STEREOCHEMISTRY OF OXIDATION BY D-GALACTOSE OXIDASE

by

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A thesis submitted to the Faculty of Graduate Studies and Research of McGill University in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

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Montreal, Quebec, Canada

November 1972

ABSTRACT

The stereochemistry of oxidation by D-galactose oxidase of the primary carbinol group is that in which the pro-S hydrogen is abstracted. This has been deduced from the primary kinetic deuterium isotope effect observed in the oxidation of substrates of predominantly R or S chirality conferred by stereoselective deuteration. In addition, an examination of the reaction products shows that the hydrogen (or deuterium) equivalent to the pro-S hydrogen is lost.

An investigation of the role of the substrate-specifying 4-hydroxy-group of D-galactose and derivatives, using a series of 4-deoxy analogues indicates that OH-4 is not involved in hydrogen-bonding in the enzyme-substrate complex.

The synthesis of various substrates utilized in this stereochemical study, and characterization of products of the oxidation is described.

Ph.D.

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RESUME

LA STEREOCHIMIE DE L'OXYDATION PAR LE D-GALACTOSE OXYDASE

La stéréochimie de l'oxydation du groupement carbinole primaire par le D-galactose oxydase implique le départ de l'hydrogène pro-S. Ceci résulte de l'effet cinétique initial de l'isotope deutérium observé dans l'oxydation de substances de prédominance configurationelle R ou S obtenue par deutération stéréosélective. De plus, l'analyse des produits de réaction met en évidence le départ de l'hydrogène (ou deutérium) correspondant à l'hydrogène pro-S.

A partir d'analogues de la série du déoxy-4, l'étude du rôle des produits possédant un groupe hydroxy-4, spécifique à la réaction du D-galactose et de ses dérivés, indique que le OH-4 ne présente pas une liaison hydrogène dans le complexe enzyme-substrat.

On y décrit aussi la synthèse de divers produits utilisés au cours de cette étude stéréochimique ainsi que la caractérisation de leurs produits d'oxydation.

PREFACE

The stereochemistry of enzyme reactions is not a new subject in itself, but has recently received increased attention in the wake of recent developments that permit the application of physical techniques to a wide variety of problems. A deeper understanding and appreciation of stereochemical principles has certainly also been a major stimulus of studies on the stereochemistry of biological and chemical systems.

This thesis has its roots in the powerful potential of the NMR technique for the solution of stereochemical problems. The objective here has been to define specifically what happens to the substrate, rather than how the enzyme directs the particular stereochemical course of the reaction. Hopefully, the conclusions reached in this thesis, by contributing to a more detailed knowledge of one enzyme reaction will aid in a broader understanding of the nature of enzymic transformations.

ACKNOWLEDGEMENTS

The author wishes to express his sincere thanks and appreciation to his research director, Dr. A.S. Perlin, for his consistent guidance and training during the entire research program over the last four years. His scrutiny, criticism and suggestions have resulted also in a considerable improvement of the manuscript presented here.

The post-doctoral fellows who came to work under Dr. Perlin's leadership are also thanked; in particular Dr. H.J. Koch who sacrificed his valuable time to record the crucial NMR spectra and in the analysis of complex spectra by spin decoupling and with the aid of an INDOR technique devised by him. The numerous suggestions and laboratory experience of Drs. B. Casu, G. Sanderson, F. Ng Kin, S.S. Bhattacharjee and N. Cyr, along with the technical assistance of Mr. R. Simoneau for running day to day routine NMR spectra, and that of N. Ness, C. Mercer and Miss Jan Richards in the early stages of the author's research program, deserve sincere appreciation.

Grateful acknowledgement is expressed to the Pulp and Paper Research Institute of Canada for generous financial support, especially for the 1969-70 Studentship and the 1970-72 C.B. Purves Memorial Fellowship Award. The outstanding services of the Institute's staff, both at 3420 University St. and at Pointe Claire, including that of the late Mr. S. Broadway, are very much appreciated.

The Department of Chemistry of McGill University kindly provided the 1968-69 and 1972 demonstratorship awards; Thanks are due also to various members of the Department, especially Mr. T. Striegler,

the glass blower, Mr. R. Stein, the carpenter, Victor Yu, the A60 NMR operator, and Messrs. P. Curie and B. Budd of the mass spectroscopy laboratory.

Much of the equipment used for kinetic studies was generously provided by Dr. J. Spencer of the Biochemistry Department, McGill University, and his graduate students.

Miss Elizabeth Szanto, who typed the original draft and three quarters of the present manuscript is gratefully thanked, along with Miss Gabriele von Chamier who completed the work started by Miss Szanto.

Sincere thanks are due to Mrs. C. Ewan for her day to day concern and assistance in many miscellaneous tasks, as well as to Dr. H.I. Bolker, fellow students in the Pulp and Paper Building and to friends in and around Montreal who contributed to a pleasant atmosphere during the author's entire stay in Canada.

Finally, special thanks are due to the Tanzania Government and to my parents for providing the stepping stone with which it was possible to come to McGill University.

TABLE OF CONTENTS

page

page

page

page

page

ABBREVIATIONS

The following abbreviations have been used in this thesis:

Ac	acetyl, CH_3CO-
ATP	adenosine triphosphate
Bn	benzyl, $C_6H_5CH_2-$
Brs	p-bromobenzenesulphonyl $BrC_6H_4SO_2-$
Bz	benzoyl, C_6H_5CO-
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulphoxide
FMN	flavin mononucleotide
I.R.; IR	infrared (spectrum, spectroscopy)
m.p.	melting point
NAD^+	nicotinamide-adenine dinucleotide
$NADP^+$	nicotinamide-adenine dinucleotide phosphate
NMR	nuclear magnetic resonance (spectrum or spectroscopy)
Ph	phenyl, C_6H_5-
ppm	parts per million (chemical shift, δ)
Py	pyridine
SO_3/Py	sulphur trioxide-pyridine complex
tlc	thin-layer chromatography
Triflyl	trifluoromethanesulphonyl, CF_3SO_2-
U.V.; UV	ultraviolet (spectrum, spectroscopy)

LIST OF FIGURES

Figure page

LIST OF TABLES

INTRODUCTION

General Remarks

The ease with which enzyme reactions catalyse complex reactions is an old standing fascination, and the urge to unravel the mystery of enzyme reactions has never faded.

Enzyme reactions proceed at low activation energies and for most, at temperature as low as 25-30°C. The yield of enzyme products (turn over number) and the specificity of enzyme reactions is unique. Certainly enzymes constitute the most effective chemical reagents available to man.

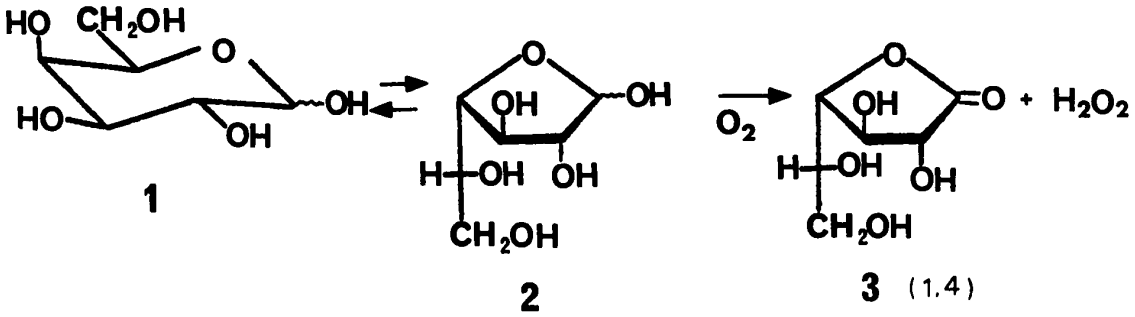
This thesis represents an attempt to provide new knowledge about one particular enzyme reaction. That is, a description of the three-dimensional spatial interaction (stereochemistry) of the enzyme D-galactose oxidase with its substrates is presented.

No attempt has been made to study the three dimensional structure of the D-galactose oxidase enzyme itself in solution or the solid state. Rather the study has been confined to the stereochemistry of substrates in solution with the assumption that the topology and stereochemistry of the active site of the enzyme will bear resemblance to that of the substrate, though in the transition state, reaction and action (Newton) will be mutual between the enzyme and the substrate (1).

INTRODUCTION

The enzyme-D-galactose oxidase

(i) Discovery - While working on D-galactose metabolising enzymes Cooper and co-workers (2) reported an enzyme from the mold Polyporous circinatus which they claimed to catalyse the conversion of D-galactose (1) to a D-galactonolactone (3) in the presence of molecular oxygen.



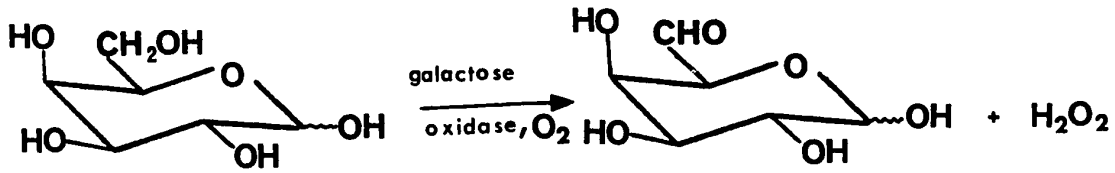
Because of the reactivity of D-galactose, and the inertness of other monosaccharides tested, this newly found enzyme was given the trivial name D-galactose oxidase. These workers were uncertain as to whether the enzyme was excreted extracellulary or had been leached out as a result of lysis of the mycelial hyphae. Horecker et al. (3,4) have suggested, however, that D-galactose oxidase is an extracellular secretion.

Although D-galactose oxidase can also achieve rapid oxidation (4) of D-galactopyranosyl residues contained in oligosaccharides such as the tetrasaccharide stachyose, or polysaccharides such as guaran, its natural function has not been described so far.

Other important features that have been noted for D-galactose oxidase are that the enzyme carries a flavin (flavin mononucleotide,

FMN) as the prosthetic group and that it is a metalloenzyme containing zinc (2,4). However, neither of these possibilities has been confirmed or refuted; in conjunction with one aspect of the present work it is assumed that D-galactose oxidase is a flavo enzyme (Chapter III).

(ii) Basic reaction - It is now certain that D-galactose oxidase from Polyporous circinatus does not catalyse conversion of D-galactose to D-galactonolactone as previously held by Cooper's group (2); instead, as shown by Horecker et al. (3,4), the enzyme specifically catalyses the aerobic oxidation of the primary carbinol of D-galactose to give an aldehyde and hydrogen peroxide.



4

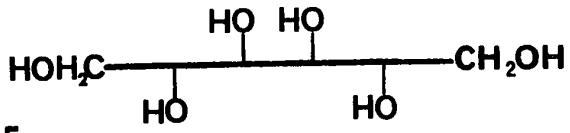
The aldehyde products isolated and characterised in this study (Chapter IV) give further support to their findings.

Under the present systematic enzyme classification, D-galactose oxidase is an O_2-oxido-reductase 1.1.3.9 (5), in that it is an oxidising enzyme acting on a -CHOH grouping, with the latter donating electrons to the molecular oxygen acceptor.

In vitro, the D-galactose oxidase reaction must be coupled to peroxidase or catalase in order to decompose the hydrogen peroxide which is produced and which inhibits the reaction (Chapter IV and V) (2-4).

(iii) Substrate specificity of D-galactose oxidase - The specificity of D-galactose oxidase for D-galactose and D-galactose derivatives unsubstituted at O-4 has stimulated the use of D-galactose oxidase for a variety of purposes. Thus the enzyme has been used to modify D-galactopyranosyl residues (6,7); to introduce tritium labelling into D-galactose oxidase-treated residues (8-10), and for systematic analysis and detection of D-galactose containing moieties (11-17). The enzyme is now commercially produced and sold as "Galactostat" (Chapter III) for medical diagnosis.

(iv) Role of OH-4 of D-galactose - That the epimer of D-galactose at C-4, D-glucose is not reactive (2-4) is a clear indication that OH-4 is critical to the enzyme reaction. The importance of OH-4 of D-galactose and its bonding characteristics to the enzyme surface is the subject of Chapter V . In addition, the investigations of Horecker's group (4) established that the D-galactopyranosyl residue is essential for neither the straight chained galactitol (5) nor D-galactofuranose (2) is reactive.



5

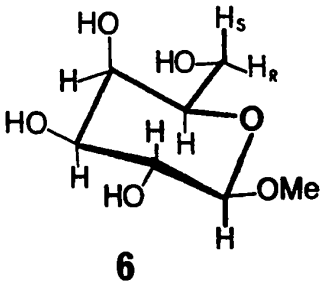
Selection of a stereochemical model for the D-galactose oxidase reaction.

The selection of a model molecule for this study was dependent upon several requirements. It was important that the substrate should react sufficiently well that reaction rate parameters could be measured easily, that substantial yields of enzyme products could be isolated

and characterised, and that the molecule be easily examined by physico-chemical techniques such as NMR, IR, UV and mass spectroscopy. It was desirable that the model substrate exist in one form. Thus D-galactose itself was not particularly well suited for the purpose because in aqueous solution it mutarotates to α and β forms. Furthermore, as has been pointed out by Perlin (18), the product of D-galactose oxidase oxidation is meso, D-galacto-dialdohexose; on equilibration, both of the terminal aldehyde groups participate in ring formation of D- and L-galacto-dialdohexose.

Methyl β-D-galactopyranoside

Methyl β-D-galactopyranoside (6) appeared to satisfy most of the above requirements. It is easily synthesized and, starting with appropriate compounds, chemical modifications can be performed on the glycon moiety. The reactivity of this substrate is three times that of D-galactose, although only half of that of stachyose (4); hence



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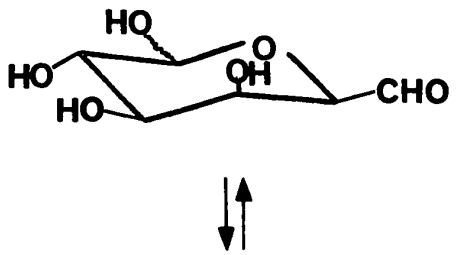
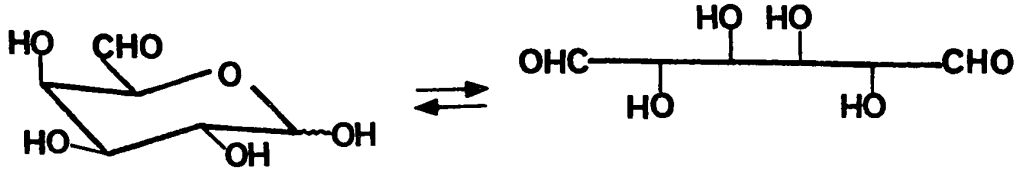




dilute substrates can be measured. Also, the methoxy-hydrogens of the aglycon, provide a ready handle for use in conjunction with NMR spectroscopy and to be retained in the identification of reaction products or in locating unreacted substrate and derivatives. Thus, throughout this project, almost all of the stereochemical aspects of D-galactose oxidase have been based on methyl β-D-galactopyranoside, and modified forms of it as substrates.

Stereochemical and topographical approach by D-galactose oxidase at the reaction centre.

The study of enzyme specificity, stereochemistry and mechanism may be simplified if a knowledge of the exact spatial arrangement of the various groups at the active site of the enzyme is available. Such information is lacking for D-galactose oxidase. An alternative method for tackling enzyme stereochemical problems is the indirect procedure of modifying substrates and observing changes in the response of the enzyme. Prelog (19) has illustrated this approach by assembling stereochemical segments of molecules which react, or do not react, with lactate dehydrogenase. In this way the geometry of the active site of the enzyme was deduced. In the present study this use of chemically-modified substrates has enabled some assessment to be made of the topological approach of D-galactose oxidase towards its substrates (Chapter V). Related methods of studying enzyme stereochemistry have been discussed (20-23).

Deuterium as a label

Topological aspects of enzyme reactions can also be studied by tagging substrate molecules with labels whose spatial arrangement is known, and by observing their fate in the course of the enzyme reaction. This is the subject of Chapter III and IV.

The discovery of a stable hydrogen isotope, deuterium (24), led to its wide application in the study of biological and chemical mechanisms. This type of application has been accelerated by the availability of reagents such as lithium aluminium deuteride ($LiAlD_4$) and sodium borodeuteride ($NaBD_4$), which reduce ketones, aldehydes, nitriles, esters, alkyl halides and a number of other functional groups.

Deuterium in a polyatomic molecule confers virtually the same physical properties as does hydrogen (protium) even though deuterium is a slightly smaller atom (25-26). However, in reactions involving cleavage of a bond containing a hydrogen atom, there is a retardation in rate when the hydrogen is substituted by deuterium. This is attributable to the low zero point energy ($1/2\ h\nu$) of deuterium as compared with hydrogen; it means that more activation energy has to be expended to loosen and break a C-D than a C-H bond. This effect of introducing deuterium is discussed in Chapter III in relation to dehydrogenation by D-galactose oxidase.

In utilizing NMR spectroscopy as in the studies described here, deuterium offers an important advantage in that it absorbs at a frequency widely different from that of protium*, and gives no interference.

*There is now a growing interest (27) in deuterium chemical shifts; but for the present purposes it was not necessary to measure deuterium spectra.

Consequently, complex proton spectra can be simplified by replacing a proton by deuterium. Thus in a system such as (Y-CH_2-X), replacement of one hydrogen by deuterium (Y-CHD-X) results in only one signal due to protium, although it is somewhat broadened due to the quadrupole effect of deuterium and a geminal coupling to protium of about (0.8-1 Hz) (28-29).

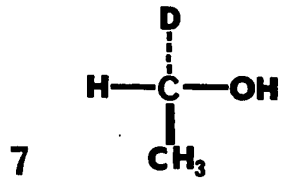
Prochirality

The subject of prochirality is well documented (30-34). Mention of this subject, to be dealt with in Chapter III and IV, will be brief here. Chiral molecules do not have rotational axes and cannot be superimposed on their mirror images. Most chiral molecules have chiral centres, also known as dissymmetric centres; a tetrahedral chiral carbon atom is bonded to four different groups.

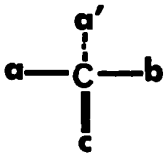
If hydrogen and deuterium are considered as two different groups then by substitution of one hydrogen atom by deuterium, as on the carbinol methylene carbon of ethanol, a chiral centre is generated (7). The methylene carbon of ethanol is therefore spoken of as a prochiral centre (35). Essentially a prochiral centre is a potential chiral centre. The ligands a, a' (a = a') attached to a prochiral centre (Aa a'bc) are termed as prochiral groups. If replacement of an a ligand gives rise to a chiral centre of R configuration (Cahn, Ingold, Prelog)(36) then a is a pro-R group (30,35). Similarly if replacement of an a' ligand gives a chiral centre of S configuration then a' is termed a pro-S group.

A pro-R or pro-S group can be an atom such as a methylene hydrogen of ethanol or a group of atoms, such as the CH_2CO_2H group of

citric acid. Thus, although the pro-R and pro-S hydrogens of ethanol are chemically and magnetically indistinguishable, they are subject to differentiation enzymically as has been demonstrated with yeast al-



7 Ethanol-1-d(S)



a prochiral centre

cohol dehydrogenase (37) and liver alcohol dehydrogenase (30).

When a prochiral centre is flanked by a chiral centre, an additional element of dissymmetry is introduced. Accordingly the prochiral groups are now magnetically non-equivalent. As will be shown later (Chapter III,IV) for methyl β-D-galactopyranoside, the two protons of C-6 are enzymically as well as magnetically non-equivalent in the D-galactose oxidase reaction.

The prochiral hydrogens of ethanol (8) and the prochiral groups of citric acid (9), or glycerol (10) become chiral or dissymmetric in the presence of an enzyme because they experience chiral environments provided by the enzyme. Thus, the phosphorylation of glycerol in rats (38) or with glycerol kinase (39) in the presence of ATP results in the formation of only L-α-glycerophosphate* (R-α-glycerophosphate)(11). Other aspects of enzyme-prochiral centre interactions also have been described (40)

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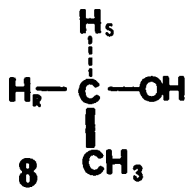
* Configuration assignment according to Swick and Nakao (38).

INTRODUCTION



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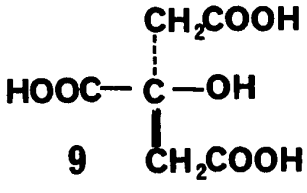
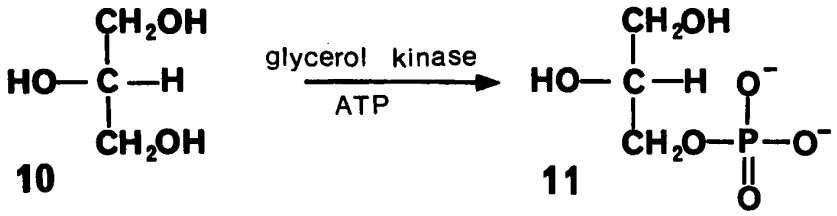


9

Prochirality also applies for double bond systems (30) of the type R R'C=X where X=O, C, N or other atom. Here prochirality exists because addition to the double bond systems can take place from either face, re (35)(re for rectus) or si (35)(si for sinister) to give two different stereoisomers. This possibility has been illustrated for acetaldehyde (30) and in Chapter III is dealt with in relation to

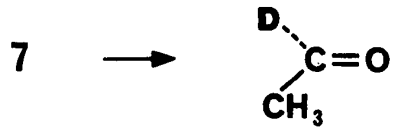


10 11

1,2:3,4-di-O-isopropylidene-α-D-galacto-hexodialdopyranose (12)

Stereospecificity

If in an enzyme or chemical reaction only one stereoisomer is consumed or formed, that reaction is considered to be stereospecific. In the reaction of ethanol-1-d (S) only the pro-R hydrogen is abstracted (37). This reaction is therefore stereospecific, as are



most enzyme reactions (33). The stereospecificity and stereochemistry of D-galactose oxidase at the prochiral centre is the subject of Chapters III and IV.

A reaction proceeding to give or consume a mixture of stereoisomers is defined as "stereoselective", and can be qualified as highly stereoselective or of low stereoselectivity depending on the proportions of stereoisomer formed or consumed (41). Stereoselectivity is more appropriate to chemical than to enzymic reactions.

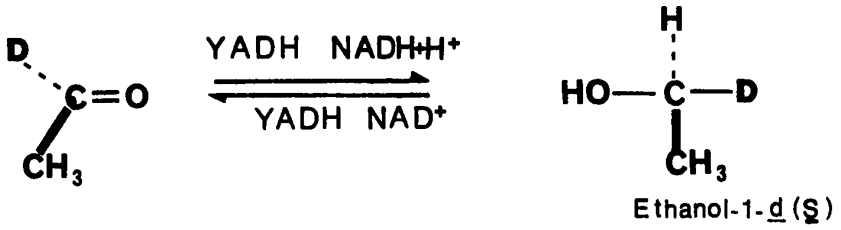
CHAPTER I

SYNTHESIS OF SUBSTRATES FOR A STEREOCHEMICAL STUDY OF THE PROCHIRAL CENTRE OF D-GALACTOSE AND METHYL β-D-GALACTOPYRANOSIDE

Having considered some stereochemical aspects of dehydrogenation by D-galactose oxidase, it was decided to synthesize 6R or 6S isomers of D-galactose and methyl β-D-galactopyranoside using deuterium to confer chirality upon the primary carbinol group. The simplest way to achieve this was to reduce an appropriate aldehyde - in this case 1,2:3,4-di-O-isopropylidene-α-D-galacto-hexodialdo-1,5-pyranose (12) or the 6-d-analogue (13) with a suitable deuteride or hydride donor. Two processes were evaluated for the reduction of 12 or 13: one involves the use of enzymes, and the other, chemical asymmetric induction. Another possibility considered involves nucleophilic displacement of suitable leaving groups at C-6 or C-1 by deuteride (see page 22).

For high optical yields (or purity)(42), enzymic methods are certainly best. Enzymes, being chiral reagents themselves, are product stereospecific and, to same extent, substrate stereospecific, and therefore do not lead to noticeable racemization. Thus Vennesland and coworkers (37) have demonstrated an *in vitro* stereospecific enzymic reduction of acetaldehyde-1-d and later carried out a large-scale preparation of chiral ethanol-1-d (43). Furthermore, Mosher and coworkers have employed alcohol dehydrogenase (YADH) from actively fermenting yeast to reduce n-butanal-1-d to n-butanol-1-d (44), trimethyl acetaldehyde-1-d

$(CH_3)_3CCDO$) to neopentyl alcohol-1-d (45), and benzaldehyde-1-d to benzyl alcohol-α-d (44). In all instances the products were of high optical purity. It appeared then that the radical attached to an aldehyde was not important. However, an attempt during the present study to apply the YADH method of Mosher et al. (44) was unsuccessful, although in a control experiment benzaldehyde was reduced.



Ethanol-1-d (S)

The failure of YADH to reduce 13 suggests that the topology of 13 does not favour formation of an enzyme-substrate complex necessary for reaction to take place. Prelog (19) has shown for example, that not all ketones are attacked by horse liver alcohol dehydrogenase, nor by an oxo-reductase enzyme isolated from the microorganism, Curvularia falcata.

Asymmetric Induction

The generation of a new chiral centre next to an existing one to give two diastereoisomers varying in proportion is usually referred to as "asymmetric induction" (46,47). In the reduction of 13 with sodium borohydride, for instance, two 6-carbinol diastereoisomers R and S are produced, with the latter predominating. This is because C-5 of 13 is a

chiral centre and its three different substituents each exert a different steric effect on nucleophiles adding to the adjacent carbonyl moiety.

With acetaldehyde, the carbonyl group of which is flanked by a symmetrical methyl group, no asymmetric induction is observed when simple nucleophiles such as deuteride or hydrogen cyanide are added. In this case both R and S isomers are produced in the same proportion and the product is therefore a racemic mixture (Fig. 1).

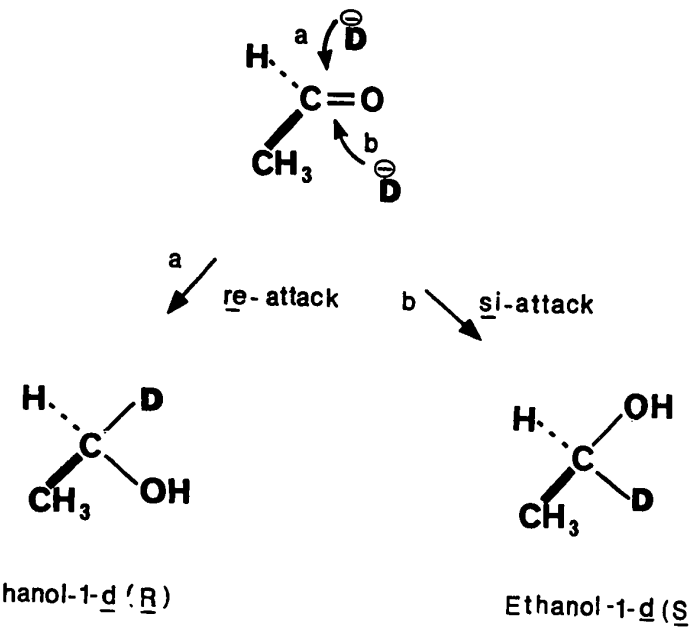


FIG. 1. Racemic product of deuteride addition to acetaldehyde.

I SYNTHESIS OF C-6 DIASTEREOISOMERS

In the reduction of acetaldehyde with sodium borodeuteride for instance, deuterium approaching from the *si*-face (35) experiences the same steric interaction with the methyl group as offered by the *re*-face (35); the result is a 1:1 ratio of *R* and *S* isomers, and the optical yield,* which is a measure of the excess of one enantiomer over the other, is zero. However, the situation can be changed if instead of using a simple nucleophile donor, a complex optically-active one is used. Asymmetric induction will then arise. Thus the use of deuterated isobornyloxy-magnesium bromide (48) on acetaldehyde affords chiral ethanol-1-*d*. A modification (49) of this reagent where a mixture of a ketone, lithium aluminium deuteride and aluminium chloride is used also affords chiral carbinols (50,51). The use of chiral Grignard reagents which transfer a β-substituted hydride in a fashion similar to the Meerwein-Ponndorf reduction, leads to synthesis of chiral n-butanol-1-*d* (52) and benzyl-1-*d* alcohols (44,53).

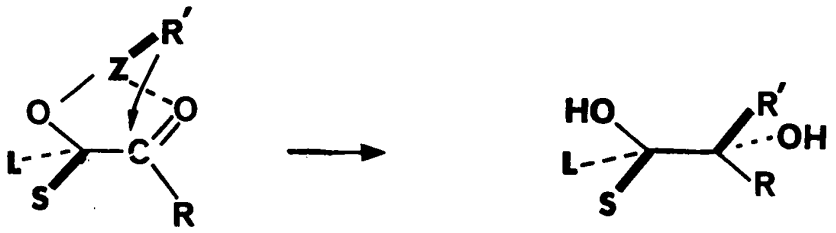
Various models (54-56) have been proposed to account for the unequal proportions of enantiomers formed in this process; most of these stress the importance of repulsive interactions on the development of the transition state. The foremost model of asymmetric induction is commonly referred to as "Cram's-rule", and has been extended by Prelog (57) to include addition to carbonyl groups separated from the chiral centre by two atoms as in the asymmetric synthesis of altrolactic acid (58).

Cram and Elhafez (54) noted that when a carbonyl group is flanked by a carbon bearing three substituents varying in size; small (S) medi-

* If F^+ and F^- are the mole fractions of the new enantiomers, and if F^+ is the predominant isomer then the enantiomeric purity is $(F^+ - F^-)/(F^+ + F^-)$ and is synonymous with optical purity $p=\alpha/A$ where α is the specific rotation of $(F^+ + F^-)$ and A that of pure enantiomer F^+ or F^- (42).

um (M) and large (L) - then the incoming nucleophile adds preferably to the carbonyl group from the side flanked by the smallest substituent (S), i.e., the group offering minimal non-bonded interaction with the incoming nucleophile (14). The orientation of the carbonyl group was not considered in detail. It was assumed that the carbonyl oxygen would be orientated between the smallest (S) and the medium sized (M) substituents, and that addition would proceed from the side nearest (S). Most compounds tested conformed to this model.

A different model was proposed by Cram and Kopecky (55) more specifically to predict the stereochemistry of addition to carbonyl groups flanked by a chiral centre that carries an amino, a hydroxyl or other group capable of complexing with the metal of the Grignard reagent or metal hydride. A favourable transition state in this case was depicted as a rigid five-membered ring (15). This model was



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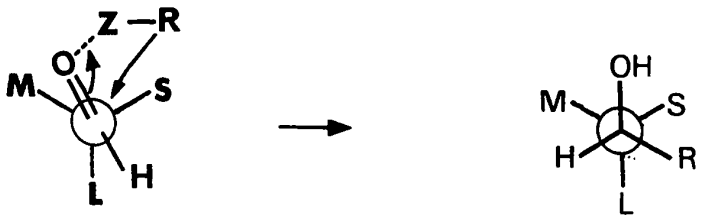
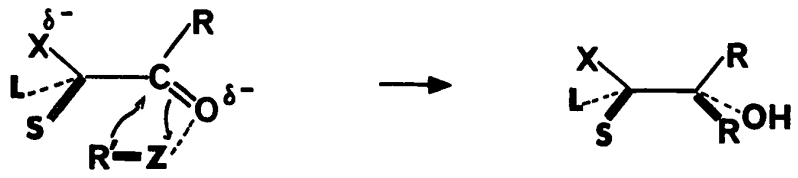


14

satisfactory in some instances, although in others the open chain model (14) appeared to have greater validity (59). In dealing with 1,2 additions to α-halo-carbonyl compounds, Cornforth et al. (56) proposed a dipolar model (16) in which the halogen and the carbonyl oxygen are anti-periplanar. With two trans-dipoles the carbonyl carbon becomes polarized and an addition reaction is facilitated.

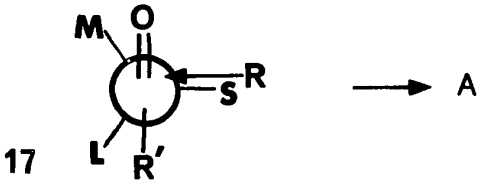


16

In attempting to account semiquantitatively for the products resulting from addition to carbonyl groups directly bonded to chiral centres, Karabatsos (60) offered a different version of the models discussed above. He regarded the stereoisomeric ratio (A/B) of addition products to be a measure of the degree of interaction between the carbonyl oxygen and the largest group (L) (L↔O)(17) on the one hand, and with the medium sized group (M) on the other (M↔O)(18) . Knowing A and B then permitted the free energy change associated with these interactions to be gauged from the equation:

$$G_A^{\neq} - G_B^{\neq} = -RT \ln A/B$$

I SYNTHESIS OF C-6 DIASTEREOISOMERS



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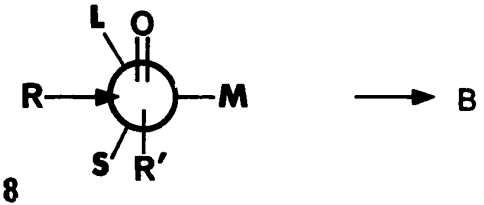
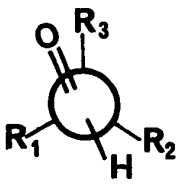


18

If Karabatsos's model (60) is valid, the diastereoisomeric ratio obtained in the reduction of (13) with sodium borohydride should be a measure of the extent of interaction between C-4 and O-5 with the carbonyl oxygen, and the expected diastereoisomeric ratio should be greater than unity. This is not observed with 13, where A < B. It must be said that problems are encountered in the actual rating of group sizes (46), also when electronegative atoms are present on the chiral centre next to the carbonyl group, such as in 13, they may exert overriding effects (61) especially on a solvation of the transition state, coupled with interactions other than those between (M↔O) and (L↔O). The rate of bond making and bond breaking in the transition state (see below) may bring about differences in the diastereoisomeric ratios.

In support of his product-specificity model, Karabatsos has consolidated his studies with those of others on rotational isomer-

ism about sp^2-sp^3 carbon-carbon bonds. Thus for acetaldehyde, microwave studies (62) show a preferred conformation in which the carbonyl oxygen nearly eclipses with a hydrogen (19), i.e. a dihedral angle of only 9° between them.



19 R_1, R_2, R_3 = H

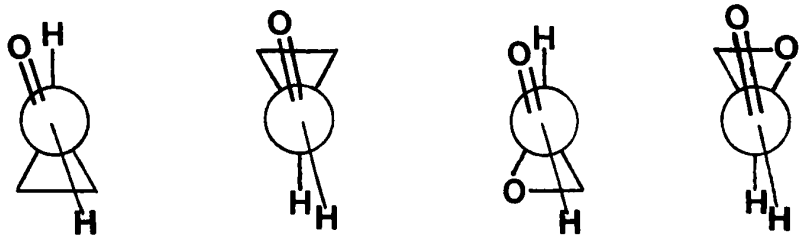
20 R_1, R_2 = H, R_3 = CH_3

21 R_1,R_3 = H, R_2 = CH_3

22 R_1,R_3 = CH_3, R_2 = H

23 R_1,R_2 = CH_3, R_3 = H

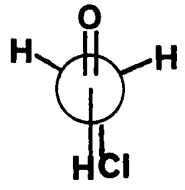
Propanal (63,64) and isobutanal (65) appear to show a preference for rotamers in which a large substituent eclipses the carbonyl oxygen; i.e. 20 rather than 21 and 22 rather than 23 respectively. According to microwave (64,66) and electron diffraction (65) spectroscopy rotamers 24 and 25 are equally populated for cyclopropane carboxaldehyde in the gas phase. For glycidaldehyde, microwave spectra suggest rotamer 26 to predominate over 27 in the gas phase as well as in the liquid state (67).



24 **25** **26** **27**

I SYNTHESIS OF C-6 DIASTEREOISOMERS

The findings of microwave spectroscopy for these compounds must be cautiously interpreted since, when it comes to complex compounds as seen above, the method begins to falter. In any case the energy difference between preferred and unpreferred rotamers is small and of the order of 1200 cals/mole at most. NMR spectroscopy, according to Karabatsos et al. (67-70) is also inadequate to provide an exact description of such sp^2-sp^3 C-C rotational isomerism. The gauche J_{HH} associated with such rotamers is usually small, usually less than 3 Hz and to some extent solvent dependent. Infrared spectroscopy (71) favours rotamer 28 over 29 for chloroacetaldehyde, which is at variance with Karabatsos NMR data, and the matter remains unresolved.



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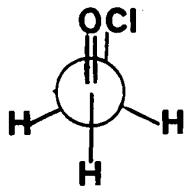


29

Compounds claimed to have a conformation analogous to 28 in the ground state were used by Cornforth et al. (56) to synthesize olefins with over 70% stereoselectivity, and led to the successful synthesis of all-trans squalene (72). Recent application of this same approach (73) at low temperature (-70 to -90°) gave olefins with over 70% stereoselectivity. As shown in Cornforth model 16 , addition to the carbonyl group is from the side of the smallest group to give a chlorohydrin in high optical yield. It is possible that in the transition state the ar-

rangement of groups in the vicinity of the carbonyl group may be quite different from that in the ground state, but this is not highly likely since extra energy has to be expended to stabilize structures already unstable in the ground state. According to the Curtin-Hammett principle (74), and as envisaged by Karabatsos (60), the conformation in the transition state has to bear resemblance to reactants and products as if no bond making or breaking has actually taken place. One can argue then that the trans-relationship between the carbonyl oxygen and the chloro-group (28) in α-chlorocarbonyls is also reflected in the chlorohydrin obtained.

The topology of 13 (page 29) in the vicinity of the carbonyl function can be taken as analogous to that of 16 when the polar group, which is also the medium group (M), is the pyranose ring oxygen, C-4 is the largest group (L), and H-5 the smallest. The anti-periplanar arrangement of O-5 and the carbonyl oxygen could then lead to attack from the re-face and to a predominantly S product. As visualized in Fig. 2, the nucleophile is a hydride and is transferred in a cyclic transition state (75).

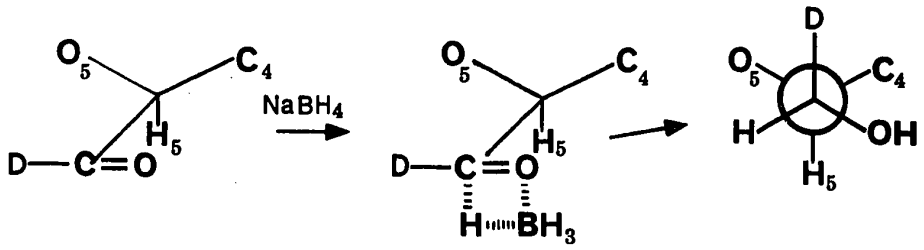
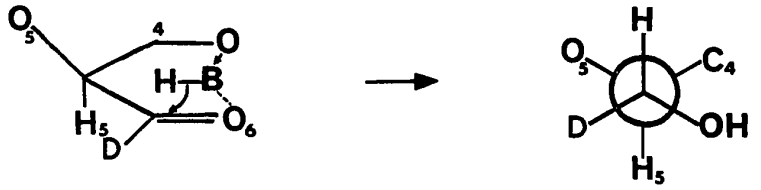


FIG. 2

By contrast, si-attack of the carbonyl function of 13 can be considered to take place as shown in 30. It is possible that 30 would be favoured by the twist (76) conformation of the pyranose moiety which, from models, appears to minimise crowding. However, this process must have a transition state that is energetically less favourable since it culminates in the minor diastereoisomer, i.e. 25% for sodium borohydride reduction, and 33% for Grignard processes (77-79). Six membered ring transition states involving Grignard reagents have been proposed (80) for other systems. However, the possibility that 13 has



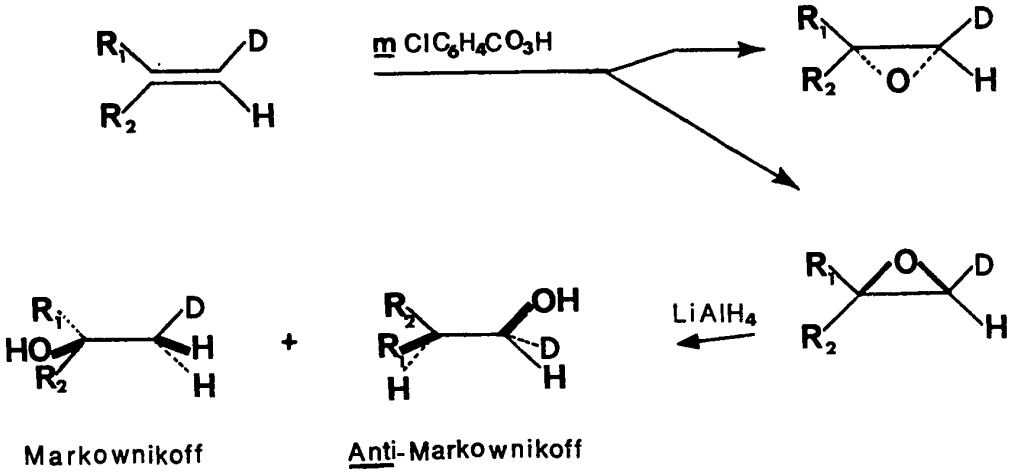
30 6R product

a small population of a rotamer with O-6 cis to O-5, such that addition to the carbonyl moiety by a nucleophile takes place from the si-face to give the 6 R product, cannot be ruled out.

S_N2 displacement methods

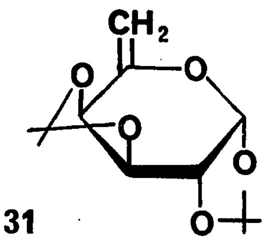
Introduction of deuterium into a secondary position can be achieved by displacing appropriate leaving groups with a deuteride donor; examples appear in the literature in which secondary halides (81) and sulphonates (81,82) are displaced with lithium aluminium deuteride. How-

ever, the incorporation of deuterium and a hydroxy-group simultaneously and stereoselectively with formation of a primary carbinol function, is difficult. Conceivably, this could be done by epoxidation of a highly stereoselectively deuterated alkene, followed by opening of the epoxide with lithium aluminium hydride (83,84) or lithium borohydride (85) also with high stereoselectivity. The deuterated alkene could likely be synthesized (86), but epoxidation of an alkene usually leads to a mixture, and the problem is compounded by the fact that ring opening of the epoxide follows Markownikoff's rule to give mainly a secondary alcohol (87). Lithium borohydride appears to slightly favour primary alcohol formation, but only a few examples of this have been reported (88).



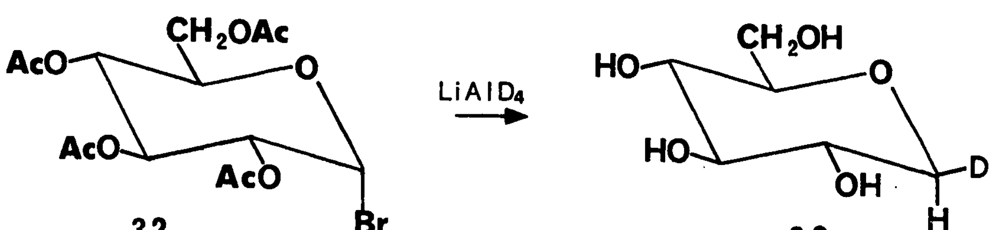
In view of these difficulties, synthesis of a highly stereoselectively deuterated D-galactose-6-d starting from a glycoseen such as 31 (89-91) was not undertaken; but the reaction is perhaps worthy of an attempt, preferably using Brown's (92) asymmetric hydroboration reaction, which circumvents formation of the unwanted secondary alcohol encountered in epoxide ring opening. Probably the main limitation here would be acqui-

sition of the specifically deuterated glycoseen.



31

A synthetic pathway which holds promise for the synthesis of highly stereoselectively deuterated primary carbinols has been described by Maradufu, Mackie and Perlin (93)(Fig. 3). Initially it involved S_N2 halide displacement at the anomeric center of a poly-O-acetyl-glycosyl halide (94). With a suitably substituted glycosyl halide such as 32 displacement of the secondary bromide at C-1 by deuteride occured (32a) with inversion. Carbon-1 was now bonded to a deuterium atom and an ether oxygen. Conversion of the ring oxygen to a hydroxy- function without affecting the chirality at C-1 was then achieved by a suitable degradation involving the C-4, C-5 bond, yielding L-threose-4-d (4S). Accordingly, by starting with L-glucose or L-galactose it should be possible to synthesize a highly stereoselectively deuterated D-galactose-6-d, i.e., via synthesis of L-threose-4-d and subsequent chain extension to the aldohexose-6-d.



32 32a

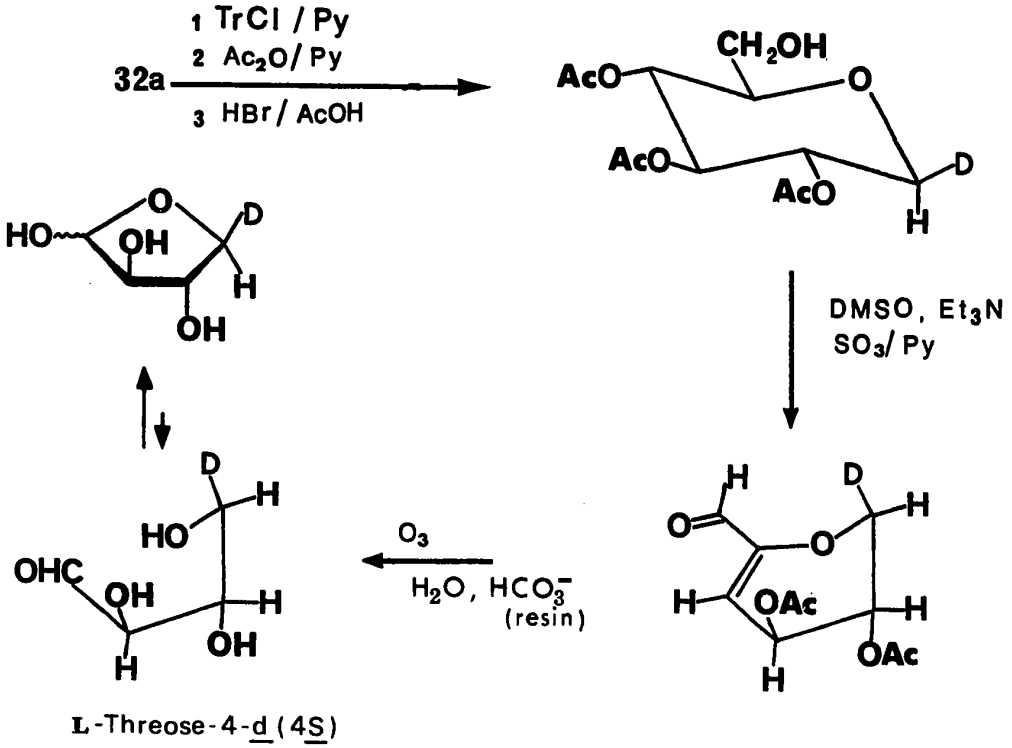


FIG. 3

Stereoselective synthesis of isomers of D-galactose-6-d

Figure 4 shows the reaction sequence taken to synthesize D-galactose-6-d predominantly 6R (33), and also its methyl β-D-glycoside-6-d (34). Oxidation of 1,2:3,4-di-O-isopropylidene-α-D-galactopyranose (35) was effected with methyl sulfoxide (DMSO) triethylamine and SO_3/pyridine, a reagent described by Parikh and Doering (95) and used for the preparation of 12 by Cree, Mackie and Perlin (96). The main product of this reaction was the required aldehyde, 1,2:3,4-di-O-isopropylidene-α-D-galacto-hexodialdose (12). However, examination of the reaction mixture showed the presence of a very small proportion

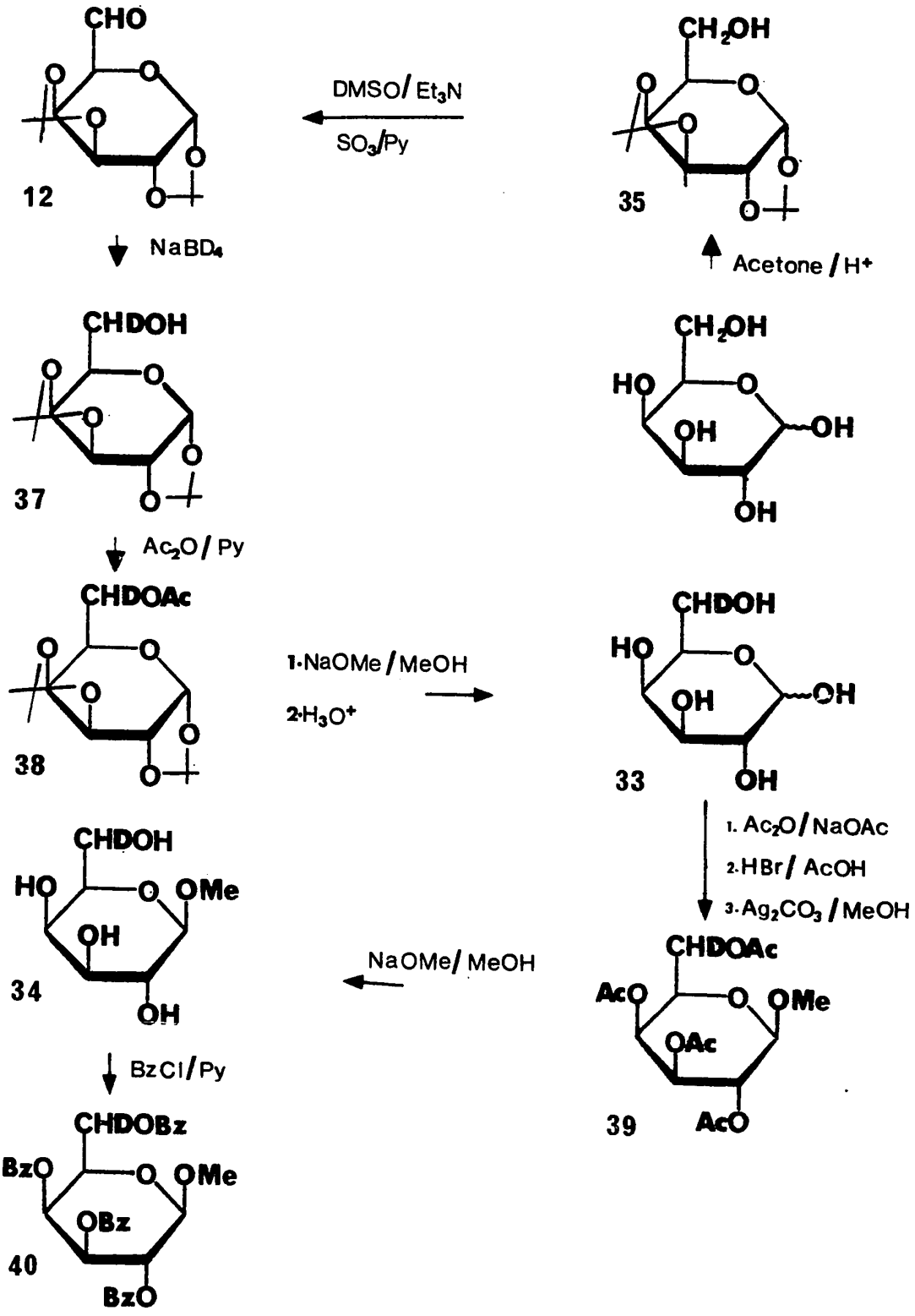
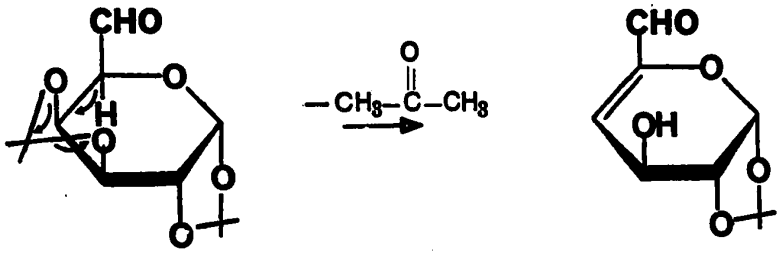


FIG. 4. Synthesis of **enzyme substrates:** D-galactose-6-d (33) and its methyl β-D-glycoside-6-d (34) and derivatives.

of another compound slow moving on tlc and staining strongly with 2,4-dinitrophenylhydrazine reagent. The NMR spectrum of this minor aldehydic byproduct was consistent with structure 36 and also with the spectra of similarly constituted compounds (97).



