STRUCTURAL STUDIES ON THE CAPSULAR ANTIGENS OF *ESCHERICHIA COLI* SEROTYPES K102 AND K47.

THESIS

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ABSTRACT

The work presented in this thesis forms part of a continuing programme concerned with the structural determination of capsular polysaccharides of some *Enterobacteriaceae*. Since bacteria of this family are pathogenic to Man, work in this laboratory has focused on the structural elucidation of *Klebsiella* and, more recently, *Escherichia coli* (*E. coli*) capsular polysaccharides (K-antigens). To date, some 74 K-antigens have been distinguished serologically within the genus *E. coli* and the structures of approximately 70% are known. In general, the K-antigens of the *E. coli* are characterized by a wide variety of constituent monosaccharides arranged in repeating units.

In this thesis the structural elucidation of the capsular polysaccharides of *E. coli* serotypes O8: K102: H^- and O8: K47: H2 is presented. A variety of chemical techniques has been employed in the structural analysis, and are discussed. The thesis also includes extensive two-dimensional n.m.r. studies on the *E. coli* K102 and K47 polysaccharides, as well as on a modified K102 polymer produced after a lithium-ethylenediamine degradation of the native polysaccharide.

1. INTRODUCTION

1.1 The Anatomy of the Bacterial Cell Wall.

Bacteria of the genus *Escherichia coli* (*E. coli*) belong to the Gram negative family of the *Enterobacteriaceae*[1]. Each rod-shaped *E. coli* cell contains a single chromosome which is centrally located and surrounded by a cytoplasm rich in ribosomes[2]. Enclosing this structure is a complex, multilayered cell wall-membrane structure.

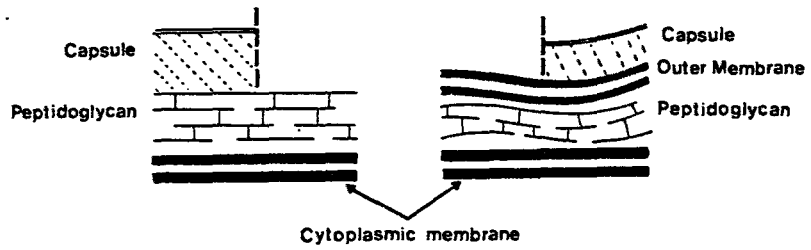


Figure 1 : Diagrammatic representation of the cell surface of Gram positive and Gram negative bacteria[3].

The inner cytoplasmic membrane, which consists of phospholipid bilayers, is semipermeable and directly encloses the cytoplasm. This membrane is a highly complex structure and contains, amongst other components, various enzyme and transport systems[3]. Surrounding the cytoplasmic membrane is the peptidoglycan layer and the outer membrane, which constitute the bacterial cell wall. The peptidoglycan layer consists of heteropolymers of alternating $\beta(1\rightarrow4)$ linked units of N-acetyl- muramic acid[4]. Peptides are attached to the muramic acid residues of the heteropolymers to yield a crosslinked structure which provides strength and rigidity to the cell.

The outer membrane is distinctive of Gram negative bacteria and contains strong

antigens, the main component being lipopolysaccharide. The complete lipopolysaccharide, which is found in all smooth *Enterobacteriaceae*, consists of three covalently bonded components, *viz.* lipid A, the core oligosaccharide and the O-specific side chain[5]. The structure of lipid A is highly conserved within the genus *E. coli* and similarly little variation is found in the monosaccharides that constitute the outer-core and the O-specific side chains (O-antigens). According to a recent publication by Dutton *et al*[1] 160 O-antigens have been distinguished serologically and the structures of 23 are known and tabulated.

Most Gram negative bacteria produce a characteristic extracellular polysaccharide which occurs either as an obvious cell capsule or as a loose slime, unattached to the cell surface[4]. The extracellular polysaccharides (Capsular / K-antigens) are high molecular weight polymers, composed of oligosaccharide repeating units. The monosaccharides that constitute the repeating units include neutral hexoses, deoxyhexoses, amino sugars and acidic components, *viz.* uronic acids, phosphate groups or ketals of pyruvic acid[3]. Pentose sugars are seldom incorporated in the oligosaccharide repeating unit. The bulk of the Gram negative capsular antigens are heteropolymers. Exceptions are the homopolymeric capsular antigens of *E. coli* K1 and *Neisseria meningitidis* B and C, which consist of N-acetylneuraminic acid (sialic acid).

Several *Enterobacteriaceae* produce a slimy acidic polysaccharide, the M-antigen (Figure 2), when grown under specific conditions, i.e. low temperatures and high osmotic pressures[6]. The M-antigen is loosely associated with the cell surface and its structure is based on a hexasaccharide backbone of colanic acid[3]. The terminal galactose of the side-chain may be substituted by either formaldehyde, acetaldehyde or pyruvate. The *Enterobacteriaceae* also produce a flagellum, the Hauch antigen (H-antigen), which consists of protein. This antigen is the predominant locomotory organelle of the bacterium and has been used in the classification and identification of flagellate bacteria, e.g. the Kaufmann-White scheme[3] for the classification of *Salmonellae*.

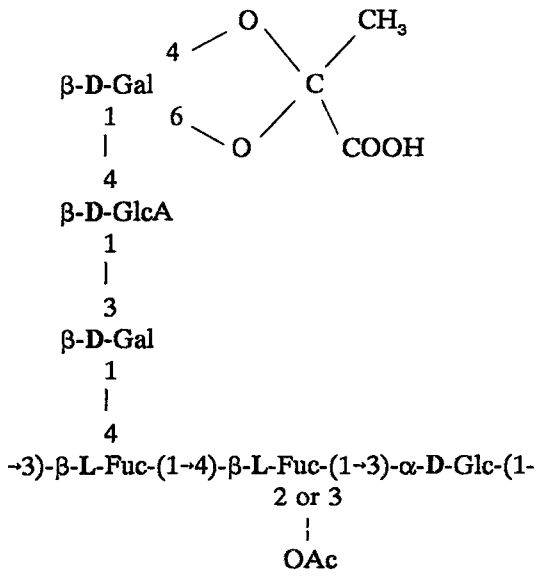


Figure 2 : Structure of the repeating unit of the M-antigen from *E coli* K12[3].

1.2 The Capsular Antigens of *Escherichia coli*.

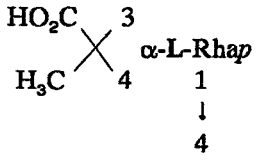
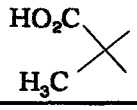
The capsular antigens (K-antigens) of *E. coli* were originally subdivided into three categories, *viz.* L, A and B[7]. This distinction was based on their O-antiserum agglutinability and their thermolability, and did not reflect any differences in chemical composition.

This classification was, however, found to be misleading and Ørskov *et al*[7] suggested that the K-antigens be divided into two groups, the protein- and the polysaccharide K- antigens. On the basis of chemical, physical and microbiological characteristics[8] the polysaccharide K-antigens of the *E. coli* are further divided into two distinct groups. Polysaccharides of group I have high molecular weights (M_r > 100 000), low electrophoretic mobilities and are heat stable (100°C) at pH 5-7[9]. These polysaccharides contain hexuronic acids as the acidic component and are usually co-expressed with the O8 and O9 antigens. Polysaccharides of group II have lower molecular weights (M_r < 50 000) and higher electrophoretic mobilities than those of group I. They are heat labile at pH 5-7 and are co-expressed with many O-antigens other than O8 and O9.

The K-antigens of group II also contain more unusual acidic components, such as N-acetylneuraminic acid, N-acetylmannos-aminuronic acid, 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo), or phosphate. To date 74 K-antigens have been distinguished serologically within the genus *E. coli*[1]. The structures of 52 are known and are listed in Table 1.

E. coli K-antigens	Structures
K1[10]	→8)-α-Neu*p*5Ac-(2-
K2, K62[11,12]	-P→4)-α-D-Gal*p*-(1→2)-Gro-(1→)$_{2n}$...(-P→5)-α-D-Gal*f*-(1→2)-Gro-(1→)$_n$... 2/3 2/3 | | OAc OAc Gro = Glycerol K2, (K2a), nonacetylated K62, (K2ab), acetylated
K3[13]	→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1- 3 3 ⋮ ⋮ 2 2 S S S= 6-O-acetyl-4-deoxy-hexulosonic acid
K4[14]	→4)-β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1- 3 ↑ 2 β-D-Fru*f*
K5[15]	→4)-β-D-Glc*p*A-(1→4)-α-D-Glc*p*NAc-(1-
K6a[16], K6b[17]	→3)-β-D-Rib*f*-(1→7)-β-Kdo*p*-(2- 2 ↑ 1 β-D-Rib*f* →2)-β-D-Rib*f*-(1→2)-β-D-Rib*f*-(1→7)-α-Kdo*p*-(2→ a b
K7 (K56)[18]	→3)-β-D-Man*p*NAcA-(1→4)-β-D-Glc*p*-(1- 6 | OAc

E. coli K-antigens	Structures
K8[19]	→3)-α-D-Glc*p*NAc-(1→3)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→2)-β-D-Gal*p*-(1 4 | OAc
K9[20]	→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-α-D-Gal*p*-(1→4)-α-Neu*p*5Ac-(2- | OAc
K11[21]	→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1-P- 3 ↑ 2 β-D-Fru*f*
K12 (K82)[22]	→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→5)-β-Kdo*p*-(2- 7/8 | OAc
K13 (K20,K23)[23,24]	→3)-β-D-Rib*f*-(1→7)-β-Kdo*p*-(2- K13, O-acetyl on 4 of Kdo K20, O-acetyl on 5 of Rib K23, nonacetylated
K14[25]	→6)-β-D-Gal*p*NAc-(1→5)-β-Kdo*p*-(2- 8 ⋮ OAc (~60%) OPropionyl (~10%)
K15[26]	→4)-α-D-Glc*p*NAc-(1→5)-β-Kdo*p*-(2-
K16[47]	→2)-β-D-Rib*f*-(1→3)-β-D-Rib*f*-(1→5)-α-Kdo*p*-(2 3 | OAc (33%)
K18, K22[28]	→2)-β-D-Rib*f*-(1→2)-D-Ribitol-(5-P- 3 ⋮ OAc K22, nonacetylated
K19[29]	OAc (~30%) ⋮ 8 →3)-β-D-Rib*f*-(1→4)-β-Kdo*p*-(2-

E. coli K-antigens	Structures
K24[30]	→7)-α-Kdo*p*-(2→1)-Gro-(3-P- Gro = glycerol 56% units acetylated at C-4 of Kdo ~28% units acetylated at C-5 of Kdo ~16% nonacetylated
K26[31]	 →3)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*A-(1→3)-α-L-Rha*p*-(1→3)- -α-L-Rha*p*-(1-
K27[32,33]	→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*A-(1→3)-α-L-Fuc*p*-(1- 3 ↑ 1 α-D-Gal*p*
K28[34]	→3)-α-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-α-L-Fuc*p*-(1- 4 2/3 ↑ ⋮ β-D-Gal*p* OAc (~70%)
K29[35]	→2)-α-D-Man*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Glc*p*A-(1→3)-α-D-Gal*p*-(1- 4 ↑  1 β-D-Glc*p*-(1→2)-α-D-Man*p*
K30[36,37]	→2)-α-D-Man*p*-(1→3)-β-D-Gal*p*-(1- 3 ↑ 1 β-D-Glc*p*A-(1→3)-α-D-Gal*p*
K31[38]	α-L-Rha*p* 1 ↓ 4 →2)-α-D-Glc*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*A-(1→2)-β-L-Rha*p*-(1→2)- -β-L-Rha*p*-(1-
K32[39]	OAc | 2 →4)-α-L-Rha*p*-(1→3)-β-D-Glc*p*-(1- 3 ↑ 1 α-D-Glc*p*A-(1→3)-α-D-Gal*p*

E. coli K-antigens	Structures
K33[40]	→3)-α-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-α-L-Fuc*p*-(1- 3 2 3 ↑ CH_3 CO_2H 1 α-D-Gal*p* contains O-acetyl groups
K34[41]	→2)-β-D-Glc*p*A-(1→4)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1- 3 ↑ 1 α-D-Glc*p*-(1→4)-β-D-Gal*p*
K36[42]	→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*A-(1→2)-α-D-Man*p*-(1- 4 ↑ 1 α-D-Man*p*
K37[43]	→3)-β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1- 4 ↑ 1 α-D-Gal*p* 6 4 (R) H_3C CO_2H
K39[44]	→6)-α-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→2)-α-D-Man*p*-(1→3)-β-D-Glc*p*-(1- 3 ↑ 1 α-D-Gal*p*
K40[45]	→4)-β-D-Glc*p*A-(1→4)-α-D-Glc*p*NAc-(1→6)-α-D-Glc*p*NAc-(1- 6 | L-Ser (amide)
K42[46]	→3)-α-D-Gal*p*-(1→3)-α-D-Gal*p*A-(1→3)-α-L-Fuc*p*-(1-
K44[47]	→4)-β-D-Glc*p*A-(1→3)-α-L-Rha*p*-(1→4)-α-D-Glc*p*NAc-(1→6)-β-D-Gal*p*NAc-(1
K49[48]	→4)-β-D-Glc*p*A-(1→6)-β-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc-(1- 6 | L-Thr (75%), L-Ser (25%) (amide)
K51[49]	→3)-α-D-Glc*p*NAc-(1→P- 4/6 | OAc

E. coli K-antigens	Structures
K52[50]	OAc (~80%) ⋮ 4 →3)-α-D-Gal*p*-(1→P- 2 ↑ 2 β-D-Fru*f* ⋮ OAc, OProp (~10%)
K53[51]	→3)-β-D-Gal*f*-(1→4)-β-D-Glc*p*A-(1- 2 | OAc
K54[52]	→3)-β-D-Glc*p*A-(1→3)-α-L-Rha*p*-(1- 6 ⋮ [L-Thr (90%), L-Ser (10%)] (~85%)
K55[53]	OAc ⋮ 2 →4)-β-D-Glc*p*A-(1→3)-β-D-Man*p*-(1- 4 6 H_3C CO_2H
K57[54]	→2)-Rib*f*-(1→4)-β-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1-
K74[55]	→3)-β-D-Rib*f*-(1→2)-β-D-Rib*f*-(1→6)-β-Kdo*f*-(2- 2 ⋮ OAc (~65%)
K85[56]	Rha 1 ↓ →2/4)-GlcA-(1→2/6)-Man-(1→3)-Man-(1→3)-GlcNAc-(1→Man-(1→3)-Man- 2/6 ↑ -(1→3)-GlcNAc-(1- GlcA Rha? ⋮
K87[57]	OAc | 3 →4)-β-D-Glc*p*A-(1→4)-α-L-Fuc*p*NAc-(1→3)-β-D-Glc*p*NAc-(1→6)-α-D-Gal*p*-(1 4 ↑ 1 β-D-Glc*p*

E. coli K-antigens	Structures
K92[58,59]	→8)-α-Neu*p*5Ac-(2→9)-α-Neu*p*5Ac-(1-
K93[51]	→3)-β-D-Gal*f*-(1→4)-β-D-Glc*p*A-(1- 5 6 | | OAc OAc
K95[60]	→3)-β-D-Rib*f*-(1→8)-Kdo*f*-(2- randomly O-acetylated
K100[61]	→3)-β-D-Rib*f*-(1→2)-D-Ribitol-(1→P-

TABLE 1. Structures of the *E. coli* K-antigens.

1.3 The Function and Immunochemistry of the Capsular Antigens.

Normally harmless *E. coli*, present as saprophytes within the intestinal lumen, can cause infections such as meningitis or peritonitis in people with weakened immunological systems[62]. Some *E. coli* also possess specific virulent properties that allow them to overcome host defence mechanisms leading to clinical infections, e.g. neonatal meningitis, urinary tract infections and diarrhoeal disease. Other important bacterial pathogens, that can cause serious diseases in their human hosts, include *Neisseria*, *Streptococcus*, *Staphylococcus*, *Haemophilus* and *Pseudomonas*[63]. Most human bacterial pathogens are encapsulated.

The capsular polysaccharides, although not critical for the growth and the survival of the bacterial cell, are important virulence factors in many bacterial infections[4]. The primary role of the capsular polysaccharides is to protect the organism from destruction by the phagocytic cells of the body which migrate to the site of infection. In addition, the capsule also masks the underlying surface antigens, thus preventing their recognition by the antibody[63]. Some capsular polysaccharides, e.g. the sialic acid polymers of *E. coli* K1

and *Neisseria meningitidis* B and C, also resemble simple molecular species of the human host, thus resulting in the suppression of antibodies towards these common structures.

On account of the capsular polysaccharides being the outermost layer of the bacterial cell, it is an obvious subject for vaccine development. To date, only three capsular polysaccharide vaccines, *viz.* those derived from *Streptococcus pneumoniae*[64], *Neisseria meningitidis*[4] and *Haemophilus influenza* type b[63], have been developed, although work on several others is well advanced[63,65].

The immune response towards polysaccharide vaccines differs from protein-driven antibody responses in that the polysaccharide antigens are T-cell independent, and do not stimulate a memory response[63]. Thus the antigens do not need the cooperation of T-cells to stimulate the B-cells to secrete the immunoglobulins, which are predominantly restricted to the IgG_2 subclass in Man[66].

Polysaccharide vaccines give adequate immune responses in adults, but are generally ineffective in children younger than two years[63]. This is a serious drawback in the development of polysaccharide vaccines since it is this age group that suffers the most from infections caused by encapsulated bacteria. An approach to overcoming this problem, and for increasing the immunogenicity of the polysaccharide antigens, is to couple the polysaccharide to a protein-carrier, thus converting it to a T-cell dependent antigen[67]. When immunised with such conjugates, the primary antibody response is generally low and of IgM specificity[63]. The T-memory cells, which are also recruited, however, stimulate an enhanced secondary anti-polysaccharide response with a shift towards IgG production.

Antibodies are antigen-specific and this specificity is dependant on the structural features of the antigens, i.e. the antibody-binding sites (epitopes). The question that present research seeks to answer, is whether these antibody-binding sites can be

mimicked by synthetic compounds of carbohydrate, or non-carbohydrate, origin[63]. This can, however, only be done once the three dimensional structures of the relevant capsular polysaccharides, and in particular the shapes of the epitopes, are known.

The work presented in this thesis forms part of a continuing program of structural studies on *Klebsiella* and *E. coli* exopolysaccharides. Whilst most of the *Klebsiella* structures have been studied, the structures of a large proportion of the *E. coli* capsular polysaccharides are still unknown. Consequently, the emphasis of research in this laboratory is on the structural elucidation of the *E. coli* capsular polysaccharides, which are proving to be more complex and have been shown to contain more unusual sugars than their *Klebsiella* counterparts.

2. ISOLATION AND PURIFICATION OF THE CAPSULAR POLYSACCHARIDES.

It is imperative to isolate the capsular polysaccharide in a suitably pure form before commencing with the structural elucidation. Numerous methods for the isolation and purification of the capsular polysaccharides have been described in the literature[17,68-70]. These methods generally involve the solubilization of the capsular material, removal of the dead bacterial cells, and the isolation of the acidic capsular polysaccharide, either by chromatography or by selective precipitation. The method described by Okutani and Dutton[70], which employs selective precipitation, is routinely used in this laboratory and will be discussed.

The serologically homogenous capsular bacteria are grown on a semi-solid agar medium at 37°C. After sufficient growth has occurred (18 to 24 hours), the bacterial cells are harvested and suspended in 1% aqueous phenol, which kills them without causing lysis. The phenolic suspension is stirred for several hours at 4°C to ensure dissolution of the capsular material after which it is ultracentrifuged to separate the polysaccharide solution from the dead bacterial cells. The crude polysaccharides, a mixture of the acidic- and neutral polysaccharides, *viz.* the K- and the O-antigens, are precipitated by pouring the supernatant into ethanol. After isolation by centrifugation the polysaccharides are dissolved in the minimum amount of water. The acidic polysaccharide is separated from the neutral O-antigens by precipitation with a quaternary ammonium salt, *viz.* cetyltrimethylammonium bromide (CTAB)[71]. The precipitate is isolated by centrifugation after which it is dissolved in 3M aqueous sodium chloride and poured into ethanol. The precipitated polysaccharide is again dissolved in a small amount of water and then dialysed against running tapwater for 48 hours. The aqueous solution is subsequently freeze-dried to yield the acidic polysaccharide. Additional purification steps include treatment with enzymes, *viz.* RNAse or proteases, or ion-exchange- and gel-permeation chromatography.

3. STRUCTURAL ELUCIDATION OF CAPSULAR POLYSACCHARIDES.

The determination of the complete primary structure of the isolated capsular polysaccharide is a complex task in which many chemical and instrumental techniques may be employed. A detailed discussion of all the analytical techniques available is beyond the scope of this review. Only the more common techniques, and those employed for the purpose of this investigation, will be discussed.

3.1. CHEMICAL ANALYSIS OF CAPSULAR POLYSACCHARIDES.

3.1.1. Determination of the Monosaccharide Composition.

The determination of the monosaccharide composition of the oligosaccharide repeating unit involves the identification and quantitative estimation of the sugars present[72]. In addition, the polysaccharide should be analysed for non-carbohydrate substituents such as O-acetyl, N-acetyl, sulphate- and phosphate groups.

The monosaccharide analysis is achieved by the acid catalysed hydrolysis of the purified polysaccharide[73]. During this reaction the polysaccharide is broken down into its constituent monosaccharides which may then be identified by various chromatographic techniques. Several factors, including the anomeric configuration, the position of linkage, and the monosaccharide composition, will influence the rate of hydrolysis. Anhydro- and deoxy-sugars such as 3,6 anhydro- galactose, sialic acids, and 2-deoxyaldoses are very sensitive to acids and are largely destroyed under extreme conditions[72], while incomplete hydrolysis is often encountered with uronosyl and 2-amino-2-deoxyglycosidic linkages. It is therefore often necessary to employ more than one set of conditions to obtain an accurate quantitative estimation of the sugars present[74].

Various acids including hydrochloric acid, sulphuric acid, and trifluoroacetic acid

(TFA)[75], have been employed to catalyse the hydrolysis reaction. Studies by Hough and co-workers[76] showed sulphuric acid and TFA to be the acids of choice since they do not effect the degradation of the monosaccharides. TFA has the additional advantage of being volatile and can therefore be easily removed from the products of hydrolysis. In this laboratory complete hydrolysis of the polysaccharide is effected by treatment with 4M aqueous TFA at 125°C for one hour[77]. Lower concentrations are often used in partial hydrolysis studies.

Methanolysis is preferred to acid hydrolysis in the analysis of polysaccharides containing uronic acid residues[78]. The uronosyl linkages are resistant to acid hydrolysis and so the polysaccharide is rather methanolysed and the resulting methyl esters reduced. During this reaction the uronic acid residues are converted to their parent aldoses which are then much more susceptible to the acid hydrolysis with aqueous TFA.

3.1.2. Determination of the Absolute Configuration of the Monosaccharides.

In Nature sugars occur in both the D- and the L- configurations[79]. The absolute configuration of the monosaccharides can be determined by several methods[80]. These include the application of specific enzymes, measurement of the optical rotation of the isolated monosaccharides and gas-liquid chromatographic (g.l.c.) analysis of acetylated glycosides formed from chiral alcohols. The latter is favoured in this laboratory since suitable enzymes are expensive and not always available whilst substantial amounts of very pure material are needed to determine the optical rotation.

Glycosides of a D- and a L-sugar, and a chiral alcohol, e.g. the secondary alcohol (-)-2-octanol are diasteriomeric and can be separated, in the acetylated form, by g.l.c.[79]. A single monosaccharide gives rise to several octyl-glycosides, which have a characteristic multiple peak pattern and, provided that the peaks of the octyl-glycosides do not overlap completely, mixtures of monosaccharides can be analysed directly.

3.1.3. Determination of the Linkage Positions.

Methylation analysis is used to determine the positions of linkage between component sugar residues in the polysaccharide[81]. This is achieved by the etherification of the free hydroxyl groups in the polysaccharide and the subsequent hydrolysis to a mixture of partially methylated monosaccharides. (The free hydroxyl groups in the partially methylated sugars mark the positions in which the sugar residues were substituted.) The partially methylated sugars are then reduced, acetylated and analysed as the methylated alditol acetates by g.l.c. or g.l.c.- m.s. (gas-liquid chromatography - mass spectrometry). The sugars are identified by their mass spectra and their g.l.c. retention times. Several factors, including undermethylation and degradation or demethylation during the acid hydrolysis, can lead to the misinterpretation of these results[82].

Many different procedures for the methylation analysis of polysaccharides have been described in the literature. In the original procedure, as used by Haworth[71], the polysaccharide was methylated with dimethylsulphate in an aqueous sodium hydroxide medium. Fresh reagents were added to the reaction mixture after certain time intervals until there was no further increase in methoxyl content. Several attempts have been made over the years to improve the yield of this reaction and to reduce the number of repetitive treatments required.

The method that is currently preferred in most structural investigations on polysaccharides is a slight modification of the one proposed by Hakomori in 1964. In the original Hakomori method[83] the polysaccharide undergoes alkoxide formation by reaction with sodium methylsulphinylmethanide (sodium dimsyl) in dimethyl sulphoxide (Equation 1). This is followed by a reaction with methyl iodide to yield a completely methylated product (Equation 2).

Equation 1 : $R\text{-}OH + CH_3\text{-}SO\text{-}CH_2^-Na^+ \rightarrow RO^-Na^+ + CH_3\text{-}SO\text{-}CH_3$

Equation 1 : $R\text{-}O^-Na^+ + CH_3I \rightarrow R\text{-}O\text{-}CH_3 + NaI$

The Hakomori methylation is carried out in one continuous process at room temperature and can be controlled by the amount of reagent added[84]. This reaction has the additional advantage in that it usually effects complete etherification in one step. Uronic acids are transformed into methyl esters and O-acetyl groups are cleaved. Acetal functions such as the pyruvic acid ketals, are however stable.

Methylation by the Hakomori method does have some serious drawbacks. The carbanion, which catalyses the first reaction step, is generated from dimethyl sulphoxide and sodium hydride. This preparation is tedious and any moisture present may inactivate the reagent and increase the amount of interfering peaks on g.l.c. analysis[85]. Commercial sodium hydride, in addition, also contains impurities which will interfere on g.l.c. analysis. Modifications such as the replacement of sodium hydride with either potassium-tert-butoxide, alkali-metal amides[86], alkali-metal hydroxides or potassium hydride[87], have been proposed. The latter is the reagent of choice in this laboratory since the preparation of dimsyl potassium is rapid and does not require any heating. The reaction products also tend to be freer of impurities when dimsyl potassium is used.

Some polysaccharides show resistance to complete O-methylation due to their free hydroxyl groups being restricted by inter- and intra-molecular hydrogen bonding. This problem can be overcome by using 1,1,3,3 tetramethylurea to cause relaxation of the hydrogen bonding[88].

Since the Hakomori-methylation does not always afford complete methylation, the product often needs to be methylated further by a different method. Purdie and Irvine[71] proposed treatment of the partially methylated material with silver oxide in gently boiling

methyl iodide. This technique is, however, not always effective and has since been greatly improved by Kuhn and co-workers[89] who carried out the reaction in the polar solvent, N,N-dimethylformamide.

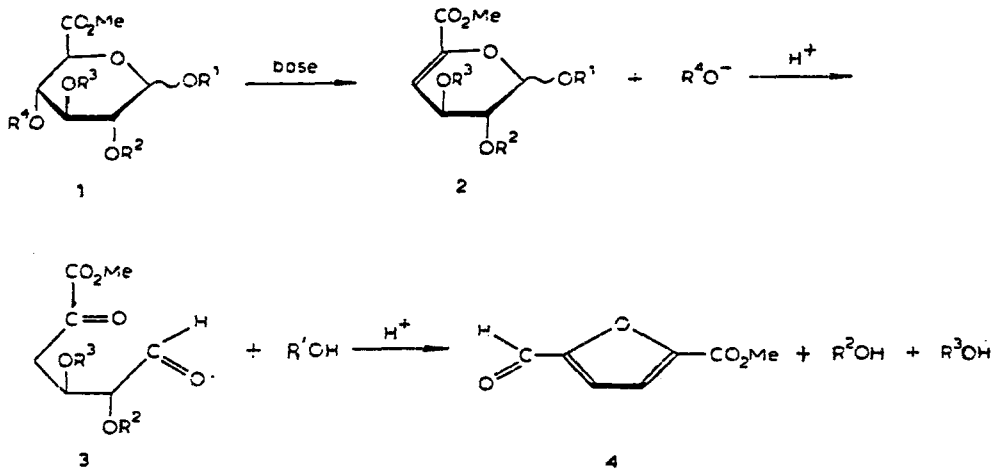
Alkali-labile substituents present a problem when methylation is attempted with the conventional reagents[90]. O-Acetyl groups are cleaved under these conditions, whereas phosphate substituents may migrate via intra-molecular cyclic esters. Methylation of low molecular weight alkali-labile carbohydrates can be achieved by reaction with methyl trifluoromethanesulphonate in trimethyl phosphate[91]. This procedure is mild and yields completely methylated products in one step.

3.1.4. The Sequencing of the Monosaccharides.

3.1.4.1. Base-catalysed degradation of Uronic Acids.

The base-catalysed degradation of methylated acidic polysaccharides yields important information on the sites of attachment of uronic acid residues. This is achieved by a single operation[92] in which the methylated polysaccharide is treated with base and then, without work-up, alkylated to label the site(s) to which the uronic acid residues were attached. This procedure avoids the intermediate isolation of degraded polysaccharides and possible loss of acid-labile substituents which might occur during the mild acid treatment proposed by Lindberg *et al*[93].

Base-catalysed β-eliminations from hexuronic acid residues occur when the uronic acid is esterified and 4-O-substituted[72] by a good leaving group, *viz.* either a derivative of a sugar residue or a methoxyl group (Scheme A, structure 1). On treatment with base these groups are eliminated and hex-4-enopyranosiduronate residues, structure **2**, are formed.



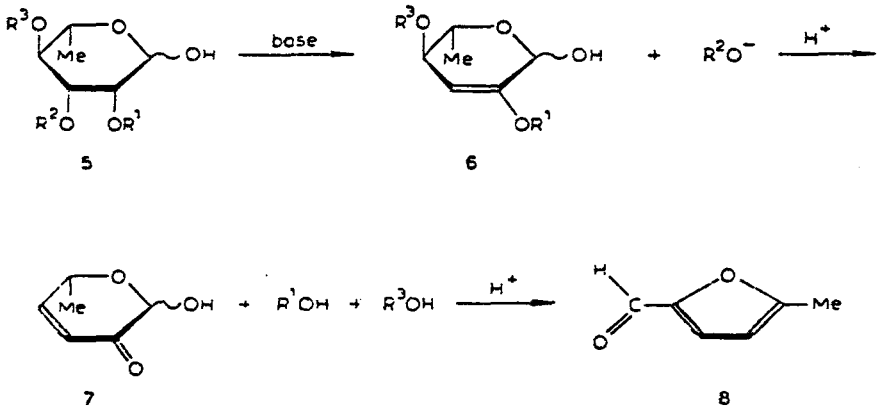
SCHEME A[93] R^1 = sugar residue

R^2, R^3, R^4 = methoxyl group (Me) or a sugar residue

In the procedure proposed by Lindberg *et al*[93] the unsaturated uronate residue is subjected to a mild hydrolysis with acid, yielding a substituted 4-deoxyhex-5-ulosuronate, structure 3, and a free aglycon. This ester then reacts further to ultimately yield a furan derivative, structure 4. In the method proposed by Aspinall and Rosell[92] however, the mild hydrolysis step is omitted, and the reaction products are directly re-alkylated with either trideuteriomethyl- or ethyl iodide after treatment with base. The modified polysaccharide is then hydrolysed and the resulting methylated sugars analysed, as the alditol acetates, by g.l.c. or g.l.c.- m.s.

