A Structural Study Of The Capsular Antigen

Of *Klebsiella* Serotype K43

Thesis

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DEDICATION

I dedicate this thesis and the enrichment of the post-graduate experience in honour of:

Mr John.C. Waberski. (dip.Pharm)

on the occasion of his 61st birthday, (24/06/1992).

TABLE OF ABBREVIATIONS USED IN THIS TEXT

Key to chemical structures:

GlcA	=	Glucuronic acid
GalA	=	Galacturonic acid
Glc	=	Glucose
Man	=	Mannose
Gal	=	Galactose
Raf	=	Raffinose
Fuc	=	Fucose
Glu	=	Glutamic acid
Pen	=	Pentulosonic acid
Lac	=	Lactyl
Py	=	Pyruvate
Ac	=	Acetyl
p	=	Pyranose
f	=	Furanose
a	=	Alpha anomeric type
ß	=	Beta anomeric type

Key to Miscellaneous

Abbreviations:

1D ^{1}H-NMR	One dimensional proton nuclear magnetic resonance
δ	Proton NMR Chemical shift in ppm
ø	Bacteriophage
P1	The oligosaccharide chemical repeating unit of the polysaccharide
P1-ol	The oligosaccharide alditol of P1
GLC	Gas Liquid Chromatography
HPLC	High Performance Liquid Chromatography
MS	Mass Spectrometry
PAAN's	Peracetylated Aldononitrile Derivatives
PMAA's	Permethylated Alditol Acetate Derivatives
TFA	Trifluoro-acetic Acid

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ABSTRACT

This thesis presents a detailed chemical and spectroscopic determination of the capsular, polysaccharide K-antigen isolated from the *Klebsiella* bacterium, serotype K43 (culture #2482). The repeating unit of the capsular polysaccharide was found to be of the "3 + 2" repeating unit type. A uronic acid was found as part of a disaccharide side chain and the main chain of the polysaccharide was found to be composed of a neutral trisaccharide of mannose and galactose. The work forms part of an ongoing research interest in bacterial polysaccharides of this laboratory and now completes the structural elucidation of all the *Klebsiella* K-antigens, bar three antigens which were originally assigned to other laboratories. These data together with the respective serological characteristics of each serotype are available to the molecular biologist, and may result in the production of: vaccine(s) against *Klebsiella* infections, diagnostic products and novel carrier molecules enabling targeted drug delivery.

1. INTRODUCTION

1.a. General Forward

Friedländer's[1] discovery of a mucin-like substance on cells of the *Diploccocus pneumonia* a century ago might have indirectly provided the impetus for elucidating bacterial polysaccharide structure. Nevertheless over the past three decades a wealth of structural data of bacterial polysaccharides has been published and today the utility of these biopolymers is being realised. These non-metabolic bacterial polysaccharides[2] are generally composed of oligosaccharide subunits which impart biological identity to the bacterium. They also serve to initiate or suppress biological processes with reference to events of cellular adhesion by pathogens, regulation of enzyme inhibitors, and reactions of antigens and antibodies of the immune system.

Parolis and Wilkinson[3] have reviewed developments and new directions within the scope of polysaccharide chemistry and clearly the pharmaceutical industry stands at the edge of an abyss filled with an exciting, growing range of biopolymers which may be modified to fulfil the industries quest for new molecules. Apart from the attractiveness of using bacteria and bio-engineering to produce polysaccharide material, the pharmaceutical industry will probably enter a new era of producing "intelligent molecules" from this material enabling either directed transit to their site of action, or carrying an agent to this site. Uhr *et al.*[4] have reviewed similar technology with regard to cancer therapy and have commented on the formation of an immunotoxin by attaching a pharmacological agent to an antigen and thereby producing specific cellular chemotherapy.

In order that the afore-mentioned understanding of the properties, functions and applications of bacterial polysaccharides can be used, methodologies and techniques as discussed in this thesis are invaluable in describing the primary structure of these polysaccharides. Most of the common bacterial O- and K-antigen primary structures have been published, but generally listings are incomplete, e.g. *Klebsiella* still have three unpublished K-antigens. Study of the published *Klebsiella* K-antigens and others reveals a need to reinvestigate many structures which were ambiguously assigned prior to the advent

of high field NMR spectroscopy. Ultimately research and development of the primary structure of bacterial polysaccharides will lead to a better understanding of the relationship between primary structure, solution conformation and physical properties necessary for producing bio-engineered vaccines.

A useful product which has emerged out of bacterial polysaccharide research is a 23-valent *Pneumococcal* vaccine and this product, the Pneumovac-23[5] is the only K-antigen vaccine registered for use in the Republic of South Africa to date. A further development on this theme was the publishing in 1988 of the results of a clinical trial, conducted on an experimental 24-valent *Klebsiella* vaccine[6] consisting of the *Klebsiella* K43[7] and it is therefore especially important that the primary structure is now published.

In conclusion, the occurrence of ever increasing microbial resistance to available antibiotic therapy necessitates a novel antimicrobial approach, and increased research and development usage of high field NMR spectroscopy will enable the determination of epitope shape necessary for the development of synthetic vaccines of medical usefulness.

1.b. Immunology of Bacterial Polysaccharide Antigens

In 1917 a "specific soluble substance" secreted by *Pneumococcus* bacteria was reported by Doches *et al.*[8] to precipitate antibodies raised to the bacterium *Pneumococcus*. Heidelberger, Avery and Goebel later showed that these secretions were polysaccharides and during the period 1968 to 1978, Heidelberger *et al.*[9-13] published cross-reactivity studies of *Klebsiella* extracelluar polysaccharide K-antigens in *Pneumococcal* and *Klebsiella* anti-sera. Chapter 7 demonstrates the utility of serological studies in enabling the determination of some structural features prior to chemical elucidation of *Klebsiella* K-antigens. The structural elucidation of *Klebsiella* K43[7] (this thesis), found structural components present in the primary structure as alluded to in the mid 1970's by Heidelberger.

Serological and chemical studies performed on capsular K-antigens, extracted from *Klebsiella* and other bacteria, have enabled immunologists to specify certain chemical determinants[14] common to polysaccharide antigens.

Bacterial Polysaccharide Antigens may be described by:

1. Polysaccharide constructed of chemical repeating units each consisting of 2-8 monosaccharides.
2. A polysaccharide with molecular weight greater than 50 000 daltons.
3. An antigenic core region of 2-4 monosaccharide units in each chemical repeating unit.
4. Antigenic specificity resident in a single monosaccharide residue at the non-reducing end of a linear repeating unit polysaccharide.
5. An antigenic core region solely in the chain, if the side chain is of oligosaccharide length.
6. An antigenic core region extending to include two or three neighbouring residues in the main chain, in a repeating unit with a single monosaccharide side chain.

It is interesting to note the subtle effect that stereochemical or minor chemical derivatisation has on antigenic character. The stereochemistry of the Me / COOH of the pyruvic acid affects the antigenic specificity of the antigen, and Jennings *et al.*[15] have reported that replacing the N-acetyl groups of sialic acid residues by propionyl groups enhanced the immune response generated by a group B *meningococcal* polysaccharide antigen.

Furthermore polysaccharide antigens show T-cell independence, i.e. only IgM antibodies are induced in children, and hence pure polysaccharide vaccines are ineffective here. Combination with an immunological adjuvant[14,16] or antigenic ligand, (e.g. when the *influenza* B polysaccharide antigen is attached to the *Diphtheria* protein antigen) enables

T-cell dependant IgG formation along with IgM, and immunological memory is produced in the paediatric population.

The study of immunological reactivities has confirmed that surface polysaccharide moieties are the serological determinants of bacteria, and polysaccharide antigens can be used to form ligand(s) which discriminate between similar receptor sites.

1.c.(i). Anatomy and Physiology of the Gram negative bacteria

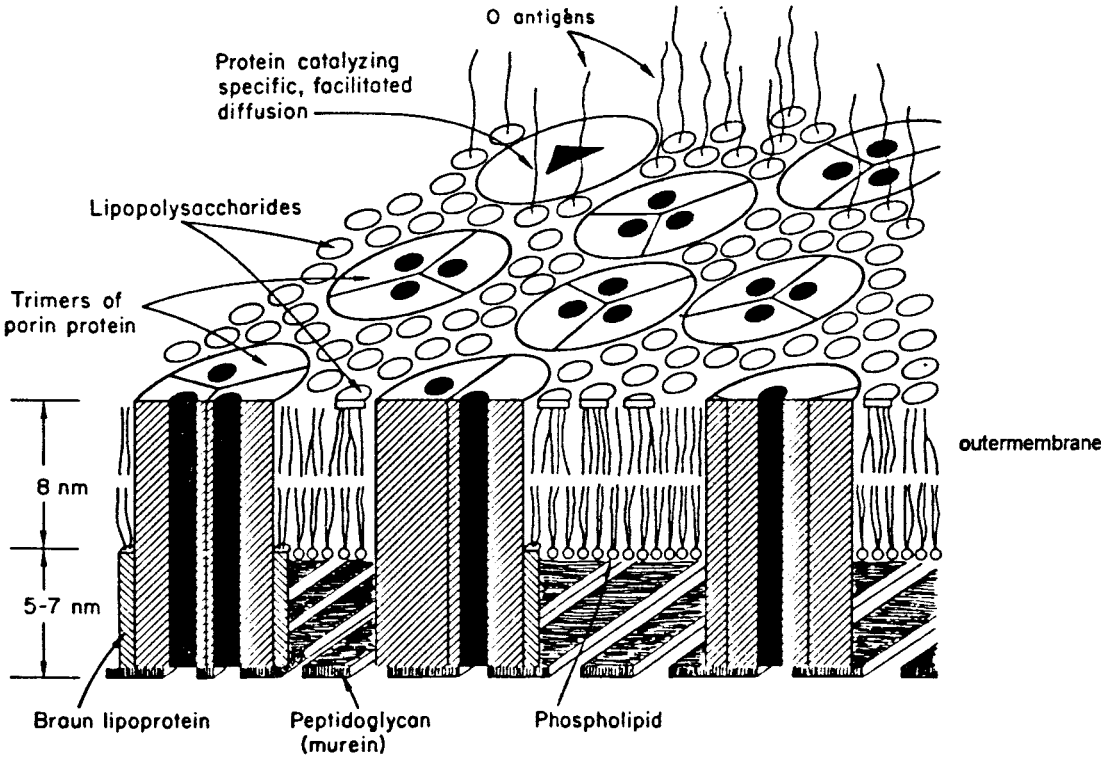


FIGURE 1. The Gram negative cell wall

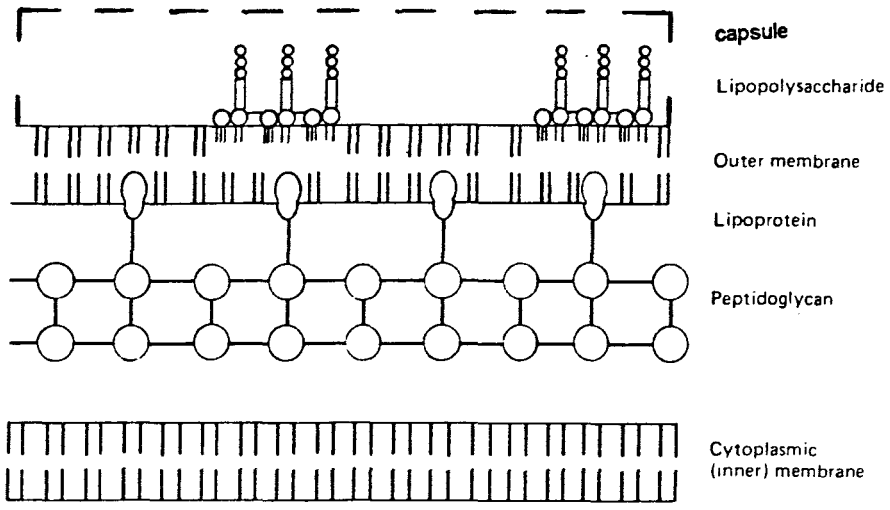


FIGURE 2. Cross-section of the gram-negative cell envelope

The gram negative cell wall content, (Figure 1. and Figure 2.) is approximately 60% of that of the gram positive wall[17]. It also consists of 10-22% lipid and an outer membrane conferring improved resistance, as seen in gram negative infections, to attack by macrocytes, enzymes, antibiotics etc.

The membrane excludes large molecules and contains protein channels called porins which regulate the entry of small molecules into the cell. Brauns lipoprotein attaches the membrane to the cell wall and the antigenic determinants are expressed outwardly from this membrane. Essentially three kinds of antigenic determinants are expressed by *Klebsiella* bacteria, *viz*. lipopolysaccharide R or O-antigen, extracelluar polysaccharide K-antigen, and M-antigen (bacterial slime). Bacteria can be defined by more than one O-antigen and interestingly the O-antigens may exibit antigenic drift[18], i.e. the O-antigen serotyping of a bacterium may change over time especially during chronic infection so as to evade the host antibody response. Figure 1. depicts O-antigen protruding from the outer membrane. Should this membrane rupture, O-antigen is released and upon hydrolysis the extremely toxic endotoxin is released.

Other antigens generally found are the M-antigen, and the proteinaceous H-antigen in motile bacteria. M-antigen is loosely attached to the capsule, serving as an adhesive agent and is formed in copious amounts under certain conditions.

Capsular K-antigen covers the surface of the outer membrane and occludes the oligosaccharide O-antigen determinants. The capsule[18] composition has been shown to be important for the degree of virulence of a bacterium, acapsular bacteria of the same species being much less pathogenic. A relationship between the quantity of capsule in some *Klebsiella* species and the species virulence, resistance to host phagocytosis or host defences, has been postulated. In the *E.coli* species it has been suggested that the quantity of capsule determines whether the bacterium will resist the bactericidal effect of serum complement or not. Again other studies believe the qualitative[19] and not the quantitative nature of the capsule to be more important. The general functions of the capsule can be summarized as:

1) protection against bacterial desiccation (hygroscopic nature of the polysaccharide)
2) possibly blocking the attachment of bacterial phages
3) conferring virulence to the bacterium (antiphagocytic ability)
4) promotion of attachment of its bacterium to hosts
5) conferring stability on the bacterial suspension by virtue of an anti-aggregatory effect induced by the anionic nature of the uronic acid content.

1.(c).ii. The bacteriology of *Klebsiella* bacteria

The genus *Klebsiella*[20] is a member of the *Enterobacteriaceae* family and are facultative, anaerobic gram negative rods which can withstand aerobic conditions. They ferment lactose, are non-motile and if encapsulated tend to produce much K-antigen. M-antigen may be exuded under conditions of higher salt concentration and decreased temperature in liquid culture.

78 K-antigens and 12 O-antigens define the whole species. *Klebsiella* bacteria are normally saprophytes but in healthy persons are found as commensal hosts of the gastro-intestinal tract. Pathogenically *K.pneumonia* K1[21] and K2[22] cause lobar pneumonia[23] and the condition of haemorrhagic necrotizing consolidation of the lung. Types K8[24], 9[25], 10[26],

24[27] are implicated in urinary tract infections and chronic hospital infections are often caused by *Klebsiella* species.

1.d. Chemistry and Serology of the *Klebsiella* antigens

The O-Antigen.

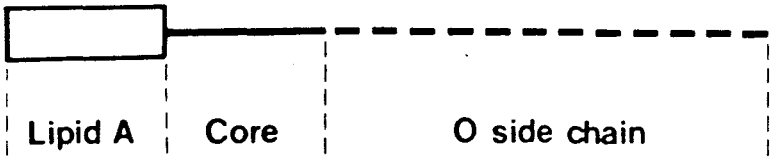


FIGURE 3. The O-Antigen

The O-antigen[18,28,29] (Figure 3), is a lipopolysaccharide moiety composed of three covalently bound subunits. Figures 4 and 5 show the antigen in greater detail.

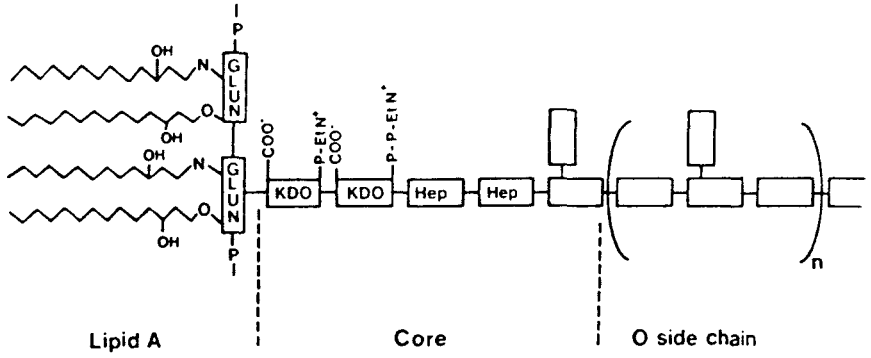


FIGURE 4. Greater Detail of the O-Antigen

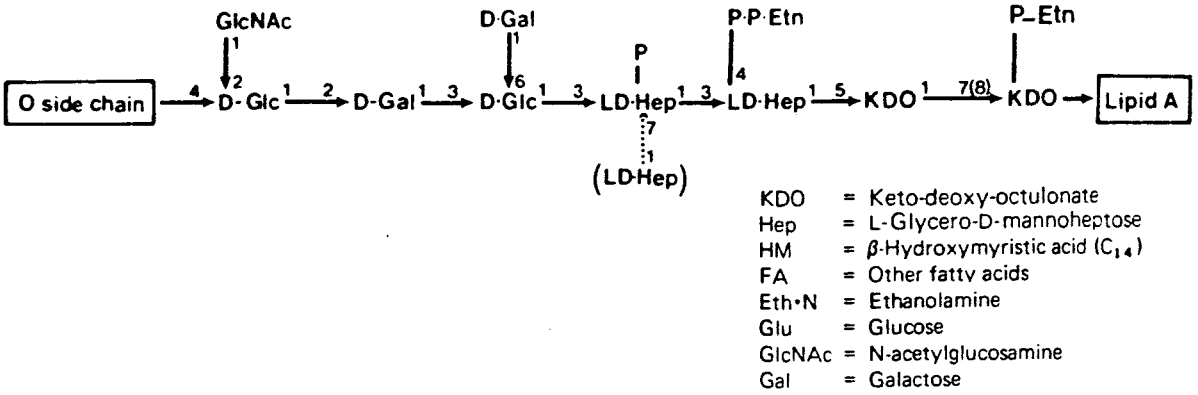


FIGURE 5. An example of an O-Antigen Core Region

The O-antigen consists of the O-specific side chain which is linked to a core region, which, in turn, is linked via its KDO, (3-deoxy-D-manno-octulosonic acid) residue to the glucosamine residue of Lipid A. The core region has been found to be generally similar in structure amongst many bacteria[18], i.e. a complex oligosaccharide of, L-glycero-D-mannoheptose along with phosphate containing groups, e.g. O-phosphorylethanolamine and O-pyrophosphorylethanolamine.

The O-side chain carries the immunological specificity of the antigen and the variety of oligosaccharide combinations is vast. *Klebsiella* have twelve known O-antigen structures. The repeating unit is built of rare and common monosaccharides and is found to exist in multiples of one to more than thirty repeating units. In 1970 Nimmich[30] characterised the 12 *Klebsiella* O-antigen serotypes chemically and alluded to the possibility that some were chemically similar. Since then O-antigen types 1, 2, 6 and 11 have been deleted because of their similarity to others.

Table 1.

The eight O-antigens[29] which currently classify the *Klebsiella* serotypes.

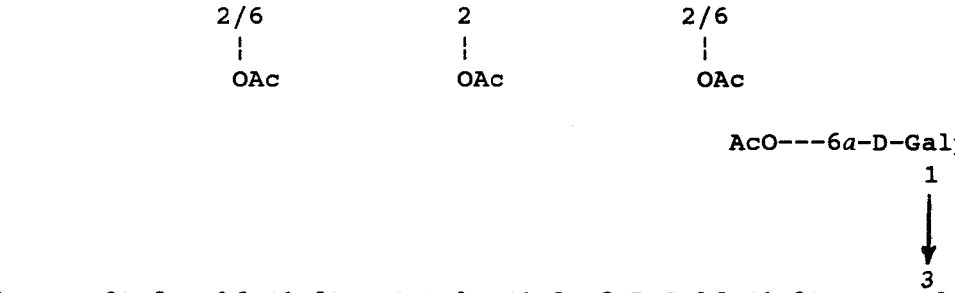
O-3 -3)-*a*-D-Manp-(1-3)-*a*-D-Manp-(1-2)-*a*-D-Manp-(1-2)-*a*-D-Manp-(1-

O-4 -2)-ß-D-Ribf-(1-4)-ß-D-Galp-(1-

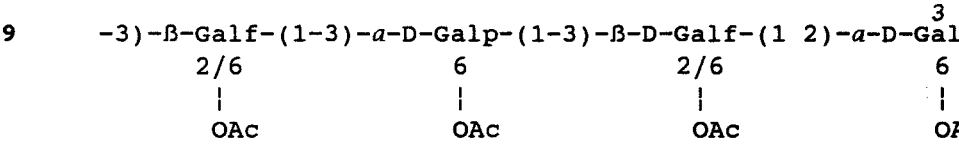
O-5 -2)-D- Manp-(1-2)-D-Manp-(1-3)-D-Manp-(1-
contains 2 *a*- and 1 ß-linkage

O-7 -2)-*a*-L-Rhap-(1-2)-ß-D-Ribf-(1-3)-*a*-L-Rhap-(1-3)-*a*-L-Rhap-(1-

O-8



O-9



O-10 -3)-*a*-L-Rhap-(1-3)-ß-D-Ribf-(1-4)-*a*-L-Rhap-(1-3)-ß-D-Ribf(1-4)-
-*a*-L-Rhap-(1-

O-12 -3)-ß-D-GlcpNAc-(1-3)-*a*-L-Rhap-(1-

The M antigen

The M-antigen [18 29] (figure 6), has a common structure of colanic acid and may be substituted at the terminal galactose monosaccharide at positions (O-3, O-4) or (O-4, O-6) with e.g. pyruvate, acetaldehyde or formaldehyde.

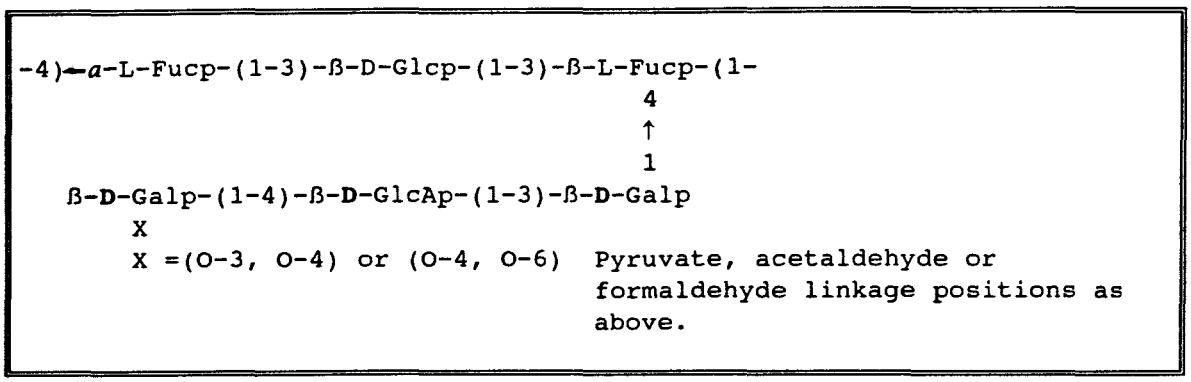


FIGURE 6.

The *Klebsiella* K-antigen

The K-antigen[18,29] is a polysaccharide composed of oligosaccharide repeating units and all the *Klebsiella* K-antigens are acidic. The acidic groups are usually uronic acids such as galacturonic or glucuronic acid. However, in some cases the acidity is due solely to pyruvic acid, e.g. K56[31], K72[32] and K32[33]. Some unusual acidic sugars have also been found and these include 4-O-lactyl-glucuronic acid (K22[34]), 4-O-lactyl-glucose (K66[35]) and 3-deoxy-L-glycero-pentulosonic acid (K38[36]).

In 1968 Nimmich[37] chemotyped the 72 serologically defined *Klebsiella* bacteria, (at that time only 72 types had been defined). The monosaccharide components were found to be of three chemical types, *viz.* acidic monosaccharide (uronic acid), neutral monosaccharide (hexoses) and neutral monosaccharide (6-deoxyhexose). In particular, the K60[38] monosaccharide content, as published by Nimmich, has been refuted and contains galactose and not fucose.

To date eighty-three *Klebsiella* K-antigens have been serologically defined, although strains[39] K73;75;76;77;78, have been deleted because of their close relationship to other known *Klebsiella* serotypes. Seventy-eight *Klebsiella* K-antigens are serologically detailed to date and Table 2. allows comparison by chemotype of a current listing of the 75 chemically elucidated structures. Over the past 30 years improved techniques, e.g. selective polysaccharide depolymerisation and the advent of high resolution NMR and highly sensitive MS instrumentation, have necessitated that certain published chemical structures be revised.

1.e. CHEMOTYPE GROUPING OF THE CURRENT *KLEBSIELLA* K-ANTIGENS

Table 2.

1) Chemotype (GlcA, Glc, Fuc)

K1[21] (1976)

repeat-4)-ß-D-GlcpA2,3Py-(1-4)-α-L-Fucp-(1-3)-ß-D-Glcp-(1-

K54[40] (1983)

ß-D-Glcp-(1-4)-┐

repeat-4)-α-D-GlcpA-(1-3)-α-L-Fucp2Ac-(1-3)-ß-D-Glcp-(1-

2) Chemotype (GlcA, Gal, Glc, Man)

K7[41] (1974)

[-3)-ß-D-GlcAp-(1-2)-α-D-Manp-(1-2)-α-Manp4,6Py-(1-3)-D-Glcp-(1-] x2

50% Gal-(1-3)-┘

K10[26] (1989)

α-D-GalpOAc(1-3)-┐

repeat-4)-ß-D-GlcpA-(1-2)-α-D-Manp-(1-3)-ß-D-Gal-(1-6)-α-D-Glcp-(1-2)-

-α-D-Galp-(1-

OAc

K13[42] (1977)

repeat-3)-ß-D-Glcp-(1-4)-ß-D-Manp-(1-4)-α-D-Glcp-(1-

ß-D-Galp3,4Py-(1-4)-α-D-GlcpA-(1-3)-┘

K26[43] (1986)

ß-D-Galp4,6Py-(1-4)-ß-D-Glcp-(1-6)-*a*-D-Glcp-(1-4)-┐
repeat-3)-ß-D-Galp-(1-2)-*a*-D-GlcpA-(1-3)-*a*-D-Manp-
-(1-2)-*a*-D-Manp-(1-

K28[44] (1975)

-2)-*a*-D-Galp-(1-3)-*a*-D-Manp-(1-2)-*a*-D-Manp-(1-3)-ß-D-Glcp-(1-
ß-D-GlcpA-(1-2)-┘
ß-D-Glcp-(1-3)-┘

K30[45] (1979)

a-D-GlcpA-(1-3)-┐
repeat-4)-ß-D-Glcp-(1-4)-ß-D-Manp-(1-4)-ß-D-Manp-(1-
ß-D-Galp3,4Py-(1-6)-┘

K31[46] (1979)

ß-D-Glcp4,6Py-(1-2)-*a*-D-Manp-(1-4)-┐
repeat-3)-ß-D-Glcp-(1-3)-*a*-D-GlcpA-(1-3)-ß-D-Galp-(1-

K33[47] (1979)

ß-D-Galp3,4Py-(1-6)-┐
repeat-4)-ß-D-Glcp-(1-4)-ß-D-Manp-(1-4)-ß-D-Manp6Ac-(1-
a-D-GlcpA-(1-3)-┘

K35[48] (1985)

repeat-3)-*a*-D-Galp4,6Py-(1-3)-*a*-D-Manp-(1-3)-*a*-D-Manp-(1-3)-ß-D-Glcp-(1-
ß-D-GlcpA-(1-2)-┘

K46[49] (1981)

repeat-3)-*α*-D-Galp-(1-3)-ß-D-Galp-(1-3)-*α*-D-GlcpA-(1-3)-*α*-D-Manp-(1-

ß-D-Glcp-(1-3)-ß-D-Manp4,6Py-(1-4)

K50[50] (1983)

ß-D-Galp-(1-6)-*α*-D-Glcp-(1-6)

repeat-3)-ß-D-Galp-(1-3)-*α*-D-Glcp-(1-4)-*α*-D-GlcpA-(1-3)-*α*-D-Manp-(1-2)-

-*α*-D-Manp-(1-

K59[51] (1975)

ß-D-GlcpA-(1-4)

repeat-3)-ß-D-Glcp-(1-3)-ß-D-Galp-(1-2)-*α*-D-Manp6Ac-(1-3)-*α*-D-Manp6Ac-

* -(1-

(only some 6 acetylation) *

K60[38] (1980)

repeat-3)-ß-D-Glcp-(1-3)-ß-D-GlcpA-(1-3)-*α*-D-Galp-(1-3)-*α*-D-Manp-(1-

α-D-Glcp-(1-4) ß-D-Glcp-(1-2) ß-D-Glcp-(1-2)

K61[52] (1979)

α-D-Galp-(1-3)

repeat-4)-ß-D-GlcpA-(1-2)-*α*-D-Manp-(1-3)-ß-D-Glcp-(1-6)-*α*-D-Glcp-(1-

---

K62[53] (1977)

α-D-Manp-(1-3)

repeat-4)-*α*-D-Glcp-(1-2)-ß-D-GlcpA-(1-2)-*α*-D-Manp-(1-3)-ß-D-Galp-(1-

K66[35](1984)

repeat-3)-α-D-Manp(1-3)-α-D-Galp-(1-2)-α-D-GlcpA-(1-3)-α-D-Manp-(1-

(3-1)-ß-D-Glcp

4OLac

Lac = Lactyl group, i.e. 4-O-Lactyl glucose

K69[54](1988)

[R]

ß-D-Galp4,6Py-(1-6)

repeat-4)-ß-D-Glcp-(1-4)-ß-D-Manp-(1-4)-ß-D-Manp-(1-

α-D-GlcpA-(1-3)

(6-OAc) 33.3%

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3) **Chemotype (GlcA, Glc, Man, Fuc)**

K6[55](1978)

repeat-3)-α-L-Fucp-(1-3)-ß-D-Glcp-(1-3)-ß-D-Manp4,6Py-(1-4)-α-D-GlcpA-

-(1-

---

4) **Chemotype (GlcA, Gal, Glc)**

K8[24](1988)

ß-D-GlcpA-(1-4)

repeat-3)-ß-D-Glcp-(1-3)-ß-D-Galp-(1-3)-α-D-Glcp-(1-

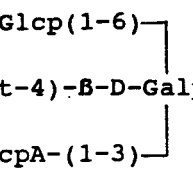
(2-1)-Galp

K11[56](1975)

α-D-Galp4,6Py-(1-4)

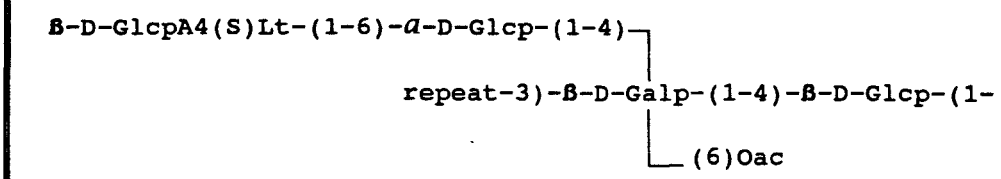
repeat-3)-ß-D-Glcp-(1-3)-ß-D-GlcpA-(1-3)-α-D-Galp-(1-

K15[57] (1992)



ß-D-Glcp(1-6)
repeat-4)-ß-D-Galp-(1-3)-α-D-Galp-(1-6)-ß-D-Galp-(1-3)-ß-D-Galp-(1-
ß-D-GlcpA-(1-3)

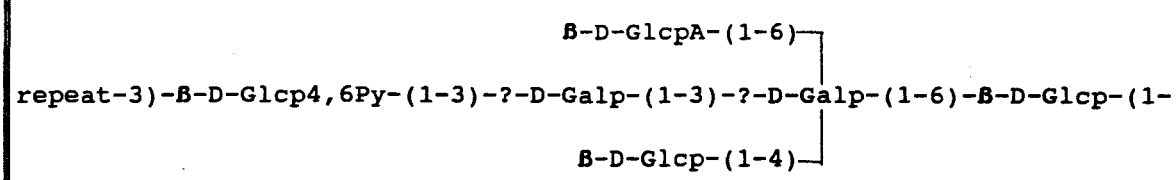
K22[34] (1988)



ß-D-GlcpA4(S)Lt-(1-6)-α-D-Glcp-(1-4)
repeat-3)-ß-D-Galp-(1-4)-ß-D-Glcp-(1-
(6)Oac

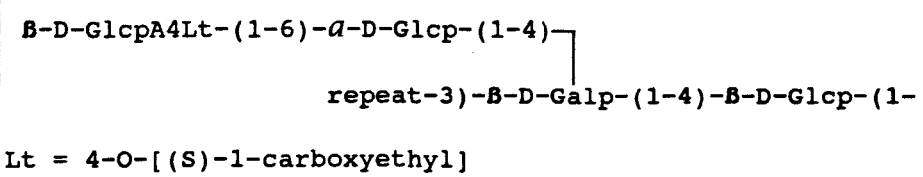
Lt = 4-O-[(S)-1-carboxyethyl]

K27[58] (1980)



ß-D-GlcpA-(1-6)
repeat-3)-ß-D-Glcp4,6Py-(1-3)-?-D-Galp-(1-3)-?-D-Galp-(1-6)-ß-D-Glcp-(1-
ß-D-Glcp-(1-4)

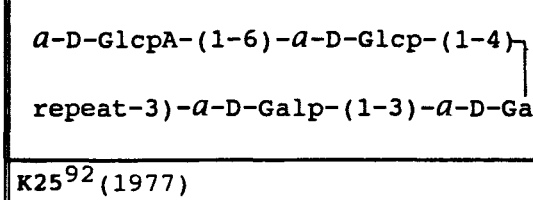
K37[59] (1977)



ß-D-GlcpA4Lt-(1-6)-α-D-Glcp-(1-4)
repeat-3)-ß-D-Galp-(1-4)-ß-D-Glcp-(1-

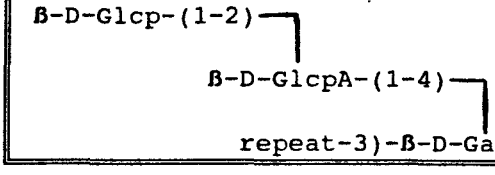
Lt = 4-O-[(S)-1-carboxyethyl]

K51[60] (1982)



α-D-GlcpA-(1-6)-α-D-Glcp-(1-4)
repeat-3)-α-D-Galp-(1-3)-α-D-Galp-(1-

K25[92] (1977)



ß-D-Glcp-(1-2)
ß-D-GlcpA-(1-4)
repeat-3)-ß-D-Galp-(1-4)-ß-D-Galcp-(1-

5) Chemotype (GlcA, Gal, Rha)

K9[25](1972)

ß-D-GlcpA-(1-4)-┐

repeat-3)-*α*-D-Galp-(1-3)-*α*-L-Rhap-(1-3)-*α*-L-Rhap-(1-2)-*α*-L-Rhap-(1-

---

K47[61](1973)

repeat-3)-ß-D-Galp-(1-4)-*α*-L-Rhap-(1-

α-L-Rhap-(1-4)-ß-D-GlcpA-(1-3)-┘

K52[62](1973)

repeat-3-?-D-Galp-(1-2)-?-L-Rhap-(1-4)-?-D-GlcpA-(1-3)-?-D-Galp-(1-4)-L-
-Rhap-(1-

└(2-1)-?-D-Galp.

K81[63](1975)

repeat-2)-*α*-L-Rhap-(1-3)-*α*-L-Rhap-(1-4)-ß-D-GlcAp-(1-2)-*α*-L-Rhap-(1-3)-
-*α*-L-Rhap-(1-3)-ß-D-Galp(1-

K83[64](1976)

repeat-3)-ß-D-Gal-(1-4)-*α*-L-Rhap-(1-

α-D-GlcpA-(1-3)-*α*-D-Galp-(1-3)-┘

6) Chemotype (GlcA, Gal, Glc, Rha)

K12[65](1980)

repeat-3)-*α*-D-Galp-(1-2)-ß-D-Galf-(1-6)-*α*-D-Glcp-(1-3)-*α*-L-Rhap-(1-

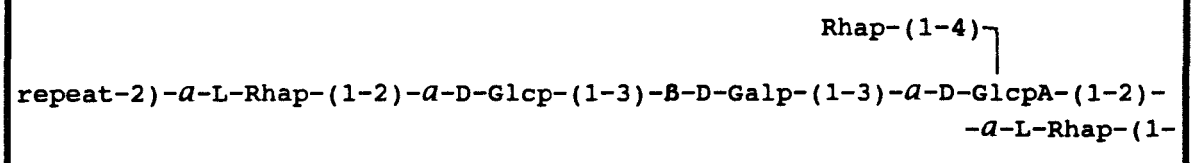
ß-D-Galp4,6Py-(1-4)-ß-D-GlcpA-(1-3)-┘

K18[66](1978)

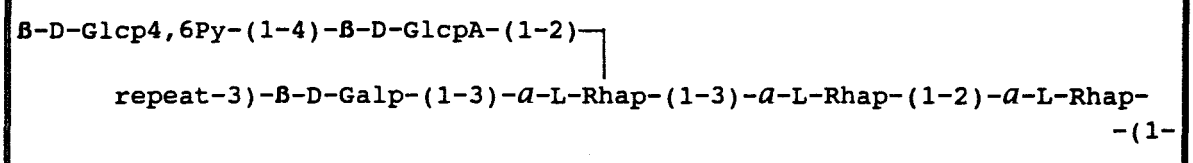
repeat-3)-*α*-L-Rhap-(1-3)-ß-D-Galp-(1-4)-*α*-D-Glcp-(1-

α-D-Glcp-(1-4)-ß-D-GlcpA-(1-2)-*α*-L-Rhap-(1-3)-┘

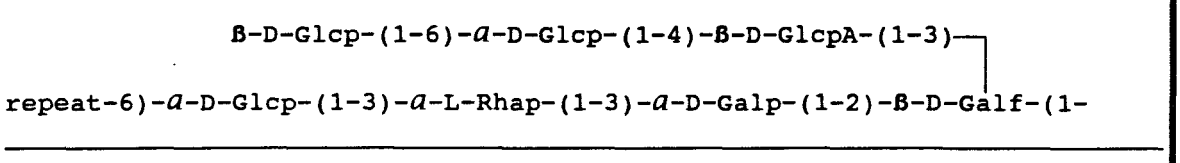
K19[67](1986)



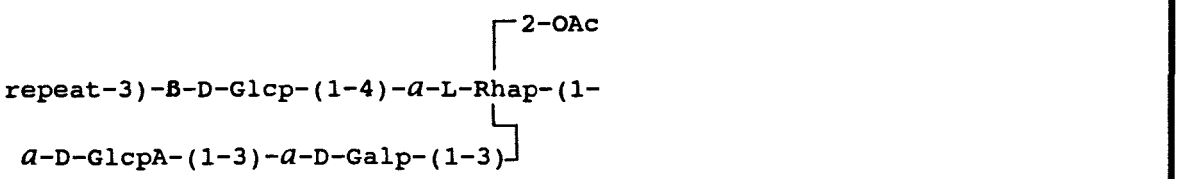
K36[68](1977)



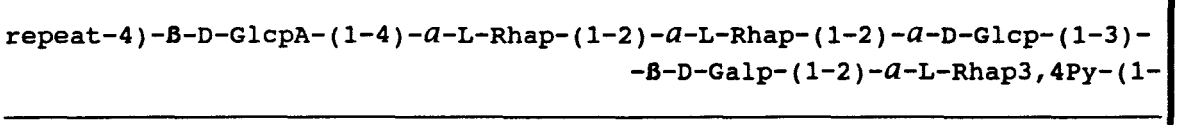
K41[69](1978)



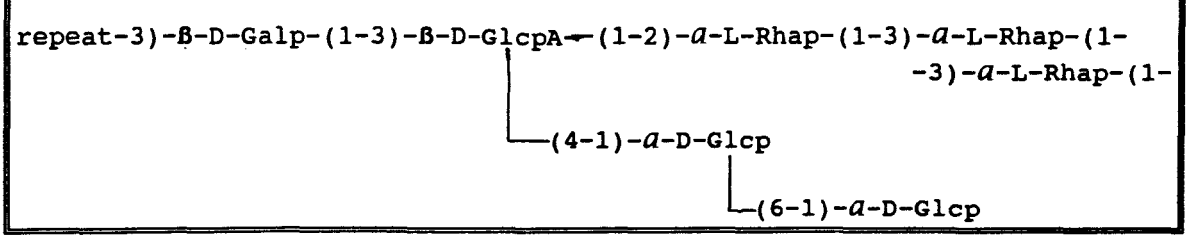
K55[70](1978)



K70[71](1978)



K79[72](1985)



7) Chemotype (GlcA, Gal, Man, Rha)

K40[73](1987)

repeat-4)-α-D-GlcpA-(1-4)-ß-D-Galp-(1-2)-α-D-Manp-(1-3)-ß-D-Galp-(1-3)-
-ß-D-Galp-(1-
ß-D-Galp-(1-2)-ß-L-Rhap-(1-3)

K53[74](1980)

repeat-4)-ß-D-GlcpA-(1-2)-α-D-Manp-(1-2)-α-D-Manp-(1-3)-ß-D-Galp-(1-2)-
-α-L-Rhap-(1-
(3-1)-Rhap-L-α

K80[75](1984)

repeat-3)-ß-D-Galp(1-2)-α-D-Manp-(1-2)-α-D-Manp-(1-
(3-1)-α-D-GlcpA
(4-1)-ß-L-Rhap4,3Py

8) Chemotype (GlcA, Glc, Man, Rha)

K64[76](1987)

α-L-Rhap-(1-3)
repeat-4)-α-D-GlcpA-(1-3)-α-D-Manp-(1-3)-ß-D-Glcp-(1-4)-ß-D-Manp-(1-
ß-D-Glcp4,6Py-(1-2)

9) Chemotype (GlcA, Gal, Glc, Man, Rha)

K14[77](1985)

repeat-4)-ß-D-GlcpA-(1-3)-ß-D-Galf-(1-3)-ß-D-Glcp-(1-4)-ß-D-Manp-(1-
α-L-Rhap-(1-3)
ß-D-Glcp4,6Py-(1-2)

K67[78](1983)

repeat-3)-α-L-Rhap-(1-3)-α-D-Manp-(1-3)-α-D-Manp-(1-3)-β-D-Glc-(1-

β-D-Galp-(1-3)-β-D-GlcA-(1-2)-┘

└-(4-1)-α-L-Rhap

10) Chemotype (GlcA, Gal, Glc, Fuc)

K16[79](1977)

β-D-Galp-(1-4)-┐

repeat-3)-α-D-Glcp-(1-4)-β-D-GlcpA-(1-4)-α-L-Fucp-(1-

K58[80](1980)

repeat-3)-α-D-Glcp-(1-4)-β-D-GlcpA2,3Py-(1-4)-α-L-Fucp-(1-

1 x OAc

α-D-Galp-(1-3)-┘

11) Chemotype (GlcA, Glc, Rha)

K17[81](1980)

repeat-4)-β-D-Glcp-(1-2)-α-L-Rhap-(1-4)-α-D-GlcpA-(1-3)-β-L-Rhap-(1-

α-L-Rhap-(1-3)-┘

K23[82](1990)

repeat-3)-β-D-GlcpA-(1-3)-α-L-Rhap-(1-

β-D-GlcpA-(1-6)-α-D-Glcp-(1-2)-┘

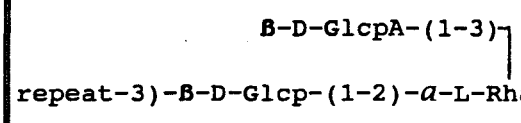
K44[83](1985)

repeat-4)-β-GlcpA-(1-2)-α-L-Rhap-(1-3)-α-L-Rhap-β-Glcp-(1-4)-α-D-Glc-

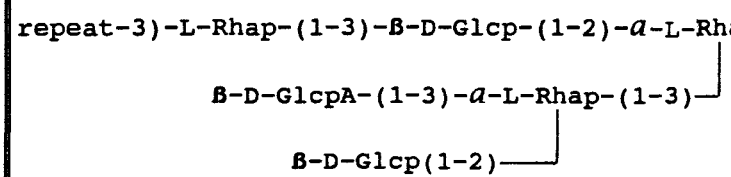
-(1-

64% (6OAc)-┘

K45[84](1982)



K71[85](1990)



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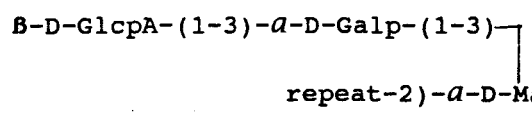
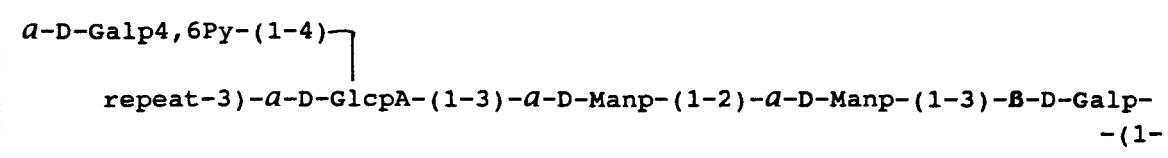
12) Chemotype (GlcA, Man, Gal)

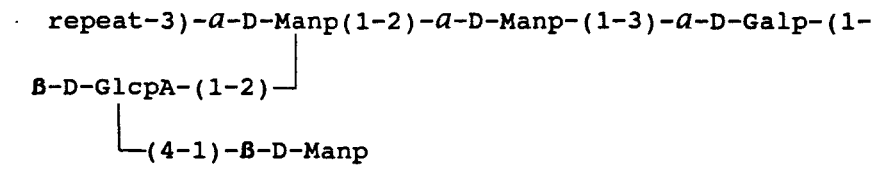
K20[86](1974)



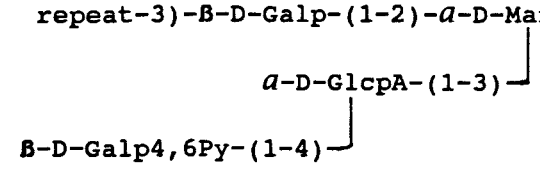
K21[86]



K43[7](1992)

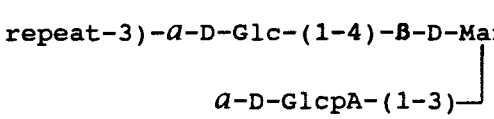


K74[88](1980)

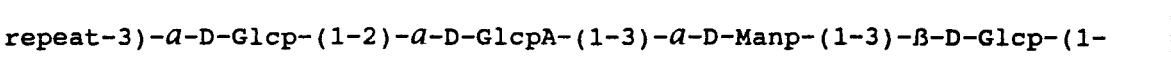


13) Chemotype (GlcA, Glc, Man)

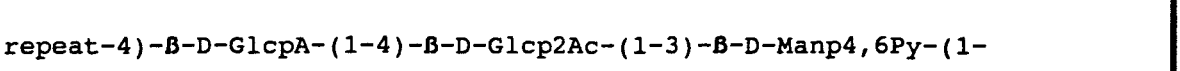
K2[22](1967)



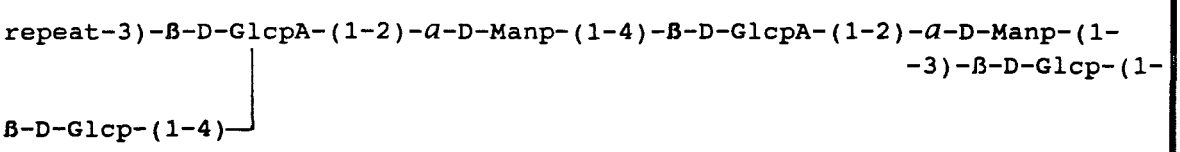
K4[89]



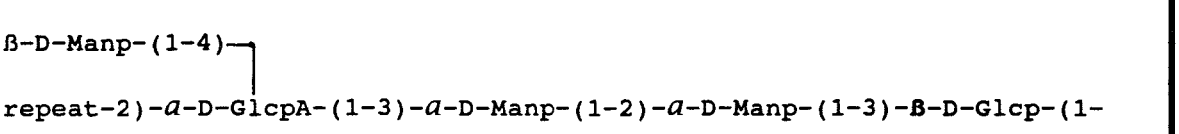
K5[90](1985)



K39[91](1987)

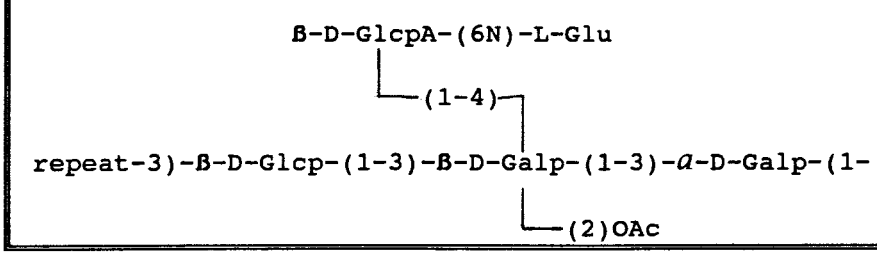


K24[27](1988)



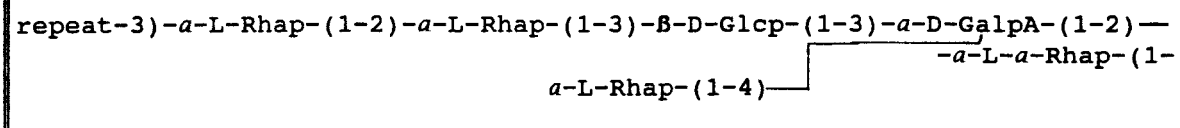
14) Chemotype (GlcA, Glc, Gal, Glu)

K82[24](1988)

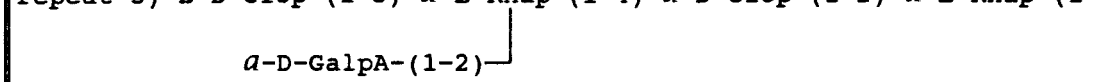


15) Chemotype (GalA, Glc, Rha)

K34[93](1982)

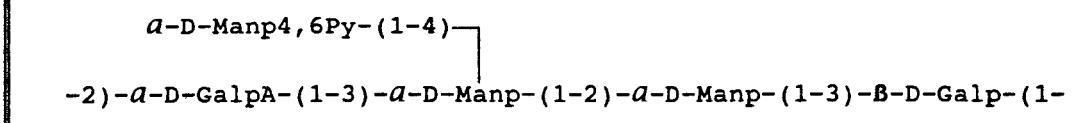


K48[94](1988)

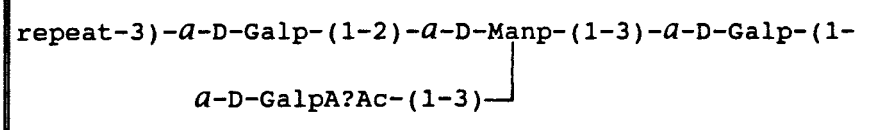


16) Chemotype (GalA, Man, Gal)

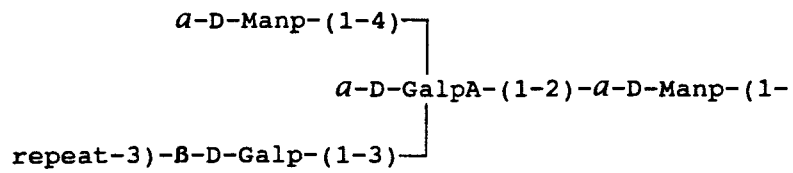
K3[95](1989)



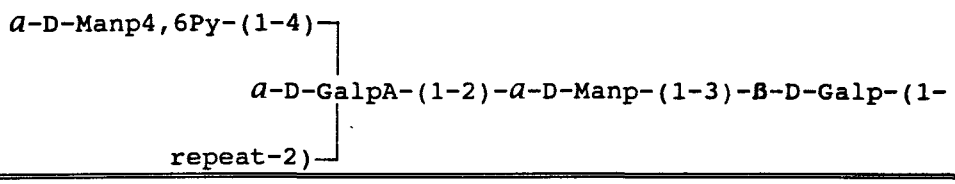
K49[96](1985)



K57[97](1975)



K68[98](1986)



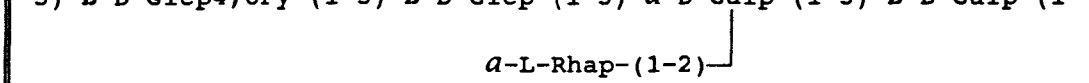
17) Chemotype (GalA, Glc, Fuc)

K63[99](1979)

repeat-3)-α-D-Galp-(1-3)-α-D-GalpA-(1-3)-α-L-Fucp-(1-

18) Chemotype (Gal, Glc, Rha)

K56[31](1973)



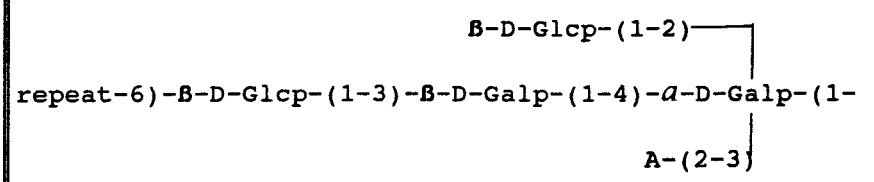
19) Chemotype (Gal, Rha)

K32[33](1978)

repeat-3)-α-D-Galp-(1-2)-α-L-Rhap3,4Py-(1-3)-β-L-Rhap-(1-4)-α-L-Rhap-
-(1-

20) Chemotype (Gal, Glc)

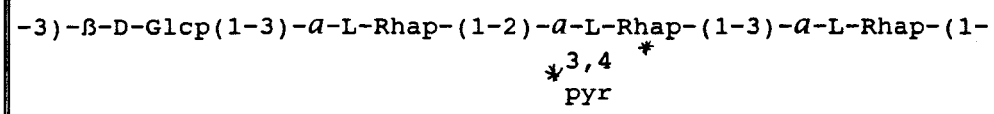
K38[36](1973)



* A = 3-deoxy-L-glycero-pentulosonic acid

21) Chemotype (Glc, Rha)

K72[32]



2. ISOLATION AND PURIFICATION OF THE *KLEBSIELLA* K-ANTIGEN

Propagation of *Klebsiella* bacteria can be carried out in liquid or on solid culture media and Duguid *et al.*[100] have reported the influence of culture conditions on polysaccharide production amongst *Klebsiella* bacteria. If grown on sucrose-rich nutrient agar the bacteria flourish and produce copious amounts of capsular polysaccharide. In order for the results of the structural analysis of the polysaccharide to be reliable, a rigorous purification procedure must be followed and the final product must be free of contaminating proteins, lipids, nucleic acids and inorganic salts. Adams[101] has described the extraction of cellular carbohydrate constituents. When separating and purifying polysaccharides it is useful to have some knowledge of the character of the polysaccharide under investigation, e.g. solubility, stability to acid and base, heat lability, and stability of labile substituents.

