STRUCTURAL STUDIES ON

SOME ENTEROBACTERIAL CAPSULAR ANTIGENS

THESIS

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For my parents

ABBREVIATIONS AND SYMBOLS

~	approximately
BIRD	bi-rotational
cw	continuous wave
CI	chemical ionisation
COSY	correlation spectroscopy
CTAB	cetyltrimethylammonium bromide
CZE	capillary zone electrophoresis
DCI	direct chemical injection
DEAE	diethylaminoethyl
δ	chemical shift in parts per million
EI	electron impact
eV	electron volt
f	furanose
FAB	fast atom bombardment
FT	Fourier transform
Fuc	fucose
FucNAc	*N*-acetyl fucosamine
g	gram
Gal	galactose
GalA	galacturonic acid
GalNAc	*N*-acetylgalactosamine
GAS	gradient accelerated spectroscopy
GLC	gas liquid chromatography
Glc	glucose
GlcA	glucuronic acid
GlcNAc	*N*-acetyl glucosamine
GPC	gel permeation chromatography

h	hours
HOHAHA	homonuclear Hartmann Hahn
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum coherence
HPAEC	high pH anion-exchange chromatography
HPLC	high performance liquid chromatography
HR	high resolution
Hz	Hertz
IE	ion-exchange
KDO	2-keto-3-deoxy*manno*-octulosonic acid
L	litre
Lac	lactyl
M	molar
Man	mannose
ManNAc	*N*-acetyl mannosamine
ManNAcA	*N*-acetyl mannosaminuronic acid
m/z	mass-to-charge ratio
mg	milligram
min	minute
mL	millilitre
mM	millimolar
MS	mass spectrometry
mol	mole
M_W	average molecular weight
NeuAc	neuraminic acid
µL	microlitre
NOESY	nuclear overhauser enhancement spectroscopy
NMR	nuclear magnetic resonance
p	pyranose

PAD	pulsed amperometric detection
PAAN	peracetylated aldononitrile
PC	paper chromatography
PFUs	plaque-forming units
PM	permethylated
PMAA	partially methylated alditol acetate
ppm	parts per million
PR	presaturated
PRC	partial reductive cleavage
pyr	pyruvate
Qui	quinovose
Rha	rhamnose
RI	refractive index
ROESY	rotating frame overhauser enhancement spectroscopy
SF	superfine
SRC	selective reductive cleavage
TOCSY	total coherence transfer spectroscopy
TFA	trifluoroacetic acid
TSR	total sugar ratio
TMS	tetramethylsilane
UV	ultraviolet

Abbreviations, chemical formulae and symbols not included in this list are defined in the text.

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ABSTRACT

The investigations presented in this thesis form part of a systematic international effort to establish the structures of the capsules produced by the bacterial genera, *Escherichia coli* and *Klebsiella* (family *Enterobacteriaceae*). These bacteria are of medical interest as they are opportunistic pathogens and are frequently responsible for serious infections in animals and man. Invasive strains are invariably surrounded by a structurally complex polysaccharide capsule which contributes to the organism's ability to attenuate non-specific host defence mechanisms or, in some instances, to completely prevent an immune response. A knowledge of the chemical composition and structure of the capsule is, therefore, of great value as it provides insight into the mechanisms involved in this process. The *E. coli*, in particular, have generated considerable interest as their capsules are more structurally diverse and cross-reactivity with other, more pathogenic bacteria has also been demonstrated.

Accordingly, the structures of three previously unstudied *E. coli* K-antigens *viz.* those produced by serotypes O20:K83:H26, O20:K84:H26, and O9:K48:H9 have been established by chemical and spectroscopic means and are presented in this thesis. In addition, a reinvestigation of the structure of the capsule produced by *Klebsiella* K15 using a novel enzymatic approach was also undertaken and a revised structure is proposed. The *E. coli* K48 polysaccharide is of special interest as it was found to contain a new diacetamido trideoxy hexose hitherto unrecorded. A synthesis for this saccharide is also presented. Finally, the application of lithium dissolved in ethylenediamine for the degradation of amino sugar-containing polysaccharides was also investigated using the capsular polysaccharides produced by *E. coli* serotypes K38 and K84 as model compounds.

1. INTRODUCTION

1.1 THE IMMUNOGENICITY AND BIOLOGICAL SIGNIFICANCE OF THE BACTERIAL CAPSULE

Bacteria of the genera *Klebsiella* and *Escherichia coli* are Gram negative rods of the family *Enterobacteriaceae*. *E. coli* is the predominant aerobic constituent of the normal colonic flora and normally colonises infants shortly after birth. It is, however, an opportunistic pathogen and certain strains are responsible for gastro-intestinal and urinary tract infections. More seriously it may cause septicaemia or meningitis in debilitated and immunosuppressed patients when host anatomical barriers have been disrupted. Infections due to *Klebsiella* usually occur in the upper respiratory or urinary tract, but wound and soft-tissue infections may occur in compromised patients. *Klebsiella* bacteraemia and pneumonia have a mortality rate which may exceed 50%[1].

The anatomy of the bacterial cell wall has been studied extensively, the singular feature of Gram negative bacteria being the presence of an additional outer membrane external to the murein sacculus which distinguishes them from Gram positive bacteria. In smooth wild-type bacteria this outer membrane layer contains specific antigenic components, the most important being lipopolysaccharide. This is comprised of three distinct moieties, *viz*. lipid A, the core oligosaccharide and the O-specific side chain. These together constitute the important somatic or O-antigens. A second important group of immunogens, if present, are the flagellae which are termed the H-antigens.

Many Gram negative bacteria, in particular those of the genera *Klebsiella* and *Escherichia* express, in addition, a characteristic acidic exocellular polysaccharide which is loosely associated with the outer membrane and encapsulates the bacterium. This capsular material is also immunogenic and elicits the production of specific anti-capsular antibodies and constitutes the K-antigen.

The expression of capsule *via* the bacterial cell wall is an exceedingly complex process. The activation and interconversion of the sugar constituents takes place in the cytoplasm after which they are transferred from their nucleotides to an acceptor by various transferases associated with the cytoplasmic membrane. The finished polysaccharide is then translocated through the cytoplasmic membrane, periplasmic space and the outer membrane to form the capsule[2].

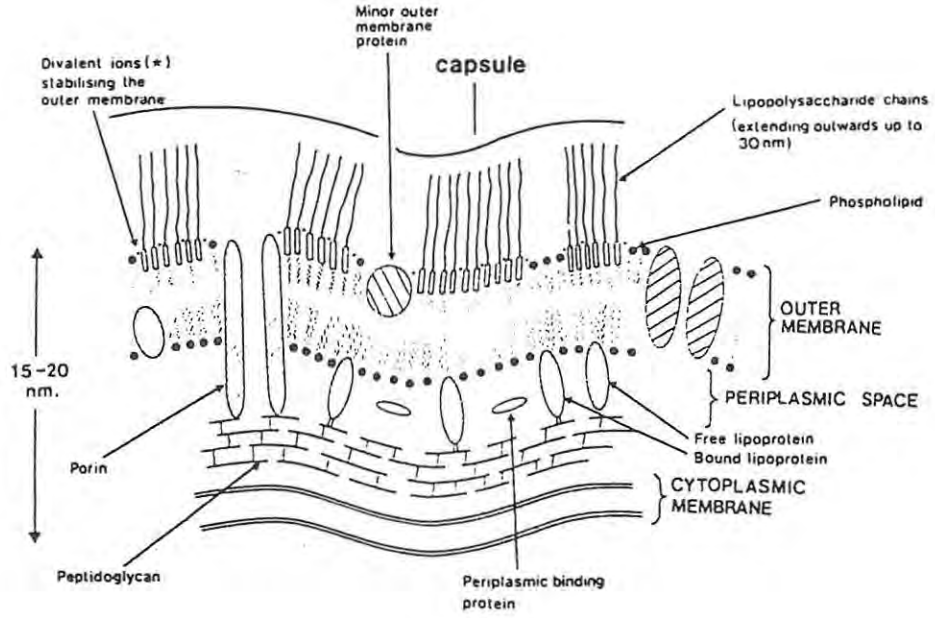


Figure 1.1 Diagrammatic representation of the envelope of encapsulated Gram-negative bacteria.

It has recently been established in the *Escherichia coli* that capsular polysaccharide chains are substituted at their reducing ends with a lipid moiety, either core lipid A or phosphatidic acid[2,3]. It is assumed that this lipid may in some way serve to anchor the capsule to the outer membrane of the bacterium by means of hydrophobic interactions and is thus important for the maintenance of the capsule. Nevertheless, the capsule is frequently lost following serial propagation *in vitro* or when the bacteria exist under ideal conditions and is not essential for the survival of the organism. Despite this tendency, the ability to produce an exocellular capsule is usually advantageous.

Encapsulated bacterial strains are almost always more resistant to phagocytosis and it is generally agreed that the capsule, in most instances, performs a protective and anti-phagocytic role enabling the bacterium to counteract non-specific host defences during the pre-immune phase of the infection. It interferes with the action of complement and impedes bacteriolysis and phagocytosis effected by the terminal membrane attack complex (MAC) and the complement component C3b respectively[4]. Normally the complement system is activated either by interaction with antibodies (classical pathway) or by triggering *via* the properdin system (alternative pathway)[5].

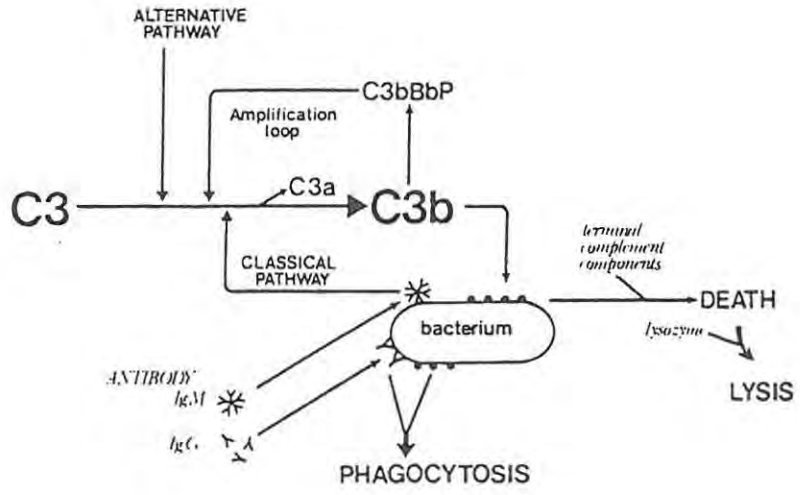


Figure 1.2 The role of complement in the elimination of microorganisms from the body. The central reaction generating the opsonically active protein C3b may be initiated by either the "classical" or "alternative" pathways[5].

The masking of the somatic (O) antigens by the capsule presents a carbohydrate surface of lower antigenic potential to the host. In some instances, capsules show structural similarity to or identity with host structures and elicit a very poor immune response, presumably due to the inability of the host to distinguish self from non-self. Such strains are highly virulent and include *Escherichia coli* K1 (resembles the carbohydrate terminus of the embryonic neural cell adhesion molecule n-CAM), K4 (resembles fructosylated chondroitin) and K5 (identical to the first polymeric intermediate in the biosynthesis of heparin)[6].

Hence the capsule enables the bacterium to counteract the host defence by interaction with complement components and by preventing immune recognition through molecular mimicry. It has also been suggested that physical factors may contribute to the capsule's ability to retard host response. The amount of capsule produced has been correlated with virulence[7], presumably due to the fact that the more voluminous the capsule the further the more recognisable surface antigenic components are from the interface. Many capsules are poorly biodegradable and the amount of capsule produced may simply overwhelm the host defence. The more hydrophilic the bacterial surface, the more resistant it is to phagocytosis by neutrophils in the absence of opsonins. The presence of uronic acids and other acidic components in many capsules suggests that the anionic nature of the polymers may also be significant in retarding phagocytic attack[5]. In addition, charged groups bind large quantities of water and ions, creating a slimy or gel-like surface that provides shock absorption and protection from mechanical damage and dehydration.

Recently Camprubi *et al.*[8] have found that, in certain strains of *Klebsiella pneumoniae*, the presence or absence of the K-antigen (capsule) did not affect susceptibility to complement-mediated phagocytosis. Instead their findings suggest that the O-antigen rather than the K-antigen plays a more crucial role in protecting the organism from complement mediated serum killing. Furthermore, recent studies[9] have shown that certain *Klebsiella* serotypes may undergo lectinophagocytosis, mediated by specific *manno-manno* disaccharide components in their capsules. These components are recognised by the mannose/*N*-acetylglucosamine-specific lectin of macrophages making these strains *more* susceptible to phagocytosis and, as a result, they are rapidly cleared from the body. It therefore appears that further study is required before the role of the capsule and its interactions with host defence systems can be fully understood.

Based on knowledge obtained from structural studies, the first capsular polysaccharide vaccines have recently been developed, however their use as immunoprophylactic agents in humans has not been overly successful. 80-90% of the *E. coli* strains implicated in cases of pyelonephritis, meningitis and diarrhoeal disease fall within K types 1, 2, 3, 5, 12, and 13[10].

For this reason researchers have concentrated on developing carbohydrate vaccines against these strains, unfortunately only with limited success. Schneerson *et al.*[11] conjugated *E. coli* K13 polysaccharide to bovine serum albumin and achieved satisfactory immunity in a rat ascending pyelonephritis model. However, no vaccines for protection against urinary tract infections in humans have been developed. *E. coli* K1 is frequently the causative agent in neonatal meningitis which is associated with a particularly high mortality rate. Attempts to develop a carbohydrate vaccine as an immunoprophylactic agent have proved unsuccessful to date, however recent advances in the design of protein-carbohydrate conjugates have led to the development of vaccines which show improved antibody responses[12]. It has been proposed that the coupling of purified capsular polysaccharide material to the major *E. coli* outer membrane proteins may result in vaccines worthy of testing in humans[10]. Capsular polysaccharide-protein conjugates for certain *Klebsiella* strains and a polyvalent *Klebsiella* vaccine have also been tested[1,13,14]. These studies have shown that purified *Klebsiella* antigens from clinically relevant strains are highly immunogenic and elicit satisfactory antibody responses in some cases, however, as with the *E. coli*, no reliable vaccines have resulted. It therefore appears that various biological, chemical and conformational parameters pertaining to these capsular antigens still remain to be understood before the first truly effective capsular polysaccharide vaccine for coliform infections can be developed.

Whilst strains such as *E. coli* K1 and K5 and certain *Klebsiella* strains are pathogenic, the majority are normally harmless to man. Exocellular polysaccharides only protect the organism during the pre-immune stage of the infection and prior to the development of specific anti-capsular antibodies and ultimate destruction by the immune system. Nevertheless they remain important antigenic determinants and display great specificity and variability of structure. A systematic study of the structures of these polysaccharides is therefore desirable as it provides insight into the role of the capsule in bacterial disease and the subsequent immune response.

1.2 THE CHEMISTRY OF THE K-ANTIGENS OF *KLEBSIELLA* AND *ESCHERICHIA COLI*

Over the last two decades the chemical structures of the capsular polysaccharides produced by these two genera have been extensively researched. This has led to a greater understanding of the relationship between structure and immunogenicity and has greatly facilitated their classification, particularly the more structurally diverse *E. coli*.

Of the above two species, *Klebsiella* is more pathogenic and tends to produce copious amounts of capsule, readily isolated in pure form, and it was therefore the first to be comprehensively studied. Cross-reactivity studies reported by Heidelberger and co-workers,[15,16] which demonstrated that several of the *Klebsiella* K-polysaccharides were cross-reactive with antipneumococcal sera, emphasised the need for a detailed knowledge of the primary structures of these K-antigens. A concerted international drive to elucidate the structures of the exopolysaccharides produced by this genus was therefore initiated in an effort to relate the serological distinctiveness of the various strains to the chemistry of the capsule and to facilitate interpretation of the observed cross-reactivity. Structural studies were further stimulated by the successful application of bacteriophage-borne enzymes for the degradation of isolated *Klebsiella* K-polysaccharides[17] to yield specific oligosaccharide fragments highly conducive to chemical and spectroscopic analysis. This programme was later extended to include the O-specific antigens and to other members of the *Enterobacteriaceae*, in particular, the more familiar *Escherichia coli*.

Qualitative analyses of capsules produced by the *Klebsiellae*, undertaken by Nimmich in 1968[18] served as the starting point and by the early 1980's all but a few remained to be elucidated with only the structures of K29, K42, K65 and K71 still unknown. The structure of the K-antigen produced by *Klebsiella* K43 being the one most recently established[19]. Several structures have been revised using more sophisticated techniques. In this thesis *Klebsiella* K15 has been re-investigated and major changes are proposed for the structure.

The *Klebsiella pneumoniae* capsular polysaccharides have been classified according to serological reactions into seventy-seven different K-antigens which are associated with approximately eight O-serotypes[20,21]. They have also been characterised according to structural multiplicity[22] which is a feature of the chemistry of these polysaccharides e.g. "5+2" or "4+2+1", the former representing a repeating unit with five residues in the main chain and two in the side chain etc., however this has not been related to immunological specificity. Characteristically, they are high molecular weight acidic heteropolysaccharides comprised of repeating units of three to seven residues. Structural studies undertaken to date have shown that the majority contain either D-GlcA or, less commonly, D-GalA, in combination with hexoses (D-Glc, D-Gal and D-Man) and 6-deoxyhexoses (L-Rha and L-Fuc). No aminodeoxy sugars are present. In nearly all cases the sugars assume the pyranose form but galactose has been found in the furanose form[23,24,25]. These glycans are largely permutations of the aforementioned sugars although some unusual sugar residues have been encountered e.g. 4-*O*-[(***S***)-1-carboxyethyl]-D-glucopyranosyluronic acid (4-*O*-(***S***)-Lac-D-GlcA) is present in a terminal position in the repeating unit of the K37[28] polysaccharide. Interestingly, K22 was originally reported to have an almost identical repeating unit, except that the terminal sugar was 4-deoxy-*threo*-hex-4-enosyluronic acid. Later studies[27] proved that this residue is formed by β-elimination of the lactyl ether substituent of 4-*O*-(***S***)-Lac-D-GlcA. It is postulated that K22 bacteria produce an enzyme capable of eliminating lactate and utilising it as a source of carbon under certain conditions. K22 and K37 therefore have the same sequence of sugars in their respective repeating units but differ in serology due to the presence of an *O*-acetyl group on position six of the galactose residue in K22.

Other novel residues that have been encountered include 3-deoxy-D-*glycero*-pentulosonic acid which occurs in K38[28], 4-*O*-(***R***)-Lac-D-Glc which is a constituent of the K66[29] antigen and recently L-glutamic acid has been reported in the K82[30] antigen. A notable feature is the presence of pyruvic acid acetals on approximately half of the known K-antigens and in some instances pyruvate is the only acidic function *viz.* in serotypes K32[31], K56[32] and K72[33]. The configuration (***R*** or ***S***) and position of the acetal and the nature of the pyruvylated residues varies with 4,6-linked acetals (six-membered ring) being the most common. However, 3,4-linked and 2,3 linked acetals in the form of a five membered dioxolane ring are also found.

O-acetyl groups are also common and, like pyruvate, are important determinants of serologic epitopes. In some instances an *O*-acetyl group is the only chemical difference between otherwise identical capsular antigens and accounts for the serological distinctiveness between K30[34] and K33[35], and K22 and K37 as discussed above. *O*-formate, originally reported to occur in these polysaccharides, has now been shown to be absent[36].

In contrast to bacteria of the genus *Klebsiella*, the *Escherichia coli* show a high degree of heterogeneity in the chemistry of their capsular (K) antigens. Many of the structures of the *E. coli* K-antigens have now been established and recent reviews by Dutton and Parolis[37] and De Bruin[38] record the structures published to date. In addition to the neutral sugars found in *Klebsiella*, the pentose D-Rib*f* and the keto-sugar D-Fru*f* also occur. Fructose has always been found in a labile, terminal position and is readily cleaved under mildly acidic conditions. This is associated with a concomitant loss of serologic specificity thus demonstrating the immunological significance of this residue[2]. These polysaccharides often contain aminodeoxy sugars and, in some cases, amino acids and phosphate esters which are not found in any of the *Klebsiella* capsules. Several different amino sugars have been reported as constituents of these polysaccharides of which D-GlcNAc and D-GalNAc are the most common. NeuAc is less frequently encountered whilst L-FucNAc has only been found in the K87 polysaccharide[39]. In addition, the unusual aminouronic acid, D-ManNAcA, is the acidic component of the K7 antigen[40] and D-Fuc3NAc is present in the K45 antigen[41]. The capsular polysaccharide from *E.coli* K48 (this thesis) contains the first diamino sugar, 2,3-diacetamido-2,3,6-trideoxy-L-mannopyranose (L-Rha2,3diNAc), to be encountered in a capsular polysaccharide in this genus. This thesis also reports, for the first time, the occurrence of the uncommon amino sugar D-ManNAc in the K-antigen produced by *E. coli* K84.

This diversity, and the occurrence of more than one hundred and sixty "O" antigens[2,10] as opposed to the eight "O" groups of *Klebsiella*, has led to a concerted effort to classify this genus. Analysis of a great number of the *Escherichia coli* capsular polysaccharides has permitted subdivision into two broad categories based on differences in chemical composition, molecular weight, mode of expression and genetic determination *viz*. groups I and II[42].

This system replaced the original A,B and L system proposed by Kaufmann[43], which was based on bacterial agglutination of unheated and heated *E. coli* by different sera.

TABLE I. GROUPING OF CAPSULAR POLYSACCHARIDE ANTIGENS OF *E. coli*

PROPERTY	CAPSULAR POLYSACCHARIDE GROUP	
	I	II
Acidic component	GlcA GalA Pyruvate	GlcA NeuAc KDO ManNAcA Phosphate
Expressed below 20°C	Yes	No
Coexpression with	O8, O9, O20	Many O antigens
Lipid at reducing end	Core Lipid A	Phosphatidic acid
Removal of lipid at pH 5-6/100°C	No	Yes
Chromosomal determination at (close to)	*rfb(his), rfc(trp)*	*kpsA(serA)*
CMP-KDO synthetase activity elevated	No	Yes
Intergeneric relationship	*Klebsiella*	*H. influenzae* *N. meningitidis*

Adapted from Jann *et al.*[2].

Group I polysaccharides have been further subdivided according to the absence or presence of amino sugars and those without have shown an intergeneric relationship with certain *Klebsiella* strains, e.g. chemical studies have shown that *E. coli* K55 has the same capsular antigen as *Klebsiella* K5[44].

The group II polysaccharides are more structurally diverse than group I and tend to include the more pathogenic strains[4,6]. They contain a wider variety of acidic components and have been further classified on this basis into four sub-categories *viz*. those containing KDO, phosphate, GlcA and NeuAc. KDO is frequently encountered together with D-Rib and is frequently acetylated at positions four, seven or eight[2]. It occurs in both the pyranosidic and the furanosidic forms and as the α or β anomers. 4-deoxy-2-hexulosonic acid, a KDO-related residue is present in the K3 antigen[45]. Phosphate-containing polymers, which resemble the teichoic acids of many gram positive bacteria, contain phosphate either as ribitol-, glycero- or sugar-phosphate esters.

TABLE II. MONOSACCHARIDES PRESENT IN THE CAPSULAR POLYSACCHARIDES OF *E. coli* AND *Klebsiella*

MONOSACCHARIDE	CAPSULAR ANTIGEN(S)	
	Klebsiella	*E. coli*
D-Glc*p*	Common	Common
D-Gal*p*	Common	Common
D-Gal*f*	K12, K14, K41	K2, K53, K93
D-Man*p*	Common	Common
L-Fuc*p*	Frequent e.g K1	K27, K28, K33, K42, K87
L-Rha*p*	Frequent e.g K32	Frequent e.g. K3
D-Fru*f*	-	K4, K52, K11
D-Rib*f*	-	K6, K13, K18, K22, K74, K95
D-GlucA*p*	Common	Common
D-GalA*p*	K34, K48, K49, K57	Frequent e.g. K36
D-Glc*p*NAc	-	Common
D-Gal*p*NAc	-	K8, K9, K14, K44,
D-Man*p*NAc *	-	K84*
L-Fuc*p*NAc	-	K87
D-Man*p*NAcA	-	K7
Kdo*p*	-	Frequent e.g. K6
Kdo*f*	-	K95
Neu*p*Ac	-	K1, K9, K92,
D-Fuc*p*3NAc	-	K45
L-Rha*p*2,3diNAc *	-	K48
4-*O*-Lac-D-Glc	K66	-
4-*O*-Lac-D-GlcA	K37, K22	-
3-deoxy-L-glycero-pentulosonic acid	K38	-
4-deoxy-2-hexulosonic acid	-	K3

* This thesis

K18, K22[48] and K100[47] are poly (ribosyl-ribitol phosphates), K2[48], K24[49] and K46[50] are glycerophosphate polymers, K51[51] is a sugar phosphate ester of α-D-GlcNAc and K52[52] of α-D-Gal substituted at position two by β-D-Fru*f*. The K7 antigen which contains the unusual aminouronic acid, ManNAcA, has been classified along with the GlcA containing polymers. Only three K-antigens have been reported to contain NeuAc viz. K1[53], K9[54] and K92[55]. K1 and K92 are homopolymers of NeuAc.

In addition to phosphate, these polysaccharides may be substituted with other non-carbohydrate substituents. *O*-propionate is present in K14[56] and K52[52] but only in small amounts. *O*-acetyl groups are far more frequently encountered and, as is the case with *Klebsiella*, they profoundly influence the antigenicity and immunological specificity of the capsule. For this reason the accurate location of *O*-acetyl groups is important and some structures have been re-examined when the location reported has been inconclusive. Pyruvate is not encountered as frequently as in the *Klebsiella* exopolysaccharides and is the sole acidic component in only four K-antigens *viz*. K37[57], K47[58], K50[59] and K103[60]. Amino acids have only been found amidically linked to uronic acids. L-Serine is present in K40[61] and both L-serine and L-threonine occur in K49[62] and K54[63].

Seventy-four of the K-antigens of *E. coli* classified to date are acidic polysaccharides and the structures of the capsular antigens of approximately sixty are known. However, two strains of *E. coli* produce proteinaceous K-antigens, *viz*. K88 and K99, and these have been reclassified as a new group, *viz*. the F-antigens[64]. Several strains are either non-encapsulated or the K-antigens have not been shown to be independent of the O-antigens, thus explaining why the K-numbers run up to one hundred and three.

2. ISOLATION AND PURIFICATION OF CAPSULAR POLYSACCHARIDES

The isolation of sufficient K-antigen in a pure form is a prerequisite for structural investigation. The procedure should be such that labile constituents of the glycan are preserved and other carbohydrate containing contaminants produced by the bacteria are excluded. A number of different methods have been employed to isolate bacterial capsular polysaccharides and these have been reviewed[65]. The method employed in this laboratory is essentially that outlined by Okutani and Dutton[66].

Serologically homogenous encapsulated bacteria, usually from a serotyped stab culture, are propagated on an appropriate sterile nutrient agar medium at 37°C until actively growing colonies are present. In this laboratory a sucrose-rich medium is used for the propagation of *Klebsiella* bacteria and either Mueller-Hinton or Luria-Bertani medium is preferred for the *E. coli*. (Appendix II). Single colonies are removed and transferred to sterile culture tubes containing the appropriate nutrient broth (~5 mL) and agitated in a water bath at 37°C to ensure good growth. The turbid solutions are then used to inoculate flasks containing ~50 mL of sterile broth. Once turbid these are poured onto stainless steel trays (60 cm x 40 cm) containing the appropriate nutrient agar, 1 cm in depth, and incubated at 37°C. After several days the cells are scraped off the agar surface and suspended in a solution of 1% phenol which kills the bacteria without lysis. The resulting cell suspension is gently stirred at 4°C for 24-48 hours to allow dissolution of the capsular polysaccharide. Cellular debris is removed by ultracentrifugation and the dissolved polysaccharide is recovered by precipitation into ethanol. The acidic K-antigen is isolated from the precipitate, which also contains small quantities of other soluble products, notably neutral O-polysaccharide, by the slow addition of a solution of 5% cetyltrimethylammonium bromide (CTAB) or other suitable quaternary ammonium salt to an aqueous solution of the precipitate. The polysaccharide-CTAB complex is then dissociated by dissolution in aqueous 3 M NaCl and reprecipitated into ethanol. The isolated precipitate, redissolved in water, is dialysed exhaustively against tap water to remove salt and residual CTAB.

The dialysed solution is then ultracentrifuged and freeze-dried to yield K-antigen as the sodium salt. Further purification, if necessary, can be achieved by ion-exchange chromatography (DEAE Sepharose CL-6B) or semi-preparative gel chromatography (Sephacryl S-400 or S-500).

Klebsiella bacteria give good yields when propagated on sucrose-rich agar using the method outlined above. In contrast to this *E. coli,* which tend to have smaller more tightly bound capsules, give far lower yields frequently contaminated with protein, lipid and nucleic acids. A modified procedure is used in this laboratory to overcome these problems. Bacteria are removed from the stab culture and streaked onto agar plates. Discrete colonies are transferred directly to tubes containing 10 mL of broth and, following incubation, are poured onto the trays and evenly spread out. This shortened procedure minimizes the possibility of the bacteria failing to express capsule due to extensive subculture. We have found that the smaller volume used to inoculate the trays (10 mL) gives higher yields of K-antigen as *E. coli* do not grow well when large volumes of liquid inoculum lie on the tray. The bacteria are harvested after approximately 18-24 hours incubation as longer periods of growth results in the medium becoming desiccated. In addition, *E. coli* bacteria sometimes produce a non-specific galactan if incubated for long periods[67]. A heat extraction procedure is sometimes used to increase yields. This facilitates the dissolution of smaller, more tightly bound capsules and involves heating the phenol suspension at 60°C for 10 min with agitation after which it is processed as usual. Unfortunately this increases the likelihood of cell rupture leading to greater contamination with nucleic acids and protein. Treatment of the isolated material with proteases and nucleases (RNAse and DNAse) is often required or alternatively ion-exchange gel chromatography can be used to remove these contaminants.

The addition of CTAB to a solution of the crude precipitate often does not produce an insoluble complex as readily as with the *Klebsiella* antigens. Gentle heating and the addition of a small quantity of sodium sulphate is sometimes beneficial, however we have found that treatment of the solution with 1% acetic acid to remove bound lipidic material prior to the addition of CTAB often results in vastly improved yields of the complex. Failing this, the acidic K-antigen can be isolated fron neutral O-antigen by ion exchange chromatography.

It is also possible to isolate capsular polysaccharide directly from a liquid culture (Westphal *et al.*[68]). A quaternary ammonium salt (e.g. CTAB) is added directly to the culture vessel and the glycan is extracted with calcium chloride. This is followed by repeated precipitation with ethanol and finally extraction with cold phenol prior to dialysis and lyophilisation.

3. METHODOLOGIES EMPLOYED IN STRUCTURAL STUDIES OF COMPLEX POLYSACCHARIDES

Over the last decade the analysis of complex polysaccharides has been revolutionised by impressive advances in instrumental technology, particularly in the fields of NMR spectroscopy, mass spectrometry and in chromatographic techniques such as gas-liquid chromatography (GLC) and high performance liquid chromatography (HPLC). Classically the analysis of polysaccharides is based on chemical degradation of the polysaccharide and subsequent characterisation of the mono- and oligo-saccharide fragments so produced by a variety of qualitative and quantitative chemical procedures involving principally glycose and methylation analysis. This "bottom up" strategy has evolved into a "top down" strategy in which the researcher is now able to elucidate the structure of the polysaccharide almost entirely by non-destructive instrumental means involving predominantly NMR spectroscopy. Chemical procedures are now aimed at generating specific oligosaccharide fragments through selective degradation of the polysaccharide. Ideally, in the case of bacterial polysaccharides, the aim is to generate an oligosaccharide analogous to the repeating unit where attached non-carbohydrate substituents are preserved as far as possible and analysis of which provides information unobtainable from studies of the intact polysaccharide. However, this is not always possible and the analyst must select an appropriate technique which generates the most useful oligosaccharide fragment(s).

This review emphasises and expands on the modern "top down" approach, in particular the use of NMR spectroscopy. The traditional chemical procedures will not be extensively covered and the reader is referred to several excellent reviews[69,70,71]. Nevertheless, routine chemical studies are still performed as they often provide invaluable supplementary information and may clarify ambiguous results obtained following extensive NMR analysis.

The study of bacterial capsular polysaccharides both chemically and spectroscopically is considerably simplified because they are comprised of regular repeating units.

When all sugars can be released by total hydrolysis of the polysaccharide, quantification will reflect the molar proportions in which the sugars occur in the repeating unit and NMR studies of the polysaccharide will to a large extent reveal its composition and structure. The sugar composition, absolute stereochemistry, ring structure, position and configuration of the linkages and the sequence of these units have to be established. Furthermore non-carbohydrate substituents (if present), need to be located and quantified and the molecular weight of the polymer determined. In some instances it is also desirable to determine the solution conformation of the polysaccharide.

3.1 DETERMINATION OF MONOSACCHARIDE COMPOSITION

The monosaccharides which comprise the repeating unit can usually be identified, and in many cases quantified, from hydrolysates of the polysaccharide by a variety of chromatographic procedures. Traditional methods such as paper chromatography (PC) and thin-layer chromatography (TLC) have largely been replaced by GLC and HPLC and, in addition to enabling excellent chromatographic resolution, these two techniques can readily be interfaced with a mass spectrometer which permits sequential separation and fragmentation analysis (section 3.8).

Sugars must first be released by cleaving the glycosidic linkages in the polysaccharide before the total sugar ratio (TSR) can be determined. In this laboratory 4M trifluoroacetic acid (TFA) is the preferred acid catalyst. It is as effective as the strong mineral acids and has the added advantage of being easily removed from the reaction mixture under reduced pressure and therefore does not have to be neutralised[72]. Nevertheless, polysaccharides are susceptible to degradation under harsh reaction conditions and hydrolysis with TFA is no exception. Anhydro sugars[73] have been identified as one of the minor degradation products which may form. Fortunately the proportion of degradation products to hydrolysed sugars is usually low.

Not all glycosidic linkages are equally susceptible to acid hydrolysis and it may be advantageous to utilise other reagents.

Deoxyhexoses e.g. 6-deoxy and 2-deoxy sugars and furanosidically linked sugars are easily cleaved and milder conditions are required to release these residues. Sugars which decompose under hydrolytic conditions e.g. sialic acids are preferably released as the methyl glycosides using 3% hydrogen chloride in anhydrous methanol (methanolysis). Enzymic hydrolysis is also a useful tool for liberating very labile residues. A method whereby sialic acids are enzymically hydrolysed and simultaneously converted into stable 2-amino-2-deoxymannose derivatives has been developed by Neeser[74].

Glycosyluronates (uronic acids) are more resistant to cleavage and have a tendency to lactonise[75]. A more profitable route involves reduction of the acidic functional group to a hydroxymethyl group prior to hydrolysis. Sodium borohydride is the reagent of choice for reducing the carboxyl groups of derivatised sugars, however aqueous carbodiimide in combination with sodium borohydride as proposed by Taylor and Conrad[76] is preferred for reduction of carboxylic acids in native polysaccharides. For a hydrophobic polymer e.g. a methylated polymer, reduction can be effected in tetrahydrofuran (THF) using lithium aluminium hydride[77] or lithium triethyl borohydride[78]. Alternatively they can be directly quantified as aldono-1,4-lactones[79] or as methyl glycosides.

The release of 2-acetamido-2-deoxy sugars is also retarded during acid hydrolysis. The acid removes the *N*-acyl group and the resulting protonated free amino function causes electrostatic shielding of the hexosamidic linkage. Polystyrene sulphonic acid is sometimes used to effect better release of amino sugars. Higher concentrations of protons are maintained around the charged sugar units which leads to increased hydrolysis[80]. Mixtures of reagents which preserve the *N*-acyl group during hydrolysis have also been advocated e.g. Wang *et al.*[81] use a mixture of acetic acid, water and trifluoroacetic acid (5:75:20) which re-*N*-acetylates hexosamine residues immediately after hydrolysis. Mercaptolysis[82] (hydrogen chloride in ethanethiol) and acetolysis/hydrolysis[83] (acetic anhydride, acetic acid and sulphuric acid) are alternative procedures which effect the release of sugars.

Once sugars have been released they must be separated and quantified. In order to achieve this by GLC, they must be rendered volatile through derivatisation. A variety of derivatives can be prepared and these have been reviewed[84,85,86]. Methyl glycosides as their acetylated and trifluoroacetylated derivatives[87], and methyl and trimethylsilyl ethers[88] are sufficiently volatile for GLC and are rapidly and conveniently prepared, however on account of the fact that the ring is preserved, they give multiple peaks in the chromatogram due to the formation of anomers in the pyranose and furanose forms. Furthermore anhydrous conditions are required in the case of the latter as water readily hydrolyses silylated products back to the parent compound. Qualitative analysis of simple mixtures is good but more heterogeneous mixtures result in complex chromatograms with overlapping peaks unsuitable for accurate quantitative analysis. Laine and Sweely[89] have described the preparation of per-trimethylsilyl oxime and per-trimethylsilyl *O*-methyloxime derivatives where sugars are converted into oximes prior to trimethylsilylation but these result in the formation of both *syn* and *anti* derivatives. Although these are frequently unresolved, effectively giving a single peak, they nevertheless still inherit the other disadvantages of trimethylsilyl ethers.

As alternatives to the aforementioned derivatives, alditol acetates[90] and aldononitrile acetates[91] are widely used. These acyclic derivatives are preferred as they give only one peak in the chromatogram and they possess excellent chromatographic properties. Alditol acetates are the most frequently prepared derivative in this class although their preparation can be problematic. The acetylation step is relatively time consuming and reduction to the alditol (usually with sodium borohydride) leaves borate which has to be removed as volatile trimethyl borate under reduced pressure by co-distillation with methanol. This method is effective only under completely anhydrous conditions as water hydrolyses methyl borate into methanol and boric acid. Acetylation using catalysts such as pyridine[92] or sodium acetate[93] is effective only in the absence of borate. Blakeney *et al.* have developed a procedure using 1-methylimidazole as catalyst which results in complete acetylation in the presence of borate in approximately 15 min and hence effectively overcomes this limitation[94].

Peracetylated aldononitrile derivatives (PAAN's) formed by the dehydration of oximes are similar to alditol acetates but in the case of the PAAN derivative C-1 is converted to the chromatographically and mass-spectrometrically distinctive nitrile group. They are conveniently prepared according to the method of Chen and M°Ginnis[96] using hydroxylamine hydrochloride in 1-methylimidazole (catalyst and solvent). Hydroxylamine converts the aldose into an oxime derivative and acetic anhydride is added to peracetylate all free hydroxyl groups and effect dehydration of the oxime to the nitrile. This method can also be used to analyse ketoses, however since dehydration to the nitrile is not possible, the resultant derivative is a peracetylated ketoxime (PAKO)[96].

Alditol acetates and aldononitrile acetates are not ideal derivatives for the determination of hexosamines because of excessively long retention times on GLC. *O*-methyl oximes, reported by Mawhinney *et al.*[97], which are intermediate between the alditol acetates and the per-trimethylsilyl *O*-methyloxime derivatives, are useful for the analysis of hexosamines. They have considerably shorter retention times and give single peaks in the chromatogram. Other derivatives for the analysis of sugars by GLC have been reported. These include: trimethylsilylated or acetylated deoxy(methoxyamino) alditols[98], *O*-benzyloximes[99], trimethysilylated or trifluoroacetylated diethyldithioacetals[100] and *n*-butyl boronates[101].

Rapid advances in the field of capillary zone electrophoresis (CZE) have led to the first reports on the application of this method for the identification and quantification of sugars. Honda *et al.* have reported the separation of twelve reducing sugars as their *N*-2-pyridylglycamine derivatives[102]. However, at this stage, it is doubtful whether this technique will be routinely used for sugar analysis as it still involves derivatisation and accordingly offers no real advantage over capillary GLC.

Conversely, HPLC is rapidly becoming the method of choice for the analysis and quantification of sugar mixtures, the principal advantage being that derivatisation can be avoided. In addition to monosaccharides such as neutral sugars, uronic acids, amino sugars, sugar-lactones etc.,

oligosaccharides can also be analysed which is a major advantage over gas chromatography. High-pH anion-exchange chromatography with pulsed amperometric detection[103] (HPAEC-PAD) represents a further advance in the field of sugar detection and quantification. Sugars can be detected and quantified directly from hydrolysates with limits of quantification in the picomole range. The reader is referred to section 3.6 (iv) where this topic will be discussed in greater detail.

Progress in the field of ^{1}H and ^{13}C NMR spectroscopy over the last two decades (see section 3.7) has revolutionised the analysis of complex oligo- and polysaccharides. It is now possible to establish the monosaccharide composition of an oligosaccharide or the repeating unit of a bacterial polysaccharide entirely by NMR spectroscopy. Each different sugar residue has a characteristic *J*-coupling pattern arising from the vicinal nature of protons attached to the sugar ring which, together with chemical shift data, enables the analyst to establish its identity. Only the first four vicinal coupling constants (H-1 to H-4) for each residue need be known to allow deduction of the basic configuration[104]. In most instances the anomeric configuration is also immediately apparent from the $^{3}J_{H\text{-}1,H\text{-}2}$ value. These characteristic *J*-connectivity patterns are shown in Table III.

Once the basic configuration has been established, the finer details of the residue's constitution can be established from its ^{1}H and ^{13}C chemical shift data. These will reveal the presence of attached non-carbohydrate substituents, the presence of acetamido groups, deoxy functions etc. NMR spectroscopy is of particular value in those cases where it is not possible to isolate sugar residues by chemical cleavage of the polysaccharide. The capsular polysaccharide produced by *E. coli* K48 (Section 4.5) contains a novel diamino trideoxy sugar which could not be released by chemical means and has been characterised entirely by NMR spectroscopy. The COSY[105] spectrum (Figure 4.21) shows the *J*-connectivity pattern for this residue. From Table III. it can easily be identified as having the *manno* configuration. The ^{1}H and ^{13}C chemical shift data also show that it is a 6-deoxy sugar and has two diacetamido functions.

TABLE III

CORRELATION OF ALL POSSIBLE VICINAL COUPLING CONSTANT PATTERNS WITH THE STEREOCHEMISTRY OF ALDOPYRANOSYL SUGAR RESIDUES

Vicinal coupling constants[a]				Aldopyranose residue stereochemistry[b]		Example residues
$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	Configuration	Conformation	
L	L	L	L	*β-gluco*	$^4C_1$	*β*-D-Glc, *β*-D-GlcNAc
L	L	L	S	*α-ido*	$^1C_4$	(*α*-D-Idose)[c]
L	L	S	L	Impossible stereochemistry		
L	L	S	S	*β-galacto*	$^4C_1$	*β*-D-Gal, *β*-L-Fuc
				α-altro	$^1C_4$	(*α*-D-Altrose)
L	S	L	L	Impossible stereochemistry		
L	S	L	S	Impossible stereochemistry		
L	S	S	L	*β-allo*	$^4C_1$	(*β*-D-Allose)
L	S	S	S	*β-gulo*	$^4C_1$	(*β*-D-Gulose)
S	L	L	L	*α-gluco*	$^4C_1$	*α*-D-Glc, *α*-D-Qui
S	L	L	S	*β-ido*	$^1C_4$	(*β*-D-Idose)
S	L	S	L	Impossible stereochemistry		
S	L	S	S	*α-galacto*	$^4C_1$	*α*-D-Gal, *α*-L-Fuc
S	S	L	L	*α-manno*	$^4C_1$	*α*-D-Man, *α*-L-Rha
				β-manno	$^4C_1$	*β*-D-Man, *β*-D-ManNAc
S	S	L	S	*α-gulo*	$^1C_4$	(*α*-D-Gulose)
S	S	S	L	*α* or *β-altro*	$^4C_1$	(*α* or *β*-D-Altrose)
				α-allo	$^4C_1$	(*α*-D-Allose)
S	S	S	S	*α* or *β-ido*	$^4C_1$	(*α* or *β*-D-Idose)
				α-gulo	$^4C_1$	(*α*-D-Gulose)
				α or *β-talo*	$^4C_1$	(*α* or *β*-D-Talose)

[a]The symbols **L** and **S** represent large (> 5 Hz) and small (< 5 Hz) vicinal proton coupling constants, respectively.

[b]The absolute stereochemistry of a residue cannot usually be established by NMR. Stereochemical designations apply as well to the D form as to the L form.

[c]Residues shown in parentheses are theoretically possible, but have not yet been encountered in Nature.

3.2 LINKAGE DETERMINATION - METHYLATION AND REDUCTIVE CLEAVAGE ANALYSIS

Methylation analysis remains one of the most important chemical methods in the analysis of complex carbohydrates and it has been extensively reviewed[106-109]. Whilst analysis of ^{1}H and, more particularly, ^{13}C chemical shift data can provide the same information in a non-destructive manner, the former is still widely used to confirm results obtained from NMR analysis and where sample sizes are such that ^{13}C NMR spectroscopy is impossible due to sensitivity considerations.

Linkage analysis involves methylation of all free hydroxyl groups in the polysaccharide, followed by hydrolysis of the permethylated polysaccharide to release the partially methylated component sugars. Hydroxyl groups which are not etherified mark the positions at which the corresponding sugar residues were substituted in the polysaccharide. Qualitative and quantitative analysis of these derivatives, usually by GLC-MS, reveals how the different sugar residues were linked. It does not provide information on sugar sequences nor on anomeric configuration.

Although the principle of the technique has remained unchanged, the process of methylation has been considerably improved. Methylation involves nucleophilic attack of alkoxides, generated by the action of strong base on the hydroxyl groups of sugars, on a methylating agent to form the permethylated product. The method most widely applied today, developed by Hakomori[110], has largely replaced the more traditional methods of Purdie and Irvine[111], Haworth[112] and Kuhn[113] as it usually affords a completely methylated product in a single treatment. The base used is the methylsulphinyl anion (dimsyl anion) generated by the reaction of sodium hydride with dimethylsulphoxide (DMSO) and the alkylating agent is methyl iodide. Undermethylation is usually related to poor solubility of the polysaccharide in DMSO. The extent of methylation can be monitored by the Zeisel-methoxyl method or excess anion, a prerequisite for complete methylation, can be ensured with the use of the indicator triphenylmethane[114]. If incomplete methylation is detected, remethylation should be carried out according to the milder Kuhn procedure as

β-elimination at the uronic acid may occur if the partially methylated polysaccharide is re-exposed to strong base (see section 3.5.1 (iii)). Several modified methylation procedures have been proposed involving the use of other reagents to generate the dimsyl anion, for example potassium hydride[115], potassium *tert*-butoxide[116] and butyl lithium[117] which afforded improvements with respect to ease of preparation and purity of product, and the use of 1,1,3,3-tetramethylurea to increase solubility in dimethylsulphoxide[118]. Further procedural improvements have been reported by Harris *et al.*[119] where methylation and subsequent hydrolysis and derivatisation are carried out in a single vessel. This is of particular value when small quantities of polysaccharide are being analysed as significant loss of methylated material may occur due to volatilisation and repeated transfers. Recently the role of the dimsyl anion during methylation has been challenged. Ciucanu and Kerek[120] have claimed that hydroxyl and hydride ions are more effective bases and proposed the use of solid NaOH or KOH in a powdered form. They reported higher yields of methylated product and fewer side reactions, however, York *et al.*[121] have reported oxidative side reactions following methylation of a polysaccharide using this procedure. Zähringer and Rietschel[122] have also reported *C*-methylation at *N*-methylacetamido groups under Hakomori conditions though this appears to be a relatively minor side reaction. It is also difficult in many instances to get full *N*-methylation of acetamido sugars and their general resistance to hydrolysis leads to low yields of fully methylated acetamido sugars in many instances.

A further disadvantage to the use of strong bases to effect alkoxide formation is the loss or migration of base-labile substituents, in particular *O*-acetyl groups. Although the presence and location of *O*-acetyl groups can readily be determined by NMR spectroscopy, methylation procedures performed under mild acidic conditions have been developed which preserve *O*-acyl groups thereby facilitating their location. Mastronardi *et al.*[123] used diazomethane in dichloromethane with boron trifluoride etherate as a Lewis acid catalyst, however few acetylated polysaccharides are soluble under these conditions. The use of methyl triflate in trimethyl phosphate in the presence of the weak, sterically hindered, base 2,6-di-(*tert*-butyl)pyridine as applied by Prehm[124] is therefore preferred. Pyruvate acetals are stable under Hakomori conditions and their position can be established directly.

The partially methylated sugars which are released on hydrolysis of the permethylated polysaccharide are usually reduced and acetylated prior to analysis by GLC-MS. Uronic acid residues, present as methyl esters, are often reduced to the corresponding hexoses prior to mass spectral analysis.

Despite its enduring popularity, methylation analysis suffers from two important disadvantages. Firstly, the carbon atom which forms part of the cyclic acetal of a particular monosaccharide is not distinguished from linked positions after hydrolysis of the permethylated polysaccharide. For example, a 4-linked aldohexopyranose gives the same product after hydrolysis as a 5-linked aldohexofuranose. Secondly, work-up after methylation is laborious, resulting in significant loss of volatile methylated constituents. Cleavage of the glycosidic linkage with triethylsilane in the presence of trimethylsilyltrifluoromethanesulphonate (TMSOTf) as catalyst, instead of acid catalysed hydrolysis, results in regiospecific reductive cleavage at the anomeric carbon atom with preservation of ring structure (see Figure 3.1). Sugars are subsequently analysed as their cyclic anhydroalditol derivatives enabling the determination of positions of linkage and ring forms simultaneously. The resultant anhydro-alditol derivatives can be readily analysed by GLC-MS with only one reaction work-up. This procedure, developed by Gray and co-workers[125], is therefore increasingly being used as an alternative to "classical" methylation analysis as described above (See also section 3.5.1 (vi)). The use of NMR spectroscopy for determining linkage positions will be discussed in section 3.7.

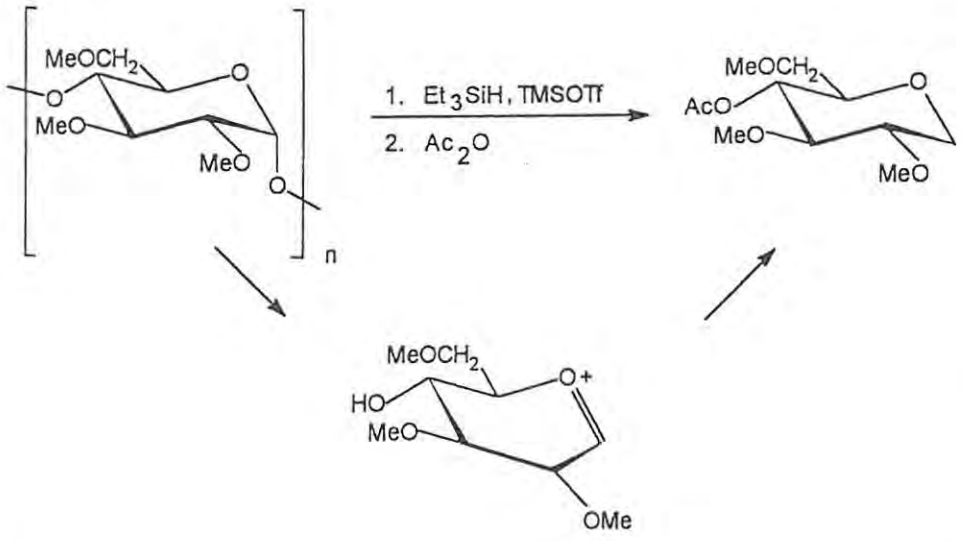


Figure 3.1 Proposed mechanism for reductive cleavage of a glycosidic linkage.

3.3 DETERMINATION OF ANOMERIC AND ABSOLUTE CONFIGURATION

Anomeric configuration:

The anomeric (α or ß) configuration of constituent sugars in a complex polysaccharide can be determined chemically or enzymatically. Unfortunately these techniques are laborious and often produce inconclusive results. Consequently, NMR spectroscopic techniques have in almost all instances superseded the former.

Acetylated methyl β-D-hexopyranosides and both α and β-furanosides are oxidised by chromium trioxide in acetic acid to the corresponding acetylated methyl hexulosonates. α-D-Hexopyranosides are resistant to oxidation[126]. The anomeric configuration of the constituent sugars of a glycan containing only hexose residues can thus be established by oxidation of the relevant acetylated methyl glycosides. Surviving sugars can be assigned the α-configuration as β-linked sugars will be prone to oxidation. Inconclusive results have been reported[127] using this technique as α-D-hexose residues which assume the $^1C_4$ configuration also have an anomeric bond in the equatorial position, a necessary prerequisite for oxidation. Examples of polysaccharides where the anomeric configurations of the glycosidic linkages have been assigned in this manner include the *E. coli* K31[128], K34[129] and K44[130] capsular polysaccharides.

Specific exo-enzymes can also reveal whether a terminal, non-reducing sugar residue is α- or β-linked as enzymatic cleavage of the glycosidic linkage may be configurationally dependant[131]. Therefore for this technique to be applicable to complex polysaccharides, oligo- or preferably di-saccharide fragments need to be generated to ensure that all constituent sugars are presented in a terminal position. This approach has been used to assign anomeric configurations in the *Klebsiella* K29[132] polysaccharide. The anomeric configuration of the terminal glucose residue in the *Klebsiella* K15 polysaccharide (see section 4.1) was confirmed as β-linked as it was released from the native polysaccharide by the action of β-glucosidase. Isolated endo-enzymes are not suitable for the configurational analysis of bacterial capsular polysaccharides due to their irregular nature,

however they have been successfully applied in glycoprotein structural analysis for the assignment of anomeric configurations. Kobata[133] has utilised an endo *N*-acetyl-β-D-glucosaminidase to hydrolyse the chitobiose section linked to asparagine. Bacteriophage-borne endo-enzymes (section 3.5.2), whilst extremely useful for the depolymerisation of bacterial polysaccharides, cannot be generally applied to establish anomeric configurations. The use of enzymes in the analysis of polysaccharides has been reviewed[134,135].

In contrast to the limitations associated with establishing anomeric configurations using chemical and enzymatic techniques, they can be easily and unambiguously established by NMR spectroscopy in virtually all instances. Chemical shift (δ), spin-spin coupling ($^3J_{1,2}$) data and the spin-lattice relaxation time (T_1) may all be used to establish anomeric configurations[136]. The reader is referred to section 3.7 where the applications of NMR spectroscopy in configurational analysis will be fully elaborated.

Absolute configuration:

The assignment of the D or L configuration to constituent monosaccharides is especially important in bacterial polysaccharides as sugars with unusual configurations are not uncommon. In cases where the sugar is labile and can be isolated in relatively large quantities the absolute configuration may be determined by measurement of its specific optical rotation[137]. Hudson's rules of iso-rotation[138] can be applied to extend this approach to establishing configurations for sugars in oligosaccharides and polysaccharides. Alternatively enzymatic methods can be used[139] or, if milligram quantities of pure sample are available, circular dichroism (c.d.) measurements of the derived alditol acetates can be performed[140]. The absolute configuration of the sugars can be determined from the c.d. band at 213 nm which may be either + or −. The experimentally determined sign is then compared with data in the literature. However, this method is not applicable for sugars that give *meso*-alditols or those that give alditol acetates with a weak c.d. This approach has been utilised in the structural elucidation of the *E. coli* K7[40], K28[141], K31[128], K44[130], and K74[142] capsular polysaccharides.

Separation of enantiomers by GC and HPLC is more widely applicable as a much smaller quantity of material is sufficient. For the chromatographic separation of enantiomers an intramolecular or intermolecular asymmetric environment must be achieved. The former can be accomplished by converting the sugars into diastereoisomeric derivatives (which can be separated on an achiral column) and the latter by the use of a chiral eluent or stationary phase to separate derivatised sugars.

The use of chiral alcohols, for example (-) and (+)-2-octanol, as developed by Leontein *et al.*[143] and (-)-2-butanol (Gerwig *et al.*[144]) to form diastereoisomers, is well established. Sugars can be identified as either D or L by comparison of their respective retention times with authentic standards on a capillary GLC column such as OV-225. Each enantiomer will give rise to four peaks i.e. two furanosides and two pyranosides, thus having the same disadvantages as the cyclic derivatives discussed above. Acyclic diastereoisomeric derivatives can be prepared to overcome this problem. Little[145] has utilised (+)-1-phenylethanethiol to produce acyclic diastereoisomeric dithioacetals which were separated as their acetylated or trimethylsilylated derivatives. Other acyclic derivatives which have been investigated include: (-)-menthyloxime pertrifluoroacetates[146], 1-(*N*-acetyl-α-methylbenzylamino)-1-deoxy-alditol acetates[147], and (-)-bornyloximes[148]. A major disadvantage to the use of chiral diastereoisomeric derivatives is their low volatility which necessitates high column temperatures and gives rise to lengthy retention times.

The separation of trifluoroacetyl and trifluoroacetyl-methylaldohexoside derivatives of enantiomeric carbohydrates by GLC using the chiral stationary phase XE-60-L-valine-*S*-α-phenylethylamide was first reported by König *et al.*[149]. Subsequently other chiral polysiloxanes have been developed for the separation of aldopentoses[150]. Recently pentylated cyclomaltohexaose has been introduced as a new stationary phase for the separation of trifluoroacetylated carbohydrate enantiomers[151]. The products of reductive depolymerisation of a polysaccharide *viz.* 1,4 and 1,5-anhydroalditols[125] can be directly resolved as D or L as their trifluoroacetyl derivatives. It is therefore possible to identify the structure and absolute configuration of the constituents of a polysaccharide in one step. Moreover, since the cyclic structure of the sugars is retained, each is represented by only one peak.

This is a significant advantage over the more conventional derivatives described above. Unfortunately these chiral columns are expensive and most laboratories establish absolute configuration by means of derivatisation to diastereoisomeric derivatives as described above. A pentylated cyclodextrin column is used in this laboratory for the separation of neutral D and L sugars, however for the analysis of amino sugars diastereoisomeric derivatisation is still preferred.

The assignment of absolute stereochemistry is one of the few structural aspects of complex polysaccharides which can not readily be assigned by NMR spectroscopy. However, Shashkov and co-workers[152] have recently reported a method for the assignment of absolute stereochemistry in disaccharides based on ^{13}C chemical shift differences, provided the absolute stereochemistry of one of the constituent sugar residues is known. Alternatively, conformational behaviour in the vicinity of the glycosidic linkage can be estimated with the help of potential energy calculations, and in combination with interresidue NMR data, can be used to determine the absolute stereochemistry of constituent monosaccharide residues in an oligo- or poly-saccharide provided the absolute configuration of the adjacent sugar is known[153]. The absolute stereochemistry of a new diacetamido sugar present in the K48 polysaccharide (this thesis) has been assigned by NMR spectroscopy. A more detailed discussion of the use of NMR spectroscopy for the assignment of absolute stereochemistry is presented in section 4.5.