When a glycosidic bond is broken on elimination with base a reducing sugar, structure 5, which may undergo further β-elimination under the conditions proposed by Lindberg *et al*[93], is formed. (Scheme B)

Addition of the alkylating agent (Aspinall and Rosell[92]) however terminates this reaction by creating a glycoside of any reducing sugar that is formed and, at the same time, alkylates the freed hydroxyl group on the sugar unit to which the uronic acid residue was linked.



SCHEME B[93]

The method proposed by Aspinall and Rosell[92] is easy to perform and does not require much material. This reaction gives considerable structural information when the results are compared with those of the methylation analysis, i.e. the identities of the sugar residues on either side of the uronic acid residue can be determined.

3.1.4.2 The Generation of Oligosaccharides.

The structural elucidation of oligosaccharides obtained from the parent polysaccharide can provide valuable information about the main structural features of the polymer. A variety of methods for the generation of these smaller oligosaccharides has been described in the literature. The most commonly employed procedure involves the acid-catalysed partial hydrolysis of the parent polysaccharide[72]. Alternative types of acid-catalysed depolymerizations, which induce different degrees of selectivity in bond cleavage, include acetolysis[94], trifluoroacetolysis[95], and solvolysis with anhydrous hydrogen fluoride[96].

The selective fragmentation of bacterial polysaccharides can also be achieved by the oxidative cleavage of sugars with vicinal hydroxyl groups. This includes reactions such as the Smith degradation[97] and lead tetraacetate oxidation[71]. Enzymes such as

bacteriophage-borne endoglycanases which afford the selective cleavage of glycosidic bonds[98], are also increasingly applied in the structural studies of bacterial polysaccharides. These enzymes are often the only method of preparing oligosaccharides that correspond to the repeating unit and/or multiples thereof. Another modern technique in the generation of oligosaccharides involves the cleavage of acidic polysaccharides at the sites of the uronic acid residues, *viz.* by treatment with lithium-metal dissolved in ethylenediamine[98]. Recently the formation of anhydrocyclitols, by the reductive cleavage of methylated polysaccharides[100], has been introduced as a method for the cleaving of glycosidic linkages. The selectivity of this method has however not been fully established.

A detailed discussion of all the above mentioned techniques is beyond the scope of this review and only those employed for the purpose of this investigation, will be discussed.

3.1.4.2.(i) Partial Hydrolysis.

Partial hydrolysis of a polysaccharide involves the termination of the hydrolysis reaction before complete depolymerization has occurred. The oligosaccharides will, on isolation, confirm certain linkage types and provide information on sequences and anomeric configurations[71].

Substantial differences in the rates of hydrolysis of the various glycosidic linkages are often encountered and advantage can be taken of these differences to achieve relatively selective fragmentations[72]. Sugars in the furanose configurations are hydrolysed more rapidly than the corresponding pyranosides. Deoxy sugars are also readily hydrolysed, especially when the deoxygenated carbon atoms are in the ring, whereas hexuronic acids and 2-amino-2-deoxy-glycosides are much more resistant to hydrolysis at low pH-values. In this laboratory the partial hydrolysis of capsular polysaccharides is effected by treatment with dilute aqueous TFA. Separation of the resulting oligosaccharides is

achieved by one, or a combination of chromatographic techniques, *viz.* paper- gel-permeation- or high-performance liquid chromatography (h.p.l.c.). The structures of the oligosaccharides are determined by both chemical and spectroscopic (mass spectrometry or nuclear magnetic resonance (n.m.r.)) methods.

The technique of partial hydrolysis has been used, for example, in the structural investigation of the *E. coli* K37 capsular polysaccharide[43] (Figure 3). Three oligosaccharides (A, B and C) were isolated after the partial hydrolysis reaction and, after being identified by methylation analysis, acid hydrolysis and n.m.r. spectroscopy, provided valuable structural information about the parent polysaccharide.

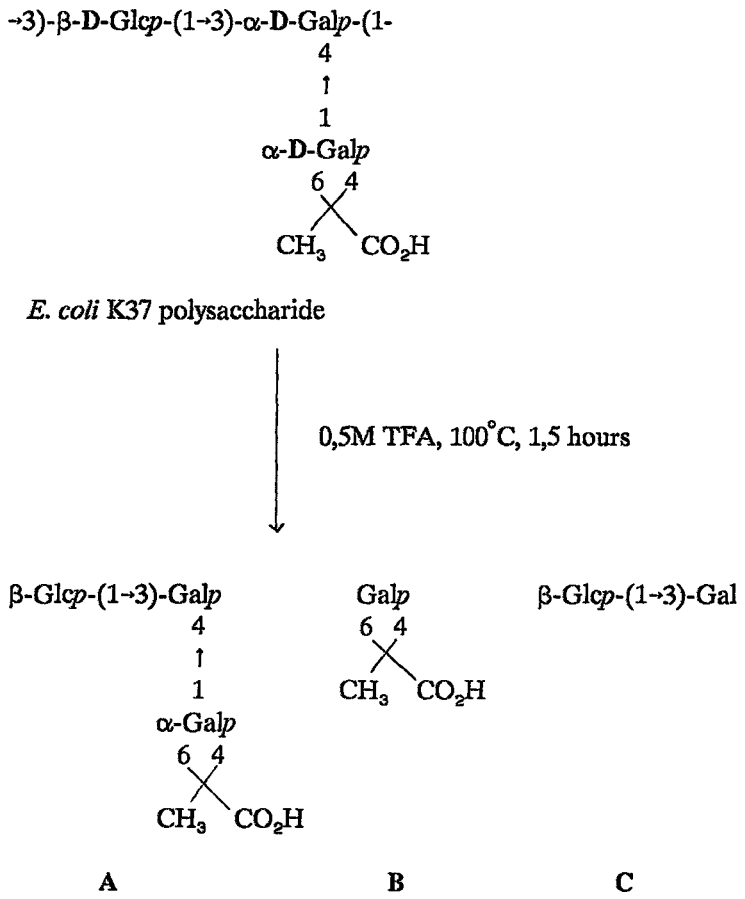


Figure 3 : Partial hydrolysis of the *E. coli* K37 polysaccharide[43].

Partial hydrolysis reactions do however have a serious drawback in their large initial

requirement of starting material[72]. The fragments that are formed during this process may also degrade further as the reaction proceeds. This will lead to a general low yield of oligosaccharides.

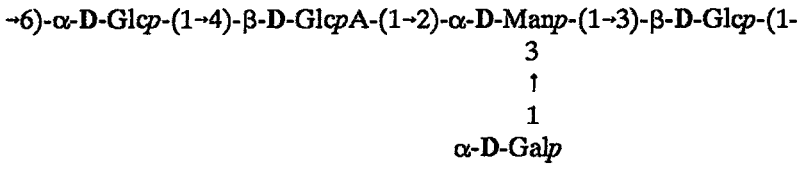
3.1.4.2.(ii) Selective Degradation by Lithium dissolved in Ethylenediamine.

Albersheim and co-workers[89] developed a general method by which underivatised carbohydrates containing uronic acid residues are cleaved by use of lithium dissolved in ethylenediamine. Lithium metal dissolved in an amine solvent is a powerful reducing agent and will cleave carbohydrates at the sites of the uronic acid residues, regardless of the position of substitution of the uronic acid. Treatment with this reagent will also result in the cleavage of methyl glycosides, the reduction of aldoses, and the cleavage of the methyl esters and pyruvyl ketals of glycosyl residues.

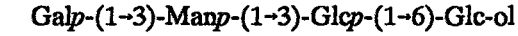
The reaction involves the dissolution of a dried carbohydrate sample in a small amount of ethylenediamine and the subsequent addition of small pieces of lithium-wire. The amount of lithium added varies according to the different constituents of the carbohydrate samples and is determined by the amount required to maintain the deep blue colour of the reaction. The reaction is left to proceed at room temperature for about one hour after which it is quenched with water. Toluene is then added to form an azeotrope with the ethylenediamine and water, and the mixture is rotoevaporated to dryness. The resulting powdery residue is dissolved in water, titrated to pH 4.5, and passed down a cation-exchange resin. The acidic fraction is collected and freeze-dried to yield the "lithium-treated carbohydrates".

This degradation reaction has been used, for example, in the structural elucidation of the *E. coli* K39 polysaccharide[44]. Treatment of a solution of the polysaccharide in ethylenediamine with lithium, followed by separation of the products by gel-permeation chromatography, afforded an oligosaccharide, A. This oligosaccharide was identified by

methylation analysis, acid hydrolysis and n.m.r. spectroscopy and provided valuable information about the structure of the polysaccharide repeating unit.



E. coli K39 polysaccharide



A

Figure 4 : Lithium in ethylenediamine degradation of the *E. coli* K39 polysaccharide[44].

The proposed mechanism of this reaction involves the cleavage of the bond between the ring oxygen and C-5 of the uronic acid residue[99]. The oxygen on C-5 moves to C-1 via α-elimination, with the concomitant loss of the oxygen of C-1 to its aglycone.

This reaction is of great value because of its ability to selectively cleave underivatised complex carbohydrates at the sites of the uronic acid residues. The reaction is rapid and relatively few side-products are formed.

3.1.4.2.(iii) Bacteriophage Degradation.

Oligosaccharides, *viz.* the repeating unit (P_1), and multiples thereof (P_2, P_3,.....), are generated from the parent capsular polysaccharide by treatment with bacteriophage-borne endoglycanases. These oligosaccharides can be subjected to further chemical analysis[91,99], however, owing to the suitability of oligosaccharides to n.m.r. study, the latter technique is very often the preferred means of analysis. The n.m.r. spectra (both ^{1}H and ^{13}C)

obtained from these low molecular weight oligosaccharides, are easier to analyse than those obtained from the parent polysaccharide, due to the signals being sharper and better resolved. The spectra, especially those obtained from the oligosaccharide repeating unit (P_1), provide valuable information on the sequencing and structure of the capsular polysaccharides.

Bacteriophages (phages) are viruses that infect bacteria[101]. They are host-specific[102], and in order to penetrate the bacterial cell, they have to degrade the cell wall. A variety of enzymic activities that catalyse the different degradation reactions are therefore associated with the bacteriophage[98]. These include the bacteriophage-borne deacetylases which catalyse the hydrolysis of the O- and N-acetyl groups substituting the capsular polysaccharides, as well as the lyases and glycanases which catalyse the hydrolysis of the glycosidic bonds, thus producing the repeating unit and/or multiples thereof. In most cases reported so far, hydrolysis occurs next to the sugar unit carrying the negative charge and the resulting reducing end sugar, is usually substituted at position 3[103]. In the majority of cases, β-glycosidic bonds are split.

This selectivity is clearly illustrated by the bacteriophage-borne endoglycanase which catalyses the hydrolysis of β-D-glucose-(1→3)-β-D-glucuronic acid bonds (arrow, ↑) in

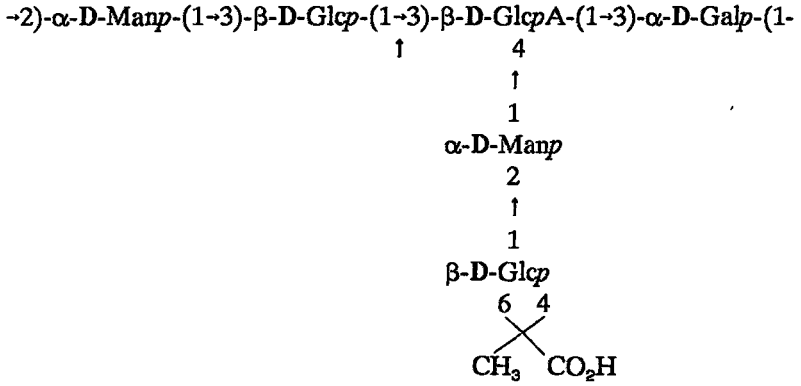


Figure 5 : Bacteriophage degradation of the *E. coli* K29 capsular polysaccharide[104].

the *E. coli* K29 polysaccharide, ultimately leading to a mixture of K29 hexasaccharide, dodecasaccharide and octadecasaccharide[104].

The phage-associated glycanases which are utilized in these degradation reactions are associated with the tail spikes of the viruses, most of which belong to Bradley's group C[103,105]. These enzymes are highly specific and have a tendency to increase their specificity upon continued repropagation of the virus.

Degradation of high molecular weight polysaccharides : The first step in the bacteriophage degradation reaction involves the isolation and purification of the phage. It is isolated from sewage water[106] and purified, on the host-strain, by successive plaque isolations. The phage is then propagated on its host in a broth medium after which the solution is assayed by the agar overlay technique[101]. This is a simple and accurate technique by which the plaques that arise from a known volume of phage suspension are counted. A final concentration of about 10^{13} plaque-forming units (pfu's) is considered adequate to afford the complete degradation of 1 g polysaccharide[98].

The polysaccharide is dissolved in the aqueous phage-solution and degradation is carried out at 37°C for three days, after which the reaction-mixture is centrifuged and freeze-dried. The material thus recovered is dissolved in a small amount of water and dialysed to exhaustion. The dialysate is collected and purified by several passages down an ion-exchange resin, after which the various components, *viz.* P_1, P_2, P_3,....., are separated by gel-permeation chromatography.

This degradation procedure has been employed in a number of structural studies on *E.coli* capsular polysaccharides, including those of *E. coli* K1[107], K5[108], K29[104], K36[42] and K39[44].

3.2. CHROMATOGRAPHIC TECHNIQUES EMPLOYED IN THE ANALYSIS OF CAPSULAR POLYSACCHARIDES.

A variety of chromatographic techniques has been employed in this investigation and are discussed. Other techniques that are commonly used in the separation of oligo- and polysaccharides include thin-layer chromatography[109], electrophoresis[110], and paper chromatography[111-115].

3.2.2. Gel-Permeation Chromatography.

Gel-permeation chromatography (g.p.c.), also known as gel filtration- or exclusion chromatography, is a technique whereby molecules are separated according to differences in their sizes[116]. The stationary phase is a three-dimensional swollen gel which is composed of porous particles[117]. On separating a mixture into its constituent components, molecules larger than the pores of the gel are eluted rapidly since they are unable to enter the gel-matrix. The smaller molecules do however enter the gel-pores to a varying extent, depending on their size and shape[116]. The components are thus eluted in order of decreasing molecular size.

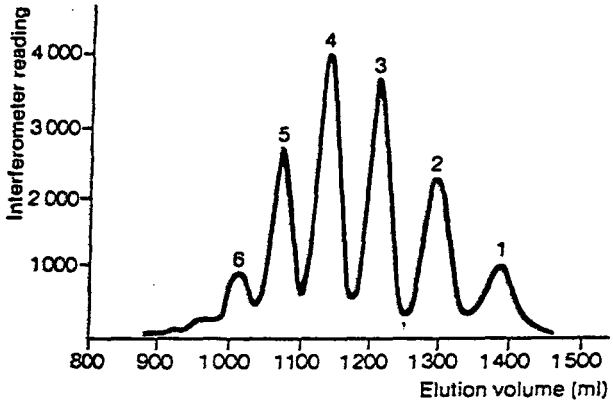


Figure 6 : Gel-permeation chromatogram of an oligosaccharide mixture from the acetolysis and hydrolysis of cellulose on Sephadex G-25. The numbers above the peaks indicate the degree of polymerization[118].

Results in gel-permeation chromatography are typically expressed in the form of an elution diagram showing the variation of solute concentration in the eluent, with the volume of eluent passed through the column (Figure 6).

The most suitable media for the gel chromatography of carbohydrates are agarose-, crosslinked dextran- and polyacrylamide gels, or porous glass- or silica beads[116]. Optimum separation is achieved when the size of the gel-pores is comparable to the sizes of the substances being chromatographed[119]. A gel of a given porosity is therefor only effective in separating substances within a certain range of molecular sizes, *viz.* the fractionation range[120]. Good separation of carbohydrate samples through g.p.c. is often dependant on the type of mobile phase employed. Underivatised carbohydrates are usually separated employing either deionized water or buffer systems, e.g. phosphate, acetate, Tris-hydrochloric acid, etc. as the mobile phase[116]. Derivatised carbohydrates, such as methylated sugars, are separated with organic solvents, e.g. acetone, methanol and/or ethanol.

G.p.c. is a simple technique and is often used as a purification step in carbohydrate chemistry. Since separation by gel filtration is based on molecular size, this technique is frequently used in molecular weight determinations of carbohydrates[121]. G.p.c. may also be employed to monitor reactions such as the acid hydrolysis of the capsular polysaccharides, *viz.* by determining the molecular weights of the products at the various stages of the reaction[122].

3.2.2. Ion-exchange Chromatography.

Ion-exchange chromatography, both resin- and gel-, has found wide application in carbohydrate chemistry. Ion-exchange resins, for example Amberlite IR-120$(H)^+$, are used for purification and exchange of ions, i.e. the removal of buffer, etc., in carbohydrate work-ups, whereas the ion-exchange gels, e.g. DEAE-Sepharose CL-6B, are

most commonly employed in the isolation and purification of the acidic polysaccharide[123]. Only the latter technique will be discussed.

Separation by ion-exchange chromatography (gel) is obtained by reversible adsorption to the gel-matrix, i.e. the stationary phase. This technique is performed in two stages, the first being sample application and adsorption. In the second stage, the compounds are separated and eluted from the column. Separation of the compounds is achieved by taking advantage of their different affinities, based on their charge properties, for the gel stationary phase. By varying conditions such as the ionic strength and pH, the various compounds are eluted from the column at different stages[124], i.e. in order of increasing ionic strength.

An ion-exchange gel consists of an insoluble matrix to which charged groups are covalently bound. The type of group determines the type and strength of the ion-exchange gel[125] and can be reversibly exchanged, with similarly charged ions, without altering the

Anion Exchangers	**Functional group**
Aminoethyl (AE-)	$-OCH_2CH_2NH_3^+$
Diethylaminoethyl (DEAE-)	$-OCH_2CH_2N^+H(CH_2CH_3)_2$
Quaternary aminoethyl (QAE-)	$-OCH_2CH_2N^+(C_2H_5)_2CH_2CH(OH)CH_3$
Cation Exchangers	
Carboxymethyl (CM-)	$-CH_2COO^-$
Phospho-	$-PO_4H^-$
Sulphopropyl (SP-)	$-CH_2CH_2CH_2SO_3^-$

Table 2 : Functional groups used in ion-exchange chromatography[125].

matrix. A variety of groups have been chosen for use in ion-exchange chromatography and are listed in Table 2.

In this laboratory this technique is performed using the anion-exchange gel DEAE-Sepharose CL-6B with gradient elution. (Ionic strength : 0 → 0.5M NaCl in 0.01M Tris buffer). Since separation is achieved by differences in the charge properties of the various compounds, the acidic capsular polysaccharides are well separated from the neutral O-antigens and other contaminants, e.g. RNA.

3.2.3. Gas-liquid Chromatography.

The chromatographic process in gas-liquid chromatography (g.l.c.) involves the partitioning of a compound between the stationary liquid phase and the mobile gas phase[126].

The sample is introduced on to a column through a specially designed inlet[127] where it is immediately vaporized. As the sample is moved down the column by the gaseous phase, the individual components partition themselves between the mobile and stationary phases[126]. Separation of the various compounds is achieved according to how each is retained by the column, i.e. depending on their relative affinities for the stationary phase. The factors that influence this separation include the column length and diameter, the column temperature, the carrier gas and its velocity, as well as the polarity and film thickness of the stationary phase.

On leaving the column, the gaseous mobile phase passes through a detector which detects the various components of the sample using one of many techniques e.g. thermal conductivity, flame ionization, etc.[128]. The retention behaviour, i.e. retention volume or retention time, is a characteristic property of a compound providing that the conditions of the separation can be duplicated exactly.

The qualitative analysis of a chromatogram involves the comparison of the retention time of a known compound to that of an unknown peak under identical chromatographic conditions[126]. To avoid slight differences that may occur due to minor variations in the operating conditions retention times are usually made relative to that of a standard compound which is assigned a value of unity. Quantitative estimations[128] are made by measuring the areas under the various peaks representative of the separated compounds.

A variety of columns have been employed in the separation of carbohydrates[129]. High resolution or capillary g.l.c. employs an open tubular column with a stationary phase film on the inner wall[130]. A large range of capillary columns, with a variety of stationary phases coated on a flexible fused silica tube, is commercially available[129]. In this laboratory wall coated columns (WCOT) of DB-1, DB-17 and DB-225 are used to separate derivatised mono-and oligosaccharides. The compositions of their various stationary phases are listed in Table 3.

Column	Composition
DB-1	100% Methyl silicone, gum
DB-17	50% Methyl silicone 50% Phenyl silicone
DB-225	25% Cyanopropyl silicone 25% Phenyl silicone 50% Methyl silicone

Table 3 : The composition of some stationary phases.

Column DB-1 is employed in the separation of alkylated glycosides and small, derivatised oligosaccharides, whereas both columns DB-17 and DB-225 are used in the separation

of the derivatised monosaccharides.

The products obtained from the various chemical reactions employed, *viz.* acid hydrolysis, methanolysis, methylation, etc., are not sufficiently volatile for g.l.c. analysis and must therefore be derivatised[131]. Several derivatization procedures such as methylation, acetylation, trimethylsilylation and butylboronate formation have been described in the literature[74,132]. A serious disadvantage however of derivatives preserving the anomeric centre, is that a single sugar may give rise to several chromatographic peaks due to the α- and β- anomers and ring isomers that are present[129]. The resulting chromatograms are therefore crowded and difficult to analyse owing to the reduced sensitivity and overlapping of signals. To overcome this problem additional derivatizations such as oxime-, methyloxime-, alditol- and aldononitrile formation, are used in conjunction with acetylation and trimethylsilylation[129]. These additional procedures reduce the incidence of multiple peaks and thus simplify the resulting chromatogram.

In this study the monosaccharide-products obtained from the acid hydrolysis of the native polysaccharide are converted to either alditol acetates[133] or peracetylated aldononitriles[134,135]. The alditol acetates have similar properties to those of the peracetylated aldononitriles, *viz.* both are thermally stable and give single and fairly well separated peaks.

G.l.c. is also used in the analysis of methylated polysaccharides. After hydrolysis of the methylated polymer alditol acetates are formed which are then separated by g.l.c. (Figure 7). The partially methylated alditol acetates have the additional advantage in that they give readily identifiable fragments when examined by mass spectrometry (combined g.l.c.- m.s.), enabling their structures to be determined[136].

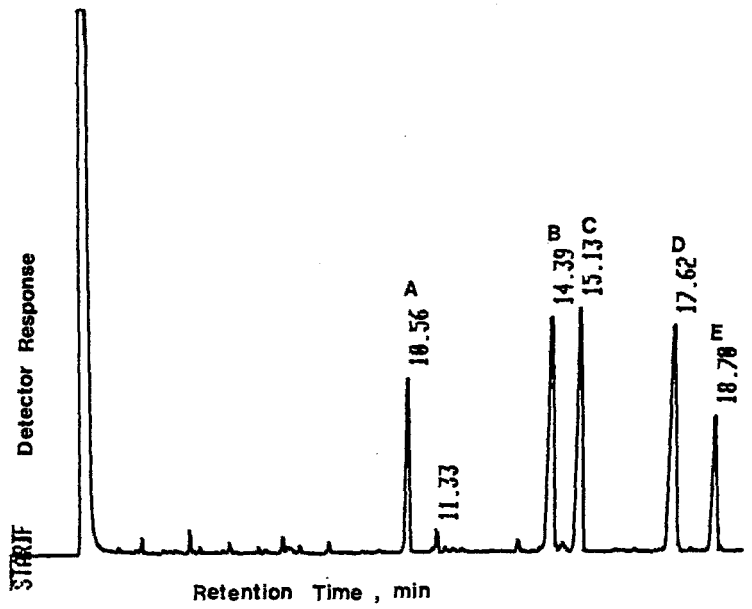


Figure 7 : The g.l.c. analysis of the partially methylated alditol acetates obtained from the *E. coli* K102 polysaccharide. **A** : 2,3,4,6-Glc, **B** : 2,3,6-Gal, **C** : 2,4,6-Gal, **D** : 2,6-Gal, **E** : 2,3-Glc
Column DB-17, 180°C for 2 min then 2°/ min to 240°C

Small oligosaccharides, i.e. di- or trisaccharides, are analysed by g.l.c. as their methyl- or trimethylsilyl derivatives[137]. The anomeric centre of the end-group sugar must however be reduced prior to methylation in order to avoid the incidence of multiple peaks.

G.l.c. is also commonly employed in the separation of enantiomers[80]. The resolution of racemic mixtures, *viz.* D- and L-sugars, is achieved by conversion of the enantiomers into diasteriomers using a chiral agent[79], and separation on a non-chiral phase, e.g. DB-17 or DB-225. Chiral stationary phases are becoming available for the separation of enantiomeric sugar residues[138].

3.2.4. High-Performance Liquid Chromatography.

High-Performance liquid chromatography (h.p.l.c.) is a popular method for the rapid separation of organic compounds in solution. Separation of the components of a mixture

is achieved according to how each is retained by the solid stationary phase of the column.

A h.p.l.c. system, or chromatograph, includes a high pressure solvent-delivery system using one or more pumps, an injection valve, a column packed with monodisperse resin- or gel particles, and a detector[139]. The chromatograph operates at elevated pressures that range from ~1 to ~30MPa[139], depending on the flow rates used and the mobile- and stationary phases employed. Many h.p.l.c. columns require elevated temperatures for optimal performance, and ovens for this purpose are available.

H.p.l.c. is well suited for the quantitative and qualitative analysis of non-volatile or thermally unstable compounds, such as carbohydrates, since the components to be separated remain in a liquid phase[140]. The sample is introduced into the h.p.l.c. system via the injection valve after which it is moved down the column by the liquid mobile phase. The mechanism by which the various components are separated, e.g. phase partitioning[139], exclusion, ligand exchange or hydrophobic adsorption[141], depends on the stationary phase employed. A large range of h.p.l.c. columns, with a variety of stationary phases including amine modified silica gels[140], reverse phased silica gels[139] and cation[142] and anion exchange resins and silica gels[143] are commercially available. The mobile phase employed is determined by both the nature of the stationary phase and the components to be separated[139].

The various components of the sample can be detected using one of the following detection systems : Ultraviolet light (U.V. light), infrared light, electrochemistry, refractive index, etc. The components of carbohydrate samples are however normally detected by their different refractive indices due to the absence of suitable chromophores.

In this laboratory a mild cation-exchange column, Pro-gel C6-11, is used to effect the separation of the monosaccharide products of hydrolysis. Water or a very weak buffer

system, *viz.* 10^{-4}M aqueous NaOH, is employed as the mobile phase (Figure 8). Oligosaccharides and low molecular weight polymers are separated on either Pro-gel TSK-Oligo-W or Pro-gel TSK-G3000-PW. Water is used as the mobile phase when the compounds are separated on Pro-gel TSK-Oligo-W, whereas the latter employs a buffer system, *viz.* 0.1M sodium acetate; 0.1M sodium sulphate; pH < 3.7.

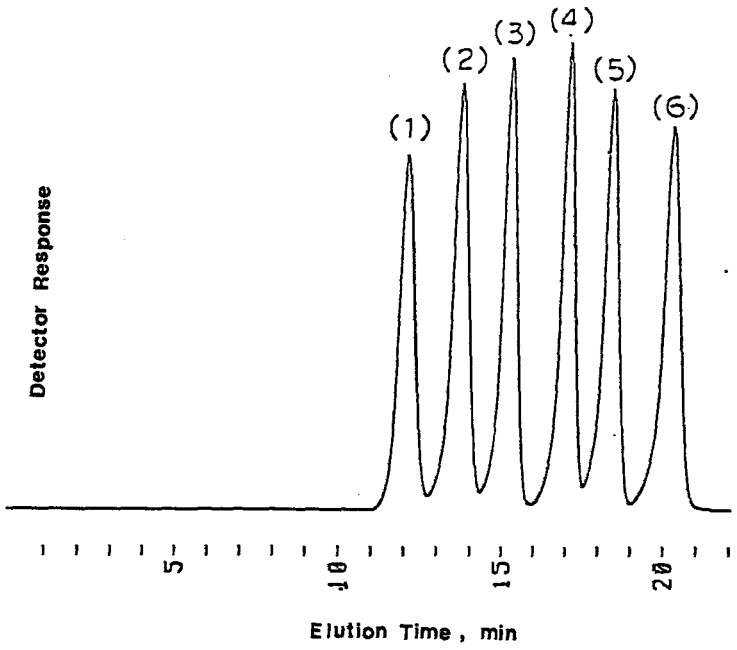


Figure 8 : The h.p.l.c. separation of a carbohydrate mixture on column Pro-gel C6-11.

1.Raffinose 2.Maltose 3.Glucose 4.Galactose 5.Mannose 6.Fructose

Eluent : 10^{-4}M aqueous NaOH

Detection : Refractive index

H.p.l.c. has many advantages over the other chromatographic methods of carbohydrate analysis. It is much faster, more sensitive and more suitable for quantitative analysis than paper- and open column chromatography[144]. H.p.l.c. is also more suitable for routine analysis than g.l.c., since it allows for the rapid separation of sugar residues without the need of pre-derivatization.

3.3. INSTRUMENTAL TECHNIQUES EMPLOYED IN THE ANALYSIS OF CAPSULAR POLYSACCHARIDES.

3.3.1. Mass Spectrometry.

Mass spectrometry (m.s.), in combination with chromatographic techniques, such as g.l.c. and h.p.l.c., has become a very important instrumental technique in the structural determination of complex carbohydrates.

M.s. is performed using a sophisticated instrument which produces, separates and detects both positive and negative gas-phase ions[145]. After volatilization of the sample in the inlet of the mass spectrometer (150°C - 180°C), the vapours are ionized either by fast electrons, a strong electrical field, or by ultraviolet light[148]. The resulting molecular and fragment ions are then separated according to their mass-to-charge ratios (m/z) and their relative abundances are displayed in the mass spectrum. Most compounds, including carbohydrates, produce characteristic patterns and can therefore be identified by their mass spectra.

Since the oligo- and mono-saccharides are thermally unstable and non-volatile, m.s. is performed using their more volatile derivatives, *viz.* the methyl[83] or trimethylsilyl ethers[147], acetates or trifluoroacetates[148]. In the methylation analysis sugar units are most often analysed as their acyclic partially methylated alditol acetates[133]. The acyclic alditols produce simple fragmentation patterns and a large bank of data has been collected in the literature, allowing for rapid identification of the sugars.

3.3.1.1. Electron-Impact Mass Spectrometry.

The most common method of ionization is by electron-impact. This usually results in the elimination of an electron from the sample molecule and thus in the formation of the

positive molecular ion[145]. The molecular ion is however seldom observed since it is involved in several fragmentation and rearrangement reactions[146].

Primary fragments are generated by fission of the alditol carbon-chain. In decreasing order, the efficiency of the fission reaction is as follows[149]:

I : Fission between two methoxylated carbon atoms. (Figure 10, A)

II : Fission between one methoxylated and one acetoxylated carbon. (Figure 10, B)

III : Fission between two acetoxylated carbon atoms. (Figure 10, C)

The primary fragments give rise to secondary fragments, mainly by elimination(s) of acetic acid (60 mass units, m.u.), ketene (42 m.u.), methanol (32 m.u.) or formaldehyde (30 m.u.)[150].