12 36

Present also in the reaction mixture, and later isolated on a silica gel column, was a fast moving fraction which was identified as 1,2:3,4-di-O-isopropylidene-6-O-(methyl thio)-methyl-D-galactopyranose (51)(page 30) inasmuch as its NMR parameters were indistinguishable from those of 51 obtained in the oxidation of 35 with DMSO-acetic anhydride (98).

Reduction of 12 with sodium borodeuteride in ethanol-water gave 1,2:3,4-di-O-isopropylidene-α-D-galactopyranose-6-d (37) purification being effected through its crystalline 6-O-acetate (38). The mass spectrum of 38 did not show the presence of the molecular ion peak but, as is usual (99) with acetonide derivatives, loss of one methyl group led to an intense peak of (M-15) mass units. Based on the relative intensities of this latter peak in the mass spectra of the deuterated and non-deuterated compound, it was found that the deuterium content at C-6 of 38

was 98%. According to the mass spectrum of 38 or 37 none of the major fragmentation patterns (99) common to compounds of this class involve loss of H_R or H_S in preference to deuterium at the prochiral centre.

Alkaline hydrolysis of 38 gave 37 which on acid hydrolysis gave D-galactose-6-d (33). Compound 33 was then converted to methyl β-D-galactopyranoside-6-d (34), a portion of which was used for the determination of relative configuration (see next part) and for the synthesis of various other derivatives for NMR spectral studies.

Synthesis of the diastereoisomeric form of D-galactose-6-d (41) started with the conversion of D-galacturonic acid (42) into 1,2:3,4-di-O-isopropylidene-α-D-galactopyranose-6,6-d_2 (43). Oxidation of the latter as above with DMSO, triethylamine, sulphur trioxide-pyridine complex gave the aldehyde, 1,2:3,4-di-O-isopropylidene-α-D-galacto-hexodialdopyranose-6-d (13). Reduction of the latter with sodium borchydride afforded 44 which was purified by acetylation to 48. From the mass spectrum of the latter, using (M-15) peak, a deuterium content at C-6 of 98% was estimated. Alkaline hydrolysis of 48 gave 44, and then acid hydrolysis liberated D-galactose-6-d (41) which is diastereoisomeric with 33. Product 41 was used for relative configuration studies (page 38) and also was converted into methyl β-D-galactopyranoside-6-d (45) and derivatives of it.

Purification of 43 was similarly effected through its acetate 49 (i.e., 1,2:3,4-di-O-isopropylidene-6-O-acetyl-α-D-galactopyranose-6,6-d_2), the mass spectrum of which showed deuterium content at C-6 of 98-99%. Conversion of 46 into methyl β-D-galactopyranoside-6,6-d_2 (47) was performed in the same way as for 33 to 34 (Fig. 5).

I SYNTHESIS OF C-6 DIASTEREOISOMERS

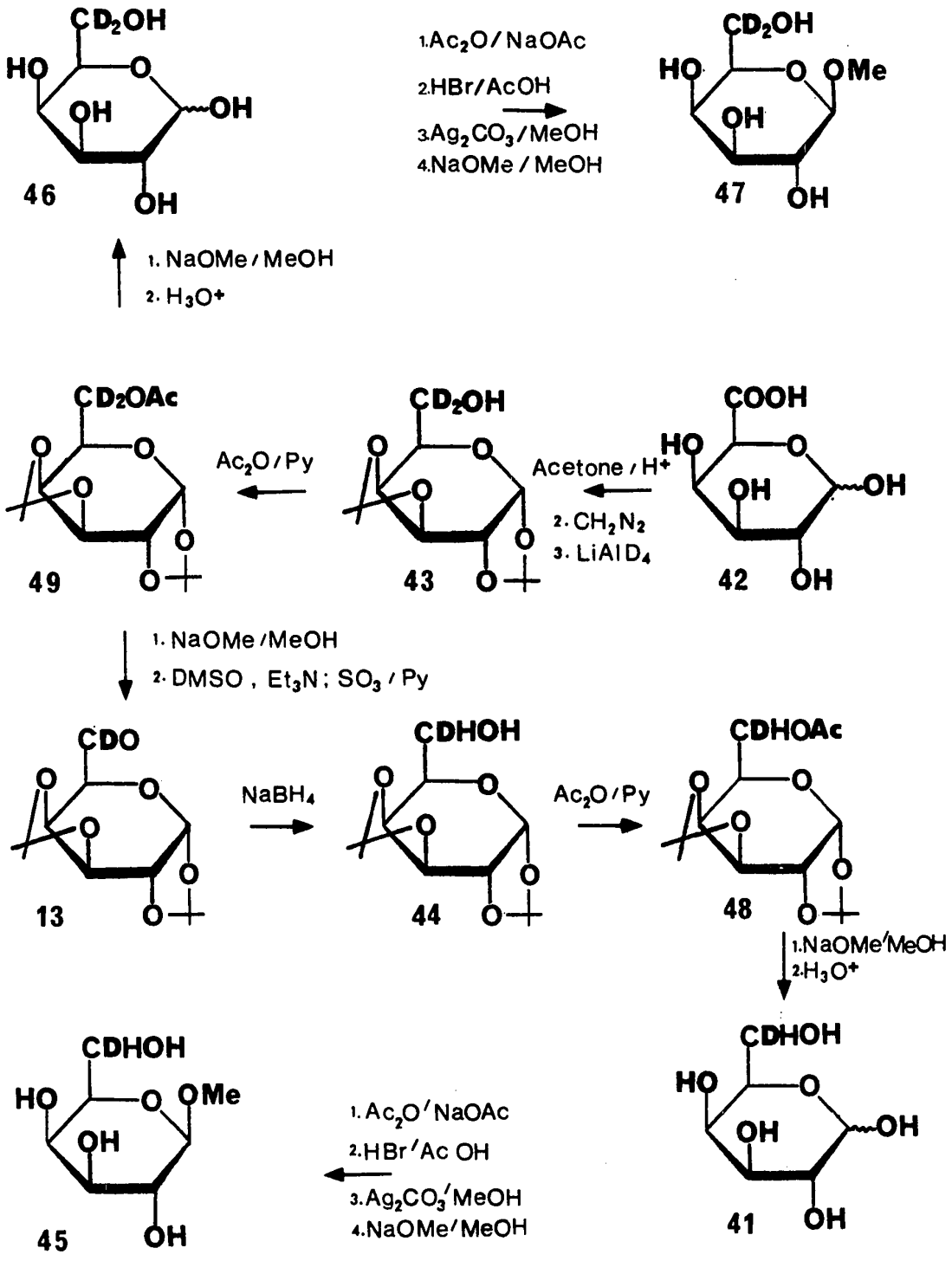
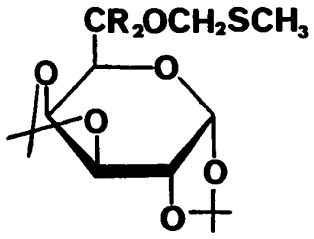


FIG. 5. Synthesis of enzyme substrates: D-galactose-6-d (41) and its methyl β-D-glycoside (45), and of D-galactose-6,6-d_2 and its methyl β-D-glycoside (47).

I SYNTHESIS OF C-6 DIASTEREOISOMERS

Some observations bearing on the mechanism of methyl sulphoxide oxidation

During the methyl sulphoxide oxidation of the 6,6-dideutero compound, 43, the dark brown colour that usually develops during these oxidations, and found with 12, was not observed; instead, the reaction mixture was pale yellow. Also, instead of the rapid evolution of heat that accompanied the oxidation of 35 only a gradual warming of the reaction mixture took place. Particularly noteworthy was the fact that with the deuterium present a higher proportion of the (methyl thio) methyl ether (50), about 20%, was produced.



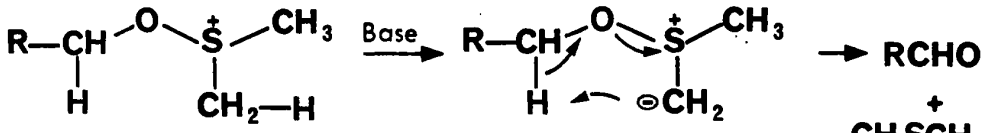
51 R = H

50 R = D

Varying proportions of this ether (100) (i.e. 51) are formed in methyl sulphoxide oxidations; depending on the activating reagent. In the presence of acetic anhydride 51 is formed almost exclusively (98) from 35, whereas relatively little is formed when the oxidation is promoted by N,N'-dicyclohexylcarbodiimide-pyridinium phosphate (91), or phosphorus pentoxide (100). These variations and the present finding that the product ratio of the sulphur-trioxide promoted oxidation is subject to an iso-

tope effect, may be considered together in terms of the mechanism of the methyl sulphoxide oxidation of alcohols (101-107).

The first step is considered to involve the formation of an alkoxyl sulphonium salt (52) and this is followed by the generation of a ylid (53). A proton is then transferred within the latter by a cyclic process as shown, with liberation of an aldehyde and dimethyl sulphide.

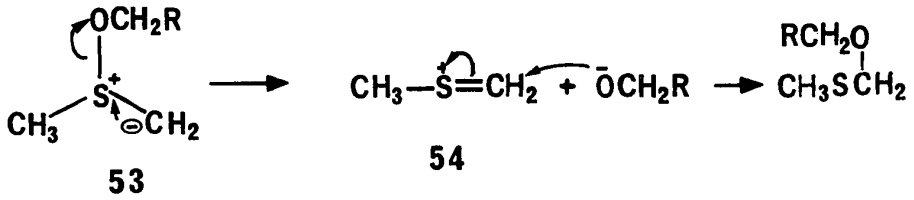


52 53

The failure of 53 to decompose into products immediately may result in the formation of side products such as the methyl thiomethyl ethers (50 or 51). It is possible that the delay imposed by the deuterium isotope effect on the oxidation of 43 to 13 alters the course of the reaction slightly to give 50 in an almost two to three parts of undeuterated 51.

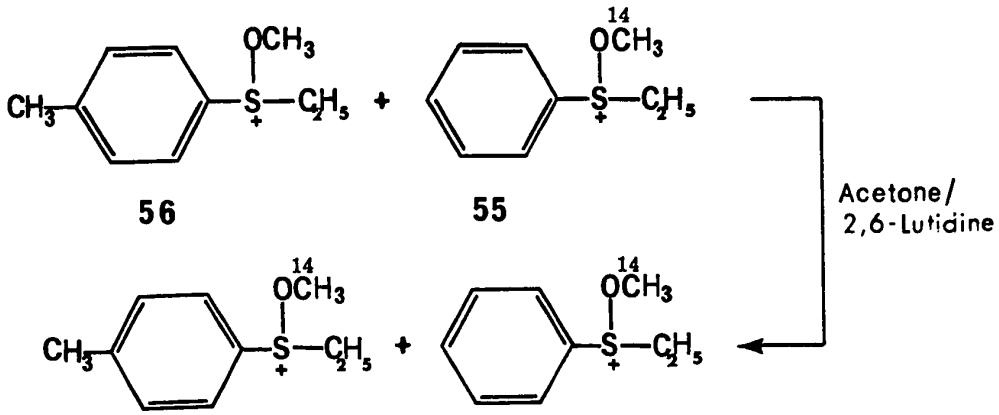
Reactions leading to side products such as 50 or 51 are collectively termed"Pummerer rearrangements"(107) and four mechanisms (108-115) have been advanced to account for them. In the first one the ylid 53 dissociates to form an intermediate 54 which is attacked by an alcohol to give a rearranged product (such as 51).

I SYNTHESIS OF C-6 DIASTEREOISOMERS



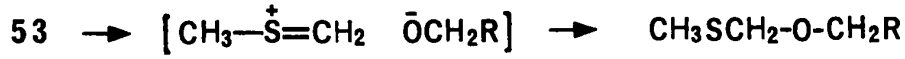
53 54

This mechanism has been substantiated by a labelling experiment (109). Using ^{14}C it was shown that labelled 55 when mixed with 56 and subjected to ylid forming conditions undergoes label-interchange.



56 55

According to the second possibility the ylid 53 dissociates but remains caged by the solvent and the rearrangement is therefore intramolecular.



53

The third mechanism is a cyclic one as illustrated in Figure 6. It could plausibly be applied to the formation of 50 because it may be ar-

gued that if the C-D bond of ylid 57 does not participate easily in the cyclic breakdown to a carbonyl product, then the alternative is an internal displacement and addition, as shown

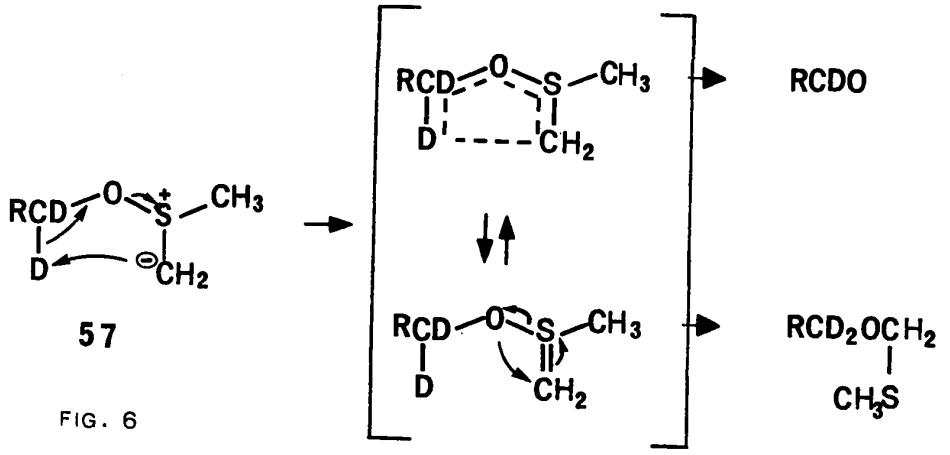


FIG. 6

It is possible therefore to suggest that both the rate determining step and the course of the reaction are associated with the rate of removal of a proton by the carbanion of the ylid.

A fourth mechanism that has been proposed has been effectively discounted by Durst (107) and will not be considered here. The second seems to hold in some other systems (116-117). The first also may not apply for 50 since the intermediate ion 54 could be attacked by other nucleophiles (such as Et_3N) which are present in excess over alcohol 43; however, it cannot be ruled out entirely.

CHAPTER II

DETERMINATION OF CONFIGURATION AT CHIRAL C-6 OF D-GALACTOSE-6-d (33) and (41)

General remarks

Absolute configuration refers to the exact arrangement of atoms of a molecule in space. The determination of absolute or relative configuration is of paramount importance in biological and organic chemistry because the reactivity of compounds especially in biological systems, depends very much on the arrangement of atoms and groups in a particular order.

There are various methods for determining configuration. A theoretical method is due to Brewster (118) and has been applied to predict the configuration of deuterated chiral centres such as those in ethanol-1-d and butanol-1-d. The classical X-ray diffraction method (119) to determine absolute configuration is of little utility for compounds whose chirality is due to the presence of deuterium for, as yet, it is not possible to differentiate between deuterium and hydrogen by X-ray diffraction. However, neutron diffraction analysis has been successfully applied to determine the absolute configuration of glycolic acid-α-d (120) and this compound has been used in turn to correlate the chirality of other deuterated compounds through comparison of their optical rotatory dispersion curves.

A chemical approach has been described (121), although it probably needs to be tested vigorously. It is based on the specific rotation of α-phenylbutyric acid produced in the esterification of a deuterated alcohol with chiral α-phenylbutyric anhydride (121). Such a me-

thod may be difficult to apply, however, to a complex compound containing several chiral centres.

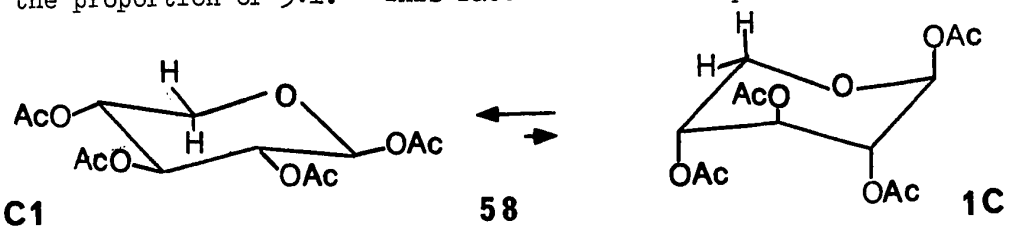
Enzymic methods can also be used to determine the configuration of a deuterated chiral centre, and are reliable. If an enzyme is known to react with an R isomer, for instance, a compound of unknown configuration will only react if it has an R configuration. A number of stereospecific enzymes and their substrates have been dealt with in recent reviews (30,33,122).

NMR spectroscopy is a method of broad applicability for the study of relative configuration. With the relative configuration determined, the absolute configuration at the chiral centre of a compound may then be defined by reference to a structurally analogous compound of known stereochemistry. As shown below, the NMR method is readily feasible when the chiral species is contained in a six-membered ring which, by virtue of its substituents, is biased to exist in one energetically favourable chair conformation. In this way it becomes possible to sort out equatorial and axial protons since their respective NMR parameters are known (123). Thus, in cyclohexane (124), tetrahydropyran (125) and pyranose monosaccharides (123), the absorption of an equatorial proton is usually at lower field than an axial one, and the vicinal splitting for axial-axial trans protons is larger than for an equatorial-axial or equatorial-equatorial arrangement.

Configuration of D-xylose-5-d at C-5

Guided by the above NMR characteristics, Lemieux and Howard (126) determined the configuration at the C-5 chiral centre of a sample of

D-xylose-5-d. This was possible because the H-5 resonance signals of 1,2,3,4-tetra-O-acetyl-β-D-xylopyranose (58) are well separated and can be distinguished as to equatorial and axial, and because the absolute configuration of the other asymmetric centres of D-xylose are known. Accordingly, the chirality at C-5 of D-xylose-5-d was worked out and found to be a mixture of R and S, with the former isomer predominating. Horton and coworkers (127) have reported that β-D-xylose tetraacetate at room temperature exists as a mixture of two chair forms C1 and 1C (58) in the proportion of 5:1. This fact modifies the quantitative interpretation of Lemieux and Howard's results, because these authors considered only the C1 conformation, but not their main conclusions.



In the present work D-xylose-5-d was used as one means of determining the absolute configuration of D-galactose-6-d. The starting material for the preparation of D-xylose-5-d differed slightly from that used by Lemieux and Howard (126). In their study, the introduction of deuterium at C-5 of D-xylose was achieved by reducing 1,2-O-isopropylidene-3-O-benzyl-α-D-xylo-pentodialdofuranose with lithium aluminium deuteride in ether. In the current work 1,2-O-isopropylidene-α-D-xylo-pentodialdose (59) was reduced with sodium borodeuteride in ethanol-water. The use of lithium aluminium deuteride in ether also gave the same results as Lemieux and Howard's as shown by converting the deuterated pentoses into β-D-xylopyranose-5-d tetraacetate and comparing their NMR spectra. In each instance, the predominant isomer was 5R, although the ratio of R:S was not exactly the same. It may be noted that 1,2-O-

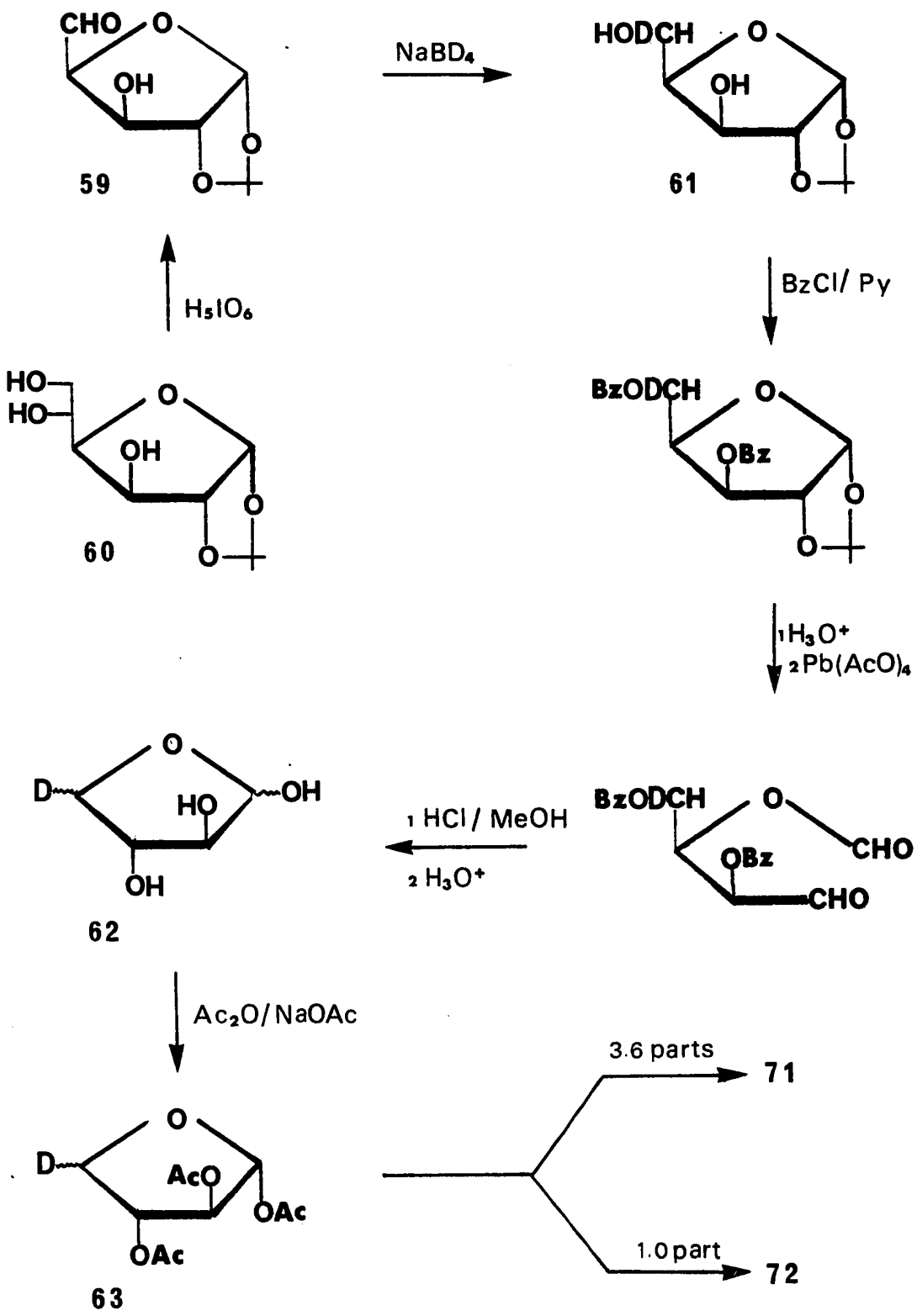


FIG. 7 Synthesis and degradation of D-xylose-5-d

isopropylidene-α-D-xylo-pentodialdofuranose resulting from the periodate cleavage of 1,2-O-isopropylidene-α-D-glucofuranose is a mixture of a dimer and an acetal; it was found, however, that reduction of either the pure dimer or the acetal with lithium aluminium deuteride, gave the predominantly R-isomer.

With the absolute configuration of D-xylose-5-d being known, correlation of the configuration at the primary carbon of D-xylose-5-d with that of D-galactose-6-d (33),(41) was mediated through degradation processes which led to the formation of D-threose-4-d from each compound. These conversions were carried out such that the deuterated chiral centres were untouched. Figure 7 shows the degradation of 1,2-O-isopropylidene-α-D-xylose-5-d (61) to D-threose-4-d (62) which, on acetylation, gave tri-O-acetyl-α-D-threose-4-d (63).

Configuration of D-galactose-6-d (33), (41) at C-6

The lead tetraacetate oxidation method of Perlin and Brice (128) was used to degrade D-galactose-6-d (33) or (41) to D-threose-4-d (64) or (65), respectively, which was acetylated to give the corresponding triacetate (66) or (67). Conversion of D-galactose-6,6'-d_2 (68) into tri-O-acetyl-α-D-threose-4,4'-d_2 (69), was also carried out for structural and NMR studies.

The NMR spectrum of tri-O-acetyl-α-D-threose (70)(Fig. 9) clearly shows the absorption signals due to the C-4 hydrogens. Assignments for the ring protons of 70 are also given, and are supported by comparison of the spectrum of 70 with that of 69 to verify the location of the H-4 signals, (Fig. 9), as well as by the pertinent splitting

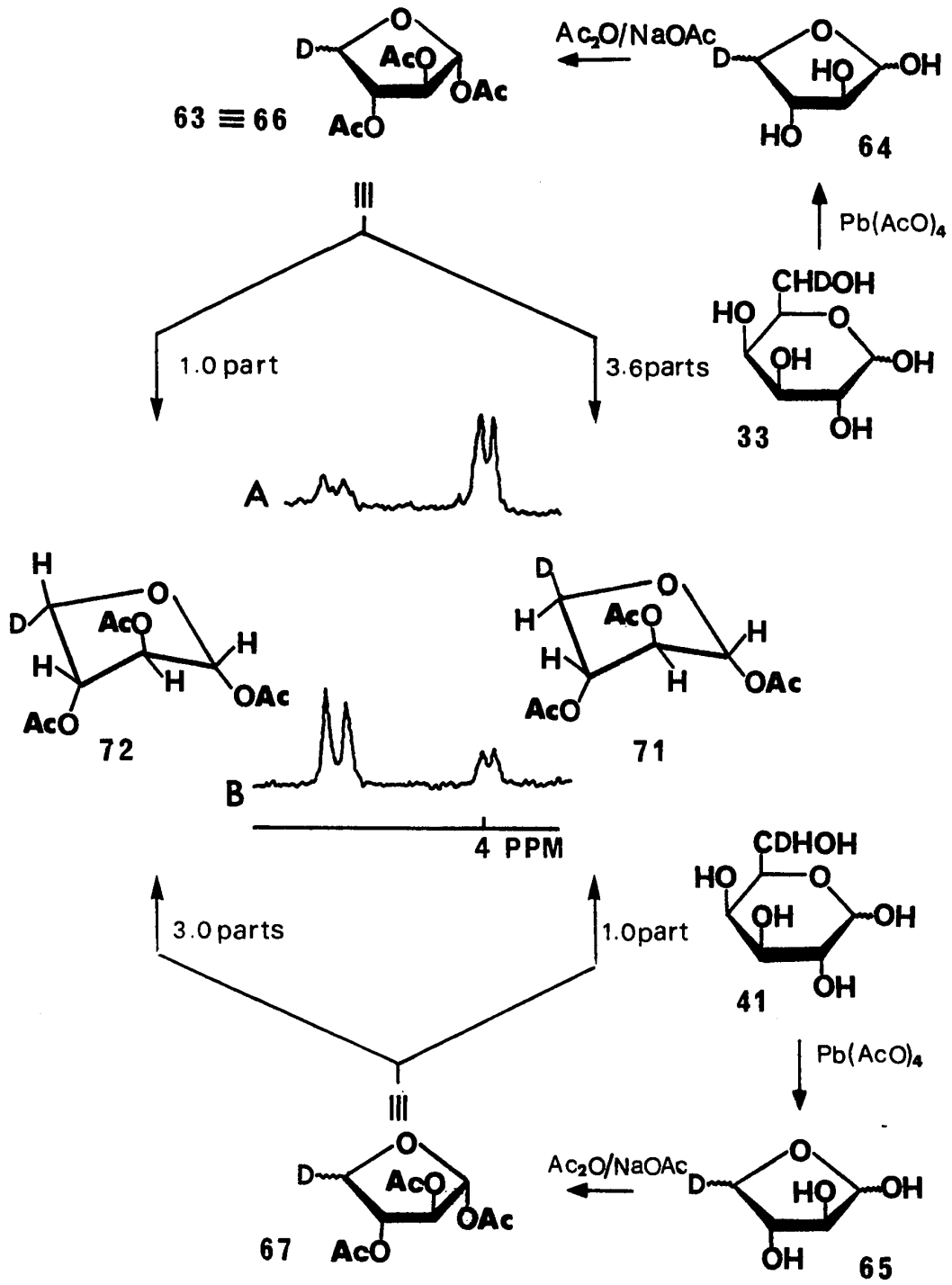


FIG. 8 Degradation of D-galactose-6-d (33) and (41) to D-threose-4-d (64) and (65) and the partial NMR spectra: A of 66 and/or 63; B of 67 in $CDCl_3$.

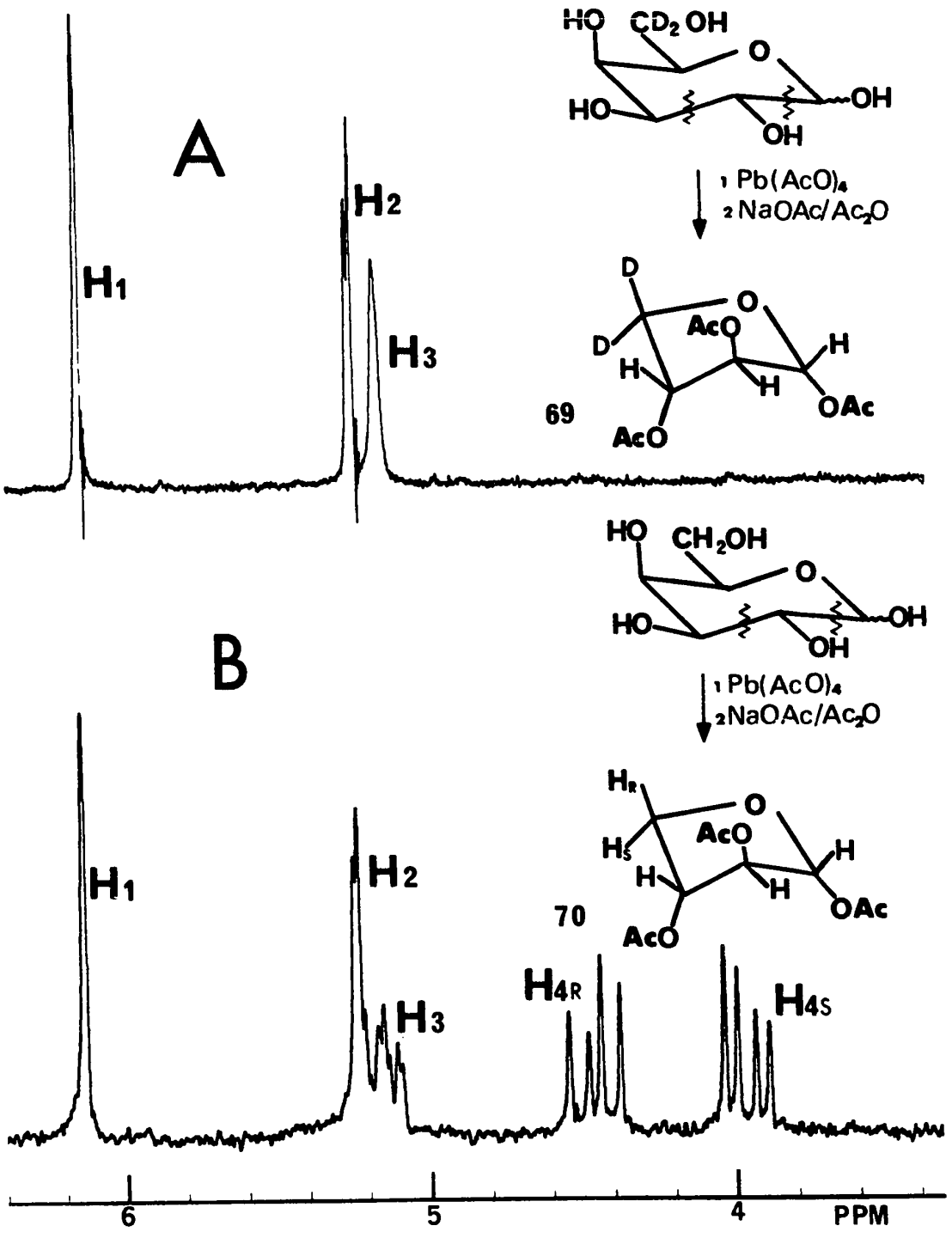


FIG. 9 Partial NMR spectra (100 Hz, 1000 Hz sweep width): A of α-D-threose-4, 4-d_2 triacetate (69); B of α-D-threose triacetate (70) in $CDCl_3$.

patterns.

In Fig. 8(A) the partial NMR spectrum of α-D-threose-4-d triacetate (63) derived from D-xylose-5-d is presented. It can be seen that (63) is composed of two isomers 71 and 72, differing in chirality at C-4. Since the predominant isomer of D-xylose-5-d is 5R, it follows that the signal centred at 4 ppm (with a splitting of 4.3 Hz) and larger in area than the signal at 4.5 ppm (with a splitting of 6.4 Hz) arises from the C-4 hydrogen of the 4R isomer (71). Hence the 4S isomer is 72 and its C-4 hydrogen producing the weaker resonance registers its signal at 4.5 ppm. The NMR spectrum of 66 is indistinguishable from that shown in Fig. 8(A). This near identity of the spectrum of 63 with that of 66 indicates that the latter also has 4R as the predominant isomer (71) and that its parent molecule, D-galactose-6-d (33), is also predominantly 6R. Therefore 33 exists as a mixture of 6R and 6S with the 6R isomer predominating in a ratio of 3.6:1.

The partial NMR spectrum of 1,2,3-tri-O-acetyl-α-D-threose-4-d (67) prepared from D-galactose-6-d (41) is shown in Fig. 8(B). Comparison of the signal at 4.5 ppm with that at 4.0 ppm clearly indicates that the predominant isomer now is 4S, and, therefore, that the parent precursor (41) is a mixture of 6S and 6R in the ratio of 3:1.

An alternative basis for establishing the chirality at C-6 of D-galactose-6-d (33 or 41) is provided by the synthesis of L-threose-4-d (4S) (93) (page 24). In this instance, a deuterated carbon of S configuration incorporated into a molecule having a stable C-1 chair conformation has been interconverted into a primary function as C-4 of L-threose-4-d. The close correspondence of the NMR spectrum of tri-O-acetyl-α-L-threose-4-d

(4S) with that of its enantiomer, tri-O-acetyl-α-D-threose-4-d (4R)(71) thus verifies the conclusion reached above with regard to the assignment of chirality.

Optical purity of D-galactose-6-d 33 and 41

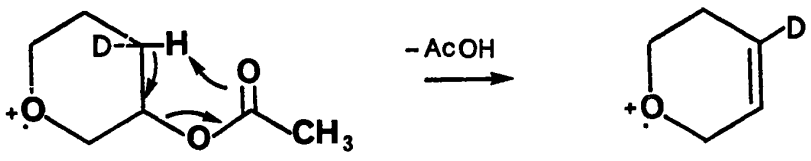
The clear separation of the H-4 signals in the spectra of 63, 66, and 67 by about 0.5 ppm permitted the measurement of optical purity of deuterated 66 and 67. Prior to this finding, examinations of the spectra of a number of derivatives of 33 and 41 and of D-galactose, namely, diacetonide, β-pentaacetate, methyl β-D-galactopyranoside and its tetraacetate, and galactitol hexaacetate, showed that the H-6 signals either overlapped or were otherwise incompletely distinguishable. Hence these compounds furnished only a qualitative estimate of the pattern of deuteration. Also, in the spectrum of methyl β-D-galactopyranoside tetrabenzoate (73) dissolved in deuterated benzene-d_6, the H-6 signals are well separated (Fig. 23), but the closeness of the H-1 signal to that of H_S interferes slightly with the exact quantitative estimation of the contribution of the latter.

The spectra of 66 and 67 revealed that the pattern of deuteration in 33 and 41 is not that of a mirror image relationship. In 33 the ratio of R and S isomers at C-6 is 3.6:1, whereas that of 41 is 1:3. The slight increase in the proportion of the R isomer in 33 as compared with that of the S isomer in 41 may be due to a slower transfer of the deuteride from $NaBD_4$, than of a hydride from $NaBH_4$, to the carbonyl compound. Presumably, this rate difference allows more time for an optimum arrangement of the reactants in the transition state. This finding accords

with the report that the rate of transfer of a deuteride is slower than of a hydride (129) and that the rate determining step in borohydride reductions involves transfer of a hydride (75,130); this being the case, higher optical yields are expected when deuteride, rather than hydride, is used as the nucleophile (51).

Mass spectra of α-D-threose triacetate and tribenzoate

Mass spectral studies on deuterated specimens of D-threose tribenzoate and triacetate were conducted to see if fragmentation involves the specific loss of deuterium or hydrogen depending on the configuration of the most likely leaving fragments. The specific departure of deuterium or hydrogen in most mass spectral studies cannot be fully assessed, in view of the widely observed deuterium-hydrogen scrambling or randomization. Nevertheless, the elimination of acetate groups for instance in the fragmentation of per-O-acetylated monosaccharides, which in most cases probably takes place through cyclic six-membered ring intermediates (131), could conceivably favour abstraction of one hydrogen atom rather than another (e.g. as in 74).

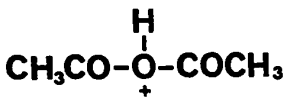


74

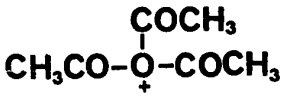
Interpretation of the mass spectrum of D-threose triacetate has been aided by use of mono and dideutero analogues, i.e., D-threose triacetate-4-d (66,67) and 4,4'-d_2. This study shows that none of the major fragmentation patterns involve loss of the C-4 hydrogens

II CONFIGURATION AT C-6

(Fig. 10). As is usual with per-O-acetylated monosaccharides (131), no molecular ion peak was detected and the most intense peak was that due to loss of an acetyl fragment (CH_3CO) m/e 43. Other peaks of mass 103 and 145 m/e representing di and tri-acetoxonium ions were observed, in agreement with the results of Biemann and co-workers (131).



m/e 103



m/e 145

Loss of an acetate radical gives rise to an intense peak at m/e 187, which shifts to 188 mass units for monodeuterated (66) and (67) and to 189 for the dideutero compound (69). Subsequent loss of acetic acid gives rise to a peak of moderate intensity at m/e 127. This latter ion suffers loss of ketene ($CH_2=C=O$) to give a product of mass 85, which is shifted to 86 and 87 for the corresponding mono- and dideuterated analogues. The resulting ketone probably does not enolize

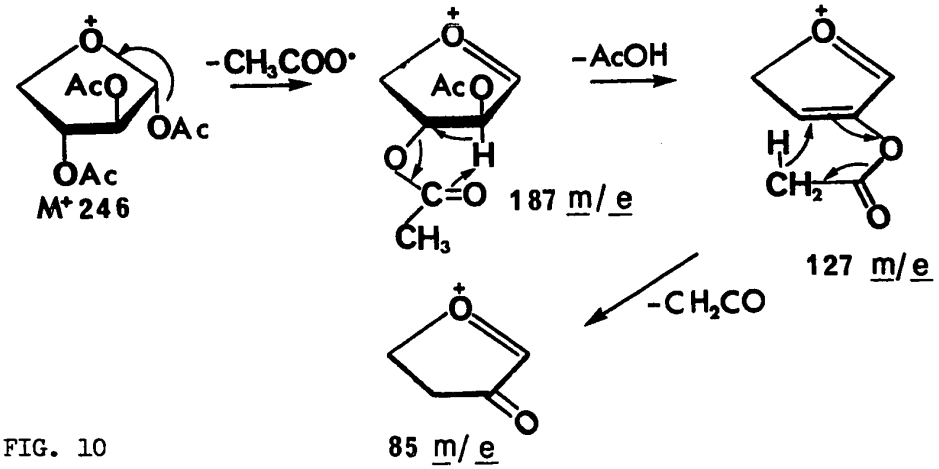


FIG. 10

with subsequent loss of water. Absence of enol-keto tautomerism in mass spectral fragments has been observed in other systems. A metastable peak of approximately m/e 93.5, probably arising from fragments of low abundance, was also detected in the spectrum of 70. Such a possibility has been discussed by Djerassi et al. (132).

The mass spectrum of D-threose tribenzoate also did not show recognisable fragments involving loss of the methylene hydrogens on C-4. The most intense peak (base peak) for this compound is the benzoyl radical (C_6H_5CO m/e 105); its appearance compares favorably with that of an acetyl group in the mass spectra of monosaccharide acetates (131). Elimination of a benzoate group most likely, as in the acetate, from the anomeric carbon, gives an intense peak of mass 311 (M-121). Subsequent loss of benzoic acid (mass 122) is characterised by a metastable peak at 114.8 m/e (calculated value 114.86) and gives a strong peak at m/e 189. No other major fragments involving the tetrose moiety are observed below mass 189. A relatively weak peak of mass 327 (M-105) representing loss of a benzoyl fragment is also observed. The suggested fragmentation pattern for D-threose tribenzoate is summarized in Fig. 11.

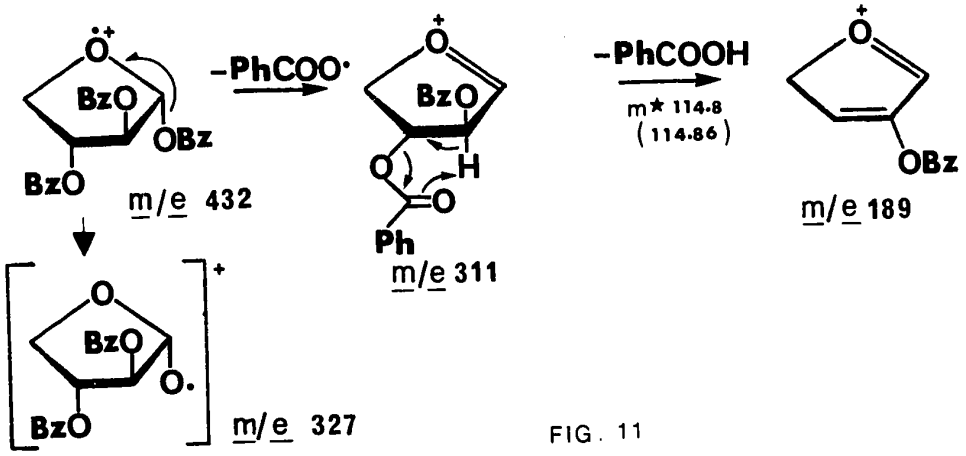
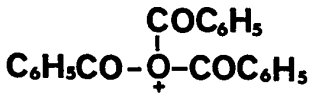


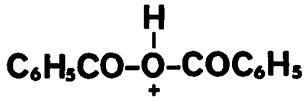
FIG. 11

II CONFIGURATION AT C-6

The mass spectrum of D-threose tribenzoate also shows a remarkably strong peak at m/e 331 which might be mistaken for a fragment containing the tetrose moiety. However, this peak is analogous to the triacetoxonium ion formed from the monosaccharide acetates and therefore is ascribed to a tribenzoxonium ion. At a lower mass number (m/e 227) a peak corresponding to a protonated dibenzoxonium ion also is registered and, similarly, parallels the protonated diacetoxonium ion of monosaccharide acetates.



m/e 331



m/e 227

Biemann and co-workers (131) have suggested that such ions arise from the elimination and protonation of acetic anhydride or the abstraction of an acetyl group by acetic anhydride to form the triacetoxonium ion; their conclusions have been substantiated by the use of deuteration techniques.

CHAPTER III

KINETIC STUDIES OF OXIDATION BY D-GALACTOSE OXIDASE

A knowledge of the relative reactivity of substrates such as methyl β-D-galactopyranoside-6-d (6R)(34) and -6-d (6S)(45), 6 and 47 towards D-galactose oxidase proved to be of primary importance in determining the stereochemistry of the enzymic dehydrogenation process.

Rate equation

It is customary in kinetic studies to formulate a rate equation expressing the manner of combination of reactants and formation of products. Before a rate equation is written down it is helpful to have a general idea of the mechanisms of a reaction. This knowledge is lacking for galactose oxidase, but a plausible mechanistic description has to include the assumption that the enzyme contains an electron acceptor. The suggestion of Cooper et al.(2) that galactose oxidase contains a flavin (FMN) as an electron acceptor is used here as a reasonable postulate to provide a working model to enable visualization of the possible mechanism. The rate equation can then be written as follows:

$$E_{ox} + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{ox}\text{—}G \xrightarrow{k_2} E_{red}\text{—}P_1 \xrightarrow{k_3} P_1 + E_{red} \qquad [1]$$

$$E_{red} + O_2 \xrightarrow{k_4} E_{red}\text{—}O_2 \xrightarrow{k_5} E_{ox} + H_2O_2\ (P_2) \qquad [2]$$

III KINETIC STUDIES

And in the presence of peroxidase and suitable chromogen, using Chance's mechanism (133):

$$E_{per} + H_2O_2 + BH_2 \longrightarrow E_{per} + B + 2H_2O \qquad [3]$$

In these equations E_{ox} is galactose oxidase in the oxidised form, G is the substrate; E_{red} is galactose oxidase reduced at the flavin moiety (or other electron acceptor); P_1 is the aldehyde product; P_2 is the second product, in this case hydrogen peroxide; E_{per} represents the peroxidase enzyme; BH_2 is the chromogen in the reduced form and B its oxidised form. $(E_{ox}\text{-}G)$ is the Michaelis enzyme-substrate complex (134); this isomerises internally in the transition state to give a product-like intermediate $(E_{red}\text{-}P_1)$ which then dissociates to give product P_1 and the reduced enzyme. The corresponding reaction constants are also shown in equations 1-3. The amount of enzyme that is reduced (E_{red}) is proportional to the product (P_1) and also to the amount of hydrogen peroxide produced, and in turn to the amount of chromogen oxidised.

Applying steady state (135) conditions for equations 1 - 3 , we have

$$k_1\left[E_{ox} - (E_{ox} - G)\right][G] = k_{-1}\left[E_{ox} - G\right] + k_3\left[E_{ox} - G\right]$$

$$\therefore k_1\left[E_{ox}\right][G] - k_1\left[E_{ox} - G\right][G] = k_{-1}\left[E_{ox} - G\right] + k_3\left[E_{ox} - G\right]$$

$$\text{i.e. } k_1\left[E_{ox}\right][G] = \left[E_{ox} - G\right]\left\{k_1[G] + k_{-1} + k_3\right\}$$

III KINETIC STUDIES

But the overall rate $v = k_3[E_{ox}\text{-}G]$ therefore

$$k_1[E_{ox}][G] = \frac{v}{k_3}\left\{k_1[G] + k_{-1} + k_3\right\} \qquad [4]$$

Applying steady state condition for equation 2, we have

$$k_4\left\{[E_{red}] - [E_{red}\text{—}P_2]\right\}[O_2] = k_5[E_{red}\text{—}P_2]$$

But overall rate $v = k_5[E_{red}\text{—}P_2]$

$$k_4[E_{red}][O_2] = \frac{v}{k_5}\left\{k_5 + k_4[O_2]\right\} \qquad [5]$$

Total amount of enzyme present $E_T = E_{red} + E_{ox}$

$$\frac{E_T}{v} = \frac{k_3 + k_5}{k_3 k_5} + \frac{k_{-1} + k_3}{k_1 k_3[G]} + \frac{1}{k_4[O_2]} \qquad [6]$$

According to equation [6] the velocity of the reaction is related to the substrate concentration [G] and also to the concentration of oxygen. Rate studies can be carried out, therefore, by varying either [G] or by observing the consumption of oxygen.

In the current study, it was assumed that the amount of substrate oxidised (G) is proportional to the amount of hydrogen peroxide produced. The latter is conveniently measured spectrophotometrically by use of a chromogen (in this instance O-toluidine which like the para-analogue (136) is oxidised to a O-nitroso toluene) giving an absorption band at 420 nm. Reaction samples were incubated at 37°, and measurements of optical density with time were obtained with a Gilford-Beckmann spectrophotometer. The rate data are reproduced in Fig. 12. These curves are virtually straight lines and their slopes give the initial rate constants (k in Table I).

It is evident from Fig. 12 and Table I that methyl β-D-galactopyranoside is oxidised faster than the dideutero carbinol 47, and

that there is an isotope effect of kH /kD = 12. A deuterium isotope effect greater than 3 or 4 is indicative of a primary kinetic isotope effect where the rate determining step involves cleavage of a C-H bond (136-148)

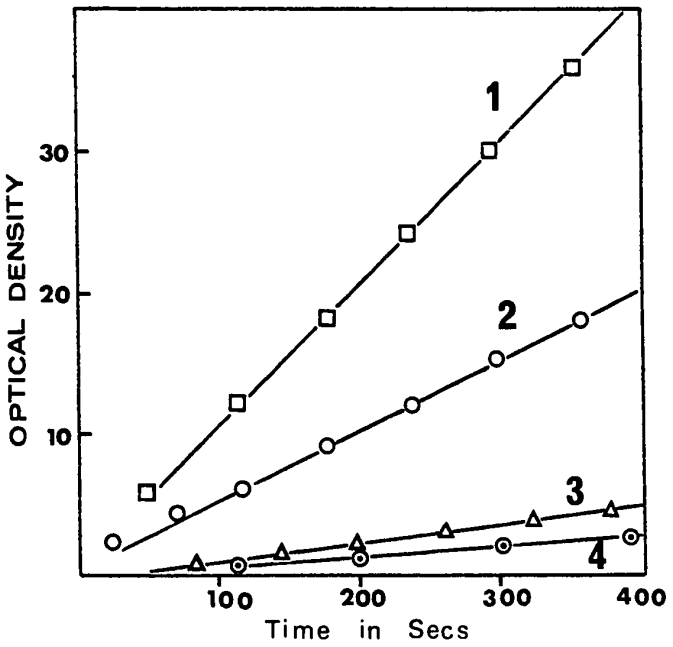


FIG. 12 Rates of reaction of D-galactose oxidase on: **1** methyl β-D-galactopyranoside (6); **2** compound 34; **3** compound 45; **4** methyl β-D-galactopyranoside-6,6-d_2 (47).

The theoretical kH/kD value calculated on the basis of vibrational zero-point energy difference between a C-H and a C-D bond is 6.9 (142). Other hydrogen-abstracting enzymes that exhibit primary kinetic isotope effects are known; these are the NADH-dependent dehydrogenases; yeast alcohol dehydrogenase (138,149), maleate dehydrogenase (150), lactate dehydrogenase (151,152) and liver alcohol dehydrogenase (138,153,154). The flavin dependent glucose oxidase shows a primary deuterium isotope effect of 10-15 (155).

Table I Action of D-Galactose Oxidase on Methyl β-D-Galactopyranoside [1]

Substrate	Rate[2]	Notation of k	Contribution of C-H bond cleavage A	Contribution of C-D cleavage B	Rate Calculated A+B
6	0.36	kH_S	-	-	-
34 (3.6 R:1 S)	0.18	kH_1	0.28	0.007	0.29
45 (1 R:3 S)	0.06	kD_1	0.09	0.008	0.10
47	0.03	kD_2	-	-	-

1 Colorimetric assay at 420 nm (experimental conditions).

2 Slope of the plot of optical density vs reaction time as shown in Fig. 12.

III KINETIC STUDIES

Stereochemistry and Stereospecificity

That methyl β-D-galactopyranoside-6-d predominantly 6R (34) should be oxidised faster than methyl β-D-galactopyranoside-6-d predominantly 6S (45) when both substrates contain one deuterium atom each at C-6, clearly suggests that the 'hydrogen' abstracted in 34 is protium and deuterium in 45 and that the enzyme is stereospecific, in its action.

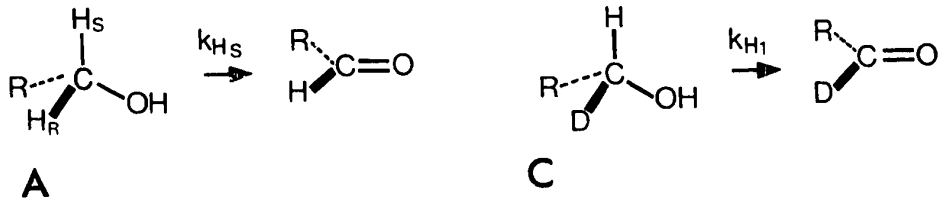
Since the protium in 34 and deuterium in 45 corresponds to the pro-S hydrogen (H_S) in methyl β-D-galactopyranoside (6), it follows therefore that D-galactose oxidase abstracts the H_S ligand irrespective of whether this position is occupied by deuterium or protium. In the oxidation of 45 where the departing 'hydrogen' is largely deuterium the rate is slow (0.06) and approaches that of the oxidation of the dideuterio compound (47)(0.03). In the oxidation of 34 the departing hydrogen is mainly protium and the oxidation rate is relatively fast (0.18), approaching that of 6 (0.36). These conclusions are supported by the constitution of the products isolated (next chapter).