On extracting the capsular polysaccharide from *Klebsiella* bacteria it is preferable to prevent cell lysis as this leads to greater ease of purification. Cells are killed[101] and the capsular polysaccharide is solubilised by stirring the suspension in 1% aqueous phenol. The cells are removed by ultracentrifugation and the supernatant is freeze-dried to yield crude capsular polysaccharide. Some lipopolysaccharide (O-antigen) is also extracted at this time. However, because this polysaccharide is neutral, the separation of K-antigen from O-antigen is simple. The acidic K-antigen is precipitated by adding a quaternary ammonium salt such as cetyltrimethylammonium bromide[101,102] (CTAB), which reacts with the acidic functions on the polysaccharide. Neutral O-antigen remains in the supernatant. Should the solution be contaminated with nucleic acids[101], increasing the CTAB concentration slowly to 0.3 M precipitates nucleic acid whilst the acidic K-antigen is still in solution. Careful control of the addition of the CTAB solution allows the different fractions present in solution to be separated. The methodology of antigen extraction in the present study is similar to that used by Okutani *et al.*[103] and enables the separation of milligram to gram quantities of acidic polysaccharide. Other techniques used on a smaller scale are a variety of chromatographic techniques, (chapter 4), e.g. ion-exchange chromatography on DEAE columns by means of gradient-elution with buffer of increasing strength, affinity chromatography, electrophoresis

and size exclusion chromatography (SEC). In this laboratory SEC is mainly used to ascertain purity and separate contaminants from capsular polysaccharides.

3. PROCEDURES USED IN THE CHEMICAL ELUCIDATION OF BACTERIAL EXOPOLYSACCHARIDES

3.0. Primary analysis

Bacterial polysaccharides are complex polymers composed of oligosaccharide repeating units. This phenomenon along with relatively high yields of bacterial polysaccharide (mg or >) permit the use of a variety of chemical and instrumental methods for structural elucidation. Certain biological techniques are also useful in depolymerising the polysaccharide. In this section the more common techniques in current use, and all techniques used in the elucidation of the structure of the *Klebsiella* K43[7] antigen will be discussed.

3.0.(i). Monosaccharide composition

When determining the sugar ratio of a bacterial polysaccharide the monosaccharide yield must be optimised and careful choice of reaction conditions, *viz*. the choice of reaction type, concentration of reactants, temperature and length of time of reaction are vitally important. Cleavage of the glycosidic linkages is associated with a degree of monosaccharide degradation and this must be kept to a minimum if an accurate sugar ratio is to be found.

Principally four reaction types may be used to determine the sugar ratio: hydrolysis[104,105] (with mineral or organic acid), methanolysis[105] (with anhydrous methanolic hydrogen chloride), acetolysis[105] (with acetic anhydride and acetic acid), and formolysis[105] (with formic acid).

Polysaccharides soluble in organic solvents e.g. methylated polysaccharide, may be subjected to acetolysis or formolysis and the water soluble products hydrolysed to their monosaccharide residues should the polymer resist hydrolysis initially. Uronic acids resist hydrolytic conditions used and polysaccharides containing uronic acids are initially methanolysed[105]. The methyl ester of the uronic acid is formed, and this is accompanied by a degree of polysaccharide depolymerisation. After reduction of the methyl ester of the uronic acid the parent monosaccharide is easily hydrolysed and subjected to analysis. Methanolysis[27] is generally carried out with 3% anhydrous methanolic hydrogen chloride solution at 80° C for 16 hours. *Cheetham et al.*[106] have published results of the degree of degradation of ten monosaccharide methylglycosides in a 1 M HCl /MeOH system at 80°C for 24 hours and Fournet *et al.*[107] have used 0.5M MeOH/HCl for 24 hours at 80° to analyse methylated glycosaminoglycans. This method allowed them to analyse neutral and amino sugars simultaneously as the methylated methylglycosides.

Hydrolysis[105] is routinely used to cleave bacterial polysaccharide glycosidic linkages and takes place in aqueous solution under the control of a proton donor *viz.* the acid. Acids used include sulphuric, hydrochloric and trifluoro-acetic acid (TFA), while the use of oxalic acid has also been reported. *Adams*[108] has discussed the acid lability of different monosaccharide forms and this information is useful when designing a hydrolytic experiment, see Table 3.

Table 3.

More acid labile	Less acid labile
Ketose sugars	Aldose sugars
Furanose cyclic forms	Pyranose cyclic forms
a-anomeric configuration	ß-anomeric configuration
Pentoses	Hexoses
Deoxysugars	
Pyruvate, acetate groups etc.	
	Uronic acid sugars

Hough[109] (1972) surveyed different acidic conditions used in polymer hydrolysis. Practically sulphuric acid is difficult to remove after hydrolysis and hydrochloric acid should be used under nitrogen to decrease the possibility of oxygen-induced monosaccharide decomposition. Trifluoro-acetic acid[27] (TFA) has become popular as it is easily removed under reduced pressure because of its volatility and is used at a strength of 2 M. Neeser *et al.*[110] have used 4 M TFA at 125°C for 1 hour, and all hydrolytic reactions performed in the study of *Klebsiella* K43[7] were conducted under these conditions.

The hydrolysate may be analysed directly, e.g. by HPLC of the products. A range of quantitative colorimetric methods[111] for the determination of monosaccharides are available, but do not discriminate between sugars of the same class and may give erroneous results depending on the products in solution. They rely on the formation of chromogens of the hydrolysate after reaction with specific reagents.

Aspinall[112] has described chemical methods for the analysis of pyruvate and other covalently bound non-carbohydrate groups, sensitive to hydrolytic conditions. Today native underivatised material may be directly studied by ^{1}H- and ^{13}C-NMR methods which are faster and non-destructive allowing labile groups to be preserved.

3.0.(ii). Molecular size determination

Polysaccharide molecular weight determinations are estimates of a repeating unit structure and are measured by one or more of the following techniques[113]: primary (direct determination), *viz*. M_n - number average or M_w - molecular weight average; Secondary technique, *viz*. M_r - relative molecular weight. The relative molecular weight is a secondary technique because it relates the polysaccharide weight to a known molecular weight polysaccharide and is quick and easily carried out on a calibrated gel permeation chromatographic column.

Primary techniques rely on a range of physico-chemical techniques[114] and the approximate weight of the polysaccharide dictates which method will be most appropriate,e.g. osmometric determination is applied to polymers which fall between 15 000 to 15 000 000 daltons, as smaller molecules diffuse through the membrane involved in this method. Isothermal distillation determines number averages between 1 000 to 20 000 daltons. The use of chemical techniques to determine the reducing end of a polysaccharide, e.g. periodate oxidation and ^{14}C labelled cyanide allows calculation of the number average on the premise that each polysaccharide sequence contains one reducing end. Other techniques available are ultracentrifugation[115] which is laborious, and computation of the degree of polymerisation by quantitative colorimetric analysis of periodate oxidised polysaccharide.

Greenwood[115,117] has evaluated inherent problems in methodology and interpretation of molecular weight determinations. Comparing M_n to M_w indicates the degree of molecular weight distribution within a system and if a polydisperse system is present the results will not be in close agreement.

3.0.(iii). Absolute configurational analysis of the monosaccharides

Monosaccharides found naturally may be either of the **D** or **L** absolute configuration e.g. glucose, mannose etc, are usually of the **D** configuration whilst rhamnose is usually **L**, but exceptions do occur and monosaccharide absolute configuration must be established for complete description of a bacterial polysaccharide.

Methods of determining the absolute configuration of a monosaccharide are: polarimetry[118], circular dichromism, enzyme catalysis and chromatographic separation of derived diastereomers.

The use of circular dichromism in measuring the derived monosaccharide alditol acetate derivatives, for the determination of absolute configuration was reported by Bebault *et al.*[119], and was conducted on milligram quantities of hydrolysed *Klebsiella* serotypes K7 and K21.

Enzymatic determination relies on the substrate specificity of the enzyme, e.g. **D**-glucose specific oxidase if shown to degrade the monosaccharide confirms that the monosaccharide was **D** glucose. Unfortunately these biological products apart from causing degradation, are difficult to acquire and are not available for the array of sugars found in bacterial polysaccharides.

Gas liquid chromatographic analysis is a popular technique for determining absolute configuration on sub-milligram quantities of the polysaccharide which is hydrolysed prior to glycosylation with a chiral reagent. The enantiomers released by hydrolysis are not distinguishable directly using conventional GLC columns and are converted to diastereomeric[120] glycosides which are separated by chromatography of their acetates. Kônig *et al.*[121] have however succeeded in determining the absolute configuration of the hydrolysed monosaccharides from a polysaccharide by GLC analysis on a chiral phase, and their report describes chiral phases suitable for analysis of a variety of volatile derivatives.

The choice of asymmetric glycosylating agent will affect the resolution of the chromatographed peaks. Monosaccharide glycosylation with a chiral alcohol usually produces overlapping peaks owing to the different forms of each glycosylated

monosaccharide, e.g. α- and ß-anomers of each of the pyranose and furanose forms. A commonly used chiral alcohol is (-)-2-octanol[122]. Comparison of a chromatogram of a standard glycosylated monosaccharide easily confirms the **D** or **L** configuration from the complicated chromatogram of the unknown monosaccharide.

Oshima *et al.*[120] have used L-(-)-methylbenzylamine to produce acyclic diastereoisomers. Multiple pyranose and furanose forms are avoided and enhanced product separation is found. An aromatic group is introduced via reductive amination and if HPLC chromatography is used, the eluting components can be detected at 230nm by a uv-detector. Another report has shown that separation of enantiomeric sugars as diasteriomeric trifluoroacetylated-(-)-bornyloximes[123] produces two GLC peaks which simplify the interpretation of the absolute configuration data.

3.1. Determination of monosaccharide linkage positions

Establishing the linkage pattern of a bacterial polysaccharide involves determining the monosaccharide linkage position(s) and the relative configuration of these linkages. Methylation analysis in tandem with one or more complementary chemical technique is necessary to describe the chemical repeating unit.

3.1.(i). Methylation analysis

Methylation has been widely used to determine monosaccharide glycosidic linkages but does not prove the sequence of these linkages.

The methylation[124] reaction assumes that all non-glycosidic hydroxyl groups are converted to methyl ethers via an alkoxide anion, formed by the action of a strong base on the cyclic monosaccharide, and that this methylation product does not undergo de-etherification on hydrolysis. The resulting free hydroxyl groups indicate the linkage positions and these positions can be determined by GLC-MS of their acetylated derivatives. Limitations of this technique are that the order of the monosaccharide linkages is not

defined, i.e. only the positions of the glycosidic linkages are found, the relative anomeric configuration is not determined, and sugars not methylated at C4 or C5 can give ambiguous ring size information.

During the period 1915-1992 a variety of solvent systems, bases and alkylating agents have been introduced, each having its own advantages under different circumstances. Overcoming solubility problems, incomplete methylation, excessive degradation or by-product formation, and the length of time for the manipulation has been the driving motivation in finding new techniques.

Methylation is carried out on milligram quantities of material and the complete methylation[125] of 1μg of a disaccharide and 5μg of an acidic polysaccharide has been reported. Other practical difficulties are the possible loss of highly volatile polymethylated monosaccharides during work up procedure. Non-carbohydrate substituents[126] e.g. O-acetyl groups are cleaved by the strong base used, but N-acetyl groups are stable.

Haworth[127] achieved methylation with a 25-30% aqueous sodium hydroxide solution in which the polymer was treated with dimethyl sulphate, but complete methylation was seldom achieved in a single reaction and Purdie developed a technique which involved refluxing the partially methylated polysaccharide in silver oxide and methyl iodide. Kuhn *et al.*[128] improved on this method by using a polar solvent e.g. N,N-dimethyl formamide with repetitive methylation in the presence of barium oxide.

In 1964 Hakomori[129] revolutionised the methylation of polysaccharides by introducing the use of sodium dimsyl and methyl iodide (MeI). Most methylation reactions are carried out by this or a derivative of the Hakamori method, i.e. that of Phillips *et al.*[130]. Initially sodium dimsyl anion[129] in DMSO was used to form the alkoxide anion. Other derivatives used are potassium dimsyl[130] which is easier to produce, less dangerous and more stable; potassium *tert*-butoxide[131] which forms an equilibrium of methylsulphinyl carbanion with *tert*-butoxide anion in solution; and lithium methylsulphinyl carbanion[132] which produces results relatively devoid of contaminating fragments, although there is considerable danger in its preparation. 1,1,3,3-tetramethyl urea[113] has been applied in a 1:1 DMSO solution, enabling intermolecular hydrogen bonding relaxation within a

polysaccharide matrix and improved MeI penetration to the polysaccharide anion. Aggregation of ribbon-like tertiary structures has been shown to decrease the extent of methylation of a galactomannan by Manzo *et al.*[134] thus reinforcing the utility of tetramethyl urea in polysaccharides present as aggregates.

Some fluorinated methylating agents have found use in polysaccharide chemistry, e.g. diazomethane-boron trifluoride[135] conserves O-acyl substituents during methylation and Arnarp *et al.*[136] offered methyl trifluoromethane sulphonate as an alternative to it where necessary. It must be noted that a nonpolar solvent is necessary if the high degree of reactivity is to be maintained when using fluorinated agents.

Methylation chemistry has advanced to a stage where a polysaccharide can be methylated in a single vessel within a few hours without intermediate work-up procedures[125]. Probably the most novel technique since the Hakomori method is that of Ciucanu[137] who replaced the dimsyl anion with a solid base of potassium hydroxide, (and others hydroxides) in methyl sulphoxide. This technique has the advantage of almost quantitative methylation in 6-7 minutes, and a clean chromatogram free of non-methylated by-products. Ciucanu *et al.*[138] have recently also published methodology used to avoid uronic acid degradation which can occur during methylation with sodium methanesulphinylmethanide in methylsulphoxide. When methylating oligosaccharide material of low molecular weight, the Prehm[139] method of methylation for small molecules may be useful.

Today methylation of polysaccharides of diverse character can be brought about by a multitude of techniques using solvent systems ranging from aqueous to non-aqueous.

3.2. Monosaccharide sequencing

One or more of the following techniques is normally used after linkage determination by methylation analysis. To confirm the structural analysis a third technique, the Smith degradation may still be necessary.

3.2.(i). ß-Elimination of uronic acid residues

The majority of bacterial polysaccharide K-antigens contain a uronic acid. This acid can be selectively cleaved by ß-elimination if the uronic acid is 4-O-substituted and the polysaccharide is methylated. Comparative GLC analysis of hydrolysed, methylated polysaccharide with that of the ß-eliminated hydrolysed products enables the site(s) of uronic acid linkage to be identified.

Polysaccharides constructed with an in-chain uronic acid degrade to produce oligosaccharides, while polymeric products are obtained from polysaccharides with a uronic acid in the side chain. Demethylation and other degradation reactions may give false information, but can be controlled.

The reaction must be performed in a basic non-aqueous solution which affords stability to the alkoxide anion, and the methyl ester of the uronic acid and methylated hydroxyl groups of the uronic acid should be stable to the ß-Elimination conditions.

Lindberg *et al.*[140] described an elimination method which takes advantage of the formation of an unsaturated bond at C4 of a uronic acid under the action of a strong base but the presence of a good leaving group at C4 is necessary. The polysaccharide is initially methylated by the modified-Hakomori method and ß-elimination will not occur under these conditions to any appreciable degree because the methyl iodide added neutralizes the dimsyl anion. On the addition of a strong base[140] and later mild hydrolysis the uronic acid degrades forming a 4-deoxyhex-5-ulosonate which may form a furan moiety through internal lactonization, (see figure 7). The substituent monosaccharide or appendage is liberated along with the intact polysaccharide or oligosaccharide.

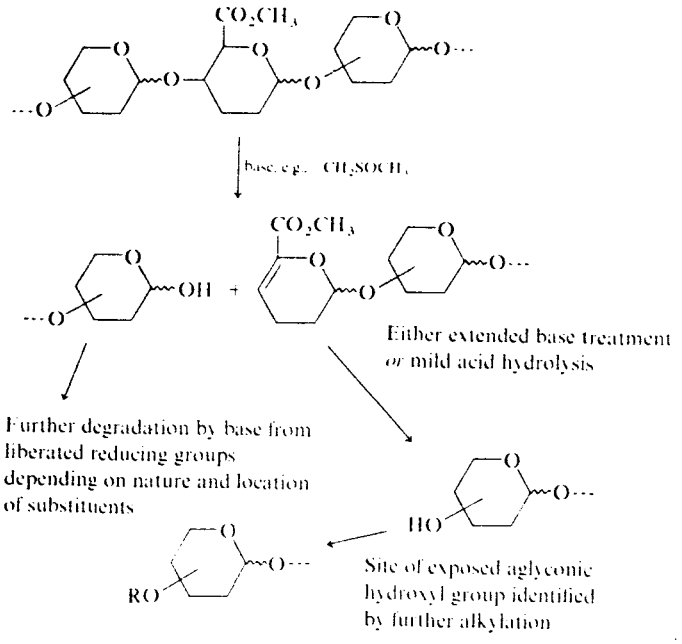


FIGURE 7. Base-catalysed ß-elimination from 4-linked hexuronate residues in permethylated polysaccharides.

Kiss[141] has reviewed the scope, mechanism and various systems susceptible to ß-elimination and provides data on these. Kenne *et al.*[142] used *Klebsiella* K47 as a model polysaccharide and methylated it selectively leaving C-3 with an underivatised hydroxyl. A carbonyl centre was generated by the oxidation of the hydroxyl under ruthium tetroxide followed by ß-elimination under suitable conditions. Aspinall *et al.*[143] reported that the elimination reaction of Lindberg[140] could be performed with the omission of the mild hydrolysis step. The point of uronic acid linkage is directly ethylated with ethyl iodide, hydrolysed and separated by GLC.

Lindberg *et al.*[144] have shown that after methylating a polysaccharide containing an uronic acid in its side chain, e.g. *Klebsiella* K28, the uronic acid could be cleaved in a 9:5 acetic-anhydride/triethylamine solution followed by treatment with 90% acetic acid. This elimination is dependant on the strength of the leaving group attached to the acid. If the group is weak, increasing the reaction conditions only serves to form intramolecular uronic ester linkages.

ß-Elimination is qualitatively carried out on milligram quantities of methylated polysaccharide and describes the relative position of the uronic acid, i.e. identifying which monosaccharides are linked to the uronic acid and gives an idea whether the acid is in the chain or not.

3.2.(ii). Reductive cleavage

Conventional analysis of methylated polysaccharides by GLC of the derived permethylated alditol acetates does not allow differentiation between O-4 linked aldopyranosyl and O-5 linked aldofuranosyl residues[124]. The technique of reductive cleavage[145] when applied to methylated polysaccharide, enables the simultaneous determination of the ring size, and linkage positions of the cleaved monosaccharides. The glycosidic C-O bond of a polysaccharide undergoes a hydride transfer by, e.g. triethylsilane in the presence of a catalyst, and the anomeric carbon of the monosaccharide cleaved is dehydrated with the formation of an anhydroalditol, (Figure 8.). GLC-MS analysis of the permethylated anhydroalditol acetates establishes the monosaccharide character unambiguously, pyranose residues are expressed as 1,5 anhydro-alditol derivatives and furanose residues as 1,4 anhydro-alditols.

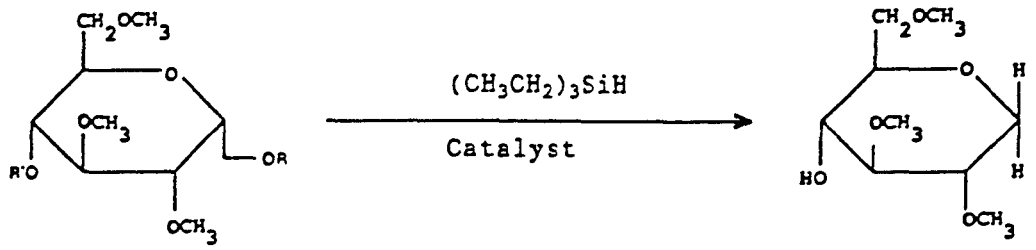


FIGURE 8. Reductive cleavage of the C-O bond via hydride transfer

The utility of following a conventional methylation reaction by reductive cleavage is witnessed by less work-up procedure, acetylation is carried out *in situ* in acetic anhydride, and the ring form is maintained along with the stable anhydro-alditol

Triethylsilane has been used as the reducing agent along with either trimethylsilyltrifluoromethanesulphonate or boron trifluoride etherate as the catalyst. By judicious choice of catalyst[146] monosaccharide residues of a polysaccharide may be preferentially cleaved, e.g. ß 1-3, ß 1-4 and 6 linked glucopyranosyl residues are stable to boron trifluoride etherate. However a solution[147] of 5:1 trimethylsilylmethanesulphonate and one equivalent of boron trifluoride etherate per equivalent of acetal has been shown to cause total polysaccharide cleavage without artifact formation.

Gray *et al.*[146] have done much to develop the method of reductive cleavage and have determined linkage position(s) and ring size amongst, e.g. D-mannans[148], 2-acetamido-2-deoxy-D-glucopyranosyl residues[149], and its application to pyruvic acid acetal containing polysaccharides[150]. They note that although the 4,6 (1-methoxycarbonylethylide) is stable to reductive cleavage conditions, eventually the S diastereoisomer of the acetal results with possible erroneous configuration determination. Slow regiospecific reductive ring-opening of the acetal may also occur with the formation of a 6-O-[1-(methoxy-carbonyl)ethyl] derivative.

Although reductive cleavage has advantages, the establishment of optimal reaction conditions of reagent/catalyst may be problematic, as described by Hackland[151] in the reductive cleavage of an *E.coli* polysaccharide.

3.2.(iii).a. **Periodate Oxidation**

Oxidation of bacterial polysaccharides by periodate is carried out in aqueous solution and the products of this reaction are used to prove points of linkage, linkage sequence, chain length and molecular weight.

In 1928 Malaprade[152] showed that α-glycols undergo quantitative oxidation with periodate to form polyaldehydes. One mole of periodate is consumed for each pair of vicinal diols present and the oxidation of an adjacent diol pair results in the release of formaldehyde. Structural information can be determined with respect to the oxidised polysaccharide being analysed, if it is assumed that all vicinal diols are oxidised. The formation of inter-residue hemi-acetals[153], after the formation of reactive carbonyls may result in a first oxidation limit, i.e. the oxidised residue has a protective effect on its nearest neighbour but reduction at this stage, followed by re-oxidation usually enables complete oxidation of the monosaccharide. Should the polysaccharide be over oxidised it is depolymerised and periodate is consumed to infinity[153].

Painter[154] has demonstrated that the "peeling reaction" taking place from the reducing end of the polysaccharide which causes depolymerisation of the polysaccharide can be eliminated. The polysaccharide is reduced after oxidation and 1-propanol is included as a radicle scavenger. Scott[155] has used divalent magnesium ions in decreasing the anionic nature of the uronic acid carboxyl. This decreases the steric repulsion toward the oxidising periodate ion and enables complete oxidation. Using a lower pH[156] has a similar effect, but causes a loss in oxidation selectivity of diol systems. Bobbit[157] indicated that the inability of a polysaccharide to undergo periodate oxidation could establish that either a vicinal diol is absent, or that an anchored *trans* configuration could be present in the structure. *Cis* diols are oxidised most quickly and therefore polysaccharides containing a greater number of *cis* diol pairs are oxidised more quickly.

Optimum conditions for selective periodate oxidation[158] include a mildly acid environment, temperature less than 6°C, darkness (periodate is decomposed by light), and a 3-4 fold excess of sodium periodate. Sterically hindered polysaccharide systems are

oxidised more slowly and HPLC analysis of oxidised polysaccharides can be useful in determining the degree of oxidation.

Periodate oxidation is useful for the quantitative determination of small quantities of polysaccharide material. Quantitative analysis of the periodate consumption relative to formaldehyde production gives an indication of the number of vicinal diol pairs, and isotachophoretic[159] analysis of periodate can be performed down to 1-2μg. Other methods[160] used in the determination of periodate, iodate and formate are spectrophotometry, titrimetry, potentiometry, manometry, and enzymic methods, depending on the particular system. Spectrophotometric[161] analysis depends on the maximum light absorbance of periodate being at 223, 260 and 290nm. However iodate absorption influences the accuracy of this technique and must be taken into account.

Preparative generation of large quantities of selectively oxidised polysaccharide is possible and the reaction is easily quenched by adding excess ethylene glycol, making periodate oxidation a very useful reaction.

3.2.(iii).b. **Smith degradation**

A Smith degradation involves initial oxidation of the polysaccharide by periodate, followed by hydrolysis under mild conditions. The Smith degradation generates an oligosaccharide which is the most elementary product of the oxidised polysaccharide. A mild hydrolytic step cleaves all the acyclic linkages present after periodate oxidation and the resulting poly- or oligosaccharide can be analysed further to yield structural information. Figure 9, depicts the Smith degradation product of the *Klebsiella* K43[7] polysaccharide.

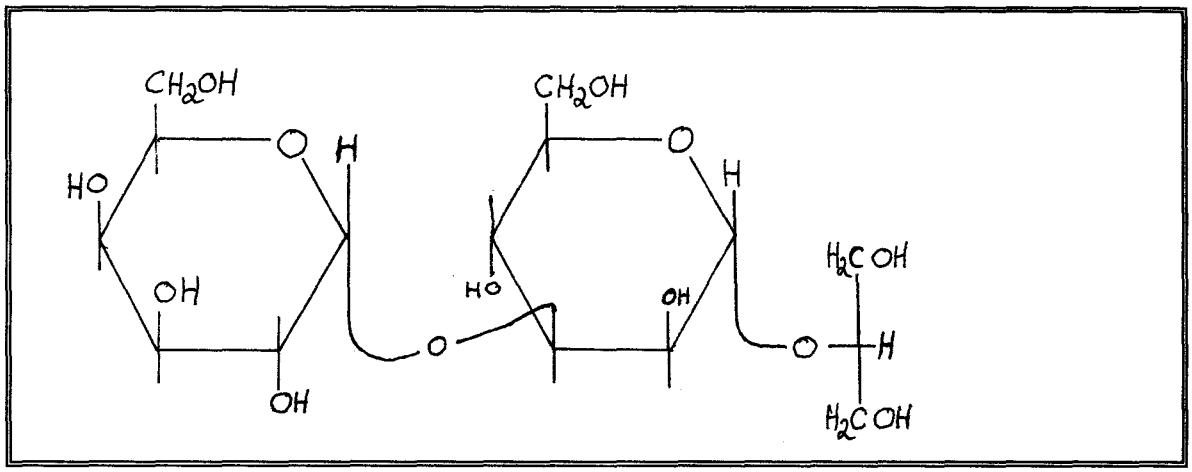


FIGURE 9. The *Klebsiella* K43 Smith fragment.

For reliable interpretation of information generated from a Smith degradation, it is necessary that all the vicinal diols of the polysaccharide undergo complete oxidation, and that hydrolysis of unoxidisable monosaccharides does not occur. Information established from the Smith degradation complements methylation and uronic acid degradation data.

Hydrolytic conditions used in the Smith degradation are mild and sensitive furanosides, if present, are not hydrolysed. Comparison of the acyclic degradative product(s) released after mild hydrolysis, with that of the Smith fragment helps to establish the polysaccharide structure. Dutton[162] surveyed hydrolytic conditions necessary for hydrolysis of glycoalditols and polysaccharide residues, whilst Baird[163] has indicated that GLC analysis of the Smith degradation products is best conducted by the formation of PAAN's derivatives, as TMS derivatives produce multiplets in the chromatogram. Quantitative determination of acyclic fragments generated can be determined relative to an internal standard[164].

1 M Trifluoroacetic acid at room temperature for 24 hours[165] has been shown to bring about complete hydrolysis of acetal linkages within the oxidised polysaccharide, but certain polysaccharides do require harsher hydrolytic conditions to cleave the acyclic aglycone. These condition can only be determined through experimentation.

3.3. Polysaccharide depolymerisation techniques

Water soluble bacterial polysaccharide K-antigens are expressed as a chain of oligosaccharide repeating units, and are often found as aggregates[166]. This gives rise to a complex quaternary structure of high molecular weight, usually several hundred thousand daltons. This material often resists attack by chemical reagents due to steric hinderance of reactive centres, but a variety of physical, chemical and biological techniques are available to decrease the molecular weight of a polysaccharide and each is useful in its own way. The degree of cleavage specificity sought, lability of certain components, the speed of the process, its availability and cost dictate the technique used.

3.3.(i). Partial Acid Hydrolysis

Aspinall[167,168] and Bouveng *et al.*[102] have reviewed the use of partial acid hydrolysis, in the structural analysis of complex polysaccharides. The polysaccharide is exposed to hydrolytic conditions that cleave only some glycosidic linkages and gel permeation chromatography is used routinely to separate the many oligosaccharides produced by partial acid hydrolysis. Structural determination of the resulting oligosaccharide allows deductions to be made about linkage location and monosaccharide sequence.

The nature of the glycosidic linkages and monosaccharide size within a polysaccharide dictate the outcome of the hydrolysis experiment. Furanose residues are hydrolysed 10^3 times faster than the pyranose forms, and uronic acids and hexosamine linkages are usually stable to partial hydrolysis. The occurrence of an aldobiouronic acid in a hydrolysate is indicative of an uronic acid linked to a neutral sugar within the polysaccharide. Furthermore the position of the glycosidic bond increases the specificity to preferential hydrolysis, e.g. (1-6) linkages are less labile than (1-2) linkages to partial hydrolysis. Changing the reaction conditions to a non-aqueous solvolysis system, e.g. acetolysis, methanolysis or mercaptolysis, reverses the relative susceptibility of different

glycosidic linkages, e.g. (1-2) linkages are then less susceptible to partial degradation than the (1-6) linkage. Careful choice of partial acid hydrolysis/solvolysis conditions will provide extra information pertinent to sequence and linkage type. Chemical manipulation, via the reduction of a uronic acid to its corresponding neutral monosaccharide, might allow the isolation of a different oligosaccharide fragment, thus yielding further structural information about the polysaccharide.

Typical aqueous partial hydrolysis conditions[169] are 0.5M H_2SO_4 at 100°C and samples are drawn at 45, 60 and 90 minutes. TFA is commonly used over a range of 0.5-2.0 M at various temperatures and periods of time.

Recently partial solvolysis[170] using liquid anhydrous hydrogen fluoride (fluorolysis), has gained popularity. It has greater scission specificity and the order of cleavage is for example,

α-D-glucopyranose residues > *α*-D-Mannopyranose > *α*-D-galactopyranose

Varying the temperature from -23 to -40 degrees increases the length of the resulting oligosaccharide. Fluorolysis is completed in 15-30 minutes and has the ability to discriminate between monosaccharide types. Negatively it is a dangerous reaction to carry out and the low temperatures involved need constant monitoring.

The reducing end of a polysaccharide has been labelled by Osman *et al.*[171] by reductive amination using aniline followed by methylation, partial hydrolysis and preparative HPLC separation of resulting oligosaccharides.

Partial hydrolysis may be selective in cleaving some polysaccharides but still remains much of a "hit and miss" method of polysaccharide depolymerisation. Large quantities of starting polysaccharide are needed and reaction conditions can be rather tedious to perfect for a given polysaccharide.

3.3.(ii). The Lithium degradation

Underivatised acidic polysaccharides undergo uronic acid cleavage by the use of lithium metal dissolved in an ethylenediamine solution of the polysaccharide[172]. The reductive strength of lithium ions dissolved in an amine solvent cleaves the polysaccharide at the point of the uronic acid linkage regardless of the linkage position. Oligosaccharides or low weight polysaccharides will form depending on the position of the uronic acid, i.e. an in-chain uronic acid will degrade into oligosaccharide length material. Unfortunately the caustic lithium ions produce a harsh reaction environment cleaving all attached ketals, and is strongly reducing to aldose sugars.

Mechanistically the lithium reaction has been suggested to be via an α-elimination of the oxygen attached between the C5 and C1 of the uronic acid, which in turn eliminates its oxygen to the aglycone liberated. The yield of neutral oligosaccharide from the lithium degraded *Klebsiella* K43[7] polysaccharide approximated 50%, but is adequate for preparative generation of large samples of uronic acid degraded polysaccharide.

Dry acidic polysaccharide is solubilised in ethylene-diamine by ultrasonication, heating and stirring. Lithium metal is introduced and vigorously stirred till a deep navy colour results. This is maintained for an hour after which the degraded polysaccharide is extracted by means of a toluene-water-ethylenediamine azeotrope as described[172]. Few by-products are formed and the reaction can be completed in a working day, although the ion-exchange of the lithium ions is time consuming.

Mort *et al.*[173] published the cleavage results of a 3-linked glycosyluronic acid in an amine solvent by lithium metal in 1982, and since then Lau *et al.*[172] have evaluated this technique by uronic acid degradation of a range of acidic biological polysaccharides. The uronic acid of the K-antigen's of *Klebsiella* K44, K17 and K62 were degraded, and this thesis has shown that the *Klebsiella* K43[7] polysaccharide glucuronic acid is also susceptible.

The lithium degradation conveniently generates uronic acid cleaved polysaccharide from underivatised polysaccharide, and the only restriction to its use is the solubility of the polysaccharide in ethylene-diamine. The lithium degradation is an important technique for generating uronic acid degraded samples of sufficient quantity for 2D NMR studies.

3.3.(iii). Enzymic Depolymerisation

Enzyme mediated polysaccharide depolymerisation has been reviewed[174-177], as well as its applicability to *Klebsiella* exopolysaccharides[175].

Many enzymes exist which catalyse the cleavage of specific glycosidic bonds. They can be divided into two main categories, *viz*. exo- and endo-enzymes. Exo-enzymes cleave the monosaccharide residues of a polysaccharide from the outside inwardly, i.e. from the non-reducing terminal of the poly- or oligosaccharide, and may be used in the step-wise identification of monosaccharides present in a saccharide. They may also aid in the determination of the linear or branched nature of a polysaccharide[174].

Endo-enzymes cleave a polysaccharide in a random pattern enabling rapid depolymerisation, but require several points of substrate attachment and these are found in regions of the polysaccharide which are unbranched or slightly branched[177]. They are particularly useful in producing large quantities of repeating unit oligosaccharide material from bacterial polysaccharides. The endo-enzyme only cleaves one type of bond, e.g. the *Klebsiella* K43[7] repeating unit oligosaccharide was produced by an endogalactosidase, cleaving an α-D-(1→3) galacto-mannose bond. All structural information of the original polysaccharide is maintained, e.g. acetate and other labile groups. It has been reported[177] that small residues may undergo transglycosylation after an endoglycanase digestion, although not common this necessitates the need to compare the repeating unit oligosaccharide produced with the native polysaccharide, e.g. by comparison of their respective methylation data.

Advantages of enzymic depolymerisation technology

1). An absolute degree of selectivity toward linkage and or sugar type is possible.

2). The method is non-contaminating and is carried out in water which allows easy dissolution of the polysaccharide and purification/separation.

3). The scission of the repeating unit or multiples thereof is possible for bacterial polysaccharides and acid labile appendages are not cleaved under the physiological conditions of the degradation.

Enzyme preparations must always be checked for purity as false information will result if impure enzyme isolates are used in structural analysis.

3.3.(iii).a. **Virus-borne enzyme depolymerisation**

Bradley[177] has reviewed the multitude of bacteriophages found and they are described as obligate viral parasites of bacteria. They depolymerise the extra-cellular polysaccharide of the encapsulated bacteria by means of the endoglycanases carried on their tail spikes, and these enzymes can be hydrolyses or lyases. Since enteric bacteria are found in sewage, phages may be easily isolated from untreated sewerage water.

Phage isolation, propagation and polysaccharide digestion used in our laboratory essentially follows the methods of Okutani *et al.*[103]. Bacteriophage propagation and concentration is carried out till a phage titre of 10^{13} plaque forming units is achieved, this being accepted as sufficient to degrade 1 g of polysaccharide.

Hydrolases[117] are reported to be most active at pH 5-6 and lyases at pH 8, however Dutton *et al.*[50] found pH 7 to be the most successful. Hydrolases have a molecular weight of near 50 000 daltons and that of lyases is higher; this physical property enables easy isolation of the resulting oligosaccharides by dialysis, with retention of the enzyme within the dialysis bag.

It has been noted[175] that amongst *Klebsiella* bacteriophages the majority bring about

hydrolysis of a ß-D-glycosidic linkage in glycosyl residues linked in the C-3 position, and the reducing-end was never found to be a uronic acid after phage digestion of the polysaccharide. In the case of K43[7], (this thesis), the endogalactosidase cleaved an *α*-linkage and not a ß-linkage. Figure 10. depicts the point of enzyme hydrolysis and the proximity of the uronic acid..

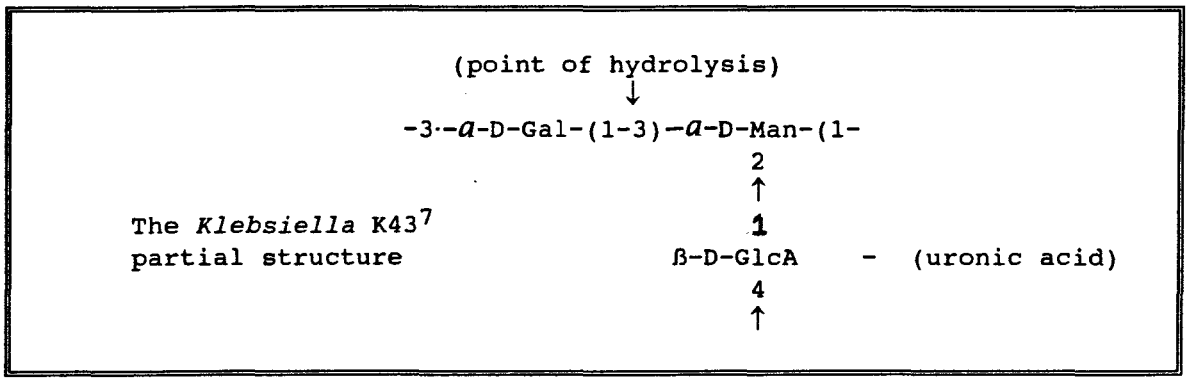


FIGURE 10. Enzyme hydrolysis

It is also not uncommon that a viral enzyme can cleave more than one exopolysaccharide into the respective oligosaccharide repeating units, e.g. phage K13[179] has been found to cleave *Klebsiella* K2, K22, K37 and K13. Phage K13 has affinity for the ß-D-glucose-(1→4)-ß-D-mannose and similarly linked galactose/glucose residues, and is unaffected by the presence of pyruvate or differences in the side chain, although the correct stereochemistry about the bond is important.

K13/K2 cleavage point: -)-ß-D-Glc-(1↓4)-ß-D-Man-(1-

K22/K37 cleavage point: -)-ß-D-Gal-(1↓4)-ß-D-Glc-(1-

The presence of pyruvate and uronic acid induce a centralised negative charge in a repeating unit and it has been demonstrated[180] that this charge is essential for the activity of the endoglycanase. K19 native polysaccharide was depolymerised by phage K19, but after the uronic acid was reduced, the endoglycanase did not cleave the polysaccharide.

The action pattern of lyases has been demonstrated[90]. Polysaccharides containing 4-linked hexuronic acids undergo lyase degradation to form an oligosaccharide with a non-

reducing terminus containing an unsaturated hex-4-enuronic acid. K5[90] and K64[76] are examples of polysaccharides which have been degraded by a lyase-containing bacteriophage.

Phage degradation is of especial utility in preserving the chemically labile pyruvate and acetate moieties found in some bacterial polysaccharides, and Dutton *et al.*[83] discovered that K44 contains an acetate appendage after work up of an enzymatic digest of the polysaccharide. This re-investigation into a published structure emphasises the importance of phage depolymerisation in the accurate determination of bacterial polysaccharides.

In conclusion phage depolymerisation yields the chemical repeating unit or multiples thereof. These oligosaccharides are then amenable for further structural analysis. In particular, they allow the acquisition of clean, well resolved NMR spectra and are excellent substrates for high resolution 2D NMR spectroscopy. Unfortunately bacteriophages are not found for all bacterial polysaccharides, e.g. *Klebsiella* K38[36] containing a pentulosonic acid was screened during initial investigations on K43[7] for a susceptible bacteriophage, but all tests were negative.

INSTRUMENTAL ANALYTICAL TECHNIQUES

4.0. Introduction

Nuclear magnetic resonance spectroscopy, mass spectrometry and different forms of chromatography are used routinely for the structural elucidation of bacterial polysaccharides. Other useful instrumental methods include polarimetry, ultra-violet spectrometry (UV), crystallography, and infra-red spectroscopy.

Polarimetry[118] was the only instrumental means of identifying the relative anomeric configuration of a monosaccharide a century ago but today is rarely used for this purpose. It is used to determine the specific light rotation of a chiral compound, i.e. specifying $[\alpha]_D$ for a polysaccharide, and for determining the absolute configuration of isolated monosaccharides.

Colorimetry and UV spectrometry, used extensively in the 1950's, has lost ground to new methods which are more accurate, but UV spectrometry[181] is still useful in the determination of phosphate in polysaccharides.

Crystallography[182] is beyond the scope of this section. It produces information on the tertiary structure (conformation) of crystallisable molecules and has been applied to the exopolysaccharide of *Klebsiella* K57[183]. Conformational molecular modelling[184] by super-computer technology has enabled mathematical, three dimensional geometrical calculations of polysaccharides and augments crystallographic studies.

Infra red (IR) analysis has been used in the determination of the carbonyl content of acidic polysaccharides and has also been used to monitor the extent of a reaction, e.g. the Smith degradation reaction.

4.1. Chromatographic Techniques

Figure 11, depicts the diversity of chromatographic techniques relevant to polysaccharide analysis.

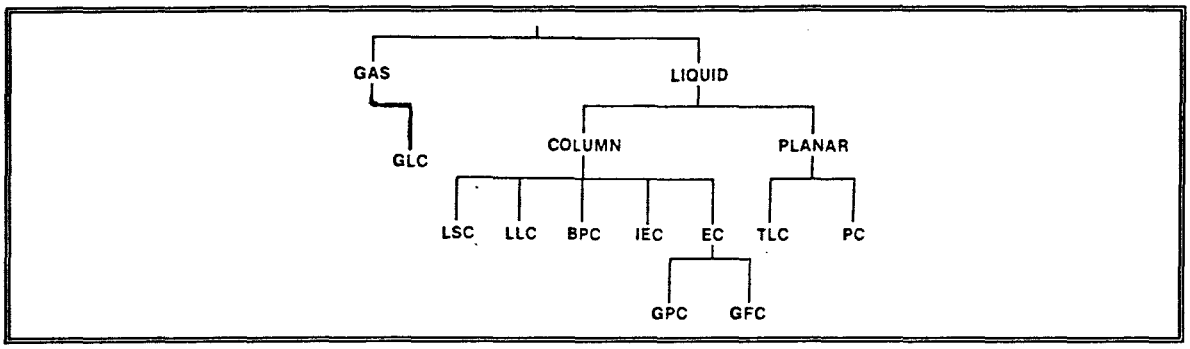


FIGURE 11. Chromatographic techniques

4.1.i. Gas Liquid Chromatography (GLC)

Gas liquid chromatography (GLC) separates complex volatile mixtures by partition between a carrier gas and a liquid stationary phase.

GLC is used to analyse the components of polysaccharides and determines the nature of monosaccharide residues, and their relative ratios. Monosaccharides are non-volatile and must undergo chemical derivatisation to increase their volatility. The volatile form chosen must be stable to the oven temperature of the chromatograph and produce well resolved chromatograms. Bishop[185] (1964) and Dutton[186] (1974) have extensively reviewed GLC (packed column technology), and the applicability of GLC to structural analysis of carbohydrates. Dutton[186] and Drozd[187] have described volatile derivatisation and its utility in polysaccharide analysis. Each derivative has its own nuance and the following are routinely used: trimethylsilyl, acetate, trifluoroacetate, methyl and aldononitrile acetate (PAAN's). Volatile cyclic derivatives[188] produce complex chromatograms because of the presence of the α- and ß-anomers of both the pyranose and furanose forms. Formation of an alditol or acyclic nitrile eliminates the multiplet pattern and produces a single well separated peak for each residue, simplifying the interpretation of the analysis. Peracetylated

aldononitriles[200] (PAAN's) are ordinarily used in the determination of monosaccharide type, and partially methylated alditol acetates[201] (PMAA's) for the determination of methylated polysaccharides. The identity of permethylated alditols should always be confirmed by mass spectrometry. PAAN's are the result of an oxime formation which stabilizes carbonyl compounds prone to producing assymetrical peaks[187]. Permethylated monosaccharides are conveniety analysed as their respective PMAA's by acetylation after hydrolysis of the polysaccharide and the derived alditol acetates[190] give distinct m/z fragments by GLC-MS analysis.

The resolving capacity[190] of a GLC system is dependant on: 1). Column variables of length, internal diameter, and stationary phase film coating thickness. 2). Operational variables of carrier gas make up, gas velocity and column temperature.

At least five types of injector valves are available, *viz.* split, splitless, on-column, direct and programmed temperature vaporising injection. Split injection allows the vast majority of sample injected after flash vaporisation to by-pass the column admitting only nanogram quantities necessary for modern fused silica capillary columns.

Fused silica capillary columns are commonly used because they exhibit inertness to sample and solvent, produce high resolution efficacy and selectivity, and are popularly used in most literature reports. Wall coated open tubular[192] columns, (WCOT) of the OV225 and OV17 types are routinely used to separate derivatised[186] monosaccharides. Differences in polarity between column coatings may be beneficial in cases where co-elution of compounds occurs and changing the stationary phase may allow better separation of the compounds of interest. The internal diameter of a capillary column ranges from 0.05mm to 0.53mm and the stationary phase film thickness from 0.1μm to 8.0μm. Stationary phase coatings are described by 9 different degrees of polarity, e.g. they vary from polymeric OV225 (viscous liquid), through gum-like OV-1 to carbowax 20M, which is solid at ambient temperature.

