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

THESIS

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ABSTRACT

The work described in this thesis forms part of an international programme concerned with the structure elucidation of the capsular antigens of some *Enterobacteriaceae*. Many of the *Klebsiella* and some of the *Escherichia coli* are pathogenic to man and, hence, they are of interest. The virulence of bacteria is a multifactorial phenomenon, in which characteristic traits of bacteria and their hosts play comparable and complementary roles. It is accepted that pathogens are more virulent when encapsulated, because, nearly all disease causing bacteria have a capsule when freshly isolated from the host. This increase in pathogenicity is related, in part, to the capsular polysaccharides' ability to avoid or attenuate the host defence mechanisms. In the majority of cases the protective aspects of the capsule are overcome in the latter stages of infection when the formation of specific antibodies by the host has occurred. However there are situations in which an immune state of the infected host is virtually never reached, and susceptiblity to the infecting bacteria is maintained even in the advanced stage of an infection. Explanation of this phenomenon becomes possible by analysing the structure of the polysaccharides. The inability of the host to raise a immune response to the capsule may be because the structure of the polysaccharide is similar or identical to the host's carbohydrates. The serological and pathogenic relatedness of encapsulated *E. coli* and *Klebsiella*, to the encapsulated strains of other genera, is based on structural identity or similarity of the respective capsules.

Capsular polysaccharides are analysed by both chemical and instrumental methods, and, at present, nuclear magnetic resonance spectroscopy is the most important analytical technique.

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1 THE BACTERIAL CELL SURFACE

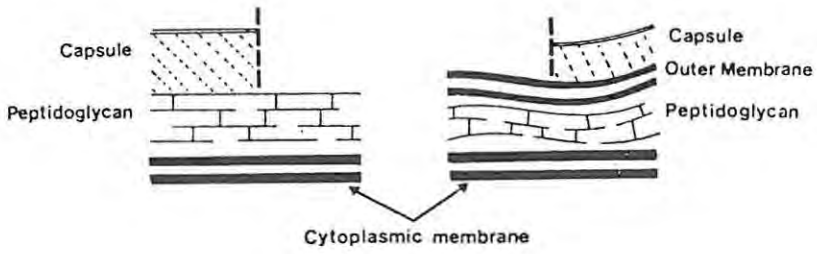


Figure 1 Diagrammatic representation of the cell surface of gram positive and gram negative bacteria[1].

The external layers of a bacterium serve as an important interface between the organism and its environment. These include the cytoplasmic membrane, peptidoglycan layer and in the case of Gram negative organisms there is also an outer membrane. The majority of bacteria also have one or more exopolysaccharides that cover the cell and give it an anionic hydrophilic surface. This covering (see Figure 2), which is referred to as either the capsule or as the slime layer, is antigenic because of its position on the outer extremity of the bacterium. These exopolysaccharides, which are second only to proteins as antigenic determinants, have great specificity and variability and form a series of related, yet distinct, antigens which produce antibodies of restricted heterogeneity[2].

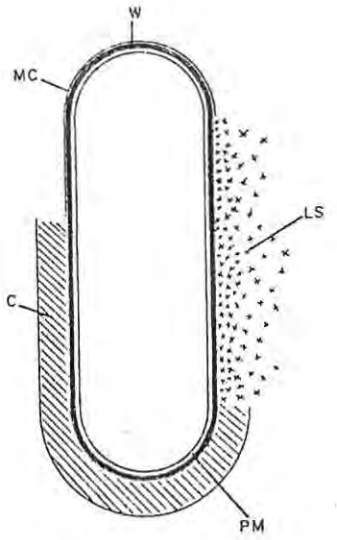


Figure 2 A diagrammatic representation of the relationship between capsules, slime, and microcapsular layers of the cell wall and protoplast membrane[3] (W = cell wall; PM = protoplasmic membrane; C = capsule; MC = Microcapsule; LS = loose slime).

Exopolysaccharides are not essential for the growth of the bacterium, but they do confer several advantages in the highly competitive conditions of its natural habitat. For example, the polysaccharide may act as an adhesive which allows the organism to resist the scouring action of water. It is also accepted that pathogens are more virulent when encapsulated simply because nearly all disease causing bacteria have a capsule when freshly isolated from the host. The increase in pathogenicity is, in part, related to the capsules ability to avoid or attenuate the host defence mechanisms.

In some cases the capsules are identical or sufficiently similar to the host structures that they are not recognised by the immune system. The best example of this phenomenon are the pathogenic *E. coli* strains exhibiting K1 or K5 capsules[4]. Their capsular polymers are non-immunogenic or, at best, only marginally so. They protect the bacterium for the duration of the infection, whereas other polysaccharides are only effective in the pre-immune stage when K specific antibodies are absent. In the instance of K1 this camouflage stems from its similarity to the carbohydrate part of the neonatal neural cell adhesion molecule (n-CAM, Figure 3).

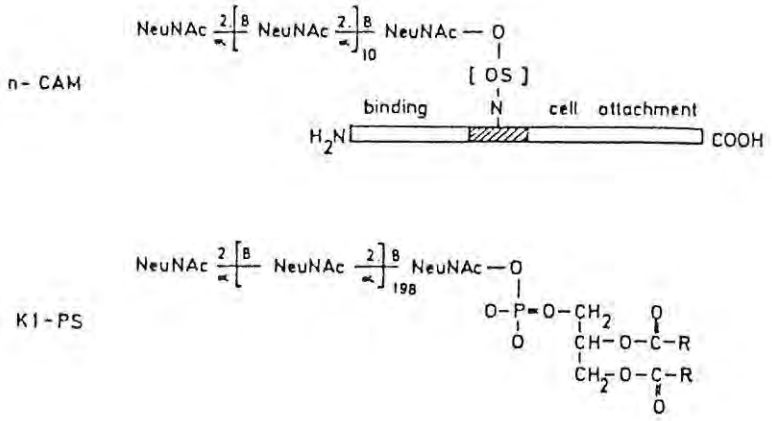


Figure 3 Structural relationship of the capsular polysaccharide (PS) of *E.coli* K1 to the carbohydrate terminal of the neonatal neural cell adhesion molecule[4] (n-CAM); NeuNAc = N-acetylneuraminic acid.

The immune system, therefore, lets it pass as a "self structure" of the body. In the same way the K5 polysaccharide mimics the first polymeric intermediate in heparin biosynthesis (Figure 4) and thus passes undetected by the body.

K5 Polysaccharide:

→4)-α-GlcNAc-(1→4)-β-GlcA-(1→4)-α-GlcNAc-(1→4)-β-GlcA-P-Diglyceride

INTERMEDIATE OF HEPARIN BIOSYNTHESIS:

→4)-α-GlcNAc-(1→4)-β-GlcA-(1→4)-α-GlcNAc-(1→)-β-GlcA-$(Gal)_2$-Xyl-Protein

Figure 4 Structural relationship between the (*E. coli*) capsular K5 polysaccharide and the first polymeric intermediate in the heparin biosynthesis[4].

Those polymers of high molecular weight that are poorly biodegradable can cause "immunological paralysis" by circulating in vast excess which overwhelms the defence mechanisms[5]. Furthermore, some encapsulated strains have enhanced ability to resist phagocytosis due, in part, to the hydrophobicity, charge and viscosity of the

polysaccharide[6]. A direct relationship exists between the anti-phagocytic activity and the amount of capsule present[7] and it has been postulated that the increased distance between the surface and the cell membrane minimises the chance of a fatal lesion resulting from the antibody-antigen complement complex[5]. Capsules also diminish the complement mediated bactericidal activity of host serum[8], an effect directly related to the chemical structure of the polysaccharide[9,10].

Gram positive and the vast majority of Gram negative bacterial capsules are heteroglycans composed of contiguous oligosaccharide repeating units[1]. These units are largely composed of acidic or neutral hexoses, 6-deoxy and aminosugars[1]; pentose units occur less frequently[1]. Acetate and a high proportion of acidic constituents such as pyruvate ketals, and phosphate groups have also been reported[8].

2 ISOLATION AND PURIFICATION OF CAPSULAR ANTIGENS FROM *Enterobacteriaceae*

One of the most important steps in the investigation of a capsular polysaccharide is the isolation and purification of sufficient pure material without inadvertently degrading the labile constituents of the glycan; therefore, extreme pH values and harsh extraction conditions must be avoided. There are numerous isolation methods which meet these requirements. They involve solubilization of the capsule, removal of the dead bacterial cells and isolation of the polysaccharide either on the basis of selective precipitation or a chromatographic separation. The latter is the method of choice where a small yield (<1g) of product is expected.

The procedure used by this author is one commonly used for isolating acidic capsular polysaccharides. A serologically pure strain is streaked onto the surface of a sterile medium and allowed to grow (usualy at 37°) for 24 hours. A single colony is selected and streaked out on another petri dish, containing sterile medium, and incubated overnight. This may be repeated several times to ensure rapid growth. A sterile broth tube is then inoculated with a single colony and shaken at 37° until the solution is turbid. This suspension of bacteria (in the log growth phase) is used to inoculate a tray of sterile medium which is allowed to grow for up to three days at 27° - 37°. The incubation temperature depends upon the characteristics of the strain. Harvested cells are diluted with an equal volume of 2% aqueous phenol to kill the bacteria and dissolve the exopolysaccharide. After stirring for 12 to 24 hours at 4° the resulting suspension is ultracentrifuged (105 000 G, 3 h) to remove the bacterial cells and other debris. The supernatant contains the O and K antigens as well as contaminants such as protein. These antigens are obtained in a semi-purified form by precipitation in a

large volume (5X) of ethanol. The precipitated mixture is dissolved in water and the acidic K antigen is selectively precipitated with a quarternary ammonium salt (QAS); cetyl trimethylammonium bromide (CTAB) is used in this laboratory. Neutral lipopolysaccharide remains in solution as the QAS only reacts with neutral glycans when the pH is high enough to ionise the hydroxyl groups. Protein precipitates with QAS at a pH alkaline of their isoelectric point and can cause a contamination problem. The selective precipitation step is most effective when the concentration of the anionic polysaccharide is between 0.1% and 1% w/v and not more than 3 mg of the QAS per mg of the polysaccharide is used[11]. Formation of the QAS-K antigen complex can be enhanced by adding sodium sulphate, but other electrolytes should be kept to a minimum to prevent redissolution of the precipitate[11]. The complex is broken in a concentrated (3M NaCl) electrolyte solution and the polysaccharide (Na salt) is finally precipitated in ethanol. It is recovered, dissolved in water and dialysed exhaustively to remove all traces of NaCl and CTAB. After lyophilisation the product purity can be determined by gel permeation chromatography, eg on Sepharose® 4BCL.

Isolation of the capsular polysaccharide from a liquid culture involves precipitating the acidic polymer (along with the bacterial cells) with a QAS. The glycan is subsequently extracted from the precipitate with calcium chloride and after repeated precipitation with ethanol from aqueous solution, extraction with cold phenol at pH 6.5. This is dialysed and the product is isolated by lyophilisation[12].

3 CHEMISTRY AND SEROLOGY OF THE *ESCHERICHIA COLI*

Escherichia coli are a group of motile saprophytic Gram negative bacilli that fall within the family *Enterobacteriaceae*. They colonise the lower gastrointestinal tract shortly after birth and perform important intra-intestinal physiological functions. In general, unless the host is severely debilitated, immunosuppressed or a critical anatomical barrier is disrupted, they remain safely confined to the colon. However, some of the *E. coli* strains are inherently pathogenic because of their ability to overcome the defence mechanisms in a healthy individual. These strains are implicated in urinary tract infections (UTI), neonatal meningitis and diarrhoeal disease[13].

The outer membrane of the bacillus contains several antigens and the predominant one is a lipopolysaccharide (LPS). This component, which is integrated into the outer membrane of the cell wall *via* a hydrophobic interaction, consists of lipid A linked to one of over 150 O-specific polysaccharides. The linkage is through an oligosaccharide (core) region of which there are five different types. The O-specific polymers from this genus can be either acidic, in contrast to those expressed by *Klebsiella* and *Salmonella* strains, or neutral. The ability to produce this O-specific polymer is lost when the bacterium undergoes a mutation from the smooth (S) to the rough (R) form[14].

The other immunogens of major interest are the capsular (K) antigen and the flagella which are denoted as the H antigen. A strain can thus be categorised into O:H:K serogroups. There are approximately 100 "K", 160 "O" and 46 "H" antigens recognised at present[4,17].

The first person to successfully classify the *E. coli* by serological means was Kauffmann[15]. Initially he identified 20 O serogroups and was later able, in conjunction with Vahlne, to identify K antigens on the basis of inagglutinability of a non-heated bacterial culture in O-antiserum[14]. This method of detecting K antigens has subsequently been replaced by immunoelectrophoresis which is more reliable. Classically, three different types of K antigen were described and designated[14] A, B, and L. This classification has been reviewed[14] by Ørskov *et al* in 1977 and they advocated its discontinuance because of the difficulties associated with this method of subdivision. They suggested restricting the nomenclature of K antigens to acidic polysaccharide and protein (only the fimbria of K88 and K99) K antigens[14]. The polysaccharides can also be grouped (either I or II) on the basis of chemical, physical and microbiologic characteristics[4]. Group I polysaccharides have a high molecular weight with a relatively low charge density/low electrophoretic mobility and contain a hexuronic acid as the acidic component. These antigens, which are stable at pH 5 to 6, are co-expressed with O8 and O9 but infrequently with O20. The growth temperature has no influence on the production of the capsule. Polysaccharides of this group resemble those of *Klebsiella* capsules. The high charge density/high electrophoretic mobility polysaccharides of group II are co-expressed with many O antigens, but not with O8 or O9 or at low temperatures, and they are thermolabile at pH 5 to 6. Several unusual acidic components, such as 2-keto-3-deoxy-D-mannooctulonic acid (KDO), N-acetyl-neuraminic acid (NeuNAc), N-acetyl-mannosaminuronic acid (ManNAcUA), or phosphate are found in group II polymers. Capsular material from this group of antigens share many properties with those from *Neisseria meningitidis* or *Haemophilus influenzae*[4].

The *E. coli*, as do other members of the family *Enterobacteriaceae*, also produce other polysaccharide antigens *viz* the enterobacterial common antigen[16] (ECA) which is elaborated by most wild type strains, and a non-specific slime (M) antigen. The latter is associated (loosely) with the outer cell surface, but is distinct from the K antigen. The M antigen is represented by a group of five acidic polysaccharides with a common backbone[14].

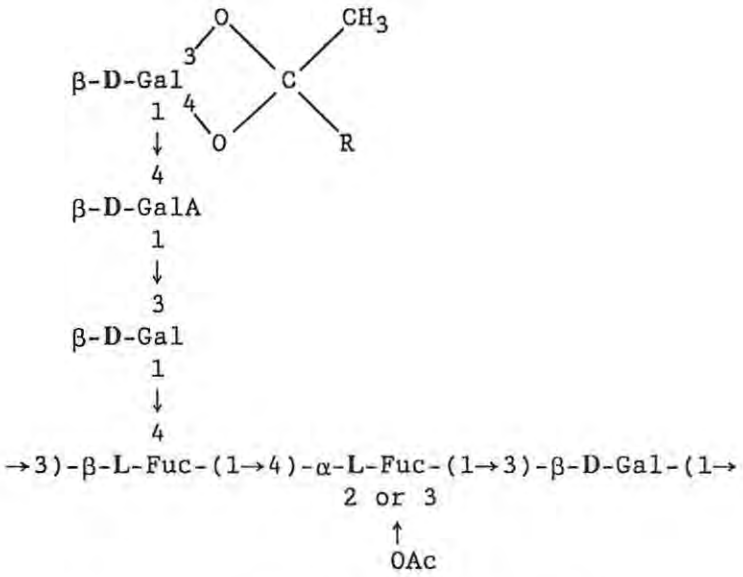


Figure 5 Structure of the M antigen. R = CH_3, isopropylidene substitution of galactose; R = H, ethylidene substitution of galactose[14].

Over forty-seven K antigens have been structurally elucidated and reported in the literature to date[17]. Neutral constituents of the glycan are most often galactose, glucose and mannose in the pyranose form. Furanosyl residues are uncommon components of these polymers, however several repeat units have been reported to contain ribofuranose (associated with KDO) or ribitol-phosphate. The predominant acidic monosaccharide is glucuronic acid, although galacturonic acid, NeuNAc and KDO are frequently reported. A D-furanosyl isomer (4-deoxy-2-hexulosonic acid) has only been found in the polymer of K3[18].

Non-carbohydrate moieties include the ubiquitous O-acetyl as well as phosphate (K2[19], K12[20], K18[21], K22[21], K52[22], K100[21]) pyruvate (K29[23] and K37[24]), glycerol (K2[19] and K62[19]) and O-propionyl (K52[22]) groups. The only capsular antigens reported to be substituted with an amino acid are K40[25] and K54[26]. With the exception of K1[27] and K92[28] all the K antigens are heteropolymers with repeating units that range in size between two and six monosaccharide units. The remaining K antigenic structures are presently being studied.

4 CHEMISTRY AND SEROLOGY OF THE *Klebsiella pneumoniae*

Klebsiella, which belong to the family *Enterobacteriaceae*, are a genus of non-motile Gram negative rods that were first described by Friedländer[29]. They have also been known under the synonyms *Aerobacter aerogenes*, *Enterobacter aerogenes* and the Friedländers bacillus, however, these names have fallen into disuse. Further confusion arose as several species assigned to this group proved to be variants of the original species. The generally accepted name for the genus is *Klebsiella pneumoniae*[29].

Nine somatic O antigens and seventy-seven K antigens[30] are recognised for the serological classification of the *Klebsiella*. They are also typed A or B depending upon whether they principally cause human or animal infections, respectively[29].

Capsular polysaccharides isolated from *Klebsiella* strains have been extensively researched and, to date, the only K antigens that remain to be characterised and reported are K29, K42, K43, K65 and K71. Nimmich[31] (1968) published the results of a comprehensive study of the monosaccharide composition of the capsules, which showed that there were approximately twenty chemotypes. More recently Kenne and Lindberg reviewed all of the *Klebsiella* K structures published up to 1982[32]. Since then the structures of K3[33], K10[34], K14[35], K15[36], K19[37], K35[38], K39[39], K45[40], K50[41], K63[42], K66[43], K67[44], K68[45], K69[46], K79[47], K80[48] and K82[49] have been published. In addition, previously published structures have been re-examined using more modern methods and corrected.

In general *Klebsiella* capsular polymers are acidic branched heteropolysaccharides which are comprised of a repeating oligomer which can range in size from three to seven monosaccharide units. The acidic component is usually a uronic acid (predominantly glucuronic), notwithstanding that in some instances pyruvate is the sole acidic constituent of the glycan. The neutral monomers are usually hexoses (galactose, glucose and mannose) or 6-deoxy hexoses such as fucose and rhammose) and unlike *E. coli* they are devoid of amino sugars, amino acids and phosphate groups. Several of the polymers, however, do contain an unusual sugar, eg K22[50] and K37[51] have a 4-O-[-(S)-1-carboxyethyl]-D-glucuronic acid, K38[52] has a 3-deoxy-L-*glycero*-pentulosonic acid and K82[49] has L-glutamic acid. The presence of O-acetyl groups is common. Approximately fifty percent of the repeating oligosaccharide units have a pyruvate moiety attached to one of the monomers. This is normally 4,6-linked, but may be 3,4-linked as found in K30[53], K33[54], K72[55] and K80[56] or 2,3-linked (eg K58[57]).

Many *Klebsiella* are pathogenic and hence of great medical interest. They infect the respiratory tract and sinuses where they produce thick mucoid (capsular) material that is difficult to dislodge and thus leads to congestion of the airways. The pneumonia caused by these bacteria is often fatal[29].

5 METHODS OF ANALYSING BACTERIAL CAPSULAR POLYSACCHARIDES

Capsules of bacteria are usually highly complex polymers composed of various monosaccharide residues joined in a number of different ways. The investigator is faced with determining:

a) the sugar composition of the repeating unit;
b) the ring structure of each component;
c) the position and configuration of linkages between the residues;
d) the sequence of sugars;
e) the attachment position of any non-carbohydrate constituents;
f) the average number of repeating units in the polysaccharide chains.

This information is derived from chemical, chromatographic and spectroscopic data obtained from analysing the polysaccharide and/or its derivatives. However, the increasing power and scope of analytical instruments for obtaining structural information from polysaccharides has, to some extent, reduced the emphasis upon chemically based analyses. The full structure of a complex polymer can now be rapidly determined by nuclear magnetic resonance spectroscopy, provided that the spectra are clear and unambiguous. There are, however, instances where chemical methods, either alone or supplemented by instrumental techniques, are more appropriate alternatives for a structural investigation. They are also invaluable for performing rapid preliminary purity and composition analysis of a sample. The main disadvantage of chemical methods is that they are destructive. This drawback has been lessened by the development of more sensitive techniques.

It is not the intention of the author to exhaustively review all of the methods available for analysing polysaccharide structures as there are several excellent and very comprehensive reviews that have already covered this material, for example, "*The Polysaccharides*" and the series "*Methods in Carbohydrate Chemistry*" and "*Advances in Carbohydrate Chemistry and Biochemistry*". In this chapter, only the newer techniques and those which we have frequently applied will be discussed. Moreover, the "specific degradation" methods, nuclear magnetic resonance (nmr), gas-liquid chromatography (glc) and mass spectrometry (ms), will also be dealt with under separate headings because of their importance in the field of structural elucidation of polymers.

5.1 Sugar Composition Analysis

5.1.1 Total Sugar Ratio Determination

The total sugar ratio (TSR) must be determined when analysing the primary structure of a polysaccharide. Identification and quantification of the component sugars is preceded by a step to cleave the glycosidic linkages. Methods described to achieve this include: hydrolysis, acetolysis, methanolysis and mercaptolysis. However, not all of these may be suitable for the glycolytic cleavage of a particular sample. For example, during hydrolysis, 2-deoxy sugars are cleaved with ease in weak acid solutions, furanosidic linkages (except those of 3,6 anhydrohexosides) are more labile than pyranosidic bonds, β- are often more stable than α-glycosides and the presence of a carboxyl (ie a uronic acid) or 2-deoxy-2-amino group, can stabilise the interglycosidic linkage to acid catalysed hydrolysis[58]. Thus, to optimise depolymerisation and minimise unwanted degradation, the configuration of the linkage, the ring form of the sugar units as well

as the polysaccharide composition, must be taken into account and a pertinent method chosen. Labile sugars can be preserved by performing the acid catalysed cleavage in a non-aqueous medium where the sugar is released as a glycoside rather than the reactive free reducing sugar. Methanolysis (glycosidic cleavage with HCl in anhydrous methanol) gives rise to a methyl glycoside of the parent sugar and these undergo fewer side-reactions than a monomer with a free reducing hydroxyl group[59,60].

Where a glycosyluronate is to be cleaved, chemical modification can be employed to make this easier; reduction of the carboxyl to a hydroxymethyl group circumvents the need for strong hydrolytic conditions to obtain complete depolymerisation of the sample. Water soluble polymers can be modified by treatment with either aqueous carbodiimide or sodium borohydride[61]. Methylated glycans and other hydrophobic polymers are reduced with lithium aluminium hydride in tetrahydrofuran (THF). This is necessary, because there is no completely satifactory volatile derivative for carboxyl containing sugars, hence, they are not usually quantified by glc. However, reduction of the carboxyl group allows these residues to be analysed by gas-liquid chromatography (glc). Glycosyluronic acids and other components can, however, be analysed and quantified by high performance liquid chromatography (hplc) without prior modification or derivatisation of any of the free sugars.

Hydrolysis of a glycosaminoglycan is retarded when the N-acetyl group is removed by the acid, leaving a free amino group which is readily protonated and thus provides an electrostatic shielding of the hexosamidic linkage. Improved hydrolysis after removal of the 2-acetamido group with hydrazine has been reported[62]. Hexosamidic linkages can be hydrolysed more rapidly, while still keeping the

overall acidity low, by using soluble polystyrene sulphonic acid which provides a high localised hydrogen ion concentration which attracts the charged sugar unit[63]. Furthermore, the glycosidic bond between an amino sugar and the sugar to which it is linked is split when 2-deoxy-2-acetamido-hexoses are deaminated with nitrous acid[62].

Trifluoroacetic acid (TFA) is the most frequently used acid catalyst because it is as effective as the mineral acids and easily removed by distillation under reduced pressure[64]; neutralisation of mineral acid is time consuming and may lead to a loss of product on recovery. Formic acid is used to hydrolyse hydrophobic polymers (eg methylated glycans) which will not dissolve in the other acids available. Formylation of the products may, however, occur as a side reaction[65].

Chromatographic methods for analysing the hydrolysate are: hplc, thin layer chromatography (tlc), high performance thin layer chromatography (hptlc), paper chromatography (pc), exchange chromatography and glc. With the exception of glc, which requires the preparation of a suitably volatile derivative, these can all be applied to the separation of free reducing sugars.

In this laboratory, the composition of the hydrolysate is initially determined using paper chromatography in several different solvent mixtures. This sensitive technique (requiring less than 1 mg) can separate monosaccharides quickly and simply. This method is followed by separation and quantification of the suitably derivatised hydrolysate components by glc and flame ionisation detection. Commonly, the free sugars are converted into either peracetylated aldononitriles[66] or O-methyl oxime acetates[67]. Where a uronic acid is present it is usualy reduced to the neutral parent sugar prior to derivatisation. These steps are, however, time consuming and as a

result glc has been replaced by high performance liquid chromatography as the method of choice from determining free sugar ratios. This technique rapidly separates any mono- and di-saccharides, sugar acids, lactones and N-acetyl-amino sugars contained in the hydrolysate[68] by using (for example) a silica gel stationary phase eluted with an aqueous mobile phase. The alkylamine-bonded silica gels are particulary useful[69]. Components are usually detected with a differential refractometer.

The ratio of the peak areas (integrals) of the anomeric proton signals, for each sugar type in the polysaccharide, can be determined from the proton magnetic resonance (^{1}H-nmr) spectrum of the polysaccharide and provides another simple and accurate method of determining the TSR.

The uniform nature of the repeat unit allows the sugar ratio to be quoted as an exact value.

5.1.2 Stereochemistry of the Monosaccharides

The commonly occurring monosaccharides are aldopentoses, ketohexoses (with three asymmetric centers) or aldohexoses (four asymmetric centers). Therefore, there are a number of optical isomers. The sixteen aldohexose isomers are divided into eight enantiomeric pairs which correspond to the naturally occurring hexoses. Thus, once a monomer is identified all that remains is to identify whether it is the **D** or **L** enantiomer.

In practice the **D/L** configuration of the sugar can be assigned by polarimetry[70], specific enzyme assay, glc analysis of a diastereomer

derivative of the enantiomer or by measuring the circular dichroism of the alditol acetate derivative[71].

Enzymic techniques rely on selective metabolism of only one of the isomers by the enzyme. After incubation of the sample and enzyme in a buffered solution, a paper chromatogram is run to determine if the sugar has been modified. The availability of pure samples of stereoselective enzymes is limited and this makes it difficult to apply as a routine technique. Furthermore, the polymer must be hydrolysed and the sugars isolated prior to the enzymic treatment, consequently there is a loss of material.

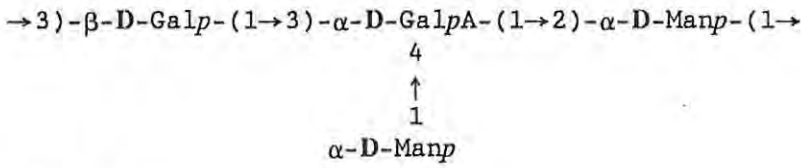
Racemic mixtures can be resolved by glc, either by conversion of the enantiomers into diastereomers with a chiral reagent and separating these on a non-chiral phase, or, by resolving the **D/L** isomers using a chiral stationary phase (eg Chirasil-val). Chiral reagents that have been recommended for use include (-)-2-butanol[72], (+)-1-phenylethanethiol[73] and (+)-2-octanol[74]. Recently a new method of establishing the sugar configuration by using a chiral stationary phase (α-cyclodextrin) was reported[75]. This techniques major advantage over the other glc methods, is that it can resolve enantiomeric anhydroalditols produced by reductive cleavage[76] of a methylated glycan. Thus, it will be possible to simultaneously identify the linkage points and stereochemistry of the residues. Furthermore, the cyclic structure is retained and each residue produces a single peak. Hence there is positive proof of the ring size and the chromatograms are less cluttered than, for example, (+)-2-octanol derivatives which produce 4 or 5 peaks for each component.

We have successfully used (-)2-octanol as a chiral reagent to produce diastereomers from all of the commonly occurring sugars and uronic

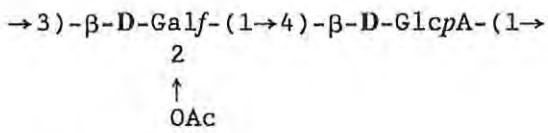
acids after reduction of the carboxyl groups. We have found that the D/L configuration of amino sugars can also be determined by this method, providing that the N groups are reacetylated prior to treatment with the chiral reagent. Separation was achieved on a fused silica capillary column coated with OV-225.

5.2 Linkage Pattern

Polysaccharides are more complex than other macromolecules (proteins and nucleic acids, for example) because their monomeric unit, the monosaccharide, has several possible points of attachment. They can also have a linear or a branched structure.



E.coli K36[77]



E.coli K53[78]

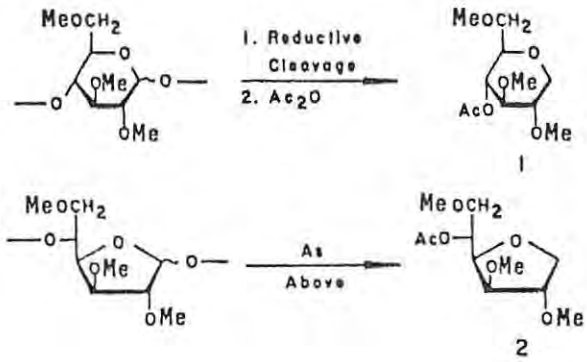
Figure 7 Example of the repeat unit of a branched and a straight chain polysaccharide.

Commonly, the linkage pattern for each sugar is resolved by "methylation analysis"; however, nuclear magnetic resonance experiments can also be used to reveal the position of the inter-glycosyl bonds.

5.2.1 Methylation Analysis

Methylation involves the etherification of all the free hydroxyl groups under either basic or mildly acidic conditions, where the

glycosidic bonds are stable. Once methylated, the glycan is depolymerised in a manner that will leave the O-methyl linkages intact. Thus, any free hydroxyl groups on the released permethylated monosaccharide indicate a former attachment point. The ring size can usually be identified by the presence of a hydroxyl at either C_4 or C_5. Hydrolysis and the conversion of the partially methylated residues to alditols suffers from the disadvantage that it is incapable of distinguishing between 4-linked aldopyranosyl and 5-linked aldofuranosyl residues in a polysaccharide, as they both produce 2,3,6, tri-O-methyl hexose residues[79]. This deficiency also applies to this method when examining 5- and 6- linked ketohexosyl residues. The reductive cleavage method[76], which involves the regiospecific cleavage of glycosidic carbon-oxygen bonds in methylated glycans, does not suffer from this disadvantage. This is because, although it is based upon the standard "methylation analysis", the fragments formed from the methylated polysaccharide are not reducing sugars. Pyranosidic residues are converted to 1,5-anhydroalditol derivatives (1) and furanosides are converted to 1,4-anhydroalditols (2). It is, therefore, possible to establish the linkage position(s) and ring size simultaneously. These stuctural entities are free of anomeric complications and can be easily characterized by glc and glc-mass spectrometry (glc-ms).



Scheme 1 Products from the reductive cleavage of a pyranose and a furanose[80].

Partially methylated products from the standard methylation analysis are separated by glc as suitably volatile derivatives, for example, as alditol acetates[64]. The components are identified on the basis of their retention time, with respect to that of a standard, and this value is collated with reference data for the column type and temperature at which it was maintained. These provisional assignments are confirmed by glc-ms. Mass spectrometry confirms the substitution pattern. It has been shown that the components of a mixture of permethylated sugars can be identified by ms *per se*[81].

5.2.2 Methylation Reagents

Method	Solvent	Base	Methylating Agent	Comments
Hakomori[82]	DMSO[a]	Na^+dimsyl[b]	MeI[c]	a) Uronic acids may undergo β-elimination b)acetamido groups may become N-methylated.
Haworth[83] ψ	Water	NaOH	dimethyl sulphate	Less β-elimination than the Hakamori method.
Kuhn[84] ψ	N-N-dimethyl formamide	Ag_2O	MeI or dimethyl sulphate	
Purdie[85] ψ	MeI	Ag_2O	MeI	Few polysaccharides are soluble in methyl iodide.

Table 1 Examples of some of the methylating procedures that use alkaline reagents.

a- dimethyl sulphoxide
b- sodium methylsulfinyl-methanide
c- Methyl iodide
ψ- These methods require repetitions to effect complete methylation.

The Hakomori method (or a modification of it) is routinely used to methylate carbohydrates, because it can effect complete O-methylation in a single treatment. This procedure involves sodium dimsyl catalysed alkoxide formation from the hydroxyl groups and the O-methyls are introduced on treatment with methyl iodide. Introduced in 1964, it has been modified in several ways. One of these is the addition of 1,1,3,3 -tetramethyl urea to restrict the intra- and inter-molecular hydrogen bonding in the polymer which can cause undermethylation[86]. Methylation is sometimes limited by the solubility of the polysaccharide in DMSO and alternative solvents have been employed. For example, N-methylmorpholine N-oxide and a mixture of this and DMSO have been shown to dissolve polymers insoluble in DMSO alone[61]. The methylsulphinyl carbanion (dimsyl), generated from DMSO and sodium hydride, can also be prepared from

butyl lithium[87] or potassium hydride[88]. The lithium and potassium dimsyl carbanions are convenient to prepare and there are fewer side reactions during their synthesis. In addition, the K^+ and Li^+ ions, as a result of ion pairing, allow a greater proportion of the free carbanion to be available for the critical alkoxide formation step[88]. It has been reported that methylation can be ensured by using an excess of the carbanion[89] and this can be detected by triphenylmethane indicator.

Harris *et al* have improved the overall methylation procedure by performing the entire operation, including any additional derivatisation steps, in a single vessel[90]. This reduces losses on glassware and avoids the need to transfer and concentrate the product with consequent depletion due to volatilisation of the product. This technique allows for rapid analysis within a single working day.

Alternative methods, which claim to give better results, have been published since the introdution of the Hakomori procedure. These are based on the assumption that OH^- and H^- ions are the effective bases and not the $CH_3SOCH_2^-$ anion[91]. Thus dimsyl has been replaced by a solid base, eg NaOH or KOH[91,92]. Short methylation times (less than one hour) and the absence of non-sugar chromatogram peaks have been reported using this approach[91].

The disadvantage of all these techniques is the strong base (eg dimsyl has a pKa of 35) required to effect deprotonisation of the hydroxyl groups leads to loss or migration of alkali labile substituents like O-acetyl groups[61]. This is avoided when using diazomethane and boron trifluoride etherate[93]. This method, which effects methylation under acidic conditions is, however, restricted to the few carbohydrates that are soluble in non-polar organic solvents. Prehm[94] reported that

methylation with methyl trifluoromethanesulphonate in trimethyl phosphate is a mild, generally applicable technique that preserves base labile substituents and effects methylation in a single step. This method is, however, limited to low molecular weight (<20 000) carbohydrates.

The absence of O-H stretching vibrations in the infra red (IR) spectrum of the methylated sample is an indication of complete O-methylation.

5.2.3 Periodate Oxidation of Polysaccharides

Periodate (periodic acid or its Na^+ or K^+ Salt) effects a 1,2-glycol scission of the 1,2 -diol and 1,2,3 -triol groups found in some sugar residues.

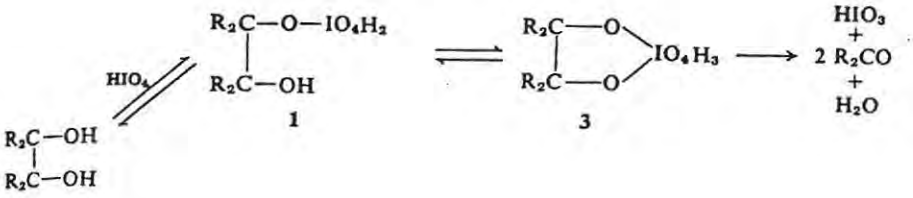


Figure 7 Action of Periodic acid on a 1,2 glycol group[95].

This is a useful analytical tool because residues substituted in a manner that leaves no hydroxyl groups on adjacent carbon atoms, for example a 3-O-linked hexopyranosyl, are resistant to cleavage.

The information yielded from this experiment depends upon the specific procedure chosen. Monitoring the uptake of oxidant (HIO_4) per mole of repeat unit, determines the number of susceptible linkages within the unit, while the rate of reaction or indentification of the reaction products gives information on the stereochemistry and the type of

structures cleaved[95]. The periodate treated sample can be subjected to a methylation analysis to provide supplementary information.

The results obtained from a periodate experiment should be treated with a measure of caution. There are examples of carbohydrates, with structures that should be susceptible to glycol scission, which have been shown to be resistant. Similarly, overoxidation is a problem, especially with glycosyluronic acid containing polysaccharides which are subject to extensive oxidation in addition to glycol cleavage[95]. A pH dependent non-specific oxidation has also been observed with N-methylated amino sugars[96]. Overoxidation can, however, be avoided provided that the reaction is carried out in the dark, at low temperatures and at a pH between 3 and 3.5[97].

5.3 Sequencing of the Repeat Unit

The monosaccharide units that make up the repeat unit can be in the linear chain or, if present, part of the side chain(s).

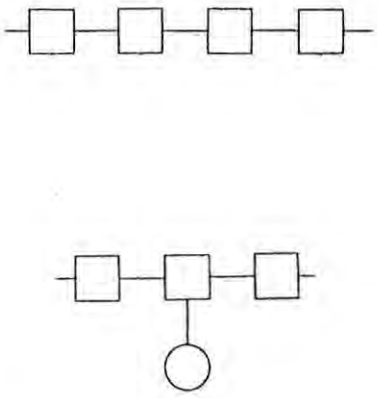


Figure 8 Two examples of patterns of the sugars in a tetrasaccharide repeat unit.
□ Sugar residue, eg 2-O linked galactopyranose.

The sequence of the repeat unit is often determined by degrading the polysaccharide to generate a suitable mixture of overlapping oligomers. This is achieved with one of the specific degradation methods. The oligosaccharides are isolated and identified by either chemical methods, for example methylation analysis, or nuclear magnetic resonance, or a combination of both. These data are used to reconstruct the pattern of the repeat unit.

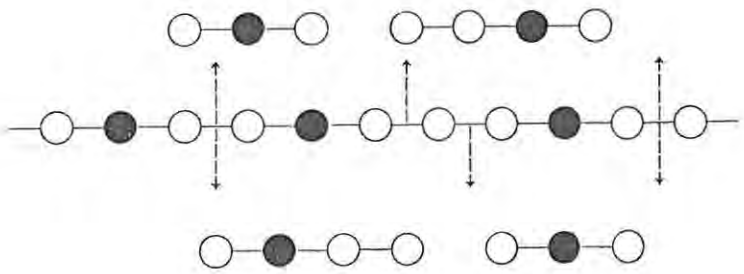


Figure 9 Oligosaccharides formed by the action of cellulase from *Streptomyces* sp. OM B814 on a mixed cereal β-D-glucan[61].

Key: ○ 4-linked β-D-glucopyranose residue
● 3-linked β-D-glucopyranose residue.

The drawback of this procedure is that it is tedious and time consuming. It also requires a substantial amount of material because most of the degradative methods give a low overall percentage yield. In addition, the relative position of a sugar in the repeat unit can be ascertained from an nmr study of the polysaccharide, which is more convenient than partially degrading the polysaccharide.

Sequencing the repeat unit by nmr is undertaken as follows. Firstly, 1D and 2D proton magnetic resonance (pmr) spectra are acquired. The protons of a sugar have a chemical shift and a coupling constant that is characteristic of its position and the sugar type. Hence,

combining the 1D ^{1}H and the 2D COSY[98] experiment data, usually allows all the proton resonances from the sugars in the repeat unit to be assigned and the residues identified. It is also notable that the resonances of those protons attached to glycosidically bonded carbons are shifted 0.1 to 0.5 ppm upfield of their normal value[100]. This information may be used to identify the linkage points, but, it is not entirely reliable.

