CHEMICAL AND SPECTROSCOPIC STUDIES OF THE CAPSULAR POLYSACCHARIDES OF SOME *KLEBSIELLA* SEROTYPES

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ABSTRACT

As part of an international collaborative programme concerned with the elucidation of the molecular structures of capsular polysaccharides (the K-antigen) produced by strains of the bacterial genus *Klebsiella*, the capsular material of serotype K71 has been investigated, and that of serotypes K36 and K64 re-examined, by novel enzymic and spectroscopic methods.

The cultivation and employment of bacteriophages which are capable of cleaving (by specific glycanase action) the isolated, cognate bacterial polysaccharide *in vitro* has yielded highly significant oligosaccharides. These may represent the repeating unit in the polysaccharide or be derivatives resulting from conversion of uronic acid to the 4,5-unsaturated analogue where, as found for serotype K64, the mode of cleavage is β-elimination not hydrolysis. The oligosaccharides thus generated have proved to be far more amenable to chemical and spectroscopic studies than their parent polymers, thereby facilitating complete characterisation of the molecular structures of the original polysaccharides.

Chemical methods applied to these oligosaccharides included specific degradations by periodate oxidation and acid-, alkali- or enzyme- catalysed hydrolysis, products being identified by methylation analysis (involving the extensive use of gas-liquid chromatography coupled to mass spectrometry) and spectroscopic studies (mass and n.m.r.).

During the course of these investigations it became apparent that the structures of the intact oligosaccharides (containing six or seven sugar residues) could be determined almost entirely from spectroscopic analysis, chiefly by detailed two-dimensional n.m.r. studies involving the use of high field spectrometers and the application of homo- and heteronuclear shift correlated spectroscopy, the sequence of sugar units being confirmed by mass spectrometric analysis of the permethylated derivatives.

Methylation analysis of the oligosaccharides derived from *Klebsiella* serotype K36 proved that the glucuronic acid residue is linked through O-2, and not O-4 as published by others; this finding was corroborated during characterisation of the monomeric oligosaccharide by mass- and n.m.r. spectroscopy. Bacteriophage Ø64 was shown to cleave the cognate K64 exopolysaccharide by a β-elimination process; the resulting hex-4-enuronic acid, present as a terminal group in the derived oligosaccharide was fully characterised by hydrogenation and g.l.c.-m.s. of acetylated products, and by detailed n.m.r. studies including long-range heteronuclear experiments.

Finally the structure of the heptasaccharide repeating unit of the *Klebsiella* K71 capsular polysaccharide was established by spectroscopic analysis of the oligosaccharides derived by bacteriophage Ø71 cleavage of the polymer; features of the proposed structure were confirmed by chemical degradation studies performed on the native polysaccharide.

Part of the work reported in this thesis has been published; *viz.*;

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ABBREVIATIONS

~	approximately
Ara	arabinose
c	concentration
cm	centimetre
d.p.	degree of polymerisation
eV	electron volt
f	furanose
f.a.b.	fast-atom-bombardment
g	gram
Gal	galactose
g.l.c.	gas-liquid chromatography
Glc	glucose
GlcA	glucuronic acid
h	hour(s)
h.p.l.c.	high performance liquid chromatography
Hz	Hertz
i.r.	infrared
L	litre
M	Molar
Man	mannose
m/e	ratio of mass to electron charge
mg	milligram
min	minute(s)
mL	millilitre
mM	millimolar

m.s.	mass spectrometry
mol	mole
mol. wt.	molecular weight
Mw	weight-average molecular weight
μL	microlitre
n.d.	not determined
nm	nanometre
n.m.r.	nuclear magnetic resonance
n.o.	not observed
n.r.	not resolved
p	pyranose
PAANs	peracetylated aldononitriles
p.m.a.a.	partially methylated alditol acetate
p.p.m.	parts per million
Rha	rhamnose
s.e.c.	steric-exclusion chromatography
s	singlet
TFA	trifluoroacetic acid
TMS	tetramethylsilane
u.v.	ultraviolet
v/v	volume per volume
w/w	weight per weight
Xyl	xylose

Abbreviations and symbols omitted from the above list are defined in the text.

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CHAPTER 1

INTRODUCTION

Bacterial cells synthesise polysaccharides which either form integral components of the cell wall or are used for storage of carbon or energy. Many cells also produce polysaccharides which lie outside the cell and are termed slime, capsular or microcapsular polysaccharides, depending on their structural relationship to the bacterial cell wall[1]. These polymers are generally referred to as exopolysaccharides; most are antigenic and are consequently used as vaccines[2,3]. Several bacterial polysaccharides are also commercially important and are produced industrially (e.g. xanthan gum)[4,5].

The main objective of the present investigation is the elucidation of the molecular structures of capsular polysaccharides produced by some strains of *Klebsiella* bacteria. These gram-negative bacteria belong to the family *Enterobacteriaceae*, a large heterogeneous group of bacilli, further classified according to tribe (*Klebsielleae*) and genus (*Klebsiella*). *Klebsiellae* are normally found in the upper respiratory, intestinal and genito-urinary tracts of healthy subjects. The bacteria may become pathogenic and are associated with urinary tract infections, meningitis and some respiratory diseases (3% of bacterial pneumonias). The pathogenic nature of bacteria is largely determined by the capsule, which protects cells from the host immune system by inhibiting phagocytosis and the action of complement[2].

Several species of the genus *Klebsiella* have been identified and named after the infection with which they are associated, *viz. K. pneumoniae, K. ozaenae* and *K. rhinoscleromatis.* Ørskov and Ørskov[6] have included non-clinical *Klebsiella* isolates and the results of DNA hybridisation studies to reclassify the species as *K. pneumoniae, K. oxytoca, K. terrigena* and *K. planticola.* However, *Klebsiella* bacteria are systematically classified according to their serological reactions (serotypes) and on this basis approximately eighty different

capsular polysaccharides (K-antigens) and nine lipopolysaccharides (O-antigens) have been recognised[6,7]. Immunochemical relationships among different *Klebsiella* K-antigens, and between these and surface antigens of other bacteria, have been established from serological cross-reactivity studies[2,8-11]. Interpretation of these findings is facilitated by a knowledge of the primary structure of the antigenic material, which is the objective of an international collaborative programme. The majority (about sixty-seven) of these polysaccharides have been characterised in terms of molecular structure, which is specific to the serotype. This programme has been extended to include structural examination of the K- and O- antigens of the *Escherichia coli* bacteria (family *Enterobacteriaceae*).

1.1 THE STRUCTURES OF KLEBSIELLA CAPSULAR POLYSACCHARIDES

The capsular polysaccharides of *Klebsiella* K-serotypes have been grouped according to their constituent sugars (chemotypes)[12,13] and on the basis of their structural patterns[14]; however, neither division accounts for their different immunological behaviour, which is specific for each molecular structure. The structures of the polysaccharides from fifty-three K-serotypes were included in a review published in 1982[7], and only recently those from the following K-serotypes have also been characterised: K3[15], K10[16], K14[17], K15[18], K19[19], K35[20], K39[21], K40[22], K50[23], K66[24], K67[25], K68[26], K69[27], K80[28] and K82[29]. Parenthetically, the last mentioned serotype was not recognised until 1977[6].

The structures known to date show that the general pattern consists of groups of sugar and uronic acid residues (3-7 per repeating unit) which are joined in a linear fashion to produce a, high molecular-weight, acidic hetero-polysaccharide. The nature of the sugar and uronic residues, their modes of linkage, and the sequence in which they are arranged, differ with the bacterial serotype. Several polysaccharides contain acetal-linked pyruvic acid which is an important immunological determinant[30]. The acidity of the K32, K56 and K72 polysaccharides is entirely determined by this substituent, as no uronic acid residues are present. The configuration and positions of acetal attachment, and the nature and location of the pyruvylated sugar residues, are varied[7]. The neutral sugars found are the common hexoses, D-glucose, D-galactose and D-mannose; and the 6-deoxysugars, L-rhamnose and L-fucose. Unique sugars (e.g. modified uronic acids) are rare and have been found only in the K22[7], K37[31], K38[32] and K66[24] polysaccharides. Acetyl substituents may also be present. The complete structural characterisation of these antigens is of vital importance in immunology as even small changes in structure result in serological differentiation. For example, K30 and K33 are serologically distinct, but the capsular polysaccharides are chemically identical except for the amount of *O*-acetate present[33].

1.2 ISOLATION AND PURIFICATION OF CAPSULAR POLYSACCHARIDES

The extraction, isolation and purification of exopolysaccharides have been reviewed[34,35] and therefore only the methods used in the present study of *Klebsiella* capsular polysaccharides will be discussed.

The *Klebsiella* bacterial specimen, isolated or obtained from the library of serotypes available (Dr I. Ørskov, W.H.O., Copenhagen), is propagated on sterile nutrient agar plates at 37°C until actively growing colonies are obtained. Contamination introduced during the recovery procedure can be obviated by the use of selective-growth media[36]. Liquid cultures are obtained by transferring a single fresh colony to capped test tubes containing sterile nutrient broth. Vigorous shaking at 37°C ensures bacterial growth which appears as solution turbidity; this can be quantified using optical density (with water or nutrient broth as blank). These cultures are then used to inoculate flasks containing large volumes of sterile broth. Once turbid, these solutions are layered into trays containing an appropriate agar medium, 1cm in depth, and incubated at 37°C. After several days the cells (slime) are scraped off the agar surfaces, treated with aqueous phenol to kill the bacteria and to dissolve the capsular polysaccharide, then ultracentrifuged to effect deposition of the cellular debris. The polymers are recovered from the supernatant fluids by precipitation with ethanol. The resulting precipitate is resuspended in water, the suspension centrifuged and the clear supernatant liquid is then freeze-dried to yield the crude polysaccharide product. Alternatively, the aqueous solution containing the acidic K-antigen can be further fractionated by the slow addition of a 5% solution of cetyltrimethylammonium bromide (Cetavlon). The acidic polysaccharide-Cetavlon complexes thus formed, precipitate, and can be separated from soluble neutral material by centrifugation. The polysaccharide is recovered by dissolution of the complex in a solution of NaCl (3M), and reprecipitation with ethanol. The product, redissolved in water, is dialysed against tap water, then distilled water, the solution is centrifuged and the resulting clear supernatant freeze-dried to yield the purified K-antigen capsular polysaccharide as the sodium salt. The protonated form of the polysaccharide can be recovered after deionisation with a cation-exchange resin. In case of heavy contamination

with neutral polysaccharides, the Cetavlon precipitation procedure can be repeated. Further purification can be achieved by anion-exchange or steric-exclusion chromatography. Protein contamination, as indicated by microanalysis (% nitrogen), can be removed using the Sevag technique[37].

1.3 STRUCTURAL DETERMINATION OF COMPLEX POLYSACCHARIDES

Structural studies of polysaccharides involve the determination of components, linkages, sequences, anomeric configurations and conformations. For bacterial polysaccharides, this task is simplified by the regular structure of these polymers, each of which is composed of uniform oligosaccharide repeating units which can be characterised[7]. The analyses involved have been greatly facilitated by advances in instrumentation of chromatographic and spectroscopic techniques which enable structural elucidation of milligram quantities of complex polysaccharides to be achieved. The chemical characterisation and structure determination of polysaccharides have been comprehensively reviewed by Aspinall (reference 38 and references therein) and will be discussed only briefly here. Details of the experimental conditions used in the present work are described in Chapter 2.

Before structural studies begin, the physical and chemical homogeneity of the isolated polysaccharide product must be established. Physical homogeneity is commonly investigated by chromatographic techniques, especially those based upon steric-exclusion principles, although ion-exchange, electrophoretic and ultracentrifugation criteria may also be applied. Chemical homogeneity is usually revealed only during the actual structural

studies, but can be estimated using spectroscopic techniques (e.g. nuclear magnetic resonance (n.m.r.), see Chapter 3) or optical rotation. The latter parameter provides a simple way of monitoring the isolation procedure and enables comparisons of different preparations of the polysaccharide to be made with ease. The synthesis of bacterial exopolysaccharides is under indirect genetic control and consequently a large measure of homogeneity is found[1,39], although variation in composition and yield of the exopolysaccharide produced by *Klebsiella* serotype K32 under different growth conditions has been reported[40].

Once the homogeneity of the polysaccharide under study is established, qualitative and quantitative analysis of the constituents is performed, after release of the sugar and non-sugar monomers by acid hydrolysis. Optimisation of the hydrolytic conditions is required to ensure the quantitative release of all the components with the minimum of free sugar degradation; this is not always possible when uronic acid residues are present because the stability of the uronosyl bonds results in the incomplete release of adjoining sugar residues. Analysis is usually by g.l.c.-m.s. of suitable derivatives, alditol and aldononitrile acetates being the most widely used sugar derivatives; these can be identified on the basis of relative retention times on g.l.c. and from interpretation of the mass spectra obtained. The detection of uronic acid residues requires prior reduction of the carboxyl group; this is performed either on the underivatised polymers (e.g. using the method of Taylor and Conrad[41,42], see 2.9) or during the analysis (e.g. methanolysis, see 2.8). Use of borodeuteride in these procedures results in the formation of a di-deuterated modified uronic acid derivative which can be readily identified by g.l.c.-m.s. The components can also be characterised using other chromatographic techniques (e.g. h.p.l.c.,

ion-exchange), spectrophotometrically or using non-destructive spectroscopic methods (in particular n.m.r.).

Identification of the absolute configuration of the constituent sugars is not possible by the methods described above, but is established by polarimetric studies after preparative separation of the components, or analytically by capillary g.l.c. after reaction with chiral reagents (e.g. (-)-2-butanol)[43] and derivatisation. Enzymic methods have also been used.

Methylation analysis[44-46] (see 2.10) involves methylation of all free hydroxyl groups in the polysaccharide and hydrolysis (or methanolysis) of the permethylated product to a mixture of partially methylated monosaccharides. Qualitative and quantitative analysis of this mixture by g.l.c.-m.s. yields information on the positions of linkage of glycosyl residues, and in some instances the ring size of the sugar residue. The methods of methylation are generally based on the Hakomori[47] procedure and improvements in efficiency, speed and safety have been reported[48-51] (including the novel use of solid base as reagent[52,53]). These advances are aided by the introduction of solvents (e.g. N-methylmorpholine N-oxide[54] and 1,1,3,3,-tetramethylurea[55]) that facilitate the dissolution of polysaccharides prior to methylation. Partially methylated alditol acetates (p.m.a.a.'s) are the most widely used derivatives in g.l.c.-m.s. analysis; mass spectra and relative retention times of these derivatives have been compiled (e.g reference 44). Asymmetrical derivatives can be prepared if required either by using $NaBD_4$ during preparation of the p.m.a.a.'s thereby putting a deuterium label on C-1, or by making the partially methylated aldononitrile derivatives.

The presence of uronic acid residues in permethylated polysaccharides results in incomplete release of contiguous sugar residues on acid hydrolysis due to the stability of the uronosyl bonds. Reduction of the methyl ester with lithium aluminium hydride (or deuteride) overcomes this difficulty and the carboxyl-reduced methylated polysaccharide can then be hydrolysed completely or remethylated prior to hydrolysis. Alternatively, the methylated product can be methanolysed and the methyl ester of the methyl glycoside reduced with sodium borohydride (or borodeuteride). Both these approaches permit identification of the uronic acid components, which can be distinguished unambiguously from other hexose derivatives if deuterium labels are used. Problems arising from base-catalysed degradation of uronic acid containing polysaccharides during Hakomori methylation have been reported[56] and can be avoided by conducting methylation analysis on the carboxyl-reduced polymer.

The location of alkali-stable substituents such as pyruvic acid acetals can be determined by comparison of the methylated products of the original and selectively de-pyruvylated polysaccharides, whereas the positions of alkali-labile groups (e.g. *O*-acetate) may be deduced from methylation achieved under acidic conditions[57] or following conversion of unsubstituted hydroxyl groups to methoxyethylacetals on reaction with methyl vinyl ether[58-60].

Sequence analysis of polysaccharides requires the specific degradation of the original polymer into fragments which are examined by chromatographic and spectroscopic techniques, coupled to methylation analysis and further depolymerisation or degradation processes. The degradation procedures used which have been extensively reviewed[38,61-63], involve acid-, alkali- or enzyme- catalysed bond cleavages, the last

-mentioned method forming the basis of the present study (see 1.4). The substrate can be either the native polysaccharide, or derivatives thereof formed by reactions which include periodate oxidation, acetylation, methylation, depolymerisation (hydrolysis, methanolysis or Smith degradation) and the reaction or introduction of specific functional groups. The success of these approaches relies upon the separation and identification of the derived oligosaccharide fragments obtained; this has been greatly facilitated by the use of h.p.l.c.-m.s. (e.g. in the characterisation of per-*O*-alkylated saccharide-alditols by Albersheim *et al.*[64-67]). If this technique is not available, as in the present study, the oligosaccharide products can be separated on a preparative scale by paper, thin-layer or steric-exclusion chromatography, then characterised as previously described or separated and identified on the analytical scale (e.g. g.l.c.-m.s. of per-*O*-alkylated di-, tri- and tetra-saccharide alditols[68-71]).

Mass spectrometry (m.s.) is used to identify monosaccharide derivatives (e.g. p.m.a.a.'s), but is also important in the characterisation of oligosaccharides[38,45,71-76], particularly as permethylated derivatives, prior to hydrolysis and g.l.c.-m.s. of the derived alditol acetates. Ionisation is most frequently achieved by electron-impact, as in this study, although softer forms of ionisation [e.g. chemical-ionisation (c.i) and fast-atom-bombardment (f.a.b.)] are used to gain information about molecular ions of higher mass[38,77-79]. Recently the application of positive- and negative- ion f.a.b.-m.s. has been extended to the analysis of underivatised oligomers and oligosaccharide derivatives of high molecular weight (~6000). The sensitivity of this method is sufficient to permit characterisation and location of substituents (e.g. acyl and sulphate), and sequence assignment of permethylated and peracetylated oligosaccharide derivatives at levels down to a few micrograms[80-82].

The nomenclature used in the present study is that of Kochetkov and Chizhov[72], as modified by Kováčik *et al.*[83,84]. Three types of e.i.-m.s. fragment ions, the A, J and the alditol-cleavage (ald) series, provide most of the information required for sequence determination[38,71-76]. The major and most useful fragments belong to the A-series, formed by fission of the glycosidic linkages and subsequent elimination(s) of methanol, where this is possible[76], while fragments obtained by fission within the alditol moiety are of low intensity and unlikely to be detected for derivatives of trisaccharides or higher oligosaccharides[73].

The studies discussed so far enable determination of sugar composition, linkages and sequence in saccharides, but do not reveal the anomeric configuration of the constituent sugar units. This can be established by chemical means, e.g. chromium trioxide oxidation of peracetylated oligo- and polysaccharides in acetic acid[85] (β-pyranosides are more rapidly oxidised than α-pyranosides, whereas acetylated furanoids are oxidised irrespective of their anomeric configurations). Alternatively physical methods, the involving either optical rotation measurements or n.m.r. studies[86] (see Chapter 3), can be used.

Finally, in order to understand the biological and physical properties of oligo- and polysaccharides, their conformation in solution must be elucidated. This may be determined by n.m.r investigations (see 3.3.5, 3.3.6) and substantiated by solid state studies and calculations[87,88].

As exemplified by the investigations described in this thesis, the structural characterisation of saccharides can follow almost entirely from spectroscopic studies, without recourse to the classical methods of carbohydrate analysis apart from initial identification of the

constituent sugars and their absolute configurations. In particular, n.m.r. spectroscopy, including 2D methods, has proved to be of crucial importance in the structural delineation of saccharides, and this topic is therefore discussed in detail in Chapter 3.

1.4 BACTERIOPHAGE DEGRADATION OF CAPSULAR POLYSACCHARIDES

The specificity afforded by enzymic degradation of polysaccharides makes this a useful tool in the structural determination of polysaccharides[38]; however, the substrate specificity required by these *endo* enzymes limits their application to the study of complex heteropolysaccharides[63]. Investigations of this nature have been revolutionised by the recognition and application of virus-associated enzymes that degrade bacterial surface carbohydrates (see review by Geyer *et al.*[89]). For *Klebsiella* capsular polysaccharides, the bacteriophage-associated enzymic activity produces oligosaccharides that correspond to one or more repeating unit(s) of the polysaccharide, all formed by fission at the same relative positions along the chain[90]. This does not necessarily coincide with the biological repeating unit, but the production and modification of these low-molecular weight products may be exploited in immunochemical and related studies. For example, investigations involving the oligosaccharides obtained from bacteriophage Ø2 cleavage of *Klebsiella* serotype K2 polysaccharide have shown that two or more repeating units coupled to suitable protein carriers may serve as immunogens, representative of the corresponding bacterial K-antigen[89]. The main advantage of bacteriophage degradation, apart from the high yield of oligosaccharides obtained, is that labile substituents remain intact during the depolymerisation reaction[38,63,89].

For each of the eighty serotypes of *Klebsiella* there exists a corresponding bacteriophage, fifty-five of which have been isolated and characterised by Rieger-Hug and Stirm[90]. The enzyme activity was found to be highly specific, although some cross-reactivity was observed; in most cases cleavage occured near the acid moiety, but uronic acid was not produced at the reducing end of the oligosaccharide. Degradation of capsular polysaccharides has been reported for *Klebsiella* K2[91], K3[15], K5[92], K6[93], K11[94], K13[95], K14[96], K17[97], K19[98], K21[99], K32[99], K36 (this thesis)[100,101], K39[21], K44[102], K46[103], K54[104], K60[105], K63[106], K64 (this thesis)[107-109], K69[27], K71 (this thesis) and K74[97]. The viral enzymes employed are generally assumed to be glycanases in the sense of promoting the hydrolysis of glycosidic bonds in a manner akin to the acid-catalysed hydrolytic process[94,97]; however, it has now become apparent that eliminative cleavages also occur. Acidic polysaccharides are ubiquitous and so are the lyases (or eliminases) that degrade them[110], but for bacteriophage activities on *Klebsiella* exopolysaccharides this mode of cleavage is nevertheless rare[38,63] and has been reported only for serotypes K5[92], K64 (this thesis)[107-109] and K14[96]. In each case, the lyase action of the bacteriophage splits a trisubstituted mannosyl residue from position 4 of D-glucuronic acid, generating an oligosaccharide terminating in a hex-4-enuronic acid group. Despite the apparent similarity of substrates, no correspondence of reactivity was found between bacteriophage Ø14 and the polysaccharides from serotypes K5 and K64[111].

The oligomers obtained from bacteriophage cleavage of *Klebsiella* exopolysaccharides are amenable to chemical and spectroscopic studies and are therefore more readily characterised than the original polymer. Consequently the structure and conformation of the exopolysaccharide may then be inferred from analysis of such products[89]. These oligosaccharides, together with derivatives obtained by chemical and enzymic means, are

also useful as standards for spectroscopy (e.g. m.s. and n.m.r.) and chromatography (e.g. calibration of steric-exclusion columns). Their application as artificial antigens has already been noted.

Isolation of the product of bacteriophage degradation of the capsular polysaccharides of *Klebsiella* serotypes K36, K64 and K71, and subsequent characterisation of the oligosaccharides constitutes the major part of the study reported in this thesis. A minor contribution, involving structural studies of the extra- and intra- cellular polysaccharides produced by the anaerobic bacterium *Clostridium acetobutylicum*, is presented in the Appendix.

CHAPTER 2

GENERAL EXPERIMENTAL CONDITIONS

2.1 PREPARATION OF CAPSULAR POLYSACCHARIDES

The isolation and purification of capsular polysaccharides produced by *Klebsiella* bacteria has already been discussed (see 1.2) and therefore only further details concerning the media used will be presented here. The liquid nutrient broth contained 5g Bactopeptone, 3g Bacto beef extract, and 2g NaCl per litre of water. The nutrient agar plates were made using a solution of nutrient liquid broth to which 15g of agar per litre had been added; 8.5 cm disposable plastic plates were used. Large scale production of capsular material was conducted in steel trays (45 x 32 x 5 cm) containing media A or B. Medium A contained: 30g sucrose, 2g NaCl, 2g yeast extract, 1g K_2HPO_4, 0.25g $MgSO_4.7H_2O$, 0.2g $CaCO_3$, and 15g agar per litre of water; whereas medium B contained Muller-Hinton broth (6g meat infusion, 17.5g casein hydrolysate and 1.5g starch) in a litre of water to which 2g NaCl, 30g sucrose and 15g agar were added. The media were sterilised by autoclaving at 121°C for 15-20 min.

2.2 PROPAGATION OF BACTERIOPHAGE CULTURES

The large quantities of bacteriophage (phage) required for the depolymerisation experiments were obtained using published procedures[94], except that the phages were propagated in nutrient broth, instead of a synthetic medium. The phage samples, isolated from sewage by Professor S. Stirm, were used to generate stock solutions in broth by plate lysis (for Ø36 and Ø64) or using the soft-agar overlay method (for Ø71).

Before conducting phage propagation experiments, the relationship of bacterial cell count to optical density (600nm) was established as follows. A flask of sterile broth (50mL) was inoculated with actively growing bacteria (5mL) and shaken vigorously at 37°C. Aliquots (3mL) were removed at 0.5h intervals, the optical density (broth blank) was read, and serial dilutions (x 10^2) were prepared in broth. A small quantity (0.1mL) of each of the appropriately diluted solutions was layered on to nutrient-agar plates and incubated overnight at 37°C. Individual bacterial colonies could then be counted and a curve constructed to relate bacterial count to optical density. This plot was used to monitor bacterial growth and lysis, and to ensure the addition of sufficient phage [usually 3 plaque-forming units (p.f.u.) per bacterial cell] during phage propagation.

The preparation of phage was conducted in tubes (3mL broth), small flasks (50mL broth) and finally large flasks (400mL broth), until 10^{13} p.f.u.'s were obtained (considered sufficient to depolymerise more than 1g of polysaccharide[94]). At each stage lysis was observed as 'clearing' of the turbid bacterial solutions and recorded as a fall in optical density. Incubation was usually continued for 1h after lysis; chloroform was then added to stop bacterial growth, the lysate was centrifuged to remove cell debris, and the resulting phage solution was assayed. For the phage assay a series of dilutions were made (usually in 0.9% NaCl in water instead of broth), and the phage potency in each diluted solution was determined, either by addition of a drop of known volume to a lawn of bacteria and counting of the p.f.u's obtained or by using the more accurate soft-agar overlay technique.

Because the purification procedures described in reference 94 usually result in poor recovery of the purified phage, the simple direct method suggested by Dutton *et al.*[97] was followed. The crude phage solutions (~1.2L) were used directly as the "reagent", after

concentration by rotary evaporation (to 50-100mL) and dialysis against tap water for 1-2 days. No loss in phage potency was detected.

2.3 PREPARATION OF OLIGOSACCHARIDES BY THE ACTION OF BACTERIOPHAGE-BORNE ENZYMES ON KLEBSIELLA CAPSULAR POLYSACCHARIDES

The depolymerisation experiments and subsequent isolation of the derived oligosaccharides were conducted using the simple procedures described by Dutton *et al.* (method 4)[97]. The phage solutions (see 2.2) were stirred with polysaccharide at 37°C in the presence of chloroform (3mL). The polysaccharide was added as a solid or as a concentrated solution in water, thereby ensuring a high concentration of polymer, a factor known to improve the yield of the monomer of the repeating unit (**P1**)[97]. After 3-5 days the reaction mixture was concentrated, then dialysed against distilled water (3 x 600mL). The dialysates were combined, concentrated and freeze-dried. The oligosaccharide product was analysed by steric-exclusion (column 2; see 2.6) or thin-layer chromatography (solvent F; see 2.4). Separation of the carbohydrate from contaminants was effected by successive passages through columns of Amberlite IR-120 (H^+) resin or using preparative chromatography. The oligosaccharides (100-300mg) were isolated and purified by preparative steric-exclusion chromatography (on columns 3, 4, or 5) and in one instance, by partition column chromatography on silica gel (solvent F).

2.4 PAPER AND THIN-LAYER CHROMATOGRAPHY (P.C. AND T.L.C.)

The following solvent systems were used: (all v/v)

A. Ethyl acetate-pyridine-water (8:2:1);

B. Ethyl acetate-pyridine-water (10:4:3);

C. 1-butanol-acetic acid-water (2:1:1);

D. Ethyl acetate-acetic acid-formic acid-water (18:3:1:4);

E. 1-butanol-ethanol-water (4:1:5, upper phase);

F. Chloroform-methanol-water (20:20:7);

G. Chloroform-methanol (97:3).

Analytical p.c. was performed on sheets of Whatman no. 1 paper and t.l.c. on Merck aluminium sheets coated with silica gel 60F, thickness 0.2 mm.

Preparative p.c. was performed on sheets of Whatman 3 MM paper, pre-washed with deionised water then dried. The sample was dissolved in water and applied in a thin band near the top of the sheet. After elution of the paper sheets and subsequent location of the components, the bands were cut from the sheets. The components were isolated by extraction with deionised water and concentration of the extract to a small volume followed by freeze-drying.

Reagents and techniques for detecting components separated by p.c. and t.l.c. were as follows:

1. Spraying with a solution of *p*-anisidinium hydrochloride in aqueous 1-butanol, followed by heating at 110°C for 5-10 minutes;

2. Dipping the paper successively through (a) a solution (0.6%) of $AgNO_3$ in acetone and, after drying (b) a solution of NaOH (2%) in ethanol;

3. Underivatised carbohydrate was detected on t.l.c.by spraying with *p*-anisaldehyde-sulphuric acid-ethanol (1:1:18, v/v) followed by heating at 110°C for 5-10 minutes;

4. Permethylated carbohydrate was detected on t.l.c. using the conditions of 3, after spraying with *p*-anisidinium hydrochloride (2%) in sulphuric acid-ethanol (1:20).

2.5 GAS-LIQUID CHROMATOGRAPHY (G.L.C.)

G.l.c. analysis of alditol acetates or partially methylated alditol acetates (p.m.a.a.'s) was carried out using a Carlo-Erba 4200 gas chromatograph coupled to a Columbia

Supergrator 3A integrator for quantitative analysis. It was fitted with one of the following columns:

column A: glass (2m x 3mm i.d.) column packed with 3% OV-225 on Chromosorb W-HP, 80-100 mesh;

column B: as A but with OV-17 (3%) as the liquid phase;

column C: fused silica capillary DB-225 (30m x 0.32mm i.d.), film thickness 0.25 microns (J & W Scientific, Inc.);

column D: glass capillary OV-225 SCOT (25m x 0.35mm i.d.).

The carrier gas was helium. Mixtures of alditol acetates were analysed on column A, isothermally at 220°C, or on column C isothermally at 215°C. Mixtures of PAANs were analysed on column B, isothermally at 220°C. Mixtures of p.m.a.a.'s were analysed on columns A and D, isothermally at 170°C or on column C isothermally at 190 or 215°C. Columns C and D were used with a splitter injection system. A flame-ionisation detector was employed. For g.l.c.-m.s., an identical gas chromatograph was coupled, through a jet separator, to a VG Micromass 16F mass spectrometer, and columns A, B and C were used.

Components in mixtures analysed were identified by comparison of relative retention times with those of standards run under identical conditions, by co-injection of standards in some cases, and by g.l.c.-m.s. Quantitative analyses were achieved by using the molar response factors of Sweet *et al.*[112] for mixtures of p.m.a.a.'s and empirically determined molar response factors for mixtures of alditol acetates.

2.6 STERIC-EXCLUSION CHROMATOGRAPHY (S.E.C.)

Average molecular weights (M_w) were estimated by steric-exclusion chromatography[113] on the following columns:

column 1: Sepharose 4B (60 x 0.9cm; eluent M NaCl);

column 2: Bio-Gel P-2 (55 x 2.5cm; eluent M NaCl);

Other columns used are described in the text.

Samples (1-2mg) were dissolved in eluent (1mL) before being applied to columns, and fractions (1-1.6mL) of the column effluent were collected. The emergence of carbohydrate in the fractions was monitored by the phenol-sulphuric acid method[114].

Preparative s.e.c was performed on the following columns:

column 3: Bio-Gel P-2 (92 x 2cm; eluent water);

column 4: Trisacryl GF05 (20 x 1.2cm; eluent water);

column 5: Trisacryl GF05 (75 x 2cm; eluent 0.1 M pyridinium acetate buffer at pH 5.0).

Fractions were monitored by the phenol-H_2SO_4 assay, polarimetry, or t.l.c. (solvent F).

2.7 GENERAL CONDITIONS

Optical rotations were measured from aqueous solutions for underivatised samples, and from chloroform solutions for methylated products, at 20°C (± 3°C) on a Perkin-Elmer Model 141 polarimeter. Infra-red spectra of methylated derivatives in chloroform were recorded on Perkin-Elmer Model 237 or 983 spectrophotometers.

Ultra-violet absorbance spectra were recorded with a Beckman UV 5260 spectrophotometer.

90, 200 and 500 MHz ^{1}H-n.m.r. and 22.6, 50.3 and 125.7 MHz ^{13}C-n.m.r. spectra were obtained on Bruker WH-90, Varian VXR-200 and Bruker WM500 spectrometers respectively. Samples were prepared for n.m.r. by dissolving them in 99.7% D_2O after freeze-drying 2-3 times from D_2O solutions. ^{1}H-n.m.r. spectra were recorded at 20°C and 80-90°C and ^{13}C-n.m.r. spectra at 25°C. ^{1}H chemical shifts were measured with reference to internal acetone, δ 2.21 (at 90 and 200 MHz) or δ 2.225 (at 500 MHz) downfield from TMS. ^{13}C chemical shifts were measured with reference to internal acetone, taken as δ 31.0 (at 22.6 and 50.3 MHz) or δ 31.07 (at 125.7 MHz) downfield from TMS. All 2D n.m.r. experiments were conducted at 25 or 30°C using the procedures described in section 3.3.7.

Mass spectrometry was performed on a VG Micromass 16F mass spectrometer, operating at 70eV or 20eV.

2.8 SUGAR ANALYSES

Neutral sugars were determined by g.l.c. as alditol acetates, prepared by the method of Albersheim *et al*[115a] or as aldononitrile acetates, as prepared by the method of Morrison[115b]. The proportions of neutral sugars constituting polymeric products were determined by two methods. In Method 1, hydrolysis in 2M TFA at 100°C for 18h or 8h, depending on whether the polysaccharide contained uronic acid or not, preceded conversion to alditol acetates and g.l.c. Results were corrected for degradation of proportions of the sugar residues during the hydrolysis[116].

Method 2 provides for a simultaneous determination of acidic and neutral sugars, following the procedure recommended by Dudman *et al*[117]. A sample of the glycan (1-2mg) was subjected to methanolysis in 1M HCl in dry methanol (1mL) at 100°C for 18h in a sealed glass tube. After cooling, the solution was neutralised with Ag_2CO_3, the resulting suspension was centrifuged and the supernatant liquor was evaporated to dryness. The residue was dissolved in 96% ethanol and $NaBD_4$ (10mg) added. The solution was sonicated for 10 minutes and then stirred overnight (~16h) to effect reduction of methyl-esterified carboxyl groups of uronic acids to 6,6-dideuterio-substituted glycosyl residues. Excess $NaBD_4$ was then decomposed by dropwise addition of glacial acetic acid and the solution was treated with Amberlite IR-120 (H^+) resin for 0.5h before filtration and evaporation of the filtrate to dryness. The residue was dissolved in 2M TFA and heated at 120°C for 1h in a sealed glass tube to hydrolyse the methyl glycosides. After cooling,

TFA was removed by freeze-drying and the aldoses were converted to alditol acetates as before.

Uronic acid was also determined colorimetrically by the method of Blumenkrantz and Asboe-Hansen[118].

2.9 CARBOXYL-REDUCTION OF ACIDIC POLYSACCHARIDES[42]

An aqueous solution of the polysaccharide was adjusted to pH 4.75 and treated with solid 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. During the ensuing reaction, the pH of the mixture was maintained at 4.75 for 1h by titration with 0.05M HCl. The reaction mixture was then treated with a 3M solution of $NaBD_4$, while the pH was maintained at 7.0 by titration with 4M HCl. After 1h the reaction mixture was rendered slightly acidic to destroy excess borodeuteride, and the solution was dialysed exhaustively against distilled water. The product was isolated by freeze-drying. It was necessary to repeat the procedure in order to achieve complete reduction. The reduced product was analysed for sugar composition as described in 2.8.

2.10 METHYLATIONS AND RELATED EXPERIMENTS

Methylations were carried out by the Hakomori method[47] as modified by Phillips and Fraser[49], using as base potassium methylsulphinylmethanide (2M), prepared by addition of dry DMSO to dry KH at 0°C[50].

Polysaccharides containing uronic acid were deionised with Amberlite IR-120 (H^+) resin and freeze-dried prior to methylation. In general, samples were vacuum-dried over P_2O_5 for at least 18h before being dissolved in dry DMSO for methylation, conducted under purified N_2. Contact time with the base ranged from 0.5h-3h, the presence of excess base being confirmed by removal of a drop of the reaction mixture for testing with triphenylmethane[50]. Solutions of the alkoxides were frozen in ice before addition of methyl or trideuteriomethyl iodide, after which solutions were stirred at room temperature for at least 0.5h. Polymeric products were recovered by addition of water and $CHCl_3$, followed by dialysis and recovery of the organic phase in the non-dialysable portion. After evaporation of $CHCl_3$, products were purified by passage through a column of Sephadex LH-20, eluted with ethanol-chloroform (2:1 v/v), the column effluent being monitored for carbohydrate with the anthrone-sulphuric acid reagent[119]. Oligomeric products were recovered from the reaction mixture by addition of chloroform and extraction of salts and DMSO with water. The products were not purified further. Where complete methylation of uronic acid-containing polysaccharides was not achieved, further methylation was carried out by the Purdie method[120].

Reduction of methyl-esterified carboxyl-groups was achieved by the method of Åman *et al.*[65], wherein the reducing agent, lithium aluminium hydride (LAH) or lithium aluminium deuteride (LAD), was heated for 30 minutes at 80°C in dry THF, the suspension was centrifuged and portions of the supernatant were added to a solution of the methylated

glycan in dry THF. The resulting solution was heated at 80°C for 18h and the reduced products were recovered by addition of moist ethyl acetate to decompose excess LAH, filtration of the suspension through Celite, washing of the residues with $CHCl_3$ and, finally, evaporation of the combined filtrate and washings to dryness.

Completeness of methylation and reduction procedures were assessed by recording i.r. spectra to monitor the disappearance of the hydroxyl and carbonyl stretches respectively.

Base-degradation of methylated uronic acid-containing polysaccharides was carried out by the method of Lindberg and Lönngren[121]. The methylated polysaccharide, previously dried over P_2O_5, was dissolved in dry DMSO containing 5% (v/v) of 2,2-dimethoxypropane and a trace of *p*-toluenesulphonic acid in a serum vial sealed with a rubber cap. The solution was treated with methyl sulphinyl anion (2M) in DMSO. After 18h at room temperature the reaction mixture was poured into 10% acetic acid. The solution was extracted with chloroform (3 times), and the combined organic phase was washed with water (3 times) and concentrated to dryness. The residue was suspended in 10% aqueous acetic acid and the suspension was kept at 100°C for 1h, then cooled and freeze-dried. The product was purified by passing through a Sephadex LH-20 column, using $CHCl_3$-acetone (2:1 v/v) as eluent.

Methylation analyses were carried out after hydrolysis of the methylated products in 2M TFA at 100°C for 8h or 18h, and removal of TFA by freeze-drying. The hydrolysates were examined in some instances by p.c. (solvent E) using the mixture of partially methylated sugars from *Virgilia oroboides* gum[122,123] as a standard. The aldoses were then converted to p.m.a.a.'s for g.l.c. analysis.

CHAPTER 3

N.M.R. SPECTROSCOPIC STUDIES OF SACCHARIDES

N.m.r. provides a convenient and non-destructive technique for determining the number and nature of the constituent sugar residues of saccharides, and for identification of substituents, e.g. pyruvic acid acetals or *O*-acyl groups[124]. The modern high-resolution multiple pulse n.m.r. techniques[125-129] have greatly facilitated interpretation of the complex spectra obtained for carbohydrates. This is the subject of many reviews and monographs[124,130,131]; generally the protocol involves an attempt to make full assignments of ^{1}H- and ^{13}C- n.m.r. signals which, by comparison with model compounds, indicate the sites of linkage and possibly the conformational properties of the saccharide[132,133]. The latter can be established using coupling constants and relaxation experiments. Since the applications of ^{1}H-, ^{13}C- and two-dimensional (2D) -n.m.r. spectroscopy in the field of carbohydrate analysis have been extensively reviewed, only a brief outline will be given here. The techniques commonly used in practice are illustrated using simple carbohydrate molecules previously isolated in our laboratory. These compounds served as models for the n.m.r. studies of the higher oligosaccharides obtained from the *Klebsiella* polysaccharides, and also initiated the use of 2D n.m.r. methods in our research programmes.

3.1 ONE-DIMENSIONAL ^{1}H-N.M.R. SPECTROSCOPY

In ^{1}H-n.m.r. spectroscopy the following spectral characteristics are measured: a) chemical shifts, b) coupling constants, c) the relative integral intensities of signals, and d) relaxation times (T1 and T2). Care should be exercised when comparing spectral data of different compounds as they can be affected by solvent, temperature and in some cases, pH[124].

3.1.1 CHEMICAL SHIFTS OF PROTONS

The chemical shift value (δ) depends on the electronic and geometric environment of the proton and is usually expressed relative to tetramethylsilane (TMS) although determined using an internal water-soluble reference compound such as sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS) or acetone[124]. The chemical shift scales of TMS and DSS differ by ~0.5 p.p.m. and so corrections should be applied when comparing spectra having different reference compounds[134]. Three main regions can be distinguished: (i) the anomeric region (δ 5.5-4.9 for α-anomers and δ 4.9-4.3 for β-anomers)[135]; (ii) the ring proton region (δ 4.5-3.0); and (iii) the high field region where the methyl groups of 6-deoxy sugars (δ 1.3)[124], pyruvate (~δ 1.5, depending on R or S stereochemistry of the acetal)[136] and acetate (δ 2.2-2.0)[124] appear. Certain ring proton resonances may appear at low field (δ 4.0-4.5), e.g. H-5 of α-uronic acids[137] and H-2's of sugars having the *manno* configuration (mannose[135] and rhamnose[138]).

3.1.2 COUPLING CONSTANTS

The coupling constant parameter (J) arises from spin-spin coupling of the proton with neighbouring protons. For vicinal protons the relationship of J to the torsional angle (Ø) is given by the Karplus equation[126]. The values are maximum when Ø is 0^{o} or 180^{o} and minimum at Ø of 90^{o} and therefore the measurement of J allows the determination of configuration and/or conformation of carbohydrate molecules[139]. If the protons are

transdiaxial a splitting of ~8-12 Hz is observed, whereas equatorial-axial or equatorial-equatorial protons have smaller coupling constants of ~1-6 and ~1-3 Hz respectively[124,139]. The effect of the relative orientation and electronegativity of substituents on the magnitude of J for pyranose rings can be predicted by additivity rules given in reference 140.

3.1.3 SIGNAL INTENSITY AND RELAXATION TIMES OF PROTONS

Under proper operating conditions[129], the relative intensities of signals due to different protons may be correlated with the relative amounts of nuclei producing the signals, thus permitting rapid quantitative analysis of the ratio of α- to β-anomers, the number of 6-deoxy sugars and of substituents such as acyl groups. Relaxation times (T1 and T2) are a measure of the relaxation environment (i.e. proximity of other magnetic nuclei and molecular motion). The spin-lattice relaxation time (T1) of a proton is more readily determined than the spin-spin relaxation time (T2). Proton T1 values have been used for assessing conformations of oligosaccharides and determining their sequence[141-144].

3.1.4 EXPERIMENTAL CONDITIONS

For ^{1}H-n.m.r. studies of carbohydrates, the samples are treated several times with deuterium oxide (D_2O) to prevent interference from protons which are exchangeable, and

the spectra are recorded in high-purity D_2O. Despite the exchanges, a residual HOD peak and sidebands are always observed in the anomeric region of the spectrum (~δ 4.8 at ambient temperature). This interference, exacerbated when low concentrations (.1mM) of sample are used, may be eliminated by recording the spectrum at 80-90°C. This shifts the HOD peak upfield (~δ 4.2) and also improves spectral resolution by reducing the viscosity of the samples. The disadvantage of this procedure is that acid-labile units may be released. If such units are present, it is preferable to suppress the solvent peak by alternative methods, for example by using suitable pulse sequences either alone (e.g. saturation decoupling[124]) or together with the addition of paramagnetic relaxation reagents[145].

3.1.5 CHEMICAL STRUCTURE DETERMINATION USING ^{1}H-N.M.R.SPECTROSCOPY

The information afforded by a one-dimensional (1D) ^{1}H-n.m.r. spectrum is usually restricted to identification of the anomeric signals (relative number and nature of the constituent sugar residues) and the high field signals (6-deoxy sugars and non-sugar substituents). Ring proton assignments, which may reveal glycosylation shifts and hence permit linkage assignments, are usually unobtainable, although some signals can be identified using spin-decoupling experiments. The spectra of polysaccharides are characterised by signal broadness (due to short proton relaxation times) and strong HOD resonance. Despite the fact that both these factors are improved by recording the spectrum at 80-90°C, little can be established about the nature of the sugar residues

present (i.e. J values and substitution pattern), although their anomeric configuration can usually be determined on the basis of chemical shift.

In the case of simple saccharides such as homopolymers (e.g. dextrans and mannans) and families of related heteropolymers, ^{1}H-n.m.r. spectroscopy has been used to establish the nature and the proportions of different glycosidic linkages present, although ^{13}C-n.m.r. analysis is more widely used because of better signal separation and the fact that signals other than those of the anomeric region can also be examined[62,124]. High-resolution ^{1}H-n.m.r. studies have proved to be of particular use in characterising the primary structure of glycopeptides[146,147]. Analysis of the spectral data at 360[146] and 500 MHz[147] for H-1 and H-2 of glycopeptides and their subunits has led to the identification of many well-defined chemical shift values for specific 'micro-environments'. These 'micro-environments' span several hexose residues and therefore their identification by 1D ^{1}H-n.m.r. analysis leads to the delineation of both the sequence of the sugars and the inter-sugar linkages within the glycopeptide[146].

The fact that the capsular polysaccharides of *Klebsiella* bacteria give interpretable spectra confirms that these heteropolysaccharides are composed of regularly repeating oligosaccharide sub-units (the size of which can usually be estimated by integration of peaks in the anomeric region)[124,148]. Despite the vast amount of published information relating chemical shift to structure, the delineation of these polysaccharides cannot be made solely by 1D ^{1}H-n.m.r. spectroscopy, but usually follows from a combination of chemical and spectroscopic studies of the polysaccharide and products of various degradative studies (e.g. partial-acid or enzymic hydrolysis, or Smith degradation). This is the approach adopted in the present investigation, with the main emphasis being on

structural studies of the oligosaccharides generated by bacteriophage cleavage of the original polymer. The structure of the capsular polysaccharide may then be inferred from characterisation of the derived oligosaccharides.

3.2 ONE-DIMENSIONAL ^{13}C-N.M.R. SPECTROSCOPY

The use of pulse Fourier transform n.m.r. spectroscopy and proton broad band decoupling has increased the sensitivity of natural abundance ^{13}C-n.m.r. techniques to such an extent that it is now widely used in carbohydrate analysis[124,149-155]. The singlets obtained are often far better dispersed than the signals of the corresponding proton spectra, although quantitative aspects of the ^{13}C spectra are generally not as satisfactory as those obtained for protons (mainly due to the nuclear Overhauser effect). Nevertheless the intensity of signals from carbons carrying the same number of protons can be compared if the correct experimental pulsing protocol is adopted[150].

3.2.1 ^{13}C CHEMICAL SHIFTS

The main parameter used for assignment of the ^{13}C spectra is the chemical shift (δ). ^{13}C-n.m.r. spectra of underivatised carbohydrates are usually run in D_2O and are referenced relative to TMS (external), DSS or a volatile internal standard, e.g. acetone, 1,4-dioxane or methanol. As in ^{1}H-n.m.r. studies, caution should be exercised when

comparing δ values of different compounds as they are known to be affected by changes in solvent, temperature (e.g. up to 0.015 p.p.m./degree for methyl α-D-glucopyranoside in D_2O) and pH (if acidic or basic functional groups are present)[124,153]. Carbohydrate spectra are recorded from 0-200 p.p.m. and four main regions can be distinguished; (i) carboxyl and carbonyl groups (~δ 170), (ii) anomeric carbons (δ 110-90), (iii) the remainder of the ring carbons (δ 80-65) and the primary alcohols (δ 65-60); and (iv) methyl groups of acetates (δ 25-20), pyruvate (δ 25-17 depending on the acetal stereochemistry)[136] and 6-deoxy sugars (δ 18-16). The anomeric region can be further divided depending on whether the sugar is free (α-pyranoses δ 90-95; β-pyranoses δ 95-98) or linked (α-pyranoses δ 98-103, β-pyranoses δ 103-106 and furanoses δ 106-109)[124,150-155].

3.2.2 CHEMICAL SHIFT ANALYSIS OF SACCHARIDES

The anomeric region is of greatest importance in the 1D analysis of saccharides as δ of an anomeric carbon (C-1) is related to the configuration and substitution of that sugar residue, and the number and intensity of the anomeric signals are indicative of the relative proportions of the constituent sugars. Generally the resonances of carbon atoms involved in linkages are shifted downfield (α-effect, 4-10 p.p.m.), while the adjacent carbon atoms are slightly shielded (β-effect). These shifts are less variable than those obtained from ^{1}H-n.m.r. , in which glycosylation of a ring hydroxyl group generally leads to a downfield shift of the geminal proton signal (~0.2 p.p.m.) and also of the signals due to protons adjacent to it (~0.15 p.p.m.) depending on stereochemistry and the nature of the substituent, and can be predicted using a set of empirical rules[155]. These empirical

regularities have been explained in terms of the spatial proton-proton interactions within the preferred conformation near the glycosidic linkage[156]. Although the validity of extending these rules to saccharides larger than trisaccharides is not known, the large body of ^{13}C shift values available[152-157] makes it possible to determine the positions of *O*-glycosylation in oligo- and polysaccharides. The use of ^{13}C-n.m.r. for examining the degree of regularity of sequences in polymers containing different types of linkages and/or sugar residues has been demonstrated for linear and branched homopolymers (e.g. mannans and dextrans) and heteropolymers (e.g. galactomannans, glycosaminoglycans, sulphated poly-saccharides etc.)[124,151,152]. From the ^{13}C-n.m.r. spectra of *Klebsiella* polysaccharides, the size of the repeating unit and possibly the anomeric configurations of the constituent sugars can be determined; however, the complexity of multi-residue saccharide spectra requires that degradative experiments and other chemical methods be applied in addition to n.m.r. studies, for a complete structural characterisation of these compounds.

3.2.3 RELAXATION TIMES

The other spectral parameters obtained from ^{13}C-n.m.r. spectroscopy are relaxation times (T1 and T2) and coupling constants. Spin-lattice relaxation times (T1) vary with orientation and relative rates of rotation of C-H dipoles in the structure[158]. ^{13}C T1 values have been used as a sequencing tool in oligosaccharide structural analysis (e.g. of stachyose, in which the ^{13}C nuclei of the internal D-galactosyl residue have more restricted motion and hence shorter T1 values than the same nuclei of the terminal D-galactose group)[124,151]. Another

application has been the differentiation between resonance signals of side-chain and main chain residues of homopolymers (e.g. glucans, dextrans and mannans), which are difficult to assign on the basis of chemical shift alone[124,151]. The paucity of T1 data available as well as the variation of T1 values with experimental conditions (e.g. viscosity and temperature) have meant that this parameter has not been used for diagnostic purposes in the study of heterosaccharides such as the *Klebsiella* capsular polysaccharides. Spin-spin relaxation times (T2) are difficult to measure and have even less applicability in the structural investigation of these polysaccharides.

3.2.4 COUPLING CONSTANTS

Coupling constants vary with the geometry of bonds linking the coupled nuclei. ^{13}C-^{13}C couplings are not observed for compounds with ^{13}C at natural abundance levels (1.1%), although measurements have been made using isotope-enriched sugars, e.g. for methyl β-lactoside[158]. The establishment of carbon-carbon connectivity allows the unambiguous assignment of ^{13}C spectra and will be discussed in the next section (3.3). ^{13}C-^{1}H couplings can be across one (^{1}J), two (^{2}J) or three (^{3}J) bonds thus giving rise to complex proton-coupled ^{13}C spectra. Splittings due to interactions with directly bonded protons are much larger than those involving more remote protons and therefore the number of attached protons can be readily determined by gated decoupling (such that the n.O.e. enhancement of signals is still obtained) or off-resonance decoupling[124,151-153]. These methods have been largely replaced by the attached proton test (APT)[159] and other experiments (e.g. SEFT, INEPT and DEPT)[128]. Single bond coupling constants are useful

in determining the anomeric configuration of sugars because $^{1}J_{C\text{-}1,H\text{-}1}$ of the axial anomer is usually ~10Hz greater than that of the equatorial anomer and therefore anomers having small chemical shift differences (e.g. mannose and rhamnose) can be distinguished by their $J_{C\text{-}1,H\text{-}1}$ values[124,151,152,160]. Long-range coupling constants may yield valuable information for molecular identification and conformational analysis, but they are usually difficult to measure. Recently both the assignment and measurement of these couplings have been facilitated by the use of new two-dimensional (2D) n.m.r. techniques[124,151,152,160]. In particular the detection and measurement of the $^{3}J_{C,H}$ across the glycosidic bond identifies both the position and possible orientation of the linkage[124,151,152,158].

3.3 MODERN PULSE METHODS IN N.M.R. SPECTROSCOPY OF SACCHARIDES

The preceding sections have suggested that the complexity of carbohydrate spectra, especially those of multi-residue saccharides, require the use of high-field spectrometers and additional investigative n.m.r. experiments (e.g. selective decoupling) in order to make individual ^{1}H and ^{13}C assignments. The ability to make these assignments has been greatly facilitated by the introduction of two-dimensional (2D) n.m.r. techniques[124-132]. 2D pulse sequences consist of one or more pulses and delays preceding the final observation of the signal. The output is usually in the form of a 2D contour plot with frequency axes F_1 and F_2. Two classes of 2D experiments exist: J-resolved and correlated 2D spectra. The first type is characterized by the F_1 axis containing the coupling information and F_2 the chemical shift information, while in the second both the axes contain chemical shift

information. The connection between the F_1 and F_2 axes is established through scalar coupling or through dipolar coupling. Scalar coupling (homo- or heteronuclear) can be direct, long-range or relayed.

3.3.1 J-RESOLVED AND CORRELATED 2D ^{1}H-N.M.R. SPECTRA

1D ^{1}H-n.m.r. spectra are complicated by the fact that both chemical shift and coupling information appears on the same axis. The homonuclear 2D J-resolved experiment separates this complexity into a plot of chemical shifts (F_2 axis) and coupling constants (F_1 axis). Projection onto the F_2 axis gives the 'fully proton-decoupled proton spectrum' thus yielding accurate proton shift values, whereas the magnitudes of all the ^{1}H-^{1}H couplings follow from the contour plot. This experiment is only applicable to first-order spectra as second-order effects result in spurious peaks.

