_5$, 6a 2.8, $J_{6a,6b}$ 12.2 Hz, H-6a), 4.18 (dd, 1 H, $J_5$, 6b 5.2, $J_{6a,6b}$ 12.2 Hz, H-6b), 4.08 (m, 4 H, H-4, H-5', H-6'a, H-6'b), 3.92 (m, 1 H, H-5), 2.16 (ddd, 1 H, $J_{3',4'e}$ 4.7, $J_{4'e,4'a}$ 12.8, $J_{4'e,5'}$ 2.0 Hz, H-4'e), 2.11, 2.08, 2.07, 2.03, 2.02, 2.00 (6 s, 18 H, 6 OAc), 1.59 (q, 1 H, H-4'a); $^{19}$F NMR (CDCl$_3$, 188 MHz): δ -149.21 (α), -133.27 (β); the compound was further characterized after anomerization.

2,3,6,2',3',6'-Hexa-0-acetyl-4'-deoxy-α-maltosyl fluoride (35).—

a) The β-fluoride 36 (130 mg, 0.224 mmol) was treated with HF-pyridine (0.50 mL) in a plastic vial for 2 h at -50°C, then the reaction quenched by adding CH$_2$Cl$_2$ and neutralized with saturated aqueous NaHCO$_3$. The aqueous phase was extracted twice with CH$_2$Cl$_2$ and the combined organic phases were washed with water. The solvent was evaporated in vacuo and the success of the anomerization was determined by $^{19}$F NMR spectroscopy. The $^{19}$F NMR spectrum displayed three singlets (δ -148.64, -149.24, -149.31 ppm), with no peak for the β-anomer. The mixture was purified by column chromatography (1:1 EtOAc-hexanes) which led to the α-maltosyl fluoride 35 and 2,3,6-tri-O-acetyl-α-D-glucosyl fluoride (-149.31 ppm). For further purification this mixture was acetylated with acetic anhydride in pyridine, subjected to aqueous extraction and passed over a column (1:1 EtOAc-hexanes) to yield pure 35 (83 mg, 0.143 mmol, 64%) as a white amorphous solid;

b) 35 could also be obtained from 33 without intermediate purification steps. A solution of the per-O-acetate 33 (850 mg, 1.37 mmol) in DMF (50 mL) was treated with hydrazine acetate (300 mg, 3.26 mmol) for 30 min when TLC showed only one product. After work up, as above, the resulting syrup was dissolved in dry CH$_2$Cl$_2$ and fluorinated with DAST (0.40 mL, 3.02 mmol). Following the usual work up the product was treated with HF-pyridine (1.00 mL) at -50°C for 1 h, then purified as described in a) to give 35 (370 mg, 0.637 mmol, 47% overall) as colourless crystals after recrystallisation, mp (ether/petrol ether) 153°C; $^1$H NMR (CDCl$_3$, 500 MHz): δ 5.60 (dd, 1 H, $J_{1,2}$ 2.7, $J_{1,F}$
53.3 Hz, H-1'), 5.48 (dd, 1 H, J_{2,3} 9.8, J_{3,4} 9.2 Hz, H-3), 5.36 (d, 1 H, J_{1,2'} 3.9 Hz, H-
1'), 5.13 (ddd, 1 H, J_{2,3} 9.0, J_{3,4}^e, J_{3,4}^a 4.9, J_{3,4}^e, J_{3,4}^a 11.2 Hz, H-3'), 4.79 (ddd, 1 H, J_{1,2} 2.8,
J_{2,F} 24.2, J_{2,3} 10.1 Hz, H-2), 4.76 (dd, 1 H, J_{1,2'} 3.9, J_{2,3'} 10.4 Hz, H-2'), 4.47 (dd, 1 H,
J_{5,6a} 1.8, J_{6a,6b} 12.3 Hz, H-6a), 4.18 (dd, 1 H, J_{5,6b} 4.3, J_{6a,6b} 12.3 Hz, H-6b), 4.10 (ddd,
1 H, J_{4,5} 10.0, J_{5,6a} 2.2, J_{5,6b} 4.1 Hz, H-5), 4.1 - 3.95 (m, 4 H, H-4, H-5', H-6'a,
H-6'b), 2.12 (ddd, 1 H, J_{3} 4.9, J_{4}^e, J_{4}^a 12.7, J_{4}^e, J_{5}^e, J_{6}^e, J_{6}^a 1.9 Hz, H-4'e), 2.08, 2.05, 2.03,
2.01 (s, 12 H, 4 OAc), 1.97 (s, 6 H, 2 OAc), 1.69 (q, 1 H, H-4'a); $^{19}$F NMR (CDCl$_3$,
188 MHz): $\delta$ -149.22; $^{19}$F NMR (CDCl$_3$, 188 MHz, coupled): -149.24 (dd, J_{1,F} 53.0,
J_{2,F} 23.9 Hz); DCIMS calcd for C$_{24}$H$_{33}$FO$_{15}$: (M+NH$_4^+$), 598.54; found: (M+NH$_4^+$),
598. Anal. Calcd. for C$_{24}$H$_{33}$O$_{15}$F: C, 49.81; H, 5.54. Found: C, 49.81; H, 5.54.

4'-Deoxy-\(\alpha\)-maltosyl fluoride (24).—The protected \(\alpha\)-fluoride 35 (83 mg, 0.143
mmol) was dissolved in dry MeOH (10 mL) and stirred with a catalytic amount of solid
sodium methoxide at room temperature. TLC analysis (7:2:1 EtOAc-MeOH-water)
showed three spots after 25 min of which only the most polar remained when the reaction
was complete. The mixture was neutralized with acidic ion exchange resin (BIO-RAD,
AG 50W-X 12, 50-100 mesh, H$^+$-form), filtered and evaporated to yield 24 (46 mg,
0.140 mmol, 98%) as a colourless glass; $^1$H NMR (D$_2$O, 500 MHz), selected data: $\delta$ 5.64
(dd, 1 H, J$_{1,F}$ 53.6, J$_{1,2}$ 2.8 Hz, H-1), 5.37 (d, 1 H, J$_{1,2'}$ 3.9 Hz, H-1'), 3.68 (dd, 1 H,
J$_{2,3}$ 9.8, J$_{3,4}$ 9.5 Hz, H-3), 3.62 (dd, 1 H, H-6), 3.60 (ddd, 1 H, J$_{1,2}$ 2.8, J$_{2,3}$ 9.8, J$_{2,F}$
26.4 Hz, H-2), 3.54 (dd, 1 H, H-6), 3.46 (dd, 1 H, J$_{1,2'}$ 3.9, J$_{2,3'}$ 9.7 Hz, H-2'), 1.93
(ddd, 1 H, J$_{3,4}^e$ 4.8, J$_{4}^e, J_{4}^a 12.6, J_{4}^e, J_{5}^e, J_{6}^e, J_{6}^a 1.9 Hz, H-4'e), 1.39 (q, 1 H, H-4'a); $^{19}$F NMR
(D$_2$O, 188 MHz): $\delta$ -149.82. Anal. Calcd. for C$_{12}$H$_{21}$O$_9$F$_2$H$_2$O: C, 39.56; H, 6.86.
Found: C, 39.81; H, 6.75.

e) The synthesis of 2-deoxy-2-fluoro-\(\alpha\)-maltosyl fluoride (22).