3.4 ANALYSIS OF NON-CARBOHYDRATE CONSTITUENTS

E. coli and *Klebsiella* capsular polysaccharides frequently contain non-carbohydrate substituents. These include *O*-acyl (*O*-acetyl or *O*-propionyl), *N*-acetyl, pyruvate, phosphate and lactyl groups and in some cases amino acids. NMR spectroscopy and mass spectrometry are powerful tools for the analysis of non-carbohydrate substituents while chemical methods are less frequently applied.

O-acyl groups, in particular *O*-acetyl groups are the most common and, as a consequence of their lability and tendency to migrate to other hydroxyl groups in the polysaccharide, are the most difficult to locate. The strongly basic conditions which prevail during Hakomori methylation remove

difficult to locate. The strongly basic conditions which prevail during Hakomori methylation remove *O*-acyl groups and hence the milder Prehm[124] methylation procedure which preserves *O*-acyl groups can be used in conjunction with Hakomori methylation to locate the substituent. Alternatively free hydroxyl groups can be converted to stable acetal[154] or phenylcarbamoyl[155] derivatives which allows selective methylation at the sites of alkali-labile substitution. The location of the *O*-acetyl group in welan, a commercial polysaccharide produced by the *Alcaligenes* species, using a reductive cleavage method has been reported by Stankowski and Zeller[156]. Acetate can also be quantified spectrophotometrically following treatment of the hydroxamic acid derivative with acidic ferric chloride to form a coloured compound[157], however integration of the methyl proton singlet in the ^{1}H-NMR spectrum is a more rapid and accurate approach.

TABLE IV.
NON-CARBOHYDRATE CONSTITUENTS PRESENT IN *E. coli* AND *Klebsiella* CAPSULAR POLYSACCHARIDES

SUBSTITUENT	STRUCTURE		^{1}H and ^{13}C NMR DATA (ppm)					
			-C=O	-CH_3	-CH_2-	-CH-	-C-	-COOH
N/*O*-acetyl	-N/O-C-CH_3 (=O)	H	-	2.0-2.3	-	-	-	-
		C	170-180	20-25	-	-	-	-
O-propionyl	-O-C-CH_2-CH_3 (=O)	H	-	1.1-1.3	2.1-2.2	-	-	-
		C	170-180	10-12	26-28	-	-	-
Pyruvate	-O(COOH)(-O-CH_3)	H	-	1.4-2.1	-	-	-	-
		C	-	17-26	-	-	107-109, 100-103*	173-175
Lactyl	-O-CH(-CH_3)-COOH	H	-	1.3-1.5	-	4.0-4.5	-	-
		C	-	19-21	-	77-79	-	178-184
Serine	-C(=O)-NH-CH(COOH)-CH_2-OH	H	-	-	2.8-3.2**	~5.0	-	-
		C	170-180	-	59-63	56-59	-	175-178
Threonine	-C(=O)-NH-CH(COOH)-CH(CH_3)-OH	H	-	1.0-2.0	-	4.1-4.5***	-	-
		C	170-180	20-22	-	58-62, 68-71****	-	175-178
Glutamic acid	-C(=O)-NH-CH(COOH)-CH_2-CH_2-COOH	H	-	-	1.0-2.2**	4.0-5.0	-	-
		C	170-180	-	25-35	50-55	-	174-180

*For 5- and 6-membered acetal rings respectively, **Ha and Hb usually close together, ***α and ß-protons close together or may overlap, ****Hα and Hß respectively.

In most cases *O*-acyl groups, even when present in non-stoichiometric quantities, can be located using NMR techniques. The presence of *O*- or *N*-acetyl groups is usually immediately apparent from 1-D ^{1}H and ^{13}C NMR spectra of the polysaccharide (Table IV). The methine proton of an acyl substituted carbon will be deshielded and analysis of proton chemical shift data is often sufficient to locate the substituent. In the ^{13}C spectrum, acylation causes significant deshielding of the α-carbon (0.6-3.5 ppm) and shielding of the adjacent β-carbons[158] and this is a more reliable indicator of the position of substitution. Furthermore, long range two- and three-bond heteronuclear connectivities obtained from an 2D-Heteronuclear Multiple Bond Coherence (HMBC) experiment[159] can unambiguously locate an *O*-acetyl substituent. If the location of the *O*-acetyl group cannot be conclusively established from studies on the polysaccharide, alternative approaches which have been successfully applied include controlled depolymerisation of the polysaccharide with anhydrous HF[160,161] or bacteriophage-borne enzymes[162] and analysis of the oligosaccharides so produced by NMR spectroscopy, FAB-MS[163,164] or chemical analysis.

NMR spectroscopy is also the method of choice for the analysis and location of pyruvic acid (1-carboxyethylidine) acetals. The methyl group and the quaternary and carbonyl carbons can be identified from 1-D ^{1}H and ^{13}C NMR data. Substituted ring carbon atoms are characteristically deshielded and resonate further downfield permitting location of the substituent. Acetal linkages are base stable and therefore the positions of substitution can also be established by methylation analysis. Pyruvate acetals are also stable under reductive cleavage conditions. Pyruvate-containing methylated polysaccharide, when subjected to total reductive cleavage in the presence of either Me_3SiOSO_2Me-BF_3.Et_2O or $Me_3SiOSO_2CF_3$ as catalyst, results in the formation of partially methylated anhydroalditol derivatives containing 1-methoxycarbonylethylidine groups[165]. These can be analysed by GLC-MS or the benzoylated products can be analysed by HPLC or ^{1}H NMR spectroscopy[166] to establish the positions of substitution of the pyruvate group. A major drawback to this approach is unavoidable isomerisation at the acetal carbon. The reductive cleavage method therefore cannot be used to establish the chirality of the original carboxyethylidene group.

When the polysaccharide is in the acidic form, the pyruvate may be more labile, particularly if it forms a five membered dioxolane ring, i.e. a 2,3 or 3,4-linked acetal. In such cases NMR analysis of the native polysaccharide is preferable as methylation analysis may not locate pyruvate conclusively. NMR spectroscopy can be used not only to locate the position of the pyruvate group but also to establish the configuration (*R* or *S*) of the quaternary acetalic carbon (C-2). This can be established from ^{13}C chemical shift[167] or NOE data[168].

The differences in chemical shifts obtained for stereoisomeric pairs of acetalic CH_3 groups are of sufficient magnitude to make possible the unequivocal determination of the configuration. Axial and equatorial CH_3 groups in 4,6-membered cyclic acetals on glucose or galactose differ by up to 7 ppm[169]. The assignment of configuration in 1,3 dioxolane (5-membered rings) is more difficult as the chemical shift differences are smaller, though nevertheless still significant. In addition there are noticeable differences in the shifts of the CH_3 protons in ^1H NMR spectra which may allow assignment of the stereochemistry. It is also possible to assign absolute stereochemistry from NOE data as the pyruvate methyl protons may show NOEs to specific ring protons. This approach has been used to establish the configuration at the chiral carbon of the pyruvate group in *E. coli* serotypes K47[58] and K103[60] and in *Klebsiella* K30[170]. This is illustrated in Figure 3.2.

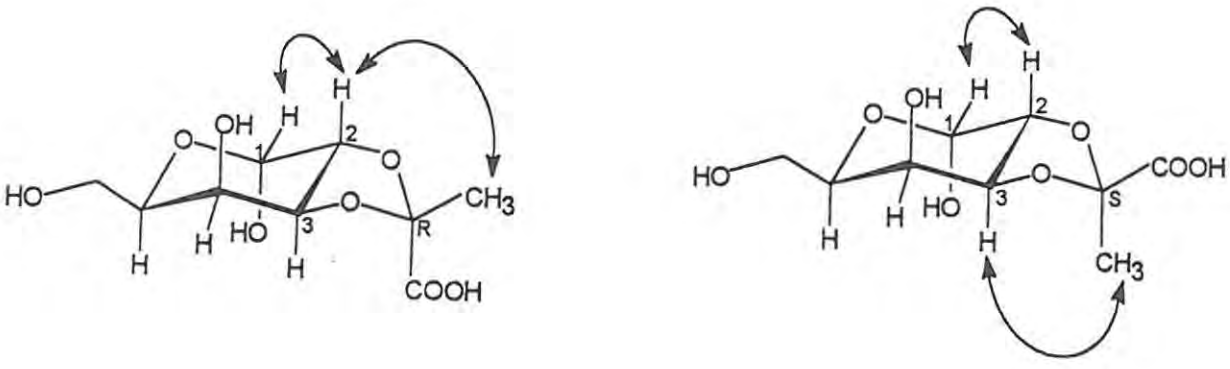


Figure 3.2 NOEs observed between specific ring protons and pyruvate methyl protons may permit assignment of the configuration of the quaternary acetalic carbon.

Lactyl (1-carboxyethyl) groups also possess a stable ether linkage allowing for identification of the position of substitution by similar spectroscopic means. The lactyl group can be hydrolysed using 48% hydrogen bromide[171] and identified by GLC as the methyl ester. Furthermore, ***R*** and ***S*** lactyl sugars are diastereomeric and therefore if the lactyl sugar can be isolated its configuration can be established by comparison with known standards using GLC.

Phosphorus occurs as glycero, ribitol or sugar phosphate. In ^{13}C spectra, phospho-substituted carbon atoms show characteristic deshielding and their corresponding signals are frequently split due to carbon-phosphorus coupling[172], see Figure 3.3. The phosphate content can be assayed colorimetrically[173,174] providing it can be completely released from the polysaccharide. Dephosphorylation can be effected with the use of cold aqueous 48% HF (0-4°C, 48-72 h)[175], and for total hydrolysis of phosphate-containing polysaccharides 38% HF (65°C, 1 h) followed by TFA hydrolysis (98°C, 16 h)[176] is appropriate.

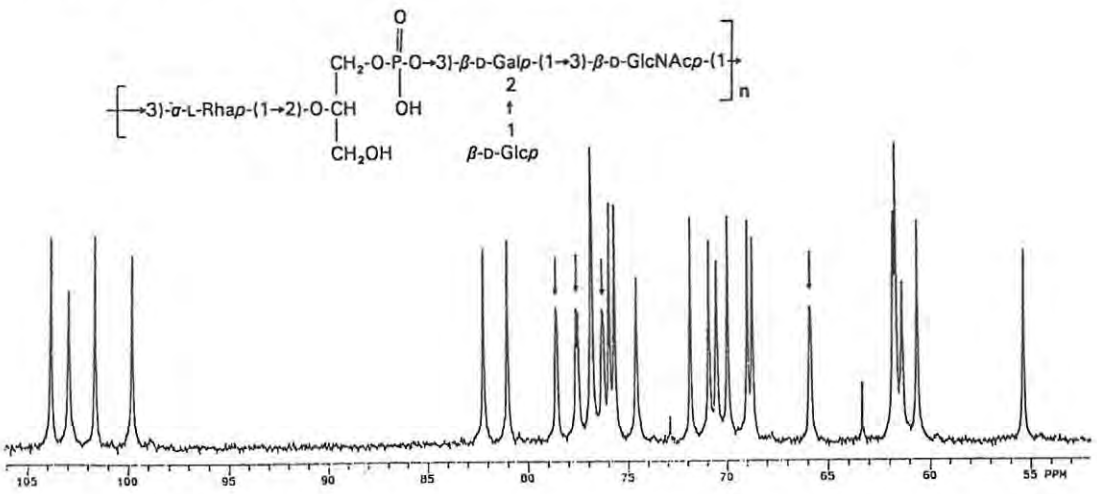


Figure 3.3 ^{13}C spectrum of the phosphorus-containing *E. coli* K46[50] capsular polysaccharide.

Amino acids have only been found amidically linked to the carboxyl group of uronosyl residues. Strongly acidic conditions are therefore required to effect release (e.g. 4 M HCl , 100°C, 18 h under N_2). Once released they can be analysed using automated amino acid analysers or by GLC. The absolute stereochemistry can be determined by preparing diastereoisomeric derivatives with a chiral alcohol, e.g. (+)-2-butanol, or by measuring optical rotations. Amino acids show characteristic chemical shift and coupling constant patterns, once again making NMR spectroscopy an invaluable tool for the analysis of amino acid-containing polysaccharides[177].

3.5 SELECTIVE DEGRADATION OF POLYSACCHARIDES - SEQUENCE ANALYSIS

Once the nature and linkage pattern of the constituent monosaccharides is known, the sequence of the sugars in the repeating unit must be established. The monosaccharides which constitute the repeating unit can be linked in several ways due to their polyhydroxyl nature. They can be arranged in a linear fashion or there may be a branch point(s) with a side chain(s) comprised of one or more sugar residues. Classically, information on the sequence of the constituent sugars is established by chemically or enzymatically degrading the polysaccharide to produce a specific oligosaccharide or mixture of overlapping oligomers which can be isolated and characterised, either by chemical means e.g. methylation analysis or by NMR spectroscopy or both. It is generally much easier to determine the structures of oligosaccharides since they usually give well defined NMR spectra and are amenable to mass spectral analysis. A major drawback to this approach is the quantity of material required, as most degradative procedures result in low yields of the desired product. Consequently NMR spectroscopic studies on the polysaccharide may be the only approach to sequencing the repeating unit if the material is only available in small quantities.

Several specific chemical degradative procedures have been developed which take advantage of structural features or differences in the lability of glycosidic linkages. Most chemical procedures require the sacrifice of one or more of the constituents which, though providing sequencing information, is a liability. Bacteriophage-mediated enzymatic depolymerisation through selective cleavage of a single glycosidic bond is therefore a very desirable method as it results in an oligosaccharide analogous to the repeating unit, analysis of which provides maximum information. This enzymatic approach, together with several chemical approaches will be discussed. Newer methods employed for the selective degradation of polysaccharides include: partial and selective reductive cleavage, solvolysis with anhydrous HF, and cleavage with lithium dissolved in ethylenediamine. These approaches will be covered in greater detail as the more established procedures have been extensively reviewed in the literature.

3.5.1. SPECIFIC CHEMICAL DEGRADATIONS

(i) PARTIAL ACID HYDROLYSIS

Graded hydrolysis of the polysaccharide with acid, followed by fractionation and identification of the resulting oligosaccharide fragments, has been the method most commonly used in the past. Information on sequences and anomeric configurations can be obtained, though often the rates of hydrolysis of the various glycosidic linkages do not differ substantially and complex mixtures of mono- and oligo-saccharides result. However, in most cases glycosidic bonds are hydrolysed at sufficiently different rates, depending on the hydrolytic conditions and the nature of the sugar residues, to produce useful products. This approach was used to great effect in structural studies of the *E. coli* K37 polysaccharide[57].

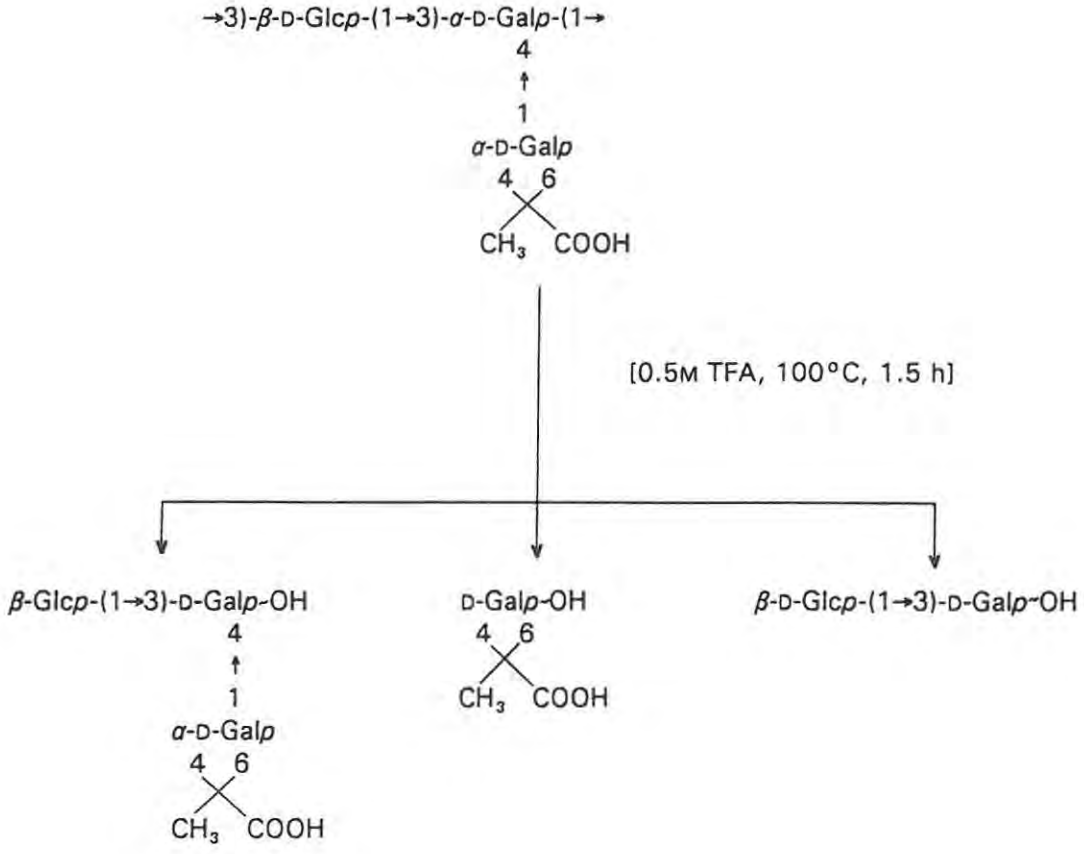


Figure 3.4 Products of partial hydrolysis of the *E. coli* K37 polysaccharide.

Weaker glycosidic linkages are most frequently associated with furanoses and deoxy residues or sugars with a deoxy function adjacent to the anomeric carbon e.g. 3-deoxyglyculosonic acids such as NeuAc and KDO[178]. Hydrolysis of polysaccharides containing more resistant sugars e.g. uronic acids and amino sugars, as is the case with many bacterial polysaccharides, should produce oligosaccharides or at least an aldobiouronic acid which can be further investigated and provide information of structural significance.

Aqueous 48% HF at very low temperatures (-23 to -40°C) is particularly useful for performing partial acid hydrolyses containing labile substituents such as *O*-acyl groups. These substituents, usually rapidly removed by other acids, are preserved by the latter[179]. Partial hydrolysis has frequently been employed in the structural analysis of bacterial polysaccharides[67,141,180,181] and the subject has been reviewed[182].

(ii) PERIODATE OXIDATION

Periodate oxidation is a well established procedure in the analysis of polysaccharides and it has found extensive application in the structural elucidation of bacterial polysaccharides[23,40,64,183]. Vicinal hydroxyl groups (1,2 diols or 1,2,3 triols) are oxidised by periodate with concomitant cleavage of the sugar ring and the formation of aldehydic groups[184]. One equivalent of periodate is consumed per *vic*-diol and formic acid (usually from triol cleavage) or formaldehyde (from exocyclic diol groups) may be liberated. Thus, depending on the linkage positions, only certain residues in the polysaccharide will be susceptible to oxidation. For example 3-linked residues will be stable whilst 4-linked residues will be susceptible to oxidation, as illustrated in Figure 3.5.

A great deal of structural information can be obtained using this approach depending on the analytical technique chosen. Analysis of the reaction products and the intact mono- or oligo-saccharide fragments remaining provides structural, stereochemical and sequencing information.

Monitoring the uptake of periodate[185-187] enables determination of the number of susceptible linkages in the repeating unit and is therefore complementary to methylation analysis. Furthermore since *cis*-1,2-diols are oxidised more rapidly than *trans* isomers[188], selective oxidation of certain sugars in the polysaccharide can be achieved.

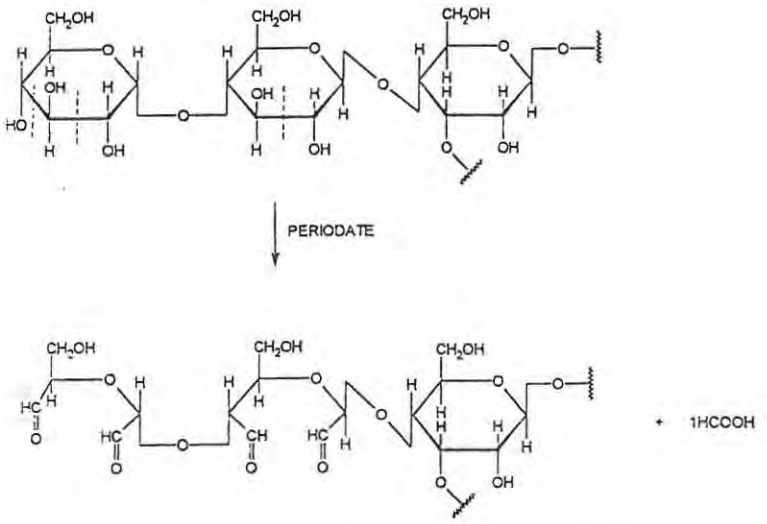


Figure 3.5 Periodate cleavage of a polysaccharide containing an adjacent triol and diol.

Cleavage of 1,2-diols results in the formation of highly reactive aldehyde groups which may form unwanted hemi-acetals[188]. For this reason, it is advisable to reduce these to the corresponding alcohols prior to analysis. Additional steps to effect further decomposition of the periodate oxidised polysaccharide can be performed, i.e. the so called Barry[190] and Smith[191] degradations. The latter, widely applied in the analysis of bacterial polysaccharides, involves the cleavage of the acyclic acetal linkages formed after periodate oxidation with mild acid to leave glycosides in which the aglycones are only fragments of the original oxidised sugars.

Despite the versatility of these reactions, the results obtained should always be interpreted with a measure of caution. Non-oxidation with periodate is not proof of the absence of *vic*-diol groups in the polysaccharide as steric constraints may preclude oxidation[192]. Over-oxidation is also a potential problem but can be avoided by excluding light and conducting the reaction at a low temperature and pH (usually 3.0-3.5).

(iii) BASE-CATALYSED DEGRADATION (β-ELIMINATION)

Treatment of methylated polysaccharides containing uronic acid residues with strong base under anhydrous conditions leads to β-elimination of the substituent at C-4 of the uronic acid and the subsequent formation of hex-4-*eno*pyranosiduronates[193]. Mild acid hydrolysis will remove the unsaturated ester and the succeeding sugar will be released as a non-reducing terminal residue with a free hydroxyl group. If the substituent at C-4 is a sugar residue substituted at O-3, it will be released in the reducing form and will be susceptible to further degradation[194]. This approach, first applied to bacterial polysaccharides by Lindberg *et al.*[195], can thus yield important sequencing information. Analysis of the oligomer or modified polysaccharide produced, usually by methylation with trideuteriomethyl iodide and analysis of the derived alditol acetates by GLC-MS, enables the residues on either side of the uronic acid to be identified.

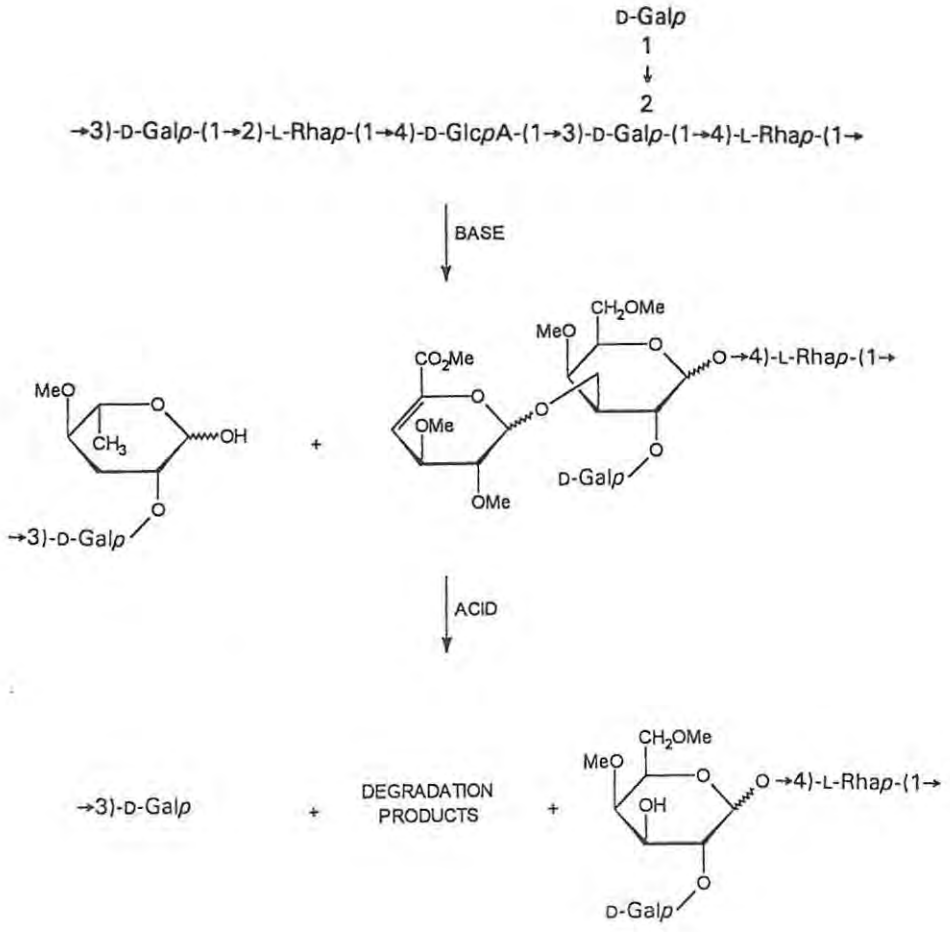


Figure 3.6 Base-catalysed degradation of the *Klebsiella* type 47 capsular polysaccharide[193].

Aspinall and Rosell[196] have introduced a modified procedure which avoids secondary eliminations. They have shown that acid hydrolysis of the products is unnecessary and advocate alkylation with trideuteriomethyl or ethyl iodide directly after treatment with base. They contend that there is complete degradation of the unsaturated hexuronic acid residue under the conditions described above[195] enabling procedural simplification. Further applications of β-elimination in the structural analysis of polysaccharides have been described[197,198] and it has been reviewed by Kiss[199] and Aspinall[200], amongst others.

(iv) ANHYDROUS HF SOLVOLYSIS

The application of anhydrous hydrogen fluoride (HF), first used to selectively cleave polysaccharides by Mort and Bauer in studies on the extracellular polysaccharides produced by *Rhizobium japonicum*[201], has become an extremely valuable technique for the selective solvolysis of polysaccharides. It is particularly useful for generating oligosaccharides from polysaccharides containing resistant linkages and labile substituents and offers certain distinct advantages over hydrolysis and methanolysis.

Glycosidic linkages are cleaved to form glycosyl fluoride intermediates with almost no decomposition of the liberated sugars. *N*-acetyl substituents are preserved and, in addition, at very low temperatures so too are *O*-acyl substituents. These qualities make anhydrous HF an extremely useful agent for degrading bacterial polysaccharides containing acetamido sugars and uronic acid residues and high yields of oligosaccharides can be isolated, frequently with labile substituents such as *O*-acetyl groups intact. Cleavage is also dependent on the nature of the constituent sugars, their anomeric configurations, and the nature of adjacent sugar residues. Furthermore, the temperature and duration of the reaction can also be controlled which provides considerable scope for achieving highly selective degradation. Knirel and co-workers[202] have reviewed the application of anhydrous hydrogen fluoride in the structural analysis of various polysaccharides.

When solvolysis is performed successively at lower and lower temperatures, more and more linkages become resistant to cleavage until, just above the freezing point of HF, only pentofuranose residues are cleaved. The glycosidic linkages of GlcNAc and GalNAc, commonly encountered in bacterial polysaccharides, are stable in HF at 0° C or lower[176], but are cleaved at higher temperatures. Deoxyhexoses are typically more labile than the corresponding hexoses. At -23° C differences in the labilities of α and β-linkages[201] become apparent in hexoses and this provides scope for further selective cleavage.

Anhydrous HF is a particularly useful agent for releasing sugars resistant to hydrolysis or methanolysis. Its ability to preserve *N*-acetyl groups prevents the formation of free amino groups which stabilise glycosidic linkages and inhibit cleavage. Several unusual amino sugars have been isolated in this manner[203-206]. However, the presence of a second acylamido group leads to additional stabilisation of the glycosidic linkages of *N*-acetyl hexosamines[202] making certain sugars extremely resistant to cleavage. For example the diacetamido uronic acid present in the *Pseudomonas aeruginosa* Lànyi O:6 polysaccharide[202] (Figure 3.7) is not released, even after stirring in liquid HF at 20° C for an extended period.

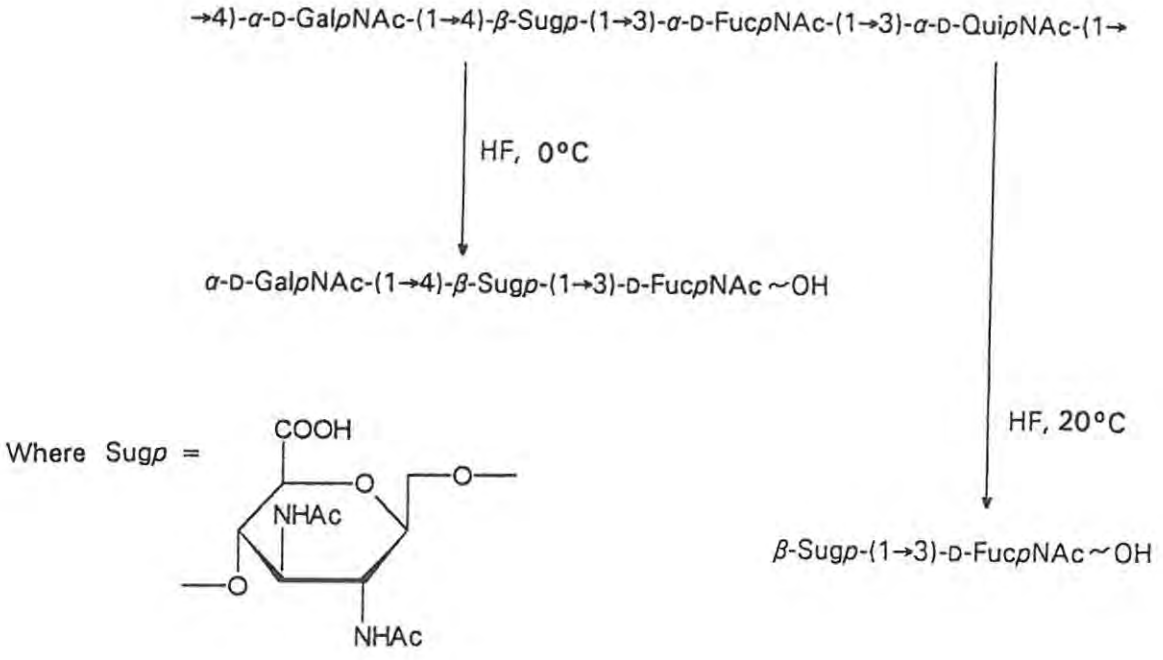


Figure 3.7 Products of the treatment of *Pseudomonas aeruginosa* Lànyi O:6 polysaccharide[202] with anhydrous HF at 0°C (1 h) and 20°C (3 h).

Studies on the O-polysaccharide produced by *Shigella sonnei*[206] and the capsular polysaccharide of *Streptococcus pneumoniae* type 1[207], which both contain 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose, showed that this residue is completely resistant to solvolysis. Likewise solvolysis of the O-specific polysaccharide produced by *Pseudomonas aurantiaca*[202], which contains 2,4-diacetamido-2,4,6-trideoxy-D-glucose and the capsular polysaccharide produced by *E. coli* K48[208] (this thesis) which contains 2,3-diacetamido-2,3,6-trideoxy-L-mannopyranose, resulted only in the isolation of disaccharides with the diacetamido sugar at the non-reducing end. Table V shows the relative labilities of sugar residues to anhydrous HF.

The behaviour of pyruvic acid acetals to HF is variable. Kuo and Mort[209] found that 4,6-linked acetals on galactose were stable, whilst pyruvate similarly linked to glucose was removed. Oligosaccharides can also be prepared by solvolysis with HF in methanol[210] and are then isolated as the methyl glycosides.

Despite its obvious advantages HF is not as widely used as one would expect. This can be attributed to its toxic properties, highly corrosive nature and its ability to cause severe burns. Apparatus for the safe handling of gaseous and liquid HF has been developed[211] and consists of a completely closed system constructed from fluorocarbon plastic attached to a vacuum line. Mort *et al.*[212] have since reported a modified device for conducting solvolysis that enables quenching of residual HF and precipitation of the products in a convenient manner together with precise control of the reaction time. Residual HF is usually removed *in vacuo* or alternatively, it can be neutralised by stirring with $CaCO_3$ in CH_2Cl_2 with cooling or the products of the reaction can be precipitated by the addition of cold ether.

TABLE 5. EXPECTED LABILITIES OF THE GLYCOSIDIC LINKAGES OF VARIOUS SUGAR RESIDUES [a]

SUGAR RESIDUE	TEMPERATURE OF HYDROGEN FLUORIDE (°C) [b]				
	< -70	-40	-23 to -20	0	20-25
Pentofuranose	+	+	+	+	+
Pentopyranose	±	±	+	+	+
6-Deoxyhexose	–	±	+	+	+
α-Hexose	–	±	+	+	+
β-Hexose	–	±	±	+	+
2-Amino-2,6-dideoxyhexose	–	–	–	±	+
3-Amino-3,6-dideoxyhexose			+	+	+
4-Amino-4,6-dideoxyhexose			+	+	+
2,4-Diamino-2,4,6-trideoxyhexose					+
2-Amino-2-deoxyhexose	–	–	–	±	±
Uronic acid	–	–	–	±	±
Galactosaminuronic acid	–	–	–	–	±
Mannosaminuronic acid	–	–	–		+
2,3-Diamino-2,3-dideoxyalduronic acids	–	–	–	–	–
5,7-Diamino-3,5,7,9-tetradeoxynonulosonic acid	–	–	–	–	–

[a] Adapted from Knirel *et al*[202]. [b] The labilities indicated are generalizations as limited data is available.

(v) LITHIUM IN ETHYLENEDIAMINE DEGRADATION

Lithium and other group 1 metals dissolved in ethylenediamine are powerful reducing agents which have been used in a variety of chemical transformations[213,214]. Mort and Bauer[201], in addition to introducing HF for the selective degradation of polysaccharides, in the same study were also the first to make use of the reductive properties of lithium in ethylenediamine to selectively cleave the polysaccharide produced by *Rhizobium japonicum* at the site of the uronic acid residue.

Albersheim and co-workers[215], in a detailed study in which they investigated the effect of lithium in ethylenediamine on a variety of model compounds and a selection of glycosyluronic acid-containing polysaccharides, were able to further demonstrate the versatility of this method and suggested procedural improvements. They confirmed the stability of neutral glycosyl residues and established that polysaccharides are cleaved by lithium treatment at the sites of glycosyluronic acid residues regardless of the anomeric configuration of the linkage or the point at which other residues are attached. 3-Linked uronic acids uniquely gave oligosaccharides terminated with a modified alditol at the reducing end, thus providing a means of confirming their linkage pattern. Methyl glycosides, methyl esters and pyruvate ketals were also shown to be susceptible to cleavage and aldoses were reduced to the corresponding alditols. Studies on the model compound 2-hydroxyhexanoic acid revealed that the oxygen atom at C-5 is lost during the reaction. This led them to propose that cleavage proceeds *via* α-elimination with the transfer of this oxygen atom to C-1 followed by the concomitant loss of the original C-1 oxygen atom to the aglycone.

The dissolution of the polysaccharide in ethylenediamine is a major limiting factor and low yields are frequently attributable to poor solubility. On dissolution of the sample, small pieces of lithium wire are added to the solution until a deep blue colour is formed. Additional pieces of wire are added to maintain the blue colour for one hour with the amount of lithium required dependent on the constituents of the polysaccharide.

Water is added to quench the reaction and solubilise the products, followed by toluene, in successive amounts, to form an azeotrope with the water and ethylenediamine. The solution is then dried *in vacuo* and the resulting powdery residue is dissolved in water and titrated with glacial acetic acid to pH 4.5. Lithium ions are then removed by passage over an excess of a suitable ion-exchange resin or alternatively the mixture can be desalted using gel chromatography or dialysis. The desalted product can then be further purified by fractionation on a suitable gel column e.g. Bio-Gel P-2.

In the aforementioned study the polysaccharides produced by *Klebsiella* serotypes K17[216] (Figure 3.8), K44[217] and K62[218], all of which contain in-chain uronic acids, were successfully degraded to produce oligo-alditols. This reaction has since been successfully used to degrade a variety of uronic acid containing bacterial polysaccharides, amongst which are *Klebsiella* K43[19] and *E. coli* serotypes K26[183], K31[128], K39[181] and K102[218].

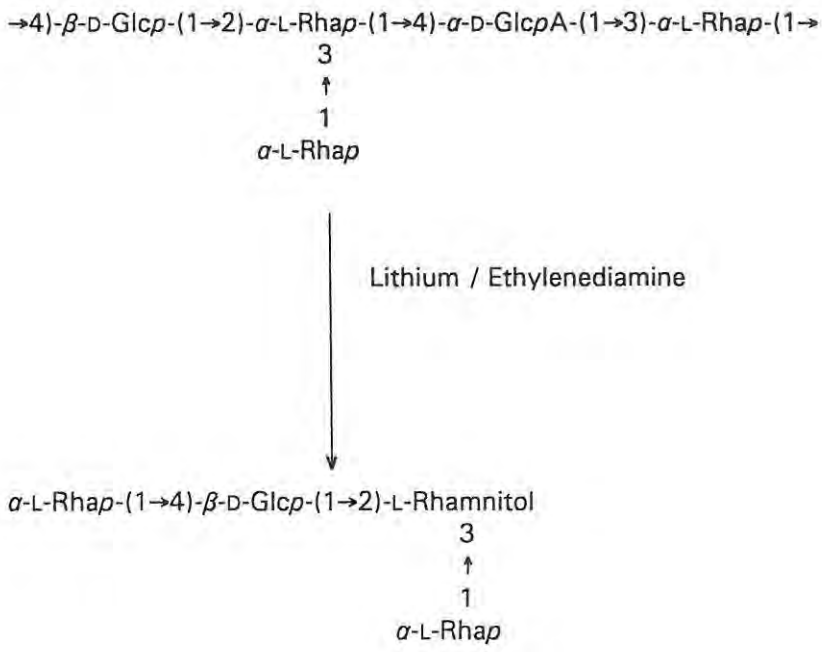


Figure 3.8 Oligo-alditol product of the lithium degraded *Klebsiella* K17 polysaccharide.

In the case of *Klebsiella* K43 and *E. coli* K102 (Figure 3.9) a modified polysaccharide was produced as the uronic acid occurs in the side chain in both instances. The use of lithium in ethylenediamine for the degradation of amino sugar containing hexosaminoglycans is discussed in section 4.4.

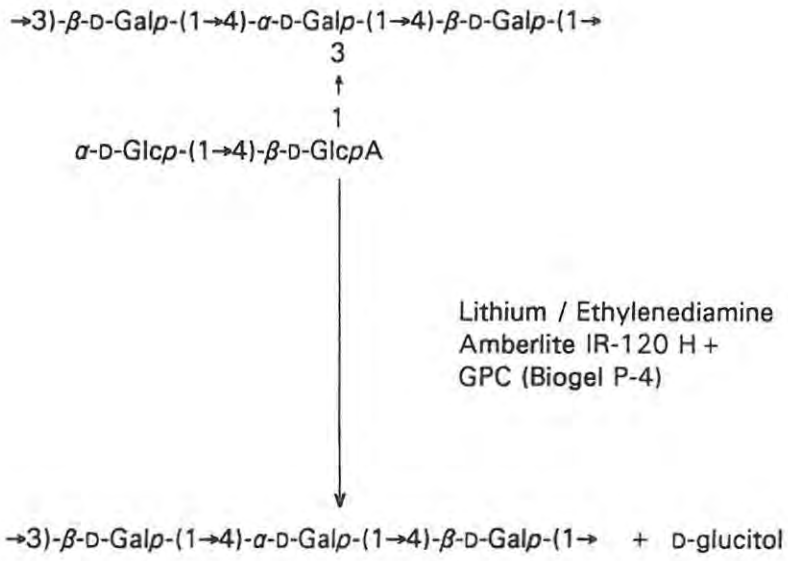


Figure 3.9 Polymeric product of the lithium/ethylenediamine degradation of the *E. coli* K102 polysaccharide[218].

(vi) PARTIAL AND SELECTIVE REDUCTIVE CLEAVAGE

The reductive cleavage method, originally developed as an alternative to methylation analysis by Rolf and Gray[220], can also be employed to selectively cleave methylated polysaccharides. The resulting methylated oligosaccharide-anhydroalditols are suitable for MS or NMR analysis without the need for further chemical derivatisation. Selectivity can be achieved by the appropriate choice of catalyst or by varying the reaction conditions.

Silicon hydrides effect reductive cleavage of glycosidic linkages *via* hydride transfer to the anomeric carbon centre after activation with a suitable catalyst.

Rolf and Gray initially used trifluoroacetic acid (TFA) as catalyst with only limited success, however they successfully reductively cleaved methylated cyclohexaamylose using a mixture of boron trifluoride etherate ($BF_3.Et_2O$) and TFA. Shortly after this trimethylsilyltrifluoromethane sulphonate (TMSOTf)[221] was found to be suitable for catalysing total reductive cleavage. Since then other catalysts have been successfully employed, some of which do not catalyse the cleavage of all glycosidic bonds thus enabling the *selective* reductive cleavage of methylated polysaccharides.

Gray *et al.*, in a study on branched mannans produced by strains of *Saccharomyces cerevisiae*[222], found that certain 2,3- and 6-linked residues at branch points were not cleaved by triethylsilane and boron trifluoride etherate. Oligosaccharides terminated by anhydroalditols were formed indicating the potential value of this catalyst for selective cleavage. In a subsequent study[223] on the glucan *pullulan* using the same catalyst, this selectivity was further demonstrated. All α-(1→4)-linked residues were cleaved whilst α-(1→6)-linked residues were shown to be stable. Recently, Jun and Gray[224] have achieved selectivity using trimethylsilylmethanesulphonate (Me_3SiOSO_2Me) and, in combination with $BF_3.EtO_2$, reported fewer side reactions and demonstrated that catalysis in this instance occurred in a synergistic manner with the formation of hybrid catalytic species of the type F_2BOSO_2Me, $FB(OSO_2Me)_2$, or $B(OSO_2Me)_3$ *via* ligand exchange.

The fate of amino sugars in polysaccharides has also been investigated[225]. They are not susceptible to reductive cleavage *per se*, however β-linked GlcNAc, when subjected to reductive cleavage using TMSOTf as catalyst, was shown to be selectively hydrolysed *via* a cyclic oxazolinium compound following the addition of aqueous sodium hydrogen carbonate to quench the reaction. α-Linked GlcNAc and GalNAc[225] are completely resistant to reductive cleavage. Pyranuronic acids have been shown to be cleaved at a slower rate than neutral sugars with the exception of 4-linked GlcA which was shown to be rapidly cleaved *via* an acyclic oxonium ion to form an isomeric furanosyl anhydroalditol[226]. Studies on *inulin*[224] have shown that furanosyl residues are particularly susceptible to reductive cleavage, thus demonstrating further scope for selective cleavage.

Ester-linked non-carbohydrate substituents and pyruvic acid acetals[166] have been shown to be stable under reductive cleavage conditions thus making it possible to identify positions of substitution. These findings, together with the selectivities demonstrated above, make reductive cleavage eminently suitable for the selective cleavage of bacterial polysaccharides. Prompted by the reported susceptibility of furanosyl residues to reductive cleavage and the stability of α-linked amino sugars, Stanley[227] selectively cleaved the methylated capsular polysaccharide produced by *E. coli* K57 at the site of the constituent D-Rib*f* residue, and isolated two oligosaccharide-anhydroalditols in sufficient yields to enable complete characterisation by NMR spectroscopy. In a similar fashion, Hackland[228] was able to isolate and characterise an anhydroribitol terminated pentasaccharide analogous to the repeating unit in the *E. coli* K38 capsular polysaccharide by CI and FAB.MS. This is illustrated in Figure 3.10.

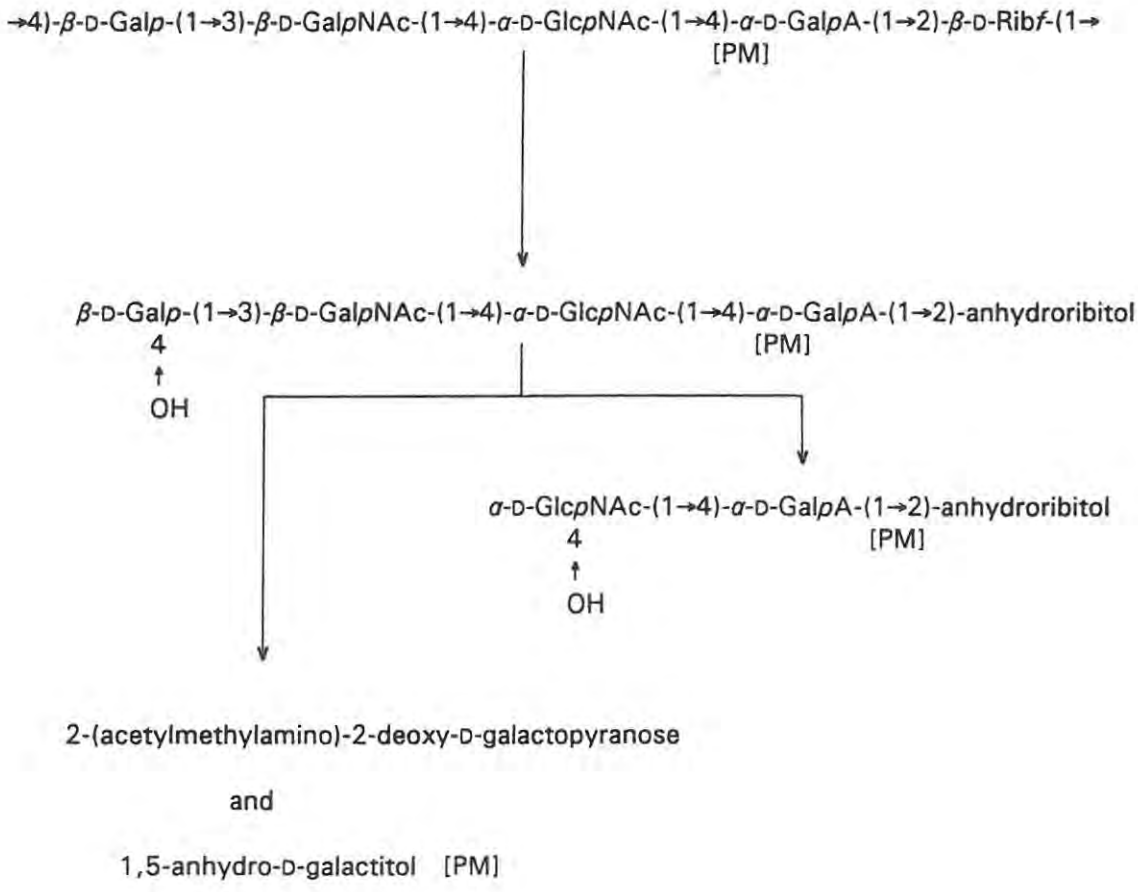


Figure 3.10 Selective reductive cleavage of the permethylated [PM] *E. coli* K38[228] polysaccharide.

A major advantage of selective reductive cleavage is the suitability of the products for further analysis by MS or NMR spectroscopy. If material is only available in small quantities, molecular mass, linkage and sequence information can be obtained from CI-, DCI- or FAB.MS studies on as little as five micrograms of methylated material. When larger quantities of an oligomer can be isolated, analysis by NMR spectroscopy may be possible. In such instances trideuteriomethylation prior to degradation facilitates NMR analysis as it ensures greatly simplified spectra. Such spectra are recorded in deuterated organic solvents and are therefore better resolved than spectra acquired in D_2O and there is no obscuring HOD signal.

In those cases where all linkages in a polysaccharide are susceptible to cleavage, oligosaccharide-anhydroalditols can still be isolated by terminating the reaction prematurely to effect *partial* reductive cleavage as linkages are rarely cleaved at the same rate. Reinhold *et al.*[229] used this approach to generate fragments from methylated β-cyclodextrin. Hence there is considerable scope for the application of partial or selective reductive cleavage as a means of generating oligomers useful for establishing linkages, positions of substitution and sequences in polysaccharides. Consequently, it is likely that this technique will become more prominent in future.

(vii) OTHER CHEMICAL METHODS

Several less frequently applied methods and techniques related to those already discussed exist for the selective chemical degradation of polysaccharides. Some of these procedures have been applied to bacterial polysaccharides and warrant further comment.

Selective cleavage of aminoglycosidic linkages in polysaccharides in order to generate oligosaccharides suitable for further structural and sequence analysis is well documented. Amino sugar-containing bacterial polysaccharides, in which the amino group is equatorial can be deaminated with nitrous acid to form a 2,5-anhydrohexose residue with simultaneous cleavage of the glycosidic linkage[230].

Despite its versatility, this reaction produces low yields of the desired product in our hands. Nevertheless, it has been successfully utilised in studies on a number of bacterial polysaccharides[130,207,231,232] and has been reviewed by Lindberg[233] and Williams[234].

A range of oxidative degradations have been used from time to time. Lead tetraacetate[235] can be used as an alternative to periodate as it also effects scission of *vic*-diols, however, a suitable solvent for both the lead tetraacetate and the polysaccharide cannot always be found. Acetic acid is the most commonly used solvent but is unsuitable for many bacterial polysaccharides. The resistance of α-linked hexopyranoses to oxidation with chromium trioxide[126] (see section 3.3) implies that this reagent can also be utilised to selectively cleave polysaccharides containing a single β- or furanosidic linkage. An oxidative procedure, developed by Svensson, using chlorine-DMSO and mild hydrolysis has been applied in structural studies on the methylated *Klebsiella* K59[236] capsular polysaccharide.

Partial acetolysis[83], usually performed in a mixture of acetic anhydride, acetic acid and sulphuric acid, is sometimes used as an alternative to acid hydrolysis. *O*-Acetylation occurs under these conditions leading to stabilisation of glycosidic bonds and the formation of different oligosaccharides from those obtained using mineral acids or TFA. For example, in contrast to acid hydrolysis, (1→6)-linkages are more susceptible to cleavage than other linkage types. Accordingly, the two methods may provide complementary information.

Trifluoroacetolysis[237], primarily used to cleave glycoconjugates[238], may have some applications in bacterial polysaccharide analysis. It is carried out in a mixture of trifluoroacetic acid and trifluoroacetic anhydride (TFA/TFAA) which initially trifluoroacetylates all free hydroxyl groups. This stabilises glycosidic linkages to a much greater extent than acetylation. If cleavage to form oligosaccharides occurs, selective removal of the *O*-trifluoroacetyl groups can be performed rendering them suitable for MS or NMR analysis. TFA/TFAA has also been used to specifically eliminate reducing 4-*O*-substituted hexose residues in oligosaccharides[239].

Honda *et al.*[240] have reported that iodotrimethylsilane in CCl_4 cleaves glycosidic linkages in pertrimethylsilylated polysaccharides at different rates. They found that glycosidic linkages were cleaved in the order, (1→4)<(1→2)<(1→3)<(1→6), making selective cleavage a possibility. However, in permethylated polysaccharides the reagent is more reactive making it very difficult for selectivity to be achieved.

There are therefore a wide range of chemical degradations available to the carbohydrate chemist which can be utilised to effect selective or specific degradation of bacterial polysaccharides. In most instances the degradative method selected should aim to provide the maximum possible amount of information on the structure of the repeating unit. As discussed, chemical methods frequently result in the alteration or destruction of one or more residues. The use of bacteriophage-borne enzymes to achieve selective cleavage, where feasible, has therefore become the method of choice. An oligosaccharide entirely representative of the repeating unit can be obtained using this approach.

3.5.2 BACTERIOPHAGE-MEDIATED ENZYMATIC DEGRADATION OF BACTERIAL POLYSACCHARIDES.

The discovery of bacteriophages, viruses that infect bacteria, is attributed to Twort[241] who, in 1915, reported that certain, normally creamy-white *Micrococcal* colonies underwent "glassy transformation" and lysis. He ascribed this phenomenon to a virus or other infective agent. This was later confirmed by d'Herelle in 1917[241] who coined the term bacteriophage meaning "bacteria-eater" and since then they have been extensively studied. Bacteriophages infect a wide variety of bacteria and those specific to encapsulated strains have proved to be of particular value to carbohydrate chemists.

In 1948, Humphries[242] noted that a bacteriophage-generated enzyme stripped the capsule from the surface of *Klebsiella pneumoniae* type 1 without destroying its immunological specificity, suggesting that depolymerisation to smaller fragments was occurring. The hydrolytic action of this enzyme was thought to expedite penetration of the capsule permitting easier adsorption of the bacteriophage to the host cell surface and/or to facilitate egress of the mature phage particles. Adams and Park, in a subsequent study on the bacteriophage specific to *K. pneumoniae* type 2[243], noted that a freely diffusible form of hydrolytic enzyme was also produced which was responsible for characteristic "halos" observed on infected agar plates of the host bacterium. They reported a marked decrease in the viscosity of an enzyme-treated bacterial suspension and noted the presence of large oligosaccharides in the degradation products. In addition, acapsular mutants were shown to be unaffected by the bacteriophages which infected their capsular parent strains, proving that the capsule was the initial receptor site for bacteriophage attachment. These capsule-specific bacteriophages were termed K-bacteriophages. Studies on the morphology of K-bacteriophages have shown that they all have a characteristic set of tail spikes with an active centre, believed to be the site of enzyme activity[244].

Sutherland and Wilkinson[17,245] were the first to make use of a bacteriophage enzyme in a partially purified form to digest a capsular polysaccharide for the purposes of structural elucidation. They showed that the K-bacteriophage specific for *Klebsiella* type 54 [A3(S1)] produced a highly specific fucosidase which cleaved the capsular polysaccharide, known to consist of a four-sugar repeating unit, into oligosaccharide products, one of which was shown to be an acetylated tetrasaccharide. Sutherland *et al.*[246] also demonstrated that this bacteriophage depolymerised the *E. coli* K27 capsular polysaccharide, which had been shown[247] to consist of almost identical repeating units, to produce a tetrasaccharide product with an *O*-acetyl group intact. Yurewicz[248], however, showed that more drastic alteration of structure, by periodate oxidation of the capsular polysaccharide from a strain of *Aerobacter aerogenes*, dramatically reduced the rate of cleavage to less than 1% of the rate recorded for the unmodified polymer thus indicating that specificity is to a large extent structure dependant.

These studies established the use of bacteriophage-borne enzymes as an extremely valuable means of selectively cleaving isolated capsular polysaccharides, especially those containing labile substituents. The fact that bacteriophages could possibly be isolated for the majority of encapsulated bacteria, particularly the *Enterobacteriaceae*, placed at the disposal of the carbohydrate chemist an array of specific agents of potential value in structural studies. Indeed, the structures of numerous *Klebsiella* and *E. coli* capsular polysaccharides have been structurally elucidated utilising bacteriophage-borne enzymes. These studies have been reviewed by Rieger-Hug and Stirm[249] and Hackland[228].

In most instances an oligosaccharide analogous to the repeating unit (P1) or its dimer (P2) is the major product, though in some cases only partial depolymerisation of the polysaccharide is achieved. The reduction in molecular weight, and consequently the viscosity of the dissolved polysaccharide, is nonetheless of value as an improvement in the resolution of subsequent NMR spectra can be expected. The use of bacteriophage-borne enzymes to generate oligosaccharides from capsular polysaccharides carrying labile substituents deserves special mention. It may be the only means of obtaining an oligosaccharide with the labile substituent intact. *E. coli* K103[60] and *Klebsiella* K32[250] (Figure 3.11) both contain extremely labile 1-carboxyethylidene groups and in both cases oligosaccharides with intact acetals were obtained permitting location of the positions of substitution.

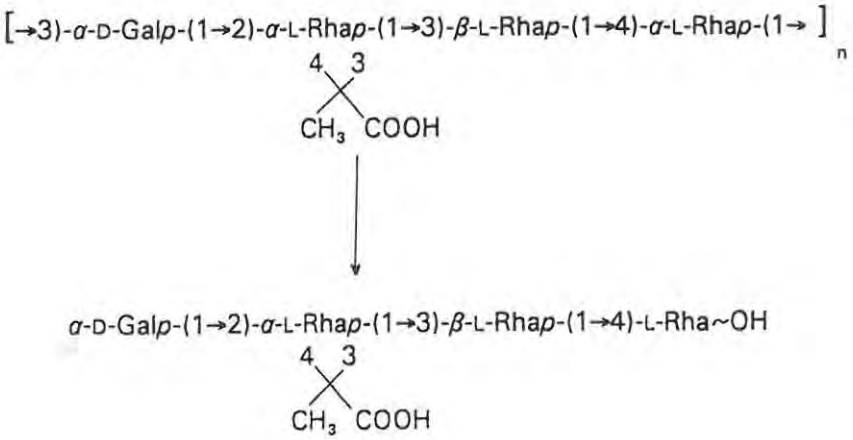


Figure 3.11 Bacteriophage degradation of the *Klebsiella* K32[250] capsular polysaccharide.

Rieger-Hug and Stirm[249], in a study which included 50 *Klebsiella* bacteriophages, established that most belong to Bradley group C[251] and, based on their depolymerisation reactions, were able to make the following general conclusions:

(1) Most cleavages occurred on either side of a negatively charged sugar unit, however reducing uronic acid residues were not usually formed.

(2) The reducing sugar was often substituted at position 3.

(3) β-Linkages were more frequently hydrolysed.

The majority of bacteriophage-borne enzymes have proved to be of the endoglycanase type, however unusual enzymes have been encountered. Several instances of lyase (eliminase) activity have been reported[252-254]. Here cleavage occurs with the formation of a terminal non-reducing hex-4-*en*uronic acid in the oligosaccharide product, presumably *via* a β-elimination reaction. This is illustrated in Figure 3.12. Endo-β-D-glucuronidase activity has been reported by Stephen[255] and is the only instance where the oligosaccharide produced has a terminal reducing uronic acid residue.

