SYNTHESIS AND ENZYMATIC EVALUATION OF ALPHA-GALACTOSIDASE INHIBITORS

by

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ABSTRACT

The compounds containing a structural motif of a cyclopropyl ring fused onto a carba-galactopyranose skeleton were synthesized and tested as potential reversible and irreversible α-galactosidase inhibitors.

(1*R*,2*S*,3*S*,4*S*,5*S*,6*S*)-5-amino-1-(hydroxymethyl)bicyclo[4.1.0]heptan-2,3,4-triol and (1*S*,2*S*,3*S*,4*S*,5*S*,6*R*)-5-amino-1-(hydroxymethyl)bicyclo[4.1.0]heptan-2,3,4-triol were synthesized from methyl α-D-galactopyranoside. A kinetic study showed the later compound is not a inhibitor. However the former is a potent reversible inhibitor of coffee bean α-galactosidase with a K_i value of 541 nM.

(1*R*,2*S*,3*S*,4*R*,5*S*,6*S*)-5-(3,5-difluorophenoxy)-1-(hydroxymethyl) bicyclo[4.1.0]heptan-2,3,4-triol and (1*S*,2*S*,3*S*,4*R*,5*S*,6*R*)-5-(3,5-difluorophenoxy)-1-(hydroxymethyl)bicyclo[4.1.0]heptan-2,3,4-triol were synthesized as irreversible inhibitors of α-galactosidases. Analysis of inhibitory kinetic data illustrated that the former compound is an irreversible inhibitor for coffee bean α-galactosidase with inactivation rate constant (k_{inact}) of 5.6×10^{-4} s^{-1} and has a dissociation constant (K_i) of 4.1 mM. Furthermore, using the competitive inhibitor, (1*R*,2*S*,3*S*,4*S*,5*S*,6*S*)-5-amino-1-(hydroxymethyl)bicyclo[4.1.0]heptan-2,3,4-triol, to protect the enzyme from inactivation confirmed that inactivation of the α-galactosidase enzyme by (1*R*,2*S*,3*S*,4*R*,5*S*,6*S*)-5-(3,5-difluorophenoxy)-1-(hydroxymethyl)bicyclo[4.1.0]heptan-2,3,4-triol occurred in the active site.

Keywords: Reversible inhibitor; Irreversible inhibitor; Alpha-galactosidase

Subject Terms: Glycosidases-inhibitors; Organic-synthesis

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δ	chemical shift
λ	wavelength
A	alanine
Ac	acetyl
Ac_2O	acetic anhydride
AcOH	acetic acid
Anal. Calcd.	analytical calculated
Ar	aromatic
Asp	aspartic acid
B	boat
Bn	benzyl
br	broad
***n*-BuLi**	*n*-butyllithium
C	chair
CE	carbohydrate esterases
Cex	A mixed-function exo-β-1.4-glucanase and β-1.4-xylanase from *Cellulomonas fimi*
COSY	COrrelation SpectroscopY
$Cu(acac)_2$	copper(II) acetylacetonate
$Cu(OTf)_2$	copper(II) trifluoromethanesulfonate
d	doublet
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
dd	doublet doublet
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DNJ	1-deoxynojirimycin
dt	doublet triplet

E	glutamic acid
ER	endoplasmic reticulum
Et_2O	diethyl ether
EtOH	ethanol
Et_3N	triethylamine
EtOAc	ethyl acetate
EtOH	ethanol
GAC	general-acid catalyst
GBC	general-base catalyst
GH	glycosyl hydrolases
GT	glycosyl transferases
HMQC	heteronuclear multiple quantum coherence
IC_{50}	concentration of inhibitor required to reduce binding by 50%
IR	fourier transform infrared
J	coupling constant
K_i	dissociation constant
k_{inact}	rate constant of enzymatic inactivation.
K_M	Michaelis constant
LSD	lysosomal storage disorder
m	multiplet
Man	mannose
Me	methyl
MeOH	methanol
mp	melting point
$NAD(P)^+$	nicotinamide adenine dinucleotide phosphate
***n*-BuLi**	*n*-butyllithium
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect spectroscopy
PDB	protein data bank
$Pd(OAc)_2$	palladium(II) acetate
Ph	phenyl
PL	polysaccharide lyases

PNPG	*p*-nitrophenyl α-D-galactopyranoside
PPh_3	triphenylphosphine
ppm	parts per million
R^2	root mean square
$Rh_2(OAc)_4$	rhodium(II) acetate dimer
$Rh_2(Cap)_4$	dirhodium(II) tetracaprolactamate
s	singlet
t	triplet
***t*-BuLi**	*tert*-butyllithium
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
TrCl	chlorotriphenylmethane
Trp	tryptophan
Try	tryrosine
TSs	transition state
UV	ultraviolet
W	tryptophan
$ZnMe_2$	dimethylzinc

1 Introduction

1.1 General Background of Carbohydrates

Whenever carbohydrates are mentioned, people immediately think of them as a source of energy. Certainly, plants and photosynthesizing bacteria provide the link between solar energy, which arrives as photons, and biochemical energy, which is often stored as polysaccharides. Approximately 3.4×10^{14} kg of carbohydrates are synthesized on the earth every year and it is estimated that carbohydrates make up about 50-60% of the calories consumed by humans.[1] In addition, it has been known for many years that carbohydrates are important structural materials in many biological systems. For example, cellulose is the main structural component of plant cell walls and the exoskeletons of insects and crustaceans are comprised of chitin. It has been stated that: "*More than 50% of the dry weight of the biomass – all plants and animals – consists of glucose polymers.*"[2]

More recently, it has been realized that carbohydrates have many other important biological functions. Mono-, oligo- or polysaccharides are attached to proteins and lipids to form glycoproteins and glycolipids, which are often referred to as glycoconjugates. There are two types of glycoproteins: That is, *N*-linked and *O*-linked glycoproteins. In an *N*-linked glycoprotein, the sugar chain is linked to the side chain nitrogen atom of an asparagine residue. In an *O*-linked glycoprotein, the oligosaccharide chain is bound to the hydroxyl group of a serine or threonine residue. *N*-linked glycoproteins are generally made in the following manner: after the protein has been

translated by the ribosome, it is transported to the endoplasmic reticulum (ER), where a dolichol-linked high mannose containing oligosaccharide precursor is transferred to the appropriate asparagine residue of the protein by a glycosyl transferase. The saccharide chains are trimmed and processed by an assembly of glycosidases and glycosyl transferases. Then vesicles transport the glycoprotein to the Golgi apparatus where further modifications of the saccharide chain take place. Finally, the glycoprotein is sent to its intended destination such as the plasma membrane.

As a hexose monosaccharide residue has four different hydroxyl groups that can be linked to other sugars, and since it can form either α- or β- stereoisomeric linkages, the sugar moieties of glycoconjugates offer an almost unlimited number of structural possibilities. As biomarkers on the cell surface, glycoconjugates play crucial roles in cell-adhesion, cellular differentiation, signal transduction, immunological recognition, and virus invasion.[3]

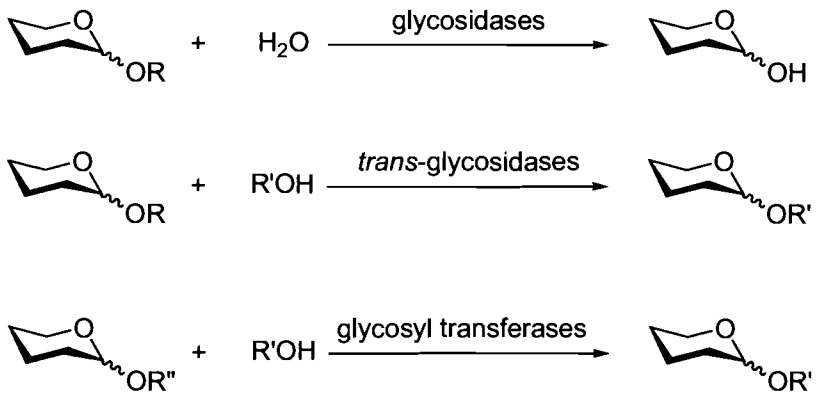
What biological functions the sugar chains in glycoconjugates perform, and how these functions are manifested is beyond the scope of this thesis. However, they are the foci of the new scientific field of glycobiology.

1.2 α-Galactosidases

1.2.1 Classification of Glycosyl Transferring Enzymes

Glycosyl transferring enzymes are divided into glycosyl transferases, *trans*-glycosidases and glycosidases (Figure 1.1).[4] Glycosyl transferases and *trans*-glycosidases catalyze the formation of glycosidic bonds. Glycosidases, on the other hand, generally hydrolyze glycosidic linkages. Glycosyl transferring enzymes control the processes of

synthesis, degradation and modification of the sugar components of glycoconjugates. This thesis focuses on α-galactosidases, a family of *O*-glycosidases.



R = a carbohydrate derivative, R'OH = another sugar

R" = a nucleoside diphosphate, a lipid phosphate or phosphate

Figure 1.1 General reactions catalyzed by glycosidases, *trans*-glycosidases and glycosyltransferases.

The International Union of Biochemistry and Molecular Biology nomenclature system classifies *O*-glycosidases with the EC numbers 3.2.1.x, where x specifies the natural substrate linkage. The problem with this kind of nomenclature is the lack of a direct relationship between EC number, enzyme structure, and enzyme mechanism. This means: (1) enzymes which have unrelated structural characteristics but the same substrate specificities have the same EC numbers, (2) enzymes employing a common mechanism do not always belong to the same family, and (3) one enzyme may have several EC numbers if it displays a variety of substrate specificities.[5,6]

In order to unify the relationship between amino acid sequence, global structure, substrate specificity and reaction mechanism, a new classification system of glycosidase

enzymes was introduce in 1991.[5] Details of this classification are available on the world wide web (see www.cazy.org).[6] This classification system has been used on glycosyl hydrolases (GH), glycosyl transferases (GT), polysaccharide lyases (PL), and carbohydrate esterases (CE).[7] The rationale behind this type of classification is that each member in a family has a similar protein sequence, so that the tertiary structure, active site residues and the catalytic mechanism of the enzymes are homologous.[8]

1.2.2 Galactosidase Mechanisms

Glycosidases, based on the anomic stereochemistry of substrate and product, have been classified as either inverting or retaining enzymes. Most of these glycosidases function by way of either a double displacement mechanism or a single displacement as originally proposed by Koshland in 1953 (Figure 1.2 and Figure 1.5).[9] Several review articles have been published concerning the mechanism of hydrolysis used by glycosidases.[10-14] Nevertheless, fundamentally different mechanisms of action have been elucidated. For example, family 4 glycosidases, which requires a $NAD(P)^+$ cofactor, operate via a mechanism involving the following steps: oxidation, elimination, addition, and reduction.[15,16] Some *N*-acetyl-β-hexosaminidases use the neighbouring *N*-acetyl group as a nucleophile to attack the anomeric carbon instead of an active site carboxylate residue.[17,18] In addition, a tyrosine residue is the catalytic nucleophile in sialidases and *trans*-sialidases.[19,20]

The enzymes used in this thesis belong to families GH-27 and GH-36. These enzymes utilize a double displacement mechanism. Therefore, only the classic and widely accepted mechanisms that were suggested by Koshland are discussed in detail in this thesis.

Retaining Glycosidases

For retaining glycosidases that employ a double displacement mechanism, one of the two catalytic carboxylic acids (generally aspartate or glutamate) acts as a general-acid catalyst (GAC) to assist departure of the aglycone, while the other nucleophilically attacks the anomeric centre (Figure 1.2). As a result, a covalent glycosyl-enzyme intermediate is formed. Next, the first carboxylate acts as a general-base catalyst (GBC) to deprotonate a nucleophilic water molecule that attacks the glycosyl-enzyme intermediate. It has been noted that the distance between these two key catalytic residues is around 5 Å.[4,11]

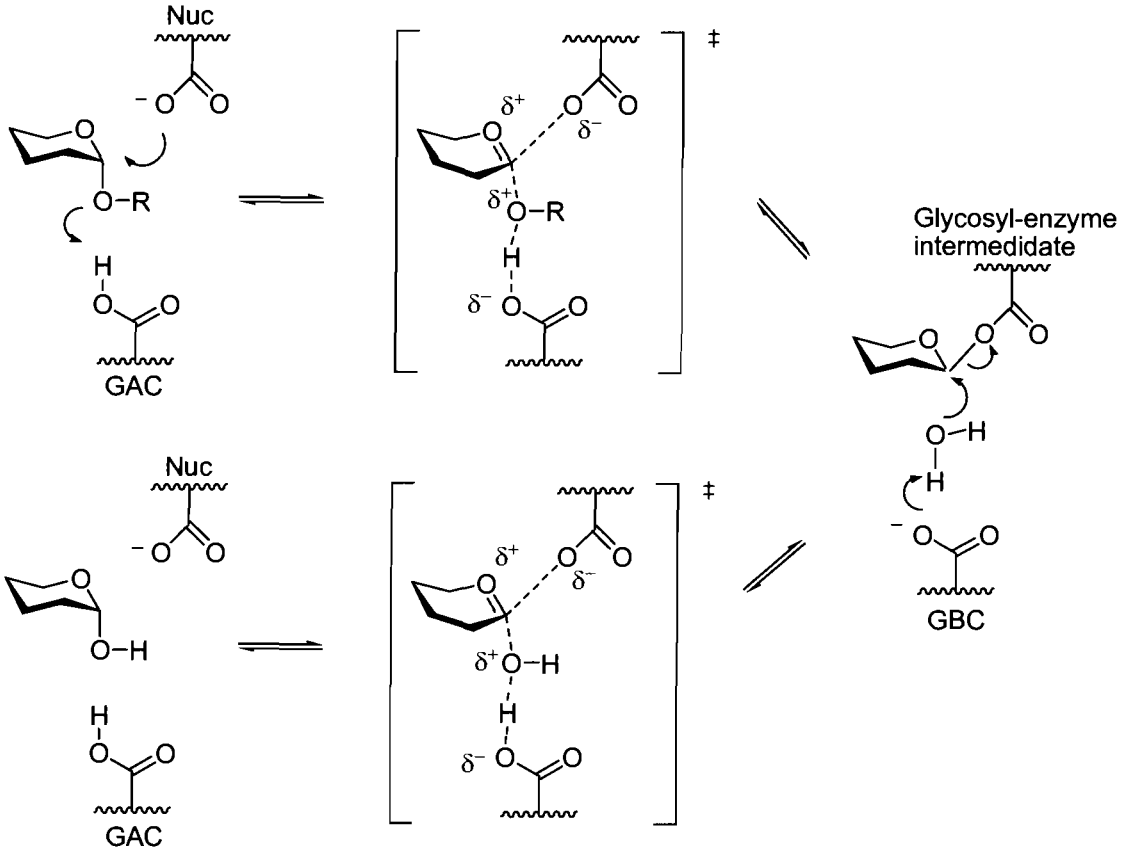


Figure 1.2 Mechanism of retaining glycosidases

Both glycosylation and deglycosylation have similar oxacarbenium ion-like transition states (Figure 1.2). In the transition state, lone pair from the endocyclic oxygen atom helps to stabilize the partial positive charge development. Other transition state characteristics include: a largely broken glycosidic bond, a partial bond between the nucleophile and the anomeric carbon atom, as well as a distorted pyranose ring so that charge delocalisation can occur onto the endocyclic oxygen atom.[21]

Withers *et al.* reported the first trapping of a glycosyl-enzyme intermediate by the use of 2-deoxy-2-fluoro-β-D-glucopyranosyl fluoride to inactivate *Alcaligenes faecalis* β-glucosidase.[22] The electron withdrawing fluorine atom attached to *C*-2 inductively destabilizes the partial positively charged oxacarbenium ion-like transition state. Furthermore, using fluorine substituent instead of the 2-OH group removes an important hydrogen bond interaction in the enzymatic transition state. These two factors result in the accumulation of a covalent intermediate, which was confirmed by electrospray ionization mass spectrometry and ^{19}F-nuclear magnetic resonance spectroscopy (NMR).[22]

The first X-ray crystallographic study that showed a covalent intermediate and its conformation was performed using the 2-deoxy-2-fluorocellobiosyl-enzyme intermediate of exo-xylanase/glucanase Cex from *Cellulomonas fim.*[23] Specifically, the mechanism based irreversible inhibitor 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-cellobioside was diffused into a crystal of Cex. The crystal structure confirms the formation of a covalent bond between the anomeric carbon and a conserved glutamate residue.[23]

Three-dimensional structure studies on mannanase 26A from *Pseudomonas cellulose* by Davies and co-workers provides "snapshots" of this enzyme at both the Michaelis complex and the covalent glycosyl-enzyme intermediate states.[24] The crystal structure of the general acid and base mutant mannanase 26A (E212A), which was incubated with 2,4-dinitrophenyl 2-deoxy-2-fluoro-β-D-mannotrioside (**1.1**) (Figure 1.3), reveals that the Michaelis complex of this substrate analogue is in a $^{1}S_{5}$ skew boat conformation. The E212A mutant mannanase 26A was then inactivated by compound **1.1** and the 3D structure shows that the covalent intermediate is trapped in a $^{O}S_{2}$ skew boat conformation.

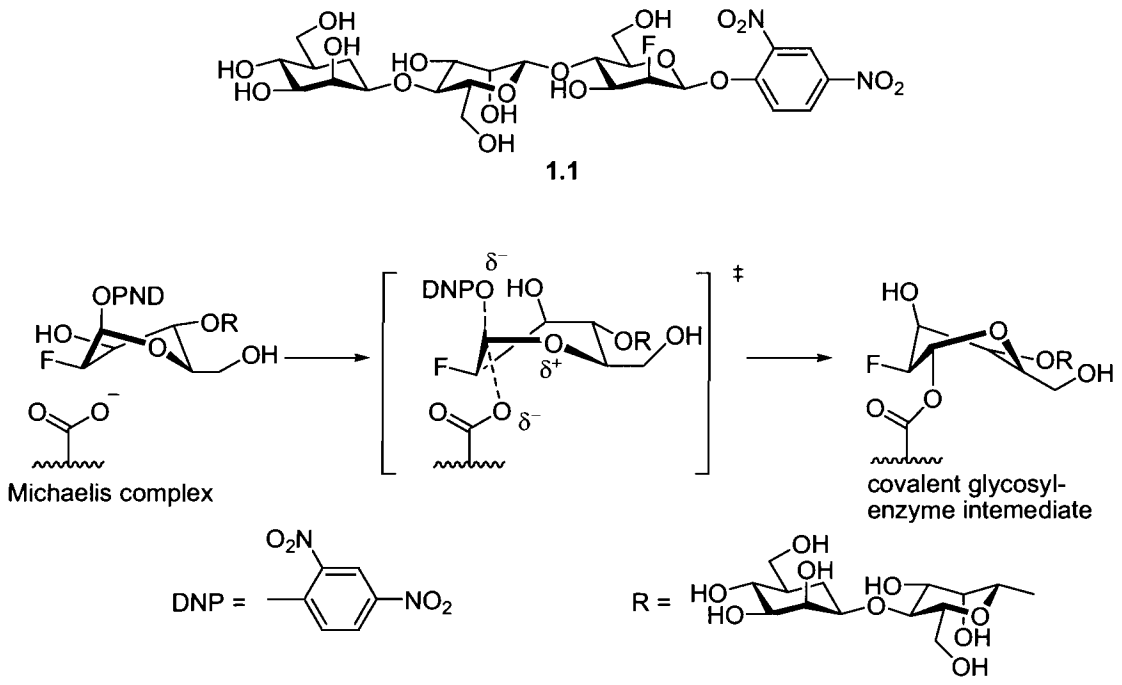


Figure 1.3 Structure of substrate **1.1** and its conformational change during the glycosylation process

This implies a ${}^{1}S_5 \rightarrow B_{2,5} \rightarrow {}^{O}S_2$ conformational itinerary for the glycosylation step (Figure 1.4).[25] Thus, Davies *et al.* suggested that mannanase 26A catalyzes hydrolysis reaction via a $B_{2,5}$ transition state.[24]

The possible configurations of glycopyranosylium ions are shown in boxes (Figure 1.4). The configuration of the two reversible inhibitors (**1.80** and **2.5**) is shown in the dash box (Figure 1.4).

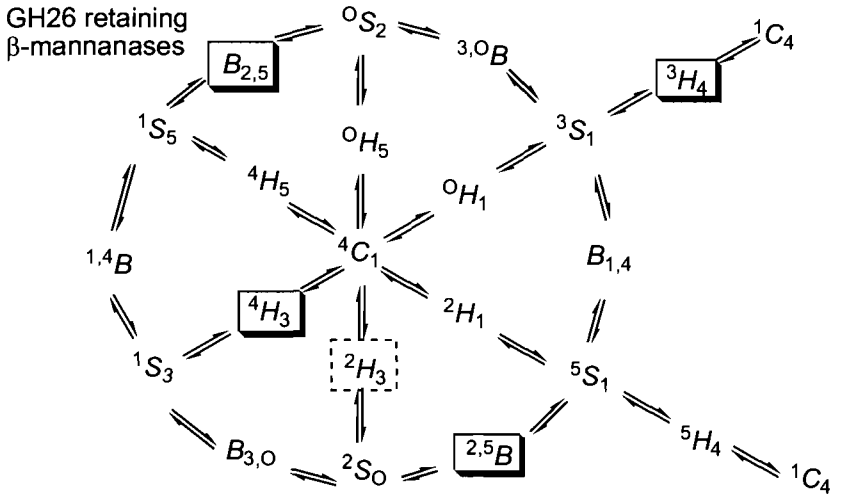


Figure 1.4 Partial map of interconversions between various pyranoside ring conformations, that indicates the mannanase 26A glycosylation step pathway

Inverting Glycosidases

Inverting glycosidases operate via a single displacement mechanism involving two active site carboxylic acid residues (generally aspartate or glutamate), that are highly conserved (Figure 1.5).[26] One residue acts as a GAC to protonate the aglycone moiety. The other carboxylate residue acts as a GBC to deprotonate an incoming water molecule as the exocyclic C-O bond is breaking. The water nucleophilically attacks the

anomeric centre from the opposite face to that occupied by the aglycone moiety. As a result, the hydrolysis product has an inverted anomeric configuration. It has been noted, that in this case, the distance between two residues are generally 7-11 Å which is bigger than in the case of retaining glycosidases.[4,11] Thus, both the water molecule and the substrate can fit between these two active site residues.

The transition state for an inverting glycosidase has similar characteristics to those for a retaining glycosidase, which are described in the section of retaining glycosidases.

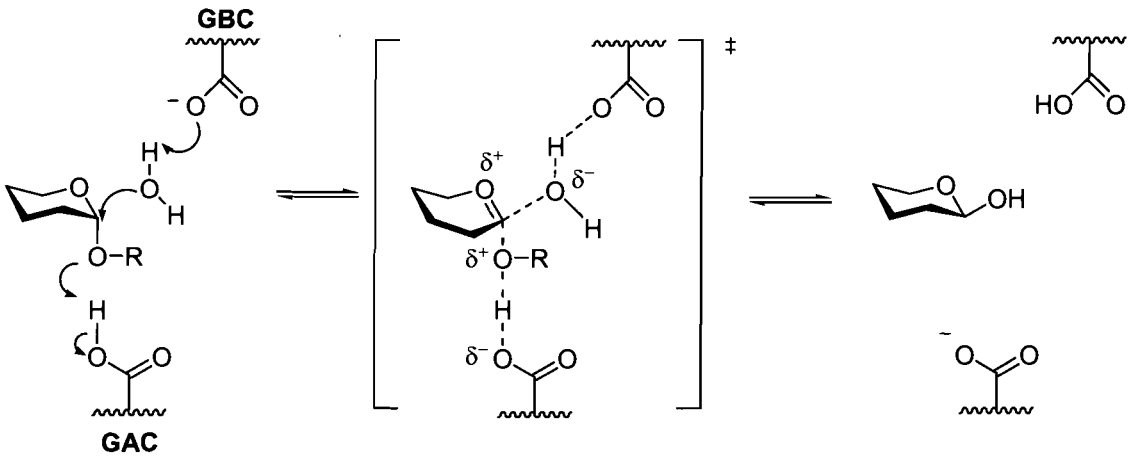


Figure 1.5 Mechanism of inverting glycosidases

1.2.3 α-Galactosidase Structures

As a result of the power of modern genome sequencing and bioinformatics, over 110 GH families have been classified. Moreover, modern X-ray single crystal diffraction methods have allowed representative members from over 60 of these families to have their 3D crystal structures solved.[7] α-Galactosidases (EC. 3.2.1.22) catalyze the cleavage of the glycosidic bond between an α-*O*-galactopyranosyl residue

and a saccharide chain. α-Galactosidases have been classified into four retaining glycosyl hydrolase families (GH-4, GH-27, GH-36 and GH-57) and one inverting family (GH-110). It had been noticed that α-galactosidases from eukaryotic organisms are mainly grouped into GH-27, while α-galactosidases from other microbial sources are generally found in one of GH-4, GH-36 or GH 57.[27]

The α-galactosidases utilized in this thesis are from *Coffea arabica* (coffee beans GH-27) and from *Escherichia coli* (*E. coli* GH-36). GH-27 and GH-36 families are thought to have the same ancestral gene.[8] In addition, the enzymes in these two families act via a double displacement mechanism.[28-30]

Although the structure of the coffee bean α-galactosidases has not yet been solved, the structures for several α-galactosidases have been reported from family 27: *Homo sapiens* (human),[28] fungus *Hypocrea jecorina* (*Trichoderma reesei*),[29] and *Oryza sativa* (rice).[30] These three α-galactosidases all have an N-terminal domain as the catalytic core and a C-terminal domain. The N-terminal domain consists of an $(\alpha/\beta)_8$ barrel basket, and it possesses several conserved residues which are involved in substrate binding. Eight antiparallel β strands make up the C-terminal domain whose size, sequence and fold are variable. It is likely that the C-domain modulates substrate specificity (Figure 1.6).[30]

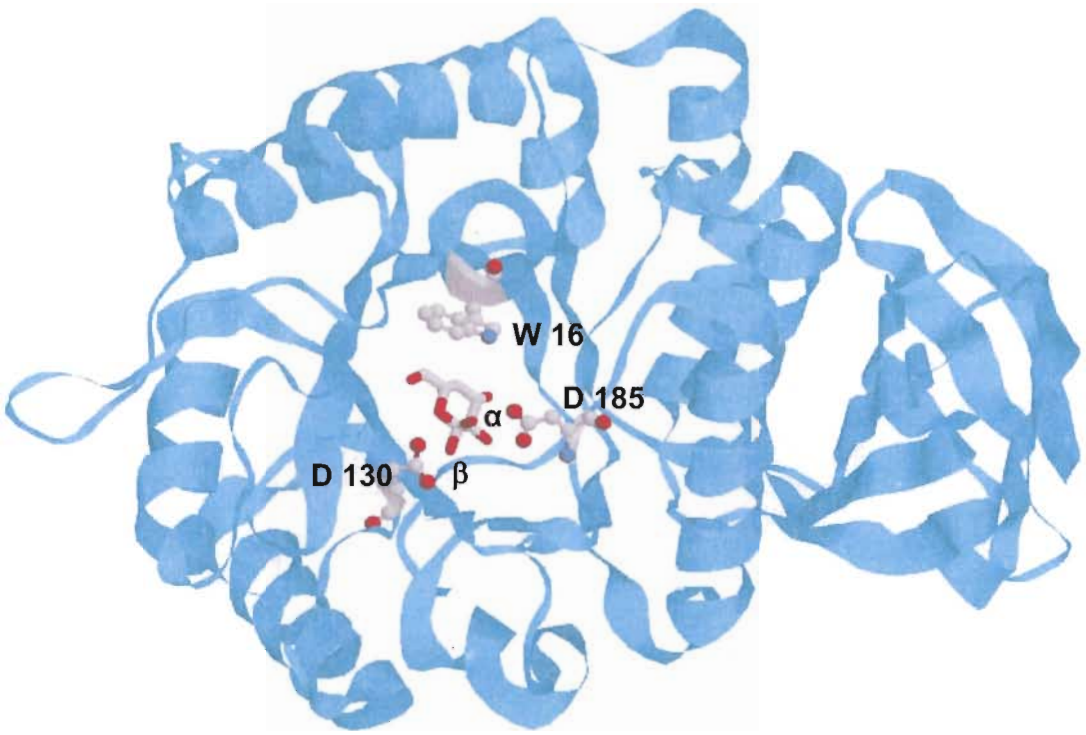


Figure 1.6 Crystal structure of α-galactosidase from rice

In the crystal structure of rice α-galactosidase and its complex with α- and β-galactose [PDB 1UAS],[30] two conserved aspartic acid residues are found in the catalytic pocket. Asp-185, which has been assigned as the general base/acid catalyst, is positioned within hydrogen bonding distance of the α-anomeric *O*-1 of the bound galactose. Asp-130, which has been identified as the nucleophile, is located near the anomeric carbon of the bound galactose at a distance of 2.5 Å. The distance of the oxygen to oxygen in these two carboxylic acids is 6.7 ± 0.3 Å. In addition, Trp-16 creates a hydrophobic interaction with the hydrogen atoms located on *C*-4, *C*-5 and *C*-6

of the pyranose ring (Figure 1.6). Analysis of the crystal structure of the α-galactosidase–galactose complex reveals that the positions of the catalytic residues is consistent with known enzymatic reaction mechanisms.

In the same situation, the crystal structure of the α-galactosidase from *E. coli* is unknown, but the tertiary structure of the α-galactosidase from *Thermotoga maritime* (*Tm*Gal), a GH-36 family member, has been recently reported [PDB 1ZY9].[27] This enzyme has three domains: An N-terminal domain, a catalytic domain and a C-terminal domain. The N-terminal domain is composed of a β-supersandwich motif whose function is to contribute a key substrate-binding residue. The catalytic domain consists of an $(\alpha/\beta)_8$ barrel basket and the C-terminal domain is made up of antiparallel β-strands whose function is unknown.

Like rice α-galactosidase, two conserved aspartate residues are located in the active site, Asp 327 and Asp 387, which have been identified as the nucleophilic and the acid/base residues, respectively. The distance of the oxygen to oxygen in these two carboxylic acids is $6.8 \pm 0.4_5$ Å, which is longer than the average of 5.5 Å for retaining glycosidases, but it is in the same range as that calculated for the rice α-galactosidase. This crystal structure provides a basis for mechanistic analysis of this retaining glycosidase.

In summary, both GH-27 and GH-36 have a common α/β barrel domain as the catalytic core; both contain two catalytic aspartic acid residues; and both operate via a double displacement retaining mechanism.

