Carbohydrate Research, 170 (1987) 193–206
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE STRUCTURE OF THE NEURAMINIC ACID-CONTAINING CAPSULAR POLYSACCHARIDE OF *Escherichia coli* SEROTYPE K9*

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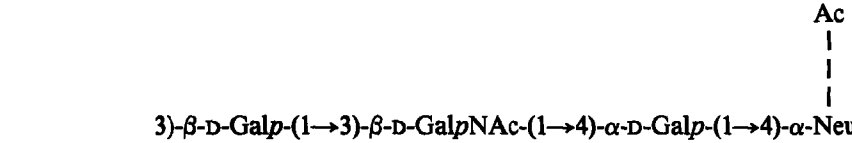
(Received February 17th, 1987; accepted for publication, June 25th, 1987)

ABSTRACT

The acidic capsular polysaccharide isolated from *Escherichia coli* O9:K9:H12 was investigated by using n.m.r. spectroscopy, methylation analysis, periodate oxidation, and bacteriophage-borne enzyme degradation. The polysaccharide, the structure of which is shown below, is the third *E. coli* capsular polysaccharide reported to contain neuraminic acid, the others being the K1 and K92 polysaccharides, and it is the first in the *E. coli* series shown to contain a 4-linked neuraminic acid unit.



INTRODUCTION

The investigation of the structure of the *Escherichia coli* serotype K9 polysaccharide forms part of a broader study of the capsular (K) antigens of *E. coli*, which involves the elucidation of their structures and the relationship between structure and immunological characteristics. The K9 polymer represents a new chemotype and, like the K1 (ref. 1) and K92 (ref. 2) antigens, possesses an *N*-acetylneuraminic acid (Neu5Ac) moiety in the repeating unit[3]. K9 is distinguished further by being the first *E. coli* K-antigen reported to contain a 4-linked Neu5Ac moiety, which is a relatively uncommon linkage in bacterial polysaccharides and to date has been found only in the polysaccharides of *Neisseria meningitidis* serotypes Y and W-135[4]. We now report on the structure of the acidic capsular polysaccharide.

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*Presented at the 13th International Carbohydrate Symposium, Ithaca, August 10–15, 1986.

0008-6215/87/$ 03.50

RESULTS AND DISCUSSION

Composition and methylation analysis. — The capsular polysaccharide from *E. coli* O9:K9:H12 was isolated from three separate growths of the bacteria by ultracentrifugation and purified *via* its cetyltrimethylammonium salt. Gel-permeation chromatography of the product in the first growth ($[\alpha]_D$ +19°) showed a single symmetrical peak on Sepharose 4B and indicated that the polysaccharide had a mol. wt. of 360,000 (calibration with dextrans). The other two growths gave products with optical rotations similar to that of the first, but n.m.r. spectroscopy showed that only the product of the first growth contained *O*-acetyl groups. The product (*A*) of the first growth was therefore kept separate from the other two which were then combined as product *B*.

The polysaccharide *B* was methanolysed and the resulting methyl glycosides were *N*-acetylated and trimethylsilylated. G.l.c. then revealed the glycosides of galactose (Gal), 2-acetamido-2-deoxygalactose (GalNAc), and *N*-acetylneuraminic acid (Neu5Ac) in the ratios 2.0:0.7:0.7. The Gal was shown to be D by g.l.c. of the acetylated (−)-2-octyl glycosides[5] and the GalNAc was shown to be D by oxidation with D-galactose oxidase[6].

The polysaccharide was methylated according to the method of Hakomori[7], as modified by Narui *et al.*[8], and, after methanolysis and acetylation of the methyl glycosides, g.l.c.–m.s. gave the results shown in Table I, column I. The methylated polysaccharide was also depolymerised by acetolysis–hydrolysis[9] (which destroyed the Neu5Ac), and g.l.c.–m.s. of the products as alditol acetates gave the results in Table I, column II. The methylation results show that the repeating unit of the

TABLE I

METHYLATION DATA FOR *E. coli* K9 POLYSACCHARIDE AND DERIVED OLIGOSACCHARIDES

Methylated sugars[a]	*I*[b]	*II*		*III*[b]	*IV*[b]	*V*[b]	*VI*[b]
		c	*d*				
2,4,6-Me_3-Gal	+	0.8	1.7				
2,3,6-Me_3-Gal	+	1.0	1.8			+	+
4,6-Me_2-GalNAcMe	+	0.6	3.5	+		+	+
1,2,7,8,9-Me_5-Neu5AcMe	+					+	
1,2,6,7,8,9-Me_6-NonNAcMe					+		+
2,3,4,6-Me_4-Gal				+	+	+	+
1,3,4-Me_3-Threitol				+			
1,2,4,7-Me_4-HeptNAcMe				+			