The number and shift of the protons present having been identified, the coupling scheme of each monosaccharide can be obtained using proton-proton 2D chemical shift correlation spectroscopy (COSY)[161]. The COSY contour plot consists of a complex diagonal with pairs of off-diagonal peaks arising from each pair of scalar-coupled protons. The pattern of coupling for a sugar residue can be traced out using an unambiguous assignment (e.g H-1) as the starting point. The overlap of proton resonances often leads to ambiguities in establishing connectivities in the COSY spectrum. These can be resolved using multiple-relay-COSY[162-165], in which correlations are transferred from nucleus to nucleus within a spin system (see 3.3.7.1, Fig. 5). This has recently been used in the ^{1}H-n.m.r.

study of cellotriose[164]: H-1 of each residue was identified by inspection, H-2 as a cross-peak in the COSY spectrum, and the remaining protons were identified by a series of multiple-relay-COSY spectra. This technique has also been used effectively in 1D n.m.r. analysis to obtain complete sets of δ and J values, the latter not being readily retrievable from the 2D experiment[165].

Another method for investigating homonuclear spin systems is spin echo correlated spectroscopy (SECSY)[127], in which the frequency differences (F_1 axis) are plotted against chemical shift (F_2 axis). Here the normal 1D spectrum appears on the centre line ($\delta = 0$) together with one off-axis cross-peak for each resonance with which it shares a scalar coupling. Proton assignments of spectra containing severe spectral overlap have been made using the homonuclear Hartman-Hahn (HOHAHA) method[166] which provides high-resolution phase-sensitive spectra that display both direct and relayed connectivities. This method has also been used to generate subspectra from which J values can be determined.

3.3.2 ASSIGNMENT OF ^{13}C SIGNALS USING 2D METHODS

The combination of these experiments (e.g. 2D J-resolved and COSY) can provide most of the ^1H assignments required for identification of the sugar residues, their anomeric configuration and possibly their positions of linkage (if the magnitude of the glycosylation shifts is sufficient). In order to confirm these findings and, in particular, establish the sites of *O*-glycosylation, ^{13}C-n.m.r. spectroscopy is necessary. Once the ^1H signals have been

identified, the ^{13}C assignments follow directly from ^{1}H-^{13}C heteronuclear chemical shift correlation spectroscopy (HETCOR)[167]. Alternatively, if the ^{13}C-n.m.r. spectrum is assigned, then the HETCOR experiment can be used to interpret the ^{1}H spectrum. The HETCOR map consists of ^{1}H chemical shifts along the F_1 axis and the ^{13}C chemical shifts along the F_2 axis. The map contains peaks which arise from connectivity between a ^{13}C nucleus and a proton. Various types of connectivity can be investigated using these shift-correlation experiments, e.g. one-bond couplings, long-range couplings or relayed correlation. The relative insensitivity of these experiments has been dramatically improved by the introduction of new ^{1}H-detected ^{1}H-^{13}C correlation maps[166,168,169], suitable for the full spectral analysis of small quantities of oligosaccharides (e.g. 3.5mg sample of a trisaccharide[168]) and polysaccharides (e.g. 10mg sample of *Haemophilus influenzae* type a capsular polysaccharide[169]). The ultimate method of tracing out the carbon skeleton is by using the 2D INADEQUATE experiment which unambiguously identifies the chemical shifts of the ^{13}C atoms that are directly bonded; unfortunately this technique is rarely used because of its poor sensitivity[125-132].

3.3.3 STRUCTURAL INFORMATION GAINED FROM IDENTIFICATION OF ^{1}H AND ^{13}C RESONANCES

Once the complete ^{1}H and ^{13}C assignments of the saccharide under investigation are obtained, comparison with literature values of model compounds should reveal the positions of glycosidic linkages (*cf.* glycosylation shifts) and possibly also information about the sequence and conformation of the constituent sugars. For example, large (0.2-0.5

p.p.m.) deshielding of protons is indicative of nonbonded interactions with oxygen atoms present in other sugar units[132], while ^{13}C shifts can be interpreted according to rules[155,156] from which the anomeric and absolute configuration and possibly the sequence of residues may be inferred.

3.3.4 TWINNING AS AN AID IN SEQUENCING SACCHARIDES

A feature of reducing oligosaccharides is that the anomeric configuration of the reducing end sugar exerts its influence on the spectral parameters of residues in its spatial neighbourhood. This causes twinning of the n.m.r. signals, especially those of the anomeric proton and carbon atoms ; the signal multiplicity and intensity are determined by the end group anomerisation[15,20,98,103,105,147,155,170-173]. The n.m.r. spectrum obtained is therefore a superposition of the subspectra of the different anomeric forms of the oligosaccharide. The twinning is usually due to α,β-pyranose equilibria; however, additional twinning due to the furanose forms of the reducing end group (e.g. for galactose[15], arabinose[171,173]) has been reported. The extent of twinning should reflect the proximity (and position of substitition) of the sugar residue to the reducing end and thus provide insight into the sequence of sugar units in the oligosaccharide. Twinning was found in all the oligosaccharides studied except where the reducing end group was 4-linked (see 3.3.7.4). The presence or absence of a substituent (e.g. pyruvic acid acetal) may influence the n.m.r. signals of the acetalated residue as well as neighbouring units and therefore can also be used as an aid in sequencing saccharides[20,103].

3.3.5 SEQUENCE AND CONFORMATIONAL ANALYSIS USING ^{1}H-N.M.R METHODS

Conformational analysis of oligosaccharides has shown that the *exo*-anomeric effect operates such that the anomeric and aglyconic protons of glycosidic bonds are in virtual van der Waals contact[143,174]. This arrangement is suitable for dipolar coupling and thus n.O.e. experiments can be used to yield intra- and inter-ring couplings. This type of experiment can either be performed in 1D, usually as n.O.e. difference spectroscopy or in 2D (NOESY)[125-131]. The contour map obtained using the NOESY experiment is analogous to the COSY plot, except that the cross-peaks are due to dipolar coupling. Comparison of the COSY and NOESY plots allows identification of J-coupling interference in the n.O.e. experiment[174]. The success of these n.O.e experiments requires that the molecule being examined should experience a measurable amount of n.O.e. and that the pertinent ^{1}H signals are adequately resolved. This technique is therefore not applicable to the n.m.r. studies of polymers of high molecular weight (e.g. *Klebsiella* capsular polysaccharides) because the slow tumbling of these molecules results in large n.O.e. effects, but poor spectral resolution due to signal broadness. In contrast, small molecules experience small n.O.e. effects, although their spectra are well resolved. Compounds of intermediate molecular weight that experience a detectable amount of n.O.e. without losing spectral resolution are most amenable to these studies, e.g. the low molecular weight *O*-polysaccharides of *Salmonella*[174,175]. In addition to establishing the sequence of sugar units in saccharides, the interatomic constraints imposed by data from the n.O.e. experiments have been shown to be consistent with the three-dimensional model obtained

by semi-empirical calculation (HSEA), i.e. the preferred conformation of the saccharide may be deduced using this method[143].

The usual way of sequencing polysaccharides is by examining the oligosaccharide fragments derived from them. These fragments are more amenable to spectroscopic studies than their parent polymers, but experience small n.O.e. effects at high fields. For this reason poor results are obtained in n.O.e. experiments of small molecules (having molecular weight 1000-2000), particularly when the transient 2D NOESY method is used[129]. Despite the difficulties associated with measuring n.O.e.'s and interference from J-coupling, this technique has been used to determine inter-ring assignments of several oligosaccharides[143,176-178]. The problems associated with n.O.e. investigations of small water-soluble molecules can be diminished by using spin-locked n.O.e. spectroscopy[166]; or by derivatisation[179] (e.g. *O*-acetylation) and use of an organic solvent. Another ^{1}H-n.m.r. method used for sequencing is the detection of the scalar coupling ($^{4}J_{H,H}$) between the anomeric and aglyconic protons using the delayed-COSY experiment[131]. This approach is complicated by interference from five-bond coupling. Both these methods rely on ^{1}H-n.m.r. spectroscopy and are therefore often complicated because of severe spectral overlap. The problems associated with spectral complexity and linkage determination can be partly overcome by the use of heteronuclear techniques which enable the superior signal separation power of ^{13}C-n.m.r. spectroscopy to be utilised.

3.3.6 SEQUENCE AND CONFORMATIONAL ANALYSIS USING HETERONUCLEAR N.M.R. METHODS

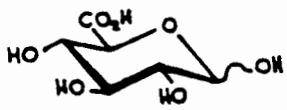
The linkage site can be determined *via* ^{1}H-^{13}C three-bond coupling ($^{3}J_{H,C}$) from the anomeric proton across the oxygen atom to the aglycon carbon atom[124,151,152,158]. Both these signals are usually well-resolved and readily identified (see 3.1 and 3.2). The coupling can be obtained from a long-range HETCOR plot (for example see 3.3.7.4, Fig. 17) or using selective proton-flip or selective heteronuclear decoupling experiments. Recently the selective INEPT experiment has been used to detect and measure intra- and inter-residue long-range ^{1}H-^{13}C couplings[131]. The sensitivity of these methods has been greatly improved by indirect observation of ^{13}C *via* ^{1}H detection[168,169]. The magnitude of coupling across the glycosidic bond relates to the angles (Ø and ψ) which describe the conformation of this bond, but more data are required before this relationship can be quantified[160]. Coupling across the glycosidic bond may possibly be detected also from the heteronuclear analogue of the relay-COSY experiment[128].

3.3.7 APPLICATION OF 2D N.M.R. SPECTROSCOPY TO SOME SIMPLE CARBOHYDRATES

N.m.r. studies of simple carbohydrate molecules (D-glucuronic acid and 2-, 3-, and 4-linked disaccharides), presented below (see 3.3.7.1-3.3.7.4) were conducted on a Varian VXR-200 spectrometer. The samples were examined using the APT[159], COSY[161] and HETCOR[167] experiments; in some cases the relay-COSY[163] and long-range HETCOR experiments were also performed to elucidate ambiguous assignments. Generally the ^{13}C resonances were well resolved and could be assigned on the basis of intensity and chemical shift analysis, whereas the proton signals were poorly resolved and could only be identified using both the COSY and HETCOR experiments. These n.m.r studies served as a useful basis for the 2D n.m.r. examination of higher oligosaccharides and in particular clearly illustrated the phenomenon of twinning (see 3.3.4) which can be used as an aid in the sequencing of oligosaccharides.

3.3.7.1 2D N.m.r. examination of D-glucuronic acid (1)

This study was undertaken in order to obtain proton chemical shift values not available in the literature. These values are required for the purpose of comparison with the chemical shifts obtained for D-Glc*p*A residues present in compounds of interest, so that glycosylation shifts can be determined and hence the mode of substitution of these units established.



1

The proton decoupled ^{13}C-n.m.r. spectrum (Fig. 1) exhibited 10 distinct signals between δ 60-100, which were assigned (Table 1) on the basis of intensity (Iβ/Iα = 3/2) and chemical shift analysis (*cf.* α- and β-D-Glc*p*A-OMe[157]).

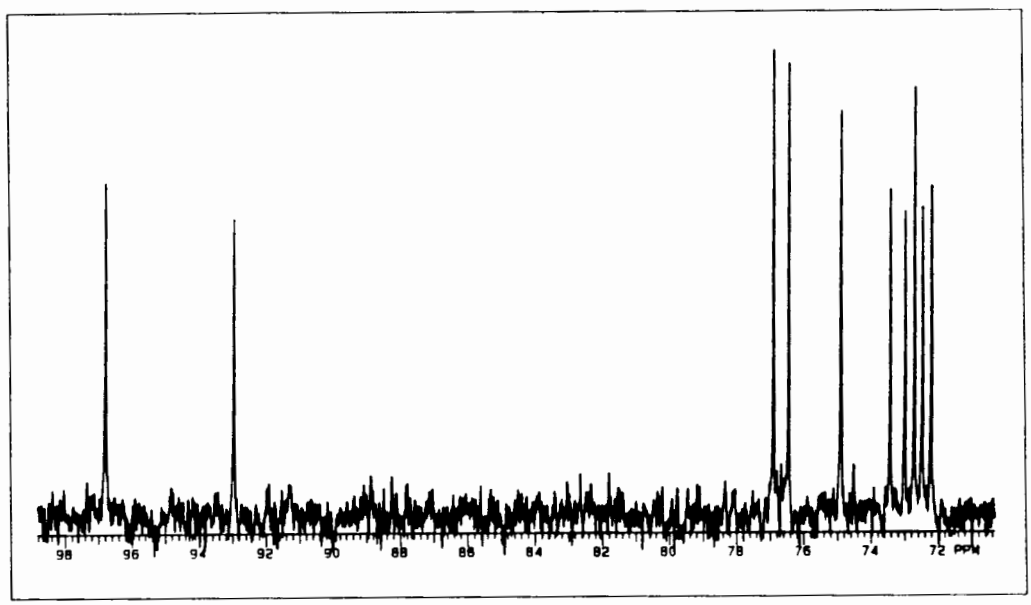


Figure 1: ^{13}C-n.m.r. spectrum (50.3 MHz) of 1 recorded at 25°C.

TABLE 1: ^{13}C-N.M.R. DATA (50.3 MHz) FOR D-GLUCURONIC ACID (1)

Compound	*Chemical shift * (p.p.m.)*					
	C-1	C-2	C-3	C-4	C-5	C-6
α-Glc*p*A-OH	92.95	72.23	73.44	72.99	72.40	178.06
β-Glc*p*A-OH	96.81	74.92	76.46	72.72	76.83	176.75

* Chemical shift relative to internal acetone (δ 31.0)

The ^{1}H-n.m.r. spectrum (see Fig. 2) clearly showed the anomeric signals of α-and β-Glc*p*A-OH at δ 5.39 ($J_{1,2}$ = 3.7 Hz, 0.4H) and 4.76 ($J_{1,2}$ = 7.9 Hz, 0.6H) respectively, while the doublet at δ 4.24 ($J_{4,5}$ = 10 Hz, 0.4H) was attributed to H-5 of α-Glc*p*A-OH.

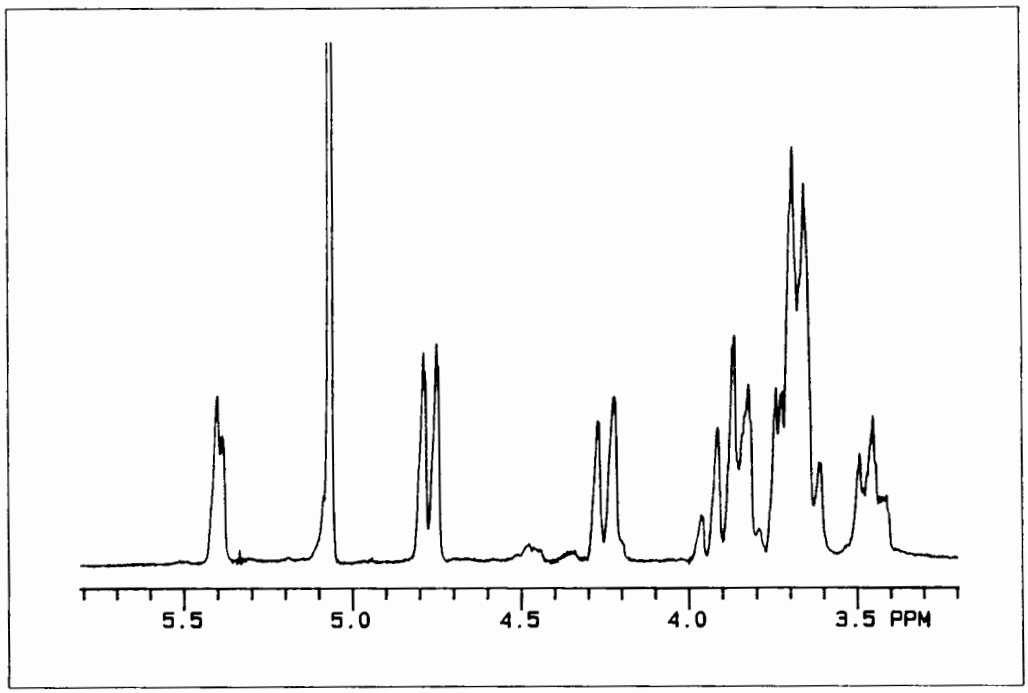


Figure 2: ^{1}H-n.m.r. spectrum (200 MHz) of 1 recorded at 25°C.

These shifts and splittings are in conformity with those reported in the literature[29,180]. Further proton assignments were made using the COSY experiment (Fig. 3) which permitted identification of H-2, H-3 and H-4 of the α-anomer and H-2 and H-3 of the β-anomer by correlations established from H-1 and H-5 of α-Glc*p*A-OH and H-1 of β-Glc*p*A-OH respectively.

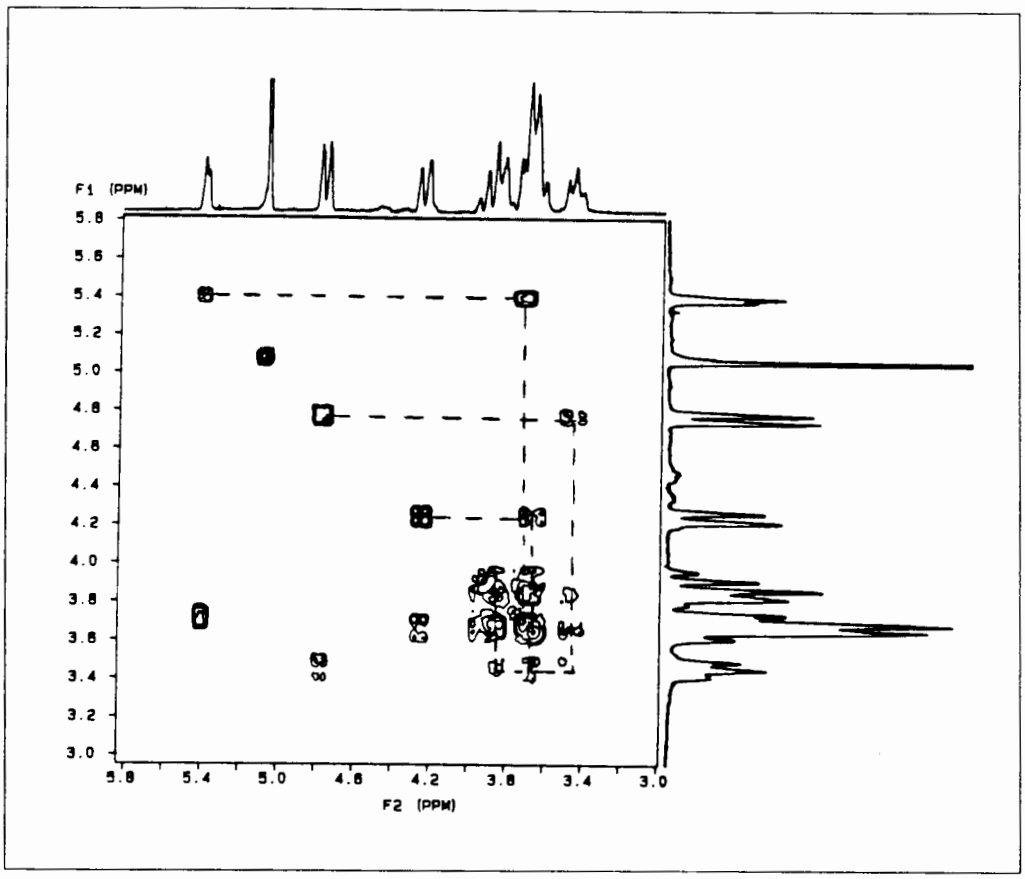


Figure 3: COSY spectrum of **1** recorded at 25°C, some of the spin systems are indicated.

These proton assignments were corroborated by a relay-COSY experiment (Fig. 4; see 3.3.1), but further connectivities could not be made unambiguously and so the remaining identifications followed from the HETCOR diagram (Fig. 5) and are presented in Table 2.

TABLE 2: ^{1}H-N.M.R. DATA[a] (200 MHz) FOR D-GLUCURONIC ACID (1)

Compound	*Chemical shift*[b] *(p.p.m.)*				
	H-1	H-2	H-3	H-4	H-5
α-Glc*p*A-OH	5.39	3.72	3.91	3.69	4.24
β-Glc*p*A-OH	4.76	3.45	3.66	3.69	3.84

[a] Coupling constants measured are given in text
[b] Chemical shift relative to internal acetone (δ 2.21)

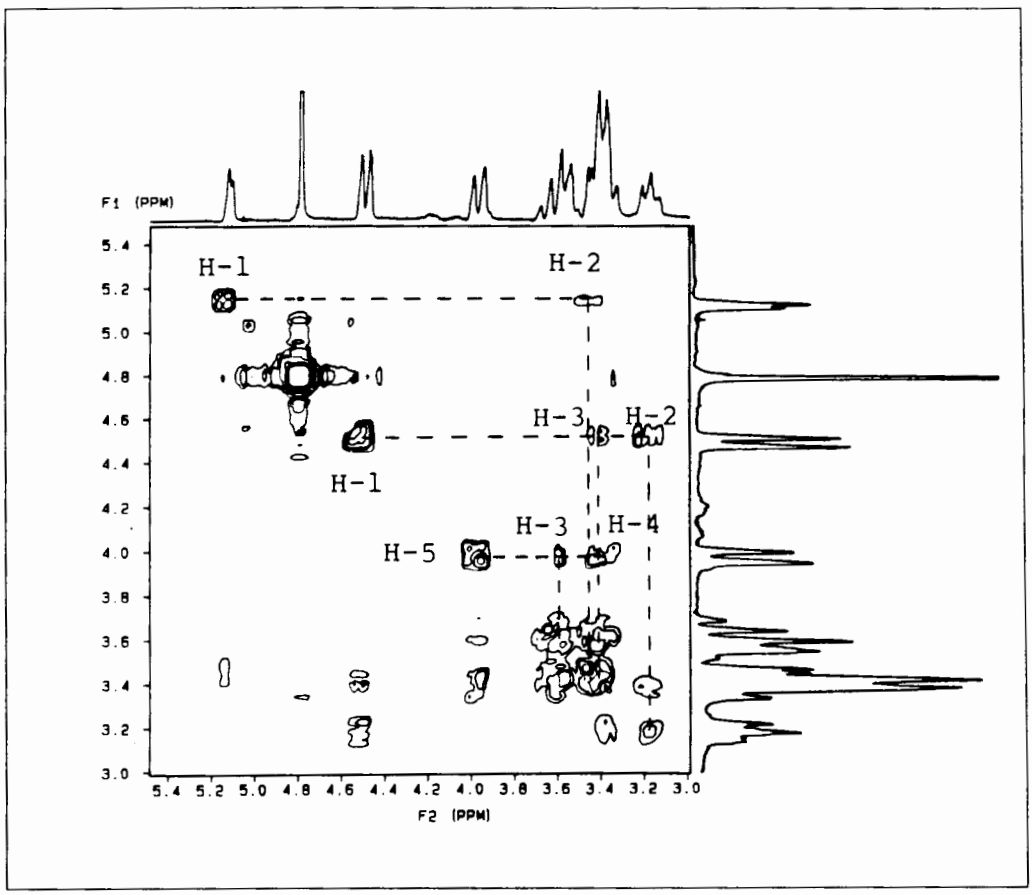


Figure 4: Relay-COSY spectrum of 1 recorded at 25°C, some of the connectivities are shown.

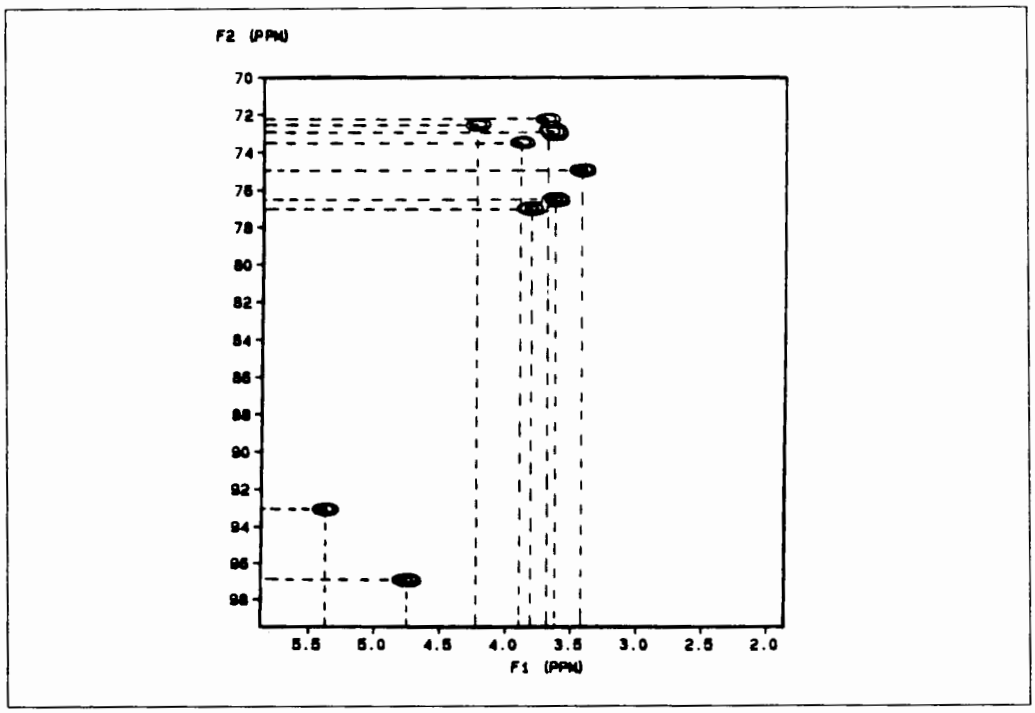


Figure 5: HETCOR spectrum of 1 recorded at 25°C, some of the C,H correlations are indicated ($J_{C,H}$ = 140 Hz).

Use of the HETCOR experiment also resulted in reversal of the assignments of C-3 and C-5 of β-Glc*p*A-OH (δ 76.46 and 76.83), which had been made previously on the basis of chemical shift analysis alone, but now followed from the unambiguous identification of H-3 of β-Glc*p*A-OH (see Figs. 4 and 5).

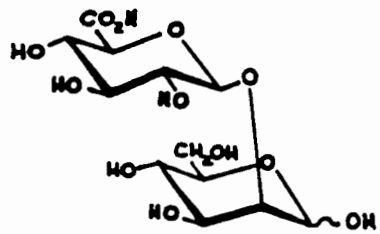
^{13}C chemical shift values reported in the literature[181] were determined using the deuterium-induced differential isotope shift (DIS) technique (pH 7.8, 15 MHz). The results in Table 1 show good agreement with the observed shifts, except that the assignment of C-5 and C-6 of the anomers is reversed (*cf.* δ 76.93 and 72.60, δ 176.85 and 177.62, for C-5 and C-6 of the α- and β-anomers respectively[181]). The C-5 assignments made in the present study (pH~8, 50.3 MHz) are supported by signal intensity (see Fig. 1) and comparison with standards (C-5 of α- and β-Glc*p*A-OMe at δ 71.9 and 75.6 respectively[157]) and follow directly from the unambiguous assignment of H-5 of α-Glc*p*A-OH (see HETCOR diagram, Fig. 5). Consequently the identification of C-5 appears unassailable. This is not the case however for the C-6 assignments, which were made solely by comparison of signal intensity.

Experimental

30mg of D-glucuronic acid (B.D.H.) was lyophilised three times from D_2O, the pH of the final solution being adjusted to ~8 with deuterated pyridine.

3.3.7.2 2D N.m.r. examination of 2-O-β-D-glucuronopyranosyl-D-mannose(2)

The aldobiouronic acid (**2**) was prepared by partial acid hydrolysis of the polysaccharide isolated from *Hakea gibbosa* (obtained by courtesy of Mr. P.F.K. Eagles). The chemical characterization of **2** is reported elsewhere[182]. This compound was studied in order to investigate the n.m.r. parameters arising from O-2 glycosylation of mannose; which is found in the hexasaccharide generated by bacteriophage Ø64 enzymic cleavage of *Klebsiella* K64 capsular polysaccharide (see Chapter 5). This aldobiouronic acid is a common constituent of acidic plant polysaccharides[183] and therefore its spectral characterisation should assist in the structural studies of these heteropolysaccharides.



2

Anomeric proton shifts and splittings of this compound have been previously reported[184] [H-1 of α- and β-D-Man*p*-OH at δ 5.27 ($J_{1,2}$ = 1.1 Hz) and 4.58 ($J_{1,2}$ = 4.4 Hz) respectively, and H-1 of β-Glc*p*A at δ 4.58 ($J_{1,2}$ = 7.5 Hz)]. The accuracy of these assignments must be questioned when compared with the n.m.r. data published for α- and β-D-Man*p*-OH [H-1 at δ 5.25 ($J_{1,2}$ = 1.7 Hz) and 4.97 ($J_{1,2}$ = 1.0 Hz) respectively][185] as both the chemical shift and coupling constant reported for β-Man*p*-OH[184] are unusual. These results[184] are also at variance with the ^{1}H-n.m.r. data more recently published[20] for **2** isolated by partial hydrolysis of *Klebsiella* K35 capsular polysaccharide. During the course of our n.m.r.

investigations the ^{13}C-n.m.r. spectrum of **2** was published[186]. These results are discussed below after the presentation of our findings from the ^{1}H-n.m.r. studies.

The ^{1}H-n.m.r. spectrum of **2** (Fig. 6) clearly showed the presence of α- and β- Man*p* with anomeric proton signals at δ 5.27 (0.75H, $J_{1,2}$ = 1.4 Hz) and 4.96 (0.25H, singlet) respectively.

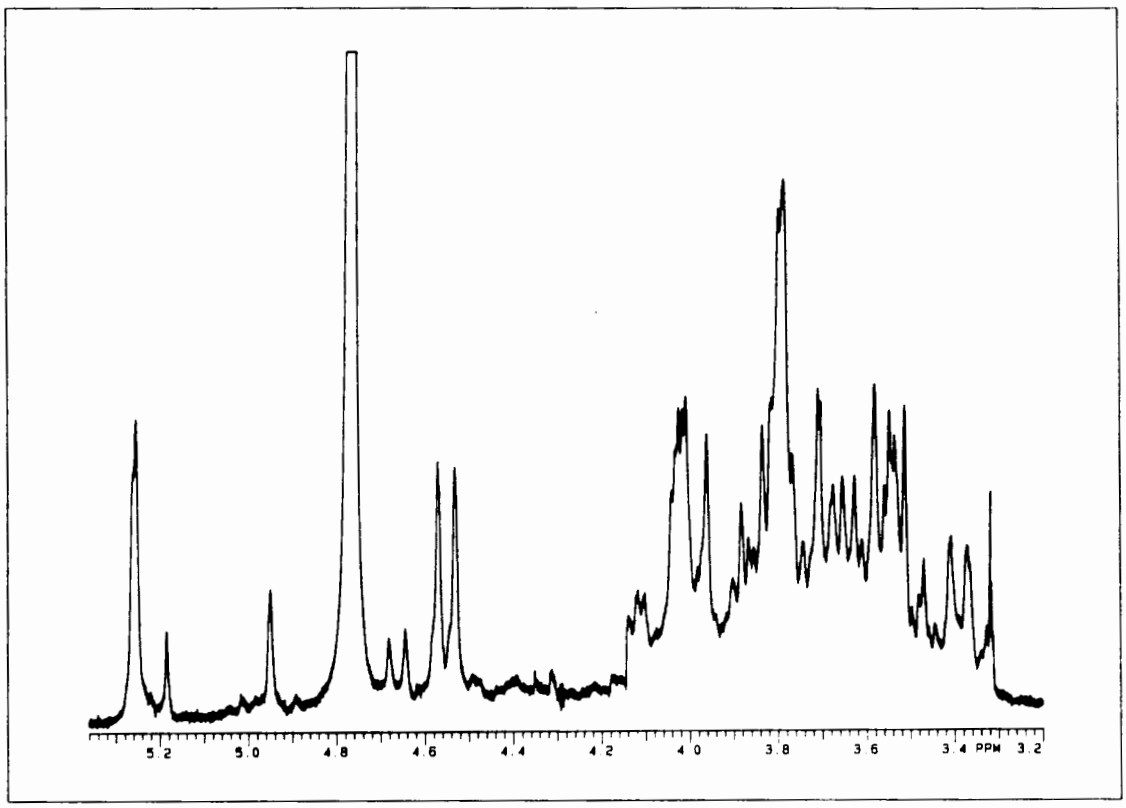


Figure 6: ^{1}H-n.m.r. spectrum (200 MHz) of **2** recorded at 25°C.

These shifts and splitting are in conformity with those reported for the free sugar[185], although the proportion of the α-anomer of **2** is slightly greater than the value quoted for the free sugar (66%)[187]. This increase in the α:β ratio upon O-2 substitution is common (e.g. 2-*O*-methyl-D-mannose contains 75% of the α-anomer) and has been rationalized in terms of an increased anomeric effect[187]. The anomeric signals at δ 4.56 (0.75H, $J_{1,2}$ = 7.6 Hz) and 4.67 (0.25H, $J_{1,2}$ = 8.4 Hz) were assigned to β-Glc*p*A of the α- and β- anomers of the disaccharide respectively, which provides a dramatic illustration of the phenomenon

of twinning (see 3.3.4). The differences in chemical shift between glycosylated residues attached to the reducing sugar anomers is indicative of the different stereoelectronic environments experienced by these residues; consequently, the relatively large shift difference (δ = 0.11 p.p.m.) between the two β-Glc*p*A signals reflects the proximity of the 2-*O*-glycosyl group to the anomeric centre of mannose. These changes in chemical shift (and effect on the α:β ratio) are diminished in the case of (1→3)- and (1→4)- linked disaccharides, as demonstrated in 3.3.7.3 and 3.3.7.4.

The anomeric signals served as the starting point for the COSY experiment (see Fig. 7) which permitted the unambiguous assignment of H-2 and some H-3 resonances (these assignments were confirmed by the use of relay-COSY experiments).

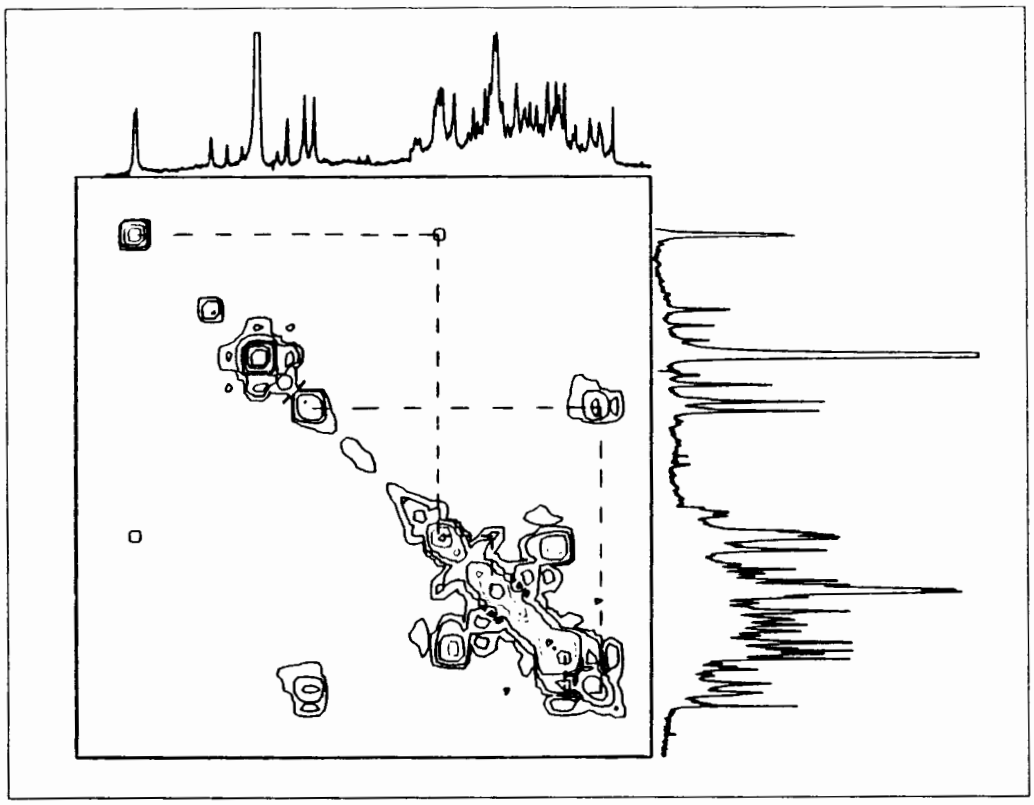


Figure 7: COSY spectrum of **2** recorded at 25°C, some of the spin systems are indicated.

The lack of sensitivity and resolution experienced at this magnetic field did not allow further connectivities to be established with confidence. The remaining proton assignments followed directly from the ^{13}C assignments *via* the HETCOR experiment (see Fig. 8) and are presented in Table 3.

TABLE 3: ^{1}H-N.M.R. DATA[a] (200 MHz) AND GLYCOSYLATION SHIFTS[b] OF 2-*O*-β-D-GLUCURONOPYRANOSYL-D-MANNOSE (2)

Sugar residue	*Chemical shift[c] (p.p.m.)*					
	H-1	H-2	H-3	H-4	H-5	H-6
α-Man*p*-OH[d]	5.27 (+.09)	4.06 (+.14)	3.86 (+.01)	3.65 (-.01)	3.79 (-.03)	3.80 -
β-Man*p*-OH[d]	4.96 (+.06)	4.12 (+.18)	3.48[e] (-.18)	3.65 (+.07)	3.41 +.04)	n.r.
β-Glc*p*A[f]	4.56[g] (-.20)	3.38 (-.07)	3.54 (-.12)	3.58 (-.11)	4.00 (-.16)	-

Glycosylation shifts given in parentheses.

[a] Coupling constants measured are given in text
[b] A downfield shift relative to the model compound is considered positive
[c] Chemical shift values relative to internal acetone (δ 2.21)
[d] Model compound Man*p*-OH (Ref. 143)
[e] Or δ 3.62
[f] Model compound β-Glc*p*A-OH (Table 2)
[g] Twin signal at δ 4.67 (see text)

The glycosylation shifts obtained by comparison of the chemical shift values for 2 with model compounds (see Table 3) were greatest for H-2 of Man*p* thus confirming that O-2 is glycosylated.

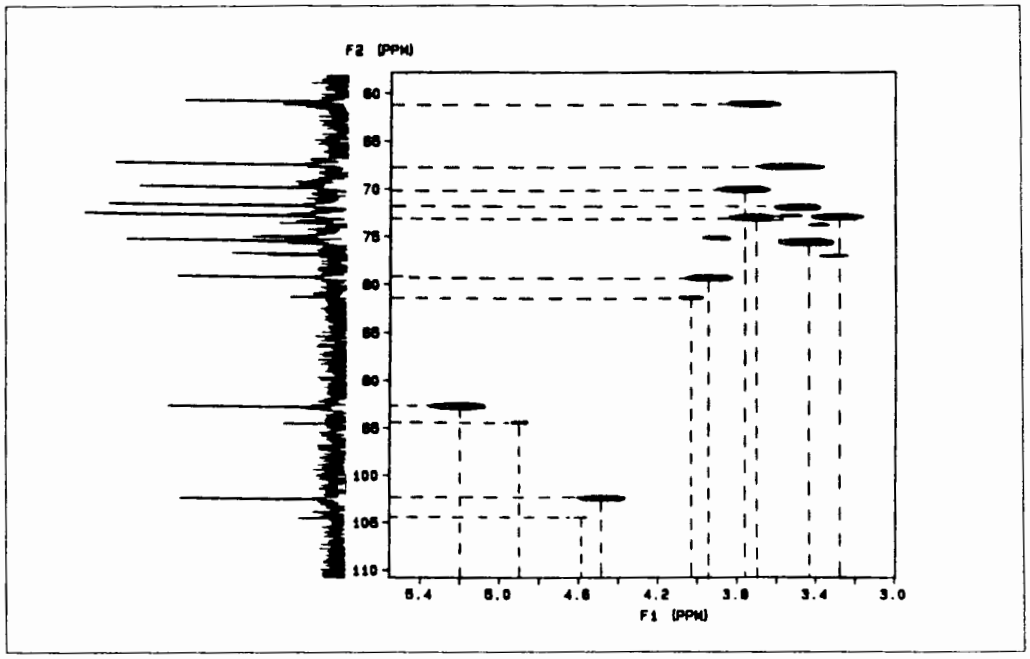


Figure 8: HETCOR spectrum of **2** recorded at 25°C, some of the C,H correlations are indicated ($J_{C,H}$ = 140 Hz).

The ^{13}C-n.m.r. spectrum (Fig. 9) was interpreted on the basis of signal intensity (Iα/Iβ = 0.75/0.25) and by comparison with chemical shifts of the constituent sugars[155] and appropriate derivatives (2-*O*-methyl-D-mannose[155] and methyl-D-glucopyranosiduronic acid[157]).

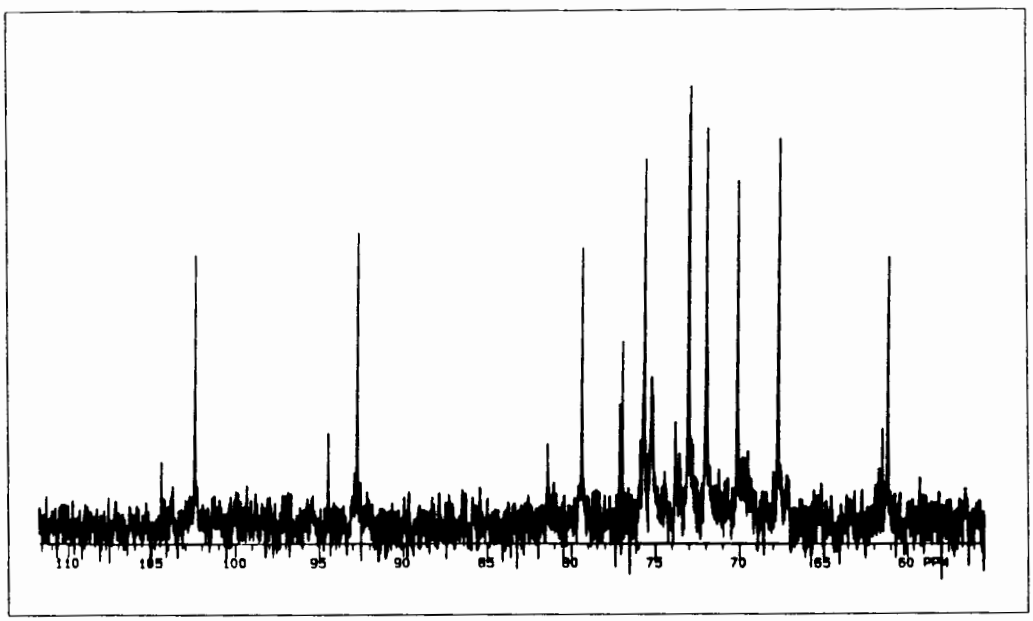


Figure 9: ^{13}C-n.m.r. spectrum (50.3 MHz) of **2** recorded at 25°C.

Some of these assigments were corroborated by use of the HETCOR experiments which established connectivity between the ^{1}H resonances previously identified and the appropriate ^{13}C signals. The ^{13}C-n.m.r. data and glycosylation shifts are presented in Table 4.

TABLE 4: ^{13}C-N.M.R. DATA (50.3 MHz) AND GLYCOSYLATION SHIFTS[a] OF 2-*O*-β-D-GLUCURONOPYRANOSYL-D-MANNOSE (2)

Sugar residue	*Chemical shift [b] (p.p.m.)*					
	C-1	C-2	C-3	C-4	C-5	C-6
α-Man*p*-OH[c]	92.77 (-2.2)	79.39 (+7.7)	70.15 (-1.2)	67.74 (-0.3)	73.06 (-0.3)	61.23 (-0.9)
β-Man*p*-OH[c]	94.47 (-0.1)	81.24 (+8.9)	73.81[d] (-.29)	67.74 (-0.1)	77.11 (-0.1)	61.53 (-0.6)
β-Glc*p*A[e]	102.46[f] (+5.6)	73.05 (-1.9)	75.68 (-0.8)	72.01 (-0.7)	75.24 (-1.6)	-

Glycosylation shifts given in parentheses.

[a] A downfield shift relative to the model compound is considered positive
[b] Chemical shift value relative to internal acetone (δ 31.0)
[c] Model compound Man*p*-OH (Ref. 155)
[d] Or δ 72.89
[e] Model compound β-Glc*p*A-OH (Table 1)
[f] Twin signal at 104.45 p.p.m.

The signals at δ 102.46 and 104.45 are attributable to C-1 of β-Glc*p*A attached to the α- and β- Man*p* respectively, the shift value being characteristic of β-linked residues (see 3.2.1) while the magnitude of the twinning of the signals indicates the close proximity of the anomeric carbon to C-1 of Man*p*-OH, i.e. a (1→2) linkage. This illustrates the high conformational sensitivity of ^{13}C-n.m.r., which is such that the change from an axial to an equatorial hydroxyl substituent at a carbon atom in a 1,4 position relative to C-1 of β-Glc*p*A induces a 2 p.p.m. change in chemical shift of the latter.

The putative linkage assignment was confirmed by the glycosylation shifts obtained from comparison of the shift values for **2** with those for the unsubstituted Man*p* anomers: this reveals a large downfield shift for C-2 of α- and β- Man*p* to δ 79.39 and 81.24 respectively (α-carbon effect) and upfield shifts of the adjacent carbon atoms (C-1 and C-3, β-carbon effect). The magnitude of the glycosylation shifts differed for the two anomers, viz. the α-effect is greater for the β-anomer (+8.9 p.p.m. compared with +7.7 p.p.m. for α-Man*p*), whereas the β-effect is larger for the α-anomer, especially for C-1 which experiences an unusually high upfield shift of 2.2 p.p.m. (*cf.* 0.1 p.p.m. for β-Man*p*). These shifts are common for O-2 substituted sugars[155] (e.g. 2-*O*-methyl-D-mannose[155], glucobioses[189], xylobioses[190] and heterodisaccharides e.g. β-D-Gal*p*-(1→2)-L-Rha[191] and 2-*O*-glycosylated L-Ara*p*[171]) and have been rationalized as arising from interaction between the axial hydroxyl on C-1 of the α-anomer and the O-2 substituent[189]. If unfavourable this interaction can lead to a change in conformation or orientation of the glycosidic bond thereby accounting for the large glycosylation shifts observed[171]. Finally, the glycosylation shift of +5.6 p.p.m. for C-1 of β-Glc*p*A (calculated using β-D-Glc*p*A-OH as the model compound) is consistent with the shift of ~5 p.p.m. reported by Kochetkov *et al.*[156] for C-1 of a β-linked D-hexose residue attached to O-2 of D-mannose, thus confirming the absolute and anomeric configurations of **2**.

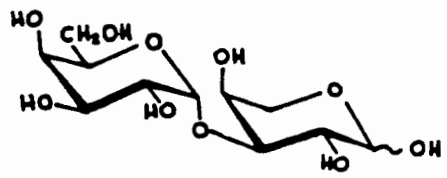
The ^{13}C assignments for **2** presented in Table 4 agree well with the published shift values[186] with one exception, namely, the assignment of C-4 of β-Glc*p*A. The value of δ 75.6 quoted (*cf.* δ 72.01 in Table 4) must be questioned as it gives rise to a glycosylation shift of +2.9 p.p.m. , which is unlikely for a carbon atom not involved in linkage formation (see 3.2.2 for expected glycosylation shifts[155]).

Experimental

Compound 2 was isolated and characterised as previously described[182]. A sample of 20mg was lyophilised from D_2O (3X) and used for the n.m.r. experiments described.

3.3.7.3 2D N.m.r. examination of 3-O-α-D-galactopyranosyl-L-arabinose (3)

The disaccharide was isolated in the form of two hepta-*O*-methyl derivatives (from arabic acid[192], derived in turn from gum arabic Turc. variety[193]) in low yield as a syrup, and from the gum exudate of stems of *Acacia cyanophylla*[194]. It was also obtained, as a crystalline product in unusually high yield, from a polysaccharide found in *Watsonia* corm-sacs[195-197]. A sample of this preparation[195] was used in our n.m.r investigation.



3

The starting points for the characterisation of the sugar were the anomeric-proton signals at δ 4.56 ($J_{1,2}$ = 7.7 Hz) and 5.26 ($J_{1,2}$ = 2.5 Hz), and 5.16 ($J_{1,2}$ = 3.4 Hz) which were assigned to H-1 of α-L-Ara*p*, β-L-Ara*p* and α-D-Gal*p* respectively, on the basis of chemical shifts and coupling constants[185].

Integration of the proton spectrum (see Fig. 10) gave a relative ratio of 3:2 for the α- and β-pyranosyl forms of the arabinose residue, which is also the ratio found for the free sugar[198], thus implying that a large substituent on O-3 does not affect the equilibrium established by mutarotation[190]. This ratio of intensities assisted in the assignment of the

other ^{1}H and the ^{13}C resonances. Evidence for the furanose forms of the reducing-sugar residue was found, as for free L-arabinose[198], but to an extent of only ~9%. This discussion relates therefore to the substituted Ara*p* anomers, a comment concerning the minor Ara*f* contribution to the spectra appears later in this section.

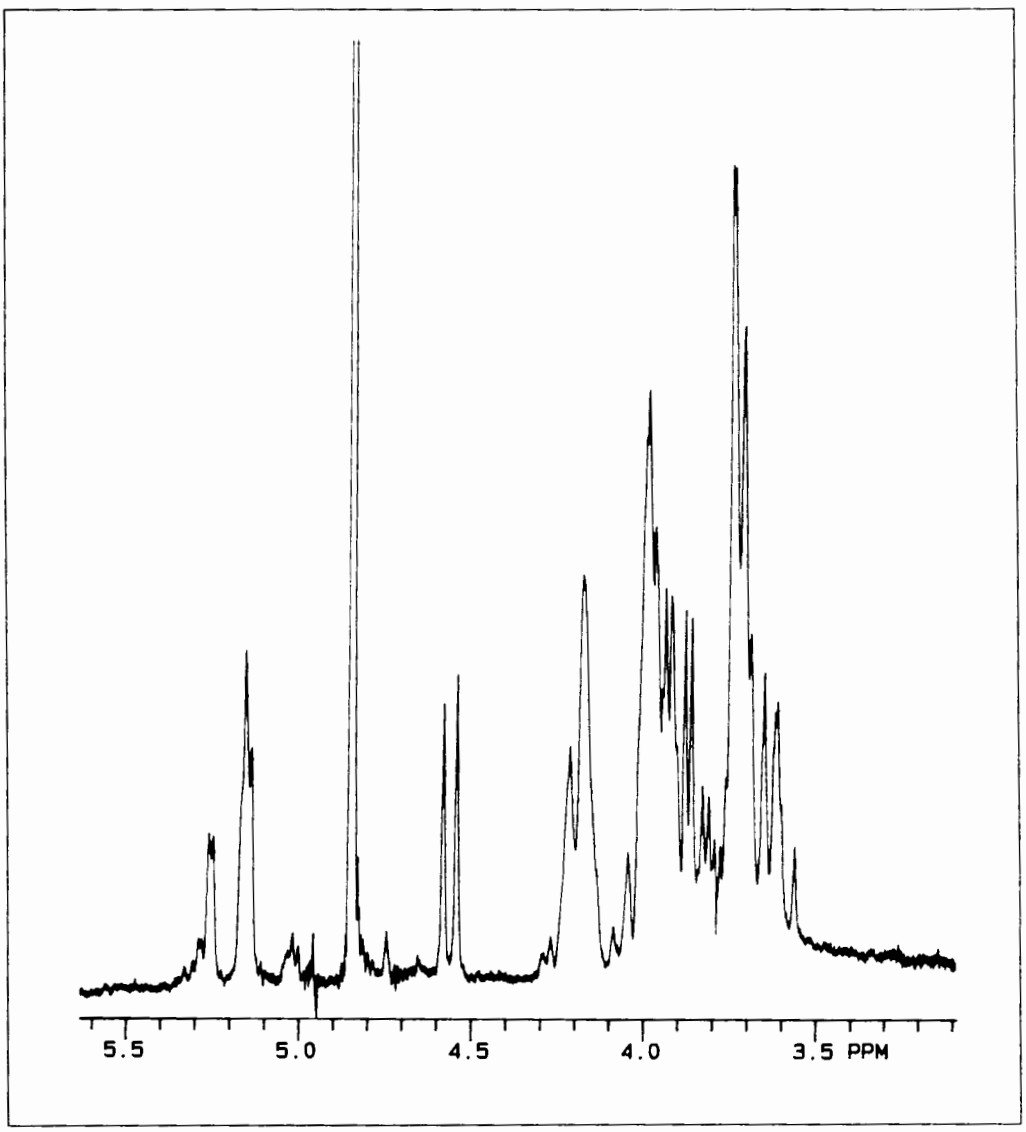


Figure 10: ^{1}H-n.m.r. spectrum (200 MHz) of **3** recorded at $25^{0}C$

From the assignments of the anomeric protons, made by using the HETCOR spectrum (see Fig. 11), the signals of the anomeric carbon atoms follow.

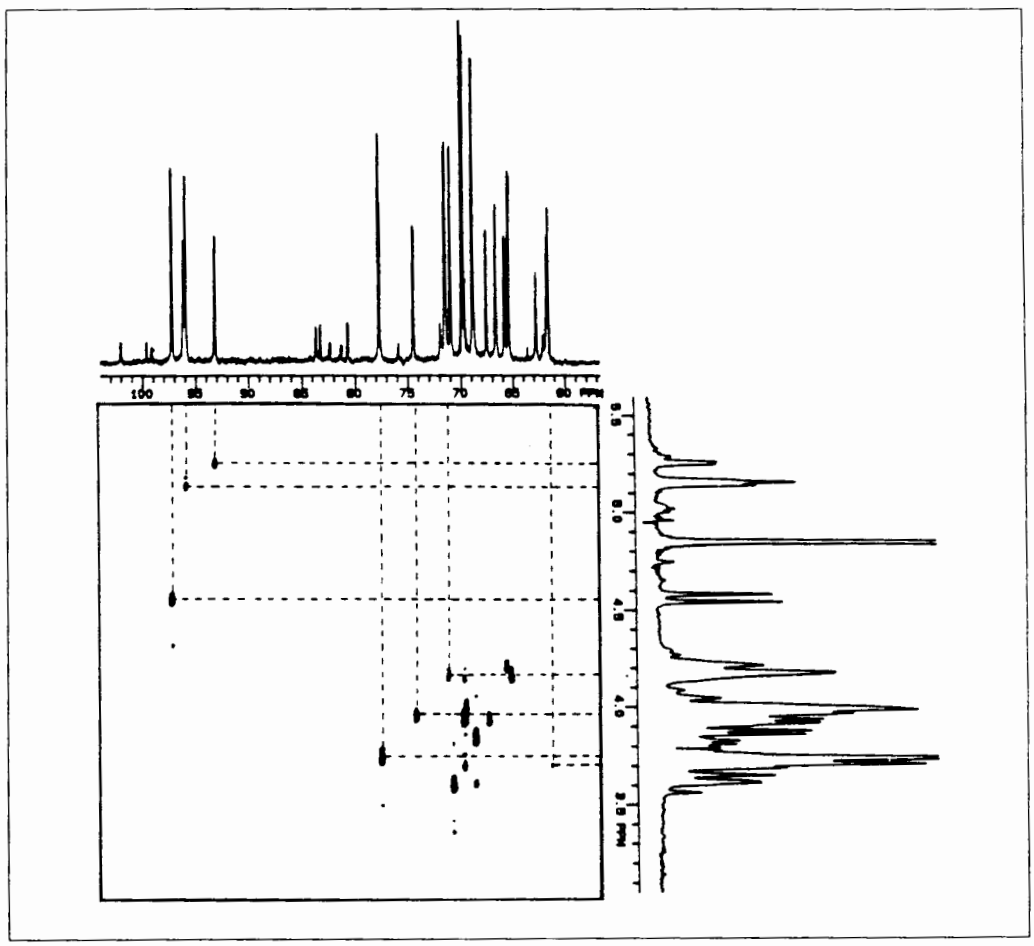


Figure 11: HETCOR spectrum of **3** recorded at 25°C, some of the C,H correlations are indicated ($J_{C,H}$ = 140Hz).

