STRUCTURAL ANALYSIS OF SOME *Escherichia coli* CAPSULAR ANTIGENS

THESIS

Submitted in Fulfilment of the
Requirements for the Degree of

DOCTOR OF PHILOSOPHY
of Rhodes University

by

PETER LINTON HACKLAND

December 1991

CONTENTS

ABSTRACT

The work presented in this thesis forms part of a collaborative effort to determine the chemical structures of the surface antigens of bacteria which belong to the *Enterobacteriaceae*. These antigens are largely polysaccharides and occur as lipopolysaccharides and capsular polysaccharides which give rise to the somatic or O antigens and the capsular or K antigens, respectively. In recent years interest has mostly been focused on the extracellular polysaccharide antigens expressed by the genus *Escherichia coli* because of the effect they exert on normal immunological processes and their structural relatedness to the surface antigens of other more pathogenic bacteria. Therefore the molecular structures of the capsular polysaccharides (K-antigens) produced by *E. coli* O9:K35 (A104a) and O9:K38 (A262a) have been determined by novel enzymic, chemical and spectroscopic procedures.

These investigations show that the structures of these polysaccharides can be determined by a combination of chemical and spectroscopic procedures, or almost entirely by n.m.r. spectroscopy alone. The *in vitro* bacteriophage mediated depolymerisation of the native *E. coli* K35 polysaccharide demonstrates the value of this method for the isolation of oligosaccharides representing the repeating- unit and multiples thereof.

Finally *E. coli* K37 and K38 capsular polysaccharides were used as model compounds for the evaluation of partial and selective reductive cleavage as methods of generating oligosaccharides for further structural analysis. The products of these reactions were analysed largely by a combination of mass spectrometric procedures.

ACKNOWLEDGEMENTS

The author would like to express his sincere gratitude to the following for assistance during the course of this doctoral study:

Professor H. Parolis, under whose guidance this research was conducted, for his support, encouragement and for adopting an "open-door" policy; Dr L.A.S. Parolis for assistance with the laboratory work conducted for this thesis and for recording n.m.r. spectra; Mr. D.L. Morley for his efficient technical support; Mr. A.W. Sonemann (Chemistry Department, Rhodes University) for assistance with mass spectrometry; Drs F. and I. Ørskov, Statens Seruminstitut, Copenhagen, for cultures of the bacteria (*E. coli* O9:K35-A104a and O9:K38-A262a); Mr. I. Antonowitz, National Chemical Research Laboratory, Pretoria, for recording the 500MHz n.m.r. spectra; Dr A.N. Anderson for isolating *E. coli* K37 capsular polysaccharide; all colleagues in the School of Pharmaceutical Sciences, Rhodes University for valuable discussion; the Foundation for Research and Development, Pretoria for financial support, and Rhodes University for the post-graduate bursary (1990).

The author would especially like to thank his wife, Janine Hackland, for so patiently and efficiently typing and proof reading the manuscript, and for keeping the home fires burning.

For my Wife

ABBREVIATIONS

~	approximately
CL	cross-linked
DEAE	diethylaminoethyl
d.p.	degree of polymerisation
δ	parts per million
e.i.	electron impact
eV	electron volt
f	furanose
f.a.b.	fast-atom-bombardment
g	gram
g.	gravity
Gal	galactose
GalA	galacturonic acid
GalNAc	N-acetylgalactosamine
g.l.c.	gas-liquid-chromatography
Glc	glucose
GlcA	glucuronic acid
GlcNAc	N-acetylglucosamine
g.p.c.	gel permeation chromatography
h	hour(s)
h.p.l.c.	high performance liquid chromatography
HR	high resolution
Hz	Hertz
Kdo	ketodeoxyoctulosonic acid
L	litre
M	molar
Man	mannose
m/z	mass-to-charge ratio
mg	milligram

min	minute(s)
mL	millilitre
mM	millimolar
m.s.	mass spectrometry
mol	mole
M_w	weight-average molecular weight
NeuNAc	neuraminic acid
µL	microlitre
n.m.r.	nuclear magnetic resonance
n.o.	not observed
p	pyranose
PAANs	peracetylated aldononitriles
p.c.	paper chromatography
PM	permethylated
p.m.a.a.	partially methylated alditol acetate
p.m.a.a's.	partially methylated alditol acetates
p.p.m.	parts per million
PRC	partial reductive cleavage
pyr	pyruvate
Rib	ribose
SF	superfine
SRC	selective reductive cleavage
TFA	trifluoroacetic acid
TMS	trimethylsilane
u.v.	ultra violet

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

LIST OF FIGURES

LIST OF TABLES PAGE

1. INTRODUCTION

The discovery of bacterial polysaccharide antigens dates back to 1917 when Dockey and Avery[1] reported that a "specific soluble substance" was secreted by *pneumococci* during growth. These microbial polysaccharides were shown to be true immunogens[2] as they induce an immune response with the generation of specific antibodies. Fig. 1.1 shows the gross anatomical differences between Gram-positive and Gram-negative bacteria and the location of the polysaccharide antigens.

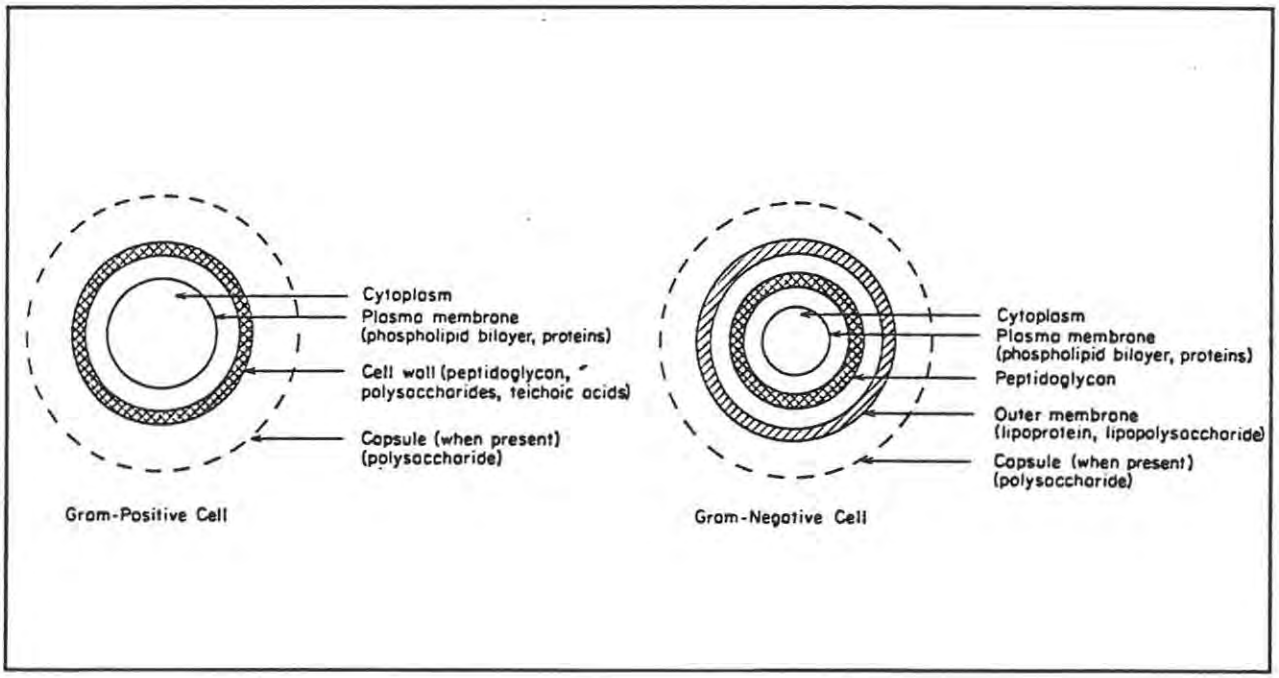


Fig. 1.1. The bacterial cell[2]

In Gram-negative bacteria the outer membrane contains a lipopolysaccharide which is a strong antigen. The high molecular weight polysaccharide portion of the lipopolysaccharide constitutes the O antigen, while the extracellular polysaccharide constitutes the K antigen. These K (kapsel) antigens are produced by both Gram-negative and Gram-positive bacteria and are usually acidic polysaccharides. They are visualised by staining with Indian ink and appear as capsules enveloping the bacterial cell. These capsules are not essential for

survival of the bacterium, however, they present a large anionic surface to host immune systems hence encapsulated pathogens have been found to be more virulent. This increase in pathogenicity is related to the capsules ability to attenuate host immune mechanisms by impeding immunoglobulin G and complement proteins preventing contact with complement activating substances (*e.g.* peptidoglycan) and initiation of complement sequence[3].

The use of these polysaccharides as antigens (immunogens) coupled with a knowledge of their chemical structure contributes significantly to the classification and identification of bacteria, to a better understanding of the immune response, to the definition of the active site in antigen-antibody interactions, and to detection and prevention of human disease[2].

For these reasons a collaborative effort to determine the structures of the capsular (K) antigens of *Escherichia coli* was initiated. The work presented in this thesis forms part of this program.

2. THE *ESCHERICHIA COLI* CAPSULAR (K) ANTIGENS

Bacteria of the genus *Escherichia* belong to the *Enterobacteriaceae* family, which are Gram-negative facultatively anaerobic rods. In all probability, more is known about the biological characteristics of *Escherichia coli* than any other bacterial species. This stems from extensive research into many aspects of these encapsulated bacteria, including the chemistry of the capsular (K) antigens, of which seventy four have been identified[4].

The majority of the capsular antigens are acidic polysaccharides with K88 and K99 being the only protein antigens in this group. The structures of the repeating-units of 54 *E. coli* K antigens have been reported, the majority of which form part of a recent review article[5]. This has been updated in Table 2.1 in this thesis. These polysaccharides are made up of regular repeating-units of 1 to 6 monosaccharides. The structures shown in Table 2.1 demonstrate the large degree of heterogeneity encountered in the chemistry of the capsular (K) polysaccharide antigens of *E. coli*. The many different monosaccharides encountered, including some less common sugars, are the major contributors to the compositional diversity of these polysaccharides. The most common neutral monosaccharides found are D-glucopyranose, D-galactopyranose, D-mannopyranose, L-rhamnopyranose, and D-ribofuranose. The less common neutral sugars include : L-fucopyranose in K27[6], K28[7], K33[5] and K42[8]; D-fructofuranose in K4[5], K11[9] and K52[10]; and D-galactofuranose in K2/K62[11] and K53/K93[12]. D-Glucopyranosyluronic acid, D-galactopyranosyluronic acid, 3-deoxy-D-*manno*-octulosonic acid (Kdo) and 5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranosonic acid (N-acetylneuraminic acid, NeuNAc) are the major acidic components, with the unusual 2-acetamido-2-deoxy-D-mannopyranosyluronic acid and 6-*O*-acetyl-4-deoxy-2-hexulosonic acid occurring in the K7[13] and K3[14] polysaccharides respectively. 2-Acetamido-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-galactopyranose are the most common amino sugars, while 2-acetamido-2-deoxy-L-fucopyranose only occurs

in the K87[15] capsular polysaccharide.

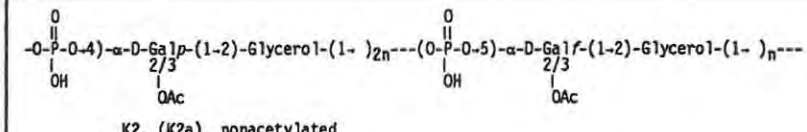
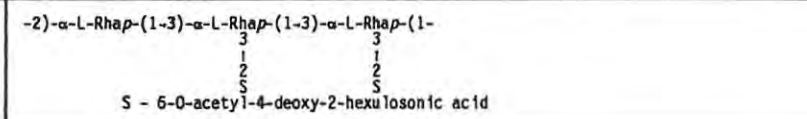
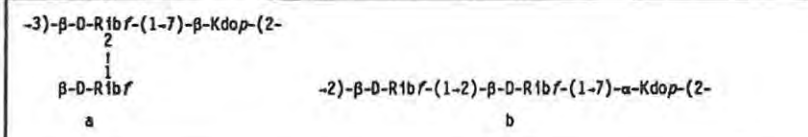
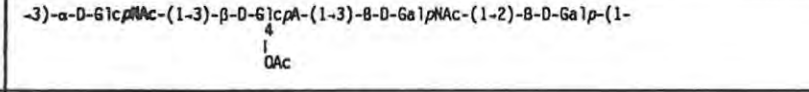
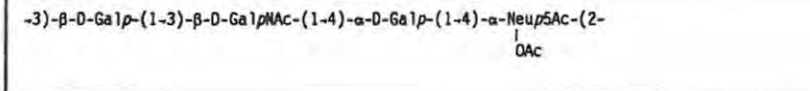
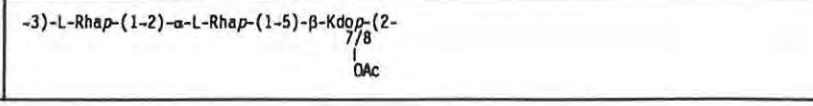
Non-sugar residues include phosphate, pyruvate, *O*-propionate, amino acids and *O*-acetate. The phosphate is present as either the ribitol or glycerol ester, and constitutes the only acidic component of several K antigens. Pyruvate is found in K26[16], K33[5], K37[17] and K55[18], and is the only acidic component of the K37 polysaccharide. Propionyl groups exist in small amounts in K14[19] and K52[10]. The amino acids are L-serine in K40[20], L-serine and L-threonine in K49[5] and K54[21], forming amide linkages with uronic acids.

The presence of *O*-acetyl groups in many of the K antigens of *E. coli* has generated considerable interest. They are responsible for small changes in polysaccharide structure, and have profound effects on immunological properties. For example, *E. coli* K18 and K22[22] are serologically distinct strains, despite having identical carbohydrate structures, differing only by the presence of one *O*-acetyl group in K18. This has prompted a re-examination of the structures of *O*-acetylated K antigens of *E. coli* (*e.g.* K87[23]) in which the location of *O*-acetylation was inconclusive.

The large number of *E. coli* capsular polysaccharides, coupled to the diversity in chemical composition, necessitates a classification system. The K antigens were, therefore, divided into three groups, A, B and L, on the basis of *O*-antiserum agglutination and heat stability[24]. Ørskov *et al.*[4] proposed that this system be discontinued due to the ambiguities that arise. Jann and Jann[25] recently proposed that the acidic capsular (K) polysaccharide antigens of *E. coli* be divided into two groups - Group I and Group II - on the basis of their physical, chemical and microbiological characteristics. Group I are high molecular weight polysaccharides, contain hexuronic acids as the acidic component, are coexpressed largely only with O8 and O9 antigens at all growth temperatures, and are heat stable at pH 5-6. These antigens share many

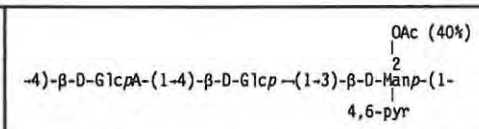
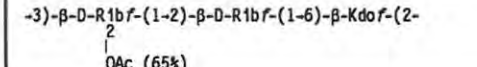
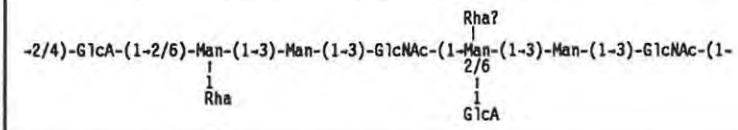
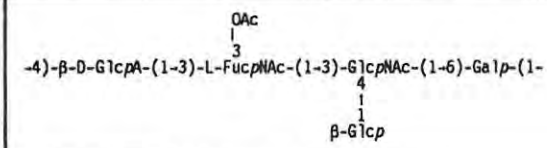
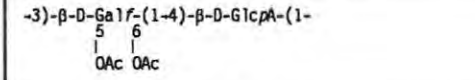
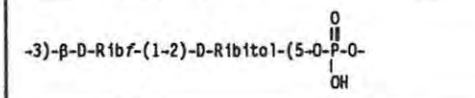
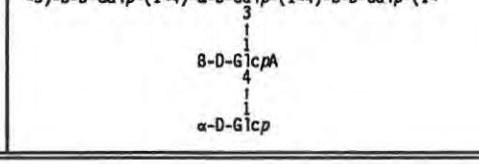
properties with those of *Klebsiella*, suggesting an intergeneric relationship. Group II are low molecular weight polysaccharides, are coexpressed with many *O*-antigens, are not expressed at low growth temperatures (17-20°), are thermolabile at pH5-6, and contain unusual acidic components (Kdo, phosphate, NeuNAc, N-acetylmannosaminuronic acid). These are similar to the capsular polysaccharides of *Neisseria meningitidis* and *Haemophilus influenzae*.

Table 2.1. *E. coli* K antigens

K antigen	Repeating-unit	Ref.
K1	→8)-α-Neu*p*5Ac-(2→	26
K2a,K2ab,K62	 K2, (K2a), nonacetylated K62 (K2ab), acetylated	11, 27, 28
K3	 S - 6-O-acetyl-4-deoxy-2-hexulosonic acid	14
K4		5
K5	→4)-β-D-Glc*p*A-(1→4)-α-D-Glc*p*NAc-(1→	29
K6		30, 31
K7 (K56)		13
K8		32
K9		33
K11		9
K12 (K82)		34

K13 (K20,K23)	→3)-β-D-Ribf-(1→7)-β-Kdop-(2→ K13, O-acetyl on 4 of Kdo K20, O-acetyl on 5 of Rib K23, nonacetylated	35, 36
K14	→6)-β-D-GalpNAc-(1→5)-β-Kdop-(2→ 8 | OAc (~60%) OPropionyl (~10%)	19
K15	→4)-α-D-GlcpNAc-(1→5)-β-Kdop-(2→	5
K16	OAc (33%) | 3 →2)-β-D-Ribf-(1→3)-β-D-Ribf-(1→5)-α-Kdop-(2→	37
K18,K22	O || →2)-β-D-Ribf-(1→2)-D-Ribitol-(5→O-P- 3 | | OH OAc K22, nonacetylated	22
K24	OAc (56%) | 4 →7)-α-Kdop-(2→1)-O-CH_2 | CHOH O | || CH_2-O-P-O- | OH	38
K19	OAc (~30%) | 8 →3)-β-D-Ribf-(1→4)-β-Kdop-(2→	39
K26	→3)-α-L-Rhap-(1→3)-β-D-Galp-(1→3)-β-D-GlcpA-(1→3)-α-L-Rhap-(1→3)-α-L-Rhap-(1→ 4 ↑ 1 3,4-pyr-α-L-Rhap	16, 40
K27	→4)-α-D-Glcp-(1→4)-α-D-GlcpA-(1→3)-α-L-Fucp-(1→ 3 ↑ 1 α-D-Galp	6,41
K28	→3)-α-D-Glcp-(1→4)-β-D-GlcpA-(1→4)-α-L-Fucp-(1→ 4 2/3 ↑ | 1 OAc (70%) β-D-Galp	7
K29	→2)-α-D-Manp-(1→3)-β-D-Glcp-(1→3)-β-D-GlcpA-(1→3)-α-D-Galp-(1→ 4 ↑ 1 4,6-pyr-β-D-Glcp-(1→2)-α-D-Manp	42, 43
K30	→2)-α-D-Manp-(1→3)-β-D-Galp-(1→ 3 ↑ 1 β-D-GlcpA-(1→3)-α-D-Galp	44, 45
K31	2)-α-D-Glcp-(1→3)-β-D-Galp-(1→3)-α-D-GlcpA-(1→2)-α-L-Rhap-(1→2)-α-L-Rhap-(1→ 4 | 1 α-L-Rhap	46
K32	→4)-α-L-Rhap-(1→3)-β-D-Glcp-1→ 3 ↑ 1 α-D-GlcpA-(1→3)-α-D-Galp	47
K33	→3)-α-D-Glcp-(1→4)-β-D-GlcpA-(1→4)-α-L-Fucp-(1→ ↑ 3 2,3-pyr ↑ 1 contains O-acetyl groups α-D-Galp	5

K34	→2)-β-D-Glc*p*A-(1→4)-β-D-Gal*p*-(1→3)-B-D-Gal*p*-(1→ 3 ↑ 1 α-D-Glc*p*-(1→4)-B-D-Gal*p*	48
K35	→4)-B-D-Man*p*-(1→4)-α-D-Glc*p*-(1→3)-B-D-Glc*p*-(1→ 3 ↑ 1 α-D-Glc*p*A 4 ↑ 1 B-D-Gal*p*	see 4.2. pg.
K36	→3)-β-D-Gal*p*-(1→3)-α-D-Gal*p*A-(1→2)-α-D-Man*p*-(1→ 4 ↑ 1 α-D-Man*p*	49
K37	→3)-β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→ 4 ↑ 4,6-pyr-α-D-Gal*p* (*R*)	17
K38	→2)-B-D-R1b*f*-(1→4)-B-D-Gal*p*-(1→3)-B-D-Gal*p*NAc-(1→4)-α-D-Glc*p*NAc-(1→4)-α-D-Gal*p*A-(1→	see 4.3. pg.
K39	→6)-α-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→2)-α-D-Man*p*-(1→3)-β-D-Glc*p*-(1→ 3 ↑ 1 α-D-Gal*p*	50
K40	→4)-β-D-Glc*p*A-(1→4)-α-D-Glc*p*NAc-(1→6)-α-D-Glc*p*NAc-(1→ 6 ↑ L-Ser (amide)	20
K42	→3)-α-D-Gal*p*-(1→3)-α-D-Gal*p*A-(1→3)-α-L-Fuc*p*-(1→	8, 51
K44	→4)-β-D-Glc*p*A-(1→3)-α-L-Rha*p*-(1→4)-α-D-Glc*p*NAc-(1→6)-β-D-Gal*p*NAc-(1→	52, 53
K46	→3)-B-D-Gal*p*-(1→3)-B-D-Glc*p*NAc-(1→3)-α-L-Rha*p*-(1→2)-O-CH(CH$_2$O-P(=O)-O-)(CH$_2$O) 2 ↑ 1 B-D-Glc*p*	Carb. Res. in press
K47	→3)-B-D-Glc*p*NAc-(1→2)-B-D-Gal*p*-(1→4)-B-D-Man*p*-(1→4)-α-D-Gal*p*-(1→ | 3,4-pyr	62
K49	→4)-β-D-Glc*p*A-(1→6)-β-D-Gal*p*-(1→6)-β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc-(1→ 6 | L-Thr (75%), L-Ser (25%) (amide)	5
K51	→3)-α-D-Glc*p*NAc-(1→O-P(=O)(OH)-O-) 4/6 | OAc	54
K52	OAc (80%) | 4 →3)-α-D-Gal*p*-(1→O-P(=O)(OH)-O-) 2 ↑ 2 β-D-Fru*f* OAc, Oprop (10%)	10
K53	→3)-β-D-Gal*f*-(1→4)-β-D-Glc*p*A-(1→ 2 | OAc	12
K54	→3)-β-D-Glc*p*A-(1→3)-α-L-Rha*p*-(1→ 6 | [L-Thr (90%), L-Ser (10%)] (85%, amide)	21

K55		18
K57	→2)-β-D-Ribf-(1→4)-β-D-Galp-(1→3)-α-D-GlcpNAc-(1→4)-α-D-GalpA-(1→	55
K74		56
K85		57
K87		15, 23
K92	→8)-α-Neup5Ac-(2→9)-α-Neup5Ac-(2→	58, 59
K93		12
K95	→3)-β-D-Ribf-(1→8)-Kdof-(2→ randomly O-acetylated	60
K100		22, 61
K102		62

3. STRUCTURAL ELUCIDATION OF THE *E. COLI* CAPSULAR (K) POLYSACCHARIDE ANTIGENS

3.1. INTRODUCTION

The majority of publications reporting on the structural elucidation of *E. coli* K antigens has been reviewed and the methods employed are listed in Table 3.1, pg. 10. From this document it is evident that the earlier work was largely reliant on chemical methods, while in more recent reports the emphasis is on spectroscopy.

The ensuing review is aimed at highlighting the methodology of the older "classical approach" to the elucidation of saccharide structure, followed by discussion of some of the modern trends. The intention is not to rigidly separate the two approaches, but rather to demonstrate their interdependence in the unambiguous elucidation of polysaccharide structure.

Chromatographic procedures are closely allied to chemical and spectroscopic methods. Detailed discussion is omitted however, as chromatographic methods have been well documented in the literature. Dutton[63,64] has reviewed the application of gas-liquid chromatography (g.l.c.) in carbohydrate analysis. High performance liquid chromatography (h.p.l.c.) of carbohydrates has been reviewed by McGinnis and Fang[65], Hicks[66], and recently by Ben-Bassat and Grushka[67]. Gel-permeation chromatography (g.p.c.) is particularly useful in purification and fractionation of poly- and oligo-saccharides and has been reviewed by Churms[68], Granath[69] and Whistler *et al.*[70]. This review is therefore not exhaustive but rather a selective one, based on the methods employed in the analysis of the *E. coli* capsular polysaccharides, as summarised in Table 3.1.

K-antigen	Chemical and enzymic methods	Chromatography	Spectroscopy
K1 (1964)[26]	periodate oxidation[71] neuraminidase (α-ketosidase) Ehrlich colour reaction[72,73] - sialic acid determination/colominic acid determination Ninhydrin procedure[74] - amino acid determination	analytical ultracentrifugation (homogeneity/molecular mass) paper chromatography (p.c.)	
K42 (1965)[8] K85 (1966)[57] K30 (1967)[44] K27 (1968)[6]	partial acid hydrolysis (H_2SO_4) ß-glucuronidase (uronic acid determination/anomeric configuration) periodate oxidation carboxyl reduction (aq. ethylene oxide/$NaBH_4$)[75] oxidation (lead tetraacetate)[76] carbazole/H_2SO_4 reagent[77] - uronic acid determination anthrone/H_2SO_4 reagent[78] - neutral hexose determination methylation[79]/methanolysis	p.c. and paper electrophoresis analytical centrifugation (homogeneity/molecular mass) gas liquid chromatography (g.l.c.) - neopentylglycol succinate - 5' X 1/8" packed open tubular column) - partially methylated methyl glycosides	polarimetry (absolute config.)
K87 (1971)[15]	periodate oxidation hydrazinolysis[15] carboxyl reduction (aq. ethylene oxide/$NaBH_4$)[75] methylation (Hakomori)[60,81] partial acid hydrolysis (0.25N H_2SO_4) Elson-Morgan reaction (amino sugar ID)[82] ß-glucosidase (anomeric configuration)	p.c. and paper electrophoresis g.l.c. (ECNSS-M) - partially methylated alditol acetates (p.m.a.a's.) ion exchange chromatography	polarimetry (absolute config.)
K29 (1971)[42]	periodate oxidation[83] carboxyl reduction[84] (aq. ethylene oxide/$NaBH_4$)[75] methylation analysis[80,81] partial acid hydrolysis (H_2SO_4) Smith degradation chemical determination of pyruvate[85] lactate dehydrogenase (pyruvate) ß-glucuronidase (anomeric configuration) RNase (purification)	analytical ultracentrifugation (homogeneity/molecular mass) paper electrophoresis g.l.c. (ECNSS-M)[86] - p.m.a.a's. column chromatography - ion exchange (DOWEX -2 acetate) p.c.[87]	

K29 (1975)[43]	periodate oxidation/Smith degradation[83] methylation analysis[80,81] partial acid hydrolysis (2MTFA) carboxyl reduction[88] (aq. ethylene oxide/$NaBH_4$)[75] α-glucosidase/ß-glucosidase/α-galactosidase[89-91] ß-glucuronidase[92] bacteriophage degradation[93] pancreatic RNase[88]	p.c. paper electrophoresis - pyruvate determination as dinitrophenylhydrazone g.l.c. (ECNSS-M) - alditol acetates[94], p.m.a.a's. column chromatography (charcoal) - oligosaccharide separation g.p.c. (Sephadex LH20/G10/G50)	^{1}H-n.m.r (220 MHz) g.l.c-m.s. (p.m.a.a's.)
K42 (1978)[51]	methylation[80,81] carboxyl reduction partial hydrolysis (0.5N H_2SO_4)	paper electrophoresis - oligosaccharide isolation p.c. - isolation of monosaccharides g.l.c. (ECNSS-M) - p.m.a.a's.	polarimetry (absolute config.) g.l.c.-m.s. (p.m.a.a's.) ^{1}H-n.m.r. (90MHz)
K92 (1977)[58]	periodate oxidation thiobarbituric acid procedure - sialic acid determination neuraminidase[95]	g.l.c. g.p.c. (Sephadex G25 (0.1M NH_4HCO_4), Sephadex G150)	^{1}H-n.m.r. (220 MHz) ^{13}C-n.m.r. (67.9 MHz - continuous wave mode)
K13 (1979)[35]	periodate oxidation/Smith degradation[96] methylation analysis[97,80] partial hydrolysis (acetic acid) thiobarbituric acid colour reaction - sialic acid determination carboxyl reduction[90] - (aq. ethylene oxide/$NaBH_4$)[75] alkaline phosphatase - phosphate hydrolysis	g.p.c. (DEAE cellulose/orcinol reagent) thin layer chromatography (t.l.c.) - cellulose paper electrophoresis p.c. - Kdo determination[98] g.l.c. (5% OV-101)	polarimetry (dispersion spectra) g.l.c.-m.s. - p.m.a.a's. (10% SE30)
K2/K62 (1980)[11,27]	periodate oxidation/Smith degradation methylation[80] dephosphorylation (aqueous 55% HF)[99] α and ß galactosidases (anomeric config.) alkaline phosphatase[100]	g.l.c. (OV-101) - p.m.a.a's., alditol acetates[94] p.c. (butanol/pyridine/water -6/4/3) paper electrophoresis g.p.c. (Biogel P2)	g.l.c.-m.s. ^{1}H-n.m.r. (anomeric config.) analytical ultracentrifugation (molecular weight determination) polarimetry
K6 (1980)[30]	colorimetric methods (TBA assay - Kdo[101,104], Orcinol assay[102] - pentoses) periodate oxidation[97]/Smith degradation methylation analysis[80,97] partial hydrolysis (1% acetic acid)[105]	g.l.c. (ECNSS-M packed column, OV-101 capillary) - TMS diethyl-dithioacetal derivatives[106] g.p.c. (Biogel P2. Sephadex G10) - desalting h.p.l.c. (Lichrosorb SI 60 - petroleum ether/acetate 5:1) - partially methylated ribitol acetates	^{13}C-n.m.r. (22.63 MHz -FT ^{1}H -decoupled) ^{1}H-n.m.r. (90 MHz - deuterated chloroform solutions)

K30 (1980)[45]	methylation[80] bacteriophage degradation (ß-galactosidase)	g.l.c.- (ECNSS-M)	^{1}H-n.m.r g.l.c.-m.s.
K5 (1981)[29]	partial acid hydrolysis[107] (2M HCl) Smith degradation[98] deamination[108,109] - nitrous acid methylation[80] carbazole method - GlcA determination/Anthrone method - glucose determination[195] RNase/DNase (purification) carboxyl reduction[110] - 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulphonate ß-glucuronidase[102]	p.c. paper electrophoresis g.l.c. - alditol acetates g.p.c. (Biogel P2 - 0.2M ammonium acetate)	^{13}C-n.m.r. ^{1}H-n.m.r. (220 MHz - continuous wave)
K6 (1982)[31]	Smith degradation[98] methylation[80] partial hydrolysis (acetic acid) periodate oxidation/Smith degradation[98] carboxyl reduction[110]	ion exchange - Rexyn 201[111] g.l.c.- (-)-2-octyl glycoside acetates[112] (absolute config.) ion exchange g.p.c. (DEAE Sephadex A25/0.1M tris buffer pH 8.5 - 0 to 0.6M NaCl gradient) g.l.c. (ECNSS-M/OV-1 180x0.15cm glass column; glass capillary SP-1000 W.C.O.T) g.p.c. (Biogel P2 - water)	^{13}C-n.m.r. (anomeric config.) ^{1}H-n.m.r. u.v. g.l.c.-m.s. (p.m.a.a's.[94], methylated oligosaccharides)
K27 (1982)[41]	O-acetyl determination[113] carbazole reagent[102] - hexuronic acid determination partial hydrolysis (0.5M H_2SO_4) carboxyl reduction (carbodiimide)[110] ß-D-glucosidase/ß-D-galactosidase/ß-D-glucuronidase/α-D-glucosidase[114] methylation[80]	p.c. (Whatman No.1) g.l.c. - p.m.a.a's. (ECNSS-M) paper electrophoresis	g.l.c.-m.s. (p.m.a.a's.)[115] ^{1}H-n.m.r. (90 MHz) analytical ultracentrifugation (molecular weight)
K2 (1982)[28]	Smith degradation/alkali hydrolysis hydrazinolysis[116] periodate oxidation/ß-elimination/hydrazinolysis/alkaline treatment	g.l.c. - TMS ethers[117] (packed column/OV-1) g.p.c. (DEAE Sephacel-water/triethylammonium carbonate buffer pH7.8; Sephadex G50)	

K7/K56 (1982)[13]	Glycose analysis : amino sugars-amino acid analyzer/neutral sugars-automated sugar analyzer[118] carboxyl reduction (carbodiimide)[110] periodate oxidation methylation[115] N-deacetylation/*O*-deacetylation/ninhydrin oxidation[119,120] D-glucose oxidase(absolute config.)	g.l.c. (OV-225/packed column)	g.l.c.-m.s. (p.m.a.a's.) ^{13}C-n.m.r. and ^{1}H-n.m.r. (300 MHz)
K12/K82 (1983)[34]	Cysteine reagent(rhamnose determination)[121] thiobarbituric acid assay(2-keto-3-deoxy-D-manno-octonic acid determination)[122] partial hydrolysis(1% acetic acid) Ruff degradation(2-keto-3-deoxy-D-*manno*-octonic acid) O-acetyl determination[123] methylation[80,124] periodate oxidation chromium trioxide oxidation(anomeric config.)[125]	g.p.c. (Sephadex G50/Biogel P2) g.l.c. (ECNSS-M/alditol acetates/octyl glycoside acetates) paper electrophoresis	^{1}H-n.m.r. (300 MHz - FT) ^{13}C-n.m.r. (gated decoupling - anomeric config.) g.l.c.-m.s. (p.m.a.a's.)
K13/K20/ K23 (1983)[36]	thiobarbituric acid assay - Kdo determination[122] methylation	g.p.c. (DEAE cellulose) immunoelectrophoresis g.l.c. - alditol acetates[36]/methyl glycosides (3% ECNSS-M)	^{13}C-n.m.r. (75 MHz)/^{1}H-n.m.r. (300 MHz) - location of *O*-acetyl groups/anomeric config.[126/31] g.l.c.-m.s.[127] - (S.E.30/glass capillary)
K14 (1983)[19]	amino acid analyzer - amino sugar determination thiobarbituric acid assay - Kdo determination[122] *O*-deacylation periodate oxidation partial hydrolysis (50mM H_2SO_4) methylation[104]	g.p.c. (Sephadex G50/Biogel P2/water) p.c. - hydroxamates[123] (*O*-acetyl and *O*-propionyl groups) g.l.c. - Porapak Q5[128] paper electrophoresis (preparative oligosaccharide isolation)	polarimetry ^{1}H-n.m.r. (300 MHz - anomeric config.) c.i.-m.s. (methylated disaccharide) ^{13}C-n.m.r. (anomeric config./linkage position/position of *O*-acetylation)

K54 (1984)[21]	uronic acid determination - carbazole reagent[102] rhamnose determination - cysteine reagent[121] periodate oxidation methylation analysis[80] - carboxyl reduction -$LiAlD_4$ partial hydrolysis (0.5M H_2SO_4) ß-glucuronidase (anomeric config.) amino acid determination - Durrum D-500 analyser	g.p.c. (Sephadex G50/Biogel P2/Sephadex LH20) g.l.c. (Carbowax/ECNSS-M/2.5% SE-30 - acetyl glycoside acetates/alditol acetates) preparative paper electrophoresis	polarimetry c.i.-m.s. (NH_3)/e.i.-m.s. (methylated oligosaccharides) g.l.c.-m.s. (p.m.a.a's.) ^{1}H-n.m.r. (300 MHz) - threonine/GlcA amide[129] ^{13}C-n.m.r. (gated decoupling - anomeric config.)[130]
K52 (1985)[10]	alkaline phosphatase[94] phosphate determination[131] acetyl and propionyl characterisation (hydroxamates)[123,128] periodate oxidation methylation analysis[80] fructose determination - 5-hydroxymethylfurfural/ß-fructosidase (invertase) partial hydrolysis (0.5M NaOH)	g.p.c. (Sephadex G50/Biogel P2-H_2O) g.l.c. (alditol acetates) p.c. (preparative) .	^{1}H-n.m.r. (300 MHz) ^{13}C-n.m.r. (selective decoupling) g.l.c.-m.s. (p.m.a.a's.) c.i.-m.s. (NH_3) - permethylated disaccharides
K28 (1985)[7]	D-galactose oxidase (absolute config.) methylation[80] carboxyl reduction (native polymer)[132] partial hydrolysis (0.01M TFA) periodate oxidation location of *O*-acetyl groups - methyl vinyl ether[7] ethylation[80]	p.c. (Whatman No.3MM preparative/analytical) g.l.c. - p.m.a.a's. (stainless steel packed columns/3% SP- 2340/3% OV-225) ion-exchange chromatography - Bio-Rad AGI-X2(formate) resin g.p.c. - molecular mass determination	polarimetry (circular dichroism - absolute config.) g.l.c.-m.s. (DB-225 capillary) alditol acetates ^{1}H-n.m.r. (twin signals - *O*-acylation/anomeric config. ^{13}C-n.m.r. (positions of *O*-acylation)
K95 (1985)[60]	Kdo determination - Thiobarbituric acid assay[122] acetate determination[128] periodate oxidation partial hydrolysis (1% acetic acid) methylation[80,133]	g.p.c. (Sephadex G50/Biogel P2 - water) g.p.c. (ECNSS-M - alditol acetates) paper electrophoresis (Whatman No.1 - analytical/Schleichter and Schüll 2043b - preparative)	^{1}H-n.m.r. (300 MHz) ^{13}C-n.m.r. (anomeric config./ring form) g.c.-m.s. (alditol acetates) c.i.-m.s. (NH_3) - methylated oligosaccharides
K92 (1985)[59]	sialic acid determination - Svennerholm method[59] methanolysis lactonisation of polysaccharide - corbodiimide periodate oxidation	g.p.c. (Sephadex CL-4B - molecular-weight)	^{13}C-n.m.r. (90 MHz - conformational analysis)[196,197] 2D-n.m.r. - HETCOR

K51 (1985)[54]	glucosamine determintion - Durrum AA analyzer phosphate determination - Ames method[131] periodate oxidation partial hydrolysis (0.1N TFA)	preparative paper elctrophoresis (pyridinium acetate) t.l.c. (butanol:pyridine:water - 6:4:3)	^{13}C-n.m.r. (gated decoupling - anomeric config.) ^{31}P-n.m.r. (P linkage position)
K40 (1986)[20]	methylation analysis[80] periodate oxidation[134] and Smith degradation glucuronic acid determination (carbazole reagent) glucosamine and serine determination (Durrum AA analyzer/Morgan-Elson reagent[135])	g.l.c. - alditol acetates (CB SE-54 25m x 0.25mm), (+)-2-butyl ester (absolute config. of serine - ECNSS-M) g.p.c. (Biogel P2)	g.l.c.-m.s. (alditol acetates) c.i.-m.s. (NH_3) - methylated oligoalditols ^{13}C-n.m.r. (linkage analysis), APT (assignments)[136,137] ^{1}H-n.m.r. (anomeric config.)
K43 (1986)[138]	periodate oxidation partial hydrolysis (0.1M TFA) methylation analysis Smith hydrolysis	g.l.c. - alditol acetates	^{1}H-n.m.r. (anomeric config.)
K32 (1987)[47]	methanolysis carboxyl reduction (carbodiimide)[132] Smith degradation methylation (dry NaOH powder as base)[139,140] bacteriophage isolation/cross reaction - *Klebsiella* K55 synthesis - partially methylated methyl rhamnosides[141]	g.l.c. - (fused silica capillary - DB-17/fully acetylated alditols/(-)2-octyl glycoside acetates - absolute config.[112]) silica gel - G60 column chromatography t.l.c.	polarimetry g.l.c.-m.s. (DB-17 capillary) ^{1}H-n.m.r. - position of *O*-acetylation 2D-n.m.r. - COSY (determination of sequence[142]) ^{13}C-n.m.r. (gated decoupling - anomeric config.) n.O.e. difference 1D-n.m.r. experiment (sequence)
K37 (1987)[17]	phosphate analysis (Molybdenum Blue method)[143] methanolysis (3% HCl/methanol) methylation analysis (Hakomori[80]/Kuhn[144]) periodate oxidation and Smith degradation[98] partial hydrolysis (0.5M TFA)	g.l.c. - peracetylated aldononitriles (PAAN's)[145], alditol acetates, (-)-2-octyl glycoside acetates[112] (DB-225 fused silica capillary column) p.c. (Whatman No.1 - descending - preparative and analytical) g.p.c. (Sepharose 4B/M NaCl - phenol-sulfuric detection[146])	polarimetry (optical rotations) i.r. spectroscopy ^{1}H-n.m.r. (500 MHz - anomeric config.) ^{13}C-n.m.r. (config. of carboxyethylidene acetal[147]) g.l.c.-m.s. (alditol acetates)

K34 (1987)[45]	methanolysis (3% HCl/methanol)[198] chromium trioxide oxidation (anomeric config.)[125] methylation analysis[80] ($LiAlH_4$ reduction) ß-elimination[146] carbodiimide reduction[132] (carboxyl reduction) Smith degradation[98] selective Smith degradation[149] bacteriophage degradation[150] α-D-glucosidase (anomeric and absolute config.)	p.c. (Whatman No.1 - analytical/Whatman 3MM - preparative) g.l.c. - alditol acetates (fused silica capillary columns - DB-17/DB-225 - analytical; stainless-steel column - 3% SP-2340 -preparative) g.p.c. (Biogel P2/Sephadex LH20)	^{1}H-n.m.r. ^{13}C-n.m.r. i.r. spectroscopy (methylation) g.l.c.-m.s. (p.m.a.a's.) circular-dichroism spectra[151] (absolute config.)
K9 (1987)[33]	methanolysis (carboxyl reduction) D-galactose oxidase[152] (absolute config.) methylation analysis[80,153] (acetolysis)[154] periodate oxidation and Smith degradation[96] bacteriophage degradation	g.l.c. - TMS methyl glycosides/(-)-2-octyl glycoside acetates - absolute config.[112] (fused silica capillary columns - OV-1,OV-17 and OV-225/packed column - ECNSS-M) g.p.c. (Biogel P4/Sepharose 4B) p.c. (Whatman No.1 - analytical - periodate-benzidene[155] and alkaline silver nitrate[156] detection)	g.l.c.-m.s. (partially methylated methyl glycoside acetates/p.m.a.a's.) ^{1}H-n.m.r. (anomeric config., α-linked Neu5Ac) ^{13}C-n.m.r. (linkage position of Neu5Ac[157]/HexNAc[158] 500 MHz) 2D-n.m.r. - COSY/HETCOR (linkage analysis)
K3 (1988)[14]	cysteine reagent - L-rhamnose determination[121] partial hydrolysis (1% acetic acid) methylation analysis[104,133] periodate oxidation	g.l.c. - alditol acetates (ECNSS-M), acetyl content[126], (+)-2-octyl glycoside acetates(absolute config.) g.p.c. (Sephadex G50/Biogel P2) paper electrophoresis (preparative)	g.l.c.-m.s. (p.m.a.a's.) ^{13}C-n.m.r. (linkage analysis, anomeric config. - gated-decoupling)[130] ^{1}H-n.m.r. (300 MHz) 2D-n.m.r. - COSY[159] (6-*O*-acetyl-4-deoxy-2-hexulosonic acid)
K53 and K93 (1988)[12]	carboxyl reduction (carbodiimide/$NaBH_4$) D-glucose and D-galactose oxidase (absolute config.)	g.l.c. - alditol acetates, (-)-2-octyl glycoside acetates	^{13}C-n.m.r. (ring form and anomeric stereochemistry - ^{1}H-coupled spectrum) ^{1}H-n.m.r. (500 MHz - positions of *O*-acetylation) 2D-n.m.r. - COSY[160]/HETCOR 1D-INEPT n.m.r. experiment[161] (linkage analysis/positions of *O*-acetylation)

K18,K22 and K100 (1988)[22]	partial hydrolysis (0.1M HCl, 0.5M NaOH) periodate oxidation methylation analysis[80,104] dephosphorylation - alkaline phosphatase orcinol test - ribose determination Ames test[131] - phosphate determination hydroxamate method[123] - acetyl content determination	g.l.c. - p.m.a.a's., alditol acetates (CB CP SIL 5), acetate content (ECNSS-M)[128] g.p.c. (Biogel P2)	g.l.c.-m.s. (p.m.a.a's.) i.r. (acetyl ester and phosphate bands) ^{13}C-n.m.r. (gated decouplin)g[130,137,162] ^{31}P-n.m.r. (determination of phosphodiester bridges) APT (attached proton test - carbon substitution[136,137])
K100 (1988)[61]	base hydrolysis/alkaline phosphatase - phosphoric diester linkage[163] periodate oxidation/aqueous Br_2 oxidation (carboxylates)/acid hydrolysis[164] neutral reducing sugar analysis - automated sugar analyzer[118]	g.l.c. - TMS ethers (SP-2100 coated glass capillary) preparative g.l.c. - octyl glycerate esters (15% SE-30)	^{1}H-n.m.r. (300 MHz/SED spin-echo difference spectra - phosphate linkage analysis) ^{13}C-n.m.r. (25 MHz) 2D-n.m.r. - COSY (^{1}H assignments) - HMQC[165,166] (^{13}C assignments) - HMBC[165,166] (linkage analysis)
K36 (1988)[49]	bacteriophage depolymerisation (endogalactosidase)[167] methylation - Hakomori[80] and Kuhn[144] methanolysis - carboxyl reduction	g.p.c. (Sepharose 4B CL/M NaCl - M_r determination) g.l.c. - (-)-2-octyl glycoside acetates (absolute config.)[112], p.m.a.a's., PAAN's[145] (DB-225 bonded-phase fused-silica capillary column) p.c. (Whatman No.1 - descending/alkaline silver nitrate detection[155])	g.l.c.-m.s. (p.m.a.a's.) ^{1}H-n.m.r. (500 MHz - anomeric config.) ^{13}C-n.m.r. - $J_{C1,H1}$ values 2D-n.m.r. - COSY - HETCOR
K44 (1988)[52]	deamination[168] carboxyl reduction - methanolysis[169]/carbodiimide[132] chromium trioxide oxidation[125] (anomeric config.) methylation analysis - Hakomori[80,170] N-deacetylation[171] ß-elimination[148] periodate oxidation[172]/Smith degradation[86] acetolysis[154]	g.l.c. - peracetylated alditol acetates, p.m.a.a's. (DB-17 bonded-phase fused-silica capillary) preparative g.l.c. (3% SP-2340/Supelcoport 100-120 mesh) p.c. (Whatman No.1 - analytical) g.p.c. (Biogel P2, Sephadex LH20)	c.d. spectra (alditol acetates - absolute config.) ^{1}H-n.m.r. (anomeric config.) ^{13}C-n.m.r. (proton-coupled SFORD experiment - anomeric config.)[173] g.l.c.-m.s. (p.m.a.a's.)

K44 (1988)[53]	bacteriophage depolymerisation (N-acetyl-ß-D-galactosaminidase) methylation analysis - Hakomori[80]	preparative p.c. (2:1:1 - butanol:acetic acid:water - oligosaccharide separation) g.p.c. (Sephadex LH20)	d.c.i.-m.s. (direct chemical ionisation) - sequence analysis (permethylated oligosaccharide)[174,175] ^{1}H-n.m.r. ^{13}C-n.m.r. f.a.b.-m.s. (positive ion mode)
K74 (1988)[56]	periodate oxidation thiobarbituric acid assay (Kdo determination)[122] methylation analysis[80,176,104,124] - modified Hakomori partial hydrolysis (1% acetic acid) methanolysis (carboxyl reduction)	g.p.c. (Biogel P2, Sephadex LH20 & G50) g.l.c. - alditol acetates (ECNSS-M)-glycose analysis/acetate determination[128] preparative paper electrophoresis (Schleichter & Schull 2043a/water)	g.l.c.-m.s. (p.m.a.a's.) immunoelectronmicroscopy[56] ^{1}H-n.m.r. (anomeric config./quantitation) ^{13}C-n.m.r. c.d. spectroscopy (config. analysis)[177]
K19 (1988)[39]	periodate oxidation methylation - Hakomori[80,104] partial hydrolysis (1% acetic acid) thiobarbituric acid assay (Kdo determination)[122] orcinol reagent (ribose determination)	g.l.c.- p.m.a.a's. (ECNSS-M) g.p.c. (Biogel P2) preparative paper electrophoresis (Schleichter & Schull 2043a paper)	^{13}C-n.m.r./attached proton test(APT)[136,137] (location of *O*-acetyl groups) ^{1}H-n.m.r.(anomeric config. 300 MHz) g.l.c.-m.s. (p.m.a.a's.) c.i.-m.s. (NH_3)
K26 (1988)[40]	methylation - modified Hakomori[80,79] partial acid hydrolysis (0.025M H_2SO_4 followed by 0.05M TFA)	p.c. - analytical (Whatman No.1) g.p.c. (Biogel P2/acidified water/phenol-sulphuric acid detection[148], Sephadex G10-desalting) g.l.c. - methylated oligosaccharides (DB-17 fused silica capillary)	g.l.c.-c.i.-m.s. (NH_3)
K39 (1989)[50]	methanolysis (carboxyl reduction) methylation analysis - Hakomori[80]/Kuhn[144] ß-elimination[178] bacteriophage depolymerisation (glucosidase) partial hydrolysis (0.5M TFA) lithium/ethylenediamine degradation[179] (-)-2-octyl glycosylation (absolute config.)[112]	g.l.c. - PAAN's, p.m.a.a's. (DB-225 bonded-phase capillary column) g.p.c. (Biogel P4/pyridinium acetate)	g.l.c.-m.s. (p.m.a.a's. - e.i. 70eV) ^{1}H-n.m.r. (anomeric config.) ^{13}C-n.m.r.

K55 (1989)[18]	bacteriophage depolymerisation[180] (lyase) (-)-2-octyl glycosylation (absolute config.) methylation analysis[124,79] - modified Hakomori & Kuhn[144] thiobarbituric acid test (4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid determination) Prehm methylation (location of *O*-acetyl groups)[181]	g.l.c. - PAAN's, p.m.a.a's. (DB-225 capillary) g.p.c. (Sephacryl S500, Biogel P4 - 0.1M pyridinium acetate)	g.l.c.-m.s. (p.m.a.a's.) ^{1}H-n.m.r. ^{13}C-n.m.r. 2D-n.m.r. - HETCOR
K8 (1989)[32]	methylation analysis[124] Smith degradation (sequence analysis) methanolysis (carboxyl reduction	g.p.c. (Sephacryl S400 - molecular weight determination/ Biogel P-2) g.l.c. - (-)-2-octyl glycoside acetates(absolute config.)[112], alditol acetates (DB-17 bonded-phase fused-silica capillary)	1D-n.m.r. - ^{1}H-n.m.r. (400 MHz - anomeric config., NAc and OAc determination) - ^{13}C-n.m.r. 2D-n.m.r. - COSY[182](location of OAc group) - RELAY COSY[183](linkage positions) - NOESY[184](sequence analysis) - HETCOR[185] g.l.c.-m.s. (p.m.a.a's. - linkage positions)
K11 (1990)[9]	glucose/fructose enzyme test (absolute config.) partial hydrolysis (0.5M NaOH) alkaline phosphatase (dephosphorylation) periodate oxidation methylation analysis - Hakomori[80]-modified[124] phosphate quantification - Ames method[131] fructose/protein/nucleic acid determination(see ref.c165 and refs. therein)	g.p.c. (Sephadex G-50, Biogel P-1 g.l.c. - alditol acetates (ECNSS-M 15mx2mm)	g.l.c.-m.s. (p.m.a.a's. - linkage positions) 1D-n.m.r. - ^{1}H-n.m.r. - ^{13}C-n.m.r./APT experiment (anomeric signal assignment)[186], gated decoupling (anomeric config.-$J_{C1,H1}$ values)[187], linkage analysis
K31 (1990)[46]	methylation analysis[80] ($LiAlH_4$ reduction) ß-elimination[148] lithium-ethylenediamine degradation[188] HF selective solvolysis[189] bacteriophage depolymerisation carboxyl reduction (carbodiimide)[132] chromium trioxide oxidation[125] (anomeric config.) partial hydrolysis (0.1M TFA)	p.c. (Whatman No.1 - analytical) preparative g.l.c.(DB-17 fused-silica capillary) g.p.c. (Biogel P-2, Sephadex LH-20 g.l.c. - alditol acetates	circular dichroism spectra (absolute config.)[151] g.l.c.-m.s. (p.m.a.a's. - DB-17 bonded-phase capillary column) c.i.-m.s. (NH_3 - methylated oligosaccharides) 1D-n.m.r. - ^{1}H-n.m.r. (400 MHz) - ^{13}C-n.m.r.(anomeric config.)[173] 2D-n.m.r. - COSY[159] - one- and two-step RELAY COSY[183] - NOESY[190,191] (sequencing)

K16 (1990)[37]	periodate oxidation thiobarbituric acid assay[122] (Kdo determination) methylation analysis - modified Hakomori[80,104,124] orcinol reagent - ribose determination[121]	g.l.c. - p.m.a.a's. (ECNSS-M) partial hydrolysis (3mM acetic acid g.p.c. (Biogel P-2, Sephadex G-50/water) high voltage paper electrophoresis (Schleichter & Schull 2043a paper)	g.l.c.-m.s. (p.m.a.a's.) 1D-n.m.r. - ^{13}C-n.m.r. (linkage analysis & position of OAc), APT[136,137], inversed gated decoupling experiment[130] - ^{1}H-n.m.r. (anomeric config.)
K57 (1990)[55]	methylation analysis[124] methanolysis (carboxyl reduction)	g.p.c. (Sepharose 4B CL) g.l.c. - *O*-methyloxime acetates[182] (DB-WAX bonded-phase capillary), p.m.a.a's. (DB-17 fused-silica capillary) g.l.c.-m.s. - p.m.a.a's.	1D-n.m.r. - ^{1}H-n.m.r. (anomeric config., quantitation) - ^{13}C-n.m.r. 2D-n.m.r. - COSY[182] - RELAY COSY[183] - NOESY[184] (sequence) - HETCOR[185] (linkage analysis)
K26 (1990)[18]	methylation analysis[80,79] ß-elimination[148] selective hydrolysis (0.1M TFA) periodate oxidation & Smith degradation[86] partial hydrolysis (1M TFA) and 0.025M H_2SO_4 followed by 0.5M TFA lithium-ethylenediamine degradation[188] carboxyl reduction (carbodiimide)	p.c. - analytical and preparative g.p.c. (Biogel P-2/acid water, Sepharose 4B, Sephadex G10 - desalting gel) g.l.c. - alditol acetates, p.m.a.a's. (DB-17 capillary), methylated oligosaccharides (DB-1 capillary), (-)-2-octyl glycoside acetates-absolute config.(DB-17)	1D-n.m.r. - ^{1}H-n.m.r. (400 MHz) - ^{13}C-n.m.r. d.c.i.-m.s. (NH_3 - methylated oligosaccharides) g.l.c.-m.s. f.a.b.-m.s.
K24 (1990)[38]	periodate oxidation thiobarbituric acid assay - Kdo determination[122] partial hydrolysis (0.5M NaOH)/alkaline phosphatase ammonium molybdate assay - phosphate determination[131]	g.p.c. (Trisacryl GF05M, Sephadex G50 - purification) g.l.c. - OAc determination (Porapak QS) paper elctrophoresis (Schleicher & Schull 2043a)	1D-n.m.r. - ^{13}C-n.m.r./ATP[136,137]/inverse-gated decoupled (OAc linkage) - ^{31}P-n.m.r. (^{13}C-^{31}P coupling - position of P linkage) - ^{1}H-n.m.r. (anomeric config.)