Disadvantages found amongst WCOT columns are a lack of film capacity for concentrated samples and less effective separation of molecules of low molecular weight. Thicker film coating is available but alternative column designs, *viz.* porous layer open tubular (PLOT), support coated (SCOT) and micro packed capillary columns[192] are

alternative possibilities.

Temperature variation, i.e. programmed temperature GLC[193] (PTGC) is a feature of all modern instruments, allowing software controlled temperature control. An initial temperature is set and throughout the GLC cycle the temperature is incrementally increased over time till a temperature plateau of choice is reached. This is advantageously used in some situations over isothermal setting because it offers a constant peak width throughout the elution, and retention times rise only at a linear rate with respect to increasing carbon number. In the case of *Klebsiella* K43[7], (this thesis) isothermal analysis proved adequate because residues were of the same carbon number and all qualitative work was conducted against standards run under identical conditions.

Five different detector[192] types are available and detector choice depends on the eluting substance. Flame ionisation detection (FID) is commonly used but the presence of helium make up gas decreases its sensitivity. Thermal conductivity detection (TCD) shows greater solution concentration sensitivity and optimal gas velocity near 5ml/minute will enable decreased band broadening. Hence the choice of carrier gas types and velocity of supply are important in optimising detector sensitivity.

Quantitative measurements are achieved by the integration of the area under the peak being assayed, relative to that of a prepared standard which is assigned the value of unity. Qualitative assurance of an eluted peak can be directly or indirectly[193] determined. The indirect method is usually preferred and consists of running a standard solution under precisely the same conditions as that of the unknown solution and the retention volumes are then compared.

Extensive tables[186] of packed column types, various monosaccharide derivatives separated and conditions used are available, but are not directly applicable to capillary columns, although may serve as a guide. Virtually every paper published on polysaccharide structure since the advent of the GLC capillary era uses some GLC application, and often it is used as a convenient technique for pilot scale research before, e.g. NMR samples are generated.

In this laboratory GLC was used for the analysis of the *Klebsiella* K43[7]

monosaccharide ratio and the Smith degradation products by means of the derived PAAN's. PMAA's were used to determine the polysaccharide linkage positions, and separately the absolute configuration of the monosaccharide residues, after polysaccharide hydrolysis and glycosylation with (-)-2-octanol.

Modern extensions to GLC analysis have been coupled instrumentation, *viz*. GLC-MS (mass spectrometry), and GLC-IR. Two dimensional[192] GLC has been used in analytical work where a separated band must be further resolved on another column of different polarity.

GLC is thus a technique of high sensitivity producing excellent resolution of mixed monosaccharide residues and is thus well suited to polysaccharide analysis. It, however, does require that the sample being eluted must be volatile and stable to the temperature of the elution.

4.1.(ii). **Liquid Chromatography (LC)**

Liquid chromatography only imposes a solubility constraint on the sample in a liquid eluent phase. Four basic chromatographic principles may apply at the stationary phase, i.e. adsorption, partition, ion-exchange or gel exclusion. LC may be either carried out by planar or column chromatography. Planar LC encompasses Paper[194,195], Thin layer[194] and Electrophoretic[194] chromatography. The former two techniques have reached maturation in application but are still readily used for quick pilot scale or qualitative separation on the bench, e.g. for determination of the extent of a reaction.

4.1.(iii). Column based liquid chromatography

This describes a range of applications and is distinguished by a stationary phase contained in a column of precise internal diameter and absolute perpendicular length. It is generally of a glass construction or solvent stable polymer material, or stainless steel. Depending on the nature of use, i.e. preparative or analytical scale, the inner diameter ranges from a fraction of a millimetre to centimetres, and length from centimetres to a metre or more. The application of HPLC in the analysis of carbohydrates has been reviewed by Hicks[196].

4.1.(iii).a. High performance liquid chromatography (HPLC)

The use of HPLC [196-198] in separating polysaccharide material has become popular. Instrumentation is cheaper than GLC, choice from a range of chromatographic modes is available, the time to elute a sample is generally short and the volatility of the mixture unimportant since separation is solution dependant.

HPLC is useful in separating small molecules (< 2 000 daltons) and may be carried out by one of many chromatographic[197] modes, *viz.* adsorption, partition (normal or reverse-phase), size exclusion, ion-exchange, affinity etc. and the separation of a mixture is achieved by high pressure (1 to >30 MPa) elution of a liquid mobile phase. It produces quantitative and qualitative data comparable with that of GLC analysis and achieves separation of a mixture without the need for residue derivatisation, although derivatisation can be useful if isomers complicate the chromatogram. Unlike GLC, both stationary and mobile phases can be optimized. Diffusion through the stationary phase may be enhanced by: decreasing the particle size of the stationary phase, increasing the column temperature, making use of a reverse stationary phase coating which increases the stationary phase surface area and changes the surface polarity. The mobile phase may be modified by choosing a solvent of different strength, (increasing the polarity of the mobile phase in an adsorption normal-

phase, or decreasing it in reverse-phase) enabling variable solute dissolution rates and allowing release from the stationary phase to be optimized.

A filtered sample dissolved in mobile phase is injected into a fixed-loop[196] injector, (analytical use) and is carried through a column[196] of typical dimensions (5 μm x 15 cm) which may be housed in an oven. The column may be packed[196] with: amine-modified silica gel, cation-exchange resins, alkylated (reverse phase) silica gel, anion-exchange resin or one of many miscellaneous stationary phase types, e.g. copper(II) silica gel achieves separation by combined ligand-exchange and normal-phase modes. A range of specific and general molecule detectors[199] are available and all require a steady, non-pulsating liquid flow.

The monosaccharide composition analysis of a lithium degraded polysaccharide sample of *Klebsiella* K43[7] (figure 12.), was established by HPLC analysis using a mild cationic-exchange column of TSK Pro-gel C611, using 1 x 10^{-4}M NaOH as the mobile phase and detected by refractive index detector.

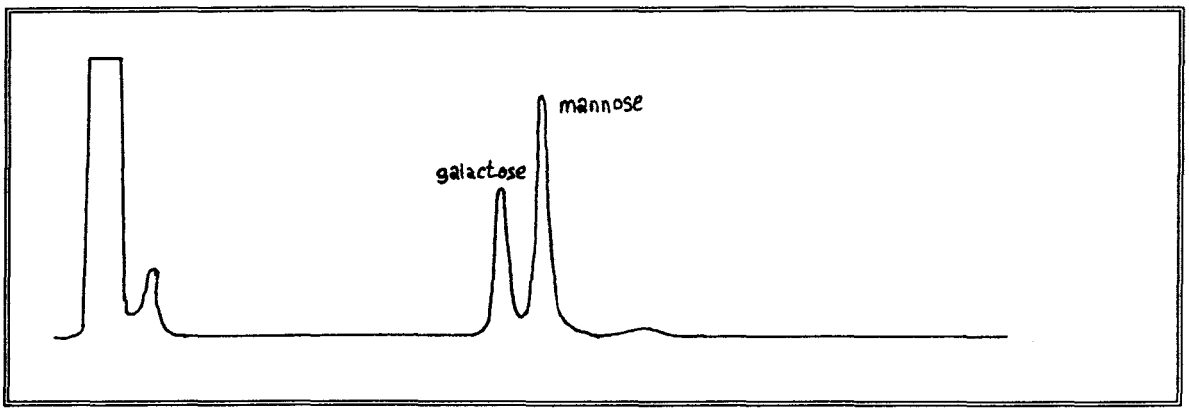


FIGURE 12. Monosacharide analysis of the lithium degraded *Klebsiella* K43 polysaccharide.

Ben-Bassat and Grushka[200] have reviewed operational conditions of column type, mobile phase and detectors used in the HPLC analysis of polysaccharides, e.g. an hydrolysate of bacterial lipopolysaccharide was separated using Nucleosil.5NH_2 eluting with MeCN-H_2O (75:25), and detected by refractive index detector. Hizukuri and Takayi[201] have used a novel method of HPLC detection by combining low-angle-laser-light-scattering photometry and differential refractometry to establish the Mw of eluting oligosaccharide

fractions.

HPLC is playing an increasing role in the elucidation of polysaccharide structure and has several advantages over other chromatographic methods, *viz.* polysaccharides can be analysed directly after hydrolysis as monosaccharides, or small oligomers and thermally unstable molecules are recoverable in the eluent. HPLC runs are fast, non-destructive, reproducible and columns have long life expectancy if used correctly.

4.1.(iii).b. **Size Exclusion Chromatography (SEC)**

Other synonyms describing the same technique are gel filtration chromatography (GFC) and gel permeation chromatography (GPC). SEC is well suited to the separation of polymeric components over a wide dispersion of molecular weights and is dependant solely on the molecular weight and conformational shape of the sample.

Depending on the nature of the stationary phase gel used, polysaccharides of molecular weights ranging from 1×10^2 to 1×10^7 daltons can be separated. Most gels will have a specific weight exclusion range. e.g. Table 4.

Table 4.

GEL FILTRATION

Technical Information - Bio-Gel P Polyacrylamide Gels

Product Polyacrylamide Gels		Applications	U.S. Standard Wet Mesh Designation (Hydrated)	Diameter of Hydrated Beads (Microns)	Fractionation Range* (Daltons)	Hydrated Bed Volume ml/g Dry Gel
Bio-Gel P-6DG	Desalting Gel	Desalting Buffer Exchange	80-170	90-180	1,000-6,000	7
Bio-Gel P-2	Fine	Analytical fractionation	200-400	40- 80	100-1,800	3.5
	Extra fine	Medium pressure fractionations	-400	<40		
Bio-Gel P-4	Coarse	Preparative industrial use	50-100	150-300	800-4,000	5
	Medium	Group separations	100-200	80-150		
	Fine	Analytical fractionation	200-400	40- 80		
	Extra fine	Medium pressure fractionations	-400	<40		
Bio-Gel P-6	Coarse	Preparative industrial use	50-100	150-300	1,000-6,000	7
	Medium	Group separations	100-200	80-150		
	Fine	Analytical fractionation	200-400	40- 80		
	Extra fine	Medium pressure fractionations	-400	<40		
Bio-Gel P-10	Coarse	Preparative industrial use	50-100	150-300	1,500-20,000	9
	Medium	Group separations	100-200	80-150		
	Fine	Analytical fractionation	200-400	40- 80		
	Extra fine	Medium pressure fractionations	-400	<40		
Bio-Gel P-30	Coarse	Group separations	50-100	150-300	2,500-40,000	11
	Fine	Analytical fractionation	100-200	80-150		
	Extra fine	Medium pressure fractionations	-400	<80		
Bio-Gel P-60	Coarse	Group separations	50-100	150-300	3,000-60,000	14
	Fine	Analytical fractionation	100-200	80-150		
	Extra fine	Medium pressure fractionations	-400	<80		
Bio-Gel P-100	Coarse	Group separations	50-100	150-300	5,000-100,000	15
	Fine	Analytical fractionation	100-200	80-150		
	Extra fine	Special applications	-400	<80		
Bio-Gel P-150	Coarse	Group separations	50-100	150-300	15,000-150,000	18
	Fine	Analytical fractionation	100-200	80-150		
	Extra fine	Special applications	-400	<80		
Bio-Gel P-200	Coarse	Group separations	50-100	150-300	30,000-200,000	25
	Fine	Analytical fractionation	100-200	80-150		
	Extra fine	Special applications	-400	<80		
Bio-Gel P-300	Coarse	Group separations	50-100	150-300	60,000-400,000	30
	Fine	Analytical fractionation	100-200	80-150		
	Extra fine	Special applications	-400	<80		

Extending the weight exclusion range for a sample with complex molecular weight distribution can be achieved by coupled column[203] SEC.

Some gel types[194] include Sephadex, Agarose, Porous glass and Styragel. At a molecular level the gel consists of a spider-web of generally polar groups which are capable of adsorbing water and other polar solvents. Gel swelling results and an expanded matrix of a specific particle size exclusion limit is generated. Elution of molecules larger than the pore diameter occurs with the column void volume. Molecules within the exclusion limit of the polymer gel diffuse into the pore cavity. Fractions of decreasing molecular weight diffuse out of the gel matrix as elution takes place, and the equilibrium of that fraction within the gel decreases. Smaller particles with less kinetic energy take longer to escape the matrix and their residence is longer. Molecular weights lower than the exclusion limit are found at the total permeation volume as an unresolved mixture[194].

Detection of compounds eluting from the column can be achieved by differential refractometry or by a method such as the phenol-sulphuric acid method[204].

SEC is useful in the separation of biological samples and may be used to determine the relative molecular weight of an oligo- or polysaccharide. It is a gentle process which can be carried out under physiological conditions of buffered pH and temperature, and has been used for the quantitative estimation of carbohydrates, i.e. Mw determination. It is simple, cheap and easy to perform giving reproducible results, and successive runs are achieved without the need for regeneration of the gel. The "softness" of the technique allows the true biological nature of a polysaccharide to be recorded. *Klebsiella* $K43^7$ (this thesis) has revealed that $K43^7$ naturally occurs as an aggregate of Mw $1{,}047 \times 10^7$, whilst the de-ionised polysaccharide had a relative Mw of $6{,}026 \times 10^4$. Churms *et al.*[166] gave further credibility to the concept of the bacterial polysaccharide repeating unit, by using SEC to separate the repeating unit and multiples thereof from an enzyme digest of the polysaccharide. They also noted the occurrence of the aggregation phenomenon.

Dulan[203] has described problems encountered by secondary retention effects found in SEC. He has also described techniques used to optimize injection volumes, and practical

sample concentration limits which ensure that column overload does not occur. Churms[205] (1970) and Whistler *et al.*[206] (1980) have reviewed the technique with reference to gel types and their respective utility to polysaccharide separation, relative molecular weight calculations, and column effects[206] experienced in unsilanised narrow columns, detector types etc.

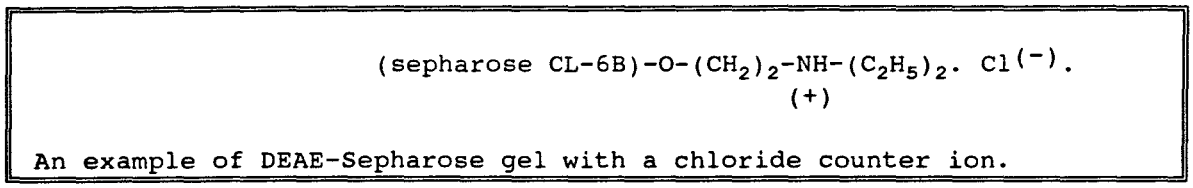
Grellert *et al.*[207] found that derivatising a monosaccharide into a methylglycoside increases the apparent retention volume by 150% and this effect was seen to be cumulative. It should be noted that apart from the sensitivity of this technique to methylated monosaccharides, comparative reference standards should approximate the type of derivatisation and shape[208] of polysaccharide material to be separated. This is especially important where a relative molecular weight determination is carried out relative to standard sugars.

Size exclusion chromatography is a most useful technique for biological polysaccharide research and has been used in this thesis to separate enzyme degraded repeating unit material, Smith degradation products and the broad molecular weight distribution of main chain oligosaccharides from a lithium degradation, along with the relative molecular weight determination of different forms of the *Klebsiella* K43[7] antigen.

4.1.(iii).c. Ion-exchange Chromatography (IEC)

Ion-exchange chromatography[194] should not be confused with ion exclusion chromatography. Ion-exchange chromatography separates molecules according to their relative difference in ionisation state and is useful for purifying the acidic bacterial polysaccharides. The instrumentation and chromatographic equipment used is similar to that in size exclusion chromatography. Two differences exist: the detection is best undertaken by means of conductivity detection and the gel used is of a different type. The stationary phase ion-exchange gel relies on the ion-exchange capability of ionised functional groups located in the cross linkages of the polymeric gel. The gel has a degree of polarity and

swells on solvation. Diethylamino ethyl (DEAE) is an example of a common anionic exchange functional group and the counter ion may be, e.g. chloride.



An example of DEAE-Sepharose gel with a chloride counter ion.

The chloride ion may be exchanged for any monovalent radicle in solution, thus maintaining the neutrality of the complex. Figure 13. clearly depicts the multi-step process of application, selective adsorption followed by selective desorption by an eluent of increasing ionic gradient.

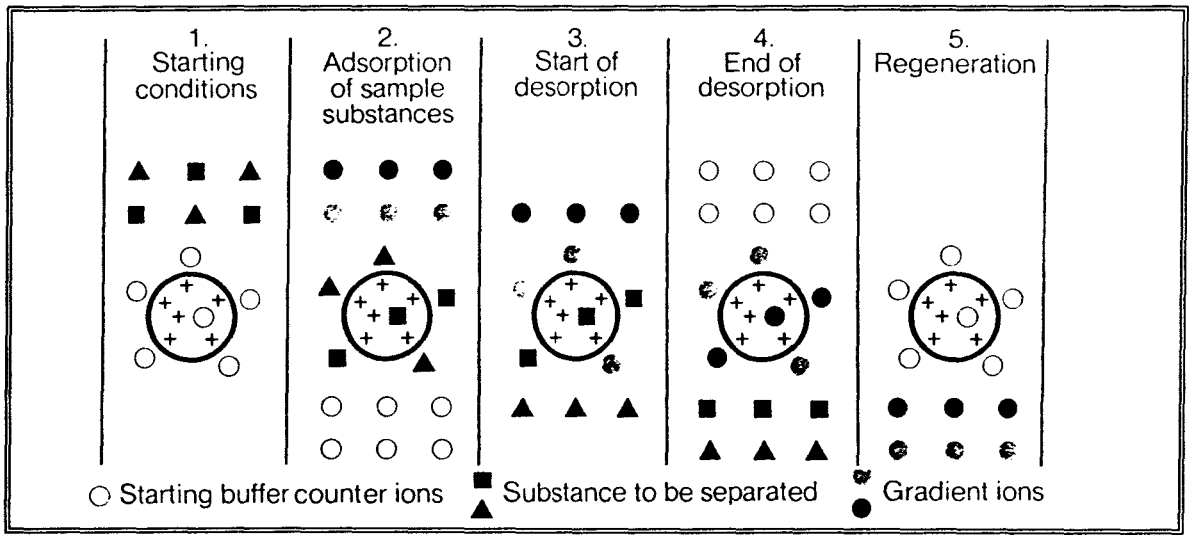


FIGURE 13. The mechanism of ion-exchange chromatography

Other adsorption interactions[209] contribute insignificantly to the principle described. Important factors when deciding on an appropriate resin include the following: size of gel particles (governs the rate of ion-exchange and sample permeation), degree of cross linkage (governs the rigidity of the support and swelling capability); functional group type (describing the type of ion-exchange capability); strength of the functional group (describing the distribution coefficient) and the number of functional groups (describing the resin capacity).

Varying the pH of the eluent phase changes the degree of ionisation experienced by the species being separated. This is advantageously used to find a buffer pH causing optimal adsorption / desorption of the species to be separated. A gradient mixing device[209] can be used to increase the ionic strength of the buffer chosen over time, and enables selective desorption of the ionically bound sample. This technique has been used to separate the acid polysaccharide antigen of *Klebsiella* K21[210], K32[210] and K26[211] from the contaminating neutral O-antigen polysaccharide.

Detection of carbohydrate has historically been conducted by detection of a colour change reaction, e.g. the Phenol-sulphuric acid test[204], although the efficiency of another non-corrosive reagent[212] has been published.

IR-120 H^+ proton exchange resin is used in this laboratory to de-ionise oligosaccharide material prior to and after work up procedures. Special utility was found in the removal of lithium ions from the polysaccharide of *Klebsiella* K43[7], after the lithium degradation.

Ion-exchange chromatography is important for the purification of contaminated bacterial polysaccharides. The technique is relatively quick, extremely sensitive in discriminating between similar ionic environments, and is economical as the resin can normally be regenerated by washing it with an electrolyte solution of appropriate polarity and counter ion concentration.

4.1.(iii).d. **Affinity Chromatography (AC).**

Affinity chromatography falls into the domain of the biochemist and is commonly employed in the separation of enzymes but has also been applied to purifying polysaccharides[213]. An analogy to ion-exchange chromatography might be drawn but instead of a specific ion, a biological ligand is covalently bound to a support and brings about the most specific reversible binding of a molecule.

Scouten[213] has concisely described the application and advances in this specialised

field, and Wood *et al.*[214] applied AC to separate oligosaccharide fractions in high yields by using an anti-I blood group immuno-adsorbent ligand.

4.2. Mass Spectrometry

4.2.(i). Introduction

Mass spectrometry (MS) is widely used in the structural determination of bacterial polysaccharides and is a highly selective, sensitive technique, e.g. 30 femto mole of a methylglycose containing-polysaccharide (M_w 3515 daltons) has been determined[215]. Often MS is the only technique sensitive enough for determining the minute quantities of polysaccharide of biological origin. Information obtained from MS techniques[216] which is useful in the structural determination of polysaccharides is the assignment of the position of linkages, determination of the reducing end monosaccharide, molecular weight determination, the assignment of an oligosaccharide with respect to the linear arrangement of monosaccharides if of different molecular weight, and the location of branching structures along a polysaccharide chain. In 1966 Kochetkov *et al.*[217] reviewed the field of MS and described fragmentation patterns produced by carbohydrate molecules.

A sample may be introduced into a spectrometer by a variety of methods. Some inlet systems used include the cold or heated inlet, the direct insertion probe and a range of chromatographic inlets. An inlet system, eg. a GLC, is interfaced[218] with the mass spectrometer and the choice of interface is dependant on the nature of the sample, the degree of volitility and sample volme. After recieving the sample from the interface, the mass spectrometer must perform three distinct functions, ie. the production, separation and detection of ions.

The production of ions is normally achieved via one of three modes (discussed over leaf) and the resulting ions are separated by an analyser, which separates ions according to their mass. A total ion chromatogram is produced which displays each fragment separated as a function of its mass-to-charge ratio versus its relative ion abundence. A characteristic

pattern is produced in the total ion chromatogram and the presence (or absence) of ions with a particular mass-to-charge ratio and, where present, their relative intensities enable structural determination.

The usage of HPLC-MS and GLC-MS in research and development environments has been documented[219]. MS systems operate under high vacuum and when coupled to capillary GLC columns, do not require a carrier gas separator. The interface is heated to 25°C above the column temperature and the detector is set at ~ 250°C, thus minimising degradation of thermally labile oligosaccharides and their volatile derivatives. Ionisation of the eluent can be finely controlled enabling detection of different positions on the eluting peak, allowing analysis of peak homogeneity.

Polysaccharides have conventionally been analysed as their derived volatile oligosaccharides or monosaccharides, e.g. PMAA's because of their acyclic form, volatility and straightforward fragmentation pattern. However these PMAA's do not allow for differentiation between stereo-isomers, e.g. 2,3-di-O-methyl-1,4,5,6-tetra-O-acetylgalactose and its manno-isomer show the same fragmentation pattern, but comparison of their GLC retention times enables their identification. Kovacik *et al.*[222] have demonstrated that glucuronic acid can be differentiated from galacturonic acid by the relative intensity responce of the derived PMAA's, but this may be regarded as an exception to the general rule.

A REVIEW OF MASS SPECTROMETRIC IONISATION MODES.

4.2.(ii). Electron ionisation mass spectrometry (e.i-MS)

This technique is considered a harsh ionisation method because of the high energy used and it produces primary fragments of an oligosaccharide or monosaccharide which may be further ionised forming secondary fragments. Large data bases of PMAA's GLC retention times and MS ion fragmentation patterns are available, and serve to finger-print compounds.

Partially methylated alditol acetates undergo specific fragmentations, producing characteristic fragments of the molecular ion. The molecular ion[220] is seldom seen because

of the low degree of secondary fragmentation which occurs. The molecular ion undergoes primary fission[221] between carbon atoms of the monosaccharide chain and in order of decreasing abundance, fission between two methoxylated carbons, is greater than that between methoxylated and acetoxylated carbons, which is greater than that between two acetoxylated carbons (Figure 14.).

Methoxylated ions give the most intense or abundant ion response and acetoxylated ions the weakest. Careful interpretation of a total ion chromatogram can supply information necessary to construct the alditol present from first principles.

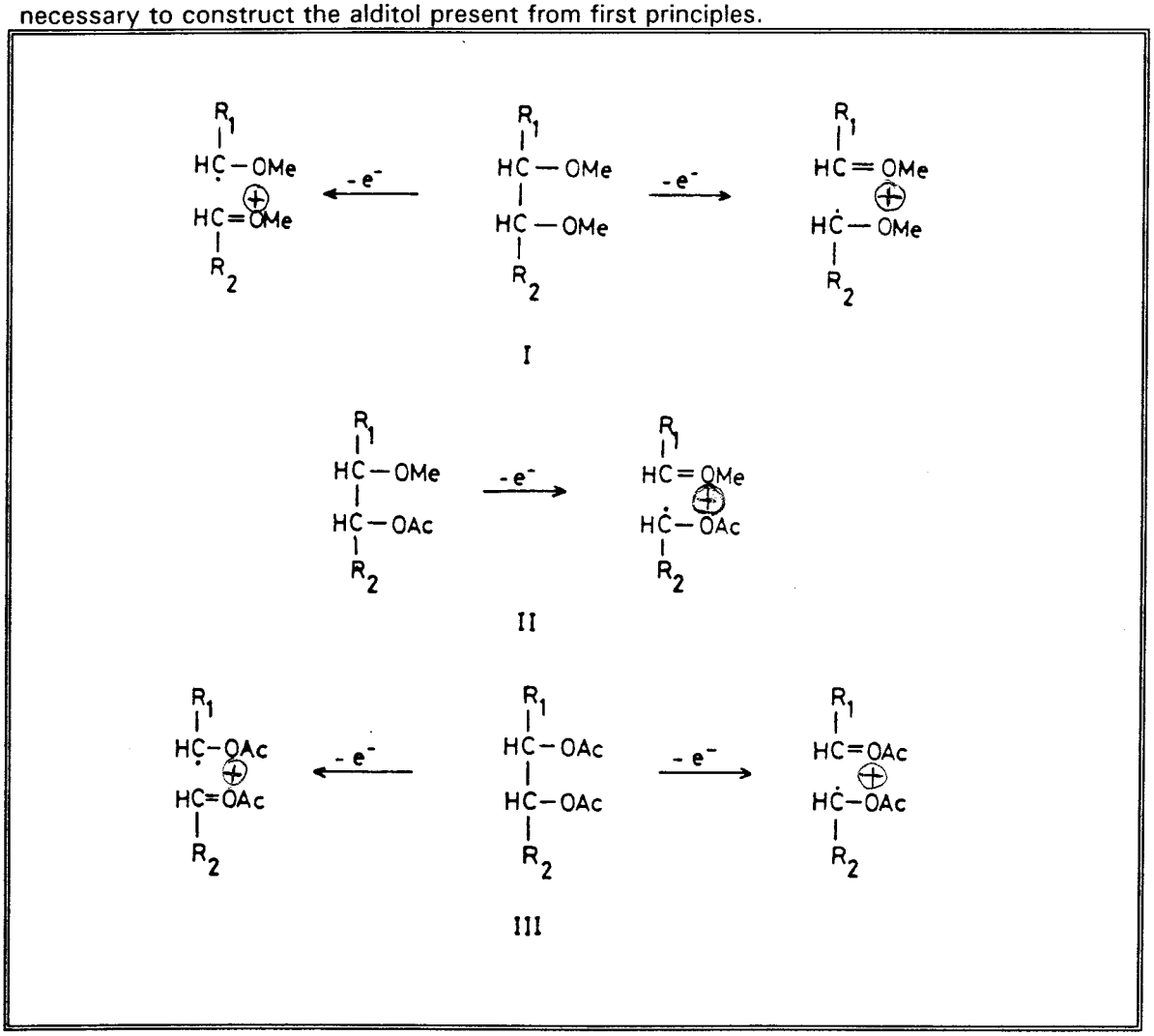


FIGURE 14. Primary ion fission

Acetic acid	(60 mass units mu)
Methanol	(32 mu)
Ketene	(42 mu)
Formaldehyde	(30 mu)

FIGURE 15. Secondary fragments observed:

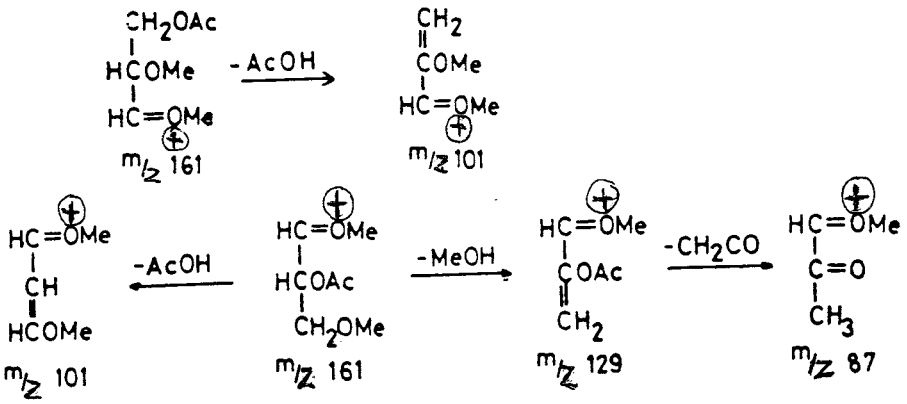


FIGURE 16. The secondary fragments observed from a primary fragment of (m/z) = 161

The use of deuterium labelling by $NaBD_4$ reduction of an oligosaccharide is indispensable in determining the reducing sugar and is useful in identifying linkage points. Björndal *et al.*[223] have discussed the application of deuterium labelling and the resultant change in ion species formed, whilst Kärkkäinen[224] has similarly reported the identification of (1→3) and (1→4) linkages at the reducing monosaccharide by $NaBD_4$ reduction and analysis of the derived PMAA's of 21 different methylated trisaccharides .

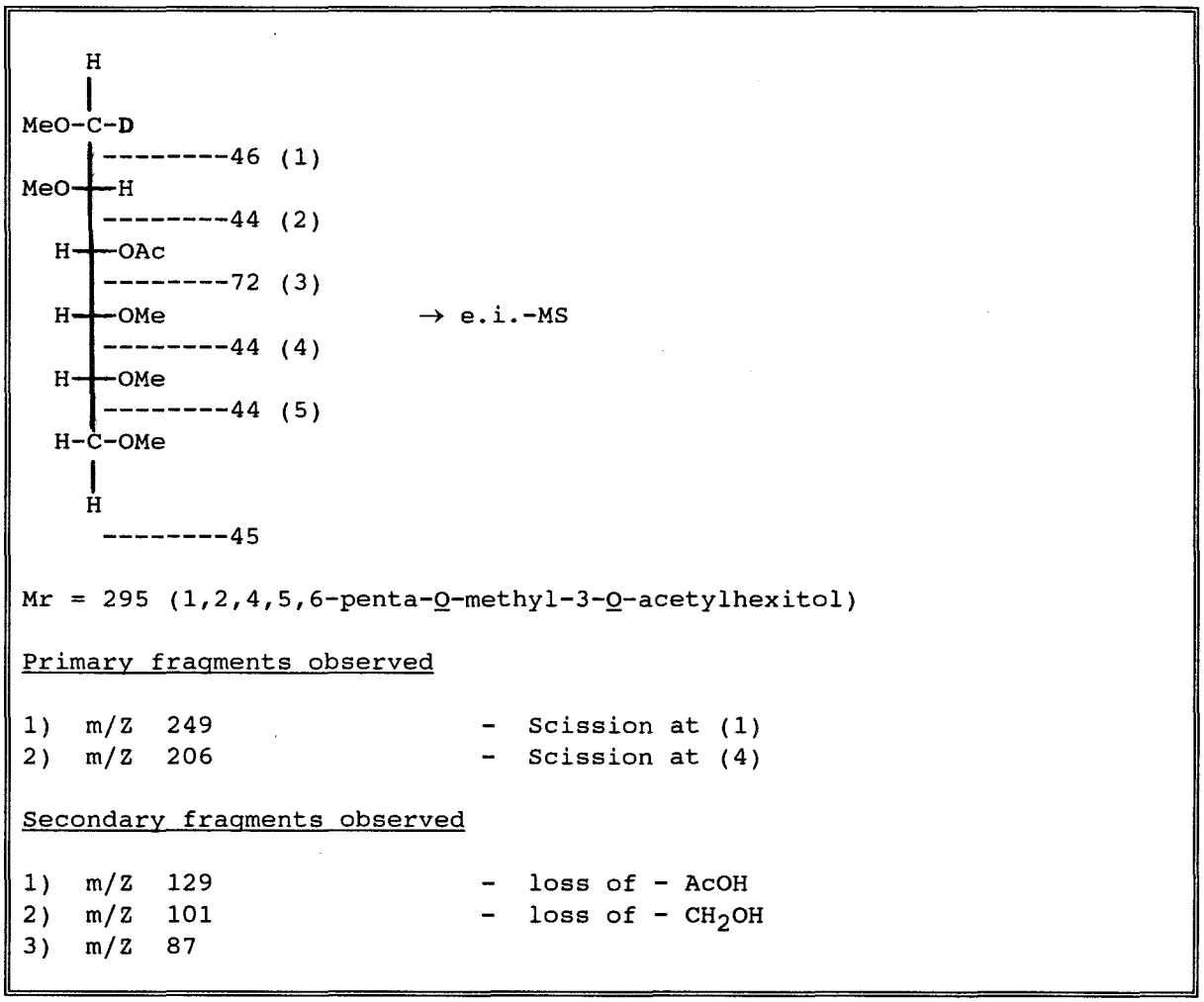
The reducing monosaccharide of the repeating unit (P1) of the *Klebsiella* K43[7] oligosaccharide was identified by $NaBD_4$ reduction of P1, followed by methylation,

hydrolysis, reduction and analysis of the derived PMAA's. A 1,2,4,5,6-penta-O-methyl-3-O-acetylhexitol was observed in the total ion chromatogram and was identified as a pentamethylated galactitol by retention time comparison to an authentic standard in GLC.

The identification of a m/z fragment of 206 and not 205 demonstrated that the C-1 of the galactose had incorporated deuterium, see Figure 17.

FIGURE 17.

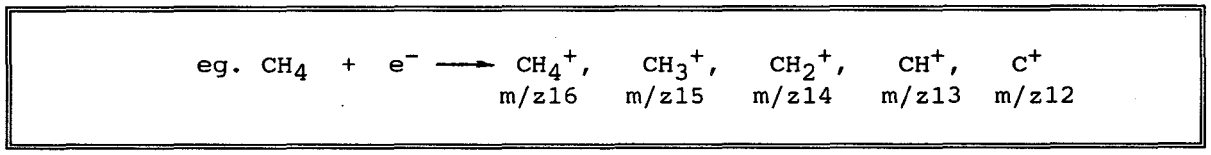
An e.i.-MS study on an isolated 1,2,4,5,6,-penta-O-methyl-3-O-acetylhexitol from the deuterium reduced *Klebsiella* K43 P1.



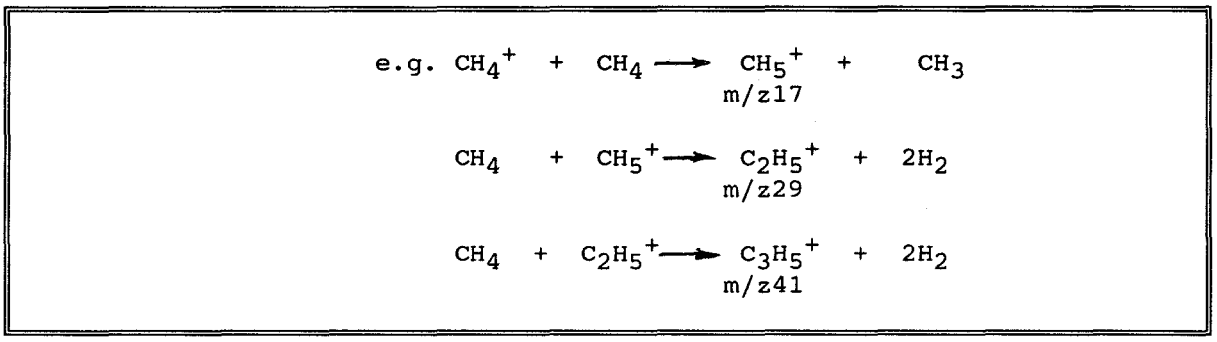
4.2.(iii) Chemical ionisation mass spectrometry (c.i.-MS)

This technique is considered to be a softer method of sample ionisation than e.i.-MS and allows the maintenance of the molecular ion (M^+). A less complex total ion chromatogram results due to less degradation of ionised species and c.i-MS has been reviewed by Munson[225,226]. An ion-molecule interaction occurs in the production of a c.i.-mass spectrum and the production of molecular weight ions enables abundant molecular ion and primary ions to be observed.

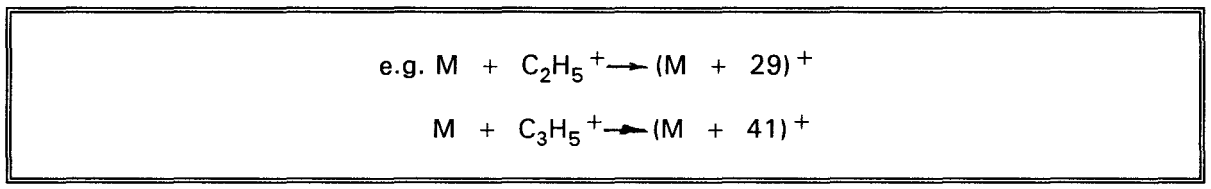
C.i.-MS total ion chromatograms are acquired by a two step mechanism[218]. An ionising reactant gas, e.g. methane, is ionised by e.i.-MS which produces the molecular ion of methane and other lesser ion fragments.



These species enter a chamber of lower vacuum allowing ion-molecule or ion-(reactant gas-molecule) reactions to occur.



The sample being analysed is bombarded by a beam of these high molecular weight ions and on collision, the sample is converted to a protonated positive ion, i.e. $(M + CH_4^+) - (M + 1)^+$. Adjunct ions are formed when less abundant ion species, i.e. m/z 29 and 41 successfully ionise the sample,



Hancock *et al.*[227] have used c.i.-MS in the assignment of O-acetyl-O-methylalditols

from methylation analysis and have reported that the $(M + 1)^+$ ion is helpful in solving the ambiguities found in the e.i.-MS total ion chromatogram. McNeil *et al.*[228] reviewed the utility of HPLC-c.i.-MS in elucidating the structure of oligosaccharides. Interestingly the HPLC solvent, after volatilization can be used as a reactant gas, permitting direct analysis of the HPLC eluent of derived PMAA's oligosaccharides. They were also able to use different concentration gradient mobile phases and analyse the eluent purity, and establish the presence of particular peracetylated oligosaccharides alditols in the HPLC eluent by selective reconstruction of ion chromatograms.

The molecular ion[215] (M + 1) is invaluable in the determination of sample molecular weight. Although c.i.-MS has advantages over e.i.-MS, the possibility of, e.g. proton abstraction $(M - 1)^+$ and charge transfer reaction, e.g. the loss of water $[(M + 1) - 18]^+$ must always be considered in the interpretation of spectra. The molecular ion may be generated by, e.g. field ionisation[229], field desorption[230,231] but each imposes prerequisites on the form of the sample, and in particular field desorption involves complicated sample preparation.

4.2.(iv). **Fast Atom Bombardment Mass Spectrometry, (f.a.b.-MS)**

F.a.b.-MS has proved invaluable in the elucidation of complex polysaccharides over the past 13 years and is the "softest" fission method used. Dell[231-233] and co-workers have periodically reviewed f.a.b.-Ms methods and through their efforts to develop special applications for the technique, have moved the frontiers of f.a.b-MS forward.

F.a.b.-MS maintains the molecular-ion in the total ion chromatogram but does not impose the need to produce a gaseous sample prior to ionisation, thus enabling analysis of liquids and solids. It is useful in the determination of thermally labile, non-volatile oligosaccharides and small polysaccharides, and is able to determine their molecular weight. Molecular weights are not artificially increased and because multi-fragmentation processes are not observed, direct characterisation of oligosaccharide mixtures[234] is possible. Both derivatised and underivatised samples can be analysed and spectra may be acquired in the positive or negative ion modes. F.a.b.-mass spectra of lower homologue oligosaccharides

have been analysed at ambient temperatures[231] but the O-acetylated oligosaccharides require higher temperatures.

A f.a.b.-mass spectrometer consists of a cold cathode discharge ion-source producing argon$^+$ ions of kinetic energy, 1-8 keV. Spluttering of ions by fast ion bombardment[235,236] (SIMS) is used to overcome the inadequacies of e.i.- and c.i-MS ionisation, and produces both negative and positive ion mass spectra. Problems encountered[237] in f.a.b.-MS are overcome by a beam of neutral atoms used as "spluttering" medium. The neutral argon atom beam is generated by the passage of the above argon$^+$ ions through a collision chamber of argon gas. The emitted beam contains argon atoms of higher velocity to that of the ions which allows ion spluttering from the sample, i.e. $(M + H^+)$ and $(M - H^+)$ ions are detected.

The sample is deposited on a stage and a matrix of glycerol, thioglycerol or 3-nitrobenzyl alcohol[238], depending on the sample character, is used, and is irradiated with high velocity argon atoms.

Polysaccharide molecular ion species may be stabilised by the addition of an alkali ion, e.g. sodium or potassium. Bosso[234] has reported MS analysis of underivatised oligosaccharide material by positive recording mode, e.g. $(M + H^+)$ and $(M + Na^+/K^+)$. Use of the negative mode molecular anion $(M - 17)^-$ was useful for acylated or charged acidic polysaccharides and a range of adduct ions, e.g. NH_4, Cu, Cl were used. The choice of adduct was found to be important for accurate determination of pseudomolecular ions. Dell[233] has reported the use of negative ion spectra which enables the elucidation of fragments from the non-reducing end of an oligosaccharide. These molecular weight ions are indicative of the sequential structure of the material, but are only useful where the polysaccharide is composed of sugars of different sizes. Dell[253] has also determined the molecular weight of *Klebsiella* polysaccharides (unspecified serotypes), and the degree of acetylation from derived permethylated and peracetylated material of 0.5 - 1.0μg. Molecular weight calculation of material > 10 000 daltons was also determined from the non-reducing end and structural elucidation performed.

Pappa *et al.*[238] have found that peracetylated polysaccharides produced superior

scission of the carbon-oxygen bonds of the backbone structure of polysaccharides and the use of f.a.b.-MS enabled both internal and external linkage scission. They also report the use of $NaBD_4$ reduction of the polysaccharide for the detection of naturally occurring hydroxyl groups, from those introduced by periodate oxidation performed on this material.

The mode of MS ionisation used is therefore dependent on the information required, and the quantity and properties of the carbohydrate under investigation. The use of e.i.-MS might complement other techniques where secondary scission is required, but interpretation of data necessitates a thorough understanding of the ion fragments produced and their natural abundance.

4.3. Nuclear Magnetic Resonance Spectroscopy (NMR)

4.3.(i). Introduction

NMR spectroscopy is the most powerful spectroscopic method for complete structural analysis of polysaccharides. Information obtained from an NMR experiment has been compared analogously to information obtainable from a person standing on a proton and looking about this position, seeing structural features, shadows and reflections of distant features.

The structural analysis of small molecules was initially conducted in the continuous wave mode (CW). However, multiple pulse-Fourier transform NMR spectroscopy (FT-NMR) is now routinely used and can achieve complete structural and conformational elucidation of complex macromolecules.

A modern spectrometer consists of a stable super-conducting magnet, a detection probe, e.g. ^1H-probe, and a radio frequency (rf) generating system coupled to a dedicated computer which controls the acquisition cycle, and the manipulation of digital information.

4.3.(ii). One Dimensional Spectroscopy (1D NMR)

In contrast to continuous wave NMR, multiple pulse Fourier transform experiments[239] stimulate a specific range of frequencies within the sample by the induction of a pulse(s) of rf energy, and the response of the system is measured as a function of time by a digital computer. The time domain is converted mathematically by the Tukey-Cooley Fast Fourier Transformation[240] producing a frequency domain f_1. After a sensitive nucleus aligns itself along the induced magnetic field, the magnetisation can be perturbed by a second field oscillation at an appropriate rf dependent on the pulse program software used, and the signal decays by free induced decay (FID). All 1D NMR acquisitions are determined by three component parameters, (Figure 18.).

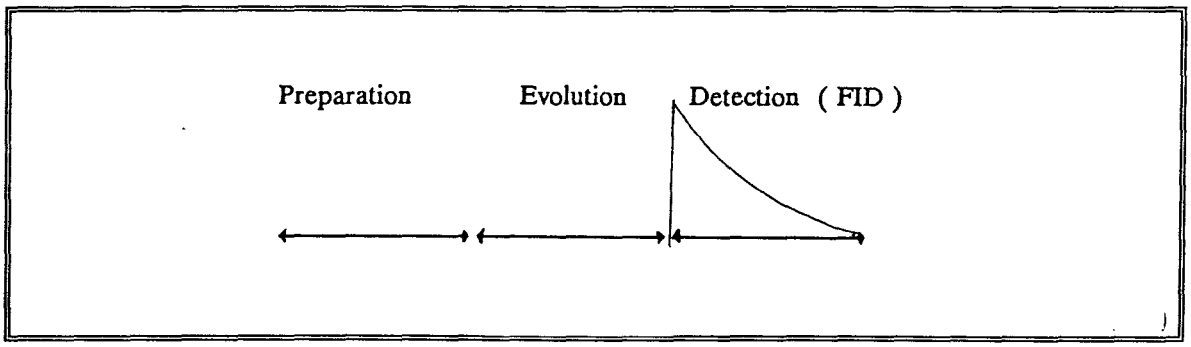


FIGURE 18.

The evolution time in a pulse FT-NMR experiment is that variable which enables the generation of useful information via a second induced rf, i.e. it provides an opportunity of refocussing the state of the spin system before detection, allowing improved spectral[241] resolution.

4.3.(ii).a. 1D ^{1}H-NMR spectroscopy of bacterial polysaccharides.

A 1D ^{1}H-NMR spectrum produces the following structural information: the monosaccharide type, anomeric form and in some cases coupling constants for some

resonances in the anomeric region. The use of certain 1D NMR pulse programs, e.g. HOHAHA[239] and INEPT[241] help to simplify 1D NMR spectra enabling chemical shifts to be identified unambiguously. The structures of small molecules are usually elucidated solely by 1D NMR, but bacterial polysaccharides usually require a variety of 2D NMR experiments to assign the sequence and linkage points, due to the heavy overlap of resonances in the ring region, i.e. H-2,H-3, H-4 etc. *Klebsiella* bacterial polysaccharides show a high degree of viscosity. Spin-spin relaxation[242] times are shortened as the viscosity of the sample increases and broad spectral lines develop leading to poor spectral resolution. Increasing the sample temperature can marginally improve resolution and for this reason 1D NMR spectra of the native polysaccharide serve mainly to determine the type and number of anomeric linkages, (Figure 19)

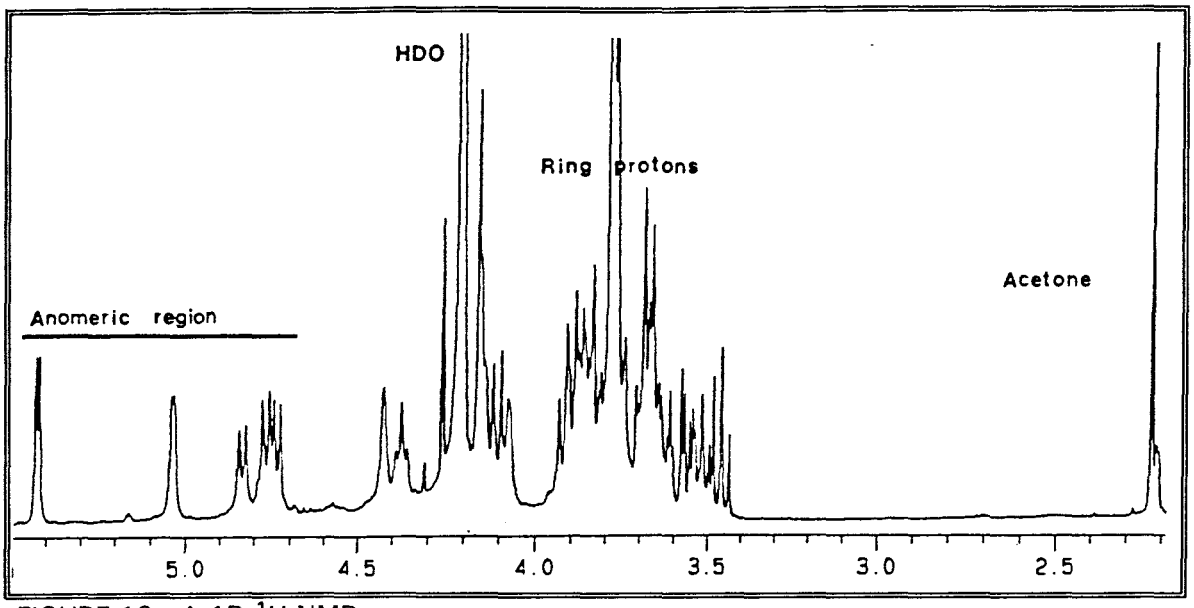


FIGURE 19. A 1D ^{1}H-NMR spectrum

Polysaccharide depolymerisation using a bacteriophage, usually produces an oligosaccharide chemical repeating unit, the spectra of which are well resolved and easier to analyse than those of the polysaccharide. However the effects of the mutarotation of the reducing end monosaccharide can complicate the spectra. Conversion of the reducing end to the alditol usually simplifies the spectrum and simultaneously identifies the reducing monosaccharide.

Tetramethylsilane (TMS), assigned a chemical shift of 0.00 ppm, is not used as an internal standard in polysaccharide spectroscopy because it is not water soluble. ^{1}H-NMR studies on polysaccharides use acetone, resonating at δ 2.23 as the internal standard. Figure 19. depicts the various proton chemical shift regions of carbohydrates. Non-carbohydrate appendages are also detected, e.g. the CH_3 of O- and N-acetate resonate as singlets at δ 1.92 - 2.05 and pyruvate at δ 1.45-1.6.

Assignment of some residues by comparison with methyl glycosides[243] is possible, but it should be noted that chemical shifts are dependent on solvent used, and the temperature of acquisition. Table 5. lists chemical shifts and coupling constants for a few methyl glycosides and that of galactitol.

Table 5.

Standard D-Hexopyranoside methyl glycoside[243] NMR shift values in ppm.