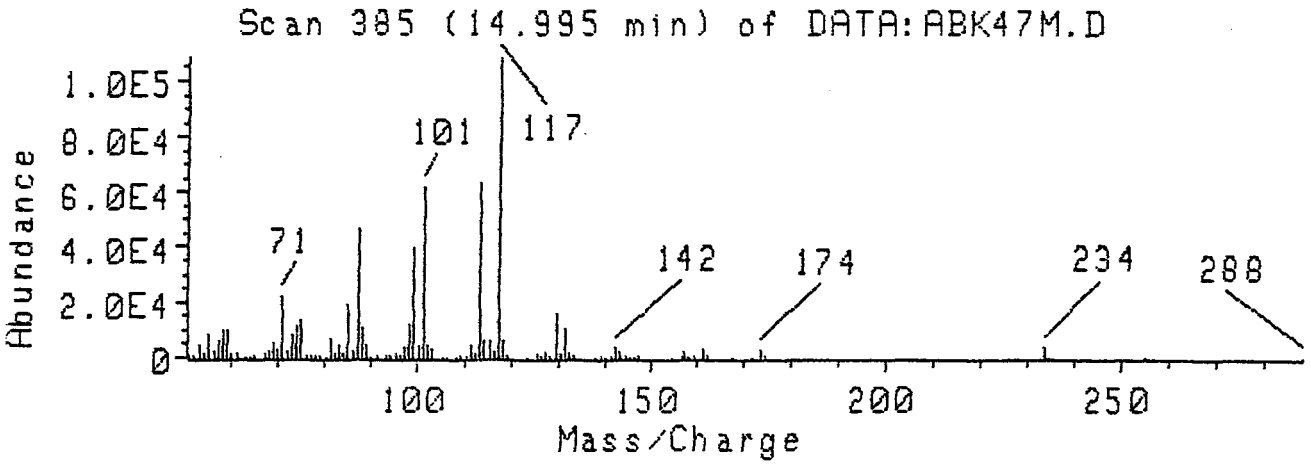
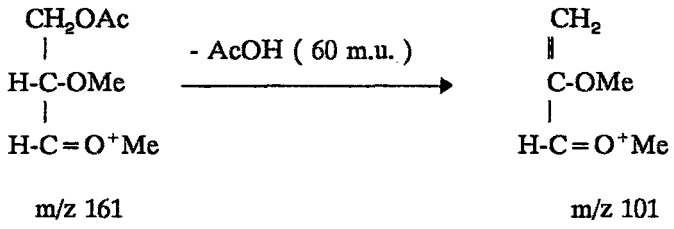


Figure 9 : The mass spectrum of a 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylhexitol.

(73 m.u.)	CH_2OAc		
	------|-------	B^1	(Fragments : 73, 277 m.u.)
(44 m.u.)	H-C-OMe		
	------|-------	A	(Fragments : 117, 233 m.u.)
(44 m.u.)	MeO-C-H		
	------|-------	B^2	(Fragments : 161, 189 m.u.)
(72 m.u.)	H-C-OAc		
	------|-------	C	(Fragments : 233, 117 m.u.)
(72 m.u.)	H-C-OAc		
	------|-------	B^3	(Fragments : 305, 45 m.u.)
(45 m.u.)	CH_2OMe		

Figure 10 : The primary fragmentation of a 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol.

An example of the secondary fragmentation of the fragment m/z 161, which arises from fission at B^2 (Figure 10), is given below[149]:



m/z 161 m/z 101

Electron-impact mass spectrometry is usually employed to provide information on the nature and position of the functional groups in a sugar unit[151]. Since the molecular ion is rarely observed molecular weights may be determined by the addition of pairs of fragments[149]. It is however impossible to distinguish between the various stereoisomers since they fragment to give similar mass spectra.

3.3.1.2. Chemical-Ionization Mass Spectrometry.

Chemical ionization is a much softer ionization procedure than electron-impact and occurs via ion-molecule interactions[145]. This results in the enhancement of the abundance of fragments with high mass-to-charge ratios, i.e. the molecular ion and the primary fragments.

Chemical-ionization mass spectrometry (c.i.- m.s.) involves the introduction of an ionizing gas, *viz.* ammonia, methane or isobutane, into the ionization chamber of the mass spectrometer[151]. The presence of the gas in the ionization chamber moderates the ion beam of the mass spectrometer and affords much larger fragment ions. When ammonia is used as the ionizing gas the protonated molecular ion, $[M + H]^+$, and the ammonium adduct molecular ion, $[M + NH_4]^+$, are observed at high intensities[152]. This pair of ions give valuable information on the molecular weights of the sugar units. C.i.- m.s. is also often used in sequencing determinations, since the ions corresponding

to the di-, tri- and tetrasaccharides, are almost always recorded as prominent peaks.

Other techniques that may be employed to observe the molecular ion, include field ionization[153], field desorption[154], and fast atom bombardment[155].

3.3.1.3. Fast Atom Bombardment Mass Spectrometry.

Fast atom bombardment mass spectrometry (f.a.b.- m.s.) involves an even softer ionization procedure than c.i.- m.s. and provides valuable information with regard to the molecular weights of large oligosaccharides and small polysaccharides[156].

F.a.b.- m.s. can be performed on both derivatised and underivatised carbohydrates. The sample is first dissolved in a suitable solvent, which may be either water, methanol, and chloroform for unmodified-, acetylated- and permethylated samples respectively, after which it is added to a drop of glycerol, or some other suitable matrix, on a stainless steel target[155]. The target is subsequently bombarded with either argon or xenon atoms, with approximately 8 KeV of energy. Both positive and negative ions are released when a compound is fast atom bombarded, and these spectra are recorded separately. Spectra recorded in the positive mode have, for example, peaks for the protonated molecular species, and positive fragment ions, whereas spectra recorded in the negative mode, have peaks for the molecular anion, and negative fragment ions.

F.a.b.- m.s., in addition, also provides complete fragmentation information, which allows for the sequencing of oligosaccharides[156].

3.3.2. Nuclear Magnetic Resonance Spectroscopy.

Carbohydrates were first investigated by nuclear magnetic resonance (n.m.r.) spectroscopy by Lemieux *et al*[157] in 1957. Since then n.m.r. spectroscopy has developed

into the most powerful spectroscopic method available for elucidating the structures and conformations of carbohydrates[158].

3.3.2.1. One-Dimensional N.M.R. Spectroscopy.

In the Fourier transform n.m.r. experiment a sequence of equally spaced rf pulses is applied to a sample[159]. These pulses simultaneously excite all spins with resonance frequencies within a certain region. The resultant free induction decay (FID) signals in the time domain are then added by a time-averaging computer and Fourier transformed to give the desired high resolution, frequency domain spectrum.

The most important feature of the Fourier transform experiment is the evolution time during which the spin system evolves[159]. The evolution time is preceded by the preparation time, while data collection only starts at the end of the evolution period, with the detection time (Figure 11).

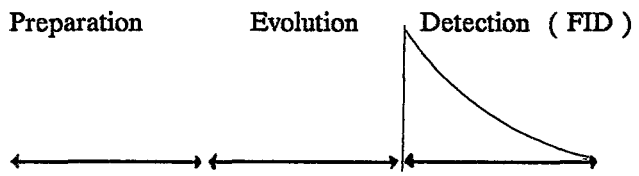


Figure 11 : Time sequence of modern pulse experiments[159].

3.3.2.1.(i) One-Dimensional ^1H N.M.R. Spectroscopy.

One-dimensional (1D) ^1H n.m.r. spectroscopy remains a valuable tool in structural determinations despite the development of sophisticated two-dimensional (2D) techniques. It reveals information about :

1. the immediate electronic environment of the protons,

2. the neighbouring molecular environment of the protons, and

3. the number of protons in a given molecule[160].

1D ^{1}H n.m.r. studies on bacterial capsular polysaccharides were first reported by Dutton *et al*[161]. Useful structural information can be obtained from these 1D spectra, even though they are fairly complex and show many overlapping signals.

Sugar residues, like all organic compounds, are characterized by their proton chemical shifts and unique spin systems. The chemical shifts are measured from a signal of an internal standard, usually tetramethylsilane (TMS)[162]. Acetone, which produces a signal 2.23 p.p.m. downfield from the TMS signal, is often added as an extra internal standard in the study of carbohydrates, since TMS is not miscible with water.

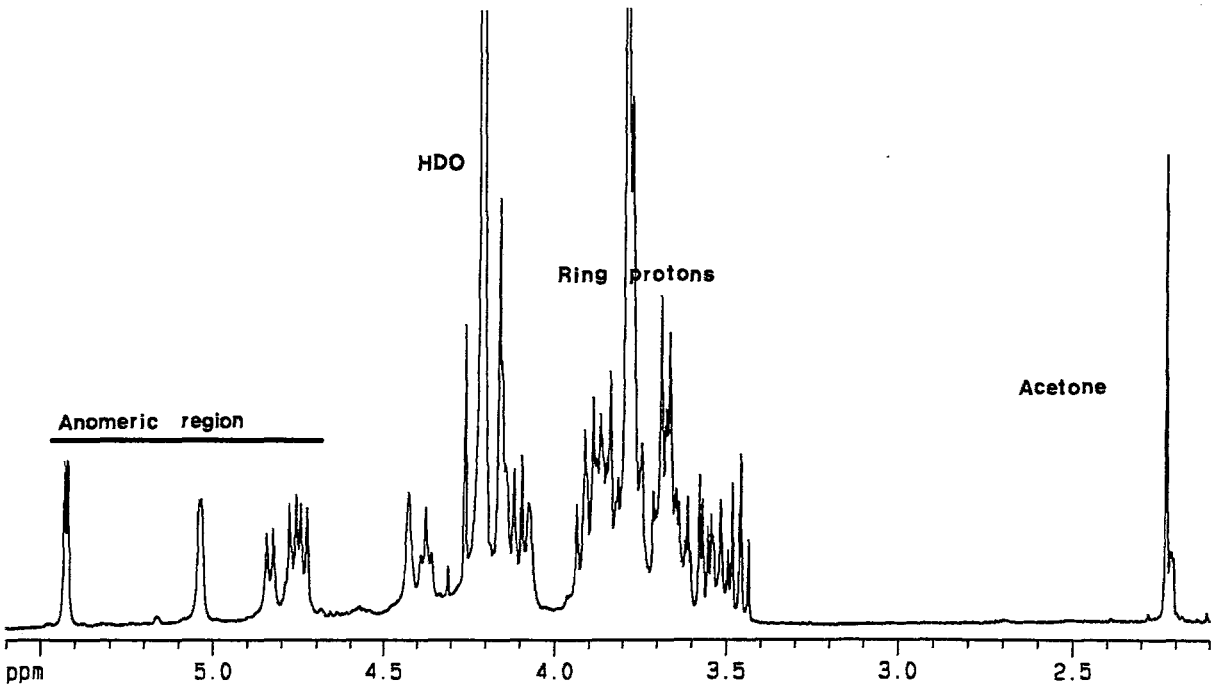


Figure 12 : The ^{1}H n.m.r. spectrum (400 MHz) of the *E. coli* K102 capsular polysaccharide.

The n.m.r. resonance characteristics of the polysaccharides are generally closely analogous to those of their constituent monosaccharides[163]. The ranges of the

characteristic proton chemical shifts have been tabulated[163], and the relevant ranges are shown in the ^1H n.m.r. spectrum of the *E. coli* K102 capsular polysaccharide (Figure 12).

The proton chemical shifts of the sugar residues are representative of their chemical nature and environment and are often used to confirm the presence of certain monosaccharides by comparison with literature values[164]. Due allowances should however be made for the protons attached to the carbon atoms involved in the linkages, since they are generally shifted downfield by a small but significant amount. These chemical shift differences are often used to determine the positions of linkage. Care should however be taken when comparing chemical shifts to literature values, since certain parameters, *viz.* the temperature at which the spectrum is obtained and the solvent system used, might affect the position of resonance. Non-carbohydrate components, e.g. N- and O-acetyl groups, as well as lactyl- and pyruvate groups, are often substituents of capsular polysaccharides and are identified by the characteristic chemical shifts of their methyl protons, e.g. the methyl protons of a pyruvate group which resonate at ~1.5 p.p.m.

The anomeric resonances of the constituent monosaccharides are well separated from the signals produced by most of the other protons[161]. This enables the determination of the number of monosaccharides that constitute the repeating unit, the estimation of their relative proportions, and the assignment of their anomeric configurations.

The value of the spin-spin coupling constant, J, depends on the size of the dihedral angle between the vicinal protons[165]. The coupling constant is large (5 - 8 Hz) when the protons are transdiaxial, and small (1 - 3.5 Hz) when they are gauche. The coupling parameters, in conjunction with the proton chemical shifts, enables the complete assignment of the anomeric region : The α-anomeric protons of pyranose sugars resonate at 5.0 - 5.5 p.p.m., and have relatively small $J_{1,2}$ values, whereas the β-anomeric protons resonate at 4.5 - 5.0 p.p.m., mostly with large $J_{1,2}$ values. Mannose residues, however,

prove to be the exception to the rule due to the anomeric protons resonating at ~5.0 p.p.m., *viz.* in between the areas of resonance of the α- and β-anomeric protons, and having small $J_{1,2}$ values. This presents a problem in assignment of the anomeric configuration, but is overcome by measurement of the spin-spin coupling constant, $^1J_{C\text{-}H}$, in the fully coupled ^{13}C spectrum. The anomeric protons of the furanose sugar residues resonate further downfield than their pyranose counterparts, and are therefore easily identified[166].

In order to minimize the obscuring of the n.m.r. signals that arise from the non-exchangeable protons of a sugar residue, the carbohydrate sample is freeze-dried three or four times from D_2O[167]. This deuterium substitution will cause the hydroxyl protons to disappear and thus, simplify the spectrum. This procedure has a minor effect on the electronic structure of the molecule and the remaining nuclei are practically unaltered. The resulting HDO-peak may however create some difficulties due to its position of resonance, *viz.* in the anomeric region, but can be shifted by a change in the operating temperature.

1D Homonuclear Hartmann-Hahn Spectroscopy : It is often very difficult to measure the coupling constants of the non-anomeric protons in the conventional 1D ^1H n.m.r. spectra. Bax *et al*[168] suggested a method whereby these coupling constants can be measured, once the proton chemical shifts are known. This is achieved by the generation of 1D subspectra of the individual spin systems, i.e. by the selective inversion of a well separated resonance, and the subsequent magnetization transfer within the coupled spin system by the homonuclear Hartmann-Hahn (HOHAHA) mechanism.

The coupling constants of the non-anomeric protons are measured with greater ease from these subspectra, due to the non-interference from the other spin systems.

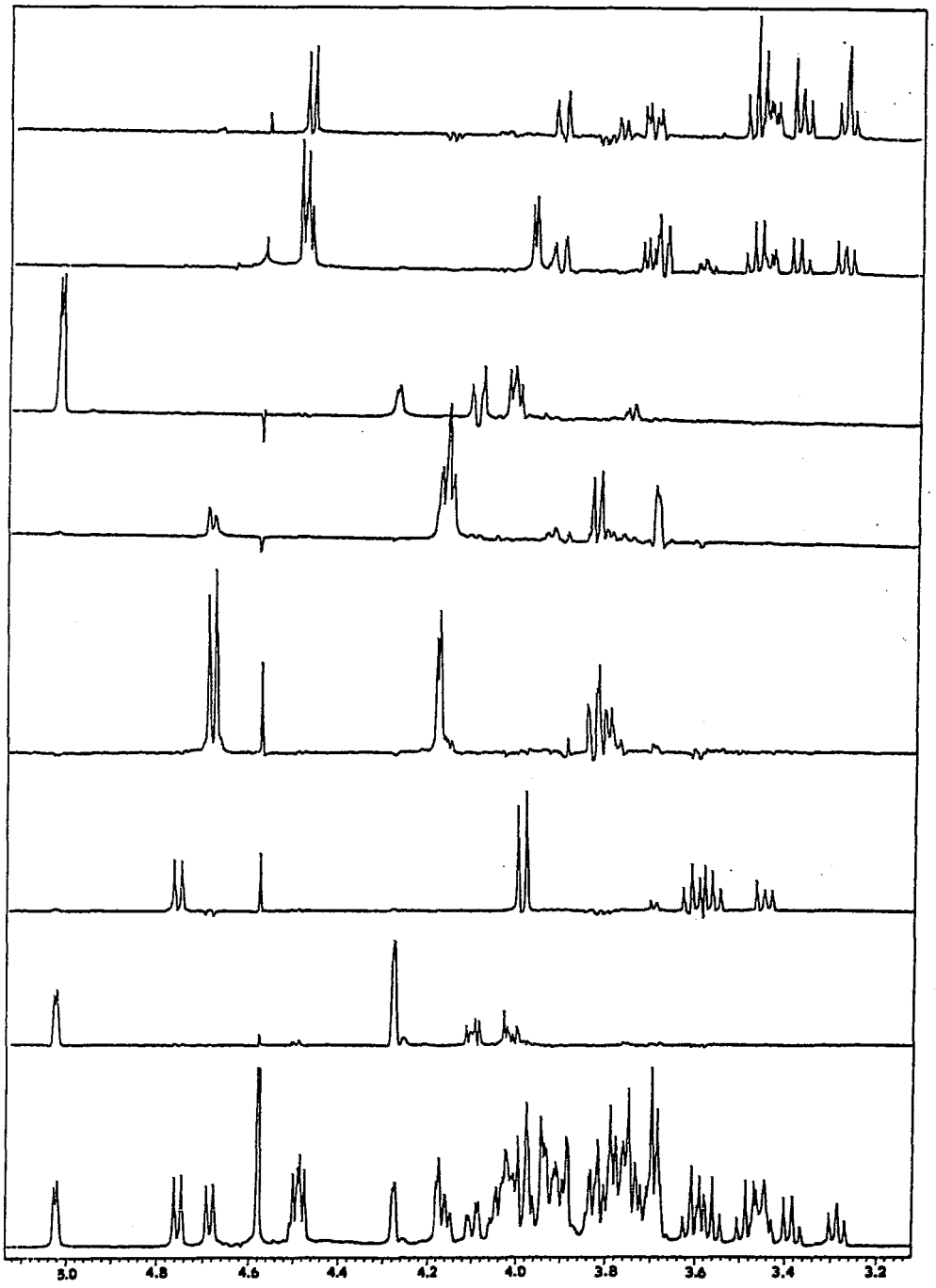


Figure 13 : Subspectra of the individual spin systems of the *Klebsiella* K15 capsular polysaccharide, obtained with a HOHAHA pulse-sequence[169].

3.3.2.1.(ii) One-Dimensional ^{13}C N.M.R. Spectroscopy.

1D ^{13}C n.m.r. spectroscopy is a powerful technique in the structural determination of polysaccharides and yields information on their composition, sequencing and conformation[170]. With the advent of modern Fourier transform techniques, spectra of the polysaccharides are obtained by using only their natural abundance ^{13}C atoms. ^{13}C spectra are usually obtained with broad band or WALTZ ^{1}H-decoupling[171]. The spectra are simple and show a single, sharp signal for each carbon in the compound. The proton-coupled spectra are much more complex, since each ^{13}C resonance is split by covalently bonded hydrogens. Additional splittings or line broadening due to long range coupling, also add to the complexity of these spectra.

^{13}C n.m.r. spectroscopy complements ^{1}H n.m.r. spectroscopy in that it gives better signal separation due to the wider range of chemical shifts involved. The ranges of the characteristic ^{13}C chemical shifts have been tabulated[163], and the relevant ranges are shown in Figure 14.

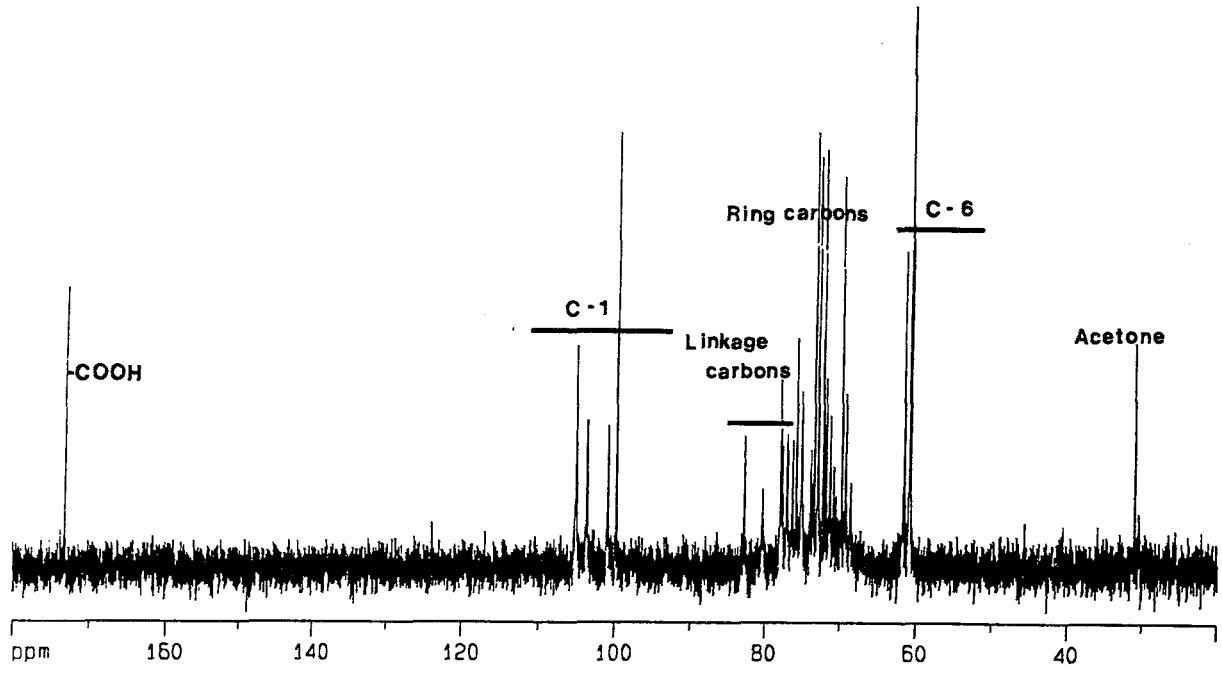


Figure 14 : One-dimensional ^{13}C n.m.r. spectrum (400 MHz) of the *E. coli* K102 polysaccharide.

The chemical shifts of the ^{13}C nuclei are characteristic of their chemical nature and environment. The monosaccharides and simple glycosides have characteristic patterns of ^{13}C chemical shifts and can generally be identified from their ^{13}C spectra. Due allowances must however be made for shifts of resonances at, and adjacent to, linkage positions[172]. The resonances of the α- and β-anomeric forms of a carbon atom involved in a linkage, or attached to a methyl group, are generally shifted downfield by 4 - 10 p.p.m.[173]. This is called the α-effect of glycosidation. The resonances of the carbon atoms adjacent to the linked carbon, are usually shifted upfield by a small, but not necessarily similar, amount (β-effect). These patterns of chemical shift differences between the monosaccharides and polysaccharides can therefore be used to determine the position of linkage.

The anomeric configuration of the glycosidic bonds may also be distinguished using ^{13}C n.m.r. spectroscopy. Resonances of the anomeric carbons having the aglycon equatorial are generally downfield to those having an axial substituent[170]. This however excludes rhamnose and mannose residues where the values of C-1 α and C-1 β are often indistinguishable. Spin-spin coupling between C-1 and H-1 can, in addition to the chemical shift, be used to assign the anomeric configuration. In pyranosides the coupling constant, $^{1}J_{C\text{-}H}$, is larger when H-1 is equatorial than when H-1 is axial.

Other parameters that can be obtained from ^{13}C n.m.r. experiments include nuclear Overhauser enhancement (n.O.e.) and nuclear relaxation times[171]. Both parameters give information on internuclear distances. The nuclear relaxation times generally increase with increased flexibility in the structure and are used to evaluate the motional properties of the polysaccharide in solution.

3.3.2.2. Two-Dimensional N.M.R. Spectroscopy.

The same time sequence of preparation, evolution and detection shown in Figure 11,

also forms the basis of 2D n.m.r. spectroscopy, and most 1D experiments can be transformed into 2D experiments by varying the evolution period[159]. In 2D n.m.r. spectroscopy, a series of FID's, S(t_2), is excited by a pulse sequence containing a time interval, t_1[174]. This time interval is varied in equal steps until a matrix, S(t_1t_2), has been built up, which describes the signal as a function of both the time after excitation (t_2) and the delay (t_1), of the pulse sequence. The matrix, S(t_1t_2), is then Fourier transformed twice to produce a 2D spectrum in the frequency domain.

There are two main classes[175] of 2D n.m.r. experiments, *viz.* those that resolve chemical shifts and coupling constants, and those that correlate nuclei based on spin-spin coupling and nuclear Overhauser enhancement. 2D J-resolved n.m.r. spectroscopy enables separation of the parameters of chemical shift and coupling constant[175]. The spectra are characterized by one frequency axis that contains coupling information, and by another, that contains chemical shifts. Correlated 2D n.m.r. spectra differ from J-resolved 2D spectra in that both frequency axes contain chemical shifts[159]. The connection between the two frequency axes is established through scalar coupling (homonuclear as well as heteronuclear), or through nuclear Overhauser enhancement. Only the latter, *viz.* correlated 2D n.m.r. spectroscopy, will be discussed in this review since the former is seldom used in carbohydrate chemistry due to the heavy overlapping of signals.

3.3.2.2.(i) Correlated Two-Dimensional N.M.R. Spectroscopy.

With the advent of modern 2D n.m.r. techniques it has become possible to elucidate the structure of the oligosaccharide repeating unit of the capsular polysaccharides entirely by n.m.r. spectroscopy. A variety of 2D experiments, which permits the characterization of the individual monosaccharides from their correlated spin systems, is available. Sequencing of the monosaccharides in the repeating unit is possible from experiments which rely on either long-range heteronuclear correlations, or homonuclear dipole coupling.

A : Homonuclear 2D N.M.R. Spectroscopy.

The ^1H - ^1H homonuclear shift-correlated (COSY)[176] experiment is one of the most important 2D n.m.r. techniques used in structural carbohydrate chemistry and yields information on the correlation between homonuclear spin-coupled systems. The basis for the COSY spectra is the classical Jeener experiment[177] where a simple sequence of two 90° pulses is applied to a proton spin system. The experiment starts with the preparation period in which thermal equilibrium is established. The first 90° pulse disturbs the spin system, after which it evolves just as in normal free induction decay. The evolution of the spin system is then interrupted by the second 90° pulse, the mixing pulse. The spin system again evolves and the signal is detected and recorded during the time after excitation. Any nuclear magnetization that is aligned along the z-axis just before the second 90° pulse, gives rise to a 1D spectrum along the diagonal of the COSY contour plot[178]. Scalar coupled protons will also show, in addition to the diagonal peaks, characteristic off-diagonal peaks (cross-peaks), depending on the size of their coupling constants. This is illustrated in Figure 15 A, where the neighbouring protons, H-1, H-2 and H-3 of a sugar residue, in addition to the diagonal peaks, also show cross-peaks at coordinates around the chemical shifts (δ_1, δ_2), (δ_2, δ_1), (δ_2, δ_3) and (δ_3, δ_2). These cross-peaks indicate a scalar spin-spin coupling between H-1 and H-2, and H-2 and H-3.

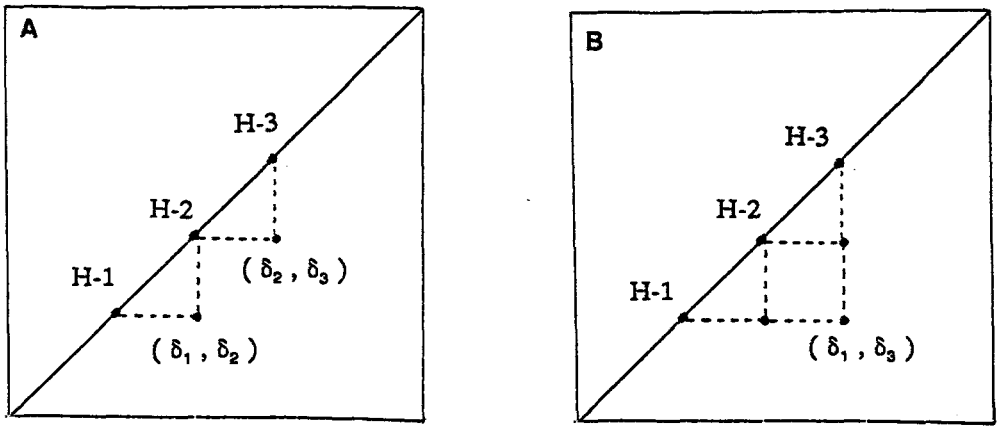


Figure 15 : Diagrammatic representation of a COSY (A) and a RELAY-COSY (B) experiment.

By using the anomeric resonances as a window into each spin system, it is therefore possible to trace the H-2, H-3, H-4, etc. connectivities belonging to each sugar residue in the repeating unit, by following the correlation map formed by the cross-peaks. This is illustrated for residue c in Figure 16.

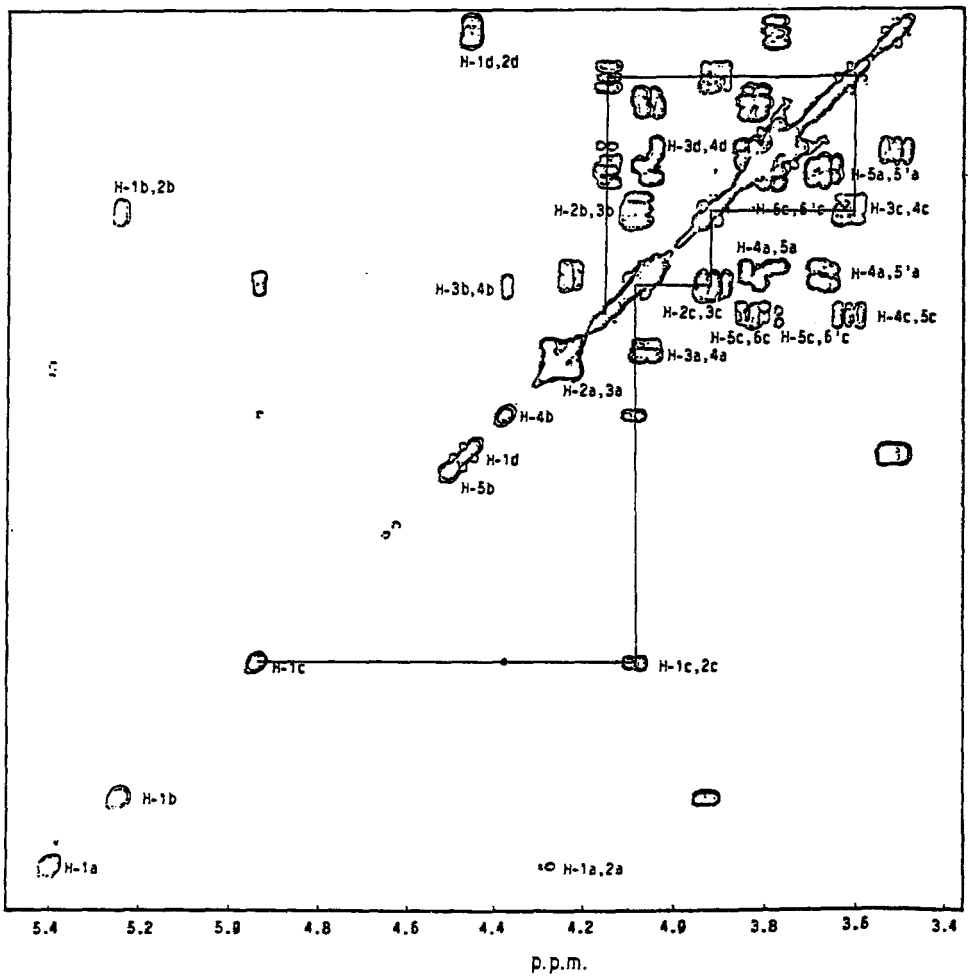


Figure 16 : The COSY spectrum of the region 5.5 - 3.4 p.p.m. of the *E. coli* K57 capsular polysaccharide[54]. The ^1H resonances of the J-coupled spin systems are labelled **a - d.**

The homonuclear relayed shift-correlated (RELAY-COSY)[178] experiment involves a second coherence transfer step, and shows correlation between two non-adjacent protons through a mutually coupled neighbouring proton. (See Figure 15 B.) This experiment provides valuable information on the chemical shifts of the non-anomeric protons in that

it resolves overlapping signals which are difficult to determine from a 1D experiment. In addition, coherence can be relayed further along the chain, e.g. from H-1 to H-4 or H-5 by a two- or three-step RELAY-COSY experiment.

