USE OF 2D N.M.R. AND BACTERIOPHAGES IN STRUCTURAL STUDIES OF SOME *E. COLI* ANTIGENS

BY

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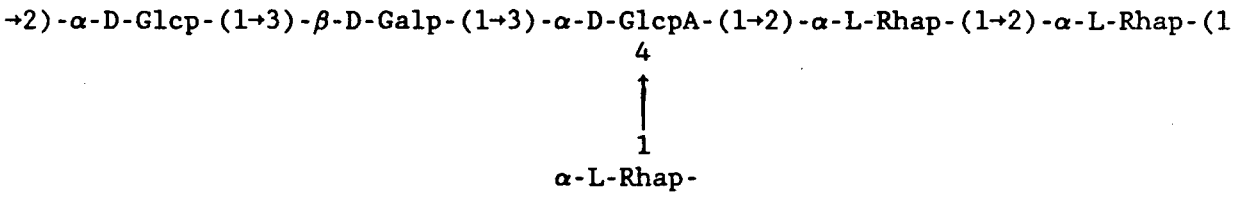
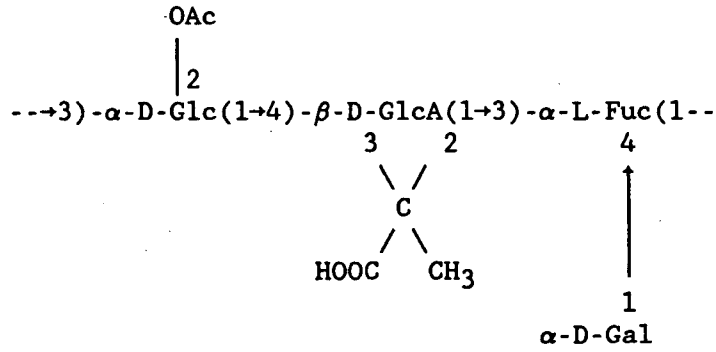
ABSTRACT

The capsular polysaccharide of Escherichia coli K31 has been found by methylation analysis and n.m.r. spectroscopy to be based on the hexasaccharide shown. The sequence of the repeating unit was deduced from the combined results of β-elimination, lithium ethylenediamine degradation, and hydrogen fluoride and selective hydrolyses. The nature of the anomeric linkages, established by chromic acid oxidation, was confirmed by ^{1}H coupled ^{13}C-n.m.r. spectroscopy. Two dimensional n.m.r. studies on a low molecular weight polymer obtained by bacteriophage depolymerization confirmed the structure given below.



The importance of bacteriophage-borne enzymes in the generation of lower polymers very suitable for homonuclear (^{1}H) two dimensional n.m.r. studies is demonstrated.

E. coli K33 has been shown to cross react with Klebsiella K58. Chemical and two-dimensional n.m.r. studies gave the K antigen of E. coli K33 as a tetrasaccharide repeating unit



and this structure is quite identical to that of Klebsiella K58. The use of homonuclear two-dimensional ^{1}H-spin correlated n.m.r. experiment as a very convenient method for the location of acetate in the repeating unit of a polysaccharide is illustrated using E. coli K33 polysaccharide as an example.

The identification of a phosphate ester in the repeating unit of E. coli K46 using n.m.r. spectroscopy is reported. Detailed n.m.r. study on this antigen is well documented in this communication. The first occurrence of a 3,6-dideoxyamino sugar residue in an E. coli K antigen (i.e. E. coli K45 K antigen) and the finger print of its proton chemical shifts is also shown.

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LIST OF ABBREVIATIONS

Glc	=	glucose; Glcp = glucopyranose
Gal	=	galactose; Galf = galactopyranose
Rha	=	rhamnose
GlcA	=	glucuronic acid
GlcNAc	=	2-acetamido-2-deoxyglucose
GalNAc	=	2-acetamido-2-deoxygalactose
PMAA	=	partially methylated alditol acetate
TFA	=	trifluoroacetic acid
DMSO	=	dimethylsulfoxide
Pyr	=	pyruvic acid acetal
Ac	=	acetyl
Me	=	methyl
mol. wt.	=	molecular weight
s	=	seconds; min = minutes; h = hours; d = days
i.r.	=	infra-red
n.m.r.	=	nuclear magnetic resonance
g.l.c.	=	gas-liquid chromatography
HPLC	=	high pressure liquid chromatography
m.s.	=	mass spectrometry
g.l.c.-m.s.	=	gas liquid chromatography - mass spectrometry
l.c.-m.s.	=	liquid chromatography - mass spectrometry
e.i.-m.s.	=	electron impact - mass spectrometry
c.i.-m.s.	=	chemical ionization - mass spectrometry

f.a.b.-m.s.	-	fast atom bombardment - mass spectrometry
ϕ	-	bacteriophage
p.f.u.	-	plaque forming units
r.t.	-	room temperature
a.m.u.	-	atomic mass unit
COSY	-	homonuclear spin correlated n.m.r. spectroscopy
Relay COSY	-	one step relayed spin coherence transfer
Two step relay COSY	-	two step relayed spin coherence transfer
NOESY	-	homonuclear dipolar correlated 2D n.m.r.
n.O.e.	-	nuclear Overhauser effect
HETCOR	-	^{1}H-^{13}C heteronuclear chemical shift correlated spectroscopy
F.t.	-	Fourier transform

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I. INTRODUCTION

Carbohydrates are polyhydroxyaldehydes, ketones (usually in the acetal or hemiacetal forms) or substances which may be hydrolyzed by dilute acid to these compounds. Carbohydrate-containing macromolecules are of widespread occurrence in most living organisms.[1] In nature, carbohydrates are often linked to other molecules such as proteins and lipids to form: (i) glycoproteins, proteoglycans and peptidoglycans, (ii) glycolipids and lipopolysaccharides, (iii) teichoic acids, and (iv) nucleic acids. Polysaccharides are carbohydrate polymers but may not be confined solely to O-glycosidically linked carbohydrates.

Polysaccharides are of widespread occurrence in nature and have significant commercial and biological importance. They have uses in numerous industries[2] ranging from foods, pharmaceuticals, textiles, papers, paints, cosmetics, and tertiary oil recovery to the treatment of environmental pollutants. The utility of polysaccharides in these industries is due to their gelling, thickening, emulsifying, binding, coating, and film forming properties. The biological importance of polysaccharides is threefold: architectural, nutritional and as specific agents. Their importance is shown in many facets of human life where they are components of antigens, enzymes, nucleic acids, and cell surface glycoconjugates.[3,4] Polysaccharides are important in the plant world, in which cellulose is the principal structural component and starch serves as the main energy bank. In insects and crustacea, chitin provides skeletal support. Carbohydrates also serve as a renewable

source of carbon, for energy storage and as a readily manipulated natural source of chirality.

I.1 Immunological importance of bacterial exopolysaccharides

The role of polysaccharides in immunology dates from 1917 with the report by Dochez and Avery[5] of a "specific soluble substance" secreted by pneumococci during growth. These "specific soluble substances" were shown to be polysaccharides and in fact this was the first time that any material other than protein had been shown to be antigenic. The protective effect exhibited by antibodies raised to pneumococcal polysaccharides against pneumococcal infections provided proof of the importance of these substances in immunology.[6,7] It was demonstrated that a relatively small portion of the polysaccharide is the major site of antibody specificity. The presence of the same determinant group in several polysaccharides was shown to be responsible for their serological cross-reactivity; that is, the capacity of a polysaccharide from one species of bacterium to precipitate the polysaccharide-specific antibodies of another. Heidelberger has illustrated the use of cross-reactions in immunological analysis of polysaccharides.[8] In his study he was able to predict the presence of structural features before they were determined. The use of polysaccharides as antigens and immunogens has contributed greatly to the classification and identification of bacteria, to a better understanding of the immune response, to the definition of the active site in antigen-antibody interactions and to

the detection and prevention of human disease caused by invasive organisms. More recently nuclear magnetic resonance spectroscopy has been applied to study the conformation of antigens during their interaction with antibodies. This chapter is not intended to be exhaustive but to provide a general overview of structural studies of polysaccharides.

Exopolysaccharide is a generic name for all forms of bacterial polysaccharides found outside the cell wall. Most bacteria, either Gram-positive or Gram-negative produce extracellular polysaccharide[9] (see Fig. I.1.1). Structure elucidation has revealed that most bacterial polysaccharides are composed of oligosaccharide repeating units.[10] Relatively little is known about the function of microbial exopolysaccharides in contrast to the extensive data on their primary chemical structures. It has been proposed[11] that they may be involved in one or all of the following:

(i) energy storage or reserve

(ii) virulence, protection against phagocytosis

(iii) protection against desiccation and predators

(iv) adhesion, and

(v) as a general barrier

Exopolysaccharides are the outermost mediators between the organism and its environment, the first portal of entry and the last barrier to excretion. The bacterial surface thus contains components which play an important role in recognition processes.[12] The cell envelope (comprising the outer membrane and capsule) contains antigens which induce the formation of antibodies in man and animals, and which react serologi-

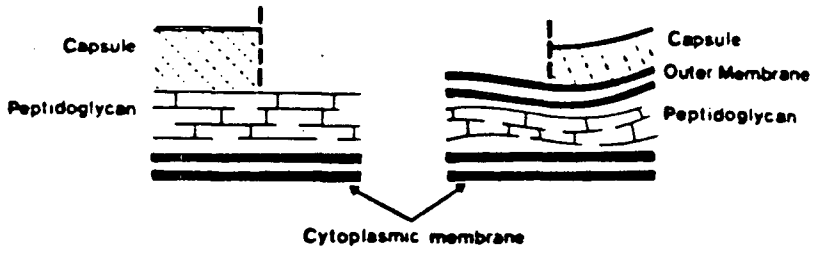


Fig. I.1.I: Diagrammatic representation of the cell surface of Gram-positive and Gram-negative bacteria

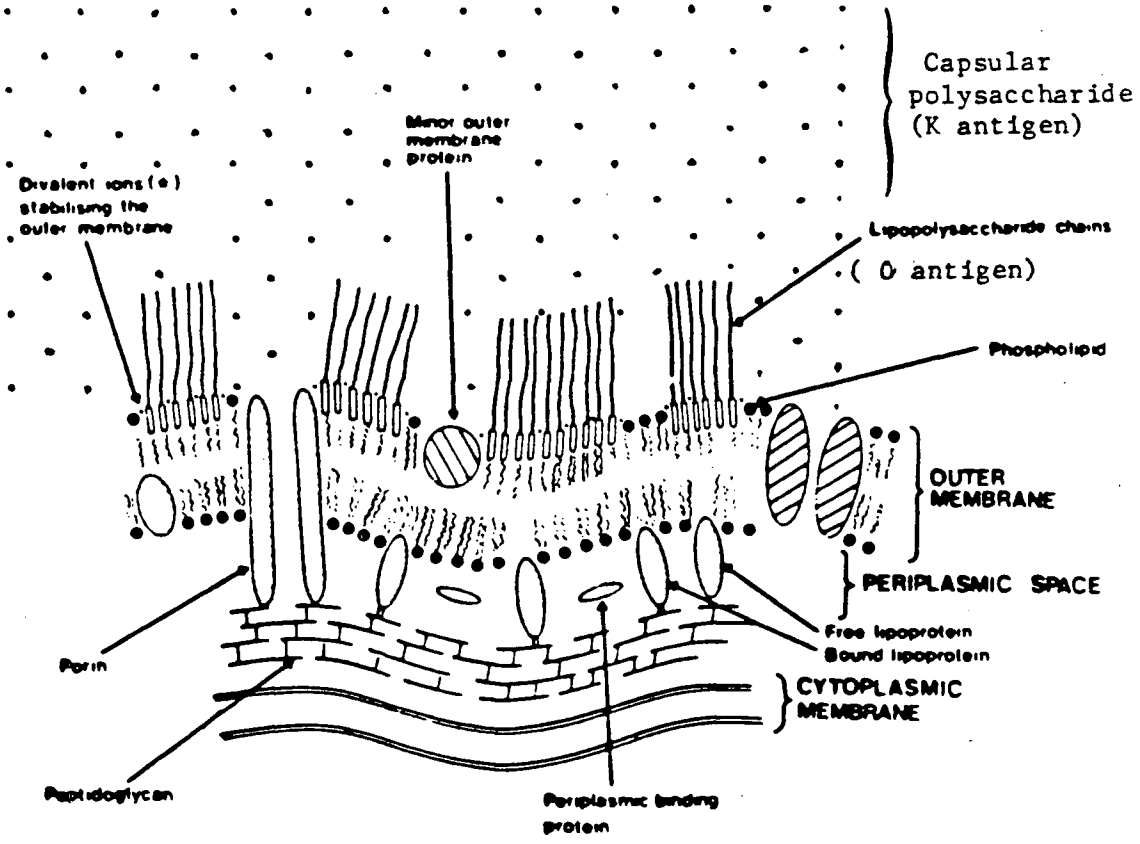


Fig. I.1.II: Diagrammatic representation of the cell surface envelope of Gram-negative bacteria

cally with these antibodies.[13]

The immune system includes all the structures and processes that provide a defense against potential pathogens. These defenses can be grouped into non-specific and specific categories. Non-specific defense mechanisms include barriers to penetration of the body and internal defense:

(a) Phagocytic cells engulf invading pathogens

(b) Interferons are polypeptides secreted by cells infected with viruses that help protect other cells from viral infections

Specific immune responses are directed against specific antibodies and are a function of lymphocytes. B lymphocytes secrete antibodies and provide humoral immunity. Killer T lymphocytes, acting through the secretion of lymphokines, provide cell-mediated immunity (i.e. they come into contact or close proximity to secrete lymphokines in order to kill victim cells).

The structure of antibodies allows them to combine with antigens in a specific fashion and thereby activate other elements of the immune system to protect against pathogens. The aim of the immunochemical analysis of polysaccharide antigens which combines serological and chemical studies is to define antigenic determinants (or immunodominant sugars) within the polysaccharide as chemical expression of its immunological character. These immunodominant residues, which form the major contributors to serological specificity, can be a monosaccharide linked in a specific manner, an oligosaccharide or even non-carbohydrate in nature, e.g. acetal-linked pyruvic acid, or O and N-acetyl groups.[14] In acidic capsular polysaccharides such as those of Klebsiella, E. coli,

and Pneumococcus, the charged constituents are often part of the antigenic determinants.[13] In branched polysaccharides, the immunodominant residues may be located in the side chain. Bacterial polysaccharides have the same antigenic determinants expressed many times over due to their repetitive structure. Molecular weight and conformation are also critical parameters in the antigenicity of a polysaccharide. The serogroup-specific polysaccharides of Neisseria meningitis (at molecular weights of 50,000-130,000) are good immunogens in man, but the response is much weaker with preparations that have a molecular weight of 30,000.[15,16] The accumulated evidence[15-18] suggests that 45,000 ± 5,000 is the molecular weight above which polysaccharides are immunogenic and below which their immunogenicity falls off rapidly.

Immunization using vaccines, has been utilized by man for a long time, for disease prevention. Vaccines derived from killed bacteria (or virus) preparations, live "attenuated" bacteria and toxoid are common and still effective. The first use of bacterial polysaccharides as a vaccine to combat pneumococcal infection was tested on U.S. Army recruits during World war II. The post World war II era saw renewed interest in prevention of diseases by immunization, due to widespread and multiple antibiotic resistance. The serious problem in developing polysaccharide vaccines is the inability of these antigens (vaccines) to develop protective levels of serum antibodies in infants and young children (i.e. they give rise to only IgM antibodies).[20,21] This difference in immune response is thought to be due to the T-cell independence of polysaccharide antigens, the participation of T cells being essential for the induction of IgG antibodies and memory cells.[21]

There have been several attempts to overcome this problem by conjugating polysaccharides to an antigenic protein in order to form a T-cell-dependent antigen.[23-25] The potential use of microbial polysaccharides in cancer research has been reported by Whistler et al.[26]

The structural studies of bacterial polysaccharides are very important in the light of bacterial infections, the production of protective vaccines and their potential as noncytotoxic antitumour drugs. To further the understanding of the chemical basis for serological differentiation, a knowledge of the primary structures of bacterial polysaccharides is required. It also forms the basis for the understanding of their three dimensional structures in the solid state and in solution and for an appreciation of the ways in which polysaccharides are biosynthesized and degraded. With these and latent potential applications of bacterial polysaccharides in mind, the task of elucidating the detailed chemical structure of seventy-four E. coli capsular polysaccharides is currently being undertaken in this and other laboratories. However, in this thesis, the emphasis is on the use of bacteriophage born enzymes and two dimensional nuclear magnetic resonance spectroscopy as a probe for investigating the structures of capsular polysaccharides, from E. coli serotypes K31, K33, K44 and K46.

I.2 Chemistry and serology of E. coli capsular polysaccharides

The organism Escherichia coli belongs to the family Enterobacteriaceae whose normal habitat is the intestinal tract of man and animals.[27]

E. coli, first isolated from faeces by Escherich in 1885, is often found in human urinary tract infections and is associated with severe infantile diarrhea.[28] Enterobacteriaceae are Gram-negative bacteria and classification of the family (Enterobacteriaceae) by Edwards and Ewing[29] (shown in Table I.1) has been updated by Kauffman.[30] An interest in Escherichia coli in recent years from both human and veterinary medicine has been followed by an interest in the surface structure of these bacteria because of their special role in pathophysiological processes, their usefulness in epidemiological studies and their importance for the normal immunological status of the host.

Within the bacterial species of E. coli there are many serotypes, each of which produces different extracellular polysaccharides. The serotyping scheme is based on the cell surface antigens (see Fig. I.1.II). These comprise the capsular or K antigens (74 polysaccharide types), the somatic O antigens (164 lipopolysaccharide types) and the flagellar H antigens (56 proteinaceous types).[30] The O antigen is the O-specific polysaccharide of the cell wall lipopolysaccharide. It is a thermostable surface antigen (the bacteria keep their immunogenic, agglutinating and agglutinin-binding capacity after boiling). The structure and known properties of some bacterial lipopolysaccharides have been reported.[13,14,31] Most E. coli strains have a unique K antigen. The K antigens are capsular and envelope antigens and all are polysaccharides except for two that are proteins (K88 and K99). These polysaccharides are made up of oligosaccharide repeating units, varying in size from one to seven sugar residues.[32] Common monosaccharides that have been reported in E. coli polysaccharides are D-hexopyranoses

(galactose, glucose, and mannose); D-pyranosyluronic acids (galacturonic and glucuronic acids); L-deoxyhexoses (fucopyranose and rhamnopyranose); 3-deoxy-D-manno-octulosonic acid (KDO) and 5-acetamino-3,5-dideoxy-D-glycero-D-galacto-nonulopyranosonic acid (NeuNAc). Immunodominant non carbohydrate substituents that occur in E. coli polysaccharides include N and O-acetyl, phosphate, and 1-carboxyethylidene (acetal-linked pyruvic acid) groups. In their review on bacterial polysaccharides Kenne and Lindberg[10] listed the proposed structures of E. coli K antigens known up until 1982. An updated list of the proposed structures for the E. coli K and O antigen has been compiled in Appendix IV. The K antigens consist of three groups (A, B and L). By electrophoretic means two groups of K antigens can be differentiated; those with high electrophoretic mobility (L antigen) and those with very low electrophoretic mobility (A and B antigens). Inspection by electron microscopy revealed that the acidic polysaccharides with low molecular weight (high electrophoretic mobility) form thin, patchy capsules while those with high molecular weight (low electrophoretic mobility) form thick and copious capsules. It has been shown that E. coli strains with O and K antigens exhibiting the same immunoelectrophoretic pattern could cause the same disease.[32]

In four cases, the chemical structure of the capsular polysaccharides of E. coli and Klebsiella were found to be identical. They are E. coli K30 and Klebsiella K20,[33] E. coli K32 and Klebsiella K55, E. coli K33 and Klebsiella K58 and E. coli K42 and Klebsiella K63.[33] Recently, the cross-reaction of the K and O antigens of E. coli with the antisera from othe micro-organisms were reported.[34-36]

I.3 Bacteriophages

Exopolysaccharases from bacteria[37] and bacteriophage-associated endoglycanases[38-40] are two main sources of enzymes that hydrolyze bacterial capsular polysaccharides. The former are isolated in low yields. Bacteriophages may be obtained in high yield and hence be used to a great advantage in our and other laboratories for the structural elucidation of bacterial capsular polysaccharides.

Bacteriophages are viruses that infect bacteria, multiply within them and eventually kill them.[41-43] Bacteriophages are classified by Bradley into morphological groups[44] (see Fig. I.3.I). The types A-C contain two strands of DNA, while type D has one. These four types are unique to phages. Types E and F contain a strand of RNA and a strand of DNA respectively. In a study on phages active on the capsular polysaccharides of the genus Klebsiella Rieger-Hug and Stirm reported that thirty of these phages belong to the Bradley type C, twelve to type B and three to type A. E. coli viruses are however known to belong to all six Bradley morphological types and these coliphage have been studied.[43]

Bacteriophages are specific regarding the species of bacteria they will infect although some have a broad or less restricted host range. This specificity depends on the presence of a specific receptor site on the cell surface. Protease can be used to demonstrate the bacteriophage receptor site for E. coli strains.[45] The viral infection of a host by bacteriophages is usually characterized by four phases (see Fig. I.3.III). These are:

(i) adsorption of the phage particle onto the susceptible host

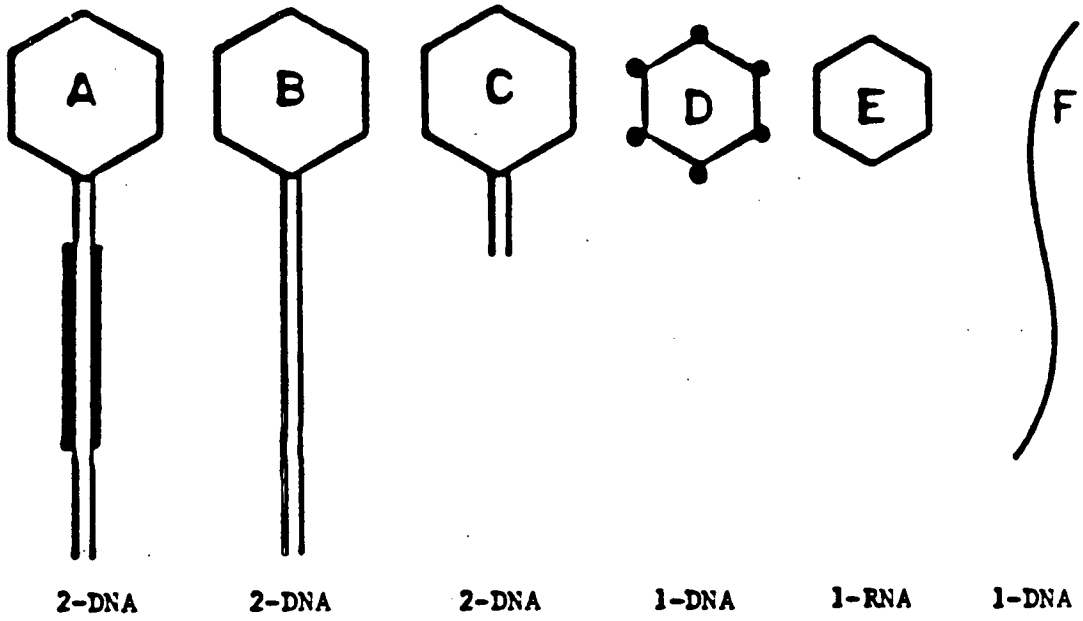


Fig. I.3.1: Basic morphological types of bacteriophage with the types of nucleic acid

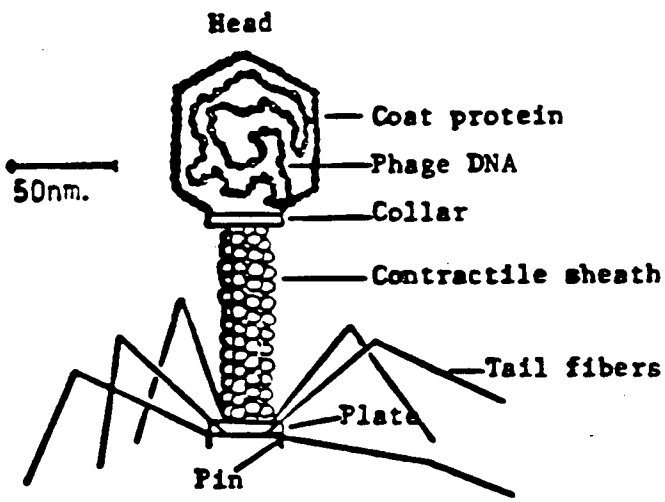


Fig. I.3.II: A diagrammatic illustration of a bacteriophage

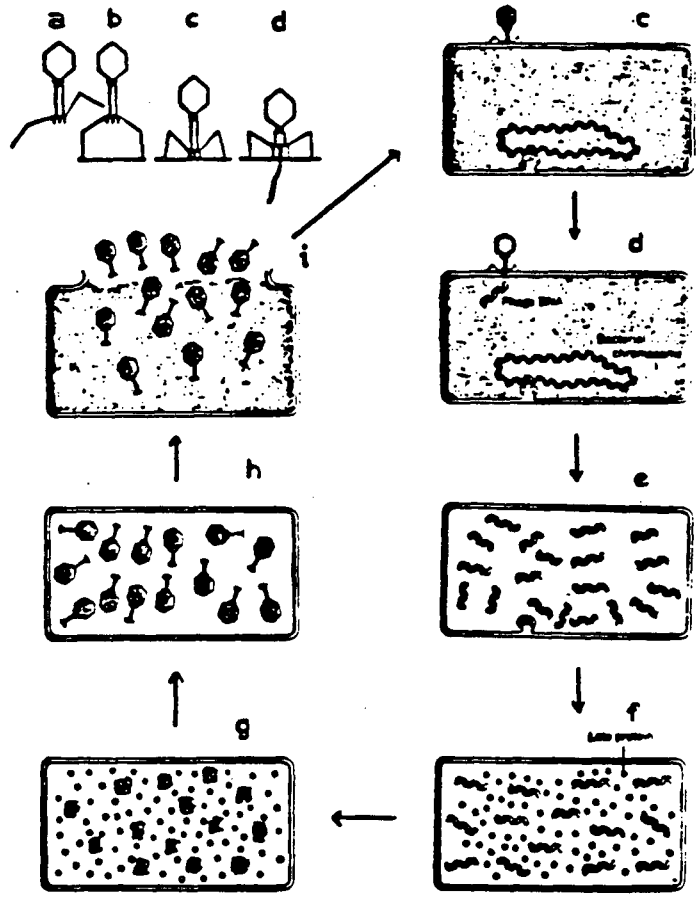


Fig. I.3.III: A schematic diagram illustrating the steps in the infection of a bacterium by a bacteriophage

(ii) injection of the viral DNA (or RNA) into the host

(iii) replication of the phage nucleic acid and phage protein at the expense of the metabolic process of the host

(iv) phage maturation and release which results in the lysis of the host cell

The presence of a phage on its host bacterial lawn can be detected by its characteristic plaque morphology, i.e. the plaque proper (clear spot with lyzed cells) is surrounded by a halo (translucent spot) in which the bacterial growth is decapsulated. The halo formation is due to the production of excess free spikes, which have been shown to contain the capsule depolymerase, during the biosynthesis of progeny virus in the host cell. These spikes diffuse from the plaque and catalyze the hydrolysis of the surrounding bacterial capsules.[46]

Bacteriophage-borne glycanases[47] may be hydrolases[48] or lyases.[49,50] These enzymes (glycanases) which occur in bacteriophages are generally specific for one or a few substrates. Kinetic studies on the bacteriophage-borne enzymes revealed that the activity is inhibited by products and high substrate concentrations.[40] Depolymerization of bacterial capsular polysaccharides by the use of phage-borne enzymes allows:

(i) the generation of selectively cleaved oligosaccharides (in high yields) corresponding to one or more repeating units.[39]

(ii) acid or base labile non-carbohydrate[51] groups (e.g. O-acetyl and acetal-linked pyruvic acid) to remain intact on the oligosaccharide repeating unit. This is difficult to achieve using chemical methods of degradation.

Bacteriophage generated oligosaccharide may be subsequently used (i) for the verification of the structures of the original polysaccharides; (ii) as substrates for mass-spectrometry and X-ray diffraction studies; (iii) in the study of conformation in solution;[53] (iv) as a source of complex and novel oligosaccharides and (v) for coupling as haptens to

immunoglobins[52] for immunological studies.

In this study bacteriophage borne enzymes were employed in generating low molecular weight polymers from E. coli capsular polysaccharides. This enables the preparation of concentrated solutions which have reasonable nuclear Overhauser effect (n.O.e.). Increasing sample concentration enhances the signal to noise ratio (i.e. sensitivity) during nuclear magnetic resonance spectroscopic studies.

I.4 Nuclear magnetic resonance spectroscopy

The first n.m.r. experiments were carried out[54,55] in 1945, but useful chemical applications became possible only after the discovery of the chemical shift effect[56,57] in 1949. Today the subject has expanded so that it is of equal importance with the old established branches of spectroscopy [e.g. vibrational (infrared) and electronic (ultraviolet)].

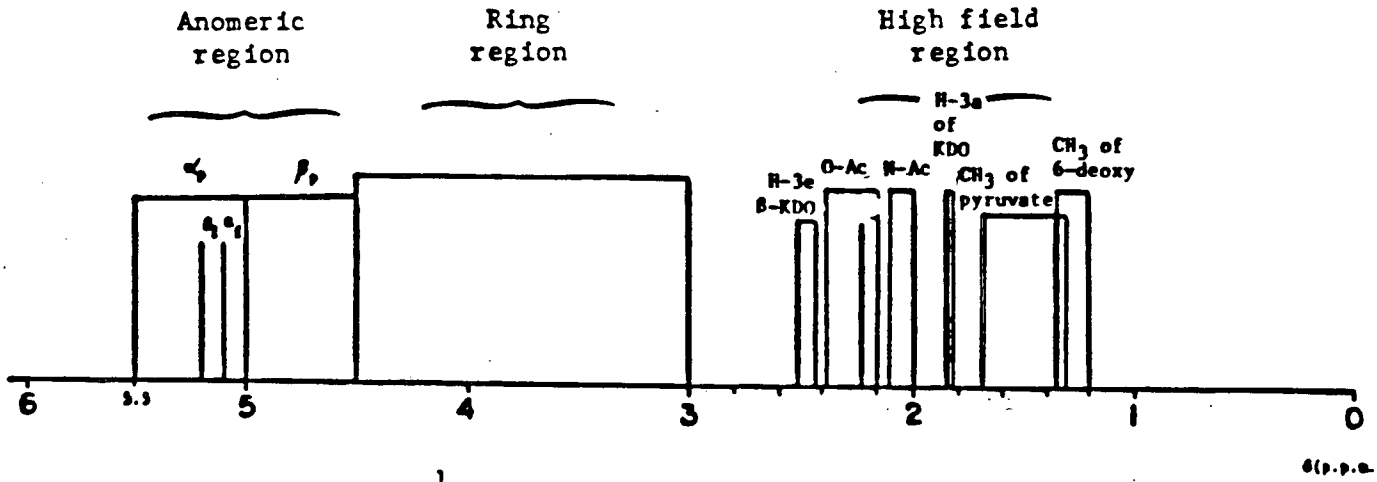
^{1}H, ^{13}C, ^{19}F, ^{31}P and ^{15}N nuclei have a spin quantum number (I) of 1/2 and because of their nuclear magnetic moment precess in an applied magnetic field. The spin of a nucleus aligns itself with one of two possible orientations with respect to the magnetic field. The parallel spin [i.e. parallel to direction of external magnetic field (B_o)] is more stable (lower energy) than the anti-parallel configuration or alignment. At resonance with an applied radio frequency field, the magnetic moment of the spins parallel to the magnetic field absorbs energy and flips to the higher-energy antiparallel spin state. The amount of energy required to execute this flip is dependent on the magnetic field

strength and the environment (i.e. electronic shielding). The induced magnetic field due to circulation of an electronic charge cloud opposes the primary applied field (B_o). Thus electrons will shield nuclei from the influence of the field B_o. This shielding can be taken into account by using an effective field B at the nucleus, given by $B = B_o (1-\sigma)$. The dimensionless number σ is a small fraction, usually listed in parts per million and is known as the shielding constant.

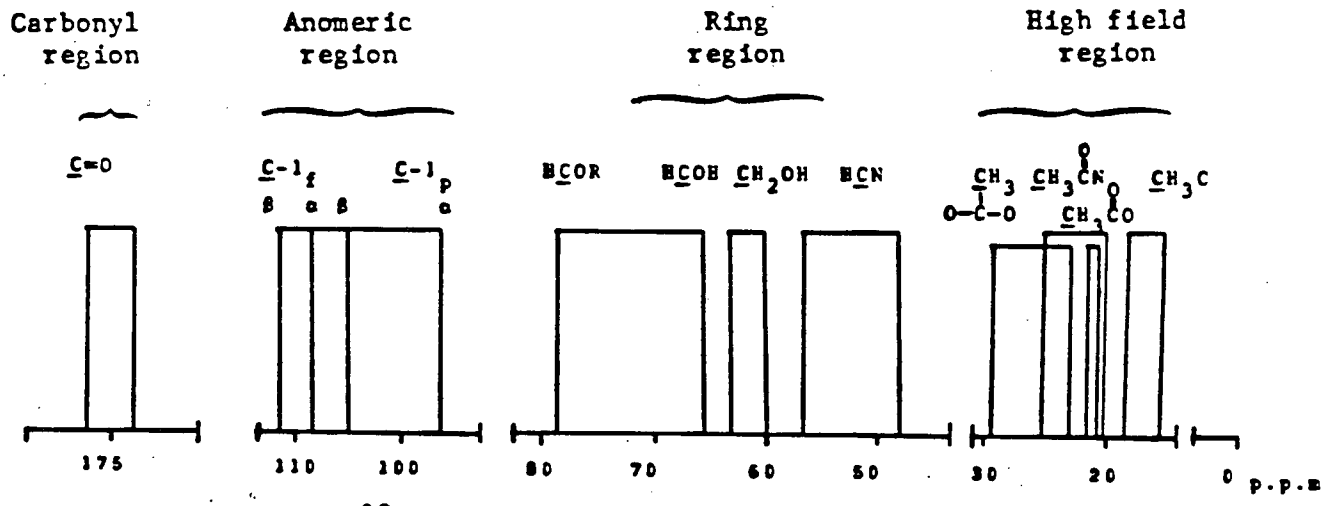
(i) Chemical shift

The phenomenon of chemical shift arises because of shielding (screening) of the nuclei from the external magnetic field by the electrons, discussed in the previous section. Since the shielding effect is caused by the electronic environment, values of σ will vary with the position of the nucleus in the molecule. The resonance condition is $\nu = |\gamma/2\pi|B_o(1-\sigma)$ where ν and σ are the Larmor (resonance) frequency and shielding constant respectively. The chemical shift (δ) is the difference between the Larmor frequency of the nucleus and that of a reference compound (e.g. TMS). The chemical shift is expressed in parts per million of the applied field and is intimately related to several effects such as orientation, electronegativity, aromatic ring effect and anisotropic effect.

The n.m.r. spectrum of a carbohydrate can be divided into three regions for a ^{1}H spectrum and four for a ^{13}C spectrum, i.e. the high field region, the ring region, the anomeric region and lastly (only for ^{13}C spectra), the carbonyl group region (see Fig. I.4.I).



^{1}H – n.m.r. spectrum



^{13}C – n.m.r. spectrum

Fig. I.4.I: Different regions in the n.m.r. (^{1}H- and ^{13}C) spectra of polysaccharides

In the high field region (between δ 1.0-2.5 in the ^{1}H-n.m.r. spectrum and δ 15-30 in the ^{13}C spectrum), the methyl resonances of acetal-linked pyruvic acid, N- and O-acetyl groups and 6-deoxy-hexoses (e.g. L-rhamnose and L-fucose) can be detected. It has been shown that the stereochemistry (R or S) of the acetalic carbon of the pyruvic acid can be differentiated by the chemical shift of the methyl groups.[58]

In the ring region, the ^{1}H-n.m.r. spectrum, (δ 3.0-4.5) is not well resolved and therefore assignments are difficult. Using 2D n.m.r. experiments[59-62] chemical shift assignment in the ring region can be simplified. In contrast to ^{1}H-n.m.r. spectra, the ring region (δ 60-85) of ^{13}C is more resolved due to a larger sweep width. O-Glycosylation and/or O-alkylation results in the carbon atom(s) involved being deshielded (by 7-11 ppm) so as to produce signals well separated from other ring carbons (δ 80 ± 5). This is called the "α-effect". However a carbon atom immediately adjacent to that carbon will be slightly shielded (1-2 ppm), and this is the "β-effect". These α and β-shifts (which are different from α and β-anomeric signals) have been used in the assignment of ^{13}C signals of oligo- and polysaccharides and consequently delineating the structural sequence.[63]

The anomeric region of ^{1}H-n.m.r. spectra is δ 4.5-5.8 and that for ^{13}C is δ 93-110. Contrary to ^{1}H-n.m.r. the α anomeric carbons appear upfield from the β-anomeric carbons due to a shielding effect. It has been found that increased shielding of a ^{13}C nucleus is accompanied by a decrease in the shielding of the appended proton, i.e. ^{13}C and ^{1}H shift are affected inversely.[63] In the n.m.r. (^{13}C and ^{1}H) spectra of an oligo or polysaccharide, the number of anomeric signals and their correspond-

ing integrals indicate the number of sugar residues per repeating unit; furthermore the linkage configuration (α and β) can be determined from the combined measurements of the chemical shift and coupling constant. Signals for some non-anomeric protons have been reported to occur in the anomeric region[64,65] but this ambiguity can be resolved by 2D homonuclear spin correlated n.m.r. experiments.[59,60]

For ^{13}C spectra, any signal in the extreme downfield region of δ 170-180 indicates the presence of a carbonyl group which could be associated with N or O-acyl group, pyruvic acid or uronic acid.

(ii) The relative area or integral of individual signals

The relative intensities of absorption signals for different nuclei are equal to the relative number of the nuclei producing the signals.[66] The number of anomeric linkages, relative amounts of 6-deoxy sugars, O-acetyl, N-acetyl and 1-carboxyethylidene substituents can be determined by computing the integrals of their corresponding signals. For oligo or poly-saccharides this parameter permits a rapid quantitative analysis of the ratio of α and β linkages. However, quantitation based on signal integration is often not reliable in the proton-decoupled ^{13}C spectrum due to saturation and n.O.e. effects. Nevertheless, comparison of integrals of ^{13}C nuclei with the same number of hydrogen atoms often yields accurate information about their relative amounts.

(iii) Coupling

Spin-spin coupling is a scalar coupling which is administered through bonds. In the simplest cases (referred to as first-order spectra) these features occur as splitting of the resonance signals due to each coupling nucleus. If there are only two nuclei with non-zero spin in a molecule under consideration, having spins I_1 and I_2, then it is found that the resonance of spin I_1 is split into $2I_2 + 1$ lines of equal intensity and that of spin I_2 is similarly split into $2I_1 + 1$ lines. From spin-spin coupling patterns structural information on nuclear environment may be obtained. The magnitude of the spin-spin coupling is given by a spin-spin coupling constant (usually simply referred to as a coupling constant and written J_{jk} for interaction between spins j and k). The variation of the spectrometer operating frequency does not affect the magnitude of spectral splitting (i.e. coupling constant) as such coupling constants are always expressed in Hertz (Hz) and never in ppm. In a first order spectrum, the magnitude of the coupling constants can be measured directly from the spectrum.

Coupling constants can also be predicted using the Karplus[67] quation and these values are usually in good agreement with observed values. However, deviations occur when substituents are of different electronegativity.[68] The Karplus equation gives an approximate relationship between the three-bond vicinal coupling constant (^3J) and the dihedral angle (ϕ) between the protons.

$$^3J\ (H1, H2) = \begin{cases} 8.5\cos^2\phi - 0.28 & 0° \leq \phi \leq 90° \\ \text{or} \\ 9.5\cos^2\phi - 0.28 & 90° \leq \phi \leq 180° \end{cases}$$

The values are maximum when the dihedral angle (ϕ) is 0° or 180°, and minimum when it is 90°. Although the coupling constant is influenced by other parameters (such as electronegativity, angle strain and bond length), it is useful for the assignment of trans-diaxial protons (ϕ = 180°, β-linked, $^{3}J_{1,2}$ 7-9 Hz) and equatorial-axial or equatorial-equatorial protons (ϕ = 60, α-linked, $^{3}J_{1,2}$ 1-3 Hz). These ^{3}J-values can be applied to hexopyranoses such as D-galactose, D-glucose, D-glucuronic acid and 2-acetamido-2-deoxyhexopyranoses (e.g. GlcNAc and GalNAc). Due to the equatorial proton at C-2, L-rhamnose and D-mannose have a different set of ^{3}J values (i.e. α-anomer $^{3}J_{1,2}$ = 2 Hz, β-anomer $^{3}J_{1,2}$ = 1 Hz). The J constants of anomeric protons are thus useful in predicting the anomeric configuration (α and β) and the overall conformation (pyranose/furanose and chair/boat forms) of the monosaccharides.

Most of the ^{13}C spectra presented in this thesis were recorded in proton-decoupled mode. However, ^{13}C spectra may also be correlated with the proton spectra to provide information on ^{13}C-^{1}H coupling constants ($^{1}J_{C1,H1}$). This is usually performed by the 'gated' decoupling or by the single frequency off resonance decoupling technique. The $^{1}J_{C1,H1}$ value is useful in differentiating anomeric pairs in the pyranose form, since they differ by 10 Hz,[68,69] e.g. $^{1}J_{C1,H1}$ for a β-rhamnoside is ~160 Hz and $^{1}J_{C1,H1}$ for an α-rhamnoside is ~169 Hz. In the structural studies on the E. coli K31 polysaccharide the anomeric configuration of the six sugar residues in the repeating unit were confirmed by their $^{1}J_{C1,H1}$ values. Recently Bock and Pedersen reported the determination of $^{1}J_{C1,H1}$ values of some carbohydrate samples through the measurements of ^{13}C satellite signals in ^{1}H-n.m.r. spectra (500 MHz).[70]

II. METHODOLOGY

E. coli capsular polysaccharides have complex and immensely diversified structural patterns[10] the structural elucidation of which necessitates the use of different chemical methods as well as spectroscopic techniques. Since the structure of these polysaccharide immunogens is intimately linked to their effectiveness as vaccines, the development of a technique for rapid structural determination would be invaluable. In this thesis, it has been demonstrated that bacteriophage borne enzymes and two dimensional nuclear magnetic resonance (2D-n.m.r.) spectroscopy are powerful tools in such an investigation. The methodology of the two dimensional n.m.r. techniques employed in this study is illustrated in this chapter. The structure of the E. coli polysaccharides determined by 2D-n.m.r. were first elucidated by chemical and mass spectrometric techniques. An overview of these techniques is also presented in this chapter.

II.1 Pulse Fourier transform nuclear magnetic resonance spectroscopy (F.T.-n.m.r.)

N.m.r. is quite insensitive compared to optical spectroscopy (e.g. UV or IR) because of the very small population excess in the lower energy state. One way to improve the sensitivity is by signal averaging in which case the rate of scan of the spectral width is very important.

In F.t.-n.m.r. the rate of scan of the spectral width far super-

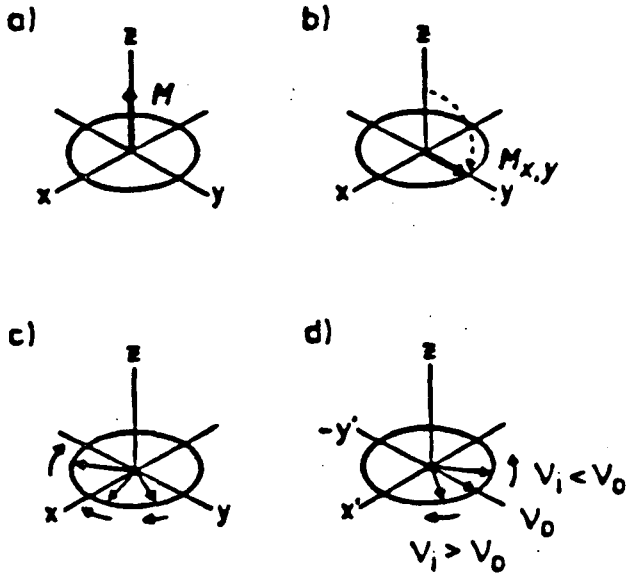
cedes that of continuous wave (CW) n.m.r. hence more experiments can be accomplished using the F.t.-n.m.r. technique. The introduction of the F.t. method has not only enhanced the sensitivity of high resolution n.m.r. spectroscopy, i.e. allowing measurements to be made on less sensitive nuclei of the periodic table, but also has paved the way for the development of a large number of new experimental techniques. The use of programmable pulse transmitters and the separation of the experiment into preparation, evolution and detection have made new n.m.r. experiments possible. In particular the concept of two-dimensional (2D) spectroscopy has opened up new possibilities important for the analysis of complicated spectra and is able to provide information not otherwise accessible.

In structural studies of carbohydrates, one dimensional (1D) n.m.r. is mostly used as a backup procedure to chemical methods. The ring proton region (~4.5 ppm to ~3.5 ppm) of 1D-n.m.r. spectra is quite complicated but may be simplified using 2D-n.m.r. spectroscopy.[118-120,149] E. coli polysaccharides are immunogens and quick structural elucidation of these immunogens may be necessary. The use of 2D-n.m.r. spectroscopy is suitable for this purpose. The use of 2D-n.m.r. in the structural studies of the capsular polysaccharides from the E. coli serotypes K31, K33, and K46 is demonstrated in this study. An overview of the n.m.r. methods used is presented in this chapter. Since a rigorous mathematical treatment is complicated and does not necessarily improve the comprehensibility, the chapter attempts to give an illustrative presentation of these techniques within the framework of the Bloch vector model.

III.1.1 The FT-n.m.r. experiment[150,151,152]

The basic principle of the Fourier transform n.m.r. experiment is recalled in this section.[150,151,152] For the nucleus of interest, the resonance signals of different Larmor frequency present in the spectral window chosen form the so-called macroscopic magnetization (M) of magnitude Mo, parallel to the external field B_o (Fig. II.1a). The strong radio frequency (RF) pulse[151] produced by a radio frequency coil on the X-axis carries M away from the Z-axis to the X, Y plane. The duration and the power of the RF pulse determine the direction of M after the pulse (with the power of the RF sources used in modern spectrometers this process requires 5-20 μs). If a so-called $90°_x$ or $\pi/2$ pulse is applied, M points along the positive Y-axis (Fig. II.1.b). The longitudinal or Z-magnetization is thus transformed into a transverse magnetization.

Within the framework of the classical macroscopic description of experiments, not only is the macroscopic magnetization M a sum of components but also each individual vector V_i is the vector sum of the various nuclear magnetic moments that have the same chemical environment and therefore precess with the same Larmor frequency. The Larmor frequencies of the various nuclear magnetic moments present vary and thus, the vector M splits into its components (Fig. II.1c). The concept of the "rotating frame"[53] is very convenient when describing an F.t.-n.m.r. experiment. It uses a coordinate system K^1 that rotates in the same sense and with the same frequency ν_o as the rotating field vector of the RF field. In the rotating frame, vectors that correspond



a) Macroscopic magnetization M in the laboratory frame; b) transverse magnetization $M_{x,y}$ after a $90°_x$ pulse; the effect of pulses is described by the "right-hand rule", where the thumb gives the direction of the pulse and the bent fingers the rotational sense of the magnetization vector; c) Larmor precession of the individual nuclear magnetic moments of different Larmor frequency in the laboratory frame; d) as c), however, in the rotating frame K'(x',y',z) (taken from R. Benn and H. Gunter's article in Angew. Chem. Int. Ed. Engl., 22, 350-380, 1983)

e)

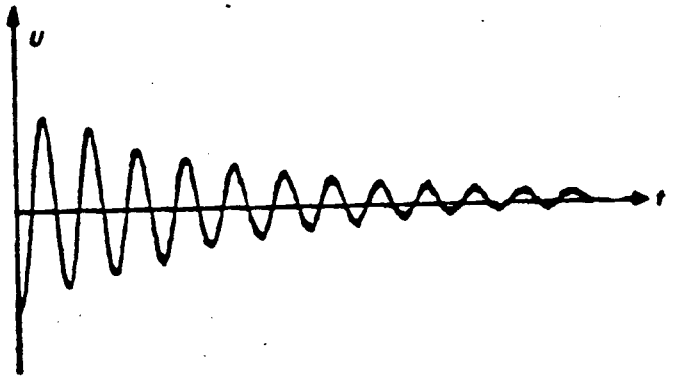


Fig. II.1: e) Free induction decay (FID) or time signal of a n.m.r. line. The damped sine wave is characterized by the time constant T_2^* and the frequency ν_i T_2^* is the effective transverse relaxation time that contains contributions from transverse relaxation and from field inhomogeneity (taken from R. Benn and H. Gunter's article in Angew. Chem. Int. Ed. Engl., 22, 350-380, 1983)

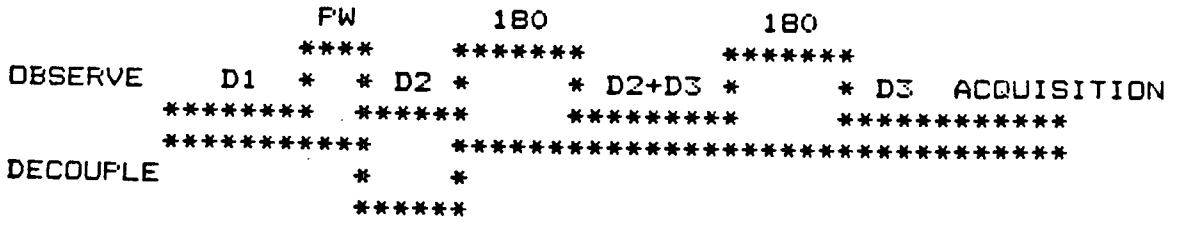
to signals with frequencies $\nu_i > \nu_o$ rotate clockwise, whereas those corresponding to signals with $\nu_i < \nu_o$ rotate anti-clockwise, a signal with $\nu_i = \nu_o$ is static in the rotating frame (Fig. III.1d). It is helpful to remember that each vector is characterized by its Larmor frequency, by its orientation in the rotating frame and by its life time. The Larmor frequency determines the position of the signal in the spectrum (i.e. chemical shift) whereas the orientation at the beginning of data accumulation determines the phase with respect to the rotating frame and hence signal phase.

The magnetic vector rotating in the X-Y plane produces a voltage in the receiver coil that is detected as the n.m.r. signal. The loss of transverse magnetization is governed by transverse relaxation processes as well as field inhomogeneity. A plot of the resultant damped oscillation with decay time is the free induced decay (FID) (Fig. II.1c). The induced voltage is converted to numerical form by analogue-to-digital converters. Fourier transform of this time domain data yields the well known n.m.r. signal or spectrum.

II.1.2 ^{13}C-n.m.r. spectroscopy via spin echo experiments. (Attached proton test)

The modulation of transverse magnetization through spin-spin coupling[176,177] can be used in a simple manner in ^{13}C-n.m.r. spectroscopy via spin echo experiments with gated ^{1}H-decoupling to distinguish between signals of quaternary C atoms and CH, CH_2 and CH_3 groups.[165,178,179] In

its simplest form, known as SEFT,[178,179] it yields singlets that differ by 180° in their phase. The SEFT pulse sequence which relies on spin echo modulation through ^{1}J (^{13}C, ^{1}H) coupling is shown.