In addition, the proton-proton (COSY) and the HETCOR spectra allow the assignments of H-2 and C-2 of α-L-Ara*p*, β-L-Ara*p* and α-D-Gal*p*. Being the position of linkage, the signal for C-3 of L-Ara*p* is shifted downfield, out of the normal range for ring-carbon atoms[155]. On the basis of intensity, the resonances at δ 77.71 and 74.48 were assigned to α-L-Ara*p* C-3 and β-L-Ara*p* C-3, respectively.

The APT experiment was used to assign the signals of α-D-Gal*p* C-6, and α- and β-L-Ara*p* C-5, these being the only methylene carbon atoms. The remaining carbon resonances were identified by comparison with the literature[155]. The final assignments are given in Table 5 and Fig. 12.

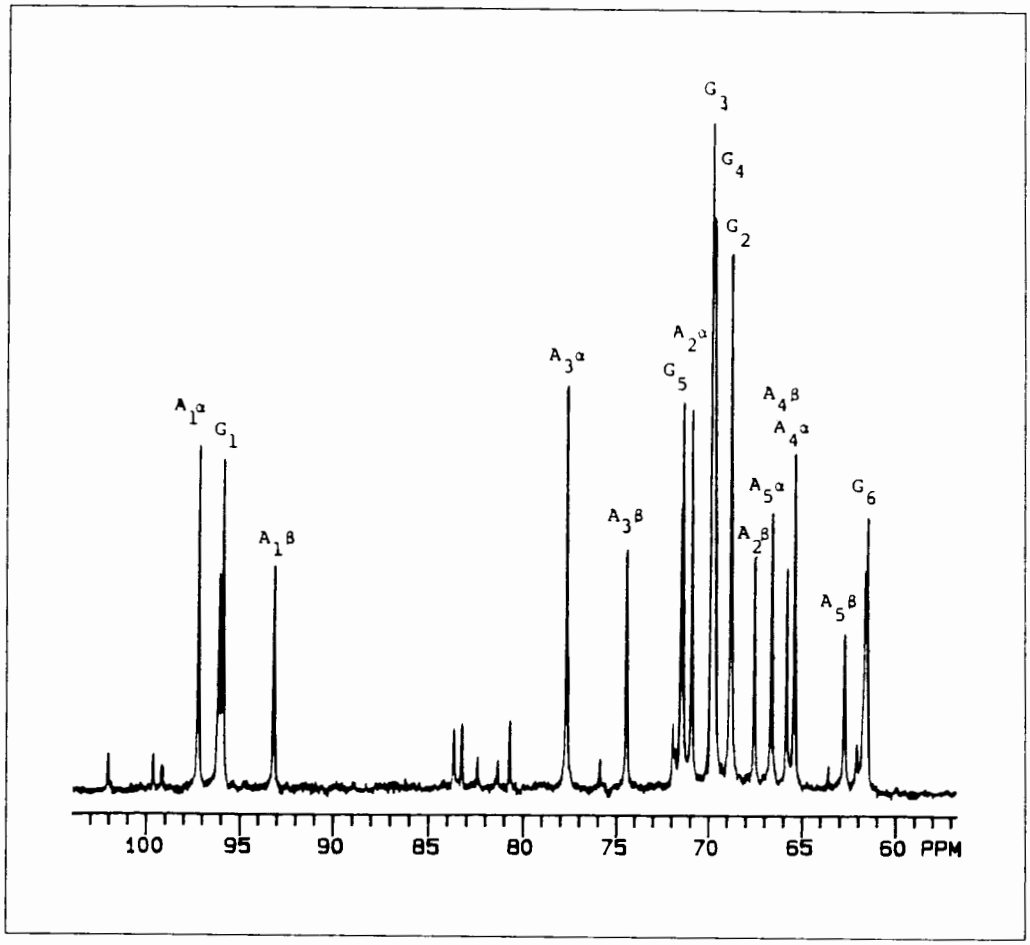


Figure 12: ^{13}C-n.m.r. spectrum (50.3 MHz) of **3** recorded at 25°C, some of the assignments are shown.

TABLE 5: ^{13}C-N.M.R. DATA (50.3 MHz) FOR 3-*O*-α-D-GALACTOPYRANOSYL-L-ARABINOSE (3) AND THE CORRESPONDING SUBSTITUTED ARABINITOL

Compound	*Chemical shift (p.p.m.)*					
	C-1	C-2	C-3	C-4	C-5	C-6
3 (α form)						
α-Gal*p*	95.95	68.87	69.85	69.73	71.38	61.52
α-Ara*p*	97.19	70.94	77.71	65.44	66.62	-
3 (β form)						
α-Gal*p*	96.14	68.87	69.85	69.73	71.49	61.64
β-Ara*p*	93.14	67.58	74.48	65.85	62.76	-
3 (α form)[a]						
α-Ara*f*	102.0	80.7	83.2[b]	83.6[b]	62.0	-
3 (β form)[a]						
β-Ara*f*	[c]	75.8	81.3[d]	82.4[d]	63.6	-
3 (reduced)						
α-Gal*p*	99.49	69.19	69.92	69.92	72.57	61.75
Ara-ol	63.20	71.41	78.65	72.24	62.95	-

[a] Only C-1 of Gal*p* could be assigned (see text)
[b] Assignments might need to be reversed
[c] Obscured by C-1 of α-Gal*p* attached to Ara*p* (see Fig. 12)
[d] Assignments might need to be reversed

Using the HETCOR experiments, complete elucidation of the proton spectrum was possible (see Table 6).

TABLE 6: ^{1}H-N.M.R. DATA (200 MHz) FOR 3-*O*-α-D-GALACTOPYRANOSYL-L-ARABINOSE (3) AND THE CORRESPONDING SUBSTITUTED ARABINITOL

Compound	*Chemical shift (p.p.m.)*					
	H-1	H-2	H-3	H-4	H-5	H-6
3 (α and β forms)						
α-Gal*p*	5.16	3.88	3.88-3.96	3.96-4.0	4.18	3.75
α-Ara*p*	4.56	3.64	3.75	4.18	3.68-3.75	-
β-Ara*p*	5.26	3.95	3.87	4.22	3.94-4.0	-
3 (reduced)						
α-Gal*p*	5.16	3.85	3.98	3.84	3.96	3.68
Ara-ol	•	3.72	3.80	3.96	3.74	-

• Not detected, due to fast relaxation resulting from geminal deuterium

These assignments were consistent with the coupling scheme shown by the COSY spectrum, a possible conformational representation of **3** being shown as Figure 13.

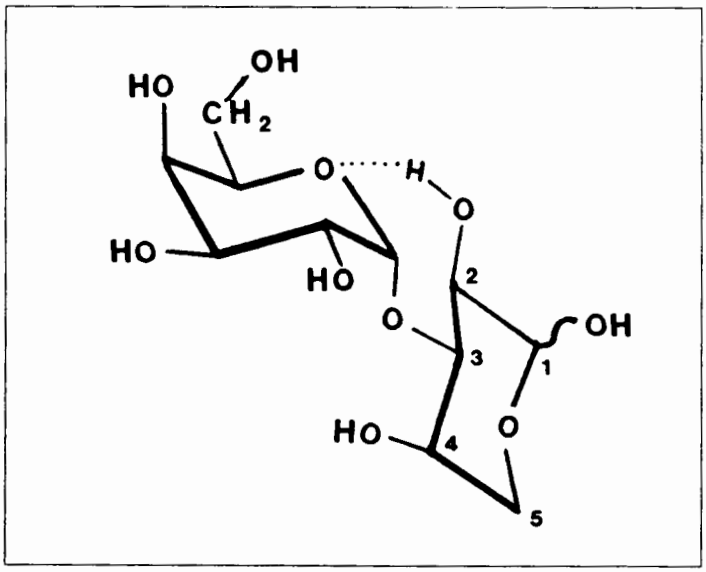


Figure 13: A possible conformation for **3** on the basis of the *exo*-anomeric effect. The conformation of L-Ara*p* here substituted at O-3 by α-D-Gal*p*, is in conformity with n.m.r. assignments.

Whereas for β-L-Ara*p* no preference is apparent for $^4C_1$ *vs.* $^1C_4$[199], the α-D-Gal*p* substituent on equatorial O-3 appears to anchor the conformation in the $^4C_1$ mode, which is the one exclusively expected for both α-L-Ara*p*[199] and 3-*O*-α-D-Gal-L-Ara*p*.

Interestingly, the effect of equilibration is expressed throughout the molecule, as some (C-1, C-5 and C-6) of the galactosyl carbon resonances are twinned in the appropriate ratio. Corresponding carbon signals have been observed as twinned for a 1,3-linked disaccharide, in dimethylsulphoxide solution[200]. Whereas twinning of the anomeric carbon signal of the non-reducing moiety of a disaccharide in which the reducing sugar is substituted by glycosylation at O-2 has been observed[189,190], the present example is remarkable in that the linkage is to O-3, and both C-5 and C-6 are implicated as well as C-1. Twinning was removed upon reduction of the disaccharide with $NaBD_4$. ^1H and ^{13}C resonances of the reduced disaccharide were fully assigned (see Tables 5 and 6) by using procedures already described. The signal of L-Arabinitol C-1 appeared as a low-intensity triplet due to ^2H coupling.

L-Ara*f* anomers were present in insufficient amount to give cross-peaks in the 2D spectra of 3-O-α-D-galactopyranosyl-L-arabinose. The proton signals at δ 5.32 ($J_{1,2}$ = 4.7 Hz) and 5.29 ($J_{1,2}$ = 1.6 Hz) are attributable to H-1 of the 3-*O*-substituted β- and α-L-Ara*f* respectively. The chemical shifts and splittings are similar to those obtained for unsubstituted arabinose (δ 5.27 and 5.26 and $J_{1,2}$ of 4.1 and 1.0 Hz)[201]. The galactosyl substituent has a small effect on the equilibrium between pyranose and furanose forms; for the substituted arabinose the furanose contribution is ~ 9%, whereas a value of 4.5% has been quoted for free arabinose[201]. The furanose forms cause the attached α-D-Gal*p* residue to experience a different electronic environment resulting in the appearance of a

broad signal at δ 5.02 due to H-1 of the α-D-Gal*p* (*cf.* δ 5.16 for H-1 of α-D-Gal*p* where L-Ara is pyranosidic). The correctness of the above interpretation was supported by recording the spectrum at 80°C, at which temperature the percentage of L-Ara*f* anomers (H-1 at δ 5.27 and 5.26) and the intensity of the signal at δ 5.02 were increased. This observation of the twinning of proton signals emanating from a glycosyl residue linked to a reducing sugar has been previously reported e.g. for an oligosaccharide containing a mannosyl unit attached to O-3 of galactose as terminal sugar[15].

The presence of the L-Ara*f* anomers in the disaccharide was most evident from the ^{13}C-n.m.r. spectrum (Fig. 12) which showed signals of low intensity (unlabelled) for all the carbon atoms of α- and β-L-Ara*f* (Table 5), with the position of glycosylation being revealed by the downfield shift of C-3. The only observable effect of the α- and β-L-Ara*f* forms on the attached α-D-Gal*p* residue was the downfield shift of the anomeric carbon atom (δ 99.6 and 99.1 respectively; *cf.* Table 5 for the chemical shifts of α-D-Gal*p* where L-arabinose is pyranosidic). These signals were modified as expected upon reduction of the disaccharide.

Experimental

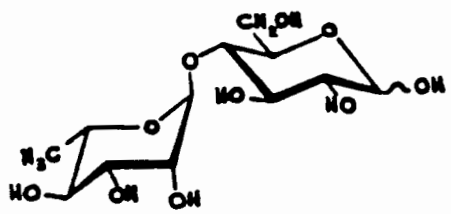
The disaccharide (**3**) was isolated from a sample of *Watsonia* corm-sac polysaccharide and identified as previously described[195]. Confirmation was obtained by using the Morrison procedure[115b], whereby the peracetylated alditol of arabinose and the peracetate of galactononitrile were characterized by g.l.c. analysis (column C at 215°C). The position of linkage was confirmed by methylation analysis of the $NaBD_4$-reduced disaccharide. G.l.c.-m.s. of the derived alditol acetates showed two peaks at retention times 0.40 (56%)

and 1.00 (44%), corresponding to the acetylated derivatives of C-1-deuterated 1,2,4,5-tetra-*O*-methylarabinitol and 2,3,4,6-tetra-*O*-methylgalactitol, respectively. E.i.-m.s. of permethylated, borodeuteride- reduced **3** showed the following diagnostic[72] fragments: m/e 219 (aA_1), 187 (aA_2), 155 (aA_3) ; 252 (abJ_1) , 192 (bA_1) ; 46, 381, 349; 45, 382, 350 (ald).

A solution of **3** in D_2O was freeze-dried, and the process was repeated three times, in order to replace exchangeable hydrogen atoms with deuterium. ^1H- and ^{13}C- n.m.r. spectra were recorded for solutions in D_2O (286 mg/mL) with a Varian VXR-200 spectrometer and with acetone as an internal reference (δ 2.21 for ^1H and 31.0 for ^{13}C). Coupling constants were determined using a diluted solution.

3.3.7.4 2D N.m.r. examination of 4-*O*-α-L-rhamnopyranosyl-D-glucose (4)

Compound **4** was isolated after acetolysis of the carboxyl reduced polysaccharide from *Acacia saligna*[202,203]. The n.m.r. study of **4** was undertaken in order to investigate the n.m.r. parameters arising from O-4 glycosylation (see Chapter 5) and to gain experience in the characterisation of rhamnosyl residues, this being the major constituent of the capsular polysaccharides of *Klebsiella* serotypes K36 (Chapter 4) and K71 (Chapter 6).



4

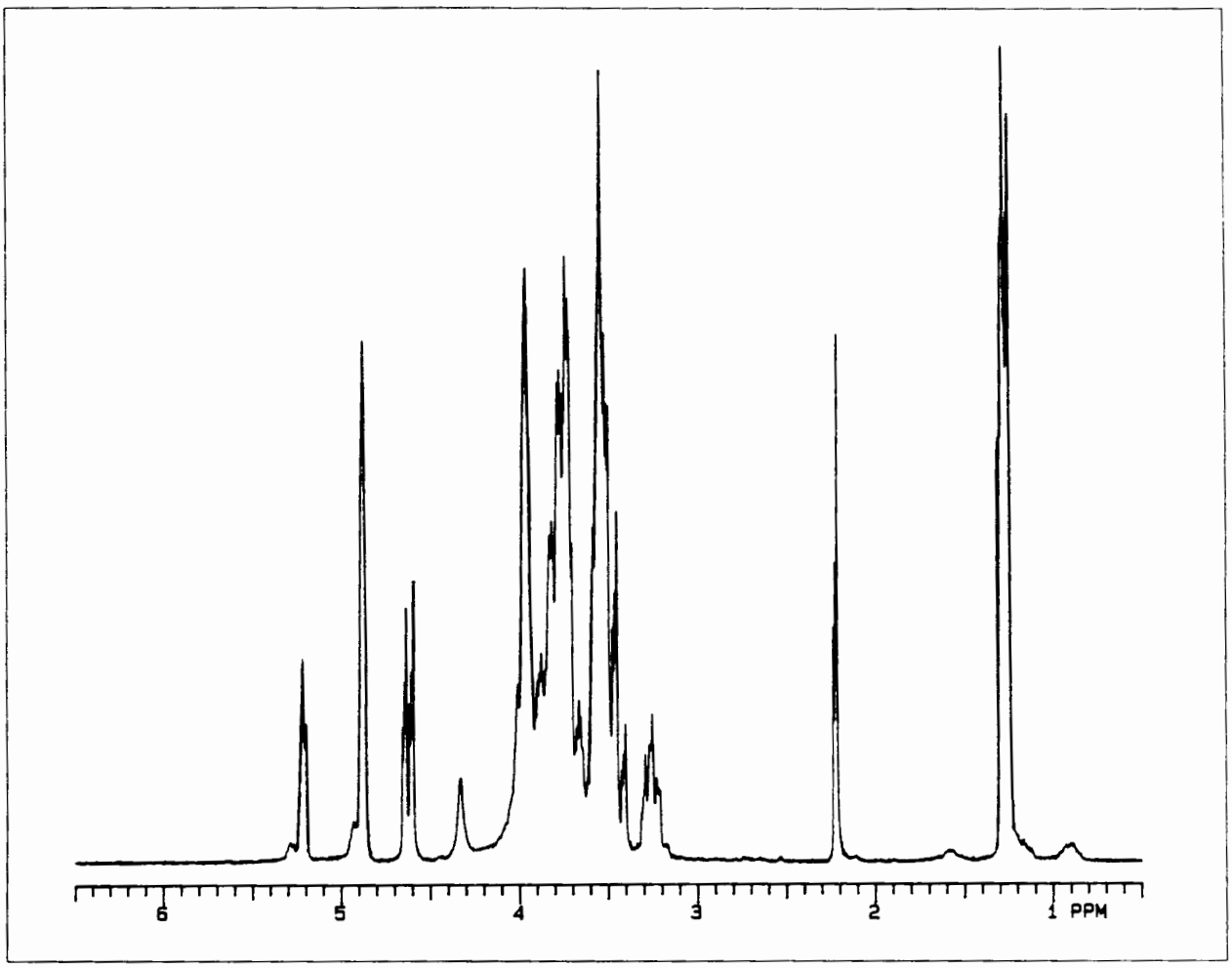


Figure 14: ^{1}H-n.m.r. spectrum (200 MHz) of **4** recorded at 25°C.

The ^{1}H-n.m.r. spectrum (Fig. 14) clearly showed the presence of α- and β- Glc*p* with anomeric proton signals at δ 5.21 (0.67H, $J_{1,2}$ = 3.5 Hz) and 4.62 (0.33H, $J_{1,2}$ = 8 Hz), together with a singlet at δ 4.85 (1H) assigned to H-1 of Rha*p*. The presence of the rhamnosyl group was confirmed by the doublet at δ 1.28 (3H, $J_{5,6}$ = 6.5 Hz) attributable to the methyl group at C-6. The shifts, splittings and ratio of α- and β- Glc*p* are similar to

those reported for unsubstituted glucose[201], thus confirming that a substituent at O-4 has little effect on the anomeric centre of the reducing unit. The anomeric configuration of the Rha*p* group was not evident at this stage, the δ value observed being attributable to either α-Rha*p* (e.g. δ 4.77 for α-Rha*p* attached to O-4 of 3-linked α-Glc*p*A)[98] or β-Rha*p* (e.g. δ 4.85 for →3)-β-Rha*p*-(1→4)-β-Glc*p*-(1→)[204], as spectra of rhamnosyl residues are known to be more affected by structure than configuration[205]. This question was settled after a detailed 2D n.m.r. examination of **4**.

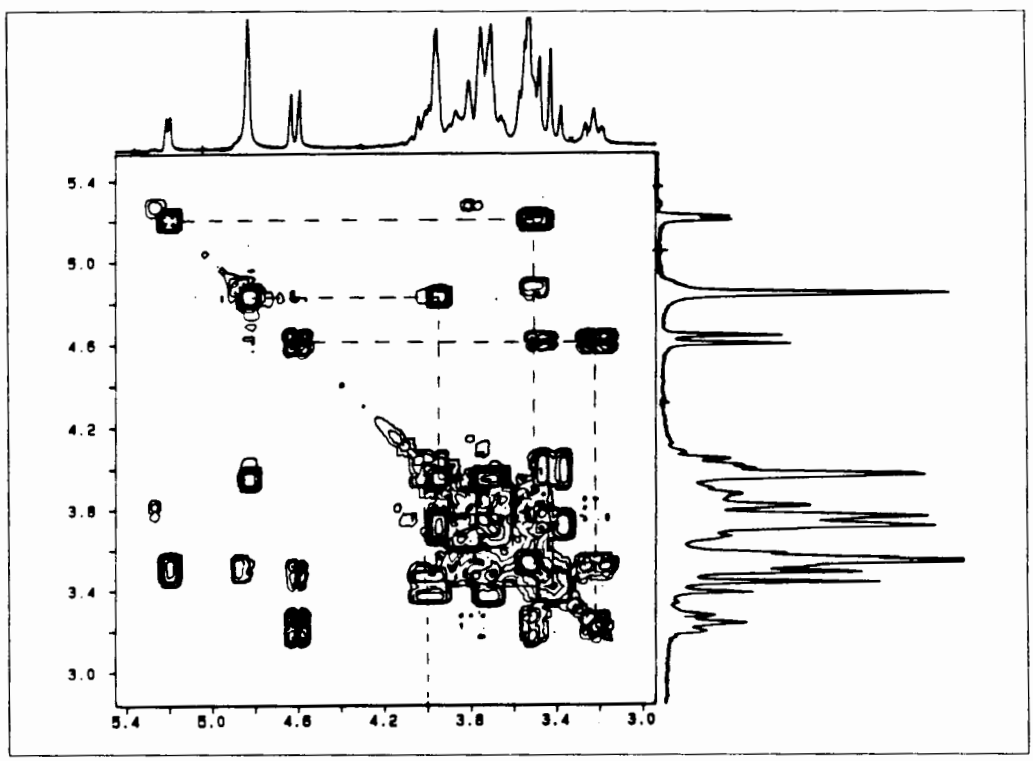


Figure 15: COSY spectrum of **4** recorded at 25°C, some of the spin systems are indicated.

The COSY diagram (Fig. 15) permitted full assignment of the Rha*p* signals to be made, using H-1 and H-6 as the starting points for establishing connectivities. Some of the signals emanating from the reducing end glucose were also identified; however, the poor resolution of the ^{1}H-n.m.r. spectrum obtained and signal overlap prevented further assignments from being made with confidence. In contrast, the proton-decoupled ^{13}C-n.m.r. spectrum (Fig.

16) was readily interpreted on the basis of signal intensity (Iα/Iβ = 2) and by comparison with chemical shift data of standards (L-Rha*p*-OMe and D-glucose)[155].

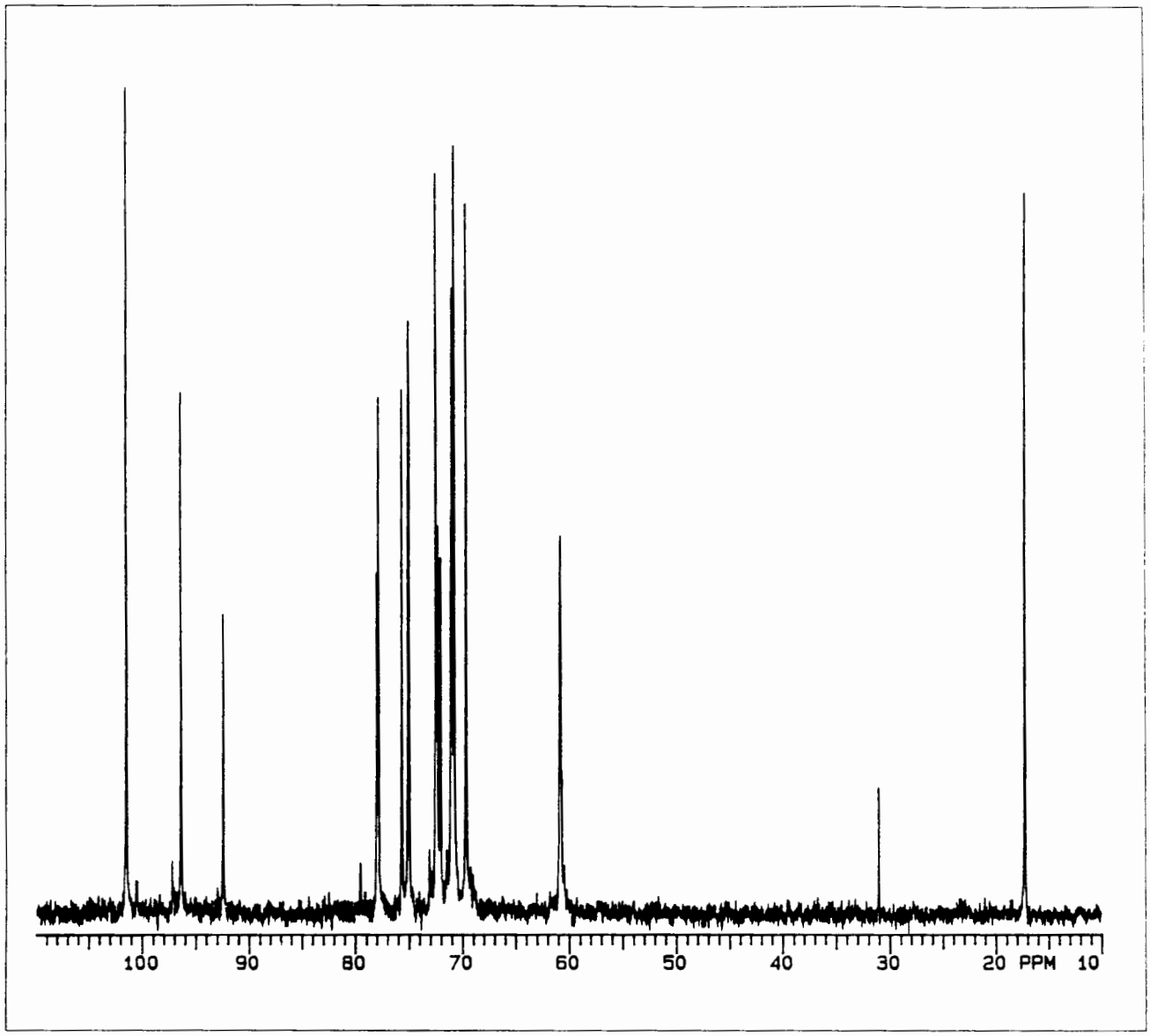


Figure 16: ^{13}C-n.m.r. spectrum (50.3 MHz) of **4** recorded at 25°C.

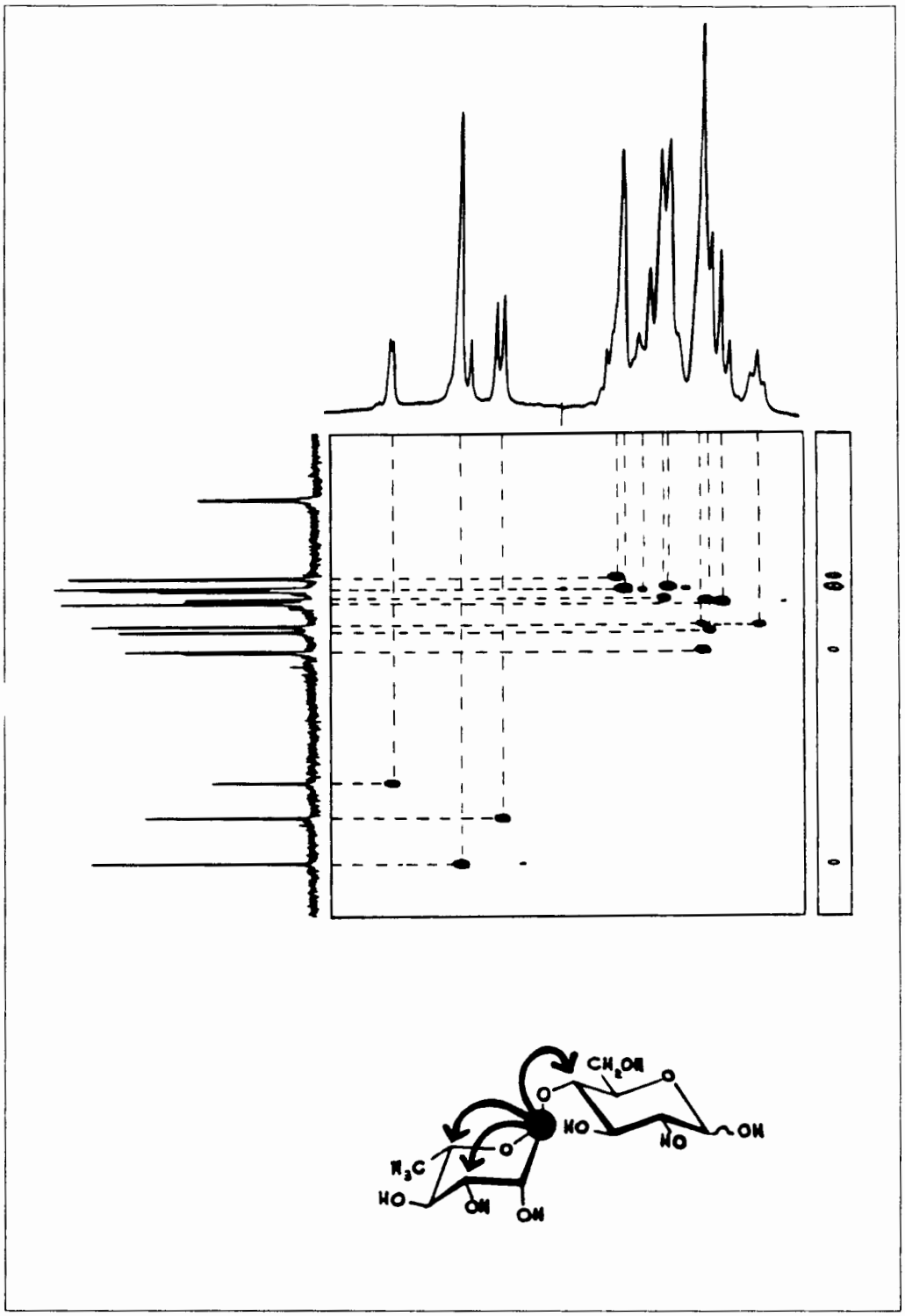


Figure 17: HETCOR spectrum of **4** recorded at 25°C, some of the C,H correlations are shown ($J_{C,H}$ = 140 Hz). Inset on right shows connectivity between H-1 of α-Rha*p* and carbons three bonds removed (long-range HETCOR, $J_{C,H}$ = 6 Hz).

Use of the HETCOR experiment (Fig. 17) confirmed some of the ^{13}C assignments (these followed from the ^{1}H signals previously identified using the COSY diagram, Fig. 15) , and enabled the remaining proton assignments to be made. The complete ^{1}H and ^{13}C assignments and glycosylation shifts for **4** are presented in Tables 7 and 8 respectively.

TABLE 7: ^{1}H-N.M.R. DATA[a] (200 MHz) AND GLYCOSYLATION SHIFTS[b] OF 4-*O*-α-L-RHAMNOPYRANOSYL-D-GLUCOSE (**4**)

Sugar residue	*Chemical shift [c] (p.p.m.)*					
	H-1	H-2	H-3	H-4	H-5	H-6
α-Glc*p*-OH[d]	5.21 (+.12)	3.52 (+.11)	3.75 (+.14)	3.54 (+.25)	3.86 (+.14)	3.55,n.o. -
β-Glc*p*-OH[d]	4.62 (+.11)	3.23 (+.10)	3.50 (+.13)	3.54 (+.24)	3.55 (+.20)	3.82,3.77 -
α-Rha*p*[a]	4.85 (+.15)	3.96 (+.03)	3.73 (+.02)	3.43 (-.01)	4.01 (+.37)	1.28 (-.02)

Glycosylation shifts given in parentheses.

[a] Coupling constants measured are given in text
[b] A downfield shift relative to the model compound is considered positive
[c] Chemical shift values relative to internal acetone (δ 2.21)
[d] Model compound Glc*p*-OH (Ref. 111)
[e] Model compound α-Rha*p*-OMe (Ref. 188)

TABLE 8: ^{13}C-N.M.R. DATA (50.3 MHz) AND GLYCOSYLATION SHIFTS[a] FOR 4-*O*-α-L-RHAMNOPYRANOSYL-D-GLUCOSE (**4**)

Sugar residue	*Chemical shift (p.p.m.)*					
	C-1	C-2	C-3	C-4	C-5	C-6
α-Glc*p*-OH[c]	92.42 (-0.5)	72.30 (-0.2)	72.02 (-1.8)	77.98 (+7.4)	71.08 (-1.2)	60.76 (-0.8)
β-Glc*p*-OH[c]	96.37 (-0.3)	75.05 (0.0)	75.66 (-1.0)	77.82 (+7.2)	74.98 (-1.8)	60.82 (-0.9)
α-Rha*p*[d]	101.46 (+6.5)	70.99 (-0.9)	70.95 (-0.1)	72.48 (-0.8)	69.65 (+0.2)	17.21 (-0.8)

Glycosylation shifts given in parentheses.

[a] A downfield shift relative to the model compound is considered positive
[b] Chemical shift relative to internal acetone (δ 31.0)
[c] Model compound Glc*p*-OH (Ref. 155)
[d] Model compound α-Rha*p*-OH (Ref. 155)

Comparison of the shift values of the Rha*p* in **4** with those in the literature[155,188,205], in particular the H-5 and H-6 resonances which are known to be diagnostic of configuration[188], established that the anomeric configuration must be α. This finding is in conformity with the result obtained by measurement of the optical rotation of **4** (observed $[\alpha]_D = +4^\circ$, whereas the values calculated on the basis of Hudson's rules of isorotation[206] are -5° and $+70^\circ$ for α-L-Rha*p*-(1→4)-D-glucose and β-L-Rha*p*-(1→4)-D-glucose respectively). The glycosylation shifts obtained for the D-glucose anomers of **4** show that the H-4 and C-4 signals are deshielded (α-effect), whereas the C-3 and C-5 resonances are slightly shielded (β-effect) relative to the values quoted for the unsubstituted glucose, thus confirming that O-4 is the site of glycosylation. The relatively large upfield β-effect (-1 p.p.m.) observed for C-3 of D-glucose in **4** provides additional evidence for the presence of α- instead of β-L-Rha*p*, as the latter is known to result in smaller β-effects (-1 to +1 p.p.m.)[156]. Finally, the proton-coupled ^{13}C spectrum of **4** yielded the expected larger $J_{C\text{-}1,H\text{-}1}$ for α-Rha (169.30 Hz) and α-Glc*p*-OH (169.75 Hz), while the smaller value of 161.87 Hz was found for β-Glc*p*-OH. The relationship between configuration factors and ^{13}C chemical shifts given in reference 156 also confirms the absolute configurations of Rha*p*(L) and glucose (D).

Some of the assignments in Tables 7 and 8 were further corroborated by a long-range HETCOR experiment. At a setting of $^3J_{C,H} = 6$ Hz (*cf.* 140 Hz for $^1J_{C,H}$) the coupling across three bonds is revealed, which showed connectivity between: H-1 and C-5 of α-Glc*p*-OH (across the ring oxygen); H-5 and C-3 of α-Rha*p*; and H-1 of α-Rha*p* and C-3 and C-5 of the same residue, as well as an inter-residue connectivity across the glycosidic bond to C-4 of D-glucose (see inset, Fig. 17).

This result clearly illustrates the usefulness of long-range connectivity experiments in sequence determination of saccharides, which is highly pertinent to this study as the oligosaccharides studied are generally not amenable to "through space connectivity" analysis by n.O.e. methods (see 3.3.5).

Finally, no twinning of the α-Rha*p* signals could be detected at the magnetic field strength employed in these n.m.r. studies. This result confirms that in contrast to 2- and 3- linked saccharides (see 3.3.7.2 and 3.3.7.3), the substituent on O-4 is relatively insensitive to changes at the anomeric centre of the reducing end sugar (for quantification of these effects, see empirical rules, reference 155).

Experimental

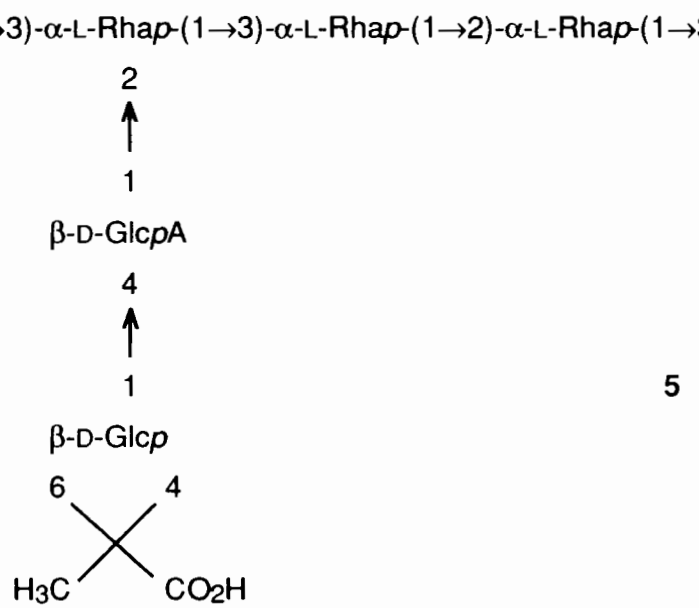
Compound **4** was isolated and characterised as previously described[203]. A sample of 250mg was lyophilised from D_2O (3X) and used for the n.m.r. experiments described.

CHAPTER 4

INVESTIGATION OF THE OLIGOSACCHARIDES PRODUCED BY BACTERIOPHAGE-BORNE ENZYME DEGRADATION OF *KLEBSIELLA* K36 POLYSACCHARIDE

4.1 INTRODUCTION

The capsular polysaccharide from *Klebsiella* serotype K36 has been classified in the chemotype containing D-glucose, D-galactose, L-rhamnose and D-glucuronic acid as the component monosaccharides. This chemotype, which was recently revised, contains the serotypes K12, K18, K19, K41, K55, K70 and K79[19]. An earlier detailed study[207] of *Klebsiella* K36 polysaccharide, involving partial acid hydrolyses and Smith degradation, led to the postulation of **5** as the structure of the repeating unit.



In order to verify this structure and to obtain novel oligosaccharides that might be clinically, chemically or spectroscopically useful, phage Ø36 depolymerisations of K36 capsular polysaccharide were conducted. The polysaccharide sample used in these experiments was not derived from the same bacterial specimen of *Klebsiella* serotype K36 as was used in the original structural investigation, that strain having lost its ability to produce capsular

material; however, chemical and spectroscopic comparison showed no difference in structure between the two polysaccharides produced by the two strains and phage Ø36 cleaved both polymers. Bacteriophage Ø36 is reported to belong to the Bradley type C class[208], and has been shown by Rieger-Hug and Stirm[90] to cause cleavage of capsular polysaccharide K36 into oligosaccharides having a galactose group at the reducing end.

Preliminary structural studies of the oligomers prepared by phage Ø36 depolymerisation (courtesy of Professor A.M. Stephen) showed that certain of the earlier linkage assignments might be incorrect, and a new structure for the repeating unit was proposed[209]. Further investigation, involving methylation analyses, mass and 2D n.m.r. spectroscopy, have proved that the glucuronic acid residue must be linked through O-2 and not O-4 as originally postulated, whereas the sequence of rhamnosyl residues is indeed as shown in **5**[100,101].

4.2 PRELIMINARY STRUCTURAL STUDIES OF THE OLIGOSACCHARIDE MIXTURES

Two depolymerisation experiments (A and B) conducted under slightly different conditions (see 4.7.1) yielded oligosaccharide products designated **A** and **B**. Steric-exclusion chromatography (s.e.c.) on Bio-Gel P-2 indicated that the oligomeric mixtures contained predominantly the monomeric repeating unit (**A**, 74%; **B**, 86%; the rest a dimer thereof). ^{1}H- and ^{13}C- n.m.r. spectra showed six discernible anomeric signals, and pyruvate and rhamnosyl methyl signals at high field, thereby confirming that **A** and **B** must be a mixture of the expected pyruvylated hexasaccharide (**P1**) and dodecasaccharide (**P2**). Acid

hydrolysis and g.l.c. analysis of the derived alditol acetates indicated residues of rhamnose (3), glucose (1) and galactose (1), while analysis of the borohydride-reduced oligosaccharides (Table 9) showed the presence of the same sugar residues (converted to per-*O*-acetylated aldononitriles) and galactitol acetate, in proportions corresponding to the relative amounts of monomer and dimer as indicated by s.e.c.

TABLE 9: DETERMINATION OF D.P. AND CHARACTERISATION OF THE REDUCING END OF OLIGOSACCHARIDE PRODUCTS A AND B

Peracetylated derivative of	*T**	*Mol %*	
		A	*B*
Rhamnononitrile	0.24	49	56
Gluconononitrile	1.00	23	20
Galactononitrile	1.08	6	3
Galactitol	1.19	22	21

* Retention times, determined on column A at 200°C, relative to 2,3,4,5,6-penta-*O*-acetylgluconononitrile

The presence of galactitol acetate amongst the PAAN's confirms the location of galactose at the reducing end of **P1** and **P2** and that the bacteriophage Ø36 enzyme is a β-D-galactopyranosidase, catalysing the cleavage of the →3)-β-D-Gal*p*-(1→3)-α-L-Rha*p*-(1→ bond.

Methylation of **B** by the Hakomori[47] method, followed by hydrolysis, derivatisation of the products as alditol acetates, and g.l.c.-m.s., gave the results shown in Table 10, column I.

TABLE 10: METHYLATION ANALYSIS OF OLIGOSACCHARIDE PRODUCTS A AND B

Partially methylated alditol acetates[a]	*T*[b]	*Mole %*[c]			
		I	II	III	IV[d]
3,4-Rha	1.00	24	28	29	58
2,4-Rha	1.12	19	17	18	6
4-Rha	1.52	6	5	6	2
3,4,6-Glc	1.89			13	3
2,5,6-Gal	1.91	7	6		
2,4,6-Gal	2.12	8	7	7	2
2,3-Glc	3.20	18	37	27	29
3,4-Glc					

[a] 3,4-Rha = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, etc

[b] Retention times, determined on column B at 200°C, relative to 3,4-Rha. Assignments confirmed by g.l.c.-m.s.

[c] Values were corrected by use of effective, carbon-response factors given by Sweet *et al.* (Ref. 112)

[d] I, methylated B; II, carboxyl ester-reduced, methylated B; III, Purdie remethylated, carboxyl ester-reduced, methylated B; IV, Hakomori remethylated, carboxyl ester-reduced, methylated A

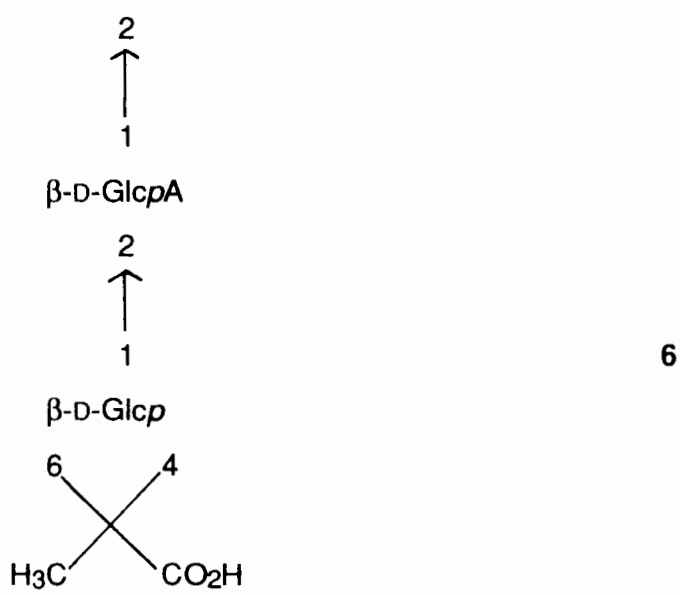
These data show that **P1** consists of a linear hexasaccharide; 2,3-di-*O*-methylglucose arises from the terminal glucose group with pyruvate attached at O-4 and O-6, and 2,4,6- and 2,5,6- tri-*O*-methylgalactose are derived from the pyranose and furanose forms of the reducing end group (the ratio of furanose/pyranose ring forms is known to increase upon changing the solvent from water to DMSO[199]). After $LiAlH_4$ reduction of methylated **B** (see Table 10, column II), the proportion of derivatised 3,4-di-*O*-methylrhamnose was slightly increased and the apparently single peak corresponding to 2,3-di-*O*-methylglucose was found to include a second component. The increase in the proportion of 3,4-di-*O*-methylrhamnose confirms that one of the 2-linked rhamnosyl residues is contiguous to the glucuronic acid residue and therefore was not completely released by hydrolysis prior to carboxyl ester-reduction (*cf.* column I). The component released only

after carboxyl-reduction must arise from the glucuronic acid residue. Careful inspection of the m.s. spectra corresponding to the peak attributed to derivatised 2,3-di-*O*-methylglucose, revealed that the second component was a derivative of 3,4-di-*O*-methylglucose. Resolution of these two partially methylated alditol acetates could be achieved using g.l.c. on capillaries coated with Carbowax 20M or OV-73. The detection of the 3,4-di-*O*-methylglucose derivative suggests that the D-glucuronic acid residue in the parent oligosaccharide must be linked through O-2 and not O-4 as in **5**. This is supported by further methylation experiments described in 4.3.

Remethylation of ester-reduced methylated **B**, using the Purdie-Irvine[120] method, was found to be incomplete (see Table 10, column III), resulting in a low yield of 3,4,6-tri-*O*-methylglucose, while the remainder of the acid derivative was detected as 3,4-di-*O*-methylglucose. For this reason oligosaccharide product **A** was subjected to methylation analysis as described for **B**, except that the re-methylation step was effected using the Hakomori procedure. The results (Table 10, column IV) indicated that degradation of certain residues had occurred, probably due to β-elimination from the reducing end, catalysed by the strong base (dimsyl) used in the Hakomori procedure[56]. Elimination of the O-3 rhamnosyl substituent from the galactose unit at the reducing end will produce an unsaturated galactose derivative and rhamnose as the new reducing end sugar, which in turn can undergo β-elimination and degradation. The detection of only trace amounts of tri-*O*-methylgalactose and 2,4-di-*O*-methylrhamnose under these conditions suggested that degradation had occured from the reducing end, and that the 3-linked and not the 2-linked rhamnosyl residue was adjacent to the galactose group. The presence of the dimer (**P2**) in **A** and **B** was made apparent by the detection of

4-mono-*O*-methylrhamnose (from the branch point, see **5**) and enhanced 2,4,6-tri-*O*-methylgalactose (attached to the branch-point rhamnosyl residue).

The results obtained from this preliminary investigation suggested that the repeating unit of *Klebsiella* K36 capsular polysaccharide should be reformulated as shown in **6**.



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Further structural studies were conducted on **P1** and **P2** in order to verify these findings; the results are presented in section 4.3-4.5.

4.3 METHYLATION STUDIES

Before further investigation of the oligosaccharide products was undertaken, samples of the two *Klebsiella* K36 polysaccharide preparations were methylated, carboxyl ester-reduced, and subjected to methylation analysis as described. As observed previously, g.l.c.-m.s. of the ester-reduced, methylated polysaccharides, indicated the

presence of derivatives of 2,3- and 3,4- di-*O*-methylglucose, whereas the latter component was not detected on examination of the methylated polysaccharide prior to reduction; it must therefore be derived from reduction of O-2 linked uronic acid residues.

These results were corroborated by repeating the methylation analyses of **P1** and **P2** (isolated by s.e.c.). The samples were reduced with $NaBD_4$ to **P1**-ol and **P2**-ol, respectively (in order to label the sugar located at the reducing end and to minimize base degradation of this group). **P1**-ol and **P2**-ol were then subjected to methylation analysis as before (see Table 11, columns I and II).

TABLE 11: METHYLATION ANALYSIS OF **P1**-OL AND **P2**-OL

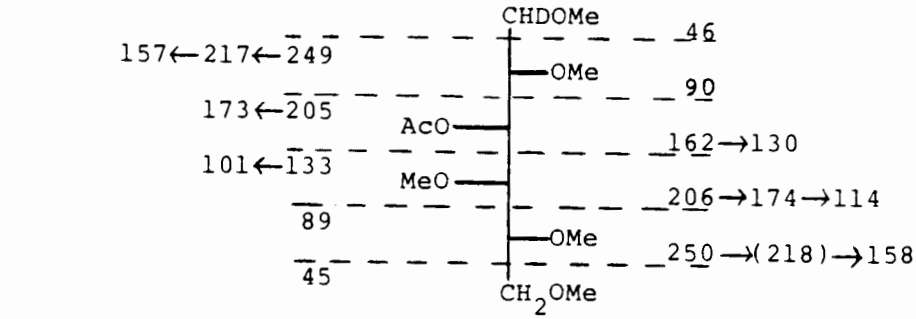
Partially methylated alditol acetates[a]	*T*[b]	*Mole %*[c]			
		I	II	III	IV[d]
1,2,4,5,6-Gal[e]	0.52	15	6	15	15
3,4-Rha	1.00	30	28	34	35
2,4-Rha	1.06	19	15	18	17
2,3,4,6-Glc[f]	1.10		5		2
4-Rha	1.59		11		
3,4,6-Glc[g]					14
2,4,6-Gal	1.91		8		
2,3-Glc	3.67	20	10	33	16
3,4-Glc[h]		[i]	[i]		

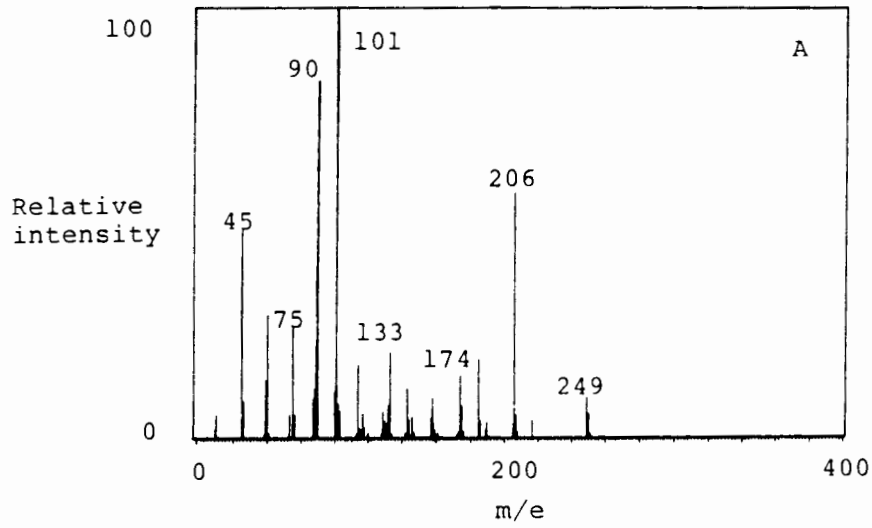
[a] 3,4-Rha = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, etc
[b] Retention times, determined on column C at 215°C, relative to 3,4-Rha. Assignments confirmed by g.l.c.-m.s.
[c] Values were corrected by use of effective, carbon-response factors given by Sweet *et al.* (Ref. 112)
[d] I, methylated **P1**-ol; II, methylated **P2**-ol; III, carboxyl-reduced, methylated **P1**-ol; IV, remethylated, carboxyl-reduced, methylated **P1**-ol
[e] C-1 deuterated according to g.l.c.-m.s. (see Fig. 18A)
[f] From some loss of pyruvic acid
[g] C-6 di-deuterated according to g.l.c.-m.s. (see Fig. 18B)
[h] C-6 di-deuterated according to gl.c.-m.s.
[i] Assumed 16%, and sugar ratios calculated accordingly

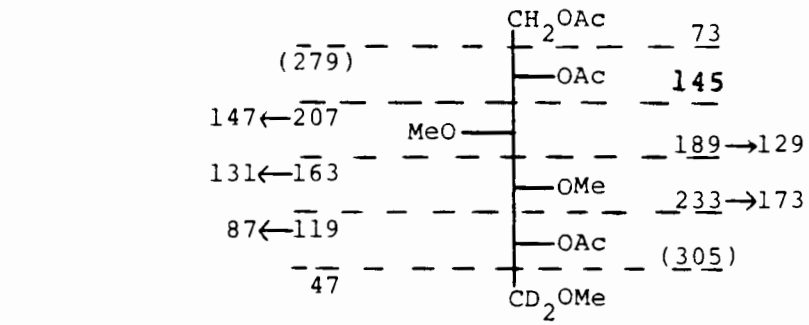
The proposed linkage assignments (*cf.* **6**) were confirmed, the deuterium label being found on the galactose residue only (detected as C-1 deuterated 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol (**7**) by g.l.c.-m.s.; see Fig. 18A for m.s. fragmentation pattern of **7**).

Carboxyl-reduction of permethylated **P1**-ol using $LiAlD_4$ (instead of $LiAlH_4$) gave rise to the expected acetylated derivative of 6,6-di-deuterated-3,4-di-*O*-methylglucitol as shown in Table 11, column III. Finally, Hakomori re-methylation of the ester-reduced, methylated **P1**-ol, yielded the acetylated derivative of 6,6-di-deuterated-3,4,6-tri-*O*-methylglucitol (**8**) as expected (Table 11, column IV; see Fig. 18B for m.s. fragmentation pattern of **8**), thereby unambiguously establishing that the D-glucuronic acid residue is linked through O-2.

The extensive degradation accompanying an earlier re-methylation of ester-reduced, methylated product (see 4.2) could not be repeated, even when the product was left in contact with base overnight, and therefore it was apparent that linkage assignments based upon this behaviour might possibly be incorrect. For this reason, the sequence of sugar residues was further investigated by mass spectrometry, as described in 4.4.







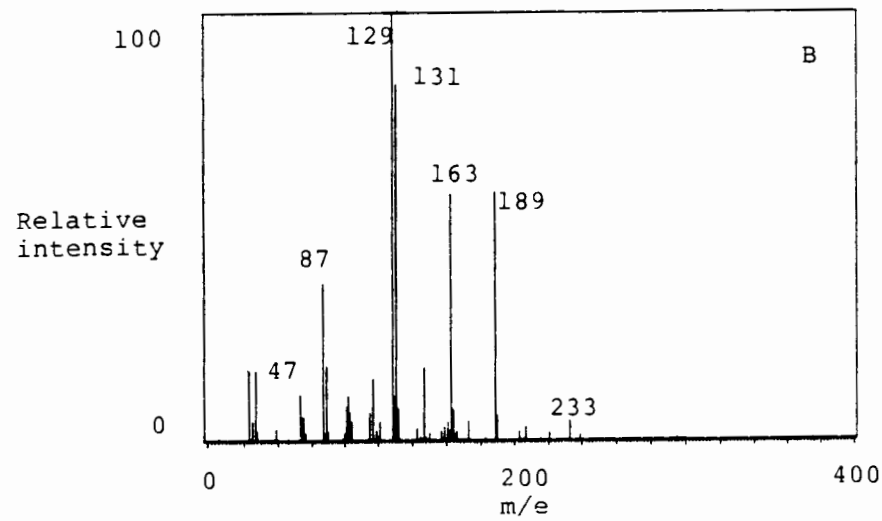
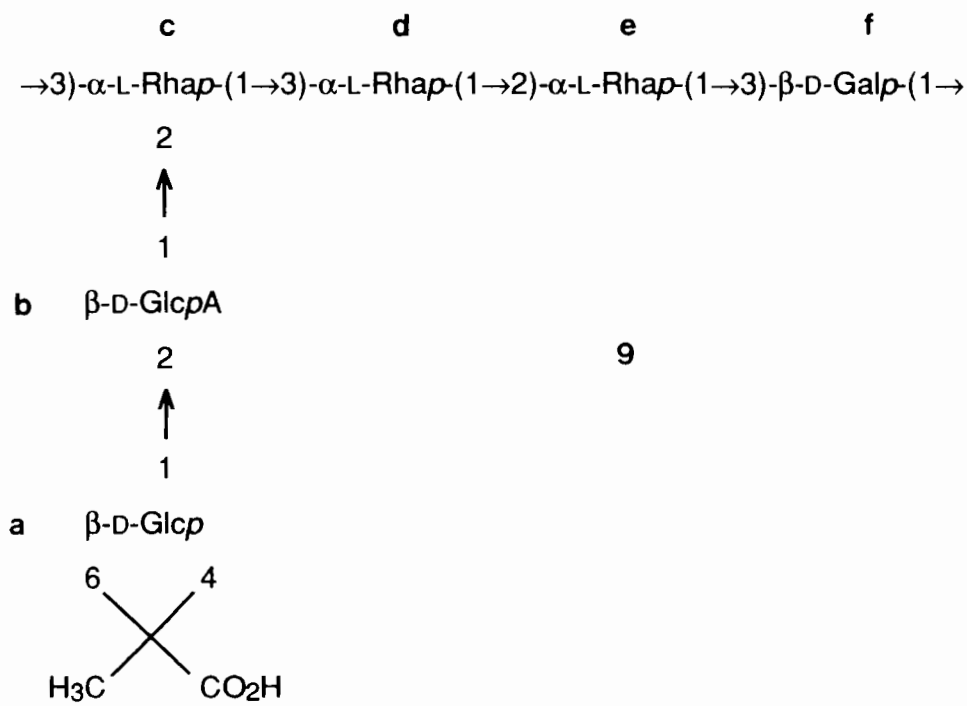


Figure 18: A and B, m.s. fragmentation patterns for **7** and **8**.