		Proton or Carbon						
		1	2	3	4	5	6a	6b
α-Gal	H	4.84	3.83	3.79	3.79	3.89	3.78	3.72
	J	3.0	9.8	2.3	1.0	-----	8.2/ 12.0	4.6
	C	100.1	69.2	70.5	70.2	71.6	62.2	
ß-Gal	H	4.31	3.50	3.64	3.92	3.68	3.80	3.75
	J	8.0	10.0	3.8	0.8	-----	7.6/ 11.2	4.4
	C	104.5	71.7	73.8	69.7	76.0	62.0	
ß-Man	H	4.58	3.99	3.64	3.57	3.38	3.94	3.74
	J	0.9	3.2	10.0	10.0	-----	2.2/ 12.2	6.7
	C	101.3	70.6	73.3	67.1	76.6	61.4	
α-Man	H	4.77	3.93	3.76	3.64	3.62	3.90	3.76
	J	1.6	3.5	10.0	10.0	-----	1.9/ 12.0	5.8
	C	101.9	71.2	71.8	68.0	73.7	62.1	
ß-Glc	H	4.38	3.26	3.49	3.38	3.47	3.93	3.57
	J	8.2	9.6	9.6	9.6	-----	2.4/ 12.8	6.4
	C	104.0	74.1	76.8	70.6	76.8	61.8	
Galactitol	C	64.5	71.5	70.7	70.7	71.5	64.5	

If coupling constants are available, residue identification can be made from first principles because these values are independent of solvent or temperature of the experiment. The coupling constant for H-1 and H-2 protons when large ($J_{1,2}$ ~ 6-8 Hz) is indicative of a trans-diaxial orientation, i.e. in gluco- and galacto- types this is indicative of a ß-linkage. Small J values ($J_{1,2}$ 1-4 Hz) confirm an α-linkage with small dihedral angle. Mannose residues because of their proton stereochemical configuration, give J values ($J_{1,2}$ 1-4Hz) for both anomers and little information is gleaned. 1D ^{1}H-NMR also allows the establishment of ring size, *viz.* the anomeric signal for α-pyranose residues is found at (δ 5.0 - 5.5) with a small J value, and ß-pyranose residues at (δ 4.5 - 5.0) with a large J value, while the anomeric signal for α-furanose residues resonates further downfield than their pyranose forms.

The polyhydroxyl nature of a monosaccharide produces a complicated ^{1}H spectrum, which can be simplified by exchanging a reactive hydroxyl proton with deuterium[245] as described in the experimental. This removes the hydroxyl proton signal, and a monosaccharide will show seven protons and an uronic acid five protons. A residual water peak, (HOD), can be observed in the anomeric region and is often superimposed on anomeric proton resonances. Varying the temperature usually shifts the HOD peak adequately to uncover the hidden resonances. Vliegenthart *et al.*[246] have described the application of 500 MHz 1D ^{1}H-NMR spectroscopy in the determination of the primary structures of polysaccharides and have emphasised the utility of structural reporter groups, i.e. signals of individually resonating protons. At this point the purity of the material, the need to depolymerise the polysaccharide, and the need for extra NMR experiments will be evident from the 1D ^{1}H-NMR spectrum.

1D Homonuclear Hartmann-Hahn Spectroscopy (HOHAHA)

The HOHAHA 1D-NMR pulse sequence has been used by Bax[247] in determining 1D NMR coupling constants after proton chemical shift assignment. It becomes useful in the determination of coupling constants of non-anomeric protons. Sub-spectra of a relayed spin

system are formed by transfer of magnetisation through the related spin -spin system by the HOHAHA pulse sequence. Morris and Freeman[248] have reported the use of the (INEPT) pulse sequence. Polarization transfer is non-selectively achieved and after the application of a 90° pulse, a characteristic spin system inversion results.

4.3.(ii).b. **1D ^{13}C-NMR Spectroscopy**

Once the 1D ^{1}H-NMR spectrum has been assigned as completely as possible, the next useful nucleus with / = (1/2) is ^{13}C, but its low natural abundance of 1.1% imposes a constraint on its detection and a large sample mass is needed. The spin-lattice relaxation rate is also longer than that of the proton nucleus and the acquisition time is longer than the 1D ^{1}H-NMR.

Broad band decoupling[239] has been used to increase the sensitivity of ^{13}C detection, i.e. by removing ^{1}H-^{13}C spin-spin coupling, detection of ^{13}C is achieved with greater sensitivity by means of the nuclear Overhauser effect. Sadler[249] has given a detailed account of the various ^{13}C pulse programs used in the acquisition of resonances.

1D NMR ^{13}C spectra[250] are simple in comparison to ^{1}H spectra because the low abundance of ^{13}C nuclei do not produce coupled signals, and polysaccharide chemical shifts are shifted over a range of (15-185 ppm). Figure 20. details regions of the ^{13}C spectrum similar to those seen in the ^{1}H spectrum, i.e. anomer region etc. A carbonyl signal corresponding to the uronic acid carboxylic acid is found far down field (160-180 ppm) and the internal standard acetone resonates at 31.07 ppm Non-carbohydrate appendages, e.g. CH_3 of acetates resonate upfield at (23-25 ppm). Anomeric signals are of strong intensity and α-pyranose residues are found at (95-100 ppm), while ß-pyranose residues are found at (100-105 ppm).

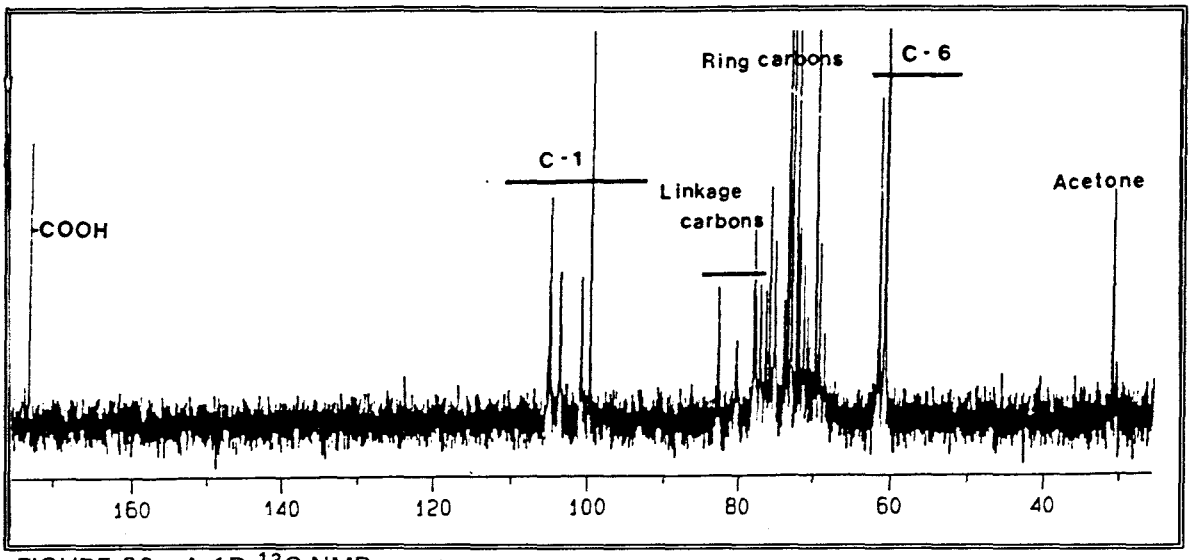


FIGURE 20. A 1D ^{13}C-NMR spectrum

^{13}C-NMR spectroscopy[251] allows identification of the anomeric configuration of the component monosaccharide residues, points of linkage and conformation. Use of this data in assigning residues is more discriminating and reliable than using the proton chemical shifts. Interpreting ^{13}C-NMR shift data by comparison with published ^{13}C-NMR chemical shift data is especially pertinent to determining linkage points and a pronounced downfield (α-effect) and upfield (ß-effect) is observed[251,252]. Carbons substituted by glycosyl residues shift downfield by (4-10 ppm), and the adjacent resonances are expected to move upfield but by ~ 1.5 ppm

Berry *et al.*[253] were the fore-runners in the use of ^{13}C-NMR in the structural elucidation of the capsular polysaccharide antigens, *viz. Klebsiella* K5, K36, and K70. Anomeric linkages were determined and the experiment was run at 35°C.

Cumming[254] used the predictability of the ^{13}C anomeric resonance (α-or ß-effect) for producing a Fortran Source code program, enabling structural analysis by relative best fitting of experimental data to a data base. Interestingly the program generates possible linkage permutations, branching combinations etc, and should the array of data generated be identical to the experimental data, a structural match is achieved. Janssen *et al.*[255] have reported the use of "CASPER", a software package which allows the elucidation of oligosaccharides from ^{13}C-NMR data in tandem with sugar and methylation analysis.

Another research group, Hounsell *et al.*[256] have constructed an extensive data base of oligosaccharide sequences of medical importance in D_2O detected by high field NMR.

4.3.(iii). Introduction to 2D NMR Correlation Spectroscopy

Since the advent of super-conducting magnets, increasing the spectrometer magnetic field has become less important than the application of specific 2D NMR experiment pulse programs[229], which are characterised by four stages, e.g. **preparation-evolution-mixing-detection** (Figure 21.).

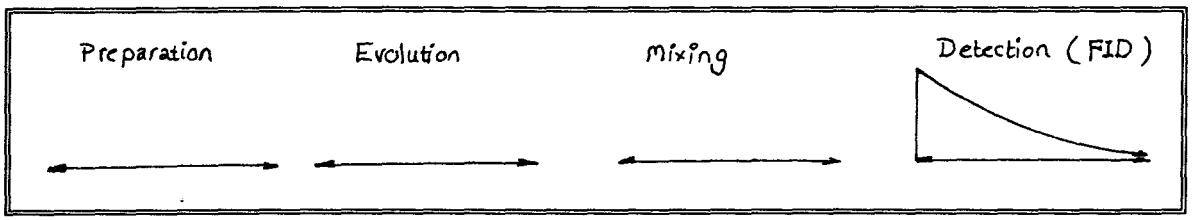


FIGURE 21. Typical pulse program

2D NMR spectroscopy may be produced as either 2D J-resolved correlation or 2D shift correlation. In 2D J correlated spectra one axis contains the chemical shift and the other coupling constants but is of little use in polysaccharide research. 2D shift (homo or heteronuclear) correlation, correlates nuclei by spin-spin coupling of the respective shifts, i.e. through bond coupling, or by nuclear Overhauser enhancement (n.O.e), e.g. correlated dipole connectivity through space and is presented on a separate axis. Homonuclear correlation experiments produce a contour map, the diagonal contains the 1D proton spectrum and off-diagonal connectivity co-ordinates provide the scalar coupling between, e.g. H-1,2 while allowing the identification of the H-2 proton. Often direct spectrometer measurement of the J value from the off-diagonal peak is the simplest means of determining the coupling constant.

4.3.(iv). 2D NMR Homonuclear Correlated Spectroscopy

4.3.(iv).a. Correlated spectroscopy (COSY).

^{1}H-^{1}H COSY was the first 2D NMR experiment and has been reviewed[242,257,258]. Other nuclei which have been correlated are ^{13}C-^{13}C, e.g. Goux *et al.*[259] successfully studied peracetylated mono- and oligosaccharides by introducing ^{13}C enrichment via an acetylation step.

In the 2D COSY contour plot the 1D ^{1}H-NMR spectrum is plotted on the diagonal and off-diagonal cross peaks depict the chemical shift of mutually coupled spin systems. Magnetisation is detected by the collection of the FID on the f_2 axis while the f_1 axis analyses the spin system during the evolution period. Subsequent Fourier transformation of this f_1 data enables the COSY contour map development (Figure 22.).

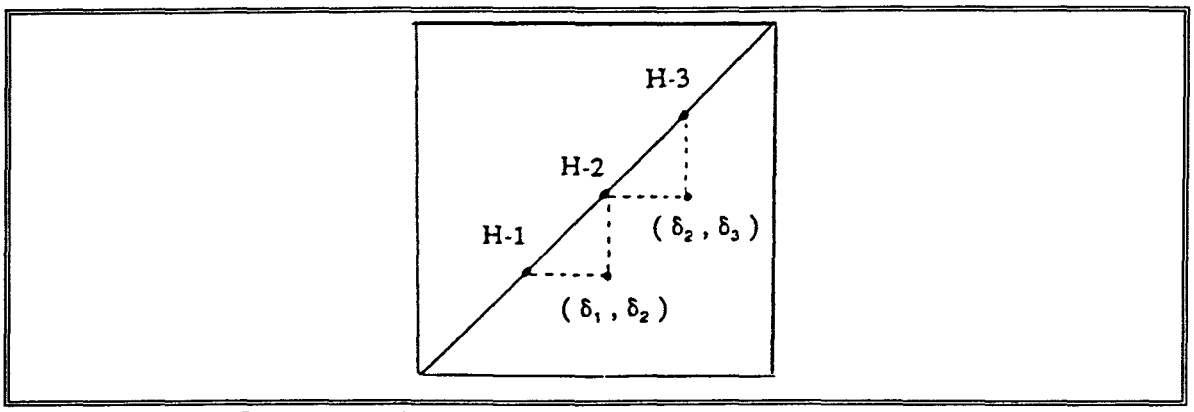


FIGURE 22. 2D Cosy correlation map

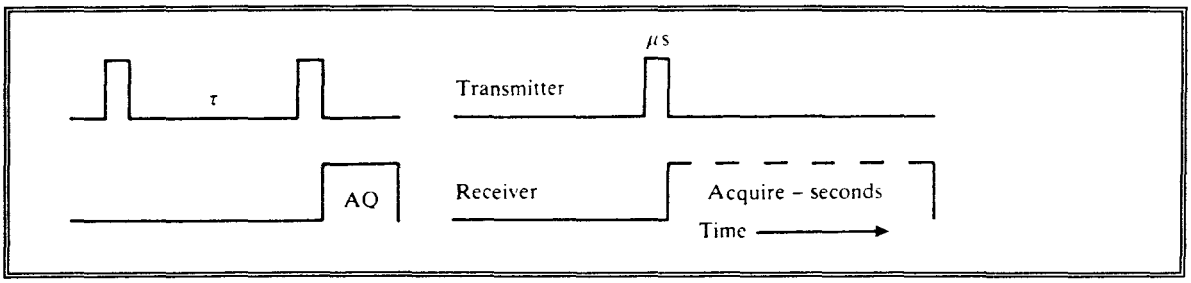


FIGURE 23. The cosy pulse sequence

The pulse sequence, (Figure 23) of the COSY 90 begins with a 90° pulse which disturbs the spin system and evolution takes place. The second pulse disturbs the relaxing spin system and detection occurs. The first pulse causes the induced vector to precess in the xy plane, after the evolution time the vector is aligned along the y axis and the second 90° pulse aligns it with the z axis. The evolution time is incrementally increased producing data in the f_2 plane describing chemical shift and coupling. The second Fourier transform enables correlation of this data with shifts on the f_1 plane.

The COSY 45 is a program using a 45° second pulse and has its advantages and problems[239,258]. Sanders[239] suggests using a yet to be recognised COSY 60, as used by them for optimal homonuclear correlation.

4.3.(iv).B. **Relay COSY.**

The relay COSY pulse program[260] is a hybrid of the original COSY. Magnetisation is transferred between protons not mutually coupled, provided both resonances are coupled to a third central proton. The acquisition parameters are adjusted to optimise the intensity of

the magnetism and minimise the intensity of the diagonal peaks, allowing optimization of the 2D spectrum. Artifacts not involved in the relay must be decreased if a well resolved spectrum is to be acquired.

Assignment of the coupling constant of H-4,5,6 for a monosaccharide residue is sometimes impossible if the COSY spectrum is complicated by superimposed signals. Use was made of a relay COSY experiment, Figure 24, for the structural determination of the *Klebsiella* K43 lithium degraded polysaccharide and the P1-ol.

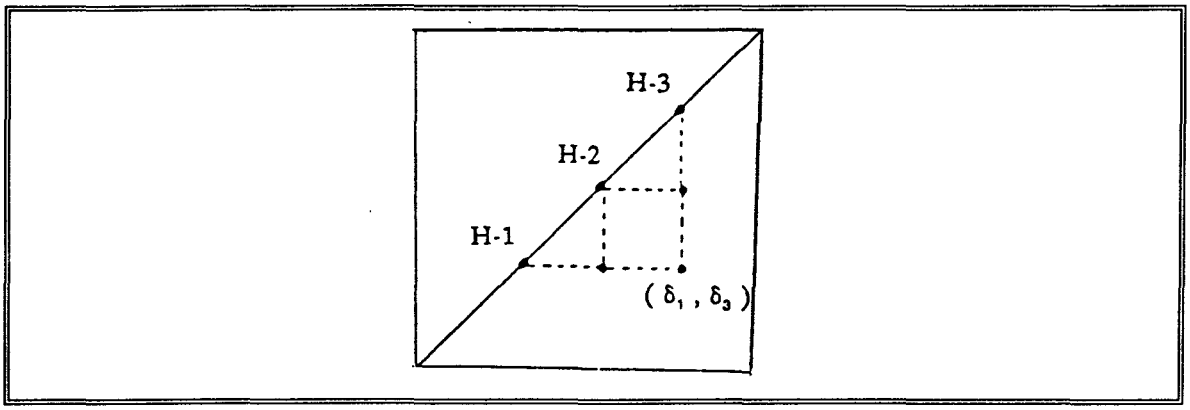


FIGURE 24. Depiction of a Relay COSY contour map

4.3.(iv).c. **Homonuclear Hartmann-Hahn (HOHAHA) spectroscopy.**

2D NMR HOHAHA spectroscopy improves resolution and sensitivity, and reveals direct and relayed J-coupled networks. Davis *et al.*[261] have reported the use of a cross-polarization HOHAHA for the determination of homonuclear scalar connectivities in polysaccharides. Relayed connectivity is transferred from one proton to another within a J-network during the first half of the mixing period, and the second half of the period enables further transfer of magnetism, if the coupling constants are greater than zero. As the mixing time lengthens single relay and multiple relay transfer occurs. Because the transfer of magnetism through a monosaccharide J network is oscillatory and the rate of propagation is proportional to the respective coupling constants, low intensity cross-peaks may result from protons having small coupling constants. Usually one coupling correlation mode will supply

data deficient in another.

All resonances of a system will seldom be relayed down to the H-1 track of the HOHAHA, but assignment of proton spectra from, e.g. the H-3 track of a studied monosaccharide might resolve the H-4 and H-5 ^{1}H chemical shifts.

4.3.(iv).d. **2D nuclear Overhauser Spectroscopy (NOESY)**

Both sequence and molecule conformation data may be established by the n.O.e correlation. The pulse sequence used to acquire a 2D NOESY experiment, (Figure 25) has been reviewed[239,242], and Bax[262] has reviewed parameters which minimise HOHAHA effects which diminish the n.O.e. correlation.

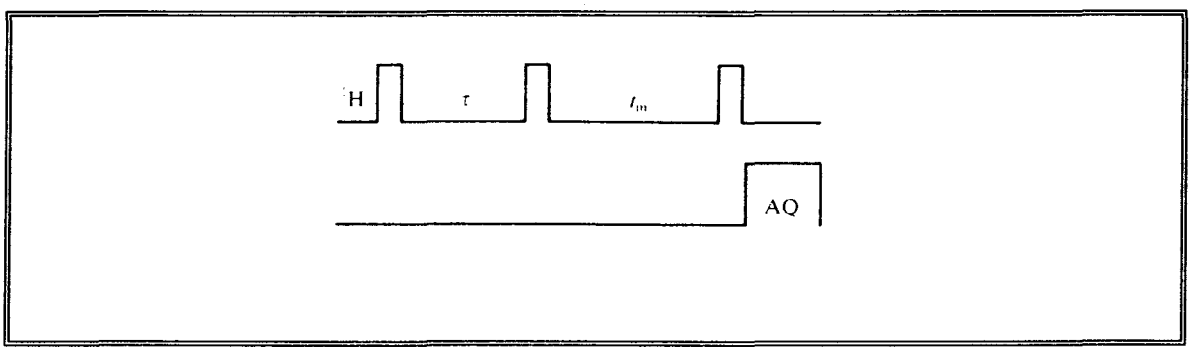


FIGURE 25. Pulse sequence of the NOESY experiment

2D NOESY experiments detect through-space correlation within the van der Waals connectivity, e.g. a distance of ~4 Å or less. After the proton shifts have been acquired via COSY etc., the three dimensional structure can be determined by use of the distance-sensitive n.O.e. in conjunction with coupling constant data[240]. The use of J resolved 2D spectra may be useful in detecting coupling constants of crowded, overlapping spectra.

Bundle *et al.*[263] have used NOESY correlation in the determination of the conformation of a polysaccharide antigen of a *Salmonella* serotype, and in this thesis the sequence of the backbone structure of the *Klebsiella* K43[7] antigen was confirmed by NOESY correlation.

4.3.(iv).e. **2D nuclear Overhauser Spectrometry, (ROESY)**

The ROESY[264-267] experiment is useful for n.O.e. observation in small molecules. It is performed by a rotating frame n.O.e. experiment known as 2D Cross-Relaxation for Mini-molecules EmuLated by spin-locking, (CAMELSPIN).

The advantage[266] of the ROESY experiment over conventional NOESY correlation is that it has a different dependence on molecular tumbling than the NOESY experiment. When an intermediate size molecule is observed, the NOESY experiment will usually produce a small or zero enhancement. ROESY on the other hand is less influenced by spin differences and better resolved correlation cross-peaks are observed, i.e. in medium sized molecules[264] cross-peak intensity is often close to zero when the correlation time approaches the vicinity of the Larmor frequency of the proton in the NOESY experiment, and here ROESY may be helpful. It may also be beneficial to larger molecules where poor n.O.e. enhancement is witnessed.

Negatively the ROESY correlation is difficult to interpret and the spectra are complicated by a non-uniform degree of spin locking, and sometimes non-uniform excitation occurs.

4.3.(v). Heteronuclear Correlated NMR Spectroscopy

4.3.(v).a. ^{13}C-detected, ^{1}H-^{13}C correlation NMR Spectroscopy

4.3.(v).a.i. Heteronuclear Correlation spectroscopy (HETCOR)

One bond ^{1}H-^{13}C nuclear coupling[242,249] is detected under the control of a proton decoupled ^{13}C-NMR pulse program, e.g. WALTZ-16[239], which alleviates the temperature gradients induced by other decoupling pulses relying on higher energy rf pulses. WALTZ brings about broadband ^{13}C decoupling during acquisition of the ^{1}H FID, so that the final proton multiplets contain only homonuclear couplings. Proton chemical shifts are depicted on the f_1 axis and ^{13}C shifts on the f_2 axis. The generated spectrum enables the identity of previously assigned shift data to be checked by their C-H connectivity, and assists in monosaccharide residue identification. Spectra are found to be well resolved if the sample mass is $>$ 15 mg, but the acquisition of spectra is time consuming.

Proton spin polarization[242] is transferred to a ^{13}C through a one bond coupling. The delay period is varied and allows the magnetisation and phase of the transferred signal to be dependent on the proton chemical shift.

Spectrometer time is often at a premium and Uhrin *et al.*[268] reported in 1992 the use of a ^{1}H chemical shift filter technique which acquires the coupled ^{13}C spectrum in a 1D experiment. No knowledge of J (C-1,H-1) values is necessary and this is a highly selective technique relying only on the proton shift responsible for the carbon connectivity.

4.3.(v).b. ^{1}H-detected ^{1}H-^{13}C Heteronuclear Correlation Spectroscopy

Inverse detection[247,268] of a ^{13}C nucleus by a more sensitive correlated ^{1}H nucleus has produced a significant advance in 2D NMR spectroscopic resolution. A smaller sample mass, e.g. 1-2 mg of oligosaccharide is sufficient to produce a well resolved spectrum, and the acquisition time[247] can be 1/10 to 1/20 of that of the HETCOR experiment. Inverse probe design has provided the impetus to novel pulse program design and Lerner[247] has

described instrument requirements for the observation of inverse detected pulse sequences. Inverse detected 2D NMR was extensively used in the structural elucidation of the oligosaccharide repeating unit and its reduced alditol form of *Klebsiella* K43[7].

4.3.(v).b.i. **Heteronuclear Multiple Quantum Coherence Spectroscopy (HMQC)**

The HMQC[247,269,270] experiment produces a spectrum (Spectrum 31, page 149) similar to the HETCOR, consisting of a 2D data matrix, with one frequency axis displaying signals as a function of their ^{13}C Chemical shifts, and another according to their ^{1}H chemical shifts. Directly linked C and H atoms are depicted by a contour level correlation point.

The generation of multiple quantum coherence between ^{1}H and ^{13}C energy levels is achieved allowing correlations between directly-bonded ^{1}H and ^{13}C resonances when ^{13}C decoupling is used. The utility of the HMQC technique is witnessed in its ability to assign carbon shifts from noted ^{1}H shifts, or *vice versa*. When ^{13}C decoupling is not used during acquisition, correlation peaks in the 2D spectrum appear as low intensity doublets in the ^{1}H dimension. The separation between signals is equal to a one-bond ^{1}H-^{13}C coupling constant, and this may be useful in determining the anomeric stereochemistry of a monosaccharide. The BIRD[247] pulse is used to decouple ^{13}C resonances, and signals originating from protons, with long-range coupling to ^{13}C atoms, are removed by phase cycling of a second 90° ^{13}C pulse.

4.3.(v).b.ii. **Heteronuclear Multiple Quantum Coherence - Total Correlated Spectroscopy, (HMQC - TOCSY)**

Through the combination of the heteronuclear correlation, HMQC, with the TOCSY pulse program, all resonances of a particular residue are coupled regardless of whether they are directly or indirectly coupled. An HOHAHA effect is obtained, allowing relayed

coherence throughout the system, and all bonded protons and carbons in the spin system are related. The result is an elaborate depiction of all H-C cross-peaks within the system.

Figure 26 depicts the complete assignment of the ^{1}H-^{13}C resonances for the terminally linked β-**D**-mannose residue of P1-ol. The rectangular area is formed by a ^{1}H-^{13}C connectivity at each corner. For example a rectangle is generated by H1C1, H1C2, H2C1, H2C2 etc.

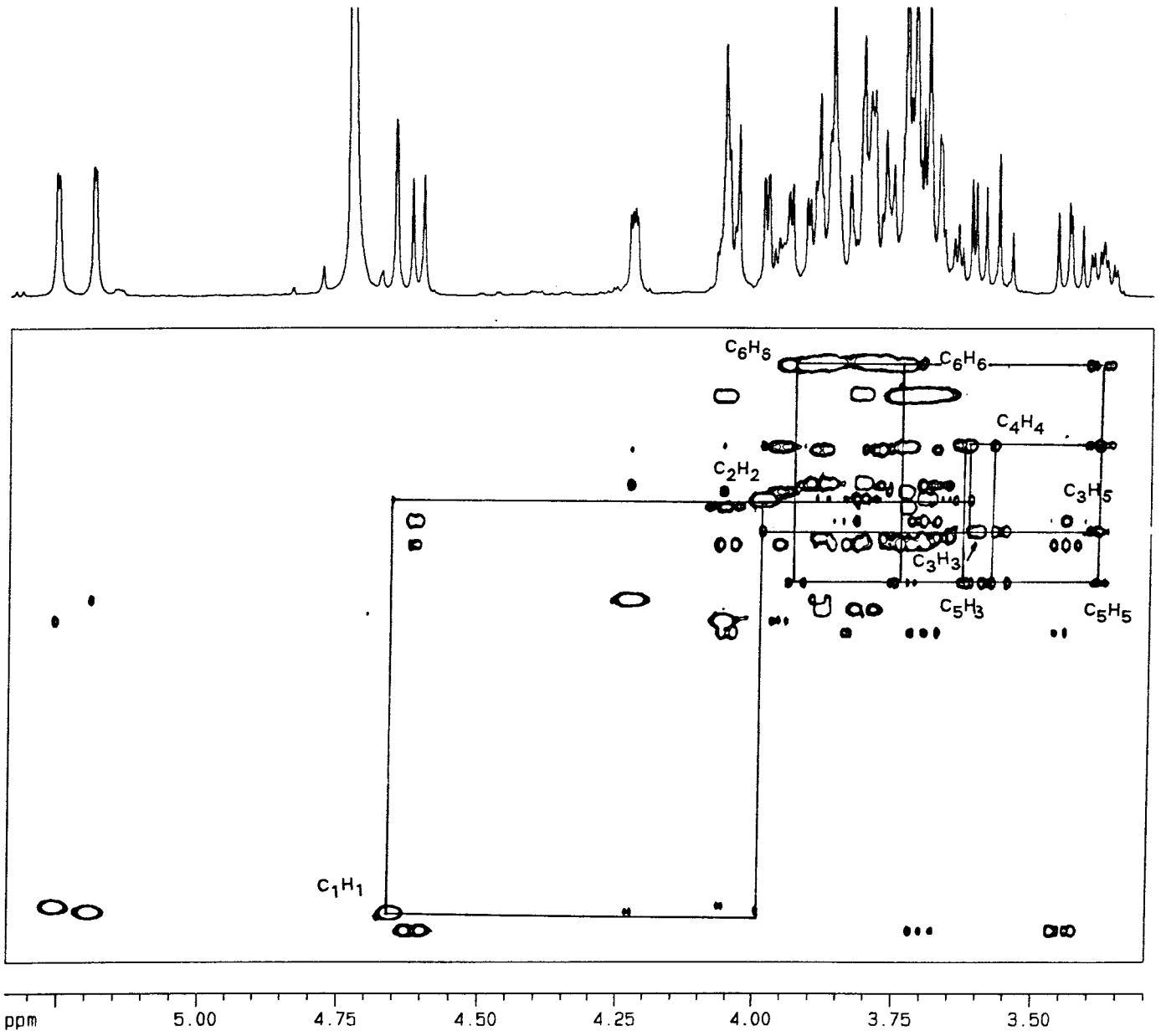


FIGURE 26. ß-D-Mannose HMQC-TOCSY correlation contour map

4.3.(v).b.iii. **Heteronuclear Multiple Bond Coherence Spectroscopy, (HMBC).**

This HMBC[269,271] experiment enables the establishment of couplings between heteronuclei over two and three bonds (Figure 27).

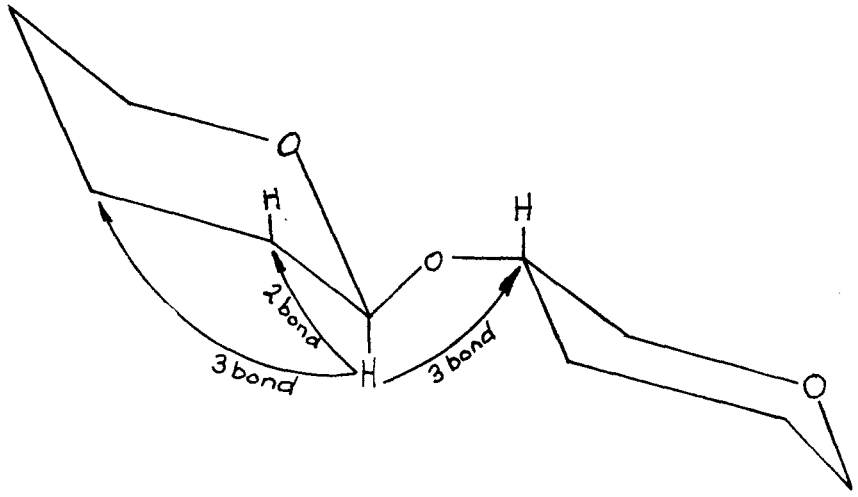


FIGURE 27. HMBC connectivities

This experiment is useful in assigning resonances unambiguously where homonuclear methods fail. It also allows assignment of the oligosaccharide sequence by means of 2 and 3 bond C-H transfer of magnitisation, and magnitisation transfer across the glycosidic bond. High resolution ^{13}C shifts are used to decrease the ^{1}H-^{1}H overlap of signals commonly observed. It is found that the relative intensity of long-range resonance detection, by a particular proton, corresponds directly to the magnitude of the coupling constant, i.e. a large coupling constant affords an intense connectivity. Naturally proton signals not coupled to ^{13}C nuclei must be suppressed and this phenomen is dependent on the stability of the spectrometer, and acquisition parameters used.

Bax *et al.*[272] have reported the structural elucidation of a mannose containing antibiotic, Deseromycin, which had overlapping 1D ^{1}H-NMR shifts. Even after 2D NMR homonuclear experimentation, the shifts were still unresolved. Use of the inverse detected HMBC experiment, however, unambiguously established all C-H correlations. *Klebsiella* K43[7] (this thesis) also depended on the HMBC experiment for the assignment of the

repeating unit, which showed several unresolved, overlapped shifts.

The use of HMBC 2D NMR is increasing and is invaluable, if inverse detection is possible on the spectrometer. It enables shift assignment of carbohydrate material with overlapping resonances and may be a useful alternative to the NOESY experiment. The correlation of heteronuclei over multiple bonds and over the glycosidic linkage permit residue sequence, linkage and previously ambiguous ^{1}H-^{13}C assignments possible.

5. STRUCTURAL ELUCIDATION OF THE CAPSULAR POLYSACCHARIDE OF *KLEBSIELLA* K43

5.0. Abstract

The repeating unit of the capsular polysaccharide (K-antigen) from the *Klebsiella* serotype K43[7], culture number 2482, was found to be of the "3+2" repeating unit type. The uronic acid was found as part of a disaccharide side chain and the backbone of the polysaccharide is a neutral trisaccharide of mannose and galactose.

repeat →3)-α-D-Manp-(1→2)- α-D-Manp-(1→3) -α-D-Galp-(1→

2
↑
1
ß-D-GlcpA

4
↑
1
ß-D-Manp

The repeating unit of the *Klebsiella* K43 polysaccharide

Structural elucidation was performed by chemical analysis, NMR and GLC-MS studies on the native polysaccharide, uronic acid degraded oligosaccharide and on the chemical repeating unit oligosaccharide produced by a bacteriophage degradation of the K43[7] polysaccharide. The repeating unit structure was confirmed by a Smith degradation experiment and by 2D NMR spectroscopy performed on both the repeating unit oligosaccharide (P1) and its reduced form (P1-Ol).

5.1. Introduction

The structural elucidation of the capsular polysaccharide (K-antigen) of the *Klebsiella* serotype K43[7] forms part of a broader, continuing international program to relate the respective bacterial antigenic characteristics to their chemical structure.

Of 78 serotyped *Klebsiella* K-antigens, K43[7] is the 75th to be determined and is the 4th member of the glucuronic acid-galactose-mannose chemotype. Other members of this group are K20[86], 21[87], and 74[88]. The acidic nature of this polysaccharide is determined by a single glucuronic acid residue found in each repeating unit. The polysaccharide does not contain non-carbohydrate appendages. Consistent with the *Klebsiella* genus, K43[7] contains no phosphate. The serological cross-precipitation reaction, as witnessed by K43[7] and K57[97] in *Pneumococcal*[12] (XVIII) antiserum, and K43[7] in *Klebsiella* K57[12] antiserum, suggests that K43[7] should be structurally close to K57[97].

5.2. Results and discussion

5.2.(i). Isolation and monosaccharide composition

Bacteria of the genus *Klebsiella,* serotype K43[7], were propagated on sucrose-rich agar as described, and the solubilised capsular material was separated from the cells by ultra-centrifugation followed by precipitation of the crude polysaccharide into ethanol. The acidic K-antigen was then separated from the neutral (O-antigen) polysaccharide by the method of Okutani *et al.*[103]. The acidic polysaccharide component was readily precipitated as the cetyltrimethylammonium bromide salt from the viscous solution of the polysaccharide mixture.

The relative molecular weight of the sodium and the acidic form of the polysaccharide was determined by GPC on a dextran-calibrated Sephacryl S-500 column (Figure 28, page 92). Both forms of the polysaccharide were found to be polydisperse with regard to molecular weight distribution. The sodium salt polysaccharide had maxima at 1.047×10^7 daltons and at 6.026×10^6 daltons, while the acidic form had maxima at 5.495×10^5 daltons and at 1.318×10^4 daltons.

All polysaccharide fractions showed identical monosaccharide ratios indicating that the extracted polysaccharide was of a homologous nature, but was probably present as aggregates.

The optical rotation of the purified sodium salt of the polysaccharide was found to be $[\alpha]_D$ +29°. An acid hydrolysate of the sodium salt of the polysaccharide tested negative for phosphate[181].

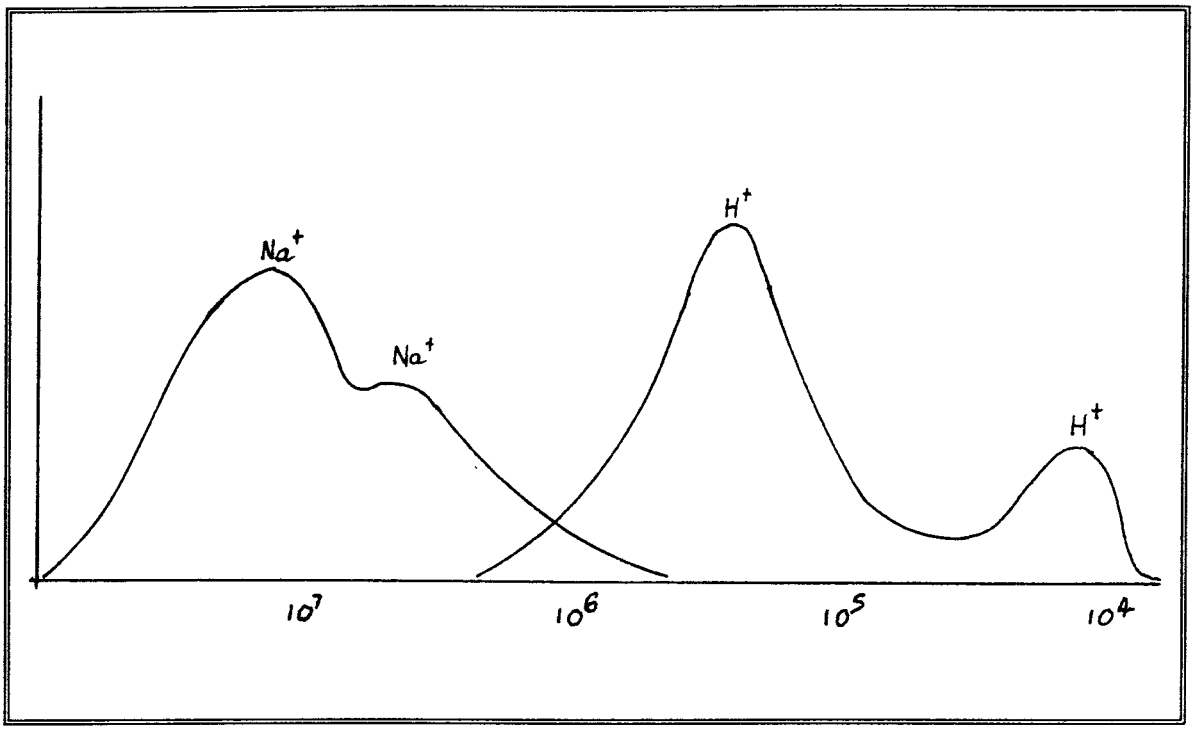


FIGURE 28. GPC on Sephacryl S500 for the Mw determination of the sodium and protonated polysaccharide forms.

Acid hydrolysed polysaccharide was converted into the peracetylated aldononitrile derivatives[189], PAAN's, and was examined by GLC. The results showed that mannose and galactose were present in the ratio 2.43 : 1. On methanolysis of the polysaccharide, reduction of the uronate ester(s), hydrolysis, and GLC analysis of the derived PAAN's, the mannose, glucose and galactose ratio was 3 : 1 : 1.1. HPLC analysis of methanolysed, reduced and hydrolysed polysaccharide yielded a ratio of mannose, glucose and galactose 2.9 : 1.0 : 1.1. The monosaccharide ratio may thus be written as mannose : glucuronic acid : galactose 3 : 1 : 1, and accords with a pentasaccharide repeating unit.

The absolute configuration of the monosaccharide residues in the polysaccharide was determined by GLC analysis of their acetylated (-)-2-octyl glycosides[122]. All were found to be of the **D** configuration.

The 1D ^{1}H-NMR spectrum of the polysaccharide, (Spectra 1-2, page 106-107), recorded at 70°C, and read directly off the spectometer contained H-1 resonances for α-residues at δ 5.29 (unresolved doublet), δ 5.27 ($J_{1,2}$ ~ 4 Hz) and δ 5.20 (unresolved doublet), and ß-residues at δ

4.69 (unresolved doublet) and δ 4.59 ($J_{1,2}$ 8 Hz). The chemical shifts at δ 5.29 and δ 5.20 were tentatively assigned to H-1 of two *α*-D-Manp residues and δ 4.69 was assigned to H-1 of a ß-D-Manp residue.

The ^{13}C-NMR spectrum, (Spectrum 3, page 108) , contained C-1 signals at (102.64, 101.92, 101.03, 100.58, and 96.02 ppm), a signal for C=O at 172.42 ppm indicative of a uronic acid, and signals for CH_2OH at 62.45, 62.04, 61.89 and 61.72 ppm. The latter chemical shifts indicated that none of the residues was 6-linked. 1D ^{1}H-NMR did not show signals for pyruvate which resonates at δ 1.45-1.6, and acetate which resonates at δ 1.92-2.05. 1D ^{1}H-NMR performed on the alkali treated polysaccharide (Spectrum 4, page 109) showed no substantial difference to that of the native polysaccharide. The results thus confirm that the polysaccharide is composed of a pentasaccharide repeating unit devoid of acetate or pyruvate moieties.

5.2.(ii). Methylation analysis

The modified Hakomori-method of Phillips and Fraser[130] was used to methylate a small quantity of polysaccharide. The product was not completely soluble in dichloromethane, indicative of incomplete methylation. The partially methylated polysaccharide was therefore methylated by the Kuhn[128] procedure to afford fully methylated polysaccharide. A methylation analysis using 1,1,3,3-tetramethyl urea to relax the hydrogen bonding, responsible for undermethylation under Hakomori conditions was abandoned when it was found that troublesome artifacts formed and interferred with the GLC analysis. One third of the methylated polysaccharide was hydrolysed with 4M trifluoro-acetic acid, and the product was then analysed as the partially methylated alditol acetate derivatives[186] (PMAA's) by GLC and GLC-MS. The results are listed in Table 6, column 1.

Table 6.

Partially Methylated Alditol Acetates	T_a	1 Polysaccharide	2 Methanolised Polysaccharide	3 ß-Elimination
2,3,4,6-tetra-O-methylglucitol	1.00	/	/	/
2,3,4,6-tetra-O-methylmannitol	0.97	0.61	0.56	/
3,4,6-tri-O-methylmannitol	1.52	1.09	1.00	0.78
4,6-di-O-methylmannitol	2.19	0.93	0.86	/
2,3-di-O-methylglucitol	3.00	/	0.49	/
2,4,6-tri-O-methylgalactitol	1.65	1.00	0.99	1.00
2,4,6-tri-O-methylmannitol	1.63	/	/	0.96

T_a retention time relative to 2,3,4,6 tetra-O-methylglucitol on an OV 225 capillary column.

Two thirds of the methylated polysaccharide was subjected to methanolysis[105], the methyl esters produced were reduced with sodium borohydride, hydrolysed and analysed by GLC as the derived PMAA's. These data are listed in Table 6, column 2. The methylation analysis thus indicates the presence of a 2-linked; 2,3-linked and terminal mannose residue, a 4-linked glucuronic acid and a 3-linked galactose residue in the repeating unit.

5.2.(iii). Sequencing of the monosaccharide residues

5.2.(iii).a. **Base catalysed ß-elimination of the uronic acid of methylated polysaccharide**

A small quantity of polysaccharide was methylated as before and was then treated with potassium dimsyl for two hours. Direct re-alkylation of the products with MeI followed in a single vessel as described by Lindberg *et al.*[140] and the product of hydrolysis was analysed as partially methylated alditol acetates by GLC-MS. The results are listed in Table 6, (column 3) and show the complete absence of 2,3,4,6-tetra-O-methylmannose and 4,6-di-O-methylmannose, and the

production of an equivalent of 2,4,6-tri-O-methylmannose. These results indicate that the glucuronic acid is found in the side chain of the repeating unit and is linked to the main chain through O-2 of a 3-linked mannose residue. Furthermore the results show that a terminal mannose residue is linked to O-4 of the glucuronic acid. The main chain of the polysaccharide thus consists of a 2-linked mannose, a 3-linked mannose and a 3-linked galactose. The partial structure (Figure 29), is thus present in the repeating unit of the polysaccharide.

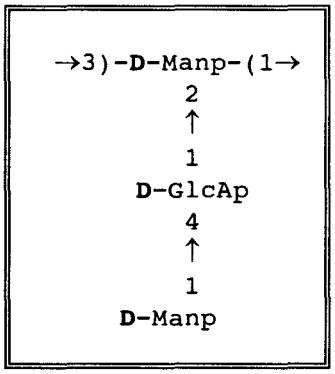


FIGURE 29. The partial structure of the repeating unit of the *Klebsiella* K43 antigen

The following two structures for the repeating unit of the *Klebsiella* K43[7] antigen (figures 30 & 31) may be postulated from the data presented.

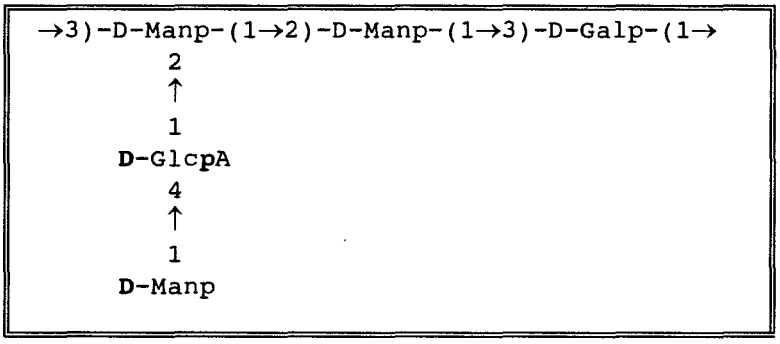


FIGURE 30.

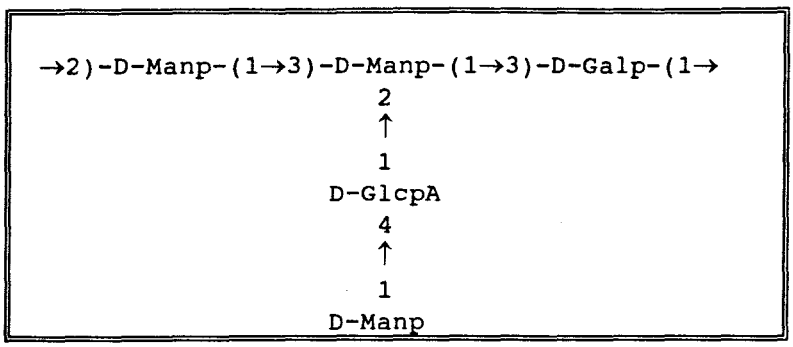


FIGURE 31.

5.2.(iii).b. **Lithium degradation of the native polysaccharide**

Polysaccharide in the sodium salt form was dissolved in ethylenediamine and small pieces of lithium metal were sequentially added, according to the method of Lau *et al.*[172]. After quenching the reaction with water and removal of the lithium ions by ion-exchange chromatography, the degraded material in the acidic form was separated on a column of Sephacryl S-200 gel. A fraction corresponding to the degraded polysaccharide was isolated, see Figure 32. The isolated material was analysed by GLC of the derived PAAN's and showed mannose and galactose in the ratio 1.9 : 1.0. This result accords with the result of the ß-elimination reaction and confirms the main chain composition as mannose and galactose in the ratio 2 : 1.

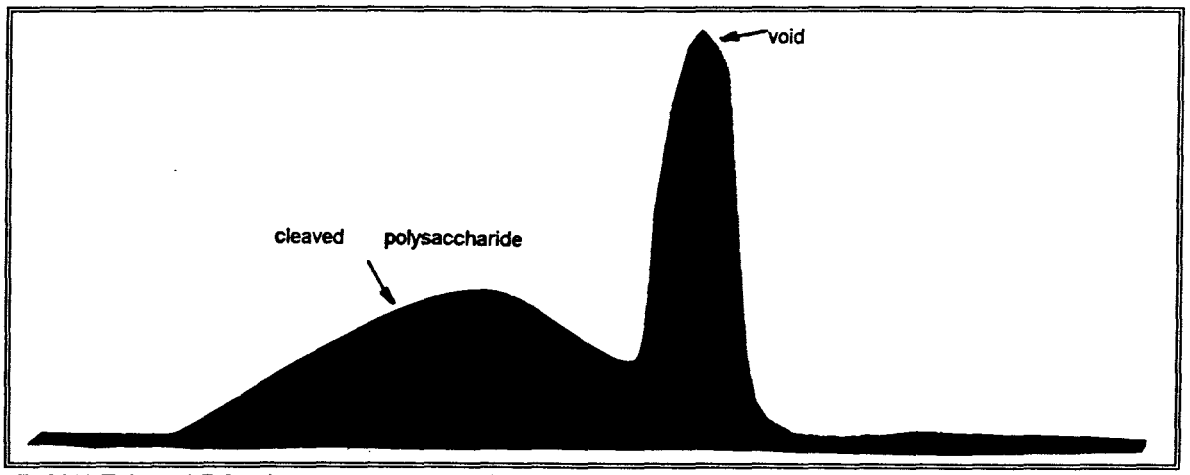


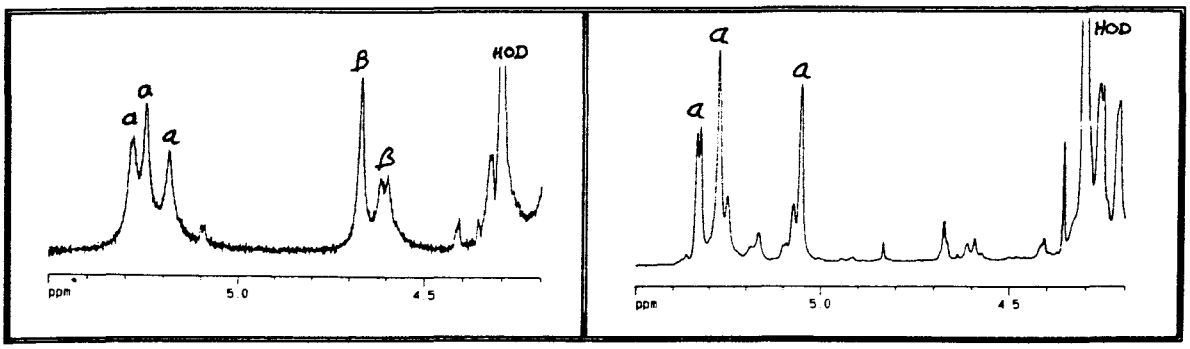
FIGURE 32. GPC chromatogram of the degraded uronic acid polysaccharide

The quantity of lithium metal necessary to produce a dark navy-blue colour for 60 minutes was found to be dependent on: the surface area of the metal, the frequency of introducing atmospheric exchange to the area above the reaction surface, or indeed a large volume of dead space above the reaction mixture. It was found that during the experiment if the glass stopper was removed from the flask, the reaction mixture changed to a creamy colour. If excessive quantities of lithium metal were added, the ion-exchange removal of lithium ions was complicated. The reaction generally needed 15-20 minutes to change colour and further lithium addition before this point resulted in excess lithium ion concentration in the solvent.