1.3 Function of α-Galactosidase Inhibitors

Glycosidases regulate the catabolism of glycosphingolipids in the lysosome. A deficiency of these hydrolases causes an accumulation of incompletely degraded carbohydrates in the lysosomes and this results in a lysosomal storage disorder (LSD) which damages the brain, liver and lungs as well as other internal organs.[31,32]

Currently, enzyme-replacement therapy is one method used to treat LSD, but it is costly. The method of substrate-reduction therapy is used to reduce the synthesis of glycosphingolipids that are involved in other important biological processes. A concern of this therapeutic method is reduction of glycosphingolipid levels may have serious consequences in many biological processes.[33]

The deficiency of α-galactosidase A causes Fabry's disease, a malady that effects the heart and kidneys. Misfolded α-galactosidases are degraded in ER. However, if they could be trafficked to the lysosome, they might still have enzymatic function. So a new remedy for Fabry's disease is to use a low concentration of α-galactosidase inhibitors as chemical chaperones to induce or to stabilize the correct conformation of these enzymes in the ER.[34,35] After this inhibitor-enzyme complex has been transported to the lysosome, it dissociates and the activity of this α-galactosidase in the lysosome increases.[34,35] Chemical chaperon therapy is also being developed to treat other LSD.[36-38]

It was reported that oral administration of 1-deoxy-*galacto*-nojirimycin (**1.35**, see Table 1.7) caused an increase of α-galactosidase A activity in the heart, kidneys and spleen of mice, and that no toxic effects were observed after 140 days.[39]

Several glycosidases are responsible for the digestion of the carbohydrates that are present in food. These enzymes sometimes cause social issues in humans. For example, a recombinant α-galactosidase is used as a treatment to mitigate the unwanted side effects of eating food, such as beans, onions and broccoli.[40,41] That is, these foods contain galacto-oligosaccharides, e.g., raffinose, stachyose and verbascose, which can not be digested by humans because we do not produce an α-galactosidase in our digestive tract. Thus, these complex carbohydrates accumulate in the large intestine, where yeast and bacteria ferment them anaerobically to give methane which leads to flatulence.[42] An α-galactosidase product known as Beano[43,44] is commercially available. Alternatively, α-galactosidase inhibitors could be used to reduce the enzymatic activity of these anaerobic microorganisms to avoid excessive gas production.

1.4 Irreversible Galactosidase Inhibitors

Irreversible inhibitors are generally designed as substrate analogues that bind to the active sites of enzymes. Most irreversible inhibitors alkylate one of the catalytic carboxylate groups of the enzyme. In this way, the enzyme is trapped in an inactive form. Thus, irreversible inhibition experiments can help to reveal details about the catalytic mechanism of the enzymatic reaction.

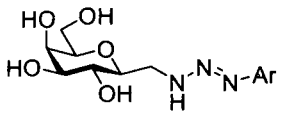
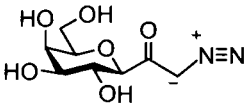
A model of inactivation by an irreversible inhibitor is shown below (Figure 1.7). Here, I is an irreversible inhibitor, EI is a non-covalent enzyme-inhibitor complex, EI_{inact}^{*} is a covalent enzyme-inhibitor complex, K_i is the dissociation constant for the non-covalent enzyme-inhibitor complex and k_{inact} is the rate constant of enzymatic inactivation.

Figure 1.7 Kinetic model for glycosidase inhibition by an irreversible inhibitor

1.4.1 Carbocation-based Irreversible Inhibitors: Galactosylmethyltriazenes 1.2 and Diazomethyl β-D-Galactopyranosyl Ketone (1.3)

Sinnott and co-workers exploited β-D-galactopyranosylmethyl-(*p*-nitrophenyl) triazene (**1.2**) as an irreversible inhibitor to inactivate α- and β-galactosidases.[45] This compound has a very weak effect on coffee bean α-galactosidase, but it inactivates β-galactosidases of *E. coli* (*lacZ*) and human liver lysosomes efficiently (Table 1.1).[45]

Table 1.1 Inhibition data of galactosylmethyltriazene **1.2** and diazoketone **1.3**

inhibitors	 **1.2**		 **1.3**
enzyme	β-galactosidase	α-galactosidase	β-galactosidase
	LacZ E. coli / human-liver lysosomes	coffee bean	*Aspergillus oryzae*
K_i (mM)	0.48 / 0.018		30.2
k_{inact} (min^{-1})	0.58 / 0.41		0.556
k_{inact} / K_i (M^{-1} min^{-1})	1.2×10^3 / 2.3×10^4	0.95	18.4
reference	45 / 45	45	49

Moreover, the [^{14}C] exocyclic methylene labelled compound **1.2** was incubated with the *E. coli* β-galactosidase to result in the completely inactivated enzyme.

Digestion and sequence analysis of this radiolabeled protein showed that the label was covalently bonded to a methionine residue,[46] which is not the catalytic nucleophile.[47]

Compound **1.2** exists as two tautomers (Figure 1.8). Upon protonation of the distal nitrogen, compound **1.2** decomposes to give a highly electrophilic carbenium ion. The sulfur atom of methionine at the active site reacts with this carbocation to form the covalent complex.[48]

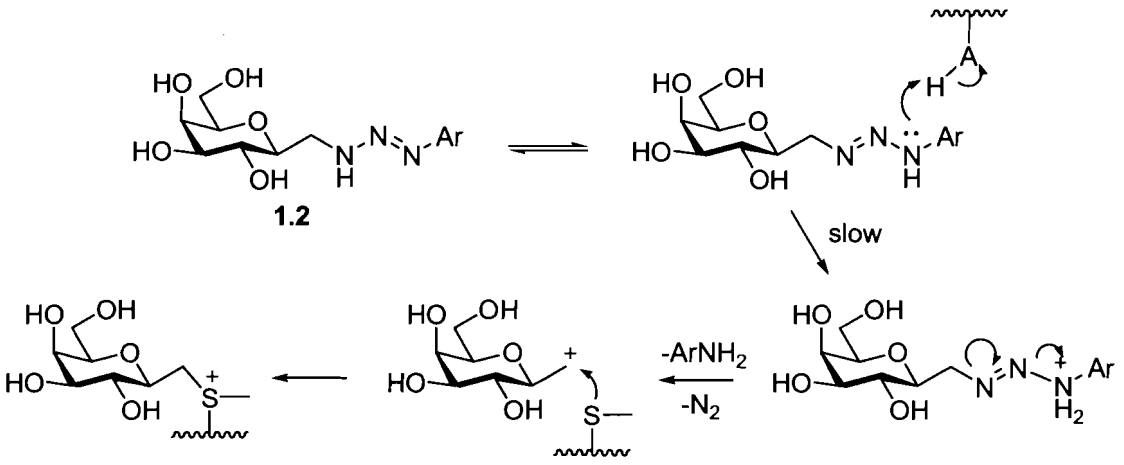


Figure 1.8 Mechanism of inactivation by galactosylmethyltriazene **1.2**

In a similar manner to the triazene **1.2**, the diazoketone **1.3**, reported by Bemiller *et al.*, also decomposes to give a reactive carbenium ion.[49] The activity of this compound depends on the pH of the medium. Thus, compound **1.3** does not inactivate *E. coli* β-D-galactosidase at pH 7.3, but it undergoes time-dependent irreversible inhibition of *aspergillus oryzae* β-D-galactosidase at pH 4.5 (Table 1.1). This indicates that compound **1.3** might inactivate the galactosidase with a more acidic active-site proton donor. Moreover, using a competitive inhibitor, methyl 1-thio-β-D-

galactopyranoside (Figure 1.9), to protect the enzyme from inactivation by compound **1.3**[49] indicated that compound **1.3** is an active site-directed irreversible inhibitor.

The proposed mechanism for inhibition using compound **1.3**, which is similar to that for the triazene **1.2**, is shown below (Figure 1.9).

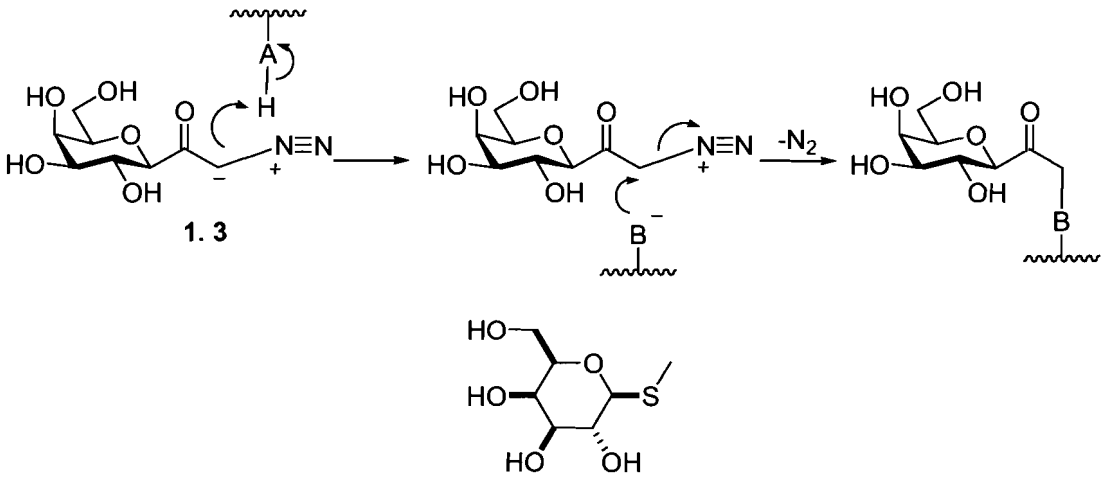
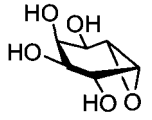
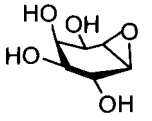
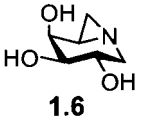


Figure 1.9 Mechanism of inhibition by compound **1.3**

1.4.2 Epoxides and Analogues: Conduritol Epoxides 1.4 and 1.5 and Aziridine Analogue 1.6

The electron-withdrawing effect of neighbouring hydroxyl groups on conduritol epoxide isomers **1.4** and **1.5** reduces the reactivity of the oxirane. However, in an enzyme's active site, protonation of the oxirane ring by an acidic residue facilitates the simultaneous attack of the catalytic nucleophile.[50] As a result, isomers **1.4** and **1.5** turn out to be efficient irreversible galactosidase inhibitors (Table 1.2). Treatment of *E. coli* β-galactosidase with ^{3}H-radio labelled conduritol epoxide **1.5** labelled the nucleophilic glutamate residue.[51] The cleaved product from the labelled enzyme was identified as *allo*-inositol.[50] This indicates a *trans*-ring opening of the epoxide.

Table 1.2 Inhibition data of conduritol epoxides **1.4**, **1.5** and aziridine **1.6**

	enzyme	K_i (mM)	k_{inact} (min^{-1})	k_{inact} / K_i (M^{-1} min^{-1})	reference
 **1.4**	α-galactosidase coffee bean	60	1.2	20	50
	α-galactosidase *E.coli*	0.14		<0.01	52
 **1.5**	β-galactosidase *E.coli*	0.23	0.052	226	52
	β-galactosidase *Aspergillus wentii*	1.0	1.4	1400	52
 **1.6**	α-galactosidase coffee bean	0.0071	0.018	2540	53

The stereochemistry of these two isomers (**1.4** and **1.5**) directs the inhibitory specificity towards the enzymes. The conduritol epoxide **1.4** deactivates only α-galactosidases, while the isomer **1.5** is specific for β-galactosidases.[51] These inhibition reactions of isomer **1.4** and **1.5** result in *trans*-diequatorial and *trans*-diaxial ring-opened products, respectively. An explanation of these results is shown below (Figure 1.10). For example, the oxirane ring of compound **1.4** has a similar orientation to the 1-OH group of α-D-galactose. Thus, the position of the oxirane ring is optimally aligned to the catalytic residues of α-galctosidases.[52]

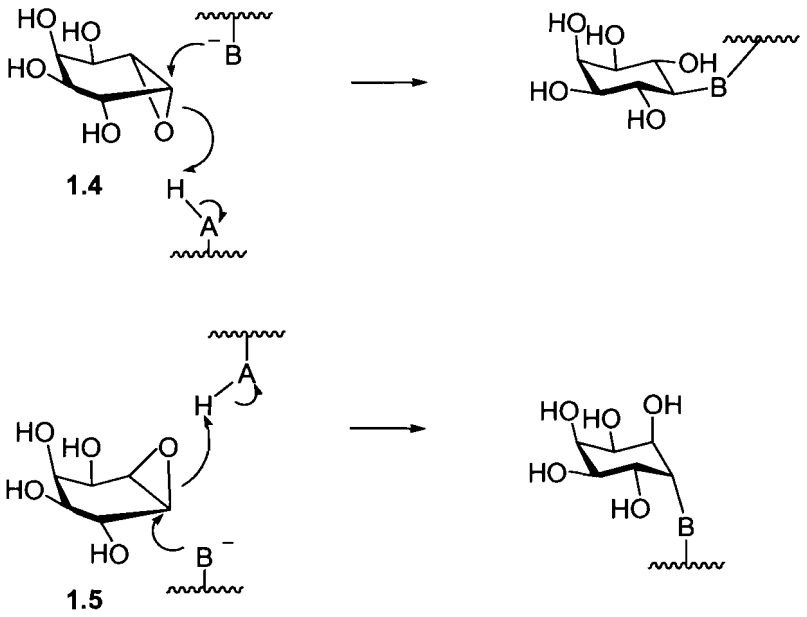


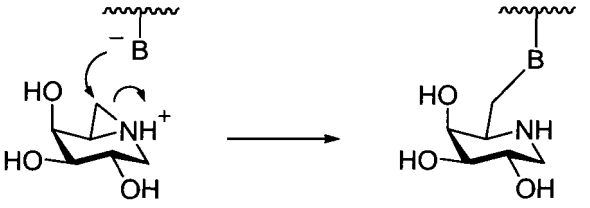
Figure 1.10 Mechanism of inhibition by conduritol epoxide isomers **1.4** and **1.5**

Ganem and co-workers prepared an aziridine analogue **1.6**, which is analogous to the conduritol epoxides (**1.4** and **1.5**), as an irreversible inhibitor.[53] Compound **1.6** is the most potent α-galactosidase irreversible inhibitor reported to date (Table 1.2).[53]

Legler suggested that the reason for the greater reactivity of aziridine **1.6** compared with epoxide **1.4** is the 10^4-fold tighter non-covalent affinity.[50] Specifically, he argued that: "*A detailed inspection of the inhibition data shows that the advantage of the aziridine*" aziridine **1.6** "*over the oxirane*" epoxide **1.4** "*lies in its almost 10^4-fold higher non-covalent affinity, which overcompensates for its ~ 60-fold lower covalent reactivity.*"

The competitive inhibitor 1-deoxy-*galacto*-nojirimycin (**1.35** see Table 1.7) protects the enzyme from inactivation by compound **1.6**. Therefore, compound **1.6** is an

active-site-directed inactivator.[53] The mechanism of inactivation of compound **1.6** is shown below (Figure 1.11).



1.6

Figure 1.11 Mechanism of inactivation by aziridine analogue **1.6**.

1.4.3 Halides: *N*-Bromoacetyl-β-D-Galactosylamine (1.7) and Glycosyl Fluorides 1.8 and 1.9

Naider *et al.* used compound **1.7**, which contained an α-bromo amido functionality, to alkylate an active site residue of *E. coli* β-galactosidase (Table 1.3).[47] The residue that was alkylated was the same methionine residue that was labelled by Sinnott and co-workers with their β-D-methylgalactopyranosyl-(*p*-nitrophenyl)triazene (**1.2**).[46] After treating the inactivated enzyme with mercaptoethanol, 94% of the original reactivity was regenerated. An explanation for this observation is illustrated below (Figure 1.12).

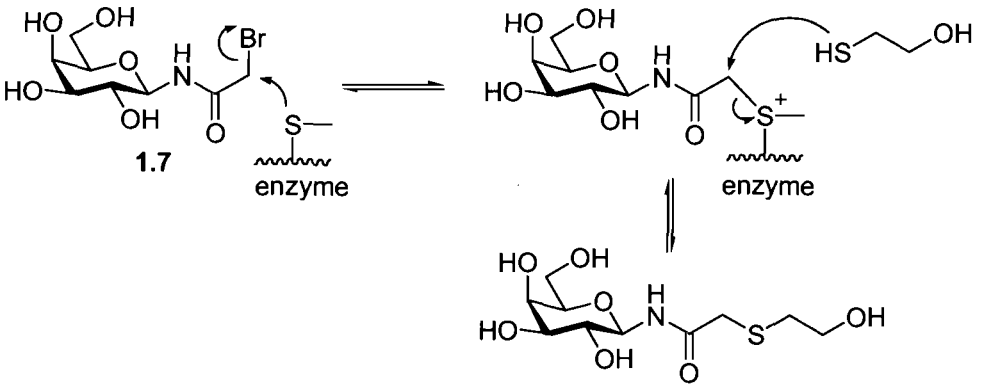
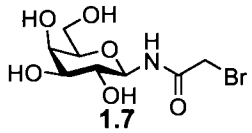
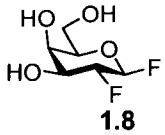
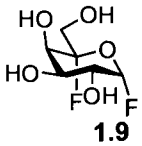
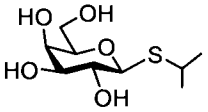


Figure 1.12 The mechanism of how mercaptoethanol rescues the inactivated enzyme

A mutant in which the methionine residue was changed to norleucine is not sensitive to alkylation by compound **1.7**, and importantly, the mutant enzyme is still catalytically active. This suggests that this methionine residue does not take part directly in the enzymatic reaction.[47]

Table 1.3 Kinetic constants for inhibitions of halides **1.7**, **1.8** and **1.9** and structure of isopropyl 1-thio-β-D-galactopyranoside

	enzyme	K_i (mM)	k_{inact} (min^{-1})	k_{inact} / K_i (M^{-1} min^{-1})	reference
 **1.7**	β-galactosidase *E. coli*	1.13	0.063	55.8	47
 **1.8**	β-galactosidase *E. coli*	13.2	1.3	1.02×10^4	54
	β-galactosidase *Aspergillus orgzae*	2.5	5.4	463	54
	β-galactosidase *Aspergillus niger*	0.8	1.25	640	54
 **1.9**	α-galactosidase coffee bean	0.0006			55
 isopropyl 1-thio-β-D-galactopyranoside					54

Using a second fluorine atom in either the *C*-2 (**1.8**) or *C*-5 (**1.9**) position of galactosyl fluoride to trap nucleophilic residues is a strategy employed by the Withers group. The electron withdrawing effect of the second fluorine atom destabilizes the cationic transition states, and thus decreases the rate of both the glycosylation and deglycosylation steps. This is because the glycosylation involves the departure of a better leaving group (fluoride) than does deglycosylation (enzyme-carbohydrate

intermediate). Glycosylation is much faster than deglycosylation, thus allowing accumulation of the glycosyl-enzyme intermediate.[54,55]

Both the kinetic studies (Table 1.3) and the use of a competitive inhibitor, isopropyl 1-thio-β-D-galactopyranoside (Table 1.3), to protect the enzyme from inactivation indicated that compound **1.8** is a time dependent, active site-directed inhibitor.[54]

For 5-fluoro-α-D-galactosyl fluoride (**1.9**), the kinetic study did not show that this compound was a time-dependent inhibitor. Instead, compound **1.9** acted as a competitive inhibitor with *K*i of 0.6 μM for the α-galactosidase from coffee bean (Table 1.3).[55] Withers and co-workers argued that although the deglycosylation was slower than glycosylation, the rate of deglycosylation was still relatively fast. By using electrospray mass spectrometry, these authors demonstrated that the proposed galactosyl-enzyme intermediate was formed during the catalytic cycle.[55]

1.5 Galactosidase Reversible Inhibitors

The design of potent glycosidase inhibitors has generally centred on trying to mimic the transition state of the enzyme-catalyzed reaction. That is, most galactosidase inhibitors have been designed to mimic the distortion of the pyranose ring, and/or the build-up of positive charge in the natural substrate.

Due to the great variety of inhibitors including carbasugars, thiosugars, *N*- and *C*-glycoside analogues (Figure 1.13),[56] only α-galactosidase inhibitors that possess either an endocyclic nitrogen or an exocyclic nitrogen atom are discussed in this thesis.

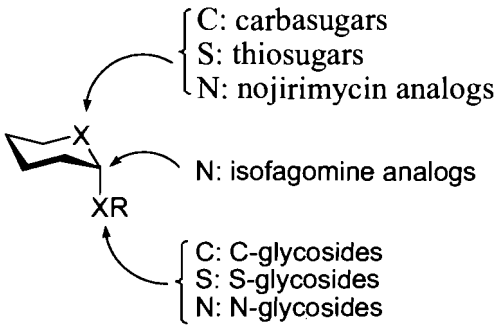


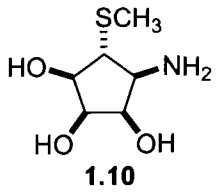
Figure 1.13 Classes of glycosidase inhibitors that contain a six-membered heterocyclic ring

1.5.1 Monosaccharide Analogues: Build-up of Positive Charge

The inhibitors detailed here all possess an amino group adjacent to the anomeric carbon or at the anomeric carbon of a monosaccharide in order to mimic the partial positive charge of the transition state (Figures 1.2 and 1.5).

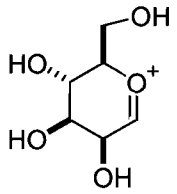
Furan Ring: Mannostatin and *Galacto*-analogues

Mannostatin A (**1.10**), a natural product (Figure 1.14), was isolated and shown to be a strong inhibitor of jack bean α-mannosidase (IC_{50} value 0.07 μM).[57] A modelling study, via which the mannostatin A structure was superimposed on a half-chair form of the mannosyl oxacarbenium ion, demonstrated that two of three OH groups of mannostatin A overlapped closely with the 2- and 3-OH groups of the mannosyl oxacarbenium ion, and the nitrogen atom of Mannostatin A was placed close to the endocyclic oxygen atom of the oxacarbenium ion.[58] Therefore, mannostatin A is assumed to be a good topographical equivalent to the mannopyranosylium cation, and it binds to mannosidase enzymes in a similar manner as would the mannosyl oxacarbenium cation.[58]



1.10

mannostatin A

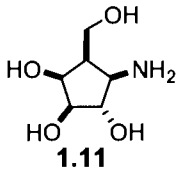
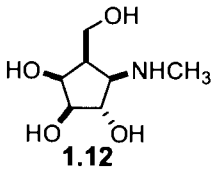
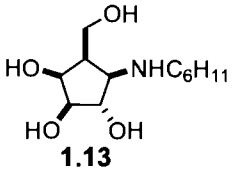
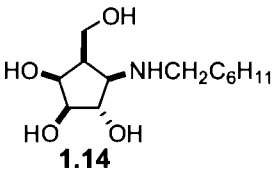
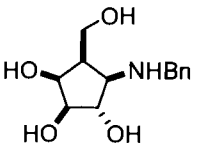


mannosyl oxacarbenium ion

Figure 1.14 Mannostatin A and the mannosyl oxacarbenium ion

Since then, a series of galactosidase inhibitors **1.11**, **1.12**, **1.13**, **1.14** and **1.15** (Table 1.4) that have similar aminocyclopentanetriol structures have been synthesized.[59] Compound **1.11** has almost the same inhibition potency with both α- and β-galactosidaes. Compound **1.15**, which is from alkylation of the primary amine of compound **1.11** dramatically increases the inhibition of *E. Coli* β-galactosidase by over 2000-fold, compared to the parent inhibitor **1.11** (Table 1.4). This effect is perhaps caused by a strong affinity between the hydrophobic pocket of β-galactosidase and the alkyl moieties of these inhibitors. On the other hand, such alkylations decrease or completely abolish activity towards α-galactosidases. These observations indicate that varying the amino substituent has a remarkable effect on the potency and selectivity of these inhibitiors.[59]

Table 1.4 Reported K_i (μM) values of some *galacto*-analogues of mannostatin

	α-galactosidase	β-galactosidase		
	coffee bean	*E. coli*	bovine liver	reference
 **1.11**	12	4.5	3.3	59
 **1.12**	NI [a]	0.22	0.09	59
 **1.13**	NI [a]	2.4	3	59
 **1.14**	22	0.02	0.004	59
 **1.15**	NI [a]	0.002	0.006	59

[a] No inhibition when IC_{50} > 1 mM

Pyranose Ring: Galactosylamine and Derivatives

In 1984, valiolamine **1.16** was identified as a potent inhibitor of maltase and sucrase (Figure 1.15).[60] Since then, many *N*-modified valiolamines have been synthesized and tested as inhibitors of various α-glucosidases. In 1994, voglibose (Basen) **1.17**,[61] a compound with an IC_{50} value of 0.0046 μM towards sucrase and

0.015 μM towards maltase, was approved as an oral antidiabetes drug in Japan (Figure 1.15).[62]

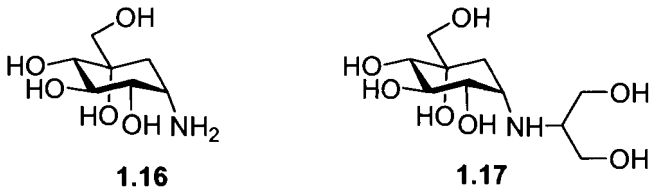


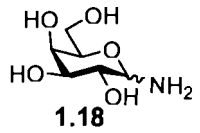
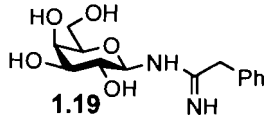
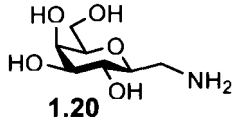
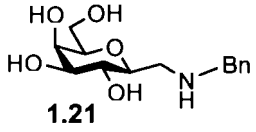
Figure 1.15 Structures of valiolamine and voglibose

Similar compounds have been made in order to obtain strong inhibitors against galactosidases. At first, galactosylamine **1.18** showed some inhibition towards both α- and β-galactosidases (Table 1.5).[63] However, this compound spontaneously undergoes hydrolysis and thus several modifications have been made in order to increase the compound's stability.

First, compound **1.19**, which contains an amidine group instead of an amino group, was reported to be stable in acidic solution between pH 5 - 7. The K_i values for galactosylamidine **1.19** towards *Aspergillus niger* α-galactosidase and *E. coli* β-galactosidase are 47 μM and 7.9 μM, respectively (Table 1.5).[64]

Second, putting a methylene group between the anomeric carbon and the exocyclic nitrogen atom to form *C*-(galactopyranosyl)methylamine (**1.20**) and *N*-substituted derivatives stabilizes the galactosamine. Although compound **1.20** is a weaker inhibitor of *E. coli lacZ* β-galactosidase, the inhibition of the *N*-benzyl derivative **1.21** increases 200-fold relative to the parent compound **1.20**, and it is specific towards β-galactosidases (Table 1.5).[65]

Table 1.5 Reported K_i (μM) values of galactosylamine and derivatives

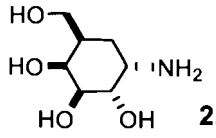
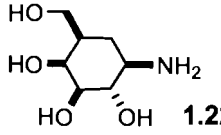
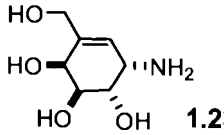
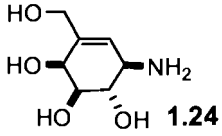
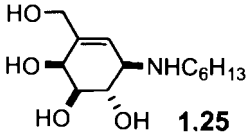
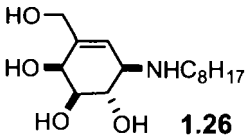
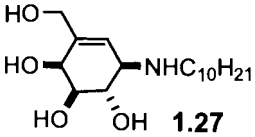
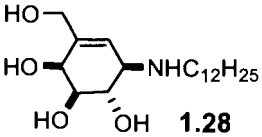
	α-galactosidase			β-galactosidase		
	coffee bean	*E. coli*	*Aspergillus niger*	*E. coli*	other species	reference
 **1.18**	7.5	86		7	13.4 [c]	63
 **1.19**			47	7.9	2.4 [b]	64
 **1.20**				505		65
 **1.21**				2.3 [a]		65

[a] *E. coli lacZ*; [b] *Aspergillus oryzae*; [c] *Aspergillus wentii*

Third, replacing the endocyclic oxygen by a carbon atom forms α- and β-*galacto-validamine* (**2.2** and **1.22**)[66] or *galacto*-valienamine (**1.23**)[67] (Table 1.6). Compound **1.22** is a mild and specific inhibitor of α-galactosidases.

Of note, α- and β-*galacto*-valienamine (**1.23** and **1.24**) and the *N*-alkyl substituted derivatives **1.25–1.28** mimic both the ring distortion and the charge present in the α-galactopyranosylium ion. Specifically, as the length of the alkyl chain gets longer, the compound becomes a more specific and a tighter binding inhibitor of β-galactosidases, while the inhibition constants against α-galactosidases do not change much.[67] This is another example that demonstrates a hydrophobic effect on the inhibitory potency and specificity.

Table 1.6 Reported IC_{50} (μM) values of *galacto*-valienamine and derivatives

	α-galactosidase coffee bean	β-galactosidase bovine liver	reference
 **2.2**	500		120
 **1.22**	2.8	NI [a]	66
 **1.23**	56	NI [b]	67
 **1.24**	12	NI [b]	67
 **1.25**	2.7	2.3	67
 **1.26**	3.1	0.87	67
 **1.27**	1.9	0.13	67
 **1.28**	4.4	0.01	67

[a] No inhibition when [inhibitor] < 10^{-3} M; [b] No inhibition is defined as IC_{50} > 0.1 mg/mL

Pyranose ring: Iminosugars and Derivatives

Several potent glucosidase inhibitors have been developed in which a nitrogen atom has replaced either the endocyclic oxygen atom or the anomeric carbon atom of

the sugar ring (compounds **1.29-1.33**). At physiological pH values, the amino group will be protonated forming a cationic inhibitor. These protonated inhibitors are set up to form an intimate ion pair with a negatively charged carboxylate group within the active site of the enzyme.

In 1967, nojirimycin (**1.29**) was isolated from *streptomyces* bacteria by Ishida *et al.* (Figure 1.16).[68] Nojirimycin displays a fairly potent inhibition towards *Aspergillus wentii* α-glucosidase with a K_i value of 6.3 μM.[69] However, nojirimycin can spontaneously mutarotate, and it is also very sensitive to the presence of oxidants. Later, the stable analogue 1-deoxynojirimycin (DNJ) (**1.30**) was isolated from the roots of the mulberry trees.[70] A synthetic *N*-substituted analogue **1.31**, called Miglitol, is a second generation of α-glucosidase inhibitor and is a commercial therapeutic for the treatment of diabetes.[62]

Meanwhile, fagomine (**1.32**) was isolated from *Fagopyrum esculentum*,[71] and isofagomine (**1.33**) was synthesized by Jespersen *et al.*[72]

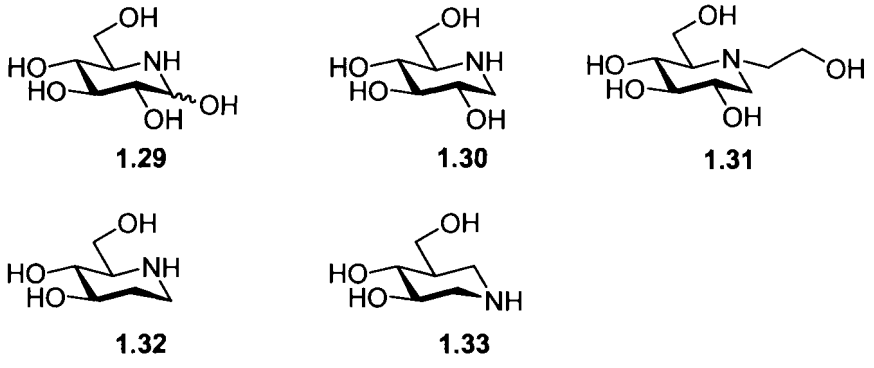
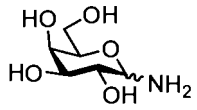
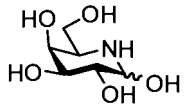
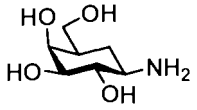
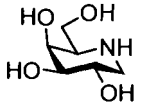
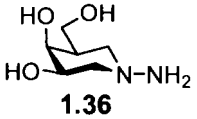
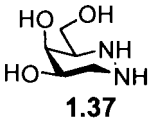


Figure 1.16 Structures of nojirimycin (**1.29**), fagomine (**1.32**) and their analogues

The analogues *galacto*-nojirimycin (**1.34**)[63] and 1-deoxy-*galacto*-nojirimycin (**1.35**) [63] have been either synthesized or isolated from natural sources.