2-Deoxy-2-fluoro-\(\alpha\)-maltosyl fluoride (22).—A solution of maltal peracetate
(Hehre et al., 1986) (500 mg, 0.89 mmol) in CFCl$_3$ was reacted with F$_2$ at -78°C
according to the general procedure to give a 1.3:1 mixture of the 2-deoxy-2-fluoro maltose (37) and \( \beta \)-addition product (38), identified by their \( ^{19} \)F NMR spectra. Flash chromatography (1:1:1 EtOAc-petroleum ether-CHCl\(_3\)) afforded the peracetate 37 (108 mg, 20\%) as a colourless gum; \(^1\)H NMR (CDCl\(_3\)): \( \delta \) 5.68 (dd, 1 H, \( J_{\text{H1},\text{F1}} \) 53.4, \( J_{\text{H1},\text{H2}} \) 2.6 Hz, H-1), and 2.14-1.95 (6 s, 6 OAc); \(^{19} \)F NMR (CDCl\(_3\)): \( \delta \) -151.2 (d, \( J_{\text{F1,F2}} \) 18.8 Hz, F-1), -205.5 (d, \( J_{\text{F2,F1}} \) 18.8 Hz, F-2). To a solution of 37 (100 mg, 0.17 mmol) in dry MeOH (10 mL) was added NaOMe (3 mL, 1.77 mmol) and the mixture was stirred under \( \text{N}_2 \) for 1 h. The mixture was neutralized with Dowex AG-50W X2 (H\(^+\)) resin and solvent was evaporated in \textit{vacuo}. Flash chromatography (5:2:1 EtOAc- EtOH-water) afforded 22 (53 mg, 90\%) as a colourless gum. \(^1\)H NMR (D\(_2\)O): \( \delta \) 5.85 (dd, 1 H, \( J_{\text{H1},\text{F1}} \) 53.9, \( J_{\text{H1},\text{H2}} \) 2.4 Hz, H-1); \(^{19} \)F NMR (D\(_2\)O): \( \delta \) -149.1 (d, \( J_{\text{F1,F2}} \) 20.0 Hz, F-1), -204.6 (d, \( J_{\text{F2,F1}} \) 20.0 Hz, F-2). Anal. Calcd. for C\(_{12}\)H\(_{20}\)F\(_2\)O\(_9\): C, 41.62; H, 5.82. Found: C, 41.28; H, 5.96.

f) The synthesis of 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-4-O-(\( \alpha \)-(1,4)-D-glucosyl)-\( \alpha \)-D-arabino-hexopyranoside (23)

\[ 3,6\text{-Di-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-\( \alpha \)-(1,4)-D-glucosyl)-2-deoxy-2-fluoro-a-glucosyl bromide} (39) \] —Maltal peracetate (17.5 g, 25.1 mmol) was fluorinated by the large scale procedure. To this reaction mixture was added CHCl\(_3\) and the organic layer was washed with water, saturated aqueous NaHCO\(_3\), and water, and dried (MgSO\(_4\)). After evaporation in \textit{vacuo}, two major products were identified by NMR: 1,3,6-tri-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-\( \alpha \)-(1,4)-D-glucosyl)-2-deoxy-2-fluoro-\( \alpha \)-glucoside, and 1,3,6-tri-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-\( \alpha \)-(1,4)-D-glucosyl)-2-deoxy-2-fluoro-\( \beta \)-mannoside, which could not be separated by flash chromatography. The syrup was dissolved in glacial acetic acid (500 mL), treated with 45\% HBr in acetic acid (50 mL) and allowed to react for 3 days at 0\textdegree C. CHCl\(_3\) was added and the organic layer was washed with water, saturated aqueous NaHCO\(_3\), and water, and dried
143

(MgSO₄). The other major product identified by NMR was 3,6-di-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-α-D-glucosyl)-2-deoxy-2-fluoro-α-mannosyl bromide; ¹H NMR (CDCl₃): δ 6.43 (d, J₁,₂ 4.4 Hz, H-1 "gluco"), 6.34 (dd, J₁,F₂a 9.6, J₁,₂ 1.5 Hz, H-1 "manno"); ¹⁹F NMR (CDCl₃): δ -182.8 (ddd, J₂,F₂a 49.6, J₂,F₂a 26.3, J₂,F₂a 9.7 Hz, F-2 "manno"), -190.3 (dd, J₃,F₂e 49.3, J₃,F₂e 10.2 Hz, F-2 "gluco"). This mixture was used without further purification.

3,6-Di-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-α-(1,4)-D-glucosyl)-2-deoxy-2-fluoro-D-glucal (40). —The mixture of 2-fluoro "gluco" and "manno" compounds was dissolved in acetonitrile (200 mL) and triethylamine (40 mL), and allowed to stir for 17 h at room temperature. The excess base was removed in vacuo, CHCl₃ was added, and the organic layer was washed with water, saturated aqueous NaHCO₃, and water, and dried (MgSO₄). By NMR analysis the "gluco" compound had completely reacted; whereas, the "manno" compound did not undergo the elimination reaction. Unfortunately the product 40 could only be partially purified (95%); ¹H NMR (CDCl₃): δ 6.68 (d, J₁,₂ 4.9 Hz, H-1); ¹⁹F NMR (CDCl₃): δ -165.3 (d, J₂,F₂ 4.3 Hz, F-2). This mixture was used without further characterization.

3,6-di-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-α-(1,4)-D-glucosyl)-2-deoxy-2,2-difluoro-a-D-arabinohexopyranose (41). —40 (3.7 g, 6.4 mmol) was fluorinated according to the large scale method. The reaction mixture was partially purified by flash chromatography (1:1 EtOAc-Hexane) to afford a syrup containing both α- and β-acetates of 1,3,4,6-tetra-O-acetyl-2-deoxy-2,2-difluoro-α/β-D-arabinohexopyranoside. The syrup was treated with hydrazine acetate (1.37 g, 14.7 mmol) in DMF (50 mL) for 3 days at 50°C. Evaporation of the solvent in vacuo, followed by flash chromatography (1:1 EtOAc-Hexane) gave only the α-hemiacetal 41 (0.70 g, 18%) as a colourless gum; ¹H NMR (CDCl₃): δ 5.63 (ddd, 1 H, J₃,F₂a 18.5, J₃,4 9.2, J₃,F₂e 6.5 Hz, H-3), 5.42 (d, 1 H, J₁,₂ 4.0 Hz, H-1'), 5.35 (dd, 1 H, J₃,2' 10.4, J₃,4' 9.6 Hz, H-3'), 5.18 (d, 1 H, J₁,F₂a 4.7 Hz, H-1), 5.05 (dd, 1 H, J₄,3' 9.8, J₄,5' 9.8 Hz, H-4'), 4.84 (dd, 1 H, J₂,3' 10.4, J₂,1' 4.0


Hz, H-2'), 4.6-4.0 (m, 7 H, H-4, H-5, H-6a, H-6b, H-5', H-6a', H-6b'), and 2.2-1.9 (5 s, 6 OAc); \(^{19}\)F NMR (CDCl\(_3\)): \(\delta\) -120.9 (dd, \(J_{F2e,F2a}\) 253, \(J_{F2e,3}\) 6.3 Hz, Fe-2), -122.7 (ddd, \(J_{F2a,F2e}\) 253, \(J_{F2a,3}\) 18.4, \(J_{F2a,1}\) 4.5 Hz, Fa-2). This compound was used without further characterization.