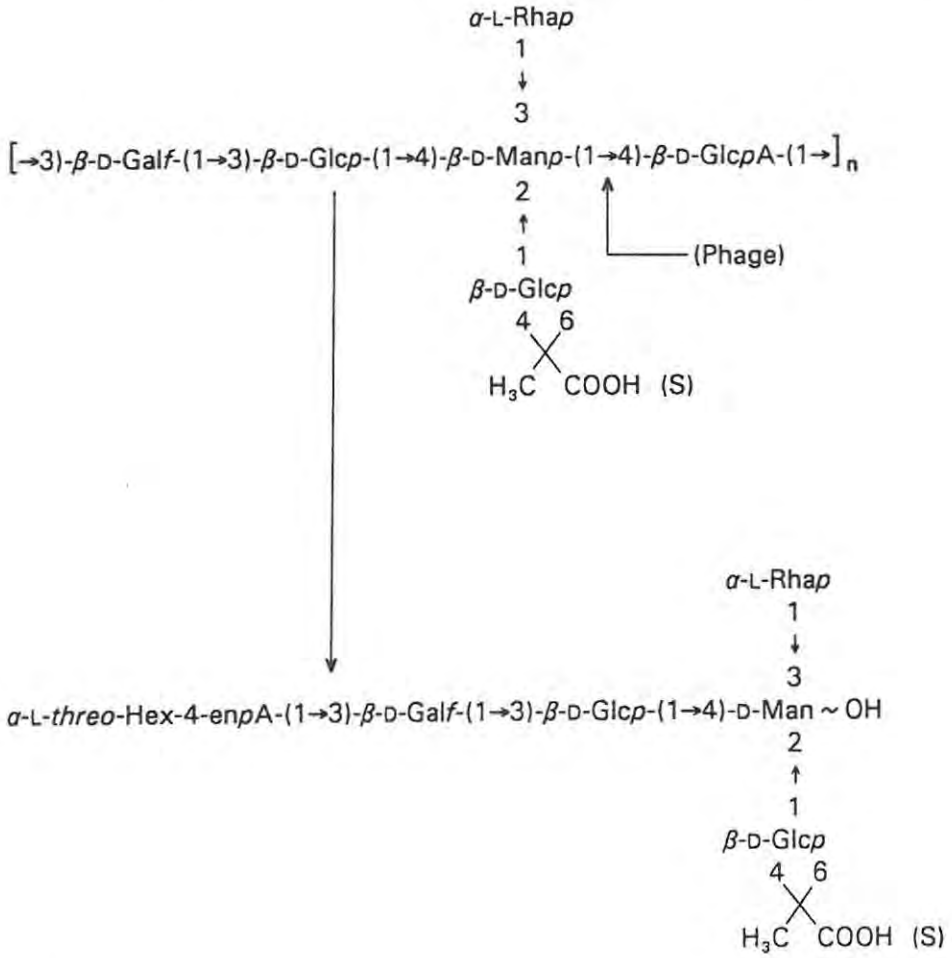


Figure 3.12 Product of bacteriophage degradation of the *Klebsiella* K14[252] capsular polysaccharide, an example of lyase activity.

Rigorous purification and concentration of bacteriophage particles can be performed by precipitation with polyethylene glycol (PEG)[256] followed by isopycnic centrifugation, however, for the purposes of depolymerisation, less exacting purification is satisfactory. Dutton *et al.*[257] have proposed four simplified procedures for the depolymerisation of polysaccharides with bacteriophages. Briefly, the bacteriophage (invariably isolated from sewage) is propagated on the host bacterium until a titre of 1×10^{13} plaque-forming units (PFUs), sufficient to depolymerise 1 gram of isolated polysaccharide[267], is achieved. The phage suspension is then purified by dialysis followed by dissolution and incubation of the isolated bacterial polysaccharide for a period of 1 - 4 days. A small amount of chloroform is usually added to prevent bacterial contamination. The resulting oligosaccharides are then isolated and purified by ion-exchange or gel chromatography.

Bacteriophage-borne enzymes continue to be utilised with great efficacy in structural studies on bacterial capsular polysaccharides. Jansson *et al.*[268] have utilised a bacteriophage to degrade the *E. coli* O8 polysaccharide and they have also been used for the selective hydrolysis of certain glycoconjugates. Bacteriophage-host interactions have also found application as models in receptor research.

3.6 CHROMATOGRAPHIC TECHNIQUES

A wide range of separation techniques are employed in complex carbohydrate structural studies for analytical and purification purposes. Several of the more important methods will therefore be reviewed, with the emphasis on GLC and HPLC.

(i) Paper Chromatography (PC)

Paper chromatography is now regarded as a rather elementary separation technique and has largely been superseded by more elegant methods such as GLC and HPLC. Nevertheless, it remains a useful method and is still occasionally employed for establishing the sugar composition of the initial hydrolysates of bacterial polysaccharides and for preparative separations such as the isolation of individual component sugars or small oligosaccharides. Sugars are separated on the basis of their differing affinities for the stationary phase (hydrated paper fibres) and the mobile phase, usually a mixture of solvents which travels along the paper in either the ascending or descending mode. The sugar composition of the *E. coli* K83 polysaccharide (this thesis) was initially established using paper chromatography and later confirmed using GLC. All component sugars were successfully separated from a TFA hydrolysate of the polysaccharide using ethyl acetate / acetic acid / pyridine / water (5:1:5:3) over 18 h in the descending mode. Grue *et al.*[41] successfully isolated Fuc3NAc which is present in a terminal position in the *E. coli* K45 capsular polysaccharide using paper chromatography. Churms[269] has published an excellent detailed handbook which outlines the different solvent systems and detection methods used for the separation of sugars by PC and the subject has recently been reviewed by Sherma[260].

(ii) Gas liquid chromatography (GLC)

GLC, first introduced by James and Martin in 1952[261], is the most widely used chromatographic technique in this field and is particularly suited for sugar analysis as it combines great sensitivity with high resolution.

Compounds are partitioned between an immobilised liquid phase (substances bonded or coated to an inert solid support or bonded directly to the inner column wall) and a mobile gaseous phase (e.g. helium, hydrogen or nitrogen) on the basis of their gas-liquid partition coefficients. Derivatisation of sugars (see sections 3.1 and 3.2) to render them suitably volatile is therefore mandatory as the sample must be presented in the gaseous form.

McInnes *et al.*[262] were the first to utilise a packed column for the separation of permethylated methyl glycosides. This was achieved on an 8 ft, 7mm internal diameter glass column packed with Apiezon M:Celite 545 (1:4 w/w). A wide variety of packed columns were subsequently developed for the purposes of sugar analysis, employing polymeric stationery phases such as the hydrocarbons Apiezon M and L, polyesters (DEGS) and polyglycols (Carbowaxes). Unfortunately the utility of these columns was somewhat limited by the relatively large samples required for injection and the generally poor peak resolution. Furthermore, low thermal stability, column bleed, and the fragile nature of the glass columns gave them relatively short useful lives. Capillary columns, originally proposed in 1977[263] as an alternative to the traditional wide diameter packed columns, became commercially available in the early 1980s and rapidly replaced classical packed columns owing to their far superior resolving capabilities.

These columns differ from the original packed columns in that they have a very much smaller internal diameter (0.25-0.75 mm) and may be up to 50 m in length. The inner surface is coated with a thin layer (0.10-0.40 μm) of the liquid phase giving them essentially an "open tubular" nature with a high specific gas permeability. These changes result in a marked increase in the number of theoretical plates and hence vastly improved separation efficiency and peak resolution. Most modern columns are now constructed from fused silica which has greater flexibility and mechanical durability than the original glass capillary columns. Silica is also a more inert surface with a reduced tendency for adsorbing active components. Wide-bore fused-silica capillaries (internal diameter $\approx$ 0.5 mm) are also available as direct alternatives to packed columns and they combine the advantages of capillary GLC with the ability to handle larger sample sizes.

Capillary columns may be classified according to the way in which the liquid phase is attached or supported and according to the type (polarity) of the liquid phase present. This is usually deposited directly onto the inner surface as is the case for "wall coated open tubular" (WCOT) columns or alternatively it may be presented on a crystalline "porous layer" (PLOT) or on a solid support *viz.* "support coated open tubular" (SCOT) columns[264]. A "micropacked" capillary column in which the entire internal diameter is completely filled with a support material is also available for specialised applications[266]. All of these methods of immobilising the stationary phase are problematic as the column cannot be back-flushed to remove impurities and there is a tendency for column bleed at higher temperatures[268]. Bonded-phase capillary columns have the liquid phase bonded directly to the silica wall thereby conferring much greater thermal stability. Temperature dependent column bleed and phase stripping is greatly reduced and repeated column washes with solvent does not damage the column.

	Packed	Capillary
Length, meters	1.5-6	5-100
I.D., millimeters	2-4	0.2-0.7
Specific Permeability, (10^{-7}) cm^2	1-10	10-1000
Flow, ml/min	10-60	0.5-15
Pressure Drop, psi	10-40	3-40
Total Effective Plates (2 meter, 50 meter)	5,000	150,000
Effective Plates per Meter	2,500 (id 2mm)	3,000 (id 0.25)
Capacity	10ug/peak	<50ng/peak
Liquid Film Thickness, um	1-10	0.05-1.0

PACKED

CAPILLARY

Figure 3.13 Differences between packed and capillary columns

Many of the liquid phases formally employed in packed columns are still used in capillary columns. They may take the form of a viscous liquid (e.g OV-225), a gum (e.g OV-1 or SE-30), or even a solid such as carbowax; however all are liquids at operating temperatures.

Those most suited for general sugar analysis are of intermediate polarity such as ECNSS-M, OV-225 and OV-17. ECNSS-M was the first stationary phase used to separate alditol acetates, however OV-225 is now preferred as the former has low thermal stability and a tendency to bleed excessively. The slightly less polar OV-17 phase has particularly good thermal stability and is useful for the separation of amino sugars. Since certain sugar derivatives emerge in a different order to OV-225, it can be used to confirm the identities of sugars separated on OV-225. DB-WAX, a very polar phase, is used in this laboratory to separate the alditol acetates of ManNAc and GalNAc as they co-elute on both OV-225 and OV-17.

Open tubular columns require relatively low flow rates (~ 0.5-1.0 ml/min) and the amount of sample entering the column is usually very much less than that required for packed columns. Column overloading can be avoided by using a split inlet system, which purges the majority of the injected sample or through careful sample dilution. A number of different detectors are used for discerning eluting peaks and these include flame ionization, thermal conductivity and electron capture detectors[267]. Eluting compounds are recorded as peaks by a chart recorder or recording integrator and are usually identified by comparison with authentic standards injected separately under identical conditions.

The linearity of the detector response is not important for qualitative analysis and molar response factors are sufficient. These can be estimated using the "effective carbon response" (ecr) theory[268] which is based on summation of the responses due to each carbon type in the molecule. More accurate determination of the response factors for each compound being assayed is essential for quantitative determinations or for sugar analyses of polysaccharides which are not comprised of repeating units. Identification of compounds solely on the basis of retention times is not always unambiguous and identity should be confirmed by other means wherever possible *viz.* utilising different derivatives and/or columns, or by hyphenated techniques such as GLC-MS.

(iii) GEL PERMEATION (GPC) AND ION-EXCHANGE GEL CHROMATOGRAPHY

Gel permeation chromatography is an important technique for the separation and purification of carbohydrates and is based on the principle of molecular size exclusion. The stationary phase consists of swollen, porous gel beads packed into a glass column and the sample is eluted using a solvent which may be water, an aqueous buffer or, infrequently, an organic solvent. Separation is dependent on the extent to which sample molecules enter the pores present in the gel. Very large molecules e.g. polysaccharides may never enter the gel matrix and simply pass directly between the gel beads to be eluted in the void volume. Smaller molecules diffuse into the pores and spend more time associated with the stationary phase and are eluted later enabling carbohydrates to be effectively chromatographed on the basis of size and shape. Eluting sugars are usually detected using a differential refractometer, collected by an automated fraction collector and traced by a chart recorder as an "elution diagram". Colorimetric methods of detection e.g. the phenol-sulphuric acid method[269] are now only used in cases where the mobile phase has a very high salt content which precludes the use of a refractive index detector e.g. when applying a salt gradient in ion-exchange gel chromatography (see below). Within limits, gel columns can be repeatedly reused without repacking and the process is sufficiently gentle to permit separation of most labile biological materials.

The range of Sephadex™ gels, formed by cross-linking dextran with epichlorhydrin[270], are among the standard gels for most applications involving molecules up to a molecular weight of 600 000 and are frequently used for carbohydrates. Sephadex G-10 and G-15 may also be used with organic solvents such as DMSO and DMF or with mixtures of water and the lower alcohols[270], however strongly hydrophobic Sephadex LH-20 is best for chloroform soluble compounds such as methylated oligosaccharides. Molecular weight determinations and the purification of higher molecular weight polysaccharides may be performed on Sephacryl™ gels such as S-400 and S-500. They are exceptionally rigid and permit high flow rates without sacrificing resolution. Polyacrylamide gels (the Bio-Gel™ series), in particular Bio-Gel P-2 and P-4 are extremely useful for

separating small oligosaccharides and are also conveniently used for desalting purposes. Sepharose™ gels, prepared from agarose and the related, more durable Sepharose CL series, are also suitable for higher molecular weight substances such as polysaccharides.

Improved sample resolution can be achieved if the gel used has ion-exchange capabilities. This is made possible by covalently bonding charged groups with mobile counter ions to the gel structure. These groups may be either anionic or cationic exchangers, however for carbohydrates anionic exchangers are the most important. Bacterial polysaccharides and their degradation products containing uronic acids can be selectively isolated from neutral materials using these gels as the negatively charged acidic function enables selective adsorption to the gel. Anion-exchange gels based on Sephadex, Sepharose CI-6B and Sephacel with diethylaminoethyl (DEAE), aminoethyl (AE) and quaternaryaminoethyl (QAE) groups are available. Separation is accomplished by reversing adsorption in a controlled fashion thus necessitating two stages in the process *viz.* sample application (adsorption), followed by sample elution (desorption) with a salt gradient. Columns are packed in a similar fashion to standard gels, however regeneration of the gel is required prior to reuse.

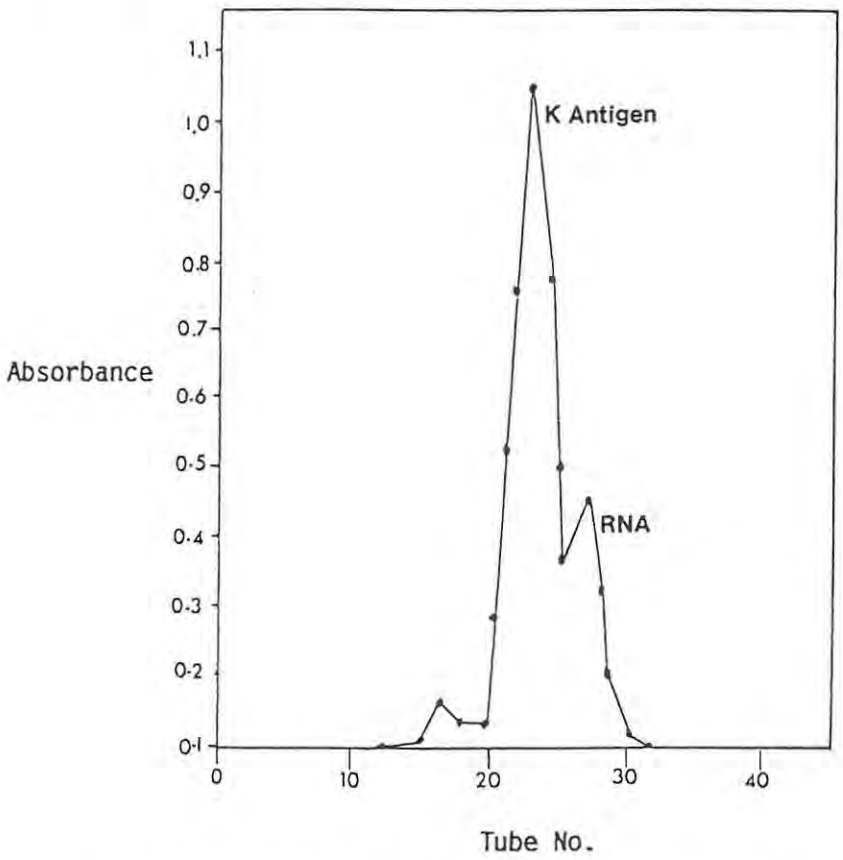


Figure 3.14 Elution diagram of the *E. coli* K83 polysaccharide on DEAE Sepharose CI-6B.

(iv) HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Modern high performance liquid chromatography was introduced in the late 1960s and since then has rapidly expanded to become one of the most important chromatographic techniques in carbohydrate research. Separation is based on a number of chromatographic principles either solely, or in combination, and may include ion-exchange, gel-permeation and partition chromatography (normal and reversed phase) as well as ligand exchange and hydrophobic absorption. The common feature is that all stationary phases are packed into short stainless steel columns and sample elution is normally achieved by pumping a mobile phase through the column under a relatively high pressure. This achieves separation in a short time (15-60 minutes), at low operating temperatures and with excellent peak resolution. Most columns are preceded by a shorter "guard column" which fulfils a protecting role thus prolonging the useful life of the column. Although not as sensitive as GLC, HPLC possesses the distinct advantage that derivatisation is usually unnecessary and the method is more amenable to preparative work.

Despite its relative insensitivity, differential refractometry is the most common detection method for sugars, however, for quantitative purposes, derivatisation to produce a chromophore or fluorophore permits more reliable and sensitive ultraviolet or fluorometric detection. HPLC should not be regarded as a universal replacement for existing chromatographic techniques but rather as an extremely powerful and versatile complementary technique. The applications of HPLC in carbohydrate structural analysis have been reviewed by Hicks[271].

A number of different stationary phases are useful for carbohydrate analysis *viz.* chemically bonded silca gels (normal and reverse-phase), ion-exchange resins and high performance size-exclusion gels. Amine modified silica gels (e.g micro Bondapak™, Partisil™ 10) separate carbohydrates by means of normal phase partitioning with acetonitrile-water as the mobile phase, however separation of derivatised e.g. perbenzoylated sugars is preferable as the aqueous component of the mobile phase eventually dissolves the stationary phase over time leaving voids in the packing.

Furthermore, reducing sugars are known to react with the amino groups on the stationary phase to form deactivated glycosylamines which ultimately leads to column failure[272]. Conversely, reverse-phase silica gels e.g octyldimethylsilyl (C_8) and octadecyldimethylsilyl (C_{18}) columns are far more popular as they are generally very stable and do not dissolve in aqueous mobile phases. They are more suitable for the analysis of less polar carbohydrates, e.g. synthetic intermediates and derivatised sugars, such as permethylated or perbenzoylated sugars, but have the disadvantage that they are able to resolve the anomeric forms of reducing sugars, leading to broadened, complex peaks.

Resin based columns are far more generally used, particularly for the separation of underivatised carbohydrates and they may contain cationic or anionic exchange resins. Sulphonated polystyrene-divinylbenzene (SDVB) in the form of finely divided spheres of ~10 μm is a commonly encountered cationic exchange resin. Carbohydrates are separated by a combination of mechanisms including size exclusion, ligand exchange and hydrophilic adsorption. Elevated temperatures are also preferred as the partitioning of solutes between the mobile and stationary phase is a diffusion limited process[273]. Resins are commonly available in the H^+ form but can be obtained in or converted to the Ca^{2+}, Pb^{2+} or Ag^{2+} form if so desired. They are robust columns which are stable over a wide pH range and they do not dissolve in aqueous media. Ion-exchange *gels* which operate on similar principles to the resins described above are also available. In this laboratory a Supelcogel™ C-611 ion-exchange column, which contains two different divalent cations, is used for the separation of both monosaccharides and small oligosaccharides with great efficacy.

High performance LC anion exchange chromatography has only just come into its own and is now an exciting new high resolution liquid chromatographic technique for sugar analysis. Silica and polystyrene-based anion exchange columns have been available for some time for the separation of acidic sugars, however neutral sugars could only be separated as their borate-complexes, after post column derivatisation for the purposes of fluorometric detection[274]. The latter approach unfortunately also gives poorly resolved peaks, is relatively insensitive, requires long analysis times,

and the columns usually have short useful lives[271]. The recent development of pulsed amperometric detection (PAD) coupled with new developments in anion exchange stationary phases has all but overcome these limitations and a new LC technique, known as high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)[103,276] is the most sensitive ultra-high resolution technique yet developed. The PAD detector generates an electrochemical response to carbohydrates by oxidising them on a gold electrode using triple pulse amperometry. The limit of reliable quantification is better than 100 pmol[276] and resolution is equal to and better than that possible with capillary GLC. Both neutral, acidic and amino sugars can be analysed as the high pH of the mobile phase (usually between 100-500 mM NaOH), converts neutral sugars into anions which are subsequently eluted using a sodium acetate gradient. This technique has revolutionised the analysis of biological samples which contain very small amounts of carbohydrates, such as glycoconjugates, and is particularly useful for the subfractionation of mixed samples isolated by gel chromatography. Several studies have recently appeared in the literature[176,276-277].

Vinyl polymer based micro gel filtration columns are also a relatively new innovation and are extremely useful for separating mixtures of oligosaccharides, either analytically or on a semi-preparative scale. Separation is accomplished largely on the basis of size exclusion principles with pure water or very weakly basic solutions e.g 10^{-4} M NaOH as the mobile phases. Operating flow rates and pressures are very much lower than normal as the gel is more fragile and less densely packed than conventional stationary phases. They possess high efficiency and capacity and resolution can be further improved by running at slightly elevated temperatures *viz.* 40-70° C. Progel™-TSK G3000 PWXL (10 μm beads), for separating both neutral and ionic samples, and Progel™ G-oligo-PW (6 μm beads), for separating and purifying non-ionic oligosaccharides, are used in this laboratory.

3.7 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Nuclear magnetic resonance (NMR) spectroscopy is the most important analytical technique in this field of research. It has undergone almost unprecedented development over the last two decades with nearly continuous publication of new techniques and applications since the early 1970s. The range of NMR experiments available to the chemist continues to grow and developments over the last decade have transformed NMR spectroscopy into a method capable of complete and independent structural analysis so that an *ab initio* assignment of the structure of an oligo- or polysaccharide without recourse to other procedures is now possible.

Two significant milestones in the history of NMR spectroscopy, since the development of the first continuous wave (cw) spectrometers, have expedited the advance of this powerful technique to its current status. The first was the introduction of pulsed Fourier transform (FT) spectroscopy which greatly reduced acquisition times and made the acquisition of spectra for low abundance nuclei such as ^{13}C a possibility. The second was the discovery that double Fourier transformation could be performed if the experiment was acquired with two time variables to produce a two dimensional spectrum possessing a second frequency dimension (2D NMR). This idea, first reported by Jeener[278], coupled with technological advances in computers and superconducting magnets, led to the development of sophisticated high frequency NMR spectrometers and explosive growth in the number of multiple pulse and 2D NMR experiments followed. The proliferation of experimental techniques has also been prompted, in part, by the need to extract well established parameters such as chemical shifts (δ), coupling constants (J), spin-lattice (T_1) and spin-spin (T_2) relaxation times from the increasingly complex spectra of biological samples. Computers now permit routine execution of experiments which require the manipulation of vast quantities of data and the accurate control of pulses and delays in the order of milli- and micro-seconds.

Whilst the developments discussed above have enabled the acquisition of high-resolution spectra in very much shorter times, sensitivity remains a pressing problem. The recent introduction of inverse detected techniques (see section 3.7 (b) (ii)) has greatly improved sensitivity in heteronuclear correlated spectroscopy and high quality spectra can now be acquired on samples as small as 0.5 mg. Larger quantities of material are preferable for most homonuclear experiments.

Polysaccharide samples are usually freeze-dried several times in high grade D_2O to replace exchangeable hydroxyl protons with deuterons prior to acquisition of the spectra in 99.995% D_2O, which serves as the lock signal. Acetone is most often included as an internal reference as it is easily removed from the sample once the spectrum has been acquired, although other compounds such as 3-(trimethylsilyl)-propionate-d_4 (TSP) and sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS), are also sometimes used. The chemical shift for acetone is taken as δ 2.225 (2.23) for ^1H and 31.07 ppm for ^{13}C spectra. However, many reports appear which quote different positions of reference for the former (δ 2.12, 2.17 and 2.22 have all been cited). The chemical shift of acetone is also influenced by temperature and the nature of the sample being acquired[279] and tetramethylsilane (TMS) and DSS, the most frequently used external references, differ in their chemical shift scales by approximately 0.5 ppm[280]. There is therefore an urgent need for standardisation of the means of referencing, and chemical shift data in the literature should be treated with a measure of caution. In this laboratory all spectra are referenced at δ 2.23 (^1H) and 31.07 ppm (^{13}C).

Polysaccharides comprised of distinct repeating units are especially suited to analysis by NMR spectroscopy for a number of reasons. Protons are, in most cases, spaced around sugar rings in a vicinally coupled manner which permits elucidation of the stereochemistry and constitution at each carbon. In addition, due to the presence of the ring oxygen atom, the anomeric proton (or carbon) is deshielded and resonates further downfield. These signals are therefore convenient windows into the spin systems of the constituent sugar residues in the polysaccharide. The severe overlap that occurs in the ring proton region and the masking of signals due to the water-derived

(HOD) peak can be overcome by a variety of multiple pulse and 2D NMR techniques, thereby allowing complete elucidation of the spin systems of individual sugar residues. In addition, proton-proton dipolar interactions and certain other techniques (see below) can be utilised to establish the sequences of sugar residues. A thorough knowledge of the applications of a wide variety of NMR experiments is therefore essential for the carbohydrate chemist.

The basic principles of modern NMR spectroscopy will not be discussed in detail since numerous texts and literature reviews[281-286] are available which adequately cover these aspects. Only the more important techniques will be discussed with attention focused on ^{1}H and ^{13}C NMR and their applications in the field of polysaccharide chemistry.

(a) ONE DIMENSIONAL TECHNIQUES

(i) 1D ^{1}H NMR Spectroscopy

This is the simplest and most widely applied Fourier transform method. It is based on the use of a single radiofrequency pulse of a few microseconds duration which momentarily excites all the protons in the sample and it results in the formation of an emission signal known as the "free induction decay" (FID). After accumulation of a set of successively obtained and combined decays, transformation yields a proton spectrum in one dimension containing resonances. The specific chemical shift of each proton resonance is determined by its chemical environment and the area beneath each signal (or multiplet) is proportional to the number of protons it represents.

Figure 3.15 Fourier transformation.

The structural complexity and purity of the sample is usually immediately apparent from the 1D spectrum and, if the sample is a polysaccharide, the effects of viscosity can be assessed. Viscous solutions lead to broader spectral lines due to shorter spin-lattice relaxation times and rapid decay of the FID. The effects of viscosity can be partly overcome by elevation of the probe temperature or slight depolymerisation of the polysaccharide, however this should be judiciously performed as labile non-carbohydrate substituents may be lost. A substantial water derived (HOD) peak can also obscure sugar resonances. Care should therefore be exercised during sample preparation and deuterium exchange to ensure that the water peak is as small as possible. Alternatively, specific pulse sequences, mostly multiple pulse techniques, have been developed which effect suppression of the HOD peak (see below). The 1D spectrum of an oligosaccharide or polysaccharide can be divided into three broad regions (see Figure 3.16), *viz.* the anomeric region (δ 4.5-5.5), the methyl group region (δ 1.2-2.3) and the ring proton region (δ 3.2-4.5).

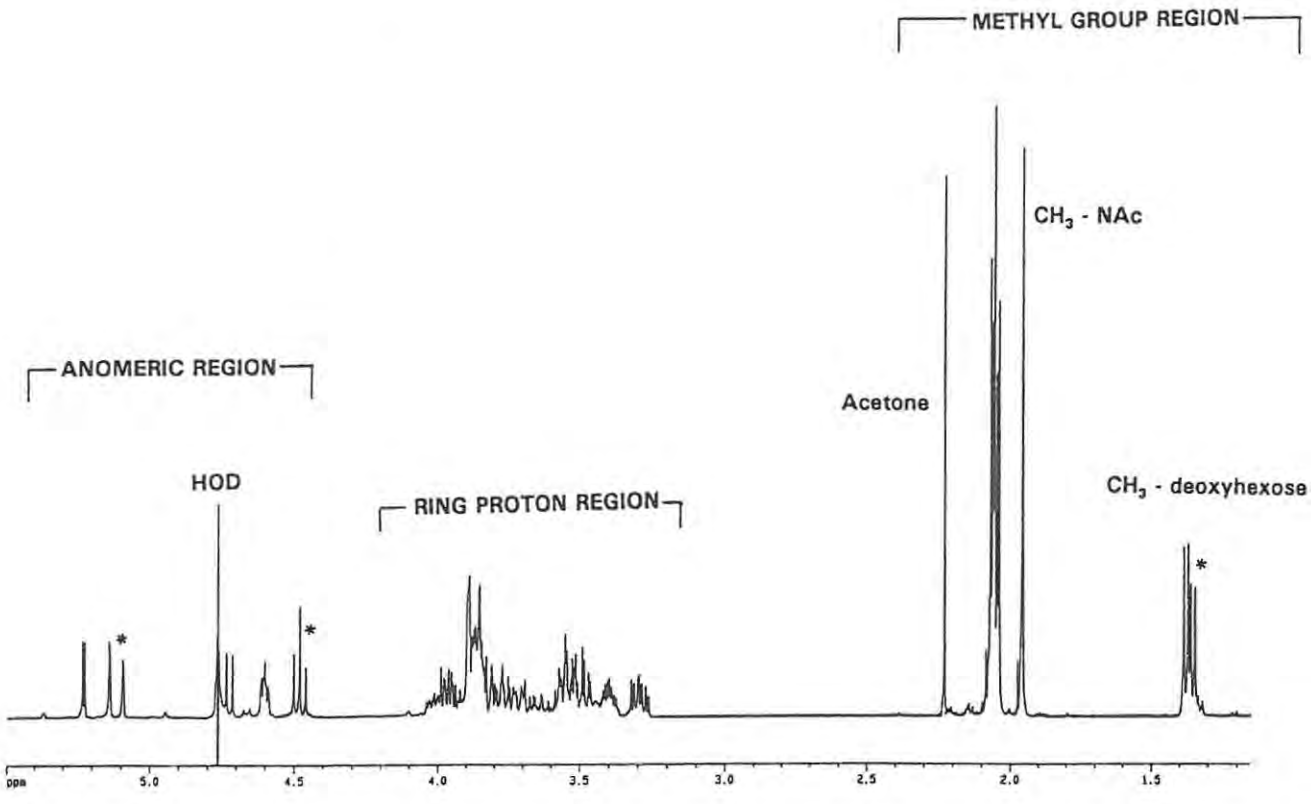


Figure 3.16 1D ^{1}H NMR spectrum (with solvent suppression) of the reducing trisaccharide produced by the action of anhydrous HF on the *E. coli* K48 capsular polysaccharide. The three principle regions are indicated, as are resonances which are noticeably "twinned".

The anomeric region usually reveals the number of monosaccharides in the repeating unit and is useful for assigning anomeric configurations to the constituent sugar residues. The anomeric protons of pyranose sugars having the *gluco* or *galacto* configuration resonate in the region δ 5.0-5.5 if they are α-linked, and between δ 4.5-5.0 if they are β-linked. In addition, these signals are usually well separated first order doublets permitting easy measurement of the $^3J_{H\text{-}1,H\text{-}2}$ coupling constants. The dihedral angle between vicinally situated protons has been shown by Karplus[286] to be proportional to the magnitude of the coupling constant. Hence β-linked sugars of this variety have coupling constants between 8-10Hz and α-linked sugars 1-4 Hz. *Manno*-sugars are problematic as the signal for the anomeric proton may resonate close to δ 5.0 and the coupling constants are small for both α and β-linked sugars. In such cases the anomeric configuration can be assigned from $J_{C\text{-}1,H\text{-}1}$ values or from NOE data (see below). The anomeric protons of furanose residues resonate slightly further downfield than their pyranosidically linked counterparts.

In certain instances non-anomeric signals may also be found between δ 4.5 and 5.5, therefore this region must be interpreted cautiously. H-5 (and sometimes H-4) of α-GalA, H-2 of ManNAc and the α-protons of *O*-acylated carbon atoms, in particular, frequently resonate in this region. Reducing sugars give rise to two signals in the anomeric region representing the α and β forms of the sugar as it mutarotates in solution. "Twinning" of other signals in the 1D NMR spectra of oligosaccharides may also be apparent due to mutarotation (see Figure 3.16). The methyl group region is less troublesome and reveals the presence of the methyl groups of 6-deoxy sugars, *O*- and *N*-acetyl substituents and pyruvate or lactyl groups. Due to severe resonance overlap, the ring proton region usually yields little direct information.

The advent of Fourier transform techniques has also greatly facilitated the measurement of T_1 and T_2 values from 1D ^{1}H NMR spectra. Grant and co-workers[287] were the first to report that proton nuclear relaxation times (T_1 values) in six-membered ring systems showed stereospecific dependencies. Based on these findings Hall and Preston[288] demonstrated that these values could be useful for assigning anomeric configurations in monosaccharides.

An axial proton at C-1 is in closer proximity to the protons at C-3 and C-5 and can therefore relax faster than an equatorial proton. Thus a β-linked glycoside can be distinguished from an α-linked glycoside on this basis. In a subsequent study[289] they extended this approach to oligo- and polysaccharides, although studies on the *Klebsiella* K24 polysaccharide showed that, for the latter, the results lacked diagnostic potential. This approach is therefore seldom used in polysaccharide chemistry for the assignment of anomeric configuration. Nevertheless, the usefulness of this method in conformational studies was demonstrated and a means of attenuating the HOD peak in proton spectra also resulted from this work. They showed that suitable delays included in the pulse sequence could lead to nulling of the HOD peak based on the fact that HOD protons have a much longer relaxation time than carbohydrate protons (see below).

(ii) 1-D ^{13}C NMR Spectroscopy

Whereas the Fourier transform mode is advantageous for ^{1}H NMR spectroscopy, it is absolutely mandatory for the routine acquisition of ^{13}C spectra due to the low natural abundance (1.1%) and inherent insensitivity (magnetogyric ratio ~ 1/4 γ_H) of this nucleus. 1D ^{13}C NMR spectroscopy is complementary to ^{1}H NMR and, in many respects, possesses distinct advantages over the latter.

The removal of ^{13}C-^{1}H coupling is imperative in most cases and greatly simplifies ^{13}C spectra by reducing the often complex, and frequently overlapping multiplets characteristic of coupled spectra, to narrow well resolved singlets. Decoupling also improves the sensitivity of the nucleus through Nuclear Overhauser Enhancement[280] and the intensity of each signal is increased in proportion to the ^{1}H splitting eliminated. Nuclear Overhauser Enhancement unfortunately precludes reliable integration of signals since the enhancement effect is not constant. This minor drawback is more than compensated for by the greater sensitivity of this nucleus to changes in its chemical and stereochemical environment, the wide range of well dispersed chemical shifts (~200 ppm for carbohydrates) and the absence of line broadening, all of which permit the acquisition of adequately

resolved spectra at frequencies as low as 100Mhz. The time required for the acquisition of a ^{13}C spectrum with an acceptable signal to noise ratio is considerably longer than that adequate for a proton spectrum, not only because of the aforementioned reasons, but also due to the fact that ^{13}C nuclei, in particular quaternary carbons, have much longer relaxation times than protons. Delays of several seconds in magnitude are sometimes required to adequately record the latter, leading to total run times which may exceed 18 hours, even on the most modern machines.

The sensitivity of ^{13}C nuclei to small changes in structure has led to numerous papers and reviews on the applications of ^{13}C NMR in the field of carbohydrate chemistry. The carbons involved in glycosidic linkages may be displaced 6-9 ppm downfield by inductive deshielding effects[291] making this an important means of establishing linkage positions in glycans. Pyruvylated and acetylated carbons also show characteristic shift changes permitting identification of the position(s) of substitution. Amongst the most useful are studies by Bock *et al.*[292,293] and Bradbury and Jenkins[291] who published extensive reviews containing data on a variety of mono- and oligo-saccharides. Jennings and Smith[294] and Barker *et al.*[295] have reviewed the application of ^{13}C NMR in the analysis of polysaccharides.

The ^{13}C spectra of bacterial polysaccharides may also be broadly divided into regions which yield structural information. Once again the anomeric resonances (90-110 ppm) are well separated from the signals produced by other carbon nuclei and should reveal the number of sugars in the repeating unit. The rest of the methine and methylene carbon resonances occur between 48 and 86 ppm. These include the methine resonances of 2-amino-2-deoxy sugars (48-58 ppm), the hydroxymethylene resonances for 6-linked (66-70 ppm) and unlinked hexoses (60-65 ppm), ring carbons (67-75 ppm) and linkage carbon atoms (75-85 ppm). In addition, low field signals in the region 167-180 ppm reflect the presence of carbonyl carbons from uronic acids, acetyl or other carbonyl-containing substituents and high field signals between 17 and 24 ppm indicate the presence of methyl groups.

The majority of ^{13}C NMR spectra are acquired in the broadband ^{1}H decoupled mode using the WALTZ-16 sequence[296], however in certain instances it may be useful to run coupled or partially decoupled spectra. These may be acquired using "gated decoupling[297]" which preserves most of the NOE enhancement or "off-resonance coherent decoupling[298]". The latter affords a spectrum with reduced one-bond splitting which provides a powerful method for confirming the number of protons attached to each carbon. The most common indication for the acquisition of a coupled ^{13}C spectrum is to establish the anomeric configurations of residues of the *manno* variety.
Bock *et al.*[299] have shown that $^{1}J_{C\text{-}1,H\text{-}1}$ for β-linked pyranoses is about 160 Hz and for α-linked pyranoses about 10 ppm greater, making it possible to distinguish between the two.

The measurement of ^{13}C spin-lattice relaxation times may also provide complementary structural information[300]. Methylene carbons (C-6's) have been shown to relax quicker than ring carbons due to the contribution of the extra proton and have T_1 values approximately half that of ring carbon atoms. In contrast the T_1 values of glycosidic methyl carbon atoms and acetyl methyl groups are approximately three times longer than the ring carbon atoms due to free rotation in solution. It is also possible to distinguish between the carbon atoms of sugars in the main backbone of a polysaccharide and those in the side chain[301] as they possess different relaxation pathways and therefore different T_1 values.

The analysis of ^{13}C chemical shift data provides a wealth of information on the conformations of carbohydrates in solution because of the exceptional sensitivity of ^{13}C nuclei to stereochemical, steric and proximity effects. In addition it has been found that two-, three- and even four-bond ^{1}H-^{13}C and ^{13}C-^{13}C coupling constants[302,303] also show dihedral angle dependence as defined by Karplus and can thus provide conformational information. ^{13}C chemical shifts are also affected by inductive effects and spatial proton-proton interactions which polarise C-H bonds resulting in unique changes to the shielding of the ^{13}C nuclei involved. Lipkind *et al.*[304] have published ^{13}C chemical shift data for a variety of reducing disaccharides and on the basis of chemical shift differences were able to compile a set of empirical rules which enables the relative absolute configuration of one of the

sugars in a disaccharide to be assigned if the absolute stereochemistry of the other is known (see section 4.5). The measurement of ^{13}C-^{13}C coupling constants is difficult at natural abundance levels, but is facilitated by modern multiple pulse techniques and most can now be obtained without the need for artificial ^{13}C enrichment.

(iii) Multiple pulse techniques

A distinct advantage of the Fourier transform method and the introduction of programmable pulse transmitters is that the single excitation pulse of the standard experiment can be replaced by a sequence of two or more pulses, separated by fixed delays. If there is a very short delay, it is likely that the transverse magnetisation vectors will have moved relative to each other by the time pulsing resumes, resulting in a final spectrum that is difficult to phase. This apparently troublesome effect is turned to advantage in multiple pulse experiments. The early multiple pulse techniques included spin-echo[305] and inversion recovery experiments but today a wide range of extremely useful pulse sequences have been developed which include sequences for water suppression, sensitivity enhancement and selective excitation. Several of the more important techniques will be briefly discussed.

Multiple pulse techniques which enable distinction between quaternary, methine, methylene and methyl carbons have been developed and they fall into two classes depending on whether or not polarisation transfer occurs. The GASPE[306] (GAted SPin-Echo) and APT[307] (Attached Proton Test) sequences do not utilise polarisation transfer and rely solely on intermittent decoupling during one of the two delay periods in the sequence and are independent of data acquisition. INEPT[308] (Insensitive Nuclei Enhancement by Polarisation Transfer) and DEPT[309] (Distortionless Enhancement by Polarisation Transfer) rely on one-bond proton-carbon couplings to distinguish between the types of proton bearing carbon atoms and effect signal enhancement through polarisation transfer from ^{1}H to ^{13}C nuclei. Quaternary carbons, which have no bonded protons are therefore not observable using the latter two pulse sequences.

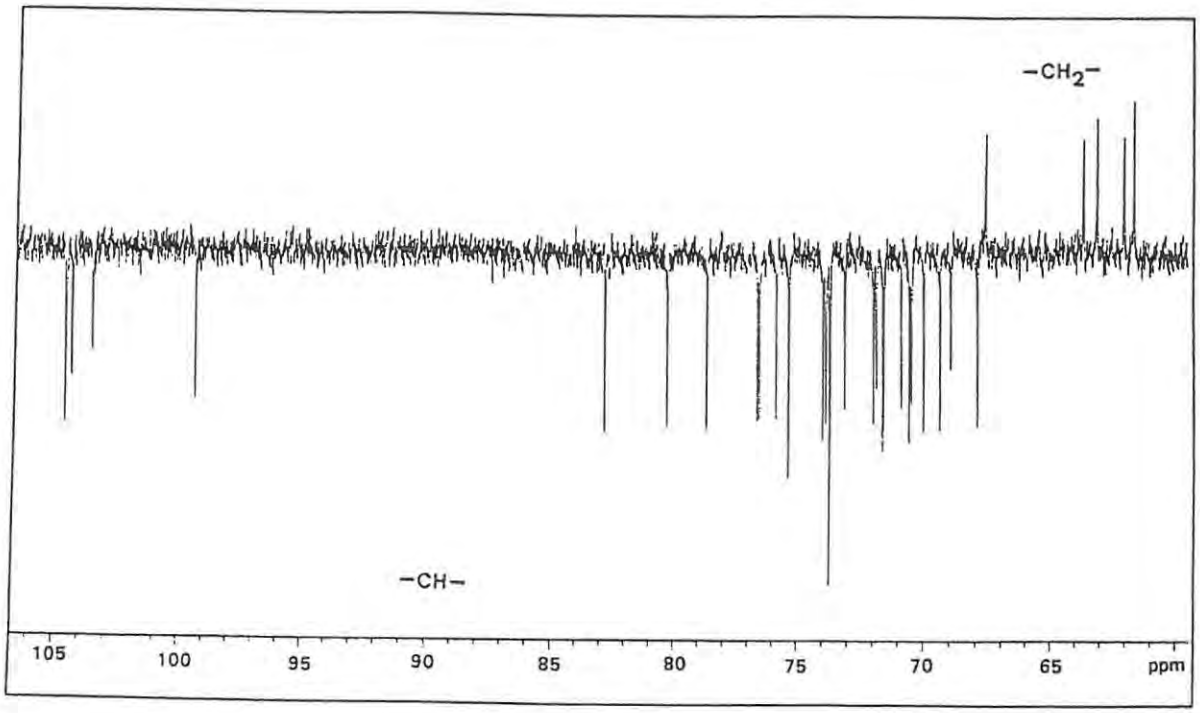


Figure 3.17 Applied Proton Test (APT) spectrum of *Klebsiella* K15 P1-OL (section 4.1).

The INADEQUATE pulse sequence[310] (Incredible Natural Abundance Double Quantum Transfer Experiment) relies on the creation of double quantum coherence between coupled ^{13}C nuclei and results in a spectrum free from uncoupled carbon signals. This "simplified" spectrum can be used as an adjunct to a conventional ^{13}C spectrum in cases of excessive overcrowding. However, it is the two-dimensional version of this experiment which is far more commonly applied as it can provide ^{13}C-^{13}C coupling constant data at natural abundances and allows the researcher to assign the entire carbon backbone of a molecule. Patt *et al.*[311] generated ^{13}C-^{13}C connectivity plots for β-cellobiose and determined the positions of the glycosidic linkage of the latter using this pulse sequence, thus demonstrating its applicability to carbohydrate chemistry.

A variety of methods have been developed to effect suppression of solvent peaks. These include techniques based on saturation decoupling, selective relaxation and selective excitation. Selective relaxation can be achieved using the WEFT[312] pulse sequence, a simple inversion-recovery sequence, which nulls the solvent peak by exploiting the large difference in the relaxation rate of the solvent (H_2O > 2s) and the constituent protons (< 0.5s). Alternatively the DANTE[313] pulse sequence can be used. The latter achieves the same effect by a combination of selective saturation and relaxation. Samples acquired in aqueous solutions require more powerful methods for water suppression. Sequences designed for such applications include the WATR[314] sequence, which achieves attenuation of the water peak by artificially increasing the T_2 relaxation rate using a chemical exchange reagent in combination with the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence, and binomial solvent suppression with, for example, the 1-3-3-1 pulse sequence[315,316] which selectively excites the region around the solvent chemical shift leaving the solvent peak in a "protected zone".

(iv) Band Selective Excitation - Shaped pulses.

All of the techniques discussed thus far employ rectangular pulses which excite the entire spectrum of frequencies of the nucleus under investigation. Several sequences have recently been introduced which use shaped pulses to achieve band selective-excitation[317-319]. Pulse shaping improves the frequency-domain response by restricting excitation to a specific region around a selected resonance.

One of the most useful examples is the 1D version[320] of the widely applied 2D HOHAHA (HOmonuclear HArtmann HAhn) experiment (see below). A single isolated resonance belonging to a closed spin system is selectively inverted using a 180° selective shaped pulse. Inverted magnetisation is then allowed to propagate through the spin system *via* the HOHAHA coherence transfer process whilst maintaining spin lock with the MLEV-17[321] composite pulse cycle. A pure phase 1D ^1H subspectrum of the protons directly or indirectly scalar coupled to the inverted

resonance can then be generated (Figure 3.18). Signals from multiple quantum transitions are removed by phase cycling and those from zero quantum transitions by using a z-filtering technique[322]. We have found that these subspectra are extremely valuable for extracting coupling constant data from the excessively overlapped ring proton region of complex bacterial polysaccharides.

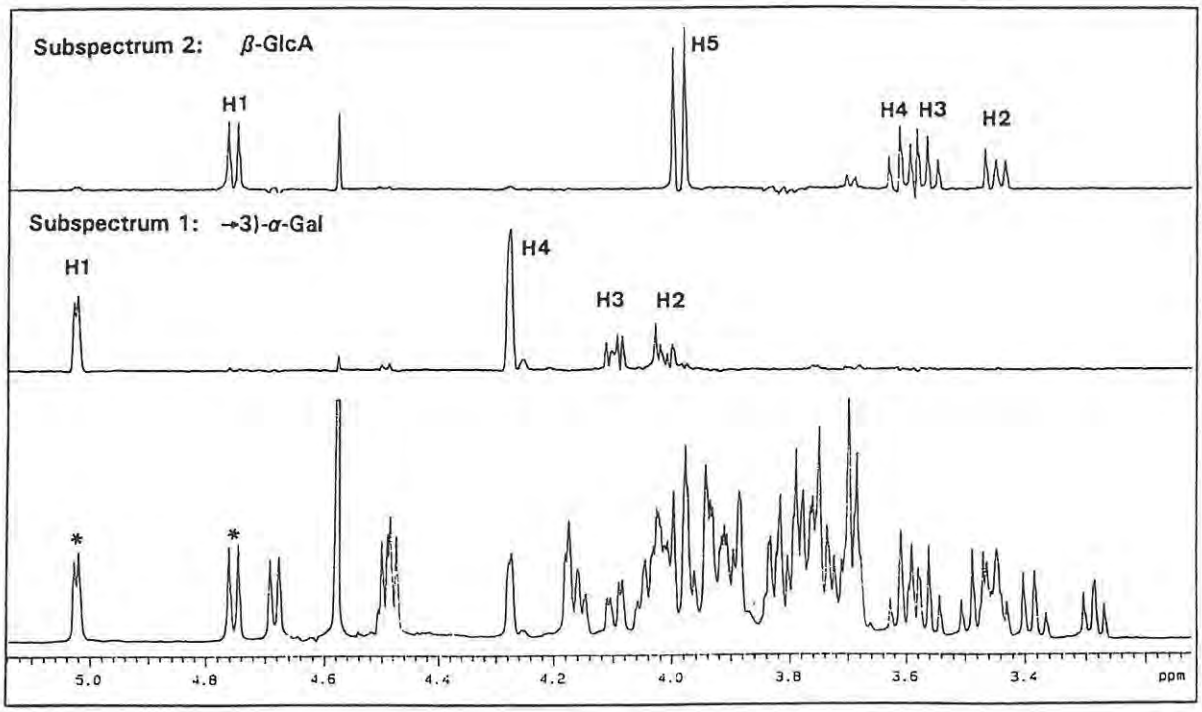


Figure 3.18 Subspectra from the 1D ^{1}H NMR spectrum of the reduced hexasaccharide (alditol) produced by bacteriophage degradation of the *Klebsiella* K15 polysaccharide. * Denotes well resolved resonances selected for inversion to produce sub-spectra 1 and 2.

Most of the other commonly employed 2D NMR experiments can be converted into 1D analogues by means of similar shaped or semi-selective pulse sequences. 1D versions of the ubiquitous COSY[323], NOESY[324] and ROESY[325] experiments have been devised. These employ semi-selective Gaussian[326], E-BURP ("Tophat")[327] or I-BURP[328] pulses to achieve selective excitation. The application of soft pulse techniques for solvent suppression has also been reported[327]. A shaped pulse directly on the solvent peak prior to the conventional hard pulse will effect nulling of the solvent peak if the two pulses are antiphase and of the same pulse angle.

(b) TWO-DIMENSIONAL TECHNIQUES

One dimensional spectra of polysaccharides are characterised by their complexity, due largely to overlap of the individual ring proton resonances between δ 3.0 and 4.0. Accordingly, if full assignment of the proton resonances of all constituent sugars is to be achieved, it is imperative that this "hidden-resonance" problem be surmounted. Limited improvement in resolution can be achieved by acquiring spectra at elevated temperatures and/or using stronger magnets with more homogeneous magnetic fields, however, due to the molecular origin of the problem, experimental rather than instrumental improvements are indicated. Several different approaches have been followed.

Chemical shifts can be spread through functional derivatisation of the hydroxyl groups (e.g esterification) or through the addition of paramagnetic (lanthanide) shift reagents[329], however, despite improvements in resolution, the resultant chemical perturbation of the polysaccharide is undesirable. Attempts to circumvent the problem using specialised one dimensional techniques, from the simple INDOR[330] (Internuclear Double-Resonance) technique through to sophisticated modern selective excitatory techniques such as the 1D HOHAHA experiment described above, have proved useful but are insufficient in most cases. The latter provides a means of generating simpler subspectra but requires well resolved resonances with sufficiently large coupling constants to enable adequate magnetisation transfer.

Two-dimensional NMR techniques, stemming from the pioneering work of *inter alia* Jeener[278], Aue and co-workers[105], and Ernst *et al.*[331], have proved to be the most successful means of overcoming the overlap problem. These techniques enable the location of obscured resonances *via* their cross peaks to coupled nuclei. Today a wide variety of two dimensional experiments are available, which allow elucidation of all but the most intractable spin systems.

In the archetypal 2D experiment free induction decays $S(t_2)$ are measured for a series of values of t_1 (the evolution period) to build up a matrix $S(t_1,t_2)$. Double Fourier transformation of this array gives a two dimensional matrix in the frequency domain $S(f_1,f_2)$.

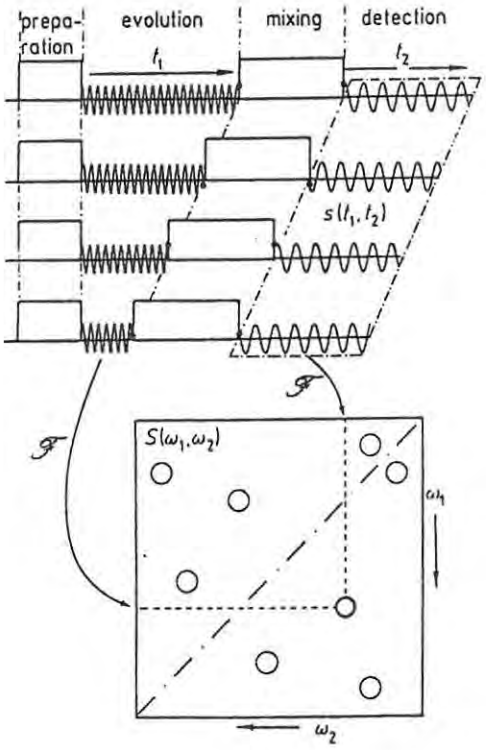


Figure 3.19 Schematic representation of the basic 2D NMR experiment.

In principle, two classes of 2D experiments are possible *viz.* *J*-resolved and correlated experiments. The former is characterised by one frequency axis (f_1) containing coupling information and the other (f_2) contains chemical shift data. In the second type both axes contain chemical shift information. *J*-resolved spectroscopy is seldom employed in carbohydrate structural analysis as it lacks sensitivity and it does not provide *J*-connectivity information[332], however, correlated spectra are extremely useful. Correlation between the two axes can be achieved through scalar coupling (homonuclear as well as heteronuclear), which involves coherent transfer of transverse magnetisation, or through dipolar coupling which is based on incoherent transfer of magnetisation. Those techniques most commonly utilised in structural studies on complex carbohydrates will be discussed briefly.

(i) Homonuclear Correlated Spectroscopy

Correlated Spectroscopy (COSY) - Jeener's original pulse sequence is epitomised in the ubiquitous COSY experiment. The basic pulse sequence is shown below:

$$90_{\phi 1} - t_1 - 90_{\phi 2} - \text{acquire}$$

An initial 90° pulse converts longitudinal magnetisation into transverse magnetisation (single quantum coherence). This is followed, prior to the detection period t_2, by a second 90° pulse which constitutes a brief mixing period during which single quantum coherences are mixed to form a range of coherences. During this crucial period, orders of coherence may be exchanged between different spins if they have a mutual scalar coupling. The net result, after Fourier transform, is the generation of a series of cross-peaks linking scalar coupled protons within the molecule under investigation. The total experimental time in the basic COSY pulse sequence is largely dependant on the number of increments in t_1 (which determines digital resolution in f_1). For most carbohydrates 256 increments are sufficient, however for crowded spectra 512 increments may be required to improve the resolution of partially overlapping cross-peaks. The form of the cross peaks is dependent on the flip angle of the second pulse. If this is reduced to 45° in order to reduce crowding on the diagonal, the cross-peaks show reduced intensity and develop asymmetry. Symmetrisation of the spectrum can be performed as a cosmetic step to improve the shape of cross peaks or to reduce the long "tails" from the solvent peak, however, it should be borne in mind that this represents non-linear manipulation of the data. The spectrum is usually presented in the form of an intensity contour plot which is a cross-section through the stacked plot parallel to the xy-plane at a given height.

An obvious limitation of the COSY experiment is that cross-peaks can only be observed if the coupling constants of the coupled nuclei involved are of a sufficient magnitude. For example, the connectivity trail for β-galactose residues can usually only be traced as far as H-4 as $J_{4,5}$ is less

than 1 Hz. Similarly, $J_{1,2}$ of mannose results in a very small cross peak between H-1 and H-2. This shortcoming, together with the common incidence of cross-peak overlap (particularly in molecules containing multiple spin systems e.g polysaccharides) and accidental degeneracy, prompted further development of the basic 2D homonuclear experiment.

Relayed Coherence Correlation Spectroscopy (RELAY-COSY) - The RELAY-COSY experiment, first introduced by Eich *et al.*[333] and later optimized by Bax and Drobny[334], permits transfer of coherent magnetisation to indirectly coupled protons *via* a mutually coupled neighbour. For example, a cross peak from H-2 to H-4 in a saccharide can be observed enabling ambiguity arising from overlap or degeneracy to be circumvented. This is made possible by introducing a second mixing pulse to enable a further stage of coherence transfer. The modified pulse sequence includes a 180° refocusing pulse as the efficiency of the second coherence transfer step is highly dependant on the choice of the fixed delay τ, and the nature of the spin system involved (size of the coupling constants).

$$90_{\phi 1} - t_1 - 90_{\phi 2} - \tau - 180_{\phi 3} - \tau - 90_{\phi 3} - \text{acquire}$$

The experiment can be extended to enable correlations to indirectly coupled protons further along the carbon skeleton by adding extra coherence transfer steps i.e. two- and three-step RELAY COSY. However, this is frequently unsuccessful as successive coherence transfer steps lead to significant signal decay, limiting the relay to two steps in most instances. Optimal signal relay once again depends on the size of the coupling constants involved.

Phase-Sensitive Correlation Spectroscopy (PS-COSY) - Remote connectivities can also be revealed by careful analysis of pure absorption, phase sensitive[335,336] cross-peaks since they reflect all the coupling information of the protons involved. Phase information, not obtainable from a conventionally acquired COSY, can be preserved in the cross-peak using TPPI phase cycling[337]. Both active (direct) coupling and passive (remote) coupling can be distinguished by analysis of the fine structure of the cross-peaks. Active coupling appears in anti-phase along both frequency axes whereas passive coupling gives rise to additional in-phase splitting of each of the anti-phase

components along the appropriate frequency axis[338]. Phase sensitive spectra of carbohydrates require at least 512 increments in t_1 to accommodate the additional phase information.

Quantum Filtered Correlation Spectroscopy (DQF- and TQF-COSY) - Improved visualisation of cross-peaks in spectra which lie close to the diagonal or in excessively crowded spectra can be obtained with the aid of quantum filtration, although there is some loss of sensitivity. Double quantum filtration[339] greatly simplifies spectra containing cross-peaks close to the diagonal as it preferentially suppresses single quantum coherence originating from the latter and from isolated spin systems such as the solvent peak and methyl groups. A COSY spectrum is generated in which the cross-peaks and the diagonal are in antiphase.

The assignment of chemical shifts to the H-5, H-6a, H-6b system in carbohydrates is frequently complicated by signal overlap. The employment of a triple quantum filter[340], which eliminates spin systems which contain less than three mutually coupled spins leads to simplified spectra which frequently assist in the assignment of the latter. This is illustrated for a ß-Glc residue below.