4.4 SEQUENCE ANALYSIS BY MASS SPECTROMETRY

At this stage of the investigation, the structure of the repeating unit of *Klebsiella* K36 polysaccharide was assumed to be as shown below (**9**), with the sequence of rhamnosyl residues under question (**d** and **e**), written as originally proposed (*cf.* 5).



Sugar moieties designated **a** - **f** occur in **P1** and **P2**. Prime letters distinguish the two halves in **P2**.

A sample of **P1** (containing only 65% of the pyruvic acetal intact, according to ^{1}H-n.m.r. integral at δ 1.5) was reduced with $NaBD_4$, then methylated. A portion of this product was analysed by g.l.c.-m.s. of the derived p.m.a.a.'s: the appearance of derivatives of both 2,3,4,6-tetra-*O*-methylglucose and 2,3-di-*O*-methylglucose confirmed that the pyruvylated glucose group is terminal (**a**), while the detection of C-1 deuterated

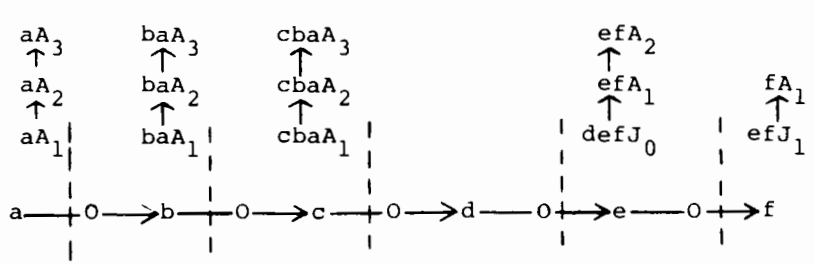
3-*O*-acetyl-1,2,4,5,6-*O*-penta-*O*-methylgalactitol established the galactose unit as the reducing end group (i.e. **f**). To confirm the remaining sequence of sugar residues (i.e. **a→b→c→d→e→f**), the permethylated product was analysed by electron impact-mass spectrometry (e.i.-m.s.). The nomenclature used and the fragment ions of use in the sequence determination of permethylated oligosaccharides have already been discussed (see 1.3).

The permethylated sample was introduced into the mass spectrometer using a deep probe attachment; gradual heating of the probe caused a slow release of the components, thus allowing partial separation of the de-pyruvylated (designated **10**) and pyruvylated (designated **11**) methylated derivatives prior to e.i. The spectra obtained were then subtracted from each other to yield fragmentation patterns pertinent to the compound of interest only. The origins of the diagnostic fragments for **10** and **11** are shown in Table I2 (see Fig. I9, A and B). Fragmentation ions common to both **10** and **11**, e.g. those formed by cleavages from the alditol end, were obtained from the unsubtracted spectrum of **10** (Table 12 and Fig I9C).

TABLE 12: M.S DIAGNOSTIC FRAGMENT IONS OF PERMETHYLATED, DE-PYRUVYLATED **10** AND PYRUVYLATED **11** REDUCED **P1**

Spectrum	*Fragment ions*												
	aA_3	aA_1	baA_3	baA_2	baA_1	$cbaA_3$	$cbaA_2$	$cbaA_1$	efA_2	efA_1	$defJ_0$	fA_1	efJ_1
A[a] B[a] C[b]	187 243	219 275	373 429	405[c] 461[c]	437 493	547 -	579 635	611 (667)[d]	378	(410)[d]	456	236	296

[a] A and B relate to the A-series fragments emanating from **10** and **11** respectively; see Fig. 19A and B
[b] C gives the J-series fragments arising from **10**; see Fig.19C
[c] Base peak
[d] Not observed



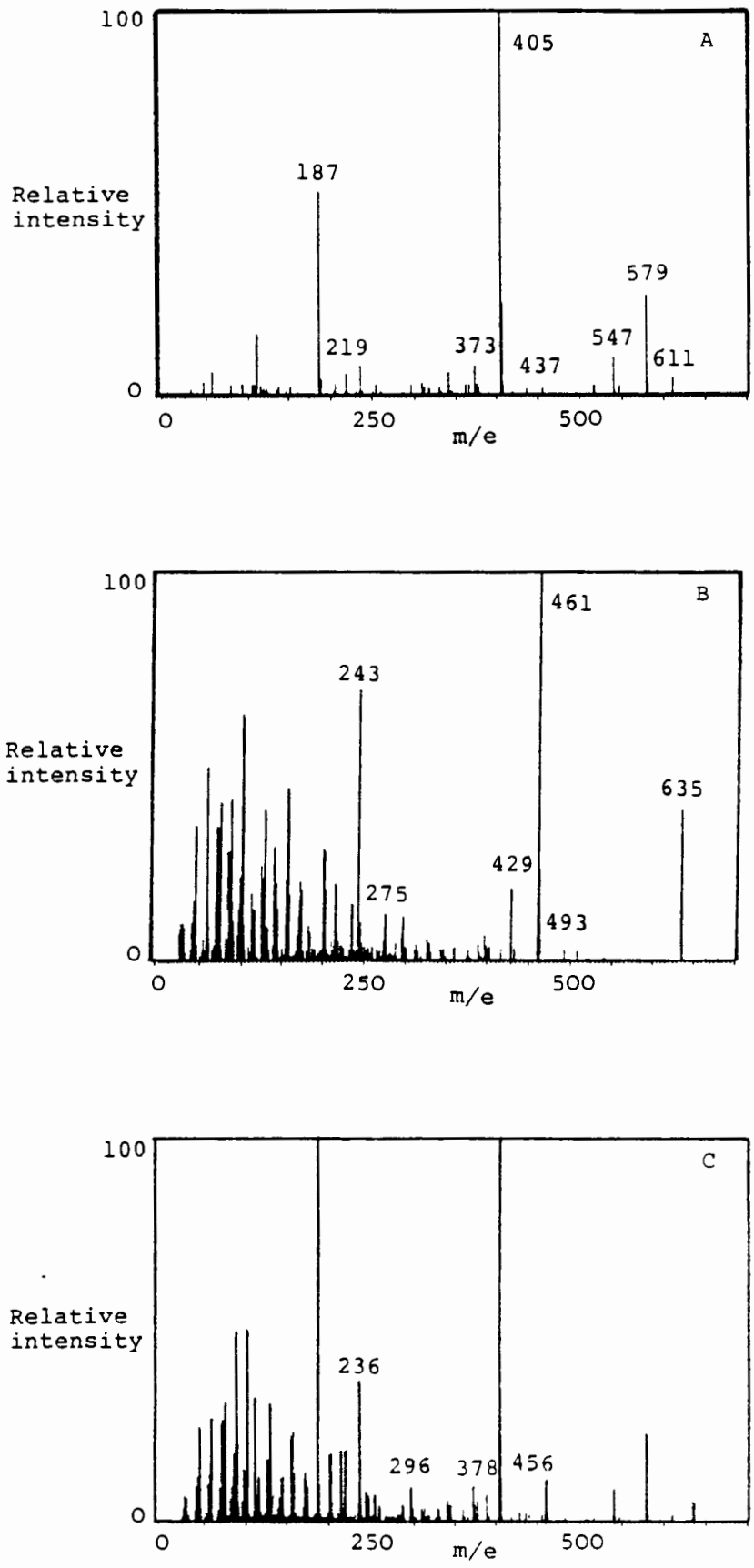


Figure 19: A and B, e.i.-m.s. fragmentation (A-series) patterns for **10** and **11**; C, fragmentation (J-series) for **10**.

The A-series ions formed from the non-reducing end of **10** (Table 12 and Fig. 19A) are characteristic of the hexose to hexuronic acid to deoxyhexose sequence. Information can also be gained concerning the inter-sugar linkages from the relative intensities of these fragments[38]. Because A_2 ions are formed by the preferential elimination of the 3-*O*-substituent, 2-linked residues readily eliminate methanol, resulting in a relatively large intensity of the A_2 ion compared with the parent A_1 ion[70]. The spectrum of **10** (Fig. l9A) shows that both the baA_2 (m/e 405) and $cbaA_2$ (m/e 579) fragment ions are of much greater intensity than their parent ions (m/e 437 and 611), confirming the **a**→2**b**→2**c** sequence. The same trend is observed for **11** (Fig. 19B) except that all the fragment ions are 56 a.m.u. higher in mass due to attachment of the methyl ester of pyruvic acid at the terminal glucose residue (**a**).

The fragmentation ions arising from cleavages from the reducing end (Table 12, Fig. 19C) are of low intensity, which is characteristic for (1→3)- and (1→2)-linked compounds[70]; however, they do confirm the deoxyhexose to deoxyhexose to deuterium-labelled hexitol sequence. The fragment efJ_1 (m/e 296) indicates a C-1 deuterated hexitol residue at the reducing end[38]. The presence of this fragment and the absence of efJo (HO=CH-O-**f** at m/e 282) shows that **e** must be 2-linked rather than 3-linked[75]. In contrast the detection of defJo (m/e 456) instead of $defJ_1$ (m/e 470) suggests that **d** is 3-linked. The position of linkage of the chain to the alditol (**f**) could not be determined using this technique because the fragments formed by cleavages within **f** would be of low intensity and in any event have a molecular mass outside the calibration range of the mass spectrometer.

Although the results of m.s. analysis confirm the sequence of sugar residues (*cf.* **9**), and support the proposed linkage assignments, they give no information regarding the

anomeric configurations of the constituent sugar units. N.m.r. analyses of the hexasaccharide **P1** and the dodecasaccharide **P2** were accordingly performed to furnish this and other structural information[124,132] (see 3.3).

4.5 N.M.R. STUDIES OF P1 AND P2

Appreciable quantities of **P1** and **P2** were isolated by preparative s.e.c. and used in the n.m.r. studies described here. A portion of **P1** was reduced with $NaBD_4$ to yield **P1**-ol, which was examined at both 200 and 500 MHz.

4.5.1 ASSIGNMENT OF ANOMERIC ^{1}H AND ^{13}C SIGNALS, AND PROOF OF →2e→3f SEQUENCE IN P1 (USING COSY AND HETCOR AT 200 MHz)

The ^{1}H- and ^{13}C-n.m.r. spectral data of **P1**, reduced **P1** (**P1**-ol), and **P2** are collected in Tables 13 and 14. The data for **P1** (Na^+ salt) confirm the hexasaccharide nature of the oligomer and show that the pyruvic acid acetal (δ 1.5, 3H) is intact. The spectrum recorded at ambient temperature shows two fractional resonances at δ 5.28 (0.4H) and 4.65 (O.6H), which represent the α- and β- configurations of the terminal D-galactopyranose residue. No evidence for the furanose forms could be seen at this temperature, although a signal at ~δ 5.29 did appear at 80°C which is characteristic of Gal*f*[15]. These signals disappeared

upon $NaBD_4$ reduction, confirming their assignment. The signals at δ 5.35, 5.18 and 4.96 are attributable to the two 2-linked and the 3-linked α-Rha*p* residues respectively, on the basis of their negligible coupling constant and by comparison with a polysaccharide containing the sequence α-Gal*p*A-(1→2)-α-Rha*p*-(1→3)-α-Rha*p*-(1→2)-α-Rha*p*-(1→(δ 5.19 and 5.16 for the 2-linked α-Rha*p* and δ 4.99 for the 3-linked α-Rha*p*)[210]. The remaining signals at δ 4.81 (obscured by the HOD signal at ambient temperature, but revealed at 80°C) and 4.75 were assigned to H-1 of the β-linked D-Glc*p* and D-Glc*p*A residues. The anomeric region of the ^{1}H-n.m.r. spectra of **P1** recorded at 25 and 80°C are displayed in Fig. 20.

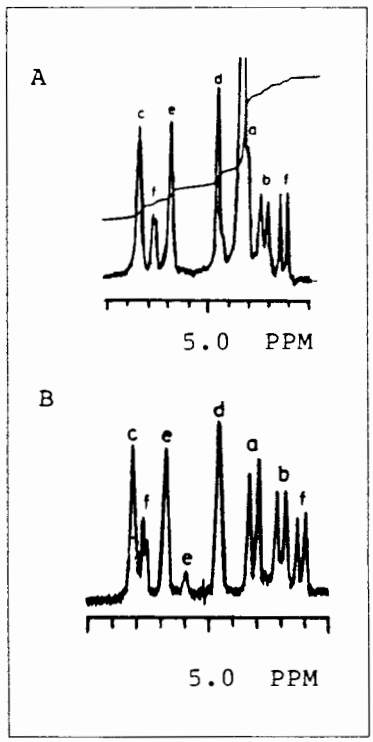


Figure 20: ^{1}H-n.m.r. spectrum (200 MHz) of the anomeric region of **P1** recorded at 25°C (A) and 80°C (B).

The correctness of the above interpretation is supported by COSY and HETCOR experiments conducted on **P1** and **P1**-ol. The anomeric ^{13}C resonances were readily assigned (Table 14) using the HETCOR diagram, which supports the α-Rha*p* assignments with the C-1 resonances of 2-linked residues at δ 101.2 and 101.4, while the C-1 resonance of the 3-linked α-Rha*p* is downfield (δ 102.7), as predicted[155]. The ^{13}C resonance at δ 101.4 is the only C-1 signal that is twinned (see 3.3.4); therefore the α-Rha*p* with H-1 at δ 5.18 must be adjacent to the reducing galactose residue and is designated α-Rha*p* (**e**). No such twinning is resolved in the proton spectrum recorded at ambient temperature, although at 80°C a small, broad signal does appear at

δ 5.09 (see Fig. 20B). This is most probably due to a different electronic environment experienced by H-1 of **e** as a consequence of the presence of furanose forms of the adjacent terminal galactose[15]. The removal of the C-1 signal twinning and the shifting of the H-1 resonance from δ 5.18 to 4.96 upon reduction of **P1** to **P1**-ol confirms this assignment of α-Rha*p* (**e**).

Consequently it follows that the other 2-linked rhamnosyl residue (δ 5.35 for H-1) must be attached to the glucuronic acid unit and is designated α-Rha*p* (**c**). The downfield shift of δ 5.35 appears to be characteristic of α-linked sugars having the *manno* configuration and a β-linked substituent at 0-2. (eg. β-Glc*p*-(1→2)-α-Rha*p*-(1→ at δ 5.31[211] and β-Glc*p*A-(1→2)-α-Man*p*-(1→ at δ 5.35[20]). The remaining α-rhamnosyl unit signal at δ 4.96 is due to the 3-linked residue, i.e. α-Rha*p* (**d**). The proton assignments of the two β-linked residues (Glc*p* at δ 4.81 and Glc*p*A at δ 4.75) could not be made by inspection alone, but followed from the ^{13}C resonance assignments. The low field signal (δ 104.9) was attributable to the pyruvylated β-Glc*p* (**a**) by comparison with the literature (δ 104.7)[136], and the signal at δ 103.8 to β-Glc*p*A (**b**). The upfield position of the acid anomeric signal when compared with the chemical shift of a 4-linked β-Glc*p*A residue (δ 104.2)[105] reflects the shielding effect of the O-2 substituent. Other distinguishing features of the spectra are presented in Tables 13 and 14, including the chemical shifts of the pyruvic acid acetal, from which the S-configuration of the acetal is evident (literature values are δ 1.50 (CH_3) and δ 25.5 (CH_3) for the S-configuration and δ 1.65 and 17.7 for the R-form)[136].

TABLE 13: ^{1}H-N.M.R. DATA (200 MHz) FOR P1, P1-OL AND P2

Compound	δ[a] (p.p.m.)	J[b] (Hz)	*Integral proton*	*Assignment*
P1 (Na^+ form)	5.35	n.r.	1.0	H-1(c) α-Rha*p*
	5.28	2.6	0.4	H-1(f) α-Gal*p*-OH
	5.18	n.r.	1.0	H-1(e) α-Rha*p*
	4.96	n.r.	1.0	H-1(d) α-Rha*p*
	4.81[c]	7.6	1.0	H-1(a) pyr-β-Glc*p*
	4.75	7.6	1.0	H-1(b) β-Glc*p*A
	4.65	6.8	0.6	H-1(f) β-Gal*p*-OH
	1.52	s	3.0	CH_3 of pyruvate
	1.28	~6.0	9.0	H-6(c,d,e) α-Rha*p*
	1.26	n.r.		
P1-ol (H^+ form)	5.35	n.r.	1.0	H-1(c) α-Rha*p*
	4.98	n.r.	2.0	H-1(d,e) α-Rha*p*
	4.81	7.6	1.0	H-1(a) pyr-β-Glc*p*
	4.72[c]	n.r.	~1.0	H-1(b) β-Glc*p*A
	1.52	s	3.0	CH_3 of pyruvate
	1.29	n.r.	9.0	H-6(c,d,e) α-Rha*p*
P2 (Na^+ form)	5.34	n.r.	1.0	H-1(c) α-Rha*p*
	5.27	n.r.	0.4	H-1(f') α-Gal*p*-OH
	5.20	n.r.	3.0	H-1(e,e') α-Rha*p*
	5.17	n.r.		H-1(c') α-Rha*p*
	5.12	n.r.	0.5	unassigned
	4.96	n.r.	2.0	H-1(d,d') α-Rha*p*
	4.90 [c]			H-1(a,a')pyr-β-Glc*p*
		complex	~5.5	H-1(b,b') β-Glc*p*A
				H-1(f) β-Gal*p*
	4.57			H-1(f') β-Gal*p*-OH
	4.53	n.r.	1.0	unassigned[d]
	1.51	s	~5.0	CH_3 of pyruvate
	1.29	n.r.	18.0	H-6(c,c',d,d',e,e') α-Rha*p*

[a] Chemical shift relative to internal acetone (δ 2.21), spectra recorded at ambient temperature

[b] Coupling constant $J_{1,2}$ for H-1; $J_{5,6}$ for H-6; s = singlet; n.r. = not resolved

[c] N.m.r. details obtained from spectrum recorded at 85°C as peak obscured by HOD at 25°C

[d] Signal attributable to either H-2 of α-Rha*p* (c') or H-3 of β-Gal*p* (f)

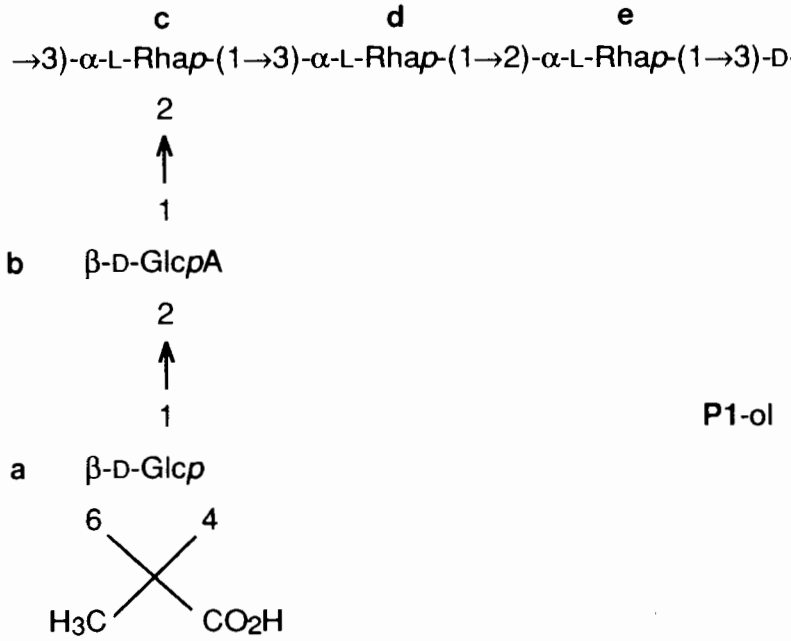
TABLE 14: ^{13}C-N.M.R. DATA (50.3 MHz) FOR P1, P1-OL AND P2

Compound	δ[a] (p.p.m.)	*Assignment*
P1 (Na^+ form)	104.9	C-1(a) pyr-β-Glc*p*
	103.8	C-1(b) β-Glc*p*A
	102.7	C-1(d) α-Rha*p*
	101.4[b]	C-1(e) α-Rha*p*
	101.2	C-1(c) α-Rha*p*
	97.0	C-1(f) α-Gal*p*-OH
	93.2	C-1(f) β-Gal*p*-OH
	25.3	CH_3 of pyruvate
	17.5	C-6(c,d,e) α-Rha*p*
	17.4	
P1-ol (H^+ form)	104.8	C-1(a) pyr-β-Glc*p*
	103.5	C-1(b) β-Glc*p*A
	102.3	C-1(d) α-Rha*p*
	101.1	C-1(e) α-Rha*p*
	100.9	C-1(c) α-Rha*p*
	25.0	CH_3 of pyruvate
	17.3	C-6(c,d,e) α-Rha*p*
P2 (Na^+ form)	105.2	C-1(f) β-Gal*p*
	105.0	C-1(a,a') pyr-β-Glc*p*
	103.9	C-1(b,b') β-Glc*p*A
	103.8	
	102.8	C-1(d,d') α-Rha*p*
	102.0	C-1(c') α-Rha*p*
	101.5	C-1(e,e') α-Rha*p*
	101.3	C-1(c) α-Rha*p*
	97.0	C-1(f') β-Gal*p*-OH
	93.0	C-1(f') α-Gal*p*-OH
	25.3	CH_3 of pyruvate
	17.9	C-6(c,c',d,d',e,e') α-Rha*p*
	17.4	

[a] Spectra recorded at ambient temperature, chemical shift values relative to internal acetone (δ 31.0)

[b] Signal split by anomerisation of the reducing galactose group

4.5.2 ESTABLISHMENT OF SUBSTITUTION PATTERNS OF SUGAR RESIDUES IN PI, FROM ASSIGNMENTS OF RING ^{1}H AND ^{13}C SIGNALS (USING COSY AND HETCOR AT 500 MHz)



Although the anomeric configuration of the constituent sugars of **P1** have been established unambiguously, the positions of linkage of the residues were inferred from the chemical shifts of the anomeric signals only. To obtain assignments of the pertinent ring protons and carbons, **P1**-ol was examined at 500 MHz. The COSY experiment (Fig. 21) aided the unambiguous assignment of the H-2 and H-3's of the α-Rha*p* residues and the H-2's of the β-linked sugars; however, further connectivities could not be made with confidence owing to the complexity of the ^{1}H-n.m.r. spectrum (Fig. 22).

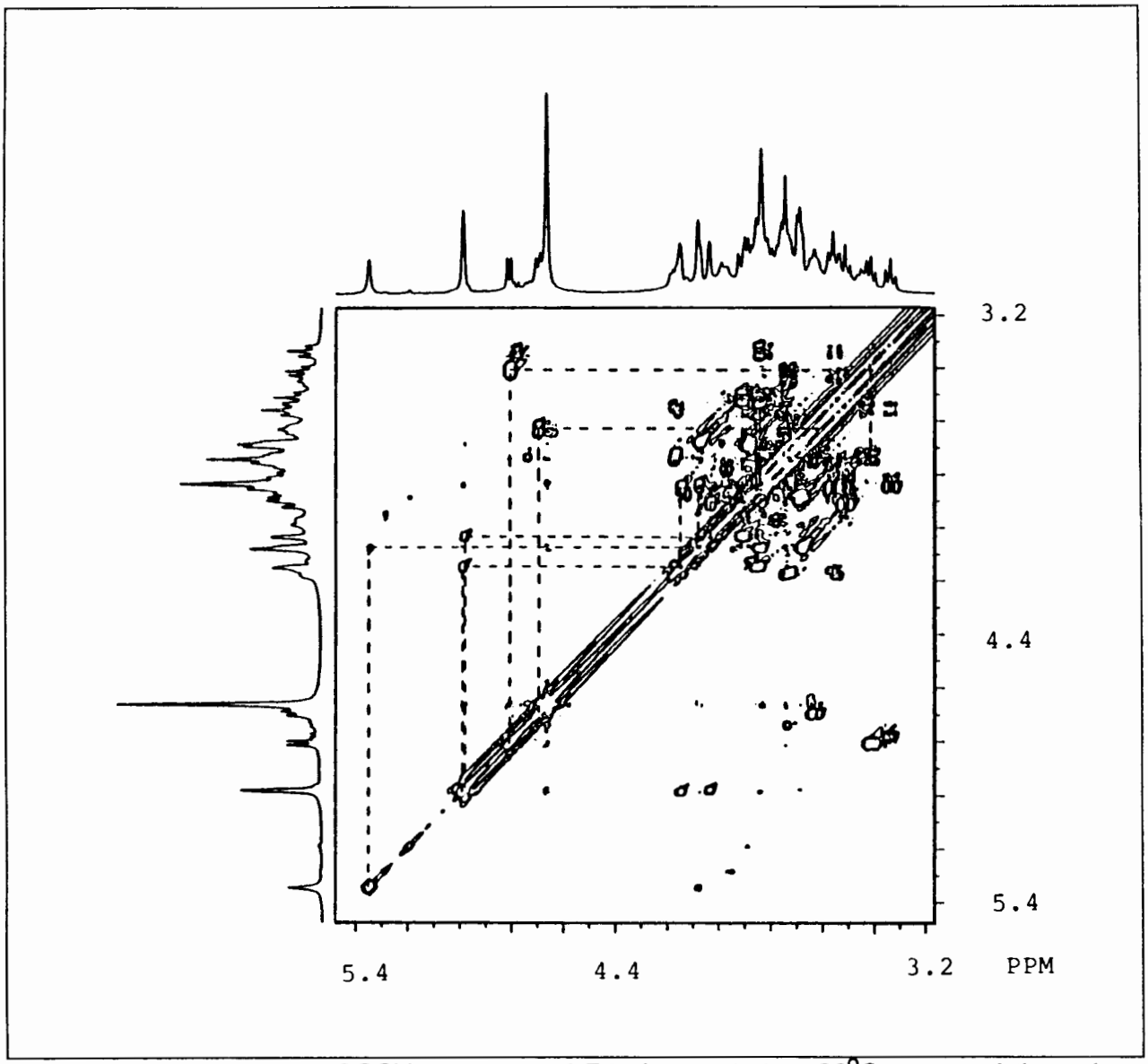


Figure 21: Expanded COSY spectrum of **P1**-ol recorded at 30°C, some of the spin systems are indicated.

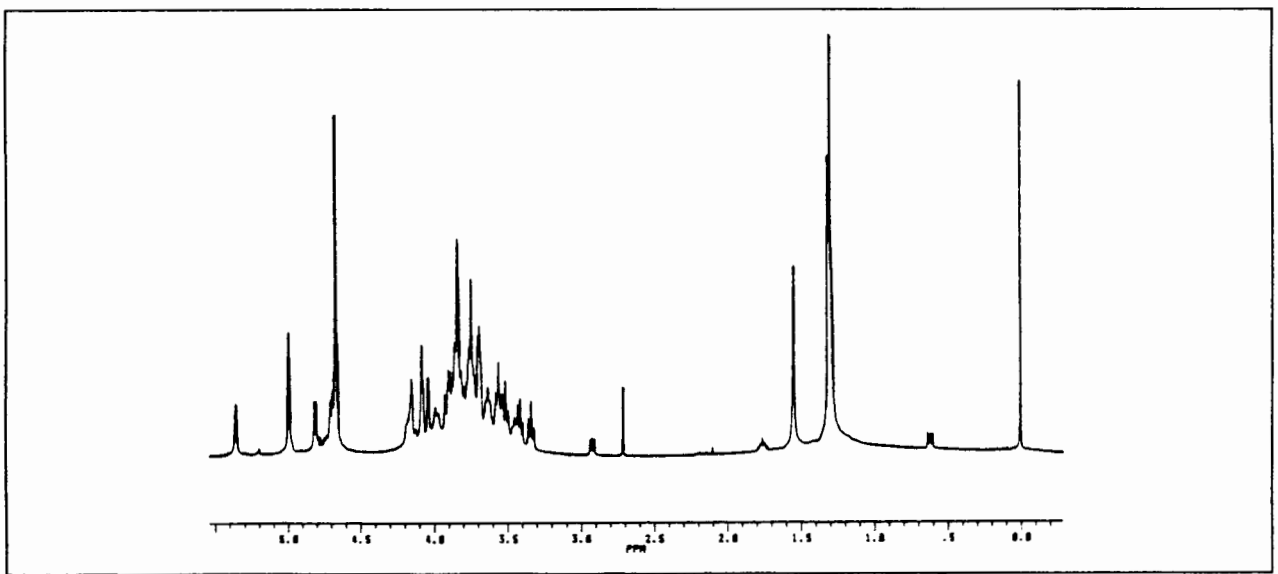


Figure 22: ^{1}H-n.m.r. spectrum (500 MHz) of **P1**-ol recorded at 25°C.

The data are presented in Table 15 and the ^{13}C assignments, which follow using the HETCOR diagram (Fig. 23), in Table 16 (see Fig. 24 for ^{13}C-n.m.r. spectrum of **P1**-ol).

TABLE 15: ^{1}H-N.M.R. DATA (500 MHz) AND GLYCOSYLATION SHIFTS[a] FOR **P1**-OL.

Sugar residue	*Chemical shift (p.p.m.)*		
	H-1	H-2	H-3
pyr-ß-Glc*p* (**a**)[b]	4.81 (+0.44)	3.33 (+0.08)	n.r.
ß-Glc*p*A (**b**)[c]	4.70 (-0.06)	3.63 (+0.18)	3.74 (-0.10)
α-Rha*p* (**c**)[d]	5.35 (+0.65)	4.08 (+0.15)	3.83 (+0.12)
α-Rha*p* (**d**)[d]	4.98 (+0.28)	4.15 (+0.22)	3.85 (+0.15)
α-Rha*p* (**e**)[d]	4.98 (+0.28)	4.03 (+0.10)	3.90 (+0.19)

Glycosylation shifts in parentheses.

[a] A downfield shift relative to the model compound is considered positive
[b] Model compound ß-Glc*p*-OMe (Ref. 177)
[c] Model compound ß-Glc*p*A-OH (Table 2)
[d] Model compound α-Rha*p*-OMe (Ref. 188)

TABLE 16: ^{13}C-N.M.R. DATA (125 MHz) AND GLYCOSYLATION SHIFTS[a] FOR **P1**-OL.

Sugar residue	*Chemical shift (p.p.m.)*		
	C-1	C-2	C-3
pyr-ß-Glc*p* (**a**)[b]	105.4 (+8.7)	74.2 (-0.9)	n.r. -
ß-Glc*p*A (**b**)[c]	104.0 (+7.2)	82.5 (+7.6)	n.r. -
α-Rha*p* (**c**)[d]	101.4 (+6.4)	82.3 (+10.4)	71.2 (+0.1)
α-Rha*p* (**d**)[d]	102.5 (+7.5)	71.4 (-0.5)	78.6 (+7.5)
α-Rha*p* (**e**)[d]	101.6 (+6.6)	79.0 (+7.1)	72.3 (+1.2)

Glycosylation shifts in parentheses

[a] A downfield shift relative to the model compound is considered positive
[b] Model compound ß-Glc*p*-OH (Ref. 155)
[c] Model compound ß-Glc*p*A-OH (Table 1)
[d] Model compound α-Rha*p*-OH (Ref. 155)

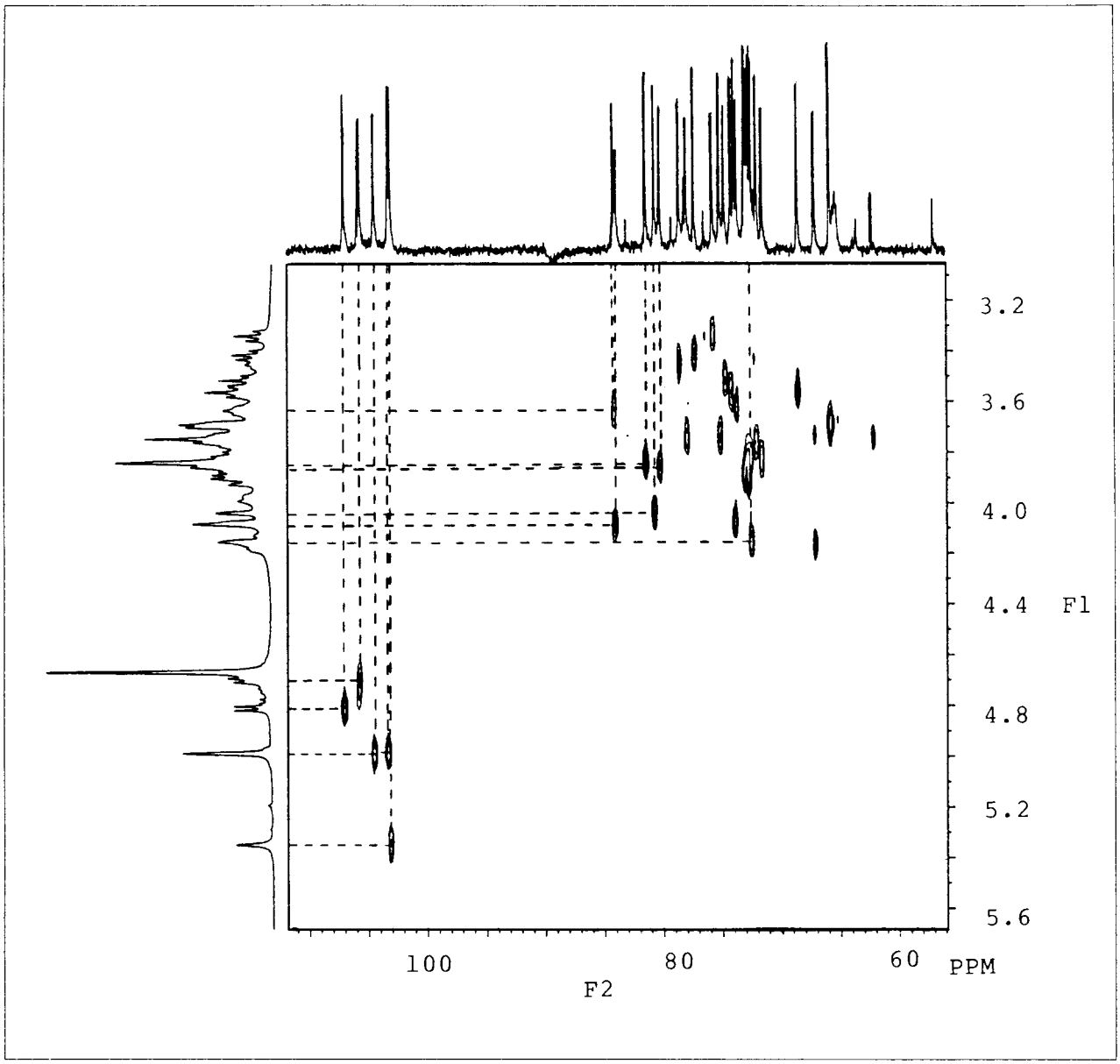


Figure 23: Expanded HETCOR spectrum of **P1**-ol recorded at 30°C, some of the C,H correlations are indicated ($J_{C,H}$ = 140 Hz).

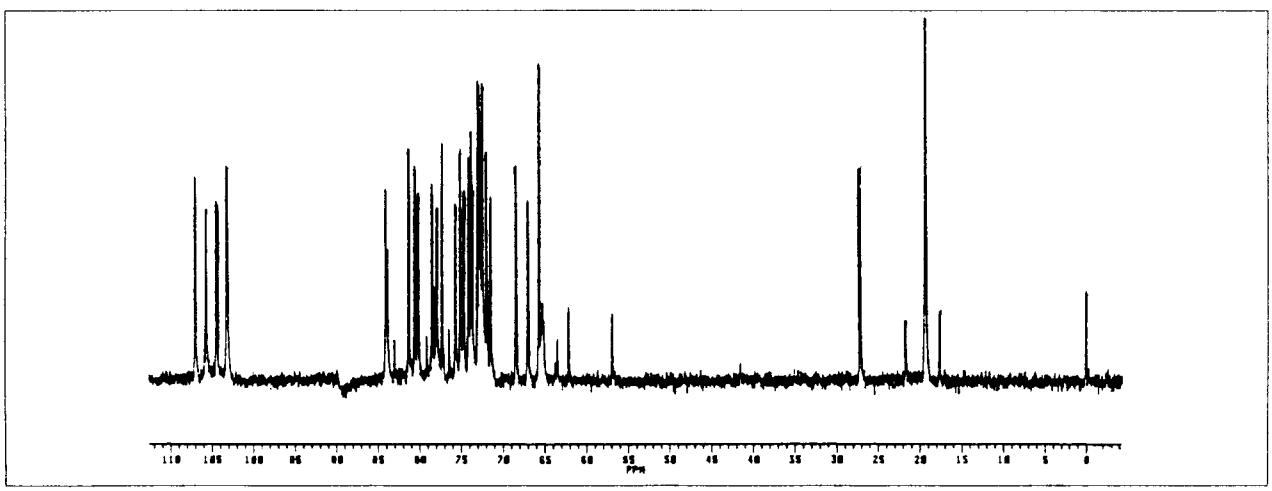


Figure 24: ^{13}C-n.m.r. spectrum (125 MHz) of **P1**-ol recorded at 25°C.

Comparison of the ^{1}H and ^{13}C chemical shifts with those of model compounds (see Tables 15 and 16) clearly revealed the glycosylation shifts and thus confirmed the putative linkage sites. It is interesting to note that the H-2 signal of the 3-linked α-Rha*p* (**d**) resonates further downfield than the corresponding protons of the 2-linked residues although the connected carbons do not. This unusual feature was also observed in the ^{1}H-n.m.r. spectrum of a linear homopolymer of alternating 2- and 3-linked α-L-Rha*p* units[138], which suggests that the H-2 of the 3-linked α-Rha*p* residue is probably involved in a non-bonded interaction with an oxygen atom in the adjacent 2-linked α-Rha*p* unit. This was confirmed by model building of the rhamnosyl residues in which the conformation, determined on the basis of the *exo*-anomeric effect, demonstrated the possibility of C-2 hydroxyl interaction of the 3-linked α-Rha*p* with the ring oxygen of the contiguous 2-linked residue such that the axial H-2 is deshielded.

4.5.3 CONFIRMATION OF THE ABSOLUTE CONFIGURATIONS OF SOME SUGAR RESIDUES OF P1-OL FROM ^{13}C-N.M.R. DATA

The correlations established between the magnitude of ^{13}C glycosylation shifts and configurational factors published by Kochetkov *et al.*[156] were used to confirm some of the absolute configuration assignments of the constituent sugars (previously determined by circular dichroism measurements); the applicability of these results are limited to 3-linked sugar residues and only certain (1→2) and (1→4) linked sugar units. Consequently only the **b**→**c**→**d**→**e** sequence could be investigated.

The glycosylation shift of 7.2 p.p.m. for C-1 of β-Glc*p*A is consistent with the shift of ~ 8 p.p.m. reported for C-1 of β-linked D-hexose residues attached to O-2 of D-hexoses carrying

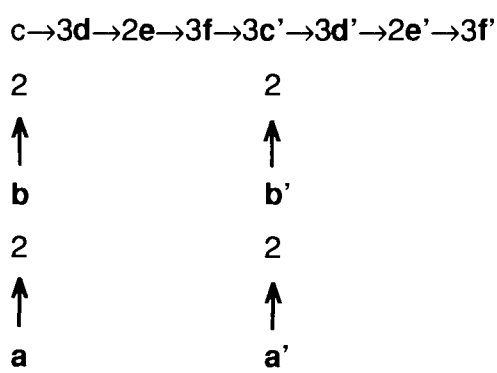
an axial substituent at C-2, thus confirming the absolute and anomeric configurations of β-D-Glc*p*A-(1→2)-α-L-Rha*p* i.e. **b**→2**c**.

The glycosylation shift of 6.4 p.p.m. for C-1 of α-Rha*p* (**c**) is slightly low in value due to O-2 glycosylation; however, it is in keeping with the relatively large α-effect (7-9 p.p.m.) characteristic of α-linked L-hexoses attached to O-3 of L-hexose units bearing an axial substituent at C-2, thus confirming the absolute and anomeric configurations of α-L-Rha*p*-(1→3)-α-L-Rha*p* i.e. **c**→3**d**. This result is supported by the glycosylation shifts (α and β) found for residue **d** *viz.* α-effect for C-3: 7.5 p.p.m. (8-10 p.p.m. predicted) and β-effect for C-2: -0.5 p.p.m. (1.5 p.p.m. predicted).

Finally, the α-effect of 7.5 p.p.m. for C-1 of α-Rha*p* (**d**) is in conformity with the value of ~ 8 p.p.m. reported for α-linked L-hexose attached to O-2 of L-hexose units having the *manno* configuration, thereby confirming the configurational factors of α-L-Rha*p*-(1→2)-α-L-Rha*p* i.e. **d**→2**e**.

4.5.4 THE →3f→3c' SEQUENCE IN P2, f BEING β-LINKED.

In order to establish the anomeric configuration of all the sugar residues in the polymer, the n.m.r. spectra of the dimer (**P2**) were also examined.



P2

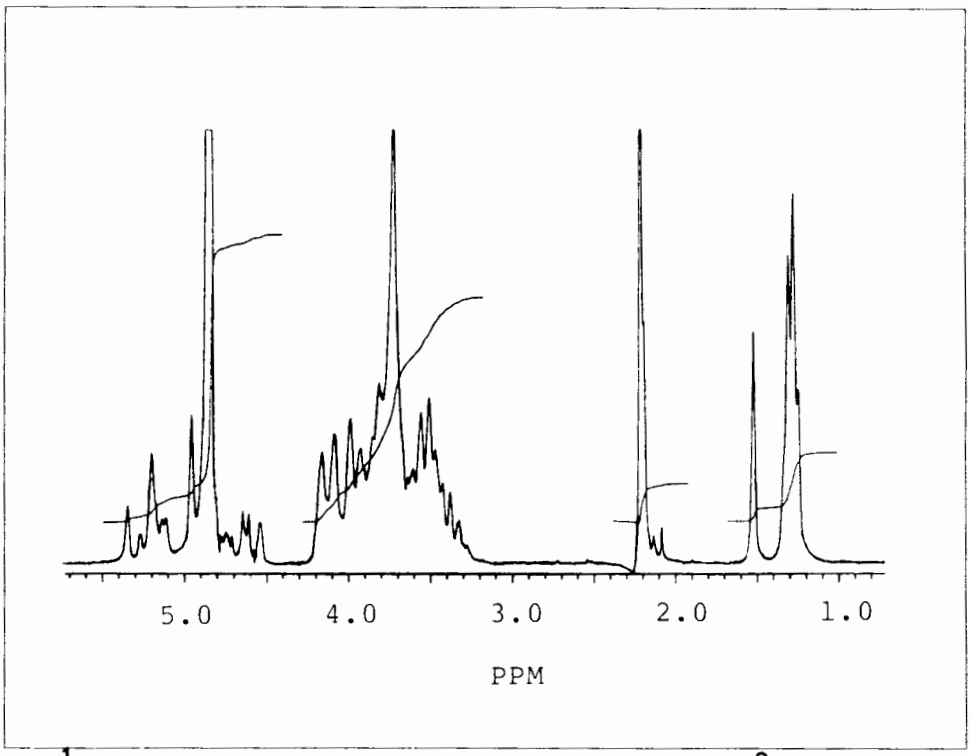


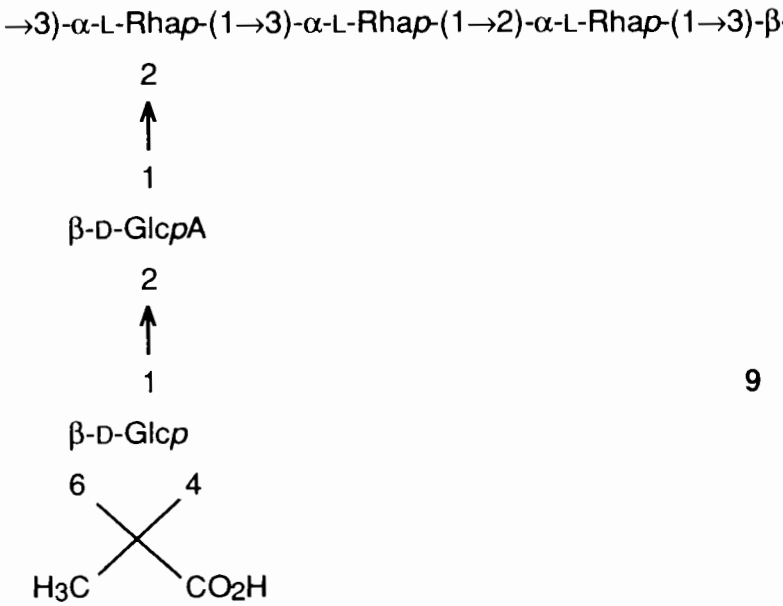
Figure 25: ^{1}H-n.m.r. spectrum (200 MHz) of **P2** recorded at 25°C

From methylation analysis (see 4.3) it is known that the Gal*p* residue of one repeating unit is joined to O-3 of a 2-linked α-Rha*p* of the second unit and therefore comparison of the spectra of **P1** and **P2** should reveal the configuration and position of attachment. The complexity of the spectra of **P2** (see Fig. 25) compared with those obtained for **P1** reflects the nonequivalence of the sugar residues. This complexity was further exacerbated by the partial loss of pyruvic acid, which caused twinning of the signals arising from the β-Glc*p* groups. Consequently no β-linked sugar proton signal assignments could be made with confidence, although all the α-linked sugar signals could be accounted for (Table 13). This proved indirectly that the in-chain Gal*p* (**f**) residue must be β-linked, which is also supported by integration of the appropriate regions of the spectrum. This assignment is corroborated by the appearance of a signal at δ 105.2 in the ^{13}C-n.m.r. spectrum of **P2** which is not present in the spectrum of **P1** (Table 14). This compares well with the chemical shift (δ 105.15) assigned to the 3-linked β-Gal*p* residue found in a polymer containing a trisaccharide repeating unit of →3)-α-Gal*p*-(1→3)-β-Gal*p*-(1→3)-β-Glc*p*-(1→[28].

The 2-linked rhamnosyl residue to which the Gal*p* in-chain unit is attached is revealed by comparison of the ^{1}H-n.m.r. spectra of **P1** and **P2** (Table 13). In the spectrum of **P1** recorded at ambient temperature the α-Rha*p* residue attached to the 3-linked α-Rha*p* resonates at δ 5.35 and the other, 2-linked α-Rha*p* at δ 5.18. In the spectrum of **P2**, however, the signal at δ 5.34 integrates for only one proton, while the signals at ~ δ 5.18 integrate for three protons. These are attributable to the two rhamnosyl residues attached to the galactose units [one at the reducing end (**e'**) and the other in-chain (**e**)] and the doubly branched α-Rha*p* residue (**c'**) not found in **P1**. The upfield shift of the signal emanating from this residue (δ 5.35 when 2-linked and δ 5.18 when 2,3-linked) is typical of the shifts observed upon increased glycosylation[212]. Thus, the anomeric configurations of all the sugar residues in the *Klebsiella* K36 polysaccharide have been established.

4.6 CONCLUSION

Structural studies of the oligosaccharides obtained by bacteriophage degradation of *Klebsiella* K36 polysaccharide have established the revised structure of the repeating unit of the polymer to be as shown in **9**.



9

Methylation studies delineated the substitution pattern of the sugar residues (implicit in **9**) and unequivocally showed that the D-glucuronic acid must be linked through O-2, and not O-4 as originally proposed (*cf.* **5**).

The sequence of sugar residues was established by mass spectral analysis of the permethylated oligosaccharides (see 4.4), while n.m.r. studies provided the anomeric configurations. In addition, detailed n.m.r. investigations (see 4.5) confirmed the inter-sugar residue linkages as determined by methylation analyses (see 4.3) and

furthermore established the sequence of rhamnosyl residues as shown in 9, thereby demonstrating the usefulness of this technique in the structural elucidation of saccharides.

4.7 EXPERIMENTAL

4.7.1 OLIGOSACCHARIDE PRODUCTS A AND B

A culture of *Klebsiella* K36 (8306) was obtained from Dr I. Ørskov, Copenhagen; polysaccharide was prepared on agar medium A, and isolated and purified as described in section 1.2 (yield, 3g). The material was shown to be homogeneous by electrophoresis and s.e.c. (mol. wt. 720 000, column 1). ^{1}H- and ^{13}C- n.m.r. spectra were recorded on Varian XL-100 (at 95°C) and Varian CFT-20 (at ambient temperature) instruments respectively; the results showed good agreement with those previously published (*viz.* ^{1}H data, ref. 207; ^{13}C data, ref. 213). Bacteriophage Ø36 was propagated as described in section 2.2, except that the phage was purified and concentrated by polyethylene glycol precipitation. The phage was resuspended in physiologically-buffered saline (P.B.S.) and used in the depolymerisation experiments: A, 30mL of phage solution was incubated with 1g of polysaccharide in 200mL of water at 37°C for 3 days; B, 40mL of phage solution was incubated with 1.34g of polysaccharide in 275mL of water at 37°C for 4 days. The oligosaccharide products (designated **A** and **B**) were isolated by centrifugation,

freeze-drying and desalting (on a column of Sephadex G-10 (100 x 25 cm) eluted with 1000:10:4 water-pyridine-acetic acid).

A and **B** were analysed by s.e.c. on column 2 and by n.m.r. spectroscopy (as described above). Sugar analyses were performed on 10mg samples (see 2.9 and Table 9 for details). Samples (50mg) of **A** and **B** were treated with Amberlite IR-120 (H^+) resin prior to methylation analysis (see 2.10). Hydrolysates of methylated products were analysed by p.c. (solvent E, spray 1) and the derived alditol acetates by g.l.c. and g.l.c.-m.s. as described in Table 10.

4.7.2 STRUCTURAL STUDIES OF P1 AND P2

P1 and **P2** were isolated from oligosaccharide products **A** and **B** by preparative s.e.c. Carbohydrate material was located by optical rotation (α_{365}) and t.l.c. (solvent F, spray 3). Column 4 yielded 35mg of **P1** which after characterisation by n.m.r., was reduced with $NaBD_4$ (10mg, overnight) prior to permethylation using the Hakomori method. This product was examined by mass spectrometry (see 4.4).

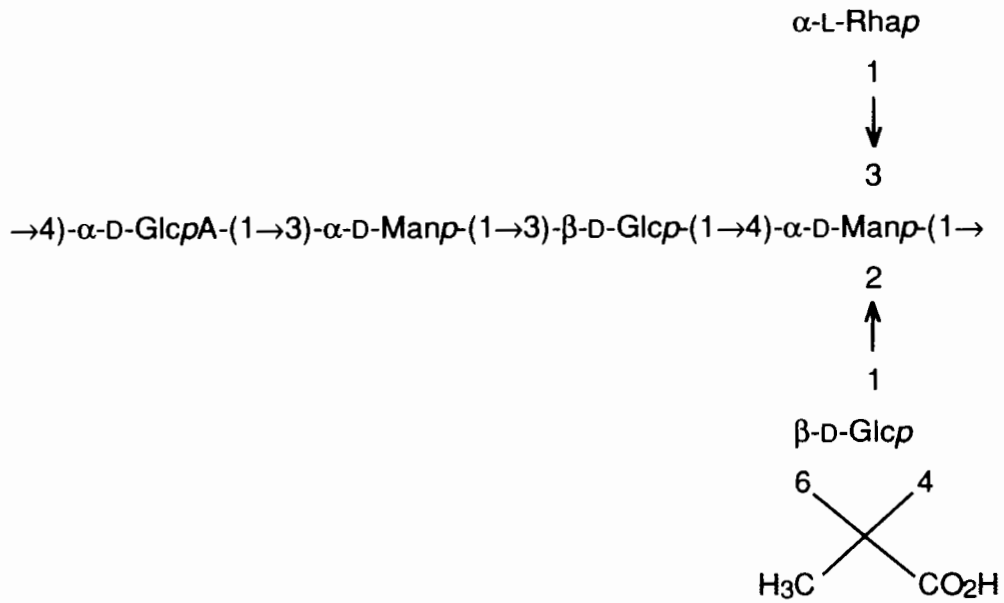
Several fractionations through column 5 yielded **P1** (270mg) and **P2** (150mg) which were used in the n.m.r. studies conducted on the Varian VXR-200 instrument. **P1** (120mg) was reduced with $NaBD_4$ (40mg) to yield **P1**-ol, which was studied using both the Varian VXR-200 and Bruker WM500 spectrometers.

CHAPTER 5

STRUCTURAL STUDIES OF THE NOVEL OLIGOSACCHARIDES PRODUCED BY BACTERIOPHAGE Ø64 LYASE ACTIVITY ON *KLEBSIELLA* K64 POLYSACCHARIDE

5.1 INTRODUCTION

The serotype K64 is one of two strains of *Klebsiella* whose capsular polysaccharides are composed of D-glucuronic acid, D-glucose, D-mannose and L-rhamnose residues[12]. Previous studies[214,215] involving partial depolymerisation by acid hydrolysis and Smith degradation have led to the formulation of a repeating unit (**12**)[215]; the unusual doubly-branched hexasaccharide structure includes one pyruvate ketal and some acetate ester.



12

Bacteriophage-borne enzymic depolymerisation of the capsular polysaccharide was conducted with a view to isolating the intact repeating unit, which would be more amenable to further chemical and spectroscopic studies and thus permit confirmation of the repeating structure and ultimately location of the *O*-acetyl substituent. Aspects of this work including

the unusual mode of enzyme cleavage and structural analysis of the oligosaccharides thereby obtained have been reported[107-109].

5.2 THE PRODUCTION AND ANALYSIS OF OLIGOSACCHARIDES P1 AND P2

Polysaccharide capsular material from *Klebsiella* K64, after isolation and purification[215], had properties and constituents (Table 17) in agreement with those found earlier.

TABLE 17: SUGAR ANALYSIS OF THE POLYSACCHARIDE FROM *KLEBSIELLA* K64 (I) COMPARED WITH PREVIOUS RESULTS (II)[215]

Sugar[a]	*Mole %*	
	I	II
Rhamnose	17	19
Mannose	32	27
Glucose	35	38
Glucuronic acid	16	[b]

[a] Neutral sugars analysed as alditol acetates by g.l.c. and uronic acid by colorimetric assay (Ref. 118)
[b] Assumed 16%, and sugar ratios calculated accordingly

Bacteriophage Ø64 was propagated on its host strain until ~ 10^{13} plaque-forming units (p.f.u.) were obtained (sufficient to degrade ~ 1g of polysaccharide[94]). Depolymerisation experiments then yielded oligomers which were isolated and separated into monomeric (**P1**) and dimeric (**P2**) fractions by s.e.c. combined with partition chromatography on silica[107]. The results of the analysis and measurement of d.p. by the method of

Morrison[115b] (Table 18) confirm that **P1** is a hexasaccharide, **P2** is the dimer, and both have a mannose residue as the terminal, reducing unit.