The next step in the procedure is to correlate the proton resonances with resonances in the ^{13}C spectrum by a heteronuclear correlation experiment (HETCOR[99]). Thus, we have fully assigned proton and ^{13}C spectra of the polysaccharide. The glycosidically linked non-anomeric carbons show a "glycosylation shift" of 6 to 9 ppm downfield[100,101] which separates them from the other non-anomeric carbon resonances, and signals from the adjacent carbons are shifted upfield. It is, thus, easy to identify the linkage carbons and hence, from the HETCOR spectrum, the protons attached to them.

The final stage is to determine the through space interactions between spin systems by using, for example, the nuclear Overhauser enhancement (nOe) difference experiment[98] or nuclear Overhauser enhancement correlation spectroscopy (NOESY[222]). These are able to detect nuclei that are interacting, but are not necessarily attached to adjacent atoms. The NOESY spectrum shows an inter-residue nOe between the anomeric proton of a sugar and a proton on the residue to which it is linked. This resonance, which is from the proton bonded to the linkage carbon, can be identified from the ^{1}H-nmr data and, therefore it is possible to simultaneously confirm the methylation results and determine the sequence of the repeating unit.

5.4 Location of Non-Carbohydrate Substituents

The residues of the polysaccharide can be substituted with O-acyl (usually O-acetyl), phosphate, pyruvate and by N-acetyl groups on amino sugars. These features can be detected and in some instances located, by nmr. Their presence influences the choice of the chemically based analytical procedures used.

The location of O-acetyl groups is the most difficult to establish because they are readily removed or undergo migration to other sites during the basic conditions used routinely for methylation. One method of determining their position(s) involves the conversion of unsubstituted hydroxyls to methoxyethyl acetals on reaction with methyl vinyl ether, followed by (simultaneous) base catalysed de-O-acetylation and methylation[102]. On hydrolysis, this modified polysaccharide gives sugar derivatives labelled with an O-methyl at the site previously occupied by the acetate. We have found that this technique is usually only effective when applied to very low molecular weight polymers. If the sample is soluble in trimethyl phosphate, the Prehm[94] methylation is an easier way of determining the O-acetyl position. This is because this methylation procedure leaves the base sensitive groups intact and therefore after hydrolysis there will be a free hydroxyl group on the permethylated residue, at the position where the O-acetate was previously situated. To avoid confusing hydroxyls arising from interglycosidic linkages and that from the O-acetyl location, a methylation is also done under basic conditions which will methylate the O-acetyl position.

All of the other substituents are reasonably base stable and their positions of attachment are identified during a methylation analysis. For example, pyruvate groups, except for those that are 3,4-O

linked[103], are stable to the reactants used in a Hakomori methylation. Therefore, after acid catalysed hydrolysis of the methylated glycan, which cleaves pyruvate groups and interglycosidic bonds, free hydroxyls indicate the positions of pyruvate attachment and the linkage points. Methylation analysis of the depyruvalated polymer distinguishes the interglycosidic linkage points from the pyruvate attachments. The removal of the pyruvic ketal can be brought about by controlled polysaccharide "autohydrolysis"[61].

Fast atom bombardment - mass spectrometry (fab-ms) can locate the sugar to which the substituent is linked[104], however, it does not reveal the linkage position(s) and can only be used for very low molecular weight carbohydrates. Nmr is an effective method of locating a substituent because they produce an observable "glycosylation shift" of the resonances from the ^{1}H and ^{13}C nuclei at the attachment point(s).

6 SPECIFIC DEGRADATIONS OF POLYSACCHARIDES

These degradations employ controlled chemical reactions that will more selectively cleave one type of (labile) glycosidic bond before the other linkages are affected. Should all the interglycosidic linkages be equally susceptible to the reagent, a random selection of all the possible oligomers will be produced in a low yield. Furthermore, it may be difficult to separate this complex mixture because of the large number of fractions and the small physico-chemical differences between them. Ideally, a high yield of a single oligomer (that corresponds almost exactly to the intact repeat unit) will be produced. Once separated by chromatography, the oligomers are primarily used to provide complementary information on the configuration of the glycosidic bonds, mode of linkage and the sugar sequence. Occasionally these degradations are used to modify a polysaccharide or oligosaccharide, in a manner that will provide further information on its structure. The combination of these methods with methylation analysis is especially valuable because the products can be analysed by mass spectrometry and the amount of material which can be analysed (1-5 μg) is much smaller than required for nmr spectroscopy. This sensitivity may be of critical importance in the structural analysis of small amounts of complex polysaccharides.

The techniques available include β-elimination at uronic acid residues, selective acetolysis, Smith degradation, selective and partial reductive cleavage, keto degradation, nitrous acid deamination of 2-amino-2-deoxyaldoses, chrominuim trioxide oxidation, as well as the widely used acid catalysed partial hydrolysis and selective enzymic hydrolysis[61,62,105-107]. Several of the currently used specific degradations are discussed.

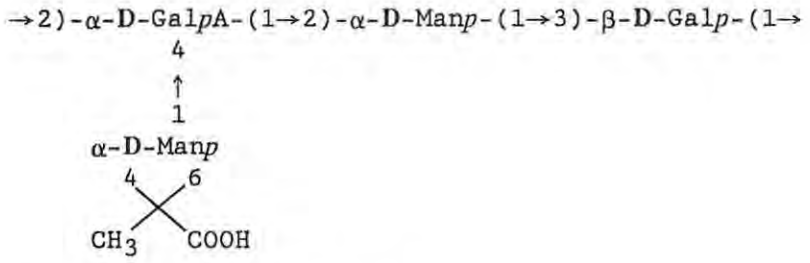
6.1 Acid Catalysed Partial Hydrolysis

This classical method of producing oligomers for glycosyl sequencing, used frequently in structural determinations[eg 108-111], has been reviewed along with other methods for partial depolymerisation[61].

The glycosidic bonds of heteropolysaccharides are hydrolysed at different rates, which depend upon the hydrolytic conditions and the nature of the sugar residue. If the reaction is stopped before complete depolymerisation is effected, oligomers of different molecular weight can be isolated by preparative chromatography. A specific "cracking" pattern occurs because some of the interglycosidic linkages of the polymer are more susceptible to acid cleavage than others. Sensitive sugars are generally furanoses, 3,6-dideoxyhexopyranoses, and residues with a deoxy function adjacent to the anomeric carbon atom (eg neuraminic and 3-deoxyglyculosonic acids[111]). Should such a monomer be present in the polymer chain its linkage will be hydrolysed rapidly with little concomitant degradation of the other bonds.

Graded hydrolysis involves, in most cases, heating approximately 0.5% of the polysaccharide in acid until the required degree of depolymerisation is achieved. Aqueous acid solutions are routinely used as the hydrolytic agent, however, non-aqueous reagents can be used where an alternative splitting pattern is desired, the polymer is insoluble in water, or the derived oligosaccharides are more stable than those produced in an aqueous solution. Liquid hydrogen fluoride (at temperatures between -40° and -23°) is also used for partial hydrolysis, because, unlike the other acids, it has the advantage of producing oligomers without removing any O-acetyl groups that may be attached[103,112].

Acid catalysed partial cleavage of a polysaccharide is a widely applied technique and has been used, for example, in the structural investigation of *klebsiella* K68. Two oligosaccharides were isolated after the partial hydrolysis[45].



Klebsiella K68 Polysaccharide

↓ H^+ (0.5m TFA, 3 h, 100°)

A1 α-D-GalpA-(1→2)-α-D-Manp-(1→3)-$^{\alpha}/_{\beta}$-D-Gal-OH
4
↑
1
α-D-Manp

A2 α-D-GalpA-(1→2)-α-D-Manp-(1→3)-$^{\alpha}/_{\beta}$-D-Gal-OH

Figure 10 Partial hydrolysis of the *Klebsiella* K68 capsular polysaccharide[45].

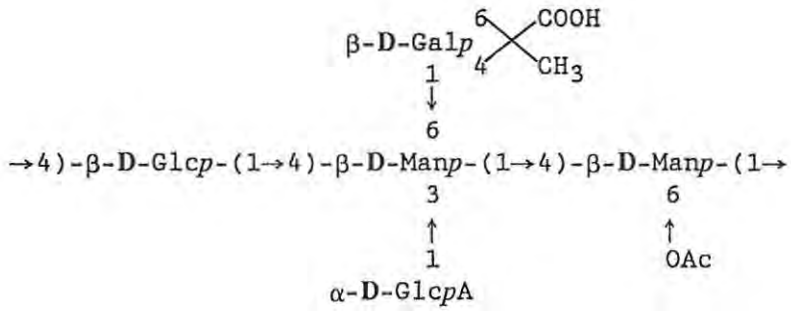
The oligosaccharides are characterised by the same methods applied to the structural elucidation of a polymer and usually provide the sequencing information.

Unfortunately, acid-catalysed polysaccharide depolymerisation initiates problems related to the exposure of the reactive hemiacetal group and the general instability of many other sugar moieties in acidic conditions. Moreover, the original pyran or furan ring form of the residue where cleavage has occurred may be lost and it is often difficult to generate a representative distribution of smaller oligomers for sequence analysis.

6.2 Selective Enzymic Hydrolysis

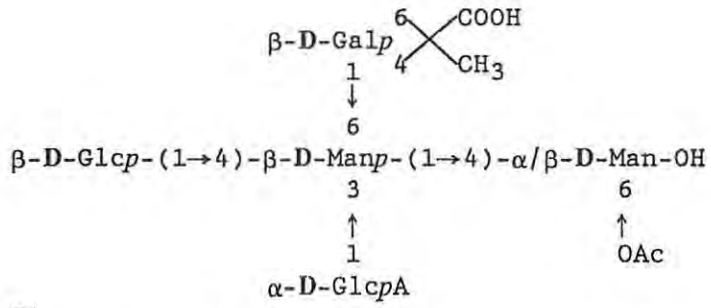
Glycolytic enzymes, particularly those that selectively cleave interglycosidic bonds, are being increasingly applied in the structural elucidation of bacterial polysaccharides[eg 33,35,42,46, 55,77,113-124]. They are, in some cases, the only method of preparing an oligosaccharide that is an analogue of the integral repeat unit. Ideally these enzymes are of the *endo* series which are specific for both the sugar residue and the linkage stereochemistry and therefore give controlled hydrolysis of only a single bond type. They produce an oligosaccharide (**P1**) that corresponds to the repeat unit. The dimer (**P2**) or trimer (**P3**) may also be products.

Some glycolases are well characterised and readily available from a commercial source, but in most instances they have to be isolated and purified by the researcher[125]. A widely applied method is to isolate a suitable bacteriophage-borne enzyme from sewage. Phages (∅) that interact specifically with acidic capsular polysaccharides (its receptor[126]), are designated **K** phages. They carry endoglycosidases associated with their thick fibres or tail spikes[126] and are host specific. We have found, with few exceptions, each of these phages will only absorb to a limited number of strains. Similarly, they will not interact with acapsular mutants[126]. This specificity is highly advantageous and ensures selective polymer hydrolysis.



Klebsiella K69

↓ Ø69



P1

Figure 11 Bacteriophage mediated depolymerisation of *Klebsiella* K69[46].

Bacteriophages with lyase activity (eg the phages that interact with *Klebsiella* K5, K14 and K64) depolymerise by a β-elimination reaction rather than the more frequently encountered glycosidic hydrolysis and produce oligomers which are not necessarily multiples of the repeat unit[35]. These are usually labelled A, B, C etc.

The polysaccharide (approximately 2 mg per 1 X 10^{10} active bacteriophages) should be hydrolysed to oligomers after several days at 29° to 37°. Thereafter, the required fragments (eg P1, P2) may be isolated by gel permeation chromatography.

Characterisation of these fragments is by the same procedures applied to the intact polysaccharide and a large amount of useful information is derived from comparing and contrasting the two sets of data. For example, methylation analysis of P1 will (by comparison with the

polymer methylation analysis results) reveal the point that the oligomer's reducing terminal sugar was attached to in the polymeric chain, and the non-reducing terminal sugar. Treatment of **P1** with the reducing agent $NaBH_4$ (or $NaBD_4$) will produce the **P1**-alditol and this labels the reducing sugar and this residue will be identified by methylation analysis. The glycosyl sequence can be determined from these data if the repeat unit is a di-, tri- or branched tetra-saccharide. The **P1**s from polymers with larger repeat units are sequenced from either nmr or fast atom bombardment - mass spectrometry (fab-ms) data.

Nuclear magnetic resonance spectra of oligosaccharides derived from a bacteriophage degradation (or any oligomer with a reducing terminal sugar) are characterised and complicated by "twinning"[127] of some of the signals. This is a result of the mutarotational equilibrium between the α and β anomeric hydroxyl orientation of the reducing sugar and the pyranose or furanose ring forms that this residue can adopt in solution. Thus, there are four different molecules present at any given instant[128] and four H-1 signals from the reducing terminus. The chemical shift difference between the signal "twins" is inversely proportional to the distance that the nucleus is from the reducing group. Therefore, the glycosyl sequence may be determined on the basis of the magnitude of the shift difference between the "twinned" signals of each residue[129].

Enzymic hydrolyses are carried out at pH 7, or thereabouts, and at 29° to 37°, and generally these conditions do not remove chemically labile structures like acetate and pyruvate groups. Therefore, when a suitable endoglycanase is available it provides an attractive method of preparing an oligosaccharide that closely resembles the repeat unit.

6.3 Partial and Selective Reductive Cleavage

The reductive cleavage (RC) method developed by Rolf and Gray[76] for the simultaneous determination of the linkage positions and ring form of each monosaccharide in a polymer, can also be used to generate methylated oligosaccharides from a methylated glycan. In this technique, glycosidic linkages are severed by the hydride transfer from silicon to the anomeric carbon center. However, unlike boron and aluminium hydrides, silicon hydrides (eg triethylsilane) require prior activation of the carbon center by either a Brønstead or Lewis acid. Initially trifluoroacetic acid (TFA) was used[130] to make the anomeric carbon electropositive, but it was not an effective catalyst[76]. At present boron trifluoride etherate ($BF_3 \cdot Et_2O$) trimethylsilyl trifluoromethane sulphonate (TMSOTf), trimethylsilyl methane sulphonate or a combination of boron trifluoride etherate and trimethylsilyl methane sulphonate (which produces[131] F_2BOSO_2Me, $FB(OSO_2Me_2)$ and $B(OSO_2Me)_3$) are used to catalyse the organosilane reduction. Some of these compounds do not catalyse the cleavage of all glycosidic bond types so, therefore, it is possible to have either a total RC or a selective reductive cleavage (SRC) of methylated glycans, by using the appropriate catalyst; the latter will produce methylated oligomers. Boron trifluoride etherate does not, for example, catalyse the cleavage of β-D-mannopyranose residues linked at both O-3 and O-6 or O-2 and O-6[132].

An example of SRC is the treatment of permethylated pullulan (a linear polysaccharide with a trisaccharide repeat unit composed of an α-1,6 and two α-1,4 linked glucopyranosyl residues) with triethylsilane and $BF_3 \cdot Et_2O$. Only the α-1,4 linkages are cleaved and the reaction

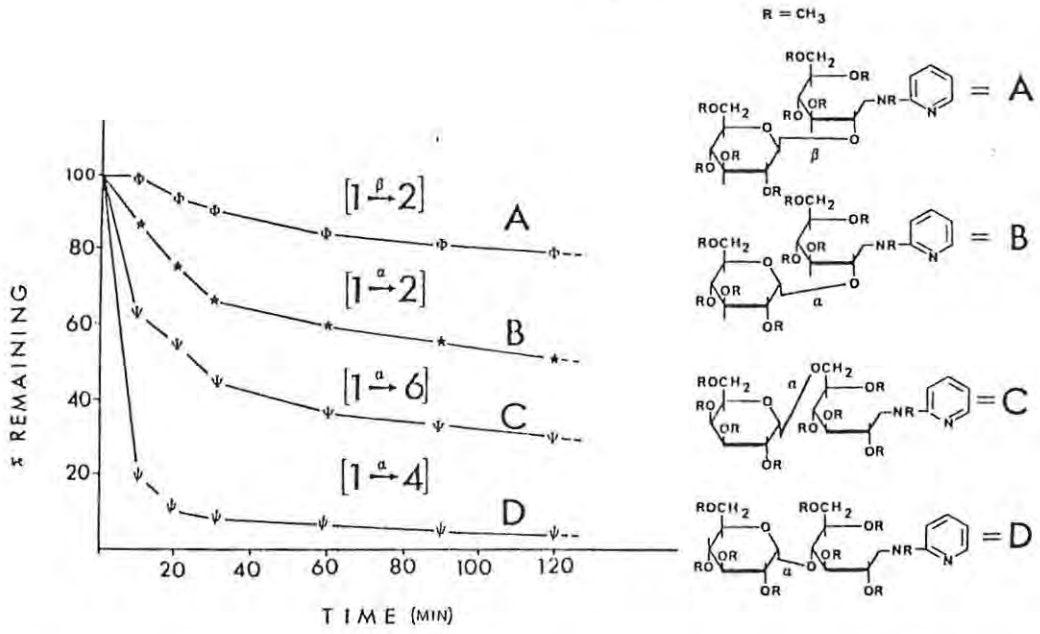


Figure 12 Linkage stability study of selected disaccharides under reductive cleavage conditions with $BF_3 \cdot Et_2O$ as the catalyst[133].

produces, after *in situ* acetylation, 4-O-acetyl-1,5-anhydro-2,3,6-tri-O-methyl-D-glucitol and a disaccharide anhydroalditol (1), 6-O-(4-O-acetyl-2,3,6-tri-O-methyl-α-D-glucopyranosyl)-1,5-anhydro-2,3,4-tri-O-methyl-D-glucitol[132].

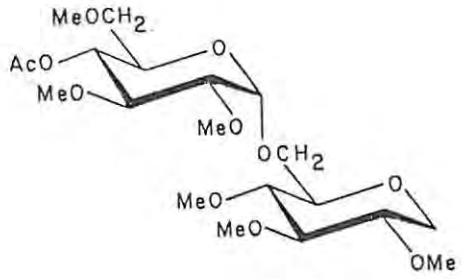


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Figure 13 A disaccharide anhydroalditol produced by the SRC of pullulan[132].

Should all the linkages in the polymer be equally susceptible to RC, oligomers can still be produced by terminating the reaction before all of the glycosidic bonds are cleaved. This is known as a partial

reductive cleavage (PRC). The first one to be reported[133] was by Reinhold *et al* in 1983. Anhydroalditol oligomers were generated from methylated β-cyclodextrin (a homoglycan) and seven fragments were isolated which corresponded to a monosaccharide and six oligomers, the largest of which was a heptamer. These were separated by hplc and eluting fractions were characterised by ammonia direct chemical ionisation - mass spectrometry (dci-ms).

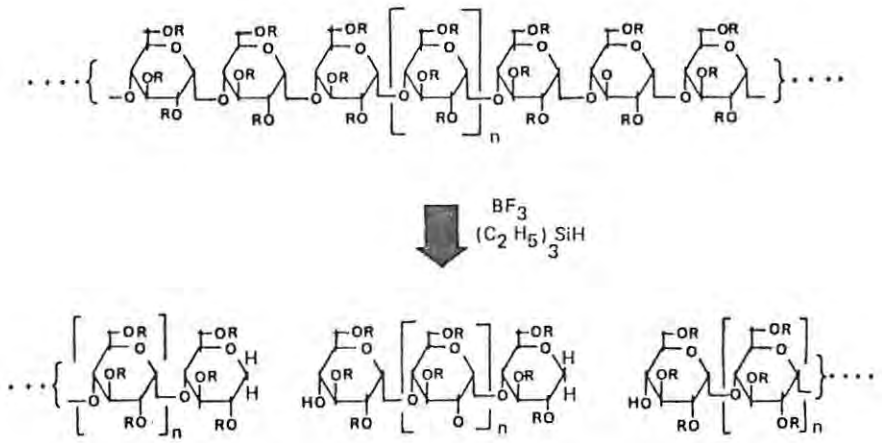


Figure 14 Partial reductive cleavage[133] of a linear glucopolymer, R=CH_3

Limited stability and, in some cases, the resistance of some sugar residue types to a particular set of reductive cleavage conditions has been reported. It is stated that the keto-furanosyl sugar, **D**-fructofuranose, is rapidly cleaved to an anhydroalditol when trimethylsilyl methanesulphonate is used as the catalyst and under these stated conditions all of these residues were cleaved within 15 minutes, whereas, no cleavage of pyranosyl residues was observed[131]. Further studies on trimethylsilyl methanesulphonate showed that it does not catalyse the RC of permethylated α-1,4-linked-**D**-glucopyranosyl, methyl 2,3,4,6-tetra-**O**-methyl-α-**D**-glucopyranoside residues or permethylated methyl β-cellobioside (a 4-linked glucopyranosyl disaccharide) when the reaction was carried out at

ambient temperature[131]. The amino sugar, 2-deoxy-2-acetamido-D-glucopyranose has been found to be totally resistant to RC if the anomeric configuration is α or the acetamido group is reduced to a methylethylamine[134]; those with the β-anomeric configuration are hydrolysed by the reagents[134]. Pyranuronic acids, it is stated[135], are likely to be cleaved more slowly than neutral sugars. The exception is 4-linked glucopyranuronic acid which undergoes a rearrangement reaction to an acyclic oxonium ion, and is rapidly reductively cleaved to an isomeric furanosyl anhydroalditol. Polysaccharides containing mannopyranose residues linked at the 2-, 3- or the 6-O position are incompletely cleaved when $BF_3 \cdot Et_2O$ is the catalyst[136]. It has also been noted that the resistance of an interglycosidic bond to RC conditions is affected by the nature of the polymer. The linear polymers amylose and cellulose are both composed of 1,4-linked glucopyranosyl residues, yet, $BF_3 \cdot Et_2O$ only catalyses the cleavage of amylose[80]. This may be due to the limited solubility of permethylated cellulose in the solvent used for RC[132].

We have found that 2-O linked β-ribofuranose and 3-O linked β-galactopyranose residue linkages are rapidly cleaved when using TMSOTf as the catalyst.

There are a number of advantages to performing either a PRC or a SRC when structurally elucidating a polysaccharide:

1 They provide per-O-methylated oligomers that can be used for polysaccharide sequencing.

2 Anhydroalditols, because they lack an anomeric hydroxyl, do not have the "anomeric complications" which can affect glc

separations and make the interpretation of nmr spectra from reducing sugar terminated molecules difficult.

3 The ring structure of all sugar types, except for 4-O-linked glucuronic acid, is retained.

4 Methylated oligosaccharides can be analysed by either dci-ms or fast atom bombardment-ms to determine their size and sugar sequence. These techniques, due to their sensitivity, require less than 5 μg of material.

5 The oligomers are soluble in organic liquids (eg chloroform) and thus, nmr spectra can be obtained for the sample in any one of several solvents. These spectra are usually better resolved than samples run in deuterium oxide (D_2O) and there is no HOD signal from the exchangeable hydroxyl groups, which can obscure some of the resonances. Further the "solvent induced shift" of signals, which occurs when the sample is dissolved in an alternative solvent, can help assign resonances with a similar chemical shift. Therefore, the interpretation of spectra from these methylated anhydroalditols is relatively simple and nmr provides a convenient, non-destructive, method of determining their structure.

6 Any free hydroxyls on the molecule indicate a former point of attachment and provide additional sequencing information.

7 We have found that some attached groups, eg pyruvate, are not reductively cleaved so their linkage points can be determined.

We have succeeded in producing several oligosaccharides from a trideuteriomethylated heteropolymer (*E. coli* K57 capsular polysaccharide) by reductive cleavage and the results are reported in this thesis. With the exception of the α-N-acetyl-glucosamine linkage, all of the residue types in the tetrasaccharide repeat unit of the glycan were, theoretically, at least partially susceptible to reductive cleavage with triethylsilane and TMSOTf. However, because each residue reacts at a different rate and the reaction time was limited, an anhydroalditol terminated tri- and tetra-saccharide were the major products. These oligomers were separated by gel permeation chromatography.

6.4 Base Catalysed Degradation

Sugar residues of poly- and oligo-saccharides are stable in alkali, except when there is a hydrogen in the α and a substituent in the β position to an electron withdrawing moiety[105]. In this case exposure to base results in the loss of the β-substituent and the reaction, consequently, is referred to as a base catalysed β-elimination. There are certain circumstances where this can be applied as a specific degradation procedure.

There are several electron withdrawing functional entities found in carbohydrates, including amides and carbonyl groups. However, the major application of β-elimination is to 4-O linked uronic acid containing polysaccharides. Once esterified (by methylating the polymer) the carboxyl group becomes electron withdrawing and the residue on the 4-position (β) will be readily removed in alkali[137], thus cleaving the uronic acid containing chain. The carboxymethyl uronic acid residue is converted to a hex-4-enopyranosiduronate[61] which can be removed by mild acid hydrolysis[105]. The uronic acids

former point of attachment is converted to a free hydroxyl group and this can be labelled by remethylation or trideuteriomethylation.

Aspinall and Rosell have reported[138] a β-elimination procedure which involves a "single flask" series of reactions. The methylated uronic acid containing polymer is treated with a base (sodium methylsulphinyl methanide) that is also effective in removing the hex-4-enopyrano siduronate residue, followed by remethylation with methyl iodide or trideuteriomethylation with trideuteriomethyl iodide. Thus eliminating the recovery steps which result in loss of material and, by avoiding acid hydrolysis, any acid labile constituents are retained.

The methylated oligosaccharides and polysaccharides (if the uronic acid is in a side chain) produced by β-elimination are hydrolysed and the permethylated residues, as suitable derivatives, are analysed by glc and glc-ms. Comparison with the results of the polysaccharide methylation analysis reveals the point that the uronic acid was formerly attached to.

The β-elimination reaction has been shown to be effective and can be used in the structural studies of acidic polysaccharides. It has been used[eg 139], to elucidate the uronic acid containing capsular polymer from several *Klebsiella* and *E. coli* strains. The disadvantages of this procedure are the low yield and numerous side reactions that occur[140].

6.5 Selective Cleavage of Aminoglycosidic Linkages by Deamination

Deamination coupled with simultaneous cleavage of the glycos-aminoglycan linkage is a highly stereospecific reaction and involves

the migration of an atom that is *trans* and coplannar to the nitrogen atom of the derived diazonium ion when the sugar is in its favoured configuration[141]. The key reaction, termed a 1→2 hydride shift, results from the attack on the intermediate diazonuim ion by the ring oxygen atom which is *trans* and antiparallel in disposition[142]. The product is a 2,5-anhydro residue. Alternative ring contractions are brought about because the same geometrical requirements are met by C-4 and, as a result, 2-C-formyl pentofuranosides are produced as a major side product (*Ca.* 25%)[61]. This side reaction also eliminates 3-O substituents when present[61].

Two common aminosugar components of glycans, 2-amino-2-deoxy- glucose and -galactose undergo deamination and yield (predominantly) 2,5-anhydro-D-mannose (chitose)[141] and 2,5-anhydro-D-talose terminated fragments respectively[61,105,141]. Analysis of these products provides information, including the position of attachment of the amino sugar. Moreover, products with a 3-O linked 2,5-anhydro hexose terminus are susceptible to a base catalysed β-elimination and this may provide a additional tool for characterising the molecule more fully.

Any N-acetyl groups present must be removed prior to deamination and this is usually accomplished by trifluoroacetolysis[143] or under basic conditions using:

a) aqueous sodium or barium hydroxide[105],
b) hydrazine in an anhydrous solvent[144]; or,
c) sodium hydroxide in aqueous methyl sulphoxide with sodium thiophenolate added to act as an oxygen scavenger and catalyst[145].

Deamination with nitrous acid in water or a water containing solvent has been used in the structural elucidation of carbohydrates with an amino sugar. Furthermore, there is scope for the reaction to be performed with alkyl nitrite in a non-aqueous solvent and also applying N-nitrosamide thermolysis, or treating triazenes with acid[141].

6.6 Lithium in Ethylenediamine Degradation of Glycosyluronates

The specific degradation of glycosyluronic acid residues in a polysaccharide by a powerful reducing agent, lithium in ethylenediamine, was reported by Mort and Bauer[140]. Hence, carbohydrates are cleaved by lithium at a uronic acid, irrespective of its linkage pattern. Furthermore, neutral glycosyl residues in the glycan are largely stable to the reaction conditions and, with the exception of the sugar attached to the glycosyluronic acid residue, which is reduced to an alditol, they are left intact. Thus, lithium/ethylenediamine is a general procedure for the selective fragmentation of underivatised polysaccharides, with uronic acid residues in the main chain, into oligosaccharides. Methylation analysis of these products, in conjunction with the methylation data from the polysaccharide, will identify the sugars that are adjacent to the uronic acid and the linkage position of the uronic acid. Consequently the sequence of these three residues will be evident. Additional information can often be obtained from an nmr study of these oligomers.

The drawback of this degradative technique is that there is also concurrent reduction of aldoses and cleavage of any methyl ethers or pyruvate ketals attached to the residues. Consequently, structural

information may be lost. We have also found this procedure to be low yielding.

The lithium/ethylenediamine degradation was applied in the determination of the primary structure of *E. coli* K39 capsular polysaccharide[146].

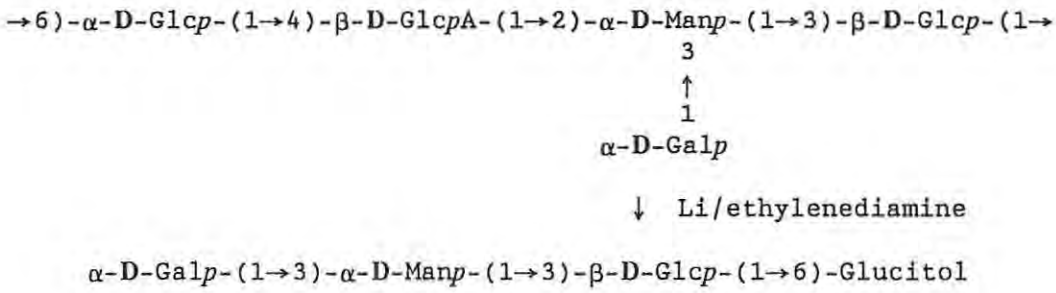


Figure 15 The selective degradation of *E.coli* K39 capsular polysaccharide by lithium in ethylenediamine[146].

Methylation analysis of this oligoalditol helped determine the overall sequence of the polysaccharide repeat unit.

7 GAS LIQUID CHROMATOGRAPHY

This is a technique where compounds are volatilised and partitioned between a moving (inert) gas phase and a liquid phase held on a support. The partition ratio, known as the distribution co-efficient, K_D, is determined only by the nature of the solute, liquid phase and the temperature[147]. This value is independant of the column type[147] and is described by the general expression:

$$K_D = \frac{\text{concentration in liquid phase}}{\text{concentration in the gas phase}},$$

therefore, those compounds with a large K_D will be more compatible with the liquid phase and spend a correspondingly longer time held stationary than a solute with a low distribution co-efficient. The net result is that the partition ratio can be directly related to the retention time of a peak and the components are eluted progressively with the increasing affinity for the liquid.

Open tubular columns, which are essentially capillary tubing coated with a liquid phase, were introduced by Golay in 1977 as an alternative to packed columns[148]. They demonstrated a dramatic increase in separatory power and are the basis of "High Resolution" gas liquid chromatography (glc). These columns differ from the conventional packed columns because they have a very small amount of stationary phase (which gives a more efficient separation) and they are more permeable to the carrier gas. This "openess" allows a longer column length (30 m *vs* 1.5 m for packed columns) which gives a larger number of theoretical plates[148]. There are several types of capillary columns:

a) those with the liquid phase deposited directly onto the capillary surface are referred to as "Wall Coated Open Tubular" (WCOT) columns and these are the most common type;

b) where the coating is deposited on a surface extended by macro elongated crystal deposits, they are known as "Porous Layer Open Tubular" (PLOT); and

c) the "Support Coated Open Tubular" (SCOT) variety where the wall is treated with a mixture of solid support and liquid phase[149]. In general, open tubular columns are only used analytically because the sample size is limited to 1 μg or less[150].

The ability of the liquid phase to effect separation and its stability to the operating conditions are prime considerations when choosing a column for a particular application. The coating can be either non-polar or polar; the non-polar are most efficient in separating solutes of differing carbon number, while compounds that fall in a narrow and low molecular weight range (e.g. a mixture of monosaccharides) are better resolved on a polar phase. Some liquid phases, like the chiral stationary phases used to separate enantiomers[75], have highly specific applications. Liquids that have found application in separating mixtures of various sugar derivatives are ECNSS-M, OV-225, OV-17, OV-1 and apiezon greases[151].

The bulk of our glc work is on a bonded, fused silica OV-225 coated column, as this, in most cases, will successfully separate volatile derivatives of monosaccharides, octylglycosides, permethylated anhydroalditols and permethylated alditols. It is also more stable, under the operating conditions that we use, than some of the other

commonly available phases, for example, ECNSS-M[150]. A column coated with OV-17 is occasionally used in preference to one with OV-225, as the elution profile for some derivatives is enhanced (for example, derivatives of amino sugars) and it has superior quantitative determination properties[151]. Moreover, some alditol acetate derivatives of permethylated sugars co-elute when using a particular stationary phase. For example, the 3,4,6- and 2,4,6-tri-O-methyl hexose alditol acetates, which cannot be resolved on OV-225, are separated on OV-17. The reverse is true of 2,3,6- and 2,3,4 tri-O-methyl hexose derivatives. Adequate resolution of all the different carbohydrate derivatives is feasible[151] when using both OV-17 and OV-225 coated columns.

Open tubular columns are characterised by low flow rates (0.5 to 10 ml/min), reduced sample capacity due to the small amount of stationary phase and lastly, fast peak profiles[152] and these factors dictate the instrumental design. The most important feature is a capillary inlet splitter, which reduces the amount of sample being introduced onto the column and prevents overloading. This divides the sample into two unequal portions, the smaller of which goes onto the column. The split ratio ranges from 50:1 to 500:1, the latter is used for a high resolution WCOT column[152].

Components to be separated by glc are usually derivatised to make them more volatile and improve their resolution. They are injected into a heated chamber, volatilisation occurs and the solutes are carried by the gas stream (N_2, He or H_2) along the column which is uniformly maintained at a temperature which will keep all the constituents volatile and afford a suitable partition ratio[151]. The column effluent is monitored with an appropriate detector and in carbohydrate analysis, flame ionisation is usually the detection method of choice

for routine analytical quantifications[153]. The output from the detector is processed by either a chart recorder or an integrator combined with a recorder, to produce a chromatogram.

Compounds are identified by comparing their retention time (ie the time taken to travel the length of the column) with the retention time of a standard or an authentic compound under the same conditions. Furthermore, the effluent can also be fed directly into a mass spectrometer to give a mass spectrum, which allows the eluting compounds to be identified from their fragmentation patterns.

Once identified, compounds can be quantified from the detector response by determining the individual molar response factor (mrf) for each component of the sample, when analysed by the detector. However, in most cases, where accuracy is not paramount and the constituents are of similar composition, the response can be considered to be equal to the weight response. The theoretical response can also be calculated from the effective carbon response[154] (ecr) of the sugar. This theory assumes that each carbon type (carboxyl, carbonyl, etc) contribute to the same extent in all molecules, regardless of the overall structure. The total response, therefore, will be the summation of all the carbon responses[154]. This is a poor substitute, however, and when accuracy is paramount the mrf of each solute in the sample should be determined[155].

In order to be effective, carbohydrate derivatives must be volatile, stable at the operating temperature of the instrument and should not absorb to the column. Those that meet these requirements include: the trimethyl-silyl and silyl ethers[155], O-methyl oxime acetates[67], aldononitrile acetates[66], acetates[156] and alditol acetates[64]. However, some of these derivatives are not as useful as others. For

instance, the trimethyl-silyl ether derivatives produce up to five derivatives (acylic, α-and β-furan, and α and β-pyran forms) for each monosaccharide and this complicates the quantitative and qualitative analysis of a sample. Others, which produce a single acyclic derivative may afford identical structures from aldo- and keto-hexose sugars, and thus, it is impossible to positively identify the starting material[79]. However, these problems are avoided if the compounds to be separated are anhydroalditols, such as the permethylated products from a reductive cleavage. We have found that, for our purposes, peracetylated aldononitriles or O-methyl oximes[67] are suitable derivatives for resolving mixtures of non-methylated sugars by glc. Permethylated sugar residues are well resolved on OV-225, as alditol acetates.

Uronic acid residues are usually carboxyl reduced to give the neutral sugar, or the COOH group is esterified to either a carboxymethyl or carboxyethyl, before being separated by glc. This is because there is no entirely satisfactory derivative for a carboxyl containing sugar. The reductive cleavage reaction is particularly useful method of cleaving the interglycosidic bonds of a (methylated) glycosyluronate because (unlike acid hydrolysis) it leaves the carboxymethyl groups intact and the uronic acid residues, as per methylated anhydroalditol acetates, can be separated by glc. Whereas a conventional "methylation analysis" usually requires the reduction of the carboxymethyl groups in the permethylated glycan, prior to hydrolysis, if the linkage pattern of a uronic acid is to be identified by glc analysis, for instance, as the alditol acetate derivative.

Amino sugar derivatives give broad peaks, long retention times, multiple peaks and are poorly resolved when chromatographed. They are often thermolabile which makes quantification difficult. Wong *et al*

have discussed the derivatization and analysis of amino sugars by glc and glc-ms[157]. During the study of the amino sugar containing capsular antigen from *E. coli* K57 (this thesis) we found that both the alditol acetate and acetate derivatives of permethylated glucosamine, gave sufficient resolution (on OV-17) to allow the identification of its linkage pattern. The mass spectrum of several partially methylated N-acetyl hexosamine acetates has been reported[158].

Gas-liquid chromatography, despite its efficacy in certain circumstances, has limited scope because of the inherent restrictions of the method. In particular, the components of the mixture being separated must be stable and volatile under the operating conditions and to achieve this they generally have to be derivatised. In structural studies of carbohydrates, glc is routinely used in the analysis of sugar mixtures, absolute configuration assignments, and, more importantly, for the analysis of partially methylated sugar residues.

8 MASS SPECTROMETRY

Mass spectra are plots of relative intensities of gaseous ions formed by ionisation and subsequent fragmentation of gaseous molecules, against their mass to charge ratio ($^{M}/_{z}$); these fragments are separated in either a magnetic or an electric field. A good correlation exists between the structure of organic molecules and their mass spectrum and traditionally mass spectrometry (ms), either alone or coupled to glc, has been a valuable technique for structure analysis. It can be used to determine: whether the sugar has a pyranose, furanose or acyclic structure; the substitution pattern of partially methylated sugar residues; the sequence of monosaccharides in small ($<10^3$) oligosaccharides and any residues to which a non-carbohydrate substituent may be attached; whether the residue is an aldose or ketose; and the position of amino or methylene groups on a sugar. Mass spectrometry is not generally able to identify stereochemical differences between compounds, therefore epimers like glucose, galactose, mannose etc cannot be distinguished. However, it has been reported that the technique of negative-ion laser desorption ionisation-Fourier transform-ion cyclotron resonance-mass spectrometry (l.d.i.-F.t.-i.c.r.-m.s.) and mass analysed ion kinetic energy (m.i.k.e.s.) mass spectrometry can identify the stereochemistry of the sugar anomeric carbon centers[159].

The requirement that a compound must be volatilised (at a temperature and pressure at which it is stable) has, to some extent limited the application of ms to the analysis of polysaccharides. When they are heated to the point that they vapourise they usually undergo extensive pyrolysis and the spectra show only the low molecular mass fragments. However, advanced mass spectrometry techniques like atom

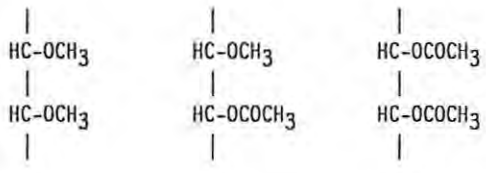
liquid sputtering ionisation and high mass high performance mass spectrometry, have the potential to be used for routine analysis of high molecular weight, intact, complex carbohydrates in the future[160]. At present ms is only effective in polysaccharide structural analysis of very small polysaccharides or if it is combined with a specific degradation technique which will produce a low molecular weight polymer or an oligomer.