The reported K_i values for *galacto*-iminosugars and several galactosylamines are listed below (Table 1.7). The difference between the pairs of compounds **1.18** and **1.34**, **1.22** and **1.35**, and **1.36**[73] and **1.37**[74] are whether an amino group is placed inside the ring or outside the ring. These comparisons clearly demonstrate that the compounds with amino group inside the six-membered ring have higher inhibition constants than those with the amino group exocyclic to the six-membered ring. As Legler proposed, "*the endocyclic location of the positive charge*" "*bears a greater resemblance with the charge distribution*" in the transition state than does the exocyclic location of the positive charge.[75]

Table 1.7 Reported K_i (μM) values of *galacto*-analogues with either endocyclic or exocyclic amino group

	α-galactosidase		β-galactosidase		reference
	coffee bean	*E. coli*	*E. coli*	other species	
 **1.18**	7.5	86	7	13.4 [c]	63
 **1.34**	0.0007	0.17	0.045	0.011 [c]	63
 **1.22**	2.8		NI [a]		66
 **1.35**	0.0016	0.24	12.5	0.16 [c]	63
 **1.36**	73			4.5 [b]	73
 **1.37**	0.28		0.30	0.04 [b]	74

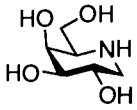
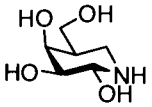
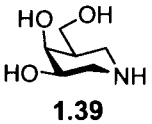
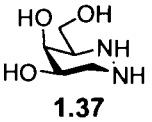
[a] No inhibition observed for bovine liver β-galactosidase; [b] *Aspergillus oryzae*; [c] *Aspergillus wentii*

The postulation that the position of the endocyclic nitrogen atom has an effect on the specificity of inhibitory activity was supported by the inhibition constants listed below (Table 1.8). The structural differences between compound **1.35**, **1.38**[76] and **1.39**[74,77] is that compound **1.35** has an amino group at the position of endocyclic oxygen, while compounds **1.38** and **1.39** have an amino group at the position of anomeric carbon. Compound **1.35** shows stronger inhibition against α-galactosidases

than β-galactosidases. On the other hand, compounds **1.38** and **1.39** show more powerful inhibition towards β-galactosidases than towards α-galactosidases. It is commonly accepted that since the nucleophilic residue of an α-galactosidase sits above the sugar ring.[75] The nucleophilic residue is nearer the amino group which replaces the endocyclic oxygen atom in compound **1.35**, thus forming a stronger electrostatic interaction. For β-galactosidases, the nucleophilic carboxylate is below the ring, thus in compounds **1.38** and **1.39**, it is closer to an amino group which replaces the anomeric carbon atom.[75] This hypothesis is further strengthened by the inhibitory activity of compound **1.37**.[74,77] Compound **1.37** has two nitrogen atoms that replace both the positions of the endocyclic oxygen and the anomeric carbon, and as a result it inhibits both α- and β-galactosidases at nearly the same level.[74,77]

A comparison of compound **1.38** with **1.39** also reveals that the presence of a 2-OH group increases the inhibition of α-galactosidases almost 70-fold. This difference is less pronounced for β-galactosidases. Indeed, the 2-OH group is thought to have a crucial interaction with some enzymes.[78]

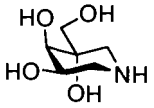
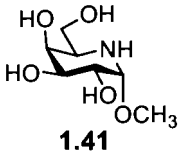
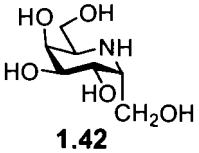
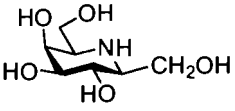
Table 1.8 Reported K_i (μM) values of *galacto*-analogues with endocyclic amino group at different position

	α-galactosidase		β-galactosidase		reference
	coffee bean	*E. coli*	*E. coli*	*Aspergillus oryzae*	
 **1.35**	0.0016	0.24	12.5	0.16[a]	63
 **1.38**	0.742		0.397	0.035	76
 **1.39**	50[b]		0.2[b]	0.004[c]	74,77
 **1.37**	0.28		0.30	0.04	74

[a] *Aspergillus wentii*; [b] Reference 74; [c] Reference 77

Compounds **1.40**,[77] **1.41**,[79] **1.42**[80] and **1.43**[80], which include other modifications that have been made to the *galacto*-iminosugar skeleton are listed below (Table 1.9).[80] Adding another OH group to give compound **1.40** does not improve the inhibition compared to the parent compound **1.39** (Table 1.8). Although *galacto*-nojirimycin (**1.34**) (see in Table 1.7) is strong inhibitor for galactosidases, it is unstable and displays a low specificity. Modified analogues **1.41**, **1.42** and **1.43** overcome these shortcomings. Moreover, compounds **1.42** and **1.43** are more specific as coffee bean α-galactosidase inhibitors.

Table 1.9 Reported K_i (μM) values of modified *galacto*-iminosugars

	α-galactosidase		β-galactosidase		reference
	Coffee bean	*E. coli*	*E. coli*	*Aspergillus oryzae*	
 **1.40**	610 [a]			17.5 [a]	77
 **1.41**		1.76	NI [c]		79
 **1.42**	0.015	1.4	NI [c]		80
 **1.43**	0.107	17% [b]	NI [c]		80

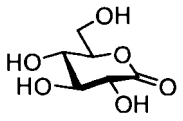
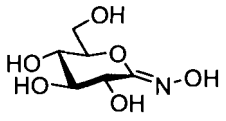
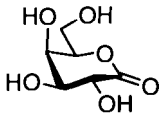
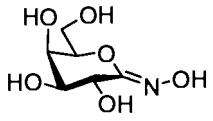
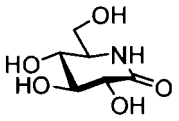
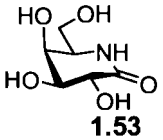
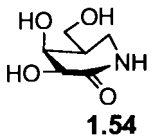
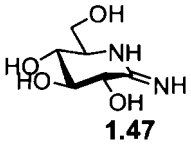
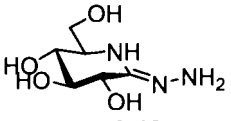
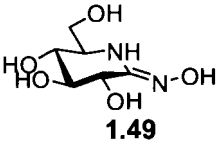
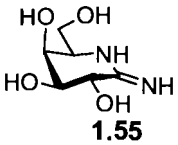
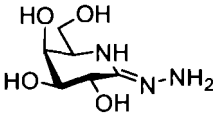
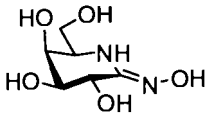
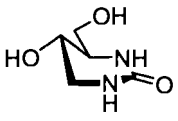
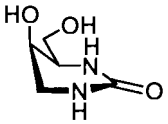
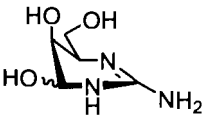
[a] IC_{50} (μM); [b] inhibition at [inhibitor] = 1 mM; [c] No inhibition observed

1.5.2 Monosaccharide Analogues: Build-up of Positive Charge and Half-chair Conformations

Although glycolactones **1.44** are the first group of compounds that were found to be glycosidases inhibitor, their instability limits their usage. Most research workers focused on the glycono-lactams and their derivatives. D-Glycono-1,5-lactams **1.46** and **1.53**, their amidine analogues **1.47** and **1.55**, amidrazone analogues **1.48** and **1.56**, and lactam oxime analogues **1.49** and **1.57** are stable compounds that mimic the conformation and electronic charge of the oxacarbenium ion (Table 1.10). The common characteristics for these compounds are: a) A positive charge at either the endo or the exocyclic nitrogen atom except compound **1.46** and **1.53**; b) an sp^2-hybridized anomeric carbon atom, and c) distortion of the pyranose ring from a chair to a flatten chair

conformation. The kinetic results demonstrate that all of these compounds are non-specific inhibitors of glycosidases.[81] All the inhibitors that are introduced in this section are listed below (Table 1.10).

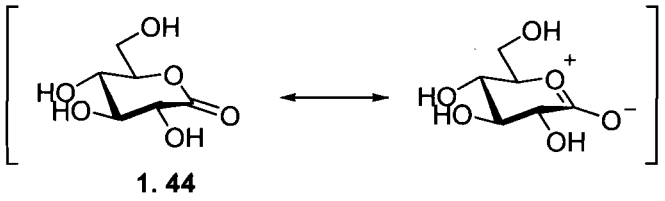
Table 1.10 Structures of D-*glucono*- and D-*galactono*-lactones, -lactams, -urea, -guanadines and derivatives

D-*glucono*-lactone and derivative	 **1.44**	 **1.45**	
D-*galactono*-lactone and derivative	 **1.51**	 **1.52**	
D-*glucono*-lactam	 **1.46**		
D-*galactono*-lactams and analogue	 **1.53**	 **1.54**	
D-*glucono*-lactam derivatives	 **1.47**	 **1.48**	 **1.49**
D-*galactono*-lactam derivatives	 **1.55**	 **1.56**	 **1.57**
D-*glucono*-urea	 **1.50**		
D-*galactono*-urea and -guanidinium	 **1.58**	 **1.59a**	

Glycono-1,5-lactones and Derivatives: Existing in a Half-chair Conformation

Glyconolactones were the first glycosidase inhibitors discovered.[56] The crystal structure of D-*glucono*-1,5-lactone **1.44** shows that it exists in a half-chair conformation,[82] and it likely exerts inhibition toward glucosidases due to its structural

similarity to the oxacarbenium ion intermediate.[82] In addition, Reese and Leaback suggested that the dipolar resonance structure of compound **1.44** is the main cause of inhibition (Figure 1.17).[83,84]



1. 44

Figure 1.17 Resonance structure of D-*glucono*-1,5-lactone **1.44**

Also, D-*galactono*-1,5-lactone **1.51** was reported to be a strong β-galactosidase (*E. coli* and *Homo sapiens*) inhibitor (Table 1.10).[85,86]

However, glycono-1,5-lactones undergo a ring opening reaction or anomerization to 1,4-lactones. Thus, it is difficult to make a quantitative explanation of glyconolactone inhibition constants.

To avoid the reactive disadvantages of lactones mentioned above, lactone oxime **1.45** (Table 1.10) was studied. Although this type of compound is stable in neutral and mildly acidic solutions, the measured inhibition is weaker than the corresponding lactones (Table 1.11).[87] This observation is interpreted that compound **1.45** is not able to form as strong a dipole as does the corresponding lactone. Therefore, the electrostatic interaction between the inhibitor **1.45** and the enzyme is weaker than that observed with compound **1.44**.[75]

Glucono-1,5-lactams and Derivatives: Effects of Positive Charge and Half-chair Conformations

Although D-*glucono*-1,5-lactam **1.46** is more stable than D-*glucono*-1,5-lactone **1.44**, its inhibitory activity doesn't increase. Therefore, several lactam derivatives were synthesized to test them as potential inhibitors (Table 1.11).[87]

It was thought that the predominant tautomer of amidine **1.49** would contain the endocyclic double bond (Figure 1.18, **1.49b**). However, ^{1}H and ^{15}N NMR spectra as well as X-ray diffraction experiments demonstrates that the major tautomer has an exocyclic double bond (**1.49a**), and that the pyranose ring adopts a flattened chair shape.[88] On the other hand, when amidine **1.49** is protonated, the ring twists into a half-chair conformation because of the strong conjugation between the two nitrogen atoms. Thus, the protonated form of amidine **1.49** not only has a half-chair conformation, but it also has positive charge that can have electrostatic interactions with the nucleophilic residue on the enzyme. Both factors contribute to amidine **1.49** being a potent inhibitor.

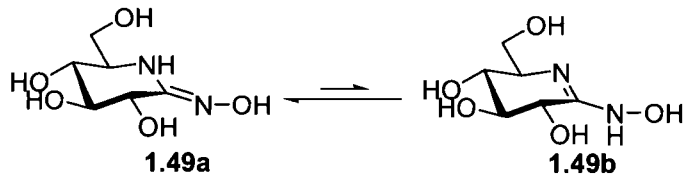
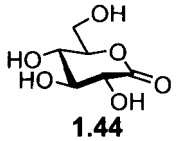
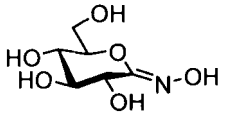
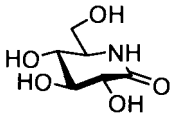
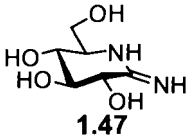
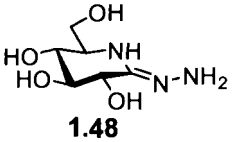
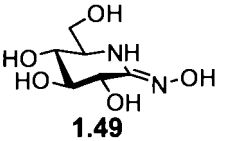


Figure 1.18 Tautomerization of amidine **1.49**

Compounds **1.47** and **1.48** should have similar distorted half-chair conformations to that of amidine **1.49** although they are less basic. The sequence of the basicities of these compounds is **1.47** > **1.48** > **1.49** (Table 1.11).[89] The inhibitory activity of compound **1.47** and **1.48** are invariant in the pH range 4.6 to 7.0, while the inhibition of compound **1.49** is dependent on pH.[90]

Furthermore, in spite of a 10^5 difference in basicity between compounds **1.47**, **1.48** and **1.49**, the inhibition constants of these compounds towards sweet almond β-glucosidase remain almost the same. Based on the assumption that compounds **1.47**, **1.48** and **1.49** inhibit sweet almond β-glucosidase in a similar manner, Ganem and co-workers postulated that the "flattened anomeric conformations" of these compounds were more important for inhibitory potency than the "full-fledged charge of the glucopyranosyl cation".[90]

Table 1.11 Reported K_i values (μM) of D-*glucono*-1,5-lactone, -lactam and various derivatives

compounds	β-glucosidase sweet almond pH 6.8	β-glucosidase sweet almond pH 5.6	pK_a	reference
 **1.44**	400			87
 **1.45**	4300			87
 **1.46**	125			87
 **1.47**		10 [a,b]	10.6 [c]	89, 90
 **1.48**		8.4 [a,b]	8.7 [c]	89, 90
 **1.49**		13.8 [a,b]	5.6 [c]	89, 90

[a] pH = 5.6; [b] Reference 90; [c] Reference 89

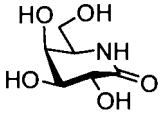
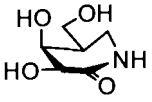
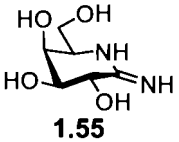
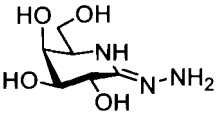
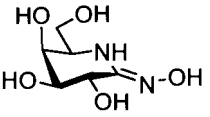
Galactono-1,5-lactams and Derivatives

The crystal structure of *galactono*-1,5-lactam **1.53** shows that it adopts a half-chair conformation.[91] Although it is not an inhibitor of α-galactosidase from *Aspergillus niger*, it displays a K_i value of 4.5 μM against β-galactosidase from *Aspergillus niger*.[91]

Lactam **1.54** proved to be a strong inhibitor with K_i value of 18 nM towards *Aspergillus oryzae* β-galactosidase (Table 1.12).[92]

Galactono-amidine **1.55**, -amidrazone **1.56** and -lactam oxime **1.57** have K_i values of 8.5 μM, 8.3 μM[90] and 5 μM[93] towards coffee bean α-galactosidase, respectively. Compound **1.56** and **1.57** have inhibition constants of 6.5 μM and 10 μM towards bovine liver β-galactosidase, respectively (Table 1.12).[90] Like *gluco*-anologues, although the basicities of these compounds are different, the inhibitory activity are in the same range.

Table 1.12 Reported K_i values (μM) of D-*galactono*-1,5-lactone, -lactam and various derivatives

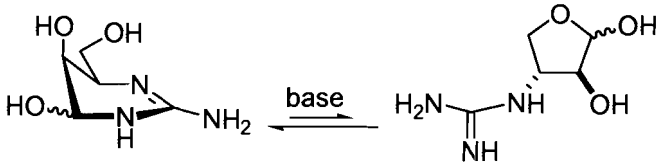
	α-galactosidase		β-galactosidase		reference
	Aspergillus niger	coffee bean	*Aspergillus niger*	bovine liver	
 **1.53**	NI [a]		4.5		92
 **1.54**			0.018 [b]		93
 **1.55**		8.5			90
 **1.56**		8.36		6.5	90
 **1.57**		5 [d]		10 [c]	90, 94

[a] No inhibition observed; [b] *Aspergillus oryzae*; [c] Reference 90; [d] Reference 94

Cyclic Urea and Guanidine Containing Compounds: Build-up of Positive Charge and Existing in Envelope Conformation

The cyclic ureas **1.50** and **1.58** and the cyclic guanidine **1.59** have envelope conformations in their ground states.[94,95,96] This kind of conformation allows *C*-5, *O*-5, *C*-1 and *C*-2 of the six-membered ring to be coplanar, a situation that is similar to the conformation of the oxacarbenium ion-like transition state. Compound **1.50** is not a potent inhibitor of *emulsin* β-glucosidase (K_i = 130 mM).[94] Its *galacto*-analogue **1.58** is a weak inhibitor of *E. coli* β-galactosidase (K_i = 15 mM) (Table 1.13).[94]

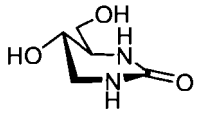
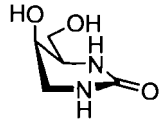
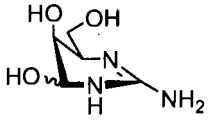
The inhibitor potency of cyclic guanidine **1.59** is pH dependent. Under basic conditions, six-membered ring conformer (**1.59**) becomes the predominate form (Figure 1.19), and the affinity of compound **1.59** for coffee bean α-galactosidase also increases (Table 1.13).[95,96] The authors argued that the interaction between compound **1.59** and enzyme caused the inhibition.[95,96]



1.59

Figure 1.19 The equilibration of compound **1.59**

Table 1.13 Reported K_i value (mM) of cyclic ureas and a cyclic guanidine

Compound	β-glucosidase *emulsin*	β-galactosidase *E. coli*	α-galactosidase coffee bean	reference
 **1.50**	130			91
 **1.58**		15		91
 **1.59**		NI [c]	1.3 [a] 0.48 [b]	95,96

[a] IC_{50} at pH of 7.5; [b] IC_{50} at pH of 10.7; [c] No inhibition observed

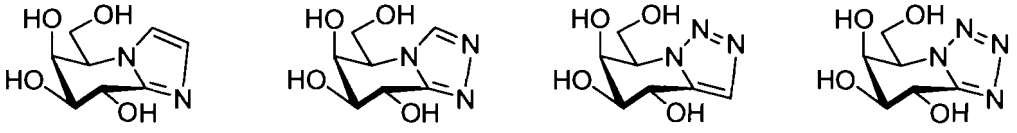
1.5.3 Bicyclic Compounds: Distorted Six-membered Ring or Build-up Positive Charge

In the classes of bicyclic compounds, the second ring, discussed below, distorts the pyranose ring conformation.

D-*Galactono*-nojirimidazole, -nojiritriazole and -nojiritetrazole

Like the previously mentioned lactams and their derivatives, D-*galactono*-nojirimidazole **1.60**, nojiri-1,2,4-triazole **1.61**, nojiri-1,2,3-triazole **1.62** and nojiritetrazole **1.63** have sp^2-hybridized *pseudo*-anomeric carbon atoms and exocyclic double bonds (Figure 1.20). These fused aromatic five-membered rings force the piperidine ring into a "*more rigid* $^4H_3$ *half-chair conformation*" than exists for the lactams and their analogues.[75] X-ray single crystal diffraction studies on D-*glucono*-analogues of compounds **1.60** and **1.63** show that both adopt half-chair conformations,[97]

and thus compounds **1.60**, **1.61**, **1.62** and **1.63** should also have similar conformations. This class of compounds are stronger inhibitors of β-galactosidases than of α-galactosidases.[97]

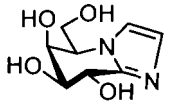
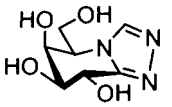
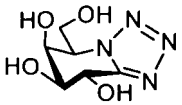
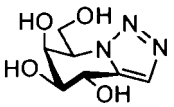


1.60 **1.61** **1.62** **1.63**

Figure 1.20 Structures of D-*galactono*-nojirimidazole (**1.60**), -nojirtriazole (**1.61** and **1.62**) and -nojiritetrazole (**1.63**)

With similar conformations, the charge development becomes the decisive factor for inhibitions of these compounds **1.60, 1.61** and **1.63**. The sequence of basicities **1.60** > **1.61** > **1.63** correlates with the compound's inhibition activity (Table 1.14).[81] However, compound **1.62**, whose basicity is in the same range as compound **1.61**, did not inhibit the enzymes at all.[81] Vasella and co-workers reasoned that the correct orientation of heteroatoms corresponding to catalytic residues on the enzyme was also necessary for inhibition.[98]

Table 1.14 Reported K_i values (μM) of D-*galactono*-nojirimidazole (**1.60**), -nojiritriazole (**1.61** and **1.62**), and -nojiritetrazole (**1.63**)

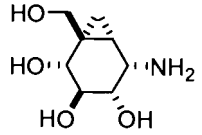
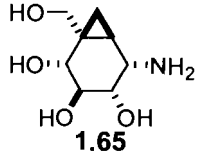
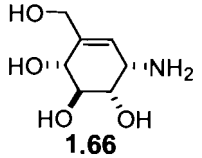
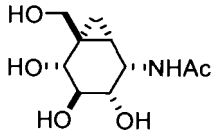
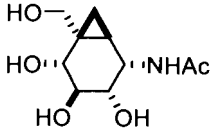
	 **1.60**	 **1.61**	 **1.63**	 **1.62**
β-galactosidase *E. coli*	0.004	0.2	1.0	NI [a]

[a] No inhibition observed

Bicyclo[4.1.0]heptane Glucose Analogues

In order to distort the pyranose ring, from a chair to one of the half-chair conformations that had been proposed as possible transition state structures, a cyclopropyl ring was fused onto a carbaglucose analogue.[99] The crystal structures of compound **1.64** and **1.65** show the six-membered rings adopt a *pseudo*-$^3H_2$ conformation. Also, the D-*gluco*-isomer **1.64** was a potent yeast α-glucosidase inhibitor with a K_i value of 107 nM (Table 1.15).[99] By comparing the D-*gluco*-isomer **1.64** with L-*ido*-isomer **1.65** and valienamine **1.66**,[60] Bennet and co-workers[99] argued that high activity of **1.64** was caused by either a favourable orientation of the hydroxymethyl group or an additional hydrophobic interaction between the cyclopropyl ring and the enzyme. These authors also emphasized the importance of charge by comparing the activities of compounds **1.64** and **1.65** with their acetamido-substituted analogues **1.67** and **1.68** which can not be protonated under physiological conditions (Table 1.15).

Table 1.15 Reported K_i values (μM) of bicyclo[4.1.0]heptane *gluco*-analogues

	α-glucosidase Yeast	α-glucosidase Rice	reference
 **1.64**	0.107	103	99
 **1.65**	820	2640	99
 **1.66**	18 [b]	NI [a]	60
 **1.67**	4100	NI [a]	99
 **1.68**	800	NI [a]	99

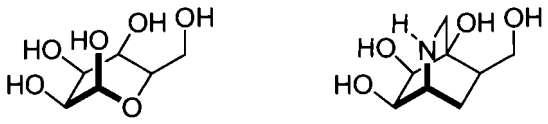
[a] No inhibition observed; [b] IC_{50} value

Isoquinuclidines and Bicyclic Mannoamidine

The compounds introduced in this section were synthesized to mimic a possible boat transition state conformation.

To accomplish this, a two-atom bridge was utilized to force the tetrahydropyran rings of isoquinuclidines **1.69** and **1.70** into boat shapes.[100] The similarity between compound **1.69** and the $^{1,4}B$ conformation of β- D-mannose is shown below (Figure 1.21). Compound **1.70** is a stronger inhibitor towards β-mannosidase than compound

1.69, likely due to a hydrophobic interaction between the enzyme and the benzyl group of compound **1.70** (Table 1.16).



$^{1,4}B$ conformer of β-D-mannose

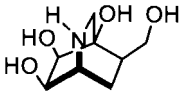
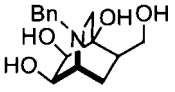
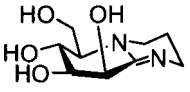
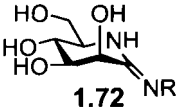
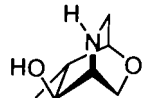
1.69

Figure 1.21 Structural similarity between compound **1.69** and the $^{1,4}B$ conformer of β-D-mannose

Heck and co-workers synthesized the bicyclic mannoamidine **1.71** to mimic a boat conformation of the transition state. The mannopyranose ring has a $B_{2,5}$ conformation due to the fused six-membered ring.[101] A comparison of the bicyclic compound **1.71** with the monocyclic compound **1.72** shows that **1.71** is a specific inhibitor towards α-mannosidase, while compound **1.72** shows no specificity. It was suggested that the boat conformation of compound **1.71** contributes to this specificity.

A bicyclic glucosylamine **1.73** with boat conformation was synthesized by Bennet and co-workers.[102] Kinetic studies revealed that compound **1.73** was a weak inhibitor of glucosidases (Table 1.16). These authors argued that the lack of both 2- and 6-hydroxyl groups may cause the weak affinity between the inhibitor and the enzyme.[102]

Table 1.16 Reported K_i values (μM) of inhibitors with boat conformations

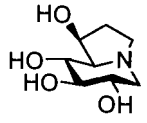
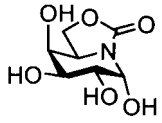
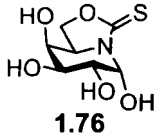
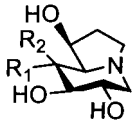
	α-mannosidase Jack bean	β-mannosidase snail	α-glucosidase rice	β-glucosidase almond	reference
 **1.69**		20			100
 **1.70**		0.17			100
 **1.71**	6.0	NI [a]			101
 **1.72** R=C_3H_7	0.11	0.19			101
 **1.73**			800	1010	102

[a] No inhibition observed

Castanospermine and Anologs

Castanospermine (**1.74**) is a natural alkaloid isolated from the seeds of *Castanospermum australe*.[103] Castanospermine is a potent inhibitor of both α- and β-glucosidases.[50] Taylor *et al.* reported that castanospermine was a potent α-glucosidase inhibitor.[104] *Galactono*-castanospermine analogues **1.75**, **1.76** and **1.77** were synthesized as potential inhibitors of galactosidases.[105,106] Although the hydroxyl groups on the pyranose ring of compounds **1.75** and **1.76** have the D-*galacto*-configuration, compounds **1.75**, **1.76** and **1.77** are only weak inhibitors of galactosidases (Table 1.17).

Table 1.17 Reported K_i values (μM) of castanospermine **1.74** and *galacto*-analogues

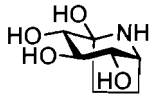
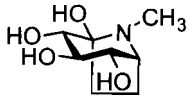
	α-glucosidase	β-glucosidase	α-galactosidase	β-galactosidase	reference
	rice	*Aspergillus wentii*	coffee bean	other species	
 **1.74**	0.015	0.9			50
 **1.75**			259		106
 **1.76**			137	267 [a]	106
 **1.77** R_1 = OH R_2 = CH_2OH			71	73 [b]	105

[a] bovine liver; [b] Aspergillus oryzae

Calystegines

Calystegines are polyhydroxylated nortropane alkaloids with both pyrrolidine and piperidine rings. They can be regarded as bicyclic isofagomine analogues. Calystegine B_2 (**1.78**) was isolated from the roots of *Calystegia sepium.*[107] Calystegine B_2 (**1.78**) and *N*-methylcalystegine B_2 (**1.79**) are specific potent inhibitors of α-galactosidases (Table 1.18).[108]

Table 1.18 Reported K_i values (μM) of calystegine B_2 and *N*-methylcalystegine B_2

	α-galactosidase coffee bean	β-galactosidase bovine liver	reference
 **1.78**	0.86	46	108
 **1.79**	0.47	NI [a]	108

[a] No inhibition observed

1.6 Rationale for the Design of Target Compounds

1.6.1 Rationale for the Design of the Reversible Inhibitors

As discussed in section 1.3.3, some compounds are designed to contain an amino group in order to set up electrostatic interactions between the inhibitor and the catalytic residues of the enzymes. In some cases, the sugar rings of the inhibitors were twisted from a chair conformation into a half-chair or a boat conformation, which are similar to that of the presumed oxacarbenium ion-like transition state.

A novel type of inhibitor, the carbocyclic bicyclo[4.1.0]heptylamine α-glucopyranose analogue **1.64** had been synthesized by Tanaka *et al.*[99] It was suggested that the fused cyclopropyl moiety introduced structural rigidity to change the conformation of the cyclohexane unit of compound **1.64** into that of a half-chair. In addition, the basic amino group replacing *O*-1 mimicked the charge development once protonated, that occurs during enzyme-catalyzed reactions.

Kinetic studies showed that compound **1.64** is a strong inhibitor of yeast α-glucosidase with sub-micromolar inhibition constant. Furthermore, the crystal structure

of compound **1.64** shows that this compound exists in a $^2H_3$ half-chair conformation as expected.[99]

Based on Tanaka *et al.*'s work,[99] we designed our target compound **1.80** (Figure 1.22). The difference between compound **1.64** and **1.80** is the stereochemistry of OH at *C*-2 which relates to the orientation of 4-OH on D-*gluco*- and D-*galacto*-pyranoside rings, respectively.

