Enzymatic Synthesis of Glycosides and Oligosaccharides

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Sincere thanks are due to my family for their kind support throughout my education.

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Declaration

The work described in this thesis is the original work of the author except where acknowledgement is made to work and ideas previously described. It was carried out in the Department of Chemistry, University of Warwick, and Colworth House, Unilever Research, between October 1987 and September 1990 and has not been submitted previously for a degree at any institution.

Memorandum

Part of this work has been published:

1. D.H.G. Crout, D.A. MacManus and P. Critchley, *J. Chem. Soc., Perkin Trans 1*, 1990, 1865.

2. D.H.G. Crout, D.A. MacManus and P. Critchley, *J. Chem. Soc. Chem. Commun.*, 1991, 376.

To my parents.

Summary

The β-D-galactosidase of *Escherichia coli* catalysed galactosyl transfer to a variety of acceptor substrates. Transfers to simple alcohols were followed by transfers to chiral alcohols, chiral diols (bearing primary and secondary hydroxyl groups) and to a *meso*-diol. In particular, the regio- and stereoselective aspects of the reactions were investigated. In general, transfer to primary hydroxyl groups was favoured over transfer to secondary hydroxyl groups, but little or no preference for the transfer to specific enantiomers in a racemic mixture was observed. The results for propane-1,2-diol and butane-1,3-diol are interpreted in terms of the possible conformations which might be adopted at the active site of the enzyme.

Transfer to *cis*-cyclohexa-3,5-diene-1,2-diol gave rise to two diastereoisomers. During the early stages of the reaction, a diastereoisomeric excess of *ca.* 80% was observed; this was reduced to *ca.* 20% as the yields of product reached their maximum values. Assignment of the structures of the products was based on a combination of the techniques of nuclear Overhauser enhancement and molecular modelling.

α-Galactosyl transfers to lactose and cellobiose using *Mortierella vinacea* α-D-galactosidase were also studied. In both cases, a single trisaccharide was isolated. Spectroscopic evidence indicated that α (1-6) linkages had been formed in both cases.

An acrylamide/acrylic acid polymer intended for use in enzymatic oligosaccharide synthesis was developed. The polymer was high swelling so as to allow permeation by the enzyme and could be easily stored. An attempt to introduce chiral cavities specific for certain monosaccharides was made by substituting part of the acrylamide for a boronate-containing acrylamide and carrying out the polymerisation in the presence of the monosaccharide. The success of the imprinting procedure was measured by the ability of the polymer to separate the components of a racemic mixture of the monosaccharide. The application of such "molecular imprinting" as an aid to oligosaccharide synthesis is discussed.

CONTENTS

List of Abbreviations

A	Absorbance
AAPB	*N*-Acryloyl-3-aminophenylboronic acid
ACPA	4,4'-Azobis(4-cyanopentanoic acid)
br	Broad
CI	Chemical ionisation
cm	centimetre
d	Doublet
d.e.	Diastereomeric excess
DMF	*N*,*N*-Dimethylformamide
EI	Electron impact
Fru	Fructose
Fuc	L-Fucose
Gal	D-Galactose
GalNAc	*N*-Acetyl-D-galactosamine
Glc	D-Glucose
GlcNAc	*N*-Acetyl-D-glucosamine
Gly	Glycosyl
Hex	Hexosyl
HPLC	High performance liquid chromatography
IPG	Isopropylideneglycerol
J	Coupling constant
LCST	Lower critical solution temperature
M	Parent molecular ion
m	Multiplet
Man	Mannose

MBA	*N,N'*-Methylenebisacrylamide
m.p.	Melting point
MurNAc	Muramic acid
M.W.	Molecular weight
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser enhancement
ONPG	*o*-Nitrophenyl galactoside
ppm	Parts per million
q	Quartet
s	Singlet
SDS	Sodium dodecyl sulphate
t	Triplet
TEMED	*N,N,N',N'*-Tetramethylethylenediamine
TLC	Thin layer chromatography
U	Units
UDP	Uridine 5'-diphosphate
UV	Ultraviolet
Xyl	Xylose

CHAPTER ONE

General Introduction: The Application of Enzymes in Oligosaccharide Synthesis.

The selective synthesis of oligosaccharides has become of increasing importance over the last few years in response to an appreciation of their role in biology.[1-23] Carbohydrate chains covalently linked to proteins and lipids are present at the periphery of all cells from every organism and in all secretions and intercellular fluids from vertebrates. It is evident that such chains in glycolipids and glycoproteins play crucial rôles in the recognition events controlling cell and tissue behaviour. Examples occur in immune systems (antigen-antibody reaction), in the reception and processing of hormones and neurotransmitters, in the recognition of molecules involved in the process of cell sorting during tissue development and in the molecular changes associated with controlled growth and differentiation. By modification of the determinants, conclusions may be drawn regarding the kind of interaction of oligosaccharides with the surface proteins. This has the potential of leading to new drugs which would, for example, prevent the adhesion of bacteria to mucosal surfaces; thus the onset of pathogenesis may be prevented by the blocking of the bacterial receptors by a carbohydrate drug. Additionally, modified glycoconjugates are indicators of diseased conditions including a number of malignancies. In this connection diagnostic kits have been developed which employ antibodies against cancer-associated carbohydrate antigens.

To explore and exploit the biological activity of carbohydrates there arises a need to develop synthetic procedures for oligosaccharide chains which can be employed following synthetic and biosynthetic work on oligopeptides and oligonucleotides.[24] Though classical methods of carbohydrate synthesis are well developed,[25-32] their inherent complexity makes this an area which provides perhaps the greatest challenge to the organic chemist. In particular, syntheses providing complete control over regio- and stereospecificity are desirable but the large number of hydroxyl groups of similar reactivity and the possibility of forming either the α- or the β-anomer necessitate many protection and deprotection steps. Thus a synthesis of a trisaccharide may require in excess of ten chemical steps whereas the synthesis of a decasaccharide is yet to be achieved. Where a synthesis is possible it is likely to be laborious and time-consuming, reducing both the yields and the chances of a practical large-scale synthesis. With these difficulties it is likely that oligosaccharide synthesis is the area where the application of enzymatic methods may have the greatest potential.

Enzymatic methods are gaining acceptance as routine tools in organic synthesis[33] and in the area of carbohydrates they offer important advantages over chemical methods. Such *biotransformations* preclude the need for hydroxyl group protections and provide stereospecific and often highly regiospecific syntheses. Additionally, they proceed under mild conditions in aqueous and often organic media. The appropriate enzymes are becoming more readily available and

immobilisations increase their lifetimes and allow their reuse. Several overviews of the types and uses of biotransformations in carbohydrate chemistry have appeared recently.[15,16,17,24] There are three main groups of enzymes dealing with oligosaccharide anabolism and catabolism *in vivo:*

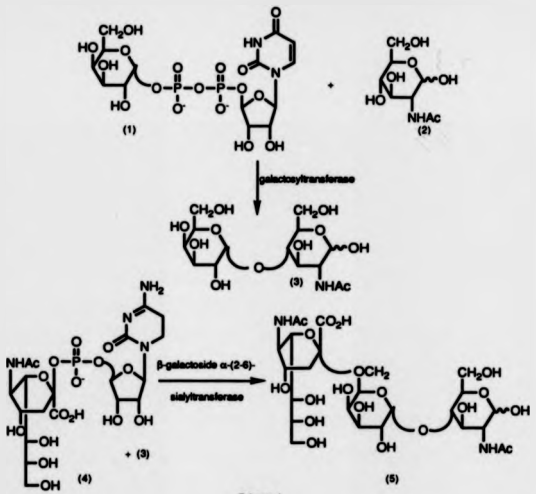
i) the enzymes of the so-called Leloir pathway

ii) the enzymes of the non-Leloir pathway

iii) the glycosidases.

Leloir glycosyltransferases

The Leloir glycosyltransferases (EC 2.4) form a section of the transferase enzymes and catalyse the transfer of a sugar moiety from a "donor" molecule to an "acceptor" molecule. In general they catalyse monosaccharide transfer from a glycosyl nucleotide (sugar nucleoside phosphate) to a protein, lipid or growing oligosaccharide. Such nucleotides are often referred to as 'activated' or 'high energy' on account of the large amount of free energy they generate on hydrolysis as a result of reduced electrostatic repulsion between negatively charged groups and an overall increase in resonance stabilisation energy. The number of activated glycosyl nucleotides used is small and each corresponds to its own set of glycosyltransferases, each enzyme being responsible for the creation of a specific glycosidic linkage. An example of the usefulness of these enzymes in synthesis is given in Scheme 1.



Scheme 1

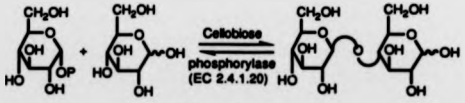
The trisaccharide 5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranolic acid-(2-6)-β-D-galactopyranosyl-(1-4)-2-acetamido-2-deoxy-D-glucopyranose[Neu5Acα(2-6)-Gal-β-(1-4)-GlcNAc, (5)], commonly found as a component of glycoproteins, was prepared by galactosyl transfer from UDP-D-galactose (1) to *N*-acetyl-D-glucosamine (2), followed by enzymatic sialyl transfer from cytidine-5'-monophosphosialate (4).[34] The enzymes employed were a galactosyltransferase [EC 2.4.1.22] and ß-D-galactoside-α(2-6)sialyltransferase [EC

2.4.99.1], the latter causing a change of configuration at the anomeric carbon of the sialic acid moiety.

Although in excess of one hundred glycosyltransferases are known and their specificity of glycosidic bond formation is particularly high, these enzymes have so far failed to fulfil their promise on account of a lack of availability and the high cost of the activated sugars necessary for their action. However, with the advent of genetic engineering, improved methods of enzyme isolation and purification as well as recent advances in enzymatic syntheses of sugar nucleotides, one can anticipate Leloir glycosyltransferases emerging as key tools in synthesis over the next few years.

Non-Leloir glycosyltransferases

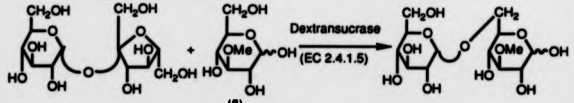
The non-Leloir pathway enzymes may also involve transfer from high energy phosphate compounds but activation is in the form of sugar phosphates rather than in the form of sugar nucleoside phosphates. An example of such a synthesis is given in Scheme 2;[35] it is interesting to note the change in anomeric configuration during the transformation.



Scheme 2

High energy phosphate compounds are not always a requirement of activity, however. Glycansucrases, in particular amylosucrase (EC 2.4.1.4), dextransucrase (EC 2.4.1.5), inulinsucrase (EC 2.4.1.9), levansucrase (EC 2.4.1.10) and alternansucrase (EC 2.4.1.125) are able to take advantage of the the relatively high bond energy in sucrose [α-D-glucopyranosyl-(1-2)-β–D-fructofuranose (Glcα(1-2)βFru)] when transferring D-fructose or D-glucose residues.

Levansucrase and inulinsucrase are D-fructosyltransferases using sucrose as substrate to synthesise β(2-6)- and β-(2-1)-D-fructans respectively by repeated D-fructose transfer. Similarly the glucosyltransferases dextransucrase, alternansucrase and amylosucrase synthesise glycogen-like polysaccharides from sucrose. As well as transferring to sucrose these enzymes have also been found to transfer to other acceptors.[36-38] Thus *Betacoccus arabinosaceous* dextransucrase effects transfer of D-glucose to the HO-6 of 3-*O*-methyl-D-glucose (6) to give mainly the (1-6)-linked disaccharide plus smaller amounts of (1-6)-linked tri- and tetrasaccharides[39](Scheme 3).



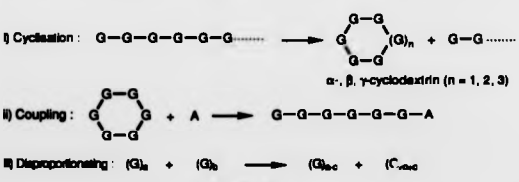
(6)

Scheme 3

Other types of glycosyltransferases have also been used in synthesis[40-42] and recently fructosyltransferases have been identified which carry out fundamentally different transfer reactions to those of levansucrase and inulinsucrase.[43-47] This serves to indicate the diversity of action of glycosyltransferases and with the large number of potential sources of enzymes it is likely that further transferases will emerge.

Certain glycosyltransferases are also be able to create glycosidic linkages *de novo* without the need for pre-existing glycosidic linkages.[48-49] Thus, for example, α-D-glucosyl fluoride may substitute for sucrose in the synthesis of glucans by amylosucrase.[50]

An important member of the transferase group of enzymes is cyclodextrin-α-(1-4)-glucosyltransferase (EC 2.4.1.19) which can carry out cyclisation, coupling and disproportionation reactions[51-53] (Scheme 4; G=D-glucose).



Scheme 4

In common with the glycosyltransferases discussed above, this enzyme also accepts fluorinated substrates.[54] Thus α-D-glucosyl fluoride is converted into a mixture of α- and β-cyclodextrins as well as malto-oligomers. In the presence of suitable acceptors[55] gluco- and malto-derivatives are produced.

Using soluble starch as donor this enzyme is also able to transfer D-glucose units to *N*-substituted moranolines[56] (to give oligoglucosyl moranolines) and the sweetener stevioside[57] to give mono-, di- and tri-α-glucosylated products.

Glycosidases

The glycosidases are classified under hydrolases (EC 3.2) as their natural function is the hydrolysis of carbohydrates; those which cleave terminal glycosidic linkages of oligo- or polysaccharides are called exoglycosidases while those cleaving at non-terminal positions are called endoglycosidases. They are subdivided according to whether they act on *O*-, *N*- or *S*-glycosyl compounds (EC 3.2.1, 3.2.2 and 3.2.3, respectively). Those acting on *O*-glycosyl compounds have been particularly valuable in synthesis, perhaps more so than their glycosyltransferase counterparts, on account of their ease of isolation and the fact that they do not involve cofactors. However, although they are generally less expensive than the transferases they usually give lower yields and do not exhibit the same high degree of regioselectivity.

About one hundred glycosidases have been identified and although not all have been used in synthesis most are likely to possess synthetic activity. Some of those which have already been used in synthesis are given in Table 1.

Table 1 : Glycosidases which have been employed in synthesis.

Enzyme EC		Systematic name	References
3.2.1.1	α-amylase	1,4-α-D-glucan glucanohydrolase	58-60
3.2.1.2	ß-amylase	1,4-α-D-glucan maltohydrolase	59,61
3.2.1.3	glucoamylase (amyloglucosidase)	exo-1,4-α-D-glucosidase	59,62-64
3.2.1.17	lysozyme	mucopeptide *N*-acetyl-muramoylhydrolase	65-73
3.2.1.20	α-D-glucosidase	α-D-glucoside glucohydrolase	125,128,129 170,179-189
3.2.1.21	ß-D-glucosidase (emulsin)	ß-D-glucoside glucohydrolase	126,127 130,158,169-178
3.2.1.22	α-D-galactosidase	α-D-galactoside galactohydrolase	136,140-142 164-168
3.2.1.23	ß-D-galactosidase	ß-D-galactoside galactohydrolase	131-135,138 140-152 154-160
3.2.1.24	α-D-mannosidase	α-D-mannoside mannohydrolase	122-124 140,141,189
3.2.1.26	ß-D-fructo-furanosidase (invertase)	ß-D-fructofuranoside fructohydrolase	137,168 190-194
3.2.1.28	α,α-trehalase	α,α-trehalase glucohydrolase	74,75
3.2.1.30	ß-*N*-acetyl-D-glucosaminidase	2-acetamido-2-hydroxy-ß-D-glucoside acetamidodeoxy-glucohydrolase	143,204,205
3.2.1.31	ß-D-glucuronidase	ß-D-glucuronide glucuronosohydrolase	76,77
3.2.1.39	*endo*-1,3-ß-D-glucanase	1,3-ß-D-glucan glucanohydrolase	78
3.2.1.41	pullulanase	pullulan 6-glucanohydrolase	79-83
3.2.1.49	α-*N*-acetyl-D-galactosaminidase	2-acetamido-2-deoxy-α-D-galactoside acetamidodeoxy-galactohydrolase	204

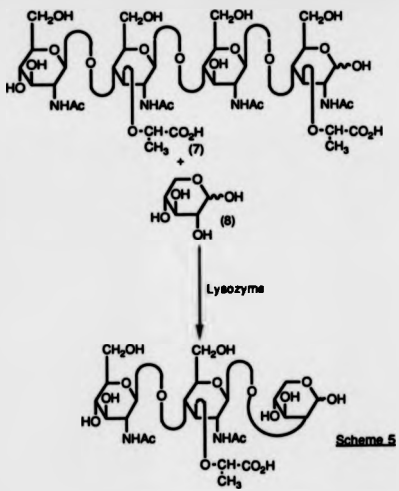
Table 1 (continued).

Enzyme EC		Other name	References
3.2.1.50	α-*N*-acetyl-D-glucosaminidase	2-acetamido-2-deoxy-α-D-glucoside acetamidodeoxy-glucohydrolase	204
3.2.1.51	α-L-fucosidase	α-L-fucoside fucohydrolase	203,204
3.2.1.52	ß-*N*-acetyl-D-hexosaminidase	2-acetamido-2-deoxy-ß-D-hexoside acetamido-deoxyhexohydrolase	204,206
3.2.1.53	ß-*N*-acetyl-D-galactosaminidase	2-acetamido-2-deoxy-ß-D-galactoside acetamidodeoxy-galactohydrolase	204
3.2.1.60	*exo*-maltotetraohydrolase	1,4-α-D-glucan maltotetraohydrolase	84
3.2.1.68	isoamylase	glycogen 6-glucanohydrolase	85-87
3.2.1.89	*endo*-1,4-ß-D-galactanase	arabinogalactan 4-ß-D-galactanohydrolase	88
3.2.1.98	*exo*-malto hexaohydrolase	1,4-α-D-glucan maltohexaohydrolase	89

Those which have been most widely used are lysozyme, pullulanase, α- and ß-amylases, isoamylase, α- and ß-D-glucosidases and galactosidases, α-D-mannosidase, ß-D-xylosidase and ß-D-fructofuranosidase. These enzymes are of two distinct types; lysozyme, pullulanase and the amylases are able to catalyse the transfer of one or more glycosidic residues whereas the "-osidases" are strictly limited to the transfer of a single sugar unit at any one time. Thus, for example, α- and ß-amylases can catalyse both glucosyl and maltosyl transfers.[59-61] These differences are reflected in the mechanism of action of these enzymes; whereas the "-osidases" are thought to possess only two binding sites (see below), lysozyme, for example, is able to accommodate six sugar residues.[71,90-92]

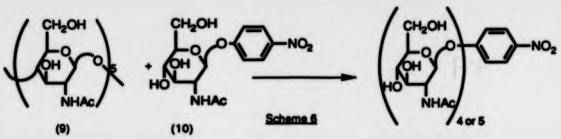
Transglycosylation reactions have been carried out with lysozyme using either the cell wall tetrasaccharides GlcNAcß(1-4)MurNAcß(1-4)GlcNAcß(1-4)MurNAc (7) or the chitin oligosaccharides $[ß(1\text{-}4)NAcGlc]_{4\text{ or }5}$ as donors and mono- or disaccharides as acceptors.

With the cell wall tetrasaccharide (7) as donor a disaccharide unit was transferred to GlcNAc, GlcNAcß(1-4)GlcNAc, GlcNAcß(1-4)MurNAc, D-galactose and D-xylose.[66,70,71] In general ß(1-4)-links were formed except in the case of D-xylose (8) where ß(1-2)-links predominated (Scheme 5).



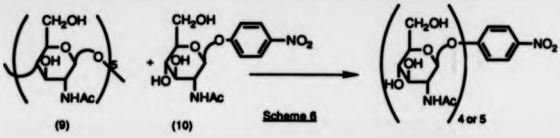
Scheme 5

With the chitin oligosaccharides transfer of two units is also common,[65,69,72,73] though examples are known where one,[65,72,73] three,[67,73] four,[67,68] and five[67] units are transferred. Two recent examples concern penta-N-acetylchitopentaose (9) as donor and *p*-nitrophenyl 2-acetamido-2-deoxy-ß-D-glucopyranoside (10) as acceptor in aqueous methanol[68] and aqueous dimethyl sulphoxide[67] media (Scheme 6).



Scheme 6

Pullulanase and isoamylase have been found to be particularly useful in modifications of cyclodextrins; α-, ß- and γ-cyclodextrins (six, seven or eight α(1-4)-linked α-D-glucopyranosyl residues joined together in a closed loop) have been receiving increasing attention over the last few years. Their hydrophobic interiors enable them to form inclusion complexes with various compounds while their hydrophilic exteriors give them relatively high solubility in aqueous media. These properties have led to them being used for stabilising labile materials, emulsifying oils, masking odours, changing viscous or oily compounds into powders and stabilising water-insoluble or slightly soluble compounds, particularly pharmaceuticals. The solubility characteristics of cyclomalto-oligosaccharides may be improved by the attachment of D-glucose or malto-oligosaccharide moieties to the 6-position(s) of the glycosyl residue(s). Such branching may be achieved with either pullulanase or isoamylase using their reverse hydrolytic or transfer properties. Reverse reactions involve condensation between malto-oligosaccharides (11) and the cyclodextrins (12) with the oligosaccharide group(s) invariably becoming attached to the 6-position(s) of the cyclodextrin [79,80,82,85-87] (Scheme 7).



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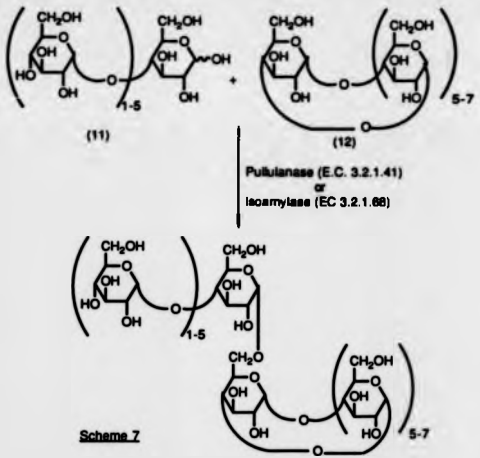


Scheme 7

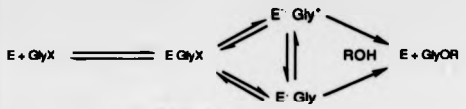
As well as the singly branched products shown in Scheme 7, these reverse hydrolytic reactions may also generate di- and tri-*O*-α-oligoglucosyl α-, ß- and γ-cyclodextrins. Mixtures of branched non-cyclic products are also produced *via* condensation of the malto-oligosaccharide.

The transfer reactions generally involve α-maltosyl fluoride as substrate and give products similar to those obtained by reverse hydrolytic reactions.[82,83] The exact composition of the product mixture depends on the source of the enzyme but yields may be significantly higher than those obtained from corresponding reactions employing maltose as substrate. Of all the enzymes so far discussed, those which

have received most attention are α- and ß-D-glucosidases and galactosidases, ß-D-xylosidase, α-D-mannosidase and ß-D-fructofuranosidase. The applications of these enzymes in synthesis are best viewed in conjunction with a consideration of their mode of action.

D-Mannosidases, D-glucosidases, D-galactosidases and ß-D-fructofuranosidase: mechanism of action.

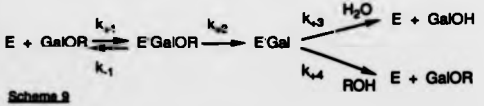
Although glycosidases have been applied in synthesis since the last century it is only in the last few years that insights have been gained into their mode of action. Despite much attention, however, no precise mechanism has yet emerged. Most theories about the mechanism involve some form of acid catalysis of the departure of the aglycone to yield a common glycosyl-enzyme intermediate, stabilised by ion pair formation or covalent bonding to an enzyme group.[93,94] The exact nature of the intermediate is still the subject of much debate but is known to be dependent on such factors as hydrophobic forces, conformational changes, entropy changes as well as the source of the enzyme and, most importantly, the structure of the substrate (both glycone and aglycone). With the possible exception of ß-D-xylosidase, where available evidence points to a single mechanism, the retention of anomeric configuration is achieved *via* a double displacement with either formation or breakdown of the intermediate being rate-determining. Work in this area has recently been reviewed by Sinnott[95,96] who considers that the double displacement mechanism involves glycopyranosyl cation-like transition states leading to and from a covalent glycosylated carboxylate as the glycosyl-enzyme intermediate with most of the catalytic power of the enzyme coming from non-covalent interactions (Scheme 8).



Scheme 8

Alternative hypotheses have recently been advanced by Fleet,[97] and Post and Karplus[98] which involve an endocyclic ring-opening between the anomeric carbon and the ring oxygen, though such proposals are not in accordance with kinetic observations.

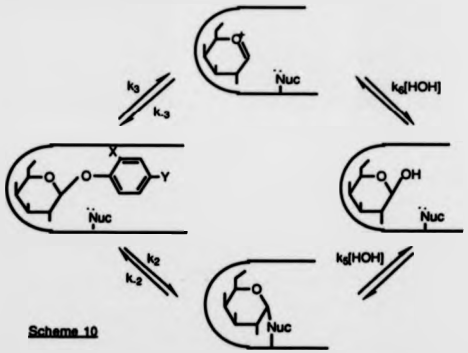
Most studies into the mechanism of action of glycosidases have centred on ß-D-galactosidase from *E. coli*. The enzyme is readily available and its amino acid sequence has been determined.[99] It is tetrameric in nature (with independent active sites) and obeys Michaelis-Menten kinetics (Scheme 9, see also Appendix 1).



Scheme 9

Since the configuration at the anomeric centre of the galactosyl moiety is unchanged during the reaction there are two possible mechanisms: a double displacement (two S_N2 steps), or a shielded oxocarbonium ion with frontside attack by the incoming nucleophile.

On the basis of studies of oxygen-18 leaving group kinetic isotope effects on the hydrolysis of *p*-nitrophenyl and *o,p*-dinitrophenyl ß-D-galactosides Rosenbirg and Kirsch[100] determined that both pathways may be operated by the enzyme depending on the reactivity of substrate. The predominant route for all but the most reactive substrates is the displacement mechanism involving S_N2 nucleophilic displacement of the aglycone to give a covalent galactosyl-enzyme intermediate followed by nucleophilic attack by water. Highly reactive substrates with acidic leaving groups form a transient enzyme-bound galactosyl oxocarbonium ion which partitions between the nucleophilic residue on the enzyme and the solvent (Scheme 10).



Scheme 10

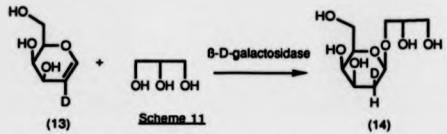
Galactosyl -enzyme

One other possibility involves neighbouring group participation of the hydroxyl group at C-2 following the realisation that *o,p*-dinitrophenyl 2-chloro-2-deoxy-ß-D-galactopyranoside is a good inhibitor of the enzyme without being a substrate.[101] The hypothesis was tested by comparing the rates of hydrolyses of ß-D-galactosides with those of the corresponding 2-deoxy-ß-D-*lyxo*-hexopyranosides.[96] The *lyxo*-derivatives exhibited extremely low rates of hydrolysis, far lower than could be accounted for by poor binding due to the absence of a C-2 hydroxyl group. Such results appear to confirm the essential rôle of the C-2 hydroxyl group in the cleavage of ß-D-galactosides by the enzyme. Unfortunately this mechanism cannot be differentiated from one involving nucleophilic attack by the enzyme on the basis of kinetic isotopic effect data.

Precise details regarding the active site of ß-D-galactosidase are yet to be defined though it is thought that there are two catalytically functioning groups; an acidic group activates the aglyconoxy group by protonation of the anomeric oxygen while a basic group is responsible for electrostatically assisting the formation of the galactosyl oxocarbonium ion and reversibly stabilising it as an α-D-galactosyl ester intermediate. Carboxylate groups have been identified at the active site[103-107] though it is uncertain whether two different carboxyl groups, one in its protonated form, the other as its conjugate base, are employed, or whether the bifunctional catalysis is executed by a single carboxyl group. The former corresponds to a bilateral model in which the carboxylic groups

act from opposite sides of the pyranoid plane whereas the latter corresponds to a monolateral model with the carboxylic group acting from only one side. Investigations into these two possibilities have been possible following the realisation that glycosidases are able to catalyse additions to the double bonds of glycals[108] and heptenitols[109] which both act as proton acceptors in the same way as glycosides.

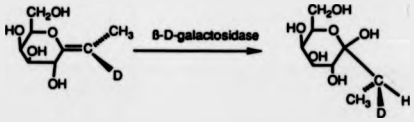
The incubation of D-galactal-2-*d* (13) with glycerol[110] yielded 1-deoxyglycerol-1-yl 2-deoxy-ß-D-*lyxo*-hexopyranoside-2(*S*)-*d* (14) which is consistent with protonation occuring from below the plane of the pyranoid ring (Scheme 11).