The method of ionisation has considerable influence over the spectrum produced in a ms analysis. The conventional method, electron impact ionisation (ei), deposits an excess amount of energy with the compound. The resultant ei spectrum is often very complex with a preponderance of low molecular mass fragments that give little indication of the molecular weight and other structural details. This drawback can be overcome by desorptive ionisation techniques that allow direct sample ionisation from a condensed phase, for example fast atom bombardment (fab), or a "soft" ionisation method, like chemical ionisation (ci) which imparts a limited amount of energy to the sample. They provide usable spectra for higher polarity and higher molecular weight compounds than ei-ms. The data is also more useful for sequencing because of the molecular-ion and partial sequence fragments that are detected.

8.1 Electron Impact - Mass Spectrometry

This involves ionisation and fragmentation of compounds by bombarding them with high energy electrons (5-70 ev), fission then occurs along specified pathways[161] to form ions which are measured on the basis of their mass to charge ratio $M/_z$; the charge is usually one. The

fragmentation of most sugars, as common sugar derivatives, has been extensively studied by ei-ms and the results reported[eg 161-163]. It is also possible to predict the major peaks in the mass spectrum of a novel sugar because the fragmentation pattern is affected by the substructures present. Take, for example[163], alditol acetate sugar derivatives, where fission occurs preferentially between the carbon atoms in partial structures 1 and 2 in preference to partial structure 3.



1 2 3

Fission also occurs more frequently between the carbons in partial structure 1 than 2[163]. Ei-ms gives its best results when performed on monosaccharides which are derivatised rather than unmodified sugars and oligosaccharides, which are usually thermolabile and non-volatile.

8.2 Chemical Ionisation-Mass Spectrometry

Chemical ionisation (ci) maximises the molecular and high molecular mass information by replacing the highly exothermic process of ei with a chemical process involving ion-molecule interactions. A reagent gas (usually methane, isobutane, or ammonia) is ionised by ei to produce a reacting species, eg CH_5^+, $C_4H_9^+$, or NH_4^+, which ionise the sample material by one of three processes[164].

a) **Charge exchange**, which can only occur when using an aprotic reagent gas like methane,

b) **Proton transfer**, where the reagent gas behaves as a Brønstead acid,

c) **Collision-stabilised adduct ion formation**, where the ionised reagent gas behaves like a Lewis acid.

The fragmentation pattern of a compound, after ci, depends upon its structure, proton affinities and the reagent gas pressure[164]. The higher the pressure, the greater the tendency to produce high mass fragments[164].

Ci-ms still requires that the sample be introduced into the ion-chamber as a gas. Therefore carbohydrates must be derivatised in a manner that will make them sufficiently volatile and avoid pyrolytic decomposition. The ci-ms spectra of several carbohydrate derivatives (eg acetylated[165-168], methylated[167,169] and trimethylsilylated[170] has been described.

The reacting species have different protonating abilities and this decreases in the order $CH_5^+ > C_4H_9^+ > NH_4^+$. Thus, fragment peaks are more abundant when using either methane or isobutane as the reagent gas[164]. Ammonia ci-ms tends to produce spectra that are dominated by ammonia clusters and molecular weight ion peaks $[M+NH_4]^+$ which give information on the molecular weight of compound. Ammonia ci-ms also produces sequence specific fragment ions and these make it fairly easy to determine the sugar sequence. It is, therefore, a useful technique for determining an oligosaccharides size and its monosaccharide sequence.

8.2.1 Direct Chemical Ionisation-Mass Spectrometry (dci-ms)

This modification to the ci technique involves the placement of the sample on an extended probe (the emitter) within the ionisation chamber. Programmed heating of the emitter (an electrically resistant wire) "distills" the material into the ionised reagent gas where ionisation takes place. Pyrolysis is minimised and therefore more structural (and sequencing) information can be obtained, even from larger molecular weight[164] (up to 3000 daltons) and more polar samples. Spectra from these compounds, when examined by some of the other ms methods, exhibit a preponderance of pyrolytic fragments so they are usually less informative. Polar samples, however, should be derivatised for the best dci-ms results and permethylation appears to be the most satisfactory[164].

Mass spectra obtained under dci conditions are distinct from those recorded for either ei- or ci-ms. There are abundant $[M+H]^+$ ions (generated by protonation of the compound M) or the ammonia adduct $[M+NH_4]^+$ ion. Sequence specific fragments are characteristic for permethylated saccharides and, thus, the technique appears to enhance sequence information. Furthermore, compared with fab-ms, dci-ms shows greater sensitivity, more specific sequence fragmentation and a better signal to noise ratio when analysing molecules below 3000 Da[164].

8.3 Fast Atom Bombardment-Mass Spectrometry

This technique can be used to measure the precise molecular weight for oligosaccharides and small polysaccharides and the sequence information (as in dci-us) can be used for structure elucidation. Fab-ms has been used, for instance, in the study of oligosaccharides with between three and nine monomers[171] and in the characterisation of

the mycobacterial O-methyl-D-glucose polysaccharide (MGP), its acetyl derivative[172] as well as O-methyl-D-glucose lipopolysaccharide (MGLP).

Samples, on either a stainless steel or copper target, are bombarded with xenon or argon atoms with approximately 8 KeV of energy. If adduct ions are required, NaCl or NH_4Cl is added to the condensed sample to produce Na^+ or NH_4^+ adducts, respectively. Positive and negative ions are both released when a compound is fast atom bombarded and these are recorded separately. The positive and negative spectra contain different structural information. Spectra recorded in the positive mode, for example, have peaks for the protonated, cationised (Na^+ or K^+) molecular species and positive fragment ions. The negative mode has a peak for the molecular anion derived from charged or acylated compounds. Fab-ms, because it is applicable to larger and more polar saccharides, is a useful tool for determining the sequence and structure of oligosaccharides and small polysaccharides.

9 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

This instrumental analytical technique (known by the acronym nmr) is a powerful, non-destructive tool for obtaining detailed structural information on poly- and oligo-saccharides. It can be used, without the assistance of other methods, to fully elucidate the structure of carbohydrates and other compounds. Moreover, the material can be recovered unaltered, which makes nmr a particularly useful analytical method for a sample that is available in a limited quantity. This is often the case with some saccharides, for instance, those derived from glycoproteins. This field has been extensively reviewed and, for example, in the period 1986 to mid 1988, over 500 books have been published on nmr and its applications[173]. A number of these text and review articles apply specifically to the nmr of carbohydrates in general, and polysaccharides and oligosaccharides in particular[eg 100,174-178]. The first review of the application of nmr to carbohydrates was in 1964[179].

Bacterial capsular polysaccharides are particularly vulnerable to nmr analysis because they generally consist of a regular repeat oligomer comprised of between two and seven well defined chemical units (monosaccharides). Each residue type, except for fructose, ketodeoxyoctonic and N-acetylneuraminic acid, consists of protons linearly spaced along the carbon skeleton of the residue. Hence, there is a proton at each optically active ring carbon and these readily accessible probes act as "reporter groups", revealing large numbers of datum on the structure and stereochemistry of the compound. The sugars, except for the three mentioned previously, have an anomeric proton that resonates in a characteristic region (the so-called "anomeric region" between about 4.2 and 5.5 ppm) which is well

separated from the other proton signals. This resonance provides a convenient starting point when following the "connectivity map" provided by the two dimensional proton J correlation techniques. One proton leads to the next along the chain, revealing the stereochemistry and constitution of each carbon and, as each residue has a characteristic J connectivity and chemical shift pattern, its identity can be determined from these data[180]. After a sugar is recognised, its anomeric stereochemistry is determined by consideration of the of the H-1 ^{3}J coupling constant and shift value. The anomeric proton is also in close proximity to the linkage site proton of the sugar to which it is linked and intra-residue interactions between the two can be identified by one of the experiments (eg nuclear Overhauser enhancement correlation spectroscopy - NOESY[181]) which correlate signals on the basis of their dipolar couplings. Hence, bacterial polysaccharides and their derived oligosaccharides, consist of a continuously coupled series of proton "reporter groups" from one end of the repeat unit to the other, making their structure readily accessible to nmr analysis.

Nmr spectra can be fully described by five sets of parameters[177], namely:

1 An **Integral**, which is proportional to the number of nuclei resonating at a particular frequency and is determined from the area under the signal. It is usually quoted as a ratio to the area of the other signals.

2 The **Chemical Shift**, defined with respect to the arbitrarily selected standard (tetramethyl silane (TMS)), and it is usually quoted in the dimensionless units of parts per million (ppm) on

the δ scale where:

$$\delta = \frac{\text{frequency separation (Hz) between the resonance and TMS} \times 10^6}{\text{spectrometer frequency (Hz)}}$$

This parameter is influenced by the chemical and electronic environment that the nucleus experiences.

3 The **Spin-Coupling Constant**, which can be determined from the splitting of the resonances caused by the interactions between spin systems, this value (in Hz) is independant of the operational frequency of the spectrometer.

4 **Spin-lattice Relaxation (T_1) Value**, the reciprocal of the rate at which the absorbed energy is transferred from a spin system to its surrounding environment (the lattice). It has been shown[182,183] that the proton T_1 value for mono-, oligo- and poly-saccharides will provide another method of evaluating the conformation of a molecule; the T_1 value for an anomeric proton has been used as a measure of the interatomic distance between H-1 and the proton on the respective linkage carbon, thus, the relative orientation of a glycosidic linkage can be determined from this parameter[100].

5 The **Spin-Spin Relaxation (T_2) Time**, a constant for the loss of phase coherence of the resonating nuclei.

There have been significant advances in nmr spectroscopy in the past two decades which have made it simpler to extract these parameters from a spectrum; the improved presentation has also made these data easier to interpret. The greatest progress, from a carbohydrate chemistry point of view, has been in resolving the "hidden resonance"

problem experienced with oligosaccharides and polysaccharides. The introduction of superconducting solenoids (which provide stronger homogeneous magnetic fields than the electromagnets used previously) allowed an increase in the operational frequency of spectrometers used for research from below 100 MHz to over 500 MHz (proton frequency). This results in a considerable increase in sensitivity and signals are separated by a larger frequency difference, whilst the coupling constant value remains invariant. Therefore, spectra are better resolved when aquired using a spectrometer with a strong magnet. The upper limit of strength for magnets giving a homogeneous field, has almost been reached and there is unlikely to be further significant advances in nmr from this area. Improvements in instrumentation are now focussed on other aspects, for example, producing instruments that can produce small pulses (eg 5°) instead of the standard 90° incremented pulses; this will give more flexibility and allow new pulse sequences to be tried.

The introduction of pulsed Fourier transform (pulsed FT) nmr as a replacement for the slower continuous wave (cw) methods also had a major impact on this analytical method. This technique involves the simultaneous excitation of all the spin systems, and the response is measured in the time domain as a free induction decay (fid). This data is manipulated mathematically to produce the conventional frequency spectrum. There are several advantages to pulsed FT nmr. One of these is the shortened experiment time which makes signal averaging a viable option which, amongst other advantages, allows insensitive nuclei (like ^{13}C and ^{15}N) to be used as the probe on a routine basis. Furthermore, the spin excitation and signal detection parts of the pulsed experiment are separated, which allows the excitation to be carried out in a manner that will produce a fid containing only selected parameters. Hence, this method can be used

to produce a simplified spectrum. In this type of experiment, termed "multiple pulse", the excitation pulse is replaced by a sequence of two or more pulses, separated by a fixed delay of a few milliseconds. If the sequence delay is systematically varied, the experiment yields a set of fid signals which, subjected to a procedure involving two Fourier transforms, will produce a spectrum with two frequency dimensions. This is termed a 2D spectrum. There are over 100 pulse sequences at present and some of these have made it feasible to measure the less accessible parameters, like T_1 values[182], on a routine basis.

Nmr has also evolved in such a way that is is no longer mandatory that a sample be in solution to record a good quality spectrum. Spectrometers capable of cross polarization (CP) and magic angle spinning (MAS), for instance, have made the measurement of spectra from solid samples possible, thereby, ensuring the true physical and chemical characteristics of a solid carbohydrate are preserved. It is also useful for polymers that have limited solvent solubility which will restrict the amount of sample that can be put into solution. The technique of ^{13}C CP/MAS nmr has been used to study the variation in chemical shift with respect to changes in the conformation of the glycosidic linkage of (1→4)-D-glucans, in a solid state[184].

Developing areas of nmr spectroscopy are the zero and multiple quantum coherence methods and the three (frequency) dimensional nmr experiments which are a combination of two 2D pulse sequences[173]. They should simplify spectral analysis for very complex molecules and provide information that cannot be obtained from other experiments. In particular zero quantum coherence has a vast potential to be developed, because it is independant of magnetic field inhomogeneity and therefore, it can be used with stronger magnets, which do not give

good magnetic field homogeneity[173]. Unfortunately, at present, no single nmr experiment allows all the parameters to be accurately determined simultaneously and, as a result, it is necessary to choose several pulsing programs that will give the required data.

Structural assignments are made for compounds on the basis of the measured parameters. These are interpreted by comparing and contrasting the data with results from other, well characterised compounds. Many data have accumulated in the literature which enable the identification of residues, their conformation and position of linkage as well as substituent locations. However, difficulties are sometimes encountered when using published values and the major one is the large number of chemicals that are used as a standard to reference the nmr signals. For instance, external carbon disulphide, internal (ie mixed with the sample) 3-(trimethylsilyl)-propionate-d_4 (TSP), sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS) and external TMS have all been used as standards[185]. These result in differing values being reported for the chemical shift of a particular nuclei. It appears, however, that in nmr analysis of carbohydrates, that acetone (internal) is the standard of choice, because it can be easily removed from the sample after the spectrum is acquired. Unfortunately, although the accepted value for acetone is ^{1}H-2.225 (rounded off to 2.23 ppm) and ^{13}C-31.07 ppm, several researchers have reported data referenced from acetone at values of 2.12[186], 2.17[37] and 2.22 ppm[36].

Furthermore, the chemical shift of acetone is influenced by the temperature, concentration and possibly the nature of the sample[100] and therefore, there is still a need for a consistent, universally accepted basis for referencing chemical shifts of samples in aqueous solutions. The chemical shifts and coupling constants of carbohydrates are affected by a solvent change[187-189] so data from

samples run in a different solvent or referenced with another standard should be used with caution.

Nmr studies of carbohydrates generally utilise either the ^{1}H or ^{13}C nuclei as probes. The choice of experiment (pulse sequence) depends on several factors, for instance, the information required, amount of sample available, viscosity and the maximum concentration possible, the sophistication of the instrument, and the available spectrometer time. Spectra are presented as either a **1D** (1 frequency dimension) or **2D** (2 frequency dimensions) plot and furnish information on the total sugar ratio, stereochemical aspects, sugar and chain conformation, nature and location of any non-carbohydrate substituent groups, as well as the sugar linkage pattern and sequence.

9.1 The Single Pulse Experiments

Sequences involving a single excitation pulse generate spectra containing the same information as a conventional ^{1}H or ^{13}C cw spectrum and are less time consuming to acquire than a multiple pulse experiment.

9.1.1 Proton Magnetic Resonance Spectroscopy

The vital step in any nmr study of a carbohydrate sample is obtaining an integrated (**1D**) proton resonance spectrum between 0 and 10 ppm; commonly this is obtained by pulsed FT nmr. The ^{1}H spectrum shows the purity and actual concentration of the carbohydrate sample, as well as the appropriateness of the run temperature for the experiment. The structural complexity will be evident, thus, once the ^{1}H spectrum has been analysed (and all its accessible information extracted) it

provides the basis on which an experimental strategy for elucidating the structure can be formulated.

The bulk of the usable information (ie the type and number of monosaccharides, their anomeric configuration, and the proportion of non-carbohydrate substituents) from the proton spectrum is derived from signals that fall outside the envelope (approximately 3.5 to 4.2 ppm) of ring proton resonances. Mostly, these are H-1 proton signals which resonate in the anomeric region of the proton spectrum. Each sugar type (eg β-galactopyranose) has a anomeric resonance with a unique chemical shift (δ) and a spin-spin coupling constant (^3J) which is characteristic of the residue type and its anomeric geometry[100] (ie the size of the dihedral angle (θ) between H-1 and H-2). The H-1 furanosyl peak occurs downfield of the signal from the pyranose form of a sugar[190]. Hence, the size and composition of the repeat unit can be determined from the δ/J data and the integral for the H-1 signal. Mannopyranose residues, however, are problematical because of the degeneration of the coupling constant for both the α and β anomeric configurations[180]. Furthermore, both have a chemical shift around 5 ppm; a signal in the region upfield of δ 5 is taken as being diagnostic of the β configuration, whilst those in the area downfield are α. The anomeric stereochemistry of *manno* residues is usually determined from the $^1J_{CH}$ coupling constant of the resonance from the C-1 nucleus in the ^{13}C-proton coupled spectra. Sialic acids and fructose, which lack an anomeric proton, have their anomeric configuration assigned from the data from the H-3 proton resonance[191].

The anomeric region is not always free of non-anomeric resonances and, thus, care should be taken to avoid confusing the two. In particular, the H-4 and H-5 proton signals from α-galacturonic acid often intrude into this area[77].

Substituents, eg pyruvate, O- and N-acetyl groups have signals that can be readily identified by their chemical shift (upfield of the ring proton resonances) and integration of these peaks, with respect to the anomeric proton signals, will determine the degree to which the repeat unit is substituted. The R or S configuration for the pyruvate ketal can be established from its shift value[192].

For reasons mentioned previously, protons act as excellent "reporter groups" for determining the structure of an unknown polysaccharide. However, if nmr is to be used (to the exclusion of all other analytical methods) for a structural study of a compound, all the ^{1}H resonances have to be assigned to the proton from which they originate. This is usually impossible to do using only a 1D proton spectrum, partly because polysaccharide solutions have high viscosity; this is also compounded by the fact that they are only soluble in viscous aqueous solvents[193]. The high viscosity limits the amount of material that can be put into solution and ultimately into the nmr tube, therefore the sample is weaker, and more importantly the viscosity results in a small T_2 value and a rapidly decaying fid which, in turn, causes broad spectral lines. The quality of the spectrum suffers as a result. Line broadening can be partially overcome by either depolymerisation or running the spectra at an elevated temperature, thereby lowering the viscosity of the solution. Nevertheless, the signal broadening is still significant and coupled with the relative insensitivity of the ^{1}H nucleus to changes in its stereochemical and chemical environment, this means that resonances from H-2 to H-6' are usually incompletely resolved in the proton spectrum, even at 600 MHz with resolution enhancement (a form of data manipulation designed to give sharper, better resolved, peaks[193]. This is termed a "hidden resonance" problem and can also affect some of the more complex oligosaccharides.

Another problem affecting proton spectra of carbohydrates run in aqueous solution is a large signal (with spinning sidebands) from the exchangeable protons (O-**H**, N-**H**) in the sample. This can obscure resonances and interfere with their integration. The signal, known as either the HOD or the water peak, can be reduced, but not removed, by several exchanges of protons with deuterons from D_2O and by running the sample dissolved in good quality deuterium oxide (ie 99.995% deuterated). Furthermore, the water peak can be shifted to higher field by raising the temperature and this may be a viable strategy for uncovering obscured signals. Nonetheless, even at high temperature, a prominent HOD makes it difficult to detect weak resonances from the solute (especially when there is less than 1mM of sample) because of the limited dynamic range of the analogue-to-digital converter used in pulsed FT nmr[173] to digitise the signal. Hence, to avoid clipping the fid, the receiver gain has to be set so that the signal stays within this range. At a low setting, which is required when the HOD peak is large, the resonances are attenuated.

A number of instrumental techniques have evolved to suppress or remove the solvent resonance peak and, although they are strictly multiple pulse sequences they have been included here because, in effect, they still produce a conventional 1D proton spectrum. A widely used technique is the "binomial solvent suppression" pulse sequence which is designed to excite the spectral region of interest but not the solvent protons; unfortunately it also suppresses some resonance peaks and gives non-uniform excitation of the spectrum[98,173]. The WEFT water suppression[98], with a time delay short enough to null the HOD, can be used for polysaccharide solutions. It is referred to as either a "saturation decoupling" or a "selective relaxation" technique and takes advantage of the difference in the relaxation rate of the solvent (H_2O >2 s) and the polysaccharide protons (<0.5 s). Methods

have also been described[173] for the measurement of spectra in aqueous solution: (a) under spin locked conditions (the so called HOHAHA experiment) with elimination of the water peak by a "lock and return" solvent suppression[194] and (b) by the WATR[195] experiment (Water Attenuation by T_2 Relaxation).

There is a derivatisation approach to avoiding the HOD signal problem which also improves the quality of the spectrum. If the hydroxyl groups are modified (eg converted to O-deuteriomethyl groups) the physiochemical properties of the polymer are dramatically altered, and the sample becomes soluble in a different range of solvents. One can be selected with a resonance peak or peaks, out of the region of interest. For example, deuteriochloroform is a good (non-viscous[193]) solvent with a signal (from the chloroform impurity) at ^{1}H-7.24 ppm. Methylated polysaccharides dissolve to give concentrated, non-viscous solutions which give a better quality ^{1}H spectrum than the polysaccharide in D_2O and, thus, the signals are easier to observe. This approach has been adopted with several polysaccharide samples[eg 196,197] which gave poor ^{1}H spectra when run in D_2O. The major disadvantage is that independant analyses have to be performed on the unmodified polymer to eliminate the possibility that any undesirable structural alteration occurred during the derivatisation procedure. Further, derivatisation usually leads to the loss of attached substituents, for example, O-acetyl groups. Consequently structural information could be lost.

The diagnostic potential of ^{1}H proton magnetic resonance spectroscopy for the analysis of carbohydrates was described[198] by Lenz *et al* in 1961, since then Choy *et al*[199] (1972) and later Bebault *et al*[200] (1973) described the effectiveness of applying ^{1}H nmr spectroscopy to the analysis of bacterial polysaccharides. The method, it was stated,

could be used to determine the number and nature of residues in the repeat unit, their anomeric configuration and the proportion of substituent groups like pyruvate and O-acetyl . It is indicative of its importance that, even after 28 years and the introduction of numerous other nmr experiments, it is still used for these purposes, in preference to more sophisticated sequences.

9.1.2 Carbon-13 Nuclear Magnetic Resonance Spectroscopy

Bacterial polysaccharides and derived oligosaccharides can contain several types of atoms (ie ^{1}H, ^{13}C, ^{15}N and ^{31}P) which can be employed as a structural probe in nmr spectroscopy, but, only carbon-13 and protons are used routinely. Compared with ^{1}H spectra there is a dramatic decrease in the sensitivity when observing ^{13}C. The first reason for this is the natural abundance of carbon-13 is 1.1%, so only a small fraction of the carbons in a sample produce a response. The second is the inherent sensitivity of a particular nucleus depends upon the cube of its magnetogyric ratio (γ) and this value is much lower for a ^{13}C than for a ^{1}H nucleus. This ratio is important because the receiver signal depends upon the magnetic moment, the rate of change in the flux in the coil (ω), and the population difference across the energy transitions, which are all proportional to γ^{201}. If a proton is given the arbitrary value of 1 for its sensitivity, then the relative sensitivity for a ^{13}C spin system is 1.59×10^{-2} (ie $(\gamma^{13}C/\gamma^{1}H)^{3}$). The signal is too weak to measure (meaningfully) by cw instruments[202] and it was only the advent of FT nmr, with its rapid repetition rate and signal averaging that less sensitive nuclei, like carbon-13, could be observed on a routine basis.

The time penalty incurred is severe when observing ^{13}C nuclei, especially if a good signal-to-noise ratio is required. This is

further exacerbated by the long ^{13}C spin-lattice relaxation times, which makes a slow pulse repetition rate essential or signals will be lost. A delay of at least 5 s between pulses is necessary. These factors, coupled with the constraint on the sample size imposed by the limited aqueous solubility of high molecular weight polymers, mean long experiment times (>16 h) to obtain a decent spectrum of a polysaccharide. The overall accumulation time can be reduced, to some degree, by using 10 mm diameter nmr tubes instead of the 5 mm tubes used for routine ^{1}H spectra. These allow four times as many nuclei in the coil for a solution of the same concentration.

These drawbacks are fortunately compensated by the enhanced sensitivity (30X) of the ^{13}C nucleus to changes in its stereochemical or chemical environment, compared with an equivalent proton. Similarly, line broadening by the viscosity effect and water peaks do not interfere with the spectra. Therefore, provided there is sufficient data accumulated, these spectra have well dispersed, sharp resonance peaks and even complex spectra can be resolved at low frequency (eg 100 MHz).

Carbon-13 nmr spectra can be obtained in several ways, each of which gives rise to a particular type of spectrum. The most widely used method is "broadband spin decoupling" (irradiation at the ^{1}H frequency) to remove ^{1}H-^{13}C spin-spin couplings and this produces a spectrum with a single sharp resonance peak for each carbon type. This is because, at natural abundance levels ^{13}C-^{13}C couplings are insignificant. Decoupling also improves the signal strength by virtue of the nuclear Overhauser effect[98] (nOe). Spectra recorded without decoupling have multiplets for 1°, 2° and 3° carbon atoms and they are further complicated by long range couplings which cause signal broadening and signal splitting. These spectra also take

longer to accumulate because each resonance is split into several smaller components and there is no nOe enhancement. Moreover, coupled spectra, even for simple compounds, are complicated as a result of the overlap of multiplets for carbon resonances whose chemical shift are in the same region of the spectrum, because of the large ($^{1}J_{CH}$) coupling constants (approximately 150 to 200 Hz for carbohydrate ^{13}C signals[100]). However, these negative aspects are offset by the increase in information that can be derived from the coupling data. "Single frequency off resonance decoupling"[203] can be used to accumulate a spectrum with the fine detail of a fully coupled ^{13}C spectrum, but reduces the size of the coupling constants and removes long range couplings. It, therefore, should be easier to interpret. Nevertheless it takes between three and five times as long to record, compared to a broad-band decoupled experiment, and assignments can be ambiguous or even impossible where there is severe overlap of adjacent multiplets or coupled patterns result from strong homonuclear proton couplings[204].

The only parameter that can be determined from ^{13}C proton nucleus decoupled spectra are the chemical shifts, because the integral of a ^{13}C signal is unreliable. The shift value is characteristic of the nuclear environment with resonances, from those carbons in the individual groups that make up the molecule, falling in well defined regions of the spectrum. There is an anomeric region, as in the proton spectrum, and this is between 98 and 106 ppm for glycosidically linked pyranoses; furanosidic sugars have anomeric signals that occur further downfield at 103 to 106 ppm. The resonances (both α and β) from C-1 of a reducing pyranosidic residue fall between 98 and 90 ppm[100]. The exact chemical shift value for the anomeric carbon depends on the particular sugar type and its C-1 stereochemistry, with the peak of the β anomeric configuration resonating downfield of the α

signal. Other carbons in the monosaccharide (for example: $\underline{C}H_2OH$, $\underline{C}OOH$, $\underline{C}$=O, and $\underline{C}$H(NH)) also display chemical shift values that make their identification unambiguous. Carbon-13 spectra furnish important data on the positions of interglycosidic bonds, because linkage carbons are strongly displaced downfield (approximately 6-9 ppm) by the inductive deshielding β-substituent effects[100,101]. Thus, the non-anomeric carbon involved in the linkage has a resonance in the area between 80-87 ppm, away from the other C-2 to C-5 signals between 65 and 75 ppm, and can be identified[100]. Carbons with an attached O-acetyl or O-phosphate also experience a glycosylation shift (downfield <3 ppm) and this may help determine their position(s) of attachment.

A proton coupled ^{13}C spectrum may be required where the anomeric configuration of a residue or residues in the repeat unit cannot be assigned from the ^{1}H or broadband proton decoupled ^{13}C spectra chemical shift data. The $^{1}J_{CH}$ coupling constant is dependant upon the C-1 configuration and it has been shown[205] that, for the α and β anomers of several of sugars, the coupling constants are about 160 Hz and 169 Hz, respectively. This datum is usually needed to assign the anomeric configuration of a mannose residue.

Carbon-13 resonance spectroscopy, therefore, provides information that is complementary to the data obtained by ^{1}H nmr spectroscopy, ie the composition of the repeat unit and the anomeric configuration of the sugars as well as the identification of any attached substituents. Furthermore, the pronounced glycosylation shift that the ^{13}C nucleus experiences provides much additional structural information.

9.2 Multiple Pulse Sequences

A spectrum from the single pulse sequence is equivalent to that from a

classical cw experiment, containing a single frequency dimension with chemical shift and coupling constant data, and, except for the convenience of signal averaging (which also allows weak nuclei to be observed) and enhancing spectral resolution by Gaussian multiplication[100] and other apodization techniques, there is no real advantage to pulsed FT nmr as a single pulse experiment. However, modern nmr spectrometers can manipulate the spin systems with multiple pulses to provide new data and allow the interactions between nuclei, such as scalar coupling and the direct magnetic effects, to be measured in a more efficient, or a more informative way. This is because the precession frequency of a peak in a multiplet is the sum of the contributions from the chemical shift and a contribution from the coupling constant. These can be separated by a sequence of pulses with suitable time delays, related to the relevant coupling constant, which removes the δ precession and uses the J precession to enhance, to remove, or to invert certain resonances. Therefore, a spectrum is produced with selected information. In order to perform these sophisticated nmr experiments the spectrometer must be capable of applying observing and decoupling pulses along the x, y, -x, and -y directions (of the rotating frame of reference) and also to be able to detect signals in these directions[204].

Multiple pulse sequences contain preparation, evolution, and detection stages and the informational content of the spectrum depends upon the nature of the preparation, and, or length of the evolution time. The spectrum can take the form of a single frequency dimension plot, known as a 1D spectrum. However, if the experiment is carried out with many different evolution times, two separate sets of spectroscopic features are obtained, ie those that influence the magnetization during the evolution time and those that influence it during acquisition.

Fourier transformation of both aspects produces a spectrum with two frequency dimensions (2D).

The multiple pulse programs have become increasingly complex, allowing more structurally revealing "dialogues" between the spectrometer and the sample magnetic nuclei.

9.2.1 One Dimensional Spectra From Multiple Pulse Experiments

There are a large number of pulsing programs that produce 1D spectra and they can combine population, chemical shift, and coupling information transfer between two coupled spin systems to provide useful analytical tools for structure elucidation of complex molecules. These sequences can be adapted to two dimensional analogues.

9.2.1.1 Distortionless Enhancement by Polarization Transfer (DEPT)

Distinguishing between the carbon-13 resonances from methyl, methylene, and methine groups is not usually possible when using a single pulse experiment; this is because of the reasons discussed previously. There are, however, several multiple pulse programs which do allow unambiguous distinction between these groups and the spectrum is frequently recorded more rapidly than in the proton coupled ^{13}C experiment. This is a result of the interaction between the coupled ^{1}H and ^{13}C which often boosts the signal strength[206].

The DEPT sequence[207,208] relies on polarization transfer from coupled protons to carbon-13 or other nuclei, therefore quarternary carbons are not observed. In theory all coupled protons should cause a response, but, in practice, it is limited to couplings over one

bond[204]. DEPT is the most productive way of obtaining a ^{13}C spectrum showing proton couplings, because it combines sensitivity enhancement with spectrum editing, thereby, giving a well resolved proton coupled ^{13}C spectrum or several sub-spectra, which can be interpreted despite the large ^{13}C-^{1}H coupling values. The intact spectrum has positive CH and CH_3 resonance peaks and negative CH_2 peaks; this can be edited into CH, CH_2, and CH_3 sub-spectra. The spectrum editing of ^{13}C and ^{15}N nmr spectra from carbohydrates by DEPT has been reported[209].

9.2.2 Two Dimensional Nmr Spectroscopy

Good spectral resolution, ie narrow spectral lines dispersed over a large frequency span, is required for the facile interpretation of the complex spectra from carbohydrates in general and polysaccharides in particular. Efforts to improve the strength, homogeneity and stability of the magnetic field throughout the nmr sample, has resulted in narrower lines. However, even at the maximum field strength available, large areas of the proton spectrum from a complex carbohydrate are only partially resolved. In fact, even a twofold increase of the maximum magnetic field strength available at present, would not result in a resolvable peak for each proton type in the molecule. 2D nmr has, however, provided a way of overcoming this problem and, also, allows two ^{1}H nuclei, in different environments, which have the identical (or very similar) chemical shift, to be identified. For example, these experiments can separate the chemical shift and coupling data onto different axes or resolve ^{1}H peaks by using the wide spectral dispersion of coupled ^{13}C nuclei. In addition, the 2D experiments provide access to information that cannot be determined from a single pulse experiment spectrum. For instance, the correlations between coupled spin systems can be identified. The advantage of 2D experiments over 1D multiple pulse sequence equivalents is that the desired information can be established simultaneously for all the spin systems, thereby, saving time if a large number of datum are required from the sample. Whereas the 1D variant may require several acquisitions.

The 1D multiple pulse sequence: preparation - evolution - detection, where the receiver signal $S(t_2)$ is a function of the detection time t_2, also forms the basis for the 2D experiment, albeit with a few

important differences. The evolution time t_1 becomes a variable within a series of pulses and it is increased by a constant time increment Δt_1. The signal S then becomes dependant upon both t_1 and t_2 (ie $S(t_1,t_2)$) and FT of the data with respect to both time variables produces the frequency variables F_1 and F_2, respectively. These are used to produce the 2D data matrix.

A 2D FT nmr experiment is possible if a systematic variation in t_1 results in a periodic change in a property of the spin system at the end of the evolution time.

Two dimensional nmr spectroscopy can be divided into (homonuclear and heteronuclear) J-resolved and correlated experiments. The resolved variant is characterised by a spectrum with a frequency axis (F_1) containing J-coupling information and another (F_2) with the chemical shift data[210]. Therefore, access to these parameters is easier and they can be accurately determined, yet, in carbohydrate structural analysis this experiment is seldom employed (except for very simple molecules). This is because it lacks sensitivity and is unable to provide J-connectivity information[180]. The second type of 2D experiment contains two frequency axes with chemical shift data and resonances are linked by correlation peaks. The program is a modified multiple pulse sequence containing an additional mixing time. Correlated 2D nmr can take one of two forms: those involving scalar couplings through the coherent transfer of transverse magnetization, or those involving dipole coupling through incoherent transfer of magnetization. The significant difference between the two is that scalar correlated spectra can be used to reveal through bond connectivities, and dipole correlated spectra can be used to reveal through space connectivities. Thus, the nature of the pulse program determines the physical meaning of the spectrum.

9.2.2.1 Homonuclear Correlation Experiments

The vast majority of 2D homonuclear correlation experiments used in the structural elucidation of bacterial polysaccharides are those that correlate the proton resonances of the sample; this is because the low sensitivity of the ^{13}C nucleus makes the carbon-13 resonance correlation experiments difficult.

9.2.2.1.1 Correlation Spectroscopy (COSY)

The COSY experiment correlates the chemical shift of a nucleus with the chemical shift of its adjoining neighbour or neighbours, through homoscalar coupling. It is, presently, the most important two dimensional technique used in the structural study of carbohydrates because it does not require a large amount of material to obtain the spectrum and the amount of usable information it yields (per unit of spectrometer time) is large. The experiment is also robust, ie tolerant to incorrectly set experimental parameters.

The basis for the COSY[211] technique is the classical Jeener sequence and it has evolved into two major variants, the COSY-90 and COSY-45[98]. The spectrum, which is measured with a two pulse sequence containing an incremented time delay, contains several peak types. Those that have the same frequency (chemical shift) in both the F_1 and F_2 dimensions are the same resonances that are found in the "conventional" (single pulse) spectrum and are known as diagonal peaks because they fall along the diagonal of the spectrum. There are also characteristic off-diagonal signals (the correlation or crosspeaks) and if two proton signals, for instance A (δ_A,δ_A) and B (δ_A,δ_A) are scalar spin-spin coupled then two peaks, one at δ_A,δ_B and the other at δ_B,δ_A will appear in the COSY spectrum. Thus, it is a simple

excercise to follow the connectivities in a monosaccharide unit, starting at H-1 and continuing around the ring, by following the correlation map formed by the crosspeaks.

There are a few instances where a full "connectivity map" cannot be constructed. This may be due to the autocorrelation peaks, that occur between the multiplets of a signal and fall close to the diagonal, which can mask the crosspeak between two resonances with a similar chemical shift. For example, the signals of H-5, H-6' of galactose residues and H-3, H-4 in N-acetyl-glucosamine residues[212]. The autocorrelations can be suppressed by using a COSY-45 sequence (ie a 45°, instead of 90° mixing pulse[98]) which only shows the directly correlated transitions[210]. A problem in constructing the map can arise where a spin-spin coupling value is small, as this can give a low intensity crosspeak. Thus, it will be difficult to see and may be indistinguishable from the background noise or couplings that originate from long-range interactions. However, the COSY experiment can be handled and processed in a manner that preserves the phase information that is usually discarded in the COSY-45 and COSY-90 experiments, for instance, the double quantum filtered COSY (DQF-COSY[213-215]). These spectra have crosspeaks with the positive and negative phase amplitude retained, instead of the dispersive COSY correlation peaks. The phase patterns in a DQF-COSY indicate whether the crosspeak arises from an active or passive coupling[98]. Hence, vicinal couplings can be identified. Further, this experiment can also be adapted to decouple the spectrum in the F_1 dimension, thereby eliminating splitting due to proton-proton couplings, making it easier to extract information from crowded spectral regions[212]. This experiment has become an important method of obtaining proton correlated spectra.

9.2.2.1.2 **Relayed Coherence Correlation Spectroscopy**

The coherences that are generated in a COSY type experiment can be redistributed *via* J-coupling, by applying an additional coherence transfer step. This gives rise to a relayed correlation experiment that produces a 2D spectrum with crosspeaks between pairs of spin systems which are not directly coupled, but are both coupled to a third spin system[216,217]. The RELAY-COSY[212,216,218], for example can show a correlation peak between two ring protons that are not adjacent to one another on the carbon skeleton of the residue. Moreover, coherence can be relayed to protons further along the chain, ie by a 2-, 3-, or 4-step RELAY-COSY.

Unfortunately the multiple-RELAY-COSY is limited by the rapid loss of magnetization during the mixing period; the rate of decay is proportional to the T_2 value of the molecule. Therefore, polysaccharides and other large molecules are not good candidates for a multiple step RELAY-COSY.

The relay peak intensities are dependant upon the precise nature of the spin systems involved and, thus, it is not uncommon for some relay correlations to be missing[202]. Generally the sensitivity of this type of experiment is rather low, so it is only employed when the J-networks in the COSY spectrum cannot be fully established and the areas of difficulty have been identified. Other methods that could be applied to solving complicated coupling networks are the 2D homonuclear Hartmann-Hahn spectroscopy[219] (the HOHAHA, which relies on cross polarization) and the TOCSY experiment[220].

9.2.2.1.3 **Nuclear Overhauser Enhancement Correlation Spectroscopy**

The 2D nuclear Overhauser enhancement (nOe) correlation experiments are a useful source of detailed information for structure elucidation, because they yield the interatomic distance between cross relaxing nuclei. Their primary use is in identifying dipolar interaction between the spins of neighbouring protons that are not necessarily connected by bonds, ie through space magnetization transfer. Heteronuclear nOe correlation spectra can also be recorded[221]. Two multiple pulse sequences are commonly used to produce nOe correlation spectra and these are known as NOESY[222] and the ROESY (rotating frame Overhauser enhancement spectroscopy[223,224]); the latter program is for oligosaccharides and other complex molecules with a molecular mass below 1500, where the NOESY experiment often fails[225]. There is, however, no appreciable difference in the type of data they yield. The emphasis of the discussion will be on the more commonly used NOESY.

Each NOESY spectrum is representative of a single, arbitrary point on the numerous nOe build up curves, so the presence or absence of a crosspeak between two resonance peaks will depend on whether or not any dipole interaction is occurring between their spins and the relationship between the speed of build up of the nOe and the selected mixing time (t_m). In other words, a correlation peak may be absent if the mixing time is either too short and the nOe has not yet evolved, or it is too long and the nOe has already reached its maximum and decayed to zero. Thus, the lack of a cross peak should not be viewed as conclusive proof of the absence of incoherent magnetization transfer. The growth rate of a NOE interaction depends on the internuclear distance (or rather $1/r^6$, where r is the separation between the nuclei), so the particular mixing time that is selected

for an experiment defines a narrow window of internuclear distances over which nOe interactions will be detectable. The shorter the mixing time, the shorter the distance over which nOes can be identified. If there are several nuclei of interest, which are separated by different interatomic distances, a number of spectra (each from an experiment with a different t_m) may be required to show all the correlations.