2,4,6-Trinitrophenyl 3,6-di-O-acetyl-4-O-(2',3',4',6'-tetra-O-acetyl-\(\alpha\)-(1,4)-D-glucosyl)-2-deoxy-2,2-difluoro-\(\alpha\)-D-arabino-hexopyranoside (42). —To a solution of 41 (535 mg, 0.87 mmol) dissolved in dry CH\(_2\)Cl\(_2\) (5 mL) was added 2,6-di-tert-butyl pyridine (0.6 mL, 2.7 mmol) and 43 (460 mg, 2.0 mmol) and the mixture was allowed to stir for 10 days at room temperature in the dark under N\(_2\). Evaporation of the solvent in vacuo, followed by flash chromatography (1:1 EtOAc-Hexane) afforded 42 (668 mg, 91%) as a yellowish gum; \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 8.85 (s, 2 H, aryl H's), 5.71 (ddd, 1 H, \(J_{3,F2a}\) 20.3, \(J_{3,4}\) 8.8, \(J_{3,F2e}\) 3.6 Hz, H-3), 5.61 (d, 1 H, \(J_{1,F2a}\) 5.3 Hz, H-1), 5.44 (d, 1 H, \(J_{1',2'}\) 4.0 Hz, H-1'), 5.33 (dd, 1 H, \(J_{3',4'}\) 10.0, \(J_{3',2'}\) 10.0 Hz, H-3'), 5.02 (dd, 1 H, \(J_{4',5'}\) 9.9, \(J_{4',3'}\) 9.9 Hz, H-4'), 4.83 (dd, 1 H, \(J_{2',3'}\) 10.5, \(J_{2',1'}\) 4.0 Hz, H-2'), 4.4-3.9 (m, 7 H, H-4, H-5, H-6a, H-6b, H-5', H-6a', H-6b'), and 2.2-1.9 (6 s, 6 OAc); \(^{19}\)F NMR (CD\(_2\)Cl\(_2\)): \(\delta\) -126.0 (ddd, \(J_{F2a,F2e}\) 264, \(J_{F2a,3}\) 19.3, \(J_{F2a,1}\) 5.1 Hz, Fa-2), -128.5 (dd, \(J_{F2e,F2a}\) 264, \(J_{F2e,3}\) 4.7 Hz, Fe-2). HRMS Calcd. for C\(_{19}\)H\(_{39}\)F\(_2\)N\(_3\)O\(_{22}\): (M+NH\(_4^+\)), 843.296; found: (M+NH\(_4^+\)), 843.18366.

\(^1\)Fluoro-2,4,6-trinitro-benzene (43) (see Shaw & Seaton, 1961). —\(^1\) Fluoro-2,4-dinitro-benzene (1.55 g, 8.33 mmol) was added to KNO\(_3\) (3.25 g, 32.1 mmol) and fuming H\(_2\)SO\(_4\) (9.7 mL, 21%), and the mixture was allowed to stir at 120°C for 5 days. The mixture was allowed to cool and then was poured over crushed ice and extracted with toluene. After evaporation of toluene in vacuo, a minimum amount of CFCl\(_3\) and toluene (1:1) was added to dissolve the gum, and ligroin (low boiling petroleum ether) was added until crystals began to form. Recrystallization from CFCl\(_3\) and toluene (1:1) afforded 43 (1.08 g, 56%) as yellowish white crystals (mp 132-134°C; Literature 131-
$132^\circ$C; $^1$H NMR (CD$_2$Cl$_2$): $\delta$ 8.84 (d, $J_{H,F}$ 5.7 Hz, aryl H's); $^{19}$F NMR (CD$_2$Cl$_2$): $\delta$ -122.2 (t, $J_{F,H}$ 5.6 Hz, F-1).

$2,4,6$-Trinitrophenyl $^{2}$-deoxy-$2,2$-difluoro-$4$-$O$-($\alpha$-($1,4$)-$D$-glucosyl)-$\alpha$-$D$-arabino-$\alpha$-hexopyranoside (23). —To 42 (96 mg, 0.12 mmol) in dry MeOH (2 mL) at 0°C was added freshly distilled acetyl chloride (80 mL) according to the general procedure. The mixture was allowed to stir at 4°C for 2 days. After workup, flash chromatography (20:2:1 EtOAc-MeOH-water; Acetone) afforded 23 (47 mg, 71%) as a yellowish gum. This was then freeze-dried to give a yellowish white solid; $^1$H NMR (D$_2$O): $\delta$ 9.14 (s, 2 H, aryl H's), 5.91 (d, 1 H, $J_{1,F2a}$ 3.6 Hz, H-1), 5.46 (d, 1 H, $J_{1',2}$ 3.9 Hz, H-1'); $^{19}$F NMR (D$_2$O): $\delta$ -122.0 (Fe-2, Fa-2). Anal. Calcd. for C$_{12}$H$_{11}$F$_2$N$_3$O$_{11}$•1.5H$_2$O: C, 36.01; H, 4.03; N 7.00. Found: C, 36.10; H, 3.96; N 6.79.

g) The synthesis of $2,4,6$-trinitrophenyl $^{2}$-deoxy-$2,2$-difluoro-$\alpha$-$D$-arabino-$\alpha$-hexopyranoside (44)

$3,4,6$-Tri-$O$-acetyl-$2$-$deoxy$-$2$-$fluoro$-$$\alpha$-$glucosyl$ bromide (47). —To 2-deoxy-$2$-fluoro-$\alpha$-glucosyl fluoride per-$O$-acetate (5 g, 16.1 mmol) was added 45% HBr in acetic acid (50 mL) and acetic anhydride (5 mL). The mixture was allowed to stir for 2 days at room temperature. CHCl$_3$ was added and the organic layer was washed with water, saturated aqueous NaHCO$_3$, and water, and dried (MgSO$_4$). Flash chromatography (1:4 EtOAc-Hexane) yielded pure 47 (4.6 g, 77%) as a colourless gum; $^1$H NMR (CDCl$_3$): $\delta$ 6.47 (dd, 1 H, $J_{1,2}$ 4.0, $J_{1,F2}$ 0.5 Hz, H-1), 5.57 (ddd, 1 H, $J_{3,F2}$ 11.0, $J_{3,4}$ 10.0, $J_{3,2}$ 10.0 Hz, H-3), 5.06 (dd, 1 H, $J_{4,5}$ 10.0, $J_{4,3}$ 10.0 Hz, H-4), 4.48 (ddd, 1 H, $J_{2,F2}$ 49.5, $J_{2,3}$ 9.5, $J_{2,1}$ 4.0 Hz, H-2), 4.3-4.0 (m, 3 H, H-5, H-6a, H-6b), and 2.1-2.0 (3 s, 3 OAc); $^{19}$F NMR (CDCl$_3$): $\delta$ -189.1 (F-2). This compound was used without further characterization.
3,4,6-Tri-O-acetyl-2-deoxy-2-fluoro-D-glucal (48). —47 (4.6 g, 12.4 mmol) was dissolved in acetonitrile (200 mL), triethylamine (18.6 mL) was added, and the reaction was allowed to stir for 20 h under reflux conditions. The excess base was removed in vacuo, CHCl₃ was added, and the organic layer was washed with water, saturated aqueous NaHCO₃, and water, and dried (MgSO₄). Flash chromatography (1:4 EtOAc-Hexane) yielded pure 48 (2.5 g, 70%) as a colourless gum; ¹H NMR (CDCl₃): δ 6.72 (d, 1 H, J₁,F₂ 5.0 Hz, H-1), 5.56 (t, 1 H, J₃,F₂ 4.0, J₃,4 4.0 Hz, H-3), 5.13 (ddd, 1 H, J₄,F₂ 4.0, J₄,3 4.0 Hz, H-4), 4.4-4.0 (m, 3 H, H-5, H-6a, H-6b), and 2.1-2.0 (3 s, 3 OAc); ¹⁹F NMR (CDCl₃): δ -166.5 (F-2). This compound was used without further characterization.