(13) (14)

Scheme 11

In a recent study[111] into the hydration products of (*Z*)-3,7-anhydro-1,2-dideoxy-2-deuterio-D-*galacto*-oct-2-enitol (15) the sole product was 2-(*S*)-1,2-dideoxy-2-deuterio-D-*galacto*-3-octulopyranose (16) which is consistent with protonation from above the plane of the pyranoside ring (Scheme 12).



Taken together, these two results indicate the location of two catalytically active groups on opposite sides of the pyranoid ring lending credence to the bilateral model in the case of ß-D-galactosidase.

As a result of various binding studies with ß-D-galactosidase the hydrolytic and transgalactosylic reactions have been found to involve two binding sites;[112-115] a "galactose" and a "glucose" site, corresponding with the two components of the natural substrate, lactose. The overall binding specificity at the "galactose" site is determined by positions 3, 4 and 6 of galactose with positions 3 and 4 being critical; the wrong orientation at either of these positions essentially eliminates binding and catalysis. The hydroxyl group at position 2 has only a small effect on the binding irrespective of its presence/absence or its orientation. As discussed above, however, it may be important in catalysis. Positions 3 and 4 are also considered to be involved in catalysis but the significance of the 5-position has not been fully elucidated.

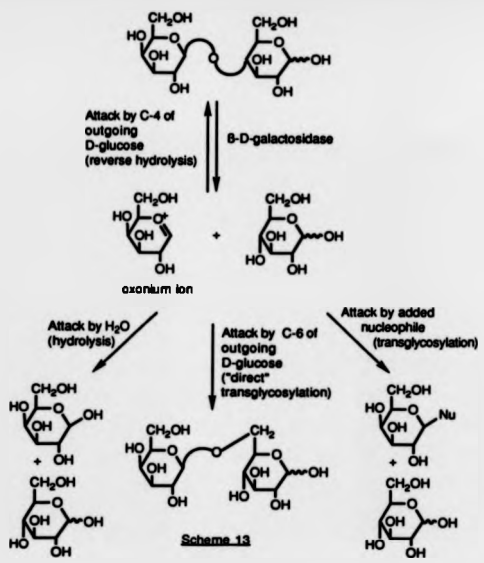
Studies involving the "glucose" site indicate that in the free state there is little affinity for glucose but that after the

glycosidic bond of the substrate has been broken the affinity for glucose greatly increases, most likely due to a conformation change of the active site of the enzyme.[99,116] This accounts for the high degree of intramolecular allolactose (Galß(1-6)Glc) production when lactose (Galß(1-4)Glc) acts as substrate. The control of levels of allolactose is the natural function of ß-D-galactosidase and is accomplished by the enzyme determining the ability of glucose to bind to the galactosyl form of the enzyme. Binding studies with a large number of sugars and alcohols have shown that it is the ability to bind rather than the reactivity which is the most important factor regarding the specificity of the "glucose" site.

Glycosidases in synthesis.

Modern applications of glycosidases in oligosaccharide manipulations stem from studies which began during the last century. The fact that enzymes were able to split oligosaccharides suggested that it may be possible to link monosaccharide units together in a reverse-type reaction. The earliest known communication on the subject describes the synthesis of maltose from glucose by the action of yeast.[116] This was followed by extensive studies and a period of prolific publication in the area of glycoside and oligosaccharide formation by Bourquelot *et al.* early this century.[118]

As discussed above, the mechanism of action of glycosidases has been greatly studied and is still the subject of much debate. Nevertheless, there is general acceptance that reactions proceed *via* a glycosyl-enzyme intermediate followed by aglycone elimination to give an oxonium ion. The oxonium ion may then undergo nucleophilic attack in one of three ways: i) attack by water (giving simple hydrolysis), ii) attack by the outgoing aglycone, or iii) attack by an added nucleophile. These various possibilities are exemplified by the action of ß-D-galactosidase on lactose (Scheme 13).



Scheme 13

In the attack by the outgoing aglycone more than one possiblity may exist depending on whether it possesses more than one nucleophilic centre. Thus in reactions where a disaccharide acts as substrate, attack may be by the newly liberated hydroxyl group (constituting the reverse reaction) or a different hydroxyl group (constituting a "direct" transglycosylation reaction).[119] If attack occurs by an added nucleophile, *eg.* an alcohol or a sugar, then a transglycosylation reaction occurs resulting in the production of a glycoside or an

oligosaccharide; it is in these types of reaction that the majority of the synthetic power of glycosidases lies.

A glycosyl-enzyme intermediate may also be formed from a free monosaccharide. By this means synthesis may be achieved from saccharides with no pre-existing glycosidic bond. Such reactions constitute "reversion" syntheses.

Reversion syntheses.

In the production of oligosaccharides from monosaccharides the equilibrium lies heavily in favour of the reactants and a practical synthesis may only be achieved by taking measures to displace the equilibrium. This is normally done by employing high concentrations of substrates, decreasing the amount of water present, removing the product or a combination of all three.

Early reversion syntheses were pioneered by Bourquelot *et al.* [118] who used high concentrations of monosaccharides in conjunction with the appropriate glycosidase (generally or α-D-glucosidase, ß-D-glucosidase, α-D-galactosidase, ß-D-galactosidase or α-D-mannosidase). A remarkable number of syntheses were achieved and these were reviewed by Oppenheimer and Kuhn.[120] A more recent review has been conducted by Stanek *et al.*[121]

α-D-Mannosidases.

α-D-Manno-oligosaccharide synthesis by reverse hydrolysis has received little attention despite the importance of D-mannose residues as elements of *N*- and *O*-linked oligosaccharides. However, two disaccharides, α-D-mannopyranosyl-(1-6)-D-mannopyranose (Manα(1-6)Man) and an unidentified isomer, were obtained by Courtois and Le Dizet[122] by the action of a plant α-D-mannosidase on a 20% w/w D-mannose solution.

Much higher yields, up to 37% disaccharides, were achieved by Johansson *et al.*[123] by using a much higher concentration of D-mannose (85% w/w) and a temperature of 75°C to speed the reaction. That such a high temperature could be tolerated by the enzyme is in accordance with an earlier discovery[124] that sugars and polyols have a stabilising effect on proteins; indeed no reduction in enzyme activity could be detected. The major product was the α(1-6)-linked isomer with smaller amounts of the α(1-2)- and (1-3)-isomers. Higher oligosaccharides were also detected but not characterised.

D-Glucosidases.

As discussed earlier, reversion reactions of D-glucosidases have been known since the last century when it was found that maltose (Glcα(1-4)Glc) could be obtained from glucose by the action of yeast. Such 'batch' reactions, *ie.* simple mixing of substrate and enzyme, give low yields as little attention is given to driving the equilibrium in favour of products. The reaction was re-investigated by Naketani *et al.*[125] In addition to maltose it was found that the yeast α-D-glucosidase also produced isomaltose (Glcα(1-6)Glc) and that yields could be increased by shaking a visking tube containing the enzyme in the glucose solution. The system, it was thought, was encouraging the synthetic reaction with the substrate being continuously provided at a high level and the products being removed by diffusion through the visking tube. It was also discovered that the presence of active charcoal in the reaction mixture further increased the yield of maltose. This was attributed to adsorption of maltose onto the charcoal once it had diffused through the visking tube, thus effectively removing it from the reaction mixture.

Tanaka and Oi also employed activated charcoal in an attempt to improve yields in the sweet almond ß-D-glucosidase-catalysed condensation of D-glucose.[126] However, rather than adding the active charcoal directly to the reaction mixture, the D-glucose solution was pumped through columns of the immobilised enzyme and active charcoal in series.

Product oligosaccharides could then be eluted from the charcoal column using progressively more concentrated solutions of ethanol in water. This so-called 'continuous' method was compared with a simple batch procedure employing the immobilised enzyme and visking tubing. Curiously the poorest yields were obtained with the continuous method. The best results were obtained by using enzyme entrapped in polyacrylamide beads in a batch process though it was still only possible to obtain 21 mg of cellobiose (Glcß(1-4)Glc) from 30 g glucose after 3 days. In all reactions cellobiose was the main product with smaller amounts of gentiobiose (Glcß(1-6)Glc). The effect of temperature, pH and substrate concentration on the production of cellobiose were also investigated in the reaction employing entrapped ß-D-glucosidase in a batch method. The yield of cellobiose could be increased by increasing temperature (to an optimum of 41°C), decreasing pH and increasing the concentration of glucose.

The same reaction in the batch mode was investigated by Ajisaka *et al.* [127] It was found that significantly higher concentrations of D-glucose (90% w/v) dramatically increased the overall yield of disaccharides to 49% thus indicating that substrate concentration is probably the most important factor in shifting the equilibrium in favour of products. In contrast to the results of Tanaka and Oi, however, gentiobiose was found to be the main product; the product mixture consisted of gentiobiose : (sophorose (Glcβ(1-2)Glc) + cellobiose) : laminaribiose (Glcß(1-3)Glc) 6:3:1. The reaction was found to

proceed fastest at pH 4 and 55°C and the yield of disaccharides increased approximately linearly with glucose concentration.

In a more recent publication Fujimoto *et al.* compared the yields and ratios of glucobioses synthesised from D-glucose by the action of an α-D-glucosidase (*Saccharomyces sp.*) two ß-D-glucosidases (almond and *Penicillium funicolosum*) and a glucoamylase (*Rhizopus sp.*) in batch and continuous processes.[128] For the batch processes yields of glucobioses of approximately 30% were obtained whereas the continuous processes gave yields lower than 10%. These differences are explained by the use of a lower monosaccharide concentration in the continuous method in order to decrease the viscosity for circulation. The factors controlling the structure and the composition of the disaccharides appeared to have a strong relationship with the substrate specificity of the enzymes. Additionally, the ratio of disaccharide compositions was markedly affected by the reaction method; such differences are most likely accounted for by transglycosylation reactions which may occur in batch reactions but which are precluded in the continuous system.

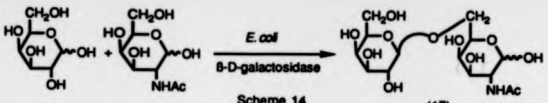
α-D-Glucosidases have also been used for the formation of α-D-glucosyl-ß-D-fructoses.[129] In a batch method *Saccharomyces sp.* α-D-glucosidase produced α-D-glucosyl-(1-1)-ß-D-fructose (Glcα(1-1)ßFru) with smaller amounts of α(1-4)-, (1-3)- and (1-6)-linked products (total 50% disaccharides). The concentration of D-glucose and D-fructose were 10% and 100% (w/v) respectively to reduce formation of glucobioses. When the α-D-glucosidase was immobilised and applied in a

continuous system with a column of activated carbon, the same saccharides were produced but in considerably lower yield (total 10% disaccharides) and with the major product being α-D-glucosyl-(1-4)-ß-D-fructose (Glcα(1-4)ßFru).

The reverse hydrolytic synthesis of long chain alkyl glucosides (important as surfactants) has also been achieved using a ß-D-glucosidase.[130] Almond ß-D-glucosidase immobilised on XAD-4 catalysed direct condensation of D-glucose with a variety of water-insoluble primary alcohols (C_4-C_{12}) by simply shaking the two phase mixture. A yield of approximately 10 g dm^{-3} for the alkyl ß-D-glucosides could be obtained after several days, though up to two weeks were required to reach thermodynamic equilibrium in the case of the long chain alcohols.

D-Galactosidases.

The most intensively studied glycosidase with respect to reversion and transglycosylation reactions is *E. coli* ß-D-galactosidase. When immobilised on Sepharose CL-4B this enzyme has been shown to produce 6-*O*-ß-D-galactopyranosyl-2-acetamido-2-deoxy-D-galactose (GalNAcß(1-6)Gal (17)) from D-galactose and 2-acetamido-2-deoxy-D-galactose[131,132] (Scheme 14).



Scheme 14

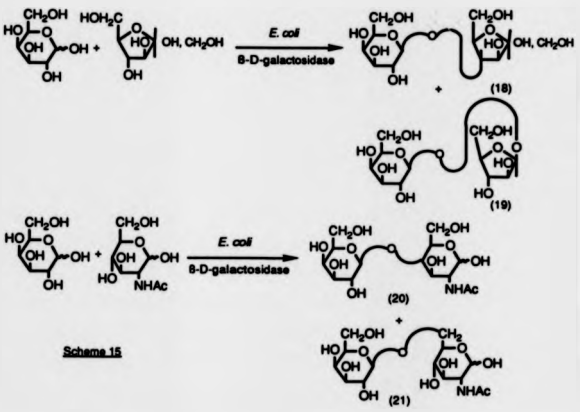
(17)

The low yield (2-3%) of the product is attributed to the use of only low substrate concentrations.

Huber and Hurlburt carried out a study into galactose/glucose and galactose/galactose condensations as well as the ability of *E. coli* ß-D-galactosidase to catalyse reversion reactions of monosaccharides other than galactose.[133]

At least ten ß-D-Gal-D-Glc's and ten ß-D-Gal-D-Gal's (including α- and ß-anomers) could be detected, their relative abundances depending on the ratio of D-glucose and D-galactose in the initial mixture. Of the various monosaccharides tested for their possible substitution of D-galactose in reversion reactions only two, D-fructose and D-arabinose, gave rise to new products as detected by GLC. No new peaks were detected for L-ribose, D-tagatose, D-lyxose, L-sorbose, D-erythrose, DL-glyceraldehyde, D-glucose, D-talose, 2-deoxy-D-galactose, or *N*-acetyl-D-galactosamine despite the fact that they bound to the "galactose" site of the enzyme or closely resembled the structure of D-galactose. These results indicate that the 6-position is least important for reactivity and that the 2-hydroxyl group must be equatorial for activity even though an axial 2-hydroxyl group also gives rise to tight binding.

Ajisaka *et al.* used immobilised *E.coli* ß-D-galactosidase in a continuous system for the condensation of D-galactose with D-fructose and *N*-acetyl-D-glucosamine (Scheme 15).[134]



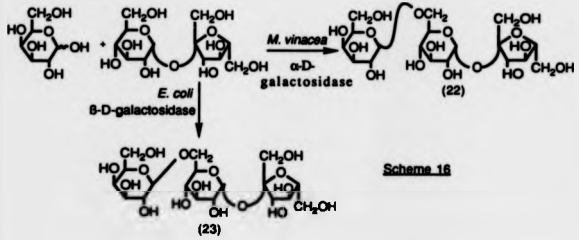
Scheme 15

Lactulose (Galß(1-4)ßFru (18)) and allolactulose (Galß(1-1)ßFru (19)) were obtained in 3.3 and 8.0% yields respectively and *N*-acetyllactosamine (Galß(1-4)GlcNAc (20)) and *N*-acetyl allolactosamine (Galß(1-6)GlcNAc (21)) were obtained in 0.9 and 9.1% yield respectively. The overall yield was found to be increased by renewing the column of activated carbon, allowing virtually all of the monosaccharides to be converted to products.

These reactions have been further studied by the same group[135] and small amounts of ß(1-5)- and ß(1-6)-linked products were also detected in the condensation of D-galactose with D-fructose. A comparison was made between batch (free enzyme) and continuous (immobilised enzyme) processes for the aforementioned synthesis and also for production of ß-D-galactosyl-D-glucoses (ß(1-2)-,(1-3)-,(1-4)- and (1-6)-linked) from D-glucose and D-galactose using the ß-D-galactosidases from *Aspergillus oryzae* and *Escherichia coli*.

In the synthesis of ß-D-galactosyl-D-glucoses and D-galactosyl-2-acetamido-2-deoxy-D-glucoses the continuous method gave yields approximately twice those obtained from the batch method though the composition of the disaccharide mixture was little affected by either the method of production or the origin of the enzyme. In the production of the ß-D-galactosyl-D-fructoses, however, the batch method gave yields over five times those obtained by the continuous method. Also, the proportions of β(1-5)-linked disaccharides were markedly different for the two enzymes and the ratios of ß(1-5)- and (1-6)-linked disaccharides associated with the batch and continuous methods were also different.

An example of trisaccharide synthesis by reversion has appeared recently.[136] D-Galactose was condensed with sucrose (α-D-glucopyranosyl-(1-2)-ß-D-fructose) using free and immobilised *M. vinacea* and *E. coli* ß-D-galactosidases by batch and continuous methods. The α– and ß-D-galactosidases produced raffinose (Galα(1-6)Glcα(1-2)ßFru (22)) and isoraffinose (Galß(1-6)Glcα(1-2)ßFru (23)) respectively (Scheme 16).



Scheme 16

Using the batch method with the free enzymes additional products were detected. The α-D-galactosidase produced planteose (Galα(1-6)Fruß(2-1)αGlc) and the ß-D-galactosidase produced the novel trisaccharide (Galβ(1-6)ßFru(2-1)αGlc); these trisaccharides were considered to be formed by transgalactosylations from the rapidly produced raffinose or isoraffinose.

ß-D-Fructofuranosidase

ß-D-Fructofuranosidase (invertase) has also been found to exhibit reverse hydrolytic activity.[137] Alkyl ß-D-fructofuranosidases were obtained using methanol, ethanol and propan-1-ol as substrates though only small yields (<3%) resulted as no attention was given to optimising the reaction (Scheme 17).

Scheme 17

Transglycosylation syntheses.

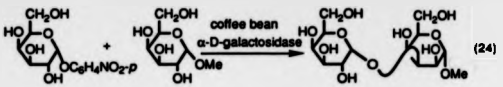
The name "transglycosylation" was originally given by Rabaté[138] to enzymatic processes by which 'a certain glycoside is converted in the presence of a suitable acceptor to a new compound corresponding to the initial substance.' With respect to glycosidases the term nowadays denotes the formation of new substances from a glycoside or oligosaccharide. The enzymes derive their names from the nature of the sugar residues they are able to transfer and the type of glycosidic linkage which is broken and formed. Thus ß-D-galactosidase catalyses reactions of sugars in which the transferred moiety is the terminal galactose attached to the rest of the donor *via* a ß-linkage. Additionally, the newly formed linkage between

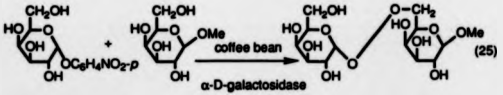
galactose and the acceptor is also ß- in type with no α-isomer being formed. It is the highly stereoselective nature of the transglycosylation reaction which makes it attractive in oligosaccharide synthesis; depending on the nature of the donor and the linkage (α- or ß-) required, the appropriate enzyme can be chosen. When more than one hydroxyl group may act as the acceptor a mixture of isomers may result . For example, when the acceptor molecule is a monosaccharide the mixture may consist of a mixture of ß(1-1)-, ß(1-2)-, ß(1-3)- and ß(1-4)- and ß(1-6)-isomers. Quite often, however, one particular isomer predominates and various methods exist of modulating the ratio of products (see below).

In contrast to equilibrium-controlled reverse reactions, transglycoslyation reactions are kinetically controlled, the optimum yields being achieved by stopping the reaction at the appropriate stage (often determined by HPLC). In general reactions are carried out in water which competes with the intended acceptor for the transferred sugar. On account of the relatively high concentration of water the equilibrium lies heavily in favour of hydrolysis. Studies with ß-D-galactosidase, however, show that the ratio of the rates of transgalactosylation and hydrolysis may be increased by increasing the acceptor concentration and that the rate of transgalactosylation is dependent on structural features of the acceptor.[139] Further, the addition of acceptor often stimulates enzymatic activity; since the extent of hydrolysis is greatly decreased this rate enhancement is due to a large increase in the rate of transfer.[140] The effect of reducing water

concentration by the addition of an organic co-solvent (*N*,*N*-dimethylformamide) was studied by Nilsson [141] using coffee bean α-D-galactosidase. Somewhat surprisingly it was found that the yield of glycoside decreased with increasing amounts of DMF despite the reduction in water activity. Thus the yield of Galα(1-3)GalαOC$_6$H$_4$NO$_2$-*p* from GalαOC$_6$H$_4$NO$_2$-*p* (donor and acceptor) was reduced from 32% to 10% on increasing the DMF co-solvent concentration from 0 to 45% (v/v). Reasons for this are unclear but it is suggested that the acceptor binds to the enzyme acceptor binding site with a lower affinity the higher the concentration of organic co-solvent. The conformation of the enzyme is apparently not distorted by the co-solvent, because the regioselectivity of the enzyme remains more or less constant. One potential advantage of miscible hydro-organic systems over purely aqueous systems, however, is that the solubilities of glycosides with hydrophobic aglycones are likely to be enhanced in the former allowing the use of higher concentrations of such glycosides as donors and acceptors.

In the same study the effect of changing the nature and configuration of the aglycones of the acceptor glycosides was also examined. Thus whereas the glycosyl acceptor GalαOMe gave Galα(1-3)GalαOMe (24) as the main product using coffee bean α-D-galactosidase and GalαOC$_6$H$_4$NO$_2$-*p* as glycosyl donor, GalßOMe gave the corresponding α(1-6)-linked product Galα(1-6)GalßOMe (25) (Scheme 18).





Scheme 18

The equivalent experiment with *E. coli* ß-D-galactosidase and Galß$OC_6H_4NO_2$-*o* as glycosyl donor gave Galß(1-6)GalαOMe and Galß(1-3)GalßOMe as the main products *i.e.* a reversal of regioselectivity. That both the structure of the aglycone and its anomeric configuration have a pronounced effect on the regioselectivity was further confirmed in a following study by Nilsson.[142] α- and ß-D-Galactosidases and α-D-mannosidases were used with *o*- or *p*-nitrophenyl ß-D-glycoside derivatives as donor and either the α– or ß-D-glycoside (aglycone = Me, $C_6H_4NO_2$-*o*, $C_6H_4NO_2$-*p*) as the acceptor. Although mixtures were always formed, a preponderance of one particular isomer could generally be obtained by careful choice of donor and acceptor though, curiously, no (1-4)-linked isomers were detected as products (Table 2).

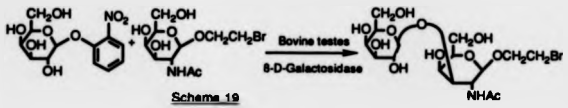
Table 2 : Effect of varying donor and acceptor on the type of linkage formed by coffee bean α-D-galactosidase.

Glycosyl donor	Glycosyl acceptor	Main Product	yield %
Galα$OC_6H_4NO_2$-*p*	GalαOMe	Galα(1-3)GalαOMe	27
Galα$OC_6H_4NO_2$-*p*	GalβOMe	Galα(1-6)GalβOMe	18
Galα$OC_6H_4NO_2$-*o*	Galα$OC_6H_4NO_2$-*o*	Galα(1-2)Galα$OC_6H_4NO_2$-*o*	6

The effect of changing only the nature of the aglycone of the acceptor was examined using jack bean α-D-mannosidase with Manα$OC_6H_4NO_2$-*p* as donor and either ManαOMe or Manα$OC_6H_4NO_2$-*p* as acceptor. In both cases the α(1-2)- and (1-6)-linked disaccharides were the major and minor products respectively but their ratio was 5:1 in the case of the former and 19:1 in the case of the latter. In the case where ManαOMe was used as acceptor the trisaccharide glycoside Manα(1-2) Manα(1-2)ManαOMe was also formed. These various differences in regioselectivity with α- and ß-D-galactosidases and α-D-mannosidase further support the conclusions regarding a hydrophobic binding site suggested by Nilsson following studies with organic co-solvents. With respect to the glycosyl acceptor the bulky, more hydrophobic aryl groups have larger effects on regioselectivity than the corresponding methyl groups.

Similar regioselectivities were obtained with α- and ß-D-galactosidases.[143] Thus using disaccharide donors (raffinose and lactose) and allyl alcohol, benzyl alcohol and trimethylsilylethanol as acceptors galactosidases were formed which when used in further transglycosylation reactions gave rise to mainly (1-3)- and (1-6)-linked disaccharide glycosides.

The effect of using 2-acetamido-2-deoxy-ß-D-glycosides as acceptors was also studied by Nilsson.[144] (Scheme 19).



Scheme 19

In general the (1-3)-linked disaccharide was the main product with nitrophenyl α- and ß-D-galactosides as donors although a significant amount of Galß(1-4)GlcNAcßOMe could be obtained using $GalßOC_6H_4NO_2$-*p* as donor and GlcNAcβOMe as acceptor.

Another way of modulating linkage formation is *via* the source of enzyme. The effect of enzyme source was highlighted by Nilsson who observed how different ß-D-galactosidases gave different disaccharide products.[24] Thus whereas *E. coli* [131,132] and yeast[145] ß-D-galactosidases catalyse mainly the formation of Galß(1-6)GlcNAc, *Lactobacillus* ß-D-galactosidase[146] and ß-D-galactosidase from rat mammary gland[147] give almost exclusively the ß(1-4)- and (1-3)-linked products respectively.

The various specificities of enzymes from different

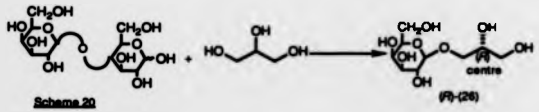
sources have been used for the preparation of a particular isomer from a mixture by specific hydrolyses of unwanted isomers.[148,149] Thus Galß(1-3)GlcNAc and Galß(1-3)GlcNAcßSEt were synthesised by transgalactosylation reactions using bovine testes ß-D-galactosidase with lactose as donor. In both cases the product mixtures contained unwanted ß(1-4)- and (1-6)-linked isomers which could be hydrolysed and subsequently removed by employing *E. coli* ß-D-galactosidase. The *E.coli* enzyme was also used in synthetic mode but only one product, Galß(1-6)GlcNAcßSEt, was obtained.

In addition to the use of more than one glycosidase in a catabolic way, it is also possible to combine glycosidases and glycosyltransferases in the synthesis and also to use enzymatic methods in conjuction with chemical methods. These possibilities have been discussed elsewhere.[24]

ß-D-Galactosidase.

Many studies with ß-D-galactosidase have involved its incubation with lactose (Galß(1-4)Glc) followed by analysis of the various oligosaccharides produced.[119,150-153] Early studies have been reviewed by Stanek *et al.*[121] A more comprehensive review has appeared more recently.[154] Common products are Galß(1-6)Gal and Galß(1-6)Glc in addition to other disaccharides, trisaccharides, tetrasaccharides, pentasaccharides and possibly higher oligosaccharides. Generally ß(1-6)- and ß(1-4)-linkages are formed with smaller amounts of ß(1-3)-linkages but the exact nature of the product spectrum is strongly dependent on the source of the enzyme.

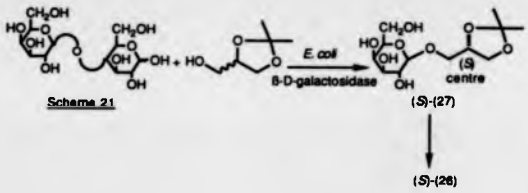
The use of non-saccharide acceptors has also been a major focus for studies with ß-D-galactosidase. Boos *et al.* have studied ß-D-galactosyl transfer to glycerol and concluded that only the (2*R*)-ß-D-galactoside (26) (in addition to smaller amounts of 2-*O*-ß-D-galactosylglycerol) is formed[155,156] (Scheme 20).



Scheme 20

Interestingly, in the reversion reaction between D-galactose and glycerol these workers determined the product mixture to consist of a diastereomeric excess of the (2*S*)-β-D-galactoside.

Similar studies have been carried out with racemic isopropylideneglycerol as acceptor which yielded diastereomerically pure isopropylideneglycerol galactoside (27). This was then hydrolysed to (2*R*)-glyceryl β-D-galactoside (26) (Scheme 21).



Scheme 21

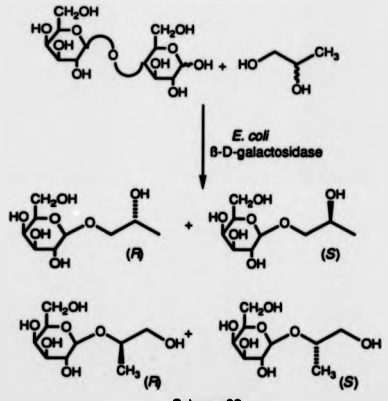
More recent studies, however, indicate that the galactosyl transfer is essentially non-stereoselective. With lactose as donor[157,158] a 1:1 diastereomeric mixture of products was obtained and with *o*-nitrophenyl ß-D-galactoside as donor[157] a 20% diastereomeric excess of the (2*S*)-ß-D-galactoside was formed. It was suggested that the pure diastereomer obtained in the Boos study was a result of inadvertent diastereomeric enrichment during the crystallisation procedure rather than any diastereoselective property of the enzyme.

Racemic 2,3-epoxypropanol has also been used as an acceptor.[157] A slight diastereomeric excess of (2*R*,3)-epoxypropyl ß-D-galactoside (*R*:*S* = 7:3) was formed when *o*-nitrophenyl ß-D-galactoside was used as donor but no diastereomeric excess could be detected when lactose was used as donor.