The NOESY and ROESY produce spectra that are complicated by the presence of COSY and HOHAHA crosspeaks, respectively, due to coherent magnetization transfer[225]. These can be identified by comparing the spectrum with the relevant 2D correlation spectrum. However, a phase sensitive NOESY[226,227] spectrum eliminates the need for this, because the coherent and incoherent magnetization transfer crosspeaks have different phase patterns. This experiment is particularly useful for crowded and complex spectra, but takes longer to accumulate the data points than the conventional magnitude presentation.

In a poly- or oligo-saccharide a nOe can be observed between an anomeric and non-anomeric proton, between pairs of non-anomeric protons, and between protons and ^{13}C nuclei. The most important of these interactions is the interglycosidic interaction between the anomeric proton on a sugar and a ring proton of the residue to which it is linked. This serves to identify the linkage position[225] of each residue and this data is also used to sequence the residues of the repeat unit. In addition, the anomeric proton, when the interatomic window of the NOESY is approximately 2.5Å, shows a crosspeak to H-2 if the anomeric configuration is α, and to H-3 and H-5 if the configuration is β[225]. Thus, the C-1 stereochemistry can be established or confirmed from this data. Furthermore besides the "obvious" nOe effects, dipolar correlations are observed between

nuclei that are more remote and these are indicative of the overall conformation of the molecule[225].

9.2.2.2. Heteronuclear Correlation Experiments (HETCOR)

Two-dimensional ^{13}C-^{1}H chemical shift correlations are helpful in assigning the crowded, overlapping non-anomeric region of the proton spectrum from a carbohydrate. This is because the carbon-13 nucleus of a (CH_n) group is more sensitive to the changes in its environment than the ^{1}H nucleus and the greater (^{13}C) spectral dispersion can be transferred to the proton spectrum by correlating the independant chemical shift parameters between bonded heteronuclei. The spectrum consists of a dimension for the proton chemical shifts and another for the carbon-13 shifts and a cross peak, with the coordinates (δ_x,δ_H), arising for each directly bonded carbon (X) and proton (H) pair in the molecule. The specificity with which a resonance, ^{13}C or ^{1}H can be characterised and assigned is therefore enhanced.

The conventional HETCOR experiment[99], where the observed nucleus is the relatively insensitive ^{13}C nucleus (with its low γ value), requires a substantial amount of pure material. The pulsing program produces signals from ^{13}C nuclei, which are modulated by their one bond scalar couplings to protons. However, the experiment is more sensitive when the ^{1}H signal is instead modulated by the coupled ^{13}C nucleus; but, until recently, indirect detection of the low γ ^{13}C nucleus *via* ^{1}H detection was complicated by unwanted signals from protons not bonded to a carbon atom. This problem has been overcome[228] and the spectra from these so called "reverse detection" sequences are the same as a conventional HETCOR, except, they can be acquired from samples $1/_{10}$th to $1/_{20}$th the size needed for a conventional (^{13}C detected) heteronuclear correlation experiment[229].

The distance over which heteronuclear shift correlation can be detected has been increased by new pulsing programs and experiments are available for establishing long range[230] and relayed heteronuclear correlation[231].

10 STRUCTURAL STUDIES ON SOME *ESCHERICHIA COLI* AND *KLEBSIELLA* CAPSULAR POLYSACCHARIDES

10.1 A Structural Investigation of the Capsular Polysaccharide of *Escherichia coli* O9:K57:H32

10.1.1 Abstract

The primary structure of the capsular polysaccharide of *Escherichia coli* K57 was fully elucidated by methylation analyses and a comprehensive nmr study which employed both 1D and 2D experiments. The polymer was found to be a linear heteroglycan with the following tetrasaccharide repeat unit:

→2)-β-D-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→

10.1.2 Introduction

The study of the *E. coli* strain K57 forms a part of a continuing international programme to elucidate the chemical structure of capsular antigens. This is partly because the bacterial capsule affects the complement mediated bactericidal activity of host serum and other important immune defensive mechanisms, effects directly related to the precise nature of the capsular polysaccharide[10,232]. Thus, revealing the construction of these polymers helps to identify the influence of chemical structures on the interactions between immune systems and antigenic determinants. Hence they provide an insight into the processes involved in infections.

Capsular (K) antigens of *Escherichia coli* are subdivided into two main groups on the basis of their molecular size, acidic component, co-expression with O-antigens and temperature regulation of their

synthesis[4]. The group I antigens have high molecular weights, low electrophoretic mobilities, heat stability at pH 5-6, and are most often co-expressed with O8 and O9 antigens; they may contain amino sugars. Group II antigens have lower molecular weights, higher electrophoretic mobilities and are heat labile at pH 5-6. They are co-expressed with many O antigens, but not with O8 or O9. In the genus *E. coli*, a hundred K antigens have been identified to date and forty nine of these have been structurally elucidated and the results reported[17]. Serotypes K6[233], K13[234], K18[21] to K20[234], K22[21], K23[234], K74[235], K95[236] and K100[21] contain ribofuranose and belong to the group II antigens. The serotypes K18, K22 and K100 incorporate ribitol phosphate, while the rest contain KDO in their repeat units. The capsular antigen of *E. coli* O9:K57:H32[237], whose structure is described here, is unique because it is the first ribose containing K antigen reported in the *E. coli* series which is not associated with ribitol phosphate or KDO and which belongs to the group I antigens.

10.1.3 Results and Discussion

10.1.3.1 Gel Permeation Chromatography, Composition and Linkage Analyses of the K57 Capsular Material

Gel permeation chromatography of the polymer on Sepharose® 4B CL gave a profile with a monodisperse peak at M_r 250 000 (Gel Profile A1).

Glc examination of O-methyloxime acetates[67] derived from a hydrolysate, with and without carboxyl reduction, showed the presence of equimolar proportions of glucosamine, galacturonic acid, galactose and ribose in the repeat unit. The free sugars were isolated by preparative paper chromatography and their optical rotation was measured; they were all found to be D.

The 1D proton nmr spectrum (Spectrum A1) of the polysaccharide contained anomeric signals at δ 5.39, δ 5.24 (^{3}J 3.5 Hz), δ 4.93 (^{3}J 2.7 Hz), and δ 4.46 (^{3}J 7.8 Hz) and a signal for a N-acetyl group at δ 2.11, (Table A1). These data showed the polysaccharide repeat unit to be a tetrasaccharide. In addition, several ring proton signals occurred in the anomeric region *viz* δ 4.51 (^{3}J, approximately 1 Hz) and δ 4.38 (^{3}J 2.5 and 1.0 Hz). The ^{1}H coupling constants in Table A1 were obtained from the 1D spectrum of the polysaccharide. The ^{13}C-nmr data (Table A1) showed C-1 signals at 107.95, 104.25, 100.07 and 98.72 ppm and a signal at 23.24 ppm for the methyl carbon of a N-acetyl (Spectrum A2). There was also a carbonyl carbon signal at 175.75 ppm. The anomeric carbon signal at 107.95 was indicative of the presence of a furanoside in the repeat unit[100]. These data complemented the results of the TSR analysis.

Methylation analysis of the polysaccharide gave the permethylated residues: 2,3,6-tri-O-methyl galactose, 2-deoxy-4,6-di-O-methyl-2-methyl-acetamidoglucose and 3,5-di-O-methyl ribose; 2,3-di-O-methyl galactose was present after a methylation analysis where the methyl esters of the methylated glycan were reduced. The residues were identified by glc-ms of their derived acetates[156] and alditol acetates[24].

10.1.3.2 Two-Dimensional Nmr Studies On the K57 Capsular Polysaccharide

The proton resonances of the repeat unit were assigned (Table A1) using data from the COSY[98], RELAY COSY[217] (Spectrum A3 and A4) and other 2D nmr experiments. The H-1 resonances were given the arbitrary labels a to d in order of their decreasing chemical shift. The connectivity maps for residues a and c were easily constructed and all their ring proton resonances were readily assigned. In the case of

residue **b**, the crosspeak between H-4 and H-5 was not observed in either the COSY or RELAY COSY spectrum. However in the spectrum (Spectrum A5) from the NOESY[222] experiment there were intra-residue nOe crosspeaks between H-3/H-4, H-4/H-5 and H-3/H-5 of **b** and this allowed the chemical shift for H-5 to be established. The residue **d** showed no correlation peaks (COSY or RELAY COSY) between resonances H-4/H-5, H-5/H-6, H-5/H-6', or H-6/H-6', so a full connectivity map could not be constructed for this residue. Nonetheless, once the proton resonances assigned from the connectivity maps of residues **a** to **c**, the intraresidue nOes and the H-1 to H-4 partial correlation map of **d**, were chemical shift correlated with their corresponding ^{13}C signal using the HETCOR[99] spectrum (Spectrum A6), only two ^{13}C signals remained. Therefore, these resonances, at 75.19 and 62.01 ppm, could be confidently assigned to C-5 and C-6 of **d** respectively on the basis of their shift values. These carbon-13 signals both correlated with a proton resonance at δ 3.78, which explained the absence of an observable crosspeak between H-5, H-6 or H-6' of **d**.

Comparison of the ^{1}H and ^{13}C-nmr data (Table A1) with literature values for methyl glycosides[186,238,239] permitted the residues **a** to **d** to be identified as: β-ribose, α-galacturonic acid, N-acetyl-α-glucosamine and β-galactose, respectively. Furthermore, the carbon-13 signals from C-2 of **a**, C-3 of **b**, C-3 of **c**, and C-4 of **d** had been "glycosidically shifted" to lower field and hence indicated that these were the linkage points. This is in accordance with the results of the methylation analyses.

The sequence of the sugars in the repeat unit of the polysaccharide was established using the NOESY spectrum data (Table A2). Inter-residue nOes were clearly observed between the anomeric protons of **a**,

b and **d** and their adjacently linked residues and these permit the following sequence to be written:

$$\mathbf{a}(1\rightarrow4)\mathbf{d}(1\rightarrow3)\mathbf{c}(1\rightarrow4)\mathbf{b}$$

The expected nOe between H-1 of **a** and one of the non-anomeric protons of **b** was not observed. There was instead an intense correlation peak between the anomeric resonances of these residues, a phenomenon that has been observed previously for α-D-hexoses that are glycosidically substituted at O-2[240-242].

In the NOESY spectrum the α-pyranose residues **b** and **c** showed a characteristic intra-molecular nOe between H-1 and H-2, while the β-residue **d** showed nOe crosspeaks between H-1 and the protons H-3 and H-5 of the residue (Table A2), thereby, confirming the anomeric assignments for **b**, **c** and **d**.

The nmr and methylation data allow the structure of the repeat unit of *Escherichia coli* K57 capsular polysaccharide to be written as:

$$\rightarrow 2)\text{-}\beta\text{-D-Rib}f\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{A-}(1\rightarrow$$

$$\mathbf{a} \qquad\qquad \mathbf{d} \qquad\qquad \mathbf{c} \qquad\qquad \mathbf{b}$$

10.1.4 Experimental

10.1.4.1 General methods. - Analytical glc was performed using a Hewlett-Packard® 5890A gas chromatograph, fitted with flame ionisation detectors, helium as carrier gas, and a 3392A recording integrator. A J+W Scientific® fused silica DB-17 bonded-phase capillary column (30 m x 0.25 mm) having a film thickness of 0.25 μm was used for separating partially methylated alditol acetates (programme I) and partially methylated methyl glycoside acetates (programme II). A J+W

Scientific® fused silica DB-WAX bonded-phase capillary column (30 m x 0.25 mm) having a film thickness of 0.15 μm was used for separating O-methyloxime acetates[67] (programme III). The temperature programmes used were: I, 180° for 1 min, then 3°/min to 240°; II, 180° for 30 min then 2°/min to 240°; and III, 80° for 1 min, then 20°/min to 180° hold for 1 min, then 2°/min to 210° hold for 1 min, then 10°/min to 230°.

The identities of all the partially methylated sugar derivatives were confirmed by glc-ms on a Hewlett-Packard® 5988A glc-mass spectrometer using the appropriate column.

Gpc of K57 polysaccharide was performed on a dextran calibrated Sepharose® 4B CL column (1.6 x 65 cm) with 1M sodium chloride at 20 mL/h as eluent. The effluent was monitored by collecting 1 mL fractions which were analysed by the phenol/sulphuric acid method.

Samples were hydrolysed with 4M trifluoroacetic acid at 120° for 1 h and the acid was co-evaporated with water under reduced pressure on a waterbath at a temperature ≤40°. Alditol acetates were prepared by reducing the hydrolysates with sodium borohydride (1 h) followed by acetylation of the alditols with 1:1 acetic anhydride-pyridine $^{v}/_{v}$ at 100° for 1 h. Carboxyl reduction (methanolysis) was acheived by treating the sample with refluxing methanolic 3% hydrogen chloride for 16 h, the acid neutralised with AG_2CO_3 and the methyl esters reduced with sodium borohydride in anhydrous methanol. O-methyloxime acetates were prepared as described by Neeser and Schweizer[67]. Methylations were carried out on the acid form of the polysaccharide using a modified[88] Hakomori method[82] employing potassium dimsyl and methyl iodide in dimethylsulphoxide. The methyl ester groups of the methylated K57 polysaccharide were reduced with lithium aluminium hydride in tetrahydrofuran.

10.1.4.2 Preparation of K57 polysaccharide.- An authentic culture of *E. coli* O9:K57:H32 was obtained from Dr I Ørskov (Copenhagen), plated out on Mueller Hinton agar and incubated at 37° overnight. A single colony was selected, replated and incubated. This was repeated twice, thereafter inocula were transferred to each of six tubes containing 5 mL of sterile Mueller Hinton broth and shaken at 37° for 6 h. The tube contents were spread on the surface of a tray (1.5 L, 1650 cm^2) of sterile Mueller Hinton agar and incubated for two days at 37°. The bacterial cells were harvested, diluted with an equal volume 2% phenol and stirred at 4° for 12 h. The solubilised material was separated from the cells by ultracentrifugation (105 000 G, 3 h) and the supernatant was precipitated into ethanol (5 vols). The precipitate (ppt) was collected by low speed centrifugation and redissolved in water. Cetyl trimethylammonium bromide (CTAB, 5% in water) was added and the ppt was isolated by low speed centrifugation. The acidic polysaccharide-CTAB was dissolved in 350 mL of aqueous 2M NaCl to break the complex and the polysaccharide precipitated into ethanol (5 vols). This was redissolved in water (300 mL), dialysed (12- 14 000 Mw cutoff) exhaustively against tap water and freeze-dried. The yield of acidic capsular polysaccharide was 1.115 g.

10.1.4.3 Nmr spectroscopy.- Samples were deuterium-exchanged by freeze-drying solutions in 99.96% D_2O then dissolved in 99.995% D_2O (0.45 mL) containing a trace of acetone as an internal reference, δ 2.23 for ^1H and 31.07 ppm for ^{13}C. Spectra were recorded at 40° on either a Bruker® WH-400 or AM-400 spectrometer, equipped with an Aspect® 3000 computer and an array processor, using standard Bruker® software. Proton spectra at 400 MHz were recorded using a spectral width of 2400 Hz and a 16K data set for a digital resolution of 0.29 Hz/point.

^{1}H homonuclear shift-correlated experiments (COSY[98] and one-step RELAY COSY[217]) and homonuclear dipolar-correlated (NOESY[222]) experiments were performed using a spectral width of 1448 Hz. Data matrices of 256 x 1024 data points were collected for 48 or 112 transients for each t_1 delay. The matrices were zero-filled in the t_1 dimension and transformed in the magnitude mode by use of a non-shifted sine-bell window function in both dimensions and symmetrised. Digital resolution in the resulting 512 x 1024 matrices was 2.8 Hz per point. Relaxation delays of 1.2 to 1.5 s were used. For the RELAY COSY experiment a fixed delay of 0.036 s was used. The mixing delay in the NOESY experiments was varied between 0.2 and 0.3s.

A ^{13}C-^{1}H shift-correlated (HETCOR)[223] experiment was recorded using a 10 000 Hz (99.4 ppm) spectral width in F_2 and 1500 Hz (3.75 ppm) in F_1. The initial matrix of 256 x 2048 data points was transformed to 512 x 2048 points and processed with Gaussian functions. Digital resolution in F_2 was 9.8 Hz/point and in F_1 5.9 Hz/point. A recycle delay of 1.5s was employed and 1600 transients per fid were collected.

TABLE A1

^{1}H-AND ^{13}C-NMR DATA-[a] FOR THE K57 POLYSACCHARIDE

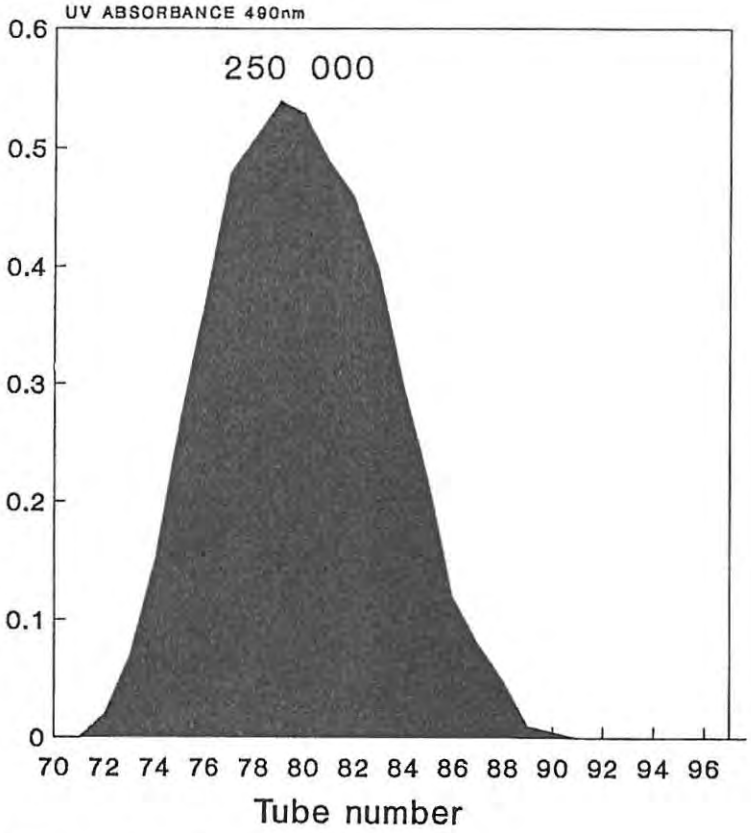
		Proton or carbon							
Residue:		1	2	3	4	5	5'	6	6'
→2)-β-Rib	H	5.39	4.28	4.24	4.07	3.67	3.83		
(a)	^{3}J[b]	<1(1,2)	4.8(2,3)	6.72(3,4)	6.7(4,5)	12.1(5,5')			
	C	107.95	80.74	70.96	83.89	63.46			
→4)-α-GalA	H	5.24	3.93	4.10	4.38	4.51			
(b)	^{3}J	3.5(1,2)	10.0(2,3)	2.5(3,4)	1.0(4,5)				
	C	98.72	68.90	69.68	80.93	72.28			
→3)-α-GlcNAc	H	4.93	4.09	3.92	3.62	4.16		3.85	3.78
(c)	^{3}J	2.7(1,2)	9.4(2,3)	9.2(3,4)	9.1(4,5)				
	C	100.07	53.36	80.85	69.21	72.76		61.16	
→4)-β-Gal	H	4.46	3.52	3.79	4.05	3.78		3.78	3.78
(d)	^{3}J	7.8(1,2)	9.9						
	C	104.25	71.56	73.69	77.22	75.19		62.01	

[a] Chemical shifts in ppm downfield from the signal for acetone at δ 2.23 and 31.07 ppm for ^{1}H and ^{13}C respectively.
[b] coupling constants are in Hz.

TABLE A2

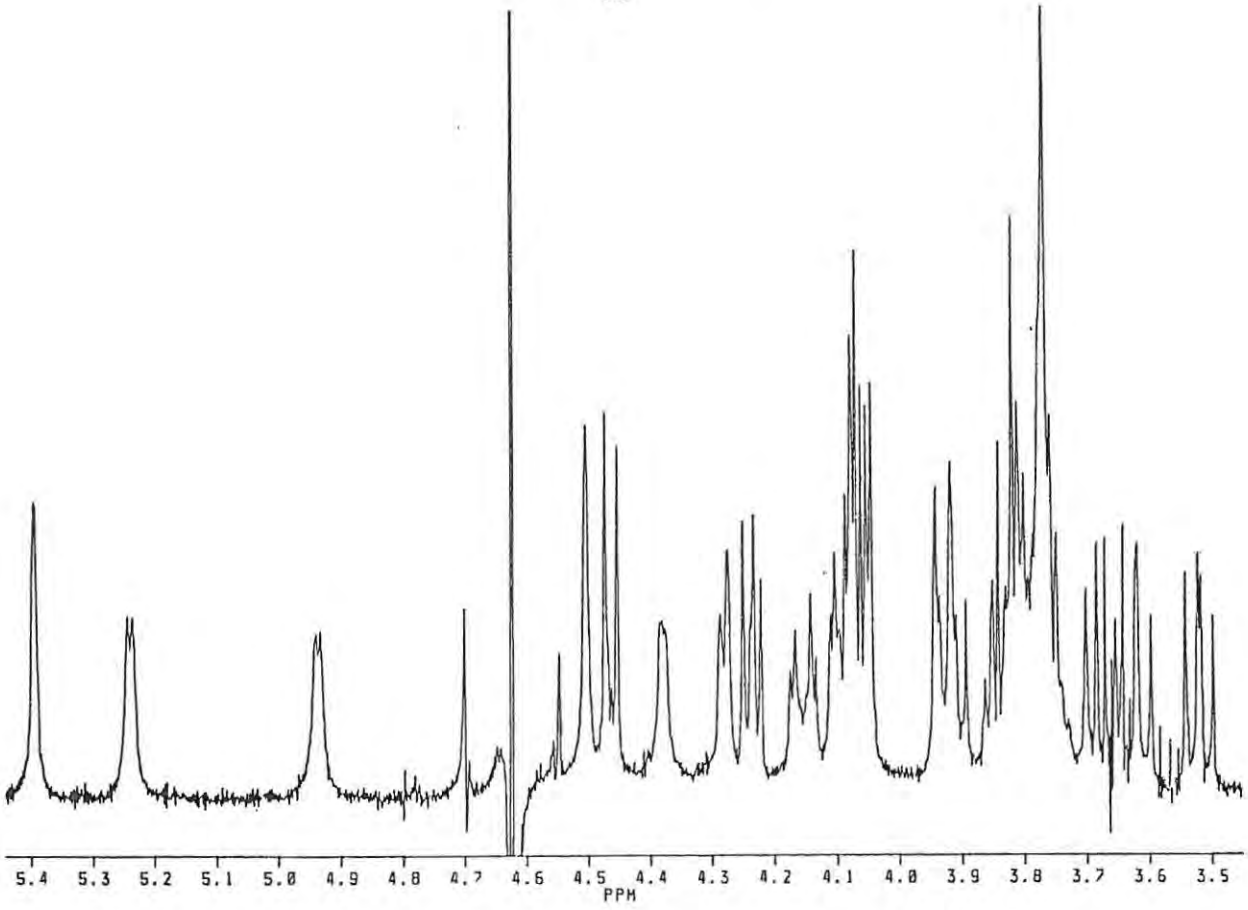
NOE CONTACTS FOR THE K57 POLYSACCHARIDE

Proton:	**NOe contact to:**
a, H-1	5.24 (b, H-1), 4.05 (d, H-4)
b, H-1	5.39 (a, H-1), 3.93 (b, H-2)
b, H-5	4.38 (b, H-4), 4.10 (b, H-3)
b, H-4	4.10 (b, H-3)
c, H-1	4.38 (b, H-4), 4.09 (c, H-2)
d, H-1	3.92 (c, H-3), 3.78 (d, H-3/or H-5)



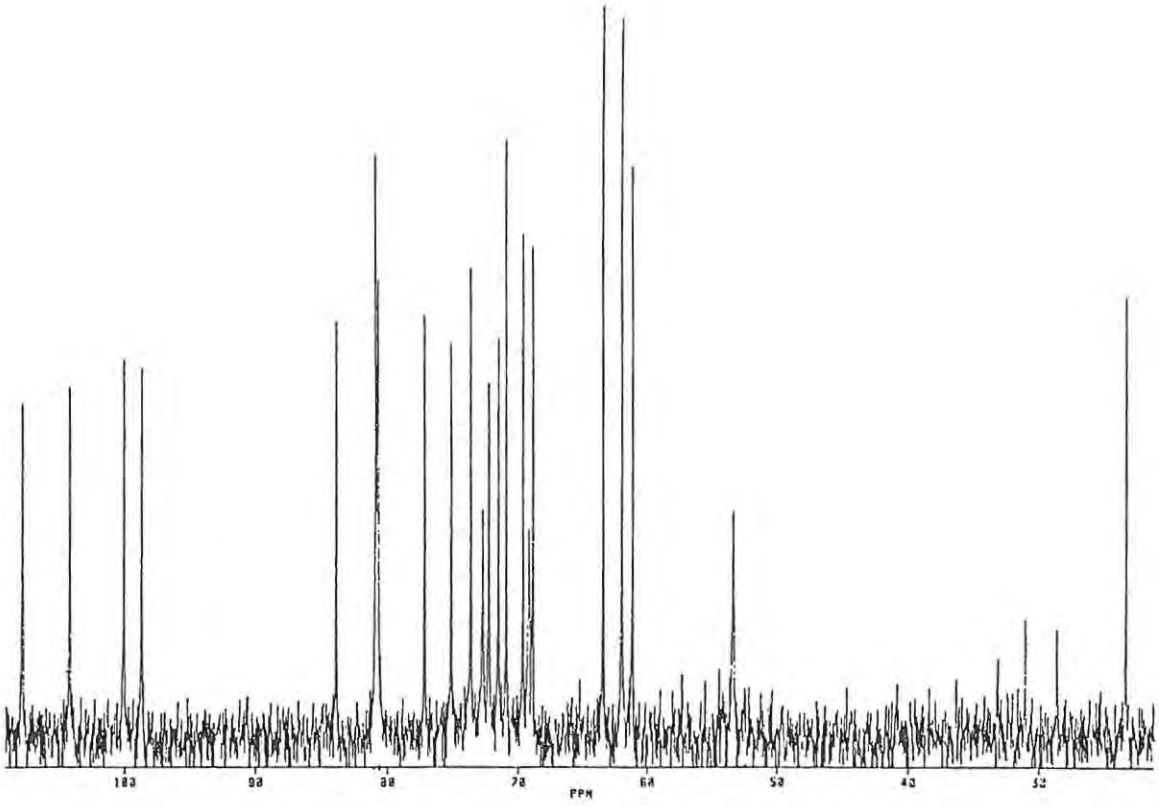
Gel Profile[a] A1 *E. coli* K57 capsular polysaccharide.

[a] Sepharose 4B-CL



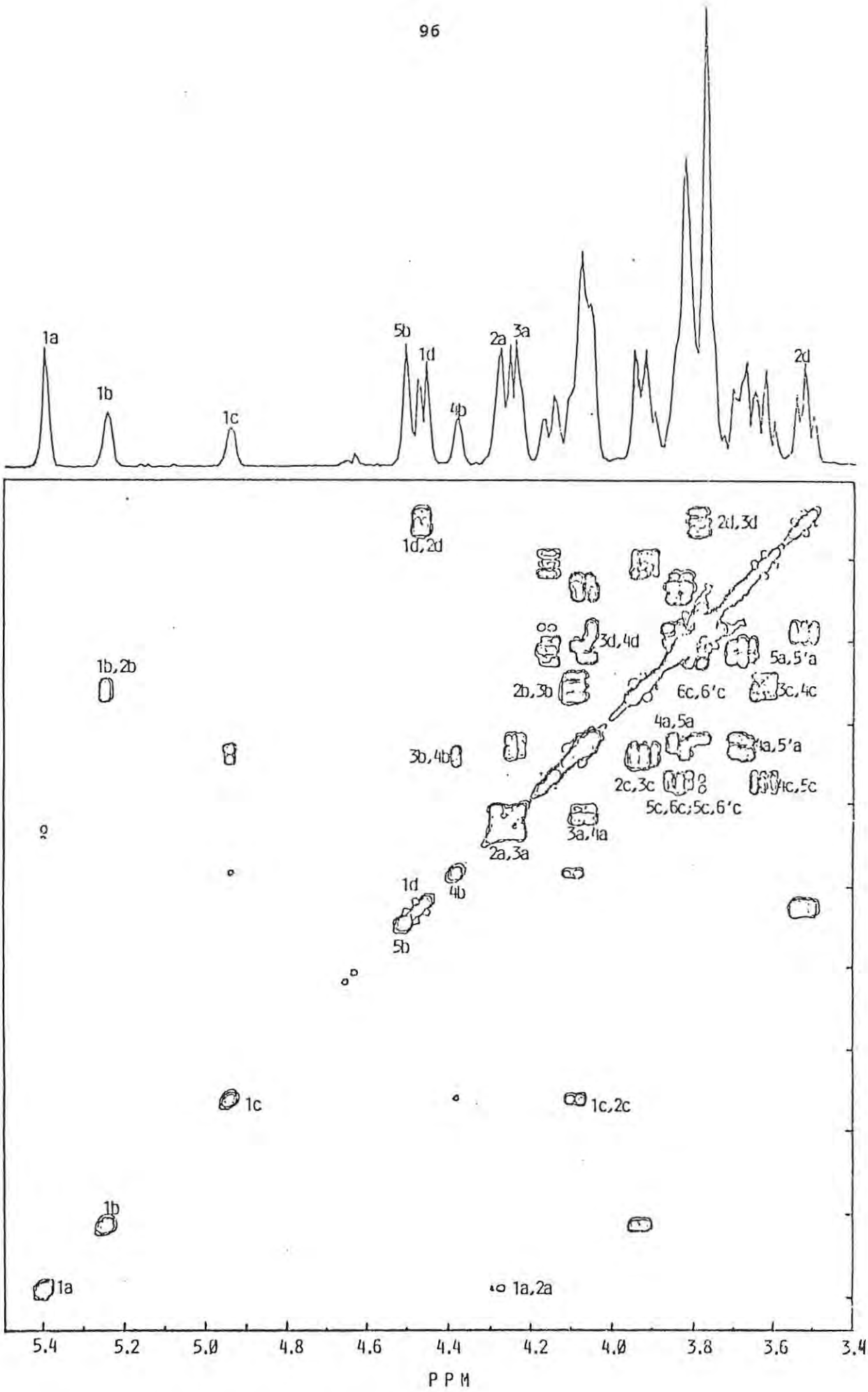
Spectrum A1 ^{1}H spectrum of the K57 polysaccharide.*

* Region shown is between approximately *3.5* and *5.5* ppm.

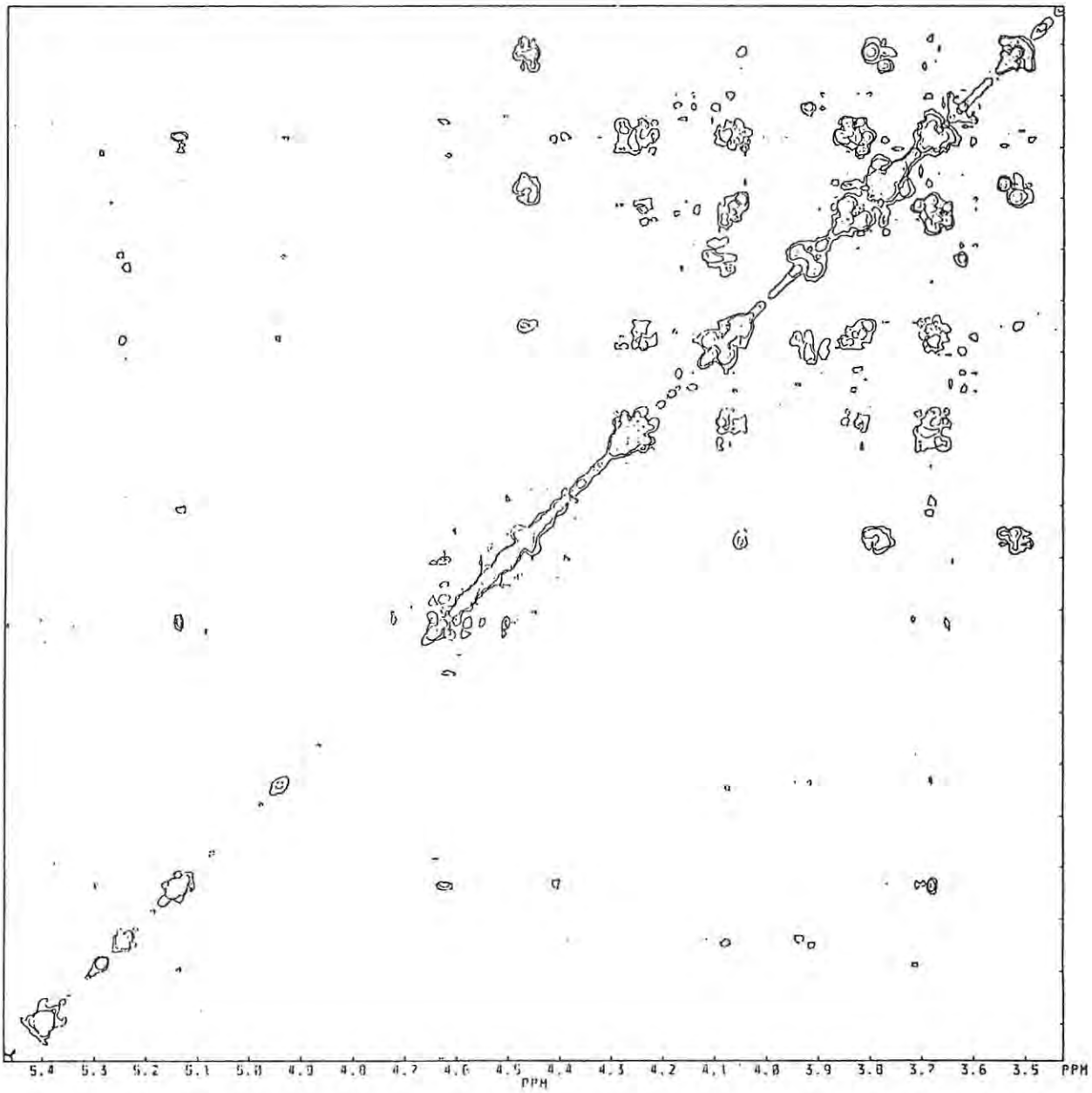


Spectrum A2 ^{13}C spectrum of the K57 polysaccharide.*

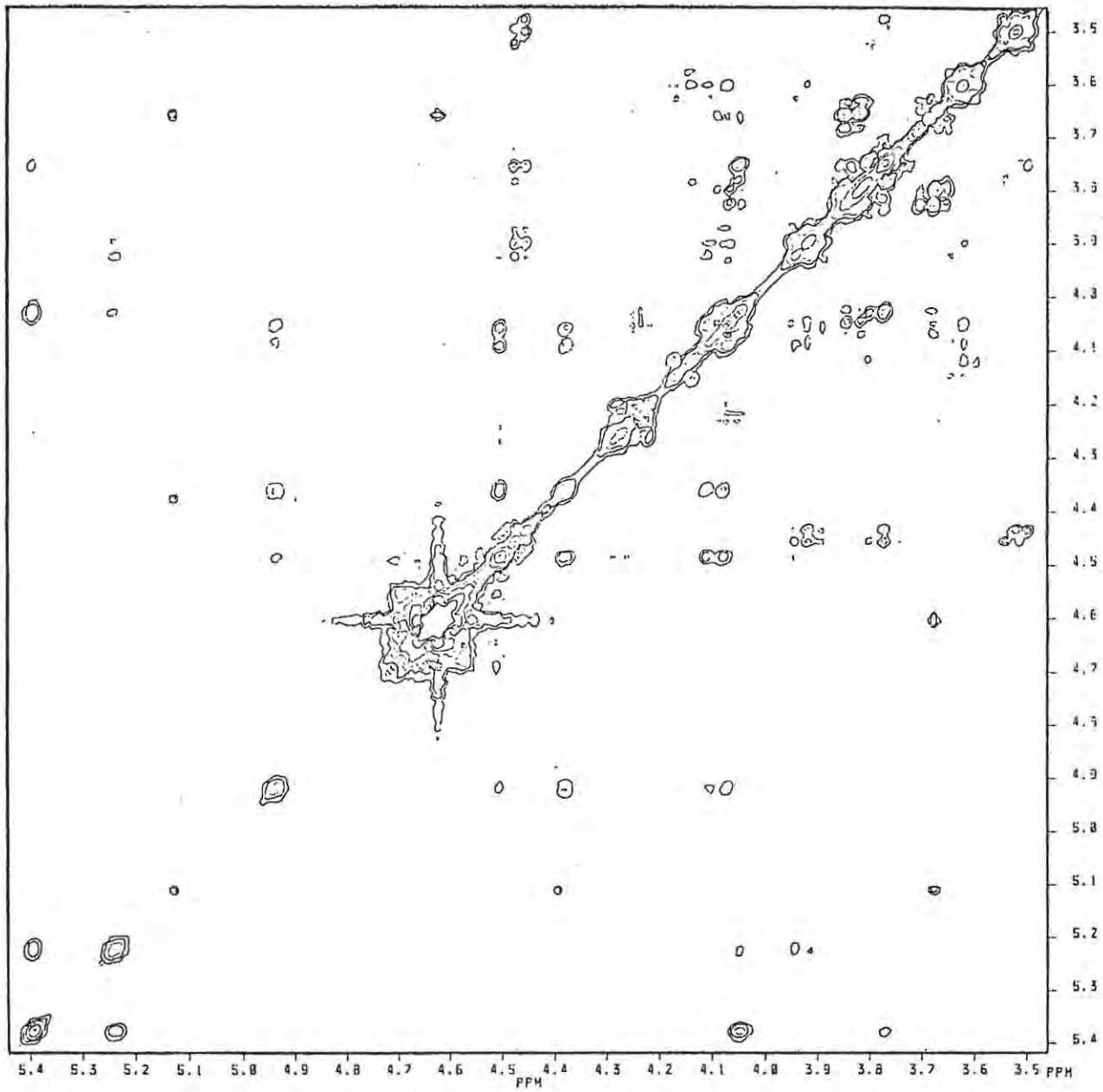
* Region shown is between approximately *20* and *110* ppm.



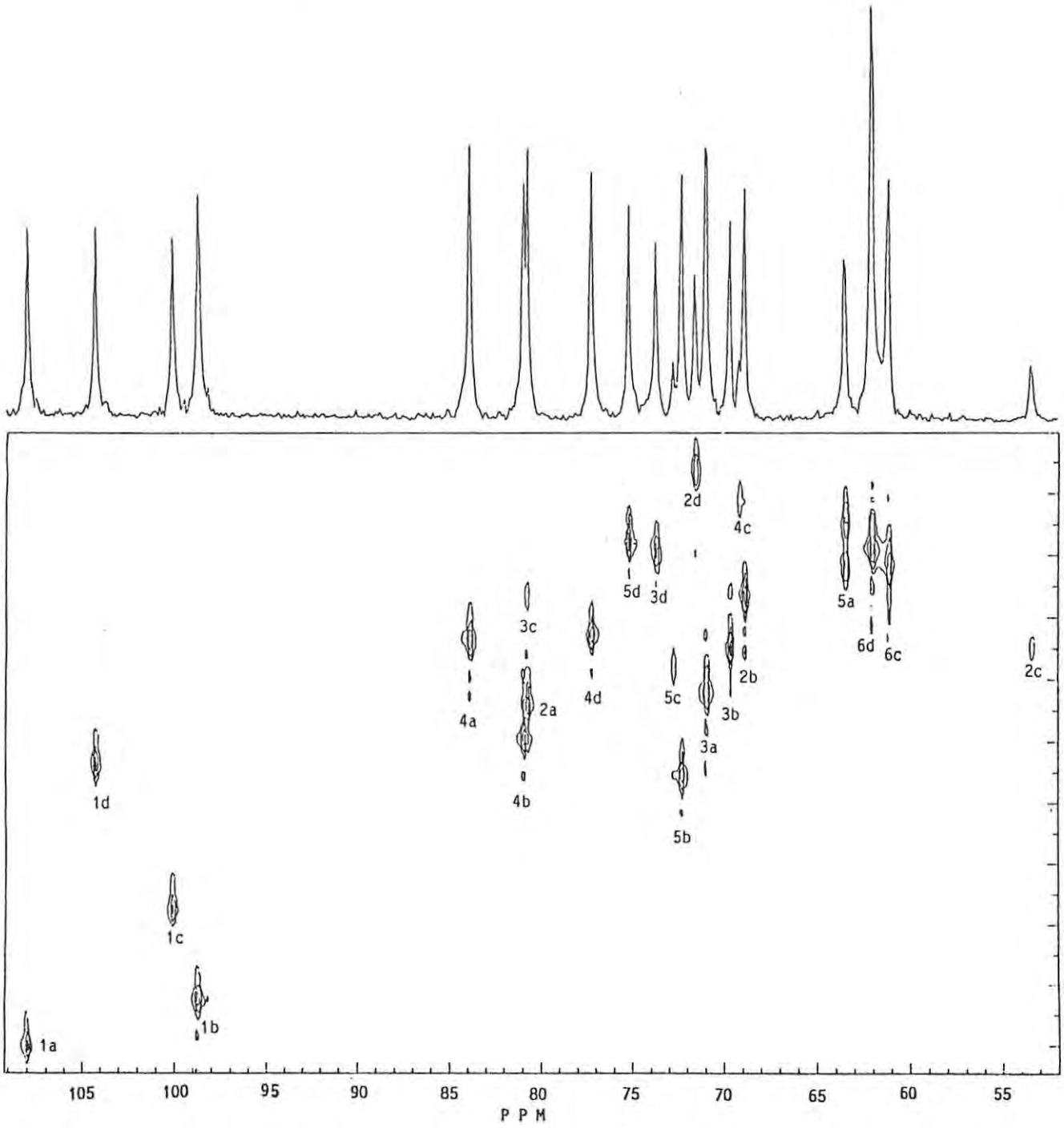
Spectrum A3 COSY spectrum of the K57 polysaccharide.