TABLE 18: DETERMINATION OF D.P. AND CHARACTERISATION OF THE REDUCING END OF **P1** AND **P2**

Peracetylated derivative of	*T**	*Mole %*	
		P1	*P2*
Rhamnononitrile	0.30	17	16
Mannononitrile	0.67	17	25
Gluconononitrile	1.00	40	44
Mannitol	1.15	26	15

* Retention times, determined on column B at 220°C, relative to 2,3,4,5,6-penta-*O*-acetylgluconononitrile

Cleavage could have occurred at either mannosidic linkage (*cf.* **12**). Reduction of **P1** to **P1**-ol with sodium borodeuteride ($NaBD_4$) followed by methylation analysis (Table 19, column II) yielded a component not present among the partially methylated alditol acetates of unreduced **P1** (column I), which corresponded to the acetylated derivative of C1 deuterium-labelled 1,5,6-tri-*O*-methylmannitol **13** (see Fig. 26 for m.s. fragmentation pattern of **13**). Thus cleavage must have occurred between the highly-substituted mannosyl residue (see structure **12**) and the glucuronic acid residue, a favoured position, with respect to the acid group, in several known cases of bacteriophage action on *Klebsiella* polysaccharides[90].

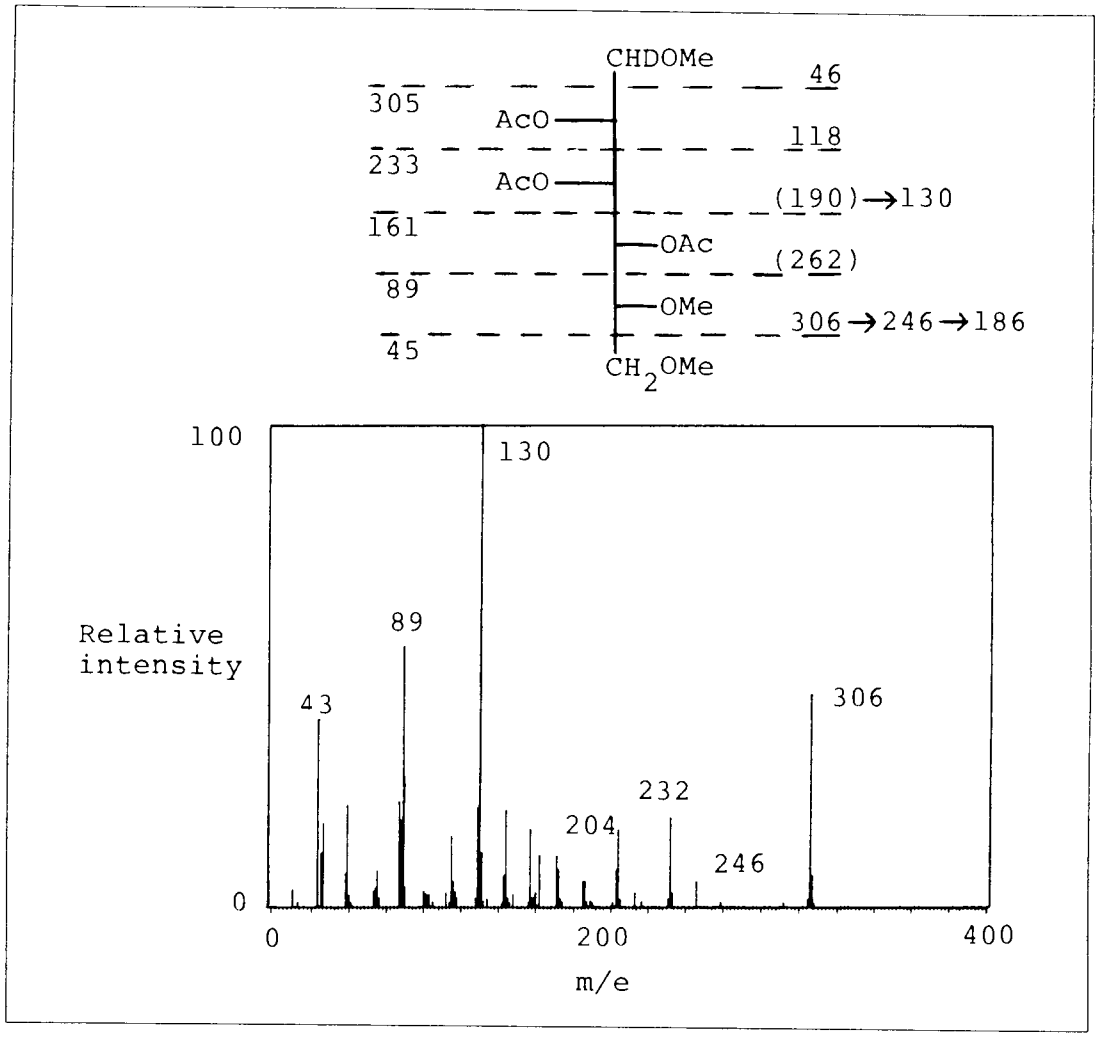


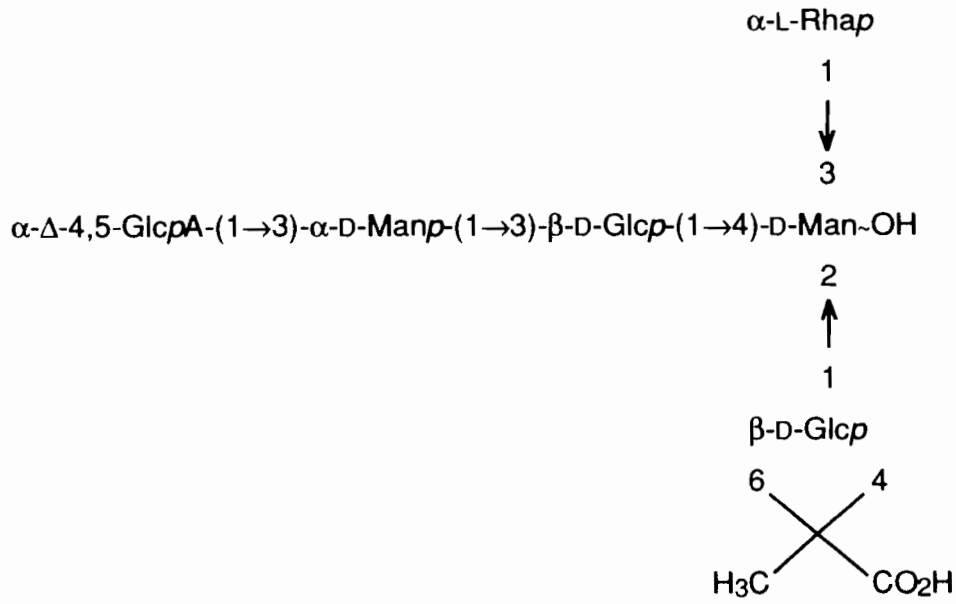
Figure 26: M.s. fragmentation pattern of 13

TABLE 19: METHYLATION ANALYSIS OF P1, P2 AND DERIVED OLIGOSACCHARIDES

Partially methylated alditol acetates[a]	T[b]	*Mole %*[c]			
		I	II	III	IV[d]
2,3,4-Rha	0.68	20	16	15	18
1,3,5,6-Man[e]	0.88	-	-	5	-
2,3,4,6-Man	0.99	9[f]	17[f]	43	26
2,3,4,6-Glc	1.00				
1,5,6-Man[e]	1.16	-	24	14	7
2,4,6-Glc	1.48	22	18	18	24
2,4,6-Man	1.52	20	16	5	10
6-Man	2.56	15	-	-	7
2,3-Glc	2.74	14	9	-	8[g]

[a] 2,3,4,6-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol,etc
[b] Retention times, determined on column C at 215°C, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol. Assignments confirmed by g.l.c.-m.s.
[c] Values were corrected by use of effective, carbon-response factors given by Sweet *et al.* (Ref. 112)
[d] I, methylated P1; II, methylated P1-ol; III, methylated autohydrolysed P1-ol; IV, carboxyl-reduced, methylated P2-ol
[e] C-1 deuterated according to g.l.c.-m.s.
[f] From some loss of acid (uronic, or pyruvic ketal)
[g] C-6 di-deuterated according to g.l.c.-m.s.

Prior to methylation analysis the sample had been used for high temperature n.m.r. studies, so that the detection of terminal glucose due to partial loss of the acid-labile pyruvic acid ketal is to be expected; there is a concomitant decrease in the peak due to 2,3-di-*O*-methyl glucose (Table 19). The appearance of 2,3,4,6-tetra-*O*-methylmannose at the expense of the 2,4,6-tri-*O*-methylmannose peak can only be explained by removal of what was the terminal glucuronic acid residue. Uronic acid glycosides are well known to be resistant to hydrolysis[38] and therefore the glycosidic link must have been destabilised during bacteriophage action. It has now become apparent that the bacteriophage Ø64 enzyme acts as a lyase[110], catalysing the β-elimination reaction of the highly substituted mannosyl residue from the 4-position of the glucuronic acid within the polysaccharide chain. In this way an unsaturated derivative of glucuronic acid is generated at the non-reducing end of the oligomers produced (**14**).



14

Degradation of heteroglycans by lyase action on bacterial products is generally common[110,216], but only two other examples of bacteriophage-associated lyases acting on *Klebsiella* polysaccharides have been reported; namely for serotypes K5[92] and K14[96].

5.3 CHARACTERISATION OF THE UNSATURATED URONIC ACID RESIDUES

Colorimetric assay for glucuronic acid[118] in **P1** gave a negligible result. Indirect evidence for a modified residue was initially obtained by the absence of detectable glucuronic acid on paper chromatography of an acid hydrolysate of **P1**; likewise methylation analysis of **P1** showed the absence of any uronic acid derivatives. These observations are consistent with the presence of the unsaturated derivative of glucuronic acid, which is known to be degraded during acid hydrolysis[38,61]. The unsaturated acid residue, as its Na^+ salt, was characterised by ultra-violet (λ_{max} 233 nm) and infrared [υ(C=C) at 1664 cm^{-1}] absorption spectroscopy, these values being in conformity with those previously reported[92,216,217]. ^{1}H-n.m.r. spectra showed downfield doublets which are not present in n.m.r spectrum of the original polymer. These signals are typical[92,217-219] of the olefinic (H-4) and anomeric (H-1) protons of an α-linked unsaturated acid residue, while C-5 (145.6 p.p.m.) and C-4 (109.3 p.p.m.) were readily identified by ^{13}C-n.m.r.[124]. Protonation of the Na^+ salt of **P1** caused the expected downfield shifts[218] of H-4 and H-1 (Table 20), confirming the assignments.

TABLE 20: ^{1}H-N.M.R. DATA (90 MHz) FOR **P1** (Na^+) AND **P1** (H^+)

Compound	δ*	$J_{1,2}$	*Assignment*	
P1 (Na^+ form)	5.76	3.2 ($J_{3,4}$)	α-Hex-4-ene*p*A	H-4
	5.33	2.8	α-Hex-4-ene*p*A	H-1
	5.24	s	α-Man*p*-OH	H-1
	5.15	1.7	α-Man*p* (c)	H-1
	4.89	s	α-Rha*p* (t)	H-1
	4.51	7.5	β-Glc*p* (c)	H-1
	4.48	7.6	pyr-β-Glc*p*	H-1
	1.44	s	CH_3 of pyr	
	1.24	6.2 ($J_{5,6}$)	CH_3 of Rha	
P1 (H^+ form)	6.20	3.2 ($J_{3,4}$)	α-Hex-4-ene*p*A	H-4
	5.36	2.8	α-Hex-4-ene*p*A	H-1
	1.50	s	CH_3 of pyr	
	Other peaks unchanged			

* Chemical shift relative to internal DSS (δ 0.0), spectra recorded at ambient temperature

The lability of the unsaturated acid residue was demonstrated during the course of high temperature n.m.r experiments in which the signals assigned to H-1 and H-4 gradually disappeared being replaced by new signals spaced between δ 6.6 and δ 8.3 (see Fig. 27).

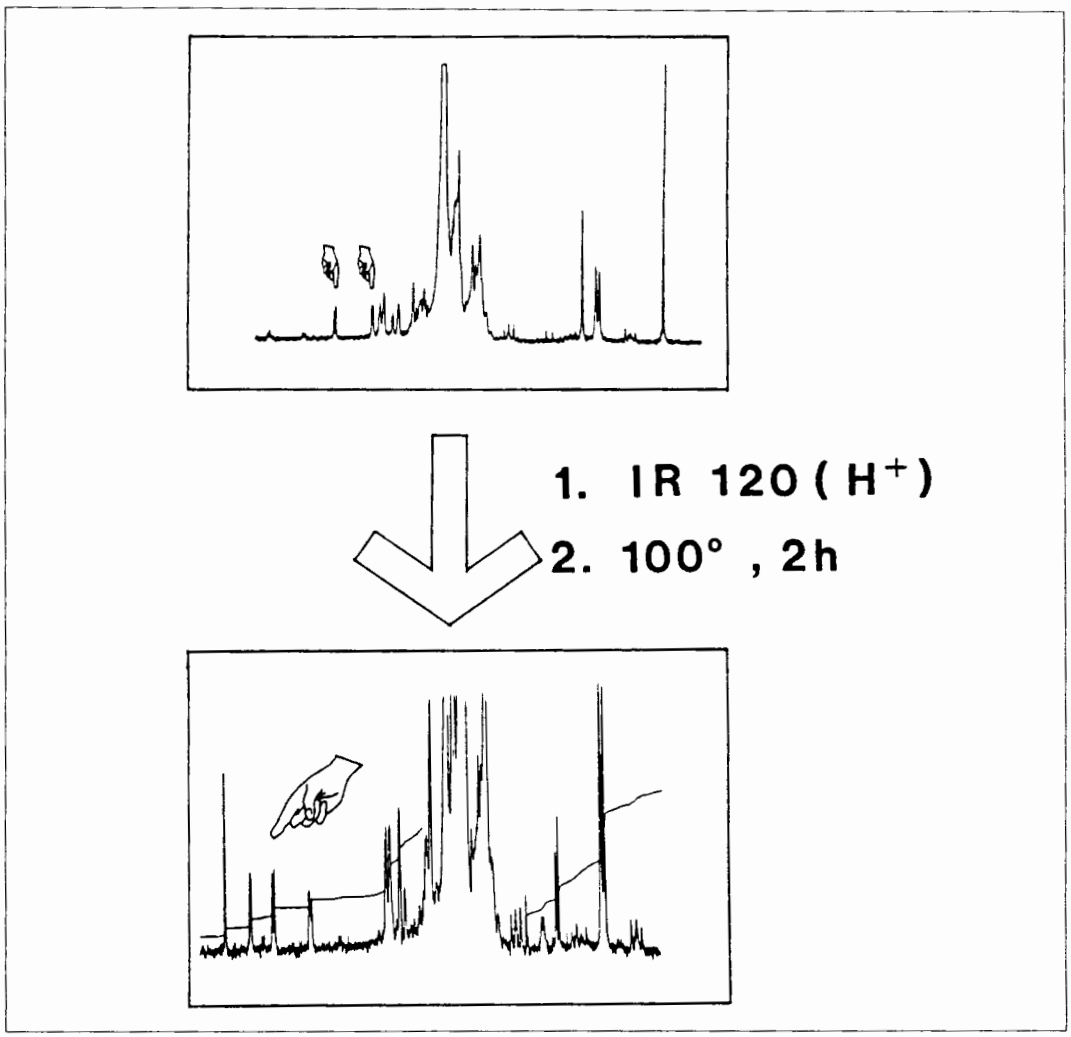


Figure 27: ^{1}H-n.m.r. spectrum (90 MHz) of **P1** and autohydrolysed **P1**

Purification of the resulting oligosaccharide product by s.e.c. yielded a neutral pentasaccharide which showed no abnormal low-field signals (Table 21); therefore, these signals must have arisen from release and further degradation of the unsaturated acid moiety[61].

TABLE 21: ^{1}H-N.M.R. DATA (90 MHz) FOR THE PENTASACCHARIDE OBTAINED BY PARTIAL HYDROLYSIS OF **P1**

Compound	δ[a]	$J_{1,2}$	*Assignment*	
Pentasaccharide	5.26	2.2	α-Man*p*-OH	H-1
	5.19	1.7	α-Man*p* (t)	H-1
	5.00	1.7	α-Rha*p* (t)	H-1
	4.90	0.8	β-Man*p*-OH	H-1
	4.49	7.6	β-Glc*p* (t)	H-1
	4.47	7.6	β-Glc*p* (c)	H-1
	1.24	6.3 ($J_{5,6}$)	CH_3 of Rha	
Reduced Pentasaccharide	5.22	1.5	α-Man*p* (t)	H-1
	5.10	1.6	α-Rha*p* (t)	H-1
	4.61	7.4	β-Glc*p* (t)	H-1
			β-Glc*p* (c)	H-1
	1.26	6.3 ($J_{5,6}$)	CH_3 of Rha	

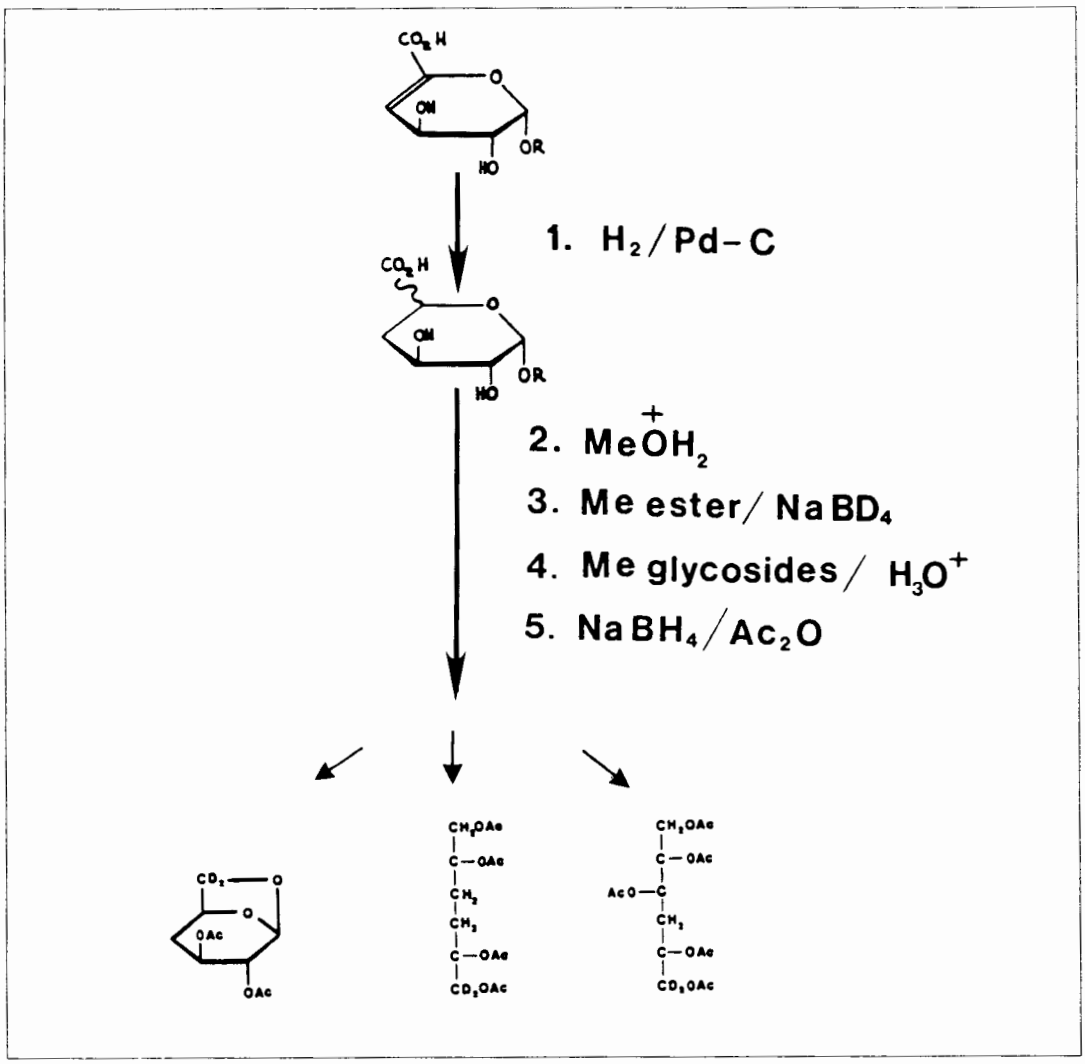
[a] Chemical shift relative to internal acetone (δ 31.0), spectra recorded at ambient temperature

P1 (as the Na^+ salt) was hydrogenated to yield a product (**15**) having no u.v. absorption at 233 nm, and for which low field doublets were absent from the ^{1}H-n.m.r. spectrum-this being indicative of the saturation of the uronic acid moiety to 4-deoxyuronate; the proportion of β-L-*arabino*- and α-D-*xylo*-C-5 epimers formed is known to vary with experimental conditions[216]. Sugar analysis of the alditol acetates (after methanolysis, methyl ester reduction with $NaBD_4$, followed by acid hydrolysis and further $NaBH_4$ treatment) gave the results shown in Table 22 (see scheme 1).

TABLE 22: SUGAR ANALYSIS OF THE HYDROGENATED **P1** (**15**)

Peracetylated derivative of	*T*[a]	*mol %*[b]	*Mass spec. m/e*
6,6-d_2-1,6-anhydro-4-deoxyhexitol	0.15	7	83,84,85,102, 130,172
Rhamnitol	0.29	16	[c]
6,6-d_2-3,4-dideoxyhexitol	0.38	3	83,85,101,103 143,145
6,6-d_2-4-deoxyhexitol	0.71	7	71,81,96,103, 131,145,156, 201,203,233, 303
Mannitol	0.83	29	[c]
Glucitol	1.00	38	[c]

[a] Retention time determined on column C at 215°C
[b] Uncorrected peak areas
[c] Identical with standards (Ref. 44)



Scheme 1: Characterisation of the unsaturated uronic acid derivative

In addition to derivatives of the neutral sugars that were expected, three components each containing deuterium label were detected: from retention times and g.l.c.-m.s. analysis[220] these were clearly identified as the 6,6-di-deuterated, acetylated derivatives of a 4-deoxyhexitol, a 1,6-anhydrol sugar, and a lesser amount of a 3,4-dideoxyhexitol (consequent upon β-elimination). 4-Deoxy-arabinohexoses are known to form 1,6-anhydrides readily[221] and were found together with the 4-deoxy and 3,4-dideoxy sugar derivatives upon acid hydrolysis of a β-(1→2)-linked 4-deoxy-D-*arabino*-hexopyranosyl homopolymer[220]. The products obtained from the present series of reactions and the

location of the deuterium labels are consistent only with hydrogenation of a hex-4-enuronic acid group in **P1**, leading to a 4-deoxy derivative, having taken place.

5.4 LINKAGE AND SEQUENCE OF SUGAR RESIDUES

Autohydrolysis of **P1** and **P2**, prior to borodeuteride reduction and methylation, ensured quantitative removal of the labile groups mentioned earlier (*viz.*, pyruvate and hexenuronate); the results of methylation analysis are shown in Table 19 (columns III and IV). The appearance of derivatives of 6-*O*-methylmannose and deuterated 2,3-di-*O*-methylglucose on analysis of **P2** (column IV) but not **P1** (column III) shows that the reducing mannopyranose residue of one repeating unit is joined to O-4 of the glucuronic acid of the next unit. Thus all the proposed linkage assignments are confirmed. Methylation analysis of autohydrolysed **P1** and **P2** shows a loss of the labile hexenuronic acid group and the appearance of greatly enhanced terminal mannose (Table 19, columns III and IV relative to I and II), which confirms the α-Δ-4,5-Glc*p*A-(1→3)-α-D-Man*p* linkage. However, the positions of attachment of the other three sugars to the mannose at the reducing end was at this stage known only by inference from the polysaccharide structure (**12**), and these were confirmed as follows.

Loss of some rhamnose during the autohydrolysis of **P1** resulted in the appearance of acetylated 1,3,5,6-tetra-*O*-methylmannitol deuterated at C-1 (**16**) [Table 19, column III; see Fig. 28 for m.s. fragmentation pattern of **16**], at the expense of the 1,5,6-tri-*O*-methylmannitol derivative.

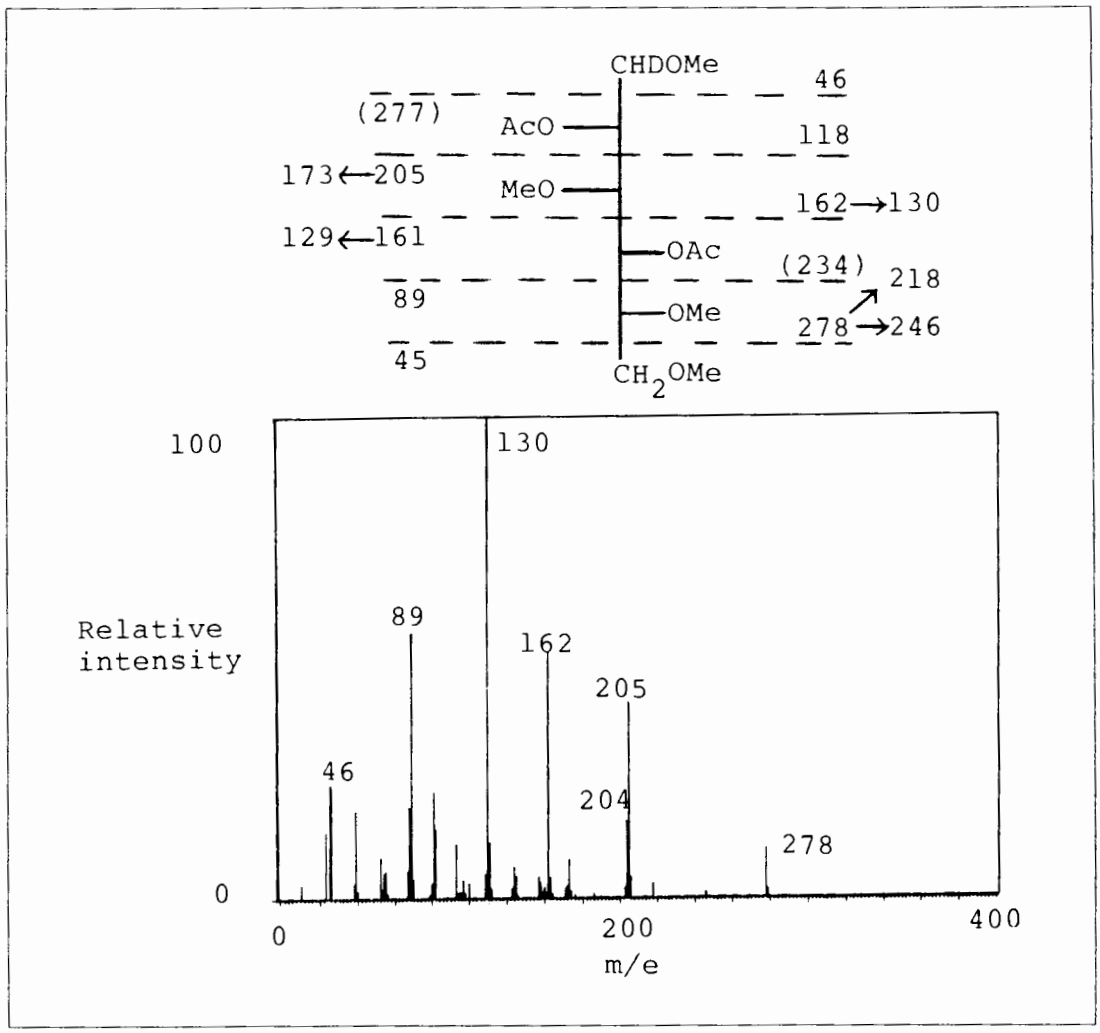
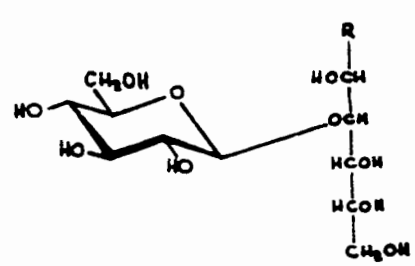


Figure 28: M.s fragmentation pattern of **16**

This result confirms that the rhamnosyl group is linked at O-3. Smith degradation of autohydrolysed **P1** yielded two oligosaccharide alditols: the major product β-D-Glc-(1→2)-pentitol (**17**), and the minor and less-degraded product β-D-Glc-(1→3)-hexitol (**18**).



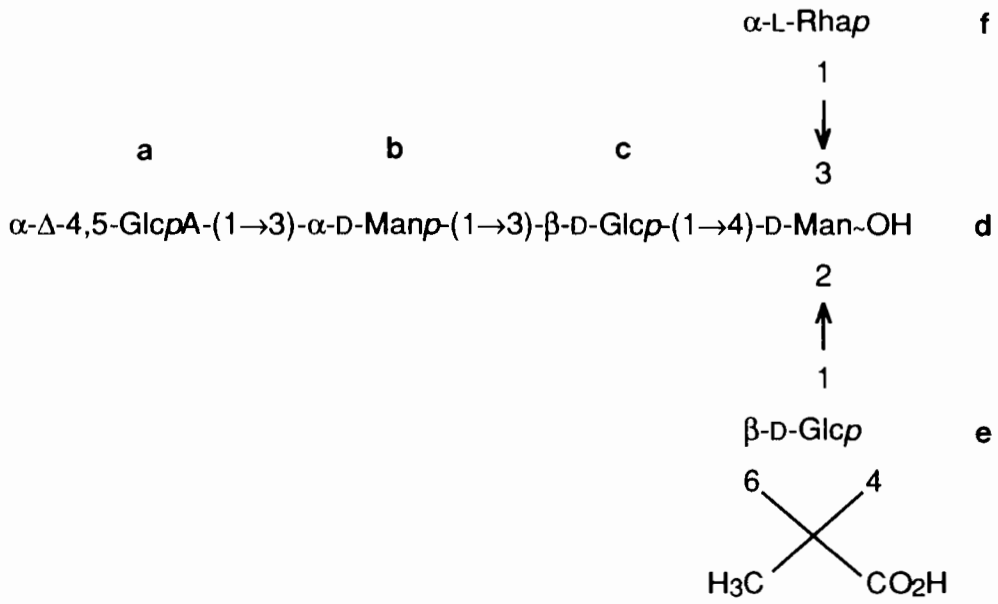
17 (R = H) **18** (R = CH_2OH)

The pentitol moiety of **17** must have arisen from oxidation causing fission between C-5 and C-6 of the ring-opened, reducing end mannose. The loss of C-6 from hexose derivative **18** shifts the position of attachment of the glucosyl group from C-3 in **18** to C-2 in pentose derivative **17**. This corresponds to glycosylation at C-4 of mannose in the original structure **12**. The 3-linked (and therefore periodate-immune) glucosyl residue in the main chain is thus linked at O-4, and the pyruvylated glucosyl group at O-2, of the mannose unit at the reducing end of **P1**.

These findings were supported by e.i.-m.s. analysis of the permethylated, autohydrolysed, $NaBD_4$-reduced **P1**, which showed the presence of terminal hexose (m/e: 219,187 and 155) and rhamnose (m/e 189) groups, in addition to the hexose → hexosyl sequence (m/e: 423,391 and 359) implicit in structure **12** (after removal of the pyruvate and hexuronate groups). Further corroboration of this structure was obtained from the detailed n.m.r. studies described in the next section.

5.5 N.M.R. STUDIES

Detailed 2D n.m.r. studies of **P1** were conducted in order to corroborate assignments made from 1D ^1H-n.m.r. spectra [recorded at 90 MHz (Table 20)] and with a view to confirming the linkages and sequence of sugar residues in **P1**, the structure of which has been previously established by classical methods of methylation analysis coupled to degradative studies (see 5.4).



P1

5.5.1 ASSIGNMENT OF ANOMERIC AND HIGH FIELD RESONANCES OF P1

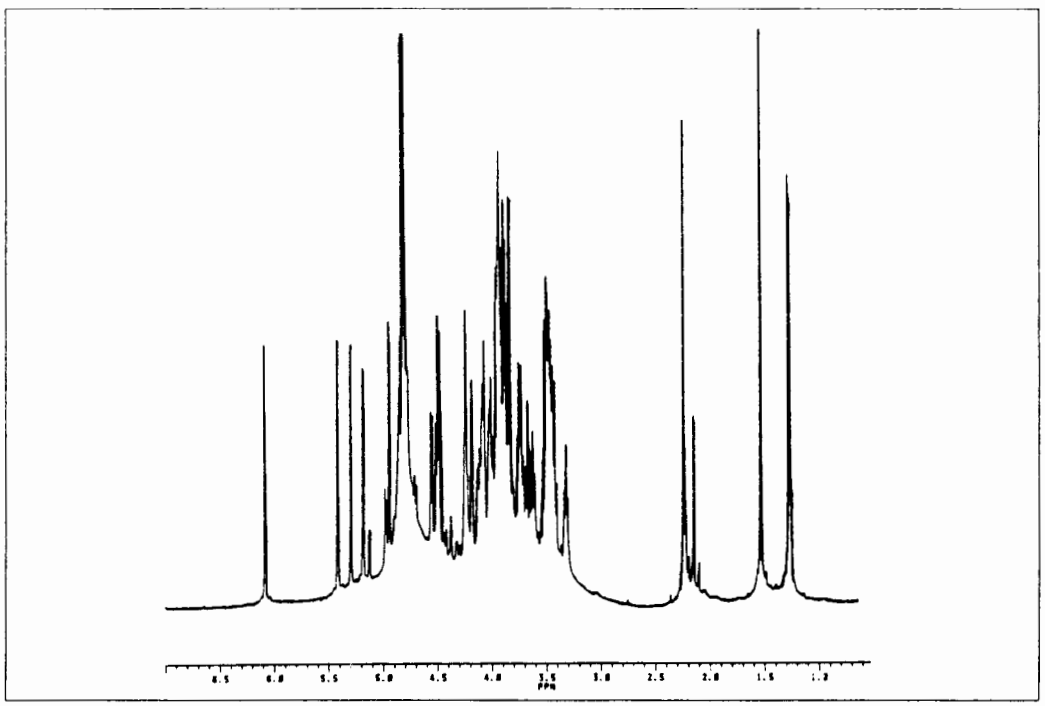


Figure 29: ^{1}H-n.m.r. spectrum (500 MHz) of **P1** recorded at 25°C

The ^{1}H-n.m.r spectrum of **P1** (Fig. 29) showed the presence of a characteristic low field signal at δ 6.07 due to H-4 of the unsaturated uronic acid (UGlc*p*A) group (see 5.3), with signals corresponding to four major α-linked anomeric protons (at δ 5.41, 5.28, 5.17 and 4.93), and two corresponding to β-linked anomeric protons (at δ 4.54 and 4.49), thereby confirming the hexasaccharide nature of **P1**. The high field signals at δ 1.52 and 1.26 confirmed the presence of the methyl groups of pyruvate (singlet; 3H) and rhamnose ($J_{5,6}$ = 5.5 Hz; 3H), while the small singlet at δ 2.14 (0.2H) was attributed to the presence of trace amounts of *O*-acetyl substituent(s). These observations were supported by inspection of the well-resolved ^{13}C-n.m.r. spectrum of **P1** (Fig. 30), the connectivities being established by use of the HETCOR experiment.

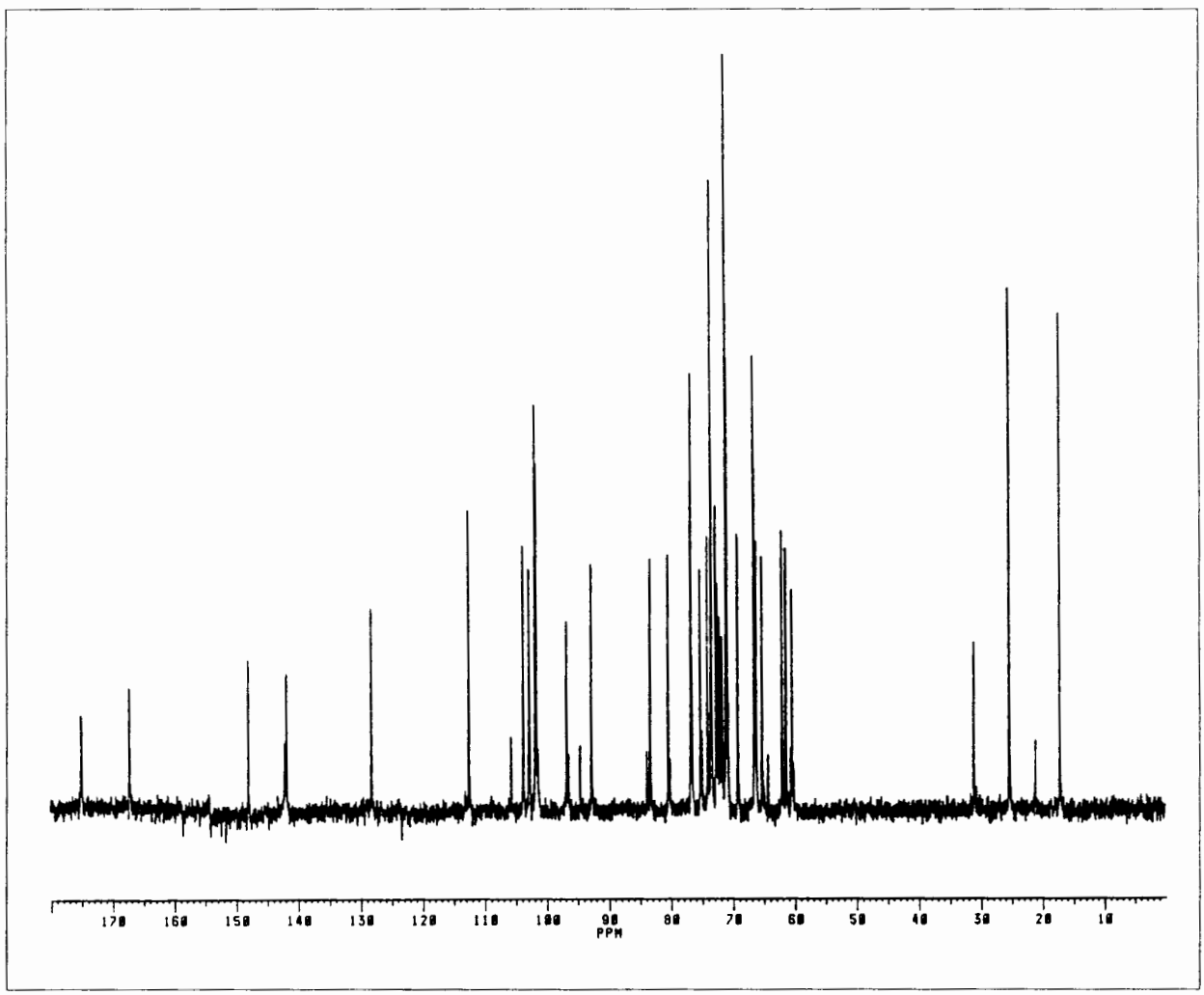


Figure 30: ^{13}C-n.m.r. spectrum (125 MHz) of **P1** recorded at 25°C

The α- and β- anomeric ^{13}C resonances were readily assigned on the basis of chemical shift analysis (see 3.2.1 for typical δ values); this data also permitted identification of the signals emanating from the reducing end mannose (**d**), which was not apparent from the ^{1}H-n.m.r. spectrum. The signals at δ 92.76 and 94.56 were accordingly assigned to α- and β- Man*p*-OH, while identification of the attached protons followed directly from the HETCOR diagram [H-1 of α- and β- Man*p*-OH (**d**) at δ 5.28 (singlet, 0.7H) and 4.97 (singlet, 0.3H)]. The ^{1}H shifts compare well with those reported for compound **2** (β-D-Glc*p*A-(1→2)-D-Mannose, see 3.3.7.2), while the proportion of the α-anomer is slightly greater than the value quoted for the unsubstituted mannose (66%)[187], due to glycosylation at O-2 (as found for **2**).

The remaining anomeric ^{1}H resonances were identified as follows. The H-4 signal of UGlc*p*A served as the starting point for the COSY experiment (see Fig. 31), which permitted the unambiguous assignment of H-3, H-2 and H-1 (δ 5.41) of this residue; this finding is in conformity with the assignments made previously (Table 20). The singlet at δ 5.17 was assigned to the 3-linked α-Man*p* (**b**) by comparison with literature values [δ 5.15 for α-Man*p* in a polymer containing the sugar sequence →3)-α-D-Gal*p*-(1→3)-α-D-Man*p*-(1→][20], and the remaining α anomeric singlet was attributed to α-L-Rha*p*(**f**) (see 3.3.7.4 for typical shift values). The assignment of the β-linked anomeric signals was not apparent at this stage of the investigation, but followed from identification of the ring ^{1}H and ^{13}C resonances described in the next section. The ^{1}H and ^{13}C assignments for **P1** are collected in Table 23 together with data concerning the peaks of low intensity found in the anomeric region. The latter signals, arising from the contribution of the β-anomer of **P1** will be further discussed in section 5.5.3. Furthermore, the ^{1}H and ^{13}C shifts of the pyruvate methyl group reveal the S-configuration of this acetal[136].

TABLE 23: ^{1}H- AND ^{13}C- N.M.R. DATA FOR **P1** (Na^{+} SALT)

^{1}H data (500 MHz)				*^{13}C data* (125 MHz)	
δ	$J_{1,2}$ (Hz)	Intensity (H)	Assignment	δ	Assignment
6.07	4[a]	1	H-4 of α-UGlcpA(**a**)	112.46	C-4 of **a**
5.41	s	1	H-1 of α-UGlcpA(**a**)	101.74	C-1 of **a**
5.28	s	0.7	H-1 of α-Manp-OH(**d**)	92.76	C-1 of **d**
5.17	s	0.7	H-1 of α-Manp(**b**)	101.54	C-1 of **b**
5.11[b]	s	0.3		101.85[b]	
4.97[b]	s	0.3	H-1 of β-Manp(**d**)	94.56	C-1 of **d**
4.96[b]	s	0.3	H-1 of α-Rhap(**f**)	96.42	C-1 of **f**
4.93	s	0.7		96.71[b]	
4.69[b]	7.0	0.3	H-1 of β-Glcp(**e**)	105.67[b]	C-1 of **e**
4.54	7.8	0.7		103.72	
4.49	8.1	n.r.	H-1 of β-Glcp(**c**)	102.74	C-1 of **c**
2.14	s	0.2	CH_3CO-	21.10	CH_3CO-
1.52	s	3	CH_3 of pyr	25.38	CH_3 of pyr
1.26	5.5[c]	3	CH_3 of α-Rhap(**f**)	17.23	CH_3 of **f**

[a] $J_{2,4}$, determined using a diluted solution
[b] Twin signals due to β-anomer of **P1**
[c] $J_{5,6}$

5.5.2 ASSIGNMENT OF RING ^{1}H AND ^{13}C RESONANCES OF P1 USING 2D N.M.R. METHODS

The proton assignments listed in Table 23 served as the starting point for correlations established using the COSY experiment (Fig 31), while the HETCOR diagram (Fig. 32) enabled identification of the attached carbon-atom resonances.

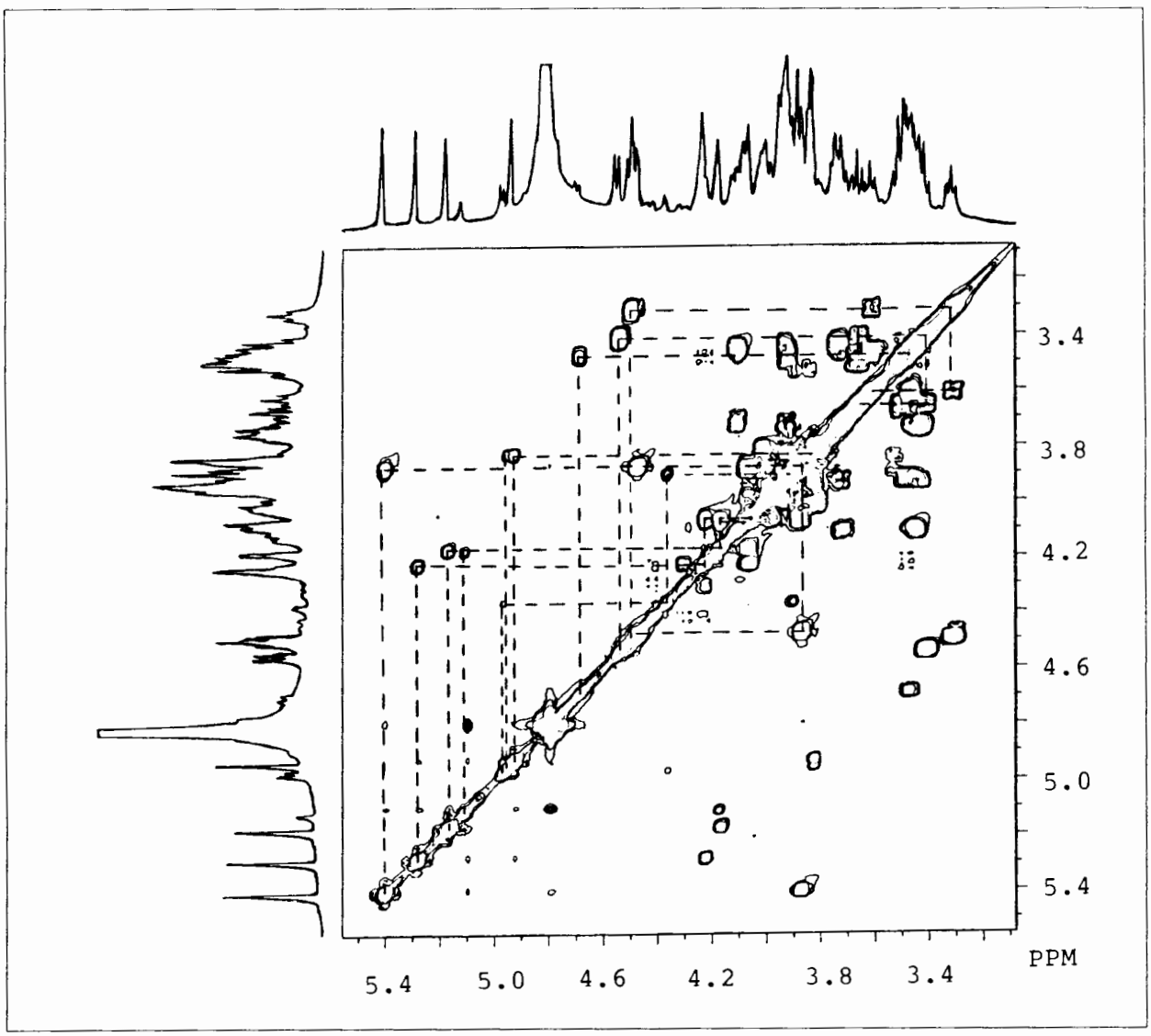


Figure 31: Expanded COSY spectrum of **P1** recorded at 30°C, some of the spin systems are indicated

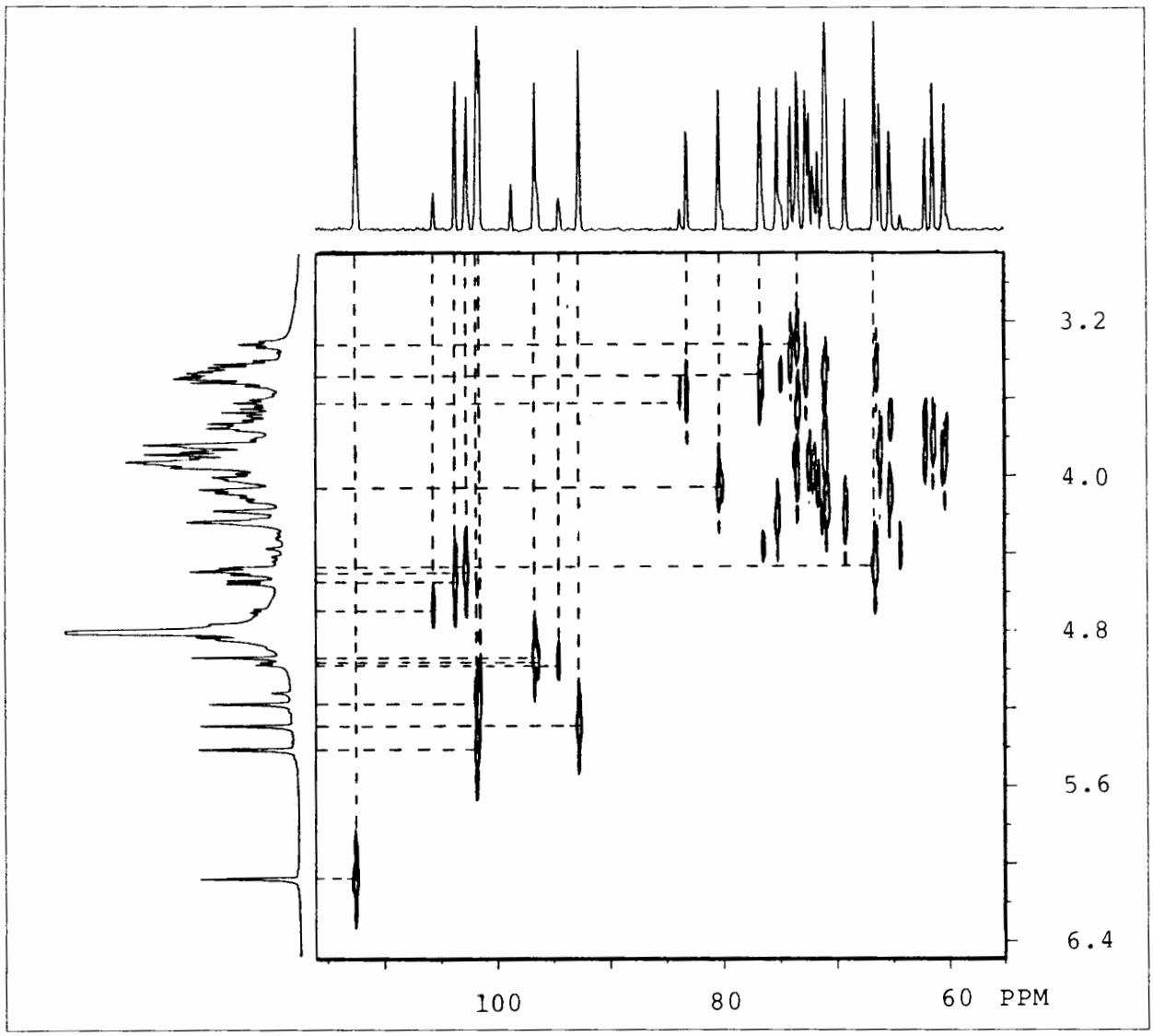


Figure 32: Expanded HETCOR spectrum of **P1** recorded at 30°C ($J_{C,H}$ = 140 Hz), some of the C,H correlations are shown

Owing to the complexity of the COSY spectrum not all the ^{1}H (and hence ^{13}C) resonances could be unambiguously assigned. Therefore some identifications were made by inspection of the 1D ^{13}C-n.m.r. spectra obtained for **P2** (see 5.5.4) and from experiments involving the investigation of long-range ^{1}H-^{13}C couplings. In addition to showing intra-residue heteronuclear correlations, the long-range coupling experiments revealed inter-residue connectivities, thereby establishing aspects of the sequence of sugar units in **P1**.

The long-range HETCOR experiment ($J_{C,H}$ = 6 Hz) gave only nine crosspeaks, most of which were due to directly bonded couplings. Therefore further investigations were carried out using a pulse sequence optimised for the detection of small couplings, namely the COLOC experiment and its 1D analogue, SPI[127,129,222]. The connectivities established using these techniques are presented in Table 24.

TABLE 24: ASSIGNMENTS MADE FROM LONG-RANGE HETERONUCLEAR CORRELATION EXPERIMENTS CONDUCTED ON **P1** *

Sugar residue	*^{1}H signal*	*δ*	*Assignment*	*Experiment*
α-UGlcpA (**a**)	H-1	66.56	C-3 of **a**	SPI
		80.38	C-3 of **b**	SPI
	H-3	112.46	C-4 of **a**	LRH
	H-4	70.95	C-2 of **a**	COLOC
α-Manp (**b**)	H-1	70.86	C-2 of **b**	SPI
		73.47	C-5 of **b**	SPI,COLOC
		80.38	C-3 of **b**	SPI
		83.27	C-3 of **c**	SPI
β-Glcp (**c**)	H-3	73.47	C-2 of **c**	LRH
		101.54	C-1 of **b**	LRH
α-Manp-OH (**d**)	H-1	71.67	C-3 of **d**	SPI
		72.44	C-5 of **d**	SPI,COLOC
pyr-β-Glcp (**e**)	H-1	75.26	C-2 of **d**	SPI
		76.68	C-5 of **e**	SPI
α-Rhap (**f**)	H-1	69.19	C-5 of **f**	SPI,COLOC
		70.95	C-2 of **f**	SPI
		71.05	C-3 of **f**	SPI,COLOC

* This investigation involved the use of the long-range HETCOR (LRH) at $J_{C,H}$ = 6 Hz, the COLOC experiment and in some instance the 1D analogue thereof (SPI)[222]. All experiments were conducted on a Bruker WM500 machine at 30°C

The SPI experiments, although time-consuming, yielded the most useful results (see Fig. 33); the coupling information was also more readily retrieved from these than from the 2D plots obtained for the long-range HETCOR and COLOC experiments.

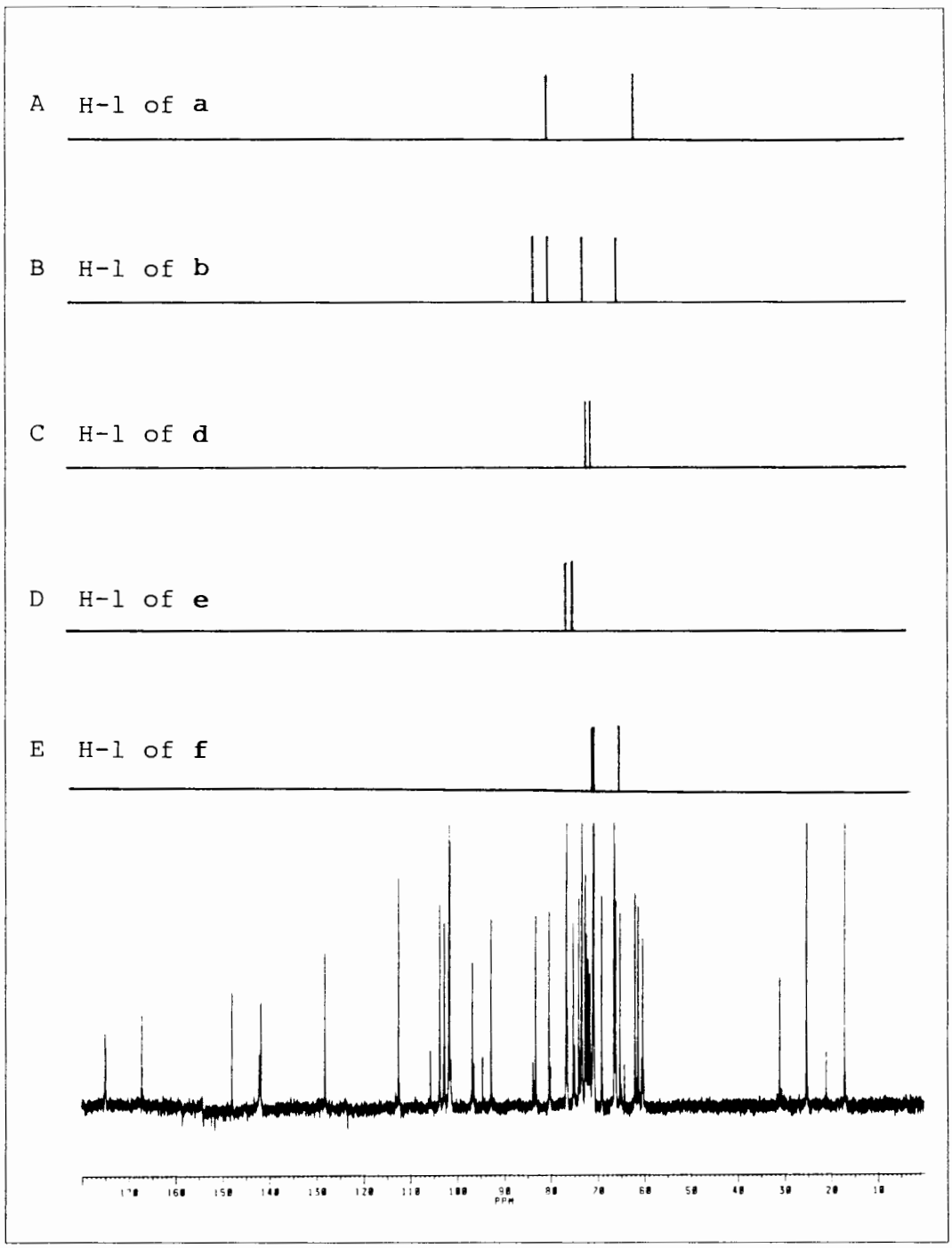


Figure 33: Connectivities established using SPI experiments, the protons investigated are indicated on the diagram

The assignments made from these n.m.r. studies, together with the glycosylation shifts determined by comparison of the shift values obtained with those of suitable standards, are displayed in Tables 25 (^{1}H data) and 26 (^{13}C data).