Regio- and stereochemical studies have been carried out by Crout *et al.* using lactose as donor with racemic butan-2-ol and a variety of diols as acceptors.[158] With racemic butan-2-ol

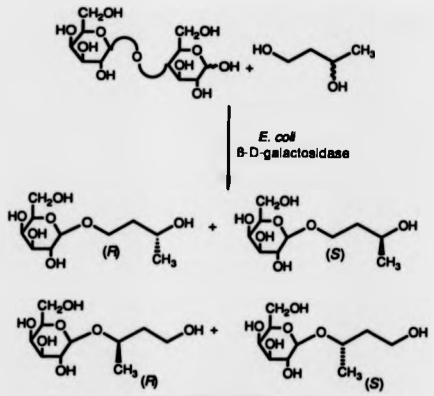
a slight selectivity for the (*S*)-enantiomer (*R*:*S* = 1.00:0.83) was observed.

When racemic propane-1,2-diol was used as acceptor all four possible products were formed (Scheme 22). The major product was the one in which the galactose was attached to the primary hydroxyl group of the diol (*R*:*S* = 1.00:0.86). A similar selectivity (*R*:*S* = 1.00:0.77) was observed in formation of the minor product by transfer to the secondary hydroxyl group. Overall, transfer to the primary hydroxyl group was favoured over the secondary by a factor of 1.00:0.35.



Scheme 22

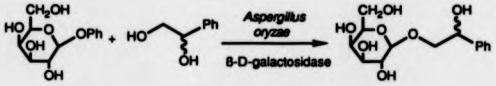
Somewhat different results were obtained when racemic butane-1,3-diol was used as acceptor (Scheme 23).



Scheme 23

In this case there was only a very slight diastereoselectivity in transfer to the primary hydroxyl group (*R*:*S*=1.00:0.99), but in transfer to the secondary hydroxyl group there was a marked selectivity in favour of the (*R*)-enantiomer (*R*:*S*=1:0.5) and overall transfer to the primary hydroxyl group was favoured over the secondary by a factor of 1.00:0.15. The various results for these diols were interpreted in terms of the possible conformations which they might adopt in the hydrophobic "glucose" binding site of the enzyme.

Racemic phenylethylene glycol has also been used as the acceptor in transgalactosylation reactions with phenyl ß-D-galactoside as donor and the enzyme from *Aspergillus oryzae* (Scheme 24).[159]

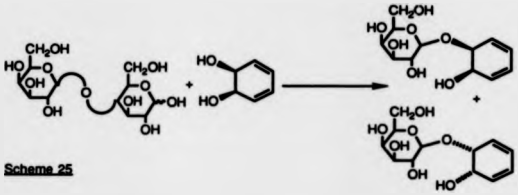


Scheme 24

Transfer occurred mainly to the primary hydroxyl group and the enzyme exhibited little or no diastereoselectivity. The diastereomers could, however, be separated by HPLC.

The ß-D-galactosidases from *E. coli* and *Aspergillus oryzae* have also been used for galactosyl transfers from lactose or phenyl ß-D-galactoside to mono- and bicyclic *meso*-1,2-diols.[160] The *Aspergillus oryzae* enzyme gave low or negligible selectivities but these could be improved considerably by using acetone as a co-solvent. Reasonable selectivities (50-90% d.e.) were obtained using the *E. coli* enzyme. Although the absolute configurations of the aglycone moieties were not determined it was observed that in the cases of *cis*-1,2-dihydroxycyclopentane and *cis*-1,2-dihydroxycyclohexane (where direct comparisons were possible) the asymmetric induction was opposite for the two enzymes.

cis-1,2-Dihydroxycyclohexa-3,5-diene has also been used as an acceptor using β-D-galactosidase from *E. coli*.[161] (Scheme 25).

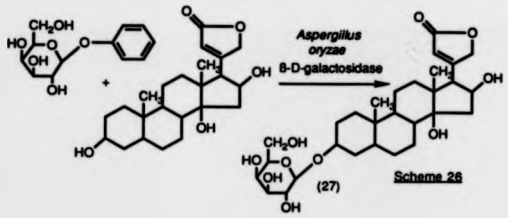


Scheme 25

When isolated in the early stages of the reaction a mixture corresponding to a diastereisomeric excess of 80% was obtained. This d.e. value was reduced to 20% if the product was isolated at the end of the reaction (when all of the lactose had been consumed). The configurations of the aglycone moieties were determined by a combination of molecular modelling and NMR techniques.

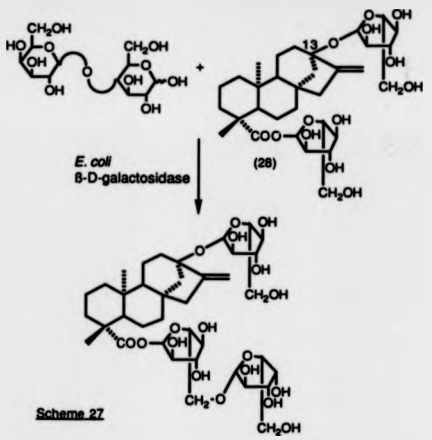
The attachment of glycosyl residues to steroids is of value for the creation of new drugs since the physiological activity of these compounds frequently depends on the type or position of attached sugars. *Aspergillus oryzae* β-D-galactosidase was able to galactosylate several steroids in good yield with solubility problems being circumvented by using organic co-solvents.[162,163] Thus the cardiac glycoside gitoxigenin (27) could be prepared in

good yield in 62.5% aqueous acetonitrile using phenyl ß-D-galactoside as donor (Scheme 26).



Scheme 26

Transglycosylation reactions have also been of value in modifying rubusoside (ß-D-glucosyl ester of 13-*O*-ß-D-glucosyl-steviol, (28)) which although being 110 times as sweet as sucrose also possesses a slightly bitter taste.[164] The sweetening properties of rubusoside may be improved by attachment of glycosyl residues to the 4- or 6-position of the glucosyl residues and this may be effected by several ß-D-galactosidases. For example, using lactose as substrate, *E. coli* ß-D-galactosidase forms the (1-6)-allolactosyl derivative (Scheme 27).

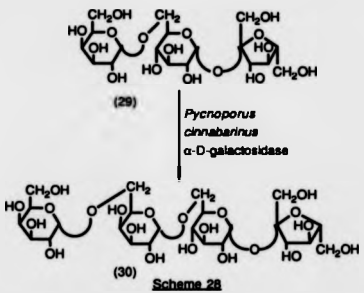


Scheme 27

α-D-Galactosidase.

The majority of transgalactosylations carried out with α-D-galactosidase have involved transfer to mono- or disaccharide acceptors and most of the early work has been reviewed by Stanek *et al.*[121] A study of the enzyme from a *Pneumococcal* source has revealed that transfer from melibiose (Galα(1-6)Glc) to D-galactose or D-glucose gives predominantly α(1-6)-linkages but also α(1-1)-linkages; a variety of other monosaccharides were also able to function as acceptors.[165]

Incubation of raffinose (Galα(1-6)Glcα(1-2)ßFru (29)) with the enzyme from *Pycnoporus cinnabarinus* also gave rise to an α(1-6)-linkage in forming stachyose (Galα(1-6)Galα(1-6)Glcα(1-2)ßFru (30)).[166]



Scheme 28

More recently the same authors characterised five further products from the reaction mixture; their structures indicated that in addition to catalysing transfer to the C-6 hydroxyl group the enzyme was also able to form α(1-3)-linkages with the terminal galactose residues.[167]

α-D-Galactosidases are also able to effect modifications of rubusoside (28) in a similar manner to ß-D-galactosidase (discussed above).[168] Raffinose and melibiose were used as donors with α-D-galactosidases from *Mortierella vinacea*, *Absidia reflexa*, *E. coli* and green coffee beans. In all cases transfer was found to occur to the C-6 hydroxyl groups of the glucosyl residues; in the case of *M. vinacea* α-D-galactosidase transfer occurred exclusively to the glycosyl residue at the

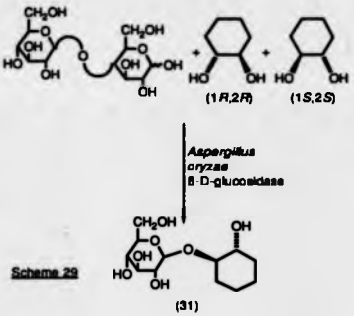
13-hydroxyl group whereas the enzymes from the other sources effected transfer to either or both glucosyl residues.

α-D-Galactosidase from *Absidia corymbifera* also gave α(1-6)-linkages when incubated with melibiose; several oligosaccharides were formed but the major products were Galα(1-6)Gal, Galα(1-6)Galα(1-6)Glc and Galα(1-6)Galα(1-6)Galα(1-6)Glc.[169]

ß-D-Glucosidases.

ß-D-Glucosidases have been used in transfer reactions to non-saccharides as well as saccharide acceptors. In the former category ß-D-glucosidase from *Aspergillus luchuensis* was able to transfer D-glucose to butan-1-ol and butan-2-ol in a column method where the enzyme was retained as the stationary phase on a column of hydrophilic solid such as cellulose.[170]

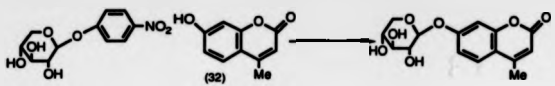
A stereospecific preparation of the mono-ß-D-glucoside (31) of (1*R*,2*R*)-*trans*-1,2-cyclohexanediol was achieved using Takadiastase (a crude enzyme mixture produced by *Aspergillus oryzae*) with cellobiose (Glcß(1-4)Glc) as donor in the presence of a racemic mixture of (1*R*,2*R*)- and (1*S*,2*S*)-*trans*-1,2-cyclohexanediols[171] (Scheme 29).



Scheme 29

Additionally, using almond β-D-glucosidase with salicin (β-D-glucoside of 2-hydroxybenzyl alcohol) as donor, *cis*-1,2-cyclohexanediol, cyclohexanol, butan-2-ol and, interestingly, *tert*-butanol could also be glycosylated.

The β-D-glucosidase of *Stachybotrys atra* has been found to transfer both D-glucosyl and D-xylosyl residues to simple alcohols.[172] However, when phenol or 4-methylumbelliferone (7-hydroxy-4-methylcoumarone (32)) were employed as acceptors, only D-xylosyl residues could be transferred (Scheme 30).[173]



Schema 30

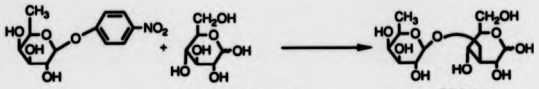
In the same study it was also found to be possible to transfer D-glucosyl residues to a variety of anilines using *p*-chlorophenyl ß-D-glucopyranoside as the donor.

ß-D-Glucosidase has also been found to glucosylate phenylethylene glycol, propylene glycol, 3-chloropropylene glycol and α-phenylethyl alcohol.[159] The transformations were carried out using a crude *Aspergillus oryzae* ß-D-galactosidase bearing ß-D-glucosidase activity. Mixtures of diastereomers were formed but could be invariably separated by HPLC.

Kusama *et al.* [174] considered that ß-D-glucosidases could be classified into two groups: hydrolases, such as those of *Aspergillus aculeatus*[175] and *Talaromyces empersonii*,[176] and transglucosidases, such as those of *Alcaligenes faecalis*,[177] *Aspergillus niger* [178] and *Streptomyces sp.*[174] depending on the ratio of hydrolysis to transfer for a given substrate concentration. The *Streptomyces sp.* enzyme produced gentiobiose (Glcß(1-6)Glc) from ß-gluco-disaccharides and also from epicellobiose (Glcß(1-4)Man); transfer products (of undetermined structure) were also obtained from *p*-nitrophenyl ß-D-glucoside, phenyl ß-D-glucoside and salicin.

A variety of commercial ß-D-glucosidase preparations were compared for their transfer activity in the study by Tanaka and Oi.[126] Using cellobiose (Glcß(1-4)Glc) as substrate, free sweet almond ß-D-glucosidase gave only hydrolysis products whereas significant amounts of the transfer products cellotriose (Glcß(1-4)Glcß(1-4)Glc) and gentiobiose (Glcß(1-6)Glc) were produced with the immobilised preparations.

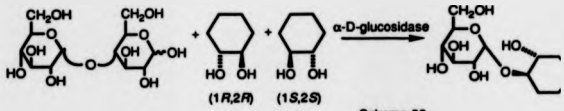
The ability of ß-D-glucosidase to transfer units other than ß-D-glucosyl was shown in a recent publication by Sakai *et al.*[179] *Bifidobacterium breve* clb ß-D-glucosidase I displayed transfucosylation activity in producing ß(1-2)-, (1-3)-, (1-4)- and (1-6)-linked D-fucopyranosyl D-glucosides (3:4:2:1, 50% yield) from *p*-nitrophenyl ß-D-fucoside and D-glucose (Scheme 31).



Scheme 31

α-D-Glucosidases.

In common with ß-D-glucosidases and most other glycosidases, α-D-glucosidases have also been used in transfer reactions to both saccharide and non-saccharide acceptors. In recent years the greatest focus has been on saccharide acceptors but Itano *et al.*[171] conducted an interesting study into the stereospecific preparation of monoglucosides of optically active *trans*-1,2-cyclohexanediols (see also above). With Takadiastase high stereospecifities could be obtained using maltose as donor in the presence of racemic mixture of the diols (Scheme 32).



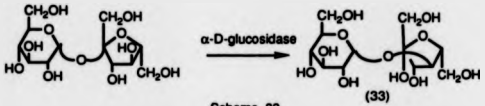
Scheme 32

cis-1,2-Cyclohexanediol was also glucosylated (structure of product not determined) but cyclohexanol did not function as an acceptor.

The action of α-D-glucosidase on maltose (Glcα(1-4)Glc) tends to give rise to α(1-4)- and (1-6)-linked di- and higher oligosaccharides though α(1-2)- and (1-3)-linked products have also been identified. Thus, for example, *Tetrahymena pyriformis* α-D-glucosidase in the presence of maltose gives maltotriose (Glcα(1-4)Glcα(1-4)Glc) and panose (Glcα(1-6)Glcα(1-4)Glc) as the main products with smaller amounts of isomaltose (Glcα(1-6)Glc), α(1-6)glucosylmaltotriose (Glcα(1-6)Glcα(1-4)Glcα(1-4)Glc) and maltotetraose (Glcα(1-4)Glcα(1-4)Glcα(1-4)Glc).[180] With D-xylose as acceptor this enzyme gave rise to 4-*O*-α-D-glucopyranosyl-D-xylose whereas D-lyxose was glucosylated to a mixture of 2-*O*-, 3-*O*-, and 4-*O*-α-D-glucopyranosyl-D-lyxoses; D-fructose, D-glucuronic acid, sucrose and methyl α-D-glucopyranoside did not, however, serve as acceptors.[181] A further study with this enzyme using phenyl α-D-glucoside as donor gave 6-*O*-α-D-glucopyranosyl-D-galactose, 6-*O*-α-D-glucopyranosyl-D-mannose, 4-*O*-α-D-glucopyranosyl-L-xylose, 1-*O*-α-D-glucopyranosylerythritol, 1-*O*-α-D-glucopyranosyl-D-mannitol, 1-*O*- and 6-*O*-α-D-glucopyranosyl-D-glucitol and 1-*O*-α-D-glucopyranosylribitol in the presence of the appropriate acceptor.[182] These results show the high preference of this enzyme for primary hydroxyl groups; with monosaccharides α(1-6)-linked disaccharides were formed but with the symmetrical and asymmetrical alditols no differentiatiation between the two possible primary hydroxyl groups was observed. When monosaccharides not bearing any primary hydroxyl groups were used as acceptors, *eg*. D-ribose, D-lyxose,

D-xylose and L-xylose, transfer to the equatorial OH at C-4 predominated.

Sucrose (Glcα(1-2)ßFru), may also act as a substrate for α-D-glucosidases. Thus maltulose (Glcα(1-4)ßFru) (33) was the main product when sucrose was treated with mucosal preparations of rats or rabbits[183] (Scheme 33).



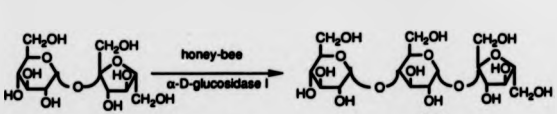
(33)

Scheme 33

The maltulose is apparently formed by transfer of D-glucose units to free D-fructose formed as result of sucrose hydrolysis.

Sucrose may also act as an acceptor in a transglucosylation reaction. Immobilised buckwheat α-D-glucosidase, using maltose as donor, is able to glucosylate sucrose to form erlose (Glcα(1-4)Glcα(1-2)ßFru (34)) as the main product, in addition to theanderose (Glcα(1-6)Glcα(1-2)ßFru) and "esculose" (Glcα(1-3)Glcα(1-2)ßFru).[184]

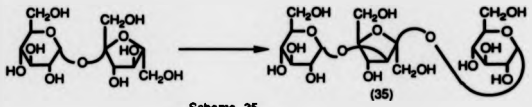
Erlose was also formed when sucrose was the sole substrate of honey-bee α-D-glucosidase I[185] (Scheme 34).



(34)

Scheme 34

These results contrast with those of Huber and Thompson, who, using the same enzyme, identified melezitose (Glcα(1-3)Fruß(2-1)αGlc (35)) as the main product by transfer of glucosyl unit to the fructosyl rather than the glucosyl residue of sucrose (Scheme 35).[186]



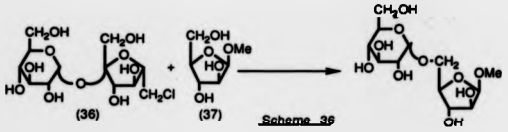
Scheme 35

When maltose and phenyl α-D-glucoside were incubated with honey-bee α-D-glucosidase the main products were maltotriose and phenyl α-maltoside further indicating the enzyme's tendency to form α(1-4)-linkages.[185]

The transglucosylating properties of the micro-organism *Protaminobacter rubrum* have been of value in the sugar industry with the discovery that it is able to convert sucrose (Glcα(1-2)ßFru) into palatinose (Glcα(1-6)ßFru), a valuable sweetener on account of its low cariogenic property.[187]

This organism has also been shown to carry out the intermolecular transfer of a glucosyl unit from sucrose to D-arabinose.[188] Recently, a more efficient method of glucosylating furanosides was discovered using immobilised *P. rubrum* and 6'-chloro-6'-deoxysucrose (36) as glucosyl donor instead of sucrose.[189] This system has proved effective for the

diastereoselective glucosylation of the hydroxymethyl group of isopropylideneglycerol[189] and in the transfer of a glucosyl unit to methyl ß-D-arabinofuranoside (37)[190] (Scheme 36).



(36) (37)

Scheme 36

D-Mannosidases.

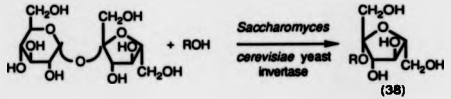
Transmannosidation reactions have hitherto received very little attention and this is somewhat surprising when one considers the degree to which they occur in glycoproteins. That such reactions may be carried out with the same ease as other transglycosylations has, however, been demonsrated by De Prijcker *et al.* who prepared methyl α-D-mannoside from *p*-nitrophenyl α–D-mannoside and methanol using *Medicago sativa* L. α-mannosidase.[191] More recently Nilsson synthesised various manno-oligosaccharides using jack bean α-D-mannosidase[141,142] (see above).

Studies with ß-D-mannosidases are even more rare than those with α-D-mannosidases.

ß-D-Fructofuranoside (invertase).

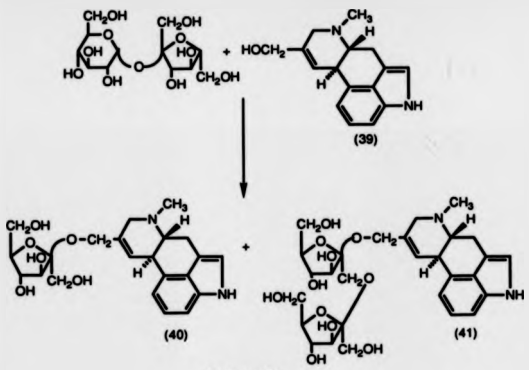
Transfructosylations with invertase have been extensive and the early work has been reviewed by Stanek *et al.*[121] and Edelman.[192] Most reactions have employed sucrose as substrate and fructosyl transfer generally gives rise to the trisaccharides 6-kestose (Glcα(1-2)Fruß(6-2)ßFru), neokestose (Fruß(2-6)Glcα(1-2)ßFru) and 1-kestose (Glcα(1-2)Fruß(1-2)ßFru) among other oligosaccharides; qualitative and quantitative analyses of reaction mixtures containing 10% sucrose and yeast invertase was carried out by Anderson *et al.* [193] That transfer occurs almost exclusively to the primary hydroxyl groups of sucrose was further confirmed by Straathof *et al.* using *Saccharomyces cerevisiae* yeast invertase.[194] The major product was 6-kestose but also detected were 6-*O*-ß-D-fructofuranosyl-D-glucose (Fruß(2-6)Glc) and inulobiose (Fruß(2-1)ßFru). More recently Jung *et al.* determined a mathematical model for the production of fructo-oligosaccharides from sucrose, 1-kestose and nystose (Glcα(1-2)Fruß(1-2)Fruß(1-2)ßFru) using a 'fructosyltransferase' from *Aureobasidium pullulans*.[195] When sucrose was employed as substrate, glucose and 1-kestose were produced and this pattern was reproduced with 1-kestose and nystose which gave sucrose and nystose, and 1-kestose and fructofuranosylnystose, respectively, as a result of fructosyl transfer to terminal fructose units.

The use of invertase for the synthesis of alkyl ß-D-fructosides was achieved by Reese and Mandels when they prepared butyl ß-D-fructoside from sucrose and butan-1-ol.[170] More recently methyl, ethyl, and propyl ß-D-fructosides (38) were prepared from sucrose and the appropriate alcohol in the presence of invertase from Baker's yeast (*Saccharomyces cerevisae*)[137] (Scheme 37).



Scheme 37

Ergot alkaloid glycosides have also been prepared by transfructosylation reactions using sucrose as donor.[196] Free and immobilised cells of *Claviceps purpurea* were able to transfer fructosyl residues to elymoclavine (39), chanoclavine, lysergol and dihydrolysergol to give mono- and difructosylated derivatives. The formation of elymoclavine-*O*-ß-D-fructoside (40) and elymoclavine-*O*-ß-D-fructofuranosyl-(2-1)-*O*-ß-D-fructofuranoside (41) from sucrose and elymoclavine is shown in Scheme 38.

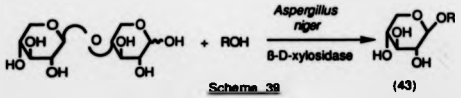


Scheme 38

D-Xylosidases

The first examples of transxylosylation reactions involved transfer from *p*-nitrophenyl ß-D-xyloside to acceptors such as methanol, glycerol and phenyl ß-D-xyloside (to give the ß(1-4)-linked phenyl ß-D-xylobioside)[197] using ß-D-xylosidase partially purified from the commercial preparation Hemicellulase.[198] The ability of *Aspergillus niger* ß-D-xylosidase to form alkyl ß-D-xylosides was recently investigated by Shinoyama *et al.*[199,200] Xylobiose (Xylß(1-4)Xyl (42)) was used as donor and ß-D-xylosides (43) were formed from a wide variety of alcohols including pentan-1-ol,

hexan-1-ol, heptan-1-ol, octan-1-ol and *tert*-butanol (Scheme 39).



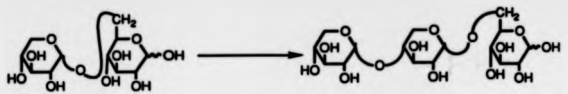
Scheme 39 (43)

With *Aspergillus niger* ß-D-xylosidase and xylobiose as the sole substrate the non-reducing disaccharide O-ß-D-xylopyranosyl-(1-1)-ß-D-xylopyranose (44) was obtained as the main product (Scheme 40).[201]

(44)

Scheme 40

Bacillus α-D-Xylosidase has been shown to exhibit transfer activity with the substrates methyl α-D-xyloside, α-1,3-xylobiose and isoprimeverose (Xylα(1-6)Glc (45)).[202] The substrates acted as both donors and acceptors and in the case of isoprimeverose the main transfer product was found to be *O*-α-D-xylopyranosyl-(1-4)-*O*-α-D-xylopyranosyl-(1-6)-D-glucopyranose (46) (Scheme 41).

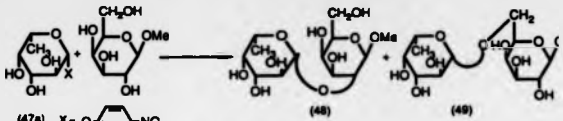


(45) Scheme 41 (46)

The ability of ß-D-glucosidase to transfer ß-D-xylosyl units in addition to ß-D-glucosyl units was discussed above.[173] *Penicillium wortmanni* ß-D-xylosidase has also demonstrated an ambivalence towards glycosyl transfer with its ability to transfer α-L-arabinopyranosyl units as well as ß-D-xylosyl units.[203]

α-L-Fucosidases.

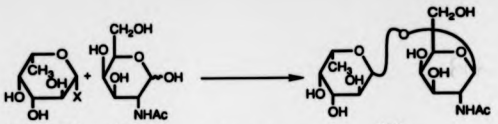
Many L-fucose-containing glycoconjugates have important biological functions but syntheses employing α-L-fucosidases are rare. A recent communication, however, cites the porcine liver α-L-fucosidase-catalysed synthesis of methyl 2-*O*- and methyl 6-*O*-(α-L-fucopyranosyl)-ß-D-galactopyranosides (L-Fucα(1-2)GalßOMe (48) and L-Fucα(1-6)GalßOMe (49) respectively) using *p*-nitrophenyl α-L-fucoside (L-Fucα$OC_6H_4NO_2$-*p* (47a)) and α-L-fucopyranosyl fluoride (L-FucαOF (47b)) as donors and methyl ß-D-galactoside (GalßOMe) as acceptor with free and immobilised enzyme[204] (Scheme 42).



Scheme 42

To improve the solubility of the glycosyl donor up to 50% organic co-solvent was added. Although the activity of the α-L-fucosidase was dramatically decreased by the co-solvent the enhanced donor solubility and minimisation of competing hydrolysis gave improved yields. Thus the best result was obtained using the free enzyme with the *p*-nitrophenyl glycoside (47a) as the donor (donor : acceptor = 1:2.5) in phosphate buffer with 30% dimethyl sulphoxide for 5 days. This yielded 16.5% of a 2:3 mixture of (48) and (49); when the glycosyl fluoride (47b) was employed as the donor the yield was shifted to 9.7% and the ratio to *ca.* 1:1. The enzymatic synthesis of (49) in good yield (25%) and with high regioselectivity has also been claimed by Nilsson.[205]

D-Glucose, L-fucose and lactose were found not to act as acceptors in the reaction but 2-acetamido-2-deoxy-D-galactose (50) yielded a small amount of the disaccharide (51) with a trehalose-type interglycosidic linkage (Scheme 43).



(47a) (47b) (50) (51)

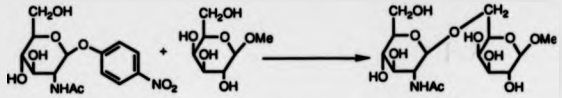
Scheme 43

N-Acetylglycosaminidase and ß-*N*-Acetylhexosaminidase.

Enzymatic syntheses involving transfer of 2-acetamido-2-deoxy-D-glucose (GlcNAc) and 2-acetamido-2-deoxy-D-galactose (GalNAc) may be carried out by *N*-acetyl-D-glucosaminidases, *N*-acetyl-D-galactosaminidases and ß-*N*-acetyl-D-hexosaminidases, the latter being able to transfer both *N*-acetyl-D-glucosyl and *N*-acetyl-D-galactosyl units. HexNAc units occur widely in glycolipids and glycoproteins with ßGlcNAc residues being situated at branch points in the core of glycoprotein glycans and imposing important effects on the conformation, rigidity and biological activity of glycans.[11] So far, however, few examples of transglycosylation reactions with these enzymes have been reported.