Spectrum A4 RELAY COSY spectrum of the K57 polysaccharide.



Spectrum A5 NOESY spectrum of the K57 polysaccharide.



Spectrum A6 HETCOR spectrum of the K57 polysaccharide.

10.2 THE SELECTIVE REDUCTIVE CLEAVAGE OF TRIDEUTERIOMETHLATED *E. coli* K57 CAPSULAR POLYSACCHARIDE

10.2.1 Abstract

The trideuteriomethylated derivative of the capsular polysaccharide from the *E. coli* K57 strain has been "selectively" reductively cleaved to produce an O-trideuteriomethylated anhydroribitol terminated tetra- and tri-saccharide. The structures of the fragments, determined by 1D and 2D nmr experiments, methylation analysis of the polysaccharide, fab-ms and a fab-ms methanolysis experiment, are:

Trisaccharide

[α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→2)-anhydroribitol]
3 per-O-trideuteriomethylated
↑
OH

Tetrasaccharide

[β-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→2)-anhydroribitol]
4 per-O-trideuteriomethylated
↑
OH

10.2.2 Introduction

A complete structural analysis of a bacterial poly- or oligo-saccharide requires the determination of the stereochemistry, sugar sequence and linkage patterns, as well as the location of any non-carbohydrate substituents in the repeat unit. This information is generally obtained from chemical and/or spectroscopic analyses of the material; nuclear magnetic resonance spectroscopy is unique in this context because all the necessary information can be derived exclusively from a few nmr experiments. However, if nmr is to yield all the relevant information the spectra must be sufficiently well resolved so that each individual resonance can be identified and

assigned to its reporter group. Where this is not possible, complete structure elucidation (by nmr alone) is either very difficult or impossible.

The high viscosity of many polysaccharide solutions means that they do not generally give good quality spectra. Take, for example, the ^{1}H spectrum of the capsular polysaccharide from *Klebsiella* K58 (Figure B1). (The reasons for the poor resolution of viscous solutions are discussed in greater detail in section 9).

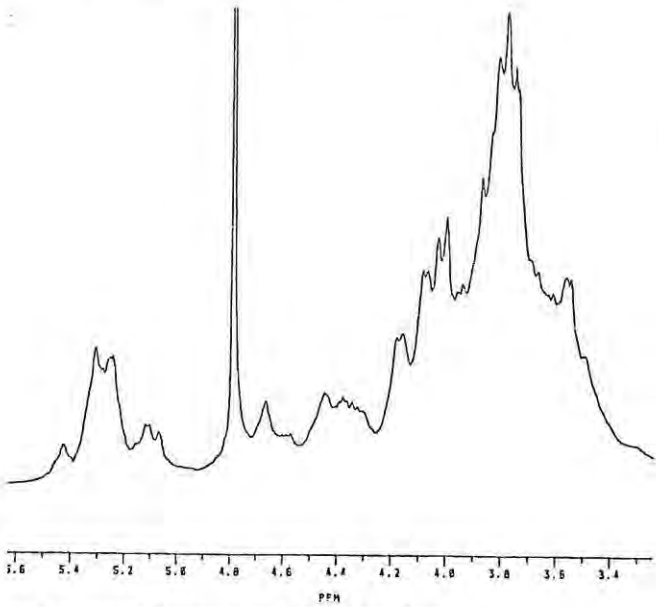


Figure B1 The 500 MHz proton magnetic resonance spectrum from *Klebsiella* K58, run at 398°K.

Chemical modification of the polysaccharide can be used to obtain a better resolved spectrum. If, for instance, the polymer is derivatised (eg O-trideuteriomethylated) the physico-chemical properties are altered and the sample can be dissolved in organic solvents like chloroform. These solutions give better spectra because the maximum sample concentration is not a limiting feature, viscosity is considerably lower than for an aqueous solution of the polymer, and there is no HOD peak to obscure signals (See Figure B2).

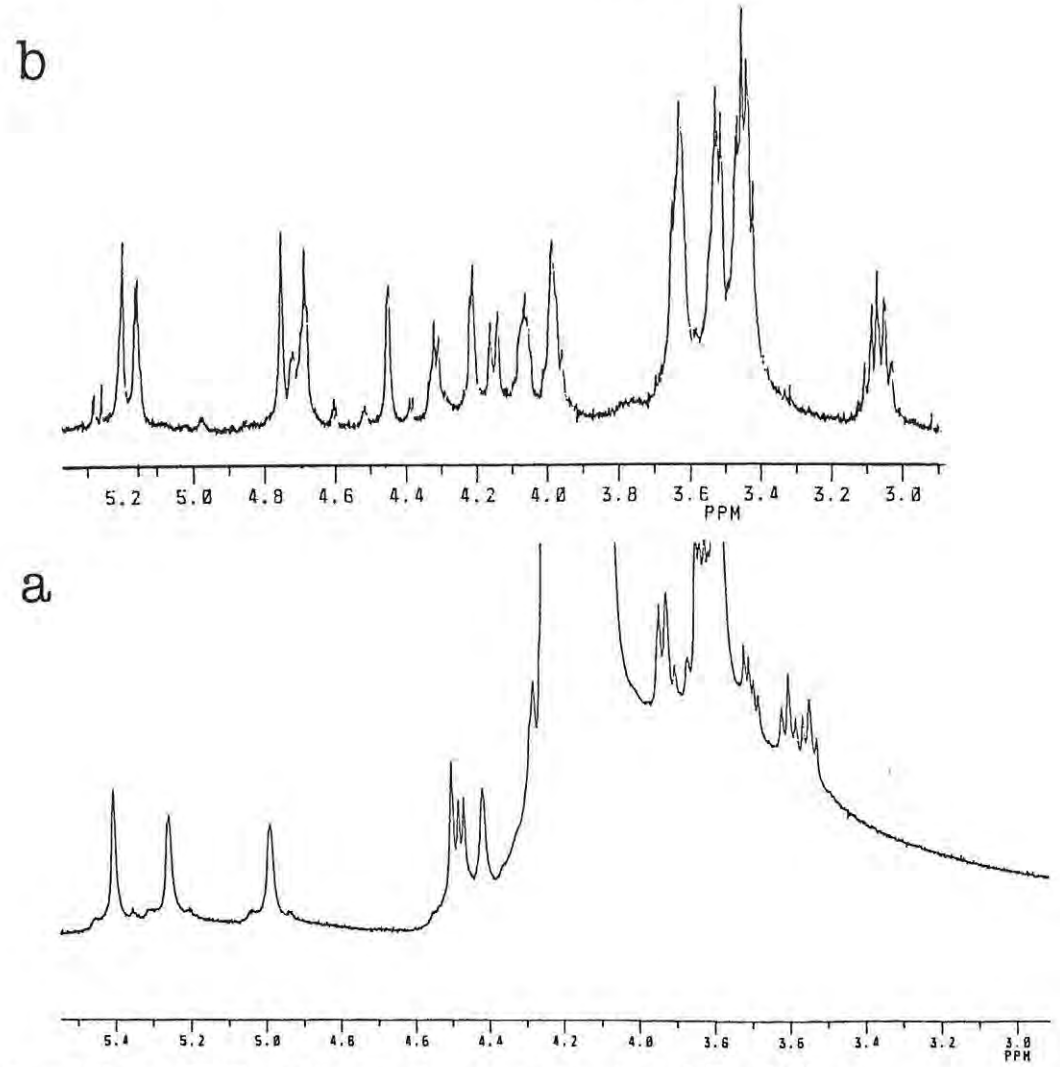


Figure B2 The 500 MHz proton magnetic resonance spectrum of *E. coli* K57
(a) The native polysaccharide (672 scans, 368°K)
(b) deuteriomethylated polysaccharide (80 scans, 303°K).

Nonetheless, this modified polysaccharide may still not give a completely resolved spectrum and so other methods of reducing the viscosity and thereby improving the quality of the spectrum are generally employed. These usually involve a specific or selective cleavage of one glycosidic bond type in the chain to produce an oligomer which will be an analogue of the repeat unit. This however represents the ideal situation and more often than not the modification of the material is more widespread because of the limited specificity of the degradation reaction. In this situation, eg a partial acid catalysed hydrolysis, the chain is cleaved into several small oligosaccharides of varying molecular weight and overlapping composition; one of which may be the analogue of the repeat unit.

The oligosaccharides usually provide only a part of the data required to fully elucidate the polysaccharide from which they were derived and although these samples do give better spectra than the polymer, there are still several disadvantages to this approach. For instance, if the oligomeric product has a reducing terminus the spectrum can be complicated by the mutarotation of this unit. This may be discerned as "twinning" of some signals and, as such, may be useful for making spectral assignments[127]. Nevertheless, this effect is also responsible for broadening some of the peaks and gives rise to several fractional signals for H-1 of the reducing sugar. The latter are sometimes masked by larger resonances. Moreover, it is impossible to define the anomeric stereochemistry of the residue where cleavage has occured. In some selective degradations (eg the Smith degradation[243]) one or more of the sugar residues in the repeat unit may be lost during the cleavage of the polymeric chain, or during work-up, and consequently all information concerning these residues is lost. Another disadvantage of selective degradation reactions is that, with the exception of the bacteriophage mediated depolymerisation, non-carbohydrate substituents are usually removed by the reaction conditions. Ideally, a selective fragmentation reaction should proceed in high yield, with the desired specificity for a single bond type and without competing side reactions. Furthermore, the generated oligosaccharides should contain all the relevant structural information and give clear nmr spectra. The (trideuterio-methylated) anhydroalditol-terminated oligosaccharides produced by a selective reductive cleavage (SRC) of a suitably derivatised polysaccharide come closer to this ideal than do the products of other degradative methods.

In 1987 Jun and Gray reported[131] that in the reductive cleavage [76] of methylated Inulin, using Me_3SiOSO_2Me as the catalyst, complete

cleavage of fructofuranosyl linkages occured within 15 minutes, whereas D-glucosyl bonds were apparently unaffected during this short reaction time. Therefore, there was a distinct possibility that this reaction, and in particular these reaction conditions, could be used for a SRC of a trideuteriomethylated polymer (with a furanose residue in its repeat unit). Furthermore, the partial reductive cleavage[133] (PRC) of methylated β-cyclodextrin produced an overlapping series of oligomers and demonstrated the potential for using methylated anhydroalditol oligomers in the structure elucidation of a polymer; these fragments were analysed by dci-ms. The advantages of a SRC are that it leaves carboxy-methyl and O-methyl groups intact, it retains the ring size and most of the stereochemistry of the cleaved unit, and the lack of an anomeric hydroxyl means that there are no anomeric complications to affect nmr spectra and glc separations. Moreover, because the starting material is derivatised, any free hydroxyls on the oligomer indicate a former point of attachment. Thus, this type of fragment contains more information than an oligosaccharide from any of the other selective degradative methods and well resolved spectra can be acquired in $CDCl_3$. Samples (>5 mg) of an anhydroalditol can be fully characterised by nmr (1D and 2D-COSY[98,211], ROESY[223] and HETCOR[99]), while smaller samples can be analysed by the more sensitive techniques of fab- or dci-ms.

The capsular material (after methylation or trideuteriomethylation) from *E. coli* K57 was selected for a SRC study because it has a repeat unit composed of a galactopyranose, ribofuranose, galacturonic acid and glucosamine. Furanoses, as mentioned previously, may be highly susceptible to RC with Me_3SiOSO_2Me as the catalyst[131]. Uronic acid residues (with the exception of those linked through O-4) may react less rapidly[135] and α-amino sugar linkages have been shown to be completely resistant to RC[134]. Therefore we had possibly only one or

two labile linkage(s) per repeat unit and, if the appropriate set of reaction conditions could be established, either an anhydroalditol-terminated analogue of the repeat unit or an overlapping series of these anhydroalditols would result.

We now report on the development and use of a SRC to produce trideuteriomethylated oligomers from K57 capsular (trideuteriomethylated) polysaccharide. The starting material was trideuteriomethylated (TDM) so that the products of the SRC could be studied by nmr spectroscopy; methylated samples give large methyl proton signals, hence the receiver gain has to be set to a low value to keep the signal within the dynamic range of the spectrometer's analogue-to-digital converter. When the gain is low all the resonances are attenuated. Methylated polysaccharide (MP) was also used during the study, however the products of these RCs were not studied by nmr. The oligosaccharides, which were separated by both thin layer chromatography (tlc) and gel permeation chromatography (gpc), were examined by nmr spectroscopy and fab-mass spectrometry. Their structures were established from these data.

10.2.3 Results and Discussion

10.2.3.1 Development of Suitable SRC Conditions and the Isolation of Trideuteriomethylated Oligomers from *E. coli* K57 TDM-Polysaccharide.

The optimal reaction conditions were determined by a series of experiments that were conducted in two stages:

ONE Samples of MP were treated for varying periods with a standard reagent mixture containing either Me_3SiOSO_2Me or $BF_3 \cdot Et_2O$ as the catalyst. The products were separated by analytical tlc (Table B1). This stage

culminated with a preparative SRC on a sample of trideuteriomethylated polysaccharide (TDMP) and the mixture of oligomers that resulted were separated by preparative tlc. The major product was a trisaccharide.

TWO The MP was treated for 30 minutes with a standard reaction mixture containing one of the following catalysts: $BF_3 \cdot Et_2O$; Me_3SiOSO_2Me; $Me_3SiOSO_2CF_3$. The fragments were separated by gpc. The $Me_3SiOSO_2CF_3$ containing reagent mixture was then evaluated further. Preparative SRC of a MP and a TDMP sample both yielded several oligosaccharides which were isolated by gpc.

STAGE ONE (A)-Optimisation of the SRC conditions

	Product				
	Polymer	Tetrasaccharide	Trisaccharide	Disaccharide[a]	Monomers
CATALYST:[b]					
(a)					
$BF_3 \cdot Et_2O$					
TIME:					
15 min	++[c]				
20 min	++				x[d]
30 min	++				x
60 min	++				x
(b)					
Me_3SiOSO_2Me					
TIME:					
15 min	++	+[e]	+	x	x
20 min	+	+	+	+	x
30 min	+	+	+	+	x
60 min	x	x	x	+	+

TABLE B1 Results of the RC of samples[f] of *E. coli* K57 MP.

a The fragments were identified by running methylated standards, for example, melezitose and cellobiose.

b 100 µl CH_2Cl_2, 25 µl triethylsilane, 40 µl catalyst.

c Intense response

d Light response

e Medium response

f 5 mg of methylated polysaccharide

The data in Table B1 suggest that $BF_3 \cdot Et_2O$ was not catalysing RC selectively and that a reagent mixture containing Me_3SiOSO_2Me could be used for a preparative SRC. The 20 minute reaction time appeared to

give the highest yield of the tetrasaccharide and trisaccharide anhydroalditols.

Note: the tlc method of evaluating the effect of the RC reagent mixtures on the MP was difficult, because analysis of the products was at best semi-quantitative and so an alternative (quantitative) method of separating the oligomers/products was developed (Stage Two).

STAGE ONE (B)-Preparative SRC

Yield:

Origin	5.5 mg	18%[a]
Tetrasaccharide	4.9 mg	16%
Trisaccharide	10.6 mg	34%
Disaccharide	7 mg	22%
Solvent front	3 mg	10%
	31 mg	100%

[a] Percentage of recovered material

The trisaccharide was examined by nmr spectroscopy.

STAGE TWO (A)-Optimisation of the SRC conditions

Profiles (Figure B3) show, as noted in Stage One, that $BF_3 \cdot Et_2O$ was catalysing the RC in a non-specific and limited fashion. The other two catalysts showed promise.

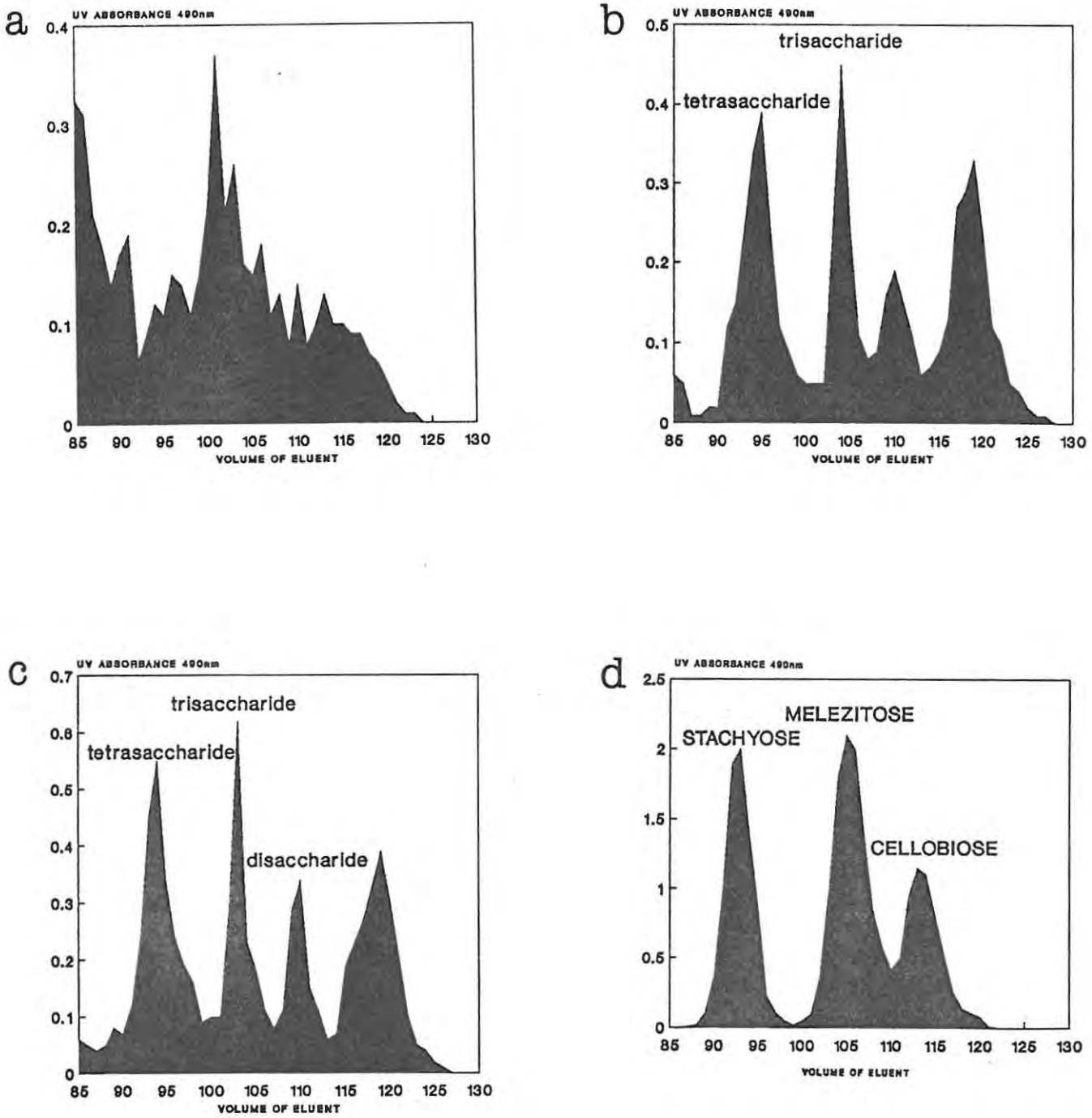


Figure B3 Gel permeation profiles[a] from the reductive cleavage[b] of methylated *E. coli* K57 capsular polysaccharidge with (a) $BF_3 \cdot Et_2O$ (b) Me_3SiOSO_2Me (c) $Me_3SiOSO_2CF_3$ (d) Standards[c].

a Small amounts of polysaccharide were present, but are not shown.

b 25 μl CH_2Cl_2, 10 μl triethylsilane, 10 μl catalyst.

c Methylated stachyose, melezitose and cellobiose.

The $Me_3SiOSO_2CF_3$ containing mixture was evaluated further because it gave a greater percentage of oligosaccharides than the Me_3SiOSO_2Me catalysed reaction. The mixture was allowed to react with samples for 20, 40 and 60 minutes (Figure B4, shows gpc profiles for the 20 and 40 minute reaction times). The yield of larger oligosaccharides decreased in proportion to the reaction time.

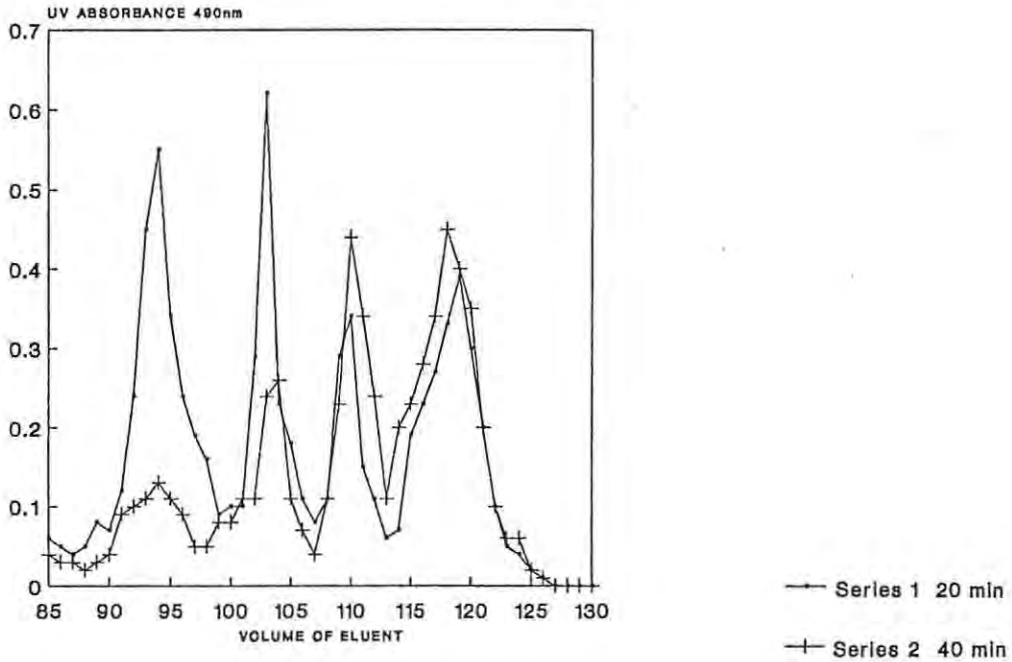


Figure B4 Gel permeation profile of a SRC after 20 minutes (series 1) and 40 minutes (series 2).

STAGE TWO (B)-Preparative SRC

Two preparative SRCs were attempted. The first, on 50 mg of MP, yielded 19 mg of the (methylated) trisaccharide and 23 mg of the (methylated) tetrasaccharide respectively. The second attempt was on 52 mg of TDMP. (The reagents were diluted in an attempt to produce a larger proportion of the tetrasaccharide relative to the trisaccharide). The products were separated by gpc and 12 mg of the TDM tetrasaccharide and 10 mg of the TDM trisaccharide anhydroalditol were recovered. Polymeric material (30%) was also recovered. The lower percentage yield of this attempt may be ascribed to the weaker reagent concentration and the possibility that the methylated and

trideuteriomethylated polymers react at different rates to the RC reagents.

10.2.3.2 Characterisation of the SRC Products

10.2.3.2.1 Nmr and Fab-ms Study of the TDM Trisaccharide

The fragments were examined by 1D and 2D nmr spectroscopy, and fab-ms. The TDM trisaccharides, isolated from the preparative SRCs in Stages One and Two, gave superimposable, extremely well resolved spectra (Spectra B1 and B2). The proton spectrum showed (Table B2), anomeric signals at δ 5.10 (^3J 3.3 Hz) and δ 4.78 (^3J 3.5 Hz) as well as peaks at δ 3.84 (^3J 9.5 Hz) and δ 4.02 (^3J 4.8 Hz); the latter are the protons attached to C-1 of the anhydroalditol. The carbon-13 spectrum had two signals (95.2 ppm, 96.2 ppm) in the anomeric region.

It was interesting to note that because the molecule was O-trideuteriomethylated the α and β effects of substituents averaged out and there was little or no observable glycosylation shift to lower field for the linkage carbons.

The residues with anomeric proton resonances at 5.1 and 4.78 ppm were given the (arbitrary) labels a and b, respectively; the anhydroalditol residue was labelled c. A COSY spectrum (Figure B5) of this oligomer allowed correlation maps from H-1 to H-5 of a, H-1 to H-6' of b, and H-1 to H-5' of c and all the correlation peaks were observed and were fully accounted for. The assigned protons were shift correlated with the signal from their bonded carbons *via* a HETCOR spectrum (Figure B6). All the ^{13}C signals showed distinct crosspeaks in the HETCOR spectrum with the exception of a peak at 75.7 ppm and this was

presumed to be C-4 of c which was the only unassigned carbon. On the

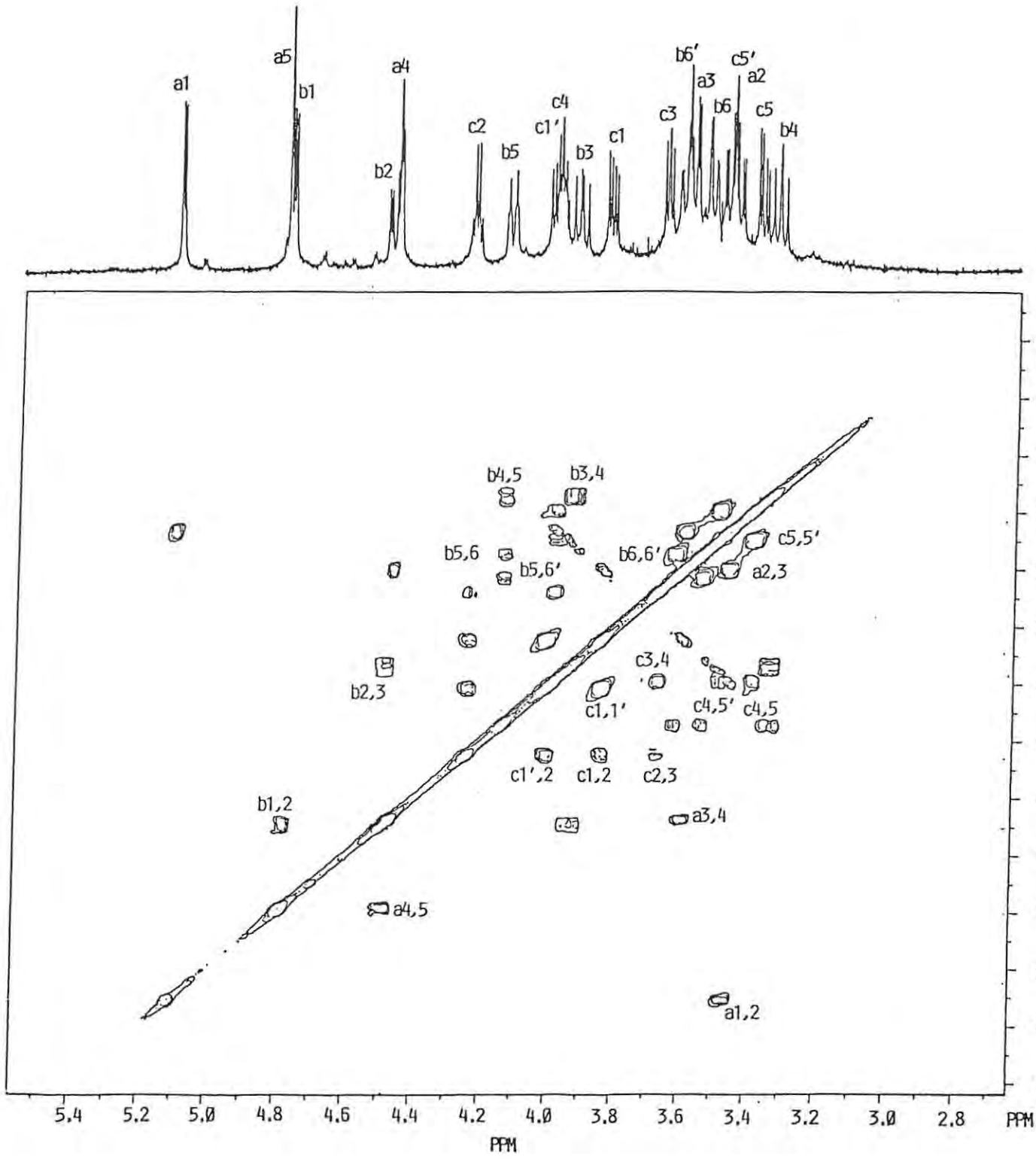


Figure B5 COSY spectrum of the trisaccharide anhydroalditol.

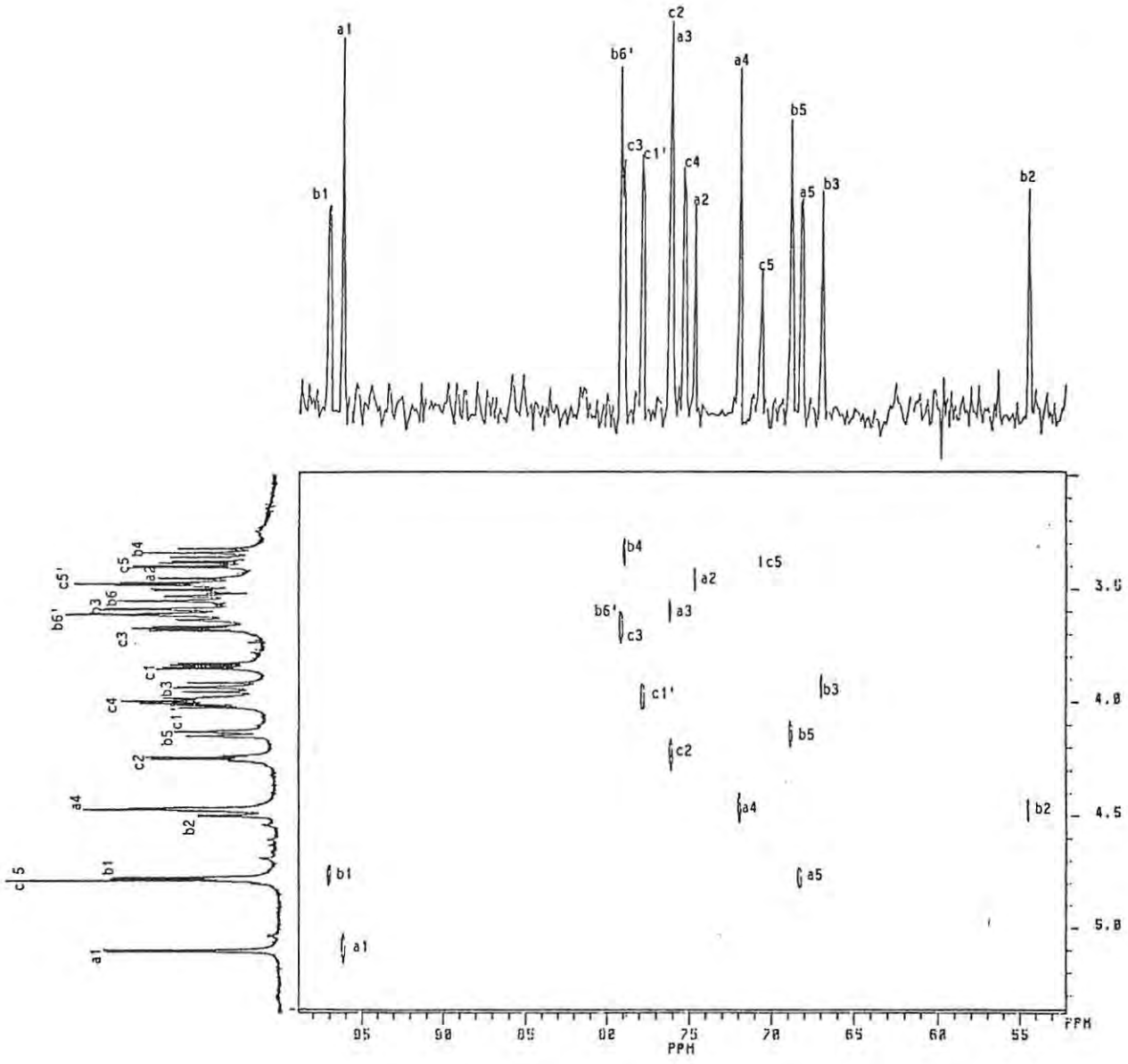


Figure B6 Heteronuclear shift correlation spectrum (HETCOR) of the trisaccharide anhydroalditol.

basis of this data, it was possible to identify residues a, b and anhydroalditol c as galacturonic acid, glucosamine and anhydroribitol, respectively. The shift differences notwithstanding, the residues have practically the same coupling constants and pattern of chemical shifts as those observed for these residues in nmr spectra of the polysaccharide (see section 10.1).

Fab-ms of the TDM trimer gave a major signal at $^{m}/_{z}$ 650 in the positive ion spectrum (Figure B7); an A-type fragment ion for the non-reducing terminal sugar (a hexNAc) with a free hydroxyl group was present at $^{m}/_{z}$ 255. The ion of highest mass indicates that this fragment is composed of a hexuronic acid, hexNAc and an anhydroribitol and hence complements the nmr data.

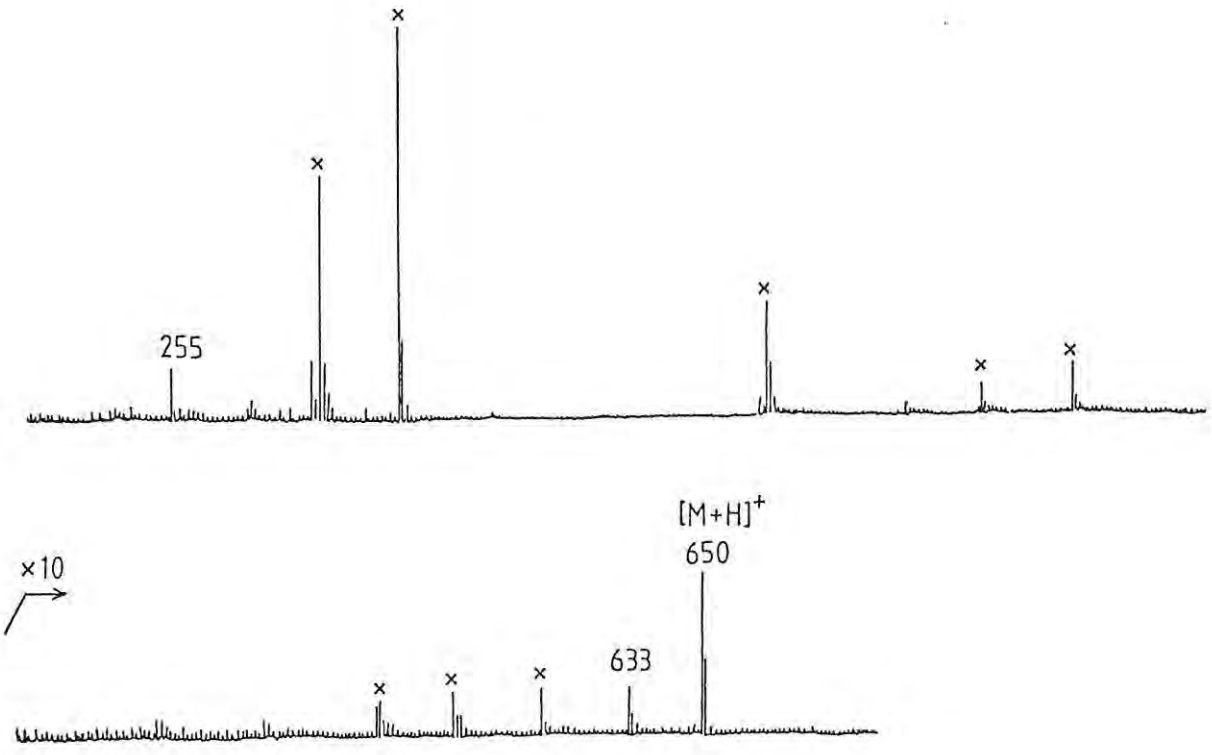


Figure B7 The fab-ms positive ion spectrum of the trisaccharide obtained from the SRC of *E. coli* K57 MP.

x- Peaks derived from the meta-nitrobenzyl alcohol matrix.

10.2.3.2.2 **Nmr and Fab-ms Study of the TDM Tetrasaccharide**

The tetrasaccharide gave an excellent proton spectrum (Spectrum B3). In addition to those signals, previously labelled a1, b1 and c1, c1', there was a further anomeric ^{1}H signal at δ 4.36 (^{3}J 7.4Hz) and this was labelled d1. The COSY spectrum (Spectrum B5) of this fragment allowed the full correlation map of the protons from residue a, b and c to be constructed, however only a partial connectivity map (H-1 to H-4) was discernible for residue d. This residue, which by difference must be a galactose, lacks a H-4/H-5 crosspeak in the COSY spectrum and this is not uncommon with this type of residue[112,212]. Nevertheless, the H-5/H-6, H-5/H-6', and H-6/H-6' crosspeaks were clearly evident and a map could be constructed for these three protons. Thus, all ^{1}H resonances could be assigned. Fab-ms positive ion spectrum of the tetramer had a major $[M+H]^{+}$ signal at $^{M}/_{z}$ 863 (one hex higher than the trisaccharide) and a signal at $^{M}/_{z}$ 885 for the sodium cationised molecular ion. There was an A-type cleavage at the hexNAc which afforded a major fragment ion at $^{M}/_{z}$ 468, ie a OH_1-hex_1-$hexNAc_1^{+}$ ion.

A fab-ms methanolysis experiment[244] on the tetrasaccharide gave the sequence of the units. The major products (after 20 min of methanolysis at 60°) gave molecular ions at $^{M}/_{z}$ 514, 650 and 727 which correspond to:

(OH)hexNAc$_1$ hexuronic acid$_1$ - methylglycoside

(OH)hexNAc$_1$ hexuronic acid$_1$ - anhydroribitol

(OH)hex$_1$ hexNAc$_1$ hexuronic acid $_1$ - methylglycoside

respectively. This indicates that the hexose is the non-reducing end and that the ribose is the anhydroalditol part of the tetramer.

Combined with the knowledge of the composition of the trisaccharide we can confidently state that the sequence of the tetramer is Gal-GlcNAc-GalA-anhydroribitol. The experimental evidence described allows the following structures to be written for the SRC products:

Trisaccharide

[α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→2)-anhydroribitol]
3 per-O-trideuteriomethylated
↑
OH

Tetrasaccharide

[β-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→2)-anhydroribitol]
4 per-O-trideuteriomethylated
↑
OH

10.2.4 Conclusions

The reductive cleavage reaction has been applied selectively to the TDMP derivative of *E. coli* K57 capsular polysaccharide resulting in the cleavage of only one or two bond types, thereby producing two oligomers as the major products. It can be seen from their structures that the 2-O β-ribofuranosyl linkages are highly susceptible to RC and that, while the 3-O linked β-galactosyl linkages are labile, they do not react as rapidly with the reagents. If they did anhydrogalactose-terminated fragments would also be present. The differential reaction rates made it possible to produce a tetrasaccharide that corresponded to an analogue of the polysaccharide repeat unit.