K87 (1990)[23]	methylation analysis - modified Hakomori[80,124,79] (-)-2-octyl glycosylation (absolute config.)[112] methanolysis - carboxyl reduction partial hydrolysis - anhydrous HF	g.l.c. - p.m.a.a's. (DB-17 bonded-phase fused-silica capillary) g.p.c. (Sepharose 4B CL - M_r determination, Sephacryl S200HR)	1D-n.m.r. - ^{1}H-n.m.r./^{13}C-n.m.r. (quantitation/anomeric config.) 2D-n.m.r. (sequence & position of OAc group) - COSY[182] - RELAY COSY[183] - NOESY[184] (sequence) - HETCOR[185] (linkage analysis) - HOHAHA[193]
K38 (1991)[194]	methylation analysis - modified Hakomori[80,124,79] (-)-2-octyl glycosylation (absolute config.)[112] methanolysis - carboxyl reduction	g.l.c. - alditol acetates, p.m.a.a's., (-)-2-octyl glycoside acetates (DB-17/DB-225/DB-WAX bonded-phase fused-silica capillary columns) g.p.c. (Sephacryl S400HR/Sephacryl S500 - 0.1M sodium acetate buffer pH 5.0) p.c. - Whatman No.1 (alkaline $AgNO_2$ detection)	g.l.c.-m.s. (e.i. 70eV - p.m.a.a's.) 1D-n.m.r. - ^{1}H-n.m.r./^{13}C-n.m.r. (quantitation/anomeric config.) 2D-n.m.r. - COSY[182] - RELAY COSY[183] - NOESY[184] (sequence) - HETCOR[185] (linkage analysis)
K35 (1991) this thesis	methylation analysis - modified Hakomori[80,124,79] (-)-2-octyl glycosylation (absolute config.)[112] methanolysis - carboxyl reduction ß elimination bacteriophage degradation	g.l.c. - p.m.a.a's., PAANs, (-)-2-octyl glycoside acetates (DB-225 bonded-phase fused-silica capillary columns) g.p.c. (Sephacryl S400HR/Sephacryl S500/Biogel P4 - 0.1M sodium acetate buffer pH 5.0)	g.l.c.-m.s. (e.i. 70eV - p.m.a.a's.) 1D-n.m.r. - ^{1}H-n.m.r./^{13}C-n.m.r. (quantitation/anomeric config.) 2D-n.m.r. - COSY[182] - HOHAHA[193] - NOESY[184] (sequence)

3.2. ISOLATION AND PURIFICATION OF CAPSULAR POLYSACCHARIDES

A suitable isolation/purification procedure must ensure a good yield of chemically pure and physically homogenous polysaccharide. The number of different procedures available are too numerous to be covered here and have been reviewed[199]. This discussion will be restricted to the methods employed in the isolation and purification of *E. coli* K antigens.

Jann, Jann, Ørskov and Ørskov are responsible for the majority of the earlier structural work on *E. coli* capsular polysaccharides (K42, K85, K30, K27, K87, K29 - see Table 3.1 and references therein). These bacteria were cultivated on ox-meat infusion and the cells were harvested with 0.9% saline containing 2% phenol (to kill the cells) followed by precipitation with ethanol. After centrifugation, the cells were washed with acetone and extracted with 45% phenol at 65° for 5 to 10 minutes. Material from the aqueous phase was ultracentrifuged (105 000 x g.) and the acidic polysaccharide isolated from the supernatant by fractional cetyltrimethylammonium bromide (CTAB) precipitation[200]. Nucleic acid contaminants were removed by RNAse treatment followed by anion-exchange column chromatography - DEAE cellulose.

In the 1970's a slightly different procedure was adopted[58]. The cells were grown in liquid culture medium (minimal medium with 0.1M dialyzed yeast extract). The bacteria and acidic polysaccharides were precipitated with CTAB before being extracted with aqueous calcium chloride. Purified polysaccharide was obtained by a sequence of steps involving alcohol precipitation and extraction with cold buffered phenol[201]. Jennings *et al.*[31] modified this procedure by disrupting the cells with glass beads in a colloid mill prior to phenol extraction.

In 1985, Dutton *et al.*[7] worked on *E. coli* K28. This was the first *E. coli* capsular polysaccharide to be investigated by that laboratory. Isolation and

purification of the acidic polysaccharide was achieved as previously described for *Klebsiella* species[202]. They experimented with a variety of growth media and found Mueller-Hinton agar to produce the best results. The slime was harvested from large trays after incubation for a suitable length of time and the capsular polysaccharide was extracted with a 1% phenol solution. Purified acidic polysaccharide was obtained by a series of steps involving ultracentrifugation, and ethanol and CTAB precipitation. This general method is applied in our laboratory followed by removal of nucleic acids by enzyme digestion and anion exchange g.p.c. (DEAE Sepharose 6B-CL).

3.3. THE "CLASSICAL APPROACH" TO THE ELUCIDATION OF SACCHARIDE STRUCTURE

3.3.1. Composition and homogeneity

The determination of the chemical composition and molecular size of polysaccharides is the initial step in their overall characterization. This requires identification and quantitation of all sugar and non-carbohydrate constituents the methods for which have been reviewed by Aspinall[203]. The task is simplified somewhat with the bacterial polysaccharides as they are composed of regular repeating oligosaccharide units, usually of heterogenous monosaccharide composition.

Monosaccharides are identified from hydrolyzates by paper chromatography and by g.l.c. The latter procedure also effects quantitation of neutral sugars. Incomplete hydrolysis is encountered with glycosiduronic acids hence carboxyl reduction prior to derivatization is required. This is accomplished in numerous ways including the ethylene oxide/$NaBH_4$ method of Aspinall[75] *et al.* , aqueous carbodiimide/$NaBH_4$ method described by Taylor[110], and methanolysis/anhydrous $NaBH_4$ reduction[204]. The derivatives most often used for g.l.c. analysis are the alditol acetates[94] and peracetylated aldononitriles (PAANs)[145]. The range of derivatives available to the researcher has been reviewed by Dutton[63,64]. It is noted that ketoses (*e.g.* NeuNAc/Kdo) can be lost by decomposition under the normal conditions of hydrolysis. These can be stabilized by methanolysis, giving the methyl glycoside methyl esters which can be quantitated by g.l.c. as acetylated or trimethylsilylated derivatives[203].

Identification of sugars is also achieved with specific enzymes and chemical reactions; quantitation is possible spectrophotometrically when a colour is produced. These procedures, when utilized in the *E. coli* series, have been noted and referenced in Table 3.1.

The non-carbohydrate substituents are identified chemically, enzymatically and chromatographically. For a review see Aminoff *et al.*[205]. Pyruvate is determined chemically by the method of Sloneker[85], enzymatically with lactate dehydrogenase[42] and by paper electrophoresis as the dinitrophenyl-hydrazone[43]. *O*-Acetyl and *O*-propionyl groups are detected by p.c. as the hydroxamate derivatives[123] and quantitated by g.l.c. on Porapak Qs according to Fromme and Beilharz[128]. Phosphate is determined according to Ames[131] or by the molybdenum blue method of Kulin *et al.*[143] Amino acids have been identified from a polysaccharide hydrolyzate using a Durrum D-500 amino acid analyzer and confirmed by c.i.-m.s.[21]

^{1}H- and ^{13}C-n.m.r. spectroscopy are extremely important spectroscopic procedures in polysaccharide analysis. They permit the non-destructive analysis of polysaccharide composition and purity, largely superceding their chemical and enzymatic counterparts. Since these one-dimensional n.m.r. techniques have been routinely applied over the last decade and have been the subject of numerous review articles[130,206-213], a detailed synopsis will not be given. The reader is referred to the "modern trends" in n.m.r. spectroscopy, section 3.4.4.

In terms of chemical composition, ^{1}H-n.m.r. spectra of polysaccharides are divided into three regions:

δ 4.4 - 5.5 - anomeric protons

δ 3.0 - 4.5 - ring protons

δ 1.5 - 2.5 - methyl protons of non-carbohydrate substituents and 6-deoxy sugars (Fig. 3.1).

The number and relative intensities of the anomeric signals provides the degree of polymerisation of the repeating-unit of the polysaccharide. To avoid ambiguous spectral interpretation, one must be aware of the fact that some non-anomeric protons resonate in this area, *e.g.* H_4 and H_5 of α-uronic acid residues[214] and H_2-mannose/rhamnose[215]. Not much information is provided by the

ring proton part of the spectrum due to signal crowding and overlap. The identification and quantitation of non-carbohydrate substituents is facilitated by their methyl proton resonances: Pyruvic acid acetals and amino acids - ~ 1.5 p.p.m., which must be distinguished from the methyl resonance of 6-deoxy sugars when present; N-acetyl substituents of acetamido groups - 1.8 to 2.1 p.p.m. ; *O*-acetyl groups - 2.0 to 2.2. p.p.m. The ratio of these signal intensities to that of an anomeric proton enables one to ascertain the degree of substitution. ^{13}C-n.m.r. spectra are divided into 5 regions. Carbonyl carbons of uronic acids, amino acids, pyruvic acid acetal and acyl groups resonate at 170 - 180 p.p.m. The anomeric carbons of pyranose sugars resonate at 98 - 106 p.p.m. , while those of furanose sugars resonate at 106 - 109 p.p.m. ^{13}C- Spectra therefore indicate the ring forms present in the polymer. The ring carbons resonate at 60 - 75

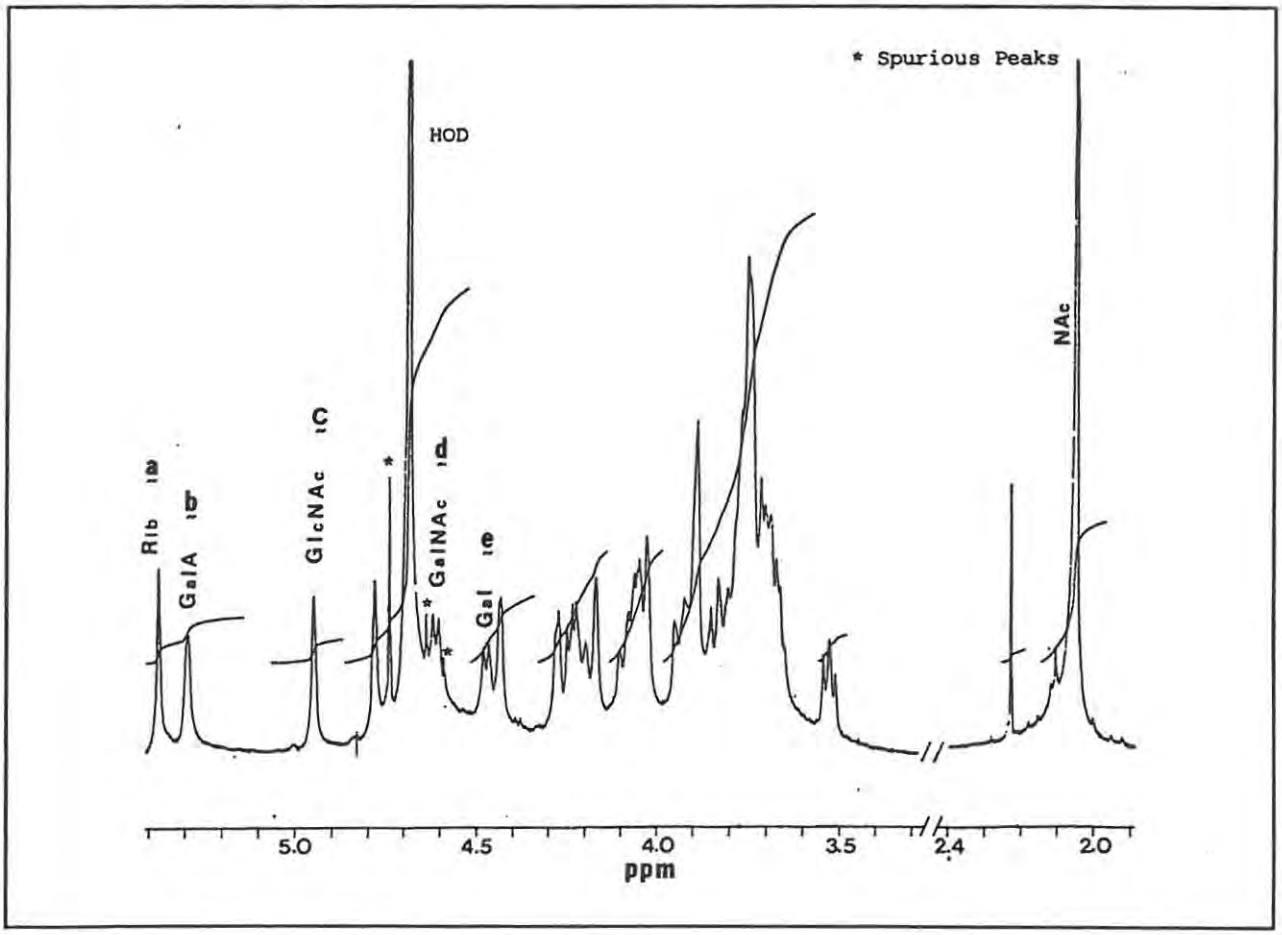


Fig. 3.1. 1D-^{1}H-n.m.r. spectrum *E. coli* K38 polysaccharide

p.p.m. with unsubstituted primary alcohol groups producing ^{13}C signals distinctively upfield at ~ 60 p.p.m. C-2 of 2-deoxy-2-acetamido sugars resonate at 50 - 55 p.p.m. The methyl carbons of acyl groups, pyruvic acid acetals, amino acids and 6-deoxy sugars resonate at 17 - 25 p.p.m. ^{13}C- Spectra are particularly useful because of the greater spectral dispersion and because a signal for each C in the repeating-unit is invariably observed. This information is complementary to that of the corresponding ^{1}H- spectrum and provides additional information as outlined by the regions defined above. It must be stressed that identification of monosaccharide constituents from simple 1D-n.m.r. spectra by comparison of δ values with literature values, must be supported by chemical evidence. This necessitates specific chemical and enzymatic degradations of the polysaccharide which are the subject of sequence analysis in section 3.2.4.

Chemical homogeneity of polysaccharides is determined by n.m.r. spectroscopy whereas the degree of physical homogeneity is indicated by molecular weight determinations. Polysaccharides are most often polydisperse with respect to molecular weight therefore experimentally determined molecular weights are recorded as weight-average values (M_w). The methods most often used for the *E. coli* capsular polysaccharides are analytical ultracentrifugation[216] and gel permeation chromatography[68]. The latter procedure is followed in this laboratory using Sephacryl S400 HR or S500 SF calibrated with dextrans (see section 4.1. for experimental details).

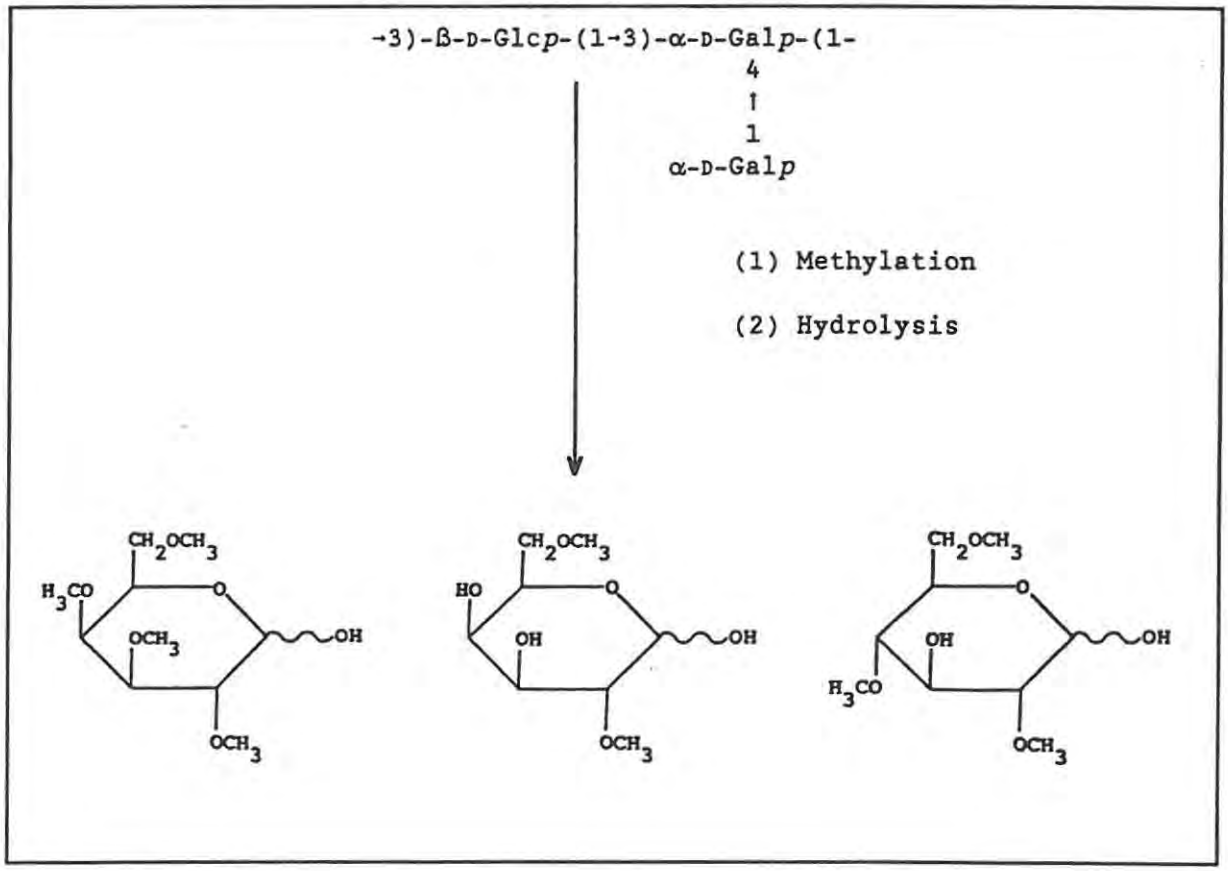
3.3.2. Linkage analysis

Having determined the chemical composition of the polysaccharide it must now be established how these components are assembled. This requires determination of the anomeric configuration and the glycosylation sites on each sugar residue.

Methylation analysis remains the most widely used procedure for linkage analysis

of carbohydrates despite the emergence of 2D-n.m.r. experiments which provide the same information, non-destructively, and often in a shorter period of time (see section 3.4.4). Methylation involves etherification of all free hydroxyl groups (methyl ethers) in the polysaccharide. Subsequent hydrolysis releases the hydroxyl groups previously involved in glycosidic linkages. Characterization of the partially methylated monosaccharides establishes which hydroxyl groups were involved in glycosidic linkages as the methyl ethers are stable under the conditions of acid catalyzed hydrolysis.

e.g. *E. coli* K37 (depyruvylated) :



The methylation methods of Purdie[140], Haworth[217] and Kuhn[144] are laborious and time consuming and have been replaced by the Hakomori procedure[80]. In this procedure the polysaccharide, dissolved in dimethyl sulfoxide (DMSO), is treated with sodium methylsulfinylmethanide (sodium dimsyl) to generate the alkoxides which are alkylated on the addition of methyl iodide. If methylation is not accomplished in a single step, the milder Kuhn[144] procedure is then used whereby

the partially methylated polymer is dissolved in the dipolar aprotic N,N-dimethylformamide (DMF) and methyl iodide is used as alkylating agent in the presence of silver oxide as base. The Hakomori procedure cannot be repeated as uronic esters are susceptible to base catalyzed ß-eliminations under the highly basic conditions [see section 3.3.3.(iii)]. This is not a major problem in the initial reaction as etherification is favoured over esterification, the latter therefore occurring at much reduced base concentration. Incomplete solubilization of polysaccharide is a major contributor to under methylation. Thus, solubility has been increased using 1:1 DMSO : 1,1,3,3-tetramethylurea as solvent[153], the latter serving to relax inter- and intra-molecular hydrogen bonding. Phillips and Fraser[124] have modified the Hakomori procedure by replacing sodium dimsyl with potassium dimsyl. The advantages include ease of preparation and a cleaner reaction product. For the linkage analysis of *E. coli* capsular polysaccharides the above procedure has been the most widely reported (Table 3.1).

Characterization of partially methylated sugars involves g.l.c. and g.l.c.-m.s. analysis of suitably volatile derivatives. The partially methylated alditol acetates (p.m.a.a's.) are the derivatives of choice[218,86]. They are identified by comparing g.l.c. retention times with those of authentic standards on various stationary phases[219,220]. These results are confirmed by g.l.c.-m.s. analysis. Interpretation of the e.i.-mass spectra is facilitated by well documented fragmentation pathways and by comparison with reference spectra[221]. The mass spectra are characteristic for specific substitution patterns but are insensitive to stereochemical differences, hence the complete characterization requires a combination of g.l.c. and g.l.c.-m.s.

On hydrolysis of methylated polysaccharides the stability of uronosyl ester and 2-acetamido-2-deoxy sugar linkages still holds. Dutton *et al.*[40] have taken advantage of this and produced methylated oligosaccharides which they

characterized by g.l.c.-c.i.-m.s. [see section 3.4.3.(i)]. Complete hydrolysis of polymers containing amino sugars is effected by the method of Neeser and Schweizer[192] (4M TFA, 125°, 1h). Carboxyl reduction of methylated polysaccharides is achieved using lithium aluminium hydride/deuteride[222] or by methanolysis followed by anhydrous sodium borohydride/deuteride reduction. Comparison of the p.m.a.a's. produced before and after carboxyl reduction provides uronic acid identification and linkage information. Pyruvate acetals are stable under the basic conditions of the Hakomori methylation hence their sites of attachment can be determined. *O*-acyl substituents, however, are base labile. In order to determine their linkage positions, milder methylation procedures have been developed which leave the *O*-acetyl groups intact[223,181]. The method described by Prehm[181] is conducted under acidic conditions using trimethylphosphate as solvent, methyl triflate as alkylating agent and 2,6-di-(*tert*-butyl)pyridine as proton scavenger. This procedure has been successfully applied to the location of *O*-acetyl groups in the *E. coli* K55[18] polysaccharide.

3.3.3. **Sequence analysis**

Composition and methylation analysis provides knowledge of the building blocks of the polysaccharides and their sites of attachment. The next step is to establish the order in which these components are assembled, the polysaccharide sequence. Various specific or selective degradation procedures have been developed, from which oligosaccharides and modified polysaccharides are generated. Characterization of these products provides the necessary sequence information. Invariably more than one degradation procedure is required to accurately sequence a polysaccharide.

The degradation procedures have been comprehensively reviewed by Bouveng and Lindberg[224], Lindberg *et al.*[225] and Aspinall[199]. Discussion will be restricted to the procedures commonly used in the structural elucidation of the *E. coli* K

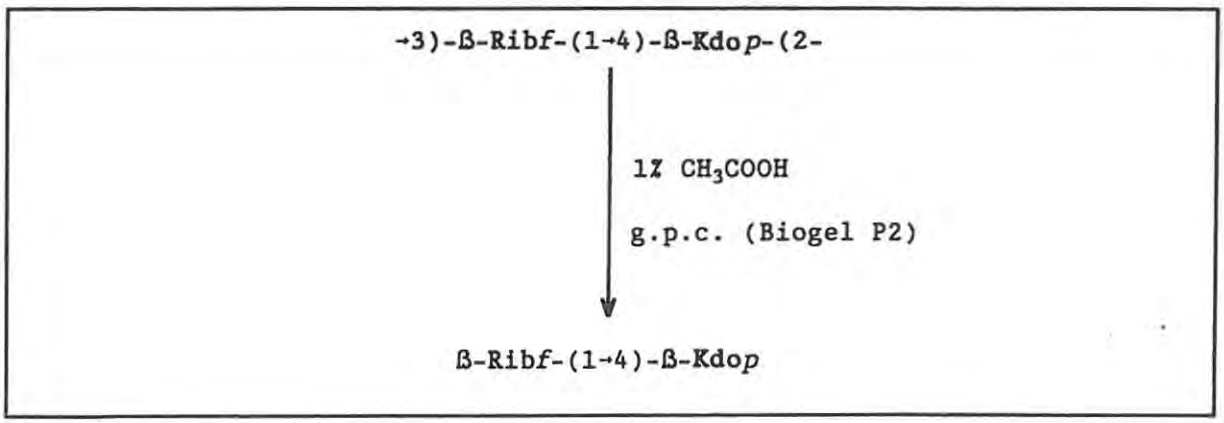
antigens. These include acid catalyzed partial hydrolysis, periodate oxidation/Smith degradation, base catalyzed ß-elimination, deamination, and bacteriophage-borne enzyme-catalyzed depolymerization. Each procedure generally involves three steps *viz.* degradation, fractionation, and characterization. Discussion is limited to an account of the degradations of the polysaccharides as procedures followed in the characterization of the products are essentially those discussed in sections 3.2.1. and 3.2.2.

3.3.3.(i) Partial hydrolysis

Fortuitously, the *E. coli* capsular polysaccharides are invariably heteropolymers whose glycosidic linkages differ significantly in their lability to acid catalyzed hydrolysis. Therefore, by judicious choice of acid, acid concentration and reaction conditions, fragmentation of the polysaccharide can be effected with a high degree of selectivity. The oligosaccharides and/or modified polysaccharides generated are isolated by various preparative chromatographic techniques and are characterized, providing useful sequence information.

Weak glycosidic linkages include those of furanose and deoxy sugars. If the deoxy function is adjacent to the glycosidic linkage, the latter is particularly weak:

e.g. Partial hydrolysis of *E. coli* K19[39] :



The extremely mild conditions effect hydrolysis of the Kdo (3-deoxy-D-*manno*-2-octulosonic acid) bond because it has a 3-deoxy function adjacent to the glycosidic linkage.

The most commonly encountered acid-resistant glycosidic linkages in the *E. coli* K antigens are those of uronic acid and 2-amino-2-deoxy-hexose residues. Good yields of the aldobiouronic acid and higher oligosaccharides are obtained by partial hydrolysis of uronic acid-containing polysaccharides (*e.g. E. coli* K26[16] capsular polysaccharide pg. 33).

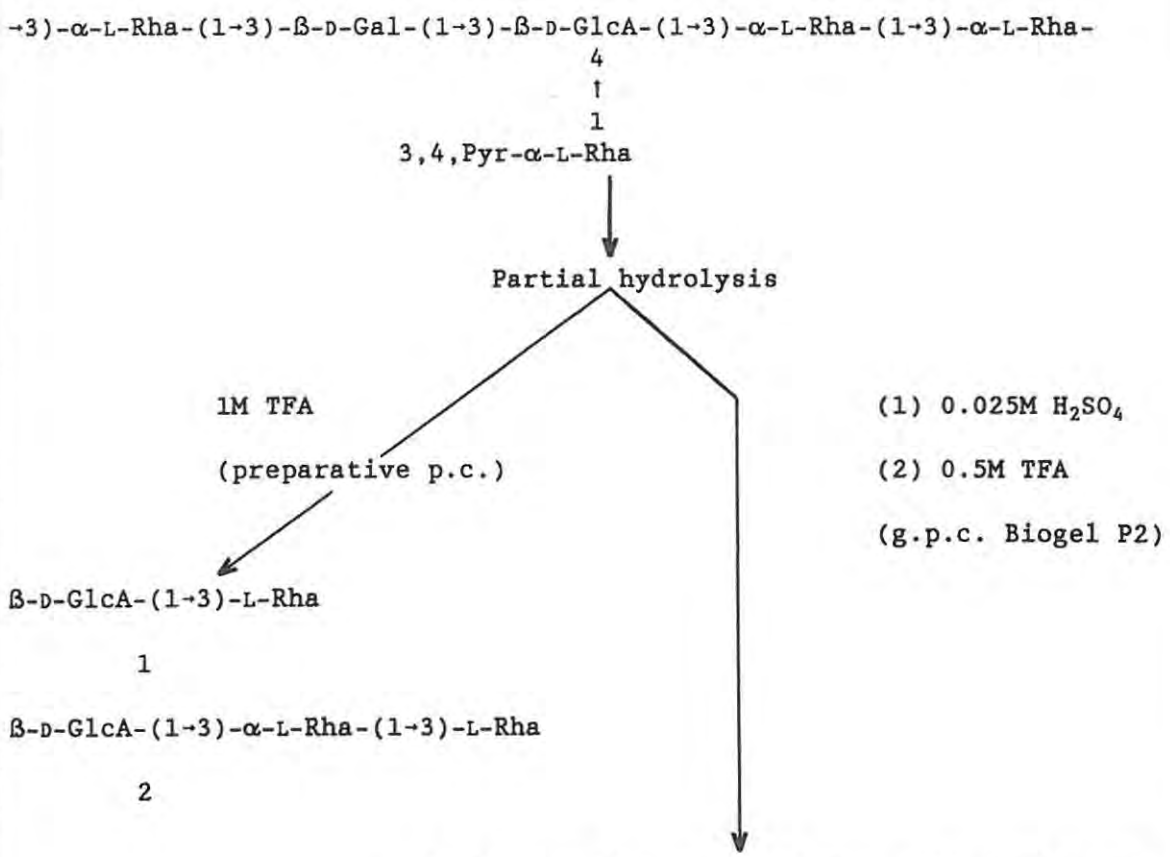
The acids most commonly used are aqueous H_2SO_4, TFA and acetic acid (Table 3.1). A major disadvantage of partial hydrolysis is that considerable degradation occurs. As a result, larger amounts of starting material are required to produce sufficient oligomeric products for chemical and spectroscopic analysis. With *E.coli* it is often difficult to isolate large amounts of pure capsular polysaccharide.

Despite the stability of amino sugar glycosidic linkages to hydrolysis, degradation involving deamination is favoured [see 3.3.3.(v)]. Non-aqueous acid catalyzed depolymerizations with different degrees of selectivity have been utilized, and are often complementary to partial hydrolysis[225]. For example, acetolysis[226], carried out in a mixture of acetic anhydride, acetic acid and sulphuric acid, readily cleaves 1-6 linkages which are relatively stable to hydrolysis. In the characterization of *E. coli* K44[52], where methylated polymers were acetolyzed[154], larger amounts of the amino sugars than the neutral sugar were released. The opposite situation is encountered with acid catalyzed hydrolysis.

3.3.3.(ii) **Periodate oxidation/Smith degradation**

The discovery, by Malaprade[227] in 1928, that 1,2 diols in carbohydrates undergo oxidative scission by periodate ion in a selective and quantitative manner, led

E. coli K26 capsular polysaccharide:



to the development of an important degradative procedure. Periodate oxidation has subsequently been utilized in the chemical characterization of 37 of the 56 *E. coli* capsular polysaccharides reviewed in Table 3.1 and several reviews on the subject have appeared in the literature[203,225,228-230].

The procedure is based on the consumption of 1 mole of periodate per molar proportion of 1,2-diol, resulting in cleavage of the carbon chain and formation of two aldehydic groups. With 1,2,3-triols, a double cleavage occurs forming two aldehydic groups and one molecular portion of formic acid[231]. With polysaccharides, 3-linked or branched sugars are therefore resistant to periodate oxidation. The periodate oxidation of such polysaccharides produces modified polymers consisting of surviving sugars and dialdehydic remnants of oxidized sugars. Goldstein *et al.*[96] found that reduction of the aldehydic functions to the primary alcohols generated acylic acetals which are more readily hydrolyzed than the glycosides. Hence, the sequence of reactions involving periodate oxidation, borohydride reduction and mild acid hydrolysis - the Smith degradation[96] - will generate oligosaccharide units glycosidically linked to the remnants of oxidized sugars - glycerol and erythritol or threitol; glycerol, if the sugar was a 2- or 6-linked hexopyranose or 5-linked pentofuranose, and erythritol or threitol if it was a 4-linked hexopyranose. Fractionation and characterization of these products then provides linkage and sequence information:

e.g. Stepwise Smith degradation of *E. coli* K8[32]

(145mg)

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

↓

(1) Periodate oxidation (aqueous sodium metaperiodate)

(2) Reduction (aqueous sodium borohydride)

(3) Hydrolysis (2% aqueous acetic acid)

(4) g.p.c. (Biogel P2)

↓

(40mg)

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

↓

Repeated steps (1) to (4)

↓

ß-D-Glc*p*A-(1→3)-ß-D-Gal*p*NAc-(1→2)-glycerol

These fragments were characterized by methylation analysis and n.m.r. spectroscopy and provided the sequence of the polysaccharide.

Fischer *et al.*[28] modified this procedure by performing sequential sodium periodate ($NaIO_4$) oxidation, ß-elimination, hydrazinolysis and alkaline treatment on the *E. coli* K2 polysaccharide. They were thus able to release the glycerophosphate portion of the polysaccharide and isolate it for stereochemical analysis. They also isolated the glycerophosphate after sequential Smith degradation/alkali hydrolysis, however, recovery was low. Prompted by the findings of Painter *et al.*[149], who demonstrated significant differences in the rate of periodate oxidation of various sugars, Dutton and Kuma-Mintah[48] performed a selective Smith degradation on the *E. coli* K34 polysaccharide. The native polysaccharide was oxidized with dilute periodate for a limited time. After reduction and Smith hydrolysis they were able to isolate a polymeric product in which only the side chain had been removed. Another variation of the Smith degradation was carried out by Egan *et al.*[163]. Here the polysaccharide *E. coli* K100, →3)-ß-D-Rib*f*-(1→2)-D-Ribitol-5-(PO_4→, was subjected to periodate oxidation, followed by oxidation of the aldehydic groups to carboxylates by means of aqueous Br_2. Subsequent acid catalyzed hydrolysis liberated L-glyceric acid, which enabled them to deduce the linkage position of the ribofuranose to the ribitolphosphate[164].

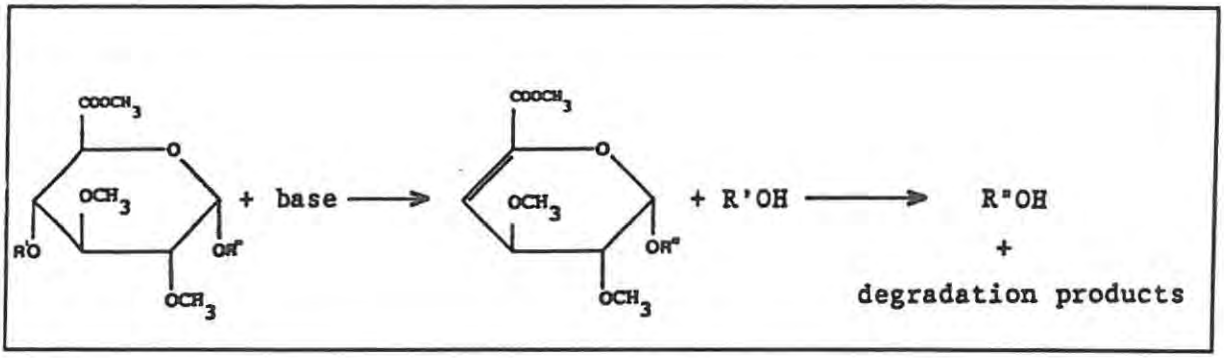
These examples illustrate the versatility and importance of periodate oxidation in the determination of polysaccharide sequence.

3.3.3.(iii) ß-elimination

When polysaccharides containing uronic acid residues are methylated, uronosyl methyl esters are formed. The carbonyl groups are therefore suitably activated to initiate base catalyzed ß-elimination reactions[203]. Lindberg *et al.*[148,232] developed a reaction sequence that leads to the degradation of methylated

polysaccharides. This widely used degradation procedure has been reviewed by Kiss[233] and updated by Lindberg *et al.*[225].

The reaction involves treating the methylated polymer with strong base under anhydrous conditions. The substituent at C-4 in the uronic acid residue (R'OH), either a methoxyl group or a sugar residue, is eliminated and an unsaturated uronic acid residue is formed. Lindberg and Lönngren[232], recognising that the unsaturated uronic acid residue would be acid labile, subjected the product to mild acid hydrolysis to release the "aglyconic" hydroxyl group (R"OH) to which the uronic acid was linked. Aspinall and Rosell[178] later proved that mild acid treatment is unnecessary as prolonged exposure to strong base produces the same result. Hence the latter method has generally been adopted.



The position of the released hydroxyl group on the terminal non-reducing residue (R"OH) is identified by further alkylation with trideuteriomethyl iodide or ethyl iodide followed by normal compositional analysis. If the uronic acid occupies a terminal position or is part of a side chain, then R"OH represents a modified polymer, and if it is "in-chain", an oligomeric product (R"OH) is formed (*e.g.* ß-elimination of the permethylated (PM) *E. coli* K39[50] polysaccharide pg. 37). The oligosaccharide was hydrolysed and each sugar was converted to the p.m.a.a. and identified by g.l.c.-m.s. By comparing the methylation data of the native polysaccharide and the base-treated polysaccharide the linkage position of the uronic acid was revealed. The permethylated oligosaccharides above could have been sequenced by c.i.-m.s.[175] or f.a.b.-m.s.[234] (see later). A mixture of

PM-*E. coli* K39 capsular polysaccharide:

PM→6)-α-D-Glc*p*-(1→4)-ß-D-Glc*p*A-(1→2)-α-D-Man*p*-(1→3)-ß-D-Glc*p*-(1→

3
↑
1
α-D-Gal*p*

(1) Dissolved in 19:1 DMSO: 2,2-dimethoxypropane (H_2O scavenger)

(2)*p*-toluenesulphonic acid (catalyst for H_2O scavenger)

(3)Sodium dimsyl

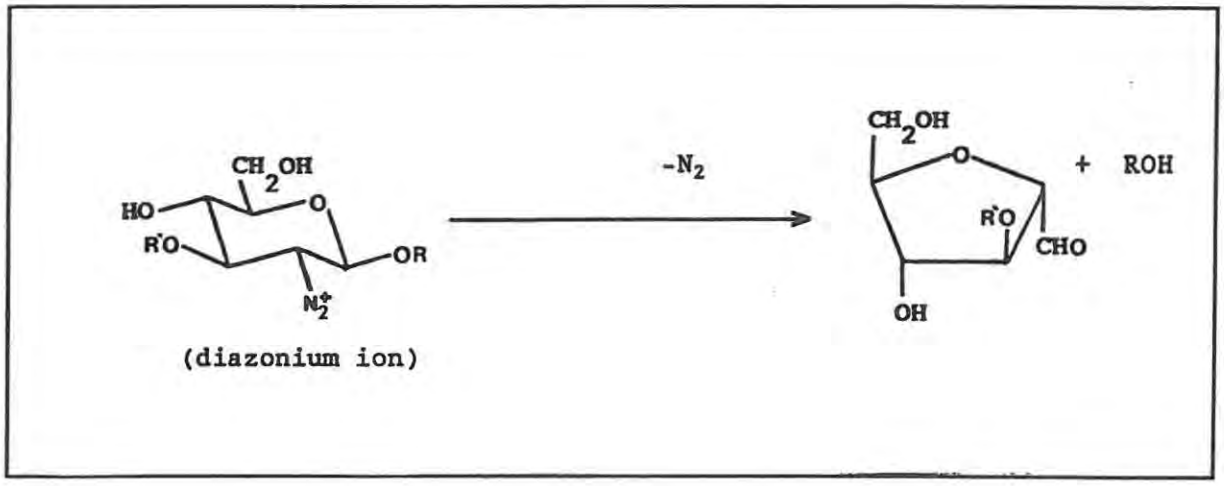
(4)CD_3I

OCD_3
2
α-D-Man*p*-(1→3)-ß-D-Glc*p*-(1→6)-α/ß-D-Glc*p*~CD_3
3
↑
α-D-Gal*p* + smaller oligosaccharides

oligosaccharides is produced in the above example because of further degradation of the terminal reducing 6-linked glucose residue. Being an aldose, a second ß-elimination reaction takes place with elimination of the methoxyl group in the three position. The methylation results of the base treated PM-K39 polysaccharide show survival of only ~20% of the reducing glucose residue. If the reducing sugar was substituted at *O*-3 with a sugar residue, another reducing sugar would be formed, which would undergo further degradation, and so on. A second problem is that only 70 - 95% of the uronic acids react[148], therefore yields of oligomeric material after fractionation are low. Selective reductive cleavage of methylated polysaccharides could be a more suitable method of producing methylated oligosaccharides for sequence analysis[235] (see section 3.4.2).

3.3.3.(iv) **Deamination**

2-Acetamido-2-deoxy-D-glucose and -galactose are the most widely occurring amino sugar constituents of polysaccharides. Deamination of these polysaccharides, in which the amino group is equatorially arranged in the most stable chair form, yields 2,5-anhydrohexose residues with the simultaneous cleavage of the 2-amino-2-deoxyglycosidic bond. The value of this reaction lies in the mild reaction conditions (pH 4, room temperature) and the oligosaccharides that are formed can be isolated and characterized, providing valuable structural information. The polysaccharides require N-deacetylation, by hydrazinolysis[236] or by treatment with sodium hydroxide in DMSO[171], prior to deamination. Rearrangement of the hexosamine to the 2,5-anhydro sugar is effected by attack of the ring oxygen on the equatorially disposed diazonium ion, which is formed on exposure of the amino sugar to nitrous acid.

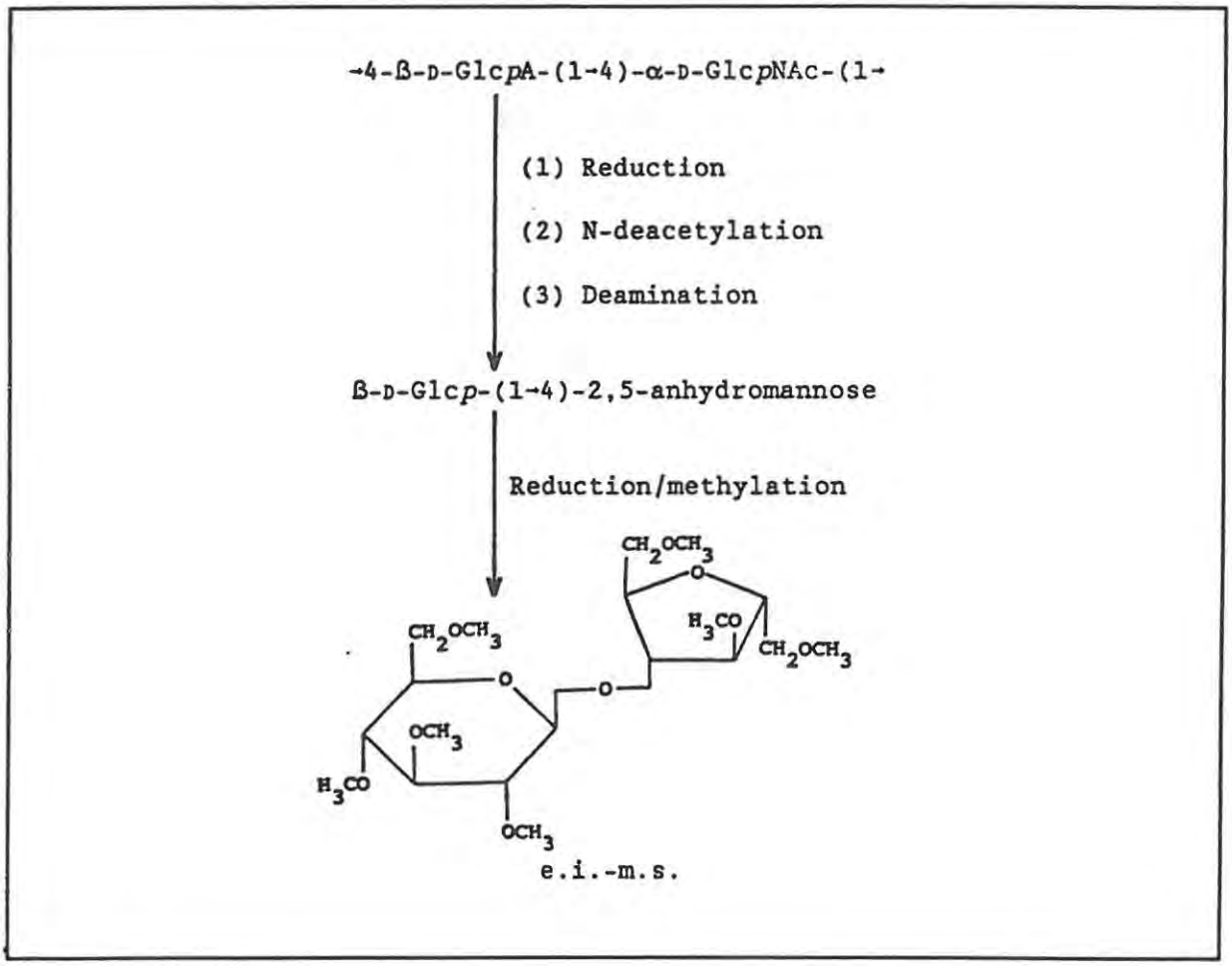


The diazonium ion formed from a 2-amino-2-deoxy-D-glucoside and 2-amino-2-deoxy-D-galactoside, yield on deamination, 2,5-anhydro-D-mannose and 2,5-anhydro-D-talose, respectively. Deamination reactions have been comprehensively reviewed by Williams[237]. Lindberg *et al.*[225] have described the cleavage of polysaccharides by nitrous acid deamination.

The carboxyl-reduced *E. coli* K5[29] polysaccharide was degraded by nitrous acid

deamination according to the method of Erbing *et al.*[109] after N-deacetylation with hydrazine/hydrazine sulphate[108]. The reaction products were chromatographed on Biogel P2 and the disaccharide obtained was reduced, methylated and analyzed by g.l.c. and e.i.-m.s. The mass spectrum was interpreted from the "A" and "J" type fragments according to Kochetkov and Chizhov[238].

e.g. E coli K5:



The *E. coli* K44[52] capsular polysaccharide was N-deactylated according to the method of Kenne and Lindberg[171] and then subjected to nitrous acid deamination. The aldobiouronic acid - ß-D-GlcA-(1→3)-L-Rha - was isolated from the reaction products and characterized by glycose and methylation analysis, providing vital sequence information.

In the structural analysis of the *E. coli* K44 polysaccharide, Dutton *et al.*[52] exhausted most of the chemical degradative procedures available to them,

including - deamination, ß-elimination and Smith degradation. It serves as a good example of the "classical approach" to the structural elucidation of an acidic bacterial polysaccharide.

3.3.3.(v) Bacteriophage depolymerization

Bacteria are subject to infection by viruses known as bacteriophages. These viral particles have been defined as "autonomous microbes analogous to plant and animal viruses but obligately parasitic on bacteria"[239]. The bacterial capsule has been recognised as the receptor for bacteriophage attachment[240] and this is supported by the fact that acapsular mutants have been found to remain unaffected by their respective bacteriophages[93].

Bradley[239] has classified the bacteriophages according to nucleic acid type and morphology. The bacteriophages infecting *E. coli* belong predominantly to Bradley group C. They possess tail spikes which are responsible for their specific interaction with the K polysaccharide antigens. Hence they are numbered according to the K-serotype with which they interact *e.g. E. coli* bacteriophage K35 ≡ *E. coli* Φ35. Some of the bacteriophages carry a glycanase activity[241], most often an endo-glycosidase, probably located in the tail spikes, which catalyzes the hydrolysis of <u>one</u> glycosidic linkage in the repeating-unit of a capsular polysaccharide.

The *in vitro* degradation of capsular polysaccharides has therefore developed into an extremely valuable, non-destructive, degradative procedure. It is a convenient means of isolating oligosaccharides corresponding to one or more repeating units (P1, P2 etc.). The procedure has been well documented[167,180] and Rieger-Hug and Stirm[150] have reviewed the depolymerases associated with *Klebsiella* bacteriophages. The majority of the *E. coli* K antigens that have been subjected to bacteriophage degradation are members of the Group I[27] (A type) antigens which

are similar to those of *Klebsiella*. The depolymerases associated with these *E. coli* bacteriophages are listed in Table 3.2.

Table 3.2. Bacteriophage depolymerisation of *E. coli* K antigens.

Serotype	Oligosaccharide released	Bond/s hydrolyzed	Ref.
K1	α-NeuNAc-(2→8)-NeuNAc	→8)-α-NeuNAc-(2→8)-α-NeuNAc-(2→ (N-acetylneuraminidase)	242, 243
K5	ß-GlcA-(1→4)-GlcNAc	→4)-ß-GluA-(1→4)-α-GlcNAc-(1→ (N-acetylglucosaminidase)	244
K9	α-Gal-(1→4)-α-Neu5Ac	→3)-ß-GalNAc-(1→4)-α-Gal-(1→ (N-acetylgalactosaminidase) →4)-α-Neu5Ac-(2→3)-ß-Gal-(1→ (N-acetylneuraminidase)	33
K20 (K13/K23)	ß-Ribf-(1→7)-ß-Kdop-(2→3)-ß-Ribf-(1→7)-Kdop	→3)-ß-Ribf-(1→7)-ß-Kdop-(1→ (ketodeoxyoctulosonidase)	245
K27 (*Klebs.*Φ54)	α-Glc-(1→4)-α-GlcA-(1→3)-Fuc 3 ↑ 1 α-Gal	→3)-α-Fuc-(1→4)-α-Glc-(→ (fucosidase)	246
K28	A: α-Fuc-(1→3)-α-Glc-(1→4)-GlcA 3 4 ↑ ↑ OAc 1 ß-Gal B: α-Glc-(1→4)-ß-GlcA-(1→4)-Fuc 4 3 ↑ ↑ 1 OAc ß-Gal	B A →3)-α-Glc-(1→4)-ß-GlcA-(1→4)-α-Fuc-(1→ 4 3 ↑ ↑ 1 OAc ß-Gal (fucosidase) (glucuronidase)	247
K29	4,6-pyr-Glc-(1→2)-Man-(1→4)-GlcA-(1→3)-Gal-(1→2)-Man-(1→3)-Glc	(mostly) →2)-α-Man-(1→3)-ß-Glc-(1→3)-ß-GlcA-(1→ (glucosidase)	43
K30 (*Klebs.* Φ20)	α-Man-(1→3)-Gal 3 ↑ 1 α-Gal 3 ↑ 1 ß-GlcA	→3)-ß-Gal-(1→2)-α-Man-(1→ (galactosidase)	45
K34	ß-GlcA-(1→4)-ß-Gal-(1→3)-Gal 3 ↑ 1 α-Glc-(1→4)-ß-Gal	→3)-ß-Gal-(1→2)-ß-GlcA-(1→ (galactosidase)	48

K35 (this thesis)	ß-Man-(1→4)-α-Glc-(1→3)-Glc 3 ↑ α-GlcA 4 ↑ 1 ß-Gal	↓ →3)-ß-Glc-(1→4)-ß-Man-(1→ (glucosidase)	this thesis
K36	α-GalA-(1→2)-α-Man-(1→3)-Gal 4 ↑ 1 α-Man	↓ →3)-ß-Gal-(1→3)-α-GalA-(1→ (galactosidase)	49
K39	α-Glc-(1→4)-ß-GlcA-(1→2)-α-Man-(1→3)-Glc 3 ↑ 1 α-Gal	↓ →3)-ß-Glc-(1→6)-α-Glc-(1→ (glucosidase)	50
K44	ß-GlcA-(1→3)-α-Rha-(1→4)-α-GlcNAc-(1→6)-GalNAc	↓ →6)-ß-GalNAc-(1→4)-ß-GlcA-(1→ (N-acetylgalactosaminidase)	53
K55 (*Klebs.* Φ5)	α-Hex-4-eneA-(1→4)-ß-Glc-(1→3)-4,6-pyr-Man	↓ →3)-4,6-pyr-ß-Man-(1→4)-ß-GlcA-(1→ (eliminase)	18
K92	α-NeuNAc-(2→9)-NeuNAc	↓ →9)-α-NeuNAc-(1→8)-α-NeuNac-(2→ (N-acetylneuraminidase)	248

These enzymes are generally highly specific. However, the inter- and intra-generic structural similarities that exist in the capsular polysaccharides means that an enzyme associated with a phage that infects one strain can be effective in the hydrolysis of the polysaccharide from another strain[249]. Hence, *Klebsiella* Φ20 and Φ5 have been used to generate the repeating-unit of *E. coli* K30[45] and K55[18] respectively. In the cleavage of the K55 polysaccharide the enzyme catalyzed an elimination type reaction with the formation of a terminal non-reducing unsaturated uronic acid. This eliminase activity has also been reported amongst the *Klebsiella* bacteriophages[250]. *E. coli* K32 was found to adsorb *Klebsiella* Φ55[47] providing confirmation that *E. coli* K32 is identical in structure to *Klebsiella* K55. Dutton *et al.*[46] have briefly treated *E. coli* K31 with bacteriophage to produce a lower molecular weight polymer, which is more amenable than the parent polymer to 2D-n.m.r. spectroscopy. Stephen *et al.*[247] reported endo-ß-D-glucuronidase activity associated with *E. coli* Φ28. This is most unusual[150] and represents the first phage degradation, in *E. coli* and *Klebsiella*, from which oligosaccharides are produced having terminal reducing uronic acid residues.

Bacteriophage depolymerization is a simple procedure and high yields of oligosaccharides are obtained. Often this is the only means of isolating oligosaccharides with labile substituents, such as *O*-acetyl groups, intact. This is significant as *O*-acetyl groups have been recognized as important immunological determinants. Characterization of these oligomers by n.m.r. spectroscopy and standard chemical analysis would provide the position/s of attachment of labile substituents as well as structural information, including sequence (see section 4.3).

3.3.4. Configurational analysis

3.3.4.(i) Anomeric configuration

Anomeric configuration can be determined enzymatically, chemically and spectroscopically. Methods used in the *E. coli* structural work will be discussed.

Exo-enzymes

Specific enzyme activities can be used as a criterion for assigning anomeric configuration. Stirm *et al.*[51] treated oligosaccharides, isolated from the partial acid hydrolysis of *E. coli* K29, with α- and ß-glucosidase, α-galactosidase and ß-glucuronidase under the appropriate conditions[89-92]. Besides confirming the monosaccharide composition, anomeric configuration is indicated by their α- or ß-glycosidase activities. Being exo-glycosidases, they only remove terminal non-reducing sugar units, therefore degradation of the polymer to expose the sugar residues to the exo-enzyme, is required. The importance of enzymes for configurational analysis has diminished in recent times, having been superseded by the convenience of n.m.r. spectroscopy (Table 3.1). The application of exo-enzymes in the analysis of glycan structure has been reviewed[251].

Chromium trioxide oxidation

Chromium trioxide in acetic acid has been shown to selectively degrade peracetylated *pyranose* sugars in the ß-anomeric configuration[252]. The methodology has been reviewed by Hoffman and Lindberg[125] and specific applications of chromic acid oxidation in carbohydrate structural analysis have been discussed by Green[253]. Caution must be exercised in the interpretation of the results of chromic acid oxidation. Schmidt and Jann[34] experienced a rapid decrease of α-linked rhamnose residues in *E. coli* K12 and ascribed this to the possibility of rhamnose occurring as the $^4C_1$ conformer in which the α-anomeric bond would be equatorial, thus subject to oxidation. The method has been successfully applied to: *E. coli* K34[48], where the only sugar to survive was a glucose which was therefore assigned the α-linkage; *E. coli* K44[52], from which the α-anomeric configuration was assigned to a rhamnose residue; and *E. coli* K31[46], a very recent account of chromium trioxide oxidation.

N.m.r. spectroscopy

N.m.r. spectroscopy is now the method of choice for the determination of anomeric configuration[254]. Three parameters provide the necessary evidence for accurately assigning α- or ß-configuration to the glycosidic bonds in a polysaccharide; chemical shift (δ), spin-spin coupling ($^3J_{1,2}$) and spin-lattice relaxation times (T_1).

Chemical shift

Pyranosyl sugars with α-glycosidic bonds have equatorial anomeric protons which resonate in the region δ 5.0-5.5, whereas axial anomeric protons of the ß-anomers resonate upfield in the region δ 4.5-5.0. Similarly, C-1 of an α-linked pyranosyl residue resonates at ~100 p.p.m. while C-1 of the ß-anomer resonates 3-4 p.p.m. downfield. The α-anomeric region is upfield of the ß-anomeric region in the ^{13}C-spectrum, opposite to the situation in the corresponding ^1H-spectrum. This is governed by differences in bond lengths and bond angles about the

anomeric centre of α- and ß-linked pyranosyl residues[208] (i.e. differences in the inductive effect of the acetal oxygens on α- and ß-linked sugars). Furanosyl residues obey the same rule, however, their anomeric carbons and protons resonate downfield of the configurationally related pyranoses[209]. Ketofuranosyl residues (*e.g.* Fruf of *E. coli* K52[10]) do not have an anomeric proton, but the chemical shift of the anomeric carbon in the ^{13}C-spectrum will allow for the assignment of anomeric configuration. When the H-1 and C-1 chemical shifts of the two anomers differ by small amounts (*e.g.* mannopyranosyl residues), unambiguous anomeric assignments can not be made. Chemical shift data must therefore be used in conjunction with spin-spin coupling information.