Werries *et al.* reported the isolation of two forms of ß-*N*-acetyl-D-glucosaminidase from bovine spleen and employed them in the synthesis of a pentasaccharide of unknown structure from a chrondoitin-4-sulphate tetrasaccharide using phenyl 2-acetamido-2-deoxy-ß-D-glucopyranoside as donor.[206]

More recently Nilsson described the syntheses of GlcNAcß(1-6)GalßOMe (52) (Scheme 44) and GlcNAcß(1-6)ManαOMe using jack bean ß-*N*-acetyl-D-glucosaminidase.[144]



(52)

Scheme 44

Subsequent studies using a hexosaminidase from mollusc *Chamelea gallina* gave a 50:50 mixture of GlcNAcß(1-6)GalßOMe and GlcNAcß(1-3)GalßOMe whereas the main product using GalαOMe as acceptor was GlcNAcß(1-6)GalαOMe.[205] These results provide further evidence of how the anomeric configuration of the acceptor and the source of the enzyme can affect regioselectivity. The structure of the donor was found to affect both yield and regioselectivity (Table 3). Thus the yield of GalNAcα(1-3)GalαOMe was greater with GalNAcα$OC_6H_4NO_2$-*o* as donor rather than GalNAcαOPh and whereas the former gave the α(1-3)-linked product almost exclusively, the phenyl glycoside gave approximately equal amounts of the α(1-6)- and α(1-3)-linked isomers. The formation of GalNAcß(1-3)GalβOMe and GlcNAcα(1-3)GalαOMe using GalNAcβ$OC_6H_4NO_2$-*p* and GlcNAcα$OC_6H_4NO_2$-*o* , respectively, as donors in the presence of the appropriate acceptor glycosides demonstrated the ability of the crude enzyme preparation to catalyse the transfer of αGlcNAc, ßGlcNAc, αGalNAc and ßGalNAc residues. The low yields obtained (5-10% based on the donor substrate) are understandable as no attempt was made to optimise the procedure. However, preliminary experiments indicated that

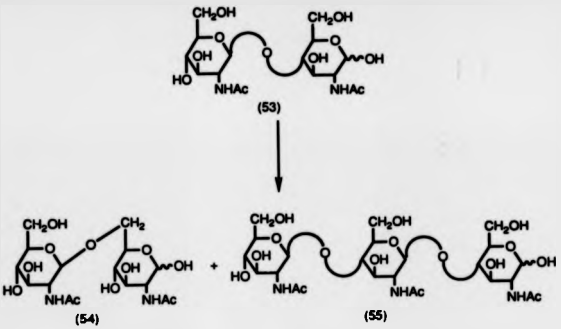
up to 50% yields of HexNAcGal glycosides might be obtained by employing a high acceptor concentration.

Table 3

Formation of disaccharide glycosides using crude enzyme preparation from *Chamelea gallina*

Donor	Acceptor	Main products	Comments
GlcNAcβOC$_6$H$_4$NO$_2$-*p*	ManαOMe	GlcNAcβ(1-6)ManαOMe	highly regioselective
	GalαOMe	GlcNAcβ(1-6)GalαOMe	>85% regioselective
	GalβOMe	GlcNAcβ(1-3)GalβOMe GlcNAcβ(1-6)GalβOMe	equal amounts
GalNAcαOC$_6$H$_4$NO$_2$-*p*	GalαOMe	GlcNAcα(1-6)GalαOMe	high yield
GalNAcαOPh	GalαOMe	GlcNAcα(1-3)GalαOMe GlcNAcα(1-6)GalαOMe	equal amounts
GalNAcβOC$_6$H$_4$NO$_2$-*p*	GalβOMe	GlcNAcβ(13)GalβOMe	highly regioselective
GalNAcαOC$_6$H$_4$NO$_2$-*o*	GalαOMe	GlcNAcα(1-3)GalαOMe	

Purified β-*N*-acetyl-D-hexosaminidase from the culture filtrate of *Nocardia orientalis* has also been shown to carry out tranglycosylation reactions.[206] Using di-*N*-acetylchitobiose (GlcNAcβ(1-4)GlcNAc (53)) as substrate two major transfer products were isolated; these were identified as GlcNAcβ(1-6)GlcNAc (54) and tri-*N*-acetylchitotriose (GlcNAcβ(1-4)GlcNAcβ(1-4)GlcNAc (55)) (Scheme 45).



Scheme 45

The enzyme was also shown to possess ß-*N*-acetyl-galactosaminidase activity (but no α-*N*-acetylhexosaminidase acitivity) though transfer reactions using this property were not explored.

Conclusion.

Until recently enzymes were the realm of the biochemist and the reluctance of chemists to explore their potential in synthesis can be understood when one considers their complex nature and the difficulties associated with their isolation and purification. Nowadays, enzymes are the domain of enzymologists but as they become more well understood and more widely available chemists are beginning to appreciate the benefits they have to offer.

In comparison to classical synthesis the study of enzyme-related transformations is in its infancy, but there can be little doubt as to their potential, particularly in the field of carbohydrate chemistry. The foregoing discussion serves to define current thinking on the mode of action of glycosidases and to collate the information hitherto gained on their application in saccharide synthesis. The advantages of these types of enzyme in forming stereo- and regiospecific linkages without the need for tedious protection and deprotection steps is clearly demonstrated. Although the enzymatic synthesis of large oligosaccharides is currently outside the scope of modern technology, the synthesis of di- and trisaccharides is common place and the barriers to more intricate syntheses may be more easily negotiable with enzymes than by applying classical synthesis.

Much research remains to be done on the carbohydrate-metabolising enzymes and indeed in all areas of biotransformations; the scale of the task is made clear when one considers the ineffable diversity of enzyme sources and the fact that each enzyme, even those bearing the same EC number, generally possesses a unique set of properties. However one can appreciate the impact that a large selection of cheap and readily available enzymes with well-defined properties will have in chemical transformations and it may not be long before enzymes form an indispensable part of the chemist's armoury.

CHAPTER TWO

Transglycosylation Studies with the β-D-Galactosidase of *Escherichia coli*.

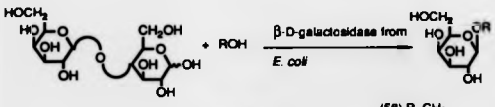
As discussed earlier, the β-D-galactosidase of *E. coli* has been the subject of a large number of enzymatic studies. The fact that it is readily available and relatively inexpensive made it attractive to us as the starting point for an investigation into the regio- and stereoselective aspects of enzymatic transglycosylation reactions. The following discussion relates to a study involving galactosyl transfer to achiral and chiral mono-ols and diols as well as to one *meso*-diol and two disaccharides.

Transfer to achiral acceptors.

Initial studies were restricted to the transfer of galactose from lactose to achiral alcohols bearing, except in the case of propane-1,3-diol, a single hydroxyl group (Scheme 46, overleaf). A list of substrates and products is given in Table 4.

Products could be easily isolated by preparative TLC, but the purest products were obtained by HPLC. A typical HPLC trace is shown in Figure 1, with the galactoside exhibiting the shortest retention time followed by glucose, galactose, lactose and allolactose (Galβ(1-6)Glc). The production of allolactose may be attributed to intramolecular or "direct" transglycosylation as a result of the glucose moiety of a lactose molecule trapping the galactosyl-enzyme intermediate at its 6-hydroxyl group before it is released from the active site of the enzyme. Alternatively, it

may be produced by free glucose acting as an acceptor in the same way as the added alcohol.



Scheme 46

(56) R=CH_3
(57) R=CH_2CH_3
(58) R=$CH_2CH_2CH_3$
(59) R=$CH(CH_3)_2$
(60) R=$CH_2CH_2OCH_3$
(61) R=$CH_2(CH_2)_2CH_3$
(62) R=$C(CH_3)_3$
(63) R=$CH_2CH(CH_3)_2$
(64) R=$CH_2(CH_2)_3CH_3$
(65) R=$CH(CH_2CH_3)_2$
(66) R=$CH_2(CH_2)_4CH_3$
(67) R=$CH_2CH_2CH_2OH$

The progress of the reaction could be easily followed by HPLC and measurement of the galactoside peak heights at different time intervals permitted the determination of the maximum product concentration (Figure 2). In general, this occurred at 36 to 48 hours, after which time it gradually declined. Thermodynamic equilibrium was reached only after several days when all glycosidic bonds had been hydrolysed by the enzyme to give a mixture consisting of only glucose, galactose and the alcohol. For each of the alcohols examined, a lag phase of two to three hours duration corresponding to a low rate of product formation was followed by a rapid rate of production where the galactoside concentration increased approximately linearly with time.

TABLE 4

Glycoside formation from lactose and various achiral alcohols catalysed by *E.coli* β-D-Galactosidase

Substrate[a]	Product galactoside	Yield[b] / %
Methanol	(56)	53
Ethanol	(57)	52
Propan-1-ol	(58)	54
Propan-2-ol	(59)	49
2-Methoxyethanol	(60)	45
Butan-1-ol	(61)	38
tert-Butanol	(62)	-
2-Methylpropan-1-ol	(63)	36
Pentan-1-ol	(64)	24
Pentan-3-ol	(65)	33
Octan-1-ol	(66)	-
Propane-1,3-diol	(67)	42

a Substrates employed as described in Method A except for propane-1,3-diol which was applied as described in Method B.

b Based on lactose with isolation by HPLC.

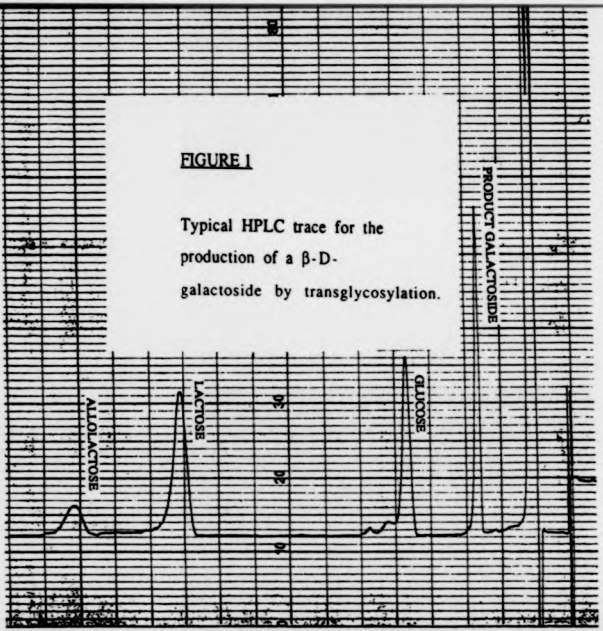
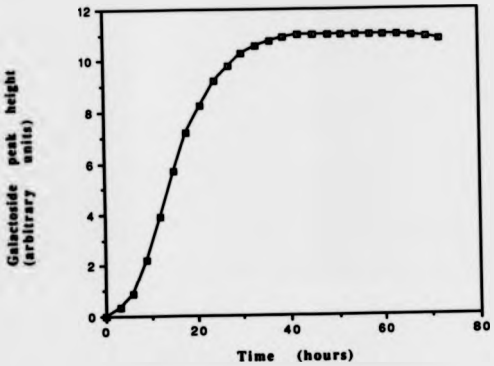


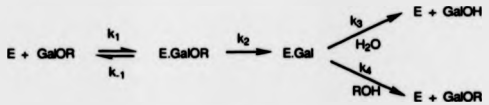
FIGURE 1

Typical HPLC trace for the production of a β-D-galactoside by transglycosylation.

Fig. 2. Graph to show the typical variation of product concentration with time.



A measure of the greater effectiveness of the alcohol in acting as the nucleophile compared with water can be gained by considering their relative concentrations. The chances of capture of the galactosyl-enzyme intermediate by the alcohol are increased by employing an 8:1 ratio of concentrations of alcohol to lactose, but the concentration of alcohol is still very small (~0.3 M) when compared to the concentration of water (55 M). This effect indicates that k_4 for the transglycosylation step (Scheme 47) is significantly greater than k_3 for the competing hydrolysis.



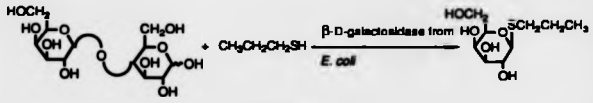
Scheme 47

A high rate of transglycosylation relative to hydrolysis is common for glycosidases and indeed, as discussed earlier, the presence of an alcohol generally results in an increase in the overall enzymatic activity.[139,140]

Of the alcohols examined, only *tert*-butanol and octan-1-ol failed to give glycosides. That *tert*-butanol did not serve as an acceptor might have been expected on steric grounds, but it should be noted that *tert*-butanol has been successfully glycosylated using the β-D-glucosidase of *Aspergillus oryzae*[171] and the β-D-xylosidase of *Aspergillus niger*.[199,200]

The reluctance of octan-1-ol to enter into the transglycosylation reaction may be due to its low solubility, although a reasonable yield of galactoside could be obtained in the case of pentan-1-ol which has a solubility of *ca.* 30 g dm^{-3} at the temperature employed in the syntheses. The involvement of octan-1-ol in transglycosylation reactions has, however, been demonstrated by the group of Shinoyama[199,200] and it has also been shown to be possible to produce long chain glycosides by reversion simply by shaking a two-phase mixture of glucose, water and the alcohol in the presence of the enzyme.[130] There is no obvious explanation of this disparity in substrate acceptance by different glycosidases, except that the K_M values (see Appendix 1) for such substrates may be an important factor.

The possibility of employing a thiol as an acceptor was also investigated (Scheme 48).



Scheme 48

Analysis by HPLC and TLC showed the production of glucose and galactose in approximately equimolar amounts corresponding to straightforward lactose hydrolysis. Further, there was no evidence of thiogalactoside formation, either on the HPLC trace or in any HPLC fractions. The inability of propane-1-thiol to act as an

acceptor is somewhat curious when one considers the nucleophilicity of the sulphur atom. However, the resistance of thiogalactosides to hydrolysis by *Escherichia coli* β-D-galactosidase is well documented and indeed this property has enabled isopropyl β-D-thiogalactoside to find application in studies of bacterial lactose transport processes.

Transfer to chiral acceptors.

A significant body of knowledge concerning transglycosylation reactions already exists, but little insight has been gained into aspects of the regio- and stereoselectivity of transfer; those studies that have been carried out were discussed earlier. The aim of the work described here was to further the understanding of transfer reactions involving chiral substrates. To this end a systematic study of galactosyl transfer to chiral acceptors catalysed by the β-D-galactosidase of *Escherichia coli* was initiated. Three chiral alcohols, butan-2-ol, pentan-2-ol and 2,2-dimethyl-1,3-dioxolane-4-methanol (isopropylidene glycerol, IPG), and two chiral diols, propane-1,2-diol and butane-1,3-diol, were examined and, except in the case of pentan-2-ol which was available only as the racemate, each was applied as a single enantiomer and as the racemate (Table 5). Determinations of the regio- and stereoselectivities of the reactions were carried out by ^{1}H NMR and, except in the case of pentan-2-ol, enantiomerically pure acceptor provided a means of ascertaining whether the enzyme displayed any stereoselectivity towards one or other enantiomer of the acceptor.

TABLE 5

Glycoside formation from lactose and various chiral alcohols catalysed by *E.coli* β-D-galactosidase

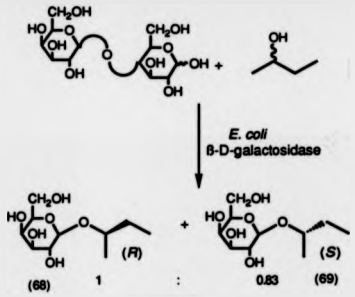
Substrate[a]	Product galactoside(s)	Yield[b] / %
Racemic Butan-2-ol	(68) + (69)	32
(*R*)-Butan-2-ol	(68)	32
racemic Pentan-2-ol	(70) + (71)	29
racemic IPG	(72) + (73)	47
(*S*)-IPG	(72)	47
racemic Propane-1,2-diol	(74) -(77)	46
(*S*)-Propane-1,2-diol	(74) + (75)	51
racemic Butan-1,3-diol	(78) -(81)	61
(*S*)-Butane-1,3-diol	(78) + (79)	63

a Substrates employed as described in Method B, except for racemic pentan-2-ol which was applied as described in Method A.

b Based on lactose with isolation by HPLC.

In the case of racemic butan-2-ol (Scheme 49), a mixture of both possible diastereoisomers ((68) and (69)) was formed, as indicated by the signals in the ^1H NMR spectrum (see also Appendix 2) attributable to the methyl group attached to the carbinol carbon atom (Figure 3).

FIGURE 3 Partial ^1H NMR spectrum for the β-D-galactosides produced from racemic butan-2-ol

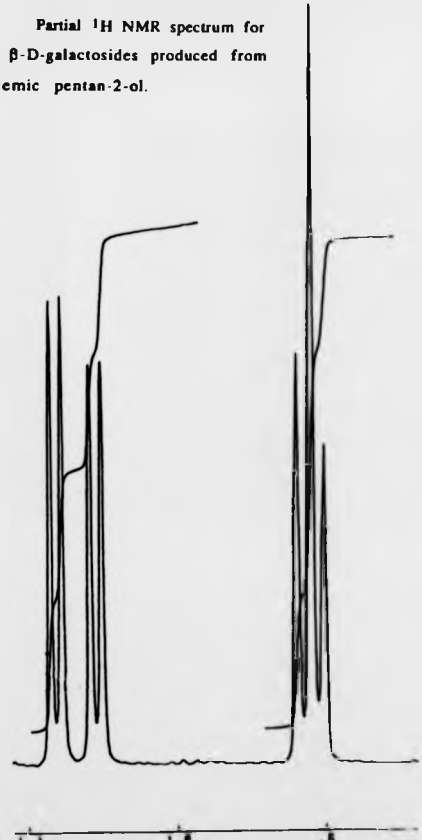


Scheme 49

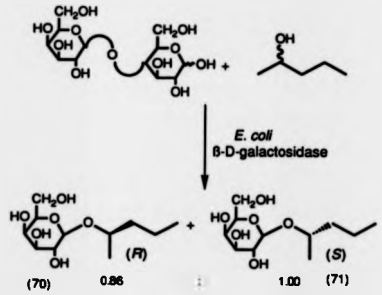
Comparison with the spectrum of the product from (*R*)-butan-2-ol showed that the products from the (*R*)- and (*S*)-enantiomers were formed in a ratio of 1.00:0.83.

With racemic pentan-2-ol, a diastereoisometric mixture of products was also formed (Scheme 50). By considering the signals in the ^{1}H NMR spectrum due to the methyl group attached to the carbinol carbon atom (in the same way as for butan-2-ol) it was clear that one product slightly predominated over the other (Figure 4). In this case, a ratio of 1.00:0.86 was indicated, but it was not possible to assign the signals to the products from the (*R*)- and (*S*)-enantiomers of pentan-2-ol as samples of the individual enantiomers were not available for comparison. However, it was noted that the ratio of the signals was in the opposite sense when compared with those in the spectrum for the galactosides of butan-2-ol. This suggested that the slight diastereoselectivity

FIGURE 4 Partial ^1H NMR spectrum for the β-D-galactosides produced from racemic pentan-2-ol.



exhibited by the enzyme was different for the two alcohols with the (*R*)-enantiomer being favoured in the case of butan-2-ol and the (*S*)-enantiomer being favoured in the case of pentan-2-ol (Scheme 50).



Scheme 50

An analogous situation was seen to exist when the ^{13}C NMR spectra were considered, *i.e.* comparable signals for the two products were also reversed in relative intensity (*cf.* Figures 5 and 6). Stereochemical influences of aglycone alcohols on ^{13}C chemical shifts (see also Appendix 2) of β-D-glucosides have been studied by several workers.[202-204,208-214] It was discovered that attachment of the anomeric carbon atom to an (*S*)-centre resulted in deshielding of the anomeric carbon atom in such a way that it gave a resonance significantly downfield of the corresponding carbon atom attached to an (*R*)-centre. This observation is

reflected in the spectra of the galactosides of (*S*)- and (*R*)-butan-2-ol (Figure 5) where the relevant resonances are at 102.8 and 101.7 p.p.m. and it may be reasonable to expect that the resonance at lowest field (104.9 p.p.m) in the spectrum of the galactosides derived from racemic pentan-2-ol is attributable to the galactoside of the (*S*)-alcohol and that the corresponding signal for the galactoside of the (*R*)-alcohol is the one at 103.7 p.p.m.. These conclusions are particularly well supported by the corresponding chemical shifts of the anomeric carbon atoms of the β-D-glucosides of (*S*)- and (*R*)-pentan-2-ol which appear at 103.9 and 102.0 p.p.m. respectively.[210]

Similar arguments may be brought to bear on the chemical shifts of the α-carbon atoms of the aglycone moieties which experience increased deshielding when attached to (*S*)-alcohols. Thus in the case of the galactosides derived from racemic pentan-2-ol, the α-carbon atom signals are situated at 76.8 and 78.1 ppm, with the latter (for the derivative of the (*S*)-alcohol) being the most intense (*cf.* Figures 5 and 6). Taken together, these results indicate that the diastereoselectivity is opposite for the two substrates although definitive assignment of the spectra of the galactosides of (*R*)- and (*S*)-pentan-2-ol is necessary before this conclusion can be substantiated.

In galactosyl transfer to racemic IPG (Scheme 51), a complete lack of diastereoselectivity was observed as shown by the pair of doublets in the ^{1}H NMR spectrum associated with the anomeric protons of the diastereoisomeric product which were of nearly equal (within 3%) intensity.

FIGURE 5 Partial ^{13}C NMR spectrum for the β-D-galactosides produced from racemic butan-2-ol.

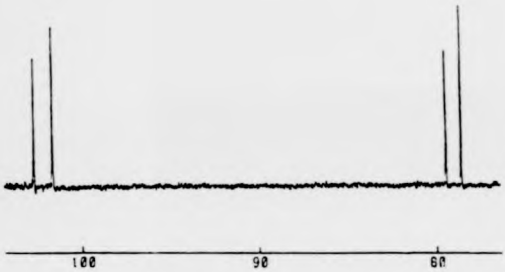
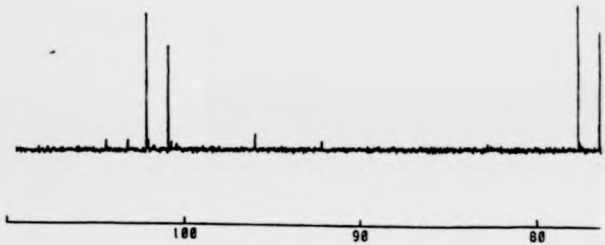
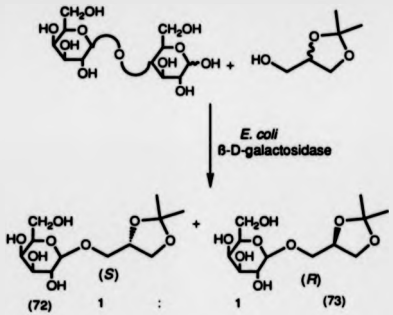


FIGURE 6 Partial ^{13}C NMR spectrum for the β-D-galactosides produced from racemic pentan-2-ol.

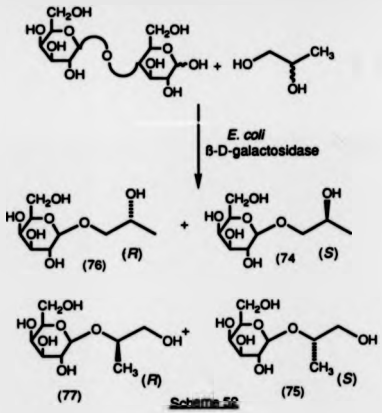




Scheme 51

Results similar to those reported here have been obtained by others[151] but they are in sharp contrast with those obtained by Boos *et al.* [155,156] which indicated that the transfer was completely stereoselective. In our study all products were examined without involving any crystallisation procedures and it is suggested that the isolation of a diastereoisomerically pure product in the Boos study was as a result of inadvertent diastereoisomeric enrichment during the crystallisation procedure.

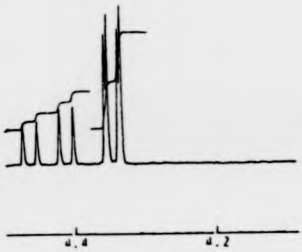
Galactosyl transfer in the presence of racemic propane-1,2-diol (Scheme 52) gave all four of the possible products as indicated by the four sets of doublets in the anomeric region of the ^{1}H NMR spectrum (Figure 7). Of the four sets, those furthest



Scheme 58

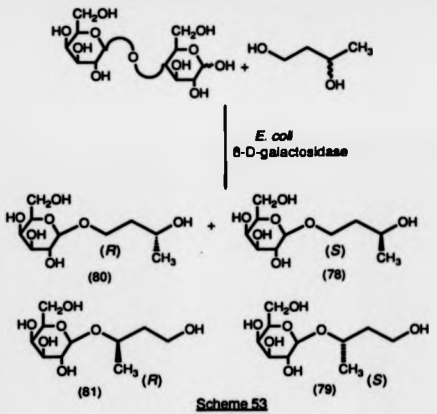
downfield were of lower intensity and corresponded to the minor products in which the galactosyl group was attached to the secondary hydroxyl group of the (*R*)- and (*S*)-enantiomers (galactosides (75) and (77)). The slight downfield shift is in accordance with the γ-effect, which predicts that anomeric protons experience greater deshielding when attached to the aglycone through a secondary hydroxyl group than when attached through a primary hydroxyl group.[210-214] The lower intensity of the doublets is a result of a smaller degree of transfer to the secondary hydroxyl groups which may be expected on steric grounds. Assignment of the signals attributable to the products from the (*R*)- and (*S*)-enantiomers of the substrate was made by

FIGURE 7 Anomeric region of the ^1H NMR spectrum for the β-D-galactosides produced from racemic propane-1,2-diol.



comparison with the product obtained from (*S*)-propane-1,2-diol and integration of the signals due to the anomeric protons showed that selectivity with respect to the enantiomeric substrates was nearly the same. In transfer to the primary hydroxyl group (to give galactosides (74) and (76)), for example, it appeared that the (*S*)-enantiomer was favoured over the (*R*)-enantiomer by a factor of 1.00:0.86, although the extreme closeness of the the pairs of doublets makes the definitive assignment of the signals difficult. In transfer to the secondary hydroxyl group, the (*R*)-enantiomer was favoured over the (*S*)-enantiomer by a factor of 1.00:0.77 and overall transfer to the primary hydroxyl group was favoured over the secondary by a factor of 1.00:0.35. When (*S*)-propane-1,2-diol was used as a substrate, a similar ratio of 1.00:0.33 was observed for the selectivity for transfer to the primary hydroxyl group.

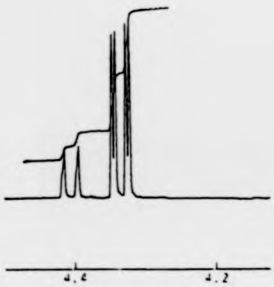
Corresponding data were obtained in a similar manner for the galactosyl transfer to racemic butane-1,3-diol, which also gave rise to four products (Scheme 53). However, in this case, the selectivity with respect to transfer to the primary and secondary hydroxyl groups was significantly greater. From the relative integrations of the signals attributable to the anomeric protons, it could be seen that the selectivity for transfer to the primary hydroxyl group was favoured over the secondary by a factor of 1.00:0.15 (Figure 8). In transfer to the primary hydroxyl group, the anomeric signals revealed that the (*R*)-enantiomer was



Scheme 53

favoured over the (*S*)-enantiomer by a factor of 1.00:0.99, indicating an almost total lack of diastereoselectivity in this mode. The corresponding information for the minor products could not be obtained from the anomeric signals as they were not distinguished at 400 MHz, but consideration of the signals attributable to the methyl groups in the product indicated that for transfer to the secondary hydroxyl group the (*R*)-enantiomer was favoured over the (*S*)-enantiomer by a factor of 1.00:0.50.

FIGURE 8 Anomeric region of the ^1H NMR spectrum for the β-D-galactosides produced from racemic butane-1,3-diol.



The selectivity of these reactions can be expressed in a different way. With racemic propane-1,2-diol, and considering the (*R*)-enantiomer, selectivity with respect to transfer to the primary hydroxyl group relative to the secondary hydroxyl group was 1.00:0.37. For the (*S*)-enantiomer, the corresponding ratio was 1.00:0.33. However, for racemic butane-1,3-diol, the corresponding ratios were 1.00:0.20 for the (*R*)-enantiomer and 1.00:0.10 for the (*S*)-enantiomer. When the (*S*)-enantiomers of propane-1,2-diol and butane-1,2-diol were used, the corresponding selectivities were found to be 1.00:0.33 and 1.00:0.13 respectively. These were not significantly different from the ratios found for the racemates.

The most comprehensive study of the structural factors affecting acceptor reactivity in β-D-galactosidase-catalysed reactions, was made by Huber *et al.*[115] who obtained kinetic data for a number of simple acceptor alcohols, monosaccharides and alditols. They interpreted their results in terms of a hypothetical glycopyranose binding site which allows for the formation of allolactose through attack of the galactosyl-enzyme intermediate by the 6-hydroxyl group of glucose (Figure 9).