1,3,4,6-Tetra-O-acetyl-2-deoxy-2,2-difluoro-a-D-arabino-hexopyranoside (49). —48 (1.0 g, 3.4 mmol) was fluorinated with acetylhypofluorite according to the large scale procedure. The reaction mixture was purified by flash chromatography (1:2 EtOAc-Hexane) to afford 49 (0.56 g, 45%) as a colourless gum; ¹H NMR (CDCl₃): δ 6.15 (t, 1 H, J₁,F₂a 3.2, J₁,F₂e 3.2 Hz, H-1), 5.52 (ddd, 1 H, J₃,F₂a 24.0, J₃,F₂e 11.6, J₃,4 10.0 Hz, H-3), 5.25 (t, 1 H, J₄,3 10.0, J₃,5 10.0 Hz, H-4), 4.3-4.0 (m, 3 H, H-5, H-6a, H-6b), 2.2-2.0 (4 s, 4 OAc); ¹⁹F NMR (CDCl₃): δ -121.0 (Fa-2, Fe-2). LRMS Calcd for C₁₄H₁₆F₄O₉: (M+NH₄⁺), 386.316; found: (M+NH₄⁺), 386.

3,4,6-Tri-O-acetyl-2-deoxy-2,2-difluoro-a-D-arabino-hexopyranose (50). —49 (0.5 g, 1.36 mmol) was dissolved in DMF (10 mL) and treated with hydrazine acetate (280 mg, 3.0 mmol) for 3 days at 50°C. Evaporation of the solvent in vacuo, followed by flash chromatography (1:1 EtOAc-Hexane) afforded 50 (280 mg, 81%) as a colourless gum; ¹H NMR (CDCl₃): δ 5.58 (ddd, 1 H, J₃,F₂a 19.0, J₃,4 11.0, J₃,F₂e 6.0 Hz, H-3), 5.3-5.1 (m, 2 H, H-1, H-4), 4.3-4.0 (m, 3 H, H-5, H-6a, H-6b), and 2.1-1.9 (3 s, 3 OAc); ¹⁹F NMR (CDCl₃): δ -120.7 (dd, J₉,F₂e,F₂a 251, J₉,F₂e,3 6.0 Hz, Fe-2), -122.7 (ddd, J₉,F₂a,F₂e 251, J₉,F₂a,3 19.8, J₉,F₂a,1 5.0 Hz, Fa-2). This compound was used without further characterization.
2,4,6-Trinitrophenyl 3,4,6-tri-O-acetyl-2-deoxy-2,2-difluoro-α-D-arabino-hexopyranoside (51). —To a solution of 50 (93 mg, 0.28 mmol) dissolved in dry CH$_2$Cl$_2$ (1 mL) was added 2,6-di-tert-butyl pyridine (0.1 mL, 0.44 mmol) and 43 (90 mg, 0.39 mmol) and the mixture was allowed to stir for 10 days at room temperature in the dark under N$_2$. Evaporation of the solvent in vacuo, followed by flash chromatography (1:2 EtOAc-Hexane) afforded 51 (76 mg, 58 %) as a yellowish gum; $^1$H NMR (CDCl$_3$): δ 8.84 (s, 2 H, aryl H's), 5.68 (ddd, 1 H, $J_{3,4}$ 9.9, $J_{3,F2a}$ 6.0 Hz, H-3), 5.62 (d, 1 H, $J_{1,F2a}$ 5.0 Hz, H-1), 5.23 (dt, 1 H, $J_{4,5}$ 10.0, $J_{4,F2e}$ 1.4 Hz, H-4), 4.3-4.0 (m, 3 H, H-5, H-6a, H-6b), and 2.2-2.0 (2 s, 3 OAc); $^{19}$F NMR (CDCl$_3$): δ -118.2 (ddd, $J_{F2a,F2e}$ 263, $J_{F2a,3}$ 19.0, $J_{F2a,1}$ 6.0 Hz, Fa-2), -121.3 (dd, $J_{F2e,F2a}$ 263, $J_{F2e,3}$ 6.0 Hz, Fe-2).

2,4,6-Trinitrophenyl 2-deoxy-2,2-difluoro-α-D-arabino-hexopyranoside (44). —To 51 (84 mg, 0.18 mmol) in dry MeOH (2 mL) at 0°C was added freshly distilled acetyl chloride (80 mL) according to the general procedure. The mixture was allowed to stir at 4°C for 2 days. After workup, flash chromatography (1:1 EtOAc-Hexane) afforded pure 44 (14 mg, 19%) as a yellowish gum. This was then freeze-dried to give a yellowish white solid; $^1$H NMR (D$_2$O): δ 9.10 (s, 2 H, aryl H's), 5.89 (d, 1 H, $J_{1,F2a}$ 4.9 Hz, H-1), 4.16 (dt, 1 H, $J_{3,F2a}$ 19.6, $J_{3,4}$ 7.5, $J_{3,F2e}$ 7.5 Hz, H-3), 3.8-3.6 (m, 4 H, H-4, H-5, H-6a, H-6b); $^{19}$F NMR (D$_2$O): δ -120.6 (dd, $J_{F2e,F2a}$ 261, $J_{F2e,3}$ 6.9 Hz, Fe-2), -122.7 (ddd, $J_{F2a,F2e}$ 261, $J_{F2a,3}$ 19.7, $J_{F2a,1}$ ~6 Hz, Fa-2). Anal. Calcd. for C$_{12}$H$_{11}$F$_2$N$_3$O$_{11}$·0.25H$_2$O: C, 34.67; H, 2.79; N 10.10. Found: C, 34.68; H, 2.91; N 9.89.
5.2. Enzyme kinetics

5.2.1. Miscellaneous procedures

Absorbance measurements were made using either a Perkin-Elmer Lambda 2 model, or a Pye-Unicam PU-8800, PU-8700, or ATI Unicam UV4 model UV-VIS spectrophotometer, each of which was equipped with a circulating water bath and thermostatted cuvette holders. In all cases absorbance measurements were made using cuvettes with a 1.0 cm pathlength.

All pH measurements were performed using a Radiometer PHM 82 pH meter equipped with an Orion combination electrode. The electrode was standardized prior to use with commercially available standard pH buffers.

All fluoride ion measurements were made using an Orion combination fluoride ion electrode. The readings were obtained from a Fisher Accumet 9500 ion meter and the data was transmitted to a computer through the program Terminal. The temperature of the experiments was regulated using a water bath.