The 2D homonuclear Hartmann Hahn (HOHAHA)[180] experiment is similar to the conventional COSY experiment in that it provides connectivities between scalar coupled protons. This technique is very powerful for the analysis of complicated coupling networks such as is the case with carbohydrates, and is often preferred to the COSY experiment since it yields information on both direct and relayed correlations. During the 2D HOHAHA experiment, magnetization is transferred from one proton to another, depending on the size of the coupling constant and, when long mixing times are used, magnetization can be distributed over most of the other protons in the same coupling network[181]. The graphical representation of the 2D HOHAHA experiment is similar to that of a multiple-step RELAY-COSY.

The 2D HOHAHA technique has one drawback in that is often difficult to determine whether the cross-peaks represent direct or relayed connectivity[182]. This difficulty is overcome by recording either a 2D HOHAHA experiment with a shorter mixing time, or a COSY experiment.

Homonuclear 2D n.m.r. spectroscopy is also often employed to determine the sequence of the sugar residues in the oligosaccharide repeating unit. This is accomplished by a 2D nuclear Overhauser (NOESY)[183] experiment which involves dipole coupling and provides information on the through-space connectivity between protons within Van der Waals contact. The through-space relationships (n.O.e. contacts) are indicated in Figure 17 as solid lines.

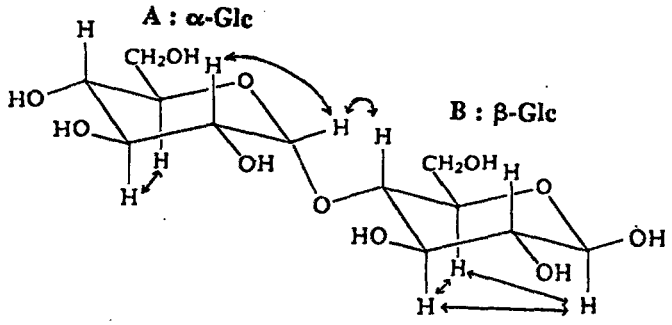


Figure 17 : Inter- and intra-residue n.O.e. contacts observed for the α-Glc*p*-(1→4)-β-Glc*p* disaccharide.

The intra-residue contacts serve to confirm the anomeric configurations of the sugar residues (compare A and B), and are also useful for assigning proton chemical shifts which are difficult to obtain from COSY spectra, e.g. H-5 of galactose residues where $J_{4,5}$ is small. The inter-residue n.O.e. contacts between protons of different J-coupled networks, e.g. from H-1 of residue A to H-4 of residue B, are however the most valuable as they provide a means of sequencing oligo- and polysaccharides.

B : Heteronuclear 2D N.M.R. Spectroscopy.

Heteronuclear 2D n.m.r. spectroscopy is a very powerful technique in the structural elucidation of complex molecules, e.g. bacterial polysaccharides, since it yields information on the correlation between heteronuclear spin systems. The ^{1}H - ^{13}C heteronuclear correlated experiments, in combination with the ^{1}H - ^{1}H homonuclear correlated experiments, can be used to assign the chemical shifts of all the carbon and proton atoms of the sugar residues present in the oligosaccharide repeating unit. With this information in hand, it is then possible to identify the sugar residues and to determine their linkage positions, by comparing the chemical shift data obtained, with published data for methyl glycosides[164].

In the conventional ^{1}H - ^{13}C heteronuclear chemical shift-correlated (HETCOR)[184] experiment the signal is acquired from the low sensitivity nucleus, ^{13}C, with correlation

to the coupled ^1H in F_1[185]. This technique of direct detection is insensitive and large amounts of sample (> 15 mg) and long experimental times are generally required. An alternative approach to heteronuclear 2D n.m.r. spectroscopy is to detect the insensitive nuclei, *viz.* ^{13}C, via the more intense ^1H signals[181]. The ^1H-detected heteronuclear multiple-quantum coherence (HMQC)[188] experiment, provides a large enhancement in sensitivity relative to the direct detection methods. Consequently, experimental times are significantly shortened and limitations, as a result of sample size, are reduced.

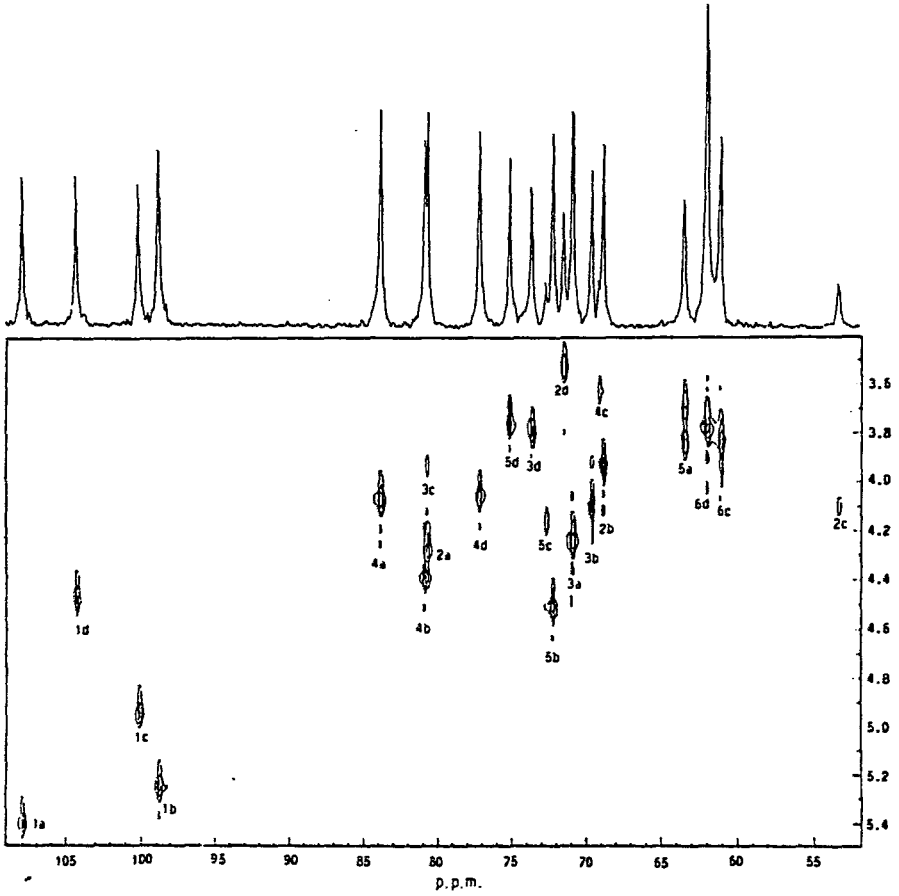


Figure 18 : Direct detection ^1H - ^{13}C correlation map of the spectral region F_2 (109 - 52 p.p.m.) and F_1 (5.5 - 3.4 p.p.m.) of the *E. coli* K57 capsular polysaccharide[54]. The ^{13}C projection is displayed along the F_2 axis, whereas the ^1H resonances can be read off the F_1 axis. The correlated spin systems are labelled a - d.

Both the HETCOR and the HMQC experiments provide a 2D data matrix with one frequency axis displaying signals as a function of their ^{13}C chemical shifts, and the other

according to their ^{1}H chemical shifts. In contour format, the peaks show correlations between the ^{1}H and ^{13}C atoms.

Another inverse-detected technique, the heteronuclear multiple bond coherence (HMBC)[187] experiment, can be used as an alternative to the NOESY experiment in sequence determinations since inter- and intra-residue heteronuclear correlations are obtained over two and three bonds. This experiment is also useful for confirming assignments of ^{1}H and ^{13}C resonances obtained from other 2D experiments.

THE STRUCTURAL ELUCIDATION OF THE CAPSULAR POLYSACCHARIDE OF *ESCHERICHIA COLI* SEROTYPE O8: K102: H$^-$.

4. THE STRUCTURAL ELUCIDATION OF THE CAPSULAR POLYSACCHARIDE OF *ESCHERICHIA COLI* SEROTYPE O8: K102: H⁻.

4.1. Abstract.

The structure of the capsular polysaccharide from *E. coli* serotype O8:K102:H⁻, culture no. 6CB10/1, was investigated by both chemical and spectroscopic techniques. The repeating unit was shown to be a branched oligosaccharide, with three sugar residues in the main chain and a disaccharide side chain.

→3)-β-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1-
3
↑
1
β-D-Glc*p*A
4
↑
1
α-D-Glc*p*

4.2. Introduction.

The structural elucidation of the *E. coli* K102 capsular polysaccharide forms part of a continuing program of structural studies on *E. coli* exopolysaccharides. These studies are undertaken in an attempt to relate chemical structure to antigenic character.

The polysaccharide capsular antigens of the *E. coli* have been divided into two groups on the basis of their chemical-, physical- and microbiological characteristics[8]. Group I antigens have high molecular weights, are heat-stable at pH 5 - 7, and are most often co-expressed with O8 and O9 antigens[9]. Group II antigens have lower molecular weights, are heat-labile at pH 5 - 7, and are co-expressed with many O-antigens other than O8 and O9.

The capsular polysaccharide of *E. coli* K102, whose structure is reported here, belongs to a subgroup of I, whose members have high molecular weights and are devoid of amino sugars, and thus resemble the *Klebsiella* capsular polysaccharides.

4.3. Results and Discussion.

(i) Monosaccharide composition.

Bacteria of the genus *E. coli* serotype O8:K102:H$^-$, culture no. 6CB10/1, were grown on Mueller-Hinton agar, and the acidic capsular polysaccharide was isolated and purified by the method described by Okutani and Dutton[70]. Difficulty was encountered with the precipitation of the acidic polysaccharide with cetyltrimethylammonium bromide (CTAB), due to the polysaccharide being lipid bound. The lipid was therefore removed from the crude polysaccharide by treatment with 1% aqueous acetic acid, after which the acidic capsular polysaccharide precipitated readily with CTAB.

Both the sodium and the acidic forms of the purified capsular polysaccharide were examined by gel-permeation chromatography on a dextran-calibrated Sephacryl S-500 column and shown to be polydisperse with regard to molecular weight distribution (Figure 4.1). A broad molecular weight distribution was observed for both forms, with peak maxima at M_r 1 x 10^7, 3 x 10^5 for the sodium salt, and at M_r 1 x 10^7, 1.3 x 10^5 for the acidic polysaccharide, respectively. The sugar ratios of the different molecular weight fractions of the capsular polysaccharide were identical. The optical rotation of the purified, acidic polysaccharide was found to be $[\alpha]_D$ +106°.

G.l.c. analysis of the peracetylated aldononitrile derivatives of the products of acid hydrolysis showed the presence of glucose and galactose in the ratio 1.0 : 2.4. On reduction of the uronic acid, after methanolysis, the sugars appeared in the ratio 1.0 : 1.75, thus indicating the repeating unit to be a pentasaccharide, consisting of glucose, glucuronic acid and galactose, in the molar ratio 1 : 1 : 3. The absolute

configurations of the monosaccharides were determined by the g.l.c. analysis of their acetylated (-)-2-octyl glycosides[79]. The constituent monosaccharides were all shown to be of the D-configuration. The native polysaccharide tested negative for the presence of phosphate groups[188].

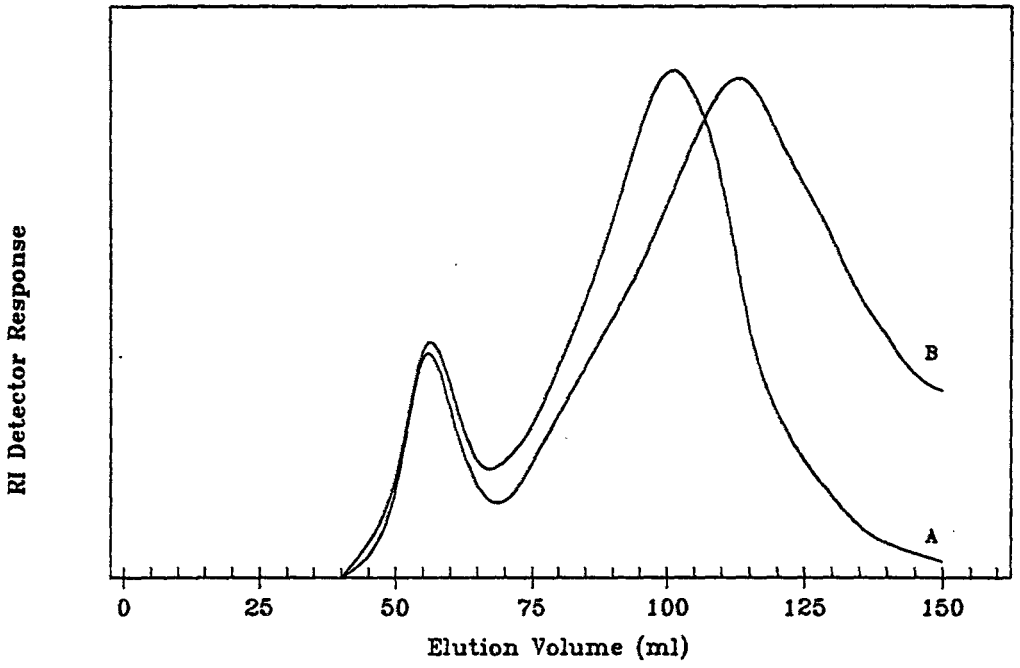


Figure 4.1 : Gel-permeation chromatography on Sephacryl S-500.

A : Sodium salt of *E. coli* K102 polysaccharide.

B : Acid form of *E. coli* K102 polysaccharide.

The 1D ^{1}H n.m.r. spectrum of the polysaccharide (Figure 12, section 3.3.2.1.(i), 353°K), contained two doublets in the α-anomeric region, at δ 5.43 (^{3}J 3.9 Hz) and δ 5.04 (^{3}J 3.4 Hz), and three doublets in the β-anomeric region, at δ 4.85 (^{3}J 7.7 Hz), δ 4.77 (^{3}J 7.7 Hz) and δ 4.74 (^{3}J 7.6 Hz). The ^{1}H- decoupled 1D ^{13}C n.m.r. spectrum (Figure 14, section 3.3.2.1.(i), 313°K), confirmed a pentasaccharide repeating unit with C-1 signals at 105.20, 105.17, 103.74, 100.99 and 99.86 p.p.m. A signal for the carbonyl carbon of the uronic acid residue was observed at 174.20 p.p.m. In addition, C-6 resonances were observed at 62.02, 61.34 and two at 61.21 p.p.m. indicating that none of the residues were 6-linked.

(ii) Methylation analysis.

A small amount of the polysaccharide, in the acid form, was methylated using potassium-dimsyl and methyl iodide according to the modified Hakomori-method[83] of Phillips and Fraser[87]. After hydrolysis the partially methylated sugars were analysed, as their alditol acetates by g.l.c. and g.l.c.- m.s. (Table 4.1, Column I). The molar quantity of 2,3,4,6-Glc was lower than expected due to the volatility of this derivative.

Table 4.1 : Methylation analysis of the *E.coli* K102 capsular polysaccharide.

Methylated sugars as alditol acetates[a].	T[b] DB-225	Molar Ratio I	II	III	IV
2,3,4,6-Glc	1.00	0.62	0.56	0.08	
2,4,6-Gal	1.69	0.99	0.98	0.96	0.96
2,3,6-Gal	1.76	1.00	0.98	2.00	2.00
2,6-Gal	2.34	0.73	1.00	0.10	
2,3-Glc	3.04		0.51		

a : 2,3,4,6-Glc = 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol

b : Retention time relative to that of 2,3,4,6-Glc

Column DB-225, 205°C (isothermal)

I : Native polysaccharide

II : Carboxyl-reduced polysaccharide

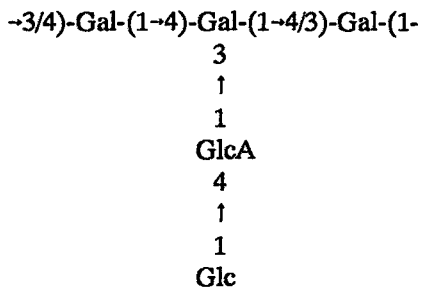
III : Base-catalysed uronic acid degradation

IV : Lithium in Ethylenediamine degradation

A small portion of the methylated polysaccharide was methanolysed and reduced, prior to hydrolysis, to reveal the linkage positions of the uronic acid residue. The results are shown in Table 4.1, Column II. (Only 51% reduction of the uronic acid was observed.) Methylation analysis thus indicates the presence of a 3- and a 4-linked galactose, a 3,4-linked galactose, a terminal glucose and a 4-linked glucuronic acid, in the oligosaccharide repeating unit.

(iii) Sequencing of the monosaccharides.

A : Base-catalysed degradations : A small portion of the acidic polysaccharide was methylated[87], treated with a strong base, *viz.* potassium-dimsyl, and alkylated with trideuteriomethyl iodide[92]. After hydrolysis, the partially methylated monosaccharides were analysed, as their alditol acetates, by both g.l.c. and g.l.c.- m.s. (Table 4.1, Column III). The results show almost complete loss of the terminal glucose and 3,4-linked galactose units, with the simultaneous production of a new methylated sugar, *viz.* 2,3,6-Gal, which had been trideuterio-methylated at position 3. The other two in-chain residues, *viz.* the 3- and 4-linked galactose units, remained intact. These results located the uronic acid in the disaccharide side chain, linked to one of the in-chain galactose residues. The loss of the terminal glucose was indicative of this unit being glycosidically linked to position 4 of the glucuronic acid. The results thus far permit the following partial structure for the polymer to be written :



A second base-catalysed degradation reaction , *viz.* treatment of the underivatised acidic polysaccharide with lithium dissolved in ethylenediamine[99], was performed to confirm

the location of the uronic acid. The products of the degradation reaction were separated on a column of Biogel P-4, and the major fraction, which proved to be polymeric, was collected and freeze-dried. A small portion of the lithium degraded polymer was methylated and analysed, as the partially methylated alditol acetates, by g.l.c. (Table 4.1, Column IV). The results indicated that the uronic acid and terminal glucose unit had been removed, leaving a linear polysaccharide consisting of one 3-linked and two 4-linked galactose residues. A Dische[189] colour reaction established the absence of uronic acid in the degraded polysaccharide. This confirms the results obtained from the first base-catalysed degradation reaction.

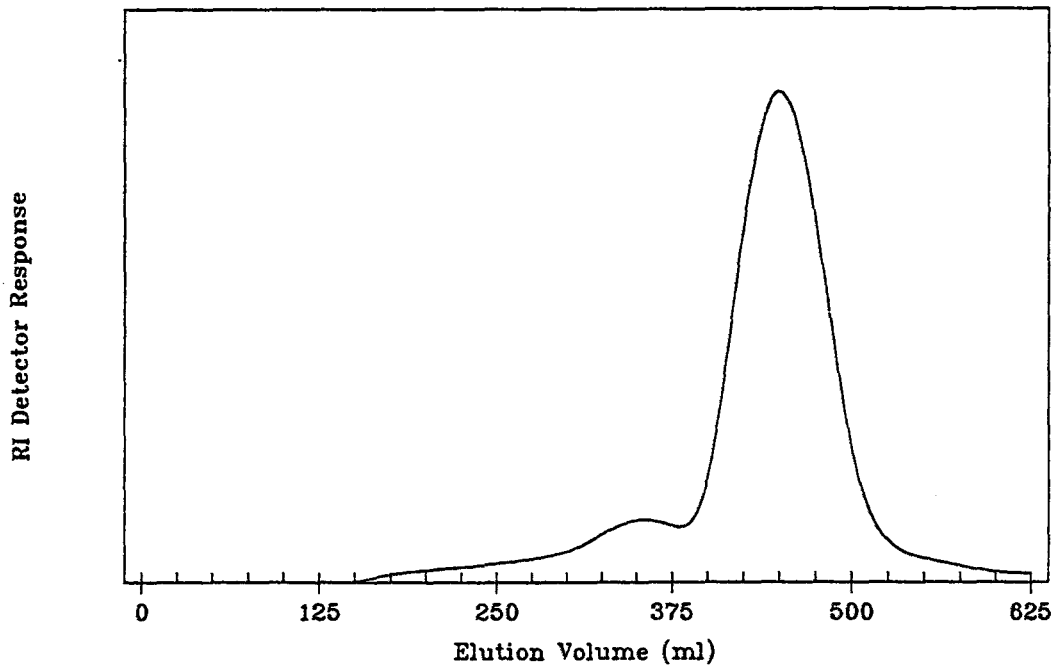


Figure 4.2 : Separation of the lithium-degraded products on a column of Biogel P-4.

A 1D and 2D n.m.r. study of the lithium degraded polymer was undertaken with the intention to sequence the galactose residues in the main chain of the polysaccharide. The 1D ^{1}H n.m.r. spectrum of the lithium degraded polymer (Spectrum 4.1) showed a marked decrease in intensity of one α- and one β-anomeric resonance, thus allowing the remaining anomeric signals, at δ 4.97, 4.71 and 4.67, to be assigned to the galactose residues in the main chain. The ^{1}H-decoupled 1D ^{13}C n.m.r. spectrum (Spectrum 4.2)

confirmed the presence of a trisaccharide repeating unit with C-1 signals at 105.45, 105.20 and 101.20 p.p.m. In addition, three C-6 resonances were observed at 61.76 and 60.92 p.p.m.(two), as well as the resonances of three linkage carbons at 83.05, 79.30 and 78.10 p.p.m. The non-anomeric ^{1}H chemical shifts of the galactose residues were established mainly from COSY-[176] and RELAY-COSY-[179] experiments. (Spectra 4.3 and 4.4) The residues were labelled **a** - **c**, in order of the decreasing chemical shifts of their anomeric protons. Starting at H-1 for each J-correlated spin system, the resonances for H-2, H-3 and H-4 (also H-5 for residue **a**) were obtained from the homonuclear shift-correlated experiments by following the correlation maps formed by the cross-peaks.

Further ^{1}H assignments were made from the 2D NOESY-experiment[159] (Spectrum 4.5) which shows through-space interactions between protons within Van der Waals contact. The observed inter- and intra-residue n.O.e. contacts are presented in Table 4.3. The α-linked galactose residue showed a characteristic intramolecular n.O.e. from H-1 to H-2, whereas the β-linked galactoses showed n.O.es from H-1 to H-3. (The expected n.O.es from H-1 to H-5 for the β-linked pyranosides were not observed.) The α-linked galactose residue **a** also showed intramolecular n.O.e. contacts between H-5 and H-4, H-5 and H-3, and H-4 and H-3. Both β-linked galactose units, **b** and **c**, showed intramolecular n.O.es between H-4 and H-3. These intra-residue n.O.es served to confirm the anomeric configurations of the sugar residues as well as the ^{1}H chemical shift assignments made from the COSY and RELAY-COSY experiments. In addition, the β-galactose residue **c**, showed a n.O.e. contact from H-4 to H-5, which permitted the chemical shift of H-5 to be established.

The ^{13}C chemical shifts of the constituent galactose residues were determined by comparing the ^{1}H resonances with the ^{1}H - ^{13}C correlation data obtained from the HETCOR experiment[184]. (Spectrum 4.6) This experiment also allowed the assignment of the H-5 chemical shift of the β-galactose unit b (by difference). Comparison of the ^{1}H- and ^{13}C-chemical shifts with the published data for methyl glycosides[184] indicated that

the α-galactose **a**, and the β-galactose **b**, are 4-linked and that β-galactose **c**, is 3-linked. The resonances of the carbon atoms involved in the linkages are shifted downfield by ~8 - 10 p.p.m., whereas the resonances of the carbon atoms adjacent to the linked carbons are shifted upfield by ~0.5 - 1.0 p.p.m. The resonances of the ring-protons involved in the linkages, are also shifted downfield.

Table 4.2 : ^{1}H- and ^{13}C n.m.r data (313°K) for the trisaccharide repeating unit of the lithium degraded *E. coli* K102 polysaccharide.

Residues	**Proton or Carbon**						
	1	**2**	**3**	**4**	**5**	**6a**	**6b**
a. →4)-α-Gal							
H	4.97	3.94	4.03	4.29	4.43	3.71	3.84
^{3}J (Hz)	3.8	10.4	2.5	< 1			
C	101.2	69.7	70.6	79.3	71.1		
b. →4)-β-Gal							
H	4.71	3.65	3.77	4.05	3.78		
^{3}J (Hz)	7.6	10.1	3.1	< 1			
C	105.45	71.7	73.2	78.1	75.9		
c. →3)-β-Gal							
H	4.67	3.78	3.88	4.15	3.73		
^{3}J (Hz)	7.5	10.1	3.0	< 1			
C	105.2	71.5	83.05	69.4	75.7		

Chemical shifts in p.p.m. relative to internal acetone at δ 2.23 for ^{1}H- and at 31.07 p.p.m. for ^{13}C n.m.r. The coupling constants were measured from a 1D ^{1}H n.m.r. spectrum at 353°K.

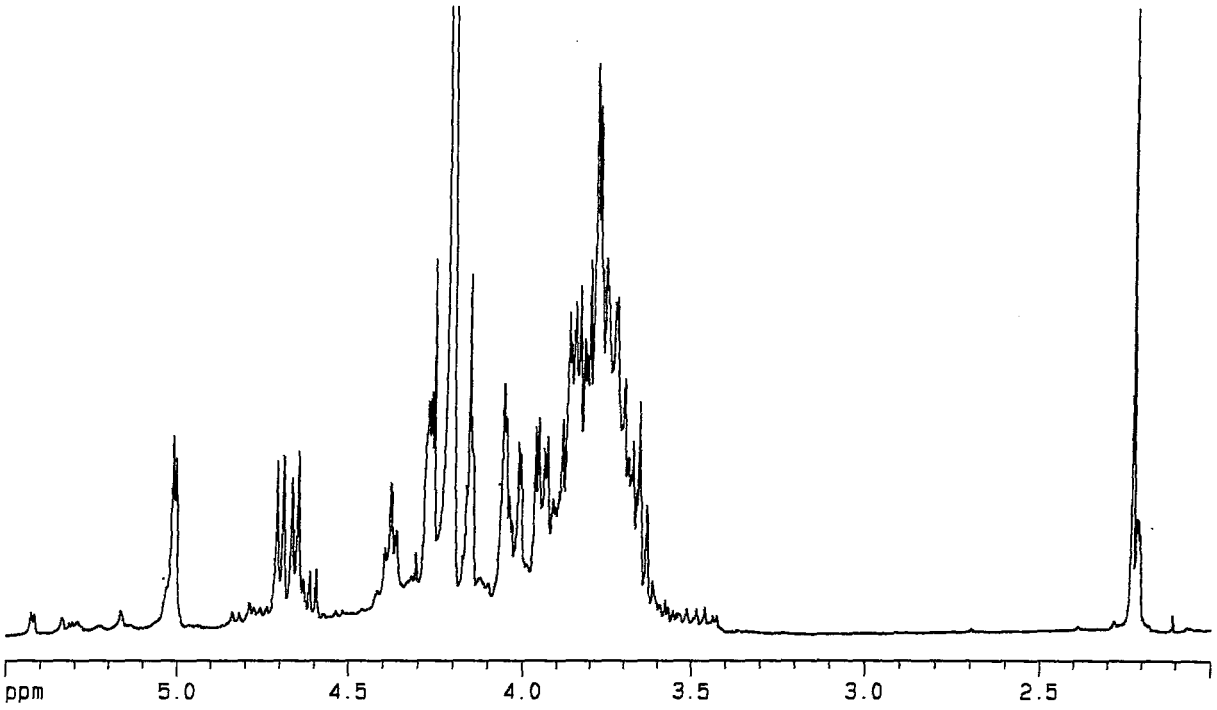
Table 4.3 : Inter- and intra-residue n.O.e. contacts for the lithium degraded *E. coli* K102 polysaccharide (313°K).