During the D2 + D3 period transverse ^{13}C magnetization is modulated through spin-spin coupling to the proton. When D2 + D3 = $^{1}/_{J}$ it is possible in only one experiment to distinguish between signals of quaternary and methylene carbon atoms, on the one hand, and those of methine and methyl carbons on the other, since for both groups of resonances a phase difference of 180° exists. The signals of quaternary carbon atoms and those of CH_2 groups have a positive and those of CH and CH_3 groups a negative phase.

In carbohydrate research the SEFT (attached proton test) experiment is used to differentiate a carbon atom at position 6 (i.e. CH_2) from the other ring carbon atoms (i.e. CH) of a sugar residue. Thus from this experiment the investigator is able to deduce the number of sugar residues in the repeating unit of a polysaccharide that are non-deoxy at C-6. This is well documented in Chapter four of this thesis.

II.1.3 Basic theory of two dimensional n.m.r. spectroscopy

The common feature of pulse F.t.-n.m.r. experiments is the time sequence preparation - evolution - detection shown in Fig. II.2. This time sequence also forms the basis of two-dimensional n.m.r. spectroscopy (2D n.m.r.);[154,155,156] with the important difference, however, that the evolution time t_1 within a sequence of pulse cycles is now a variable. In 2D-n.m.r. experiments the receiver signal is also dependent on the evolution period t_1 because over n experiments each t_1 is increased by a constant time increment Δt_1. The receiver signal is thus a function of t_1 and t_2. Fourier transformation with respect to t_2 initially yields n conventional spectra whose data points on the time axis t_1 define the modulation frequency, which can be determined by a second Fourier transformation (Fig. II.3). Two frequency variables F_1 and F_2 are obtained in 2D-n.m.r. experiments. The first Fourier transformation with respect to t_2 (F_2) yields the resonance frequency and the second Fourier transformation with respect to t_1 the modulation frequency (i.e. coupling constant, chemical shift due to coherence transfer etc.).

For the graphical display of 2D-n.m.r. the procedures currently used are stacked plot[157,158] and contour plot.[159] The stacked plot is, in most cases, aesthetically appealing, but often badly arranged and therefore difficult to analyze. The contour plot is a cross-section through the stacked plot parallel to the X,Y-plane at a chosen height. In this way one obtains a clearly arranged diagram of contour lines that is easy to analyze, in particular for correlated spectra.

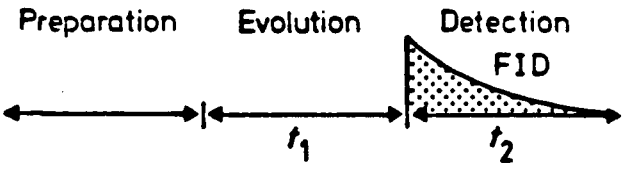


Fig. II.2: Time sequence of modern pulse experiments

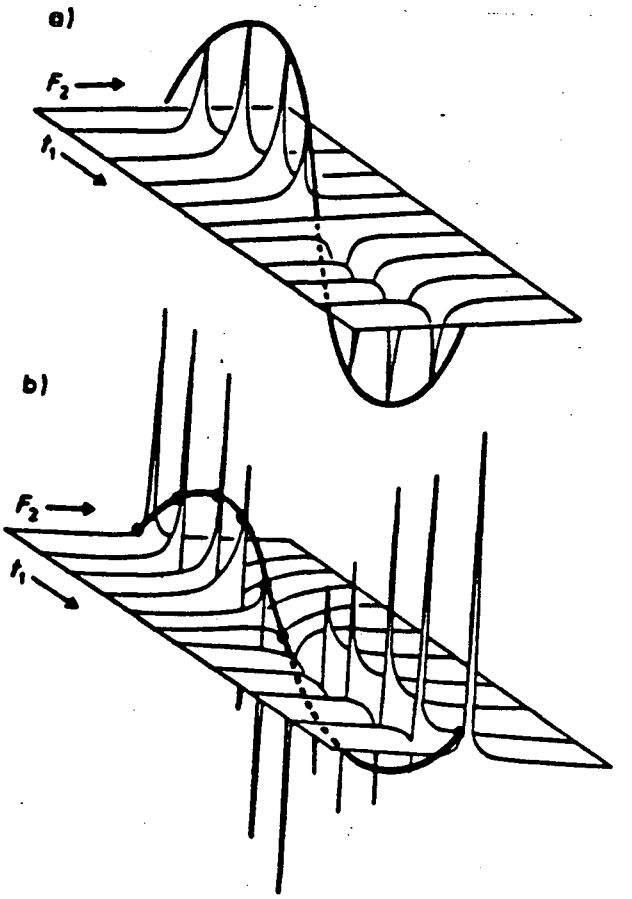


Fig. II.3: Two-dimensional n.m.r. spectroscopy through amplitude and phase modulation (a, b respectively). The figure shows the situation after the first Fourier transformation. The modulation of the signals results from a periodic perturbation of the spin system during the evolution period. Repeated Fourier transformation of the time signal $S(t_1)$ yields the frequency F_1 (taken from R. Benn and H. Gunter's article in Angew. Chem. Int. Ed. Engl. 22, 350-380, 1983).

II.1.4 Two dimensional spin correlated n.m.r. spectroscopy

Maudsley and Ernst[160] were among the first to propose and realize two dimensional spin correlated n.m.r. experiments. Experimentally they require an additional time interval, the so-called mixing time,[161,119] between evolution and detection periods. In correlated 2D-n.m.r. spectra both frequency axes (F_1 and F_2) contain chemical shifts. Two types of 2D correlated spectra can be distinguished: both dimensions may be coupled through coherent transfer of transverse magnetization (scalar correlation[59-62,160]) or through incoherent transfer of magnetization (two-dimensional n.O.e. spectra,[119] two-dimensional chemical exchange spectra).

II.1.4a Homoscalar-correlated 2D-n.m.r. spectroscopy

Two-dimensional correlations for homonuclear spin systems (for example A = X = ^{1}H) are known as COSY[59,118] and SECSY[60]. Both frequency axes F_1 and F_2 contain the Larmor frequencies of the same nucleus, e.g. the δ (^{1}H) values. The COSY experiments done in this study involved using pre-saturation of solvent with two power levels (i.e. S1 and S2). In these experiments a modified Bruker program[162] with a pulse sequence

D1-D3-90°$_X$-D0-D2-90°$_X$-F1D

HG(S1)-(S2).....D0

(see appendix II) was used. After the preparation period (i.e. D1 and D3), a 90°$_X$ pulse produces transverse magnetization. The magnetization

vectors precess in the X,Y plane according to their Larmor frequencies and their spin-spin coupling constant J. For the case ($J_{(A,X)} \neq 0$), the A magnetization through the scalar A,X- interaction in the x,y plane also depends on the Larmor frequencies ν_X and ν_A. The 2D spectrum therefore contains the characteristic off-diagonal signals (cross peaks) with coordinates δ_A, δ_X and δ_X δ_A, which indicate a scalar spin-spin coupling between A and X.

The coupling scheme of each monosaccharide can be obtained using proton-proton 2D chemical shift correlation spectroscopy (COSY)[163] based on the fact that vicinal protons exhibit off-diagonal cross peaks in the 2D spectrum due to scalar coupling. The pattern of coupling for a sugar residue can be traced out using an unambiguous assignment (e.g. H-1) as the starting point. The overlap of proton resonances often leads to ambiguities in establishing connectivities in the COSY spectrum. These can be resolved using multiple-relay-COSY, in which correlations are transferred from nucleus to nucleus within a spin system. In this study (Chapter 4) H-1 of each sugar residue was identified by inspection, H-2 as a cross-peak in the COSY spectrum and the remaining protons were identified by a series of multiple-relay-COSY experiments.

Another method for investigating homonuclear spin systems is spin echo correlated spectroscopy (SECSY),[60] in which the frequency differences (F_1 axis) are plotted against chemical shift (F_2 axis). Proton assignment of spectra containing severe spectral overlaps have been made using the homonuclear Hartman-Hahn (HOHAHA) method[164,169] which provides high-resolution phase-sensitive spectra that display both direct and relayed connectivities.

II.1.4b Heteronuclear correlated 2D-n.m.r. spectroscopy

In heteronuclear correlated 2D-n.m.r. (HETCOR) experiments[120] the Larmor frequencies of two different types of nuclei e.g. ^{1}H and ^{13}C are related through scalar coupling. The fundamental concept of heteronuclear correlated (HETCOR) experiments has been documented by Benn and Gunter.[165] The HETCOR (^{1}H and ^{13}C) map consists of ^{1}H chemical shifts along the F_1 axis and the ^{13}C chemical shifts along the F_2 axis. Various types of connectivity can be investigated using these shift-correlated experiments, e.g. one-bond couplings, long-range couplings or delayed correlation. The relative insensitivity of these experiments has been dramatically improved by the introduction of new ^{1}H-detected ^{1}H-^{13}C correlation maps[164,166,167] suitable for full spectral analysis of small quantities of oligosaccharides (e.g. 3.5 mg sample of a trisaccharide[166]) and polysaccharides (e.g. 10 mg sample of Haemophilus influenzae type capsular polysaccharide[167]).

Homonuclear 2D-correlated experiments (e.g. COSY, multiple relay COSY) can provide most of the ^{1}H assignments required for identification of the sugar residues. Once the ^{1}H signals have been identified, the ^{13}C assignments follow directly from ^{1}H-^{13}C heteronuclear chemical shift correlation spectroscopy (HETCOR). Alternatively, if the ^{13}C-n.m.r. spectrum is assigned then the HETCOR experiment can be used to interpret the ^{1}H spectrum.

^{1}H-^{13}C three-bond coupling ($^{3}J_{H-C}$) from the anomeric proton across the oxygen atom to the aglycone carbon skeleton could provide information on linkage site.[168,63,170] Recently the selective INEPT experiment

has been used to detect and measure intra- and inter-residue long-range ^{1}H-^{13}C couplings.[171] The sensitivity of these methods has been greatly improved by indirect observation of ^{13}C via ^{1}H detection.[168,167] Coupling across the glycosidic bond may possibly be detected also from the heteronuclear analogue of the relay-COSY experiment.[172] In this study, the investigator only had access to a spectrometer lacking reverse detection capability and this technique, therefore, could be not be employed.

II.1.5 Sequence analysis using ^{1}H-n.m.r. methods

Conformational analysis of oligosaccharides has shown that the exoanomeric effect operates such that the anomeric and aglyconic protons of glycosidic bonds are in virtual van der Waals contact.[173,174] This makes possible the use of nuclear Overhauser effect (n.O.e.) experiments to yield intra and inter-ring couplings, because the arrangement of the protons is suitable for dipolar coupling. This type of experiment can either be done in 1D, usually as n.O.e. difference spectroscopy, or 2D (NOESY)[119]. The contour map obtained for a NOESY experiment is analogous to the COSY plot, except that the cross-peaks are due to dipolar coupling. The identification of inter-ring coupling in the n.O.e. experiment[174] may be obtained by comparing COSY and NOESY plots. The success of these n.O.e. experiments requires that the molecule being examined should experience a measurable amount of n.O.e. and that the pertinent ^{1}H signals are adequately resolved. Compounds of intermediate

molecular weight that experience a detectable amount of n.O.e. without losing spectral resolution are most amenable to these studies. In this investigation low molecular weight polymer generated by bacteriophage-borne glycanase degradation was employed in the NOESY experiments.

The usual way of sequencing polysaccharides is by examining the oligosaccharide fragments derived from them. These fragments are more suitable to spectroscopic studies than their parent polymers, but experience small n.O.e. effects at high fields. The problems associated with n.O.e. investigations of small water-soluble molecules can be diminished by using spin-locked n.O.e. spectroscopy[164] or by derivatization (e.g. O-acetylation)[175] and use of an organic solvent.

In delayed COSY experiments, scalar coupling ($^4J_{HH}$) between the anomeric and aglyconic protons may provide sequencing information.[171] This approach is complicated by interference from five-bond coupling.

II.2 Chemical methods

The methodology of structural studies of polysaccharide using conventional chemical methods includes:

i) qualitative and quantitative estimation of the sugar constituents

ii) analysis for non-carbohydrate substituent groups (O-acetyl, N-acetyl, phosphate etc.)

iii) determination of the linkage configuration

iv) determination of the position of linkage

v) determination of the sugar sequence

Some known analytical methods employed for these goals are described in the following sections.

II.2.1 Isolation and purification[71-75]

A major task in polysaccharide chemistry is obtaining the material under investigation in a pure form. In this study the polysaccharide of interest is the K antigen. The isolation and purification process involves three stages:

i) bacterial growth and harvest of crude polysaccharide components;

ii) the isolation of the polysaccharide such that it is free from low molecular weight material and other high molecular weight material, and

iii) isolation of a single, monodispersed, polysaccharide species. The purity of the polysaccharide, the absence of heterogeneity, rather than the presence of homogeneity can be demonstrated by many independent techniques such as nuclear magnetic resonance spectroscopy, optical rotation measurements, electrophoresis[71,72] and gel-permeation chromatography.[73]

Escherichia coli bacteria serotypes K31, K33, K44 and K46 were received as stab cultures from Dr. Ida Orskov (Copenhagen). These bacterial cultures were streaked on Mueller-Hinton agar plates at 37°C until large, individual capsular colonies were obtained. E. coli K31 was grown by inoculating a Mueller-Hinton broth medium with single

colonies and incubating the resultant liquid culture at 37°C on Mueller-Hinton agar for four days. The lawn was scraped off and the capsular bacteria were killed by adding phenol solution and stirring the mixture for five hours. The phenol in this mixture (polysaccharide component plus bacterial debris) was dialyzed out.

The polysaccharide components were separated from the high molecular components (e.g. bacterial cells) by ultracentrifugation. Isolation from aqueous solution by the addition of a water-miscible solvent[74] (e.g. ethanol, acetone) resulted in the precipitation of neutral and acidic polysaccharides. The resultant stringy precipitate was dissolved in water and treated with Cetavlon[75] (cetyltrimethylammonium bromide) solution, which selectively precipitated the acidic polysaccharide. The neutral polysaccharide present remained in solution and the precipitate (acidic polysaccharide) was separated from the supernatant by centrifugation. Dissolution of the precipitate in 4M sodium chloride, precipitation with ethanol and centrifugation were carried out twice. The final precipitate was dissolved in water, dialyzed for two days and freeze dried to yield the purified K31 capsular polysaccharide. Isolation and purification of E. coli serotypes K33, K44 and K46 were carried out as described for E. coli K31. Chromatographic techniques such as gel-permeation and ion-exchange chromatography may be used to enhance the purity and homogeneity of capsular polysaccharides. Traces of low molecular weight contaminants in the capsular polysaccharides were removed by gel-permeation chromatography (Bio-Gel P2).

II.2.2 Sugar analysis

II.2.2a Total hydrolysis[76-81]

The initial step in the structural study of a polysaccharide is the quantitative acid hydrolysis of the polysaccharide into individual monosaccharides with minimum degradation. Dutton[76] reviewed the advantages and disadvantages in the use of different acids. Sulfuric acid, hydrochloric acid, formic acid and trifluoroacetic acid are the most commonly used acids. Trifluoroacetic acid is easily removed under diminished pressure and thus is being used increasingly instead of the mineral acids. The conditions of hydrolysis must be carefully chosen and controlled. To attain the correct hydrolysis condition the hydrolyzate may be monitored by paper chromatography high performance liquid chromatography (h.p.l.c.) or gas liquid chromatography (g.l.c.). The quantitative hydrolysis of *E. coli* K31 polysaccharide into its individual monosaccharides with minimum degradation was attained using 2M trifluoroacetic acid for 20 hours.

The hydrolytic rates of glycosidic linkages vary greatly. Uronosyl linkages in acidic polysaccharides are more resistant to acid hydrolysis because of the presence of electron acceptor carboxyl groups which stabilize the uronosyl linkages through the heterocyclic oxygen. One of the means of overcoming the resistance of the uronosyl linkage to acid hydrolysis, is the reduction of all carboxyl functionalities in the acidic polysaccahride by conversion into carbodiimide[77] derivatives followed by sodium borohydride reduction. An alternative method of

reduction developed in our laboratory[74] to overcome the resistance of uronosyl linkages to acid hydrolysis, involves methanolysis of the glycosyl linkages with the simultaneous esterification of the carboxylic acid, which is then reduced to the corresponding alcohol. The resulting mixture of neutral glycosides is hydrolyzed with 2M trifluoroacetic acid, to ensure complete release of sugar residues. Methanolic hydrogen chloride has the advantage of being less destructive to deoxy sugars and sialic acids.[80,81] Karunaratne has discussed the hydrolytic conditions for the hydrolysis of polysaccharides containing amino sugars[78] which will therefore not be duplicated in this thesis.

II.2.2b Characterization and quantification of sugars[76,82-91]

The sugars released upon acid hydrolysis can be analyzed qualitatively using paper chromatography,[82,83] high performance liquid chromatography,[84] paper electrophoresis,[85] and thin layer chromatography.[86] Colorimetric[87,88] analysis can be used in the classification of sugars into broad classes (hexoses, pentoses, uronic acids, deoxy or amino sugars and sialic acid) but has limited applications for individual characterization. High performance liquid chromatography (h.p.l.c.) can also be used for quantitative analysis of underivatized sugars. H.p.l.c. however has a limited number of stationary phases compared to gas-liquid chromatography.

The monosaccharides, released upon acid hydrolysis of a polysaccharide, can be converted to volatile derivatives and analyzed using

gas-liquid chromatography (g.l.c.). G.l.c. offers reproducible quantification and characterization of sugar residues of polysaccharides. An extensive review of this technique has been reported by Dutton.[76,89] Sugars can be analyzed as their volatile trimethylsilyl (TMS) derivatives but the existence of anomeric forms of sugars at equilibrium yields a complicated chromatogram of multiple peaks. This problem was solved by converting the acyclic sugar alditols into the volatile acetates, trifluoroacetates or trimethylsilyl ethers. Alditol trifluoro acetates show partial de-esterification on the column and the TMS derivatives of the alditols give poor resolution[90] on g.l.c. Alditol acetates have good resolution and short retention times[91] and therefore, were used throughout this investigation. Sugars separated by g.l.c. are usually confirmed by g.l.c.-mass spectrometry.

II.2.2c Determination of the configuration (D or L) of sugars[92-95]

In general, chromatographic separation methods and spectroscopic analyses do not distinguish between enantiomers. However enantiomers can be separated by g.l.c. using a chiral column or converting the enantiomers into diastereomers using chiral reagents (for example, (-)-2-butanol, (+)-2-octanol, or (+)-1-phenylethanethiol) and separation on a non-chiral phase.[92,93,94]

The D and L configuration of sugars can be determined by the isolation of the different monosaccharides and measurement of their optical rotation $[\alpha]_D$. Specific oxidases (e.g. D-glucose and

D-galactose oxidases) and enzymes can be used for the determination of D and L configuration of sugars.

The method employed in determining the D and L configuration of sugar in this investigation was by circular dichroism[47] measurement at 213 nm on alditol acetates, acetylated aldononitrile or partially methylated alditol acetates, where the acetoxy group acts as a chromophore.

II.2.3 Position of linkage

II.2.3a Methylation analysis[96]

This technique involves the complete etherification of the free hydroxyl groups of the sugar residues in oligo- and polysaccharides[96,97] which acts as a label in distinguishing the original unlinked positions from the linked position. Methylation analysis is routinely employed in the structural characterization of complex carbohydrates as a means to establish (i) linkage positions; (ii) number and types of sugar per repeating unit; (iii) identity of terminal unit(s), branching unit(s); and (iv) the position of base-stable substituents (e.g. pyruvate).

In the early days, methyl ethers were formed by repeated reaction with dimethyl sulfate and sodium hydroxide.[98] Treatment of a polysaccharide with silver oxide in boiling methyl iodide according to Purdie and Irvine[99] gives a fully methylated polysaccharide. Purdie's

method was considerably improved by Kuhn and colleagues[100] who used N,N-dimethylformamide as a solvent in conjunction with methyl iodide and silver oxide. A more convenient method for methylating polysaccharides was devised by Hakomori.[101] This involves the treatment of a polysaccharide with sodium methylsulfinyl methanide (dimsyl sodium) and methyl iodide.[102] Most undermethylations, by the Hakomori method or not, are due to incomplete dissolution of the sample. The solubility of a polysaccharide in the appropriate organic solvent may be enhanced by careful de-ionization of the polysaccharide, (for example, using Amberlite IR-120 (H^+) resin). In cases where the Hakomori methylation gives an "undermethylated" product, complete methylation can be achieved by using the Purdie method. A second Hakomori methylation is never conducted on a methylated acidic oligo- or poly- saccharide as this will result in β-elimination. Methyl trifluoromethane sulfonate is a milder base and, in the presence of 2,6-ditertiarybutylpyridine and trimethylphosphate as solvent,[103] effects methylation without cleavage of acyl groups. Polysaccharides containing uronic acids may be reduced with lithium aluminum hydride after the permethylation step.

The methylated material is usually purified by dialysis, extraction and gel-permeation chromatography (Sephadex LH 20). The completeness of methylation can be verified by i.r. spectroscopy (absence of hydroxyl absorption at 3600 cm^{-1}) or by analysis of the methoxyl content. The methylated polymer is usually hydrolyzed using 2M trifluoroacetic acid at 95°C for about 18 h. The partially methylated monosaccharides released on hydrolysis are reduced to their alditols and acetylated to give volatile partially methylated alditol acetate derivatives for

g.l.c. and g.l.c.-m.s. analyses. Uronic acids may be identified by comparison of methylation analysis results of the acidic polysaccharide with those of the methylated-reduced polysaccharide.

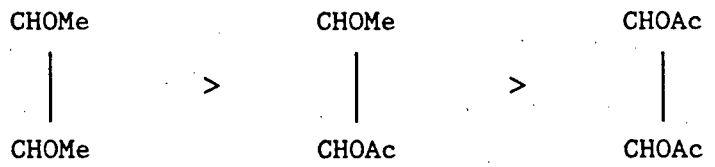
II.2.3b Characterization and quantitation of methylated sugars[76,89,104,105,44]

Partially methylated monosaccharides released on total hydrolysis of the permethylated oligo- or polysaccharide can be characterized using paper chromatography.[104] The methylated sugars are detected with p-anisidine hydrochloride spray[44] following by heating at 110°C for 5 min. These sugars are then characterized according to their relative mobilities (R_f values) and the different colours formed.

Gas-liquid chromatography (g.l.c.) is the most widely used technique for quantitative and qualitative analysis of methylated sugars. A review of the applications of g.l.c. to carbohydrate analysis has been published by Dutton.[76,89] The methylated sugars were analyzed as their partially methylated alditol acetates during this work. The identification and quantitation of these partially methylated alditol acetates were made by consideration of the relative retention times and co-chromatography with authentic samples.

For unambiguous identification of partially methylated alditol acetates, g.l.c. results should be confirmed using g.l.c.-m.s. Studies done on the fragmentation of partially methylated alditol acetates have been reported.[105] The primary fragments are formed as a result of

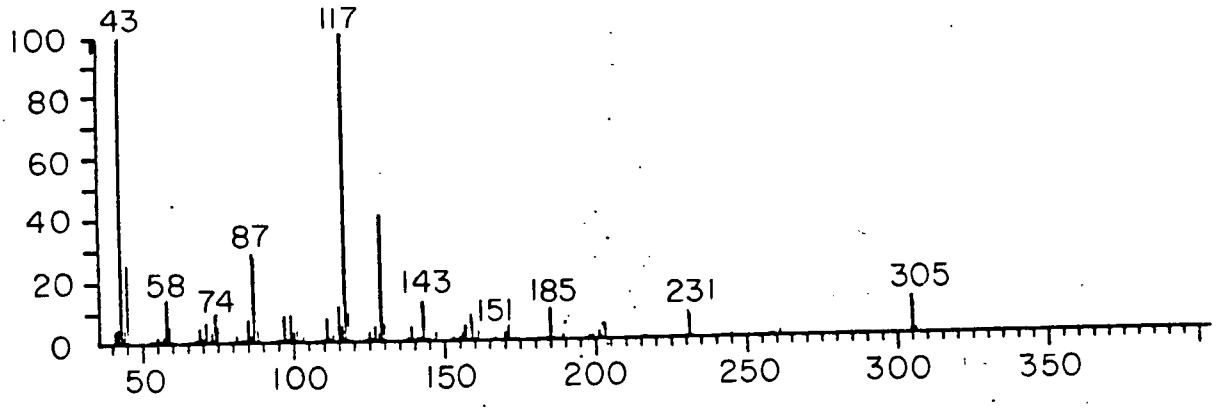
fission of the C-C bond in the alditol chain and this cleavage follows the preferential order indicated.



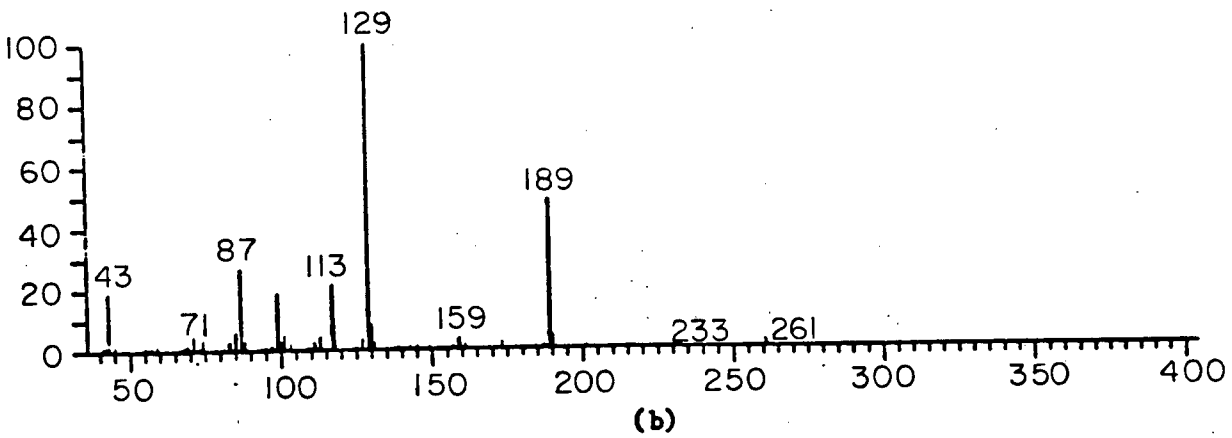
The intensity of the primary ion decreases with increasing molecular weight. Secondary ions can be obtained by loss of acetic acid (m/z 60), ketene (m/z 42), methanol (m/z 32), methyl acetate (m/z 74), methoxymethyl acetate (m/z 104) or acetoxymethyl acetate (m/z 132). Fig. II.1 illustrates the differences in the mass spectra of 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-D-glucitol and 1,2,5,6-tetra-O-3,4-di-O-methyl-D-glucitol.

II.2.4 Sugar sequence

The elucidation of the sequence of sugars in a polysaccharide involves the isolation and characterization of oligosaccharide fragments. Lindberg and coworkers[106] have reviewed the various techniques employed in the specific degradation of polysaccharides. The degradation techniques employed in this investigation are discussed in this section.



(a)



(b)

Fig. II.1: **Mass spectra of (a) 1,3,4,5-tetra-O-acetyl-2,6-dimethyl-glucitol. (b) 1,2,5,6-tetra-O-acetyl-3,4-dimethylglucitol**

II.2.4a Periodate oxidation and Smith degradation[107-112]

Oxidative cleavage of the C-C bond of vicinal diols by sodium metaperiodate is of importance in the structural determination of polysaccharides[107] and its uses are two fold. First, as an analytical technique using small amounts of material and secondly, as a preparative technique namely Smith degradation.[112] Oxidations are usually carried out in aqueous media with the water soluble metaperiodate ion. Over-oxidation may be prevented by performing the reaction in the dark at about 4°C.[108] The periodate consumption can be monitored spectrophotometrically[108] and the results illustrate the number of periodate sensitive sugars per repeating unit in a polysaccharide. Except for terminal side-chains and 1-6 linked sugars, one mole of periodate is consumed for every oxidizable hexose in a repeating unit.[109] The "polyaldehyde" produced, on periodate oxidation of a polysaccharide, is usually reduced with sodium borohydride into the polyol.

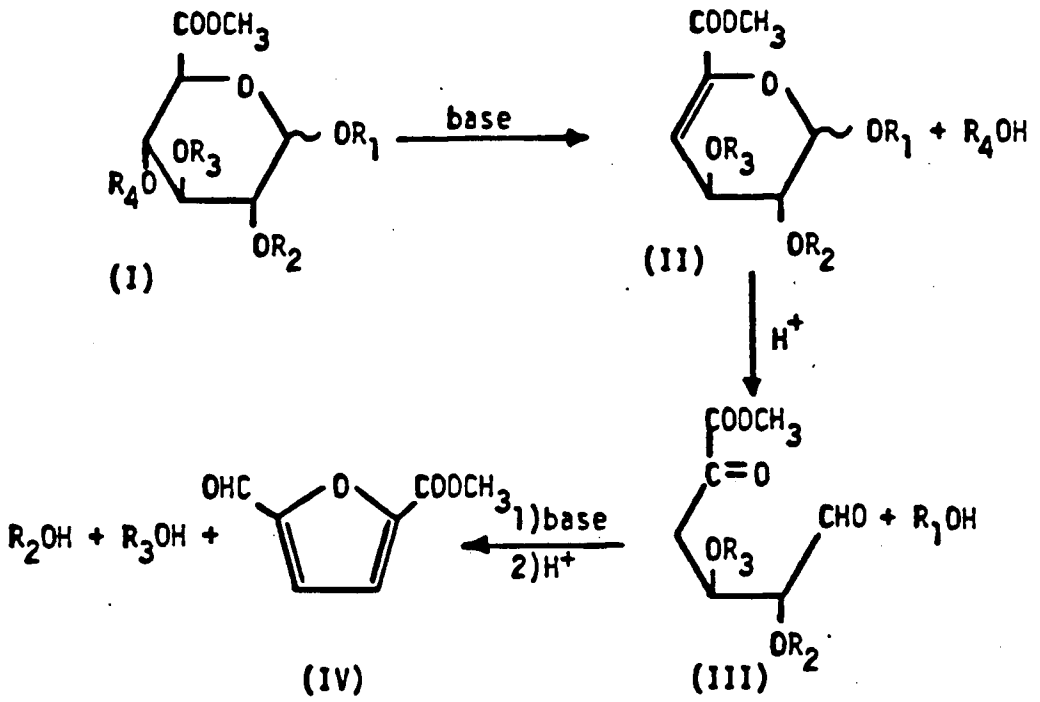
Cis glycols are observed to oxidize faster than trans and some trans glycol are resistant to oxidation if fixed in an unfavourable conformation.[110] Ebisu et al. selectively oxidized the terminal β-D-galactopyranosyl residues in the Pneumococcus S-14 polysaccharide leaving the 1→4 linked β-D-glucose units in the main chain intact.[111] In a similar exercise, the selective oxidation of the terminal D-glucose, and 1→4 linked galactose over 1→2 linked glucuronic acid in E. coli K34 was achieved.[113]

For analytical purpose, methylation analysis or sugar analysis is mostly performed on small quantities of the polyol.

Smith degradation is an important modification of periodate oxidation devised by Smith and coworkers.[112] It gives valuable information on the sequence of sugar residues in a polysaccharide. Smith degradation involves periodate oxidation followed by mild acid hydrolysis on large quantities of the resultant polyol. This mild acid hydrolysis of the polyol results in the cleavage of the acetal linkages leaving the glycosidic linkages intact. Smith degradation yields oligo- or polysaccharides and these oligo- or polysaccharides may be characterized by sugar or methylation analysis.

II.2.4b Uronic acid degradation (β-elimination)[106,114,115,116,117]

Base catalyzed degradation can be employed to generate defined oligosaccharide fragments from an acidic polysaccharide.[106,114,115] The carboxylic functionality of the uronic acid in an acidic polysaccharide is esterified on methylation. The esterified uronic acid residue is a strong electron-withdrawing group and thus enhances the acidity of the ring proton at C-5. When the methylated polysaccharide is treated with base (sodium methylsulfinyl methanide), the proton at C-5 is removed followed by a β-elimination of the 4-O-substituent with the formation of hex-4-eno-pyranosiduronate residues.[116] The main steps of this degradation are outlined as follows:



Aspinall and Rosell have shown in their experiments that, under conditions normally used for base degradations, complete loss of uronic acid residues occurs and that an acid hydrolysis is unnecessary.[117] The free hydroxyl group exposed after β-elimination, can be labelled by alkylation with methyl iodide, ethyl iodide or trideuteromethyl iodide. The resultant alkylated oligosaccharide is analyzed by g.l.c.-m.s. of the partially methylated alditol acetates. In the structure elucidation of E. coli K31, the site of attachment of the uronic acid unit was determined by comparing g.l.c.-m.s. results of the β-elimination and that of the methylation analysis of the native polysaccharide.

II.2.4c Partial hydrolysis

Partial hydrolysis followed by characterization of the product(s) is often utilized in structural studies of carbohydrates. The method is of particular value when a polymer contains a limited number of acid-labile glycosidic linkages, which may be cleaved without significant hydrolysis of the other glycosidic linkages. It is advisable, therefore, to perform some pilot experiments in order to determine optimal conditions for the partial acid hydrolysis. Many factors seem to influence the rate of hydrolysis, including the ring size, configuration, conformation, and polarity of the sugar as well as the size and polarity of the aglycon.[121] Capon has reviewed the rate constants for the acid-catalyzed hydrolysis of a large number of glycosides.[122] In general, furanosides are hydrolyzed more readily than pyranosides. Deoxyglycopyranosides are more acid labile than glycopyranosides and aminosugars. In this investigation selective hydrolysis of a side chain rhamnosyl linkage over an in chain one was attained using 0.1M TFA for 25 min (see Chapter 4). Uronic acids are relatively resistant to hydrolysis compared to neutral sugars and graded hydrolysis of acidic polysaccharides leads to isolation of acidic disaccharides (aldobiouronic acids) and higher oligosaccharides. In the structural studies of *E. coli* K31 capsular polysaccharide the uronic acid degradation (β-elimination) result was confirmed by isolation of an aldobiouronic acid (GlcAα1$\rightarrow$2Rha; see Chapter 3).

Mort and co-workers[123] have reported that using anhydrous liquid hydrogen fluoride (HF), there was a large enough variation in the rates

of glycosidic bond cleavage of amino and neutral sugars at 0°C, that amino sugar linkages remained intact while those of neutral sugars were cleaved. It has also been illustrated that differential cleavage of neutral and acidic sugars could be obtained using HF at below zero temperatures.[124] Kuo and Mort[125] using extremely mild conditions (-40°) to cleave gellan gum, obtained preferential cleavage of α over β linkages in HF and the resultant tetrasaccharide had its acetate and glycerate substituents intact. In the present study partial hydrolysis using HF below -40°C was used in the structural investigation of E. coli serotypes K31 and K46 polysaccharides.

II.2.4d Lithium ethylenediamine degradation

Mort and Bauer[126] have shown that 3-linked glycosyluronic acid-containing polysaccharides can be cleaved using lithium metal dissolved in ethylenediamine. Subsequently it was demonstrated by Albersheim et al.[127] that treatment of carbohydrates with lithium cleaves the glycosyluronic acid residues regardless of their positions of substitution by other glycosyl residues. The lithium reaction is similar to a β-elimination in that the polysaccharide is cleaved at the glycosyluronic acid residues. The lithium degradation is particularly valuable because it cleaves underivatized polysaccharides thus allowing the products to be used for both structural analysis and studies on their biological activity. This type of degradation was used in the structural elucidation of E. coli K31 capsular polysaccharide.

II.3 Mass spectrometry

Mass spectrometry is mostly applied in carbohydrate chemistry as a backup procedure to "wet chemical" methods. In the last two decades the growth in chemical instrumentation has been phenomenal, largely due to the advances in the electronic industry. Mass spectrometry is playing an increasingly important role in carbohydrate research.[128] This technique is based on the fragmentation of ionized molecules and differentiation of the resulting particles by use of the mass-to-charge ratio.

Mass spectrometry in carbohydrate analysis provides information on:

i) the substitution patterns in partially methylated alditol acetate (PMAA) derivatives

ii) the position of the methyl and methylene groups in deoxy sugars and the amino group in amino deoxy sugars

iii) the sugar sequence in oligosaccharides, and

iv) the various forms of the monosaccharides, e.g. between cyclic and acyclic, pyranose and furanose, pentose and hexose and aldoses and ketose.

Mass spectrometry, however, does not differentiate between diastereomeric compounds, e.g. glucose, galactose and mannose.

All mass spectrometers have four components: (a) the ion source, where the sample under investigation is ionized and transferred into the gas phase; (b) the analyzer, where the molecular or fragment ions are mass analyzed according to their mass to charge ratio; (c) the

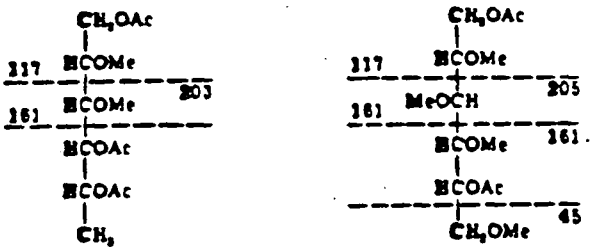
detector, where the resolved ions are detected, amplified and their intensity measured; (d) the vacuum system, which provides a stable environment for the above mentioned processes. There are numerous methods for ion production and their relative importance is continuously changing with the advent of new techniques. The choice of ionization technique is dictated by the nature of sample and information sought. The main objective is to produce as many ions as possible with a beam composition which accurately represents the structural features of the sample.

II.3.1 Electron impact-mass spectrometry

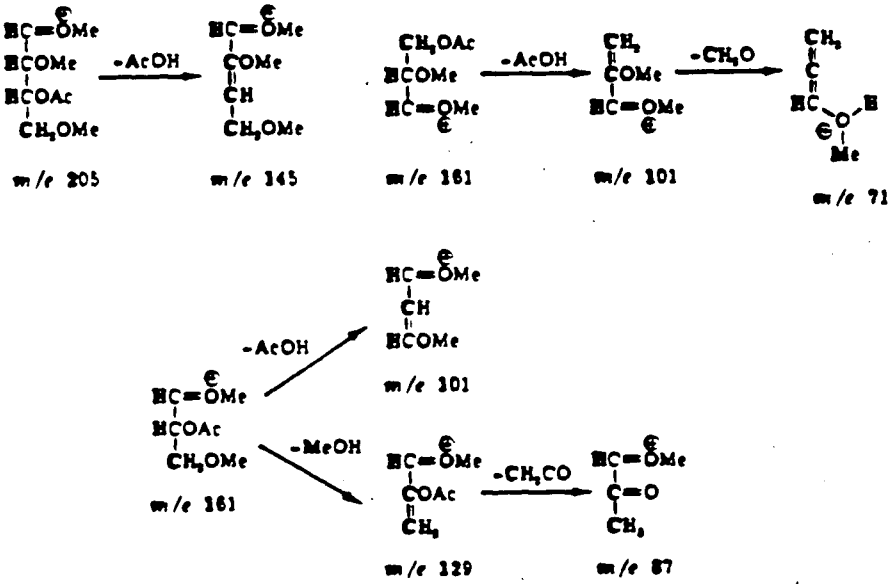
This is the most popular technique for generating ions in the gas phase. An electron beam of 70 eV is the maximum for most ion efficiency curves and the excess energy acquired by the molecule causes it to undergo single or multi-stage fragmentation. Hence, the molecular ions are often not observed in the case of carbohydrate mass spectrometric analysis. EIMS is usually performed on volatile, low mass samples like alditol acetates.

Fragmentation patterns typical of partially methylated alditol acetates are illustrated in Fig. II.5. Primary fragment ions undergo a series of subsequent eliminations, including β-eliminations, to give secondary fragments, by loss of acetic acid (m/z 60) or methanol (m/z 32). Losses by α-elimination of acetic acid (m/z 60), but not of methanol, and losses via cyclic transition states of formaldehyde

Primary fragmentation:



Secondary fragmentation:



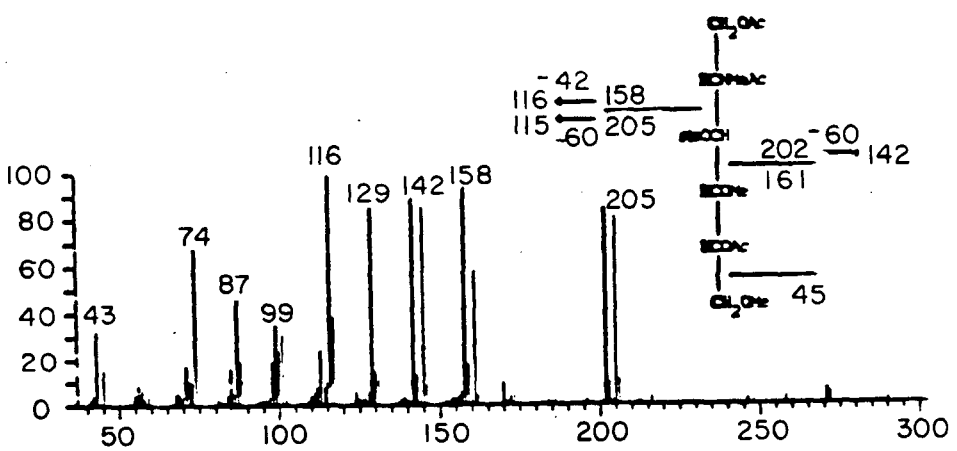


Fig. II.5: Fragmentation pathways of some partially methylated alditol acetates

methoxymethyl acetate or acetomethyl acetate may also occur.

The mass spectra of partially methylated alditol acetates of 2-amino-2-deoxyhexoses have also been reported,[129,130,131] the characteristic base peak being that of m/z 116. The primary fragmentation is dependent on the position of the acetamido group, and fission is almost exclusively between the methylacetamido group and the adjacent methoxyl or acetoxyl group. The secondary fragments are similarly formed as illustrated in Scheme II.3.1.

II.3.2 Chemical ionization (CI) mass spectrometry

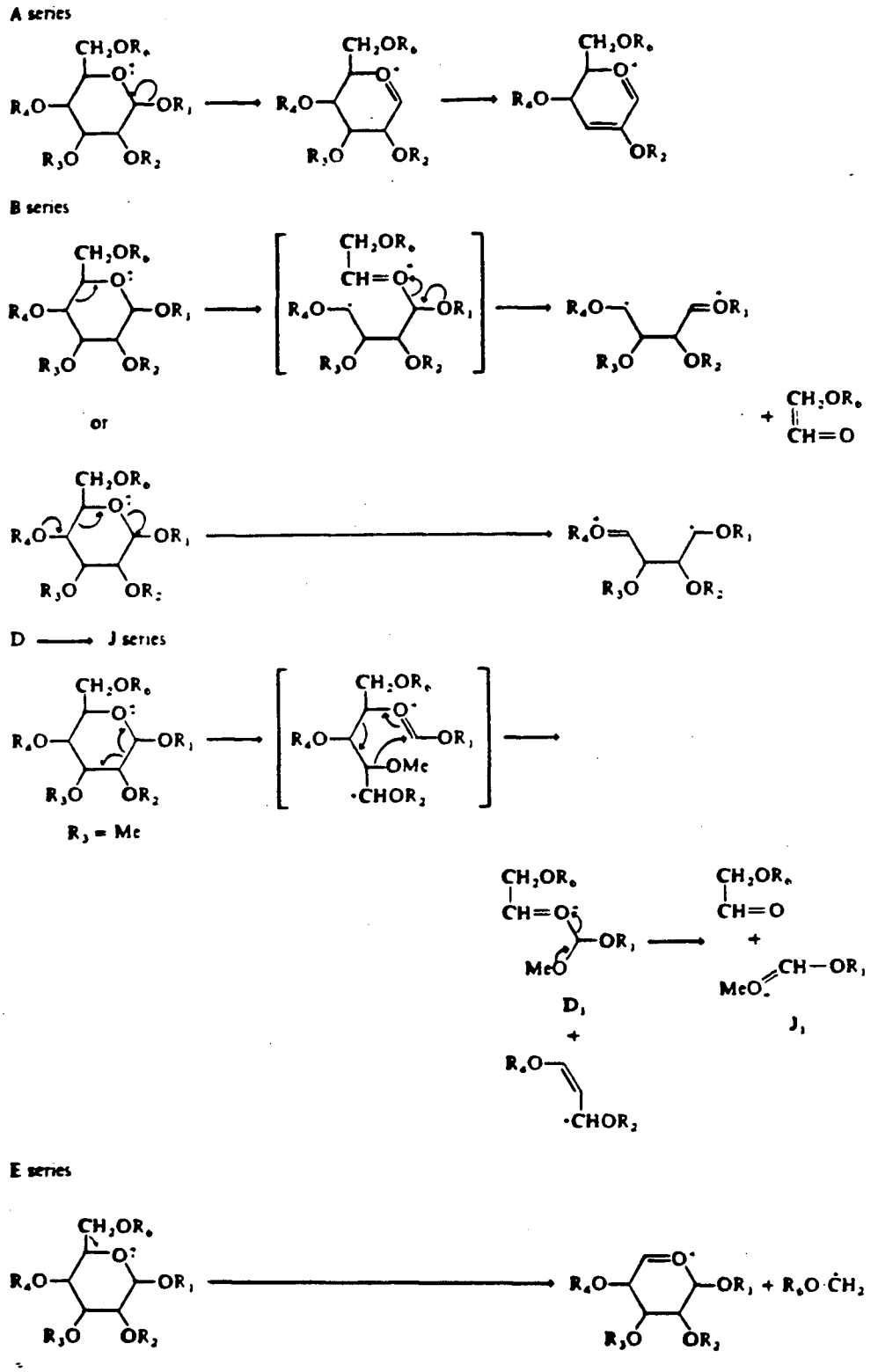
Chemical ionization was first introduced by Munson and Field in 1966.[132] Ionization is achieved by gas phase ion/molecule reactions. Thus the reagent gas, introduced into the ionization chamber in large excess, is ionized by energetic electrons. This is followed by ion/molecule reactions between the primary ions and the neutral reagent gas which produces the CI reagent ions. Therefore the CI reagent ions have much lower energy than the ionizing electrons. Consequently CI is a milder form of ionization, where frequently the molecular ion can be observed in abundance. The extent of fragmentation is dependent on the reagent gas used. The most common reagent gases used in carbohydrate research are methane and ammonia.[133] The CI ionization mode employed in this study was desorption chemical ionization (i.e. to desorb material from external probes which are positioned directly within the CI reagent ion plasma).

C.i.-m.s. is particularly useful for analyzing derivatized oligosaccharides and oligosaccharide alditols (i.e., C.i.-m.s. ionization is in gas phase so conversion to volatile derivatives such as acetates, methyl esters etc. is essential for C.i.-m.s. analysis). C.i.-m.s. was used in the structural studies of the capsular polysaccharides for E. coli serotypes K31 and K46.

The mass spectrometry behaviour of permethylated oligosaccharides has been extensively investigated,[134,135] and the fragmentation pathways proceed in a manner similar to those of the monosaccharide derivatives. Scheme II.5.II shows the fragmentation nomenclature first devised by Chizhov and Kochetkov[136] and later modified by Kovacik et al.[137] C.i.-m.s. provides relevant information on the sequences of sugar units when these are of different types. On the whole, very little information can be obtained concerning the stereochemistry, either of individual residues or of glycosidic linkages. Nevertheless some information can be obtained on linkage types from an examination of the fragmentation pathways.

II.3.3 Fast atom bombardment - mass spectrometry (f.a.b.-m.s.)

F.a.b.-m.s. is gaining a wide application in carbohydrate research.[138] Typically, a sample is dissolved in a suitable solvent (e.g. water, glycerol, thioglycerol, methanol or chloroform) and is introduced as a matrix onto the probe. The sample is then bombarded with argon or xenon atoms possessing 7-8 keV of energy and the positive



Scheme II.3.I: Some mass spectral fragmentation pathways for permethylated glycopyranosides

and/or negative ions are released and analyzed.

The fragmentation information provided by f.a.b.-m.s. is similar to that of c.i.-m.s. The advantage of this technique is the ability to analyze underivatized samples. Lam[139] obtained excellent sequencing information on some bacteriophage generated oligosaccharides using this technique.

II.3.4 Laser desorption ionization Fourier transform ion cyclotron resonance (L.d.i.-F.t.-i.c.r.) mass spectrometry

Laser radiation is coherent, monochromatic, directional and intense. The high intensity of a laser beam enables sample vaporization to be on a short time scale. After vaporization, collision between neutral and primary ions in the plasma can lead to formation of secondary ions. The dominant process is cationization by alkali ions.[140]

The most popular ionization techniques for oligosaccharide m.s. analysis are desorption chemical ionization and fast-atom bombardment (f.a.b.). These desorption techniques give abundant sequence information, but provide little or no information on the positions of linkage or the anomeric configuration at the linkage. Mass analyzed ion kinetic energy experiments have been used to confirm the anomeric configuration[141] with some success and tandem-m.s. experiments (like collision-activation dissociation) have been used to distinguish the position of linkage.[142] The disadvantage of both methods is that the oligosaccharide, unless of very low molecular weight, must be derivatized prior to

analysis. Positive ion laser desorption ionization Fourier transform ion cyclotron resonance spectroscopy (l.d.i.-f.t.-i.c.r.)[143-146] has been used for the mass-spectral analysis of glycosides. It has recently been reported that positive ion l.d.i.-f.t.-i.c.r. provides both sequence information and a tentative indication of some linkage position.[147] It has also been suggested that negative l.d.i.-f.t.-i.c.r. on underivatized oligosaccharides may provide potential information on the anomeric configuration of the individual monosaccharides.[148]

III. STRUCTURAL STUDIES OF E. COLI CAPSULAR POLYSACCHARIDES BY CHEMICAL METHODS

In this section the chemical study on the K antigen of E. coli serotype K31 is presented. Detailed n.m.r. study on this K antigen is reported in the next chapter.

III.1 The structure of Escherichia coli K31 antigen

III.1.1 Introduction

The capsular (K) antigen of Escherichia coli K31 has been designated[30] as heat stable (type A) and may, therefore, in the absence of amino sugars, be expected to resemble those of Klebsiella. A partial structure of E. coli K31 has been proposed earlier by other workers[31] and we now report the complete structural elucidation of this antigen.

III.1.2 Results and discussion

Composition. Analysis of the native polysaccharide before and after reduction[77] of the uronic acid gave galactose, glucose, and rhamnose in the ratios of 1.00:0.98:2.95 and 1.00:1.89:2.98, respectively. A composition of galactose, glucose, glucuronic acid, and rhamnose in the ratios of 1:1:1:3 was consistent with n.m.r. data

suggesting a hexasaccharide repeating unit.

Methylation. Methylation[101] analyses without and with reduction of the uronic acid gave the results shown in Table III.1, columns I and II, from which it may be deduced that a rhamnose residue occupies a terminal position and the glucuronic acid unit constitutes the branch point. Methylation analysis of the product (PH) obtained by selective partial hydrolysis indicates (Table III.1, column III) that the lateral rhamnose unit is linked to the glucuronic acid at C-4. The glucuronic acid was shown by a β-elimination experiment[115] to be linked to C-2 of a rhamnose unit (Table III.1, column IV).