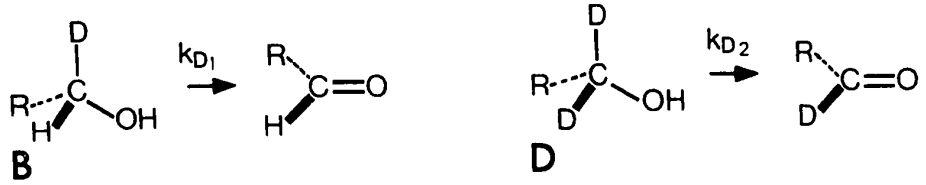
Secondary deuterium isotope effect

Ideally the rate of oxidation of 34 should be the same as that of 56, because in both cases, the departing 'hydrogen' is protium; similarly that of 45 should be the same as that of 47 because the species abstracted from both substrates is deuterium. Considering that the contribution to the total rate of oxidation of 34 by the 1/4.6 molar fraction of the 6S isomer is negligible (0.01), and to that of 45 by 1/4 molar

fraction of the 6R isomer is also negligible (0.02); that the substrate concentration always exceeds that of the enzyme, and that the proportion of the enzyme engaged at any moment of the reaction in complexing either the 6S isomer in 34 or the 6R isomer in 45 is negligible; it follows that the factor responsible for lowering the rate of oxidation of 34 relative to that of methyl β-D-galactopyranoside (0.36 vs 0.18) must be a secondary deuterium isotope effect. Hence, $kH_S/kH_1 = 0.36/0.18 = 2$ is the magnitude of the secondary deuterium isotope effect.

An estimation of the primary deuterium isotope effect may be made from the relative rates of cleavage of the specific C-H and C-D bonds, assuming that the constitution of the rest of each molecule, and subsequently the product, is the same. This is illustrated in reactions A-D below, (A stands for the oxidation of 6, B for that of 45, C for that of 34 and D for that of 47. Thus the true primary isotope effect is kH_S/kD_1, which equals 6; and it can be seen from Table I that this is also equal to kH_1/kD_2.





III KINETIC STUDIES

From reactions A-D the secondary deuterium isotope effect can also be estimated, and may simply be considered as the effect of deuterium rather than protium becoming the aldehydic 'hydrogen', or the effect of the mass of a geminal deuterium in the vicinity of the departing 'hydrogen'. This is simply kH_S/kH_1 or kD_1/kD_2. The two ratios are equal and have a value of 2.

Spectroscopic studies have shown that there is a change in molecular geometry associated with isotopic substitution. According to electron diffraction studies, a C-D bond in deuterated methane or ethane is about 0.008 atomic units shorter than a C-H bond in the same compound (156,157). Full deuteration of CH_3Cl and CH_3Br causes shortening of the C-Cl and C-Br bonds (158)(secondary isotope effect). From Raman spectra it has been suggested (159) that, on the average, a C-H bond in CH_3D is shorter than in CH_4 (secondary isotope effect). In NH_3 (160-162) and PH_3 (163), deuteration results in a decrease in the H-N-H and H-P-H bond angles. Recent NMR spectral studies on the first row compounds, have shown that a geminally bonded deuterium (as in CHD-X) deshields hydrogen (29). For a CH_2O-CHDO system the deshielding parameter of H by D is 0.023-0.029 ppm (apparently the only system of the compounds examined that was solvent dependent)(29), and for a CH_3D the deshielding parameter is 0.001 (29,164). Furthermore, it has been suggested that chemical shifts of about 0.02 ppm correspond to an average bond angle-change of 10' or a bond length change of 0.0012 atomic units (29).

In the NMR spectrum of enzyme enriched (or resolved) methyl β-D-galactopyranoside-6-d (6S) benzoylated (page 90), the H-6 doublet is more unfield than the corresponding H_R quartet of methyl β-D-galactopyranoside tetrabenzoate by 0.05 ppm and calls for a bond angle change at C-6 in the former substrate of 25' and a C-H bond length change of 0.003 atomic units.

III KINETIC STUDIES

Apart from the geometrical change introduced by the isotope, the inductive effect (165-168, 169-170) of deuterium may lower the acidity of the departing hydrogen. Also the rehybridisation from sp^3 to sp^2 takes place more readily when the carbinol bears H rather than D (171,172).

One is therefore led to assume that bond angles, bond lenths and electron distribution at the deuterated carbinols 34, 45, 47 are significantly different from those of the non-deuterated glycoside (6). These geometrical changes are sensed by the enzyme and bring about retardation of reaction rates in a secondary role.

In the oxidation of free D-galactose and its C-6 deuterated isomers, the calculated rates were in agreement with those observed (172)(Table II); and it was concluded that secondary isotope effects are marginal. But in the oxidation of glycosides 6, 34, 45 and 47 there is a relatively large difference between rates calculated and observed (Table I). Perhaps this anomaly arises because D-galactose, being less reactive than 6, conceals a deep-seated secondary isotope effect.

Mechanism of D-galactose oxidase action

In the D-galactose oxidase reaction two main steps are recognised; first, the abstraction of the hydrogen and its transfer to oxygen and, secondly, the decomposition of hydrogen peroxide by peroxidase or catalase. The latter reaction is known to proceed via a free radical mechanism (174), whereas the former might involve a free radical, hydride or proton pathway.

According to Cooper et al. (2), D-galactose oxidase contains

Table II Rates of oxidation of D-galactose and deuterated D-galactoses by D-galactose oxidase[1]

Substrate	Rate[2] (obs.)	Contribution due to C-H	Contribution due to C-D	Rate (calc.)
D-galactose	0.77	-	-	-
D-galactose-6-d (33) (3.6 R:1S)	0.66	0.60	0.02	0.62
D-galactose-6-d (41) (1R:3S)	0.28	0.19	0.08	0.27
D-galactose-6,6'-d_2 (46)	0.10	-	-	-

1. Colorimetric assay at 420 nm (experimental reaction).

2. Slope of the plot of optical density vs reaction time from 2 to 12 min.; during this initial period each plot was virtually a straight line. The initial concentration in these reactions is higher than that described in Fig. 12 and Table I.

III KINETIC STUDIES

flavin-mononucleotide (FMN) as the prosthetic group. This is suggestive evidence that the oxidation, as for other (175,176) flavin-mediated electron transfers, does not proceed via a free radical mechanism (177). Brown and Hamilton (177) have suggested that flavins promote a proton-abstraction process, possibly as depicted in Fig. 13. According to this mechanism the carbinol oxygen attacks the flavin at C-4a of 75 to give 76; (not C-2 as was tentatively suggested for a similar process for D-glucose oxidase (155)).

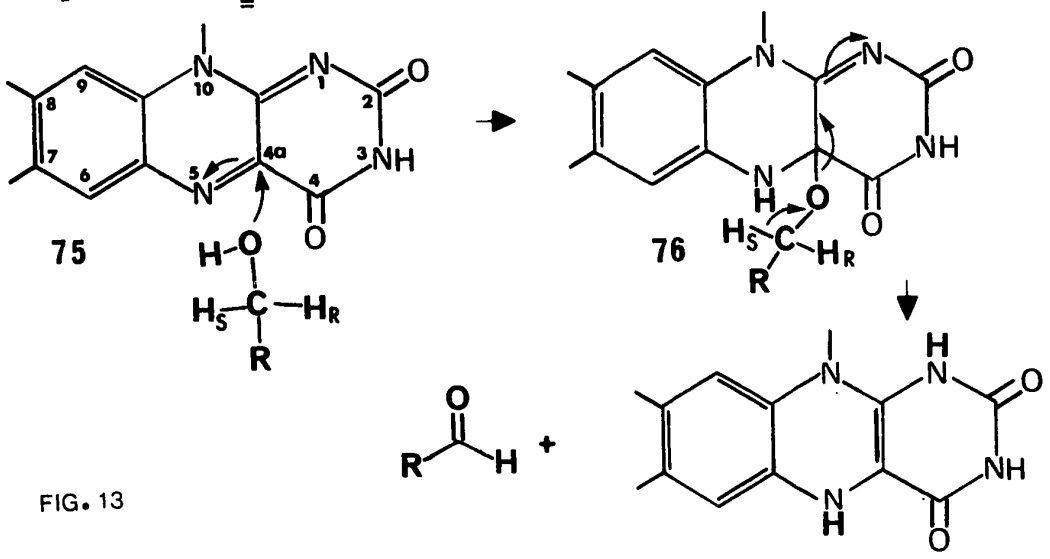


FIG. 13

Such a reaction should be subject to a solvent deuterium isotope effect. In the reaction of D-galactose oxidase, the rate in D_2O is substantially slower for 6 than in H_2O (kH_2O/kD_2O = ca. 2) This is an additional indication that the reaction proceeds in a manner resembling that shown in Fig. 13.

In the flavin-catalysed oxidation of L-amino acids, a primary and also a solvent deuterium isotope effect have been observed (178). Brown and Hamilton (177) attribute this solvent effect to the stage at which protonation of N-1 of the flavin moiety occurs. Once again, since both primary and solvent deuterium isotope effects are observed in the D-galactose oxidase reaction, it is reasonable to propose that this reaction also proceeds with proton abstraction.

III KINETIC STUDIES

Since the nature of the proton abstraction process by the enzyme is not yet known, electron spin resonance (ESR) spectroscopy should serve to help identify the type of species involved in the D-galactose oxidase reaction i.e., a free radical process should be readily detectable; otherwise negative evidence would be obtained.

CHAPTER IV

PRODUCTS OF THE ACTION OF GALACTOSE OXIDASE ON METHYL β-D-GALACTOPYRANOSIDE AND ITS 6-DEUTERO ANALOGUES

Although the kinetic studies described in the previous chapter provide a clear indication as to which of the 6-hydrogens of D-galactose or methyl β-D-galactopyranoside is specifically removed by galactose oxidase, the stereochemistry of the reaction has been confirmed by examining the products of the isotopically-labelled substrates used in those studies. In addition, this chapter deals with some characteristics of the oxidation products themselves.

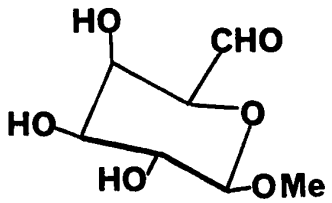
Reaction conditions used for the preparation of products were similar to those of the kinetic experiments, although on a larger scale. In addition, galactose oxidase was coupled with catalase in order to decompose enzyme-inhibiting hydrogen peroxide formed, and thereby shift the overall oxidation reaction to the right. The course of reaction was followed conveniently by thin layer chromatography.

Undeuterated methyl β-D-galactopyranoside (6) was examined initially in order to find optimum experimental conditions. In a typical reaction, the concentration of substrate was 5×10^{-2} M. By following the reaction by tlc, four products were detected. The fastest-moving of these was a minor component (ca. 5%) having R_f 0.75 which stained strongly with 2,4-dinitrophenylhydrazine and absorbed U.V. light. This behaviour suggested that the product is an α,β-unsaturated aldehyde, and a further indication of this was obtained

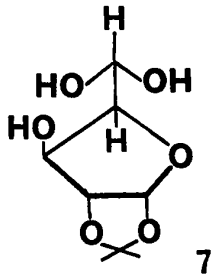
IV OXIDATION OF SUBSTRATES

by isolating the compound from a cellulose column and recording its U.V. spectrum; it showed λ_{max} at 250 nm in water characteristic of compounds of this class(97). It is of interest to note that no α,β- unsaturated aldehydes have been detected previously among D-galactose oxidase products. This compound was further investigated (see below) to clearly establish its identity. After allowing the enzyme reaction to proceed for three hours, the reaction product was worked up so as to minimise degradation of sensitive carbonyl compounds. Thus the reaction mixture was stirred with a mixed resin bed (H^+ and HCO_3^-) to remove buffer cations and anions as well as some protein material. The ion-free solution, on evaporation gave an amorphous solid contaminated with soluble protein material.

The major fraction of the D-galactose oxidase reaction product was undoubtedly methyl β-D-galacto-hexodialdo-1,5-pyranoside (77). Its mobility on a tlc plate was slightly higher (R_f. 0.63) than that of

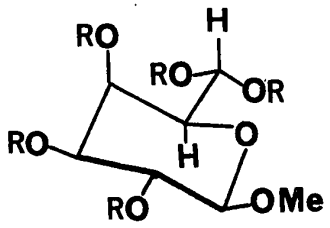


77



78 $J_{4,5}$ = 7.5 Hz

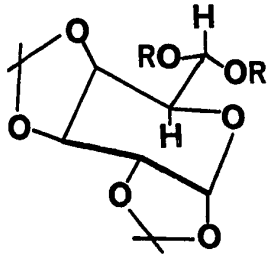
δ H-5 = 5.35 ppm



79 R = H, $J_{5,6}$ = 7.5 Hz

δH-6 = 5.22 ppm

79a R = Ac



80 $J_{5,6}$ = 7.1 Hz

δH-6 4.95 ppm

unreacted 6 (R_f. 0.47), and it stained with 2,4-dinitrophenylhydrazine at room temperature giving a yellow hydrazone which turned brick-red upon heating. The intensity of 77 was seen by chromatography to reach its maximum in about one hour reaction time. A third component, present in minute proportion in some preparations had nearly the same mobility as 6 and, also since it did not give a colour reaction with 2,4-dinitrophenylhydrazine, undoubtedly was unreacted substrate.

The fourth component (R_f. 0.46) was relatively major and had staining properties closely similar to those of aldehyde 77. This inferred that the product was either a dimer of 77, or a hydrated form of the aldehyde (aldehydrol compound 79), either of which would be expected to react with 2,4-dinitrophenylhydrazine, although its low chromatographic mobility favoured the dimeric assignment; this latter possibility is substantiated by evidence presented below.

NMR spectrum of products resulting from the enzyme oxidation of 6

The NMR spectrum of the amorphous solid total product in deuterium oxide showed no signal in the region of 9-10 ppm corresponding to a free aldehyde proton. Instead, a doublet with a splitting of 7.5 Hz centred at 5.22 ppm was observed. The signal due to H-6 in the original substrate was diminished considerably and those of the other protons, with the exception of H-1 and H-4, were not clearly resolved. However, when the solvent was DMSO-d_6, the NMR spectrum showed a moderately strong singlet at 9.51 ppm diminishing with D_2O exchange and represented a free aldehyde signal. In this aldehydic proton resonance region, there was also another weak signal at 9.22 ppm, which suggested the pres-

ence of an α,β-unsaturated aldehyde for it was accompanied by a broad doublet at 6.02 ppm typical of compounds of this class (97). This implies that in anhydrous DMSO a fair proportion of 77 exists as a free aldehyde and that the presence of an α,β-unsaturated aldehyde cannot be ignored.

According to Perlin (179), compound 78 in an aqueous solution is hydrated, and in this instance also a large vicinal splitting for H-4, H-5 ($J_{4,5}$ = 7.5 Hz) suggests that H-4, H-5 are trans-diaxially disposed.

Horton et al (180) have studied the spectral behaviour of 1,2:3,4-di-O-isopropylidene-α-D-galacto-hexodialdo-1,5-pyranose (12) in deuterium oxide, in chloroform, and in slightly moist chloroform. In aqueous media, 12 is easily hydrated to form an aldehydrol (80), and the large coupling constant (J = 7.1 Hz) observed for H-6, H-5 suggests that H-5 and H-6 have a trans-diaxial relationship. The tendency of aldehydes to hydrate in aqueous medium or moist solvents is now a well-known phenomenon (181, 182), and NMR spectroscopic studies of various other hydrated aldehydes have been described (180, 182).

Comparison of NMR parameters for the enzyme product (77) with those of 78 and 80 suggests that 77 exists mainly as the aldehydrol (79). Since there were no other prominent signals in the NMR spectrum of 77, it is clear that the compound behaving on tlc as a dimer exists mainly as a hydrated aldehyde when in an aqueous system.

Compounds such as 78, 79, and 80 have prochiral centres at C-6 just as do the parent monosaccharides. It should be interesting in future to use ^{17}O NMR spectroscopy (183) to tackle the orientation problem of prochiral groups at C-6 of these compounds, and possibly the mechan-

ism of hydration.

Acetylation of the enzyme products

Acetylation of the enzyme products was carried out in the cold with acetic anhydride-pyridine so as to minimize side reactions or decomposition. An attempt to use sodium acetate at 100° as the catalysing agent resulted in a poor yield of product. Tlc examination of the acetylated reaction mixture showed a fast moving, U.V.-absorbing spot suggesting the presence of an α,β-unsaturated aldehyde, a slow moving major spot, and a very weak spot corresponding to unreacted substrate (as its acetate).

These acetylated products were separated by chromatography on a column of silica gel. The fastest-moving component (82), comprising about 25 percent of the total, gave an NMR spectrum (Fig. 14A) indistinguishable from that of methyl 2,3-di-O-acetyl-6-aldehydo-4-deoxy-L-*threo*-hex-4-eno-1,5-pyranoside prepared by a different procedure (97), and its identity was confirmed by preparation of the crystalline 2,4-dinitrophenylhydrazone. Since this compound in a non-acetylated form (i.e. 81) had been detected as a much more minor component of the original enzymic digest, it must have arisen mainly from aldehyde 77 by an α,β-elimination during acetylation. The isolation of this aldehydic derivative gave further proof that the product of the D-galactose oxidase reaction is not a D-galactonolactone as claimed originally by Cooper *et al.* (2) but a D-galactohexodialdose as reported by Horecker *et al.* (3,4).

IV OXIDATION OF SUBSTRATES

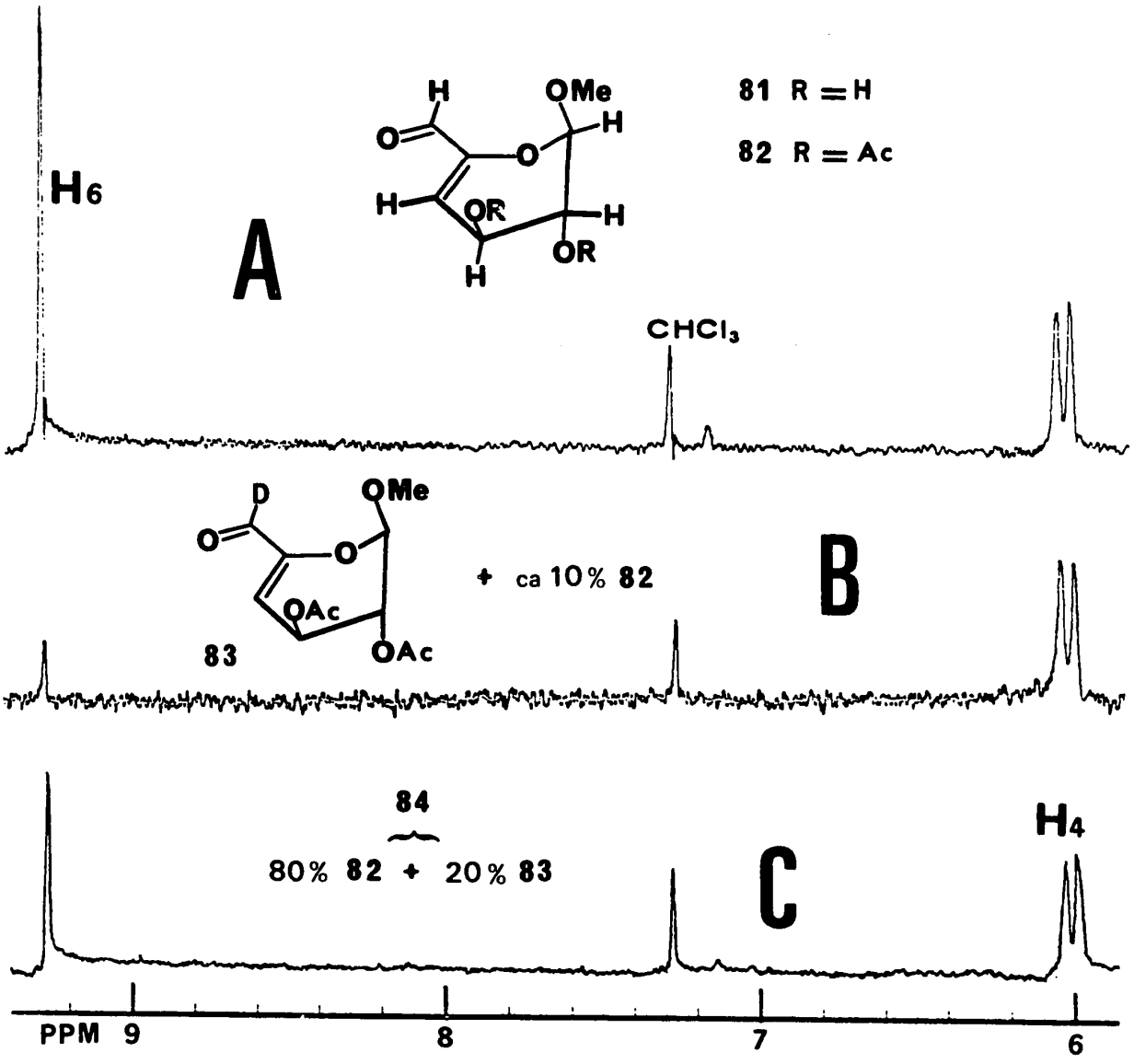


FIG. 14 Partial NMR spectrum (100 MHz, 1000 Hz sweep width) in $CDCl_3$, of the olefinic aldehyde isolated from the acetylated enzyme product: A, spectrum of 82 from non-deuterated 6; B, spectrum of 83 from substrate 34 showing the highly diminished aldehydic H-signal due to the presence of deuterium; C, spectrum of the olefinic product from substrate 45 showing 80% of an aldehydic H-signal.

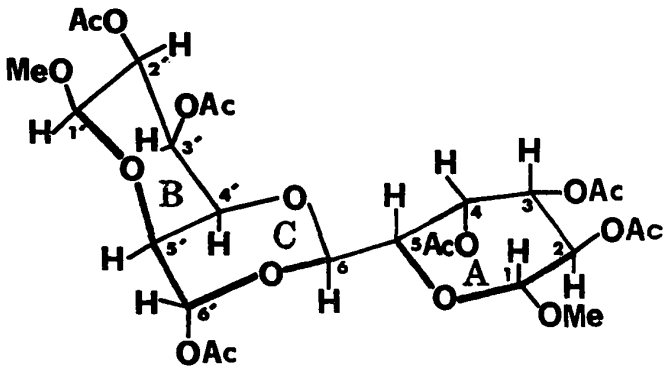
IV OXIDATION OF SUBSTRATES

Noteworthy in Fig. 14A is the low field singlet (at 9.26 ppm) ascribable to H-6. Since this signal is well separated from all others, and its integral corresponds to one proton, it provided a particularly suitable standard with which to compare the corresponding signal produced by the deuterated oxidation products.

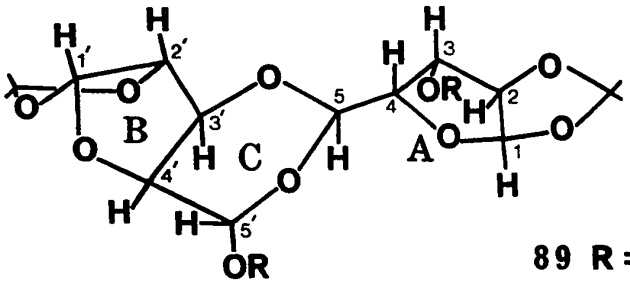
Aside from a few percent (≤ 5) of acetylated 6 the only other product found was the slowest moving fraction, amounting to about 70% of the total. This material (85) was crystalline (mp 206-8°) and its mass spectrum (see below) corresponded to that expected of a dimer of aldehyde 77.

NMR spectrum of dimer 85

The NMR spectrum of 85 (Fig. 15) showed the presence of six acetate and two methoxy-groups. This indicated that two molecules of compound 77 had been fused together unsymmetrically so that each parent monomer contributed nonequivalent methoxy and acetate groups. The presence of five pairs of proton signals retaining the splittings of the parent monomer suggests that the original stereochemistry of the pyranose ring is retained. Thus the possibility of having a product resulting from an intramolecular hemiacetal linkage between the OH-3 group and the C-6 aldehyde can be ruled out, since this would entail a complete change of stereochemistry of the parent monomer from C 1 to 1 C (86).

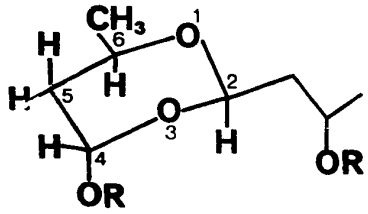


85



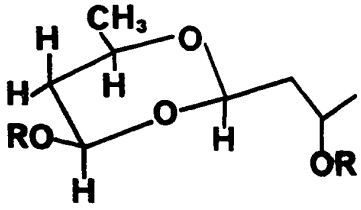
89 R = H

90 R = Ac



91 R = H

92 R = Ac



93 R = H

94 R = Ac

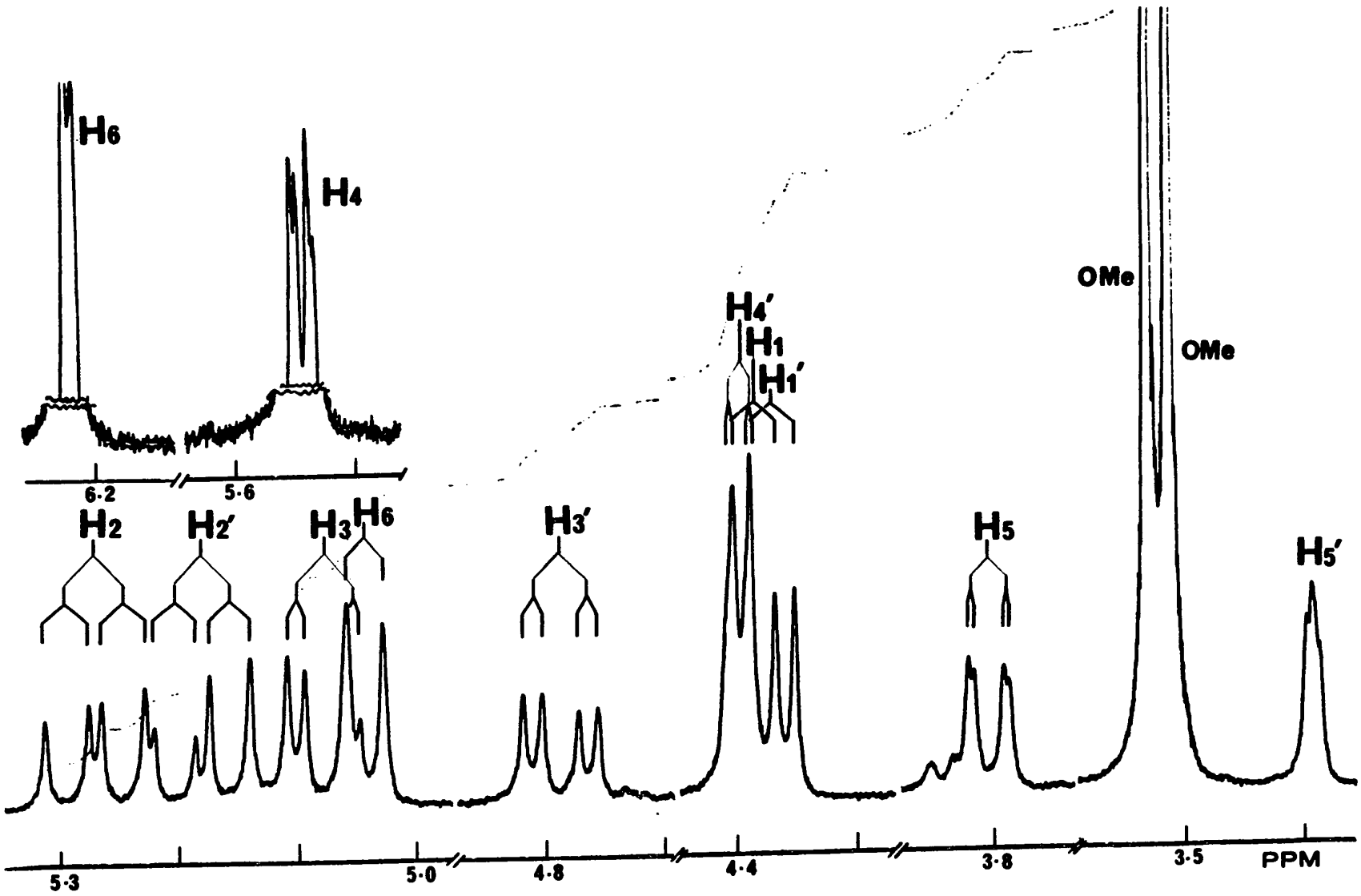
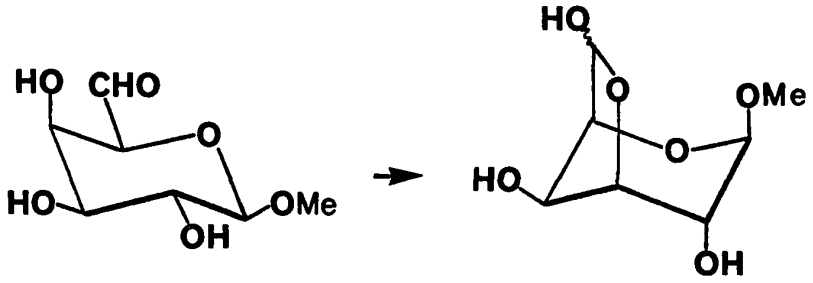


FIG. 15 Partial NMR spectrum (220 MHz, 500 Hz sweep width) of the crystalline dimer 85 in $CDCl_3$. H-6' and H-4 insets are recorded at a much higher gain than the rest of the spectrum.

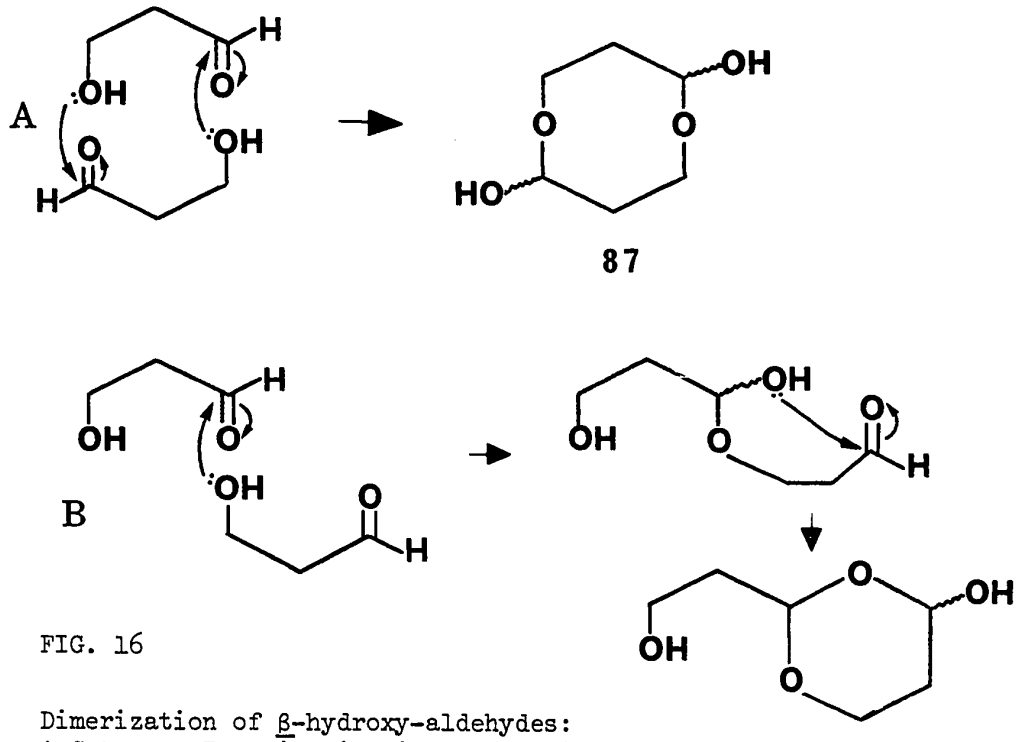
IV OXIDATION OF SUBSTRATES



C1 1C

86

Different ways in which 77 might dimerize can be visualized (Fig. 16): the process could take place with each monomer contributing a hydroxyl linked to an aldehyde group of the other (A), or else, only one monomer need contribute a hydroxy-group and two aldehyde groups, one from each monomer, could be involved (B).

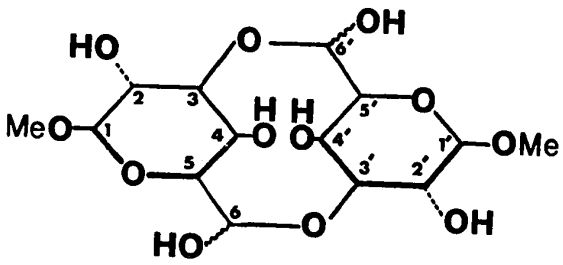


87

FIG. 16

Dimerization of β-hydroxy-aldehydes:
A Category I; dimerization proceeding to give a symmetrical 1,5-oxocane; B: Category II, formation of an unsymmetrical 1,3-dioxane

Dimerization of the first category would result in the formation of a symmetrical ten-membered ring if OH-3 participates, or an eight-membered ring (1,5-oxocane)* if OH-4 participates. Participation of OH-2 appears to be impossible because of the distance required, and OH-3 participation appears unlikely because the dimer so formed would incorporate a strong repulsive interaction between its C-4 substituents (88).



88

This therefore leaves OH-4 as the probable candidate for the dimerisation process. In this event, it can be seen that OH-4 is β to the 6-aldehydo group and hence 77 should behave as a β-hydroxy-aldehyde. Bergmann et al (184,185) have noted the possibility that a β-hydroxy-aldehyde may dimerise to form an eight-membered ring (186)(a 1,5-oxo-

* 1,5-Oxocanes need not be symmetrical because the two hemiacetal hydroxy-groups can assume different orientations; the impact of this difference on the overall geometry and therefore NMR spectrum would probably be small.

cane)(87), i.e. dimerisation of the first category. Later Späth et al. (187-189) showed experimentally that dimerisation of some β-hydroxy-aldehydes can proceed to give six membered 1,3-dioxanes and not 1,5-oxocanes. Nevertheless Dreiding Models indicate that dimerisation of 77 could in fact give a rigid 1,5-oxocane in contrast to simple saturated and heterocyclic eight-membered rings (190-192), which are flexible.

Dimerisation of the second category (Fig. 16, B) proceeds to give a 1,3-dioxane in which only one of the hydroxy-groups is anomeric. Schaffer and Isbell (193) noted that the acetate groups of dimeric 1,2-O-isopropylidene-α-D-xylo-pentodialdofuranose acetate (90) exhibited different behaviour on saponification. Accordingly, one acetate group, presumably the anomeric one because it would be activated by electronegative neighbouring groups, was hydrolysed faster than the other hydroxy-group. The latter, at C-3, is sterically hindered as shown by the fact that it is difficult to benzoylate 1,2-O-isopropylidene-α-D-xylofuranose.* It is therefore reasonable to conclude that the dimerisation of 1,2-O-isopropylidene-α-D-xylo-pentodialdofuranose , which is also a β-hydroxy-aldehyde, proceeds in this manner. Although four isomers are possible for the Schaffer-Isbell dimer it appears that steric interactions greatly favour one of them, as pointed out by Inch (194) in an NMR spectral study of the acetate. The NMR spectrum of paraldol (91, 93) formed from β-hydroxy-butanal, is also consistent with a 1,3-dioxane ring form (195).

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* Forcing conditions, i.e. long reaction time and excess benzoyl chloride achieved 3,5-di-O-benzoylation of 1,2-O-isopropylidene-α-D-xylofuranose-5-d (61) (page 37).

IV OXIDATION OF SUBSTRATES

By comparing NMR spectral data for dimeric 1,2-O-isopropylidene-α-D-xylo-pentodialdofuranose (89) in DMSO and pyridine-d_6, and for its acetate (90) in chloroform-d_6, and also, of β-hydroxy-butanal (91, 93) and its acetylated forms (92, 94)(195) with the spectrum of the acetylated dimeric D-galactose oxidase product, the assignment of structure (85) to the latter dimer appears to be a logical conclusion.

Structure 85 requires that ring A be independent of the 1,3-dioxane ring C. Thus the chemical shifts for H-1, -2, -3, -4 and -5 should be nearly the same as those found for methyl β-D-galactopyranoside tetraacetate itself (95); this is seen to be the case (Table III). Table III also shows

Table III Chemical Shifts in PPM of Ring Protons of 85 and Methyl β-D-Galactopyranoside Tetraacetate 95 in Chloroform-D

95	dimer 85 ring A	dimer 85 ring B
H-1 4.40	4.37	H-1' 4.39
H-2 5.21	5.27	H-2' 5.18
H-3 5.00	5.08	H-3' 4.79
H-4 5.49	5.55	H-4' 4.40
H-5 3.90	3.81	H-5' 3.39

that with the exception of H-1' and H-2', which are far from the point of fusion of ring B and C, the chemical shifts of the other protons of ring B diverge from those of 95. In particular, H-4' absorbs at approximately 1 ppm upfield from H-4 of 95. That is, the OH-4' on acquiring an ether function through linkage to ring A attains a tendency

to release electrons and in this way H-4' as well as the trans-disposed protons H-3' and H-5' adjacent to it are shielded much more than when OH-4 is esterified. The shielding effect on H-4 of an electron releasing substituent at C-4 as well as on the protons trans to the substituent, i.e. H-3 and H-5 in methyl β-D-galactopyranoside derivatives, has also been observed with methyl 4-deoxy-4-iodo-β-D-galactopyranoside and derivatives (Chapter VI). These chemical shift differences suggest that only OH-4' participates in the dimeric linkage and that OH-4 (of ring A) does not. In addition, H-4' of ring B constitutes equatorial H-5 with respect to the 1,3-dioxane ring C. Such protons are known to absorb at higher fields than the axial protons on the same carbon (196,197) although in cyclohexanes and pyranoses, equatorial protons of a methylene group resonate at lower fields than axial protons (124,123).

In ring A of 85 the coupling parameter $J_{5,6}$ is 7.1 Hz which suggests that H-5 is trans-axial to H-6, as in aldehydrol 79 where $J_{5,6}$ is 7.5 Hz. Therefore, the two oxygens of the 1,3-dioxane ring are disposed with respect to pyranose ring A in the same way as in the aldehydrol 79. The corresponding protons in 89 give $J_{4,5}$ 7 Hz*. The chemical shift for H-6 of 85 is 5.04 ppm, whereas the corresponding proton of 90 (H-5) resonates at 5.11 ppm. The chemical shift for H-6' is 6.22 ppm. This is the region for anomeric proton signals of α-D-hexopyranose acetates (198), e.g. for α-D-glucose pentaacetate H-1 resonates at 6.34 ppm, for α-D-mannose pentaacetate at 6.09 ppm, and for α-D-galactose pentaacetate at 6.36 ppm. In dimer 92, which has an axial acetoxy-group, the anomeric

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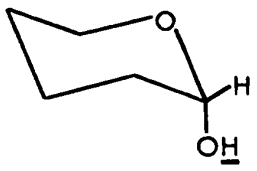
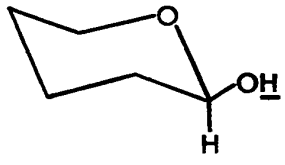
* Inch (194) reported that $J_{4,5}$ of 90 is 5.4 Hz, and suggested that free rotation accounts for this small value. It is felt here that this value could have been erroneously reported, as there is no obvious basis for such a decrease.

proton resonates at 6.15 ppm whereas the anomeric proton of 94, having an equatorial acetoxy-group, resonates at 5.75 ppm. The reported value for the corresponding proton in 90 is 6.36 ppm (194). In addition, considering that the Karplus curve holds for 1,3-dioxanes (199,200) then the coupling parameter J= 1.3 Hz observed for H-5', H-6' supports a gauche disposition for these protons. This value (1.3 Hz) falls in the range that is generally encountered with 1,3-dioxanes for $J_{4e,5e}$ (i.e., 0.6-1.9 Hz)(196,197,201,202), whereas $J_{4a,5e}$ is usually larger (2.6 to 3.2 Hz). The anomeric effect (203,204) should also favour an axial acetoxy-group at C-6'.

The anomeric centre, C-5' of 90, has been assigned an axial orientation based on a value of $J_{4',5'}$ of 0.5 Hz (194). In the current study the NMR spectrum of the non-acetylated dimer (89) in DMSO was recorded. A doublet found at 7.44 ppm with a splitting of 4.5 Hz was ascribed to the anomeric (O-5') hydroxy-proton. This is characteristic of an axial anomeric hydroxy-proton of pyranoses (205-207). An equatorial hydroxy-proton has $J_{\underline{OH},H-1}$ of 6.7 Hz. Thus on going from



$J_{\underline{OH},H-1}$ = 4.0 - 4.5 hz



$J_{\underline{OH},H-1}$ = 6.0 - 6.7 Hz

89 to 90 there is no basic change in ring structure nor orientation at the anomeric centre.

When methyl β-D-galactopyranoside-6-d predominantly 6R (34) was the enzyme substrate, the isolated product corresponding to 85 gave virtually no signal ascribable to H-6 and H-6', and this gave further evidence on the type of proton abstracted by D-galactose-oxidase, i.e., on the basis of the stereochemistry of hydrogen abstraction defined earlier by the kinetic results, the 6R diastereoisomer loses hydrogen equivalent to H_S in the non-deuterated 6; deuterium which takes the place of H_R is retained to form H-6 and H-6' of the dimer (85).

Stereochemistry of dimerisation

A stepwise mechanism may be envisaged for dimerisation of the β-hydroxy-aldehyde 77, analogous to that discussed briefly by Jedlinski and Majnusz (208) for α-chloro-β-hydroxyaldehydes. In the formation of a compound such as 85 from 77, Dreiding models suggest that steric factors favour the addition of ring B OH-4 to the C-6 carbonyl group from the si-face, so that the carbonyl oxygen becomes cis with respect to O-5 of ring A (Fig. 17). The resulting hemiacetal hydroxy-group then adds to the carbonyl, which becomes C-6' of ring B, again from the si-face, but with O-6' trans to O-5'. In this way a perfect chair conformation is elaborated for the 1,3-dioxane ring without twisting any bonds. The same mechanism can be envisaged to account for the dimerisation of 1,2-O-isopropylidene-α-D-xylo-pentodialdofuranose (59), in this instance OH-3 and C-5 being

involved in the addition reactions.

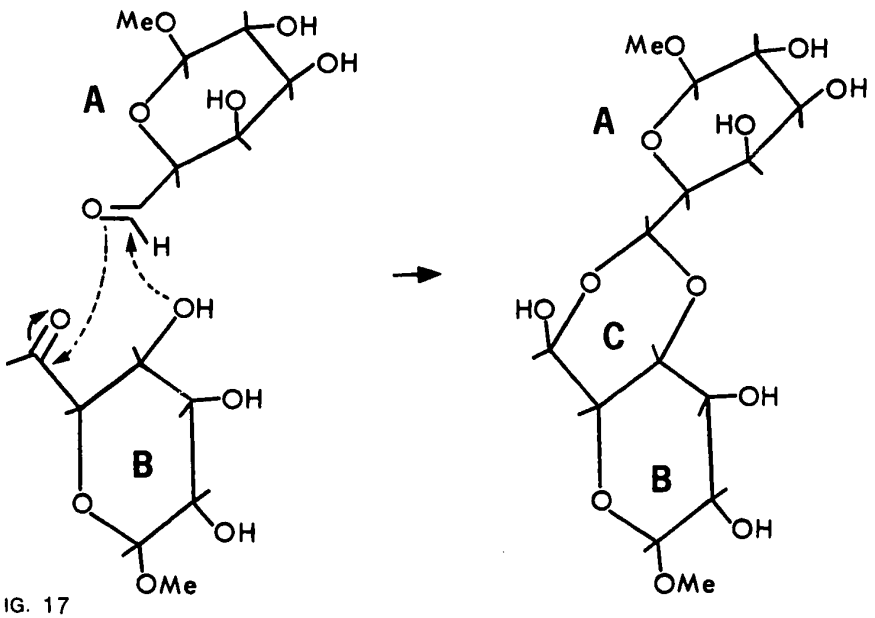


FIG. 17

The NMR spectrum of syrupy dimer 85, i.e., before purification is the same as that of the pure crystalline dimer, hence the mother liquours contained only 85 and no other anomer. Therefore, unlike the acetylation of 93 (195), only a single anomer is formed during the esterification process leading to 85.

The mass spectrum of 85*

Additional information on the structure of 85 was obtained from mass spectral data which showed characteristic features common to per-O-acetylated hexopyranosides (209) and hexoses (131). Thus, there were peaks at 637 and 679 m/e corresponding to M+1 and M+43 of hexopyranoside acetates. Also, strong peaks at 145 and 103 m/e due to acetoxonium ions, found in the spectra of per-O-acetylated hexopyranoses but not of hexopyranosides, were also absent from the spectrum of 85.

* The partial mass spectrum of 85 is shown as Appendix A p. 211.

IV OXIDATION OF SUBSTRATES

Several fragmentation patterns have been recognized, and are in close accord with the structure assigned to dimer 85. These patterns have been arbitrarily designated A to F, and some appear more than once due to the complexity and multiplicity of acetate functional groups.

Fragments leading to A ions are schematically shown in Fig. 18. An intense peak (A_1) appears at (M-59) mass units and corresponds to a loss of an acetoxy-radical (CH_3-COO). It appears that the latter is derived from the anomeric acetoxy-group at C-6' because glycosidic acetates are commonly lost as radicals rather than as neutral acetic acid. The A_1 ion $(M-59=577)^+$ loses acetic acid, most likely from ring A to give ion A_2 (m/e 517). This transition gives rise to an intense metastable peak at 463.3 mass units (calculated 463.3). The fact that glycosidic acetates are not eliminated in the form of neutral acetic acid suggests that there are not two anomeric acetates, as would be required for a 1,5-oxocane structure. The third fragment A_3 could arise from the loss of ketene (CH_2=C=O, mass 42) from A_2 which suggests that the conversion of A_1 into A_2 takes place in the neighbourhood of another acetoxy-group, most likely that of C-3 or C-3'. In this way a double bond is generated next to an acetate group, which then leaves as ketene, according to the mechanism proposed by Biemann et al. (131). Glycosidic acetoxy-groups whether in a 1,5-oxocane structure or 85 would still lack an adjacent acetate group, and their departure would not create conditions for loss of ketene. Ion A_3 (m/e 475) loses acetic acid to give ion A_4, presumably from ring B, which in turn eliminates ketene followed by acetic acid giving ions A_5 and A_6, respectively. Particularly noteworthy in this A series of ions is the fact that in the spectrum of 85 bearing deuterium at C-6 and C-6', the lines are all shifted upward by two mass units.

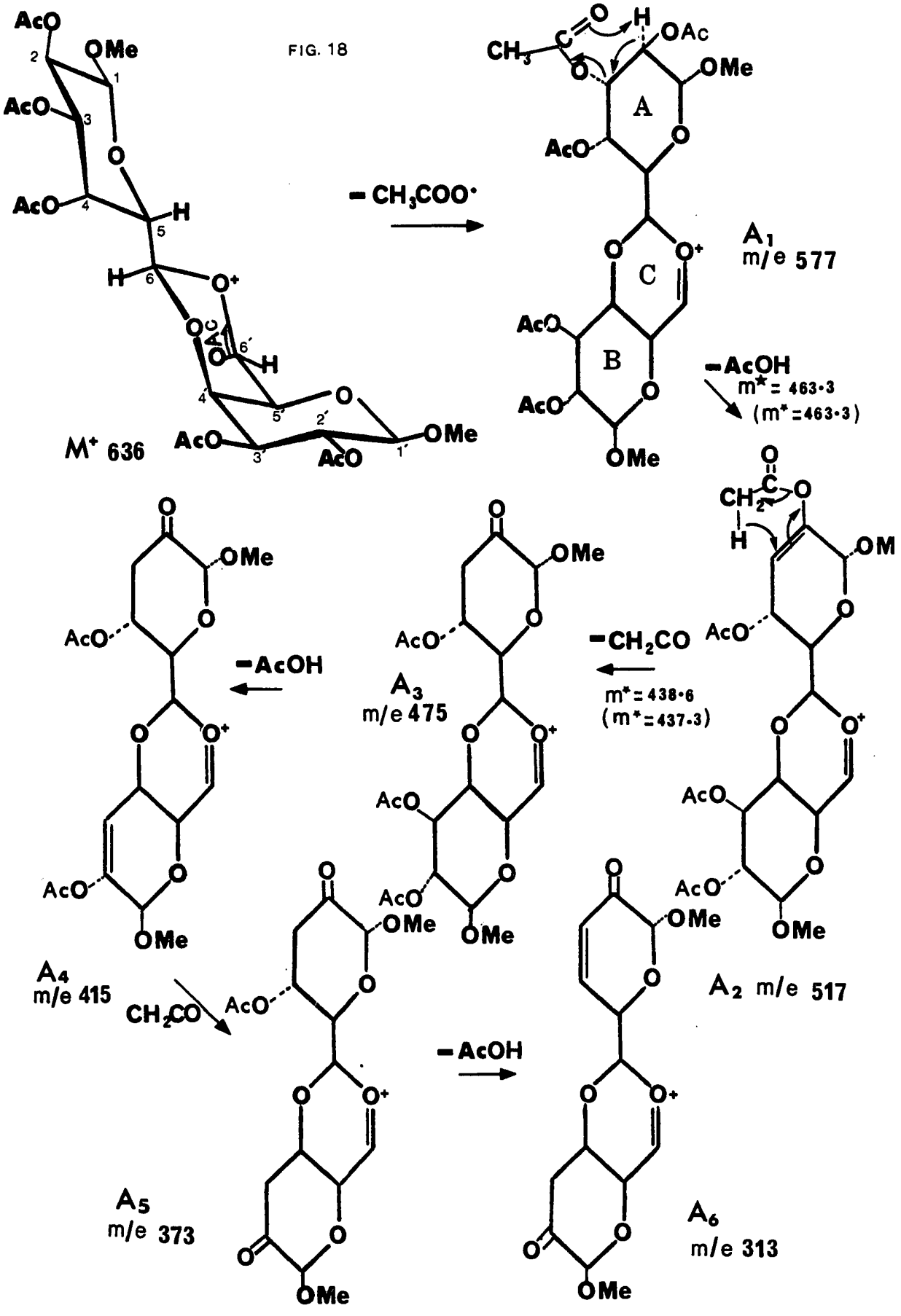


FIG. 18
M+ 636
- CH3COO·
A1 m/e 577
-AcOH
m* = 463·3
(m* = 463·3)
-AcOH
A3 m/e 475
-CH2CO
m* = 438·6
(m* = 437·3)
-AcOH
A4 m/e 415
CH2CO
A2 m/e 517
-AcOH
A5 m/e 373
A6 m/e 313

IV OXIDATION OF SUBSTRATES

The B series of ions, shown schematically in Fig. 19, starts with the cleavage of the C-6, C-5 bond to give B_1, an ion of strong intensity having m/e 347, or m/e 349 in the spectrum of the C-6, C-6' deuterated dimer. The latter value indicates that there is no hydrogen-deuterium scrabbling in this process. Loss of acetic acid then occurs (B_2) followed by ketene to give B_3. Presumably, the glycosidic acetate is spared in these transformations, but is then removed as acetic acid when B_3 is converted into B_4. Probably this occurs due to enhanced stabilization afforded by the 1,3-dioxane ring; it cannot be lost as an acetate radical since this would increase the charge on B_4. Rearrangement of B_4 with subsequent fragmentation then gives ions B_{6-7} as shown in Fig. 19. The analogous B_4 ion among the deuterated fragments is at 187 mass units, but there is also a strong peak at 186 mass units which is absent in the spectrum of 85 suggesting that there is hydrogen-deuterium scrambling at this step.