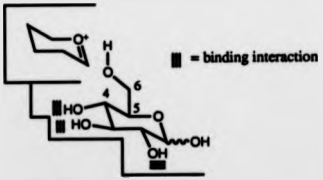


Figure 9

Although this model must be regarded as tentative, it does nevertheless provide a starting point for a discussion of the results obtained with butane-1,3-diol. The corresponding conformations with respect to binding at the active site are (i) and (ii) (Figure 10) for attack by the primary and secondary hydroxyl groups of (*R*)-butane-1,3-diol and (iii) and (iv) for the corresponding reaction modes for the (*S*)-enantiomer. These conformations have been chosen to mimic as closely as possible the conformation of the C-4 to C-6 component of glucopyranose, as this part of the molecule is considered to be the most important in relation to recognition by the active site.

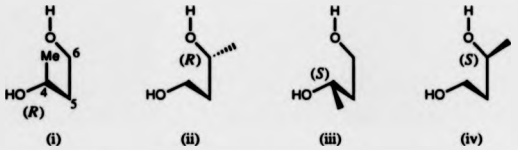


Figure 10

The relationship of the structures in Figure 10 to the bound glucopyranose system in Figure 9 is indicated in the structure (i) (Figure 10) in which the most important carbon atoms are numbered. For attack by the primary hydroxyl group it is conformation (iii) of the (*S*)-enantiomer that most closely resembles that of the model (Figure 9). However, since there was essentially no discrimination between the (*R*)- and (*S*)-

enantiomers, the alternative conformation (i) for the (*R*)-enantiomer in which the methyl group is in an 'axial' conformation in terms of the glucopyranose model must be equally acceptable.

For attack by the secondary hydroxyl group, the only difference in conformation for the two enantiomers is at the chiral centres (*cf.* (ii) and (iv)). The preference for attack by the primary as opposed to secondary hydroxyl groups suggests that steric factors are important in this transglycosylation reaction, but the explanation for attack by the secondary hydroxyl group of the (*R*)-enantiomer (ii) being twice as favoured as attack by the secondary hydroxyl group of the (*S*)-enantiomer may also lie in the highly hydrophobic nature of the glucose binding site of β-D-galactosidase. This discrimination suggests that the orientation of the methyl group in (iv) is disfavoured for steric reasons or that the orientation of the methyl group in conformation (ii) of the (*R*)-enantiomer is favoured by a hydrophobic interaction. It is possible, of course, that both factors operate simultaneously.

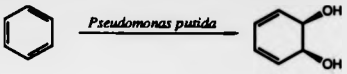
Interpretation of the results with propane-1,2-diol is less informative since the 1,2-diol structure mimics the C-4 to C-6 component of glucopyranose less well than butane-1,3-diol. Also, there is essentially no discrimination between the enantiomers. One significant difference between the two systems, however, is in the greater discrimination between the primary and secondary hydroxyl groups of butane-1,3-diol (1.00:0.15) compared to propane-1,2-diol (1.00:0.35). The source of the difference may be the difference in binding ability imparted to the two diols by the methyl groups when they adopt conformations for attack by the

primary hydroxyl groups. Thus the greater binding of (i) and (iii) relative to the equivalent conformations for propane-1,2-diol has the effect of more than halving the participation of the secondary hydroxyl group of butane-1,3-diol relative to propane-1,2-diol in the transglycosylation reaction.

The conclusions of Huber *et al.*, although highly informative, did not have the benefit of product analysis, so the importance of competing modes of reaction could not be appreciated. Our results do take this factor into account and also indicate that stereochemical discrimination is greatest for galactosyl transfer to secondary hydroxyl groups. If used for biotransformations, with high diastereoselectivity as the goal, transgalactosylation is therefore likely to give better results when secondary hydroxyl groups act as the acceptors.

Transfer to *cis*-cyclohexa-3,5-diene-1,2-diol.

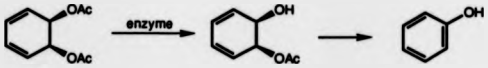
The ability of micro-organisms to dihydroxylate aromatic substrates has led to considerable interest in dihydrocatechols as building blocks in organic synthesis. The archetypal example of the oxidation is the conversion of benzene by strains of *Pseudomonas putida* to *cis*-cyclohexa-3,5-diene-1,2-diol ("benzene-*cis*-glycol"(82), Scheme 54).[215]



(82)

Scheme 54

In recent years, this product has found application in the synthesis of polyphenylene[216], (±)-,[217] (+).[218]and (-)-pinitol[218] as well as *myo*-inositol-1,4,5-triphosphate.[219] One possible way of enhancing the synthetic value of benzene-*cis*-glycol (82) is to destroy the plane of symmetry by introducing a substituent at one of the hydroxyl positions and create a chiral building block. The obvious method for achieving this would be by using the "*meso* trick",[220] that is, enantioselective enzymatic hydrolysis of the diacetate (83) (Scheme 55).



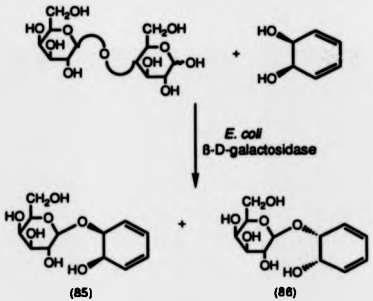
(83) (84)

Scheme 55

However, although selective hydrolysis occurs, the intermediate monoester (84) rapidly undergoes elimination of acetic acid to give phenol as the sole product.[221]

In order to produce a stereoselectively modified derivative of (82) which would be less likely to undergo elimination, galactosyl transfer was investigated. Incubation of lactose and the

meso-diol with the β-D-galactosidase of *Escherichia coli* was found to produce both of the possible products (Scheme 56).

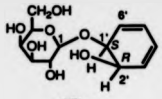


Scheme 56

Analysis of the reaction mixture by HPLC during the early stages of the reaction (3 to 4 hours) indicated a diastereoisomeric excess of *ca.* 80%; this could be confirmed by NMR analysis of the isolated product. In order to achieve optimum yields, however, it was necessary to allow the reaction to continue until almost all of the lactose had been consumed. This resulted in a decline in the diastereoisomeric ratio to *ca.* 20%, though it was found possible to separate the diastereoisomers by HPLC. The changes in the ratios of the products may be explained by the relative rates of formation and hydrolysis of the products, with the fastest-formed diastereoisomer also being the one most susceptible to hydrolysis. The diastereoisomeric excess of the product is therefore dependent on the incubation time. As with all the galactosyl

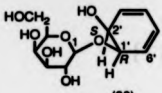
transfers so far considered, however, hydrolysis of the products is relatively slow compared to the rate of transfer, so that isolation of the products prior to extensive hydrolysis is quite simple. Another possible explanation for the variation in product ratios might have been the enzyme-catalysed transfer of the galactosyl residue between the two hydroxyl groups. This possibility is eliminated, however, since incubation of the pure diastereoisomers did not result in any interconversion.

Once the products had been separated an attempt was made to deduce their structures. Such an assignment presents some problems and the difficulty of the task is shown by the work of Gais *et al.*[160] who also carried out galactosyl transfer to *meso*-diols but postponed elucidation of the stereochemistry of the products. The method used in the present case combined nuclear Overhauser enhancement (NOE) studies with molecular modelling. First, the minimum energy conformations of the two structures were determined using the molecular mechanics programme PCMODEL.[222] In order to ensure that all of the reasonable energy minima had been located, the MULTOR routine was used to generate a series of conformations by 60° rotations about the C-1/O glycosidic bond. For each of these conformations, a second set was generated by 60° rotations about the O/C-1' bond. From the thirty six conformations thus generated for each of the structures, thirteen meaningful conformations were obtained for the (1'-*S*)-galactoside (85) and twelve for the (1'-*R*)-galactoside (86) (Figure 11).



(85)

minor galactoside (B)



(86)

minor galactoside (A)

Figure 11

Each of these conformations was then minimised in two ways: first, permitting intramolecular hydrogen bonding and second, with intramolecular hydrogen bonding prevented, to simulate the situation in aqueous solution. In both cases, the lowest energy conformation for the (1'-*R*)-galactoside (86) was the one with a 1-H/6'-H distance of 2.2 Å (Figures 12 and 13). For the (1'*S*-galactoside (85), the lowest energy conformation with intramolecular hydrogen bonding permitted had a 1-H/6'-H distance of 2.5 Å. However, this conformation was stabilised by a hydrogen bond between the hydrogen atom of the 2'-hydroxyl group and the ring oxygen of the galactosyl residue. When this possibility was prevented, this conformer had a 1-H/6'-H distance of 3.9 Å (Figures 14 and 15). The difference between the 1-H/6'-H distances for the two structures is quite significant, with that for the (1'-*S*)-galactoside being in the range where NOE effects might be expected. Examination of the NOE spectra of the major isomer (A) with the longer retention time on HPLC showed that when the anomeric proton 1-H (4.48 p.p.m) was irradiated, there was a significant NOE to 6'-H at 5.9 p.p.m. (Figure 16). Conversely, irradiation of 6'-H produced a strong NOE to 1-H (Figure 17). The corresponding experiments with the minor isomer (B) which had the shorter retention time failed to give any NOEs (Figures 18 and 19). These results were supported by further NOE data in cases

FIGURE 12 PCMODEL-generated diagram of the (1'*R*)-β-D-galactoside of *cis*-cyclohexa-3,5-diene-1,2-diol.

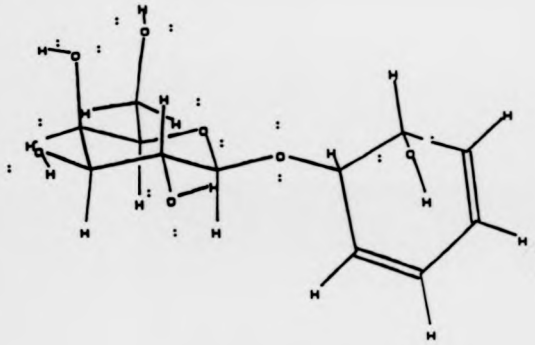


FIGURE 13 3D Representation of the (1'*R*)-β-D-galactoside of *cis*-cyclohexa-3,5-diene-1,2-diol.

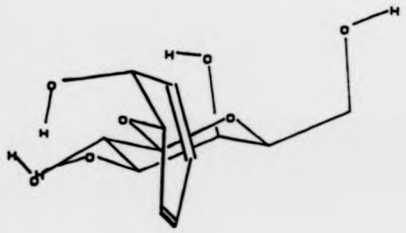


FIGURE 14 PCMODEL-generated diagram of the (1'*S*)-β-D-galactoside of *cis*-cyclohexa-3,5-diene-1,2-diol.

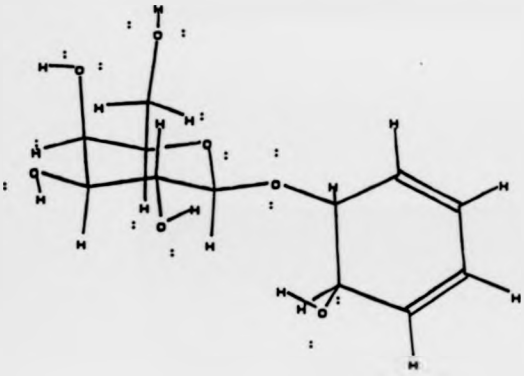


FIGURE 15 3D Representation of the (1'*S*)-β–D-galactoside of *cis*-cyclohexa-3,5-diene-1,2-diol.

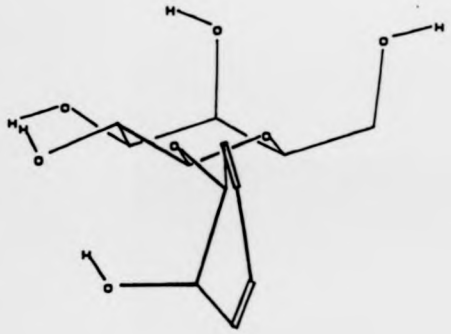
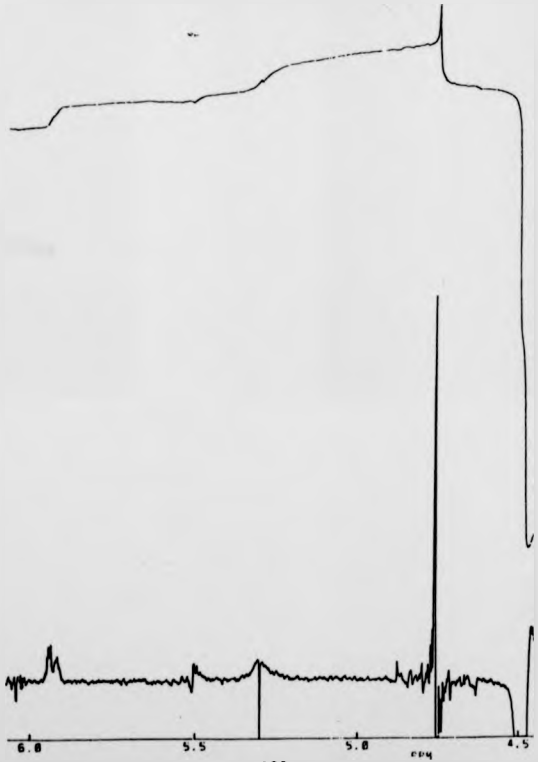


FIGURE 16 NOE Spectrum obtained by irradiation of the H-1 proton of the major isomer of the galactoside mixture obtained from *cis*-cyclohexa-3,5-diene-1,2-diol.



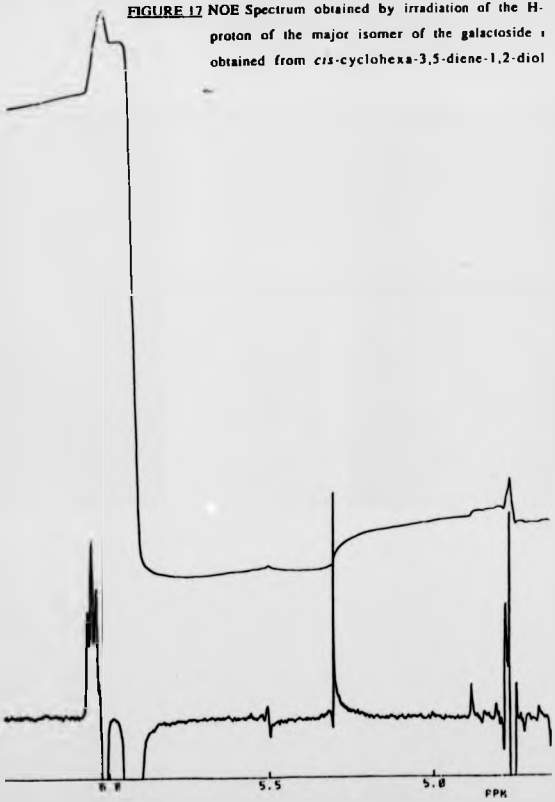


FIGURE 17 NOE Spectrum obtained by irradiation of the H-proton of the major isomer of the galactoside 1 obtained from *cis*-cyclohexa-3,5-diene-1,2-diol

FIGURE 18 NOE Spectrum obtained by irradiation of the H-1 proton of the minor isomer of the galactoside mixture obtained from *cis*-cyclohexa-3,5-diene-1,2-diol.

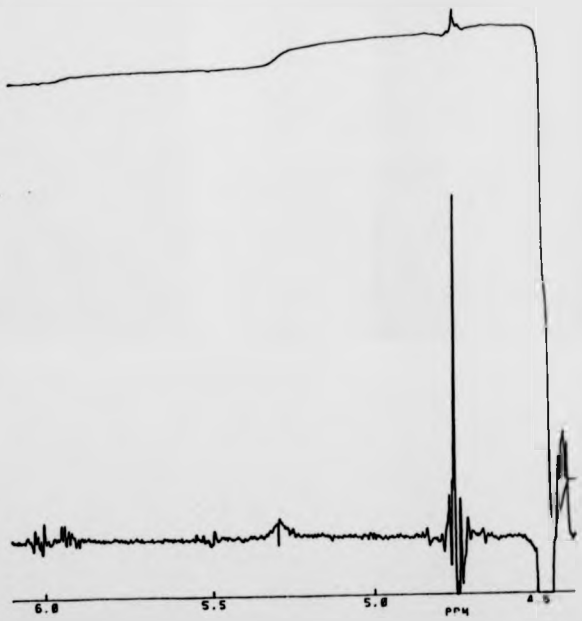
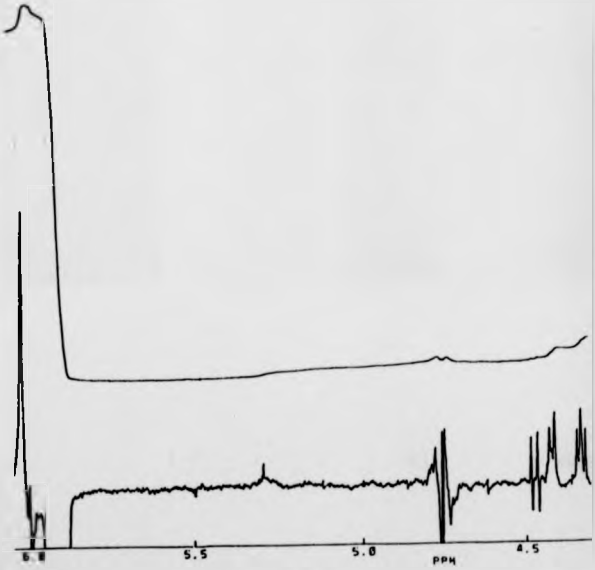


FIGURE 19 NOE Spectrum obtained by irradiation of the H-6' proton of the minor isomer of the galactoside mixture obtained from *cis*-cyclohexa-3,5-diene-1,2-diol.



where proton-proton distances were significantly different for the two diastereoisomers and permitted the preliminary conclusion to be drawn that galactoside (B) is the (1'*S*, 2'*R*)-galactoside (85) and that galactoside (A) is the (1'*R*, 2'*S*)-galactoside (86).

While the reliability of the conclusions derived in the above manner is open to question, the potential of the method is clearly demonstrated. However, although the galactosides displayed a remarkable amenability for study by this method, a definitive assignment of configuration is still needed.

Although the enzyme applied in this transfer reaction failed to give an overall high yield of a single diastereoisomer, the high diastereoselectivity exhibited during the early part of the reaction indicates that there may be other β-D-galactosidases which would give higher diastereoselectivities. For example, the enzyme from *Aspergillus oryzae* has proved superior to the *E. coli* enzyme in transfers to mono- and bicyclic *meso*-diols.

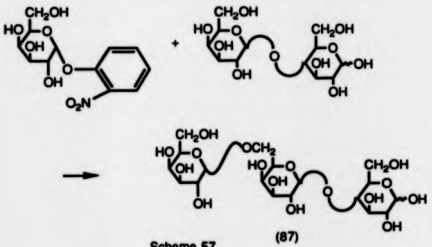
Transfer to disaccharide acceptors.

The foregoing discussion relating to glycosyl transfer to non-saccharides is valuable for gaining an understanding of these types of reactions. However, the most important application of these enzymes is in the synthesis of oligosaccharides. Enzymatic disaccharide synthesis is now commonplace, but little study has been made of transfers to disaccharide acceptors. Our attempts at trisaccharide synthesis employed the α-D-galactosidase of

Mortierella vinacea using *o*-nitrophenyl α-D-galactoside as donor with lactose and cellobiose as acceptors.

(i) Transfer to lactose (Galß(1-4)Glc).

α-Galactosyl transfer to lactose (Scheme 57) was carried out with an acceptor:donor ratio of 4:1.

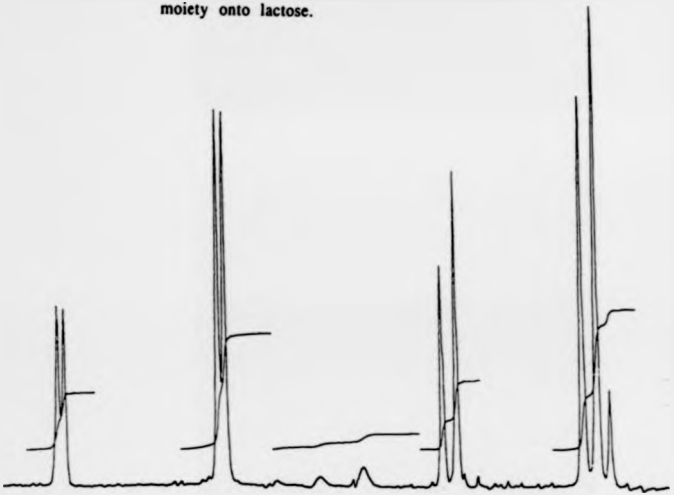


Scheme 57

(87)

HPLC analysis of the product mixture showed a single trisaccharide peak which could not be resolved by varying the solvent composition. Isolation of this fraction and analysis by ^{1}H and ^{13}C NMR confirmed that a single isomer had been formed. Thus, for example, the ^{1}H NMR spectrum showed four anomeric signals (Figure 20). The signal at δ 4.94 is typical of protons attached to anomeric carbon atoms involved in α-linkages, while the signal at δ 4.42 corresponded to the anomeric proton on the C-7 carbon atom. This latter resonance is complicated by a small amount of lactose impurity. The signals at δ 4.61 and δ 5.17 have a combined integration corresponding to a single proton and are

FIGURE 20 Anomeric region of the ^1H NMR spectrum of the trisaccharide obtained by transfer of a galactose moiety onto lactose.



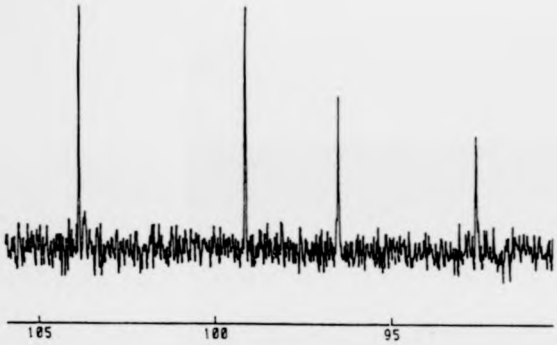
associated with the anomeric protons of the reducing moiety of the product.

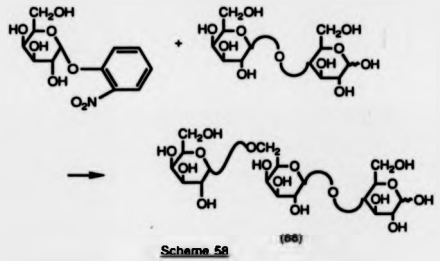
Establishment of the newly-formed linkage was made by considering the ^{13}C NMR spectrum (Figure 21). As expected, there were four resonances corresponding to the four anomeric carbon atoms. The resonance at δ 99.1 corresponded to the anomeric carbon atom of the newly-introduced α-galactosyl moiety. Its position at δ 99.1 was indicative of an α(1-6)-linkage, but the strongest evidence in favour of this conclusion was the resonance at δ 67.2 associated with the C-12 carbon atom. In general, such carbon atoms give signals at *ca.* δ 61-62 when bearing free hydroxyl groups, but when involved in forming α-linkages to glycosyl residues they experience a downfield shift of several ppm.[223] Such shifts would not result from attachment of a glycosyl moiety at any other positions. That the galactosyl residue was attached to the C-12 rather than the C-6 carbon atom of lactose (which would give a branched trisaccharide) was confirmed by the splitting of the resonance at δ 60.9/61.0 attributable to the C-6 of the reducing glucosyl unit. These values are very close to the corresponding values for lactose (*cf.* δ 60.8/60.9) and had the galactosyl residue been attached at this position the split resonance would have been observed at *ca.* δ 67.

(ii) Transfer to cellobiose (Glcβ(1-4)Glc).

α-Galactosyl transfer to cellobiose was also found to produce a single product ((88), Scheme 58).

FIGURE 21 Anomeric region of the ^{13}C NMR spectrum of the trisaccharide obtained by transfer of a galactose moiety onto lactose.





(86)

Scheme 58

The ^{1}H NMR spectrum was reminiscent of that of Galα(1-6)Galβ(1-4)Glc (87) prepared above, with the anomeric protons of the transferred galactosyl residue giving a signal at δ 4.46 (*cf.* 4.42, above). Determination of the nature of the newly-formed linkage was carried out by applying the same approach as that in the foregoing case. The conclusion that attachment was *via* C-12 of the non-reducing component of the acceptor was supported by the observation of a resonance at δ 66.7 (*cf.* 67.2, above).

These results with the α-D-galactosidase of *M. vinacea* parallel those of Kitahata *et al.* who, in similar transfers to the sweetener rubusoside also found that this enzyme had a tendency to form only α(1-6)-linkages.[168]

The ease with which these trisaccharides have been prepared demonstrates the value of glycosidases in synthesis, since the classical synthesis of such compounds would require many steps. However, there remains the question of whether higher oligosaccharides may be prepared with similar facility.

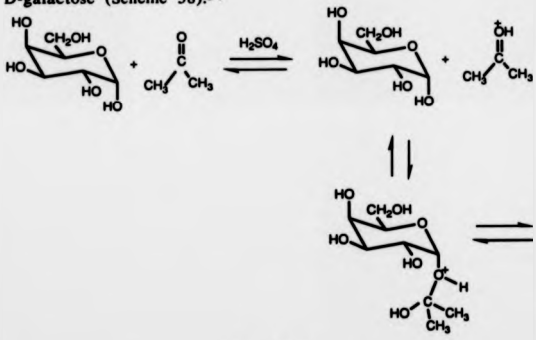
Further, glycosidases will only realise their full potential when it becomes possible to select a certain variety of enzyme capable of catalysing the formation of the specific linkage required. For example, the α-D-galactosidases for which information is available have been found capable only of forming α(1-6) linkages and it is necessary to characterise other varieties which would form specifically α(1-4), α(1-3) or α(1-2) linkages. If such a feat could be accomplished for all types of linkage, the problem of separation of product mixtures would be obviated and one might envisage enzymatic oligosaccharide synthesis superseding classical oligosaccharide synthesis.

CHAPTER THREE

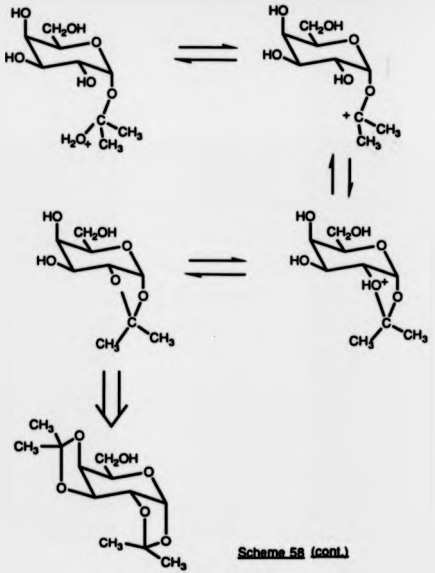
Molecular Imprinting as an Aid to Oligosaccharide Synthesis

A common problem in oligosaccharide synthesis is control over the regioselectivity of bond formation. As discussed above, it may be possible to modulate the nature of the linkage by the judicious choice of the aglycone of the acceptor and the enzyme source. Other approaches include the classical chemical protection, the addition of complexing agents, immobilisation of the acceptor and relatively new technique of "molecular imprinting."

The application of classical protecting groups such as isopropylidene, benzylidene and acetate, *etc.*, is well known in carbohydrate chemistry; acetone, for example, forms 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranoside with the α-anomer of D-galactose (Scheme 58).[57]



Scheme 58

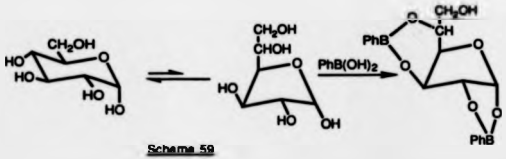


Scheme 58 (cont.)

Since the 6-hydroxyl group is not involved in the protection one could envisage its participation in a transglycosylation reaction.

The same rationale applies to the addition of complexing agents, *i.e.* the specific binding of certain hydroxyl groups so that glycosidic bond formation is restricted to a reduced number of hydroxyl groups. Much work has been concerned with complexing agents and in particular with boronate agents

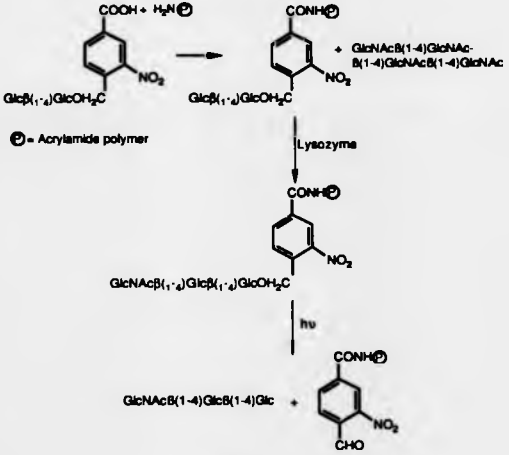
which yield crystalline derivatives.[224] D-Glucose, for example, gives a crystalline 1,2:3,5-bis(phenylboronate) with phenylboronic acid (Scheme 59).[225]



Scheme 59

Although boronates have great potential for application in many areas of chemistry, advances have been hampered by the diversity of linkages formed by boron with polyols. As a result the possibility of transglycosylation reactions employing carbohydrate boronates as acceptors has yet to be explored. Boronic acids have, however, been of great value in areas such as molecular imprinting (see below).