Spin-spin coupling

Lemieux *et al.*[255] discovered that vicinal, spin-spin coupling in pyranosyl residues is a function of the dihedral angle (θ). Therefore, the coupling constant ($^3J_{1,2}$) between H-1 and H-2 is large when they are antiparallel (~10 Hz) and small when gauche disposed [axial-equatorial (~3 Hz) and equatorial-equatorial (~1 Hz)]. Karplus[256] rationalized this change in coupling constants with θ by the relationship: $J=J_o\cos^2\theta+K$ where J_o and K are constants (K = -0.28 and J_o = 8.5. when $0° \leq \theta \leq 90°$ and J_o=9.5 when $90° \leq \theta \leq 180°$). Karplus was quick to caution against relying solely on θ in calculating J as it also depends on other criteria (*e.g.* bond lengths etc.).

The magnitudes of $J_{1,2}$ can be used to confirm the anomeric configuration of glycoconjugates. Generally, α-linked pyranosyl residues have H-1 and H-2 gauche disposed with $J_{1,2}$ 1-4 Hz, whereas in ß-linked sugars they are antiparallel with $J_{1,2}$ 8-10 Hz. Sugars with mannopyranosyl stereochemistry have H-2 equatorial, therefore $^3J_{1,2}$ is small whether α- or ß-linked. Coupling between C-1 and H-1 ($^1J_{C1',H1}$) is then a reliable criterion of configuration. Double-resonance (spin-decoupling) techniques are utilized to determine $^1J_{C,H}$. The gated decoupling experiment described by Bock and Pedersen[173] has been applied in the structural

analysis of *E. coli* K12[34], K54[21], K52[10], K51[54], K32[47], K3[14] and K24[38]. Pyranosyl residues that are ß-linked have $^{1}J_{C1,H1}$ ~160 Hz while the α-anomers have $^{1}J_{C1,H1}$ ~170 Hz[210]. The major advantage of working in the gated-decoupling mode is that most of the n.O.e. is retained. Dutton *et al.*[52] working on *E. coli* K44 obtained a proton-coupled ^{13}C-spectrum of the native polysaccharide by the single frequency off-resonance decoupling (SFORD) technique. In this way they were able to determine the anomeric configuration of all the sugar residues in the tetrasaccharide repeating-unit.

Spin-lattice relaxation times (T_1)

Spin-lattice relaxation involves a transfer of magnetization between the nuclei of interest (the spins) and the surrounding environment (the lattice). The rate at which this occurs will provide a measure of T_1. The T_1 values of anomeric protons of pyranose sugars have a configurational dependence[257]. The relaxation of an axially orientated (ß-linked) anomeric proton is influenced by contributions from axial protons at 3 and 5, therefore T_1 values are lower than those of equatorially orientated anomeric protons (α-linked). Proton spin-lattice relaxation rates in the structural analysis of carbohydrates have been reviewed by Dais and Perlin[258]. N.m.r. is the preferred approach to configurational analysis because the technique is non-destructive and provides accurate results in a relatively short period of time.

3.3.4.(ii) **Absolute configuration**

Assignment of the absolute configuration of the constituent sugars is required to complete the structural elucidation of a polysaccharide. This is one of the few parameters that cannot be readily determined by n.m.r. spectroscopy. Therefore, D- or L- sugars are identified from the optical rotation of the isolated monosaccharides and by enzymatic methods[251] (*e.g.* D-galactose oxidase - *E. coli* K9[33]). The disadvantages of these procedures are that relatively large

amounts of material are required and suitable enzymes may not be available. Hence, assigning absolute configuration from circular dichroism (c.d.)[151] of alditol acetates or by separation of enantiomers by g.l.c.[259], are more popular methods as they are rapid and reliable and require small amounts of material. Resolution of enantiomers by g.l.c. has been achieved by conversion of the racemate to a mixture of glycosides of a chiral alcohol[112], or by separation on a chiral stationary phase. The former type of separation is reported more often in the literature as capillary columns coated with chiral stationary phases are expensive.

Circular dichroism

Bebault *et al.*[151] have shown that sugar configuration (D or L) may be determined unambiguously, using milligram quantities of monosaccharide derivatives, from the c.d. band at 213 nm of the corresponding alditol acetates. C.d. bands may be either sign (+ or -), analogous to the specific rotation of a sugar. Therefore, this sign must be determined experimentally and compared with data in the literature.

The method is not applicable to *meso*-alditols, such as galactitol, but does apply to their chiral partially methylated alditol acetates. Dutton and Stephen originally applied this technique in the structural study of *Klebsiella* K7[112]. It has subsequently been reported in the characterization of *E. coli* K28[7], K31[46], K44[52], K74[56] and K31[46].

G.l.c.

The procedure described by Leontein *et al.*[112], is based on the formation of glycosides of a chiral alcohol [(+)-2-octanol], and the diastereomers are separated by g.l.c. on non-chiral stationary phases. Each enantiomer will give four characteristic peaks, i.e. , the two pyranosides and the two furanosides. The relative retention times are constant and are compared with those of

authentic standards, and the D or L configurations are thus assigned. Provided that the peaks do not completely overlap, mixtures of monosaccharides can be analyzed directly, as for the constituents of *E. coli* K35 and K38 capsular polysaccharides (this thesis).

Gerwig *et al.*[260] described a similar procedure in which the multiple peak patterns of trimethylsilylated (-)-2-butyl glycosides are used for the assignment of absolute configuration. They illustrated the method on a mixture of the constituent monosaccharides of *Klebsiella* K57, and on 2-acetamido-2-deoxy sugars and uronic acids. In the structural analysis of *E. coli* K40[20] the absolute configuration of an amino acid constituent, serine, was determined by g.l.c. analysis of the (+)-2-butyl ester. To overcome the multiplicity of peaks, Little[261] converted pairs of sugar enantiomers into acyclic diastereomeric dithioacetals by reacting them with (+)-1-phenylethanethiol. The diastereomers are separated by g.l.c. as volatile acetylated or trimethylsilylated derivatives. Each enantiomer produces only one peak in the chromatogram. Schweer describes g.l.c. separation of carbohydrate enantiomers as (-)-menthyloxime[262] or (-)-bornyloxime[263] pertrifluoroacetates, where each enantiomer produces two peaks.

The g.l.c. separation of chiral enantiomers using a chiral stationary phase was first described by König *et al.*[264]. They attached L-valine-s-α-phenyl-ethylamide to the functionalized cyanoethyl side-chains of polysiloxane and coated glass capillaries with this modified polymer. Trifluoroacetylated sugars or their methylglycosides were separated, each enantiomer producing four peaks - two anomers for each of two cyclic sugars. The major advantage here being the resolution of simple derivatives of carbohydrate enantiomers, however, these columns are excessively costly. The glycosylation reactions described above are relatively laborious and the results suffer from errors arising from incomplete purity of the chiral reagents.

3.4. "*MODERN TRENDS*" IN THE ELUCIDATION OF SACCHARIDE STRUCTURE

As demonstrated by the investigation of the *E. coli* K38 capsular antigen described in this thesis, the structural characterization of saccharides can be accomplished almost entirely from spectroscopic studies. In particular n.m.r. spectroscopy, notably 2-D experiments, is of vital importance. Where degradations of polysaccharides are carried out to generate material more amenable to n.m.r. spectroscopy, some modern trends are evident, notably, the application of hydrofluorinolysis, lithium in ethylenediamine degradation and selective reductive cleavage.

3.4.1. Routine chemical analysis

"*Classical methods*" of determining polysaccharide composition, homogeneity and absolute configuration are routinely applied in conjunction with spectroscopic studies. These procedures have been discussed in section 3.

3.4.2. Specific degradations

3.4.2.(i) Anhydrous hydrofluorinolysis

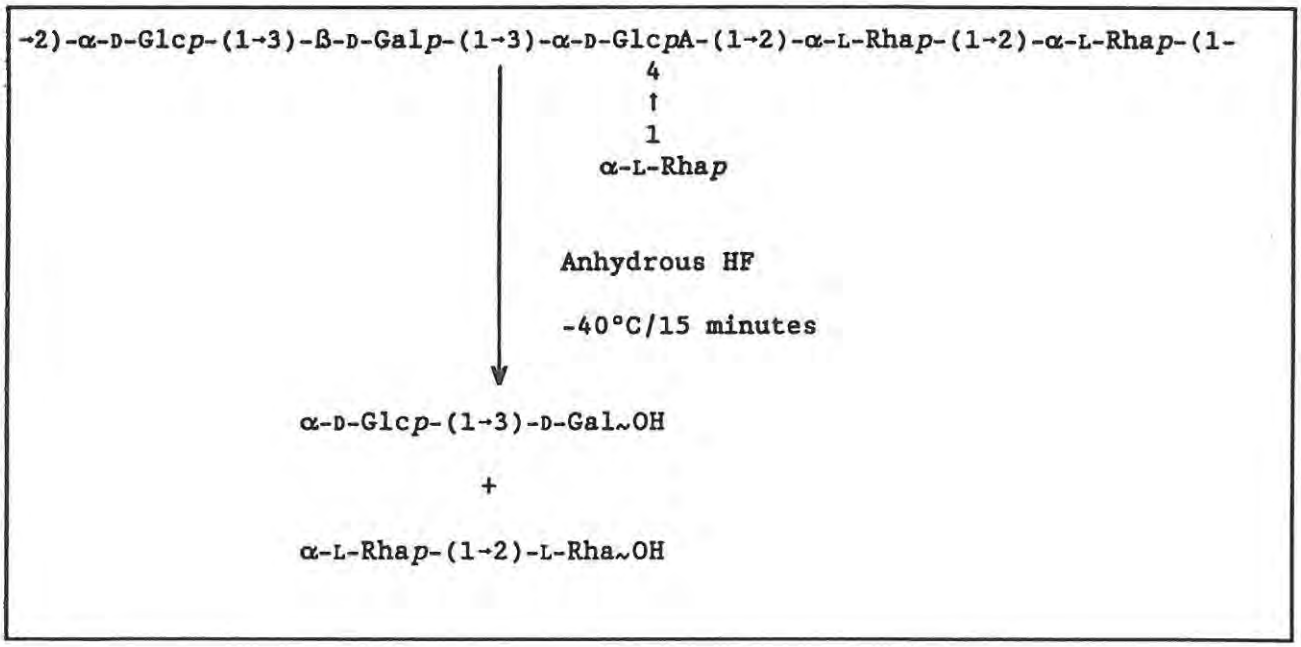
Anhydrous hydrogen fluoride (HF) was initially used as an alternative to aqueous acid and methanolic HCl for the complete cleavage of polysaccharides to their constituent monosaccharides. The advantages of HF solvolysis include negligible sugar decomposition, retention of the integrity of N-acyl substituents, and, under certain conditions, the retention of *O*-acyl substituents. Furthermore, not all sugars are equally susceptible to cleavage, hence experiments can be designed (primarily at sub-zero temperatures) to yield oligosaccharides. In a review, Knirel *et al.*[265] have discussed the mechanism of the reaction and its application in the structural analysis of polysaccharides.

Preparation of oligosaccharides

The lability of glycosidic linkages of different sugars to HF varies which allows for the complete cleavage of one or more linkages in a polysaccharide, with little or no cleavage of others. As the reaction temperature is lowered, so more glycosidic linkages become resistant. Therefore, with bacterial polysaccharides of repeating structure high yields of oligosaccharides can be obtained.

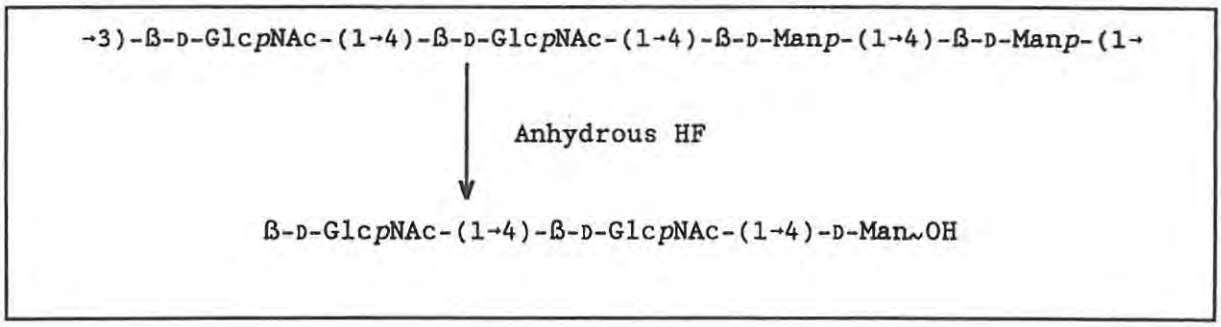
Generally, the glycosyl linkages of amino sugars, uronic acids and their derivatives are more resistant to HF than neutral sugars[265]. This is significant in the structural analysis of bacterial polysaccharides as amino sugars, uronic acids and their derivatives are common constituents thereof. However, HF solvolysis of the acidic capsular polysaccharide of *E. coli* K31[46] yielded two neutral disaccharides in significant amounts:

e.g. *E. coli* K31 repeating unit



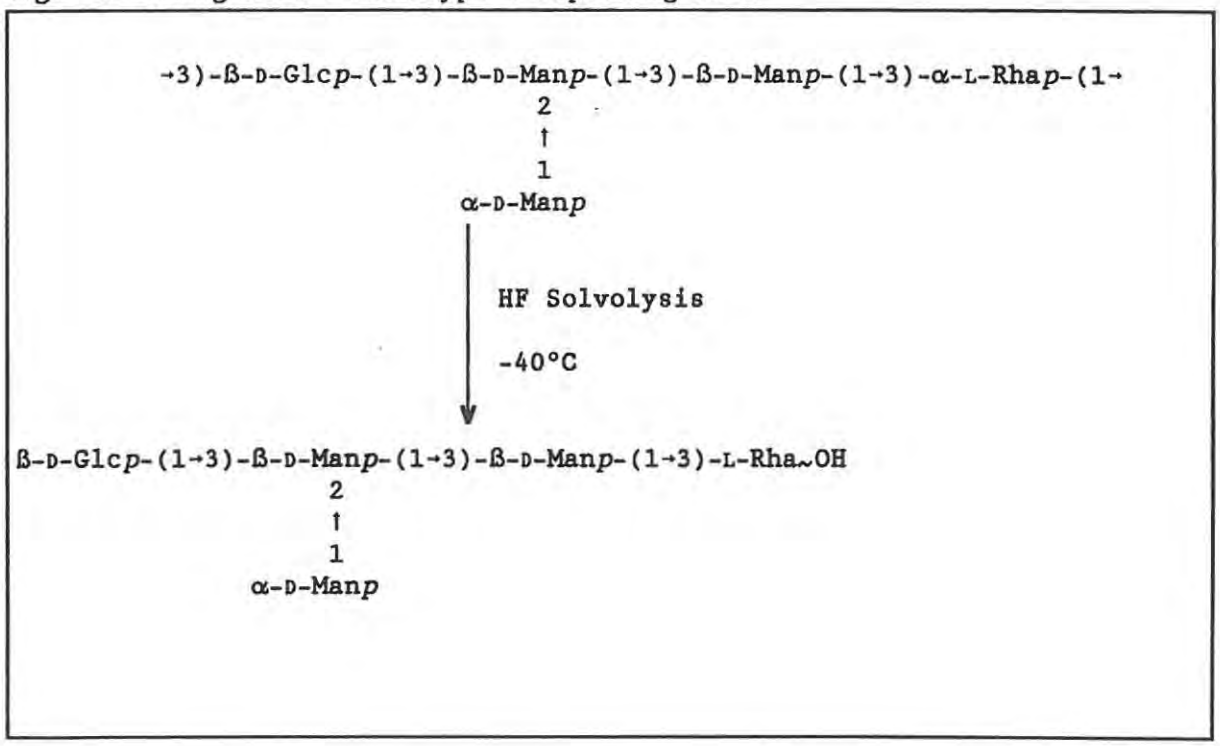
The glycosidic linkages of N-acetyl-D-glucosamine and D-galactosamine are stable in HF at 0° and below[189]. Partial HF solvolysis of *E. coli* O78[266] O-antigen gave the trisaccharide shown:

e.g. *E. coli* 078 repeating unit



6-Deoxyhexoses are more labile than their corresponding hexoses. The selective cleavage of the rhamnose linkage in the extracellular polysaccharide of *P. aeruginosa* immunotype 4 produced a pentasaccharide representative of this repeating unit[267]:

e.g. *P .aeruginosa* immunotype 4 repeating unit



Mort[268] demonstrated significant differences in the susceptibility of α and ß linkages of hexoses at -23°. Mort and Kuo[269], working on the extracellular polysaccharides of *Rhizobium* spp., were therefore able to isolate the repeating-units as only an α- linked-D-glucosyl residue was cleaved at -40°. Behaviour of the pyruvic acid acetals was not consistent, some were removed while others remained intact[269]. A useful table, listing the expected labilities of the

glycosidic bonds of various sugars at various HF temperatures, has been compiled by Knirel *et al.*[265].

HF solvolysis has also been used to decrease the molecular weight of large polysaccharides. Aqueous solutions of the modified polymers have much reduced viscosities thus permitting the acquisition of n.m.r. spectra of greater resolution[270,271,23]. This technique has obvious advantages over hydrolysis with aqueous acid, and has the potential to find wide application.

3.4.1.(ii) **Lithium/ethylenediamine degradation**

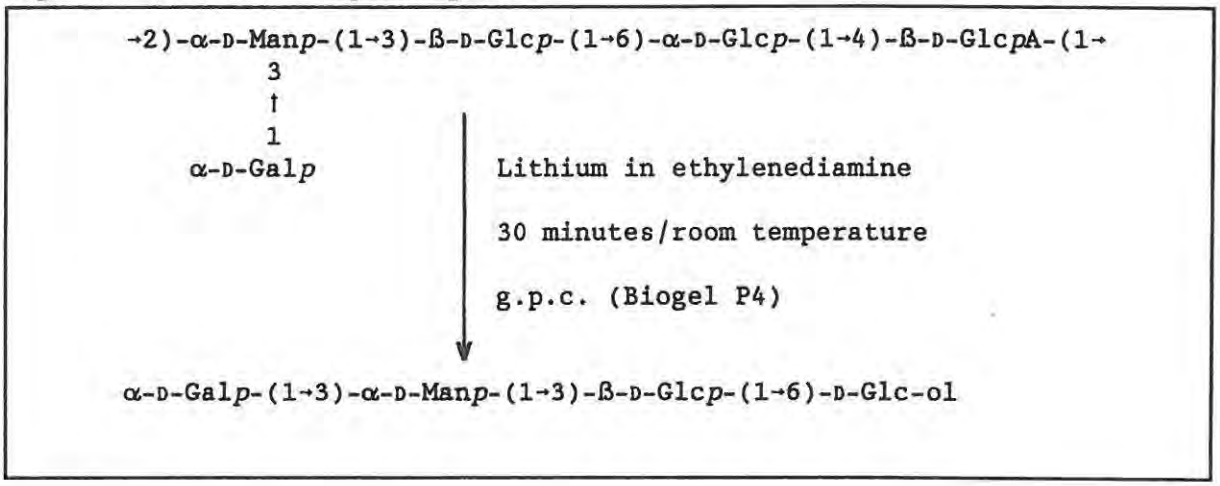
Selective degradation of uronic acid residues of polysaccharides by lithium dissolved in ethylenediamine was reported by Mort and Bauer[188]. Lau *et al.*[179] have subsequently developed a general method for the selective cleavage of underivatized glycosyluronic acid-containing polysaccharides by lithium treatment. Lithium dissolved in an amine is a powerful reducing agent, accounting for the generation of oligosaccharide-alditols[179]. The authors determined the effects of lithium treatment on other glycosyl residues and non-glycosyl substituents, and identified the products of the cleavage reaction.

The method involves dissolving dry polysaccharide in dry ethylenediamine and adding sufficient lithium wire to maintain the dark blue colour of the reaction for the desired reaction time. The reaction is quenched with anhydrous methanol or water. The excess ethylenediamine and methanol are removed under vacuum over H_2SO_4 and NaOH. Excess ethylenediamine and water is evaporated under diminished pressure as the azeotrope with toluene. Acetic acid is added to neutralize the lithium methoxide or lithium hydroxide formed. The material is desalted by g.p.c. or ion-exchange, and fractionated.

If the uronic acid residue is: (1) In-chain, an oligosaccharide-alditol is

formed (*e.g. E. coli* K26[16], K31[46] and K39[50]); (2) in a side chain, a modified polysaccharide and an alditol or oligosaccharide-alditol are formed; and (3) terminal non-reducing, only a modified polymer is formed (*e.g. E. coli* K102[62] and *Klebsiella* K45[272]).

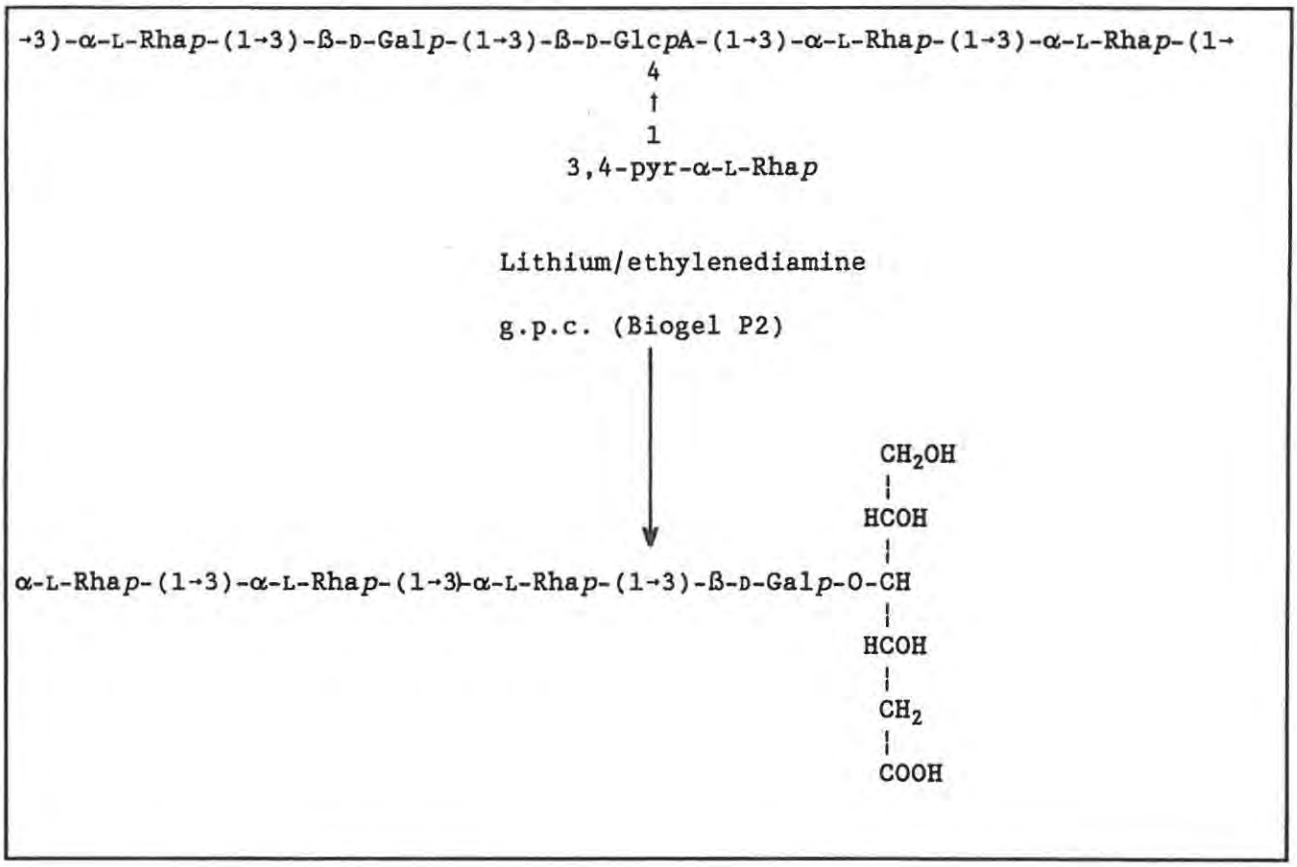
e.g. E. coli K39[50] repeating unit



Lau *et al.*[179], using model compounds, established that methyl glycosides, methyl ethers and pyruvyl ketals are also cleaved under these reaction conditions, while the glycosyl bonds of neutral sugars are largely stable. The solubility of a polysaccharide in ethylenediamine is of prime importance. When polymers are partially soluble, both starting material and cleavage product are recovered from the reaction.

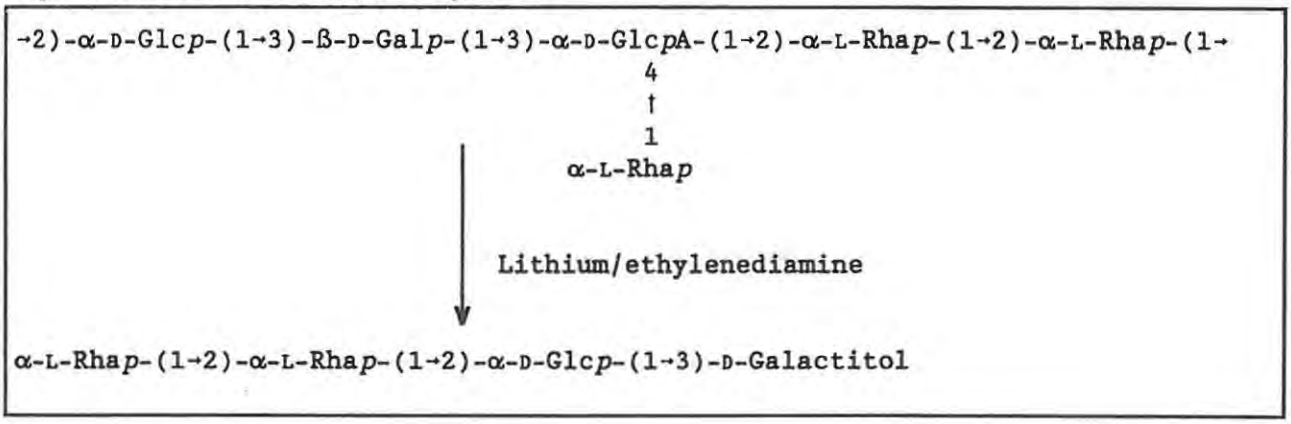
When the uronic acid is substituted at *O*-4 and/or *O*-2 it is completely degraded and no portion thereof remains attached to the product. Lithium treatment of such polysaccharides releases the glycosyl residues attached at *O*-2 and *O*-4 and *O*-1 of the uronic acid. Lau *et al.*[179] showed that glycosyl residues linked at *O*-3 are not released, therefore, polysaccharides containing in-chain uronic acid residues substituted at *O*-3 yield oligosaccharides terminated by modified uronic acid residues (i.e. 2-deoxy derivatives):

e.g. *E. coli* K26[16] repeating unit



In the lithium degradation of the *E. coli* K31[46] capsular polysaccharide however, the glycosidic linkage of the hexosyl residue linked at *O*-3 of the uronic acid was cleaved:

e.g. *E. coli* K31[46] repeating unit



Because the oxygen at C-5 of the uronic acid is lost during the lithium reaction, Lau *et al.*[179] proposed that the oxygen on C-5 may have moved to C-1 via alpha elimination, concomitant with the loss of the oxygen at C-1 to its aglycone.

Furthermore, this may be the mechanism of the lithium degradation of all uronic acid containing polysaccharides.

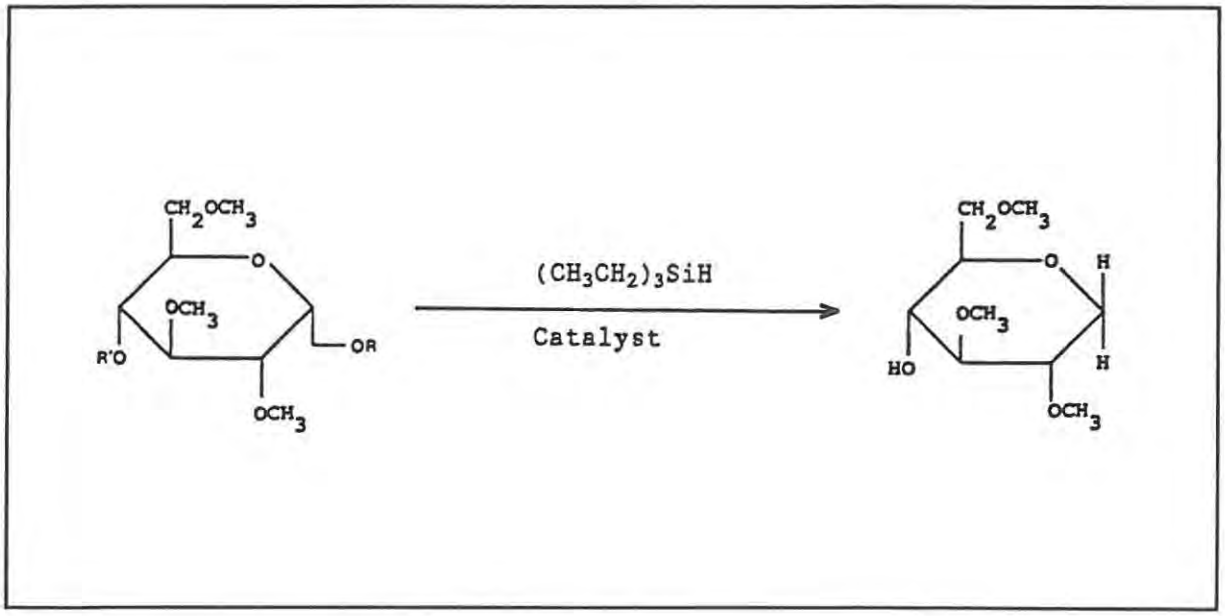
3.4.2.(iii) **Partial and Selective reductive cleavage**

Reductive cleavage of methylated glycans was developed as an alternative to traditional methylation analysis because the latter procedure suffers from two significant disadvantages[273]:

(1) Ambiguities arise in assigning linkage positions and ring form to aldopyranosyl and aldofuranosyl residues linked at *O*-4 and *O*-5, respectively, and ketohexopyranosyl and ketohexofuranosyl residues linked at *O*-5 and *O*-6, respectively.

(2) The method is laborious.

Reductive cleavage of the glycosidic C-O bond is effected by a hydride transfer from triethylsilane to the anomeric carbon in the presence of a Lewis-acid catalyst.



Rolf and Gray[273] initially used trifluoroacetic acid as catalyst under conditions known to reduce hemiacetals[274], however the reaction was not successful. Boron

trifluoride etherate ($BF_3.Et_2O$), trimethylsilyltrifluoromethanesulphonate (TMSOTF) and trimethylsilylmethanesulphonate (Me_3SiOSO_2Me) are effective activators of the anomeric carbon centre promoting the hydride transfer from $(CH_3CH_2)_3SiH$. Recently, Jun and Gray[275] described a new catalyst for the reductive cleavage of methylated glycans *viz.* 5 equivalents of Me_3SiOSO_2Me plus 1 equivalent of $BF_3.Et_2O$ per equivalent of acetal, and reported stronger catalysis with fewer side reactions. The experimental procedures and applications of reductive cleavage have been reviewed by Gray[276]. Pyranosides are converted to 1,5-anhydroalditol derivatives and furanosides to 1,4-anhydroalditol derivatives, allowing for the simultaneous determination of ring form and linkage position by g.l.c. and g.l.c.-m.s. Van Langenhove and Reinhold[277] have published g.l.c. and g.l.c.-m.s. reference data. Bowie and Gray[278] have published ^{1}H-n.m.r., c.i.- and e.i.-m.s. data for a series of 1,5-anhydroalditol-D-mannitol residues. Besides the above-mentioned advantages over standard methylation analysis the method also shows selectivity[275]. Hence, by judicious choice of catalyst and reaction conditions, both total reductive cleavage and selective reductive cleavage (SRC) can be achieved. Certain selectivities have been demonstrated:

(1) Gray *et al.*[279], using $BF_3.Et_2O$ as catalyst, found that -2, -3 and -6 linked residues were incompletely cleaved, while branch point residues linked at both *O*-2 and *O*-6 or *O*-3 and *O*-6 were not cleaved at all. Anhydroalditol terminated oligosaccharides (oligosaccharide-anhydroalditols), thus generated would be useful in sequence analysis.

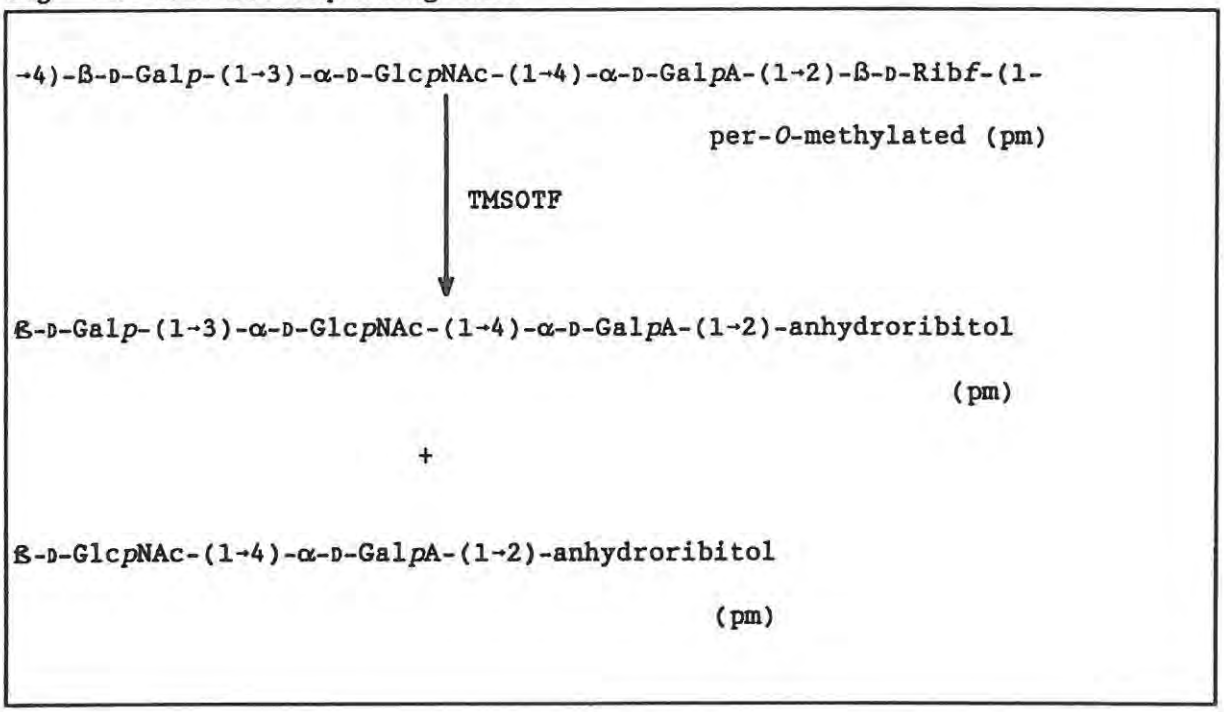
(2) Reductive cleavage of a permethylated pullulan[280] (a glucan) catalyzed by $BF_3.Et_2O$ cleaved α-(1→4) linkages but not α-(1→6) linkages. A methylated disaccharide-anhydroalditol derivative containing the intact α-(1→6) linkage was identified as the major product.

(3) α-linked 2-acetamido-2-deoxy-D-hexopyranosyl residues are completely resistant to reductive cleavage, irrespective of which catalyst is utilized[281], whilst the ß-anomers are readily cleaved. The presence of an α-linked amino sugar should therefore guarantee the formation of a disaccharide-anhydroalditol.

(4) Jun and Gray[275], conducting a reductive cleavage study on inulin which contains a large proportion of furanosyl residues, found that Me_3SiOSO_2Me catalyzes the reductive cleavage of D-fructofuranosyl residues while the D-glucopyranosyl residues remained unaffected.

(5) Stanley[235], prompted by the findings of Jun and Gray[275] above, effected the SRC of the ribofuranose containing permethylated capsular polysaccharide of *E. coli* K57 : (see also section 5.2.)

e.g. *E. coli* K57 repeating unit



These oligosaccharide-anhydroalditols were isolated in sufficient yields for complete characterization of the repeating unit by n.m.r. spectroscopy and f.a.b.-m.s.

(6) Esters of carboxylic acid groups were found to be stable to reductive cleavage conditions[282], suggesting that the characterization of carbohydrates containing uronic acids, pyruvic acid acetals and lactic acid ethers could be achieved by this procedure. As uronic acids are stable to hydrolysis it was anticipated that they would resist reductive cleavage. If this were so, most acidic capsular polysaccharides would be amenable to SRC. However, they are susceptible to reductive cleavage and furthermore undergo a pyran to furan

rearrangement[282].

(7) The fate of pyruvylated sugars was explored by Zeller and Gray[283]. When $BF_3.Et_2O$ or TMSOTF are used as catalyst very little reductive cleavage of the glycoside of methyl 4,6-*O*-[(R)- and (S)-1-methoxycarbonylethylidene]-2,3-di-*O*-methyl-α-D-glucopyranoside occurred. Isomerization of the initial R,S mixture of diastereoisomers to the more stable S diastereoisomer was noted. These findings could be an indication for SRC of polysaccharides containing pyruvylated monosaccharides (see section 5.1).

When all the linkages in a polysaccharide are equal or near equal in their susceptibility to reductive cleavage, oligomers may still be produced by terminating the reaction before all glycosidic bonds have been cleaved. This has been referred to as partial reductive cleavage (PRC)[235]. Invariably a more complex mixture of oligosaccharide-anhydroalditols would be produced which would be more difficult to fractionate. Reinhold *et al.*[284] reported the PRC of ß-cyclodextrin from which they isolated monosaccharide- through to heptasaccharide-anhydroalditols by h.p.l.c. (Fig. 3.2). The oligosaccharide-anhydroalditols were characterized by direct chemical ionization (ammonia) - mass spectrometry.

The major advantage of SRC and PRC is that oligosaccharides are isolated as derivatives suitable for m.s. and n.m.r. analysis without further chemical manipulation. When small amounts of material are available, c.i.-m.s. and f.a.b.-m.s. provide molecular mass, linkage and sequence information on 1-5µg of product. With larger amounts of material, the polysaccharide can be trideuteriomethylated prior to depolymerization and milligram quantities of oligo-anhydroalditols are isolated for n.m.r. spectroscopy. The spectra are recorded in organic solvents , typically deuterated chloroform. Spectra are usually better resolved than for samples acquired in deuterium oxide (D_2O) and are not complicated by the presence of an HOD signal. The oligosaccharides are terminated by anhydroalditols, hence chromatograms and n.m.r. spectra do not

suffer from the "anomeric complications" experienced with molecules terminated by a reducing sugar residue. Therefore, n.m.r. spectra are readily interpreted providing a convenient, non-destructive technique for structural elucidation.

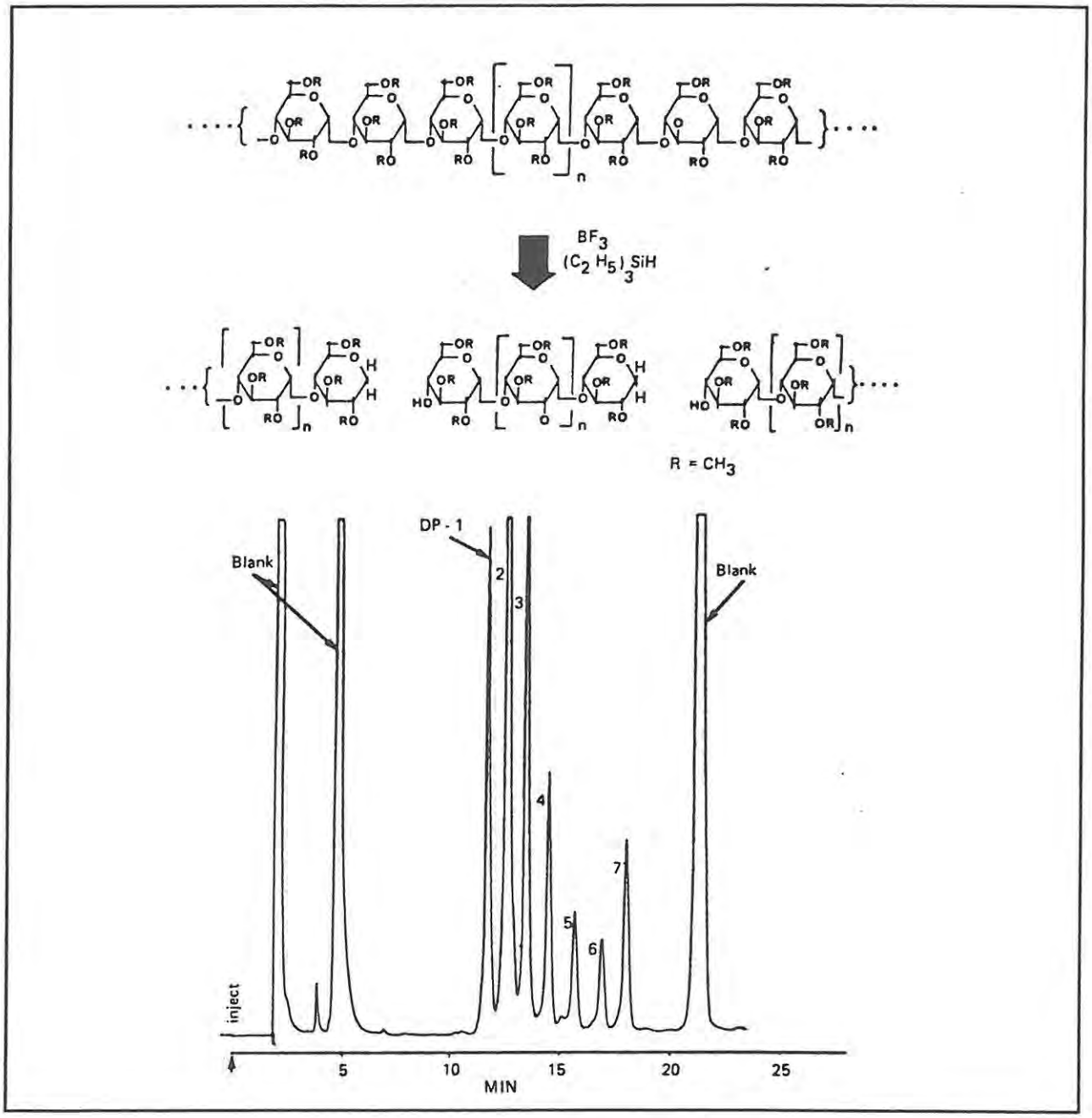


Fig. 3.2. Mixture of products from PRC of permethylated ß-cyclodextrin[284]

3.4.3. Mass spectrometry

Electron impact-mass spectrometry (e.i.-m.s.) is a valuable analytical tool which is well established in carbohydrate chemistry. Therefore, the application of e.i.-m.s. to carbohydrates in general and to various derivatives (*e.g.* partially methylated alditol acetates) has been well documented[203,218,221,285]. Recently, m.s. has established itself as a reliable procedure for the sequencing of oligosaccharides and short-chain polysaccharides. E.i.-m.s. of permethylated oligosaccharides can provide sequence information from the A-type fragmentation pathways[203,238], however, the results are generally unreliable because of the low relative abundance of structurally informative high-mass fragments and molecular ions (M^+). Chemical ionization- and fast atom bombardment mass spectrometry (c.i.-m.s. and f.a.b.-m.s.) have largely overcome these limitations as considerably less energy is transferred to the sample molecule being ionized than with e.i. Hence, c.i.- and f.a.b.-m.s. may be regarded as "soft" ionization techniques, providing usable spectra of compounds of increased polarity and higher molecular weight, and are complementary to e.i.-m.s. This approach has increased the versatility of m.s. in the delineation of saccharide structure.

3.4.3.(i) Chemical ionization-mass spectrometry (c.i.-m.s.)

C.i.-m.s. requires the introduction of a sample into an ion-plasma which is generated by e.i. of a reagent gas maintained at near-atmospheric pressure. The reagent gasses used include ammonia, methane and isobutane. The sample is then ionized by one of three possible processes[286,287]:

(1) Charge exchange

(2) Proton transfer

(3) The formation of collision-stabilized adduct ions

The base peak is often represented by the molecular adduct ion thus providing the molecular weight/s of the sample. Ammonia, being the softest ionizing reagent

gas, is most suitable for the determination of molecular weights. C.i.-m.s. has therefore been used in conjunction with e.i.-m.s. for the analysis of products of partial degradation of *E. coli* capsular polysaccharides.

The disaccharide, ß-D-GalNAc-(1→5)-Kdo, isolated from mild acid hydrolysis of *E. coli* K14[19] was reduced, methylated and characterized by a combination of e.i.- and c.i.-m.s. The former providing sequence and linkage information, the latter providing the molecular mass from the quasimolecular ion (M+1) generated by a proton transfer. Disaccharides isolated from the partial hydrolysis of *E. coli* K54[21] were characterized as above and showed that the amino acid, threonine, was attached to the uronic acid residue via an amide linkage. Similarly, a disaccharide isolated from *E. coli* K52[10] was found to be a ß-D-Fruf-(2→2)-Gal. C.i.-m.s. was also conducted on oligosaccharides derived from Smith degradation and partial hydrolysis of *E. coli* K40[20] and K95[60]. Beynon and Dutton[40,16] were able to characterize a series of oligosaccharides, generated by partial hydrolysis of *E. coli* K26, using g.l.c.-c.i.-m.s. They were thus able to deduce the sugar sequence of the polymeric backbone of E. coli K26 capsular polysaccharide, which is complicated by containing three rhamnose residues all having 1→3 glycosidic linkages. Jann *et al.*[39] determined the structure of the disaccharide ß-Ribf-(1→4)-Kdo, representative of the repeating-unit of *E. coli* K19 capsular polysaccharide, by combined g.l.c.-c.i.- and -e.i.-m.s.

C.i. requires vaporization of the sample by heating, which leads to the unavoidable pyrolytic decomposition of underivatized material. The derivatives used to increase the volatility of carbohydrates include per-*O*-acetyl, per-*O*-methyl and per-*O*-trimethylsilyl analogues. Horton *et al.*[288] have reviewed c.i.-m.s. of the various sugar derivatives. The introduction of direct chemical ionization mass spectrometry (direct c.i.-m.s.)[175], has decreased the problem of pyrolytic decomposition and provides molecular weight and sequence information. Therefore, the technique can be successfully applied to larger and more polar

compounds than conventional c.i.-m.s.

Using stable isotopes in the reagent gas or incorporated in the sample, Reinhold and Carr[289] were able to study the direct c.i. fragmentation patterns of permethylated-oligosaccharides. Enhanced glycosidic cleavage is observed with ammonia d.c.i. providing significant sequence information[175]. Direct c.i.-m.s. involves placing the sample directly into the ionization chamber on an extended probe which is heated to volatilize the sample. This is referred to as heat desorption of the sample from the direct c.i. emitter. G.l.c.-c.i.-m.s. is effective for the characterization of di- and tri-saccharides. Tetra- and higher oligo-saccharides have excessively long g.l.c. retention times and are therefore subjected to direct c.i.-m.s. A major limitation of direct c.i.-m.s. is that large molecules require excessive probe temperatures to volatilize, hence spectra are dominated by pyrolytic fragments. As molecular weight increases so too does fragmentation. Therefore, there exists a molecular weight beyond which direct c.i.-m.s. becomes less informative. This is exemplified by a comparison of the c.i. mass spectra of the trisaccharide anhydroalditol and pentasaccharide anhydroalditol in section 5.2.2. Sequence information provided by c.i.-m.s. is associated with cleavage on either side of the glycosidic oxygen followed by hydrogen transfer. Methylation of oligosaccharides stabilizes the sugar rings and enhances glycosidic rupture. The terminal, T, and reducing, R, ends of the oligomer (Fig. 3.3) produce two separate ion series, accounted for as hydrogen or methyl transfer products. i.e. T fragments arise from cleavage on the reducing side of the glycosidic bond followed by transfer of a hydrogen (or methyl group), and association with an ammonium ion. R fragments are described in a similar fashion. Fragments occur as protonated or ammonium adduct ions depending on the oligosaccharide basicity, hence, N-containing compounds result in protonated fragments whilst neutral saccharides are characterized by ammonium adduct ions. Dutton *et al.*[53,46] determined the sequence of the tetrasaccharide repeating unit (P1) of *E. coli* K44[53] (Fig. 3.4), isolated from bacteriophage

degradation, and the backbone sequence of *E. coli* K31[46] capsular polysaccharide by direct c.i.-m.s.

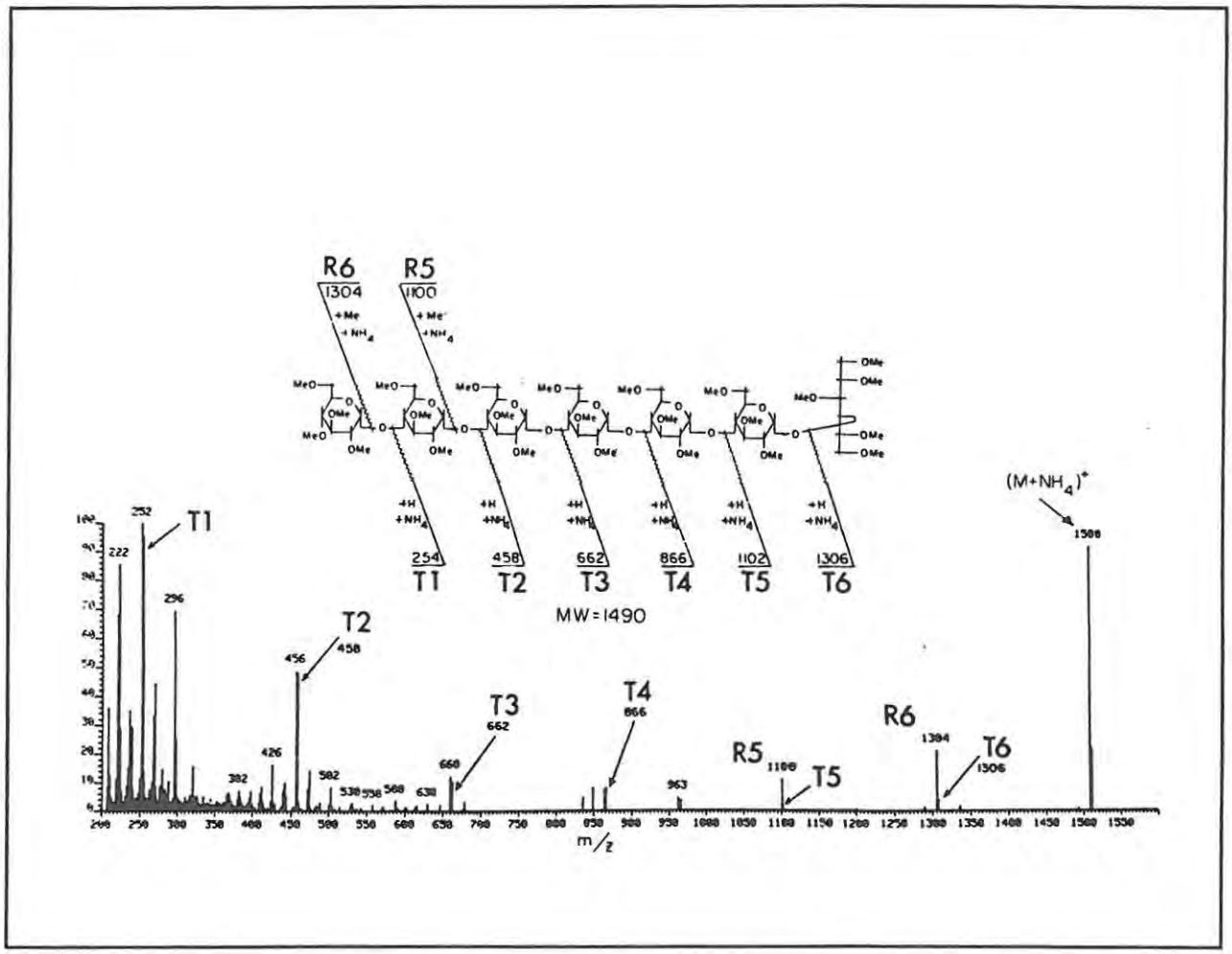


Fig. 3.3. Direct c.i.-m.s. of a reduced, methylated heptasaccharide[175]

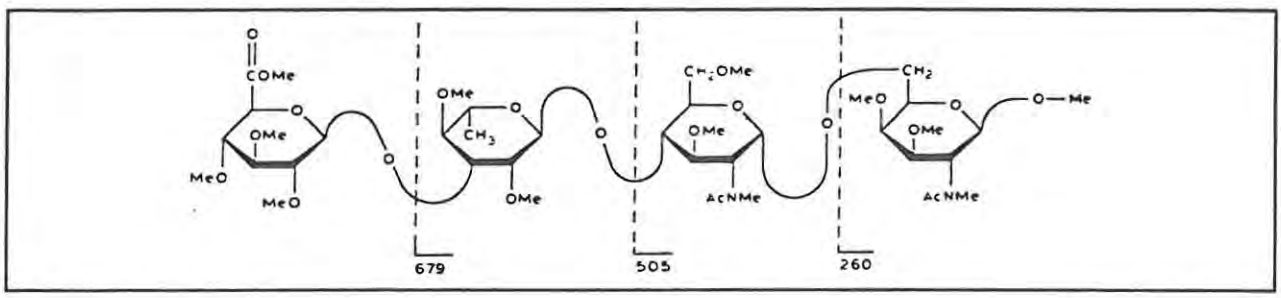


Fig. 3.4. Structure of methylated oligosaccharide P1 from *E. coli* K44 showing its fragmentation on direct c.i.-m.s.

ion-mass spectrometry (s.s.i.-m.s.)[291] where the ion beam has been replaced by an atom beam, hence it has become known as fast atom bombardment mass spectrometry (f.a.b.-m.s.). F.a.b.-m.s. has subsequently largely replaced s.s.i.-m.s. and field desorption mass spectrometry (f.d.-m.s.[292,293]) for the analysis of non-volatile and thermally labile compounds. It has developed into an extremely valuable analytical tool for the structural analysis of carbohydrate compounds and is the subject of a comprehensive review[234].

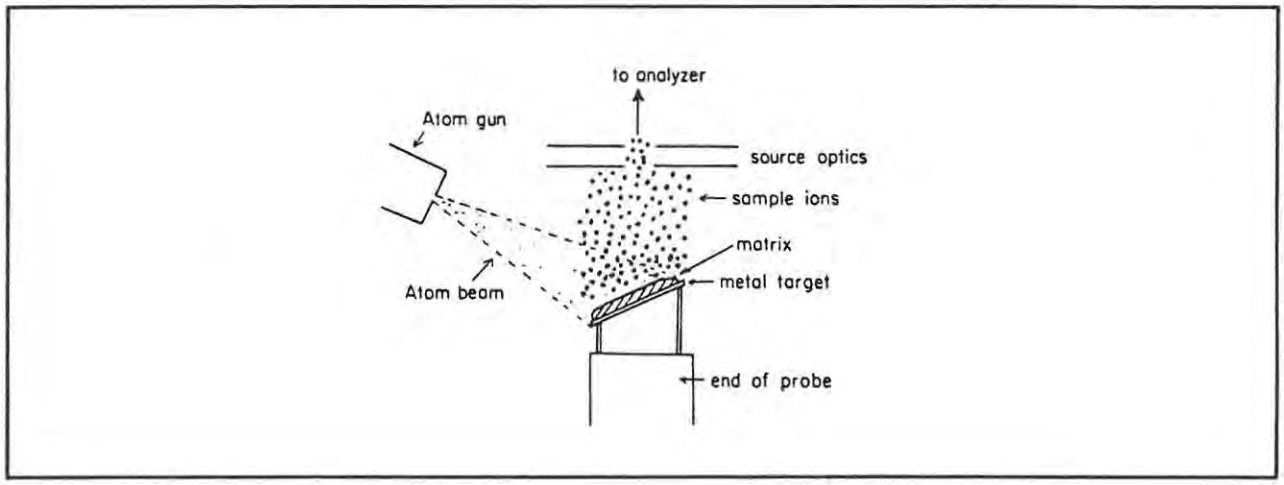


Fig. 3.6. Schematic representation of a f.a.b. source[234]

A schematic representation of a f.a.b. source is provided by Fig. 3.6. The sample to be analyzed is dissolved in a viscous liquid matrix, typically glycerol for polar compounds and 1-thioglycerol for hydrophobic compounds. The matrix is loaded on to a metal target, which constitutes the tip of a probe insert, and introduced into the source of the spectrometer. The target is bombarded with an accelerated beam of atoms (*e.g.* Ar) or ions[290,291]. On collision with the matrix, kinetic energy is transferred to the surface molecules, some of which are released into the ion-source with a significant number being simultaneously ionized. Subsequently ionization is achieved by a sputtering process analogous to that in s.s.i.-m.s. Gas phase ions are therefore produced without heating the target and long lasting spectra are obtained which are not wrought with the

problems of pyrolysis. Both positive and negative ions are generated, hence spectra may be recorded in the positive or the negative ion mode. Solubility of the sample in the matrix is of prime importance and numerous liquids have been used[234]. The inclusion of certain additives has been found to enhance the sensitivity of f.a.b.-m.s. of carbohydrates:

(1) Dilute aqueous HCl - increases $(M+H)^+$ abundance.