Ions forming the C series are considered to arise as shown in Fig. 20. Ion A_1 yields ion C_1 of mass 259 and a neutral fragment (D_o) which probably picks up an acetyl radical* to give ion D_2 of m/e (318 + 43) = 361; C_1 loses acetic acid to give ion C_2 (m/e 199) and loss of ketene from C_2 gives C_3 of mass 157. Fragment C_1 can also lose a formyl radical to give ion C_4 of 230 mass units, and sequential elimination of acetic acid and then ketene gives ions C_5 and C_6. Other ions C_7 and C_8 are also shown.

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* The triacetoxonium and diacetoxionium ions are formed as a result of neutral acetic anhydride picking up an acetyl radical or undergoing protonation, respectively (131).

FIG. 19

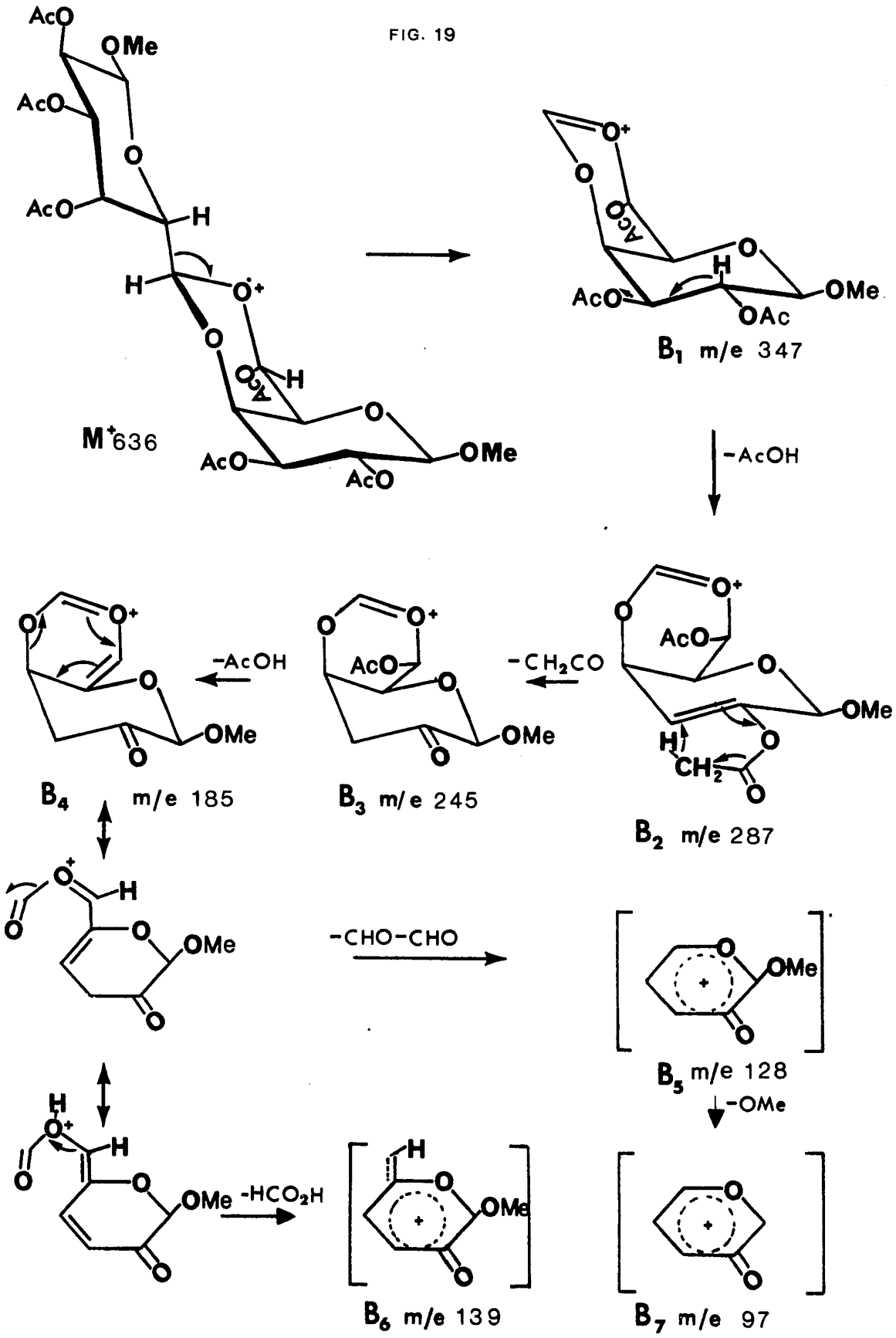
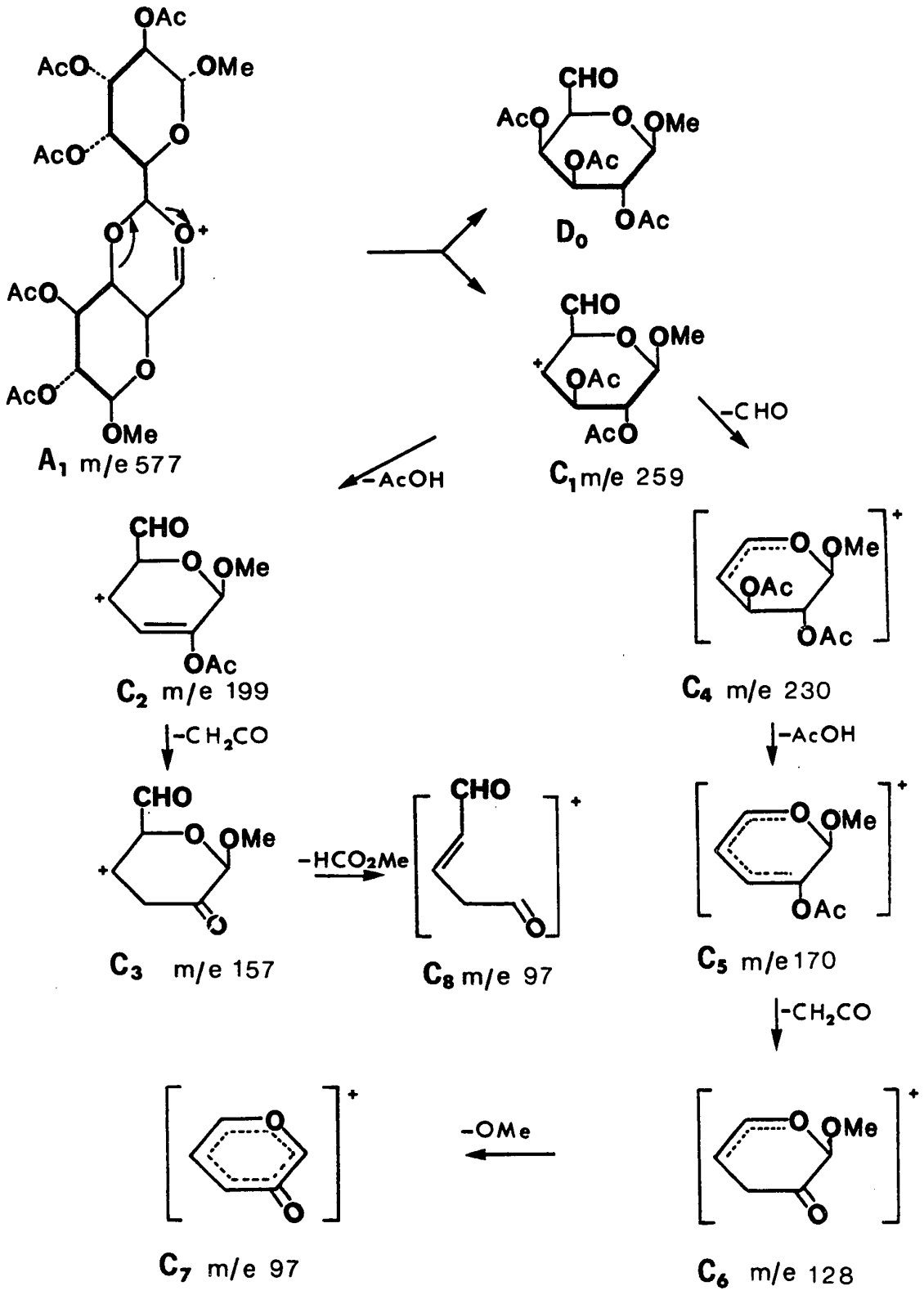

M+636
B1 m/e 347
-AcOH
B2 m/e 287
-CH2CO
B3 m/e 245
-AcOH
B4 m/e 185
-CHO-CHO
B5 m/e 128
-OMe
B6 m/e 139
-HCO2H
B7 m/e 97

FIG. 20


A1 m/e 577
D0
C1 m/e 259
-CHO
-AcOH
C2 m/e 199
C4 m/e 230
-CH2CO
-AcOH
C3 m/e 157
-HCO2Me
C8 m/e 97
C5 m/e 170
-CH2CO
-OMe
C7 m/e 97
C6 m/e 128

IV OXIDATION OF SUBSTRATES

If the ion leading to fragment D_o carries a charge located on the pyranose ring A oxygen then D_1 rather than D_o could arise. Fragmentation of D_1 is depicted in Fig. 21. Ion D_1 is intense whereas D_3, D_4, D_5 are of moderate intensity, and D_6 is slightly more intense than the others. Of less significance to the structural study of 85 is the fragmentation pathway leading to the E series of ions. This route closely resembles a fragmentation scheme described by Heyns and Müller (210). Here (Fig. 22) an initial loss of methyl acetate from the parent molecule 85 gives an ion of m/e 562 $(M-74)^+$ which further suffers loss of an acetate radical to give rise to ion E_2 of m/e 503. Alternatively, cleavage could occur to give an ion of small mass (E_3, m/e 157) which loses ketene and gives rise to a stronger peak at m/e 115 (E_4). E_2 can further disintegrate into smaller ions involving loss of formic acid and acetate.

One other fragmentation pattern to be considered is similar to that observed by Biemann and DeJongh (209) for glycosides. A positive ion centered on the ring oxygen of ring A of 85 leads to rupture of the C-1, C-2 bond of ring A with subsequent loss of methyl formate to give ion F_1, m/e 576. F_1 can then lose an acetoxy-radical to give ion F_2 (m/e 517) or acetic acid to give F_3 (m/e 516). Cleavage of the C-5, C-6 bond of F_2 gives an intense ion F_4 at m/e 170 and also one of m/e 169. This by no means exhausts the number of fragmentation patterns that must occur. Others that are less prominent but possibly equally important may have been overlooked.

FIG. 21

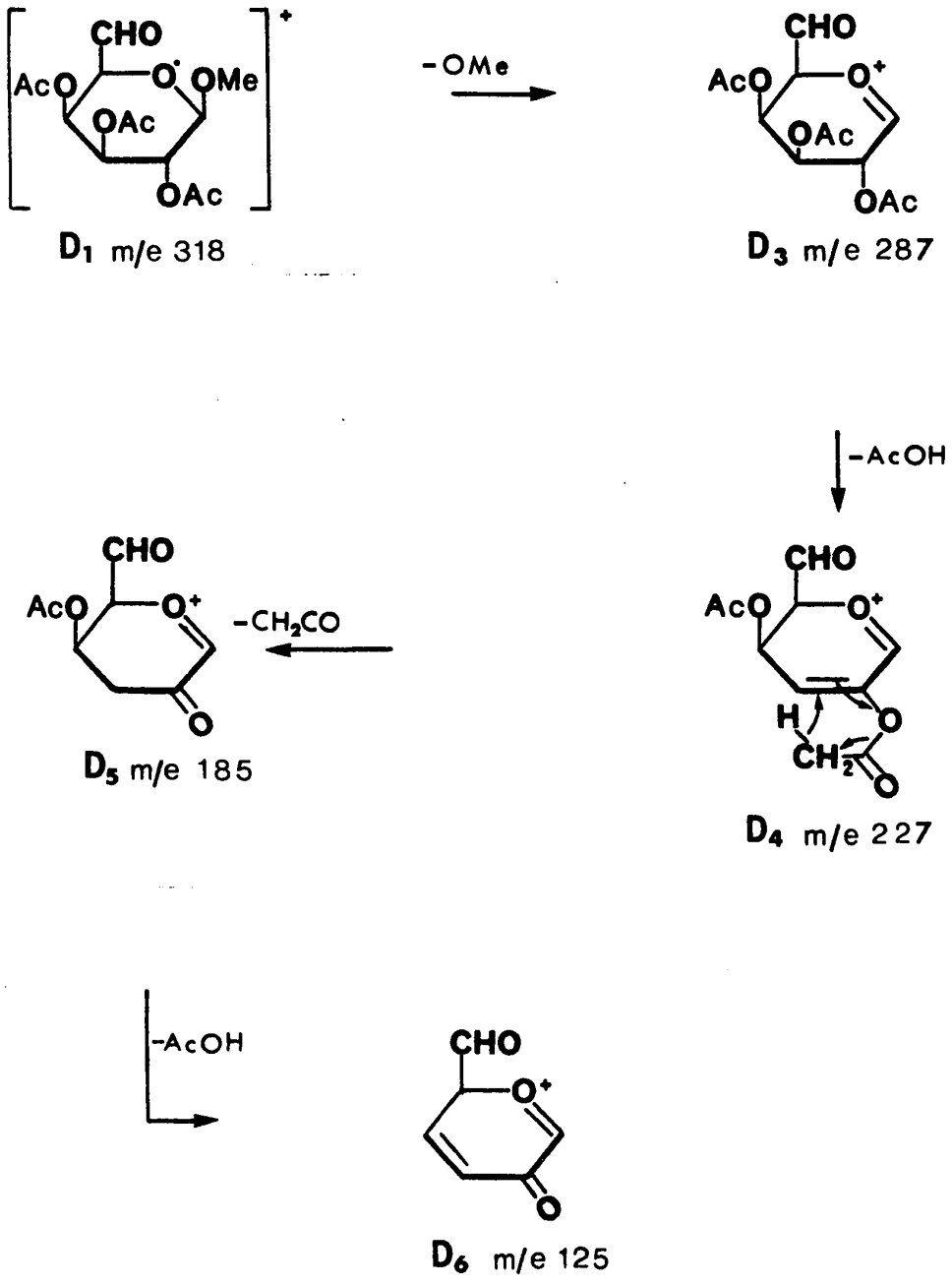
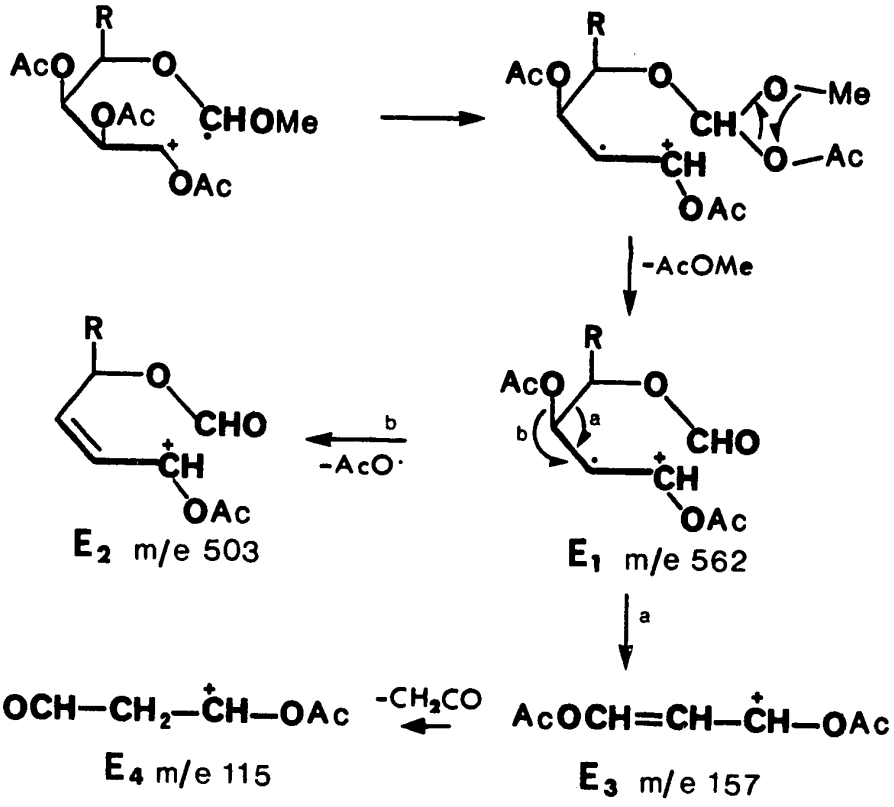
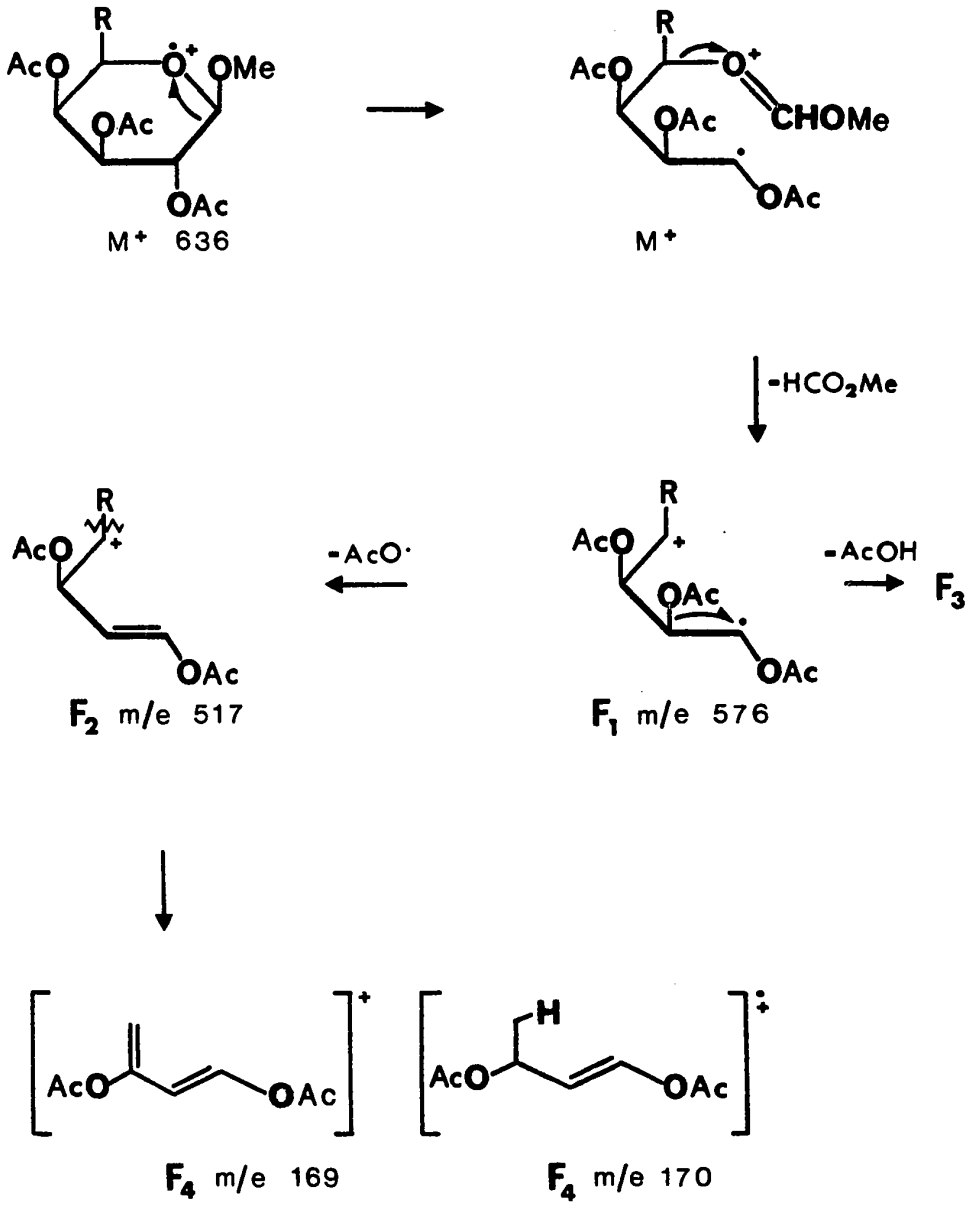

CHO
AcO
O
OMe
OAc
OAc
+
D1 m/e 318
-OMe
CHO
AcO
O+
OAc
OAc
D3 m/e 287
-AcOH
CHO
AcO
O+
H
O
CH2
O
D4 m/e 227
-CH2CO
CHO
AcO
O+
O
D5 m/e 185
-AcOH
CHO
O+
O
D6 m/e 125

FIG. 22



R = rings B and C of **85**

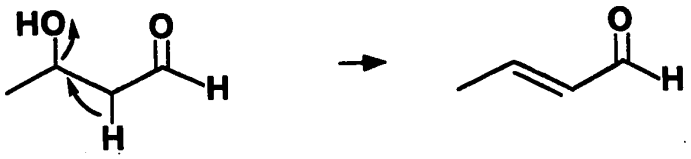

R
AcO
OMe
OAc
OAc
M+ 636
CHOMe
M+
-HCO2Me
-AcO·
-AcOH
F3
F2 m/e 517
F1 m/e 576
F4 m/e 169
F4 m/e 170
R=rings B and C of 85

IV OXIDATION OF SUBSTRATES

The presence of an α,β-unsaturated aldehyde in D-galactose oxidase products

The presence of an α,β-unsaturated aldehyde (81) among the products of enzymic oxidation, and subsequently among those of the acetylation process, merited closer study.

The presence of a carbonyl group at C-6 of 77 and also a ring oxygen attached to C-5 makes H-5 more acidic and prone to undergo elimination along with the trans-disposed C-4 hydroxy-group, even in the enzyme reaction medium. Thus, facile elimination is observed with β-hydroxybutanal (211)(and other aldols) which loses a molecule of water to form crotonaldehyde.



An enzyme-induced elimination is also possible, if D-galactose oxidase is coupled to a dehydrating enzyme present as an impurity. Such an enzyme could be part of a chain of enzymes, the purpose of which is to synthesize 4-deoxy-pyranoses. Hence, the C-6 carbonyl group would induce elimination, and subsequent reduction of the double bond of 81 (page 64) by another enzyme using $NADH+H^+$ or $NADPH+H^+$ would give a 4-deoxy-L or -D-glycoside*. An analogous type of reaction has been

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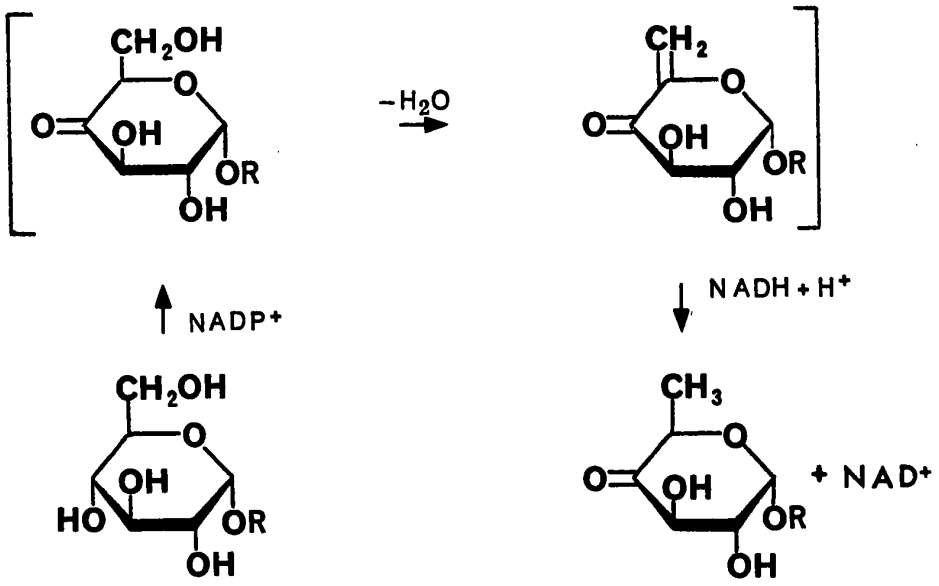
* Hanessian (214) has pointed out that 4-deoxy-hexoses are not common in nature; thus it is possible that D-galactose oxidase could be linked to another process the nature of which is unknown at the moment.

observed in the microbial conversion (212) of D-glucose to 6-deoxy-D-glucose through the intermediacy of an α,β-unsaturated ketone and a model for this transformation has been worked out (213).



Facile elimination during acetylation of the D-galactose oxidase products was also investigated. Separation of the enzyme products, i.e. the free aldehyde and the dimer, was achieved on preparative tlc plates. Acetylation of these two fractions separately with acetic anhydride–pyridine, followed by purification on silica gel gave 82 and dimer 85 in a ratio of about 1:2 from the free aldehyde, whereas the dimeric fraction gave predominantly dimer 85, with 82 as a minor fraction. Thus these two experiments suggest that elimination can take place

with the dimer as well as 77 and that dimerisation of the latter can occur before or during acetylation; although it is possible also that the dimer reverts to the monomer in the acetylating medium.

Reaction of D-galactose oxidase with methyl β-D-galactopyranoside--6-d (34)

Methyl β-D-galactopyranoside-6-d predominantly 6-R (34) was treated with D-galactose oxidase under the same conditions as for the unlabelled substrate 6. The reaction was also followed by tlc and the same pattern of products was observed. That is, present in the reaction mixture were small quantities of an α,β-unsaturated aldehyde, the free aldehyde, the unreacted substrate and the dimer. However, the reaction was slow relative to that of the undeuterated glycoside and the spot corresponding to the unreacted substrate was correspondingly larger.

Acetylation of the enzyme products in the usual way followed by column chromatographic separation on silica gel afforded the α,β-unsaturated aldehyde 83 (page 64) having the same U.V. parameters as 82. However, the NMR spectrum of 83 showed only a weak signal ascribable to the aldehydic proton (Fig. 14B).

It is evident therefore that D-galactose oxidase had specifically abstracted a protium atom. In the unlabelled substrate (6) H_S is equivalent to H-6 of pure 6R of 34. Since the predominant C-6 isomer of 34 is 6R (R:S=4.6:1) then the relatively small size of the aldehyde proton signal at 9.26 ppm was the result of abstraction primarily of deuterium from the 6S diostereoisomer leaving only the hydrogen equivalent to H_R

in unlabelled 6. The mass spectrum of the 2,4-dinitrophenylhydrazone derivative of 83 confirmed that deuterium was present to a level of over 85%.

The second fraction to be separated from the column was, again, not the tri-O-acetyl derivative of the free aldehyde but the acetylated unreacted substrate, amounting to about 10% of the total product.

The dimer corresponding to 85, but now containing mostly deuterium at C-6 and C-6' was the last major fraction to be separated from the column. The NMR spectrum of this deuterated dimer has already been discussed (page 65).

Reaction of D-galactose oxidase with methyl β-D-galactopyranoside-6-d (45).

Compound 45 which is predominantly 6S, was treated in the same way as were 34 and 6, but in accord with the results of the kinetic experiments was oxidized very slowly. Acetylation of the enzyme products in the usual way followed by column chromatographic separation afforded the α,β-unsaturated aldehyde 84, the unreacted acetylated substrate and a small quantity of the dimer corresponding to 85.

Compound 84 now comprised about 7-8% of the total product; its IR and U.V. spectra and the m.p. of its 2,4-dinitrophenylhydrazone identified it with 82. Its NMR spectrum (Fig. 14C) showed an aldehydic proton signal at 9.26 ppm slightly less intense than the same signal in Fig. 14A, i.e. approximately 3/4 of a proton. Therefore the missing 1/4 was due to the presence of the aldehydic deuterium originating in the oxi-

dation of the 6R isomer present in 45. Abstraction of the rest of the deuterium by oxidation of the 6S component of 45 meant that the enzyme had again reacted stereospecifically: that is, deuterium which occupied the position of H_S in the unlabelled substrate had been removed.

Considering that the oxidation of 45 was much slower than of 6 or 34, it was of interest to examine the unreacted substrate. The NMR spectrum (Fig. 23C) of this glycoside after benzoylation showed that the enzyme had achieved virtually a clean resolution of the 6S diastereoisomer from the 6R in 45. That is the reactive 6R isomer was specifically used up completely, leaving behind the unreactive 6S diastereoisomer. This is demonstrated by comparison with the NMR spectrum of benzoylated 6 and 45 (Fig. 23A, and 23B respectively); only the region of the C-6 hydrogens is shown. Signal H_A arises from the predominant 6S diastereoisomer and H_B from the 6R component, the two together amounting to one proton of 45. In the spectrum of the non-deuterated glycoside (Fig. 23A) H_R (equivalent to H_A in 45) is a quartet due to geminal coupling with H_S and to vicinal coupling with H-5, and the signal of the latter (H_S) equivalent to H_B in 45 also is a quartet. Introduction of the deuterium replaces the strong H-6 geminal coupling in 6 with weak coupling to the isotope, and results in essentially the doublets observed. The NMR spectrum (Fig. 23C) of enzyme treated and benzoylated 45, however, shows the absence of the signal due to H_B of the 6R diastereoisomer, and thus confirms the kinetic enzymic resolution of the deuterated 6R and 6S diastereoisomers admixed in 45.

The accessibility of this enzyme-purified methyl β-D-galactopyranoside-6-d (6S) proved to be of value in the study of rotational iso-

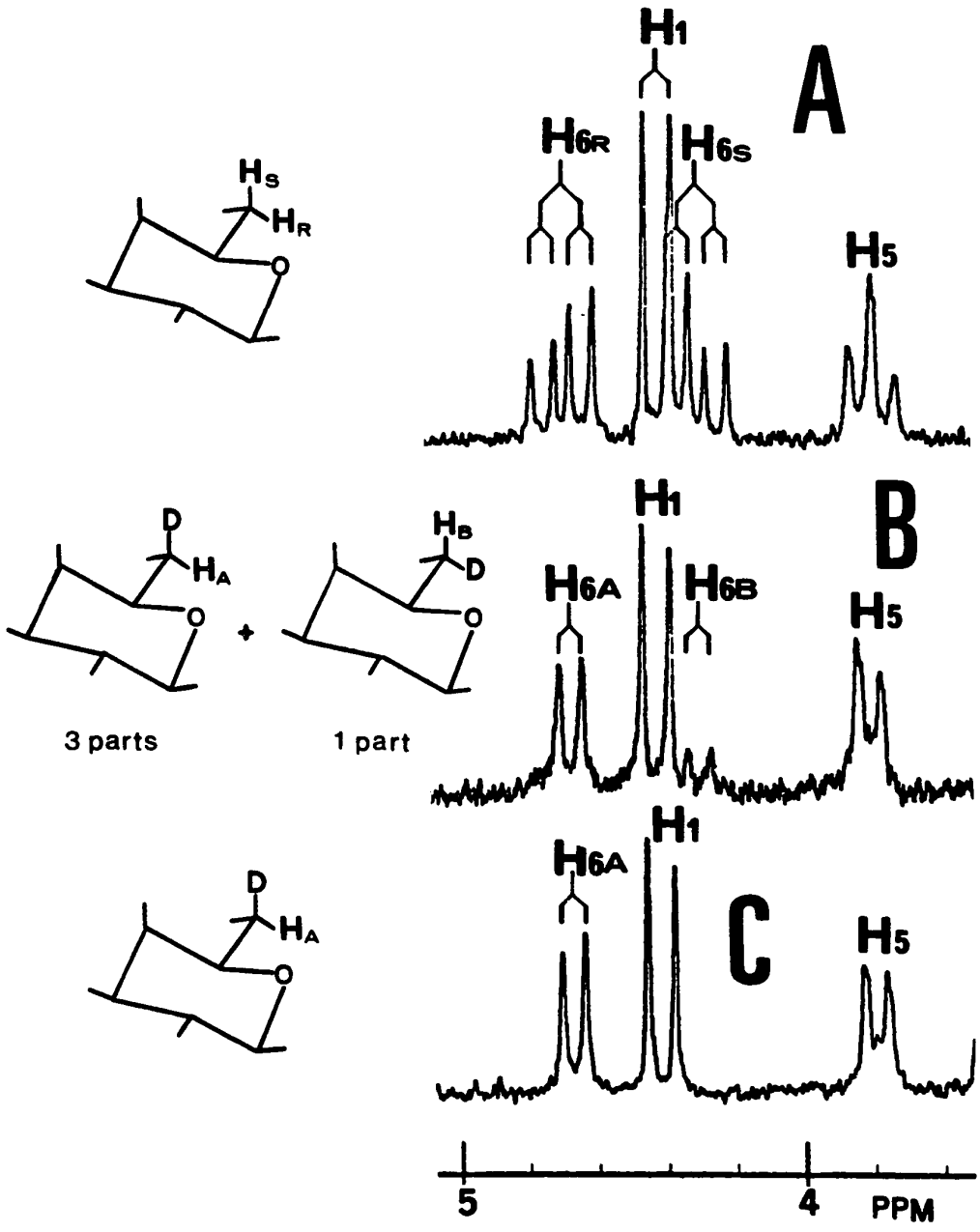


FIG. 23 Partial NMR spectra (100 MHz, 1000 Hz sweep width) in C_6D_6 of: A, methyl β-D-galactopyranoside (6) tetrabenzoate; B, methyl β-D-galactopyranoside-6-d (45) benzoylated; C, enzyme purified (or resolved) 45, benzoylated.

merism about the C-5, C-6 bond of methyl β-D-galactopyranoside derivatives (Chapter VII). With sufficient quantities of the enzyme available for a larger-scale preparation it could be used also for reinvestigation of the postulated secondary deuterium isotope effect in the D-galactose oxidase reaction (Chap. III).

Reaction of D-galactose oxidase with methyl β-D-galactopyranoside-6,6'-d_2 (47)

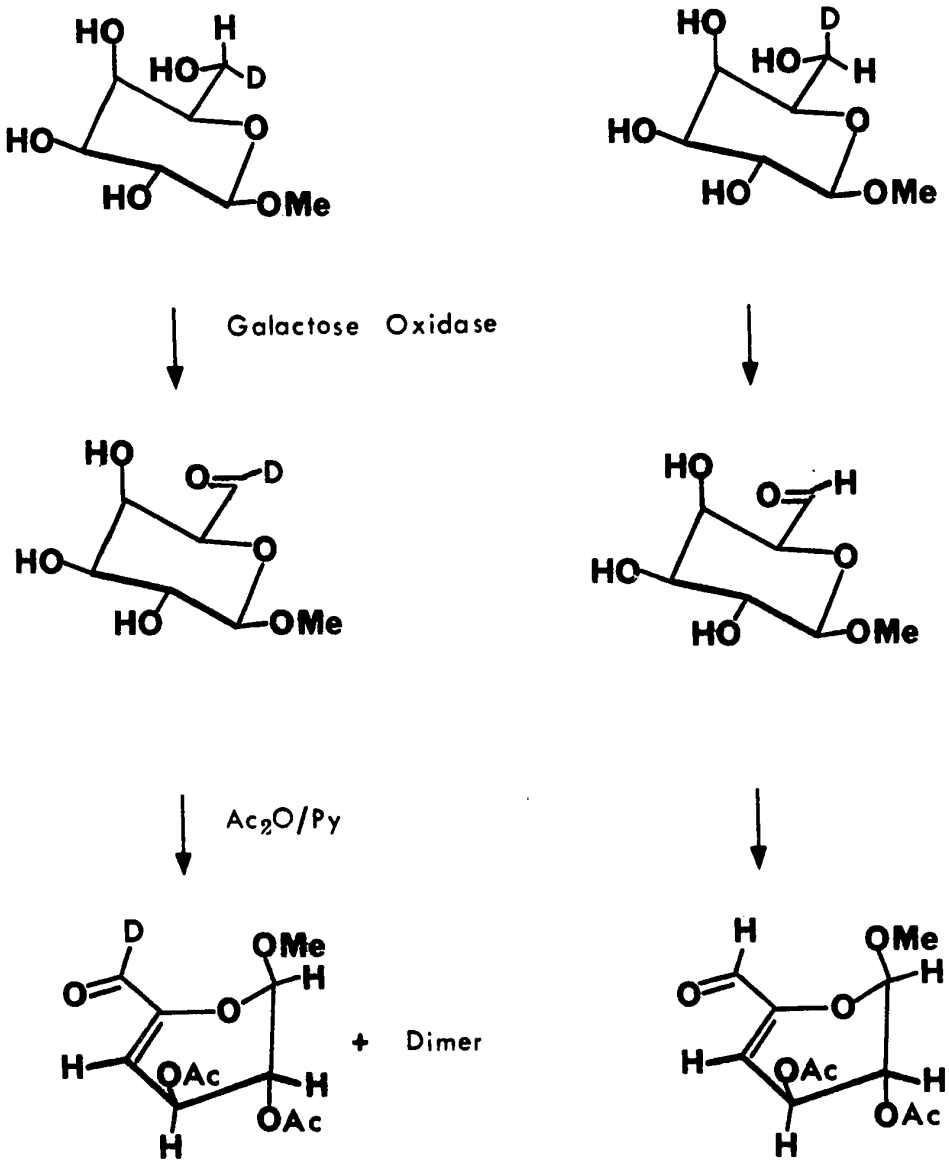
Treating 47 with the enzyme in the usual way gave only a very small yield of aldehydic products, and since this reaction had no stereochemical significance it was not pursued further.

Summary of the stereospecific hydrogen abstraction by D-galactose oxidase

The preceding discussion is summarised in Fig. 24. Of the two prochiral hydrogens at C-6 of methyl β-D-galactopyranoside only the pro-S hydrogen is abstracted. Although there may be free rotation about the C_5-C_6 bond, this is restricted in the transition state leading to the enzyme-substrate complex. In this way the enzyme causes the prochiral centre to become chiral, permitting abstracting groups on the enzyme surface to be orientated towards H_S.

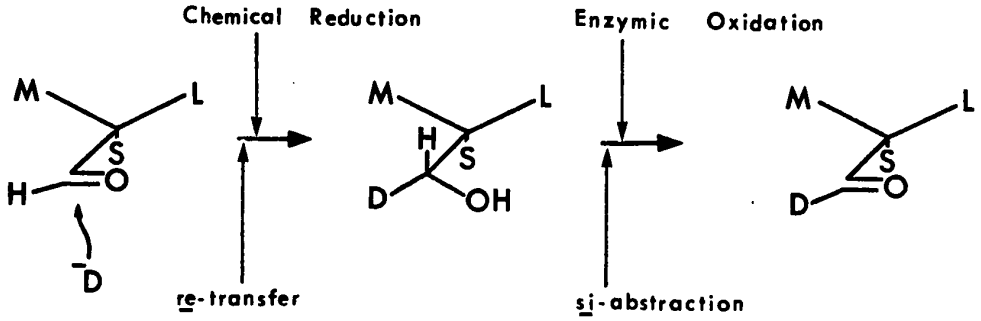
Although the exact orientation of H_S in the transition state can only be speculated upon, it is logical to assume that the energy of activation leading to the enzyme-substrate complex could be lower if the transition state bears resemblance to the reactants in the ground state.

FIG. 24


HO
H
HO
D
HO
O
HO
OMe
HO
D
HO
H
HO
O
HO
OMe
Galactose Oxidase
HO
O
D
HO
O
HO
OMe
HO
O
H
HO
O
HO
OMe
Ac2O/Py
D
O
OMe
H
O
H
OAc
H
OAc
H
+ Dimer
H
O
OMe
H
O
H
OAc
H
OAc
H

IV OXIDATION OF SUBSTRATES

The NMR spectra of a number of methyl β-D-galactopyranoside derivatives in non-polar solvents and of methyl 4-deoxy-4-iodo-β-D-galactopyranoside in deuterium oxide (Chapter VII) suggests that orientation of $H\text{-}6_S$ lies in a 1,3 *cis* relationship with the substrate specifying C-4 hydroxy-group (Chapter V). Therefore it can be suggested that whereas in the reduction of aldehyde 12 the deuteride added preferentially from the less-hindered *re*-face, the reverse takes place in the enzymic oxidation with H_S leaving from the more hindered *si*-face. The driving force of the enzyme action may be derived from the steric congestion of groups of atoms within the vicinity of the reaction sites in that the reacting groups are held within a short distance of each other.



The analysis of enzyme products permits the assertion that there is no deuterium-hydrogen scrambling in the abstraction process, or exchange with the medium. This suggests that the process is concerted.

The stereochemical course of a number of NAD-dependent dehydrogenases such as alcohol dehydrogenase (37), liver alcohol dehydrogenase (126) and muscle lactate dehydrogenase (215) favours abstraction of *pro*-R hydrogen, in contrast to the *pro*-S nature of D-galactose oxidase. Hence the stereochemical requirements of these other enzymes must be different from those of D-galactose oxidase.

CHAPTER V

ACTION OF D-GALACTOSE OXIDASE ON SUBSTRATES CHEMICALLY MODIFIED AT C-4

Reactivity of D-galactose analogues

Chemical modification of enzymes and substrates can furnish a wealth of information concerning structure-function relationships. Usually such modifications are designed to gain an understanding of the mode of enzyme action at a level of conformational features, the nature of enzyme-substrate binding, and of the chemical constitution of the active site. The subject of chemical modification of enzymes for structural studies has been reviewed recently (216,217).

Modification of the enzyme substrate is relatively the easier procedure. Its significance in the carbohydrate field has been discussed by Gottschalk (218). The design of drugs, enzyme substrate inhibitors, and immunological antigens, and other areas of important application involve compounds that have structural features closely resembling those of natural substrates.

In this stereochemical study of D-galactose oxidase action it was of interest to investigate the significance of the 4-carbinol group as a substrate specifying group. Earlier studies on D-galactose oxidase established that chemical modification at C-1, C-2 or C-3 of D-galactose had no drastic effect on the enzyme reaction (4, 219). Thus replacement of OH-1 by a hydrogen atom, as in 1,5-anhydro-galactitol (96), had no effect on the enzyme reaction (4). An anomeric change at C-1 also is not critical, since both α and β anomers are oxidised (4). How-

ever, lactose (97) was found not to be oxidisable whereas melibiose (98) is.

The reactivity of 2-deoxy-D-galactose (99) means that the hydroxy-function at C-2 is not critical. Also, blocking of the hydroxy-function at C-2 or C-3, or both, by etherification as in 2-O-methyl and 3-O-methyl-D-galactopyranose (100 and 101, respectively) does not affect the D-galactose oxidase reaction (219). Methylation of OH-4 to give 102, however, renders the substrate unreactive (219).

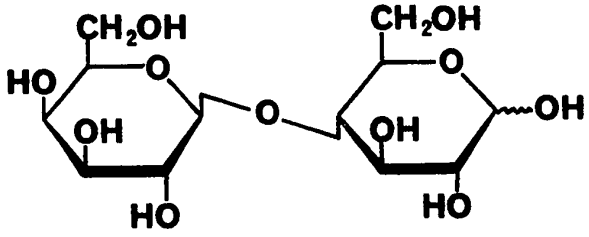
The unreactivity of 102 suggests either that the methoxy-substituent is too large for the space on the enzyme surface, usually occupied by the hydroxy-group or that a hydroxyl hydrogen that is normally essential for hydrogen-bonding with the enzyme is no longer available. If by contrast, the enzyme donates a hydrogen for coordination with O-4 of the substrate, then this type of binding should be less drastically affected. In other words two types of hydrogen-bonding can take place between the enzyme and the substrate. In the first one, A, the hydrogen donor is the substrate and in B it is the enzyme.

A Enzyme-X---H—O—Substrate

X = N, or O

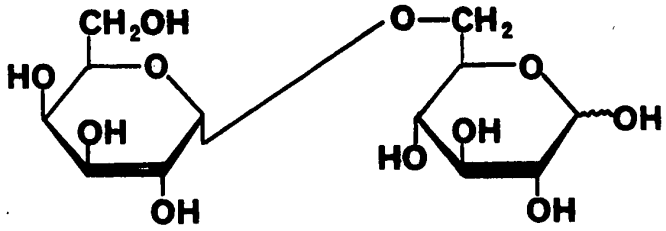
B Enzyme-X—H---O—Substrate
H

Forces other than hydrogen bonding may be important in the bonding of enzymes to substrates (220, 221). According to Jencks (220) these forces are responsible for most noncovalent intermolecular interactions in aqueous solutions and he has collectively called them 'hydrophobic forces'; they are probably of the van der Waals-London dispersion type.



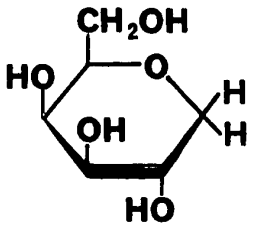
Lactose

97

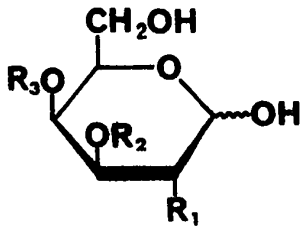


Melibiose

98



96



99 $R_1 = R_2 = R_3 = H$

100 $R_1 = OMe$, $R_2 = R_3 = H$

101 $R_1 = OH$, $R_2 = Me$, $R_3 = H$

102 $R_1 = OH$, $R_2 = H$, $R_3 = Me$

V REACTIVITY OF C-4 MODIFIED SUBSTRATES

To assess the sensitivity of the enzyme towards the size of the substituent at C-4 and the type of affinity that the enzyme exercises towards it, substrates were prepared in which OH-4 of D-galactose is replaced by protic and aprotic groups. The protic groups chosen were: amino and thio, both of which are larger than a hydroxy-group. The aprotic groups were: hydrogen, the halogens and the azido group; these were expected to participate in weak hydrogen bonding, or none at all.

The substrates thus prepared were methyl 4-deoxy-β-D-xylo-hexopyranoside (methyl 4-deoxy-β-D-galactopyranoside) (103), methyl 4-deoxy-4-fluoro (104), 4-amino (105), 4-chloro (106), 4-bromo (107), 4-iodo (108), 4-thio (109) and 4-azido (110), -β-D-galactopyranosides. With the exception of the thio compound (109) of which more will be said later, all were pure crystalline compounds and readily soluble in water.

Kinetic studies on substrates 103-110 were conducted with "Galactostat" as described in Chapter III for methyl β-D-galactopyranoside and the deuterated C-6 diastereoisomers. For less reactive substrates high concentrations were used, and it was assumed that in all cases the rate equations of Chapter III hold also for these substrates.

Samples containing substrates were monitored continuously and the absorbance was directly recorded at intervals of 9 seconds at 420 nm using the Beckmann-Gilford coupled spectrophotometer provided with a constant temperature compartment. In this way plots of optical density against time were obtained directly, and are reproduced in Fig. 25 for the substrates which were found to be reactive. Straight line plots were obtained for the interval from two to 20 min., at which time the reaction was stopped. Initial rate constants (k) were obtained from the gradient

of the lines, and by following each reaction at different concentrations it was possible through use of the Lineweaver-Burk (222) plot to determine the Michaelis-Menten constant, Km, and the maximum velocity, Vm (Table IV). Within experimental error the reactive substrates obeyed the Michaelis-Menten equation, although at high concentrations compound 47 deviated slightly and the Lineweaver-Burk plot for 105 was not a straightforward case as was apparent for 6 and 104 (Fig. 26).*

No reaction with D-galactose oxidase was observed for the bromo, iodo, thio and azido substrates.** Methyl 4-deoxy-β-D-*xylo*-hexopyranoside showed very low reactivity and Km and Vm values were not obtained for this compound.

Of the deoxy substrates, 103, 104, 105 and 106, the amino compound 105 was the most reactive (k=0.22) followed in order by fluoro 104 (k=0.17), chloro (106) and 103 (Table IV). The affinity of the enzyme towards these substrates is given by the reciprocal of Km (223) and it can be seen that it is highest for methyl β-D-galactopyranoside and lowest for the chloro compound (106). Table IV shows also that Vm, which is a measure of the rate of decomposition of the enzyme-substrate complex to give products, is highest for the fluoro derivative 104 and lowest for methyl β-D-galactopyranoside-6,6-d_2 (47).

---

* Aqueous solutions of 105 have a pH greater than 7 (ca. 8-9). At the constant buffer concentration used, the pH fluctuations arising from use of different substrate concentrations may not have been sufficiently compensated for by the buffer. The isoelectric point of the enzyme might therefore have been affected by this slight pH fluctuation.

** The thio (109) bromo (107) and iodo (108) derivatives also showed no reaction under the conditions used for preparative work (Chap. IV) using D-galactose oxidase-catalase system.

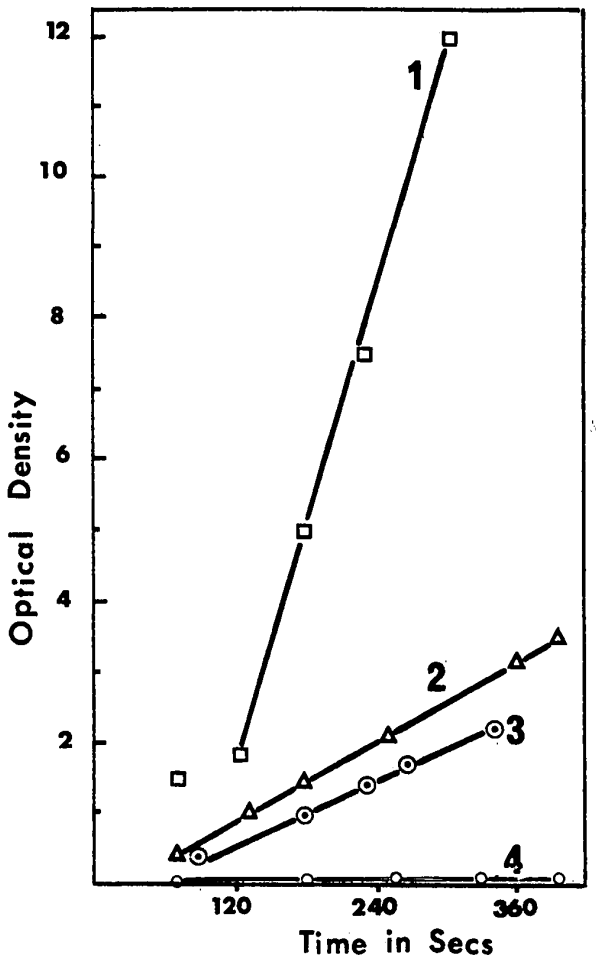


FIG. 25 Initial rate reaction of D-galactose oxidase on: **1** methyl β-D-galactopyranoside (6), **2** methyl 4-amino-4-deoxy-β-D-galactopyranoside (105), **3** methyl 4-deoxy-4-fluoro-β-D-galactopyranoside (104), **4** methyl 4-chloro-4-deoxy-β-D-galactopyranoside. All substrates were at a concentration of 2.9×10^{-5} M.