5.2.2. Enzymes used in this study and their assays

a) Glycogen debranching enzyme

Glycogen debranching enzyme (EC 3.2.1.33 + EC 2.4.1.25) was prepared from rabbit muscle by Shirley Shechosky of Dr. Madsens' laboratory at the University of Alberta. The isolation procedure followed the protocol outlined by Takrama & Madsen (1988) and the concentration of this protein in stock solutions was determined from $A_{280}$ measurements using an $E_{m}^mL = 1.75$ cm$^{-1}$. The specific activity of the enzyme was 11 $\mu$mol min$^{-1}$mg$^{-1}$, measured by the coupled activity assay of Gillard and Nelson (1977).
b) α-Amylase

α-Amylase (EC 3.2.1.1) was prepared from human pancreas using the procedure outlined by Burke et al. (1993). The enzyme was shown to be pure by polyacrylamide gel electrophoresis. Preparation of α-amylase was assisted by Mrs. Yili Wang. The concentration of this protein in stock solutions was determined from A$_{280}$ measurements using an E$^{	ext{mg/mL}}$=2.33 cm$^{-1}$ (Burke et al., 1993). The specific activity of the enzyme was 388 U/mg. One unit of activity yields 1 mg of maltose from soluble starch in 3 min at 30°C (Burke et al., 1993).

α-Amylase cloned from baby hamster kidney cell lines was prepared by Dr. Helen Côté. The cDNA was cloned by PCR on human pancreatic mRNA. The cDNA was cloned into the expression vector pNUT and was transfected into BHK cells. The method used to purify HPA was used to purify the recombinant HPA (Burke et al., 1993).

c) Yeast α-glucosidase

Yeast α-glucosidase (typeIII) was purchased from the Sigma Chemical Company, and the catalogue number of this product was G 7256.

5.2.3. Evaluation of substrates

a) Determinations of $K_m$ and $k_{cat}$ for various substrates

Approximate values of $K_m$ and $k_{cat}$ (see Appendix II.1 for theory and calculations) were determined by measuring initial reaction rates using 3 widely different concentrations of the substrate. Accurate values were then determined by using 5-8 different concentrations of the substrate that bracketed the approximately determined $K_m$ value (typically a range of substrate concentration from 0.3-5 times the $K_m$). Values of $K_m$ and $k_{cat}$ were calculated by fitting the data to the Michaelis-Menten equation by
initial rate data were also plotted according to the method of Lineweaver and Burk (1934), but this method was not used for calculating kinetic parameters, but rather as a tool for recognizing deviations from linear behaviour.

b) Detailed assay conditions

Glycosyl fluoride substrates for glycogen debranching enzyme and α-amylase were kinetically evaluated using the following procedure: enzyme (10 μL) was added to a solution (240 μL) containing substrate and buffer incubated at 30°C. The initial rates were determined by following the release of fluoride ion up to a maximum of 10% substrate depletion. The buffer used for assaying the glucosidase activity of glycogen debranching enzyme was 100 mM sodium phosphate, and 1 mM EDTA, pH 6.9. The buffer used for assaying the transferase activity of glycogen debranching enzyme was 100 mM sodium phosphate, and 1 mM EDTA, pH 6.0. Rabbit liver glycogen (type III) used in the assay of the transferase activity of glycogen debranching enzyme was purchased from Sigma Chemical Company and was purified by Reneé Mosi with AG-1X8 (200-400 mesh, Cl− form) ion-exchange resin. The buffer for α-amylase studies was 20 mM sodium phosphate, and 25 mM sodium chloride, pH 6.9.

α-Glucosidase from yeast was assayed by following the release of p-nitrophenol from the substrate p-nitrophenyl α-D-glucoside (Sigma Chemical Company) in 1 mL reaction volumes using a UV-VIS spectrophotometer and the temperature of the experiments was maintained at 37°C. Except as noted, the phosphate buffer (50 mM), pH 6.8, contained BSA (0.1%). The molar extinction coefficient for p-nitrophenol at pH 6.8 and 37°C is 7280 M⁻¹cm⁻¹ (Street, 1988). p-Nitrophenyl α-D-glucoside was also evaluated as a substrate of the glucosidase activity of the debranching enzyme and the temperature of the experiment was maintained at 37°C. The buffer used was 50 mM
sodium phosphate, pH 6.9. 2FaGF was evaluated as a substrate of yeast α-glucosidase using the procedure outlined above for glycosyl fluorides.

5.2.4. Evaluation of reversible inhibitors

Approximate values for $K_i$ (range-finding or RF $K_i$) were determined by measuring initial reaction rates at a single substrate concentration in the presence of 5 or 6 different inhibitor concentrations. An accurate $V_{\text{max}}$ value was also determined using several different concentrations of substrate. The data from the first experiment were then plotted as a Dixon plot ($1/v$ vs. $[I]$) (Dixon, 1972). The RF $K_i$ value was determined from the intercept ($-K_i$) of the line through the plotted data and the inverse of the maximum velocity ($1/V_{\text{max}}$) (Dixon, 1972).

Deoxynojirimycin (DNJ) was tested as an inhibitor of the glucosidase activity of Glyx. RF $K_i$ was determined by following the release of fluoride from reaction mixtures (250 μL) containing α-glucosyl fluoride (1.8 mM), enzyme (21 μg) and DNJ (0, 1, 4, 10, 30 μM) in the appropriate buffer. The results were used to help determine the mechanism of action of Glyx on α-maltosyl fluoride.

5.2.5. Evaluation of irreversible inhibitors

a) Detailed assay conditions (see Appendix II.2 for theory and calculations)

2-Deoxy-2-fluoro-α-maltotriosyl fluoride was tested as an inactivator of glycogen debranching enzyme by incubating a solution (100 μL) of 2-deoxy-2-fluoro-α-maltotriosyl fluoride (12.8 mM), 50 mM sodium phosphate buffer (pH 6.8), and 0.9 mg of enzyme for 3 days at room temperature. Aliquots (10 μL) of the inactivation mixture (Imix) were removed over the course of the experiment and added to a reaction mixture which contained α-maltotriosyl fluoride (15.0 mM), 0.09% glycogen in 100 mM sodium phosphate buffer (pH 6.8). Initial reaction rates were compared with a control experiment, which lacked inhibitor, to check for time dependent inactivation.
2-Deoxy-2-fluoro-α-maltosyl fluoride was tested as an inactivator of α-amylase by incubating a solution (60 μL) of 2-deoxy-2-fluoro-α-maltosyl fluoride (7.7 mM) and enzyme (4.2 μg) in 50 mM sodium phosphate buffer (pH 6.8) for 3 days at room temperature. Aliquots (10 μL) of the Imix were removed over the course of the experiment and added to a reaction mixture which contained α-maltosyl fluoride (13.3 mM) and 100 mM sodium phosphate buffer (pH 6.8). Initial reaction rates were compared with a control experiment, which lacked inhibitor, to check for time dependent inactivation.