(2) Sodium acetate - where $(M+Na)^+$ pseudomolecular ions are generated.

(3) Ammonium thiocyanate - $(M+NH_4)^+$ pseudomolecular ions.

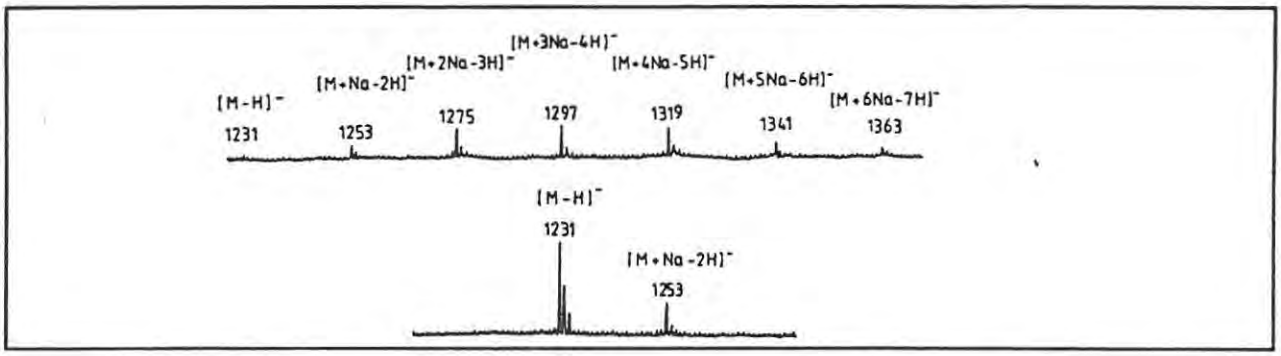


Fig. 3.7. Molecular-ion regions of a negative f.a.b.-m.s.[234] showing the complexity of this region of the f.a.b. spectrum

Spectra are characterized by abundant pseudomolecular ions, a high level of chemical noise, cluster ions (largely matrix-derived) and fragment ions. Pseudomolecular ions appear as clusters (Fig. 3.7) largely due to the presence of the ^{13}C isotope, and oxidation and reduction that occur in the matrix during f.a.b. Magnetic sector mass spectrometers typically have a mass range of 4000, operating at accelerating voltages of 4-8 kV. Above a mass range of 4000 sensitivity drops off dramatically because the accelerating voltage is dropped below 4 kV to allow detection. Some modern spectrometers have the ability to detect compounds having molecular weights in excess of 4000.

F.a.b.-m.s. of carbohydrates provides two types of information. Firstly, the molecular weight of the oligosaccharide or glycoconjugate, from which the composition may be ascertained (i.e. types and numbers of sugars, type of

aglycon, types and number of non-carbohydrate substituents), and secondly, sequence information. The molecular weight is determined from interpretation of the pseudomolecular ion region. This is often complex, especially if the sample is contaminated with K^+ or Na^+, where $(M+K)^+$, $(M+Na)^+$ and $(M+H)^+$ are generated. Here, addition of acid to the matrix is particularly useful as $(M+H)^+$ abundance is increased. Fragmentation pathways have been identified[234], however, reliable sequence information requires derivatization of the material. Derivatization also permits f.a.b.-m.s. analysis of impure compounds, improves sensitivity, enables molecules in excess of M_r 4000 daltons to be analyzed by f.a.b.-"mapping"[234], and aids in the location of *O*-acetyl substituents. The latter, is particularly useful in the structural analysis of bacterial polysaccharides as they often contain immunodominant *O*-acetyl groups. The per-*O*-acetyl[294] and per-*O*-methyl derivatives, besides being well established in carbohydrate analysis, are the derivatives of choice. The possibility of underderivatization occurring must be borne in mind to avoid ambiguous spectral interpretation. Dell[234] has outlined a protocol for acquiring spectra on nanogram quantities of derivatised sample. Furthermore, monitoring partial methanolysis of permethylated sample by f.a.b.-m.s., provides valuable sequence information, including branching patterns[295].

The versatile nature of the technique in carbohydrate structure analysis is highlighted by the variety of carbohydrate compounds amenable to f.a.b.-m.s:

(1) Glycolipids - glycosphingolipids, for example gangliosides, readily produce pseudomolecular ions and sequence specific fragments when a suitable matrix is chosen[296]. Egge[297] *et al.* were able to detect a $(M+Na)^+$ signal at m/z 6184 for a permethylated 25-sugar glycosphingolipid after sodium acetate dosing of the matrix. Glycosphingolipids present in granulocytes, leukaemia cells, cancer cells, erythrocytes, insects and human lungs[234 and references therein] have been characterized by f.a.b.-m.s. Recently, the f.a.b.-m.s.-assisted structural elucidation of the glycolipids isolated from the protozoan parasite *Leishmania*

major[298] and from the liver of the Rainbow trout[299], was reported by Dell *et al.*

(2) Glycoproteins - lactosaminoglycans of human granulocytes[300], and glycoproteins in the brains of Springer spaniels suffering from a progressive nervous disorder, have been identified by f.a.b.-m.s. F.a.b. mapping procedures, recently reviewed by Dell *et al.*[301], are extremely valuable in this area as the technique permits the characterization of these compounds with molecular weights as large as 20 000. Thomas-Oates and Dell[302] have reviewed the structure analysis of the sugar portion of glycoproteins by a combination of f.a.b.-m.s., and enzymatic and chemical reactions. Hypotheses regarding the biosynthesis of glycoproteins have been monitored by f.a.b.-m.s.[303] and Dell *et al.*[304] have developed a protocol for rapidly screening glycoproteins by f.a.b.-m.s., employing simple purification steps, and the applications of this procedure in the biotechnology industry are reviewed by Dell and Rogers[305].

(3) Bacterial polysaccharides - f.a.b.-m.s. has been used to determine the composition (including *O*-acyl substituents) and the sequence of microbial saccharides. Significant progress in f.a.b.-m.s. was initiated from the investigation of the mycobacterial methyl glucose polysaccharide (MGP)[306]. The enhanced quality of the spectra, compared to those of compounds without *O*-methyl groups, prompted the study of per-*O*-methyl derivatives. F.a.b.-m.s. of the *Klebsiella* K54[307] repeating unit proved that it is not formylated, but did demonstrate the presence of acetyl groups. Bacteriophage degradation products of the acidic polysaccharides secreted by *Rhizobium* spp. were characterized by f.a.b.-m.s., indicating the type of enzyme activity associated with the phage[308]. Oligosaccharides derived from *Meningococcal* lipopolysaccharides[309], a group B *Streptococcus*[310], the *Salmonella thompson* serogroup C1(6,7)[311] O-antigen and *E. coli* K26[16] polysaccharide were partially characterized by f.a.b.-m.s. This short list exemplifies the variety of bacterial saccharides amenable to f.a.b.-m.s. Furthermore, it was used to locate the site of *O*-acylation on the *Klebsiella* K69

capsular polysaccharide[312] and to determine the sequence of the methylated oligosaccharides from SRC of *E. coli* K57[235] and K38 capsular polysaccharides (this thesis).

(4) Plant cell-wall polysaccharides *e.g.* Soybean cell-walls[313], gellan gum[314].

(5) Cyclic polysaccharides[234] and various other carbohydrate compounds.

The advantages of f.a.b.-m.s. in the structural analysis of carbohydrates are numerous and include the rapid screening of cell surface glycoconjugates, the analysis of glycopeptides as large as 20 000 dalton by f.a.b.-"mapping", monitoring of the progress of chemical and enzymatic degradation, rapid screening of genetically engineered glycoproteins, the detection of minor components due to the sensitivity of f.a.b.-m.s., and reliable routine composition and sequence analysis of saccharides.

3.4.4. Modern nuclear magnetic resonance (n.m.r.) spectroscopy in carbohydrate structure determination

Lemieux *et al.*[255] reported the application of n.m.r. spectroscopy in carbohydrate structural analysis as early as 1957. However, it is only during the last decade that it has emerged as the major analytical tool in carbohydrate chemistry. This was made possible by the commercial availability of instruments having powerful superconducting magnets (400-600 MHz) capable of operating in the pulsed Fourier transform (Ft) mode, and dedicated computers driving sophisticated soft-ware permitting the acquisition of data from multiple pulse sequences. This trend is depicted in Table 3.1, where we find limited application of the technique in earlier structural analysis. A period then followed where 1D, single pulse, ^{1}H- and ^{13}C-n.m.r. experiments were routinely used in conjunction with chemical and other instrumental analytical procedures ("classical approach"). In several of

the latest publications we find that the structures of these polysaccharides can be determined almost exclusively by n.m.r. techniques ("modern trends").

N.m.r. spectroscopy is now so widely used that Rabenstein and Guo[315] reported a literature search for the period 1985-1987 consisting of over 500 books and review articles on the subject. It is the proliferation of multiple pulse experiments that has contributed largely to this situation. It is beyond the scope of this thesis to present an exhaustive review of all the multiple pulse experiments applied to carbohydrate structure elucidation. Some of these techniques will be discussed, with emphasis being placed on 2D experiments which provide unambiguous resonance assignments, thus enabling structural elucidation of compounds without the need for chemical methods (see Bax *et al.*[161]). Several general review articles[137,161,315-319], a review of the application of 2D n.m.r. spectroscopy to the structural elucidation of oligosaccharides by Dabrowski[320], and books by Derome[321], and Schraml and Bellama[322] have been extremely useful.

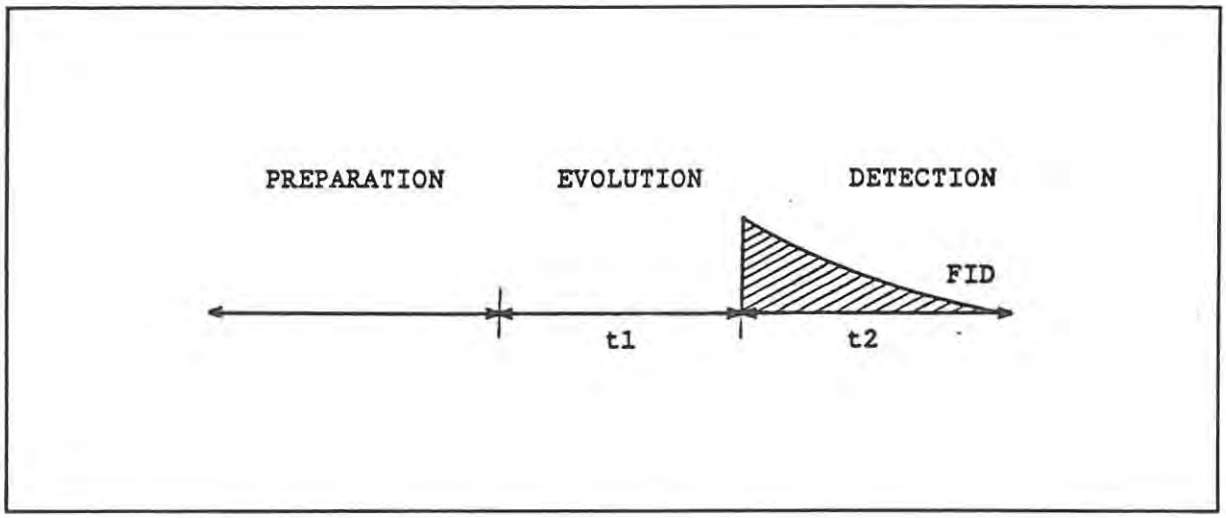
The various structural features of saccharides that need to be determined, and some of the n.m.r. methods used to provide this information, are summarised in

Table 3.3. (adapted from Dabrowski[320])

Structural features	Required n.m.r. data	N.m.r. experiments providing this data
1. Number and identity of sugar residues	Anomeric ^{1}H- and ^{13}C chemical shifts All ^{1}H and ^{13}C chemical shifts	Integrated 1D ^{1}H spectrum; decoupled 1D ^{13}C spectrum 2D ^{1}H correlation spectroscopy - COSY, RCT COSY, TOCSY (HOHAHA) 2D ^{1}H-^{13}C correlation spectroscopy - HETCOR
2. Anomeric configuration	Vicinal ^{1}H coupling constants ($^{3}J_{H,H}$) ^{1}H-^{13}C coupling constants	Phase sensitive COSY, 1D ^{1}H spectrum, 1D HOHAHA spectra Distortionless enhancement by polarization transfer (DEPT - 1D multiple pulse experiments)
3. Sequence and linkage sites	Interresidue n.O.e.	2D - NOESY, ROESY
4. Spatial arrangement (conformation)	n.O.e. ; cross-relaxation rates	NOESY, ROESY

3.4.4.(i) **Multiple pulse n.m.r. spectroscopy**

One-dimensional (1D) and two-dimensional (2D) multiple pulse n.m.r. experiments in general can be divided into three time intervals:



During the preparation time a period of thermal equilibrium is established among the nuclear spins in the sample, and hence the spin systems are prepared for evolution. The evolution time (t1) is the most important feature as it is during this time that the spin system evolves after being disturbed by a radio-frequency (rf) pulse. Further rf pulses may be applied after various t1 intervals giving rise to the multiple pulse techniques. The variety of pulse sequences which can be applied provide the n.m.r. spectroscopist with considerable latitude in controlling the information provided by the experiment. During the detection period (t2) the motion of the spin system is followed, and it is this motion that induces a signal which is detected. The time dependence of the signal obtained, s(t), is known as the FID (free induction decay).

In the 1D experiments the signal is recorded as a function of the detection period, S(t2), and it is this single time response that is Fourier transformed (Ft) to produce a frequency dependence of the signal, S(f2), and hence the n.m.r. spectrum.

In 2D-n.m.r. experiments it is the Ft of the signal dependence on two independent time variables, t1 and t2, S(t1,t2), that produces a two-dimensional frequency spectrum, S(f1,f2). After a single pulse, a 1D dependence of the signal on time is obtained, therefore the use of two pulses was proposed by Jeener, to obtain a 2D dependence of the signal on time.

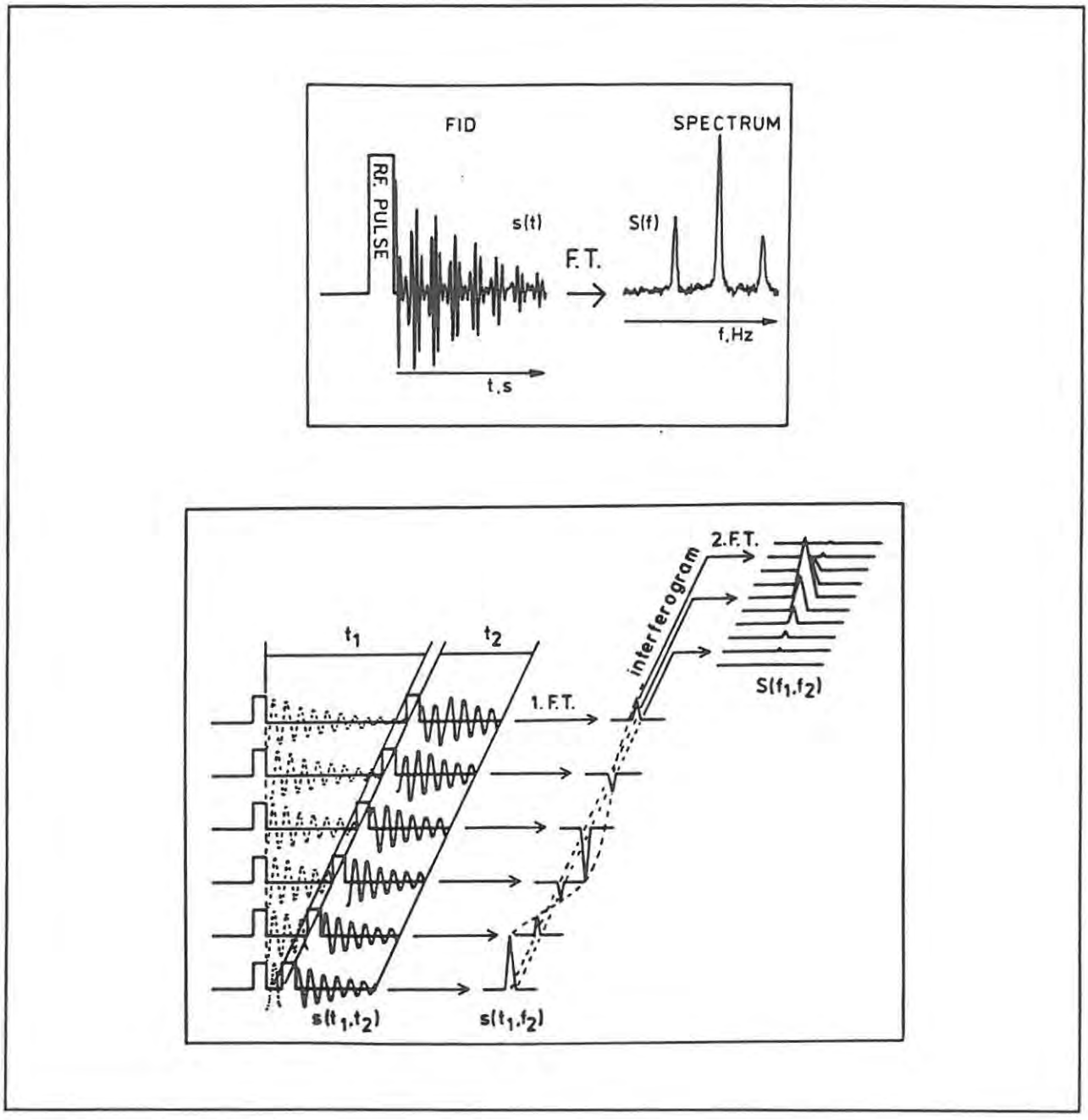


Fig. 3.8. Pulsed 1D n.m.r. measurement and the Jeener experiment[322]

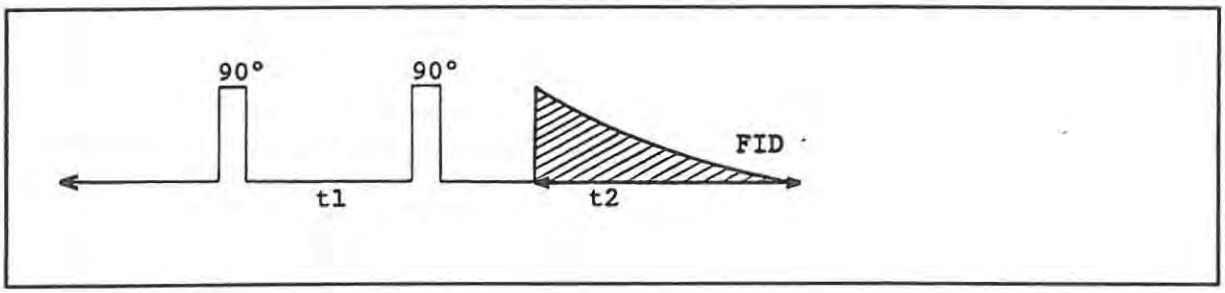
Ernst *et al.*[323] have shown that Jeener's original idea has a much broader application in n.m.r. spectroscopy, resulting in many 2D and now even 3D experiments. The major advantage of these techniques is the vast improvement in spectral resolution which assists greatly in overcoming the hidden resonance problem encountered in 1D n.m.r. spectroscopy. Hence, the researcher is now able to unravel the complicated network of spin systems within large biomolecules such as polysaccharides.

Identification and quantitation of constituent sugar residues

The number of sugar residues can be ascertained by integrating the signals of anomeric protons, which are usually well resolved, in the 1D ^{1}H-spectrum. This information is complemented by the number of anomeric ^{13}C signals in the 1D ^{13}C-spectrum (see section 3.3.1). A through-bond connectivity analysis maps out the number of individual spin systems that correspond to the number of sugar residues in a carbohydrate molecule. This is achieved by performing scalar coupling-correlated (J-correlated) spectroscopy. This takes the form of 2D homonuclear (^{1}H-^{1}H) J-correlated spectroscopy - COSY[182,323,324], RCTCOSY[183](relayed coherence transfer correlated spectroscopy), and TOCSY[325](total correlation spectroscopy) or HOHAHA[193](homonuclear Hartmann-Hahn spectroscopy) - from which all ^{1}H resonances may be assigned. 2D Heteronuclear ^{1}H-^{13}C correlation spectroscopy (HETCOR)[185] permits the assignment of all ^{13}C resonances, from previously determined ^{1}H chemical shifts, of each directly bonded carbon-proton pair. Dabrowski[320] demonstrated the ability to establish remote connectivities from pure-absorption, phase-sensitive COSY (PS-COSY). This lies in the capability of COSY cross-peaks to display the entire coupling information concerning the protons in a saccharide. Proton-proton spin-spin coupling constants ($^{3}J_{H,H}$) can also be obtained from a 1D version of the HOHAHA[326] experiment. These experiments have therefore largely replaced 2D J-resolved spectroscopy for the acquisition of coupling parameters[320(and references therein)]. The sugars can then be identified by comparing the chemical shift and coupling data with literature values[211,327,328] for methyl glycosides.

COSY

The COSY experiment is based on the Jeener[323] sequence of two 90° pulses separated by the evolution period (t1) and followed by the detection period.



The first 90° pulse rotates the equilibrium z magnetization of the protons into the x, y plane (transverse magnetization). During the evolution period the various magnetization components precess with different frequencies which correspond to the lines in the ^{1}H-n.m.r. spectrum. The second 90° pulse (mixing pulse) results in exchange of magnetization between nuclei. If this transfer of magnetization takes place between components belonging to the same protons, then after Ft F1 will equal F2, resulting in "diagonal-peaks". However, transfer between scalar spin-spin coupled protons results in "cross-peaks", which correlate lines belonging to different protons. From these "cross-peaks" one can trace the connectivities of complete spin systems (Fig. 3.9).

When dealing with complex carbohydrates, it is seldom that the spin systems of all sugar residues are traced from a COSY-90 experiment alone, as there is considerable overlap of cross-peaks in the vicinity of the ring proton chemical shifts. Fortunately, other 2D and selective 1D methods are available to resolve this problem.

RCTCOSY

The COSY provides one-step coherence transfer, providing evidence of direct coupling

between nuclei only. Bax and Freeman[182] reported that the introduction of a delay prior to data acquisition enhances the relative intensity of cross-peaks arising from long-range coupling. This is referred to as relayed coherence transfer COSY (RCTCOSY).

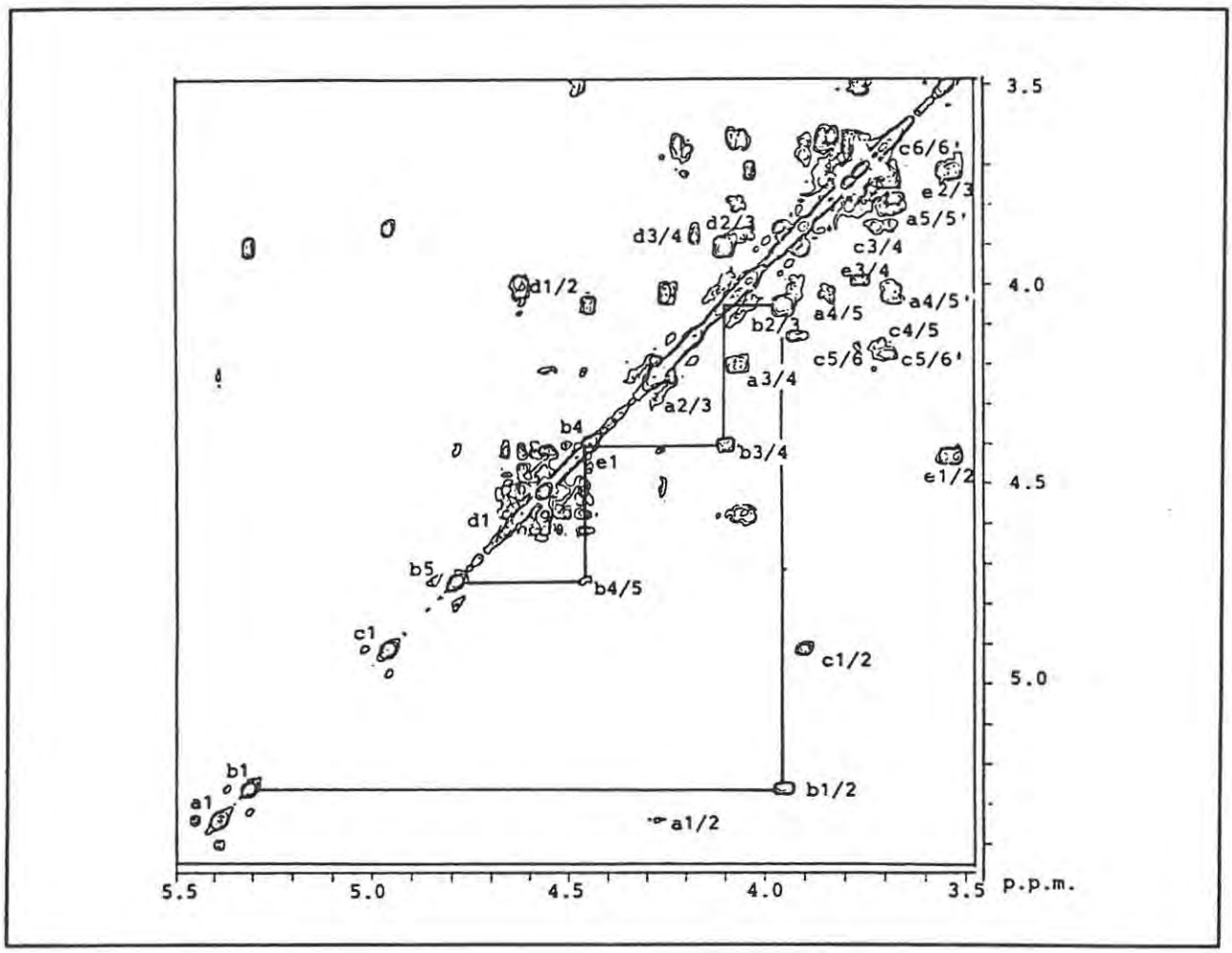
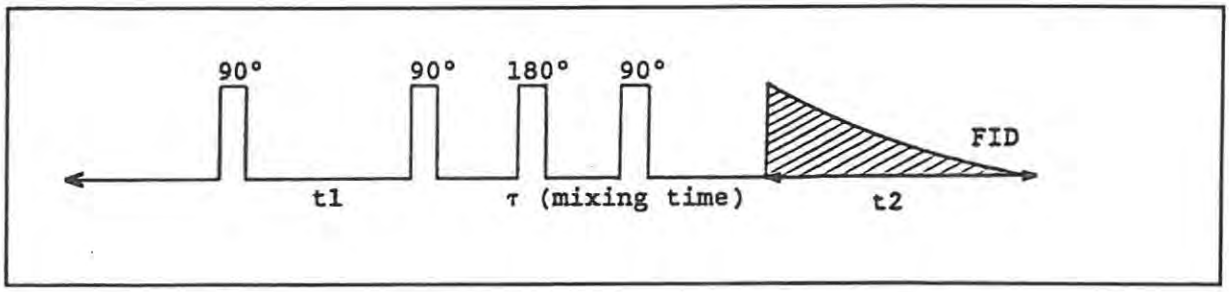


Fig. 3.9. 2D-COSY spectrum of *E. coli* K38 polysaccharide showing the connectivities of H-1 through to H-5 and H-5' of a ß-D-Rib*f* residue

Bax and Drobny[183] later reported on the optimization of 2D homonuclear RCTCOSY (or Relay COSY). The basic pulse scheme of the homonuclear RELAY experiment is as follows:



Magnetization is transferred between noncoupled nuclei using one or more nuclei as mediators. Therefore in a RCT experiment, H-1 of a sugar residue will have a cross-peak with H-2 and H-3. In a doubly relayed (RCT2) experiment we generated H1/2, H1/3 and H1/4 cross-peaks. Similarly, a RCT3 spectrum will display correlation signals for five nuclei, and in a RCT4 spectrum, H-1 of a typical hexopyranose sugar residue can be expected to display correlations along the entire connecting path to H-6 and H-6'. In practice however this is difficult to accomplish as RCT2COSY (double relay) is usually the practical limit of this experiment[318], hence we need to look to other n.m.r. experiments, *e.g.* TOCSY.

TOCSY

Total correlation spectroscopy provides multistep correlations similar to RCTCOSY. However, continuous strong proton irradiation is used which induces strong spin-spin coupling, so that, if the irradiation is maintained for the correct length of time, all coherences from a given spin system will mix[318] (i.e. for each sugar residue). Homonuclear Hartmann-Hahn spectroscopy (HOHAHA)[193,329] is a variation of TOCSY which uses a "spin-lock" or Hartmann-Hahn condition for mixing[330]. The net magnetization transfer obtained permits the recording of phase-sensitive spectra, often giving better resolution and sensitivity than found in COSY experiments. 2D-HOHAHA spectroscopy has been successfully applied by this laboratory in the structural analysis of the *E. coli* K87 capsular polysaccharide[23].

HETCOR

Heteronuclear chemical shift correlation (HETCOR)[185] is a valuable technique in that it combines the good resolution of decoupled ^{13}C-n.m.r. with the ease of interpretation of proton chemical shifts. The experiment involves transferring spin polarization to ^{13}C through one-bond ^{1}H-^{13}C couplings. Hence a 2D HETCOR spectrum contains one peak for each distinct CH group in a molecule, with ^{1}H and ^{13}C chemical shift frequencies in F1 and F2 respectively. HETCOR experiments are routinely utilized in conjunction with the homonuclear correlated experiments, introduced above, in the elucidation of polysaccharide structure[140,33,12,49,18,32,55,23,194].

HMQC

A major limitation of the HETCOR experiment is that the magnetization, which arises as ^{1}H polarization, is detected as the insensitive ^{13}C signal. The experiment therefore requires relatively large amounts of material and makes excessive demands on expensive spectrometer time. This problem has been solved by the development of proton-detected heteronuclear correlation experiments such as HMQC (heteronuclear multiple quantum coherence spectroscopy[331]). Lerner and Bax[165] applied this experiment to a trisaccharide. It has subsequently been applied to capsular polysaccharides of *Haemophilus influenzae*[166] and *E. coli*[61], demonstrating the power of this technique.

Determination of anomeric configuration

Anomeric configuration can be ascertained from the chemical shifts and coupling constants ($^{3}J_{H1,H2}$) of the well resolved anomeric signals in high resolution 1D-^{1}H-n.m.r. spectra, as discussed in more detail in section 3.3.4. When anomeric signals are not well resolved, $^{3}J_{H1,H2}$ values can be obtained from PSCOSY, as described by Dabrowski[320] for oligosaccharides, or from the 1D version of the 2D-HOHAHA experiment, as described by Bax and Davis[326]. In the 1D HOHAHA experiment subspectra

of each spin system (i.e. each sugar residue) are generated by inverting the resonances of an isolated spin multiplet with a selective 180° rf pulse and allowing this magnetization to propagate through the ^1H coupling network. This experiment was applied to a hexasaccharide isolated from the bacteriophage degradation of the *Klebsiella* K15 polysaccharide (Fig. 3.10).

Linkage and sequence analysis

Sequence and linkage sites of saccharides can be established from nuclear Overhauser enhancement (n.O.e.) signals generated by through-space dipolar interactions between

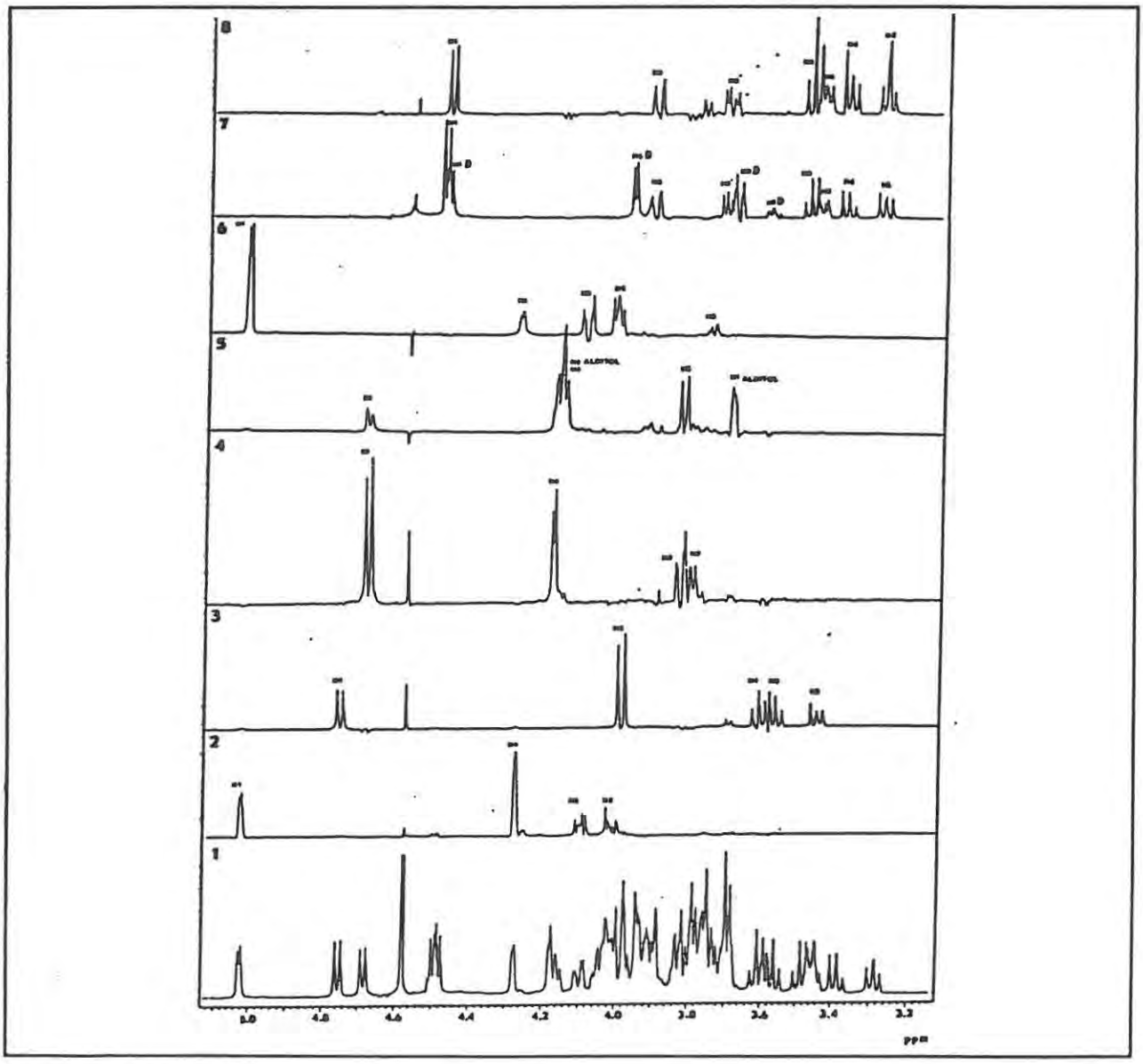


Fig. 3.10. 1D-HOHAHA spectra of *Klebsiella* K15 P1-alditol showing the spectrum of P1-alditol (1) and subspectra of individual spin systems (2-8)

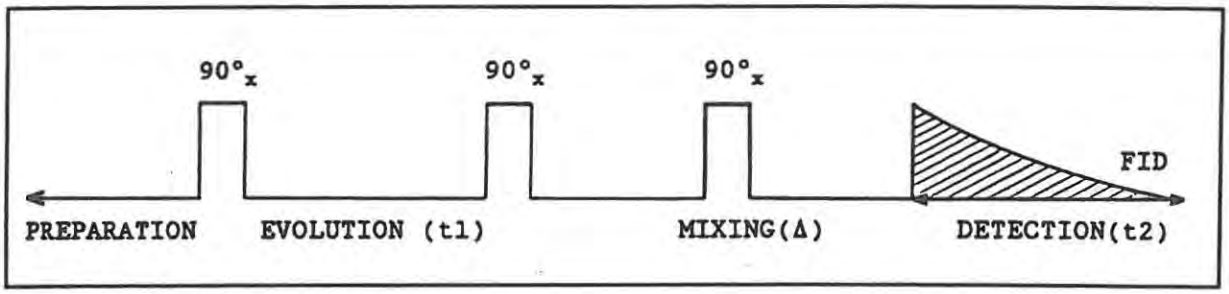
the anomeric and the transglycosidic protons[320]. 2D Nuclear Overhauser enhancement spectroscopy (2D-NOESY), besides providing sequence and linkage information, confirms ^{1}H assignments and anomeric configuration. Dua *et al.*[332] found some uncertainty in the use of n.O.e. to determine linkage positions as H-4 of a 3-glycosylated Gal residue showed greater n.O.e. than H-3 itself. Dabrowski[320] contends that this is of no consequence because if the galactose residue is 4-linked, only the 1,4 interglycosidic n.O.e. would be observed as H-3 is axial. Furthermore, in all recorded cases, a 3-linked galactose residue has resulted in either a 1/3- or 1/3- and 1/4-interglycosidic n.O.es and never just the 1/4-n.O.e. Confirmation of linkage positions is also provided by the downfield glycosylation shifts of 4-10 p.p.m. of the respective ^{13}C signals[327].

A 2D heteronuclear multiple-bond correlation (HMBC) experiment has also been applied to obtain sequence and linkage information[166]. This experiment is based on chemical shift correlations which depend on two- or three-bond couplings, *e.g.* , the coupling between a proton and a carbon nucleus across an *O*-linked glycosidic linkage. This is a ^{1}H-detected experiment, which therefore is very sensitive, and does not make excessive demands on spectrometer time. It has largely replaced the 1D-selective INEPT experiment (insensitive nuclei enhanced by polarization transfer) introduced by Morris and Freeman[333], which also relies on long-range scalar coupling interactions.

NOESY

All the experiments discussed above are based on coherent magnetization transfer between spin-spin coupled nuclei (through-bond coupling). 2D NOESY, on the other hand, is a form of 2D exchange spectroscopy involving magnetization transfer via dipole-dipole interactions (through-space coupling). The n.O.e. is measured as a fractional change in intensity, by cross-relaxation, of one n.m.r. line when another resonance is perturbed by irradiation[322]. The n.O.e. is inversely proportional to

the sixth power of the internuclear distance, providing information on the spatial proximity of observed and perturbed nuclei. When measuring 2D n.O.es relaxation delays of three to five times T1 are necessary. The technique is therefore better suited to macromolecules (*e.g.* polysaccharides) having small T1 values than to small molecules (*e.g.* oligosaccharides) with large T1 values.



The first $90°_x$ pulse produces transverse magnetization which precesses during the evolution time (t1), as with COSY. The $90°_x$ mixing pulse is then followed by the delay Δ to permit magnetization transfer arising from dipolar interactions. The third $90°_x$ pulse transforms the resulting magnetization into transverse magnetization, which is measured during the detection phase. It is this exchange of magnetization due to dipolar interaction between nuclei which shows up as cross peaks in the 2D NOESY spectrum. The information provided by the 2D NOESY experiment complements that of COSY.

3.4.4.(ii). ^{31}P-N.m.r. spectroscopy

Several of the *E. coli*. capsular polysaccharides have been shown to contain phosphate (Table 3.1). Jann *et al.*[54] were able to determine the presence of a phosphodiester bridge in the native K51 antigen from a ^{31}P-n.m.r. spectrum. Using external phosphoric acid as the reference, the only other signal was at -1.21 p.p.m., indicative of a monophosphodiester, as phosphomonoesters show a signal between 3-4 p.p.m. They were also able to determine the linkage position of the phosphate to N-acetylglucosamine from the 2- and 3-bond ^{31}P-^{13}C coupling constants. Recently, these techniques were applied to the *E. coli*. K24 antigen[38], which is a

polymer consisting of α-Kdo*p* and glycerol phosphate.

Byrd *et al.*[166] utilized a ^{1}H-detected, ^{1}H-^{31}P HMQC 2D experiment, to determine the phosphoric diester linkage sites in the *Haemophilus influenzae* Type a capsular polysaccharide. In this experiment protons were correlated with phosphorous *via* long-range ^{1}H-^{31}P scalar coupling. ^{31}P-N.m.r. spectroscopy adds another dimension to the power of n.m.r. spectroscopy in structural analysis.

3.4.4.(iii). Computer-assisted structural analysis of polysaccharides on the basis of n.m.r. data

A computer programme, CASPER (Computer Assisted Spectrum Evaluation of Regular polysaccharides), has been developed by P-E. Jansson *et al.*[334] for the determination of the structure of polysaccharides composed of regular repeating-units. The program is based on the ^{13}C-n.m.r. chemical shift data and information from glycose and methylation analysis. The database contains ^{13}C-n.m.r. chemical shifts obtained from disaccharides and simulates spectra for all possible monosaccharide sequences. The best fit with the observed spectrum of the polysaccharide in question, is chosen. The potential of CASPER to predict polysaccharide structures has been demonstrated[334], however, the structures proposed cannot be taken as conclusive and the database cannot support branched structures. A similar computer program has been described by Knirel *et al.*[335].

polymer consisting of α-Kdo*p* and glycerol phosphate.

Byrd *et al.*[166] utilized a ^{1}H-detected, ^{1}H-^{31}P HMQC 2D experiment, to determine the phosphoric diester linkage sites in the *Haemophilus influenzae* Type a capsular polysaccharide. In this experiment protons were correlated with phosphorous *via* long-range ^{1}H-^{31}P scalar coupling. ^{31}P-N.m.r. spectroscopy adds another dimension to the power of n.m.r. spectroscopy in structural analysis.

3.4.4.(iii). Computer-assisted structural analysis of polysaccharides on the basis of n.m.r. data

A computer programme, CASPER (Computer Assisted Spectrum Evaluation of Regular polysaccharides), has been developed by P-E. Jansson *et al.*[334] for the determination of the structure of polysaccharides composed of regular repeating-units. The program is based on the ^{13}C-n.m.r. chemical shift data and information from glycose and methylation analysis. The database contains ^{13}C-n.m.r. chemical shifts obtained from disaccharides and simulates spectra for all possible monosaccharide sequences. The best fit with the observed spectrum of the polysaccharide in question, is chosen. The potential of CASPER to predict polysaccharide structures has been demonstrated[334], however, the structures proposed cannot be taken as conclusive and the database cannot support branched structures. A similar computer program has been described by Knirel *et al.*[335].

4. STRUCTURAL ANALYSIS OF SOME *E. COLI* CAPSULAR (K) ANTIGENS

4.1. GENERAL EXPERIMENTAL PROCEDURES

The following general procedures were followed in the experimental work presented in sections 4.2, 4.3, 5.1 and 5.2. Additional methods will be discussed in detail under the relevant sections.

Isolation and purification of acidic capsular polysaccharides

Authentic cultures of the *E. coli* serotypes studied were obtained from Dr. I. Ørskov of the WHO's designated *Escherichia* centre in Copenhagen. The bacteria were subcultured on Mueller-Hinton agar (Biolab) by incubation at 37° overnight. Individual colonies were picked, transferred to 5 ml sterile Mueller-Hinton broth (Merck) and incubated in a waterbath-shaker at 37° for 6 to 8h. These broth cultures were used for the large-scale growth of the bacteria on Mueller-Hinton agar (1.0 L) in 6-12 stainless steel trays (40 x 60 cm). The bacteria were spread over the surface of the culture medium by means of a sterile glass rod and incubated at 37° for 36-72h. The cells and slime were harvested from the surface of the agar in each tray, pooled, then suspended in an equal volume of 2% aqueous phenol. This suspension was stirred overnight at 4° to extract the capsular material. The cells were removed by ultracentrifugation (35k r.p.m., 3h) and the supernatant was slowly added to 5 volumes of ethanol to precipitate the polysaccharides. These polysaccharides were recovered by centrifugation (2000 r.p.m., 20 min), were washed with ethanol, and then redissolved in a minimum volume of water. A 5% aqueous cetyltrimethylammonium bromide (CTAB) solution was slowly added to the stirred polysaccharide solution until no further precipitation of the acidic capsular polysaccharide-CTAB complex occurred. The precipitate was isolated by centrifugation (2000 r.p.m., 20 min), was washed with water, and was then dissolved in the minimum volume of aqueous 3M NaCl to break the complex. The acidic polysaccharide was precipitated from this solution by

slowly adding it to 5 volumes of ethanol. The precipitate was isolated by centrifugation (2000 r.p.m., 20 min), was washed (ethanol), and then dissolved in a minimum volume of water. The polysaccharide solution was dialyzed exhaustively against running tap-water (1200-1400 Mw cutt-off, 3d). The retentate was ultracentrifuged (40k r.p.m., 1h) and the supernatant was lyophilized to yield acidic capsular polysaccharide material.

All culture media were sterilized by autoclaving at 121° for 20 min and the bacteria were checked for the presence of capsule at each step by phase contrast microscopy of the cells mounted in Indian ink.

Glycose analysis

Unless otherwise stated, all solutions were evaporated under diminished pressure on a waterbath at temperatures not exceeding 40°. A sample of polysaccharide (~1 mg) was hydrolyzed in 4M trifluoroacetic acid (~1 mL) at 125° for 1h, under nitrogen, in a sealed reactivial. The acid was co-evaporated with water and the released monosaccharides were identified by g.l.c. analysis of the derived peracetylated aldononitriles (PAAN's) or alditol acetates. PAAN's were prepared by the method[145] of McGinnis and alditol acetates by reduction of the hydrolyzate with sodium borohydride (1h) in water, followed by acetylation in 1:1 acetic anhydride/pyridine (1h, 100°). To identify uronic acid residues, a sample of the polysaccharide (~3 mg) was methanolyzed with anhydrous 3% methanolic HCl at 80° overnight. After neutralization of the acid (Ag_2CO_3), the methanolyzate, in anhydrous methanol, was carboxyl reduced overnight with sodium borohydride (excess), then hydrolyzed (4M TFA, 1h, 125°) and the sugar residues analyzed as above. Oligosaccharides were analyzed by the Morrison method[336] in which the reducing sugar is identified by formation of the oligosaccharide alditol ($NaBH_4$, water) prior to hydrolysis, derivatization (PAAN's)[145] and g.l.c. analysis.

Methylation analysis

Samples of polysaccharide or oligosaccharide alditols (produced by reduction with $NaBD_4$) were methylated by the Hakomori[80] procedure as modified by Phillips and Fraser[124]. A dried sample in the free acid form was dissolved in anhydrous DMSO (10 mg saccharide/mL) with stirring under dry nitrogen. Dissolution of high molecular weight polysaccharides was aided by ultrasonic agitation and heat (60°). Potassium methylsulfinyl (dimsyl) anion was added (half of DMSO volume) and the gel that formed was dispersed with stirring and intermittent ultrasonication (1h, room temperature). The reaction mixture was cooled on ice and an excess of methyl iodide (half of DMSO volume) was slowly added with stirring. The reaction mixture was allowed to come up to room temperature and was stirred for a further 1h prior to being quenched with water. Methylated material was recovered by dialysis (12000-14000 Mw cut-off) followed by freeze-drying (polysaccharides) or by extraction with dichloromethane (oligosaccharides). Purification was achieved by passing the material through a column of Sephadex LH20 using chloroform as eluent. Partially methylated material was subjected to a Kuhn methylation[144] to ensure complete etherification. The material was dissolved in dimethylformamide (DMF, 0.5 mL) and was stirred in the presence of methyl iodide (1.0 mL) and silver oxide for 48h, with two further additions of methyl iodide and silver oxide at 12h intervals. The permethylated product was isolated after removal of the silver salts (centrifugation) and DMF (high vacuum, 70°), and was then purified as above. The permethylated product was analyzed as follows: A dry sample was methanolyzed in refluxing 3% methanolic HCl (80°, 16h) and after neutralization (Ag_2CO_3) the methanolyzate was split into two portions. One portion was hydrolyzed (4M TFA, 1h, 125°), reduced ($NaBH_4$, water) and acetylated with 1:1 acetic anhydride/pyridine (1h, 100°). The other portion was reduced ($NaBH_4$, anhydrous methanol, 24h), hydrolyzed, reduced and acetylated as above. The partially methylated alditol acetate (p.m.a.a.) derivatives were analyzed by g.l.c. and g.l.c.-m.s. as described below.

Determination of absolute configuration

The absolute configuration of the sugar residues was determined by the method of Leontein *et al.*[112]. A sample of the polysaccharide (~8 mg) was first carboxyl reduced (as above), then hydrolyzed (4M TFA, 1h, 125°) and the aqueous solution of the hydrolyzate was transferred to a 2 mL glass ampoule. The monosaccharides were recovered by freeze-drying and were then suspended in (-)-2-octanol (0.5 mL) with the aid of ultrasonic agitation. A small teflon coated stirrer bar and a drop of TFA were added, the ampoule was flushed with dry nitrogen and sealed. The reaction was then stirred for 16h at 130°. After removal of the excess (-)-2-octanol under vacuum at 55°, the residue was acetylated (pyridine-acetic anhydride) and the resulting peracetylated (-)-2-octyl glycosides were analyzed by g.l.c.

N-acetylation

Amino sugars were N-acetylated prior to formation of the (-)-2-octyl glycosides. The hydrolyzate was dissolved in 0.5 mL water and 2 mL each of saturated $NaHCO_3$ and 5% acetic anhydride were added. The reaction was held in ice (~5 min). A further 2 mL each of saturated $NaHCO_3$ and acetic anhydride were added and the reaction was left to stand overnight (16h, room temperature). The reaction was quenched with cation exchange resin (Amberlite IR 120 H^+) and the monosaccharides were recovered after concentration to dryness.

Gas-liquid chromatography (g.l.c.)

Analytical g.l.c. was performed using a Hewlett-Packard 5890A gas chromatograph, fitted with flame-ionization detectors and a 3392A recording integrator, with helium as carrier gas. A J & W Scientific fused silica DB-17 bonded-phase capillary column (30 m x 0.25 mm) having a film thickness of 0.25 μm was used for separating p.m.a.a's. (program I), alditol acetates (program II) and peracetylated (-)-2-octyl glycosides of amino sugars (III). A J & W Scientific DB 225 bonded-phase capillary column (30 m x 0.25 mm) having a film thickness of 0.25 μm was

used for separating PAAN's (230° isothermal) and peracetylated (-)-2-octyl glycosides of neutral sugars (220° isothermal). A J & W Scientific fused-silica DB-WAX bonded-phase capillary column (30 m x 0.25 mm) having a film thickness of 0.15 μm was used to separate the alditol acetates of N-acetyl mannosamine, N-acetyl glucosamine and N-acetyl galactosamine (240° isothermal). The temperature programs used were I, 180° to 240° at $3°.min^{-1}$; II, 180° for 1 min, then $3°.min^{-1}$ to 250°; and III, 180° for 2 min, then $3°.min^{-1}$ to 240°. The identities of all derivatives were confirmed by g.l.c.-m.s. on a Hewlett-Packard 5988A g.l.c.-mass spectrometer using the appropriate column with an ionization energy of 70 eV and an ion-source temperature of 200°.

Nuclear magnetic resonance spectroscopy

N.m.r. spectra were recorded on either a Bruker WM-500 or a Bruker AMX-400 spectrometer, using standard Bruker software. Samples were deuterium exchanged several times by freeze-drying from D_2O, and were examined as solutions in 99.99% D_2O (0.45 mL) containing a trace of acetone as internal standard (δ 2.23 for ^1H and 31.07 p.p.m. for ^{13}C).

Gel-permeation chromatography

G.p.c. was performed using a Pharmacia FRAC-100 fraction collector, a P-1 peristaltic pump with a constant pressure Mariotte flask as the eluent reservoir, with a Waters differential refractometer (Model R401) and a Rikadenki flat bed recorder (Model R-01). Analytical g.p.c. profiles for polysaccharides were obtained using dextran calibrated columns (1.6 x 65 cm) of Sephacryl S400 HR or Sephacryl S500 using 0.1 M sodium acetate buffer (pH 5.0) as eluent (20 mL/h). Semi-preparative g.p.c. was performed on columns (2.6 x 65 cm) of Sephacryl S400 HR (30 mL/h) for polysaccharides and (2.5 x 90 cm) Biogel P4 (20 mL/h) for oligosaccharides (d.p. 3-12) using the same mobile phase. Partially methylated oligosaccharide anhydroalditols were separated on a column of Sephadex LH20 (1.0 x 45 cm), with methanol as eluent, at a flow rate of 9 mL/h.

Ultracentrifugation

Ultracentrifugation was performed using a Beckman L8-80M ultracentrifuge with a type 70 Ti rotor (35-40k r.p.m.) for large volumes, a type 70.1 Ti rotor (40k r.p.m.) for small volumes and a type SW28 (25k r.p.m.) for large volumes not requiring > 28 000 r.p.m.

4.2. THE STRUCTURAL ELUCIDATION OF THE CAPSULAR POLYSACCHARIDE OF *E. COLI* 09:K35 (A104a)

4.2.1. Introduction

The capsular antigens of *E. coli* have been divided into two groups, Group I and Group II, depending on their thermal lability[24], or their physical, chemical and microbiological characteristics[25] (section 2). The group I antigens are high molecular weight acidic polysaccharides, are heat stable at pH5-6, and may contain amino sugars[4] (section 4.3). The capsular polysaccharide of *E. coli* 09:K35 (A104a) has been found to belong to a subgroup of I whose capsules do not contain amino sugars and which closely resemble those of *Klebsiella*. Of the 54 *E. coli* capsular antigens reported to date[337] only ten serotypes (K26, K29, K30, K31, K32, K34, K36, K37, K39 and K55) belong to this subgroup. The structure of the *E. coli* K35 repeating-unit, which we now report, is almost identical to that of *Klebsiella* K13[338], the only difference being the absence of the 1-carboxyethylidene acetal in the K35 repeating-unit.

4.2.2. Results and Discussion

Composition and n.m.r. spectra

E. coli K35 bacteria were grown on an agar medium and the acidic polysaccharide was isolated and purified by the method of Altman and Dutton[7] (section 4.1). The purified polysaccharide showed a broad distribution of molecular weights (> 2×10^7 - 1×10^6) in gel-permeation chromatography on Sephacryl S500, with an average M_r at 10^7.

G.l.c. analysis of the acetylated aldononitriles derived from the products of an acid hydrolysate of the polysaccharide, with and without prior reduction of the acidic function, indicated that it is composed of glucose, mannose, galactose and

glucuronic acid in the molar ratio 2:1:1:1. All the sugars were shown to have the D configuration by g.l.c. of their acetylated (-)-2-octyl glycosides[112]. Preliminary n.m.r. investigations were unsuccessful. This was attributed to the presence of a large amount of impurities in the isolated material, evident as a white precipitate when the material, in the free acid form, was dissolved in D_2O. In the ^1H-n.m.r. spectrum there was no evidence of the presence of acid or base labile non-carbohydrate substituents (*O*-acetyl, 1-carboxyethylidene acetal). The material was therefore treated with acid (1% acetic acid, 60°, 1h), then with base (0.25M NaOH, 60°, 1h) and finally subjected to autolysis (water, 100°, 15 min). ^1H-n.m.r. spectra of the material thus treated, either in the sodium or acid form, were not well resolved.

A considerable amount of the capsular material did not form a complex with CTAB during the isolation procedure. This material was isolated as a mixture with the neutral polysaccharide portion of the O9 antigen. In order to remove any protein contaminants the polysaccharides (O9:K35) were treated with a protease enzyme in a phosphate buffer (see experimental). In an attempt to separate the neutral and acidic polysaccharides the following experiments were performed: G.p.c. on Sephacryl S400 HR; treatment with CTAB; acid treatment (as above) followed by treatment with CTAB; ion-exchange g.p.c. (DEAE Sepharose 6B-CL) [Fig. 4.1]. G.p.c. (Sephacryl S400 HR) and the additional attempts at CTAB complexation were not successful. The ion-exchange g.p.c. was relatively successful and n.m.r. spectra of this material were of a reasonable quality.