The role of the 4-hydroxy-group in the binding of D-galactose by the enzyme

Two properties of the 4-hydroxy-group of D-galactose may be considered in relation to its role as a determinant in the dehydro-

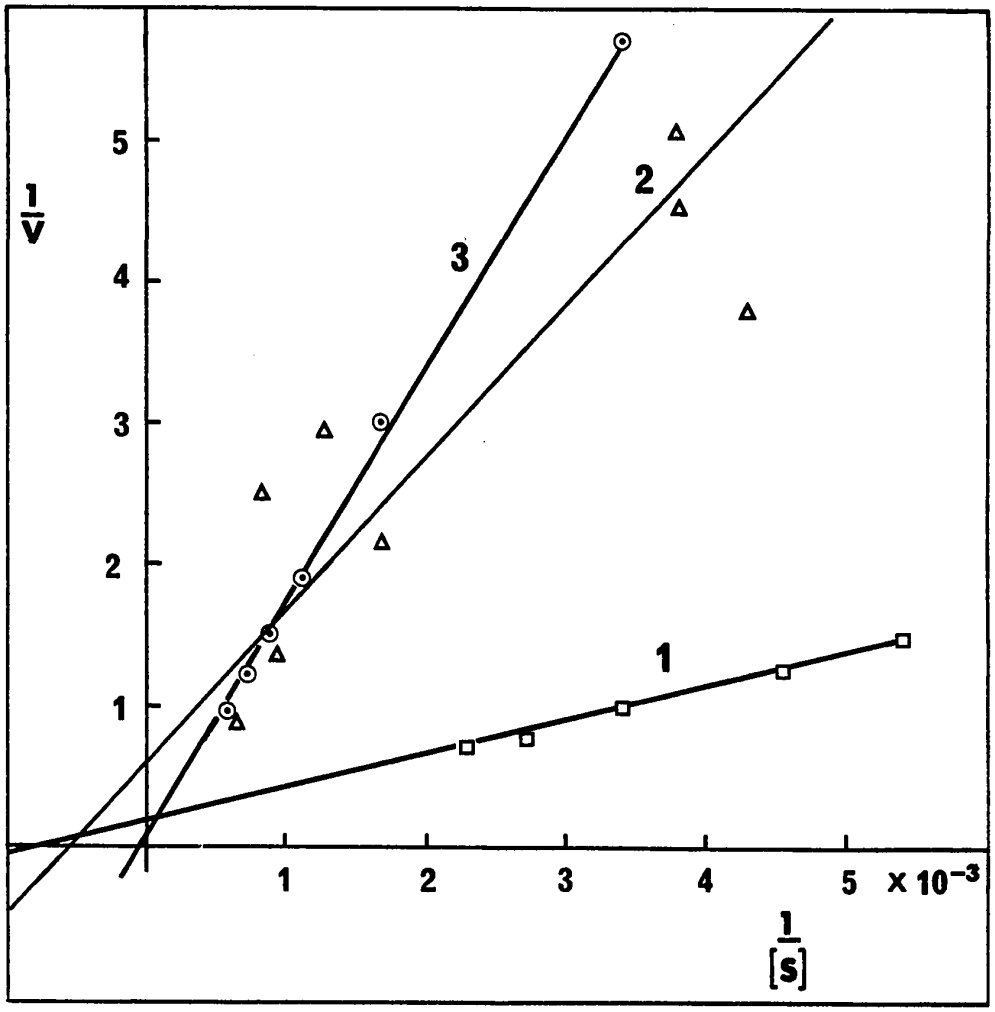


FIG. 26 Lineweaver-Burk plot for initial rate of reaction of D-galactose oxidase on: **1** methyl β-D-galactopyranoside (6), **2*** methyl 4-amino-4-deoxy-β-D-galactopyranoside (105), and **3** methyl 4-deoxy-4-fluoro-β-D-galactopyranoside (104).

*plotted from values obtained by the least square fit method

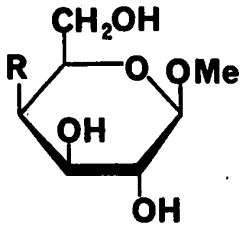
genation of the sugar by galactose oxidase: namely, its ability to hydrogen bond, and its size.

The fact that methyl 4-deoxy-β-D-galactopyranoside (103) reacts at all, even though very slowly, shows that OH-4 of D-galactose has

V REACTIVITY OF C-4 MODIFIED SUBSTRATES

Table IV Action of D-Galactose Oxidase on Chemically Modified Substrates (111)

Substrate	1/Km	Vm	k x 10
6 R = OH	650	6.7	9.67
103 = H	-	-	0.015
104 = F	50	10.0	1.74
105 = NH_2	553	1.7	2.22
106 = Cl	40	3.3	0.04
107 = Br	-	-	-
108 = I	-	-	-
109 = SH	-	-	-
110 = N_3	-	-	-
Methyl β-D-gal. 6,6'd_2 (47)	180	1.6	0.98



111

no essential hydrogen-bonding function; for it is improbable that hydrogen bonded to carbon can participate in hydrogen bonding.

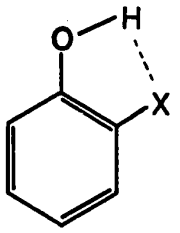
It is claimed that a fluoro atom bonded to carbon cannot act as a hydrogen acceptor (224). Hence the relatively high reactivity of the 4-deoxy-4-fluoro derivative (104) also indicates that H-bonding is not a prerequisite for its oxidation. The enzyme has a much lower affinity for 104 than for the D-galactoside (1/Km 50 vs 650, Table IV), but once the enzyme substrate complex is formed its breakdown to products is highly favourable (as suggested by the large value of V_{max} for 104. Table IV). The other halogens are known to engage in weak intramolecular hydrogen bonding, as in the ortho-halophenols(112), but only the chloro-analogue (106) shows a detectable reaction with the enzyme, and it is far less reactive than 104.

These various data concur, therefore in showing that hydrogen bonding is not an important function of the 4-hydroxy-group in the oxidation of D-galactose.

The reactivity of methyl 4-amino-4-deoxy-β-D-galactopyranoside (105) as measured by the initial rate constant (k) and the maximum velocity (Vm) is higher than that of methyl β-D-galactopyranoside 6,6-d_2 (47).

That is, although 47 satisfies the steric requirement at C-4, and accordingly shows a high affinity, yet the removal of the deuteride at C-6 retards the overall reaction. These results indicate that the varying reactivity of D-galactose oxidase substrates depends

V REACTIVITY OF C-4 MODIFIED SUBSTRATES



112

First overtone of weak intramolecular hydrogen bonding (225)

X = Cl	6890 cm^{-1}	X = I	6800 cm^{-1}
X = Br	6860 cm^{-1}	X = OMe	6930 cm^{-1}

on factors other than the rate of H-6 abstraction, such as on the extent of formation of an enzyme-substrate complex. The latter expresses itself in the magnitude of Km and it appears that it is related to the size of substituent R of 111. When R is a hydroxy-group 1/Km is large. 1/Km is also fairly large when R is a group with a size approaching that of a hydroxy-group (e.g. an amino-group). The rate of breakdown of the enzyme-substrate complex to give products also is important, as expressed by the magnitude of Vm, and it has a low value when there is a deuterium isotope effect, as in the oxidation of 47. The high value of Vm in the oxidation of the fluoro compound 104 probably finds an explanation in the ease of internal isomerisation of the enzyme-substrate complex to give a product-like intermediate (E_{red}-P_1) of equation [1] (page 47).

V REACTIVITY OF C-4 MODIFIED SUBSTRATES

The D-galactose oxidase-D-galactose complex

It appears then, that the difference in oxidation rates shown by D-galactose and its 4-deoxy-analogues lies mainly in the ease of formation of the enzyme-substrate complex. The rate at which the complex decomposes is relatively much more uniform.

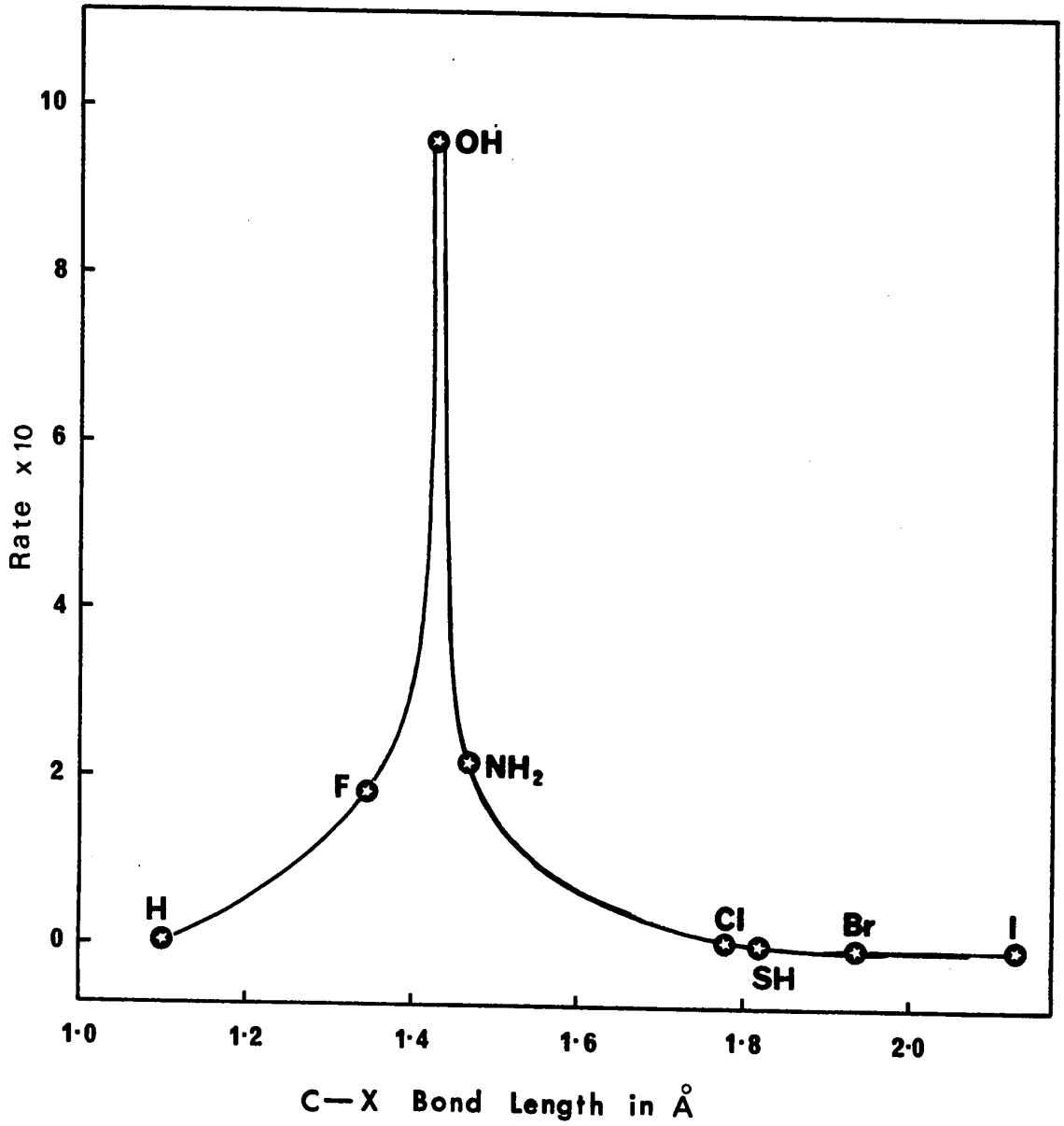
Now, factors that facilitate formation of an enzyme substrate complex must be the same as those that make the 4-carbinol group of D-galactose crucial in the D-galactose oxidase reaction. It is proposed that these factors bear a connection with the size of the substituent at C-4. If the velocities of substrate oxidation given in Table IV are plotted against the size, or length, of C-X (where X is the central atom bonded to carbon), of the substituent at C-4 of III, it is seen that the hydroxy-group sits at the apex of the curve (Fig. 27). That is, on passing from a hydrogen substituted substrate, through the fluoro to the ideal hydroxy-group the rate increases, and then falls off as the substituents become larger. Hence the closer in size the group R of III is to the hydroxy-group, the more reactive is the substrate.

Orbital Steering

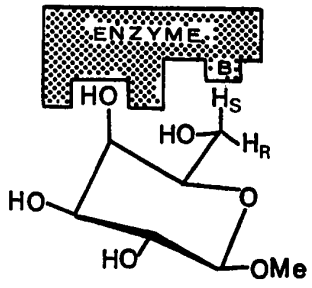
In terms of the stereochemical requirements of the enzyme, the effect of having a large or small substituent at C-4 of substrates of D-galactose oxidase presumably is to alter the geometry of the enzyme at that particular site, so that groups normally juxtaposed in their

V REACTIVITY OF C-4 MODIFIED SUBSTRATES

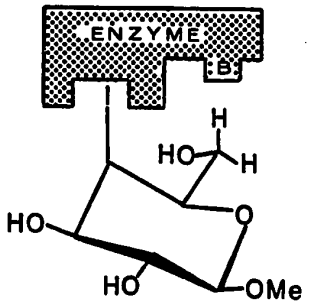
FIG. 27 Effect of substituent size at C-4 of 111 towards the D-galactose oxidase action. X = central atom of the substituent R.



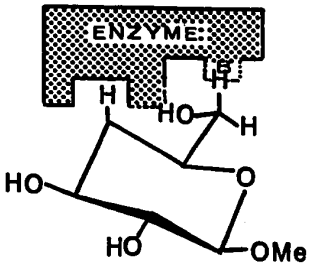
proper orientation are distorted. Thus as visualized in 113 for the OH-4 of methyl β-D-galactopyranoside the group has the ideal size to direct or 'steer'* the basic group :B on the enzyme surface into a proper allignment with the reactive hydrogen (H_S) at C-6.



113



114



115

In 114 however, the iodine substituent at C-4 is large. If the enzyme attempts to incorporate such a substrate it will be found that its basic group :B will be ineffectively remote from H_S; hence there will be no reaction. At the other extreme, a small group such as a hydrogen atom at C-4 will also lead to poor contact with the enzyme surface and :B again will not coincide ideally with H_S. As a result if the reaction takes place, it will be slow as in the case of methyl 4-deoxy-β-D-xylo-hexopyranoside (103). Structure 115 illustrates this third case.

* Terminology according to Storm and Koshland (226)(see below).

V REACTIVITY OF C-4 MODIFIED SUBSTRATES

In essence, illustrations 113-115 correspond in theory to Storm and Koshland's theory of orbital steering (226). This theory attempts to offer an explanation of the special catalytic power of enzymes. It suggests that apart from the importance of proximity (227)(or propinquity)(221) effects in enzyme reactions, the reactivity of the enzyme towards the substrate will be affected by a slight change in the size of the orbital introduced by the new atom (or group of atoms) in the enzyme or substrate. The change can be in the direction of maximising or minimising proper overlap of reacting groups. Thus in 114 for instance, the iodo substituent steers the reacting groups at the reaction centre away from H_S.

Criticism directed against the validity of Storm and Koshland's orbital model (226) has come from Capon (228) and Bruice et al. (229). The latter group claims for instance that changes in bond length of only 0.03 to 0.16 Å should not bring about drastic rate changes; also, for polyatomic molecules such a change should be buffered or dissipated in the entire molecule without having any noticeable effect on the orientation of the groups taking part in the reaction. Nevertheless, in the D-galactose oxidase reaction, methyl 4-deoxy-4-chloro-β-D-galactopyranoside is reactive whereas the bromo analogue is not; yet the difference in bond length between C-Cl and C-Br is only 0.145Å.

If OH-4 has no hydrogen bonding function it may therefore be regarded as a filler or anchor for a particular cleft on the enzyme surface. The nature of its bonding with the enzyme, which must also apply for the amino, fluoro, hydrogen and chloro substituents, is proposed here to be hydrophobic (220). Hydrophobic bonding has been described for certain sites of the papain enzyme-substrate complex (230-232) and it

seems likely that the bonding of the methyl group of acetaldehyde and ethanol in the yeast alcohol dehydrogenase reaction (37) is also hydrophobic.

It is noteworthy that the totally unreactive and slightly reactive substrates are not competitive inhibitors of D-galactose oxidase. Thus, experiments showed that when methyl β-D-galactopyranoside is mixed with, e.g., methyl 4-bromo-4-deoxy, 4-iodo, or 4-chloro-β-D-galactopyranoside, the rate of the oxidation reaction was as fast as in the control experiment without the deoxy substrates.* This suggests that the unreactive substrates do not tie up the enzyme in a non-productive enzyme-substrate complex.

Finally, it can be said that a multipoint contact theory (233) operates for D-galactose oxidase. The orientation of the crucial OH-4 of D-galactose and its D-glycoside derivatives with respect to the enzyme reaction suggests that the enzyme binds the upper side of the pyranose moiety occupied by OH-4. The configuration of OH-3 is also important since D-gulose , the epimer of D-galactose at C-3 is very slowly oxidised, (0.08 units compared to 100 units of D-galactose)(4). Similarly, the unreactivity of D-glycero-D-galactoheptose (219), and the slight rate retardation observed with methyl β-D-galactopyranoside 6-d-(6R)(for which a secondary deuterium isotope effect is operative) suggests that the enzyme is sensitive to the steric requirements at C-6. In view of these facts it appears that Ogston's theory (234) of a three centered enzyme binding with the substrate is inadequate to relate to D-galactose oxidase. Gottschalk (233), in discussing the multipoint-contact theory in relation

* A small rate increase observed with the chloro-substrate, was due to the extra contribution of the latter to the total rate.

to carbohydrate substrates, concluded that an enzyme may bind the lower or upper side of a pyranose (or furanose) ring; as for D-galactose and its glycoside derivatives this must be the upper side. As suggested in Chapters IV and VII, a minimum energy conformation of the 6-carbinol group also places H_S on the upper side of the pyranose ring and hence, presumably, in an orientation ideally suited for abstraction by the enzyme.

CHAPTER VI

SYNTHESIS OF ANALOGUES OF METHYL β-D-GALACTOPYRANOSIDE CHEMICALLY MODIFIED AT C-4

Analogues of methyl β-D-galactopyranoside substituted at C-4 with azido, halogeno, thio- and thiocyano groups were synthesized through nucleophilic S_N2 displacement of a 4-O-sulphonyl group of a suitably protected derivative of methyl β-D-glucopyranoside (116).

Displacement reactions of sulphonyloxy-groups in carbohydrate chemistry (235-238) and other systems (239) have been very widely used. The triflouromethanesulphonyloxy-group (triflyl)(240-243), which has not been utilized previously as the moiety in such carbohydrate reactions, has been employed in an exploratory way in one synthesis described below. Because of its extreme reactivity, this group permits the use of relatively gentle reaction conditions and, hopefully, will make feasible the introduction of functional groups hitherto inaccessible by nucleophilic displacement of sulphonyloxy-derivatives now in general use.

Methyl 2,3,6-tri-O-benzoyl-β-D-glucopyranoside (117)

Synthesis of most of the compounds described in this chapter began with methyl β-D-glucopyranoside. Taking advantage of the unreactivity of OH-4 of glycopyranosides and the ease of alkaline hydrolysis of benzoates, selective trimolar benzoylation at low tempera-

ture affords methyl 2,3,6-tri-O-benzoyl-β-D-glucopyranoside. Byproducts are the tetrabenzoate (116b), 3,4,6-tribenzoate (118) 3,6-and 2,6-dibenzoates (119) and (120) respectively. Aside from differences in by-product formation (244,245), this parallels the results that have been obtained by other workers using the α anomers of gluco-(246, 247) and galactopyranosides (248).

4-O-Sulphonylation of 117

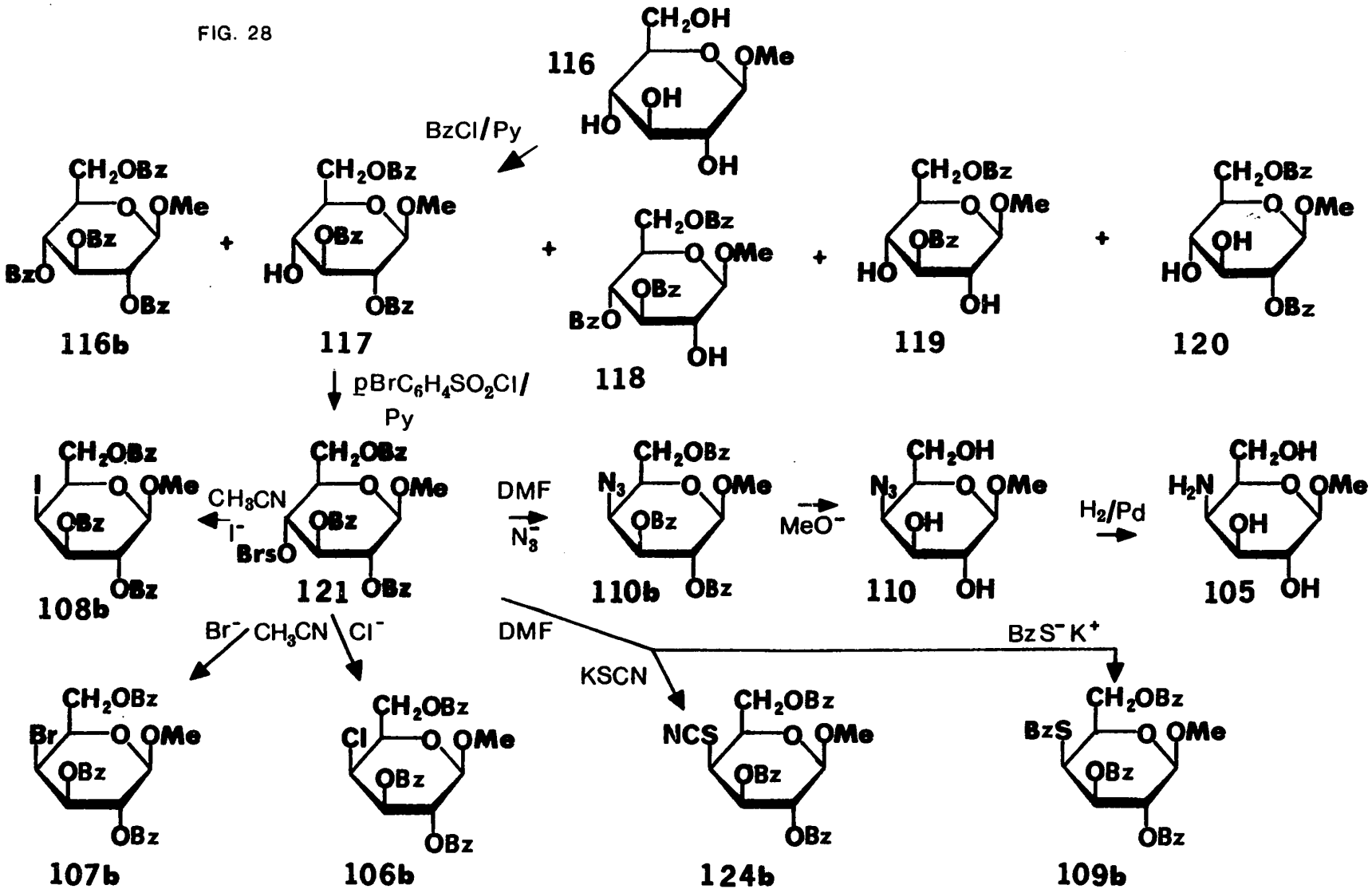
Since relatively weak nucleophiles were to be used in several of the sulphonyloxy displacement reactions projected, the p-bromobenzenesulphonyl (brosyl) group, rather than the widely-used, but less reactive, methane- or p-toluenesulphonyl, was used in the esterification of 117.* Using pyridine as solvent and the sulphonyl chloride at 45-55°, methyl 2,3,6-tri-O-benzoyl-4-O-(p-bromobenzenesulphonyl)-β-D-glucopyranoside (121) was obtained in quantitative yield. This product, then, served as the focal point for most of the reactions represented in Figure 28.

Methyl 4-azido-4-deoxy-β-D-galactopyranoside (110) and methyl 4-amino-4-deoxy-β-D-galactopyranoside (105).

Compound 121 in N,N-dimethylformamide (DMF) was solvolyzed with a suspension of sodium azide at 95°-100° for 35-48 h. affording methyl 2,4,6-tri-O-benzoyl-4-azido-4-deoxy-β-D-galactopyranoside (110b). Under the same reaction conditions, methyl 2,3,6-tri-O-benzoyl-4-O-tri-

* Use of the even more reactive triflyl group was not considered until after most of this work had been completed.

FIG. 28


116
BzCl/Py
116b
117
118
119
120
pBrC6H4SO2Cl/
Py
108b
121
CH3CN
I-
DMF
N3-
110b
MeO-
110
H2/Pd
105
Br- CH3CN Cl-
DMF
KSCN
BzS- K+
107b
106b
124b
109b

flyl-β-D-glucopyranoside (122) gave a comparable yield of 110b in only 2-3 hours reaction time. Alkaline de-O-benzoylation converted 110b into crystalline methyl 4-azido-4-deoxy-β-D-galactopyranoside (110) and hydrogenation over palladium black at room temperature and pressure then afforded crystalline methyl 4-amino-4-deoxy-β-D-galactopyranoside (105). The NMR spectrum of 110b (Fig. 29A), as well as those of the acetates (110a and 105a of 110 and 105), was consistent with a galacto configuration; this is evident most clearly from the small values observable (e.g. Fig. 29A) for $J_{3,4}$ and $J_{4,5}$. In the spectrum of 105a, signal H-4 showed a coupling of 8 Hz with the acetamido hydrogen (the latter could not be exchanged with D_2O).

Methyl 4-deoxy-4-thio-β-D-galactopyranoside (109)

The synthesis of thio sugars by nucleophilic displacement has been briefly reviewed by Horton and Hutson (249). The most common nucleophiles used in these syntheses are thioacetate, thiobenzoate and thiocyano anions.

An attempt to displace the brosylate of 121 in DMF with thioacetate resulted in the formation of a mixture of difficultly-separable compounds which was not characterised. However, with thiobenzoate as the nucleophile solvolysis was achieved in 4-6 h. at a bath temperature of 95-100°, and purification of the reaction product on silica gel afforded crystalline methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-S-benzoyl-β-D-galactopyranoside (109b). As noted above in conjunction with the synthesis of 110b, the NMR spectrum of 109b (Fig. 29C) was consistent with the expectation that there had been an inversion of

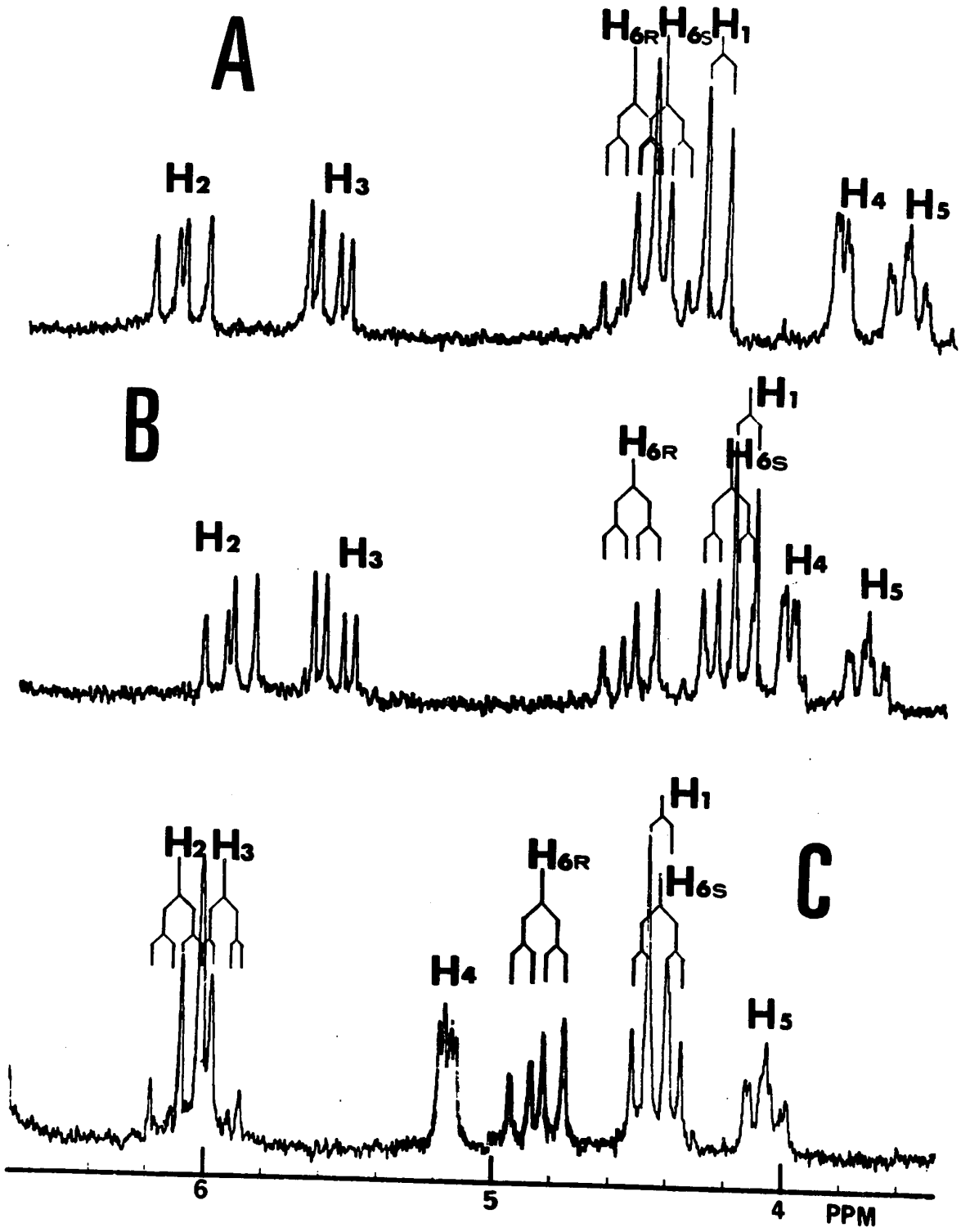


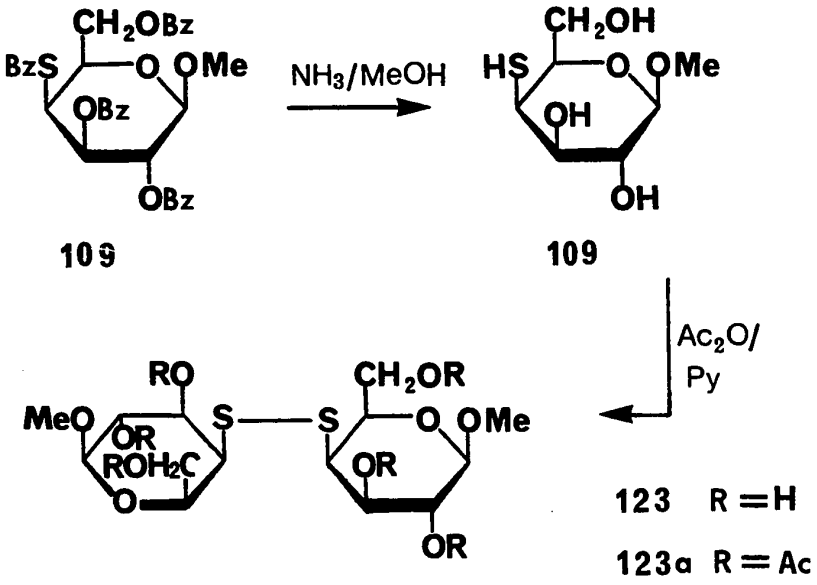
FIG. 29 Partial NMR spectra (100 MHz at 1000 Hz sweep width) in C_6D_6 of A: methyl 4-azido-2,3,6-tri-O-benzoyl-4-deoxy-β-D-galactopyranoside (110b); B: compound 124b; C: compound 109b.

the gluco to galacto configuration. One effect of having a sulphur atom bonded to C-4 in 109b was a shift of signal H-4 from 6.2 ppm (in methyl β-D-galactopyranoside tetrabenzoate) to 5.17 ppm, with benzene-d_6 as the solvent. Probably, in 109b the lone pair orbitals of sulphur are orientated more directly towards H-4 than occurs in the O-analogue.

De-O- and S-benzoylation of 109b to obtain methyl 4-deoxy-4-thio-β-D-galactopyranoside (109) was not straightforward. When ammonia gas was bubbled through the methanolic solution of 109b, a mixture of four compounds - reducing to three later - resulted, and these persisted even after treatment with ammonia at room temperature or at 30° for several days. Only after nearly two weeks was 109b converted into a single major compound. This crystalline product (109), presumably methyl 4-thio-β-D-galactopyranoside could not be purified by recrystallization. The use of lithium aluminium hydride in benzene-ether, or of sodium methoxide in methanol gave results similar to those obtained with ammonia.

Compound 109 was soluble in methanol, ethanol and very readily in water. Its NMR spectrum in D_2O was complex but the H-1 and H-5 signals were typical of the galacto-configuration. Acetylation of 109, at room temperature with pyridine-acetic anhydride gave a crystalline compound (123a) whose NMR spectrum (Fig. 30) was consistent with a galactopyranose structure. The presence of 9 acetoxy-proton signals (or 18, if the H-4 or H-5 signal is counted as two protons), and the absence of S- Ac absorption at (ν 1700 cm^{-1}) in its infrared spectrum, clearly indicated that the sulfur atom did not carry an acetoxy-group. Of the ring protons H-4 was the most shielded (Fig. 30), an indication that the thio group of 109 had acquired an ether or disulphide function,

and was not esterified as in 109b. The mass spectrum of 123a confirmed that it has a disulphide-linked dimeric structure. It is most likely that prior to acetylation, 109 had been oxidised to the disulphide 123, because oxidation of free thiol groups of monosaccharides to form S-S linked dimers is of frequent occurrence (250-253).



Cook and Overend (254) reported synthesis of methyl 4-deoxy-4-thio-α-D-galactopyranoside, involving alkaline hydrolysis of methyl 2,3-di-O-benzoyl-4-deoxy-4-thiocyano-α-D-galactopyranoside. Their product was a syrup which turned blue on exposure to air, but was desulphurised to give methyl 4-deoxy-α-D-xylo-hexopyranoside. However, alkaline hydrolysis of methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-thiocyano-β-D-galactopyranoside (124b), synthesized in the current study through displacement of the brosyl group of 121 with thiocyanate ion in DMF, gave products (isolated as an acetylated mixture), indis-

VI SUBSTRATES MODIFIED AT C-4

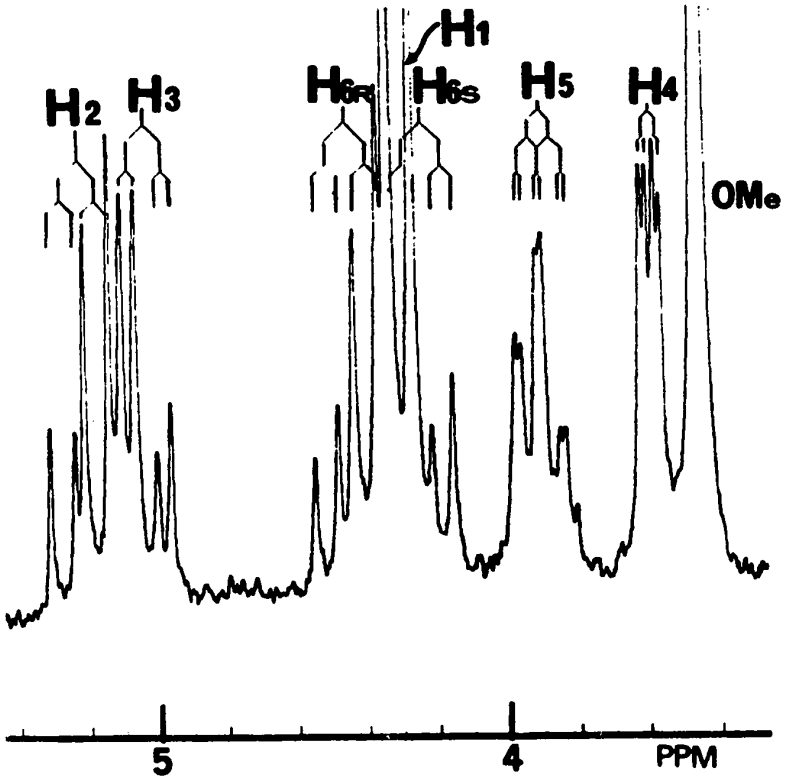
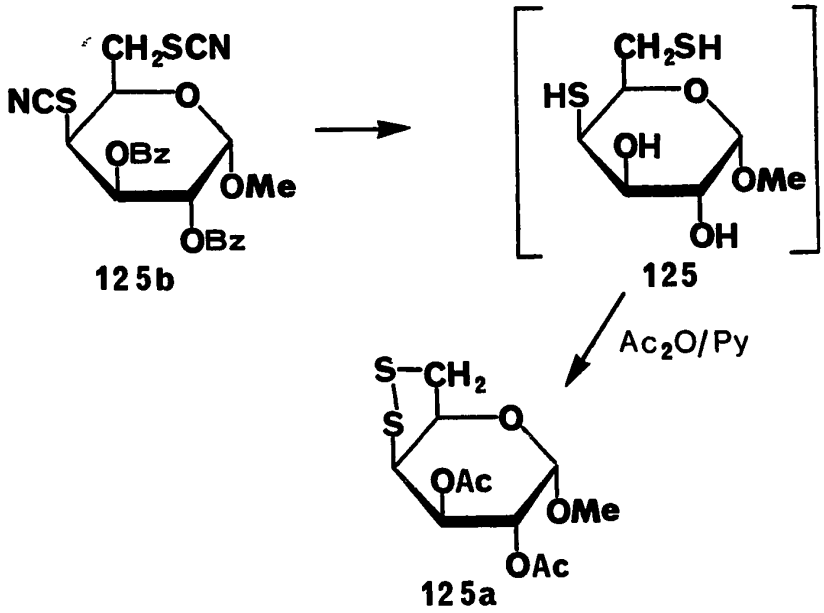


FIG. 30 Partial NMR spectrum (100 MHz, at 1000 Hz sweep width) of 123a in $CDCl_3$.

tinguishable from those already described for 109b. In contrast to 109b, the hydrolysis of 124b proceeded readily. Although the free thiol was not isolated, no complication was reported in the alkaline hydrolysis of methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-thiocyano-α-D-glucopyranoside (255). Thus the reluctance of the axial thiobenzoate grouping in 109b to hydrolyse smoothly is probably a stereochemical problem. It has been reported that alkaline hydrolysis of methyl 2,3-di-O-benzoyl, 4,6-di-deoxy-4,6-dithiocyano-α-D-galactopyranoside (125b) gave a product which on acetylation yielded 125a; the intermediate compound 125 was probably formed but then

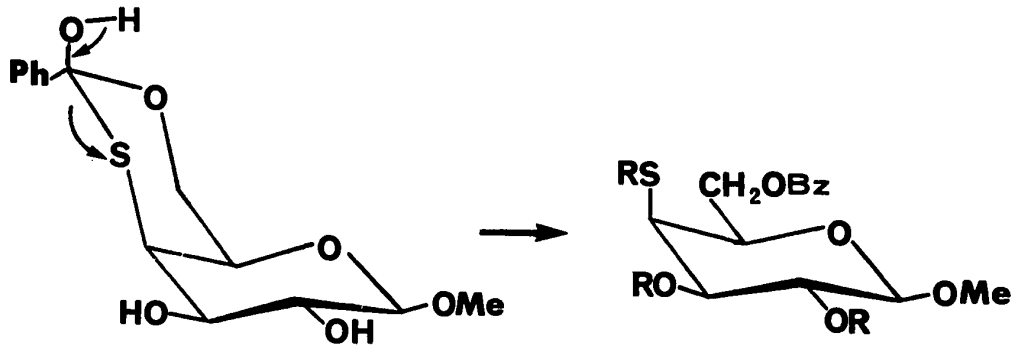
oxidised in air to a disulphide moiety before acetylation (256). In the NMR spectrum of 125a, as in that of 123a, the H-4 signal is at high field (4.18 ppm) whereas the other four ring protons resonate at low field.



The marked stability of an O-benzoyl and/or S-benzoyl group in 109b invited closer examination, since the hydrolysis of similar compounds has not been studied in detail. After treatment of 109b with sodium methoxide-methanol for two days, the mixture of products was fractionated on a silica gel column. The major compound is probably 126, the triacetate of which (126a) gave an NMR spectrum showing both aromatic and acetyl proton resonances. Compared to the spectrum of 109b, the signals of H-2, H-3 and H-4 were shifted upfield whereas there was little change in the chemical shifts of the 6-protons; this suggests that O-6 of 126a is benzoylated and that O-2, O-3 and S-4 are acetylated. In addition

one acetate resonance appeared at unusually low field (2.2 ppm), and was deshielded further on addition of benzene-$\underline{d}_6$. The other acetate resonances were centered at 1.87 and 1.96 ppm and were little affected by the change of solvent.

That a benzoate group should remain on a primary carbinol when the secondary ones are cleaved is surprising. Gluco- or galacto-hexopyranosides tetrabenzoates or tetraacetates present no such problem on saponification. Hence the obstacle to the hydrolysis of 109b probably lies in the strong nucleophilicity of the thio anion for carbon and the axial orientation of the thiobenzoate group. An axial ester on a cyclohexane system is expected to hydrolyse slowly (257). Thus it appears that the 4-S derivative retains the benzoyl group but hands it to the primary carbinol in the final stages of the hydrolysis work up, probably through the intermediacy of a six-membered ring orthoester (126c)



126c

126 R = H

126a R = Ac

This is analogous to the well-known migration of secondary acyl groups to primary unsubstituted carbinols of partially acetylated monosaccharides in acid or alkaline media (258-262).

VI SUBSTRATES MODIFIED AT C-4

Methyl 4-deoxy-4-halo-β-D-galactopyranosides

Reviews on synthetic methods leading to halogeno sugars have been given by Barnett (263) and Hanessian (264). Few of these methods involve secondary O-sulphonyl groups, and this in itself is a reflection of the difficulty commonly encountered in the displacement of secondary sulphonyloxy-groups by halide ions. The reaction closely follows the degree of nucleophilicity of the ion (265) although there is an indication that, as expected (266), the smaller, chloro- and fluoro ions in an aprotic solvent are also good nucleophiles. In view of the success with which sulphonyloxy displacement is effected by fluoride ion in acetonitrile (267), similar conditions were used in the preparation of the compounds described here.

Methyl 4-deoxy-4-iodo-β-D-galactopyranoside

Solvolysis of 121 in acetonitrile at 95-100° with a six-fold excess of tetra-n-butylammonium iodide caused gradual displacement of the brosylate but the reaction was not carried to completion because another compound (on tlc benzene:ether 9:1) was subsequently formed at the expense of the initial product. The latter was isolated by chromatography on silica gel and was crystallized from methanol. Its NMR spectrum is consistent with a galacto configuration at C-4 which, together with analytical data, shows that the compound is methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-iodo-β-D-galacto-

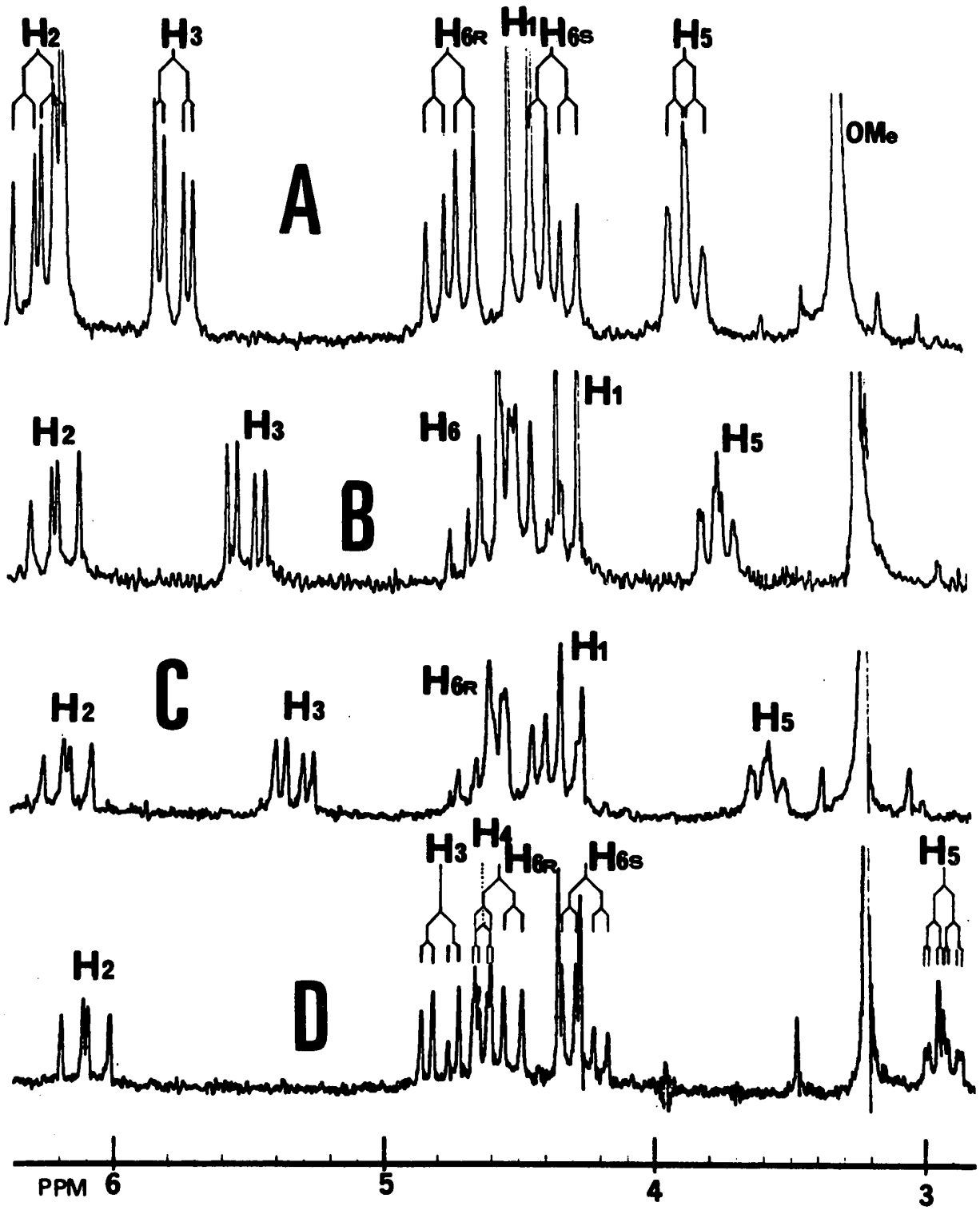


FIG. 31 Partial NMR spectra (100 MHz, at 1000 Hz sweep width) in C_6D_6 A: methyl β-D-galactopyranoside tetrabenzoate. B: methyl 2,3,6-tri-O-benzoyl-4-chloro-4-deoxy (106b), C: 4-bromo-4-deoxy (107b) and D: 4-deoxy-4-iodo (108b)-β-D-galactopyranoside tribenzoates.

pyranoside (108b). Signals for H-3, H-4 and H-5 of (108b) are at higher field (Fig. 31.D) than those of methyl β-D-galactopyranoside tetrabenzoate (Fig. 31.A). The upfield shift of H-4 - and this was observed as well for C-4 in ^{13}C NMR spectrum - is expected because of the relatively high electron density at C-4; that of H-3 or H-5 must be due to the electronic field of the iodo orbital. The configuration of the iodo-group in 108b seems to be important in this context since no comparable upfield displacements for signals H-3 and H-5 are observed in the NMR spectrum of methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-iodo-β-D-glucopyranoside (127b)(succeeding paragraph).

The second compound (127b) produced together with 108b was purified by chromatography and crystallization from ethanol. Its NMR spectrum (Fig. 32.C) is consistent with that of a glucopyranoside configuration (198). That is, the H-4 signal, which in most O-acyl glucopyranose compounds including the starting material 121 absorbs at low field, is upfield of the H-2, H-3, H-6 signals. That 127b and 108b should be produced at the same time, does not require that an S_N1 mechanism was operative but rather that the iodine of the galacto isomer was displaced by iodide ion to give a product (127b) with overall retention of configuration. Racemization of chiral centres during S_N2 reactions involving halogen exchange (268,269) is quite well known (270), and radioactive iodine (^{131}I) exchange studies have shown that an iodine atom at C-4 is much more easily displaced in the galacto than in the gluco series (271).

Solvolysis of 121 at a higher bath temperature (100-120°) in acetonitrile proceeded to give, in addition to compounds 108b and 127b a third compound (128b) as a minor fraction. The NMR spectrum

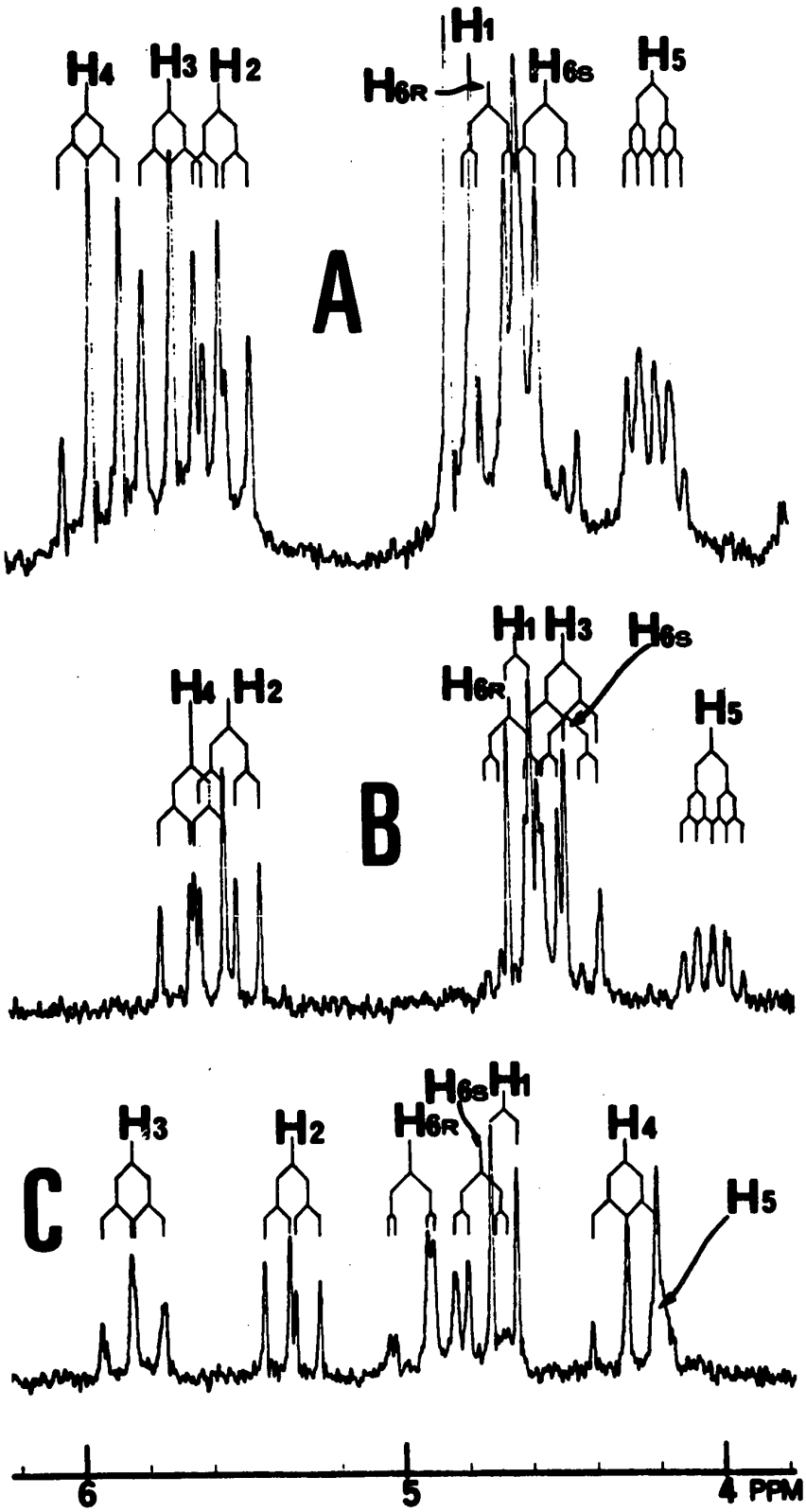
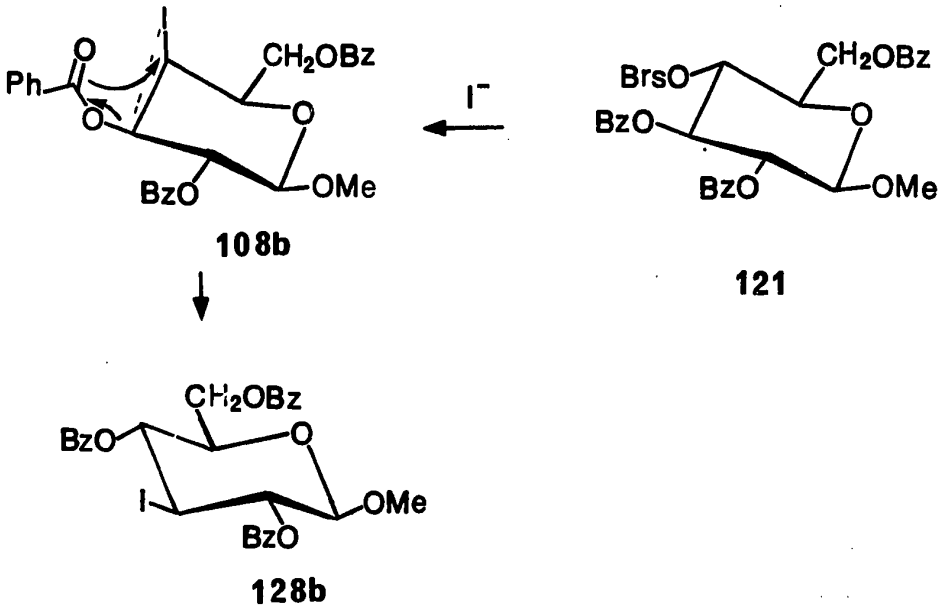


FIG. 32 Partial NMR spectra in $CDCl_3$ of A: methyl β-D-glucopyranoside tetrabenzoate (116b). B: of 128b and C: of 127b (100 MHz, 1000 Hz sweep width).