Residue	Proton	N.O.e. contact to
a. →4)-α-Gal	H-1	4.05 (**b.** H-4), 3.94 (**a.** H-2)
	H-4	4.03 (**a.** H-3)
	H-5	4.29 (**a.** H-4), 4.03 (**a.** H-3)
b. →4)-β-Gal	H-1	3.88 (**c.** H-3), 3.77 (**b.** H-3)
	H-4	3.77 (**b.** H-3)
c. →3)-β-Gal	H-1	4.29 (**a.** H-4), 3.88 (**c.** H-3)
	H-4	3.88 (**c.** H-3), 3.73 (**c.** H-5)

The sequence of the galactose residues in the degraded polysaccharide was established by a NOESY-experiment[159]. (Spectrum 4.5 and Table 4.3) The inter-residue n.O.es between the anomeric protons and the relevant protons of the adjacently linked residues, permitted the structure of the trisaccharide repeating unit of the lithium degraded *E. coli* K102 polysaccharide, to be written as :

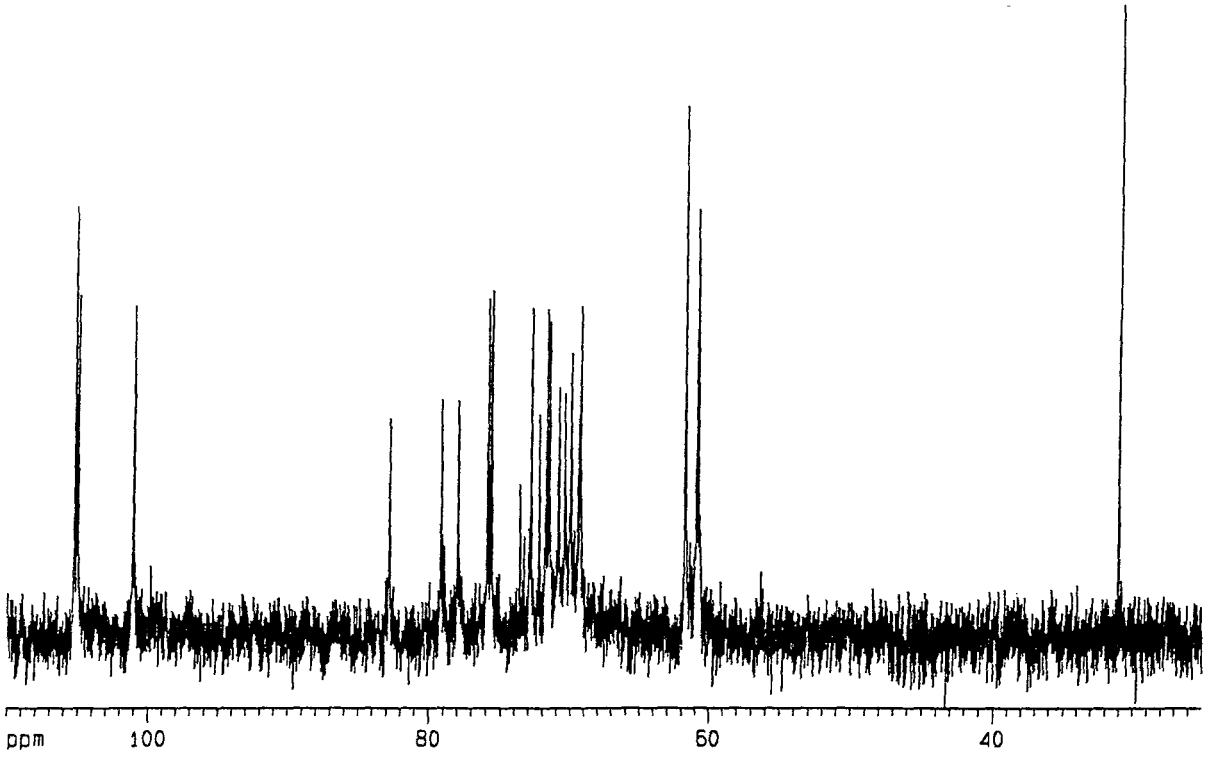
→3)-β-**D**-Gal*p*-(1→4)-α-**D**-Gal*p*-(1→4)-β-**D**-Gal*p*-(1-

Spectrum 4.1



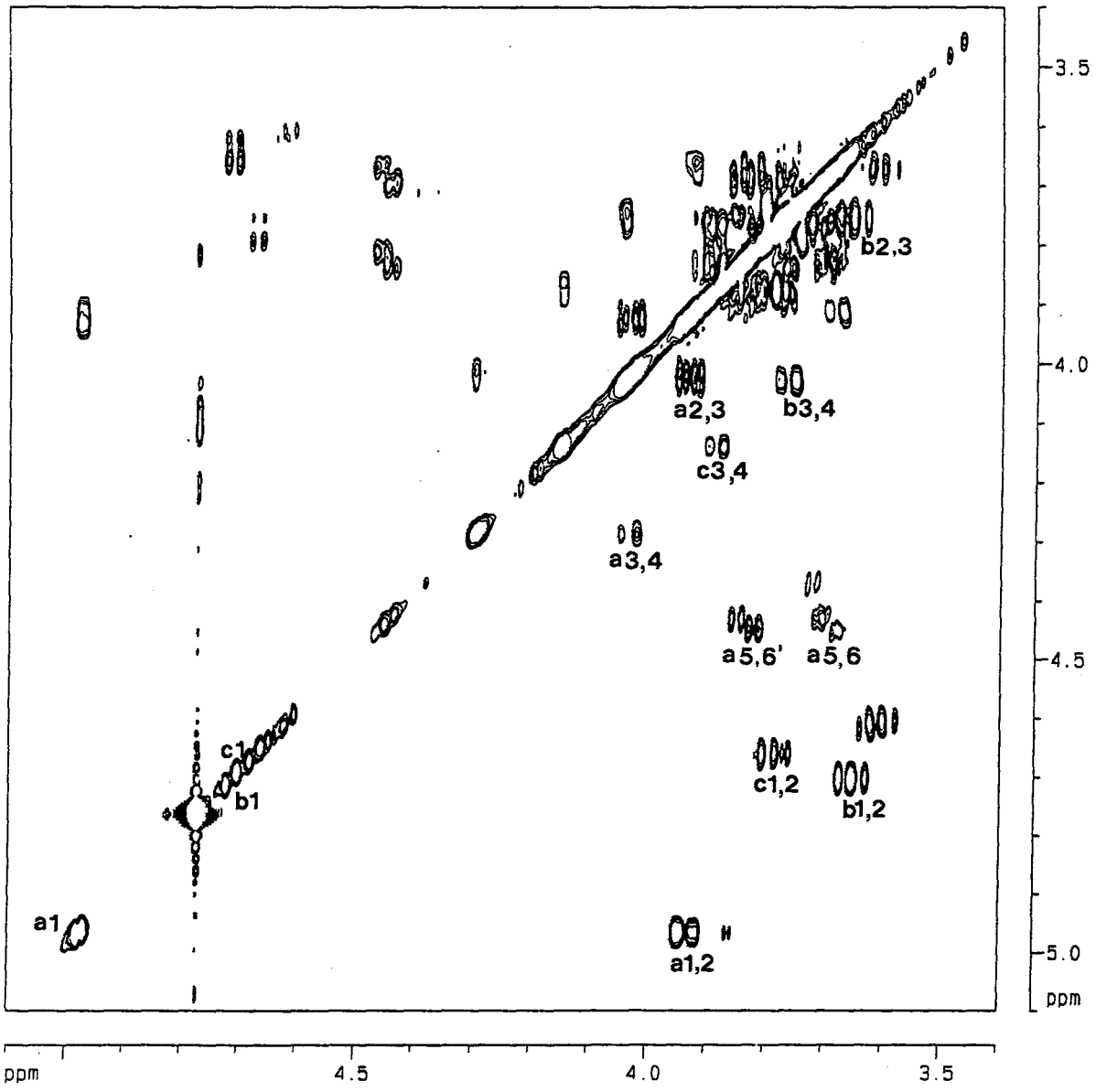
1D ^{1}H n.m.r. spectrum (400 MHz) of the lithium degraded *E. coli* K102 polysaccharide.

Spectrum 4.2



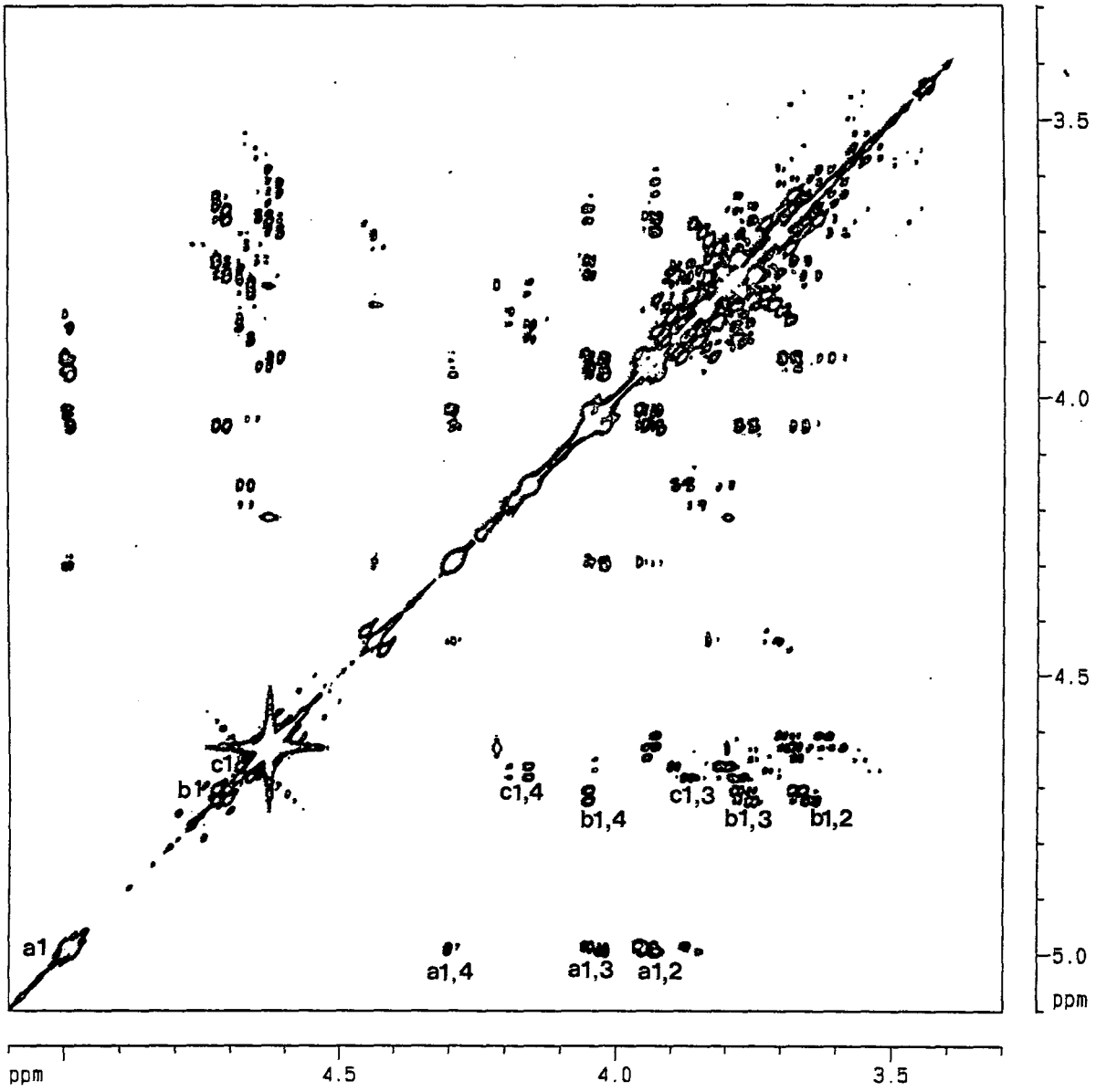
1D ^{13}C n.m.r spectrum (400 MHz) of the lithium degraded *E. coli* K102 polysaccharide.

Spectrum 4.3



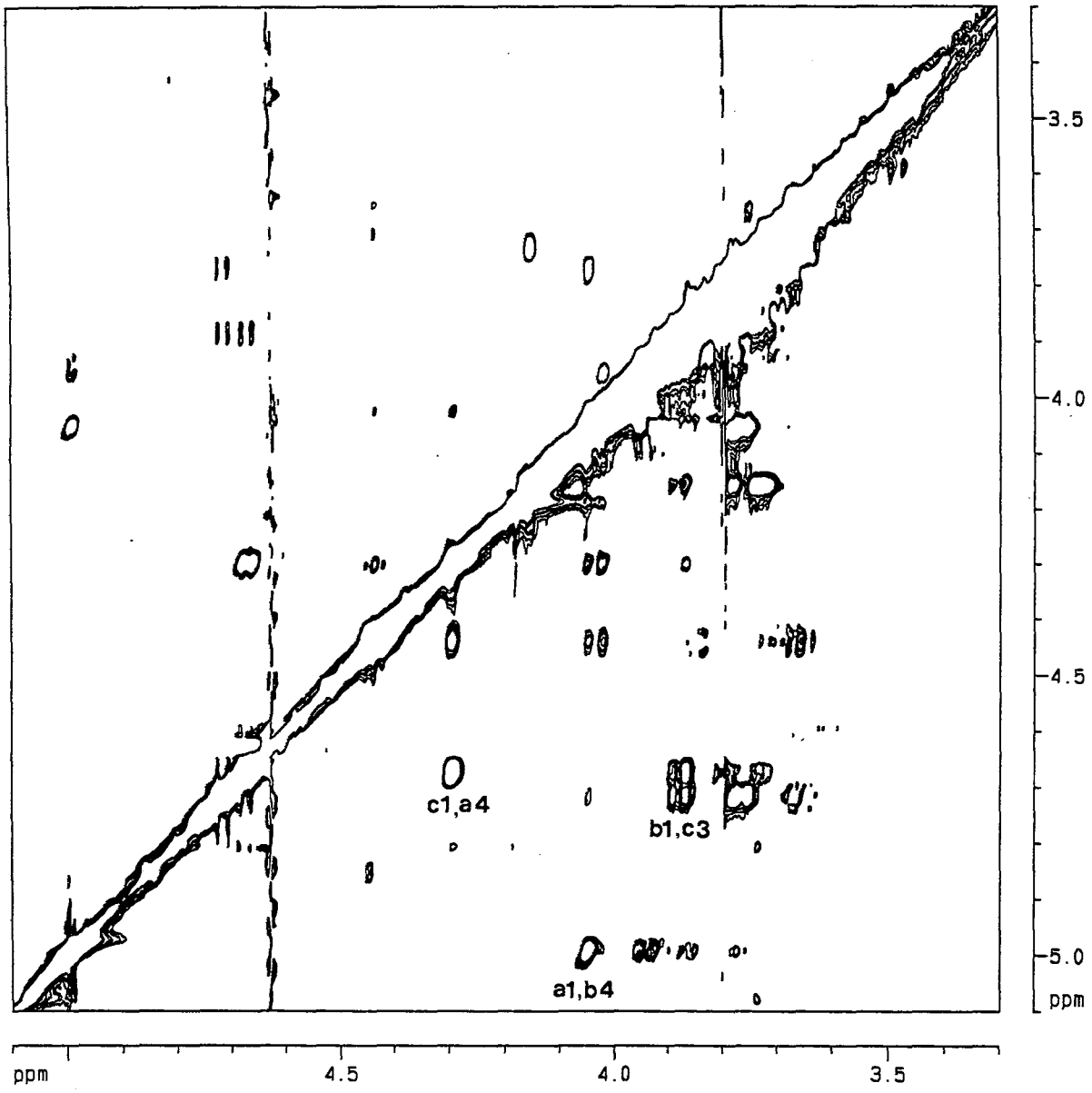
The COSY-spectrum (400 MHz) of the region 5.1 - 3.4 p.p.m. of the lithium degraded *E. coli* K102 capsular polysaccharide. The ^{1}H resonances of the J-coupled spin systems are labelled **a** - **c**. **a1** connotes H-1 of residue **a** and **a1,2** connotes the cross-peak between H-1 and H-2 of residue **a**, etc.

Spectrum 4.4



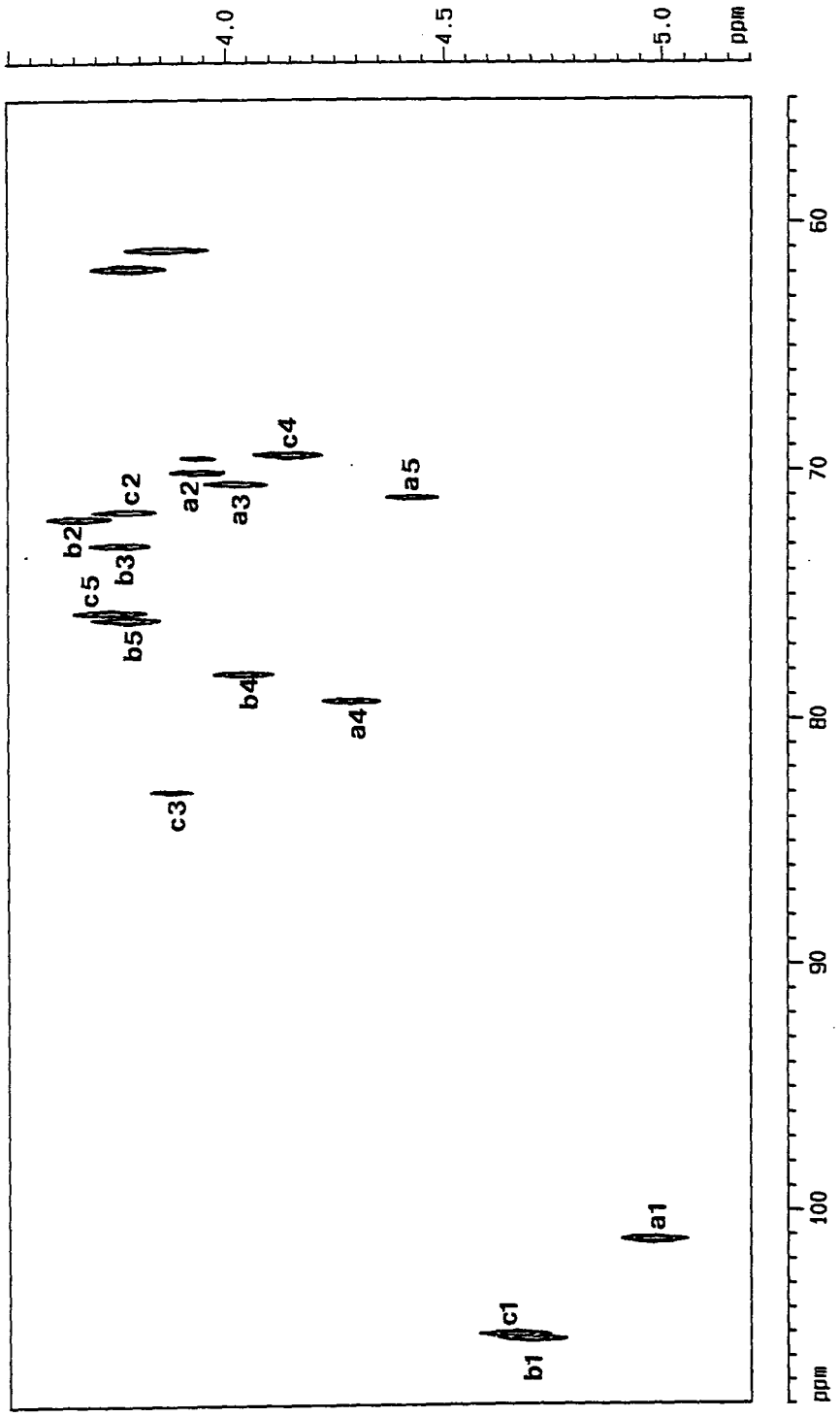
The two-step RELAY-COSY spectrum (400 MHz) of the region 5.1 - 3.3 p.p.m. of the lithium degraded *E. coli* K102 polysaccharide. The coupled spin systems are labelled **a - c** as before.

Spectrum 4.5



The 2D NOESY-spectrum (400 MHz) of the region 5.1 - 3.3 p.p.m. of the lithium degraded *E. coli* K102 polysaccharide. The inter-residue n.O.e. contacts between the anomeric protons and the relevant protons of the adjacently linked residues are labelled. **a1,b4** connotes the n.O.e. contact between H-1 of residue **a** and H-4 of residue **b**, etc.

Spectrum 4.6



The HETCOR-spectrum (400 MHz) of the lithium degraded *E. coli* K102 polysaccharide. The ^{13}C projection is displayed along the F_2 axis, whereas the ^{1}H resonances can be read off the F_1 axis. The correlated spin systems are labelled a - c.

B : Partial Hydrolysis : A partial hydrolysis study was carried out on a portion of the native polysaccharide in order to obtain fragments which could aid in the sequencing and placement of the disaccharide side chain. This reaction however produced complex mixtures of oligosaccharides, and was not pursued further. We therefore resorted to 2D homo- and heteronuclear n.m.r. studies on the capsular polysaccharide, for assignment and sequencing of the constituent monosaccharides.

C : The n.m.r. study of the *E. coli* K102 polysaccharide : As in the case with the lithium degraded polysaccharide, the ^1H resonances were allocated from COSY[178] (Spectrum 4.7) and RELAY-COSY[179] (Spectrum 4.8) experiments. In addition, a 2D HOHAHA experiment[180] (Spectrum 4.9) provided valuable information on the chemical shifts of the non-anomeric protons that were overlapped in the 1D ^1H- and COSY experiments. The residues in the repeating unit were labelled **a** - **e** in order of decreasing chemical shift of the resonances of their anomeric protons. Using the anomeric resonances as a window into each spin system, the corresponding H-2 resonances of all of the constituent monosaccharides, and H-3, H-4, H-5, H-6a and H-6b of residue **a**, were readily established from the COSY experiment. These chemical shift assignments were confirmed by both the RELAY-COSY and the 2D HOHAHA experiments. The chemical shifts for H-3 of residue **b**, and H-3 and H-4 of residue **c**, were established from the two-step RELAY-COSY experiment. These results were confirmed, and the chemical shift for H-4 of residue **b** established, by the 2D HOHAHA experiment. Returning to the COSY spectrum, the chemical shifts for H-5, H-6a and H-6b of residue **b** could be established through the H-4/H-5, H-5/H-6a and H-5/H-6b cross-peaks.

The chemical shifts of the anomeric protons of residues **d** and **e** coincide at δ 4.74 (313°K), and heavy overlapping of signals was observed in the two-step RELAY-COSY and 2D HOHAHA experiments. However, since the H-2 chemical shifts of both residues were known from the COSY spectrum, it was possible to trace the spin system belonging to residue **d** from the H-2 and H-5 tracks, of the 2D HOHAHA spectrum. (Spectrum 4.9)

Table 4.4 : ^{1}H- and ^{13}C n.m.r. data for the *E. coli* K102 polysaccharide. (313°K)

Residue	Proton or Carbon						
	1	2	3	4	5	6a	6b
a. α-Glc							
H	5.43	3.55	3.67	3.46	3.60	3.80	3.80
^{3}J (Hz)	3.9	9.85	9.4	9.95			
C	99.86	72 36	73.54	69.84	73.04		
b. →4)-α-Gal							
H	5.01	4.16	4.13	4.44	4.41	3.64	3.78
^{3}J (Hz)	3.4	9.85	3.0				
C	100.99	68.57	80.35	77.17	70.80	60.79	
c. →3)-β-Gal							
H	4.85	3.75	3.88	4.15	3.68		
^{3}J (Hz)	7.7	11.35	3.75	<1			
C	103.74	71.4	82.84	69.27	75.26		
d.→4)-β-GlcA							
H	4.74	3.52	3.83	3.91	4.11		
^{3}J (Hz)	7.7*	9.0	8.9	9.15			
C	105.17	73.96	76.47	77.82	75.19		
e. →4)-β-Gal							
H	4.74	3.67	3.77	4.05	3.78		
^{3}J (Hz)	7.6 *	9.73	2.9	0.9			
C	105.20	71.95	72.97	78.0	75.89		

Chemical shifts in p.p.m. relative to internal acetone at δ 2.23 for ^{1}H- and at 31.07 p.p.m. for ^{13}C n.m.r. (* = interchangeable)

The chemical shifts of the protons belonging to residue e were assigned by difference, and the results confirmed by both the COSY and the NOESY[159] (Spectrum 4.10) experiments. The coupling constants were measured from a 1D ^{1}H n.m.r. spectrum at 353°K.

Table 4.5 : Inter-and intra-residue n.O.e. contacts for the *E. coli* K102 polysaccharide. (313°K)

Residue	Proton	N.O.e. contact to
a. α-Glc	H-1	3.91 (d. H-4), 3.55 (a. H-2)
b. →4)-α-Gal	H-1	4.16 (b. H-2), 4.05 (e. H-4)
	H-4	4.13 (b. H-3)
	H-5	4.13 (b. H-3)
c. →3)-β-Gal	H-1	4.44 (b. H-4), 3.88 (c. H-3),
		3.68 (c. H-5)
	H-3	3.68 (c. H-5)
	H-4	3.68 (c. H-5)
d. →4)-β-GlcA	H-1	4.13 (b. H-3), 4.11 (d. H-5),
		3.83 (d. H-3)
	H-5	3.83 (d. H-3)
e. →4)-β-Gal	H-1	3.88 (c. H-3), 3.78 (e. H-5),
		3.77 (e. H-3)
	H-4	3.78 (e. H-5)

Chemical shifts in p.p.m. relative to internal acetone at δ 2.23 for ^{1}H n.m.r.

The observed inter- and intra-residue n.O.e. contacts are presented in Table 4.5. The α-

linked residues, **a** and **b**, showed the expected n.O.e. contacts from H-1 to H-2, whereas the β-linked residues, **c**, **d** and **e**, showed characteristic n.O.es from H-1 to H-3 and H-5 respectively. In addition, intramolecular n.O.e. contacts from H-3 to H-5 (residue **c**), H-4 to H-3 (residue **b**), H-4 to H-5 (residue **c** and **e**) and H-5 to H-3 (residues **b** and **d**) were observed, which confirmed the conformations of the residues. The intra-residue n.O.e. contacts from H-1, H-3 and H-4 to H-5, allowed the chemical shift for H-5 of residue **c**, to be established.

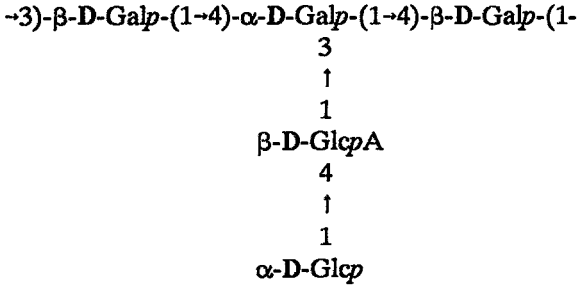
The ^{13}C chemical shifts for the constituent sugar residues were determined by comparing the ^{1}H resonance data, with the data obtained from a ^{1}H - ^{13}C shift-correlated experiment[184] (HETCOR, Spectrum 4.11). Comparison of the ^{1}H- and ^{13}C chemical shift data with the published data for methyl glycosides[184], permitted residues **a** - **e** to be identified (Table 4.4), and their linkage positions to be established. The results indicate that **a** is terminal, **b** is 3,4-linked, **c** is 3-linked, and **d** and **e** are 4-linked. This is in accordance with the results of the methylation analysis.

The sequence of the monosaccharides in the repeating unit was established from the NOESY-experiment[159]. (Spectrum 4.10) The strong inter-residue n.O.e. contacts that were observed (Table 4.5), permitted the sequence of the residues in the repeating unit to be written as :

→3) - **c** - (1→4) - **b** - (1→4) - **e** - (1-
3
↑
1
d
4
↑
1
a

4.4. Conclusion.

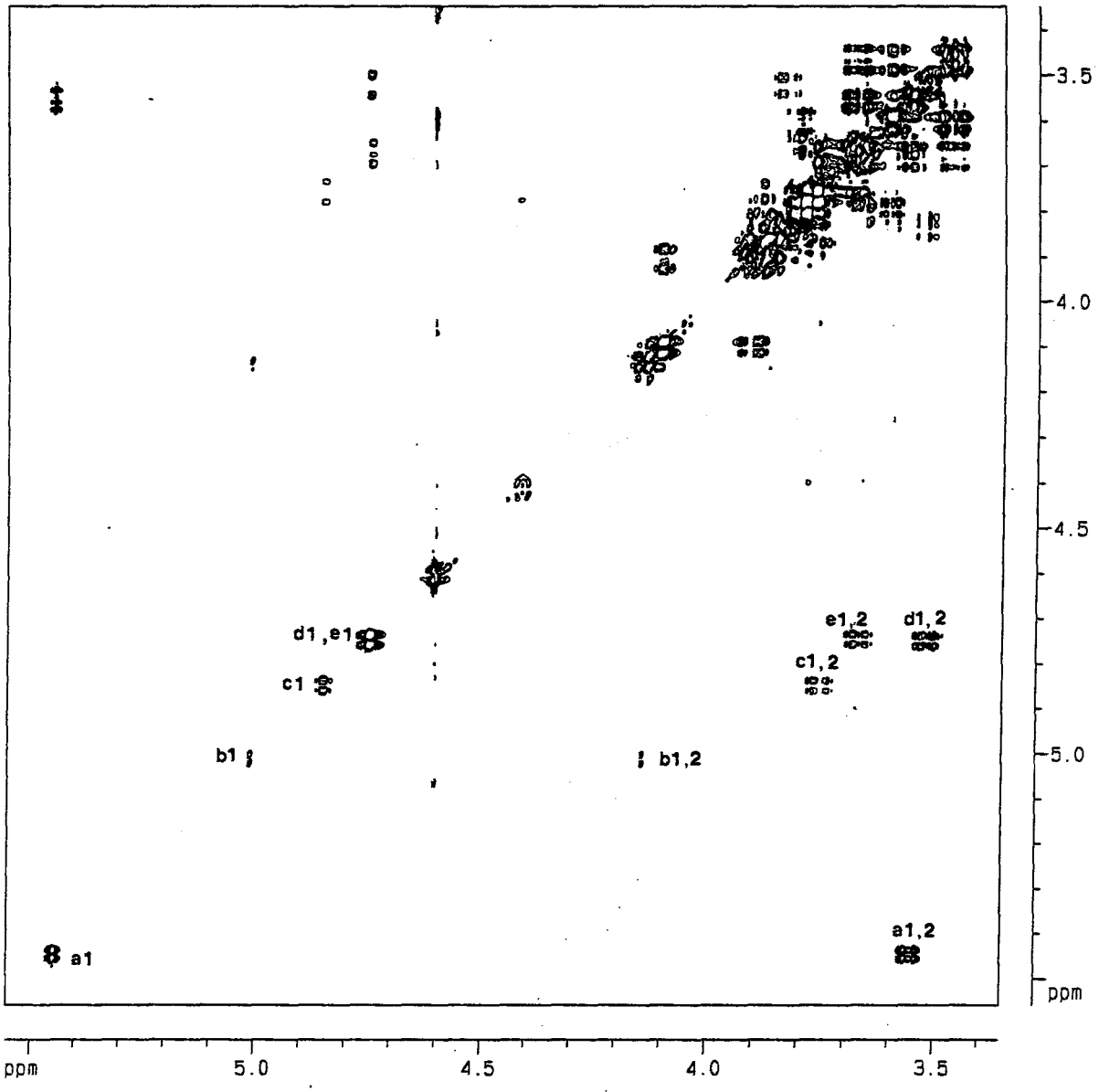
The combined n.m.r. and methylation data of the polysaccharide and the lithium degraded polymer permitted the structure of the pentasaccharide repeating unit of the capsular polysaccharide of *E. coli* K102 to be written as :



The structure of the *E. coli* K102 polysaccharide corresponds to the well-established *Klebsiella* pattern, *viz.* "3 + 2", as found in *Klebsiella* K13[190], K31[191], K74[192] and K80[193]. The pattern however differs in detail from those observed in the *Klebsiella* series in that they all have pyruvic acid incorporated in the terminal sugar residue of the disaccharide side-chain.

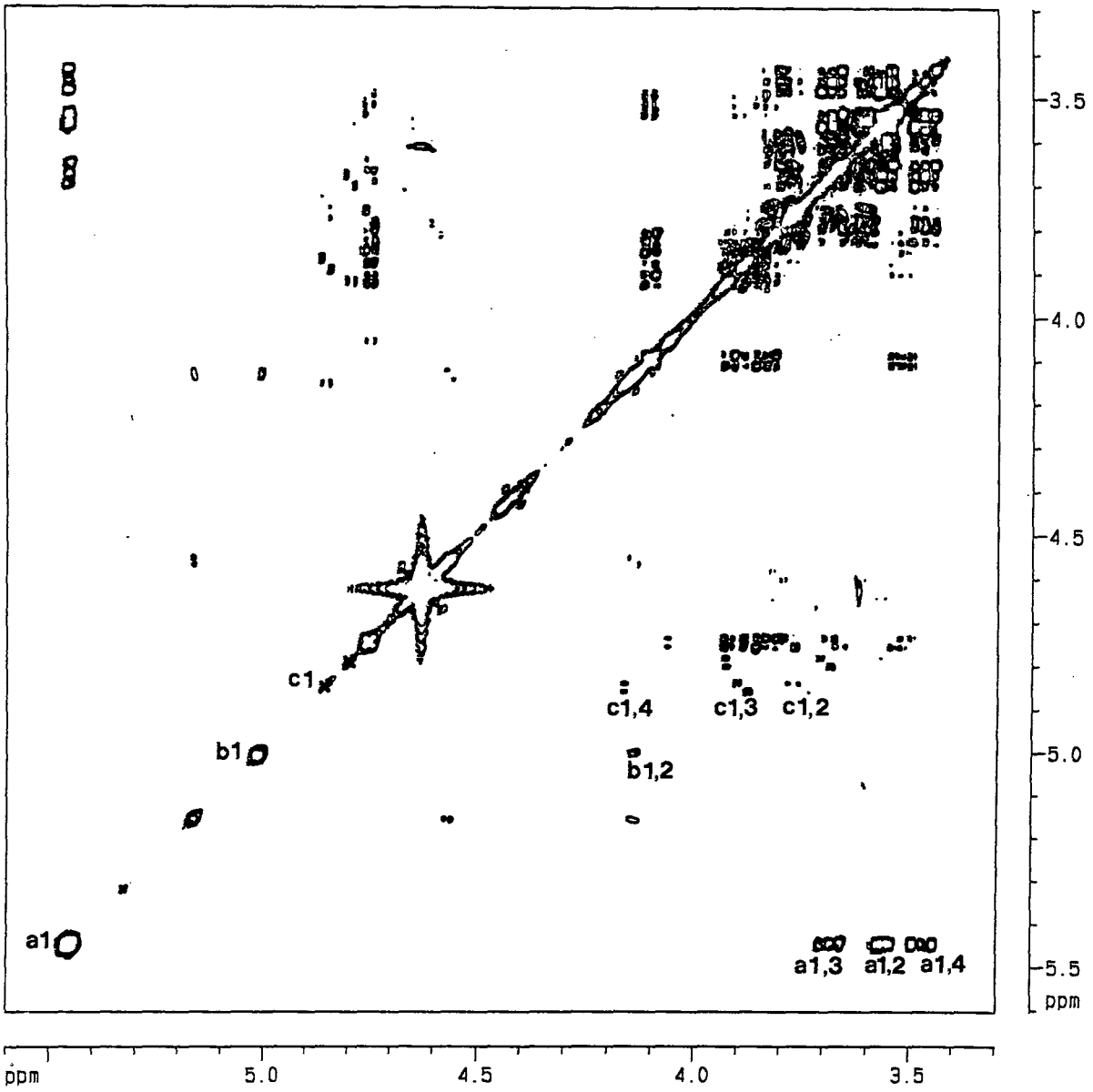
The repeating unit of the *E. coli* K34[41] polysaccharide is the only other "3 + 2" structure in the *E. coli* series and has, in addition, an identical monosaccharide composition to the *E. coli* K102 repeating unit. The *E. coli* K102 capsular polysaccharide is, however, the only structure known within the *E. coli* series to date, with a backbone consisting only of galactose.