In order to confirm the identity of the sugar to which the glucuronic acid is linked a portion of the hydrolyzate used to determine the sugar composition of the native polysaccharide was methylated and analyzed directly by g.l.c. Subsequent to the elution of a mixture of permethylated monosaccharide methyl glycosides a single peak appeared which was shown by c.i.-m.s. to give a molecular ion $[M + \overset{+}{NH_4}] = 456$ consistent with the formulation of the aldobiouronic acid as GlcA — Rha (Fig. III.1). The potential of capillary columns to separate neutral and acidic materials has been noted previously[180] and avoids tedious separations on ion-exchange resins.

On the basis of these methylation experiments the partial structure

$$\begin{array}{l} \rightarrow 3)\text{-GlcA-}(1\rightarrow 2)\text{-Rha-}(1\text{-} \\ \qquad\qquad\quad\ 4 \\ \qquad\qquad\quad\ \uparrow \\ \qquad\qquad\quad\ 1 \\ \qquad\qquad\quad\ \text{Rha} \end{array}$$

may be written.

Table III.1: Methylation analyses of *E. coli* K31 polysaccharide and derived products

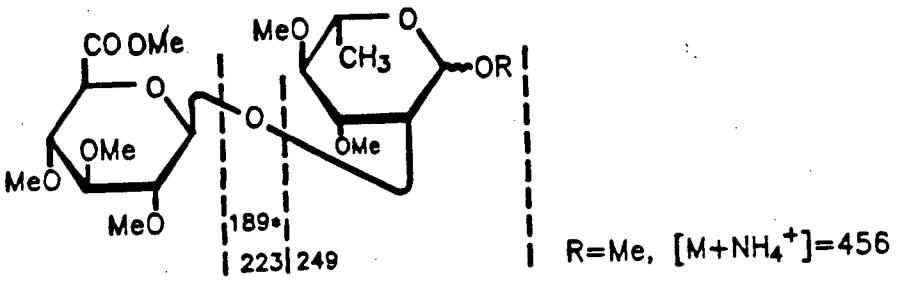
Methylated sugars[a] (as alditol acetates)	Mole %[b]						
	I[c]	II	III	IV	V	VI	VII
3,4-Rha	39.7	33.9	43.6	26.8	-	41.54	27.6
2,3,4-Rha	14.0	16.9	5.1	31.1	-	22.72	29.3
2,4,6-Gal	20.8	17.3	16.2	20.4	50.9	-	21.4
2-Glc	-	13.8	5.2	-	-	17.54	-
2,4-Glc	-	-	9.0	-	-	-	-
3,4,6-Glc	18.2	18.1	21.0	21.8	-	19.54	21.7
2,3,4,6-Glc	-	-	-	-	49.1	-	-

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

b Values are corrected using the effective carbon response factors given by Sweet et al.; determined on a DB 17 column programmed for 1 min at 180° then to 250° at 2°/min.

c I, K31 polysaccharide; II, K31 polysaccharide, uronic ester reduced after methylation; III, product PH from selective partial hydrolysis; IV, product from β-elimination and remethylation; V, product A2 from HF hydrolysis; VI, product from chromium trioxide oxidation; VII, product F1 from Li-ethylene-diamine degradation.

The configuration of the rhamnose was established as L and of the other sugars as D by comparison of the circular dichroism spectra of their methylated derivatives with standards.[95]



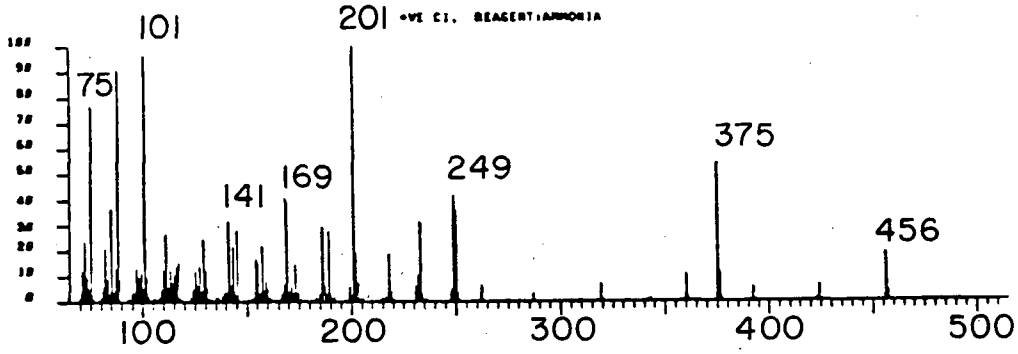


Fig. III.1: C.i.-mass spectrum and fragmentation pattern of methylated aldobiouronic acid (GlcA — Rha)

Lithium-ethylenediamine degradation. The polysaccharide was treated with lithium in ethylenediamine and the reducing oligosaccharide (F1) present in the mixture was isolated by gel-permeation and paper chromatography. N.m.r. spectroscopy (^{1}H and ^{13}C) and methylation data (Table III.1, column VII) showed the product F1 to be a tetrasaccharide

containing two deoxy sugar residues and the reducing end was determined by the method of Morrison[200] to be galactose which therefore in the polymer is linked to glucuronic acid at C-3.

The tetrasaccharide F1, is, therefore, (Glc,Rha,Rha)-Gal and since two of the three rhamnose residues have already been located it remains to establish whether the sequence in F1 and, hence, in the native polysaccharide is Rha-Rha-Glc-Gal or Rha-Glc-Rha-Gal. Partial hydrolysis of the native polysaccharide with hydrofluoric acid distinguished between these possibilities.

Hydrofluoric acid hydrolysis. From this hydrolysis two disaccharides (A2 and A3) were isolated, the latter in only small amount. Methylation analyses of A2 (Table III.1, column V) showed it to be Glc-(1→3)Gal and c.i.-m.s. gave $[M + NH_4^+] = 472$ consistent with A2 being a hexose-hexose disaccharide (Fig. III.2). Methylated A3 similarly gave $[M + NH_4^+] = 412$ (Fig. III.3) indicative of a disaccharide composed of two deoxyhexose units which, from the methylation results of the original polysaccharide, must be Rha-(1→2)-Rha.

It therefore follows that the sequence in the main chain is

Glc — Gal — GlcA — Rha — Rha

N.m.r. spectroscopy. The proton spectrum of the native polysaccharide showed six signals in the anomeric region between δ 5.24 and δ 4.82 (Table III.2 and Appendix III). Each signal integrated to approximately 1 H but only the signal at δ 4.82 showed a measurable coupling

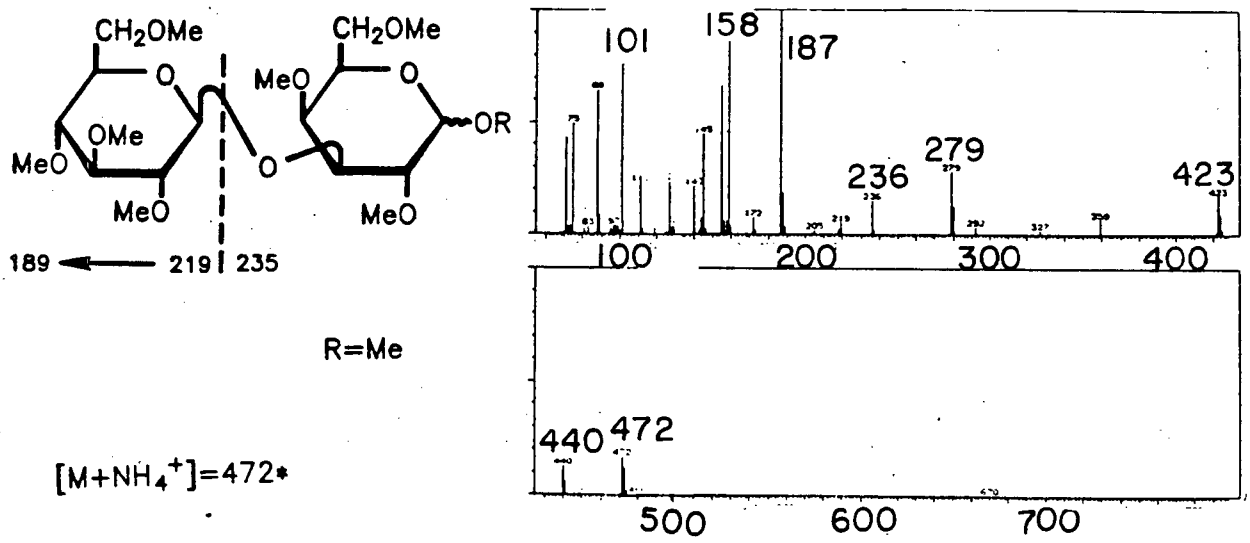


Fig. III.2: C.i.-mass spectrum and fragmentation pattern of methylated HF product A2

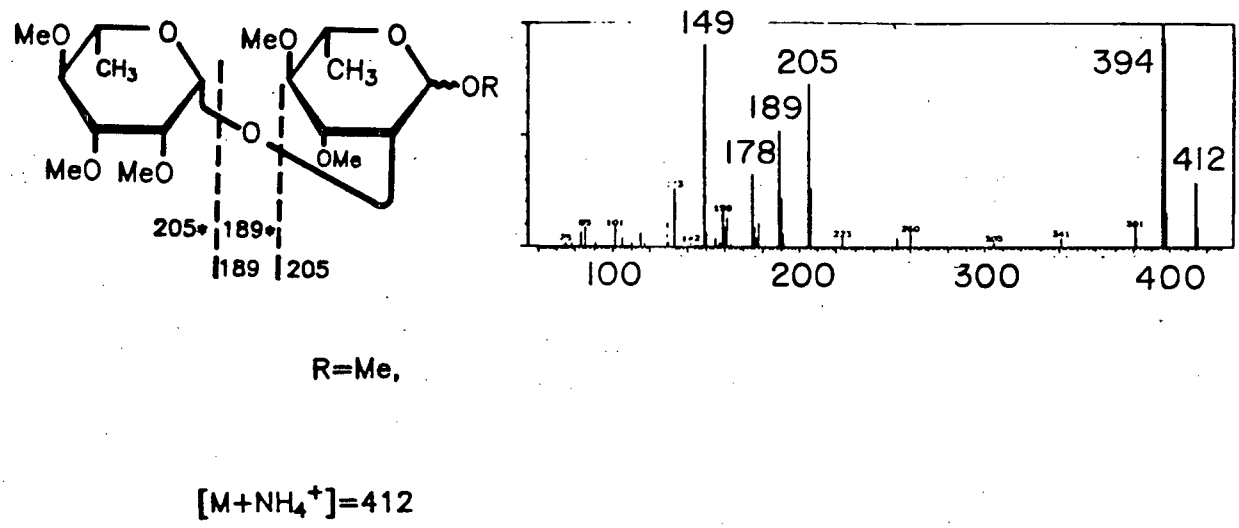


Fig. III.3: C.i.-mass spectrum and fragmentation pattern of methylated HF product A3

constant of 8 Hz, the others were broad due, presumably, to the viscosity of the solution. In addition two signals at δ 1.31 and δ 1.28 integrating to approximately 9 H were observed (Table III.2 and Appendix III).

The proton decoupled ^{13}C-spectrum likewise showed six signals in the anomeric range between 104.73 and 95.76 p.p.m. together with signals at 176.94, 17.57 and 17.45 p.p.m. indicative of uronic acid and 6-deoxysugar (Table III.2 and Appendix III). In a proton coupled spectrum the signal at 104.73 p.p.m. exhibited a coupling constant ($J_{C,H}$) of 160.35 Hz whereas the others gave values between 168.52-172.88 Hz (Table III.3). Both the proton and the ^{13}C spectra suggest that the K31 polysaccharide is composed of a hexasaccharide repeating unit with a single β-glycosidic linkage. This was confirmed by a chromium trioxide oxidation[184] when galactose was the only sugar completely degraded.

Detailed n.m.r. studies on a lower molecular weight polymer (generated by depolymerization of native polysaccharide with viral endoglycanase) are illustrated in the next Chapter. These studies afforded the confirmation of the structure of E. coli K31 capsular antigen using modern pulse n.m.r. techniques.

III.1.3 Experimental

General methods. Solutions were concentrated on a rotary evaporator with bath temperatures not exceeding 40°C. Frozen solutions were obtained using a dry ice-acetone mixture and lyophilized on a

Table III.2: N.m.r. data for derived products of *E. coli* K31 capsular polysaccharide

Compound	^{1}H-n.m.r. data δ^a	$J_{1,2}$[b] (Hz)	Integral	Assignments	^{13}C-n.m.r. data p.p.m.[d]	Assignments[c]
Native polysaccharide	5.24	b	1	2-Rhaα-	176.94	CO of uronic acid
	5.13	b	1	2-Glcα-		
	5.11	b	0.9	2-Rhaα-	104.73	3-Galβ-
	5.03	b	0.9	3,4-GlcAα-	101.80	Rhaα-
	4.87	b	0.9	Rhaα-	100.80	3,4-GlcAα-
	4.82	8	0.9	3-Galβ-	100.21	2-Rhaα-
	1.31				96.47	2-Glcα-
	1.28	CH_3 of Rha's			95.76	2-Rhaα-
					17.57/17.45	CH_3 of Rha's
$Rha^{1\to2}_{\alpha}Rha^{1\to2}_{\alpha}Glc^{1\to3}_{\alpha}Gal$-OH	5.25	b	1	2-Rhaα-		
	5.13	3	1.2	2-Glcα-		
	4.99	<1	1.3	Rhaα-		
	4.95	b	0.2	3-Galα-		
	4.65	8	0.8	3-Galβ-		
	1.31	8	3			
	1.29	8	3	CH_3 of Rha's		

a Chemical shift downfield from sodium-4,4-dimethyl-4-silapentane-1-sulfonate (DSS)

b Key: b = broad, unable to assign accurate coupling constant

c For example 3-Galβ- refers to anomeric proton of a 3-linked galactosyl residue in the β-anomeric configuration

d Chemical shift in ppm downfield from DSS

Table III.3: Determination of anomeric configuration (^{13}C, ^{1}H coupled)

Chemical shift decoupled nuclei (ppm)	Chemical shift downfield (ppm)	Chemical shift upfield (ppm)	Coupling constant	Configuration
104.73	105.794	103.586	165.60	β
101.80	102.952	100.647	172.88	α
100.80	101.923	99.650	170.46	α
100.21	101.165	98.874	171.82	α
96.47	97.592	95.345	168.52	α
95.72	96.847	94.595	168.90	α

* G.l.c.-m.s. analysis on methylated product from chromium trioxide oxidation indicated that the sugar residue with β-configuration is galactose and the rest of the sugars have the α configuration.

Unitrap II freeze-dryer. Circular dichroism spectra (c.d.) were recorded on a Jasco J-500A automatic recording spectropolarimeter, with a quartz cell of 0.3 mL capacity and a path length of 0.1 cm. C.d. samples were prepared by dissolving the appropriate alditol acetate in spectroscopic grade acetonitrile. The c.d. spectra were recorded in the range 210-240 nm. The infrared (i.r.) spectra of methylated derivatives were recorded on a Perkin Elmer model 457 spectrophotometer. The solvent used for sample preparation was spectroscopic grade carbon tetrachloride.

Analytical paper chromatography was performed by the descending method using Whatman No. 1 paper and the following systems: (1) 18:3:1:4 ethyl acetate-acetic acid-formic acid-water; (2) 8:2:1 ethyl acetate-pyridine-water; and, (3) upper phase of 4:1:5 1-butanol-ethanol-water. Preparative paper chromatography was carried out using Whatman No. 3 paper and solvent system 1. Chromatograms were either developed with alkaline silver nitrate[194] or by heating at 100°C for 10 min after being sprayed with p-anisidine hydrochloride[44] in aqueous 1-butanol. Sugars and oligosaccharides were detected by these methods.

A Bio-Gel P-2 (400 mesh) column (2.5 x 100 cm) was used for preparative gel-permeation chromatography. The void volume of the column and the efficiency of packing were determined using blue dextran (0.2%). The concentration of the samples applied to the column ranged from 40-100 mg/mL. Eluant was distilled water at a flow rate of approximately 6 mL/h. Fractions were collected, freeze-dried, weighed and the elution profile was obtained. Sephadex LH-20 was used to purify large molecular weight carbohydrate material that is soluble in organic

solvent, e.g. permethylated oligo- and polysaccharides.

A Hewlett-Packard 5890 instrument equipped with dual flame-ionization detectors was used for analytical g.l.c. separations. A Hewlett-Packard 3392A integrator was used to quantify the peak areas. Open tubular (capillary) columns were used with a helium carrier-gas flow rate of 48 mL/min. The columns used were: (A) fused silica capillary column (DB-17-15N); (B) fused silica capillary column (DB-225-15N). Preparative g.l.c. was carried out with F & M model 720 dual column instrument fitted with thermal conductivity detectors. Stainless-steel columns (1.8 m x 6.3 mm) were used with carrier-gas helium flow-rate of 60 mL/min. This column was packed with 3% of SP-2340 on Supelcoport (100-200 mesh) and programmed from 175°C to 240°C at 1°/min. G.l.c.-m.s. analyses were performed with a V.C. Micromass 12 instrument equipped with a Watson-Biemann separator. Spectra were recorded at 70 eV with an ionization current of 100 A and an ion source at 200°C. The columns used for the separation were (A) and (B).

^{13}C-N.m.r. and ^{1}H-n.m.r. spectra were recorded on Varian XL-300 and Bruker WH-400 instruments respectively. Acetone was used as an internal standard for both ^{1}H-n.m.r. (2.23 p.p.m.) and ^{13}C-n.m.r. (31.07 p.p.m.) spectroscopy. ^{1}H-N.m.r. spectra were recorded at elevated temperature and chemical shift values are given relative to that of external sodium - 4,4-dimethyl-4-silapentanesulfonate (taken as zero). ^{1}H-N.m.r. samples were prepared by dissolving in D_2O and lyophilizing three times from D_2O solutions. These samples were dissolved in D_2O and submitted in 5 mm diameter n.m.r. tubes. ^{13}C-N.m.r. spectra were recorded at ambient temperature. Samples were dissolved in the minimum of D_2O and submitted

in n.m.r. tubes of diameter size 5 mm.

In our laboratory, we generally proceed as follows for the isolation of E. coli bacteriophages. Large samples of sewage are mixed with concentrated Mueller-Hinton broth and then with an actively growing culture of the bacteria for which a virus shall be isolated. This mixture is then incubated (37°C) overnight. The bacteria in this mixture are killed by adding chloroform and shaking vigorously. The crude bacteriophage solution is separated from the bacterial debris by centrifugation.

The bacterial lawn needed for bacteriophage assay is prepared as follows. A Mueller-Hinton agar plate was dried, upside down at 37°C in an incubator for 2 hours. Actively growing bacterial culture (3 mL) was pipetted on to the agar surface and after 20 min at 37°C, excess liquid was drained off. The plate was then incubated at 37°C for 1 hour to produce the bacterial lawn.

An assay for determining bacteriophage concentration is described as follows: a 0.3 mL portion of bacteriophage suspension was diluted ten-fold by adding 2.7 ml of sterile broth. A 0.3 mL portion of the resultant solution was further diluted ten-fold (in a similar manner) and the process repeated on subsequent solutions until a dilution range of 10^{-1} - 10^{-10} was obtained. One small drop of bacteriophage suspension was spotted on the bacterial lawn by means of a sterile pipette drawn to a fine tip. After overnight incubation at 37°C, the number of plaques observed for the highest dilution are counted. The counts of plaque-forming units (p.f.u.) per mL of undiluted phage suspension were calculated based on the volume of the bacteriophage

solution applied, the number of plaques and the dilution that gave those plaques.

The methods employed in building up the concentration of bacteriophage to a level sufficient for degrading the polysaccharide in question are tube lysis and flask lysis.

(a) Tube lysis

An actively growing culture of E. coli was obtained by successive replating on agar plates. A colony of this actively growing bacteria was picked up into sterile broth (5 mL) and incubated at 37°C until the bacterial culture became turbid (4 hours). Sterile broth (5 x 5 mL) in culture test-tubes was then inoculated with the bacterial culture (0.5 mL) and incubated at 37°C. After 30 minutes of incubation, bacteriophage suspension was added to the test-tubes consecutively at 30 minutes interval. Continued incubation results in gradual clearing of the cloudy solution due to cell lysis. After the last tube had cleared (about 5 hours after the first addition of bacteriophage) a few drops of chloroform were added to each tube to prevent bacterial growth. The last two tubes were combined and the bacteriophage solution was separated from the bacterial debris by centrifugation.

(b) Flask lysis

This technique is quite similar to the tube lysis except that large volumes of bacteriophage are produced. Aliquots (48 mL) of sterile

Mueller Hinton broth, in six Erlenmeyer flasks (125 ml), were each inoculated with 1 mL of actively growing culture. At 30 minute intervals, bacteriophage suspension (1 mL from the tube lysis or otherwise) was consecutively added to the flasks. The procedure was then continued as described for tube lysis.

Isolation and purification of E. coli K31 capsular polysaccharide. The medium used for the growth of the bacteria was Mueller Hinton agar:beef extract (3.0 g), acid hydrolyzate of casein (17.5 g), starch (1.5 g), and agar (12.0 g) per liter of water. Sterilization of glassware and Mueller Hinton medium was done in an American Sterilizer model 57-CR for 15 minutes at 121° and 15-20 p.s.i.

E. coli K31 culture was obtained from Dr. Ida Ørskov (WHO International Escherichia Center, Copenhagen). Actively growing colonies of E. coli K31 were propagated by replating several times on to Petri dishes (layered with sterile Mueller Hinton agar); a single colony being selected each time the bacteria were to be plated. Overnight growth of bacteria at 37°C was sufficient. Broth (100 mL) was inoculated with E. coli K31 bacteria and incubated for 4 hours. Actively growing E. coli K31 bacteria were poured onto a sterile, Mueller Hinton agar medium (in a metal tray 60 x 40 cm) and incubated for four days at 37°C. The K31 bacteria were scraped from the agar surface, diluted with 1% phenol solution and stirred at 4°C for 5 hours. The mixture of polysaccharide, bacterial cells and other debris was ultracentrifuged (for 4 hours at 15°C on a Beckman L3-50 ultracentrifuge using rotor 45 Ti at 31000 r.p.m. or 80000 g) to separate the dead bacterial cells from the

polysaccharide solution. The viscous honey-coloured supernatant was precipitated with ethanol. The resultant stringy precipitate was dissolved in water and treated with Cetavlon[193] (cetyltrimethylammonium bromide). The Cetavlon-polysaccharide complex was dissolved in 4M NaCl solution, precipitated into ethanol, redissolved in water and dialyzed against distilled water (two days). The polysaccharide was isolated as a styrofoam-like material by lyophilization. The isolated polysaccharide was further purified by gel permeation chromatography using a Bio-Gel P2 column (100 cm x 2.5 cm).

Sugar analysis and composition. Hydrolysis of a sample (20 mg) of K31 polysaccharide with 2 M trifluoroacetic acid (TFA) for 20 h at 95°, removal of excess acid by coevaporation with water, followed by paper chromatography (solvent 1) showed glucose, galactose, rhamnose, and an aldobiouronic acid. The neutral sugars released were analyzed as alditol acetates by g.l.c. column DB 17, programmed from 180° to 220°C at 5°/min.

A portion of this hydrolyzate was methylated[101] and subjected to g.l.c. using the program 145°, 1 min then 2°/min to 155°, 1 min, 3°/min to 220°, 1 min and then to 250°C at 3°/min. Under these conditions the permethylated monosaccharides were eluted between 1.9 and 4.9 min and the methylated aldobiouronic ester methyl glycoside at 16.0 min. When this component was analyzed by c.i.-m.s. a value of $[M + NH_4^+] = 456$ was obtained.

A portion (49.5 mg) of K31 polysaccharide (H^+ form) was dissolved in water (30 mL), 1-cyclo-hexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-

toluenesulfonate (CMC, 423 mg) was added and reduction was achieved by the addition of aqueous sodium borohydride (3M, ca 100 mL) with continuous adjustment of the pH to 7. The product (61 mg), isolated by dialyses and lyophilization, was analyzed as alditol acetates following hydrolysis with 2 M TFA.

Methylation analysis. The capsular polysaccharide (30 mg, H^+ form) was methylated by the method of Hakomori,[101] dialyzed, partitioned between dichloromethane and water and purified on Sephadex LH 20 to give a product which showed no absorption at 3625 cm^{-1}. Analytical results on this material are given in Table III.1, column I and on a portion which was refluxed overnight with lithium aluminum hydride in oxolane in Table III.1, column II.

Uronic acid degradation. A sample (25 mg) of methylated K31 polysaccharide was dried and then, with a trace of p-toluenesulfonic acid, was dissolved in 19:1 dimethyl sulfoxide--2,2-dimethoxypropane (12 mL) and the flask was sealed under nitrogen. Dimethylsulfinyl anion (5 mL) was added and allowed to react for 18 h at room temperature. Methyl iodide (3 mL) was added to the cooled reaction mixture and stirring was continued for 7 hours. The methylated degraded product was isolated by partition between chloroform and water and was then purified by gel permeation chromatography on Sephadex LH-20. The degraded product was hydrolyzed with 2 M TFA for 8 h at 95° and analyzed by g.l.c. (Table III.1, column IV).

Selective partial hydrolysis. Polysaccharide (60 mg) was hydrolyzed with 0.1 M TFA for 25 min at 95°C. After dialysis the retentate (PH) was methylated[101] followed by reduction of the carboxyl function with lithium aluminum hydride. The analytical results are presented in Table III.1, column IV.

Reaction with lithium in ethylenediamine. Dry K31 polysaccharide (150 mg) was suspended in dry ethylenediamine (21 mL) and six pieces of lithium wire (3 mm x 3 mm, hexane washed) were added. The intense blue color obtained was maintained for 1 h by the addition of smaller pieces of lithium. The reaction was terminated by the addition of dry methanol (4 mL) with the flask cooled in ice water. The excess ethylenediamine and methanol were removed *in vacuo* over sulfuric acid and sodium hydroxide. Glacial acetic acid (3 mL) was added to the residue, with external cooling to decompose lithium methoxide followed by the addition of an equal volume of water. The resultant product was purified by ion-exchange chromatography (Bio-Rad AG 50X8 resin) and gel permeation chromatography (Bio Gel P2). A pure oligosaccharide (F1) was isolated by preparative paper chromatography. Methylation data on F1 is reported in Table III.1, column VII. The proton n.m.r. spectrum showed signals at δ 5.25 (b, 1H), 5.13 (3 Hz, 1.2H), 4.99 (b, 1.3H), 4.95 (b, 0.2H), and 4.65 (8 Hz, 0.8H).

Hydrofluoric acid hydrolysis. The native polysaccharide was hydrolyzed by HF at -40°C for 15 mins and the products A2 (~8 mg) and A3 (~2 mg) were separated by paper chromatography. Methylation data for A2

are given in Table III.1, column V. G.l.c.-c.i.-m.s. of methylated A2 gave $[M + NH_4^+] = 472$ and methylated A3 gave $[M + NH_4^+] = 412$ (Fig. III.3)

Chromium trioxide oxidation. Acetylated polysaccharide (25 mg) was dissolved in acetic acid and treated with chromium trioxide (100 mg) for 2 h at 50°C. The results of methylation analysis of the product are shown in Table III.1, column VI.

Bacteriophage depolymerization. The phage was isolated from Vancouver sewage and propagated by tube and flask lysis to a concentration of 1.2×10^{11} p.f.u. mL^{-1}. Bacteriophage was added to an aqueous solution of 150 mg of K31 polysaccharide. Depolymerization was carried out for 5 h at 37°C after which the solution was heated to 85°C for 5 min and lyophilized. The crude product was deionized by passage through a column of Amberlite IR 120 (H^+) and the eluate was concentrated and added to a column of Bio-Gel P2 (400 mesh) which was eluted with water at 20 mL h^{-1}. The low molecular weight polymer (Pn) was collected between 133-150 mL to give 80 mg.

IV. STRUCTURAL STUDIES ON THREE *E. coli* CAPSULAR POLYSACCHARIDES USING MODERN N.M.R. TECHNIQUES

IV.1 Introduction

Until recently n.m.r. was used as a back-up technique in carbohydrate research. With the introduction of modern computers pulse n.m.r. techniques have been employed extensively in structural elucidation of carbohydrates. The advantages of using principally n.m.r. techniques in structural studies of carbohydrates are that the method:

(i) is non-destructive

(ii) requires smaller samples than chemical methods

(iii) is fast.

Capsular polysaccharides are immunogenic and the use of fast techniques, such as modern n.m.r. methods, in their structural elucidation may be essential. As an on-going process in this and other laboratories we wish to build a good data base and hence establish n.m.r. as a reliable routine technique for structural studies of polysaccharides. In this investigation, mostly low molecular weight polymer, generated by viral glycan depolymerization of natural capsular polysaccharide, was used for n.m.r. studies. The advantage of using these lower molecular weight polymers is that they give good resolution as well as n.O.e.'s.

IV.2 ^{1}H Chemical shift assignment of the sugar residues in E. coli K44 capsular polysaccharide

IV.2.1 Introduction

The structure of the capsular polysaccharide from Escherichia coli 08:K44(A):H- (K44 antigen) has been established[196] using the techniques of methylation β-elimination, deamination, Smith hydrolysis and n.m.r. spectroscopy.

4)β-D-GlcAp-(1→3)-α-L-Rhap-(1→4)-α-D-GlcpNAc-(1→6)-β-D-GalpNAc-(1→

This capsule is unique because it has two amino-sugar residues linked to each other as well as uronic acid in its repeating unit.

This is a model study to assign the ^{1}H chemical shifts of the sugar residues in the repeating unit of the K44 polysaccharide.

IV.2.2 Results and Discussion

The ^{1}H-n.m.r. spectrum of the native polysaccharide (Table IV.1) showed four anomeric proton signals:[198] an α-linkage at δ 4.95 ($J_{1,2}$ 3 Hz), two β-linkages at δ 4.72 ($J_{1,2}$ 8 Hz, 1H) and δ 4.60 ($J_{1,2}$ 8 Hz, 1H) and a borderline signal at δ 4.89 (b, 1H). In the ^{1}H-n.m.r. spectrum of the native polysaccharide, methyl protons of rhamnose appeared at δ 1.34 (b, 3H) and two signals attributed to methyl protons of the N-acyl groups were observed at δ 2.09 (s, 3H) and δ 2.06 (s, 3H), respectively.

Table IV 1: N.M.R. data for P1 and the native E. coli K44 polysaccharide.

Compound[a]	^{1}H-N.m.r. data				^{13}C-N.m.r.	
	δ	$J_{1,2}$ (Hz)	Integral proton	Assignment	P.p.m.	Assignment
$\underset{\beta}{\overset{4}{\text{GlcA}}}\underset{}{\overset{1}{}}\underset{\alpha}{\overset{3}{\text{Rha}}}\underset{}{\overset{1}{}}\underset{\alpha}{\overset{4}{\text{GlcNAc}}}\underset{}{\overset{1}{}}\underset{\alpha}{\overset{6}{\text{GalNAc}}}\underset{}{\overset{1}{}}\underset{\beta}{}$	4.96	3	1.0	$\overset{4}{\text{GlcNAc}}\underset{\alpha}{\overset{1}{}}$	175.80	C=O of GlcNAc and GalNAc
	4.89	b	1.0	$\overset{3}{\text{Rha}}\underset{\alpha}{\overset{1}{}}$	175.00	
K44 polyssaccharide (Na^+)	4.70	8	1.0	$\overset{4}{\text{GlcA}}\underset{\beta}{\overset{1}{}}$	172.30	C=O (GlcA)
	4.61	8	1.0	$\overset{6}{\text{GalNAc}}\underset{\beta}{\overset{1}{}}$	104.77	$\overset{4}{\text{GlcA}}\underset{\beta}{\overset{1}{}}$
	2.09	s	3.0	CH_3 of N-Ac (GlcNAc)	102.89	$\overset{6}{\text{GalNAc}}\underset{\beta}{\overset{1}{}}$
	2.06	s	3.0	CH_3 of N-Ac (GalNAc)	101.46	$\overset{3}{\text{Rha}}\underset{\alpha}{\overset{1}{}}$
	1.33	5	3.0	CH_3 (Rha)	98.90	$\overset{4}{\text{GlcNAc}}\underset{\alpha}{\overset{1}{}}$
					61.08	C-6 (GlcNAc)
					54.79	C-2 (GlcNAc)
					52.90	C-2 (GalNAc)
					23.21	N-$\overset{O}{\overset{\|}{C}}$-$CH_3$ (GalNAc)
					22.67	N-$\overset{O}{\overset{\|}{C}}$-$CH_3$ (GlcNAc)
					17.52	C-6 (Rha)

The ^{13}C-n.m.r. spectrum of the native polysaccharide showed (Table IV.1) a total of twenty eight carbon signals and these, coupled with the presence of only four anomeric carbon signals,[197] confirmed a tetrasaccharide repeating unit for the K44 polysaccharide. The three C=O signals in the downfield region of the spectrum (between 170-180 ppm) agree with the presence of two acetamido and one carboxyl group associated with the polysaccharide. The signals at 54.84 ppm and 53.08 ppm can be attributed to C-2 of glucos- and galactos-amine. In the upfield region of the spectrum, three signals corresponding to the methyl carbon of the two N-acyl groups and the rhamnose residue were observed. The anomeric configuration of the sugar residues in the repeating units were established[196] by a proton-coupled ^{13}C-n.m.r. experiment on the native polysaccharide.

^{1}H chemical shift assignments: The sugar residues were arbitrarily labelled A to D in the order of decreasing chemical shift of their H-1 resonances. H-1 and H-2 resonances of all the sugar residues were established by a COSY n.m.r. experiment (Fig. IV.1 and Table IV.2). H-3 and H-4 of residue C were established by one and two step relay experiments (Figs. IV.2, IV.3 and Table IV.2). Complete assignment of ^{1}H resonances of the rhamnosyl residue was achieved by taking F_2 slides in the F_1 dimension and walking through the 2D COSY spectrum. H-3, H-4 and H-5 of residue D were established by walking through a 2D COSY spectrum (Fig. IV.1). H-6 and H-6' of residue A were established from a heteronuclear correlated experiment. This assignment made possible for the assignment of H-5, H-4 and H-3 of residue A.

Table IV.2: ^{1}H N.m.r. data for E. coli K44 native polysaccharide

Symbol	Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6/H-6'
A	→4)GlcNAc1→	4.94	3.96	3.71	4.17	3.90	3.87/3.74
B	→3)α-Rha(1→	4.89	4.29	3.75	3.54	3.46	1.33
C	→4)β-GlcA(1→	4.71	3.46	3.64	3.82	-	-
D	→6)β-GalNAc(1→	4.58	3.95	3.78	3.63	3.47	-

Based on the composition and methylation results[196] for the native polysaccharide and on comparison of ^{1}H-n.m.r. data for the native polysaccharide with those of monosaccharide methyl glycosides, residue A was identified as the 4-linked α-glucosamine, residue B as the 3-linked α-rhamnose, C as the β-glucuronic acid and D as the 6-linked β-galactosamine.

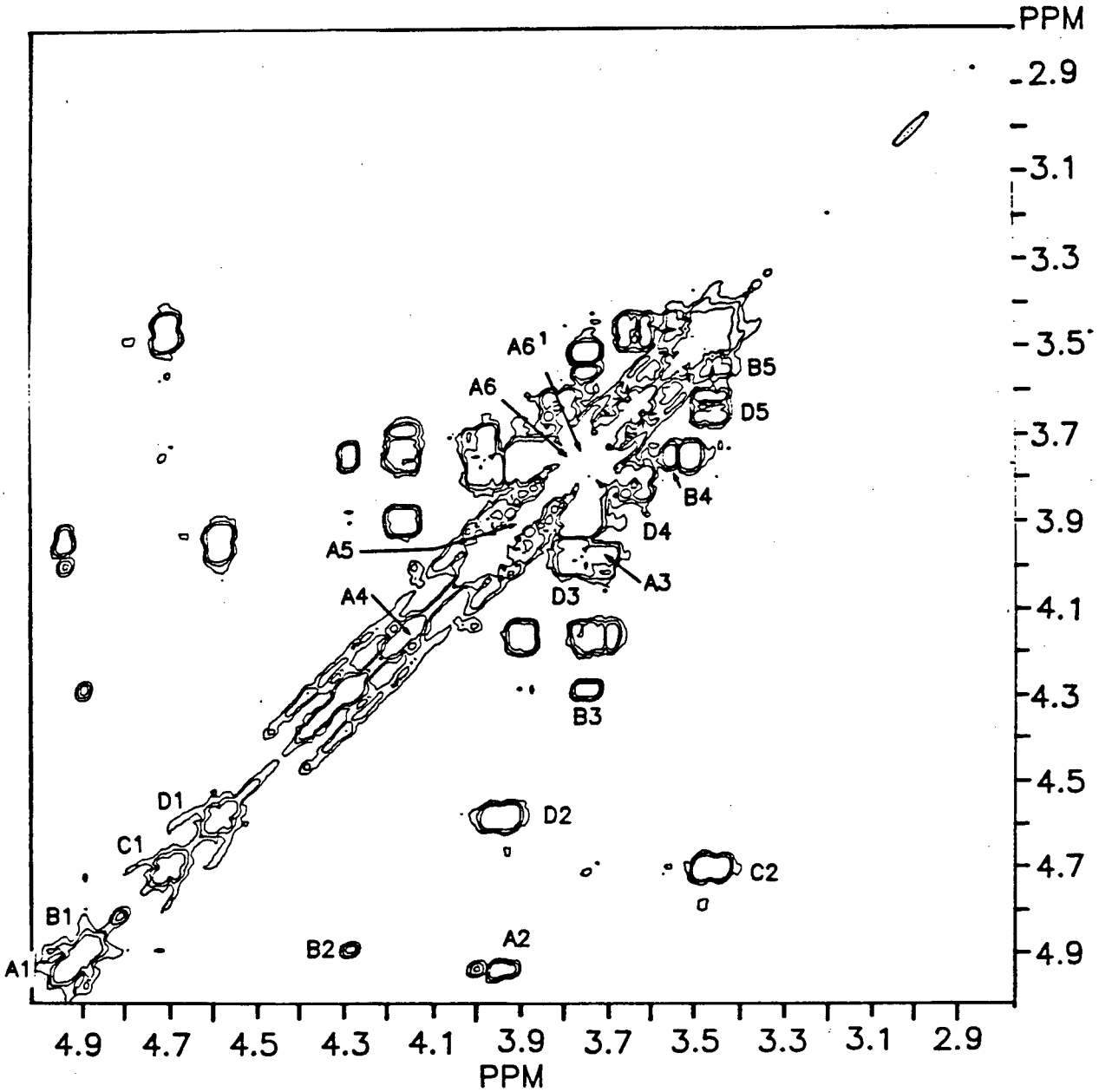


Fig. IV.1: Homonuclear ^{1}H-spin correlated (COSY) n.m.r. spectrum of native polysaccharide (K44).

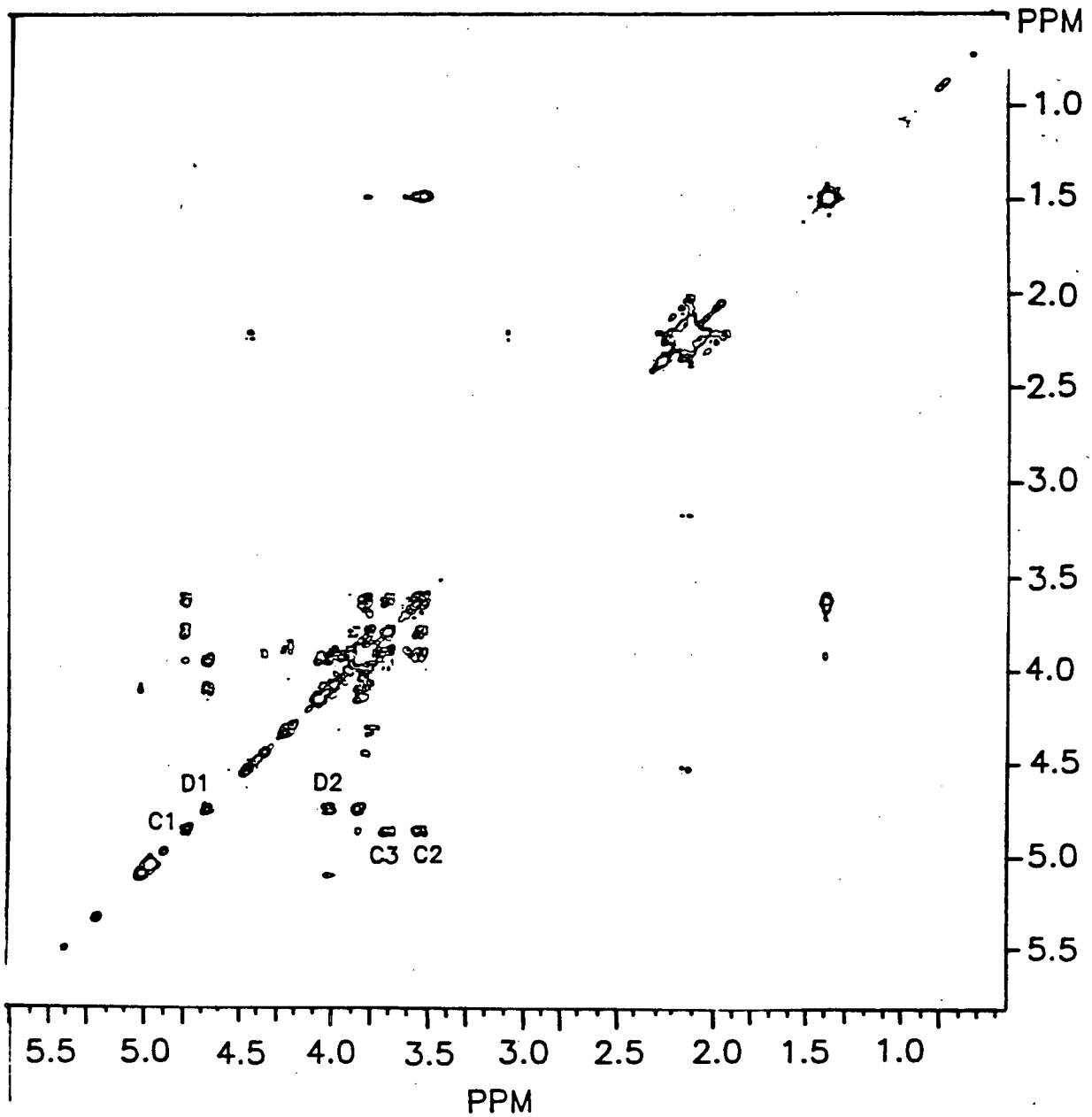


Fig. IV.2: One step ^{1}H-spin coherence transfer (COSY HGR1) n.m.r. spectrum of native polysaccharide (K44).

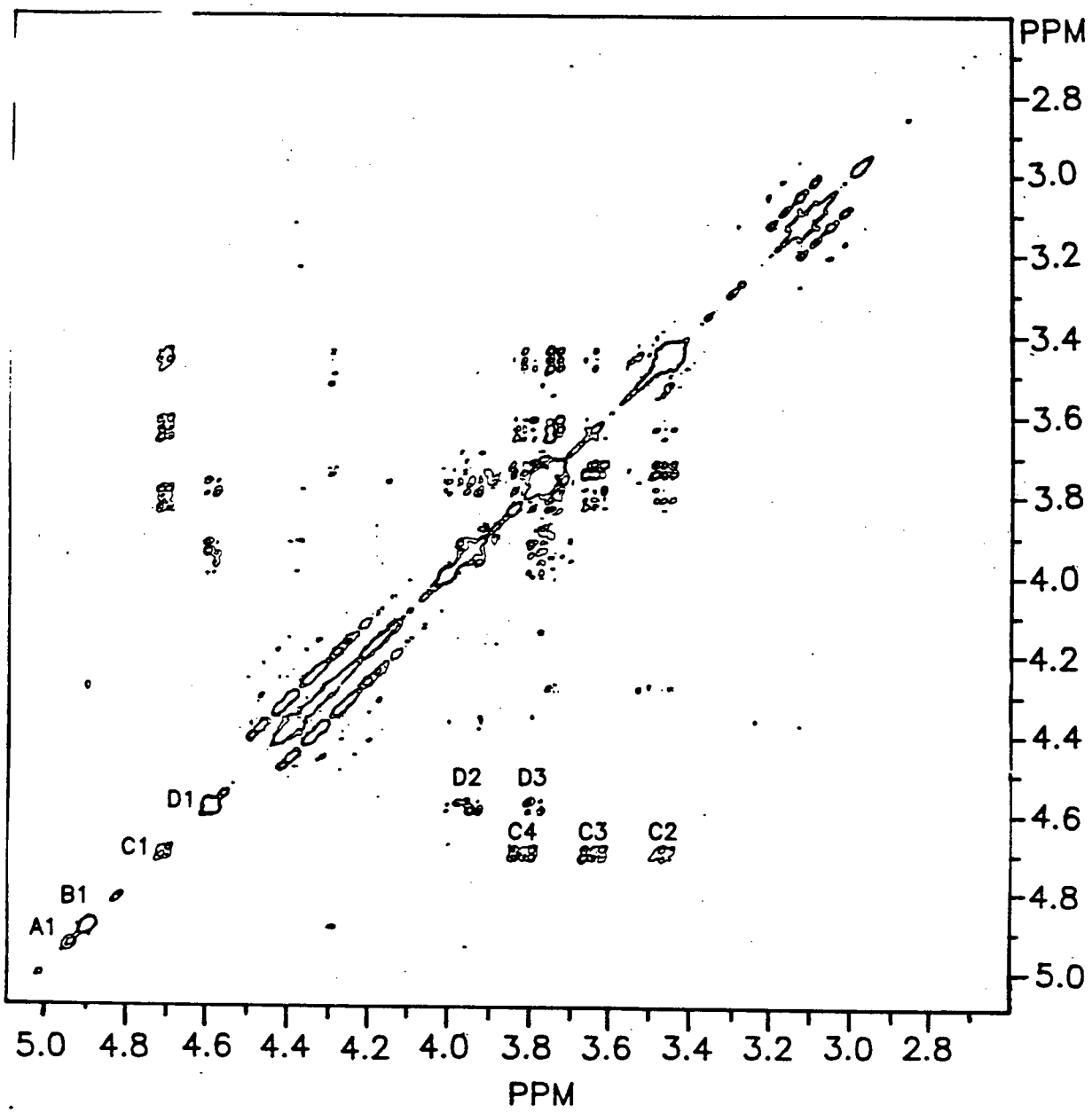


Fig. IV.3: Two step ^{1}H-spin coherence transfer (COSY HGR2) n.m.r. spectrum of native polysaccharide (K44).

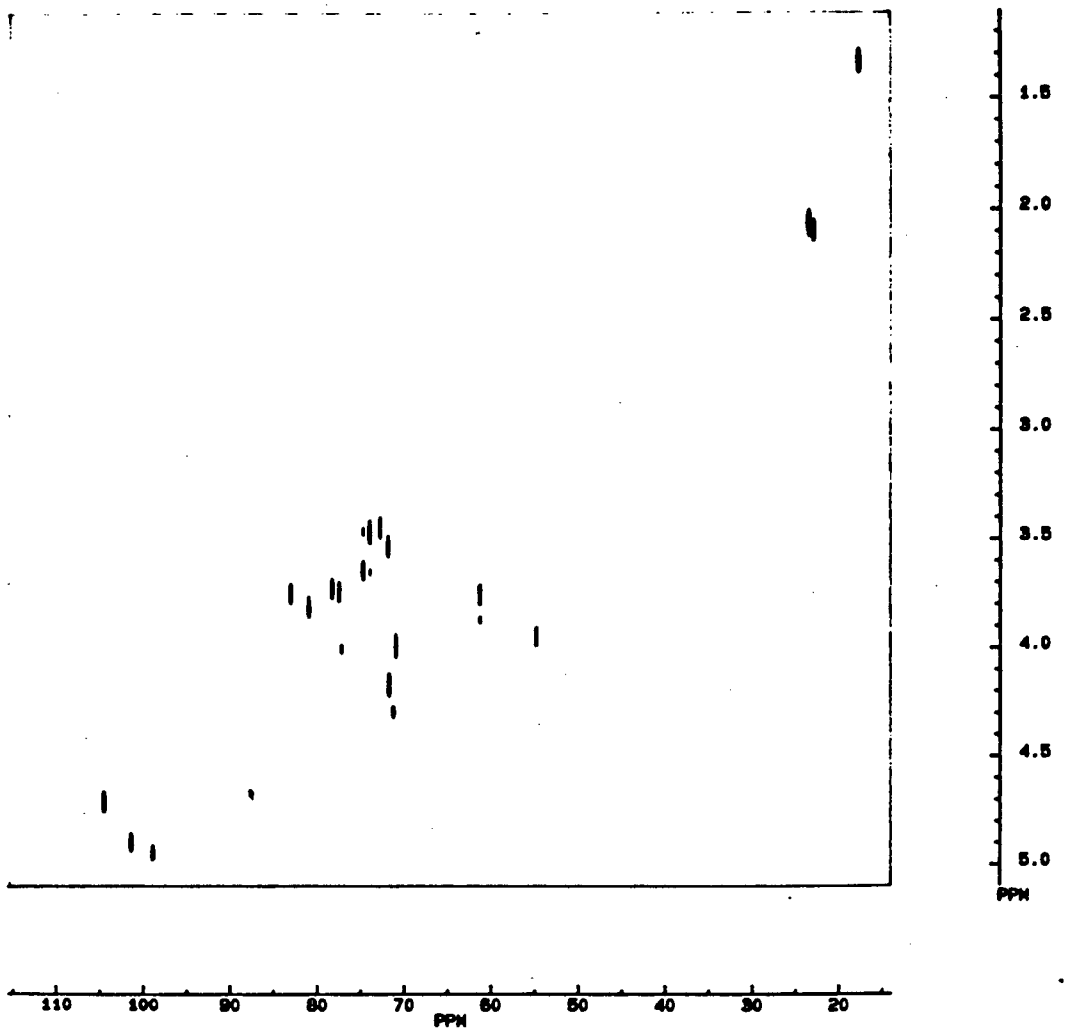


Fig. IV.4: Heteronuclear (^{13}C-^{1}H) correlated n.m.r. spectrum of native polysaccharide (K44)

IV.2.3 Experimental

General methods: Spectra were recorded for samples in 5 mm diameter tubes at 400 MHz using a Bruker WH400 spectrometer equipped with an Aspect 3000 computer. Homonuclear two-dimensional spectroscopy was performed using Bruker D15B871 software. All 2D experiments were performed with suppression of HOD resonance. All homonuclear experiments were performed with quadrature detection in the F1 dimension and a total of 256 t_1 increments of 96 or 112 scans each were recorded with minimum delay between pulses of 1.2 s and 1024 real data points in t_2. All 2D time domain data sets were symmetrized after Fourier transformation. Symmetrized 2D spectra were compared with the unsymmetrized one to check for any artifacts introduced during symmetrization.