The fragments generated in a SRC are terminated by a non-reducing and an anhydroalditol sugar and hence they do not have the same "anomeric complications" as the reducing oligomers. The etherification of all the free hydroxyl groups in the polysaccharide means that the SRC products are essentially non-polar and soluble in organic liquids, which has several advantages when examining these molecules by nmr

spectroscopy. Furthermore, their volatility prevents extensive pyrolysis occurring during the acquisition of mass spectra by one of the desorptive techniques and allows the formation of prominent molecular ions and large mass fragments. Therefore, methylated anhydroalditols can either be fully characterised by nmr spectroscopy or partially elucidated by mass spectrometry which has the advantage of being extremely sensitive, so μg amounts will suffice.

The selectivity of a RC reaction depends upon the nature of the catalyst in the reagent mixture. Fortunately, because a wide range of saccharides (containing commonly encountered interglycosidic bond types) are being treated with RC reagents containing different catalysts and the products characterised, the data concerning the various susceptiblities of the different bond types grows continually[131,135,136,245]. It will, therefore, become easier to choose the appropriate reagent combination for the SRC of a polysaccharide.

10.2.5 Experimental

10.2.5.1 General Methods. - The capsular polysaccharide used for this SRC was isolated and characterised as described previously in 10.1. The polysaccharide was methylated *via* a modified[88] Hakomori[82] method using either potassium dimsyl and methyl iodide to give MP or trideuteriomethylated using potassium dimsyl and trideuteriomethyl iodide. The derivatised material was purified by passage down a Sephadex® LH-20 gel column (1 x 15cm) eluted with $CHCl_3$. It was filtered through a 0.45 μm Millipore® filter and examined by IR to confirm that O-methylation or O-trideuteriomethylation was complete. The oligosaccharides cellobiose, melezitose and stachyose were methylated by the same procedure. Hydrolysis of the derivatised

polysaccharide (4M TFA, 120°, 1 h) followed by O- and N-acetylation (1:1 acetic anhydride $^{v}/_{v}$, 100°, 1 h) of the residues gave the expected products from a 3-O linked glucosamine, 2-O linked ribose and a 4-O linked galactose. Analytical glc was performed using a Hewlet-Packard® model 5890A gas-liquid chromatograph equipped with a Hewlet-Packard® model 3392A integrator, a flame-ionisation detector, a J+W scientific® fused silica capillary column (0.25 mm x 30 m) coated with OV-17 (0.25 μm film thickness). The column was maintained isothermally at 205° and the carrier gas was helium.

10.2.5.2 Reductive cleavage. - The reagents $Me_3SiOSO_2CF_3$, $BF_3 \cdot Et_2O$ and Et_3SiH for the RC were purchased from Fluka®, Aldrich® and Merck® respectively and Me_3SiOSO_2Me was prepared as described previously[131]. All reagents were dried over CaH_2. Samples (5 mg unless otherwise stated) were weighed into a 5 ml flask with a teflon covered stirrer bar and dried for 24 hours at 40° *in vacuo*. Thereafter a serum cap was fitted to the flask and the air was expelled with dry N2, the CH_2Cl_2, triethylsilane and a catalyst were then added sequentially. The reaction was allowed to proceed for a timed interval before 1 ml of methanol was added. The composition of the standard reagent mixtures was: Stage One- 100 μl CH_2Cl_2, 25 μl triethylsilane and 40 μl catalyst, and Stage Two- 25 μl CH_2Cl_2, 10 μl triethylsilane and 10 μl of catalyst. The preparative SRC in Stage One used 51 mg of TDMP sample, 100 μl CH_2Cl_2, 25 μl triethylsilane, 40 μl Me_3SiOSO_2Me. The first preparative SRC in Stage Two used 50 mg of MP, 250 μl CH_2Cl_2, 100 μl triethylsilane and 100 μl $Me_3SiOSO_2CF_3$ (20 min) and the second used 52 mg of TDMP, 350 μl CH_2Cl_2, 50 μl triethylsilane and 50 μl of $Me_3SiOSO_2CF_3$ (20 min).

10.2.5.3 Thin layer chromatography (tlc). - Mixtures to be separated by analytical or preparative tlc were deionised using an Amberlite®

MB-1 resin column (1 x 15 cm) and evaporated to dryness. The samples were made up in a small volume and spotted onto either an analytical (2.5 x 5 cm) glass backed Kieselguhr 60 tlc plate or a aluminium backed preparative plate (20 x 5 cm, Merck®) coated with GF_{254}, dried for 1 h at 110°. The solvent system used was a 9:1 $^{v}/_{v}$ mixture of freshly distilled $CHCl_3$ and methanol. Methylated components were visualised on the developed plate by spraying with 10% H_2SO_4 in ethanol and heating the plate at 110° until the spots appeared. The compounds on the GF_{254} plate were visualised with uv 254 lamp.

10.2.5.4 Gel permeation chromatography (gpc).- Mixtures were separated on a Sephadex® LH-20 column (1.6 cm x 70 cm) eluted with freshly distilled methanol at a flow rate of 4 mL/h; the reservoir was sparged with helium to remove dissolved air. The column was calibrated with methylated cellobiose, melezitose and stachyose (Figure B3), 1 mL fractions were collected, evaporated to dryness in a vacuum oven at 40°, and then 1.0 mL of methanol, 1.0 mL of phenol and 1.0 mL of sulphuric acid were added sequentially. The uv absorbance of these solutions were measured at 490 nm. The first preparative run of Stage Two was analysed by evaporating the fractions to dryness in a vacuum oven at 40°, they were made up to 2.0 mL and a 10% aliquot was removed and examined by the phenol/sulphuric acid method. From these data, gel permeation profiles (uv absorbance *vs* elution volume) could be plotted. For the second preparative run 1.0 mL fractions were collected and analysed by tlc.

10.2.5.5 Nmr spectroscopy - The ^{1}H spectra were recorded at 500 MHz with a Bruker® WM-500 spectrometer fitted with an Aspect® 2000 computer using standard Bruker® software. The spectra were measured in 0.4 mL $CDCl_3$ in a 5 mm tube at 303°K and referenced to $CHCl_3$ at δ 7.24. The COSY spectrum was recorded at 500 MHz using a COSY-45 pulse

sequence, with 512 scans and 40 experiments and a relaxation delay of 2 s. The spectra were symmetrised. ^{13}C-nmr and HETCOR spectra were recorded at 75 MHz on a Bruker® AM-300 spectrometer fitted with an Aspect® 3000 computer using standard Bruker® software and referenced to $CHCl_3$ at 77.0 ppm.

10.2.5.6 Fab-mass spectrometry. - Spectra were run in either a meta-nitrobenzyl alcohol matrix (the trisaccharide) or a thioglycerol matrix (the tetrasaccharide). The methanolysis experiment involved incubating the tetrasaccharide under methanolytic conditions at 60° for 40 minutes; aliquots were removed at 2 minutes, 10 minutes, 20 minutes and 40 minutes and then analysed by fab-ms.

TABLE B1

(a) ^{1}H AND ^{13}C-NMR DATA FOR THE METHYLATED TRI-SACCHARIDE FROM METHYLATED K57 CAPSULAR POLYSACCHARIDE

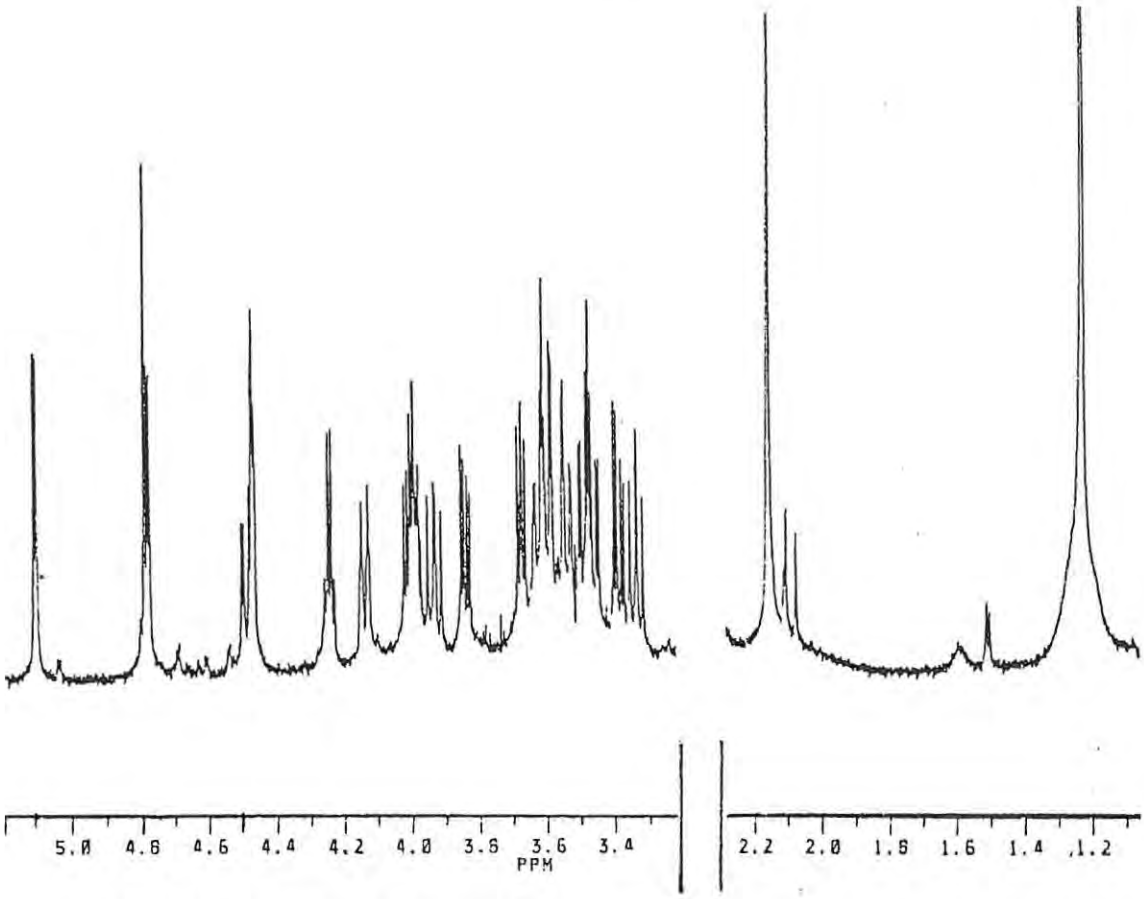
Residue:		1	1'	2	3	4	5	5'	6	6'	N-acetate
→4)-α-GalA	H	5.10[a]		3.46	3.60	4.47	4.79				
(a)	^{3}J	3.3		10.3	2.8	<1					
	C	95.2		74.8	76.6	72.0	68.4		172.9		
α-GlcNAc	H	4.78		4.50	3.94	3.34	4.14		3.55	3.64	2.16
(b)	^{3}J	3.5		10.7	9.5	10.4	(2.0,2.0)		10.5		
	C	96.2		54.7	67.0	78.9	68.9		79.4	23.87	
→2)-Anr1b[b]	H	3.84	4.02	4.25	3.67	3.98	3.39	3.50			
(c)	^{3}J	(9.5,4.8)	4.8	4.8	5.8	(4.5,3.8)	10.5				
	C	77.9		76.7	79.2	75.7	70.8				

(b) ^{1}H-NMR DATA FOR THE METHYLATED TETRASACCHARIDE FROM METHYLATED K57 CAPSULAR POLYSACCHARIDE

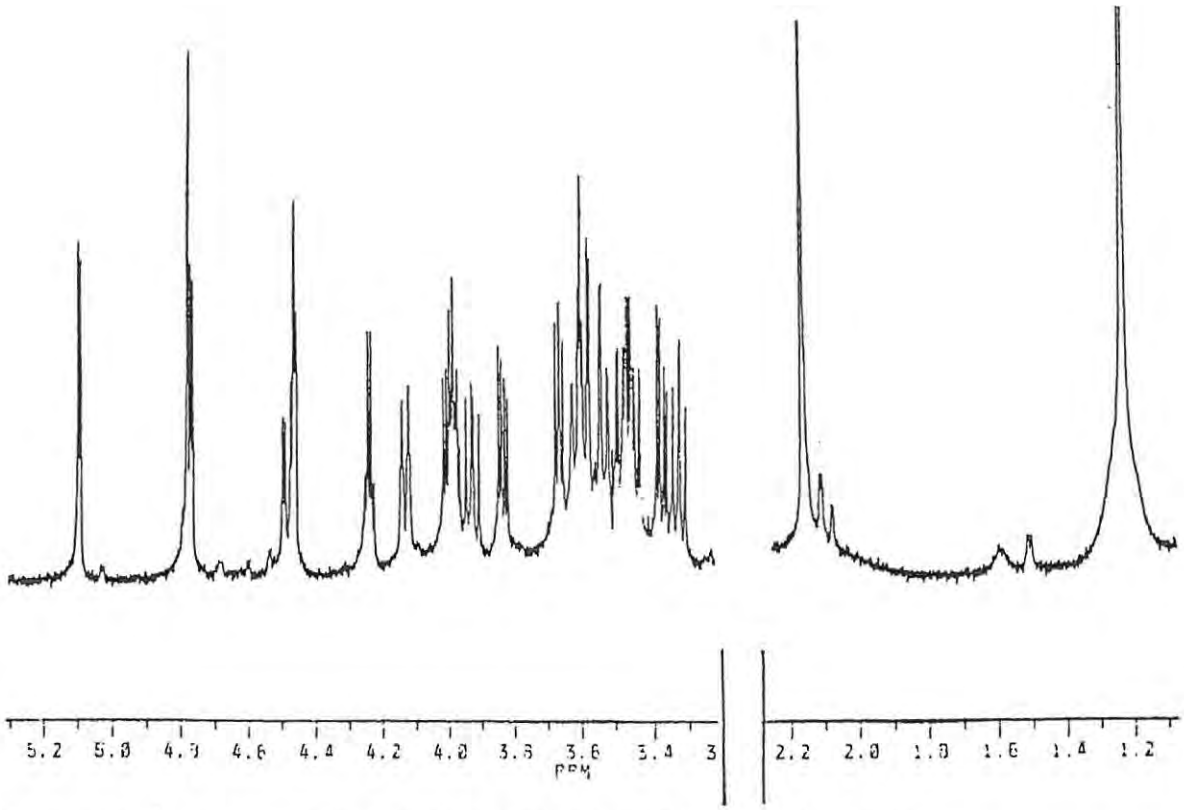
Residue:		1	1'	2	3	4	5	5'	6	6'	N-acetate
→4)-α-GalA	H	5.09[a]		3.46	3.58	4.45	4.80				
(a)	^{3}J	3.8		10.3	3.2	1.1					
α-GlcNAc	H	4.71		4.67	4.01	3.47	4.18		3.65	3.54	2.16
(b) 3	^{3}J	4.0		10.7	10.2	(2.3,2.3)			10.5		
↑											
→2)-Anr1b[b]	H	3.84	4.02	4.24	3.67	3.98	3.40	3.50			
(c)	^{3}J	(9.5,4.7)	4.7	4.7	5.6	(4.3,3.8)	10.5				
β-Gal	H	4.36		3.17	3.10	4.05	3.49		3.70	3.59	
(d)	^{3}J	7.4		9.5	3.3	<1	(6.4,4.8)		9.5		

[a] Chemical shifts in ppm relative to the signal for chloroform at δ 7.24, and coupling constants in Hz.

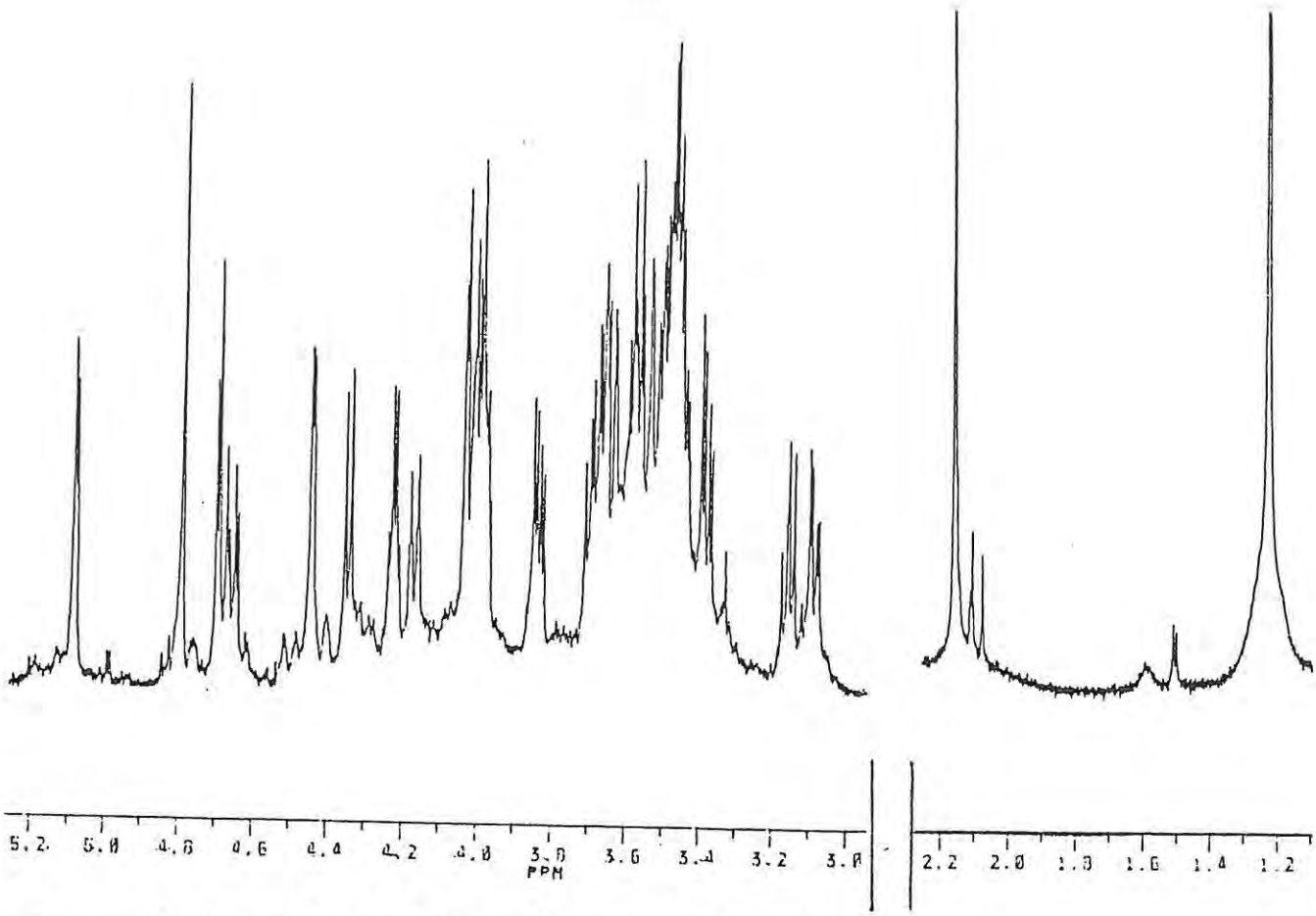
[b] Anrib = 1,4-anhydro ribose



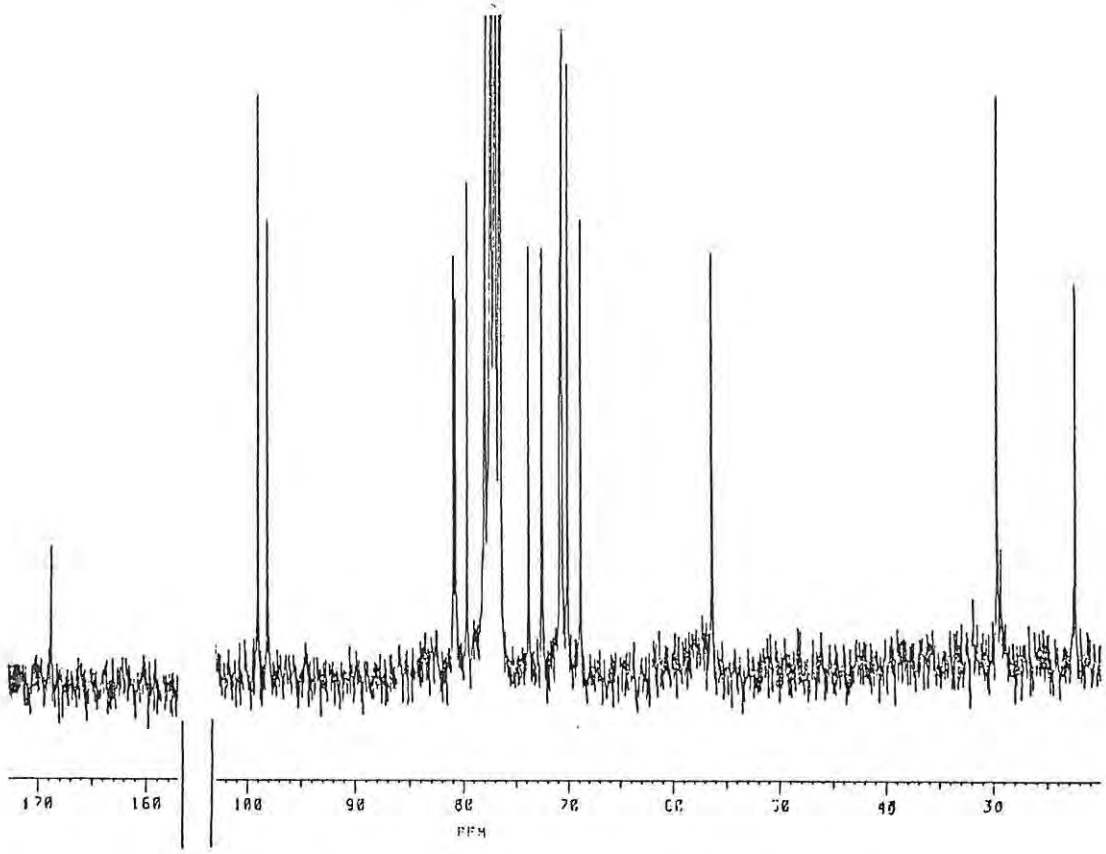
Spectrum B1 ^{1}H spectrum of the methylated trisaccharide isolated in stage one.



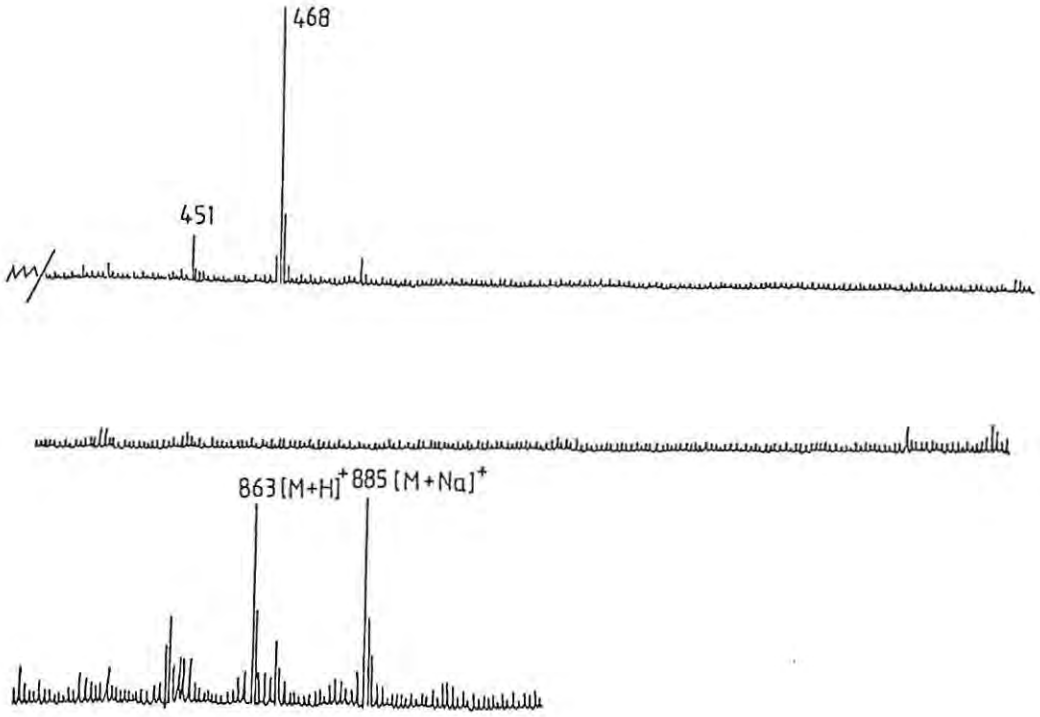
Spectrum B2 ^{1}H spectrum of the methylated trisaccharide isolated in stage two.



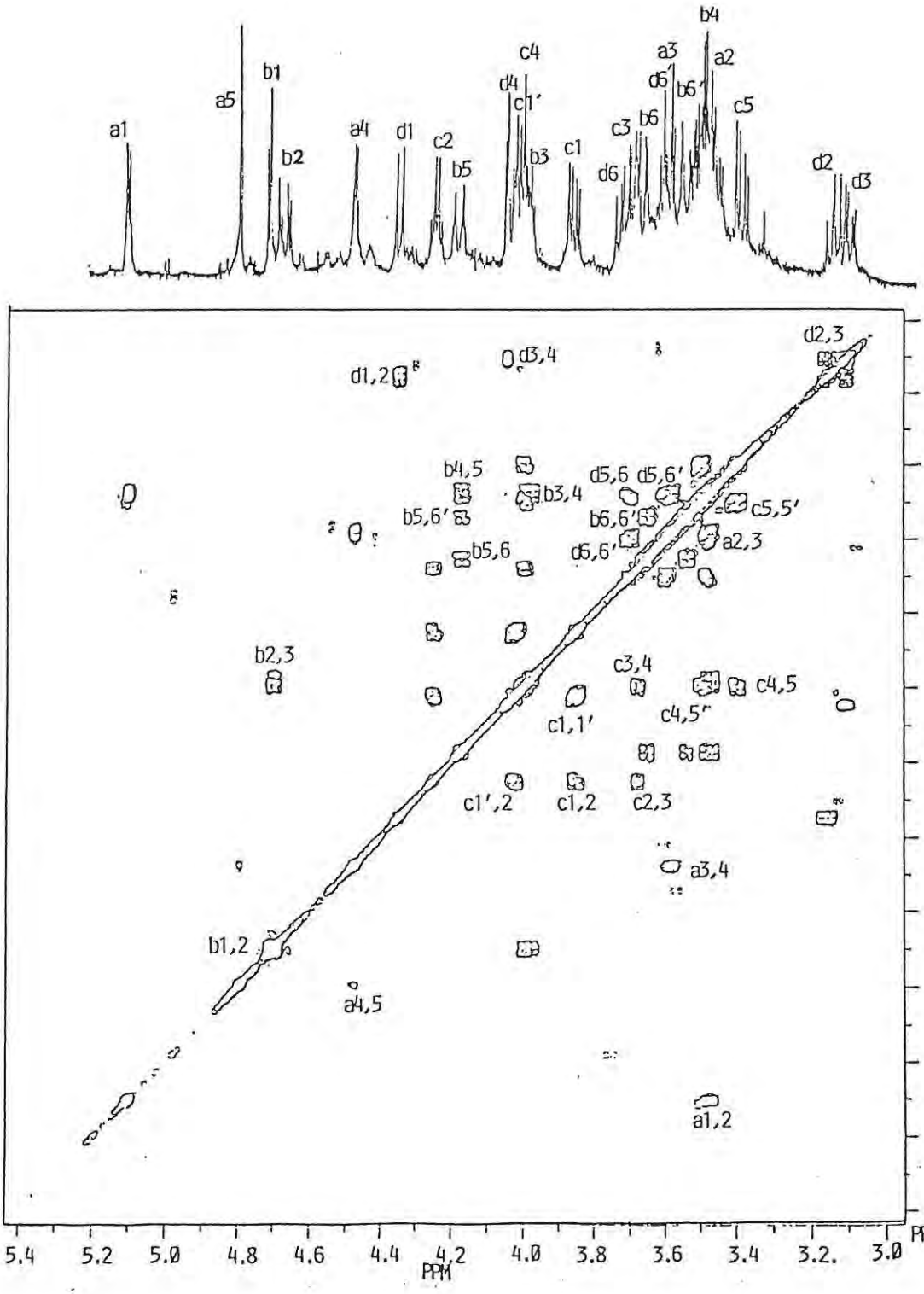
Spectrum B3 ^{1}H spectrum of the methylated tetrasaccharide isolated in stage two.



Spectrum B4 ^{13} spectrum of the methylated trisaccharide isolated in stage two.



Mass Spectrum B1 Fab-ms spectrum of the methylated tetrasaccharide isolated in stage two.[a]
[a] The protonated and sodium cationised molecular ions are at M/z 863 and 885 respectively.

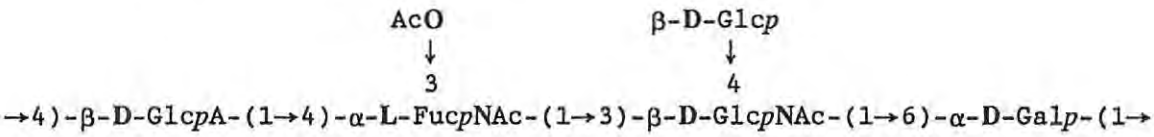


Spectrum B5 COSY spectrum of the methylated tetrasaccharide isolated in stage two.

10.3 THE STRUCTURE OF THE REPEAT UNIT OF THE CAPSULAR ANTIGEN FROM *ESCHERICHIA COLI* 08:K87:H19

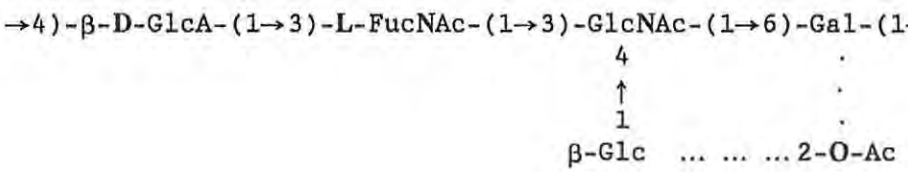
10.3.1 Abstract

The structure of the capsular polysaccharide from *Escherichia coli* 08:K87:H19 was investigated by methylation analysis and by one- and two-dimensional ^{1}H- and ^{13}C-nmr spectroscopy of the polysaccharide and a derived low molecular weight (M_r 15 000) polymer obtained from a HF catalysed partial hydrolysis. The repeat unit was shown to be a branched pentasaccharide with the structure:



10.3.2 Introduction

The acidic capsular antigen of *Escherichia coli* K87 has been reported[246] to consist of equimolar amounts of N-acetyl-L-fucosamine, and N-acetyl glucosamine, glucuronic acid, glucose, and galactose, and to have the partial structure:



This proposed structure was the same as that reported[4] for the polysaccharide component of *E. coli* O32 lipopolysaccharide and was thought to be an example of a polymer that occurs as either a group I capsular polysaccharide or as part of an acidic lipopolysaccharide[4].

Serological studies[246] of K87 have examined the influence of the K

antigen's chemical structure by monitoring the effect of oligosaccharides, obtained from an acid catalysed partial hydrolysis of the polysaccharide, on the inhibition of the immune precipitation in O8:K87 antiserum. An analogue of the repeat unit was, as expected, the best inhibitor of precipitation as this contained all the structural components of the polysaccharide except for a Gal-(1→4)-GlcA bond. Other oligosaccharides of different size and structure exerted varying degrees of inhibition and it was concluded from these data that the O-acetyl group was important and the major determinant of the K87 polysaccharide (in O8:K87 antiserum) was the pendant glucose.[246]

The present study completes the structure elucidation of the capsular polysaccharide from *E. coli* K87 (also known as G7[246]) and establishes the location of the O-acetyl group in the repeat unit. The K87 capsular polymer is one of six *E. coli* antigens (ie K27[247], K28[248], K33[17], K40[25], K42[249] and K87) that contain a FucNAc residue and it belongs to a small sub-group of polymers that have two amino sugars in the repeat unit; the others are K8[250], K44[251] and K85[252]. The K87 capsular polysaccharide belongs to the subgroup in group I which contain amino sugars.

10.3.3 Results and Discussion

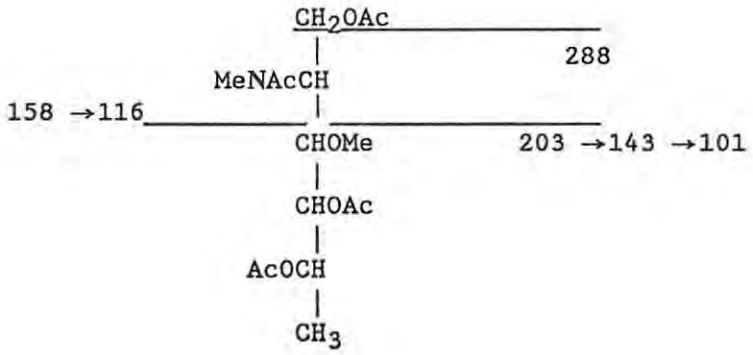
The *E. coli* strain O8:K87:H19 was grown on Mueller Hinton agar and seven trays in the first growth yielded 1.11 g of pure capsular material, however subsequent growths had up to 10% RNA contamination and were not used in this study. The gel permeation profile of the capsular polysaccharide, separated on a dextran calibrated Sephacryl® S400 HR gel column showed (Gel Profile C1) a monodisperse peak with a M_r of 300 000.

Previous results[246] that showed FucNAc, GlcNAc, Glc, Gal and GlcA as the sugar components of K87 capsular polymer were confirmed by glc analyses of the peracetylated aldononitrile (PAAN) derivatives[255] of the free sugars in a hydrolysate and methanolysate. The L stereochemical configuration of the FucNac was established previously[246], and the D configuration of the other residues was determined by glc analyses of the derived (-)-2-octyl glycoside acetates[74].

A portion of the polysaccharide from the first growth was partially depolymerised with liquid HF at -23° for 20 minutes. The major products had M_rs of approximately 15 000 and 20 000 respectively and were isolated by gel filtration on Sephacryl® S200 HR. These were labelled (Gel Profile C2) Fraction A and Fraction B respectively.

Methylation analysis of the polysaccharide, with and without reduction of the methyl-esters of the methylated product, gave the sugars listed in Table C1 (columns II and I). The results showed that the Glc was terminal, the GlcA, Gal, and FucNAc were 4-, 6- and 4-linked respectively and that the GlcNAc was linked through O-3 and O-4. Previously[246] the FucNAc was reported to be 3-linked, however the absence of primary fragments at $^{m}/_{z}$ 131 and 274 and the presence of fragments at $^{m}/_{z}$ 203 (primary), 143, and 101 in the ms of the derived alditol acetate confirmed that it was 4-linked. Some of the fragment

observed in the mass spectrum of the methylated FucNAc derivative are indicated in 1.



1

10.3.3.1 **Nmr study**

10.3.3.1.1 **1D Nmr Spectroscopy**

The 400 MHz ^{1}H-nmr spectrum of the polysaccharide (Spectrum C1) recorded at 40° contained eight one-proton signals in the δ 4.37 - 5.48 region of the spectrum. The signals at δ 5.48 (^{3}J 2.0 Hz), δ 5.18 (^{3}J 3.5 Hz), δ 4.73 (^{3}J 7.7 Hz), δ 4.52 (^{3}J 7.5 Hz), and δ 4.41 (^{3}J 7.8 Hz) were clearly anomeric signals while those at δ 4.91 (^{3}J 11.1 and 2.3 Hz), δ 4.86 (^{3}J 6.5 and <1 Hz), and δ 4.37 (^{3}J 3.8 and 11.1 Hz) arose from non-anomeric protons. Signals were also observed for the methyl group of a 6-deoxy sugar at δ 1.31 (^{3}J 6.5 Hz), for two NAc groups at δ 1.97 and δ 1.99 and for an OAc group at δ 2.09. The ^{13}C-nmr (Spectrum C2) data complemented the ^{1}H-nmr results showing *inter alia* five signals for anomeric carbons at 104.67, 102.47, 102.24, 99.35, and 97.85 ppm, a signal for a C-6 carbon of a 6-deoxyhexose at 16.05 ppm, signals for an OAc group and two NAc groups at 21.36 and 22.98 ppm respectively, for carbonyl carbons at 174.45, 174.64, and 174.80 ppm and for two C-2 carbons of amino sugars at

47.88 and 56.49 ppm. An APT spectrum[253] (Spectrum C3) of the polysaccharide showed signals for three hexopyranose C-6 (methylene) carbons at 60.47, 62.44, and 67.64 ppm. The latter signal suggested the presence of a 6-linked hexopyranose residue because of its downfield resonance position. The above data indicated that the polysaccharide consists of a pentasaccharide repeating unit composed of one acidic, two neutral and two amino sugars. According to the ^{1}H-nmr data (Table C2), three residues are β-linked and two are α-linked.

10.3.3.2 2D Nmr Spectroscopy

The location of the O-acetyl group and the sequence of residues in the repeating unit of the polysaccharide were established by 2D-nmr experiments. Furthermore, these experiments confirmed the glycosylation sites in the polysaccharide. Proton resonance assignments were made mostly from COSY[98,211], one- and two-step RELAY COSY[212,216], and NOESY[222] experiments at 400 MHz on the native polysaccharide and from COSY and 2D HOHAHA[219] experiments at 600 MHz on a partially depolymerised sample of the polysaccharide (Fraction B). The residues in the repeating unit were labelled a to e in order of decreasing chemical shift of the anomeric protons. The COSY spectrum (Figure C1) of the polysaccharide and Fraction B (Spectrum C5) did not allow a full correlation map to be constructed for any of the residues in the repeat unit. Thus, ^{1}H assignments were made, as follows, from a combination of the nmr experimental data.