(Fig. 32.B) of 128b is consistent with a glucopyranose stereochemistry, i.e. the coupling parameters for the ring protons are all large (8-10 Hz). In addition, it is clear that the brosyl group of 121 has been removed (three groups of benzoate proton signals in the NMR). The H-3 signal is at higher field than the corresponding proton in 127b in the same solvent, and H-4 is at relatively low field as in glucopyranose compounds esterified at O-4. Thus 128b is formulated as a 3-deoxy-3-iodo-4-benzoate. It must be formed as a result of intramolecular benzoyl migration and displacement by an iodo anion, presumably involving the reactive 4-iodide of the galacto compound as in the following sequence.



108b

121

128b

De-O-benzoylation of 108b afforded crystalline methyl 4-deoxy-4-iodo-β-D-galactopyranoside 108. The NMR spectrum of the triacetate derivative (108a) was consistent with the galacto configuration;

again H-3 and H-5 signals are at higher field (Table V) than in methyl β-D-galactopyranoside tetraacetate. Therefore on going from 108b to 108, the iodo group was not affected. The conformation of 108 as seen from its NMR spectrum in D_2O is C_1; an axial iodide does not change the pyranose conformation. Similarly, de-O-benzoylation of 127b gave a product which after subsequent acetylation gave an NMR spectrum consistent with the gluco configuration.

Compound 127b, containing a little of the galacto analogue (108b), was de O-benzoylated and the product dehalogenated by hydrogenolysis over palladium black.* Purification of the hydrogenolysis product through conversion into methyl 2,3,6-tri-O-acetyl-4-deoxy-β-D-xylo-hexopyranoside (103a), followed by de-O-acetylation, afforded another required enzyme substrate, i.e., methyl 4-deoxy-β-D-xylo-hexopyranoside (103). The NMR spectrum of 103a (Table V) was consistent with the assigned structure.

Methyl 4-bromo-4-deoxy-β-D-galactopyranoside (107)

Solvolysis of 121 with tetra-n-butylammonium bromide in acetonitrile gave results similar to those described for the iodo compound 108. However, S_N2 halogen exchange was not as pronounced as in the synthesis of 108 and the yield of methyl 2,3,6-tri-O-benzoyl-4-bromo-4-deoxy-β-D-galactopyranoside (107b) was relatively much higher. De-O-benzoylation of 107b proceeded without complication to give methyl

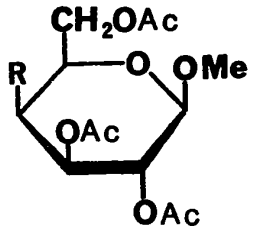
* Hydrogenolysis proceeded more rapidly with the gluco than with the galacto diastereoisomer (as monitored by tlc).

Table V Chemical Shifts (δ, ppm) of Derivatives of 111a in $CDCl_3$

Compound		H-1	H-2	H-3	H-4	H-5	H-6R	H-6S	OMe	OAc		
103a	R = H	4.33	4.83	5.00	2.12 1.60[a]	3.78	4.23	4.10	3.47	2.07	2.04	2.01
104a	F	4.40	5.28	4.98	4.85	3.80	4.42	4.24	3.50	2.08	2.07	2.05
106a	Cl	4.43	5.30	5.03	4.51	4.00	4.39	4.22	3.51	2.12	2.10	2.08
107a	Br	4.45	5.22	4.88	4.57	3.82	4.45	4.25	3.54	2.12	2.10	2.08
108a	I	4.47	5.32	4.34	4.66	3.17	4.39	4.11	3.52	2.11	2.08	2.07
110a	N_3	4.37	5.28	5.08	4.05	3.81	4.37	4.16	3.47	2.10	2.08	2.04
105a	$NHCOCH_3$[b]	4.20	5.01		4.69	3.90	4.20		3.51	2.09	2.05	2.01
6a	OAc	4.42	5.22	5.02	5.41	3.92	4.25	4.13	3.52	2.17	2.06	2.05

a H-4 axial

b NH signal at 6.11 ppm



111a

4-bromo-4-deoxy-β-D-galactopyranoside (107). The NMR spectrum (Table V) of its triacetate (107a) confirmed the structure assigned.

Methyl 4-chloro-4-deoxy-β-D-galactopyranoside (106)

When treated with tetra-n-butylammonium chloride in acetonitrile for two days, 121 gave only one product, identified by analysis and from its NMR spectrum (Fig. 31B) as methyl 2,3,6-tri-O-benzoyl-4-chloro-4-deoxy-β-D-galactopyranoside (106b). The absence of halogen exchange in this reaction is as expected due to the fact that chlorine as well as fluorine atoms are not only bad leaving groups but also of low nucleophilicity (272). De-O-benzoylation of 106b afforded crystalline methyl 4-chloro-4-deoxy-β-D-galactopyranoside (106), characterized further by the NMR spectrum of its triacetate (106a)(Table V).

Methyl 4-deoxy-4-fluoro-β-D-galactopyranoside (104)

The use of tetra-n-butylammonium fluoride prepared according to Foster et al. (267) in the solvolysis of 121 resulted in virtually complete loss of the benzoate and brosyl groups. A sample of tetra-n-butylammonium fluoride prepared at a pH adjusted to 4.7 or 4.8 gave the same results.

Marcus and Westwood (273) have recently reported the synthesis of methyl 4-deoxy-4-fluoro-α-D-galactopyranoside from methyl 2,3-di-O-benzyl-4-O-(methanesulphonyl)-6-O-trityl-α-D-glucopyranoside. The β-anomer (104) was prepared for the present purpose using the cor-

responding β-D-gluco derivative (129), as shown in Figure 33, except that the brosyl group was used instead of mesyl and a reaction time of two, rather than five, days was required.

Hydrogenolysis of 130 over palladium black at room temperature and pressure was slow (40 h. having been reported for the α- analogue). However, hydrogenolysis after de-O-tritylation was rapid and required less than two hours, to give readily crystalline 104. This modification also avoided the relatively tedious removal of triphenylmethane which would be formed if hydrogenolysis of 130 was undertaken. The NMR spectrum (Fig. 34) of the triacetate (104a) of 104 is consistent with a galacto configuration at C-4. Accordingly, as was observed for the α-analogue (273), the geminal coupling between H-4 and ^{19}F is 50 Hz; and the trans-disposed H-5 and H-3 both show a $J_{^{19}F,\,^{1}H}$ value of 25 Hz. Spacings for the two H-4 doublets that arise from coupling with H-3 and H-5 are as expected for the galacto configuration. The absence of any gluco isomer among the products of hydrogenolysis clearly indicated that the conversion of 129 to 130 did not involve racemization at C-4.

Of special interest to the D-galactose oxidase study are two novel features in the NMR spectrum of methyl 2,3,6-tri-O-acetyl-4-deoxy-4-fluoro-β-D-galactopyranoside (104a). Whereas no long range coupling was observed in the NMR spectrum of the α-anomer (273), 104a shows two such interactions each amounting to 1.1 Hz over four bonds involving the fluoro atom and H-2 on one hand, and one H-6 on the other. Long-range ^{19}F-^{1}H and ^{1}H, ^{1}H coupling over four bonds in systems in which the nuclei have "W" or "M" coplanar relationship is well known (274-277). When the nuclei are in a 1,3-axial-equatorial relationship,

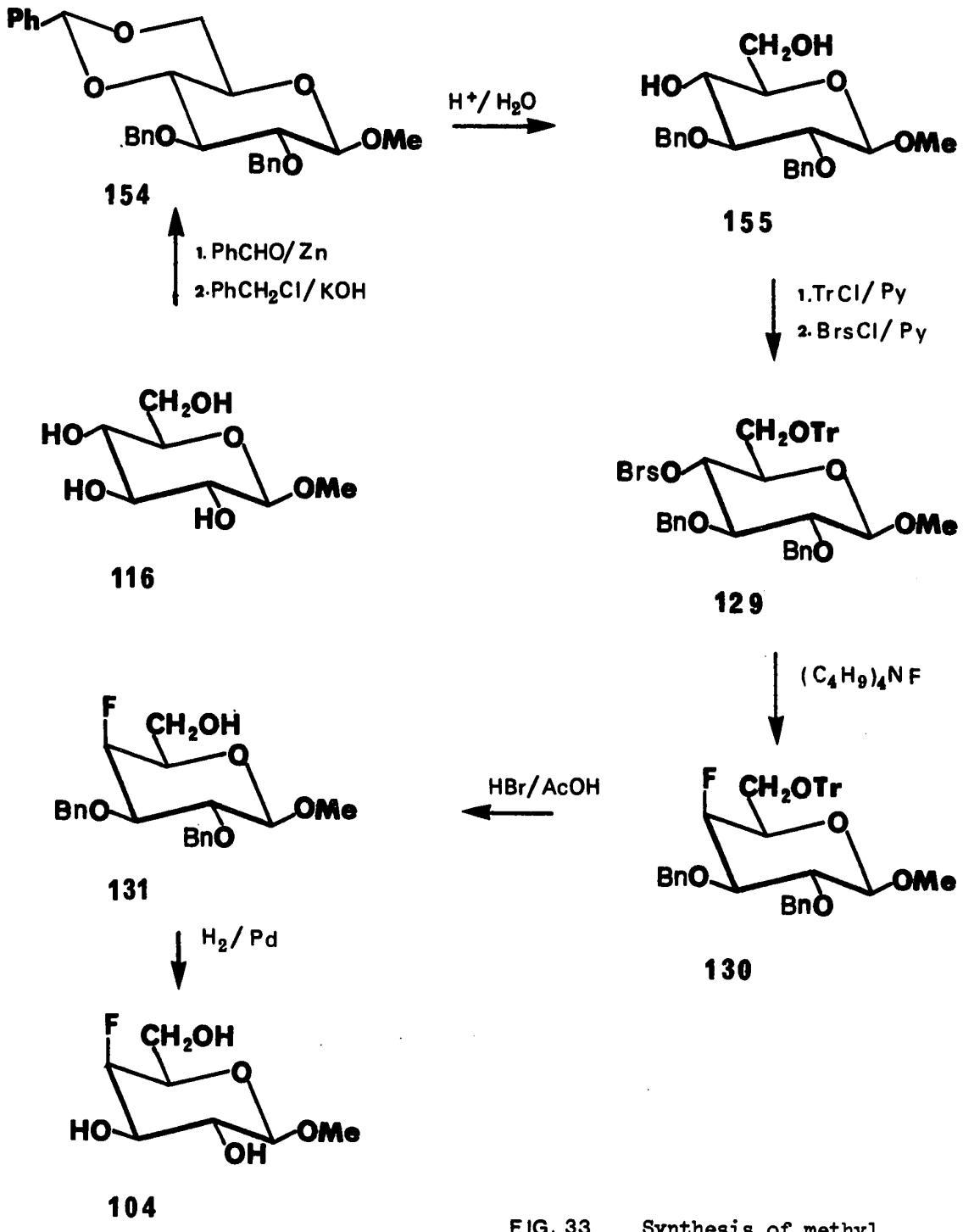


FIG. 33 Synthesis of methyl 4-deoxy-4-fluoro-β-D-galactopyranoside (104)

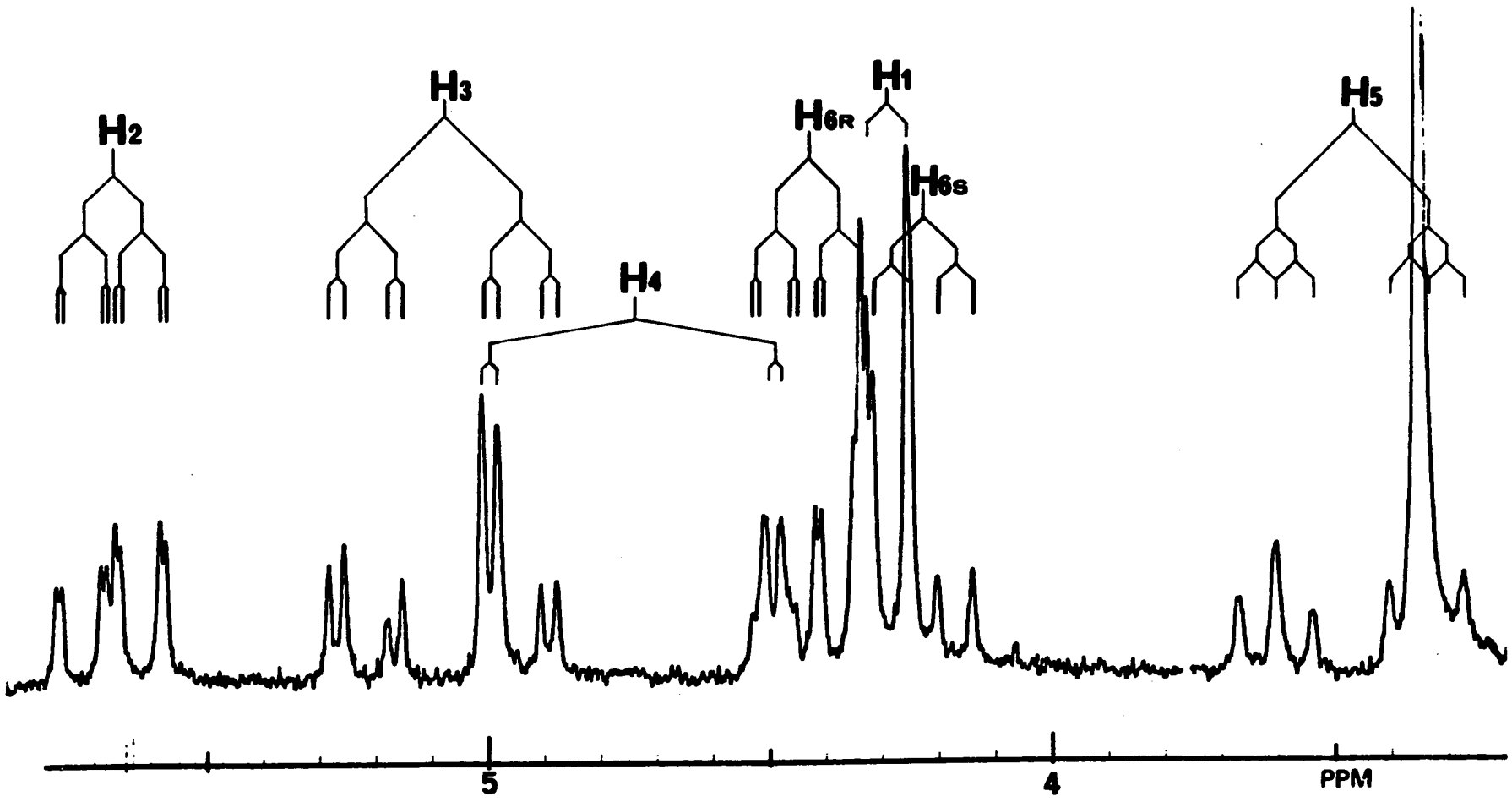
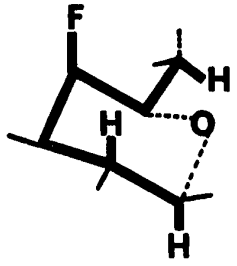


FIG. 34 Partial NMR spectrum (100 MHz) of methyl 2,3,6-tri-O-acetyl-4-deoxy-4-fluoro-β-D-galactopyranoside (104a) in C_6D_6 at 500 Hz sweep width.

coupling is less common. However, Hall et al (278) have recently reported a value of 1.1 Hz for $^{4}J_{^{19}F,^{1}H\text{-}5}$ in compound 131a and 1.5 Hz for compound 132 in addition to one $^{5}J_{^{19}F,^{1}H}$ over five bonds. These interactions observed with 104a are also found with methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-fluoro-β-D-galactopyranoside (104b) although the splittings are now smaller (0.7 Hz). Since the coupling constants for the pyranose ring protons do not change, in comparing 104a and 104b, these long range couplings speak of a combination of stereo-chemical, electronic and substituent dependence rather than of stereochemistry alone.

Another, novel, axial-axial five bonds coupling was observed in NMR spectra recorded at 100 and 220 MHz for compound 104b. This involved, axial ^{19}F-4 and axial H-1 and a magnitude of $\leq$ 0.5 Hz. Equatorial ^{19}F-4 and equatorial H-1 coupling over five bonds, amounting to 3.4 - 4 Hz, has been already reported in derivatives of methyl 4-deoxy-4-fluoro-α-D-glucopyranoside (279) and other systems (280). Structure 133 summarises the long-range coupling observed in both 104a and 104b.

The fact that only one H-6 of 104a and 104b is long-range coupled with the fluoro atom raises the question of the orientation of substituents at C-6, where the possibility of rapid, free rotation about the C-5, C-6 bond exists. That $^{4}J_{^{19}F,^{1}H\text{-}6}$ should have the same value as $^{4}J_{^{19}F,^{1}H\text{-}2}$ when H-2 is fixed on the pyranose ring clearly suggests that only one rotamer is overwhelmingly preferred. The matter is pursued further in the following chapter.



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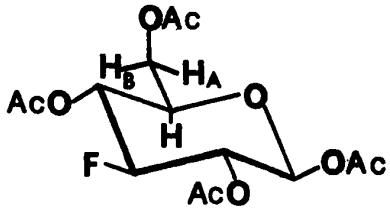
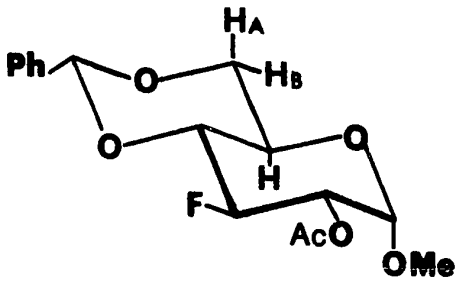


131a

$J_{F,\ H\text{-}5} = 1.1$ Hz

$J_{F,\ H\text{-}6B} = 1.5$ Hz



132

$J_{F,\ H_B} = 2.0$ Hz

$J_{F,\ H_5} = 1.5$ Hz

CHAPTER VII

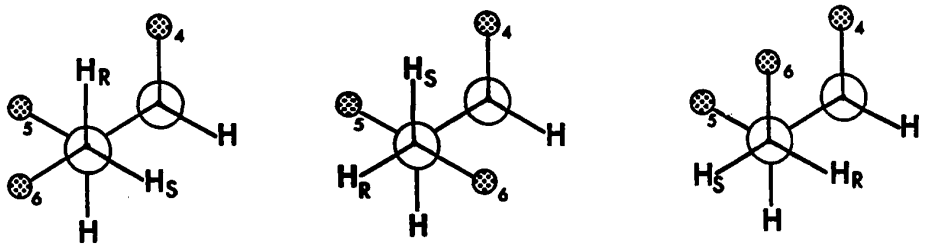
ROTATIONAL ISOMERISM ABOUT THE C-5, C-6 BOND OF D-GALACTOSE AND ITS DERIVATIVES

A. General Remarks

Of possible significance to this stereochemical study of D-galactose oxidase action, is the question of the rotational conformation of the 6-carbinol group of methyl β-D-galactopyranoside, and of the modified substrates described in the previous Chapter. Although an exact conformation of D-galactose oxidase substrates cannot be defined for the transition state of the dehydrogenation step, nevertheless a knowledge of the ground state stereochemistry may assist in a visualization of the geometry of the transition state; for in the initial stages of enzyme-substrate complex formation, the ground state conformation of the substrate will be important.

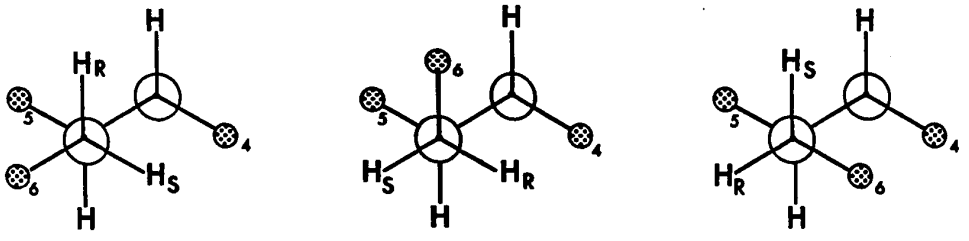
The detailed structure of methyl α-D-galactopyranoside monohydrate in the solid state has recently been described by Gatehouse and Poppleton (281). In this molecule the primary carbinol oxygen, which is involved also in hydrogen bonding, adopts a disposition that is gauche relative to O-5 and *trans* to C-4 (134). In attempting to explain the abnormal melting point of the same compound, however, these authors consider rotamer 135 to play a significant role in the transition from the solid state to the liquid phase.

VII ROTATIONAL ISOMERISM OF SUBSTRATES



134 **135** **136**

Model calculations also have been advanced in support of rotamer 134 for both gluco and galacto pyranoses (282). In trying to rationalize the low solvolytic reactivity of 6-O-sulphonyl derivatives of D-galactose as compared with their gluco analogues, it has been suggested that the D-gluco compounds can adopt rotamers 137-139 easily (283). This should provide the nucleophile with more ready access to C-6 than could occur with the D-galactopyranose ester, because O-4 of the latter would strongly interact with the nucleophile. Also, only rotamers 134 and 135 should contribute appreciably to the transition state of solvolysis of D-galacto-6- sulphonates.



137 **138** **139**

VII ROTATIONAL ISOMERISM OF SUBSTRATES

The conformation of the 6-carbinol group of D-galacto- and D-glucopyranose and derivatives in solution has been the subject of controversy. There is no doubt that because of the chiral nature of C-5 of these hexoses the 6-hydrogens are chemically and magnetically non-equivalent. Hall et al.(284) have presented NMR spectral data in favour of rotamer 135, for the substituents of the hydroxymethyl group of α-D-galactopyranose derivatives. This was based on vicinal couplings of $J_{5,6_2}$ (7 Hz), $J_{5,6_1}$ (5·5 Hz) and geminal $J_{6_1,6_2}$ (10.8 Hz), and after taking electronegativity effects into consideration. Our data (173) also appear to favour rotamer 135 for methyl β-D-galactopyranoside derivatives in aprotic solvents although they suggest further that H_S is turned about 25° towards O-5 from the plane bisecting the C_5-H_5 bond because the observed coupling of 6-7 Hz is not truly representative of trans diaxial vicinal couplings. In addition this slight deviation would alleviate eclipsing between H_S and O-4.

Lemieux and Martin (285) have used polarimetry to argue in favour of rotamers 134 and 135 (in the ratio of 2:1) for D-galactose derivatives and 137 for the D-glucose derivatives. Although their proposals, based also on O/O, O/C gauche interactions, seem to hold for oxygen substituents on the pyranose ring the situation is not as clear cut for the rotamer population of the hydroxymethyl group. Furthermore, Lemieux and Martin (285) tended to discount rotamer 139 on the grounds that O-6 interacts 1,3-diaxially with O-4. This is reasonable, and is supported by NMR data (173,198,284). Nevertheless, to account for solvent variable optical rotations in D-glucose derivatives they agree with Yamana's proposal (286,287) that rotamer 139 may experience

stabilization through hydrogen bonding.

If hydrogen bonding is indeed important in rotamer 139 in aprotic solvents, then the observed coupling parameters for methyl 2,3,4-tri-O-acetyl-β-D-glucopyranoside (140) should be different from those of methyl-2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (116a) in the same solvent; for only in 140 can the primary carbinol easily participate in hydrogen bonding with O-4. However, this is not observed, because the vicinal coupling parameters for 140 and 116a remain the same (Table VI - footnote); only the relative chemical shifts of H_R and H_S are interchanged for 140 and 116a, although this is probably due to the close alignment of the carbonyl of the 6-O-acetyl group relative to H_S in 116a.

TABLE VI

Effect of O-6 substituents on the chemical shifts of prochiral (H_{6R} and H_{6S}) hydrogens of D-glucose derivatives 141.

COMPOUND [1]	δH_R [2]	δH_S [3]
140 [4]	3.62	3.44
116a	4.08	4.27
141a	4.57	4.39
142	3.62	3.42
143	4.05	4.25
144	3.28	3.02

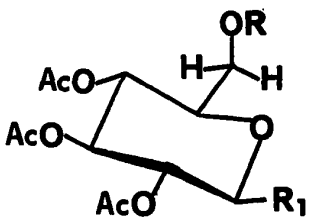
1 In $CDCl_3$ containing 25% C_6D_6

2 Signal with narrow splittings: $J_{5,6R}$ 2.5 Hz, $J_{6R,6S}$ 12.5 Hz

3 Signal with splittings: $J_{5,6S}$ 4.7 Hz, $J_{6R,6S}$ 12.5 Hz

4 D_2O exchanged

VII ROTATIONAL ISOMERISM OF SUBSTRATES



141

140 R = H, R_1 = OMe

116a R = Ac, R_1 = OMe

141a R = Bz, R_1 = OMe

142 R = H, R_1 = D

143 R = Ac, R_1 = D

144 R = trityl, R_1 = D

In addition, as reported below, in the NMR spectrum of methyl 4-deoxy-4-iodo-β-D-galactopyranoside (108) in D_2O (Fig. 35) the prochiral hydrogens (H_{6R}, H_{6S}) show coupling with H-5 to the same extent as with the tribenzoate or triacetate of 108 in deuterobenzene or deuterochloroform (Tables VII-IX). This implies that the basic composition of rotamer populations does not change significantly with change of solvent for these compounds.

B. Factors affecting J values of 6-protons of methyl β-D-galactopyranoside derivatives and analogues

In an effort to resolve these ambiguities concerning rotamers 134-136 a comparison was made of the impact of different C-4 substituents on the coupling and chemical shift parameters of H-5, H-6 of methyl β-D-galactopyranosides; for the main difference between the D-galacto and D-gluco compounds displaying difference in H-5, H-6 couplings lies in the configuration of the substituents at C-4. If then the C-4 substituents

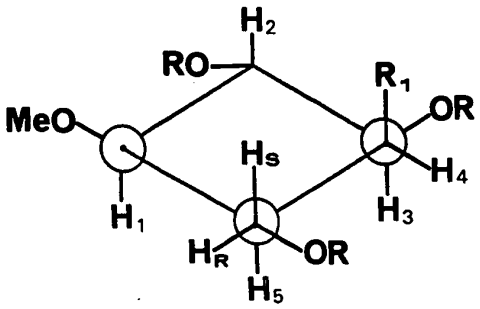
VII ROTATIONAL ISOMERISM OF SUBSTRATES

are systematically modified in both series, intermediate coupling parameters should be obtained for J_{H_5,H_6}. The NMR parameters for $H_{\underline{R}}$ and $H_{\underline{S}}$ of chemically-modified methyl β-D-galactopyranoside, and others, are shown in Tables VII-IX.

The chemical shift of $H_{6\underline{R}}$ of non-deuterated methyl β-D-galactopyranoside tetrabenzoate was deduced from the NMR spectrum of enzyme-resolved methyl β-D-galactopyranoside 6-d (6S) tetrabenzoate (Chapter IV), and found to be at lower field than $H_{6\underline{S}}$. It is seen that coupling between $H_{6\underline{R}}$ and H_5 maintains a relatively constant value for the C-4 modified substrates.

TABLES VII-IX

Some NMR spectral data (at 100 MHz) for derivatives of methyl β-D-galactopyranoside and C-4 substituted analogues (145)



145

TABLE VII

R_1	$J_{5,6\underline{R}}$	$J_{5,6\underline{S}}$	$J_{6\underline{R},6\underline{S}}$	$\delta H_{\underline{R}}$	$\delta H_{\underline{S}}$	$\Delta\delta$(ppm)	δH-2
F	7.0	6.0	11.5	4.64	4.45	0.19	6.15
Cl	6.7	5.4	11.5	4.67	4.43	0.24	6.20
Br	6.6	5.1	11.4	4.62	4.36	0.26	6.18
I	7.0	5.0	11.6	4.58	4.25	0.33	6.10
OBz	6.4	6.2	11.2	4.74	4.36	0.38	6.27
SBz	7.0	5.5	11.2	4.84	4.43	0.41	6.08
S-CN	7.0	5.0	11.6	4.53	4.20	0.33	5.92
N=N=N	6.8	6.1	11.2	4.54	4.40	0.12	6.10

Values of J are in Hz; those of δ in ppm

Solvent, C_6D_6

R = benzoate (145)

TABLE VIII

R_1	$J_{5,6\underline{R}}$	$J_{5,6\underline{S}}$	$J_{6\underline{R},6\underline{S}}$	$\delta H_{\underline{R}}$	$\delta H_{\underline{S}}$	$\delta H_{\underline{R}}-\delta H_{\underline{S}}$ $=\Delta\delta$	δH-2
Cl	6.0	5.9	11.8	4.76	4.58	0.17	5.83
Br	6.6	5.8	11.7	4.75	4.55	0.20	5.83
I	6.6	5.9	11.6	4.73	4.46	0.27	5.83
OBz [1]	5.2	6.1	9.8	4.68	4.45	0.23	5.85
OBz *[1]	9.0	6.0	13.4	4.55	4.23	0.32	5.62
SBz	7.9	5.4	12.2	4.78	4.52	0.26	5.60

* CS_2 as solvent

Solvent: $CDCl_3$

R = benzoate (145)

couplings in Hz, chemical shifts in ppm

[1] compound 6b

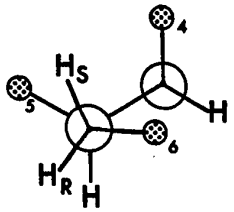
TABLE IX

R_1	$J_{5,6\underline{R}}$	$J_{5,6\underline{S}}$	$J_{6\underline{R},6\underline{S}}$	$\delta H_{\underline{R}}$	$\delta H_{\underline{S}}$	$\delta H_{\underline{R}} - \delta H_{\underline{S}} = \Delta\delta$	δH-2
H	6.0	4.5	11.5	4.23	4.10	0.11	4.86
F	6.4	6.2	11.5	4.42	4.24	0.18	5.28
Cl	6.6	6.0	11.6	4.39	4.22	0.17	5.31
Br	6.5	6.0	11.7	4.45	4.25	0.20	5.22
I	6.8	5.8	11.5	4.39	4.11	0.27	5.32
Ac	7.5	5.5	11.4	4.25	4.13	0.12	5.22

Solvent: $CDCl_3$ and R = OAc (in 145)

J in Hz and δ in ppm

This consistency helps to identify the H-6R and H-6S resonances presented in Tables VII-IX. Since, as noted above, the resonances of H-6R and H-6S interchange positions when a comparison is made between 140 and 116a, caution was exercised in assigning these resonances. Thus only parameters for fully benzoylated, or acetylated, or completely non-esterified compounds and in one particular solvent were used. It may be noted that, with a few exceptions, $J_{5,6R}$ is always larger than $J_{5,6S}$ as generally assumed. That is, the larger coupling constant of 7 Hz reported by Hall and Manville (284) for (135) does not arise from trans diaxial H-5, H-6S but from gauche disposed H-5, H-6R*. If the Karplus curve (288,289) applies fairly closely for these systems, H-6S should be rotated even more than the value of 25° towards O-5 (cited above) such that the torsional angle (dihedral angle) between H-6R and H-5 is about 25° (146).



146

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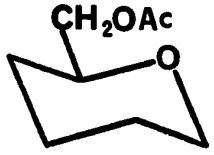
* According to the assignment of Hall et al (284), the H-6 proton with $J_{5,6} = 4.2$ Hz in the gluco derivative is H_S; therefore H_R has a coupling of 2.5 Hz with H-5 in the same compound.

VII ROTATIONAL ISOMERISM OF SUBSTRATES

That exocyclic rotamers deviate from the ethane staggered conformation, where substituents are disposed at angles of 180° or 60° with respect to each other, seems to be true of several compounds (290).

The NMR spectral data (Table IX) for methyl 4-deoxy-β-D-xylo-hexopyranoside triacetate 103a are noteworthy. This compound is the point of convergence between the D-gluco and D-galacto diastereoisomers; and the absence of an equatorial OH-4 in the gluco compounds should permit O-6 to relax from its position in rotamer 138 to that in 146. In this latter position H-6S originally showing a coupling of $J_{5,6S} = 4.5$ Hz in rotamer 138 now gives a J value of 4.2 Hz in 147. It is interesting to note that Lemieux and Martin (285) have suggested both a rotamer corresponding to 135 and one close to 146 for compound 147, which in the vicinity of C-4 and O-5



147

$J_{5,6} = 6.2$ Hz

$J_{5,6'} = 4.2$ Hz

$J_{6,6'} = 11.2$ Hz

is akin to methyl 2,3,6-tri-O-acetyl-4-deoxy-β-D-xylo-hexopyranoside, and which also shows nearly the same couplings for the 5 and 6-protons.

The values of $J_{5,6R}$ and $J_{5,6S}$ (6.4 and 6.2 Hz respectively) become nearly the same in methyl β-D-galactopyranoside tetrabenzoate in deuterobenzene-d_6, suggesting that there is a degree of flexibility (perhaps of about 5°) in the rotation about the C-5, C-6 bond of these D-galacto compounds.

VII ROTATIONAL ISOMERISM OF SUBSTRATES

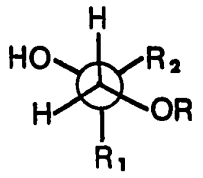
For most of the compounds listed in Tables VII-IX, the coupling constants for the prochiral hydrogens approximate to about 6 Hz. It appears that there is a common position for these prochiral hydrogens. In the particular case of methyl 2,3,6-tri-O-acetyl-4-deoxy-4-fluoro-β-D-galactopyranoside (104a) the fluorine atom exhibits long-range coupling of 1.1 Hz with only one H-6 proton, i.e. H_R. Hence the residence time of one particular rotamer, about C-5, C-6 in this instance, must be long enough to minimize the contribution of other rotamers. Five-bond long range coupling involving pyranose ring fluoro atoms and C-6 hydrogens has been observed (277,278), but its stereochemical implications has not been rationalized. Therefore it may be premature here, due to the limited data available, to suggest an origin for the stereochemical dependence of this type of ^{19}F-^{1}H long range coupling in methyl 2,3,6-tri-O-acetyl-4-deoxy-4-fluoro-β-D-galactopyranoside.

C. Factors contributing to anisochronism of 6-protons of D-glycopyranose derivatives.

a. Other saturated aliphatic systems.

Recently Dabrowski and Ejchart (291) have conducted ^{1}H and ^{13}C NMR spectral studies on the magnetic non-equivalence of carbinols of the general formula $R_1R_2C(OH)CH_2OR$ (the overall evidence indicates that conformer 148 is the most favoured one). Their results indicate that variation of R_1 and R or R_2 and R will cause a difference in the anisochronism of the methylene protons. Thus if R_2 = Butyl, R_1 = R = Me, the chemical shift difference $\Delta\delta$ between the geminal protons in 148 is

VII ROTATIONAL ISOMERISM OF SUBSTRATES



148

0.14 ppm, whereas when R is changed to isopropyl, $\Delta\delta$ = 0.169 ppm. In short their results indicate that anisochronism in compounds of general formula 148 is brought about by the gauche interaction between R and either R_1 or R_2.

b. Methyl β-D-galactopyranoside derivatives and analogues.

In like manner, the substituents at C-4 of methyl-β-D-galactopyranoside and derivatives would be expected to induce anisochronism for the prochiral hydrogens of the compounds listed in Tables VII-IX.

The differences in chemical shifts in ppm between H_R and H_S for the compounds in which R_1 and R of O-6 of 145 are varied are shown in Tables VII-IX. The accompanying coupling constants are shown also. Several points emerge from these data*. First, the gradual increase of anisochronism as R_1 varies from F to I suggests that there is interaction

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* One should consider here the possibility that the rotamer population changes, giving rise to different average values of δ. However, it should be noted that in 145, R_1 is equidistant from H-2 and H_S (or from H_R of rotamer 134). Accordingly, since δH-2 is nearly constant (Tables VII-IX) $\Delta\delta H_R, H_S$ would then be the result of the $R_1 \leftrightarrow$ OR interaction.

between the substituents at C-4 and C-6, i.e. between R_1 and OR-6 or R of O-6. This interaction cannot be that of an axial substituent deshielding an axial proton in a 1,3 cis diaxial relationship, as is known (198) for the effect of an acetoxy or hydroxy-group in carbohydrate derivatives, or cyclohexanes (292). For if this was the case, H-2 of 145 which is in a 1,3 cis diaxial relationship with respect to R_1, also should be significantly affected. The data in Tables VII-IX show that for most of the compounds listed the chemical shift for H-2 remains nearly constant, except where R_1 is thiobenzoate*. Therefore changes in chemical shifts of H_R and H_S are less likely to arise from interaction of R_1 with these protons in a 1,3 cis diaxial relationship, as implied for rotamers 134 and 135, than from its interaction with O-6 substituents. R_1 and R of O-6 can better interact if C-4 and O-6 are gauche , as in rotamers 135 and 136 than in 134 where they are anti. Rotamer 136 can most easily be discounted because of the particularly unfavourable non-bonded interaction it incorporates. This leaves rotamer 135 and intermediate rotamers such as 146 as more plausible. The long-range coupling $^4J_{19F,\,6R}$ detected in methyl 2,3,6-tri-O-acetyl-4-deoxy-4-fluoro-β-D-galactopyranoside(104a)is reduced from 1.1 Hz to 0.7 Hz in the corresponding tribenzoate derivative in the same solvent. This is an indication that the 6-benzoate group interacts differently than the 6-acetate group with neighbouring substituents.

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* There is an indication that the lone pairs on sulphur point away from the ring and are probably directed parallel to H-4, causing the latter to resonate upfield (Fig. 29, p.114).

VII ROTATIONAL ISOMERISM OF SUBSTRATES

When R_1 (in 145) contains oxygen bonded to C-4 several anomalies arise. In D-galactose or methyl β-D-galactopyranoside, R_1 = OH and R = H, and OH-4 and OH-6 should not interact strongly enough to produce a field gradient (293) at C-6. As a result, in D_2O the prochiral hydrogens appear almost as a broad singlet. However, when R_1 = Iodo and R = H, there is a sufficient field gradient at C-6 to enable the NMR spectrometer to resolve the H_R and H_S chemical shifts (Fig. 35). When R_1 = OAc and R = $CH_3\overset{O}{\overset{\|}{C}}$, anisochronism for H_R and H_S is slightly larger (0.12 Hz, Table IX). The greater bulkiness of the acetoxy, as compared with the OH-group together with its carbonyl group anisotropy is likely responsible for this slight increase of anisochronism. When R_1 = OBz and R = Ph-$\overset{O}{\overset{\|}{C}}$-, there is an additional increase of anisochronism (0.23 ppm, Table VIII). The benzoate groups are larger and carry an appreciably greater anisotropic magnetic field than the OH or acetoxy-groups.

To sum up, it appears that when R_1 = OR, anisochronism for H_R and H_S is small and is solvent-dependent (compound 6b in Tables VII and VIII); for solvents with a large internal anisotropic magnetic field such as benzene or carbon disulphide, anisochronism increases and is suggestive of an appreciable participation by the solvent in the interaction of an OR_1 substituent at C-4 and R at O-6. This solvent interaction, probably involving O-4 and O-6, may even change the dihedral angle slightly between H-5 and 6-protons, as is probably the case in methyl β-D-galactopyranoside tetrabenzoate with carbon disulphide as a solvent, and contrasts sharply ($J_{6S,6R}$ = 13.4 Hz) with the parameters of the same compound in deuterochloroform (Table VIII, Compound 6b). Alternatively, it is possible

VII ROTATIONAL ISOMERISM OF SUBSTRATES

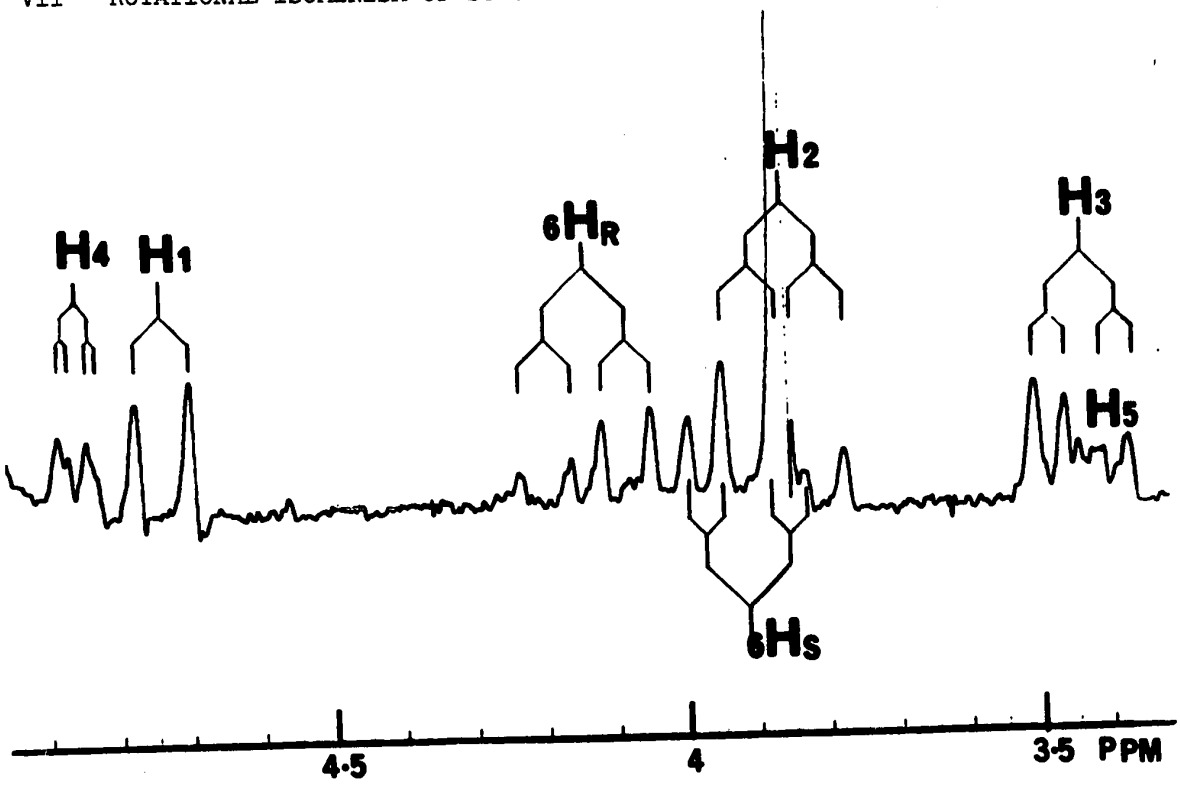

FIG. 35 Partial NMR spectrum (100 MHz) of methyl 4-deoxy-4-iodo-β-D-galactopyranoside in D_2O at 500 Hz sweep width.

that a solvent can induce interaction of the lone pairs on O-6 with the H-6 protons resulting in a change in the geminal coupling, because geminal coupling of methylene protons is usually affected by lone pair orbitals of a heteroatom attached to the methylene carbon (294,295). By contrast, coupling of the H_R and H_S with H-5 in methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-S-benzoyl-β-D-galactopyranoside remains the same in deuterochloroform and benzene-d_6. This suggests that the complexation of the solvent with atoms bonded to C-4 and C-6 is different for oxygen than for sulphur.

VII ROTATIONAL ISOMERISM OF SUBSTRATES

Use of different proportions of deuterochloroform and benzene-$\underline{d}_6$ reveals a strong dependence of D-galacto rotamers upon the solvent when R_1 = OR (in 145). In methyl β-D-galactopyranoside tetrabenzoate, when deuterochloroform is the solvent, $J_{5,6\underline{S}}$ is slightly larger (6.1 Hz) than $J_{5,6\underline{R}}$ (5.2 Hz) (Table VIII) and the geminal coupling $J_{6\underline{R},6\underline{S}}$ is about 10 Hz. As the proportion of benzene-$\underline{d}_6$ increases both $H_{\underline{R}}$ and $H_{\underline{S}}$ are deshielded. In pure benzene-$\underline{d}_6$, $J_{5,6\underline{R}}$ becomes slightly larger (6.4 Hz) than $J_{5,6\underline{S}}$ (6.2 Hz) and the geminal coupling also increases (to 11.2 Hz). With carbon disulphide as the solvent, the observed coupling becomes atypical, i.e. $J_{5,6\underline{R}}$ = 9 Hz, $J_{5,6\underline{S}}$ = 6 Hz and $J_{\underline{gem}}$ = 13.4 Hz. Thus carbon disulphide must play a significant role in the complexation of groups on O-4 and O-6.

In the NMR spectrum of methyl 2,3,4-tri-O-acetyl-6-O-trityl-β-D-galactopyranoside in carbon disulphide and deuterochloroform (and assuming that the 6-O-trityl group of D-galacto derivatives like that of D-glucose derivatives (page 136) does not interchange $H_{6\underline{R}}$ and $H_{6\underline{S}}$ signals from 6-O-benzoyl derivative signals) $H_{\underline{S}}$ resonates at 3.01 ppm and $H_{\underline{R}}$ at 3.32 ppm. The coupling parameters are $J_{5,6\underline{S}}$ = 8 Hz, $J_{5,6\underline{R}}$ = 5.7 Hz and $J_{6\underline{R},6\underline{S}}$ = 9 Hz. These data suggest that the trityl group, in trying to avoid C-4 adopts rotamer 135* such that $H_{\underline{S}}$ is almost antiperiplanar to H-5.

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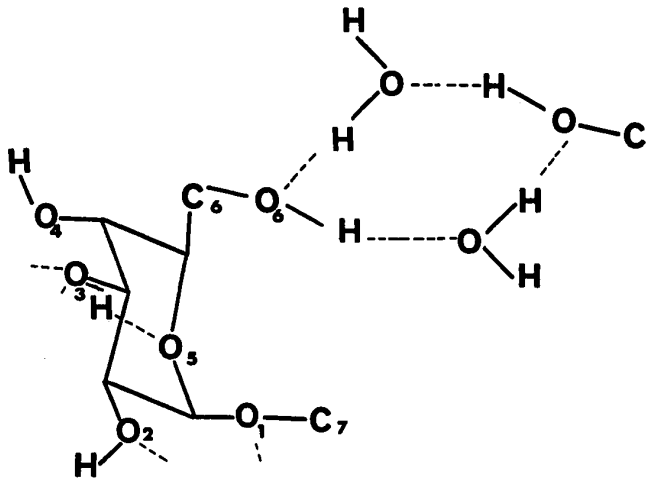
* NMR spectral studies in this laboratory (101) on 6-O-trityl derivatives of D-glycopyranose acetates have shown that the 6-O-trityl group produces diamagnetic shielding of 4-O-acetyl protons, i.e. there is an interaction between the trityl group and the 4-O-acetyl group. The absence of a significant 6-O-trityl diamagnetic shielding of the 4-O-acetyl signals of D-galactopyranose derivatives is consistent with a steric restriction of the 6-O-trityl group to rotamer 135 as discussed above. Polarimetric studies on the same compounds were also consistent with rotamer 135 for D-galactopyranose derivatives and rotamer 138 for D-glucopyranose derivatives (101). With the polarimetric method known as .(cont'd)

These couplings were found not to be solvent-dependent, an indication that the trityl group, unlike the 6-O-benzoyl group, has no room for flexibility at O-6.

In conclusion, it appears from this interpretation of the data of Tables VII-IX that the substituents of the hydroxymethyl group of D-galactose and derivatives in solution are orientated such that O-6 is always predominantly gauche relative to C-4 (135).The preference for rotamer 134 in methyl α-D-galactopyranoside monohydrate, shown by X-ray crystallography (281), may well be due therefore to strong hydrogen bonding in the solid state, as seen in the partial structure 149.



149

"aromatic chirality" (296,297), introduced recently by Nakanishi et al (298), into carbohydrate chemistry, an additional tool has been provided for studying rotational isomerism of benzoylated D-glycopyranose derivatives and might usefully be examined for the compounds described here.

VII ROTATIONAL ISOMERISM OF SUBSTRATES

c. Methyl β-D-glucopyranose analogues.

Anisochronism has been observed also for the prochiral hydrogens of the gluco-derivatives and this is in accordance with rotamer 138 in which O-6 interacts with the C-4 substituents. The gluco compounds examined were methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-iodo and 4-bromo-4-deoxy, β-D-glucopyranosides, methyl 2,3,6-tri-O-benzoyl-4-O-trifly1-β-D-glucopyranoside, and methyl β-D-glucopyranoside tetrabenzoate. With the exception of the O-triflate derivative, which exhibited vicinal couplings of 4.4 and 3.9 Hz for the prochiral hydrogens, these compounds all showed the same geminal and vicinal couplings, i.e. $J_{6R,6S}$ = 12.5 Hz, $J_{5,6R}$ = 2.5 Hz and $J_{5,6S}$ = 4.5 Hz.

EXPERIMENTAL

PART I
GENERAL METHODS

Melting points were determined with a Fisher-Johns hot-plate apparatus, and are uncorrected.

Optical Rotations, $[\alpha]_D$, were measured with a Carl Zeiss polarimeter (Model 367732), using the solvents indicated, and at room temperature.

Microanalyses were performed by Alfred Bernhardt, Elbach über Engelskirchen, West Germany.

Spectroscopy

Infrared spectra were recorded with solutions, using the solvents indicated, and also films on KBr or NaCl discs. Solid samples were examined as KBr discs. A Unicam SP-200G grating infrared spectrometer was used.

Electronic spectra were recorded with a Unicam SP-800 Ultraviolet spectrometer; unless otherwise indicated the solvent was diethyl ether.

Mass spectra were recorded with a double focusing MS 902 AEl spectrometer operating at 70 eV with an ion source temperature varying

from 100 - 200° C, a trap current of 500 μA, and an acceleration voltage of 8000.

NMR spectra were recorded with a Varian HA-100 spectrometer using an internal lock signal, and also with Varian A60 and T60 spectrometers. Spectra at 220 MHz were provided by the Canadian 220 Hz NMR Centre, Sheridan Park, Ontario.

Chromatography

Column chromatography was performed using cellulose powder for aqueous systems and silica gel (Macherey, Nagel and Co.) for non-aqueous systems. Solvents were dried over sodium wire where appropriate or over molecular sieves.

Thin layer chromatography (tlc) plates were prepared from silica gel G according to Stahl, and were dried at 100°C for one hour and then cooled in a vacuum desiccator. Preparative tlc plates were prepared in the same way but with a thicker coating. Visualization of tlc plates was effected with 5% conc. sulphuric acid in ethanol or 2,4-dinitrophenylhydrazine in phosphoric acid and ethanol (reagent C) (299); with both reagents, carbohydrates and derivatives showed up as dark spots on a lighter background; α,β-unsaturated aldehydes and benzoates were also visualized with an ultraviolet lamp. The solvents were: A, benzene: ether (9:1 v/v); B, n-propanol: ethyl acetate: water (3:2:1 v/v); Reagent C is 2,4-dinitrophenylhydrazine in ethanol containing phosphoric acid (299).

Descending chromatography on Whatman No. 1 paper was used for all chromatography work; reducing sugars were detected with silver nitrate (300) or with aniline oxalate (301). Solvents for paper chromato-

grams were butanol:ethanol:water (4:1:5 upper layer) or n-propanol:acetic acid:water (6:1:2) (4), unless otherwise specified.

Gas liquid chromatography (glc) was conducted with a Hewlett Packard F & M 402 gas chromatograph using a silicone gum column (4% U.C.W.) on chromosorb. W.

Acetylation

Unless otherwise stated, acetylation was conducted at about 4° with pyridine-acetic anhydride in the ratio 2:1. Pyridine and excess acetic anhydride were evaporated off, toluene was added to the residual syrup and evaporated off several times to facilitate the removal of pyridine. The procedure used for the preparation of compound 104a is typical of acetylations conducted.