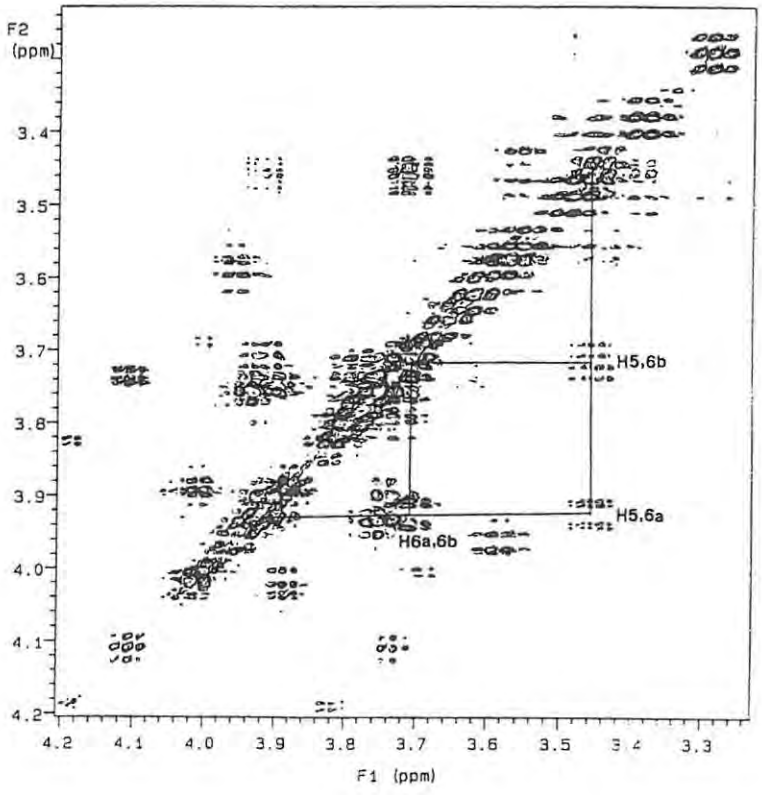


Figure 3.20 TQF COSY spectrum of *Klebsiella* K15 (P1-OL). The connectivity pattern of the geminally coupled protons for a ß-Glc residue are shown.

Homonuclear Hartmann Hahn (HOHAHA) Spectroscopy - This technique overcomes the problem of signal decay which plagues conventional relayed coherence transfer spectroscopy and is based on the principle of polarisation transfer first introduced by Hartmann and Hahn[341]. The net magnetisation transfer that occurs permits the acquisition of a phase sensitive spectrum and hence improved resolution and sensitivity is achieved. In this experiment, developed by Davis and Bax[342], magnetisation is transmitted throughout a scalar coupled proton spin system whilst the system is under the influence of a spin locking pulse. However, if one of the couplings is small it will once again block the flow of HOHAHA magnetisation. Only in the case of sugars having the *gluco* configuration where all couplings are of a similar order of magnitude can magnetisation be transferred from H-1 to the rapidly relaxing H-6 protons. Inagaki *et al.*[343], have recently described a RELAYED variation of the HOHAHA experiment which includes an additional conventional relay step in an attempt to overcome this limitation. They report full correlation from H-1 to H-5 for both *α*- and *β-galacto* residues.

The TOCSY (TOtal Correlation SpectroscopY) experiment is closely related to the HOHAHA experiment. Multistep correlations under conditions of isotropic mixing are obtained in the absence of a phase alternated spin-lock field by eliminating the Zeeman term from the Hamiltonian[344] but without removing spin-spin couplings. In most instances the 2D spectrum that results is identical to that obtained under spin-lock and the two are frequently confused in the literature.

2D Nuclear Overhauser Effect Spectroscopy (NOESY) - The correlations described thus far are based on through-bond connectivity which arises as a consequence of coherent magnetisation transfer between scalar coupled protons. NOE spectroscopy is based on incoherent magnetisation transfer and dipolar coupling which arises as a consequence of the proximity of protons to each other. Through space coupling is therefore dependant on internuclear distances and is inversely proportional to the sixth power of interproton distance, with the cut-off being in the vicinity of 3 Å. The length of the mixing time in the pulse sequence is therefore very important as the presence or absence of a correlation peak will depend on whether there is sufficient time for NOE build up

to occur. The pulse sequence for the NOESY experiment is similar to the COSY pulse sequence, except it detects changes in *z*-magnetisation during τ, which leads to the detection of signals during t_2 which have f_1 modulation frequencies different from their larmor frequencies and hence the formation of cross-peaks in the transformed spectrum[345].

$$90_{\phi 1} - t_1 - 90_{\phi 2} - \tau - 90_0 - \text{acquire}$$

NOE spectroscopy has proved to be immensely useful for structural studies on poly- and oligo-saccharides for a number of reasons: It provides complementary information on assignments made on the basis of through-bond analysis as *intra-molecular* NOE cross-peaks are usually observable for diaxial and equatorial-axial protons. It is also a means of assigning resonances which show weak or no scalar coupling e.g. a 1,5-diaxial correlation enables location of the H-5 resonance in *β-galacto* sugars as an H-4/H-5 cross peak is normally absent in the COSY due to the small $J_{4,5}$ coupling constant. Since, by definition, *α* and *β* sugars differ in the axial or equatorial configuration at C-1, the anomeric configuration of a sugar residue can be easily assigned from a 2D NOESY spectrum. However, the most useful benefits lie in the fact that *inter-residue* NOEs are observable between protons on adjacent sugars, providing a means of establishing the sequencing of sugar residues in oligo- and poly-saccharides. In certain cases correlations to more remote protons can be detected providing valuable information on solution conformations.

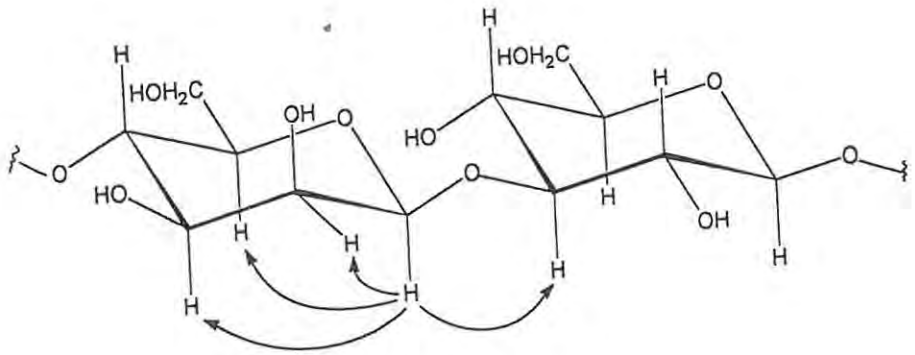


Figure 3.21 Expected inter- and intra-residue NOE cross-peaks for the disaccharide →4)-ß-Man*p*-(1→3)-ß-Glc*p*-(1→

The magnitude of a NOE peak also depends on the rotational correlation time of the molecule which in turn is dependent on the size of the molecule and the viscosity of the medium[346]. A maximum enhancement of 50% is possible for small molecules ranging through to -100% at the other extreme (large molecules) e.g. polysaccharides. Consequently, the conventional NOESY sequence may fail for rapidly tumbling molecules of intermediate size such as small oligosaccharides (MW ~ 1500), as NOEs may be small or non-existent. This limitation can be overcome by performing the NOE experiment in the rotating frame (ROESY)[347,348]. This is achieved by applying a spin-locking pulse (as for the TOCSY experiment above) to enable measurement of transient NOEs.

Both NOESY and ROESY spectra are frequently complicated by the presence of COSY or HOHAHA cross-peaks arising from coherent magnetisation transfer (zero quantum coherence). This can be reduced by including an additional 180° pulse during the delay τ in the conventional NOESY pulse sequence[346], by recording the spectrum in the phase sensitive mode[349] or simply by reducing the mixing time to approximately 200 ms to reduce spin diffusion (most 2D NOESY spectra are acquired at ca. 300 ms.). HOHAHA cross-peaks can be eliminated in ROESY spectra by setting the offset frequency at the low end of the spectrum.

(ii) Heteronuclear Correlated Spectroscopy

Chemical shift correlation between protons and other coupled nuclei of spin quantum number ± ½ is also readily achievable and represents a powerful means of overcoming many of the problems common to the homonuclear correlated experiments discussed above. Experiments correlating ^{1}H and ^{13}C chemical shifts are typical in the case of most organic molecules, although versions correlating protons to nuclei such as ^{14}N and ^{31}P are not uncommon. The principal advantage of a ^{1}H-^{13}C correlated spectrum is that it combines the excellent resolving power of the decoupled ^{13}C spectrum of a molecule with its more easily interpreted, though frequently overlapped, ^{1}H chemical shifts, thus greatly facilitating chemical shift assignment. There are two methods for acquiring such spectra, both of which are extensively used in structural studies on complex carbohydrates.

^{13}C-Detected Heteronuclear Correlation Spectroscopy (HETCOR) - In this classic version of the experiment, magnetisation originating as *proton* spin coherence, is transferred to ^{13}C nuclei and detected as a ^{1}H-decoupled ^{13}C free induction decay. The FID is modulated as a function of t_1 by the directly coupled ^{1}H chemical shift frequencies[350] resulting in a 2D spectrum containing ^{13}C-^{1}H correlations with the f_1 axis displaying proton chemical shifts and the f_2 axis ^{13}C chemical shift information. As a consequence of the low natural abundance of ^{13}C nuclei (1.1%), the experiment lacks sensitivity and requires larger sample sizes (preferably >15 mg) and long experimental acquisition times. Despite this drawback, the HETCOR experiment provides a means of identifying proton chemical shifts impossible to assign from homonuclear correlated spectra due to overlap or coupling constant constraints. A variation of the HETCOR experiment utilising long-range coupling (COLOC[351]) has been used to determine sugar sequences *via* heteronuclear correlations across glycosidic linkages. However, the time penalty is severe and several days may be required to successfully acquire a spectrum. For this reason proton detected heteronuclear correlated spectra have largely replaced the HETCOR experiment.

^{1}H-Detected Heteronuclear Correlation Spectroscopy - This approach achieves heteronuclear chemical shift correlation *via* proton detection of heteronuclear *multiple* quantum coherence (HMQC), thus overcoming the sensitivity constraints of the ^{13}C detected analogue. Although this approach was pioneered by Müller[352] prior to the development of the conventional experiment based on single quantum coherence described above, the technical difficulties associated with HMQC prompted the initial development of the former technique. The HMQC experiment, and related pulse sequences, are founded on the creation of heteronuclear multiple quantum coherence followed by reconversion of magnetisation to single quantum coherence for the purposes of detection[363]. A major difficulty associated with this approach is that strong signals arising from ^{1}H-^{12}C couplings need to be suppressed. For small molecules, such as oligosaccharides, this can be achieved by applying a bilinear rotational (BIRD) pulse[364] prior to the generation of multiple quantum coherence which selectively inverts the magnetisation of all protons not coupled to ^{13}C nuclei. Bax *et al.*[365] have proposed the insertion of an additional delay after the BIRD pulse optimised with

respect to proton T_1 relaxation times so that ^1H-^{12}C signals are strongly attenuated when pulsing begins. Residual signals not eliminated by this saturation process are removed by phase cycling. An HMQC spectrum acquired without ^{13}C decoupling (HMQC-ND)[366] has recently been proposed for the purposes of obtaining $J_{C\text{-}1,H\text{-}1}$ coupling constants. In studies on the *E. coli* O1A polysaccharide the authors demonstrate that this approach overcomes sensitivity and signal overlap problems which limit the use of ^1H-coupled ^{13}C or *J*-resolved spectra.

In those instances where chemical shifts cannot be unambiguously assigned from an HMQC spectrum, a 2D HMQC-TOCSY (HOHAHA) experiment may be helpful. This variation, described by Lerner and Bax[367] and extensively applied in this laboratory, includes an MLEV-17 isotropic mixing period after restoration of single quantum coherence to permit further propagation of magnetisation. A similar pulse sequence, HMQC-RELAY, reported by Brühwiler and Wagner[368], incorporates a relayed coherence transfer step instead of the MLEV-17 mixing interval. Related hybrid techniques which are sometimes used include the HMQC-COSY[359], HMQC-NOESY[360], and DEPT-HMQC[361] experiments.

The low sensitivity and long acquisition times which severely limit the use of long range ^{13}C-detected heteronuclear experiments, are no longer a liability in the case of their proton detected equivalent, the HMBC (heteronuclear multiple bond correlation) experiment[159]. The pulse sequence for the latter does not contain a BIRD pulse to effect nulling of ^1H-^{12}C signals but relies instead on extensive phase cycling. High spectrometer stability is therefore a prerequisite for the successful application of this experiment. Its ability to reveal long-range connectivities across glycosidic linkages has established it, together with the NOESY experiment, as an important spectroscopic technique for sequencing oligo- and poly-saccharides. Martin and Zektzer have published a detailed review[362] on ^{13}C and ^1H-detected long-range heteronuclear chemical shift correlation techniques.

(iii) Nuclei other than ^{1}H and ^{13}C

The vast majority of NMR experiments employed in structural studies on complex carbohydrates and other natural products involve observation of either ^{1}H or ^{13}C nuclei. However, many of the techniques described above can be extended to other nuclei both non-metallic (e.g ^{31}P, ^{14}N, ^{33}S, ^{17}O and ^{18}F) and metallic (e.g ^{27}Al, ^{25}Mg and ^{195}Pt)[283]

^{31}P-NMR is of particular relevance to the analysis of bacterial polysaccharides as a large number (including several *E. coli* capsules) contain phosphate, usually present as a diester. ^{31}P NMR has been utilised in studies on the *E. coli* K51[61] and K24[49] capsular antigens which contain phosphorus linked as a phosphodiester and glycerol phosphate respectively. Following confirmation of the presence of phosphorus from ^{31}P spectra, phosphate linkage positions were established in both instances from the observed two- and three-bond ^{31}P-^{13}C coupling. Inverse detected ^{1}H-^{31}P HMQC-TOCSY experiments have been used to determine the site(s) of phosphate substitution *via* long range coupling, for example in the capsular polysaccharides produced by *Actinobacillus pleuropneumonia* serotype 9[363] and *Haemophilus influenza* type a[364].

^{15}N NMR is also occasionally used e.g. Hermansson *et al.*[365] found a ^{15}N-^{1}H HMQC experiment useful for the structural elucidation of the *Fusobacterium necrophorum* polysaccharide.

(iv) Recent Advances in NMR Spectroscopy

Such is the power and versatility of NMR spectroscopy that new advances and applications continue to be reported on a regular basis. Certain developments relevant to carbohydrate structural analysis merit some discussion.

Advanced two-dimensional spectra are often insufficient for the complete elucidation of complex biomolecules, such as polysaccharides and proteins, if severe resonance overlap or degeneracy

exists. In such cases three dimensional (3D) NMR can be extremely useful. Instead of a single mixing period in which only two frequency variables are related, two sequential mixing periods enable correlation *via* a third frequency axis. A large number of such experiments are therefore conceivable using this approach. A 3D COSY-NOESY[366] spectrum, for example, would therefore permit the entire structural assignment of an oligosaccharide from a single data set. 3D experiments are also extremely useful for conformational studies. De Waard *et al.*[367] applied both 2D and 3D NMR in a conformational study of a diantennary oligosaccharide. They employed 3D NOE-HOHAHA and 3D HMQC-NOE experiments to extract NOEs which were hidden in conventional 2D NOESY spectra. These techniques have been accompanied by advances in integrated software designed to facilitate spectral analysis. Computer aided spectral assignment *via* sophisticated graphics programs now enables detailed multidimensional analysis impossible to achieve on paper.

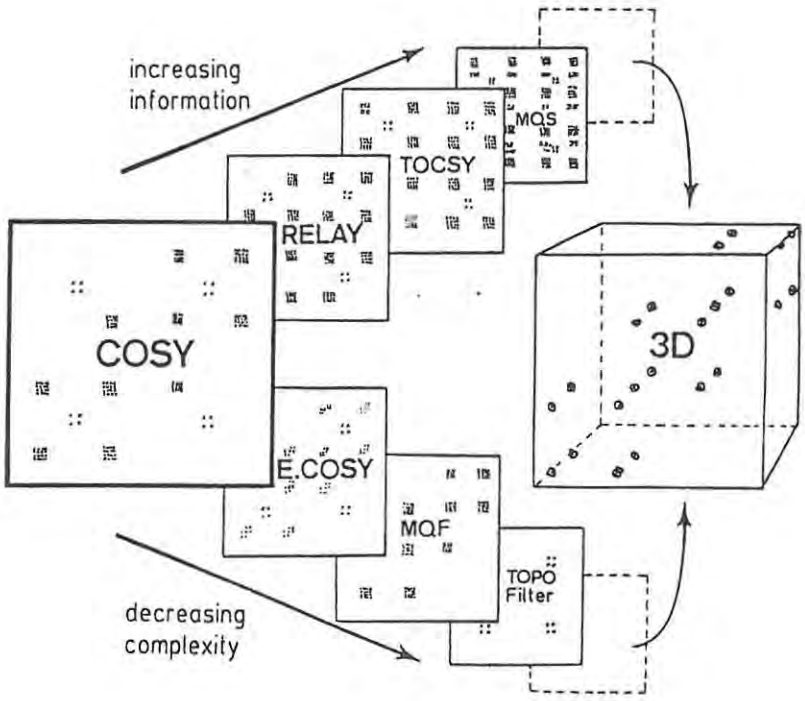


Figure 3.22 Extensions of the COSY experiment logically point toward 3D NMR spectroscopy.

A new development of particular relevance to the field of bacterial polysaccharide research has been the introduction of computerised methods for the structural elucidation of polysaccharides composed of regular repeating units. The first program, developed by Jansson *et al.*[368] and known by the acronym CASPER (Computer Assisted Spectrum Evaluation of Regular Polysaccharides) utilises the ^{13}C NMR spectrum of the polysaccharide as well as information from sugar and methylation analyses to assist in structural analysis. An improved version of this program[368] soon followed with an expanded database capable of assigning structures from the ^{1}H and ^{13}C chemical shift data or the completely unassigned HETCOR spectrum. Baumann *et al.*[370] subsequently demonstrated its utility by successfully elucidating the structure of the *E. coli O1A* polysaccharide and Hermansson and co-workers[371] have used it to study oligosaccharides from human milk. The second program, introduced at approximately the same time as CASPER, was developed by Lipkind *et al.*[372]. This version uses only ^{13}C data and has been successfully employed in structural studies on several branched polysaccharides[372]. A third program was recently developed and reported by Van Kuik *et al*[373]. The latter comprises a ^{1}H NMR database comprised of carbohydrate structures, ^{1}H NMR data, and literature references and generates possible structures following input of ^{1}H chemical shift values. These programs appear to be the forerunners of more sophisticated databases which are likely to revolutionise complex carbohydrate structural analysis.

One of the major limitations of two dimensional NMR experiments, whether homonuclear or heteronuclear, is the time required for data acquisition. Two dimensional spectra of polysaccharides invariably require acquisition times of at least 12 hours to produce adequately resolved spectra. A revolutionary new approach known as *Gradient-Accelerated Spectroscopy (GAS)*[374] has recently been introduced.

A pulsed field *gradient* is introduced in episodes into the 2D sequence which enables ω_1 quadrature detection[281] in a single acquisition with excellent suppression of unwanted coherences e.g. solvent signals. This results in the acquisition of well resolved spectra with a time saving of up to a factor of 20 compared to conventional methods. For example the COSY spectrum of a polysaccharide which would normally require an acquisition time of approximately 12 hours can now be acquired in 30 minutes.

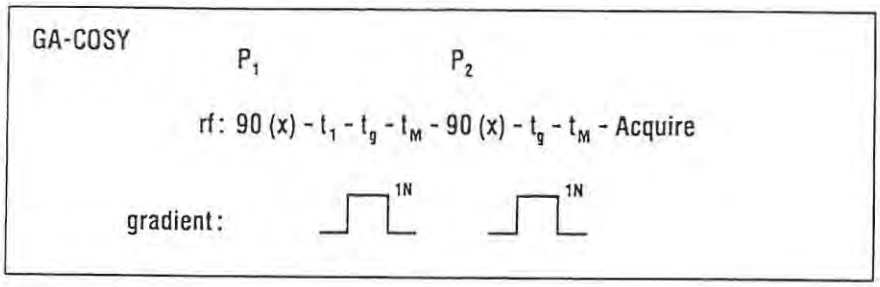


Figure 3.23 Pulse sequence for a 2D gradient-accelerated COSY.

Gradient accelerated versions of COSY (Figure 3.23), DQF-COSY, INADEQUATE, and HMQC are described[374]. However, several modifications are required to both hardware and software to maintain spectral resolution. The gradient pulse must be shaped to eliminate the effects of induced eddy currents (gradient pre-emphasis), induced B_0 shifts must be corrected by means of a decaying DC current through the field setting coil of the shim system, and gradient trimming is required to ensure that precise ratios of gradient strengths are used.

3.8 MASS SPECTROMETRY

Mass spectrometry (MS) is an important and versatile instrumental technique in carbohydrate structural analysis and its applications in this field are well documented[375-378]. It involves the ionisation of a molecule with a stream of high velocity particles (usually electrons) to form a molecular ion which subsequently fragments to smaller charged species. This is followed by separation and differentiation of these fragments *via* an electric or magnetic field based on their mass to charge ratios. For most carbohydrates fragmentation occurs in a definite, often predictable, fashion providing valuable structural information on the starting compound. The most common approach, electron impact mass spectrometry (EI-MS), involves bombardment of the sample with electrons to produce spectra dominated by small, stable fragment-ions. However, the need to produce fragments of higher molecular weight has lead to the emergence of related techniques which achieve "softer" ionisation by means of condensed phases or matrices e.g. chemical ionisation (CI-MS) and fast atom bombardment mass spectrometry (FAB-MS). These techniques impart less energy to the substrate resulting in larger fragments suitable for molecular weight determination and which, in the case of oligosaccharides and small polysaccharides, may provide information on the sequencing of constituent residues or enable the location of labile substituents.

The principle advantage of the mass spectrometric technique lies in the fact that a great deal of structural information can be obtained from small quantities of material which would be impossible to analyse by NMR spectroscopy. However, it is a destructive technique and, for carbohydrates, requires chemical derivatisation of the sample to improve volatility and thermal stability.

(i) Electron-Impact Mass Spectrometry (EI-MS)

The ease with which a gas-liquid chromatographic system can be linked to a mass spectrometer has made EI-MS a useful complementary technique for the analysis and identification of monosaccharides. Volatile derivatives prepared for glycose or methylation studies can be directly introduced into the mass spectrometer following separation on a GLC column. Distinctive fragmentation patterns enable the analyst to readily distinguish, for example, between (1) pyranosidically and furanosidically linked sugars, (2) aldoses and ketoses and (3) between hexose and pentose sugars. Furthermore, methyl and methylene functions signifying deoxy sugars and substituents such as acetyl groups can also be easily detected. Volatile derivatives, as previously discussed in section 3.1, may be cyclic e.g. methylated or trimethylsilylated methyl glycosides, or acyclic such as alditol acetates. The fragmentation of methylated methyl glycosides, initiated by ionisation at the ring oxygen, is proposed[378] to proceed *via* several possible pathways depending on the positions of substitution on the ring. These pathways have been further classified on the basis of where ring cleavage occurs *viz.* cleavage of the C1-C2 bond generates the C,D,F,H and K series, C4-C5 cleavage the J,H,B and D series and cleavage of the C1-exocyclic oxygen bond the A-series[380]. However, direct derivatisation to form the glycoside generates a mixture of compounds due to anomerisation so it is generally advantageous to reduce the ring to an alditol.

Acyclic derivatives produce greatly simplified mass spectra and the reduction step presents an opportunity to include an isotopic label e.g. deuterium by reducing with a deuterated agent such as $NaBD_4$. Partially methylated alditol acetates (PMAAs) are the derivatives of choice for methylation studies and analysis by EI-MS is a valuable supplementary step to GLC as the two techniques are frequently directly complementary to one another. For example MS can readily detect the presence of identically co-eluting compounds and GLC can distinguish between similarly linked stereoisomeric sugars, such as galactose and glucose, which would give practically identical mass spectra.

The fragmentation pathways of PMAAs have been well studied[381,382] and the substitution pattern can readily be determined from the resultant primary and secondary fragments. A molecular ion is rarely evident (though molecular mass can be inferred by the addition of pairs of fragments). Cleavage occurs most readily between adjacent methoxyl functions, followed by cleavage between acetylated and methoxylated carbons with cleavage least likely to occur between two acetylated carbon atoms. Secondary fragments are formed by the loss of acetic acid (60 m.u.) and methanol (32 m.u) *via* β-elimination, and ketene (42 m.u.) or formaldehyde (30 m.u.) *via* α-elimination.

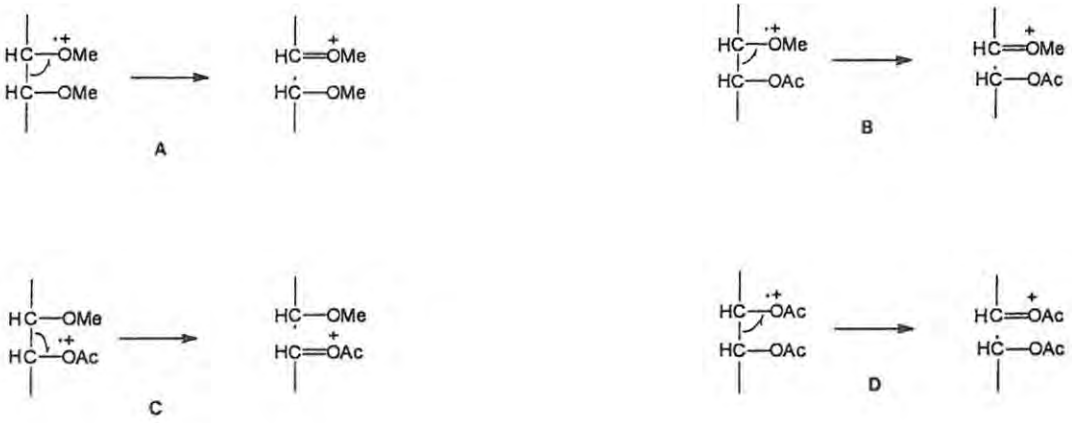


Figure 3.24 Possible fragmentation patterns for PMAA derivatives.

Fragmentation pathways for amino sugars[383], uronic acids[384], and sialic acids[385,386] have also been reported as well as those for less frequently used derivatives of the neutral hexoses such as partially methylated aldononitrile acetates[387], alditol acetates[388], alditol trifluoroacetates[389], dithioacetals[390], peracetylated oxime derivatives[391] and partially ethylated alditol acetates[392].

EI-MS is not considered to be the ideal technique for studies on oligosaccharides as they frequently undergo excessive fragmentation to produce complex spectra, even when the energy of impacting electrons is reduced (e.g. to < 20eV).

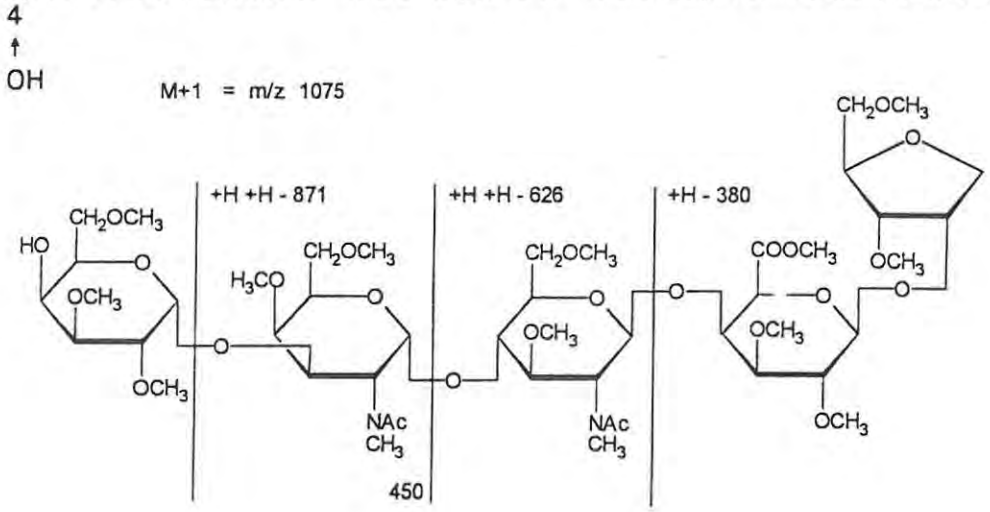
However, the fragmentation of smaller oligosaccharides (usually di- or tri-saccharides) has been studied[393-395]. Permethylated oligosaccharide alditols introduced directly or from a gas-liquid or high performance liquid chromatograph are the derivatives of choice. Their mass spectra predictably contain characteristic fragments arising from the acyclic sugar and the glycosidically linked sugar(s) and the sequence of the constituent sugars can be established if they have different molecular masses. EI-MS spectra of oligosaccharides larger than tetrasaccharides are generally too complex and softer techniques such as CI-MS are preferred.

(ii) Chemical Ionisation Mass Spectrometry (CI-MS)

CI-MS achieves softer and more controlled ionisation through a process involving low energy ion-molecule reactions. A dilute gaseous solution ($\approx$ 0.1%) of the sample in a reactant gas is introduced into the spectrometer and subjected to electron impact. Due to the low concentrations of sample, the reagent gas is ionised to a far greater extent and the primary ions thus formed undergo a series of secondary deactivating collisions which result in the ionisation of the sample molecule. This may occur *via* charge or proton transfer, or the formation of a low energy charged adduct with the neutral sample molecule. These charged species may then undergo further fragmentation to produce mass spectra containing relatively few ions, generally of higher molecular weight and, most notably, a pseudo-molecular ion, formed simply by capture of an ionic species, which provides the molecular mass of the compound. The reagent gas most commonly employed for studies involving carbohydrates is ammonia, followed by isobutane and methane which produce the ionic species NH_4+, C_4H_9+ and CH_5+ respectively. In certain instances mixtures of reagent gases have also been used, usually a combination of isobutane and ammonia. Methane and isobutane spectra of carbohydrates usually contain more fragment ions than those generated using ammonia. Greater fragmentation can also be induced by raising the temperature and repeller voltage within the source[396].

CI-MS has been widely applied in carbohydrate structural analysis[397-399] and the technique is frequently used in studies on bacterial polysaccharides. For example CI-MS or a combination of EI- and CI-MS were key techniques in the structural analysis of the capsular polysaccharides produced by *E. coli* serotypes K19[400], K26[183], K37 and K38[228], K40[61], and K95[401].

ß-D-Gal*p*-(1→3)-ß-D-Gal*p*NAc-(1→4)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→2)-anhydroribitol [PM]



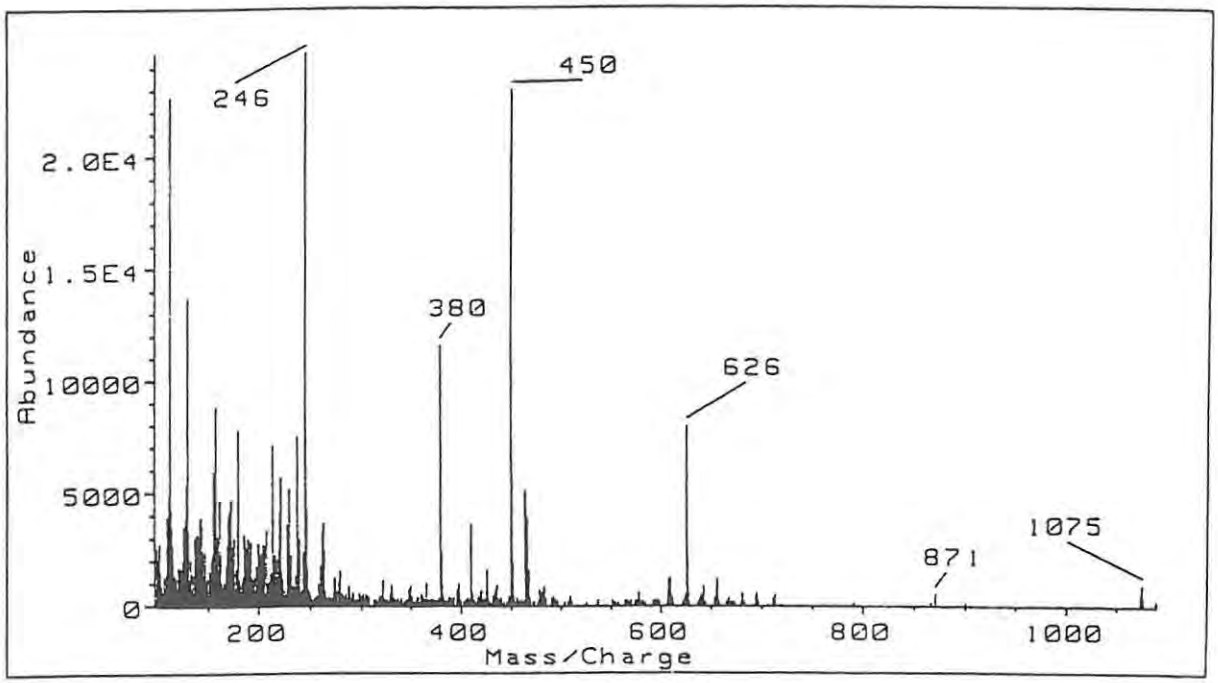


Figure 3.25 CI-MS spectrum of the anhydroribitol terminated pentasaccharide anhydroalditol product obtained following SRC of the *E. coli* K38 polysaccharide.

The tendency for larger, more polar molecules of low volatility to undergo undesirable pyrolysis prompted the development of a modified CI-technique known as direct chemical ionisation mass spectrometry (DCI-MS). Pyrolysis is reduced by placement of the sample directly within the ionisation chamber on a probe from which heat induced desorption occurs directly into the ion plasma[402] instead of subjecting the material to lengthy retention on a column at elevated temperatures. DCI-MS, using ammonia as the reagent gas, has the added advantage that it preferentially cleaves glycosidic linkages in larger oligosaccharides to produce high molecular weight fragments eminently suitable for sequencing purposes. This approach was successfully employed in studies on a tetrasaccharide isolated by means of phage degradation of the *E. coli* K44[130] polysaccharide. Notwithstanding the advantages direct injection confers, larger oligosaccharides containing more than five sugars still require high probe temperatures to achieve volatilisation which may result in some degree of pyrolysis or the loss of labile substituents. Fast atom bombardment mass spectrometry (FAB-MS), which does not employ heating, is the method of choice for extending the mass range of oligosaccharides accessible to mass spectrometric analysis.

(iii) Fast atom bombardment mass spectrometry (FAB-MS)

This method, as the name implies, involves bombardment of the sample with a stream of atoms or ions (usually argon or xenon) rather than electrons. The sample is dissolved in a viscous liquid (called the matrix) and coated onto a small metal target. The stream of atoms achieves ionisation of the sample by the addition of a proton or cation (detected in the +ve ion mode) or the loss of a proton (-ve ion mode) and the resultant quasi-molecular ions fragment further and are sputtered out of the matrix and into the high vacuum source of the spectrometer[403]. High molecular weight (up to 4000 m.u.), polar, non-volatile or thermally labile compounds can therefore be analysed successfully without the need for volatilisation. FAB spectra contain peaks for every mass within the desired range, generated by sample fragmentation (major peaks) and low frequency side

reactions (minor peaks) involving degradation and ionisation of the matrix and other small molecular fragments. Sample peaks characteristically appear as clusters due to isotopic mass differences e.g. the 1.1% abundance of ^{13}C and the presence of less intense by-products generated together with the major ion. The most widely used matrix is glycerol, however thioglycerol or a 1:1 mixture of glycerol / thioglycerol is sometimes more effective for hydrophobic samples e.g. permethylated and peracetylated derivatives. In certain cases matrix additives, such as 0.1 M hydrochloric acid, which increases $(M+H)^+$ abundance, and salts such as sodium acetate and ammonium thiocyanate[404] may be used to simplify the appearance of FAB spectra. Cationic salts stabilise the molecular species formed as adduct ions i.e. as $(M+Na)^+$ and $(M+NH_4)+$ ions respectively.

The fragmentation of oligosaccharides frequently involves cleavage at the glycosidic linkages, thus if the oligosaccharide contains sugar constituents of different molecular masses such as hexosyl, deoxy-hexosyl or amino sugars, it provides a means of confirming the molecular composition of the sample and is a valuable source of sequencing information. Furthermore supplementary structural information can be obtained from smaller molecular fragments. As is the case with other mass spectrometric techniques, the method cannot generally distinguish between epimers and the anomeric nature of the linkages, and is not useful for determining linkage positions.

FAB-MS has been widely applied in the analysis of carbohydrates and their conjugates, notably by Dell and co-workers[405-407]. It has been used to determine the composition and sequences of bacterial polysaccharides[227,228,408,409] as well as to locate labile substituents (e.g. *O*-acetyl groups)[163,164]. Most carbohydrate containing samples are derivatised, usually by acetylation or methylation prior to analysis. This protocol stemmed from studies on the *mycobacterial O*-methyl-D-glucose (MGP) polysaccharide[410] which displayed enhanced desorptive properties. Methylation was shown to reduce H-bonding interactions within the sample solution thereby promoting desorption and fragmentation. The broad applicability of this technique is demonstrated in its successful application in numerous structural studies on other carbohydrate containing materials isolated from *inter alia* glycolipids[411], glycoproteins[412], and gums[413].

In certain instances it may be advantageous to couple a liquid chromatographic (HPLC) system directly to a FAB mass spectrometer. This is advantageous as temperature or base sensitive biological samples which cannot be derivatised frequently require chromatographic separation prior to MS analysis, for example mixtures of oligosaccharides containing labile substituents. However, the interface between the two machines is far more problematic than is the case for GLC-MS. Introduction of the sample is usually engineered using a thermospray process. A fine jet of the HPLC column effluent is split off and sprayed directly into the atom stream. Peralkylated sugars give reasonably good responses, however for underivatised carbohydrate samples sensitivity is generally low and the spectra show a high degree of secondary fragmentation and a low abundance of molecular ion peaks[414]. Santikarn *et al.*[415] have recently reported enhanced sensitivity and a decrease in fragmentation using a polyimide "moving belt" interface for the introduction of eluting sample into the atom stream.

Finally, a relatively new mass spectrometric method, laser desorption ionisation Fourier transform ion cyclotron resonance mass spectrometry[416] (LDI-FT-ICR-MS), has been shown by Lam *et al.*[417,418] to be a particularly valuable hybrid technique for the analysis of bacterial polysaccharides. In addition to providing sequencing information, it is also possible to identify linkage positions from smaller fragment-ions, and in the negative ion mode to distinguish the anomeric nature of the sugar linkages.

STRUCTURAL STUDIES ON SOME ENTEROBACTERIAL CAPSULAR ANTIGENS

4.1 RE-INVESTIGATION OF THE CAPSULAR POLYSACCHARIDE PRODUCED BY *Klebsiella* K15 USING BACTERIOPHAGE DEGRADATION AND HIGH RESOLUTION NMR SPECTROSCOPY

ABSTRACT

The structure of the capsular polysaccharide produced by *Klebsiella* K15 has been re-investigated, principally by 1D and 2D ^{1}H- and ^{13}C-NMR spectroscopic studies of the borohydride reduced hexasaccharide (alditol) obtained *via* bacteriophage-mediated enzymatic degradation of the polysaccharide. The polysaccharide was shown to be comprised of the following repeating units:

ß-D-Glc*p*
1
↓
6
→4)-*β*-D-Gal*p*-(1→3)-*α*-D-Gal*p*-(1→6)-ß-D-Gal*p*-(1→3)-ß-D-Gal*p*-(1→
3
↑
1
ß-D-Glc*p*A

INTRODUCTION

Klebsiella K15 formed part of a programme in our laboratory to screen for the antigenically important[2], yet often unreported presence of *O*-acetyl groups on capsular polysaccharides. This included bacterial polysaccharides whose structures had been established principally by chemical, rather than spectroscopic means, as these methods often inadvertently remove labile substituents such as *O*-acetyl groups. Preliminary NMR studies in this regard[227] on the K15 polysaccharide and an oligosaccharide, obtained through bacteriophage degradation of the former, showed that the polysaccharide was only acetylated to a very small extent (~15%). However, it soon became apparent that the NMR spectra did not accord with the structure **A** reported[419] for the repeating unit and prompted a complete structural reinvestigation. Selective depolymerisation of bacterial capsular polysaccharides with a linkage-specific bacteriophage-borne enzyme is a particularly useful technique as it can afford an oligosaccharide analogous to the repeating unit which is eminently suitable for NMR analysis, and it is also a mild enough procedure to preserve any labile substituents.

β-D-Glc*p*-(1→4)-α-D-Glc*p*A
1
↓
2
→4)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→

A

RESULTS AND DISCUSSION

Composition and 1D NMR spectra of the polysaccharide — *Klebsiella* K15 bacteria were grown on sucrose-rich agar and the acidic capsular polysaccharide (**PS**) was isolated using cetyltrimethylammonium bromide (CTAB) and further purified by GPC. GLC analysis of the derived peracetylated aldononitriles with and without prior reduction of the uronic acid confirmed the presence of GlcA, Glc, and Gal in the molar ratio 1:1:4. The D-configuration for all residues was established by GLC analysis of the derived acetylated (-)-2-octylglycosides.

The ^{1}H NMR spectrum of **PS** (at 340K) contained an anomeric signal at δ 5.02 ($^3J_{1,2}$ = 3.8 Hz) for an α-linkage, anomeric signals at δ 4.82 ($^3J_{1,2}$ = 7.9 Hz) and 4.48 ($^3J_{1,2}$ = 7.8 Hz) for ß-linked sugars, and a complex of overlapping signals at ~ δ 4.7 integrating for 3 protons, consistent with a hexasaccharide repeating unit. In addition, a small signal at δ 2.15 for the methyl protons of an *O*-acetyl group indicated **PS** was ~ 15% *O*-acetylated.

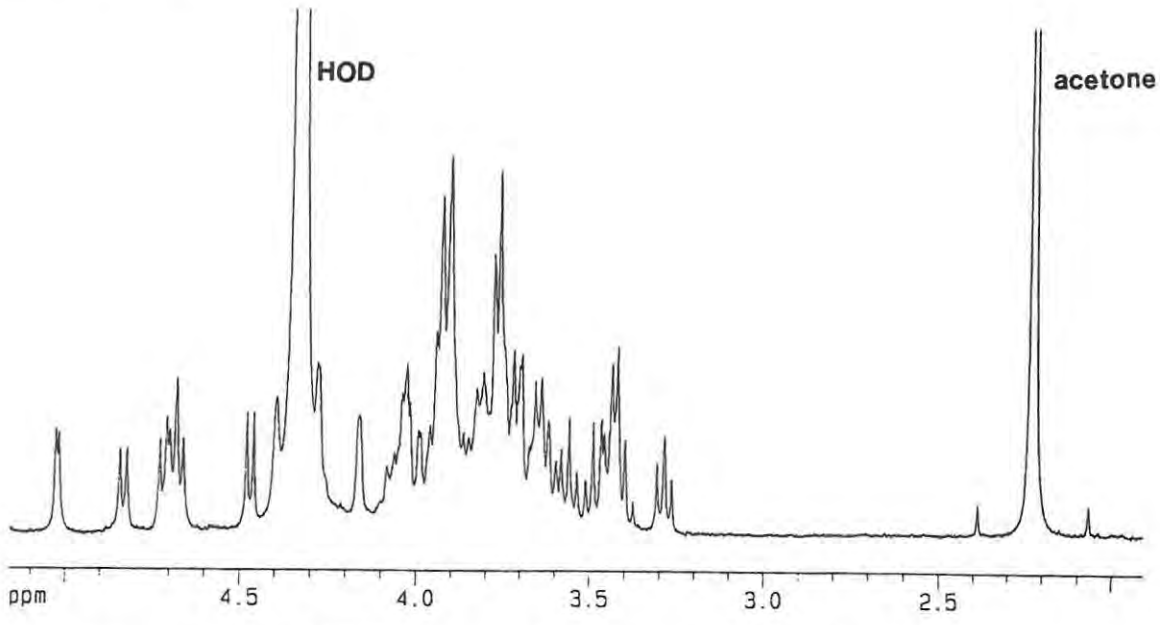


Figure 4.1 400 MHz ^{1}H-NMR spectrum of *O*-deacetylated **PS** at 340K.

Enzymatic studies — A bacteriophage was isolated from Grahamstown sewage water and propagated on *Klebsiella* K15 bacteria by means of serial tube and flask lyses until a solution containing 1.3×10^{13} PFU was obtained. **PS** was incubated in this solution for four days followed by isolation of the products of bacteriophage-mediated depolymerisation by dialysis and GPC. The principal product, a reducing hexasaccharide analogous to the repeating oligosaccharide (**P1**), was isolated in high yield. No fragment containing an *O*-acetyl group was isolated. Comparison of the ^{1}H-NMR spectra of **PS** and **P1** clearly established that a β-linkage had been cleaved by the enzyme. Furthermore, considerable amounts of glucose were obtained after incubation of both **PS** and **P1** with β-glucosidase. This indicated that β-glucose was present in a terminal position in both compounds.

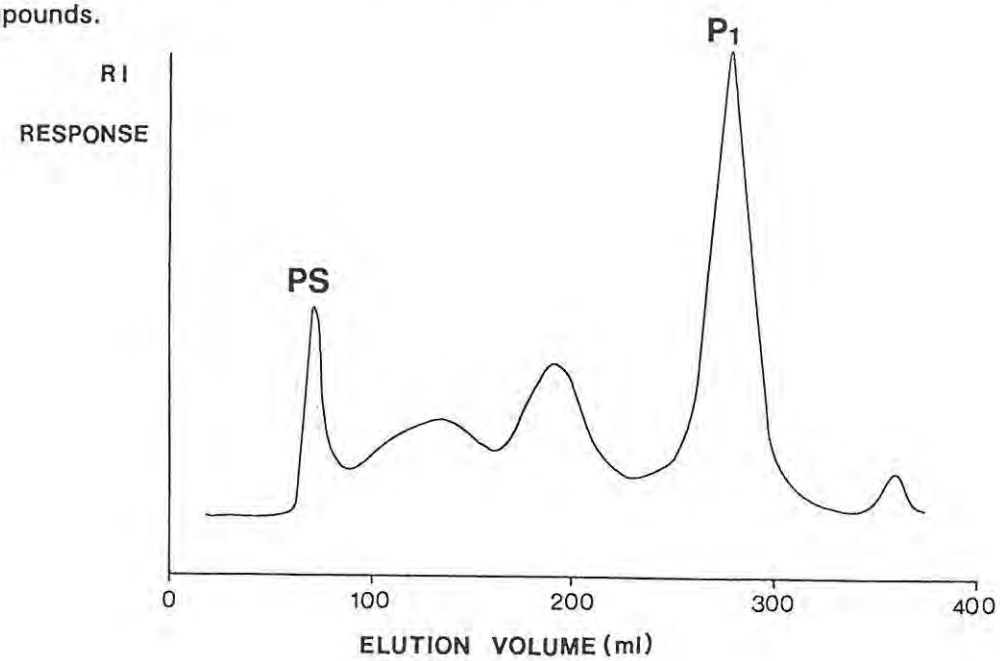


Figure 4.2 Elution diagram of the products of bacteriophage-mediated enzymatic degradation of **PS**

Methylation analyses — **PS** was methylated by a modified Hakomori procedure[116] using potassium dimsyl. GLC and GLC-MS analysis of the partially methylated alditol acetates derived from a hydrolysate of methylated **PS**, without and with carboxyl reduction, gave the results shown in Table I (columns I and II respectively). **P1** was reduced with sodium borohydride, and the derived oligosaccharide alditol (**P1-OL**) was also methylated, the carboxymethyl group reduced and the derived partially methylated alditol acetates examined by GLC and GLC-MS (Table I, column III).

These results indicated that **PS** contained terminal Glc and GlcA, a 6-linked Gal, a 3,4,6-linked Gal and two 3-linked Gal residues. Furthermore, the reducing saccharide of **P1** was clearly shown to be a 3-linked Gal residue which must have been linked to the 4-position of the 3,4,6-linked Gal in the polysaccharide. These data therefore show that the bacteriophage endoglycanase is a *β*-galactosidase.

TABLE I

METHYLATION ANALYSES OF PS AND P1-OL

Methylated Sugar[a] (as alditol acetate)	T[b] on DB-225	Molar Ratios[c]			
		I	II	III	IV
1,2,4,5,6-Gal	0.60	–	–	0.63	0.54
2,3,4,6-Glc	1.00	1.00	1.00	1.00	1.00
2,4,6-Gal	1,61	1.93	2.04	1.03	0.93
2,3,4-Glc	1,73	–	0.63	0.62	–
2,3,4-Gal	2.05	0.98	1.00	0.86	1.46
2,4-Gal	3.16	–	–	0.78	–
2-Gal	3.81	0.91	1.23	–	–

[a] 1,2,4,5,6-Gal = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol, etc.
[b] Retention times relative to 2,3,4,6-Glc on column DB-225 (see experimental).
[c] I, methylated **PS**; II, methylated reduced **PS**; III, methylated carboxyl-reduced **P1-OL**; IV, methylated base-degraded **P1-OL**.

*Base-catalysed degradation (β-Elimination) of **P1-OL*** — Methylated **P1-OL** was degraded with methylsulphinyl carbanion in DMSO and then alkylated with methyl iodide[196]. The partially methylated alditol acetates derived from a hydrolysate of the product were examined by GLC-MS. The results (Table I column IV) established that the terminal GlcA was linked to *O*-3 of the 3,6-linked Gal in **P1-OL** and thus the 3,4,6-linked Gal in **PS**. These findings are consistent with the following partial structure for the repeating unit of **PS**:

D-GlcA
↓
3
→3)-ß-D-Gal-(1→4)-D-Gal-(1→
6
↑

Further information for the sequence of the residues in **PS** was obtained from 2D-NMR experiments on **P1-OL**.

*2D-NMR studies of **P1-OL*** — Complete assignment of the ^{1}H and ^{13}C resonances of the sugar residues and the terminal alditol were made from COSY[105], two-step RELAY COSY[334], HMQC[355], and HMQC-TOCSY[367] experiments. The H-1 resonances for the five aldoses in **P1-OL** are labelled **a-e** in decreasing order of chemical shift. COSY and two-step RELAY COSY contour plots of **P1-OL** are shown in Figure 4.3 and Figure 4.4 respectively and the ^{1}H assignments are presented in Table II. Commencing from the resonance for H-1, the chemical shifts of all the ^{1}H resonances for residues **a**, **b** and **e** were traced *via* the cross peaks in the COSY contour map. All ambiguities for these residues could be resolved by referring to the two-step RELAY COSY spectrum. In the case of residues **c** and **d**, only H-1 to H-4 could be assigned from the ^{1}H-^{1}H correlation spectra.

The ^{1}H resonances assigned for residues **a-e** were then compared with the ^{13}C-^{1}H correlation data obtained from the HMQC experiment (Figure 4.5 and Table II.). This permitted the unambiguous assignment of all the ^{13}C resonances for residues **a**, **b** and **e**, and C-1 to C-4 for residues **c** and **d**. The assignment of the chemical shifts for the C-5/H-5 and C-6/H-6a, H-6b resonances for residues **c** and **d**, and the chemical shifts for the $^{13}C/^{1}H$ resonances of the alditol **f** was accomplished as follows: On the basis of literature values, which show that the chemical shifts of primary carbon atoms of alditols occur approximately 1-1.5 ppm to lower field than do those of hexopyranoses, the sets of $^{13}C/^{1}H$ resonances (HMQC, Figure 4.5 and Table II.) at 63.10 ppm/δ 3.80 and 63.64 ppm/δ 3.70 were assigned to the methylene groups in the alditol **f** and are labelled **f1** and **f6**.

The chemical shifts for C-2/H-2 (f2) and C-5/H-5 (f5) could now be established from their respective relayed connectivities to C-1/H-1a, H-1b (f1) and C-6/H-6a, H-6b (f6) in the HMQC-TOCSY spectrum (Figure 4.6 and Table II.). Returning to the COSY spectrum, all the ^{1}H chemical shifts for the alditol could now be assigned.

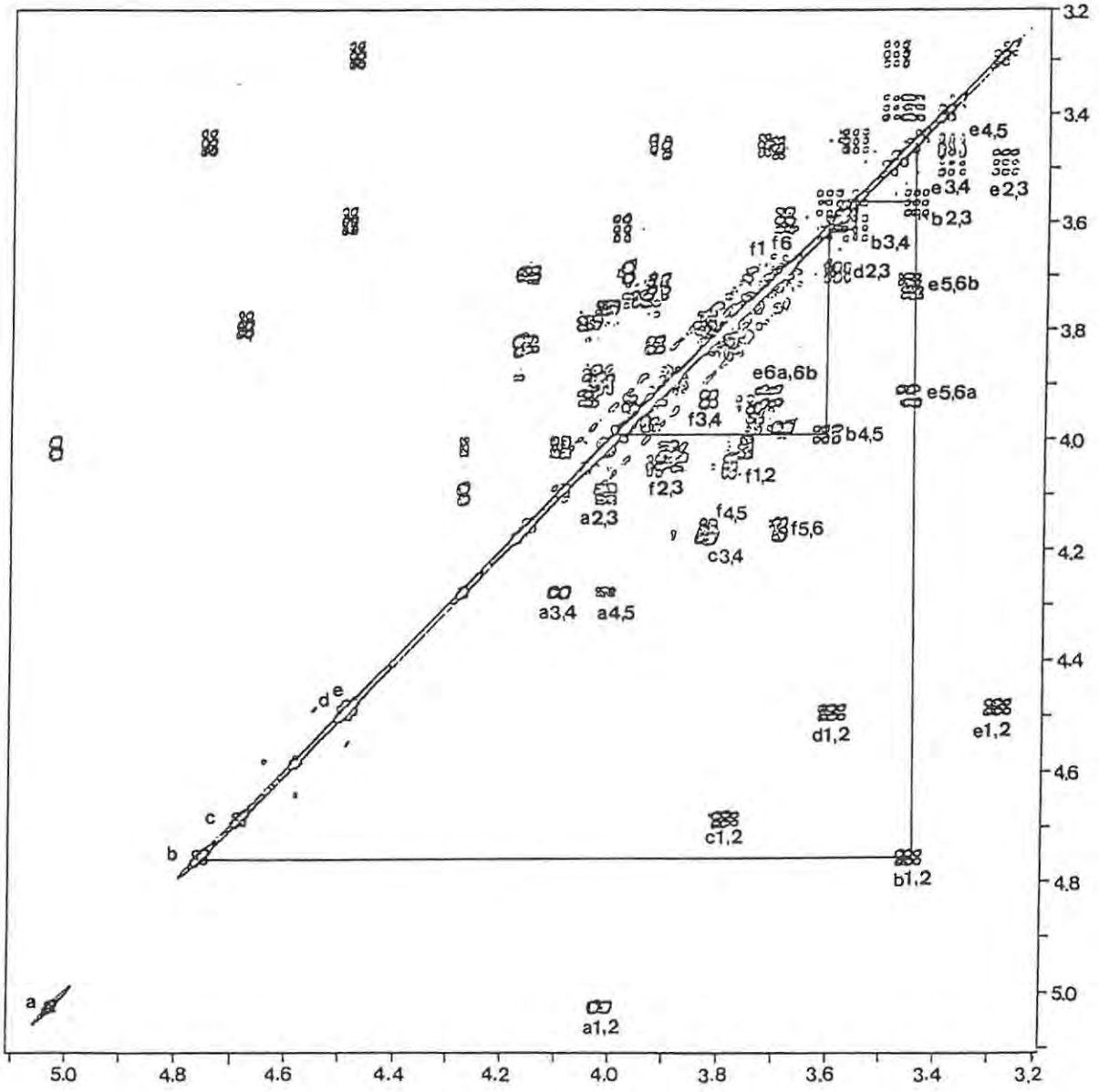


Figure 4.3 COSY contour plot of the region δ 5.1-3.2 of **P1-OL**. The ^{1}H resonances of the *J*-coupled spin systems are labelled a-f. a1 connotes H-1 of residue a, and a1,2 connotes the cross-peak between H-1 and H-2 of residue a, etc.

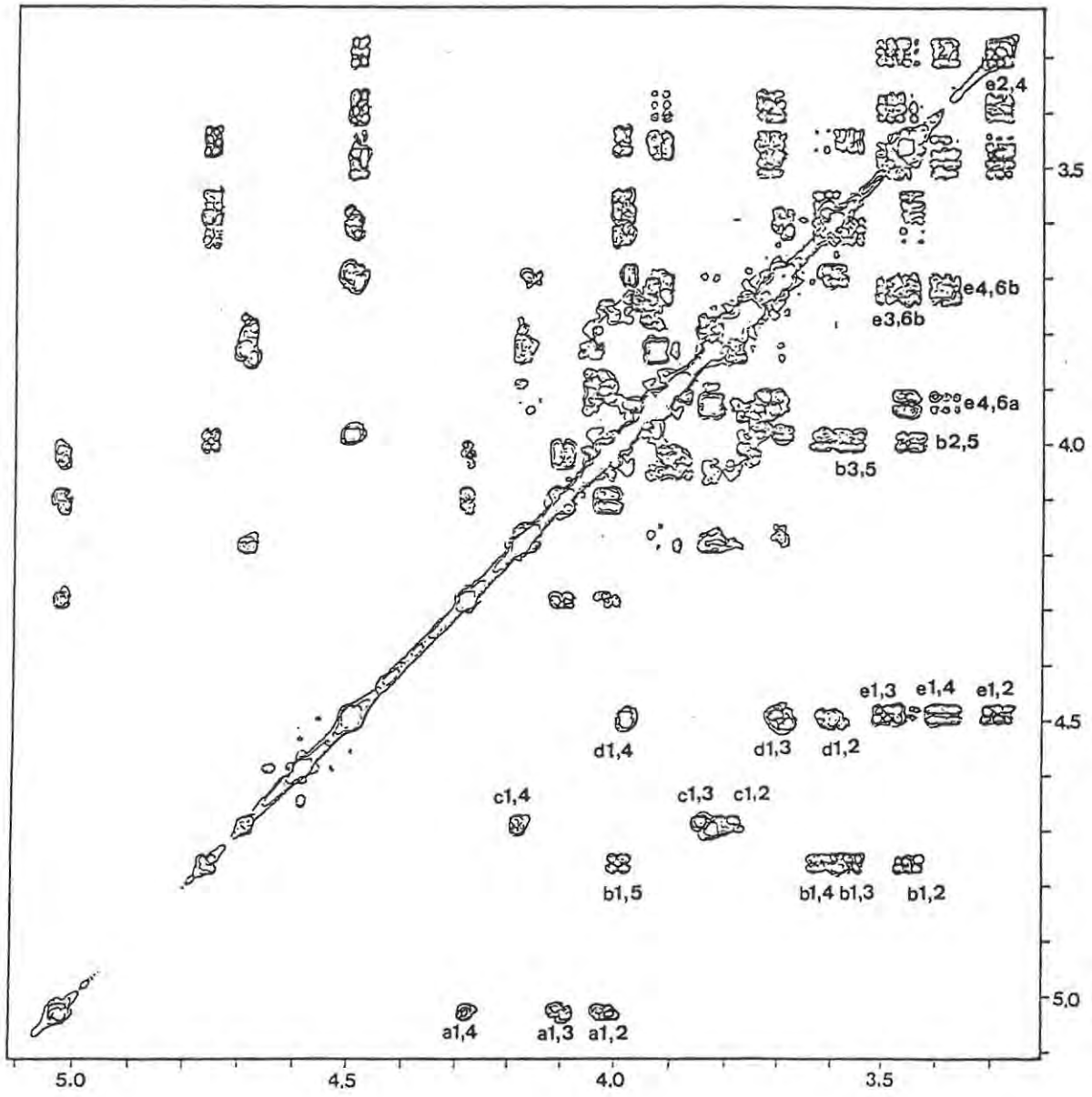


Figure 4.4 Two-step RELAY COSY contour plot of the region δ 5.1-3.2 of **P1-OL**. Correlated resonances are labelled **a-f**. a1 connotes H-1 of residue **a**, and a1,3 connotes the cross-peak between H-1 and H-3 of residue **a**, etc.