TABLE 25: ^{1}H-N.M.R. DATA (500 MHz) AND GLYCOSYLATION SHIFTS[a] FOR **P1**

Sugar residue	*Chemical shift (p.p.m.)*					
	H-1	H-2	H-3	H-4	H-5	H-6
α-UGlc*p*A(**a**)[b]	5.41 (+.02)	3.86 (+.14)	4.46 (+.55)	6.07 (+2.38)	-	-
α-Man*p*(**b**)[c]	5.17 (+.40)	4.17 (+.23)	4.08 (+.31)	n.r.	n.r.	3.83 -
β-Glc*p*(**c**)[d]	4.49 (+.12)	3.31 (+.06)	3.61 (+.13)	n.r.	3.50 (+.05)	3.74,3.92 (+.02), (-.01)
α-Man*p*OH(**d**)[c]	5.28 (+.10)	4.23 (+.31)	4.06 (+.21)	n.r.	3.96 (+.14)	3.91 -
β-Glc*p*(**e**)[d]	4.54 (+.17)	3.41 (+.16)	3.66 (+.18)	n.r.	3.44 (-.01)	3.72,4.12 (0.0), (+.21)
α-Rha*p*(**f**)[e]	4.93 (+.23)	3.85 (-.08)	3.94 (+.23)	3.49 (+.05)	4.23 (+.59)	1.26 (-.04)

Glycosylation shifts given in parentheses.

[a] A downfield shift is considered positive
[b] Model compound α-Glc*p*A-OH (Table 2)
[c] Model compound α-Man*p*-OMe (Ref. 188)
[d] Model compound β-Glc*p*-OMe (Ref. 177)
[e] Model compound α-Rha*p*-OMe (Ref. 188)

TABLE 26: ^{13}C-N.M.R. DATA (125 MHz) AND GLYCOSYLATION SHIFTS[a] FOR **P1**

Sugar residue	*Chemical shift (p.p.m.)*					
	C-1	C-2	C-3	C-4	C-5	C-6
α-UGlc*p*A (**a**)[b]	101.74 (+8.8)	70.95 (-1.3)	66.56 (-6.9)	112.46 (+39.5)	142.14 (+69.7)	167.13 (-10.9)
α-Man*p* (**b**)[c]	101.54 (+6.0)	70.86 (-1.4)	80.38 (+8.5)	n.r.	73.47 (-0.4)	61.41 (-1.2)
β-Glc*p* (**c**)[d]	102.74 (+6.0)	73.47 (-1.6)	83.27 (+6.6)	n.r.	76.77 (0.0)	62.06 (+0.4)
α-Man*p*-OH(**d**)[c]	92.76 (-2.7)	75.26 (+3.0)	71.67 (-0.2)	n.r.	72.44 (-1.5)	60.38 (-2.2)
β-Glc*p*(**e**)[d]	103.72 (+7.0)	74.08 (-1.0)	73.40 (-3.3)	n.r.	76.68 (-0.2)	65.25 (+3.6)
α-Rha*p* (**f**)[e]	96.71 (+1.7)	70.95 (-0.9)	71.05 (0.0)	72.72 (-0.6)	69.19 (-0.2)	17.23 (-0.8)

Glycosylation shifts given in parentheses.

[a] A downfield shift is considered positive
[b] Model compound α-Glc*p*A-OH (Table 1)
[c] Model compound α-Man*p*-OH (Ref. 155)
[d] Model compound β-Glc*p*-OH (Ref. 155)
[e] Model compound α-Rha*p*-OH (Ref. 155)

5.5.3 ESTABLISHMENT OF THE SEQUENCE AND SUBSTITUTION PATTERNS OF SUGAR RESIDUES IN P1

The glycosylation shifts presented in Tables 25 and 26, especially the data obtained for the ^{13}C resonances, confirm the putative linkage sites (*cf.* **14**), although the shifts found for the doubly-branched mannose are small (possibly due to unusual shielding effects experienced under conditions of heavy glycosylation). Where possible the data published by Kochetkov *et al.*[156], which correlates the ranges of ^{13C} α- and β- effects of glycosylation and δ values of C-1 signals with configurational factors, were applied. This analysis, together with the results of long-range coupling experiments (see Table 24) and the twinning of specific n.m.r. signals, permitted complete elucidation of the sequence of sugar residues in **P1**. This is discussed for residues **a**→**f** below.

(i) α-D-**UGlc*p*A(a)**: The location of this unsaturated group at the terminal non-reducing end is implicit in its formation. The presence of the double bond is confirmed by the dramatic deshielding of the H-4, C-4 and C-5 resonances and the absence of the H-5 signal, whereas the upfield shift of the C-3 resonances is expected for the γ-carbon-atom of this α,β-unsaturated acid moiety[219]. The absence of downfield shifts for C-2 and C-3 confirms that neither position is glycosylated and proves that **a** is indeed terminal. The SPI experiment (Fig. 33A) revealed connectivity between H-1 of **a** and a ^{13}C resonance at δ 80.36, previously assigned to C-3 of α-Man*p* (**b**), thereby establishing the **a**→3**b** sequence. Furthermore, the anomeric carbon shift at δ 101.74 is diagnostic for α-linked D-hexoses having the *gluco* configuration and attached to O-3 of D-Man*p* (100.5-102

p.p.m.)[156]. Thus the configurational factors for α-D-UGlc*p*A-(1→3)-D-Man*p* sequence are confirmed.

(ii) **α-D-Man*p*(b)**: The large α-effect of glycosylation at C-3 (+8.5 p.p.m.) and the β-effect of -1.4 p.p.m. found for C-2 (the C-4 signal could not be identified unambiguously) clearly shows that **b** is 3-linked. This result was corroborated by the large downfield shift found for H-3 (+0.31 p.p.m.), this proton being geminal to the site of glycosylation. The ^{13}C glycosylation shifts for **b** also provided further proof of the α-D-Hex*p*-(1→3)-D-Man*p* fragment (α-effect for C-3 of D-Man*p*, 8.0 - 8.5 p.p.m.; β-effect for C-2 of D-Man*p*, -1.5 to +1.5 p.p.m.[156]). In addition to showing intra-residue connectivities, the SPI experiment (Fig. 33B) indicated pertubation of the ^{13}C signal at δ 83.27 imputed to C-3 of β-Glc*p*(**c**), thus establishing the **b**→3**c** sequence.

(iii) **β-D-Glc*p*(c)**: The ^{1}H glycosylation shift data (Table 25) suggests that O-3 is the position of linkage (+0.13 p.p.m. for H-3). This was confirmed by the α-effect of glycosylation for C-3 (+6.6 p.p.m.) and the β-effect for C-2 (-1.6 p.p.m.); C-4 could not be assigned unambiguously. The β-effect of -1.6 p.p.m. for C-2 is also consistent with the range given for the sugar sequence α-D-Man*p*-(1→3)-D-Glc*p* (-1.1 to -2.0 p.p.m.)[156], thus the absolute and anomeric configurations and the linkage sites of a→3**b**→3**c** are established.

(iv) **D-Man-OH(d)**: The location of this residue at the reducing end of **P1** has already been discussed (see 5.5.1). The unusual shielding effects arising from the heavy glycosylation of **d** results in ambiguous glycosylation shifts. Nevertheless, the upfield shifts (β-effect of glycosylation) of -2.7 and -1.5 p.p.m. observed for C-1 and C-5 of **d** confirm that **d** is

glycosylated at O-2 and O-4, while the small α-effect of glycosylation found for C-2 (+ 3.0 p.p.m. instead of ~ +8 p.p.m.[155]) suggests the presence of an O-3 substituent. These assumptions are supported by the appearance of twinning of the n.m.r. signals imputed to the residues attached to O-2 and O-3 of **d** [see (v) and (vi)].

(v) pyr-β-D-**Glc*p*(e)**: The attachment of the pyruvic acid acetal to O-4 and O-6 of this residue is partly confirmed by the positive glycosylation shifts of + 3.6 p.p.m. found for C-6 (C-4 could not be assigned unambiguously) and the shielding of the adjacent carbon atoms [β-effects of -3.3 p.p.m. (C-3) and -0.2 p.p.m. (C-5)]. The twinning of the n.m.r. signals emanating from H-1, H-2 and the attached carbon atoms of this residue confirm that **e** is in close proximity to the reducing end group of **P1**, while the relatively large differences in the chemical shift values between the twin signals [*viz.* H-1 (0.15 p.p.m.), C-1 (1.95 p.p.m.), H-2 (0.06 p.p.m.) and C-2 (0.88 p.p.m.)] suggest that **e** is linked to O-2 (and not O-3) of mannose (**d**). This proposition was supported by the finding of smaller differences in chemical shift between the twin signal of the anomeric resonances of α-Rha*p*(**f**) and finally confirmed by the SPI experiment (Fig. 33D), which showed connectivity between H-1 of **e** and δ 75.26 (the ^{13}C resonance previously assigned to C-2 of **d**).. No conclusions regarding the configurational aspects of the **e**→2**d** bond could be drawn as the correlations reported by Kochetkov *et al.* are not applicable to branched sugar residues.

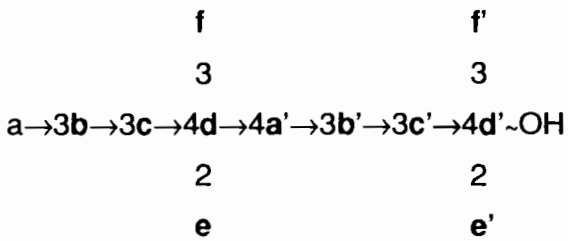
(vi) α-L-**Rha*p*(f)**: The small ^{1}H and ^{13}C glycosylation shift values obtained for this residue confirm that **f** is terminal. As noted above, the small differences in chemical shift between the twin signals of the anomeric resonances [H-1 (0.03 p.p.m.) and C-1 (0.29 p.p.m.)] verify that **f** is attached to O-3 of the reducing end mannose (**d**). Although this residue is attached to a doubly-branched sugar residue and therefore the configurational correlations cannot

be used with confidence, the C-1 signal at δ 96.71 lies within the range of 97 to 98.5 p.p.m. given for C-1 of α-linked L-hexoses having the *manno* configuration and which are linked to O-3 of D-Man*p* units, thereby affirming the α-L-Rha*p*-(1→3)-D-mannose sequence (i.e. **f**→3**d**).

Thus the anomeric configurations, substitution pattern and sequence of sugar units **a** to **f** in **P1** have been verified solely by n.m.r. methods. In most cases the putative absolute configurations of the sugar residues were also confirmed.

5.5.4 DETERMINATION OF THE ANOMERIC CONFIGURATION OF THE DOUBLY-BRANCHED MAN RESIDUE PRESENT IN P2

In order to establish the anomeric configuration of all of the sugar residues in the original polysaccharide, n.m.r. spectra of the dimer (**P2**) were also examined.



P2

Although n.m.r. evidence obtained after Smith degradation of the polysaccharide[215] suggested that both mannose residues were α-linked (*cf.* **12**), preliminary ^{1}H-n.m.r. studies

conducted at 90 MHz indicated that the signal at δ 4.74 in the spectrum of **P2**, which persisted after $NaBH_4$ reduction, is attributable to a β-D-mannosyl residue. Inspection of the ^{1}H-n.m.r. spectra obtained earlier[215] allows a similar interpretation to be made of an unresolved signal at δ 4.76, on the shoulder of the HOD peak. In order to verify this finding, **P2** was examined at higher field strength, using a Varian VXR-200 spectrometer; the ^{1}H- and ^{13}C- n.m.r spectra obtained are displayed in Figs. 34 and 35 respectively.

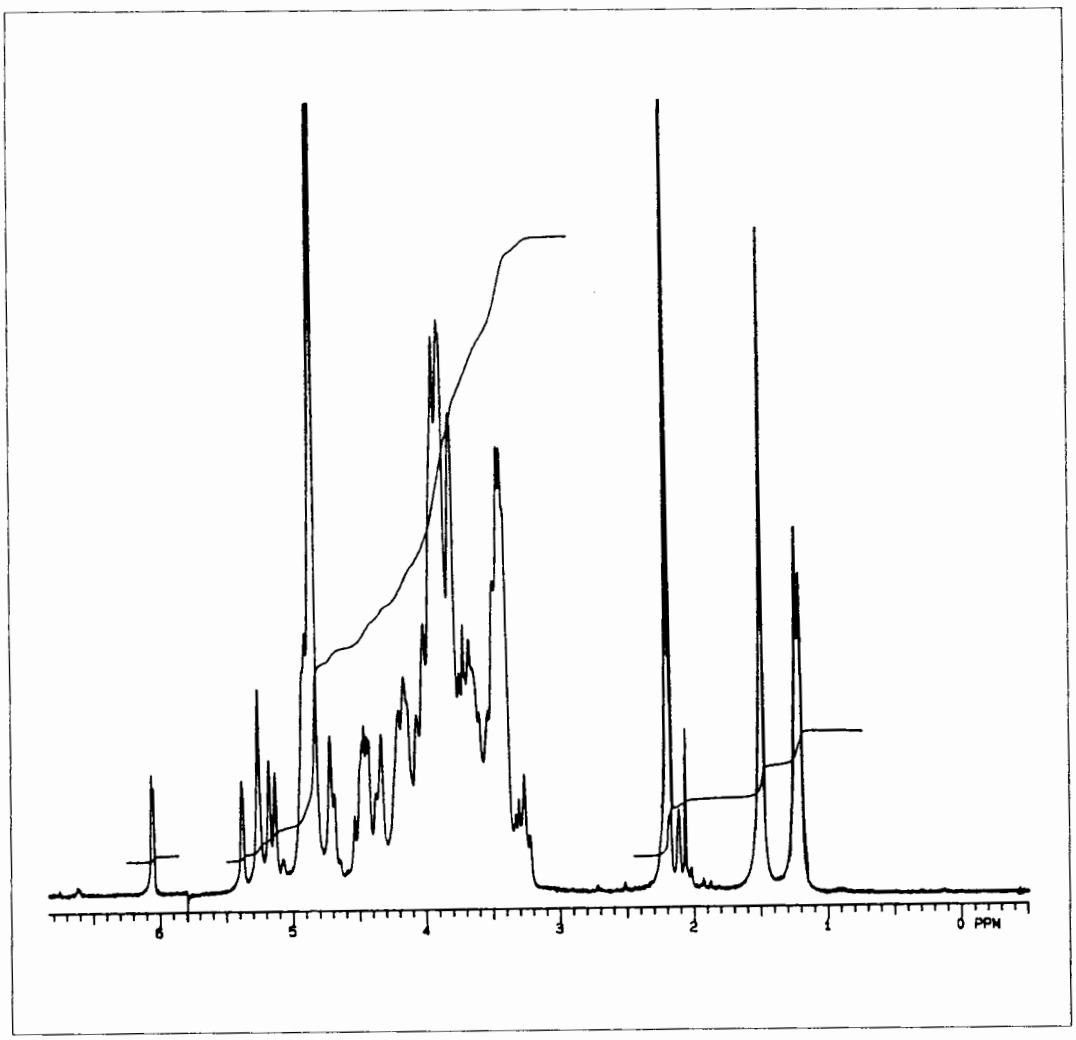


Figure 34: ^{1}H-n.m.r. spectrum (200 MHz) of **P2** recorded at 25°C

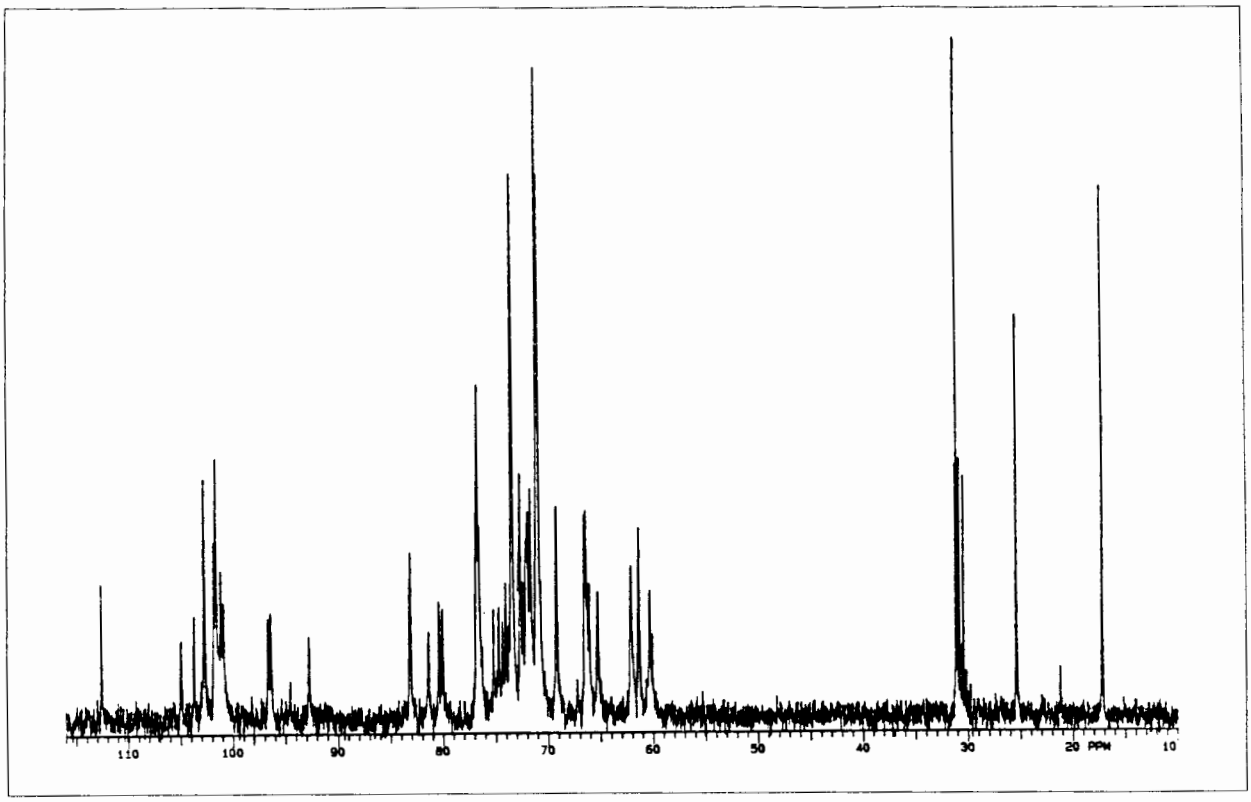


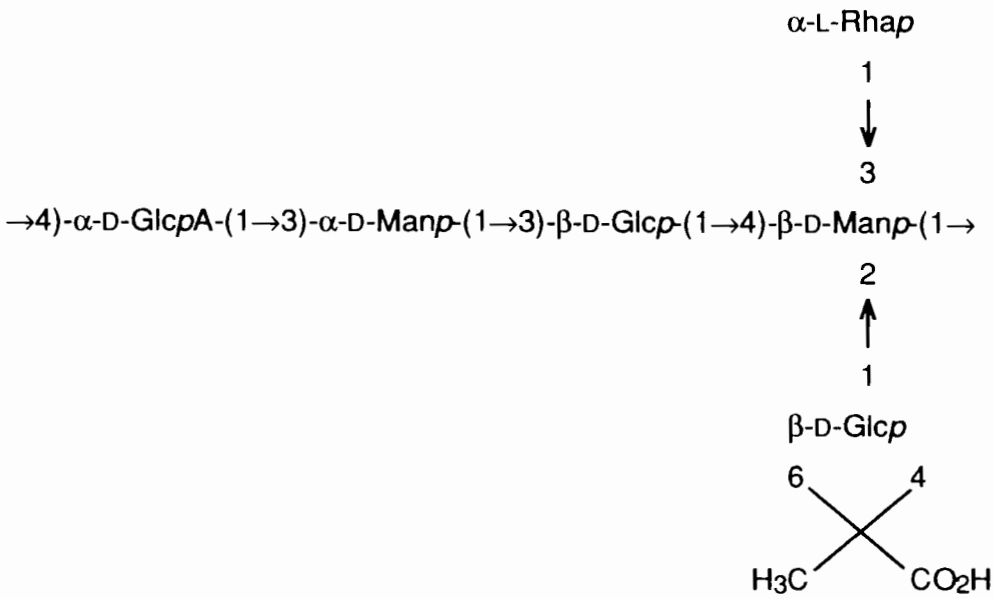
Figure 35: ^{13}C-n.m.r. spectrum (50,3 MHz) of **P2** recorded at 25°C

Assignment of the anomeric ^{1}H and ^{13}C resonances was made by comparison of these spectra with those obtained for **P1** (see Figs. 29 and 30) and supported by use of the HETCOR experiment. Final proof of the anomeric configurations of the ^{13}C signals was achieved by measurement of the $J_{C\text{-}1,H\text{-}1}$ values obtained from the n.O.e. enhanced fully coupled ^{13}C spectrum of **P2**.

TABLE 27: ^{1}H- AND ^{13}C- N.M.R. DATA* FOR P2 (Na^+ SALT)

^{1}H data (200 MHz)				*^{13}C data* (50.3 MHz)		
δ	$J_{1,2}$ (Hz)	Intensity (H)	Assignment	δ	$J_{C\text{-}1,H\text{-}1}$	Assignment
6.04	4 ($J_{3,4}$)	1	H-4 of α-UGlc*p*A(a)	112.58	167.26 ($J_{C\text{-}4,H\text{-}4}$)	C-4 of **a**
5.38	s	1	H-1 of α-UGlc*p*A(a)	101.79	170.1	C-1 of **a**
5.26	s	1.7	H-1 of α-Man*p*-OH(d')	92.76	170.8	C-1 of **d'**
			H-1 of α-Glc*p*A(a')	101.12	169.4	C-1 of **a'**
5.17	s	1	H-1 of α-Man*p*(b)	101.59	171.5	C-1 of **b,b'**
5.12	s	1	H-1 of α-Man*p*(b')			
5.07	s	0.3	H-1 of β-Man*p*-OH(d')	94.58	n.o.	C-1 of **d'**
~4.9	n.o.	n.o.	H-1 of α-Rha*p*(f)	96.41	171.3	C-1 of **f**
			H-1 of α-Rha*p*(f')	96.69	171.6	C-1 of **f'**
4.72	s	1	H-1 of β-Man*p*(d)	100.89	163.9	C-1 of **d**
			H-1 of pyr-β-Glc*p*(e)	104.96	n.o.	C-1 of **e**
~4.5	n.o.	~4	H-1 of pyr-β-Glc*p*(e')	103.71	161.4	C-1 of **e'**
			H-1 of β-Glc*p*(c,c')	102.73	160.4	C-1 of **c,c'**
1.50	s	6	CH_3 of pyr	25.40	-	CH_3 of pyr
1.21	6 ($J_{5,6}$)	6	CH_3 of α-Rha*p*(f,f')	17.25	-	CH_3 of α-Rha*p* (f,f')

* Chemical shift relative to internal acetone at δ 2.21 (^{1}H) and 31.0 (^{13}C)

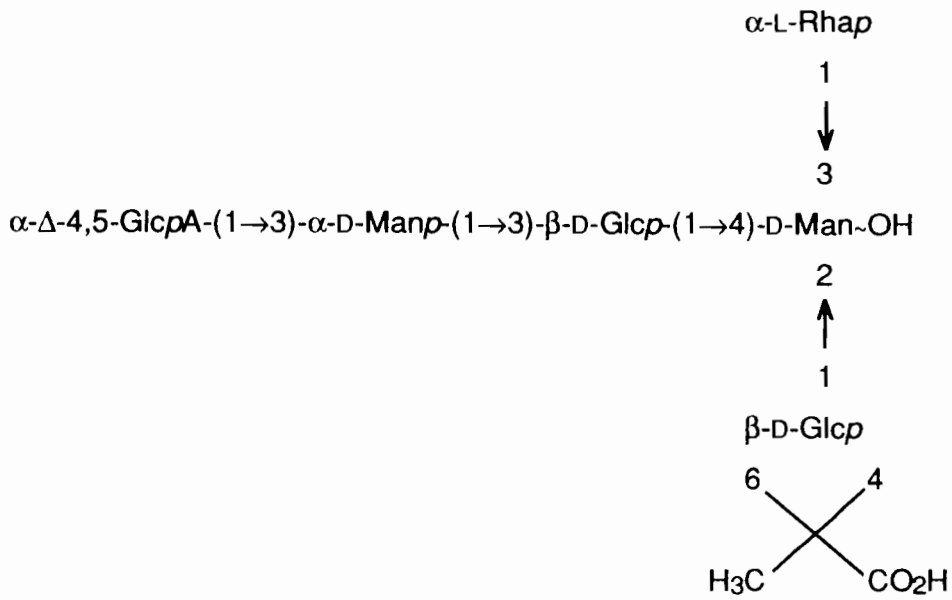


19

The fact that the residues attached to O-2 and O-3 of the doubly branched Man*p* (**d**) unit [i.e. pyr-β-Glc*p* (**e**) and α-Rha*p* (**f**)] have the same chemical shift as was found for these residues in the β-anomer of **P1** (see Table 23) verifies that **d** is indeed β-linked. It is of interest to note that this is the anomeric configuration of the substituted mannoses in both of the other *Klebsiella* polysaccharides (K5[92] and K14[96]) that have also been reported to undergo elimination by lyase action of the appropriate bacteriophage.

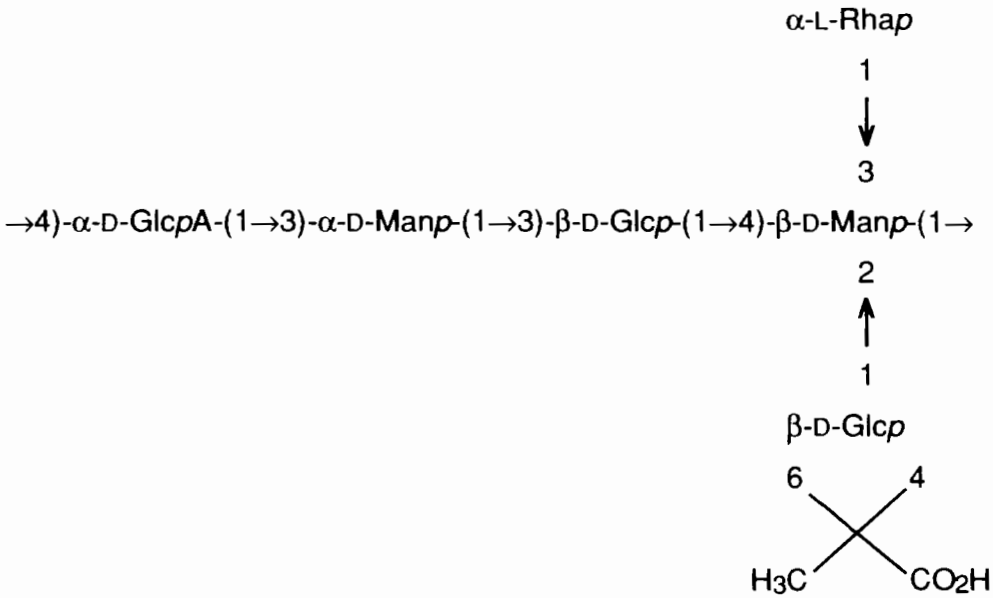
5.6 CONCLUSION

Structural studies of the oligosaccharides obtained by bacteriophage degradation of *Klebsiella* K64 polysaccharide have revealed that phage Ø64 acts as a lyase, generating a hexasaccharide having an unsaturated derivative of glucuronic acid at the non-reducing end (**14**).



14

The unsaturated hex-4-enuronic acid residue generated was characterised spectroscopically (u.v. and n.m.r.) and by g.l.c.-m.s. of acetylated products formed after hydrogenation of the double bond. Partial hydrolysis and Smith degradation coupled to methylation analysis and n.m.r. spectroscopy have been used to establish the structures of the oligosaccharides produced from the polysaccharide. Once again, detailed 2D n.m.r. studies confirmed the configuration, linkages and sequence of sugar residues as shown in **14**, while n.m.r. analysis of **P2** (the dimer of **14**) established that the in-chain doubly-branched Man*p* residue is β-linked. This finding necessitates reformulation of the structure of the repeating unit of the polymer to be as shown overleaf (**19**).


α-L-Rhap
1
3
→4)-α-D-GlcpA-(1→3)-α-D-Manp-(1→3)-β-D-Glcp-(1→4)-β-D-Manp-(1→
2
1
β-D-Glcp
6
4
H3C
CO2H

19

5.7 EXPERIMENTAL

5.7.1 PREPARATION AND PROPERTIES OF K64 CAPSULAR POLYSACCHARIDE

A culture of *Klebsiella* K64 was grown as previously described in sections 1.2 and 2.1 on medium A and the polysaccharide was purified by precipitation once with cetyltrimethylammonium bromide. The purified polysaccharide (~ 3g from four batches of four trays [each 38 x 27 x 1.5 cm]) typically had $[\alpha]_D$ +28° (*c* 0.1); N, found, 0.4%; and sugar constituents as shown in Table 17. S.e.c. (column I) indicated an average molecular weight of ~ 1.6×10^6 (calibration with dextran standards).

5.7.2 PROPAGATION OF BACTERIOPHAGE Ø64

Bacteriophage Ø64 (obtained courtesy of Professor S. Stirm) was propagated on host strain *Klebsiella* K64 in nutrient broth until 1L of lysate containing ~ 10^{13} p.f.u. had been obtained. The phage solution was concentrated and dialysed against running tap-water (2days), the dialysate finally being concentrated (to 100mL). Two such preparations were conducted.

5.7.3 DEPOLYMERISATION OF POLYSACCHARIDE AND ISOLATION AND PURIFICATION OF THE DERIVED OLIGOSACCHARIDES

(A) Phage solution (100mL containing 3×10^{13} p.f.u.) was added to the polysaccharide (570mg) in water (150mL), and the mixture was stirred for 3 days at 37°C.

(B) Polysaccharide (0.8g) was added directly to the phage solution (5×10^{13} p.f.u. in 100mL) and stirred for 5 days at 37°C in the presence of chloroform (3mL). The oligomers

produced were isolated by dialysis and purified by successive passages through a column of Amberlite IR-120 (H^+) resin. Treatment with resin was repeated until a colourless solution was obtained which, on freeze-drying after neutralisation with $NaHCO_3$, afforded oligosaccharides (Na^+ form) in yields greater than 50% (A 300mg; B 620mg). The oligosaccharide products typically had $[\alpha]_D$ $+17^o$ (*c* 0.5; calculated, on the basis of Hudson's rules applied appropriately, $+22^o$) and contained 60% of the monomer (according to s.e.c. on column 2). Paper chromatography (solvent A) showed traces of uronic acid in an hydrolysate, while g.l.c. analysis (column A at 220°C) of the derived alditol acetates confirmed the presence of rhamnose (1 mol), mannose (2) and glucose (2). A portion (100mg) of the oligomers was fractionated on column 3, resulting in incomplete separation of **P1** and **P2**, as shown by t.l.c. analysis (solvent F). Passage of the **P1**-rich fraction through column 4 yielded **P1** (42mg). A further portion (300mg) was successfully separated on a silica gel column (30 x 1.5 cm), eluted with solvent F) followed by passage through column 4 to yield purified **P1** (135mg) and **P2** (96mg).

5.7.4 ANALYSIS OF THE OLIGOSACCHARIDES

A sample (5mg) of each oligosaccharide was dissolved in water (2mL) and treated with sodium borohydride (10mg) overnight. The reduced oligosaccharides recovered were hydrolysed (2M TFA 18h at 100°C) and the products converted into peracetylated aldononitrile (PAAN) derivatives for analysis (Table 18). All methylations were conducted on 2-20mg of sample as described in section 2.10 (see Table 19). The process of autohydrolysis of **P1** and **P2**, carried out by heating the deionised sample (10-20mg in 1mL D_2O) at 100°C for 2h, was followed by t.l.c. analysis (solvent F) and ^1H-n.m.r. spectroscopy. Hydrogenation experiments were conducted under pressure (30 bar), the samples (20mg in 3mL water) being stirred with palladium-on-charcoal (10mg) for 2h. The solutions were filtered through Celite, centrifuged, and freeze-dried, yielding hydrogenated products (~ 15mg). Low pressure hydrogenation (1bar) using Pd/C or Pt as catalyst was

unsuccessful. Hydrogenated **P1** was treated with $NaBH_4$ (5mg), and the product (15mg) was subjected to methanolysis as described in section 2.8. The results of g.l.c.-m.s. analysis are shown in Table 22.

5.7.5 SMITH DEGRADATION

A portion (120mg) of oligosaccharide product (B) was acidified using Amberlite IR-120 (H^+) resin, then autohydrolysed for 2h at 100°C. The product was purified by passage through column 4. The collected product (90mg) was treated with 0.1M sodium periodate (12.5mL) in the dark at room temperature. After 3 days the periodate consumption[38] became constant at 1.2 mole of periodate per sugar residue (value expected, 1.35, based on a molar ratio of **P1**/**P2** = 4). Excess of periodate was decomposed and the oxidised material reduced by $NaBH_4$ (1.35g) over-night. Following decomposition of the hydride with acetic acid, the solution was freeze-dried, methanol was added to the residue and removed by evaporation (three times), and the product was fractionated on column 4. The major component of low molecular weight (~ 350) was kept in M TFA (4mL) at room temperature. Hydrolysis was monitored by t.l.c. which indicated a single component (R_{Rha} 0.73, solvent F) after 4 days. ^{1}H-n.m.r. of the recovered material (30mg) showed a single β-anomeric signal (δ 4.58, $J_{1,2}$ 7.4 Hz). The permethylated product gave a single spot on t.l.c. analysis (solvent F) but g.l.c. analysis (column C at 215°C) showed the presence of two components **17** and **18** at retention times 0.66 (80%) and 1.03 (20%) relative to permethylated cellobiitol. G.l.c.-m.s. analysis showed **17** to be Hex*p* (1→2) pentitol with characteristic fragments (m/e: 45, 88, 89, 101, 127, 155, 159, 187, 191, 219 and 251), and **18** to be Hex*p* (1→3) hexitol (m/e: 45, 88, 89, 101, 155, 171, 187, 219, 235 and 295). The β-configuration of the terminal linkage was confirmed by ^{1}H-n.m.r. analysis of the permethylated product in $CDCl_3$ (δ = 4.36, $J_{1,2}$ 7.2 Hz). Hydrolysis (2M TFA for 6h at 100°C) followed by g.l.c.-m.s. analysis (column C at 215°C) of the derived alditol acetates showed 3 peaks at relative retention times of 0.47 (24%), 0.61 (9%) and 1.00 (67%) corresponding to the acetylated

derivatives of 1,3,4,5-tetra-*O*-methylpentitol, 1,2,4,5,6-penta-*O*-methylhexitol and 2,3,4,6-tetra-*O*-methylglucitol respectively.

5.7.6 N.M.R. ANALYSES

The ^{1}H-n.m.r. data presented in sections 5.2 to 5.4 was conducted on a Bruker WH-90 spectrometer, whereas the detailed n.m.r. studies described in section 5.5 were conducted on Varian VXR-200 and Bruker WM500 machines. Sample preparation and n.m.r. operating conditions are described in section 2.7.

CHAPTER 6

THE STRUCTURAL ELUCIDATION OF THE CAPSULAR POLYSACCHARIDE OF *KLEBSIELLA* SEROTYPE K71

6.1 INTRODUCTION

The capsular polysaccharide produced by *Klebsiella* serotype K71 bacteria has been classified in the chemotype containing D-glucose, L-rhamnose and D-glucuronic acid as the component monosaccharides. This chemotype includes the polysaccharides of serotypes K17, K23, K44 and K45, the structures of which have been published[7,223]. Although the structure of the *Klebsiella* K71 polysaccharide has not been published, Heidelberger and Nimmich[10], on the basis of serological cross-reactions, have predicted the presence of terminal or (1→2) linked D-glucuronic acid units, while Rieger-Hug and Stirm[90] have shown that bacteriophage Ø71 causes cleavage of the polymer into oligosaccharides having a rhamnose group at the reducing end. In the present study the structure of the *Klebsiella* K71 polysaccharide has been elucidated by both chemical and spectroscopic investigation of the polymer and of the oligomers obtained by phage Ø71 depolymerisation.

6.2 ISOLATION AND ANALYSIS OF KLEBSIELLA K71 POLYSACCHARIDE

The bacteria of serotype *Klebsiella* K71 were grown on agar medium B (Mueller-Hinton) instead of the standard agar medium A because the latter resulted in poor yields of capsular material. The acidic polysaccharide was isolated and purified by precipitation with Cetavlon. The purified polysaccharide, which had $[\alpha]_D$ -40° (*c.* 0.1, water) and contained 12.5% (by weight) of uronic acid, was shown by steric-exclusion chromatography to have $M_W \sim 1.5 \times 10^6$. Paper chromatography of the acid hydrolysate showed rhamnose, glucose and glucuronic acid. Determination by g.l.c. of the relative proportions of the neutral sugars, both as alditol acetates and as the peracetylated aldononitriles, indicated that glucose and rhamnose were present in the molar ratio of 2.0 : 3.6, whereas methanolysis of the polysaccharide, followed by reduction with $NaBD_4$ and subsequent hydrolysis and g.l.c. analysis of the derived alditol acetates, gave glucose and rhamnose in the ratio 3.0 : 3.9, indicating that the uronic acid is glucuronic acid.

The absolute configurations of each of the sugar components were confirmed by measurement of their optical rotation after isolation by preparative paper chromatography of the hydrolysate. The ^{1}H-n.m.r. spectrum of the polysaccharide indicated the presence of seven anomeric protons, corresponding to four α- and three β- linked sugar residues, while the signals at high field (~ δ 1.3) established the presence of C-6 methyl groups of the rhamnosyl units.

6.3 METHYLATION ANALYSIS OF KLEBSIELLA K71 POLYSACCHARIDE

A sample of the original polysaccharide was methylated by the Hakomori method; hydrolysis of a portion and g.l.c.-m.s. of the derived alditol acetates gave the results shown in Table 28, column I. After reduction of another portion of the methylated polysaccharide with $LiAlD_4$, similar analysis of the product gave an additional peak, (containing the deuterium label) due to the derivative (**20**) from the ester-reduced uronic acid (see Table 28, column II); Fig. 36 shows the m.s. fragmentation pattern of **20**. Remethylation of the ester-reduced methylated polymer using the Hakomori method, followed by analysis as previously described, gave the results shown in Table 28, column III.

TABLE 28: METHYLATION ANALYSIS OF *KLEBSIELLA* K71 POLYSACCHARIDE AND DERIVED POLYSACCHARIDES

Partially methylated alditol acetates[a]	T[b]	*Mole %*[c]			
		I	II	III	IV[d]
3,4-Rha	0.89	14	14	17	26
2,4-Rha	0.95	12	12	12	19
2,3,4,6-Glc	1.00	17	18	30[e]	15
4-Rha	1.46	25	26	24	15
2,4,6-Glc	1.65	18	17	17	25
2,3,4-Glc	1.94	[f]	13[g]	-	-

[a] 3,4-Rha = 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol, etc
[b] Retention times, determined on column C at 190°C, relative to 2,3,4,6-Glc
[c] Values were corrected by use of effective, carbon-response factors given Sweet *et al.* (Ref. 112)
[d] I, methylated native polysaccharide; II, carboxyl ester-reduced, methylated polysaccharide; III, remethylated, carboxyl ester-reduced, methylated polysaccharide; IV, remethylated, base-degraded, methylated polysaccharide
[e] Peak contains ~ 50% of the C-6 di-deuterated derivative according to g.l.c.-m.s.
[f] Assumed 14%, sugar ratios calculated accordingly
[g] C-6 di-deuterated according to g.l.c.-m.s. (see Fig. 36)

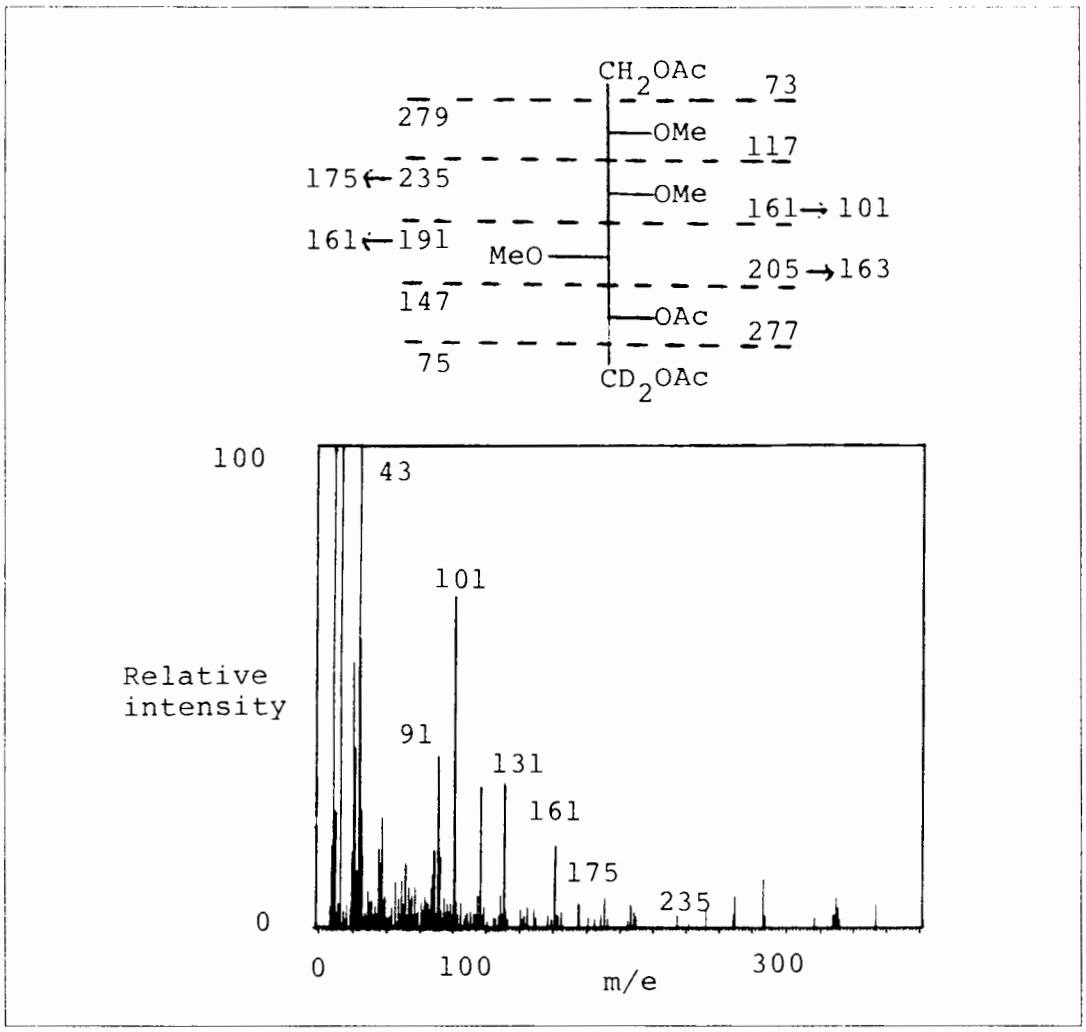


Figure 36: M.s. fragmentation pattern of **20**

These data indicate that there are single branch points on each of two L-rhamnosyl residues, while the other rhamnosyl residues are chain units linked through O-2 and O-3 respectively. One glucosyl residue is a chain unit linked through O-3, whereas the other glucose and the glucuronic acid group are terminal. The occurrence of glucuronic acid as a terminal group confirms the prediction made from immunological cross-reactivity studies[10].

Another portion of the methylated polysaccharide was subjected to a base-catalysed β-elimination by treatment with dimethylsulphinyl anion, followed by re-alkylation with methyl iodide. This degradation caused loss of the glucuronic acid (the absence of which

was evident from paper chromatography), and after remethylation an increase in the proportion of the 3,4-di-*O*-methylrhamnosyl derivative was observed with a concomitant decrease in the amount of the 4-*O*-methylrhamnosyl derivative (see Table 28, column IV). This result indicates that the terminal glucuronic acid group is attached to O-3 of a rhamnosyl branch point.

6.4 THE PRODUCTION AND ANALYSIS OF OLIGOSACCHARIDES P1 AND P2

Bacteriophage Ø71 action on the cognate polysaccharide yielded oligomers which were isolated and separated by s.e.c. into monomeric (**P1**) and dimeric (**P2**) fractions. Reduction of **P1** with $NaBH_4$ (to **P1**-ol) followed by methylation analysis (Table 29, column I) yielded components not present among the partially methylated alditol acetates of the original polymer (Table 28, column I), which corresponded to the acetylated derivatives of 1,3,4,5-tetra-*O*-methylrhamnitol (**21**) [see Fig. 37 for m.s. fragmentation pattern of **21**] and 2,3,4-tri-*O*-methylrhamnitol.

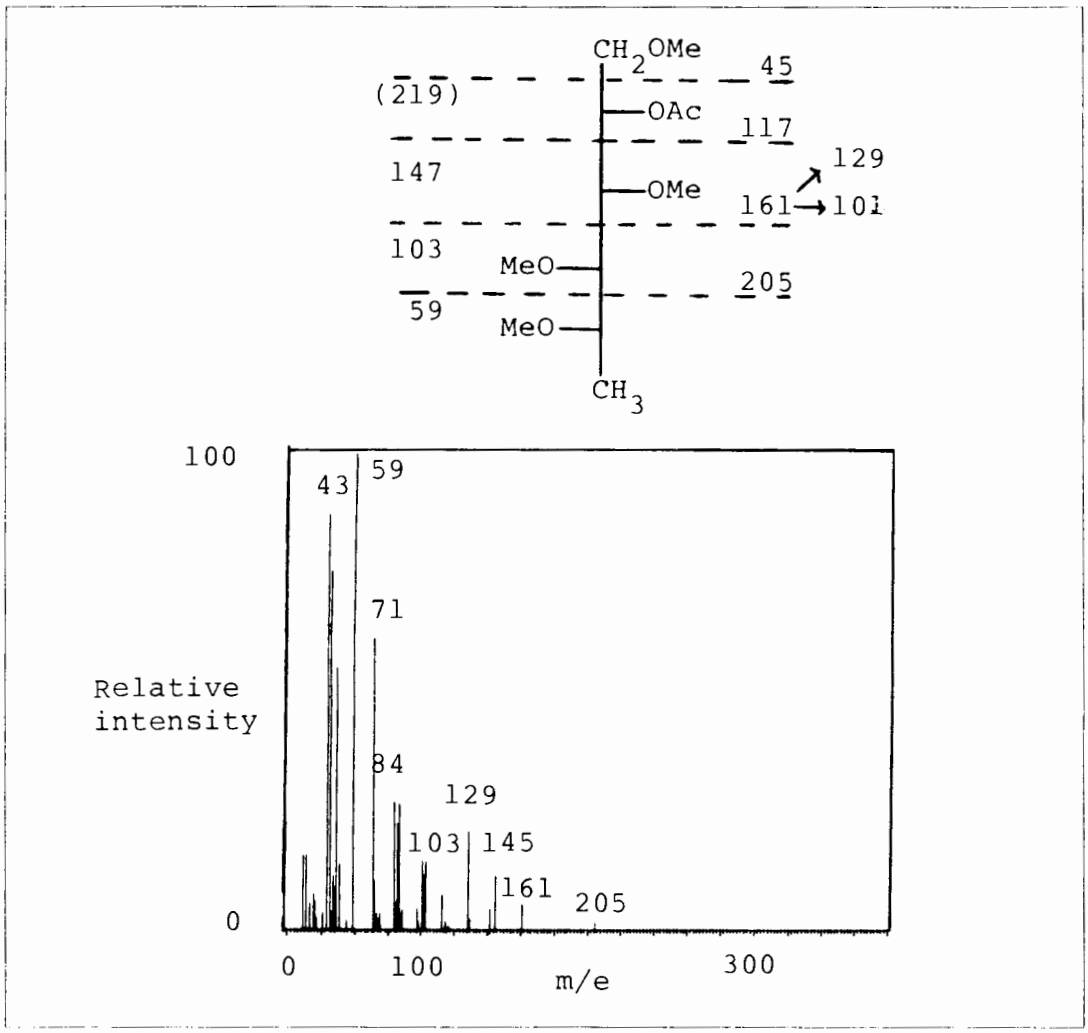


Figure 37: M.s. fragmentation pattern of **21**

These results suggest that cleavage must have occurred between the O-2 and O-3 linked rhamnosyl residues. This finding is supported by the absence of 2- and 3- linked Rha*p* units in **P1**-ol, although these residues are present in **P2**-ol (Table 29, column II) and the polysaccharide.

TABLE 29: METHYLATION ANALYSIS OF P1-OL AND P2-OL

Partially methylated alditol acetates[a]	T[b]	*Mole %*[c]			
		I	II	III	IV[d]
1,3,4,5-Rha[e]	0.49	6	3	4	2
2,3,4-Rha	0.53	14	7	12	6
3,4-Rha	0.89	-	10	-	4
2,4-Rha	0.95	-	7	-	-
2,3,4,6-Glc	1.00	12	15	18	20[f]
4-Rha	1.46	35	29	38	19
2,4,6-Glc	1.65	19	15	15	8
2,3,4-Glc	1.94	[g]	[g]	13[h]	-

[a] 1,3,4,5-Rha = 2-*O*-acetyl-1,3,4,5-tetra-*O*-methylrhamnitol, etc

[b] Retention times, determined on column C at 190°C, relative to 2,3,4,6-Glc. Assignments confirmed by g.l.c.-m.s.

[c] Values were corrected by use of effective, carbon-response factors given Sweet *et al.* (Ref. 112)

[d] I, methylated P1-ol ($NaBH_4$-reduced); II, methylated P2-ol ($NaBH_4$-reduced); III, carboxyl ester-reduced, metnylated P1-ol; IV, remethylated, carboxyl ester-reduced, methylated P1-ol

[e] See Fig. 37

[f] Peak contains ~ 50% of the C-6 di-deuterated derivative according to g.l.c.-m.s.

[g] Assumed 14%, sugar ratios calculated accordingly

[h] C-6 di-deuterated according to g.l.c.-m.s. (see Fig. 36)

The results of g.l.c.-m.s. analyses of the partially methylated alditol acetates derived from carboxyl ester-reduced, methylated **P1**-ol and the remethylated product (given in Table 29, columns III and IV) verify the presence of terminal glucuronic acid groups. These data confirm that **P1** is a heptasaccharide, and **P2** the dimer thereof, and that both have a 2-linked rhamnose residue as the terminal reducing unit.

The identification of rhamnose as the reducing terminal group is further supported by n.m.r. evidence (see 6.5), and finally by the presence of per-*O*-acetylated rhamnitol among the PAAN derivatives formed upon analysis of **P1** by the method of Morrison[115b]. This result is in accordance with the original findings reported by Rieger-Hug and Stirm[90].

Detailed n.m.r. and mass spectroscopic studies were conducted on **P1** and **P2** with the objective of achieving the complete structural elucidation of these oligomers without

recourse to further chemical degradative studies. These investigations are discussed in sections 6.5 and 6.6 respectively.

6.5 N.M.R. STUDIES

P1 and $NaBH_4$-reduced **P1** (**P1**-ol) were examined using 2D n.m.r. methods with a view to establishing the structure of the heptasaccharide repeating unit solely by n.m.r. methods.

6.5.1 ASSIGNMENT OF ANOMERIC AND SOME RING ^1H AND ^{13}C RESONANCES OF P1

The ^1H-n.m.r. spectrum of **P1** (Fig. 38) shows four α-linked anomeric signals (δ 5.4-5.1, singlets) attributable to sugar units having the *manno* configuration (i.e. the rhamnosyl residues), while recording the spectrum at 80°C revealed the presence of three β-linked sugar residues (δ 4.8-4.6; J ~ 8 Hz) ascribed to the hexose and uronic acid sugar units; the assignments were made on the basis of chemical shift and coupling constant analysis (see 3.1.1. and 3.1.2).

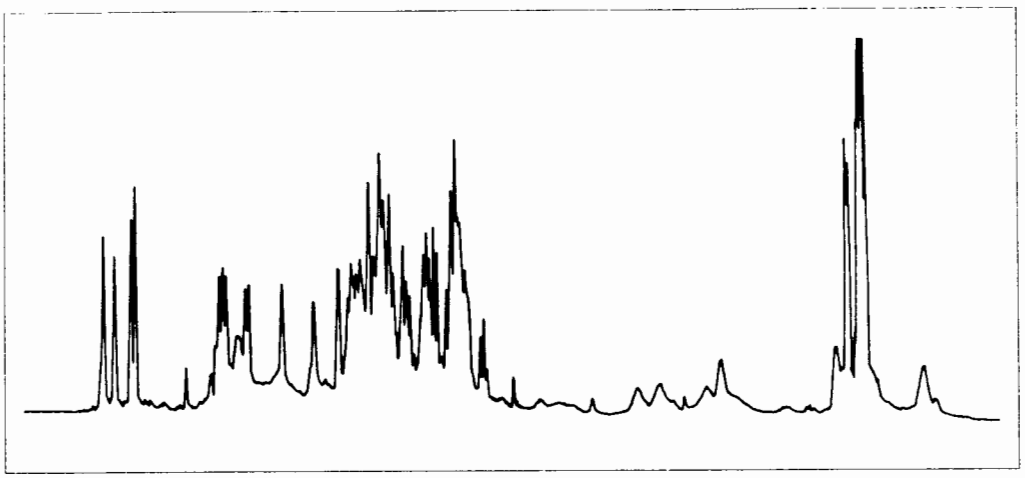


Figure 38: ^{1}H-n.m.r. spectrum (500 MHz) of **P1** recorded at 30°C

The sugar residues giving rise to the anomeric resonances were designated **a** to **g** in order of decreasing anomeric proton chemical shift. The signal at δ 4.85 (0.2H, singlet) was assigned to β-Rha*p*-OH of the reducing end. The assignments were confirmed by inspection of the ^{13}C-n.m.r. spectrum (Fig. 39, see 3.2 for typical δ values), connectivities being established using the HETCOR experiment.