The lithium reaction is effective in cleaving uronic acid residues and has been utilized by DeBruin[273] in the elucidation of the structure of the repeating unit of *E.coli* K102 capsular polysaccharide.

5.3. NMR study on the lithium degraded polysaccharide

This study established the anomeric configuration of the residues in the polysaccharide and allowed the sequencing of the main chain residues. A 1D NMR study on the degraded material confirmed the anomeric configuration of the repeating unit, and a 2D study allowed the sequencing of the main chain residues. The anomeric configurations of the monosaccharide residues in the backbone structure of the K43[7] polysaccharide was assigned by comparing the proton 1D ^{1}H NMR spectra of the native (Spectrum 5) and lithium degraded polysaccharides (Spectrum 6).



SPECTRUM 5. 1D ^{1}H-NMR of the polysaccharide

SPECTRUM 6. 1D ^{1}H-NMR of the degraded polysaccharide

Spectrum 6, shows 3 α-anomeric signals for the degraded polysaccharide while the spectrum of the native polysaccharide has 3 *α*- and 2 ß-signals in the anomeric region.

The repeating unit is thus composed of:

side chain residues ß-D-Manp(1→, →4)-ß-D-GlcpA

main chain residues →2)-*α*-D-Manp-, →3)-*α*-D-Manp-
2
↑
→3)-*α*-D-Galp-

1D ^{1}H-NMR spectrum, (Spectra 7,8, page 110-111) recorded at 50°C contained H-1 resonances for the *α*-linked residues at δ 5.342 ($J_{1,2}$ 4.00 Hz), δ 5.295 ($J_{1,2}$ unresolved) and δ 5.048 ($J_{1,2}$ 1.4Hz), and the ^{13}C-NMR spectrum, (Spectrum 9, page 112) contained C-1 signals at 103.03, 101.20 and 95.44 ppm. The chemical shifts of most of the ^{1}H and ^{13}C resonances of the residues (Table 7, page 100) were established mainly from ^{1}H-^{1}H correlation experiments, *viz.* COSY, Relay COSY and HOHAHA, and a ^{1}H-^{13}C heteronuclear correlation experiment, *viz.* HETCOR. Use of through space n.O.e. couplings confirmed the linkage positions and established the residue sequence. The residues were labelled arbitrarily **a** to **c** in order of decreasing chemical shift of the H-1 resonances.

Residue a The chemical shifts for the H-1/2 resonances were established from the COSY-45 spectra, (Spectra 10,11, page 113-114) and the connectivities up to H-4 were noted in the H-1 track of the HOHAHA contour map, (Spectrum 12, page 115). The chemical shift for the H-5 resonance was determined from the n.O.e between H-4 and H-5 in a NOESY experiment, (Spectrum 13, page 116). The H-6a and H-6b resonances could then be identified from the COSY spectrum (Spectrum 11, page 114).

Residue b The chemical shifts for the H-1/2 resonances were established from the COSY spectrum. The chemical shift for H-3 was established from the H-1 track in the double Relay COSY contour plot, (Spectrum 14, page 117), while those for the H-4 and H-5 resonances were noted in the H-2 and H-3 track of this Relay COSY.

Residue c The chemical shifts for the H-1 to 3 resonances were traced in the COSY spectrum and that for the H-4 in the H-3 track of the double Relay COSY contour map. The chemical shift for the H-5 resonance was established from the n.O.e. between H-3 and H-5 in the NOESY experiment. The ^{13}C resonances for residues **a** to **c** (Table 7, page 100) were assigned by comparing the ^{1}H assignments with the ^{1}H-^{13}C correlation data obtained from the HETCOR, (Spectrum 15, page 118). The identity of the residues **a** to **c**, their linkage positions, and anomeric configurations followed from a comparison of the NMR data with those of methyl glycosides, (Table 5, page 74).

Identification of an unknown residue may be conducted either by a comparative study with standard monosaccharide derivatives[243] (methyl glycosides), or from first principles using the unknown residue's coupling constant values, if available. The latter method is especially useful where standard shift data is not available. Each chiral centre's relative configuration is determined on the premise that large "J" values indicate a trans stereochemistry, e.g. a ß-**D**-Glc will exhibit large coupling constants for H1,2; H2,3 and H 3,4 of approximately 8-10 Hz.

Use of standard methyl glycoside shift data is a quick and convenient means of identifying unknown residues. Both the ^{1}H and ^{13}C data acquired for the unknown residue are compared to that of a range of standards. ^{13}C shifts are less variable than the ^{1}H shifts and are used first in identifying the unknown resonance. An almost text book example of identifying an unknown residue, is that of the terminal ß-**D**-mannose residue, (residue **c**, which is known to be terminally linked from methylation results) of the repeating unit of *Klebsiella* K43.[7] The magnetic environment of the resonances of a terminal residue are not affected appreciably by neighbouring groups, and show ^{1}H and ^{13}C shift data virtually identical to those of, e.g. a methyl glycoside of the particular sugar residue. Table 7 monosaccharide shift values (page 100) compares standard methyl glycoside NMR shifts with those of the unknown residue (**c**).

Points of monosaccharide linkage are detectable by the degree of ^{13}C chemical shift, up or down field, in comparison to the standard methyl glycoside residues. Generally these shift differences can be up to 10 ppm for ^{13}C resonances, e.g. the unknown residue **c** of the lithium degradation product of *Klebsiella* K43[7] can be identified as a 3-linked ④-**D**-mannose by use of

the standard methyl glycoside shifts, (Table 8, page 100).

Table 7

NMR shift details for residue **c** of the reduced repeating unit of *Klebsiella* K43.

	1	2	3	4	5		
c	**H**	4.65	3.99	3.63	3.58	3.39	3.93	3.74
c	**C**	100.9	71.35	73.57	67.44	77.26	61.49	
S	**H**	4.58	3.99	3.64	3.57	3.38	3.94	3.74
S	**C**	101.3	70.6	73.3	67.1	76.6	61.4	

S = standard methyl -ß-**D**-mannopyranoside chemical shifts[243], see Table 5, page 74.

Table 8

NMR shift details for residue **c** of the lithium degraded polysaccharide.

c	**H**	5.048	4.280	3.973	3.831	3.834
c	**C**	103.03	70.48	79.03	67.19	74.16
S	**H**	4.77	3.93	3.765	3.64	3.62
S	**S**	101.9	71.2	71.8	68.0	73.7

S = standard methyl-*a*-**D**-mannopyranoside chemical shifts[243], see Table 5, page 74

- underlined carbon shifts indicate points of linkage

The sequence of the residues was established from a phase-sensitive NOESY experiment (Spectrum 13, page 116). The protons attached to the carbons across the glycosidic linkage are in van der Waals contact and therefore display a n.O.e (Figure 33). From Figure 33, it will become apparent that the *a*-mannose and *a*-galactose residues should show the following n.O.e's: *a*-mannose H1,2; H2,3 and H3,5 and *a*-galactose H1,2; H3,4, H3,5 and H4,5.

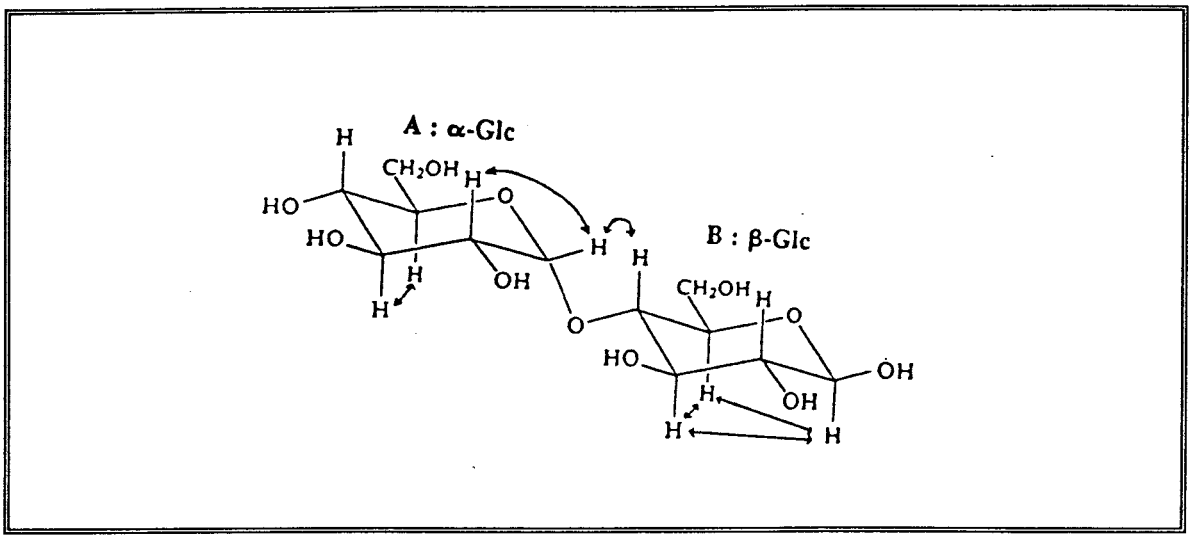


FIGURE 33. n.O.e's possible between adjacent protons

The n.O.e's. observed are listed in (Table 10, page 103). H-1 of residue **a** showed an intense n.O.e. cross-peak at δ 3.96. This cross-peak probably consists of an intra-residue n.O.e from H-1 to H-2 of **a**, and an overlapping inter-residue n.O.e. from H-1 of **a** to H-3 of **c** . H-1 of residue **b** showed an inter-residue n.O.e. to H-4 of **a** and an intense cross-peak centred at δ 4.036. The latter n.O.e. could have arisen from an inter-residue n.O.e. to H-3 of **a** and/or from an intra-residue n.O.e. to H-2. It is not uncommon for both H-3 and H-4 of a galactose residue to show n.O.e. to H-1 of the glycosylating residue. H-1 of residue **c** showed the expected intra-residue n.O.e to H-2 and an inter-residue n.O.e. to H-2 of **b**. Although both the inter-residue n.O.e. to H-3 and that of the intra-residue n.O.e. to H-2 were overlapped, both n.O.e.'s were expected, the former because galactose was shown to be 3-linked by the methylation results and the latter because residue **b** is *a*-linked.

Table 9. ^{1}H- and ^{13}C-NMR data for the *Klebsiella* K43 lithium degraded polysaccharide. (50 °C)

		1	2	3	4	5	6a	6b
a	**H**	**5.342**	**3.954**	**4.040**	**4.223**	**4.057**	**3.783**	**3.783**
	S	4.84	3.83	3.79	3.97	3.89	3.783	
	C	**101.20**	**67.96**	**79.93**	**66.12**	**72.02**	**62.13**	
	S	100.1	69.2	70.5	70.2	71.6		
b	**H**	**5.294**	**4.036**	**4.028**	**3.738**	**3.907**		
	S	4.77	3.93	3.76	3.64	3.62		
	C	**95.44**	**74.41**	**70.87**	**67.83**	**73.64**		
	S	101.9	71.2	71.8	68.0	73.7		
c	**H**	**5.048**	**4.280**	**3.973**	**3.831**	**3.834**		
	S	4.77	3.93	3.76	3.64	3.62		
	C	**103.03**	**70.48**	**79.03**	**67.19**	**74.16**		
	S	101.9	71.2	71.8	68.0	73.7		

Chemical shifts in ppm relative to internal standard acetone δ 2.23 for ^{1}H- and at 31.07 ppm for ^{13}C-NMR.

Standards are: S_a methyl-*a*-D-galactopyranoside

$S_b = S_c$ methyl-*a*-D-mannopyranoside

- Underlined carbon shifts indicate points of linkage

Table 10.

n.O.e.'s for the *Klebsiella* K43 Lithium degradation

Proton		n.O.e. to
a, H-1	5.342	3.954(a,H-2); 3.973(c,H-3)
a, H-3	4.040	4.223(a,H-4)
a, H-4	4.223	4.057(a,H-5)
b, H-1	5.295	4.223(a,H-4); 4.036(b,H-2)
c, H-1	5.048	4.280(c,H-2); 4.036(b,H-2)
c, H-3	3.973	4.280(c,H-2)

The sequence for the lithium degraded polysaccharide may thus be written:

→3)c-(1→2)-b-(1→3)-a(1→

Thus the backbone structure of the polysaccharide of the *Klebsiella* K43[7] antigen may be written:

→3)-*α*-D-Manp-(1→2)-*α*-D-Manp-(1→3)-*α*-D-Galp(1→

The main chain of the *Klebsiella* K43[7] antigen oligosaccharide repeating unit.

5.4. Smith degradation

Polysaccharide, in the sodium salt form, was selectively oxidised with a 3 fold excess of sodium periodate and a 4 fold excess of sodium perchlorate. The addition of sodium perchlorate decreases the anionic nature of the uronic acid thereby decreasing the steric hinderance to the oxidising periodate ion. The course of the reaction was followed by sampling small aliquots of the reactants over time and analysing an hydrolysate of the oxidised material by HPLC. A first oxidation limit was reached and the material was freeze dried and re-oxidised as before. Finally the oxidised material was reduced, and then hydrolysed using 1 M TFA at room temperature for two hours. This acid strength was higher than normally reported for the Smith degradation, but was necessary because the material showed resistance to hydrolysis under milder conditions. The products of the Smith degradation were chromatographed on a calibrated column of Biogel P2 gel (Figure 34), and a peak corresponding to the expected molecular weight of the Smith fragment was isolated.

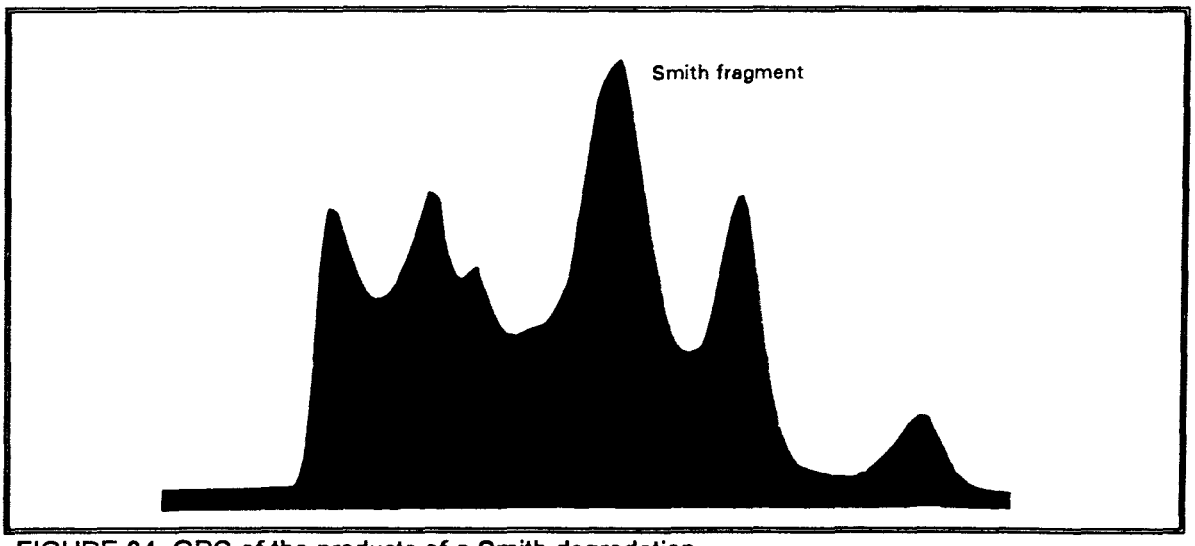


FIGURE 34. GPC of the products of a Smith degradation

Hydrolysis of the methylated Smith degradation product, and GLC analysis of the derived PMAA's showed the presence of 2,3,4,6-tetra-O-methylgalactose and 2,4,6-tri-O-methylmannose. The expected di-O-methylglycerol (from the oxidation of a 2-linked mannose residue) was not detected and was probably lost on evaporation in the work-up procedure. The

Smith degradation scheme is shown in Figure 35.

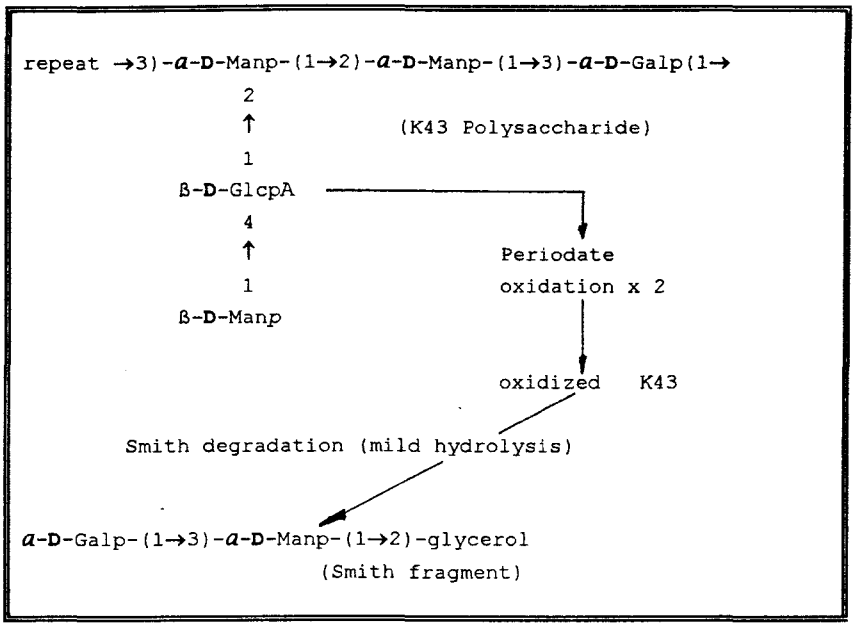
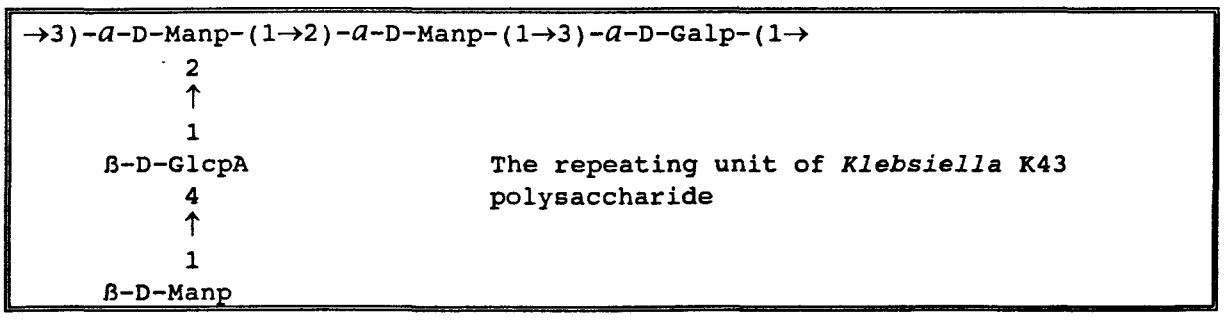
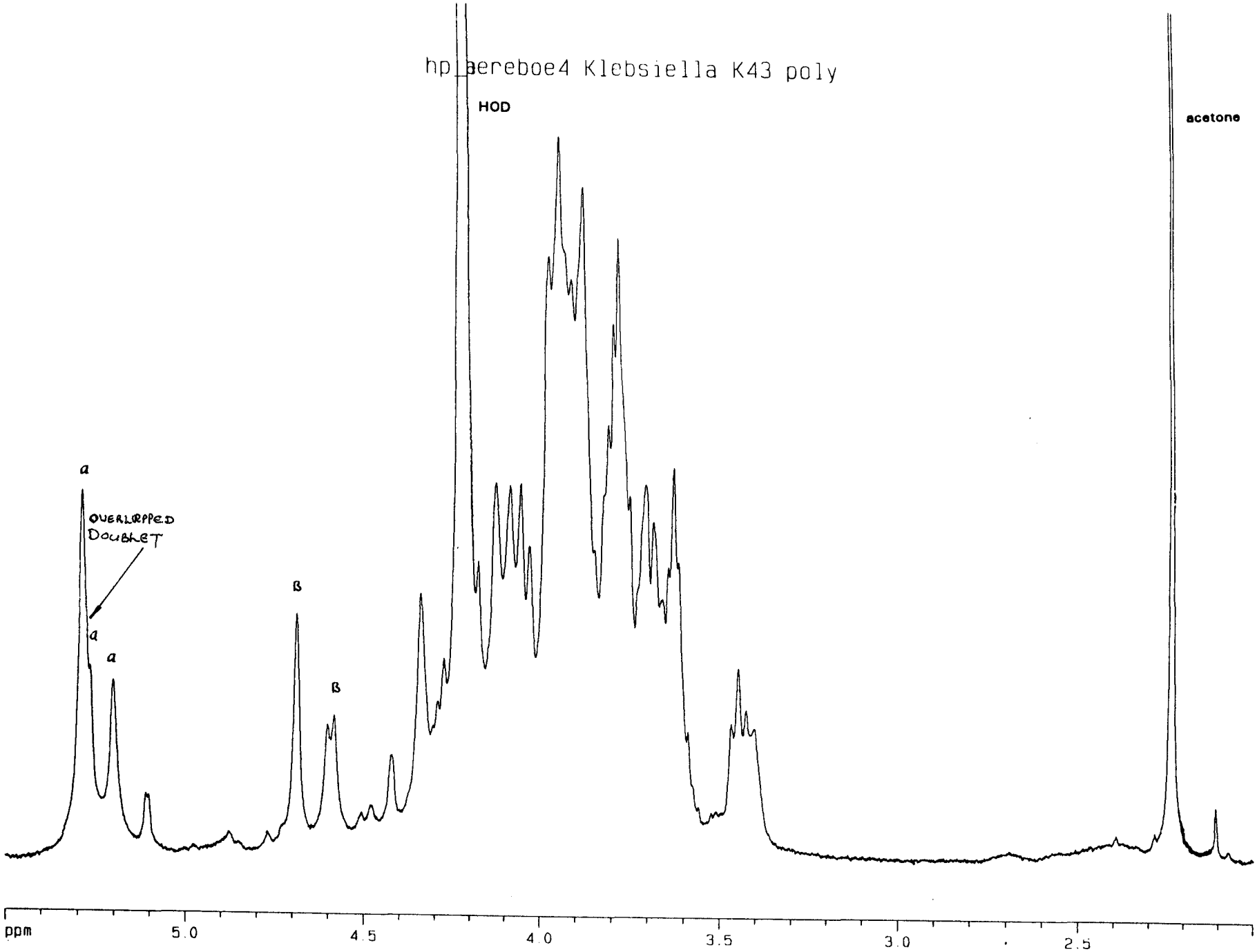


FIGURE 35.

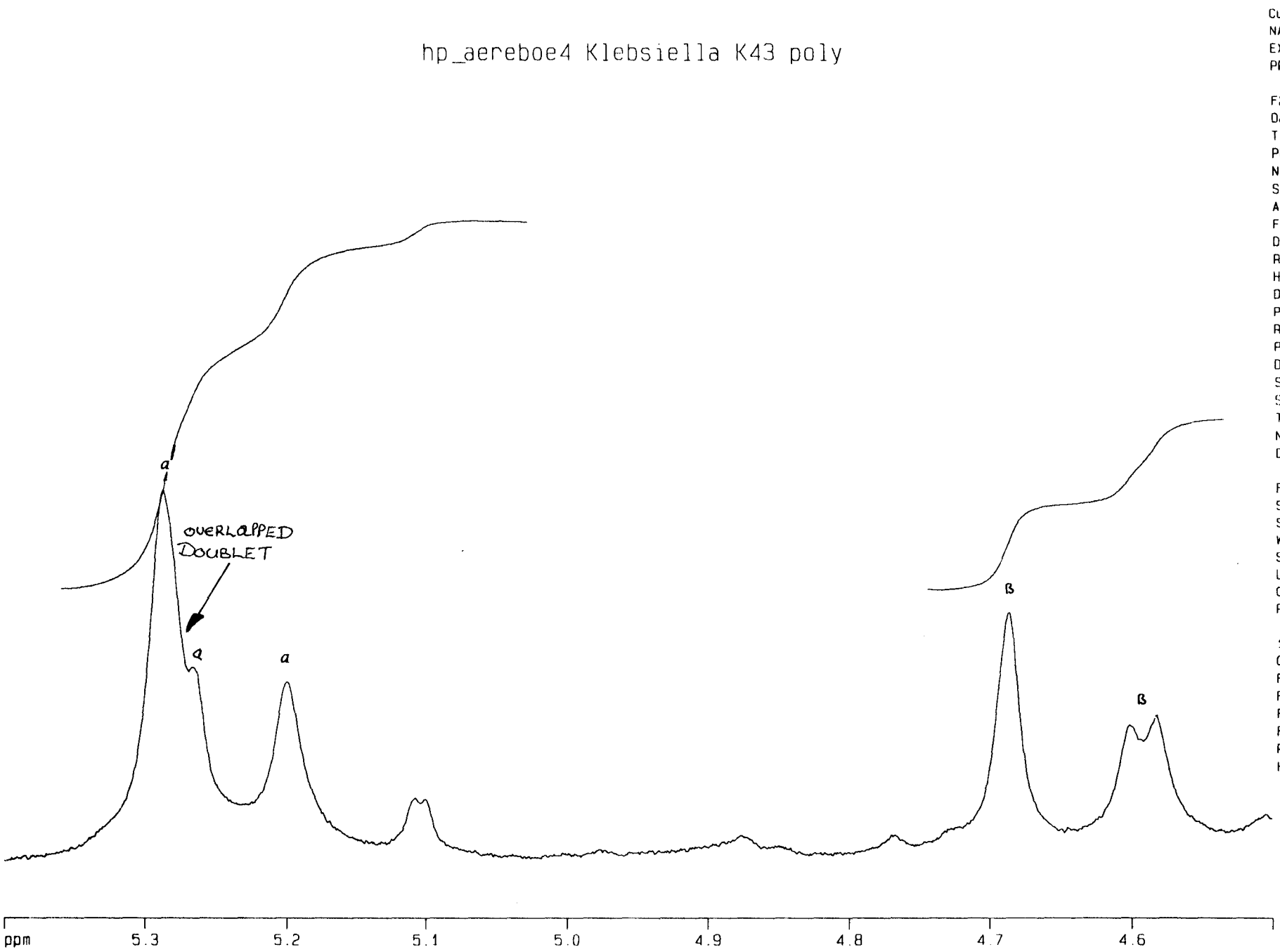
5.5. Conclusion

The combined methylation data from the native polysaccharide, ß-elimination and Smith degradation analysis along with NMR studies performed on native polysaccharide and on the lithium degraded polysaccharide, enabled the pentasaccharide repeating unit of the capsular polysaccharide from *Klebsiella* serotype K43[7] to be written as:





Spectrum 1. 1D ^{1}H-NMR of the *Klebsiella* K43 polysaccharide



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PROCNO	1

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D1	1.0000000 se
P1	13.0 us
RD	0.0000000 se
PW	0.0 us
DE	130.0 us
SFO1	400.1368820 MH
SWH	4807.69 Hz
TD	32768
NS	128
DS	2

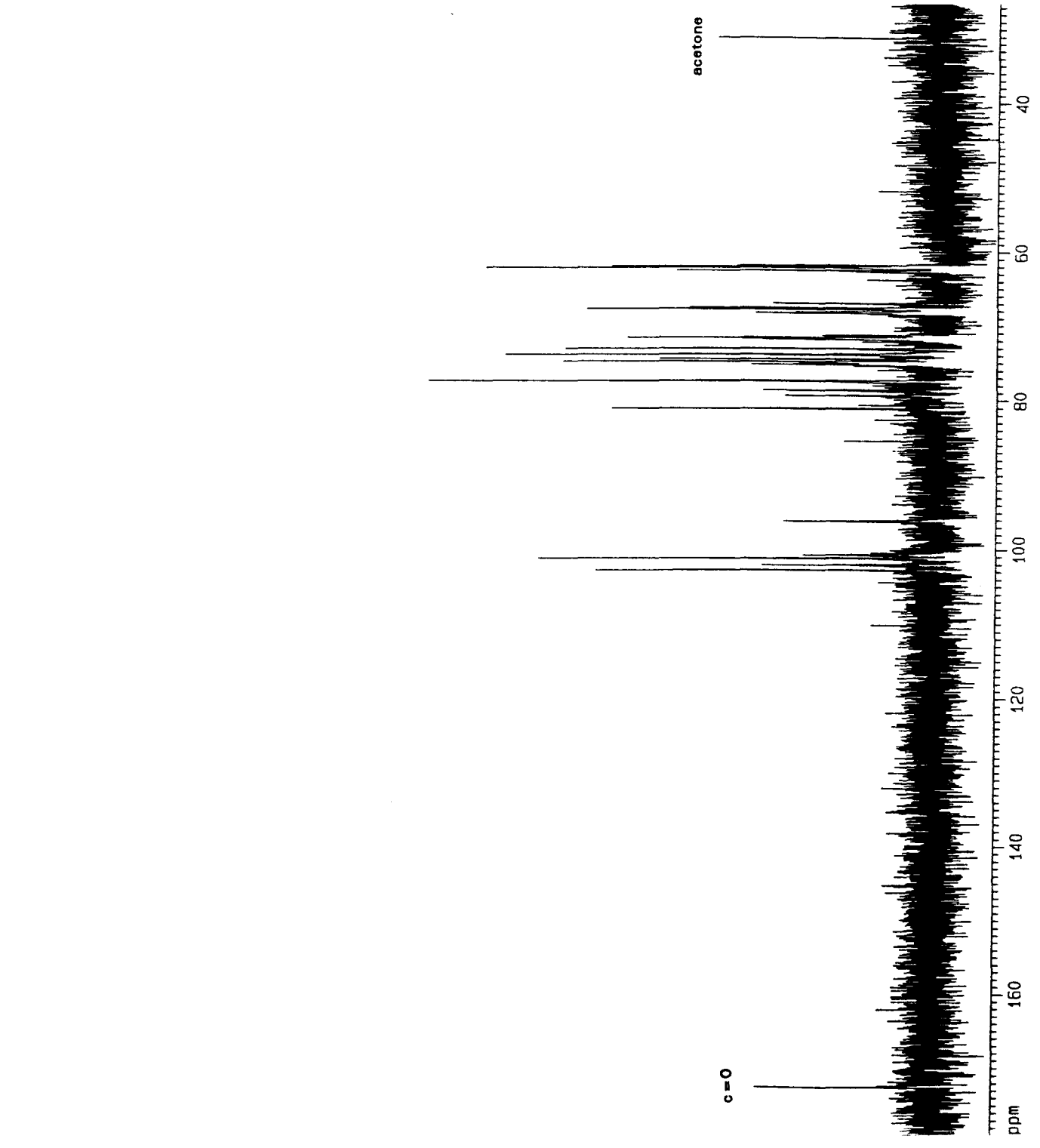
F2 - Processing parameters

SI	16384
SF	400.1355973 MH
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

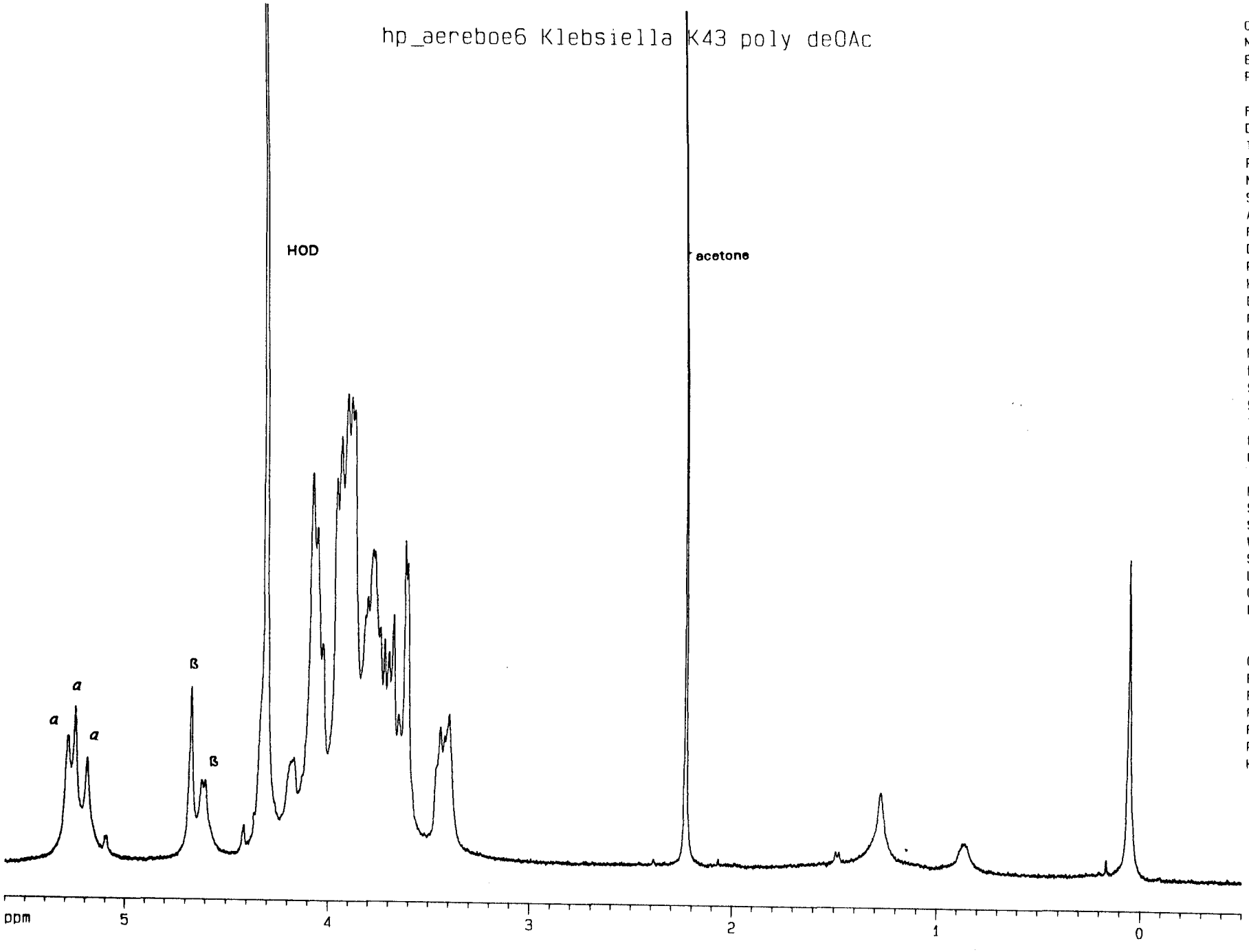
1D NMR plot parameters

CX	23.90 cm
F1P	5.400 pp
F1	2160.73 Hz
F2P	4.500 pp
F2	1800.61 Hz
PPMCM	0.03766 pp
HZCM	15.06787 Hz

Spectrum 2. **1D ^{1}H-NMR anomeric region expansion of the *Klebsiella* K43 polysaccharide**



pectrum 3. 1D ^{13}C-NMR of the *Klebsiella* K43 polysaccharide



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PROCNO	1

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D1	1.0000000 se
P1	13.0 us
RD	0.0000000 se
PW	0.0 us
DE	130.0 us
SFO1	400.1368820 MH
SWH	4807.69 Hz
TD	32768
NS	64
OS	2

F2 - Processing parameters

SI	16384
SF	400.1355630 MH
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

1D NMR plot parameters

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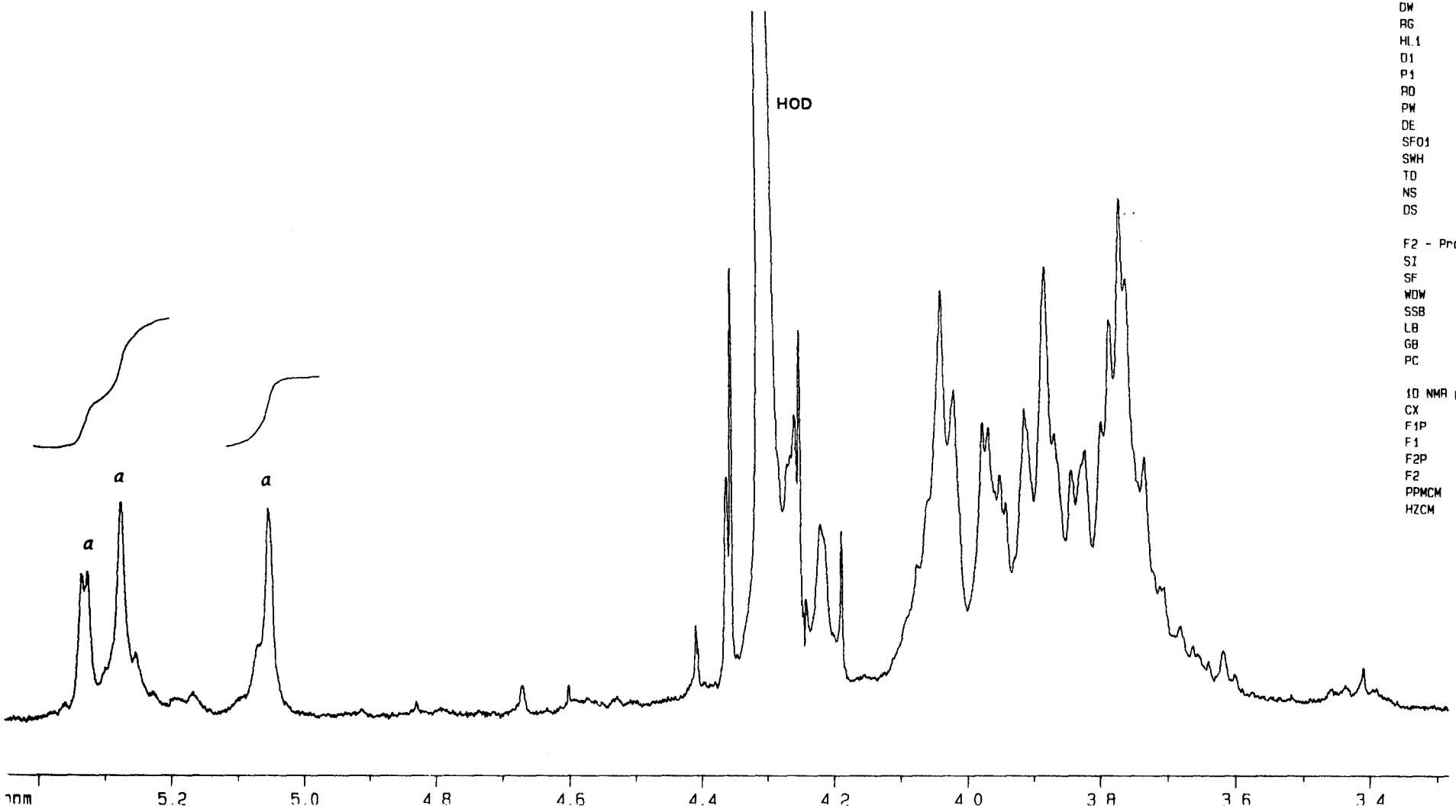
F1P	5.600 pp
F1	2240.76 Hz
F2P	-0.500 pp
F2	-200.07 Hz
PPMCM	0.25523 pp
HZCM	102.12665 Hz

Spectrum 4. 1D ^{1}H-NMR of the de-O-acetylated *Klebsiella* K43 polysaccharide

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PROCNO                  1

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FIDRES      0.097813 Hz
DW              156.0 usec
RG                  1024
HL1                    1 dB
D1          0.7000000 sec
P1                 13.0 usec
RD          0.0000000 sec
PW                  0.0 usec
DE                195.0 usec
SFO1      400.1367556 MHz
SWH          3205.13 Hz
TD                 32768
NS                   128
DS                     2

F2 - Processing parameters
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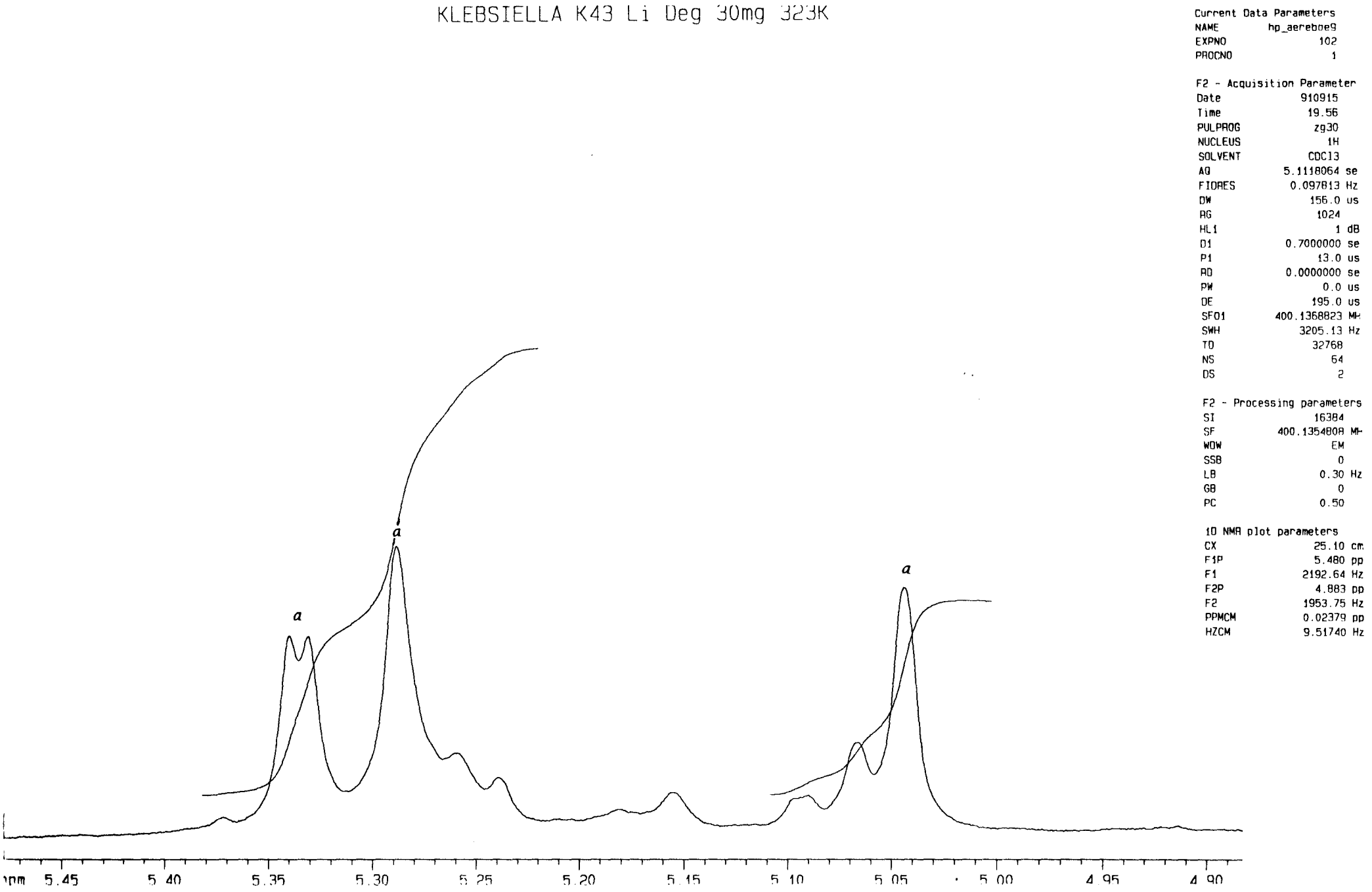
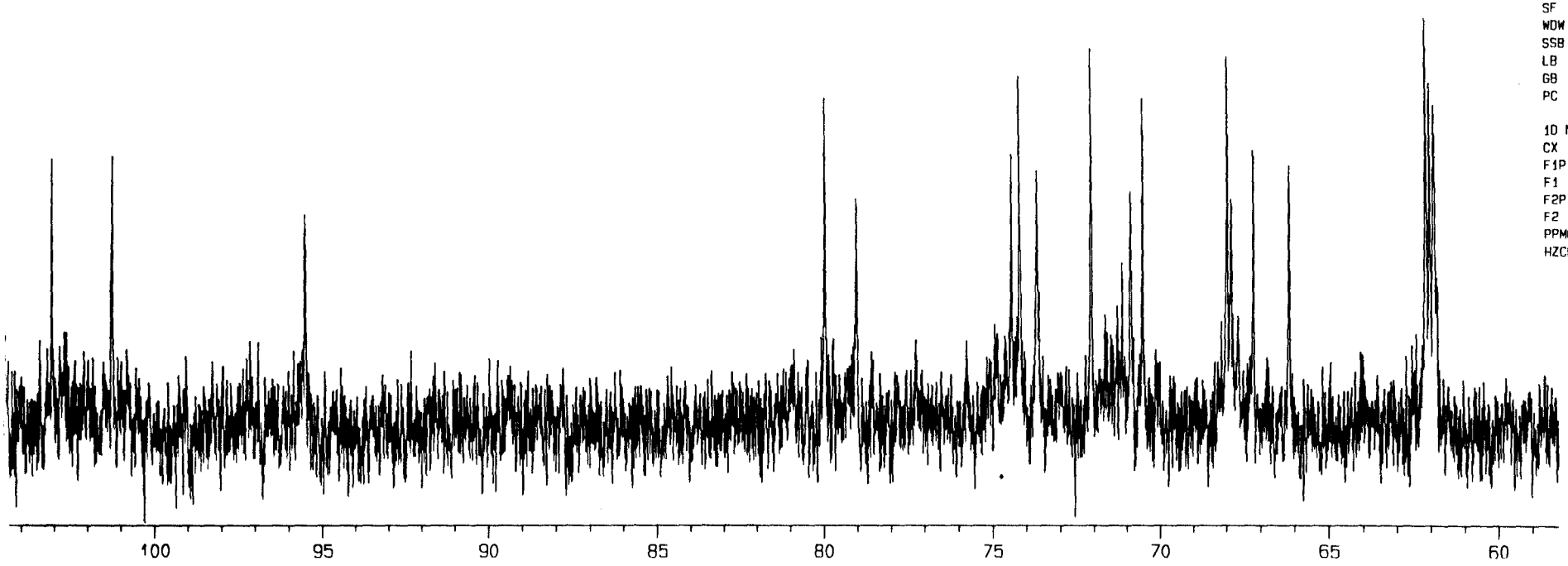
SF        400.1355645 MHz
WDW                   EM
SSB                    0
LB              0.30 Hz
GB                     0
PC                  0.50