Oligosaccharide synthesis by immobilisation of the acceptor on a polymer support has attracted considerable interest in recent years,[65,72,226-229] although this method has yet to become competitive with classical solution chemistry. As discussed by Zehavi *et al.*[72] the major reasons for this are i) incomplete glycosylation, ii) scarcity of saccharide building blocks and iii) the necessity to introduce protecting groups. The application of enzymes serves to overcome the difficulties mentioned under ii) and iii) and a recent example involves the lysozyme-catalysed transfer of a monosaccharide unit to a cellobio-polymer (Scheme 60).[65]

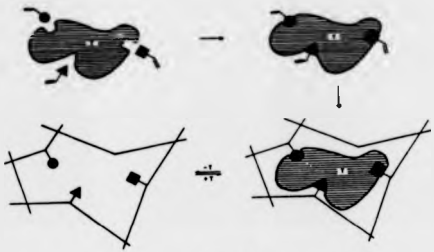


Scheme 60

The *o*-nitrophenyl cellobiose was attached to an aminoethyl-substituted acrylamide polymer *via* amide bonds and following the transglycosylation reaction the product was released by UV irradiation.

"Molecular imprinting" was the name coined by Wulff to describe the production of a polymer bearing tailor-made cavities for specific types of molecules.[230-233] One characteristic of enzyme catalysis is the binding of the reacting substrate in the active centre which is a perfectly fitting cavity

containing, in the correct stereochemistry, functional groups for binding and catalysis. The creation of such tight-fitting cavities in a polymer matrix may be achieved by binding suitable polymerisable groups to a template or "print" molecule and then copolymerising in the presence of a large amount of cross-linking agent. This gives a rigid polymer with cavities filled with the tightly-fitting template molecules. Subsequent removal of the template molecules generates cavities with an affinity for the original template molecules (Scheme 61, T = template molecule).



Scheme 61

Much of the work in this area has been focussed on phenyl α-D-mannopyranoside (template) and 4-vinylphenylboronic acid which reversibly binds with the template in a well-defined manner; two molecules of the latter bind each template molecule at four hydroxyl groups *via* ester linkages (Figure 22).

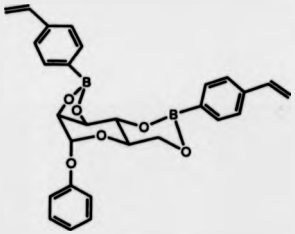
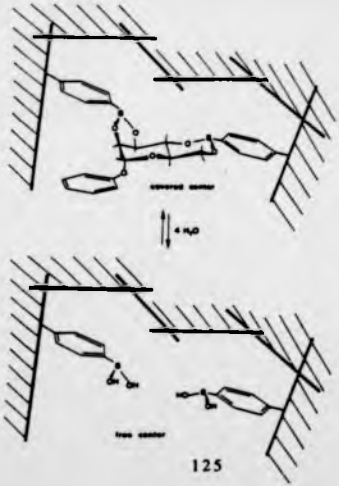


Figure 22

After complexation, polymerisation generates a structure in which the template molecules are bound in rigid cavities. Treatment with water or methanol then leaches out the print molecules to give the imprinted polymer (Scheme 62).



Scheme 62

If as in this case the print molecule is chiral, a measure of the success of the imprinting method can be gained from the ability of the polymer to separate the racemate of the print molecule, *i.e.* phenyl α-L and α-D-mannopyranosides in the case described. Another possibility, which has only recently met with success,[234] is to measure the optical activity of the polymer.

The application of imprinted polymers in the enzymatic synthesis of oligosaccharides has, as far as the author is aware, yet to be investigated. Since glycosidases are characterised by their ability to transfer glycosyl moieties to the non-reducing ends of oligosaccharides little advantage can be expected to be gained by embedding the acceptor in a polymer cavity. However, it might be supposed that the embedding of the acceptor may have an effect on the regioselectivity of the transfer reaction and thus offer an alternative way to modulate linkage-formation.

A proposed synthesis might begin with the formation of an imprinted polymer bearing cavities specific for the reducing end of target molecule. For example, if it were proposed to synthesise the trisaccharide Galß(1-4)Galß(1-4)Glc then glucose (but see below) would be employed as the print molecule, with glucose complexation being envisaged as being carried out by a boronate monomer (as above). Following polymerisation and washing, the polymer would be equilibrated with the acceptor molecule lactose (Galß(1-4)Glc) in the expectation that the glucose moieties would be bound in the cavities and the

galactose moieties would remain exposed. The exposed galactose moieties would then be used as the acceptors in a transgalactosylation reaction to give the trisaccharide product which would be easily removed by washing. Such a possibility raises many questions, the answers to which are likely to require a considerable amount of research. The ensuing study can only begin to explore the possibility of using imprinted polymers as tools in the enzymatic synthesis of oligosaccharides. The following discussion attempts to draw attention to some of the strengths and weaknesses of the strategy.

For a polymer to be of any value in enzymatic oligosaccharide synthesis it would need to possess several attributes. In the first instance it must exhibit a powerful memory effect and be easily permeable to the enzyme. Development of a polymer to this end involves many factors; enzyme permeability may be achieved using macroporous (macroreticular) polymers consisting of a network of polymer chains and large pores while optimisation of the memory effect requires that the influences of rigidity, flexibility and accessibility be considered.

The polymer should also possess high rigidity in order to preserve the shape of the cavities. This can be done by high cross-linking and it is known that for some polymers less than 10% cross-linking agent results in a complete lack of specificity for the template.[235] In contrast to the requirement for rigidity, the polymer should also exhibit a degree of flexibility in or

around the cavities since cavities of accurate shape but lacking flexibility show a kinetic hindrance to reversible binding. Flexibility is greatly influenced by the type of cross-linking agent and a balance needs to be reached between a polymer with highly specific cavities which cannot be split from their hosts and one which possesses less selective cavities but allows fast binding and splitting of print molecules. In the case of boronate polymers it may be possible to exert some degree of control over the binding/splitting process by altering the pH; studies by Barker *et al.*[236] indicate that with phenylboronic acid carbohydrates are essentially uncomplexed below pH 5 and fully complexed beyond pH 9.

Finally the polymer should possess a degree of mechanical stability and have as many as possible of its cavities accessible to acceptor, donor and, most importantly, the enzyme; the co-existence of the acceptor, donor and enzyme at the same area of the polymer structure is dependent on the flexibility of the polymer and the pore size distribution.

In the hypothetical trisaccharide synthesis outlined above D-glucose was proposed as the print molecule; careful consideration of the interaction of areneboronic acids with D-glucose, however, indicates that this would be unsuitable as the print molecule as it reacts in the furanose form (Scheme 59). A more appropriate print molecule would be 4-*O*-methyl D-glucose which, like the glucose moiety of lactose, is unable to adopt the furanose form. Further, the specificity of cavities

formed by 4-*O*-methyl D-glucose would not be dependent on the 4-position.

As discussed above pH has an important effect on binding. To optimise the number and specificity of cavities, polymerisation should be carried out at elevated pH. Control of pH would also be important when rebinding lactose and carrying out the transglycosylation reaction; a balance would need to be reached between the degree of lactose binding and the enzyme activity. Although most glycosidases exhibit their greatest activity at approximately pH 7 or less, considerable activity may still be maintained at pH 8 or greater which would favour binding of the acceptor (lactose).

Accessibility of the bound lactose may be a problem in syntheses of this nature, *i.e.* the enzyme may be too large or the cavity may be too "buried" for the enzyme to be able to "see" the lactose molecule. A possible way of circumventing such a problem would be to increase the proportion of each acceptor molecule not involved in binding in the cavity. Since the acceptor molecule is predetermined, the easiest way of regulating the amount of each acceptor exposed to the enzyme is *via* the size of the cavity which is controlled by the choice of the print molecule. In trisaccharide syntheses, variation of the print molecule is not possible since a monosaccharide must be used, but as the size of the target molecule increases, so does the choice of print molecule.

A possible advantage in the synthesis of higher oligosaccharides is in the increased specificity that the deeper cavities may have for the acceptor; the greater specificity conferred on the cavities by an increased number of boronate-carbohydrate interactions would decrease the chances of the acceptor molecule going into the cavity backwards. However, with deeper cavities, unfavourable entropy effects may become prohibitive to the uptake of acceptor molecules by the cavities.

CHAPTER FOUR

Molecular Imprinting Using Acrylamide Polymers.

In the development of a saccharide-imprinted polymer for use in enzymatic oligosaccharide synthesis the primary considerations are i) that the polymer will allow free movement of the enzyme and ii) that it should bear groups capable of binding saccharide molecules. On account of the high molecular weights of enzymes it is perhaps the first of these considerations which would be likely to pose the greater problem since the incorporation of suitable binding groups may be achieved *via* small modifications in the composition of the monomer mixture used in polymerisation.

In our efforts to overcome the problem of the enzyme accessibility we were attracted to acrylamide polymers which are known for their capacity for large changes in swelling, *i.e.* their ability to adopt a very open structure of large pore sizes. Further, acrylamide polymers are amenable to substitution by boronate-containing monomers which can bind saccharide molecules during polymerisation. The possibility for altering the swelling of the polymer was also attractive to us as a means of physically altering the structure of the polymer on a microscale to facilitate sugar binding/release. The transition of an imprinted structure from small volume to high volume, for example, gives the possibility for distortion of the cavities and a consequent change in the degree of sugar binding.

With these considerations in mind our approach was to consider first *N*-isopropylacrylamide polymers and then acrylamide/acrylic acid co-polymers.

N-Isopropylacrylamide polymers.

Polymers of *N*-isopropylacrylamide exhibit large changes in swelling (see Appendix 2) when their temperatures are raised or lowered through their lower critical solution temperature (LCST).[237-239] Thus these 'hydrogels' shrink or deswell in aqueous solutions as the temperature is raised through a few degrees at their LCST (generally 30 to 40°C) and reswell or expand on cooling below the LCST. These properties have allowed these thermally reversible gels to be used to deliver drugs, to selectively or non-selectively remove undesirable toxins, to assay for specific substances, to recover selected products of a bioprocess or chemical process, to separate *via* affinity binding a substance from a complex mixture and to act as temperature-sensitive catalysts and reaction control systems.

Our aim was to develop a polymer which would exhibit a swelling great enough to permit free movement of the enzyme at temperatures above the transition range of temperatures. In a typical experiment it was envisaged that the enzyme would execute the transfer of a glycoside moiety onto a bound acceptor molecule at, for example, 40°C and then the temperature would be reduced to below the transition range to facilitate product removal.

Experiments were conducted to examine the effects of various factors on the swelling of *N*-isopropylacrylamide polymers in distilled water at 20°C and 50°C, *i.e.* temperatures significantly below and above the transition range. With the swelling at the upper temperatures being the most important in determining the suitability of the polymer for use in conjunction with polymers, efforts were made to maximise this value by variation of cross-linking agent, initiator and monomer concentrations.

Cross-linking agent (*N,N'*-methylenebisacrylamide, MBA) was varied from 0.1 to 3.0 mole %.* With increasing amounts of MBA the swelling at 20°C was found to decrease approximately exponentially from *ca*.30 $cm^3\ g^{-1}$ to *ca*.6 $cm^3\ g^{-1}$, but the swelling at 50°C was found to remain approximately constant at *ca*.3 $cm^3\ g^{-1}$.

Variation of the concentration of the initiator mixture (*N,N,N',N'*-tetramethylethylenediamine (TEMED): ammonium persulphate, 1:1) was found to have only a small effect on swelling. Thus a fivefold increase in initiator concentration increased the swelling at 20°C from *ca*.6 $cm^3\ g^{-1}$ to *ca*.8 $cm^3\ g^{-1}$ but had little or no effect on the swelling at 50°C which remained approximately constant at *ca*.3 $cm^3\ g^{-1}$.

* Cross-linking agent concentrations are expressed with respect to the total concentration of monomer.

The effects of varying the concentration of monomer (from 5 to 20 g cm^{-3}) were similar to those observed in the variation of cross-linking agent. With increasing monomer concentration the swelling at 20°C was found to decrease approximately exponentially from *ca.*35 $cm^3\ g^{-1}$ to *ca.*6 $cm^3\ g^{-1}$, but the swelling at 50°C was found to remain approximately constant.

From these experiments the difficulty of increasing the swelling at 50°C of *N*-isopropylacrylamide polymers was clearly demonstrated. Gels with swellings greater than *ca.*4 $cm^3\ g^{-1}$ could not be obtained and since this value was considered to be too low for use of the polymer in conjunction with enzymes, an alternative type of acrylamide polymer was examined.

Acrylamide/acrylic acid co-polymers

Acrylamide/acrylic acid co-polymers are known for their capacity for large changes in swelling in aqueous media and, under the trade name Agrigel,[240,241] have found application in agriculture as slow releasers of moisture in soil. The swellings of these types of polymer are found to be dependent on the ionic strength of the swelling medium. The origin of this effect is the repulsion between the negatively charged carboxyl groups. In distilled water there are no barriers to the repulsion and the swelling is at a maximum, but by increasing the ionic strength of the medium the carboxyl groups are shielded from one and another causing a reduction in swelling. The ability to

impose a control over swelling by varying the ionic strength was attractive to us for the reasons outlined above.

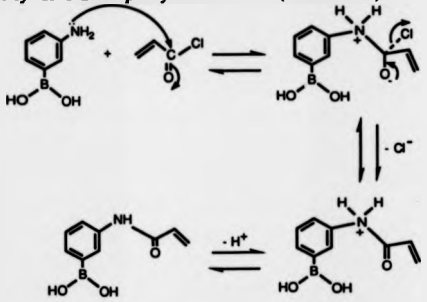
Preliminary studies into acrylamide/acrylic acid-type co-polymers were concerned with methacrylic acid as the acid component on account of the greater rigidity it was expected to impart to the polymer compared to acrylic acid. Polymerisations involving methacrylic acid were, however, slow and unpredictable and it was superseded in favour of acrylic acid which gave sufficiently rigid yet reproducible co-polymers.

Development of the acrylamide/acrylic acid co-polymer was based on a 50:50 molar amide/acid composition and the first experiments were designed to define approximate levels of cross-linking agent (MBA) and initiator (TEMED/ammonium persulphate). Below 0.1% MBA the polymer was so sticky as to be unworkable, but at MBA levels of 1.0% sufficiently rigid polymers with reasonable swelling values in water and 1 M NaCl* were obtained (*ca*.65 and *ca*.25 $cm^3 g^{-1}$, respectively).

---

* Initially 1 M NaCl was used for measuring swellings in high ionic strength media but it later became clear that values obtained in 0.1 M phosphate, *i.e.* the medium in which the enzyme and polymer would likely be employed in synthesis, would provide a better guide as to the suitability of the polymer.

An attempt was then made to substitute acrylamide for *N*-acryloyl-3-aminophenylboronic acid (AAPB). The synthesis of AAPB was in accordance with the method of Igloi and Kössel[242,243] but with some modifications on account of the low solubility of 3-aminophenylboronic acid (Scheme 63).

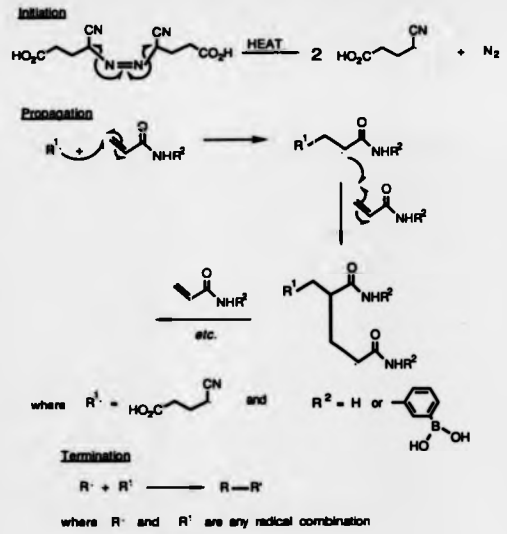


Scheme 63

Several attempts to improve the yield were made *eg.* by the addition of triethylamine to quench the liberated HCl (as successfully achieved by Schott[244] in his synthesis of the methacryloyl derivative) and the use of a slight excess (1:1 equivalents) of acryloyl chloride. The addition of triethylamine had an adverse effect on the reaction; a small amount of a very dark unidentified material was obtained. For the purposes of identification the AAPB was recrystallised from water but in the preparation of polymers it was employed in its crude form.

In the substitution of acrylamide for AAPB a change of initiator from TEMED/ammonium persulphate to the thermal

initiator 4,4'-azobis(4-cyanopentanoic acid) (ACPA) was made. The purpose of this change was to preclude oxidation problems which may have occurred during polymerisation in the presence of monosaccharides on account of the powerful oxidising properties of ammonium persulphate and to alleviate the solubility problems of AAPB as result of the increased temperatures (*ca.*80°C) used for polymerisation. Unlike the TEMED/ammonium persulphate system which generates free radicals by a complex, poorly understood mechanism, ACPA functions in a well-defined manner (Scheme 64).



Schema 64

Boron was incorporated into the polymers by substitution of acrylamide by 5 to 25% AAPB. The degree of incorporation was determined by plasma emission spectrometry (see Appendix 3) and in each case approximately half of the AAPB was taken up into the polymer. It was also found that the presence of glucose during polymerisation did not affect boron uptake and that values of swelling were largely unaffected.

The effect of the incorporation of AAPB on swelling was measured in 1 M NaCl and found to be independent of the levels AAPB. At this stage of the gel development, an arbitrary AAPB level of 15% was chosen for subsequent studies. Also, all further swellings were carried out in 0.1 M sodium phosphate buffer rather than 1 M NaCl for the reasons described in the footnote on page 130. The effect of varying the pH of the buffer (from 6.3 to 8.8) on swelling for 15% AAPB gels at three levels of cross-linking was determined. The swelling values of *ca*.43, *ca*.33 and *ca*.29 $cm^3\ g^{-1}$ obtained for gels of 0.46, 0.58 and 0.70 mole % cross-linking agent, respectively, were found to be independent of pH and compared with values of *ca*.110, *ca*.70 and *ca*.60 $cm^3\ g^{-1}$ determined in distilled water.

An indication as to the readiness of *E. coli* ß-D-galactosidase to permeate these gels was gained by immersion of the fully swollen gel in an enzyme/buffer solution (pH 7) followed by filtration, re-emersion in a solution of *o*-nitrophenyl ß-D-galactoside (ONPG) and the measurement of the rate of the liberation of *o*-nitrophenol. Somewhat surprisingly, there was no increase in the absorption of the supernatant ONPG solution. This result was unexpected because gels with swellings greater than 18 $cm^3\ g^{-1}$ are known to be permeable to globular proteins with molecular masses of up to 400,000 and yet these gels had swelling values in excess of 30 $cm^3\ g^{-1}$ but were not permeable to *E. coli* ß-D-galactosidase with a molecular mass of approximately 540,000. Similar studies were then carried out with *A. oryzae* ß-D-galactosidase (M.W. = 100,000) and similar results obtained.

The reluctance of these enzymes to permeate the gels may be explained in terms of i) charged repulsion between the gel and the enzyme, ii) enzyme aggregation, or iii) a combination of i) and ii). The first of these possibilities concerns repulsion between the negatively charged polymer ions (on account of the carboxylate groups) and the enzyme which is also negatively charged at the pHs employed in the experiment. The negative charges on the enzyme are explained by a preponderance of carboxylate groups on the exterior of the enzyme (as evidenced by the isoelectric point of *ca.*4 of the enzyme) which are deprotonated at the elevated pH of the experiment. Aggregation to dimers, trimers, tetramers, *etc.*, is commonly experienced with enzymes and there is a strong possibility that this may be a contributing factor to the reluctance of the gels to allow free passage of the enzyme. A common method of reducing aggregation is the addition of sodium dodecyl sulphate (SDS); some preliminary studies were carried out into the effects of SDS on enzyme activity but it soon became clear that the best way of ensuring uptake of the enzyme by the gel would be to allow the dehydrated gel to swell in the enzyme solution. Under these conditions the osmotic forces favouring entry of the enzyme into the gel were sufficient to overcome the aggregation or electrical effects which may have existed.

By allowing the gel to swell in a volume of a solution of enzyme in buffer equivalent to one and a half times its normal swelling volume and measuring the increase (or otherwise) in activity of the supernatant enzyme solution, it was possible to

gauge the degree to which the enzyme had permeated the gel. In the event that there was no permeation of the gel by the enzyme the activity of the supernatant solution would have been expected to have increased threefold whereas unrestricted permeation would have resulted in no change in activity. When a gel cross-linked with 0.46 mole % MBA was tested in this manner with *E. coli* ß-D-galactosidase the activity of the supernatant after four hours was increased from 2.1 to 2.7 Ucm^{-3} implying that the enzyme activity of the gel-absorbed solution was 1.8 Ucm^{-3}. In the corresponding experiment with *A. oryzae* ß-D-galactosidase the enzyme activity of the supernatant solution was increased from 6.5 to 9.5 Ucm^{-3} so that the solution contained within the gel had an activity of 5.0 Ucm^{-3}. The levels of enzyme uptake were sufficiently great for there to have been little further concern about the compatibility of these gels with enzymes of fairly high molecular weight.

Should uptake of the enzyme by the gel still have remained a problem a possible solution may have been to have allowed the gel to swell in a solution of the enzyme in distilled water (which would have caused maximum swelling of the gel and therefore a greater chance of permeation by the enzyme) and then deswell by increasing phosphate concentration.

At this stage of the polymer development the realisation was made that the imprinting procedure was being carried out at small volumes and that the gels were then being expected to exhibit selectivity for the print molecule after swelling in

buffer solution. Since such a swelling would be likely to cause a distortion of the cavities and a concomitant reduction in selectivity, an attempt was made to prepare the polymer at a volume which would be more consistent with that at which it would be employed in an oligosaccharide synthesis. To this end a study was carried out to determine the highest dilution which would give rise to a workable gel. At this point an attempt was also made to improve the imprinting method by carrying out the polymerisation at elevated pH. Thus a 0.1 M solution of disodium orthophosphate (Na_2HPO_4, natural pH *ca.*8.9) was used as the solvent for the monomers. This provided a sufficiently high pH to greatly encourage complexation of the print molecule and therefore increase the number and selectivity of cavities formed. The monomer concentration was minimised at 0.58 mmol cm^{-3} using an initiator concentration of 0.4 mole % and a greater level of cross-linking agent (5.5 mole % MBA); below this monomer concentration polymerisation was unpredictable with viscous solutions often being formed.

The complexing ability of a gel is at its maximum when its volume corresponds to the volume used in the polymerisation since this swelling reflects the conditions under which the imprinting took place. In general, however, enzyme experiments are carried out in 0.1 M phosphate buffer which would cause a significant swelling of the gel. A measure of the distortion of the polymer caused by swelling in 0.1 M phosphate buffer may be gained from the ratio of the volumes it occupies at the point of polymerisation and in the buffer

solution. Under ideal circumstances the value of this quotient will tend to unity but in the case of the boronate gels the quotients are significantly less than unity. For example, in the case where the monomer concentration was 0.58 mmol cm^{-3} polymerisation took place at a volume of 27 cm^3 but in 0.1 M phosphate the gel swelled to *ca*.50 cm^3 giving a quotient of 0.54. One way of increasing quotients and thereby bringing gels closer to the volumes they occupied during the imprinting procedure is to enforce a decrease in swelling by increasing the phosphate concentration. Thus by soaking the gel in 1.0 M phosphate it was possible to reduce the swelling from a value of *ca*.50 $cm^3\ g^{-1}$ to a value of *ca*.40 $cm^3\ g^{-1}$ which corresponded with a quotient of 0.68. However, since only small improvements in the quotients could be achieved with higher concentrations of phosphate and concentrations approaching 1.0 M led to crystallisation of the solute, it was decided to conduct subsequent experiments with 0.1 M phosphate buffer.

In an effort to gauge the affinity of glucose-imprinted polymers for glucose, two polymers, one with and one without AAPB, both prepared in the presence of glucose, were washed repeatedly with buffer solutions of varying pH and the concentration of glucose in the filtrate measured by the phenol-sulphuric acid method. When both polymers were washed with 0.1 M phosphate buffer at pH 8.9 the expected gradual decrease in the glucose concentration of the filtrate was observed, but when the pH of the buffer solution was changed to pH 4.0 the concentration of glucose in the filtrate from the boronate polymer increased threefold compared to slight

decrease in glucose concentration in the filtrate from the polymer devoid of AAPB. Although these results serve only as an indicator of the affinity the boronate polymer may exhibit for glucose, it is interesting that the effect is in the direction one would expect considering the higher degree of saccharide binding at higher pH's.

An improved method for determining the selectivity of boronate polymers was to measure the degree to which they were able to separate the enantiomers of the print molecule used in their formation. For each print molecule comparisons were made between four polymers *i.e.* with and without boronate and in the presence and absence of the print molecule.

When a racemic mixture of D-(+)- and L-(-)-glucose was passed through columns of the polymers random fluctuations in the rotations of the eluted fractions occurred but in the case of the polymers containing boronate and prepared in the presence of D-(+)-glucose there was slight tendency towards more negative rotations. A similar effect was observed in the attempted separation of L-(+)- and D-(-)-arabinoses but care should be exercised when interpreting these results particularly when one considers the small differences which one is attempting to measure and the tendency of the polarimeter to give inconsistent readings.

These results are further complicated by the fact that i) the proportion of boronate molecules successfully involved in

binding monosaccharide is not known and ii) the relative selectivities of the cavities for the enantiomers is also unknown. Thus, for example, it may be that only a small number of boronate groups became part of accessible cavities so that the polymer is effectively saturated by the monosaccharide. Conversely, the majority of boronate molecule may be available for, and involved in, binding, but exhibit a distinct lack of enantioselectivity so that all monosaccharide molecules are bound regardless of chirality. In order to distinguish between these possibilities and properly assess the abilities of these polymers to bind/separate D- and L-sugars, a significant amount of further, more specific experimentation is required.

The above discussion charts the attempted development of a boronate polymer for use in enzymatic oligosaccharide synthesis. The development reached the stage whereby a polymer was prepared which allows permeation by high molecular weight enzymes and shows promise in exhibiting a degree of affinity and possibly enantioselectivity for monosaccharides. Much of the work described is qualitative in nature on account of the nature of the goal and the reluctance that acrylamide/acrylic acid polymers show for accurate measurement. As a result, emphasis has been put on the thought processes surrounding the polymer development rather than recital of experimental data.

This study has only begun to investigate the potential that polymers may have in oligosaccharide synthesis but it is

hoped that some progress has been made towards a better understanding of the possibilities which these types of polymer may offer.

CHAPTER FIVE

Experimental for Chapter 2.

General

All transgalactosylations were carried out in 0.1 M sodium phosphate buffer; 0.1 M Na_2HPO_4 was adjusted to the correct pH using 0.1 M NaH_2PO_4. Analytical TLC was carried out using Kieselgel 60 PF-254 plates with development in the solvent system propan-1-ol:nitromethane:water (10:9:3 v/v). Products were visualised by spraying the plates with a solution of phosphomolybdic acid in ethanol (70 gdm^{-3}) and heating to 150°C for 5 minutes. Preparative TLC plates were prepared using Kieselgel 60 PF-254. Development was with propan-1-ol:nitromethane:water (10:9:2 v/v) and visualisation was, unless otherwise stated, as described above. Product galactosides were isolated from the silica by treatment with hot distilled water followed by filtration through Celite. The filtrate was lyophilised and the product again treated with a small amount of hot distilled water. Filtration through Celite followed by lyophilisation gave virtually silica-free product. Recrystallisations were carried out by a mixed solvent method (water/ethanol).

HPLC was carried out using a Waters 6000A solvent delivery system with an R401 differential refractometer. A Magnasil 5H aminopropyl column (25 cm x 4 mm) was used with the solvent system acetonitrile:water (77:23 v/v, unless otherwise stated).

^1H NMR was carried out at 400 MHz using a Bruker WH400 spectrometer. ^{13}C NMR spectra were determined at 100.62 MHz using the Bruker WH400 spectrometer. NMR measurements were carried out with D_2O as solvent and all NMR data is expressed in parts per million (ppm).

Infrared spectra were carried out using a Perkin Elmer Infrared Spectrophotometer 197 using Nujol as the mulling agent.