Spectrum 4.7



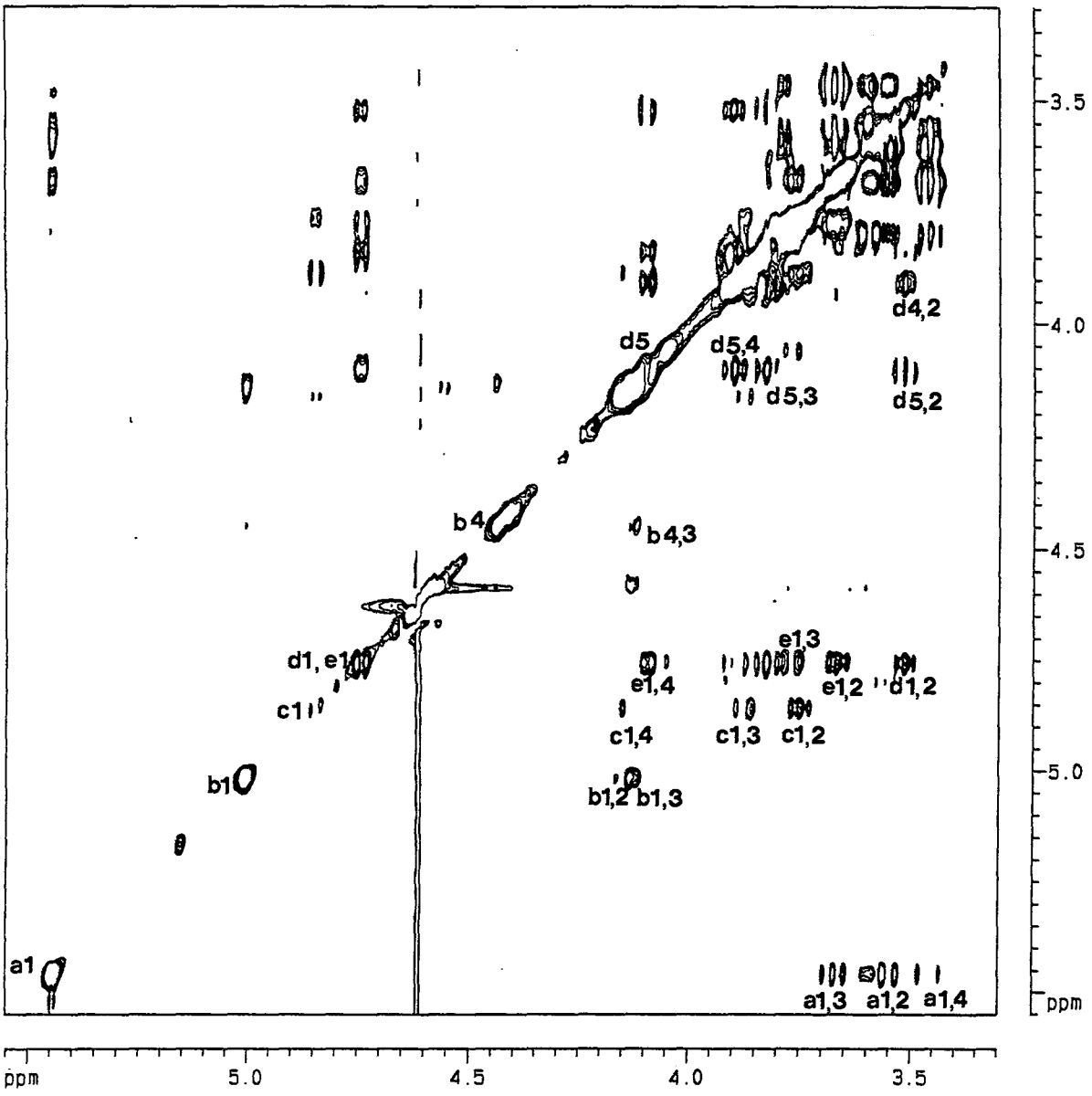
The COSY-spectrum (400 MHz) of the region 5.5 - 3.4 p.p.m. of the *E. coli* K102 capsular polysaccharide. The ^{1}H resonances of the J-coupled spin systems are labelled a - e. a1 connotes H-1 of residue a and a1,2 connotes the cross-peak between H-1 and H-2 of residue a, etc.

Spectrum 4.8



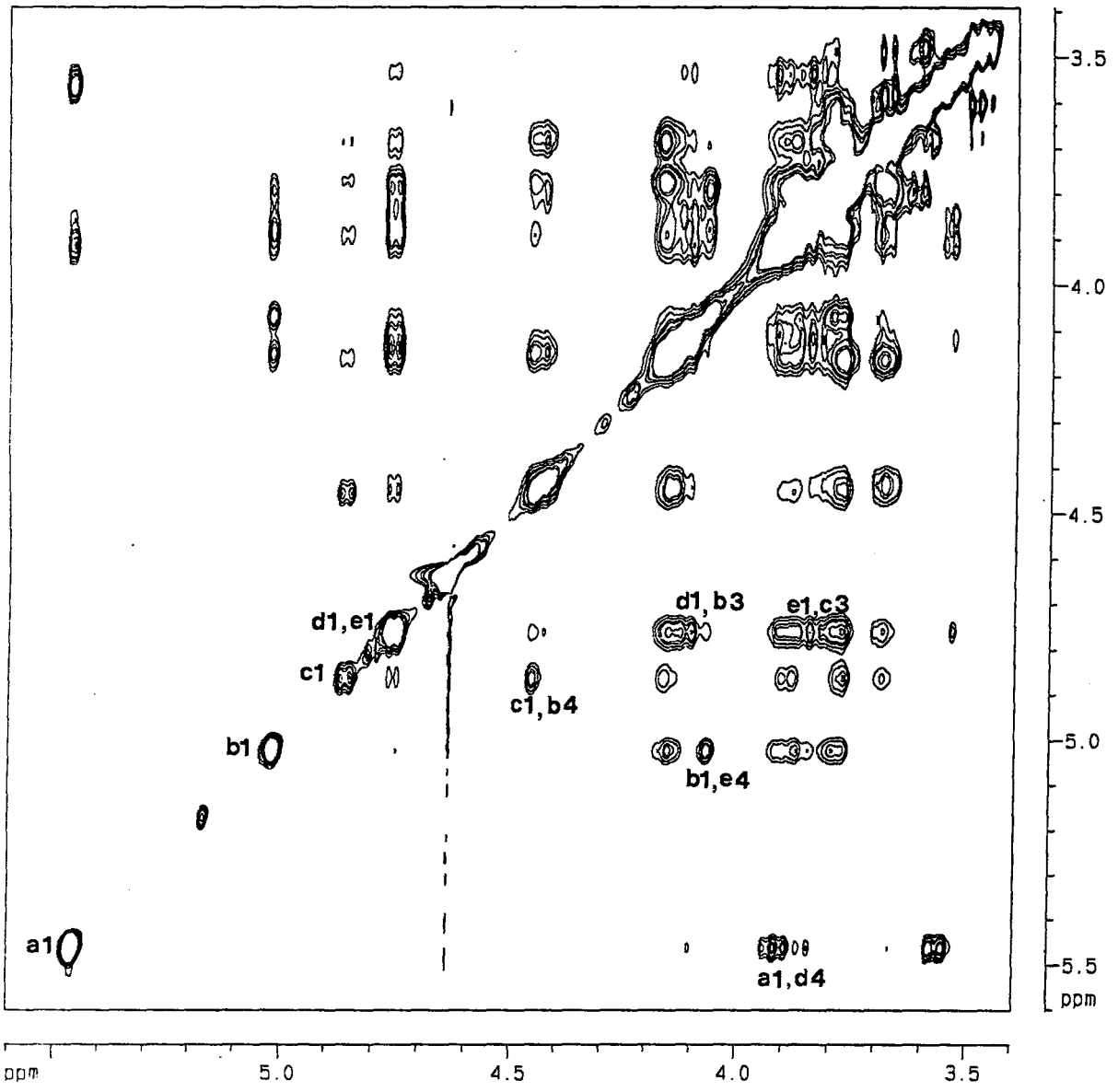
The two-step RELAY-COSY spectrum (400 MHz) of the region 5.6 - 3.3 p.p.m. of the *E. coli* K102 capsular polysaccharide. The coupled spin systems are labelled a - e, as before.

Spectrum 4.9



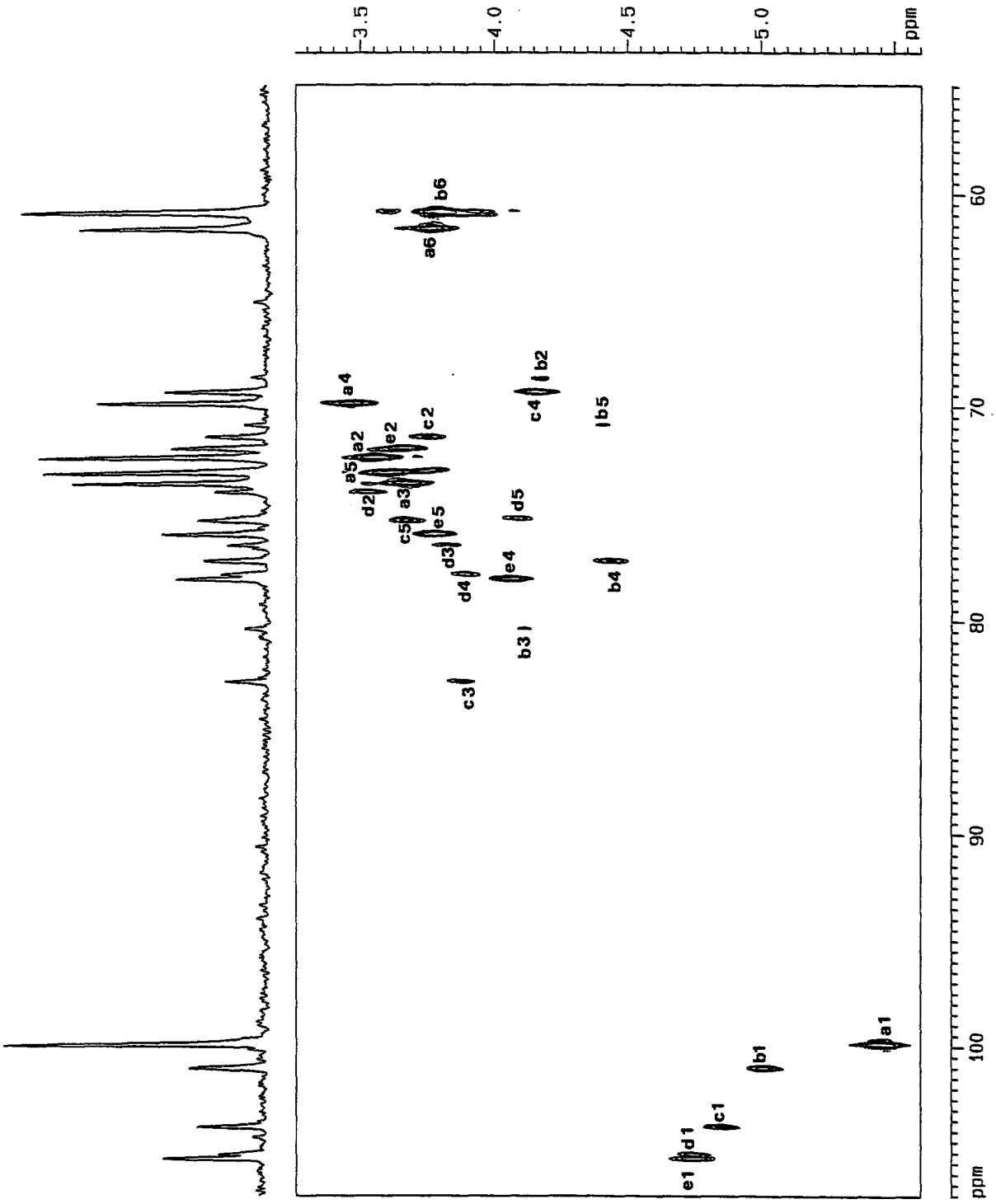
The 2D HOHAHA-spectrum (400 MHz) of the region 5.55 - 3.3 p.p.m. of the *E. coli* K102 capsular polysaccharide. The ^{1}H resonances of the J-coupled spin systems are labelled a - e. a1 connotes H-1 of residue a, a1,2 connotes the cross-peak between H-1 and H-2 of residue a, etc.

Spectrum 4.10



The NOESY-spectrum (400 MHz) of the region 5.6 - 3.4 p.p.m. of the *E. coli* K102 capsular polysaccharide. The inter-residue n.O.e. contacts are labelled as before. **a1,d4** connotes the n.O.e. contact between H-1 of residue **a** and H-4 of residue **d**, etc.

Spectrum 4.11



The HETCOR-spectrum (400 MHz) of the *E. coli* K102 capsular polysaccharide. The ^{13}C projection is displayed along the F_2 axis, whereas the ^{1}H resonances can be read off the F_1 axis. The correlated resonances are labelled a - e.

4.5. Experimental.

4.5.(i) General Methods.

Unless otherwise stated, solutions were concentrated under reduced pressure at temperatures not exceeding 40°C (bath). Optical rotations were measured in a 1 cm cell at 23°C (± 2°C), using a Perkin-Elmer model 141 polarimeter. Gel-permeation chromatography was performed on columns of I : Biogel P-2 (eluent : water), II : Biogel P-4 (eluent : 0.1M sodium acetate buffer, pH 5), III : Sephacryl S-200 (eluent : 0.1M sodium acetate buffer, pH 5) and IV : Sephacryl S-500 (eluent : 0.1M sodium acetate buffer, pH 5). The columns were coupled to a Waters Differential Refractometer R401 and the signals recorded on a Rikadenki flat-bed recorder. Analytical gas-liquid chromatography was performed using a Hewlett-Packard 5890A gas-liquid chromatograph, fitted with a flame ionization detector and a Hewlett-Packard 3392A recording integrator. A wall-coated, fused silica, capillary column, *viz.* DB-225 (30 m x 0.252 mm, film thickness of bonded liquid phase : 0.25 μm), was used and operated at 220°C (isothermal) or 205°C (isothermal) unless otherwise stated. G.l.c.- m.s. was conducted with a Hewlett-Packard 5988A GC/MS system. Spectra were recorded at 70eV and an ion-source temperature of 200°C. ^{1}H- and ^{13}C n.m.r. spectra were recorded on a Bruker AMX 400 spectrometer at 40°C, and in some cases at 80°C for ^{1}H n.m.r. The samples were deuterium-exchanged by freeze-drying solutions in D_2O, and then dissolved in ~0.5 mL 99.995% D_2O. In all cases acetone which resonates at δ 2.23 for ^{1}H- and at 31.07 p.p.m. for ^{13}C n.m.r., was used as the internal standard.

4.5.(ii) Isolation and purification of the polysaccharide.

An authentic culture of *E. coli* O8:K102:H^- (culture no. 6CB10/1) was obtained from Dr. I. Ørskov (Copenhagen), plated out on Mueller-Hinton agar (Formula 1, Annexure A) and incubated overnight at 37°C. Once the bacteria were growing vigorously, a single colony was removed carefully, replated on Mueller-Hinton agar, and again incubated overnight at 37°C (carried out in duplicate). Single colonies were

transferred from these plates to eight test tubes, each containing 5 mL of Mueller-Hinton broth (Formula 2, Annexure A), which were then incubated overnight at 37°C in a shaker-waterbath. These 5 mL broth cultures were subsequently used to inoculate eight small Erlenmeyer flasks, each containing 50 mL of Mueller-Hinton broth. After an overnight incubation period at 37°C in the shaker-waterbath, the 55 mL broth cultures were used to inoculate eight stainless steel trays, each containing 1.5 L of sterile Mueller-Hinton agar. The trays were incubated at 37°C for 18 hours after which the bacteria were scraped off the agar carefully and suspended in a 1% phenol solution. The phenolic suspension was stirred overnight at 4°C to ensure dissolution of the capsular material, and then ultracentrifuged (35 000 r.p.m. on a Beckman L8-M ultracentrifuge, rotor type 70 Ti) for 3 hours. The supernatants were combined and the polysaccharide (both the K- and the O-antigens) precipitated into 5 volumes of ethanol, after which it was centrifuged. The clear yellow supernatant was poured off and the polysaccharide pellets combined, and dissolved in the minimum amount of water (~ 60 mL). This fairly viscous solution was divided into two portions, A and B, which were worked up separately.

A : 5 mL of a 5% cetytrimethylammonium bromide (CTAB) solution was added slowly, with stirring, to 25 mL of the aqueous polysaccharide solution (Portion A). The mixture was allowed to stand for several hours, after which the precipitate, i.e. the cetyltrimethylammonium salt of the acidic polysaccharide, was separated from the supernatant by centrifugation. The precipitate was dissolved in 25 mL of 3M sodium chloride (to allow dissociation of the CTAB-complex), after which the polysaccharide was precipitated into 5 volumes of ethanol. After centrifugation, the polysaccharide was dissolved in water and dialysed (12 000 - 14 000 Mw cut-off) for 48 hours against running tapwater. The solution was freeze-dried to yield 155 mg of the acidic polysaccharide. G.l.c. analysis of the acetylated aldononitrile derivatives of an acid hydrolysate however showed this material to be contaminated by the neutral O-antigen, O8.

B : Portion B, *viz.* 35 mL of the aqueous polysaccharide solution, was adjusted to 1% acetic acid with 50% aqueous acetic acid, and treated at 100°C for 1 hour to sever the lipid from the acidic capsular polysaccharide. The solution was cooled to room temperature and ultracentrifuged (40 000 r.p.m. on a Beckman L8-M ultracentrifuge, rotor type 70 Ti) for 2 hours. The lipid-containing pellets were discarded, and the clear supernatants were combined and dialysed (12 000 - 14 000 Mw cut-off) for 24 hours against running tapwater. The solution was freeze-dried overnight, dissolved in the minimum amount of water, and was then treated in the same way as portion A, with 5% CTAB to give 158 mg of the purified acidic polysaccharide.

Additional capsular material was obtained from the bacterial cells by a heat extraction (60°C / 10 min) and the acidic polysaccharide was isolated as described in B. The overall yield of the acidic polysaccharide from this first growth was 788 mg. The entire procedure, route B, was repeated and yielded 665 mg, which brought the total yield of the acidic capsular polysaccharide to 1.453 g. The purified polysaccharide had an optical rotation of $[\alpha]_D$ +106° and was shown to be polydisperse with regard to molecular weight distribution (g.p.c. column IV).

4.5.(iii) Monosaccharide composition.

A small portion (3 mg) of the native polysaccharide was hydrolysed with 2 mL 4M trifluoroacetic acid (TFA) at 125°C for 1 hour. The reaction product was concentrated under reduced pressure after which it was co-concentrated several times with small aliquots of water to ensure that all the acid had evaporated. The liberated sugars were transformed into their acetylated aldononitrile derivatives (PAAN'S)[135] and analysed by g.l.c., on the capillary column DB-225 at 220°C (isothermal).

To identify the uronic acid, the native polysaccharide (5 mg) was dried in a drying pistol (50°C), for 6 hours after which it was treated with methanolic 3% HCl at 80°C for 16 hours. The solution was neutralized with Ag_2CO_3 and stirred for 1 hour. The salts

were removed by centrifugation after which the solution was reduced with $NaBH_4$ in dry methanol (overnight). The solution was subsequently passed down an Amberlite IR-120$(H)^+$ ion-exchange resin, and the acidic fraction was collected and co-concentrated with several small aliquots of methanol. The residue was hydrolysed (4M TFA, 125°C, 1 hour), after which the free reducing sugars were converted into their PAAN'S. G.l.c. analysis was conducted on column DB-225 at 220°C (isothermal).

4.2.(iv) Absolute configuration of the monosaccharides.

The absolute configurations of the constituent monosaccharides were determined by g.l.c. analysis of their acetylated (-)-2-octyl glycosides[79]. The native polysaccharide (10 mg) was methanolysed (methanolic 3% HCl, 80°C, 16 hours), reduced ($NaBH_4$, overnight), and hydrolysed with 4M TFA (125°C, 1 hour). The hydrolysate was freeze-dried in an ampoule, and to the dried material was added 1 drop of concentrated TFA, 0.5 mL (-)-2-octanol and a small magnetic stirrer bar. The ampoule was sealed and heated, with stirring, in an oilbath at 130°C for 16 hours. The reaction mixture was then concentrated to dryness (55°C, vacuum pump attached to the rotary evaporator) and the derived octyl glycosides acetylated with pyridine : acetic anhydride 1 : 1 v/v at 100°C for 1 hour. G.l.c. analysis was performed using column DB-225 with the following temperature programme : 220°C for 5 min then 1°/min to 235°C.

4.5.(v) Methylation analysis.

A portion (18 mg) of the acidic polysaccharide was dried for 16 hours in a drying pistol prior to methylation with potassium-dimsyl and methyl iodide[87]. The methylated polysaccharide was divided into two portions, I and II :

I : One third of the methylated polysaccharide was hydrolysed with 4M TFA (125°C, 1 hour). After evaporation of the acid, the hydrolysate was reduced ($NaBH_4$, 1 hour), and passed down Amberlite IR-120$(H)^+$ resin. The acidic fraction was collected and co-concentrated with small aliquots of methanol (3 x 2 mL) to remove the borates.

Acetylation of the partially methylated alditol acetates was achieved by treatment with equal volumes of pyridine and acetic anhydride at 100°C for 1 hour. The partially methylated alditol acetates were analysed by g.l.c. (column DB-225, 205°C, isothermal) and g.l.c.- m.s. The results are tabulated in Table 4.1, Column I.

II : Two thirds of the methylated polysaccharide was dried (6 hours) in a drying pistol prior to methanolysis (methanolic 3% HCl, 80°C, 16 hours). The reaction mixture was neutralized with Ag_2CO_3, centrifuged, and the supernatant concentrated to dryness. The residue was dissolved in methanol (~5 mL), filtered (0.22 μm Millipore filter), and reduced ($NaBH_4$) overnight. The alkaline solution was subsequently passed down Amberlite IR-120(H)$^+$ resin. The acidic fraction was collected and concentrated with small aliquots of methanol. The residue was hydrolysed (4M TFA, 125°C, 1 hour), and the partially methylated monosaccharides converted into their alditol acetates as before, and analysed by g.l.c. (column DB-225, 205°C, isothermal) and g.l.c.- m.s. The results are tabulated in Table 4.1, Column II.

4.5.(vi) Uronic acid degradation.

The methylated polysaccharide (15 mg) was subjected to a base-catalysed degradation, following a modification of the method as proposed by Aspinall and Rosell[92].

A mixture (1 mL) containing dry dimethylsulphoxide : 2,2-dimethoxypropane (19 : 1 v/v) was added to the methylated polymer under nitrogen. The mixture was stirred for 1 hour after which potassium-dimsyl (1 mL) was added. After stirring for another 2 hours, the polymer was re-alkylated with trideuteriomethyl-iodide (0.5 mL) whilst keeping the mixture cool in an ice-bath. The reaction mixture was stirred for another hour after which it was diluted with water (3 mL) and the reaction product extracted with dichloromethane (4 x 3 mL). The dichloromethane fractions were pooled, washed with water (4 x 3 mL), and concentrated to dryness. The residue was hydrolysed (4M TFA, 125°C, 1 hour), and the partially methylated monosaccharides converted into

their alditol acetates, as previously described. The partially methylated alditol acetates were analysed by g.l.c. (Column DB-225, 205°C, isothermal) and g.l.c.- m.s. The results are tabulated in Table 4.1, Column III.

4.5.(vii) Selective degradation by lithium dissolved in ethylenediamine.

The acidic polysaccharide (20 mg) was selectively degraded by lithium dissolved in ethylenediamine, following the method described by Albersheim and co-workers[99]. The dried polysaccharide sample was dissolved in ethylenediamine (25 mL). Upon dissolution of the sample, 2 - 5 mm pieces of lithium-wire were added to the solution. The solution turned deep blue and the colour was maintained, at room temperature, for 1 hour by the addition of more lithium pieces. The reaction was quenched with water (20 mL) after which the resulting clear solution was transferred to a bigger flask. Toluene (25 mL) was added to form an azeotrope with the ethylenediamine and water, and the mixture was rotoevaporated to dryness. The residue was co-concentrated with another two aliquots (2 x 25 mL) of toluene after which the resulting powdery residue was cooled in an ice-bath and titrated to pH 4.5 with glacial acetic acid. The acidic solution was passed down an Amberlite IR-120(H)$^+$ resin column to remove the lithium ions. The acidic fraction was collected and freeze-dried to yield the lithium-treated product.

An aqueous solution of the lithium-treated product was passed down g.p.c. column II.The carbohydrate fraction was collected, passed down an Amberlite IR-120(H)$^+$ resin column and freeze-dried. The product was further purified by a passage down g.p.c. column III. The carbohydrate fraction was collected and worked up as before to remove the buffer. The purified lithium-treated polymer was tested for the presence of uronic acids, following the method as proposed by Dische[189].

The lithium-treated polysaccharide (2.4 mg) was methylated, methanolysed and hydrolysed, as previously described. The partially methylated monosaccharides were

converted into their alditol acetates, as before, and analysed by g.l.c. (Column DB-225, 205°C, isothermal) The results are tabulated in Table 4.1, Column IV.

The lithium degradation reaction was repeated on 100 mg of the acidic polysaccharide and yielded 19.7 mg of product. 14.7 mg of the lithium-treated polysaccharide was deuterium-exchanged and subjected to n.m.r. analysis.

4.5.(viii) 2D n.m.r. spectroscopy.

Lithium degraded polymer : The ^{1}H homonuclear shift-correlated experiment (COSY)[178] at 400 MHz was performed using a spectral width of 2008.03 Hz. A data matrix of 256 x 2048 data points was collected with 96 transients for each delay . The data matrix was zero-filled in the t_1 dimension and transformed after the application of a non-shifted sine-bell window function in both dimensions and symmetrised. Digital resolution in the resulting 1024 x 2048 matrix was 0.98 Hz/point. One- and two-step RELAY-COSY[179] experiments at 400 MHz were performed using a spectral width of 801.28 Hz. Data matrices of 256 x 1024 data points were collected with 64 transients for each t_1 delay. The data matrices were zero-filled to 512 data points in t_1 and transformed after the application of a non-shifted sine-bell window function. Digital resolution in the 512 x 1024 matrices were 0.78 Hz/point. Fixed delays of 0.036 s were used. A homonuclear dipolar-correlated (NOESY)[159] experiment (400 MHz) was performed using a spectral width of 801.28 Hz. A data matrix of 256 x 2048 data points was collected with 144 transients for each t_1 delay. The matrix was zero-filled in the t_1 dimension and transformed after the application of a shifted sine-square window function in both dimensions and symmetrised. Digital resolution in the resulting 1024 x 2048 matrix was 0.39 Hz/point. The mixing delay in the NOESY experiment was 0.3 s. A ^{13}C - ^{1}H shift-correlated experiment (HETCOR)[184] was recorded, using a spectral width in f_2 of 9090.91 Hz (90.36 p.p.m.) and 1400.5 Hz (3.5 p.p.m.) in f_1. The initial data matrix of 58 x 2048 data points was zero-filled in the t_1 dimension to 256 x 2048 points and transformed after the application of a shifted sine-square window function in both

dimensions. Digital resolution in f_2 was 4.44 Hz/point and in f_1 5.47 Hz/point. A recycle delay of 0.5 s was employed and 4000 transients per f.i.d. were collected.

E. coli K102 capsular polysaccharide : ^{1}H Homonuclear shift-correlated (COSY and RELAY-COSY)[178,179] and homonuclear dipolar-correlated (NOESY)[159] experiments at 400 MHz were performed using a spectral width of 1805.05 Hz. In the COSY and RELAY-COSY experiments, data matrices of 512 x 2048 points were collected with 128 or 64 transients for each t_1 delay respectively. The matrices were zero-filled in the t_1 dimension and transformed after the application of non-shifted sine-bell (RELAY-COSY) and shifted sine-square (COSY) window functions and symmetrised. Digital resolution in the resulting 1024 x 2048 matrices was 0.88 Hz/point. For the RELAY-COSY experiment fixed delays of 0.036 s were used. In the NOESY experiment an initial data matrix of 256 x 2048 data points was collected with 160 transients for each t_1 delay. The matrix was zero-filled in the t_1 dimension (1024 x 2048 points) and transformed after the application of a shifted sine-square window function and symmetrised. Digital resolution was 0.88 Hz/point. The mixing delay in the NOESY experiment was 0.3 s. The 2D homonuclear Hartmann-Hahn (HOHAHA)[180] experiment at 400 MHz was performed using a spectral width of 1805.05 Hz. The mixing period consisted of 50 MLEV-17 cycles. A data matrix of 526 x 2048 was acquired with 112 transients for each t_1 delay. The matrix was zero-filled in the t_1 dimension (1024 x 2048 data points) and multiplied in both dimensions with a shifted sine-square window function prior to phase sensitive Fourier transformation. A ^{13}C - ^{1}H shift-correlated experiment (HETCOR)[184] was recorded using a spectral width in f_2 of 9090.91 Hz (90.36 p.p.m.) and 1520.5 Hz (3.8 p.p.m.) in f_1. The initial matrix of 64 x 2048 data points was zero-filled in the t_1 dimension and transformed to 256 x 2048 points after the application of a shifted sine-square window function. Digital resolution in f_2 was 4.44 Hz/point and in f_1 5.94 Hz/point. A recycle delay of 0.5 s was employed and 4000 transients per f.i.d. were collected.

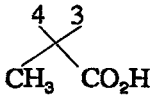
THE STRUCTURAL ELUCIDATION OF THE CAPSULAR POLYSACCHARIDE OF *ESCHERICHIA COLI* SEROTYPE O8: K47: H2.