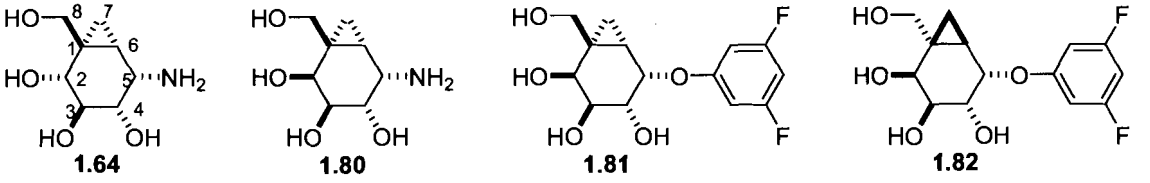
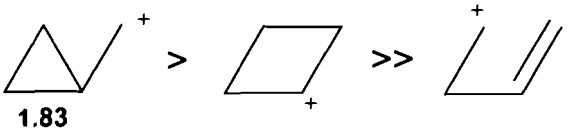


Figure 1.22 The structures of target compounds **1.80**-**1.82** and glucosidase inhibitor **1.64**

1.6.2 Rationale for the Design of the Irreversible Inhibitors

It has been known that the solvolysis rate of cyclopropylcarbinyl chloride is 27 times that of cyclobutyl chloride, and that allylcarbinyl chloride does not react at a measurable rate in aqueous ethanol.[109] This means cyclopropylmethyl carbonium ion **1.83** is formed faster than those of the corresponding acyclic homologues (Figure 1.23).[109]



1.83

Figure 1.23 The relative rates of formation of cyclopropylmethyl carbonium ion and some of its C_4H_7 homologues

Moreover, the cyclopropylmethyl cation **1.83** is a non-classical bicyclobutenium ion **1.84** which is rapid interconverting to the other cations (Figure 1.24). Therefore, nucleophilic capture can occur of carbons **a**, **b** and **c** of cation **1.84**.

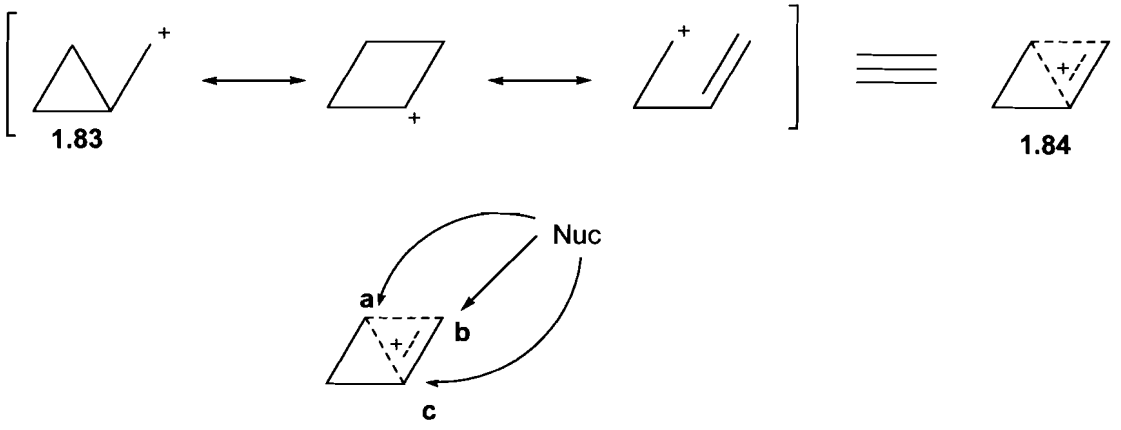


Figure 1.24 Resonance structure of bicyclobutenium ion **1.84**

Two isomers **1.81** and **1.82** (Figure 1.22) were proposed as potential irreversible inhibitors. Generally, *p*-nitrophenoxide is used as a leaving group, but in the synthesis of compound **1.81** and **1.82**, the last step is debenzylation using H_2/Pd, this will result in the reduction of nitro group. Therefore, the 3,5-difluorophenoxide was chosen as a leaving group based on the electronic withdrawing effect of two fluorine atoms. We postulate that while the acidic residue catalyzes the departure of difluorophenoxy group of compound **1.81** and **1.82**, the developing positive charge will be delocalized by sigma-bond participation of a suitably-oriented strained cyclopropyl C-C bond.

2 A Potent Bicyclic Inhibitor of a Family 27 α-Galactosidase

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Wang, Y.and Bennet, A. J.

Organic & Biomolecular Chemistry **2007**, *5*, 1731-1738.

2.1 Introduction

An important field in glycobiology involves design, synthesis, and biological evaluation of inhibitors for various glycosidase enzymes.[62] Tight-binding reversible inhibitors of glycosidases have the potential to be therapeutic agents for the treatment of various medical conditions, including diabetes,[110] cancer,[111] and influenza.[112] Of particular importance with respect to this report, it is known that Fabry disease is a lysosomal storage disorder caused by a deficiency of α-galactosidase A (α-Gal A).[113] This enzyme activity deficiency results from impaired trafficking of misfolded α-Gal A variants to the lysosome.[113] Of note, the use of low concentrations of galactosidase inhibitors, as chemical chaperones, ameliorates the trafficking of mutant enzymes presumably by stabilizing the correctly folded enzymatic structure.[37,39]

At the present time, α-galactosidase enzymes are classified into five glycosidase families based on protein sequence alignment methods.[5] These families have been

assigned glycosyl hydrolase (GH) numbers 4, 27, 36, 57 and 110. Most of these categorized α-galactosidases (EC 3.2.1.22) are retaining hydrolases, that is, the first product formed during the catalysed hydrolysis of an α-galactopyranoside is α-galactopyranose. Moreover, as family GH 4 members operate via an atypical mechanism involving oxidation of the 3-OH group followed by an El_{CB} elimination reaction,[114] compounds that are designed to inhibit glycopyranosylium ion-like transitions states are not expected to inhibit enzymes from glycosidase family GH 4. Consequently, discussion will be limited to retaining glycosidases that hydrolyse sugar acetal linkages via a glycosylated enzyme intermediate.[9,115] Both enzyme-catalysed glycosylation and deglycosylation steps occur via transition states (TSs) that have oxacarbenium ion character and a distorted six-membered ring.

While no single compound effectively inhibits all galactosidases[74,77,92] most inhibitors possess structural features that mimic certain facets of the galactopyranosylium ion (**2.1**), a high-energy intermediate formed during the acid-catalysed hydrolysis of galactosides in aqueous solution.[116-118] Regardless of whether the enzyme-catalysed glycosylation and deglycosylation steps are dissociative (D_N * A_N)[119,120] or "exploded" associative (A_ND_N)[120,121] reactions, it is clear that there is a sizable degree of cationic character on the sugar moiety at the critical enzymatic TSs,[122,123] which therefore must bear some resemblance to the galactopyranosylium ion. Thus, 1-deoxy-*galacto*-nojirimycin (**1.35**),[63,124] *galacto*-isofagomine (**1.39**)[77,125] and *galacto*-validamine (**2.2**)[126] mimic charge development that occurs along the reaction coordinate by incorporating a basic nitrogen atom in place of *O*-5, *C*-1 and *O*-1,

respectively (Figure 2.1). Whereas, *galacto*-valienamine (**1.23**) mimics both the ring distortion and charge development present in galactopyranosylium ions.[67]

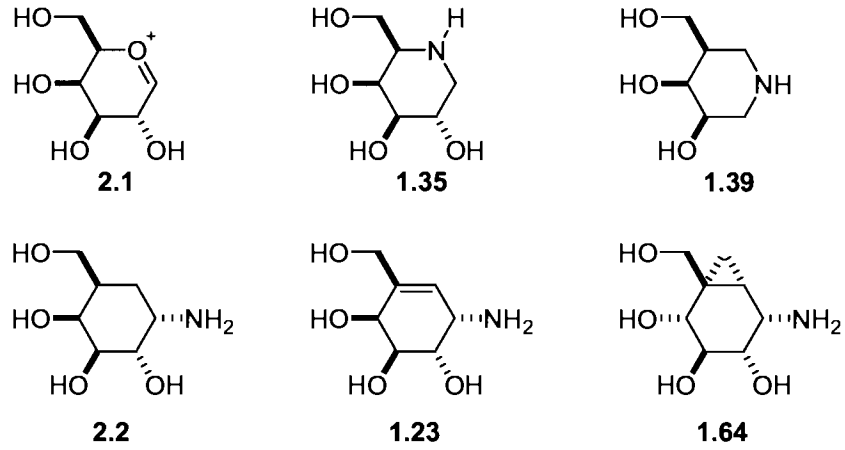


Figure 2.1 Structures of the galactopyranosylium ion (**2.1**) and selected glycosidase inhibitors that contain a basic amino group

In 2001, Tanaka *et al.* reported that sub-micromolar inhibition was obtained for yeast α-glucosidase using the carbocyclic bicyclo[4.1.0]heptylamine α-glucopyranose mimic **1.64**.[99] The current report details the synthesis and inhibitory activity of the four structurally similar cyclopropane containing bicyclic galactose analogues **1.80**, **2.3**-**2.5** (Figure 2.2).

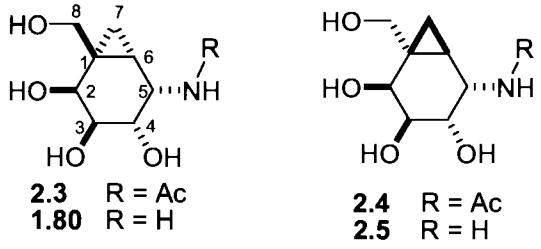
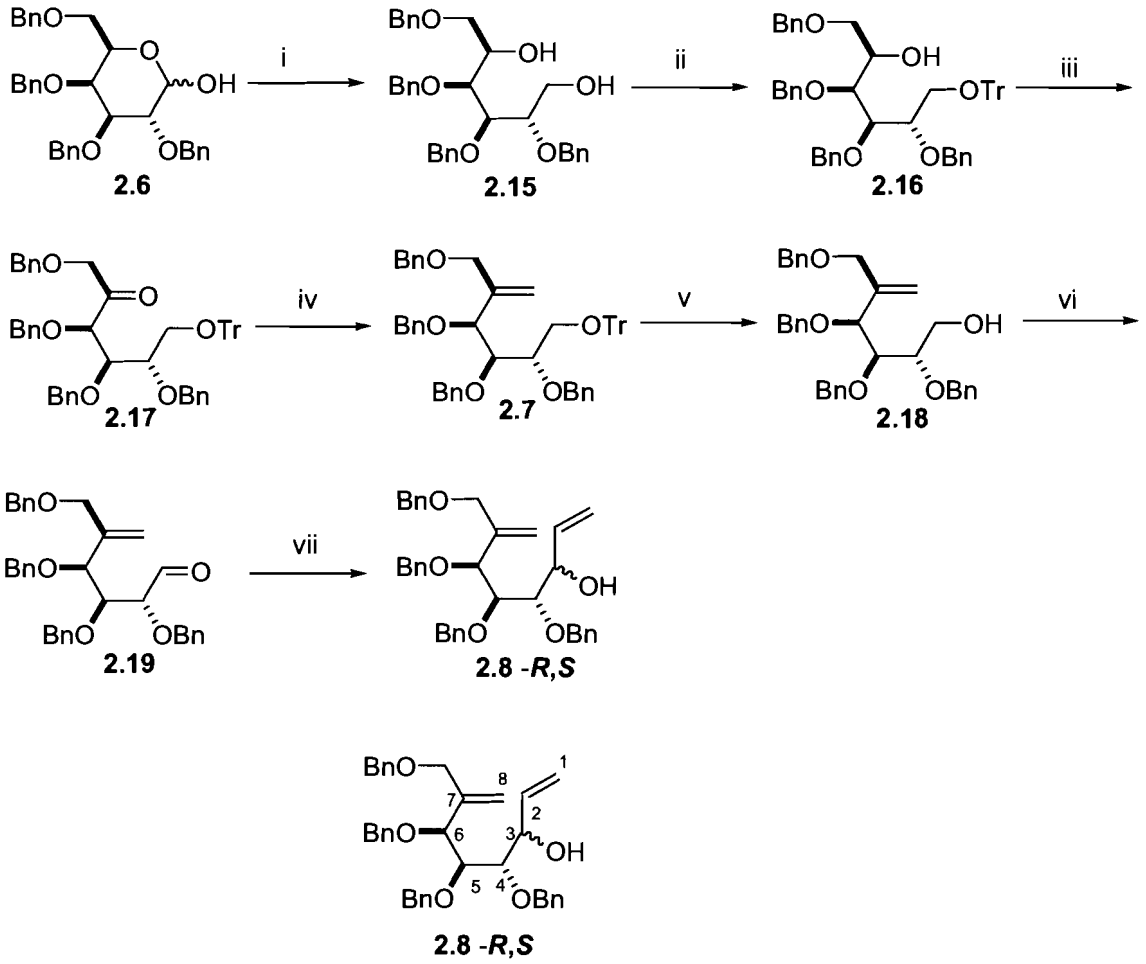


Figure 2.2 Structures of the designed bicyclo[4.1.0]heptane-based potential galactosidase inhibitors **1.80** and **2.3** - **2.5**

2.2 Results and Discussion

The route chosen for the synthesis of these inhibitors utilized 2,3,4,6-tetra-*O*-benzyl-D-galactose (**2.6**), which can be made in two steps from methyl α-D-galactopyranoside,[127] as the starting material.

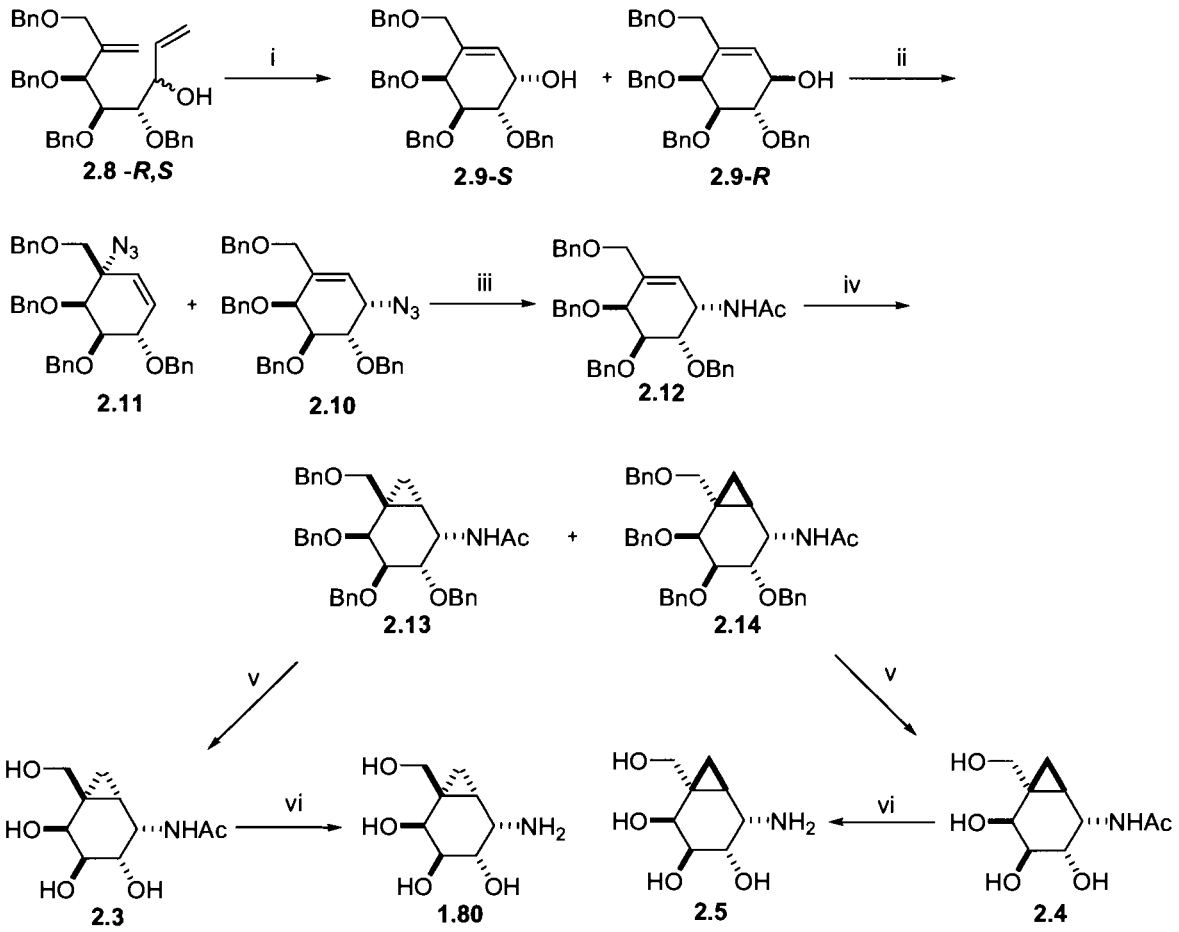
After reduction of the protected hemiacetal, selective protection of the primary alcohol as a trityl ether, followed by a Swern oxidation and a Wittig reaction gave olefin **2.7** in a yield of 47% over these four steps (Scheme 2.1). Removal of the trityl protecting group using mild acid-catalysis and subsequent Swern oxidation and addition of vinyl magnesium bromide gave a 1:2 ratio of the *R*:*S* diastereoisomers of an acyclic octadienol **2.8-*R*,S** (Scheme 2.1). Of note, the assignment of stereochemistry is based on the ratio of compound **2.9-*R*** to **2.9-*S*** that is formed by a ring closing metathesis reaction catalysed by a second generation Grubbs' catalyst.[128] Specifically, the stereochemistry of compound **2.9-*R*** was assigned based on the larger coupling constant observed between H-1 and H-6 in the ^{1}H NMR spectrum (6.4 Hz) relative to that of the **2.9-*S*** diastereoisomer (4.3 Hz).[129] In addition, an NOE contact was observed for compound **2.9-*R*** between H-1 and H-5. Following separation of the *R*-diastereoisomer **2.9-*R*** from the *pseudo*-axial isomer **2.9-*S***, a process that was accomplished by the use of radial chromatography,[130] incorporation of an azide functionality was accomplished with inversion of configuration to give the allylic azide **2.10**, along with a small amount of the S_N2' product **2.11**. Reduction of the azide, followed by an acylation gave the acetamide **2.12** (Scheme 2.2).



Scheme 2.1 Synthesis of (3*R*or* 3*S**,4*S*,5*S*,6*S*)-4,5,6-tribenzyloxy-7-[(benzyloxy)methyl]-octa-1,7-dien-3-ol (**2.8-*R*,*S***). *Reagents and Conditions*: i, $NaBH_4$, EtOH, rt, 93%; ii, TrCl, pyridine, rt, 74%; iii, DMSO, Ac_2O, rt, 86%; iv, Ph_3PCH_3I, *n*-BuLi, –78 °C to 0 °, 79%; v, HCl, H_2O, AcOH, rt, 61%; vi, $(COCl)_2$, DMSO, –78 °C, then Et_3N; vii, $CH_2CHMgBr$, –78 °C, *R*:*S* 1:2, 60%

Subsequent use of the Furukawa modification of the Simmons-Smith reaction gave the two cyclopropyl isomers in a total yield of 88%.[131] Of note, the ratio of the D-*galacto* (**2.13**) and L-*altro* (**2.14**) diastereoisomers formed in this reaction was unpredictable, that is, it varied between 2.5:1.0 and 1.0:1.5 (Scheme 2.2). The absolute stereochemistry for the two diastereoisomers was assigned based on observed NOE

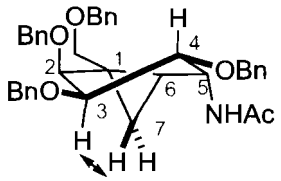
contacts between one of the H-7 protons and either H-3 or H-4 in the 1D NOE difference spectra (Figure 2.3).



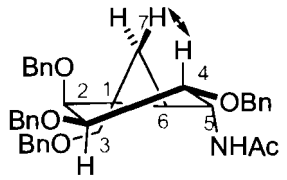
Scheme 2.2 Synthesis of reversible inhibitors **1.80** and **2.5**. *Reagents and Conditions*: i, Grubb's, CH_2Cl_2, 40 °C, *R*-32%, *S*-54%; ii, $(PhO)_2P(O)N_3$, $PhCH_3$, DBU, 60 °C, 15-76%, 16-21%; iii, H_2S, Et_3N, H_2O, rt, then AcCl, pyridine, 80%; iv, $ZnMe_2$, CH_2I_2, -10 °C, 88%; v, 10% Pd-C, H_2, rt; vi, LiOH, THF:H_2O 1:1, 70 °C

Removal of the benzyl protecting groups was accomplished using conventional hydrogenation conditions to give the amides **2.3** and **2.4** in good yields. Subsequent hydrolysis of the amide groups, using LiOH in THF/H_2O gave the corresponding

amines **1.80** and **2.5** (Scheme 2.2). It was not possible to obtain crystals of either compound **1.80** or **2.5**, however, their conformations in solution are likely similar to their respective *gluco*-analogues[99] because of the similarity of ^{1}H-^{1}H coupling constants for the *pseudo*-anomeric hydrogen atom.



2.13

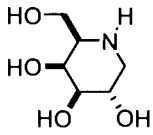
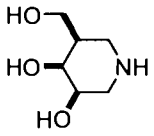
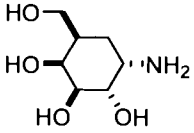
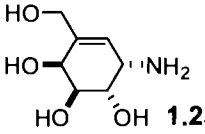
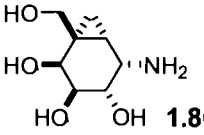
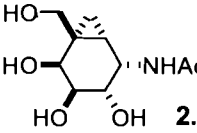
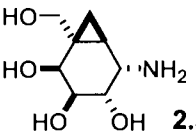
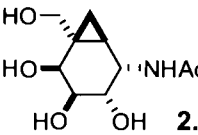


2.14

Figure 2.3 Observed NOE contacts for the protected bicyclo[4.1.0]heptane-based galactoside analogues (**2.13** and **2.14**)

Amines **1.80** and **2.5**, and their respective acetamido compounds **2.3** and **2.4** were tested as inhibitors against two commercially available α-galactosidase enzymes. Measured inhibition parameters (K_i or IC_{50} values) for these four bicyclo[4.1.0]heptane derivatives and the reported values for compounds **1.35**, **1.39**, **2.2** and **1.23** are listed below (Table 2.1).

Table 2.1 Inhibitory activity (IC_{50} μM) values to two α-galactosidases

	α-galactosidases		reference
	coffee bean	*E. coli*	
 **1.35**	0.0016	0.24	63
 **1.39**	200		125
 **2.2**	500	890	126
 **1.23**	56		67
 **1.80**	0.541 ± 0.018 [c]	80 ± 6	
 **2.3**	343 ± 14	NI [a]	
 **2.5**	286 ± 15	2460 ±130	
 **2.4**	NI [a]	NI [b]	

[a] No inhibition observed at 1.0 Mm; [b] No inhibition observed at 5.0 mM; [c] K_i value

The plot (Figure 2.4) for inhibition of 4-nitrophenyl α-D-galactopyranoside hydrolysis catalysed by coffee bean α-galactosidase on the addition of compound **1.80**

shows that this compound binds competitively to the active site of the enzyme. Specifically, fitting the kinetic data displayed in Figure 2.4 to three standard models of inhibition, namely: competitive, non-competitive, and uncompetitive results in the following "goodness of fit" (R^2 values) of 0.999, 0.983, and 0.956, respectively.

Moreover, the measured K_i value (541 ± 18 nM) for inhibition of coffee bean α-galactosidase shows that compound **1.80** is the tightest binding inhibitor of those in which the basic nitrogen atom of the inhibitor is located in the position of a substrate's glycosidic oxygen atom (*cf.*, compound **2.2**, **1.23**, and **1.80** in Table 2.1), an observation that matches the inhibition of yeast α-glucosidase by compound **1.64**, valienamine and validamine.[99]

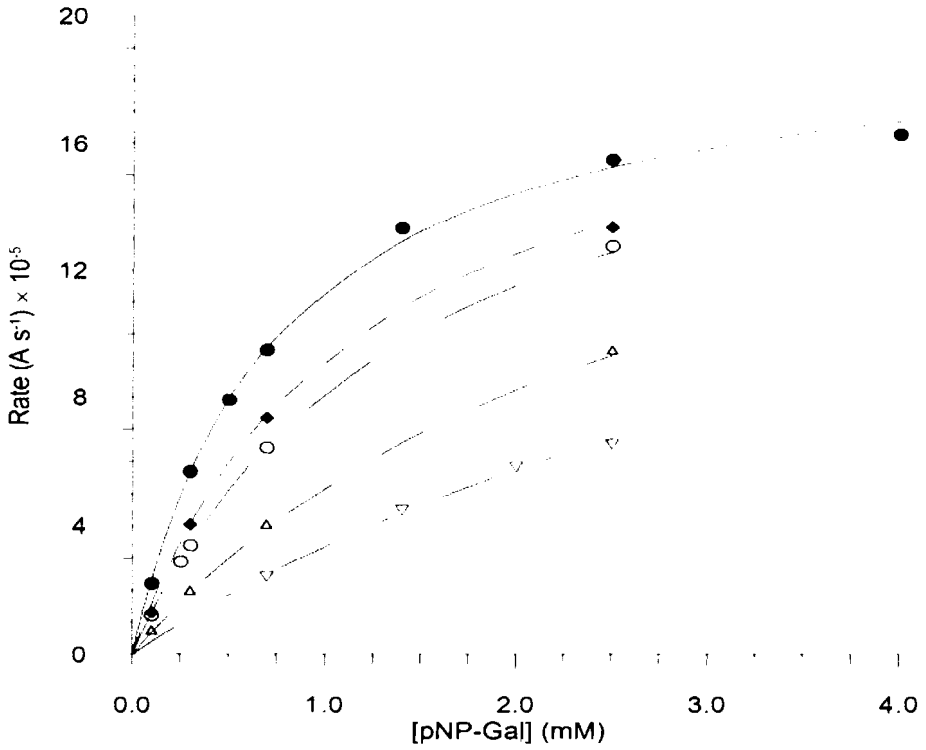


Figure 2.4 Plot for inhibition of coffee bean α-galactosidase by compound **1.80**. Drawn lines are for the fit of the data to a competitive inhibition model. Concentrations of inhibitor **1.80** are: ● 0.0 μM, ♦ 0.3 μM, o 0.5 μM, Δ 1.5 μM, and ∇ 3.0 μM.

Given that all IC_{50} values were measured at low substrate concentration (between 0.20 and 0.25 times K_m) the reported IC_{50} values are between 1.20 and 1.25 times larger than the true inhibition constant K_i,[132] assuming that all inhibitors bind competitively to the enzyme. With respect to inhibition of the GH27 enzyme (coffee bean), the comparison between the *galacto*-bicyclic inhibitor **1.80** and both compounds **1.23** and **2.5** is particularly interesting since these compounds are structurally very similar, and compound **1.80** is ~100 fold (11.9 kJ/mol) tighter binder than either the alkene **1.23** or the *altro*-isomer **2.5**. This observation suggests that in compound **1.80** the hydroxymethyl group (CH_2OH) has more optimal interactions with the coffee bean

enzyme, but that in both compound **1.23** and **2.5** no severe steric interactions are introduced by changing the position of this group relative to the carbasugar portion of the inhibitors. Also, the contrast between the potency of amine **1.80** and acetamide **2.3** supports the idea that a positive charge enhances inhibitor binding to the enzymatic active site, in this case the difference in the free energy of binding is 14.5 kJ/mol.

Given that both glycosyl hydrolase families GH27 and GH36 share a commonality of mechanism,[27] it is not surprising that inhibitor **1.80** is also the tightest binder to the GH36 enzyme from *E. coli.* (*cf.*, **2.2**, **1.80**, and **2.5** in Table 2.1). An extended discussion on the differences in binding affinity of compound **1.80** between the two GH family members is unwarranted until structural data is available.

Also of note, the weakest binding inhibitor of the three compounds that are present in $^4C_1$ conformations (**1.35**, **1.39** and **2.2**; Table 2.1) is compound **2.2**, the inhibitor where the basic nitrogen atom is incorporated in place of *O*-1. It remains to be seen whether analogues of compound **1.35** and **1.39** in which the pyranosyl ring is held in non-chair conformations are tighter binding inhibitors than the parent compounds themselves as is the case for compound **1.80**.

2.3 Conclusions

A tight binding competitive inhibitor of coffee bean α-galactosidase (**1.80**) was prepared in 13 steps from 2,3,4,6-tetra-*O*-benzyl-D-galactose in an overall yield of 0.6%.

2.4 Experimental

All the chemicals were purchased from Aldrich company and used without further purification. Thin-layer chromatography (TLC) was performed on aluminium-backed TLC plates pre-coated with Merck silica gel 60 F_{254}. Compounds were visualized with UV light and/or staining with phosphomolybdic acid (5% solution in EtOH). Flash chromatography was performed using Avanco silica gel 60 (230-400 mesh). Radial chromatograph was performed on a Harrison Research Inc. model 8924 chromatotron. Melting points were recorded on a Gallenkamp melting point apparatus and are uncorrected. Solvents used for anhydrous reactions were dried and distilled immediately prior to use. Methanol was dried and distilled over magnesium methoxide. Dichloromethane was dried and distilled over calcium hydride. Glassware for anhydrous reactions was flame-dried and cooled under a nitrogen atmosphere immediately prior to use. NMR spectra were recorded on a Varian Unity 500 MHz spectrometer and operated at a frequency of 499.767 MHz. Chemical shifts (δ) are listed in ppm downfield from TMS using the residual solvent peak as an internal reference. ^{1}H and ^{13}C NMR peak assignments are made based on ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMQC experiments. Coupling constants are reported in Hz. IR spectra were recorded on a Bomem IR spectrometer and samples were prepared as cast evaporative films on NaCl plates from CH_2Cl_2. Optical rotations were measured using a Perkin-Elmer 341 polarimeter and are reported in units of deg cm^2 g^{-1} (concentrations reported in units of g/100 mL).

Green coffee bean (GH27) and *E. coli* (GH36) α-galactosidases were purchased from Sigma-Aldrich and Calbiochem, respectively.

2,3,4,6-Tetra-*O*-benzyl-D-galactitol (2.15)

$NaBH_4$ (2.8 g, 74 mmol) was added to a solution of compound **2.6**[127] (17.5 g, 32.4 mmol) in ethanol (EtOH) (180 mL). After the reaction mixture has been stirred for 15 h, it was diluted with ether (Et_2O) (300 mL). Then it was washed with water (2 × 100 mL), brine (100 mL), dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane:EtOAc, 2:1 v/v) to give compound **2.15** as a colourless syrup (16.3 g, 93%): $[\alpha]^{20}_D$ –5.5 (*c* = 1.85, CH_2Cl_2); IR 3443 (br, OH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.50 (dd, 1 H, $J_{6,6'}$ = 9.1, $J_{5,6}$ = 6.7, H-6), 3.54 (dd, 1 H, $J_{5,6'}$ = 5.5, H-6'), 3.68–3.73 (m, 2 H, H-3, H-1), 3.79 (m, 1 H, H-1'), 3.86–3.91 (m, 2 H, H-2, H-4), 4.03 (m, 1 H, H-5), 4.42 (d, 1 H, $J_{A,B}$ = 11.9, $CH_AH_BC_6H5$), 4.47 (d, 1 H, $CH_AH_BC_6H_5$), 4.49 (d, 1 H, $J_{C,D}$ = 11.3, $CH_CH_DC_6H_5$), 4.60 (d, 1 H, $J_{E,F}$ = 11.6, $CH_EH_FC_6H_5$), 4.64 (d, 1 H, $CH_CH_DC_6H_5$), 4.67 (d, 1 H, $CH_EH_FC_6H_5$), 4.68 (d, 1 H, $J_{G,H}$ = 11.3, $CH_GH_HC_6H_5$), 4.75 (d, 1 H, $CH_GH_HC_6H_5$), 7.22–7.36 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 60.8, 69.7, 70.8, 72.2, 73.1, 73.6, 74.2, 77.3, 79.0, 79.9, 127.6, 127.7, 128.0, 128.2, 128.3, 137.7, 137.8, 137.9.