Comparison of these data with the ^{13}C-^{1}H correlation data from the HMQC experiment permitted the assignment of all the resonances for **f**. Finally, the connectivities between the two sets of C-6/H6a, H-6b resonances and their respective C-5/H-5 neighbours were obtained from the HMQC-TOCSY spectrum (Figure 4.6). As no connectivity between H-4/C-4 and H-5/C-5 in these residues was observed in any of the correlation spectra, a long range COSY[420] experiment was performed with the delays optimised for small couplings[421]. A connectivity between H-4 and H-5 for residue **c** was observed and allowed the assignment of the remaining chemical shifts for residues **c** and **d**.

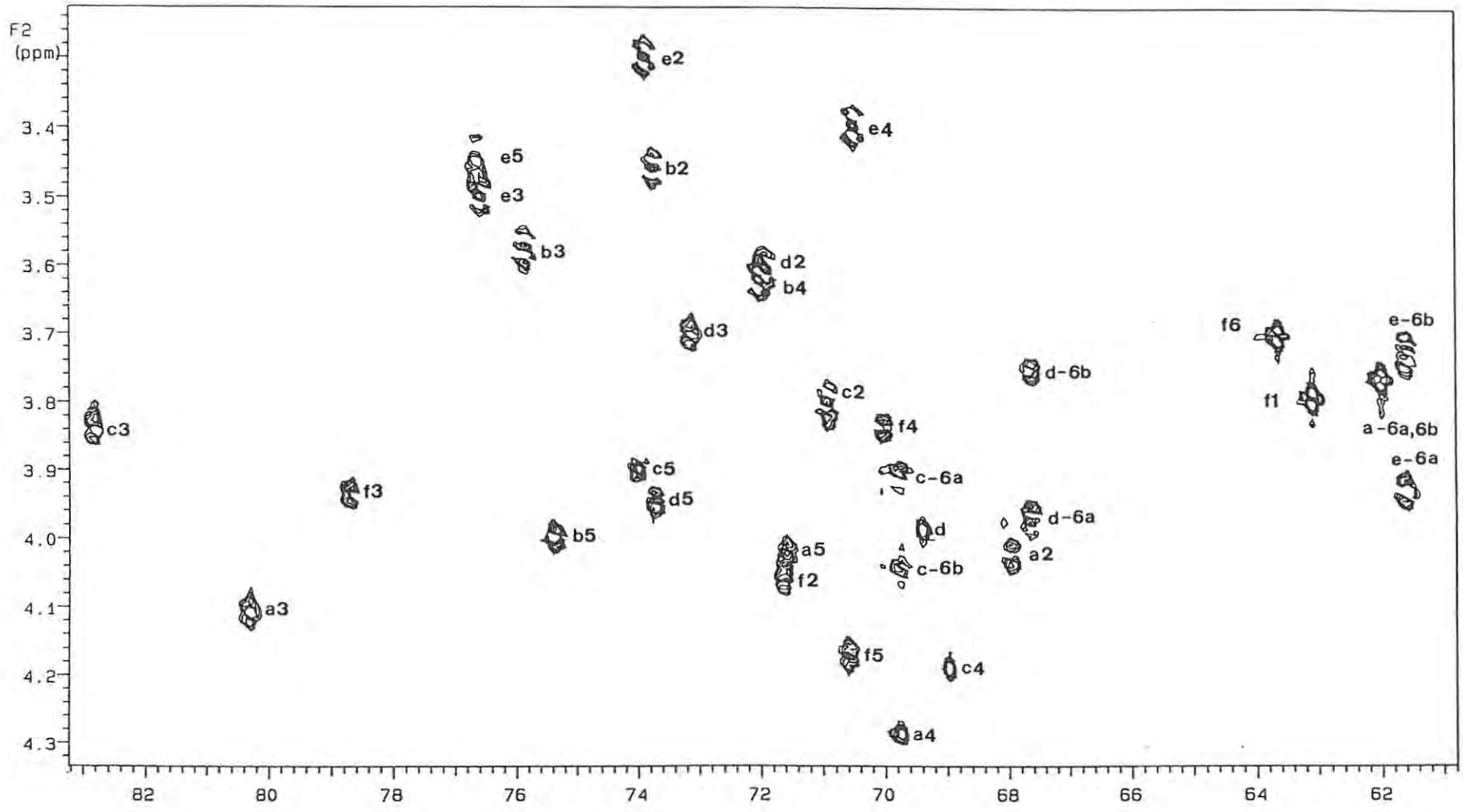


Figure 4.5 HMQC ^{1}H-^{13}C shift correlation map of the spectral region f_2 83-61 ppm (^{13}C) and f_1 δ 3.2-4.3 (^{1}H) of **P1-OL**. Correlated resonances are labelled **a-f**.

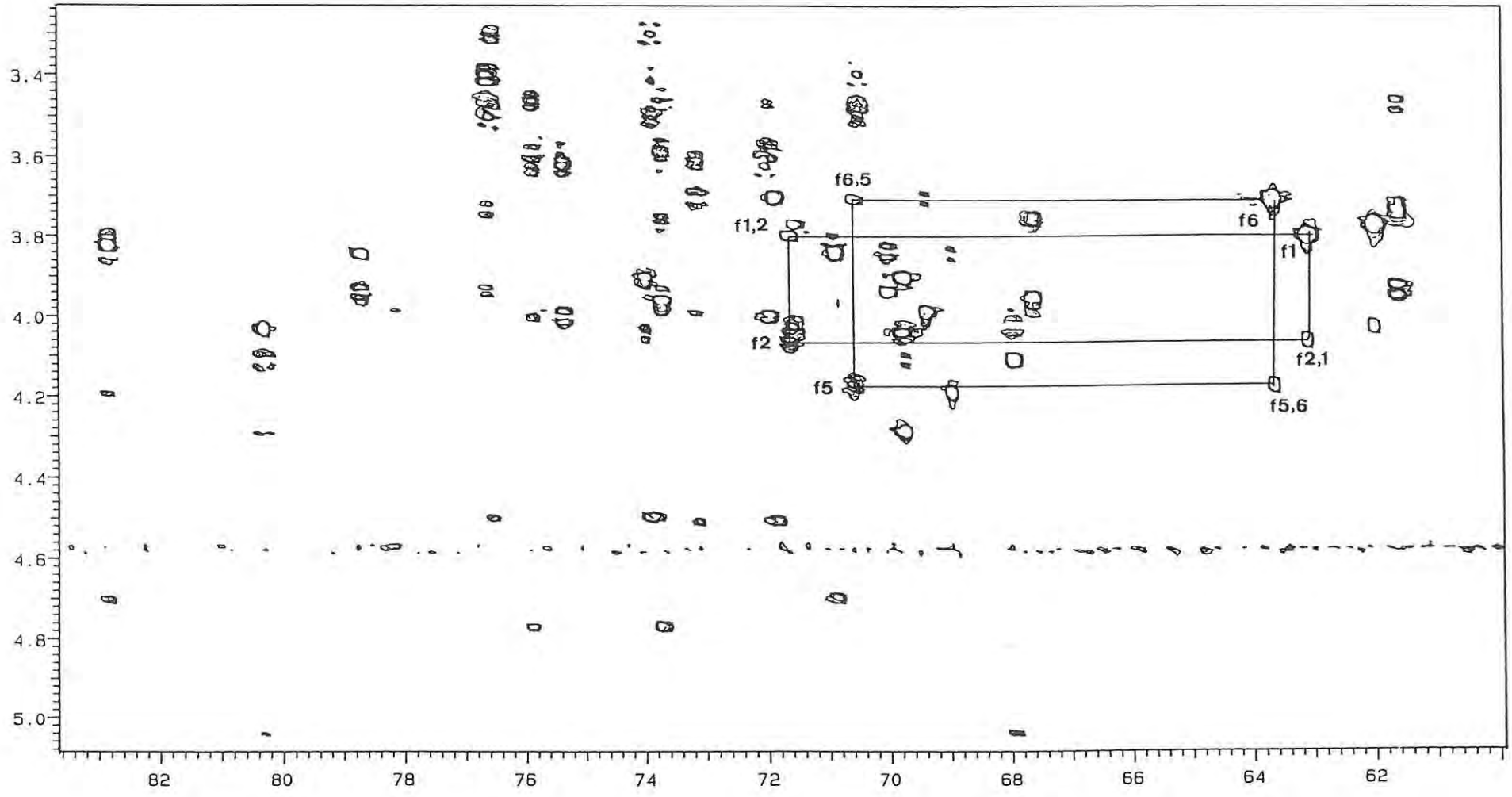


Figure 4.6 HMQC-TOCSY ^{1}H-^{13}C contour plot of the spectral region f_2 83-61 ppm (^{13}C) and f_1 δ 3.2-4.3 (^{1}H) of **P1-OL**. The respective connectivities between f6 and f5, and f1 and f2 are indicated, where f1 = C-1/H-1a, H-1b, etc.

TABLE II NMR DATA[a] FOR P1-OL

Residue		1a	1b	2	3	4	5	6a	6b
a									
	H	5.02	-	4.01	4.10	4.28	4.02	3.77	3.77
→3)-α-Gal	^3J[b]	3.9		10.2	3.2	0.9			
	C	99.44	-	68.00	80.35	69.80	71.59	62.00	-
b									
	H	4.75	-	3.45	3.57	3.62	4.00	-	-
ß-GlcA	^3J	7.9		8.9	8.9	9.4			
	C	104.49	-	73.77	75.89	71.90	75.40	-	-
c									
	H	4.68	-	3.79	3.83	4.18	3.89	4.04	3.91
→3,6)-β-Gal	^3J	7.6		10.0	3.8				
	C	104.70	-	70.95	82.80	69.00	74.00	69.70	-
d									
	H	4.49	-	3.59	3.70	3.98	3.95	3.98	3.76
→6)-ß-Gal	^3J	7.8		9.4	3.6				
	C	104.70	-	71.93	73.14	69.42	73.70	67.80	-
e									
	H	4.48	-	3.29	3.49	3.40	3.46	3.93	3.73
ß-Glc	^3J	7.9		9.3	9.2	9.2	9.7	2.0/11.8	7.8
	C	103.64	-	73.80	76.59	70.50	76.72	61.64	-
f									
	H	3.80	3.80	4.06	3.94	3.84	4.16	3.70	3.70
→3)-Gal-ol	C	63.10	-	71.62	78.70	70.00	70.60	63.64	-

[a] Chemical shifts with acetone as internal reference, δ 2.23 and 31.07 ppm respectively for ^1H and ^{13}C.
[b] ^1H-^1H coupling constants in Hz.

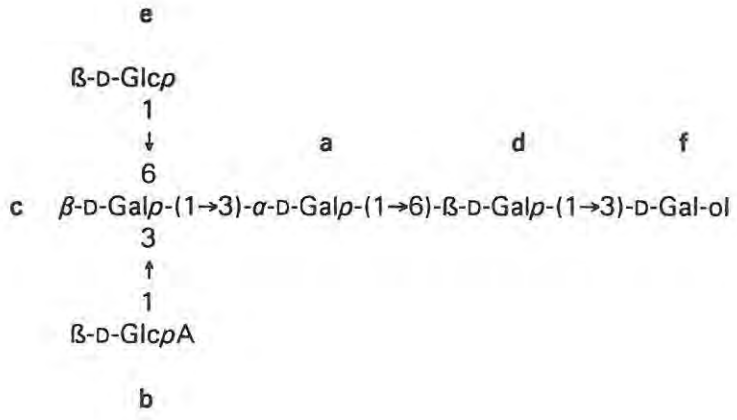
The ^{1}H-^{1}H coupling constants for the sugar residues were obtained from a resolution enhanced 1D spectrum of P1-OL and from 1D HOHAHA[320] subspectra obtained by selectively inverting a well separated multiplet from each spin system (see Figure 3.18, page 74). Comparison of the NMR data for residues **a**-**f** with those for model compounds[293,422,423] identified the residues as indicated in Table II. The significant deshielding displayed by C-3 of **a**, C-3,6 of **c**, C-6 of **d**, and C-3 of **f** identified the linkage positions of the residues and were in accordance with the methylation results from **P1-OL**.

Sequencing of the residues — In order to establish the sequence of the residues in **P1-OL**, an HMBC[169] experiment was acquired. The relevant through-bond inter-residue connectivities are shown in Table III.

TABLE III: OBSERVED THREE-BOND INTERRESIDUE ^{13}C-^{1}H SCALAR COUPLING (HMBC EXPERIMENT)

Anomeric proton of residue	Long-range through bond connectivity to carbon (ppm)
a	67.80 (**d**, C-6)
b	82.80 (**c**, C-3)
c	80.35 (**a**, C-3)
d	78.70 (**f**, C-3)
e	69.70 (**c**, C-6)

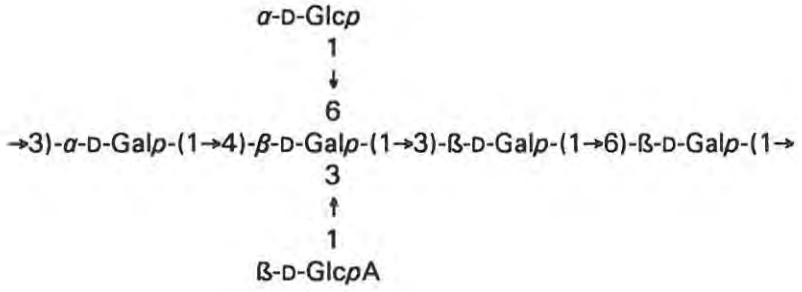
These data establish the following structure for **P1-OL**:



CONCLUSION

The combined NMR, chemical and enzymatic studies on **P1-OL** and **PS** therefore prove the structure of the repeating unit of the *Klebsiella* K15 capsular polysaccharide is as shown in the abstract. The location of the *O*-acetyl group was not established.

At approximately the same time this work was published[424], a different research group in India also published a revised structure for the K15 polysaccharide[425]. Their structure, shown below, contains *two* α-linked sugars and the sequence of the residues, which also differs from that determined above, was established largely from methylation and enzymatic studies on oligomers generated by Smith degradation of the polysaccharide. Only 1D ^{1}H and ^{13}C spectra were obtained.



The high resolution 1D spectra of **PS** and **P1-OL** obtained in this study clearly show that the polysaccharide only contains *one* α-linked residue and the sequence of the residues in the repeating unit was unambiguously obtained from the HMBC experiment.

EXPERIMENTAL

General methods — Analytical GLC was performed with a Hewlett-Packard 5890A gas chromatograph, fitted with a flame-ionisation detector and a 3392A recording integrator, with helium as carrier gas. A J&W Scientific fused silica DB-225 bonded-phase capillary column (30 m x 0.25 mm) having a film thickness of 0.25 μm, operated isothermally at 210°C and 100 KPa head pressure, was used for separating aldononitrile acetates and partially methylated alditol acetates, and at 230°C and 140 KPa to separate acetylated octyl glycosides. A Hewlett-Packard 5988A GLC-MS using the same column was used to confirm the identities of the methylated derivatives.

Isolation and purification of the Klebsiella K15 polysaccharide — An authentic stab culture of *Klebsiella* K15, obtained from Dr. I. Ørskov (Copenhagen), was plated on sucrose-rich agar (Appendix II) and incubated overnight. A single actively growing colony was selected and replated, and the process was repeated a third time with a colony selected from the latter to ensure strain purity. Inocula were then transferred to each of 8 culture tubes containing 5 mL of sterile nutrient broth and shaken at 37°C for 6 h. The contents of the tubes were then added to 8 x 100 mL conical flasks containing 50 mL of sterile broth and incubated for a further 4 h after which the contents were spread evenly over the surfaces of 8 sterile stainless steel trays (60 cm x 40 cm) containing sucrose-rich agar and the trays (glass-covered) were incubated at 37°C. After 3 days of growth, the bacterial slime was harvested (290 mL), diluted with an equal volume of 2% phenol solution, and gently stirred at 4°C for 24 h to dissolve the capsular material. The dead bacterial cells were separated from the supernatant by ultracentrifugation (105 000 x g, 3 h) and the supernatant was precipitated into ethanol (5 vols). The precipitate was isolated using low-speed centrifugation (3000 rpm, 20 min) and was redissolved in water (200 mL). Cetyltrimethylammonium bromide (CTAB, 5% in water) was added dropwise (12 mL) and the resulting precipitate was isolated by low-speed centrifugation as above. The acidic polysaccharide-CTAB complex was dissolved in 3 M NaCl (170 mL) and was precipitated into ethanol (5 vols).

The precipitate was redissolved in water (200 mL) and dialysed exhaustively (12-14 000 mw cut-off) against tap-water and lyophilised. The yield of crude acidic capsular polysaccharide was 940 mg. 200 mg of this material was further purified by GPC on a Sephacryl S500 column (100 cm x 2.5 cm), NaOAc buffer (0.1 M) as eluent.

Bacteriophage isolation and purification — A host-specific bacteriophage (Ø15) was isolated by incubating a mixture of Grahamstown sewage water (25 mL), sterilised double-strength nutrient broth (25 mL), and a 6 h culture (5 mL) of the host bacterium at 37°C for 12 h. Bacterial growth was terminated by the addition of $CHCl_3$ (5 mL), the cellular material was removed by low-speed centrifugation and the clear supernatant was retained. The resulting impure phage suspension was assayed using the "sloppy layer" plaque technique[241] and the titre was found to be 7,3 x 10^7 PFU/mL. The phage was purified and the titre increased for the purposes of depolymerisation as follows:

A single plaque possessing a distinct "halo" was picked from one of the agar plates and added to nutrient broth (5 mL) which had been freshly inoculated with host bacteria. This was incubated with shaking at 37°C for 6 h after which the bacterial cells were killed by the addition of a few drops of $CHCl_3$ and removed by centrifugation as described above. This process was repeated twice to ensure a pure phage line. The titre and volume of the pure phage suspension was then increased by a series of tube and flask lyses. 10 culture tubes each containing 5 mL of nutrient broth were inoculated with host bacteria and, at 15 min intervals, phage solution (0.1 mL) was successively added to the tubes to ensure an increasing concentration of bacteria from tubes 1 to 10. The tenth tube was assayed as described above and the titre was found to be ~ 10^7 PFU/mL. To increase the titre to the desired 10^{13} PFU[267], the process was repeated on a larger scale (10x scale up) inoculating with phage from the 10th tube. This resulted in an increase in the titre to 3.2 x 10^{10} PFU/ml. 400 mL of phage solution containing 1.3 x 10^{13} PFU was prepared and was purified exhaustively by dialysis against tap water (12-14 000 mw cut off).

Bacteriophage-mediated depolymerization of PS — **PS** (Na^+ salt) (500 mg) was dissolved in the phage solution (400 mL) prepared above and was gently agitated at 37°C in the presence of chloroform (4 mL) to prevent bacterial growth. After 4 days, the mixture was lyophilised and the resultant lyophilisate was redissolved in water (30 mL) and dialysed (mw cut off <3500) against 6 x 50 mL distilled water fractions for 24 h periods. The combined diffusate was lyophilised, redissolved in 15 mL of water, and successively passed down an amberlite IR-(120)H^+ column and re-freeze dried until a constant mass was obtained. The total yield of low molecular weight material was 410 mg. Finally, the material was fractionated on a Bio-Gel™ P4 column (2.6 cm x 100 cm) using NaOAc buffer (0.1 M) as the eluent to afford the purified hexasaccharide, **P1** (130 mg). A sample (50 mg) was dissolved in water and was reduced with $NaBH_4$ to produce the reduced hexasaccharide-alditol (**P1-OL**).

Enzymatic treatment with β-glucosidase — **P1** (2 mg) and **PS** (2 mg) were each dissolved in NaOAc buffer (pH 5.00, 2 mL) containing *β*-glucosidase (Sigma Chemical Corporation, 0.5 mg). The solutions were gently stirred for 3-4 h at 37°C after which the enzyme was denatured by raising the temperature of the solutions to 60°C for 10 min. Both solutions were concentrated on a rotary evaporator prior to analysis by paper chromatography (Whatman™ No. 1, descending mode, ethyl acetate:acetic acid:pyridine:water (18:3:1:4) as mobile phase). Liberated glucose was identified in both instances by development of the chromatogram with ethanolic $AgNO_3$ solution.

Glycose analysis — The sugar composition of **PS** was determined according to the method of Chen and McGinnis[95]. **PS** (3 mg) was hydrolysed (4 M TFA, 1mL, 125°C, 1 h) and after evaporation of the acid, water (0.2 mL) and 2.5% hydroxylamine in *N*-methylimidazole (0.4 mL) were added and the flask sealed and heated at 80°C for 10 min. Acetylation of the aldononitrile derivatives was effected by the dropwise addition of Ac_2O with cooling, and the resulting aldononitrile acetates were recovered by extraction into $CHCl_3$ and analysed by GLC. To ascertain the identity of the uronic acid, **PS** (5 mg) was methanolysed (refluxing 3% methanolic HCl, 16 h)

and, after neutralisation of the acid with $AgCO_3$, the methyl esters were reduced with $NaBH_4$ in anhyd. MeOH and the resulting methyl glycosides were hydrolysed and converted into aldononitrile derivatives as described above.

Preparation of (-)-2-octylglycoside acetates — Acetylated octyl glycosides were prepared according to the procedure of Leontein *et al.*[143]. A carboxyl reduced methanolysate of **PS** (5 mg) was transferred to a 5 mL ampoule and lyophilised. To the lyophilised residue was added concentrated TFA (1 drop) and (-)-2-octanol (0.5 mL) and the ampoule was sealed and heated in an oil bath at 130°C for 12 h. After concentration to dryness under vacuum at 55°C, the residue was acetylated (pyridine-acetic anhydride, 100°C, 1 h) and the peracetylated octyl glycosides were analysed by GLC.

*Methylation of **PS** and **P1-OL*** — Permethylation of **PS** was achieved by the Hakomori method as modified by Narui *et al.*[118]. A dried sample of **PS** (acid form, 15 mg) was dissolved in a mixture of DMSO (1 mL) and tetramethylurea (1 mL) and, whilst flushing with N_2, methylsulphinyl (dimsyl) anion (1.7 mL) was added and the mixture stirred at room temperature for 1-2 h. The reaction mixture was cooled in ice and MeI (0.8 mL) was added dropwise with stirring. After 3-4 h this mixture was dialysed against running water (12-14 000 mw cut off) and freeze-dried. A sample of the permethylated product (5mg) was hydrolysed (4 M TFA, 1mL, 125°C, 1 h), reduced ($NaBH_4$) and the resulting partially methylated alditols were acetylated with 1:1 Ac_2O-pyridine (1mL, 100°C, 1 h). In order to identify the linkage positions on the uronic acid, a second sample (10 mg) was first methanolysed and the resulting methyl esters of the uronic acids were reduced with $NaBH_4$, prior to acid hydrolysis, reduction and acetylation as described above. Methylation of **P1-OL** was carried out essentially as described above except that no tetramethylurea was added to the DMSO and the permethylated product was not dialysed. Instead the mixture was extracted with $CHCl_3$ and the product purified by passage down a column of Sephadex™ LH-20.

Base catalysed degradation (β-elimination) — Methylated **P1-OL** (10 mg) was dissolved in a 19:1 mixture of DMSO:2,2-dimethoxypropane (5 mL) containing 1 mg of *p*-toluenesulphonic acid. To this was added dimethylsulphinyl anion (5 mL) and the flask was allowed to stir under N_2 for 2 h after which MeI (3 mL) was slowly added with cooling. The degraded polysaccharide was then recovered by extraction into CH_2Cl_2 (4 x) and washed with water (4 x). The CH_2Cl_2 fraction was dried (anhyd. Na_2SO_4), filtered and the solvent removed under a stream of N_2. The residue was hydrolysed (4 M TFA, 1mL, 125°C, 1 h), reduced ($NaBH_4$) and the resulting partially methylated alditols were acetylated with 1:1 Ac_2O-pyridine (1mL, 100°C, 1 h). This is essentially the "single flask method" as described by Aspinall and Rosell[196].

NMR spectroscopy — Samples were deuterium-exchanged with D_2O, and then examined as solutions in 99.99% D_2O (0.6mL) containing a trace of acetone as internal standard (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). Spectra were recorded on either a Bruker WM 500, AMX 400 or a Varian Unity 500 MHz spectrometer, using standard Bruker or Varian software. All 2D experiments were carried out at 318K. The parameters used were as follows: COSY and COSY LR: 512 x 2048 data matrix, 16 scans per t_1 value, 1s recycle delay, non-shifted sine-bell filtering in t_1 and t_2; two-step RELAY-COSY: 512 x 2048 data matrix (zero-filled to 1024 data points in t_1), 64 scans per t_1 value, 1s recycle delay, fixed delays of 0.036 s. HMQC: 256 x 2048 data matrix (zero-filled to 1024 data points in t_1), 8 scans per t_1 value, 1 s recycle delay. HMQC-TOCSY: 256 x 2048 data matrix, 16 scans per t_1 value, 25 ms MLEV-17 mixing time, 1 s recycle delay. HMBC 256 x 2048 data matrix, 32 scans per t_1 value, $\Delta 1$ and $\Delta 2$ durations of 3.45 and 60 ms respectively, 1 s recycle delay, and a sine-squared filter.

4.2 STRUCTURAL ELUCIDATION OF THE CAPSULAR POLYSACCHARIDE EXPRESSED BY *Escherichia coli* O20:K83:H26 BY HIGH RESOLUTION NMR SPECTROSCOPY

ABSTRACT

The structure of the capsular polysaccharide produced by *Escherichia coli* O20: K83: H26 has been investigated by one- and two-dimensional ^{1}H- and ^{13}C-NMR spectroscopy and by glycose and methylation analysis. The results show that the polysaccharide is comprised of *O*-acetylated linear pentasaccharide repeating units of the following structure:

→4)-*α*-D-Man*p*-(1→4)-*α*-D-Gal*p*A-(1→3)-ß-D-Glc*p*NAc-(1→2)-*α*-D-Gal*p*-(1→3)-*α*-L-Rha*p*-(1→
2
↑
OAc

INTRODUCTION

The presence of *O*-acetyl groups in many of the capsular (K) antigens of *E. coli* has generated considerable interest. They are responsible for small changes in polysaccharide structure and are the immunodominant parts of serologic epitopes[2]. In certain instances an *O*-acetyl group is the only chemical difference between otherwise identical capsular antigens[426]. Although *O*-acetylation does not necessarily occur on every repeating unit, it is not a random occurrence and usually the same hydroxyl group on the same sugar residue is acetylated. Owing to the lability of *O*-acetyl groups they are often inadvertently removed during chemical manipulations or they may migrate to other positions in the polymer. NMR spectroscopy offers a rapid, unambiguous means of locating *O*-acetyl groups even when the degree of acetylation is low. This study presents the structural elucidation of the *E. coli* K83 K-antigen and the location of the *O*-acetyl group.

RESULTS AND DISCUSSION

Isolation, composition and linkage analysis — *E. coli* O20:K83:H26 bacteria were grown on Mueller-Hinton agar and the capsular polysaccharide was isolated essentially as described for *Klebsiella* K15 (see section 4.1). The isolated material was further purified by treatment with RNAse followed by ion-exchange chromatography on DEAE-Sepharose CL-6B. The polysaccharide (**PS**) was shown to have an average M_r of 1.25×10^6 by GPC on a dextran-calibrated column of Sephacryl S500-HR. Analytical GLC of the alditol acetates derived from a hydrolysate of purified **PS** revealed the presence of Rha, Man, Gal and GlcN. Methanolysis and uronic-ester reduction prior to hydrolysis increased the ratio of Gal, indicating that GalA was present. Sugars were present in approximately equimolar proportions. GLC analysis of the derived (-)-2-octylglycoside acetates showed that all sugars were of the D-configuration except for Rha which had the L-configuration. Methylation of **PS** followed by GLC-MS of the derived methylated alditol acetates (with carboxyl reduction) revealed the presence of 3-linked Rha, 4-linked Man, 2-linked Gal, 4-linked GalA and 3-linked GlcN. These results accord with a linear pentasaccharide repeating unit for **PS**.

NMR spectroscopy — The ^{1}H-NMR spectrum of native **PS** at 323K (Figure 4.7a) contained five signals in the anomeric region (δ 4.50 - 5.50) at δ 4.87, 4.97, 5.21, 5.25 and 5.32. Furthermore a doublet at δ 1.30, typical of a 6-deoxy sugar, and two singlets for methyl protons of acetyl groups, at δ 1.97 and 2.26, were observed. Examination of the same sample at 343K revealed that an additional β-anomeric signal, hidden by the HOD peak at 323K, was present at δ 4.58. Since glycose analysis had indicated the presence of only one aminodeoxy sugar, the signal at δ 2.26 was attributed to the methyl protons of an *O*-acetyl group. The ^{13}C-NMR spectrum of **PS** showed five signals for pyranosidically linked hexoses, supportive of a pentasaccharide repeating unit, in the anomeric region (95 - 105 ppm) at 97.30, 98.28, 101.53, 102.25 and 104.29 ppm, (Figure 4.8a). In addition a signal at 55.30 ppm confirmed the presence of a single aminodeoxy sugar. The ^{1}H-NMR spectrum of the *O*-deacetylated polysaccharide (**DPS**) (Figure 4.7b) lacked the resonance at δ 2.26 and one of the resonances (δ 5.21) in the anomeric region. The latter therefore must have arisen from the methine proton of an acetoxylated carbon.

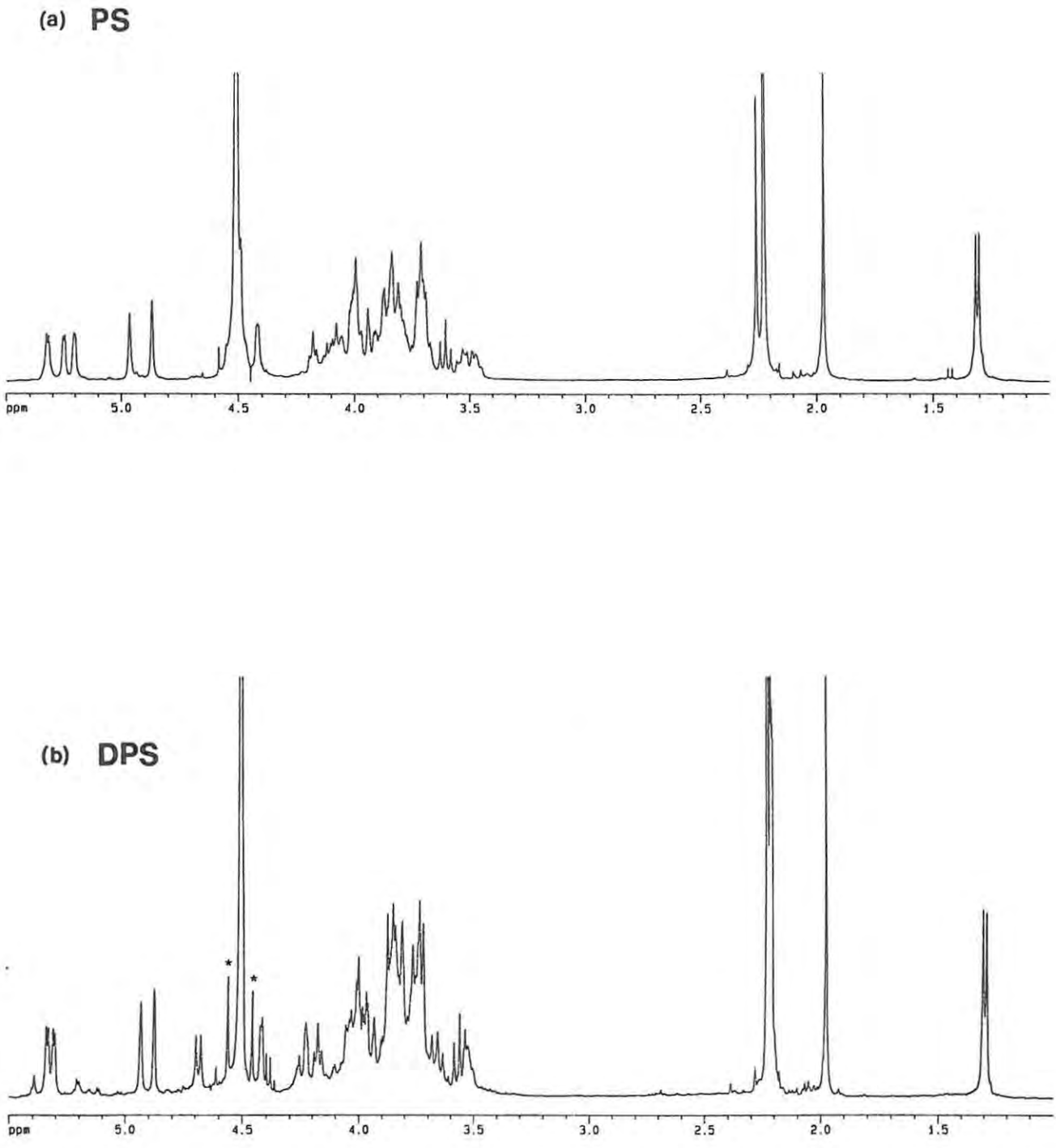


Figure 4.7 400 MHz ^{1}H-NMR spectra of **PS** (a) and **DPS** (b) at 323K. (* = Spinning side bands)

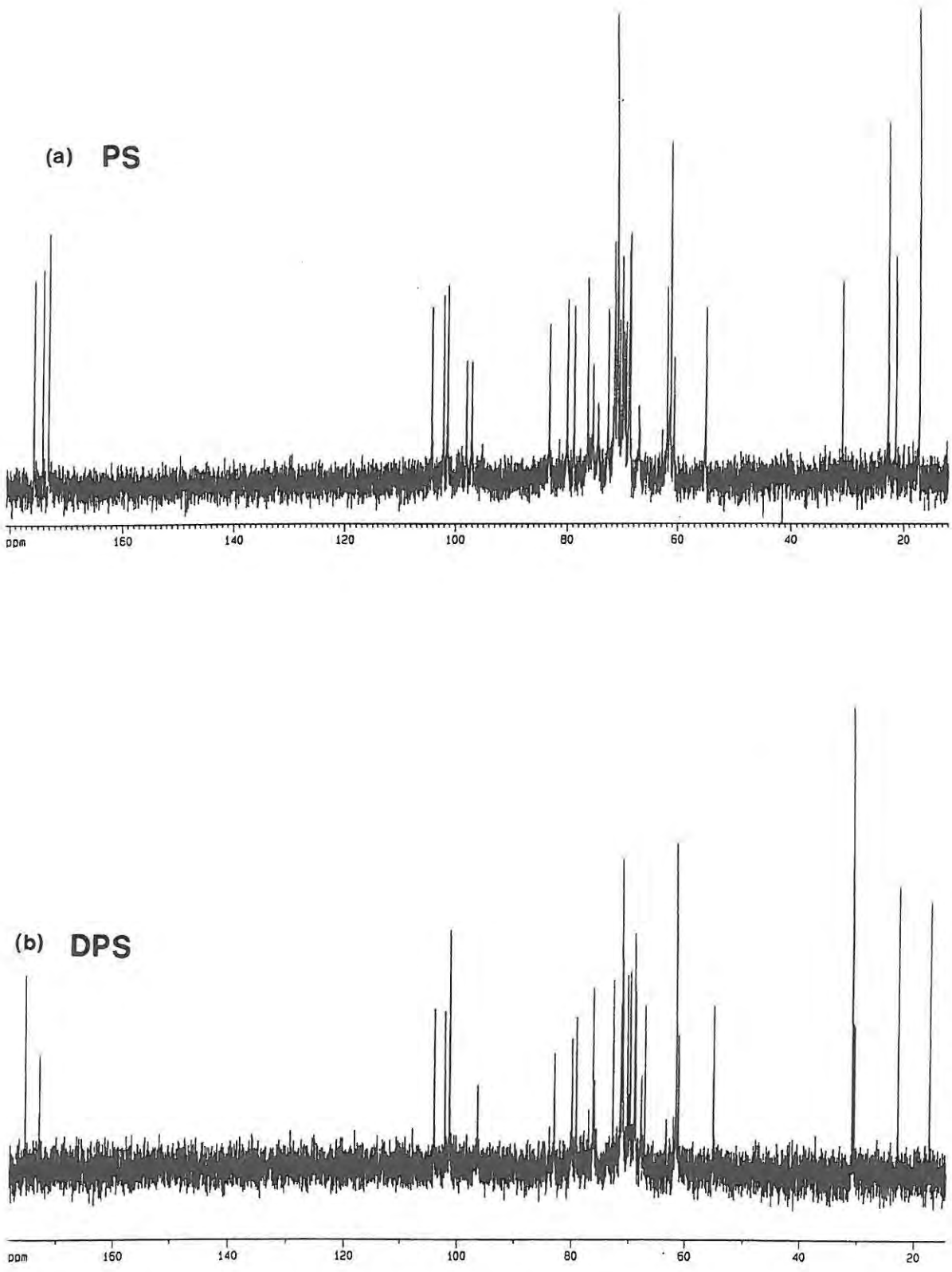


Figure 4.8 ^{13}C-NMR spectra of K83 **PS** (a) and **DPS** (b) at 323K.

The ^{13}C-NMR spectrum of **DPS** (Figure 4.8b) showed four signals, representing the resonances of five carbons in the anomeric region, at 96.47, 101.41 (2C), 102.21 and 104.05 ppm (Table I). The signal at 173.11 ppm was assigned to the carbonyl carbon of the uronic acid and signals at 55.37, 23.03 and 175.54 ppm were assigned to C-2 of GlcNAc and to the methyl and carbonyl carbons of the NAc substituent, respectively. These assignments were confirmed from long-range two- and three-bond connectivities observed in an HMBC experiment performed on a sample of **DPS** (see below). A two-bond correlation between H-5 of the uronic acid (δ 4.51) and the carbonyl carbon at 173.11 ppm confirmed the assignment made above and the remaining resonance at 175.54 ppm could then be assigned to the carbonyl carbon of the NAc substituent.

The $^{3}J_{1,2}$ coupling constants for the anomeric protons, measured from a resolution enhanced proton spectrum of **DPS**, were consistent with one ß-signal (8.2 Hz) and two α-signals (4.1 and 3.8 Hz). The remaining two signals, (1.5 and < 1 Hz) could not be assigned as either α or ß on the basis of chemical shift or $^{3}J_{1,2}$ values being of the *manno* type; both were assigned as α from observed NOE peaks in the NOESY spectrum (see later). COSY experiments were performed on both **PS** and **DPS** and since the latter showed less resonance overlap, all further NMR studies were performed on **DPS**.

The chemical shifts for the ^{1}H- and ^{13}C-NMR resonances of the various residues were assigned using COSY, HOHAHA[342], HMQC[365] and HMQC-TOCSY[367] experiments, and the data are presented in Table I. The sugar residues are labelled **a**-**e** in order of the decreasing chemical shifts of their H-1 resonances. Following the cross-peaks in the contour plots, all the ^{1}H resonances for residues **a**, **c** and **e** together with H-1 to H-5 of residue **b** and H-1 to H-4 of residue **d** could be traced from COSY (Figure 4.9) and HOHAHA spectra of **DPS**. The ^{13}C resonances for these residues were assigned by comparison with ^{1}H-^{13}C correlation data from an HMQC experiment (Figure 4.10) and the outstanding chemical shift for C-5/H-5 of residue **d** could now be assigned by inspection. C-6/H-6a,H-6b for **b** and **d** were difficult to assign owing to excessive overlap, however assignments were made from data obtained from an HMQC-TOCSY experiment.

TABLE I
NMR DATA FOR *E. coli* K83 DPS

Atom	*Residue*[a]				
	a →4)-α-GalA	b →2)-α-Gal	c →3)-α-Rha	d →4)-α-Man	e →3)-ß-GlcNAc
H-1	5.34 (4.1)[b]	5.31 (3.8)	4.94 (1.5)	4.88	4.69 (8.2)
C-1	101.40	96.47	101.40	102.20	104.06
H-2	3.86 (10.2)	3.86 (10.2)	4.23 (3.3)	3.85	3.83 (8.9)
C-2	69.19	79.35[c]	67.92	71.58	55.38
H-3	3.99 (3.0)	3.97 (2.5)	3.82 (9.5)	3.89	3.75 (9.2)
C-3	69.00	69.12	77.20	70.00	83.16
H-4	4.42	4.01	3.56 (9.6)	3.81	3.66 (9.2)
C-4	80.09	70.43	71.25	76.18	71.37
H-5	4.51	4.18	4.04 (6.2)	4.04	3.52
C-5	71.25	71.37	69.93	72.94	76.42
H-6a	-	3.75	1.30	3.81	3.95
H-6b	-	3.73	-	3.76	3.75
C-6	173.10	61.75	17.49	61.30	61.75

[a] Chemical shifts in ppm with acetone as internal reference, δ 2.23 and 31.07 ppm for ^{1}H and ^{13}C respectively. [b] $^{3}J_{H,H}$ values in Hz. [c] Linkage carbons underlined.

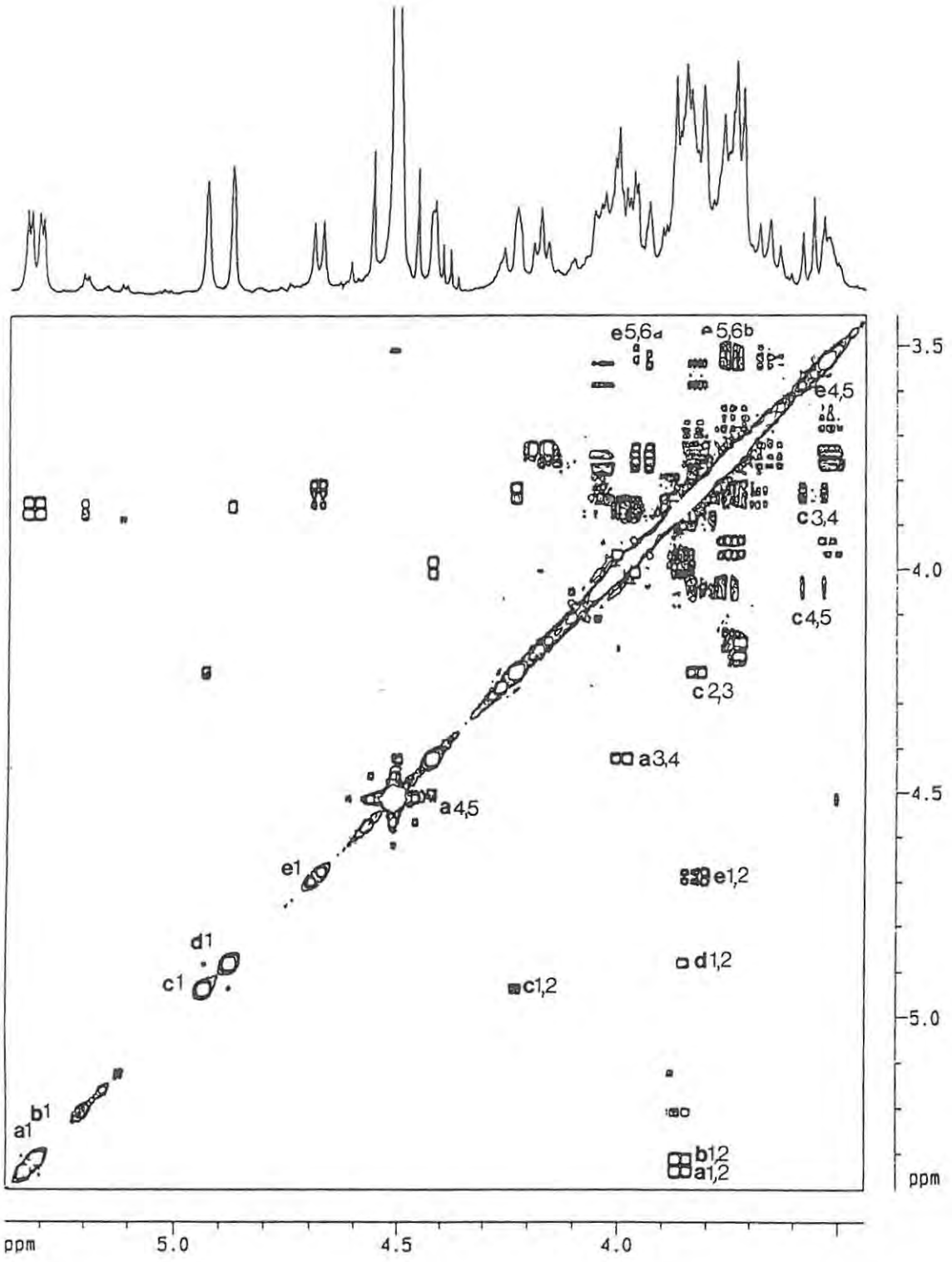


Figure 4.9 COSY contour plot of the region δ 5.5-3.5 of **DPS** at 323K. The proton resonances of the sugar residues are labelled a-e: a1 connotes H-1 of residue a and a1,2 connotes the cross-peak between H-1 and H-2 of residue a, etc.

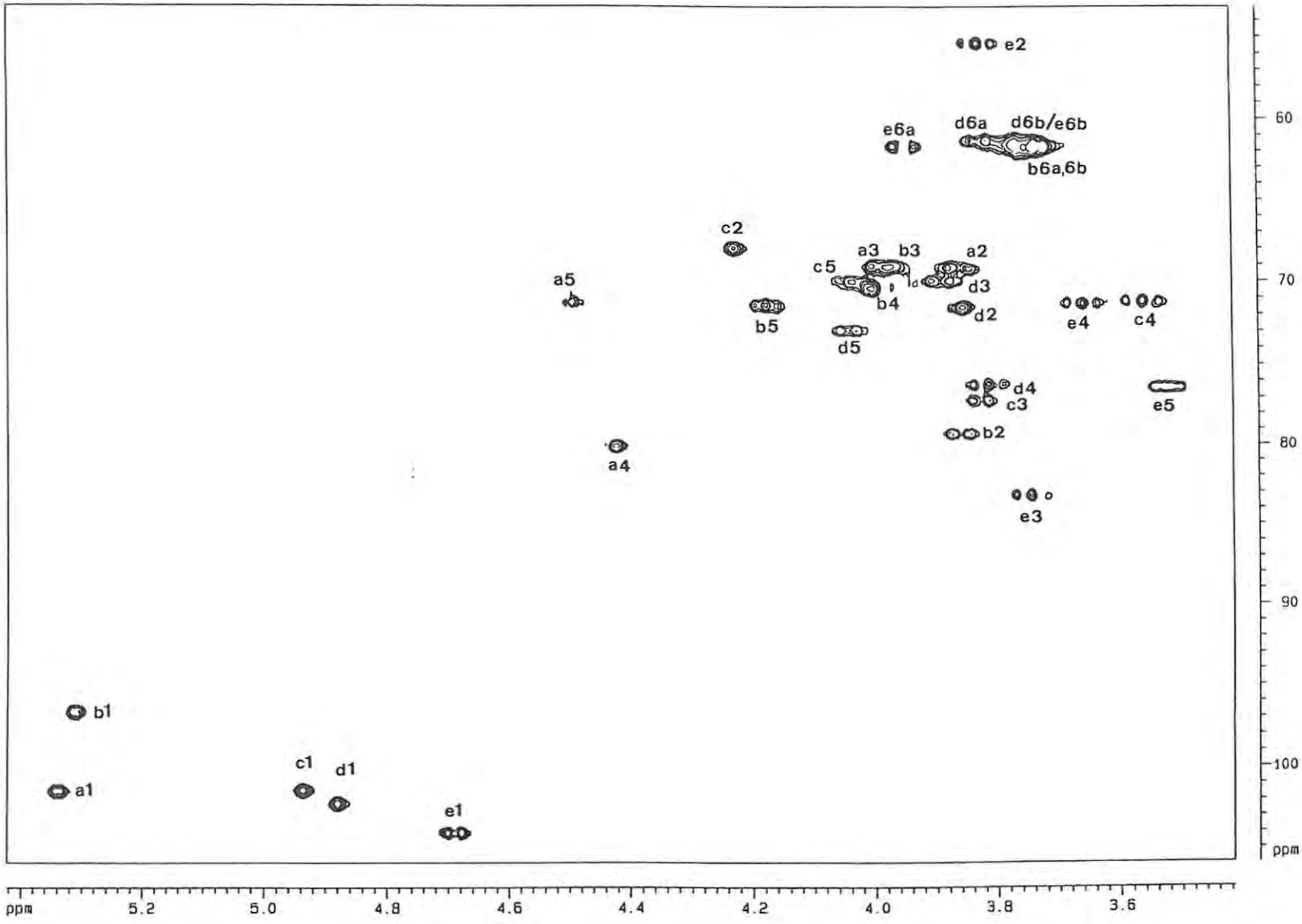


Figure 4.10 HMQC ^{1}H-^{13}C shift correlation map of the region f_1 106-53 ppm and f_2 δ 5.4-3.4 for **DPS** at 323K. Correlated resonances are labelled a-e.

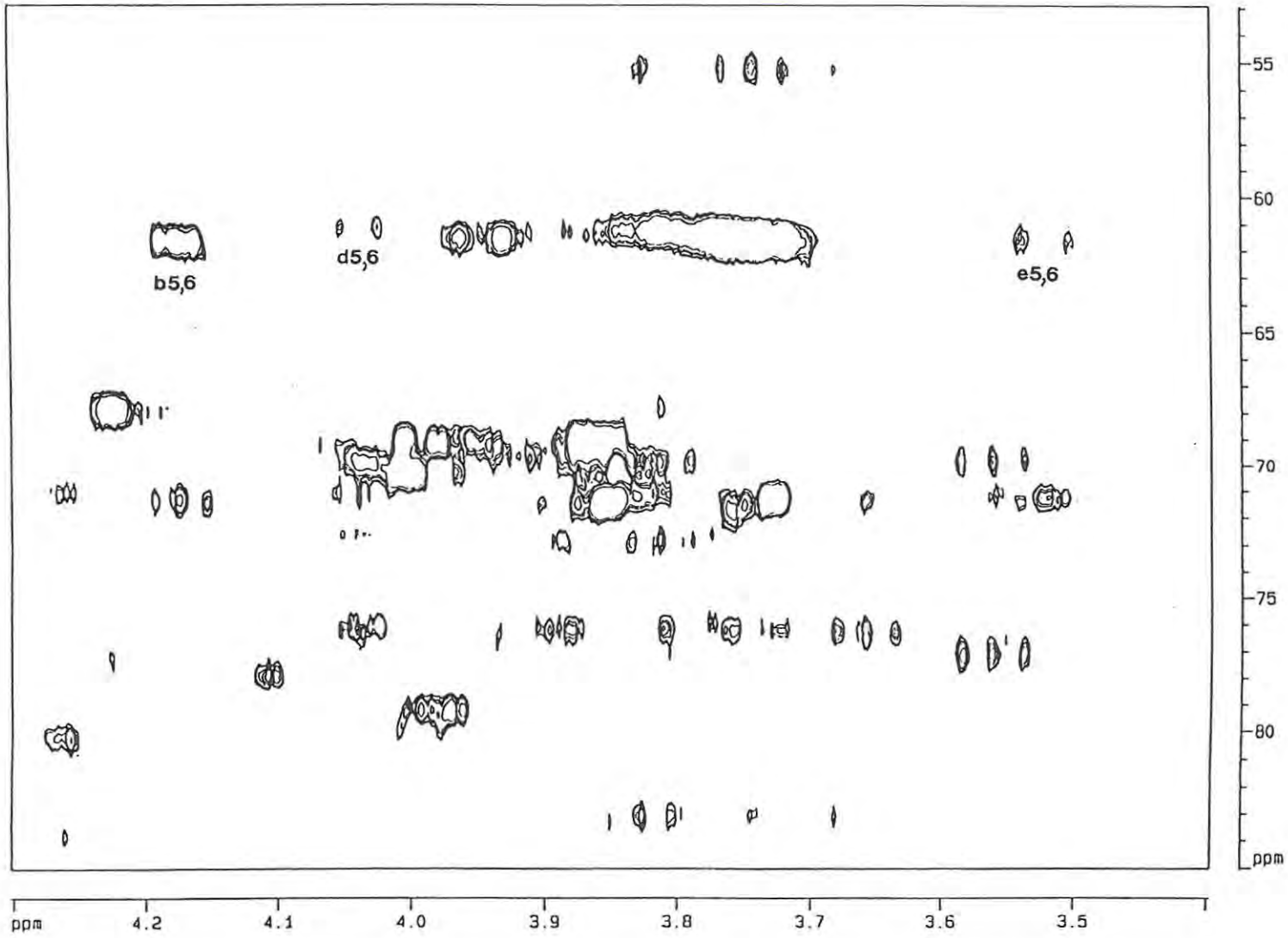


Figure 4.11 HMQC-TOCSY ^{1}H-^{13}C contour plot of the spectral region f_1 85-53 ppm and f_2 δ 4.3-3.4 for **DPS** at 323K. The respective connectivities between H-5 and C-6 are indicated for residues **b**,**d** and **e**.

The latter showed C-6/H-5 relays for both residues which permitted the assignment of the C-6 chemical shifts, (Figure 4.11). Comparison of the ^{1}H- and ^{13}C-NMR data for residues a-e with literature values for methyl glycosides[292,293] permitted the residues in the repeating unit to be identified as indicated in Table I, and their linkage positions to be established. In agreement with the results from the methylation analysis, C-4 of **a**, C-2 of **b**, C-3 of **c**, C-4 of **d** and C-3 of **e** experienced significant deshielding.

Sequencing — The sequence of the sugar residues in the repeating unit was established from an HMBC experiment[168]. The relevant inter- and intra-residue long range heteronuclear correlations are listed in Table II and establish the sequence shown below for the repeating unit. A NOESY experiment performed on the same sample supplied further proof of the sequence of the residues and showed that both residues **c** and **d** were α-linked since both residues showed strong H-1/H-2 intra-residue NOEs.

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

A strong NOE between the anomeric protons of residues **b** and **e** was also observed. This uncommon NOE confirms a 1→2 link between the latter two residues and has been observed previously for α-D-hexose residues substituted by a glycosyl group at *O*-2[427].

Location of the O-*acetyl group* — The position of the *O*-acetyl group was established from the COSY spectrum of **PS** and further confirmed by an HMBC experiment. The spin system for the 3-linked α-Rha residue, readily traced from the COSY spectrum, showed a connectivity between the signal at δ 5.21 and the anomeric signal at δ 4.97 which located the acetyl substituent at *O*-2 of this residue. A downfield shift of similar magnitude has previously been reported[428,429] for a rhamnose residue carrying a 2-*O*-acetyl group. The HMBC experiment showed a three-bond long-range correlation between the methine proton at δ 5.21 and the carbonyl carbon of the acetyl group at 174.17 ppm. A two-bond heteronuclear correlation between the carbonyl carbon at 174.17 ppm and the methyl protons of the acetyl group at δ 2.26 was also observed.

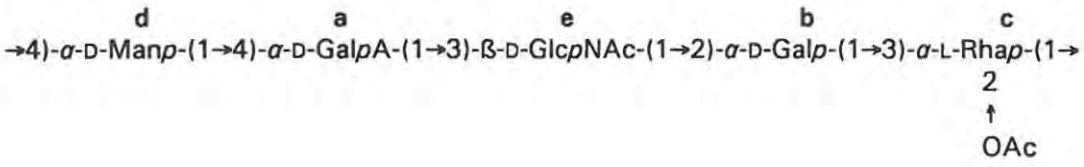
TABLE II TWO- AND THREE-BOND CORRELATIONS (HMBC) FOR K83 DPS

Residue	Anomeric proton	Long range contact to
a	5.34	69.00 (a, C-3) 71.25 (a, C-5) 83.16 (e, C-3)[a]
b	5.31	69.12 (b, C-3) 71.37 (b, C-5) 77.20 (c, C-3)
c	4.94	77.20 (c, C-3) 69.93 (c, C-5) 76.18 (d, C-4)
d	4.88	70.00 (d, C-3) 72.94 (d, C-5) 80.09 (a, C-4)
e	4.69	79.35 (b, C-2)

[a] Interresidue (linkage) connectivities underlined.

CONCLUSION

The combined NMR and methylation data permit the structure of the pentasaccharide repeating unit of the capsular polysaccharide of *E. coli* K83 to be written as:



The rhamnosyl residues in the *E. coli* K32[428] and K98[430] polysaccharides are also acetylated at *O*-2.