COSY: Native polysaccharide (K44) was lyophilized twice from deuterium oxide and dissolved at a concentration of 410 mg mL^{-1}. The deuterium exchange was done at 338°K to enhance polysaccharide solubility. This sample was used for the 2D n.m.r. experiments which were conducted at 338°K. The 1D n.m.r. (^1H) experiment was done at 368°K and ^{13}C n.m.r. at 300°K. The pulse sequence used in the COSY experiment is given in section II.4.1 (appendix II). The acquisition parameters are given below:

Sweep width in F1 dimension (SW1) = 1000 Hz
Fixed delay to enhance effects from small J(D2) = 0.000003
Relaxation delay (D1) = 1.3 s
Delay for evolution of shut and coupling (D0) = 0.000003 (with increments of 0.5/SW1 for 256 times)
Pre-saturation with power S1 = 40L

Power S2 for evolution = 50L
Additional delay for switching (D3) = 0.002 s
90° excitation pulse (P1) = 19.5 μs
Mixing pulse (145°)/90° = 9.8 μs/19.5μs
Number of scans per experiment (NS) = 96
Number of experiments (NE) = 256

Relay COSY (1 step and 2 step): The pulse sequence for the relay COSY experiments is given in Appendix II. The acquisition parameters for the one step relay COSY n.m.r. experiment are as follows:

Sweep width in F1 dimension (SW1) = 1000 Hz
Relaxation delay (D1) = 1.2 s
Pre-saturation with power S1 = 30 L
Delay for switching (D3) = 0.000003
Power S2 for evolution = 45 L
Delay for evolution of shifts (D0) = 0.000003 (with increments of 0.5/SW1 for 256 times)
Second coherence period (D2) = 0.035, etc.
90° pulse creating XY-magnetization = 14.3 μs
180° pulse for refocus chemical shifts = 28.6 μs
Number of experiments (NE) = 256
Number of scans per experiment (NS) = 96

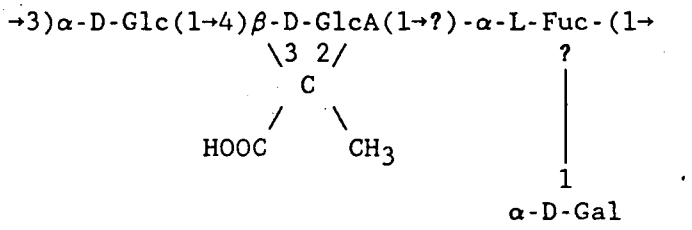
The acquisition parameters for the two step relay COSY n.m.r. experiment are illustrated below.

Sweep width in F1 dimension (SW1) = 1000 Hz
Relaxation delay (D1) = 1.5 s
Pre-saturation with power S1 = 30 L
Delay for switching (D5) = 0.002
Power S2 for evolution = 63 L
Delay for evolution of shifts (D0) = 0.000003 (with increments of 0.5/SW1 for 256 times)
Second coherence period (D2) = 0.035
Third coherence period (D3) = 0.035
90° pulse creating XY-magnetization (P1-PH2) = 14.3 μs
90° pulse applied to complete 1st coherence transfer (P1-PH2) = 14.3 μs
180° pulse to refocus chemical shifts (P2-PH2) = 28.6 μs
90° pulse applied to complete 2nd coherence transfer (P1-PH2) = 14.3 μs
90° pulse for third coherence transfer (P1-P4) = 14.3 μs
Number of experiments (NE) = 256
Number of scans per experiment (NS) = 96

IV.4 Sequencing and location of acetate in E. coli K33 capsular polysaccharide using N.M.R.

IV.4.1 Introduction

The capsular (K) antigen of Escherichia coli K33 has been designated as heat stable (type A) and may, therefore, in the absence of amino sugars, be expected to resemble those of Klebsiella. E. coli K33 has been shown to cross react with Klebsiella K58. In collaboration with Dr. B.A. Lewis, structural studies [195] using conventional chemical techniques gave a partial structure of K33 polysaccharide



In this section of the thesis, the use of 2D n.m.r. spectroscopy as a convenient method for the location of acetate in the polysaccharide is demonstrated. The use of 2D n.m.r. for sequencing the sugar residues in the repeating unit of the polysaccharide is also reported.

IV.4.2 Results and discussion

^{13}C-N.m.r.: ^{13}C-n.m.r. data of the native polysaccharide are given in Table IV.3. The ^{13}C broad band ^{1}H decoupled n.m.r. spectrum (75 MHz) of the deacetylated polysaccharide (Fig. IV.5 and Table IV.3) showed

Table IV.3: ^{13}C N.m.r. data of K33 polysaccharide and derived product

Product	Chemical shift (ppm)	Assignments
Native polysaccharide	174.74	CO of ketal pyruvic acid
	174.70	CO of acetyl group
	174.07	CO of GlcA
	104.60	C-1 of β-GlcA
	101.40	
	101.30	
	100.30	
	99.50	
	97.73	
	62.64	C-6 of β-Gal
	61.14	C-6 of α-Glc
	23.58	CH_3 of acetyl group
	21.27	CH_3 of pyruvic acid ketal
	16.07	CH_3 of Fuc
Deacetylated polysaccharide	174.70	CO pyruvic acid ketal
	173.14	CO of GlcA
	104.48	—GlcA-α
	101.23	—Gal-α
	100.11	—Fuc-α
	99.98	—Glc-α
	62.46[a]	C-6 of Gal
	60.89[a]	C-6 of Glc
	22.99	CH_3 of pyruvic acid ketal
	16.10	CH_3 of Fuc

[a] These assignments have been confirmed by the attached proton test (ATP) experiment

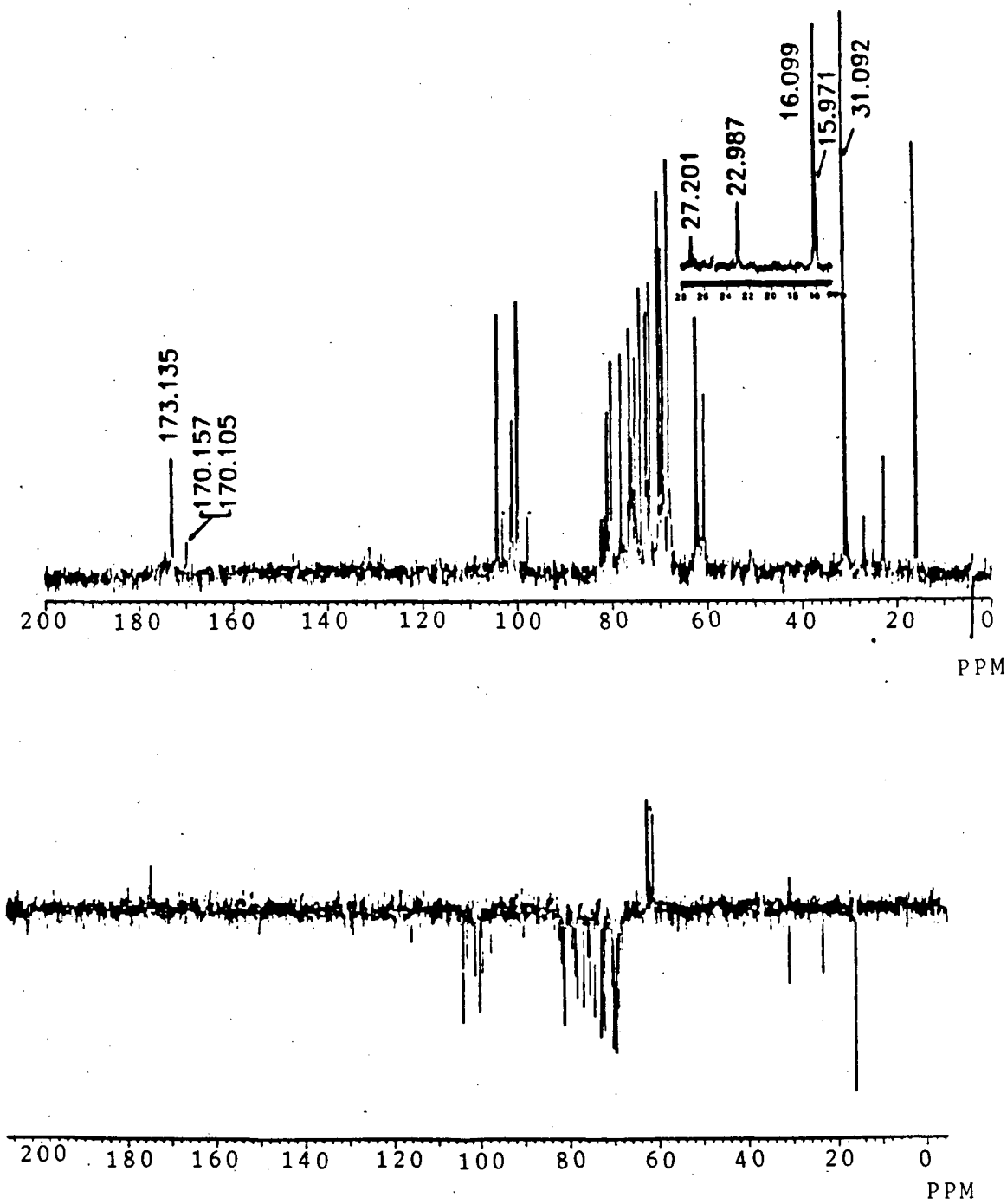


Fig. IV.5: ^{13}C N.m.r. spectra of deacetylated polysaccharide (K33)
(a) Broad band decoupled
(b) Attached proton test experiment (ATP)

* incomplete deacetylation

four major signals for anomeric carbons at 104.476, 101.225, 100.107 and 99.976 ppm. Among other ^{13}C signals, are ^{13}C signals at 22.987 and 16.099 ppm corresponding to CH_3 of pyruvate and fucose residues respectively. The C-6 methylene carbons were clearly differentiated from methine ring carbons in the ^{13}C attached proton test (APT) n.m.r. spectrum (Fig. IV.5). The ^{13}C signals at 174.70 and 173.135 ppm correspond to carbonyl carbons of pyruvate and glucuronic acid residues respectively.

A proton-coupled ^{13}C-n.m.r. spectrum of the deacetylated, depyruvylated polysaccharide (see spectrum in Appendix III) showed signals at 104.46 ppm ($^1J_{C1,H1}$ 162.7125 Hz), 101.214 ppm ($^1H_{C1,H1}$ 174.1875 Hz), 100.103 ppm ($^1J_{C1,H1}$ 171.1425 Hz) and 99.476 ($^1J_{C1,H1}$ 171.7575 Hz) (Table IV.4). The $^1J_{C1,H1}$ values reported for the α and β glycosidic linkages of 6-deoxy and hexopyranoses are ~169 and ~160 Hz respectively.[68,69] Hence there are one β and three α linkages in the repeating unit of the K33 polysaccharide. 2D N.m.r. experiments (see later) afforded the assignment of the glucuronic acid anomeric linkage as β and the rest of the sugar residues in the repeating unit as α.

^1H Chemical shift assignments (deacetylated polysaccharide). The sugar residues were arbitrarily labelled by their H-1 resonances. H-1, H-2, H-3 resonances of all the sugar residues were established by COSY (Fig. IV.7) and one relay COSY (Fig. IV.8) n.m.r. experiments. The H-4 resonance of the glucuronic acid residue was assigned from the two step relay COSY spectrum (Fig. IV.9). The H-5 resonance (δ 4.0096) was located based on the diagonal cross peak for H-4 (δ 3.9162) in the COSY

Table IV.4: ^{13}C-^{1}H Coupled n.m.r. experiment data

Chemical shift ^{1}H-decoupled resonance) ppm and sugar residue	Chemical shift ^{1}H-coupled		Chemical shift difference between downfield & upfield resonance	^{13}C-H coupling constant $^{1}J_{CH}$	Anomeric configuration
	downfield resonance	upfield resonance			
104.46 (Glc A)	105.2246	103.0548	2.17	162.71	β
101.21 (Gal)	101.9939	99.6715	2.32	174.19	α
100.10 (Fuc)	100.8915	98.6096	2.28	171.14	α
99.48 (Glc)	100.3368	98.0467	2.29	171.76	α

spectrum. The H-5 signal for the fucose was assigned by the additional window provided by the methyl resonances.

Based on the composition, methylation results and comparison of the ^{1}H-n.m.r. data of the deacetylated polysaccharide with those for monosaccharide methyl glycosides, residue D was identified as 4-linked β-glucuronic acid, residue B as branched point fucose, residue A as 3-linked α-glucose, and residue C as terminal galactose.

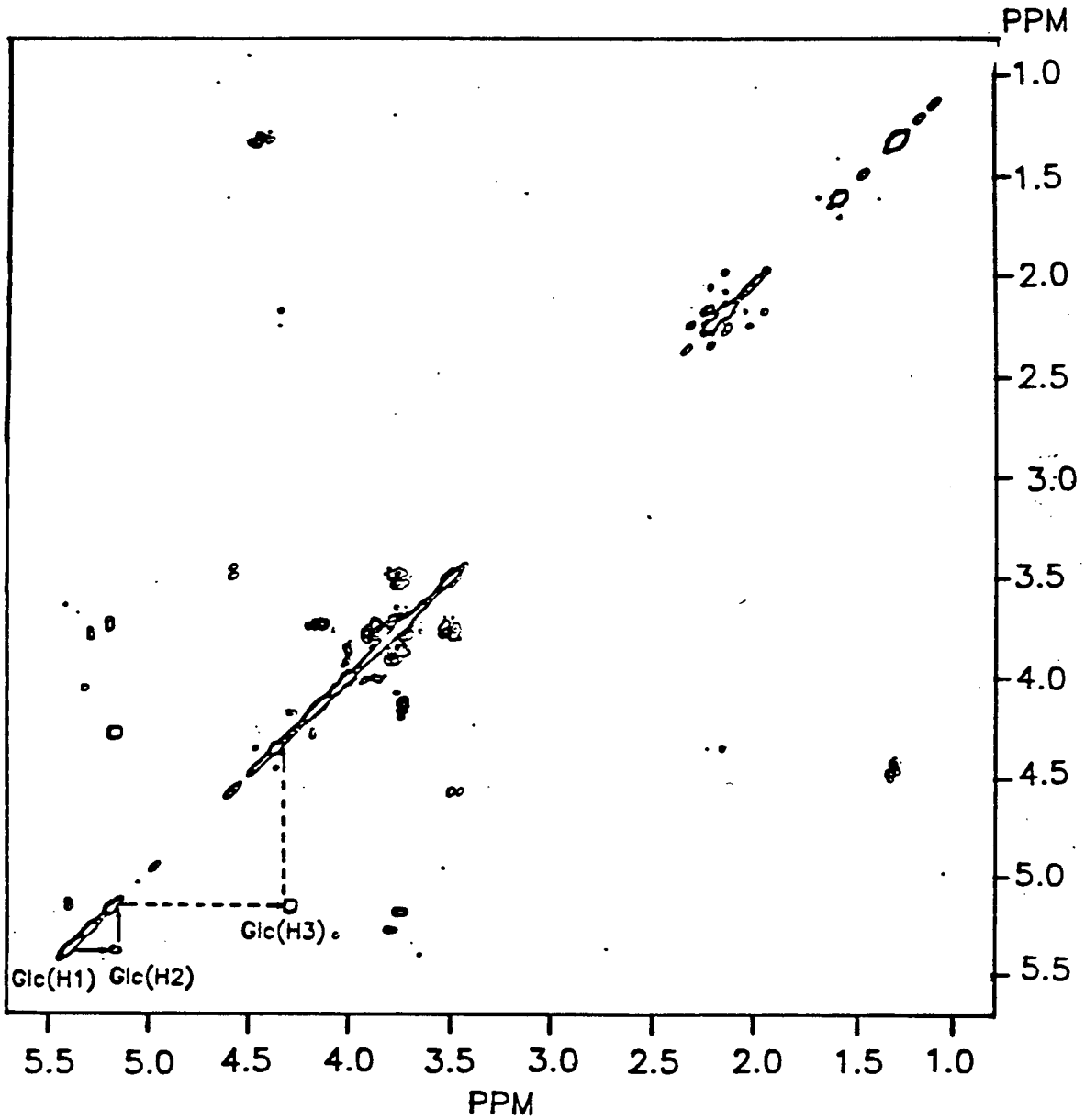


Fig. IV.6: Homonuclear ^{1}H-spin correlated (COSY) n.m.r. spectrum of 'native' polysaccharide (K33)

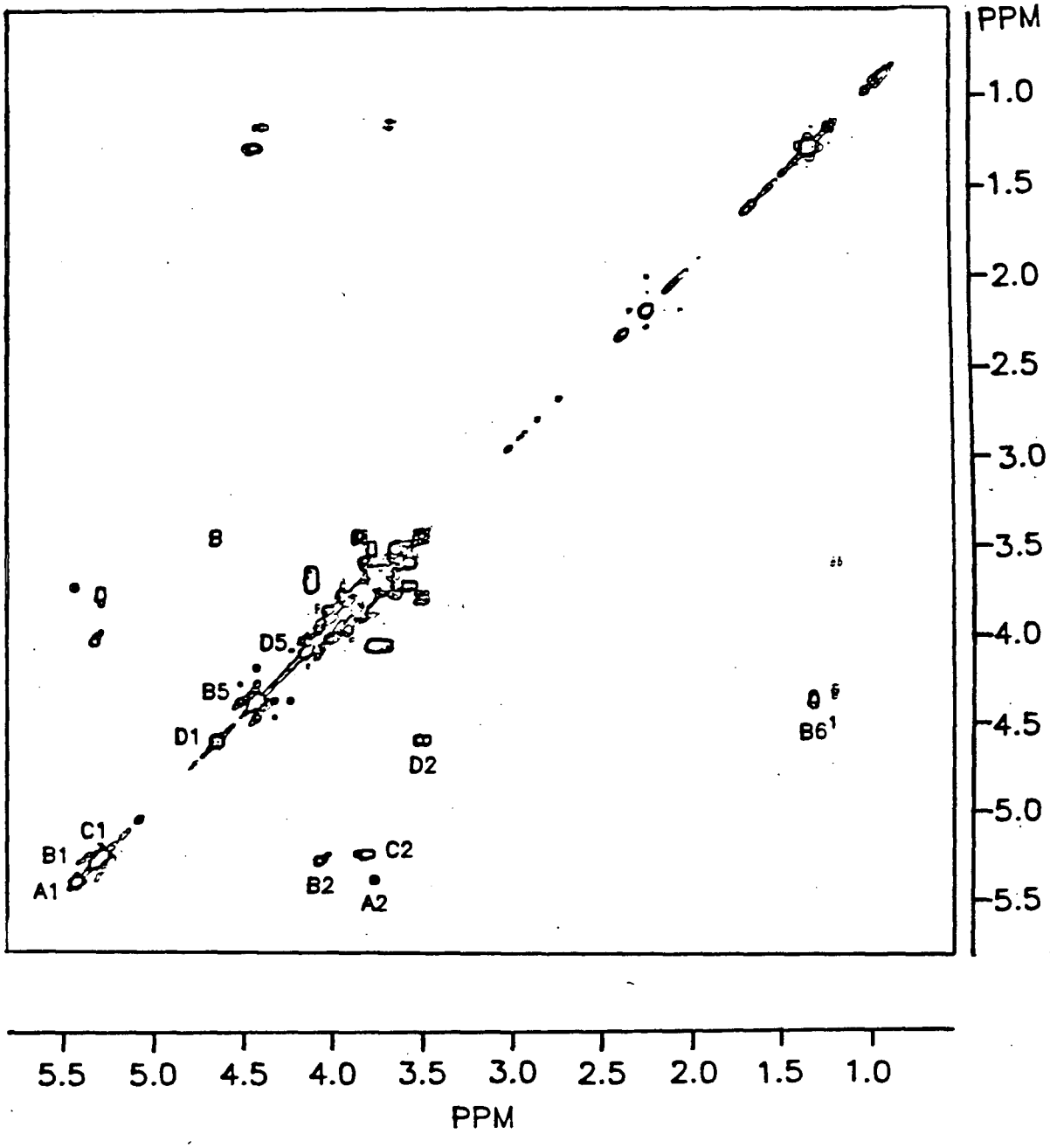


Fig. IV.7: Homonuclear ^{1}H-spin correlated (COSY) n.m.r. spectrum of deacetylated polysaccharide (K33)

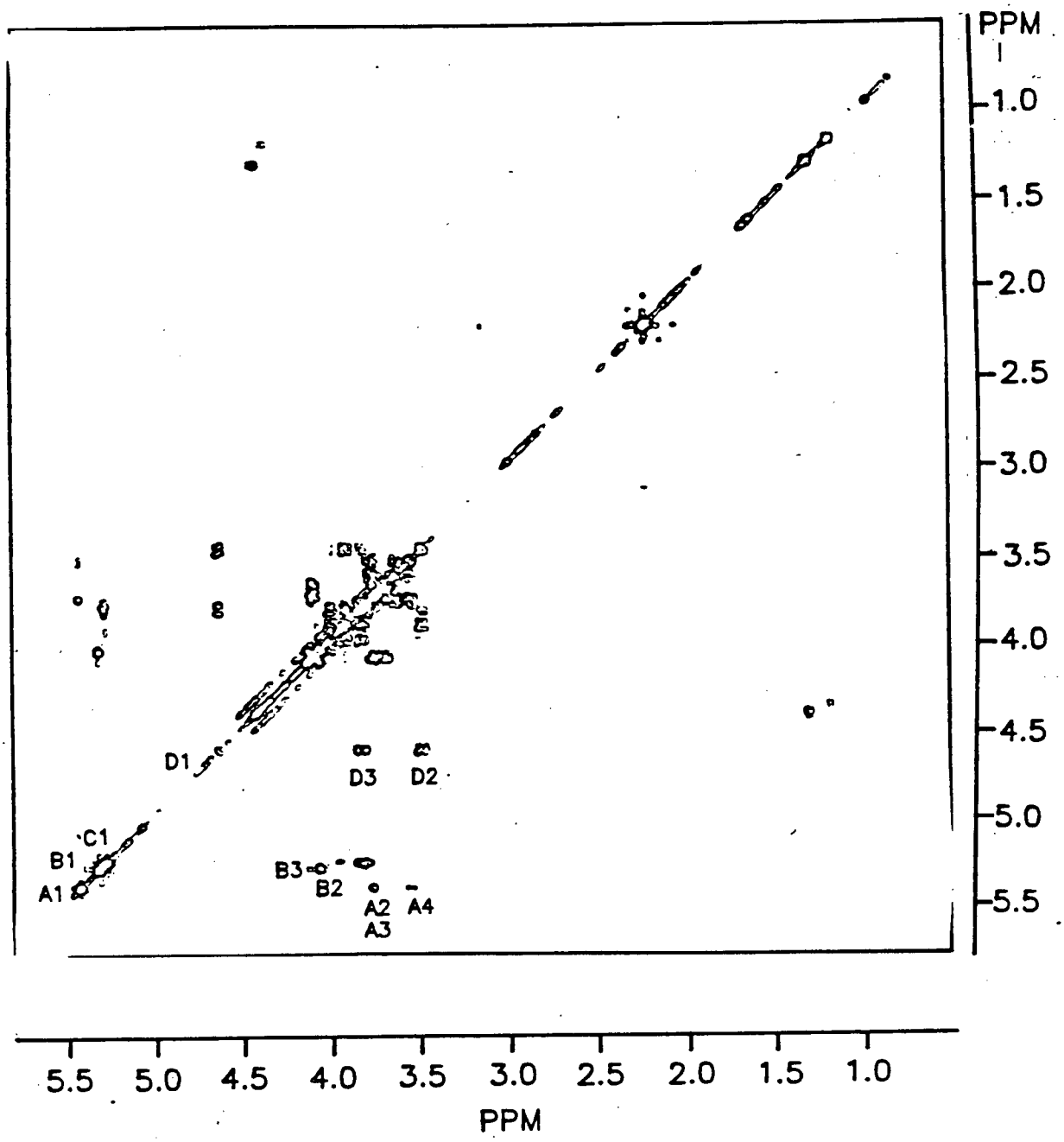


Fig. IV.8: One step relay ^{1}H spin coherence transfer (COSYHGR1) spectrum of deacetylated polysaccharide (K33)

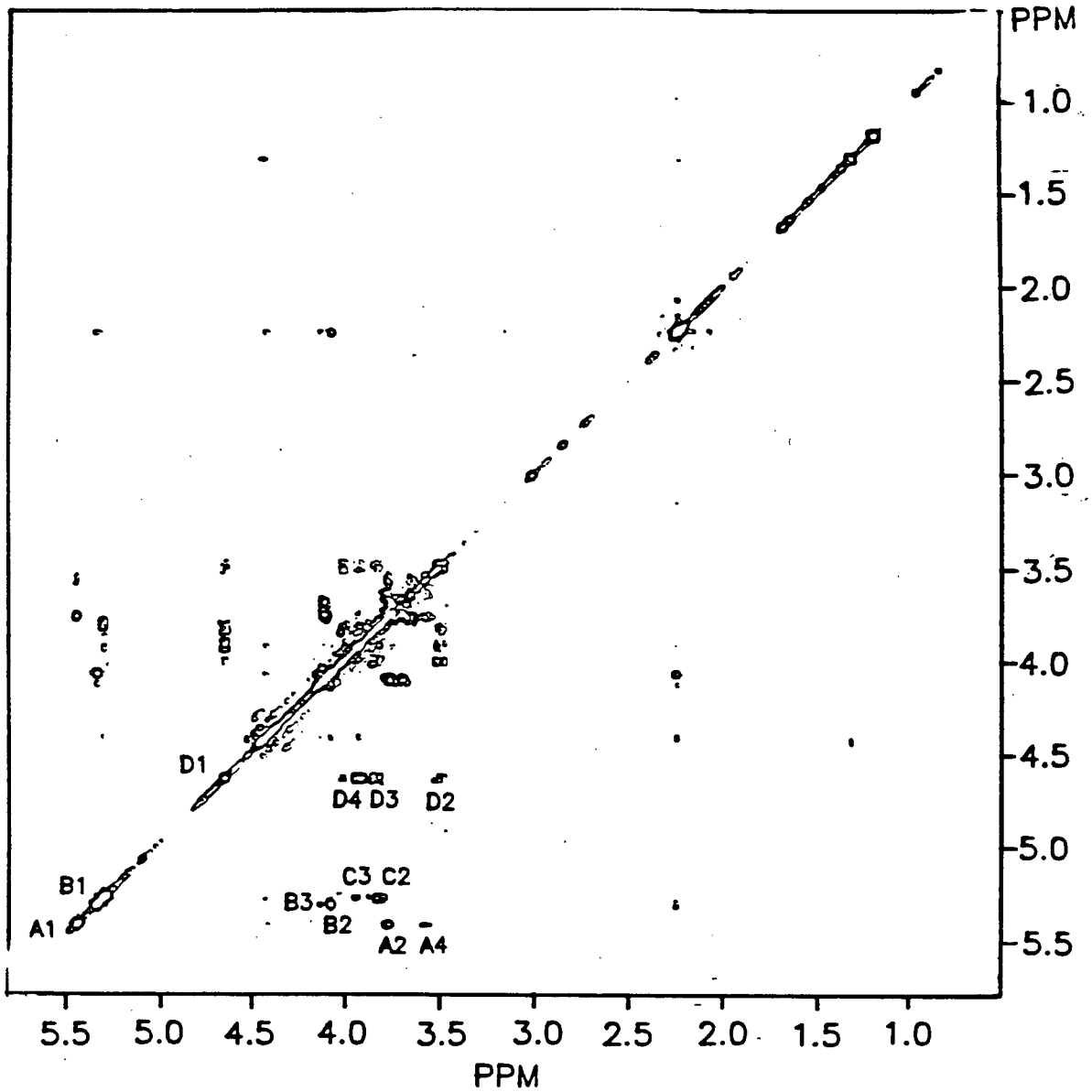


Fig. IV.9: Two step relayed ^{1}H spin coherence transfer (COSYHGR2) spectrum of deacetylated polysaccharide (K33)

Location of acetate. The glucose residue (A) shows a diagonal cross peak between H-1 (δ = 5.3780) and H-2 (δ = 5.1482) in the COSY spectrum (Fig. IV.6 and Table IV.6) of the native polysaccharide. This diagonal cross peak is however absent in the COSY spectrum (Fig. IV.7 and Table IV.5) of the deacetylated polysaccharide. This is an illustration that the acetyl group is located on position 2 of the glucose residue. The down field shift of a ring proton to the anomeric region (δ 6.00 to 4.5) due to acetylation is known.[65]

Sequencing. The sequence of the sugar residues in the polysaccharide back bone was obtained from a NOESY experiment[185] (Fig. VI.10 and Table IV.7). Interresidue n.O.e. contacts established that A was linked to D and that D was linked to B, thus giving the following partial sequence.

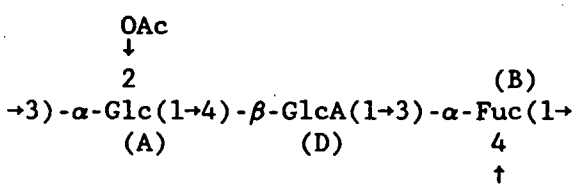


Table IV.5: ^{1}H-N.m.r. data for *E. coli* K33 deacetylated polysaccharide

Symbol	Sugar Residue	H1	H2	H3	H4	H5	H6/H6'
A	Glc	5.42	3.76	3.76	3.56	3.63	-
B	Fuc	5.28	4.06	4.12	-	4.42	1.32/1.30
C	Gal	5.27	3.81	3.95	-	-	-
D	GlcA	4.63	3.49	3.82	3.92	4.01	-

Table IV.6: ^{1}H-N.m.r. data 2D (COSY n.m.r. experiment) for *E. coli* K33 polysaccharide

Symbol	Sugar Residue	H1	H2	H3	H4
A	Glc	5.38	5.15	4.27	4.19
B	Fuc	5.28	4.06	-	-
-	-	5.25	3.78	-	-
-	-	5.16	3.72	-	-
D		4.56	3.47	-	-

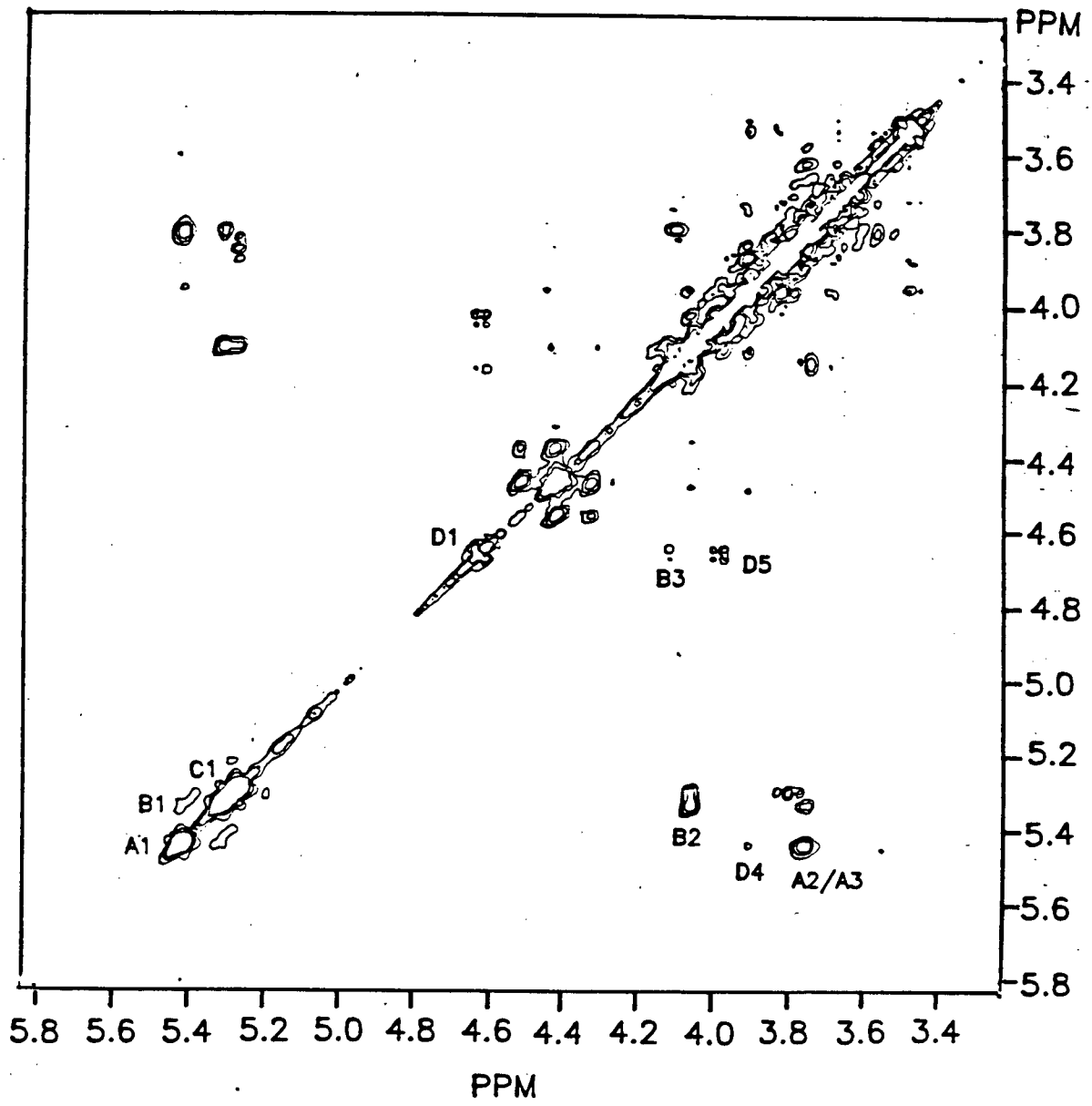


Fig. IV.10: **Homonuclear dipolar correlated 2D n.m.r. (NOESYHG) spectrum of deacetylated polysaccharide (K33) at 338°K**

Table IV.7: N.O.e. data for *E. coli* K33 polysaccharide

Sugar Residue	Symbol	Interresidue contact	Intraresidue contact
→3)-α-Glc-(1→	A	3.92, H-4 (D)	3.76, 3.56
→3)-α-Fuc(1→ 4 |	B	3.76 [a]	4.06
→1)-α-Gal	C	- [b]	3.81
→4)-β-GlcA(1→	D	4.12, H-3 (B)	4.01

[a] suspect H2 and H3 coinciding hence this n.O.e. contact could not be assigned

[b] poor resolution; the linkage between Gal and Fuc was established by β-elimination experiment

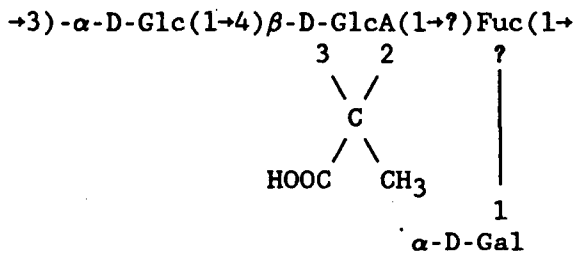
The COSY, relay COSY and NOESY n.m.r. experiments were repeated on another n.m.r. spectrometer (AM 400 MHz Bruker n.m.r. spectrometer) for the following reasons.

(a) to check the validity of n.O.e. contacts

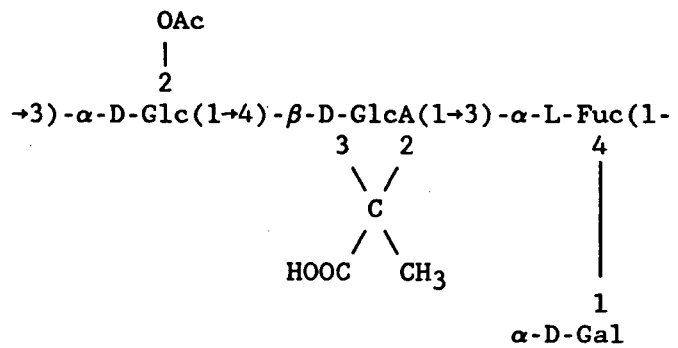
(b) to obtain additional sequencing information.

The sequencing information (Table IV.8, Table IV.9 and Fig. IV.11) is identical to what was previously obtained.

Chemical studies[195] conducted on this polysaccharide suggest the structure,



Based on 2D n.m.r. and chemical data we report the complete structure of E. coli K33 K antigen as:



The K antigens of E. coli K33 and Klebsiella K58 are similar and these two strains have been shown to cross react.

Table IV.8: ^{1}H-N.m.r. data for *E. coli* K33 deacetylated polysaccharide (on AM400 Bruker n.m.r. spectrometer and experiment performed at 300°K)

Symbol	Sugar Residue	H1	H2	H3	H4	H5
A	Glc	5.40	3.72	3.72	3.52	3.64
B	Fuc	5.28	4.05	4.12	3.93	-
C	Gal	5.23	3.82	3.93	-	-
D	GlcA	4.58	3.46	3.79	3.88	-

Table IV.9: N.O.E. data for *E. coli* K33 deacetylated polysaccharide (on AM400 Bruker n.m.r. spectrometer and experiment performed at 300°K)

Sugar Residue	Symbol	Interresidue contact	Interresidue contact
→3)-αGlc(1→	A	3.88 H-4 (D)	3.72
→3)-α-Fuc(1→ 4 ↑	B	3.72[a]	4.01
→1)-α-Gal	C	- [b]	3.81, 3.75
→4)-β-GlcA(1→	D	4.12 H-3 (B)	3.96

[a] suspect H2 and H3 coinciding and this n.O.e. contact could not be assigned

[b] poor resolution

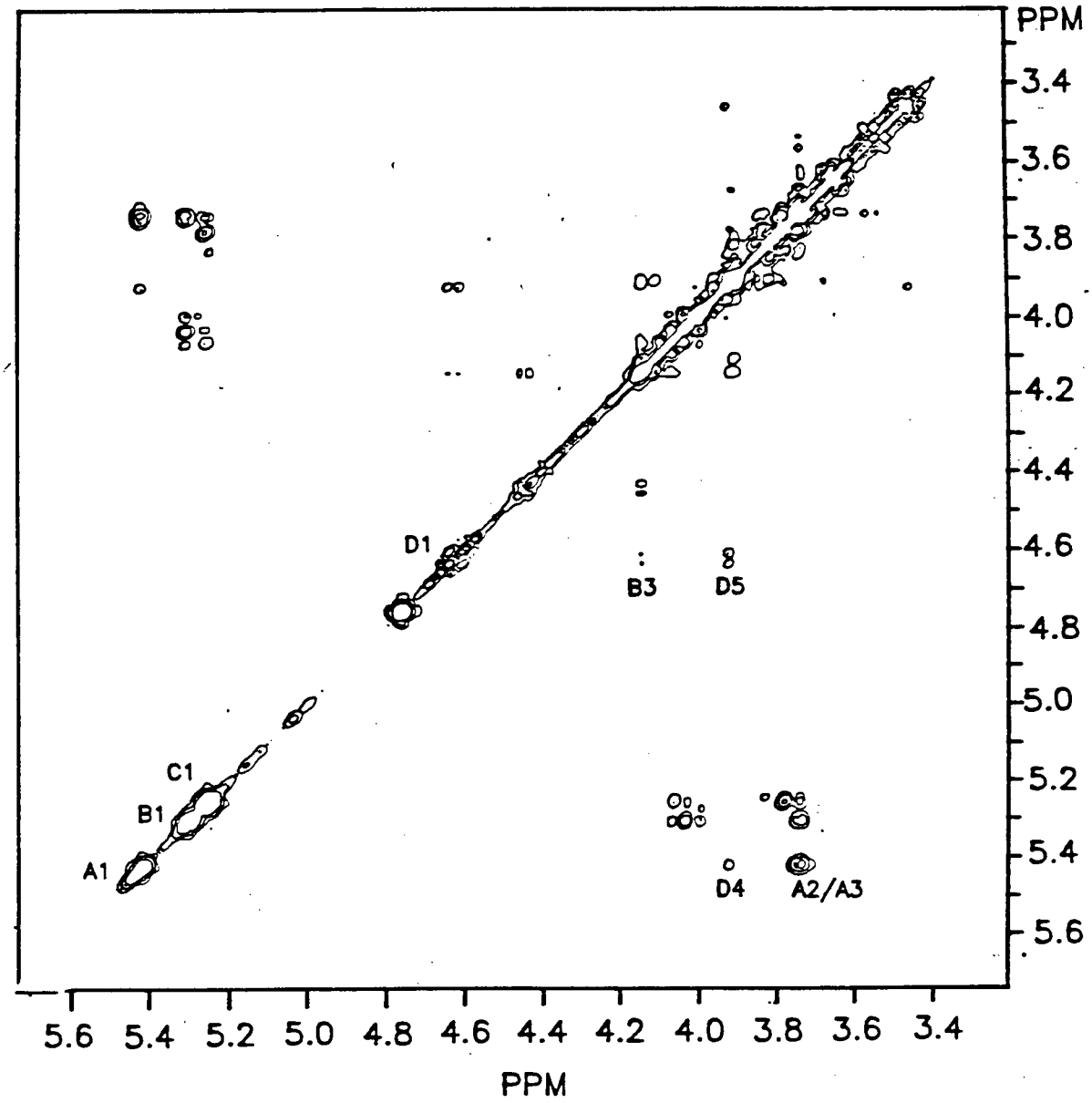


Fig. IV.11: Homonuclear dipolar correlated 2D n.m.r. (NOESYHG) spectrum of deacetylated polysaccharide(K33) at 300°K

IV.3.3 Experimental

General methods. The general experimental procedures are as described in Section III.1.3.

Isolation and purification of E. coli K33 capsular polysaccharide. E. coli K33 culture was obtained from Dr. Ida Ørskov, WHO International Escherichia Centre, Copenhagen). Actively growing colonies of E. coli K33 were propagated and grown as described in Section III.1.3. Isolation and purification of polysaccharide were done as previously described (Section III.1.3).

Deacetylation. 150 mg of K33 native polysaccharide was dissolved in 0.1 M NaOH solution and heated at 80°C for 4 h. The resultant product was dialyzed (mol. wt. cut off 3500) against distilled water, followed by lyophilization of the retentate. The retentate was purified by gel permeation chromatography (Bio-Gel P2, column size 92 cm x 2.6 cm).

Depyruvylation. 70 mg of deacetylated polysaccharide was dissolved in 0.1% acetic acid and heated at 80°C for 20 h. The resultant product was dialyzed and the retentate was lyophilized. The resultant product was purified on a Bio Gel P2 column (92 cm x 2.6 cm).

N.m.r. studies. General methods are as discussed in Section IV.2.3.

COSY Experiment on native polysaccharide. Native polysaccharide was

lyophilized twice from deuterium oxide and dissolved at 20 mg mL^{-1}. The experiment was performed at 338°K. The acquisition parameters were identical to those of the COSY experiment in Section IV.2.4 apart from using 32 transients per experiment.

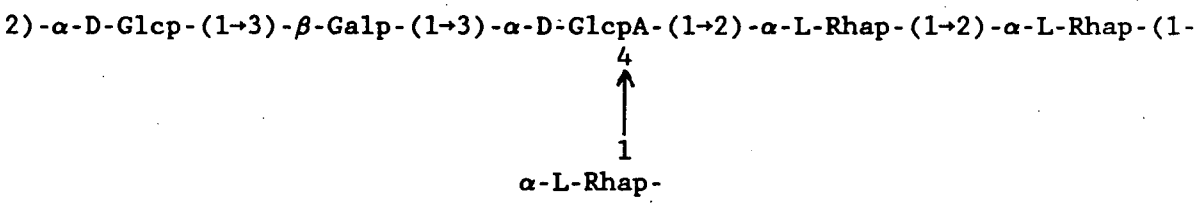
2D Experiment on deacetylated polysaccharide. K33 native polysaccharide (100 mg) was dissolved in 0.1 M NaOH and heated at 80°C for 6 h. Dialysis (mol. wt. cut off 3500) against distilled water was followed by lyophilization of the retentate which was further purified by gel permeation chromatography. The resultant product was lyophilized twice from deuterium oxide and dissolved at a concentration of 30 mg mL^{-1}. This sample was used for all 2D n.m.r. and ^{13}C-n.m.r. experiments for deacetylated polysaccharide. The acquisition parameters for the COSY and relay COSY experiments were identical to those given in Section IV.2.4. A number of NOESY experiments were performed and the best results were attained using a mixing time (D9) of 300 ms with no random variation (i.e. V9 = 0). The other NOESY parameters are:

Sweep width in F1 dimension (SW1) = 920 Hz
Relaxation delay (D1) = 1.2 s
Pre-saturation with power S1 = 40 L
Delay for evolution of shifts (D0) = 0.0002 (with increments of 0.5/SW1 for 256 times)
90° excitation pulse (P1) = 16μs
Mixing pulse 90° (P2) = 16 μs
Detection pulse 90° (P3) = 16 μs
Number of scans per experiment (NS) = 112
Number of experiments (NE) = 256

IV.4 Sequencing of a hexasaccharide repeating unit (*E. coli* K31 polysaccharide) by homonuclear 2D n.m.r. spectroscopy

IV.4.1 Introduction

The capsular antigen of *Escherichia coli* K31 has been designated[30] as heat stable (type A) and may, therefore, in the absence of amino sugars be expected to resemble those of *Klebsiella*. A partial structure of *E. coli* K31 has been proposed earlier by other workers.[31] Structural studies on this polysaccharide using conventional chemical methods suggested the repeating unit given below.



This structure was confirmed by 2D n.m.r. studies on lower molecular weight polymer (Pn) generated by bacteriophage glycanase degradation of the native polysaccharide.

IV.4.2 Results

A polysaccharide (Pn) of significantly lower molecular weight and viscosity than the native polysaccharide which was prepared by depolymerization with a viral endoglycanase was used in all the 2D experiments.

The sugar residues were arbitrarily labelled A to F in order of decreasing chemical shift of their H-1 resonances. The results of COSY[163] (Fig. IV.12 and Table IV.10) afforded the assignments of H-1 and H-2 resonances of all the sugar residues in the repeating unit. Further assignments were made using COSY data after interpreting the data for the relay COSY experiments. One and two step relay COSY spectra (Figs. IV.13, IV.14 and Table IV.10) afforded the assignment of H-3 and H-4 resonances of most of the sugar residues in the repeating unit. The assignment of the ^{1}H signals for the α-6-deoxy residues A, C, and E were greatly facilitated by the additional window provided by the methyl resonances. Thus following the cross-peaks from H-1 of A, C and E the respective H-2, H-3 and H-4 resonances were established while from the H-6 resonances, the H-5 resonances and their respective connectivities to H-4 were established (Fig. IV.12). The assignment of the resonances for the glucuronic acid residues was likewise facilitated by the easily recognized H-5 doublet (^{3}J 4.0 Hz) at δ 4.3538 (Figs. IV.12 and IV.16). Only the resonances of F could not be completely assigned.

Based on the composition and methylation results for the native polysaccharide and on the comparison of the ^{1}H-n.m.r. data for Pn with those for monosaccharide methyl glycosides, residue E was identified as the terminal α-rhamnose, residues A and C as 2-linked α-rhamnoses, B as the 2-linked α-glucose residue, D as the 3,4-linkaged α-glucuronic acid and F as the 3-linked β-galactose residue.

The sequence of the sugar residues in the repeating unit was confirmed by data from a NOESY experiment[185] (Table IV.11). Interresidue n.O.e. contacts[186] established that A was linked to B, D to C, and E and

Table IV.10: ^{1}H-N.m.r. data for *E. coli* K31 polysaccharide

Symbol	Residue	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
A	2-Rhaα-	5.25[a] (<1)	4.11	3.86	3.50	3.71	1.32	
B	2-Glcα-	5.13 (3)	3.69	3.96	3.50	3.95	3.78	3.86
C	2-Rhaα-	5.10 (<1)	4.10	3.89	3.56	3.75	1.32	
D	3,4-GlcAα-	5.03 (3)	3.86	4.13	3.77	4.35	-	
E	Rhaα-	4.84 (<1)	3.96	3.85	3.42	4.42	1.29	
F	3-Galβ-	4.82	3.66	3.74	4.09	-	-	

[a] Determined at 400 MHz, measured from internal acetone at δ 2.23. Spectra were recorded at 343°K. The sample used was the low M.W. polymer Pn, see text for details.

Table IV.11: N.O.E. data for *E. coli* K31 polysaccharide

Sugar Residue	Symbol	Inter-residue contact[a]	Inter-residue contacts
2-Rhaα-	A	3.69, H-2 (B)	4.11
2-Glcα-	B	3.74, H-3 (F)	3.69
2-Rhaα-	C	-	4.10
3,4-GlcAα-	D	4.10, H-2 (C)	3.86
Rhaα-	E	3.79, H-4 (D)	3.96
3-Galβ-	F	4.13, H-3 (D)	3.66[b]

[a] See footnote a, Table II.

[b] May be due to scalar correlation effect.