2,3,4,6-Tetra-*O*-benzyl-1-*O*-trityl-D-galactitol (2.16)

To a solution of **2.15** (5.4 g, 10 mmol) in dry pyridine (17 mL) was added chlorotriphenylmethane (3.1 g, 11 mmol) was added. The reaction mixture was stirred at rt for 72 h, and then it was poured onto ice (200 mL). The resultant slurry was stirred for 2 h at 0 °C, and the aqueous layer was decanted from the syrup. The syrup was then dissolved in chloroform (120 mL) and washed with aqueous acetic acid (AcOH) (10%, 30 mL), saturated aqueous $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated under reduced pressure. The resultant residue was purified by flash column chromatography (hexane: EtOAc, 6:1 v/v) to give compound **2.16** as a light yellow syrup (5.8 g, 74%):

$[\alpha]^{20}_D$ +22.5 (c = 1.32, CH_2Cl_2); IR 3529 (br, OH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.23 (dd, 1 H, $J_{1,1'}$ = 10.3, $J_{1,2}$ = 4.9, H-1), 3.41 (dd, 1 H, $J_{6,6'}$ = 9.4, $J_{6,5}$ = 6.0, H-6), 3.45 (dd, 1 H, $J_{1',2}$ = 4.7, H-1'), 3.53 (dd, 1 H, $J_{6',5}$ = 6.4, H-6'), 3.69 (dd, 1 H, $J_{4,5}$ = 1.7, $J_{4,3}$ = 5.2, H-4), 3.84 (q, 1H, $J_{2,3} + J_{2,1} + J_{2,1'}$ = 14.5, H-2), 4.00 (dt, 1 H, H-5), 4.18 (t, 1 H, H-3), 4.31 (d, 1 H, $J_{A,B}$ = 11.7, $CH_AH_BC_6H_5$), 4.37 (d, 1 H, $J_{C,D}$ = 12.0, $CH_CH_DC_6H_5$), 4.42 (d, 1 H, $CH_CH_DC_6H_5$), 4.44 (d, 1 H, $CH_AH_BC_6H_5$), 4.51 (d, 1 H, $J_{E,F}$ = 11.7, $CH_EH_FC_6H_5$), 4.62 (d, 1 H, $J_{G,H}$ = 11.0, $CH_GH_HC_6H_5$), 4.66 (d, 1 H, $CH_EH_FC_6H_5$), 4.73 (d, 1 H, $CH_GH_HC_6H_5$), 7.09–7.44 (m, 35 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 63.3 (C-1), 70.2 (C-5), 71.5 (C-6), 72.8, 73.0, 73.4, 75.2 (4 × $CH_2C_6H_5$), 77.1 (C-4), 78.9 (C-2), 79.8 (C-3), 87.1 (CPh_3), 127.2, 127.7, 127.8, 127.9, 128.0, 128.2, 128.3, 128.4, 128.4_4, 128.8, 138.2, 138.2, 138.4, 138.5, 144.0. Anal. Calcd For $C_{53}H_{52}O_6$: C, 81.1; H, 6.7. Found: C, 80.5; H, 6.6.

(3*R*,4*R*,5*S*)-1,3,4,5-tetrabenzyloxy-6-trityloxy-2-hexanone (2.17)

Acetic anhydride (17 mL, 180 mmol) was added to a solution of **2.16** (6.7 g, 8.5 mmol) in dry dimethylsulfoxide (DMSO) (27 mL). The reaction mixture was stirred for 16 h at rt under N_2. Following the addition of water (40 mL) to the reaction mixture, aqueous NH_3 (28%) was added. The resultant mixture was extracted with Et_2O (3 × 30 mL) and the combined organic layers were washed with brine (60 mL), dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by flash column chromatograph (hexane: EtOAc, 9:1 v/v) to give compound **2.17** as a yellow syrup (5.7 g, 86%): $[\alpha]^{20}_D$ +0.17 (c = 0.60, CH_2Cl_2); IR 1731 (CO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.33 (dd, 1 H, $J_{6,6'}$ = 10.4, $J_{5,6}$ = 5.3, H-6), 3.46 (dd, 1 H, $J_{5,6'}$ = 3.7, H-6'), 3.84 (m, 1 H, H-5), 3.88 (d, 1 H, $J_{3,4}$ = 3.9, H-3), 4.22 (dd, 1 H, $J_{4,5}$ = 5.7, H-4), 4.25–4.46 (m, 6 H,

$CH_2C_6H_5$), 4.51 (d, 1 H, $J_{A,B}$ = 11.6, $CH_AH_BC_6H_5$), 4.58 (d, 1 H, $CH_AH_BC_6H_5$), 4.60 (bs, 2 H, H-1, H-1'), 7.01–7.44 (m, 35 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 63.3 (C-6), 72.4, 73.0, 73.0_5, 74.4 (4 × $CH_2C_6H_5$), 74.5 (C-1), 79.0 (C-5), 80.4 (C-4), 82.6 (C-3), 86.8 (CPh_3), 127.0, 127.4, 127.5, 127.6, 127.7, 127.7_7, 127.8, 127.9, 128.1, 128.2, 128.2_6, 128.2_8, 128.3, 128.4, 128.6, 137.3, 137.6, 137.9, 138.3, 143.9, 207.4 (C-2). Anal. Calcd For $C_{53}H_{50}O_6$: C, 81.3; H, 6.4. Found: C, 80.95; H, 6.7.

(3*S*,4*S*,5*S*)-3,4,5-tribenzyloxy-2-[(benzyloxy)methyl)]-6-trityloxy-1-hexene (2.7)

To a solution of methyltriphenylphosphonium iodide (1.1 g, 2.8 mmol) in tetrahydrofuran (THF) (10 mL), was added *n*-butyllithium (2.5 M in hexane, 1.1 mL, 2.8 mmol) at –78 °C. After stirring the reaction at this temperature for 1 h, the mixture was allowed to warm to 0 °C and a solution of **2.17** (1.0 g, 1.3 mmol) in THF (5 mL) was added dropwise. The resulting mixture was allowed to warm to rt over a period of 16 h. The reaction mixture was quenched by the addition of a solution of saturated aqueous NH_4Cl, and it was then diluted with water (20 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were washed with aqueous H_2SO_4 (10%), saturated aqueous $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated at reduced pressure. The resultant crude syrup was purified by flash column chromatography (hexane: EtOAc, 9:1 v/v) to give the product **2.7** as a light yellow syrup (0.78 g, 79%): $[\alpha]^{20}_D$ +22.5 (*c* = 1.32, CH_2Cl_2); IR 1650 (C=C) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.25 (dd, 1 H, $J_{6,6'}$ = 10.0, $J_{6,5}$ = 5.6, H-6), 3.27 (dd, $J_{6',5}$ = 5.3, H-6'), 3.81 (br q, 1 H, $J_{6,5} + J_{6',5} + J_{5,4}$ = 14.7, H-5), 3.86 (dd, 1 H, $J_{4,5}$ = 3.9, $J_{4,3}$ = 7.1, H-4), 4.04–4.16 (m, 4 H, H-3, H-7, H-7', $CH_AH_BC_6H_5$), 4.43 (d, 1 H, $J_{C,D}$ = 11.0, $CH_CH_DC_6H_5$), 4.47 (d, 1 H, $J_{B,A}$ = 11.6, $CH_AH_BC_6H_5$), 4.48 (d, 1 H, $J_{E,F}$ = 12.0, $CH_EH_FC_6H_5$), 4.49 (d, 1 H, $J_{G,H}$ = 11.6,

$CH_GH_HC_6H_5$), 4.52 (d, 1 H, $CH_EH_FC_6H_5$), 4.57 (d, 1 H, $CH_CH_DC_6H_5$), 4.64 (d, 1 H, $CH_GH_HC_6H_5$), 5.13 (bs, 1 H, H-1), 5.44 (m, 1 H, H-1'), 7.07–7.43 (m, 35 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 63.7 (C-6), 70.4 (C-7), 71.0, 72.8, 73.7, 74.8 (4 × $CH_2C_6H_5$), 78.6 (C-5), 79.8 (C-3), 81.2 (C-4), 87.0 (CPh_3), 115.9 (C-1), 127.0, 127.4, 127.5, 127.6, 127.7, 127.8, 127.9, 128.1, 128.3, 128.3_8, 128.4, 128.8, 138.5, 138.5_3, 138.6, 139.0, 144.0 (C-2), 144.1. Anal. Calcd For $C_{54}H_{52}O_5$: C, 83.05; H, 6.7. Found: C, 82.8; H, 6.8.

(2*S*,3*S*,4*S*)-2,3,4-tribenzyloxy-5-[(benzyloxy)methyl]-5-hexen-1-ol (2.18)

Aqueous HCl (1 M, 1 mL) was added to a solution of compound **2.7** (10 g, 13 mmol) in aqueous AcOH (90%, 150 mL). After stirring this mixture for 40 min, the reaction was quenched by the addition of saturated aqueous $NaHCO_3$. The resultant solution was extracted with Et_2O (3 × 50 mL). The combined organic layers were washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure. The crude product was purified by flash column chromatography (hexane:EtOAc, 3:1 v/v) to give compound **2.18** as a light yellow syrup (4.2 g, 61%): $[\alpha]^{20}_D$ + 22.8 (c = 0.71, CH_2Cl_2); IR 3470 (br, OH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.59 (dd, 1 H, $J_{1,2}$ = 4.8, $J_{1,1'}$ = 11.7, H-1), 3.66 (dd, 1 H, $J_{1',2}$ = 4.5, H-1'), 3.70–3.76 (m, 2 H, H-2, H-3), 4.06–4.18 (m, 3 H, H-4, H-7, H-7'), 4.22 (d, 1 H, $J_{A,B}$ = 11.4, $CH_AH_BC_6H_5$), 4.48–4.62 (m, 7 H, $CH_2C_6H_5$), 5.45 (bs, 1 H, H-6), 5.55 (bs, 1 H, H-6'), 7.20–7.34 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 62.0 (C-1), 70.6 (C-7), 70.5, 72.8, 73.2, 74.4 (4 × $CH_2C_6H_5$), 79.7, 81.0 (C-3, C-2), 80.1 (C-4), 116.2 (C-6), 127.6, 127.7, 127.7, 127.9, 128.0, 128.3, 128.4, 138.0, 138.2, 138.3, 138.6, 143.7 (C-5). Anal. Calcd For $C_{35}H_{38}O_5$: 78.0; H, 7.1. Found: C, 77.78; H, 6.87.

(2*S*,3*S*,4*S*)-2,3,4-tribenzyloxy-5-[(benzyloxy)methyl]-5-hexenal (2.19)

To a solution of oxalyl chloride (0.23 mL, 2 M in CH_2Cl_2, 0.46 mmol) in CH_2Cl_2 (2.6 mL) at –78 °C, DMSO (0.11 mL, 1.6 mmol) was added slowly. This mixture was stirred for 10 min, and then a solution of compound **2.18** (206 mg, 0.38 mmol) in CH_2Cl_2 (1.5 mL) was added dropwise. After 10 min, triethylamine (Et_3N) (0.26 mL, 1.9 mmol) was added to the reaction mixture, which was kept at –78 °C for 40 min. The solvent was then evaporated under reduced pressure to give compound **2.19** as a light yellow syrup (196 mg), which was used in the next step without further purification.

(3*R,4*S*,5*S*,6*S*)-4,5,6-tribenzyloxy-7-[(benzyloxy)methyl]-octa-1,7-dien-3-ol (2.8-*R* and 2.8-*S*)**

To a solution of the freshly prepared aldehyde **2.19** in dry THF (50 mL) at –78 °C under N_2 was slowly added a solution of vinylmagnesium bromide in THF (0.30 mL, 1.0 M, 0.30 mmol). The reaction mixture was then stirred for 1 h at rt, after which it was quenched by the addition of saturated aqueous NH_4Cl. The resultant solution was extracted with EtO_2 (3 × 60 mL) and the combined organic layers were washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure to give crude product as an orange coloured syrup. This material was purified by flash column chromatography (hexane: EtOAc, 4:1 v/v) to give compound **2.8-*R*** and **2.8-*S*** as a pale yellow syrup (129 mg, ratio **2.8-*R*** : **2.8-*S*** = 1:2, 60% over 2 steps): IR 3463 (br, OH) cm^{-1}; Anal. Calcd For $C_{37}H_{40}O_5$: C, 78.7; H, 7.1. Found: C, 78.7; H, 7.05. The NMR spectral assignments given below were made on the mixture of diastereoisomers (Scheme 2.1), therefore, some peaks were difficult to be assigned.

Compound **2.8**-*R*: ^1H NMR ($CDCl_3$) δ 2.66 (d, 1 H, $J_{OH,3}$ = 5.6, OH), 3.66 (t, 1 H, $J_{4,5} + J_{4,3}$ = 9.0, H-4), 3.80 (dd, 1 H, $J_{5,4}$ = 4.8, $J_{5,6}$ = 7.1, H-5), 4.05–4.18 (m, 3 H, H-

6, H-9, H-9'), 4.24–4.31 (m, 2 H, H-3, $CH_AH_BC_6H_5$), 4.45–4.70 (m, 7 H, $CH_2C_6H_5$), 5.15 (dt, 1 H, $J_{1E,2}$ = 10.5, $J_{1E,1Z} + J_{1E,3}$ = 2.7, H-1*E*), 5.26 (dt, 1 H, $J_{1Z,2}$ = 17.2, $J_{1Z,1E} + J_{1Z,3}$ = 2.9, H-1*Z*), 5.44 (bs, 1 H, H-8), 5.56 (d, 1 H, $J_{8,8'}$ = 1.5, H-8'), 5.86 (m, 1 H, H-2), 7.36–7.21 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 70.5 (C-3), 81.1 (C-5), 82.1 (C-4), 116.3 (C-1), 116.4 (C-8), 138.2 (C-2), 143.8 (C-7).

Compound **2.8-*S***: ^{1}H NMR ($CDCl_3$) δ 2.62 (d, 1 H, $J_{OH,3}$ = 5.8, OH), 3.71 (dd, 1 H, $J_{4,5}$ = 3.1, $J_{4,3}$ = 5.5, H-4), 3.86 (dd, 1 H, $J_{5,6}$ = 8.0, H-5), 4.05–4.19 (m, 4H, H-6, H-9, H-9', $CH_AH_BC_6H_5$), 4.41 (m, 1 H, H-3), 4.45–4.70 (m, 7 H, $CH_2C_6H_5$), 5.21 (dt, 1 H, $J_{1E,2}$ =10.6, $J_{1E,1Z} + J_{1E,3}$ = 3.2, H-1*E*), 5.37 (dt, 1 H, $J_{1Z,2}$ = 17.2, $J_{1Z,1E} + J_{1Z,3}$ = 3.3, H-1*Z*), 5.47 (bs, 1 H, H-8), 5.58 (d, 1 H, $J_{6,8'}$ = 1.5, H-8), 5.94 (m, 1 H, H-2), 7.36–7.21 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 72.2 (C-3), 80.4 (C-5), 80.6 (C-4), 116.2 (C-1), 116.5 (C-8), 138.1 (C-2), 143.8 (C-7). Because this is mixture of

(1*R,4*S*,5*S*,6*S*)-4,5,6-tribenzyloxy-3-[(benzyloxy)methyl]cyclohex-2-enol (2.9-*R* and 2.9-*S*)**

To a solution of the diastereoisomeric mixture of **2.8-*R*** and **2.8-*S*** (2.3 g, 4.1 mmol) in dry CH_2Cl_2 (1.1 L) was added second generation Grubbs' catalyst (0.17 g, 0.21 mmol). This reaction mixture was heated to reflux for 3 h under N_2, and then the volatiles were removed under reduced pressure. The resultant residue was purified by flash column chromatography (hexane:EtOAc, 2:1 v/v) to give a mixture **2.9-*R*** and **2.9-*S*** as a colourless syrup. The two diastereoisomers were separated by radial chromatography (CH_2Cl_2:EtOAc, 25:1 v/v) to give compound **2.9-*R*** (0.72 g, 32%) and compound **2.9-*S*** (1.2 g, 54%) as colourless syrups:

Compound **2.9-*R***: $[\alpha]^{20}_{D}$ +15.8 (*c* = 1.61, CH_2Cl_2); IR 3420 (br, OH) cm^{-1}; 3.64 (dd, 1 H, $J_{5,4}$ = 3.5, $J_{5,6}$ = 9.2, H-5), 3.88 (d, 1 H, $J_{7,7'}$ = 12.1, H-7), 3.96 (dd, 1 H, $J_{6,1}$ = 6.4, H-6), 4.08 (d, 1 H, H-7'), 4.14 (m, 1 H, H-1), 4.27 (d, 1 H, H-4), 4.38 (d, 1 H, $J_{A,B}$ = 11.8, $CH_AH_BC_6H_5$), 4.44 (d, 1 H, $CH_AH_BC_6H_5$), 4.58 (d, 1 H, $J_{C,D}$ = 11.3, $CH_CH_DC_6H_5$), 4.75 (d, 1 H, $J_{E,F}$ = 11.6, $CH_EH_FC_6H_5$), 4.78–4.81 (m, 2 H, $CH_2C_6H_5$), 4.89 (d, 1 H, $CH_CH_DC_6H_5$), 4.91 (d, 1 H, $CH_EH_FC_6H_5$), 5.76 (d, 1 H, $J_{2,1}$ = 2.7, H-2), 7.25–7.40 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 70.6 (C-7), 71.1 (C-1), 72.4 ($CH_AH_BC_6H_5$), 73.3 ($CH_GH_HC_6H_5$), 73.5 (C-4), 74.3 ($CH_EH_FC_6H_5$), 74.4 ($CH_CH_DC_6H_5$), 79.5 (C-5), 80.6 (C-6), 127.6, 127.6_4, 127.6_6, 127.7, 127.8, 128.0, 128.0_1 (C-2), 128.1, 128.3, 128.4, 128.4_2, 128.4_8, 135.4, 138.1, 138.4, 138.6, 138.7. Anal. Calcd For $C_{35}H_{36}O_5$: C, 78.3; H, 6.8. Found: C, 78.0; H, 6.85.

(3*S*,4*S*,5*S*,6*S*)-3-azido-4,5,6-tribenzyloxy-1-[(benzyloxy)methyl]cyclohexene (2.10) and (3*S*,4*S*,5*R*,6*S*)-3-azido-4,5,6-tribenzyloxy-3-[(benzyloxy)methyl]cyclohexene (2.11)

To a solution of compound **2.9-*R*** (754 mg, 1.4 mmol) in dry toluene (60 mL) was added diphenyl phosphoryl azide (1.8 mL, 8.4 mmol) followed by the slow addition of 1,8-diazabicyclo[5.4.0]undec-7-ene (1.2 mL, 8.0 mmol) at 0 °C under a N_2 atmosphere. The reaction mixture was stirred for 1.5 h at 0 °C, after which sodium azide (374 mg, 5.8 mmol) and 15-crown-5 (0.6 mL, 3 mmol) were added. The reaction mixture was subsequently heated to 60 °C for 14 h. The resultant mixture was then diluted with EtOAc (120 mL). The mixture was washed with aqueous H_2SO_4 (10%), saturated aqueous $NaHCO_3$, brine, dried (Na_2SO_4), and concentrated under reduced pressure. The syrupy residue was purified by flash column chromatography (hexane:

EtOAc, 8:1 v/v) to give compound **2.10** as a colourless syrup (600 mg, 76%), and the S_N2' tertiary azide product **2.11** as a colourless syrup (160 mg, 21%):

Compound **2.10**: $[\alpha]^{20}_D$ +76.0 (*c* = 1.23, CH_2Cl_2); IR 2030 (N_3) cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.86 (dd, 1 H, $J_{5,6}$ = 3.5, $J_{5,4}$ = 8.1, H-5), 3.89 (d, 1 H, $J_{7,7'}$ = 12.9, H-7), 4.07–4.15 (m, 3 H, H-7', H-3, H-4), 4.24 (d, 1 H, H-6), 4.39 (d, 1 H, $J_{A,B}$ = 11.9, $CH_AH_BC_6H_5$), 4.46 (d, 1 H, $CH_AH_BC_6H_5$), 4.52 (d, 1 H, $J_{C,D}$ = 11.3, $CH_CH_DC_6H_5$), 4.68–4.79 (m, 4 H, $CH_2C_6H_5$), 4.81 (d, 1 H, $CH_CH_DC_6H_5$), 5.72 (d, 1 H, $J_{2,3}$ = 3.7, H-2), 7.19–7.42 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 58.5 (C-3), 70.1 (C-7), 72.0 ($CH_AH_BC_6H_5$), 73.4 ($CH_2C_6H_5$), 73.8 (C-6), 73.8$_3$ ($CH_2C_6H_5$), 74.3 ($CH_CH_DC_6H_5$), 76.5 (C-4), 76.6 (C-5), 120.1, 120.1$_4$, 122.2 (C-2), 125.6, 127.6$_3$, 127.6$_5$, 127.7$_1$, 127.7$_3$, 127.7$_8$, 127.8, 127.9, 128.2, 128.3$_1$, 128.3$_8$, 128.4, 129.8, 138.0, 138.1, 138.4, 138.5, 139.0, Anal. Calcd For $C_{35}H_{35}N_3O_4$: C, 74.8; H, 6.3; N, 7.5. Found; C, 74.5; H, 6.2; N, 7.3.

Compound **2.11:** IR 2099 (N_3) cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.65 (d, 1 H, $J_{7,7'}$ = 9.7, H-7), 3.69 (d, 1 H, H-7'), 3.91 (m, 1 H, H-4), 3.98 (dd, 1H, $J_{5,6}$ + $J_{5,4}$ = 9.6, H-5), 4.40 (m, 1 H, H-6), 4.52 (d, 1 H, $J_{A,B}$ = 12.0, $CH_AH_BC_6H_5$), 4.56 (d, 1 H, $CH_AH_BC_6H_5$), 4.59 (d, 1 H, $J_{C,D}$ = 11.2, $CH_CH_DC_6H_5$), 4.64–4.76 (m, 4 H, $CH_2C_6H_5$), 4.90 (d, 1 H, $CH_CH_DC_6H_5$), 5.61–5.67 (m, 1 H, H-2), 6.01 (dd, 1 H, $J_{1,2}$ = 10.0, $J_{1,6}$ = 2.2, H-1), 7.19–7.40 (m, 20 H, H-Ar). Because compound **2.11** is a byproduct, we didn't fully analyze this compound.

(3*S*,4*S*,5*S*,6*S*)-3-acetamido-4,5,6-tribenzyloxy-1-[(benzyloxy)methyl]cyclohexene (2.12)

H_2S gas was bubbled through a solution containing compound **2.10** (77 mg, 0.14 mmol) in pyridine, Et_3N, and H_2O (4:1:1 v/v/v, 2 mL) for 3 h at rt. Then N_2 gas was used to purge the aqueous reaction mixture, and the resultant mixture was concentrated under reduced pressure. The syrupy residue was dissolved in dry toluene (2.6 mL) that contained pyridine (0.26 mL, 3.2 mmol). Acetyl chloride (0.052 mL, 0.73 mmol) was then added slowly. The reaction mixture was stirred for 1 h at rt, after which it was diluted with EtOAc (10 mL). The resultant mixture was washed with aqueous H_2SO_4 (10%), saturated aqueous $NaHCO_3$, brine, dried (Na_2SO_4), and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane:EtOAc, 2:1 v/v) to give compound **2.12** as a colourless syrup (63 mg, 80%): $[\alpha]^{20}_D$ +69.7 (*c* = 3.76, CH_2Cl_2); IR 3291 (NH) cm^{-1}, 1650 (CO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.92 (s, 3 H, CH_3), 3.81 (dd, 1 H, $J_{5,4} + J_{5,6} = 9.8$, H-5), 3.88–3.93 (m, 2 H, H-7, H-4), 4.25 (d, 1 H, $J_{7',7} = 12.1$, H-7'), 4.30 (br s, 1 H, H-6), 4.42 (d, 1 H, $J_{A,B}$ = 11.8, $CH_AH_BC_6H_5$), 4.43 (d, 1 H, $J_{C,D} = 11.8$, $CH_CH_DC_6H_5$), 4.45 (d, 1 H, $CH_AH_BC_6H_5$), 4.48 (d, 1 H, $J_{E,F} = 11.5$, $CH_EH_FC_6H_5$), 4.49 (d, 1 H, $CH_CH_DC_6H_5$), 4.60 (d, 1 H, $CH_EH_FC_6H_5$), 4.67 (d, 1 H, $J_{G,H} = 12.3$, $CH_GH_HC_6H_5$), 4.71 (d, 1 H, $CH_GH_HC_6H_5$), 4.91–4.97 (m, 1 H, H-3), 5.63 (bs, 1 H, H-2), 6.73 (d, 1 H, $J_{NH,3} = 8.9$, NH), 7.16–7.37 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ: 23.4 (CH_3), 45.8 (C-3), 70.1 (C-7), 72.4 ($CH_EH_FC_6H_5$), 72.8 ($CH_CH_DC_6H_5$), 72.9 ($CH_GH_HC_6H_5$), 73.0 ($CH_AH_BC_6H_5$), 73.2 (C-6), 73.7 (C-5), 75.8 (C-4), 125.4 (C-2), 127.5, 127.6, 127.7, 127.8, 128.0, 128.0_2, 128.0_5, 128.0_8, 128.3, 128.4, 128.5, 135.9 (C-1), 137.8, 138.3, 138.3_2, 138.4, 169.4 (C=O). Anal. Calcd For $C_{35}H_{39}NO_5$: 76.9; H, 6.8; N, 2.4. Found: C, 76.7; H, 7.0; N, 2.5.

(1*R,2*S*,3*S*,4*S*,5*S*,6*S**)-5-acetamido-2,3,4-tribenzyloxy-1-[(benzyloxy)methyl]**

bicyclo[4.1.0]heptane (2.13: 1*R*,6*S*) and (2.14: 1*S*,6*R*)

Under N_2, a solution of compound **2.12** (523 mg, 0.91 mmol) in dry toluene (125 mL) was cooled to –10 °C. Dimethyl zinc (2 M in toluene, 8.2 mL, 16 mmol) was added dropwise. The reaction was stirred at –10 °C for 15 min, then CH_2I_2 (1.7 mL, 2.1 mmol) was added slowly. The reaction mixture was stirred for 16 h while being allowed to warm to 20 °C. The reaction was quenched by the addition of aqueous H_2SO_4 (10%, 4 mL), and subsequently EtOAc (200 mL) was added. The resultant organic layer was washed with saturated aqueous $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated under reduced pressure. The two cyclopropyl isomers were separated and purified by radial chromatography (CH_2Cl_2:MeOH 200:1 v/v) to give compound **2.13** and **2.14** as colourless syrups (total weight 470 mg, 88%):

Compound **2.13**: $[\alpha]^{20}_D$ +67.2 (*c* = 0.661, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.63–0.71 (m, 2 H, H-7, H-7'), 1.33 (m, 1 H, H-6), 1.92 (s, 3 H, CH_3), 2.72 (d, 1 H, $J_{8,8'}$ = 9.5, H-8), 3.54 (dd, 1 H, $J_{3,2}$ = 2.6, $J_{3,4}$ = 6.8, H-3), 3.95 (t, 1 H, $J_{4,5}$ + $J_{4,3}$ = 13.7, H-4), 4.09 (d, 1 H, H-8'), 4.29 (d, 1 H, H-2), 4.38–4.42 (d, 2 H, $CH_2C_6H_5$), 4.45 (d, 1 H, $J_{A,B}$ = 12.0, $CH_AH_BC_6H_5$), 4.49 (d, 1 H, $J_{C,D}$ = 11.5, $CH_CH_DC_6H_5$), 4.60 (d, 1 H, $J_{E,F}$ = 11.3, $CH_EH_FC_6H_5$), 4.65 (d, 1 H, $J_{G,H}$ = 11.9, $CH_GH_HC_6H_5$), 4.74 (m, 1 H, H-5), 4.76 (d, 1 H, $CH_GH_HC_6H_5$), 4.83 (d, 1 H, $CH_EH_FC_6H_5$), 6.00 (d, 1 H, $J_{5,NH}$ = 7.1, NH), 7.18–7.37 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 13.8 (C-7), 20.2 (C-6), 23.6 (CH_3), 26.1 (C-1), 45.4 (C-5), 72.6, 73.1, 73.3 (3 × $CH_2C_6H_5$), 74.3 (C-8), 74.7 ($CH_2C_6H_5$), 75.0 (C-2), 77.1 (C-4), 79.3 (C-3), 127.5, 127.6$_2$, 127.6$_7$, 127.7, 127.9$_5$, 127.9$_7$, 128.0, 128.4, 128.5, 128.6, 138.0, 138.4, 138.6, 139.2, 169.8 (C=O). Anal. Calcd For $C_{38}H_{41}NO_5$: C, 77.1; H, 7.0; N, 2.4. Found: C, 76.9; H, 7.1; N, 2.65.

Compound **2.14**: $[\alpha]^{20}_D$ +18.7 (c = 1.20, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.63 (dd, 1 H, $J_{6,7}$ = 9.4, $J_{7,7'}$ = 5.1 H-7), 0.91–0.96 (m, 1 H, H-6), 1.28 (t, 1 H, $J_{7',6} + J_{7',7}$ = 10.8, H-7'), 1.93 (s, 3 H, CH_3), 2.81 (d, 1 H, $J_{8,8'}$ = 10.2, H-8), 3.66 (m, 1 H, H-4), 3.75–3.80 (m, 2 H, H-3, H-8'), 4.32 (d, 1 H, $J_{2,3}$ = 4.3, H-2), 4.37–4.43 (m, 3 H, H-5, 2 × $CH_2C_6H_5$), 4.45–4.60 (m, 6 H, $CH_2C_6H_5$), 6.10 (d, 1 H, $J_{NH,5}$ = 8.5, NH), 7.22–7.36 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 13.1 (C-7), 22.7 (C-6), 23.6 (CH_3), 24.2 (C-1), 45.4 (C-5), 72.6 ($CH_2C_6H_5$), 71.9 (C-2), 72.5, 72.6, 72.7 (3 × $CH_2C_6H_5$), 74.8 (C-3), 75.9 (C-8), 76.8 (C-4), 127.6, 127.7, 127.8, 128.0, 128.1, 128.4, 128.5, 128.7, 138.0, 138.3, 138.6, 138.7, 169.6 (C=O). Anal. Calcd For $C_{38}H_{41}NO_5$: C, 77.1; H, 7.0; N, 2.4. Found: C, 77.2; H, 7.0; N, 2.1.