EXPERIMENTAL

General methods — Instrumentation used was as described for *Klebsiella* K15. A DB-17 bonded phase capillary column (30 m x 0.25 mm) having a film thickness of 0.25μm was used to separate alditol acetates and partially methylated alditol acetates (temperature programme : 180°C for 2 min then 3°.min^{-1} to 240°C, head pressure 100KPa). Acetylated octyl glycosides were prepared according to the procedure of Leontein *et al.*[143], (see pg 112) with an additional re *N*-acetylation step after hydrolysis and prior to octyl glycoside formation and acetylation. The resulting acetylated octyl glycosides were separated by GLC on a DB-225 column (temperature programme: 220°C for 5 min then 1°.min^{-1} to 235°C, column head pressure 150 KPa). *O*-Deacetylation was effected by heating a solution of **PS** in 0.1 M NaOH at 40°C for 4 h followed by passage over an amberlite IR-120(H^+) resin and recovery of *O*-deacetylated **PS** (**DPS**) by freeze-drying. Methanolyses were carried out with refluxing methanolic 3% HCl at 80°C for 16 h. Samples were carboxyl-reduced with NaBH4 in anhyd. MeOH and hydrolyses were performed with 4 M TFA at 125°C for 1 h. The molecular weight determination of **PS** was performed on a dextran-calibrated column of Sephacryl S500-HR (70 x 1.6 cm) using 0.1 M NaOAc buffer (pH 5.0) as eluent. Material was detected by refractive index.

Isolation and purification of K83 polysaccharide — An authentic culture of *E. coli* O20:K83:H26 bacteria (Culture No. CDC-134-51) was obtained from Dr. I. Ørskov (Copenhagen), and the capsular polysaccharide was isolated essentially as described for *Klebsiella* K15 (pg 109) with the following differences: 8 sterile culture tubes each containing a 6 h culture of encapsulated bacteria (10 mL) were poured directly onto 8 stainless steel trays (60 cm x 40 cm) containing 1L Mueller-Hinton agar (Appendix II) and the bacteria were propagated at 37°C for 18 h. The bacterial slime was harvested and the acidic capsular polysaccharide was isolated using CTAB as before. The crude material was found to be contaminated with RNA and this was removed by treatment with RNAse (Sigma Chemical Corporation) followed by ion-exchange chromatography on a DEAE-Sepharose CL-6B column (2.5 x 28 cm) with a NaCl gradient of 0.1-0.5 M.

Methylation analysis — Methylation was carried out according to the Hakomori procedure as modified by Phillips and Fraser[115]. **DPS** (15 mg), in the acid form, was dissolved in DMSO (1 mL) and potassium dimsyl (0.8 mL) was added, dropwise, under N_2 gas and allowed to stir for 1 h. The flask was cooled in an ice-bath and CH_3I (0.5 mL) was added dropwise over 3 min. Excess CH_3I was removed by displacement with N_2 gas and the contents of the flask were poured into chilled water and extracted with $CHCl_4$ (3x). The $CHCl_3$ extract was washed with 5% sodium thiosulphate solution (10 mL) prior to drying the $CHCl_3$ solution over anhyd. Na_2SO_4. PMAAs, with and without reduction of the carboxyl group of the uronic acid, were then prepared as previously described (section 4.1 pg 112).

NMR spectroscopy — Samples were deuterium-exchanged by freeze-drying solutions in D_2O and then dissolved in 99.99% D_2O (0.6 mL) containing a trace of acetone as internal reference (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). Spectra were recorded on a Bruker AMX-400 NMR spectrometer equipped with an X32 computer. All experiments were carried out at 323K and a 1 s recycle delay was used in each case. ^1H-Homonuclear shift-correlated (COSY) experiments on **PS** and **DPS** were performed using a spectral width of 2008 Hz, and data matrices of 256 X 2048 data points were collected with 128 transients for each t_1 increment. The matrices were zero-filled in the t_1 dimension to 1024 data points and, following the application of a non-shifted sine-bell window function, the data were transformed and symmetrised. Homonuclear Hartmann-Hahn (HOHAHA) and NOESY spectra were obtained using the same spectral width but with initial data matrices of 512 X 2048 data points prior to zero-filling. For the HOHAHA experiment a mixing time of 84 ms was used with sine-squared filtering in t_1 and t_2 and for the NOESY experiment a 0.2 s mixing delay was used and a phase-shifted sine-squared window function was applied during transformation. Inverse experiments: HMQC, 512 X 4096 data matrix with 48 scans per t_1 increment ; HMQC-TOCSY, 256 X 4096 data matrix, 96 scans per t_1 increment, mixing time 25 ms ; HMBC, 256 X 4096, 112 scans per t_1 increment, $\Delta 1$ and $\Delta 2$ durations of 3.45 and 60 ms respectively and a sine squared filter. All three inverse experiments were processed with a final data matrix of 1024 X 2048 points.

4.3 STRUCTURAL ELUCIDATION OF THE CAPSULAR POLYSACCHARIDE PRODUCED BY *Escherichia coli* O20: K84: H26

ABSTRACT

The structure of the capsular antigen produced by *E. coli* K84 has been established, primarily by 1D and 2D ^{1}H- and ^{13}C NMR studies of the polysaccharide, and by glycose and methylation analysis and found to be comprised of linear hexasaccharide repeating units of the following structure:

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

INTRODUCTION

Bacteria of the genus *Escherichia coli* have been broadly subdivided into two groups, based primarily on differences in chemical composition, but also in terms of other factors such as mode of expression and genetic determination[42]. Group I polysaccharides, which are co-expressed only with O8, O9, and O20, tend to be less structurally diverse than the Group II antigens and have been further sub-divided according to the presence or absence of amino sugars. The *E. coli* K84 capsular antigen may now be classified as an amino sugar-containing Group I polysaccharide. It resembles the K83 capsular polysaccharide (section 4.2), which is also co-expressed with O20 and H26, in that it too is a linear polysaccharide. A hexasaccharide repeating unit is, however, unusual as the average number of residues per repeating unit in this genus is four, and larger repeating units are frequently branched. It is also the first *E. coli* capsular antigen found to contain ManNAc. Interestingly, the *E. coli* K50[69] polysaccharide, the structure of which was established in this laboratory shortly after this study, also contains ManNAc, although it is α-linked in this instance.

RESULTS AND DISCUSSION

Isolation, composition and linkage analysis — The bacteria were grown on Mueller-Hinton agar and the capsular polysaccharide was isolated in the conventional manner using CTAB. The acidic K-antigen precipitated as a gel-like mass only after the crude polysaccharide was treated with 1% acetic acid and the lipid so released was removed by ultracentrifugation. After dissolution of the CTAB-polysaccharide complex in 3 M NaCl, the material was reprecipitated into ethanol and the precipitated polysaccharide was further purified by dialysis and GPC on Sephacryl S-500. The polysaccharide (**PS**) was resistant to acid hydrolysis and the constituent sugars were not released in molar proportions. Man, Glc, GalA, GlcN and ManN were determined to be the constituent monosaccharides by analytical GLC-MS of the derived alditol or *O*-methyloxime acetates following hydrolysis, and methanolysis with carboxyl reduction. GLC analysis of the derived acetylated (-)-2-octyl glycosides showed that all the sugars had the D-configuration. **PS** was methylated according to a modified Hakomori[115] procedure, and the partially methylated alditol acetates derived from an acid hydrolysate of methylated **PS**, with and without carboxyl reduction, revealed the presence of 4-linked GalA, 3-linked Man, 3-linked GlcN, 3-linked ManN, 2-linked Man, and 4-linked Glc. This linkage pattern is consistent with a linear hexasaccharide repeating unit.

NMR spectroscopy — The proton NMR spectrum of **PS** at 313K contained nine resonances in the anomeric region (δ 4.5-5.5) at δ 5.29, 5.25, 5.17, 4.93, 4.83, 4.75, 4.72, 4.57 and 4.55. In addition, signals for methyl protons of acetyl functions were present at δ 2.10 and 2.04. Coupling constants could only be measured for the signals at δ 5.29 (4.1 Hz) and 5.17 (3.4 Hz), indicative of two α-linked sugars, and for a ß-signal at δ 4.57 (8.1 Hz), after acquiring a 1D spectrum at 343K to improve resolution. The anomeric configurations of the remaining sugars were established from the NOESY spectrum (see below). The ^{13}C spectrum of **PS** showed only six signals for pyranosidically linked hexoses at 97.13, 99.02, 100.34, 101.46, 101.96 and 103.44 ppm, thus proving that three of the nine resonances in the anomeric region of the proton spectrum were non-anomeric.

Signals at 50.57 and 53.70 ppm for acetamido substituted carbons and resonances for carbonyl carbons at 175.80 and 175.29 ppm, and for methyl carbons (2xC) at 22.94 ppm, were consistent with the presence of two *N*-acetylated amino sugars in the repeating unit. An additional signal for a carbonyl carbon at 173.27 ppm was assigned to C-6 of a uronic acid.

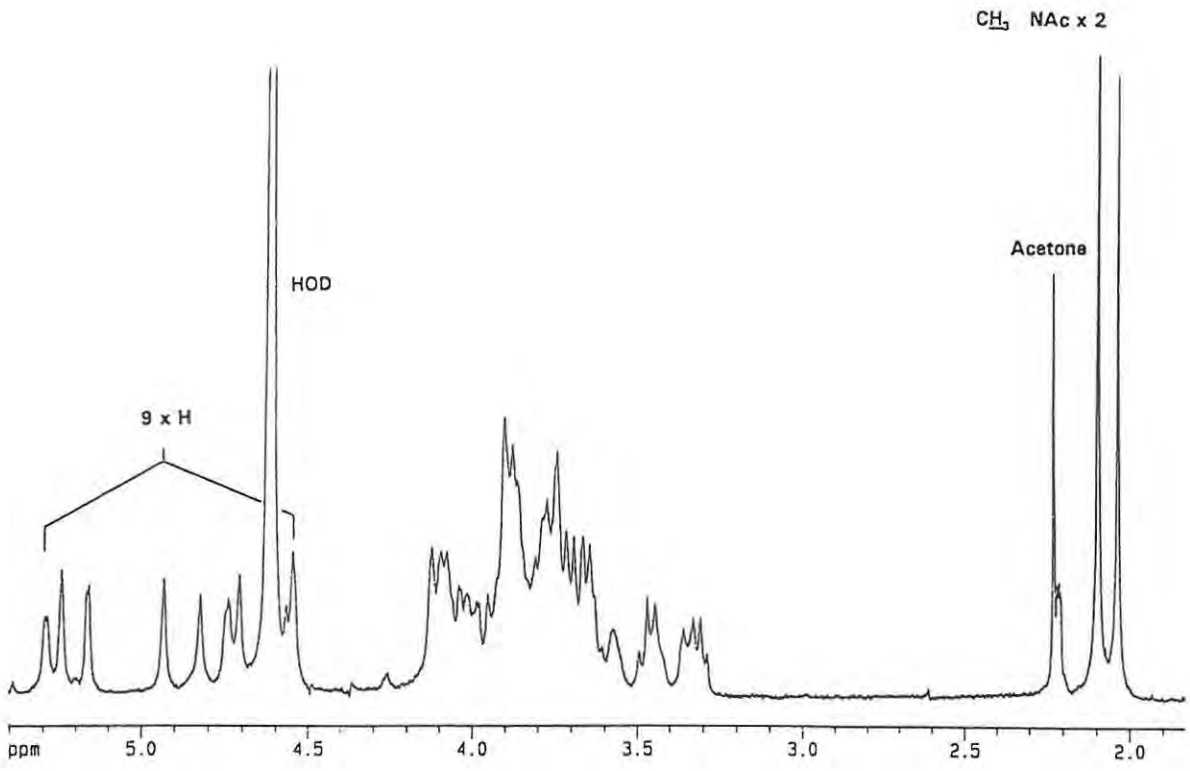


Figure 4.12 ^{1}H NMR spectrum of K84 **PS** at 313K.

The remaining ^{1}H and ^{13}C resonances for the spin systems of the six sugars in the repeating unit were assigned using COSY[105], HOHAHA[342], HMQC[365], HMQC-TOCSY[357] and NOESY[346] experiments and these data are presented in Table I. Sugars are labelled **a**-f in decreasing order of the chemical shifts of their H-1 resonances.

TABLE I

^{1}H and ^{13}C CHEMICAL SHIFT DATA FOR K84 PS AT 313K

Residue		1	2	3	4	5	6a	6b
a	H	5.29[a]	3.83	4.09	4.55	4.72	-	-
→4)-α-GalA	C	101.46	69.14	70.16	78.68	71.34	173.27	-
b	H	5.25	4.13	3.88	3.86	3.74	*	*
→3)-α-Man	C	101.96	70.92	79.9	66.52	74.55	*	
c	H	5.17	4.03	4.07	3.67	4.10	3.92	3.73
→3)-α-GlcNAc	C	99.02	53.70	81.20	68.78	71.71	60.82	
d	H	4.93	4.75	4.01	3.47	3.35	3.88	3.80
→3)-β-ManNAc	C	100.34	50.57	77.93	65.76	77.08	*	
e	H	4.83	3.91	3.77	3.70	3.43	3.97	3.78
→2)-β-Man	C	97.13	77.08	74.33	68.09	77.61	61.82	
f	H	4.57	3.32	3.65	3.63	3.57	3.90	3.77
→4)-β-Glc	C	103.44	74.00	76.82	77.08	75.41	*	
CH_3 (NAc)	H	2.10 / 2.04[b]						
	C	22.94 / 22.94						
C=O (NAc)	C	175.80 / 175.29						

[a] Chemical shifts in ppm with acetone as internal reference, δ 2.23 and 31.07 ppm for ^{1}H and ^{13}C respectively. [b] Methyl proton assignments interchangeable. * Not assigned.

*Residue **a*** [→4)-α-GalA] — The ^{1}H resonances for residue **a** were readily established *via* their cross-peaks in the COSY spectrum and further confirmation followed from cross-peaks observed in the HOHAHA spectrum. The chemical shifts of the corresponding directly coupled ^{13}C nuclei were then assigned by comparing the ^{1}H resonances obtained above with the ^{1}H-^{13}C correlation data obtained from an HMQC spectrum of **PS** (Figure 4.14). H-4 (δ 4.55) and H-5 (δ 4.72) of this residue account for two of the three non-anomeric signals present in the anomeric region. Such low-field chemical shifts for H-4 and H-5 are a characteristic of →4)-α-GalA residues.

*Residue **b*** [→3)-α-Man] — Assignment of the ^{1}H resonances for this residue was problematic due to considerable overlap with the cross-peaks of other spin systems in the COSY spectrum, as well as partial overlap within its own spin system. Only H-1 to H-3 could be assigned from the COSY spectrum. Once these signals and all the ^{1}H-^{13}C correlation data for residues **a**, **c**, **d**, **e** and **f** (see below) had been assigned in the HMQC spectrum, two sets of ^{1}H-^{13}C correlations (excluding H-6/C-6 correlations) remained unassigned, therefore by default these had to represent H-4/C-4 and H-5/C-5 for residue **b**. The set at 3.86/66.52 was assigned to the former and the set at 3.74/74.55 to the latter on the basis of literature values which show that C-5 is usually to lower field in 3-linked mannose residues.

*Residue **c*** [→3)-α-GlcNAc] — The proton spin system for this sugar (H-1 through H-6a/6b) could be assigned from the COSY spectrum, but only with assistance from the HOHAHA experiment, as partial signal overlap between H-3 and H-5 lead to a measure of confusion. All the corresponding carbon resonances were assigned from the HMQC spectrum.

*Residue **d*** [→3)-ß-ManNAc] — The entire spin system for this residue was readily established by following the cross-peaks in the COSY (Figure 4.13) and HOHAHA spectra and the ^{13}C data for C-1 to C-5 were then assigned from the ^{1}H-^{13}C correlation data obtained from the HMQC spectrum. H-2 (δ 4.75) of this residue could clearly be identified as the third non-anomeric signal between δ 4.5 and 5.5. Due to excessive overlap in the HMQC spectrum and the absence of an H-5/C-6 relay in the HMQC-TOCSY spectrum, the chemical shift for C-6 could not be assigned with certainty.

Residue e [→2)-ß-Man] — The assignment of resonances for this residue was also problematic due to overlap in the COSY spectrum which obscured the H-2/H-3 cross-peak. Only the H-2 chemical shift could be assigned initially. This remaining resonances for this residue were assigned as follows: The ^{13}C chemical shift for C-2 (77.08 ppm) correlated to H-2 (δ 3.91) was obtained from the HMQC spectrum and, using this as a window, relayed peaks were sought in the HMQC-TOCSY spectrum. Long range heteronuclear correlations were observed from C-2 (77.08 ppm) to proton signals at δ 3.70 and 3.77 (see Figure 4.15). Returning to the COSY spectrum, the signal at δ 3.70 could be assigned to H-4, as the cross-peak linking it to H-5 and the rest of the correlated spin system was clearly visible. The signal at δ 3.77 therefore clearly belonged to H-3. The remaining ^{13}C signals could then be assigned by inspection from the HMQC spectrum. Intra-residue NOEs observed for this residue in the NOESY spectrum (see Table II) provided further supportive evidence for these assignments. Strong H-1/H-3 and H-1/H-5 NOEs confirmed the H-3 (δ 3.77) and H-5 (δ 3.43) chemical shifts assigned above.

Residue f [→4)-ß-Glc] — ß-Glc residues are normally easy to assign as their stereochemistry is conducive to strong magnetisation transfer; however in this instance almost complete resonance overlap between H-3 and H-4 and poor magnetisation transfer between H-5 and H-6a/6b precluded the unambiguous assignment of the entire spin system from the COSY spectrum. Examination of a 1D slice through the cross-peaks relaying to H-1 in the HOHAHA spectrum revealed overlap of H-3 and H-4 and permitted elucidation of the proton spin system. Correlation of these resonances with their corresponding carbon nuclei *via* the correlation data in the HMQC spectrum permitted assignment of C-1 through C-5. It is interesting to note that the ^{13}C chemical shifts for C-3 and C-4 are also very close together. Surprisingly, C-6 could not be assigned with certainty due to the absence of an H-5/C-6 relay in the HMQC-TOCSY spectrum. There therefore appears to be a conformational constraint of some kind, possibly due to glycosylation at C-4, which is affecting magnetisation transfer.

Comparison of the ^{1}H and ^{13}C chemical shift data for residues a through f with literature values for methyl glycosides[292,293] permitted identification of the six sugar residues and, in agreement with

the linkage pattern obtained from methylation analysis, C-4 of **a**, C-3 of **b**, C-3 of **c**, C-3 of **d**, C-2 of **e**, and C-4 of **f** experienced significant deshielding. It is interesting to note that the proton chemical shifts, particularly those for residues **b** and **e** (the two mannose residues) do not reliably indicate the linkage positions e.g H-2 of residue **b** experiences far greater deshielding than H-3, the linkage site. This underlines the necessity for obtaining full ^{13}C chemical shift information in structural studies, especially in the absence of methylation data.

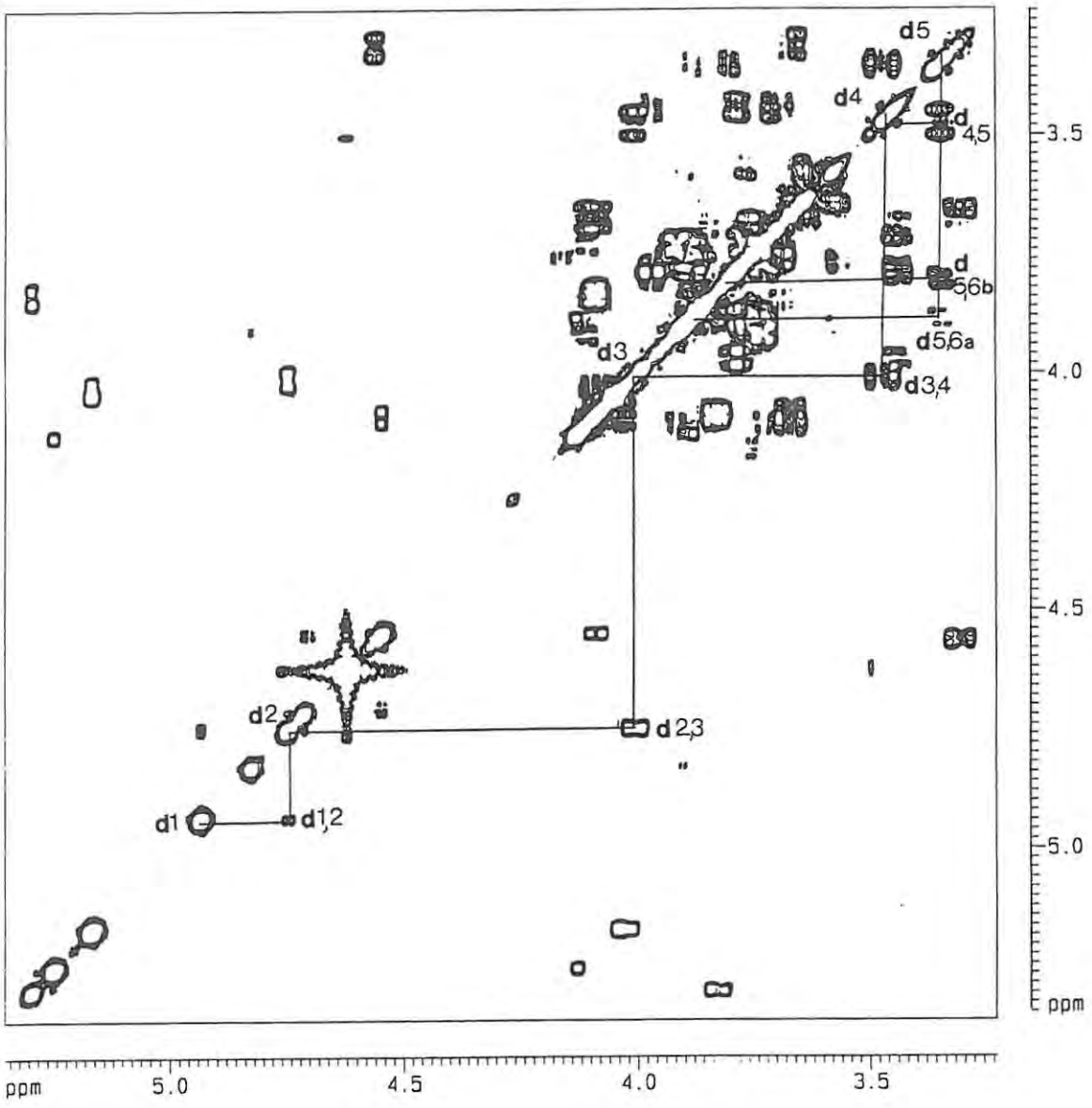


Figure 4.13. COSY contour plot of the region δ 5.5-3.3 of K84 PS at 313K. The *J*-coupled spin system for residue **d** (→3)-ß-ManNAc) is shown. d1 connotes H-1 and **d**1,2 connotes the cross-peak between H-1 and H-2 of residue **d**, etc.

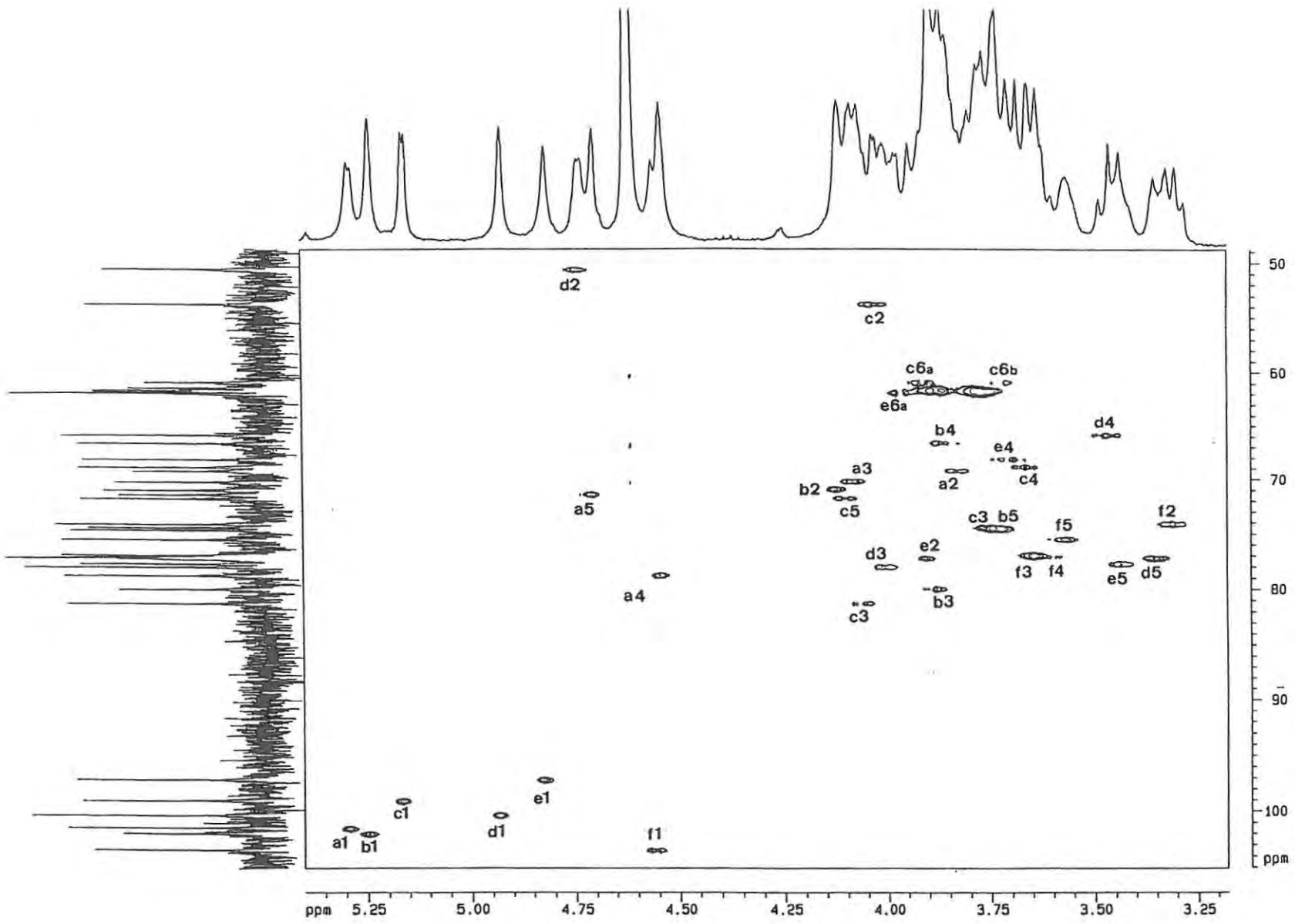


Figure 4.14 HMQC ^{1}H-^{13}C shift correlation map of the region f_1 110-48 ppm and f_2 5.4-3.2 for K84 **PS** at 313K. Correlated resonances are labelled a-f.

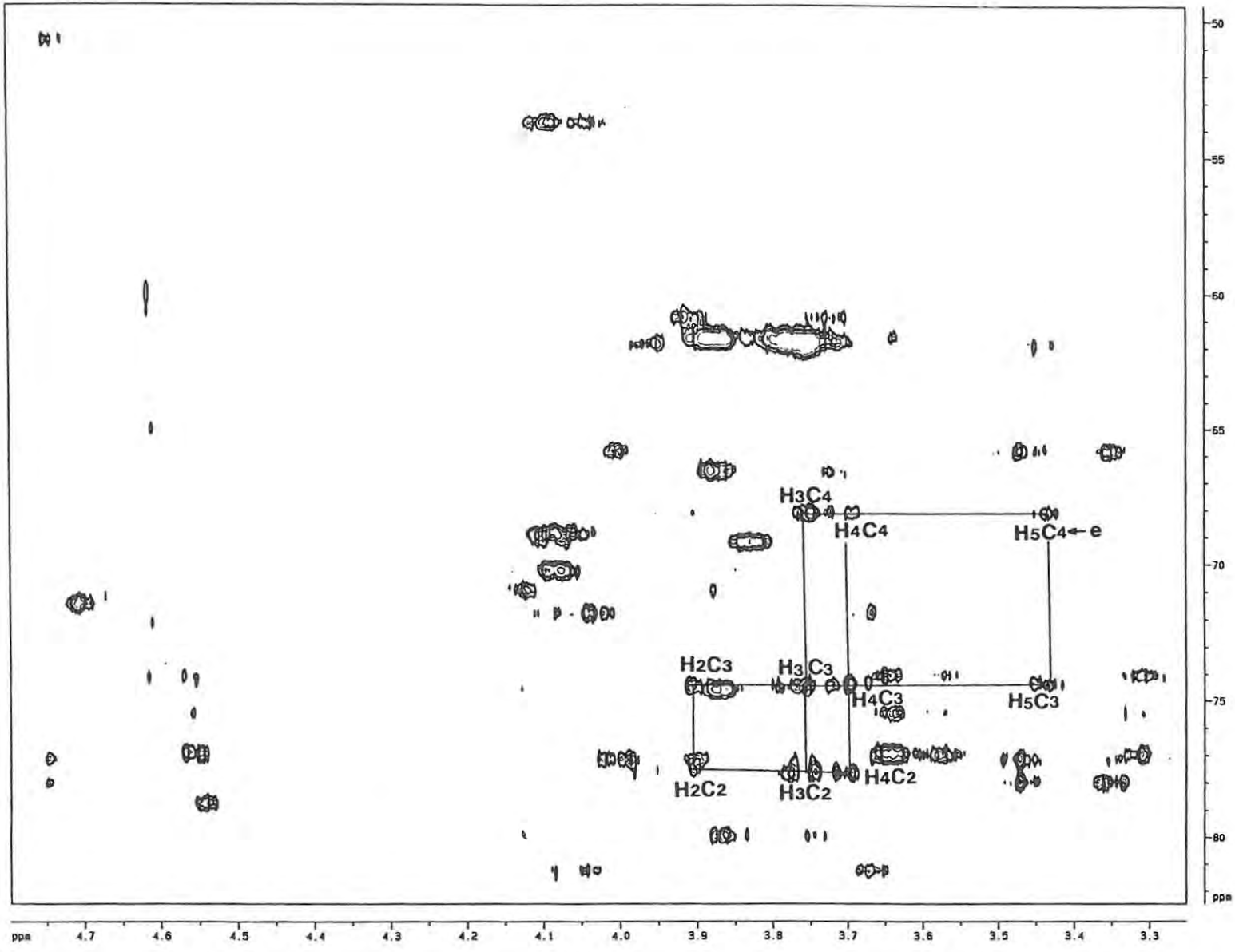


Figure 4.15 HMQC-TOCSY ^{1}H-^{13}C shift correlation map of the region f_1 84-50 ppm and f_2 4.8-3.3 for K84 **PS** at 313K. Relayed connectivities for residue **e** are indicated.

Sequencing the repeating unit — The sequence of the residues in the repeating unit was established from inter-residue NOEs obtained from the NOESY spectrum of **PS**. (Figure 4.16).

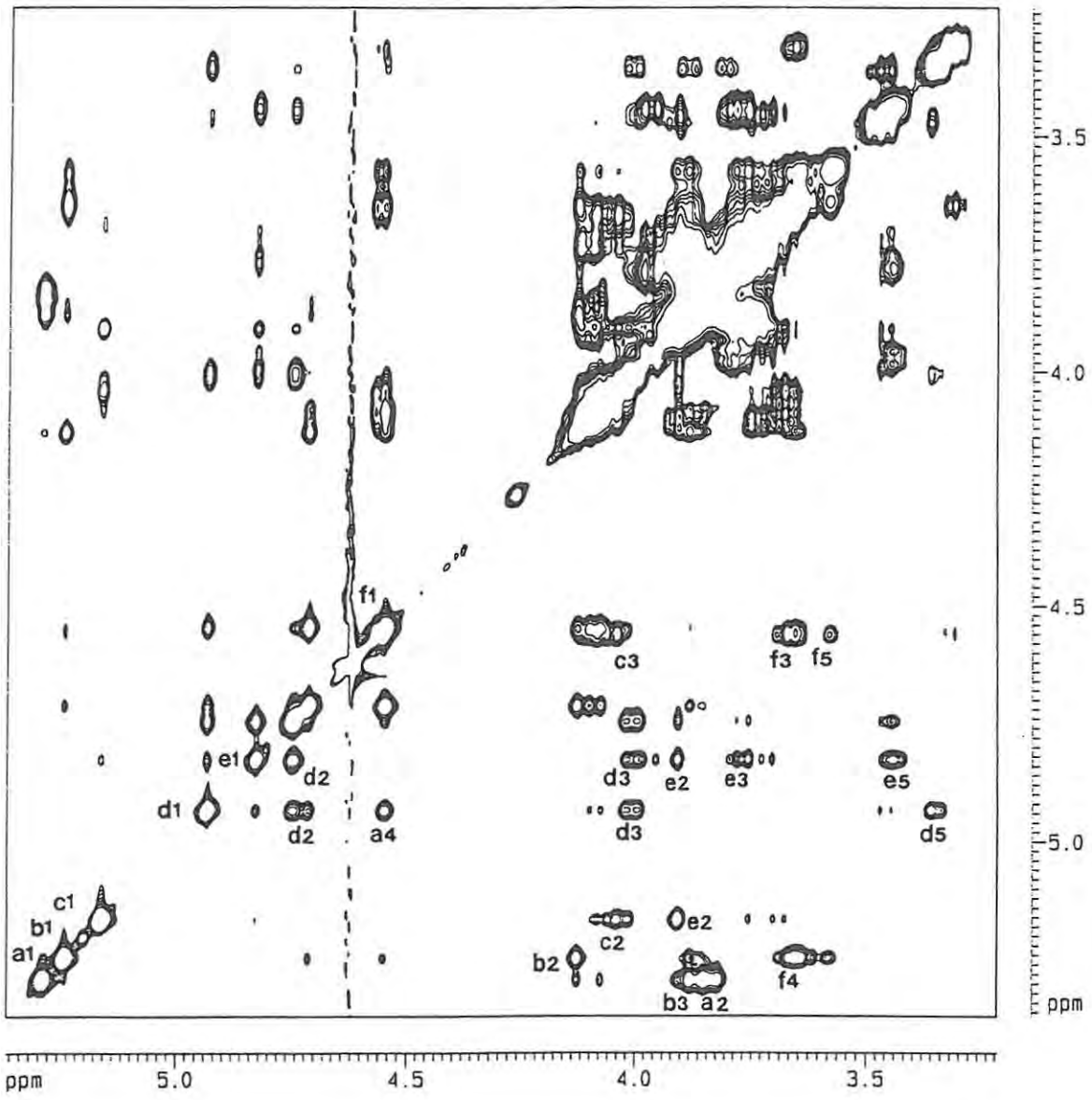


Figure 4.16 2D NOESY spectrum of K84 **PS** at 313K. Relevant intra- and inter residue NOEs are denoted on the spectrum and are recorded in Table II.

TABLE II

OBSERVED NOEs FOR K84 PS

RESIDUE	ANOMERIC PROTON	NOE AT
a	H-1 (5.29)	3.83 (**a**, H-2) 3.88 (**b**, H-3)
b	H-1 (5.25)	4.13 (**b**, H-2) 3.63 (**f**, H-4)
c	H-1 (5.17)	4.03 (**c**, H-2) 3.91 (**e**, H-2)
d	H-1 (4.93)	4.75 (**d**, H-2) 4.55 (**a**, H-4) 4.01 (**d**, H-3) 3.35 (**d**, H-5)
e	H-1 (4.83)	4.75 (**d**, H-2) 4.01 (**d**, H-3) 3.91 (**e**, H-2) 3.77 (**e**, H-3) 3.43 (**e**, H-5)
f	H-1 (4.57)	4.07 (**c**, H-3) 3.65 (**f**, H-3) 3.57 (**f**, H-5)

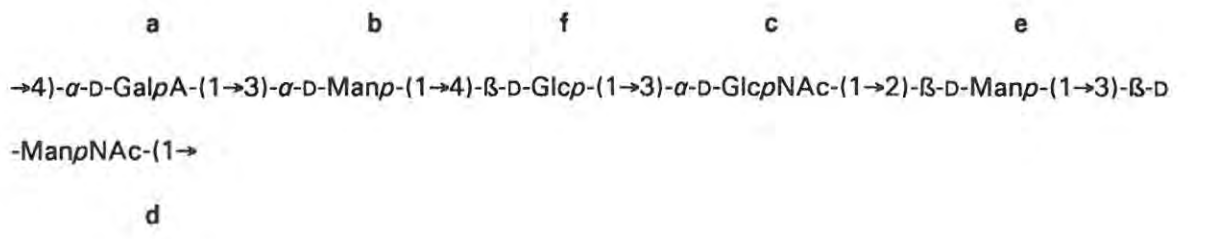
These connectivities are supportive of the following sequence for the repeating unit:

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

The linkage NOE between H-1 of **e** and H-3 of **d** was noted to be weaker than the NOE observed to H-2 of **d**. A stronger NOE to H-2 in 3-linked sugars having the *manno* configuration is frequently observed due to the fact that H-2 and H-3 are *gauche* with respect to each other. The anomeric configurations for residues **b**, **d** and **e**, which could not be assigned from $^{3}J_{1,2}$ coupling constant values being of the *manno* type, were established from intra-residue NOEs present in the NOESY spectrum. Residues **d** and **e** were clearly ß-linked as both residues showed H-1/H-2, H-1/H-3 and H-1,H-5 NOEs, whilst Residue **b**, which showed only an H-1/H-2 intra-residue NOE, was assigned the α-configuration.

CONCLUSION

The combined data obtained from chemical and NMR analysis of the polysaccharide are consistent with the following structure for the hexasaccharide repeating unit of the *E. coli* K84 capsular polysaccharide:



Further confirmatory studies were, however, considered desirable in view of the difficulty encountered in analysing the NMR spectra (see section 4.4).

EXPERIMENTAL

General methods — The general methods were essentially as described in section 4.2. The fused silica DB-17 capillary column was used to separate alditol acetates and partially methylated alditol acetates (operated on a temperature programme of 180°C for 2 min then $2°.min^{-1}$ to 240°C, head pressure 100 KPa) on account of the excessively long retention times for amino sugar derivatives on a DB-225 column. ManNAc, which co-elutes with GalNAc on both DB-225 and DB-17 was identified as its *O*-methyloxime derivative on a DB-Wax capillary column operated with the following temperature programme: 180°C for 2 min then $3°.min^{-1}$ to 240°C, column head pressure 140 KPa. Where necessary, samples of **PS** were converted into the acid form by passage down an amberlite IR-120 (H^+) resin column and subsequently freeze-dried prior to analysis.

Isolation and purification — An authentic culture of *E. coli* O20:K84:H26 bacteria (Culture No. CDC-2292-55) was obtained from Dr. I. Ørskov (Copenhagen). The bacteria were propagated (two batches of eight trays, Mueller-Hinton agar (Appendix II), 18 h, 37°C) and the capsular polysaccharide isolated, as described for *E. coli* K83 (pg 125), giving a total yield of 474 mg. An additional step to remove attached lipid (1% AcOH, 60°C, 1 h) was required prior to precipitation with CTAB as its presence was found to impair CTAB complex formation. Further purification of the isolated capsular polysaccharide was achieved by GPC on Sephacryl S-500 (100 x 2.5 cm) using aqueous acetate buffer (0.1 M) as eluent.

Sugar composition — **PS** (2 mg) was hydrolysed and the liberated sugars were converted into alditol acetates and analysed by GLC as previously described for *E. coli* K83. To establish the identity of the uronic acid **PS** (3 mg) was first methanolysed (3% methanolic HCl, 80°, 12 h), and the uronate ester reduced ($NaBH_4$) prior to derivatisation and analysis as described above. *O*-methyloxime derivatives were prepared as described by Neeser and Schweizer[72] to confirm the presence of ManNAc. An acid hydrolysate of **PS** (3 mg) was added to 0.5 mL of a mixture [prepared by dissolving *O*-methylhydroxylamine hydrochloride (300 mg) in dry CH_3OH (1 mL), pyridine (1.78 mL) and 1-dimethylaminopropanol (0.22 mL)], and heated (70-80°C, 20 min) with gentle agitation. After cooling to room temperature and drying under a stream of N_2, the resulting *O*-methyl oximes were acetylated (1:3 pyridine-Ac_2O), 80°C, 30 min) and analysed by GLC. The absolute stereochemistry of the sugars was established by analysis of the derived acetylated (-)-2-octyl glycosides as described for *E. coli* K83.

Methylation Analysis — **PS** (15 mg), in the free acid form, was methylated according to the Hakomori procedure as modified by Phillips and Fraser[115], and a portion (6 mg) of methylated **PS** was converted into PMAAs and analysed as previously described. The other portion (9 mg) of methylated **PS** was methanolysed (3% methanolic HCl, 80°C, 16 h), neutralised (Ag_2NO_3), and reduced ($NaBH_4$) prior to derivatisation and analysis as described above to ascertain the identity of the uronic acid.

NMR spectroscopy — Samples were deuterium exchanged by freeze-drying solutions in D_2O and then dissolved in 99.99% D_2O (0.6 mL) containing a trace of acetone as internal reference (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). Spectra were recorded on a Bruker AMX-400 NMR spectrometer equipped with an X32 computer and standard Bruker software. Experiments on **PS** were performed at 313K and the parameters used for the 2D experiments were as follows: COSY [256 x 2048 data matrix, zero-filled to 1024 data points in t_1, 1.0 s recycle delay, 112 scans per t_1 value, spectral width 2008 Hz and unshifted sine-bell filtering in t_1 and t_2 prior to transformation and symmetrisation]. HOHAHA [512 x 2048 data matrix, zero-filled to 1024 data points in t_1, 1.0 s recycle delay, 64 scans per t_1 value, mixing time 84 ms, spectral width 2008 Hz and shifted sine-squared filtering in t_1 and t_2]. NOESY [512 x 2048 data matrix, zero-filled to 1024 data points in t_1, 60 scans per t_1 value, 0.3 s mixing delay, and a phase-shifted sine-squared window function was applied during transformation. HMQC [512 x 4096 data matrix, zero-filled to 1024 data points in t_1, 52 scans per t_1 value, spectral width 14085 Hz in t_1 and 2008 Hz in t_2, and 1.0 s recycle delay. HMQC-TOCSY [512 x 4096 data matrix, zero-filled to 1024 data points in t_1, 48 scans per t_1 value, MLEV-17 mixing time 25 ms, spectral width 14085 Hz in t_1 and 2008 Hz in t_2, and a 1.0 s recycle delay.

4.4 DEGRADATIVE STUDIES ON THE AMINO SUGAR-CONTAINING POLYSACCHARIDES PRODUCED BY *Escherichia coli* SEROTYPES K38 AND K84 WITH LITHIUM DISSOLVED IN ETHYLENEDIAMINE

ABSTRACT

The amino sugar-containing polysaccharides produced by *E. coli* K38 and K84 have been selectively cleaved at the sites of the in-chain uronic acid residues using lithium dissolved in ethylenediamine. The reaction, initially performed on the K38 polysaccharide (Study A), resulted in the formation of the expected tetrasaccharide terminated by glucosaminitol (oligosaccharide **1**), together with a secondary product due to subsequent loss of the terminal ribofuranose residue (oligosaccharide **2**). Degradation of the K84 polysaccharide (Study B) yielded only a single pentasaccharide terminated by mannosaminitol *viz.* oligosaccharide **3**. All structures were determined using 1D and 2D ^{1}H NMR spectroscopy.

STUDY A:

ß-D-Rib*f*-(1→4)-ß-D-Gal*p*-(1→3)-ß-D-Gal*p*NAc-(1→4)-D-GlcNAc-ol ..... **(1)**

ß-D-Gal*p*-(1→3)-ß-D-Gal*p*NAc-(1→4)-D-GlcNAc-ol ..... **(2)**

STUDY B:

α-D-Man*p*-(1→4)-ß-D-Glc*p*-(1→3)-*α*-D-Glc*p*NAc-(1→2)-ß-D-Man*p*-(1→3)-D-ManNAc-ol ..... **(3)**

INTRODUCTION

Owing to the difficulty experienced in analysing the NMR spectra used to establish the primary structure of the K84 polysaccharide (section 4.3), selective degradation to produce a fragment suitable for further confirmatory NMR studies was considered desirable.

Lithium metal dissolved in ethylenediamine has been shown to be a useful means of selectively cleaving acidic bacterial polysaccharides at the sites of the uronic acid residues[216] and has found application in structural studies on several *Klebsiella* and *E. coli* capsular polysaccharides (see section 3.5.1 (v)). However, a literature search revealed that, in all instances, only acidic polysaccharides containing neutral and deoxyhexoses had previously been degraded by this method. No example of its application to an amino sugar-containing acidic polysaccharide was found thus prompting the present study. Prior to degradation of the K84 polysaccharide, a modified reaction procedure was developed using the K38 capsular polysaccharide as a substrate. It resembles the K84 polysaccharide in that it too is linear and contains two amino sugars, one of which is also linked to *O*-4 of an *α*-GalA residue.

Structure of the *E. coli* K38 repeating unit:

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

Proposed structure of the *E. coli* K84 repeating unit:

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

RESULTS AND DISCUSSION

STUDY A: Degradation of the E. coli K38 polysaccharide — A sample of *E. coli* K38 polysaccharide was degraded, essentially as previously described[216], but with certain modifications. The sample was dissolved in ethylenediamine and sufficient lithium wire was added to maintain a deep blue colour for one hour. It is noteworthy that for this polysaccharide considerably more lithium wire was required to maintain the blue colour than hitherto observed for amino sugar-free polysaccharides degraded in this laboratory. Thereafter, as before, the reaction mixture was cooled, quenched with water and, after prior removal of the ethylenediamine and water under reduced pressure as the toluene azeotrope, an aqueous solution of the dried residue was titrated

to pH 4.5 with acetic acid and concentrated. The large quantity of lithium wire required in the first step (converted to lithium acetate by the addition of acetic acid), resulted in the solution becoming progressively more viscous during concentration. Consequently, this had a limiting effect on the amount of polysaccharide which could be degraded at any one time. In the published procedure[216], this salt is removed by passage down a cation-exchange resin or alternatively by dialysis if the lithium degraded product is polymeric. However, both of these techniques were inappropriate in this instance due to the low molecular weight of the product and the probability of the *N*-deacetylated amino sugars formed during the strongly basic degradation binding to the exchange resin. Therefore desalting on a Bio-Gel P-2 column was performed in two stages, with the sample suitably diluted to reduce the viscosity, and the products of the reaction were isolated, re *N*-acetylated, and refractionated (see Figure 4.17).

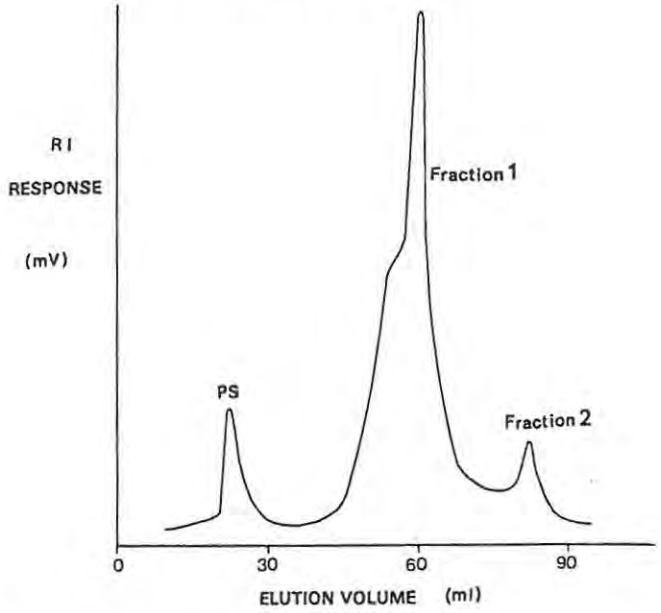


Figure 4.17. Elution diagram of the products of lithium degradation of the K38 polysaccharide on Bio-Gel P-2.

Two principle fractions were isolated, *viz.* a mixed oligosaccharide fraction and a fraction eluting in the monosaccharide region. Examination of the former by 1D ^{1}H NMR spectroscopy revealed the presence of two oligosaccharides which were mostly in the non-reducing form. This fraction was therefore subjected to a further reduction step and the two components (oligosaccharides 1 and 2) were separated using semi-preparative HPLC, prior to re-examination by NMR spectroscopy. The second fraction was identified as ribitol by GLC of its pentaacetate thus indicating that some loss of the ribofuranose residue from the primary oligosaccharide product had occurred.

NMR spectroscopy — Comprehensive NMR studies of oligosaccharides 1 and 2 were not undertaken, however 1D ^1H and COSY spectra of the latter permitted the assignments detailed in Table I. The sequences were assigned by comparison with the structure and the assignments reported for the *E. coli* K38 polysaccharide[431].

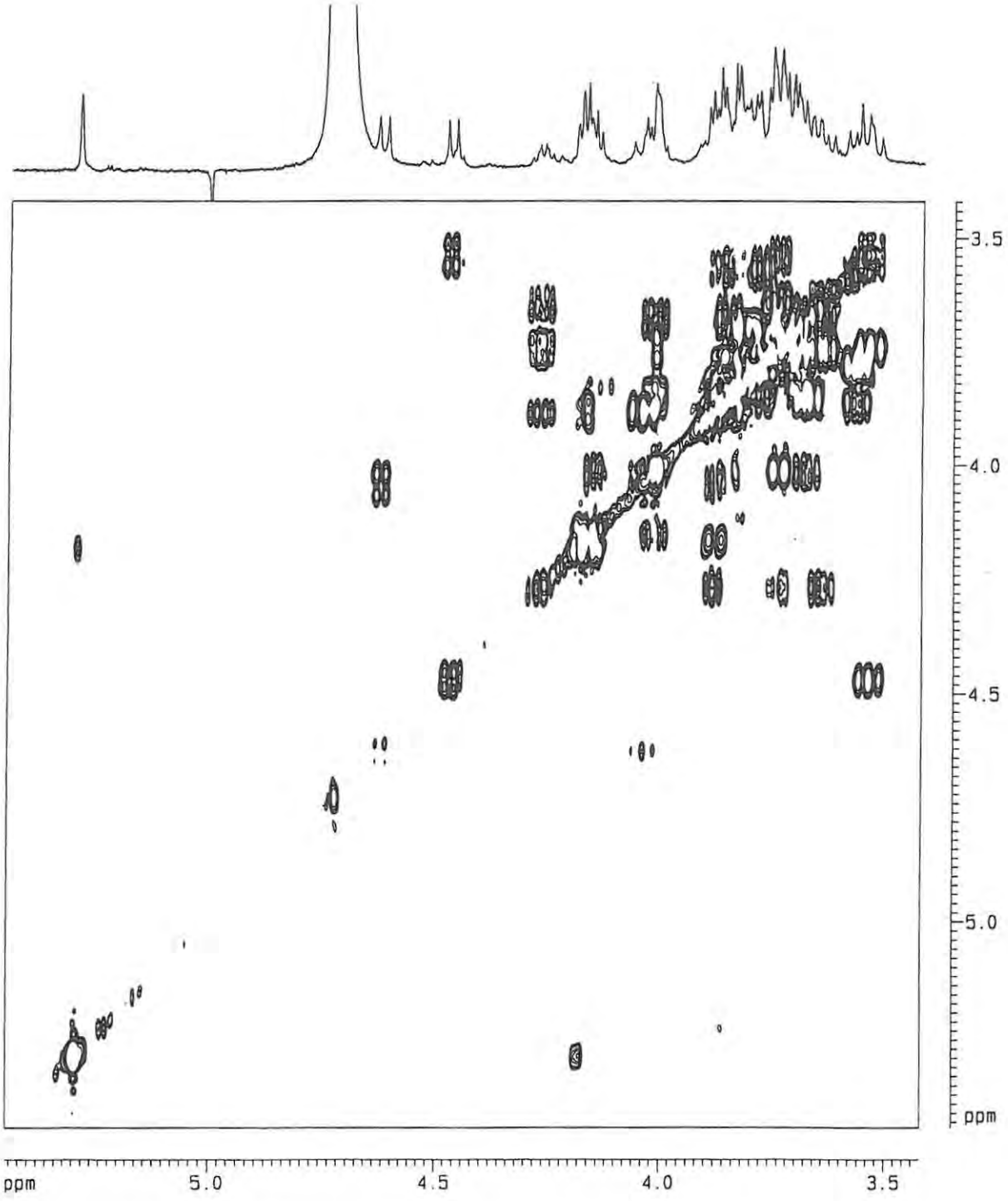


Figure 4.18. COSY contour plot of oligosaccharide 1 for the region δ 3.5-5.5. A 1D projection is displayed along the f_2 axis.

TABLE I ^{1}H CHEMICAL SHIFT VALUES[1] FOR OLIGOSACCHARIDES 1 and 2

Residue	*Oligosaccharide* 1		*Oligosaccharide* 2	
ß-D-Rib*f*	H1	5.30 (5.40)[2]		
	H2	4.18 (4.29)		
	H3	4.15 (4.27)		
	H4	4.02 (4.09)		
	H5a	3.85 (3.88)		
	H5b	3.69 (3.69)		
→3)-ß-D-GalNAc	H1	4.63 (4.63)	→3)-ß-D-GalNAc	4.63 (4.63)
	H2	4.03 (4.07)		4.03 (4.07)
	H3	3.88 (3.92)		3.89 (3.92)
	H4	4.16 (4.20)		4.18 (4.20)
	H5	* (3.73)		* (3.73)
	H6a	* (3.81)		* (3.81)
	H6b	* (3.81)		* (3.81)
→4)-ß-D-Gal	H1	4.47 (4.46)	ß-D-Gal	4.46 (4.46)
	H2	3.54 (3.54)		3.54 (3.52)
	H3	3.74 (3.79)		3.63 (3.79)
	H4	4.05 (4.05)		3.92 (4.05)
	H5	* (3.77)		* (3.77)
	H6a	* (3.77)		* (3.77)
	H6b	* (3.77)		* (3.77)
→4)-D-GlcNAc-ol	H1a	3.74 (3.73)[3]	→4)-D-GlcNAc-ol	3.74 (3.73)
	H1b	3.65 (3.64)		3.65 (3.64)
	H2	4.25 (4.07)		4.25 (4.08)
	H3	3.88 (3.96)		3.88 (3.96)
	H4	3.82 (3.61)		3.82 (3.61)
	H5	3.57 (3.76)		3.57 (3.76)
	H6a	3.87 (3.82)		3.87 (3.82)
	H6b	3.78 (3.65)		3.78 (3.65)

[1] Chemical shifts with acetone as internal reference at δ 2.23. [2,3]Chemical shifts in parentheses are those previously assigned for the corresponding residues in the *E. coli* K38 polysaccharide[431] and for *N*-Acetyl glucosaminitol respectively. * = not assigned.

STUDY B: Degradation of the E. coli K84 polysaccharide — A sample of the K84 polysaccharide was degraded exactly as outlined for the K38 polysaccharide above. This polysaccharide also required desalting in two stages due to the amount of lithium initially required and the resultant increase in the viscosity of the concentrate. A single oligosaccharide-alditol (oligosaccharide **3**) was isolated using GPC, was further purified using semi-preparative HPLC, and was subjected to detailed NMR analysis.

^{1}H NMR studies on oligosaccharide 3 — The 1D ^{1}H NMR spectrum of oligosaccharide **3** (Figure 4.19) contained four signals in the anomeric region at δ 5.29, 5.26, 4.81 and 4.57. $^3J_{1,2}$ coupling constants could be measured for the signals at δ 5.26 (2.9 Hz) and 4.57 (7.9 Hz), consistent with an α- and a ß-linked residue respectively. Assignment of the chemical shifts of the remaining protons in the *J*-coupled spin systems of the four sugar residues and the terminal alditol was accomplished using COSY-PR (COSY with presaturation during relaxation delay), HOHAHA[342] and ROESY[348] experiments (Table II). The five constituent residues are labelled **a** to **e** in decreasing order of the chemical shifts of their H-1 resonances. The anomeric configurations for residues **a** and **c** were obtained from a ROESY spectrum of oligosaccharide **3**. Residue **a** was determined to be α from the observed intra-residue NOE to H-2, and residue **c**, which showed strong intra-residue connectivities between H-1 and H-2, H-1 and H-3, and H-1 and H-5, was clearly ß-linked.

The sequence of the residues was also established from the ROESY spectrum of oligosaccharide **3**. Inter-residue NOEs were observed between H-1 of **a** and H-4 of **d**, H-1 of **d** and H-3 of **b**, H-1 of **b** and H-2 of **c**, and between H-1 of **c** and H-3 of **e**, thus enabling the structure of the lithium degraded product to be written as:

a **d** **b** **c** **e**

α-D-Man*p*-(1→4)-ß-D-Glc*p*-(1→3)-α-D-Glc*p*NAc-(1→2)-ß-D-Man*p*-(1→3)-D-ManNAc-ol

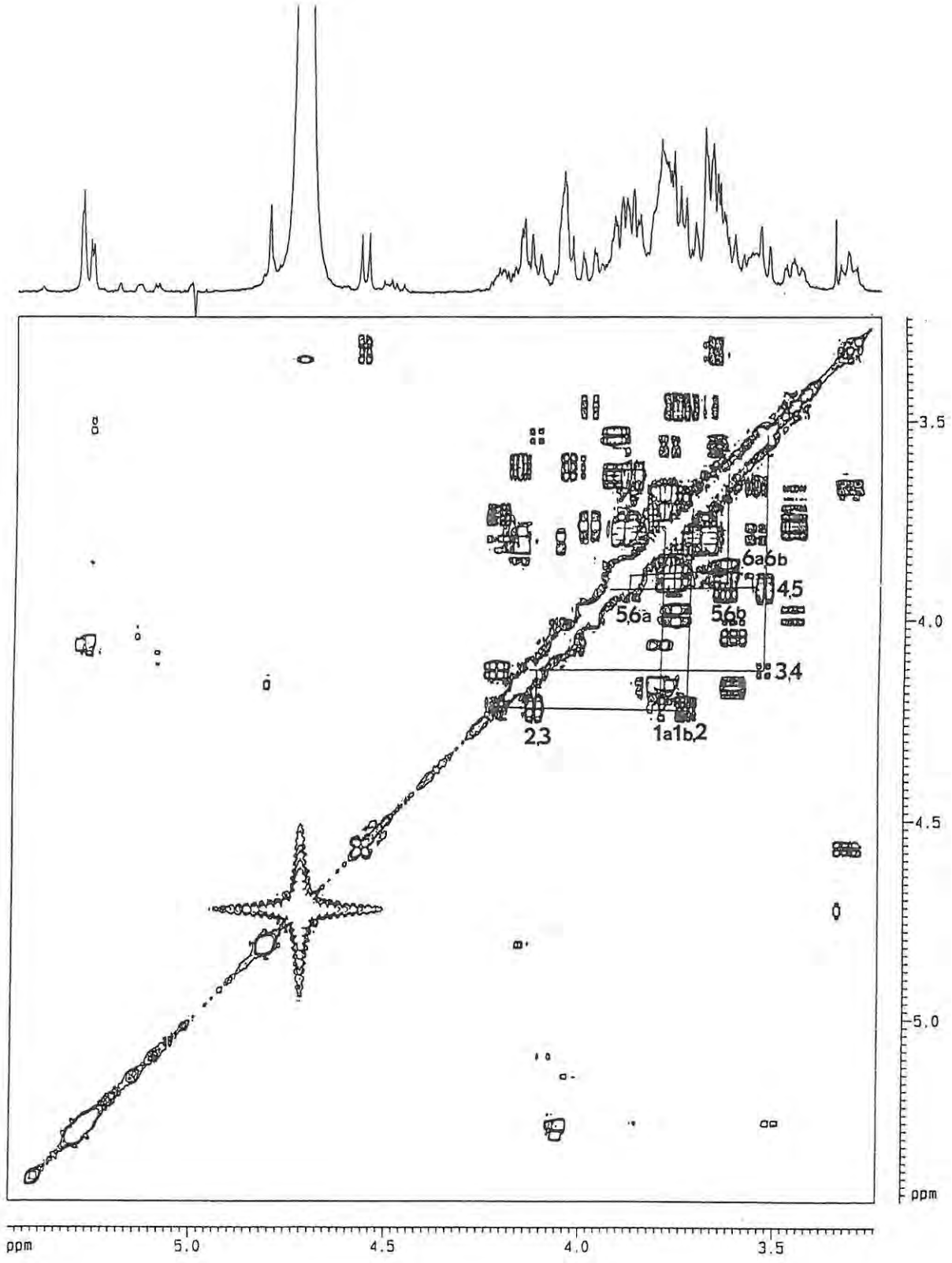


Figure 4.19 COSY contour plot of oligosaccharide **3** at 303K together with a 1D projection on the f_2 axis. The spin system for the terminal 3-linked ManNAc-ol is shown on the spectrum. e 1a/2 connotes the cross-peak between H-1a and H-2 of residue **e**, etc.