Previous work on the *E. coli* K35 serotype in this laboratory demonstrated inconsistent capsule expression. Anderson[18] demonstrated that expression of the *E. coli* K55 capsular polysaccharide was dependent on incubation time. Despite applying the short (36h) incubation times suggested, two subsequent isolates of the *E. coli* K35 serotype yielded a galactan and a 50:50 mixture of capsular polysaccharide and galactan. The fourth large scale growth of the bacteria was

initiated from a culture of *E. coli* K35 on a nutrient agar slope covered with sterile mineral oil which had been kept at 4° for one year. A good yield (1100 mg) of acidic polysaccharide was isolated and the n.m.r. spectra of this material were well resolved.

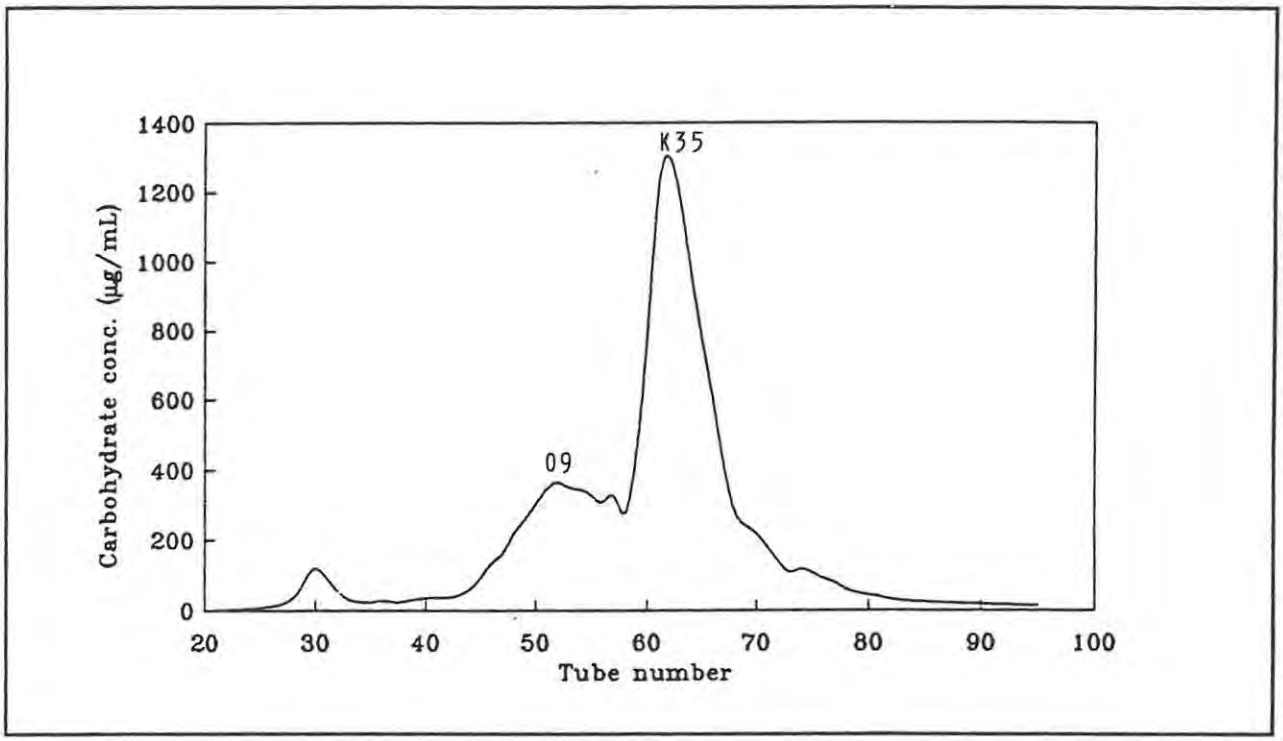


Fig. 4.1. DEAE Sepharose 6B-CL gel permeation profile of *E. coli* 09/K35 mixture

The ^{1}H-n.m.r. spectrum (Table 4.1) of an acid treated (see experimental) sample of the polysaccharide showed, *inter alia*, the presence of five anomeric protons corresponding to two α-linkages (δ 5.30 and 5.23) and three ß-linkages (δ 4.79, 4.55 and 4.49). The unresolved doublet at δ 4.79 was assigned to H-1 of a ß-D-mannopyranosyl residue. The ^{13}C-n.m.r. data showed four anomeric signals at 103.23, 102.09, 100.62 and 99.90 p.p.m. However, from the intensity of the signal at 103.23 p.p.m. it was taken to be representative of two overlapping anomeric carbon resonances, hence the ^{13}C-n.m.r. data were in agreement with the 1H-n.m.r. results.

Table 4.1. ^{1}H-N.M.R. data for *E. coli* K35 polysaccharide and derived products

Compound	δ[a] (p.p.m.)	^{3}J (Hz)	Integral (No. of ^{1}H)	Assignment[c]
K35 Polysaccharide[d]	5.30	3.7	1	4-α-Glc
	5.23	3.8	1	4-α-GlcA
	4.79	n.o.[b]	1	3,4-ß-Man
	4.55	7.9	1	3-ß-Glc
	4.49	7.6	1	ß-Gal
	4.51	9.2	1	H-5 of α-GlcA
P1	5.38	3.9	0.4	4-α-Glc-(1→3)-α-Glc
	5.35	3.9	0.6	4-α-Glc-(1→3)-ß-Glc
	5.29	3.9	1	4-α-GlcA
	5.24	3.8	0.4	3-α-Glc
	4.79	n.o.	0.4	3-ß-Man
	4.78	n.o.	0.6	3-ß-Man
	4.67	8.0	0.6	3-ß-Glc
	4.48	7.8	1	ß-Gal
	4.47	9.7	1	H-5 of 4-α-GlcA
P1-alditol (Contaminated with unreduced P1)	5.30	3.8	1	4-α-GlcA
	5.14	3.9	1	4-α-Glc
	4.79	n.o.	1	3-ß-Man
	4.50	9.3	1	H-5 of 4-α-GlcA
	4.48	7.7	1	ß-Gal

P2	5.37	3.8	0.4	4-α-Glc
	5.35	3.8	0.6	4-α-Glc
	5.30	3.8	1	4-α-Glc
	5.28	3.9	1	4-α-GlcA
	5.24	3.8	0.4	3-α-Glc
	5.20	3.9	1	4-α-GlcA
	4.79	n.o.	0.4	3,4-ß-Man
	4.78	n.o.	0.6	3,4-ß-Man
	4.78	n.o.	1	3-ß-Man
	4.67	8.0	0.6	3-ß-Glc
	4.54	8.0	1	3-ß-Glc
	4.47	7.5	1	ß-Gal
	4.46	7.5	1	ß-Gal
	4.41	10.0	1	H-5 of 4-α-GlcA
	4.40	10.0	1	H-5 of 4-α-GlcA

[a] Chemical shift relative to internal acetone at δ 2.23.

[b] Not observed

[c] 4-α-Glc connotes the anomeric proton of a 4-linked α-D-glucopyranosyl residue, etc. The absence of a numerical prefix indicates a terminal non-reducing group.

[d] Recorded at 70°

Methylation analysis

The native polysaccharide was methylated and the alditol acetates prepared from an acid hydrolysate of the methylated polysaccharide, with and without reduction of the methoxycarbonyl function. G.l.c. and g.l.c.-m.s. analysis of the derived p.m.a.a. derivatives gave the results shown in Table 4.2, columns I and II.

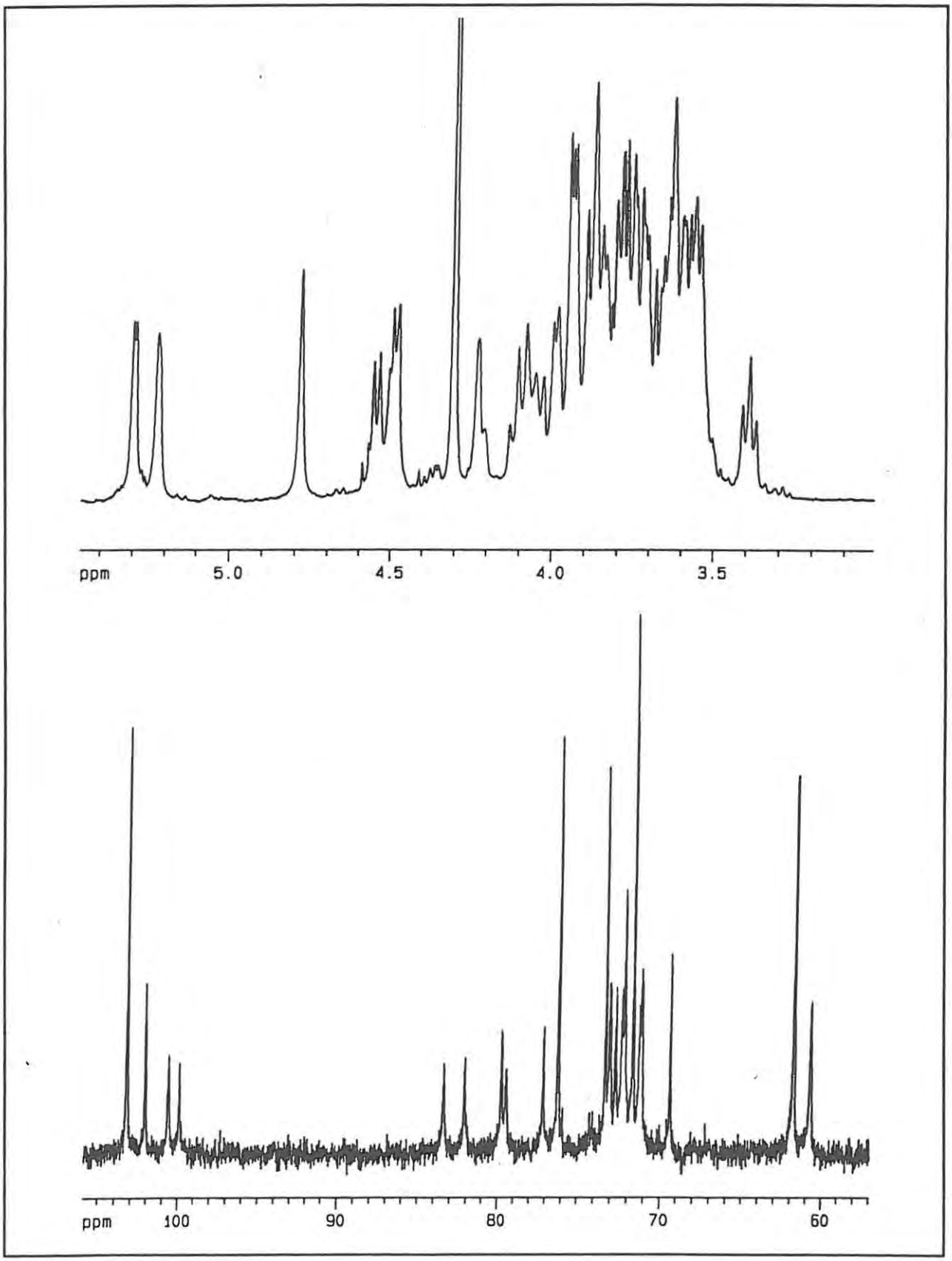


Fig. 4.2. 400-MHz ^{1}H- and ^{13}C-n.m.r. spectrum of the *E. coli* K35 capsular polysaccharide at 70°

The results show that galactose occurs as a terminal group, glucuronic acid is linked through *O*-4, mannose is linked through *O*-3 and *O*-4 (branch point), one glucose is linked through *O*-4, and the other through *O*-3. These data confirm that the polysaccharide has a pentasaccharide repeating-unit.

Table 4.2. Methylation analysis of *E. coli* K35 polysaccharide and derived products

Methylated sugar[a] (as alditol acetate)	Molar ratio[b,c]						
	I	II	III	IV	V	VI	VII
1,2,4,5,6 - Glc			0.47	0.62			
2,3,4,6 - Gal	0.40	0.34	0.33	1.44	0.14	0.14	0.13
2,4,6 - Glc	1.00	0.78		1.00	0.96	0.87	0.63
2,4,6 - Man			0.73	0.94			
2,3,6 - Man					0.38	0.57	0.68
2,3,6 - Glc	1.00	1.00	1.00	2.15	1.00	1.00	1.00
2,6 - Man	0.79	0.84		0.91	0.55	0.38	0.21
2,3 - Glc		0.31	0.44	1.52			

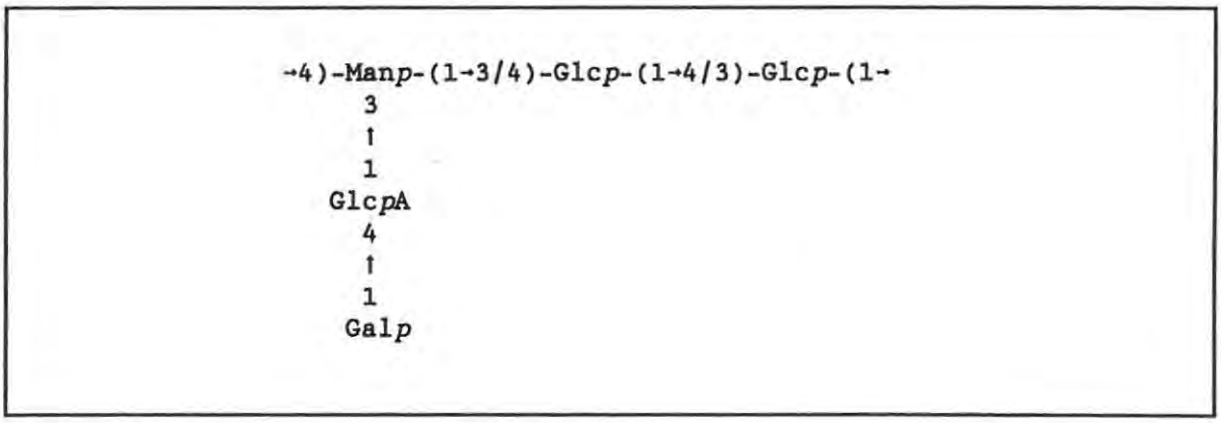
[a] 1,2,4,5,6-Glc = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylglucitol, etc.; all substitution patterns were confirmed by g.l.c.-m.s.

[b] Determined on a DB-225 capillary column at 210°, molar ratios corrected using molar response-factors according to the equal weight-response theory[339].

[c] I, methylated K35 polysaccharide; II, methylated, carboxyl-reduced K35 polysaccharide; III, methylated, carboxyl-reduced P1-alditol; IV, methylated, carboxyl-reduced P2-alditol; V, base-degraded (1h) methylated polysaccharide, re-methylated; VI, base-degraded (4h) methylated polysaccharide, re-methylated; VII, base-degraded (6h) methylated polysaccharide, re-methylated. Carboxyl-reduction, where indicated, was performed after methylation.

Uronic-acid degradation[178]

The methylated polysaccharide was subjected to a series of partial ß-elimination reactions (see experimental section 4.2.4) using potassium dimsyl as base[124]. It was thought that the rate of the reaction with potassium dimsyl would be faster than that with sodium dimsyl due to the greater basicity of the former, therefore the reaction was allowed to proceed for 1, 4 and 6h, respectively, and the alditol acetates derived from acid hydrolysates of the re-methylated products were analyzed by g.l.c.-m.s. (Table 4.2, columns V, VI and VII). The concomitant decrease in the yield of 2,6-di-*O*-methylmannose and 2,3,4,6-tetra-*O*-methyl galactose and the production of 2,3,6-tri-*O*-methylmannose demonstrate that the glucuronic acid is linked to *O*-3 of the mannose and that the terminal galactose residue is linked to the uronic acid. The following partial structure can therefore be written for the repeating-unit:



Bacteriophage-mediated degradation of K35 polysaccharide

A bacteriophage which infects *E. coli* K35 (Φ35) was isolated from sewage, was purified by successive single plaque pickings and propagated on the host bacteria in Luria-Bertani broth. The bacteriophage suspension was added to a solution of impure K35 polysaccharide from batches 1 and 3 (450 mg) and the degradation was performed as described (section 4.2.4). G.p.c. (Fig. 4.3) of a dialysate of the digest yielded two pure oligosaccharides, P1 and P2. The degree of polymerisation (d.p.) was determined for each oligosaccharide by the Morrison

method[336] and indicated that P1 and P2 were penta- and deca-saccharides, respectively. Furthermore, P1 and P2 had a glucose reducing terminus and were composed of glucose, galactose, mannose, and glucuronic acid in the same ratios as in the native polysaccharide. N.m.r. spectroscopy (Table 4.1) and methylation analysis (Table 4.2) confirmed the above results.

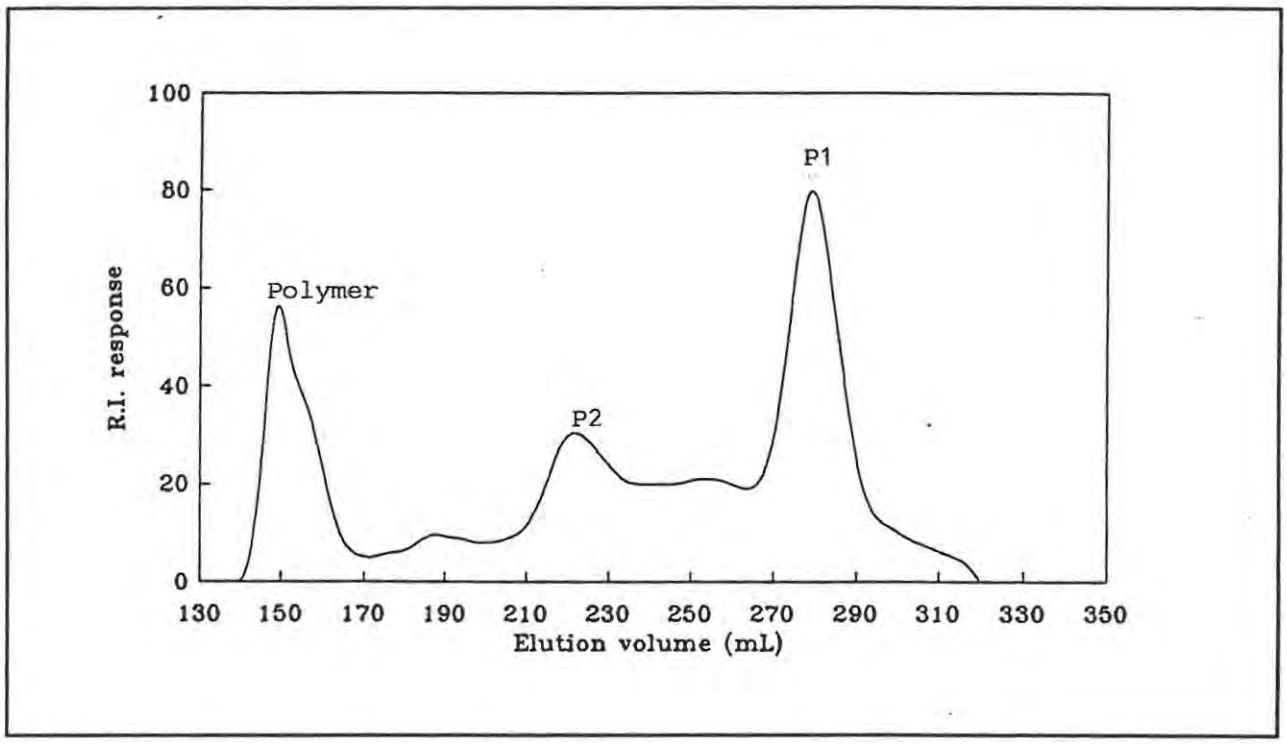


Fig. 4.3. Biogel P4 gel permeation profile of the products of the bacteriophage degradation of the *E. coli* K35 capsular polysaccharide

Methylation analysis.- P1 and P2 were reduced to their respective alditols with sodium borodeuteride and then methylated. The p.m.a.a's. prepared from hydrolysates of the permethylated, carboxyl-reduced P1- and P2-alditols were analyzed by g.l.c. and g.l.c.-m.s. The results for P1-alditol (Table 4.2, column III) show a terminal galactose, glucuronic acid linked through *O*-4, glucose linked through *O*-4, mannose linked through *O*-3, and a glucitol linked through *O*-3. Comparing these results with those for P2-alditol (Table 4.2, column IV), the polysaccharide (Table 4.2, column II), and the base degraded polysaccharide demonstrates that the bacteriophage borne enzyme cleaved →3)-Glc*p*-(1→4)-Man*p*-(1→

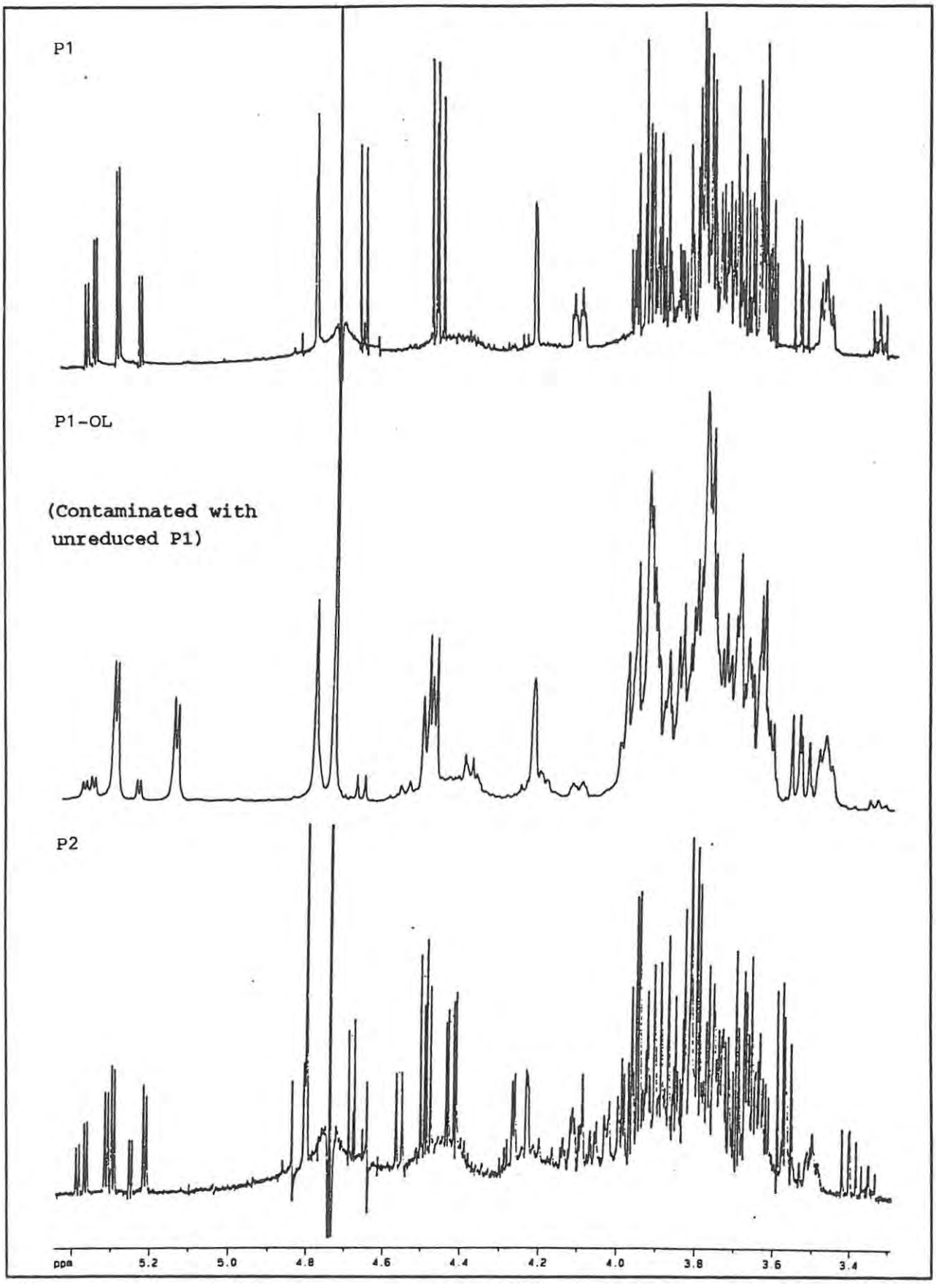
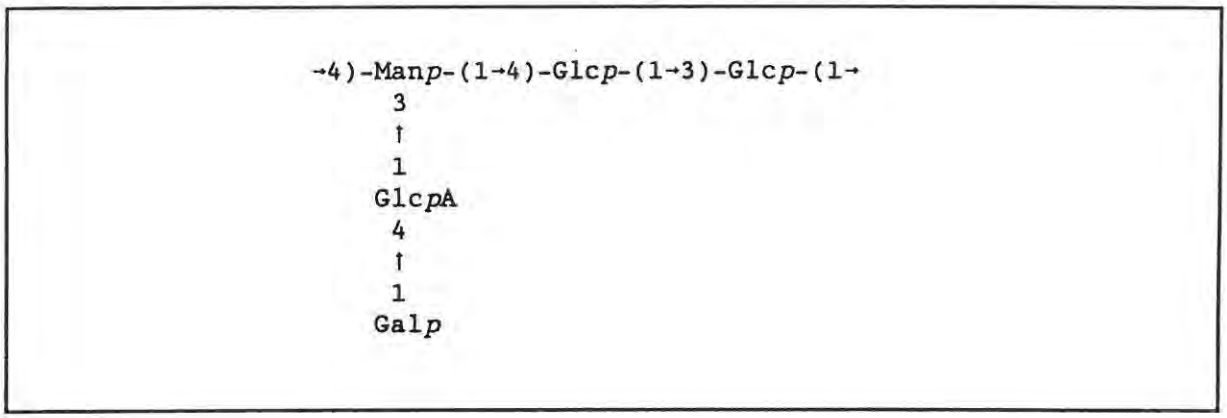


Fig. 4.4. 500 MHz ^{1}H-n.m.r. spectra of *E. coli* K35 P1, P1-OL and P2

linkages in the native K35 polysaccharide and permits the following sequence to be written for the repeating-unit of K35 polysaccharide:



P1 and P2 are, therefore, representative of a single and double repeating-unit of the K35 polysaccharide, respectively.

Comparison of the ^{1}H-n.m.r. data (Table 4.1 and Fig. 4.4) shows that the ß-anomeric signal at δ 4.55 in the spectrum of the polysaccharide has been replaced by partial signals at δ 5.24 and δ 4.67 in the spectrum of P1. This demonstrates that the 3-linked glucose residue cleaved by the phage enzyme has the ß configuration. The absence of a ß-anomeric signal at δ 4.55 in the spectrum of P1-alditol supports the above assignment.

Two further sets of partial signals are observed in the ^{1}H spectrum of P1. These are due to the α and ß anomers of P1 present in solution, caused by mutarotation of the reducing glucose residue. The set of partial signals at δ 5.38 and δ 5.35 (resolved doublets) were assigned to H-1 of the 4-linked α-D glucopyranosyl residue which is linked to *O*-3 of the reducing glucose residue. The partial signals at δ 4.79 and δ 4.78 (unresolved doublets) were therefore assigned to the 3,4-linked-ß-D-mannopyranose residue because this residue is linked to the α-D-glucopyranose residue through which it experiences the twinning effect of the mutarotating centre. The H-5 signal of α-glycopyranosyluronic acid residues frequently resonates in, or close to, the anomeric region in ^{1}H-n.m.r.-spectra[340].

This resonance displays the largest coupling constant ($J_{4,5}$~10Hz) in this region and, thus, it is possible to assign the signal at δ 4.47 ($J_{4,5}$ 9.7 Hz) to H-5 of an α-glucopyranosyluronic acid residue. The remaining α-anomeric signal at δ 5.29 in the spectrum of P1 was therefore assigned to the 4-linked glucopyranosyluronic acid residue. By difference then, the signal at δ 4.48 was assigned to the terminal ß-D-galactopyranosyl residue.

2D-n.m.r. studies of the E. coli K35 polysaccharide

With the exception of H-6 and H-6' of the ß-galactopyranose residue all of the ^{1}H resonances of the five sugar residues in the repeating-unit were established from COSY[182] (Fig. 4.5), HOHAHA[193] (Fig. 4.6) and NOESY[184] (Fig. 4.7) experiments (Table 4.3). These data confirmed the assignments in Table 4.1 above and provided further evidence of the sequence of the sugar residues in the repeating-unit. The H-1 resonances of the sugar residues were arbitrarily labelled A to E in order of decreasing chemical shift.

Residue A.- Connectivities from H-1 to H-4 were established from the COSY spectrum. These connectivities were confirmed from the H-1 track in the HOHAHA spectrum and an additional connectivity from H-1 to a proton resonating at δ 4.07 was noted. This signal led to the identification of an H-4,5 cross peak in the COSY spectrum, from which connectivities from H-5 to H-6 and H-6' were established.

Residue B.- The chemical shifts for H-1 to H-5 were easily established from the COSY spectrum, with the downfield H-5 resonance of this α-Glc*p*A residue providing a second window into the spin system.

Residue C.- The connectivities from H-1 to H-4 were established from the COSY spectrum. The H-5 resonance was established from the H-2 track in the HOHAHA spectrum and this assignment was confirmed by the intra-residue n.O.e. between H-1 and H-5 in the NOESY experiment. Returning to the COSY spectrum, a weak H-4/H-5 cross-peak was noted, from which cross-peaks for H-5/H-6, H-5/H-6'and H-

Table 4.3. ^{1}H-N.m.r. data for *E. coli* K35 polysaccharide

Symbol	Residue	Chemical shifts in p.p.m.[a]						
		H-1	H-2	H-3	H-4	H-5	H-6	H-6'
A	4-α-Glc*p*	5.30	3.64	3.92	3.70	4.07	3.76	3.84
B	4-α-Glc*p*A	5.23	3.65	3.98	3.91	4.53		
C	3,4-ß-Man*p*	4.79	4.24	3.88	4.12	3.61	3.88	4.03
D	3-ß-Glc*p*	4.55	3.39	3.67	3.58	3.58	3.77	3.99
E	ß-Gal*p*	4.49	3.58	3.67	3.97	3.74		

[a]Determined at 400 MHz, measured from internal acetone at δ 2.23. Spectra were recorded at 343K. The sample used for these 2D experiments was the acid treated polysaccharide used in the 1D experiment (Table 4.1).

6/H-6' were identified. Hence, the entire spin system of residue C was mapped out in the COSY spectrum (Fig. 4.5).

Residue D.- The chemical shifts of H-1 to H-4 were established from the COSY spectrum and H-5 from the expected intra-residue n.O.e. between H-1 and H-5 in the NOESY spectrum. It was established that H-4 and H-5 had the same chemical shifts. Therefore, on returning to the COSY spectrum, the chemical shifts of H-6 and H-6' were established. These assignments were confirmed by following the connectivities of the H-2 track in the HOHAHA spectrum (Fig. 4.6).

Residue E.- Connectivities from H-1 to H-4 were established from the COSY spectrum. The chemical shift of H-5 was established from the intra-residue n.O.e. between H-1 and H-5 in the NOESY spectrum.

The inter- and intra-residue n.O.e. contacts observed in the 2D-NOESY spectra of *E. coli* K35 polysaccharide are listed in Table 4.3. Intense inter-residue n.O.es between the anomeric protons and the protons across the glycosidic linkage were

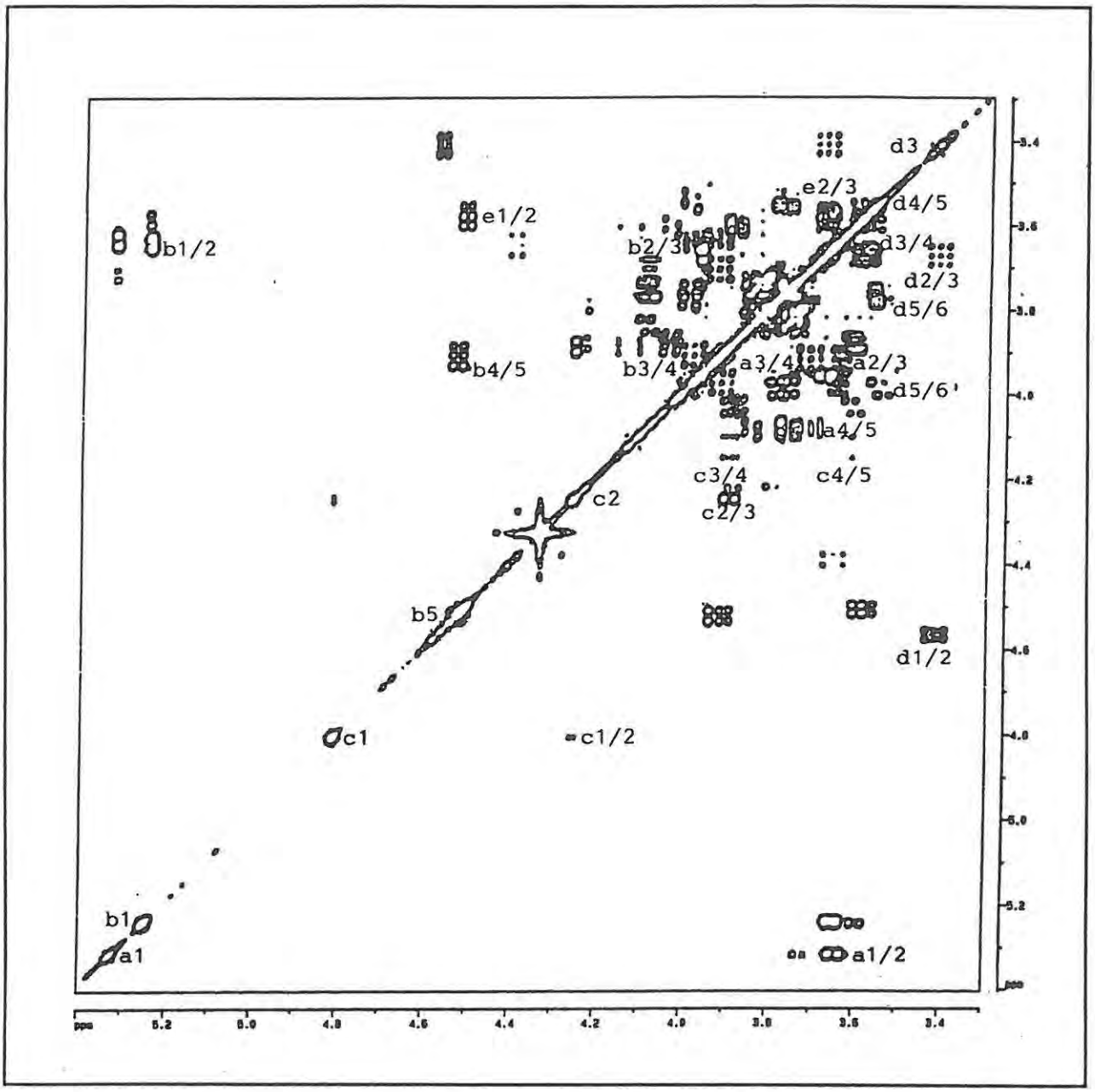


Fig. 4.5. COSY contour plot of the region δ 3.3-5.4 for the *E. coli* K35 capsular polysaccharide

observed for residues B, C and D, while that for residue A shows some overlapping with the expected H-1/H-2 intra-residue n.O.e. A less intense inter-residue n.O.e. was observed for residue E. A further inter-residue n.O.e. was observed between H-1 of residue B and H-2 of residue C. A molecular model of residue B linked to residue C in the three position suggests that an n.O.e. effect between H-1 of B to H-2 and H-3 of C is possible. However, a molecular model of residue

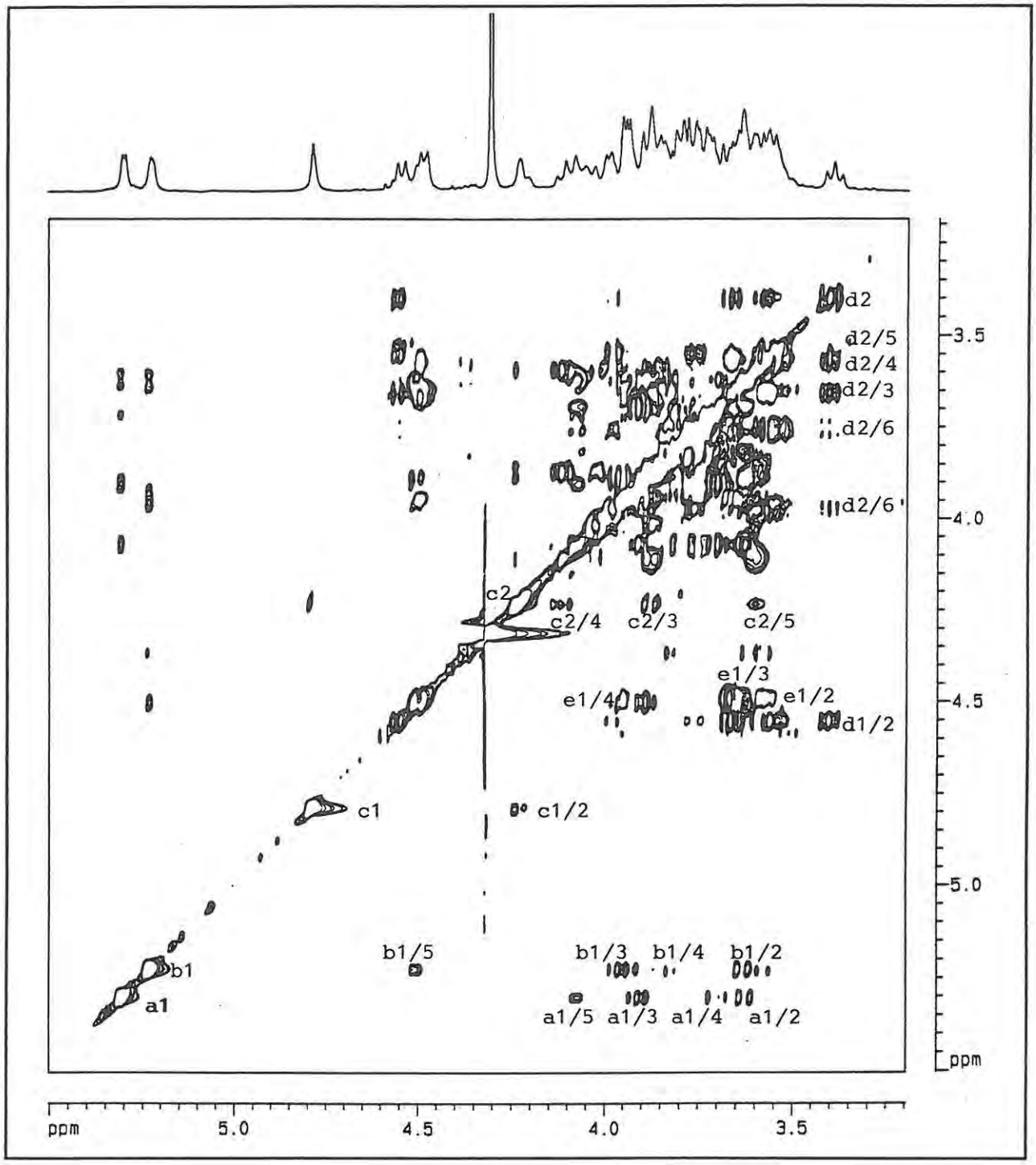
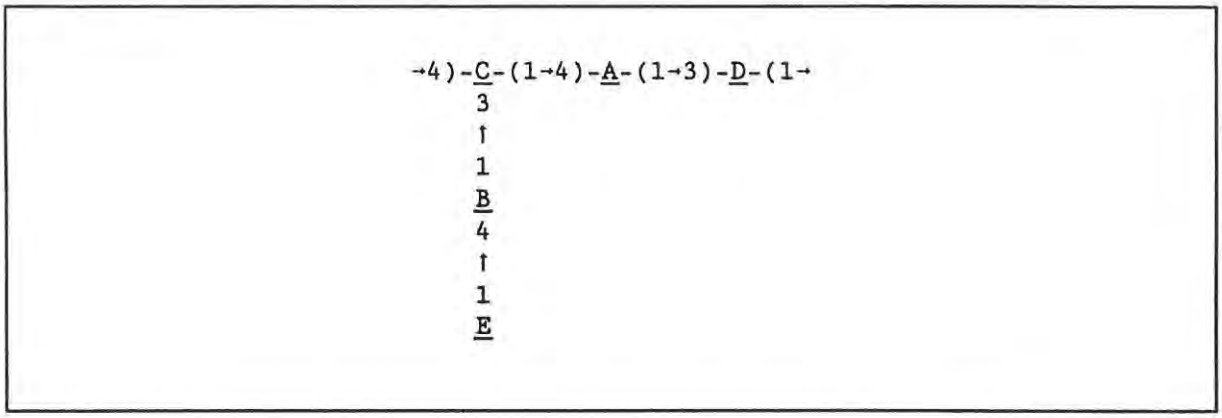


Fig. 4.6. HOHAHA contour plot of the region δ 3.3-5.5 for the *E. coli* K35 capsular polysaccharide

B linked to residue C in the two position suggests that the only expected inter-residue n.O.e. would be between H-1 of B and H-2 of C. This confirmed that residue B is linked to residue C in the three position.

These inter-residue n.O.es confirm the following sequence for the repeating unit of the polysaccharide:



These findings support the results of the methylation analysis (Table 4.2). The α-linked pyranoside residues A and B showed characteristic intra-residue n.O.es between H-1 and H-2 while the ß-linked pyranoside residues showed expected n.O.es from H-1 to H-3 and H-5. Residue C also showed intra-residue n.O.es between H-1 and H-2, and H-2 and H-3, which is further confirmation that C is the ß-D-mannopyranose residue.

Table 4.3a. n.O.e. contacts for the K35 polysaccharide

Proton	N.O.e. contact to
a, H-1	3.67 (d, H-3), 3.64 (a, H-2)
b, H-1	4.24 (c, H-2), 3.88 (c, H-3), 3.65 (b, H-2)
c, H-1	4.24 (c, H-2), 3.88 (c, H-3), 3.70 (a, H-4), 3.61 (c, H-5)
d, H-1	4.12 (c, H-4), 3.67 (d, H-3), 3.58 (d, H-5)
e, H-1	3.91 (b, H-4), 3.74 (e, H-5)

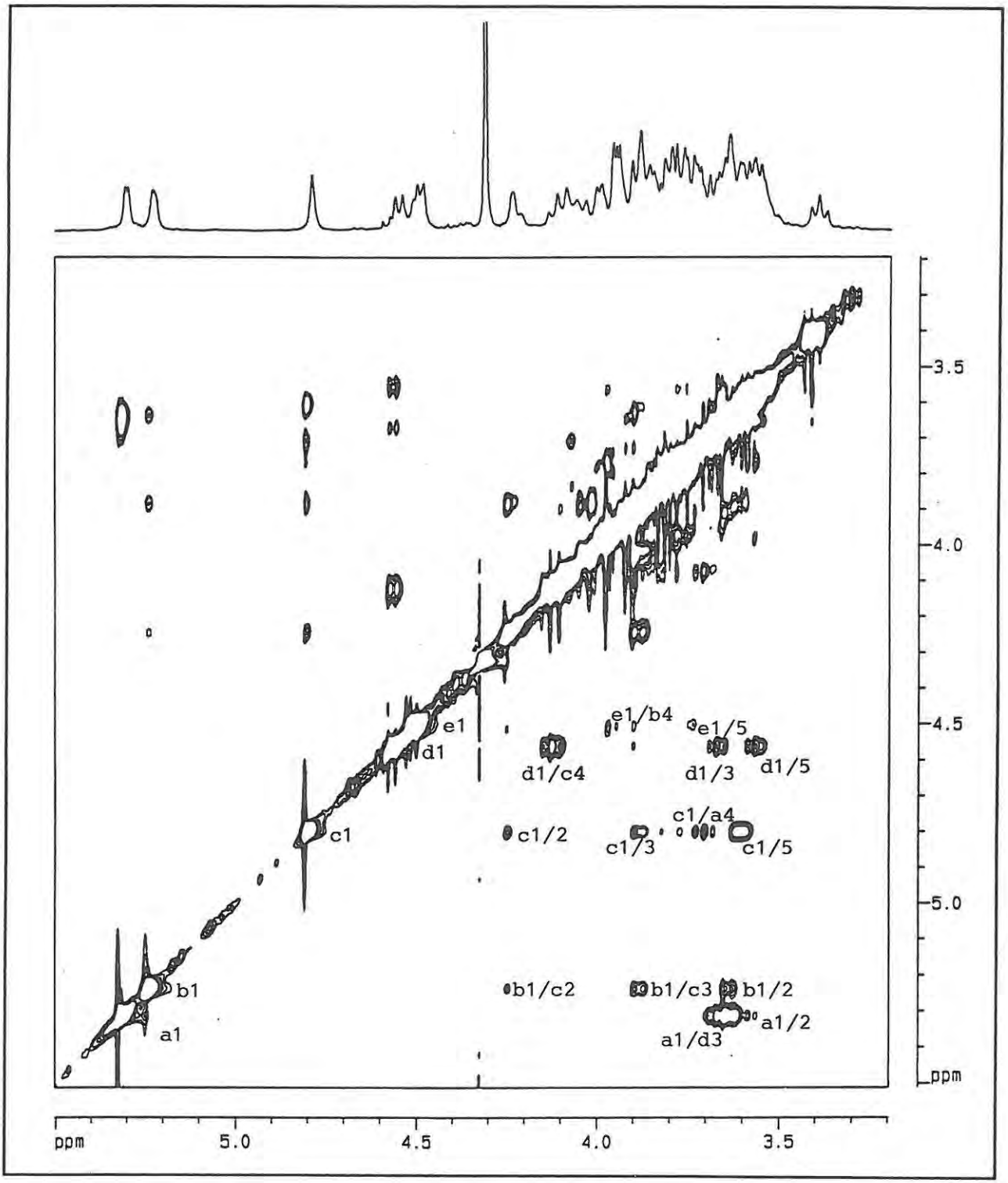
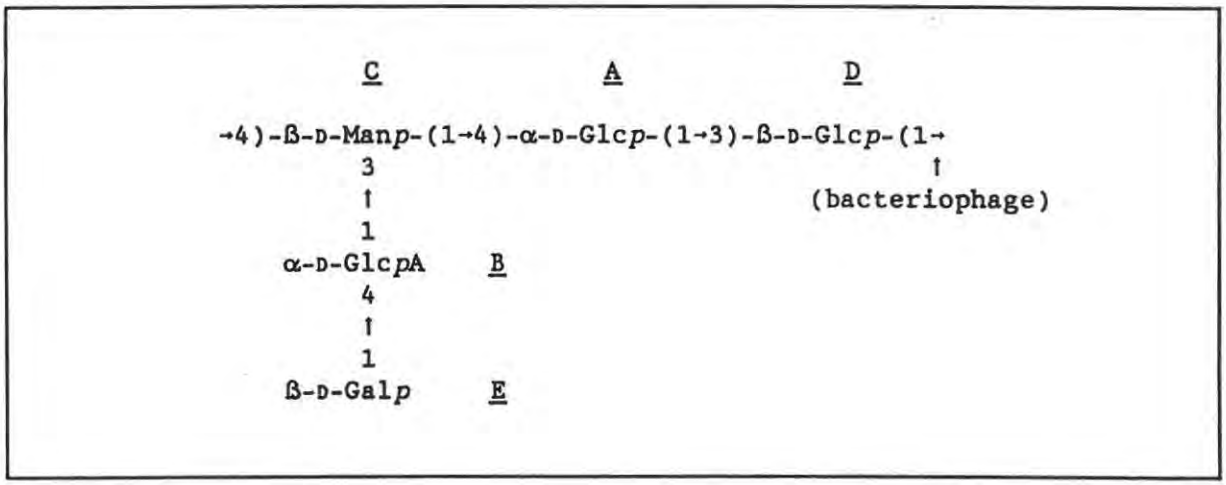


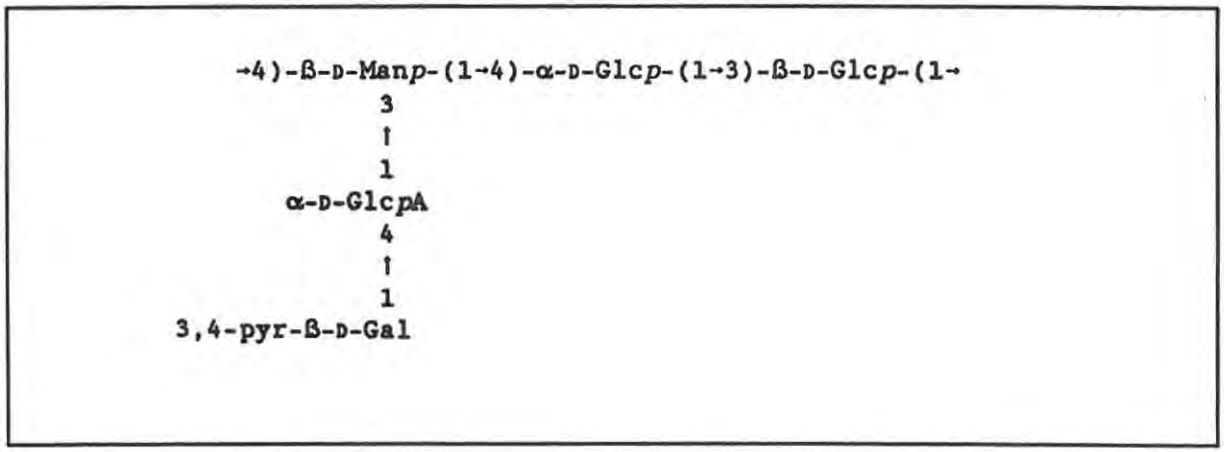
Fig. 4.7. NOESY contour plot of the region δ 3.4-5.5 for the *E. coli* K35 capsular polysaccharide

4.2.3. Conclusion

The results of methylation analysis and n.m.r.-spectroscopy of the polysaccharide and oligosaccharides isolated from a bacteriophage enzyme-mediated depolymerisation, permit the structure of the repeating-unit of *E. coli* K35 capsular polysaccharide to be written as



The primary structure differs from that of the *Klebsiella* K13[338] polysaccharide by not having a pyruvate acetal as shown below:



In the bacteriophage degradation of *Klebsiella* K13 polysaccharide the ß-glucosidase activity associated with the phage particles also catalysed the hydrolysis of the →3)-ß-D-Glcp-(1→4)-ß-D-Manp-(1→ linkage.

↑ 3
(bacteriophage) ↑

The value of the bacteriophage degradation technique has been illustrated in the

study of several capsular polysaccharides [Table 3.2 section 3.3.3 (V)]. The ability of these enzymes to tolerate minor structural variations has been demonstrated by Anderson[18] who showed that *Klebsiella* Φ5 catalyzed the hydrolysis of the *E. coli* K55 capsular polysaccharide. Furthermore, the above study demonstrates the ability of a bacteriophage (*E. coli* Φ35) to depolymerise impure substrate (*E. coli* K35/galactan) permitting the isolation of pure oligosaccharides (P1 and P2) suitable for further structural analysis.

4.2.4. Experimental

General methods

See section 4.1. G.p.c. of the isolates from batches 1-4 (~1.0 mg) was performed on a column of Sephacryl S500 as described in section 4.1.

Isolation and purification of K35 polysaccharide

An authentic culture of *E. coli* 09:K35 (A104a) was obtained from Dr. I. Ørskov (Copenhagen). Four batches of the bacteria were grown on Mueller-Hinton agar as described (section 4.1) and incubated for 72h, 40h, 48h, and 18h respectively. The acidic polysaccharide was isolated by CTAB precipitation to give yields of 650mg (K35 - contaminated), 100 mg (galactan), 150 mg (50:50 K35/galactan), and 1100 mg (K35 - pure) for batches 1-4 respectively. A further 240 mg of 40:60 09/K35 was isolated from batch 1 (for origin see section 4.2.2).

Additional purification steps

A sample of material from batch 1 (150 mg) was dissolved in 25 mL of distilled water, 25 mL of 2% acetic acid was added and the solution was heated at 60° in a waterbath for 1h. The resulting precipitate was removed by ultracentrifugation at 35k r.p.m. for 2.5h and the material was recovered by freeze-drying (80 mg). A sample of this material (45 mg) was treated with 0.25M NaOH (10 mL) at 60° for 1h. The solution was dialysed against distilled water for three days (12 000 -

14 000 Mw cut-off) and the base treated material was recovered by freeze-drying (30 mg). The K35 capsular polysaccharide was isolated from the O9/K35 mixture by the following procedure: The material was dissolved in 50 mL 0.06M phosphate buffer pH 7.5 (0.06M Na_2HPO_4 41.85 mL + 0.06M KH_2PO_4 8.15 mL) and incubated with ~2 mg of dispase (Boehringer) at 37° for 30 min to digest any protein contaminants. The protease enzyme was deactivated by heating the solution at 60° for 30 min. The material was dialysed (12 000 - 14 000 Mw cut-off, 24h), centrifuged (20k r.p.m., 1h), recovered by freeze-drying, and then subjected to preparative g.p.c. on a column of Sephacryl S400 HR (see section 4.1). The polymer was recovered from a major peak near the void volume of the column, dialysed and freeze-dried. An aqueous solution of the material was treated with 5% CTAB, however no complexation was evident. The material was recovered by precipitation (5 volumes ethanol) and was treated with acetic acid as above, then with 5% CTAB, and again there was no complexation. Finally, the material was subjected to anion-exchange g.p.c. on a column of DEAE-Sepharose 6B-CL (2.5 x 20 cm). The sample (50 mg) was applied and the column was washed with 240 mL 0.01M Tris/HCl buffer (pH 8.5) and the material was eluted with an ionic gradient of 0-0.5M NaCl in 0.01M Tris/HCl buffer (20 mL/h). Fractions were analyzed by the phenol-sulphuric acid method (Fig. 4.1). Partially purified K35 polysaccharide (90:10 K35/O9) was recovered by dialysis followed by freeze-drying (30 mg). Sugar analysis of the various isolates was carried out on the PAAN derivatives as described in section 4.2.

Uronic acid degradation

Three samples of methylated polysaccharide (3 mg) were dried *in vacuo* and dissolved in 19:1 dimethylsulphoxide/2,2-dimethoxypropane (1 mL), containing a trace of *p*-tolouene-sulphonic acid, by stirring under nitrogen. Potassium dimsyl[124] (0.5 mL) was added and the mixtures were stirred for 1h, 4h and 6h, respectively, with brief ultrasonic agitation. The solutions were cooled on an ice bath, trideuteriomethyl iodide (0.25 mL) was added, and stirred for 1h under

nitrogen. Excess trideuteriomethyl iodide was blown off with nitrogen and the products were extracted into chloroform (3 x 2 mL). The chloroform extract was washed three times with water (6 mL), dried over anhydrous Na_2SO_4, and the uronic acid degraded methylated material was recovered by evaporation under diminished pressure. The products were analyzed by g.l.c. analysis of the p.m.a.a. derivatives as described in section 4.2.