(1*R*,2*S*,3*S*,4*S*,5*S*,6*S*)-5-acetamido-1-(hydroxymethyl)bicyclo[4.1.0]heptane-2,3,4-triol (2.3)

A mixture of compound **2.13** (467 mg, 0.79 mmol) and 10% Pd-C (217 mg) in MeOH (90 mL) was stirred at room temperature under an atmosphere of H_2 for 14 h. The mixture was filtered through a celite pad, which was washed thoroughly with MeOH (50 mL). The filtrate and washings were combined and concentrated under pressure to give a colourless oil. This material was purified by flash column chromatography (CH_2Cl_2/MeOH, 4:1 v/v) to give compound **2.3** (151 mg, 83%) as a colourless syrup: $[\alpha]^{20}_D$ +92.7 (c = 0.52, MeOH); ^{1}H NMR (D_2O) δ 0.58–0.70 (m, 2 H, H-7, H-7'), 1.35 (m, 1 H, H-6), 2.01 (s, 3 H, CH_3), 2.89 (d, 1 H, $J_{8,8'}$ = 11.5, H-8), 3.48 (dd, 1 H, $J_{3,4}$ = 10.0, $J_{3,2}$ = 3.0, H-3), 3.91 (dd, 1 H, $J_{4,5}$ = 7.3, H-4), 4.0 (d, 1 H, H-8'), 4.34 (d, 1 H, H-2), 4.60 (t, 1 H, H-5); ^{13}C NMR (D_2O) δ 10.9 (C-7), 19.9 (C-6), 22.0 (CH_3), 28.0 (C-1), 46.5 (C-5), 65.9 (C-8), 66.6 (C-4), 68.6 (C-2), 68.8 (C-3), 174.3

(C=O). Anal. Calcd For $C_{10}H_{17}NO_5$: C, 51.9; H, 7.4; N, 6.1. Found; C, 51.6; H, 7.6; N, 6.1.

(1*S*,2*S*,3*S*,4*S*,5*S*,6*R*)-5-acetamido-1-(hydroxymethyl)bicyclo[4.1.0]heptane-2,3,4-triol (2.4)

A mixture of compound **2.14** (533 mg, 0.90 mmol) and 10% Pd-C (247 mg) in MeOH (100 mL) was stirred at rt under an atmosphere of H_2 for 14 h. The reaction mixture was filtered through a celite pad, which was then washed thoroughly with MeOH (50 mL). The filtrate and washings were combined and concentrated under pressure to give a colourless syrup. The resultant crude product was purified by flash column chromatography (CH_2Cl_2/MeOH, 4:1 v/v) to yield compound **2.4** (185 mg, 88%) as a colourless syrup: $[\alpha]^{20}_D$ +64.5 (c = 0.53, H_2O); ^{1}H NMR (D_2O) δ 0.60 (dd, 1 H, $J_{7,7'}$ = 5.7, $J_{7,6}$ = 9.8, H-7), 0.83 (t, 1 H, $J_{7',6} + J_{7,7'}$ = 11.7, H-7'), 1.10 (ddd, 1 H, $J_{6,5}$ = 1.4, H-6), 2.03 (s, 3 H, CH_3), 3.06 (d, 1 H, $J_{8,8'}$ = 11.7, H-8), 3.65–3.67 (m, 2 H, H-3, H-4), 3.71 (d, 1H, H-8'), 4.42 (m, 1 H, H-2), 4.48 (br s, 1 H, H-5); ^{13}C NMR (D_2O) δ 9.7 (C-7), 22.2 (CH_3), 22.6 (C-6), 27.6 (C-1), 48.7 (C-5), 66.0 (C-4), 68.1 (C-2), 68.2 (C-8), 69.2 (C-3), 174.4 (C=O). Anal. Calcd For $C_{10}H_{17}NO_5$: C, 51.9; H, 7.4; N, 6.1. Found; C, 52.2; H, 7.5; N, 5.9.

(1*R*,2*S*,3*S*,4*S*,5*S*,6*S*)-5-amino-1-(hydroxymethyl)bicyclo[4.1.0]heptan-2,3,4-triol (1.80)

LiOH (32 mg, 0.76 mmol) was added to a solution of amide **2.3** (82 mg, 0.35 mmol) in THF/H_2O (1:1, 12 mL). This mixture was heated at 70 °C for 32 h. The solution was then neutralized by the addition of Amberlite IR resin (H^+ form), and

filtered. The resin was washed by thoroughly with water and the product was eluted with aqueous NH_4OH (5%, 100 mL). The eluant was concentrated under pressure to give a light yellow oil. This substance was purified further by flash chromatography (MeOH/$CHCl_3$/28% NH_3 in H_2O 5:5:1 v/v/v) to give compound **1.80** as a colourless syrup (35 mg, 52%): $[\alpha]^{20}_D$ +76.5 (*c* = 0.51, MeOH); ^{1}H NMR (CD_3OD) δ 0.67 (dd, 1 H, $J_{7,7'}$ = 5.5, $J_{7,6}$ = 9.3, H-7), 0.75 (m, 1 H, H-7'), 1.33 (m, 1 H, H-6), 3.41 (d, 1 H, $J_{8,8'}$ = 11.3, H-8), 3.54 (dd, 1 H, $J_{3,4}$ = 8.9, $J_{3,2}$ = 3.1, H-3), 3.74 (d, 1 H, H-8'), 3.82 (t, 1 H, $J_{5,4}$ + $J_{5,6}$ = 13.9, H-5), 3.93 (dd, 1 H, $J_{4,5}$ = 6.6, H-4), 4.30 (d, 1 H, H-2); ^{13}C NMR (CD_3OD) δ 11.6 (C-7), 20.8 (C-6), 29.1 (C-1), 48.7 (C-5), 67.6 (C-8), 68.5 (C-4), 70.5 (C-3), 70.5_2 (C-2). Found 190.1074 (ESI-HRMS) for $C_8H_{16}NO_4$ ($M+H^+$), requires 190.1079.

(1*S*,2*S*,3*S*,4*S*,5*S*,6*R*)-5-amino-1-(hydroxymethyl)bicyclo[4.1.0]heptan-2,3,4-triol (2.5)

LiOH (15 mg, 0.36 mmol) was added to a solution of amide **2.4** (30 mg, 0.13 mmol) in THF/H_2O (1:1, 4 mL). This mixture was heated at 70 °C for 32 h, when it was neutralized by adding Amberlite IR resin (H^+ form). After filtration the resin was washed thorough by water. The product was eluted using aqueous NH_4OH (5%, 20 mL) and the eluant was concentrated under pressure to give a light yellow oil. This material was purified by flash chromatography (MeOH/$CHCl_3$/28% NH_3 in H_2O 5:5:1 v/v/v) to give compound **2.5** as a colourless syrup (21 mg, 84%): $[\alpha]^{20}_D$ +72.7 (*c* = 0.85, MeOH:H_2O 1:1 v/v); ^{1}H NMR (CD_3OD) δ 0.54 (dd, 1 H, $J_{7,7'}$ = 5.2, $J_{7,6}$ = 9.7, H-7), 0.92 (t, 1 H, $J_{6,7'}$ + $J_{7,7'}$ = 11.3, H-7'), 1.05 (m, 1 H, H-6), 2.99 (d, 1 H, $J_{8,8'}$ = 11.2, H-8), 3.49 (m, 1 H, H-5), 3.61 (dd, 1 H, $J_{4,5}$ = 4.2, $J_{4,3}$ = 9.2, H-4), 3.66 (dd, 1 H, $J_{3,2}$ = 4.7, H-

3), 3.79 (d, 1 H, H-8'), 4.37 (d, 1 H, H-2); ^{13}C NMR (CD_3OD) δ 9.2 (C-7), 22.3 (C-6), 28.0 (C-1), 49.5 (C-5), 66.1 (C-4), 66.5 (C-2), 68.0 (C-8), 68.9(C-3). Found 190.1074 (ESI-HRMS) for $C_8H_{16}NO_4$ ($M+H^+$), requires 190.1079.

2.5 Enzyme Kinetics

The activity of both the coffee bean and *E. coli* enzymes were assayed by monitoring the rate of hydrolysis of *p*-nitrophenyl α-D-galactopyranoside (PNPG). For the measurement of K_i values 4 different concentrations of substrate from 0.1 mM to 2.5 mM and 4 different concentration of inhibitor from 3×10^{-4} to 3×10^{-3} mM were used. Whereas, for IC_{50} values a single concentration of PNPG was utilized and this was 0.14 and 0.01 mM for the coffee bean and *E. coli* enzymes, respectively. The corresponding Michaelis constants (K_m) for the PNPG substrate are 0.7 and 0.04 mM for the coffee bean and *E. coli* enzymes, respectively.

The coffee bean α-galactosidase activity assay solutions contained 0.62 mU/mL enzyme in 50 mM sodium phosphate buffer (pH 6.52) with 0.1% bovine serum albumin in total volume of 0.400 mL. Each experiment was initiated by the addition of enzyme to an equilibrated assay solution held at 37 °C.

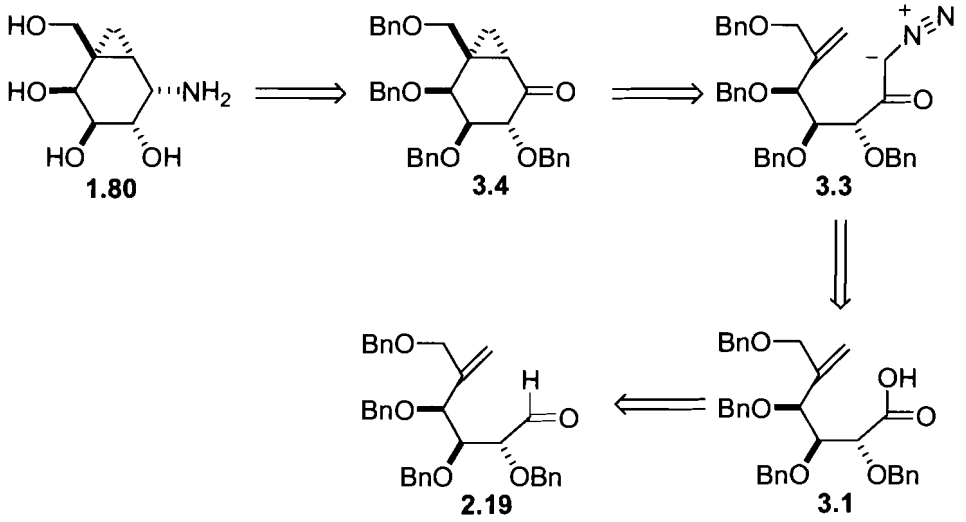
The activity of the *E. coli* enzyme was assayed using a solution that comprised of 5 mU/mL enzyme containing 0.1% bovine serum albumin in 50 mM sodium phosphate buffer (pH 7.26), the total volume of the assay solution was 0.400 mL. Each analysis began by the addition of a stock solution of enzyme to an equilibrated assay solution held at 25 °C.

For both enzymes, the absorbance at 400 nM was monitored for 10 min using a Cary 3E spectrophotometer equipped with a Peltier temperature controller. The measured initial rate versus inhibitor concentration data were fit to standard enzyme kinetic equations using a nonlinear least squares program (Prism).

3 Synthesis of Carbocyclic Bicyclo[4.1.0]heptylamine α-Galactopyranose Mimic by Intramolecular Cyclopropanation

3.1 Introduction

The initial route chosen for the synthesis of the target compound **1.80** involved a metal-catalyzed carbenoid reaction addition using diazo ketone **3.3** in an intramolecular cyclopropanation reaction (Scheme 3.1).

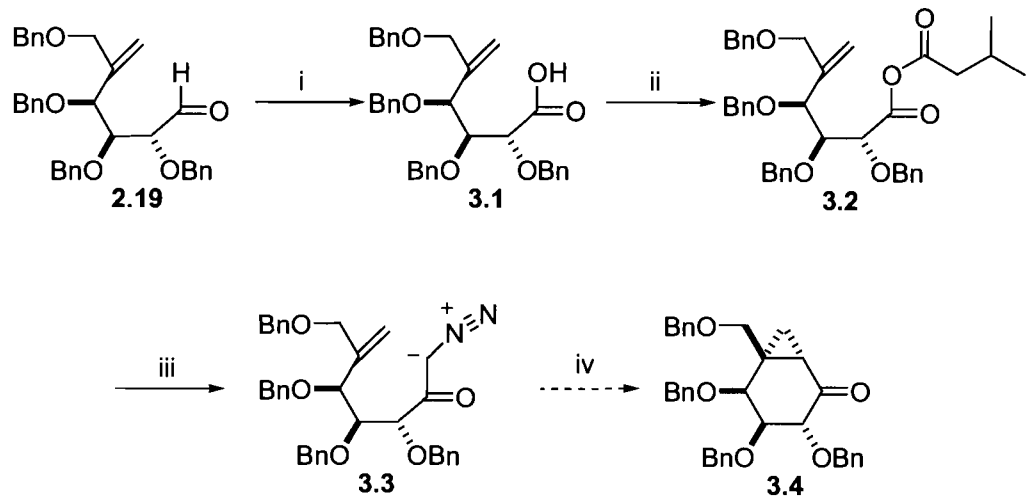


Scheme 3.1 Proposed intramolecular cyclopropanation route for the synthesis of compound **1.80**

3.2 Results and Discussion

The details for synthesis of compound **2.19** are given in Chapter 2. Oxidation using sodium chlorite cleanly converted aldehyde **2.19** into carboxylic acid **3.1** (Scheme

3.2). The ^{1}H NMR spectrum of compound **3.1** clearly showed the disappearance of the CHO signal of precursor **2.19** at δ 9.53.



Scheme 3.2 The synthetic route for the desired compound **3.4**. *Reagents and Conditions*: i, $NaClO_2$, THF/i-BuOH/2-methyl-2-butene/water, rt, 67%; ii, isobutyl chloroformate, Et_3N, THF, 0 °C; iii, diazomethane, 0 °C to rt; iv, CH_2Cl_2 or toluene, metal catalyst, rt or reflux

Immediately following the formation of the mixed anhydride **3.2** from carboxylic acid **3.1** using isobutyl chloroformate, an ethereal solution of diazomethane (produced from *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide)[133] was added to give compound **3.3** as a pale yellow syrup. This material possesses a diagnostic ^{1}H NMR signal at δ 5.79 ppm, a ^{13}C NMR signal at δ 54.2 ppm and an IR signal at 2105 cm^{-1}, which are all associated with the diazomethyl group of compound **3.3**.

Various metal species were used to try and form the required cyclopropane. These metal catalysts included copper(II) trifluoromethanesulfonate ($Cu(OTf)_2$),[134] Cu(II) acetylacetonate ($Cu(acac)_2$),[135] Cu(I) iodide (CuI),[136] Cu(bronze) activated by iodine in acetone,[137,138] palladium(II) acetate ($Pd(OAc)_2$),[139] rhodium(II) acetate dimer

($Rh_2(OAc)_4$), [140] and dirhodium(II) tetracaprolactamate ($Rh_2(Cap)_4$).[141] However, none of these reactions gave the desired product **3.4** (Table 3.1). Even using $Rh_2(Cap)_4$, which has been reported to favour C=C addition reactions over C-H insertions,[141] failed to give the desired product **3.4**. Instead, in most cases, the C-H insertion reaction occurred.

UV light was also used in one instance to try to induce the desired reaction of the diazoketone. In addition, one metal-catalyzed reaction using $Rh_2(OAc)_4$ was carried out in the dark to avoid the possible occurrence of free radical reactions. However, no desired product was formed in either of these experiments (Table 3.1).

Table 3.1 The reaction conditions and the results of cyclopropanation experiments

catalyst	solvent	temperature (°C)	procedure	result
$Cu(OTf)_2$ $PhNHNH_2$	CH_2Cl_2	rt	**II**	Insertion
$Cu(acac)_2$	benzene	80	**I**	Insertion
CuI	benzene	80	**I**	Insertion
Activated Cu (bronze)	toluene	110	**III**	No cyclopropane [a]
$Rh_2(OAc)_4$	CH_2Cl_2	rt	**I**	Insertion
$Rh_2(OAc)_4$	CH_2Cl_2	rt [c]	**I**	No cyclopropane [a]
$Rh_2(Cap)_4$	CH_2Cl_2	rt	**I**	Insertion
$Pd(OAc)_2$	CH_2Cl_2	rt	**I**	No reaction [b]
$Pd(OAc)_2$	benzene	80	**I**	No cyclopropane [a]
$Pd(OAc)_2$	cyclohexane	80	**I**	No cyclopropane [a]
UV (254 nM)	toluene	80		No cyclopropane [a]

[a] No cyclopropanation reaction occurred, and no starting material was left. In these cases, the results of the reactions were not analyzed; [b] Starting material was unreacted; [c] The reaction was performed in the dark.

Some reactions formed several products. Two major isomers were partially purified, and identified as C-H insertion products **3.5** and **3.6** based on NMR spectroscopic data (Figure 3.1)

One isomer has two doublet and doublet signals of ^{1}H NMR spectrum at δ 2.51 ppm and δ 2.76 ppm which correlate each other with the coupling constant of 17.9 Hz. These two signals must be associated with the two protons at *C*-5 of compound **3.5** or *C*-4 of compound **3.6**. Furthermore, both protons couple with one proton which has a multiply signal of ^{1}H NMR spectrum at δ 5.51 ppm and which does not couple with any other proton. This proton is considered at *C*-6 of compound **3.5** or at *C*-5 of compound **3.6**.

Similarly another isomer shows two signals in the ^{1}H NMR spectrum appearing as doublet of doublet at δ 2.41 ppm and δ 2.78 ppm that couple to each other with a coupling constant of 17.8 Hz, and each signal can be integrate as one proton. Both protons couple with one proton, which has a doublet of doublet signal at δ 5.16 ppm and does not couple with any other protons, with coupling constants of 10.9 and 5.9 Hz, respectively.

Both isomers show two single signals in ^{1}H NMR spectrum at δ 5.48 and δ 5.55 ppm. These two signals represent the two protons attached to a double bond.

Based on these NMR data, we proposed that the metal induced carbene of diazoketone **3.3** inserted into a C-H bond of one of the methylene unite of a benzyl protecting group to give isomers **3.5** and/or **3.6**.

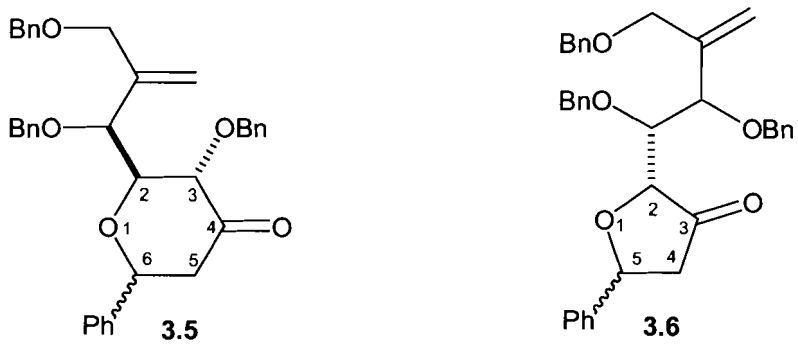


Figure 3.1 Structures of possible C-H insertion products

After these efforts did not afford the desired cyclopropane **3.4**, we abandoned this synthesis route. Therefore, we did not fully analyze all of the intermediates prepared by this synthetic route.

A new synthetic route was later utilized which was introduced in Chapter 2.

3.3 Conclusion

Metal-catalyzed intramolecular cyclopropanation of diazo ketone **3.3** failed to give C=C insertion, instead, it led to unwanted C-H insertion reactions.

3.4 Experimental

(2*R*,3*S*,4*S*)-2,3,4-tris(benzyloxy)-5-[(benzyloxy)methyl]hex-5-enoic acid (3.1)[142]

The freshly prepared aldehyde **2.19** (55 mg, 0.10 mmol) was dissolved in a solution (2.4 mL, 1:1:0.2:0.2, THF:i-BuOH:1.8 M NaH_2PO_4:2-methyl-2-butene) followed by slow addition of aqueous sodium chlorite (1.9 M, 0.4 mL, 7.6 mmol) solution. The reaction mixture was stirred at room temperature for 3 h. The mixture was poured into 2 mL water. The resultant solution was extracted with either (3 × 1.5 mL)

and the combined organic layers were washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure to give the crude product as a pale yellow syrup. This material was purified by flash column chromatography (hexane: EtOAc, 3:1 v/v) to give compound **3.1** as a colourless syrup (38 mg, 67%): ^{1}H NMR ($CDCl_3$) δ 4.03-4.13 (m, 4 H, H-2, H-7, H-4, $CH_AH_BC_6H_5$), 4.15 (d, 1H, $J_{7',7}$ = 9.1, H-7'), 4.39-4.53 (m, 6 H, H-3, $CH_AH_BC_6H_5$, $CH_2C_6H_5$), 4.53 (d, 1 H, $J_{C,D}$ = 12.3, $CH_CH_DC_6H_5$), 4.58 (d, 1 H, $CH_CH_DC_6H_5$), 5.49 (s, 1H, H-6), 5,60 (m, 1H, H-6'), 7.17–7.37 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 70.0$_4$ (C-7), 70.1, 72.8, 73.9, 74.6 ($CH_AH_BC_6H_5$), 78.1 (C-3), 78.6 (C-4), 81.4 (C-2), 117.1 (C-6), 127.5, 127.7,127.9, 128.0, 128.1, 128.3, 128.3$_5$, 128.4, 128.4$_4$, 136.6, 137.4, 137.8, 138.1, 143.1 (C-5), 174.7 (C=O).

(3*R*,4*S*,5*S*)-1-diazo-3,4,5-tris(benzyloxy)-6-[(benzyloxy)methyl]-hept-6-en-2-one (3.3)[143]

To carboxylic acid **3.1** (275 mg, 0.50 mmol) in dry THF (5 mL) was added Et_3N (0.12 mL, 0.83 mmol) at -20 °C. Then isobutyl chloroformate (0.11 mL, 0.83 mmol) was added. The reaction mixture was stirred for 30 min under N_2, then warmed to 0 °C. A freshly prepared ethereal diazomethane solution (8 mL, about 2 mmol), produced from the reaction of *N*-methyl-*N*-nitrosotoluene-*p*-sulphonamide with potassium hydroxide in ethanol, was added dropwise.[144] The reaction was slowly warmed to room temperature, when the colour of the mixture changed to yellow. The reaction was continued for 5 h. The mixture was evaporated to around half of its volume under water aspirator vacuum, and the yellow colour of mixture disappeared. Ether (5 mL) was added to dilute the reaction. The reaction mixture was washed with water, saturated aqueous $NaHCO_3$ solution, brine, dried (Na_2SO_4) and concentrated under reduced

pressure to give the crude product as a pale yellow syrup, The residue was purified by flash column chromatography (hexane: Et_2O, 3:1) to give compound **3.3** as a yellow syrup compound (245 mg, 0.43 mmol, 85%): IR 2105 (N_2) cm^{-1}, 1627 (CO) cm^{-1}; ^1H NMR ($CDCl_3$) δ 5.79 (s, 1 H, H-1); ^{13} C NMR ($CDCl_3$) δ 54.0 (C-1), 197.1 (C=O)

Attempted Synthesis of (1*R*,2*S*,3*S*,4*S*,6*S*)-2,3,4-tris(benzyloxy)-1-[(benzyloxy)methyl]bicyclo[4,1,0]heptan-5-one (3.4)

General cyclopropanation procedure **I** with a metal catalyst rhodium(II) acetate as an example:[140]

To a solution of rhodium(II) acetate in dry dichloromethane (0.6 mM, 3 mL, 1.8 μmol) was added α-diazo ketone **3.3** (46 mg, 0.073 mmol) in dichloromethane (2 mL) over 5 h via a syringe pump at rt under N_2. Then the reaction continued for another 12 h. One experiment was carried out in the dark. If benzene or toluene was used as the solvent instead of dichloromethane, the reaction was generally heated to reflex. After reaction, the mixture was concentrated under reduced pressure to afford a syrup which was further purified by column chromatography (hexane:EtOAc, 9:1) which often gave partially separated isomer **3.5** and **3.6**.

Cyclopropanation procedure **II** with copper(II) triflate as catalyst:[134]

To a solution of copper(II) triflate (4.0 mg, 0.011 mmol) in dry dichloromethane (3 mL) was added phenylhydrazine (0.002 mL, 0.02 mmol) at room temperature under N_2. The resulting solution was stirred for 5 min, and a pink colour formed. Diazo ketone **3.3** (32.5 mg, 0.056 mmol) in dichloromethane (2 mL) was added over the course of 90 min via syringe pump. The colour of the reaction mixture changed to orange-red. The

reaction was stirred for another 14 h under N_2, and concentrated under reduced pressure to give the crude compound as a brown syrup, which was further purified by column chromatography (hexane: EtOAc, 9:1) to give two partially separated isomer **3.5** and **3.6**.

Cyclopropanation procedure **III** with activated copper bronze:

Copper bronze (200 mg) was treated with a solution of iodine (2 mL, 2% iodine in acetone) for 5 min. Then the metal was filtered, and transferred to the solution of aqueous HCl (37%, 0.5 mL) in acetone (0.5 mL) for 30 min. After filtration, the powder was washed with acetone (3 × 2 mL) and dried in a vacuum desiccator. The activated copper bronze (2 mg) was added to the solution of α-diazoketone **3.3** (11 mg, 0.016 mmol) in toluene (5 mL). The reaction mixture was refluxed for 40 min, then the copper was filtered. The filtrate was concentrated under reduced pressure to give a product as a syrup. A COSY NMR spectrum of the crude product showed that no cyclopropane was present in the reaction mixture, that is, no correlated protons with chemical shifts < 1 ppm were present.

4 Irreversible Inhibition of α-Galactosidases: A Bicyclobutenium Ion Equivalent in an Enzymatic Active Site

4.1 Introduction

In 2001, Tanaka *et al.* reported the aforementioned structural motif **1.64**, containing a cyclopropyl ring fused onto a carbaglucose analogue, that is a potent glycosidase inhibitor.[99] This bicyclo[4.1.0]heptyl *glucose*-analogue turned out to be a potent yeast α-glucosidase inhibitor with a K_i value of 107 nM.[99] The *galacto*-analogue **1.80** containing the same structural motif was shown to be a inhibitor of coffee bean α-galactosidase with a K_i value of 541 nM (chapter 2).[145] These reports led to a common conclusion that the bicyclo[4.1.0]heptane structural motif has a high affinity for binding to the corresponding α-glycosidase.

As part of a continuing effort towards the use of this structural motif in glycosidase inhibitors it was decided that the introduction of a good leaving group at the *pseudo C*-1 position might enhance the propensity for the formation of carbocationic intermediates during the enzymatic reaction, and that this may result in the labeling of an active site amino acid residue of the α-galactosidase. Thus, compounds **1.81** and **1.82** were designed as potential irreversible inhibitors of α-galactosidase enzymes (Figure 1.22).

As Koshland proposed, retaining glycosidase enzymes function via a double displacement mechanism that includes the formation of a glycosyl-enzyme covalent

intermediate.[9] The enzyme-catalyzed glycosylation can proceed either by dissociative (D_N * A_N)[119,120] or associative (A_ND_N)[120,121] reactions. Nevertheless, a significant degree of positive charge is developed at the anomeric center in the enzyme-catalyzed transition states.

Given that the known compound **4.12** has a half-time of reaction in 70:30 v/v acetone:water at 100 °C of ~ 20 min (Figure 4.1),[146] the reaction of compound **1.81** and **1.82** under physiological condition is expected to be slow. However, when compound **1.81** and **1.82** are bound to an α-galactosidase, the active-site general-acid catalyst may facilitate the reaction.

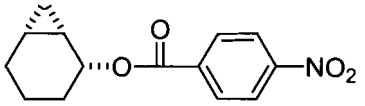
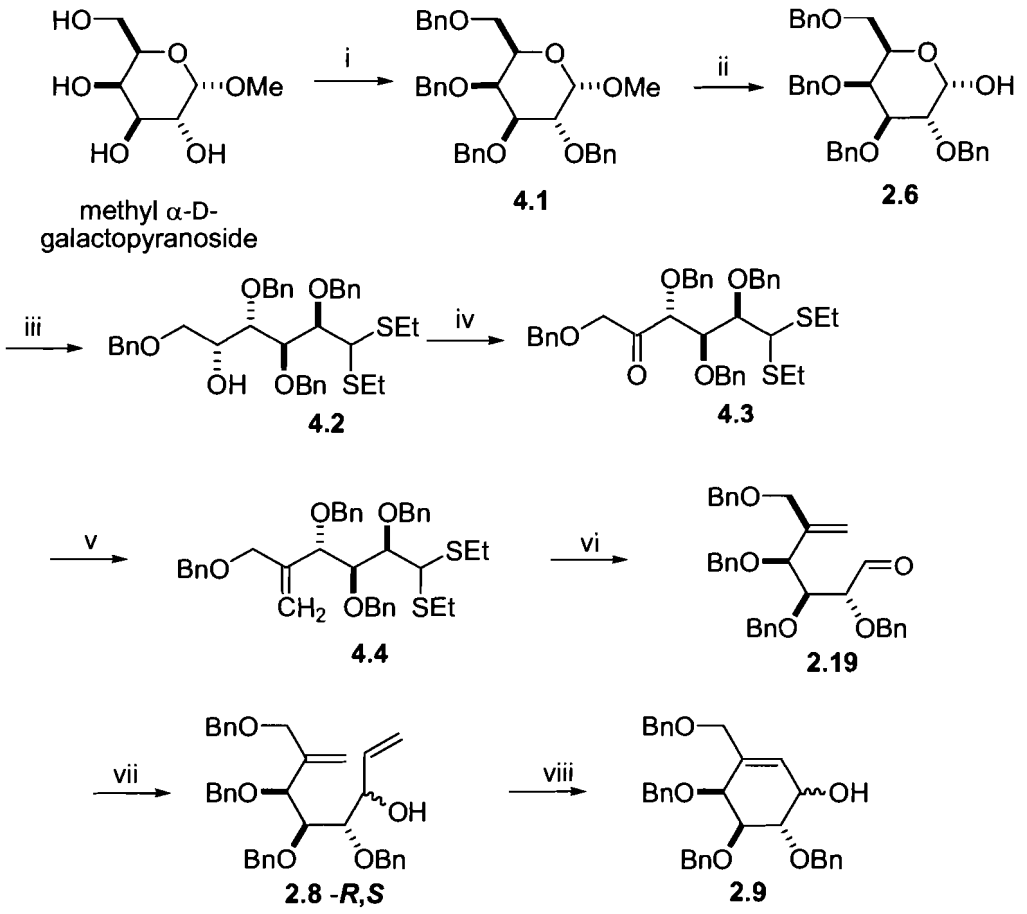


Figure 4.1 The structure of compound **4.12**

4.2 Results and Discussion

The route chosen to synthesize the designed inhibitors **1.81** and **1.82** was modified from the one used to make the reversible α-galactosidase inhibitor **1.80**. Both routes utilized 2,3,4,6-tetra-*O*-benzyl-D-galactose (**2.6**), which can be made in two steps using methyl α-D-galactopyranoside as a starting material (Scheme 4.11).[127,145] From compound **2.6** to compound **2.9-*S*,*R***, as compared to the synthetic route to compound **1.80** (Chapter 2), this route (Scheme 4.21) was shorter by two steps, and the overall yield was increased from 17% to 28%.