Residue a

This sugar with a H-1 resonance peak at δ 5.475 had its proton signals assigned as far as H-3 using the COSY spectrum. The H-4 resonance was identified from the 2D HOHAHA experiment (Spectrum C4),

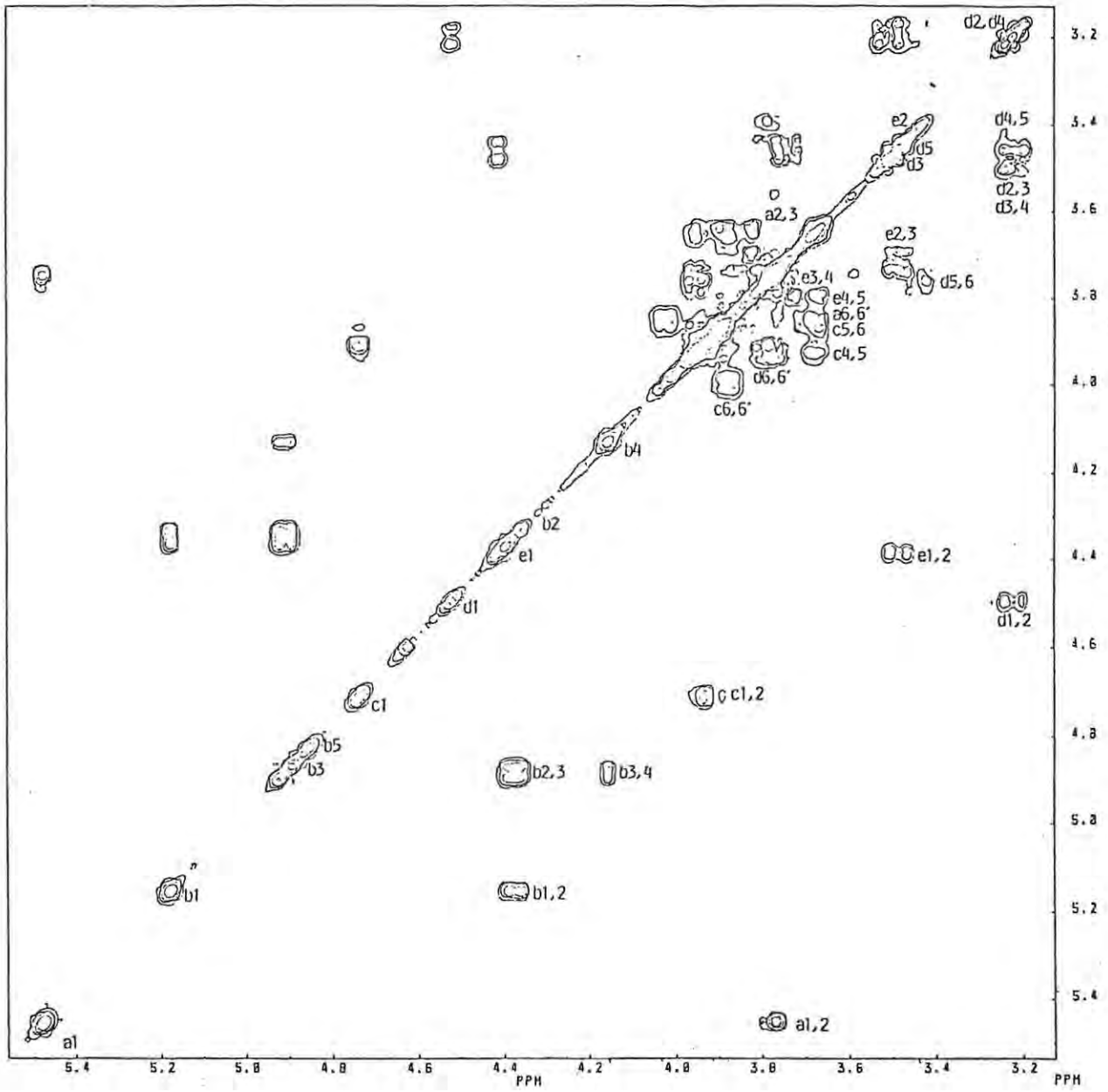


Figure C1 COSY spectrum of the polysaccharide from *E. coli* K87.

and the remaining protons were assigned as follows. The APT spectrum showed three hexopyranose methylene resonances and two of these were assigned to residues by identifying their H-6 and H-6' protons (from COSY, RELAY COSY, or the 2D HOHAHA spectra) and shift-correlating these to a C-6 signal using the HETCOR spectrum (Figure C2). This left a methylene carbon peak at 67.64 ppm unassigned and this was assumed to be C-6 of **a**, the remaining hexopyranose residue. This peak shift-correlates with ^{1}H resonances at δ 3.680 and δ 3.897, the H-6 and H-6' signals. There was no H-5/H-6 or H-5/H-6' crosspeak in the COSY spectrum. However, the C-6 ^{13}C resonance of residue **a** was shifted significantly downfield and indicated that this residue was linked at the 6 position and hence identified **a** as the 6-O linked galactopyranose. The C-5 resonance of this galactose was one of two signals remaining after the ^{13}C resonances were assigned using the HETCOR spectrum and assigned ^{1}H signals. The resonance at 68.91 ppm was closest to the literature value[186,238,239] for C-5 of a galactose; this ^{13}C signal shift-correlates with a ^{1}H peak at δ 3.890.

Residue **b**

The partial COSY correlation map for the ^{1}H signals of **b** extended from H-1 (δ 5.178) to H-4. The chemical shift of H-5 was established from the observed intramolecular nOe between H-4 and H-5 in the NOESY experiment (Table C2) and was confirmed by the presence of a H-4/H-5 connectivity peak in the one-step RELAY COSY spectrum (Spectrum C6). The remainder of the COSY connectivity map could be constructed by continuing from H-5.

Table C4 compares the ^{13}C-nmr data for the residue **b** and methyl-α-L-Fuc*p*NAc[254]. These data establish **b** as the 4-O linked α-L-Fuc*p*NAc.

Residue c

The COSY spectrum had a crosspeak between H-1 (δ 4.733) and H-2, and the H-3 value was established from the one-step RELAY COSY. However, the H-3/H-4 crosspeak in the COSY spectrum and H-1/H-4 in the two-step RELAY COSY (Spectrum C7) were absent. This peak was eventually assigned as follows: after the ^{13}C signals in the HETCOR spectrum were assigned and C-5 of **a** was identified from its shift value, the remaining resonance was, by default, C-4 of **c**. The H-4 resonance was then identified *via* the HETCOR spectrum. Evidently the COSY crosspeak was not apparent because the H-4 resonance overlapped those of H-2 and H-3 of **c**. The H-5 peak was identified from the intramolecular nOe observed between H-1 and H-5 and, from this point, H-5/H-6 and H-6/H-6' crosspeaks could be located in the COSY spectrum.

Residue d

The H-1 peak of **d** at δ 4.520 correlated with H-2. The H-2/H-3 connectivity peak was, however, difficult to identify in the COSY spectrum. The H-3 chemical shift was assigned from the one-step RELAY COSY, but the H-1/H-4 correlation peak was not apparent in the two-step RELAY COSY spectrum. The peaks H-4, H-5, H-6 and H-6' could, however, be identified from a 2D HOHAHA spectrum of Fraction **B**. These data showed (Table C2) that resonances H-2 and H-4 overlap and explained why the H-1/H-4 crosspeak was not observed in the two-step RELAY COSY spectrum.

Residue e

The COSY spectrum of the polysaccharide showed a crosspeak between H-1 (δ 4.417) and H-2, and the chemical shifts of H-3 and H-4 were readily

identified from the one- and two-step RELAY COSY spectra. The shift value of H-5 was obtained from the intramolecular nOe betwen H-1 and H-5 of this β-residue in the NOESY spectrum (Table C3).

Comparison of the ^{1}H and ^{13}C-nmr data (Table C2) for residues **a**, **c**, **d**, and **e** with literature values for methyl glycosides[186,238,239] permitted the residues to be identified as:

a →6)-α-Gal

c →3,4)-β-GlcNAc

d β-Glc

e →4)-β-Glc A

Examination of the ^{13}C-nmr data revealed that as expected from the methylation analysis results (Table C1), C-6 of **a**, C-3 and C-4 of **c**, and C-4 of **e** were significantly deshielded and therefore indicated linkage carbons. There was also a downfield shift of approximately 2 ppm for C-3 and an upfield shift of approximately 3 ppm for C-2 of **b** and this indicated that the O-acetyl was linked to the 3 position of this sugar. This was confirmed by the ^{1}H-nmr data (Table C2) which showed that the H-3 proton of **b** is about 1.1 ppm downfield from its more usual position of resonance around δ 3.8[186].

Data from the NOESY spectrum (see Table C3 for the nOe correlations) confirmed the anomeric stereochemistry and established the sequence of the sugars in the repeat unit. The α-residues (**a**, **b**) showed, as expected[225], intramolecular nOes from the anomeric proton to H-2,

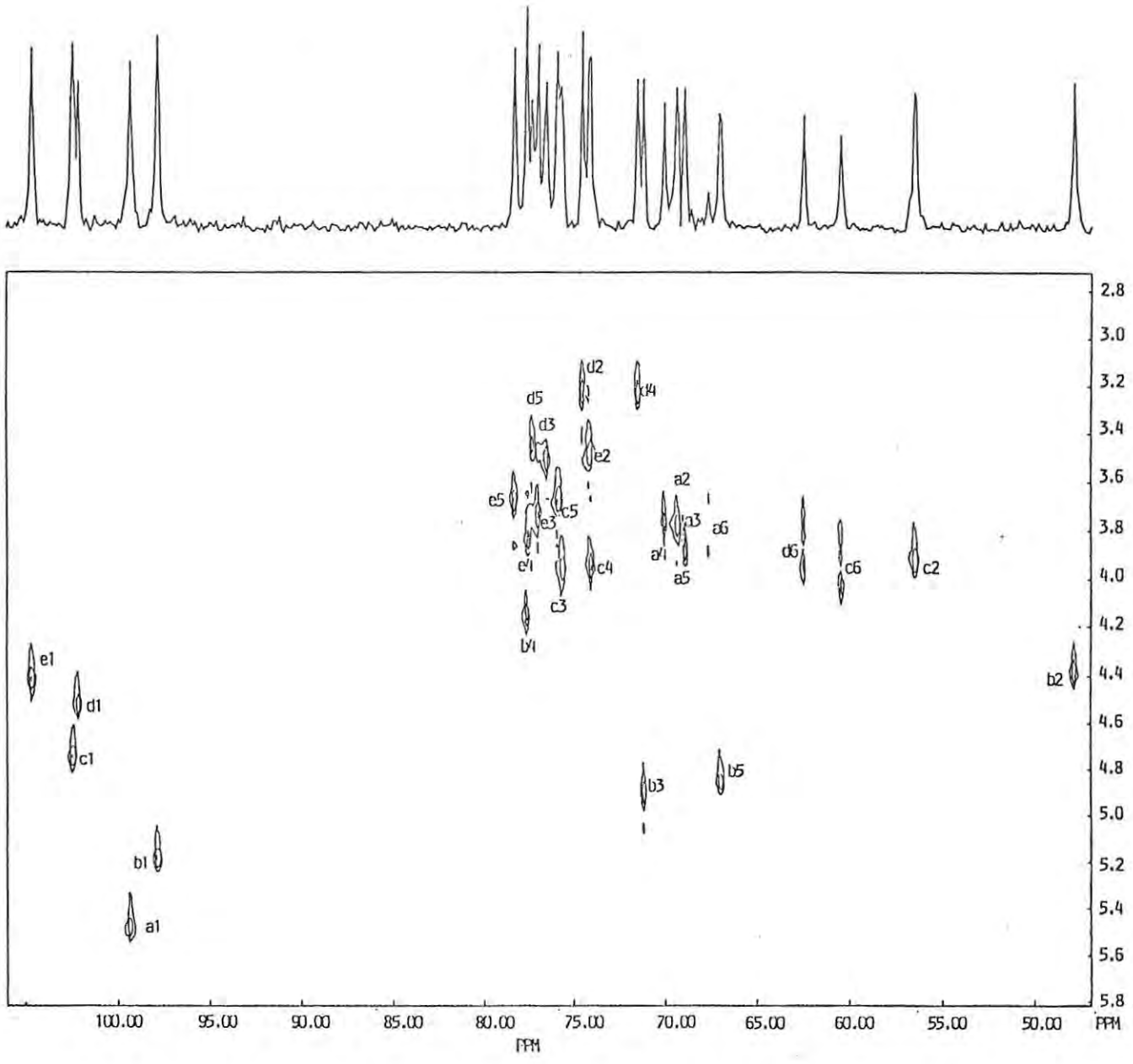
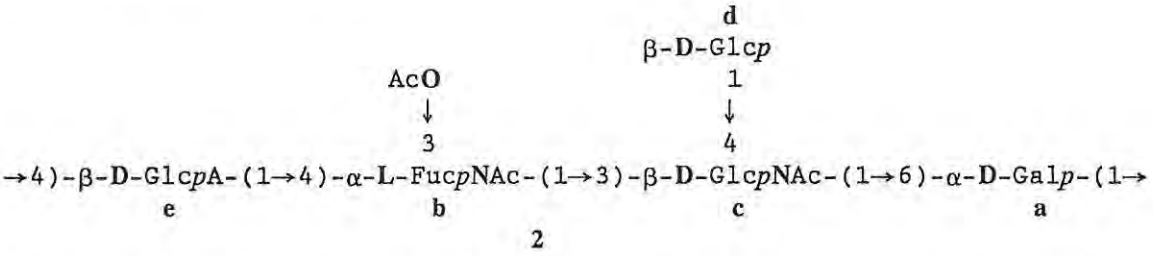


Figure C2 HETCOR spectrum of *E. coli* K87 capsular polysaccharide.

while the β-residues (c, d, e) showed characteristic[225] contacts between H-1 and the peaks from H-3 and H-5. Intraresidue nOe crosspeaks were also clearly observed (Table C3) and hence the sugar sequence could be established.

The combined nmr and methylation analysis data permitted the structure of the pentasaccharide repeating unit of the capsular polysaccharide of K87 to be written as **2**.



The structure **2** established for the repeating unit for the *E. coli* K87 polysaccharide differs in two major respects from that previously reported[246]. Structure **2** shows the α-L-Fuc*p*NAc residue 3-O-acetylated and linked in position 4 whereas the previously reported partial structure had the α-L-FucNAc 3-linked and the OAc group tentatively assigned to C-2 of either the glucose or galactose residue.

10.3.4 Experimental

10.3.4.1 General methods. - Analytical glc was performed using a Hewlett-Packard® 5890A gas chromatograph fitted with flame ionisation detectors and a 3392A recording integrator; helium was used as the carrier gas. A J+W Scientific® fused silica DB-17 bonded-phase capillary column (30 m x 0.25 mm) with a film thickness of 0.25 μm was used for separating methylated alditol acetates with the temperature programmed from 180° for 1 minute to 240° at 3°/min. Glc-ms was conducted using a Hewlett-Packard® 5988A mass spectrometer. A J+W

Scientific® fused silica DB-225 bonded-phase capillary column (30 m x 0.25 mm) with a film thickness of 0.25 μm was used for separating acetylated aldononitrile derivatives with the temperature maintained isothermally at 210°. The polysaccharide was hydrolysed at 120° (4M TFA) for 1 h and the acid was removed by co-distillation with water under a vacuum at a temperature ≤40°. Carboxyl reduction (methanolysis) was achieved by treating the sample with refluxing methanolic 3% hydrogen chloride for 16 h, the acid was neutralised with AG_2CO_3 and the methyl esters formed were reduced with sodium borohydride in anhydrous methanol. This sample was hydrolysed with 4M TFA for 30 minutes at 120° and the sugars were transformed into per-acetylated aldononitriles according to the method of McGinnis.[255] Methylation was carried out using the Hakomori method as modified by Sandford and Conrad[256] using potassium dimsyl. The methylated product was divided into two portions. One was hydrolysed with 4M TFA for 1 h at 120°, the hydrolysate reduced with sodium borohydride (1 h) and the derived alditols acetylated with 1:1 $^{v}/_{v}$ acetic anhydride-pyridine (1 h, 100°) to produce alditol acetates. The other portion was methanolysed and converted to the alditol acetate derivatives. Analysis of both by glc-ms[161-163] allowed the assignments in Table C1, columns I and II. Octylglycoside acetates were derived as described in section 10.1.4.

10.3.4.2 Preparation of *E. coli* K87 polysaccharide. - An authentic culture of *E. coli* O8:K87:H19 was obtained from Dr I Ørskov (Copenhagen) and the bacteria were propagated at 37° on Mueller-Hinton agar (1.5 L, 1650 cm^2 per tray). The cells were harvested and the polysaccharide purified as described in section 10.1.4. The yield per tray was 159 mg. The polysaccharide gave a single peak at M_r 300 000 on gel-permeation chromatography using a dextran calibrated Sephacryl® S400 HR column eluted with Na acetate buffer (pH 5), the effluent was

monitored with a Waters® model R401 differential refractometer coupled to a Rikadenki® model R-01 flatbed recorder.

10.3.4.3 Partial HF hydrolysis of polysaccharide. - Polysaccharide (200 mg) in liquid HF (approximately 2 mL) was maintained at -23° for 20 minutes, the reaction was quenched with ether and concentrated to dryness. Fluoroglycosides were cleaved with 0.5M TFA at ambient temperature for 24 hours and then dialysed (12-14 000 MW cut-off) against distilled water. The retentate was freeze dried and separated on a column (70 x 2.6 cm) of Sephacryl® S200 HR eluted with 0.1M sodium acetate buffer (pH 5) at 35 mL/h. The effluent was monitored by differential refractometry and 1 mL fractions were collected. The peaks with a M_r of 20 000 (A) and 15 000 (B) were collected, desalted on an Amberlite® IR 120H$^+$ resin column and then freeze dried. The Fraction B (M_r 15 000) was re-separated.

10.3.4.4 Nmr spectroscopy. - Samples were deuterium-exchanged by freeze-drying in D_2O then dissolved in 99.99% D_2O (0.45 mL) containing a trace of acetone as internal reference, δ 2.23 for ^1H and 31.07 ppm for ^{13}C. Spectra were recorded at 40° on either a Bruker® WH-400, AM-400 or AM-600 spectrometer, equipped with an Aspect® 3000 computer and an array processor, using standard Bruker® software.

^1H Homonuclear shift-correlated experiments (COSY[98,211] and one- and two-step RELAY COSY[212,216]) and homonuclear dipolar-correlated (NOESY[222]) experiments at 400 MHz were performed using a spectral width of 1838 Hz. Data matrices of 256 x 1024 data points were collected for 32 or 112 transients for each t_1 delay. The matrices were zero-filled in the t_1 dimension and transformed in the magnitude mode by use of a non-shifted sine-bell window function in both dimensions and symmetrised. Digital resolution in the resulting 512 x

1024 matrices was 3.6 Hz per point. A COSY experiment at 600 MHz was performed using a spectral width of 2994 Hz. A data matrix of 256 x 1024 data points was collected for 16 transients for each t_1 delay. The matrix was zero-filled in both dimensions and transformed in the magnitude mode by use of a non-shifted sine-bell window function. Digital resolution in the resulting 512 x 2048 maxtrix was 5.8 Hz and 2.9 Hz per point in the F_1 and F_2 dimensions respectively. Relaxation delays of 1.0 to 1.3 s were used. The mixing delay in the NOESY experiment was 0.3 s. Homonuclear Hartmann-Hahn (HOHAHA) spectra were obtained according to ref 219. The spectral width was 2994 Hz, the 180° pulse width was 54 μs and the mixing periods used consisted of 15 and 60 MLEV-17 cycles respectively. Data matrices of 512 x 2048 were acquired for 32 transients for each t_1 delay. The matrices were zero-filled in the t_1 dimension and multiplied in both dimensions with a phase-shifted sine-square function prior to phase-sensitive FT to obtain 1024 x 2048 K data matrices.

A ^{13}C-^{1}H shift-correlated (HETCOR)[99] experiment was recorded using a spectral width in F_2 of 11900 Hz (117.9 ppm) and 2500 Hz (6.25 ppm) in F_1. The initial matrix of 256 x 2048 was transformed to 512 x 2048 data points and processed with Gaussian functions. Digital resolution in F_2 was 11.6 Hz/point and in F_1 9.8 Hz/point. A recycle delay of 1.5 s was employed and 1200 transients per fid were collected.

TABLE C1

METHYLATION ANALYSIS OF POLYSACCHARIDE

Sugar[a]	T[b]	Molar ratio[c]	
		I	II
2,3,4,6-Glc	1.00	1.28	0.90
2,3,4-Gal	1.40	1.00	1.00
2,3-Glc	1.58		0.60
2,3-Fuc-NAc	1.82	0.93	0.71
2,6-GlcNAc	2.49	1.05	0.59

[a] 2,3,4,6-Glc = 2,3,4,6-tetra-methyl-D-glucose, etc.
[b] Relative retention time of the derived alditol acetate.
[c] I, methylated polysaccharide; II, methylated, carboxyl-reduced polysaccharide.

TABLE C2

NMR DATA[a] FOR *E. coli* K87 POLYSACCHARIDE

Atom	Unit **a** →6)-α-Gal	Unit **b** →4)-αFucNAc	Unit **c** →3,4)-β-GlcNAc	Unit **d** β-Glc	Unit **e** →4)-β-GlcA
H-1	5.475	5.178	4.733	4.520	4.417
C-1	99.35	97.85	102.47	102.24	104.67
H-2	3.792	4.375	3.914	3.221	3.483
C-2	69.31	47.88	56.49	74.57	74.17
H-3	3.801	4.908	3.948	3.513	3.746
C-3	69.03	71.22	75.67	76.59	77.00
H-4	3.745	4.153	3.935	3.321	3.848
C-4	70.06	77.69	74.17	71.51	77.52
H-5	3.890	4.855	3.678	3.439	3.675
C-5	68.91	67.00	75.90	77.29	78.38
H-6	3.680	1.306	3.877	3.790	
C-6	67.64	16.05	60.47	62.44	
H-6'	3.897		4.024	3.951	

[a] Chemical shifts with acetone as internal reference, 2.23 and 31.07 ppm for ^{1}H and ^{13}C respectively.

TABLE C3

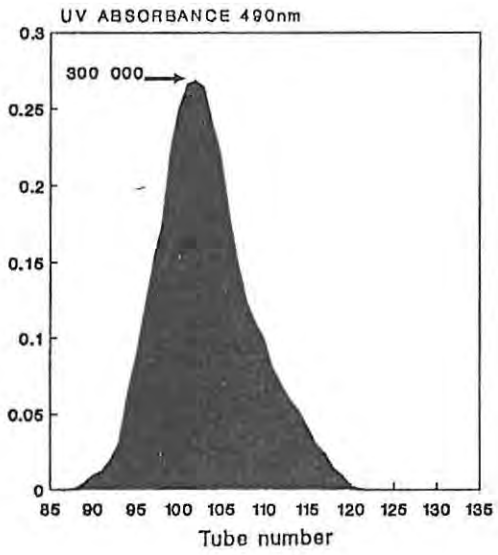
NOE CONTACTS FOR *E. coli* K87 POLYSACCHARIDE

Proton:	NOe contact to:
a, H-1	3.85 (**e**, H-4), 3.79 (**a**, H-2)
b, H-1	3.95 (**c**, H-3), 4.38 (**b**, H-2)
H-4	4.86 (**b**, H-5)
c, H-1	3.90 (**a**, H-6'), 3.95 (**c**, H-3), 3.68 (**c**, H-5)
d, H-1	3.94 (**c**, H-4), 3.51 (**d**, H-3), 3.44 (**d**, H-5)
e, H-1	4.15 (**b**, H-4), 3.75 (**e**, H-3), 3.68 (**e**, H-5)

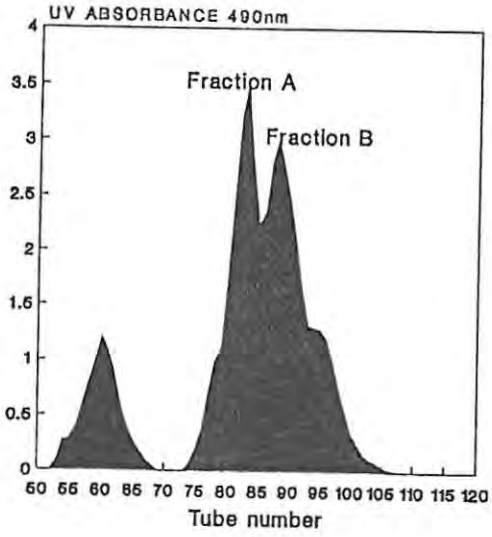
TABLE C4

COMPARISON OF THE ^{13}C-NMR DATA FOR RESIDUE b AND Me α-L-Fuc*p*NAc

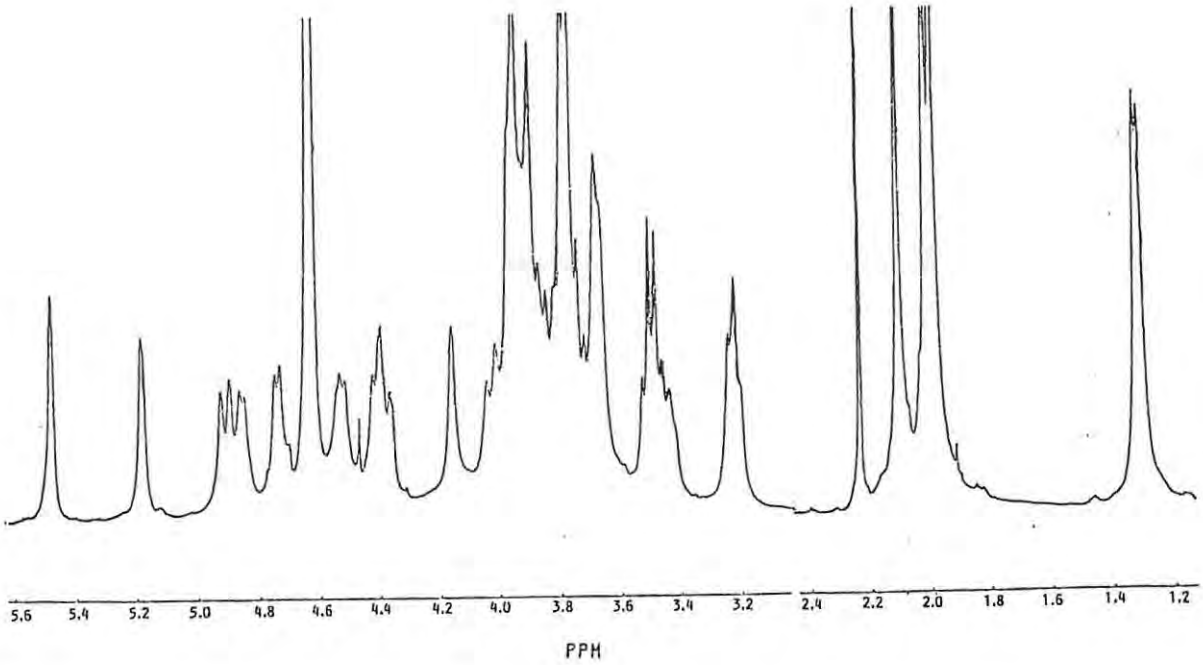
	C-1	C-2	C-3	C-4	C-5	C-6
Me α-L-Fuc*p*NAc[254]	99.3	50.8	69.1	72.2	67.6	16.6
Residue **b**	97.85	47.88	71.22	77.69	67.00	16.05



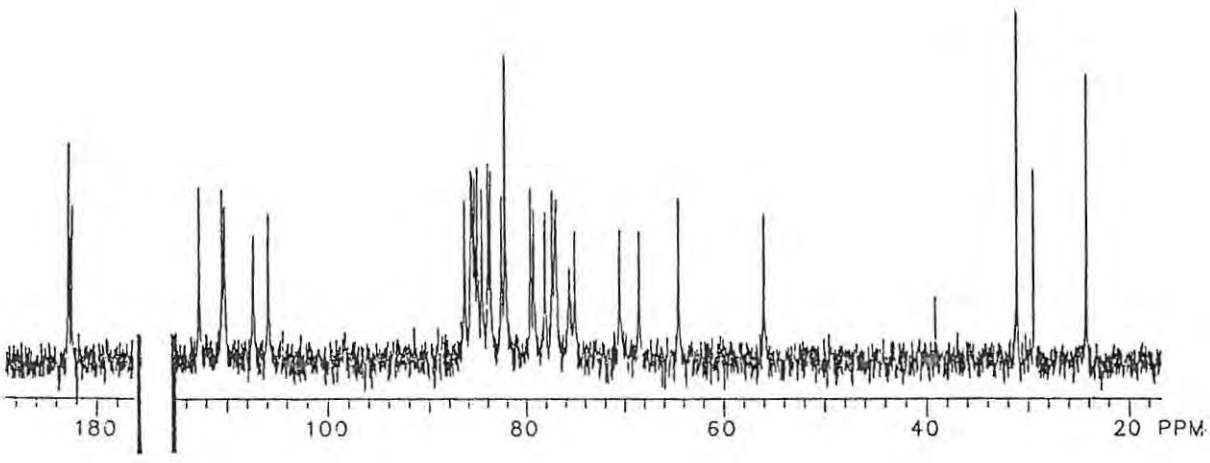
Gel Profile[a] C1 *E. coli* K87 capsular polysaccharide.
[a] Sephacryl S400 HR



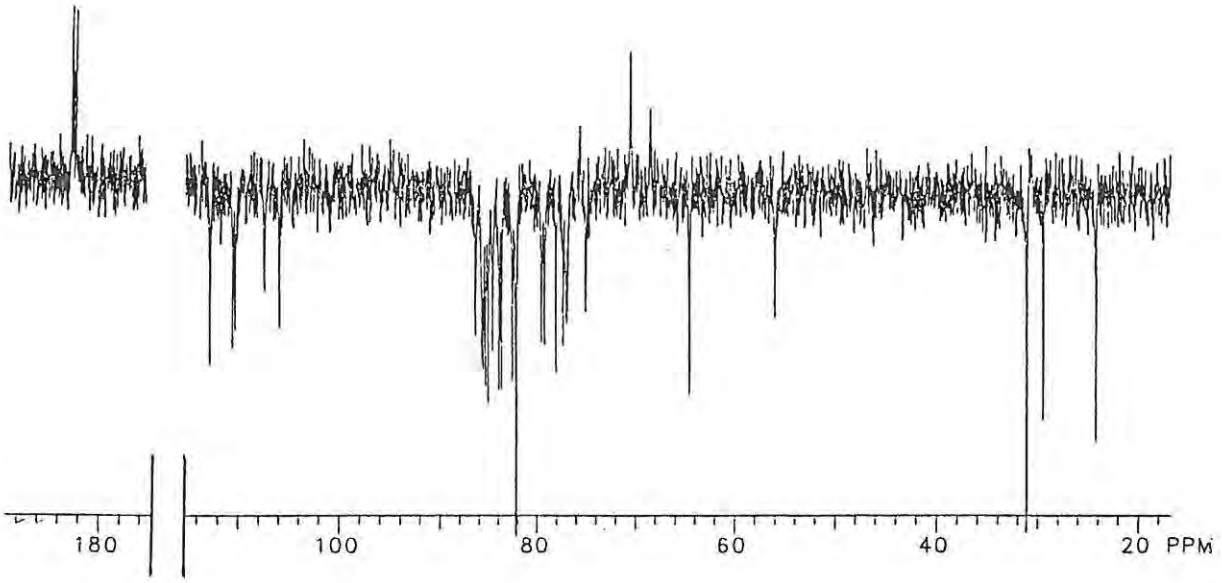
Gel Profile[a] C2 Products of the HF catalysed partial acid hydrolysis of K87 capsular polysaccharide.
[a] Sephacryl S200 HR



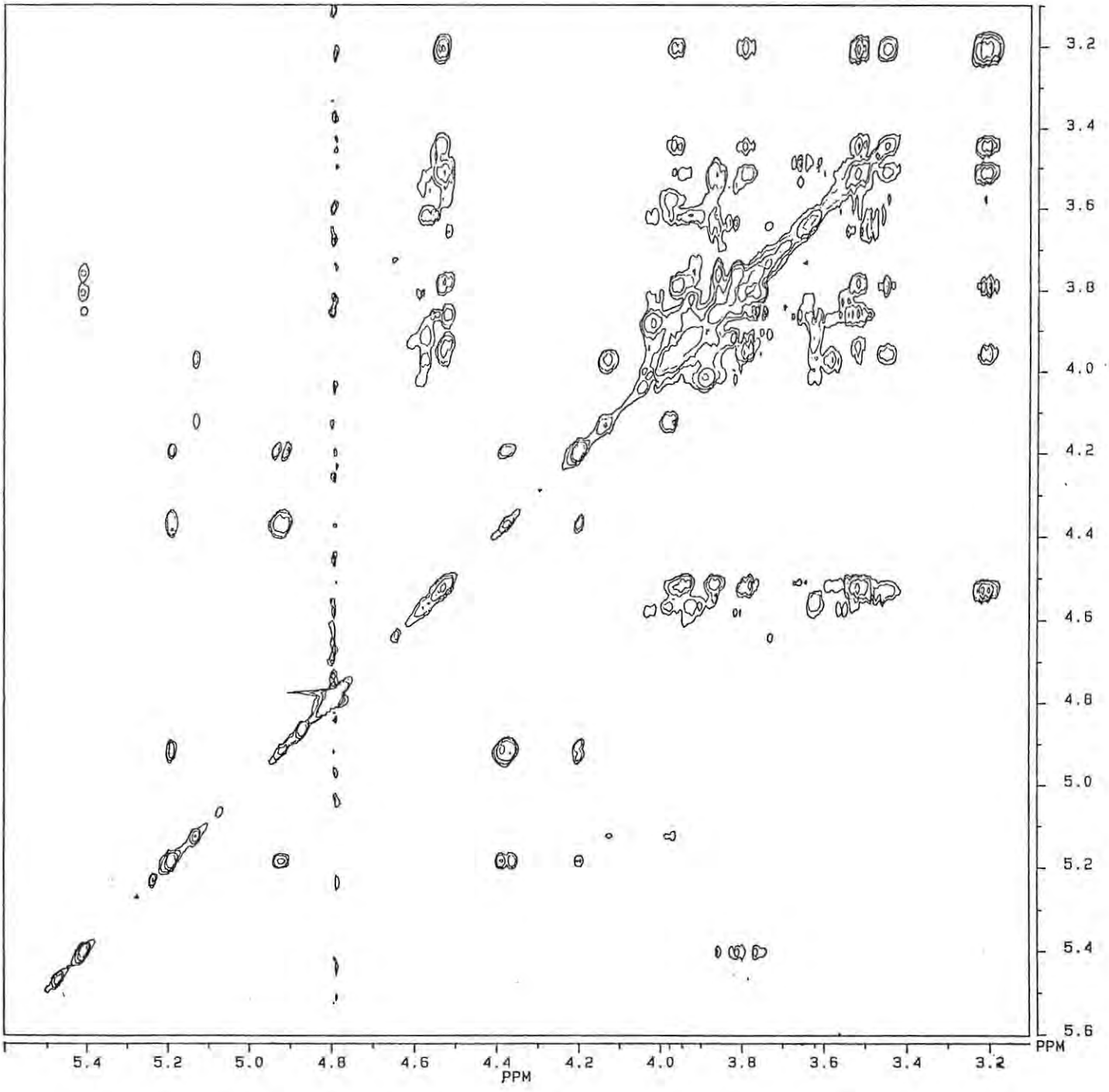
Spectrum C1 ^{1}H spectrum of the K87 polysaccharide.



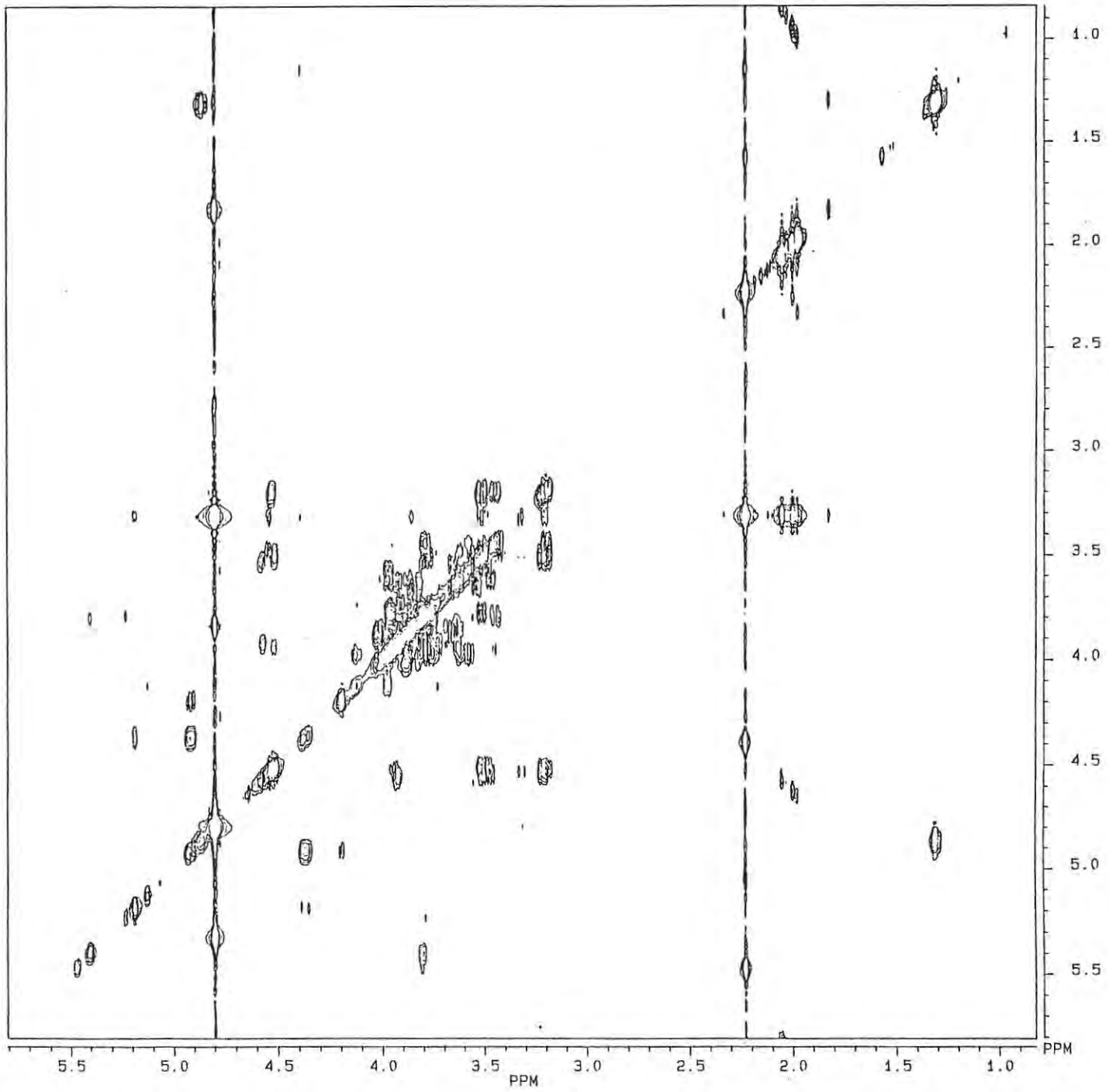
Spectrum C2 ^{13}C spectrum of the K87 polysaccharide.



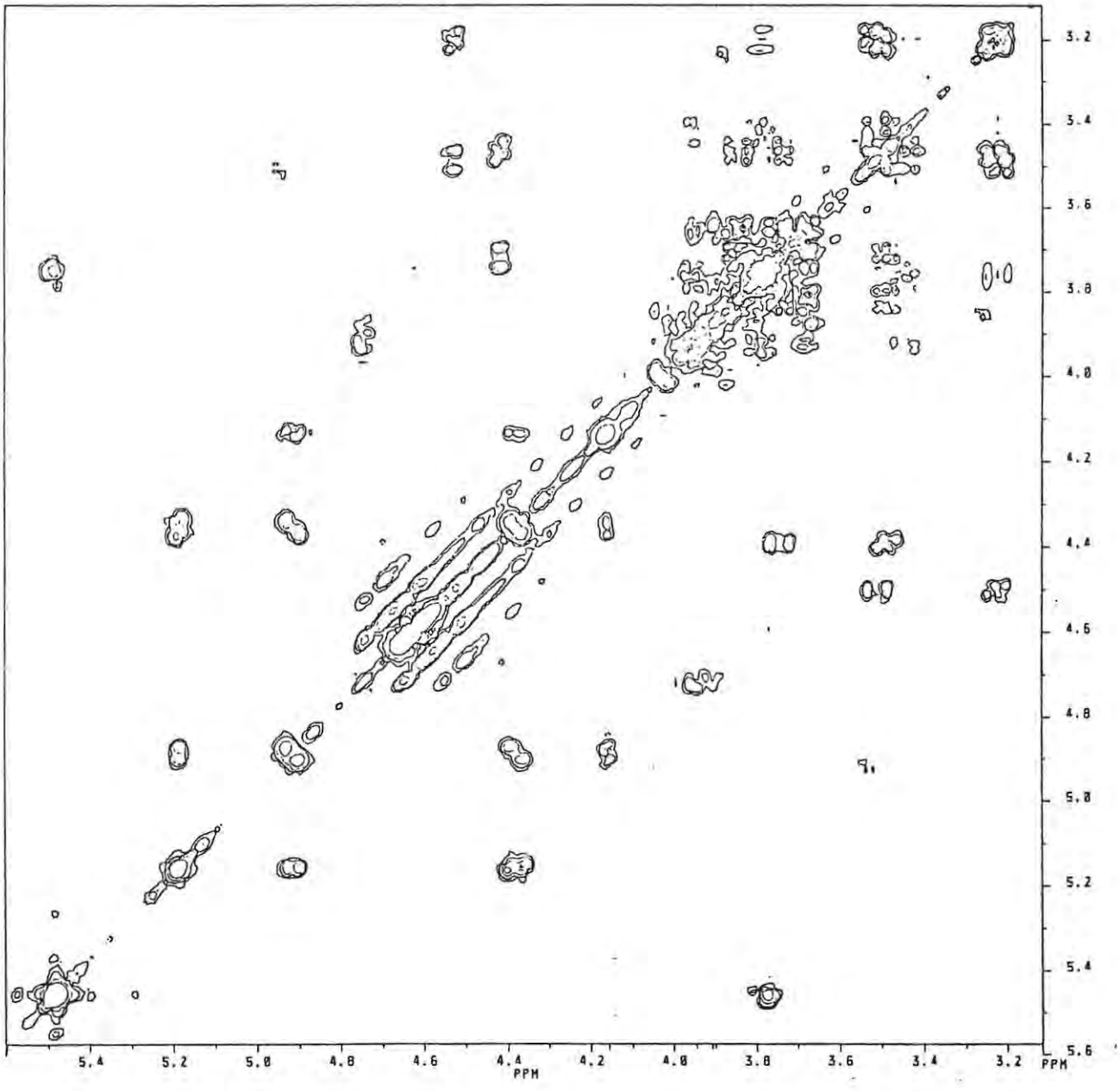
Spectrum C3 APT spectrum of the K87 polysaccharide.