The following inactivation tests were performed with either α-amylase or α-glucosidase (yeast). In these tests, inactivation mixtures were each set up containing one of several different inactivator concentrations in the appropriate buffer system, and incubated at room temperature. To each mixture was added the same amount of enzyme and the residual activity was measured at various time intervals. Small aliquots (typically 5 or 10 μL) were removed and added to the assay mixture which contained the substrate in a relatively large volume (0.25 or 1 mL) of the same buffer system. The residual α-amylase activity was measured by following the release of fluoride ion from a 2.1 mM α-maltosyl fluoride ($K_m = 4.3$ mM) solution. The residual α-glucosidase activity was measured spectrophotometrically by following the release of p-nitrophenol at 400 nm from a 1.0 mM p-nitrophenyl α-glucoside ($K_m = 0.1$ mM) solution (0.02% BSA). 22FαGTNP was tested as an irreversible inactivator of yeast α-glucosidase. 22FαG2TNP was tested as an irreversible inactivator of α-amylase.

b) Protection against inactivation

To protect against inactivation of yeast α-glucosidase (3.4 μg) by 22FαGTNP (1.6 mM), DNJ (45 μM) was added (total volume 110 μL, 0.1% BSA). Aliquots (5 μL) of this mixture over the time course of the experiment were added to an assay mixture. The results were plotted in the $\ln(A/A_0)$ vs. time graph and compared with inactivation
results obtained with no DNJ present. Different batches of α-glucosidase were used in
the protection experiment (with and without DNJ) and in the inactivation experiment
above. In the protection against inactivation of α-amylase (0.55 μg) by 22FoG2TNP
(5.6 mM), acarbose (64 μM) was added (total volume 90 μL). Residual activity was
followed as described earlier.

c) Reactivation of irreversibly inactivated enzymes

The inactivated enzyme α-amylase was placed in an Amicon® Centricon® 30000
Da molecular weight cutoff membrane to remove excess inactivator. The enzyme was
diluted into the appropriate buffer and the concentration of enzyme determined
spectrophotometrically. To the reactivation mixture, maltose (50 mM) was added to help
in the reactivation. Aliquots of the reactivation mixture were removed over a period of
time and added to the same reaction mixture used in the inactivation experiment and the
initial rates observed. A control experiment was also set up and treated in the same way
as the inactivated enzyme, but no inactivator was present.

d) Burst experiments

The burst experiment for glycogen debranching enzyme with 4-deoxy-α-
maltotriosyl fluoride was performed by following the release of fluoride ion using an
Orion ion selective fluoride ion electrode. To 4-deoxy-α-maltotriosyl fluoride (150 μL,
3.1 mM) in 100 mM sodium phosphate and 1 mM EDTA buffer, pH 6.0 at 30°C was
added glycogen debranching enzyme (100 μL, 28.3 mg/ml). Maltotriose (6 mg) was
added after the burst had been achieved to act as an appropriate acceptor. Purification of
products was performed using a Dextropak® HPLC column from Waters®.
Identification of products was achieved using a Kratos® matrix assisted laser desorption
ionization mass spectrometer. The spectrometer was calibrated using an external standard
maltotetraose.
The burst experiment for α-amylase with 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-4-O-(α-(1,4)-D-glucosyl)-α-D-arabinopyranoside was performed by following the release of trinitrophenol using a UV/VIS spectrophotometer. To 2,4,6-trinitrophenyl 2-deoxy-2,2-difluoro-4-O-(α-(1,4)-D-glucosyl)-α-D-arabinopyranoside (10 μL, 0.98 mM) in 20 mM sodium phosphate, 25 mM NaCl buffer, pH 6.9 at 25°C was added α-amylase (10 μL, 5.2 mg/ml). Extrapolation of the linear portion of the burst phase and steady state phase back to time zero determined the size of the burst.

The extinction coefficient of 2,4,6-trinitrophenol was determined by measuring the absorbance at 400 nm of a known concentration of sample in pH 6.9 buffer. The 2,4,6-trinitrophenol was recrystallized from CHCl3. The following four concentrations were used: 0.218 mM (A400 2.516); 0.150 mM (A400 1.725); 0.115 mM (A400 1.337); and 0.078 mM (A400 0.899). The extinction coefficients were averaged and the value for 2,4,6-trinitrophenol ε = 11.55 mM−1cm−1 was obtained.

e) Contamination experiments

Two experiments were performed to check for contaminants which could have been responsible for the inactivation of α-glucosidase (yeast) with 2FoGF and 22FoGTNP. In the first experiment (1) the concentration of 2FoGF was kept constant and the concentration of enzyme was varied. In the second experiment (2) the concentration of 22FoGTNP was varied and the concentration of enzyme was kept constant.

1) Yeast α-glucosidase + 2FoGF

Imixes (110 μL) were set up containing 2FoGF (10 mM), BSA (0.05%), and α-glucosidase (0.26 μM, 1.3 μM, 4.3 μM, and 17.1 μM) in sodium phosphate (50 mM) buffer (pH 6.8). Aliquots (10 μL) of appropriate dilutions were added to the reaction mixture (1 mL) containing αPNPG (1.0 mM) and BSA (0.05%) in buffer. The same
concentration of enzyme (0.26 mM) was added from each of the different Imixes by making the following dilutions before addition to the reaction mixture: 0.26 μM no dilution; 1.3 μM 5-fold (10 μL/50 μL); 4.3 μM 16-fold (10 μL/160 μL); and 17.1 μM 64-fold (10 μL/640 μL). Residual activity was monitored over time.

Aliquots were also assayed for total fluoride ion released. After 15.5 min a 25-fold dilution (10 μL/250 μL) of the 17.1 μM α-glucosidase Imix was used to determine that 3.5 mM 2FaGF was still present in the Imix. After 23.5 min a 25-fold dilution (10 μL/250 μL) of the 4.3 μM α-glucosidase Imix was used to determine that 6.5 mM 2FaGF was still present in the Imix.

2) Yeast α-glucosidase + 22FaGTNP

A solution (180 μL) of 22FaGTNP (0.93 mM) and α-glucosidase (49 μM) were incubated in buffer (50 mM phosphate, 0.005% BSA, pH 6.8) for 30 min at room temperature. The sample was spun through an Amicon® Centricon® 30000 Da molecular weight cutoff membrane. The supernatant was checked to see if it could inactivate fresh enzyme. An Imix (110 μL) was set up containing 22FaGTNP (2-fold dilution of supernatant), α-glucosidase (0.18 mg/ml), and BSA (0.005%) in buffer. Residual activity was checked and a $k_{\text{obs}}$ of 0.056 min$^{-1}$ was found.

5.2.6. Determination of product distribution and kinetic parameters by HPLC

a) HPLC instrument

HPLC analyses were performed on an instrument from Waters®, including the following instrumentation, Model 712 WISP® auto-sampler, Model 410 differential refractive index detector, a Novapak® C$_{18}$ Guard-Pak® precolumn, and an analytical Dextropak® column (100 x 8 mm; water as eluent). A SIM® interface module was used to allow the data to be collected on the computer using the Baseline® 810...
chromatography workstation. Chromatographs were exported from the workstation as ASCII data files to the computer program Grafit™ (Leatherbarrow, 1990) for printing.

b) Product distribution of the amylase catalyzed malto-oligosaccharide hydrolysis

To separate solutions (160 μL) of maltotetraose (0.5 mM), maltopentaose (0.5 mM), maltohexaose (0.5 mM), and maltoheptaose (0.5 mM) in 50 mM sodium phosphate buffer (pH 6.8) was added α-amylase (21, 0.84, 2.1, and 0.42 μg, respectively). The reaction mixture was injected onto the HPLC 1 min after enzyme was added, and the product distribution was determined. The assignment of peaks was achieved by comparing retention times with the standard malto-oligosaccharides, glucose to maltoheptaose. The amount of each product formed was determined by measuring the area under the peak as determined by the Baseline® 810 chromatography workstation. The peak areas were converted to concentrations by determining standard curves for each of the standards, maltose, maltotriose, and maltotetraose. The cleavage sites were also determined from the product distribution experiments.