1D NMR plot parameters
CX                25.10 cm
F1P                5.456 ppm
F1              2182.94 Hz
F2P                3.283 ppm
F2              1313.58 Hz
PPMCM          0.08656 ppm
HZCM          34.63595 Hz/cm
```


Spectrum 7. 1D ^{1}H – NMR of the lithium degraded *Klebsiella* K43 polysaccharide



Spectrum 8. **1D ^{1}H-NMR anomeric region expansion of the *Klebsiella* K43 lithium degraded polysaccharide**



Current Data Parameters

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PROCNO	1

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FIDRES	0.293438
DW	52.0
RG	32768
HL1	1
D1	0.7000000
D11	0.0300000
P1	7.5
RD	0.0000000
PW	0.0
DE	65.0
SFO1	100.6213932
SWH	9615.38
TD	32768
NS	1733
DS	2

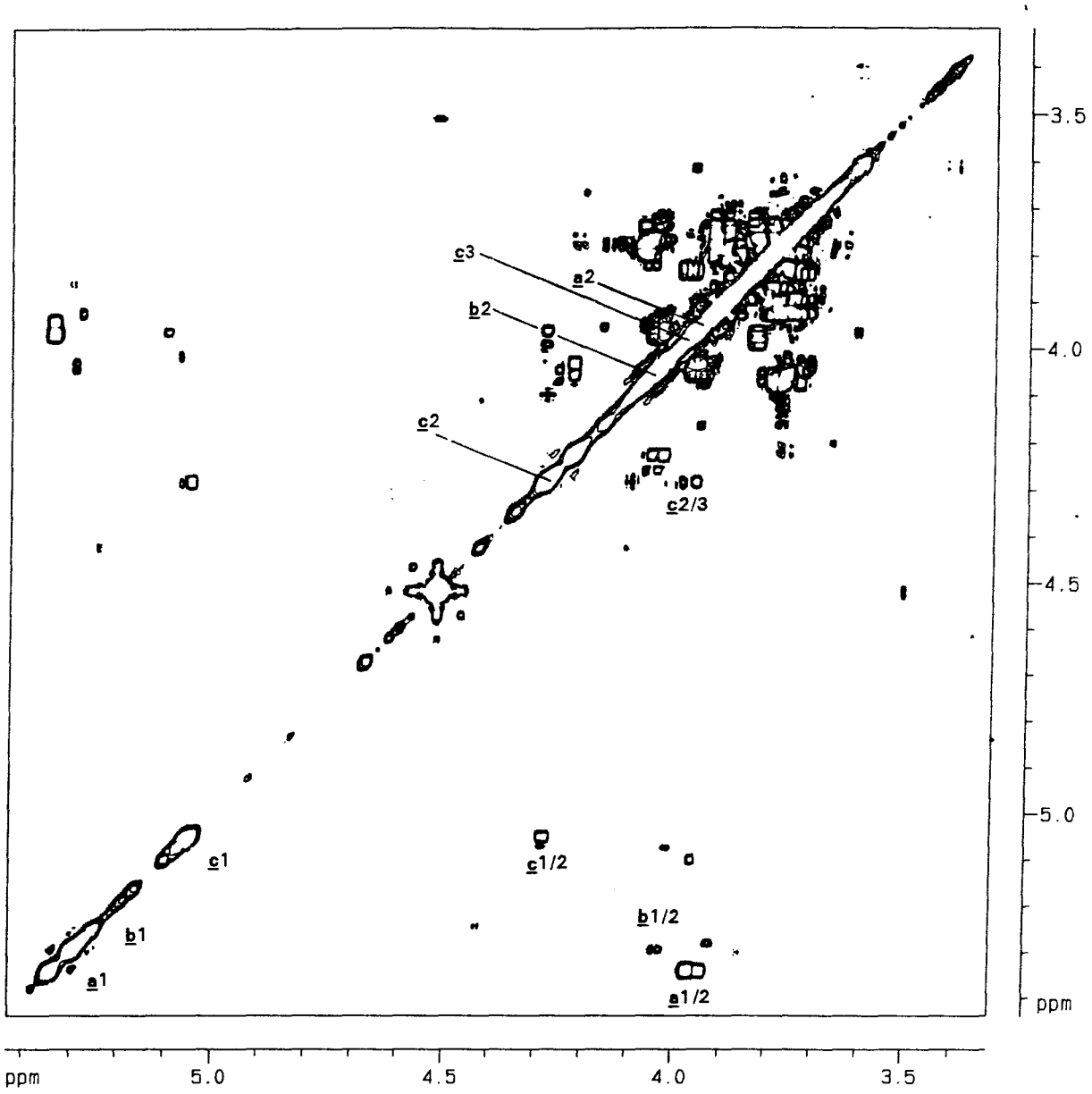
F2 - Processing paramet

SI	32768
SF	100.6140739
WDW	EM
SSB	0
LB	1.00
GB	0
PC	1.40

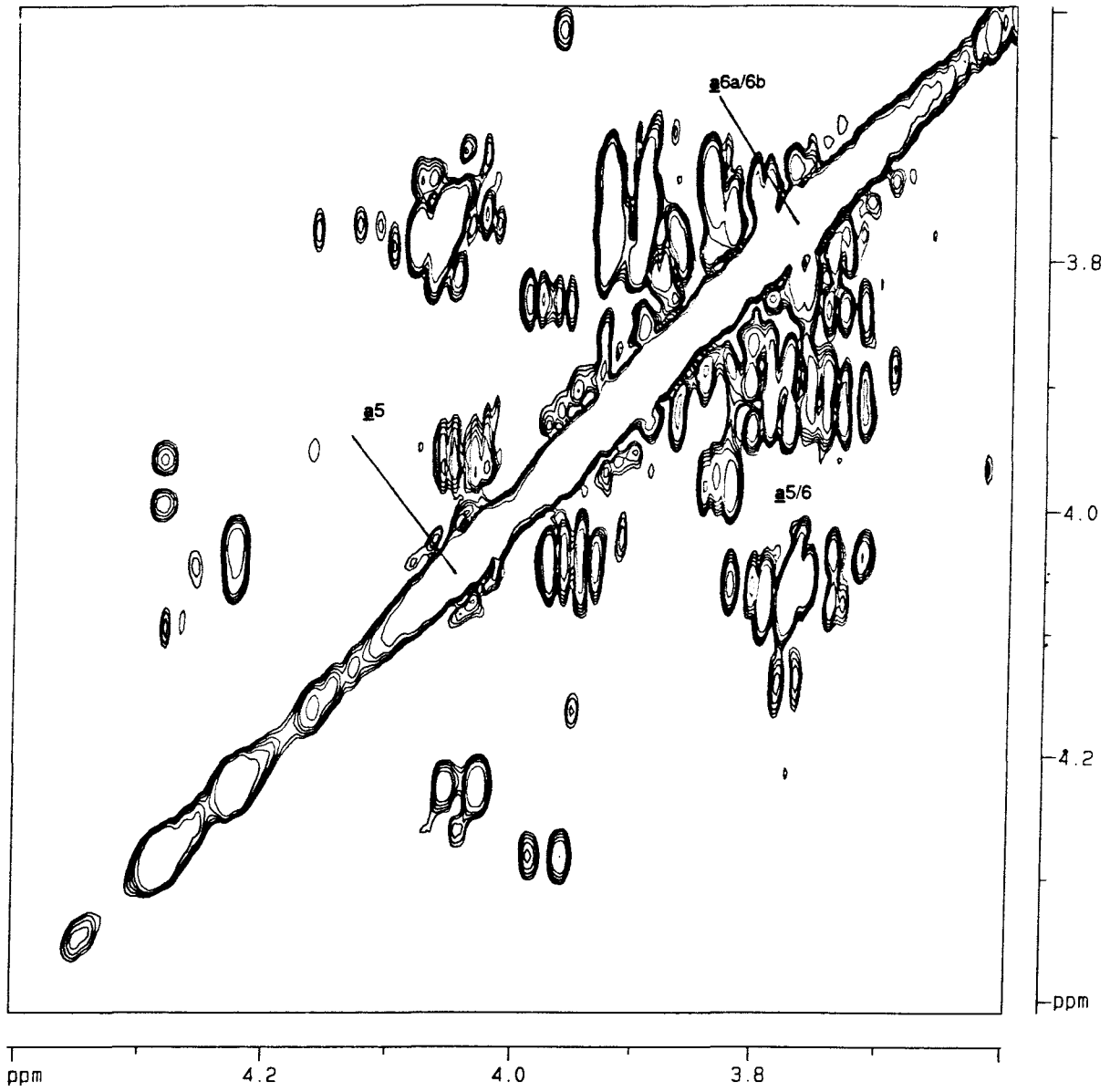
1D NMR plot parameters

CX	25.00
F1P	104.652
F1	10529.47
F2P	58.257
F2	5861.51
PPMCM	1.85579
HZCM	186.71851

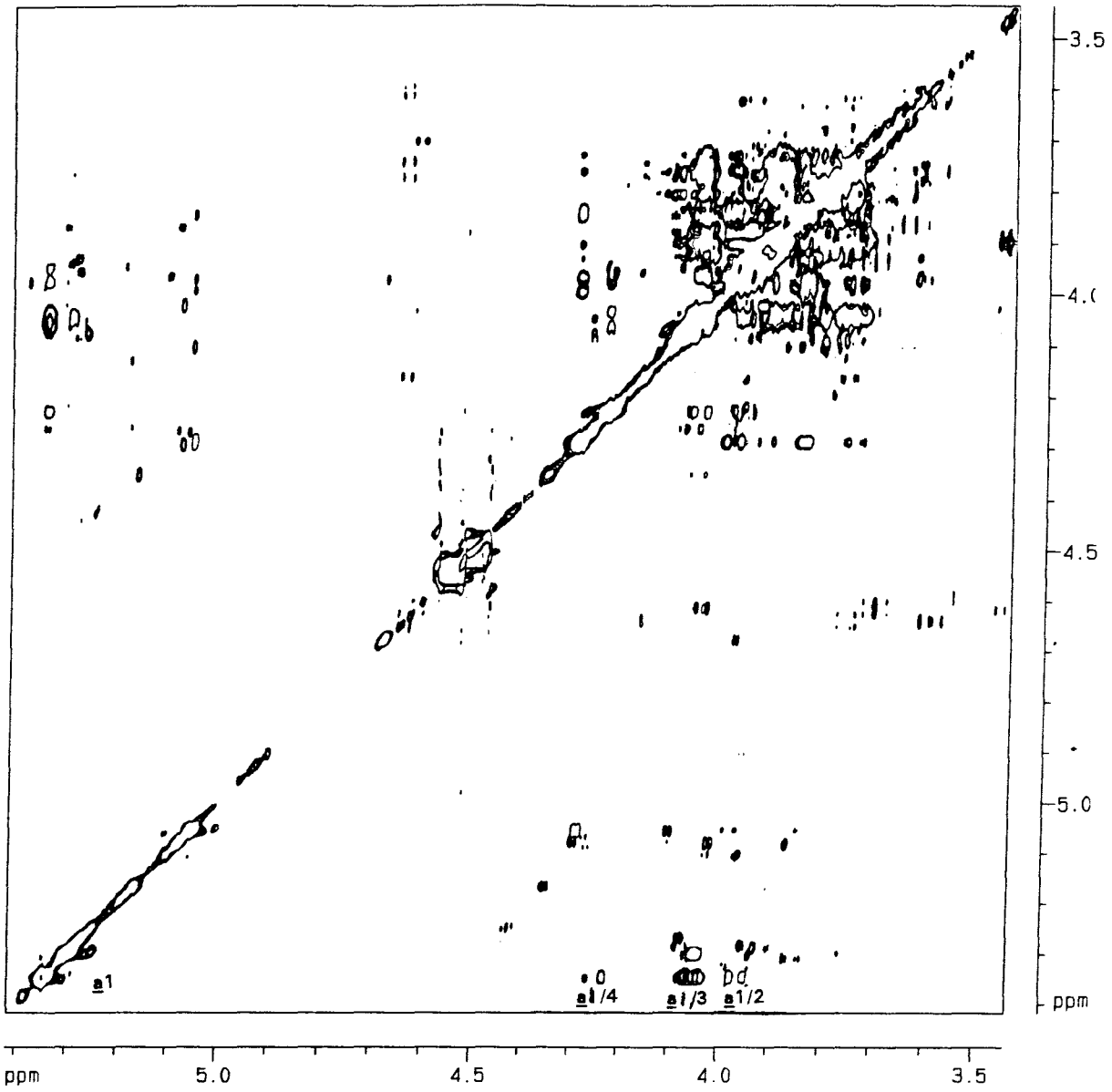
Spectrum 9. **1D ^{13}C-NMR of the *Klebsiella* K43 lithium degraded polysaccharide**



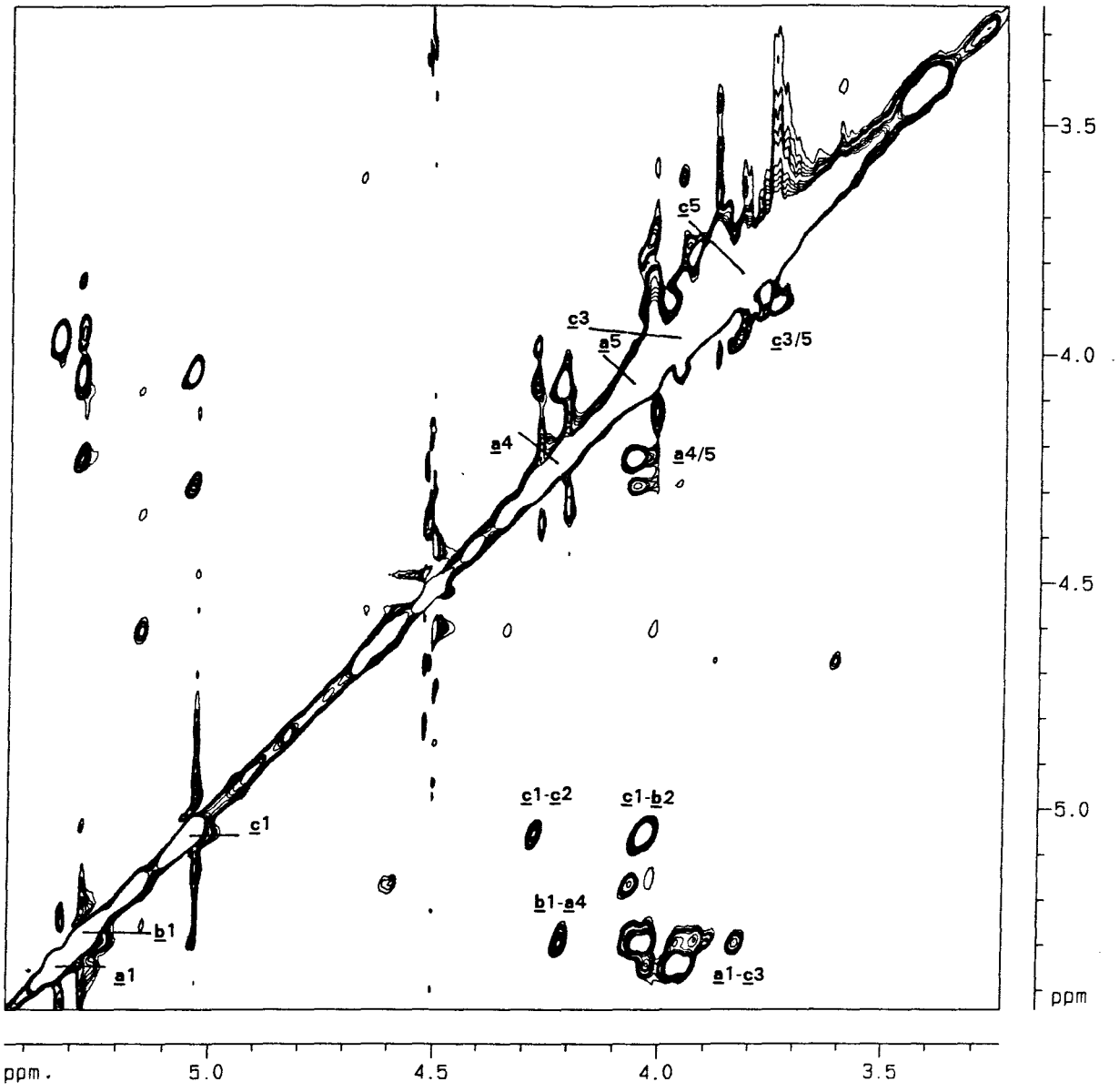
Spectrum 10. 2D ^{1}H-^{1}H-NMR COSY contour map of the *Klebsiella* K43 lithium degraded polysaccharide



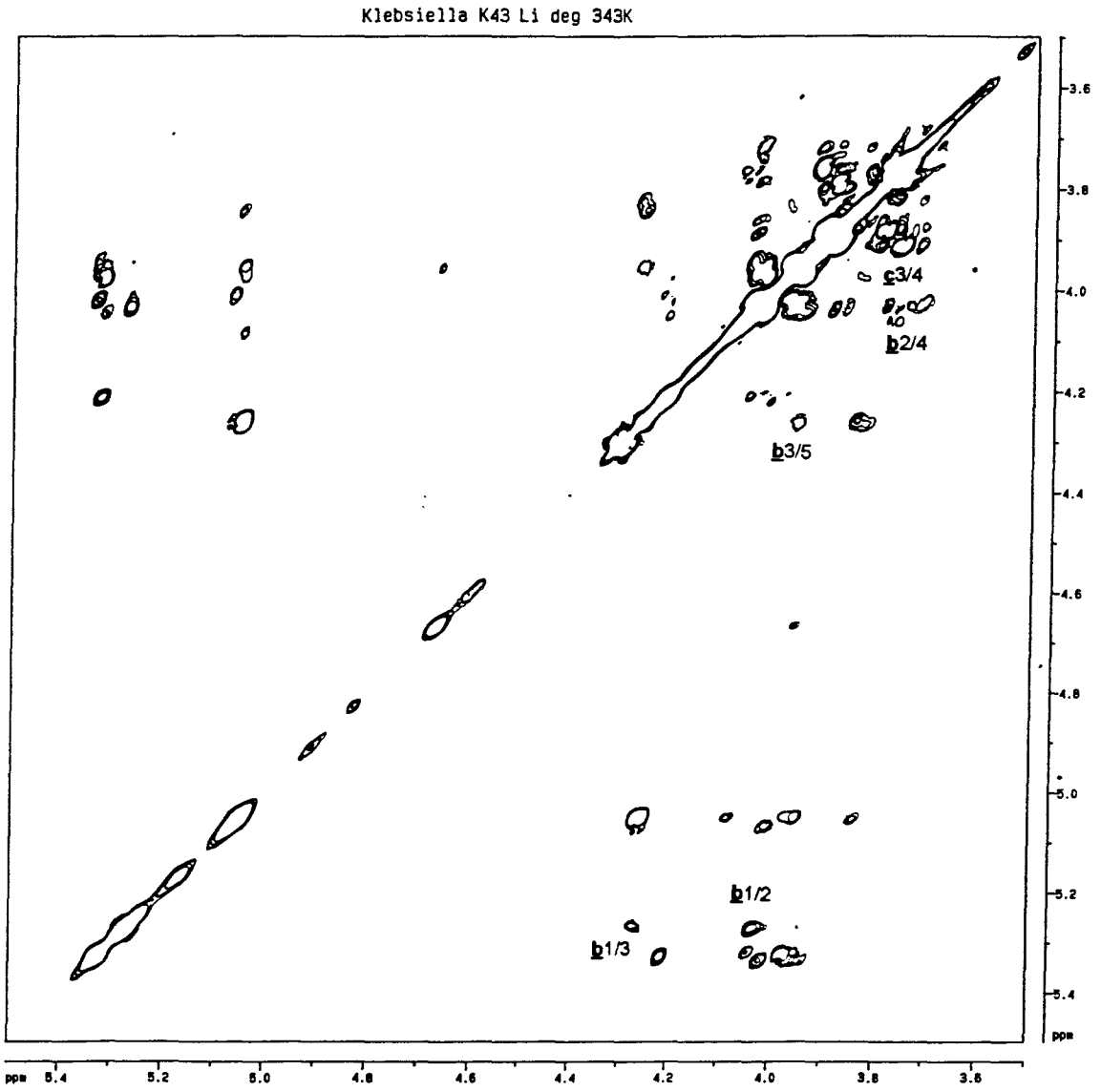
Spectrum 11. 2D ^{1}H-^{1}H COSY contour map expansion of the *Klebsiella* K43 lithium degraded polysaccharide



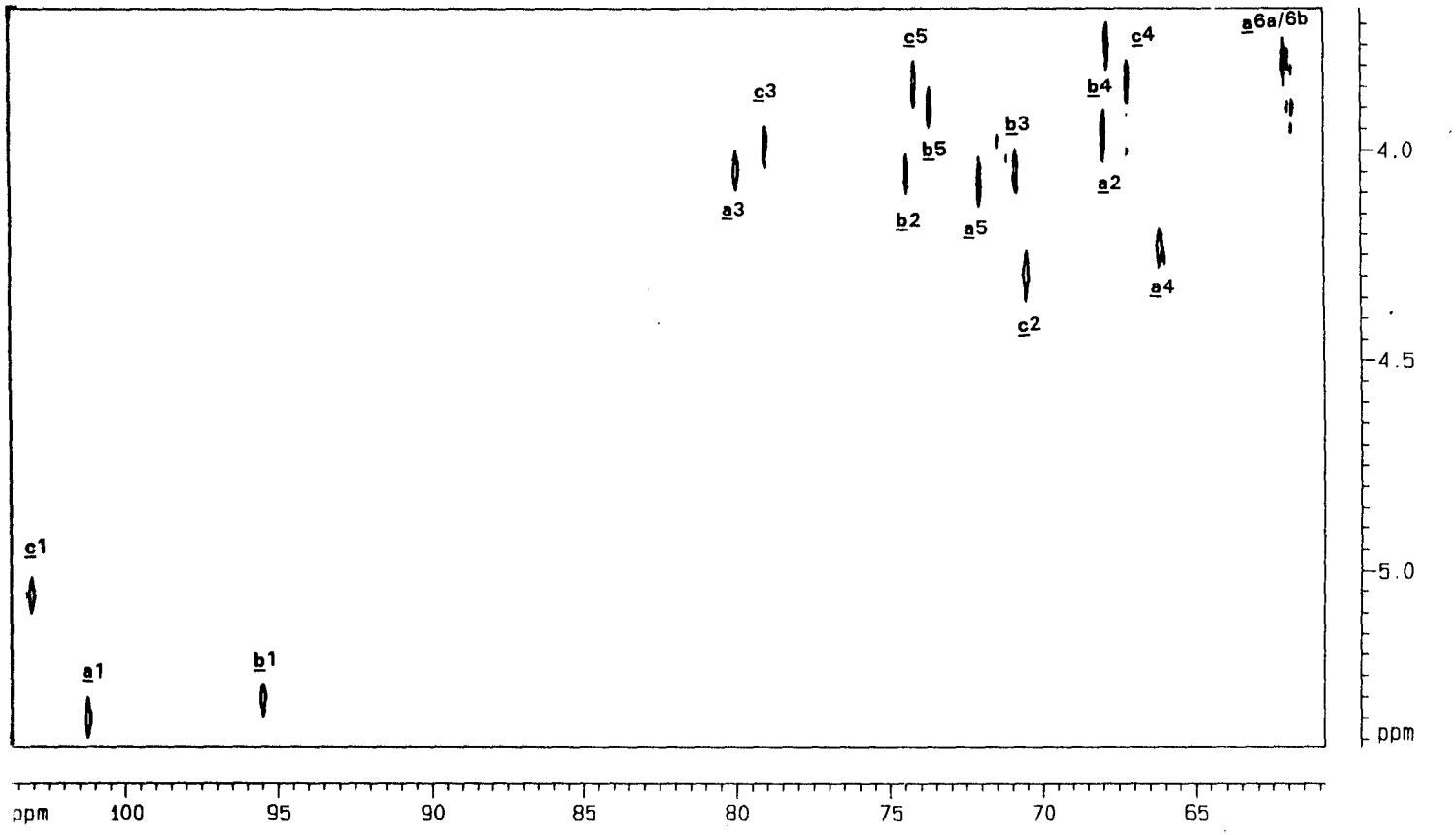
Spectrum 12. 2D ^{1}H-^{1}H – NMR HOHAHA contour map of the *Klebsiella* K43 lithium degraded polysaccharide



Spectrum 13. 2D ^{1}H-^{1}H-NMR NOESY contour map of the *Klebsiella* K43 lithium degradation



Spectrum 14. 2D ^{1}H-^{1}H-NMR Relay COSY contour map of the *Klebsiella* K43 lithium degraded polysaccharide



Spectrum 15. 2D ^{1}H-^{13}C-NMR HETCOR contour map of the *Klebsiella* K43 lithium degraded polysaccharide

6. PREPARATION AND NMR STUDY OF THE OLIGOSACCHARIDE REPEATING UNIT OF THE *KLEBSIELLA* K43 ANTIGEN

6.1. Introduction

Aqueous solutions of the native polysaccharide are viscous and this results in broad lines[242] in the proton NMR spectra, making complete structural analysis by 2D NMR methods difficult. Bacteriophages may carry endoglycanases[177] which are capable of depolymerising host capsular polysaccharide to oligosaccharidesof repeating unit size. The oligosaccharide produced retains all the structural information of the polysaccharide and gives excellent, well resolved spectra amenable to detailed 2D NMR structural studies. The *Klebsiella* K43[7] polysaccharide was depolymerised by its bacteriophage, and the resulting oligosaccharide P1 and reduced form P1-ol, were analysed by 2D NMR methods.

6.2. Results and discussion

6.2.(i). Depolymerisation of the exopolysaccharide antigen of *Klebsiella* K43 by a specific bacteriophage-borne endoglycanase

The *Klebsiella* K43[7] polysaccharide was depolymerised by a bacteriophage active on the host bacteria, by the method published by Okutani and Dutton[103].

The *Klebsiella* ø 43 (phage) was isolated by inoculating a liquid culture of *Klebsiella* bacteria with raw sewage water from Grahamstown sewerage works. The cellular material was precipitated the following day and the supernatant containing possible phage was accumulated. This potential phage solution was serially diluted and added to an equal volume of *Klebsiella* K43 suspension in nutrient broth (Annexure 1, formula 9.2), mixed with sloppy agar (Annexure 1, formula 9.3), and overlaid over nutrient agar (Annexure 1, formula 9.4). After incubation over night large haloes were seen on the plates, indicative of vigorous phage activity. The phage was isolated by "picking" a small amount of agar from the centre of a halo, extracting the phage, and

repeating the overlay and picking technique three times. Bulk phage propagation was carried out in order that a volume of phage could be generated that had approximately 10^{13} plaque forming units (PFU's), that concentration estimated to be sufficient to depolymerise 1 g of bacterial polysaccharide. The phage was assayed for PFU number by comparison of the phage ability to form haloes on solid agar inoculated with a known serial dilution of *Klebsiella* bacteria. The crude phage solution was then dialysed (12-14 000 Mw) against running water for 48 hours and assayed again for strength.

The purified *Klebsiella* K43[7] exopolysaccharide was then committed to the phage solution for three days in a mechanical shaking water bath set at 37°C. The viscosity of the polysaccharide solution dropped rapidly overnight as the polysaccharide was digested. After 3 days the solution was freeze-dried, reconstituted with a minimum volume of water and dialysed for a further 3 days against distilled water. The dialysate was freeze-dried, subjected to gel permeation chromatography and showed the following profile, (Figure 36). P1 refers to the

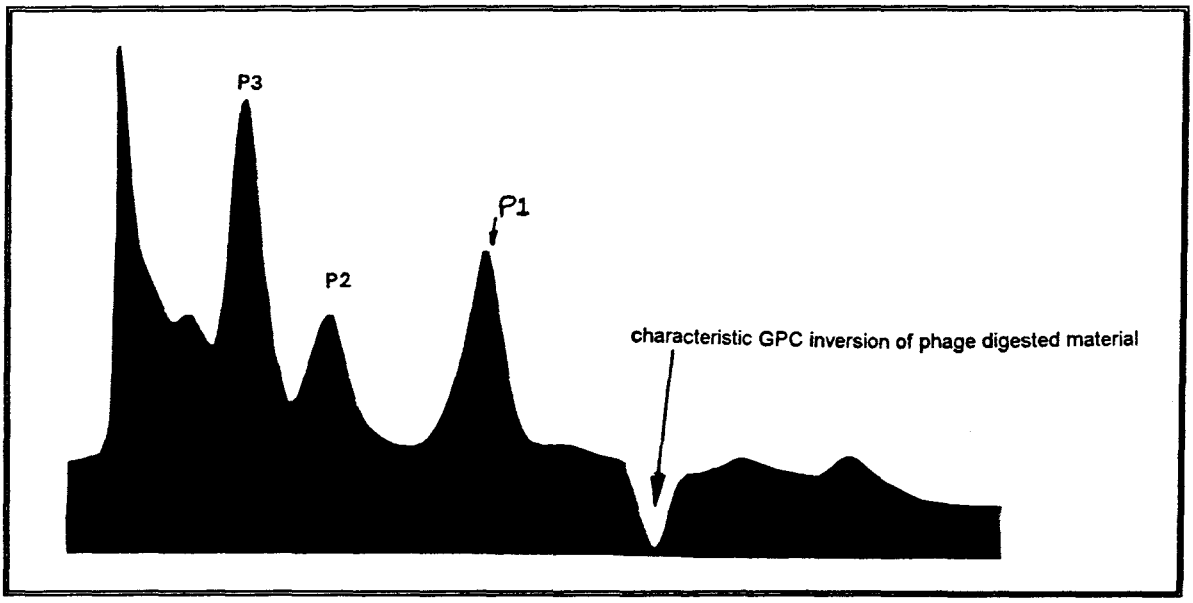


FIGURE 36. GPC chromatograph of the bacteriophage mediated depolymerisation of the K43 antigen

The degraded material was then committed to a preparative GPC column of Biogel P4, eluted with a buffer and an oligosaccharide fraction corresponding to the retention volume of a

pentasaccharide was isolated.

6.2.(ii). **Methylation analysis of the repeating unit (P1)**

A portion of the oligosaccharide P1 was reduced with $NaBD_4$, to label[223] the reducing end, and was then methylated according to the modified Hakomori[130] method. The product was then methanolysed, reduced, hydrolysed and converted to the PMAA's which were analysed by GLC-MS (Table 11, column 1).

Table 11.

		1
Partially Methylated Alditol Acetates	Ta	Reduced repeating unit (P1-ol)
2,3,4,6-tetra-O-methylglucitol	1.00	
2,3,4,6-tetra-O-methylmannitol	0.97	0.46
3,4,6-tri-O-methylmannitol	1.52	2.00
2,3-di-O-methylglucitol	3.00	0.60

T_a retention time relative to 2,3,4,6-tetra-O-methylglucitol.

All material was run on a OV 225 capillary column.

A small quantity of deuterium labelled pentamethylated hexitol, (lost due to volatility, was not integrated in the GLC analysis, Table 11), was detected by GLC-MS, and allowed the galactose to be assigned as the reducing end. The results accord with a pentasaccharide repeating unit and indicate that the depolymerised polysaccharide (P1), is present as a linear oligosaccharide, having a terminal mannose residue, a 4-linked glucuronic acid, two 2-linked mannose residues and a 3-linked galactose residue. Thus far the accumulated data suggest that the material isolated from the bacteriophage depolymerisation is the repeating unit.

6.2.(iii). Reduction of P1 and NMR investigation of the repeating unit alditol P1-ol

P1 was reduced ($NaBH_4$) to form the alditol P1-ol, the formation of which simplified the 2D NMR spectrum by replacing the α- and ß-anomers of galactose with the alditol, thereby removing complications due to mutarotation. This enabled assignment of the P1-ol structure.

The ^{1}H-NMR spectrum of the P1-ol, (Spectra 16-17, page 128-129) recorded at 30°C, contained H-1 resonances for *a*-residues at δ 5.26, 5.20, and ß-residues at δ 4.65 and δ 4.62. The resonances at δ 5.26 and δ 5.20 were assigned to two *a*-D-Manp residues, and δ 4.62 was assigned to a ß-D-GlcAp which relayed as far as C-5, (expected for uronic acid residues). The ^{13}C-NMR spectrum, (Spectrum 18, page 130) contained C-1 signals at 100.46, 100.84, 100.92, 102.19 ppm, and signals for CH_2OH at 61.47, 61.72, 61.78, 63.83 and 63.98 ppm. The observed ^{31}C-NMR signals are thus consistent with the presence of 3 hexoses, a hexuronic acid and an alditol.

The chemical shifts of most of the ^{1}H and ^{13}C resonances, (Table 13, page 126) were established mainly from ^{1}H-^{1}H homonuclear correlation spectroscopy (COSY, Relay COSY, and HOHAHA), heteronuclear ^{1}H-^{13}C (HETCOR) and inverse detected ^{1}H-^{13}C correlation, (HMQC-TOCSY and HMBC) experiments. The residues were labelled arbitrarily **a** to **e** in order of decreasing chemical shift of the H-1 resonances.

2D-NMR INVESTIGATION INTO THE REDUCED REPEATING UNIT STRUCTURE

Residue a. The chemical shifts for H-1/2 resonances were established from the COSY contour map, (Spectrum 19, page 131) and the connectivities up to H-4 were noted in the HOHAHA contour map (Spectrum 20, page 132).

Residue b The chemical shifts for the H-1/2 and H-2/3 connectivities were established from the COSY contour map, while resonances to H-4 were noted in the Relay COSY, (Spectrum 21, page 133) and in the H-1 track of the HOHAHA experiment. In addition connectivities to H-5 were observed in the H-2 track of the HOHAHA spectrum.

Residue c The chemical shifts for the H-1 to H-6a/6b resonances were established completely from the COSY spectrum.

Residue d The chemical shifts for the H-1/2 and H-2/3 connectivities were established from the COSY contour map, while resonances to H-4 were established in the H-2 track of the Relay COSY contour map. Returning to the COSY spectrum the H-4/H-5 cross peak was identified and the chemical shift for H-5 established. The assignments were confirmed in the H-1 track of the HOHAHA spectrum. The ^{1}H chemical shifts, above, were compared with the ^{1}H-^{13}C correlation data obtained from the HETCOR experiment (Spectra 22 + 23, page 134-135), and permitted the unambiguous assignment of all the ^{13}C resonances for residue **c** and **d**, C-1 to C-4 of residue **a**, and C-1 to C-5 of residue **b**. The assignment of the chemical shifts for C-5/H-5 and C-6/H-6a,6b for residue **a**, C-6/H-6a,6b for residue **b**, and all of the $^{13}C/^{1}H$ resonances for the $\rightarrow$ 3)-galactitol, residue **e**, were accomplished mainly from an HMBC (Heteronuclear Multiple Bond Correlation) experiment, (Spectrum 24, page 136) measuring through-bond connectivity between C and H atoms, two and three bonds distant. Thus H-4 of **b** showed a correlation to ^{13}C resonance at 61.47 ppm which was assigned to C-6 of this residue. The chemical shifts for H-6a and H-6b of **b** followed from the ^{1}H-^{13}C correlation data.

Residue e On the basis of literature values, which show that the chemical shifts for unlinked primary carbon atoms of alditols occur between 1-1.5 ppm lower field than those of hexopyranoses, the unassigned sets of $^{13}C/^{1}H$ resonances: 63.83 ppm/δ 3.709 and 63.98 ppm/δ 3.726 were assigned to the C-1/H-1 and C-6/H-6a,6b resonances of **e**. The remaining $^{13}C/^{1}H$

resonances for **e** were established by comparing the correlations established from the HMBC experiment (Spectrum 24, page 136) with the ^{1}H-^{13}C correlation data form the HECTOR experiment (Spectra 22,23, page 134-135). The correct sequence of the carbon atoms in **e** was established by assigning the most downfield ^{13}C resonance of **e** to the linkage carbon, C-3 (known from methylation analysis). The HMQC-TOCSY spectrum (Spectrum 25, page 137) supported the sequence of the carbon atoms in the alditol **e**.

Comparison of the NMR data for residues **a** - **e** with those for model compounds identified the residues in P1-ol as indicated on. The significant deshielding of the C-2 of **a**, C-2 of **b** and C-4 of **d**, identified these as residue linkage positions. These results accord with the methylation results for P1-ol. The remaining two sets of $^{1}H/^{13}C$ chemical shifts could now be assigned by inspection to C-5/H-5 and C-6/H-6a,6b of residue **a**.

The sequence of the residues **a** - **e** (P1-ol) was established from an HMBC experiment, (Spectrum 24, page 136). The relevant 3 bond relayed connectivities are listed in table 12.

Table 12.

HMBC Correlation data for P1-ol

Residue	Proton Shift(δ)	correlated residue (carbon)	
a (H-1)	5.26	**e** (C-3)	79.16
b (H-1)	5.20	**a** (C-2)	80.00
c (H-1)	4.65	**d** (C-4)	80.80
d (H-1)	4.611	**b** (C-2)	78.46

Table 13 shows a comparison of chemical shift and coupling constant data for P1-ol with that for methyl glycosides and galactitol(see table 5 page 74), and allows residues **a** and **e** to be identified:

a →2)-*a*-**D**-mannose

b →2)-*a*-**D**-mannose

c β-**D**-mannose

d →4)-β-**D**-glucuronic acid

e →3)-galactitol

The following sequence may be written for the P1-ol oligosaccharide

P1-ol	**c**-(1→4)-**d**-(1→2)-**b**-(1→2)-**a**-(1→3)-**e**

or

β-D-Manp-(1→4)-β-D-GlcAp-(1→2)-*a*-D-Manp-(1→2)-*a*-D-Manp-(1→3)-galactitol

The reduced oligosaccharide of the *Klebsiella* K43 exopolysaccharide degraded by a bacteriophage enzyme

Table 13. P1-ol ^{1}H- and ^{13}C-NMR shift data for *Klebsiella* K43. (30°C)

		1	2	3	4	5	6a	6b
a	H	**5.256**	**4.059**	**3.956**	**3.740**	**3.730**	**3.800**	**3.905**
	S	4.77	3.93	3.76	3.64	3.62		
	C	**100.46**	**80.00**	**70.74**	**67.54**	**74.53**	**61.72**	
	S	101.9	71.2	71.8	68.0	73.7		
b	H	**5.192**	**4.228**	**3.884**	**3.692**	**3.786**	**3.785**	**3.883**
	S	4.77	3.93	3.76	3.64	3.62	3.76	3.90
	C	**100.84**	**78.46**	**70.29**	**67.75**	**74.04**	**61.49**	
	S	101.9	71.2	71.8	68.0	73.7	62.1	
c	H	**4.651**	**3.988**	**3.630**	**3.572**	**3.387**	**3.734**	**3.930**
	S	4.58	3.99	3.64	3.57	3.38	3.74	3.94
	C	**100.92**	**71.35**	**73.57**	**67.44**	**77.26**	**61.78**	
	S	101.3	70.6	73.3	67.1	76.6	61.4	
d	H	**4.611**	**3.445**	**3.697**	**3.839**	**4.053**		
	S	4.38	3.26	3.49	3.38	3.47		
	C	**102.19**	**72.84**	**74.53**	**80.81**	**74.53**		
	S	104.0	74.1	76.8	70.6	76.8		
e	H	**3.71**	**3.83**	**3.82**	**3.89**	**4.058**	**3.726**	**3.726**
	S							
	C	**63.83**	**71.26**	**79.16**	**70.10**	**71.84**	**63.98**	
	S	64.5	71.5	70.7	70.7	71.5	64.5	

Chemical shifts in ppm relative to internal acetone at δ 2.23 for ^{1}H- and at 31.07 ppm for ^{13}C-NMR

* Note residue **d** standard is methyl glucoside and not glucuronic acid, but serves as an indicator of shift for α-methyl glucuronic acid.

"S" refers to standard methyl glycosides :

a S= methyl-α-**D**-mannose

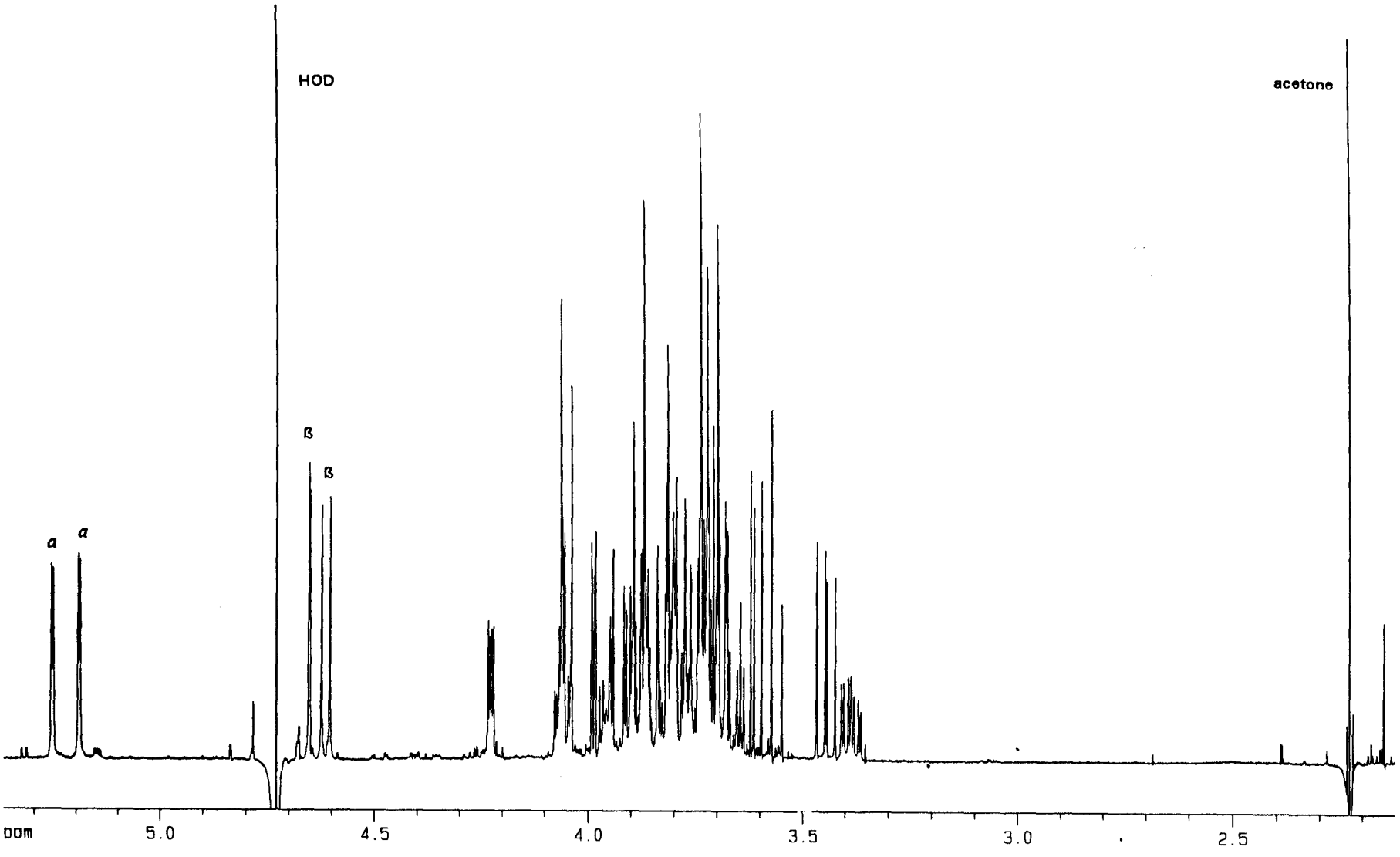
b S= methyl-α-**D**-mannose

c S= methyl-β-**D**-mannose

d S= methyl-β-**D**-glucose

e S= galactitol

- Underlined carbon shifts indicate points of linkage



Current Data Parameters

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EXPNO	100
PROCNO	1

F2 - Acquisition Parameter

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FIDRES	0.097813 Hz
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RG	1024
NUCLEUS	1H
TE	303.0 K
HL1	1 dB
D1	1.0000000 se
P1	13.0 us
DE	195.0 us
SFO1	400.1368010 MH
SWH	3205.13 Hz
TD	32768
NS	1024
DS	2

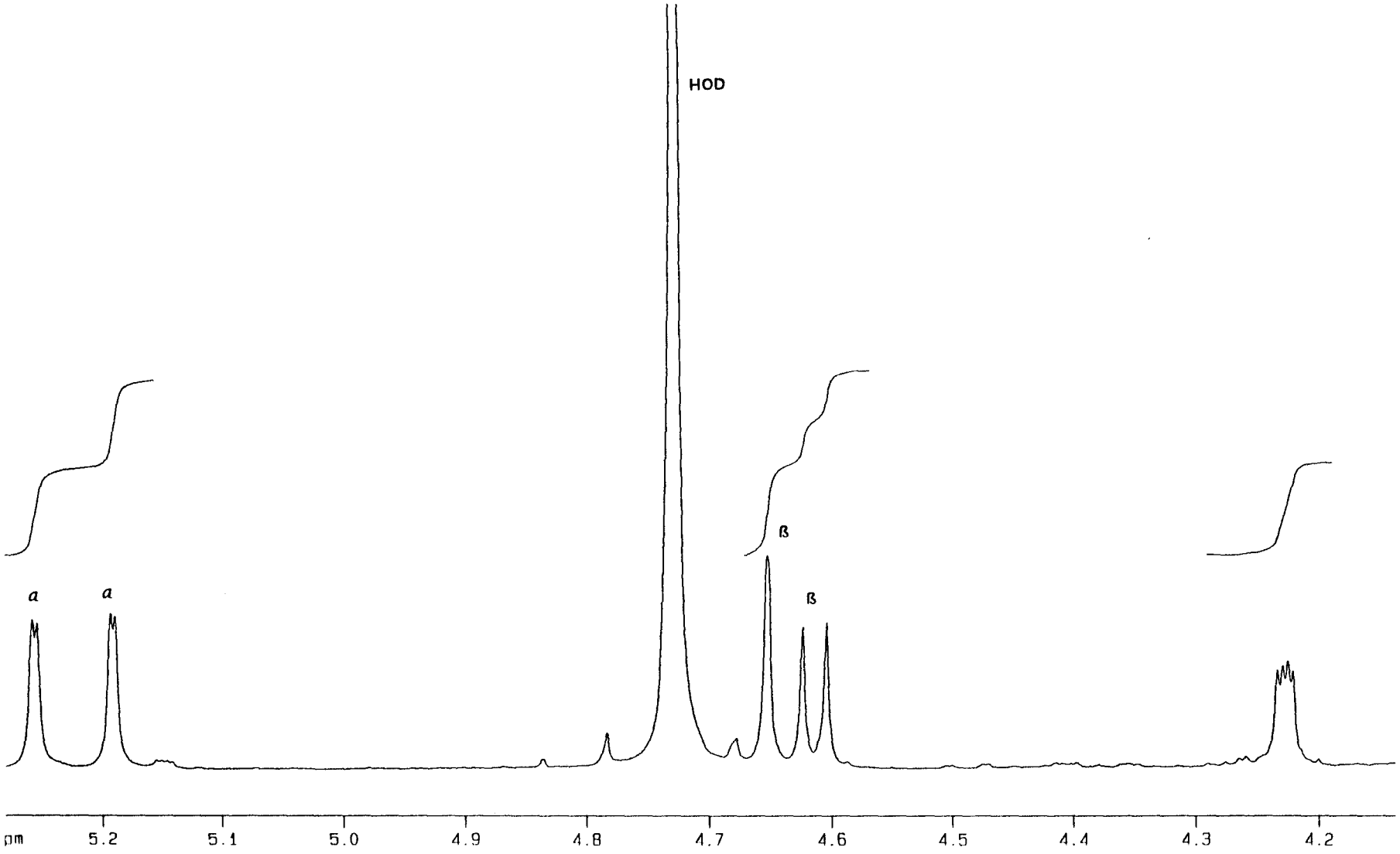
F2 - Processing parameters

SI	16384
SF	400.1353895 MH
WDW	GM
SSB	0
LB	-1.00 Hz
GB	0.2
PC	1.00

1D NMR plot parameters

CX	24.00 cm
F1P	5.374 pp
F1	2150.50 Hz
F2P	2.126 pp
F2	850.51 Hz
PPMCM	0.13537 pp
HZCM	54.16631 Hz

Spectrum 16. 1D ^{1}H-NMR of the *Klebsiella* K43 P1-ol



Current Data Parameters

NAME	hp_aereboe14
EXPNO	100
PROCNO	1

F2 - Acquisition Parameter

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SOLVENT	D2O
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FIDRES	0.097813 Hz
DW	156.0 us
RG	1024
NUCLEUS	1H
TE	303.0 K
HL1	1 dB
D1	1.0000000 se
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SWH	3205.13 Hz
TD	32768
NS	1024
DS	2

F2 - Processing parameters

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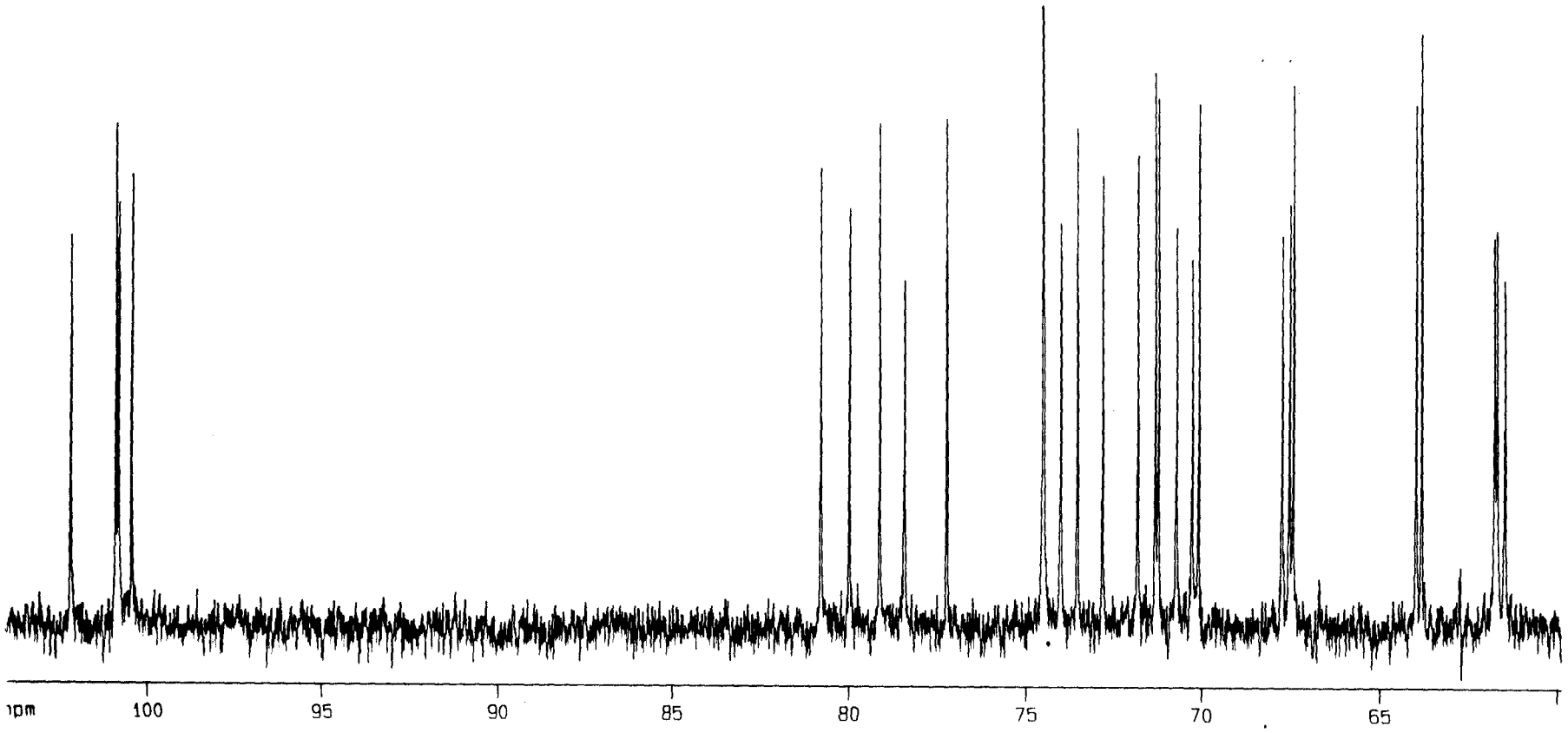
SF	400.1353895 MH
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	24.00 cm
F1P	5.290 pp
F1	2116.68 Hz
F2P	4.137 pp
F2	1655.25 Hz
PPMCM	0.04805 pp
HZCM	19.22609 Hz

1D ^{1}H NMR anomeric region expansion of the *Klebsiella* K43 P1-ol



Current Data Parameters
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EXPNO 200
PROCNO 1

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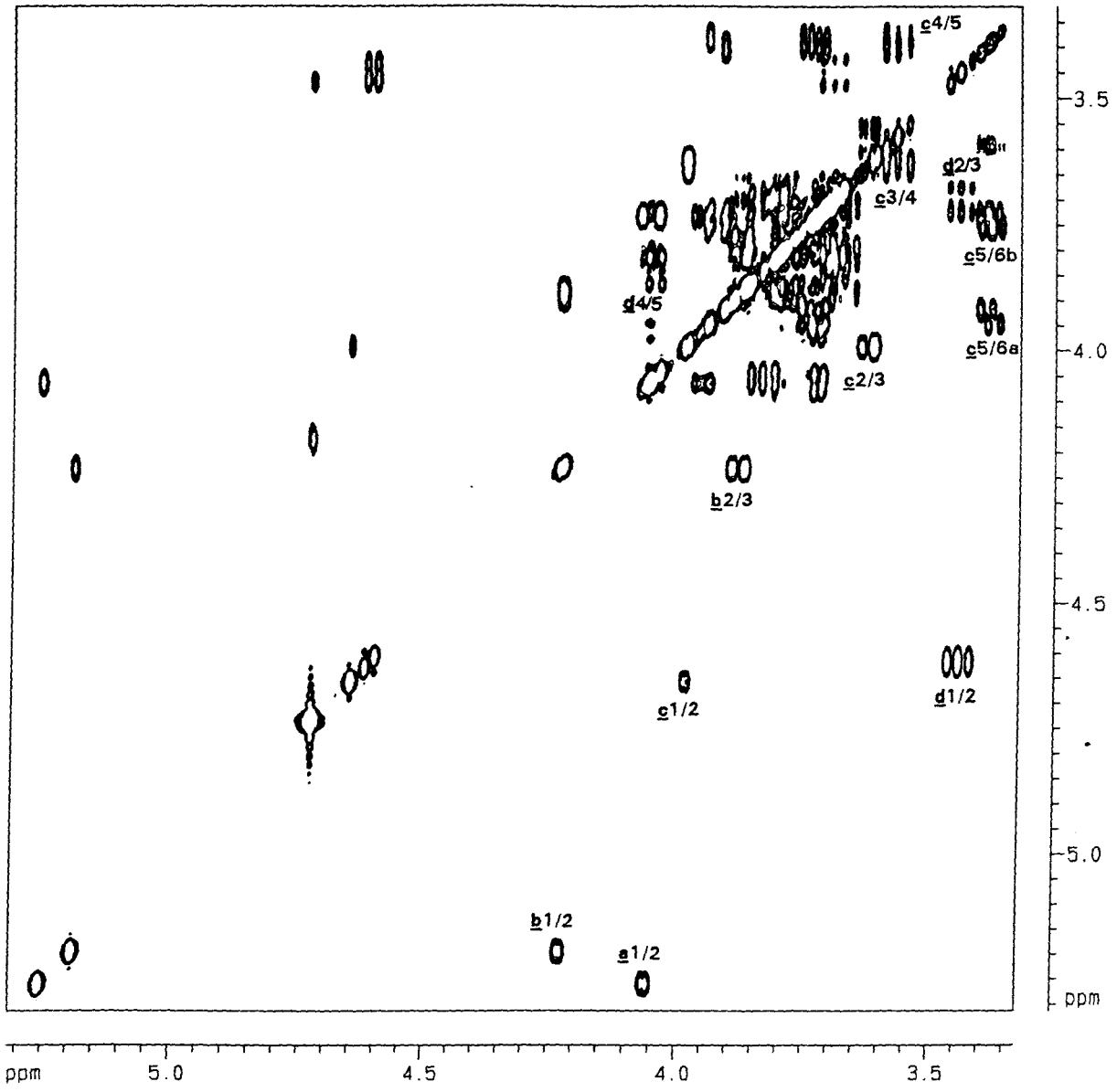
FIDRES 0.803094 Hz
DW 19.0 use
RG 32768
NUCLEUS 13C
TE 303.0 K
HL1 1 dB
D4 1.0000000 sec
P31 100.0 use
S4 26 dB
D11 0.0300000 sec
S2 26 dB
P1 7.5 use
DE 23.8 use
SFO1 100.6271180 MHz
SWH 26315.79 Hz
TD 32768
NS 39229
DS 2

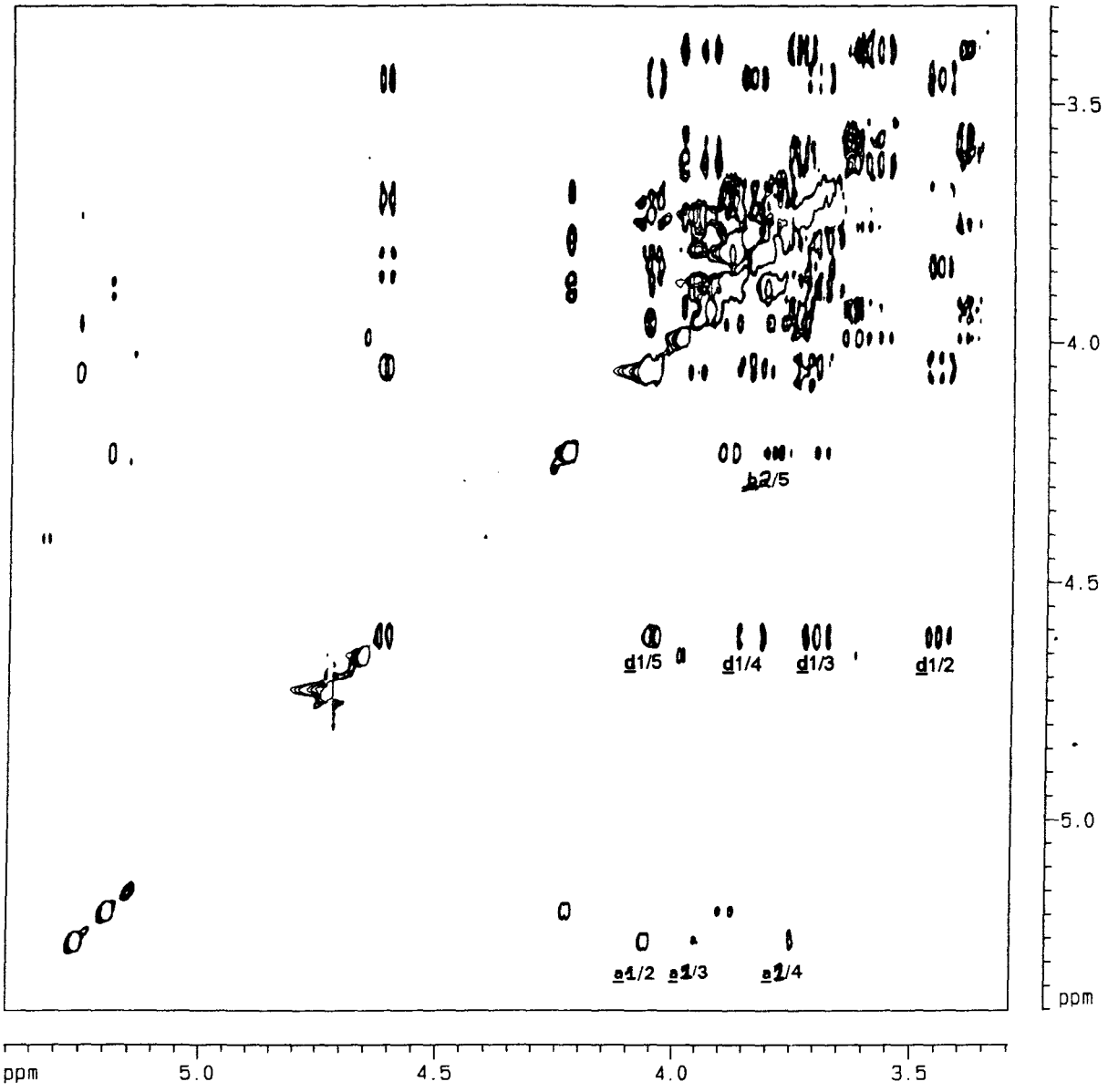
F2 - Processing parameters
SI 32768
SF 100.6140480 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

1D NMR plot parameters
CX 23.90 cm
F1P 103.078 ppm
F1 10371.07 Hz
F2P 99.487 ppm
F2 10009.75 Hz
PPMCM 0.15026 ppm
HZCM 15.11812 Hz/

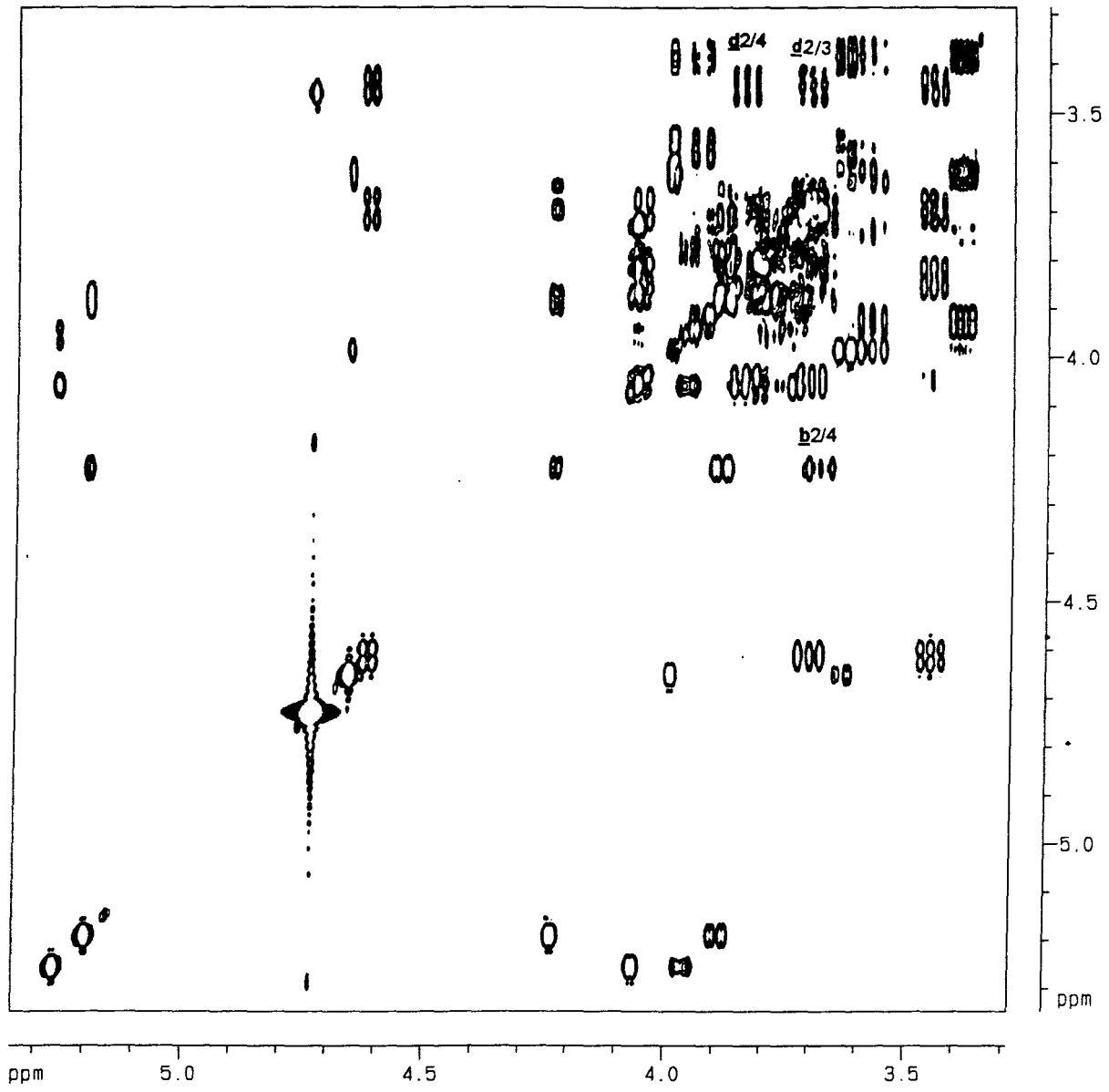
1D ^{13}C NMR of the *Klebsiella* K43 P1-ol



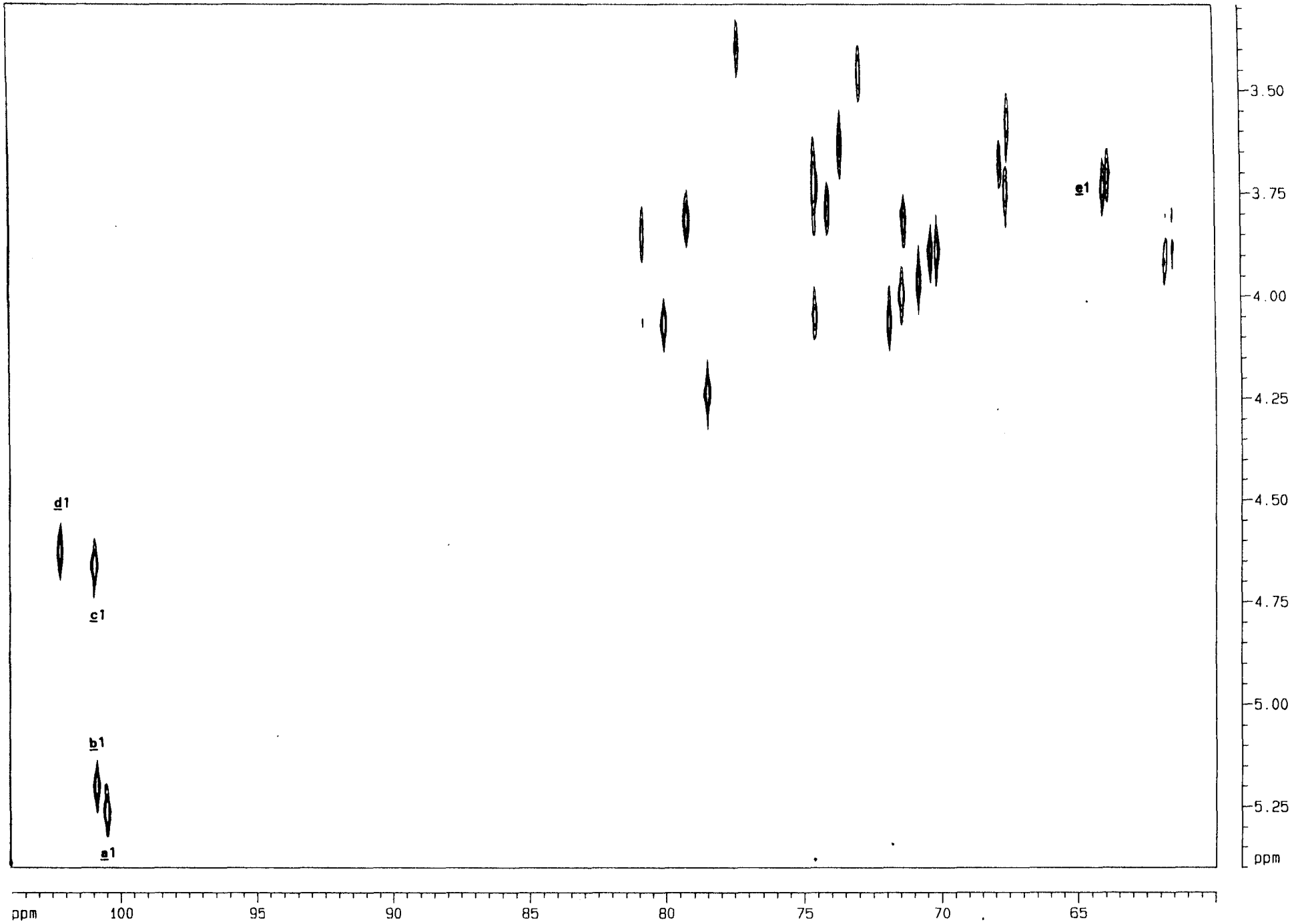
Spectrum 19. 2D ^{1}H-^{1}H-NMR COSY contour map of the *Klebsiella* K43 P1-ol



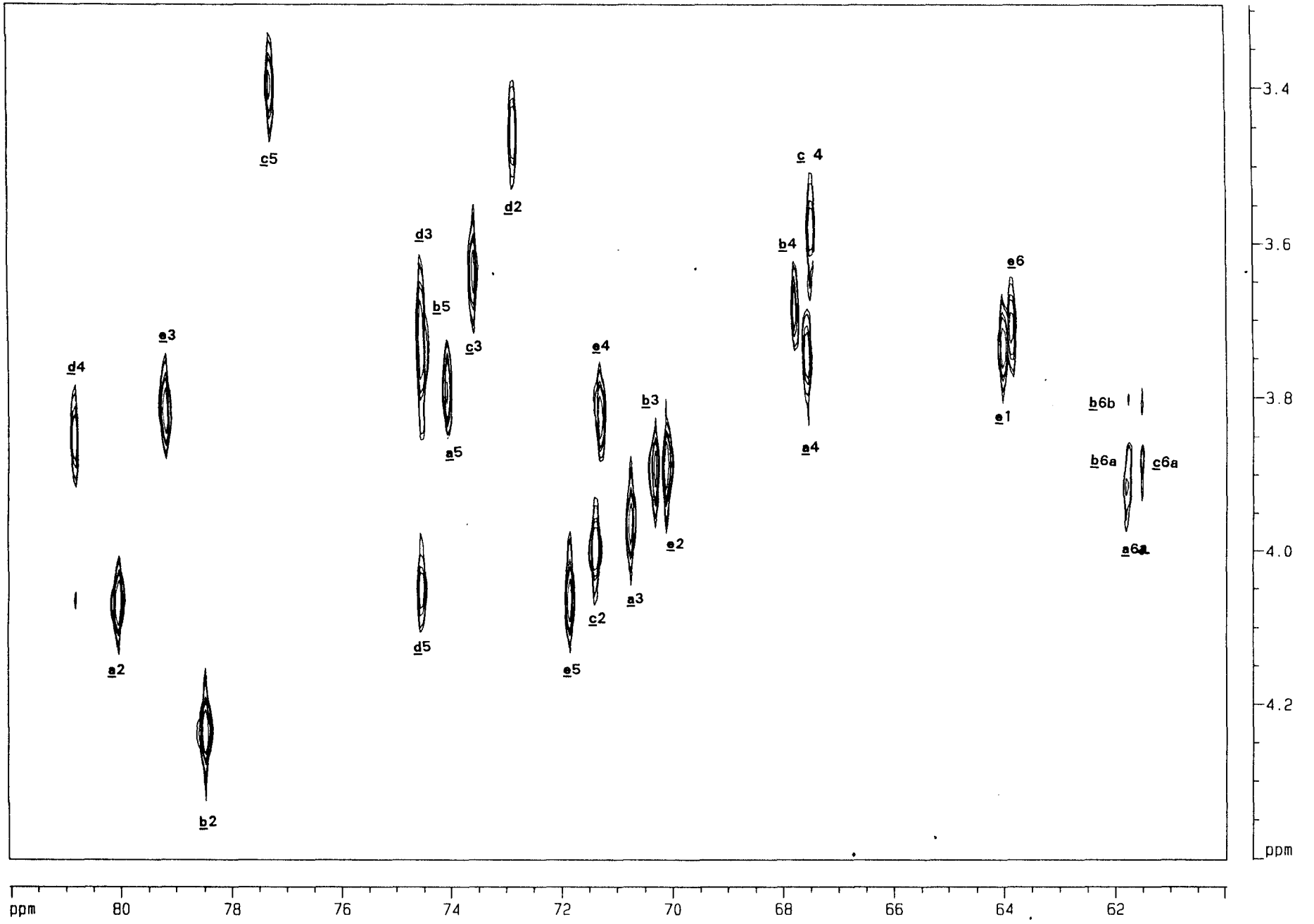
Spectrum 20. 2D H-^{1}H-NMR HOHAHA contour map of the *Klebsiella* K43 P1-ol



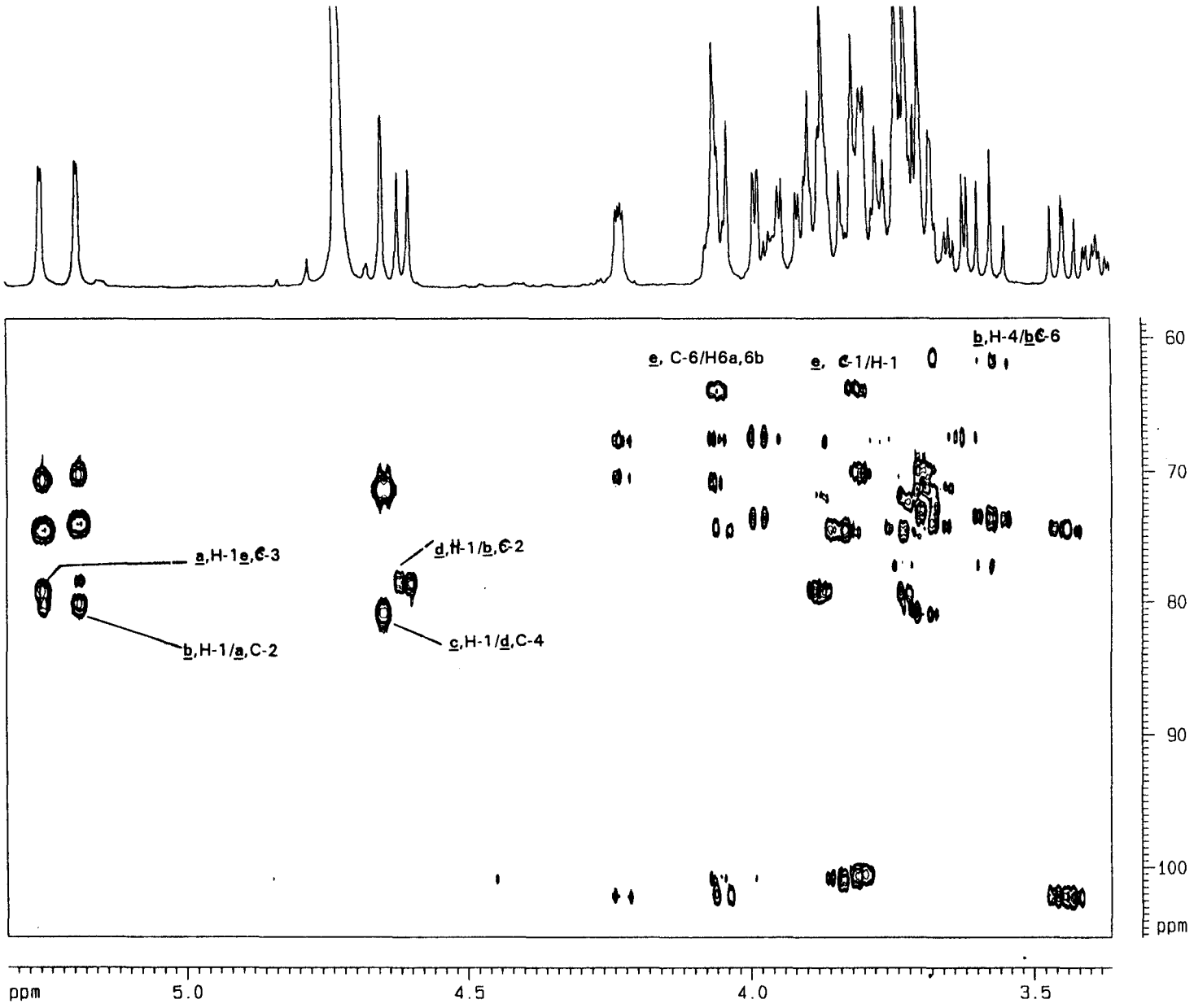
Spectrum 21. 2D ^{1}H-^{1}H-NMR Relay COSY contour map of the *Klebsiella* K43 P1-ol



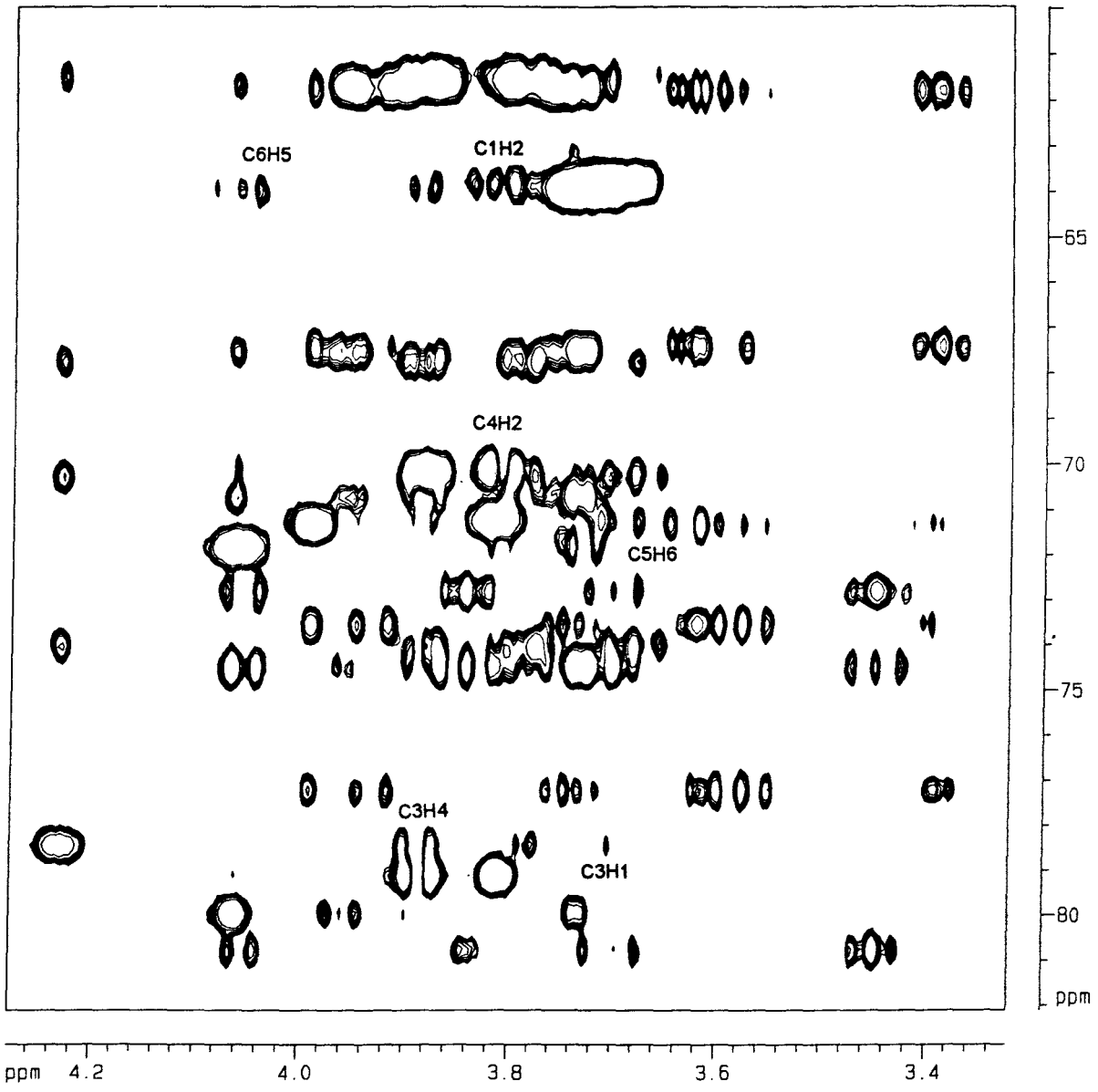
Spectrum 22. 2D $^{1}H^{13}C$-NMR HETCOR contour map of the *Klebsiella* K43 P1-ol



Spectrum 23. 2D ^{1}H-^{13}C-NMR HETCOR contour map expansion of the *Klebsiella* K43 P1-ol



Spectrum 24. 2D ^{1}H-^{13}C-NMR HMBC contour map of the *Klebsiella* K43 P1-ol



Spectrum 25. 2D ^{1}H-^{13}C-NMR HMQC-TOCSY contour map of the *Klebsiella* K43 P1-ol galactitol

6.2.(iv). NMR investigation into the oligosaccharide P1

The assignment of the ^{1}H- and ^{13}C-NMR chemical shifts for P1 was complicated by the occurence of two populations of oligosaccharide causing cluttered spectra which

contained overlapping resonances. The *a*-anomeric region of the 1D ^{1}H-NMR was initially confusing, because of inherent partial superimposition of two apparently different signals. Use of data aquired from the P1-ol NMR study was helpful in assigning shifts which were either poorly relayed in 2D experiments, or partially overlapped by other resonances.

The ^{1}H-NMR spectrum of P1, (Spectra 26+27, page 144-145) recorded at 30°C, contained H-1 resonances for *a*-residues at δ 5.294 ($J_{1,2}$ 1.37 Hz), δ 5.281 ($J_{1,2}$ 1.96 Hz), δ 5.290 ($J_{1,2}$ 3.72 Hz), δ 5.164 ($J_{1,2}$ 1.76 Hz) and β-residues at δ 4.637 ($J_{1,2}$ 0.97 Hz), δ 4.63 ($J_{1,2}$ 8.02 Hz) and δ 4.595 ($J_{1,2}$ 7.83 Hz).

The resonances at δ 5.290 and δ 5.164 were assigned to *a*-**D**-Galp and *a*-**D**-Manp respectively. Those at δ 5.294 and 5.281 were tentatively assigned to an *a*-**D**-Manp. Resonances at δ 4.637, 4.631 and 4.595 were assigned to ß-**D**-Manp, ß-**D**-Galp and ß-**D**-GlcpA.

The ^{13}C-NMR spectrum, (Spectrum 28, page 146) contained C-1 signals at 95.14, 95.33, 92.99, 100.90, 100.71, 97.06 and 102.03 ppm, and signals for CH_2OH at 61.78, 61.71, 62.04, 61.61, 61.82 and 61.84 ppm, and a signal at 174.95 ppm indicative of the uronic acid carbonyl.

The chemical shifts of most of the ^{1}H and ^{13}C resonances as listed in (Table 15, page 142), were established mainly from ^{1}H-^{1}H homonuclear correlation experiments, (COSY and HOHAHA), and inverse-detected ^{13}C-^{1}H correlation (HMQC, HMQC-TOCSY and HMBC) experiments. The residues were labelled arbitrarily **a** to **f** in order of decreasing chemical shift of the H-1 resonances.