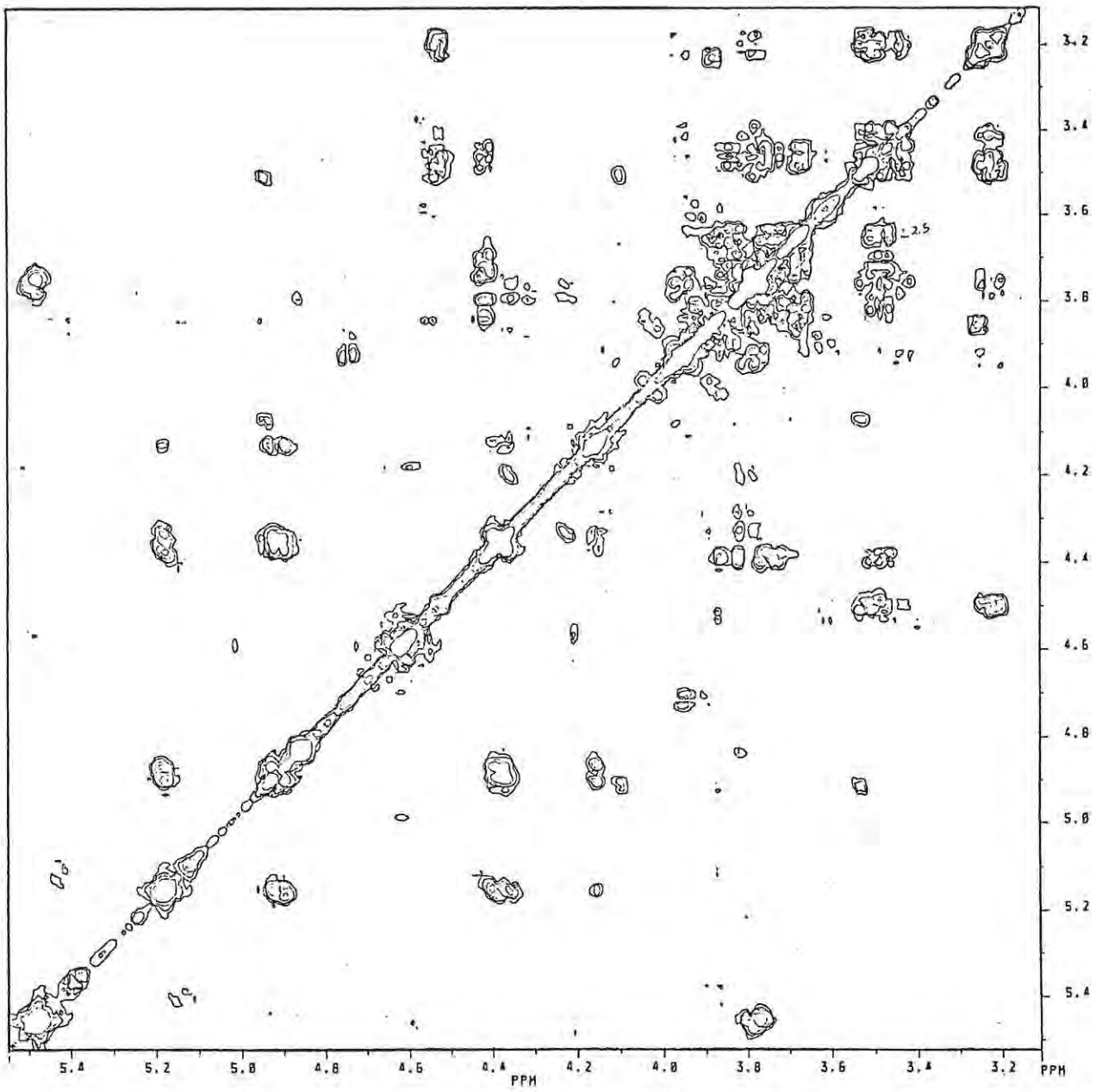
Spectrum C4 HOHAHA spectrum of Fraction **B.**



Spectrum C5 COSY spectrum of Fraction B.



Spectrum C6 RELAY COSY spectrum of the K87 polysaccharide.

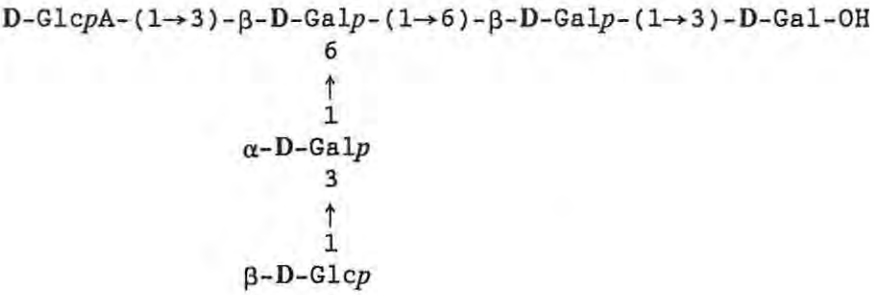


Spectrum C7 Two-step RELAY COSY spectrum of the K87 polysaccharide.

10.4 THE PREPARATION AND STRUCTURAL STUDY OF AN OLIGOSACCHARIDE FROM *KLEBSIELLA* K15 CAPSULAR POLYSACCHARIDE

10.4.1 Abstract

The capsular polysaccharide from *Klebsiella* K15 has been depolymerised by a bacteriophage-borne endoglycanase to produce a hexasaccharide (**P1**) which was an analogue of the repeat unit. This fragment has been characterised by β-elimination of the methylated reduced oligomer, methylation analyses, and 1D and 2D nmr spectroscopy. These data permit the structure of **P1** to be written as follows:

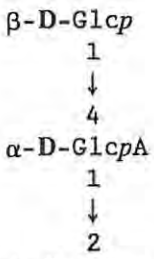


10.4.2 Introduction

Oligosaccharides required for conformational[257] and immunochemical[258] studies may be synthesised[eg 259] or produced by the selective depolymerisation of a polysaccharide with a regular repeat unit. The latter alternative is particularly attractive when a bacteriophage is available, which can be used to depolymerise a bacterial polysaccharide. Such phages, which have glycanases associated with their tail spikes or thick fibres[126], are used to prepare oligosaccharides that corresponds to one or more repeat unit(s), formed by fission at the same relative positions along the chain. The method has greatly improved the selection of oligomers available. It is in this context that we report on the preparation and structural

study of a hexasaccharide (P1) obtained from the depolymerisation of the *Klebsiella* K15 capsular polysaccharide by a phage-borne β-galactosidase.

The *Klebsiella* K15 capsular polymer has been reported[36] to have the structure 1 and belong to a chemotype which includes the capsular antigens from *Klebsiella* K8, K25, K27 and K78, the structures of which have been published[260-263].



→4)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→

1

A comparative study[264] of host capsule depolymerases associated with *Klebsiella* K bacteriophages, allowed the following general conclusions to be stated:

(1) in most cases cleavage occurs on either side of the sugar in the repeat unit with a negative charge, but reducing glycuronic acids are not produced;

(2) most often the reducing end sugar of the derived oligosaccharide is substituted at O-3;

(3) in the majority of cases β-glycosidic linkages are hydrolysed.

These observations have been substantiated by many other phage induced polysaccharide depolymerisations that have been reported[eg 127,265,266] since the results of this study were published in 1981. Hence, in

terms of structure 1, we expected a β-residue linkage in the main chain to be hydrolysed by the host (K15) specific phage, Ø15, and the 2,3-linked galactose's linkage was the most likely cleavage point.

10.4.3 Results and Discussion

The capsular polysaccharide isolated from *Klebsiella* K15, grown on Mueller Hinton agar, was composed of galactose, glucose, and glucuronic acid. The D configuration of the sugars was established by glc of their octylglycoside acetates. The 1D proton nmr spectrum of this material showed that, except for a single α sugar, the residues all had a β-anomeric configuration.

Klebsiella Ø15 was isolated from sewage, purified, and grown on its host strain in nutrient broth until there were 4×10^{12} plaque forming units (PFU). This phage solution was used to depolymerise the K15 polysaccharide and a hexasaccharide (P1) was recovered by gel permeation chromatography on Biogel® P6 (Gel Profile D1). A sample of P1 was sodium borohydride reduced to give the oligoalditol, P1-reduced.

Methylation analysis of P1-reduced gave the sugars listed in Table D1 (column I). This data identifies the linkage positions of the sugars and shows that there is a 3,6-linked galactose branch point and that the GlcA and Glc are non-reducing termini. The cyclic residues were all of the pyranose ring form. The alditol could be identified as arising from a 3- and not a 4-linked residue because, when this data was compared to the methylation analysis results from the polysaccharide, it was evident that a mole of 2,4,6-tri-O-methyl-galactose had been lost. This assignment was confirmed by the nmr data (see later) from this terminus. The GlcA, which was identified

as 4-linked from the results of the methylation analysis of the carboxyl reduced polysaccharide, was shown to be the position at which cleavage occurred.

10.4.3.1 Nmr Study of P1 and P1-reduced

The numerical data from the ^{1}H spectra are listed in Table D2.

10.4.3.1.1 1D Nmr Experiments

The proton spectrum of **P1** (Spectrum D1) was characterised by five anomeric resonances *viz* δ 5.018 (^{3}J 3.8 Hz), δ 4.74 (^{3}J 8.1 Hz), δ 4.659 (^{3}J 7.3 Hz), δ 4.635 (^{3}J 8.0 Hz), δ 4.487 (^{3}J 8.0 Hz), with an integral of 1 and by two fractional signals at δ 5.289 (^{3}J 2.7 Hz, 0.4 H) and δ 4.631 (^{3}J 6.6 Hz, 0.6 H).

The ^{13}C spectrum of **P1** (Spectrum D2) showed anomeric signals at 104.86, 104.59, 104.47, 103.63, 99.12, 97.01 and 92.97 ppm and a resonance peak for a carboxyl group at 173.69 ppm. The anomeric resonance at 104.59 was twinned.

These data suggest that a β-linkage in the polysaccharide has been cleaved by the phage depolymerisation and the product, **P1**, was a reducing hexasaccharide.

The **P1**-reduced sample gave a spectrum (Spectrum D3) with resonances at δ 5.027 (^{3}J 3.5 Hz), δ 4.752 (^{3}J 7.8 Hz), δ 4.687 (^{3}J 7.5 Hz), δ 4.507 (^{3}J 7.7 Hz), δ 4.479 (^{3}J 7.9 Hz). Hence, as expected, the fractional anomeric resonances were lost on reduction of the oligomer. It is noteworthy that the δ 4.635 resonance in the **P1** spectrum has

been shifted upfield by 0.128 ppm after reduction of the reducing terminus.

10.4.3.1.2 **COSY Experiments**

The anomeric signals from the **P1** sample, excluding those from the reducing sugar, were labeled **a** to **e** in order of decreasing chemical shift.

The proton resonances of residues **b** and **e** could be fully assigned from the connectivity maps in the COSY[98,211] spectrum of **P1** (Spectrum D4). However, only the signals from H-1 to H-4 of **a**, **c** and **d** could be assigned from this spectrum. Nonetheless, this data (Table D2), when compared with literature values for methyl glycosides[186,238,239], made it possible to identify **a** to **e** as: α-galactose, β-glucuronic acid, β-galactose, β-galactose, and β-glucose respectively. It was also evident that the Glc and GlcA are non-reducing termini as none of their signals were glycosidically shifted. This was in accordance with the methylation results. The H-3 and adjacent proton resonances of the reducing galactose residue were shifted to lower field and thus, confirmed that it was linked at position 3.

The COSY spectrum of **P1**-reduced (Spectrum D5) allowed a full connectivity map to be constructed for the α-galactose residue **a**, as well as sugars **b** and **e**. The assignments for **a** were compared with literature values[186] for methyl-α-galactose and the pattern of glycosylation shifts suggested that this residue was the 3-linked galactose. Hence the two remaining residues, **c** and **d**, are the unassigned sugars, 3,6- and 6-linked galactose. The chemical shift of the signal from H-3 of **d** corresponds to within 0.05 ppm of the literature value[186] (δ 3.64) for a methyl-β-galactose H-3 and this excludes the possibility that **d** is 3-linked. The residue **c**, where H-3

is deshielded, is therefore the sugar linked at the 3,6- positions and, by default, **d** is the 6-linked galactose.

The following assignments were thus made from the methylation analysis and nmr data:

a →3)-α-Gal

b β-GlcA

c →3,6)-β-Gal

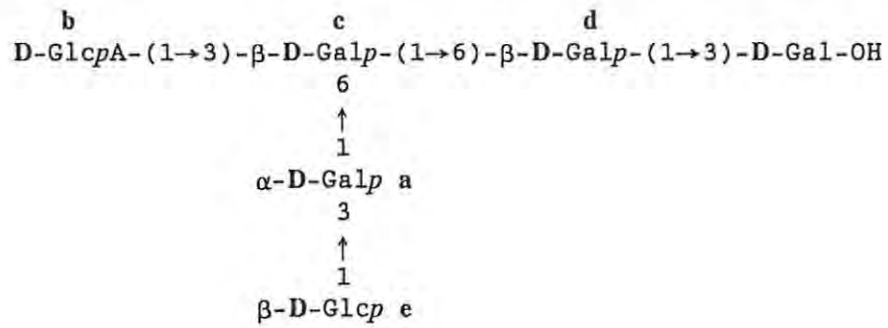
d →6)-β-Gal

e β-Glc

There was twinning of several signals in the COSY and 1D spectra of **P1** and the resonances of residues **c** and, in particular, **d** were the most affected. This effect, coupled to the observed upfield shift that the anomeric resonance of **d** experienced on reduction of **P1**, identifies the 6-linked galactose as the sugar adjacent to the reducing terminus and **c** as the residue next in the chain.

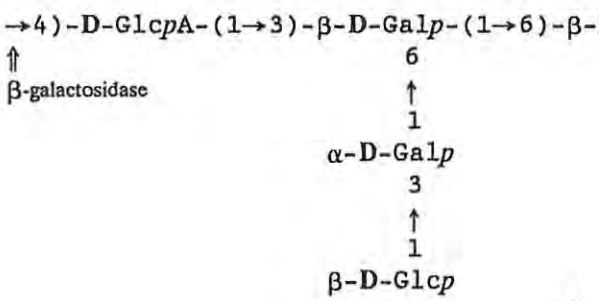
A β-elimination and methylation analysis of methylated **P1**-reduced showed (Table D1, column II) that the GlcA was linked to the 3-position of the branch point.

These results enable the structure of **P1** to be written as follows:



This is incompatible with the structure published[36] for *Klebsiella* **K15** capsular polysaccharide. We thus propose the following revised

structure (2) for this K antigen, based on the results of this study and data from the polysaccharide methylation analysis.



2

It can be seen from these results that the ∅15 cleaves a β-linkage next to the negatively charged sugar unit to produce an oligosaccharide that has a reducing end sugar substituted at O-3. This is consistent with the conclusions on the behavior of *Klebsiella* K bacteriophage depolymerases stated by Rieger-Hug and Stirm[264].

10.4.4 Experimental

10.4.4.1 General methods. - These are the same as the methods described in 10.1.4.1.

10.4.4.2 Preparation of *Klebsiella* K15 polysaccharide. - An authentic culture of *Klebsiella* K15 was obtained from Dr I. Ørskov (Copenhagen). This was grown and the capsular material isolated and purified as described in section 10.1.4.2. The yield was 37 mg/tray. The polysaccharide required an additional ultracentrifugation step (120 000 G, 3 h) to remove particulate matter that was apparent when the sample was dissolved in water. The sugar composition of the polysaccharide was determined by qualitative paper chromatography of a hydrolysate on Whatman® Number 1 paper, developed with 5:1:5:3 v/v/v/v (ethyl acetate, acetic acid, pyridine, and water).

10.4.4.3 Bacteriophage isolation. - A host specific bacteriophage, Ø15, was isolated by adding 10 mL of sewage water (Grahamstown sewage works, RSA) to 50 mL of unsterilised nutrient broth (NB) and 10 mL of a 6 h old K15 NB culture. This was shaken at 37° for six h and 5 mL of $CHCl_3$ was then added to stop further bacterial growth. Centrifugation at low speed removed the bacterial cells and other debris and the supernatant, containing the bacteriophages, was retained. Assays for the phage were performed by applying a measured volume (approximately 0.01 mL) from each tube of a saline serial dilution (10^{-2} to 10^{-8}) of the suspension, to a fresh lawn of *Klebsiella* K15. These were incubated overnight at 37°. Plates with between 30 and 300 plaques were counted and after correcting for the dilution, the phage titre was quoted as plaque forming units (PFU) per mL.

A large single phage colony was selected on the basis of the size of the plaque and its halo, and this was picked from the plate and added to 5 mL of NB which had been freshly inoculated with K15. This was shaken at 37° (6 h) and centrifuged at low speed after 1 mL of $CHCl_3$ was added. A pure phage line was established by successive single plaque isolations.

The titre and volume of the phage suspensions were increased by a tube lysis and several flask lyses. The former involved inoculating six 10 ml NB tubes with 1 mL of 6 h bacterial cell NB culture and successive tubes were inoculated with 0.1 mL (1×10^8 PFU/ml) at 0, 15, 30, 45, 60 and 75 min. Tubes that cleared after shaking at 37° for several hours were treated with $CHCl_3$ and centrifuged at low speed. A flask lysis was the same procedure on a five times larger scale. These lyses were repeated until there was 4×10^{12} PFU in 400 mL. This was dialysed exhaustively against tap water (12 - 14000 Mw cut off).

10.4.4.4 Bacteriophage depolymerisation. - A sample (176 mg) of purified *Klebsiella* K15 capsular antigen (Na^+ salt) was treated with 4 x 10^{12} phage in 400 mL. After incubation at 37° with stirring for 3 days the mixture was freeze-dried. The lyophilisate (801 mg) was made up to 50 mL in distilled water and dialysed (Mw cutoff <3500) against 6 x 100 mL distilled water for 24 h periods, the dialysate was freeze-dried. The total yield of of low molecular weight material was 401 mg. The dialysate from days one and two were combined (254 mg) and a 6.5 mg sample was analysed by analytical gpc on Biogel® P6 (1.6 x 60 cm) calibrated with stachyose and dextrans, using pyridinium acetate (water, acetic acid, pyridine - 500:2:5 v/v/v) as the eluent. One mL fractions were collected and analysed by the phenol/sulphuric acid method. The elution profile (Gel Permeation Profile D1) was plotted from these data. The remaining material was separated using the P6 column and a 10% aliquot of each 1 mL fraction was analysed as described before. The yield of **P1** was 50.3 mg.

The oligosaccharide **P1** (22 mg in 5 mL of water) was reduced to an oligoalditol with $NaBH_4$ (200 mg). Excess borohydride was destroyed by adding acetic acid. The solution was passed down an Amberlite® IR-120H^+ column (1 x 15 cm) followed by co-evaporation with methanol under reduced pressure at ≤40° to remove methyl borate.

10.4.4.5 Nmr spectrosopy. - Samples were deuterium exchanged by freeze-drying in 99.96% D_2O, then dissolved in 99.995% D_2O (0.45 mL) containing a trace of acetone as the internal reference, δ 2.23 for ^1H and 31.07 ppm for ^{13}C. Spectra were recorded from samples in a 5 mm tube on a Bruker® WM-500 (^1H) or AM-300 (^{13}C) fitted with an Aspect® 2000 and 3000 respectively. Standard Bruker® software was used.

The 1D ^1H spectra were recorded at 500MHz as follows: (a) the sample

P1 was run at 30° using a spectral width of 5494 Hz and a 16K data set, gave a digital resolution of 0.671 Hz/point; (b) the **P1**-reduced spectrum which was acquired at 85°, gave a digital resolution of 0.259 Hz/point by using a 16K data set and a spectral width of 2111 Hz.

The ^{13}C spectrum of **P1** was recorded at 30° using a spectral width of 33 333 Hz. The digital resolution was 2.035 Hz/point.

The ^{1}H COSY[98,211] experiment on **P1** at 30° was performed at 500 MHz using spectral widths of 2906 and 1453 Hz with 48 scans and 512 experiments. The COSY experiment on **P1**-reduced at 85° was performed using spectral widths of 2118 and 1059 Hz with 48 scans and 512 experiments. Relaxation delays of 2 s were used and the data were symmetrised.

10.4.4.6 **β-Elimination.** - The methylated **P1**-reduced was was β-eliminated and remethylated using the single flask method of Aspinall and Rosell[138].

TABLE D1

METHYLATION ANALYSIS OF P1-reduced AND P1-reduced AFTER β-ELIMINATION

Sugar[a]	T[b]	Molar ratio[c]	
		I	II
1,2,4,5,6-Gal	0.68	0.63	0.54
2,3,4,6-Glc	1.00	1.00	1.00
2,4,6-Gal	1.38	1.03	0.93
2,3,4-Glc	1.52	0.62	
2,3,4-Gal	1.43	0.86	1.46
2,4-Gal	1.92	0.78	

[a] 2,3,4,6-tetra-methyl-D-glucose, etc.

[b] Relative retention time of the derived alditol acetate on OV-17.

[c] I, methylated, carboxy ester reduced **P1**; II, β-eliminated, methylated, carboxy ether reduced methylated **P1**

TABLE D2

NMR DATA[a]

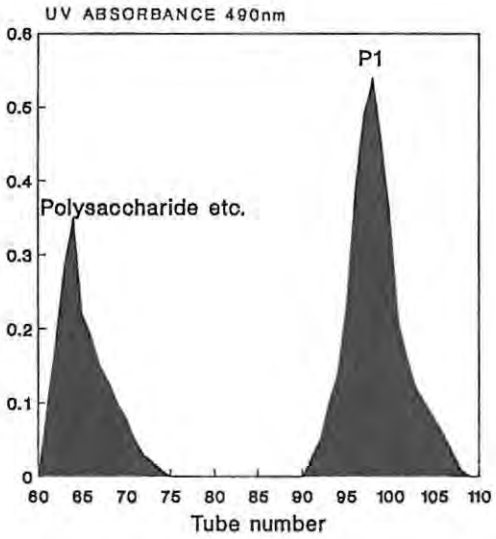
(a) **P1**

Atom	Unit **a** →3)-α-Gal	Unit **b** β-GlcA	Unit **c** →3,6)-β-Gal	Unit **d** β-Gal 6↑	Unit **e** β-Glc	Reducing sugar α-Gal-OH	Reducing sugar β-Gal-OH
H-1	5.018	4.74	4.659	4.635	4.487	5.289	4.62
^3J	3.8	8.1	7.3	8.0	8.0	2.7	6.6
H-2	4.02	3.44	3.80	3.63	3.29	3.98	3.64
^3J		9.0	9.0	9.0	9.0		
H-3	3.96	3.57	3.83	3.70	3.49	4.23	
^3J		9.0	2.7		9.0		
H-4	4.28	3.59	4.19	4.00	3.38		
^3J		9.0			9.0		
H-5		3.94			3.47		
^3J							
H-6					3.72		
^3J							
H-6'					3.92		
^3J							

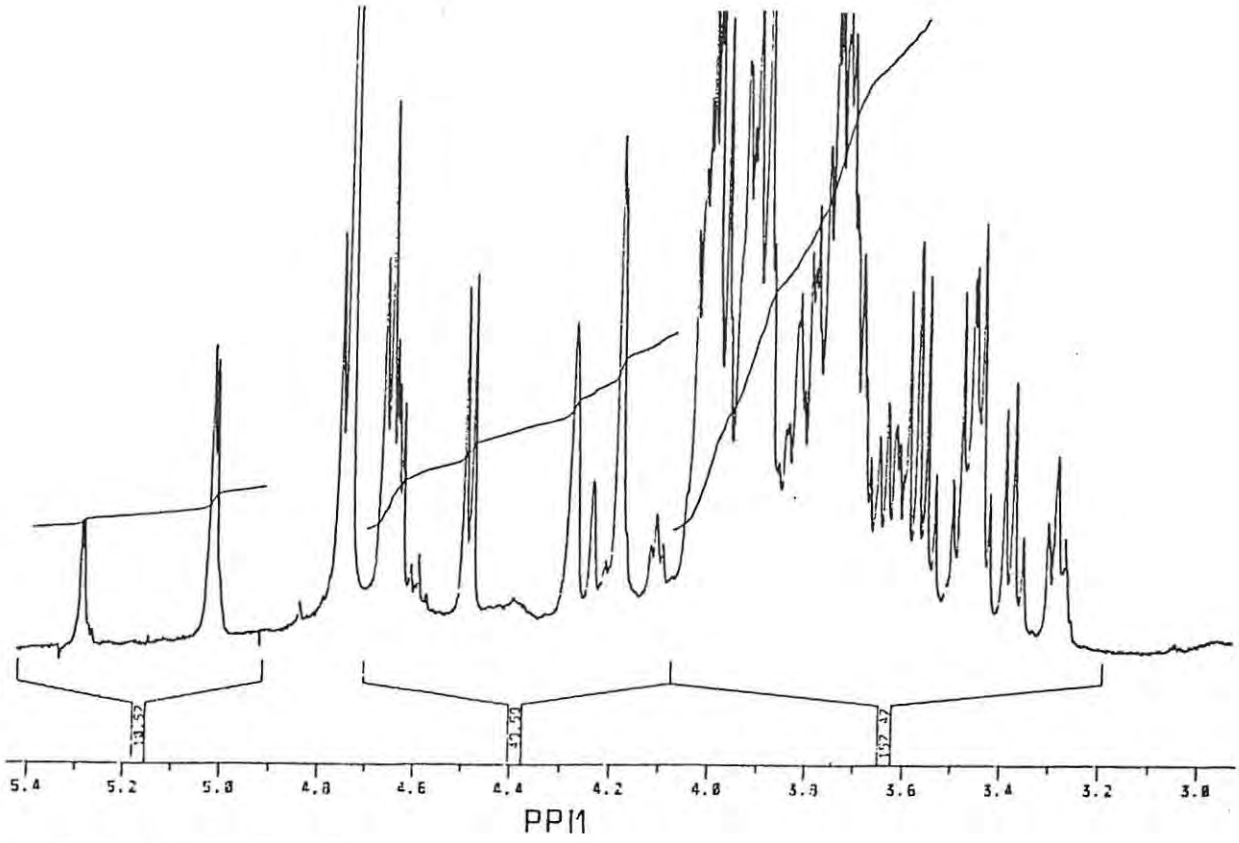
(b) **P1-reduced**[b]

Atom	Unit **a** →3)-α-Gal	Unit **b** β-GlcA	Unit **c** →3,6)-β-Gal	Unit **d** β-Gal 6↑	Unit **e** β-Glc
H-1	5.027	4.752	4.687	4.507	4.479
^3J	3.5	7.8	7.5	7.7	7.9
H-2	4.03	3.46	3.82	3.62	3.30
^3J					
H-3	4.08	3.57	3.84	3.69	3.49
^3J					
H-4	4.27	3.63	4.18	3.98	3.42
^3J		9.0			9.0
H-5	3.99	3.94			3.47
^3J					
H-6	3.77				3.73
^3J					
H-6'	3.79				3.93
^3J					

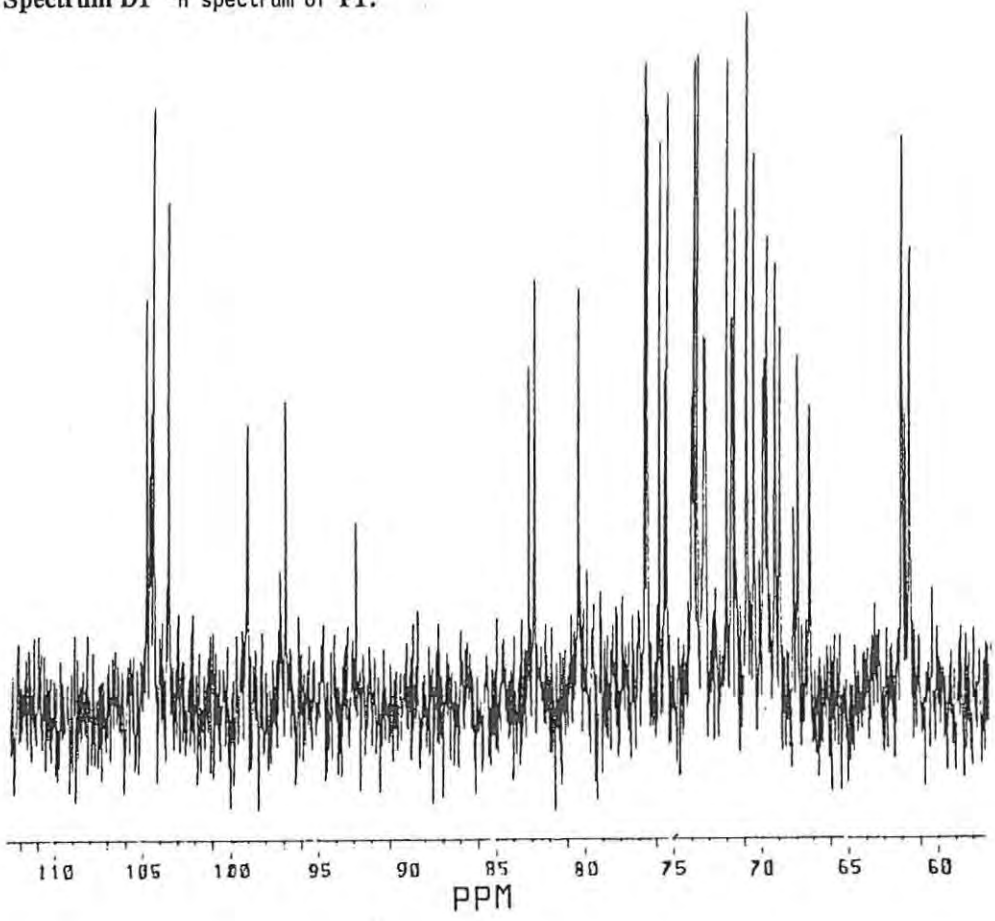
[a] Coupling constants in Hz and chemical shifts relative to acetone as the internal reference, 2.23 ppm.
[b] Data from the alditol is not shown.



Gel Profile[a] **D1** Products from the ∅15 depolymerisation of **K15**.
[a] Separated on Biogel® P6.

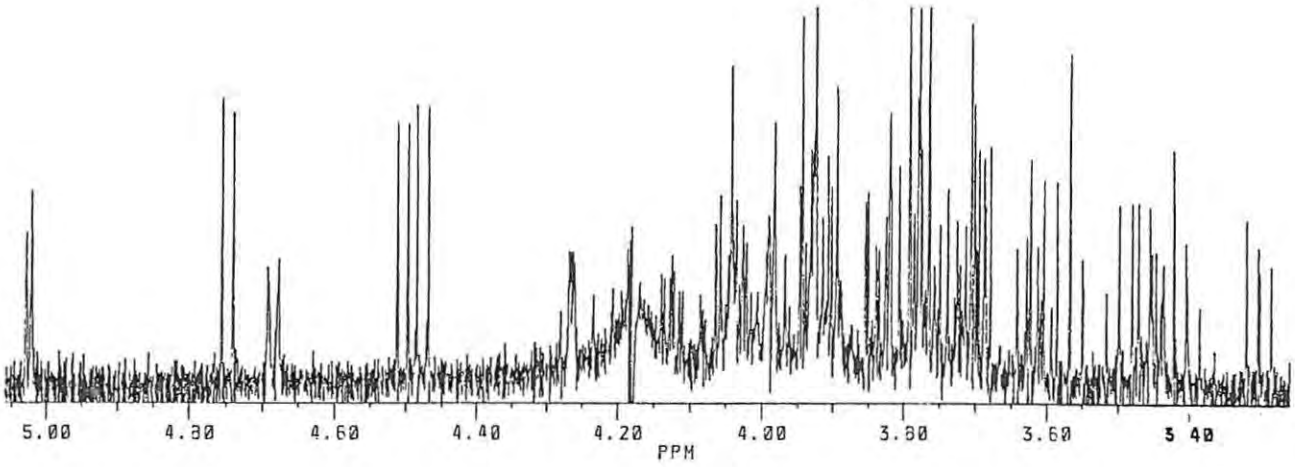


Spectrum D1 ^{1}H spectrum of **P1.**

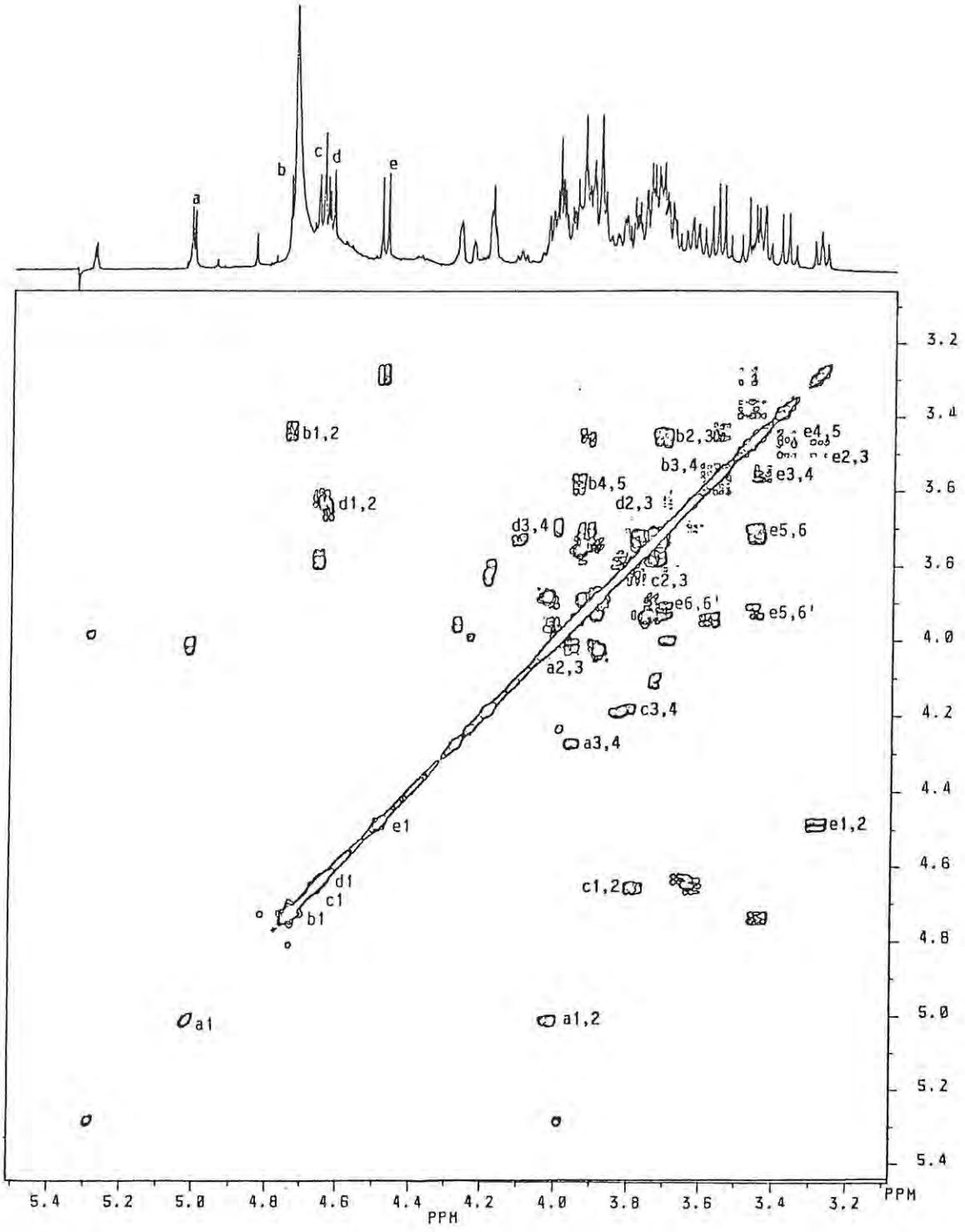


Spectrum D2 ^{13} spectrum of **P1.***

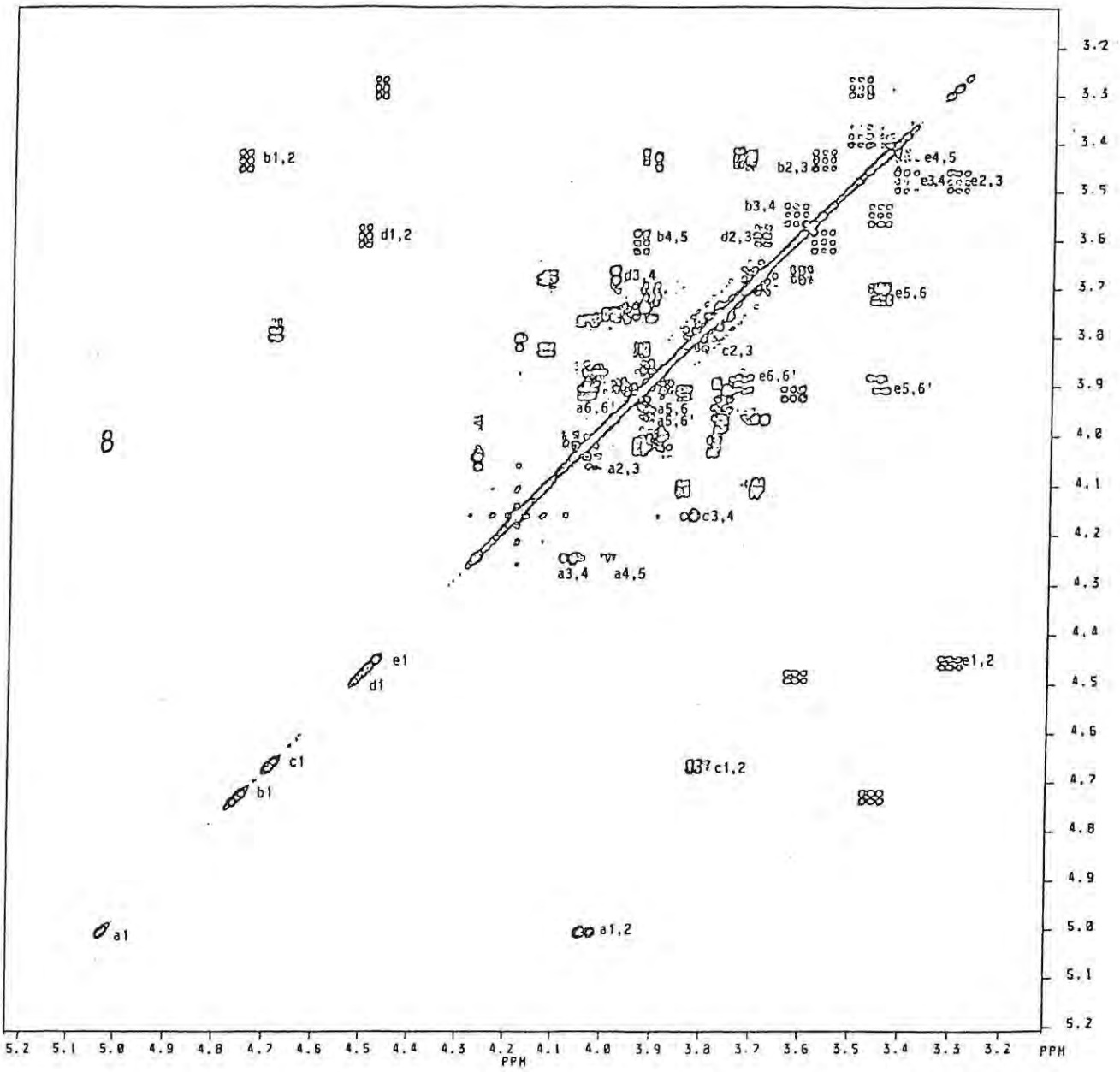
* Region shown is between approximately *55* and *110* ppm.



Spectrum D3 ^{1}H spectrum of **P1**-reduced.



Spectrum D4 COSY spectrum of **P1**.



Spectrum D5 COSY spectrum of **P1**-reduced.

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