Bacteriophage degradation

The bacteriophage (Φ35) was isolated from sewage water using the following enrichment procedure - double strength nutrient broth (25 mL) + *E. coli* K35 nutrient broth suspension (5 mL) + sewage water (Grahamstown) (25 mL) were incubated in a waterbath-shaker (18h, 37°). A sample (10 mL) was sterilised with chloroform (1 mL) and centrifuged (2000 r.p.m.) to remove cellular material. The phage suspension was serially diluted (10^{-2} - 10^{-8}) in sterile SM medium (Appendix 4.1), and an aliquot (0.1 mL) from each dilution was added to *E. coli* K35 Luria-Bertani broth (LBB - Appendix 4.1) (0.2 mL) suspension and was allowed to stand for 5 min to allow phage adsorption. Thereafter, 4.0 mL of sloppy Luria-Bertani agar (LBA: LBB + 0.7% agar) at 37°was added, the solution was vortexed gently, then poured over LBA plates (LBB + 1.5% agar) and incubated at 30° overnight. A single plaque was isolated and stored in 1 mL SM medium. This phage suspension was serially diluted (10^{-2} - 10^{-8}) and the above procedure was repeated three times. The phage count was increased, by successive test tube and small flask lyses, until 400 mL of phage suspension (1 x 10^{11} p.f.u./mL), in LBB, was obtained. This was dialysed against running deionized water (3500 Mw cut-off, 48h), then assayed (1 x 10^{11} p.f.u./mL) by the sloppy overlay technique described above. The material to be degraded (650 mg - batch 1 and 3) was dissolved in 75 mL of water, then dialysed (12 000 - 14 000 Mw cut-off, 48h), ultracentrifuged (25k r.p.m., 1h) and recovered by freeze-drying (450 mg). This material was dissolved in ~5 mL water and added to 250 mL of dialysed Φ35 suspension (2.5 x 10^{13} p.f.u.). Depolymerisation was carried out for 72h in a waterbath-shaker at

37° in the presence of chloroform (20 mL) to keep the solution sterile. The solution was then centrifuged (3000 r.p.m., 30 min) and the supernatant freeze-dried. The recovered material was dissolved in water (10 mL) and dialysed against distilled water (4 x 120 mL, 3500 Mw cut-off, 24h). The dialysates were pooled and freeze-dried (547 mg), then dissolved in 3 mL water and passed through a column of Amberlite I.R.-120 (H^+) cation exchange resin at 4°. The eluent was ultracentrifuged (25k r.p.m., 1h) and the supernatant freeze-dried (304 mg). The material was applied to a Biogel P4 column (2.6 x 90 cm) in two equal fractions and eluted with sodium acetate buffer (section 4.2). The two oligosaccharide fractions (Fig. 4.3) were desalted by passage through a column of Amberlite I.R.-120 (H^+) cation exchange resin at 4° to desalt, and lyophilised to yield P1 (55 mg) and P2 (35 mg).

2D-N.m.r. spectroscopy

All 2D experiments were recorded at 70° on a Bruker AMX-400 spectrometer, equipped with an X32 computer and an array processor, using standard Bruker software.

A ^{1}H-homonuclear shift-correlated experiment (COSY[182]) was performed using a spectral width of 2202 Hz. Data matrices of 512 x 2048 data points were collected for 64 transients for each t_1 delay. The matrix was zero-filled in the t_1 dimension and transformed by use of a non-shifted sine-bell window function in both dimensions and symmetrised. Digital resolution in the resulting 1024 x 2048 matrices was 1.07 Hz per point. A relaxation delay of 0.7 s was used. A phase sensitive homonuclear dipolar-correlated (NOESY[184]) experiment was performed using a spectral width of 1400 Hz. A data matrix of 256 x 1024 data points was collected for 112 transients for each t_1 delay. The matrix was zero-filled in the t_1 dimension and transformed by use of a non-shifted sine-square function in both dimensions and symmetrised. Digital resolution in the resulting 1024 x 2048 matrix was 0.68 Hz per point. The mixing delay in the NOESY spectrum was 0.3 s.

A homonuclear Hartman-Hahn (HOHAHA[193]) experiment was performed with a spectral width of 1400 Hz. The 180° pulse width was 51 μs and the mixing period consisted of 50 MLEV-17 cycles. A data matrix of 256 x 2048 was acquired for 128 transients for each t_1 delay. The matrix was zero-filled in the t_1 dimension and multiplied in both dimensions with a phase-shifted sine-square function prior to Ft to obtain a 512 x 2048 data matrix.

Appendix 4.1.

Recipes of media used in bacteriophage work

SM media:

5.8 g NaCl + 2 g $MgSO_4.7H_2O$ + 50 mL 1M Tris/HCl pH 7.5 + 5 mL 2% gelatin + distilled water to 1000 mL.

LBB:

Bacto tryptone 10 g + yeast extract 5 g + NaCl 10 g + maltose 2 g + distilled water to 1000 mL.

4.3. *ESCHERICHIA COLI* 09:K38 CAPSULAR ANTIGEN : ANOTHER RIBOFURANOSE-CONTAINING GLYCAN

4.3.1. Introduction

The structures of 54 *Escherichia coli* capsular antigens have been reported; of these K6, K13, K16, K18-20, K22, K23, K57, K74, K95, and K100 (Fig. 2.1) contain ribofuranose in their repeating units. Serotype K57 is the only antigen among these which belongs to the Group 1 antigens[25], the rest belong to the Group 11 antigens[25]. The former tend to be heat stable and are most often co-expressed with 08 and 09 antigens; the latter have lower molecular weights and are thermolabile.

4.3.2. Results and Discussion

Isolation, composition, and linkage analysis of the capsular antigen

E. coli 09:K38 bacteria were grown on Mueller-Hinton agar, and the acidic capsular polysaccharide was isolated and purified by precipitation with cetyltrimethylammonium bromide. Further purification of the polysaccharide was effected by RNAse and DNAse treatment followed by separation by gel-permeation chromatography on Sephacryl S400-HR. The purified polysaccharide showed a broad distribution of molecular weights in gel-permeation chromatography on Sephacryl S500 with an average M_r at 10^7 (Fig. 4.8).

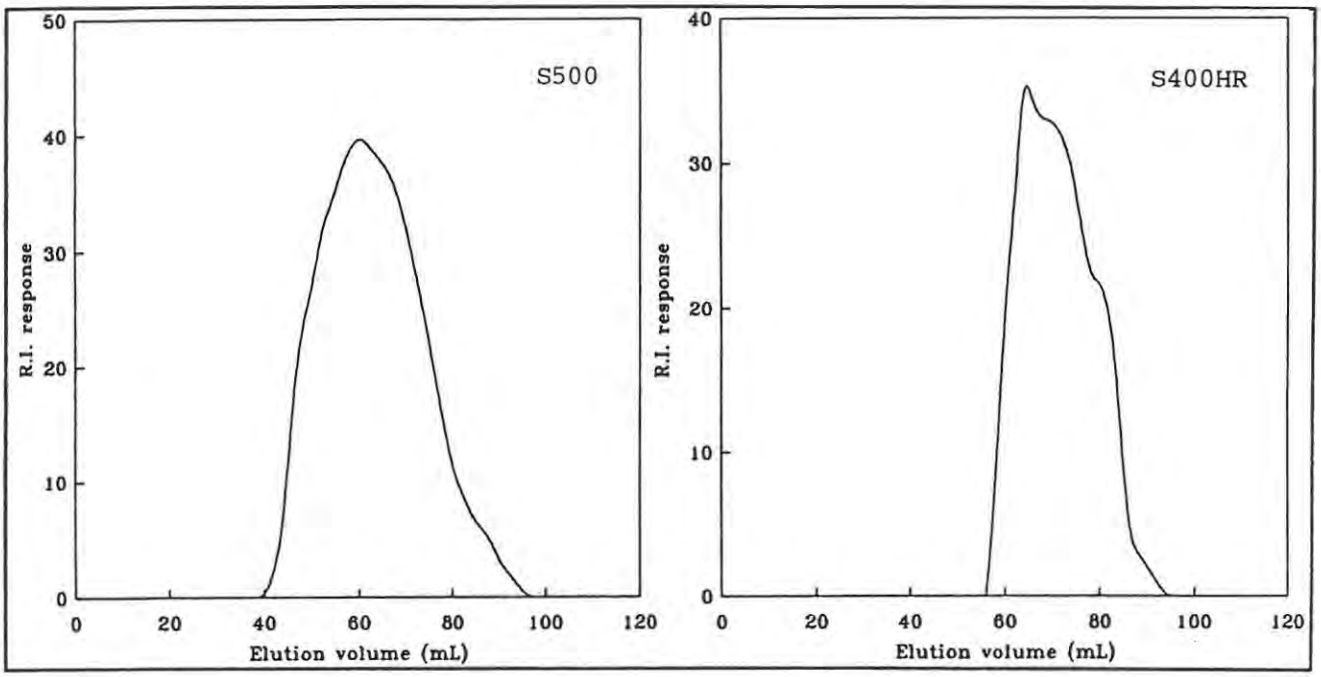


Fig. 4.8. Sephacryl S400HR and S500 gel permeation profiles of the *E. coli* K38 capsular polysaccharide

G.l.c. examination of the derived alditol acetates showed the polysaccharide to be composed of Rib, Gal, GalA, GalNAc, and GlcNAc. The constituent sugars were shown to have the D configuration by g.l.c. of the derived acetylated (-)-2-octyl glycosides[112]. The ^{1}H-n.m.r. spectrum of the purified polysaccharide was complex due to the presence of numerous partial resonances resulting from variable *O*-acetylation. The polymer was therefore treated with base and all subsequent n.m.r. experiments were performed on the *O*-deacetylated polysaccharide. The ^{1}H-n.m.r. spectrum (Fig. 4.1) contained H-1 signals at δ 5.40 (^{3}J < 1 Hz), 5.31 (^{3}J 3.5 Hz), 4.97 (^{3}J 3.0 Hz), 4.63 (^{3}J 8.0 Hz), and 4.48 (^{3}J 7.8 Hz), and a signal for the methyl protons of two NAc at 2.05 (6H). The ^{13}C-n.m.r. data complemented the ^{1}H-n.m.r. results and confirmed the pentasaccharide repeating-unit for the polysaccharide, with signals for C-1 at 107.84, 105.74, 102.24, 98.92, and 98.39 p.p.m., and a signal at 23.00 p.p.m. for the methyl carbons of NAc groups. In addition, signals for carbonyl carbons occurred at 175.64, 175.22, and 172.66 p.p.m. The C-1 signal at 107.84 p.p.m. indicates the presence of a furanoside (Fig. 4.9).

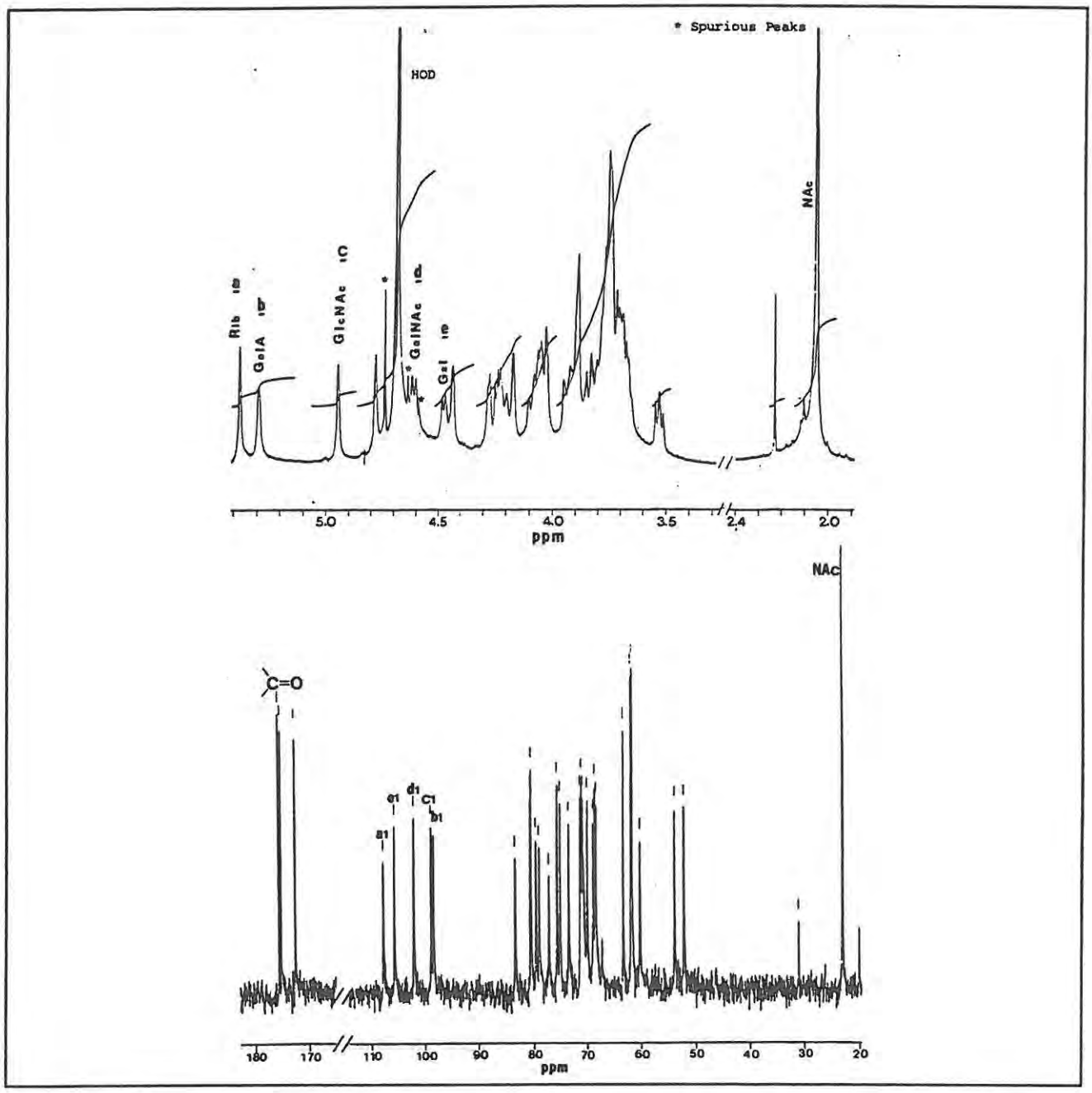


Fig. 4.9. 500 MHz ^{1}H- and ^{13}C-n.m.r. spectra of base treated *E. coli* K38 capsular polysaccharide

Methylation analysis of the polysaccharide gave 3,5-di-*O*-methylribose, 2,3,6-tri-O-methylgalactose, 2-deoxy-3,6-di-*O*-methyl-2-methylacetamidoglucose, 2-deoxy-4,6-di-*O*-methyl-2-methylacetamidogalactose,and 2,3-di-*O*-methylgalactose (after carboxyl reduction). These results accord with a linear pentasaccharide repeating-unit for the polysaccharide.

2D-N.m.r. studies of the E. coli K38 polysaccharide

The sequence of the residues in the repeating-unit was established by 2D-n.m.r. experiments, which also confirmed the glycosylation sites in the polysaccharide. Most of the ^{1}H resonances of the five sugar residues in the repeating-unit were established from COSY[182] and one- and two-step relay COSY experiments[183]. The residues in the repeating-unit were labelled a - e in order of decreasing chemical shift of their anomeric protons (Fig. 4.10). All the ^{1}H resonances of residues a, b, and, c and H-1 to H-4 of residues d and e were assigned by following the cross-peaks in the contour maps of the COSY (Fig. 4.10) and relay COSY experiments (Fig. 4.11 shows the one-step relay COSY contour map). The ^{1}H resonances for H-5 of residues d and e were assigned from the intramolecular n.O.es observed between H-1 and H-3 and H-1 and H-5 of d and e in the NOESY[184] experiment (see later). Cross peaks between H-5 and H-6 and H-6' for residues d and e could not be distinguished because of the proximity of these resonances.

The ^{1}H resonances for each residue were then compared with data obtained from a ^{1}H-^{13}C shift-correlated experiment[185] (HETCOR) (Fig. 4.12 and Table 4.4). In this way, all the ^{13}C resonances of a - c and C-1 to C-5 of residues d and e could be assigned. The two remaining sets of $^{13}C/^{1}H$ resonances from the HETCOR experiment at 61.67 p.p.m./δ 3.81 and 62.02 p.p.m./δ 3.77 were assigned to C-6/H-6 and H-6' of residues d and e respectively (see later for a discussion of these assignments).

Comparison of the ^{1}H and ^{13}C-n.m.r. data for residues a - e with literature values for methyl glycosides[211,327,328] permitted the residues in the repeating-unit to be identified, as indicated in Table 4.4, and their linkage positions to be established. In agreement with the results of methylation analysis, C-2 of a, C-4 of b, C-4 of c, C-3 of d, and C-4 of e experienced significant deshielding.

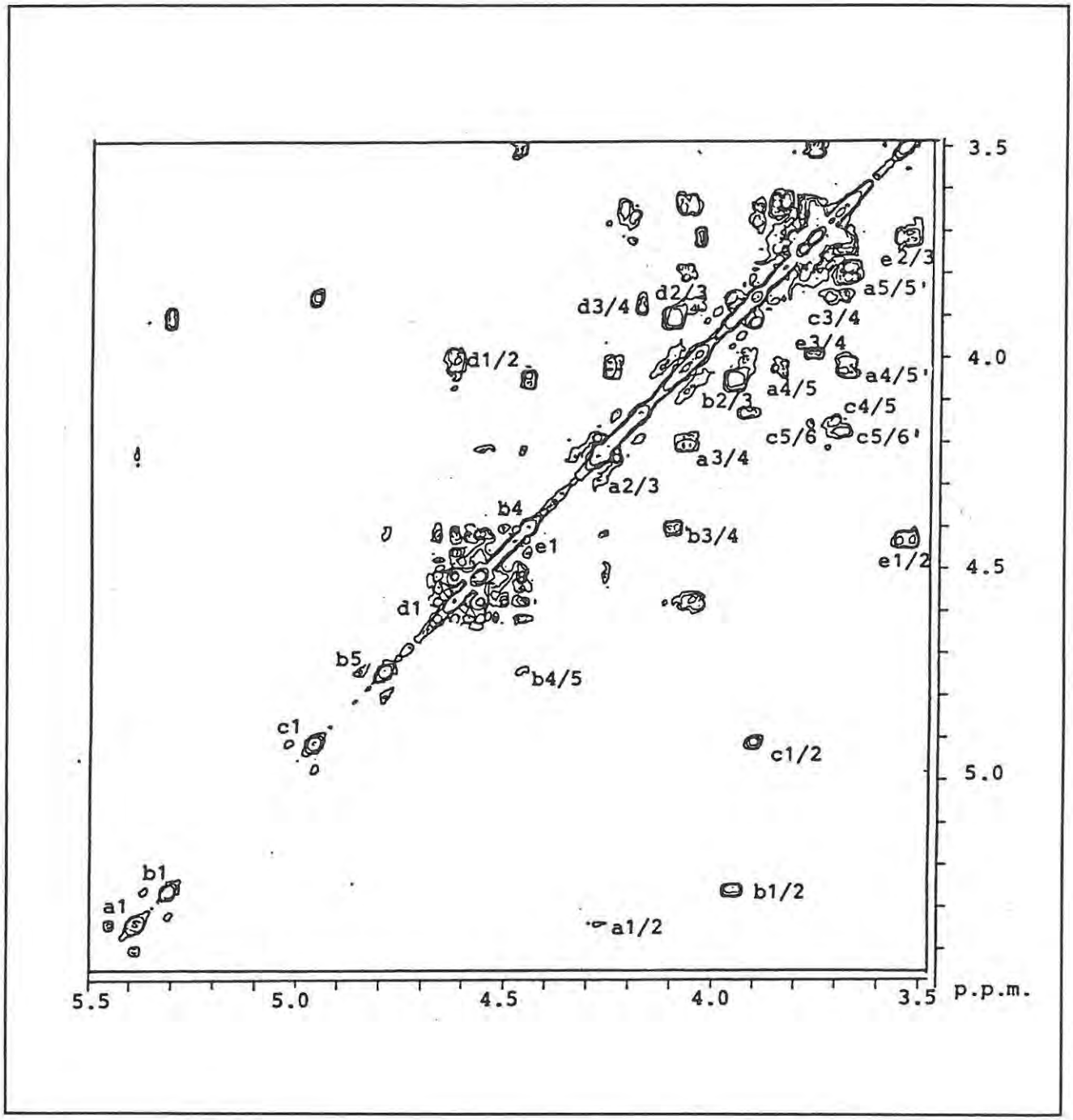


Fig. 4.10. COSY contour plot of the region δ 3.5-5.5 for the *E. coli* K38 capsular polysaccharide. The ^{1}H resonances of the *J*-coupled spin systems are labelled a-e; a1 connotes H-1 of residue a, and a1/2 connotes the cross-peak between H-1 and H-2 of residue a, *etc.*

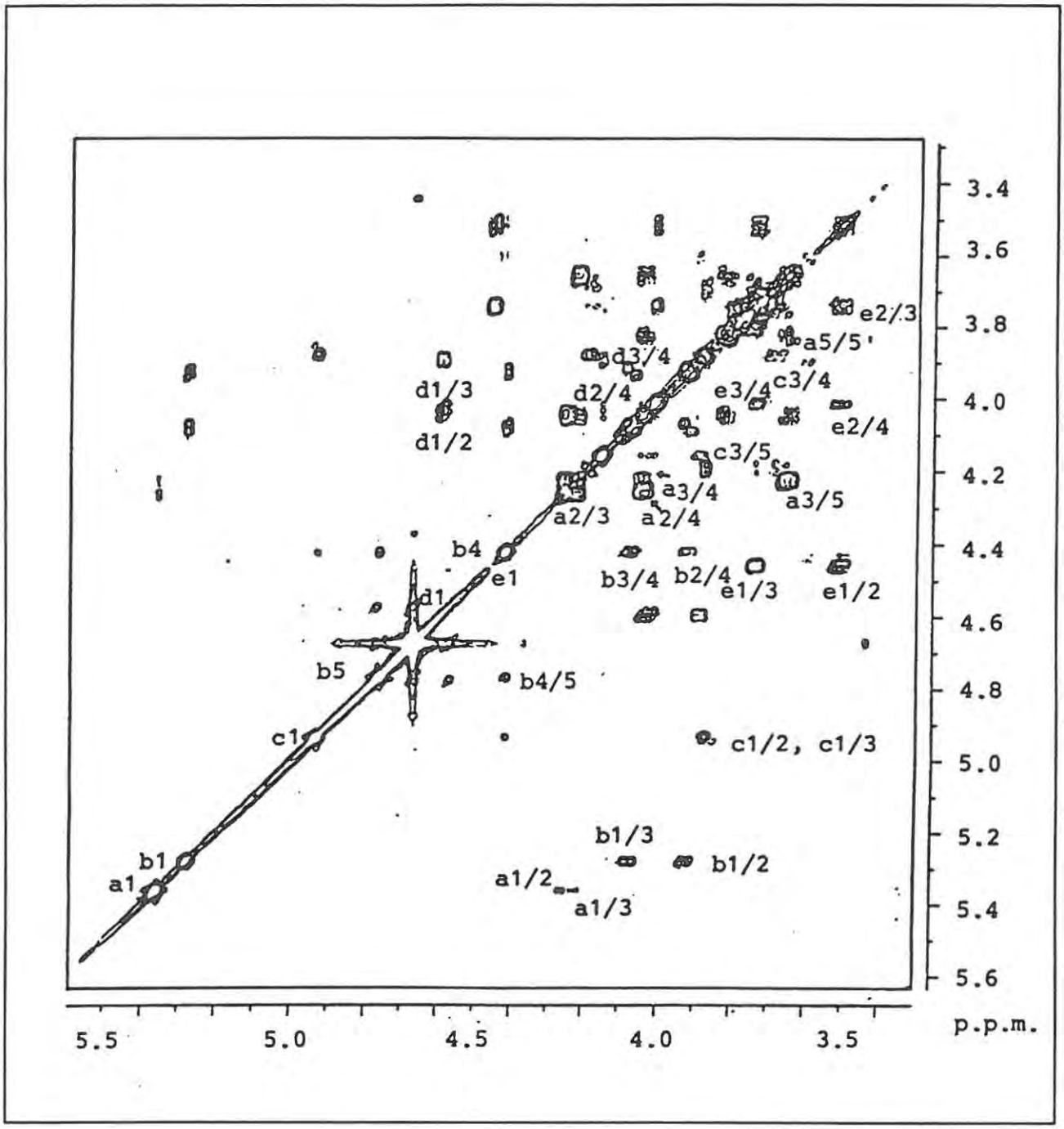


Fig. 4.11. One step relay COSY contour plot of the region δ 3.3-5.6 for the *E. coli* K38 capsular polysaccharide.

Table 4.4. N.m.r. data for de-*O*-acetylated *E. coli* K38 polysaccharide

	Residue							
	a →2)-ß-Rib		b →4)-α-GalA		c →4)-α-GlcNAc	d →3)-ß-GalNAc	e →4)-ß-Gal	
H-1	5.40	(5.39)[b]	5.31	(5.24)	4.97	4.63	4.48	(4.46)
C-1	107.84	(107.95)	98.39	(98.72)	98.92	102.24	105.74	(104.25)
H-2	4.29	(4.28)	3.94	(3.93)	3.92	4.07	3.54	(3.52)
C-2	80.73	(80.74)	68.32	(68.90)	53.98	52.23	71.46	(71.56)
H-3	4.27	(4.24)	4.12	(4.10)	3.92	3.92	3.79	(3.79)
C-3	70.76	(70.96)	69.02	(69.68)	70.07	80.56	73.56	(73.69)
H-4	4.09	(4.07)	4.44	(4.38)	3.75	4.20	4.05	(4.05)
C-4	83.35	(83.89)	78.98	(80.93)	79.68	68.67	77.23	(77.22)
H-5	3.69	(3.67)	4.80	(4.51)	4.22	3.73	3.77	(3.78)
H-5'	3.88	(3.83)						
C-5	63.25	(63.46)	70.94	(72.28)	71.11	75.14	75.66	(75.19)
H-6					3.71	3.81	3.77	(3.78)
H-6'					3.81	3.81	3.77	(3.78)
C-6					60.27	61.67	62.02	(62.01)

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

[b] Values in parentheses are those previously reported for *E. coli* K57 capsular polysaccharide.

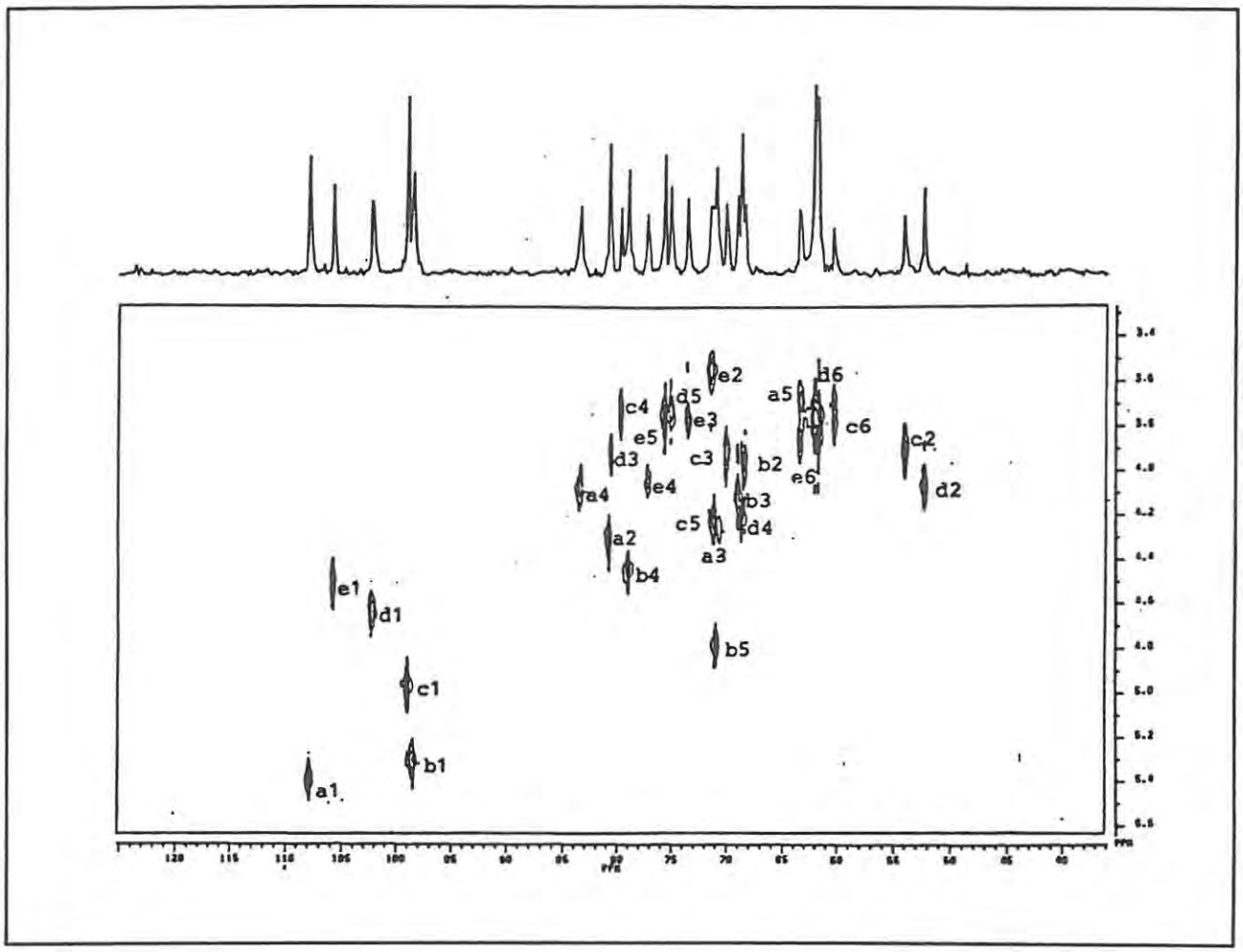


Fig. 4.12. ^{1}H-^{13}C shift correlation map of the spectral region *f*2 125-36 p.p.m., and *f*1 5.6-3.3 p.p.m. The ^{13}C projection is displayed along the *f*2 axis. The *f*1 axis represents the proton resonances. The correlated resonances are labelled a-e.

The sequence of the residues a - e in the repeating-unit was established by a NOESY[184] experiment. The inter- and intra-residue n.O.e. contacts are listed in Table 4.5. The α-linked pyranoside residues b and c showed characteristic intramolecular n.O.es between H-1 and H-2 while the β-linked pyranoside residues showed the expected n.O.es from H-1 to H-3 and H-5. Residue b also showed intra-residue n.O.es between H-3 and H-4, H-4 and H-5, and H-3 and H-5. These contacts are further confirmation that residue b has the *galacto* configuration. Intense inter-residue n.O.es between the anomeric protons and the protons across the

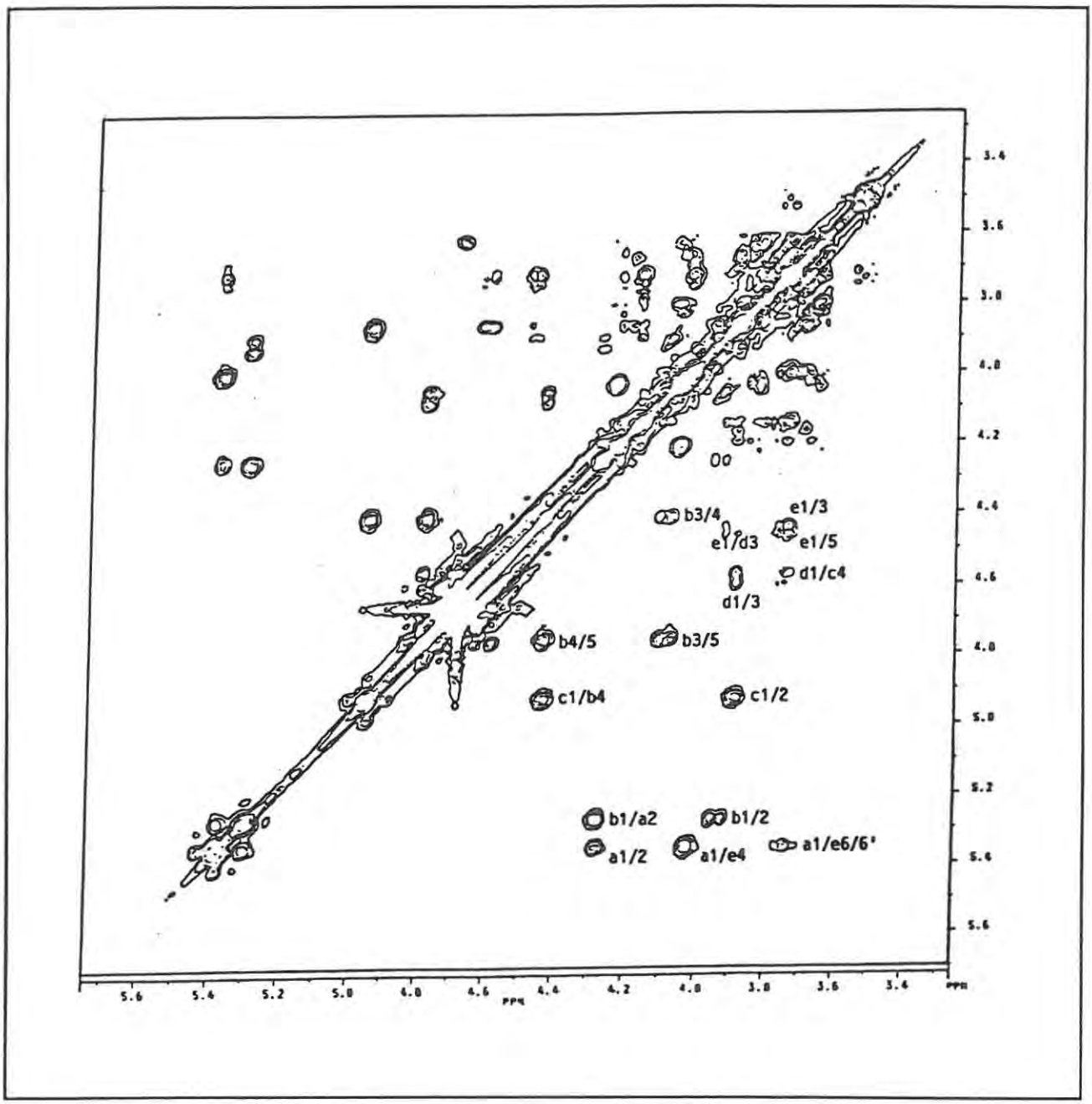


Fig. 4.13. NOESY contour plot of the region δ 3.3-5.6 for the *E. coli* K38 capsular polysaccharide.

glycosidic linkages were observed for residues a, b, and c while less intense n.O.es were observed for d and e (Fig. 4.13). These inter-residue n.O.es establish the sequence

→4)c(1→4)b(1→2)a(1→4)e(1→3)d(1→

for the repeating-unit of the polysaccharide. An intense inter-residue n.O.e. was

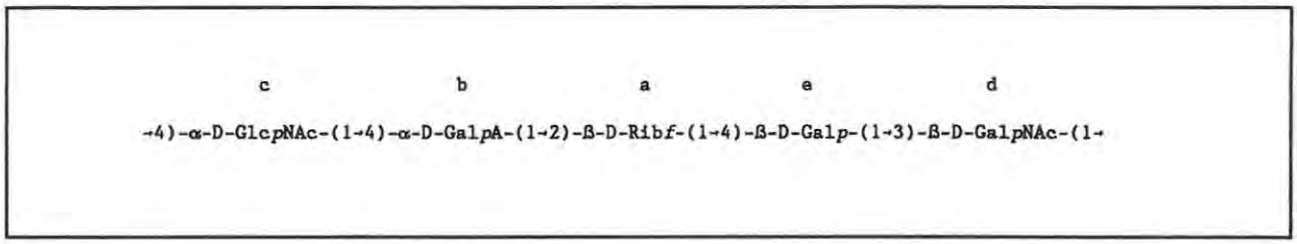
also noted between the anomeric protons of residues a and b. A similar n.O.e. was reported[55] to occur between these residues in the capsular polysaccharide of *E. coli* K57 and has also been observed[341-343] for other α-D-hexopyranose residues glycosylated at position 2. A further, but less intense, n.O.e. was noted between the anomeric proton of a and a proton resonating at δ 3,77, which could either be H-5 or H-6 and H-6' of residue e. Inspection of a molecular model of the repeating-unit of the polysaccharide suggests that the n.O.e. effect from the anomeric proton of a is to H-6 and/or H-6' rather than to H-5 of e. This unexpected n.O.e. permitted the assignment of the set of $^{13}C/^{1}H$ resonances at 62.02 p.p.m./δ 3.77 to C-6 and H-6 and H-6' of residue e and thus the set at 61.67 p.p.m./δ 3.81 was assigned to C-6 and H-6 and H-6' of residue d.

Table 4.5. N.O.e. contacts for the K38 polysaccharide

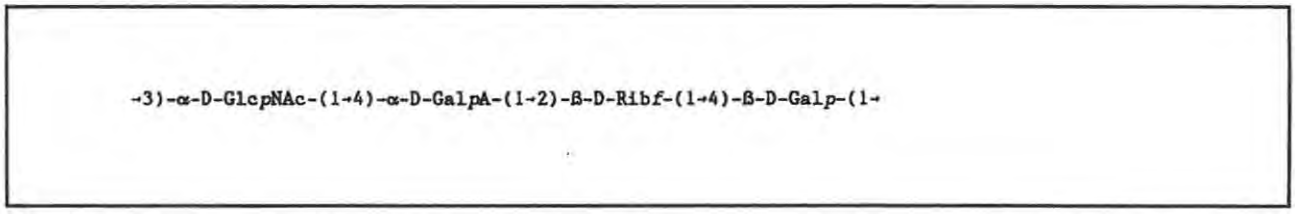
Proton	N.O.e. contact to
a, H-1	5.31 (b, H-1), 4.29 (a, H-2), 4.05 (e, H-4), 3.77 (e, H-6,H6')
b, H-1	4.29 (a, H-2), 3.94 (b, H-2)
b, H-5	4.44 (b, H-4), 4.12 (b, H-3)
b, H-4	4.12 (b, H-3)
c, H-1	4.44 (b, H-4), 3.92 (c, H-2)
d, H-1	3.92 (d, H-3), 3.73 (d, H-5), 3.75 (c, H-4)
e, H-1	3.79 (e, H-3), 3.77 (e, H-5), 3.92 (d, H-3)

4.3.3. Conclusion

The combined chemical and n.m.r. data permit the structure of the pentasaccharide repeating-unit of *E. coli* K38 to be written as



The above repeating-unit closely resembles that determined[55] for the capsular polysaccharide of *E. coli* K57 which is shown below.



Comparison of the n.m.r. data for the two repeating-units reveals that the ^{1}H and ^{13}C chemical shifts for the α-GalA, ß-Rib, and ß-Gal residues are almost identical (the values in parentheses in Table 4.4 are those previously reported for the K57 repeating unit). The principal deviations noted are for H-4/C-4 and H-5/C-5 of the α-GalA. These deviations are readily accounted for by the different pH conditions under which the two sets of n.m.r. data were acquired; the sodium salt of K57 capsular polysaccharide was used while the acid form of K38 polysaccharide was employed.

4.3.4. Experimental

General Methods

See section 4.2. G.p.c. of K38 polysaccharide was performed on dextran calibrated columns (1.6 x 65 cm) of Sephacryl S500 and Sephacryl S400 HR using 0.1M sodium acetate buffer (pH 5.0) as eluent (Fig. 4.8). Semi-preparative g.p.c. was performed on a column (2.6 x 65 cm) of Sephacryl S400 HR using the same eluent.

Alditol acetates were prepared by reduction of aqueous solutions of hydrolysates with sodium borohydride followed by acetylation with 1:1 acetic anhydride:pyridine at 100° for 1 h. Samples were methanolysed by treatment with refluxing methanolic 3% hydrogen chloride for 16 h. Native and methylated polysaccharides were carboxyl-reduced with $NaBH_4$ in dry methanol after methanolysis. Methylations were carried out on the acid form of the polysaccharide, using potassium dimsyl[124] and methyl iodide in dimethylsulphoxide.

Preparation of the K38 polysaccharide

An authentic culture of *E. coli* 09:K38 was obtained from Dr. I. Ørskov (Copenhagen), and the bacteria were grown on Mueller-Hinton agar. The capsular polysaccharide was separated from the cells by ultracentrifugation and isolated by precipitation with cetyltrimethylammonium bromide. One batch of 12 trays produced 1130 mg of material. This isolate was further purified by dissolving it in 100 mL 0.06M Na_2HPO_4/KH_2PO_4 buffer (pH 7.06) and incubating the solution with 0.1 mg each of RNASE and DNASE for 20 min at 37°. Enzymes were deactivated by heating the solution to 60° and holding it there for 10 min. The polysaccharide was recovered by lyophilisation after ultracentrifugation (25k r.p.m., 30 min) and dialysis (12 000 - 14 000 Mw cut-off). Samples for n.m.r. studies were subjected to preparative g.p.c. on Sephacryl S400 HR as described in section 4.2. *O*-Deacetylation of the polysaccharide was effected by heating the polysaccharide for 2 h at 40° in 0.1M sodium hydroxide. The *O*-deacetylated polysaccharide was purified by dialysis and

recovered by freeze-drying.

N.m.r. spectroscopy

All two-dimensional experiments were carried out at 33°. A COSY[182] experiment was performed using a spectral width of 2024 Hz. A data matrix of 256 x 1024 data points was collected for 32 scans per t_1 value. The initial matrix was zero-filled to 512 data points in the t_1 dimension and was transformed in both domains with a non-shifted sine-bell window function and symmetrised. Digital resolution in the f_1 domain was 3.95 Hz per point. One-step and two-step relay COSY[183] and NOESY[184] experiments were carried out using a spectral width of 2500 Hz; initial data matrices of 512 x 2048 data points were zero-filled to 1024 x 2048 data points to provide digital resolution of 2.44 Hz per point in the f_1 domain. Fourier transformation was as for the COSY experiment. Relaxation delays of 1.1 - 1.2 s were used. For the relay COSY experiments, fixed delays of 0.036 s were used. The mixing delay in the NOESY experiment was 0.3 s.

A ^{13}C-^{1}H shift-correlated (HETCOR)[185] experiment was recorded using a spectral width in f_2 of 13,513 Hz (108.1 p.p.m.) and 1440 (2.88 p.p.m) in f_1. The initial matrix of 256 x 2048 was zero filled to 512 x 2048 data points and processed with Gaussian functions. Digital resolution in f_2 was 13.2 Hz per point and in f_1 5.6 Hz per point. A recycle delay of 1.5 s was employed and 1600 scans per f.i.d were collected.

5. PARTIAL AND SELECTIVE REDUCTIVE CLEAVAGE STUDIES (PRC/SRC) OF THE *E. COLI* K37 AND K38 CAPSULAR POLYSACCHARIDES

The structural analysis of polysaccharides is simplified when an overlapping series of oligosaccharides is generated by partial depolymerisation thereof. Ideally, the fragmentation reaction should proceed with sufficient selectivity to produce oligosaccharides in high yield. Reductive cleavage has been discussed in section 2.4.2.(iii), and although this reaction was developed as an alternative to standard methylation analysis, it has been shown that by judicious choice of reaction conditions partial depolymerization of permethylated polysaccharides can be achieved. Characterization of the resulting oligosaccharides by chemical and/or spectroscopic analyses can provide the complete ring-form, linkage and sequence information.

Jun and Gray[275], conducting a reductive cleavage study on inulin which contains a large proportion of furanosyl residues, found that Me_3SiOSO_2Me catalyzes the reductive cleavage of D-fructofuranosyl residues while the D-glucopyranosyl residues remained unaffected. Stanley[235], prompted by the findings of Jun and Gray[275] above, effected the selective reductive cleavage (SRC) of the ribofuranose containing permethylated *E. coli* K57 capsular polysaccharide. The anhydroribitol terminated tri- and tetra-saccharides thus produced were characterised by n.m.r. spectroscopy and f.a.b.-m.s. Polysaccharides with glycosidic linkages of similar susceptibility to reductive cleavage can be subjected to partial reductive cleavage (PRC). This was demonstrated by Reinhold *et al.*[284] who produced an overlapping series of oligomers by PRC of permethylated ß-cyclodextrin which were analysed by direct c.i.-m.s.

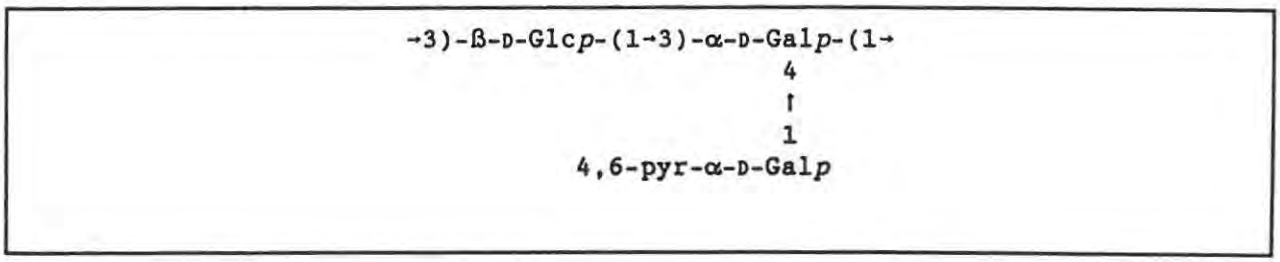
The advantages of SRC and PRC are that carboxy-methyl and *O*-methyl groups are left intact, the ring size and most of the stereochemistry of the cleaved unit is retained, and the lack of an anomeric hydroxyl means that there are no

anomeric complications to affect n.m.r. spectra and g.l.c. separations. Therefore, to determine the prospects of applying the technique in the structural analysis of the bacterial polysaccharides in general, *E. coli* K37 and K38 capsular polysaccharides, having repeating-units of significantly different composition, were chosen as model polymers.

5.1. PARTIAL REDUCTIVE CLEAVAGE (PRC) OF THE PERMETHYLATED *E. COLI* K37 POLYSACCHARIDE

5.1.1. Introduction

The capsular polysaccharide of *E. coli* 09:K37 (A84a) was previously found to be composed of the following trisaccharide repeating-units[17]:



The findings of Zeller and Gray[283] suggest that the glycosidic linkage of a pyruvylated sugar is relatively resistant to reductive cleavage in the presence of $BF_3.Et_2O$ or TMSOTf. Therefore, under the appropriate reaction conditions, pyruvylated Gal*p* residues in the permethylated K37 polysaccharide may result in the SRC thereof.

Permethylated (pm) K37 polysaccharide was subjected to reductive cleavage under various reaction conditions (see experimental section 5.1.4). The products were isolated by g.p.c. (Sephadex LH20) and were characterized by g.l.c.-m.s. operating in both e.i. and c.i. modes. The results, however, demonstrate negligible selectivity with a large variety of fragments being produced under partial reductive cleavage conditions.

5.1.2. Results and discussion

Native *E. coli* K37 polysaccharide isolated and purified by Anderson *et al.*[17] as described in section 4.1. was methylated by the Hakomori procedure as modified by Phillips and Fraser[124].

Reductive cleavage experiments

Method 1[276]: Dry samples of permethylated K37 polysaccharide dissolved in dichloromethane were treated with triethylsilane (Et_3SiH) and catalyst (TMSOTf or $BF_3.Et_2O$) for varying lengths of time (see experimental section 5.1.4). The reactions were quenched with acetic anhydride, which acetylated the free hydroxyl groups generated during the reductive cleavage reaction.

(i) A sample of permethylated K37 polysaccharide was subjected to complete reductive cleavage (**Method 1**) using $BF_3.Et_2O$ as catalyst. The products were isolated by g.p.c. on Sephadex LH20 and analyzed by g.l.c.-m.s., spectra recorded both in the e.i. and c.i. mode. As expected, there were three major products (Table 5.1, column I) which were identified as 3-*O*-acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-glucitol (**1**), 3,4-di-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-galactitol (**2**), and 1,5-anhydro-4,6-*O*-[(S)-1-methoxycarbonylethylidene]-2,3-di-*O*-methyl-D-galactitol (**3**), from their respective e.i.- and c.i.-mass spectra (Fig. 5.1). The e.i.-m.s. data were consistent with those reported in the literature[278,284]. Although compounds **2** and **3** have the same molecular weights of 256 (M+18, m/z 294), they are distinguished by the characteristic base peak at m/z 217 (Fig. 5.2) in the e.i.-m.s. of compound **3**.

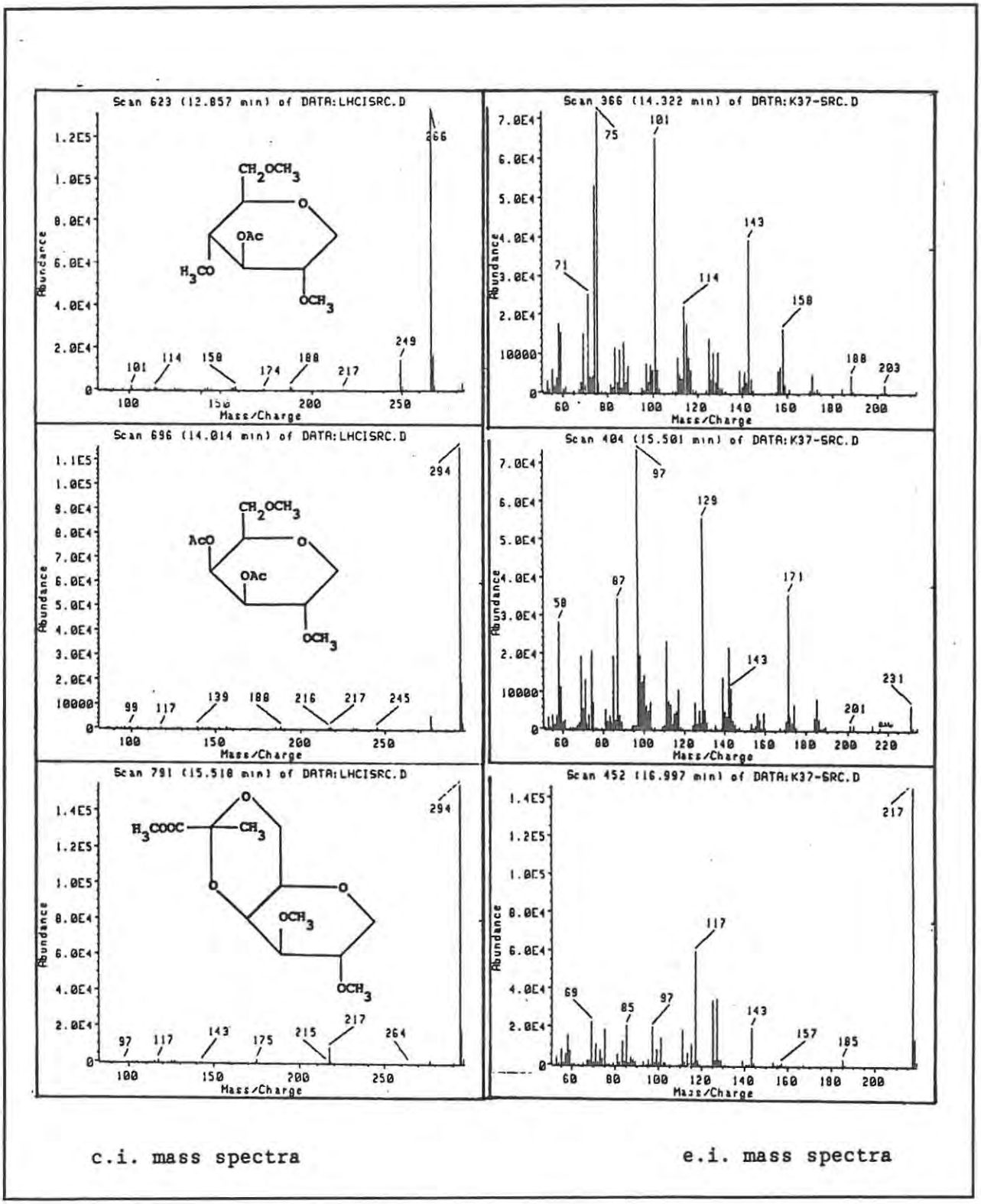


Fig. 5.1. C.i.- and e.i.-mass spectra of compounds **1**, **2** and **3** generated by complete reductive cleavage of the permethylated *E. coli* K37 capsular polysaccharide.

These results are in agreement with the published data[17] and confirm the linkage positions and ring forms assigned to the constituent monosaccharides.

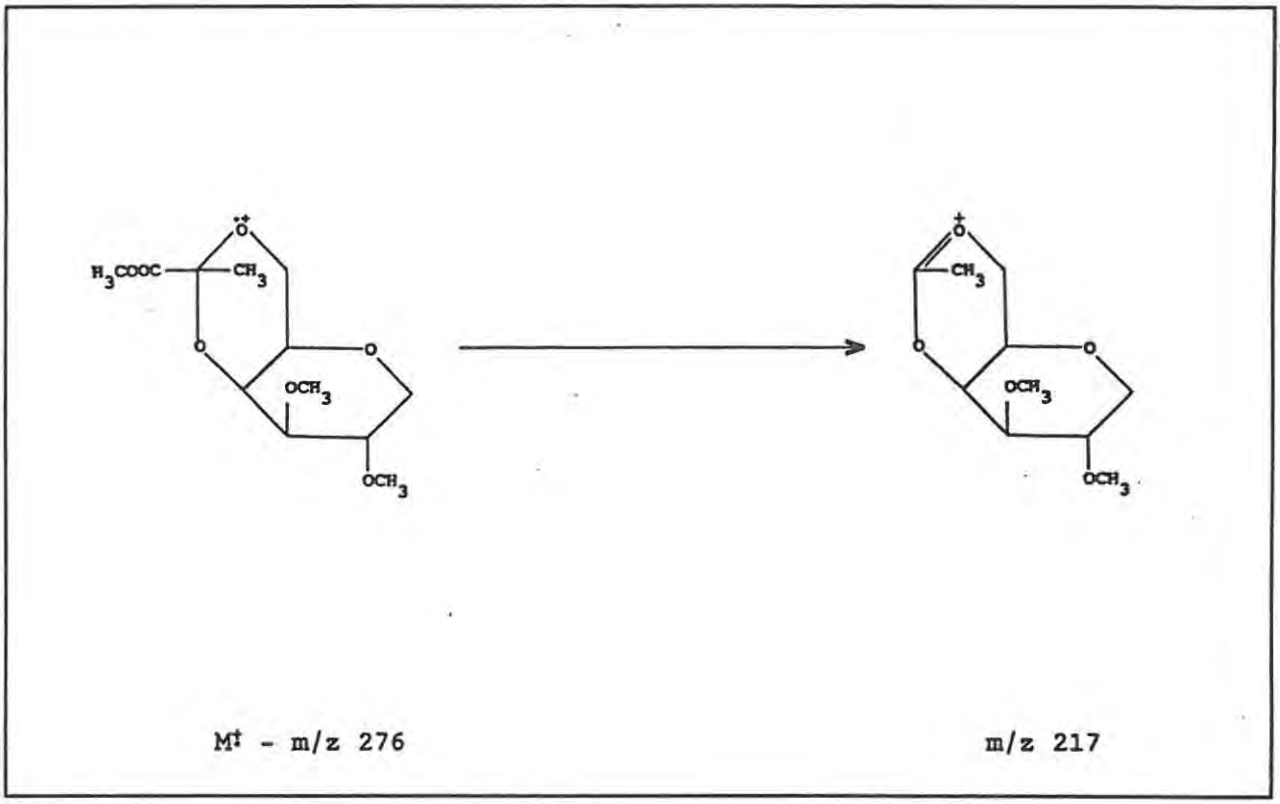


Fig. 5.2. Characteristic fragmentation of compound 3 producing the intense ion at m/z 217 (Fig.5.1).

(ii) *TMSOTf as catalyst.*- Four reactions were carried out at room temperature for 5, 10, 15 and 20 min, respectively. The products of each reaction were separated by g.p.c. (Sephadex LH20, methanol) and the gel chromatograms from each reaction were found to be identical despite the different reaction times employed. Two fractions [**(ii)A** and **(ii)B**, Fig. 5.3] were isolated and analyzed by g.l.c.-.m.s. as above. Fraction **(ii)B** was identified as a mixture of monosaccharide anhydroalditols (1,2 and 3) and their 1-*O*-acetyl analogues in the molar ratios shown in Table 5.1, column (II). Similarly, fraction **(ii)A** was shown to be a mixture of disaccharide anhydroalditols and their 1-*O*-acetyl analogues. G.l.c.-c.i.m.s. showed that these compounds originated from the two in-chain sugars →3)-ß-D-Glc*p*-(1→3)-α-D-(1→4)-Gal*p*-(1→] having molecular weights

of 480 and 538 (M+18 - m/z 498 and 556) respectively. Fig 5.4 shows the e.i.- and c.i.-m.s. of the major compound in the total ion chromatogram of fraction **(ii)A**, and identifies it as 3-*O*-(3-*O*-acetyl-2,3,6-tri-*O*-methyl-ß-D-glucopyranosyl-)4-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-galactitol **(7)** from the A-type fragmentation. These results suggest that in this system the terminal pyruvylated galactose residue is the most susceptible to reductive cleavage with the glucose residue being the most stable.