5. THE STRUCTURAL ELUCIDATION OF THE CAPSULAR POLYSACCHARIDE OF *ESCHERICHIA COLI* SEROTYPE O8: K47: H2.

5.1. Abstract.

The structure of the repeating unit of the capsular polysaccharide from *E. coli* serotype O8:K47:H2, culture no. A282a, was investigated using methylation analysis, degradation by bacteriophage-borne endoglycanases, mass spectrometry and 1D and 2D n.m.r. spectroscopy. The repeating unit was shown to be a linear tetrasaccharide, with pyruvate as the only acidic component :

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



(R)

5.2. Introduction.

Pyruvic acid, linked as a cyclic ketal to a pyranosyl residue, is a common component of bacterial polysaccharides[194]. In the *E. coli* series, the pyruvic acid ketal is most often linked to O-4 and O-6 of the pyranosyl residue, for example in the capsular polysaccharides of *E. coli* K29[35], K37[43] and K55[53]. Pyruvate has also been found linked to O-2 and O-3 of D-glucopyranosyl residues (*E. coli* K33[40]), and to O-3 and O-4 of L-rhamnopyranosyl residues (*E. coli* K26[31]).

The pyruvic acid ketal is often part of the immunological determinant, and the immunological specificity is affected by the configuration of the monosaccharide to which the pyruvate is linked, the positions of the two hydroxyl groups that are bridged by the ketal, and the configuration of the methyl- and carboxyl groups at the asymmetric ketal carbon atom, *viz.* C-2 of the pyruvic acid moiety[4]. The determination of this latter

configuration is thus very important in immunological studies[195,196].

The structural determination of the capsular polysaccharide of *E. coli* K47, which contains a pyruvic acid ketal linked to O-3 and O-4 of a D-galactopyranosyl residue, is now reported. The capsular polysaccharide of the *E. coli* K47 belongs to a subgroup of Group I[9], due to the polymer containing an amino sugar and being of high molecular weight. In addition, the *E. coli* K47 polysaccharide is co-expressed with O-antigen 8 and does not contain any labile sugars.

5.3. Results and Discussion.

5.1.(i) Monosaccharide composition.

Bacteria of the genus *E. coli* serotype O8:K47:H2, culture no. A282a, were grown on Luria Bertani agar, and the acidic capsular polysaccharide was isolated and purified by the method described by Okutani and Dutton[70]. A low yield of capsular material was obtained and examination of the bacterial cells by the Indian Ink Method[197], showed sparse encapsulation. The growth procedure was thus repeated twice to ensure that sufficient capsular material was available to conduct the chemical and instrumental analysis.

The polysaccharide was further purified by passage down a DEAE-Sepharose CL-6B anion-exchange gel. The purified material had an optical rotation of $[\alpha]_D$ +11.7 and, in gel-permeation chromatography on Sephacryl S-400, showed a broad molecular weight distribution with a peak maximum at M_r 6.5 x 10^5 (Figure 5.1).

G.l.c. analysis of the acetylated aldononitriles[135], derived from the products of an acid hydrolysate of the polysaccharide, indicated the presence of mannose, galactose and glucosamine in the molar ratio 1 : 2 : 1. The ratio did not change after a methanolysis, reduction sequence, indicating the absence of a uronic acid residue. The monosaccharide

composition was confirmed by h.p.l.c. analysis of the liberated sugars from the acid hydrolysis. The monosaccharides, were all shown to have the D-configuration, by g.l.c. analysis of their acetylated (-)-2-octyl glycosides[79]. The purified polysaccharide tested negative for the presence of any phosphate groups[188].

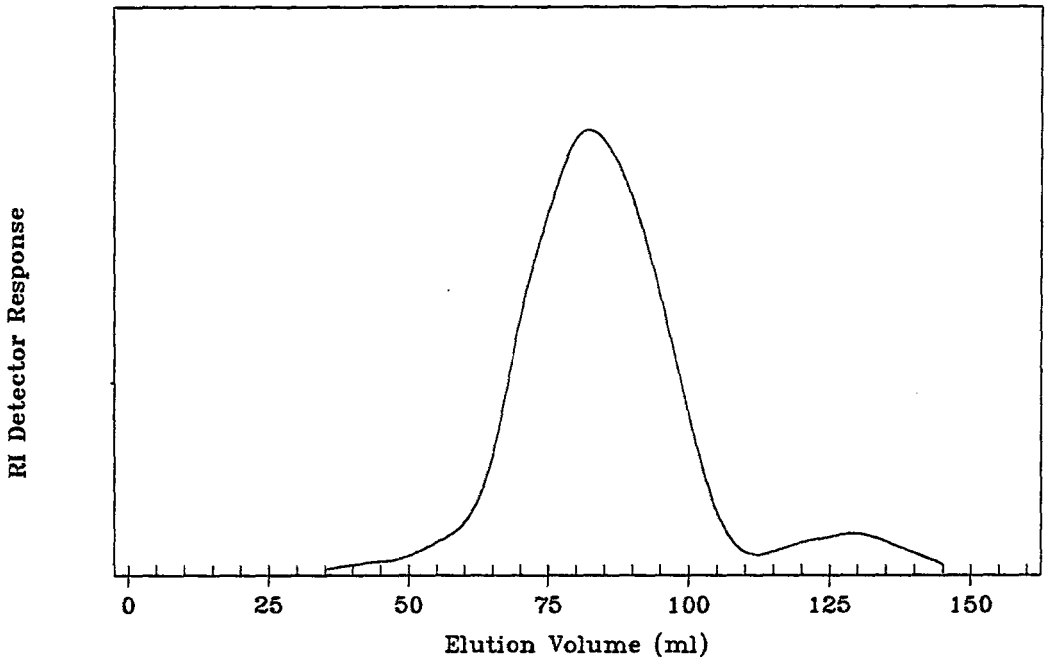
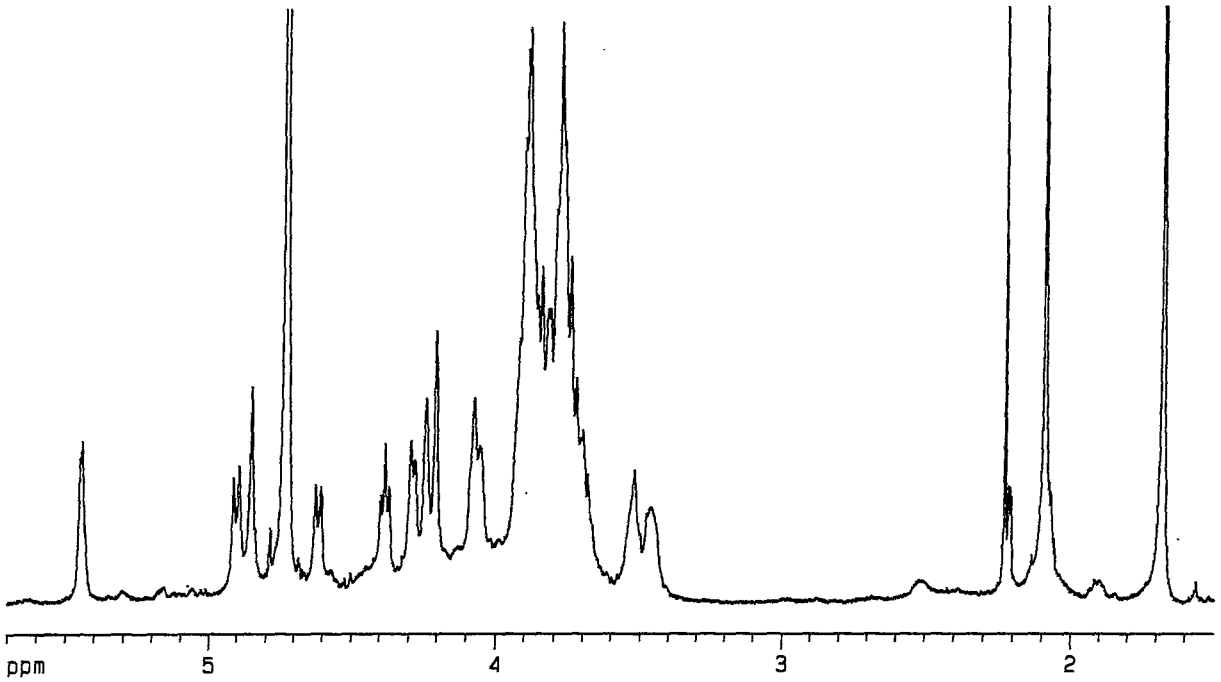


Figure 5.1 : Gel-permeation chromatogram (Sephacryl S-400) of the *E.coli* K47 polysaccharide.

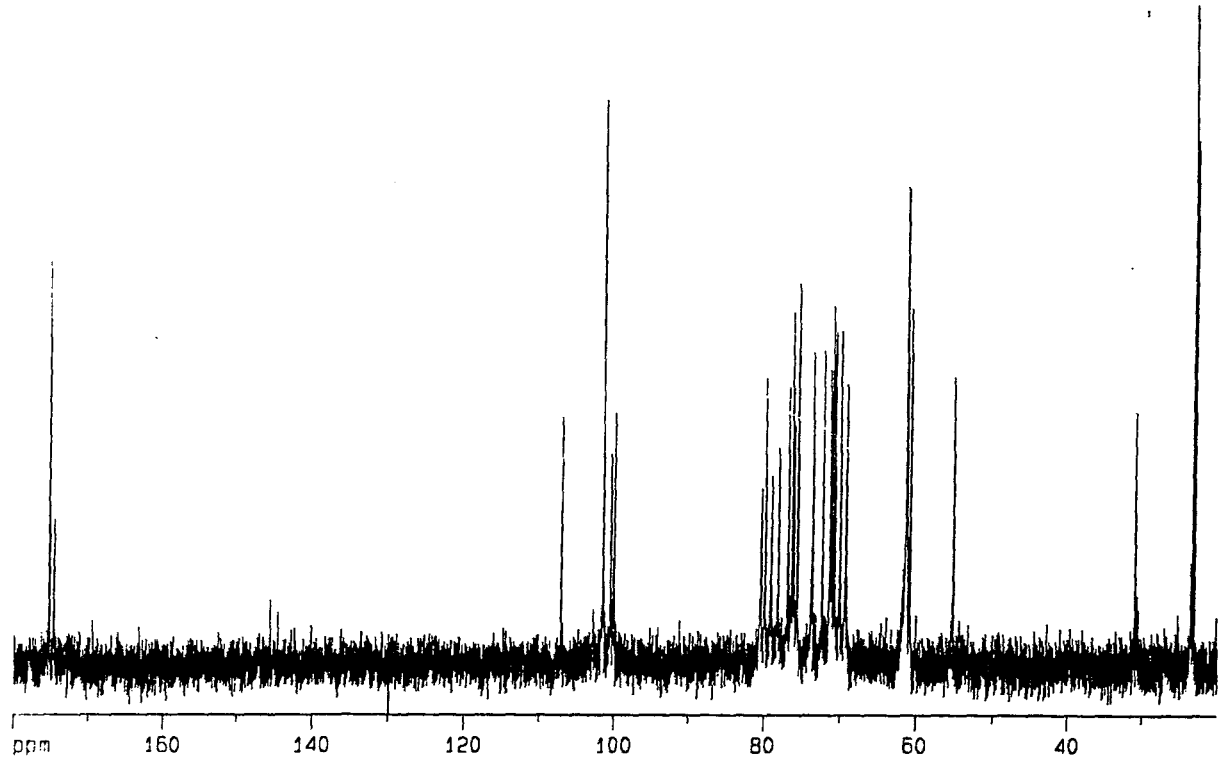
The 1D ^{1}H n.m.r. spectrum (Spectrum 5.1) of the polysaccharide contained one H-1 signal in the α-anomeric region, at δ 5.46 ($J_{1,2}$ 3.2 Hz), and three H-1 signals in the β-anomeric region, at δ 4.91 ($J_{1,2}$ 8.0 Hz), δ 4.85 (unresolved doublet) and δ 4.62 ($J_{1,2}$ 7.6 Hz). In addition, the spectrum also contained signals, integrating for three protons, for the methyl groups of a N-acetate and a pyruvic acid ketal at δ 2.09 and δ 1.68 respectively. The ^{1}H-decoupled 1D ^{13}C n.m.r. spectrum (Spectrum 5.2) complemented the ^{1}H n.m.r. data with signals for C-1 at 101.65 (two), 100.60 and 100.11 p.p.m. A signal for the assymetric ketal carbon atom of the pyruvic moiety was also observed at 107.22 p.p.m. Signals for methyl carbons of the N-acetate and pyruvate groups were observed at 23.45 and 23.15 p.p.m., and signals for two carbonyl carbons at 175.31 and 174.64 p.p.m. The results confirmed a tetrasaccharide repeating unit for the

Spectrum 5.1



The 1D ^{1}H n.m.r. spectrum (400 MHz) of the *E. coli* K47 capsular polysaccharide.

Spectrum 5.2



The 1D ^{13}C n.m.r. spectrum (400 MHz) of the *E. coli* K47 capsular polysaccharide.

polysaccharide and are consistent with the presence of one mole of pyruvate as the acidic function.

5.3.(ii) Methylation analysis.

A portion of the polysaccharide, in the acid form, was methylated with potassium-dimsyl and methyl iodide[87]. This however did not afford complete methylation and the partially

Table 5.1 : Methylation analysis of the *E. coli* K47 capsular polysaccharide.

Methylated sugars as alditol acetates[a]	T[b]		Molar Ratio			
	DB-225	DB-17	I	II	III	IV
2,3,6-Man	1.61	1.38	1.00	1.00	1.00	1.00
2,3,6-Gal	1.70	1.39	0.63	0.49	0.51	0.35
6-Gal	2.97	2.10	0.64	0.59	0.83	0.74
4,6-GlcNAc[c]		3.09		0.24		0.12

a. 2,3,6-Man = 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylmannitol

b. Retention times relative to that of 2,3,4,6-Glc

DB-225 : Isothermal at 205°C

DB-17 : 180°C for 2 min then 2°/min to 250°C

I : methylated polysaccharide (DB-225)

II : methylated polysaccharide (DB-17)

III : methylated, carboxyl-reduced polysaccharide (DB-225)

IV : methylated, carboxyl-reduced polysaccharide (DB-17)

c. The retention time of 4,6-GlcNAc is too long on column DB-225 and the peak too small to integrate.

methylated polymer was methylated further by the method proposed by Kuhn and co-workers[89]. After hydrolysis, the partially methylated monosaccharides were analysed, as their alditol acetates, by g.l.c. and g.l.c.- m.s. (Table 5.1, Columns I and II). A portion of the methylated polysaccharide was methanolysed and reduced prior to hydrolysis and analysed, as before (Table 5.1, Columns III and IV).

The results are in accordance with a tetrasaccharide repeating unit for the polysaccharide and indicate that the polymer is linear with the mannose linked at position 4, the glucosamine at position 3, and one galactose unit at position 4. The position of linkage of the 6-O-methyl galactose residue is however uncertain since this is the sugar unit to which the acidic pyruvate group is linked. The pyruvic acid can be linked as a ketal to either O-2 and O-3, or to O-3 and O-4 of the D-galactopyranosyl residue. These linkage positions, together with that of the glycosidic linkage to the next sugar in sequence, will be established through 2D n.m.r. spectroscopy.

5.3.(iii) Bacteriophage degradation.

The native polysaccharide was subjected to degradation by bacteriophage-borne enzymes. The objective of this reaction was to obtain oligosaccharides, *viz.* the repeating unit and/or multiples thereof, which would then provide valuable information on the sequencing and structure of the polysaccharide. The degraded material was applied to a column of Biogel P-4, and the major product was isolated and freeze-dried.

Due to the low activity of the bacteriophage enzymes, the depolymerization reaction did not afford oligosaccharides corresponding to P_1, P_2, P_3,...etc. The lower molecular weight material that was produced, however, had identical sugar ratios to those of the undegraded polymer. Owing to the suitability of the lower molecular weight material for n.m.r. study, it was deuterium-exchanged and subjected to ^1H- and ^{13}C n.m.r. analysis.

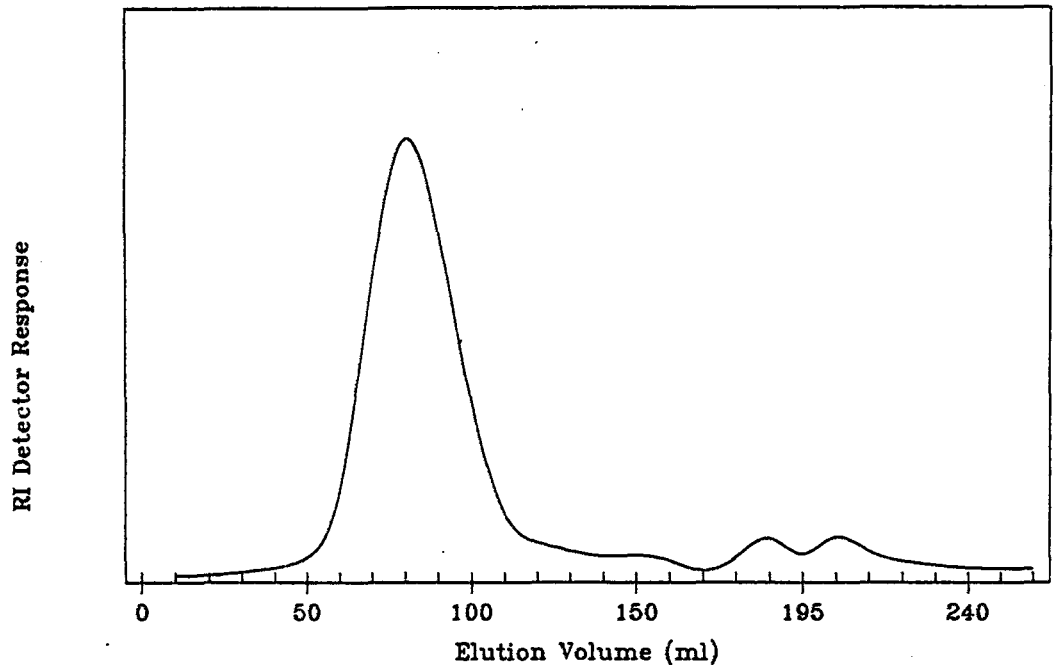


Figure 5.2 : The chromatogram of the phage-degraded *E. coli* K47 polysaccharide.

(Biogel P-4)

5.3.(iv) The n.m.r. study of the bacteriophage degraded *E. coli* K47 polysaccharide.

The ^1H resonances of the four monosaccharides in the repeating unit were established (Table 5.2) from COSY[176] (Spectrum 5.3), 2D HOHAHA[180] (Spectra 5.4 and 5.5), and phase sensitive NOESY[159] (Spectrum 5.6) experiments. The residues in the repeating unit were labelled a - d in order of decreasing chemical shift of the anomeric protons. Using the H-1 resonances as a window into each spin system, the corresponding H-2 connectivities of all the constituent monosaccharides were readily traced via their H-1/H-2 cross-peaks. (See Spectrum 5.3.) The chemical shift of H-3 for residue c was also assigned from the COSY spectrum. These results were confirmed and the chemical shifts for H-4 of c, H-3 and H-4 of a and d, and H-3, H-4 and H-5 of b were established from the two 2D HOHAHA experiments. These two experiments differ only in their mixing times, i.e. the time allowed for magnetization to transfer through the spin system. The 2D HOHAHA experiment with the mixing time of 28 ms approximates the one-step RELAY-COSY experiment and showed, in addition to the H-1/H-2 cross-peaks, a relayed coherence from H-1 to H-3. The experiment with the longer mixing time of 87 ms,

corresponds to a multiple-step RELAY-COSY experiment and showed virtually all the correlations for each spin system in the H-1 track.

Table 5.2 : ^{1}H- and ^{13}C n.m.r. data (303°K) for the tetrasaccharide repeating unit of the *E. coli* K47 capsular polysaccharide.

Residue	Proton or Carbon						
	1	2	3	4	5	6a	6b
a. →4)-α-Gal							
H	5.46	3.89	3.94	4.26	3.91	~3.78	~3.70
^{3}J (Hz)	3.2			1.2			
C	100.11	69.30	70.10	76.97	71.18	60.88	
b. →3)-β-GlcNAc							
H	4.91	3.86	3.77	3.74	3.45	~3.76	~3.76
^{3}J (Hz)	8.0						
C	100.60	55.21	80.49	71.46	76.45	61.28	
c. →4)-β-Man							
H	4.85	4.22	3.79	3.77	3.52		
^{3}J (Hz)	1.0						
C	101.65	70.83	72.39	78.25	75.71		
d. →2)-β-Gal | (3,4) Pyr							
H	4.62	3.81	4.39	4.29	4.07	~3.91	~3.84
^{3}J (Hz)	7.6	6.1	6.1	~0.8			
C	101.65	79.16	79.96	76.21	73.76		

Chemical shifts in p.p.m. relative to internal acetone at δ 2.23 for ^{1}H- and at 31.07 p.p.m. for ^{13}C n.m.r.

Returning to the COSY spectrum the chemical shift for H-5 of residue a was established via the H-4/H-5 cross-peak. Further ^1H assignments were made from the phase sensitive NOESY experiment. The observed inter- and intra-residue n.O.e. contacts are presented in Table 5.3.

Table 5.3 : Inter- and intra-residue n.O.E contacts for the *E. coli* K47 polysaccharide.

Residue	Proton	N.O.e. contact to
a. →4)-α-Gal	H-1	3.89 (a. H-2), 3.77 (b. H-3)
b. →3)-β-GlcNAc	H-1	3.81 (d. H-2), 3.77 (b. H-3), 3.45 (b. H-5)
c. →4)-β-Man	H-1	4,26 (a. H-4), 4.22 (c. H-2), 3.79 (c. H-3), 3.52 (c. H-5)
	H-2	3.79 (c. H-3)
	H-3	3.52 (c. H-5)
d. →2)-β-Gal | (3,4) Pyr	H-1	4.39 (d. H-3), 4.07 (d. H-5), 3.77 (c. H-4)
	H-3	4.29 (d. H-4), 4.07 (d. H-5)
	H-4	4.07 (d. H-5)
	CH_3 (Pyr)	3.81 (d. H-2)

^1H chemical shifts in p.p.m. relative to internal acetone at δ 2.23.

The α-linked residue, a, showed the expected intra-residue n.O.e. contact from H-1 to H-2 whereas the β-linked residues, b, c and d, showed characteristic n.O.es from H-1 to H-3 and H-5 respectively. The n.O.e. contacts from H-1 to H-5 allowed the chemical shifts of H-5 for both residues c and d to be established. In addition, intramolecular

n.O.e. contacts from H-1 to H-2, H-2 to H-3 and H-3 to H-5 (residue **c**) and from H-3 to H-4, H-3 to H-5 and H-4 to H-5 (residue **d**) were observed. These contacts served to confirm the chemical shift assignments made from the COSY and 2D HOHAHA experiments.

The resonances for H-6a and H-6b of residues **a**, **b** and **d**, were established from a triple quantum filter, phase sensitive COSY-experiment (Spectrum 5.7) which only shows subspectra due to isolated three-spin systems[198], *viz.* from H-5 to H-6a and H-6b. Subspectra due to the two-spin systems, e.g. from H-1 to H-2, are eliminated from the resulting contour plot. The coupling constants were measured from a 1D ^{1}H n.m.r. spectrum at 323°K.

The ^{13}C resonances for residues **a** - **d** were determined by comparing the ^{1}H chemical shift assignments with the ^{1}H - ^{13}C correlation data obtained from the HETCOR[184]-experiment.(Table 5.2 and Spectrum 5.8).

Comparison of the ^{1}H and ^{13}C chemical shift data with published data for methyl glycosides[184,198], permitted the residues to be identified as **a**, α-galactose; **b**, β-glucosamine; and **c**, β-mannose. Due to the coupling constants and ^{1}H- and ^{13}C chemical shifts of residue **d** not being comparable to literature values[184], it was impossible to identify this sugar from n.m.r. data (Table 5.4). **D** was identified as β-galactose mainly from the methylation analysis data. Furthermore, the significant deshielding of C-4 of **a** and **c**, C-3 of **b**, and C-2,3,4 of **d**, indicated that these were the linkage positions. This is in accordance with the results of the methylation analysis.

The sequence of the residues in the repeating unit was established from the NOESY experiment and inter-residue n.O.e. contacts between the anomeric protons and the relevant protons of the adjacently linked residues were clearly observed. (Table 5.3)

The sequence is thus :

→3) - **b** - (1→2) - **d** - (1→4) - **c** - (1→4) - **a** - (1-

Table 5.4 : Comparison of the observed n.m.r. data (303°K) for residue **d** and the literature values for methyl α-D-galactopyranoside.

Residue	**Proton or Carbon**						
	1	**2**	**3**	**4**	**5**	**6a**	**6b**
d. →2)-β-Gal | (3,4) Pyr							
H	4.62	3.81	4.39	4.29	4.07	~3.91	~3.84
	(4.31)	(3.50)	(3.64)	(3.92)	(3.68)	(3.80)	(3.75)
^3J (Hz)	7.6	6.1	6.1	~0.8			
	(8.0)	(10.0)	(3.8)	(0.8)	-	(7.6, 11.2)	(4.4)
C	101.65	79.16	79.96	76.21	73.76		
	(104.5)	(71.7)	(73.8)	(69.7)	(76.0)	(62.0)	

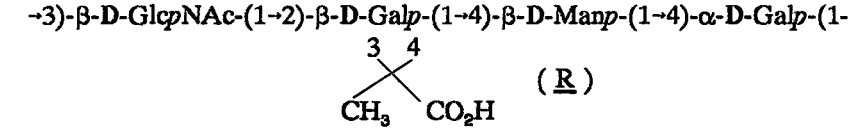
The values in parenthesis are the literature values.

The pyruvic acid ketal group was located on O-3 and O-4 of residue **d** on the basis of the deshielding shown by the resonances for C-3 and C-4, in comparison with their chemical shifts in methyl β-D-galactopyranoside[164]. (Table 5.4) The occurrence of the ^{13}C resonance for the asymmetric carbon atom of the pyruvic acid ketal at 107.22 p.p.m. is indicative of the presence of the ketal in a five-membered ring and is supportive of the above assignment. The orientation of the methyl group in the 3,4 ketal was established from the dipolar interaction observed between the methyl group and H-2 of residue **d** in

the NOESY spectrum. (Table 5.3) This defines the configuration of the ketalic carbon as R.

5.4. Conclusion.

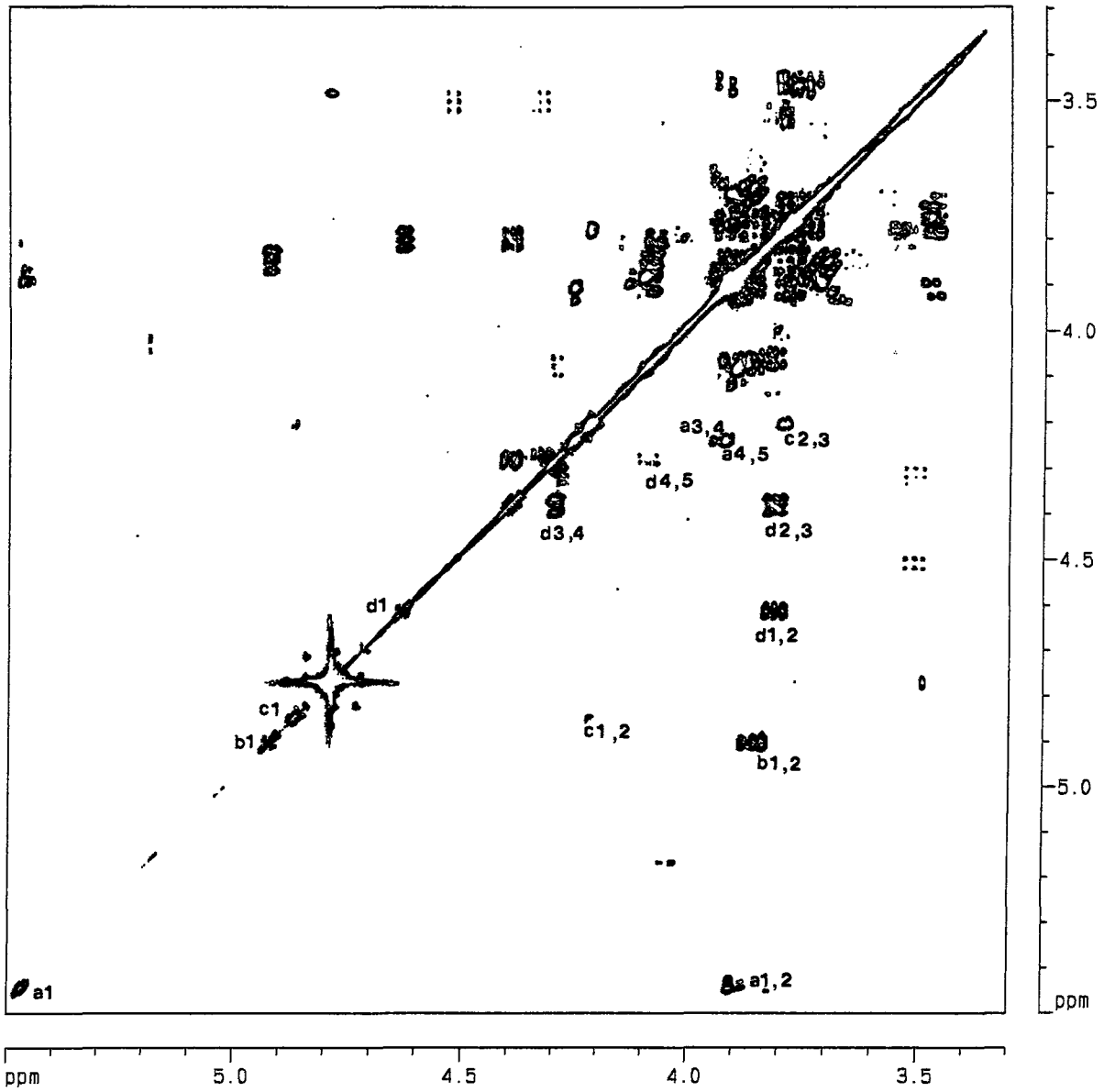
The combined n.m.r. and methylation analysis data permit the tetrasaccharide repeating unit of the *E. coli* K47 capsular polysaccharide to be written as :



The capsular polysaccharide of *E. coli* K47 is only the second in the *E. coli* series shown to owe its acidity to a pyruvic acid ketal, the other being *E. coli* K37[43].