TABLE II ^{1}H CHEMICAL SHIFT DATA[1] FOR OLIGOSACCHARIDE 3

Residue		1a	1b	2	3	4	5	6a	6b
a α-D-Man*p*	H	-	5.29	4.06	3.80	3.68	3.61	3.88	3.78
b →3)-α-D-Glc*p*NAc	H	-	5.26	4.06	4.03	3.62	4.17	3.83	3.80
c →2)-ß-D-Man*p*	H	-	4.81	4.16	3.78	3.70	3.46	3.98	3.76
d →4)-ß-D-Glc*p*	H	-	4.57	3.32	3.65	3.65	3.57	3.91	3.78
e →3)-ManNAc-ol	H	3.77	3.73	4.22	4.12	3.53	3.92	3.87	3.64

[1] Chemical shifts with acetone as internal reference at δ 2.23.

CONCLUSION

Degradation of the polysaccharides produced by *E. coli* K38 and K84, which both contain in chain →4)-α-GalA residues, with lithium in ethylenediamine results in the formation of the expected oligosaccharide-alditol derivatives. However, the yields (10-15%) of the relevant oligosaccharides were very low, and in the case of the K38 product, 40 % of the primary product was further degraded *via* cleavage of the terminal furanose residue under the harsh reducing conditions which prevail. These polysaccharides required the addition of considerably more lithium wire to maintain the blue colour than hitherto observed for amino sugar free polysaccharides degraded in this laboratory. It is therefore likely that the presence of amino sugars in some way influences complex formation. The formation of the pentasaccharide-alditol (**3**) following degradation of the K84 polysaccharide is supportive of the structure of the repeating unit established by NMR studies on the polysaccharide (section 4.3).

EXPERIMENTAL

General procedure for the lithium/ethylenediamine degradation — A sample of the polysaccharide (25 mg, acid form) was dried *in vacuo* (60°C, 12 h) and dissolved in ethylenediamine (15 mL). Clean, dry lithium wire (8-12 x 3 mm) lengths were added at intervals to the solution and the contents of the flask were gently swirled to maintain the deep blue colour for a period of 1 h. Gentle, efficient swirling of the contents of the flask as opposed to rapid stirring or agitation was found to promote the development and maintenance of the blue colour. After 1 h the reaction was cooled (ice/water bath) and quenched by the slow addition of water (25 mL), and the contents of the flask were stirred until a clear solution was obtained. The water and ethylenediamine were then removed as the toluene azeotrope *via* successive additions of toluene (6 x 10 mL aliquots), followed by evaporation under reduced pressure after each addition until a white powdery residue of LiOH and carbohydrate remained. The residue was cooled (ice/water bath) and dissolved in a minimum quantity of water and titrated to pH 4.5 with glacial acetic acid. The resulting acidic solution was concentrated under reduced pressure and desalted on a Bio-Gel P-2 column (70 cm x 1.6 cm) in two stages using water as eluent. The carbohydrate fractions were isolated, freeze-dried, re *N*-acetylated (2 mL H_2O - 0.1 mL MeOH - 0.1 mL Ac_2O, 3 h, 20°C followed by the addition of 0.1 mL 25% (aq) NH_3), and rechromatographed on an identical column. Further purification or separation was carried out using semi-preparative HPLC (Progel TSK-oligo-PW column, 10^{-4} M NaOH as eluent). Prior to NMR analysis, a final reduction step ($NaBH_4$) was performed to ensure complete reduction of the product to the corresponding alditol. The yields of the products were as follows: K38 degradation (oligosaccharide **1**, 3.1 mg; oligosaccharide **2**, 0.9 mg). K84 degradation (oligosaccharide **3**, 4.6 mg)

NMR spectroscopy — Samples were prepared as previously described and spectra were recorded on a Bruker AMX-400 NMR spectrometer equipped with an X32 computer. All experiments were carried out at 303K and the parameters used for the 2D experiments were as follows:

COSY-90 and COSY-PR [256 x 2048 data matrix, zero-filled to 1024 data points in t_1, 1.0 s recycle delay, 300 scans per t_1 value (K38) and 112 scans per t_1 value (K84), spectral width 2604 Hz and unshifted sine-bell filtering in t_1 and t_2 prior to transformation and symmetrisation]. HOHAHA [512 x 2048 data matrix, zero-filled to 1024 data points in t_1, 1.0 s recycle delay, 136 scans per t_1 value, mixing time 84 ms, spectral width 2008 Hz and shifted sine-squared filtering in t_1 and t_2]. ROESY [256 x 2048 data matrix, zero-filled to 1024 data points in t_1, 112 scans per t_1 value, acquired with the offset frequency at the lower end of the spectrum. A phase-shifted sine-squared window function was applied during transformation].

4.5 STRUCTURAL ELUCIDATION OF THE *Escherichia coli* K48 CAPSULAR POLYSACCHARIDE: A GLYCAN CONTAINING A NOVEL DIACETAMIDO SUGAR

ABSTRACT

The structure of the exocellular capsular polysaccharide expressed by *Escherichia coli* O8:K48:H9 has been investigated by hydrolysis and methylation analysis, and by NMR spectroscopic studies of the polysaccharide and of the oligosaccharides generated by solvolysis with anhydrous HF. The capsular polysaccharide was shown to have the repeating unit:

→3)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A-(1→
3
↑
1
β-L-Sug*p*

Where β-L-Sug*p* represents 2,3-diacetamido-2,3,6-trideoxy-β-L-mannopyranose.

INTRODUCTION

Bacteria of the genus *Escherichia coli*, of which the structures of the capsular antigens of sixty of the seventy four[432] encapsulated strains identified are now known, show a high degree of heterogeneity in the chemistry of their capsular (K) antigens. Several different amino sugars have been reported as constituents of these polysaccharides. GlcNAc and GalNAc are frequently present, and ManNAc has now been shown to be a constituent of the *E. coli* K84 capsular polysaccharide (section 4.3). L-FucNAc and D-Fuc3NAc occur in the K87[39] and K45[41] antigens respectively, and the unusual aminouronic acid, 2-acetamido-2-deoxy-D-mannopyranosyluronic acid (ManNAcA), is the acidic component of the K7[40] antigen. *N*-acetylneuraminic acid (NeuAc) is also infrequently encountered.

The capsular polysaccharide from *E. coli* K48, which is another example of a K-antigen which contains no simple hexose residues, contains the first diamino sugar to be encountered in a capsular polysaccharide in this genus.

RESULTS AND DISCUSSION

Isolation and purification — The bacteria (*E. coli* O8: K48: H9, culture no. A290a) were grown on Mueller-Hinton agar and the acidic capsular polysaccharide (**PS**) was isolated by selective precipitation using cetyltrimethylammonium bromide in the conventional manner. Further purification of **PS** was effected by dialysis and ion-exchange chromatog aphy on DEAE-Sepharose Cl-6B. The purified material showed a broad distribution of molecular weights on Sephacryl S500 with an average M_r of 5×10^5.

Sugar and methylation analysis — The polymer was found to be resistant to acid hydrolysis (4 M TFA) and the constituent sugars were not released in molar proportions. GlcA and GlcN were identified by analytical GLC-MS of the derived alditol acetates following methanolysis, carboxyl reduction and hydrolysis of a sample of **PS**. Analysis of the derived acetylated (-)-2-octyl glycosides by GLC showed that GlcA and GlcN had the D-configuration. Methylation of **PS** followed by GLC-MS analysis of the derived methylated alditol acetates revealed the presence of 4-linked GlcA, 3-linked GlcN and a very small amount of 3,4-linked GlcN, indicative of a branch point. No methylated alditol acetate for a terminal residue was detected.

NMR spectroscopy — The ^1H and ^{13}C NMR spectra of the polysaccharide were consistent with a tetrasaccharide repeating unit thus indicating the presence of a fourth sugar residue. The ^1H NMR spectrum of **PS** showed, *inter alia,* a doublet at δ 1.36 of a 6-deoxyhexosyl residue, three signals at δ 1.97 (3H), 2.02 (3H) and 2.05 (6H), for methyl protons of four NAc groups, and in the anomeric region, a signal with a small coupling constant at δ 5.07 and a complex of overlapping peaks integrating for four protons at δ 4.5-4.6.

The ^{13}C-NMR spectrum of **PS** (Figure 4.20) showed the presence of four anomeric carbons (99.83, 101.20, 101.95 and 103.46 ppm), five signals for carbonyl carbons (171.74, 174.87, 175.02, 175.27 and 175.61 ppm), four signals for methyl carbons (22.68, 22.94, 23.02 and 23.42 ppm), four signals attributable to C-N carbons of aminodeoxy sugars (52.19, 54.61, 54.89 and 55.83 ppm), and one signal for the methyl carbon of a 6-deoxyhexosyl sugar (18.00 ppm). These data indicated the presence of four acetamido functions in the repeating unit and suggested that the fourth residue was a diacetamido-trideoxy hexose.

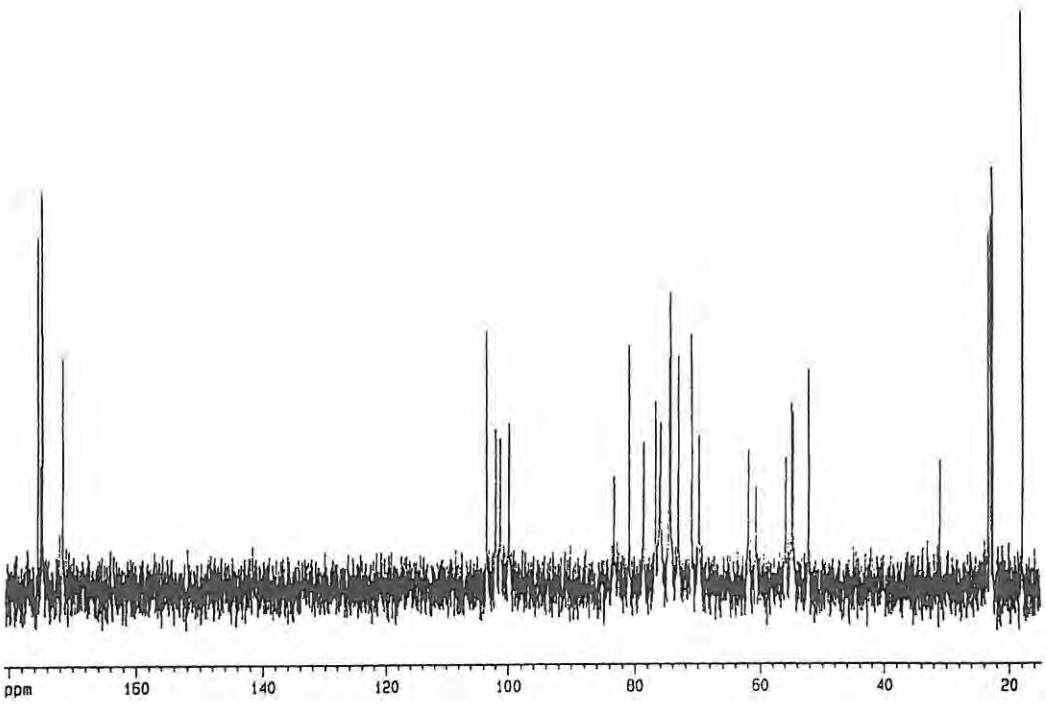


Figure 4.20 ^{13}C-NMR spectrum of K48 **PS** at 328K for the spectral region 18-170 ppm.

The chemical shifts for the ^{1}H and ^{13}C resonances of the residues in the repeating unit of **PS** were assigned from COSY[105], HOHAHA[342], HETCOR[350] and NOESY[345] experiments. The four residues in the repeating unit were labelled a-d in order of decreasing chemical shift of their H-1 resonances (Table I). The ^{1}H-chemical shifts for residues a,b and d could readily be traced from the COSY (Figure 4.21) and HOHAHA spectra of **PS**, however, for unit c the connectivity pattern could not be traced with certainty beyond H-2 due to poorly defined cross-peaks for this residue.

These ^{1}H resonances were compared with ^{13}C chemical shift values obtained from a ^{1}H-^{13}C shift correlation (HETCOR) experiment (Figure 4.22) and once all known ^{1}H chemical shifts had been correlated with their respective ^{13}C chemical shifts, four unassigned sets of ^{1}H-^{13}C chemical shifts remained.

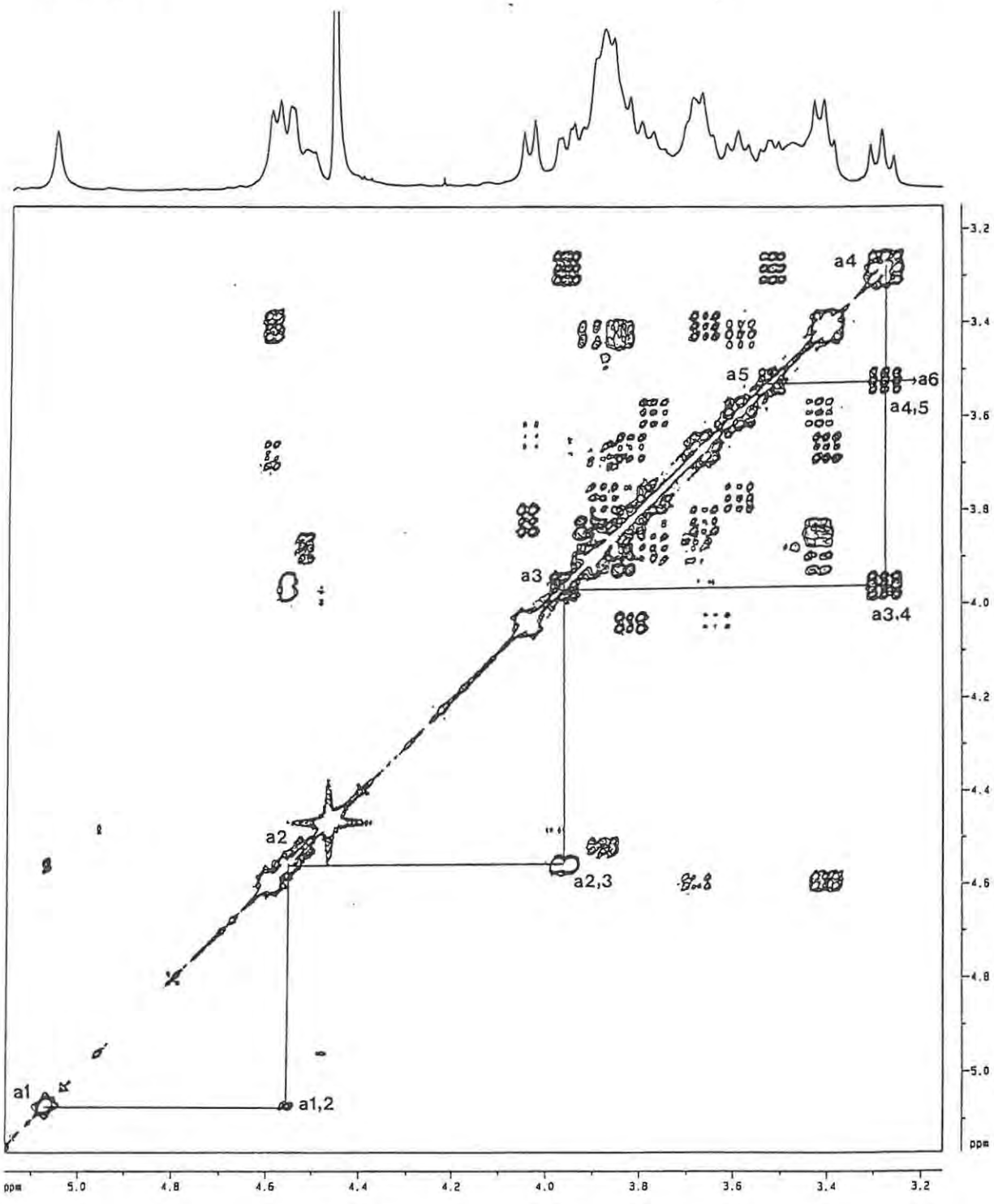


Figure 4.21 COSY contour plot of K48 **PS** for the spectral region δ 3.3-5.3. The ^{1}H resonances of the *J*-coupled spin systems for Sug are illustrated on the spectrum. a-1 connotes H-1 of residue a, and a-1,2 connotes the cross-peak between H-1 and H-2 of residue a, etc. The 1D spectrum is displayed along the f_2-axis.

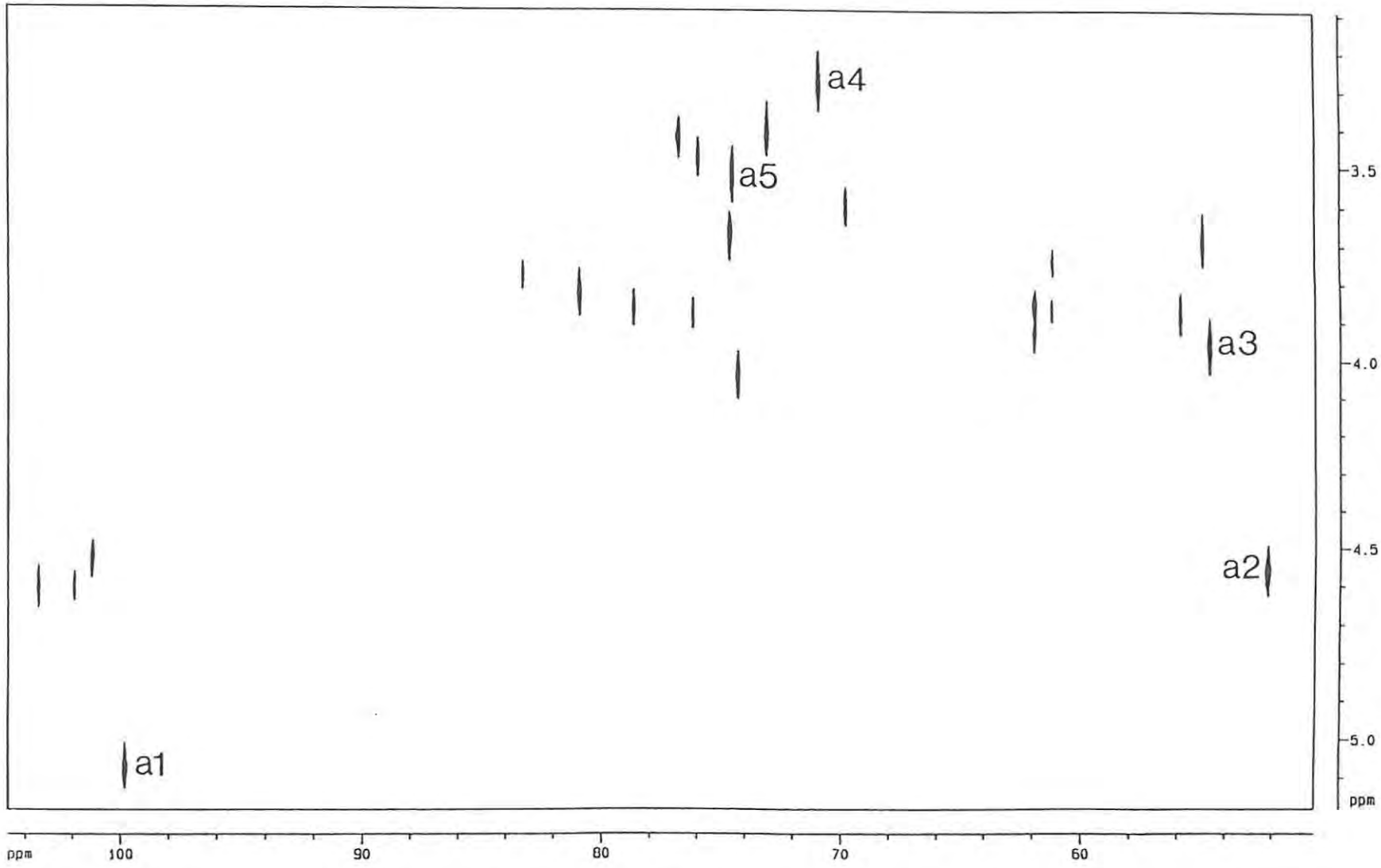


Figure 4.22 ^{1}H-^{13}C shift-correlation (HETCOR) map of the spectral region f_2 (120-10 ppm) and f_1 (3.1-5.2 ppm) for K48 **PS**. Correlated cross-peaks assigned to Sug are illustrated on the spectrum. a1 represents H-1/C-1, etc.

TABLE 1

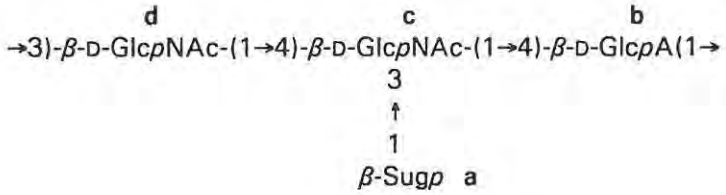
NMR DATA[a] (400 MHz) FOR PS

Residue		1	2	3	4	5	6a	6b
a	H	5.07	4.55	3.96	3.28	3.52	1.36	-
ß-Sug*p*	^3J [b]	1.2	4.1	10.0	9.6	5.7	-	
	C	99.83	52.19	54.61	70.87	74.46	18.00	-
b	H	4.60	3.40	3.66	3.81	4.04	-	-
→4)-ß-GlcA	C	103.46	73.01	74.60	<u>80.91</u>[c]	74.25	171.74	-
c	H	4.60	3.68	3.87	3.88	3.47	3.73	3.88
→3,4)-ß-GlcNAc	C	101.95	54.89	<u>78.64</u>	<u>76.73</u>	75.91	60.70	-
d	H	4.52	3.88	3.78	3.59	3.42	3.85	3.91
→3)-ß-GlcNAc	C	101.20	55.83	<u>83.28</u>	69.76	76.73	61.88	

[a] Chemical shifts with acetone as internal reference, δ 2.23 and 31.07 ppm respectively. [2] ^{1}H-^{1}H coupling constants in Hz for Sug. [3] Linkage carbons underlined.

The set of low intensity at δ 3.73, 3.88 /60.70 clearly belonged to H-6a, H-6b/C-6 for residue **c** and of the remaining three sets (δ 3.47/75.91, δ 3.87/78.64 and δ 3.88/76.73) the first two were assigned to H-5/C-5 and H-3/C-3 of **c** from observed NOE cross peaks in the NOESY spectrum between H-1 and H-5 and H-3 and H-5. The remaining set therefore belonged to H-4/C-4 of **c**. Comparison of the ^{1}H- and ^{13}C-NMR data for residues **b**, **c** and **d** with literature values for methyl glycosides[293,433,434] permitted the residues in the repeating unit to be identified as indicated in Table I, and their linkage positions were confirmed by the observed ^{13}C glycosylation shifts[291] for C-4 of **b**, C-3 and C-4 of **c** and C-3 of **d**. The NMR data therefore accord with the presence of 3-linked β-GlcNAc, 3,4-linked β-GlcNAc, 4-linked β-GlcA and a terminal 2,3-diacetamido-2,3,6-trideoxy hexose (Sug). The fact that unit **c** gave such poorly defined cross peaks could be ascribed to short T_2 values for this residue resulting from reduced mobility at the branch point[338].

The ^{1}H-^{1}H coupling constants measured for Sug (residue **a**) indicated that it had the *manno* configuration (Tables I and II). A coupled HMQC spectrum[356] of **PS** gave a $J_{C\text{-}1,H\text{-}1}$ value of 166 Hz for Sug, identical to the $J_{C\text{-}1,H\text{-}1}$ value recently reported for a terminal β-ManNAc[356]. A value of 175 Hz was recorded for methyl 2,3-diacetamido-2,3,6-trideoxy-α-D-mannopyranoside synthesized in our laboratory (see section 4.6) suggesting a β-linkage for Sug. This was conclusively proved from the strong interresidue NOEs observed between H-1 and H-3, H-1 and H-5, and H-3 and H-5 in the NOESY spectrum of **PS**. The sequence of residues **a**-**d** in the repeating unit was established from the 2D NOESY experiment and was confirmed by an HMBC[159] experiment. The relevant inter- and intra-residue NOEs are presented in Table III. The observed interresidue NOEs between anomeric protons and the relevant protons of the adjacent glycosidically linked residues are consistent with the following structure for the repeating unit:



Where Sug is 2,3-diacetamido-2,3,6-trideoxy-β-mannopyranose.

TABLE II OBSERVED NOEs FOR K48 PS

RESIDUE	PROTON	NOE AT
a	H-1 5.07[b]	4.55(**a**,H-2) ; 3.96(**a**,H-3)
		3.52(**a**,H-5) ; <u>3.87</u>[a](**c**,H-3)
	H-2 4.55	3.96(**a**,H-3)
	H-3 3.96	3.52(**a**,H-5)
	H-6 1.36	3.28(**a**,H-4)
b	H-1 4.60	3.66(**b**,H-3) ; 4.04(**b**,H-5)
		<u>3.78</u>(**d**,H-3)
	H-3 3.65	4.04(**b**,H-5)
c	H-1 4.60	3.87(**c**,H-3) ; 3.47(**c**,H-5)
		<u>3.81</u>(**b**,H-4)
	H-3 3.87	3.47(**c**,H-5)
d	H-1 4.52	3.78(**d**,H-3) ; 3.42(**d**,H-5)
		<u>3.88</u>(**c**,H-4)
	H-3 3.78	3.42(**d**,H-5)

[a]Interresidue (linkage) NOEs are underlined. [b]Chemical shift in ppm.

Long-range heteronuclear correlation data from the HMBC experiment accorded with this structure and in addition served to confirm the assignments for H-3/C-3 and H-4/C-4 of the disubstituted GlcNAc residue. Three-bond correlations were observed from H-1 of **b** to C-3 of **d** (83.28 ppm), H-1 of **c** to C-4 of **b** (80.91 ppm), H-1 of **a** to C-3 of **c** (78.64 ppm) and from H-1 of **d** to C-4 of **c** (76.73 ppm).

Attempts to isolate Sug using established procedures involving hydrolysis, methanolysis, and acetolysis were unsuccessful. In order to generate smaller fragments for further study, a sample of **PS** was therefore subjected to solvolysis with anhydrous HF. This method is particularly suitable for polysaccharides of this nature as it preserves *N*-acyl substituents and under suitable conditions can be highly selective. Amino sugars and uronic acid residues are known to show greater stability to anhydrous HF than hexoses and deoxyhexoses, and the presence of a second *N*-acyl group is known to cause additional stabilisation of the glycosidic linkages of amino sugars[202].

Accordingly **PS** was expected to be highly resistant. HF solvolysis of **PS** afforded two principal oligosaccharides, **TS** and **DS** (Figure 4.23) which were isolated by GPC. GlcNAc was the only monosaccharide isolated.

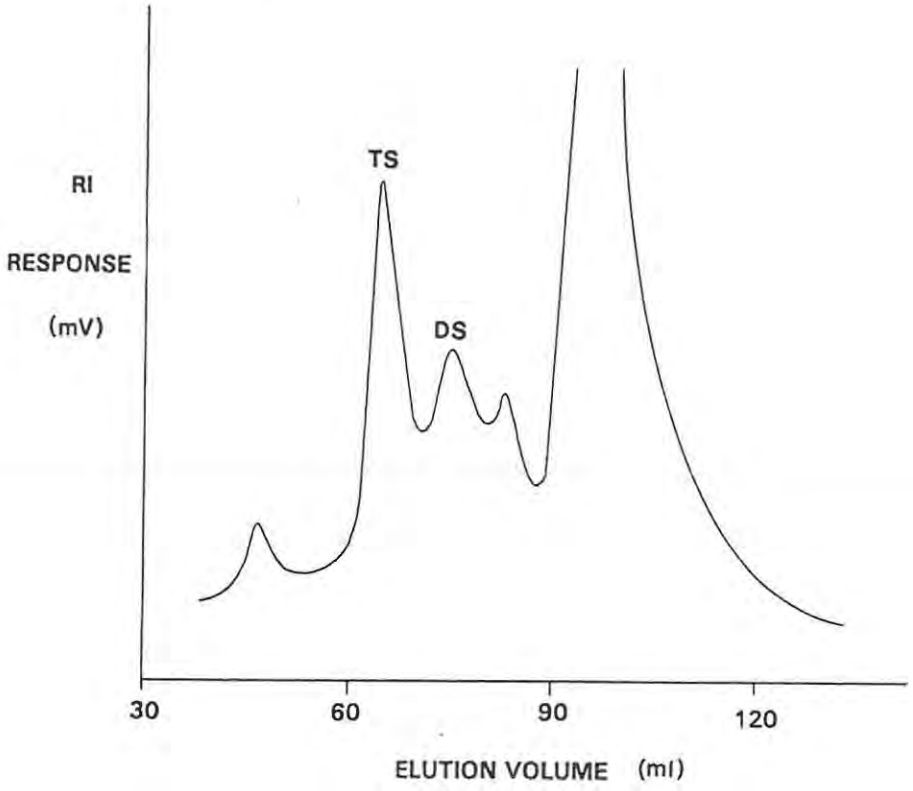


Figure 4.23 Elution diagram of the products of anhydrous HF solvolysis of K48 **PS**

*NMR analysis of **TS** and **DS**.* — The ^{1}H NMR spectrum of **TS** (Figure 4.24) contained an α-signal, δ 5.23 ($^3J_{1,2}$ = 3.6 Hz) and a ß-signal, δ 4.73 ($^3J_{1,2}$ = 8.5 Hz) indicative of the presence of a reducing sugar in solution (α:ß = 3:2). In addition, two sets of twinned anomeric signals (ascribed to muta-rotational effects) occurred at δ 5.10/5.14 ($^3J_{1,2}$ = 1.5 Hz) and δ 4.47/4.49 ($^3J_{1,2}$ = 8.1 Hz). The presence of a twinned doublet at δ 1.36/1.38 for a 6-deoxyhexosyl residue and an additional twinned peak at δ 4.60/4.62, which displayed a coupling pattern consistent with H-2 of a sugar having the *manno*-configuration, showed that Sug was a component of **TS**. A series of overlapping twinned methyl proton resonances at ~ δ 2.05 indicated that *N*-acyl functions had been preserved during HF solvolysis. These data are consistent with a reducing trisaccharide containing Sug.

The ^{13}C NMR spectrum showed five anomeric carbon signals between 91 and 102 ppm, two of which (99.81 and 99.91 ppm) appeared to be a twinned pair. Of the three remaining anomeric carbon signals (91.39, 95.50 and 101.69 ppm), two had to represent the α and ß carbons of the reducing sugar, making these data consistent with the presence of three sugars and supportive of **TS** being a reducing trisaccharide. The anomeric region of the ^{1}H NMR spectrum of **DS** (Figure 4.25) lacked the twinned signal for a ß-linked sugar present in the proton spectrum of **TS** but still contained twinned pairs at δ 4.97/4.99 and δ 4.48/4.51 representative of H-1 and H-2 of Sug respectively, and an α- and ß-signal at δ 5.24 ($^{3}J_{1,2}$ = 3.4 Hz) and 4.74 ($^{3}J_{1,2}$ = 8.6 Hz) for a reducing residue. The ^{13}C spectrum of **DS** contained four signals in the anomeric region *viz.* at 91.76, 95.59, 100.42 and 100.55 ppm, representing one twinned pair of signals (100.42 and 100.55), and signals for α and ß C-1 carbons of a reducing sugar, which is consistent with a reducing disaccharide. A detailed study of **TS** and **DS** using 2D NMR spectroscopy (COSY, HMQC, HMQC-TOCSY[367] and HMBC) confirmed these assumptions and gave the data listed in Table II. These data show that **TS** and **DS** have the following structures:

TS

d **c**
β-D-Glc*p*NAc-(1→4)-D-GlcNAc
3
↑
1
β-Sug*p* **a**

DS

a **c**
β-Sug*p*-(1→3)-D-GlcNAc

The chemical shifts for the ^{1}H resonances of residues **a** and **d** could be readily traced by following the cross-peaks in the COSY spectrum of **TS**. The disubstituted reducing GlcNAc residue, however, could only be fully assigned from HMQC and HMQC-TOCSY spectra (Figure 4.26) due to considerable signal overlap. The remaining ^{13}C chemical shifts for **TS** were also obtained from the HMQC spectrum. The ^{1}H and ^{13}C resonances for the residues in **DS** were fully assigned from COSY and HMQC experiments. Long range three-bond ^{1}H-^{13}C correlations (Fig. 4.27) in the HMBC experiment performed on **TS** enabled it to be sequenced as indicated.

The HMBC experiment also served to confirm the acyl substitution pattern of Sug and the two GlcNAc residues in that connectivities were observed between the ring protons linked to the acetamido substituted carbons and the carbonyl carbons of the acetamido substituents.

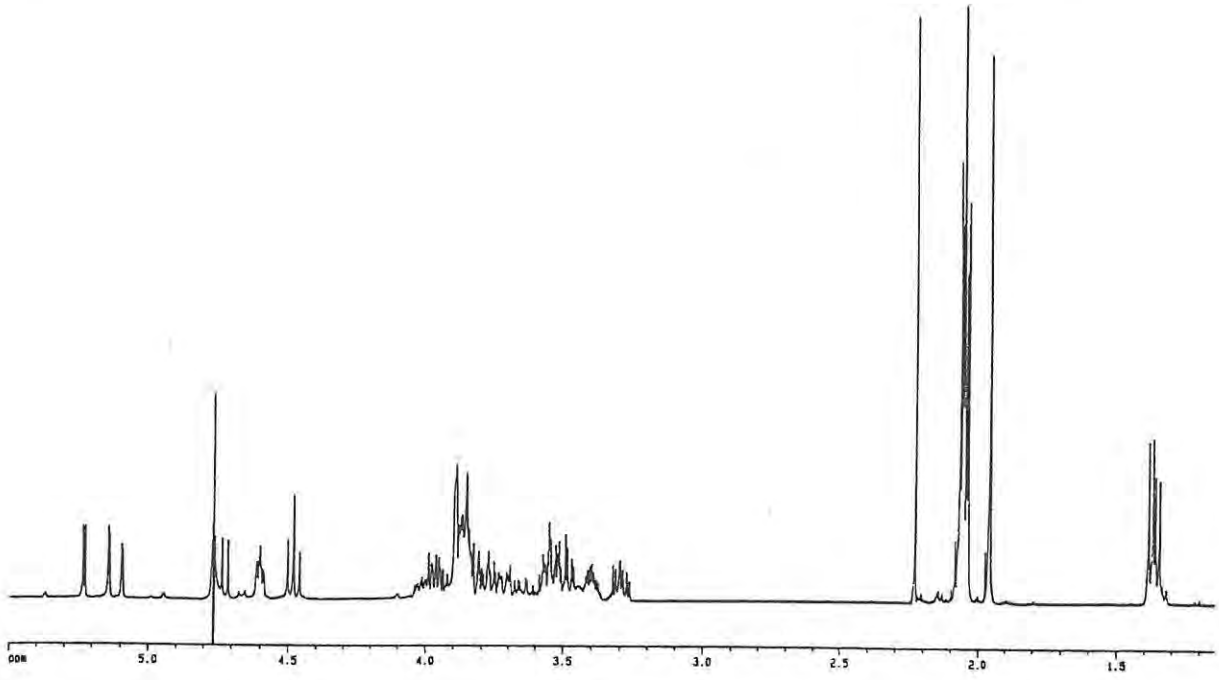


Figure 4.24 ^{1}H NMR spectrum of **TS** at 303K.

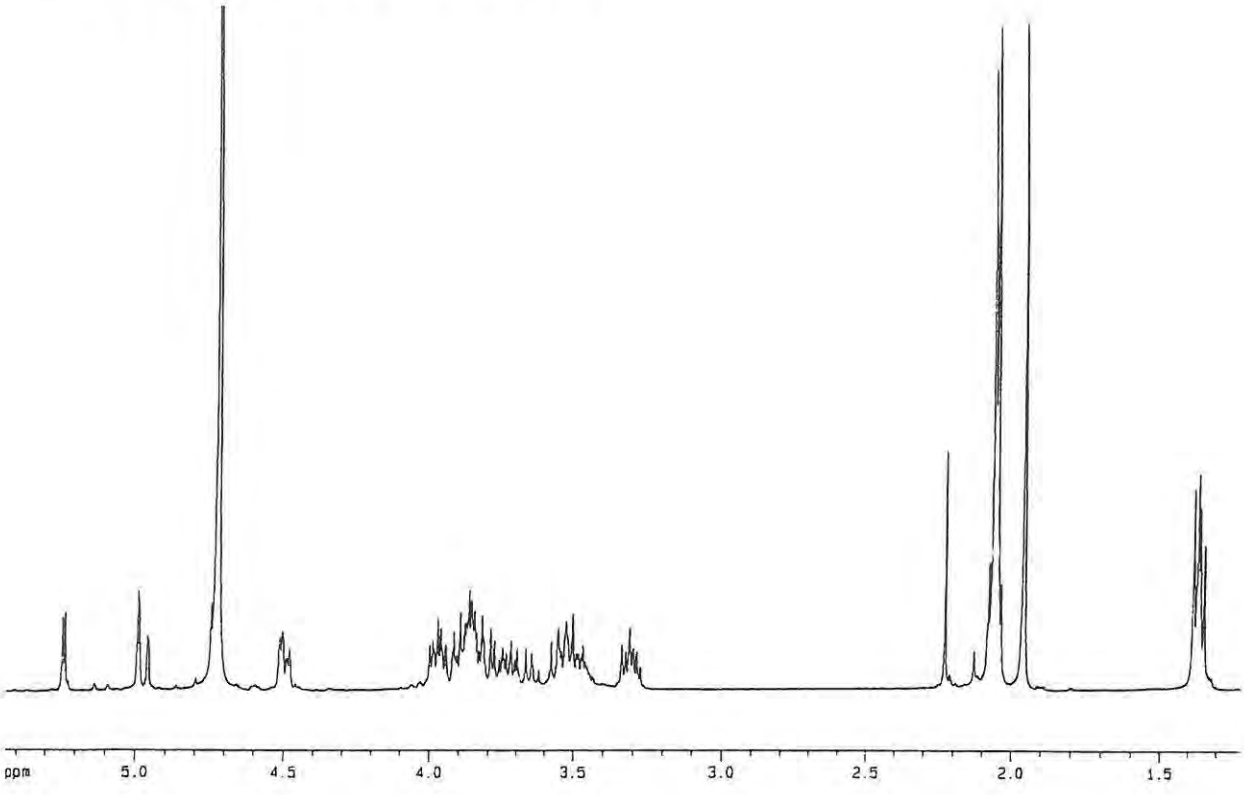


Figure 4.25 ^{1}H NMR spectrum of **DS** at 303K.

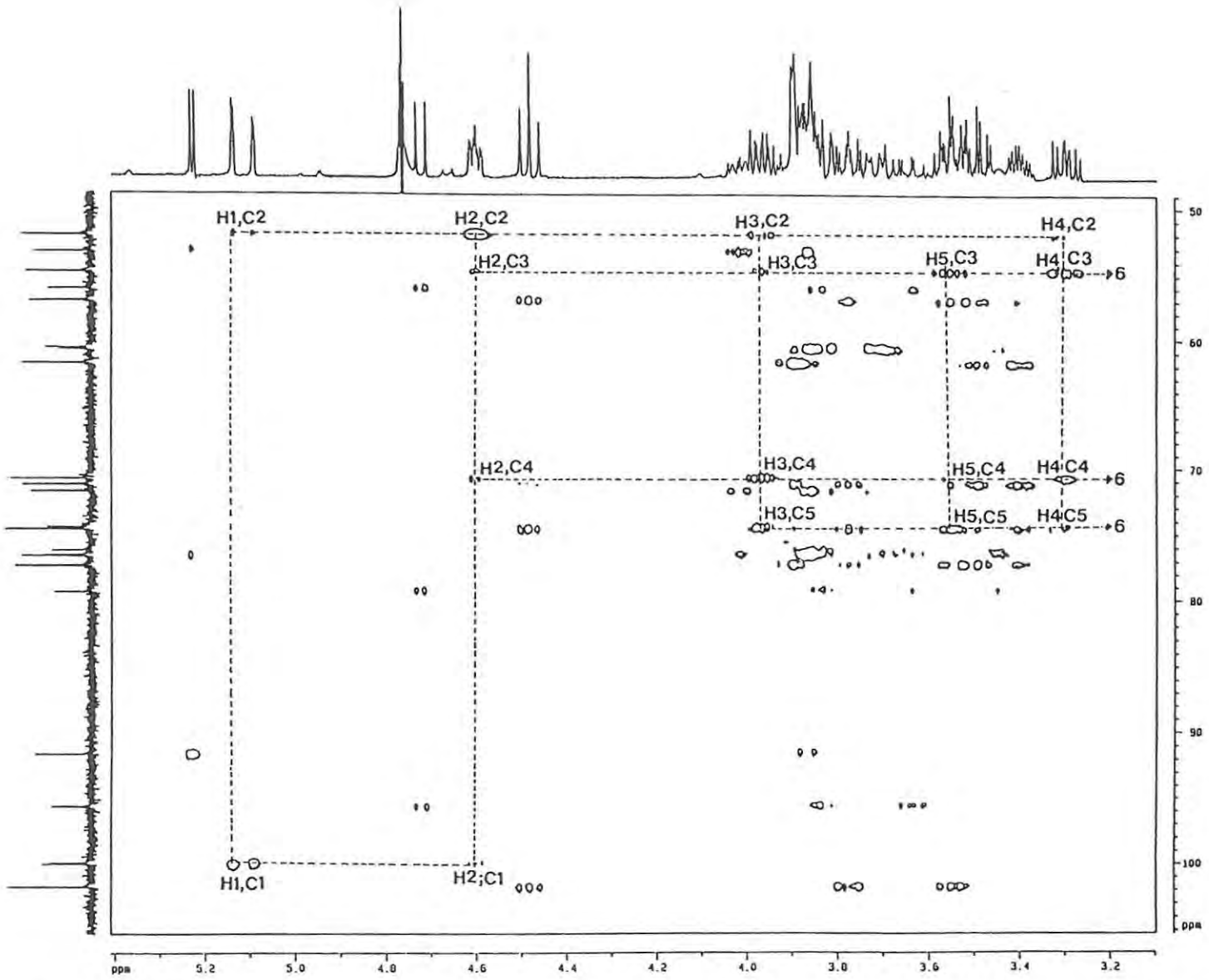


Figure 4.26 HMQC-TOCSY ^{1}H-^{13}C contour plot of the spectral region f_1 50-105 ppm (^{13}C) and f_2 3.2-5.4 ppm (^{1}H) for the reducing trisaccharide, **TS**. Heteronuclear connectivities for Sug are connected by broken lines where H1,C2 represents a connectivity from H-1 of Sug to C-2, etc.

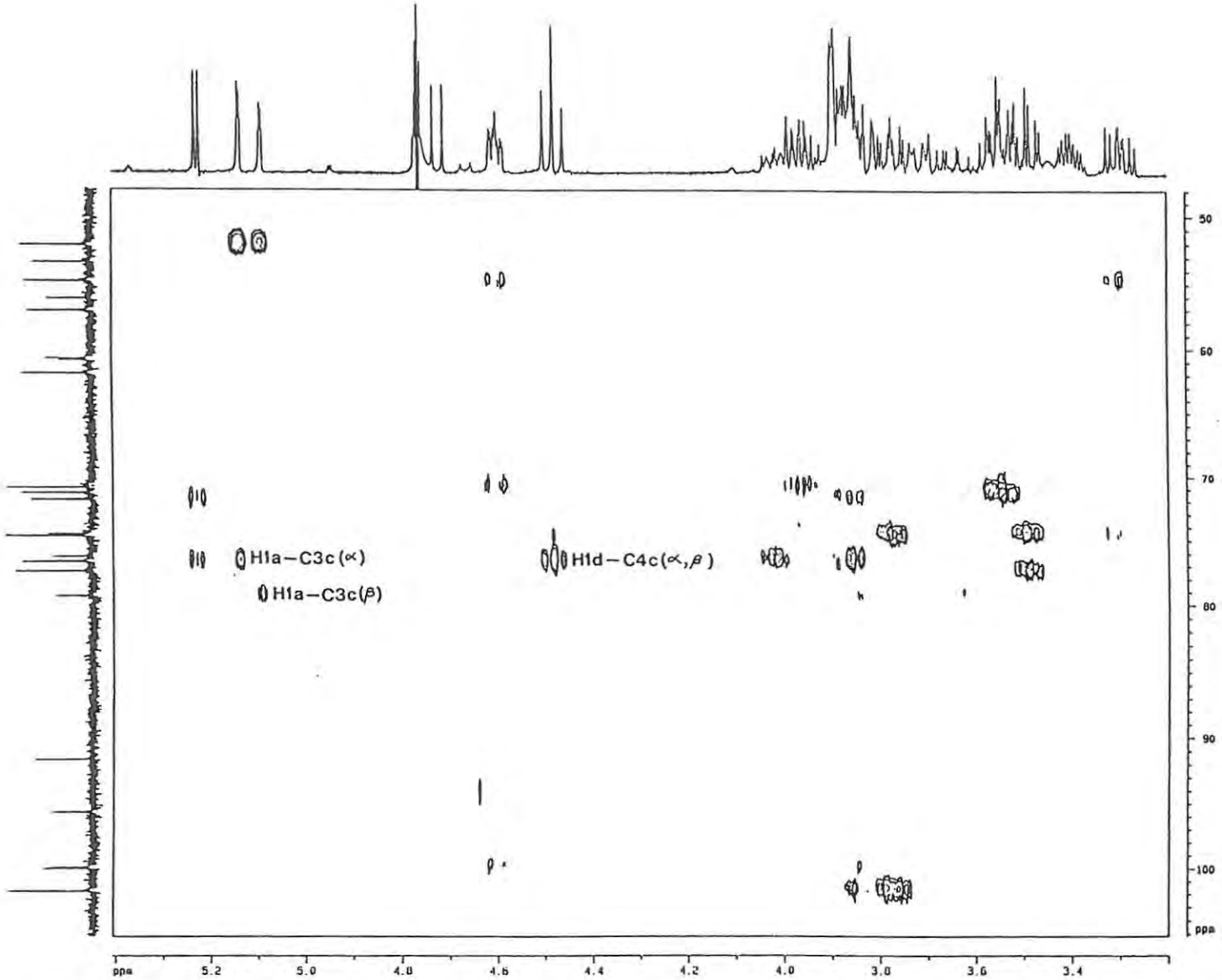


Figure 4.27 HMBC spectrum of **TS** for the spectral region f_1 48- 105 ppm (^{13}C) and f_2 3.2-5.4 ppm (^1H). Only the three-bond interresidue connectivities are indicated. H1a-C3c (*α*) connotes the through bond connectivity from H-1 of residue a to C-3 of residue c when c exists as the *α*-anomer.

TABLE III ^{1}H and ^{13}C NMR data[a] for the oligosaccharides TS AND DS.

^{1}H	TS	DS	^{13}C	TS	DS
a	H1 5.10/5.14[b](1.5)[c]	4.97/4.99 (1.8)		C1 99.81/99.91	100.42/100.55
	H2 4.60/4.62 (4.1)	4.48/4.51 (4.1)		C2 52.06/52.07	52.28/ 52.29
	H3 3.96/3.97 (10.5)	3.96/3.98 (10.2)		C3 54.68/54.71	54.38/ 54.42
β-Sug*p*	H4 3.29/3.31 (9.5)	3.30/3.31 (9.4)		C4 71.15/71.16	70.56/ 70.57
	H5 3.54/3.56 (6.2)	3.49/3.52 (6.1)		C5 74.50/74.53	74.53/ 74.64
	H6 1.36/1.38	1.35/1.38		C6 17.92/18.01	17.97/ 18.03
c	H1 5.23 (3.6)	5.24 (3.4)		C1 91.39	91.7
	H2 3.86 (9.3)	3.85 (9.9)		C2 53.26	53.41
	H3 4.02 (10.0)	3.93 (9.7)		C3 <u>76.39</u>[d]	<u>79.96</u>
α-GlcNAc	H4 3.86	3.55 (9.4)		C4 <u>76.45</u>	70.29
	H5 3.86	3.87 (5.54)(1.8)		C5 71.69	72.29
	H6a 3.83	3.82 (12.0)		C6 60.61	61.27
	H6b 3.72	3.77			
c	H1 4.73 (8.5)	4.74 (8.6)		C1 95.50	95.59
	H2 3.64 (10.4)	3.64 (9.9)		C2 56.02	56.03
	H3 3.85	3.73 (9.7)		C3 <u>79.11</u>	<u>82.36</u>
β-GlcNAc	H4 3.85	3.52 (9.1)		C4 <u>76.42</u>	70.35
	H5 3.44	3.45 (5.75)(2.1)		C5 76.03	76.52
	H6a 3.87	3.90 (12.2)		C6 60.70	61.45
	H6b 3.68	3.74			
d	H1 4.47/4.49 (8.1)			C1 101.69	
	H2 3.77/3.78 (10.0)			C2 56.95/56.98	
	H3 3.54/3.55 (9.1)			C3 74.3	
β-GlcNAc	H4 3.48/3.49 (9.1)			C4 71.18	
	H5 3.40			C5 77.16/77.17	
	H6a 3.89			C6 61.78	
	H6b 3.91				

[a]Chemical shifts with acetone as internal standard, δ 2.23 and 31.07 ppm respectively for ^{1}H and ^{13}C. [b]Signals twinned due to the reducing GlcNAc residue. [c] ^{1}H-^{1}H coupling constants in Hz. [d]Linkage carbons underlined.

Absolute Configuration of Sug. — Due to the resistance of Sug to hydrolysis its absolute configuration could not be determined using conventional methods. A tentative assignment was however made on the basis of ^{13}C chemical shift differences, as described by Lipkind *et al.*[304], from the disaccharide β-Sug*p*-(1→3)-D-GlcNAc. In a disaccharide, the formation of the glycosidic linkage between the two units leads to predictable changes in the ^{13}C NMR spectra of both the glycone and the aglycone (in this instance Sug and GlcNAc respectively). These effects depend on the position and configuration of the glycosidic linkage, the general configuration of the aglycone, and the relative absolute configurations of both residues[304]. If the absolute stereochemistry of the aglycone is known, the absolute stereochemistry of the glycone can be deduced from the influence it has on the ^{13}C chemical shifts of the aglycone.

The largest differences in chemical shift are observed for those carbons involved in the glycosidic linkage (the α-effect, with a large downfield shift) and the carbons immediately adjacent to the substituted carbon on the aglycone which experience smaller upfield shifts (the β-effect)[152]. ^{13}C chemical shifts can also be affected by inductive effects and by spatial proton-proton interactions which polarise C-H bonds resulting in unique changes in the shielding of the carbon nuclei involved[152]. The latter effect only occurs when the aglycone carries at least one equatorial proton at a β-carbon. In the present study, since the aglycone has the *gluco* configuration, any changes in the ^{13}C chemical shifts at the β-carbons can be attributed to inductive effects which occur when one of these carbons is in close proximity to the ring oxygen of the glycone[152].

In disaccharides of this nature it is known that the aglycone tends to orientate itself away from the side of the glycone which carries substituents i.e. at C-2, C-3 and C-4 and towards the side of the ring where the ring oxygen is situated which is less sterically hindered[152]. Accordingly the β-carbon, for which a relatively larger (in absolute value) negative β-effect of glycosylation is observed, should be closer to O-5 of the glycone ring. It is therefore possible to determine the absolute stereochemistry of the glycone in this class of disaccharides, if the absolute stereochemistry of the aglycone is known, by observing which β-carbon experiences greater

deshielding. A ^{13}C-spectrum of GlcNAc was run under identical conditions to the spectrum acquired for β-Sug*p*-(1→3)-D-GlcNAc and the chemical shift differences (Table IV) between the GlcNAc residues were compared. The results show that C-2 of the reducing GlcNAc in the oligosaccharide experiences greater deshielding than C-4. This deshielding is attributed to the closer proximity of C-2, rather than C-4, to O-5 of the glycone. The minimum energy conformations established for the disaccharides β-L-Sug*p*-(1→3)-D-GlcNAc and β-D-Sug*p*-(1→3)-D-GlcNAc show that this is only possible when Sug has the L-configuration. This evidence suggests that Sug has the L-configuration.

TABLE IV: ^{13}C Chemical shift glycosylation effects for **DS** at 303K

	D-GlcNAc	β-Sug*p*-(1→3)-D-GlcNAc	Chemical shift difference
α-ANOMER			
C1	91.67	91.76	0.09
C2	54.91	53.41	-1.50
C3	71.52	79.96[a]	**8.44**
C4	70.91	70.29	-0.62
C5	72.40	72.29	-0.11
C6	61.43	61.27	-0.16
β-ANOMER			
C1	95.76	95.59	-0.17
C2	57.55	56.03	-1.52
C3	74.73	82.36	**7.63**
C4	70.69	70.35	-0.34
C5	76.78	76.52	-0.26
C6	61.59	61.45	-0.14

[a]Linkage carbons underlined.

CONCLUSION

The structure of the repeating unit of the capsular polysaccharide of *E. coli* K48 is as shown in the abstract. Although several diamino uronic acids, diamino dideoxy and diamino trideoxy sugars have been encountered in Nature[206,435-437], this is the first report of the occurrence of 2,3-diacetamido-2,3,6-trideoxy-β-L-mannopyranose. Recently Anderson *et al.* reported[438] the presence of 2-acetamido-3-formamido-2,3,6-trideoxy-D-mannopyranose in the O-antigen of *E. coli* O119.

It is interesting to note that in this genus unusual sugars frequently occur as a single terminal residue linked to the main polysaccharide chain. e.g. *E. coli* serotypes K4[2] and K45[42]. K45 contains a Fuc3NAc residue linked in this manner and K4 contains a labile fructofuranose residue, the loss of which results in a drastic reduction in the antigenic specificity of the latter K-antigen[2]. It is therefore likely that the Fuc3NAc in K45 and Sug in K48 are also immunodominant constituents.

EXPERIMENTAL

General methods. — The instrumentation and general methods were essentially as previously described. The DB-17 capillary column was used to separate alditol acetates and partially methylated alditol acetates using the same programme employed for the studies on *E. coli* K83. Acetylated octyl glycosides were again prepared as before and were separated by GLC on the above column on the same temperature programme, but with a column pressure of 140 KPa. Methylation analysis was carried out as described for *E. coli* K83 and K84. The molecular weight determination of **PS** was performed on a dextran-calibrated column of Sephacryl S500 (70 cm x 1.6 cm) using 0.1 M NaOAc buffer (pH 5.00) as eluent. Material was detected by refractive index.

Isolation and purification K48 ***PS***. — An authentic culture of *E. coli* O8:K48:H9 (A290a) was obtained from Dr I.Ørskov (Copenhagen), and the bacteria were propagated on Mueller-Hinton agar at 37°C for 18 h. The harvested bacterial slime was suspended in aqueous 1% phenol and stirred at 4°C for 48 h, after which the cells were removed by ultracentrifugation and the polysaccharide isolated by precipitation of the supernatant into EtOH. Acidic polysaccharide was purified as before by selective precipitation using CTAB and then further purified by dialysis (12000 mw cut off) and finally by ion-exchange chromatography on DEAE-Sepharose Cl-6B using gradient elution with NaCl (0.1-0.5 M).

Degradation of K48 ***PS*** *with anhydrous HF*. — **PS** (20 mg) was subjected to solvolysis with anhydrous HF (introduced under vacuum using the apparatus described by Sanger and Lamport[211]) and the sample was left to stir for 3 h at 25°C. Excess HF was removed under a stream of N_2 after which 50% AcOH (2 mL) was added and the mixture stirred for 1 h in order to hydrolyse the resultant glycosyl fluorides. The acetic acid was removed at 40°C under reduced pressure and the residue reconstituted in water (1 mL). This was applied to a column of Bio-Gel P-2 (70 cm x 1.6 cm) using water as eluent and the resulting fractions were isolated and freeze-dried.

NMR spectroscopy. — Samples were deuterium-exchanged several times by freeze drying from D_2O, and then examined as solutions in 99.99% D_2O (0.6 mL) containing a trace of acetone as internal standard (δ 2.23 for ^1H and 31.07 for ^{13}C). Spectra were recorded on a Bruker AMX-400 spectrometer equipped with an X32 computer. Experiments on **PS** were carried out at 328K and all experiments on **TS** and **DS** at 303K. COSY-45 experiments were performed on **PS** and **TS** whilst a COSY-90 pulse sequence was used for **DS**. Data matrices were typically 512 x 2048 data points with 64 transients per t_1 delay and were zero-filled to 1024 data points in the t_1 dimension. Prior to transformation and symmetrisation, a non-shifted sine-bell window function was applied and the digital resolution of the resulting matrices was typically 3.5 Hz per point. The NOESY experiment was performed using a similar data set with a 0.3 s mixing delay and a phase-shifted

sine-squared window function was applied during transformation. For the HOHAHA spectrum of **PS** a mixing time of 84 ms was used and for **TS** 89 ms with shifted sine-squared filtering in t_1 and t_2. The ^1H-^{13}C shift-correlated (HETCOR) experiment was recorded using a spectral width of 12.02 KHz in t_2 and 1.8 KHz in t_1 and processed with Gaussian functions. Inverse experiments: HMQC, 256 x 4096 data matrix with 72 scans per t_1 value, zero-filled to 1024 data points in t_1, 1 s recycle delay ; HMQC-TOCSY, 512 x 4096 data matrix, 80 scans per t_1 value, mixing time 25 ms with a 1 s recycle delay ; HMBC, 256 x 2048, 112 scans per t_1 value, $\Delta 1$ and $\Delta 2$ durations of 3 and 60 ms respectively, 1 s recycle delay and a sine-squared filter.