Mass spectra were determined using a Kratos MS80 mass spectrometer in the electron impact mode or the chemical ionisation mode with ammonia as the ionising gas. For accurate mass determination the acetate of the product was used. Acetates were obtained by treating the galactoside (10 mg) with a mixture of pyridine and acetic anhydride (1:1 v/v, 2 cm^3) for 12 h at 20°C followed by reagent removal under reduced pressure.

All yields are based on the galactosyl donor *i.e.* lactose or *o*-nitrophenyl ß-D-galactopyranoside.

Melting points, where applicable, were determined using a Stuart Scientific Melting Point Apparatus (SMP 1).

Trans-ß-galactosylations

Method A

Lactose (0.18 g, 0.5 mmol) and the alcohol (4 mmol) were dissolved in 0.1 M phosphate buffer (pH 7.3, 3.0 cm^3). ß-D-Galactosidase from *E. coli* (Sigma Grade X, 25 units) was added to the stirred mixture at t=0, 3 and 6 h (75 units total). The mixture was incubated at 25°C for 48 h at which time HPLC examination showed that most of the lactose had been consumed. The reaction mixture was heated to 90°C for 5 minutes and filtered. The filtrate was lyophilised and the product isolated by either preparative TLC or HPLC, as described above.

Method B

Lactose (0.27 g, 0.75 mmol) and the alcohol (6 mmol) were dissolved in 0.1 M phosphate buffer (pH 7.3, 2.5 cm^3). ß-D-Galactosidase from *E. coli* (Sigma, Grade X, 50 units) was added to the stirred mixture at t=0, 3 and 6 h (150 units total). The mixture was incubated and worked up as described in Method A.

Methyl β-D-galactopyranoside (56)

Method A, using methanol, yielded methyl β-D-galactopyranoside (51 mg, 53%), m.p. 177-180°C; R_f(HPLC)=3.2min; δ_H 3.43 (1H, dd, $J_{2,1}$ 7.9 Hz, $J_{2,3}$ 9.9 Hz, 2-H), 3.51 (3H, s, Me), 3.58 (1H, dd, $J_{3,2}$ 9.9 Hz, $J_{3,4}$ 3.5 Hz, 3-H), 3.63 (1H, ddd, $J_{5,4}$ 1.0 Hz, $J_{5,6A}$ 7.9 Hz, $J_{5,6B}$ 4.3 Hz, 5-H), 3.68 (1H, dd, $J_{6B,5}$ 4.3 Hz, $J_{6B,6A}$ 11.6 Hz, 6-H), 3.73 (1H, dd, $J_{6A,5}$ 7.9 Hz, $J_{6A,6B}$ 11.6 Hz, 6-H), 3.85 (1H, dd, $J_{4,3}$ 3.5 Hz, $J_{4,5}$ 0.9 Hz, 4-H), 4.25 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_c 57.9 (C-1'), 61.7 (C-6), 69.5 (C-4), 71.5 (C-2), 73.6 (C-3), 75.9 (C-5), 104.6 (C-1);

EI, m/z 195 (M^++1,23%), 163 (M^+-OCH_3,62), 91 (M^+-$C_4H_7O_3$,37), 73 (M^+-$C_4H_9O_4$,49), 60 (M^+-$C_5H_{10}O_4$,100);

IR(cm^{-1}), 3300(br, OH), 2870(m, CH), 1400-1270(m,-OH), 1150-1040(m, C-O).

Ethyl β-D-galactopyranoside (57)

Method A, using ethanol, yielded ethyl β-D-galactopyranoside (54 mg, 52%), m.p. 114-116°C; R_f(HPLC)=3.4min; δ_H 1.17 (3H, t, $J_{2',1'}$ 7.1 Hz, 2'-H), 3.40-3.85 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), 3.7 (1H, 1"-H), 3.91 (1H, dd, $J_{1',2'}$ 7.1 Hz, $J_{1',1"}$ 9.8 Hz, 1'-H), 4.34 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δc 15.1 (C-2'), 61.7 (C-6), 66.8 (C-1'), 69.4 (C-4), 71.5 (C-2), 73.6 (C-3), 75.8 (C-5), 103.2 (C-1);

EI, m/z 209 (M^++1,52%), 163 (M^+-C_2H_5,100), 91 (M^+-$C_5H_9O_3$,26), 73 (M^+-$C_5H_{11}O_4$,48), 60 (M^+-$C_6H_{12}O_4$,73);

IR(cm^{-1}), 3290(br, OH), 2870(m, CH), 1410-1270(m,-OH), 1150-1040(m, C-O).

Propyl ß-D-galactopyranoside (58)

Method A, using propan-1-ol yielded propyl ß-D-galactopyranoside (60 mg, 54%) m.p. 103-105°C;

R_f(HPLC)=3.5min; δ_H 0.85 (3H, t, $J_{3',2'}$ 7.5 Hz, 3'-H), 1.56 (2H, m, 2'-H), 3.41-3.86 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), 3.56 (1H, 1"-H), 3.86 (1H, dq, $J_{1',2'}$ 6.9 Hz, $J_{1',1"}$ 9.8 Hz, 1'-H), 4.33 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 10.5 (C-3'), 23.0 (C-2'), 61.7 (C-6), 69.4 (C-4), 71.6 (C-2), 72.9 (C-1'), 73.6 (C-3), 75.8 (C-5), 103.5 (C-1);

CI (NH_3), m/z 240 ($M+NH_4^+$,71%), 223 (M^++1,61), 180 (M^+-OC_3H_7+NH_4^+,52), 163 (M^+-OC_3H_7,100), 91 (M^+-$C_6H_{11}O_3$,31), 73 (M^+-$C_6H_{13}O_4$,33), 60 (M^+-$C_7H_{14}O_4$,44);

IR(cm^{-1}), 3300(br, OH), 2870(m, CH), 1390-1270(m,-OH), 1160-1040(m, C-O).

1-Methylethyl ß-D-galactopyranoside (59)

Method A, using propan-2-ol, yielded 1-methylethyl ß-D-galactopyranoside (54 mg, 49%), m.p. 125-128°C;

R_f(HPLC)=3.8min; δ_H 1.14 (3H, d, $J_{2',1'}$ 6.3 Hz, 2'-H), 1.17 (3H, d, $J_{2",1'}$ 6.14 Hz, 2"-H), 3.36-3.85 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), 4.04 (1H, m, 1'-H), 4.40 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 21.8/23.2 (C-2'/C-2"), 61.6 (C-6), 69.4 (C-4), 71.6 (C-2), 73.7 (C-3), 73.7 (C-1'), 75.8 (C-5), 101.7 (C-1);

EI, m/z 223 (M^++1,45%), 163 (M^+-OC_3H_7,100), 91 (M^+-$C_6H_{11}O_3$,54), 73 (M^+-$C_6H_{13}O_4$,29), 60 (M^+-$C_7H_{14}O_4$,35);

CI (NH_3), m/z 240 (M+NH_4^+,61%), 223 (M^++1,39), 180 (M^+-OC_3H_7+NH_4^+,100), 163 (M^+-OC_3H_7,39), 91 (M^+-$C_6H_{11}O_3$,13), 73 (M^+-$C_6H_{13}O_4$,68), 60(M^+-$C_7H_{14}O_4$,21);

IR(cm^{-1}), 3300(br, OH), 2850(m, CH), 1410-1270(m,-OH), 1150-1040(m, C-O).

2-Methoxyethyl ß-D-galactopyranoside (60)

Method A, using 2-methoxyethanol, yielded 2-methoxyethyl ß-D-galactopyranoside (53 mg, 45%); R_f(HPLC)=4.0min; δ_H 3.32 (3H, s, Me), 3.42-3.84 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), ≈ 3.6 (2H, 2'-H), ≈3.7 (1H, 1"-H), 3.99 (1H, dt, $J_{1',1"}$ 11.5 Hz, $J_{1",2'}$ 4.3 Hz, 1'-H), 4.32 (1H, d, $J_{1,2}$ 7.8 Hz, 1-H);

δ_C 58.6 (C(Me)), 61.6 (C-6), 69.2 (C-2'), 69.2 (C-4), 71.4 (C-1'), 71.7 (C-2), 73.3 (C-3), 75.7 (C-5), 103.4 (C-1);

CI (NH_3), m/z 256 (M+NH_4^+,47%), 239 (M^++1,61), 180 (M^+-OCH_2CH_2OMe+NH_4^+,100), 163 (M^+-OCH_2CH_2OMe,61), 91 (M^+-$C_6H_{11}O_4$,26), 73 (M^+-$C_6H_{13}O_5$,42), 60 (M^+-$C_7H_{14}O_5$,25);

IR(cm^{-1}), 3300(br, OH), 2870(m, CH), 1410-1270(m,-OH), 1150-1040(m, C-O).

Butyl ß-D-galactopyranoside (61)

Method A, using butan-1-ol, yielded butyl ß-D-galactopyranoside (45 mg, 38%), m.p. 103-105°C; R_f(HPLC)=4.5min; δ_H 0.85 (3H, t, $J_{4',3'}$ 7.4 Hz, 4'-H), 1.30 (2H, m, 3'H), 1.55 (2H, m, 2'-H), 3.41-3.86 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), ≈3.6 (1H, 1"-H), 3.87 (1H, dt, $J_{1',2'}$ 6.8 Hz, $J_{1',1"}$ 9.9 Hz, 1'-H), 4.33 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 13.8 (C-4'), 19.1 (C-3'), 31.5 (C-2'), 61.5 (C-6), 69.2 (C-4), 70.9 (C-1'), 71.4 (C-2), 73.5 (C-3), 75.7 (C-5), 103.4 (C-1);

CI (NH_3), m/z 254 ($M+NH_4^+$,29%), 237 (M^++1,7), 180 (M^+-OC_4H_9+NH_4^+,28), 163 (M^+-OC_4H_9,100), 91 (M^+-$C_7H_{13}O_3$,54), 73 (M^+-$C_7H_{15}O_4$,4), 60 (M^+-$C_8H_{16}O_4$,5);

IR(cm^{-1}), 3290(br, OH), 2870(m, CH), 1410-1270(m,-OH), 1160-1050(m, C-O).

2-Methylpropyl ß-D-galactopyranoside (63)

Method A, using 2-methylpropan-1-ol, yielded 2-methylpropyl ß-D-galactopyranoside (43 mg, 36%), m.p. 118-120°C; R_f(HPLC)=4.5min; δ_H 0.826 (3H, d, $J_{3",2'}$ 6.7 Hz, 3"-H), 0.834 (3H, d, $J_{3',2'}$ 6.7 Hz, 3'-H), 1.81 (1H, m, 2'-H), 3.32-3.84 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), 3.35 (1H, dd, $J_{1",1'}$ 9.8 Hz, $J_{1",2'}$ 6.9 Hz, 1"-H), ≈3.6 (1H, 1'-H), 4.29 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 19.1 (C-3', C-3"), 28.4 (C-2'), 61.5 (C-6), 69.3 (C-4), 71.4 (C-2), 73.4 (C-3), 75.7 (C-5), 77.7 (C-1'), 103.7 (C-1);

EI, m/z 237 (M^++1,41%), 163 (M^+-OC_4H_9,69), 91 (M^+-$C_7H_{13}O_3$,57), 73 (M^+-$C_7H_{15}O_4$,23), 60 (M^+-$C_8H_{16}O_4$,100);

IR(cm^{-1}), 3300(br, OH), 2870(m, CH), 1420-1270(m,-OH), 1150-1040(m, C-O).

Pentyl β-D-galactopyranoside (64)

Method A, using pentan-1-ol, yielded pentyl β-D-galactopyranoside (30 mg, 24%); R_t(HPLC)=5.1min; δ_H = 0.8 (3H, t, $J_{5',4'}$ =7 Hz, 5'-H), 1.24 (4H, m, 3'-H + 4'-H), 1.55 (2H, m, 2'-H), 3.38-3.84 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), =3.6 (1H, 1"-H), 3.84 (1H, dt, $J_{1',1"}$ 9.8 Hz, $J_{1',2'}$ 6.9 Hz, 1'-H), 4.30 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 13.9 (C-5'), 22.4 (C-4'), 27.9 (C-3'), 29.1 (C-2'), 61.5 (C-6), 69.2 (C-4), 71.2 (C-1"), 71.3 (C-2), 73.4 (C-3), 75.6 (C-5), 103.3 (C-1);

CI (NH_3), m/z 268 ($M+NH_4^+$,33%), 251 (M^++1,22), 180 (M^+-OC_5H_{11}+NH_4^+,100), 163 (M^+-OC_5H_{11},61), 91 (M^+-$C_8H_{15}O_3$,43), 73 (M^+-$C_8H_{17}O_4$,20), 60 (M^+-$C_9H_{18}O_4$,25);

IR(cm^{-1}), 3300(br, OH), 2900(m, CH), 1410-1270(m,-OH), 1170-1040(m, C-O).

1-Ethylpropyl β-D-galactopyranoside (65)

Method A, using pentan-3-ol, yielded 1-ethylpropyl β-D-galactopyranoside (41 mg, 33%); R_t(HPLC)=4.9min; δ_H 0.816 (3H, t, $J_{3",2"}$ 7.5 Hz, 3"-H), 0.821 (3H, t, $J_{3",2"}$ 7.4 Hz, 3"-H), =1.5 (4H, m, 2'-H + 2"-H), 3.38-3.84 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), =3.6 (1H, 1'-H), 4.37 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 8.9/9.6 (C-3'/C-3"), 25.6/26.8 (C-2'/C-2"), 61.4 (C-6), 69.2 (C-4), 71.6 (C-2), 73.6 (C-3), 75.5 (C-5), 83.9 (C-1'), 102.5 (C-1);

CI (NH_3), m/z 268 ($M+NH_4^+$,25%), 251 (M^++1,41), 180 ($M^+-OC_5H_{11}+NH_4^+$,57), 163 ($M^+-OC_5H_{11}$,100), 91 ($M^+-C_8H_{15}O_3$,54), 73 ($M^+-C_8H_{17}O_4$,24), 60 ($M^+-C_9H_{18}O_4$,35);

IR(cm^{-1}), 3300(br, OH), 2880(m, CH), 1410-1280(m,-OH), 1150-1040(m, C-O).

3-Hydroxypropyl ß-D-galactopyranoside (67)

Method B, using propane-1,3-diol, yielded 3-hydroxypropyl ß-D-galactopyranoside (75 mg, 42%); R_f(HPLC)=4.8min; δ_H 1.81 (2H, m, 2'-H), 3.41-3.86 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), 3.64 (2H, t, $J_{3',2'}$ 6.4 Hz, 3'-H), ~3.7 (1H, 1"-H), 3.95 (1H, dt, $J_{1',1"}$ 10.1 Hz, $J_{1',2'}$ 6.4 Hz, 1'-H), 4.33 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 32.2 (C-2'), 59.3 (C-3'), 61.7 (C-6), 67.9 (C-1'), 69.4 (C-4), 71.5 (C-2), 73.5 (C-3), 75.8 (C-5), 103.6 (C-1);

EI, m/z 239 (M^++1,47%), 163 ($M^+-OCH_2CH_2CH_2OH$,45), 91 ($M^+-C_6H_{11}O_4$,100), 73 ($M^+-C_6H_{13}O_5$,37), 60 ($M^+-C_7H_{14}O_5$,49);

CI (NH_3), m/z 466.1881 ($M(Ac)_5+NH_4^+$);

IR(cm^{-1}), 3290(br, OH), 2870(m, CH), 1410-1270(m,-OH), 1150-1040(m, C-O).

1-Methylpropyl ß-D-galactopyranoside (68)

Method B, using racemic butan-2-ol, yielded a diastereomeric mixture of 1-methylpropyl ß-D-galactopyranoside (56 mg, 32%); R_t(HPLC)=4.8min.

CI (NH_3), m/z 254 ($M+NH_4^+$,100%), 237 (M^++1,76), 180 (M^+-OC_4H_9+NH_4^+,22), 163 (M^+-OC_4H_9,18), 91 (M^+-$C_7H_{13}O_3$,24), 73 (M^+-$C_7H_{15}O_4$,76), 60 (M^+-$C_8H_{16}O_4$,47);

CI (NH_3), m/z 422.2050 ($M(Ac)_4+NH_4^+$);

IR(cm^{-1}), 3300(br, OH), 2860(m, CH), 1400-1270(m,-OH), 1140-1040(m, C-O).

(*R*)-1-Methylpropyl ß-D-galactopyranoside (68)

Method B, using (*R*)-butan-2-ol, yielded (*R*)-1-methylpropyl ß-D-galactopyranoside (57 mg, 32%), m.p. 118-121°C; R_t(HPLC)=4.8min; δ_H 0.83 (3H, t, $J_{3'2'}$ 7.5 Hz, 3'-H), 1.12 (3H, d, $J_{2''2'}$ 6.2 Hz, 2''-H), 1.44 (1H, m, 2''-H), 1.58 (1H, m, 2'-H), 3.38-3.87 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), 3.83 (1H, qt, $J_{1'2'}$ 6.3 Hz, $J_{1'2''}$ 6.3 Hz, 1'-H), 4.40 (1H, d, $J_{1,2}$ 8.0 Hz, 1-H);

δ_c 9.9 (C-3''), 19.1 (C-2''), 30.0 (C-2'), 61.7 (C-6), 69.4 (C-4), 71.6 (C-2), 73.8 (C-3), 75.8 (C-5), 78.6 (C-1'), 101.7 (C-1);

EI, m/z 237 (M^++1,7%), 163 (M^+-OC_4H_9,6), 91 (M^+-$C_7H_{13}O_3$,100), 73 (M^+-$C_7H_{15}O_4$,71), 60 (M^+-$C_8H_{16}O_4$,27);

CI (NH_3), m/z 254 ($M+NH_4^+$,15%), 237 (M^++1,39), 180 ($M^+-OC_4H_9+NH_4^+$,16), 163 ($M^+-OC_4H_9$,73), 91 ($M^+-C_7H_{13}O_3$,17), 73 ($M^+-C_7H_{15}O_4$,43), 60 ($M^+-C_8H_{16}O_4$,100);

CI (NH_3), m/z 422.2050 ($M(Ac)_4+NH_4^+$);

IR(cm^{-1}), 3300(br, OH), 2860(m, CH), 1410-1270(m,-OH), 1140-1040(m, C-O).

NMR data for (*S*)-1-Methylpropyl ß-D-galactopyranoside (69)

δ_H 0.83 (3H, t, $J_{3',2'}$ 7.4 Hz, 3'H), 1.16 (3H, d, $J_{2'',1'}$ 6.3 Hz, 2''-H), ≈1.4 (1H, 2''-H), ≈1.6 (1H, 2'-H), 3.38-3.86 (ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), ≈3.8 (1H, 1'-H), 4.40 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 9.5 (C-3'), 20.7 (C-2''), 29.1 (C-2'), 61.7 (C-6), 69.4 (C-4), 71.8 (C-2), 73.7 (C-3), 75.8 (C-5), 79.4 (C-1'), 102.8 (C-1).

1-Methylbutyl ß-D-galactopyranoside (70),(71)

Method A, using racemic pentan-2-ol, yielded a diastereomeric mixture of 1-methylbutyl ß-D-galactopyranoside (36 mg, 29%); R_t(HPLC)=5.2min; δ_H 0.82 (6H, t, $J_{4',3'}$ 7.3 Hz, 4'-H), 1.11 (3H, d, $J_{2'',1'}$ 6.1 Hz, 2''-H), 1.16 (3H, d, $J_{2'',1}$ 6.3 Hz, 2''-H), 1.29 (4H, 3'-H), 1.38 (2H, 2''-H), 1.51 (2H, 2'-H), 3.36-3.84 (12H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), ≈3.9 (2H, 1'-H), 4.38 (2H, d, $J_{1,2}$ 7.9 Hz, 1-H);

CI (NH_3), m/z 268 ($M+NH_4^+$,41%), 251 (M^++1,40), 180 (M^+-OC_5H_{11}+NH_4^+,81), 163 (M^+-OC_5H_{11},69), 91 (M^+-$C_8H_{15}O_3$,100), 73 (M^+-$C_8H_{17}O_4$,29), 60 (M^+-$C_9H_{18}O_4$,48);

IR(cm^{-1}), 3290(br, OH), 2870(m, CH), 1410-1270(m,-OH), 1150-1040(m, C-O).

δ_C Assignments*

(*R*)-1-Methylbutyl ß-D-galactopyranoside (70); δ_C 13.9/14.0 (C-4'), 18.4/18.7 (C-3'), 19.4 (C-2"), 39.0 (C-2'), 61.4 (C-6), 69.2 (C-4), 71.4/71.6 (C-2), 73.5/73.6 (C-3), 75.6 (C-5), 76.8 (C-1'), 101.4 (C-1).

(*S*)-1-Methylbutyl ß-D-galactopyranoside (71); δ_C 13.9/14.0 (C-4'), 18.4/18.7 (C-3'), 21.1 (C-2"), 38.4 (C-2'), 61.4 (C-6), 69.2 (C-4), 71.4/71.6 (C-2), 73.5/73.6 (C-3), 75.6 (C-5), 78.1 (C-1'), 102.7 (C-1).

* Assignments based on studies by Kasai *et al.*[208] and comparisons with 1-methylpropyl ß-D-galactopyranoside.

2,2-Dimethyl-1,3-dioxolane-4-methyl ß-D-galactopyranoside (72),(73)

Method B, using racemic 2,2-dimethyl-1,3-dioxolane-4-methanol, yielded a diastereomeric mixture of 2,2-dimethyl-1,3-dioxolane-4-methyl ß-D-galactopyranoside (103 mg, 47%); R_f(HPLC)=4.7min.

EI, m/z 295 (M^++1,54%), 163 (M^+-$C_6H_{11}O_3$,100), 91 (M^+-$C_6H_{15}O_5$,19), 73 (M^+-$C_6H_{17}O_6$,26), 60 (M^+-$C_7H_{18}O_6$,87);

IR(cm^{-1}), 3280(br, OH), 2850(m, CH), 1400-1270(m,-OH), 1140-1040(m, C-O).

(*S*)-2,2-Dimethyl-1,3-dioxolane-4-methyl ß-D-galactopyranoside (72)

Method B, using (*S*)-2,2-dimethyl-1,3-dioxolane-4-methanol, yielded (*S*)-2,2-dimethyl-1,3-dioxolane-4-methyl ß-D-galactopyranoside (103 mg, 47%); R_f(HPLC)=4.7min; δ_H 1.33 (3H, s 5'-H), 1.40 (3H, s, 5"-H), 3.43-3.85 (6H, sugar ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), =3.7 (1H, 1"-H), 3.77 (1H, dd, $J_{3",3'}$ 8.7 Hz, $J_{3",2'}$ 6.8 Hz, 3"-H), 3.88 (1H, dd, $J_{1',1"}$ 11.1 Hz, $J_{1',2'}$ 6.5 Hz, 1'-H), 4.10 (1H, dd, $J_{3',3"}$ 8.7 Hz, $J_{3',2'}$ 6.4 Hz, 3'-H), 4.34 (1H, d, $J_{1,2}$ 7.8 Hz 1-H), 4.40 (1H, m, 2'-H);

δ_C 25.0/26.4 (26.4 (C-5', C-5"), 61.7 (C-6), 66.3 (C-3'), 69.4 (C-4), 70.6 (C-1"), 71.5 (C-2), 73.5 (C-3), 75.1 (C-2"), 75.9 (C-5), 103.8 (C-1), 110.8 (C-4');

EI, m/z 295 (M^++1,32%), 163 (M^+-$C_6H_{11}O_3$,100), 91 (M^+-$C_6H_{15}O_5$,65), 73 (M^+-$C_6H_{17}O_6$,43), 60 (M^+-$C_7H_{18}O_6$,54);

IR(cm^{-1}), 3300(br, OH), 2870(m, CH), 1410-1270(m,-OH), 1150-1040(m, C-O).

NMR Data for (*R*)-2,2-Dimethyl-1,3-dioxolane-4-methyl ß-D-galactopyranoside (73)

δ_H 1.33 (3H, s, 5'-H), 1.40 (3H, s, 5"-H), 3.44-3.85 (6H, sugar ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), ≈3.7 (1H, 1"-H), 3.77 (1H, 3"-H), 3.96 (1H, dd, $J_{1',1"}$ 10.9 Hz, $J_{1',2'}$ 3.8 Hz, 1'-H), 4.10 (1H, dd, $J_{3',3"}$ 8.7 Hz, $J_{3',2'}$ 6.4 Hz, 3'-H), 4.34 (1H, d, $J_{1,2}$ 7.8 Hz, 1-H), ≈4.4 (1H, m, 2'-H);

δ_C 25.0/26.4 (C-5', C-5"), 61.7 (C-6), 66.3 (C-3'), 69.4 (C-4), 71.2 (C-1'), 71.6 (C-2), 73.4 (C-3), 75.4 (C-2'), 75.9 (C-5), 103.9 (C-1), 110.9 (C-4').

ß-D-Galactosides of Propane-1,2-diol (74),(75),(76),(77)

Method B, using racemic propane-1,2-diol, yielded a mixture of four ß-D-galactosides (82 mg, 46%); R_t(HPLC)=3.7min.

CI (NH_3), m/z 256 ($M+NH_4^+$,100%), 239 (M^++1,80), 180 ($M^+-C_3H_7O_2+NH_4^+$,21), 163 ($M^+-C_3H_7O_2$,65), 91 ($M^+-C_6H_{11}O_4$,30), 73 ($M^+-C_6H_{13}O_5$,63), 60 ($M^+-C_7H_{14}O_5$,44);

CI (NH_3), 466.1920 ($M(Ac)_5+NH_4^+$).

IR(cm^{-1}), 3300(br, OH), 2860(m, CH), 1400-1270(m,-OH), 1150-1040(m, C-O).

ß-D-Galactosides of (*S*)-Propane-1,2-diol (74),(75)

Method B, using (*S*)-propane-1,2-diol, yielded a mixture of two ß-D-galactosides (91 mg, 51%); R_t(HPLC)=3.6min.

CI (NH_3), m/z 256 ($M+NH_4^+$,79%), 239 (M^++1,87), 180 (M^+-$C_3H_7O_2$+NH_4^+,65), 163 (M^+-$C_3H_7O_2$,32), 91 (M^+-$C_6H_{11}O_4$,19), 73 (M^+-$C_6H_{13}O_5$,100), 60 (M^+-$C_7H_{14}O_5$,47);

CI (NH_3), 466.1916 ($M(Ac)_5+NH_4^+$);

IR(cm^{-1}), 3280(br, OH), 2860(m, CH), 1400-1270(m,-OH), 1150-1040(m, C-O).

NMR Data for (*S*)-2-hydroxypropyl ß-D-galactopyranoside (74)

δ_H 1.09 (3H, d, $J_{3',2'}$ 6.5 Hz, 3'-H), 3.41-3.84 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), 3.43 (1H, dd, $J_{1'',1'}$10.7 Hz, $J_{1'',2'}$ 7.8 Hz, 1''-H), 3.84 (1H, dd, $J_{1',1''}$ 10.7 Hz, $J_{1',2'}$ 3.1 Hz, 1'-H), ≈3.9 (1H, m, 2'-H), 4.34 (1H, d, $J_{1,2}$ 7.8 Hz, 1-H);

δ_C 18.8 (C-3'), 61.7 (C-6), 67.4 (C-2''), 69.4 (C-4), 71.7 (C-2), 73.5 (C-3), 75.9 (C-1''), 75.9 (C-5), 104.1 (C-1).

NMR Data for (*S*)-1-methyl-2-hydroxyethyl ß-D-galactopyranoside (75)

δ_H 1.16 (3H, d, $J_{2''',1'}$ 6.5 Hz, 2'''-H), 3.41-3.84 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), ≈3.5 (1H, 2''-H), 3.84 (1H, 2'-H), ≈3.9 (1H, m, 1'-H), 4.45 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 17.8 (C-2"), 61.7 (C-6), 65.6 (C-2'), 69.4 (C-4), 71.8 (C-2), 73.5 (C-3), 75.9 (C-5), 78.1 (C-1'), 103.2 (C-1).

NMR Data for (*R*)-2-hydroxypropyl ß-D-galactopyranoside (76)

δ_H 1.10 (3H, d, $J_{3',2'}$ 6.5 Hz, 3'-H), 3.42-3.88 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), =3.5 (1H, 2"-H), =3.85 (1H, 2'-H), =3.9 (1H, 1'-H), 4.35 (1H, d, $J_{1,2}$ 7.8 Hz, 1-H);

δ_C 18.8 (C-3'), 61.7 (C-6), 67.1 (C-2'), 69.4 (C-4), 71.6 (C-2), 73.5 (C-3), 75.6 (C-1'), 75.9 (C-5), 103.7 (C-1).

NMR Data for (*R*)-1-methyl-2-hydroxyethyl ß-D-galactopyranoside (77)

δ_H 1.12 (3H, d, $J_{2'',1'}$ 6.7 Hz, 2"'-H), 3.42-3.88 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), 4.41 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H).