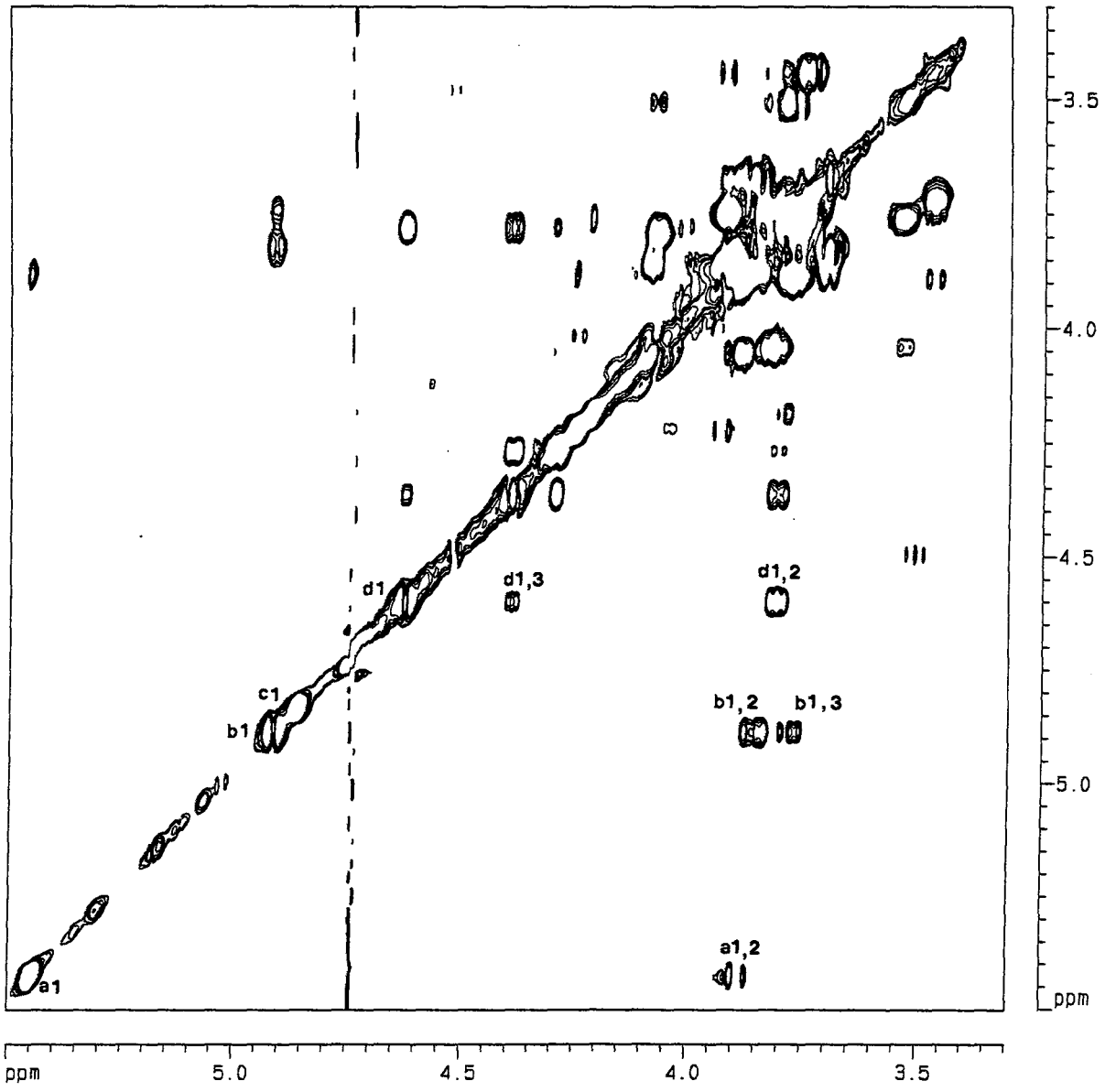
Examination of molecular models showed that in forming a 3,4 ketal, the $^4C_1$ conformation of β-galactopyranose was distorted and approached that of a skew half-chair. This conformational change was accompanied by a lessening of the dihedral angles between H-2, H-3 and between H-3, H-4. According to the Karplus relationship for vicinal protons[200], this should have resulted in a smaller $J_{2,3}$ and a larger $J_{3,4}$ than for β-galactopyranose. This was found to be the case for both $J_{2,3}$ and $J_{3,4}$. (Table 5.4) These results suggest that the presence of a pyruvic acid ketal linked to O-3 and O-4 of a galactopyranosyl residue may be established by ^1H n.m.r. spectroscopy.

Spectrum 5.3



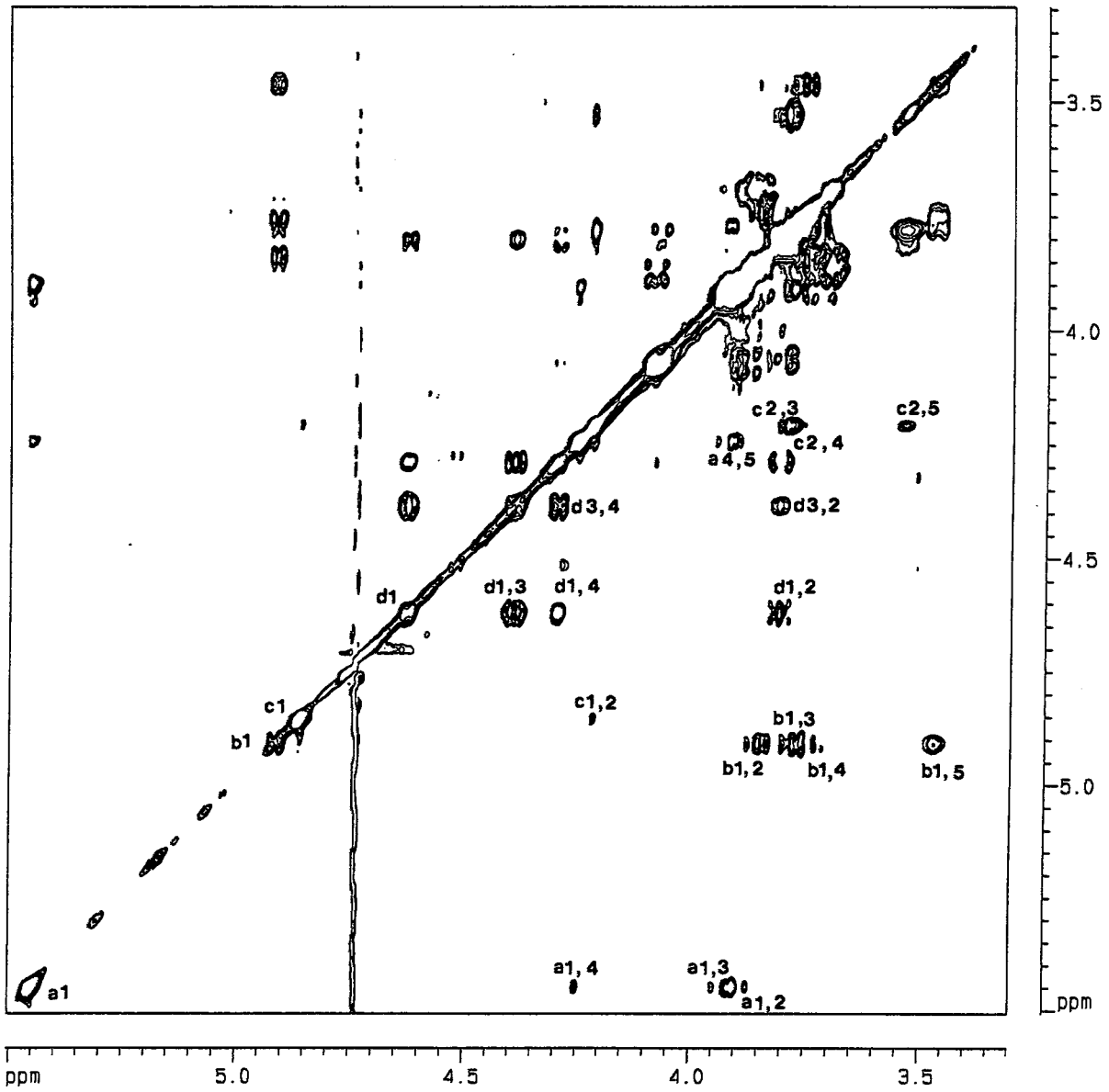
The COSY-spectrum (400 MHz) of the region 5.5 - 3.3 p.p.m. of the *E. coli* K47 capsular polysaccharide. The ^{1}H resonances of the J-coupled spin systems are labelled a - d. a1 connotes H-1 of residue a and a1,2 connotes the cross-peak between H-1 and H-2 of residue a, etc.

Spectrum 5.4



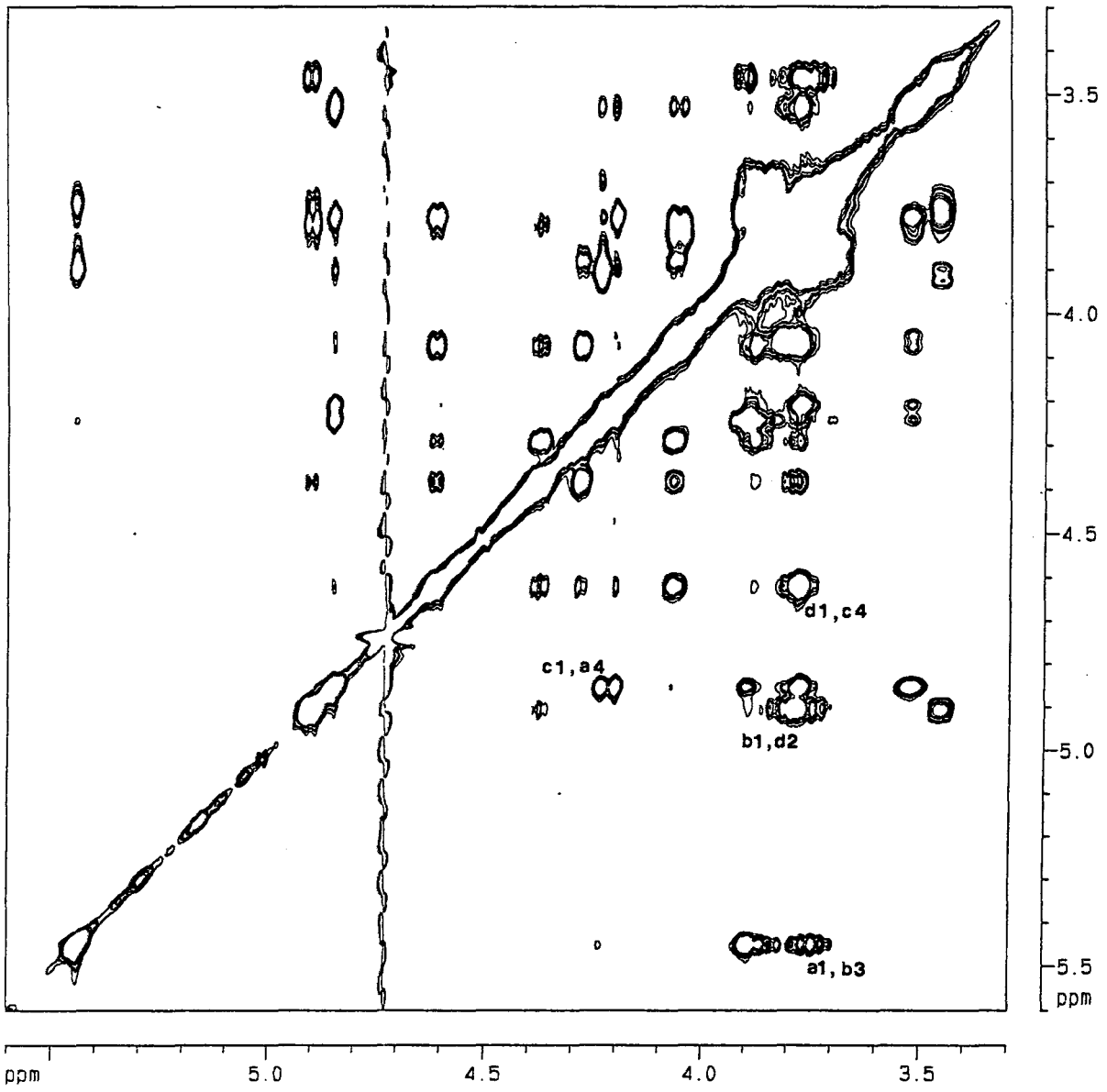
The 2D HOHAHA spectrum (Mixing time : 28 ms, 400 MHz) of the region 5.5 - 3.3 p.p.m. of the *E. coli* K47 capsular polysaccharide. The coupled spin systems are labelled **a** - **d** as before.

Spectrum 5.5



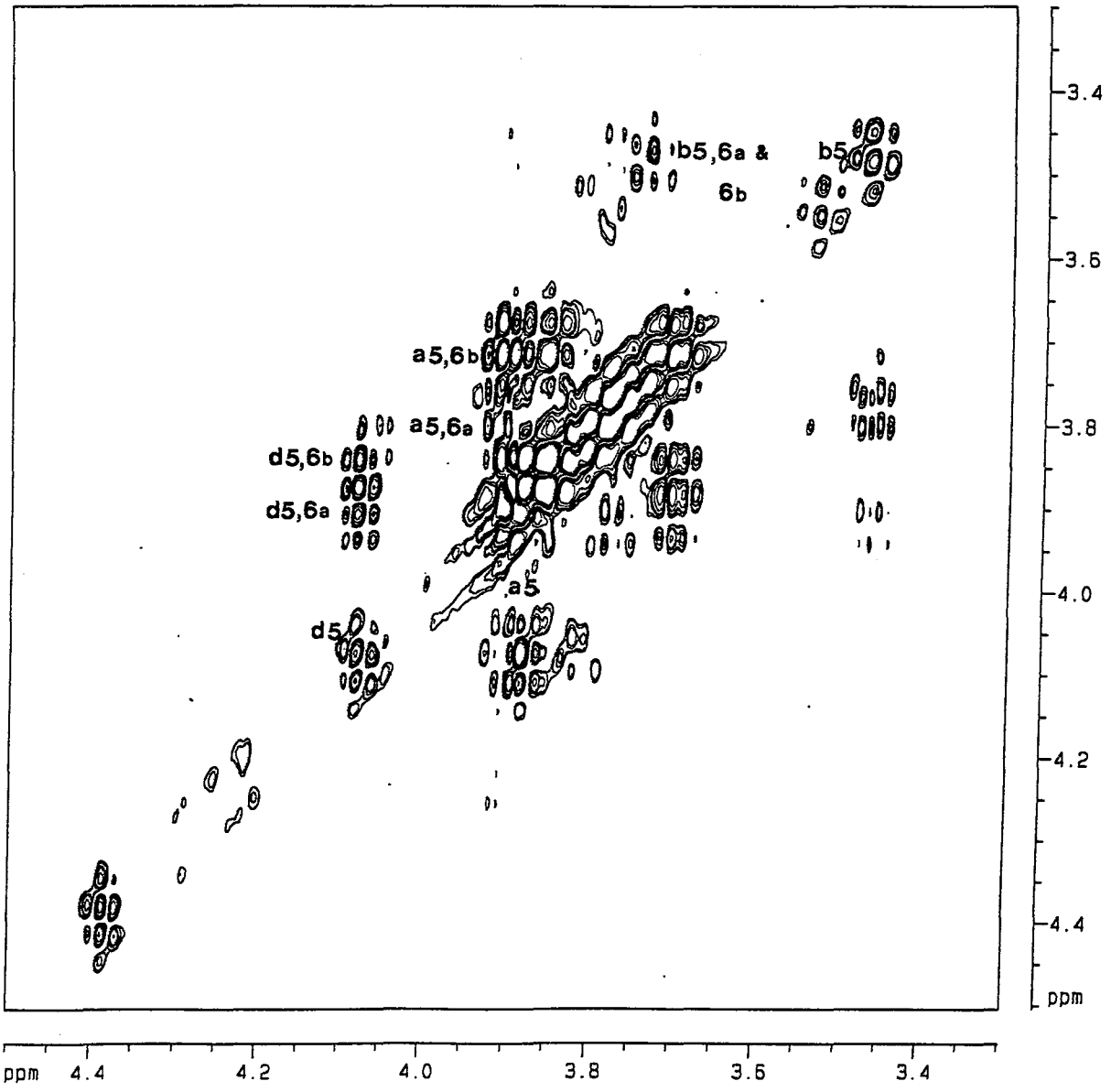
The 2D HOHAHA spectrum (Mixing time : 87 ms, 400 MHz) of the region 5.5 - 3.3 p.p.m. of the *E. coli* K47 capsular polysaccharide. The coupled spin systems are labelled **a - d** as before.

Spectrum 5.6



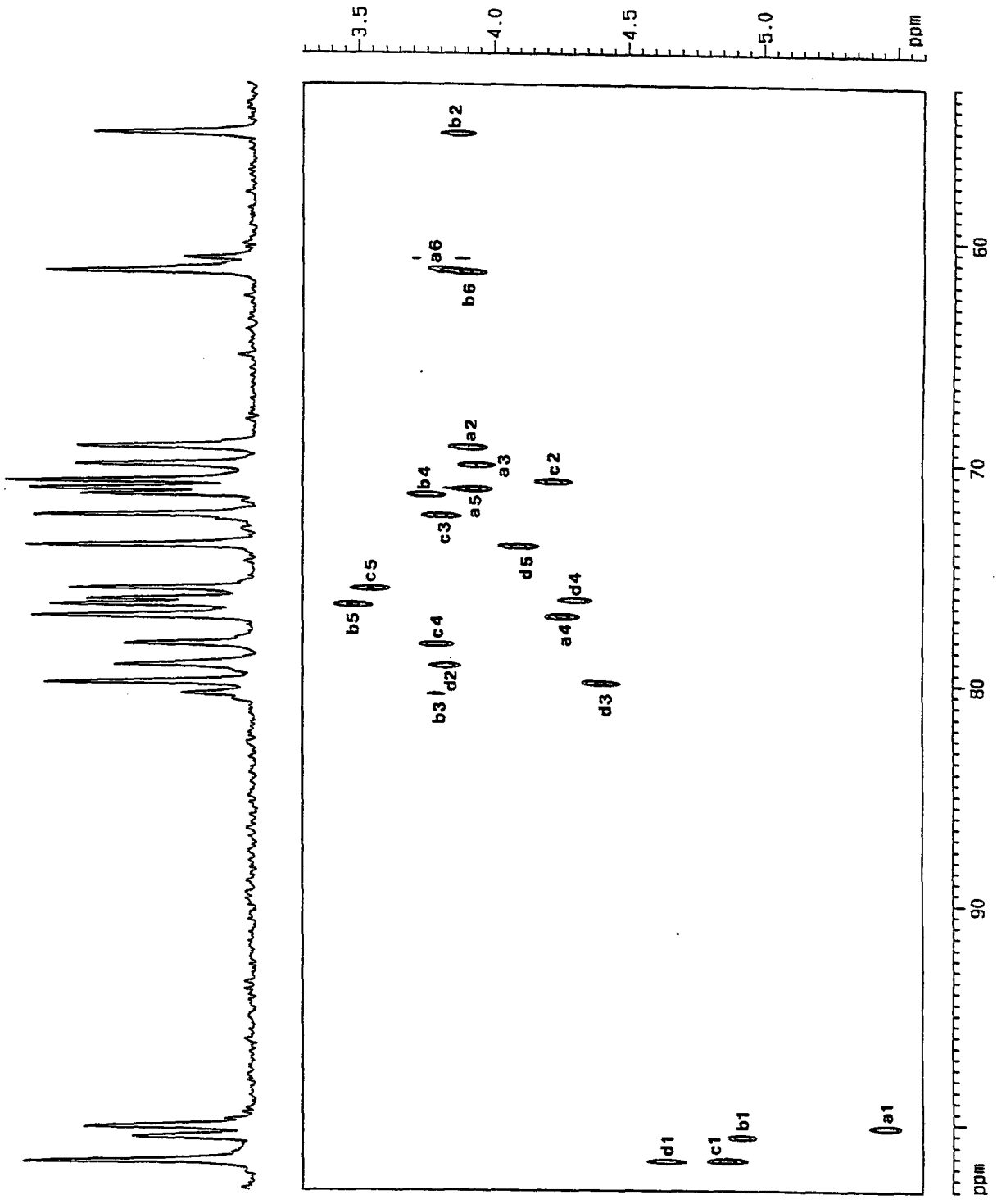
The 2D NOESY-spectrum (400 MHz) of the region 5.6 - 3.3 p.p.m. of the *E. coli* K47 capsular polysaccharide. The inter-residue n.O.e. contacts are labelled as before. a1,b3 connotes the inter-residue n.O.e. contact between H-1 of residue a and H-3 of residue b, etc.

Spectrum 5.7



The triple quantum filter, phase sensitive COSY-spectrum (400 MHz) of the region 4.5 - 3.3 p.p.m. of the *E. coli* K47 capsular polysaccharide. The coupled spin systems are labelled **a** - **d** as before.

Spectrum 5.8



The HETCOR-spectrum (400 MHz) of the *E. coli* K47 capsular polysaccharide. The ^{13}C projection is displayed along the F_2 axis, whereas the ^{1}H resonances can be read off the F_1 axis. The correlated spin systems are labelled a - d.

5.5. Experimental.

The general methods used were identical to those for the *E. coli* K102 capsular polysaccharide. For gel-permeation chromatography however, columns of I : Biogel P-4, II : Sephacryl S-200 and III : Sephacryl S-400 were employed. 0.1M Sodium acetate buffer, pH 5, was used as the eluent. Ion-exchange chromatography was performed using a DEAE-Sepharose CL-6B anion-exchange resin, with gradient elution, *viz.* 0 - 1.0M NaCl in 0.01M Tris(hydroxymethyl)-aminomethane, and a flow rate of 0.35 mL/min. Fractions were analysed by the phenol-sulphuric acid method[201]. Analytical gas-liquid chromatography was performed using both DB-17 and DB-225 bonded phase, fused silica capillary columns, 30 m x 0.252 mm and 30 m x 0.255 mm respectively. Unless otherwise stated, column DB-225 was operated at 205°C (isothermal) and column DB-17 at 180°C for 2 min, then 2°/min to 250°C. Analytical high-performance liquid chromatography was performed using a Spectra-Physics h.p.l.c. system. The chromatograph included an IsoChrom LC pump, a SSI 505 LC oven, an ERC-7515 RI detector and an SP4290 integrator. Separations were achieved at 60°C on a column of Pro-gel C6-11 and eluted with 10^{-4}M NaOH at 0.5 mL/min.

5.5.(i) Isolation and purification of the polysaccharide.

An authentic culture of *E. coli* O8:K47:H2, culture no. A 282a, was obtained from Dr. I. Ørskov, Copenhagen. The bacteria were propagated in the same manner as for *E. coli* K102 (route A), with the exception that Luria Bertani broth and -agar (Formulae 3 and 4, Annexure A) were used. The acidic capsular polysaccharide was isolated from the neutral O-antigens via its cetyltrimethylammonium salt. The isolated polysaccharide tested positive for the presence of phosphate[188]. An additional purification step, *viz.* passage down a DEAE-Sepharose CL-6B ion-exchange gel with gradient elution (0 - 1.0M NaCl in 0.01M Tris buffer), was performed. Three fractions were collected : a at 0.15M, b at 0.24M and c at 0.80M NaCl in the 0.01M Tris buffer. A small portion of each fraction was hydrolysed (4M TFA, 125°C, 1 hour), and the

hydrolysates tested for phosphate[184]. Only fraction c tested positive for the presence of phosphate. G.l.c. analysis of the acid hydrolysates, a, b and c, showed a to be composed of very little carbohydrate material, b to be the acidic capsular polysaccharide of *E. coli* K47, and c to be composed of galactose only. Fractions a and c were not investigated further. The polysaccharide material from fraction b had an optical rotation of $[\alpha]_D$ +11.7 and a broad molecular weight distribution (g.p.c. column III) with a maximum at M_r 6.5 x 10^5.

5.5.(ii) Monosaccharide composition.

In order to establish the monosaccharide composition of the capsular polysaccharide, the purified acidic polysaccharide (3 mg) was hydrolysed (4M TFA, 125°C, 1 hour), and the liberated sugars analysed as their PAAN derivatives by g.l.c. on column DB-17 at 180°C for 2 min, then 3°/min to 250°C. The results were confirmed by the h.p.l.c. analysis of the hydrolysate on a column of Pro-gel C6-11. To determine the presence of a uronic acid residue, the purified polysaccharide (5 mg)was dried, and then methanolysed (methanolic 3% HCl, 80°C, 16 hours), reduced ($NaBH_4$, 16 hours), hydrolysed (4M TFA, 125°C, 1 hour) and the liberated sugars converted into their PAAN derivatives. The PAAN'S were analysed by g.l.c. on column DB-17 at 180°C for 2 min, then 3°/min to 250°C.

5.5.(iii) Absolute configuration of the monosaccharides.

The absolute configuration of the four monosaccharides in the repeating unit were determined by the g.l.c. analysis of their acetylated (-)-2-octyl glycosides, as described before. Since no uronic acid group was present in the repeating unit, the purified polysaccharide (5 mg) was hydrolysed with 4M TFA (125°C, 1 hour), and the methanolysis- and reduction steps omitted. The acid was removed from the hydrolysate by evaporation under reduced pressure. The hydrolysis reaction causes N-deacetylation of the aminosugar, and the hydrolysate was subsequently re-N-acetylated, following the procedure of Hase and co-workers[202] : Saturated $NaHCO_3$ (200 µL) and acetic anhydride

(8 μL) was added to the dried hydrolysate. The mixture was allowed to stand for 5 minutes after which another aliquot each of $NaHCO_3$ and acetic anhydride was added. The mixture was allowed to stand at room temperature for 30 minutes after which Amberlite IR-120(H)$^+$ resin beads were added to the mixture until a pH of ~3 was reached. The acidic solution was then passed down an Amberlite IR-120(H)$^+$ resin, and the acidic fraction was collected and concentrated. The residue was dissolved in 0.5 mL water and freeze-dried in an ampoule. The sugars were treated with (-)-2-octanol and TFA as before and, after acetylation of the glycosides, were analysed on column DB-225 at 220°C for 5 min, then 1°/min to 235°C and on column DB-17 at 240°C, isothermal.

5.5.(iv) Methylation analysis.

The polysaccharide, in the acid form (15 mg), was methylated using methyl iodide and potassium-dimsyl[87]. This procedure did however not afford complete methylation and the partially methylated polysaccharide was methylated further by the method proposed by Kuhn and co-workers[89]. 30% of the methylated product was hydrolysed (4M TFA, 125°C, 1 hour), and the liberated partially methylated monosaccharides transformed into their alditol acetates (pyridine - acetic anhydride 1 : 1 v/v, 100°C, 1 hour). The partially methylated alditol acetates were analysed by g.l.c. (columns DB-17 and DB-225) and g.l.c.- m.s. The results are tabulated in Table 5.1, Columns I and II.

70% of the methylated product was methanolysed (methanolic 3% HCl, 80°C, 16 hours) and reduced ($NaBH_4$, 16 hours) prior to hydrolysis with 4M TFA (125°C, 1 hour). The liberated partially methylated sugars were subsequently transformed into their alditol acetates, as before, and analysed by g.l.c. (columns DB-17 and DB-225) and g.l.c.- m.s. The results are tabulated in Table 5.1, columns III and IV.

5.5.(v) Degradation by bacteriophage-borne enzymes.

A bacteriophage for *E. coli* K47 was isolated from Grahamstown sewage water and propagated on its host bacteria in Luria Bertani broth. A final titre of ~1.5×10^{13}

pfu/mL was obtained after which the solution was dialysed against running tapwater for 48 hours. The phage solution was assayed[101] again to ensure that the titre had not dropped.

The native polysaccharide (150 mg) was dissolved in the phage solution and degradation was carried out for 96 hours at 37°C in a shaker-waterbath. A small amount of chloroform was added to the solution to prevent bacterial growth. The solution was freeze-dried after which the residue was dissolved in the minimum amount of water and dialysed (~ 3 500 Mw cut off) against distilled water. The dialysates (4 x 80 mL) were collected, pooled and freeze-dried. The dried material was dissolved in ~ 5 mL water and passed down an Amberlite IR-120(H)$^+$ resin (4°C). This procedure was repeated (2x) and the phage-degraded material freeze-dried. The degraded material was applied to a column of Biogel P-4 and eluted with 0.1M sodium acetate buffer, pH 5. Three fractions were collected and passed down Amberlite IR-120(H)$^+$ to remove the buffer. The major fraction was applied to a column of Sephacryl S-200 and eluted with 0.1M sodium acetate buffer, pH 5. One fraction was collected and the buffer removed, as before. The dialysate of the bacteriophage degradation yielded 12.9 mg of the lower molecular weight material which was deuterium-exchanged and subjected to n.m.r. analysis.

The bacteriophage degraded retentate, *viz.* the material retained in the dialysis tubing, was also freeze-dried and the impurities removed by several passages down an Amberlite IR-120(H)$^+$ resin (4°C). A small portion (2 mg) was hydrolysed and, and analysed by h.p.l.c. (column Pro-gel C6-11). The phage-degraded retentate yielded 35 mg of pure material which was deuterium-exchanged and subjected to ^{1}H- and ^{13}C n.m.r. analysis.

5.5.(vi) 2D n.m.r. spectroscopy.

Unless otherwise stated ^{1}H- and ^{13}C n.m.r. spectra were recorded at 303°K on a Bruker AMX 400 spectrometer. Samples were deuterium exchanged and then dissolved in 99.99%

D_2O containing a trace of acetone as internal standard, δ 2.23 for ^1H and 31.07 p.p.m. for ^{13}C.

Homonuclear shift-correlated experiments : The COSY[176] experiment was performed using a spectral width of 2024.29 Hz and a data matrix of 512 x 2048 data points was collected with 48 transients for each delay. The data matrix was zero-filled in the t_1 dimension to 1024 data points and transformed after the application of a non-shifted sine-bell window function in both dimensions and symmetrised. Digital resolution in the resulting matrix was 0.98 Hz/point. The triple quantum phase sensitive COSY[198] experiment was performed using a spectral width of 2040.82 Hz. A data matrix of 256 x 2048 data points was collected with 120 transients for each delay. The matrix was zero-filled in the t_1 dimension and transformed after the application of a shifted sine-square window function in both dimensions and symmetrised. Digital resolution in the resulting 1024 x 2048 data matrix was 0.99 Hz/point.

The homonuclear dipolar-correlated (NOESY)[159] experiment was performed using a spectral width of 2032.52 Hz. An initial data matrix of 512 x 2048 data points was collected with 112 transients for each delay. The matrix was zero-filled to 1024 data points in t_1 and transformed after the application of a shifted sine-square window function in both dimensions and symmetrised. Digital resolution in the resulting matrix was 0.99 Hz/point. The mixing delay in the NOESY experiment was 0.3 s.

Homonuclear Hartmann-Hahn (HOHAHA)[180] spectra were obtained using a spectral width of 2032.52 Hz. The mixing periods consisted of 14 MLEV-17 (28 ms) and 50 MLEV-17 (87 ms) cycles respectively. Data matrices of 512 x 2048 were acquired with 128 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 Fourier transformation to obtain 1024 x 2048 data matrices.

A ^{13}C - ^{1}H shift-correlated (HETCOR)[184] experiment was recorded using a spectral width of 1640 Hz in t_1 and 10204 Hz in t_2. The initial matrix of 74 x 2048 was zero-filled to 256 x 2048 data points and transformed afte the application of a shifted sine-square window function. Digital resolution in f_1 was 6.41 Hz/point and in f_2 4.98 Hz/point. A recycle delay of 0.7 s was employed and 3000 transients per f.i.d. were collected.

6. ANNEXURE A

Formulae for Nutrient Media.

Formula 1 : Mueller-Hinton Agar (g/L, in water)

Meat infusion	5.0
Casein hydrolysate	17.5
Starch	1.5
Agar	14.0

pH = 7.4 (- 0.2)

Formula 2 : Mueller-Hinton Broth (g/L, in water)

Meat infusion	2.0
Casein hydrolysate	17.5
Starch	1.5

pH = 7.4 (- 0.2) at 25°C

Formula 3 : Luria Bertani Broth (g/L, in water)

Bacto-tryptone	10.0
Yeast extract	5.0
NaCl	10.0
Maltose	2.0

Formula 4 : Luria Bertani Agar (g/L, in water)

Bacto-tryptone	10.0
Yeast extract	5.0
NaCl	10.0
Maltose	2.0
Agar	15.02

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