Benzoylation

The procedure used to benzoylate compound 104 is typical of the benzoylation steps used in this work.

De-O-acylation

De-O-acylation was effected with 0.1 M sodium methoxide in methanol, the base being introduced dropwise until the solution was just alkaline. Unless otherwise stated, de-O-acylation was conducted at temperatures ranging from 0° - 25°. Compounds poorly soluble in methanol were solubilized in dry benzene. When de-O-acylation was complete, the solution was neutralized with Amberlite IR-120 (H^+) resin. Conversion of 108b to 108 is a typical example.

Evaporations

All evaporations were carried out under reduced pressure, usually at 45°C or below.

VIII EXPERIMENTAL

Petroleum-ether (pet-ether) refers to the material of boiling range of 65 - 110°, and ether to diethyl ether.

PART II
SUBSTRATES FOR THE STEREOCHEMICAL STUDY AT C-6 OF D-GALACTOSE, THE CENTRE OF THE OXIDATION REACTION

A. METHYL β-D-GALACTOPYRANOSIDE (6)

A.1 Methyl β-D-galactopyranoside was synthesized by well established procedures (302-306).

A.2 Benzoylation of 6 afforded the tetrabenzoate (6b), m.p. 135 - 6° $[\alpha]_D$ + 105.1° (c, 2.03 chloroform).

B. METHLY β-D-GALACTOPYRANOSIDE-6-d (PREDOMINANTLY 6R) (34)

B.1 1,2:3,4-di-O-isopropylidene-α-D-galacto-hexodialdo-1,5-pyranose (12)

1,2:3,4-Di-O-isopropylidene-α-D-galactopyranose (35) (prepared by de-O-acetylation of the crystalline 6-acetate, m.p. 109 - 110° (307) was oxidized with methyl sulfoxide in the presence of triethylamine-sulfur trioxide (96). The diketal (1.5 g) was dissolved in methylsulfoxide (10 ml), triethylamine (3 ml) was then added, followed by a solution of sulfur trioxide-pyridine (2.8 g) in methylsulfoxide (10 ml). On shaking, the mixture rapidly became warm and darkened (reaction was complete within a few min., as indicated by tlc) (benzene:ether (1:1)). It was

then cooled in acetone-CO_2 and slowly diluted with chloroform (200 ml), then washed successively with 2N hydrochloric acid, water, dilute sodium bicarbonate, water (2x). The dried chloroform solution was concentrated to a syrup (1.14 g), which was chromatographed on a column of silica gel with benzene:ether (1:1) as eluant to separate the aldehyde (12) (1.0 g) from a small amount (50 mg) of accompanying 1,2:3,4-di-O-isopropylidene-6-O-(methylthio)-methyl-α-D-galactopyranose (51), and also a small quantity of the α,β-unsaturated aldehyde (36) (90 mg). Compounds 49 and 36 were isolated in a syrupy form and were characterized mainly by NMR spectroscopy, 51 from the close correspondence of its spectrum with published data (98). The glycal (36), which absorbed in the U.V. region, afforded the following NMR data ($CDCl_3$ as solvent): δ 9.24, singlet (H-6); δ 5.96 quartet ($J_{3,4}$ = 5.4 Hz, $J_{2,4}$ = 1 Hz; H-4); δ 5.62 doublet ($J_{1,2}$ = 3 Hz, H-1); δ 4.52 quartet slightly broad due to coupling with OH proton ($J_{3,4}$ = 5.4 Hz, $J_{3,2}$ = 1.5 Hz, H-3); δ 4.30 broad singlet (H-2), δ 2.70 broad multiplet (D_2O exchangeable, OH-3), δ 1.4 doublet $(CH_3)_2$.

B.2 6-O-Acetyl-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose-6-d (3.6 R : 1 S). (38)

To a stirred solution of 1,2:3-4-di-O-isopropylidene-α-D-galacto-hexodialdose-1,5-pyranose (12) (1.0 g) in ice-cold ethanol:water (1:1) (15 ml), a solution of sodium borodeuteride (0.2 g) in ice water (5 ml) was slowly added, and stirring was continued for 30 min (reduction complete, as shown by tlc) (benzene:ether (1:1)). Excess of Amberlite

IR-120 (H^+) resin was added, the solution was evaporated to dryness, and boric acid was removed by repeatedly dissolving the residue in methanol, followed by concentration to dryness. The reduction product (0.9 g) was acetylated with acetic anhydride (2.5 ml) in pyridine (5 ml), affording crystalline 6-O-acetyl-1,2:3,4-di-O-isopropylidene-α-D-galacto-pyranose-6-d (0.7 g) (38); m.p. 107 - 108°, $[\alpha]_D$ − 50.7° (c, 2.15 chloroform).

B.3 Methyl β-D-galactopyranoside-6-d (34)

1,2:3,4-Di-O-isopropylidene-α-D-galactopyranose-6-d (37) (prepared from the above 6-acetate (38)) (1.0 g) was dissolved in 0.1 N sulfuric acid (40 ml), the solution was heated at 95° for 45 min, cooled, and neutralized with barium carbonate. Filtration was facilitated by use of celite, the filtrate was treated with Amberlite IR-120 (H^+) resin, then concentrated, and the residue was crystallized with cold methanol. Yield of D-galactose-6-d (33) 0.5 g, m.p. 164 - 166°. The sugar (1.0 g) was converted successively by well-established procedures (302-306) into 1,2,3,4,6-penta-O-acetyl-β-D-galactopyranose-6-d, 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl-6-d bromide, methyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside-6-d and finally to methyl β-D-galactopyranoside-6-d (34) m.p. 176 - 177° (0.3 g). $[\alpha]_D$ 0.0° (c, 1 water).

Benzoylation of (34) afforded the tetrabenzoate (40) m.p. 134 - 135° $[\alpha]_D$ + 111.5° (c, 2.14 chloroform).

VIII EXPERIMENTAL

C. METHYL β-D-GALACTOPYRANOSIDE-6-d (PREDOMINANTLY 6S) (45)

C.1 6-O-Acetyl-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose-6,6'-d_2 (49)

1,2:3,4-Di-O-isopropylidene-α-D-galacturonic acid (308) (m.p. 158.5 - 159.5°) was converted to the methyl ester with diazomethane. The ester (7.5 g) in ether (100 ml) was reduced with lithium aluminium deuteride (1.0 g) dissolved in ether (150 ml), the deuteride being added over a period of 30 min with good stirring and under gentle reflux. Ethyl acetate (2 ml) and water (50 ml) were added in succession, followed by acidification with 2 N hydrochloric acid and dilution with water (100 ml). The aqueous layer was extracted with chloroform, and the combined chloroform and ether solutions (after washings with sodium bicarbonate and water) were dried, and concentrated. Acetylation of the residue (6.1 g), as above, afforded 6-O-acetyl-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose-6,6'-d_2 (6.5 g), m.p. 110°, $[\alpha]_D$ − 47.4° (c, 2.04 chloroform).

C.2 Methyl β-D-galactopyranoside-6-d (45)

1,2:3,4-Di-O-isopropylidene-α-D-galactopyranose-6,6'-d_2 (43) (5.2 g) (prepared from the 6-acetate, previous section) was oxidized to the 6-deuterio-dialdose (13) as described under (12) above*; yield, 4.0 g. This syrupy aldehyde, in ethanol:water (1:1) (30 ml) was reduced at 10° with sodium borohydride (0.4 g), the product was purified by chromato-

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* In this reaction the proportion of 6-O-methylthiomethyl ether (50) was much higher (by almost 25%) than in the oxidation of the undeuterated carbinol.

graphy on silica gel (benzene:ether (1:1) as eluant) (yield, 2.8 g), and then acetylated with acetic anhydride-pyridine to give 6-O-acetyl-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose-6-d; m.p. 109 - 110°, $[\alpha]_D$ - 48.5° (c, 2.0 chloroform). The latter was deacetylated and hydrolysed, and the D-galactose-6-d (41) (0.9 g) obtained was converted via the acetobromo derivative, as described above to methyl β-D-galactopyranoside-6-d (45) (0.3 g); m.p. 167°, $[\alpha]_D$ 0.0° (c, 1 water).

Benzoylation of 45 afforded the tetrabenzoate (45b) m.p. 133 - 134° $[\alpha]_D$ + 106.6°.

D. METHYL β-D-GALACTOPYRANOSIDE-6,6'-d_2 (47)

Hydrolysis of 43 as above (37 to 33) afforded D-galactose-6,6'-d_2 (46). Conversion of 46 as above (33 into 34), via the acetobromo derivative into methyl β-D-galactopyranoside-tetraacetate-6,6'-d_2, followed by de-O-acetylation, gave methyl β-D-galactopyranoside-6,6'-d_2 (47), m.p. 175 - 176° $[\alpha]_D$ 0.0° (c, 1 water).

PART III

CONFIGURATION OF THE PRIMARY CARBINOL OF THE SUBSTRATES

E.1 1,2,3-Tri-O-acetyl-α-D-threofuranose-4-d (63) from 1,2-O-isopropylidene-α-D-xylose-5-d (61)

1,2-O-Isopropylidene-α-D-xylofuranose-5-d (309) (6.0 g) was converted into 3,5-di-O-benzoyl-D-xylofuranose-5-d, using conditions for

benzoylation and partial hydrolysis described by Baker and Schaub (310). The syrupy dibenzoate obtained (5.6 g), in acetic acid (300 ml), was oxidized with lead tetraacetate (8.5 g) in acetic acid (250 ml) for one h., and after removal of most of the solvent the product was extracted into benzene; yield 4.5 g of syrup. Methanolysis of this syrup was effected with methanol (200 ml) containing acetyl chloride (1.6 ml), under reflux for 18 h., the solution was neutralized with silver carbonate, concentrated, and the product was hydrolysed with 0.1 N sulfuric acid (30 ml) at 95° for one h. The hydrolysate, after neutralization with barium carbonate and **Amberlite** IR-120(H^+) resin, was found by chromatography to consist mainly of threose; a small proportion of xylose was detected, as well as some other minor, unidentified, components. By column chromatography on cellulose (eluant: one-quarter saturated butanol), the threose was separated out (0.3 g) and then acetylated to yield, finally, 1,2,3-tri-O-acetyl-α-D-threofuranose-4-d (63); m.p. 118 - 120°.

E.2 1,2,3-Tri-O-acetyl-α-D-threofuranose-4-d (66 and 67)

D-Galactopyranose-6-d (33 or 41) was oxidized in acetic acid solution with 2 moles of lead tetraacetate per mole (128). The syrupy formate ester obtained was hydrolysed, and the product acetylated with acetic anhydride-sodium acetate, yielding 1,2,3-tri-O-acetyl-α-D-threofuranose-4-d (66), m.p. 120 - 121°, $[\alpha]_D$ + 35° ($CHCl_3$); or 67, m.p. 120 - 121°, $[\alpha]_D$ + 37 °($CHCl_3$) .

VIII EXPERIMENTAL

E.3 α-D-Threofuranose triacetate (70) and tribenzoate (70b)

D-Threose was synthesized according to Perlin and Brice (128), and was benzoylated to give 70b, m.p. 176 - 178° $[\alpha]_D$ 0.0°(c, 5 chloroform), or acetylated to give 70, m.p. 120 - 121°, $[\alpha]_D$ + 34.5°.

E.4 1,2,3-Tri-O-acetyl-α-D-threofuranose-4,4'-d_2 (69)

Compound 69 was synthesized from D-galactose-6,6'-d_2 using the procedure for converting 33 into 66 above; m.p. 121 - 122°, $[\alpha]_D$ + 35° (c, 5 chloroform).

E.5 Oxidation of 1,2-O-isopropylidene α-D-glucofuranose with lead tetraacetate

Essentially, the conditions used were those described by Inch (194). 1,2-O-Isopropylidene-α-D-glucofuranose (20 g) was dissolved in glacial acetic acid (600 ml) and to the solution lead tetraacetate (recrystallized from acetic acid) (42.0 g in 1 l. acetic acid) was added. At 30 min reaction time, tlc (benzene:ether:methanol 8:1:1) showed the presence of two major compounds. The rest of the work up was carried out as in the oxidation of 33, (Section E·2) and the resulting syrup was transferred onto a column of silica gel and eluted with ether. Fractions of the fastest moving component, i.e. 1,2-O-isopropylidene-3,5-O-methylene (5-hydroxy -α-D-xylofuranose) (150), were eluted first, and the dimeric material was subsequently eluted with the same solvent. The crystalline dimer (89), after recrystallization from chloroform-pet-ether had m.p. 169 - 171°, $[\alpha]_D$ + 25.1° (c, 2 chloroform) (lit. (193,194), m.p. 178 - 180°, $[\alpha]_D$ + 21.0° (c, 1.8 chloroform).

VIII EXPERIMENTAL

E.6 Reduction of 1,2-O-isopropylidene-3,5-O-methylene (5-hydroxy-α-D-xylofuranose) (150)

Compound 150 (5.0 g) dissolved in ether (50 ml) was added to a solution of lithium aluminium deuteride (1 g) in ether (200 ml) maintained under gentle reflux. When the reaction was over (tlc, benzene: ether:methanol (8:1:1)), the reductant was decomposed with ethyl acetate, (2 ml) and water (20 ml). The ethereal layer was decanted off, the aqueous mixture centrifuged and the supernatant neutralized with Amberlite IR-120 (H^+) resin and then concentrated to a syrup. Removal of the isopropylidene group by hydrolysis afforded a syrupy residue (1.2 g) which, after freeze drying, was acetylated with acetic anhydride-sodium acetate. This gave crystalline β-D-xylose-5-d tetraacetate (151) m.p. 125 - 126° (ethanol) (lit. (311) m.p. 128°). The NMR spectrum of (151) was closely similar to that described by Lemieux and Howard (126) for the deuterated compound prepared in a slightly different fashion.

E.7 Reduction of 89 - (dimeric 1,2-O-isopropylidene-α-D-xylopentodialdofuranose).

The same procedure as for 150 was followed. Starting with 0.5 gm of 89 0.4 g of β-D-xylose-5-d tetraacetate was obtained. The NMR spectrum of the tetraacetate closely resembled that of 151.

PART IV

KINETICS OF DEHYDROGENATION
BY D-GALACTOSE OXIDASE

F. MEASUREMENT OF REACTION RATES FOR D-GALACTOSE OXIDASE

VIII EXPERIMENTAL

Kinetic measurements were carried out using "Galactostat" (Worthington). The "Galactostat" solution consisted of D-galactose oxidase (20 units), peroxidase (2.5 mg) and the chromogen O-tolidene (2.5 mg) in 0.01 M phosphate buffer (50 ml; pH 7.0). One and a half ml of this solution was added to substrate solutions (2 ml) at concentrations ranging from 3×10^{-4}M to 1.3×10^{-3}M for methyl β-D-galactopyranoside and its deuterated analogues, and from 5×10^{-4}M to 1.04×10^{-2}M for the deoxy substrates. Enzyme reaction mixtures were incubated at 37°C, and absorbance was monitored at 420 nm with a Beckmann spectrophotometer coupled to Gilford automatic recording accessories, having a constant temperature compartment.

PART V

OXIDATION OF SUBSTRATES WITH D-GALACTOSE OXIDASE

G.1 Oxidation of methyl β-D-galactopyranoside and its deuterated analogues (34, 45, 47)

In a typical experiment, the substrate (40 mg) was dissolved in phosphate buffer (4 ml; 0.2 M, pH 7.0) and incubated with D-galactose oxidase* (5 mg, 125 units) and catalase* (5 mg) at 37°C for 3 h. Tlc examination (solvent B) showed the presence of a major product R_f 0.63 as compared with R_f 0.47 for the intact glycoside , and two minor products (R_f 0.75 and R_f 0.46, respectively). A mixture of Amberlite IR-120 (H^+) and Dowex-1 (bicarbonate) resin was added to the digest with stirring,

*Purchased from Worthington Biochemicals, New Jersey.

the suspension was filtered, the filtrate concentrated and then lyophilized. The product (36 mg) was treated with pyridine (0.8 ml) and acetic anhydride (0.4 ml) at room temperature for 18 h., the reaction mixture was concentrated, and the syrupy residue chromatographed on a column of silica gel (eluant-benzene:ether (9:1)). This afforded methyl 2,3-di-O-acetyl-6-aldehydo-4-deoxy-L-threo-hex-4-eno-1,5-pyranoside (82) (10 mg) followed by methyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (3 mg from the undeuterated glycoside; 7 mg from 34; 12 mg from 45). The subsequent use of benzene:ether (1:4) caused elution of the dimer 85 (30 mg) m.p. 206 - 208° (ethanol) $[\alpha]_D$ + 41.6° (c, 2.07 chloroform)

Anal. Calc. for $C_{26}H_{36}O_{18}$: C, 49.1; H, 5.7

Found: C, 48.4; H, 5.7

The deuterated dimer resulting from the oxidation of 34 had m.p. 210 - 211°(ethanol) $[\alpha]_D$ + 47.0° (c, 0.4 chloroform)

Anal. Calc. for $C_{26}H_{34}O_{18}$ (deuterium)$_2$: C, 48.9; H, 5.4; deuterium, 0.6

Found: C, 49.0; H(+ deuterium), 5.8

G.2 Oxidation of methyl 4-deoxy-4-fluoro-β-D-galactopyranoside (104)

The experimental procedure under section G.1 for the oxidation of 6 was followed, 15 mg of 104 being used. The reaction mixture, after the enzyme reaction and work up, showed (tlc, solvent B) the presence of an α,β-unsaturated aldehyde (81) (R_f 0.62) as the major fraction, unreacted 104 (R_f 0.46) and an unidentified compound (R_f 0.29). Under

these conditions the products of oxidation of methyl β-D-galactopyranoside showed R_f 0.62 (compound 81), R_f 0.46 (free aldehyde) and R_f 0.27 (dimeric product).

G.3 Oxidation of methyl 4-amino-4-deoxy-β-D-galactopyranoside (105)

The experimental procedure under Section G.2 was used. Tlc (solvent B) examination of the enzyme products showed the presence of a slow-moving compound with aldehydic staining properties; its low mobility suggested that the aldehyde was involved in an intermolecular acetal formation. Compound 105 had R_f 0.09.

G.4 α,β-Unsaturated aldehydes among the enzyme products

Components of the enzyme products from the oxidation of methyl β-D-galactopyranoside were separated on a preparative tlc plate, acetylated (without isolating them from the silica gel) and purified by column chromatography. The α,β-unsaturated aldehyde zone afforded (82). Fractions corresponding to the free aldehyde gave 82 and the dimer (85), whereas slow moving dimeric fractions afforded 85 and small quantities of 82. Column chromatographic separation on cellulose of the enzyme products afforded 81; $\lambda_{max}^{H_2O}$ 253 nm ($\varepsilon = 6.96 \times 10^{-2}$)

G.5 2,4-Dinitrophenylhydrazone of methyl 2,3-di-O-acetyl-α-L-threo-4-deoxy-hex-4-enodialdopyranoside (82)

The α,β-unsaturated aldehyde (10 mg) was dissolved in ether (0.5 ml) and to this solution was added 2,4-dinitrophenylhydrazine (0.1 M

in phosphoric acid-ethanol (299) (3-4 drops). Orange-yellow crystals which soon formed on standing, were washed with ethanol and ether; m.p. 148 - 152°, undepressed on admixture with a specimen prepared from an authentic sample of the α,β-unsaturated aldehyde (97). The NMR and mass spectra of the two hydrazones were indistinguishable. A higher melting form of this hydrazone (m.p. 167°) which, however, is indistinguishable from the current product by NMR and mass spectrometry also has been obtained (97).

PART VI

SELECTIVE TRIMOLAR BENZOYLATION OF METHYL β-D-GLUCOPYRANOSIDE AND SYNTHESIS OF 121

H.1 Methyl 2,3,6-tri-O-benzoyl-β-D-glucopyranoside (117)

Methyl β-D-glucopyranoside (116) (18 g) dissolved in pyridine (60 ml) was cooled to -10° and benzoyl chloride (31 ml) added dropwise to the solution with continued cooling. The reaction mixture was stirred at -10° for 10 h. and then the temperature allowed to rise to 25°. Tlc (benzene:ether, 1:1) showed the presence of five major compounds*. Water was added, the reaction mixture extracted with chloroform, and the chloroform layer was washed successively with 2N HCl, water, 1 N $NaHCO_3$, then water and then dried over $MgSO_4$. Evaporation of the chloroform

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* J.M. Williams (248) has noted that at least four major compounds are detectable from this same synthesis; however isolation of the benzoylation products has not been reported.

afforded a crystalline mass which was dissolved in benzene and transferred onto a column of silica gel. Elution with benzene:ether (49:1) afforded methyl β-D-glucopyranoside tetrabenzoate (116b) (4.0 g, 7.1%) m.p. 161 - 162°, $[\alpha]_D$ + 26.9° (c, 2.15 chloroform) (lit.(312,313), m.p. 158 - 160°, $[\alpha]_D$ + 30.9° ($CHCl_3$)). Elution with benzene:ether (9:1) gave methyl 2,3,6-tri-O-benzoyl-β-D-glucopyranoside (117) (16 g, 34%), m.p. 148.5 - 150.5° (chloroform, pet-ether) $[\alpha]_D$ + 76.6° (c, 2.0 chloroform) (lit.(314) m.p. 145.5 - 146.5°, $[\alpha]_D$ + 82.0° (chloroform)).

H.2 Methyl 3,4,6-tri-O-benzoyl-β-D-glucopyranoside (118)

Continued elution of the chromatogram (in Section H.1) with benzene:ether (9:1) afforded a syrup (1.5 g, 3%), $[\alpha]_D$ − 29.1° (c, 14.07 chloroform), identified by NMR spectroscopy as 118*: δ 7.0 - 8.26 ppm (three benzoate groups); δ 6.26 ppm, triplet ($J_{3,4} = J_{4,5}$ = 9.3 Hz, H-4); δ 6.01, triplet ($J_{3,4} = J_{3,2}$ = 9.5 Hz, H-3); δ 4.89, doublet ($J_{1,2}$ = 8.0 Hz, H-1); δ 4.83, multiplet (H-6R and H-6S); δ 4.4, multiplet (H-5); δ 4.27, quartet ($J_{2,1}$ = 8 Hz, $J_{2,3}$ = 9.5 Hz, H-2); δ 3.62, singlet (OCH_3). Solvent C_5D_5N.

Acetylation of 118 afforded a monoacetate (NMR), m.p. 162.5 - 163.5 °(crystallized from methanol), $[\alpha]_D$ − 42.0° (c, 2.26 chloroform).

H.3 Methyl 3,6-di-O-benzoyl-β-D-glucopyranoside (119)

Further elution with benzene:ether (9:1) afforded a compound (2.7 g, 4%) identified by NMR (in pyridine-d_5) as methyl 3,6-di-O-benzoyl-

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* Trimolar benzoylation of the α-anomer affords besides the 2,3,6-tribenzoate, the 2,4,6-tribenzoate (248), rather than the 3,4,6-isomer.

β-D-glucopyranoside; m.p. 155 - 156° (crystallized from chloroform-pet-ether) $[\alpha]_D$ + 14.8° (c, 2.34 chloroform).

Acetylation of 119 afforded the diacetate (identified by NMR) m.p. 106 - 107° (crystallized from ethanol), $[\alpha]_D$ + 21.5° (c, 2.02 chloroform).

H.4 Methyl 2,6-di-O-benzoyl-β-D-glucopyranoside (120)

Elution of the silica gel chromatogram with benzene:ether (1:1) afforded a compound identified by NMR in pyridine-d_5 as (120) (1.1 g, 4%). Recrystallization from chloroform-pet-ether afforded pure 120; m.p. 177 - 178° $[\alpha]_D$ − 42.1° (c, 1.16 chloroform).

Acetylation of 120 afforded a diacetate (NMR); m.p. 167 - 168° (crystallized from methanol), $[\alpha]_D$ + 54.8° (c, 2.0 chloroform) (lit.(315) m.p. 166°, $[\alpha]_D$ + 54.8° (c, 1.34 acetone)).

H.5 Methyl 2,3,6-tri-O-benzoyl-4-O-(p-bromobenzenesulphonyl)-β-D-glucopyranoside (121)

p-Bromobenzenesulphonyl chloride (15 g) was added to a solution of 117 (18 g) in pyridine (30 ml) at 45 - 50°. The reaction mixture was stirred for 24 h., at which time tlc (benzene:ether, 1:1) showed that the reaction was complete; water was then introduced dropwise with cooling followed by ice water. Solid material that precipitated out was collected, washed with water, and dissolved in chloroform. The chloroform layer was washed successively with 2N HCl, water, 1 N $NaHCO_3$, and water, and then dried over $MgSO_4$. Evaporation of the solvent gave crystalline 121. Recrystallization from chloroform-pet-ether afforded pure 121 (12.5 g),

m.p. 164 - 165° (decomp.); $[\alpha]_D$ + 23.2° (c, 2.1 chloroform).

Anal. Calc. for $C_{34}H_{29}O_{11}SBr$: C, 56.3; H, 4.0; S, 4.4; Br, 11.0

Found:* C, 56.3; H, 3.7; S, 4.3; Br, 11.1

PART VII

SOME OTHER 4-DEOXY-SUBSTRATES

I. METHYL 4-AZIDO-4-DEOXY-β-D-GALACTOPYRANOSIDE

I.1 Methyl 4-azido-2,3,6-tri-O-benzoyl-4-deoxy-β-D-galactopyranoside (110b)

Compound 121 (5.0 g) was dissolved in N,N-dimethylformamide (DMF) (16 ml) and the solution stirred with a suspension of sodium azide (3 g) at a bath temperature of 95 - 105°. Tlc (benzene:ether, 9:1) after 12 h. showed that the reaction was about 90% complete, the main product 110b staining strongly with reagent C. It was found that a more prolonged reaction caused the formation of a slower moving fraction. After filtration, and the removal of more salts by addition of ether, the solution was evaporated to dryness. Chromatography of the product on silica gel and elution with benzene:ether (49:1) afforded crystalline 110b (3.5 g, 96%), m.p. 157 - 158°. After recrystallization from benzene-pet-ether or $CHCl_3$-pet-ether, the m.p. was 158 - 159°; $[\alpha]_D$ - 69.9° (c, 2 chloroform), λ max 2100 cm^{-1} (N=N=N) (KBr). NMR spectrum: δ 6.8 - 8.2 (three benzoates, δ 3.20, (OCH_3) H-1 to H-6, H-6' (Fig. 29, p. 114).

Anal. Calc. for $C_{28}H_{25}O_8N_3$: C, 63.3; H, 4.7; N, 7.9

Found: C, 62.4; H, 4.7; N, 7.5.

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* Analysis by Daessle Microanalyses, Montreal, Quebec.

VIII EXPERIMENTAL

I.2 Methyl 4-azido-4-deoxy-β-D-galactopyranoside (110)

De-O-benzoylation of 110b (1.0 g) employing the general procedure above gave 110 (0.20 g, 49%) which, on recrystallization from methanol-ether containing a little n-hexane, had m.p. 169 - 172°; $[\alpha]_D$ − 59.2°(c, 1 water).

Anal. Calc. for $C_7H_{13}O_5N_3$: C, 38.4; H, 6.0; N, 19.2
Found: C, 39.0; H, 6.0; N, 19.7.

I.3 Methyl 2,3,6-tri-O-acetyl-4-azido-4-deoxy-β-D-galactopyranoside (110a)

Acetylation of 110 (40 mg) gave 110a (50 mg, 79%); recrystallization from ethanol afforded crystals m.p. 116 - 118°, $[\alpha]_D$ − 60.9° (c, 1.86 chloroform)

Anal. Calc. for $C_{13}H_{19}O_8N_3$: Mol. wt. 345
Found: $(M-59)^+$, i.e. $(M-CH_3COO)^+$ at m/e 286
followed by $(M-59-N_3)^+$ at m/e 244

I.4 Methyl 2,3,6-tri-O-benzoyl-4-O-triflyl*-β-D-glucopyranoside (122)

Methyl 2,3,6-tri-O-benzoyl-β-D-glucopyranoside (117) (1.0 g) dissolved in pyridine (16 ml) was cooled to 0° and to this solution was added dropwise with cooling, trifluoromethanesulphonyl anhydride ($(CF_3SO_2)_2O$) (0.63 g) (prepared by distillation of trifluoromethanesulphonic acid over P_2O_5 and collecting the fraction boiling at 80 - 82°). Tlc (benzene: ether (9:1)) showed that a considerable proportion of 121 had been

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*trifluoromethanesulphonyl

esterified*. The stirred solution was treated with cold water, then shaken with chloroform and the organic layer was washed successively with 2N HCl, with water, 1 N $NaHCO_3$, water, and finally dried over $MgSO_4$. Evaporation of the solvent gave a syrup that darkened on standing. This syrup was transferred onto a column of silica gel and eluted with benzene: ether (9:1). Fractions of 122 obtained also darkened on standing, but crystallized from ethanol. Recrystallization from the same solvent afforded pure 122 (0.6 g, 48%) m.p. 124-125° (decomp.); $[\alpha]_D$ + 25.7° (c, 1.83 chloroform).

Anal. Calc. for $C_{29}H_{25}O_{10}F_3S$: C, 56.0; H, 4.1; F, 9.2; S, 5.2.

Found: C, 56.4; H, 4.0; F, 8.3; S, 5.0.

I.5 Displacement of the triflyl group of 122 by azide ion to give 110b

To compound 122 (80 mg) dissolved in N,N -dimethylformamide (5 ml) sodium azide (0.2 g) was added and the suspension heated at 95 - 100° (bath temp.). The reaction was carried out for 6 h. but tlc (solvent A) after 2 h. showed that the reaction was nearly complete. The reaction mixture was concentrated and the resulting syrup was chromatographed on silica gel (elution with benzene:ether (9:1)). The purified product crystallized from chloroform-pet-ether (50 mg, 75%) and had mixed m.p., $[\alpha]_D$, NMR and IR spectra indistinguishable from those of 110b.

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* A prolonged reaction time or addition of more $(CF_3SO_2)_2O$ caused formation of a number of side products among which methyl 2,3,6-tri-O-benzoyl-4-O-triflyl-β-D-galactopyranoside $[\alpha]_D$ + 35.9°($CHCl_3$) and methyl 2,3,6-tri-O-benzoyl-β-D-galactopyranoside (converted into its 4-acetate $[\alpha]_D$ + 40.2°($CHCl_3$)) have been isolated.

VIII EXPERIMENTAL

J. METHYL 4-AMINO-4-DEOXY-β-D-GALACTOPYRANOSIDE (105)

Compound 110 (0.20 g) was dissolved in water (5 ml), to this solution palladium black (60 mg) was added, and the mixture was hydrogenated at atmospheric pressure for 13 h. Tlc (n-propanol:ethyl acetate:water, 3:2:1) showed the presence of a slow moving fraction (relative to 110). Filtration through a celite pad and removal of the solvent gave a solid product) (0.15 g, 85%) m.p. 188 - 189°. Recrystallization (of 0.1 g) from ethanol gave 105 as granular crystals (86 mg) m.p. 194 - 195° (decomp.), $[\alpha]_D$ ca. + 0.0° (c, 1 water).

Anal. Calc. for $C_7H_{15}O_5N$: C, 43.5; H, 7.8; N, 7.3.
Found: C, 43.6; H, 7.8; N, 7.2.

J.2 Methyl 2,3,6-tri-O-acetyl-4-acetamido-4-deoxy-β-D-galactopyranoside (105a)

Acetylation of 105 (15 mg) with acetic anhydride-pyridine as above gave 105a, purified on silica gel with ethyl acetate as eluant. The NMR spectrum of 105a is described in Table V) p. 126.

K. METHYL 4-DEOXY-4-THIO-β-D-GALACTOPYRANOSIDE

K.1 Methyl 2,3,6-tri-O-benzoyl-4-deoxy-S-benzoyl-β-D-galactopyranoside (109b)

To compound 121 (5.0 g) in DMF (20 ml) was added potassium thiobenzoate (2.2 g) (recrystallized from ethanol). The solution was heated at 100 - 105° (bath temp.) for three hours (tlc (benzene:ether, 9:1) showed that the reaction was then complete), cooled and filtered.

Ether was added to the filtrate until there was no further precipitation, and the solution was recovered and concentrated. A yellow syrup obtained was chromatographed on silica gel by eluting with benzene. The fast-moving fraction (3.0 g) crystallized from ethanol (2.4 g, 56%) m.p. 85 - 87°. Recrystallization from cellosolve-methanol raised the m.p. to 87 - 90° , $[\alpha]_D$ + 36.8° (c, 2.2 chloroform).

Anal. Calc. for $C_{35}H_{30}O_9S$: C, 67.1; H, 4.8; S, 5.1.

Found: C, 66.9; H, 4.8; S, 5.0.

K.2 Methyl 4-deoxy-4-thio-β-D-galactopyranoside (109), and its acetate dimer (123a) De-O-benzoylation of 109b

(i) With NH_3 in methanol

Dry ammonia gas was bubbled into a solution of 109b (1 g) in methanol (200 ml) and the reaction monitored by tlc: a) benzene:ether, 9:1) - absence of 109b; b) n-propanol:ethyl acetate:water (3:2:1) - presence of 109. After one week the reaction was incomplete; therefore, the temperature was raised to 30° and after 14 days, when a major spot was detected, the reaction mixture was concentrated. The addition of ether caused precipitation of crude 109 (0.3 g, 89%). Recrystallization from methanol or methanol-ether was unsuccessful.

(ii) With lithium aluminium hydride

Compound 109b (0.5 g) in benzene (5 ml) was added slowly to a suspension of $LiAlH_4$ (excess) in ether. Decomposition of excess $LiAlH_4$ with ethyl acetate and the addition of water followed successively by

centrifugation, treatment of the supernatant with Amberlite IR-120 (H^+) resin, and freeze-drying, afforded a clear syrup (0.14 g). Efforts to crystallize it failed. The NMR in D_2O of this syrup showed the absence of benzoate groups: δ 3.7, OCH_3; δ 4.48, H-1; δ 4.0, H-6R, H-6S (unresolved).

(iii) With sodium methoxide in methanol

(a) Prolonged reaction: De-O-benzoylation of 109b (0.6 g) at room temperature for ten days with sodium methoxide-methanol gave an impure compound whose NMR spectrum (in D_2O) showed weak aromatic absorption (δ 8 - 8.4). Other resonances discernable were a doublet at δ 4.8 (J = 7.8 Hz, H-1); a broad triplet typical of H-5 of galacto-derivatives at δ 3.6 (H-5).

(b) Short interval de-O and de-S-benzoylation of 109b: The de-O-benzoylation of 109b (1.0 g) as above was stopped after two days, the solution being neutralized with Amberlite IR-120 (H^+) resin, and concentrated. Benzoates were extracted into benzene and the aqueous layer was then extracted with chloroform. Concentration of the aqueous layer gave a residue weighing 14 mg, whereas the chloroform soluble material (0.17 g), by chromatography on silica gel (benzene:ether (3:1) as eluant), afforded a minor fraction (7 mg) m.p. 165 - 166° and a major fraction (70 mg). The latter, on acetylation and purification on silica gel (benzene:ether (9:1) as eluant) afforded a crystalline compound tentatively designated as methyl 2,3-di-O-acetyl-6-O-benzoyl-4-deoxy-4-S-acetyl-β-D-galactopyranoside (126a). Recrystallized from ethanol, 126a had m.p. 146 - 147°, $[\alpha]_D$ − 50.9° (c, 2.52 chloroform).

VIII EXPERIMENTAL

The NMR spectrum (in $CDCl_3$) of 126a showed: δ 7.2 - 8.15 (one benzoate group); δ 5.25, quartet ($J_{3,4}$ = 1 Hz, $J_{2,3}$ = 10 Hz, H-3); δ 5.06 quartet ($J_{1,2}$ = 7 Hz, $J_{2,3}$ = 10 Hz, H-2); δ 4.28 doublet ($J_{1,2}$ = 7 Hz, H-1); δ 4.56 quartet ($J_{6R,5}$ = 6.8 Hz, $J_{6R,6S}$ = 11 Hz, H-6R); δ 4.40 quartet ($J_{4,5}$ = 1 Hz, $J_{3,4}$ = 4 Hz, H-4); δ 4.29 quartet ($J_{5,6S}$ = 5.6, $J_{6R,6S}$ = 11 Hz, H-6S); δ 4.0 H-5; δ 3.39 singlet (OCH_3); δ 2.20 singlet (probably S-Ac); singlets at δ 1.97, 1.88 (two-OAc).

Anal. Calc. for $C_{20}H_{24}O_9S$: C, 54.5; H, 5.5; S, 7.3
Found: C, 53.5; H, 4.9; S, 7.0.

(iv) Acetylation of 109

Acetylation of the combined products of de-O-benzoylation (0.12 g) from reactions i, ii and iii above (believed to be methyl 4-deoxy-4-thio-β-D-galactopyranoside, 109) gave, after purification on silica gel (with benzene:ether (1:1) as eluant), crystalline 123a. Recrystallization from ethanol afforded 123a (34 mg); m.p. 202 - 203°, $[\alpha]_D$ − 180.5° (c, 1.13 chloroform).

Anal. Calc. for $C_{26}H_{38}O_{16}S_2$: C, 46.6; H, 5.7; S, 9.6.
Found: C, 46.6; H, 5.7; S, 10.4.

K.3 Methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-thiocyano-β-D-galactopyranoside (124b)

To compound 121 (1.0 g) dissolved in N,N-dimethylformamide (10 ml) potassium thiocyanate (1.1 g) was added. The reaction mixture at 95°- 100° (bath temp.) was stirred for 14 h., after which tlc (benzene:

ether (9:1)) showed that the reaction was complete. Ether was used to precipitate most of the salts, and soluble material was chromatographed on silica gel. Elution with benzene:ether (9:1) afforded a syrupy fraction; this crystallized from carbon tetrachloride-pet-ether (0.23 g, 31%); m.p. 151 - 152°, $[\alpha]_D$ - 41.6°(c, 2 chloroform; $\lambda_{max}^{CCl_4}$ 2150 cm^{-1}. (-S-C≡N).

K.4 De-O-benzoylation and -S-CN hydrolysis of 124b

Treatment of 124b (0.15 g) with sodium methoxide-methanol gave one major spot on tlc (solvent C). Evaporation of the solvent was followed immediately by acetylation of the residue, and then purification of the product on silica gel (elution with benzene:ether, 9:1). This gave a fast moving fraction (21 mg) obtained as a syrup, and a crystalline material (19 mg) (eluted with benzene:ether, 1:1). The R_f of the latter was the same as that of 123a.

PART VIII

METHYL HALOGENO-β-D-GALACTOSIDES

L. METHYL 4-DEOXY-4-FLUORO-β-D-GALACTOPYRANOSIDE

L.1 Methyl 4,6-O-benzylidene-β-D-glucopyranoside (152)

Methyl β-D-glucopyranoside (14 g) was stirred vigorously with zinc chloride (10.5 g) and benzaldehyde (40 mls), affording a solid mass. The crystalline material was washed with water and pet-ether, and

recrystallized from hot ethyl acetate; m.p. 198 - 202°, $[\alpha]_D$ - 98.9° (c, 2 pyridine)(lit.(316), m.p. 199 - 201°; $[\alpha]_D$ - 62.3°(c, 1.2 chloroform)).

L.2 Methyl 2,3-di-O-benzyl-4,6-O-benzylidene-β-D-glucopyranoside (153)

The method of Bell and Lorber (317) for the preparation of the α-anomer was used. Compound 152 (7.2 g) was stirred with toluene (125 ml), powdered potassium hydroxide (63 g) and benzyl chloride (14 ml) at a bath temperature of 110 - 120° for 24 h. Tlc (benzene ether, 9:1) showed the presence of only one compound. On cooling, the reaction mixture was washed with water, tartaric acid solution, sodium bicarbonate and water, and dried over magnesium sulfate. Concentration of the toluene solution to a minimum volume and addition of pet-ether gave a crystalline mass. Recrystallization from ethanol gave crystals; m.p. 122 - 123°, $[\alpha]_D$ - 34.7° (c, 2 chloroform) (lit.(318), m.p. 119 - 120°, $[\alpha]_D$ - 35.8° (c, 3.02 chloroform)).

L.3 Methyl 2,3-di-O-benzyl-β-D-glucopyranoside (154)

The method of Bell and Lorber (317) for preparation of the α-anomer was used. A solution containing compound 153 (3.5 g), acetone (45 ml), water (10 ml) and 1 N HCl (3 ml), was heated under reflux, the course of the reaction being followed by tlc (benzene:ether (1:1)) - absence of 153 and ether - presence of 154 On cooling the solution was neutralized with $BaCO_3$ and treated with a little Amberlite IR-120 (H^+). Evaporation of solvents gave a syrup which crystallized from chloroform

pet-ether (b.p. 30 - 60°) to give fine needles (1.5 gm); m.p. 124 - 126°, $[\alpha]_D$ - 20.1° (chloroform) (lit.(318), m.p. 122 - 123°, [α] - 13.3° (chloroform)).

L.4 Methyl 2,3-di-O-benzyl-4-O-(p-bromobenzenesulphonyl)-6-O-trityl-β-D-glucopyranoside (129)

A solution of compound 154 (1 g) in pyridine (15 ml) was stirred with triphenylchloromethane (trityl chloride) (1 gm) at a bath temperature of 40 - 50° C for 36 hours, and the reaction monitored by tlc (benzene). When tritylation was complete, p-bromobenzenesulphonyl chloride (1.2 gm) was added and the reaction mixture was heated and stirred for an additional 36 h. Ice water was added to the cooled solution, followed by chloroform, and the organic layer was treated with hydrochloric acid, water, sodium bicarbonate and water, and dried ($MgSO_4$). Evaporation of the solvent gave a syrup (contaminated with by-products from the trityl chloride) which was transferred onto a silica gel column and eluted with benzene:pet-ether:ether (1:3:5). A clear syrup of 129 (4.5 g) was recovered; $[\alpha]_D$ - 7.1° (c, 3.3 chloroform).

L.5 Methyl 2,3-di-O-benzyl-4-deoxy-4-fluoro-6-O-trityl-β-D-galactopyranoside (130)

(i) Tetra-n-butylammonium fluoride

Tetra-n-butylammonium hydroxide (Eastman-Kodak) (10% solution in water) was titrated to about pH 4.6 (methyl orange) and then to pH 4.8 (pH meter) with hydrofluoric acid (HF) (20% solution in water). The

solution was concentrated *in vacuo* and then freeze-dried for 4 days.

(ii) Solvolysis of 129 with tetra-n-butylammonium fluoride

Compound 129 (1 g) was dissolved in acetonitrile (distilled over P_2O_5) (10 ml). To this solution tetra-n-butylammonium fluoride (5.5 g in acetonitrile 10 ml) was added and the solution refluxed for two days at a bath temperature of 95 - 100°. The solvent was evaporated off and the residual syrup dissolved in benzene, washed with water, dried ($MgSO_4$), recovered by concentration, and chromatographed on a column of silica gel with pet-ether: ether (9:1) as the eluant. This afforded crystalline 130 (0.22 g) which, after recrystallization from pet-ether (b.p. 30 - 60°):ether, gave 130 (0.175 g, 30%), m.p. 137 - 138°, $[\alpha]_D$ + 21.6° (c, 3 chloroform).

L.6 Methyl 2,3-di-O-benzyl-4-deoxy-4-fluoro-β-D-galactopyranoside (131)

To a solution of compound 130 (0.5 g) in cold glacial acetic acid (15 ml) cold hydrobromic acid (32% in acetic acid) was added. Crystalline trityl bromide which formed was filtered off, the solution was poured onto a water-ice mixture and the resulting suspension was filtered. The filtrate was extracted with chloroform and the latter layer treated with 1 N $NaHCO_3$, washed with water, and dried over magnesium sulphate. Evaporation of solvent afforded a syrup which crystallized readily from toluene:pet-ether. Recrystallization from the same solvent gave 131 (0.23 g, 76%), m.p. 105 - 106°, $[\alpha]_D$ - 15.6° (c, 2 chloroform).

VIII EXPERIMENTAL

L.7 Methyl 4-deoxy-4-fluoro-β-D-galactopyranoside (104)

Compound 131 (0.20 g) in ethanol (8 ml) was treated with palladium black (0.10 g) and hydrogen at room temperature and pressure. Tlc (benzene:ether (1:1)) after 2 h. showed that the reaction was complete. The mixture was filtered over celite, and the combined filtrate and washings were evaporated to give a crystalline residue. Recrystallization from ethyl acetate-ethanol afforded pure 104 (0.10 g, 96%) m.p. 155 - 156°, $[\alpha]_D$ - 21.3° (c, 1 water).

Anal. Calc. for $C_7H_{13}O_5F$: C, 42.9; H, 6.7; F, 9.7
Found: C, 43.3; H, 6.4; F, 9.5.

L.8 Methyl 2,3,6-tri-O-acetyl-4-deoxy-4-fluoro-β-D-galactopyranoside (104a)

A portion of 104 (ca. 25 mg) was acetylated in pyridine-acetic anhydride at room temperature. Evaporation of the acetylating medium *in vacuo* afforded a solid product which was purified by passage through a silica gel column, using benzene:ether as the eluant (4:1). The crystalline product (30 mg, 72%) obtained after recrystallization from ethanol had m.p. 95 - 97°, $[\alpha]_D$ - 6.20° (c, 1.7 chloroform).

Anal. Calc. for $C_{13}H_{19}O_8F$: Mol. wt. 322;
Found: $(M-60)^+$, i.e. $(M-CH_3CO_2H)^+$ at 262.

VIII EXPERIMENTAL

L.9 Methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-fluoro-β-D-galactopyranoside (104b)

To a stirred solution of 104 (30 mg) in pyridine maintained at 0°, benzoyl chloride (0.2 ml) was added dropwise, the reaction being monitored by tlc (benzene:ether (9:1)).

Excess of benzoyl chloride was decomposed by dropwise addition of water accompanied with stirring, and the solution was extracted with chloroform. The chloroform layer was washed with 2 N HCl, water, $NaHCO_3$ and water, and dried over $MgSO_4$. The residue obtained after removal of the chloroform was passed through a short silica gel column and eluted with benzene:ether (9:1). A crystalline material (104b) was obtained, which upon recrystallization from methanol (40 mg, 51%) had m.p. 134 - 135°, $[\alpha]_D$ + 63.2° (c, 2 chloroform)

M. METHYL 4-CHLORO-4-DEOXY-β-D-GALACTOPYRANOSIDE (106)

M.1 Methyl 2,3,6-tri-O-benzoyl-4-chloro-4-deoxy-β-D-galactopyranoside (106b)

To methyl 2,3,6-tri-O-benzoyl-4-O-(p-bromobenzenesulphonyl)-β-D-glucopyranoside (121) (2.0 g) dissolved in acetonitrile (30 ml), tetra-n-butylammonium chloride (5 g) was added, the solution was refluxed for 60 h. and then evaporated to dryness. The residue was chromatographed on a column of silica gel with benzene:ether (49:1); the major fraction (106b) obtained, crystallized from methanol (1.2 g, 83%); m.p. 131 - 132°, $[\alpha]_D$ + 60.7° (c, 3 chloroform). NMR spectral analysis showed δ 8.08, 7.02 (3 benzoates), δ 3.22 (OCH_3), δ H-1 to H-6R, H-6S (Fig. 31, p.121).

Anal. Calc. for $C_{28}H_{25}O_8Cl$: C, 64.1; H, 4.8; Cl, 6.8.
Found: C, 64.3; H, 5.0; Cl, 7.4.

M.2 Methyl 4-chloro-4-deoxy-β-D-galactopyranoside (106)

De-O-benzoylation of 106b was carried out as for the procedure described for 108b. Compound 106b (1.0 g) gave 106 (0.32 g, 79%), m.p. 158 - 160°. Recrystallization from methanol-ether gave needles, m.p. 158 - 159°, $[\alpha]_D$ + 8.7° (c, 1 water).

Anal. Calc. for $C_7H_{13}O_5Cl$: C, 39.5; H, 6.2; Cl, 16.7
Found: C, 37.9; H, 6.2; Cl, 15.2.

M.3 Methyl 2,3,6-tri-O-acetyl-4-chloro-4-deoxy-β-D-galactopyranoside (106a)

The acetylation procedure above for 104 was used. Compound 106 (50 mg) gave (106a) (60 mg, 76%); m.p. 96 - 97° (ethanol), $[\alpha]_D$ + 30.9° (c, 1 chloroform).

Anal. Calc. for $C_{13}H_{19}O_8Cl$: Mol. wt. 338 for ^{35}Cl, and Mol. wt. 340 for ^{37}Cl.

Found: $(M-59)^+$, i.e. $(M-CH_3COO)^+$ for ^{35}Cl (intense) centered at m/e 279;

$(M-59)^+$, i.e. $(M-CH_3COO)^+$ for ^{37}Cl (less intense) centered at m/e 281;

Also $(M-60)^+$, i.e. $(M-CH_3COOH)^+$ for both isotopes.

VIII EXPERIMENTAL

N. METHYL 4-BROMO-4-DEOXY-β-D-GALACTOPYRANOSIDE

N.1 Methyl 2,3,6-tri-O-benzoyl-4-bromo-4-deoxy-β-D-galactopyranoside (107b)

(a) Solvolysis of 121 at 110 - 120°C bath temperature: The conditions for the iodo compound (108b) were used. To compound 121 (2 g) dissolved in acetonitrile (12 ml), tetra-n-butylammonium bromide (6.0 g) was added and the solution refluxed for 48 h.; at this time tlc indicated the disappearance of ca. 95% of 121. The solution was then concentrated to a small volume, ether was added to precipitate salts and the filtrate concentrated and transferred onto a column of silica gel (eluant; benzene:ether (99:1)). The fastest fraction (0.63 g) crystallized from methanol (m.p. 148 - 150°). Recrystallization from the same solvent raised the m.p. to 151 - 153°. The mother liquors plus the second fraction to emerge were rechromatographed on silica gel and eluted with benzene; in this instance the major fraction was compound 155, the epimer of 107b. Crystallization was effected from ethanol; m.p. 132 - 134°. The third fraction (0.20 g) was crystallized from methanol, m.p. 132 - 133°; this is compound 156, corresponding to 128b.

(b) Solvolysis of 121 at 95 - 100°: Compound 121 (6.0 g) was dissolved in acetonitrile (30 ml) and heated under reflux with tetra-n-butylammonium bromide (recrystallized from acetone-ether) (25 g) for 30 h., after which time tlc (benzene:ether (9:1)) suggested that 95% of 121 had reacted. On cooling, the solution was concentrated to a small volume and ether added to precipitate out salts. The filtrate was concentrated and transferred onto a column of silica gel and separated as above. The

fast-moving, major, fraction (1.47 g, 31%) crystallized from methanol; (0.8 g, 17%) m.p. 150 - 151°, with recrystallization from methanol raising the m.p. to 151-153°. This is compound 107b, $[\alpha]_D$ + 52.7° (c, 2 chloroform).

Anal. Calc. for $C_{28}H_{25}O_8Br$: C, 59.1; H, 4.4; Br, 14.0
Found: C, 59.7; H, 4.3; Br, 13.6

The second major fraction contaminated with 107b (NMR), crystallized from ethanol (0.75 g); it had m.p. 120°. Rechromatography with benzene as eluant and recrystallization from ethanol gave pure 155, m.p. 132 - 134°, $[\alpha]_D$ + 19.4° (c, 1.23 chloroform). NMR spectrum ($CDCl_3$: C_6D_6, 3:1) δ 8.0, 7.30 (3 benzoates), δ 5.83 (H-3); δ 5.40 (H-2); δ 4.80 (H-6R) δ 4.60 (H-6S); δ 4.46 (H-1); δ 4.1 (H-4); δ 3.84 (H-5); δ 3.35 (OCH_3).

Anal. Calc. for $C_{28}H_{25}O_8Br$: C, 59.1; H, 4.4; Br, 14.0
Found: C, 59.7; H, 4.3; Br, 13.8.

N.2 Methyl 4-bromo-4-deoxy-β-D-galactopyranoside (107)

De-O-benzoylation of 107b (1.5 g) afforded 107 (0.51 g, 75%), m.p. 173°; recrystallization from methanol-ether gave crystals, m.p. 184 - 185°, $[\alpha]_D$ + 15.2° (c, 1 water).