2D NMR INVESTIGATION INTO THE REPEATING UNIT STRUCTURE P1

Residue a. The chemical shifts for H-1 to H-6a,6b were established from the Cosy-45 contour map, (Spectrum 29, page 147) and the weak H-4,5, cross-peak was detectable.

Residue b. The chemical shifts for H-1 to H-4 were established from the COSY contour map and H-4 from the H-2 track in the HOHAHA, (Spectrum 30, page 148).

Residue c. The chemical shifts for the H-1 to H-3 resonances were established from the COSY contour map, whilst H-2 to H-5 were established in the H-2 track of the HOHAHA spectrum. H-6a,6b could not be determined with any certainty.

Residue d. The chemical shifts for the H-1 to H-6 were easily established from the COSY contour map.

Residue e. The chemical shifts for the H-1 to H-5 resonances were established from the COSY contour map, the H-4,5 cross-peak was weak but discernable.

Residue f. The chemical shifts for the H-1 to H-3 resonances were established from the COSYcontour map and H-1 to H-5 in the HOHAHA contour map.

The ^{13}C data was established by comparing the ^{1}H assignments with the ^{1}H-^{13}C HMQC experiment (Spectrum 31, page 149). Assignment of C-6's was difficult, however data obtained from the HMQC-TOCSY experiment (Spectrum 32, page 150) enabled C6 determination from H5C5 to H5C6 relayed connectivty, e.g. residue d H5C5 at (δ 3.384)(77.22 ppm) showed a relayed connectivity to H5C6 at (3.384)(61.82 ppm), thus establishing the chemical shift for d C-6.

The sequence of the residues was established from the HMBC correlation experiment, (Spectra 33 page151). The pertinent correlations are presented in Table 14.

Table 14.

HMBC Correlation data for P1.

Residue	Proton Shift (*)		Correlated residue (carbon)	
b (H-1)	5.281	uncertain to	**a** (C-3)	73.90
b (H-1)	5.281		**e** (C-3)	77.30
b (H-1)	5.294	weak to	**b** (C-2)	80.19
c (H-1)	5.164		**c** (C-3)	70.24
c (H-1)	5.164		**c** (C-5)	74.10
c (H-1)	5.164		**b** (C-2)	80.19
c (H-1)	5.164	weak to	**c** (C-2)	78.21
d (H-1)	4.637		**f** (C-4)	80.92
d (H-1)	4.637		**d** (C-2)	71.36
e (H-1)	4.631		**e** (C-3)	77.30
f (H-1)	4.595		**c** (C-2)	78.21
f (H-5)	3.923		**f** (C-6)	174.95
f (H-4)	3.785		**f** (C-6)	174.95

Table 14 shows a comparison of chemical shift and coupling constant data with that for methyl glycosides (see Table 5, page 74), and allows residues **a** to **f** to be identified as:

a →3)-*α*-**D**-galactose

b →2)-*α*-**D**-mannose

c →2)-*α*-**D**-mannose

d β-**D**-mannose

e →3)-β-**D**-galactose

f →4)-β-**D**-glucuronic acid

The following sequence may be written for the P1 oligosaccharide

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

OR

β-D-Manp-(1→4)-β-D-GlcAp-(1→2)-*a*-D-Manp-(1→2)-*a*-D-Manp-(1→3)-, β-D-Gal

The P1 oligosaccharide of the *Klebsiella* K43 exopolysaccharide degraded by a bacteriophage enzyme

Table 15. ^{1}H- and ^{13}C-NMR data for the *Klebsiella* K43 P1. (30°C)

		1	2	3	4	5	6a	6b
a	**H**	**5.290**	**3.908**	**3.961**	**4.210**	**4.062**	**3.735**	**3.735**
	J	**(3.72)**		**(3.13)**	**(1.18)**			
	S	4.84	3.83	3.79	3.97	3.89	3.78	3.72
		(3.0)		(2.3)	(1.0)			
	C	**92.99**	**67.56**	**73.90**	**65.89**	**71.14**	**62.04**	
	S	100.1	69.2	70.5	70.2	71.6	62.2	
b	**H**	**5.294**	**4.054**	**4.034**	**3.733**	**3.877**	**3.878**	
	J	**(1.37)**		**(8.9)**			**(11.63)**	
	S	4.77	3.93	3.76	3.64	3.62	3.90	
		(1.6)		(10.0)			(12.0)	
	C	**95.14**	**80.19**	**70.67**	**67.73**	**73.48**	**61.78**	
	S	101.9	71.2	71.8	68.0	73.7	62.1	
b	**H**	**5.281**	**4.052**	**4.034**	**3.726**	**3.877**	**3.878**	
	J	**(1.96)**	**(3.33)**	**(8.9)**			**(11.63)**	
	S	4.77	3.93	3.76	3.64	3.62	3.90	
		(1.6)	(3.5)	(10.0)			(12.0)	
	C	**95.33**	**80.14**	**70.67**	**67.65**	**73.48**	**61.71**	
	S	101.9	71.2	71.8	68.0	73.7	62.1	
c	**H**	**5.164**	**4.252**	**3.891**	**3.653**	**3.809**	**3.878**	
	J	**(1.76)**	**(3.52)**	**(9.39)**	**(9.39)**		**(11.63)**	
	S	4.77	3.93	3.76	3.64	3.62	3.90	
		(1.6)	(3.5)	(10.0)	(10.0)		(12.0)	
	C	**100.90**	**78.21**	**70.24**	**67.87**	**74.10**	**61.61**	
	S	101.9	71.2	71.8	68.0	73.7	62.1	

* Table continues overpage

		1	2	3	4	5	6a	6b
d	**H**	**4.637**	**3.989**	**3.634**	**3.565**	**3.384**	**3.731**	**3.929**
	J	**(0.98)**	**(3.13)**	**(9.59)**	**(9.59)**	**(2.35,6.46)**		
	S	4.58	3.99	3.64	3.57	3.38	3.76	3.94
		(0.9)	(3.2)	(10.0)	(10.0)			
	C	**100.71**	**71.36**	**73.43**	**67.45**	**77.22**	**61.82**	
	S	101.3	70.6	73.3	67.1	76.6	61.4	
e	**H**	**4.631**	**3.573**	**3.751**	**4.154**	**3.678**		
	J	**(8.02)**	**(9.98)**	**(3.33)**	**(0.98)**			
	S	4.31	3.50	3.64	3.92	3.68	3.80	
		(8.0)	(10.0)	(3.8)	(0.8)			
	C	**97.06**	**71.10**	**77.30**	**65.29**	**75.77**	**61.84**	
	S	104.5	71.7	73.8	69.7	76.0	62.0	
f	**H**	**4.595**	**3.436**	**3.674**	**3.785**	**3.923**		
	J	**(7.83)**	**(9.39)**	**(9.39)**	**(9.95)**			
	S	4.38	3.26	3.49	3.38	3.47		
		(8.2)	(9.6)	(9.6)	(9.6)	(---)		
	C	**102.03**	**72.91**	**74.58**	**80.92**	**75.54**	**174.95**	
	S	104.0	74.1	76.8	70.6	76.8	61.8	

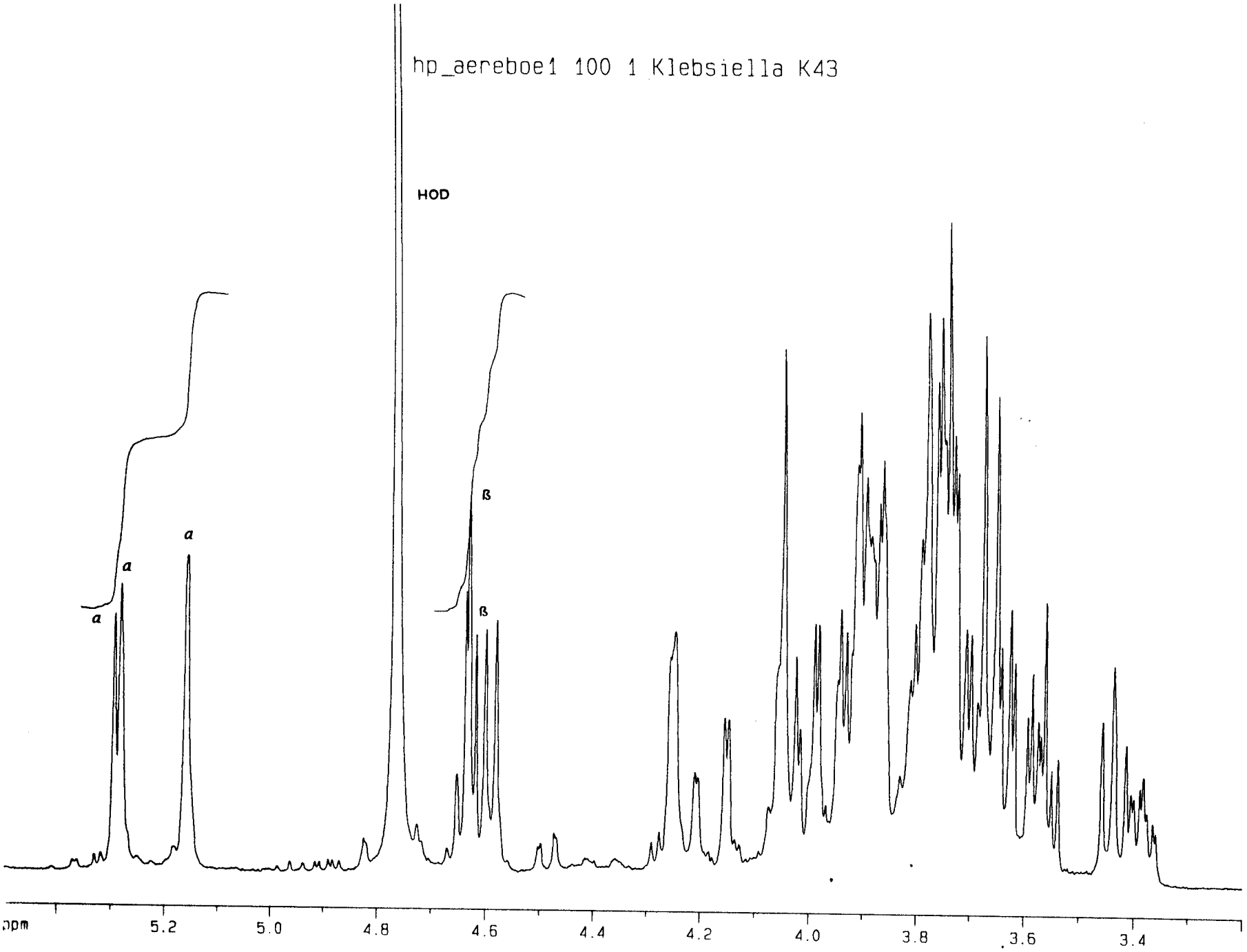
Chemical shifts in ppm relative to internal acetone at δ 2.23 for ^{1}H- and at 31.07 ppm for ^{13}C-NMR.

* Note residue **f** standard is methyl glucoside and not glucuronic acid, but serves as an indicator approximating ß-methyl glucuronic acid.

"S" refers to standard methyl glycosides:

a S = methyl-*α*-D-galactose
b S = methyl-*α*-D-mannose
c S = methyl-*α*-D-mannose
d S = methyl-ß-D-mannose
e S = methyl-ß-D-galactose
f S = methyl-ß-D-glucose

- Underlined carbon shifts indicate points of linkage



Current Data Parameters

NAME	hp_aereboe1
EXPNO	100
PROCNO	1

F2 - Acquisition Parameter

Date	910509
Time	10.59
PULPROG	zg30
NUCLEUS	1H
SOLVENT	CDCl3
AQ	3.4078720 se
FIDRES	0.146719 Hz
DW	104.0 us
RG	2048
HL1	1 dB
D1	1.0000000 se
P1	13.0 us
RD	0.0000000 se
PW	0.0 us
DE	130.0 us
SFO1	400.1368820 MH
SWH	4807.69 Hz
TD	32768
NS	128
DS	2

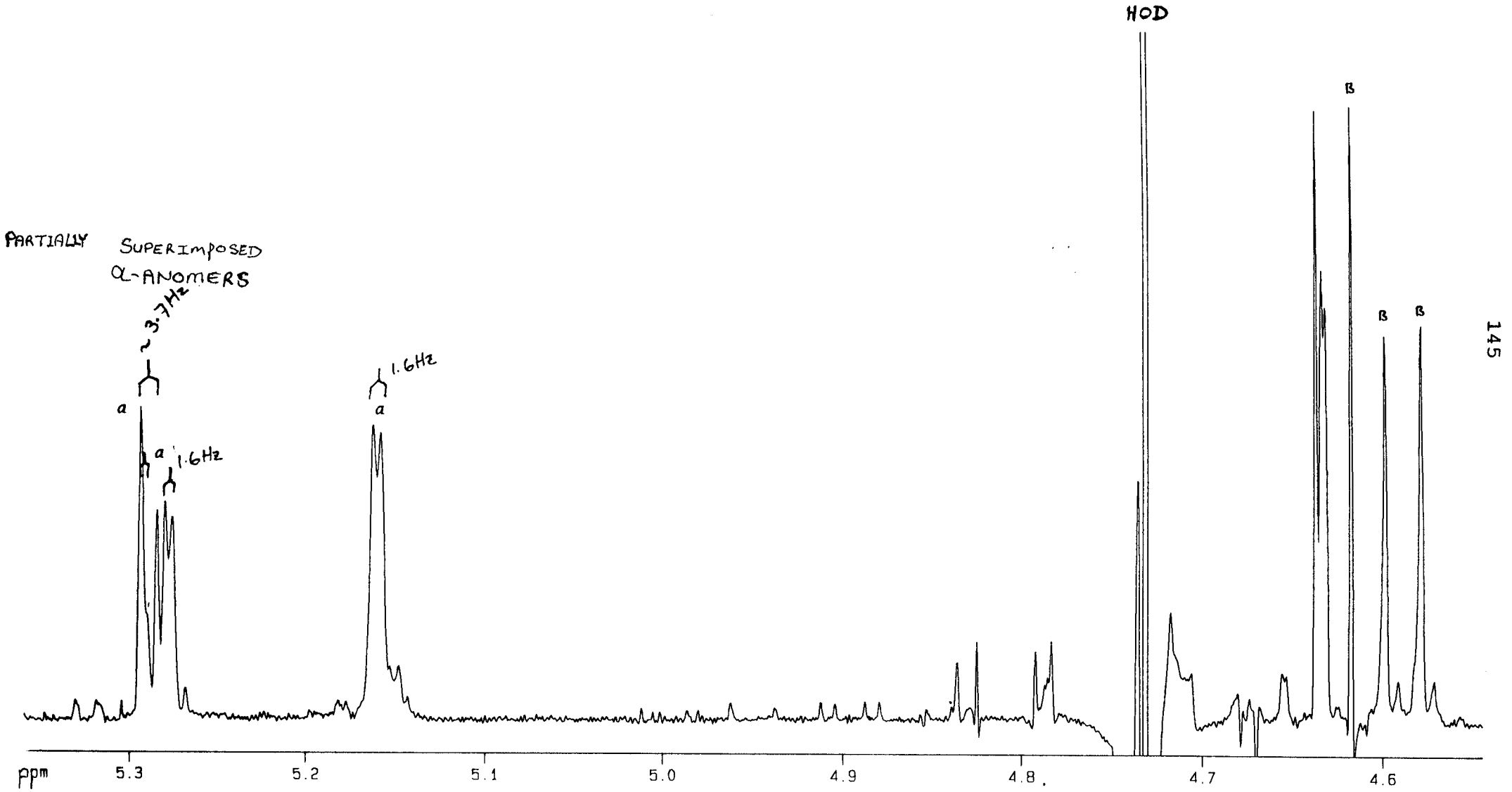
F2 - Processing parameters

SI	16384
SF	400.1353749 MH
WDW	EM
SSB	0
LB	0.30 Hz
GB	0
PC	1.00

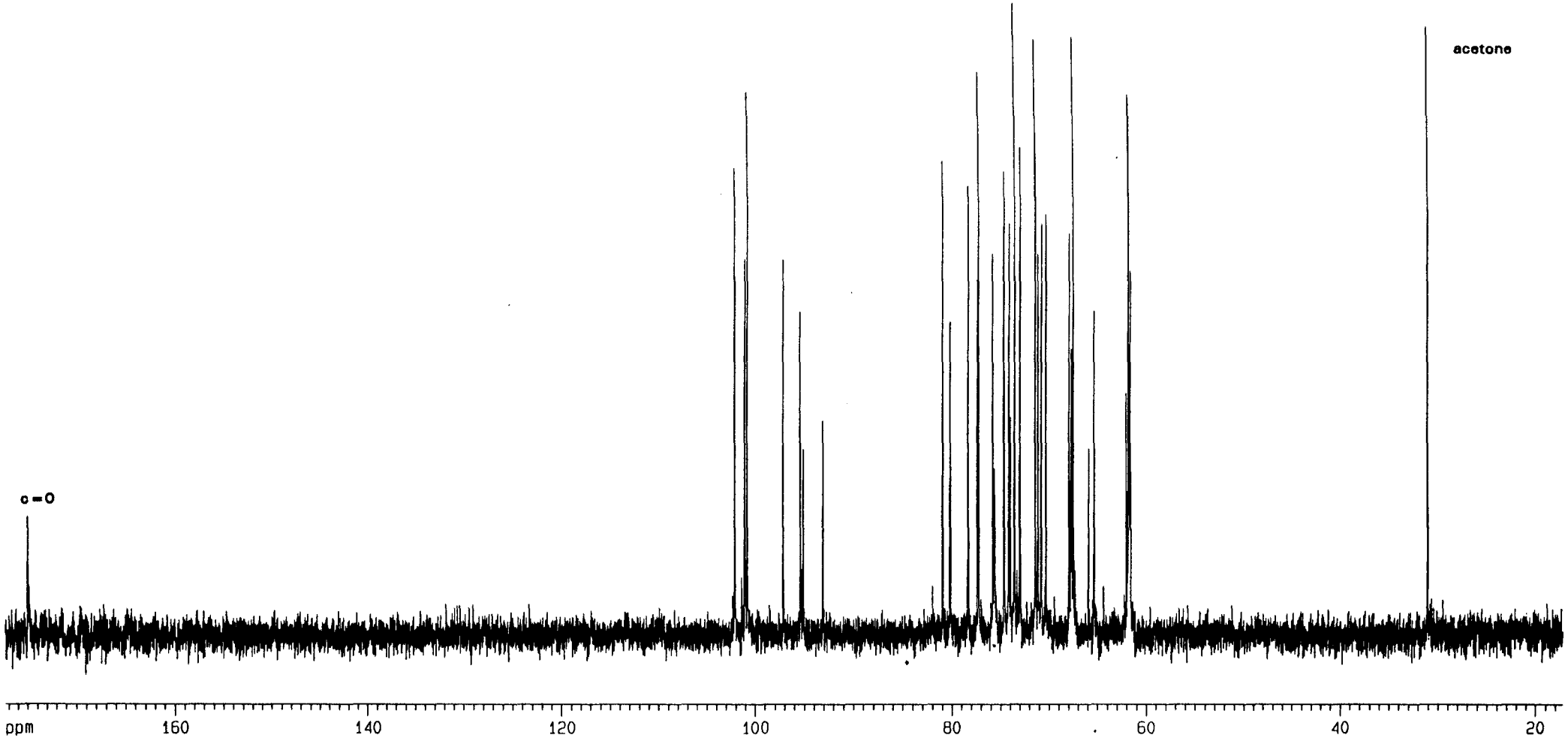
1D NMR plot parameters

CX	23.90 cm
F1P	5.500 pp
F1	2200.74 Hz
F2P	3.200 pp
F2	1280.43 Hz
PPMCM	0.09623 pp
HZCM	38.50675 Hz

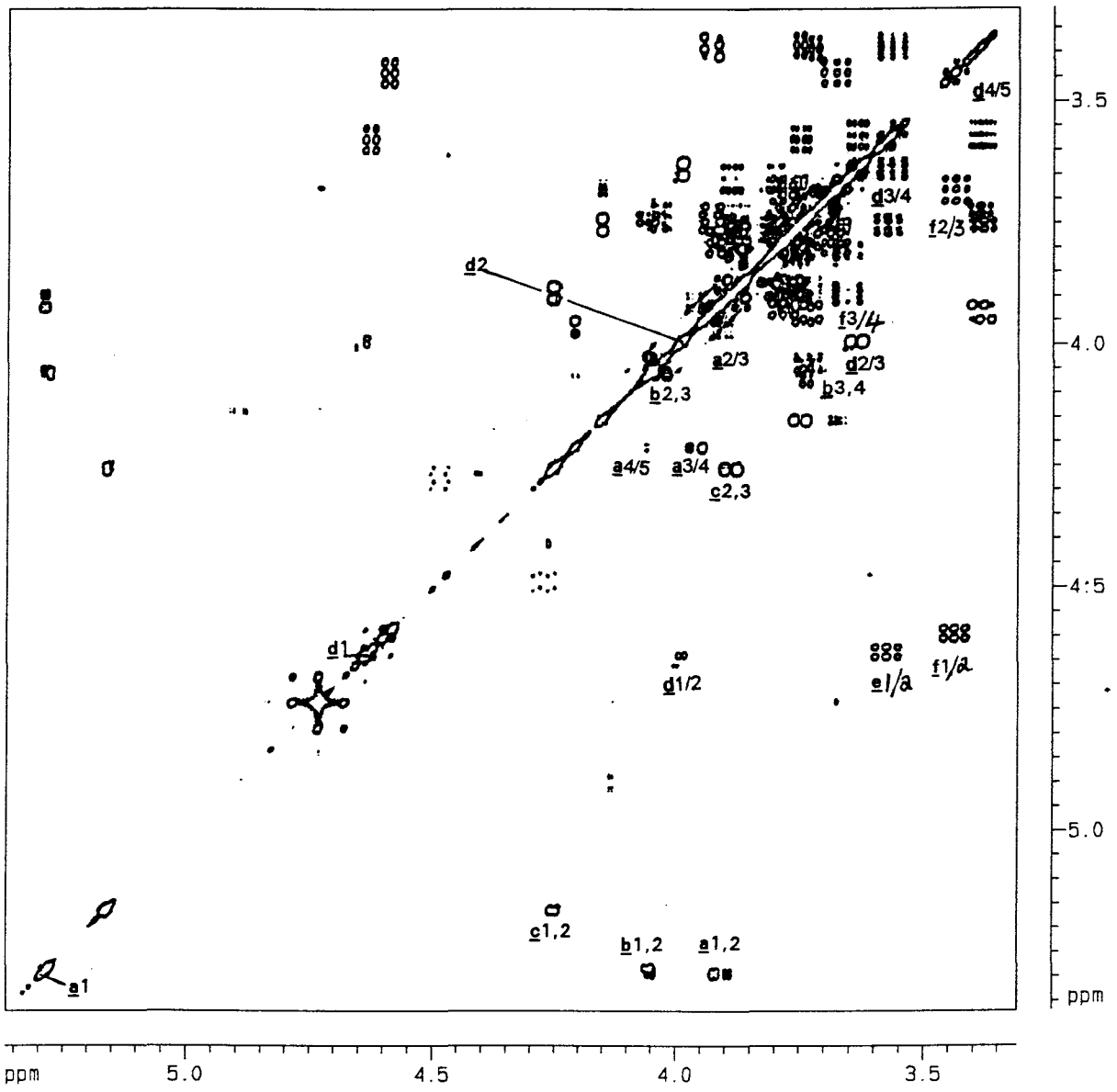
Spectrum 26. 1D ^{1}H-NMR of the *Klebsiella* K43 P1



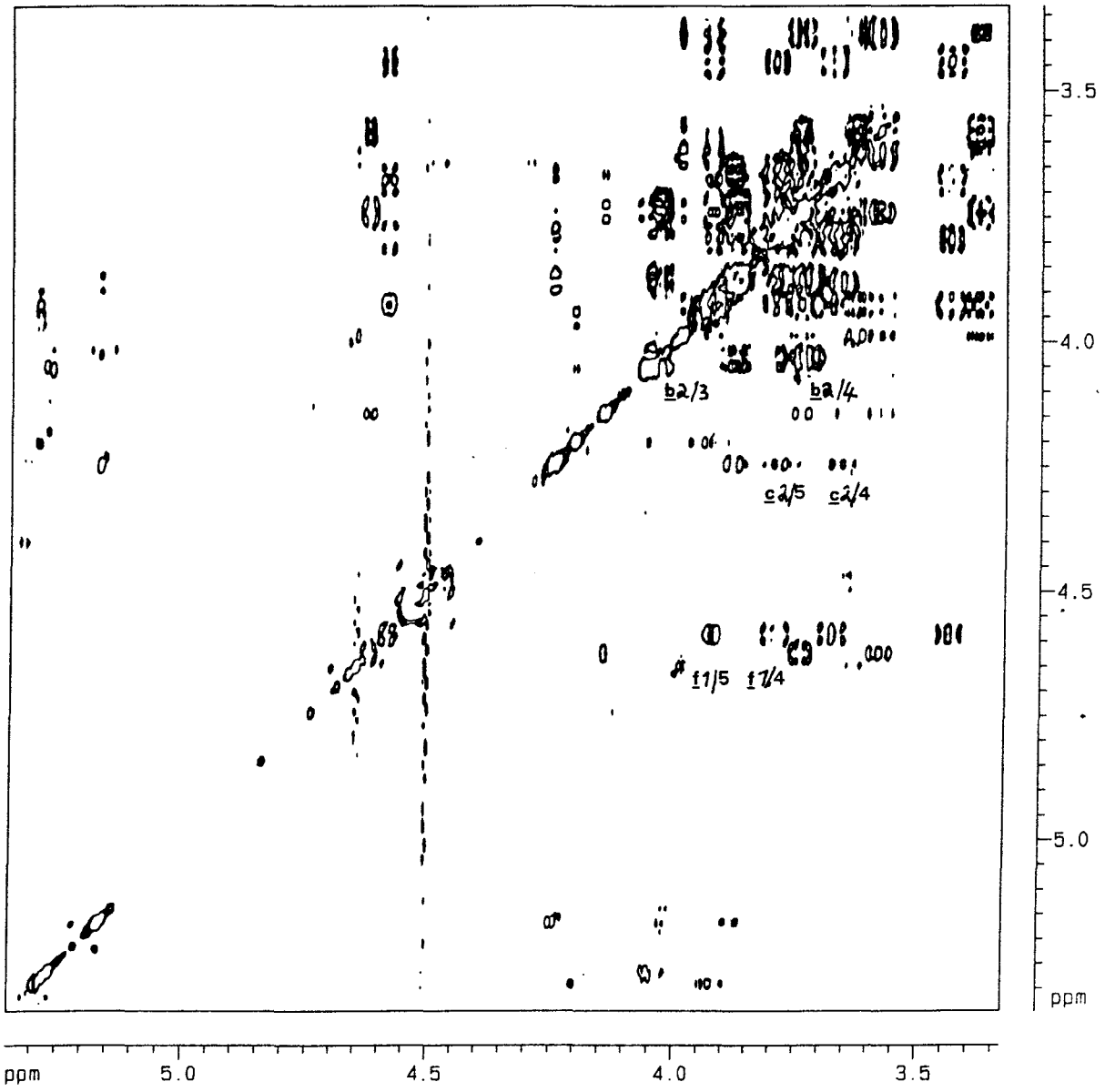
Spectrum 27. 1D ^{1}H-NMR anomeric region expansion of the *Klebsiella* K43 P1



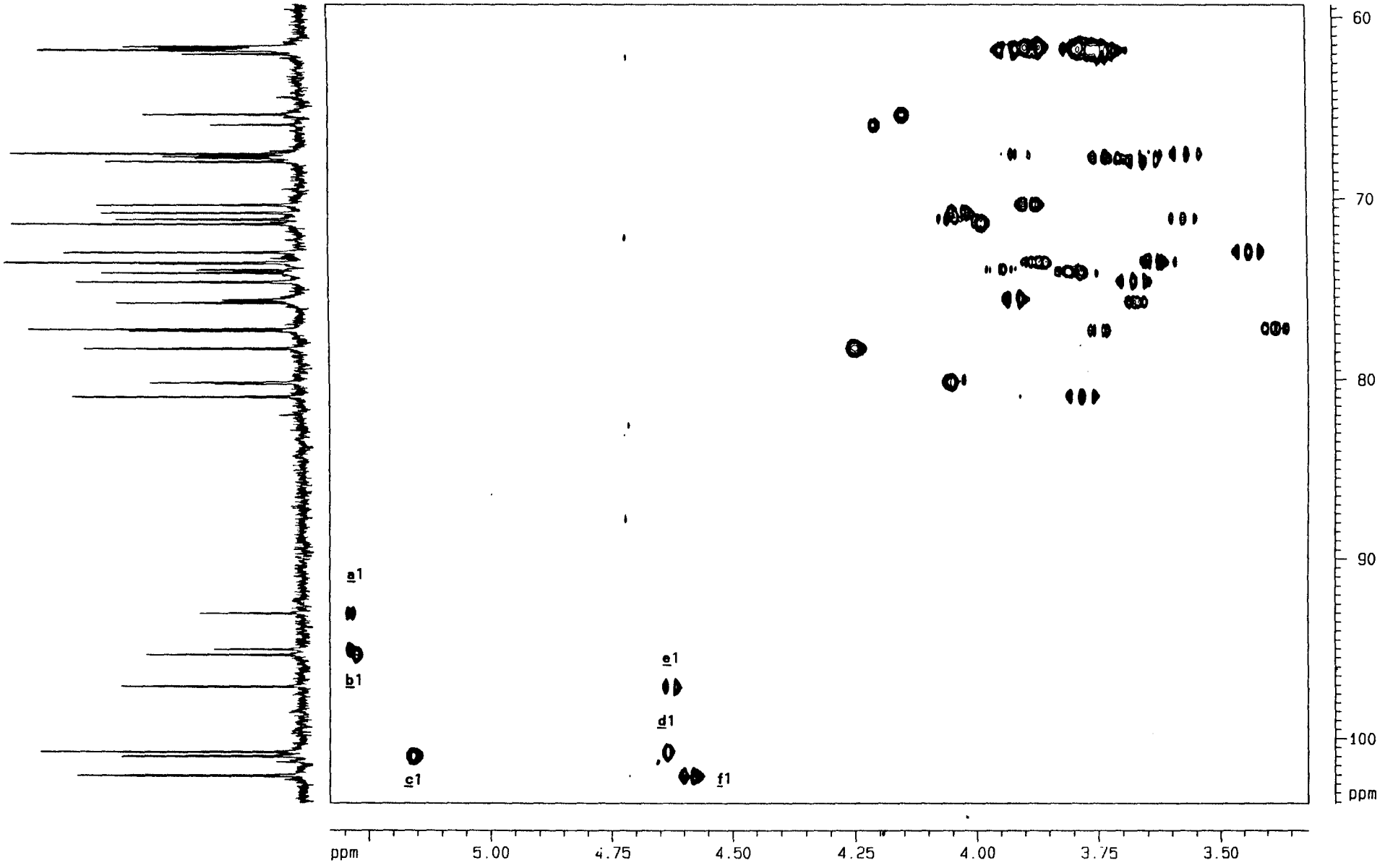
Spectrum 28. 1D ^{13}C-NMR of the *Klebsiella* K43 P1



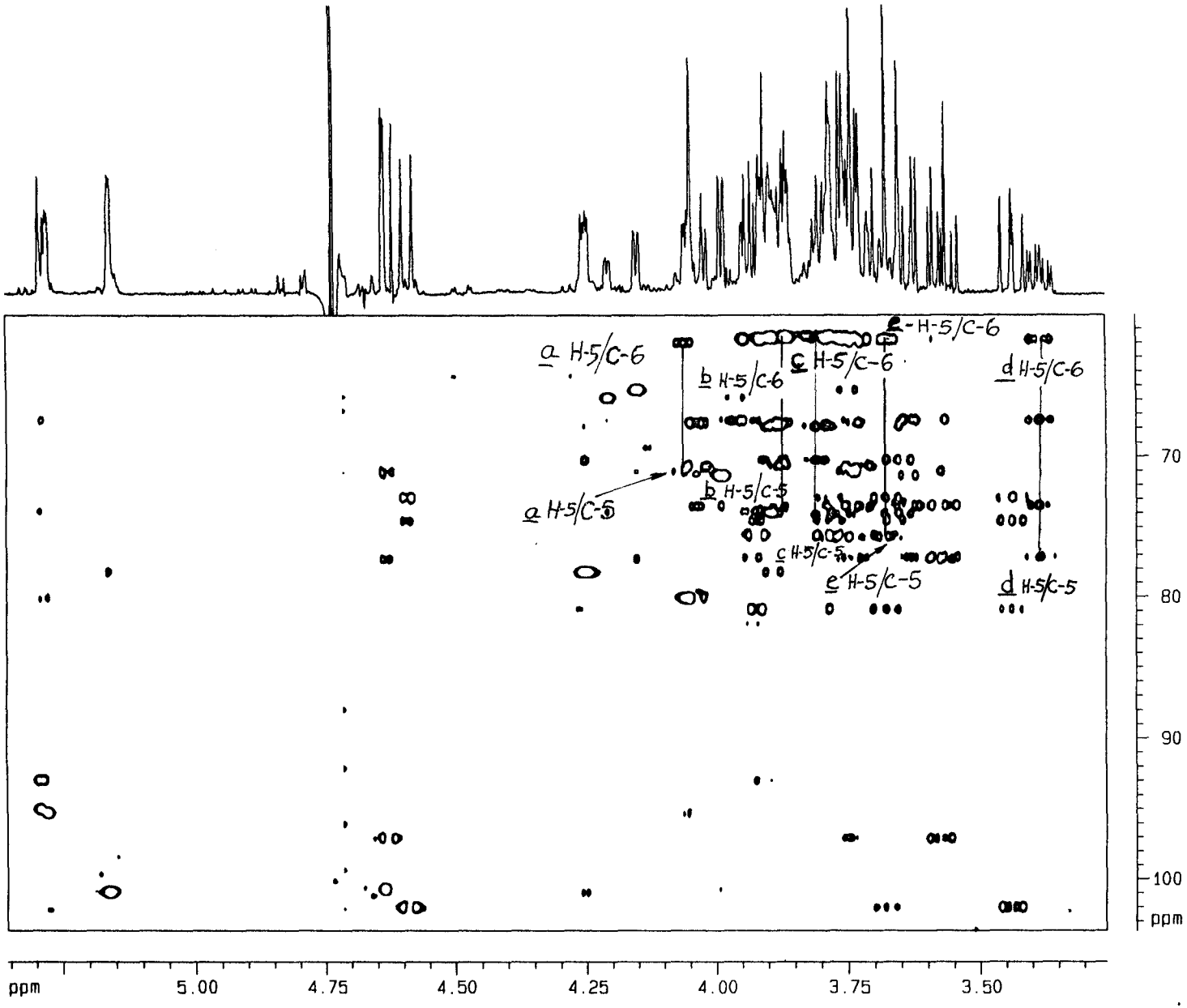
Spectrum 29. 2D ^{1}H-^{1}H-NMR COSY contour map of the *Klebsiella* K43 P1



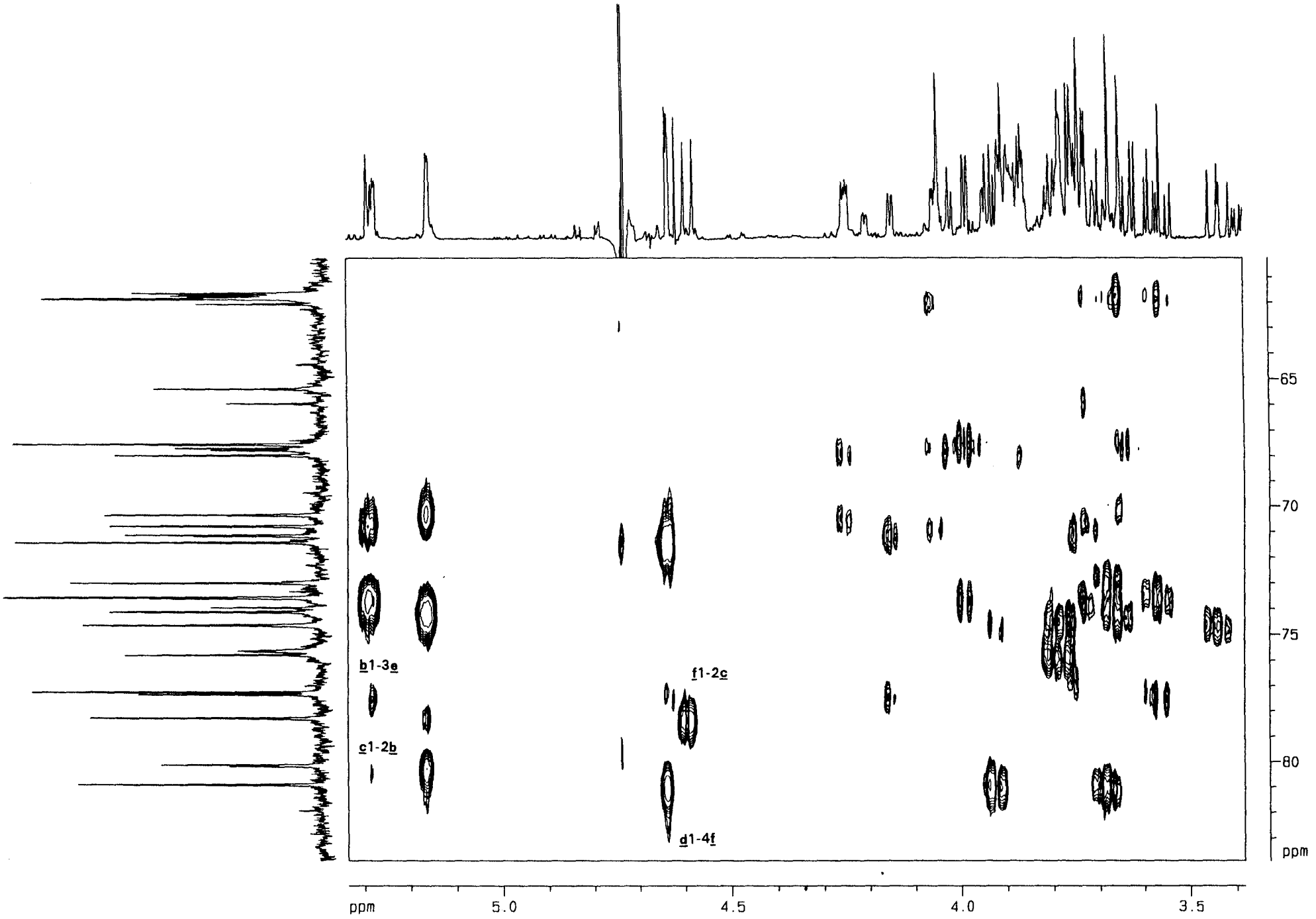
Spectrum 30. 2D ^{1}H-^{1}H-NMR HOHAHA contour map of the *Klebsiella* K43 P1



Spectrum 31. 2D ^{1}H-^{13}C-NMR HMQC contour map of the *Klebsiella* K43 P1



Spectrum 32. 2D ^{1}H-^{13}C-NMR HMQC-TOCSY contour map of the *Klebsiella* K43 P1



Spectrum 33. 2D ^{1}H-^{13}C-NMR HMBC expansion contour map of the *Klebsiella* K43 PI

6.3. Conclusion

The bacteriophage degradation of the *Klebsiella* K43 capsular polysaccharide afforded the repeating unit oligosaccharide P1. Both P1 and the borohydride reduced oligosaccharide alditol P1-ol were studied by NMR spectroscopy. These results along with those of the lithium degradation conclusively allow the repeating unit structure to be described by (Figure 37).

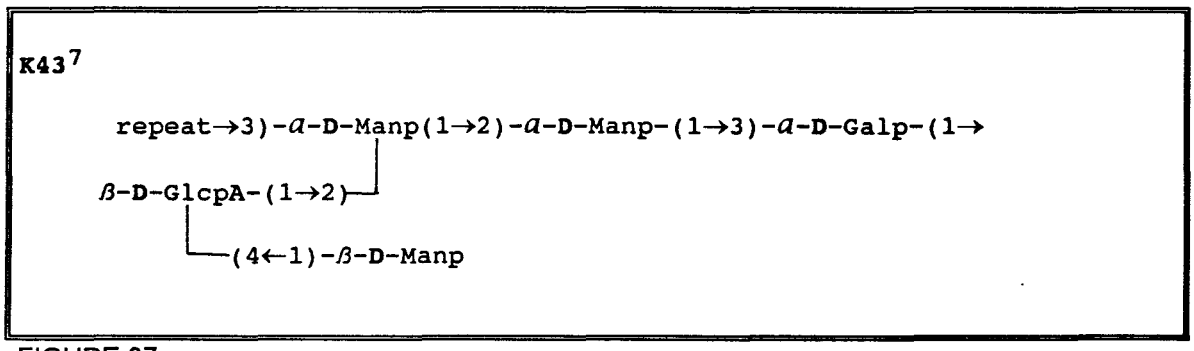


FIGURE 37.

7. A STUDY OF DATA ACCUMULATED FROM SEROLOGICAL STUDIES ON *KLEBSIELLA* K-ANTIGENS, PERTINENT TO PREDICTING THE PARTIAL STRUCTURE OF THE *KLEBSIELLA* K43 ANTIGEN

This section illustrates the usefulness of cross-reactive fitting of an unknown polysaccharide antigen, by antibodies generated from antigens of known chemical structure, as advised by Van Regenmorten[274]. This serological technique is useful in choosing which antigens might be dedicated to further chemical elucidation, and reveals elements which may be expected in an unknown structure.

Heidelberger, Dutton and Nimmich amongst others conducted cross-reactivity studies on *Klebsiella* K-antigens in anti-*Pneumococcal*, anti-*Salmonella* and anti-*Klebsiella* sera. By quantitative and qualitative analysis of antibody-antigen precipitation they were able to build a database, enabling the chemical constitution of unknown *Klebsiella* polysaccharide K-antigens to be predicted by their respective immunological specificities.

The most valuable chemical predictions arising out of their work are: monosaccharide type, linkage type and tentative partial structures to unknown serotypes, indications of the reactive centre of the polysaccharide and the linkage positions of various monosaccharides, information regarding the reducing monosaccharide of an isolated repeating unit, and attachment of non-carbohydrate appendages.

Problems encountered in the research were: a lack of complete chemical structure of certain *Pneumococcal* and *Salmonella* serotypes, and the lack of an explanation for the fact that, *Klebsiella* K-antigens contained **D**-Mannose, but *Pneumococcal* serotypes did not, and *Pneumococcal* serotypes contained amino sugars which *Klebsiella* Serotypes did not.

A precise' based on Heidelberger *et al.*[9-13] research data of cross-reactive studies of *Klebsiella* K-antigens in *Pneumococcal* and *Klebsiella* antisera, pertinent to predicting the *Klebsiella* K43 partial structure

Heidelberger's findings:

1). *Klebsiella* Serotypes K2, K22, K33, K43 and K57 are related.

2). Antibody induced by the *Pneumococcal* XV antigen was used to test *Klebsiella* K-antigen serotypes. This study showed that of those *Klebsiella* K-antigens tested, an increasing order of precipitation in anti-*Pn* XV was shown by: K2 < K22 < K33 < K43. It was suggested that the end group i.e. the reducing end monosaccharide of an isolated oligosaccharide repeating unit would be **D**-Galactose if the chemotype contained galactose, and **D**-Glucose would be linked in various ways.

Data accumulated :

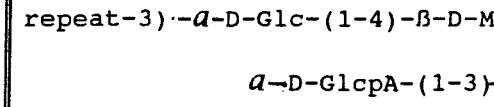
1)

The chemical composition of the *Pneumococcal* XV antigen on which the induced antibody predictions were based in 1976:

Gal; Glc; GalN; GlcN; Glycerol and PO_4^{2-}

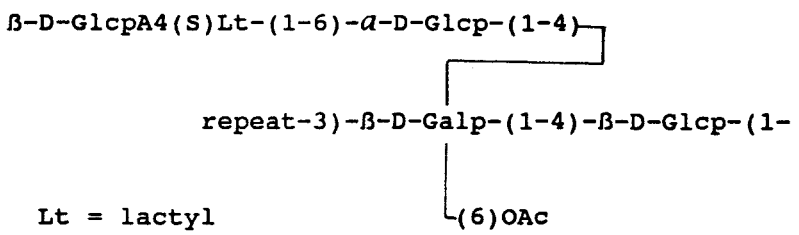
2). The repeating unit structure of *Klebsiella* K2

The structure of the **K2**[22] antigen.



3.a. Antibody induced by *Klebsiella* K22 was used to test *Klebsiella* K-2, 22, 33, 43 and 57 antigens.

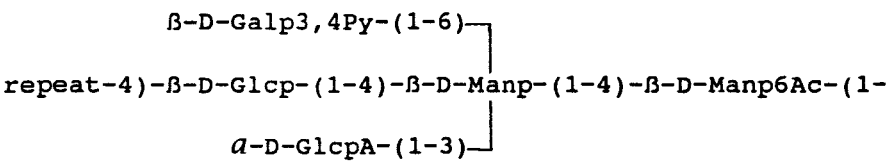
The structure of the K22[34] antigen used to induce its corresponding antibody.



Lt = lactyl

3.b. The repeating unit structure of *Klebsiella* K33

The structure of the K33[47] antigen.



Ac = acetyl

3.c. The chemotype[37] of the *Klebsiella* K43 antigen

Glucuronic acid; Mannose; Galactose.

3.d. **<u>Possible elements in common from this study:</u>**

3.d.i. The presence of α- or ß-GlcA in the side chain.

3.d.ii. The presence of 6-O-Ac in the structure.

3.d.iii. If mannose is present in the backbone of the polymer, the uronic acid side chain may be linked to position 3 of the mannose.

4.a. Antibody induced by the *Pneumococcal* XVIII[12] antigen as used to test 60 *Klebsiella* K-antigens

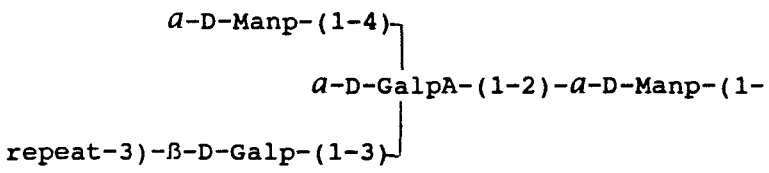
The structure of the *Pneumococcal* XVIII repeating unit, used to generate its corresponding antibody.

-)-D-Gal-(1-4)-*a*-D-(1-6)-D-Glc-(1-3)-L-Rham-(1-4)-D-Glc-(-

4.b. Of all *Klebsiella* K-antigens tested, K57 and K43 were the only to cause precipitation of this antibody. It was suggested that because K57 was cross-reactive with certain other polysaccharide antigens[12], it may be acetylated and was structurally the closest *Klebsiella* K-antigen to that of the unknown K43.

5.a. Antibody induced by the *Klebsiella* K57 antigen was used to test *Klebsiella* K43

The structure of the *Klebsiella* **K57**[97] repeating unit, used to induce its corresponding antibody.



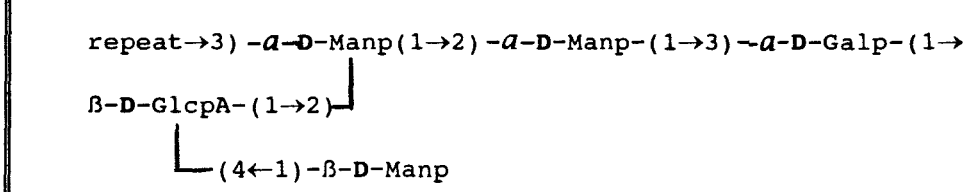
5.b. *Klebsiella* K43 reacted strongly in anti-K57 and this result lead Heidelberger to postulate that the mannose linkages in K43 would be similar to that in K57, and because K43 was strongly cross-reactive in anti-K57 serum, the chemical repeating unit of K57 and K43 would be similar, although their uronic acids were galacturonic acid in K57 and glucuronic acid in K43.

Deductions made from this study allowing the most likely partial structure for the *Klebsiella* K43 antigen to be postulated:

1. **D**-galactose may be the reducing end monosaccharide of an isolated repeating unit oligosaccharide of K43.
2. **D**-glucuronic acid is present in the main chain or side chain.
3. The repeating unit may be acetylated, although probably not because K57 is not acetylated.
4. Mannose residues will be present and may be (1→2), (1→3) or (1→4) linked
5. Mannose residue(s) might be linked (1→4) to an uronic acid in the side chain.
6. If a mannose side chain is present, it could be linked to glucuronic acid.
7. If the uronic acid is in the side chain, the monosaccharide to which it is attached will be of the inverse anomeric configuration to its self, i.e. an ***α***-monosaccharide in the main chain will probably be attached to a ß-uronic acid.

The chemical repeating unit of *Klebsiella* K43 as determined in this work.

K43[7]



Conclusion to this literature survey.

Many of the deductions of bacteria polysaccharide structure made in the middle 1970's, through cross-reactive serological studies on *Klebsiella and Pneumococcal* serotypes, aided by their induced antibodies, correlate directly with many of the features found for the elucidated structure of the *Klebsiella* K43 antigen. In particular, attention is drawn to the mannose linkage types and the attachment of the uronic acid.

Whilst cross-reactivity precipitation studies may provide valuable structural information and give direction to a study of an unknown polysaccharide antigen, they suffer because of the need for a range of chemically known polysaccharides, availability of pure antibodies of known mechanistic activity, and the difficulty in quantifying marginal precipitation reactions.

Although a conformational study on the *Klebsiella* K43 antigen is beyond the scope of this thesis, it is interesting to note that a crystallographic[183] study on K57 has revealed a trifolded helix with an axial projection. This helix was also found to be highly extended in spite of a 1,2-diaxial linkage being present in the main chain. It is important to note that, whilst the chemical repeating unit may be fundamental to the antigenic character, it may not be the effective biological repeating unit. Crystallographic or computer simulated conformational studies in tandem with the observed serological reactivities are necesssary to adequately describe the biological sequence of the antigen.

8. EXPERIMENTAL

8.1. General Methods.

Unless otherwise stated, solutions were concentrated under reduced pressure, at a water-bath temperature not exceeding 40°C using a rotary evaporator. Optical rotations were measured in a cell of pathlength 1cm, at 23°C (± 2°C), using a Perkin-Elmer model 141 polarimeter. Gel-permeation chromatography was performed on columns each having different length and internal diameter, dimensions for an analytical column were 1.8 by 65 cm, and preparative columns had dimensions 3.0 by 88 cm. Depending on the application, gels used included: Biogel P-2 (eluent - water), Biogel P-4 (eluent - 0.1M sodium acetate buffer, pH 5.0), Sephacryl S-200 (eluent - 0.1M sodium acetate buffer, pH 5.0) and Sephacryl S-500 (eluent - 0.1M sodium acetate buffer, pH 5.0). The columns were coupled to a Waters Differential Refractometer (R401) and the signals were recorded on a flat-bed recorder.

Analytical gas-liquid chromatography was performed using a Hewlett-Packard 5890A gas chromatograph fitted with flame-ionisation detectors and a 3392A recording integrator, with helium as the carrier gas. A J + W Scientific wall-coated, fused-silica, bonded-phase capillary column of OV-225 dimensions (30m x 0.252mm), having a film thickness of 0.25 μm, was used for separating partially methylated alditol acetates or aldononitrile derivatives. The temperature settings were always isothermal and varied between 190°C and 210°C. The identities of all derivatives were confirmed by GLC-MS on a Hewlett-Packard 5988A GLC-Mass spectrometer using the OV-225 column. Mass spectrometric studies were recorded at 70 eV and an ion-source temperature of 200°C.

All ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker AMX 400 spectrometer at 30°C, 50°C or 70°C. The samples were deuterium-exchanged by freeze-drying solutions three times in 99.6% D_2O and finally dissolving in 99.995% D_2O. Acetone was used as an internal standard (δ 2.23) for ^{1}H and at 31.07 ppm for ^{13}C.

8.2. Preparation of the K43 polysaccharide

An authentic culture of *Klebsiella* K43 (culture #.2482) was obtained from Dr.I.Ørskov (Copenhagen), and the bacteria were plated out on sucrose-rich agar (Formula 9.4, Annexure 1) and incubated overnight at 37°C (triplicate). Single colonies were transferred from these plates to eight test tubes, each containing 5 mL of nutrient broth (Formula 9.1, Annexure 1), which were then incubated overnight at 37°C in a mechanical shaking-waterbath. These broth cultures were then used to inoculate evenly eight stainless steel trays, each containing 1.5 L of sterile sucrose-rich agar, (Formula 9.4, Annexure 1). The trays were covered and incubated at 37°C for 86 hours after which the bacterial slime was scraped off the agar surface carefully and suspended in a 1% phenol solution. This suspension was stirred overnight at 4°C ensuring bacterial death and dissolution of the capsular polysaccharide. Capsular polysaccharide was then separated from the cellular debris by ultracentrifugation (Beckman L8-M ultracentrifuge at 35 000 rpm for 3 hours using a type 70 Ti rotor). The supernatants were combined and the polysaccharide (both the K- and O-antigens) precipitated into 5 volumes of ethanol, after which the mixture was centrifuged at 3 000 rpm. The clear, off-white supernatant was poured off and the polysaccharide sediments combined, and dissolved in the minimum volume of water (≈50 ml). The resultant mixed antigen solution of high viscosity was subjected to further purification.

Approximately 15 mL of a 5% cetyltrimethylammonium bromide (CTAB) solution was slowly added with stirring to the solution of impure polysaccharide. CTAB was added till no more polysaccharide appeared to precipitate, the system was allowed to stand overnight and the precipitate was centrifuged at low speed. The CTAB salt of the polysaccharide was then dissolved in 25 ml of a 3M aqueous NaCl solution, to form the sodium salt of the polysaccharide and dissociate the CTAB-complex, after which the polysaccharide was precipitated into 5 volumes of ethanol. After centrifugation, the polysaccharide was dissolved in 50 ml of water and dialysed (12 000 - 14 000 Mw cut-off) against running tape water over 48 hours. The solution was freeze-dried to yield 542 mg of the sodium salt polysaccharide.