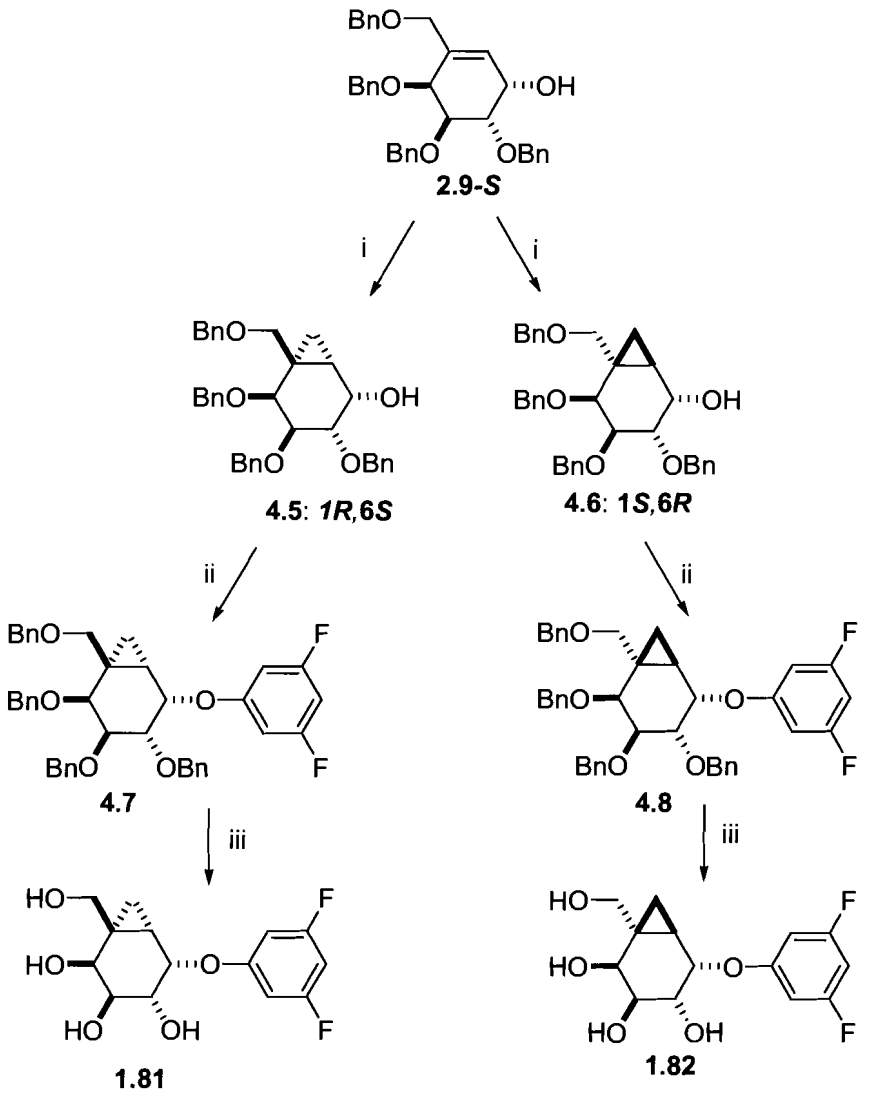
Scheme 4.1 Synthesis route of compound **2.9**. *Reagents and conditions*: i, NaH, BnBr, DMF, 40 °C, 91%; ii, H_2SO_4, AcOH, 120 °C, 60%; iii, EtSH, CF_3CO_2H, rt, 71%; iv, DMSO, Ac_2O, rt, 87%; v, Ph_3PCH_3I, n-BuLi, –78 °C to 0 °C, then add ketone, 0 °C, 75%; vi, Hg_2O, CH_3CN, H_2O, rt, then $HgCl_2$; vii, $CH_2CHMgBr$, THF, –78 °C to rt, *R*:*S* 1:2, 61% in two steps; viii, Grubb's catalyst, CH_2Cl_2, 40 °C, *R*:32%, *S*:54%.

After the reaction of the protected galactose **2.6** with ethanethiol under acidic conditions, Swern oxidation followed by a Wittig reaction gave the acyclic dithioacetal **4.4** containing a methylene unit in a yield of 46% over three steps. Hydrolysis of dithioacetal **4.4**, and addition of vinyl magnesium bromide gave a 1:2 ratio of the ***R*:*S*** diastereoisomers of the acyclic octadienol (**2.8-*R*,S**). Of note, the assignment of stereochemistry is based on the ratio of **2.9-*R*** to **2.9-*S*** that is formed by a ring closing

metathesis reaction catalyzed by second generation Grubbs' catalyst (see Chapter 2).[128,145]

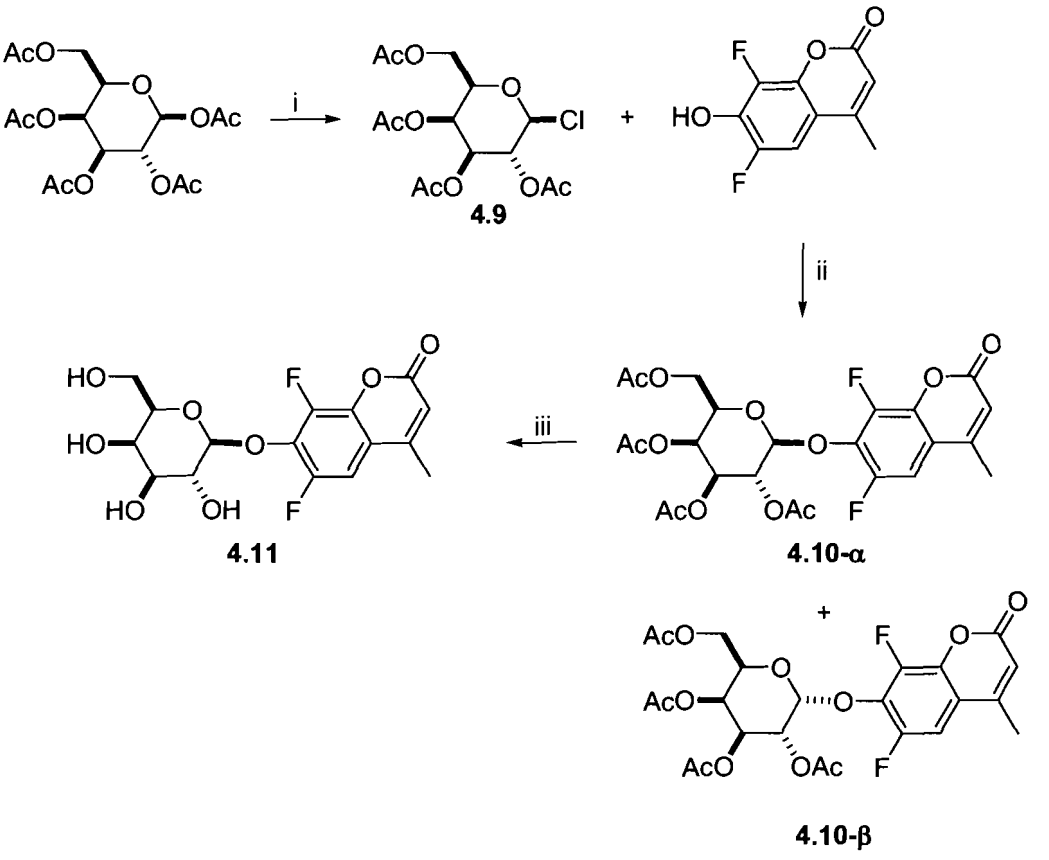
Following separation of the *S*-diastereoisomer **2.9-*S*** from the *pseudo*-equatorial isomer **2.9-*R***, a process that was accomplished by the use of radial chromatography, use of the Furukawa modification of the Simmons-Smith reaction gave the two cyclopropyl isomers in a total yield of 90%.[131] Of note, the ratio of the D-*galacto* (**4.5**) and L-*altro* (**4.6**) diastereoisomers formed in this reaction varied between 1:3 and 1.0:1.2. The reasons for the capricious nature of this cyclopropanation reaction are not clear.[145]

Coupling of a leaving group onto the *pseudo*-anomeric centre of a compound with retention of configuration was accomplished by use of a nucleophilic aromatic substitution reaction. Considering of last proposed synthetic step is debenzylation, 1,3,5-trifluorobenzene was chosen as the electrophilic aromatic substrate instead of 1-fluoro-4-nitrobenzene. That is, treatment of the two diastereoisomers **4.5** and **4.6** with sodium hydride in DMSO followed by the addition of 1,3,5-trifluorobenzene gave the difluorophenyl compounds **4.7** and **4.8** in yields of 69 and 75%, respectively. Under standard hydrogenation conditions the benzyl protecting groups were removed in good yields.



Scheme 4.2 Synthesis of irreversible inhibitor **1.81** and **1.82**. *Reagents and condition*: i, $ZnMe_2$, CH_2I_2, $PhCH_3$, –10 °C to 15 °C, 90%; ii, NaH, DMSO, $PhCO_2K$, 1,3,5-$C_6H_3F_3$, 18 °C, 69% (**4.7**), 75% (**4.8**); iii, 10% Pd-C, H_2, MeOH, RT, 91% (**1.81**), 73% (**1.82**)

For kinetic studies, a fluorogenic substrate 6,8-difluoro-4-methylumbelliferyl α-D-galactopyranoside (**4.11**) was also synthesized from commercial 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose in three steps with the yield of 16% (Scheme 4.3).[147]



Scheme 4.3 Synthesis of compound **4.11**. *Reagents and conditions*: i, $AlCl_3$, $CHCl_3$, rt, 75%; ii, $(CH_3)COK$, MeOH, rt; then DMF, 18-crown-6, rt; iii, MeONa, MeOH, 0 °C, 22% for over two steps

Incubation of coffee bean (GH 27) α-galactosidase with inhibitor (1*R*,2*S*,3*S*,4*R*,5*S*,6*S*)-5-(3,5-difluorophenoxy)-1-(hydroxymethyl)bicyclo[4.1.0]heptan-2,3,4-triol (**1.81**) resulted in time-depended inactivation of the enzyme (Figure 4.2).

A control experiment in which the enzyme was not exposed to the inhibitor was used to monitor the stability of enzyme under these reaction conditions (0 mM inhibitor in Figure 4.2).

[compound **1.81**]

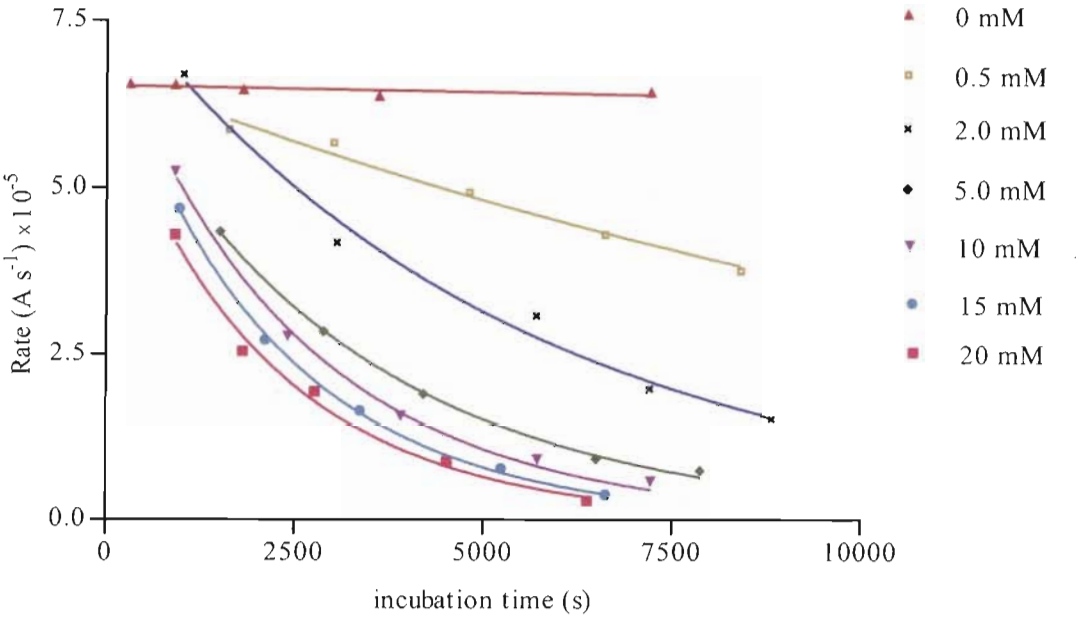


Figure 4.2 Time-depended inactivation of coffee bean α-galactosidase by compound **1.81**

Analysis of the enzyme-inactivation rate with the various concentration of compound **1.81** (from 0.5 mM to 20 mM) allowed for the calculation of the inactivation rate constant (k_{inact} = 5.6 × 10^{-4} s^{-1}) and of the dissociation constant (K_i = 4.1 mM) for coffee bean α-galactosidase with the following "goodness of fit" (R^2 values) of 0.999 (Figure 4.3).

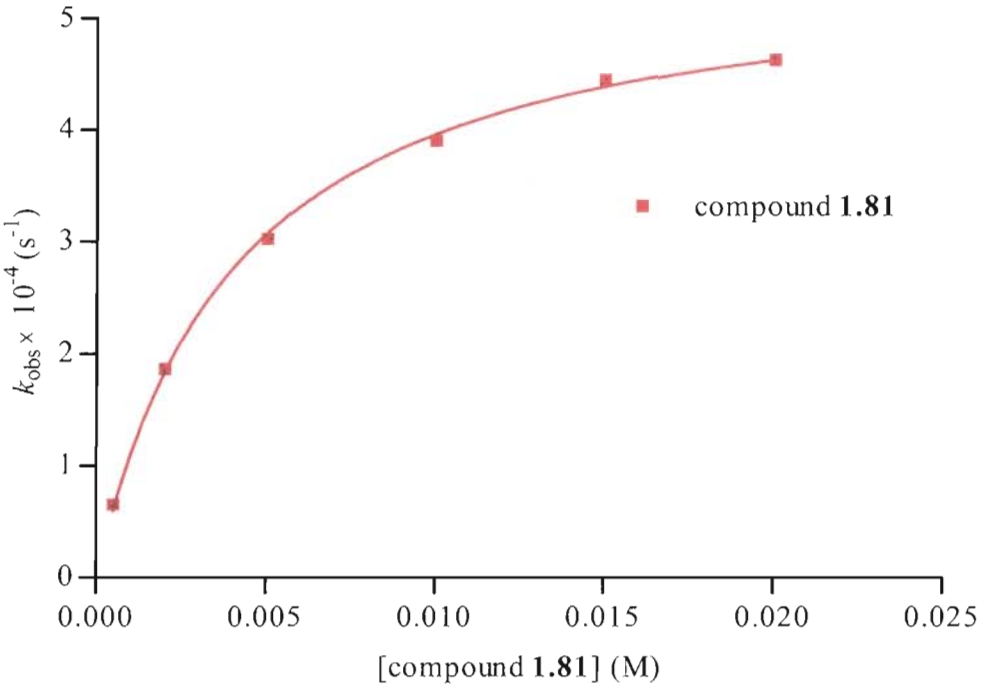


Figure 4.3 Inactivation rate of coffee bean α-galactosidase by compound **1.81**. $K_i = 4.1 \pm 0.2$ mM; $k_{inact} = (5.6 \pm 0.1) \times 10^{-4}$ s^{-1}; $k_{inact} / K_i = 0.14 \pm 0.01$ M^{-1} s^{-1}

Incubation of the enzyme with **1.81** (4 mM, K_i = 4.1 mM) in the presence of the competitive inhibitor **1.80** (1 μM, K_i = 0.54 μM) resulted in a decrease in the inactivation rate constant from 3.3×10^{-4} s^{-1} in the absence of compound **1.80** to 7.4×10^{-5} s^{-1} (Figure 4.4). Protection from inactivation using compound **1.80** confirmed that inactivation of the α-galactosidase by compound **1.81** occurs at the active site.

To confirm that binding of compound **1.81** with the enzyme was weak, the sensitive fluorogenic compound **4.11** was used as substrate so that the activity of the enzyme could be monitored quickly (18 seconds). Thus, it was shown that compound **1.81** has a low affinity to the enzyme. Specifically, the measured enzyme activity was

reduced to 24% when [**1.81**] = 1 mM at a constant substrate concentration ([**4.11**] = 0.001 mM).

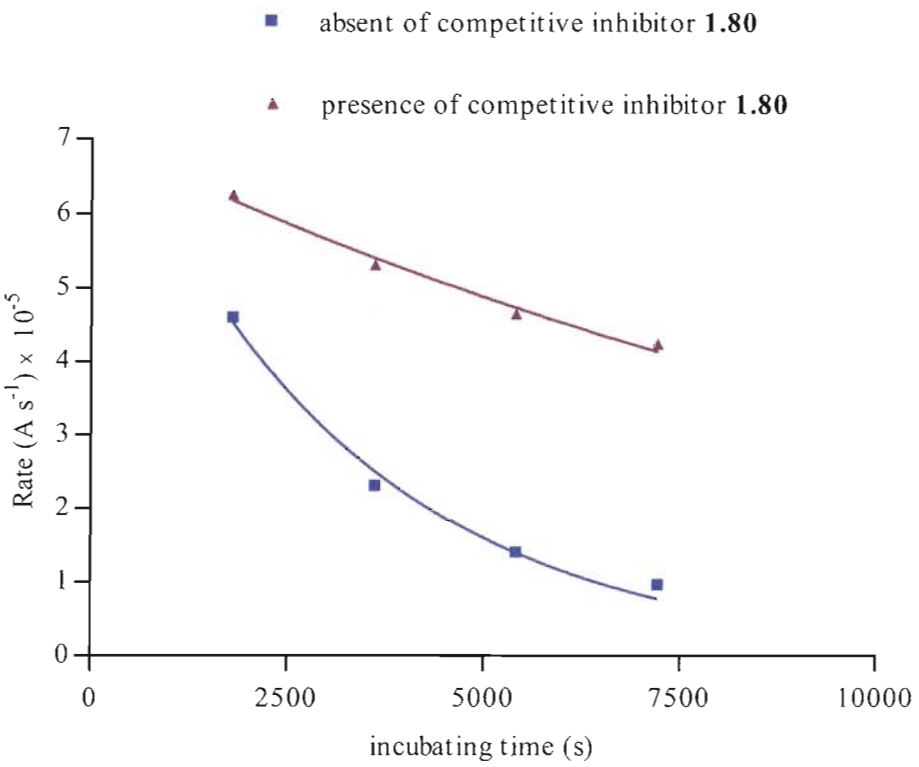


Figure 4.4 The inactivation of compound **1.81** towards coffee bean α-galactosidase in the presence and the absence of competitive inhibitor **1.80**

Comparing the K_i values of irreversible inhibitor **1.81** and competitive inhibitor **1.80** shows that compound **1.81** binds to the enzyme much more weakly than does compound **1.80** although both of them have the same bicyclo[4.1.0]heptyl *galactose* structural motif. This difference in affinity once again points to the importance of a positive charge for tight binding inhibition.

In addition, if the kinetic data collected during the first 5 minute of incubation is included in the analysis of the enzyme-inactivation parameters (k_{inact} = 6.3 × 10^{-4} s^{-1}, K_i = 5.2 mM) for coffee bean α-galactosidase a poorer correlation is observed (R^2 value of 0.970). The likely reason for this discrepancy is that using the PNPG substrate requires a long data acquisition time period (10 min) and during this time some of the enzyme in the cuvette is inactivated. Nevertheless, compound **1.81** is a good irreversible inhibitor of the coffee bean enzyme.

(1*S*,2*S*,3*S*,4*R*,5*S*,6*R*)-5-(3,5-difluorophenoxy)-1-(hydroxymethyl)-bicyclo[4.1.0] heptan-2,3,4-triol (**1.82**) showed very weak inactivation of the coffee bean α-galactosidase (Table 4.1).

Table 4.1 The inactivity parameters of compound **1.81** and **1.82** towards various enzymes

	compound	k_{inact} (s^{-1})	K_i (mM)	k_{inact}/K_i (M^{-1} s^{-1})
α-galactosidase coffee Bean	**1.81**	(5.6 ± 0.1) × 10^{-4}	4.1 ± 0.2	0.14 ± 0.01
α-galactosidase coffee Bean	**1.82**	ND [a]	ND [a]	
α-galactosidase coffee Bean	**1.80**		5.41 × 10^{-4}	

[a] None detected at 20 mM inhibitor

4.3 Possible Products of Inhibition

Since the stereochemistry of compounds **1.81** and **1.82** are different, the correctly aligned sigma-bonds which can participate in formation of a bicyclobutenium ion are

different (see labels in Figure 4.5). Therefore, the two stereoisomers can result in the formation of different reaction products (Figure 4.5).

It is postulated that the general-acid residue facilitates the cleavage of the exocyclic C-O bond simultaneously with participation from the strained C-C bond (red colour), which is antiperiplanar with the exocyclic C-O band.

If nucleophilic attack at the pseudo aromatic carbon centre (see route a in Figure 4.5) occurs before further cationic rearrangement then compound **1.81** will be the first designed irreversible inhibitor that alkylates the general-acid / -base residue of a glycosidase.

In cases where cation rearrangement is faster than capture, then it is likely that many other possible products can be formed (see routes b and c in Figure 4.5).

Thus, to understand the mechanism of this type of irreversible inhibition a more detailed investigation will need to be done.

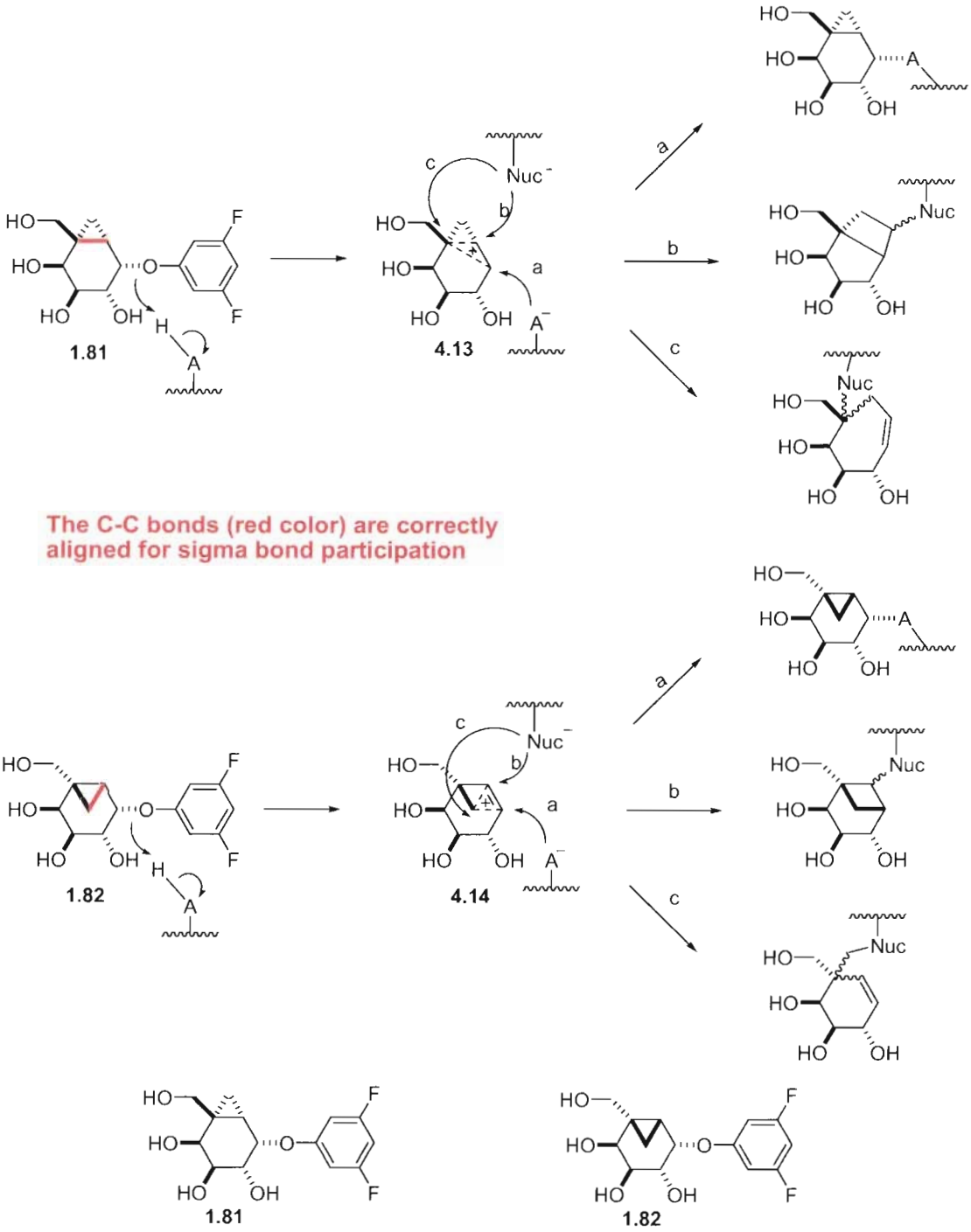


Figure 4.5 Possible mechanisms for enzymatic inactivation by compounds **1.81** and **1.82**

4.4 Conclusion

Compound **1.81** is active-site-directed and time-dependant inactivator of coffee bean α-galactosidase

4.5 Experimental

Methyl 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranoside (4.1)

After a suspension of NaH (60% in mineral oil, 21.0 g, 0.53 mol) had been washed with hexane (3 × 200 mL), it was transferred in dry *N,N*-dimethylformamide (DMF) (275 mL) into a 1 L flask that had been cooled to 0 °C. To this mixture a solution of methyl α-D-galactopyranoside (10.0 g, 0.051 mol) in DMF (100 mL) was added dropwise over a period of 20 min. This mixture was stirred at rt for 2 h. Then, tetra-*N*-butylammonium iodide (1.9 g, 5.1 mmol) was added followed by the slow addition of benzyl bromide (BnBr) (31 mL, 0.26 mol) over a period of 30 min. The temperature of the reaction mixture was kept below 40 °C during the addition, after which it was stirred at rt for 15 h. Following the addition of MeOH (70 mL) the reaction mixture was poured into Et_2O (1 L), and it was washed with water (3 × 300 mL), brine (300 mL), dried ($MgSO_4$), and concentrated at reduced pressure. The resultant crude product was purified by flash column chromatography (hexane:EtOAc, 4:1 v/v) to give compound **4.1** as a colourless syrup (25.5 g, 91%): ^{1}H NMR ($CDCl_3$) δ 3.35 (s, 3 H, CH_3), 3.45–3.52 (m, 2 H, H-6, H-6'), 3.86–3.94 (m, 3 H, H-3, H-4, H-5), 4.02 (m, 1 H, H-2), 4.37 (d, 1 H, $J_{A,B}$ = 11.8, CH_AH$_B$C$_6$H$_5$), 4.46 (d, 1 H, CH$_A$$H_BC_6H_5$), 4.55 (d, 1 H, $J_{C,D}$ = 11.5, CH_CH$_D$C$_6$H$_5$), 4.66 (d, 1 H, $J_{1,2}$ = 3.6, H-1), 4.67 (d, 1 H, $J_{E,F}$ = 12.1, CH_EH$_F$C$_6$H$_5$), 4.72 (d, 1 H, $J_{G,H}$ = 11.7, CH_GH$_H$C$_6$H$_5$), 4.82 (d, 1 H, CH$_E$$H_FC_6H_5$), 4.83

(d, 1 H, $CH_G\mathit{H}_HC_6H_5$), 4.92 (d, 1 H, $CH_C\mathit{H}_DC_6H_5$), 7.21–7.39 (m, 20 H, H-Ar); The ^{13}C NMR spectrum was identical to that reported in the literature.[148]

2,3,4,6-Tetra-*O*-benzyl-α- and β-D-galactopyranose (2.6)

A solution of compound **4.1** (3.0 g, 5.4 mmol) in acetic acid (100 mL) was heated to 120 °C. Then an aqueous H_2SO_4 solution (1 M, 25 mL, 25 mmol) was added over a period of 1 h. The reaction was maintained at this temperature for 30 min and, then it was poured into water (100 mL). The resulting mixture was extracted with CH_2Cl_2 (3 × 60 mL) and the combined organic layers were washed with saturated aqueous $NaHCO_3$ solution, brine, dried ($NaSO_4$), and concentrated under reduced pressure. The resultant crude syrup was purified by flash column chromatography (toluene: EtOAc, 6:1) to give an inseparable anomeric mixture **2.6** as a light yellow syrup (1.83 g, α/β = 5:3, 63%): IR 3419 (br, OH) cm^{-1}; the ^1H NMR and ^{13}C NMR spectra were identical to those reported in the literature.[149]

2,3,4,6-Tetra-*O*-benzyl-D-galactose diethyl dithioacetal (4.2)

Trifluoroacetic acid (TFA) (10 mL, 134 mmol) was added to the solution of compound **2.6** (19.3 g, 36 mmol) in ethanethiol (50 mL). After the reaction mixture was stirred at rt overnight, it was quenched by the addition of a saturated aqueous $NaHCO_3$ (150 mL), and this mixture was extracted with Et_2O (3 × 50 mL). The combined organic layer was washed with brine, dried ($MgSO_4$), and concentrated at reduced pressure. The resultant crude compound was purified by flash column chromatography (hexane:EtOAc, 6:1) to give compound **4.2** as a light yellow syrup (16.5 g, 71%): $[\alpha]^{20}_D$ –24.7 (c = 1.97, CH_2Cl_2); IR 3500 (br, OH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.08 (t, 3 H, CH_3),1.12 (t, 3 H,

CH_3), 2.41–2.53 (m, 2 H, CH_2-CH_3), 2.58–2.67 (m, 2 H, CH_2-CH_3), 3.42–3.51 (m, 2 H, H-6), 3.71 (dd, 1 H, $J_{4,5}$ = 1.5, $J_{4,3}$ = 3.9, H-4), 3.84 (d, 1 H, $J_{1,2}$ = 4.0, H-1), 3.92 (dd, 1 H, $J_{2,3}$ = 6.8, H-2), 3.98 (m, 1 H, H-5), 4.25 (d, 1 H, $J_{A,B}$ = 11.6, $CH_AH_BC_6H_5$), 4.32 (dd, 1 H, H-3), 4.35 (d, 1 H, $CH_AH_BC_6H_5$), 4.45 (d, 1 H, $J_{C,D}$ = 11.8, $CH_CH_DC_6H_5$), 4.63 (d, 1 H, $CH_CH_DC_6H_5$), 4.73 (d, 1 H, $J_{E,F}$ = 11.0, $CH_EH_FC_6H_5$), 4.76 (d, 1 H, $J_{G,H}$ = 11.7, $CH_GH_HC_6H_5$), 4.77 (d, 1 H, $CH_EH_FC_6H_5$), 4.83 (d, 1 H, $CH_GH_HC_6H_5$), 7.12–7.34 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 14.4 (CH_3), 14.4$_6$ (CH_3), 24.9 (CH_2-CH_3), 25.4 (CH_2-CH_3), 53.5 (C-1), 70.1 (C-5), 70.5 (C-6), 73.3, 73.2, 75.0, 75.6 ($CH_2C_6H_5$), 76.2 (C-4), 81.8 (C-3), 83.1 (C-2), 127.4, 127.6, 127.6$_3$, 127.7, 127.8, 128.0, 128.1$_7$, 128.2, 128.2$_4$, 128.3, 138.0, 138.2, 138.5 (Ar-C). Anal. Calcd For $C_{38}H_{46}O_5S_2$: C, 70.55; H, 7.17. Found: C, 70.69; H, 7.13.