Anal. Calc. for $C_7H_{13}O_5Br$: C, 32.7; H, 5.1; Br, 31.1
Found: C, 33.7; H, 4.8; Br, 28.0

VIII EXPERIMENTAL

N.3 Methyl 2,3,6-tri-O-acetyl-4-bromo-4-deoxy-β-D-galactopyranoside (107a)

Acetylation of 107 (0.1 g) and purification as noted above, gave crystals (0.14 g) (recrystallized from methanol-ether), m.p. 99 - 100°, $[\alpha]_D$ + 39.2° (c, 1.55 chloroform).

Anal. Calc. for $C_{13}H_{19}O_8{}^{79}Br$: Mol. wt. 382

Found: $(M-60)^+$ i.e. $(M-AcOH)^+$ at m/e 322

also $(M-60-H\ {}^{79}Br)^+$ = m/e 242 (very intense)

and $(M-60-{}^{79}Br)^+$ = m/e 243 (very intense).

Similarly:

Anal. Calc. for $C_{13}H_{19}O_8\ {}^{81}Br$: Mol. wt. 384

Found: $(M-60)^+$ at m/e 324

and $(M-60-H\ {}^{81}Br)^+$ = m/e 242

also $(M-60-{}^{81}Br)^+$ = m/e 243

O. METHYL 4-DEOXY-4-IODO-β-D-GALACTOPYRANOSIDE

O.1 Methyl 2,3,6-tri-O-benzoyl-4-deoxy-4-iodo-β-D-galactopyranoside (108b)

(a) Solvolysis of 121 at 100 - 120°C (bath temp.)

To compound 121 (2 g) dissolved in acetonitrile (12 mls), tetra-n-butylammonium iodide (4 g) was added and the solution refluxed at the bath temperature. The reaction was monitored by tlc (benzene: ether, 9:1) which showed that a fast-moving compound formed initially was being converted into two other, slow-moving compounds. After 30 h.

the incomplete reaction was stopped, the solvent evaporated off, and the residue taken up in benzene. Addition of ether caused precipitation of salts. The filtrate was concentrated and transferred onto a column of silica gel. Elution with benzene:ether (99:1) afforded in sequence crystalline (108b) (0.34 g), m.p. 163 - 164° (from methanol); crystalline 127b, m.p. 130 - 131° (from ethanol), $[\alpha]_D$ + 17.8° (c, 1 chloroform); crystalline 128b, m.p. 146 - 147° (from methanol), $[\alpha]_D$ + 3.7° (c, 0.04 chloroform); and unreacted 121 (0.11 g).

(b) Solvolysis of 121 with tetra-n-butylammonium iodide at 96-100°

Compound 121 (6 g) dissolved in acetonitrile (30 ml), was refluxed with tetra-n-butylammonium iodide (recrystallized from acetone-ether) (25 g) for 30 h. Work up of the reaction mixture was as above (Section 0.1). Column chromatographic separation afforded:

(i) Syrupy 108b(1.51 g, 30%), crystallizing from methanol to give 1.1 g (22%) of material which after recrystallization from methanol, had m.p. 163 - 164°, $[\alpha]_D$ + 47.7° (c, 2 chloroform).

Anal. Calc. for $C_{28}H_{25}O_8I$: C, 54.6; H, 4.1; I, 20.6.
Found: C, 55.5; H, 4.0; I, 20.5.

(ii) A mixture of 108b and 127b (NMR). A portion of this mixture was rechromatographed on silica gel with benzene as the eluant. Pure 127b crystallized from ethanol to give crystals with m.p. 131 - 133°; $[\alpha]_D$ + 19.9° (c, 0.78 chloroform).

Anal. Calc. for $C_{28}H_{25}O_8I$: C, 54.6; H, 4.1: I, 20.6
Found: C, 54.3; H, 4.0; I, 18.3.

VIII EXPERIMENTAL

0.2 Methyl 4-deoxy-4-iodo-β-D-galactopyranoside (108)

A solution of compound 108b (1.0 g) in benzene (10 ml) was diluted with methanol (dried over molecular sieves type A-4) and the solution was made weakly basic with 0.1 M sodium methoxide. Tlc (benzene:ether, 9:1 - absence of 108b; ethanol:ethyl acetate, 1:1 - presence of 108) showed that the reaction was complete in 12 h. at room temperature. The solution was treated with Amberlite IR-120 (H^+) resin, diluted with water, evaporated to remove most of the methanol, and extracted with benzene. The aqueous layer on concentration gave a sticky crystalline material which gave crystals from methanol-ether (0.39 g, 79%), m.p. 195 - 196° (decomp.). Recrystallization from methanol afforded pure 108, m.p. 200° (decomp.), $[\alpha]_D$ + 25.5°, (c, 1.0 water).

Anal. Calc. for $C_7H_{13}O_5I$: C, 27.7; H, 4.3; I, 41.7
Found: C, 28.1; H, 4.2; I, 42.4

0.3 Methyl 2,3,6-tri-O-acetyl-4-deoxy-4-iodo-β-D-galactopyranoside (108a)

Compound 108 (60 mg) was acetylated as for compound 104. This gave crystals (70 mg, 83%), m.p. 87 - 88°(from ethanol-water), $[\alpha]_D$ + 76.0° (c, 1 chloroform).

Anal. Calc. for $C_{13}H_{19}O_8I$: Mol. Wt. 430
Found: $(M-60)^+$ at m/e 370
and also $(M-59-128)^+$ at 243 (very intense)
i.e. $(M-CH_3COO-HI)^+$

VIII EXPERIMENTAL

P. METHYL 4-DEOXY-β-D-XYLO-HEXOPYRANOSIDE (103)

Compound 127b, containing a small proportion of 108b (NMR analysis) (0.60 g) was de-O-benzoylated according to the procedure for 108b above. This afforded a syrup containing crystals (0.20 g). The mixture was dissolved in water and stirred with palladium black (0.10 g) under hydrogen at 5 - 10° (normal pressure) for 24 h. Tlc (ethyl acetate:n-propanol, 1:1) showed that the reaction was almost complete. The acidic solution (due to HI) was filtered through a celite pad, and neutralized by stirring it with Dowex-1 (HCO_3^-) resin, filtered and then freeze-dried. Acetylation with acetic anhydride-pyridine followed by purification on a silica gel column (eluant, benzene:ether (9:1)) afforded methyl 2,3,6-tri-O-acetyl-4-deoxy-β-D-xylo-hexoside (103a) (30 mg), $[\alpha]_D$ - 23.2° (c, 2.2 $CHCl_3$) (lit.(319), m.p. 59 - 60°, $[\alpha]_D$ - 23.3° (c, 1.2 chloroform)).

De-O-acetylation of 103a with sodium methoxide-methanol as above afforded crystalline 103, m.p. 146 - 147° (ethyl acetate) (lit.(319) m.p. 145 - 147°)).

PART IX

OTHER COMPOUNDS PREPARED FOR STUDIES OF ROTATIONAL CONFORMATION ABOUT THE EXOCYCLIC C-5, C-6 BOND OF HEXOPYRANOSIDES

Q.1 Methyl 2,3,4-tri-O-acetyl-6-O-trityl-β-D-galactopyranoside

Methyl 6-O-trityl-β-D-galactopyranoside (320) was acetylated

with acetic anhydride-pyridine. Purification of the crude product was achieved on silica gel with benzene:ether (9:1) as the eluant. Pure fractions crystallized from ethanol giving m.p. 141 - 142°; $[\alpha]_D$ - 50.9° (c, 2.07 chloroform) (lit.(320), m.p. 138°, $[\alpha]_D$ - 50.3°)).

Q.2 Attempt to synthesize Methyl 2,3,4-tri-O-acetyl-β-D-galactopyranoside

To methyl 2,3,4-tri-O-acetyl-6-O-trityl-β-D-galactopyranoside (0.49 g) dissolved in cold acetic acid, hydrobromic acid (32% HBr in acetic acid) was added dropwise until tlc (benzene:ether, 5:1) showed the complete absence of starting material. Solid trityl bromide was filtered off and the filtrate poured into water. The resulting suspension was again filtered off, and the filtrate concentrated to a small volume and extracted with chloroform. This extract was washed successively with 1 N sodium bicarbonate, water, and dried over anhydrous sodium sulphate. The residue obtained on evaporation of the solvent was transferred onto a column of silica gel and eluted with benzene:ether (1:1). Fractions having the same mobility on tlc (benzene:ether, 1:1) were combined; $[\alpha]_D$ - 0.8° (c, 14.01, chloroform), and the NMR spectrum (C_6D_6 exchanged with D_2O) showed overlapping signals with peaks showing presence of two OCH_3 groups.

Q.3 Methyl 2,3,4-tri-O-acetyl-6-O-benzoyl-β-D-glucopyranoside (141a)

Methyl 2,3,4-tri-O-acetyl-β-D-glucopyranoside* (140) (0.6 g)

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* Kindly provided by Dr. D.M. Mackie

was benzoylated according to the procedure outlined above. A crystalline product (141a) was isolated and recrystallized from ethanol; m.p. 131 - 132°, $[\alpha]_D$ + 16.6° (c, 2 chloroform) (lit.(321), m.p. 127°, $[\alpha]_D$ + 15.5° (chloroform) and (315) m.p. 130 - 131°, $[\alpha]_D$ + 15.8° (chloroform)).

REFERENCES

1. D.E. Koshland Jr. and K.E. Neet, Ann. Rev. Biochem., 37, 359 (1968).
2. J.A.D. Cooper, W. Smith, M. Bacila and H. Medina, J. Biol. Chem., 234, 445 (1959).
3. G. Avigad, C. Asensio, D. Amaral and B.L. Horecker, Biochem. Biophys. Research Communs., 4, 474 (1961).
4. G. Avigad, D. Amaral, C. Asensio and B.L. Horecker, J. Biol. Chem., 237, 2736 (1962).
5. Report of the Commission on Enzymes of the International Union of Biochemistry, Oxford, Pergamon Press, 1961; Enzyme Nomenclature, Elsevier Publishing Company, Amsterdam, 1965.
6. J.C. Robinson and J.E. Pierce, Arch. Biochem. Biophys., 106, 348 (1964).
7. G.F. Springer, Y. Nagai and H. Tegtmeyer, Biochemistry, 5, 3254 (1966).
8. G. Avigad, Carbohyd. Res., 3, 430 (1967).
9. J.E. Barnett, Carbohyd. Res., 4, 267 (1967).
10. A.G. Morell, C.J.A. van den Hamer, I.H. Scheinberg and G. Ashwell, J. Biol. Chem., 241, 3745 (1966).
11. S.A. Barker, G. Pardoe and M. Stacey, Nature, 197, 231 (1963).
12. R.M. Bradley and J.N. Kanfer, Biochim. Biophys. Acta., 84, 210 (1964).
13. C.H. DeVerdier and M. Hjelm, Clin. Chim. Acta., 7, 742 (1962).
14. W.Fischer and J. Zapf, Z. Physiol. Chem., 337, 186 (1964).

15. M. Hjelm, Clin. Chim. Acta, 15, 87 (1967).

16. E. Rorem and C. Lewis, Anal. Biochem., 3, 230 (1962).

17. J. Sempere, C. Gancedo and C. Asensio, Anal. Biochem., 12, 590 (1965).

18. A.S. Perlin, personal communication to authors of ref. 3.

19. V. Prelog, Pure Appl. Chem., 9, 119 (1964).

20. J.M.H. Graves, A. Clark and H.J. Ringold, Biochemistry, 4, 2655 (1965).

21. H.M. Kagan, L.R. Manning and A. Meister, Biochemistry, 4, 1063 (1965).

22. A. Meister, Advan. Enzymol., 31, 183 (1968).

23. J. Rétey, A.Umani-Ronchi and D. Arigoni, Experientia, 22, 72 (1966).

24. H.C. Urey, F. Brickwedde and G.M. Murphy, Phys. Rev., [2] 39, 164 (1932).

25. L.S. Bartell, J. Am. Chem. Soc., 83, 3567 (1961).

26. L.S. Bartell, Tetrahedron, 17, 177 (1962).

27. J.R. Campbell, L.D. Hall and P.R. Steiner, Can. J. Chem., 50, 504 (1972).

28. R.A. Niedrich, D.M. Grant, and M. Barfield, J. Chem. Phys., 42, 3733 (1965).

29. R.A. Bernheim and H. Batiz-Hernandez, J. Chem. Phys., 45, 2261 (1966).

30. D. Arigoni and E.L. Eliel. In Topics in Stereochemistry. N.L. Allinger and E.L. Eliel, Eds. Vol. 4 Interscience, New York, N.Y., 1969, p. 120.

31. R. Bentley, Molecular Assymetry in Biology, Vol. 1, Academic Press New York, N.Y. 1969, p. 148.

32. L. Verbit, Progr. Phys. Org. Chem., 7, 51 (1970).

33. G. Popjak, Stereospecificity of Enzymic Reactions. In "The Enzymes" edited by P.D. Boyer, Vol. II, 3rd edition, Academic Press, New York, N.Y., 1970, p. 115.

34. H.R. Levy, P.B. Talalay and B. Vennesland, Prog. Stereochem., 3, 299 (1962).

35. K.R. Hanson, J. Am. Chem. Soc., 88, 2731 (1966).

36. R.S. Cahn, C.K. Ingold and V. Prelog, Angew Chem., 78, 413 (1966); Angew, Chem. Intern. Ed. Eng., 5, 385 (1966).

37. F.A. Loewus, F.H. Westheimer and B. Vennesland, J. Am. Chem. Soc., 75, 5018 (1953).

38. R.W. Swick and A. Nakao, J. Biol. Chem., 206, 883 (1954).

39. C. Bublitz and E.P. Kennedy, J. Biol. Chem., 211, 951 (1955).

40. G. Popjak and J.W. Cornforth, Biochemistry J., 101, 553 (1966).

41. E.L. Eliel, "Stereochemistry of Carbon Compounds," McGraw Hill, New York, N.Y., 1962, p. 436.

42. M. Raban and K. Mislow, Determination of Optical Purity, in "Topics in Stereochemistry," N.L. Allinger and E.L. Eliel, eds. vol. 2, Interscience, New York, 1967, p.199.

43. H.R. Levy, F.A. Loewus and B. Vennesland, J. Am. Chem. Soc., 79, 2949 (1957).

44. V.E. Althouse, D.M. Feigl, W.A. Sanderson and H.S. Mosher, J. Am. Chem. Soc., 88, 3595 (1966).

45. V.E. Althouse, K. Ueda and H.S. Mosher, J. Amer. Chem. Soc., 82, 5938 (1960).

46. D.R. Boyd and M.A. McKervey, Quart. Rev., 22, 95 (1968).

47. A. McKenzie, J. Chem. Soc., 1249 (1904) and later papers.

48. A. Streitwieser, Jr. and M.R. Granger, J. Org. Chem., 32, 1528 (1967).

49. E.L. Eliel and M.N. Rerick, J. Am. Chem. Soc., 82, 1367 (1960).

50. E.L. Eliel and D. Nasipuri, J. Org. Chem., 30, 3809 (1965).

51. D. Nasipuri, C.K. Ghosh and R.J.L. Martin, J. Org. Chem., 35, 657 (1970).

52. A. Streitwieser Jr., J. Am. Chem. Soc., 75, 5014 (1953).

53. A. Streitwieser Jr. and J.R. Wolfe Jr., J. Am. Chem. Soc., 79, 903 (1957).

54. D.J. Cram and F.A. Abd Elhafez, J. Am. Chem. Soc., 74, 5828 (1952).

55. D.J. Cram and K.R. Kopecky, J. Am. Chem. Soc., 81, 2748 (1959).

56. J.W. Cornforth, R.H. Cornforth and K.K. Mathews, J. Chem. Soc., 112 (1959).

57. V. Prelog, Helv. Chim. Acta, 36, 308 (1953).

58. A. McKenzie and H.B. Thompson, J. Chem. Soc., 1004 (1905).

59. J.H. Stocker, P. Sidisunthorn, B.M. Benjamin and C.J. Collins, J. Am. Chem. Soc., 82, 3913 (1960).

60. G.J. Karabatsos, J. Am. Chem. Soc., 89, 1367 (1967).

61. D.J. Cram and D.R. Wilson, J. Am. Chem. Soc., 85, 1245 (1963).

62. R.W. Kilb, C.C. Lin, and E.B. Wilson, J. Chem. Phys., 26, 1695 (1957).

63. S.S. Butcher and E.B. Wilson Jr., J. Chem. Phys., 40, 1671 (1964).

64. R.J. Abraham and J.A. Pople, Mol. Phys., 3, 609 (1960).

65. L.S. Bartell, B.L. Carrol and J.P. Guillory, Tetrahedron Letters, 705 (1964).

66. L.S. Bartell, and J. P. Guillory, J. Chem. Phys., 43, 647 (1965).

67. G.J. Karabatsos and D.J. Fenoglio, J. Am. Chem. Soc., 91, 3577 (1969).

68. G.J. Karabatsos and D.J. Fenoglio, J. Am. Chem. Soc., 91, 1124 (1969).

69. G.J. Karabatsos and N. Hsi, J. Am. Chem. Soc. 87, 2864 (1965).

70. G.J. Karabatsos, D.J. Fenoglio and S.S. Lande, J. Am. Chem. Soc., 91, 3572 (1969).

71. L.J. Bellamy and R.L. Williams, J. Chem. Soc., 3465 (1958).

72. J.W. Cornforth, R.H. Cornforth and K.K. Mathew, J. Chem. Soc., 2539 (1959).

73. S.F. Brady, M.A. Ilton and W.S. Johnson, J. Am. Chem. Soc., 90, 2882 (1968).

74. D.Y. Curtin, Rec. Chem. Progr. (Kresge-Hooker Sci. Lib.), 15, 111 (1954).

75. H.C. Brown, O.H. Wheeler and K. Ichikawa, Tetrahedron, 1, 214 (1957).

76. C. Cone and L. Hough, Carbohyd . Res., 1, 1 (1965).

77. D. Horton, J.B. Hughes and J.M. J. Tronchet, Chem. Commun ., 481 (1965).

78. G.B. Howarth, D.G. Lance, W.A. Szarek and J.K.N. Jones, Can. J. Chem., 47, 75 (1969).

79. D.G. Lance, W.A. Szarek, J.K.N. Jones and G.B. Howarth, Can. J. Chem., 47, 2871 (1969).

80. K.W. Bentley, D.G. Hardy and B. Meek, J. Am. Chem. Soc., 89, 3273 (1967).

81. G.K. Helmkamp and B.F. Rickborn, J. Org. Chem., 22, 479 (1957).

82. E.R. Alexander, J. Am. Chem. Soc., 72, 3796 (1950).

83. L.W. Trevoy and W.G. Brown, J. Am. Chem. Soc., 71, 1675 (1949).

84. D.K. Murphy, R.L. Alumbaugh and B. Rickborn, J. Am. Chem. Soc., 91, 2649 (1969).

85. R.H. Cornforth, J. Chem. Soc., (C), 928 (1970).

86. H. Eggerer, W. Buckel, H. Lenz, P. Wunderwald, G. Gottschalk, J.W. Cornforth, C. Donninger, R. Mallaby and J.W. Redmond. Nature, Lond. 226 517 (1970).

87. E.L. Eliel, Substitution at Saturated Carbon Atoms , in"Steric Effects in Organic Chemistry," M.S. Newman, ed., John Wiley and Sons, Inc., New York, 1956, p. 61.

88. R. Fuchs and C.A. Vanderwerf, J. Am. Chem. Soc., 76, 1631 (1954).

89. B. Helferich and E. Himmen, Ber., 62, 2136 (1929).

90. G. Siewert and O. Westphal., Ann., 720, 161 (1968).

91. J. Stanek, M. Cerny, J. Kocourek, J. Pacak, "The Monosaccharides", Academic Press, New York, N.Y., 1963, p. 395.

92. G. Zweifel, N.R. Ayyangar, T. Munekata, and H.C. Brown, J. Am. Chem. Soc., 86, 1076 (1964).

93. A. Maradufu, D.M. Mackie and A.S. Perlin, Can. J. Chem., 50, 2617 (1972).

94. L.J. Haynes and F.H. Newth, Adv. Carbohyd. Chem. 10, 207 (1955).

95. J.R. Parikh and W. von E. Doering, J. Am. Chem. Soc., 89, 5505 (1967).

96. G.M. Cree, D.M. Mackie and A.S. Perlin, Can. J. Chem. 47, 511 (1969).

97. A.S. Perlin, D.M. Mackie and C.P. Dietrich, Carbohyd . Res., 18, 185 (1971).

98. J.L. Godman and D. Horton, Carbohyd . Res., 6, 229 (1968).

99. D.C. DeJongh and K. Biemann, J. Am. Chem. Soc., 86, 67 (1964).

100. D.J. Ward, W.A. Szarek and J.K.N. Jones, Carbohyd. Res., 21, 305 (1972).

101. D.M. Mackie, Ph.D. Thesis, McGill University, 1971

102. K. Onodera, S. Hirano and N. Kashimura, J. Am. Chem. Soc., 87, 4651 (1965).

103. J. Onodera, S. Hirano, N. Kashimura, F. Masuda, T. Yajima and N. Miyazaki, J. Org. Chem., 31, 1291 (1966).

104. K. Onodera, S. Hirano, N. Kashimura, Carbohyd. Res., 6, 276 (1968).

105. A.H. Fenselau and J.G. Moffatt, J. Am. Chem. Soc., 88, 1762 (1966).

106. J.D. Albright and L. Goldman, J. Am. Chem. Soc., 89, 2416 (1967).

107. T. Durst, Dimethyl Sulphoxides in Organic Synthesis, in "Advan. in Org. Chem., Method and Results.," E.C. Taylor, H. Wynberg, eds. vol. 6, Interscience, New York (1969) p. 285.

108. F.G. Bordwell and B.M. Pitt, J. Am. Chem. Soc., 77, 572 (1955).

109. C.R. Johnson, J.C. Sharp and W.G. Phillips, Tetrahedron Letters, 5299 (1967).

110. W.E. Parham and M.D. Bhavsar, J. Org. Chem., 28, 2686 (1963).

111. L. Horner and P. Kaiser, Ann., 626, 19 (1959).

112. W.E. Parham and R. Koncos, J. Am. Chem. Soc., 83, 4034 (1961).

113. S. Oae, T. Kitao, S. Kawamura and Y. Kitaoka, Tetrahedron, 19, 817 (1963).

114. S. Oae, M. Kise, Tetrahedron Letters, 1409 (1967).

115. S. Oae, T. Kitao, and S. Kawamura, Tetrahedron, 19, 1783 (1963).

116. K.W. Ratts and A.N. Yao, J. Org. Chem., 33, 70 (1968).

117. K.W. Ratts and A.N. Yao, J. Org. Chem., 31, 1689 (1966), 31, 1185 (1966).

118. J.H. Brewster, Tetrahedron Letters, 20, 23 (1959).

119. J.M. Bijvoet, A.F. Peerdeman and A.J. van Bommel, Nature, 168, 271 (1951).

120. C.K. Johnson, E.J. Gabe, M.R. Taylor and I.A. Rose, J. Am. Chem. Soc., 87, 1802 (1965).

121. A. Horeau and A. Nouaille, Tetrahedron Letters, 1966, 3953.

122. J.W. Cornforth and G. Ryback, Ann. Rept., 62, 428 (1965).

123. R.U. Lemieux, R.K. Kullnig, H.J. Bernstein and W.G. Schneider. J. Am. Chem. Soc., 80, 6098 (1958).

124. E.W. Garbisch and M.G. Griffith. J. Am. Chem. Soc., 90, 6543 (1968).

125. G. Gatti, A.L. Segre, and C. Morandi, J. Chem. Soc., (B), 1203 (1967).

126. R.U. Lemiux and J. Howard. Can. J. Chem., 41, 308 (1963).

127. P.L. Durette, D. Horton and N.S. Bhacca, Carbohyd. Res., 10, 565 (1969).

128. A.S. Perlin and C. Brice, Can. J. Chem., 34, 541 (1956).

129. G.E. Dunn and J. Warkentin, Can. J. Chem., 34, 75 (1956).

130. R.E. Davis and J.A. Gottbrath, J. Am. Chem. Soc., 84, 895 (1962).

131. K. Biemann, D.C. DeJongh and H.K. Schnoes, J. Am. Chem. Soc., 85, 1763 (1963).

132. H. Budzikiewicz, C. Djerassi and D.H. Williams. "Structure Elucidation of Natural products by Mass Spectrometry." Vol. II. Holden-Day, 1964, p. 203.

133. B. Chance, J. Biol. Chem., 151, 553 (1943).

134. L. Michaelis and M.L. Menten, Biochem. Z., 49, 333 (1913).

135. M. Dixon and E.C. Webb "Enzymes" 2nd Ed. Academic Press, New York,. N.Y., 1964 p. 105.

136. K.B. Wiberg, Chem. Rev. 55, 713 (1955).

137. L. Melander, "Isotope Effects on Reaction Rates". The Ronald Press, New York, N.Y. 1960.

138. V.J. Shiner, Jr., H.R. Mahler, R.H. Baker, Jr., and R.R. Hiatt, Ann. N.Y. Acad. Sci., 84, 583 (1960).

139. S.V. Rieder and I.A. Rose, J. Biol. Chem., 234, 1007 (1959).

140. I.A. Rose, J. Biol. Chem., 236, 603 (1961).

141. R.H. Abeles, W. Frisell and C. Mackenzie, J. Biol. Chem., 235, 853 (1960).

142. R.P. Bell, "The Proton in Chemistry" Cornell University Press, Ithaca, New York, N.Y., 1959, p. 183.

143. E.A. Halevi, Progr. Phys. Org. Chem., 1, 109 (1963).

144. H. Simon and D. Palm, Angew.Chem. Internat Edn., 5, 920 (1966).

145. W.H. Saunders, Surv. Prog. Chem., 3, 109 (1966).

146. W.P. Jencks "Catalysis in Chemistry and Enzymology", McGraw Hill, New York, N.Y. 1969, p.243.

147. F.H. Westheimer, Chem. Rev., 61, 265 (1961).

148. B.C. Challis, Ann. Reports 64, 99 (1967); 63, 277 (1966); 62, 251 (1965).

149. H.R. Mahler and J. Douglas, J. Am. Chem. Soc., 79, 1159 (1957); D. Palm., Z. Naturforsch. 21b, 540, 547 (1966).

150. J.F. Thomson, D.A. Bray, J.J. Bummett, Biochem. Pharmacol., 11, 943 (1962).

151. J.F. Thomson, J.J. Darling, L.F. Bordner, Biochim. Biophys. Acta, 85, 177 (1964); J.F. Thomson and S.L.Nature, ibid. 99, 369 (1965).

152. D. Palm, Eur. J. Biochem., 5, 270 (1968).

153. R.H. Baker Jr.,Biochemistry, 1, 41(1962).

154. D. Palm, T. Fielder, D. Ruhrseitz., Z. Naturforsch., 23b, 623 (1968)

155. H.J. Bright and Q.H. Gibson, J. Biol. Chem., 242, 994 (1967).

156. L.S. Bartell, K. Kuchitsu and R.J. de Neui, J. Chem. Phys., 35, 1211 (1961).

157. L.S. Bartell and H.K. Higginbotham, J. Chem. Phys., 42, 851 (1965).

158. R.H. Schwendeman and J.D. Kelly, J. Chem. Phys., 42, 1132 (1965).

159. E.H. Richardson, S. Brodersen, L. Krause and H.L. Welsh, J. Mol. Spectry. 8, 406 (1962).

160. M.T. Weiss and M.W.P. Strandberg, Phys. Rev., 83, 567 (1951).

161. W.S. Benedict and E.K. Plyler, Can. J. Phys. 35, 1235 (1257).

162. O. Bastiansen and B. Beagley, Acta Chem. Scand., 18, 2077 (1964).

163. M.H. Sirvetz and R.E. Weston Jr., J. Chem. Phys., 21, 898 (1953).

164. M.J. Lacey, C.G. MacDonald, A.Pross, J.S. Shannon and S. Sternhell, Aust. J. Chem., 23, 1421 (1970).

165. E.A. Halevi, Tetrahedron 1, 174 (1957).

166. E.A. Halevi, Trans. Faraday Soc., 54, 1441 (1958).

167. A. Streitwieser Jr., and H.S. Klein, J. Am. Chem. Soc., 85, 2759 (1963).

168. E.A. Halevi, M. Nussim and A. Ron. J. Chem. Soc., 866 (1963).

169. H.C. Brown and G.J. McDonald, J. Am. Chem. Soc., 88, 2514 (1966).

170. H.C. Brown, M.E. Azzaro, J.G. Koelling and G.J. McDonald, J. Am. Chem. Soc., 88, 2520 (1966).

171. E.R. Thornton, Ann. Rev. Phys. Chem., 17, 349 (1966).

172. A. Streitwieser Jr., R.H. Jagow, R.C. Fahey and S. Suzuki, J. Am. Chem. Soc., 80, 2326 (1958).

173. A. Maradufu, G.M. Cree and A.S. Perlin, Can. J. Chem., 49, 3429 (1971).

174. I. Yamazaki, H.S. Mason, and L. Piette, J. Biol. Chem., 235, 2444 (1960).

175. G.A. Hamilton, in Progress in Bioorganic Chemistry, edited by E.T. Kaiser and F.J. Kezdy, Vol. I, Interscience, New York, N.Y. 1971.

176. G.A. Hamilton, Advan. Enzymol., 32, 55 (1969).

177. L.E. Brown and G.A. Hamilton, J. Am. Chem. Soc. 92, 7225 (1970) and reference therein

178. D.J.T. Porter and H.J. Bright, Biochem. Biophys. Res. Commun. 36, 209 (1969).

179. A.S. Perlin, Can. J. Chem., 42, 1365 (1964).

180. D. Horton, M. Nakadate and J.M.J. Tronchet, Carbohyd. Res., 7, 56 (1968).

181. R.P. Bell, Adv. Phys. Org. Chem., 4, 1 (1966).

182. Y. Ogata and A. Kawasaki, in "The Chemistry of Carbonyl Compounds," J. Zabicky, ed. Vol. 2, Interscience, London, 1970, p.1.

183. P. Greenzaid, Z. Luz and D. Samuel, J. Am. Chem. Soc., 89, 749 (1967).

184. M. Bergmann and M. Miekeley, Ber., 62, 2297 (1929).

185. M. Bergmann, M. Miekeley and E. von Lippman, Ber., 62, 1467 (1929).

186. L.N. Owen, Annual Repts., 41, 142 (1944).

187. E. Späth and H. Schmid, Ber., 74, 859 (1941).

188. E. Späth, R. Lorenz, and E. Freund, Ber., 76, 57, 1196 (1943); 77, 354 (1944).

189. E. Späth, and I. v. Szilágy, Ber., 76, 949 (1943).

190. F.A.L. Anet in "Conformational Analysis," G. Chiurdoglu, ed., Academic Press, New York, N.Y., 1971, p.15.

191. J. Dale, T. Ekeland, J. Krane, J. Am. Chem. Soc., 94, 1389 (1972).

192. F.A.L. Anet and P.J. Degen, J. Am. Chem. Soc., 94, 1390 (1972).

193. R. Schaffer and H.S. Isbell, J. Am. Chem. Soc., 79, 3864 (1957).

194. T.D. Inch, Carbohyd. Res., 5, 53 (1967).

195. M. Vogel and D. Rhum, J. Org. Chem., 31, 1775 (1966).

196. A. Anteunis, D. Tavernier and F. Borremans, Bull, Soc. Chem. Belges, 75, 396 (1966).

197. H. Booth, Progr. Nucl. Magn. Resn. Spectr., 5, 353 (1969).

198. R.U. Lemieux and J.D. Stevens, Can. J. Chem., 43, 2059 (1965).

199. C. Barbier, J. Delmau and J. Ranft., Tetrahedron Letters, 3339 (1964).

200. Y. Fujiwara and S. Fujiwara, Bull. Chim. Soc. Japan, 37, 1010 (1964).

201. H. Booth, Tetrahedron Letters, 411 (1965).

202. R.J. Abraham, L. Cavalli and K.G.R. Pachler, Mol. Phys., 11, 471 (1966).

203. J.T. Edward, Chem. Ind. London, 1102 (1955).

204. R.U. Lemieux and N.J. Chü, 133rd Meeting, Am. Chem. Soc., Abstract of Papers 31N (1958); R.U. Lemieux in P. de Mayo, "Molecular rearrangements", Interscience, New York, N.Y. 1963, p. 713.

205. O.L. Chapman and R.W. King, J. Am. Chem. Soc., 86, 1256 (1964).

206. B. Casu, M. Reggiani, G.G. Gallo and A. Vigevani, Tetrahedron Letters, 2839 (1964).

207. W. Mackie and A.S. Perlin, Can. J. Chem., 44, 2039 (1966).

208. Z. Jedlinski and J. Majnusz, Tetrahedron, 25, 2963 (1969).

209. D.C. De Jongh and K. Biemann, J. Am. Chem. Soc., 85, 2289 (1963).

210. K. Heyns and D. Müller, Tetrahedron Letters, 6061 (1966).

211. L.C. Gruen and P.T. McTigue, Australian J. Chem., 17, 953 (1964).

212. A. Melo, W.H. Elliot and L. Glaser, J. Biol. Chem., 243, 1467 (1968); O. Gabriel and L.C. Lindquist, J. Biol. Chem., 243, 1479 (1968).

213. O. Gabriel, Carbohyd. Res., 6, 111 (1968).

214. S. Hanessian, Adv. Carbohyd. Chem., 21, 143 (1966).

215. I.A. Rose, J. Am. Chem. Soc., 80, 5835 (1958) and references therein.

216. E. Shaw, Chemical Modification by Active-Site Directed Reagents in "The Enzymes", ed. P.D. Boyer, vol. I, 3rd. edn., Academic Press, New York, N.Y., 1970, p. 91.

217. L.A. Cohen, Chemical Modification as a Probe of Structure and Function in "The Enzymes", ed. P.D. Boyer, Vol. I, 3rd. edn., Academic Press, New York, N.Y., 1970, p. 147.

218. A. Gottschalk, Advan. Carbohyd. Chem., 5, 49 (1950).

219. R.A. Schlegel, C.M. Gerbeck and R. Montgomery, Carbohyd. Res., 7, 193 (1968).

220. W.P. Jencks,"Catalysis in Chemistry and Enzymology", McGraw-Hill, 1969, p. 393.

221. T.C. Bruice, Proximity Effects and Enzyme Catalysis in "The Enzymes", P.D. Boyer, ed., Vol. II, 3rd. edn., Academic Press, New York, N.Y., 1970, p. 217.

222. H. Lineweaver and D. Burk, J. Am. Chem. Soc., 56, 658 (1934).

223. M. Dixon and E.C. Webb, "Enzymes" 2nd. edn., Academic Press, New York, N.Y., 1964, p. 67.

224. L. Pauling,"The Nature of the Chemical Bond," 3rd. edn., Cornell University Press, Ithaca, N.Y., 1960, p. 460.

225. L. Pauling,"The Nature of the Chemical Bond," 3rd. ed., Cornell University Press, Ithaca, N.Y., 1960, p. 490; J. Am. Chem. Soc., 58, 94 (1936).

226. D.R. Storm and D.E. Koshland, Jr., Proc. Nat. Acad. Sci., U.S.A., 66, 445 (1970).

227. D.E. Koshland Jr., J. Theor. Biol., 2, 75 (1962).

228. B. Capon, J. Chem. Soc., (B), 1207 (1971).

229. T.C. Bruice, A. Brown, and D.O. Harris, Proc. Nat. Acad. Sci. U.S.A., 68, 658 (1971).

230. A.L. Fink and M.L. Bender, Biochemistry, 8, 5109 (1969).

231. E.C. Lucas and A. Williams, Biochemistry, 8, 5125 (1969).

232. A. Williams, E.C. Lucas, A.R. Rimmer and H.C. Hawkins, J. Chem. Soc., Perkin II 627 (1972).

233. A. Gottschalk, Rev. Pure and Appl. Chem., 3, 179 (1953).

234. A.G. Ogston, Nature, 162, 963 (1948).

235. R.S. Tipson, Advan. Carbohyd. Chem., 8, 107 (1953).

236. D.H. Ball and F.W. Parrish, Advan. Carbohyd. Chem., 23, 233 (1968); 24, 139 (1969).

237. J. Hill, L. Hough and A.C. Richardson, Proc. Chem. Soc., 346 (1963); Carbohyd. Res., 8, 7 (1968).

238. A.C. Richardson, Carbohyd. Res., 10, 395 (1969).

239. R.M. Coates and J.P. Chen, Tetrahedron Letters, 2705 (1969).

240. T. Gramstad and R.N. Haszeldine, J. Chem. Soc., 4069 (1957).

241. R.L. Hansen, J. Org. Chem., 30, 4322 (1965).

242. A. Streitwieser, Jr., C.L. Wilkins and E. Kiehlmann, J. Am. Chem. Soc., 90, 1598 (1968).

243. T.M. Su, W.F. Sliwinski, and P.v.R. Schleyer, J. Am. Chem. Soc., 91, 5386 (1969).

244. Ref. 251, p. 105.

245. Z. Jedlinski, Roczniki Chemji., 32, 1257 (1958); Chem. Abstr. 53, 10046 (1959).

246. E.J. Reist, R.R. Spencer, D.F. Calkins, B.R. Baker, and L. Goodman, J. Org. Chem., 30, 2312 (1965).

247. A.C. Richardson and J.M. Williams, Chem. Comm., 104 (1965).

248. J.M. Williams and A.C. Richardson, Tetrahedron, 23, 1369 (1967).

249. D. Horton and D.H. Hutson, Advan. Carbohyd. Chem., 18, 123 (1963).

250. V. Prey and F. Grundschober, Monatsh. Chem., 91, 358 (1960).

251. J. Stanek, M. Cerny, J. Kocourek, J. Pacak, "The Monosaccharides", Academic Press, New York, N.Y. 1963 (p. 615).

252. C.G. Youngs and A.S. Perlin, Can. J. Chem., 45, 1801 (1967).

253. J.M. Heap and L.N. Owen, J. Chem. Soc., (C) 707 (1970); 712 (1970).

254. A.F. Cook and W.G. Overend, J. Chem. Soc., (C), 1549 (1966).

255. S.D. Gero and R.D. Guthrie, J. Chem. Soc., (C), 1761 (1967).

256. J. Hill, L. Hough, and A.C. Richardson, Carbohyd. Res., 8, 19 (1968).

257. E.L. Eliel, N.L. Allinger, S.J. Angyal and G.A. Morrison, Eds. "Conformational Analysis", Interscience 1965, p.71 and 269 and references therein.

258. J.M. Sugihara, Advan. Carbohyd. Chem., 8, 4 (1953).

259. J.S.D. Bacon, D.J. Bell and H.W. Kosterlitz, J. Chem. Soc., 1248 (1939).

260. B. Helferich and W. Klein, Ann., 455, 173 (1927).

261. A. Thompson, M.L. Wolfrom and M. Inatome, J. Am. Chem. Soc., 77, 3160 (1955).

262. M.L. Wolfrom, A. Thompson and M. Inatome, J. Am. Chem. Soc., 79, 3868 (1957).

263. J.E.G. Barnett, Advan. Carbohyd. Chem., 22, 177 (1967).

264. S. Hanessian, Advan. Chem. Ser. (Am. Chem. Soc.) No. 74, 159 (1968).

265. C.G. Swain and C.B. Scott, J. Am. Chem. Soc., 75, 141 (1953).

266. J. Miller and A.J. Parker, J. Am. Chem. Soc., 83, 117 (1961);
A.J. Parker, J. Chem. Soc., 1328 (1961);
A.J. Parker, Adv. Phy. Org. Chem., 5, 173 (1963).

267. A.B. Foster, R. Hems, and J.M. Webber, Carbohyd. Res., 5, 292 (1967).

268. E.D. Hughes, F. Juliusburger, S. Masterman, B. Topley and J. Weiss, J. Chem. Soc., 1525 (1935).

269. P.B.D. de la Mare, J. Chem. Soc., 3169 (1955); 3180 (1955).
P.B.D. de la Mare, L. Fowden, E.D. Hughes, C.K. Ingold and J.D.H. Mackie, J. Chem. Soc., 3196 (1955); 3200 (1955).

270. M. Duffy, M. Jeffries, G. Pass and G.O. Phillips, J. Chem. Soc., Perkin II, 821 (1972).

271. C.L. Stevens, K.G. Taylor and J.A. Valicenti, J. Am. Chem. Soc., 87, 4579 (1965).

272. E.S. Gould,"Mechanism and Structure in Organic Chemistry," Holt, New York, N.Y. 1959, p. 258.

273. D.M. Marcus and J.H. Westwood, Carbohyd. Res., 17, 269 (1971).

274. S. Sternhell, Rev. Pure Appl. Chem., 14, 15 (1964).

275. L.D. Hall, J.F. Manville and A. Tracey, Carbohyd. Res., 4, 514 (1967).

276. M. Barfield and B. Chakrabarti, Chem. Rev., 69, 757 (1969).

277. A.B. Foster, R. Hems, L.D. Hall and J.F. Manville, Chem. Commun., 158 (1968).

278. A.B. Foster, R. Hems, and L.D. Hall, Can. J. Chem., 48 3937 (1970).

279. A.B. Foster, R. Hems and J.H. Westwood, Carbohyd. Res., 15 41, (1970).

280. C.W. Jefford, D.T. Hill, L. Ghosez, S. Toppet and K.C. Ramey, J. Am. Chem. Soc., 91, 1532 (1969).

281. B.M. Gatehouse and B.J. Poppleton, Acta Crystallogr. Sect (B), 27, 654 (1971).

282. V.S.R. Rao, personal communication.

283. J.M. Sugihara, and W.J. Teerlink, J. Org. Chem., 29, 550 (1964).

284. L.D. Hall, J.F. Manville and N.S. Bhacca, Can. J. Chem., 47, 1 (1969).

285. R.U. Lemieux and J.C. Martin, Carbohyd. Res., 13, 139 (1970).

286. S. Yamana, Bull, Chem. Soc., Japan, 30, 920 (1957).

287. S. Yamana, Bull, Chem. Soc., Japan, 31, 558 (1958).

288. M. Karplus, J. Chem. Phys., 30, 11 (1959).

289. F.A.L. Anet, Can. J. Chem., 39, 789 (1961).

290. M. Sundaralingam, J. Am. Chem. Soc., 87, 599 (1965).

291. J. Dabrowski and A. Ejchart, Org. Magn. Reson., 4, 131 (1972).

292. K. Tori and T. Komeno, Tetrahedron, 21, 309 (1965).

293. T.H. Siddall and W.E. Stewart, Progr. Nucl. Magn. Reson., 5, 33 (1969).

294. R.C. Cookson, T.A. Crabb, J.J. Frankel and J. Hudec, Tetrahedron Supplement, 7, 355 (1966).

295. R.C. Cookson and T.A. Crabb, Tetrahedron, 28, 2139 (1972); M. Anteunis, G. Swaelens and J. Gelan, Tetrahedron, 27, 1917 (1971).

296. N. Harada and K. Nakanishi, J. Am. Chem. Soc., 91, 3989 (1969).

297. N. Harada, K. Nakanishi and S. Tatsuoka, J. Am. Chem. Soc., 91, 5896 (1969).

298. N. Harada, H. Sato and K. Nakanishi, Chem. Commun., 1691 (1970).

299. G.D. Johnson, J. Am. Chem. Soc., 73, 5888 (1951).

300. S.M. Partridge, Nature, 158, 270 (1946).

301. R.M. Horrocks, Nature, 164, 444 (1949).

302. C.S. Hudson and J. Johnson, J. Am.Chem. Soc., 38, 1223 (1916).

303. H. Ohle, W. Marecek and W. Bourjau, Ber., 62, 849 (1929).

304. J.K. Dale and C.S. Hudson, J. Am. Chem. Soc., 52, 2534 (1930).

305. W. Koenigs and E. Knorr, Ber., 34, 957 (1901).

306. F.J. Bates and Associates, "Polarimetry and Saccharimetry and the Sugars", Circ. Natl. Bur. Stand. C440, Washington D.C.,1942, p.516.

307. H. Ohle and G. Berend, Ber., 58, 2585 (1925).

308. O. Schier and E. Waldman, Mh. Chem. Bd., 88, 847 (1957).

309. A.S. Perlin, Can. J. Chem., 44, 1757 (1966).

310. B.R. Baker and R.E. Schaub, J. Am. Chem. Soc., 77, 5900 (1955).

311. C.S. Hudson and J.M. Johnson, J. Am. Chem. Soc., 37, 2748 (1915).

312. J.M. Sugihara and J.C. Petersen, J. Am. Chem. Soc., 78, 1760 (1956).

313. E. Fischer and B. Helferich, Ann., 383, 68 (1911).

314. P.A. Levene and A.L. Raymond, J. Biol. Chem., 97, 763 (1932).

315. P. Brigl and H. Grüner, Ann., 495, 60 (1932).

316. D.S. Mathers and G.J. Robertson, J. Chem. Soc., 696 (1933).

317. D.J. Bell and J. Lorber, J. Chem. Soc. 453 (1940).

318. J.C. Dennison and D.I. McGilvray, J. Chem. Soc., 1616 (1951).

319. E.J. Hedgley, W.G. Overend and R.A.C. Rennie, J. Chem. Soc., 4701 (1963).

320. A. Muller, Ber., 64, 1820 (1931).

321. H. Ohle and K. Spencker, Ber., 59, 1836 (1926).

CLAIMS TO ORIGINAL RESEARCH

1. The synthesis of D-galactose-6-d of predominantly 6R (and also 6S) configuration has been carried out by asymmetric induction, and its absolute configuration has been determined.

2. In steps leading to the synthesis of D-galactose-6-d, it has been observed that dimethyl sulphoxide oxidation is subject to a deuterium isotope effect that leads to an increase in the yield of the methyl thio-methyl ether byproduct. This furnishes novel information about the mechanism of oxidation.

3. Kinetic studies of measurements of the action of D-galactose oxidase on D-galactose-6-d show that there is a primary deuterium isotope effect, and that the oxidative process involves abstraction of the pro-S hydrogen. This is confirmed by the results of analysis of products of the oxidation. A kinetic enzymic resolution of a mixture of 6R and 6S diastereoisomers has been effected through selective oxidation of the 6R carbinol, resulting in the isolation of material of pure 6S configuration.

4. Secondary deuterium isotope effects encountered in the oxidation of the C-6 deuterated substrates are interpreted in terms of the steric sensitivity of the enzyme to the components of the reaction centre, and to the chemical behaviour of the deuterated moieties. There is also a solvent deuterium isotope effect, implying that the hydrogen abstraction process proceeds by proton removal, and is concerted.

5. The presence of α-β-unsaturated aldehydes among enzyme products has been demonstrated. Their occurrence is attributed to chemical α-β-

CLAIMS TO ORIGINAL RESEARCH

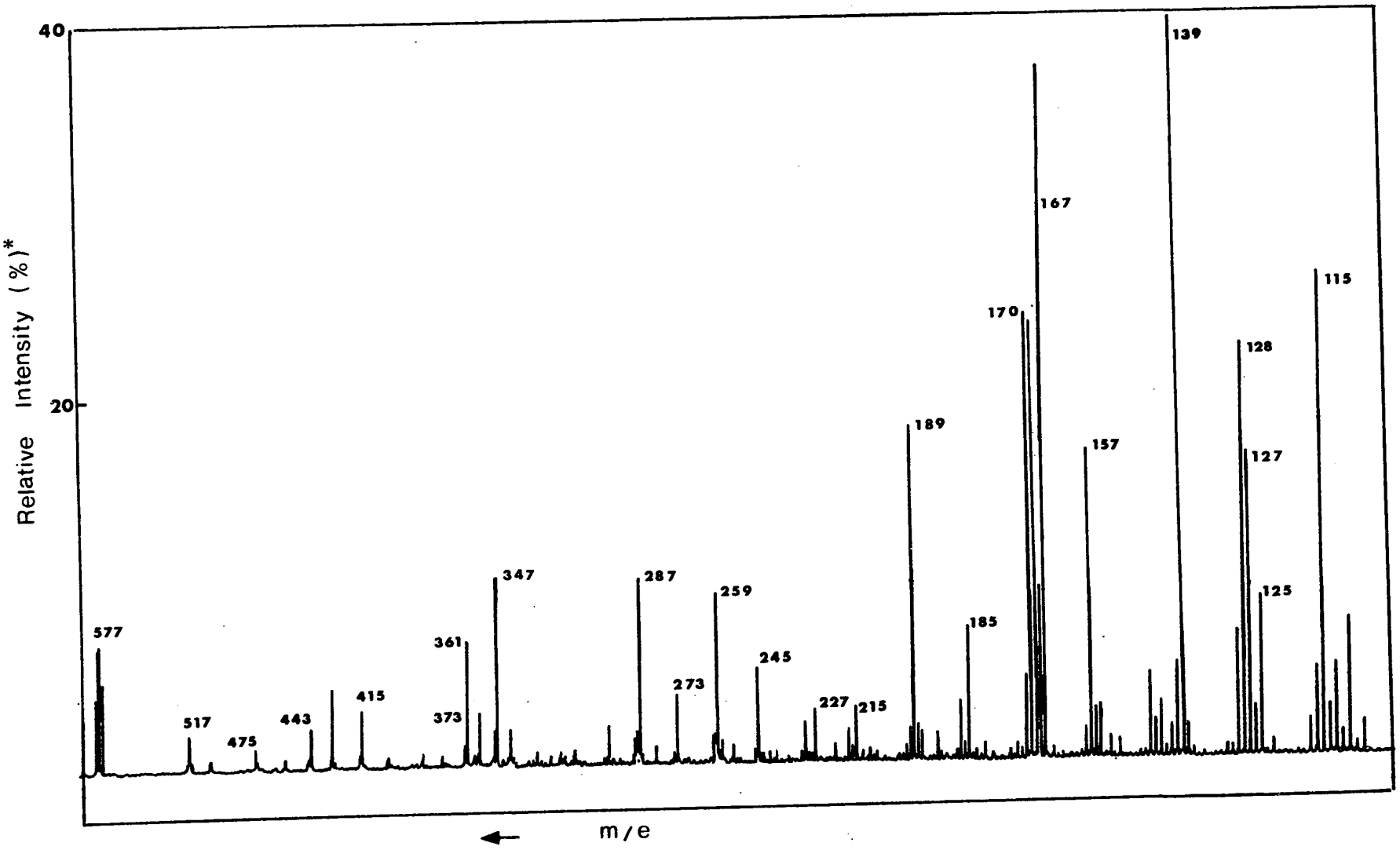
elimination initiated through the 6-carbonyl group formed, rather than to the presence of an α-β-eliminase in the enzyme preparation.

6. Dimeric enzyme products resulting from the oxidation of methyl β-D-galactopyranoside have been isolated and characterised. The initial 6-aldehydo products dimerize as β-hydroxy-aldehydes in such a way that two aldehyde-groups and one β-hydroxy-group (OH-4) participate in the dimeric linkages.

7. The rate of oxidation of 4-deoxy substrates (analogues of methyl β-D-galactopyranoside) bearing aprotic and protic groups at C-4, depends much more on the size of the substituent than on its characteristic as a donor or proton acceptor. This indicates that OH-4 of D-galactose acts primarily as a filler of a particular cleft on the enzyme surface. It is proposed that the type of bonding between OH-4 and the enzyme surface is 'hydrophobic'.

8. The orientation of OH-4 of D-galactose oxidase substrates and relative to that of H_S-6 is shown to be predominantly 1,3-cis, and this is probably the most favourable arrangement for abstraction of this proton by the enzyme.

9. A description of the synthesis and characterisation of the various 4-deoxy-substrates (analogues of methyl β-D-galactopyranoside) which were utilized for the stereochemical study of D-galactose oxidase is presented. Some of these new synthetic compounds are methyl 4-amino-4-deoxy-, 4-azido-4-deoxy, 4-bromo-4-deoxy, 4-chloro-4-deoxy, 4-deoxy-4-fluoro, -4-iodo, -β-D-galactopyranoside and derivatives.



* Relative to the base peak of CH_3CO^+, at m/e 43.