[a] 2,4,6-Me_3-Gal = 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol or methyl 3-*O*-acetyl-2,4,6-tri-*O*-methylgalactoside, *etc.* [b] Acetylated methyl glycosides on OV-17; temperature programme: 170° for 10 min, 3°/min to 240°; I + II, native polysaccharide; III, Smith-degraded product SD; IV, phage product PA-alditol; V, phage product P1; VI, P1-alditol. [c] Molar ratios of alditol acetates on 3% ECNSS-M (180°). [d] Retention times relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol.

polysaccharide contained a 3- and 4-linked Gal, 3-linked GalNAc, and 4-linked Neu5Ac. The e.i.-mass spectrum of the 4-*O*-acetyl-7,8,9-tri-*O*-methylneuraminic acid derivative compares well with the data reported[10].

N.m.r. spectroscopy. — The ^{1}H-n.m.r. spectrum of polymer *A* contained (Table II) three signals for anomeric protons [δ 4.5–5.5; two β (δ 4.52, $J_{1,2}$ 7 Hz; δ 4.75, $J_{1,2}$ 8 Hz), and one α (δ 5.06, $J_{1,2}$ not observed)] as well as the signals for two NAc groups (δ 2.04) and one OAc group (δ 2.16). Two other 1-proton multiplets were present at δ 1.75 (J 12.5 Hz) and 2.91 (unresolved). These values accord with those reported[11] for H-3*a* and H-3*e* of α-Neu5Ac, thus indicating the presence of an α-linked Neu5Ac in the K9 polysaccharide. The ^{1}H-n.m.r. data suggest a tetrasaccharide repeating-unit for the polysaccharide.

The proton-decoupled ^{13}C-n.m.r. spectrum for polymer *B* contained four signals for anomeric carbons at 96.15, 96.74, 100.44, and 103.30 p.p.m. The last two signals were assigned to C-1 of the two β-linked sugars and that at 96.15 p.p.m. to C-1 of the α-linked sugar. The signal at 96.74 p.p.m. was of much lower intensity than the other three and thus is assigned to C-2 of Neu5Ac, which has no α-protons and therefore will experience less n.O.e. and have a longer relaxation time. The spectrum also contained signals for C-3 and C-5 of Neu5Ac (37.51 and 50.22 p.p.m.)[12], indicating a 4-linkage on the following basis. The chemical shifts of the signals for C-3 and C-5 in unsubstituted Neu5Ac occur at 39.9 and 53.2 p.p.m, respectively[4], whereas C-3 and C-5 resonate at ~41 and ~53 p.p.m. when Neu5Ac is 8- or 9-linked as in colominic acid and *N. meningitidis* type B and C polysaccharides[12]. When Neu5Ac is 4-linked as in *N. meningitidis* type Y and W-135 polysaccharides[4], then C-3 and C-5 resonate further upfield (β-effect) at 37.8 and 50.7 p.p.m., respectively.

The ^{13}C-n.m.r. spectrum of polymer *B* also contained a resonance at 52.22 p.p.m. consistent[13] with C-2 of a 3-linked HexNAc. Methyl carbon resonances (23.00 and 23.29 p.p.m.) of the NAc groups and the carbonyl carbon resonances of the carboxylic acid group of Neu5Ac (174.39 p.p.m) and the carbonyl groups of the NAc groups (175.05 and 175.65 p.p.m) were also observed. Thus, the n.m.r. data confirmed that the K9 polysaccharide consists of a repeating unit containing two neutral sugars, a 3-linked HexNAc, and a 4-linked α-Neu5Ac.

Periodate oxidation and Smith degradation. — Periodate-oxidation studies[14] were carried out on the *O*-acetylated polymer *A* and the polymer *B* which was devoid of *O*-acetyl groups. The former consumed 2.1 mol of periodate per mol of trisaccharide repeating-unit and the latter, 3.3 mol. In the absence of *O*-acetyl groups, 2 mol of periodate were consumed rapidly and the third mol was consumed slowly. The rapid uptake is attributed to cleavage of the C-7–C-8 and C-8–C-9 bonds of Neu5Ac, and the slow uptake to oxidation of the 4-linked Gal. Periodate-oxidation studies[15] on Neu5Ac, 9-*O*-acetylNeu5Ac, and 4,9-di-*O*-acetylNeu5Ac, and the corresponding methyl glycosides, have shown that non-*O*-acetylated Neu5Ac is rapidly oxidised by periodate but that 9-acetylation severely retards oxidation. *O*-Acetylated K9 polysaccharide rapidly consumed 1 mol of periodate followed by