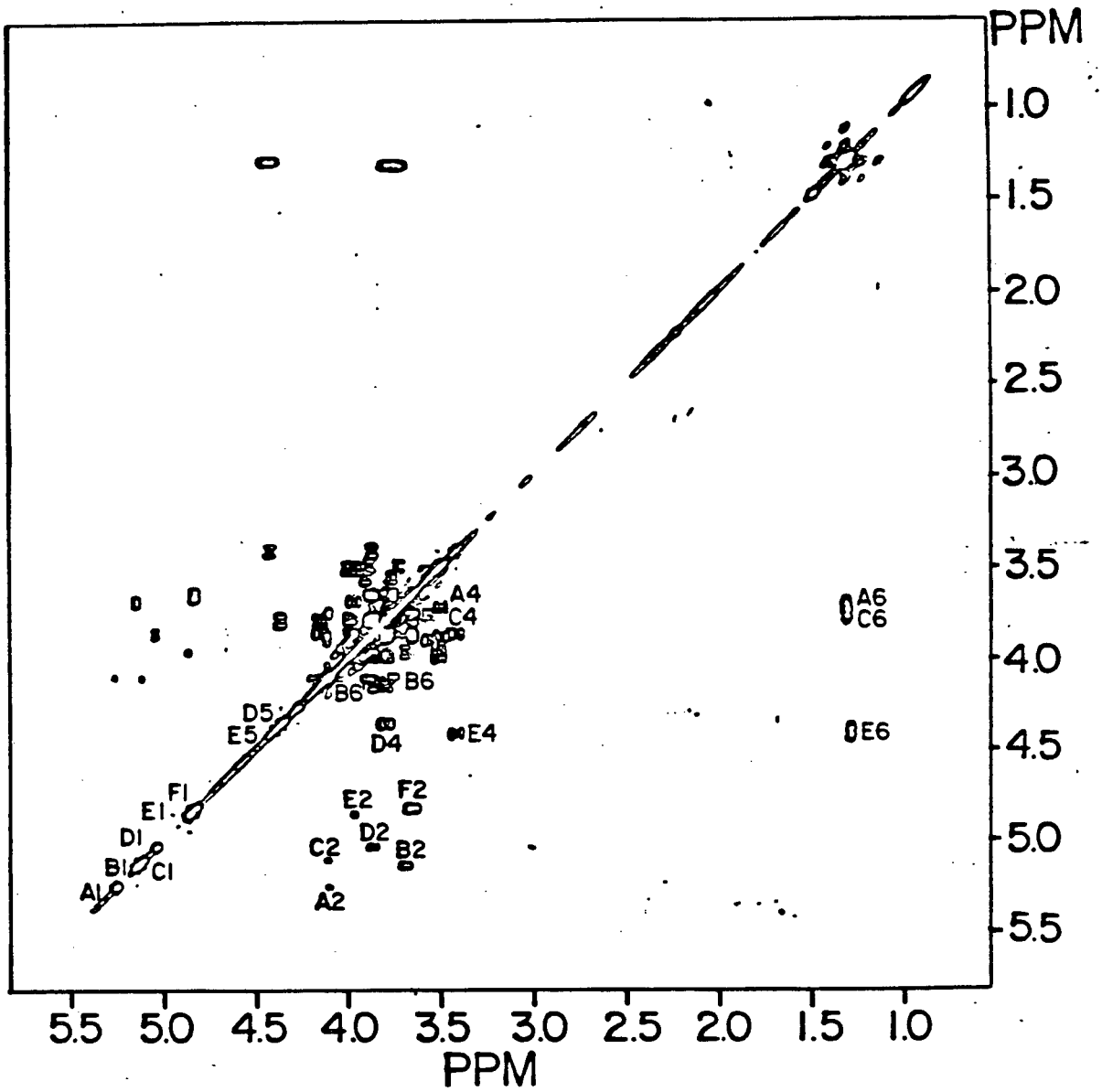


Fig. IV.12: 2D H-H spin-correlated (COSYHG45) spectrum of Pn

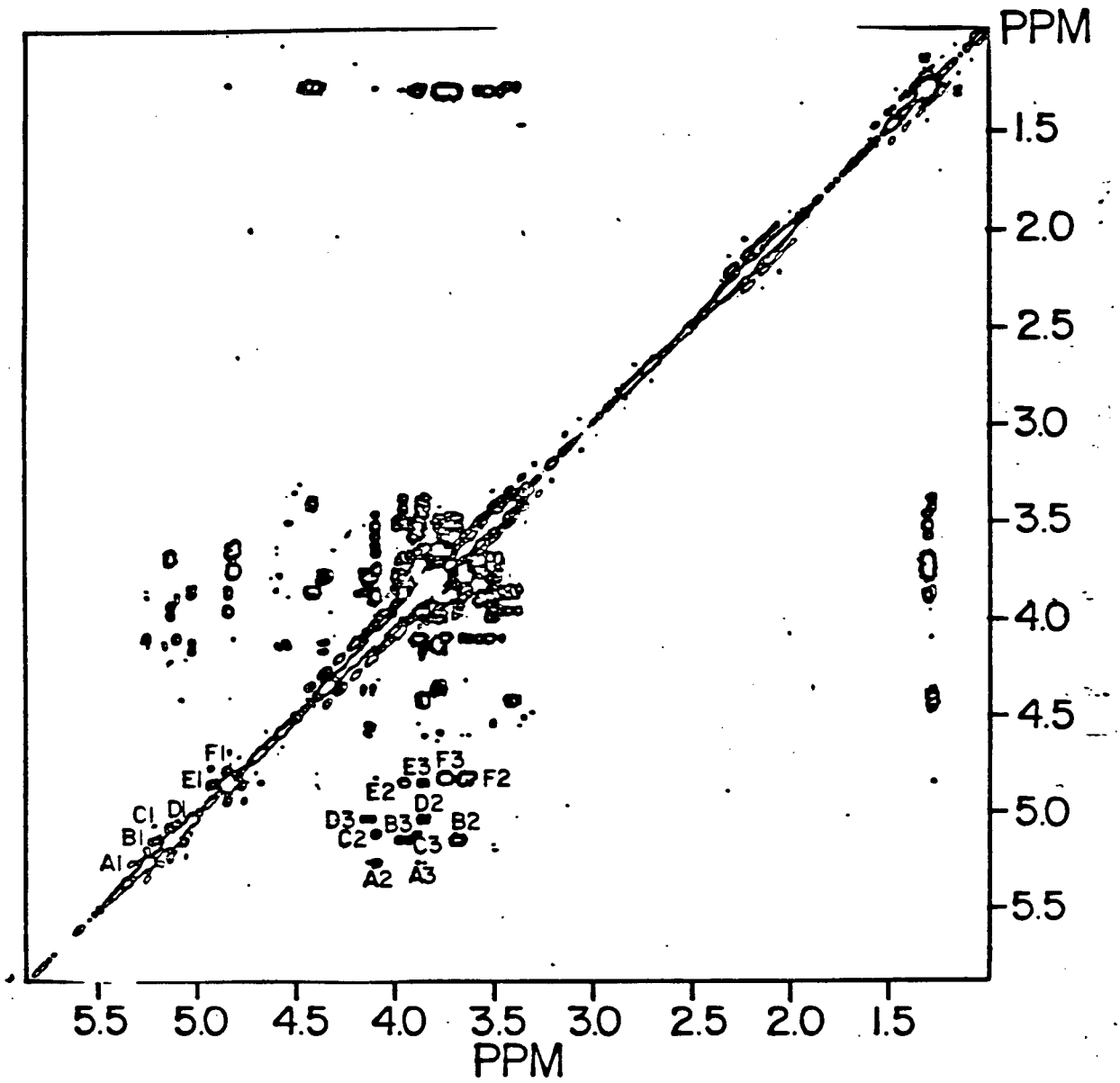


Fig. IV.13: One step relayed ^{1}H spin coherence transfer (COSYR1HG) spectrum of Pn

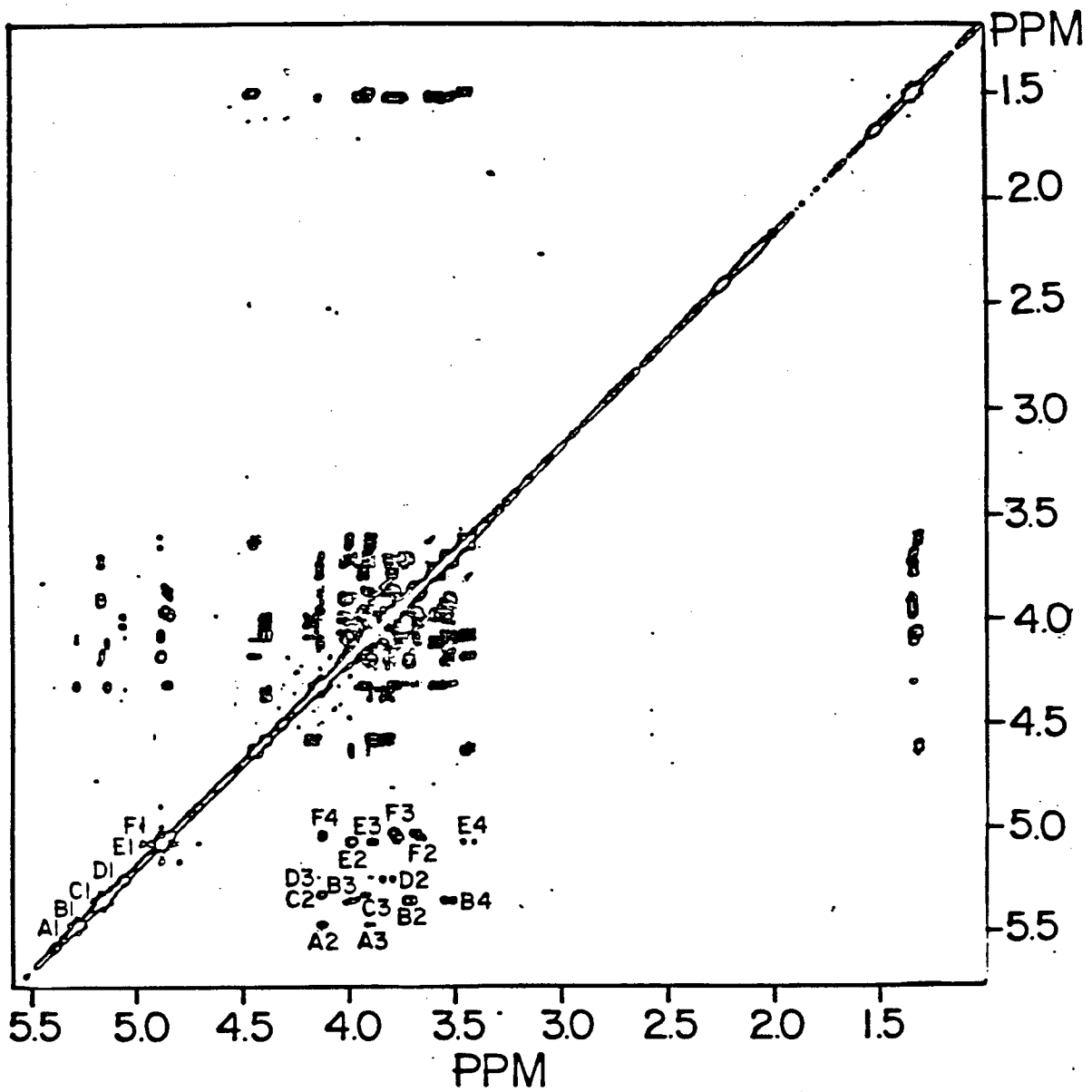


Fig. IV.14: Two step relayed ^{1}H spin coherence transfer (COSYHGR2) spectrum of Pn

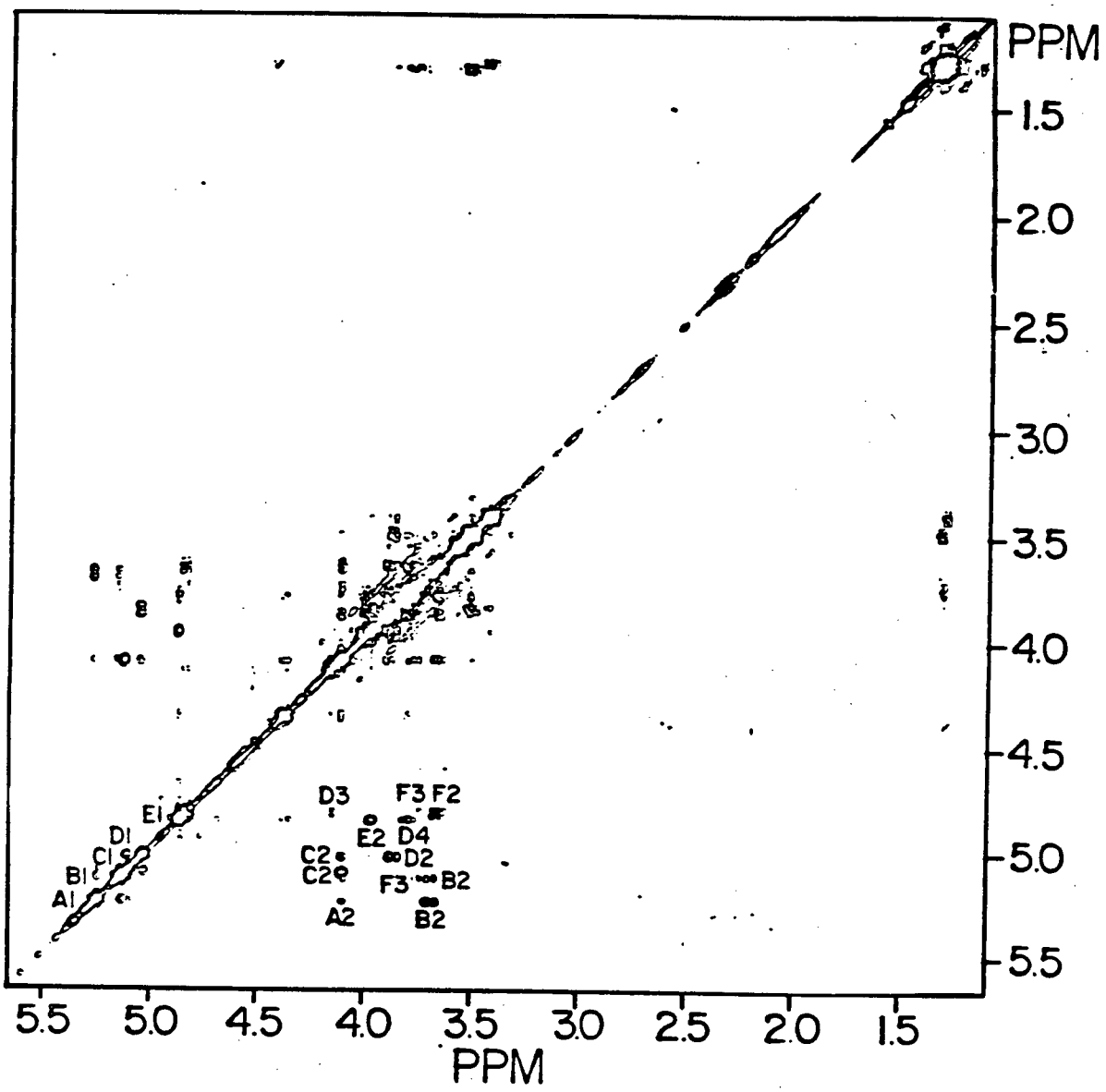


Fig. IV.15: Homonuclear dipolar correlated 2D-n.m.r. (NOESY) spectrum of Pn

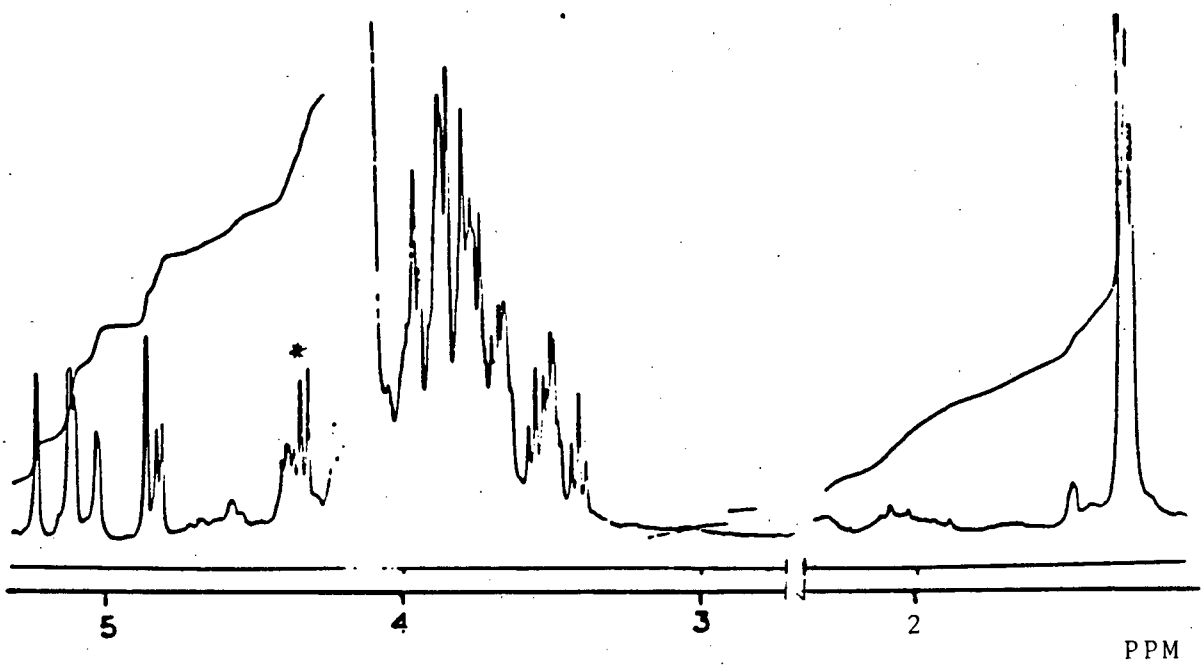
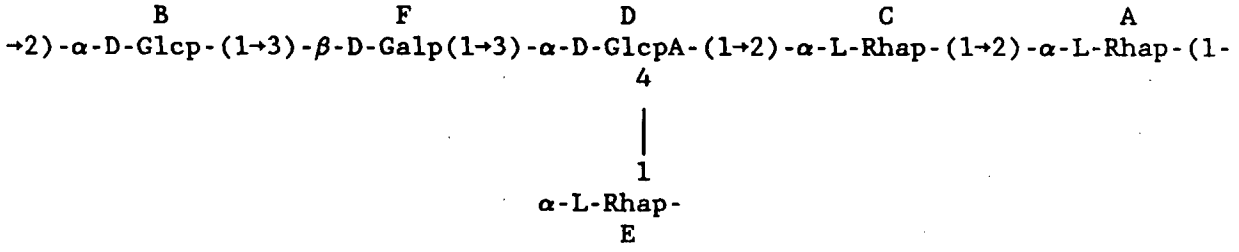


Fig. IV.16: ^{1}H-n.m.r. spectrum of Pn

* indicating H5 of GlcA

F were linked to D, thus giving the sequence shown. Interestingly, n.O.e.s were observed between A-1 and C-1, and C-1 and D-1. In both cases, H-1 of the glycosyl residue is located between the proton at the bridge (H-2) and the equatorial neighbouring proton (H-1) of the glycosyl-linked sugar residue. This observation is common for α-D-mannose-type residues linked by a glycosyl group at O-2.[187-192]

The proposed structure differs in several respects from the partial one published previously.[31] The main difference is the absence in the latter of a lateral rhamnose unit although a cross-reaction with Pneumococus suggested its presence.[31] It is known, however, that lateral rhamnose residues are sometimes fortuitiously eliminated during purification steps.



IV.4.3 Experimental

General methods are as discussed in Section IV.2.4.

COSY. Phage degraded polymer Pn was lyophilized twice from deuterium oxide and dissolved at a concentration of 25 mg mL^{-1}. This sample was used for all the 2D n.m.r. experiments which were conducted at 343°K. The pulse sequence used in the COSY experiment is given in

Section II.4.1 (Appendix II). The acquisition parameters were identical to those of the COSY experiment in Section IV.2.4 apart from using 112 transients per experiment.

Relay COSY (one and two step). The pulse sequence for the relay COSY experiments is given in Appendix II. The acquisition parameters for the one step and two step relay COSY n.m.r. experiment were identical to those of the relay COSY experiments given in Section IV.2.4.

NOESY. Several NOESY experiments were performed and the best results were attained using a mixing time (D9) 0.25s with no random variation (i.e. V9 = 0). The other NOESY parameters were identical with those given in Section IV.2.3.

CONCLUDING REMARKS

Knowledge of the primary structure of bacterial polysaccharides besides explaining the serology of the different bacterial strains on a molecular level, has important biological, medical and commercial significance. E. coli capsular polysaccharides like other bacterial polysaccharides consist of regular repeating units. N.m.r. has originally been used as a supportive technique to chemical methods in structural studies of carbohydrates. The goal in this study is to obtain complete structures of E. coli antigens using only n.m.r. methods and hence to establish this as a reliable routine process in this laboratory.

During this investigation:

i. the complete structure of E. coli K31 antigen was established using chemical methods.

ii. the complete sequence and linkage of the sugar residues in the hexasaccharide repeating unit of E. coli K31 capsular polysaccharide was established by homonuclear 2D n.m.r.

iii. the use of 2D n.m.r. as a convenient method for locating the position of acetate in the repeating unit of capsular polysaccharides is also demonstrated.

iv. the complete sequence and linkage of the sugar residues in the repeating unit of E. coli K46 polysaccharide was established by 2D n.m.r. (the use of n.m.r. methods for the identification and location of the position of phosphate diester in the repeating

unit of polysaccharides is illustrated using *E. coli* K46 polysaccharide as an example).

While major advances in the application of n.m.r. spectroscopy to structural studies of carbohydrate biopolymers have been made in recent years, the ability to identify sugar residues in the repeating unit of complex bacterial polysaccharides using only n.m.r. data has always been a difficult task for carbohydrate n.m.r. spectroscopists. It is hoped that efforts in using only n.m.r. spectroscopy in the complete structure elucidation of bacterial polysaccharides will be achieved in the next few years.

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APPENDIX I

^1H Chemical Shift Assignments of 3,6-dideoxy Amino Sugar Residue in *E. coli* K45 Capsular Polysaccharide and Structural Studies on *E. coli* K46 K Antigen

STRUCTURAL STUDIES ON E. COLI K46 POLYSACCHARIDE

Introduction

The occurrence of amino-sugars in the capsular polysaccharides of E. coli is known. Most of the reported K antigens of E. coli are acidic. In this investigation structural studies on a K antigen containing an amino sugar and a phosphate ester are reported.

Results and Discussion

Composition. Analysis of the native polysaccharide before and after reduction[77] both gave glycerol, rhamnose, glucose, galactose and glucosamine in the ratio 0.5:0.7:1.08:1.08:1. N.m.r. studies (see later) suggest the presence of phosphate ester and a three carbon fragment in addition to four sugar residues in the repeating unit of the K46 polysaccharide, thus suggesting that this polysaccharide consists of a pentasaccharide repeating unit with a composition of rhamnose, glucose, galactose, glucosamine, glycerol in a ratio of 1:1:1:1:1.

Methylation. Methylation[101] analyses gave the results shown in Table VII.1 from which it may be deduced that a glucose residue occupies a terminal position and a galactose unit constitutes the branch point.

Table VII.1: Methylation analysis of E. coli polysaccharide

Sugar Residue	Methylated sugar[a] (as alditol acetates)	Mole %[b]
→3)-Rha-(1→	2,4-Rha	27.28
Glc(1→	2,3,4,6-Glc	30.10
→2)-Gal(1→ 3 |	4,6-Gal	21.77
→3)-GlcNAc(1→	4,6-GlcNAc	20.10

[a] 2,3,4,6-Glc = 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol etc.

[b] Values are corrected using the effective carbon response factors given by Sweet et al.; determined on a DB-17 column programmed for 1 min. at 180°C then 250°C at 2°/min.

Dephosphorylation. 60 mg of dephosphorylated product was obtained by treating 100 mg of native polysaccharide with 48% aqueous hydrogen fluoride (for 72 h at 4°C) and purifying by gel permeation chromatography (Bio Gel P2).

5 mg of dephosphorylated product was treated with anhydrous hydrogen fluoride at 20°C for 6 h. The resultant product was converted to alditol acetates by sodium borodeuteride reduction and acetylation. G.l.c. results indicate that the dephosphorylated product contains

glycerol, rhamnose, glucose, galactose and glucosamine in ratios of 1:1:1:1:1.

Detailed n.m.r. studies were performed on the dephosphorylated product (see later). The dephosphorylated product is awaiting FAB-MS analysis.

N.m.r. studies. ^{1}H-N.m.r. (400 MHz, 300°K) spectrum of native polysaccharide showed four signals in the anomeric region at δ 4.97 (s, 1H), 4.79 (d, 1H, J_{HH} = ~8 Hz), 4.75 (d, 1H, J_{HH} = ~8 Hz), and 4.66 (d, 1H, J_{HH} = ~8 Hz), also signals at 2.05 (s, 3H, CH_3 of NAc) and ~1.30 (d, 3H, J_{HH} = ~7.5 CH_3 of rhamnose). Consistent with the results, the ^{13}C-n.m.r. (75 MHz, 300°K) spectrum showed characteristic signals at 103.730, 102.864, 101.553 and 99.706 ppm corresponding to four anomeric carbons. The ^{13}C-n.m.r. spectrum of the native CPS contains 28 carbon resonances (Fig. VII.1) but the heteronuclear correlated spectrum (Fig. VII.8) indicated 29 cross-peaks (see later) (i.e. two carbon resonances coincided). This observation suggests the presence of a three carbon fragment (glycerol) in the repeating unit in addition to four sugar residues. Due to splittings in four of the ^{13}C signals[183,199] (Fig. VII.1 i.e. ^{13}C broad band ^{1}H decoupled spectrum), the presence of an element with spin = 1/2 apart from ^{1}H and ^{13}C in the repeating unit of the native polysaccharide was suspected. ^{31}P-N.m.r. (122 MHz) (see Fig. VII.4) indicated the presence of a phosphorus ester in the repeating unit of the native polysaccharide. 2D N.m.r. studies (see later) suggested that the phosphorus ester is linked at C-3 to a galactose residue.

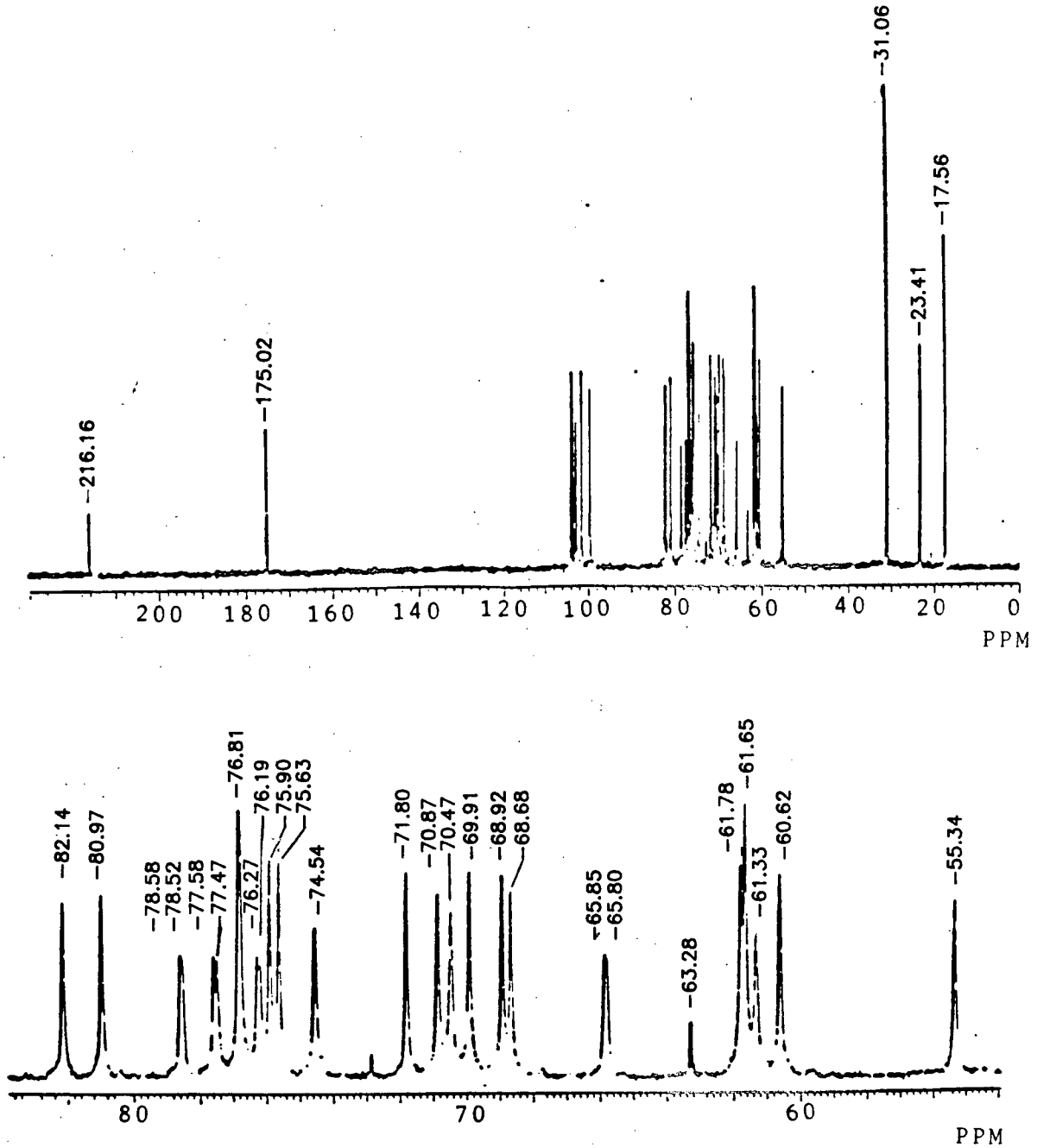


Fig. VII.1: ^{13}C-N.m.r. (^{1}H decoupled) spectrum of K46 native polysaccharide (i) full spectrum (ii) ring region (90-60 ppm)

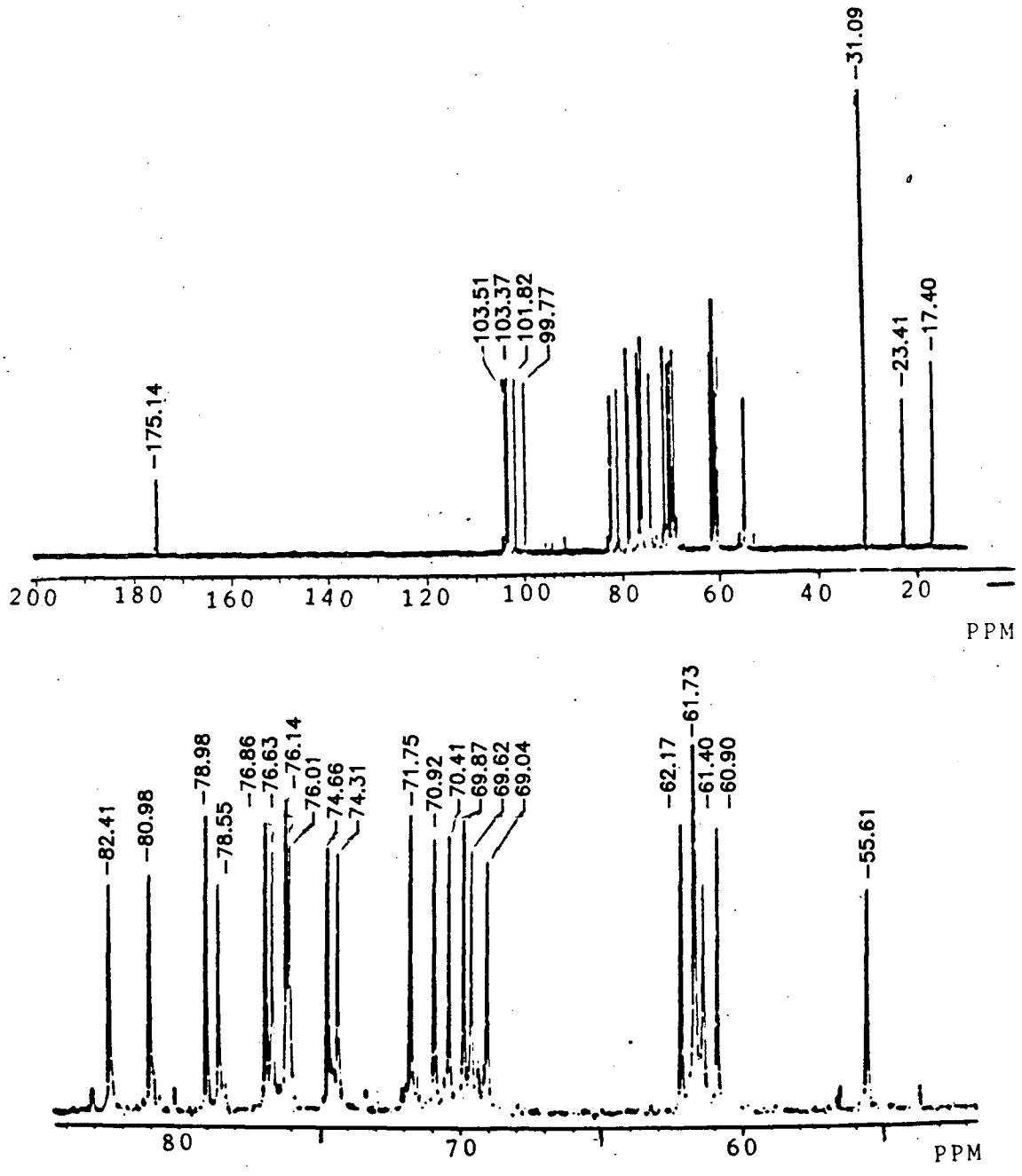


Fig. VII.2: ^{13}C-N.m.r. (^{1}H decoupled) spectrum of K46 dephosphorylated product. (i) full spectrum (ii) ring region.

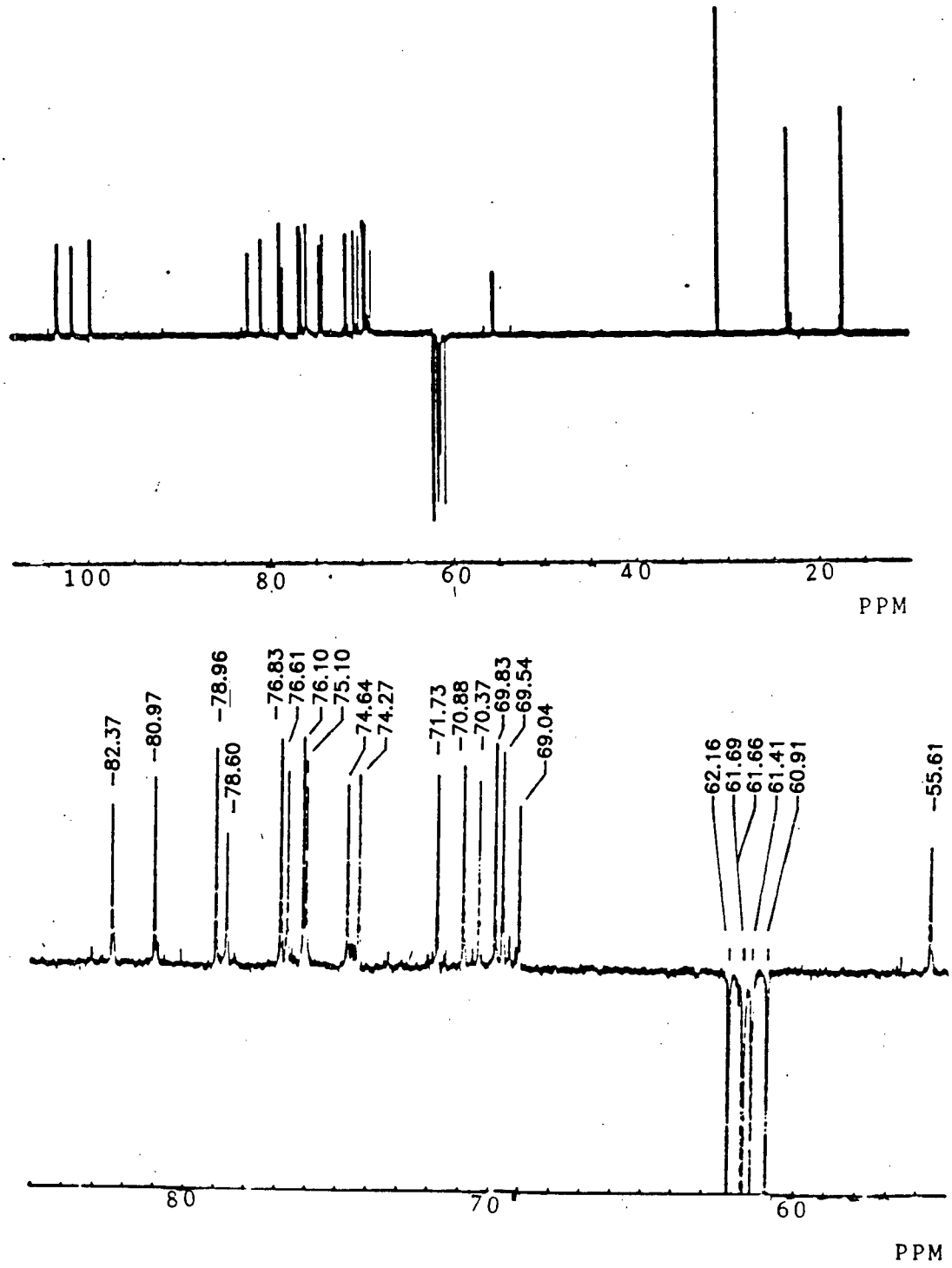


Fig. VII.3: ^{13}C-N.m.r.-APT spectrum of K46 dephosphorylated product (i) full spectrum; (ii) ring region

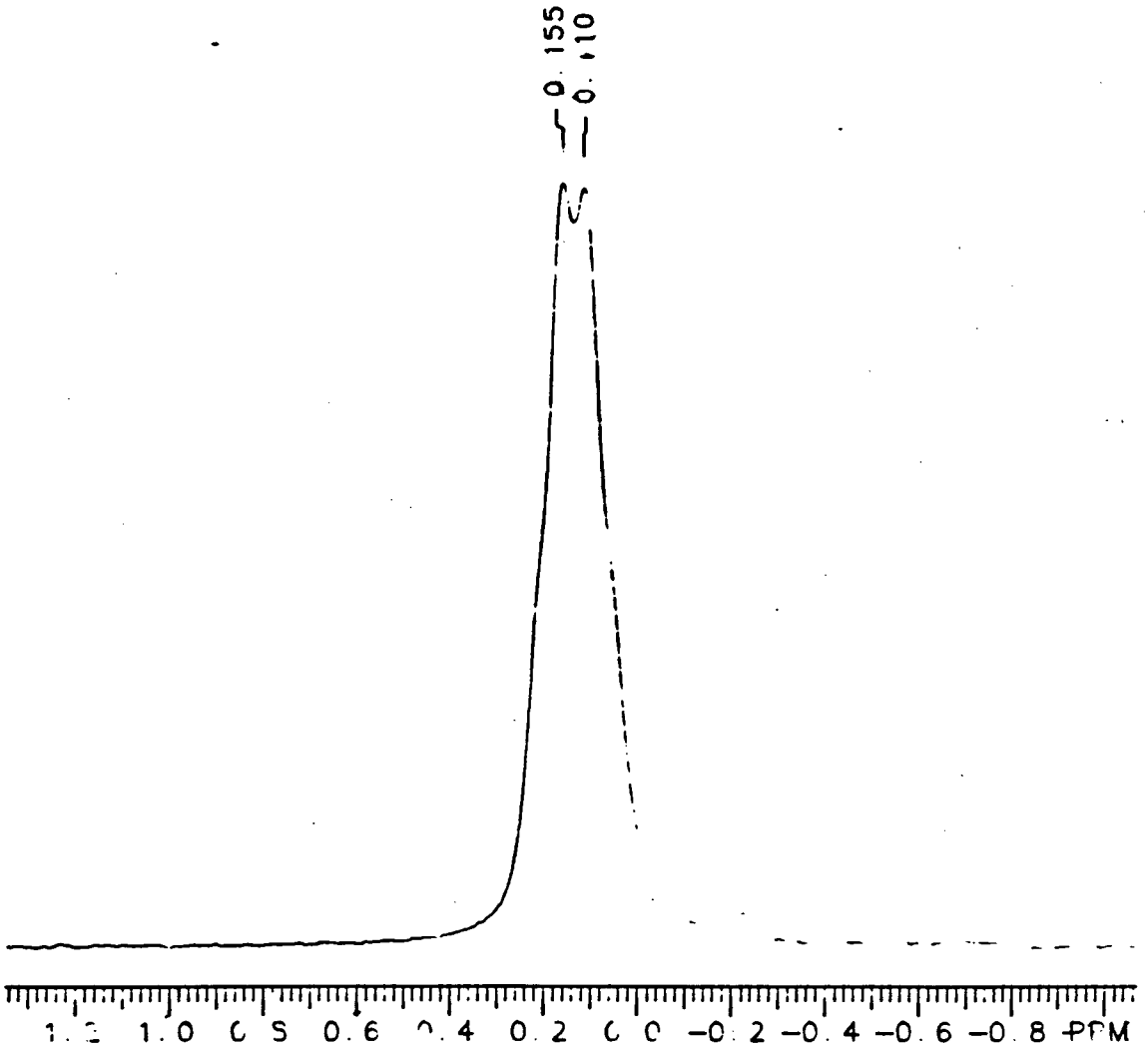


Fig. VII.4: ^{31}P-n.m.r. (^{1}H coupled) of native polysaccharide (K46)

The sugar residues were arbitrarily labelled A to D in order of decreasing chemical shift of their H-1 resonances. The results of COSY[163] (Fig. VII.5 and Table VII.2) afforded the assignment of H-1 and H-2 resonances of most of the sugar residues in the repeating unit. Further assignments were made using COSY results after interpreting the data for the heteronuclear shift correlated 2D n.m.r. experiment. One and two step relay[62] spectra (Fig. VII.6 and Table VII.2) afforded the assignments of H-3 and H-4 resonances of most of the sugar residues in the repeating unit. The entire J system of the residue B was relayed in the two step relayed COSY experiment (Fig. VII.6). This may be due to long T1 and T2 since B is the lateral residue. However, this was not further investigated because of machine time.

The assignment of the ^{1}H signals for the α-6-deoxy residue A was greatly facilitated by the additional window provided by the methyl resonance. The heteronuclear shift correlated 2D n.m.r. experiment using the CHORTLE technique[182] was then performed and permitted the unambiguous assignments of most carbons in the repeating unit (Fig. VII.8 and Table VII.4). The correlation of the methylene carbons with the corresponding proton signal via the CHORTLE experiment led to assignment of some of the resonances of the ^{1}H-n.m.r. spectrum (Table VII.2).

C-2 of the aminohexosyl residue has a characteristic resonance at about 55 ppm.[201] H-2 of the residue C correlated with carbon resonance at 55.340 ppm in the heteronuclear correlated spectrum (Fig. VII.8, Table VII.2 and Table VII.4). Thus C was identified as glucosamine. H-2 resonance (3.247 ppm) of residue B (Table VII.2) is typical of

Table VII.2: ^{1}H-n.m.r. data for *E. coli* K46 polysaccharide

Symbol	Sugar Residue	H-1	H-1	H-2	H-3	H-4	H-5	H-6	H-6^{1}
A	→3)α-Rha(1→	~4.97	-	4.28	3.88	3.49	3.91	1.30	
B	β-Glc(1→	4.79	-	3.25	3.50	3.38	3.43	3.74*	3.91*
C	→3)-β-GlcNAc(1→	4.75	-	3.89	3.92	~3.59	3.49	3.78*	3.77*
D	→2)-β-Gal(1→	4.66	-	3.94	4.31	4.12	3.78*	3.84*	3.92*
E	glycerol		4.05*	3.93*	3.74	-			

* Chemical shift values obtained from HET-COR

β-glucose.[174] Based on this evidence and methylation results (see later) residue B was identified as terminal β-glucose. Methyl residue resonance at 1.298 ppm showed ^{1}H spin correlation (i.e. COSY spectrum Fig. VII.5 and Table VII.2) to H-5 of residue A. Based on this evidence, the fact that H-1 resonance is a singlet and methylation data suggests that residue A is 3-linked α-rhamnose. Residue D was then assigned as 2,3-linked β-galactose residue and E as a glycerol. The anomeric configurations of the sugar residues in the repeating unit were established by a ^{13}C-^{1}H coupled n.m.r. experiment (Table VII.5 and spectrum in Appendix III).

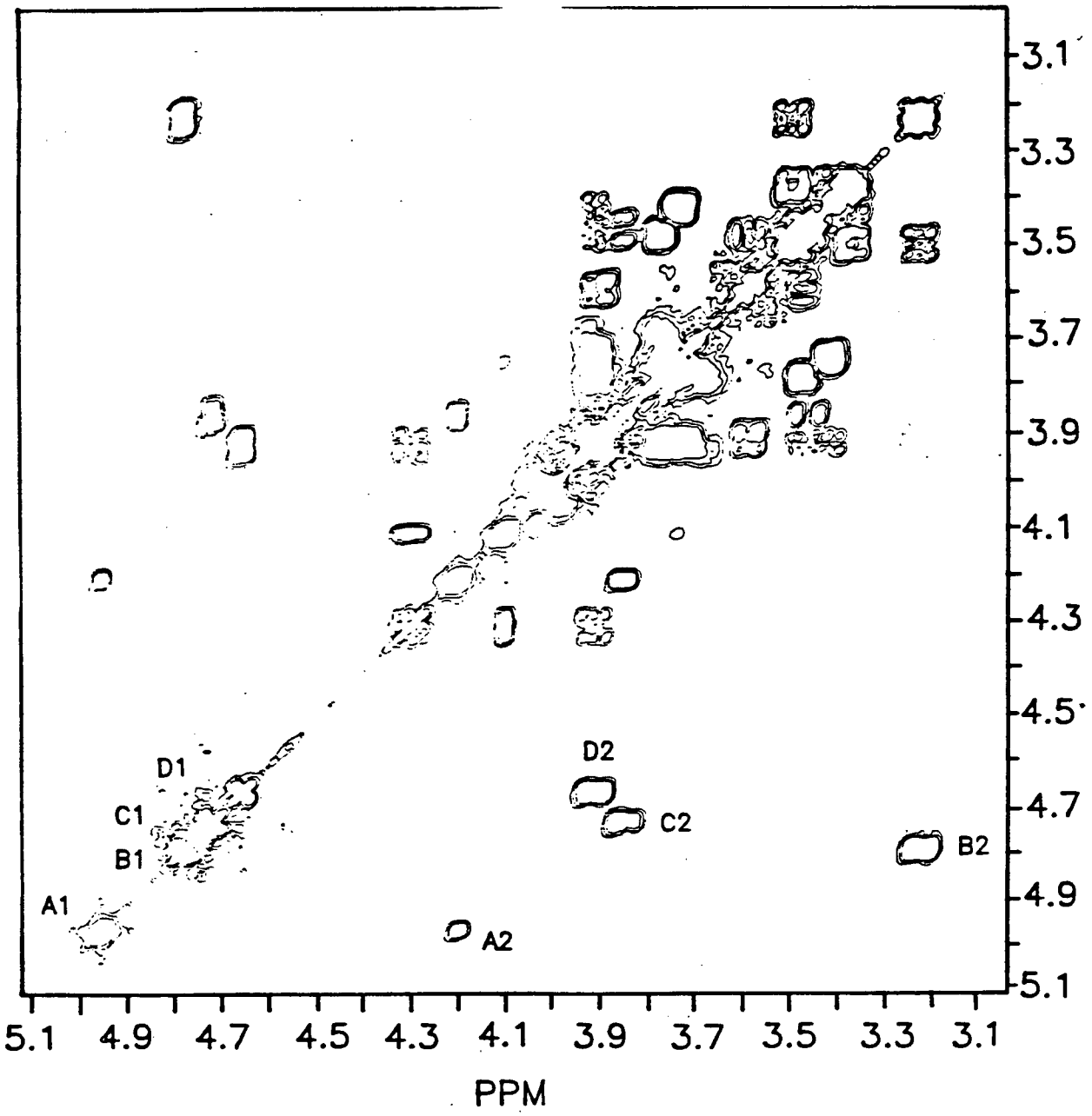


Fig. VII.5: Homonuclear ^{1}H-spin correlated (COSY) n.m.r. spectrum of native polysaccharide (K46)

Table VII.3: N.O.e. data for E. coli K46 polysaccharide

Symbol	Sugar Residue	Interresidue n.O.e.	Intraresidue[a] n.O.e.
A	→3)-α-Rha-(1→	3.94 (H2 of D)	4.22
B	-β-Glc-(1→	3.92	3.25, ~3.50, 3,38
C	→3)-β-Glc-NAc-(1→	3.87 (H3 of A)	~3.50
D	→2)-β-Gal-(1→ 3 |	3.93	4.31, 3.90

[a] could be scaler correlated effect

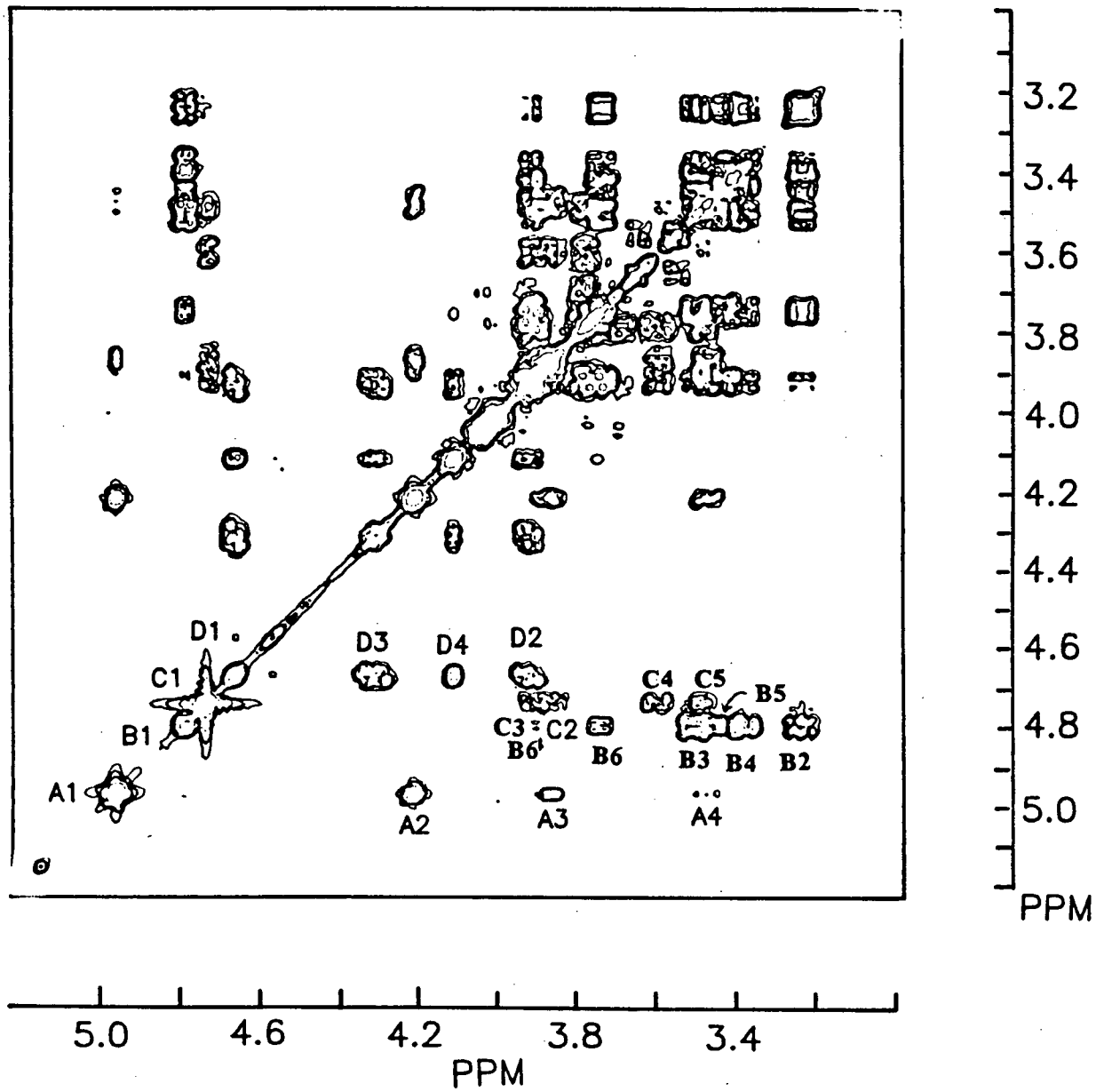


Fig. VII.6: Two step relay ^{1}H spin coherence transfer (COSYHGR2) spectrum of native polysaccharide