δ_C 16.3 (C-2"'), 61.8 (C-6), 66.1 (C-2'), 69.4 (C-4), 71.6 (C-2), 73.6 (C-3), 75.9 (C-5), 77.2 (C-1'), 101.9 (C-1).

ß-D-Galactosides of Butane-1,3-diol (78),(79),(80),(81)

Method B, using racemic butane-1,3-diol, yielded a mixture of four ß-D-galactosidases (116 mg, 61%); R_t(HPLC)=4.4min.

CI (NH_3), m/z 270 ($M+NH_4^+$,100%), 253 (M^++1,32), 180 (M^+-$C_4H_9O_2$+NH_4^+,54), 163 (M^+-$C_4H_9O_2$,33), 91 (M^+-$C_7H_{13}O_4$,89),

73 (M^+-$C_7H_{15}O_5$,10), 60 (M^+-$C_8H_{16}O_5$,49);

CI (NH_3), m/z 480.2077 ($M(Ac)_5 + NH_4^+$);

IR(cm^{-1}), 3300(br, OH), 2870(m, CH), 1410-1270(m,-OH), 1150-1040(m, C-O).

<u>β-D-Galactosides of (*S*)-Butane-1,3-diol (78),(79)</u>

Method B, using (*S*)-butane-1,3-diol, yielded a mixture of two β-D-galactosides (119 mg, 63%); R_t(HPLC)=4.4min.

CI (NH_3), m/z 270 ($M+NH_4^+$,100%), 253 (M^++1,49), 180 (M^+-$C_4H_9O_2$+NH_4^+,32), 163 (M^+-$C_4H_9O_2$,43), 91 (M^+-$C_7H_{13}O_4$,87), 73 (M^+-$C_7H_{15}O_5$,57), 60 (M^+-$C_8H_{16}O_5$,41);

CI (NH_3), m/z 480.2085 ($M(Ac)_5 + NH_4^+$);

IR(cm^{-1}), 3290(br, OH), 2870(m, CH), 1410-1270(m,-OH), 1150-1040(m, C-O).

<u>NMR Data for (*S*)-3-hydroxybutyl β-D-galactopyranoside (78)</u>

δ_H 1.14 (3H, d, $J_{4',3'}$ 6.1 Hz, 4'-H), 1.72 (2H, m, 2'-H), 3.41-3.86 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), ≈3.7 (1H, 1"-H), ≈3.9 (1H, 1'-H), ≈3.9 (1H, 3'-H), 4.34 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 23.0 (C-4'), 38.5 (C-2'), 61.7 (C-6), 65.8 (C-3'), 68.1 (C-1'), 69.4 (C-4), 71.6 (C-2), 73.6 (C-3), 75.8 (C-5), 103.7 (C-1).

NMR Data for (*S*)-1-methyl-3-hydroxypropyl ß-D-galactopyranoside (79)

δ_H 1.13 (3H, d, $J_{2'',1''}$ 6.3 Hz, 2''-H), 1.66 (2H, m, 2'-H), 3.41-3.86 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), ≈3.6 (2H, 3'-H), ≈3.9 (1H, 1'-H), 4.40 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 21.7 (C-2''), 38.9 (C-2'), 59.2 (C-3'), 61.7 (C-6), 69.4 (C-4), 71.6 (C-2), 73.6 (C-3), 75.8 (C-5), 76.1 (C-1'), 103.3 (C-1).

NMR Data for (*R*)-3-hydroxybutyl ß-D-galactopyranoside (80)

δ_H 1.14 (3H, d, $J_{4'',3''}$ 6.3 Hz, 4''-H), ≈1.72 (2H, m, 2'-H) 3.41-3.86 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), ≈3.7 (1H, 1''-H), ≈3.9 (1H, 1'-H), ≈3.9 (1H, 3''-H), 4.33 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 22.9 (C-4''), 38.4 (C-2'), 61.7 (C-6), 65.8 (C-3''), 68.1 (C-1'), 69.4 (C-4), 71.6 (C-2), 73.6 (C-3), 75.8 (C-5), 103.6 (C-1).

NMR Data for (*R*)-1-methyl-3-hydroxypropyl ß-D-galactopyranoside (81)

δ_H 1.17 (3H. d, $J_{2'',1}$ 6.3 Hz, 2''-H), ≈1.66 (2H, m, 2'-H), 3.41-3.86 (6H, ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), ≈3.6 (2H, 3'-H), ≈ 3.9 (1H, 1'-H), 4.40 (1H, d, $J_{1,2}$ 7.9 Hz, 1-H);

δ_C 20.0 (C-2''), 39.4 (C-2'), 59.2 (C-3'), 61.8 (C-6), 69.4 (C-4), 71.8 (C-2), 73.7 (C-3), 75.8 (C-1'), 75.8 (C-5), 101.8 (C-1).

Transfer to a *meso*-diol

cis-2-Hydroxycyclohexa-3,5-dienyl ß-D-galactopyranoside (85),(86)

Lactose (0.54 g, 1.5 mmol) and *cis*-cyclohexa-3,5-diene-1,2-diol (0.67 g, 6 mmol) were dissolved on 0.1 M phosphate buffer (pH 7.3, 5 cm^3). ß-D-Galactosidase from *E. coli* (Sigma, Grade X, 100 units) was added to the stirred mixture at t=0 and t=3 h (200 units total). The mixture was incubated at 25°C for 48 h with the levels of lactose and products being monitored by HPLC. Excess of the diol acceptor was removed by extraction into ethyl acetate and the product galactosides were isolated as a diastereomeric mixture by HPLC (standard conditions) or preparative TLC (using UV for visualisation). The galactoside mixture was separated into the component diastereomers by further HPLC purification using the same column but with the solvent mixture MeCN:H_2O, 90:10 v/v. This process gave rise to a major isomer of longer retention time (87 mg, 21%), m.p. 125-129°C and a minor isomer of shorter retention time (54 mg, 13%), m.p. 134-136°C.

The minor isomer was identified as (1*S*,2*R*)-2-hydroxycyclohexa-3,5-dienyl ß-D-galactopyranoside (85); R_f(HPLC)=4.6min; δ_H 3.46-3.85 (6H, sugar ring protons 2-H, 3-H, 5-H, 6A-H, 6B-H, 4-H), 4.33 (1H, dd, $J_{2',1'}$ 5.1 Hz, $J_{2',3'}$ 0.9 Hz, 2'-H), 4.42 (1H, dd, $J_{1',2'}$ 5.0 Hz, $J_{1',6'}$ 1.2 Hz, 1'-H), 4.47 (1H, d, $J_{1,2}$ 7.8 Hz, 1-H), 5.94 (2H, m, 3'-H + 6'-H), 6.02 (2H, m, 4'-H + 5'-H);

δ_C 61.6 (C-6), 66.3 (C-2'), 69.3 (C-4), 71.8 (C-2), 73.4 (C-3), 75.9 (C-5), 76.7 (C-1''), 103.4 (C-1), 126.1/126.3/127.9/129.1 (C-3', C-4', C-5', C-6');

CI (NH_3), m/z 292 ($M+NH_4^+$,100%), 275 (M^++1,57), 180 ($M^+-C_6H_7O_2+NH_4^+$,65), 163 ($M^+-C_6H_7O_2$,19), 91 ($M^+-C_9H_{11}O_4$,65), 73 ($M^+-C_9H_{13}O_5$,43), 60 ($M^+-C_{10}H_{14}O_5$,23);

CI (NH_3), m/z 502.1925 ($M(Ac)_5+NH_4^+$).

IR(cm^{-1}), 3300(br, OH), 3000(s, C=C), 2860(m, CH), 1640(s, C=C), 1600(s, C=C), 1400-1270(m,-OH), 1140-1040(m, C-O).

The major isomer was identified as (1*R*,2*S*)-2-hydroxycyclohexa-3,5-dienyl ß-D-galactopyranoside (86); R_f(HPLC)=4.9min; δ_H 3.46-3.85 (6H, sugar ring protons 2-H, 3-H, 5-H, 6A-H, 6B, 4-H), 4.29 (1H, dd, $J_{2',1'}$ 5.3 Hz, $J_{2'3'}$ 0.9 Hz, 2'-H), 4.45 (1H, m, 1'-H), 4.48 (1H, d, $J_{1,2}$ 7.7 Hz, 1-H), 5.94 (2H, m, 3'-H + 6'-H), 6.04 (2H, m, 4'-H + 5'-H);

δ_C 61.7 (C-6), 66.4 (C-2'), 69.4 (C-4), 71.5 (C-2), 73.4 (C-3), 75.8 (C-5), 76.8 (C-1'), 102.6 (C-1), 126.2/126.5/127.3/128.5 (C-3', C-4', C-5', C-6');

CI (NH_3), m/z 292 ($M+NH_4^+$,78%), 275 (M^++1,100), 180 ($M^+-C_6H_7O_2+NH_4^+$,34), 163 ($M^+-C_6H_7O_2$,23), 91 ($M^+-C_9H_{11}O_4$,45), 73 ($M^+-C_9H_{13}O_5$,87), 60 ($M^+-C_{10}H_{14}O_5$,71);

CI (NH_3), m/z 502.1925 ($M(Ac)_5+NH_4^+$);

IR(cm^{-1}), 3300(br, OH), 3000(s, C=C), 2860(m, CH), 1640(s, C=C), 1600(s, C=C), 1400-1270(m,-OH), 1140-1040(m, C-O).

Transfer to disaccharide acceptors

α-D-Galactopyranosyl-(1-6)-β-D-galactopyranosyl-(1-4)-glucopyranose (Galα(1-6)Galβ(1-4)Glc) (87)

o-Nitrophenyl α-D-galactopyranoside (0.031 g, 0.1 mmol) and lactose (0.144 g, 0.4 mmol) were dissolved in 0.1 M phosphate buffer (pH 6.0, 2 cm^3). α-D-Galactosidase from from *Mortierella vinacea* (Hokkaido Sugar Co., Ltd., 2 units) was added and the mixture incubated at 40°C for 24 h. Levels of trisaccharides were monitored by HPLC using the solvent mixture MeCN:H_2O, 73:27 v/v; a single trisaccharide peak was evident and the same HPLC conditions were used to isolate the product (21 mg, 42%); R_t(HPLC)=17min; δ_H 4.42 (1H, d, $J_{7,8}$ 7.8 Hz, 7-H), 4.61 (=1/2 H, d, $J_{1,2}$ 8.0 Hz, β-1-H), 4.94 (1H, d, $J_{13,14}$ 3.9 Hz, 13-H), 5.17 (=1/2 H, d, $J_{1,2}$ 3.7 Hz, α-1-H);

δ_C 60.9 (αC-6), 61.0 (βC-6), 62.0 (C-18), 67.2 (C-12), 69.2/69.4/70.1/71.6/71.9/73.3/73.9 (C-8, C-9, C-10, C-11), 69.2/69.4 (C-14), 70.1 (C-15), 70.1 (C-16), 70.7 (αC-2), 75.2 (βC-3), 75.4 (β-C-5), 80.1 (βC-4), 80.3 (αC-4), 92.6 (αC-1), 96.5 (βC-1), 99.1 (C-13), 103.9 (C-7);

CI (NH_3), m/z 522 ($M+NH_4^+$,29%), 505 (M^++1,100), 343 (M^+-$C_6H_{11}O_6$+NH_4^+,55), 325 (M^+-$C_6H_{11}O_6$,34), 180 (M^+-$C_{12}H_{21}O_{11}$+NH_4^+,15), 163 (M^+-$C_{12}H_{21}O_{11}$,57);

IR(cm^{-1}), 3270(br, OH), 2870(m, CH), 1400-1270(m,-OH), 1150-1040(m, C-O).

α-D-Galactopyranosyl-(1-6)-β-D-glucopyranosyl-(1-4)-glucopyranose (Galα(1-6)Glcβ(1-4)Glc) (88)

o-Nitrophenyl α-D-galactopyranoside (0.031 g, 0.1 mmol) and cellobiose (0.137 g, 0.4 mmol) were dissolved in 0.1 M phosphate buffer (pH 6.0, 2cm^3). α-D-Galactosidase from *Mortierella vinacea* (Hokkaido Sugar Co., Ltd., 2 units) was added and the mixture incubated at 40°C for 24 h. Levels of trisaccharide were monitored by HPLC using the solvent mixture MeCN:H_2O, 73:27 v/v; a single trisaccharide product was evident and the same HPLC conditions were used to isolate the product (12 mg, 24%); R_t(HPLC)=19min; δ_H 4.46 (1H, d, $J_{7,8}$ 8.0 Hz, 7-H), 4.58 (≈2/3 H, d, $J_{1,2}$ 8.0 Hz, β-1-OH), 4.93 (1H, d, $J_{13,14}$ 3.8 Hz, 13-H), 5.14 (≈1/3 H, d, $J_{1,2}$ 3.8 Hz, α-1-OH);

δ_C 66.7 (C-12), 92.5 (αC-1), 96.3 (βC-1), 98.9 (C-13), 103.4 (C-7);

CI (NH_3), m/z 522 (M+NH_4^+,57%), 505 (M^++1,44), 343 (M^+-$C_6H_{11}O_6$+NH_4^+,100), 325 (M^+-$C_6H_{11}O_6$,67), 180 (M^+-$C_{12}H_{21}O_{11}$+NH_4^+,33), 163 (M^+-$C_{12}H_{21}O_{11}$,96);

IR(cm^{-1}), 3280(br, OH), 2870(m, CH), 1410-1270(m,-OH), 1150-1040(m, C-O).

CHAPTER SIX

Experimental for Chapter 4.

After polymerisation all gels were broken up into small particles and washed copiously with water to remove residual monomers and initiator fragments. Swelling measurements were then made as follows; the gel was allowed to equilibrate in the swelling medium and then portions (>1 g) were pressed between absorbent tissues to remove excess swelling medium. The gel was then weighed, dried overnight in an oven at 105°C, and reweighed. The swelling was then calculated using the equation derived in Appendix 3. Gels could be stored by exposing them to gradually increasing concentrations of acetone (up to 100%), filtering and drying under vacuum. The hard granules formed in this way could be easily hydrated to gels by immersion in an aqueous medium.

Enzymatic solutions were assayed at 37°C by measuring the increase in absorption at 410 nm of a solution of *o*-nitrophenyl ß-D-galactopyranoside. For this purpose the following reagents were pipetted into a 3 cm^3 cuvette (1 cm light path):

Reagents
- A 0.1 M Sodium phosphate buffer (pH according to the pH maximum of the enzyme, 2.7 cm^3).
- B 0.03 M $MgCl_2$ (0.1 cm^3).
- C Enzyme solution (0.1 cm^3).
- D 0.068 M *o*-Nitrophenyl ß-D-galactopyranoside in Reagent A (0.1 cm^3).

The variation of A_{410} nm at 37°C was measured as a function of time using a thermostatted spectrophotometer with Reagent A as reference.

Poly(*N*-isopropylacrylamide)

N-Isopropylacrylamide (2 g, 17.7 mmol) and N,N'-methylenebisacrylamide (0.0273 g, 0.177 mmol) were dissolved in distilled water (28.6 cm^3). Ammonium persulphate (0.2 g, 0.88 mmol) was added and after dissolution the mixture was purged with argon for ten minutes. *N,N,N',N'*-Tetramethylethylenediamine (0.154 g, 0.2 cm^3, 1.3 mmol.) was added and the mixture rapidly stirred. After one hour the resultant polymer was washed with distilled water and swelling measurements made in distilled water at 20°C and 50°C.

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N-Acryloyl-3-aminophenylboronic acid[242,243]

Sodium hydrogen carbonate (8.4 g, 0.1 mol) was added slowly to a stirred solution of 3-aminophenylboronic acid hemisulphate (9.3 g, 0.05 mol) in distilled water (350 cm^3). The resulting precipitate was dissolved by warming and the solution cooled to 10°C. Acryloyl chloride (4.98 g, 4.5 cm^3, 0.055 mol) was added slowly over a 20 minute period with the temperature being maintained at 10-15°C. The mixture was stored at 4°C overnight, filtered under suction and the precipitate was washed with ice-cold water. Recrystallisation from water gave a fawn solid (7.48 g, 79%), mp. 256-260°C; δ_H 5.82 (1H, dd, CH_2=C*H*), =6.3 (2H, m, CH_2=CH), 7.38 (1H, t, 5H), 7.52 (2H, d, 4-H + 6-H), 7.67 (1H, s, 2-H);

δ_c 124.8/127.1/131.0/131.3 (C-2,4,5,6), 129.0 (CH=CH_2), 129.4 (*C*H=CH_2), 137.0 (C-3), 167.4 (CO).

IR(cm^{-1}), 3500(OH, br), 3300(NH, s), 3260(NH, s), 1670(CO, s), 1620(C=C, s), 1510(aryl-H, s).

50:50 Acrylamide/acrylic acid polymer

Acrylamide (0.621 g, 8.74 mmol), acrylic acid (0.63 g, 0.6 cm^3, 8.74 mmol) and *N*,*N'*-methylenebisacrylamide (0.15 g, 0.97 mmol) were dissolved in aqueous solution of disodium orthophosphate (0.1 M Na_2HPO_4, natural pH *ca*.8.9, 30 cm^3). Sodium hydrogen carbonate (0.77 g, 9.17 mmol) was added slowly and after the effervescence had ceased a solution of 4,4'-azobis(4-cyanopentanoic acid) (0.0196 g, 0.07 mmol) and sodium carbonate (0.076 g, 0.072 mmol) in aqueous disodium orthophosphate (0.1 M Na_2HPO_4, natural pH ≈8.9, 0.4 cm^3) was added. The mixture was purged with argon for ten minutes in a sealed tube and then polymerisation allowed to take place by gently shaking in a water bath at 80°C for one hour. After washing with water, the gel was stored as described above.

This method was also employed to form two other 50:50 acrylamide/acrylic acid polymers but with the additional ingredients of D-glucose (0.589 g, 3.27 mmol) and L-arabinose (0.491 g, 3.27 mmol).

35:15:50 Acrylamide/*N*-acryloyl-3-aminobenzeneboronic acid/acrylic acid polymer

Acrylamide (0.528 g, 7.43 mmol), *N*-acryloyl-3-aminophenylboronic acid (0.25 g, 1.31 mmol), acrylic acid (0.63 g, 0.6 cm^3, 8.74 mmol) and *N*,*N'*-methylenebisacrylamide (0.15 g, 0.97 mmol) were copolymerised as described above in the preparation of the 50:50 acrylamide/acrylic acid polymer.

35:15:50 Acrylamide/*N*-acryloyl-3-aminophenylboronic acid/acrylic acid polymers imprinted with D-glucose and L-arabinose

The above procedure for the preparation of a 35:15:50 acrylamide/*N*-acryloyl-3-aminophenylboronic acid/acrylic acid polymer was used for the preparation of imprinted boronate polymers by the addition of D-glucose (0.589 g, 3.27 mmol) and L-arabinose (0.491 g, 3.27 mmol) to the monomer mixture.

Measurements of the rate of leaching of monosaccharides from acrylamide polymers

Each polymer was divided approximately in half and the portions stirred for a few minutes with 0.1 M sodium phosphate buffer (20 cm^3) at either *ca.*pH 4 (natural pH of NaH_2PO_4) or ~pH 8.9 (natural pH of Na_2HPO_4) with washings being collected by filtration. After six successive washings, the polymers were washed twice with the reversed buffer, *i.e.*, a polymer which had been washed with buffer at ~pH 4 was now washed with buffer at *ca.*pH 8.9, and *vice versa*.

The concentration of the monosaccharides in the filtrates were determined by comparison with a standard curve obtained as follows: a solution of phenol in water (40 $mgcm^{-3}$, 1 cm^3) and the sugar solution (5-200 $\mu g cm^{-3}$, 1 cm^3) were pipetted into a test-tube. Concentrated sulphuric acid (5 cm^3) was added rapidly and the solution mixed by inversion. After cooling the absorbance at 485 nm was measured; a reference was prepared by substituting distilled water for the sugar solution.

Permeation of gel by enzymes

The hydrated gel (*ca*.0.5 g) was allowed to swell and equilibrate in a solution of the enzyme (5-10 units cm^{-3}) in 0.1 M phosphate buffer (pH according to the pH maximum of the enzyme). The volume of the enzyme solution used was one and a half times the volume which the gel would normally absorb ie. mass of gel x swelling x 1.5 cm^3. A measure of the permeation of the gel by the enzyme was determined by assaying the original enzyme solution and comparing it with the values obtained for the supernatant enzyme solution.

Separation of enantiomers by acrylamide polymers

The fully-washed polymer (~0.5 g) was allowed to swell in 0.1 M sodium phosphate buffer (~pH 9) and placed in a chromatography column (3 cm x 30 cm). The same buffer solution was passed through the gel until monosaccharide could no longer be detected (phenol-sulphuric acid method) in the washings; the column was then run 'dry'. A solution of the appropriate D-monosaccharide (50 mg) and L-monosaccharide (50 mg) in buffer (10 cm^3) was introduced into the column and allowed to pass through the gel slowly. Fractions of 2 cm^3 were collected and their optical rotations were measured.

Fraction	Rotation / degrees
Original solution	0.000
1	0.002
2	-0.005
3	-0.003
4	-0.030
5	-0.047
6	-0.034
7	-0.018
8	-0.026

Appendix 1

Derivation and meaning of K_M

Most enzymes function in accordance to the following equation:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} \text{products} + E \qquad (1)$$

for which the following symbols may be used:

[E] = total concentration of enzyme

[S] = total concentration of substrate

[ES] = concentration of enzyme-substrate complex

[E]-[ES] = concentration of free enzyme

and k_1, k_2, k_3 represent the rate constants for the reactions.

For the rate of formation of ES one obtains:

$$\frac{d[ES]}{dt} = k_1([E] - [ES])[S] \qquad (2)$$

since the rate of formation of ES is proportional to the concentration of substrate and free enzyme.

The rate of disappearance of ES is given by:

$$-\frac{d[ES]}{dt} = k_2[ES] + k_3[ES] \qquad (3)$$

Thus in the steady state:

$$k_1([E] - [ES])[S] = k_2[ES] + k_3[ES] \qquad (4)$$

which on rearrangement gives:

$$\frac{[S]([E] - [ES])}{[ES]} = \frac{k_2 + k_3}{k_1} = K_M \qquad (5)$$

Solving for [ES] one obtains an equation for the steady state concentration of ES:

$$[ES] = \frac{[E][S]}{K_M + [S]} \qquad (6)$$

The observed initial rate v of the reaction is proportional to [ES].

Thus:

$$v = k_3[ES] \qquad (7)$$

When the substrate concentration is high in relation to the enzyme concentration all the enzyme is present as ES and the rate of the reaction is maximal and given by:

$$V_{max} = k_3[E] \qquad (8)$$

Substituting the value of [ES] in equation 6 for [ES] in equation 7 and dividing by equation 8 gives the Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_M + [S]} \qquad (9)$$

When $v=V_{max}/2$, $K_M=[S]$ and K_M is the substrate concentration which will give half maximum rate. For any enzyme-substrate system K_M will have a characteristic value independent of the enzyme concentration. If an enzyme acts on several substrates it will have a characteristic K_M for each substrate.

K_M is also a function of a substrate's ability to bind with a low value reflecting strong binding and *vice versa*.

Appendix 2

NMR of Carbohydrates

i). Proton NMR

^1H NMR spectroscopy is a powerful technique for the elucidation of carbohydrate structures. In the case of monosaccharides, where the problem of overlapping signals is a minor one, a study of the splitting patterns of the different signals allows determination of which protons are coupled to which. Complete assignment may be made *via* decoupling experiments which consist of the consecutive irradiation of the individual signals and observing the collapse in the splitting pattern of the signals of the adjacent protons. This is made easier by the fact that the anomeric proton is readily identified as the one resonating at the lowest field. Thus saturation of the anomeric proton signal will lead to collapse, or partial collapse, of the H-2 signal. Irradiation of the H-2 signal will then allow identification of the H-3 signal and so on around the ring. The configuration of the monosaccharide may then be determined by examination of the coupling constants which provide information about the dihedral angle between adjacent protons.

Finally, the closeness in space of particular protons may be studied using the phenomenon of nuclear Overhauser enhancement (NOE). Briefly, NOE derives from the dipolar interactions between two nuclei. Perturbation of the population of spin states of one of the nuclei (by selective irradiation) causes more efficient dipolar relaxation of the other nucleus leading to an increase in its intensity. Since the degree of enhancement is dependent on the proximity of the nuclei

this method may provide useful structural information.

A complete assignment of proton signals for methyl β-D-galactoside was given by Welti[245]. The most characteristic signal in the spectrum, and in the spectra of all β-D-galactosides, is the doublet associated with the anomeric proton (H-1) which resonates at least 0.4ppm downfield of any other ring proton. The coupling constant between H-1 and the adjoining proton H-2 is usually about 8Hz reflecting the transdiaxial relationship. The corresponding coupling constant for α-D-galactosides is 3-4Hz. Assignment of protons in the aglycon is straightforward and recourse to more complicated experiments is unnecessary in these simple cases.

ii). Carbon-13 NMR

With the advent of Fourier transform techniques the measurement of ^{13}C spectra has become routine and the information it provides is invaluable to the chemist. The spectra obtained are generally much more simple than the corresponding ^{1}H spectra because there are fewer signals spread over a wider of frequency. In their most simple forms, the spectra are of three types: i). undecoupled, where each carbon resonance is split by its attached protons, ii). off-resonance decoupled, where partial proton decoupling produces considerable simplification but without removing all of the decoupling information and iii). proton noise decoupled, where each carbon gives rise to a single resonance. In practice only the latter two are usually used and nowadays it is common to glean coupling information from DEPT (distortionless enhancement by polarisation transfer) experiments rather than

via off-resonance decoupling. DEPT experiments enable the distinction between different types of carbon atoms *i.e.* between methyl, methylene and methine carbon atoms, and have the advantage over off-resonance techniques of producing simple spectra thus eliminating the possibility of any ambiguities when determining multiplicities.

The assignment of resonances in the carbon-13 spectrum of β-D-galactosides is facilitated by the fact that the values for each ring carbon (C-2, C-3, C-4, C-5 and C-6) vary by no more than 0.2ppm irrespective of the aglycon.[202-204,208-214] The chemical shift for C-1 lies considerably downfield of all other ring carbons on account of the greater deshielding caused by attachment to two oxygen atoms rather than one. Any ambiguities in assignment of the carbon resonances in the aglycon are eliminated by DEPT135 experiments which distinguish carbon atoms bearing two protons from those bearing one or three protons.

iii). Two Dimensional NMR

Greater information than is available from standard one dimensional NMR experiments may be obtained *via* two dimensional NMR (2D NMR). 2D NMR experiments involve subtle changes in the way in which the magnetic field is applied to the sample and provide a means of separating chemical shift and coupling effects along two axes. Thus a three dimensional representation of the data may be obtained with the chemical shift on the conventional axis and the coupling information on a new axis stretching behind the conventional spectrum. Commonly, the information is displayed as a contour plot and the technique is termed

correlation spectroscopy (COSY). The 2D COSY spectrum thus consists of a set of resonances along the diagonal (corresponding to the normal one dimensional spectrum) and off-diagonal "cross peaks" which signify spin couplings between the related multiplets on the diagonal.

The most common types of COSY experiments are ^{1}H-^{1}H 2D COSY (homonuclear correlation spectroscopy) which give proton-proton coupling information and ^{1}H-^{13}C COSY (heteronuclear correlation spectroscopy) which correlate carbon resonances with proton resonances. With these and other more elegant NMR techniques it is possible to assign the spectra of relatively complex carbohydrate molecules.

Appendix 3

Calculations of values of swelling

Values of swelling are defined as the volume of aqueous medium absorbed by one gramme of fully swollen polymer. All values are approximate and are calculated as follows:

Let A = Mass of polymer + mass of salt.

B = Mass of polymer + mass of salt + mass of water.

C = Mass of polymer.

Then swelling, $S = \frac{B - C}{C}$

Now, C = A-mass of salt

=A-x(B - A),

where x = mass of salt per gramme of water

$$\therefore \quad S = \frac{B-(A - x(B - A))}{A - x(B - A)}$$

For distilled water $x=0 \Rightarrow S = \frac{B - A}{A}$

For 1 M NaCl x = 0.05844

For phosphate buffers values of x could be determined by evaporation of known volumes of the buffer.

Appendix 4

Boron analyses for polymers of increasing AAPB[a]

Samples were digested in HNO_3/H_2O_2 and analyses carried out by inductively-coupled plasma emission spectrometry. Levels of boron are given in p.p.m.

Sample[b]	% AAPB	Found	Expected
A	0	0	0
B	5	1680	3540
C	10	3610	7080
D	15	5100	10620
E	20	6960	14160
F	25	7860	17700
G	15	5230	10620

[a]0→25% with respect to acrylamide.

[b]Sample G was prepared in the presence of glucose.

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