The kinetic constants were determined by HPLC analysis using a Dextropak® column. Solutions of maltotriose to maltoheptaose, at five different concentrations, were incubated with α-amylase at 30°C in 20 mM sodium phosphate, 25 mM sodium chloride buffer (pH 6.9). Aliquots of a reaction mixture were removed at time intervals and boiled for 2 min (irreversibly denatures the enzyme) and then injected onto the HPLC for analysis. Initial rates were determined by monitoring the increase in concentration of a particular product over time. The kinetic constants, $K_m$ and $k_{cat}$, were thus determined as described above.

c) Determination of product stereochemistry by HPLC

To a solution (160 μL) of maltopentaose (0.52 mM) in 20 mM sodium phosphate, 25 mM sodium chloride buffer (pH 6.9) was added α-amylase (0.84 μg). After 2 min, an
aliquot (50 µL) of the reaction mixture was injected onto the HPLC Dextropak® column and the products analyzed. Simultaneously, another aliquot (100 µL) was boiled for 2 min, then 50 µL injected onto the HPLC Dextropak® column. The only difference between the two samples was that any products with just one anomer present in the first sample will have both present in the second sample.

5.2.7. Determination of the product stereochemistry by NMR

Both natural and unnatural (ie. glycosyl fluorides) substrates were utilized in the determination of the stereochemistry at the anomeric center in the reaction products formed by glycogen debranching enzyme, and α-amylase. All of the unnatural substrates tested were prepared in the laboratory and the syntheses described elsewhere. Maltoheptaose was purchased from Boehringer Manheim, and limit dextrin was provided by Dr. Madsen at the University of Alberta. The substrates were first exchanged two times in D2O, and then dissolved in 0.5 mL D2O-exchanged buffer (the same buffer used in the enzyme assay was used in this experiment). A 1H NMR spectrum was obtained of the substrate before enzyme was added. Enzyme exchanged in D2O buffer was then added to begin the reaction. Spectra were acquired at various time intervals to first see the increase in amount of one anomer and then followed long enough to see the formation of the other anomer due to mutarotation. Based on chemical shift and coupling constants it was possible to assign the stereochemistry of the reaction products.

5.3. Electrospray mass spectrometry

Electrospray protein mass spectrometry was carried out on a PE-Sciex API III triple quadrupole instrument (Sciex, Thornhill, Ontario) at the Biomedical Research Centre of the University of British Columbia.

Labeling of the glycosidases was achieved by incubating the enzyme with excess inactivator. The enzyme (final concentration 1-3 mg/mL) was digested by adding 50 mM
sodium phosphate buffer (pH 2), pepsin (1/10) and/or dithothreitol (2 mM) in pH 2 buffer. The mixture was incubated at room temperature until SDS-PAGE gel analysis confirmed that the enzyme was completely digested.

Analysis of the proteolytic digests was carried out using a Sciex electrospray mass spectrometer (ESMS). In each MS experiment the digest was loaded onto a C18 column (Reliasil, 1 x 150 mm), then eluted with a gradient of 0-60% solvent B over 20 minutes followed by 100% B over 2 minutes at a flow rate of 50 μL/min (solvent A: 0.05% trifluoroacetic acid (TFA), 2% acetonitrile in water; solvent B: 0.045% TFA, 80% acetonitrile in water).

a) Experiments with glycogen debranching enzyme

The single quadrupole mode (normal LC/MS) MS conditions: the quadrupole mass analyzer was scanned over a m/z range 300-2400 Da with a step size of 0.5 Da and a dwell time of 1 ms/step. The ion source voltage (ISV) was set at 5 kV and the orifice energy (OR) was 80 V. The neutral loss MS/MS spectra were obtained in the triple quadrupole neutral loss scan mode searching for the loss of m/z 235.5, corresponding to the loss of inhibitor label from a peptide in the doubly charged state. Thus, scan range = 300-1200 m/z; step size = 0.5 Da; dwell time = 1 ms/step; ISV = 5 kV; OR = 80 V; RE1 = 115; DM1 = 0.16; R1 = 0 V; R2 = -50 V; RE3 = 115; DM3 = 0.16; collision gas thickness (CGT) = 3.2-3.6 X 10^{14} molecules/cm^2. To maximize the sensitivity of neutral loss detection, normally the resolution (RE and DM) is compromised without generating artifact neutral loss peaks. The MS/MS daughter ion spectrum was obtained in the triple quadrupole daughter scan mode by selectively introducing the m/z 1081 (peptide 1) and 1329 (peptide 2) doubly charged peptides from Q1 into the collision cell (Q2) and observing the daughter ions in Q3. Thus, Q1 was locked on m/z 1081 or 1329; Q3 scan range 300-2400 m/z; step size = 1.0 Da; dwell time = 1 ms/step; ISV = 5 kV;
OR = 80 V; RE1 = 112; DM1 = 0.18; R1 = 0 V; R2 = -50V; RE3 = 112; DM3 = 0.18; CGT = 4.5 X 10^{14} molecules/cm^2.

For aminolysis of the labeled peptic digest (10 μL, 5 mg/ml), 2 μL of concentrated ammonium hydroxide was added. The mixture was incubated at 50°C for 15 min. After acidification with TFA, the mixture was analyzed by ESMS.

b) Experiments with yeast α-glucosidase

In the digests of α-glucosidase inactivated with 22FxαGTNP and 2C12FxαGCl the respective peaks at m/z 927 and 943 were obtained using the single quadrupole mode (normal LC/MS) MS conditions. The MS/MS daughter ion spectrum for the 2C12FxαGCl labeled sample was obtained in the triple quadrupole daughter scan mode by selectively introducing the m/z 943. The conditions were the same as the experiment with Glyx but the CGT = 5.5 X 10^{14} molecules/cm^2.
Appendix I: Supplementary Data

Figure 5.1. The Lineweaver-Burk plot for the hydrolysis of αGF. [Glyx] = 0.038 mg/mL

Figure 5.2. The Lineweaver-Burk plot for the cleavage of αG3F. [Glyx] = 0.37 mg/mL

Figure 5.3. The Lineweaver-Burk plot for the cleavage of αG3F. [Glyx] = 0.73 mg/mL
Figure 5.4. The Lineweaver-Burk plot for the hydrolysis of G3. [HPA] = 40 μg/mL

Figure 5.5. The Lineweaver-Burk plot for the hydrolysis of G4. [HPA] = 2.4 μg/mL

Figure 5.6. The Lineweaver-Burk plot for the hydrolysis of G5. [HPA] = 0.91 μg/mL

Figure 5.7. The Lineweaver-Burk plot for the hydrolysis of G6. [HPA] = 0.12 μg/mL

Figure 5.8. The Lineweaver-Burk plot for the hydrolysis of G7. [HPA] = 0.91 μg/mL
162

Figure 5.9. The Lineweaver-Burk plot for the cleavage of $\alpha$G2F. $[\text{HPA}] = 0.24 \, \mu\text{g/mL}$

Figure 5.10. The Lineweaver-Burk plot for the cleavage of $\alpha$G2F. $[\text{BHKHPA}] = 0.11 \, \mu\text{g/mL}$

Figure 5.11. The Lineweaver-Burk plot for the hydrolysis of 4D$\alpha$G2F. $[\text{BHKHPA}] = 0.17 \, \mu\text{g/mL}$

Figure 5.12. The Lineweaver-Burk plot for the cleavage of $\alpha$G3F. $[\text{BHKHPA}] = 0.11 \, \mu\text{g/mL}$

Figure 5.13. The Lineweaver-Burk plot for the hydrolysis of 4D$\alpha$G3F. $[\text{BHKHPA}] = 0.11 \, \mu\text{g/mL}$
Figure 5.14. The Lineweaver-Burk plot for the hydrolysis of αPNPG. [Glyx] = 82 μg/mL

Figure 5.15. The Lineweaver-Burk plot for the cleavage of 2FaGF. [yeast α-glucosidase] = 15 μg/mL

A sample of the raw data ([2FaGF] = 3.5 mM) is shown in Figure 4.1
Appendix II: Theory

A-II.1. Enzyme catalysis

Michaelis & Menten (1913) developed a theory for enzyme kinetics. Briggs & Haldane (1925) later generalized this theory further by introducing the idea of steady-state. The general expression for an enzymic reaction is shown as follows:

\[
\begin{align*}
E + S & \xrightleftharpoons[k_{-1}]{k_1} ES \\
& \xrightarrow{k_2} E + P
\end{align*}
\]

The free enzyme (E) combines with the substrate to form an enzyme-substrate complex (ES), which is then turned over to form free enzyme and product (P).