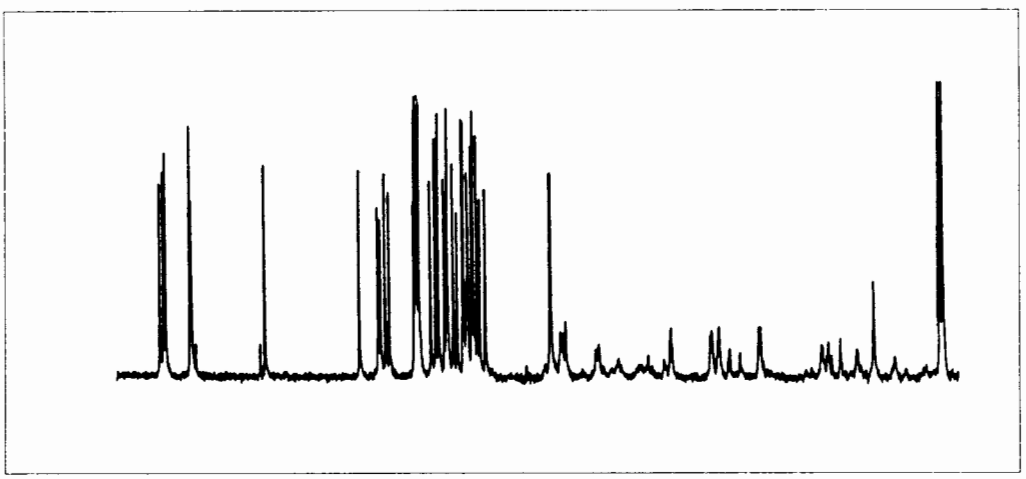


Figure 39: ^{13}C-n.m.r. spectrum (125 MHz) of **P1** recorded at 30°C

The anomeric proton signal of the α-Rha*p*-OH residue (**c**) was not immediately assignable by inspection alone, but followed from the ^{13}C-n.m.r. assignment of C-1 at δ 93.58. The effect of anomerisation of this terminal rhamnose on adjacent residues (i.e. "twinning" of

signals, see 3.3.4) could not be discerned in the ^{1}H-n.m.r. spectrum, although it is apparent in the ^{13}C-n.m.r. spectrum (see Figure 39).

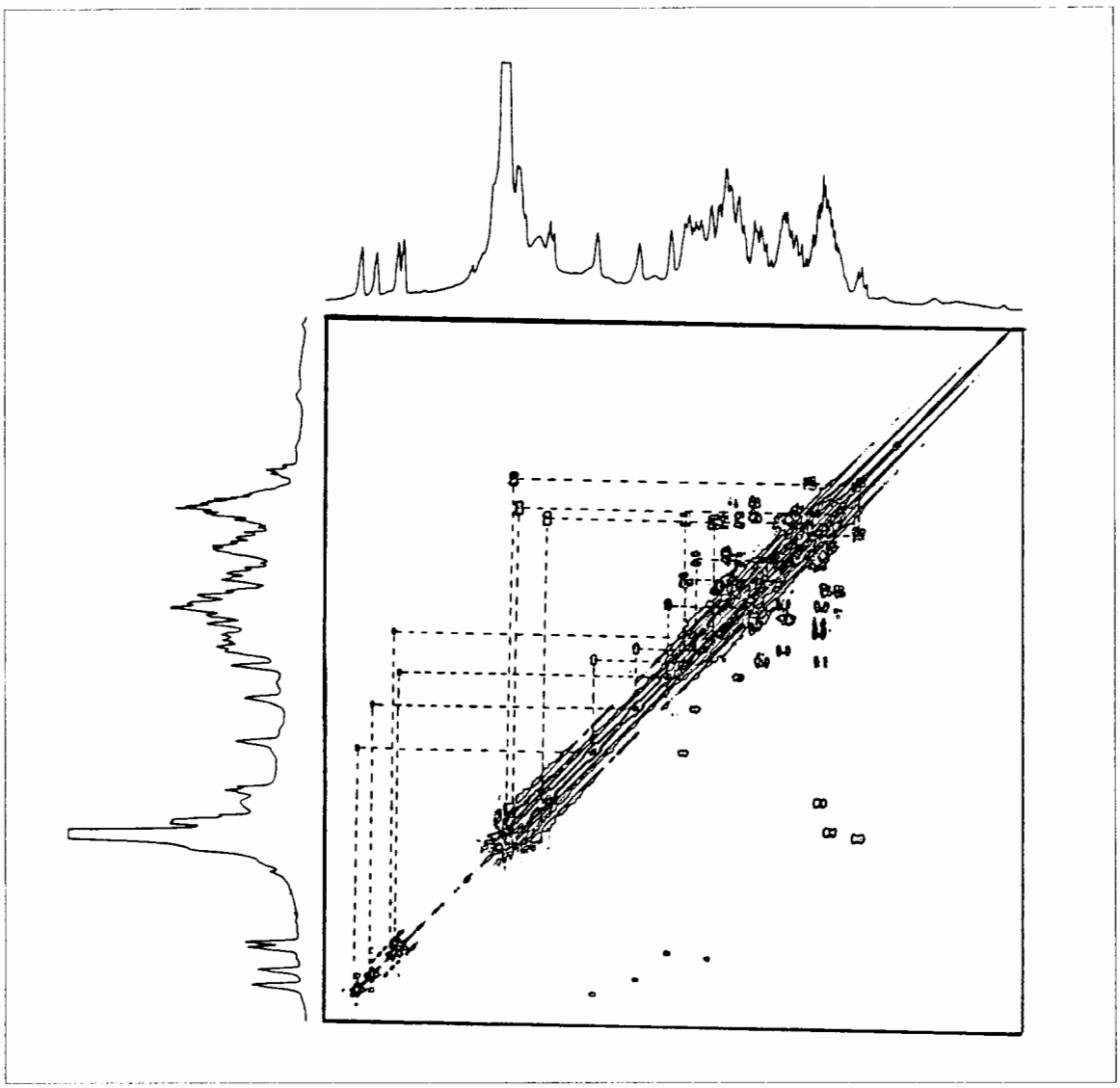


Figure 40: COSY spectrum of **P1** recorded at 30°C, some of the spin systems are indicated

The COSY experiment (Fig. 40) aided the unambiguous assignment of most of the H-2 and H-3 resonances of the sugar residues, while the ^{13}C assignments followed using the HETCOR diagram (Fig. 41). The ^{1}H and ^{13}C data are given in Tables 30 and 31 respectively.

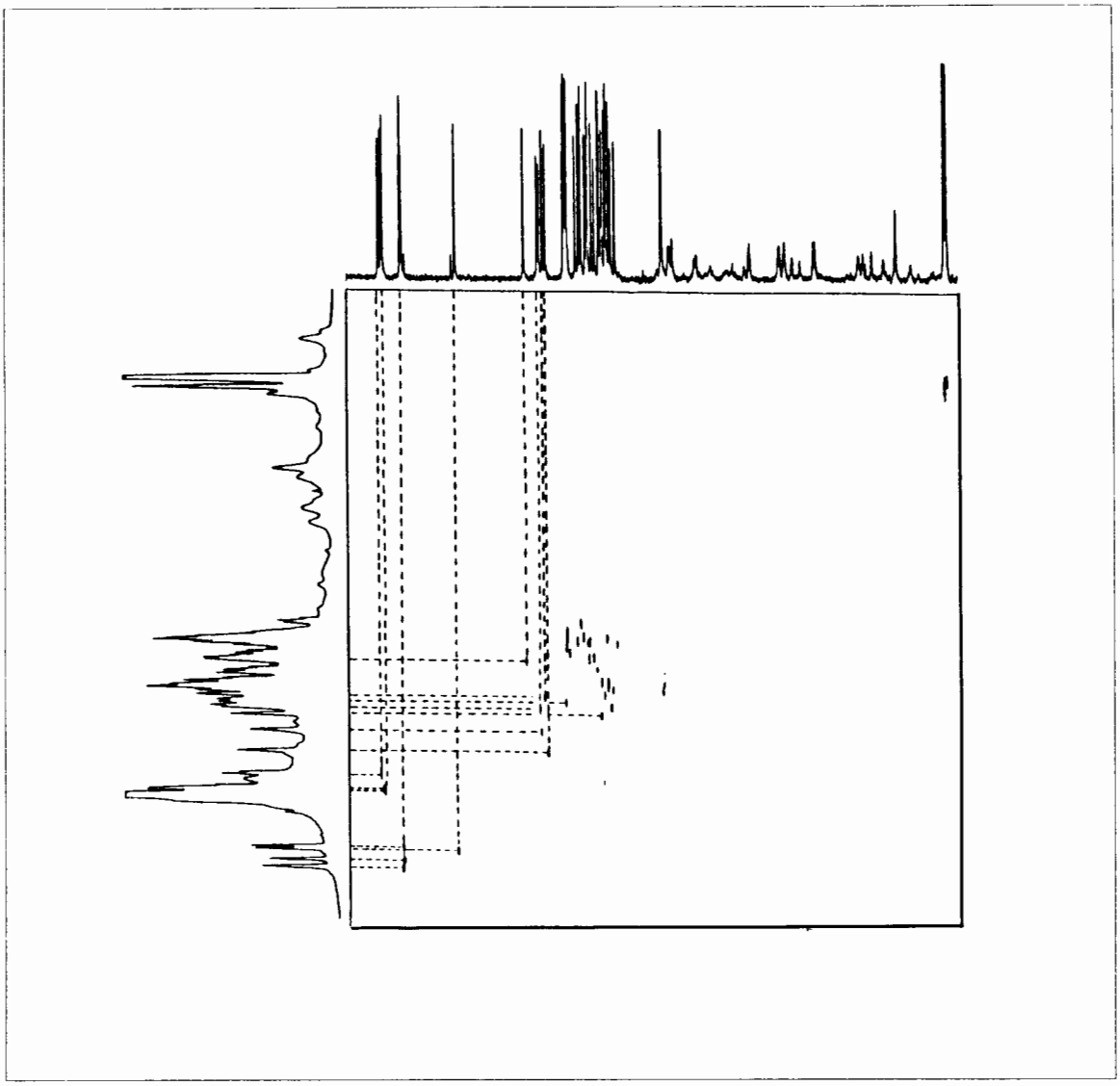


Figure 41: HETCOR spectrum of **P1** recorded at 30°C ($J_{C,H}$ = 140 Hz), some of the C,H correlations are indicated

TABLE 30: ^{1}H-N.M.R. DATA (500 MHz) AND GLYCOSYLATION SHIFTS * FOR P1

Sugar residue	*Chemical shift (p.p.m.)*		
	H-1	H-2	H-3
a*	5.36 (+0.63)	4.40 (+0.47)	4.03 (+0.32)
b*	5.30 (+0.60)	4.23 (+0.30)	3.98 (+0.27)
c*	5.21 (+0.10)	3.93 (+0.01)	n.r.
d*	5.19 (+0.49)	4.10 (+0.17)	3.79 (+0.08)
e*	4.73 (+0.36)	3.32 (+0.07)	3.52 (+0.04)
f*	4.71 (-0.05)	3.43 (-0.02)	3.55 (-0.11)
g*	4.60 (+0.23)	3.48 (+0.23)	3.64 (+0.16)

Glycosylation shifts are given in parentheses.

* A downfield shift relative to the model compound is considered positive
* Model compound α-Rha*p*-OMe (Ref. 188)
* Model compound α-Rha*p*-OH (Ref. 188)
* Model compound β-Glc*p*-OMe (Ref. 177)
* Model compound β-Glc*p*A-OH (Table 2)

TABLE 31: ^{13}C-N.M.R. DATA (125 MHz) AND GLYCOSYLATION SHIFTS [a] FOR **P1**

Sugar residue	*Chemical shift (p.p.m.)*		
	C-1	C-2	C-3
a [b]	102.02 (+7.0)	79.58 (+7.7)	80.83 (+9.7)
b [b]	101.80 [c] (+6.8)	80.56 (+8.7)	76.82 (+5.7)
c [b,d]	93.58 (-1.4)	80.04 (+8.1)	n.r. -
d [b]	101.95 (+7.0)	71.32 (-0.6)	27.07 (-0.4)
e [e]	104.68 (+8.0)	74.41 (-0.7)	~76.7 (0.0)
f [f]	104.94 (+8.1)	74.05 (-0.9)	76.21 (-0.3)
g [e]	105.34 [g] (+8.6)	74.94 (-0.2)	82.95 (+6.2)

Glycosylation shifts are given in parentheses.

[a] A downfield shift relative to the model compound is considered positive
[b] Model compound α-Rha*p*-OH (Ref. 155)
[c] Twin signal at δ 101.35
[d] Twin signals at δ 94.10 and 79.95 due to C-1 and C-2 of β-Rha*p*-OH (c)
[e] Model compound β-Glc*p*-OH (Ref. 155)
[f] Model compound β-Glc*p*A (Table 1)
[g] Twin signal at δ 105.42

Comparison of the chemical shift values obtained with those of model compounds (see Table 30 and 31) clearly showed the positions of linkage to be: 2,3-**a**; 2,3-**b**; 2-**c**~OH and 3-**g**, with residues **d**,**e** and **f** present as terminal groups. The assignments are consistent with the results obtained from methylation analysis (see Table 29); however the sequence of sugar residues was not yet apparent. The significance of the glycosylation shifts found for the sugar residues and their relationship to the configurational factors present in **P1** will be discussed later (see 6.5.3).

Examination of the ^{13}C-n.m.r. spectrum of **P1** (Fig. 39) revealed the twinning of certain signals which were tentatively attributed to those emanating from the residues at or near the reducing end. In addition to the resonances due to C-1 ($\Delta\delta$ = 0.52 p.p.m.) and C-2 ($\Delta\delta$ = +0.09 p.p.m.) of α-Rha*p*(**c**), twinning was also observed for the signals ascribed to C-1

($\Delta\delta$ = 0.45 p.p.m.) of **b** and C-1 ($\Delta\delta$ = 0.08 p.p.m.) of **g**. The chemical shift differences between the twin signals of the anomeric carbons, given in parentheses, suggest the sequence **g**→**b**→2**c**~OH. **g** can be linked to O-2 or O-3 of **b**, the former position of attachment being more probable because the effects of anomerisation are greater for O-2 than O-3 linked substituents (see 3.3.4 and 3.3.7). In order to determine the remaining sequence and possibly also conformation of the sugar residues in **P1**, the $NaBH_4$-reduced **P1** (**P1**-ol) was examined in detail as described in the next sections (6.5.2 and 6.5.3).

6.5.2 ASSIGNMENT OF THE ^1H AND ^{13}C RESONANCES OF P1-OL

The ^1H- and ^{13}C- n.m.r. spectra of **P1**-ol are given in figures 42 and 43.

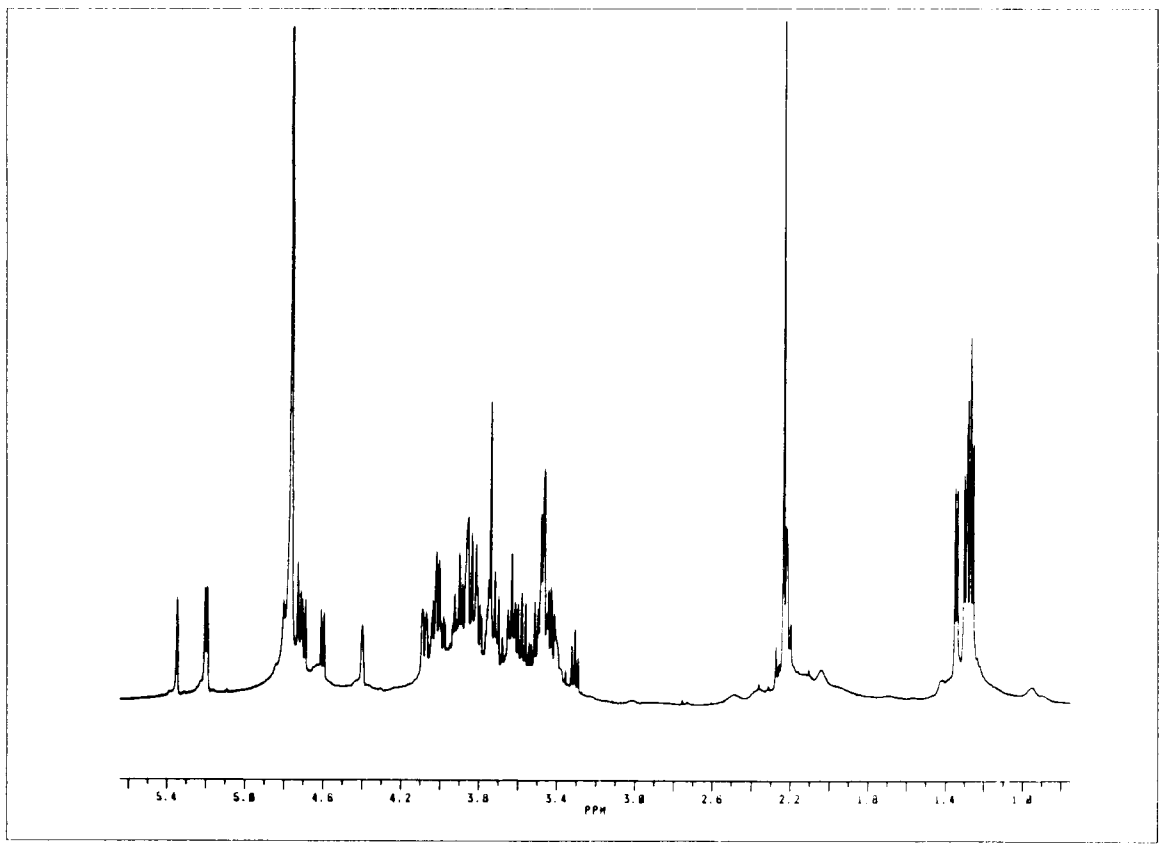


Figure 42: ^1H-n.m.r. spectrum (500 MHz) of **P1**-ol recorded at 25°C

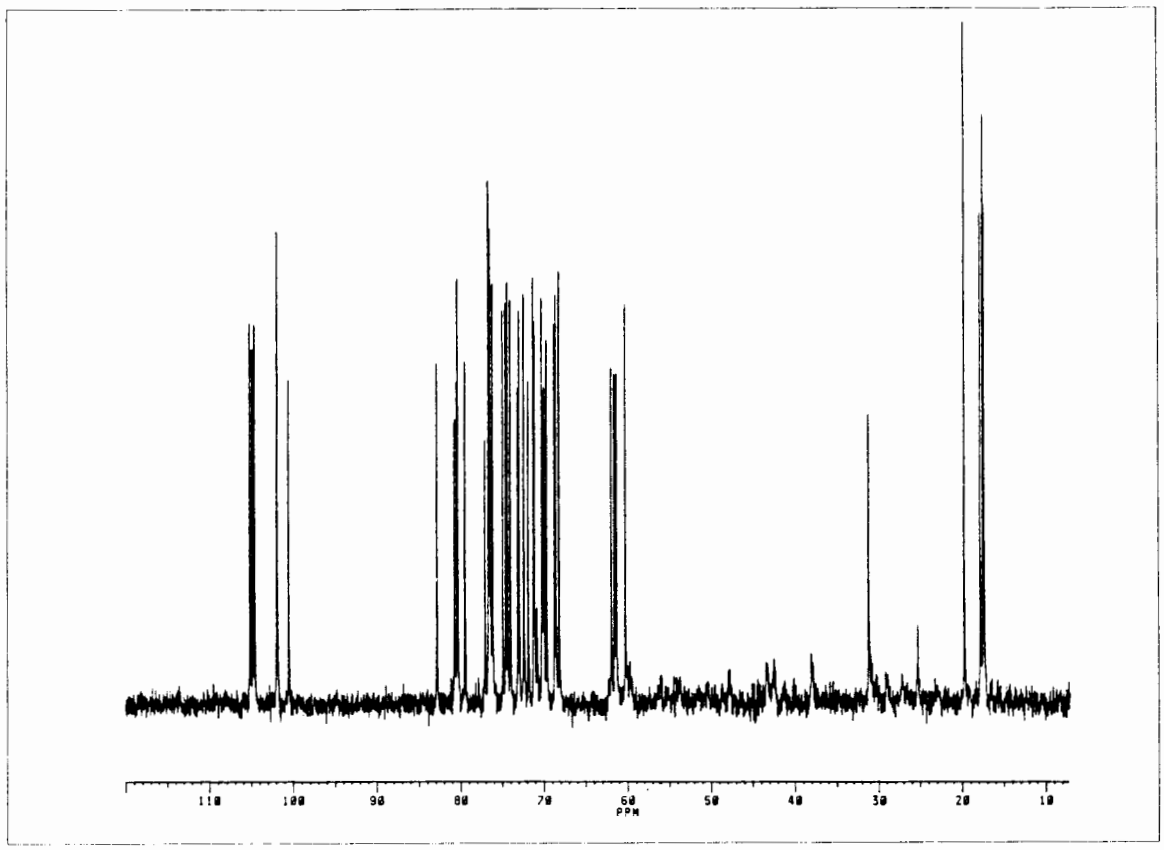


Figure 43: ^{13}C-n.m.r. spectrum (125 MHz) of **P1**-ol recorded at 25°C

Comparison of the ^{1}H-n.m.r. spectra of **P1** and **P1**-ol shows that the signal attributed to H-1 of C (δ 5.21) disappears upon reduction, while the resonance at δ 5.30 (**b**) is shifted upfield to δ 5.20 in **P1**-ol. These changes in chemical shift confirm the sequence **b**→2**c**. This result was corroborated by inspection of the ^{13}C-n.m.r. spectrum of **P1**-ol (Fig. 43) which showed the disappearance of the twin signals for residue **b**, while the resonances due to the reducing end (**c**) were modified as expected. Moreover the spectrum also showed the absence of twinning for the ^{13}C signal assigned to residue **g**. These observations support the postulate that the sugar sequence **g**→2**b**→2**c**, is present at the reducing end of **P1**.

The anomeric proton assignments served as the starting point for correlations established using the COSY experiment (Fig. 44), while the HETCOR diagram (Fig. 45) enabled

identification of the attached carbon atoms. In addition the H-6 signals of the rhamnosyl residues **a** to **d** permitted assignment of the corresponding H-5 (and hence C-5) resonances.

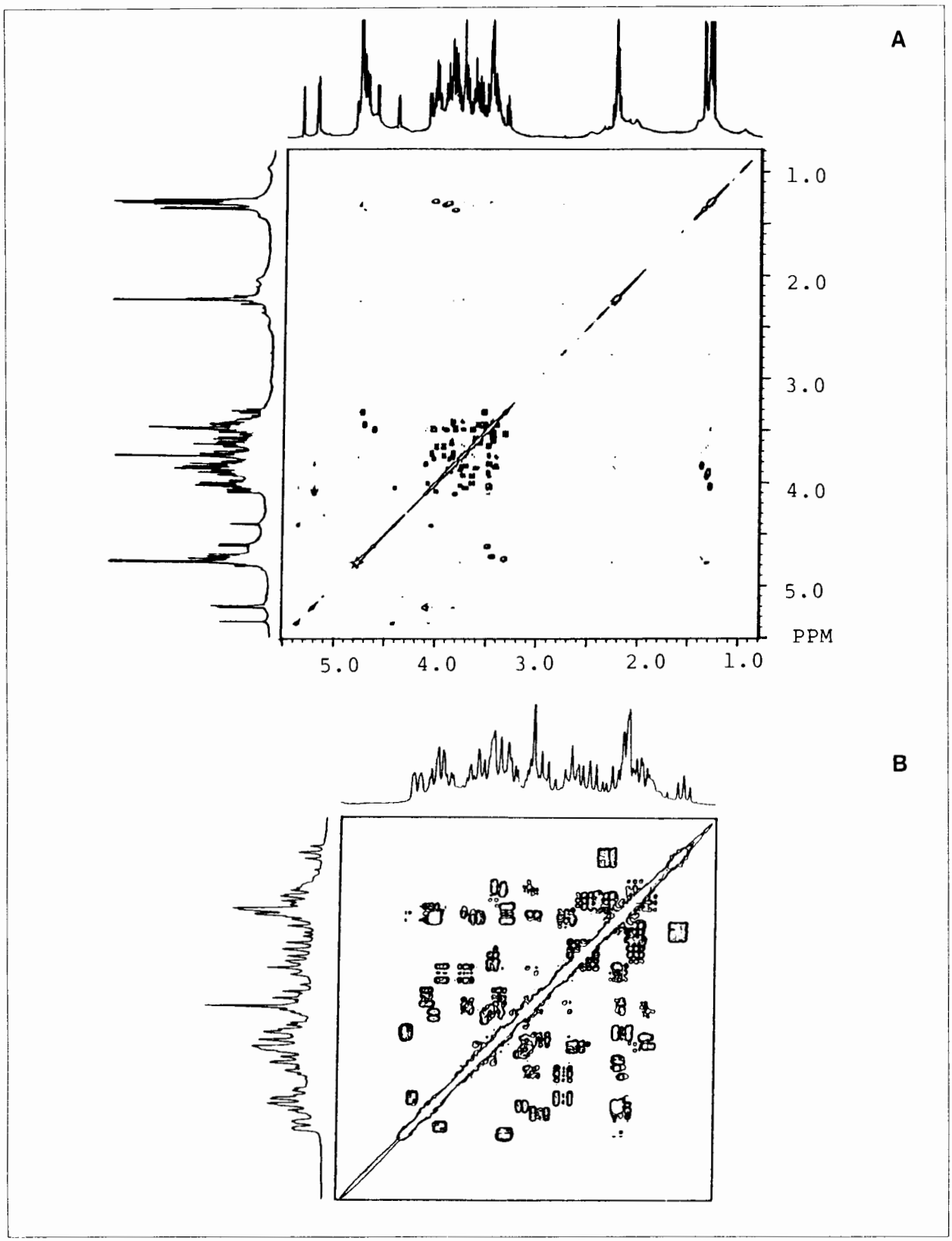


Figure 44: The full (A) and expanded (B) COSY spectra of **P1**-ol recorded at 30°C

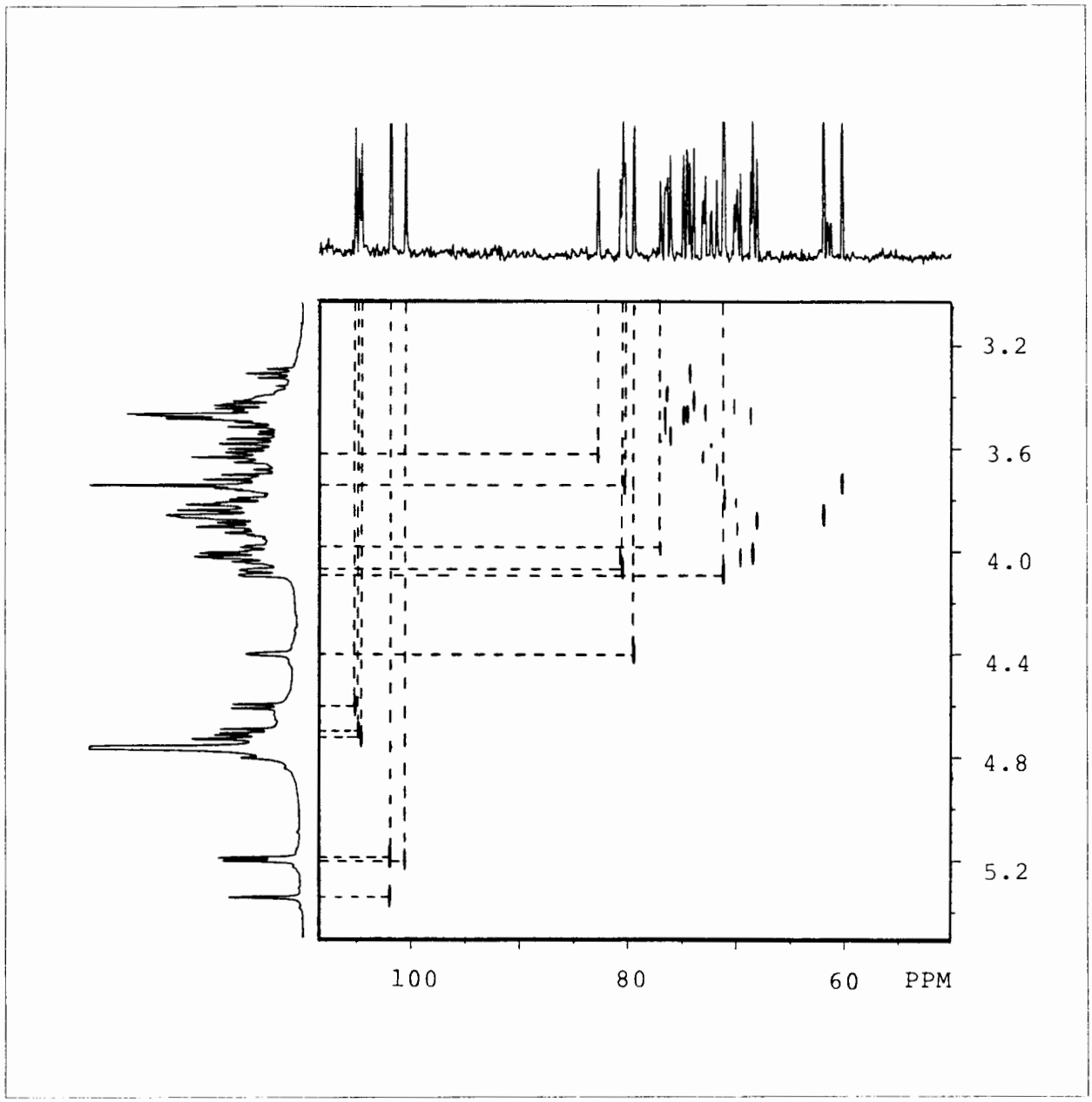


Figure 45: Expanded HETCOR spectrum of **P1**-ol recorded at 30°C ($J_{C,H}$ = 140 Hz), some of the C,H correlations are shown

Connectivities established using the long-range HETCOR experiment (Fig. 46) were corroborated by inspection of the 1D plots, the results are presented in Table 32 together with the data obtained from the COLOC experiment[222] (Fig. 47, see 5.5.2 for details).

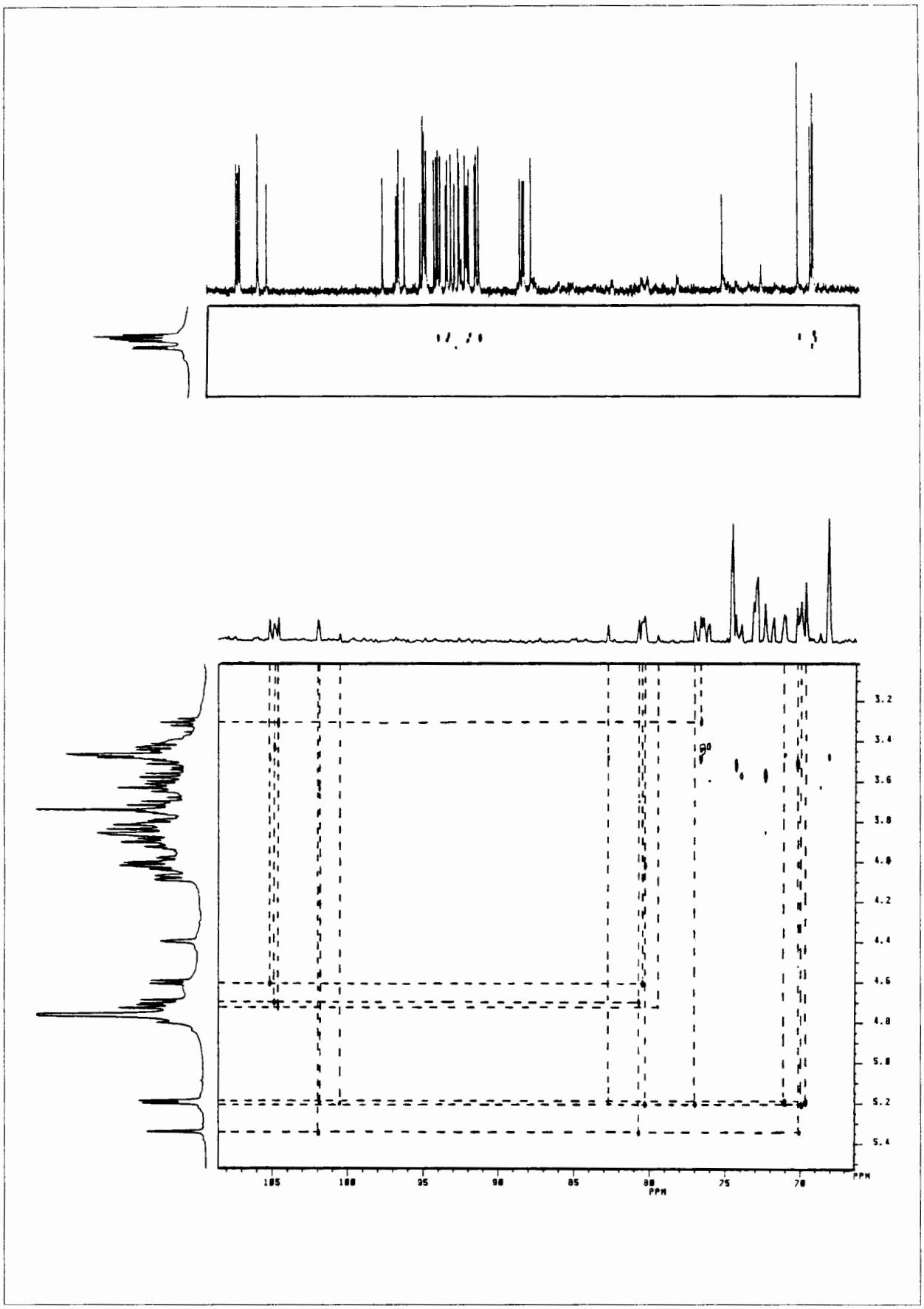


Figure 46: Expanded long-range HETCOR spectrum of **P1**-ol recorded at 30°C ($J_{C,H}$ = 6 Hz), some of the C,H correlations are indicated

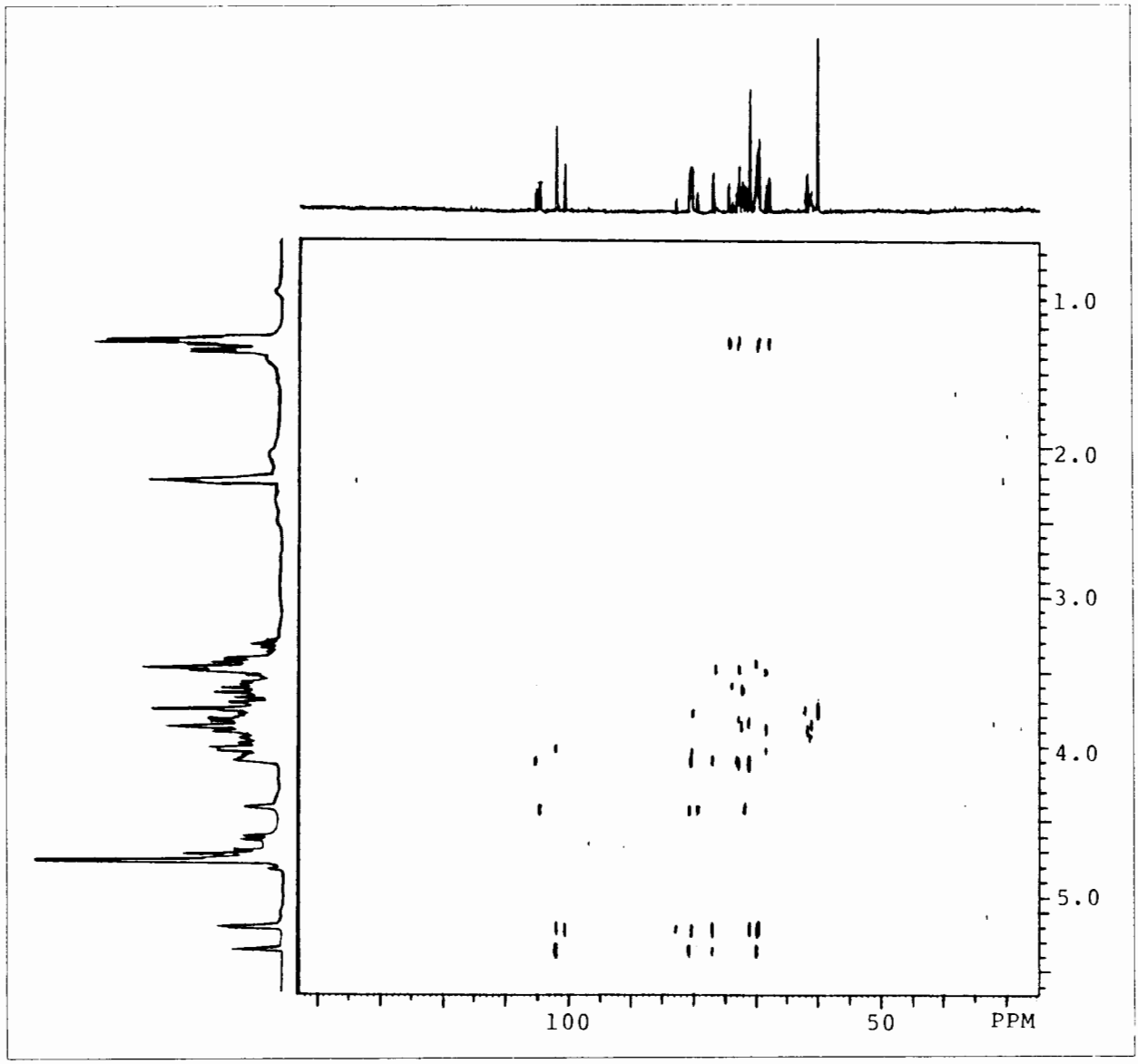


Figure 47: COLOC spectrum of **P1**-ol recorded at 30°C

The long-range heteronuclear correlation experiments established connectivity between H-1 and C-3 and C-5 of the α-Rha*p* units. These results confirmed the C-3 assignments (made previously from the H-3 identifications) and united the assignments made from H-1 with those made from correlations established from H-6. Figure 46 also revealed connectivity between H-6 of the rhamnosyl residues and C-6, C-5 and C-4 of the same residues, thus permitting identification of the H-4's (using the HETCOR diagram). These experiments enabled the full assignments of residues **a**, **b** and **d** to be made [see Tables 33 (^{1}H) and 34 (^{13}C)].

TABLE 32: ASSIGNMENTS ESTABLISHED USING THE LONG-RANGE HETERONUCLEAR CORRELATION EXPERIMENTS (SEE FIGS. 49 AND 50)

Sugar residue	*^{1}H signal*	*δ*	*Assignment*	*Experiment*[a]
a	H-1	101.86	C-1 of **a**	LRH,COLOC
		80.64	C-3 of **a**	LRH,COLOC
		76.96	C-3 of **b**	LRH(1D),COLOC
		69.98	C-5 of **a**	LRH,COLOC
	H-2	104.51	C-1 of **e**	COLOC
		80.64	C-3 of **a**	COLOC
		79.36	C-2 of **a**	COLOC
		71.74	C-4 of **a**	COLOC
	H-6	71.24	C-4 of **a**	LRH,COLOC
		69.98	C-5 of **a**	LRH,COLOC
		17.72	C-6 of **a**	LRH
b	H-1	100.46	C-1 of **b**	LRH,COLOC
		80.25	C-2 of **b**	LRH,COLOC
		76.96	C-3 of **b**	LRH,COLOC
		69.85	C-5 of **b**	LRH,COLOC
	H-2	105.06	C-1 of **g**	COLOC
		80.36	C-2 of **b**	COLOC
		76.96	C-3 of **b**	COLOC
		73.02	C-4 of **b**	COLOC
	H-6	73.02	C-4 of **b**	LRH,COLOC
		69.85	C-5 of **b**	LRH,COLOC
		17.24	C-6 of **b**	LRH
c	H-6	76.53	C-4 of **c**	LRH,COLOC
		68.07	C-5 of **c**	LRH,COLOC
		19.62	C-6 of **c**	LRH
d	H-1	101.81	C-1 of **d**	LRH,COLOC
		82.72	C-2 of **g**	LRH,COLOC
		71.00	C-3 of **d**	LRH,COLOC
		69.56	C-5 of **d**	LRH,COLOC
	H-2	72.83	C-4 of **c**	COLOC
		~71.0	C-2 and C-3 of **c**	COLOC
	H-6	72.83	C-4 of **c**	LRH,COLOC
		69.56	C-5 of **c**	LRH,COLOC
		17.35	C-6 of **c**	LRH
e	H-1	104.51	C-1 of **e**	LRH
		79.36	C-2 of **a**	LRH
	H-2	104.51	C-1 of **e**	LRH
		76.53	C-3 of **e**	LRH
f	H-1	104.77	C-1 of **f**	LRH
		80.64	C-3 of **a**	LRH
	H-2	76.03	C-3 of **f**	LRH
g	H-1	105.06	C-1 of **g**	LRH
		80.36	C-2 of **b**	LRH
	H-2	105.06	C-1 of **g**	LRH
		82.72	C-3 of **g**	LRH

[a] LRH = long-range HETCOR experiment ($J_{C,H}$ = 6 Hz), COLOC = COrrelation spectroscopy *via* LOng-range Couplings, see Ref. 222). The experiments were conducted on a Bruker WM500 machine at 30°C

The n.m.r. assignments of residue **c** were made as follows. Inspection of the ^{13}C-n.m.r. spectra of **P1** and **P1**-ol permitted the assignments of C-2 at δ 80.25 (δ 80.04 and 79.95 in **P1**) and C-6 at δ 19.62 (all signals between δ 17.90 and 17.42 in **P1**, Fig. 39). The C-6 assignment permitted the identification of H-6 (at δ 1.28) from which the C-5 and C-4

resonances of **c** were assigned (see long-range HETCOR, Fig. 46). Figure 46 also revealed connectivity between H-2 of residue **c** and the ^{13}C signals at δ 60.17 and 61.88 (not present in the ^{13}C-n.m.r. spectrum of **P1**), these resonances were assigned to C-1 and possibly C-1 or C-3 of **c** respectively. However, the ^{13}C signal at δ 68.47 (also not present in the ^{13}C-n.m.r. spectrum of **P1**) may also be attributed to C-3 of **c**, on the basis of chemical shift analysis (*cf.* 2-linked rhamnitol[224]) and connectivity suggested by the COSY diagram (Fig. 44). The ^{13}C assignments as depicted in Table 34 were partly verified by identification of the attached protons (using the HETCOR diagram, Fig. 45) and inspection of the connectivities indicated by the COSY plot (Fig. 44).

The β-linked sugars (**e**, **f**, **g**) proved to be less amenable to n.m.r. analysis because of the small chemical shift differences found between the signals of the same atoms of different residues. Consequently some of the assignments were made by chemical shift analysis only. The COSY experiment (Fig. 44) enabled identification of the H-2 and H-3 signals to be made, while the attached carbon assignments followed from the HETCOR plot. The long-range HETCOR experiment (Fig. 46) showed coupling between the following: H-2 to C-1 and C-3 of **e**, H-3 and C-2 of **f** and finally H-2 and C-4 of **g**. Position **f** was assigned to the glucuronic acid residue, for the reasons discussed in the next section [see 6.5.3 (v)].

The assignments made from the n.m.r. studies described above, together with the glycosylation shifts determined by comparison of the shift values obtained with those of suitable model compounds, are displayed in Tables 33 (^{1}H data) and 34 (^{13}C data).

TABLE 33: ^{1}H-N.M.R. DATA (500 MHz) AND GLYCOSYLATION SHIFTS [a] FOR **P1**-OL

Sugar residue	*Chemical shift [c] (p.p.m.)*					
	H-1	H-2	H-3	H-4	H-5	H-6
α-Rha*p* (a) [b]	5.34 (+.64)	4.40 (+.47)	4.03 (+.32)	3.70 (+.26)	3.85 (+.21)	1.34 (+.04)
α-Rha*p* (b) [b]	5.20 (+.50)	4.07 (+.14)	3.99 (+.28)	3.63 (+.19)	3.92 (+.28)	1.29 (+.01)
Rhamnitol (c) [b]	3.73 (-.97)	3.75 (-.18)	4.00 [e] (+.29)	3.49 (+.05)	3.90 (+.26)	1.28 (-.02)
α-Rha*p* (d) [b]	5.19 (+.49)	4.09 (+.16)	3.79 (+.08)	3.45 (+.01)	4.02 (+.38)	1.24 (-.06)
β-Glc*p* (e) [d]	4.72 (+.35)	3.32 (+.07)	3.52 (+.12)	n.r.	n.r.	3.77 [e] -
β-Glc*p*A (f) [f]	4.69 (-.07)	3.43 (-.02)	3.53 (-.13)	n.r.	n.r.	-
β-Glc*p* (g) [d]	4.60 (+.23)	3.49 (+.24)	3.62 (+.22)	n.r.	n.r.	3.71,3.92 -

Glycosylation shifts given in parentheses.

[a] A downfield shift relative to the model compound is considered positive
[b] Model compound α-Rha*p*-OMe (Ref. 188)
[c] Or δ 3.88
[d] Model compound β-Glc*p*-OMe (Ref. 177)
[e] Assignments might be reversed
[f] Model compound β-Glc*p*A-OH (Table 2)

TABLE 34: ^{13}C-N.M.R. DATA (125 MHz) AND GLYCOSYLATION SHIFTS [a] FOR **P1**-OL

Sugar residue	*Chemical shift (p.p.m.)*					
	C-1	C-2	C-3	C-4	C-5	C-6
α-Rha*p* (a) [b]	101.86 (+6.9)	79.36 (+7.5)	80.64 (+9.5)	71.74 (-1.6)	69.98 (+0.6)	17.72 (-0.3)
α-Rha*p* (b) [b]	100.46 (+5.5)	80.36 (+8.5)	76.96 (+5.9)	73.02 (-0.3)	69.85 (+0.4)	17.24 (-0.76)
Rhamnitol (c) [c]	60.17 [d] (-5.5)	80.25 (+6.5)	68.47 [d] (-3.7)	76.53 (+0.6)	68.07 (-1.3)	19.62 (-1.8)
α-Rha*p* (d) [b]	101.81 (+6.8)	71.14 (-0.7)	71.00 (-0.1)	72.83 (-0.5)	69.56 (+0.2)	17.35 (-0.6)
β-Glc*p* (e) [e]	104.51 (+7.8)	74.23 (-0.9)	76.53 (-0.2)	n.r.	n.r.	61.23 [f] (-0.5)
β-Glc*p*A (f) [g]	104.77 (+8.0)	73.88 (-1.0)	76.03 (-0.4)	n.r.	n.r.	174.50 (-2 2)
β-Glc*p* (g) [e]	105.06 (+8.4)	74.45 (-0.6)	82.72 (+6.0)	n.r.	n.r.	61.44 [f] (-0.3)

Glycosylation shifts given in parentheses.

[a] A downfield shift relative to the model compound is considered positive
[b] Model compound α-Rha*p*-OH (Ref. 155)
[c] Model compound L-rhamnitol (Ref. 224)
[d] Or δ 61.88
[e] Model compound β-Glc*p*-OH (Ref. 155)
[f] Assignments might be reversed
[g] Model compound β-Glc*p*A-OH (Table 1)

6.5.3 ESTABLISHMENT OF THE SEQUENCE AND SUBSTITUTION PATTERNS OF SUGAR RESIDUES IN P1

The glycosylation shifts presented in Tables 33 and 34, especially the ^{13}C shifts, confirm the linkage assignments made for **P1** (see 6.5.1). The establishment of the sequence of sugar residues **a** to **g** is discussed below. Where possible the correlations involving ^{13}C chemical shift and configurational factors, as promulgated by Kochetkov *et al.*[156], were applied.

(i) α-**Rha*p*** **(a)**

The small differences in chemical shift values observed for **a** in **P1** and **P1**-ol suggests that this residue is not in close proximity to the reducing end group (previously identified as **c**). The large glycosylation shifts found for C-2 (+ 7.5 p.p.m.) and C-3 (+ 9.5 p.p.m.) confirms that residue **a** is 2,3-linked and furthermore suggests that the attached substituents must be β-linked (**e**, **f** or **g**) rather that α-Rha*p* units (**b** to **d**). [*cf.* ranges of glycosylation shifts given for α- and β- linked sugar residues, Ref. 155]. This supposition was justified by the long-range ^{1}H-^{13}C correlation experiments (Table 32, Figs. 46 and 47) which showed cross-peaks between the signals assigned to the anomeric protons of units **e** and **f** and those imputed to C-2 and C-3 of **a**, thereby establishing the **e**→2**a**→ and **f** →3**a**→ fragments of **P1**. The **e**→2**a**→ sequence was corroborated by connectivity found between the H-2 resonance of **a** and the signal previously assigned to C-1 of **e** (Fig. 47, COLOC experiment). Furthermore, both the long-range HETCOR (1D) and COLOC spectra revealed

connectivity between H-1 of **a** and the ^{13}C signal at δ 76.96, attributed to C-3 of **b**. Thus the sequence of sugar residues **a**,**b**,**e** and **f** are established as shown below; however, the configurational factors of these linkages could not be determined because the data provided by Kochetkov *et al.*[156] does not include correlations for branched sugar units.

f→3**a**→3**b**
2
↑
e

Regarding the H-2 resonance at δ 4.40, a downfield shift appears to be common for the H-2's of 3-linked Rha*p* units (see 4.5.2). In this case the trend is further enhanced by O-2 glycosylation, resulting in a large downfield shift of +0.47 p.p.m. for H-2 of this 2,3-linked residue.

(ii) α-Rha*p* (b)

The ^{1}H and ^{13}C glycosylation shifts observed for **b** clearly show that this residue is 2,3-linked (see Tables 34 and 35). The attachment of **b** to O-2 of the reducing end group (**c**) has already been discussed; this linkage assignment was verified by the detection of long-range connectivity between H-1 of **b** and C-2 of the rhamnitol moiety (**c**). The attachment of **a** to O-3 of **b** has already been proved [see (i), and Table 32], while the twinning of signals assigned to C-1 of **g** (in **P1**) indicated that this residue is linked to O-2 of **b** (see 6.5.2). These linkage assignments are supported by the magnitudes of the ^{13}C glycosylation shifts (+8.5 p.p.m. for C-2 and +5.9 p.p.m. for C-3; see ranges for α- and β-linked substituents given in Ref. 155). The **g**→2**b** sequence was corroborated by the

presence of a cross-peak between the anomeric proton signal of **g** and the ^{13}C resonance at δ 80.36, previously attributed to C-2 of **b** (Fig. 46) and finally by connectivity between H-2 of **b** and C-1 of **g** as revealed in the COLOC spectrum (Fig. 47). Although no configurational factors could be confirmed (*cf.* Ref. 156), the following structure could be formulated (solely on the basis of n.m.r. evidence).

g→2**b**→2**c**-ol
3
↑
f→3**a**
2
↑
e

(iii) Rha-OH (c)

The presence of this residue at the reducing end of **P1** was evident from the ^{13}C anomeric signals at δ 93.58 and 94.10 (for the α- and β- anomers respectively). The upfield shifts of these signals are diagnostic of O-2 glycosylation (β-effects of -1.4 and -0.5 p.p.m. for the α- and β- anomers respectively)[155,191]; this position of linkage was confirmed by the downfield shifts of +8.1 and 7.6 p.p.m. detected for C-2 of α- and β-Rha*p*-OH (see Table 31). These signals were modified as expected upon reduction of **P1** to **P1**-ol.

(vi) β-Glc*p*A (f)

The assignment of **f** as the terminal glucuronic acid group follows from the inputation of **e** as described above. The attachment of this group to O-3 of **a** has been shown by long-range coupling experiments (Table 32) and also by chemical means [involving base-degradation of the permethylated polymer (see 6.3)]. Apart from the major factors of absolute and anomeric configurations of the sugar residues and the nature of the aglycone, the chemical shift value of the anomeric carbon involved in linkage appears to be also influenced by the C-2 substituent of the same residue[156]; consequently it may be assumed that the anomeric centres of β-D-Glc*p*A and β-D-Glc*p* are sufficiently similar to permit use of the configurational correlations described in reference 156. The C-1 signals at δ 104.94 (**P1**) and 104.77 (**P1**-ol) observed for **f** fall within the range reported for β-linked D-hexosyl groups that are attached to O-3 of L-Rha*p* units (104-105.5 p.p.m.), thereby confirming the configurational factors of the **f**→3**a** fragment.

(vii) β-Glc*p* (g)

The ^{13}C glycosylation shifts observed for **g** (+6.2 and +6.0 p.p.m. for C-3 in **P1** and **P1**-ol) confirm that this residue is 3-linked, while the small shielding found for C-2 (the β-effect of glycosylation) is within the range given for C-2 of β-D-glucose substituted at O-3 by α-L-Rha*p* (-0.9 to +0.6 p.p.m.)[156], thus establishing the configurational factors of the **d**→3**g** linkage. The position of **g** in the structure of the heptasaccharide repeating unit, as revealed by long-range connectivity experiments (Table 32), has already been proved [see (ii) & (iv)].

Thus the anomeric configurations, substitution pattern and sequence of sugar units **a** to **g** in **P1** have been established unambiguously solely by n.m.r. methods. In some cases the putative absolute configurations of the sugar residues were also confirmed.

d **g** **b** **c**

α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→2)-L-Rha-OH

3 ↑ 1

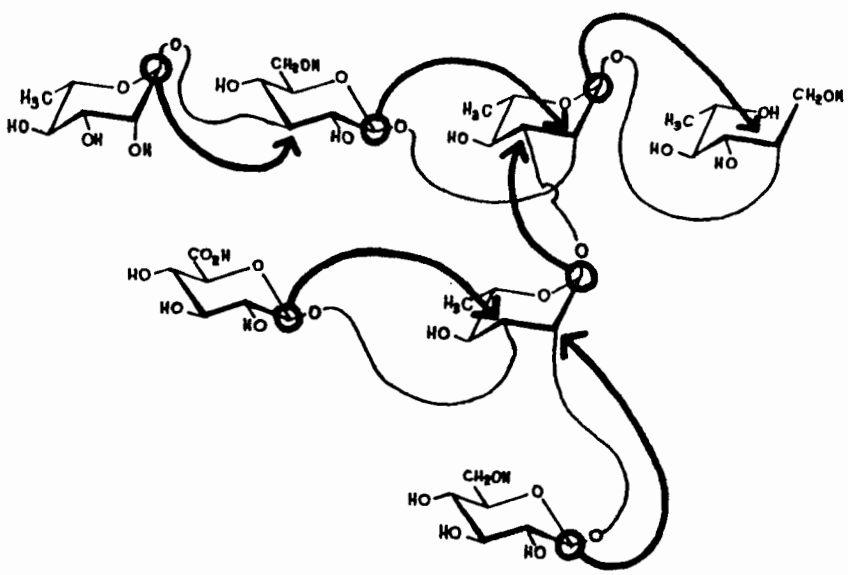
β-D-Glc*p*A-(1→3)-α-L-Rha*p* **a**

f 2 ↑ 1

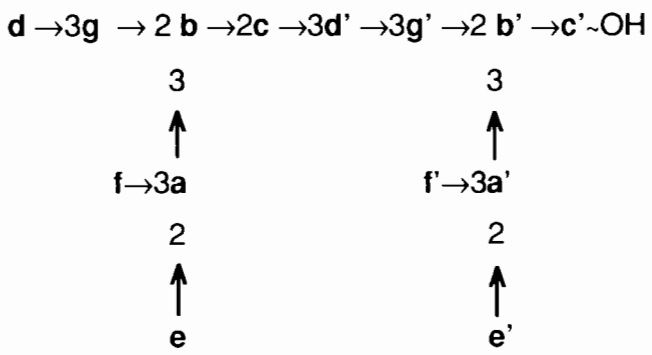
β-D-Glc*p* **e**

P1

A summary of the connectivity revealed by the long-range ^{1}H-^{13}C correlation experiments (Table 32, Figs. 46 and 47) is depicted below. The successful application of these methods in the structural delineation of **P1** is largely due to the preponderance of the rhamnosyl units, these being particularly amenable to n.m.r. studies of this nature.