The purified polysaccharide (sodium salt) was shown to be polydisperse with regard to molecular weight distribution . GPC was performed on a column of Sephacryl S500 with

dimensions 30 mm x 88 mm.

8.3. Monosaccharide composition.

A small portion (3.1 mg) of the polysaccharide was hydrolysed with 2 mL 4M trifluoroactetic acid (TFA at 125°C for 1 hour). The reaction mixture was concentrated under reduced pressure after which it was co-concentrated several times with small aliquots of water, (to ensure the removal of traces of TFA). The liberated monosaccharide residues were transformed into their acetylated aldononitrile derivatives[189] (PAAN'S) and analysed by GLC on an OV-225 capillary column at 220°C.

The uronic acid was identified as follows: dried native polysaccharide was refluxed with methanolic 3% HCL at 80°C for 16 hours. The solution was neutralised with Ag_2CO_3 and stirred for 1 hour and the resultant precipitate was removed by low speed centrifugation after which the solution was reduced with $NaBH_4$ in dry methanol (overnight). The solution was subsequently passed down an Amberlite IR-120(H+) resin ion-exchange column, and the acidic fraction was collected and co-concentrated with several aliquots of methanol to remove borate. The product was hydrolysed (4M TFA, 125°C, 1 hour), after which the free reducing sugars were converted into their PAAN'S and examined by GLC as before.

8.4. Absolute configuration of the monosaccharides

The absolute configuration of each constituent monosaccharide was determined by GLC analysis of their acetylated (-)-2-octyl glycosides[122]. The native polysaccharide (5.5 mg) was methanolysed (methanolic 3% HCL, 80°C, 16 hours), reduced ($NaBH_4$, overnight), and hydrolysed with (4M TFA, 125°C, 1 hour). The hydrolysate was freeze-dried in an ampoule to which 1 drop of TFA, 0.5 mL (-)-2-octanol and a small magnetic flea was added. The sealed ampoule was stirred in an oil bath at 130°C for 16 hours. The reaction solution was then concentrated to dryness. The resulting octyl glycosides were acetylated using pyridine : acetic anhydride (1 : 1 v/v at 100°C for 1 hour), after which they were examined by GLC at 220°C and

their retention times compared to reference **D** and **L** monosaccharides derivatives.

8.5. Methylation analysis

A portion (22mg) of the acidic polysaccharide was dried for 16 hours in a drying pistol and then methylated by the modified Hakamori method of Phillips *et al.*[130] with potassium-dimsyl and methyl iodide, followed by the Khun[128] method using Ag_2O and methyl iodide in DMF to fully methylate the polysaccharide. The product was divided into two portions in the ratio 1:2.

One third of the methylated polysaccharide was hydrolysed with (4M TFA, 125°C, 1 hour). After evaporation of the acid the hydrolysate was reduced ($NaBH_4$), and passed down an Amberlite IR-120(H+) resin column. The acidic fractions were collected and co-concentrated with repeated aliquots of methanol to remove borates. Acetylation of the partially methylated alditol residues was achieved by treatment with equal volumes (1ml) of acetic anhydride and pyridine at 100°C for 1 hour. The partially methylated alditol acetates[186] were analysed by GLC (205°C). Results are presented in Table 6 (column 1, page. 94).

Two thirds of the methylated polysaccharide was dried (6 hours) in a drying pistol prior to methanolysis (methanolic 3% HCL, 80°C, 16 hours). The solution was neutralised with $AgCO_3$, centrifuged, and the supernatant co-concentrated to dryness. The residue was dissolved in methanol ($\sim$ 3 mL), filtered (0.22µm filter), and reduced ($NaBH_4$) overnight. The alkaline solution was subsequently passed down Amberlite IR-120(H+) resin and the acidic fraction eluted was collected and concentrated with small aliquots of methanol. The residue was hydrolysed (4M TFA, 125°C, 1 hour), and the partially methylated monosaccharides converted into their alditol acetates[186] as previously described, and analysed by GLC (205 °C), Table 6 (Column 2, page 94).

8.6. Uronic acid degradation

8.6.(a). ß-Elimination of permethylated polysaccharide

The methylated polysaccharide (10 mg) was subjected to a base-catalysed degradation,

following a modified method introduced by Lindberg *et al.*[144].

A mixture (1 mL) containing dry dimethylsulphoxide : 2,2-dimethoxypropane (19 : 1 v/v) was added to the methylated polysaccharide under positive nitrogen pressure. The mixture was stirred for 1 hour after which potassium-dimsyl (1 mL) was added. After stirring for another 2 hours, the polysaccharide was re-alkylated with MeI (0.5 mL) whilst keeping the mixture cool in an ice-bath. The reaction solution was stirred for another hour after which it was diluted with water (3 mL) and the reaction products were extracted with dichloromethane (4 x 3 mL). The dichloromethane fractions were pooled, washed with water (4 x 3 mL), and concentrated to dryness. The residue was hydrolysed with (4M TFA, 125°C, 1 hour), and the partially methylated alditol acetates were analysed by GLC (205°C). The results are presented in Table 6 (column 3 page 94).

8.6.(b). **Lithium degradation of underivatised polysaccharide**

Dry acidic polysaccharide (100mg) was dissolved in ethylenediamine (80 mL) by the method described by Lau *et al.*[172]. To the solution was added two small lengths (3-5 mm) of lithium wire. The round-bottom flask chosen had little head space above the reaction surface and was securely stoppered. The solution and wire were vigorously stirred till a navy-blue colour formed (20 minutes), thereafter as the colour faded to a creamy-yellow, more wire was cut and added. After an hour the reaction mixture was slowly quenched with water (80 mL) and transferred to a larger flask. The product mixture was then roto-evaporated to dryness as an azeotrope, (addition of 100 mL toluene). The residue was co-concentrated with two aliquots (2 x 100 mL) of toluene after which the powdery residue was cooled in an ice-bath and adjusted to pH 4.5 with glacial acetic acid. The acidic solution was passed down an Amberlite IR-120(H+) resin column, (to exchange lithium ions for protons). The rate of elution of the acidic fraction from the column was slow (resin has a low affinity for lithium ions) and 90 mg of degraded polysaccharide product was freeze dried.

A concentrated aqueous solution of the freeze-dried, degraded polysaccharide was chromatographed down a column 18 mm by 88 cm of Sephacryl S-200 gel. The carbohydrate

component was detected using the phenol-sulphuric acid assay[204].

The polysaccharide fraction was freeze-dried and 24mg of degraded material was isolated. A small quantity (1 mg) of the product was hydrolysed, (4M TFA, 125°C, 1 hour), and the products were converted to PAAN's, and analysed by GLC at (220°C).

The lithium degraded material (9mg) was deuterium-exchanged and subjected to thorough NMR analysis.

8.6.b.(i). 2D NMR spectroscopy acquisition parameters of the lithium degraded polysaccharide

^1H- and ^{13}C-NMR spectra were recorded at 50°C on a Bruker AMX 400 spectrometer. Samples were deuterium exchanged and then dissolved in 99.99% D_2O containing a trace of acetone as an internal standard, δ 2.23 for ^1H and 31.07 ppm for ^{13}C.

The 2D ^1H homonuclear shift-correlated experiment (COSY)[242] was aquired over a spectral width of 2008.03 Hz. A data matrix of 256 x 2048 data points was collected with 128 transients for each delay. The data matrix was zero-filled in the t_1 dimension to 1024 data points and transformed after the application of an un-shifted sine-bell window function in both dimensions and symmetrised. Digital resolution in the resulting 2048 x 1024 matrix was 0.98 Hz/point in f_2.

The 2D Relay-COSY[260] experiment at 400 MHz was performed using a spectral width of 1483.68 Hz. Data matrices of 256 x 2048 data points were collected with 176 transients for each t_1 delay. The data matrices were zero-filled to 1024 data points in t_1 transferred after the application of a non-shifted sine-bell window function. Digital resolution in the 2048 x 1024 matrices were 0.72 Hz/point in f_2. Fixed delays of 0.036 s were used.

The 2D homonuclear Hartmann-Hahn (HOHAHA)[261] experiment was performed using a spectral width of 840.34 Hz. The mixing period consisted of 50 MLEV-17 cycles. A data matrix of 2048 x 256 was aquired with 116 transients for each t_1 delay. The matrix was zero-filled in the t_1 dimension (2048 x 1024 data points) and multiplied in both dimensions with a shifted sine-square window function prior to phase sensitive Fourier transformation.

The 2D homonuclear dipolar-correlated (NOESY)[239] experiment was performed using a spectral width of 1483.68 Hz. An initial data matrix of 2048 x 256 data points was collected with 128 transients for each delay. The matrix was zero-filled to 1024 data points in t_1 and transformed after the application of a shifted sine-square window function in both dimensions and was not symmetrised. Digital resolution in the resulting matrix was 0.72 Hz/point, in f_2. The mixing delay in the NOESY experiment was 0.30 s.

The 2D ^{13}C - ^{1}H heteronuclear shift-correlated (HETCOR)[249] experiment was recorded using a spectral width of 960.31 Hz in t_1 and 5555.56 Hz in t_2. The initial matrix of 2048 x 128 was zero-filled to 2048 x 512 data points and transformed after the application of a shifted sine-square window function. Digital resolution in f_1 was 1.87 Hz/point and in f_2 2.7 Hz/point. A recycle delay of 1 s was employed and 1000 transients per FID were collected.

8.7. Smith degradation of the K43 polysaccharide

Klebsiella K43 polysaccharide (100 mg) in the sodium salt form was dissolved in 25ml water, 29ml of a 0.2M sodium perchlorate solution, and 14ml of a 0.1 Molar sodium metaperiodate solution was added. The mixture was kept in the dark (4°C, 96 hour), the reaction was then quenched with 2ml of ethylene glycol, dialysed for 24 hours, freeze-dried and reduced ($NaBH_4$ 150mg) for 24 hours. After destruction of excess sodium borohydride by the addition of 10% acetic acid, the oxidised product was dialysed (Mw cut off 3500, for 24 hours) against running water, then freeze-dried.

A small sample mass was hydrolysed and analysed as the PAAN's[189] derivatives by GLC analysis. A higher ratio than that expected (ie.1 : 1, mannose to galactose) was found indicating that incomplete oxidation had occured, (see pages 39-41). The oxidation step was then repeated. The polysaccharide was dissolved in 1 M TFA and was kept at room temperature for 24 hours. TFA was then evaporated off and the product was reduced ($NaBH_4$). The $NaBH_4$ was quenched with Amberlite IR-120(H^+) resin and the solution was passed down a small de-ionizing column which was eluted with methanol. The solvent was removed by evaporation and the borate esters removed by co-distillation with methanol (4x). The product of the reaction was

then separated on Biogel P2 using water as eluent. Material eluting with the retention volume of a trisaccharide was isolated and freeze-dried. The product was methylated and analysed by GLC-MS as the derived PMAA's.

8.8. Bacteriophage depolymerisation of the K43 polysaccharide

An active bacteriophage forming large haloes on a lawn of *Klebsiella* K43[7] bacteria was isolated from Grahamstown sewerage and was used to depolymerise the K43 capsular polysaccharide. The bacteriophage was propagated on bacteria in nutrient broth (Annexure 1, Formula 9.1) until a total of 5.28×10^{12} plaque forming units (PFU) was obtained. Polysaccharide (500mg) was dissolved in the phage solution (80ml, 5.28×10^{12} PFU) and the solution was shaken gently at 37°C for 79 hours in the presence of a small amount of chloroform. Thereafter the digest was freeze-dried, re-dissolved in a small amount of water and dialysed against distilled water. The diffusates were combined and freeze-dried yielding depolymerised material (393mg). The product was passed down an ice-jacketed column of Amberlite IR 120 (H^+) resin and then subjected to chromatography on Bio-gel P-4 (eluted with 0.1M sodium acetate buffer pH 5.0). Four fractions labelled P1-5 were collected and the mass of each fraction in order of decreasing oligosaccaride size was P4 (15.6mg); P3 (50mg); P2 (8.5mg) and P1 (14mg). D_2O solutions of all 4 fractions were examined in by ^{1}H-NMR and intensive studies were performed on the P1 and reduced P1 alditol.

8.8.a. Methylation analysis of the oligosaccharide repeating unit P1

A portion of reduced P1 ($NaBD_4$) (4.4 mg) was dried overnight at 60°C in a drying pistol. The oligosaccharide was dissolved in DMSO to which 0.3 ml of potassium dimsyl and later 0.3 ml MeI was added at 10°C, according to the modified Hakomori method[130]. The reaction was quenched with 5 ml of water and extracted four times with 3 ml of methylene chloride. The methylated oligosaccharide had to be isolated and purified by extraction in organic solvent because the size of the oligosaccharide prohibited the use of a dialysing membrane. The

methylene chloride phases were combined and washed three times with 3 ml of water. Finally a 5 % sodium thiosulphate wash removed residual iodine and the organic phase was dried with sodium sulphate, filtered and concentrated to dryness.

The methylated oligosaccharide was reduced for (16 hours) in dry methanol and was then hydrolysed (4 M TFA, 125°C, 1 hour). The derived PMAA's[186] were examined by GLC at 220°C and yielded the partially methylated alditol acetates listed in Table 11 (page 121)

8.8.b. **Reduction of the oligosaccharide repeating unit P1**

P1 was reduced with $NaBH_4$ and the derived alditol was exchanged with D_2O (99.6%), made up in 99.995% D_2O and examined by NMR.

8.8.b.(i). **NMR acquisition parameters of the repeating unit alditol P1-oL**

^1H- and ^{13}C-NMR spectra were recorded at 30°C on a Bruker AMX 400 spectrometer. Samples were deuterium exchanged and then dissolved in 99.99% D_2O containing a trace of acetone as internal standard, δ 2.23 for ^1H and 31.07 ppm for ^{13}C.

The 2D ^1H homonuclear shift-correlated experiment (COSY)[242] was performed using a spectral width of 1805.05 Hz. A data matrix of 256 x 2048 data points was collected with 152 transients for each delay. The data matrix was zero-filled in the t_1 dimension to 1024 data points and transformed after the application of a non-shifted sine-bell window function in both dimensions and symmetrised. Digital resolution in the resulting 2048 x 1024 matrix was 0.88 Hz/point.

The 2D Relay-COSY[260] experiments at 400 MHz was performed using a spectral width of 1805.05 Hz. Data matrices of 2048 x 256 data points were collected with 144 transients for each t_1 delay. The data matrices were zero-filled to 1024 data points in t_1 transformed after the application of a shifted sine-bell window function. Digital resolution in the 2048 x 1024 matrices were 0.88 Hz/point in f_2. Fixed delays of 0.078 s were used.

The 2D homonuclear Hartmann-Hahn (HOHAHA)[261] experiment was performed using a

spectral width of 1805.05 Hz. The mixing period consisted of 50 MLEV-17 cycles. A data matrix of 1024 x 371 was acquired with 112 transients for each t_1 delay. The matrix was zero-filled in the t_1 dimension to (1024 x 1024 data points) giving a digital resolution of 1.76 Hz/point in f_2 and multiplied in both dimensions with a shifted sine-square window function prior to phase sensitive Fourier transformation.

The 2D ^{13}C - ^{1}H heteronuclear shift-correlated (HETCOR)[249] experiment was recorded using a spectral width of 1600 Hz in t_1 and 10204.08 Hz in t_2. The initial matrix of 4096 x 128 was zero-filled to 2048 x 256 data points and transformed after the application of a shifted sine-square window function. Digital resolution in f_1 was 0.16 Hz/point and in f_2 4.98 Hz/point. A recycle delay of 1 s was employed and 1100 transients per f.i.d. were collected.

The 2D heteronuclear multiple quantum coherence-total correlated spectroscopy (HMQC-TOCSY) experiment was recorded using: 4096 x 512 data matrix, 48 scans per t_1 value, 50 MLEV-17 cycles, with a 1-s recycle delay.

The 2D heternuclear multiple bond coherence spectroscopy (HMBC)[269] experiment was recorded using: 4096 x 256 data matrix, 96 scans per t_1 value, $\Delta 1$ and $\Delta 2$ durations of 3.45 ms and 60 ms, respectively, and a sine-bell filter.

8.8.c. NMR acquisition parameters of the repeating unit P1

The 2D ^{1}H homonuclear shift-correlated experiment (COSY)[242] was performed using a spectral width of 1602.56.Hz. A data matrix of 512 x 2048 data points was collected with 64 transients for each delay. The data matrix was zero-filled in the t_1 dimension to 1024 data points and transformed after the application of a non-shifted sine-bell window function in both dimensions and symmetrised. Digital resolution in the resulting 2048 x 1024 matrix was 0.78Hz/point.

The 2D homonuclear Hartmann-Hahn (HOHAHA)[261] experiment at 400 MHz was performed using a spectral width of 1602.56 Hz. The mixing period consisted of 50 MLEV-17 cycles. A data matrix of (2048 x 512) was acquired with 64 transients for each t_1 delay. The matrix was zero-filled in the t_1 dimension (2048 x 1024 data points) giving a digital resolution of

0.78 Hz/point and multiplied in both dimension with a shifted sine-square window function prior to phase sensitive Fourier transformation.

The 2D heternuclear multiple coherence correlated spectroscopy (HMQC)[247] experiment was recorded: (4096 x 512) data matrix, 32 scans per t_1 value, 1-s recycle delay, zero-filled to 1024 data points in t_1

The 2D heteronuclear multiple quantum total-coherence correlated spectroscopy (HMQC-TOCSY) experiment was recorded using: 4096 x 512 data matrix, 48 scans per t_1 value, 12-MLEV-17 cycles, with a 1-s recycle delay.

The 2D heternuclear multiple bond coherence spectroscopy (HMBC)[269] experiment was recorded using: (4096 x 256) data matrix, 112 scans per t_1 value, $\Delta 1$ and $\Delta 2$ durations of 3.45 and 60 ms, and a sine-bell filter.

9. ANNEXURE 1

9.0. Formulae for Nutrient Media.

9.1. Formula 1: Luria Bertani Broth (LBB)

Bacto-Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
Maltose	2 g
Water to	1 L

9.2. Formula 2: Luria Bertani Agar (LBA)

LBB + 1.5% agar.

9.3. Formula 3: Luria Bertani Sloppy Agar

LBA + 0.7% agar.

9.4. Formula 4: Sucrose-Rich Medium

Sucrose	75.0g
NaCl	5.0g
Yeast Extract	5.0g
$K_2HPO_4 . H_2O$	2.5g
$MgSO_4 . 7 H_2O$	0.62g
$CaCO_3$	0.5g
Agar	37.5g
Water to	2.5 L

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