Under steady-state conditions:

\[
d\frac{[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] = 0 \quad (1)
\]

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

a) The concentration of the substrate is much greater than that of the enzyme, thus the formation of the enzyme-substrate complex essentially does not lower the concentration of substrate.

b) Since the initial rate is determined, only the forward reaction is considered as the [P] is very low.

The total concentration of enzyme, \([E]_0\), is the sum of the free enzyme plus the enzyme-substrate complexes.

\[
[E]_0 = [E] + [ES] \quad (3)
\]

Combining Equations 2 and 3, one obtains:

\[
[ES] = \frac{[E]_0 [S]}{[S] + (k_{-1} + k_2)/k_1} \quad (4)
\]
c) Assuming the formation of products is the rate-limiting step ($k_2$).

The initial velocity ($v$) is thus equal to the rate of the forward reaction.

$$v = \frac{d [P]}{dt} = k_2 \left[ ES \right]$$  \hspace{1cm} (5)

By substituting Equation 4 into 5, one obtains:

$$v = \frac{k_2 \left[ E \right]_0 \left[ S \right]}{\left[ S \right] + \left( k_{-1} + k_2 \right) / k_1}$$  \hspace{1cm} (6)

where $k_2 = k_{\text{cat}}$ (turnover number)

and $K_m = \left( k_{-1} + k_2 \right) / k_1$

When $k_2 << k_{-1}$ then $K_m = k_{-1} / k_1 = K_S$ (dissociation constant)

The equation is now in the form of the Michaelis-Menten equation:

$$v = \frac{k_{\text{cat}} \left[ E \right]_0 \left[ S \right]}{K_m + \left[ S \right]}$$  \hspace{1cm} (7)

At low substrate concentrations, where $[S] << K_m$, the initial reaction rate is proportional to the substrate concentration and the slope is the specificity constant ($k_{\text{cat}}/K_m$):

$$v = \frac{k_{\text{cat}} \left[ E \right]_0 \left[ S \right]}{K_m}$$  \hspace{1cm} (8)

At high concentrations of substrate, where $[S] >> K_m$, the initial reaction rate becomes independent of substrate concentration:

$$v = \frac{k_{\text{cat}} \left[ E \right]_0}{2} = \frac{V_{\text{max}}}{2}$$  \hspace{1cm} (9)

When the substrate concentration is equal to $K_m$, $[S] = K_m$, the initial rate of reaction is equal to one-half the maximum rate ($V_{\text{max}}$):

$$v = \frac{k_{\text{cat}} \left[ E \right]_0}{2} = \frac{V_{\text{max}}}{2}$$  \hspace{1cm} (10)

The Michaelis-Menten equation is often transformed into the linear, double-reciprocal plot (Lineweaver & Burk, 1934) of $1/v$ vs. $1/[S]$ as follows:

$$\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}} \left[ S \right]}$$
Deviations in the Michaelis-Menten equation are often better observed in this plot and is only used for visual purposes as the kinetic parameters are calculated from the original equation using non-linear regression analysis by the computer program Grafit™ (Leatherbarrow, 1990).

A-II.2. Irreversible inhibition of enzyme catalysis

The inactivation of a glycosidase can be expressed as follows:

\[ E + I \rightleftharpoons EI \rightarrow E-I \]

The first step involves a reversible binding (\( K_i \)) of the inactivator (I) and the free enzyme (E). The second step which is rate limiting, involves an irreversible, bond forming step (\( k_i \)) that forms an inactivated-enzyme intermediate (E—I). If the concentration of inactivator is much greater than the enzyme concentration ([I] >> [E]), then the amount of inactivator is essentially constant over the course of the reaction, and the kinetics are pseudo first-order with respect to the enzyme concentration. The inactivation equation can then be written in the Michaelis-Menten form as:

\[
v = k_i [E]_0 [I] / (K_i + [I]) \tag{11}
\]

where: \( v \) = rate of inactivation

\( k_i \) = rate constant of inactivation

\( K_i \) = the apparent dissociation constant for all species of enzyme bound inactivator

\[
K_i = [E][I]/[EI] \tag{12}
\]

If [I] is constant, Equation (11) becomes:

\[
v = k_{obs} [E]_t \tag{13}
\]

where:

\[
k_{obs} = k_i [I] / (K_i + [I]) \tag{14}
\]

where: \( k_{obs} \) = the pseudo first-order rate constant of inactivation at one [I]
The \( k_{\text{obs}} \) values, at the different \([I]\), were calculated for each time-dependent, first-order decay in residual activity by fitting the initial rates to Equation 15, using the computer program Grafit\textsuperscript{TM} (Leatherbarrow, 1990).

\[
[E] = [E]_0 e^{-\left(k_{\text{obs}} t\right)}
\]  

(15)

where: \([E]_0\) = the initial enzyme concentration

\([E]\) = the active enzyme concentration

The pseudo first-order rate constant obtained at each concentration of inactivator was fitted to Equation 14 using the computer program Grafit\textsuperscript{TM} (Leatherbarrow, 1990) to calculate the dissociation constant, \( K_i \), and the inactivation rate constant, \( k_i \). As with the Lineweaver-Burk plot this double reciprocal plot was presented for visual purposes only.
A-II.3. Protection against inactivation: calculations

The rate equation for competitive inhibition is as follows:

\[ v = \frac{V_m [S]}{K_m \left(1 + \frac{[\text{Inh}]}{K_i}\right) + [S]} \]  (16)

Equation 16 can be rewritten as:

\[ \frac{1}{v} = \frac{K_m [\text{Inh}]}{V_m K_i [S]} + \frac{1}{V_m} \left(1 + \frac{K_m}{[S]}\right) \]

where:  
- Inh = inhibitor
- \( K_i \) = the apparent dissociation constant for all species of enzyme-bound inhibitor

By substituting \( k_{\text{obs}} \) for \( v \), \( k_i \) for \( V_m \), \( I \) for \( S \), and \( K_i \) for \( K_m \), Equation 16 becomes:

\[ \frac{1}{k_{\text{obs}}} = \frac{K_i [\text{Inh}]}{k_i K_i [I]} + \frac{1}{k_i} \left(1 + \frac{K_i}{[I]}\right) \]  (17)

where:  
- \( I \) = inactivator
- \( k_i \) = rate constant of inactivation
- \( K_i \) = the apparent dissociation constant for all species of enzyme-bound inactivator

\( k_i \) was estimated from the slope of \( 1/k_{\text{obs}} \) vs. \( 1/[I] \)
REFERENCES