6.5.4 N.M.R. ANALYSIS OF P2; PROOF THAT THE 2-LINKED RHA*P* UNIT IS α-LINKED



P2

In order to establish the anomeric configurations of all the sugar residues in the polysaccharide, n.m.r. spectra of the dimer (**P2**) and the $NaBH_4$-reduced dimer (**P2**-ol) were examined. Assignments of the anomeric signals of **P2** were substantiated by analysis of the ^1H- and ^{13}C- n.m.r. spectra of **P2**-ol (see Figs. 48 and 49 respectively and Table 35).

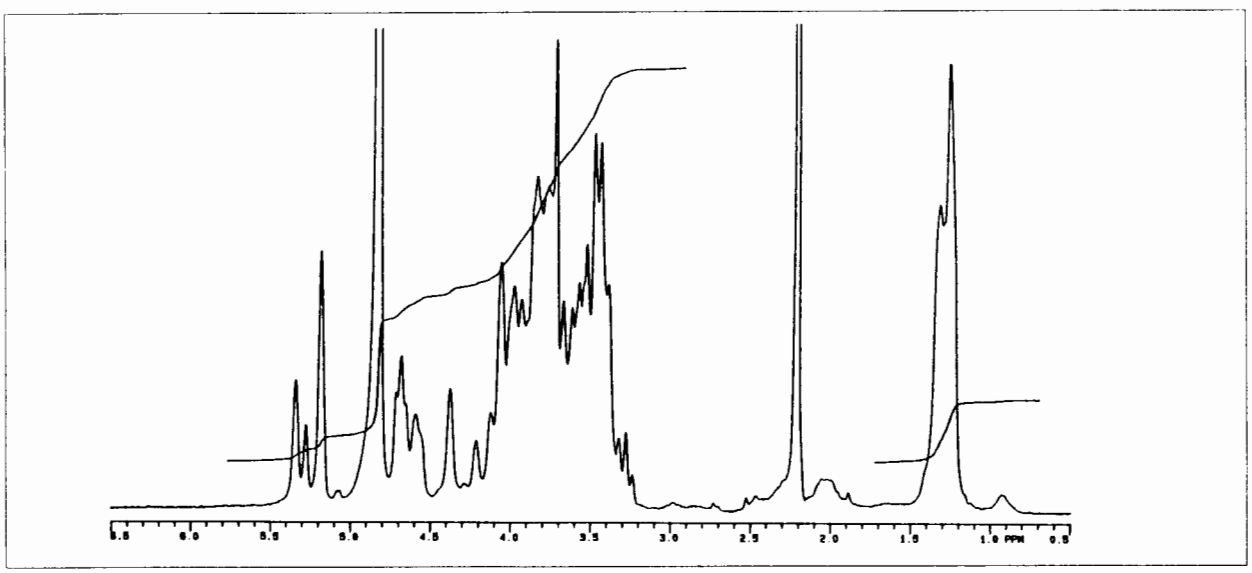


Figure 48: ^1H-n.m.r. spectrum (200 MHz) of **P2**-ol recorded at 25°C

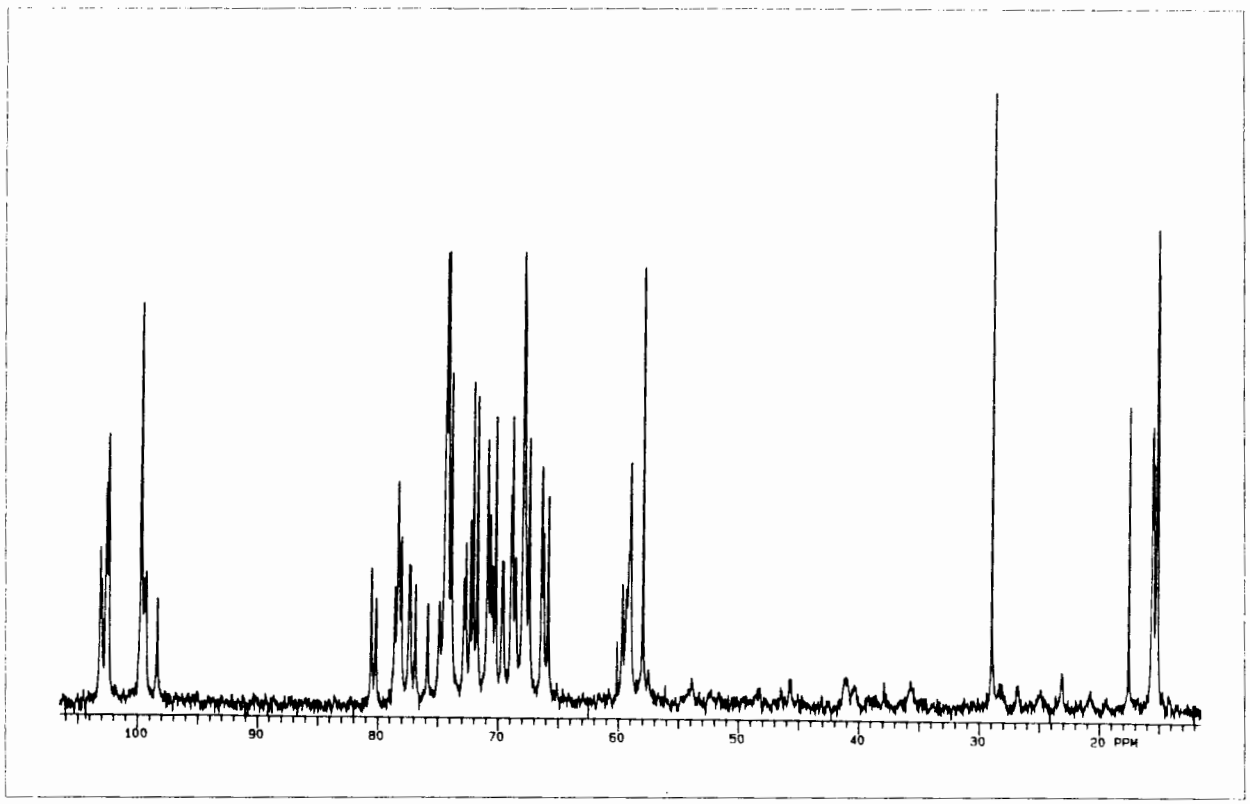


Figure 49: ^{13}C-n.m.r. spectrum (50.3 MHz) of **P2**-ol recorded at 25°C

TABLE 35: ^{1}H- AND ^{13}C- N.M.R. DATA FOR **P2**-ol

^{1}H data (200 MHz)				*^{13}C data* (50.3 MHz)	
δ (p.p.m.)	$J_{1,2}$ (Hz)	Integral Proton	Assignment	δ (p.p.m.)	*Assignment*
5.34	s	2	H-1 of α-Rha*p* (**a**,**a'**)	105.12	C-1 of **g**,**g'**
5.28	s	1	H-1 of α-Rha*p* (**b**)	104.99	
5.18	s	4	H-1 of α-Rha*p* (**b'**,**d**,**d'**,**c**)	104.62	C-1 of **f**,**f'**
4.70[b]	~6.4	2	H-1 of β-Glc*p* (**e**,**e'**)	104.59	
4.67[b]	~5.4	2	H-1 of β-Glc*p*A (**f**,**f'**)	104.49	C-1 of **e**,**e'**
4.58[b]	~5.4	2	H-1 of β-Glc*p* (**g**,**g'**)	101.72	C-1 of **a**,**a'**,**d**
4.38	s	2	H-2 of α-Rha*p* (**a**,**a'**)	101.45	C-1 of **d'**
4.21	s	1	H-2 of α-Rha*p* (**b**)	101.29	C-1 of **c**
~1.30	n.r.	24	H-6 of α-Rha*p* (**a-d**,**a'-d'**)	100.35	C-1 of **b'**

[a] Chemical shift values relative to internal acetone at δ 2.21 (^{1}H) and 31.0 (^{13}C)

[b] Revealed when the spectrum was recorded at 80°C

The anomeric signals at δ 5.18 (^{1}H) and 101.29 (^{13}C) assigned to **c** are diagnostic for α-linked Rha*p* units, thus confirming the original anomeric assignment made from examination of the ^{1}H-n.m.r. spectrum of the K71 polysaccharide, while the ^{13}C signal at 101.45 (not present in the ^{13}C-n.m.r. spectrum of **P1**-ol) was ascribed to the 3-linked Rha*p* residue (**d'**).

6.6 SEQUENCE ANALYSIS OF P1 BY MASS SPECTROMETRY

Confirmation of structural aspects of the proposed **P1** structure was obtained from e.i.-m.s. analysis of permethylated **P1**-ol (**22**) [see Fig. 50]. The fragment ions formed are described using the letters **a** to **g** as used in the n.m.r. study.

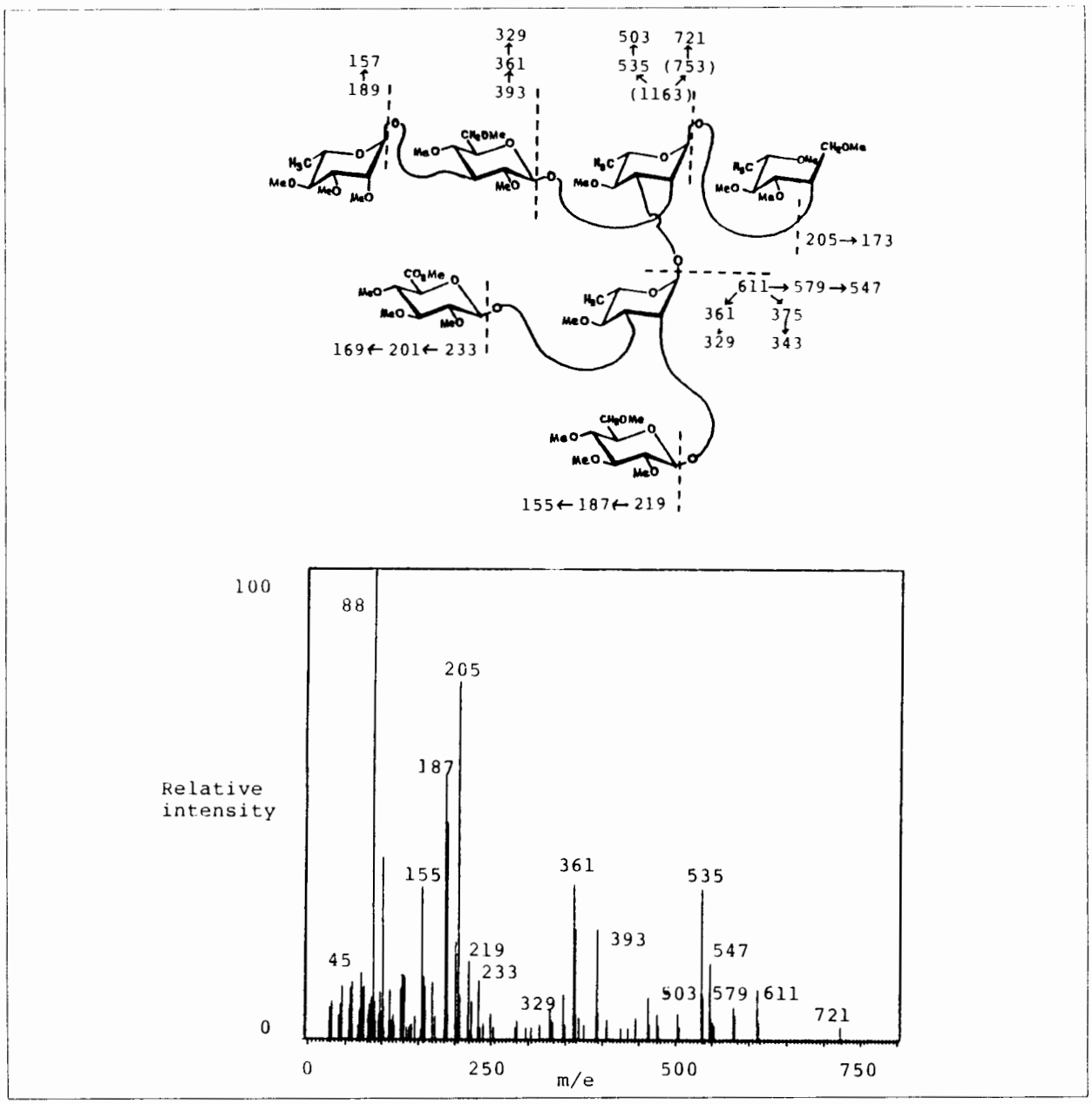


Figure 50: E.i.-m.s. fragmentation pattern of permethylated **P1**-ol (**22**), recorded at 20eV

The A-series ions formed from the non-reducing end of **22** are characteristic of terminal deoxyhexose (**d**), hexose (**e**) and hexuronic acid (**f**) groups, while the fragment ions at m/e 393 and 361 can be attributed to the deoxyhexose to hexose sequence (gdA_1 and gdA_2 ions repectively). The peak at m/e 611 was ascribed to the A-fragment formed by cleavage of the **a**→2**b** bond (i.e. the $afeA_1$ ion) which gives rise to the fragment ion at m/e 579 by loss of methanol. Alternatively, loss of the O-2 and O-3 substituents should result in fragment ions at m/e 375 (and 343) and 361 (and 329), as observed. The low intensity of the peak at m/e 375 compared to that found for the m/e 361 ion (although the latter is also attributed to the gdA_2 ion), suggests that the larger substituent (**f**) is preferentially eliminated, thereby indicating that it is attached to O-3 of **a** (*cf.* relative intensities of fragment ion, see 4.4). The peak of high intensity at m/e 205 confirms the presence of rhamnitol (**c**) at the reducing end (cA_1); no J-series ions could be detected. Although the A-series fragment generated by cleavage of the **b**→**c** bond was not observed (calibration range of mass spectrometer ~750 a.m.u.), the ions at m/e 535 and 503 could be assigned to the A_2 (and A_3) fragments formed by elimination of the O-3 substituent (followed by the loss of methanol). Elimination of the O-2 substituent followed by the loss of methanol would account for the fragment ion detected at m/e 721. The relative intensities of the fragments formed by elimination of the O-2 and O-3 substituents can be rationalised as before.

The correctness of the assignments and interpretation described above was substantiated by m.s. analysis of the ester-reduced permethylated derivative of **P1**-ol (**23**) [see Fig. 51].

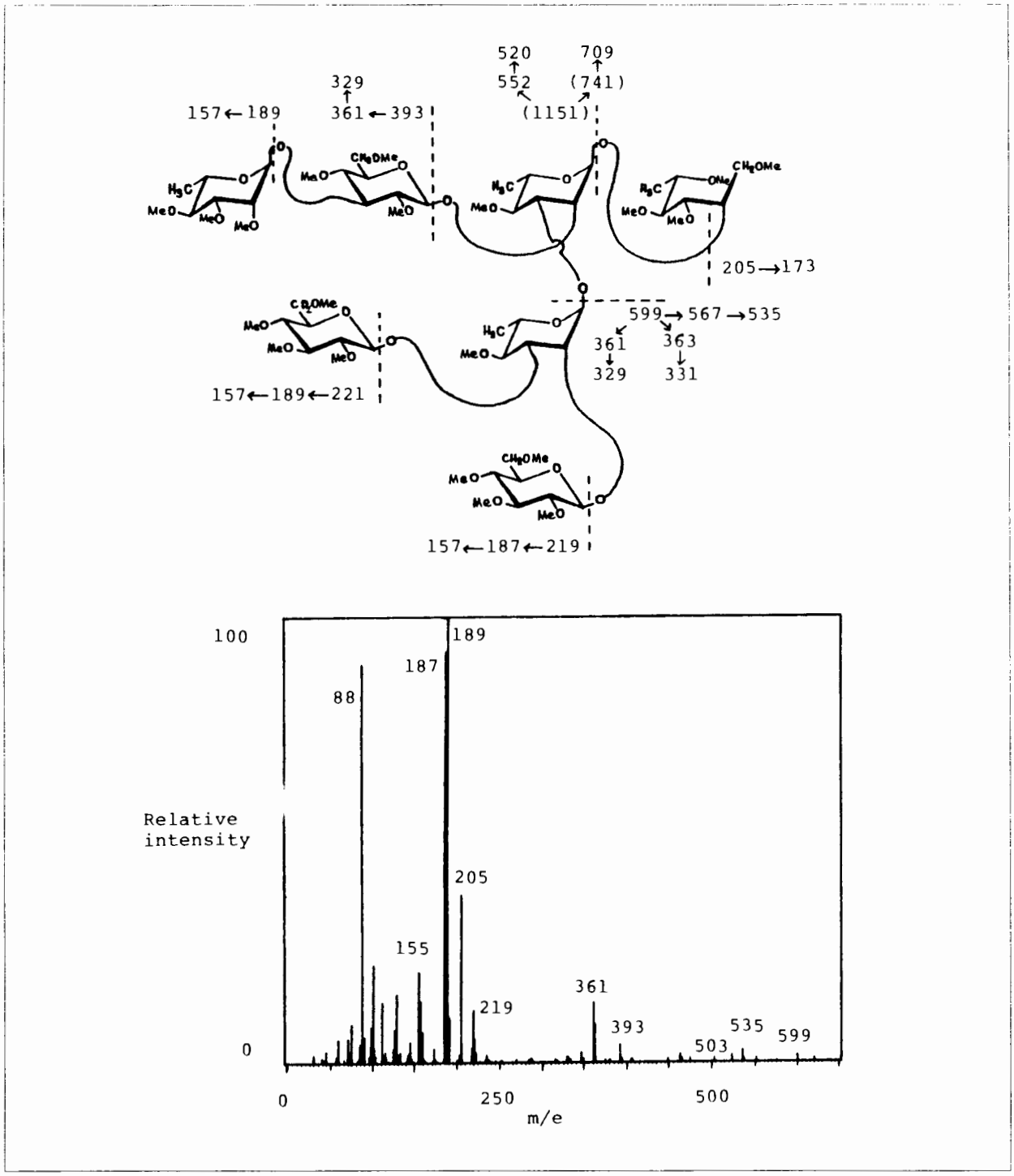
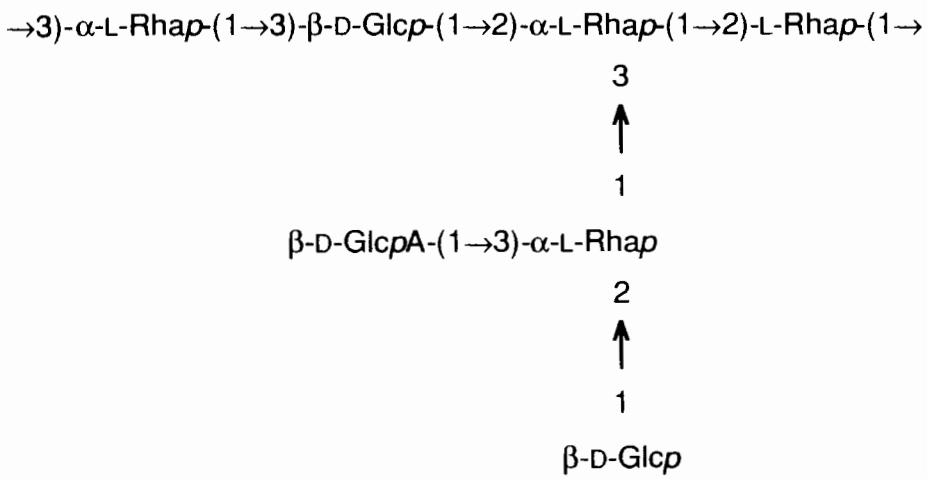


Figure 51: E.i.-m.s. fragmentation pattern of **23**, recorded at 20eV

The presence of the $LiAlD_4$-reduced glucuronic acid (**f**) derivative was indicated by the peaks at m/e 221 (fA_1) and 189 (fA_2, of enhanced intensity compared to Fig. 49).Fragments formed as described for **22**, containing the modified glucuronic acid derivative, showed the expected decrease in molecular mass (see Fig. 50) thus verifying the assignments made for **22**.

The structure of the heptasaccharide repeating unit of *Klebsiella* K71 polysaccharide based on detailed spectroscopic analysis of **P1** and **P2** is given below (**24**).



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Corroboration of these findings was sought from chemical degradative studies of the original polysaccharide, described in 6.7.

6.7 CHEMICAL DEGRADATIVE STUDIES

The polysaccharide was subjected to partial acid hydrolyses and Smith degradation experiments with the objective of confirming the sequence of the constituent sugar residues (*cf.* **24**) as established by analysis of the oligosaccharides produced by bacteriophage Ø71 cleavage of the *Klebsiella* K71 polysaccharide (see 6.3-6.6).

6.7.1 PARTIAL ACID HYDROLYSES

Mild partial acid hydrolysis of the K71 polysaccharide yielded a non-dialysable product (**25**) together with traces of glucose and rhamnose. Methylation analysis of **25**, compared to that of the original polymer (Table 28, column I), revealed a decrease in the proportions of the terminal glucose and 2,3-linked rhamnosyl residues, and an increase in the number of 2-linked rhamnosyl units, which can be explained as follows. Partial removal of the side chain from O-3 of **b** results in a decrease of the peaks due to residues **a**,**e** and **f** and a concomitant increase in the amount of the 2-linked Rha*p* residues.

Partial acid hydrolysis, under more vigorous conditions of hydrolysis, yielded an oligosaccharide product (**26**) and polymeric material. Reduction of the hydrolysate with $NaBD_4$

was followed by methylation (including remethylation after $LiAlD_4$ reduction of the methyl ester). The products were fractionated on a column of silica gel to yield the permethylated alditol derivative of **26** (designated **M26**). Hydrolysis of a portion of **M26** followed by g.l.c.-m.s. analysis of the derived alditol acetates gave the results shown in Table 36 (column I).

TABLE 36: METHYLATION ANALYSIS OF SACCHARIDES DERIVED FROM *KLEBSIELLA* K71 POLYSACCHARIDE

Partially methylated alditol acetates[a]	T[b]	*Mole %*[c]		
		I	II	III[d]
1,2,4,5-Rha[e]	0.46	12	-	-
2,3,4-Rha	0.53	-	11	34
3,4-Rha	0.89	24	18	25
2,4-Rha	0.95	8	22	2
2,3,4,6-Glc	1.00	41[f]	-	11
4-Rha	1.46	16	22	10
2,4,6-Glc	1.65	-	27	18

[a] 1,2,4,5-Rha = 3-*O*-acetyl-1,2,4,5-tetra-*O*-methylrhamnitol, etc
[b] Retention times, determined on column C at 190°C, relative to 2,3,4,6-Glc. Assignments confirmed by g.l.c.-m.s.
[c] Values were corrected by use of effective, carbon-response factors given Sweet *et al.* (Ref. 112)
[d] I, acid degraded polysaccharide product **M26**; II and III, Smith degradation products **SD** and **SDA**
[e] C-1 deuterated according to g.l.c.-m.s. (see Fig. 52)
[f] Peak contains some of the C-6 di-deuterated derivative according to g.l.c.-m.s.

Comparison of the data in Table 36 with that obtained for methylation analysis of the polymer (Table 28) reveals the absence of the 2,4,6-tri-*O*-methylglucose derivative and the presence of a new component corresponding to the acetylated derivative of C-1 deuterium-labelled 1,2,4,5-tetra-*O*-methylrhamnitol (**27**) [see Fig. 52 for the m.s. fragmentation pattern of **27**].

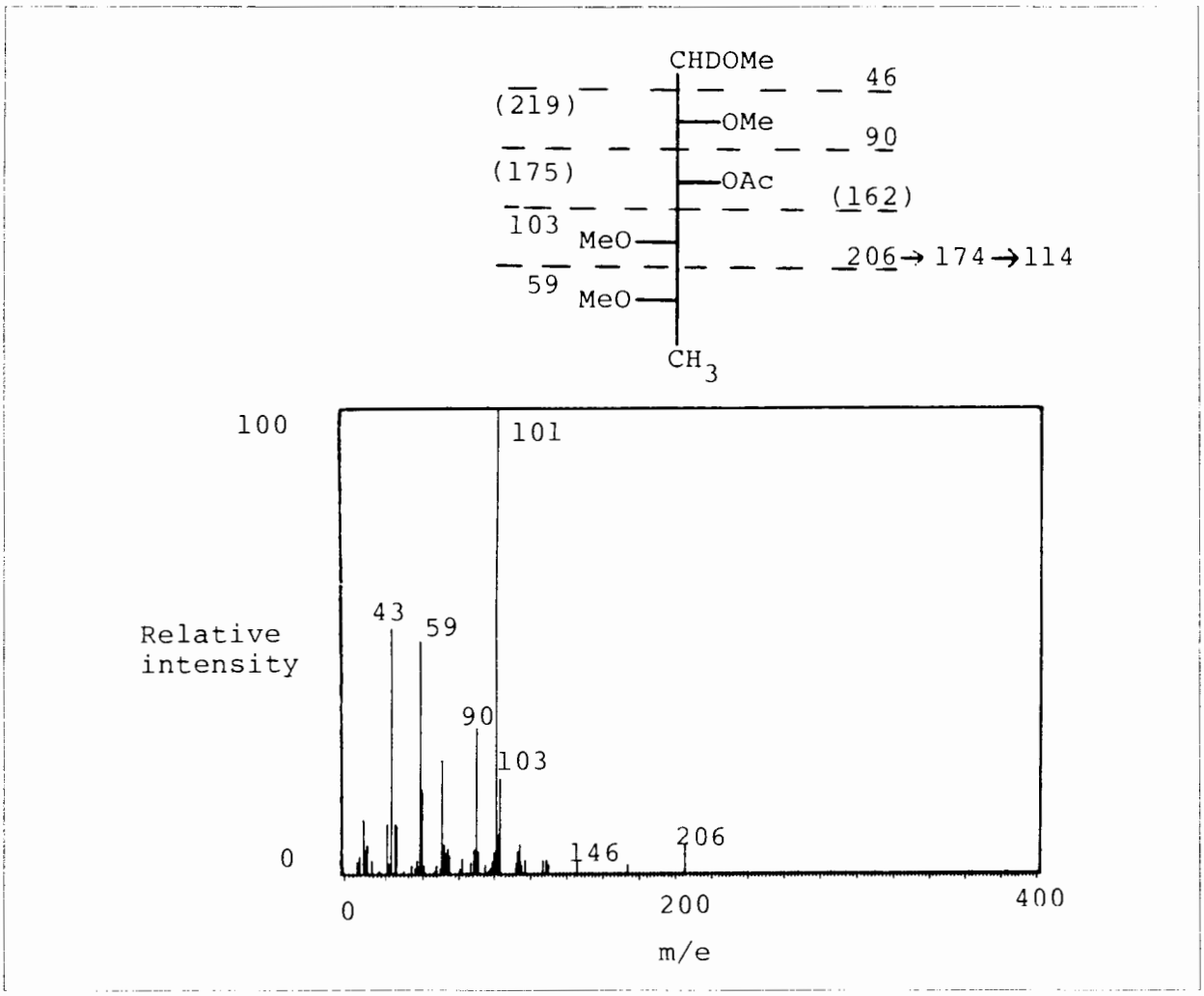
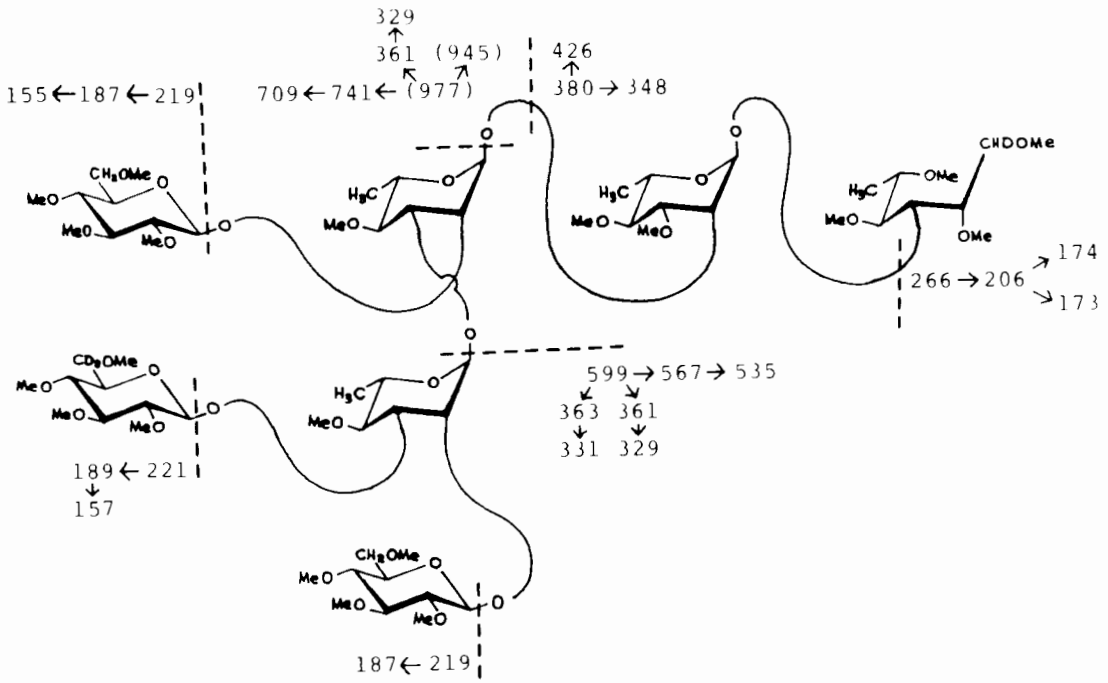


Figure 52: M.s. fragmentation pattern of **27**

These results imply that it is the →3) Rha*p*-(1→3)-Glc*p* bond (i.e. **c**→**g**) that is cleft by acid hydrolysis. Mild acid hydrolysis of the *Klebsiella* K45 polysaccharide yielded the pentasaccharide repeating unit as the major product upon cleavage of this bond thereby demonstrating the acid-lability of the 3-linked rhamnosidic linkage[223]. In the present study mild acid hydrolysis generated mainly the heptasaccharide repeating unit , the structure of which was indicated by e.i.-m.s. analysis of **M26** (Fig. 53).



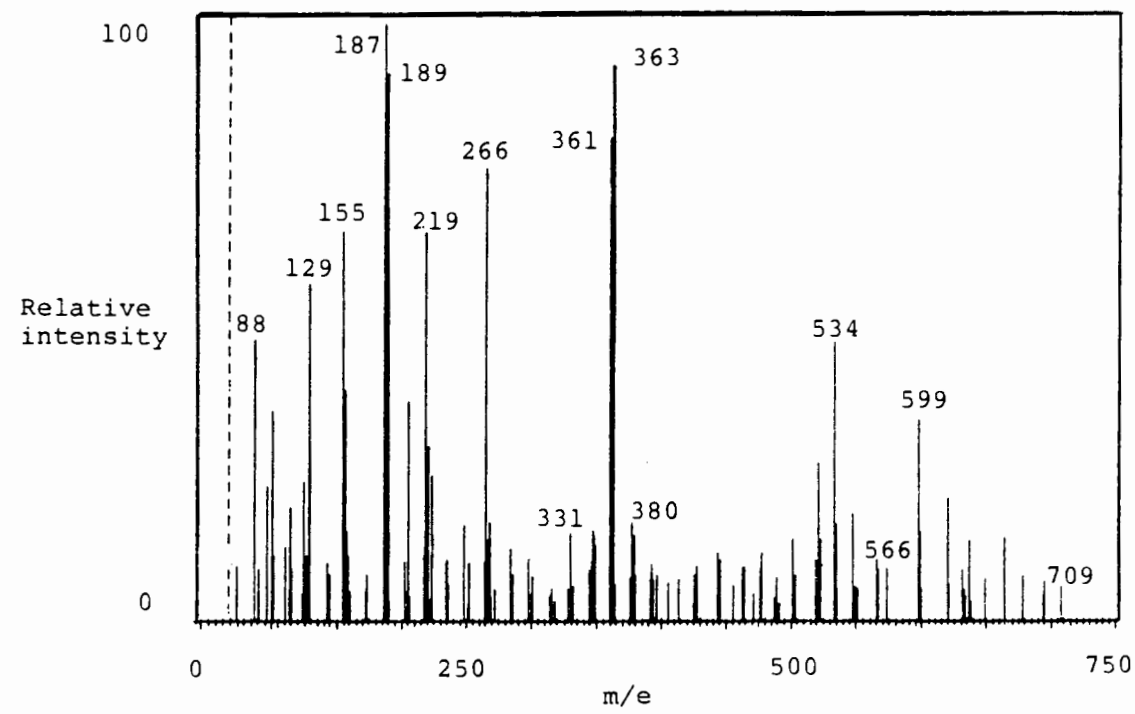


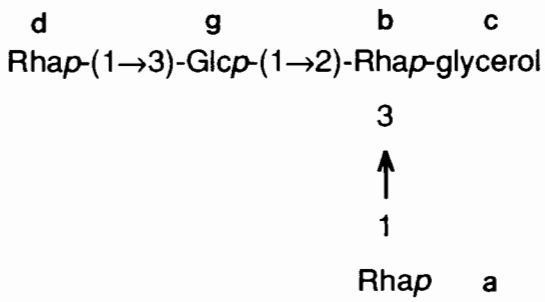
Figure 53: M.s. fragmentation pattern of **M26**, recorded at 20eV

6.7.2 SMITH DEGRADATION EXPERIMENTS

Further structural investigations, involving Smith degradation experiments, were conducted on the *Klebsiella* K71 polysaccharide and the carboxyl-reduced derivative. A sample of polysaccharide was oxidised with sodium metaperiodate for 5 days. Reduction with $NaBH_4$, followed by Smith hydrolysis of the resulting acetal gave a polymeric product (**SD**) and trace amounts of oligomers. Since an oligomeric product is to be expected from Smith degradation of structure **24**, due to cleavage of the in-chain 2-linked Rha*p* residue (**c**), its paucity in this experiment was attributed to incomplete oxidation of unit **c** rather than incomplete hydrolysis of the reduced oxidised polysaccharide under the conditions used. This supposition was verified by methylation analysis of **SD** which showed (by g.l.c.-m.s. of the alditol acetates derived from the hydrolysate: Table 36, column II) the presence of 2-linked Rha*p* as well as terminal rhamnose groups, 3-linked Rha*p* and Glc*p* residues and 2,3-linked Rha*p* units in approximately equimolar amounts. Terminal glucose and glucuronic acid groups were found (by p.c. of the hydrolysate) to be absent. The 2-linked Rha*p* residue present in **SD** was assumed to be derived from the unoxidised Rha*p* unit in the chain and the terminal rhamnose group from the loss of terminal glucose and glucuronic acid groups from the doubly branched rhamnosyl residue (**a**) present in the side-chain of **24**.

Partial Smith degradation of other rhamnosyl-containing *Klebsiella* polysaccharides (K18, K36 and K55)[207] have been reported, although in these instances it was the hydrolysis step which was assumed to be incomplete. With the aim of effecting complete Smith degradation of the *Klebsiella* K71 polysaccharide, the procedure was modified to include

two treatments with metaperiodate prior to reduction and mild acid treatment. These reactions, performed on a sample of polysaccharide submitted to prior carboxyl-reduction, readily yielded an oligomeric product (**SDA**). Methylation analysis of **SDA** (Table 36, column III) confirmed that the acetal linkage generated within the chain had been cleaved (implicit in the formation of **SDA**) to yield terminal rhamnose groups from the previously 3-linked Rha*p* unit (**d**) present in **24** and **SD**. The 1,3-di-*O*-methylglycerol derivative expected from the oxidised 2-linked Rha*p* residue was not detected, possibly due to loss encountered under diminished pressure. These results indicate that **SDA** has the structure shown below.

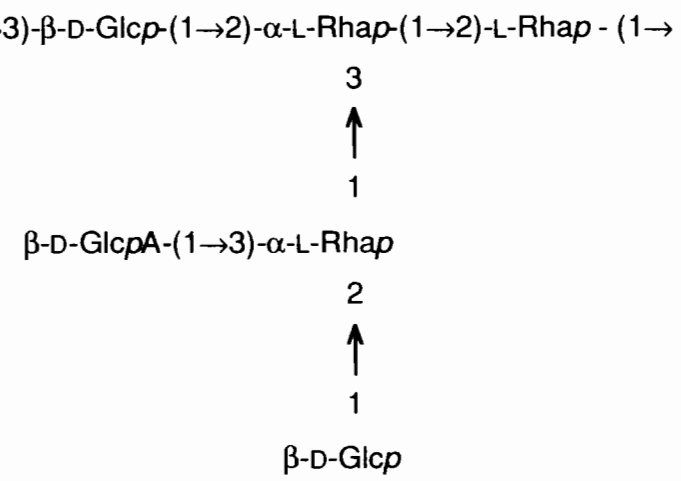


The presence of low amounts of terminal glucose groups and 2,3-linked Rha*p* units can be explained by partial loss of the acid-labile rhamnose end groups (**d** and **a**) during the Smith hydrolysis step.

Although these results are consistent with the heptasaccharide repeating unit structure proposed for the *Klebsiella* K71 polysaccharide, these experiments should be repeated on a larger scale so that the reaction products can be separated and completely characterised.

6.8 CONCLUSION

The structure of the heptasaccharide repeating unit of the capsular polysaccharide from *Klebsiella* serotype K71 has been elucidated mainly from methylation analysis and spectroscopic studies (in particular n.m.r.) of the oligosaccharides obtained on depolymerisation of the polysaccharide with a bacteriophage-borne *endo*rhamnosidase.



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Extensive use of 2D n.m.r. spectroscopy established the substitution pattern of sugar residues (implicit in **24**), while the sequence of sugar units suggested by glycosylation shift data and the twinning of pertinent signals in the n.m.r spectra of the derived oligosaccharides, was confirmed by application of long-range ^{1}H-^{13}C correlation experiments.

Structural aspects of **24** were confirmed by chemical degradative studies performed with the whole polysaccharide as starting material. These involved partial acid hydrolyses and Smith degradation experiments.

The pattern exhibited by the heptasaccharide repeating structure is of the "4+2+1" type, *Klebsiella* serotype K67 being the only other example in this series to have a capsular polysaccharide with a branched side chain[25]. Within the chemotype, the structure of the capsular polysaccharide from serotype K45 is identical to that postulated for K71 with respect to the main chain; however, the side-chain contains only a single β-D-Glc*p*A group, attached directly to O-3 of the 2-linked in-chain α-L-Rha*p* residue ("4+1" type)[223].

6.9 EXPERIMENTAL

6.9.1 ISOLATION AND ANALYSIS OF *KLEBSIELLA* K71 POLYSACCHARIDE

A culture of *Klebsiella* K71 was grown as previously described in sections 1.2 and 2.1 on medium B and the polysaccharide was purified by precipitation once with cetyltrimethylammonium bromide. The purified polysaccharide (~ 5g from three batches of six trays [each 45 x 32 x 5cm]) typically had the properties described in the text. Sugar analyses were conducted on 10mg samples as described in section 2.8, the g.l.c. analysis were performed on columns B (PAAN's at 220°C) and C (alditol acetates at 215°C).

The absolute configurations of the sugar components were established as follows. A portion of polysaccharide (200mg) was hydrolysed (2M TFA, 100°C, 18h). P.c. using solvent A gave good separation of the components; the procedure was repeated on the preparative scale (see 2.4) and the sugars were isolated by extraction into water. Optical rotations were measured and found to agree with values obtained for authentic D-glucuronic acid, D-glucose and L-rhamnose.

6.9.2 METHYLATION ANALYSIS OF *KLEBSIELLA* K71 POLYSACCHARIDE

Methylation analysis was conducted on a portion of polysaccharide (100mg) as described in section 2.10 (see Table 28, columns I-III). A sample of methylated polysaccharide (40mg) was base-degraded (see 2.10), and then directly re-alkylated with methyl iodide (without isolation and subsequent work-up as described in section 2.10) to give results shown in Table 28, column IV.

6.9.3 PROPAGATION OF BACTERIOPHAGE Ø71

Bacteriophage Ø71 (obtained courtesy of Professor S.Stirm) was propagated on host strain *Klebsiella* K71 initially on plates and then in solutions of nutrient broth until 2L of lysate containing ~ 10^{13} p.f.u. had been obtained. The phage solution was concentrated and dialysed against running tap-water (2 days), the dialysate finally being concentrated (to 200mL).

6.9.4 PRODUCTION AND ANALYSIS OF OLIGOSACCHARIDES P1 AND P2

Polysaccharide (1.5g) was added directly to the phage solution (~ 10^{13} p.f.u. in 200mL) and stirred for 3 days at 37°C in the presence of chloroform (3mL). The oligomers produced were isolated by dialysis and preparative s.e.c. (column 5) to yield **P1** (760mg)

and **P2** (200mg). A sample (5mg) of each oligosaccharide was dissolved in water (2mL) and treated with sodium borohydride (10mg) overnight. The reduced oligosaccharides recovered were hydrolysed (2M TFA 18h at 100°C) and the products converted into peracetylated aldononitrile (PAAN) derivatives for analysis by g.l.c.-m.s. (column B at 220°C).

Methylations (see 2.10) were conducted on **P1** (40mg) and **P2** (5mg) after reduction with $NaBH_4$ to give the results shown in Table 29. E.i.-m.s. analysis of permethylated derivatives of **P1**-ol was performed on a VG Micromass 16F mass spectrometer operating at 20eV. The samples were introduced into the mass spectrometer using the deep probe attachment, to give the results presented in section 6.6.

6.9.5 N.M.R. STUDIES

N.m.r. analysis of **P1** (80mg) and **P1**-ol (60mg) were conducted on a Bruker WM500 spectrometer using standard conditions (see 3.3.7 and references therein). The n.m.r. spectra of **P2** (not presented) and **P2**-ol were recorded using a Varian VXR-200 machine.

6.9.6 PARTIAL ACID HYDROLYSIS STUDIES

Mild partial acid hydrolysis of K71 polysaccharide (100mg) was performed using 0.01M TFA for 12h at 100°C. The hydrolysate was freeze-dried then dialysed against distilled water (3 x 400 mL) for 3 days. The non-dialysable product (80mg) was subjected to methylation analysis as described in section 2.10.

Acid hydrolysis of the polysaccharide (60mg) using 0.25M TFA at 100°C was monitored by t.l.c. analysis (solvent F). After 2.5h the hydrolysate was freeze-dried and reduced with $NaBD_4$. Methylation followed by $LiAlD_4$-reduction of the methyl ester and subsequent remethylation with methyl iodide yielded a permethylated mixture which was fractionated on a column of silica gel (30 x 0.8 cm; using solvent F and finally methanol as the eluent) to yield **M26** (28mg) as the major product. E.i.-m.s. analysis of **M26** gave results shown in Figure 53, while hydrolysis of a portion of **M26** (5mg) gave the results presented in Table 36 (column I).

6.9.7 SMITH DEGRADATION EXPERIMENTS

A sample of polysaccharide (100mg) was oxidised with sodium metaperiodate (0.1M in the dark at room temperature for 5 days). The periodate consumption (6.5 mol per mol repeating unit) was monitored by the arsenite titration method[225]. Ethan-1,2-diol was added to the reaction mixture and the solution was dialysed for 2 days. Reduction of the oxidised polysaccharide sample with an excess of sodium borohydride over 3 days followed by Smith hydrolysis (M TFA at room temperature, 10 days) and fractionation of the products by s.e.c. (column 5) yielded a polymeric product (**SD**, 30mg) and trace amounts of oligomers. Hydrolysis of **SD** followed by p.c. analysis (solvent **A**) indicated the

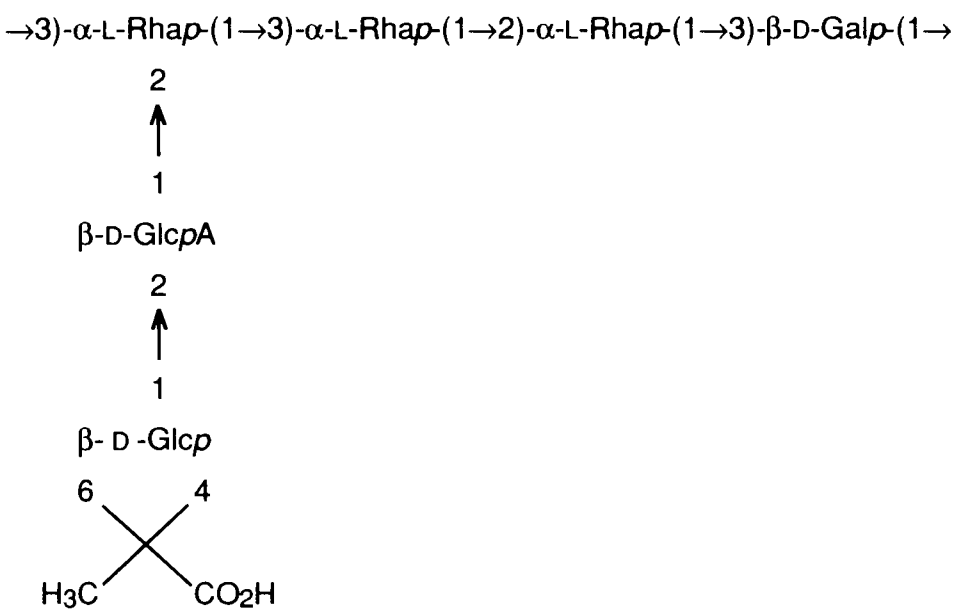
presence of rhamnosyl (4) and glucosyl (1) residues, while methylation analysis gave the results shown in Table 36, column II.

A sample of polysaccharide (150mg) was carboxyl-reduced (see 2.8) then oxidised with metaperiodate as described above. After 5 days the reaction was terminated with ethan-1,2-diol (2mL) and the resulting aldehydes reduced with sodium borohydride. After dialysis and freeze-drying the product was again exposed to periodate (4 days) and reduced as before. After dialysis and freeze-drying the reduced, oxidised polymer was hydrolysed with M TFA at room temperature. After 3 days t.l.c analysis (solvent F) indicated mainly the presence of an oligosaccharide product (**SDA**). The TFA was removed by freeze-drying and the solution reduced with sodium bordeuteride prior to methylation analysis (see Table 36, column III).

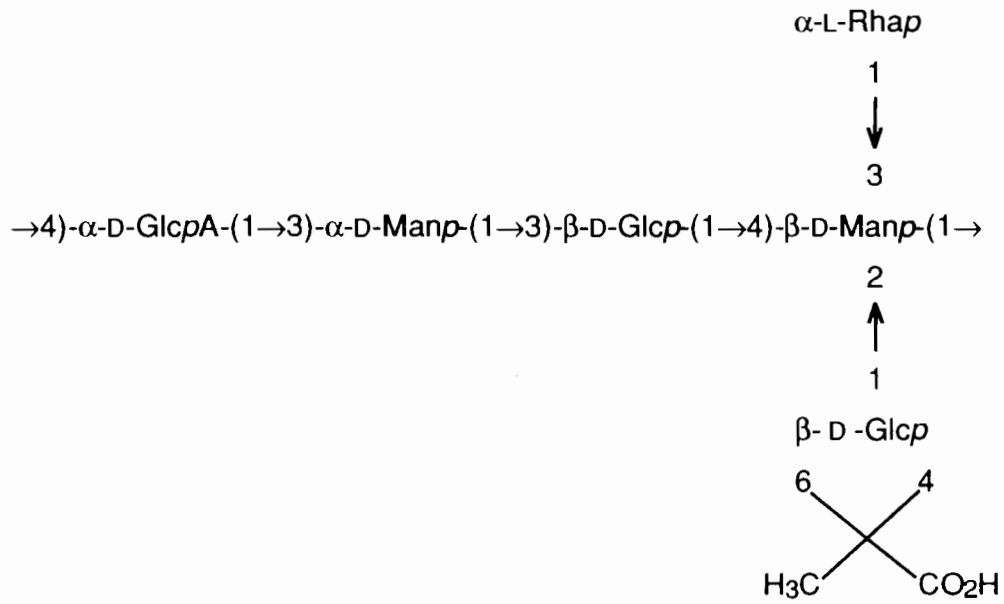
CHAPTER 7

CONCLUSION

Elucidation of the structures of the capsular polysaccharides produced by *Klebsiella* serotypes K36, K64 and K71 has been achieved by examination of the oligosaccharides derived by bacteriophage-borne enzymic depolymerisation of the cognate polysaccharides. The usefulness of this approach is emphasised by the fact that the results obtained necessitated certain reformulation of the structures, previously published for the polysaccharides from serotypes K36 and K64, as shown below.



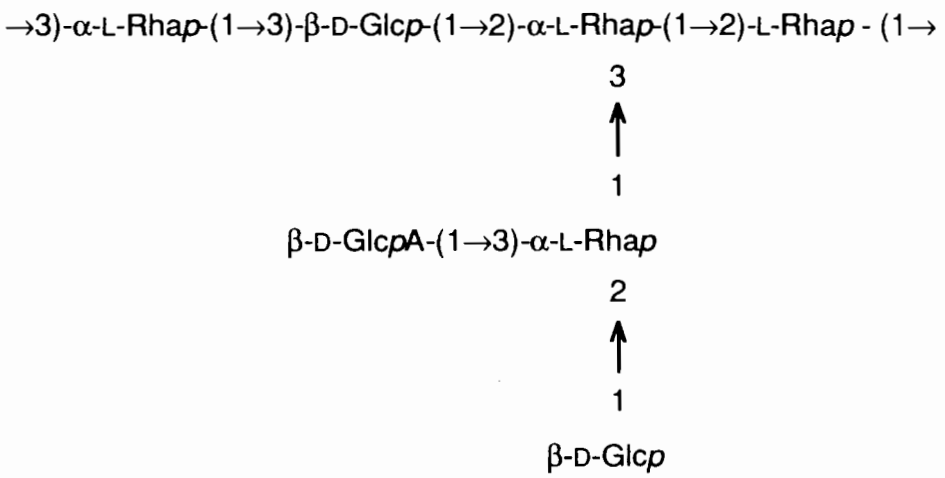
***Klebsiella* K36**



***Klebsiella* K64**

In addition to the structural delineations outlined above, these studies also showed that bacteriophage Ø64 possesses a lyase that depolymerises the polysaccharide of *Klebsiella* K64 into oligosaccharides having an unsaturated derivative of glucuronic acid at the non-reducing end. The unsaturated hex-4-enuronic acid residue generated was characterised spectroscopically (u.v. and n.m.r.) and by g.l.c.-m.s. after hydrogenation of the double bond.

The structure of the heptasaccharide repeating unit postulated for the capsular polysaccharide from serotype K71 has been elucidated *de novo*, mainly by the use of spectroscopic methods (in particular n.m.r.) to examine the intact oligosaccharide (**P1** and **P2**).



***Klebsiella* K71**

The application of spectroscopic studies has been a major facet of this investigation. The extensive use of two-dimensional n.m.r. methods has proved to be of crucial importance in making assignments in ^{1}H and ^{13}C spectroscopy of the oligosaccharides obtained from all the *Klebsiella* polysaccharides studies. Possibilities for further work in this field include conformational investigations involving model building of the oligomers based on the quantification of the long-range ^{1}H-^{13}C couplings and possibly the measurement of pertinent n.O.e.'s.

APPENDIX

Polysaccharides produced by *Clostridium acetobutylicum*.

The anaerobic bacterium *Clostridium acetobutylicum* P262 which is used for the industrial production of acetone, butanol and ethanol from molasses[226], is known to produce both intra-and extracellular polysaccharides, both of which have been investigated.

The accumulation of intracellular granules of reserved material is common amongst the Clostridia. In the majority of *Clostridium* species investigated, the reserve material has been shown to be a polyglucan, which has been given the generic name granulose[227]. The characterisation of the granulose described here is part of a larger programme involving the study of the biosynthesis and regulation of granulose in *Clostridium acetobutylicum*[228], which is being carried out in the Department of Microbiology at the University of Cape Town.

The granulose was isolated and analysis by s.e.c. indicated a high molecular weight (6 x 10^6), while hydrolysis yielded glucose only (upon analysis by p.c. and g.l.c. of the derived alditol acetates). Methylation of the polymer followed by hydrolysis gave a single component (according to p.c. and t.l.c. analysis), which was characterised by g.l.c.-m.s. as the acetylated derivative of 2,3,6-tri-*O*-methylglucitol. Finally the ^{1}H-n.m.r. spectrum recorded at 80°C yielded a single anomeric signal at δ 5.23 ($J_{1,2}$ = 3 Hz) characteristic for α-Glc*p* residues[139]. These results show that the granulose produced by *C. acetobutylicum* P262 consists of a high molecular weight, linear polymer of 4-linked α-Glc*p* units. This finding is similar to that reported for the granulose produced by strains of *C. pasteurianum*[229] except that no traces of α-(1→6)-Glc*p* units or branch points were detected in the present case.

The bacterium is also known to produce a slime, of hitherto unknown composition, under nutrient-limited conditions. Two samples of this slime, provided by the Department of Microbiology, University of Cape Town, have been examined. The first of these, isolated by centrifugation,, was shown to contain more than one component, and therefore the second sample was isolated under conditions which prevented contamination by cell-wall constituents. The crude polysaccharide was further purified by an ammonium sulphate precipitation. The polysaccharide material isolated had 0.5% N, $[\alpha]_D$ + 46°, and a molecular weight 10^6. Acid hydrolysis followed by g.l.c. analysis of the derived alditol acetates showed the presence of rhamnose (2), galactose (1) and glucose (2) as the constituent sugars of the polymers. The ^{1}H-n.m.r. spectrum of the polysaccharide in D_2O at 85°C confirmed the presence of rhamnose (δ 1.3) and showed four discernible peaks in the anomeric region, indicating that the repeating unit in the polysaccharide structure consists of at least four monosaccharide residues. Attempts to confirm the nature of the repeating unit have not been entirely successful due to incomplete methylation of the polymer when standard procedures, involving Hakomori and Purdie treatments, have been employed.

Mild alkali treatment of the material prior to methylation appeared to improve the solubility of the polymer in DMSO, so that complete methylation was achieved. Hydrolysis of the permethylated product followed by g.l.c.-m.s. of the derived alditol acetates showed the presence of 3-linked rhamnosyl (1), galactosyl (1) and glucosyl (2) residues and terminal rhamnose (2) and glucose () groups, together with a 3,4,6-linked hexosyl unit (1). Paucity of material has necessitated the further production of slime upon which further structural studies will be carried out.

Experimental

The *C. acetobutylicum* P262 wild-type strain has been described previously[230]. Cells grown in buffered *Clostridium* basal medium (CBM)[231] were harvested during early growth phase and the pellets were washed and resuspended in 25mM Tris-HCl buffer, pH 8.0 (0.5g wet weight/mL). The cells were disrupted by passage through a Yeda Press (Yeda Scientific Instruments, Rehovot, Israel). A preparation of granulose granules was obtained from the extract by differential centrifugation as described by Robson *et al.*[232]. The polysaccharide was extracted by the KOH and ethanol procedure of Darvill *et al.*[229]. Analysis by s.e.c (column 1) gave a single peak in the void volume. Complete methylation was achieved on a lyophilised sample (10mg) using the modified Hakomori and Purdie treatments (see 2.10), while sugar analyses were performed on column C, as described in section 2.8 . The ^{1}H-n.m.r. spectrum of granulose (20mg in D_2O) was recorded on the Bruker WH-90 spectrometer.

The extracellular slime (0.6g) was isolated using the cold aqueous extraction method described by Sutherland and Wilkinson[34] and purified by an ammonium sulphate precipitation. A sample (80mg) of the polysaccharide was treated with alkali (M NaOH for 1.5h at 25^{o}C), dialysed (3 days) and freeze-dried to yield a product (62mg), which showed improved solubility in water and DMSO. Per-methylation of this alkali-treated material (40mg) was achieved using the Hakomori procedure followed by the Purdie treatment[120] (5 days). Sugar analyses were performed on samples (5mg) of the slime polysaccharide, as described in section 2.8 and the ^{1}H-n.m.r. spectrum was recorded on the Bruker WH-90 n.m.r. machine.

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