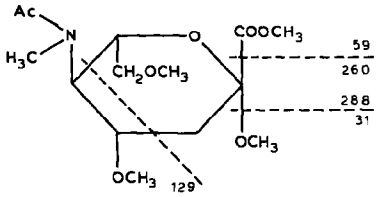
TABLE II

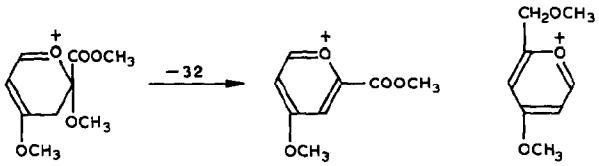
N.M.R. DATA FOR *E. coli* K9 POLYSACCHARIDE AND DERIVED OLIGOSACCHARIDES

Compound[a]	*^{1}H-N.m.r. data*				*^{13}C-N.m.r. data*	
	δ[b] *(p.p.m.)*	$J_{1,2}$ *Hz*	*Integral (No. of H)*	*Assignment*[c]	*P.p.m.*[d]	*Assignment*[e]
[→3)-β-Gal-(1→3)-β-GalNAc-(1→4)--α-Gal-(1→4)-α-Neu5Ac-(2→]$_n$	1.75	12.5[f]10.5[g]	1	H-3*a* →4)-α-Neu5Ac	23.00	CH_3 NAc
	2.04		6	2 NAc	23.29	CH_3 NAc
Polysaccharide	2.16		3	OAc[i]	37.51	C-3 →4)-α-Neu5Ac
	2.91	unresolved[h]	1	H-3*e* →4)-α-Neu5Ac	50.22	C-5 →4)-α-Neu5Ac
	4.52	7	1	→3)-β-Gal	52.22	C-2 →3)-β-GalNAc
	4.75	8	1	→3)-β-GalNAc	80.50	C-3 →3)-β-GalNAc
	5.06	n.o.	1	→4)-α-Gal	96.15	C-1 →4)-α-Gal
					96.74	C-2 →4)-α-Neu5Ac
					100.44	C-1 →3)-β-GalNAc
					103.30	C-1 →3)-β-Gal
					174.39	C=O Neu5Ac
					175.05	C=O NAc
					175.65	C=O NAc
β-Gal-(1→3)-β-GalNAc-(1→2)-threitol	4.70	8	1	→3)-β-GalNAc	22.89	CH_3 NAc
Smith-degradation product (SD1)	4.48	7	1	β-Gal	52.32	C-2 →3)-β-GalNAc
	2.06		3	NAc	102.02	C-1 →3)-β-GalNAc
					105.51	C-1 β-Gal
					175.10	C=O NAc
α-Gal-(1→4)-α-Neu5Ac	5.06	3	1	α-Gal	23.03	CH_3 NAc
Phage product (PA)	2.04		3	NAc	36.65	C-3 →4)-Neu5Ac
	1.76		1	H-3*a* →4)-Neu5Ac	50.61	C-5 →4)-Neu5Ac
	2.46		1	H-3*e* →4)-Neu5Ac	95.44	C-1 α-Gal
					175.09	C=O NAc

[a] See text. [b] Chemical shift relative to that of internal acetone (δ 2.23 downfield from signal for DSS). [c] →3)-β-Gal refers to the anomeric proton of a 3-linked β-Gal*p*, *etc.* [d] Chemical shift in p.p.m. relative to that of internal acetone (31.07 p.p.m. downfield from DSS). [e] As for *c*, but for ^{13}C. [f] $J_{3a,3e}$. [g] $J_{3a,4}$. [h] $J_{3e,4}$ 4.4 Hz. [i] Found only in one of the three batches of polysaccharide prepared.

slow uptake by the 4-linked Gal. These results indicate that the *O*-acetyl groups are located at positions on the Neu5Ac residues. The consumption of ~3 mol of periodate per repeating unit by polysaccharide *B* is also consistent with the linkage positions of the sugars as determined by methylation analysis.





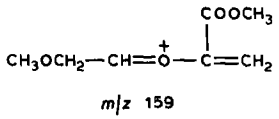


Fig. 1. Mass fragmentation scheme of methyl [methyl 3,5-dideoxy-4,7-di-*O*-methyl-5-(*N*-methylacetamido)-L-*arabino*-heptulosid]onate (methylated H).

The product (SD) of Smith degradation[14] of polymer *B* was methylated and methanolysed, and the products were acetylated. G.l.c.-m.s. then gave the results in Table I, column III, which indicated SD to be a mixture of a trisaccharide-alditol (SD1) and an aminodideoxyheptose (H) derived from periodate oxidation of Neu5Ac (Fig. 1). The linkage of this heptose is acid-labile and must have been cleaved during the Smith-hydrolysis step. The formation of 2,3,4,6-tetra-*O*-methylgalactose in the methylation analysis of SD indicated the Neu5Ac to be linked to the 3-linked Gal in the polysaccharide since the 4-linked Gal was oxidised by periodate. SD1 was separated from the mixture SD by p.c. Both the ^{1}H- and ^{13}C-n.m.r. spectra (Table II) of SD1 showed the presence of two β-anomeric signals and an NAc group. The ^{13}C-n.m.r. spectrum also contained a resonance for C-2 (52.32 p.p.m.) of GalNAc and the carbonyl carbon resonance of the NAc group. The results are consistent with the structure β-Gal-(1→3)-β-GalNAc-(1→2)-threitol for SD1.