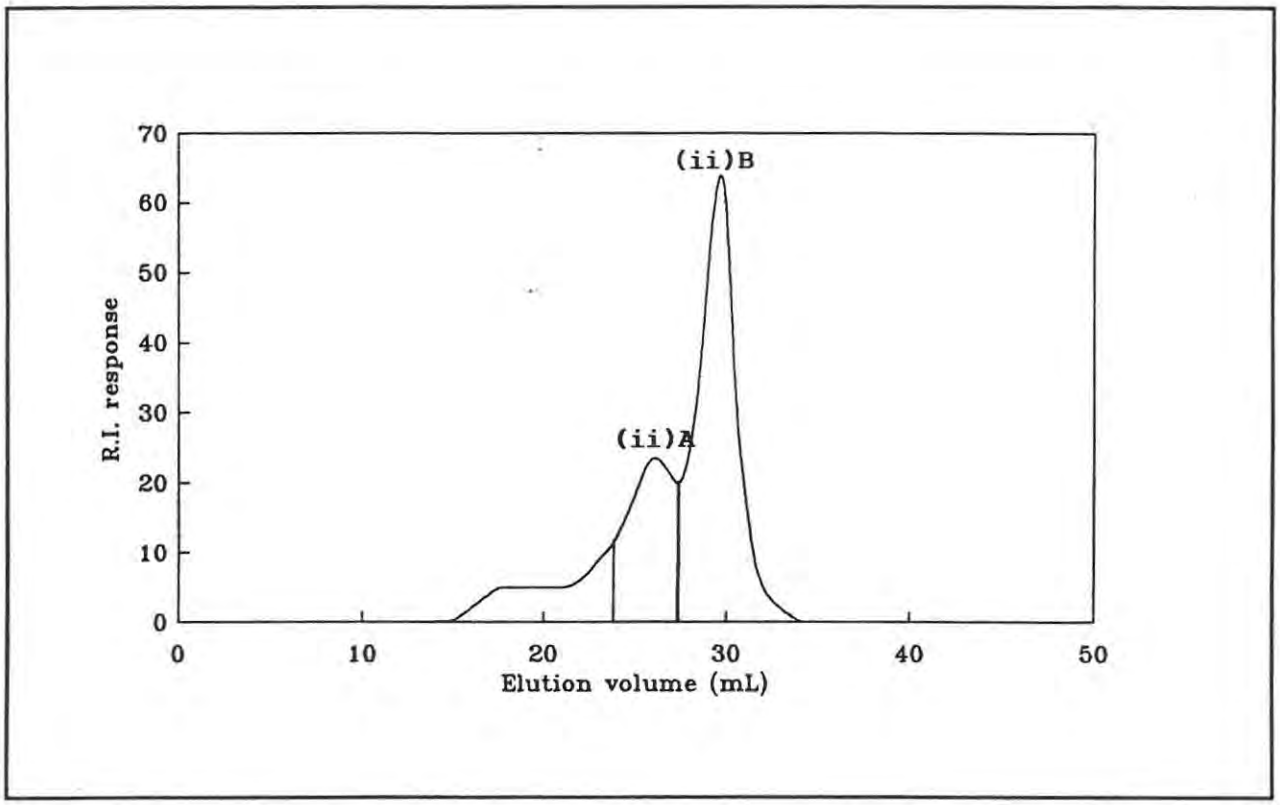
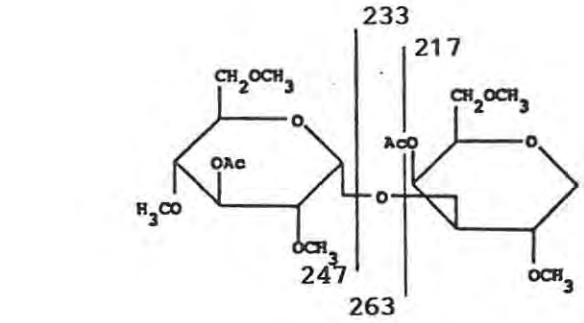
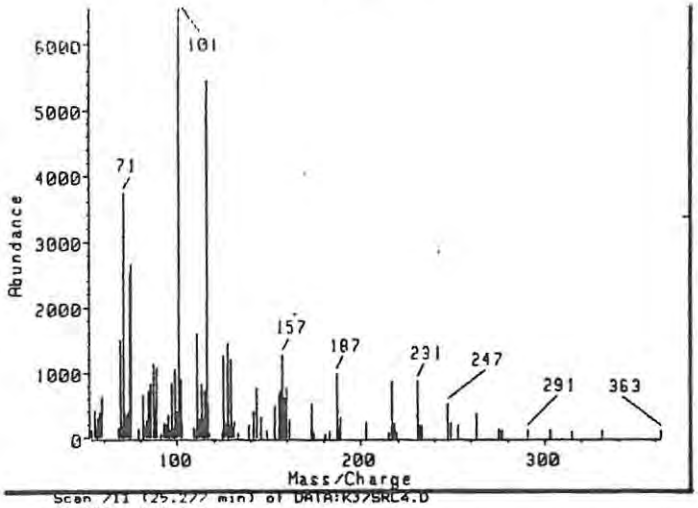


Fig. 5.3. Sephadex LH20 gel permeation profile of the products of reaction **(ii)**.





Scan 711 (25.277 min) of DATA:K37SRC4.D
K37SRC-1.1*1.2 A

m/z	abund.	m/z	abund.	m/z	abund.	m/z	abund.
53.00	123	91.05	61	124.80	1271	182.95	101
55.00	410	92.95	213	126.00	213	186.90	993
56.00	153	93.95	203	127.00	1453	187.90	143
56.90	309	94.90	353	128.00	187	188.90	316
57.95	390	95.90	126	129.00	1204	202.90	229
59.05	630	97.00	841	130.00	163	214.90	69
68.00	150	98.00	148	130.90	240	217.00	861
68.90	1495	98.90	1061	132.85	77	218.00	219
70.00	170	100.00	404	138.95	189	218.90	97
71.00	3729	101.00	6535	140.90	394	230.90	876
72.00	336	102.00	905	142.90	763	231.80	185
72.95	381	102.95	206	144.90	325	232.90	185
73.95	2520	109.05	152	148.85	105	247.00	515
74.95	2653	110.00	59	152.95	496	248.90	230
79.05	131	110.90	1595	154.90	656	252.85	190
80.90	640	111.90	287	155.90	734	262.80	363
81.80	180	112.90	835	156.90	1266	274.80	135
83.00	266	113.90	306	158.00	622	276.80	115
84.00	719	115.00	718	159.00	755	290.90	122
85.00	829	116.00	5451	161.00	262	303.00	115
87.00	1142	116.90	519	173.00	519	314.75	90
87.95	449	118.05	72	174.00	84	330.65	102
88.95	1073	124.05	83	180.85	52	362.70	108

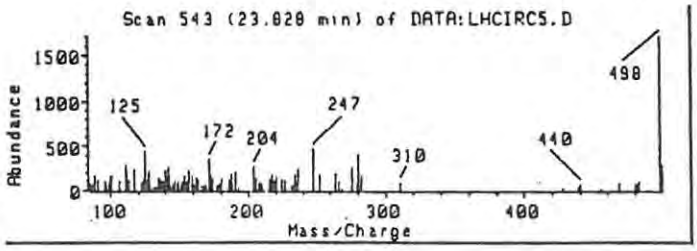


Fig. 5.4. C.i.- and e.i.-mass spectra of the major component of **(ii)A** (compound **7**) (Fig. 5.3)

Table 5.1.

Sugar residue[a]	T[b]	Molar ratios			
		I[c]	II	III	IV
1	1.00	1.00	0.43	0.04	0.15
2	1.09	0.70	0.75	0.03	0.07
3	1.20	0.91	1.00	0.27	1.00
4	1.28	-	0.35	0.12	-
5	1.38	-	0.34	0.07	-
6	1.51	-	0.39	1.00	-

[a] Sugar residues labelled 1-6 with-

1: 3-*O*-acetyl-1,5-anhydro-2,4,6-tri-*O*-methyl-D-glucitol

2: 3,4-di-*O*-acetyl-1,5-anhydro-2,6-di-*O*-methyl-D-galactitol

3: 1,5-anhydro-4,6-*O*-[(S)-1-methoxycarbonylethylidene]-2,3-di-*O*-methyl-D-galactitol

4: 1,3-di-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucose

5: 1,3,4-trio-*O*-acetyl-2,6-di-*O*-methyl-D-galactose

6: 1-*O*-acetyl-4,6-*O*-[(S)-1-methoxycarbonylethylidene]-2,3-di-*O*-methyl-D-galactose

[b] Retention times relative to that of residue **1**

[c] Molar ratios of monosaccharide products:

I, reaction **(i)** - complete reductive cleavage of permethylated *E. coli* K37 polysaccharide (**Method 1**);

II, reaction **(ii)** - PRC of permethylated *E. coli* K35 polysaccharide using TMSOTf as catalyst (**Method 1**);

III, reaction **(iii)** - PRC of permethylated *E. coli* K35 polysaccharide using $BF_3.Et_2O$ as catalyst (**Method 1**);

IV, reaction **(iv)** - PRC of permethylated *E. coli* K35 polysaccharide using $BF_3.Et_2O$ as catalyst (**Method 2**).

(iii) *$BF_3.Et_2O$ as catalyst*.- A reaction was carried out at -13° (ice/acetone) for 5 min (method 1) and the products were worked up as described above. Two fractions were isolated by g.p.c. [**(iii)A** and **(iii)B**, Fig 5.5] and were analyzed by g.l.c. and g.l.c.-.m.s. Fraction **(iii)B** was shown to be a mixture of compounds **1-6** (Table 5.1, column III). Fraction **(iii)A** was found to be a mixture of disaccharides as for **(ii)A**. Both fractions, however, are dominated by the 1-*O*-acetyl analogues of the anticipated anhydroalditols. The major components of **(iii)B** are compounds **3** and **6** (Table 5.1), while the major component of **(iii)A** is the 1-*O*-acetyl analog (Fig. 5.6) of compound **7** (Fig. 5.4). Using $BF_3.Et_2O$ as catalyst a significantly larger proportion of the terminal pyruvylated galactose residue was cleaved than the in-chain sugar residues, indicating a greater degree of selectivity than with TMSOTf.

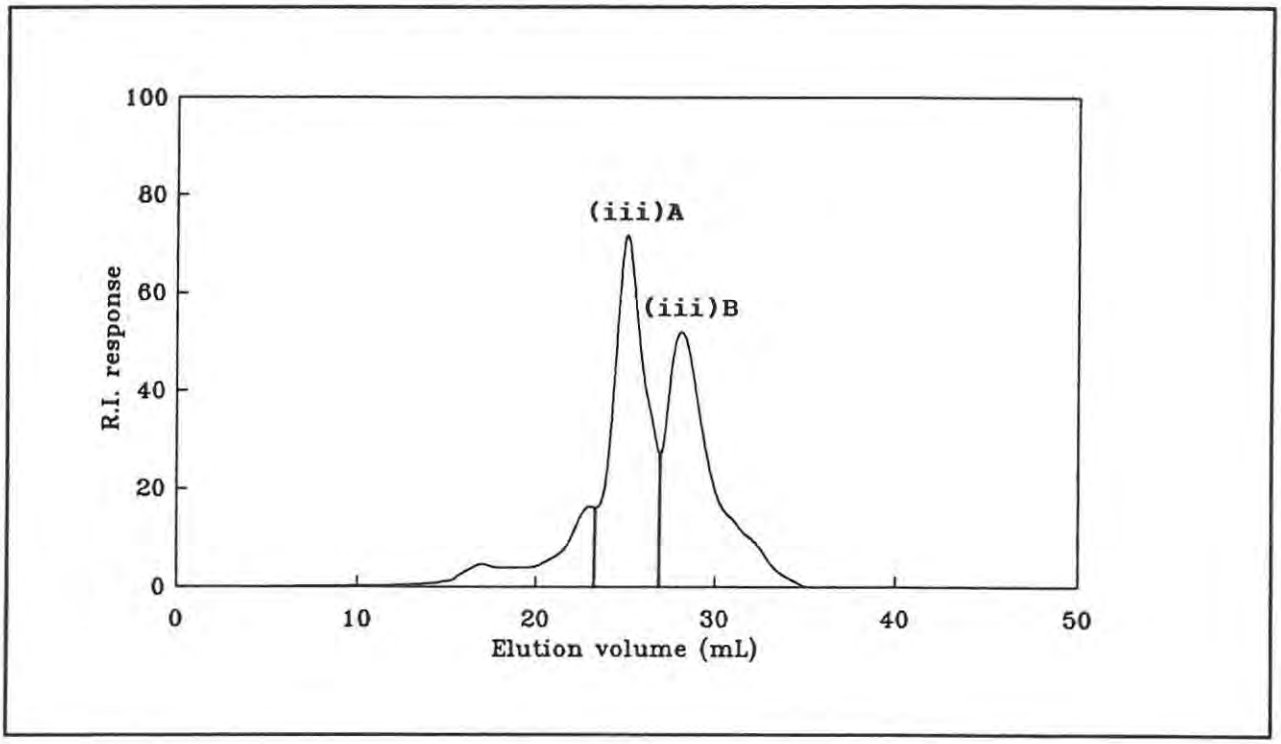
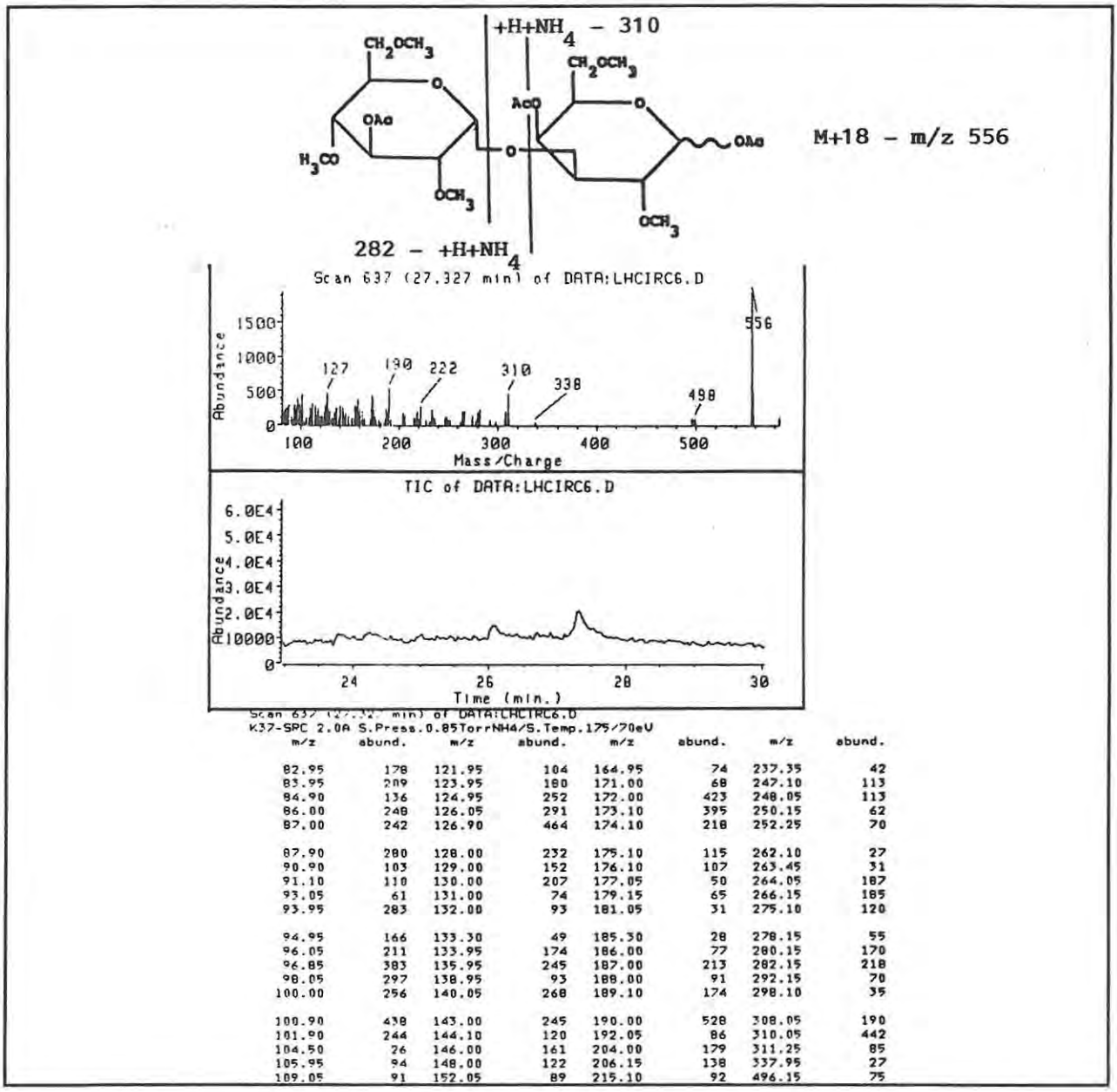


Fig. 5.5. Sephadex LH20 gel permeation profile of the products of reaction **(iii)**.

The large amounts of 1-*O*-acetyl derivatives that were generated in reactions **(ii)** and **(iii)** above suggest that the reaction was not quenched on addition of acetic anhydride. Further proof of this was the fact that the g.p.c. profiles in **(ii)** above were identical despite the different reaction times employed. It would appear that both TMSOTf and $BF_3.Et_2O$ catalysed acetolysis of the polymer occurred when the reaction mixtures were heated in the presence of acetic anhydride. This prompted a modification of **method 1** described above.



m/z	abund.	m/z	abund.	m/z	abund.	m/z	abund.
82.95	178	121.95	104	164.95	74	237.35	42
83.95	209	123.95	180	171.00	68	247.10	113
84.90	136	124.95	252	172.00	423	248.05	113
86.00	248	126.05	291	173.10	395	250.15	62
87.00	242	126.90	464	174.10	218	252.25	70
87.90	280	128.00	232	175.10	115	262.10	27
90.90	103	129.00	152	176.10	107	263.45	31
91.10	110	130.00	207	177.05	50	264.05	187
93.05	61	131.00	74	179.15	65	266.15	185
93.95	283	132.00	93	181.05	31	275.10	120
94.95	166	133.30	49	185.30	28	278.15	55
96.05	211	133.95	174	186.00	77	280.15	170
96.85	383	135.95	245	187.00	213	282.15	218
98.05	297	138.95	93	188.00	91	292.15	70
100.00	256	140.05	268	189.10	174	298.10	35
100.90	438	143.00	245	190.00	528	308.05	190
101.90	244	144.10	120	192.05	86	310.05	442
104.50	26	146.00	161	204.00	179	311.25	85
105.95	94	148.00	122	206.15	138	337.95	27
109.05	91	152.05	89	215.10	92	496.15	75

Fig. 5.6. C.i.-mass spectrum of the major component (1-*O*-acetyl derivative of compound **7**) of fraction **(iii)A** (Fig. 5.5).

Method 2: The reagents were mixed prior to being added to the dried methylated sample of permethylated K37 polysaccharide. Triethylsilane (20 μL) and $BF_3.Et_2O$ (20 μL) were brought into solution with CH_2Cl_2 (360 μL) in a dried, silanized reactivial. One hundred microlitres of this reaction mixture was added to samples of permethylated K37 (~5 mg). Reactions were carried out at various temperatures for various lengths of time. The reactions were quenched with methanol (500 μL) and the products deionized with Amberlite MB-1 mixed ion-exchange resin. The products were separated by g.p.c. as before and were recovered by evaporation of the methanol under-diminished pressure. The products were acetylated in 1:1 acetic anhydride/pyridine (100°, 1h) prior to g.l.c.-m.s.

(iv) Reactions were carried out at -13° for 1, 2 and 5 min; 0° for 5 min; and 22° for 5 and 20 min. Only polymeric material was recovered from all the above reactions, indicating that no significant cleavage had taken place. These results confirm that, under the conditions employed in reactions **(ii)** and **(iii)** above, the acetic anhydride did not quench the reactions and the polymer underwent further degradation.

Method 3: A sample of permethylated K37 polysaccharide was dissolved in CH_2Cl_2 (200 μL). Triethylsilane (20 μL) and $BF_3.Et_2O$ (20 μL) were sequentially added to the solution. The reactions were allowed to proceed at room temperature and were quenched with methanol and worked up as described in **method 2** above.

(v) Reactions were carried out for 20 min and 1h at room temperature. The gel permeation profiles of the products of these reactions (Fig. 5.7) show peaks corresponding to monosaccharide- and disaccharide-anhydroalditols, higher oligomers and polymer. This broad distribution of products was isolated and labelled **(v)A** and **(v)B** (Fig. 5.7).

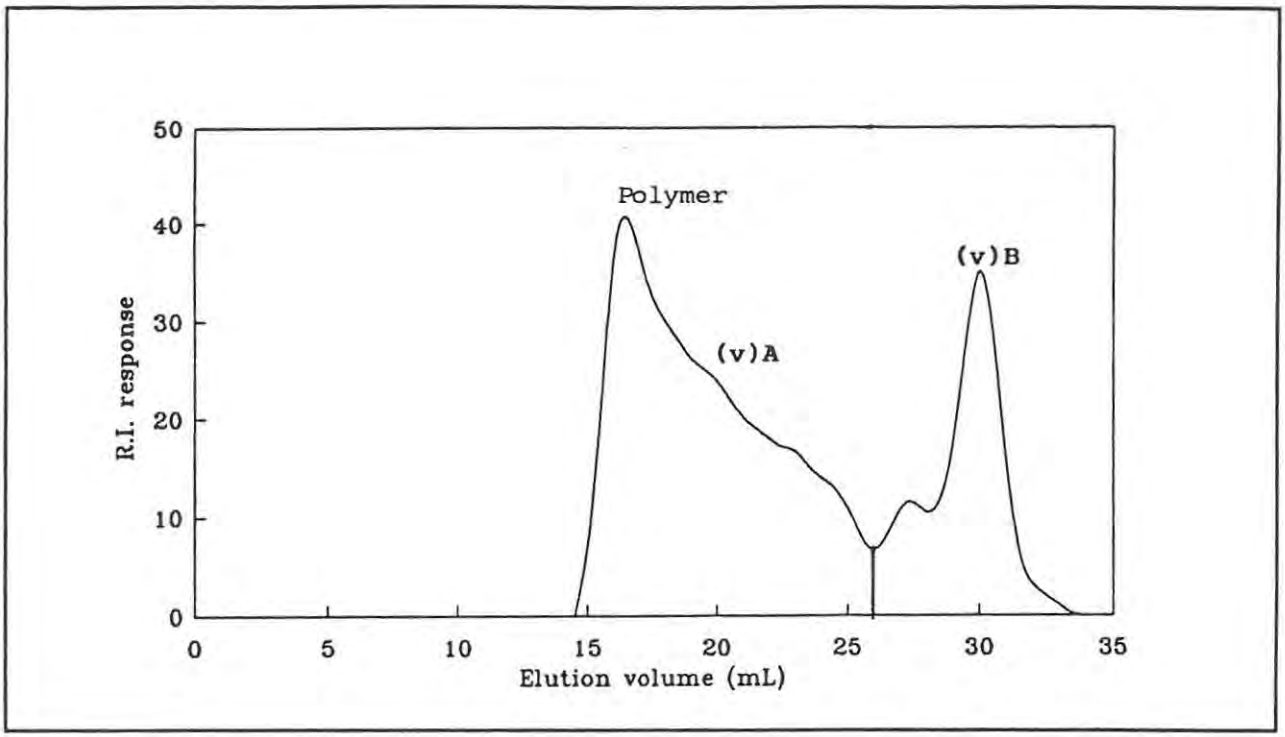


Fig. 5.7. Sephadex LH20 gel permeation profile of the products of reaction (v)

Fraction **(v)A** was acetylated and analyzed by g.l.c. and g.l.c.-m.s. It was found to be a mixture of derivatives **1** - **3** (Table 5.1, column IV) and disaccharide anhydroalditols. The major disaccharide anhydroalditol was identified as compound **7** from the A, R and T^{175} type fragments in the c.i.-m.s. (Fig. 5.4). Fraction **(v)B**, which corresponded to higher oligomeric and polymeric material, was hydrolysed, reduced, acetylated, and the derived p.m.a.a's. were analyzed by g.l.c. (Table 5.2, column II). The large amount of compound **3** in fraction **(v)B** (Table 5.1, column IV), and the small amount of 2,3-Gal produced on hydrolysis of fraction **(v)A** (Table 5.2, column II) is further confirmation that the pyruvylated galactose is readily cleaved.

Table 5.2.[a]

Sugar derivative[b]	Molar ratio[c]	
	I	II
2,4,6-Glc	0.93	0.81
2,6-Gal	1.00	1.00
2,3-Gal	0.78	0.40

[a] G.l.c.-analysis, on column DB-225 (J & W fused-silica bonded-phase capillary, 0.25 μm film thickness, 30 m x 0.25 mm) isothermal at 220°.

[b] 2,4,6-Glc = 1,3,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol, etc.

[c] Molar ratios: I, methylated native polysaccharide

II, methylated, polymeric and oligomeric products of PRC [reaction (v)]

5.1.3. **Conclusion**

The study has shown that oligosaccharide-anhydroalditols were readily generated by PRC of the permethylated *E. coli* K37 polysaccharide in the presence of both $BF_3.Et_2O$ and TMSOTf as catalyst. Therefore, employing more efficient chromatographic procedures than g.p.c. (*e.g.* preparative h.p.l.c.) one could isolate milligram quantities of these oligosaccharides, which could subsequently be characterised by n.m.r. spectroscopy as well as m.s. Furthermore, the glycosidic linkage of the terminal residue was shown to be more susceptible to reductive cleavage than those of the in-chain residues. This was more pronounced in the presence of $BF_3.Et_2O$ than with TMSOTf, suggesting that $BF_3.Et_2O$ catalysed reductive cleavage was more selective than TMSOTf catalysed reductive cleavage. The procedure is simplified considerably when the reductive cleavage is selective in nature resulting in one or two oligosaccharide products only (see section 5.2).

5.1.4. Experimental

General methods.- The *E. coli* K37 capsular polysaccharide used for the reductive cleavage study was previously isolated and characterised in this laboratory[17]. Samples of the polysaccharide (~5 mg) were dissolved in water (~20 mL), and ultracentrifuged (25k r.p.m., 1h). The clear supernatant was passed through a column of Amberlite IR 120 (H^+) cation exchange resin and the polysaccharide, in the free acid form, was recovered by freeze-drying. This polymer was dried *in vacuo* (~60°) overnight, then dissolved in 1:1 1,1,3,3-tetramethylurea: dimethylsulphoxide[153] (5 mL) and methylated as described in section 4.2. A sample of permethylated K37 polysaccharide (~2 mg) was hydrolyzed (4M TFA, 125°, 1h), the hydrolyzate was converted to the p.m.a.a. derivatives, as described in section 4.2., and these were analyzed by g.l.c. and g.l.c.-m.s. giving the results in Table 5.2, column I. 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 was used for separating p.m.a.a's. isothermally at 220°.

Reductive cleavage reaction.- The reducing agent triethylsilane (Et_3SiH), and catalysts trimethylsilyltrifluoromethanesulphonate (TMSOTf) and boron trifluoride etherate ($BF_3.Et_2O$), were purchased from Merck^R, Fluka^R and Aldrich^R, respectively. All reagents were stored over calcium hydride. All reactions were carried out in 2 mL reactivials which were previously silylated with Aquasil^R solution. Samples of permethylated polymer (~5 mg) were transferred to these reactivials, containing teflon coated stirrer bars, and dried for ~16h at 60° *in vacuo*. The reactivials were then stoppered with serum caps and the air was expelled with dry N_2.

Method 1: Dichloromethane (200μL), stored over CaH_2, triethylsilane (10 μL) and TMSOTf or BF3.Et_2O (10 μL) were sequentially added *via* dry syringes to samples of dry permethylated polymer (5 mg). The reactions were stirred for timed intervals

(section 4.1.2), acetic anhydride (50 μL) was then added and the reactions were heated at 40° for 10 min. The reactions were quenched by the addition of saturated aqueous $NaHCO_3$ (0.5 mL) and were stirred for 1h at room temperature. The product was extracted from the biphasic reaction mixture with of CH_2Cl_2 (3 mL), which was washed with water (2 x 3 mL), dried over anhydrous Na_2SO_4 and concentrated to dryness. The product was then subjected to g.p.c. as described below.

Method 2: A dry, silanized 5 mL reactivial, containing a teflon coated stirrer bar, was stoppered with a serum cap and the air expelled with N_2. CH_2Cl_2 (360 μL), triethylsilane (20 μL) and $BF_3.Et_2O$ (20 μL), were sequentially added to the reactivial and brought into solution at -13° (ice/acetone) with stirring. Samples were treated with 100 μL of this stock solution for varying lengths of time, at various temperatures (see section 5.1.2). The reactions were quenched by adding methanol (0.5 mL), and the mixture was deionized by adding Amberlite MB-1 resin (~1.0 mL). The resin was removed by filtration and was washed with methanol (~3 mL). The filtrate was collected in a 25 mL round-bottom flask and the reaction products were concentrated to dryness, then subjected to g.p.c.

Method 3: Samples were dissolved in CH_2Cl_2 (200 μL), then triethylsilane and $BF_3.Et_2O$ (20 μL) were sequentially added. The reactions were allowed to proceed for 20 min and 1h at room temperature, followed by the work-up procedure described in method 2 above. The fractions isolated by g.p.c. were acetylated in 1:1 pyridine/acetic anhydride, as described in section 4.2, prior to g.l.c. analysis.

Gel permeation chromatography.- Reaction products were dissolved in methanol (0.5 mL), applied to a column of Sephadex LH 20 (1 cm x 45 cm) and eluted with methanol at a flow rate of 9 mL/h. The methanol reservoir was periodically sparged with helium to remove dissolved air. Fractions (1 mL) were collected.

The column effluent was monitored by a Waters model R401 differential refractometer coupled to a flat-bed recorder.

G.l.c. and g.l.c.-m.s.- A Hewlett-Packard fused-silica HP-1 bonded-phase capillary column (12 m x 0.5 mm) having a film thickness of 0.65 μm, was used to separate the products of reductive cleavage . The oven was maintained at 75° for 2 min, then programmed to heat at $2°.min^{-1}$ to 250°. G.l.c.-m.s. was carried out on a Hewlett-Packard 5988A g.l.c.-mass spectrometer using the same column and temperature programme. E.i.-m.s. was carried out with an ionization energy of 70 eV and an ion source temperature of 200°. C.i.-m.s. was carried out at 50 eV in the positive ion mode, using ammonia as the reagent gas at a source pressure of 0.7 torr, and a source temperature of 175°.

5.2. SELECTIVE REDUCTIVE CLEAVAGE (SRC) OF THE PERMETHYLATED *E. COLI* K38 POLYSACCHARIDE

5.2.1. Introduction

The primary structure of the K-antigen isolated from *E. coli* O9:K38 (A262a) was established by glycose analysis, methylation analysis and n.m.r. spectroscopy (see section 4.3). The polysaccharide was found to have the following linear pentasaccharide repeating-unit:

→4)β-D-Gal*p*-(1→3)-β-D-GalpNAc-(1→4)-α-D-GlcpNAc-(1→4)-α-D-GalA-(1→2)-β-D-Rib*f*-(1→

This structure is closely related to the linear tetrasaccharide repeating-unit of the *E. coli* K57 capsular polysaccharide[55]:

→4)-β-D-Gal*p*-(1→3)-α-D-GlcpNAc-(1→4)-α-D-GalpA-(1→2)-β-D-Rib*f*-(1→

Working in this laboratory, Stanley was able to effect the SRC of the permethylated K57 polysaccharide to produce the following methylated anhydroribitol terminated tetra- and tri-saccharides:

[β-D-Gal*p*-(1→3)-α-D-GlcpNAc-(1→4)-α-D-GAlpA-(1→2)-anhydroribitol] (per-*O*-methylated)
4
|
OH

+

[α-D-GlcpNAc-(1→4)-α-D-GalpA-(1→2)-anhydroribitol] (per-*O*-methylated)
3
|
OH

These oligosaccharide anhydroalditols were produced in high yield, primarily due to the presence of the ribofuranosyl residue, which is highly susceptible to reductive cleavage[275]. Furthermore, α-amino sugar linkages are completely resistant to reductive cleavage[281]. Therefore, the α-D-Glc*p*NAc residue in the K57 polysaccharide would have contributed substantially to the selectivity of the

above reaction.

Similar selectivity was encountered in the reductive cleavage of the K38 polysaccharide which also has the ß-D-Rib*f* and α-D-Glc*p*NAc residues in its repeating-unit. The polysaccharide served as a good model of SRC from which high yields of oligosaccharide anhydroalditols were obtained. This is in contrast to PRC of the K37 polysaccharide (section 5.1), where little selectivity was encountered, resulting in a large mixture of products. We now report on SRC of the permethylated K38 polysaccharide and characterization of the resulting oligosaccharide anhydroalditols by mass spectrometry.

5.2.2. Results and discussion

E. coli K38 polysaccharide, which had previously been isolated and purified (section 4.3), was methylated by the modified Hakomori method[124] discussed in section 4.1. Samples of permethylated K38 (~5 mg) were dissolved in CH_2Cl_2 (250 µL), then Et_3SiH (10 µL) and catalyst (10 µL) were sequentially added. The reactions were allowed to proceed for timed intervals, at room temperature, and were then quenched with methanol (0.5 mL). The reaction mixtures were deionized with Amberlite MB-1 resin and the reaction products were recovered by concentrating them to dryness.

(i) *TMSOTf as catalyst*.- Five reactions were carried out for 10, 15, 20, 30 and 60 min respectively. The products were subjected to g.p.c. (Sephadex LH20, methanol) and gave the profiles shown in Fig. 5.8. Three low molecular weight fractions, labelled **A**, **B** and **C** (Fig. 5.8), were isolated by g.p.c. and **B** and **C** were acetylated with 1:1 pyridine/acetic anhydride (100°, 1h). **Fraction C** was analyzed by g.l.c.-m.s. and was found to be a mixture of two monosaccharide derivatives (**C1** and **C2**). The compound with the shorter g.l.c. retention time (**C1**) produced the e.i.- and c.i.-mass spectra shown in Fig. 5.9.

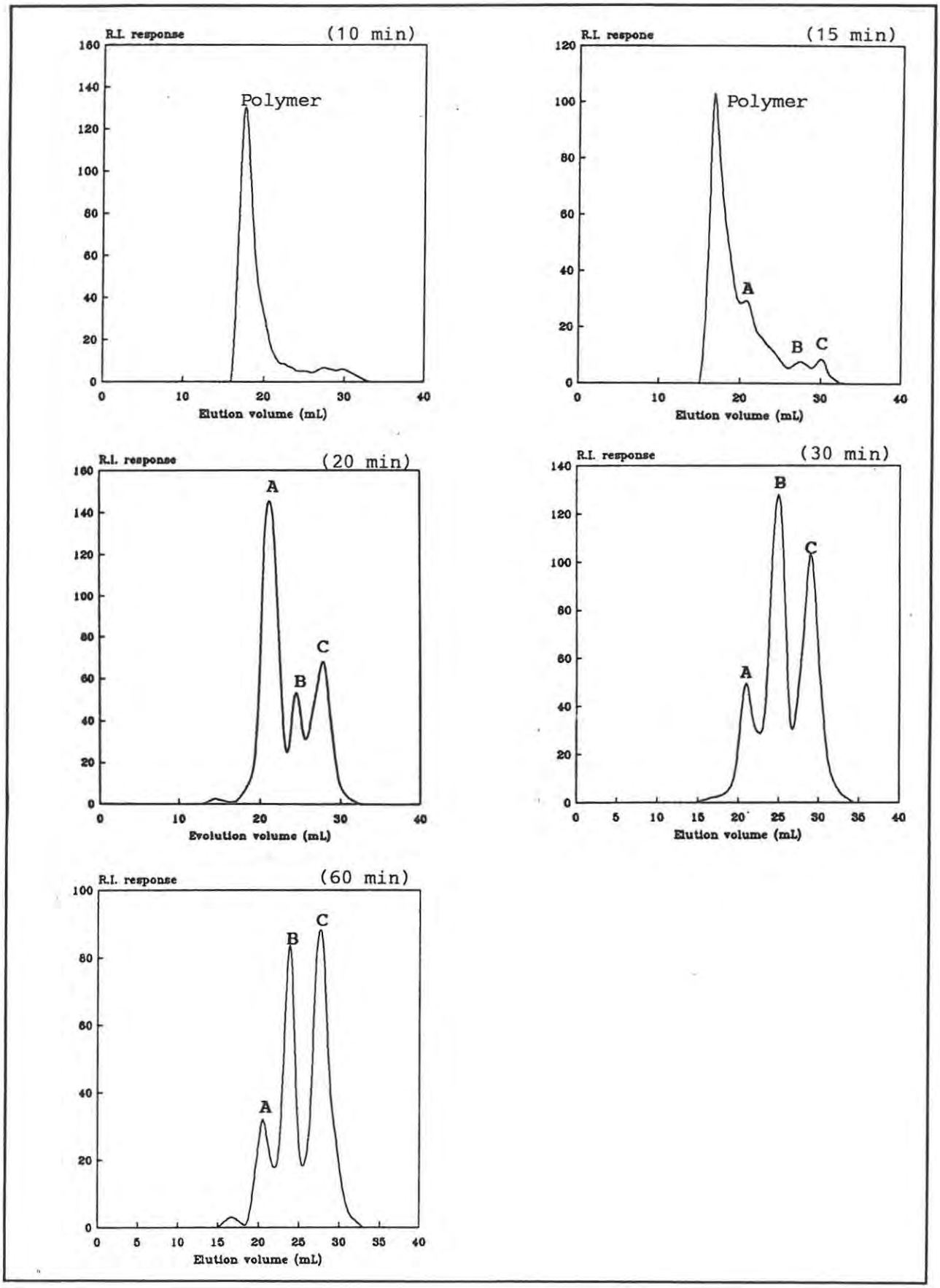


Fig. 5.8. Sephadex LH 20 gel permeation profiles of the products from reaction (i) above

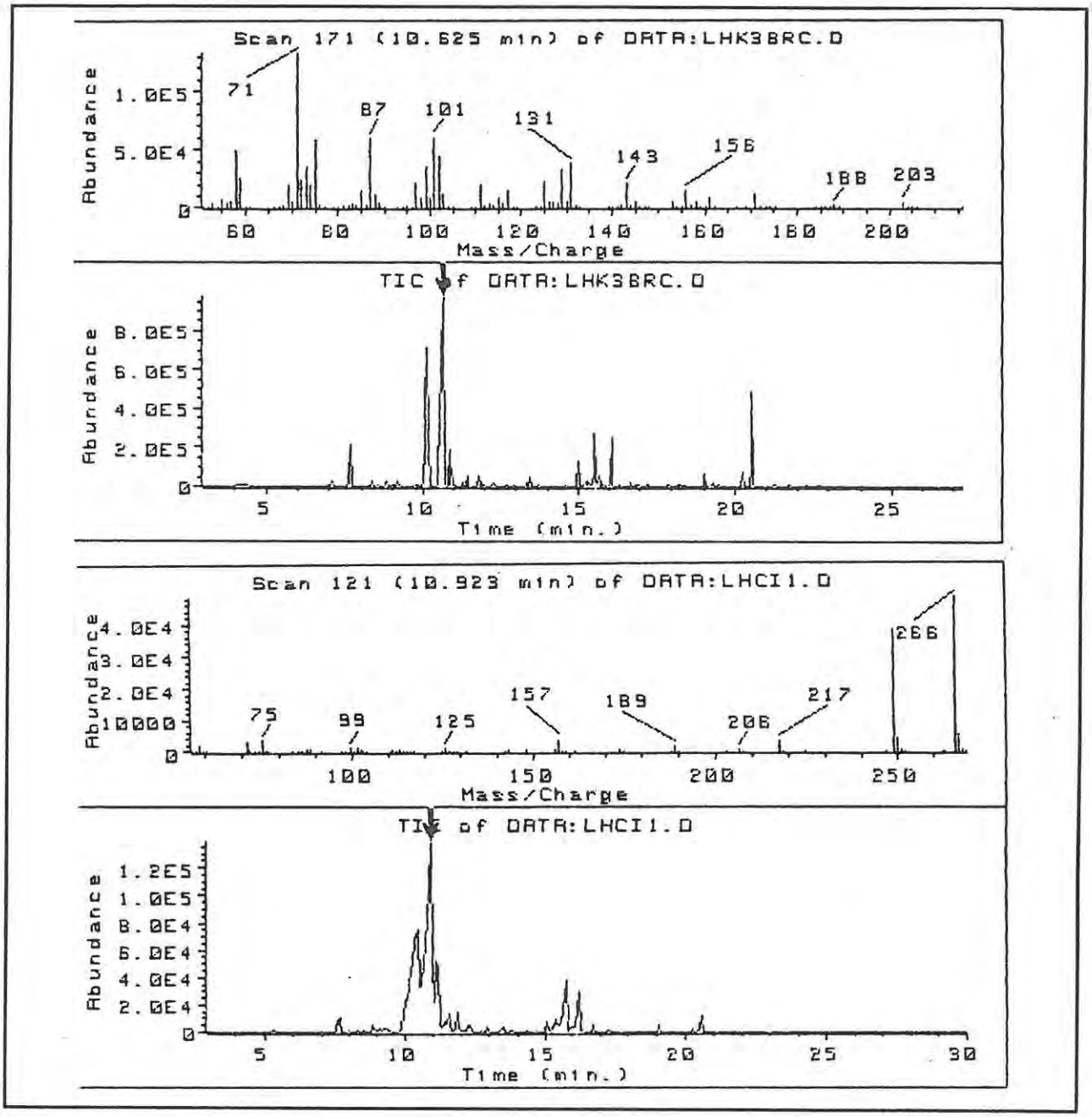


Fig. 5.9. E.i- and c.i.-mass spectra of compound **C1** of **Fraction C** Fig. 5.8

Compound C1 was identified as 4-*O*-acetyl-1,5-anhydro-2,3,6-tri-*O*-methyl-D-galactitol from the e.i.-mass spectrum by comparison with reference spectra[277]. The c.i.-mass spectrum is consistent with the above assignment showing a peak for the ammonium adduct ion (M+18) at m/z 266 and a pseudomolecular ion (M+1) at m/z 249.

Bennek *et al.*[281] have shown that methyl 2-(acetylmethylamino)-2-deoxy-3,4,6-tri-*O*-methyl-ß-D-glucopyranoside is converted to (1,2-dideoxy-3,4,6-tri-*O*-methyl-α-D-glucopyrano)-2,3-dimethyl-[2,1-d]-2-oxazolinium trifluoromethanesulphonate when treated with Et_3SiH in the presence of TMSOTf as catalyst. Subsequent quenching of the reaction with saturated aqueous $NaHCO_3$ resulted in hydrolysis of this oxazolinium salt to form 2-(acetylmethylamino)-2-deoxy-3,4,6-tri-*O*-methyl-D-glucopyranose. Therefore, compound **C2** was similarly derived from the ß-D-GalNAc residue in the permethylated K38 polysaccharide as follows:

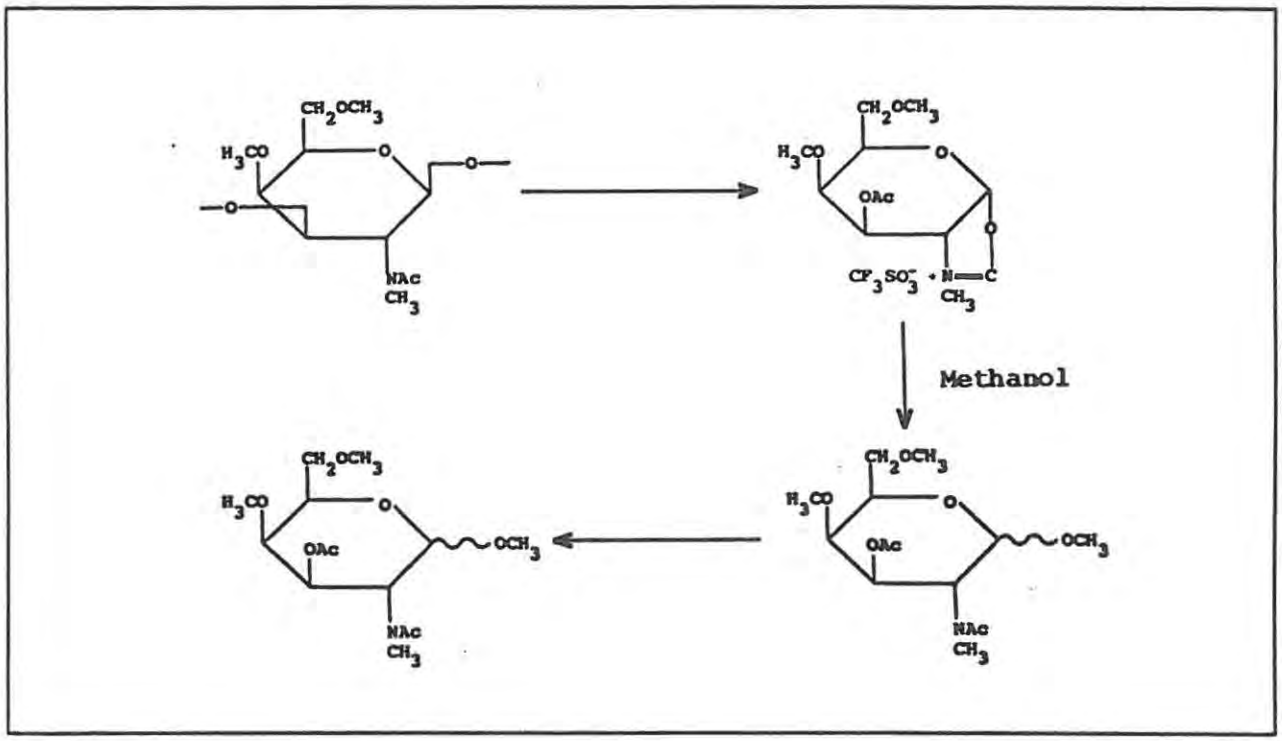
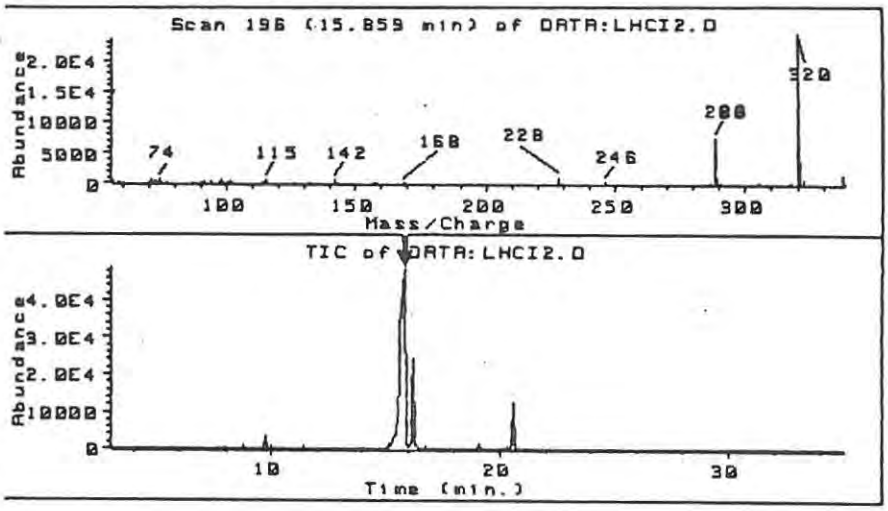
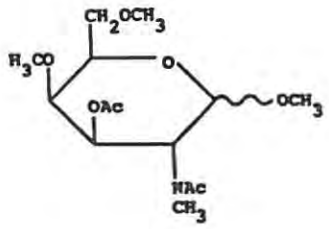


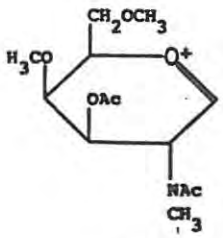
Fig. 5.10. **C2**: methyl 3-*O*-acetyl-2-(acetylmethylamino)-2-deoxy-4,6-di-*O*-methyl-D-galactopyranoside

This assignment is consistent with the results of c.i.-m.s. (Fig. 5.11) producing a pseudomolecular ion (M+1) at m/z 320. The intense ion at m/z 288 is attributed to the stable oxazolinium ion that forms *via* the facile displacement of the 1-*O*-methyl group[281]:





C2 - M+1, m/z 320



m/z 288

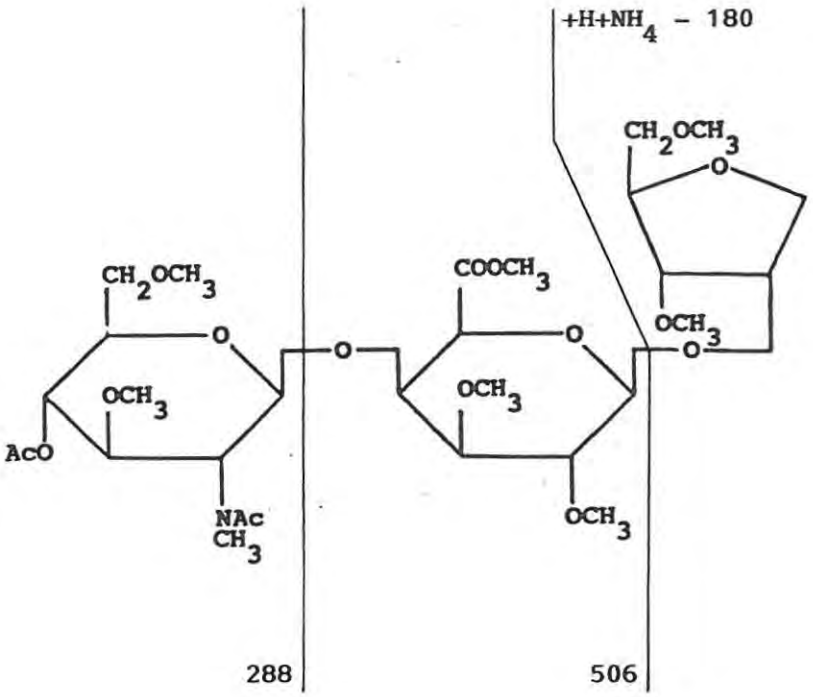
Fig. 5.11. C.i.-mass spectrum of compound **C2**

Fraction B was analyzed by c.i.-m.s. and produced the spectrum shown in Fig. 5.12. The intense M+1 and M+18 ions at m/z 668 and 685, respectively, indicate that the major component of **fraction B** is the trisaccharide anhydroalditol:

α-D-Glc*p*NAc-(1→4)-α-D-Glc*p*A-(1→2)-anhydroribitol (per-*O*-methylated)
4
↑
OAc

M+1, m/z 668

This is further substantiated by the fragment ions at m/z 180, 288, and 506 as indicated in Fig. 5.12.



M+1 - m/z 668

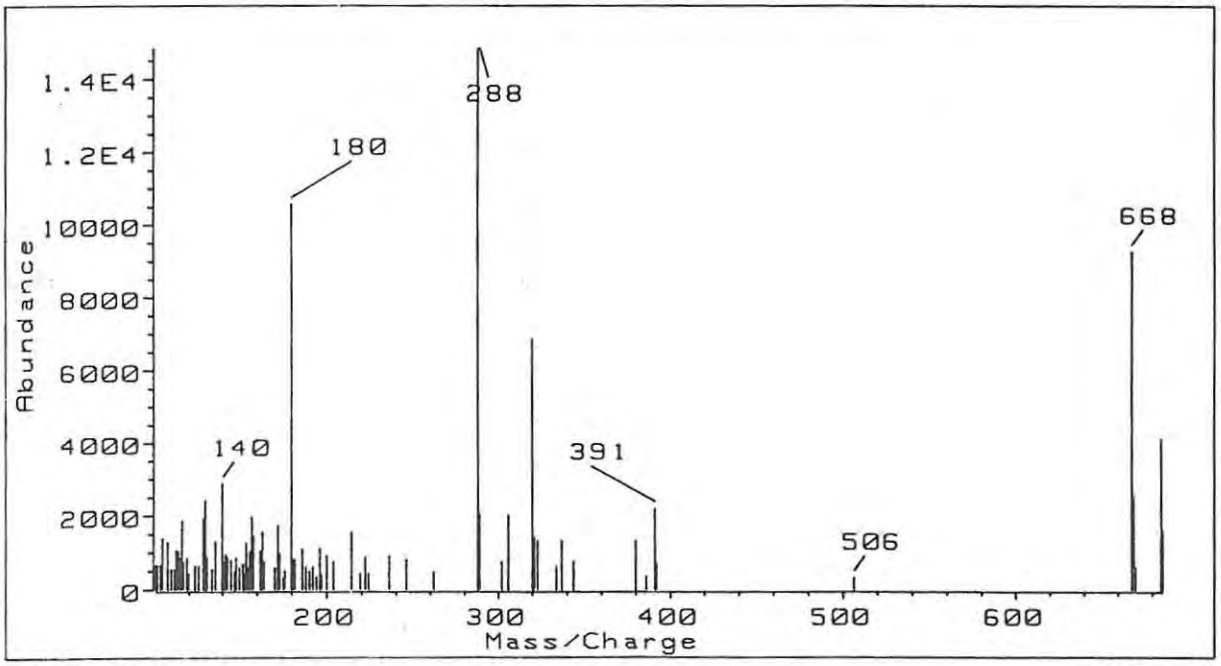


Fig. 5.12. C.i.-mass spectrum of compound B (fraction B Fig. 5.8)

Fraction A, which was not acetylated, was analyzed by c.i.-m.s. (Fig. 5.13) and f.a.b.-m.s. (Fig. 5.14) and the major component was found to be the anhydroribitol terminated pentasaccharide anhydroalditol (compound **A**) representative of the repeating-unit of the K38 polysaccharide:

ß-D-Galp-(1-3)-ß-D-GalpNAc-(1-4)-α-D-GlcpNAc-(1-4)-α-D-GalpA-(1-2)-anhydroribitol
4 (per-*O*-methylated)
1
OH

M+1, m/z 1075

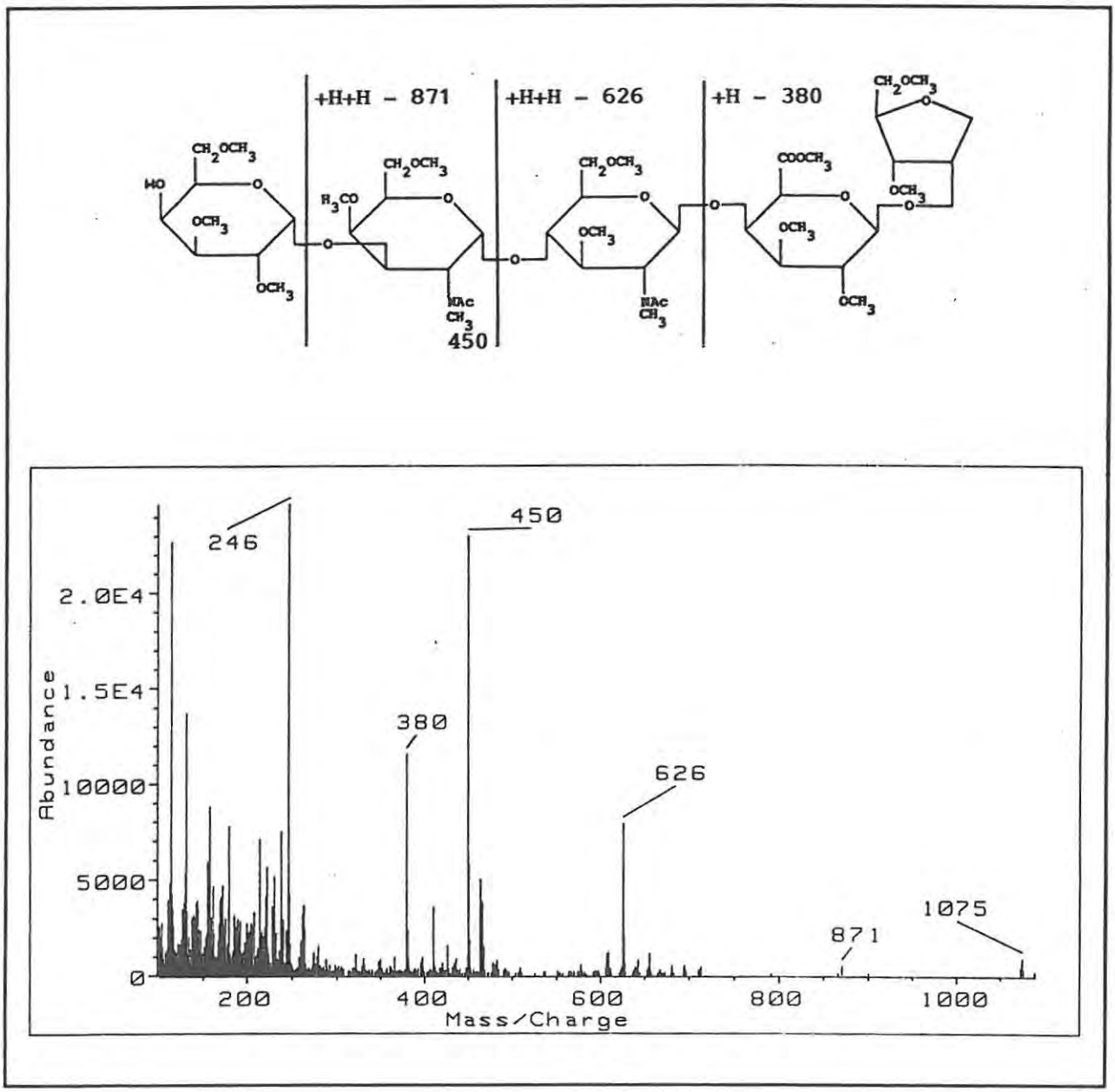


Fig. 5.13. C.i.-mass spectrum of compound **A** (**fraction A** Fig. 5.8)

F.a.b.-m.s. (Fig. 5.14) shows strong (M+Na) at m/z 1097, the (M+1) at m/z 1075, (M-1) at m/z 1073, and A-type fragment ions at m/z 450 $[HO\text{-}Hex\text{-}HexNAc]^+$ and m/z 695 $[HO\text{-}Hex\text{-}HexNAc\text{-}HexNAc]^+$, confirm that **fraction A** consists largely of the anhydroribitol terminated pentasaccharide:

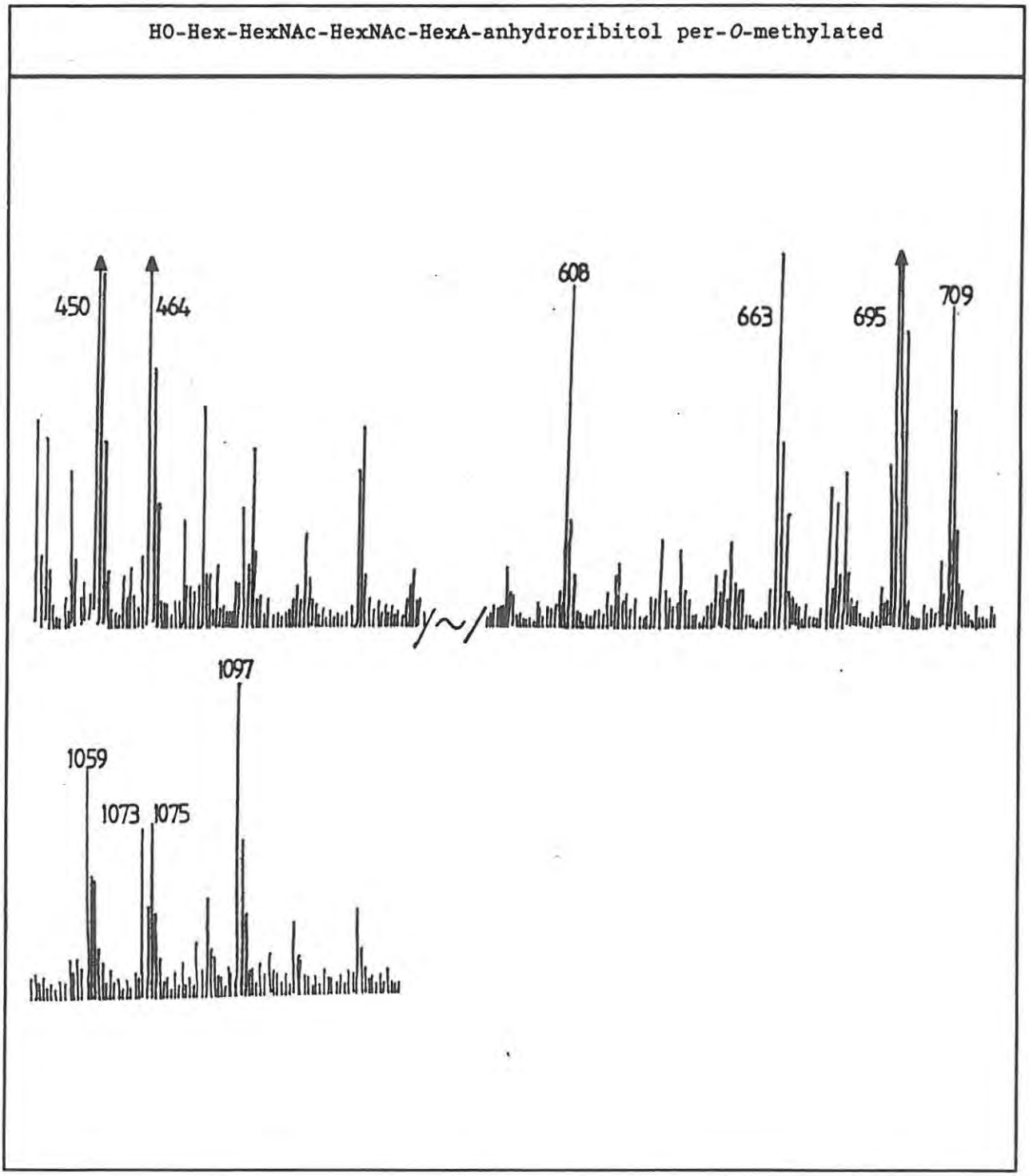


Fig. 5.14. F.a.b.-m.s. of compound **A**

(ii) *$BF_3.Et_2O$ as catalyst.*- Two reactions were carried out at room temperature for 30 and 60 min respectively. The products from these reactions are depicted in the gel permeation profiles in Fig. 5.15. The reaction products were found to be identical to those produced in **(i)** above. A comparison of the g.p.c. profiles in Fig. 5.9 and Fig. 5.15, shows that the $BF_3.Et_2O$ catalysed reactions proceed at a slower rate than the TMSOTf catalysed reactions. Hence we find a greater proportion of **fraction A** (pentasaccharide) after 1h in the presence of $BF_3.Et_2O$, than after 1h in the presence of TMSOTf (Fig. 5.15).