2,3,4,6-Tetra-*O*-benzyl-L-arabino-hexos-5-ulose diethyl dithioacetal (4.3)

Compound **4.2** (3.5 g, 5.4 mmol) was dissolved in DMSO (10 mL) and then acetic anhydride (6 mL, 63 mmol) was added slowly. The resulting reaction mixture was stirred 16 h at rt under a N_2 atmosphere. Then water (20 mL) and aqueous NH_4OH (28%, 15 mL) were added to quenched reaction, and the reaction mixture was then extracted with ether (3 × 30 mL). The combined organic layer was washed with brine, dried ($MgSO_4$), and concentrated down under reduced pressure. The residue was purified by flash column chromatography (hexane: EtOAc,8:1) to give compound **4.3** as a colourless syrup (3.0 g, 87%): $[\alpha]^{20}_D$ +8.93 (c = 0.63, CH_2Cl_2); IR 1728 (CO) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.22 (t, 1 H, CH_3), 1.28 (t, 1 H, CH_3), 2.64–2.76 (m, 4 H, CH_2-CH_3), 4.03 (dd, 1 H, $J_{2,1}$ = 3.4, $J_{2,3}$ = 7.3, H-2), 4.09 (d, 1 H, H-1), 4.26 (d, 1 H, $J_{4,3}$ = 3.1, H-4), 4.32 (dd, 1 H, H-3), 4.45 (brs, 2 H, H-6), 4.48 (d, 1 H, $J_{A,B}$ = 11.9, $CH_AH_BC_6H_5$), 4.54 (d, 1 H, $J_{C,D}$ = 11.9,

$CH_CH_DC_6H_5$), 4.57 (d, 1 H, $CH_AH_BC_6H_5$), 4.58 (d, 1 H, $CH_CH_DC_6H_5$), 4.72 (d, 1 H, $J_{E,F}$ = 11.2, $CH_EH_FC_6H_5$), 4.75 (d, 1 H, $CH_EH_FC_6H_5$), 4.76 (d, 1 H, $J_{G,H}$ = 10.9, $CH_GH_HC_6H_5$), 4.92 (d, 1 H, $CH_GH_HC_6H_5$), 7.22–7.40 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 14.1 (CH_3), 14.5 (CH_3), 24.7 (CH_2-CH_3), 25.6 (CH_2-CH_3), 63.3 (C-1), 71.9, 73.0 ($CH_2C_6H_5$), 74.4 (C-6), 75.0, 75.8 ($CH_2C_6H_5$), 81.9 (C-3), 82.5 (C-2), 83.1 (C-4), 127.1, 127.3, 127.4, 127.6, 127.6_3, 127.7, 127.8, 127.9, 128.0, 128.1, 128.2 136.8, 137.0,137.9, 138.4 (Ar-C), 208.2 (C=O). Anal. Calcd For $C_{38}H_{44}O_5S_2$: C, 70.77; H, 6.88. Found: C, 70.78; H, 6.99.

2,3,4,6-Tetra-*O*-benzyl-5-methylene-L-arabino-hexose diethyl dithioacetal (4.4)

To a solution of methyltriphenylphosphonium iodide (1.7 g, 4.3 mmol) in THF (20 mL) at –78 °C, n-BuLi (2.5 M in hexane, 1.6 mL, 4.0 mmol) was added slowly. After 1 h, the reaction mixture was warmed to 0 °C, and a solution of the compound **4.3** (0.92 g, 1.4 mmol) in THF (10 mL) was added dropwise. The reaction mixture was then allowed to warm slowly to rt over 16 h. The reaction was quenched by the addition of saturated aqueous NH_4Cl (20 mL), the solution was then diluted with water (50 mL), and extracted with CH_2Cl_2 (2 × 50 mL). The combined organic layer was washed with brine, dried ($MgSO_4$), and concentrated at reduced pressure. The resultant crude compound was purified by flash column chromatography (hexane:EtOAc, 9:1) to give the product **4.4** as a light yellow syrup (0.68 g, 75%): $[\alpha]^{20}_D$ +13.7 (c = 1.31, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 1.17 (t, 3 H, CH_3), 1.18 (t, 3 H, CH_3), 2.53–2.62 (m, 2 H, CH_2-CH_3), 2.62-2.69 (m, 2 H, CH_2-CH_3), 3.93 (m, 1 H, H-2), 4.07 (d, 1 H, $J_{1,2}$ = 5.8, H-1), 4.08–4.21 (m, H-4, H-3, H-6), 4.26 (d, 1 H, $J_{A,B}$ = 11.6, $CH_AH_BC_6H_5$), 4.49–4.60 (m, 3 H, $CH_2C_6H_5$), 4.66–4.73 (m, 3 H, $CH_2C_6H_5$), 4.83 (d, 1 H, $J_{C,D}$ = 11.1, $CH_CH_DC_6H_5$), 5.43 (s, 1 H, C=CH_2), 5.54 (s, 1 H, C=CH_2), 7.20–7.37 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 14.76, 14.81 (CH_3), 25.2,

25.8 (CH_2CH_3), 54.2 (C-1), 70.6 ($CH_2C_6H_5$), 70.8 (C-6), 73.0, 74.8, 75.6 ($CH_2C_6H_5$), 81.1, 81.6 (C-4, C-3), 83.1 (C-2), 116.2 (C=CH_2), 127.6, 127.6$_2$, 127.8, 127.9, 128.0$_2$, 128.0$_6$, 128.3, 128.4, 128.6, 128.6$_3$, 128.7, 128.8, 129.0, 138.4, 138.6, 139.0, 139.1 (Ar-C). 144.3 (*C*= CH_2). Anal. Calcd For $C_{39}H_{46}O_4S_2$: C, 72.86; H, 7.21. Found: C, 72.79; H, 7.12

(2*S,3*S*,4*S*)-2,3,4-tribenzyloxy-5-[(benzyloxy)methyl]-5-hexenal (2.8-*S*,*R*)**

Mercury (II) oxide (4.97 g, 22.9 mmol) was added to a solution of compound **4.4** (3.7 g, 5.7 mmol) in CH_3CN (150 mL) and H_2O (15 mL). After the solution was stirred for 5 min at rt, mercury (II) chloride (2.92 g, 10.7 mmol) was added. The reaction mixture was stirred for another 1.5 h, quenched by the addition of saturated aqueous $NaHCO_3$ (50 mL), and filtered through Celite. The filtrate was added water (50 mL), and extracted with ether (3 × 100 mL). The combined organic layer was washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure to give the crude aldehyde **2.19**, which was carried on to next step without further purification.

To a solution of compound **2.19** in dry THF (200 mL) at –78 °C under N_2 was slowly added vinyl magnesium bromide (6 mL, 1.0 M in THF, 6 mmol). The reaction mixture was stirred for 1 h at rt following which saturated aqueous NH_4Cl solution was added and the resultant solution was extracted with ether (3 × 100 mL). The combined organic layer was washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure to give an orange coloured syrup. This residue was purified by flash column chromatography column chromatography (hexane:EtOAc, 4:1) to give mixture of compound **2.8** as an almost colourless syrup (1.96 g, **2.8-*R*** : **2.8-*S*** = 1:2, 61%). The full characterization of these two compounds is described in Chapter 2.[145]

(1*S,4S,5S,6S)-4,5,6-tribenzyloxy-3-[(benzyloxy)methyl]cyclohex-2-enol (2.9-S)**

See the protocol in Chapter 2

(1*R*,2*S*,3*S*,4*S*,5*S*,6*S*)-3,4,5-tribenzyloxy-6-[(benzyloxy)methyl]bicyclo[4.1.0]heptan-2-ol (4.5) and (1*S*,2*S*,3*S*,4*S*,5*S*,6*R*)-3,4,5-tribenzyloxy-6-[(benzyloxy)methyl]bicycle [4.1.0]heptan-2-ol (4.6)

Under N_2, a solution of **2.9-*S*** (1.3 g, 2.5 mmol) in dry toluene (270 mL) was cooled to –10 °C. Dimethyl zinc (2 M in toluene, 6 mL, 12 mmol) was then added dropwise. The reaction was stirred at –10 °C for 15 min, then CH_2I_2 (2.0 mL, 25 mmol) was added slowly over 30 min. The reaction mixture was stirred for 16 h while being allowed to warm to 15 °C. The reaction was quenched by the addition of aqueous H_2SO_4 (10%), and it was diluted by adding EtOAc (300 mL). The isolated organic layer was washed with saturated aqueous $NaHCO_3$ solution, brine, dried ($MgSO_4$), and concentrated under reduced pressure. The two cyclopropyl isomers were separated and purified by radial chromatography (EtOAc/hexane 1:6) to give compound **4.5** and **4.6** as a colourless syrup (total weight 1.2 g, 90%).

Compound **4.5**: $[\alpha]^{20}_D$ +55.9 (c = 2.40, CH_2Cl_2); IR 3530 (br, OH) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.72 (dd, 1 H, $J_{7,7'}$ = 9.4, $J_{7,1}$ = 5.7, H-7), 0.88 (m, 1 H, H-7'), 1.21 (m, 1 H, H-6), 2.61 (d, 1 H, $J_{8,8'}$ = 9.3, H-8), 2.87 (d, 1 H, $J_{OH,5}$ = 1.5, OH), 3.53 (dd, 1 H, $J_{3,4}$ = 10.0, $J_{3,2}$ = 2.5, H-3), 3.99 (dd, 1 H, $J_{4,5}$ = 5.5, H-4), 4.14 (dd, 1 H, H-8'), 4.40 (m, 1 H, H-5), 4.42–4.49 (m, 3 H, H-5, $CH_2C_6H_5$), 4.58 (d, 1 H, $J_{A,B}$ = 10.7, $CH_AH_BC_6H_5$), 4.66 (d, 1 H, $J_{C,D}$ = 11.6, $CH_CH_DC_6H_5$), 4.70 (d, 1 H, $J_{E,F}$ = 11.8, $CH_EH_FC_6H_5$), 4.75 (d, 1 H, $CH_EH_FC_6H_5$), 4.78 (d, 1 H, $CH_CH_DC_6H_5$), 4.99 (d, 1 H, $CH_AH_BC_6H_5$), 7.25–7.38 (m, 20

H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 11.6 (C-7), 20.1 (C-1), 27.5 (C-6), 64.5 (C-2), 72.9, 73.0, 73.5, ($CH_2C_6H_5$), 74.3 (C-8), 75.2 (CH_2C_6H), 76.4 (C-5), 77.2 (C-3), 77.3 (C-4), 127.3, 127.4, 127.4_2, 127.6, 127.7, 127.8, 127.9, 128.0, 128.2, 128.3, 128.4, 138.2_2, 138.2_5, 138.8, 139.2 (Ar-C). Anal. Calcd For $C_{36}H_{38}O_5$: C, 78.52; H, 6.96. Found: C, 78.38; H, 7.13.

Compound **4.6**: $[\alpha]^{20}_D$ +42.9 (c = 2.37, CH_2Cl_2); IR 3560 (br, OH) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.65 (dd, 1 H, $J_{7,7'}$ = 10.2, $J_{7,1}$ = 5.3, H-7), 0.86 (t, 1 H, $J_{7,7'} + J_{7',1}$ = 11.9, H-7'), 1.24 (m, 1 H, H-6), 2.91 (d, 1 H, $J_{OH,5}$ = 1.6, O*H*), 3.17 (d, 1 H, $J_{8,8'}$ = 10.2, H-8), 3.30 (d, 1 H, H-8'), 3.67 (dd, 1 H, $J_{4,3}$ = 10.0, $J_{4,5}$ = 3.6, H-4), 3.84 (dd, 1 H, $J_{3,2}$ = 4.1, H-3), 4.22 (d, 1 H, H-2), 4.36 (m, 1 H, H-5), 4.44 (d, 1 H, $J_{A,B}$ = 12.1, $CH_AH_BC_6H_5$), 4.52 (d, 1 H, $CH_AH_BC_6H_5$), 4.58 (d, 1 H, $J_{C,D}$ = 12.6, $CH_CH_DC_6H_5$), 4.61–4.66 (m, 3 H, $CH_2C_6H_5$), 4.71 (d, 1 H, $CH_CH_DC_6H_5$), 4.84 (d, 1 H, $J_{E,F}$ = 11.5, $CH_EH_FC_6H_5$), 7.22–7.37 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 9.2 (C-7), 24.0 (C-1), 25.1 (C-6), 68.2 (C-2), 70.5, 72.3, 72.4 ($CH_2C_6H_5$), 73.3 (C-5), 73.3_2 ($CH_2C_6H_5$), 74.5(C-3), 76.3 (C-4), 76.6 (C-8), 127.2, 127.3, 127.5, 127.6, 127.6_3, 127.7, 127.8, 128.1, 128.2, 128.3, 128.4, 138.3_6, 138.4, 138.8, 138.9 (Ar-C). Anal. Calcd For $C_{36}H_{38}O_5$: C, 78.52; H, 6.96. Found: C, 78.35; H, 7.13.

(1R,2S,3S,4S,5S,6S)-2,3,4-tribenzyloxy-1-[(benzyloxy)methyl]-5-(3,5-difluorophenoxy)bicyclo[4.1.0]heptane (4.7)

After a suspension of NaH (60% in mineral oil, 99 mg, 2.5 mmol) was washed with hexane (2 × 5 mL), it was transferred in dry DMSO (20 mL) into 200 mL flask maintained at 18 °C. To this mixture a solution of compound **4.5** (176 mg, 0.32 mmol) in dry DMSO (25 mL) was added dropwise. The mixture was stirred for 30 min at 18 °C.

Potassium benzoate (97 mg, 0.61 mmol) was then added, and stirring was continued for a further 30 min. At which time, 1,3,5-trifluorobenzene (624 μL, 6.4 mmol) was added slowly. After 30 min the reaction was quenched by the addition of a saturated aqueous NH_4Cl. Following the addition of brine (100 mL), the resultant mixture was extracted with Et_2O (3 × 100 mL). The combined organic layer was dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by flash column chromatography (EtOAc/ Hexane 1:12) to give compound **4.7** as a colourless syrup (145 mg, 69%): $[\alpha]^{20}_D$ + 93.4 (c = 3.34, CH_2Cl_2); ^{1}H NMR ($CDCl_3$) δ 0.67 (dd, 1 H, $J_{7,7'}$ = 5.7, $J_{7,6}$ = 9.3, H-7), 0.76 (m, 1 H, H-7'), 1.25 (m, 1 H, H-6), 2.64 (d, 1 H, $J_{8,8'}$ = 9.3, H-8), 3.72 (dd, 1 H, $J_{3,2}$ = 2.7, $J_{3,4}$ = 9.7, H-3), 4.05 (dd, 1 H, $J_{8',7'}$ = 1.5, H-8'), 4.12 (dd, 1 H, $J_{4,5}$ = 5.1, H-4), 4.35–4.40 (m, 2 H, H-2, $CH_AH_BC_6H_5$), 4.44 (d, 1 H, $J_{A,B}$ = 12.0, $CH_AH_BC_6H_5$), 4.61 (d, 1 H, $J_{C,D}$ = 10.7, $CH_CH_DC_6H_5$), 4.62 (d, 1 H, $J_{E,F}$ = 12.1, $CH_EH_FC_6H_5$), 4.69 (d, 1 H, $CH_EH_FC_6H_5$), 4.73 (d, 1 H, $J_{G,H}$ = 11.8, $CH_GH_HC_6H_5$), 4.78 (dd, 1 H, $J_{5,6}$ = 7.3, H-5), 4.86 (d, 1 H, $CH_GH_HC_6H_5$), 4.99 (d, 1 H, $CH_CH_DC_6H_5$), 6.34–6.45 (m, 3 H, H-4', H-2', H-6'), 7.20–7.39 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 12.1 (C-7), 19.1 (C-6), 27.7 (C-1), 72.0 (C-5), 72.9, 73.0, 73.3 ($CH_2C_6H_5$), 73.6 (C-8), 75.2 ($CH_2C_6H_5$), 75.7 (C-4), 76.6 (C-2), , 76.7 (C-3), 96.4 (t, $^2J_{C,F}$ = 25.9, C-4'), 99.6–99.9 (m, C-2', C-6'), 127.4, 127.4$_5$, 127.5, 127.6$_5$, 127.7, 127.8, 128.0, 128.2, 128.2$_2$, 128.3, 128.4, 138.1, 138.4, 138.9, 139.1, 160.4 (t, $^3J_{C,F}$ = 13.7, C-1'), 163.8 (dd, $^1J_{C,F}$ = 245.8, $^3J_{C,F}$ = 15.9, C-3', C-5'); ^{19}F NMR ($CDCl_3$) δ –110.1 (t, $^3J_{H,F}$ = 8.6). Anal. Calcd For $C_{42}H_{40}F_2O_5$: C, 76.11; H, 6.08. Found: C, 76.15; H, 5.88.

(1S,2S,3S,4S,5S,6R)-2,3,4-tribenzyloxy-1-[(benzyloxy)methyl]-5-(3,5-difluorophenoxy)bicyclo[4.1.0]heptane (4.8)

After a suspension of NaH (60% in mineral oil, 11 mg, 0.28 mmol) was washed with hexane (2 × 1 mL), it was transferred in dry DMSO (3 mL) into 25 mL flask held at 18 °C. To this mixture a solution of compound **4.6** (20 mg, 0.036 mmol) in dry DMSO (2 mL) was added dropwise. The mixture was stirred for 30 min at 18 °C. Potassium benzoate (11 mg, 0.068 mmol) was then added, and stirring was continued for a further 30 min. At which time, 1,3,5-trifluorobenzene (0.13 mL, 1.3 mmol) was added slowly. After 45 min the reaction was quenched by the addition of a saturated aqueous NH_4Cl. Following the addition of brine (40 mL), the resultant mixture was extracted with Et_2O (3 × 20 mL). The combined organic layer was dried ($MgSO_4$), and concentrated under reduced pressure. The residue was purified by flash column chromatography (EtOAc/ Hexane 1:12) to give compound **4.8** as a colourless syrup (18 mg, 75%): $[\alpha]^{20}_D$ +57.2 (*c* = 5.30, CH_2Cl_2); IR 1115 (CF) cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.69 (dd, 1 H, $J_{7,7'}$ = 5.1, $J_{7,6}$ = 9.5, H-7), 1.10 (t, 1 H, $J_{7,7'} + J_{7',6}$ = 11.2, H-7'), 1.17 (m, 1 H, H-6), 2.98 (d, 1 H, $J_{8,8'}$ = 10.5, H-8), 3.61 (d, 1 H, H-8'), 3.85 (dd, 1 H, $J_{4,3}$ = 7.7, $J_{4,5}$ = 3.4, H-4), 3.95 (dd, 1 H, $J_{3,2}$ = 4.4, H-3), 4.37 (d, 1 H, H-2), 4.39 (d, 1 H, $J_{A,B}$ = 12.0, $CH_AH_BC_6H_5$), 4.54 (d, 1 H, $CH_AH_BC_6H_5$), 4.56–4.67 (m, 5 H, H-5, H-$CH_2C_6H_5$), 4.69 (br s, 2 H, $CH_2C_6H_5$), 6.42 (m, 1 H, H-4'), 6.46-6.53 (m, 2 H, H-2', H-6'), 7.19–7.39 (m, 20 H, H-Ar); ^{13}C NMR ($CDCl_3$) δ 11.1 (C-7), 22.2 (C-6), 25.4 (C-1), 71.6, 72.3, 73.1 ($CH_2C_6H_5$), 73.1_4 (C-2), 73.7 ($CH_2C_6H_5$), 75.0 (C-4), 75.7 (C-8), 76.1, 76.2 (C-3, C-5), 96.5 (t, $^2J_{C,F}$ = 26.0, C-4'), 99.7–99.9 (m, C-2', C-6'), 127.6, 127.6_4, 127.6_8, 127.0, 127.8, 127.8_3, 128.1, 128.4, 128.4_2, 128.4_3, 128.4_4, 138.6, 138.7, 138.7_1, 138.9, 160.4 (t, $^3J_{C,F}$ = 13.7, C-1'), 163.8 (dd, $^1J_{C,F}$ = 245.9, $^3J_{C,F}$ = 15.8, C-3', C-5'); ^{19}F NMR ($CDCl_3$) δ –109.9 (t, $^3J_{H,F}$ = 8.8). Anal. Calcd For $C_{42}H_{40}F_2O_5$: C, 76.11; H, 6.08. Found: C, 75.86; H, 5.82.

(1R,2S,3S,4R,5S,6S)-5-(3,5-difluorophenoxy)-1-(hydroxymethyl)bicyclo[4.1.0]heptan-2,3,4-triol (1.81)

A mixture of compound **4.7** (149 mg, 0.22 mmol) and 10% Pd-C (60 mg, 10%, w/w) in MeOH (30 mL) was stirred at room temperature under a H_2 atmosphere for 6 h. The mixture was filtered through a Celite pad, which was subsequently washed thoroughly with MeOH (100 mL). The filtrate and washings were combined and concentrated under pressure to a solid. The product **1.81** was crystallized from MeOH (62 mg, 91%): $[\alpha]^{20}{}_D$ + 106.6 (c = 0.41, MeOH); ^{1}H NMR (D_2O) δ 0.58 (dd, 1 H, $J_{7,7'}$ = 5.7, $J_{7,6}$ = 9.5, H-7), 0.72 (td, 1 H, $J_{7,7'}$ + $J_{7',6}$ = 11.0, $J_{7',8'}$ = 1.5, H-7b), 1.58 (m, 1 H, H-6), 2.99 (d, 1 H, $J_{8,8'}$ = 11.5, H-8), 3.71 (dd, 1 H, $J_{3,2}$ = 3.3, $J_{3,4}$ = 11.1, H-3), 3.93 (dd, 1 H, $J_{4,5}$ = 5.2, H-4), 4.03 (dd, 1 H, H-8'), 4.43 (d, 1 H, H-2), 5.00 (dd, 1 H, H-5), 6.58 (m, 1 H, H-4'), 6.63–6.70 (m, 2 H, H-2', H-6'); ^{13}C NMR (D_2O) δ 10.4 (C-7), 19.3 (C-6), 29.2 (C-1), 66.0 (C-8), 66.6 (C-3), 67.2 (C-4), 69.2 (C-2), 72.7 (C-5), 96.6 (t, $^3J_{C,F}$ = 26.2, C-4'), 99.7–99.9 (m, C-2', C-6'), 159.1 (t, $^3J_{C\text{-}F}$ = 14.1, C-1'), 163.6 (dd, $^1J_{C,F}$ = 243.7, $^3J_{C,F}$ = 16.1, C-3', C-5'); ^{19}F NMR ($CDCl_3$) δ –112.1 (t, $^3J_{H,F}$ = 9.1). Anal. Calcd For $C_{14}H_{16}F_2O_5$: C, 55.63; H, 5.34. Found: C, 55.65; H, 5.50.

(1S,2S,3S,4R,5S,6R)-5-(3,5-difluorophenoxy)-1-(hydroxymethyl)-bicyclo[4.1.0] heptan-2,3,4-triol (1.82)

A mixture of compound **4.8** (164 mg, 0.25 mmol) and 10% Pd-C (67 mg, 10%, w/w) in MeOH (33 mL) was stirred at room temperature under a H_2 atmosphere for 9 h. The mixture was filtered through a Celite pad, which was subsequently washed thoroughly with MeOH (200 mL). The filtrate and washings were combined and

concentrated under pressure to give a white solid. The product was purified by flash column chromatography (CH_2Cl_2/MeOH, 10:1) to give compound **1.82** as a white solid, which was recrystallized from MeOH (54 mg, 73%): $[\alpha]^{20}_D$ + 97.7 (*c* = 0.62, MeOH); m.p 160.5 - 160.7 °C; ^{1}H NMR (CD_3OD) δ 0.65 (dd, 1 H, $J_{7,7'}$ = 5.1, $J_{7,6}$ = 9.6, H-7), 0.97 (t, 1 H, $J_{7,7} + J_{7',6}$ = 11.1, H-7'), 1.21 (ddd, 1 H, $J_{7',6}$ = 6.0, $J_{6,5}$ = 1.6, H-6), 3.13 (d, 1 H, $J_{8,8'}$ = 11.4, H-8), 3.68 (dd, 1 H, H-8'), 3.81 (dd, 1 H, $J_{4,5}$ = 3.3, $J_{4,3}$ = 8.5, H-4), 3.94 (dd, 1 H, $J_{3,2}$ = 5.0, H-3), 4.39 (d, 1 H, H-2), 4.73 (dd, 1 H, H-5), 6.50 (m, 1 H, H-4'), 6.63–6.68 (m, 2 H, H-2', H-6'); ^{13}C NMR (CD_3OD) δ 10.8 (C-7), 22.1 (C-6), 29.7 (C-1), 68.0 (C-2), 68.9 (C-4), 69.0 (C-8), 70.8 (C-3), 77.6 (C-5), 96.9 (t, $^3J_{C,F}$ = 26.4, C-4'), 100.5–100.7 (m, 2 C, C-2', C-6'), 162.1 (t, $^3J_{C,F}$ = 13.9, C-1'), 165.2 (dd, $^1J_{C,F}$ = 244.6, $^3J_{C,F}$ = 15.9, C-3', C-5'); ^{19}F NMR ($CDCl_3$) δ –112.0 (t, $^3J_{H,F}$ = 9.1). Anal. Calcd For $C_{14}H_{16}F_2O_5$: C, 55.63; H, 5.34. Found: C, 55.53; H, 5.26.

2,3,4,6-Tetra-O-acetyl-β-d-galactopyranosyl chloride (4.9)[150]

To a solution of 1,2,3,4,6-penta-*O*-acetyl β-D-galactopyranose (5.77 g, 14.8 mmol) in dry chloroform (300 mL) was added anhydrous aluminium chloride (10.0 g, 77 mmol), and the mixture was stirred at rt for 1 h. After filtering the reaction solution, dry hexane (700 mL) was added and the white solid was removed by filtration. The solvent was removed from the resultant solution under reduced pressure to give a white solid. This material was crystallized using ether and petroleum ether to give compound **4.9** as white crystal (4.1 g, 75%). All spectral data matched that reported in the literature.[151]

6,8-Difluoro-4-methylumbelliferyl α-D-galactopyranoside (4.11)

To a solution of 4-methyl-6,8-difluoroumbelliferone [152,153] (223 mg, 1.1 mmol) in dry MeOH (4 mL) potassium *tert*-butoxide (80 mg, 0.72 mmol) was added. After 10 min the yellow-coloured solution was evaporated to dryness, and the resultant salt was dried under high vacuum. To a flask containing dried, 3 h at 150 °C, molecular sieves (2 g) anhydrous DMF (4 mL) was added and the resulting suspension was stirred for 30 min under N_2. To this mixture was added the potassium 6,8-difluoro-4-methylumbelliferone salt made before and 18-crown-6 (186 mg, 0.56 mmol). This mixture was stirred for a further 30 min under N_2. Following the addition of compound **4.9** (184 mg, 0.50 mmol) the resultant solution was stirred at rt for 14 h. After adding a saturated aqueous $NaHCO_3$ (10 mL) the mixture was extracted with EtOAc (2 × 10 mL). The combined organic layers were washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure. The resultant crude compound was purified by flash column chromatography (hexane: EtOAc, 3:2) to give compound **4.10-β** as a white solid (22 mg) which was identified according to the literature,[147] a mixture of mainly compound **4.10-β** with slightly compound **4.10-α** (43 mg), and a slightly impure white solid **4.10-α** (105 mg) that was deprotected as follows.

To the partially purified solution of the product **4.10-α** (105 mg) in methanol (10 mL) a catalytic amount of a sodium methoxide solution was added at 0 °C. After 1 h freshly regenerated Rexyn Resin (H^+ form) was added in order to neutralize the solution. The volatiles were removed under reduced pressure and, the resultant crude product was purified by flash column chromatography (chloroform:methanol 5:1) to give compound **4.11** as white solid (41 mg, about 22%): m.p 164.5 – 166.0 °C; ^{1}H NMR (CD_3OD) δ 2.48 (d, 3 H, $J_{Mc,2'}$ =1.2, CH_3), 3.65 (dd, 1 H, $J_{6,6'}$ = 11.3, $J_{6,5}$ = 6.5, H-6), 3.71 (dd, 1 H, $J_{6',5}$ =

5.8, H-6'), 4.04–4.09 (m, 3 H, H-2, H-3, H-4), 4.20 (m, 1 H, H-5), 5.77 (d, 1 H, $J_{1,2}$ = 1.8, H-1), 6.41 (d, 1 H, H-3'), 7.49 (dd, 1 H, $^{5}J_{5',F}$ = 2.1, $^{3}J_{5',F}$ = 11.4, H-5'); ^{13}C NMR (CD_3OD) δ 18.9 (CH_3), 62.2 (C-6), 70.0 (C-4), 70.8 (C-3), 70.9 (C-2), 74.5 (C-5), 104.0 (C-1), 107.7 (C-5'), 115.7 (C-3'). Due to lack of solubility, multiple C-F couplings and the lack of NOE enhancements the other aromatic; ^{13}C resonances could not be definitively assigned. Anal. Calcd For $C_{16}H_{16}F_2O_8$: C, 51.34; H, 4.31. Found; C, 51.40; H, 4.43.

4.6 Enzyme Kinetics

The kinetic parameters K_i and k_{inact} were determined by classic dilution assay method. The inactivation of coffee bean α-galactosidase was monitored by incubating the enzyme (0.27 μM) with various concentrations (0.5 mM – 20 mM) of inhibitors **1.81** and **1.82**, respectively, in 50 mM sodium phosphate buffer, pH 6.52, at 37 °C in total volume of 40 μL. The fraction of remaining enzyme activity was measured as a function of time by addition of an aliquot (5 μL) of the inactivation mixture (40 μL, made above) to a solution of *p*-nitrophenyl α- D-galactopyranoside (PNPG) (0.15 mM, 395 μL). Pseudo-first-order rate constants at each inactivator concentration (k_{obs}) were determined by fitting each inactivation curve to a first-order rate equation. The second-order rate constant for the inactivation process was determined by fitting each k_{obs} value to the Michaelis-Menten equation.

The active-site-directed nature of the inactivation was proven by demonstrating protection against inactivation by a competitive inhibitor **1.80**. Inactivation mixtures (40 μL) containing 0.27 μM enzyme and 4 mM compound **1.81** were incubated in the

absence and presence of competitive inhibitor **1.80** (0.001 mM, K_i = 0.541μM). At various time intervals, an aliquots (5 μL) were removed and assayed for residual activity as described above.

For *p*-nitrophenyl α-D-galactopyranoside as substrate, the absorbance at 400 nM was monitored for 10 min using a Cary 3E spectrophotometer equipped with a Peltier temperature controller.

For 6,8-Difluoro-4-methylumbelliferyl α-D-galactopyranoside (**4.11**) as substrate, the activity of coffee bean enzymes was assayed by monitoring the rate of hydrolysis of compound **4.11**. The coffee bean α-galactosidase activity assay solutions contained 0.001 mM of compound **4.11** and 0.63 mU/cm^3 enzyme in 50 mM sodium phosphate buffer (pH 6.52) with 0.1% bovine serum albumin in total volume of 400 μL. Each experiment was initiated by the addition of enzyme to an equilibrated assay solution held at 37 °C. the fluorescence signals were measured for 18 seconds with a Cary Eclipse fluorometer equipped with a Peltier temperature controller using excitation detection at 360 nm and emission detection at 460 nm.

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