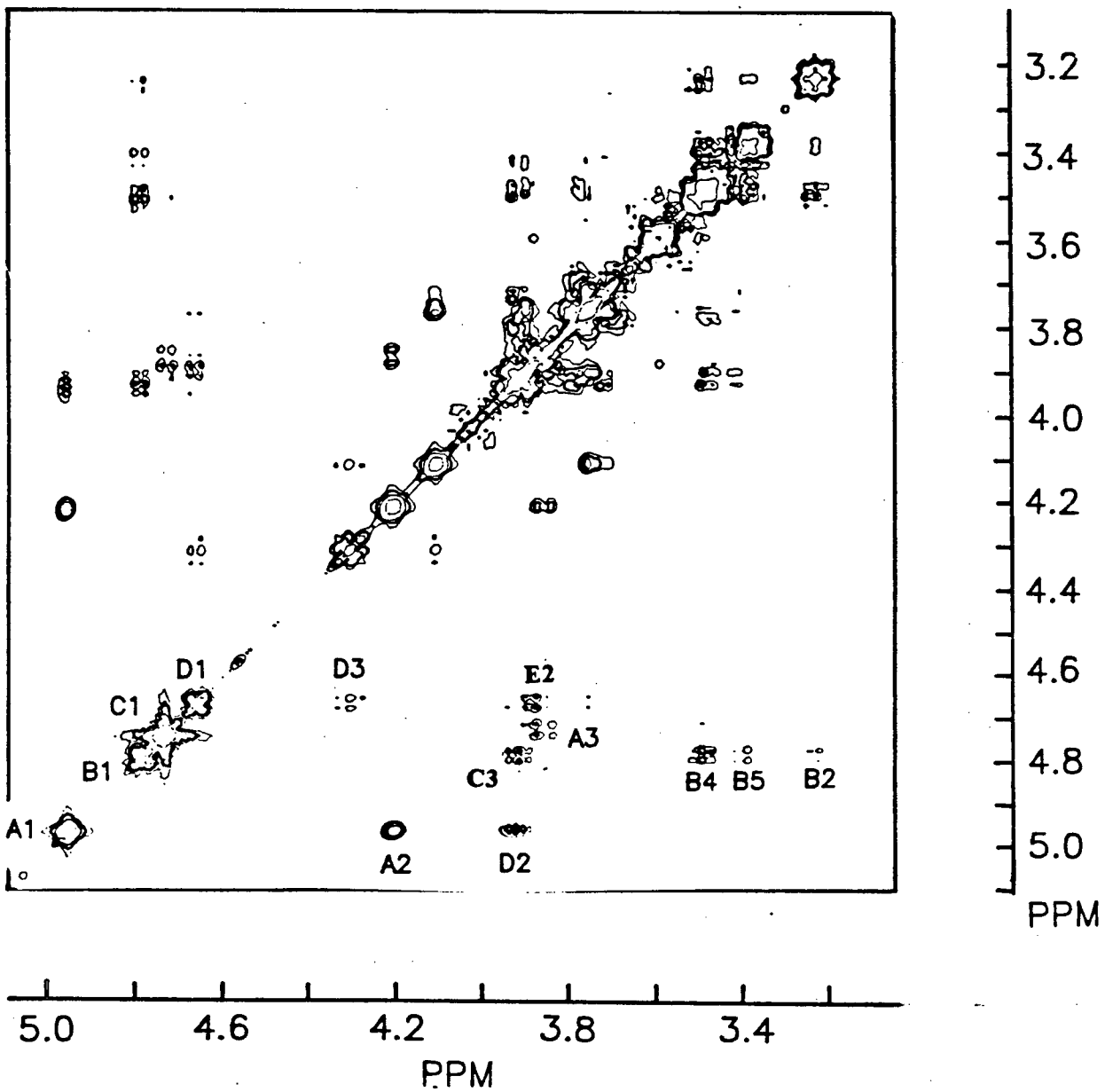


Fig. VII.7: Homonuclear dipolar correlated 2D n.m.r. (NOESYHG) spectrum of native polysaccharide (K46)

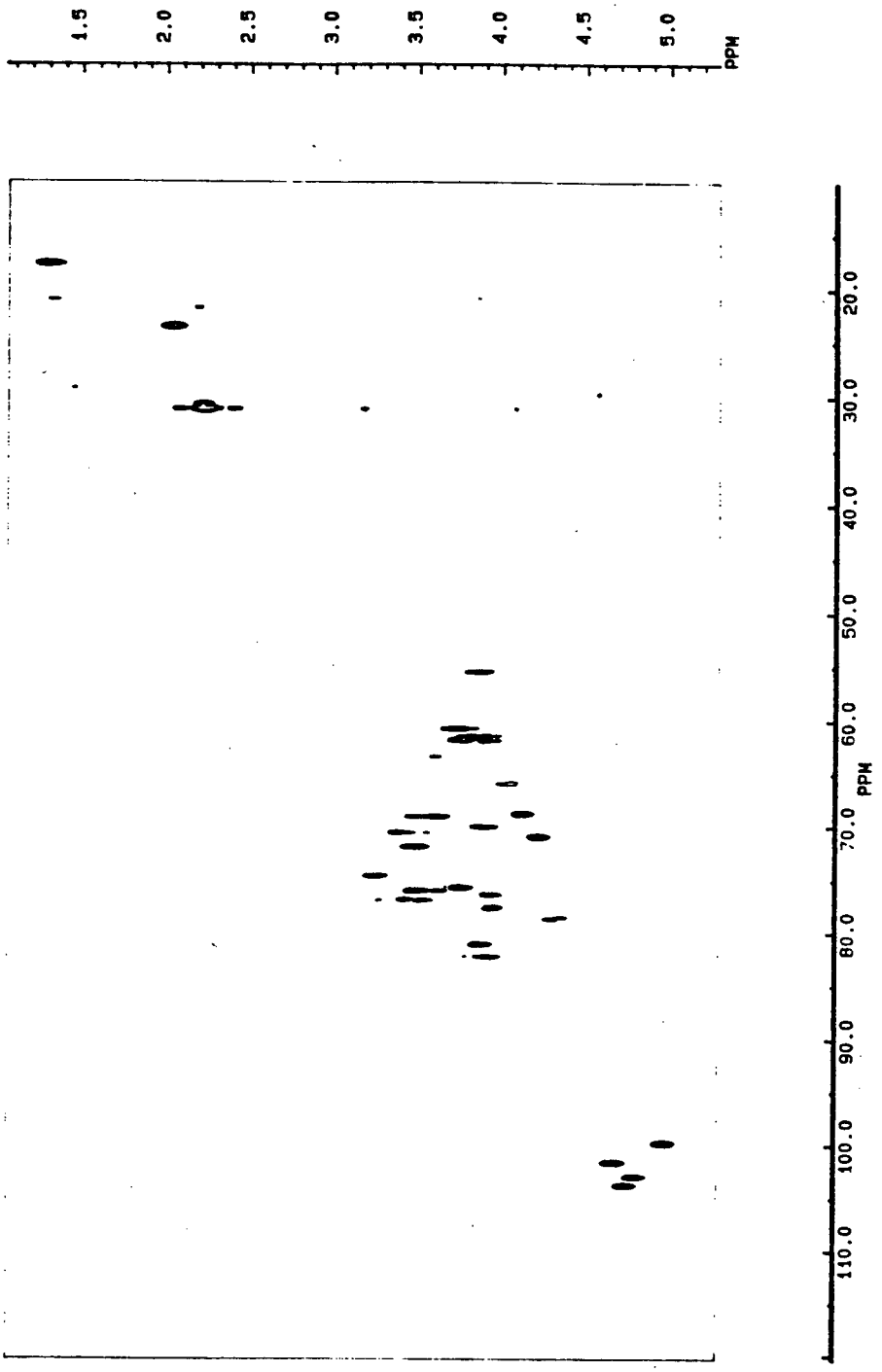


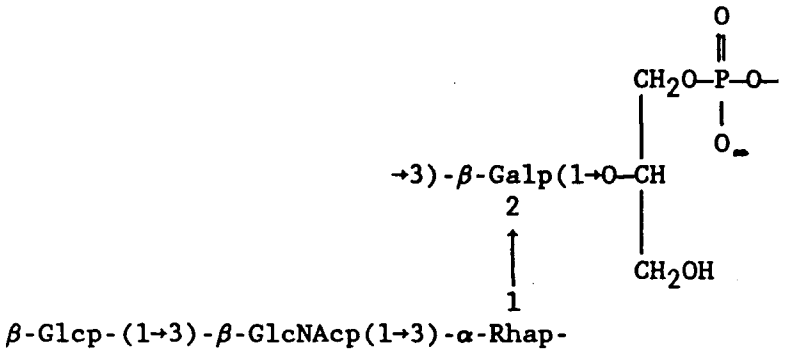
Fig. VII.8: Heteronuclear (^{1}H-^{13}C) correlated spectrum of native polysaccharide (K46)

Table VII.4: ^{13}C-N.m.r. chemical shifts[a] and ^{31}P-^{13}C coupling constants (in parentheses) for *E. coli* K46 native polysaccharide

Symbol	Sugar Residue	C-1	C-2	C-3	C-4	C-5	C-6
A	→3)-α-Rha(1→	99.71	70.81	80.97	71.80	80.97	17.57
B	β-Glc(1→	101.55	74.54	76.81	70.47	76.81	61.78
C	→3)-β-GlcNAc(1→	102.86	55.34	82.14	68.92	75.90	61.65
D	→2)-β-Gal(1→ 3 ↑	103.73	~77.52 (7.95 Hz)	~78.55 (4.4 Hz)	68.68	75.63	61.33
E	Glycerol	65.83 (3.7 Hz)	76.23 (5.25 Hz)	60.62			

[a] In ppm from internal acetone (1% 31.070 ppm)

The sequence of the sugar residues in the repeating unit was obtained by data from a NOESY experiment[189] (Table VII.3 and Fig. VII.7). Inter-residue n.O.e. contacts[186] established that A was linked to D, C to A, B to C and D to E, thus giving the sequence below.



Methylation analysis data indicate that the galactose residue is linked at C-2 and C-3. The splitting of ^{13}C resonances[199,201] of C-2 and C-3 of the galactose residue gave an indication that the phosphate diester residue is scalar coupled to these carbons. Sugar analysis and ^{13}C-n.m.r. data on the dephosphorylated product (P_D) indicate that the P_D is a pentasaccharide repeating unit. The ^{1}H-n.m.r. spectrum of P_D does not show anomerization of the galactose residue. These evidences and NOESY results (i.e. galactose is linked at C-2 to the rhamnose residue) suggests that the phosphate diester is linked to C-3 of the galactose residue and C-1 of the glycerol.

The H-1 and H-2 resonances of most of the sugar residues in the repeating unit of the dephosphorylated product (P_D) are obtained from COSY data (Fig. VII.9 and Table VII.6). The H-3 and H-4 resonances of most of the sugar residues in P_D were established from one and two step

Table VII.5: ^{13}C-^{1}H Coupled n.m.r. experimental data

Chemical shift (^{1}H-decoupled resonance) ppm and sugar residue	Chemical shift ^{1}H-coupled		Chemical shift difference between downfield & upfield resonances	^{13}C-H coupling constant $^{1}J_{CH}$
	downfield resonance	upfield resonance		
103.73 (β-Gal)	104.799	102.629	~2.17	162.75
102.86 (β-GlcNAc)	103.903	101.707	~2.20	164.70
101.55 (β-Glc)	102.629	100.438	2.19	164.31
99.71 (α-Rha)	100.776	98.512	2.26	169.80

Table VII.6: ^{1}H chemical shift data of K46-dephosphorylated polysaccharide

Symbol	Sugar Residue	H-1	H-2	H-3	H-4	H-5	H6/H6
A	→3)α-Rha(1→	~4.97	4.22	3.88	3.51	3.86	1.29
B	β-Glc(1→	4.81	3.30	3.51	3.42	-	-
C	→3)-β-GlcNAc(1→	4.80	3.82	3.93	3.60	-	-
D	→2)-β-Gal(1→ 3 |	4.60	3.79	3.86	3.92	-	-
E	Glycerol	-	-	-	-	-	-

Table VII.7: ^{13}C-N.m.r. chemical shifts[a] for *E. coli* dephosphorylated product

Symbol	Sugar Residue	C-1	C-2	C-3	C-4	C-5	C-6
A	→3)-α-Rha(1→	99.77	70.92	74.31	71.75	69.81	17.40
B	β-Glc(1→	101.82	74.66	76.63	76.01	-	-
C	→3)-β-GlcNAc(1→	103.37	55.61	69.62	69.04	-	-
D	→2)-β-Gal(1→ 3 ↑	103.51	78.55	80.99	82.41	-	-
E	Glycerol	-	-	-	-	-	-

[a] In ppm from internal acetone (1% 31.070 ppm)

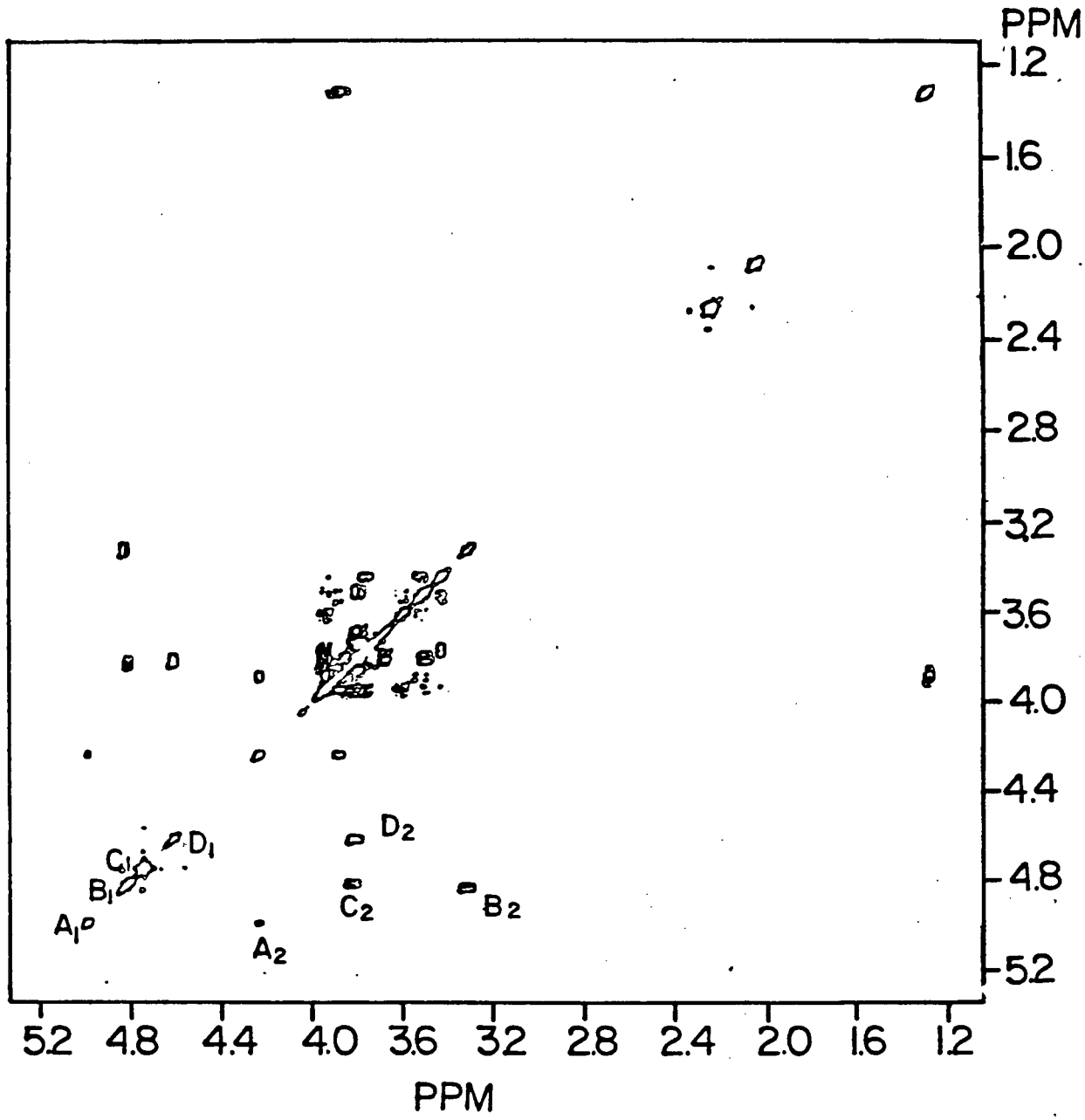


Fig. VII.9: Homonuclear ^{1}H-spin correlated (COSY) n.m.r. spectrum of dephosphorylated product (K46)

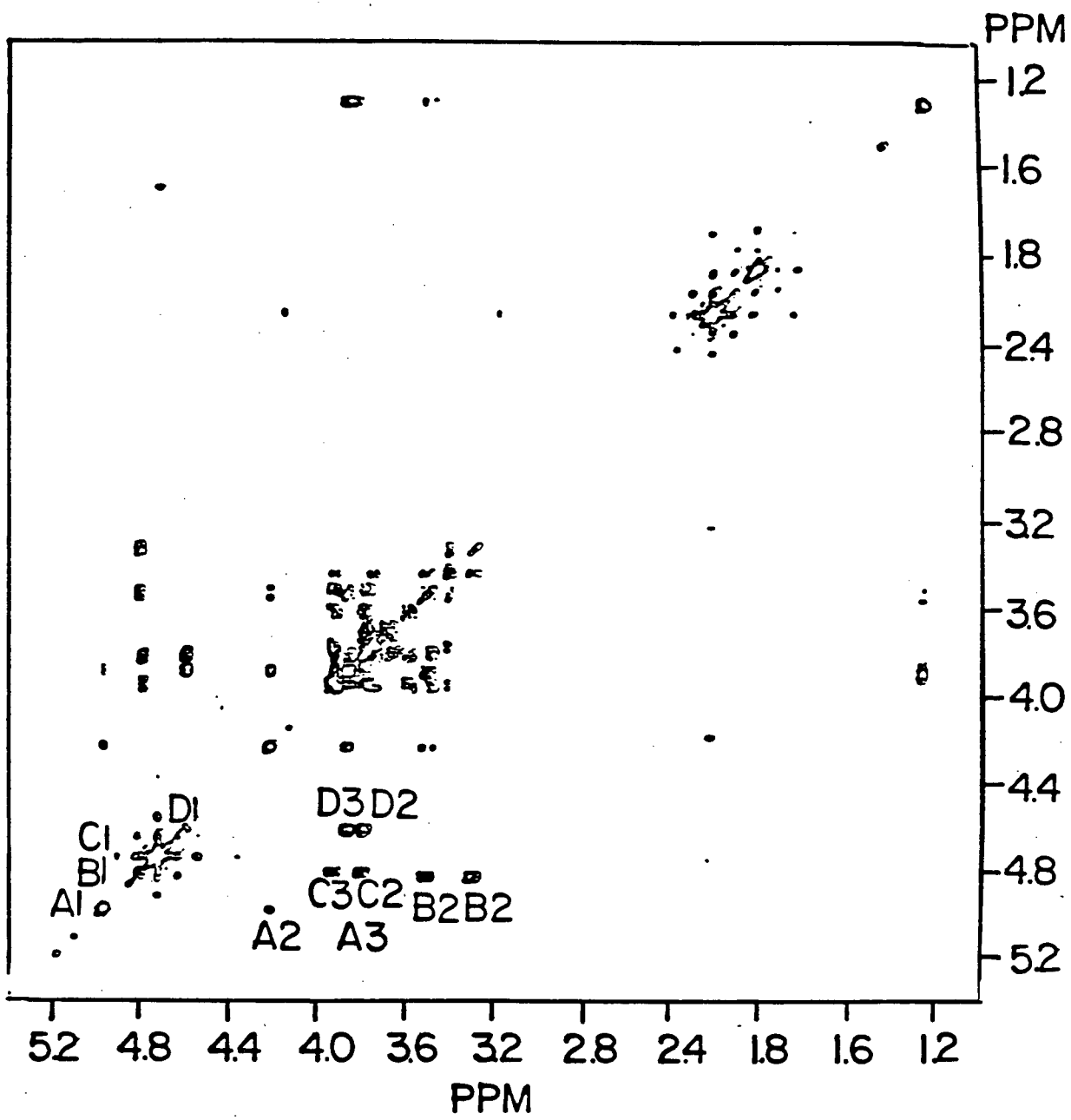


Fig. VII.10: One step relay ^{1}H spin coherence transfer (COSYRCT) spectrum of dephosphorylated product (K46)

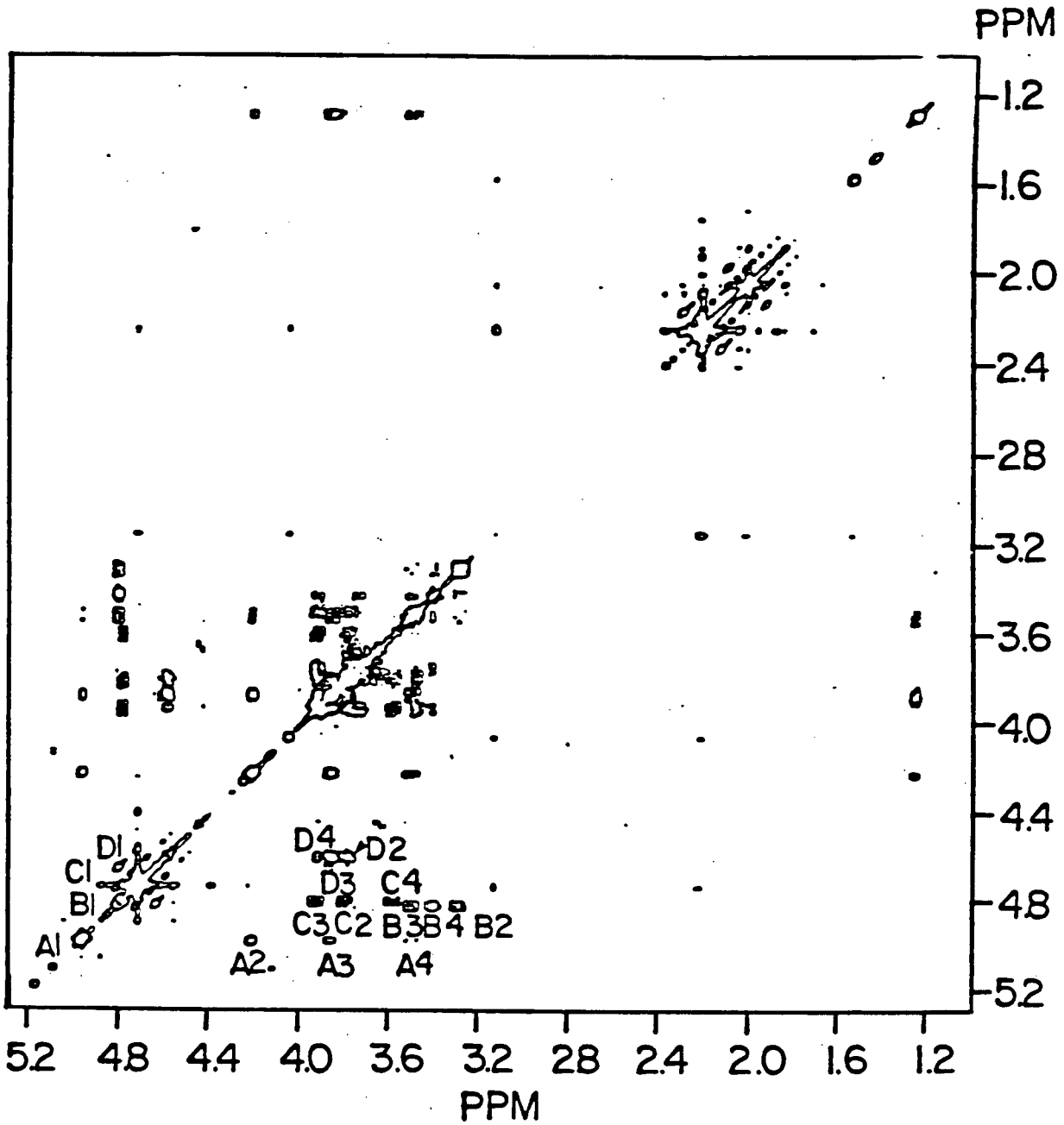


Fig. VII.11: Two step relay ^{1}H spin coherence transfer (COSYRCT) spectrum of dephosphorylated product (K46)

relay COSY experiments (Figs. VII.10, VII.11 and Table VII.6). The extra window provided by the methyl group resonance afforded the assignment of the chemical shift of ^1H resonance of residue A. Residue A was assigned as 3-linked α rhamnose, B as lateral β-glucose, C as 3-linked glucosamine, D as 2,3-linked β-galactose and E as three carbon fragment (Table VII.6). This assignment is based on previous results (Table VII.2). A heteronuclear correlated n.m.r. experiment on P_D afforded the assignment of carbon resonances, C-1 to C-4 of most of sugar residues. P_D is awaiting FAB mass spectrometric analysis.

A polysaccharide (Px), of significantly lower molecular weight and viscosity than the native polysaccharide was prepared by depolymerization with a viral endoglycanase and was used for 2D n.m.r. studies. The use of Px enabled the preparation of more concentrated samples than was used for the previous 2D n.m.r. study. In fact this 2D n.m.r. study was an attempt to reproduce the n.O.e. contacts in Table VII.3 using a lower molecular weight polymer.

The sugar residues were arbitrarily labelled A' and D' in order of decreasing chemical shift of their H-1 resonances. The results of COSY[163] (Fig. VII.13 and Table VII.8) gave the assignments of H-1 and H-2 resonances of all the sugar residues in the repeating unit. One and two step relay COSY[62] spectra (Figs. VII.14, VII.15 and Table VII.8) afforded the assignments of H-3 and H-4 resonances of the rhamnosyl, glucosyl and 2-acetamido-2-deoxyglucosyl residues. The assignment of H-5 of the rhamnosyl residue was achieved by the additional window provided by methyl resonances.

Partial sequencing of the sugar residues in the repeating unit was

Table VII.8: ^{1}H-N.m.r. data for E. coli K46 bacteriophage polysaccharide (Px)

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6	H6[1]
A' (Rha)	4.96	4.20	3.86	3.50	3.85	1.33[a]	-
B' (Glc-NHAc)	4.82	3.82	3.95	3.52	-	-	-
C' (β-Glc)	4.78	3.28	3.53	3.42	-	-	-
D' (β-Gal)	4.64	3.91	-	-	-	3.93	3.81

[a] chemical shift values of CH_3 of rhamnosyl residue

Table VII.9: N.O.e. data of lower molecular weight polymer (Px)

Symbol	Sugar residue	Interresidue n.O.e.	Intraresidue* n.O.e.
A'	→3)-α-Rha-(1→	-	4.20
B'	→3)-β-GlcNAc-(1→	3.87 (H3 of A)	-
C'	β-Glc-(1→	-	3.28
D'	→2,3)-Gal-(1→	-	3.95

* scalar effect

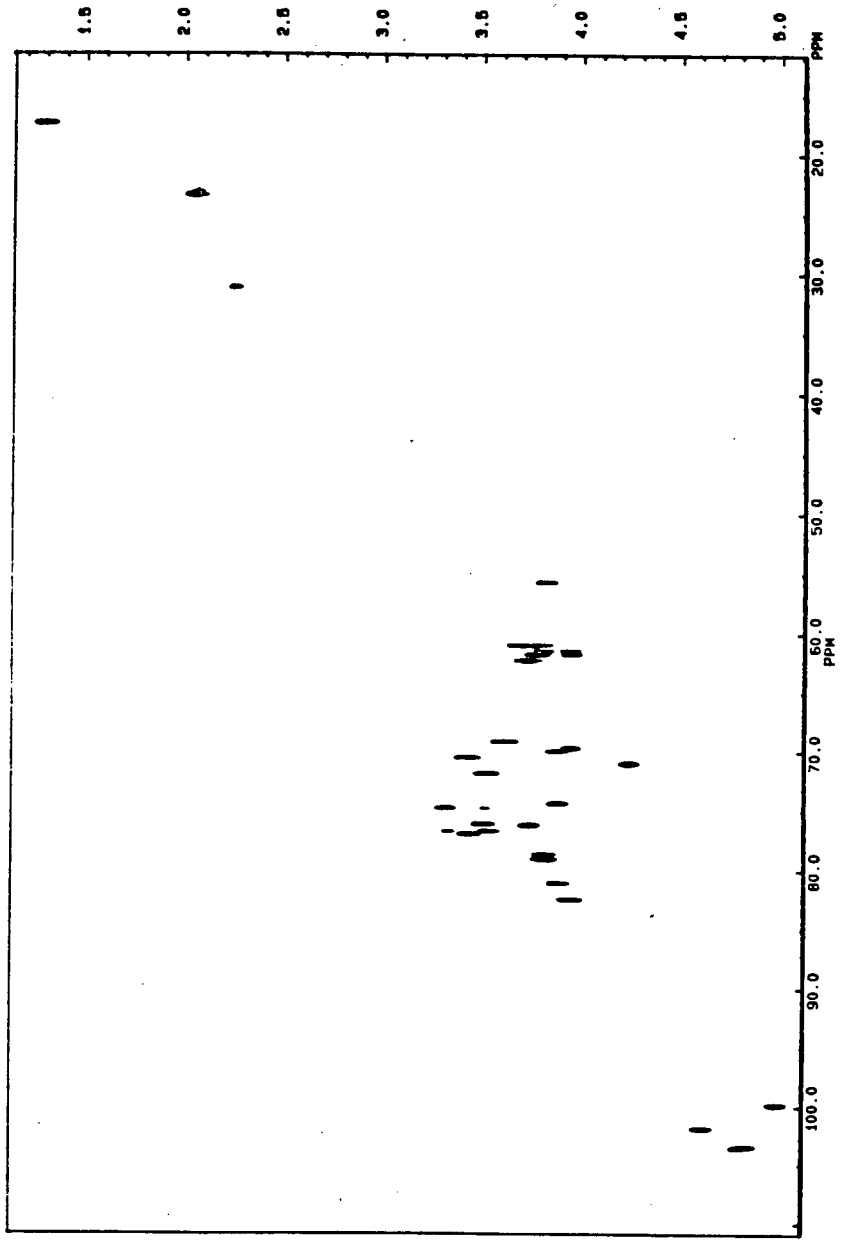


Fig. VII.12: Heteronuclear (^{13}C-^{1}H) correlated n.m.r. spectrum of K46 dephosphorylated product

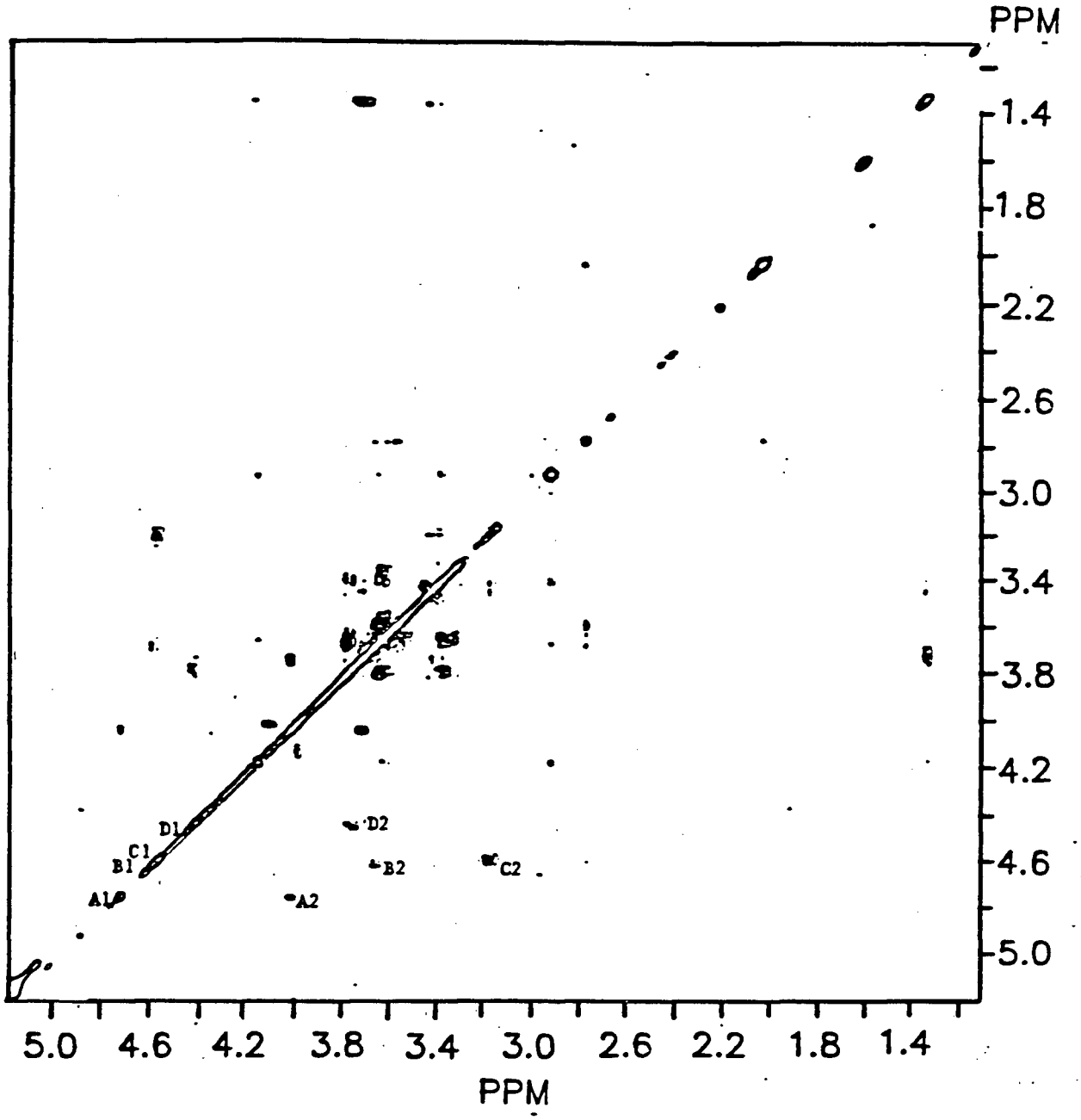


Fig. VII.13: Homonuclear ^{1}H-spin correlated (COSY) n.m.r. spectrum of a lower molecular weight polymer (Px) derived from K46 native polysaccharide

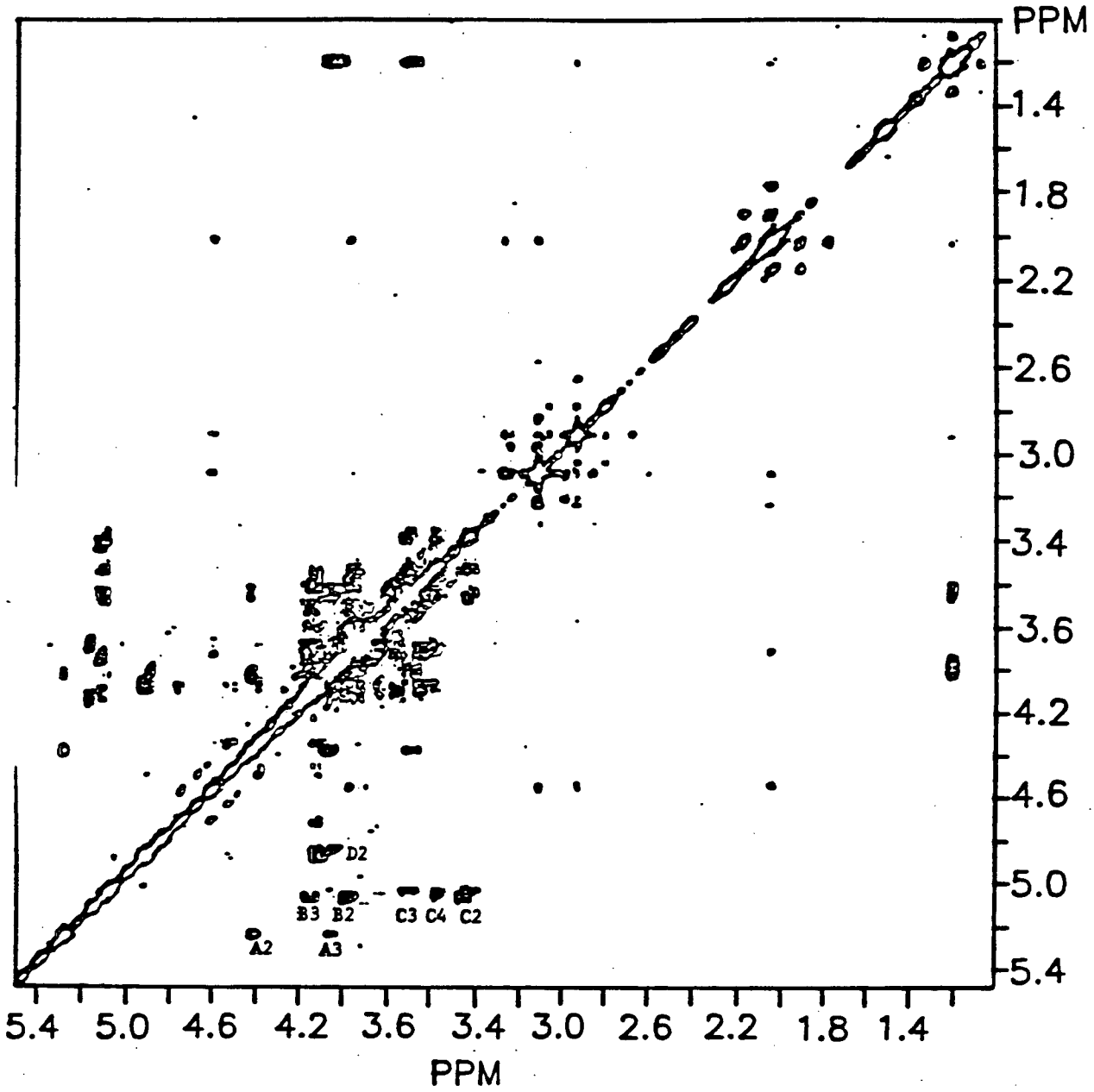


Fig. VII.14: One step relayed ^{1}H spin coherence transfer (COSYR1HG) n.m.r. spectrum of a lower molecular weight polymer (Px) derived from K46 native polysaccharide

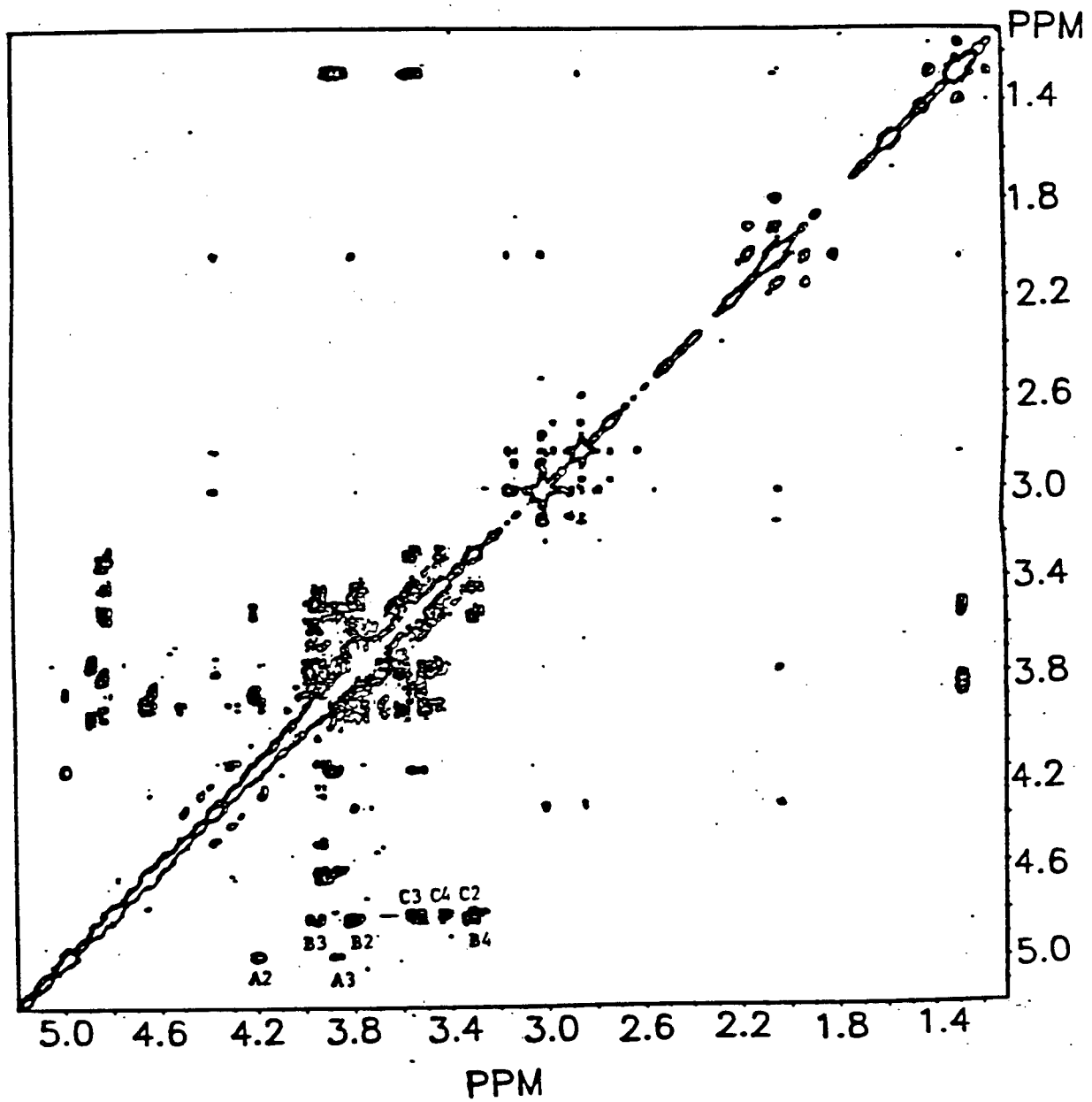


Fig. VII.15: Two step relayed ^{1}H spin coherence transfer (COSYHGR2) n.m.r. spectrum of a lower molecular weight polymer (Px) derived from K46 native polysaccharide

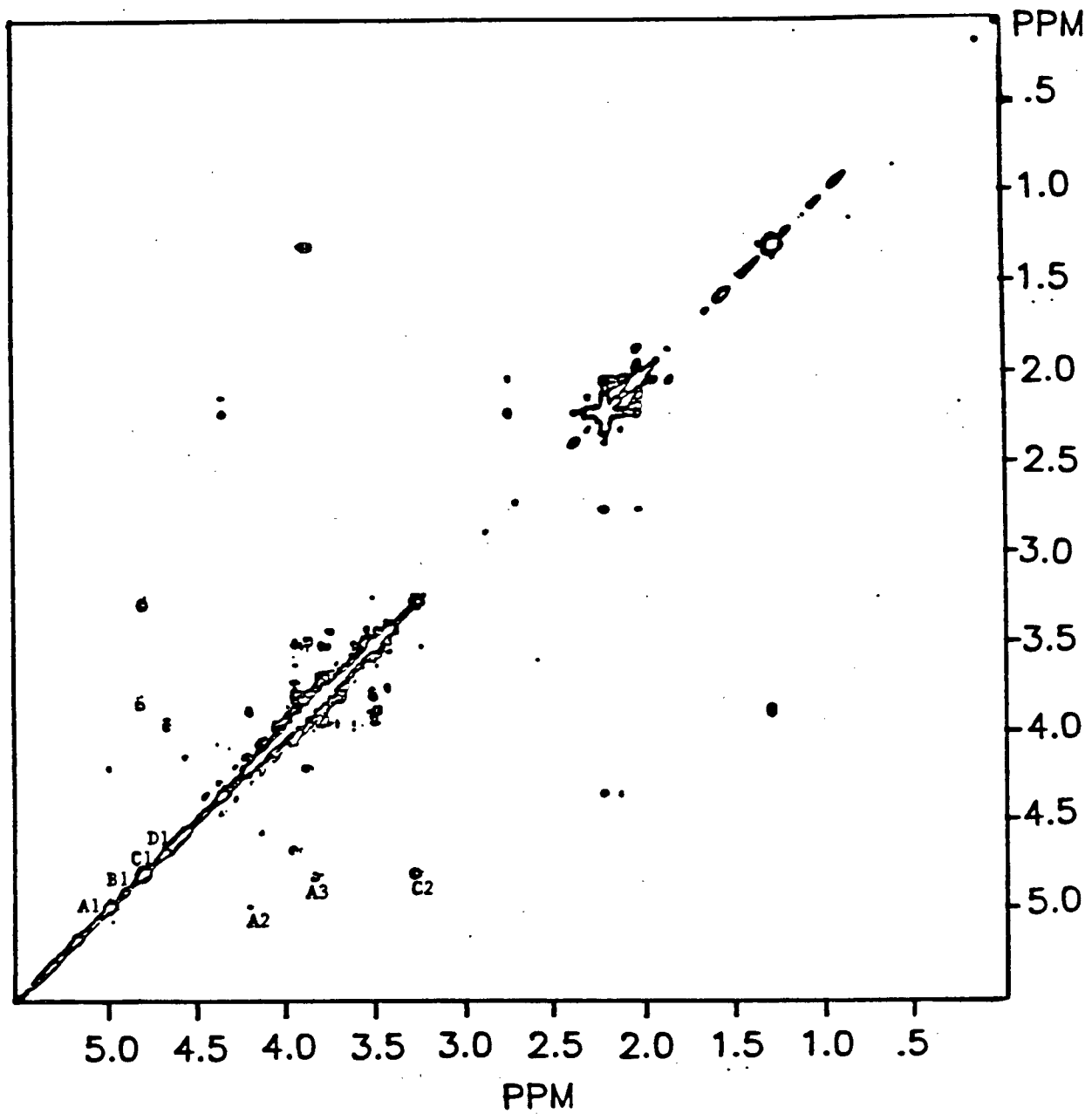
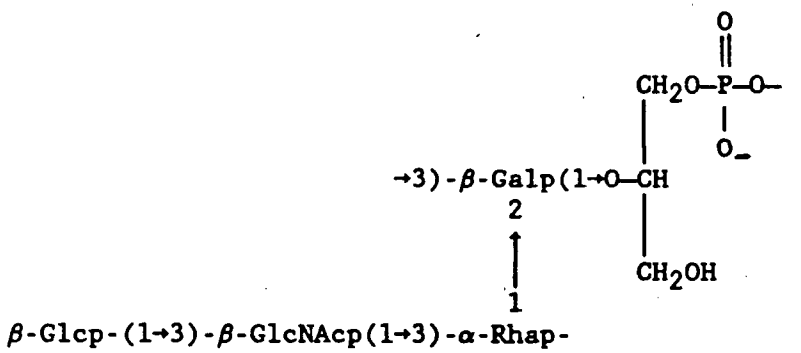


Fig. VII.16: Homonuclear dipolar correlated 2D n.m.r. (NOESY) spectrum of a lower molecular weight polymer (Px) derived from K46 native polysaccharide

obtained by data from a NOESY experiment[185] (Table VII.9 and Fig. VII.16). Interresidue n.O.e. contacts[186] established that B' was linked to A' thus giving the sequence shown.

$$\overset{(B)'}{\text{GlcNAc}}\overset{1\ 3}{\text{---}}\overset{(A)'}{\text{Rha}}$$

Chemical studies and n.m.r. studies on the native polysaccharide and bacteriophage product (Px) afforded the total sequence of the sugar residues in the repeating unit of the polysaccharide as shown below.



Experimental

General methods. The general experimental procedures are as described in Section III.1.3.

Isolation and purification of E. coli K46 capsular polysaccharide. E. coli K46 culture was obtained from Dr. Ida Ørskov, WHO International Escherichia Centre, Copenhagen). Actively growing colonies of E. coli

K46 were propagated by replating several times onto Petri dishes (layered with sterile Mueller Hinton agar), a single colony being selected each time the bacteria were to be plated. This culture was grown as described in Section III.1.3. The K46 bacteria were scraped from the agar surface, diluted with 1% phenol solution and stirred at 4°C for 6 h. The mixture of polysaccharide bacterial cells and other debris was ultracentrifuged (for 4 h at 15°C on a Beckman L3-50 ultracentrifuge using rotor 45 Ti at 31,000 r.p.m. or 80,000 g) to separate the polysaccharide from the dead bacterial cells. The viscous honey-colored supernatant was precipitated with ethanol. The resultant stringy precipitate was dissolved in water. The resultant solution was treated with a few drops of Cetavlon (cetyltrimethylammonium bromide) and was centrifuged. The supernatant was further treated with Cetavlon and kept at 4°C overnight. This fractional precipitation was adapted because the first precipitate was impure. The Cetavlon-polysaccharide complex was dissolved in 4M NaCl solution, precipitated into ethanol, redissolved in water and dialyzed against distilled water (for two days). The polysaccharide was isolated as a styrofoam-like material by lyophilization. The isolated polysaccharide was further purified by gel permeation chromatography using Sephadex S-400 column (100 cm x 2.5 cm).

Sugar analysis and composition. 10 mg of K46 polysaccharide was treated with anhydrous hydrogen fluoride for 6 h at 25°C, then the acid was removed by evaporation and co-distilled with 5% acetic acid. The neutral sugars were analyzed as alditol acetates by g.l.c.-m.s. A portion of K46 polysaccharide (10 mg) was methanolyzed overnight in 3%

HCl-CH_3OH, and the resultant product was reduced with sodium borohydride, hydrolyzed and analyzed by g.l.c.-m.s. as alditol acetates.

Methylation analysis. The native polysaccharide (20 mg) was methylated by the method of Hakomori,[101] dialyzed, partitioned between dichloromethane and water and purified on a column of Sephadex LH20 to give a product which showed no absorption at 3625 cm^{-1}. The methylated polymer was converted to alditol acetates and analyzed by g.l.c.-m.s.

Determination of reducing end.[200] A sample (7 mg) of compound 2 (i.e. lower molecular weight compound) was converted to its alditol by $NaBH_4$ reduction. This alditol was hydrolyzed using 4M TFA for 0.5 h at 122°C. After removing the excess TFA, the anhydrous residue was treated with 5% hydroxylamine hydrochloride in pyridine (1 mL) and heated at 95°C for 20 min. Anhydrous acetic anhydride (1 mL) was added to the cooled reaction mixture and further heated (at 95°C for 25 min). Removal of excess acetic anhydride and pyridine was attained by the addition of ethanol and repeated codistillation with water. Pure alditol acetate and acetylated aldononitriles were obtained by water/chloroform extraction and evaporation of the chloroform phase. The alditol acetate and acetylated aldononitriles were analyzed by g.l.c.-m.s. on a DB-17 column programmed at 180°C, 5°C per minute to 220°C.

Dephosphorylation of native polysaccharide. Native polysaccharide (100 mg) was dissolved in 48% aqueous hydrogen fluoride and stirred at

4°C for 72 h. The reaction mixture was then neutralized using 50% ammonium hydroxide solution. The reaction mixture was desalted on Sephadex G10. The isolation of dephosphorylated polysaccharide was by gel permeation chromatography on Bio-Gel P2 column (100 cm by 2.5 cm). This resultant product (60 mg) was analyzed by ^{13}C, ^{1}H, ^{31}P n.m.r. and 2D n.m.r. 5 mg of dephosphorylated product was dried under vacuum for 18 h and treated with anhydrous hydrogen fluoride for 6 h. The excess hydrogen fluoride was removed by evaporation and co-distillation with 5% acetic acid aqueous solution. The resultant product was reduced by sodium borodeuteride and analyzed by g.l.c.-m.s. as alditol acetates.