Energy minimisation calculations — Minimum energy conformation calculations for **DS** were kindly performed by Dr J-R. Brisson, National Research Council, Ottawa, Canada, using the MM3 programme.

4.6 SYNTHESIS : Methyl 2,3-diacetamido-2,3,6-trideoxy-α-D-*manno*pyranoside

ABSTRACT

The synthesis of methyl 2,3-diacetamido-2,3,6-trideoxy-α-D-*manno*pyranoside, commencing from methyl 2,3-anhydro-4,6-*O*-benzylidene-α-D-*allo*pyranoside (**2**), is reported. The key diazido-*manno* intermediate, methyl 4,6-*O*-benzylidene-2,3-diazido-2,3-dideoxy-α-D-*manno*pyranoside (**5**), was prepared by opening the epoxide in **2** with sodium azide, followed by triflation of the product, methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-α-D-*altro*pyranoside (**3**), and displacement of the introduced 3-triflate group with sodium azide. Treatment of **5** with *N*-bromosuccinamide (NBS) gave methyl 4-*O*-benzoyl-6-bromo-2,3-diazido-2,3,6-trideoxy-α-D-*manno*pyranoside (**6**) which, after prior removal of the benzoyl ester group, was catalytically reduced to furnish methyl 2,3-diamino-2,3,6-trideoxy-α-D-*manno*pyranoside (**8**). *N*-acetylation of **8** gave the title glycoside (**9**).

INTRODUCTION

The recent report[438] of the presence of 2-acetamido-3-formamido-2,3,6-trideoxy-D-mannopyranose in the O-antigen of *E. coli* O119, and the presence of a trideoxy diacetamido sugar possessing the *manno* configuration in the capsular polysaccharide of *E. coli* K48, prompted the synthesis of the title compound. The synthesis of this sugar has not been reported previously to the best of our knowledge, although synthetic routes for a number of other diacetamido trideoxy sugars have been published[439-442].

RESULTS AND DISCUSSION

Ring opening of sugar epoxides, such as methyl 2,3-anhydro-4,6-*O*-benzylidene-α-D-*allo*pyranoside using azide as the nucleophile, is a well established method of synthesizing 2-amino-2-deoxy sugars[443]. Moreover, a second azido function at C-3 can be introduced *via* SN_2 displacement, after prior conversion of the resulting hydroxyl group at C-3 to a trifluoromethylsulphonyloxy group[444]. This approach was chosen in order to generate the key 2,3-diazido-*manno* intermediate (**5**) in the synthesis of methyl 2,3-diacetamido-2,3,6-trideoxy-α-D-*manno*pyranoside.

Methyl 2,3-anhydro-4,6-*O*-benzylidene-α-D-allopyranoside (**2**) was prepared, in 90% yield, according to the standard procedure[443] by the addition of methanolic sodium methoxide to a solution of methyl 4,6-*O*-benzylidene-2,3-di-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside (**1**) in chloroform. Treatment of **2** with sodium azide in ethanol as described afforded methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-α-D-*altro*pyranoside (**3**) in high yield. Triflation[445] of the free hydroxy group at C-3 of **3** was achieved by the dropwise introduction of a solution of trifluoromethanesulphonic (triflic) anhydride in dichloromethane into a solution of **3** in dichloromethane containing pyridine at -20°C. The trifluoromethylsulphonyl derivative methyl 4,6-*O*-benzylidene-2-azido-2-deoxy-3-*O*-trifluoromethylsulphonyl-α-D-*altro*pyranoside (**4**) was isolated in 69% yield and was recrystallised from dicloromethane-methanol. In the ^1H NMR spectrum of **4** the H-3 signal as expected showed a significant downfield shift compared to its position of resonance in the spectrum of **3**. Treatment of a solution of **4** in dry *N,N*-dimethylformamide (DMF) with a saturated solution of sodium azide in dimethylsulphoxide (DMSO), followed by heating at 60°C for 90 min, afforded methyl 4,6-*O*-benzylidene-2,3-diazido-2,3-dideoxy-α-D-*manno*pyranoside (**5**) in 68% yield. The absence of the resonance at δ5.07 in the ^1H spectrum of **5** confirmed displacement of the trifluoromethylsulphonyloxy group and the $^3J_{H,H}$ values measured for H-1 to H-4 confirmed the *manno*-configuration of the product.

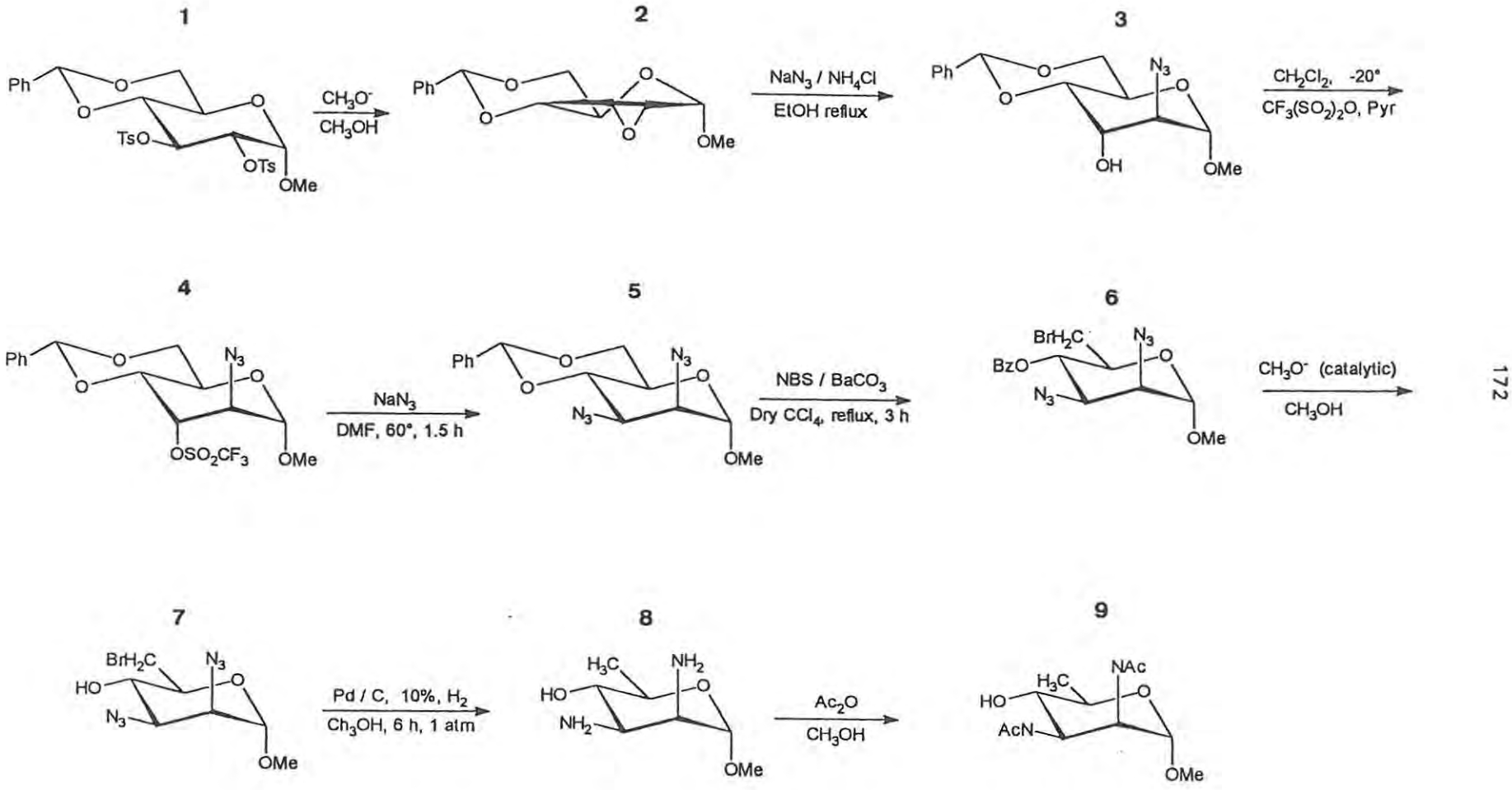


Figure 4.28 Flow diagram of the synthetic route followed.

Benzylidene ring-opening using NBS[446] was selected as the means of achieving the desired 2,3,6-trideoxy glycoside since the 6-bromo-6-deoxy function so formed can be readily reduced to a methyl group[447]. Reduction of the azido functions in **5** prior to opening the 4,6-benzylidene ring proved to be problematic as the benzoate ester formed migrated readily from C-4 to the amino function at C-3. Reduction of the azido groups followed by *N*-acetylation of the amino functions prior to ring opening, on the other hand, rendered the diacetamido 4,6-benzylidene derivative insufficiently soluble in either carbon tetrachloride or tetrachloroethane, a prerequisite for the ring opening reaction with NBS[446]. Compound **5** was therefore treated with NBS in carbon tetrachloride, as described[446] to produce methyl 4-*O*-benzoyl-6-bromo-2,3-diazido-2,3,6-trideoxy-α-D-*manno*pyranoside (**6**), in 57 % yield. The ^{1}H NMR spectrum predictably showed a large downfield shift for the H-4 double doublet (δ5.36) due to the deshielding effects of the benzoyl group. The absence of a resonance for the benzylidene ring proton confirmed that cleavage of the ring had occurred. The benzoate ester at C-4 was then removed using a catalytic amount of sodium methoxide to produce methyl 6-deoxy-6-bromo-2,3-diazido-2,3,6-trideoxy-α-D-*manno*pyranoside (**7**). Complete debenzoylation was confirmed by the absence of resonances for aromatic protons in the ^{1}H NMR spectrum of **7** and from the chemical shift for H-4 (no longer far downfield). Hydrogenation of a solution of **7** in dry methanol containing 10% palladium on carbon effected simultaneous reduction at C-2, C-3 and C-6 and gave methyl 2,3-diamino-2,3,6-trideoxy-α-D-*manno*pyranoside (**8**), as a syrup. The presence of a doublet at δ 1.35, $^3J_{5,6}$ = 6,3 Hz confirmed reduction of the 6-bromo-6-deoxy function. *N*-acetylation of **8** in dry methanol with acetic anhydride gave the title compound (**9**). The ^{1}H NMR spectrum showed signals for methyl protons at δ 1.97 and 2.05, and the ^{13}C spectrum showed signals for methyl carbons at 22.69 and 22.64 ppm, and signals for carbonyl carbons at 175.28 and 175.21 ppm (see Table I).

In addition to ^{1}H and ^{13}C spectra, a COSY (Figure 4.29) and a coupled HMQC[356] spectrum (Figure 4.30) was also acquired to further characterise the final product. Comparison of these data (Table I), with those obtained for Sug (the 2,3-diacetamido-2,3,6-trideoxy-ß-L-*manno*-residue in the *E. coli* K48 capsular polysaccharide), showed that both residues had similar connectivity patterns and possessed very similar $J_{H,H}$ values. The marked differences in chemical shifts for H-2, H-3 and H-5 are attributed to the deshielding influence of the axial *O*-methyl group.

TABLE I

^{1}H and ^{13}C NMR data[1] for methyl 2,3-diacetamido-2,3,6-trideoxy-*manno*pyranoside

	1	2	3	4	5	6
H	4.6 (5.07)[2]	4.36 (4.55)	4.16 (3.96)	3.39 (3.28)	3.81 (3.52)	1.32 (1.36)
$3J_{H,H}$	1.3 (1.2)	4.3 (4.1)	10.8 (10.0)	9.6 (9.6)	6.27 (5.7)	-
C	99.90 (99.83)	51.25 (52.19)	51.37 (54.61)	69.62 (70.87)	70.21 (74.46)	17.49 (18.00)
$^{3}J_{H\text{-}1,C\text{-}1}$	175 (166)					
$C\underline{H}_3$	1.97 (NAc)	2.05 (NAc)	3.42 (OCH_3)			
$\underline{C}H_3$	22.64	22.69	55.47			
$\underline{C}=O$	175.21	175.28				

[1] Chemical shifts with acetone as internal reference, δ 2.23 and 31.07 ppm for ^{1}H and ^{13}C respectively. [2] Values in parentheses are those for Sug, the 2,3-diacetamido-2,3,6-trideoxy-ß-L-*manno*-residue in the *E. coli* K48 capsular polysaccharide.

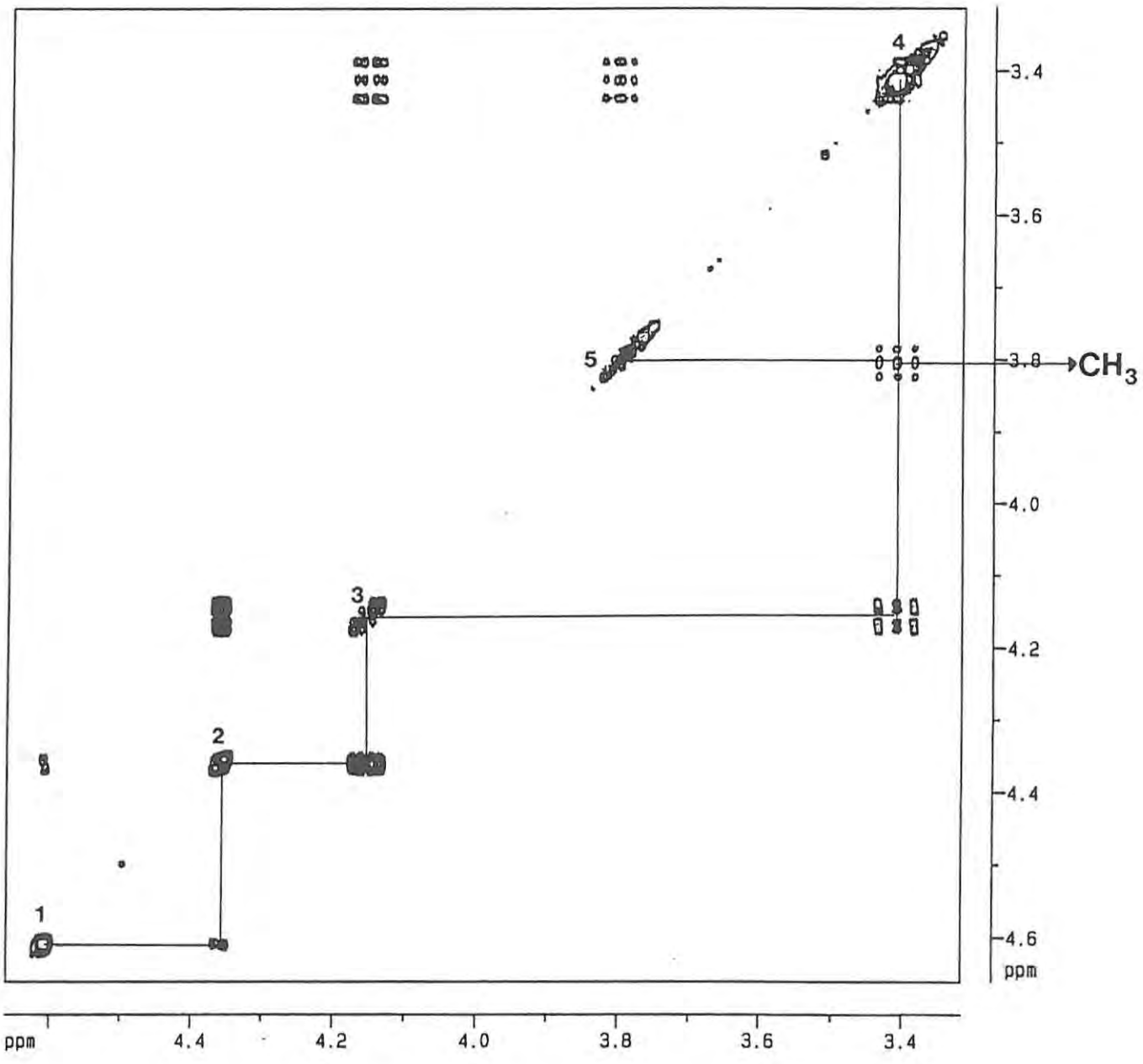


Figure 4.29 COSY contour plot of methyl 2,3-diacetamido-2,3,6-trideoxy-*α*-D-mannopyranoside for the region δ 3.3 - 4.7.

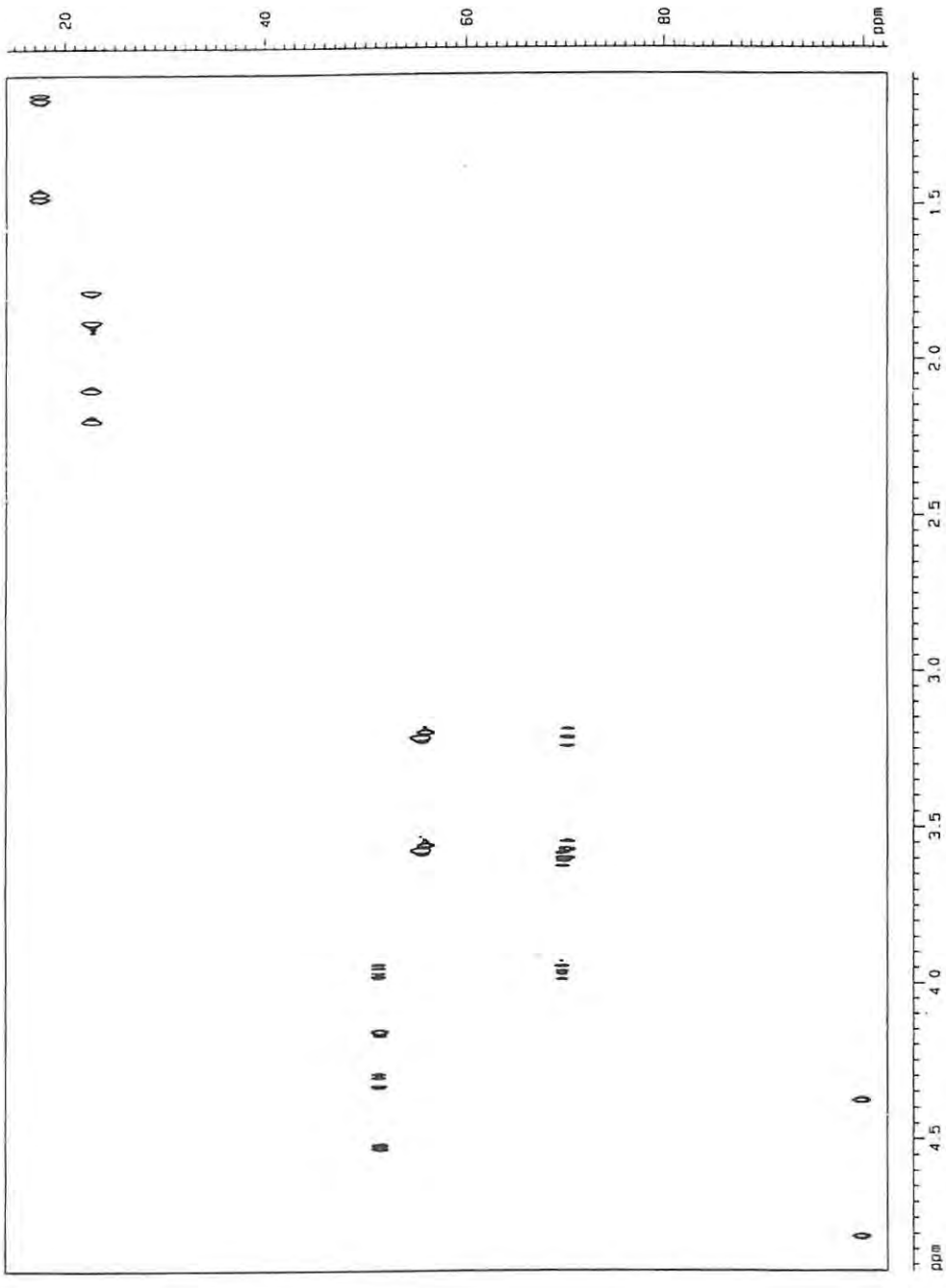


Fig 4.30 Coupled HMQC spectrum of methyl 2,3-diacetamido-2,3,6-trideoxy-α-D-mannopyranoside.

EXPERIMENTAL

General methods — Melting points were measured in capillary tubes on a Gallenkamp electrically heated melting-point apparatus and are not corrected. TLC was performed on glass plates coated with silica gel 60G (*E. Merck*) using *ethyl acetate: hexane* as the mobile phase, either *A* (4:1) or *B* (1:10) and were developed with alcoholic sulphuric acid (10%) followed by heating. All compounds were verified from their ^{1}H NMR spectra (see Appendix I) recorded on a Bruker AMX 400 spectrometer in chloroform-*d* (referenced at δ 7.25), or in D_2O with acetone as internal reference (δ 2.23 and 31.07 ppm for ^{1}H and ^{13}C respectively). H-6a refers to the equatorial proton on C-6, and H-6b to the axial proton linked to the same carbon. Evaporations were performed under reduced pressure with the bath temperature not exceeding 40°C. Light petroleum ether refers to the fraction boiling at 40-60°C. Solvents were dried over 0.4 nm molecular sieves (CH_2Cl_2, DMF, and DMSO) or by passage over neutral alumina (CCl_4).

*Methyl 2-azido-4,6-*O*-benzylidene-2-deoxy-α-*D*-altropyranoside* (**3**) — Methyl 2,3-anhydro-4,6-*O*-benzylidene-α-D-*allo*pyranoside (1.45 g) (**2**), prepared from methyl 4,6-*O*-benzylidene-2,3-di-*O*-toluene-*p*-sulphonyl-α-D-glucopyranoside (**1**) as described[443], was suspended in hot EtOH (18 mL) and an aqueous solution (4.5 mL) containing NaN_3 (1.45 g) and NH_4Cl (1.45 g) was added and the mixture was refluxed (22 h) with vigorous stirring. After cooling, water (30 mL) was slowly added and the product, which precipitated as an oil, was extracted with $CHCl_3$ (2 vols). The $CHCl_3$ layer was separated, dried (Na_2SO_4) and evaporated to dryness. Crystallisation of the syrupy residue from ether/ light petroleum ether afforded **3**. (Yield 1.39 g, 82%), m.p. 74-76°C (literature[444] 77-80°C).

*Methyl 4,6-benzylidene-2-azido-2-deoxy-3-*O*-trifluoromethanesulphonyl-α-*D*-altropyranoside* (**4**) — Trifluoromethanesulphonic anhydride (6.5 mmol) in dry CH_2Cl_2 (22 mL) was added dropwise to a stirred solution of **3** (1 g, 3.25 mmol) in dry CH_2Cl_2 (15 mL) at -20°C (dry ice/acetone bath). Stirring was continued at 0°C for 15 min and then at ambient temperature for a further 30 min. TLC (solvent *B*) showed a single major product (R_F 0.42). The mixture was shaken with an equal volume of water, and the aqueous phase was extracted twice with CH_2Cl_2.

The combined organic phase was washed with aqueous $NaHCO_3$ (5%), dried (Na_2SO_4), and evaporated to give a crystalline residue from which portions of CH_2Cl_2 and toluene were evaporated to remove residual pyridine. The residue was recrystallised from MeOH-CH_2Cl_2 and the resulting yellow crystals were collected, washed with chilled MeOH and dried. (Yield 0.992 g, 69%). m.p. 110-112°C (literature[448] 113°C). ^{1}H NMR data ($CDCl_3$) — δ 4.75 (d, 1H, $J_{1,2}$ < 1Hz, H-1), 4.05 (dd, 1H, $J_{2,3}$ 2.8 Hz, H-2), 5.07 (unresolved multiplet, 1H, H-3), 4.00 (dd, 1H, $J_{4,5}$ 9.7 Hz, H-4), 4.22 (m, 1H, H-5), 4.32 (dd, 1H, $J_{5,6a}$ 5.2 Hz, $J_{6a,6b}$ 10.25 Hz, H-6a), 3.78 (dd, 1H, $J_{5,6b}$ 10.3 Hz, H-6b). Other - 7.34-7.45 (2 x m, 5H aromatic, *Ph*-CH), 5.61 (s, 1H, Ph-C*H*), 3.44 (s, 3H, *O*-CH_3).

Methyl 4,6-O-benzylidene-2,3-diazido-2,3-dideoxy-α-D-mannopyranoside (**5**) — A saturated solution of NaN_3 (0.5 g) in dry DMSO (5 mL) was added to a solution of **4** (0.5 g) in dry DMF (5 mL). The reaction mixture was warmed in a water bath (60° for 1.5 h) with vigorous stirring and then poured into iced water (50 mL). The precipitate which formed was isolated and dissolved in CH_2Cl_2 (5 mL) and the residual reaction mixture was extracted twice with the same solvent. The combined organic phases were washed with aqueous $NaHCO_3$ (5%) and water, and dried (Na_2SO_4). The residue remaining after removal of the CH_2Cl_2 under reduced pressure was recrystallised from CH_2Cl_2-EtOH to afford **5**. (Yield 0.242g, 64%), m.p 148-150°C. ^{1}H NMR data ($CDCl_3$) — δ 4.70 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1), 3.88 (dd, 1H, $J_{2,3}$ 3.7 Hz, H-2), 4.12 (dd, 1H, $J_{3,4}$ 10.25 Hz, H-3), 4.06 (m, 1H, $J_{4,5}$ 8.6 Hz, H-4), 3.80-3.84 (m, 2H, H-5/H-6b), 4.27 (dd, 1H, $J_{5,6a}$ 5.5 Hz, $J_{6a,6b}$ 10.7 Hz, H-6a). Other - 7.35-7.49 (2 x m, 5H, *Ph*-CH), 5.64 (s, 1H, Ph-C*H*), 3.40 (s, 3H, *O*-CH_3).

Methyl 4-O-benzoyl-6-bromo-2,3-diazido-2,3,6-trideoxy-α-D-mannopyranoside (**6**) — A suspension of **5** (100 mg), NBS (60 mg), and $BaCO_3$ (100 mg) in dry CCl_4 (7 mL) was refluxed for 2.5 h with stirring. The reaction mixture turned brick red after 15 min and then slowly faded to a golden colour over the 2.5 h reflux period. The hot suspension was then filtered to remove the salts, and the filtrate was concentrated under reduced pressure to give a pale yellow syrup. The syrup, dissolved in ether (15 mL), was gently heated until the first turbidity and then allowed to stand for 12 h. The resulting crystals (**6**) were isolated and dried. (Yield 70 mg, 57%), m.p. 111-112°C.

^{1}H NMR data ($CDCl_3$) — δ 4.81 (d, 1H, $J_{1,2}$ 1.7 Hz, H-1), 3.95 (dd, 1H, $J_{2,3}$ 3.6 Hz, H-2), 4.17 (dd, 1H, $J_{3,4}$ 10.0 Hz, H-3), 5.36 (dd, 1H, $J_{4,5}$ 9.9 Hz, H-4), 4.05 (m, 1H, H-5), 3.71 (dd, 1H, $J_{5,6a}$ 6.7 Hz, H-6a), 3.42 (m, 1H, $J_{6,6b}$ 11.3 Hz, H-6b). Other - 7.40-7.60, 8.0-8.1 (3 x m, 5H, -O*Bz*), 3.49 (s, 1H, *O*-C*H*$_3$).

Methyl 6-deoxy-6-bromo-2,3-diazido-2,3,6-trideoxy-α-D-mannopyranoside (**7**) — A small piece of sodium metal was added to a solution of compound **6** (50 mg) in dry MeOH (10 mL) at 0°C, and left to stir at ambient temperature overnight. The solution was neutralised with IR-120(H+) resin beads, filtered and evaporated. The resulting syrup (which could not be crystallised) (Yield 29 mg, 80%) was used as such in the following step. ^{1}H NMR data ($CDCl_3$) — δ 4.75 (d, 1H, $J_{1,2}$ < 1 Hz, H-1), 3.70 (dd, 1H, $J_{2,3}$ = 2.3 Hz, H-2), 3.73-3.93 (4H, H-3, 4, 5, 6b), 3.42 (m, 1H, $J_{5,6a}$ 6.5 Hz, $J_{6a,6b}$ 11.3 Hz, H-6b).

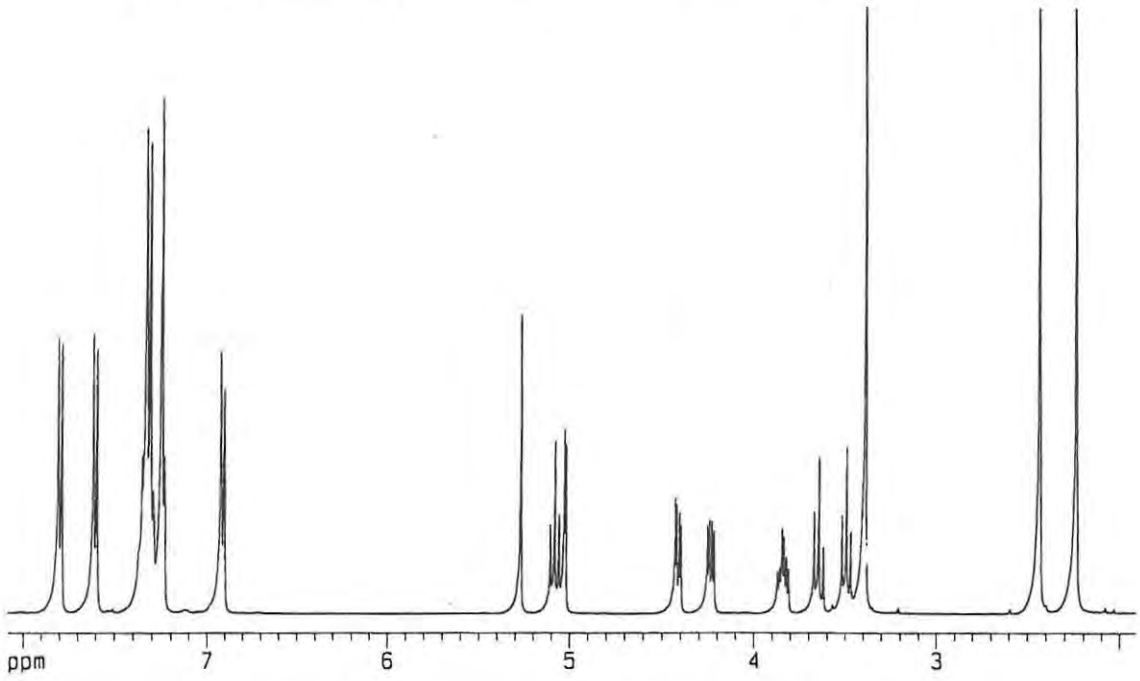
Methyl 2,3-diamino-2,3,6-trideoxy-α-D-mannopyranoside (**8**) — A stirred, dry methanolic suspension (10 mL) containing compound **7** (20 mg), 10% Pd/C (60 mg) and $BaCO_3$ (200 mg) was hydrogenated (1 atm) for 8 h. The suspension was filtered, and the filtrate evaporated to dryness to furnish a colourless syrup, (Yield 9 mg, 79%) which was used directly in the following step without further purification. ^{1}H NMR data (D_2O) — 4.71 (partially obscured by HOD, J <1Hz, H-1), 3.79 (unresolved dd, H-2), 3.25-3.65 (H-3, 4, 5, 6a, 6b, OC*H*$_3$), 1.35 (d, $J_{5,6}$ 6.3 Hz, 3H, H-6).

Methyl 2,3-diacetamido-2,3,6-trideoxy-α-D-mannopyranoside (**9**) — To a chilled solution (0°C) of compound **8** (9 mg) in dry MeOH (5 mL), acetic anhydride (0.4 mL) was added and the solution was allowed to stir for 4 h. The MeOH was evaporated and the excess acetic anhydride was removed by repeated codistillation with toluene. The resulting syrup (yield 9.7 mg, 81%) was dissolved in water (2 mL), purified by HPLC (Supelcogel C-611, 10^{-4} NaOH), and was examined by ^{1}H and ^{13}C NMR spectroscopy (D_2O) (see Table I).

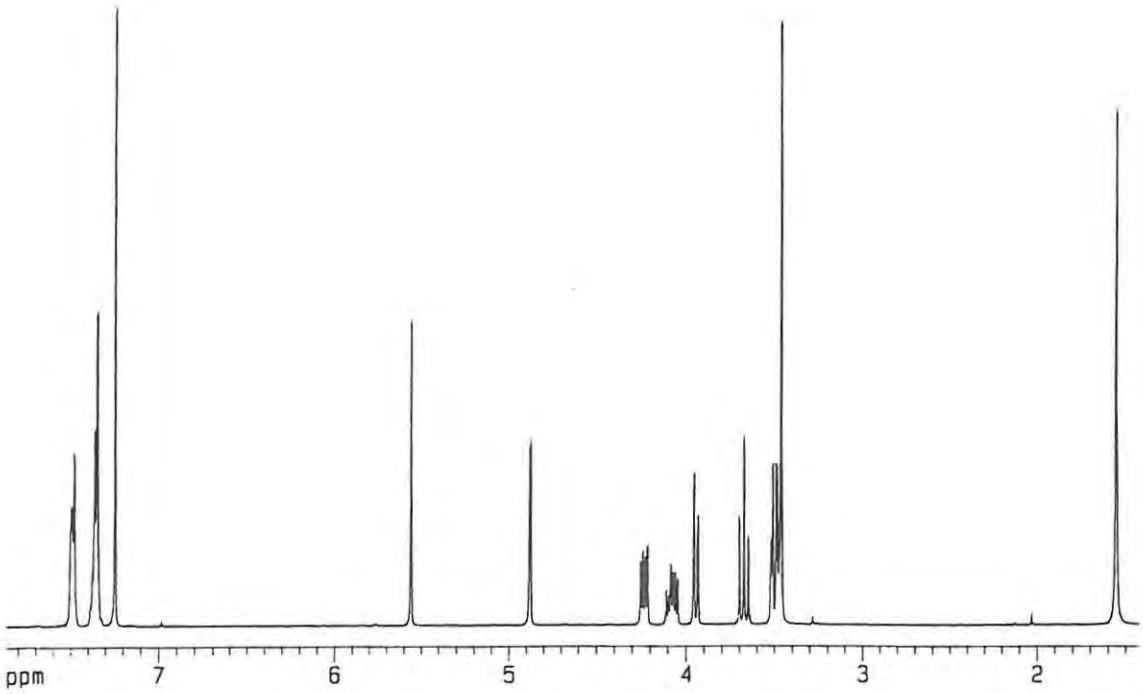
APPENDICES

APPENDIX I

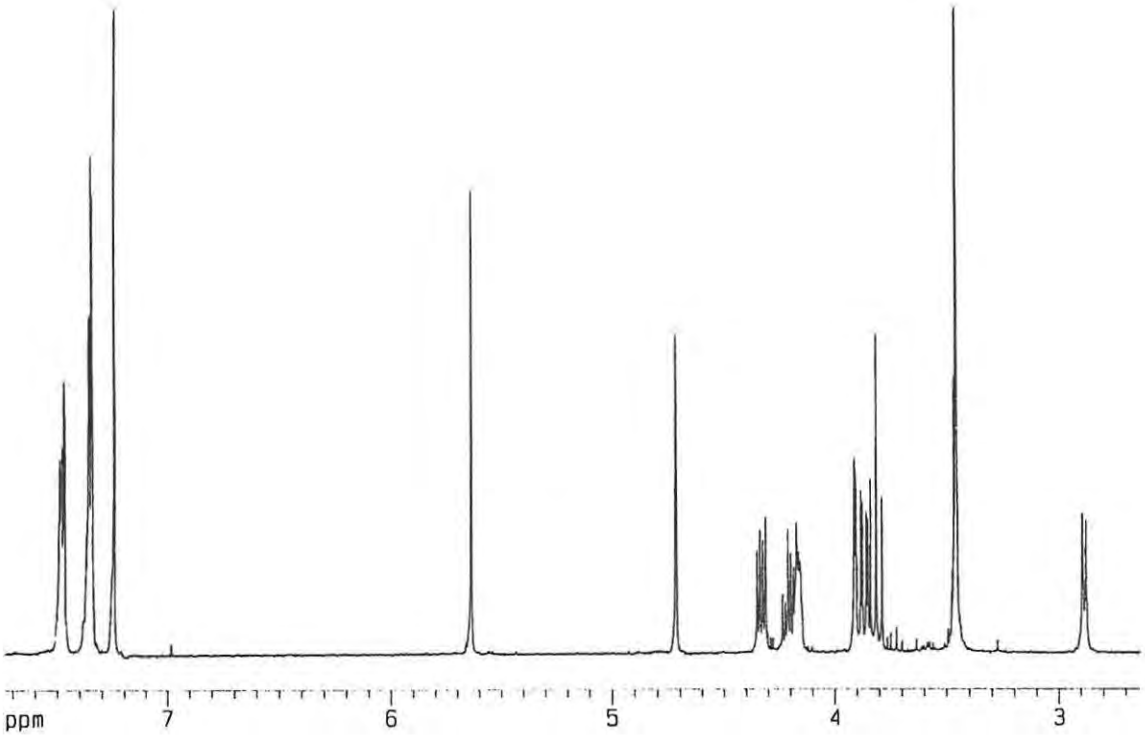
1D ^{1}H NMR spectra of synthetic intermediates 1 to 9 (Section 4.6)



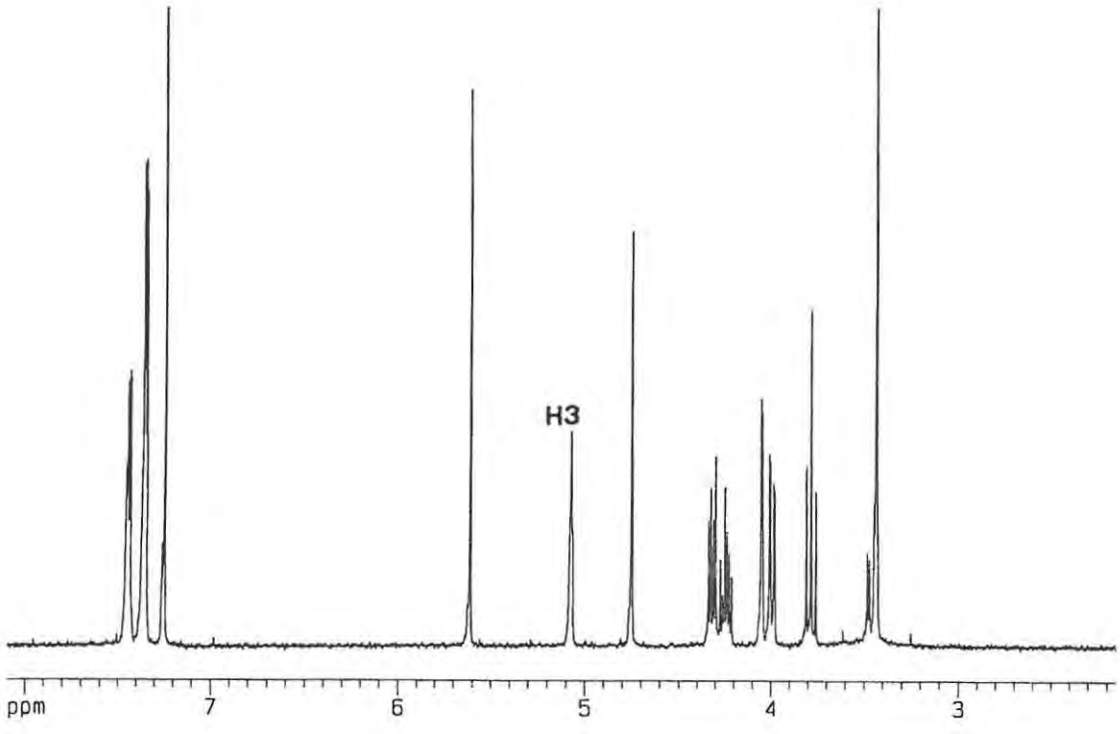
Methyl 4,6-O-benzylidene-2,3-di-O-p-toluenesulphonyl-α-D-glucopyranoside **(1)**



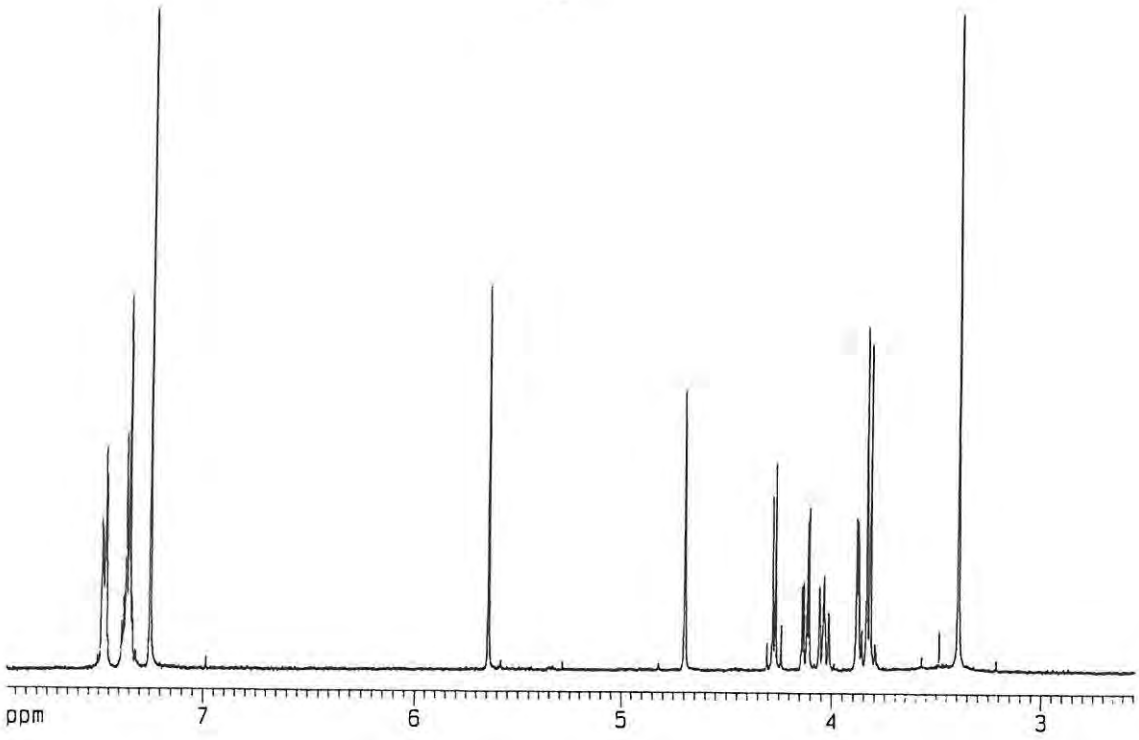
Methyl 2,3-anhydro-4,6-O-benzylidene-α-D-allopyranoside **(2)**



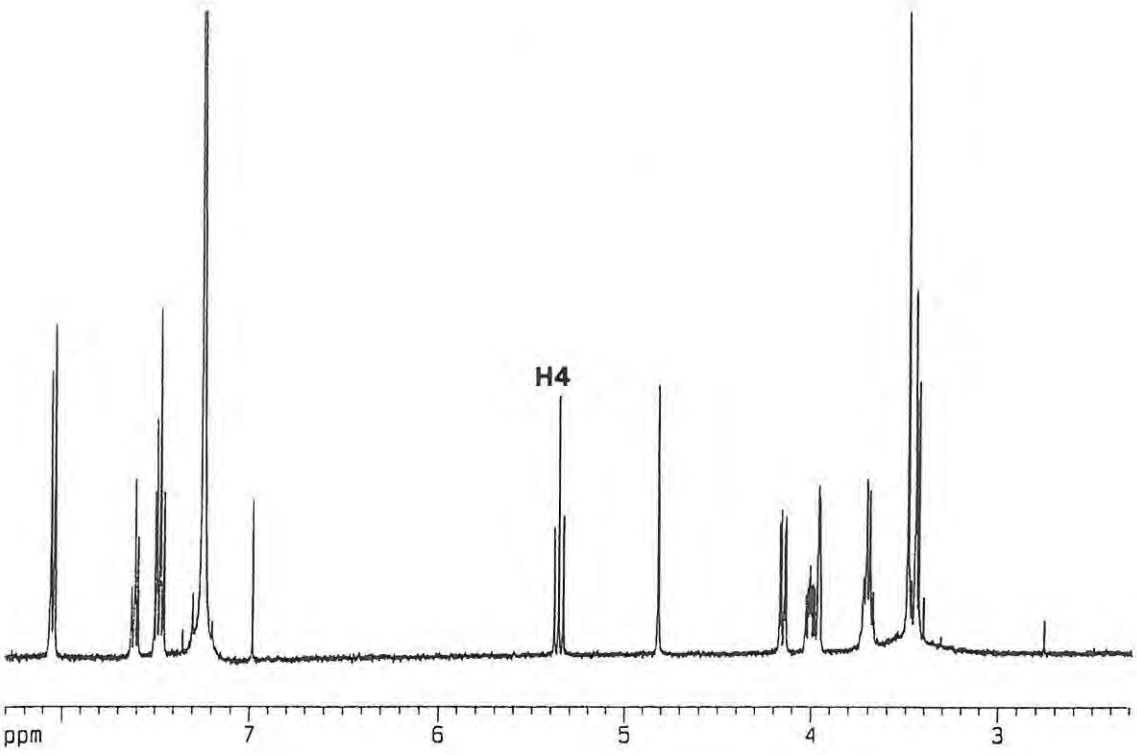
Methyl 2-azido-4,6-O-benzylidene-2-deoxy-α-D-altropyranoside **(3)**



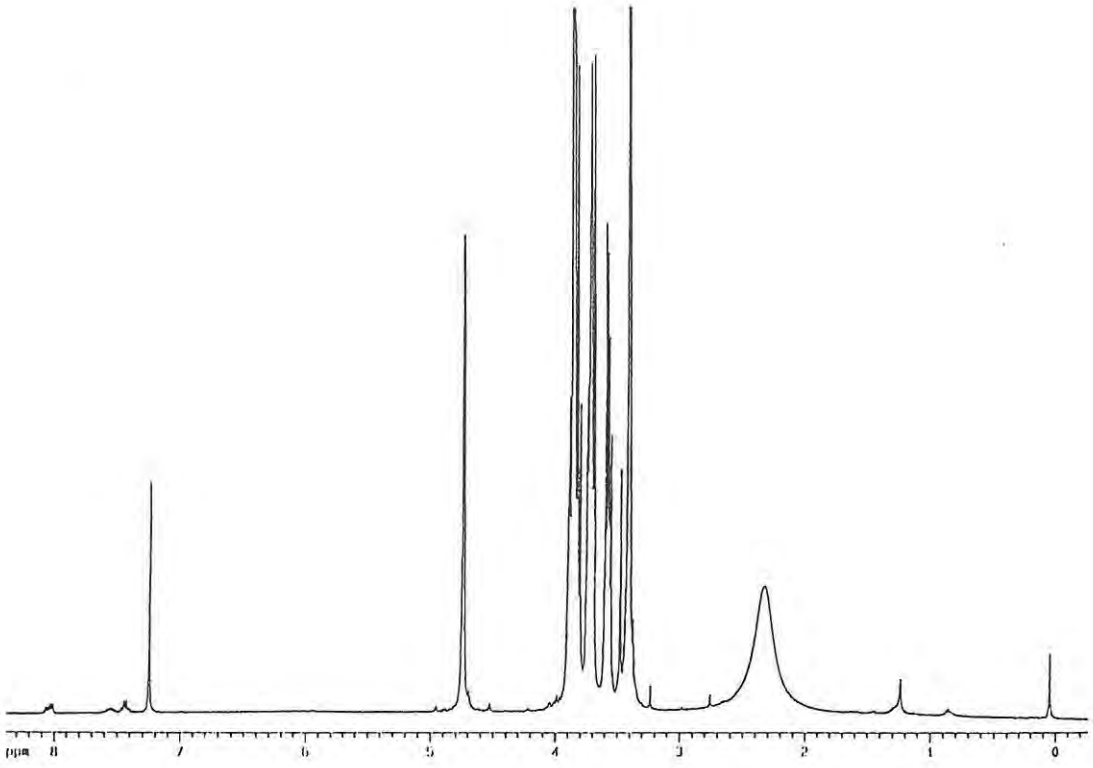
Methyl 4,6-O-benzylidene-2-azido-2-deoxy-3-O-trifluoromethylsulphonyl-α-D-altropyranoside **(4)**



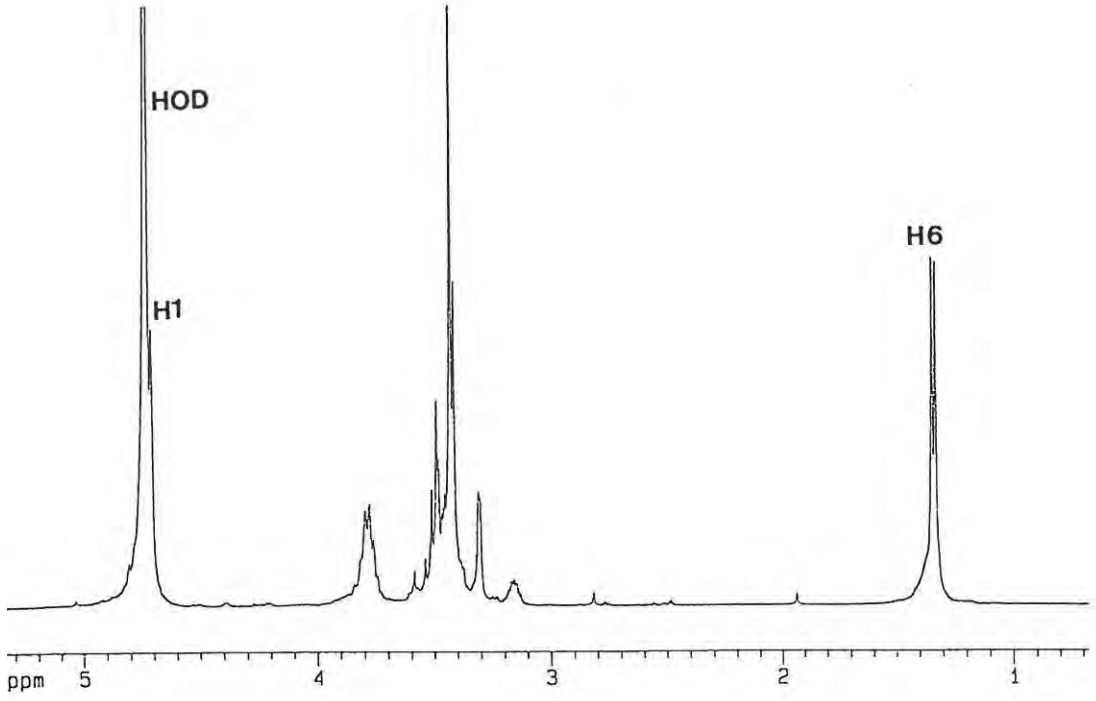
***Methyl 4,6-O-benzylidene-2,3-diazido-2,3-dideoxy-α-D-mannopyranoside* (5)**



***Methyl 4-O-benzoyl-6-bromo-2,3-diazido-2,3,6-trideoxy-α-D-mannopyranoside* (6)**

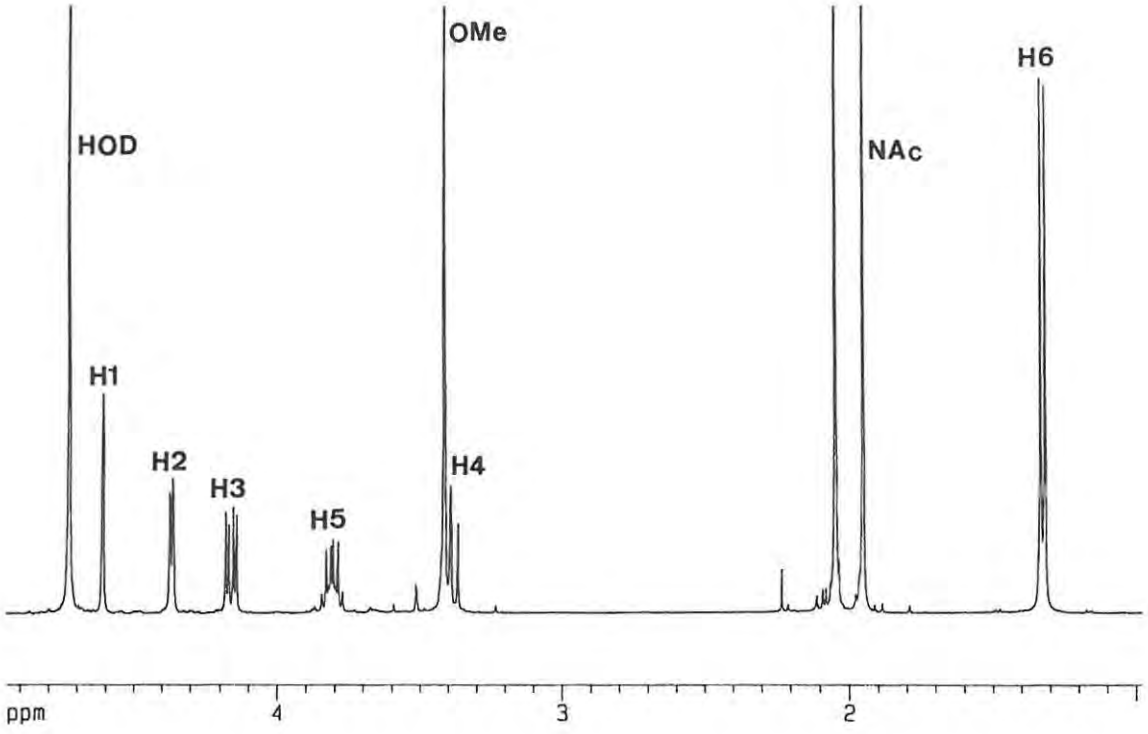


Methyl 6-deoxy-6-bromo-2,3-diazido-2,3,6-trideoxy-α-D-mannopyranoside (7)

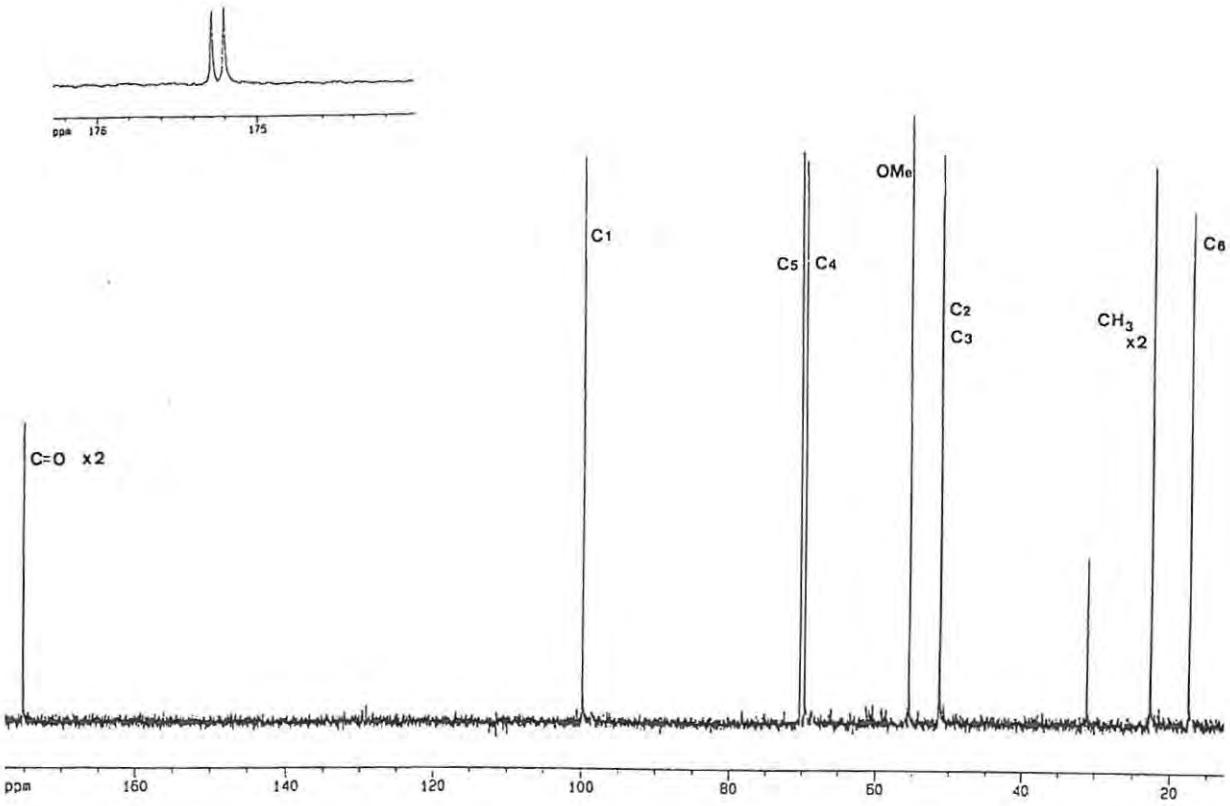


Methyl 2,3-diamino-2,3,6-trideoxy-α-D-mannopyranoside (8)

^{1}H NMR spectrum



^{13}C NMR spectrum



Methyl 2,3-diacetamido-2,3,6-trideoxy-α-D-mannopyranoside (9)

APPENDIX II

CULTURE MEDIA

Sucrose-rich Agar

Sucrose	30g
NaCl	2.0g
Yeast Extract	2.0g
K_2HPO_4	1.0g
$MgSO_4.7H_2O$	0.25g
$CaCO_3$	0.2g
Agar	15.0g
Aqua ad	1.0L

Mueller-Hinton Agar

Meat Infusion	5.0g
Casein hydrolysate	17.5g
Soluble starch	1.5g
Agar	14.0g
Aqua ad	1.0L

Nutrient Agar

Meat extract	1.0g
Peptone	5.0g
Yeast extract	2.0g
NaCl	8.0g
Agar	15.0g
Aqua ad	1.0L

In those instances where broth media were required, agar was omitted from the above formulae.

All media were autoclaved at 121° for 20 min prior to use.

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