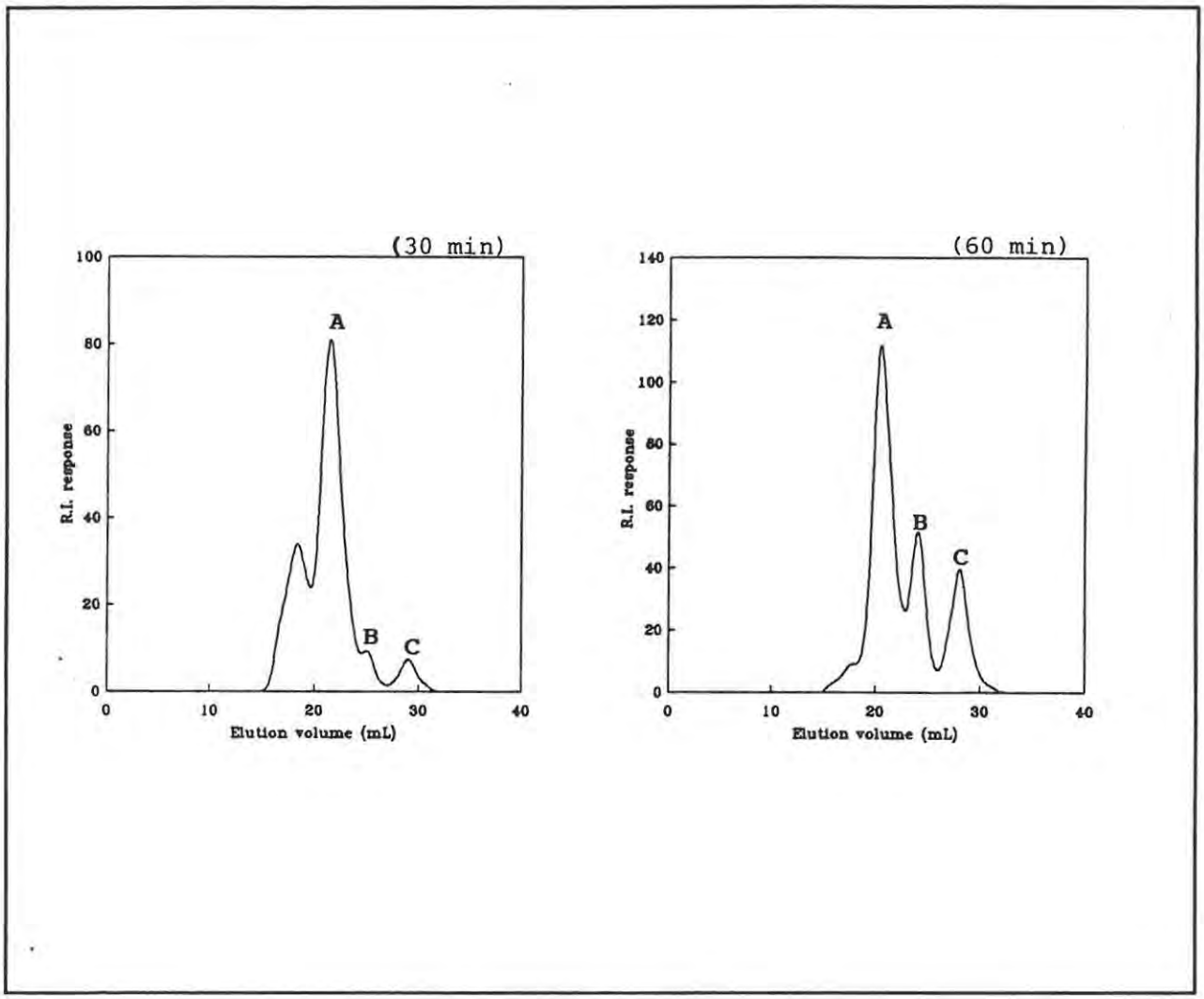


Fig. 5.15. Sephadex LH20 gel permeation profiles of the products of the $BF_3.Et_2O$ catlaysed SRC of the permethylated *E. coli* K38 capsular polysaccharide

5.2.3. Conclusion

Selective reductive cleavage of permethylated *E. coli* K38 polysaccharide was readily effected in the presence of both TMSOTf and $BF_3.Et_2O$ as catalyst. The pentasaccharide anhydroalditol **(A)**, trisaccharide anhydroalditol **(B)** and two monosaccharide derivatives **(C)** generated were readily isolated by g.p.c. and subjected to m.s. C.i.-m.s. of the pentasaccharide derivative produced spectra with high mass fragments of low intensity. This was attributed to pyrolytic decomposition at the high temperatures required to volatilize the sample. F.a.b.-m.s., however, produced spectra with pseudomolecular ions and structurally diagnostic A-type fragments of high-intensity. The glycosidic linkages of the ß-Gal*p* and ß-Glc*p*NAc residues are similarly susceptible to cleavage under reductive cleavage conditions, irrespective of catalyst, because no significant amounts of HO-Hex-HexNAc-*O*-Me or HO-HexNAc-HexNAc-HexA-anhydroribitol were detected. The ß-Rib*f* was therefore the most labile residue followed by the ß-Gal*p* and ß-Gal*p*NAc residues, which were equally susceptible to cleavage, under the reaction conditions employed in the above study.

The results of c.i. and f.a.b.-m.s. confirm that the *E. coli* K38 capsular polysaccharide is made up of linear pentasaccharide repeating-units with the general structure -Hex-HexNAc-HexNAc-HexA-Pent-. Furthermore, these results indicate that SRC is preferred to PRC because of the complexity of the reaction products obtained by PRC (see section 5.1). However, these studies demonstrate that reductive cleavage can be applied to polysaccharides in general and that selectivity, although advantageous, is not essential for the experiment to succeed. The technique is particularly valuable when small amounts of material are available, as sequence can be determined by a f.a.b.-m.s. methanolysis experiment[295].

5.2.4. Experimental

General experimental methods.- The capsular polysaccharide used for this reductive cleavage study was previously isolated and purified as described in section 4.3. The polymer was methylated by a modified Hakomori procedure[80] using potassium dimsyl[124] and methyliodide to produce permethylated polysaccharide as described in section 4.1.

Reductive cleavage.- Reactions were carried out on 5 mg samples of permethylated K38 polysaccharide in silanized reactivials, as described in section 5.1.4 (**Method 3**). The reagent mixture was standardised throughout the experiments and was made up of 250 μL CH_2Cl_2, 10 μL catalyst ($BF_3.Et_2O$ or TMSOTf) and 10 μL Et_3SiH.

G.p.c.- see section 5.1.4

G.l.c.- Instrumentation used for g.l.c. and g.l.c.-m.s. is described in section 4.1. In each case a Hewlett Packard fused-silica HP-1 bonded-phase capillary column (12 m x 0.2 mm) with a film thickness of 0.33 μm was used to separate the monosaccharide derivatives (fraction C). The penta- and trisaccharide derivatives were introduced into the mass spectrometer *via* the d.i.p. inlet or *via* the g.c. using a 3 m length of uncoated fused-silica capillary (oven temperature 250°). Ammonia was used as the reagent gas when operating in the c.i. mode. The instrument was set at 70 eV, with a source temperature of 200° and total source pressure of 0.6 torr for acquisition of the c.i.-mass spectra discussed above (section 5.2.2).

REFERENCES

1. A.P. Dochez and O.T. Avery, *J.Exp.Med.*, 26 (1917) 477-493.
2. C.T. Bishop and H.J. Jennings, *The Polysaccharides*, Vol.1, pg.291-330, Ed. G.O.Aspinall, Academic Press, N.Y.
3. S.M. Hammond, P.A. Lambert, and A.N. Rycroft, *The Bacterial Cell Surface*, pg.147-193, Croom Helm, London and Sydney.
4. I. Ørskov, F. Ørskov, B. Jann, and K. Jann, *Bacterial Reviews* 41 (1977) 667-710.
5. G.G.S. Dutton and L.A.S. Parolis, *Biomedical and Biotechnological Advances in Industrial Polysaccharides*, pg. 223-240, V.Cresceni, I.C.M. Dea, S. Paoletti, S.S. Stivala, and I.W. Sutherland (Ed.), Gordon and Breach, N.Y., 1989.
6. K. Jann, B. Jann, F. Ørskov, I. Ørskov, and K.F. Schneider, *European J. Biochem.*, 5 (1968) 456-465.
7. E. Altman and G.G.S. Dutton, *Carbohydr. Res.*, 138 (1985) 293-303.
8. K. Jann, B. Jann, F. Ørskov, I. Ørskov, and O. Westphal, *Biochem. Zeits.*, 342 (1965) 1-22.
9. M.-L. Rodriguez, B. Jann, and K. Jann, *Carbohydr. Res.*, 196 (1990) 101-109.
10. P. Hofmann, B. Jann, and K. Jann, *European J. Biochem.*, 147 (1985) 601-609.
11. K. Jann and A. Schmidt, *FEMS Micro. Letters*, 7 (1980) 79-81.
12. A. Bax, M.F. Summers, W. Egan, N. Guirgis, R. Schneerson, J.B. Robbins, F. Ørskov, I. Ørskov and W.F. Vann, *Carbohydr. Res.*, 173 (1988) 53-64.
13. F.P. Tsui, R.A. Boykins, and W. Egan, *Carbohydr. Res.*, 102 (1982) 263-271.
14. T. Dengler, K. Himmelspach, B. Jann, and K. Jann, *Carbohydr. Res.*, 178 (1988) 191-201.
15. L. Tarcsay, B. Jann, and K. Jann, *European J. Biochem.*, 23 (1971) 505-514.
16. L.M. Beynon and G.G.S. Dutton, *Carbohydr. Res.*, 200 (1990) 457-468.
17. A.N. Anderson, H. Parolis, and L.A.S. Parolis, *Carbohydr. Res.*, 163 (1987) 81-90.
18. A.N. Anderson and H. Parolis, *Carbohydr. Res.*, 188 (1989) 157-168.
19. B. Jann, P. Hofmann, and K. Jann, *Carbohydr. Res.*, 120 (1983) 131-141.
20. T. Dengler, B. Jann, and K. Jann, *Carbohydr. Res.*,150 (1986) 233-240.
21. P. Hofmann, B. Jann, and K. Jann, *Carbohydr. Res.*, 138 (1984) 1-11.
22. M.L. Rodriguez, B. Jann, and K. Jann, *Carbohydr. Res.*, 173 (1988) 243-253.
23. H. Parolis, L.A.S. Parolis, S.M.R. Stanley, and G.G.S. Dutton, *Carbohydr. Res.*, 205 (1990) 361-370.
24. F. Kaufmann, *Acta Path. Microbiol. Scand.*, 20 (1943) 21-44.
25. K. Jann and B. Jann, *Reviews Inf. Dis.*, 9 (1987) S517-S526.
26. E.J. McGuire and S.B. Binkley, *Biochemistry*, 3 (1964) 247-251.
27. K. Jann, B. Jann, A.M. Schmidt, and W.F. Vann, *J. Bacteriol.*, 143 (1980) 1108-1115.
28. W. Fischer, M.A. Schmidt, and B. Jann, *Biochemistry*, 21 (1982) 1279-1284.
29. W.F. Vann, A.M. Schmidt, B. Jann, and K. Jann, *European J. Biochem.*, 116 (1981) 359-364.
30. P. Messner and F.M. Unger, *Biochem. Biophys. Res. Comm.*, 96 (1980) 1003-1010.
31. H.J. Jennings, K.G. Rossell, and K.G. Johnson, *Carbohydr. Res.*, 105 (1982) 45-56.
32. L.A.S. Parolis, H. Parolis, and G.G.S. Dutton, *Carbohydr. Res.*, 193 (1989) 157-163.

33. G.G.S. Dutton, H. Parolis, and L.A.S. Parolis, *Carbohydr. Res.*, 170 (1987) 193-206.
34. M.A. Schmidt and K. Jann, *European J. Biochem.*,131 (1983) 509-517.
35. W. Vann and K. Jann, *Inf. and Immun.*, 25 (1979) 85-92.
36. W.F. Vann, T. Sodderstrom, W. Egan, F.-P. Tsui, R. Schneerson, I. Ørskov, and F. Ørskov, *Infect. Immun.*, 30 (1983) 623-629.
37. M. Lenter, B. Jann, and K. Jann, *Carbohydr. Res.*, 197 (1990) 197-204.
38. M.C. Lenter, B. Jann, and K. Jann, *Carbohydr. Res.*, 208 (1990) 139-144.
39. B. Jann, R. Ahrens, T. Dengler, and K. Jann, *Carbohydr. Res.*, 177 (1988) 273-277.
40. L.M. Beynon and G.G.S. Dutton, *Carbohydr. Res.*, 179 (1988) 419-423.
41. A.K. Chakraborty, *Makromol. Chem.*, 183 (1982) 2881-2887.
42. L.B. Lhan, B. Jann, and K. Jann, *European J. Biochem.*, 21 (1971) 226-234.
43. Y-M Choy, F. Fehmel, N. Frank, and S. Stirm, *J. Virol.*, 16 (1975) 581-590.
44. D. Hungerer, K. Jann, B. Jann, F. Ørskov, and I. Ørskov, *European J. Biochem.*, 2 (1967) 115-126.
45. A.K. Chakraborty, H. Friebolin, and S. Stirm, *J. Bacteriol.*, 141 (1980) 971-972.
46. G.G.S. Dutton, A. Kuma-Mintah, and H. Parolis, *Carbohydr. Res.*, 197 (1990) 171-180.
47. G. Annison, G.G.S. Dutton, and E. Altman, *Carbohydr. Res.*, 168 (1987) 89-102.
48. G.G.S. Dutton and A. Kuma-Mintah, *Carbohydr. Res.*, 169 (1987) 213-220.
49. H. Parolis, L.A.S. Parolis, and S.M.R. Stanley, *Carbohydr. Res.*, 175 (1988) 77-83.
50. H. Parolis, L.A.S. Parolis, and R.D. Venter, *Carbohydr. Res.*, 185 (1989) 225-232.
51. H. Niemann, A.K. Chakraborty, H. Friebolin, and S. Stirm, *J. Bacteriol.*, 331 (1978) 390-391.
52. G.G.S. Dutton, D.N. Karunaratne, and A.V.S. Lim, *Carbohydr. Res.*, 183 (1988) 111-122.
53. G.G.S. Dutton, Z. Lam, and A.V.S. Lim, *Carbohydr. Res.*, 183 (1988) 123-125.
54. B. Jann, T. Dengler, and K. Jann, *FEMS Micro. Letters*, 29 (1985) 257-261.
55. H. Parolis, L.A.S. Parolis, S.M.R. Stanley, and G.G.S. Dutton, *Carbohydr. Res.*, 200 (1990) 449-456.
56. R. Ahrens, B. Jann, K. Jann, and H. Brade, *Carbohydr. Res.*, 179 (1988) 223-231.
57. K. Jann, B. Jann, F. Ørskov, and I. Ørskov, *Biochem. Zeits.*, 346 (1966) 368-385.
58. W. Egan, T-Y Liu, D. Dorrow, J.S. Cohen, J.D. Robbins, E.C. Gotschlich, and J.R. Robbins, *Biochemistry*, 16 (1977) 3687-3692.
59. M.R. Lifely, J.C. Lindon, J.M. Williams, and C. Moreno, *Carbohydr. Res.*, 143 (1985) 191-205.
60. T. Dengler, B. Jann, and K. Jann, *Carbohydr. Res.*, 142 (1985) 269-276.
61. F-P. Tsui, W., M.F. Summers, R.A. Byrd, R. Schneerson, and J.B. Robbins, *Carbohydr. Res.*, 173 (1988) 65-74.
62. A.H. De Bruin, *M.Sc. Thesis*, Rhodes University, 1991.
63. G.G.S. Dutton, *Adv. Carbohydr. Chem. Biochem.*, 28 (1973) 11-160.
64. G.G.S. Dutton, *Adv. Carbohydr. Chem. Biochem.*, 30 (1974) 9-110.

65. G.D. McGinnis and P. Fang, *Methods Carbohydr. Chem.*, 8 (1980) 33-43.
66. K.B. Hicks, *Adv. Carbohydr. Chem.*, 46 (1988) 17-72.
67. A.V. Ben-Bassat and E. Grushka, *J. Liquid Chromatog.*, 14 (1991) 1051-1111.
68. S.C. Churms, *Adv. Carbohydr. Chem. Biochem.*, 25 (1970) 13-51.
69. K.A. Granath, *Methods Carbohydr. Chem.*, 5 (1965) 20-28.
70. R.L. Whistler and A.K.M. Anisuzzaman, *Methods Carbohydr. Chem.*, 8 (1980) 45-53.
71. J.R. Dyer, *Methods Biochem. Analy.*, 3 (1956) 111.
72. L. Warren, *J. Biol. Chem*, 234 (1959) 1971.
73. I. Werner and L. Odin, *Acta. Soc. Med. Upsalien*, 57 (1952) 230.
74. S. Moore and W.H. Stein, *J.Biol. Chem.*, 211 (1954) 907.
75. G.O. Aspinall and A. Canas-Rodriguez, *J. Chem. Soc.*, (1958) 4020.
76. A.J. Charlson and A.S. Perlin, *Can. J. Chem.*, 34 (1956) 1200.
77. Z. Dische, *J. Biol. Chem.*, 167 (1947) 189.
78. R. Shields and W. Burnett, *Anal. Chem.*, 32 (1960) 885.
79. P.A. Sandford and H.E. Conrad, *Biochemistry*, 5 (1966) 1508-1517.
80. S.J. Hakomori, *Biochemistry.*, 55 (1964) 205.
81. C.G. Hellerquist, B. Lindberg, S. Svensson, T. Holme, and A.A. Lindberg, *Carbohydr. Res.*, 8 (1968) 43.
82. J.L. Strominger, T.J. Park, and R.E. Thompson, *J. Biol. Chem.*, 234 (1959) 3263.
83. G. Avigad, *Carbohydr. Res.*, 11 (1969) 119.
84. H. Penel, *Helv. Chim. Acta.*, 30 (1947) 1523.
85. J.H. Sloneker and D.G. Orentas, *Nature*, 194 (1962) 478.
86. H. Björndal, B. Lindberg, and S. Svensson, *Acta. Chem. Scand.*, 21 (1967) 1801-1804.
87. F.G. Fischer, H. Dörfel, *Hoppe-Seyler's Z. Physiol. Chem.*, 301 (1955) 224.
88. D. Hungerer, K. Jann, B. Jann, F. Ørskov, and I. Ørskov, *European J. Biochem.*, 2 (1967) 115-126.
89. P.M. Dey and J.B. Pridham, *Adv. Enzymol.*, 36 (1972) 91-131.
90. H.Halvorson, *Methods Enzymol.*, 8 (1966) 559-562.
91. S. Hestrim, D.S. Feingold, and M. Schramm, *Methods Enzymol.*, 1 (1955) 231-257.
92. G.A. Levvy and C.A. Marsh, *The Enzymes*, 4 (1961) 392-407, Ed. P.D. Boyer, H. Lardy and K. Myrback, Academic Press, New York.
93. S. Stirm and E. Freund-Molbert, *J. Virol.*, 8 (1971) 330-342.
94. J.S. Sawardeker, J.H. Sloneker, and A. Jeanes, *Anal. Chem.*, 12 (1965) 1602-1604.
95. T-Y Liu, E.C. Gotschlich, F.T. Dunne, and E.K. Jönssen, *J. Biol. Chem.*, 246 (1971) 4703.
96. I.J. Goldstein, G.W. Hay, B.A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1965) 361-370.
97. K. Reske and K. Jann, *European J. Biochem.*, 31 (1972) 320-328.
98. P.J. Anderson, *J. Chromatogr.*, 21 (1966) 163-164.
99. P. Prehm, S. Stirm, B. Jann, and K. Jann, *European J. Biochem.*, 56 (1975) 41-55.
100. B. Jann, K. Jann, G. Schmidt, I. Ørskov, and F. Ørskov, *European J. Biochem.*, 15 (1970) 29-39.
101. L. Warren, *J. Biol. Chem.*, 234 (1959) 1971-1975.

102. Z. Dische, *Methods Carbohydr. Chem.*, 1 (1962) 484-488.
103. W. Dröge, V. Schman, O. Lüderitz, and O. Westphal, *European J. Biochem.*, 14 (1970) 175-184.
104. W. Dröge, V. Lehmann, O. Lüderitz, and O. Westphal, *European J. Biochem.*, 14 (1970) 175-184.
105. M.J. Osborne, *Proc. Natl. Acad. Sci.*, 50 (1963) 499-506.
106. S. Honda, N. Yamauchi, and K. Kakehi, *J. Chromatogr.*, 169 (1979) 287-293.
107. S. Roseman and I. Daffmer, *Anal. Chem.*, 28 (1956) 1743-1746.
108. B.A. Dmitriev, Y.A. Knirel, N.K. Kotchetkov, and I.L. Hofman, *Carbohydr. Res.*, 44 (1975) 77-85.
109. C. Erbing, K. Granath, L. Kenne, and B. Lindberg, *Carbohydr. Res.*, 47 (1976) C5-C7.
110. R.L. Taylor, J.E. Schively, and H.E. Conrad, *Methods Carbohydr. Chem.* Carbohydr. Chem., 7 (1979) 149-151.
111. A.K. Bhattacharjee, H.J. Jennings, and C.P. Kenny, *Biochemistry*, 17 (1978) 645-651.
112. K. Leontein, B. Lindberg, and J. Lönngren, *Carbohydr. Res.*, 62 (1978) 359-362.
113. J. Ludowieg and A. Dorfman, *Biochim. Biophys. Acta.*, 38 (1960) 212.
114. H. Thurow, Y-M Choy, N. Frank, H. Niemann, S. Stirm, *Carbohydr. Res.*, 41 (1975) 241-256.
115. H.Björndal, C.C. Hellerqvist, B. Lindberg, S. Svensson, *Angew. Chem.*, 82 (1970) 643.
116. J. Le Cocq and C.F. Ballou, *Biochemistry.*, 3 (1964) 976-980.
117. W. Fischer, I. Ishizuka, H.R. Landgraf, and J. Hermann, *Biochim. Biophys. Acta.*, 296 (1973) 527-545.
118. R.A. Boykins and T.-Y. Liu, *J. Biochem. Biophys. Methods*, 2 (1980) 71-78.
119. F.-P. Tsui, R. Schneerson, and W. Egan, *Carbohydr. Res.*, 88 (1981) 85-92.
120. F.-P. Tsui, R. Schneerson, R.A. Boykins, A.B. Karpas, and W.Egan, *Carbohydr. Res.*, 97 (1981) 293-306.
121. E.A. Kabat and M.M. Mayer, *Exp. Immunochem.*, 2nd Edition pg. 520-525, C.C. Thomas, Springfield.
122. V.S. Waravdekar and L.D. Saslaw, *J. Biol. Chem.*, 234 (1959) 1945-1950.
123. F. Snyder and N. Stephens, *Biochim. Biophys. Acta.*, 34 (1959) 244-254.
124. L.R. Phillips and B.A. Fraser, *Carbohydr. Res.*, 90 (1981) 149-152.
125. J. Hofman and B. Lindberg, *Methods Carbohydr. Chem.*, 8 (1980) 117-122.
126. P.A.J. Gorin and M. Mazurek, *Carbohydr. Res.*, 48 (1976) 171-186.
127. B. Lindberg, *Methods Enzymol.*, 28 (1972) 178-195.
128. I. Fromme and H. Beilharz, *Anal. Biochem.*, 84 (1978) 347-353.
129. G.G.S. Dutton and T.E. Folkman, *Carbohydr. Res.*, 78 (1980) 305-315.
130. K. Bock and C. Pedersen, *Adv. Carb. Chem. Biochem.*, 41 (1983) 27-66.
131. B.N. Ames, *Methods Enzymol.*, 8 (1966) 115-118.
132. H.E. Conrad and R.L. Taylor, *Biochemistry*, 11 (1972) 1383-1388.
133. B. Lindberg and J. Lönngren, *Methods Enzymol.*, 50 (1978) 3-33.
134. S. Hanessian and T. Haskell, *J. Biol. Chem.*, 329 (1964) 2758-2764.
135. J.L. Strominger, J. L. Park, and R.E. Thompson, *J. Biol. Chem.*, 234 (1959) 3263-3274.
136. S.L. Patt, *J. Magn. Reson.*, 46 (1982) 535-539.
137. R. Benn and H. Günther, *Angew. Chem. Int. Ed. Engl.* , 22 (1983) 350-381.
138. Y.M. Choy and G.G.S. Dutton, *Abstracts, XIII Int. Carb. Symp.*, (1986).
139. A. Isogai, A. Ishizu, J. Nakano, S. Eda, and K. Kato, *Carbohydr. Res.*, 138 (1985) 99-108.
140. T. Purdie and J.C. Irvine, *J. Chem. Soc.*, 83 (1903) 1021-1037.
141. A. Liptàk, P. Nànàsi, A. Neszmelyi, and H. Wagner, *Tetrahedron*, 36 (1980) 1261-1268.
142. O.A. Gansow and W. Schittenhelm, *J. Am. Chem. Soc.*, 93 (1971) 4294-4295.
143. A.I. Vogel, *Textbook of Quantitative Inorganic Analysis*, 4th edn., Longmans, London, (1979) 756.

144. R. Kuhn, H. Trischmann, and I. Low, *Angew. Chem.*, 67 (1955) 32.
145. G.D. McGinnis, *Carbohydr. Res.*, 108 (1982) 284-292.
146. M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350.
147. P.J. Garegg, P.-E. Jansson, B. Lindberg, F. Lindh, J. Lönngren, I. Kvarnström, and W. Nimmich, *Carbohydr. Res.*, 78 (1980) 127-132.
148. B. Lindberg, J. Lönngren, and J.L. Thompson, *Carbohydr. Res.*, 28 (1973) 351-357.
149. K.M. Aalmo, M.F. Ishak, and T.J. Painter, *Carbohydr. Res.*, 63 (1978) c3-c7.
150. D. Rieger-Hug and S.Stirm, *Virology*, 113 (1981) 363-378.
151. G.M. Bebault, J.W. Berry, Y.M. Choy, G.G.S. Dutton, N. Funnell, L.D. Hayward, and A.M. Stephan, *Can. J. Chem.*, 51 (1973) 324-326.
152. J.H. Pazur, H.R. Knull, and G.E. Chevalier, *J. Carbohydr. Nucleosides Nucleotides*, 4 (1977) 129-146.
153. T. Narui, K. Takahashi, M. Kobayashi, and S. Shibata, *Carbohydr. Res.*,103 (1982) 293-295.
154. K. Stellner, H. Saito, and S.-I. Hakomori, *Arch. Biochem. Biophys.*, 155 (1973) 464-472.
155. W.E. Trevelyan, D.P. Proctor, and J.S. Harrison, *Nature (London)*, 166 (1950) 444-445.
156. H.T. Gordon, W. Thornburg, and L.N. Werum, *Anal. Chem.*, 28 (1956) 849-855.
157. A.K. Bhattacharjee, H.J. Jennings, C.P. Kenny, A. Martin, and I.C.P. Smith, *Can. J. Biochem.*, 54 (1976) 1-8.
158. A.S. Shashkov, A.J. Evstigneev, and V. Derevitskaya, *Carbohydr. Res.*, 72 (1979) 215-217.
159. R. Bax and R. Freeman, *J. Magn. Reson.*, 42 (1981) 164-168.
160. A. Bax, *Two Dimensional Nuclear Magnetic Resonance in Liquids*, D. Reidel Press, Boston, (1982).
161. A. Bax, W. Egan, and P. Kováč, *J. Carbohydr. Chem.*, 3 (1984) 593-611.
162. L.D. Hall and G.A. Morris, *Carbohydr. Res.*, 82 (1980) 175-184.
163. W. Egan, F-P. Tsui, and R. Schneerson, *Carbohydr. Res.*, 79 (1980) 271-277.
164. A.R. Archibald, J. Baddiley, and J.G. Buchanan, *Biochem. J.*, 81 (1961) 124-134.
165. L. Lerner and A. Bax, *Carbohydr. Res.*, 166 (1987) 35-46.
166. R. A. Byrd, W. Egan, M. F. Summers, and A. Bax, *Carbohydr. Res.*, 166 (1987) 47-58.
167. H. Thurow, H. Nieman, and S. Stirm, *Carbohydr. Res.*, 41 (1975) 257-271.
168. J.A. Cifonelli, *Methods Carbohydr. Chem.*, 7 (1976) 139-141.
169. G.G.S. Dutton and M.-T. Yang, *Can. J. Chem.*, 59 (1977) 179-192.
170. H.E. Conrad, *Methods Carbohydr. Chem.*, 6 (1972) 361-364.
171. L. Kenne and B. Lindberg, *Methods Carbohydr. Chem*, 8 (1980) 295-296.
172. G.O. Aspinall and R.J. Ferrier, *Chem. Ind. (London)*, (1957) 1216.
173. K. Bock and C. Pederson, *J.Chem.Soc., Perkin Trans.*, 2 (1974) 293-297.
174. G.G.S. Dutton, G. Eigendorf, Z. Lam, and A.V.S. Lim, *Biomed. Environ. Mass Spectrom.*, 15 (1988) 459-460.
175. V.N. Reinhold, *Methods Enzymol.*, 138 (1987) 59-84.
176. A. Tacken, E.T. Rietschel, and H. Brade, *Carbohydr. Res.*, 149 (1986) 263-277.
177. G. Snatzke, *Angew. Chem.*, 91 (1979) 380-393.
178. G.O. Aspinall and K.G. Rosell, *Carbohydr. Res.*, 57 (1977) c23-c26.
179. J.M. Lau, M. McNeil, A.G. Darvill, and P. Albersheim, *Carbohydr. Res.* 168 (1977) 219-243.
180. G.G.S. Dutton, J.L. Di Fabio, D.M. Leek, E.H. Merrifield, J.R. Nunn, and A.M. Stephen, *Carbohydr. Res.*, 97 (1981) 127-138.
181. P. Prehm, *Carbohydr. Res.*, 78 (1980) 372-374.
182. A. Bax and R. Freeman, *J. Magn. Reson.*, 44 (1981) 542-561.
183. A. Bax and G. Drobny, *J. Magn. Reson.*, 61 (1985) 306-320.

184. R. Baumann, G. Wider, R.R. Ernst, and K. Wüthrich, *J. Magn. Reson.*, 44 (1981) 402-406.
185. A. Bax and G. Morris, *J. Magn. Reson.*, 42 (1981) 501-505.
186. D. Brown, T.T. Nakashima, and D.L. Rabenstein, *J. Magn. Reson.*, 45 (1981) 302-314.
187. R. Freeman and G. Morris, *Bull. Magn. Reson.*, 1 (1979) 5-26.
188. A.J. Mort and W.D. Bauer, *J. Biol. Chem.*, 257 (1982) 1870-1875.
189. A.J. Mort and D.T.A. Lamport, *Anal. Biochem.*, 82 (1979) 289-309.
190. J. Dabrowsky, P. Hanfland, H. Egge, and N. Dabrowski, *Arch. Biochem. Biophys.*, 210 (1981) 405-411.
191. A. Kumar, R.R. Ernst, and K. Wuethrich, *Biochem. Biophys. Res. Commun.*, 95 (1980) 1-6.
192. J.-R. Neeser and T.F. Schweizer, *Anal. Biochem.*, 142 (1984) 58-67.
193. A. Bax and D.G. Davis, *J. Magn. Reson.*, 65 (1985) 355-360.
194. P.L. Hackland, H. Parolis, and L.A.S. Parolis, *Carbohydr. Res.*, In Press.
195. G. Ashwell, *Methods Enzymol.*, 3 (1957) 73-105.
196. J.C. Lindon, J.G. Vinter, M.R. Lifely, and C. Moreno, *Carbohydr. Res.*, 133 (1984) 59-74.
197. D. Doddrell, V. Glushko, and A. Allerhand, J. Chem. Phys., 56 (1972) 3683-3689.
198. G.G.S. Dutton and M.-T. Yang, *Carbohydr. Res.*, 59 (1977) 179-193.
199. G.O. Aspinall in *The Polysaccharides*, Vol. 1, pg. 19-34, G.O. Aspinall (Ed.), Academic Press, N.Y., 1982.
200. J.E. Scott, *Methods Carbohydr. Chem.*, 5 (1965) 38-44.
201. O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83-91.
202. G.G.S. Dutton and K. Okutani, *Carbohydr. Res.*, 86 (1980) 259-271.
203. G.O. Aspinall in *The Polysaccharides*, Vol. 1, pg.35-131, G.O. Aspinall (Ed.), Academic Press, N.Y., 1982.
204. G.O. Aspinall, *Methods Carbohydr. Chem.*, 5 (1965) 397-400.
205. D. Aminoff, W.W. Binkley, R. Schaffer, and R.W. Mowry, in *The Carbohydrates, Chem. and Biochem.* , Vol. 2B, pg. 739-807, W. Pigman and D. Horton (Ed.), Academic Press, N.Y., 1970.
206. L.D. Hall, *Adv. Carbohydr. Chem.*, 19 (1964) 51-93.
207. B. Coxon, *Adv. Carbohydr. Chem. Biochem.*, 27 (1972) 7-83.
208. L.D. Hall in *The Carbohydrates, Chem. and Biochem.*, Vol. 1B, pg. 1299-1326, W. Pigman and D. Horton (Ed.), Academic Press, N.Y., 1980.
209. A.S. Perlin and B. Casu in *The Polysaccharides*, Vol. 1, pg. 133-193, G.O. Aspinall (Ed.), Academic Press, N.Y., 1982.
210. P.A.J. Gorin, *Adv. Carbohydr. Chem. Biochem.*, 38 (1981) 13-104.
211. K. Bock and H. Thøgerson, *Annu. Rep. N.M.R. Spectr.*, 13 (1982) 1-57.
212. H.J. Jennings and I.C.P. Smith, *Methods Carbohydr. Chem.*, 8 (1980) 97-105.
213. H.J. Jennings and I.C.P. Smith, *Methods Enzymol.*, 50 (1978) 39-50.
214. B. Matsuhiro, A.B. Zanlungo, and G.G.S. Dutton, *Carbohydr. Res.*, 144 (1985) c1-c5.
215. J.P. Carver, A.A. Grey, F.M. Winnik, J. Hakimi, C. Ceccarini, and P.H. Atkinson, *J. Biochem.*, 20 (1981) 6600-6606.
216. R. St J Manley, *Methods Carbohydr. Chem.*, 3 (1963) 289-302.
217. W.N. Haworth, *J. Chem. Soc.*, 83 (1903) 1021.
218. J. Lönngren and S. Svensson, *Adv. Carbohydr. Chem. Biochem.*, 29 (1974) 41-106.
219. H. Parolis and D. McGarvie, *Carbohydr. Res.*, 62 (1978) 363-367.
220. E. Barreto-Bergter, L. Hogge, and P.A.J. Gorin, *Carbohydr. Res.*, 97 (1981) 147-150.

221. P.-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, and L. Lönngren, *Chem. Commun.*, 8 (1976) 1-75.
222. H.O. Bouveng, *Acta Chem. Scand.*, 15 (1961) 96.
223. A.N. de Belder and B. Norrman, *Carbohydr. Res.*, 8 (1968) 1-6.
224. H.O. Bouveng and B. Lindberg, *Adv. Carbohydr. Chem.*, 15 (1960) 53-89.
225. B. Lindberg, J. Lönngren, and S. Svensson, *Adv. Carbohydr. Chem. Biochem.*, 31 (1975) 185-240.
226. R.D. Guthrie and J.F. McCarthy, *Adv. Carbohydr. Chem.*, 22 (1967) 11-23.
227. L. Malaprade, *C.R. Acad. Sci., Paris*, 186 (1928) 382-384.
228. J.M. Bobbitt, *Adv. Carbohydr. Chem.*, 11 (1956) 1-41.
229. R.D. Guthrie, *Adv. Carbohydr. Chem.*, 16 (1961) 105-158.
230. A.S. Perlin in *The Carbohydrates, Chem. and Biochem.* , Vol. 1B, pg.1167-1215, W. Pigman and D. Horton (Ed.), Academic Press, N.Y., 1980.
231. G.W. Hay, B.A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1965) 357-361.
232. B. Lindberg and J. Lönngren, *Methods Carbohydr. Chem.*, 7 (1976) 142-148.
233. J. Kiss, *Adv. Carbohydr. Chem.*, 29 (1974) 229-303.
234. A. Dell, *Adv. Carbohydr. Chem. Biochem.*, 45 (1987) 19-72.
235. S.M.R. Stanley, PhD Thesis, Rhodes University, 1990.
236. Z. Yosizawa, T. Sato, and K. Schmid, *Biochim. Biophys. Acta*, 121 (1966) 417-420.
237. J.M. Williams, *Adv. Carbohydr. Chem. Biochem.*, 31 (1975) 9-79.
238. N.K. Kotchetkov and O.S. Chizhov, *Adv. Carbohydr. Chem.*, 21 (1966) 39-93.
239. D.E. Bradley, *Bacteriological Reviews*, 31 (1967) 230-314 (and references therein).
240. S. Stirm, *J. Virol.*, 2 (1968) 1107-1114 (and references therein).
241. S. Stirm, W. Bessler, F. Fehmel, and E. Freund-Molbert, *J. Virol*, 8 (1971) 343-346.
242. B. Kwiatkowski, B. Boschek, H. Thiele, and S. Stirm, *J. Virol.*, 43 (1982) 697-704.
243. P.C. Hallenbeck, E.R. Vimr, F. Yu, B. Bassler, and F.A. Troy, *J. Biol. Chem.*, 262 (1987) 3553-3561.
244. D.S. Gupta, B. Jann, and K. Jann, *FEMS Micro. Letters*, 16 (1983) 13-17.
245. S. Stirm, F. Altmann, and F.M. Unger, Abstract A2-15P, Europ. Carb. Symp., 1985.
246. I.W. Sutherland, K. Jann, and B. Jann, *Eur. J. Biochem.*, 12 (1970) 285-288.
247. E. Altman, G.G.S. Dutton, and A.M. Stephen, *S.A. J. Sci.*, 82 (1986) 45-46.
248. B. Kwiatkowski, B. Boschek, H. Thiele, and S. Stirm, *J. Virol.*, 45 (1983) 367-374.
249. B.V. McCleary and N.K. Matheson, *Adv. Carbohydr. Chem. Biochem.*, 44 (1986) 217-230.
250. N. Ravenscroft, pHd Thesis, University of Cape Town, 1988 (and references therein).
251. N.K. Matheson and B.V. McCleary in *The Polysaccharides*, Vol.3, pg. 1-105, G.O. Aspinall (Ed.), Academic Press, N.Y., 1980.
252. S.J. Angyal and K. James, *Aust. J. Chem.*, 23 (1970) 1209-1215.
253. J.W. Green in *The Carbohydrates, Chem. and Biochem.*, Vol. 1B, pg. 1139-1142, W. Pigman nad D. Horton (Ed.), Academic Press, N.Y., 1980.
254. D.R. Bundle and R.U. Lemieux, *Methods Carbohydr. Chem.*, 7 (1976) 79-86.
255. R.U. Lemieux, R.K. Kullnig, H.J. Bernstein, and W.G. Schreider, *J. Am. Chem. Soc.*, 79 (1957) 1005.
256. M. Karplus, *J. Chem. Phys.*, 30 (1959) 11.
257. L.D. Hall and C.M. Preston, *Carbohydr. Res.*, 37 (1974) 267.

258. P. Dais and A.S. Perlin, *Adv. Carbohydr. Chem. Biochem.*, 45 (1987) 125-168.
259. C.H. Lochmuller and R.W. Souter, *J. Chromatogr.*, 113 (1975) 283-302.
260. G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349-357.
261. M.R. Little, *Carbohydr. Res.*, 105 (1982) 1-8.
262. H. Schweer, *J. Chromatogr.*, 243 (1982) 149-152.
263. H. Schweer, *J. Chromatogr.*, 259 (1983) 164-168.
264. W.A. König, I. Benecke, and S. Sievers, *J. Chromatogr.*, 217 (1981) 71-79.
265. Y.A. Knirel, E.V. Vinogradov, and A.J. Mort, *Adv. Carbohydr. Chem. Biochem.*, 47 (1989) 167-202 (and references therein).
266. P.-E. Jansson, B. Lindberg, G. Widmalm, and K. Leontein, *Carbohydr. Res.*, 165 (1987) 87-92.
267. N.A. Kocharova, Y.A. Knirel, A.S. Shashkov, N.K. Kotchetkov, and G.B. Pier, *J. Biol. Chem.*, 263 (1988) 11291-11295.
268. A.J. Mort, *Abstr. Pap. Am. Chem. Soc. Meeting*, 181 (1981) carb.49.
269. M.-S. Kuo and A.J. Mort, *Carbohydr. Res.*, 145 (1986) 247-265.
270. A. van der Kaaden, G.J. Gerwig, J.P. Kamerling, J.F.G. Vliegenthart, and R.H. Tiesjema, *Eur. J. Biochem.*, 152 (1985) 663-668.
271. K. Leontein, B. Lindberg, J. Lönngren, and D.J. Carlo, *Carbohydr. Res.*, 114 (1983) 257-266.
272. M. Aereboe, Rhodes University, S.A., 1991 (personal communication).
273. D. Rolf and G.R. Gray, *J. Am. Chem. Soc.*, 104 (1982) 3539-3541.
274. M.P. Doyl, D.J. De Bruyn, and D.A. Kooistra, *J. Am. Chem. Soc.*, 94 (1972) 3659-3661.
275. J.-G. Jun and G.R. Gray, *Carbohydr. Res.*, 163 (1987) 247-261.
276. G.R. Gray, *Methods Enzymol.*, 138 (1987) 26-38.
277. A. Van Langenhove and V.N. Reinhold, *Carbohydr. Res.*, 143 (1985) 1-20.
278. J.U. Bowie and G.R. Gray, *Carbohydr. Res.*, 129 (1984) 87-97.
279. G.R. Gray and D. Rolf, *Carbohydr. Res.*, 125 (1984) 301-307.
280. D. Rolf, J.A. Bennek, and G.R. Gray, *Carbohydr. Res.*, 137 (1985) 183-196.
281. J.A. Bennek, M.J. Rice, and G.R. Gray, *Carbohydr. Res.*, 157 (1986) 125-137.
282. S.A. Vodonik and G.R. Gray, *Carbohydr. Res.*, 175 (1988) 93-102.
283. S.G. Zeller and G.R. Gray, *Carbohydr. Res.*, 198 (1990) 285-303.
284. V.N. Reinhold, E. Coles, and S.A. Carr, *J. Carbohydr. Chem.*, 2 (1983) 1-18.
285. T. Radford and D.C. De Jongh, *Biochem. Appl. Mass Spectrom.*, 1 (1980) 255-310.
286. B. Munson, *Anal. Chem.*, 43 (1971) 28A-43A.
287. B. Munson, *Anal. Chem.*, 49 (1977) 772A-778A.
288. D. Horton, J.D. Wander, and R.L. Foltz, *Carbohydr. Res.*, 36 (1974) 75-96.
289. V.N. Reinhold and S.A. Carr, *Mass Spectrom. Rev.*, 2 (1983) 153.
290. M. Barber, R.S. Bordoli, R.D. Sedgwick, and A.N. Tyler, *J.C.S. Chem. Commun.*, 063 (1981) 325-327.
291. D.J. Surman and J.C. Vickerman, *J.C.S. Chem. Commun.*, 1357 (1981) 324-325.
292. H.D. Beckey and H.-R. Schulten, *Angew Chem. Int. Ed.*, 14 (1975) 403-415.
293. J. Van der Greef, *Trends Anal. Chem.*, 5 (1986) 241-246.
294. E.J. Bourne, M. Stacey, J.C. Tatlow, and J.M. Tedder, *J. Chem. Soc.*, (1949) 2976-2979.
295. A. Dell, *Methods Enzymol.*, 193 (1990) 647-660.
296. M. Arita, M. Iwamori, T. Higuchi, and Y. Nagai, *J. Biochem. (Tokyo)*, 93 (1983) 319-322.
297. H. Egge, J. Dabrowski, and P. Hanfland, *Pure Appl. Chem.*, 56 (1984) 807-819.

298. M.J. McConville, S.W. Homans, J.E. Thomas-Oates, A. Dell, and A. Bacic, *J. Biol. Chem.*, 265 (1990) 7385-7394.
299. G.K. Ostrander, M. Bozlee, M. Fukuda, A. Dell, J.E. Thomas-Oates, S.B. Levery, H.L. Eaton, S.I. Hakomori and E.H. Holmes, *Arch. Biochem. Biophys.*, 284 (1991) 413-421.
300. M. Fukuda, E. Spooncer, J.E. Thomas-Oates, A. Dell, and J.C. Klock, *J. Biol. Chem.*, 259 (1984) 10925-10935.
301. H.R. Morris, A. Dell, M. Panico, J. Thomas-Oates, M. Rogers, R. Mc Dowell, and A. Chatterjee, *Pract. Spectrosc.*, 8(mass spectrom. biol. mater) (1990) 137-167.
302. J.E. Thomas-Oates and A. Dell, *Biochem. Soc. Trans.*, 17 (1989) 243-245.
303. L. Ballou, E. Alvarado, P. Tsai, A. Dell, and C.E. Ballou, *J. Biol. Chem.*, 264 (1989) 11857-11864.
304. A.Dell, J.E. Thomas-Oates, M.E. Rogers, and P.R. Tiller, ***Biochimie***, 70 (1988) 1435-1444.
305. A. Dell and M.E. Rogers, *Anal. Chem.*, 8 (1989) 375-378.
306. A. Dell and C.E. Ballou, *Carbohydr. Res.*, 120 (1983) 95-111.
307. A. Dell, G.G.S. Dutton, P.-E. Jansson, B. Lindberg, U. Lindquist, and I.W. Sutherland, *Carbohydr. Res.*, 122 (1983) 340-343.
308. M. McNeil, J. Darvill ,A.G. Darvill, P. Albersheim, R. van Veen, P. Hooykaas, R.Schilperoort, and A. Dell, *Carbohydr. Res.*, 146 (1986) 307-326.
309. A. Dell, P. Azadi, P.R. Tiller, J.E. Thomas-Oates, H.J. Jennings, P. Beurret, and F. Michon, *Carbohydr. Res.*, 200 (1990) 59-76.
310. F. Michon, J.R. Brisson, A. Dell, D.L. Kasper, and H.J. Jennings, *Biochemistry*, 27 (1988) 5341-5351.
311. B. Lindberg, K. Leontein, U. Lindquist, S.B. Svenson, G. Wrangsell, A. Dell and M. Rogers, *Carbohydr. Res.*, 174 (1988) 313-322.
312. P.L. Hackland, H. Parolis, A. Dell, and P.R. Tiller, *Carbohydr. Res.*, 181 (1988) 153-162.
313. E.A. Nothnagel, M. McNeil, P. Albersheim, and A. Dell, *Plant Physiol.*, 71 (1983) 916-926.
314. M.S. Kuo, A.J. Mort, and A. Dell, *Carbohydr. Res.*, 156 (1986) 173-187.
315. D.L. Rabenstein and W. Guo, *Anal. Chem.*, 60 (1988) 1R-28R.
316. L.W. Jelinski, *AT & T Bell Laboratories*, *C & EN*, 1984, 26-47.
317. I.H. Sadler, *Nat. Prod. Reports*, 1988, 101-127.
318. G.A. Morris, *Magnetic Resonance in Chem.*, 24 (1986) 371-403.
319. T.C. Farrar, *Anal. Chem.*, 59 (1987) 749A-761A.
320. J. Dabrowski, *Methods Enzymol.*, 179 (1989) 122-156 (and references therein).
321. A.E Derome, *Modern NMR Techniques for Chemistry Research*, Pergamon Press, Oxford, 1987.
322. J. Schraml and J.M. Bellama, *Two Dimensional NMR Spectroscopy*, Wilet-Interscience, N.Y., 1988.
323. W.P. Aue, E Bartholdi, and R.R.J. Ernst, *J. Chem. Phys.*, 64 (1976) 2229-2246.
324. M.A. Bernstein and L.D. Hall, *J. Am. Chem. Soc.*, 104 (1982) 5553-5555.
325. L. Braunschweiler and R.R. Ernst, *J. Magn. Reson.*, 53 (1983) 521.
326. A. Bax and D.G. Davis, *J. Am. Chem. Soc.*, 107 (1985) 7197-7198.
327. J.H. Bradbury and G.A. Jenkins, *Carbohydr. Res.*, 126 (1984) 125-156.
328. K. Izumi, *Carbohydr. Res.*, 170 (1987) 19-25.
329. D.G. Davis and A. Bax, *J. Am. Chem. Soc.*, 107 (1985) 2820-2821.
330. S.R. Hartman and E.L. Hahn, *Phys. Review*, 128 (1962) 2042-2053.
331. A. Bax and S. Subramanian, *J. Magn. Reson.*, 67 (1986) 565-569.

332. V.K. Dua, B.N.N. Rao, S.-S. Wu, V.E. Dube, and C.A. Bush, *J. Biol. Chem.*, 261 (1986) 1599-1608.
333. G.A. Morris and R. Freeman, *J. Am. Chem. Soc.*, 101 (1979) 760-762.
334. P.-E. Jansson, L. Kenne, and G. Widmalm, *Carbohydr. Res.*, 168 (1987) 67-77.
335. G.M. Lipkind, A.S. Shashkov, Y.A. Knirel, E.V. Vinogradov, and N.K. Kotchetkov, *Carbohydr. Res.*, 175 (1988) 59-75.
336. I.M. Morrison, *J. Chromatogr.*, 108 (1975) 361-364.
337. G.G.S. Dutton, A. Kuma-Mintah, S.K. Ng, H. Parolis, A. Dell, and A. Reason, *Carbohydr. Res.*, In Press.
338. H. Nieman, N. Frank, and S. Stirm, *Carbohydr. Res.*, 59 (1977) 165-177.
339. D.P. Sweet, R.H. Shapiro, P. Albersheim, *Carbohydr. Res.*, 40 (1975) 217-225.
340. P.L. Hackland, L.A.S. Parolis, and H. Parolis, *Carbohydr. Res.*, 172 (1988) 209-216.
341. G.G.S. Dutton, S.K. Ng, L.A.S. Parolis, H. Parolis, and A.K. Chakraborty, *Carbohydr. Res.*, 193 (1989) 147-155.
342. J. Dabrowski, M. Hauck, E. Romanowska, and A. Gamian, *Carbohydr. Res.*, 180 (1988) 163-174.
343. J.-R. Brisson and J.P. Carver, *J. Biol. Chem.*, 258 (1983) 1431-1434.