Bacteriophage depolymerization. The phage was isolated from Vancouver sewage and propagated by tube and flask lysis to a concentration of 1.3×10^{11} p.f.u. mL^{-1}. Depolymerization and isolation of low molecular weight polymer (Px) were conducted as described in Section III.1.3. 140 mg of K46 polysaccharide was used in depolymerization and 60 mg of Px was isolated.

N.m.r. studies

General methods for 2D homonuclear experiments are as discussed in Section IV.2.4.

The native polysaccharide was lyophilized twice from deuterium oxide and dissolved at a concentration of 30 mg mL^{-1}. ^{13}C-N.m.r. spectra were obtained using samples in 5 mm diameter tubes at 300°K on a Varian XL 300 spectrometer operating at 75 MHz in the pulsed Fourier transformed

mode with complete proton decoupling. ^{13}C-^{31}P Coupling constants were obtained from the expanded ring region (90 ppm to 60 ppm) of the ^{13}C-n.m.r. spectrum at 75 MHz. A heteronuclear ^{13}C-^{1}H shift correlated experiment was done on a Bruker AM 400 spectrometer using the CHORTLE technique (carbon-hydrogen correlations from one-dimensional polarization-transfer spectra by least-square analysis).[200] The experiment was performed at 300°K on a sample of a native CPS in deuterium oxide (300 mg mL^{-1}). 128 experiments were performed using 1072 transients per experiment.

The acquisition parameters of the COSY and relay COSY (i.e. one and two step) experiments were identical to that given in Section IV.2.4. These experiments were performed on the AM 400 Bruker spectrometer.

The pulse sequence for the NOESY experiments is given in Appendix II. Several NOESY experiments were performed and the best results were obtained using a mixing time (D9) of 0.255s with no random variation (V9=0). The other details are:

Sweep width in F1 dimension (SW1) = 920 Hz
Relaxation delay (D1) = 1.2 s
Pre-saturation with power S1 = 40 L
Delay for evolution of shifts (D0) = 0.0002 (with increments of 0.5/SW1 for 256 times)
90° excitation pulse (P1) = 16 μs
Mixing pulse 90° (P2) = 16 μs
Detection pulse 90° (P3) = 16 μs
Number of scans per experiment (NS) = 112
Number of experiments (NE) = 256

^{31}P-N.m.r. experiments were performed on a Varian XL300 at 122 MHz in the pulse Fourier transform mode with no proton decoupling.

Phage degraded polymer Px was lyophilized twice from deuterium oxide and dissolved at a concentration of 35 mg mL^{-1}. This sample was used

for the 2D n.m.r. experiments all of which were conducted at 343° K. The acquisition parameters of the COSY experiment were identical to those given in Section IV.2.3 except for using 112 transients per experiment. Apart from the second coherence period (D2) and third coherence period (D3), the acquisition parameters for relay COSY experiments were identical to those given in Section IV.2.3. The second coherence period (D3) was varied in a number of experiments to attain the best relayed spin coherence transfer. Several NOESY experiments were performed and the best results were attained using a mixing time (D9) of 0.26 s with no random variation (i.e. V9 = 0). The other NOESY parameters were identical with those given in this section.

^{1}H Chemical shift assignment of 3,6-dideoxyamino sugar residue in E. coli K45 capsular polysaccharide

Introduction

The repeating unit of the K antigen of E. coli K45 includes a 3,6-dideoxyamino sugar residue but the complete structure has not been established. Further work will be done to establish the structure. The ^{1}H chemical shift assignment of the 3,6-dideoxyamino sugar residue is reported.

Results and discussion

The repeating unit of E. coli K45 polysaccharide contains a 3,6-di-

deoxy amino sugar and the mass spectrum of the alditol acetate[202] of this amino sugar is shown below.

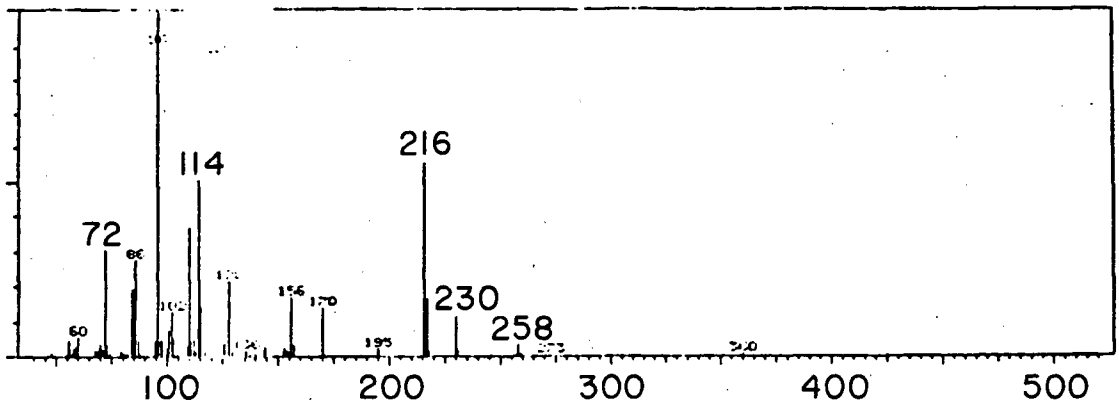


Table VII.10: ^{1}H N.m.r. data for E. coli K45 native polysaccharide

Symbol	Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6/H-6'
A	-	4.94	4.04	3.84	3.63	3.51	-
B	-	4.62	3.88	3.56	4.30	3.83	-
C	3,6-dideoxy amino sugar	4.54	3.62	3.57	3.81	3.95	1.26
D	-	4.48	3.53	3.92	3.74	3.66	-

The H-1, H-2, H-3, and H-4 resonances of all the sugar residues in the repeating unit were assigned from the COSY n.m.r. experiment data (Fig. V.1 and Table V.1). Complete assignment of the proton resonances of residue C was afforded by the additional window provided by CH_3 resonance. Residue C was assigned as 3,6-dideoxyamino sugar.

An attempt to isolate a pure form of the 3,6-dideoxyamino sugar for K45 polysaccharide by paper chromatography was unsuccessful. It was anticipated that a 2D-COSY phase-sensitive double quantum filter n.m.r. experimental result on the pure form of the 3,6-dideoxyamino sugar could have provided complete structure of this amino sugar. The isolation of alditol acetates of the 3,6-dideoxyamino sugar and its analysis by X-ray crystallographic will be pursued.

Although the complete structure of the 3,6-dideoxyamino sugar is unknown, this study provides the finger print of the proton chemical shifts of this amino sugar.

Experimental

General methods for 2D homonuclear experiments are as discussed in Section IV.2.4.

The acquisition parameters of the COSY experiment were identical to those given in Section IV.2.4 except for using 48 transients per experiment. COSY-45 (45° mixing pulse P_2 = 9.8 μs) and COS 90 (90° mixing pulse P_2 = 19.5 μs) experiments were performed. COSY-45 provided better results (see Table V.1 and Fig. V.1).

Native polysaccharide (K45) was lyophilized twice from deuterium oxide and dissolved at 25 mg mL^{-1}. COSY experiment (Table V.1 and Fig. V.1) were performed at 338°K.

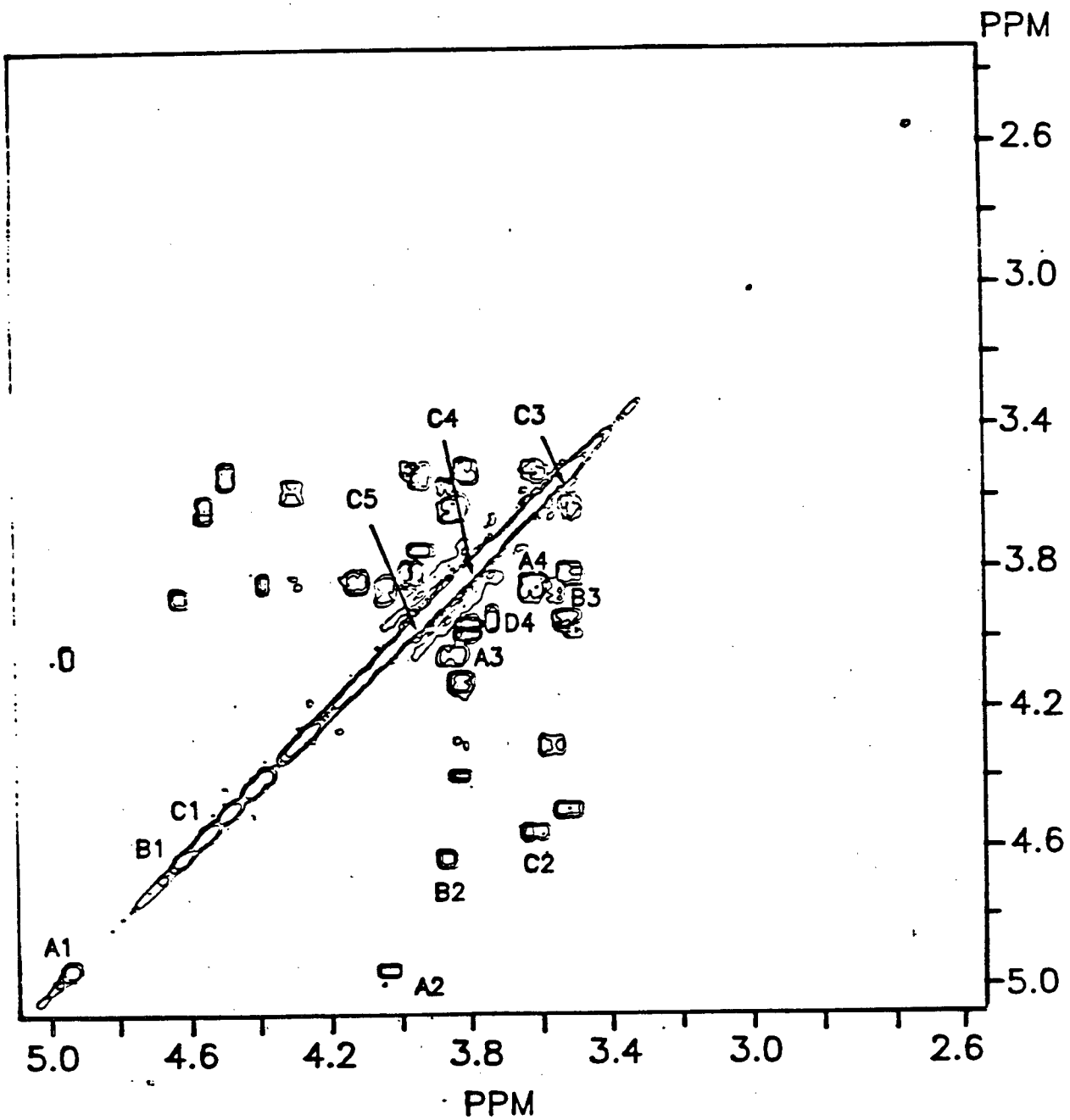


Fig. VII.17: Homonuclear ^{1}H-spin correlated (COSY) n.m.r. spectrum of native polysaccharide (K45)

APPENDIX II

Bruker 2D Files Employed in this Study

```
;  COSY.AU
; HOMONUCLEAR SHIFT-CORRELATED 2-D NMR (JEENER)
; W.P.AUE, E.BARTHOLDI, R.R.ERNST, J.CHEM.PHYS. 64, 2229 (1976)
; K.NAGAYAMA ET AL, J.MAGN.RES. 40, 321 (1980)

;  D1 - 90 - D0 - 90 OR 45 - FID

; SYMMETRIC MATRIX WITH SHIFTS AND COUPLINGS IN F1, F2
; OFF-DIAGONAL PEAKS CORRELATE SPINS WHICH SHARE A
; SCALAR COUPLING J.

.1 ZE
 2 D1              ;RELAXATION
 3 P1 PH1          ;90 DEG EXCITATION PULSE
 4 D0              ;EVOLUTION OF SHIFTS AND COUPLINGS
 5 P2 PH2          ;MIXING PULSE, 90 OR 45 DEG
 6 GO=2            ;ACQUIRE FID
 7 WR #1           ;STORE FID
 8 IF #1           ;INCREMENT FILE NUMBER
 9 IN=1            ;INCREMENT D0 AND LOOP FOR NEXT EXPER.
10 EXIT

PH1=A0 A0 A0 A0 A1 A1 A1 A1      ;PHASE PROGRAMS CANCEL AXIAL
    A2 A2 A2 A2 A3 A3 A3 A3      ;PEAKS (SCANS 1-2), SELECT N-TYPE
                                 ;PEAKS (SCANS 3-4), SUPPRESS F2
PH2=A0 A2 A1 A3 A1 A3 A2 A0      ;QUAD IMAGES (SCANS 5-8), AND CANCEL
    A1 A3 A2 A0 A2 A0 A3 A1      ;ARTEFACTS FROM P1 (SCANS 9-16).

;PROGRAM REQUESTS FILENAME WITH .SER EXTENSION
;NE DEFINES NUMBER OF FIDS = TD1
;USE QF, NS = 4,8, OR 16 (COMPLETE PHASE CYCLE)
;DS = 2 OR 4
;RD=PW=0
;D1 = 1-5*T1
;P1 = 90 DEG
;P2 = 90 DEG FOR MAX. SENSITIVITY
;   = 45 DEG FOR MINIMAL DIAGONAL (GOOD FOR TIGHT AB SYSTEMS)
;        AND 'TILTED' CORREL. PEAKS (SIGNS OF COUPLINGS).
;D0 = 3E-6 INITIAL DELAY
;IN = 0.5/SW1 = 2*DW
;NDO = 1
;I2D = 1, SW1=SW/2

;CHOOSE SW AND SI SO THAT HZ/PT = CA. 2-6 HZ
;TYPICALLY USE TD = SI, NO ZERO-FILLING IN F2
;              NE = SI/4, ZERO-FILL IN F1
;MATRIX CAN BE SYMMETRIZED ABOUT DIAGONAL
```

```
*LIST COSYHG.AU

------ FILE: COSYHG  .AU

;  COSYHG.AU
; HOMONUCLEAR SHIFT-CORRELATED 2-D NMR (JEENER)
; USING  PRE-SATURATION OF SOLVENT WITH TWO POWER LEVELS.
; ALSO ALLOWS FOR FIXED DELAY AS IN COSYLR.AU

;  HG(S1)-(S2)-------------------------- DO
;  D1 -   D3 - 90 - D0 -D2-90 OR 45-D2 - FID

; SYMMETRIC MATRIX WITH SHIFTS AND COUPLINGS IN F1, F2
; OFF-DIAGONAL PEAKS CORRELATE SPINS WHICH SHARE A
; SCALAR COUPLING J.

1 ZE
2 D1 HG S1        ;RELAXATION, PRE-SATURATION WITH POWER S1
  D3 S2           ;SWITCH TO MIN. POWER S2 FOR EVOLUTION
3 P1 PH1          ;90 DEG EXCITATION PULSE
4 D0              ;EVOLUTION OF SHIFTS AND COUPLINGS
  D2              ;FIXED DELAY TO ENHANCE EFFECTS FROM SMALL J
5 P2 PH2          ;MIXING PULSE, 90 OR 45 DEG
  D2
6 GO=2 DO         ;ACQUIRE FID WITH DEC. GATED OFF
7 WR #1           ;STORE FID
8 IF #1           ;INCREMENT FILE NUMBER
9 IN=1            ;INCREMENT DO AND LOOP FOR NEXT EXPER.
10 EXIT


PH1=A0 A0 A0 A0 A1 A1 A1 A1
    A2 A2 A2 A2 A3 A3 A3 A3

PH2=A0 A2 A1 A3 A1 A3 A2 A0
    A1 A3 A2 A0 A2 A0 A3 A1

;PROGRAM REQUESTS FILENAME
```

```
------ FILE: COSYRCT .AU

; COSYRCT.AU
; COSY WITH 1-STEP RELAYED COHERENCE TRANSFER (MAGNITUDE MODE)
; G.WAGNER, JMR 55, 151 (83).
; A.BAX & G.DROBNY, JMR 61,306 (85)

; D1-90-D0-90-D2-180-D2-90-FID

; CORRELATION CROSS-PEAKS CAN BE OBTAINED FROM SPINS A AND X
; IN AN AMX SYSTEM WHEN J(AX) IS TOO SMALL.

1 ZE
2 D1              ;RELAXATION DELAY
3 P1 PH1          ;90 DEG PULSE CREATES XY-MAGN.
4 D0              ;EVOLUTION OF SHIFTS
5 P1 PH2          ;COMPLETE FIRST COHERENCE TRANSFER, E.G.
                  ; SPIN A TO M DEPENDS ON SIN(PI*J(AM)*D0)
6 D2              ;SECOND COHERENCE PERIOD
7 P2 PH2          ;REFOCUS CHEMICAL SHIFTS
8 D2
9 P1 PH3          ;COMPLETE SECOND TRANSFER (EG. M TO X)
                  ; EFFICIENCY DEPENDS ON
                  ; SIN(PI*2D2*J(AM))*SIN(PI*2D2*J(MX)
10 GO=2 PH4       ;ACQUIRE FID
11 WR #1          ;STORE FID IN .SER FILE
12 IF #1
13 IN=1           ;LOOP FOR NEXT EXPERIMENT
14 EXIT

PH1=A0 A0 A0 A0 A0 A0 A0 A0      ;SCANS 1-2 SUPPRESS AXIAL PEAKS
    A1 A1 A1 A1 A1 A1 A1 A1      ;SCANS 3-4 FOR F1 QUAD (N-TYPE)
    A2 A2 A2 A2 A2 A2 A2 A2
    A3 A3 A3 A3 A3 A3 A3 A3
PH2=A0 A0 A1 A1 A2 A2 A3 A3      ;SCANS 5-8 SUPPRESS NOESY PEAKS
    A1 A1 A2 A2 A3 A3 A0 A0      ;FURTHER CYCLING FOR F2 QUAD (QF)
    A2 A2 A3 A3 A0 A0 A1 A1
    A3 A3 A0 A0 A1 A1 A2 A2
PH3=A0 A2 A1 A3 A0 A2 A1 A3
    A1 A3 A2 A0 A1 A3 A2 A0
    A2 A0 A3 A1 A2 A0 A3 A1
    A3 A1 A0 A2 A3 A1 A0 A2

PH4=R0 R0 R2 R2 R0 R0 R2 R2
    R1 R1 R3 R3 R1 R1 R3 R3
    R2 R2 R0 R0 R2 R2 R0 R0
    R3 R3 R1 R1 R3 R3 R1 R1

;D2 = CA. 0.5/(J(AM)+J(MX)) WHEN COUPLINGS DO NOT DIFFER BY
;      MORE THAN FACTOR 2
;    = CA. 0.5/( 1.6*J(MAX) ) OR AT MOST 0.5/( 1.3*J(MAX) )
;      TO COVER A WIDER RANGE OF J.
;      NULLING CONDITIONS OF D2=0.5/J SHOULD BE AVOIDED

;NS=8*N
;P1=90, P2=180
;  OTHERWISE PARAMETERS AS FOR COSY.

;SEE ALSO 'RECOSY2.AU'
```

```
------ FILE: COSYR1HG.AU

; COSYR1HG.AU
; COSY WITH 1-STEP RELAYED COHERENCE TRANSFER (MAGNITUDE MODE)
; WITH 2 LEVELS SOLVENT SIGNAL SUPPRESSION BY PRESATURATION
; G.WAGNER, JMR 55, 151 (83).
; A.BAX & G.DROBNY, JMR 61,306 (85)

; HG(S1)-(S2)----------------------DO
; D1  -  D3-90-D0-90-D2-180-D2-90-FID

; CORRELATION CROSS-PEAKS CAN BE OBTAINED FROM SPINS A AND X
; IN AN AMX SYSTEM WHEN J(AX) IS TOO SMALL.

1 ZE
2 D1 HG S1        ;RELAXATION DELAY, PRE-SAT WITH POWER S1
  D3 S2           ;SWITCH TO MIN. POWER S2 FOR EVOLUTION
3 P1 PH1          ;90 DEG PULSE CREATES XY-MAGN.
4 D0              ;EVOLUTION OF SHIFTS
5 P1 PH2          ;COMPLETE FIRST COHERENCE TRANSFER, E.G.
                  ; SPIN A TO M DEPENDS ON SIN(PI*J(AM)*D0)
6 D2              ;SECOND COHERENCE PERIOD
7 P2 PH2          ;REFOCUS CHEMICAL SHIFTS
8 D2
9 P1 PH3          ;COMPLETE SECOND TRANSFER (EG. M TO X)
                  ; EFFICIENCY DEPENDS ON
                  ; SIN(PI*2D2*J(AM))*SIN(PI*2D2*J(MX)
10 GO=2 DC PH4    ;ACQUIRE FID WITH DEC. GATED OFF
11 WR #1          ;STORE FID IN .SER FILE
12 IF #1
13 IN=1           ;LOOP FOR NEXT EXPERIMENT
14 EXIT

PH1=A0 A0 A0 A0 A0 A0 A0 A0      ;SCANS 1-2 SUPPRESS AXIAL PEAKS
    A1 A1 A1 A1 A1 A1 A1 A1      ;SCANS 3-4 FOR F1 QUAD (N-TYPE)
    A2 A2 A2 A2 A2 A2 A2 A2
    A3 A3 A3 A3 A3 A3 A3 A3
PH2=A0 A0 A1 A1 A2 A2 A3 A3      ;SCANS 5-8 SUPPRESS NOESY PEAKS
    A1 A1 A2 A2 A3 A3 A0 A0      ;FURTHER CYCLING FOR F2 QUAD (QF)
    A2 A2 A3 A3 A0 A0 A1 A1
    A3 A3 A0 A0 A1 A1 A2 A2
PH3=A0 A2 A1 A3 A0 A2 A1 A3
    A1 A3 A2 A0 A1 A3 A2 A0
    A2 A0 A3 A1 A2 A0 A3 A1
    A3 A1 A0 A2 A3 A1 A0 A2

PH4=R0 R0 R2 R2 R0 R0 R2 R2
    R1 R1 R3 R3 R1 R1 R3 R3
    R2 R2 R0 R0 R2 R2 R0 R0
    R3 R3 R1 R1 R3 R3 R1 R1


;D3 = 2 MSEC TO SWITCH DEC POWER
;S1 = 30L, S2 = 45L
;D2 = CA. 0.5/(J(AM)+J(MX)) WHEN COUPLINGS DO NOT DIFFER BY
;       MORE THAN FACTOR 2
;    = CA. 0.5/( 1.6*J(MAX) ) OR AT MOST 0.5/( 1.3*J(MAX) )
;       TO COVER A WIDER RANGE OF J.
;       NULLING CONDITIONS OF D2=0.5/J SHOULD BE AVOIDED

;NS=8*N
;P1=90, P2=180
;  OTHERWISE PARAMETERS AS FOR COSY.
```

```
------ FILE: COSYRCT2.AU

; COSYRCT2.AU
; COSY WITH 2-STEP RELAYED COHERENCE TRANSFER (MAGNITUDE MODE)
; G.WAGNER, JMR 55, 151 (83).
; A.BAX & G.DROBNY, JMR 61,306 (85)

;  90-D0-90-D2-180-D2-90-D3-180-D3-90-FID

; CORRELATION CROSS-PEAKS CAN BE OBTAINED FOR SPIN A
; FROM SPINS M,Q,X IN AN AMQX SPIN SYSTEM.

1 ZE
2 D1              ;RELAXATION DELAY
3 P1 PH1          ;90 DEG PULSE CREATES XY-MAGN.
4 D0              ;EVOLUTION OF SHIFTS
5 P1 PH2          ;COMPLETE FIRST COHERENCE TRANSFER, E.G.
                  ; SPIN A TO M DEPENDS ON SIN(PI*J(AM)*D0)
6 D2              ;SECOND COHERENCE PERIOD
7 P2 PH2          ;REFOCUS CHEMICAL SHIFTS
8 D2
9 P1 PH3          ;COMPLETE SECOND TRANSFER (EG. M TO Q)
10 D3
11 P2 PH2
12 D3
13 P1 PH4         ;THIRD TRANSFER FROM Q TO X
14 GO=2 PH5       ;ACQUIRE FID
15 WR #1          ;STORE FID IN .SER FILE
16 IF #1
17 IN=1           ;LOOP FOR NEXT EXPERIMENT
18 EXIT

PH1=A0                              ;SCANS 1-2 SUPPRESS AXIAL PEAKS
                                    ;SCANS 3-4 FOR F1 QUAD (N-TYPE)
                                    ;SCANS 5-8 SUPPRESS NOESY PEAKS
PH2=A0 A0 A0 A0 A1 A1 A1 A1
    A2 A2 A2 A2 A3 A3 A3 A3         ;FOR F2 QUAD PHASE CYCLING (QF,
                                    ;CYCLOPS) ALL PHASES MUST BE
PH3=A0 A0 A2 A2 A1 A1 A3 A3         ;INCREMENTED IN 90 DEG STEPS.

PH4=A0 A2 A0 A2 A1 A3 A1 A3

PH5=R0 R0 R0 R0 R2 R2 R2 R2

;FOR LINEAR SPIN SYSTEM AMQX, THE TRANSFER FUNCTION IS
; SIN(PI*J(AM)*2D2)SIN(PI*J(MQ)*2D2)*
; SIN(PI*J(MQ)*2D3)SIN(PI*J(QX)*2D3)
;SET D2 = CA. 0.5/(1.6*J), WHERE J = LARGER OF J(AM), J(MQ)
;SET D3 =       "     "          "                J(MQ), J(QX)

;NS=16*N
;P1=90, P2=180
;  OTHERWISE PARAMETERS AS FOR COSY.

;SEE ALSO RECOSY3.AU
```

```
------ FILE: COSYR2HG.AU

; COSYR2HG.AU
; COSY WITH 2-STEP RELAYED COHERENCE TRANSFER (MAGNITUDE MODE)
; AND 2-LEVEL PRESATURATION OF SOLVENT SIGNAL
; MODIFICATION MADE BY S. ORSON CHAN, DEPT OF CHEM, UBC
; G.WAGNER, JMR 55, 151 (83).
; A.BAX & G.DROBNY, JMR 61,306 (85)

; HG(S1)-(S2)-----------------------------------DO
; D1  -  D5-90-D0-90-D2-180-D2-90-D3-180-D3-90-FID

; CORRELATION CROSS-PEAKS CAN BE OBTAINED FOR SPIN A
; FROM SPINS M,Q,X IN AN AMQX SPIN SYSTEM.

1 ZE
2 D1 HG S1        ;RELAXATION DELAY, PRESATURATION WITH POWER S1
  D5 S2           ;SWITCH TO MIN. POWER S2 FOR EVOLUTION
3 P1 PH1          ;90 DEG PULSE CREATES XY-MAGN.
4 D0              ;EVOLUTION OF SHIFTS
5 P1 PH2          ;COMPLETE FIRST COHERENCE TRANSFER, E.G.
                  ; SPIN A TO M DEPENDS ON SIN(PI*J(AM)*D0)
6 D2              ;SECOND COHERENCE PERIOD
7 P2 PH2          ;REFOCUS CHEMICAL SHIFTS
8 D2
9 P1 PH3          ;COMPLETE SECOND TRANSFER (EG. M TO Q)
10 D3
11 P2 PH2
12 D3
13 P1 PH4         ;THIRD TRANSFER FROM Q TO X
14 GO=2 PH5 DO    ;ACQUIRE FID WITH DEC. GATED OFF
15 WR #1          ;STORE FID IN .SER FILE
16 IF #1
17 IN=1           ;LOOP FOR NEXT EXPERIMENT
18 EXIT

PH1=A0                              ;SCANS 1-2 SUPPRESS AXIAL PEAKS
                                    ;SCANS 3-4 FOR F1 QUAD (N-TYPE)
                                    ;SCANS 5-8 SUPPRESS NOESY PEAKS
PH2=A0 A0 A0 A0 A1 A1 A1 A1
    A2 A2 A2 A2 A3 A3 A3 A3         ;FOR F2 QUAD PHASE CYCLING (OP,
                                    ;CYCLOPS) ALL PHASES MUST BE
PH3=A0 A0 A2 A2 A1 A1 A3 A3         ;INCREMENTED IN 90 DEG STEPS.

PH4=A0 A2 A0 A2 A1 A3 A1 A3

PH5=R0 R0 R0 R0 R2 R2 R2 R2

;FOR LINEAR SPIN SYSTEM AMQX, THE TRANSFER FUNCTION IS
; SIN(PI*J(AM)*2D2)SIN(PI*J(MQ)*2D2)*
; SIN(PI*J(MQ)*2D3)SIN(PI*J(QX)*2D3)
;SET D2 = CA. 0.5/(1.6*J), WHERE J = LARGER OF J(AM), J(MQ)
;SET D3 =      "     "         "                 J(MQ), J(QX)

;D1=1-5*T1
;D5=2 MSEC TO SWITCH DEC. POWER
;NS=16*N
;P1=90, P2=180
;  OTHERWISE PARAMETERS AS FOR COSY.

;SEE ALSO RECOSY3.AU
```

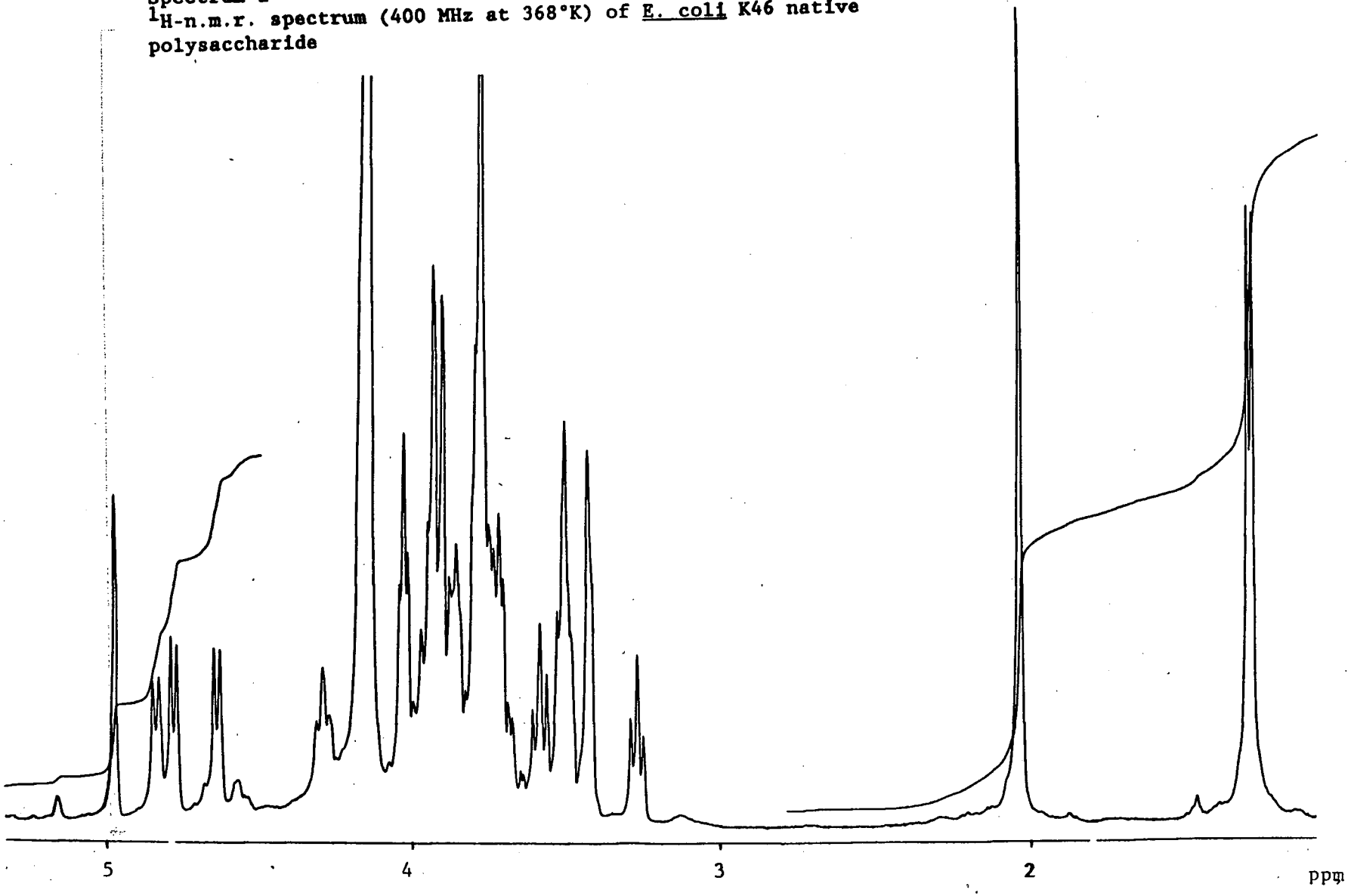
```
------ FILE: NOESYHG .AU

;  NOESYHG.AU
; HOMONUCLEAR DIPOLAR-CORRELATED 2-D NMR (MAGNITUDE MODE)
; WITH PRE-SATURATION OF SOLVENT.
; DIPOLAR COUPLING MAY BE DUE TO NOE OR CHEMICAL EXCHANGE.

;  D1 - 90 - D0 - 90(OR 45) - D9 - 90(OR 45) - FID

; SYMMETRIC MATRIX WITH SHIFTS AND COUPLINGS IN F1, F2
; OFF-DIAGONAL PEAKS CORRELATE SPINS WHICH SHARE A
; DIPOLAR COUPLING.
; SCALAR COUPLING CORRELATIONS ARE STRONGLY REDUCED BY
; RANDOM VARIATION OF THE MIXING TIME D9.

1 ZE
2 D1 HG S3        ;RELAXATION WITH PRE-SATURATION
3 P1 PH1          ;90 DEG EXCITATION PULSE
4 D0              ;EVOLUTION OF SHIFTS AND COUPLINGS
5 P2 PH2          ;MIXING PULSE, 90 (OR 45) DEG
6 D9              ;MIXING TIME FOR Z-MAGN. EXCHANGE
7 P3 PH3          ;DETECTION PULSE, 90 (OR 45) DEG
8 GO=2 PH4 D0     ;ACQUIRE FID WITH DEC. GATED OFF
9 WR #1           ;STORE FID
10 IF #1          ;INCREMENT FILE NUMBER
11 IN=1           ;INCREMENT D0 AND LOOP FOR NEXT EXPER.
12 EXIT

PH1=A0                              ;SCANS 1-2 SUPPRESS AXIAL PEAKS
PH2=A0 A2 A1 A3                     ;SCANS 3-4 GIVE F1 QUAD (N-TYPE)
PH3=A0 A0 A1 A1 A2 A2 A3 A3         ;SCANS 5-8 SUPPRESS DBL. QUANTUM
    A1 A1 A2 A2 A3 A3 A0 A0         ;SCANS 9-16 FOR QF
PH4=R0 R2 R2 R0 R2 R0 R0 R2
    R1 R3 R3 R1 R3 R1 R1 R3

;PROGRAM REQUESTS FILENAME WITH .SER EXTENSION
;NE DEFINES NUMBER OF FIDS = TD1
;NS = 4, 8 OR 16 (COMPLETE PHASE CYCLE)
;DS = 2 OR 4
;FD=FW=0
;D1 = 1-5*T1
;S3 = DEC. POWER FOR PRE-SATURATION, SHOULD BE AS LOW AS POSSIBLE
;      TO AVOID BLOCH-SIEGERT EFFECTS (30-40L).
;P1 = 90 DEG, P2 AND P3 = NORMALLY 90 DEG BUT CAN BE 45 DEG
;    TO GIVE REPRESENTATION LIKE COSY-45.
;D0 = 3E-6 INITIAL DELAY
;IN = 0.5/SW1 = 2*DW
;NDO = 1
;I2D = 1, SW1=SW/2

;D9 = MIXING TIME = CA. T1 FOR SMALL MOLECULES (EXTREME
;  NARROWING LIMIT) OR CA. 50-200 MSEC FOR LARGE MOLECULES
;  WITH CROSS-RELAXATION (SPIN-DIFFUSION).
;V9: D9 WILL BE VARIED RANDOMLY BY MAX. +/- V9 % OF ITS VALUE
;  TO SUPPRESS ZERO-QUANTUM J-CROSS PEAKS (COSY); CHOOSE V9
;  SO THAT D9 IS VARIED BY CA. +/- 20 MSEC TO SUPPRESS J-CROSS
;  PEAKS BETWEEN SPINS WHOSE SHIFTS DIFFER BY >50 HZ.
;TYPICALLY USE TD = SI, NO ZERO-FILLING IN F2
;             NE = SI/4, ZERO-FILL IN F1
;MATRIX CAN BE SYMMETRIZED ABOUT DIAGONAL
```

```
------ FILE: XHCORR  .AUR

;  XHCORR.AUR
; HETERONUC. SHIFT-CORRELATED 2-D NMR (CPD DECOUPLING)
; USING POLARIZATION TRANSFER FROM 1H TO X VIA J(XH).
; A.BAX & G.MORRIS, J.MAGN.RES. 42, 501 (81)

;  1H: D0 - 90 - D0 -   - D0 - D3 - 90        BB
;   X: D1           -180-          90 - D4 - FID

; F2 DOMAIN: BB DEC. X-NUCLEUS SPECTRUM
; F1 DOMAIN: X-NUCLEUS DECOUPLED 1H SPECTRUM WITH J(HH)
; J(XH) MUST BE > 1/T2

1 ZE
2 D1 D0 S1          ;1H RELAXATION, SET DEC. FOR PULSING
3 P1:D PH1          ;90 DEG 1H PULSE
4 D0                ;EVOLUTION OF 1H SHIFTS AND COUPLINGS
5 P4 PH4            ;180 DEG X PULSE TO DECOUPLE X FROM 1H
6 D0                ;FURTHER EVOLUTION
7 D3                ;WAIT FOR OPTIMUM POLARIZATION OF X-H
                    ;  1H DOUBLET
8 P1:D PH2  P3 PH3      ;90 DEG 1H PULSE COMPLETES POLAR.
                        ; TRANSFER, 90 DEG X PULSE CREATES
                    ;DETECTABLE X,Y-MAGN.
9 D4 S2             ;WAIT FOR ANTI-PHASE X-NUCLEUS MULTIPLETS
                    ;  TO REPHASE
10 GO=2 PH5 CPD   ;ACQUIRE BB DEC. X-NUCLEUS FID, MODULATED BY
                    ;1H SHIFTS AND J(HH).
11 D4 D0            ;GATE DEC. OFF
12 WR #1            ;STORE FID
13 IF #1            ;INCREMENT FILE NUMBER
14 IN=1             ;INCREMENT D0, LOOP FOR NEXT EXPER.
15 EXIT

PH1=B0
PH2=B0 B2 B1 B3
PH3=A0 A0 A0 A0 A0 A0 A0 A0
    A1 A1 A1 A1 A1 A1 A1 A1
    A2 A2 A2 A2 A2 A2 A2 A2
    A3 A3 A3 A3 A3 A3 A3 A3

PH4=A0 A0 A0 A0 A2 A2 A2 A2

PH5=R0 R2 R1 R3 R0 R2 R1 R3
    R1 R3 R2 R0 R1 R3 R2 R0
    R2 R0 R3 R1 R2 R0 R3 R1
    R3 R1 R0 R2 R3 R1 R0 R2

;NS=4*N

;PROGRAM REQUESTS FID FILENAME WITH .SER EXTENSION
;NE DEFINES THE NUMBER OF EXPERIMENTS =TD1 FOR 1H
;D1 = 1-5*T1 FOR 1H
;S1 = 0H, MAX. POWER FOR PULSING
;S2 = NORMAL POWER FOR CPD DECOUPLING
;D0 = 3E-6 INITIAL DELAY
;P1 = 90 DEG 1H PULSE
;P3,P4 = 90,180 X PULSE
;D3 = 0.5/J(XH) FOR MAX. POLARIZATION TRANSFER
;D4 = 0.25/J(XH) TO OBSERVE ALL MULTIPLICITIES
;   = 0.5/J(XH) TO OBSERVE XH DOUBLET MULTIP. ONLY
;RD=PW=0
```

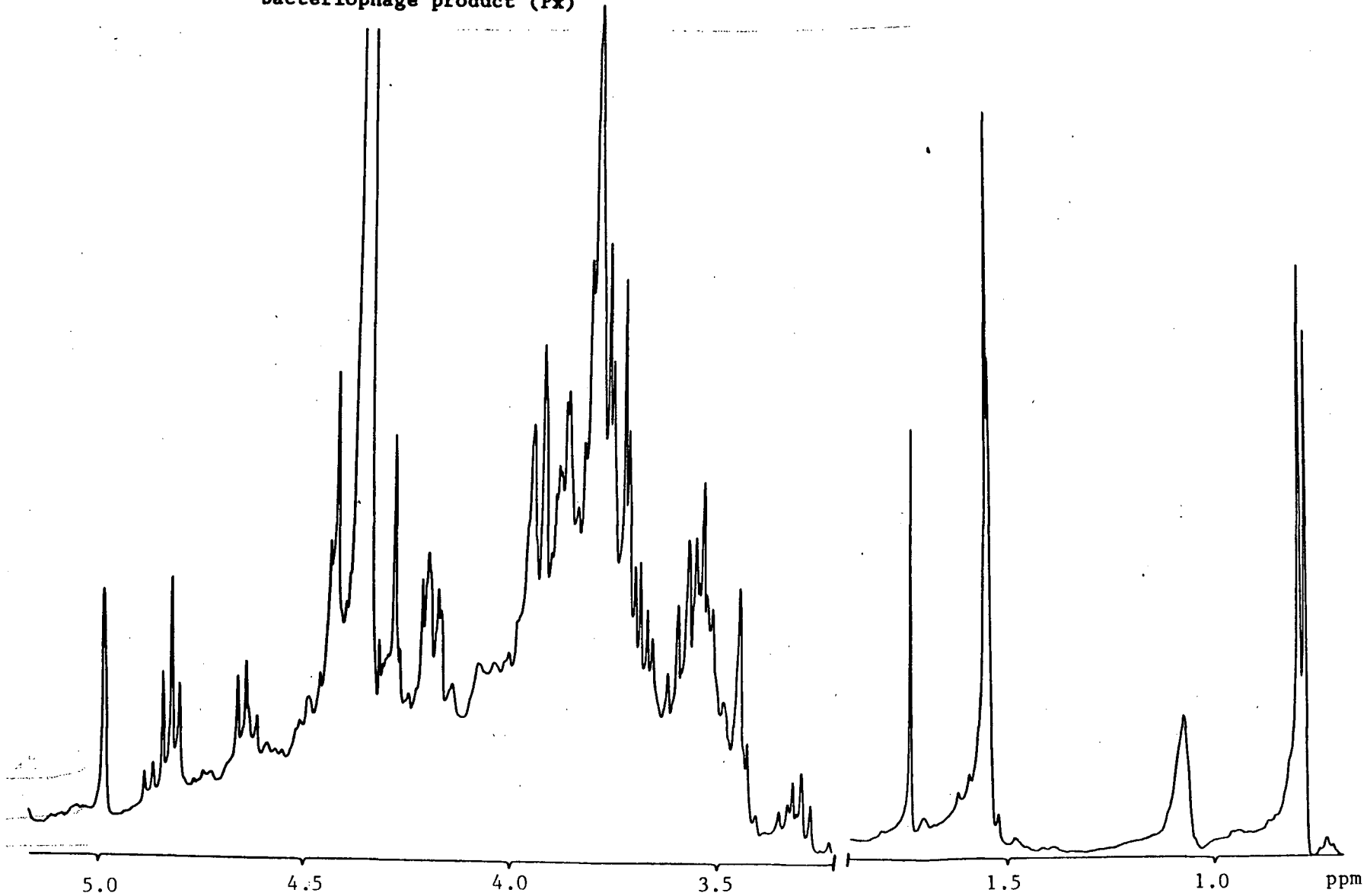
APPENDIX III

N.m.r. Spectra

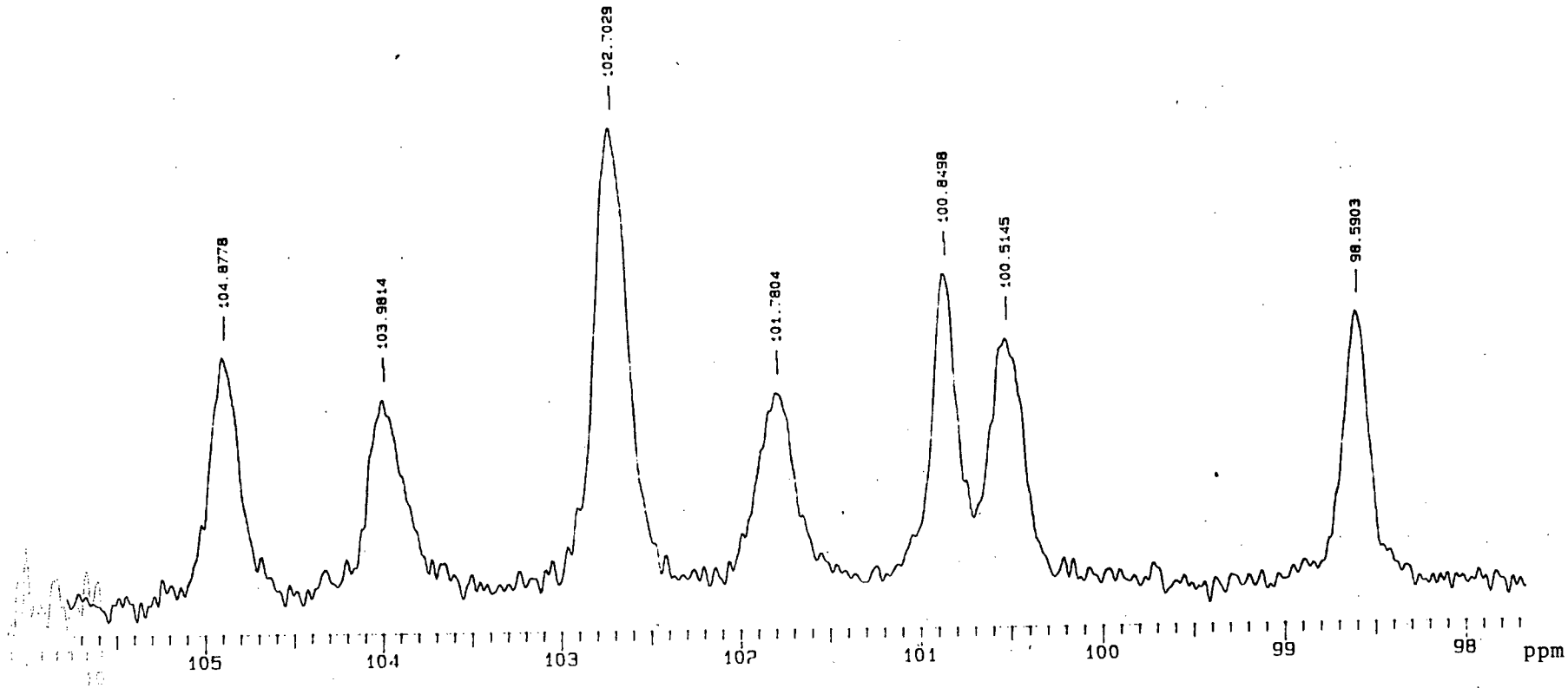
Spectrum 1
^{1}H-n.m.r. spectrum (400 MHz at 368°K) of E. coli K46 native polysaccharide



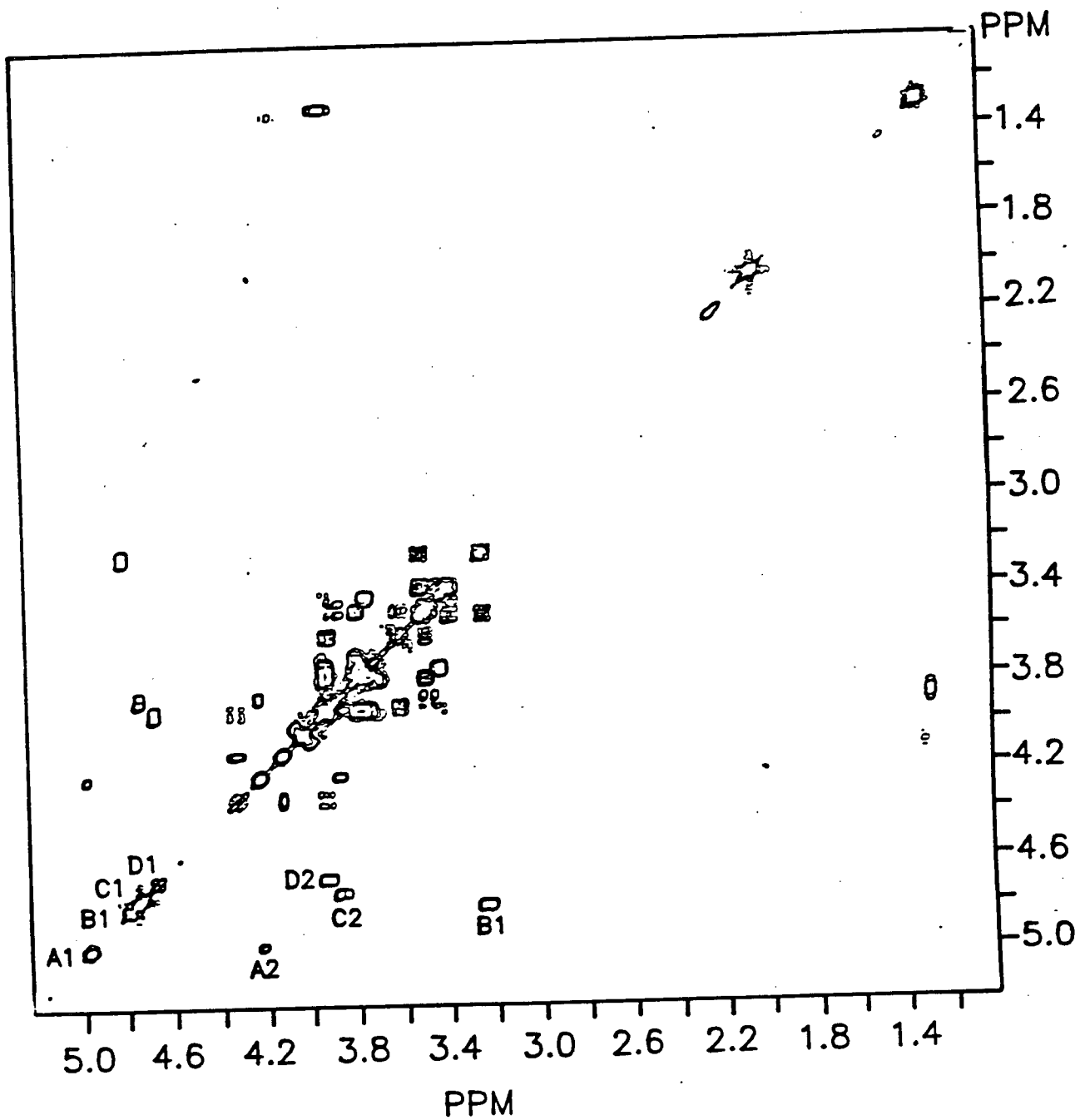
Spectrum 2
^{1}H-n.m.r. spectrum (400 MHz at 343°K) of E. coli K46 bacteriophage product (Px)



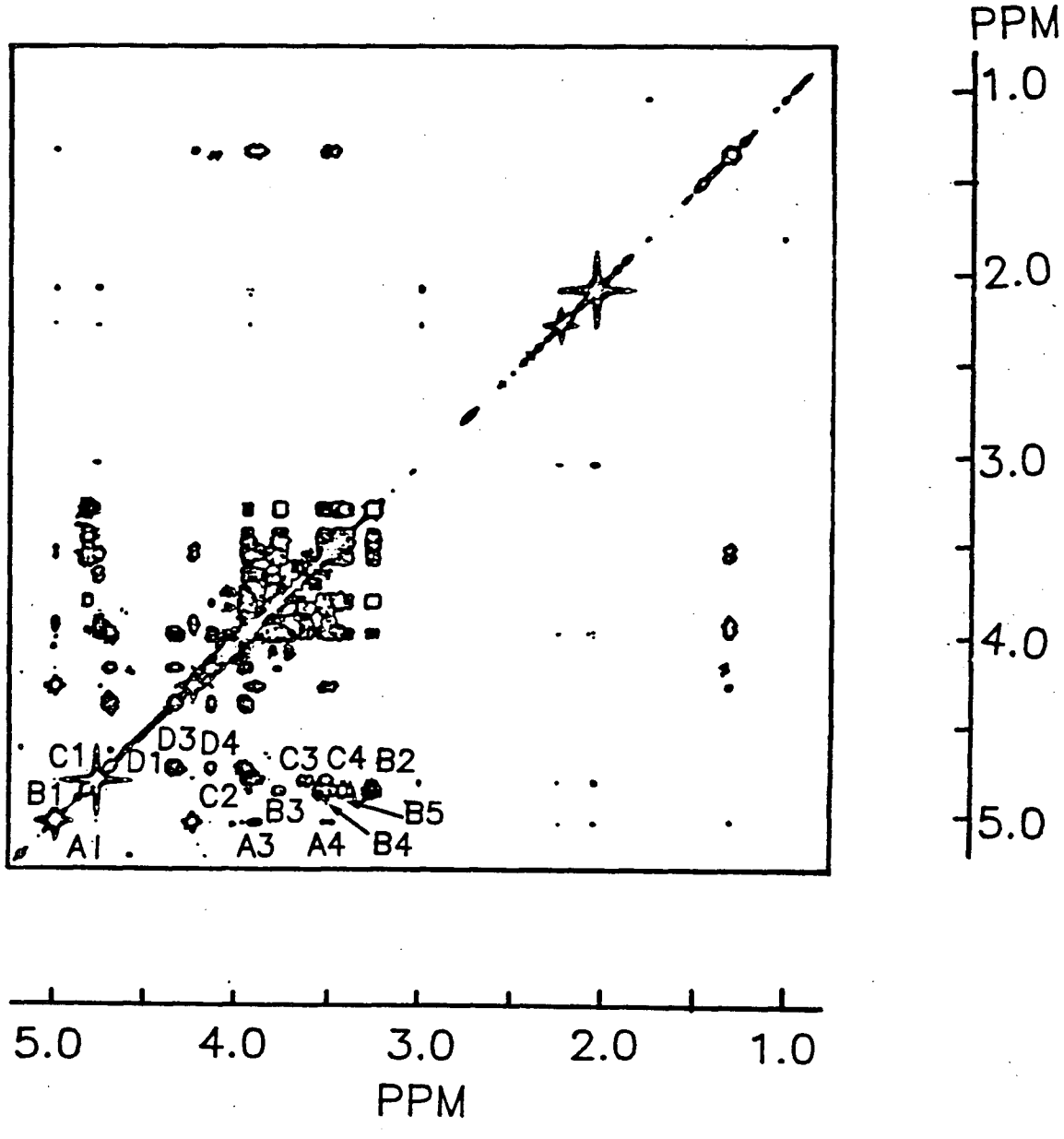
Spectrum 3
^{13}C (^{1}H coupled spectrum)n.m.r. (75 MHz at 300°K) spectrum of E. coli K46 native polysaccharide



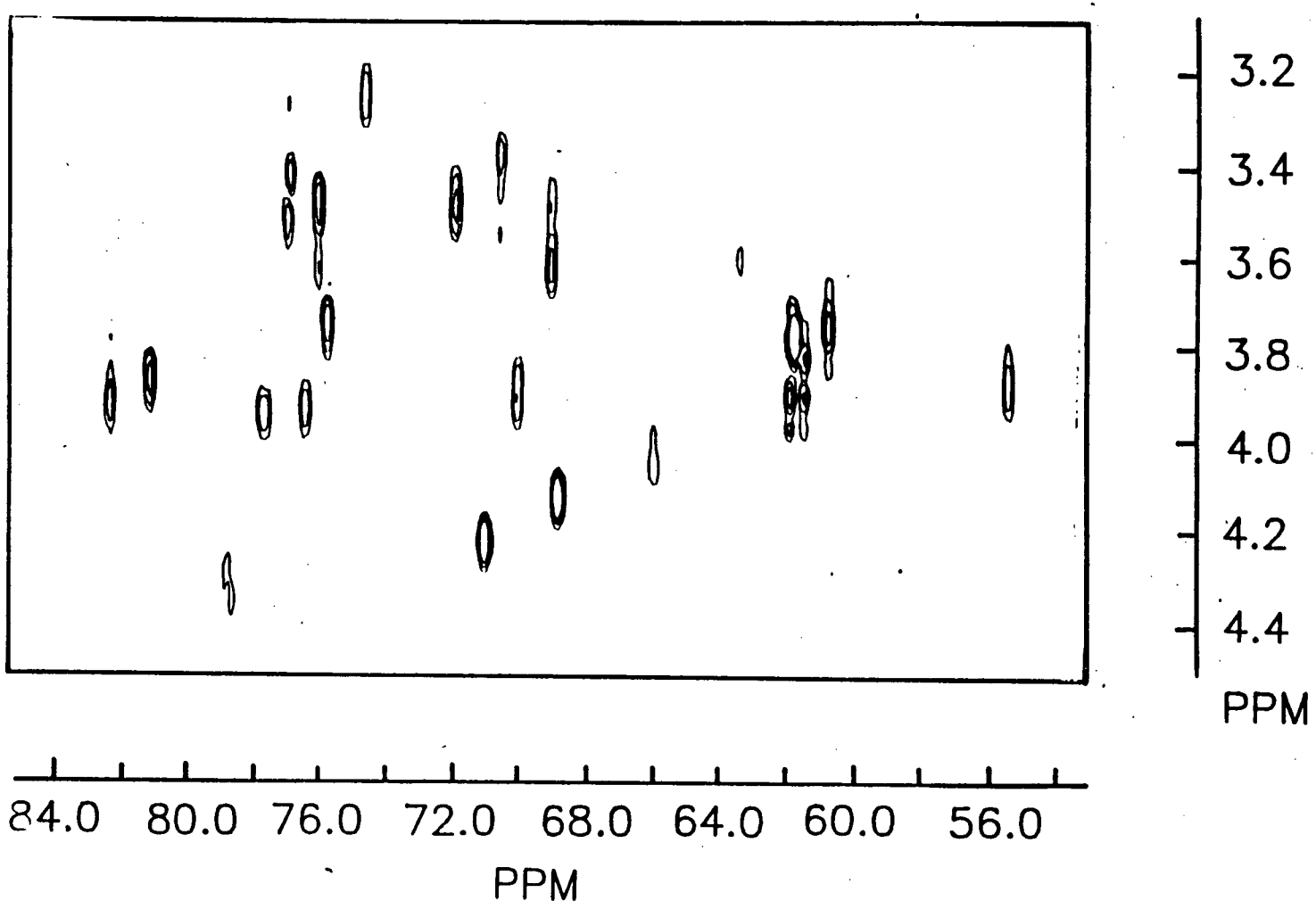
Spectrum 4
2D COSY n.m.r. spectrum of E. coli K46 native polysaccharide (at 300°K)



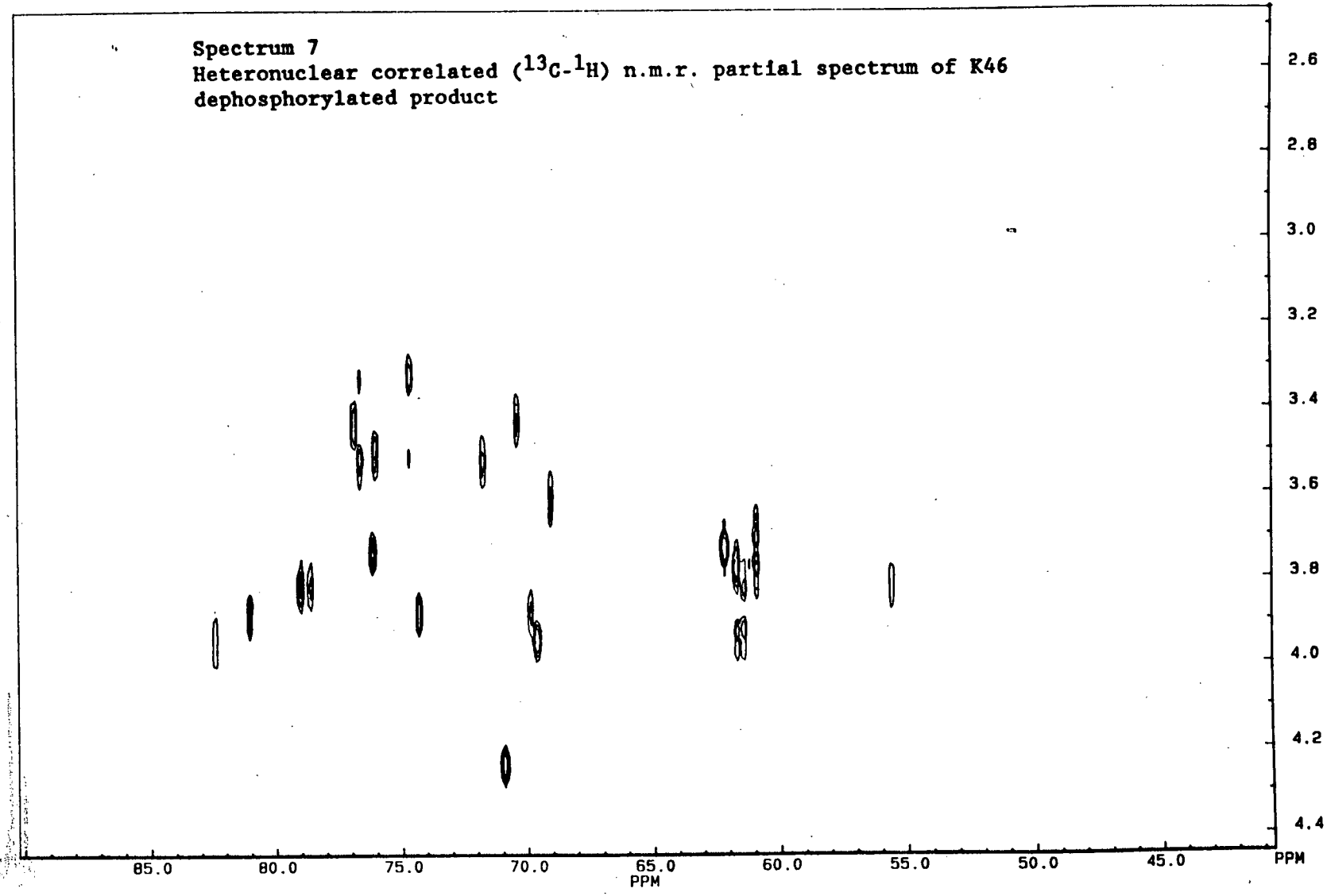
Spectrum 5
2D 2 step relay COSY n.m.r. spectrum (COSYRCT2) spectrum of native polysaccharide (300°K)



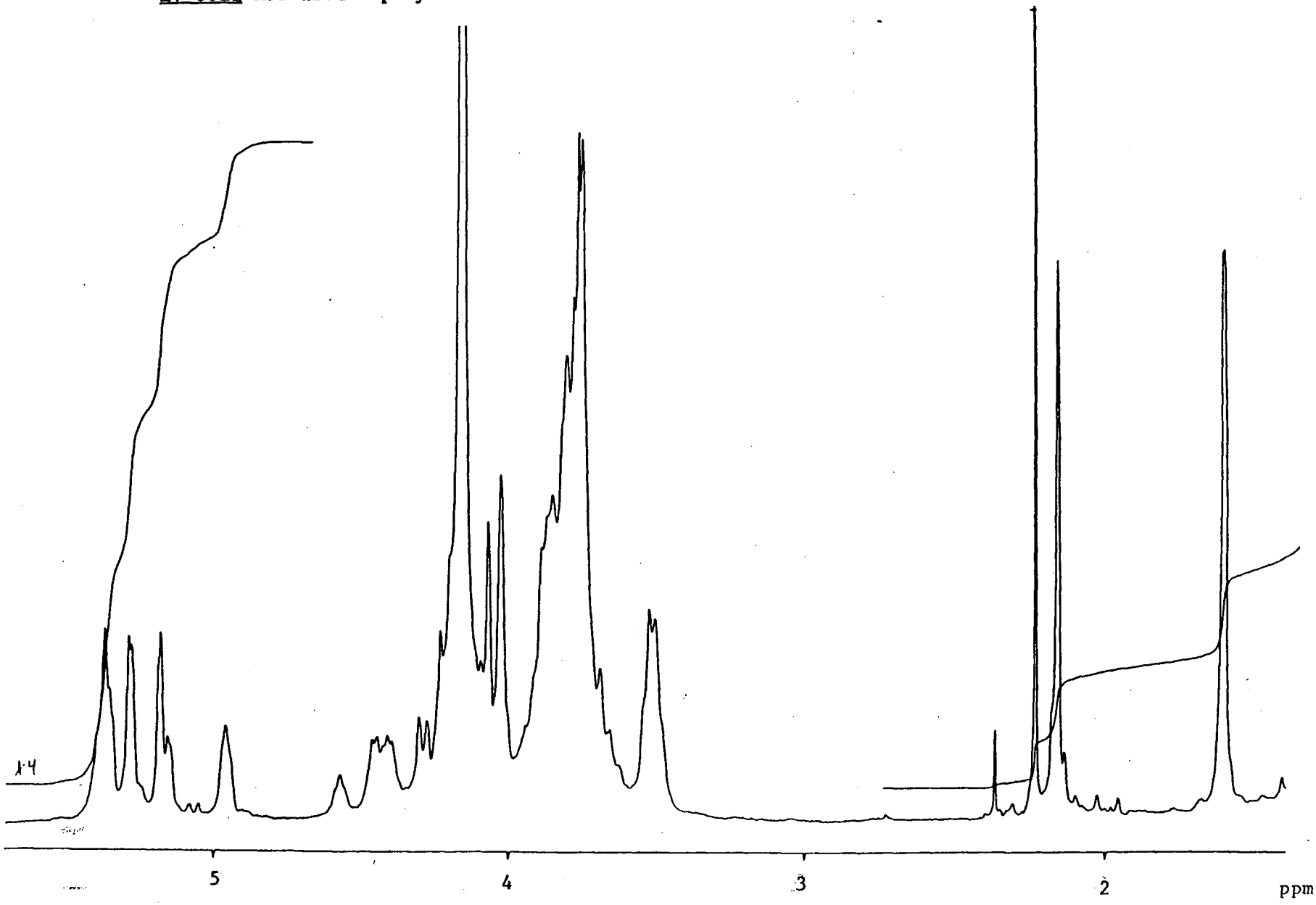
Spectrum 6
HETCOR n.m.r. partial spectrum of E. coli K46 native polysaccharide



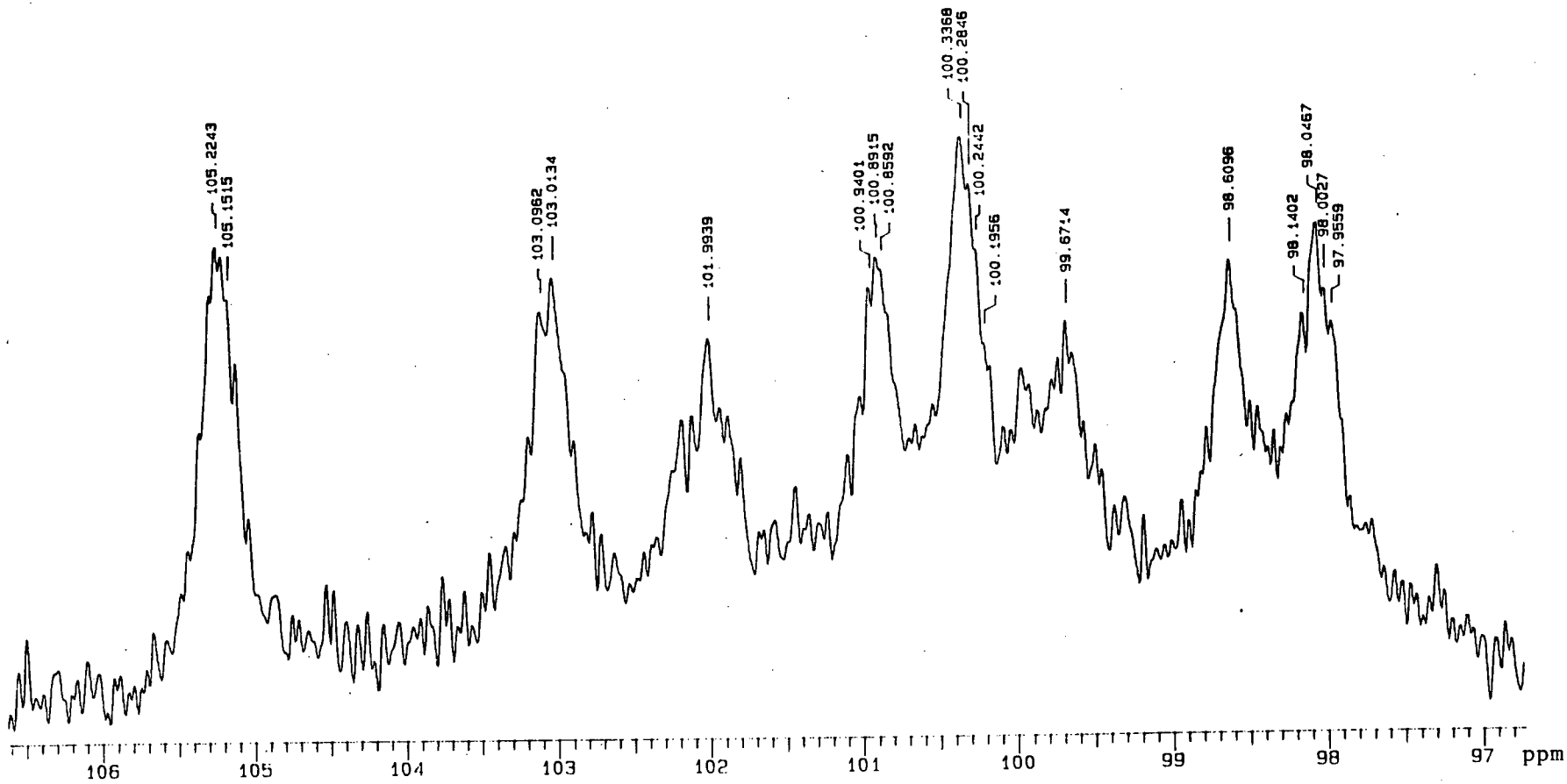
Spectrum 7
Heteronuclear correlated (^{13}C-^{1}H) n.m.r. partial spectrum of K46 dephosphorylated product



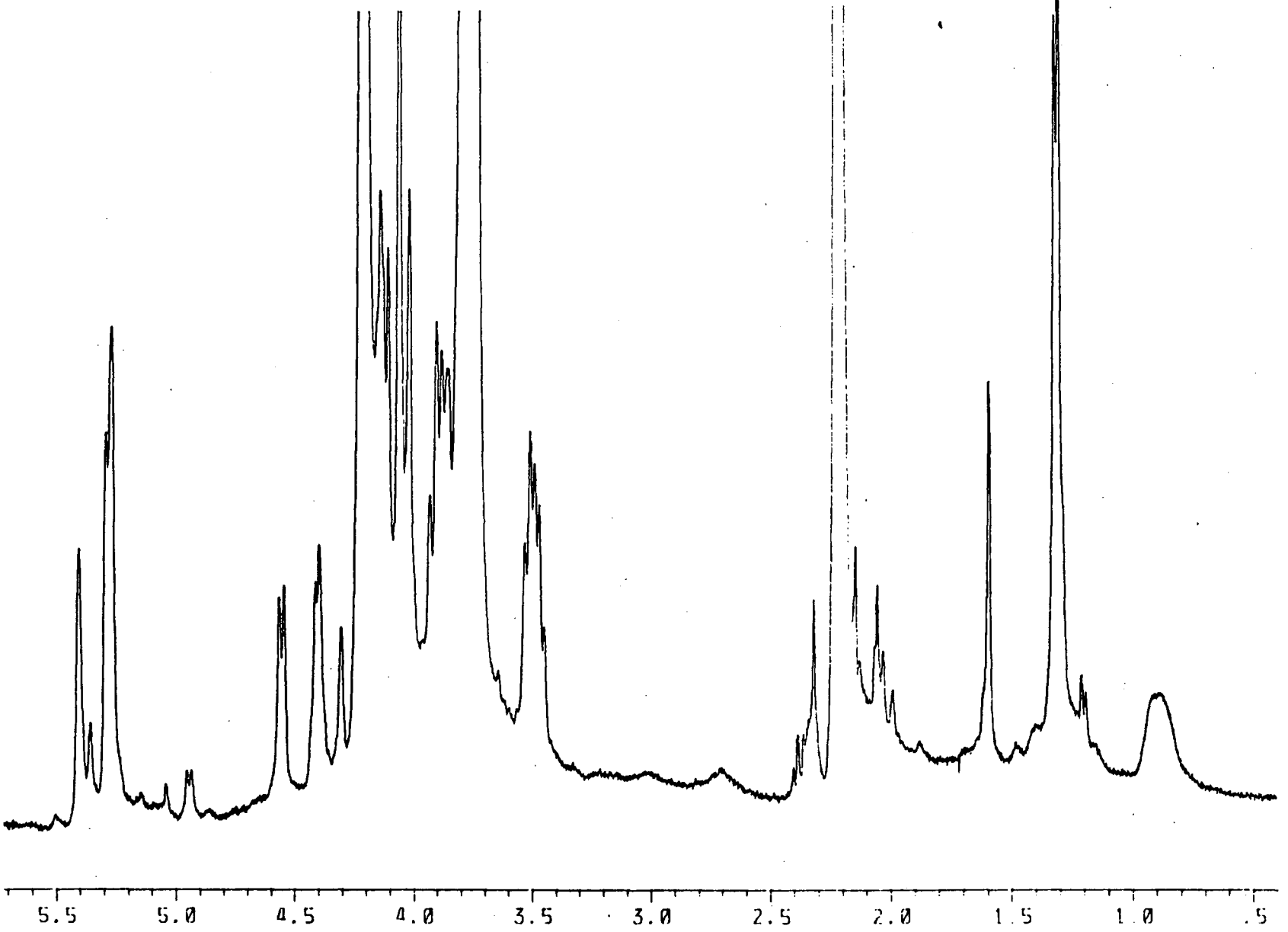
Spectrum 8
^{1}H-n.m.r. spectrum (400 MHz 368°K) of
E. coli K33 native-polysaccharide



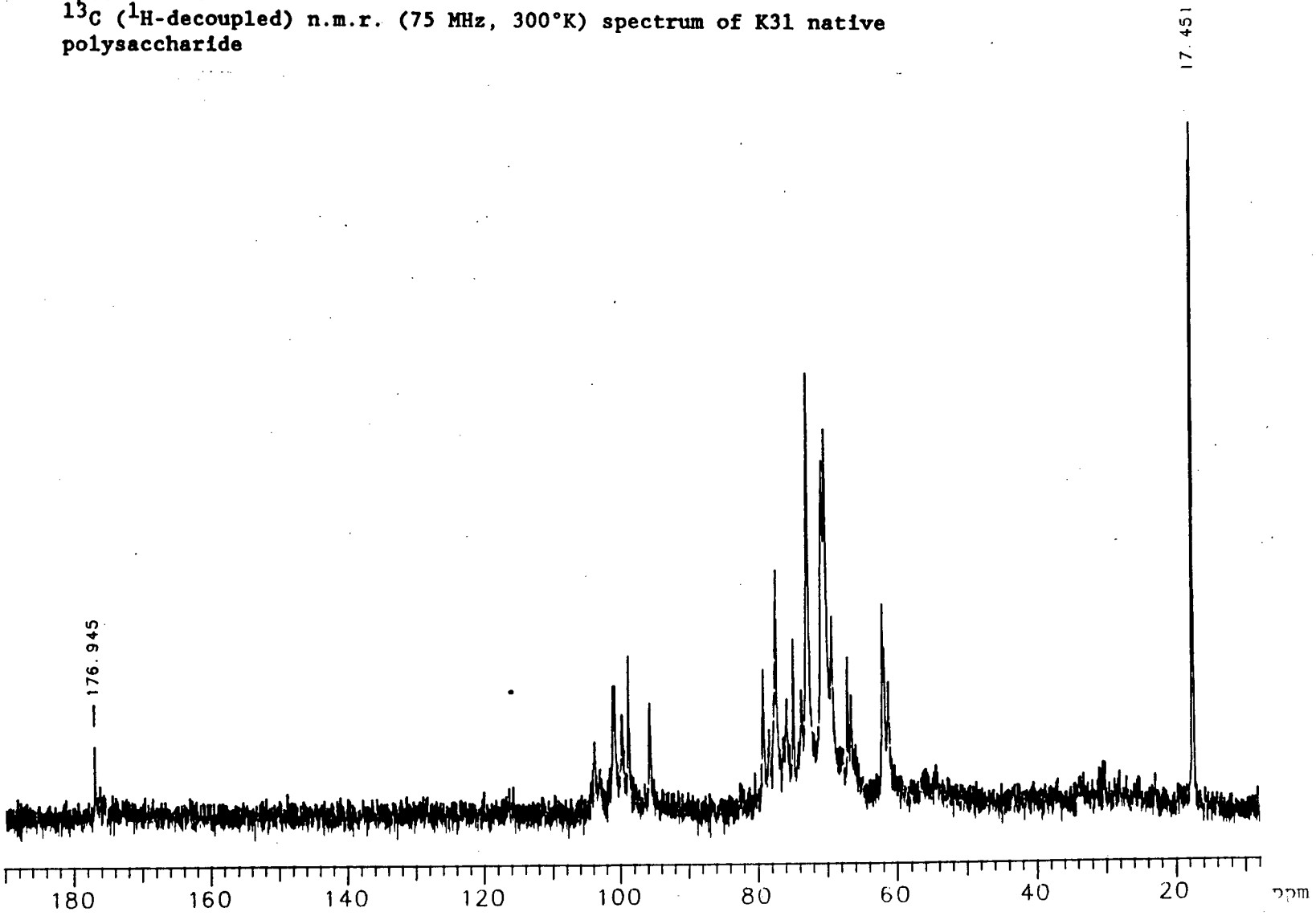
Spectrum 9
^{13}C (^{1}H-coupled) n.m.r. (75 MHz at 300°K) of E. coli K33 deacetylated, depyruvylated polysaccharide.



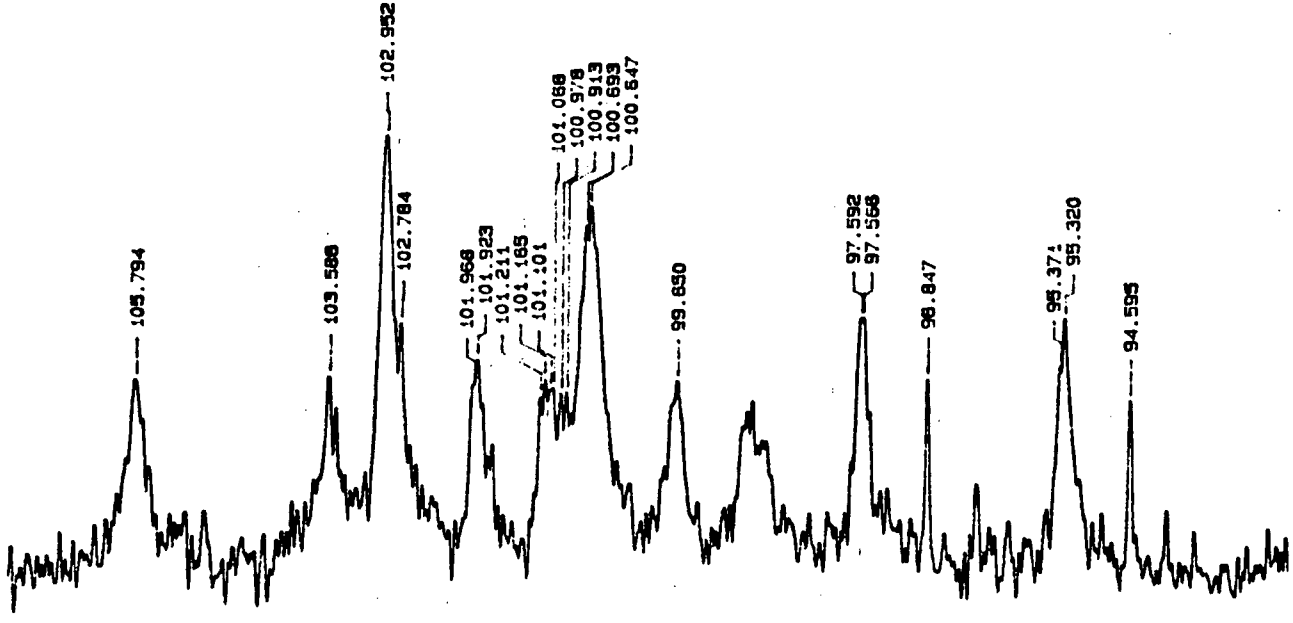
Spectrum 10
^{1}H-n.m.r. spectrum (400 MHz, 368°K) of E. coli K33 deacetylated polysaccharide



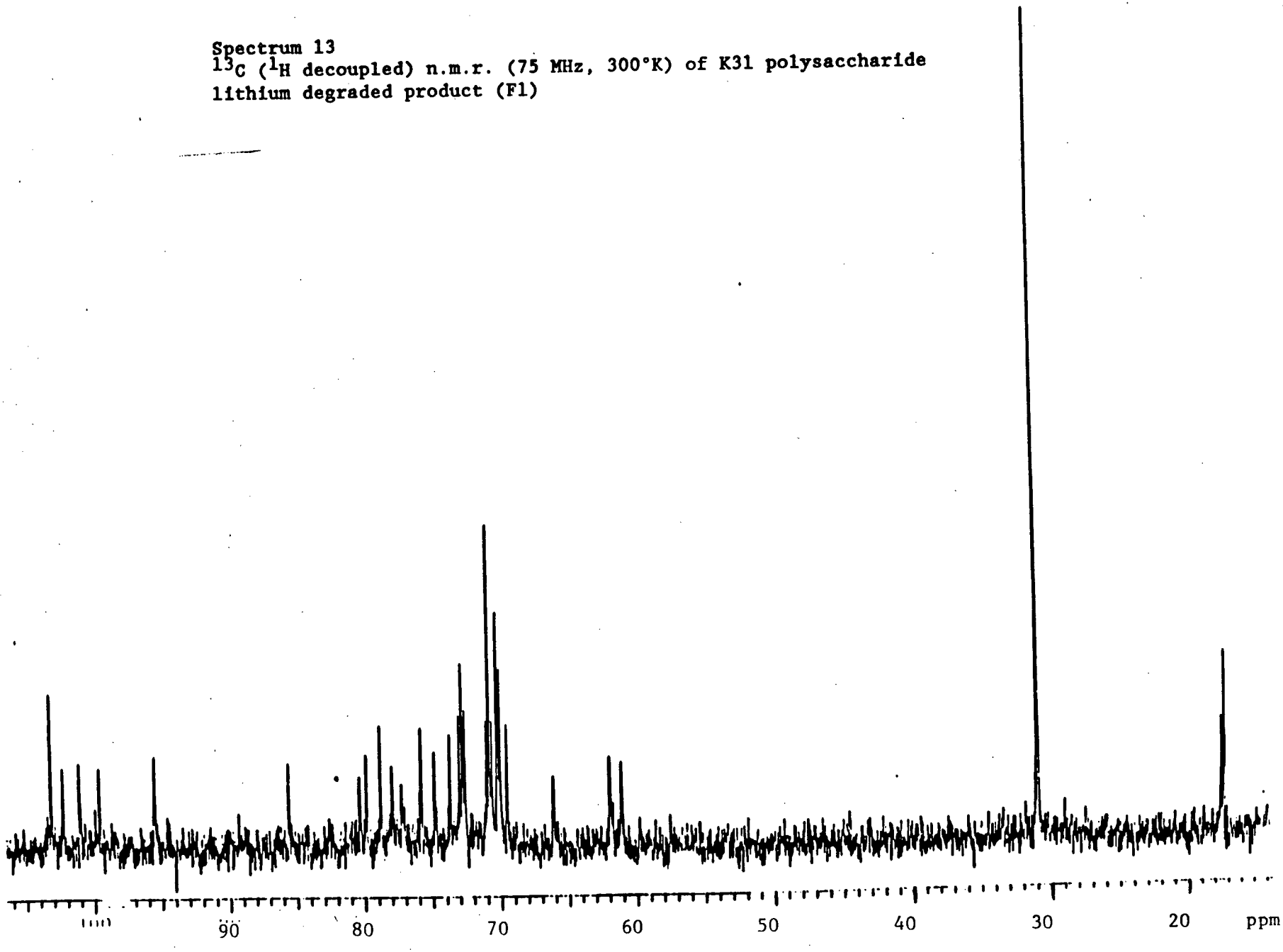
Spectrum 11
^{13}C (^{1}H-decoupled) n.m.r. (75 MHz, 300°K) spectrum of K31 native polysaccharide



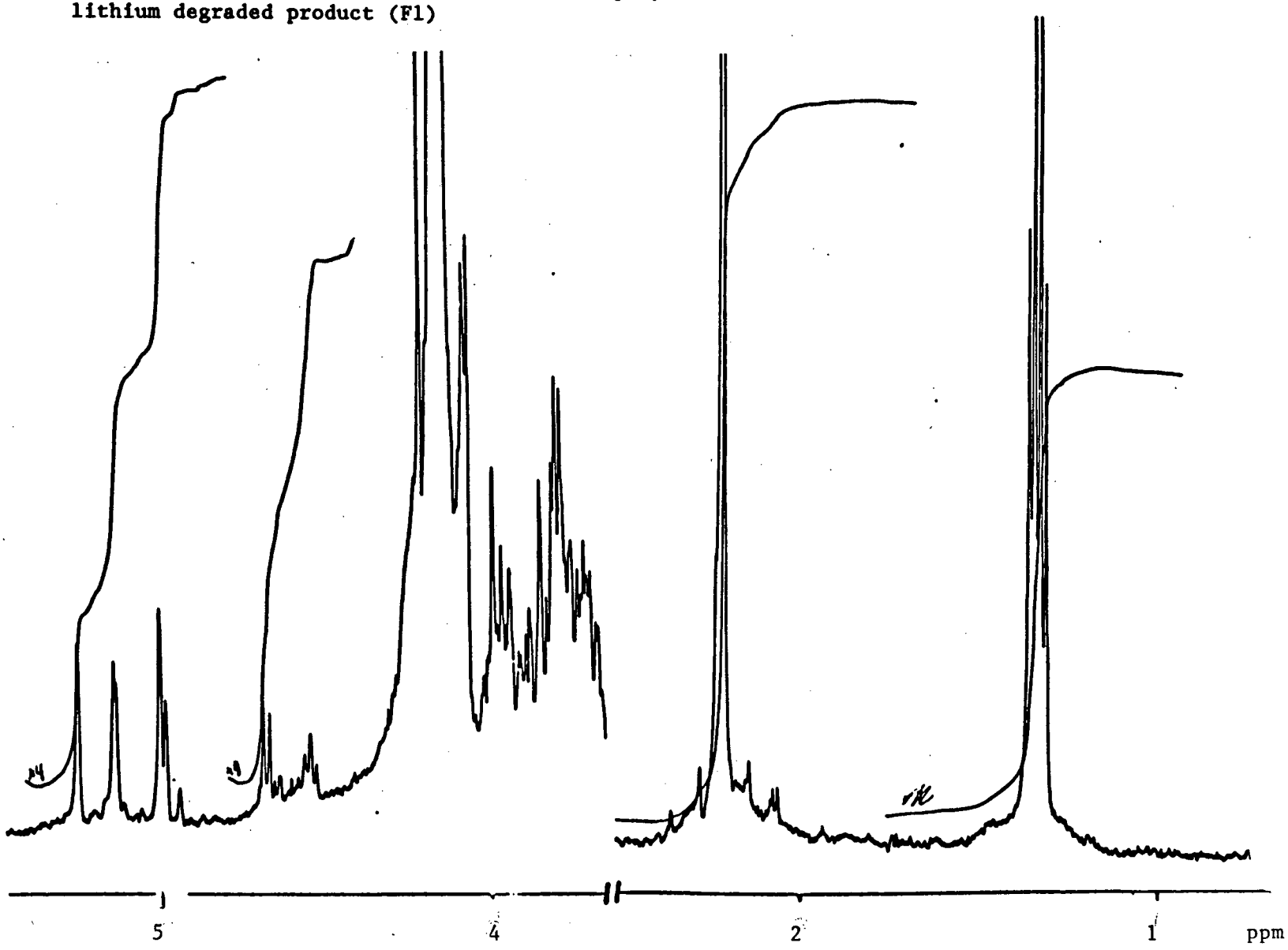
Spectrum 12
^{13}C (^{1}H-coupled) n.m.r. (75 MHz, 300°K) spectrum of K31 native polysaccharide



Spectrum 13
^{13}C (^{1}H decoupled) n.m.r. (75 MHz, 300°K) of K31 polysaccharide lithium degraded product (F1)



Spectrum 14
^{1}H-n.m.r. (400 MHz, 368°K) spectrum of K31 polysaccharide lithium degraded product (F1)



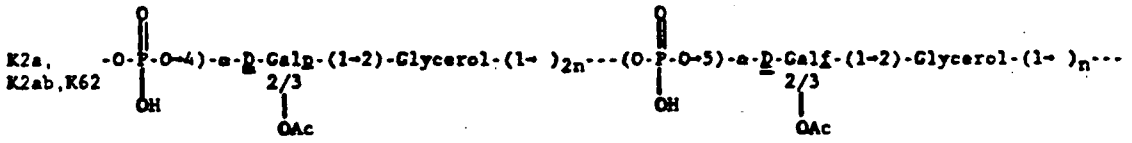
APPENDIX IV

POLYSACCHARIDE ANTIGENS OF *ESCHERICHIA COLI*

STRUCTURES OF *E. coli* K-ANTIGENS

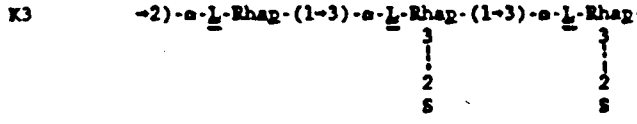
K1 →8)-α-Neup5Ac-(2-

K2a, K2ab, K62



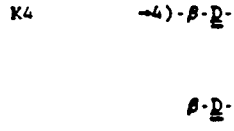
K2, (K2a), nonacetylated
K62 (K2ab), acetylated

K3



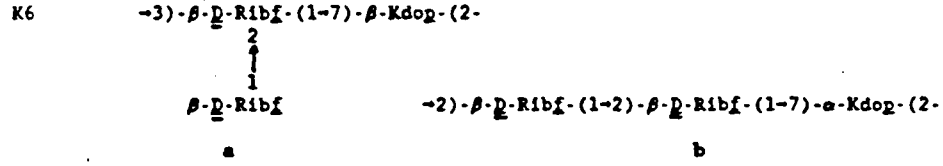
S = 6-O-acetyl-4-deoxy-2-hexulosonic acid

K4

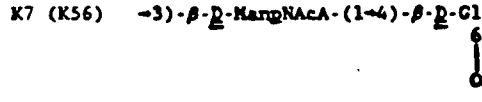


K5 →4)-β-D-GlcpA-(1→4)-α-D-GlcpNAc-(1-

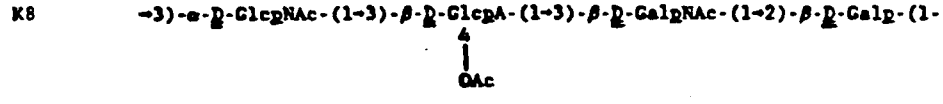
K6



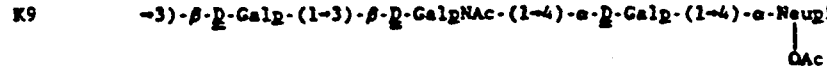
K7 (K56)



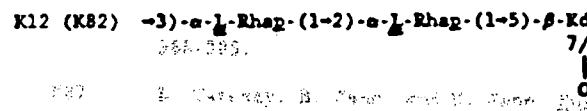
K8



K9



K12 (K82)



K13 (K20,K23) →3)-β-D-Ribf-(1→7)-β-Kdop-(2- K13, O-acetyl on 4 of Kdo; K20, O-acetyl on 5 of Rib; K23, nonacetylated

K14 →6)-β-D-GalpNAc-(1→5)-β-Kdop-(2-
8
OAc (~60%), OPropionyl (~10%)

K15 →4)-α-D-GlcpNAc-(1→5)-β-Kdop-(2-

K18, K22 →2)-β-D-Ribf-(1→2)-D-Ribitol-(5-O-P(=O)(OH)-O-
3
OAc K22, nonacetylated

OAc (~30%)
8
K19 →3)-β-D-Ribf-(1→4)-β-Kdop-(2-

K26 →3)-α-L-Rhap-(1→3)-β-D-Galp-(1→3)-β-D-GlcpA-(1→3)-α-L-Rhap-(1→3)-α-L-Rhap-(1-
4
1
HO2C 3
H3C 4
α-L-Rhap

K27 →4)-α-D-Glcp-(1→4)-α-D-GlcpA-(1→3)-α-L-Fucp-(1-
3
1
α-D-Galp

K28 →3)-α-D-Glcp-(1→4)-β-D-GlcpA-(1→4)-α-L-Fucp-(1-
4 2/3
1 OAc (~70%)
β-D-Galp

K29 →2)-α-D-Manp-(1→3)-β-D-Glcp-(1→3)-β-D-GlcpA-(1→3)-α-D-Galp-(1-
4
1
HO2C 4
H3C 6
β-D-Glcp-(1→2)-α-D-Manp

K30 →2)-α-D-Manp-(1→3)-β-D-Galp-(1-
3
1
β-D-GlcpA-(1→3)-α-D-Galp

K31 →2)-α-D-Glcp-(1→3)-β-D-Galp-(1→3)-β-D-GlcpA-(1→2)-β-L-Rhap-(1→2)-β-L-Rhap-(1-
4
↑
1
α-L-Rhap

OAc
|
2
K32 →4)-α-L-Rhap-(1→3)-β-D-Glcp-(1-
3
↑
1
α-D-GlcpA-(1→3)-α-D-Galp

K33 →3)-α-D-Glcp-(1→4)-β-D-GlcpA-(1→4)-α-L-Fucp-(1-
3 2 3
H3C CO2H ↑
1
α-D-Galp

contains O-acetyl groups

K34 →2)-β-D-GlcpA-(1→4)-β-D-Galp-(1→3)-β-D-Galp-(1-
3
↑
1
α-D-Glcp-(1→4)-β-D-Galp

K36 →3)-β-D-Galp-(1→3)-α-D-GalpA-(1→2)-α-D-Manp-(1-
4
↑
1
α-D-Manp

K37 →3)-β-D-Glcp-(1→3)-α-D-Galp-(1-
4
↑
1
α-D-Galp
6 4
H3C CO2H (R)

K39 →6)-α-D-Glcp-(1→4)-β-D-GlcpA-(1→2)-α-D-Manp-(1→3)-β-D-Glcp-(1-
3
↑
1
α-D-Galp

K40 →4)-β-D-GlcpA-(1→4)-α-D-GlcpNAc-(1→6)-α-D-GlcpNAc-(1-
6
|
L-Ser (amide)

K42 →3)-α-D-Galp-(1→3)-α-D-GalpA-(1→3)-α-L-Fucp-(1-

K44 →4)-β-D-GlcpA-(1→3)-α-L-Rhap-(1→4)-α-D-GlcpNAc-(1→6)-β-D-GalpNAc-(1-

K49 →4)-β-D-GlcpA-(1→6)-β-D-Galp-(1→6)-β-D-Glcp-(1→3)-β-D-GlcpNAc-(1→
6
|
L-Thr (75%), L-Ser (25%)
(amide)

K51 →3)-α-D-GlcpNAc-(1→O-P(=O)(OH)-O-
4/6
|
OAc

K52 →3)-α-D-Galp-(1→O-P(=O)(OH)-O-
OAc (~80%)
4
2
↑
2
β-D-Fruf
OAc, OProp (~10%)

K53 →3)-β-D-Galf-(1→4)-β-D-GlcpA-(1→
2
|
OAc

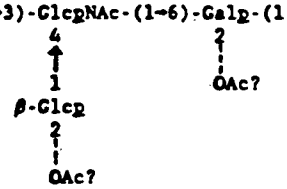
K54 →3)-β-D-GlcpA-(1→3)-α-L-Rhap-(1→
6
[L-Thr (90%), L-Ser (10%)] (~85%, amide)

K55 →4)-β-D-GlcpA-(1→4)-β-D-GlcpA-(1→3)-β-D-Manp-(1→
OAc (40%)
2
4,6
H_3C CO_2H

K74 →3)-β-D-Ribf-(1→2)-β-D-Ribf-(1→6)-β-Kdof-(2→
2
OAc (~65%)

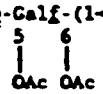
K85 →2/4)-GlcA-(1→2/6)-Man-(1→3)-Man-(1→3)-GlcNAc-(1→4)-Man-(1→3)-Man-(1→3)-GlcNAc-(1→
Rha?
2/6
1
Rha
1
GlcA

K87 →4)-β-D-GlcpA-(1→3)-L-FucpNAc-(1→3)-GlcpNAc-(1→6)-Galp-(1-



K92 →8)-α-Neup5Ac-(2→9)-α-Neup5Ac-(2-

K93 →3)-β-D-Galf-(1→4)-β-D-GlcpA-(1-



K95 →3)-β-D-Ribf-(1→8)-Kdof-(2-

randomly O-acetylated

K100 →3)-β-D-Ribf-(1→2)-D-Ribitol-(5→O-P(=O)(OH)-O-

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STRUCTURES OF O-ANTIGENS OF *E. coli*

O2 →4)-β-D-GlcpNAc-(1→3)-α-L-Rhap-(1→2)-α-L-Rhap-(1→3)-β-L-Rhap-(1→

2
↑
1
α-D-Fucp3NAc

O4 →3)-β-D-GlcpNAc-(1→2)-α-L-Rhap-(1→6)-α-D-Glcp-(1→3)-α-L-FucpNAc-(1→

3
↑
1
α-D-Glcp

O6 →3)-β-D-Manp-(1→4)-β-D-Manp-(1→3)-α-D-GlcpNAc-(1→4)-α-D-GalpNAc-(1→

2
↑
1
β-D-Glcp

O7 →3)-α-D-GlcpNAc-(1→3)-β-D-Quip4NAc-(1→2)-α-D-Manp-(1→4)-β-D-Galp-(1→

3
↑
1
α-L-Rhap

D-QuipNAc = 4-acetamido-4,6-dideoxy-D-glucopyranose

O8 α-D-Manp3Me-(1→3)-β-D-Manp-(1→2)-α-D-Manp-(1→2)-α-D-Manp-(1→$_n$

D-Man3Me = 3-O-methyl-D-mannose n = ~10

O9 →3)-α-D-Manp-(1→3)-α-D-Manp-(1→2)-α-D-Manp-(1→2)-α-D-Manp-(1→2)-α-D-Manp-(1→

O9a →3)-α-D-Manp-(1→3)-α-D-Manp-(1→2)-α-D-Manp-(1→2)-α-D-Manp-(1→

010 →3)-β-D-GlcpNAc-(1→3)-α-L-Rhap-(1→3)-α-L-Rhap-(1→3)-α-D-Galp-(1-
2
↑
1
α-D-Fucp4NAcyl

Acyl = Acetyl (60%) or D-3-hydroxybutyryl (40%)

018ac →2)-α-Rhap-(1→4)-α-Galp-(1→6)-α-Glcp-(1→3)-α-GlcpNAc-(1-
3
↑
1
β-GlcpNAc

020 →2)-β-D-Ribf-(1→4)-α-D-Galp-(1-

020ac →4)-α-D-Galp-(1→2)-β-D-Ribf-(1-
3
↑
1
α-D-Galp

α-L-Rhap
1
↓
3
025 →3)-α-L-FucpNAc-(1→3)-β-D-GlcpNAc-(1→4)-α-D-Glcp-(1-
6
↑
1
β-D-Glcp

055 →3)-α-D-Galp-(1→3)-β-D-GalpNAc-(1→6)-β-D-GlcpNAc-(1-
3
↑
1
α-Colp-(1→2)-β-D-Galp

Col = 3,6-dideoxy-L-xylo-hexose

058 →3)-β-D-GlcpNAc-(1→4)-α-D-Manp-(1→4)-α-D-Manp-(1-
3 2/3
↑ |
1 OAc
α-S

S = 3-O-[(R)-1-carboxyethyl]-L-rhamnopyranose

069 →3)-β-D-GlcpNAc-(1→2)-α-L-Rhap-(1→2)-α-L-Rhap-(1→2)-α-D-Galp-(1-

075 →3)-β-D-GlcpNAc-(1→3)-α-D-Galp-(1→4)-α-L-Rhap-(1-
4
↑
1
β-D-Manp

O78 →3)-β-D-GlcpNAc-(1→4)-β-D-GlcpNAc-(1→4)-β-D-Manp-(1→4)-α-D-Manp-(1-

O86 →4)-α-L-Fucp-(1→2)-β-D-Galp-(1→3)-α-D-GalpNAc-(1→3)-β-D-GalpNAc-(1-
3
↑
1
α-D-Galp

α-Colp
1
↓
3
O111 →3)-β-D-GlcpNAc-(1→4)-α-D-Glcp-(1→4)-α-D-Galp-(1-
6
↑
1
α-Colp

Col = 3,6-dideoxy-L-xylo-hexose

O114 →3)-α-D-GlcpNAc-(1→4)-β-D-Quip3N-(1→3)-β-D-Ribf-(1→4)-β-D-Galp-(1-
|
N-acetyl-L-Ser (amide)

Quip3N = 3-amino-3,6-dideoxy-β-D-glucopyranose

O124 →3)-β-D-GalpNAc-(1→3)-α-D-Galp-(1→6)-β-D-Galf-(1-
4
↑
1
β-S-(1→6)-α-D-Glcp

S = 4-O-[(R)-1-carboxyethyl]-D-glucopyranose

O149 →4)-β-D-GlcpNAc-(1→3)-β-D-GlcpNAc-(1→3)-β-L-Rhap-(1-
4 6
X
H3C CO2H
S

O157 →3)-α-D-GalpNAc-(1→2)-α-D-PerpNAc-(1→3)-α-L-Fucp-(1→4)-β-D-Glcp-(1-

Per = 4-amino-4,6-dideoxy-α-D-mannopyranose

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