Bacteriophage degradation of K9 polysaccharide. — Treatment of polysaccharide *B* with bacteriophage φ9, which infects *E. coli* K9 bacteria, gave (p.c.)

only one major reducing oligosaccharide (PA). The ^{1}H-n.m.r. spectrum of PA (Table II) contained signals for an α-anomeric proton, NAc, and H-3*e* and H-3*a* of Neu5Ac[11], and the ^{13}C-n.m.r. spectrum contained signals for an α-anomeric carbon, the methyl carbon of NAc, and C-3 and C-5 of Neu5Ac.

The oligosaccharide PA was reduced, methylated, and methanolysed, and the products were acetylated. The results are shown in Table I, column IV. The formation of methyl 4-*O*-acetyl-3,5-dideoxy-2,6,7,8,9-penta-*O*-methyl-5-(methylacetamido)nononate (Fig. 2) is consistent with Neu5Ac occupying the reducing terminus of the oligosaccharide. Only one peak for the nononate was observed in the chromatogram, which could be either or both C-2 epimers. The e.i.-mass spectrum of the methylated nononate gave the primary fragment ions shown in Fig. 2, and secondary ions[16] at *m/z* 287, 257, 243, 206, 200, 170, 158, 145 (base peak), 142, 130, 112, 103, 101, and 59. Thus the phage product PA was α-Gal-(1→4)-Neu5Ac.

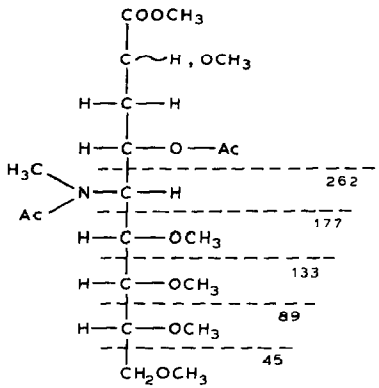


Fig. 2. Fragmentation pattern, showing only primary fragments, of methyl 4-*O*-acetyl-3,5-dideoxy-2,6,7,8,9-penta-*O*-methyl-5-(*N*-methylacetamido)nononate.

The isolation of a disaccharide and not the expected tetrasaccharide repeating-unit[17] is unusual and may be due to ϕ9 possessing two types of cleaving enzymes or to cleavage of the tetrasaccharide during isolation and/or purification. The repetition of the phage degradation of polysaccharide *B* under more stringent conditions (see Experimental) gave the tetrasaccharide P1, an octasaccharide (P2), and higher oligomers which were separated on Bio-Gel P-4.

P1 was studied by ^{1}H- and ^{13}C-n.m.r. spectroscopy as well as by methylation analysis. The ^{1}H-n.m.r. spectrum of P1 contained (Table III) signals for H-1 of β-Gal, β-GalNAc, and α-Gal. The signals of H-3*a* and H-3*e* of Neu5Ac were observed at δ 1.83 and 2.48, respectively, indicating[11] the Neu5Ac to be β and thus occupying the reducing terminus since Neu5Ac is α in the polysaccharide. Only one set of signals for H-3 was observed because the terminal Neu5Ac in P1, like free Neu5Ac, exists almost entirely as the β anomer[18]. The presence of two NAc groups was indicated by the signals at δ 2.06 and 2.04. P1 was reduced with $NaBH_4$, P1 and

P1-alditol were methylated and methanolysed, and the products were acetylated. G.l.c. (see Table I, columns V and VI) then confirmed P1 to be a tetrasaccharide with Neu5Ac as the reducing terminus and Gal as the non-reducing terminus. Thus, P1 was β-Gal-(1→3)-β-GalNAc-(1→4)-α-Gal-(1→4)-Neu5Ac. The above data accord with the structure of the repeating unit of the *E. coli* K9 polysaccharide shown in the Abstract.

2D-N.m.r. spectroscopy of P1. — The 2D ^{1}H–^{1}H spin-correlated spectra were obtained using the COSY-45 programme, and contour plots are shown in Fig. 3. The use of COSY allowed many of the signals in the ^{1}H-n.m.r. spectrum of P1 to be assigned and thence the coupling constants from the expanded 1-D spectrum for non-overlapping peaks.

TABLE III

^{13}C- AND ^{1}H-N.M.R. DATA FOR K9 P1

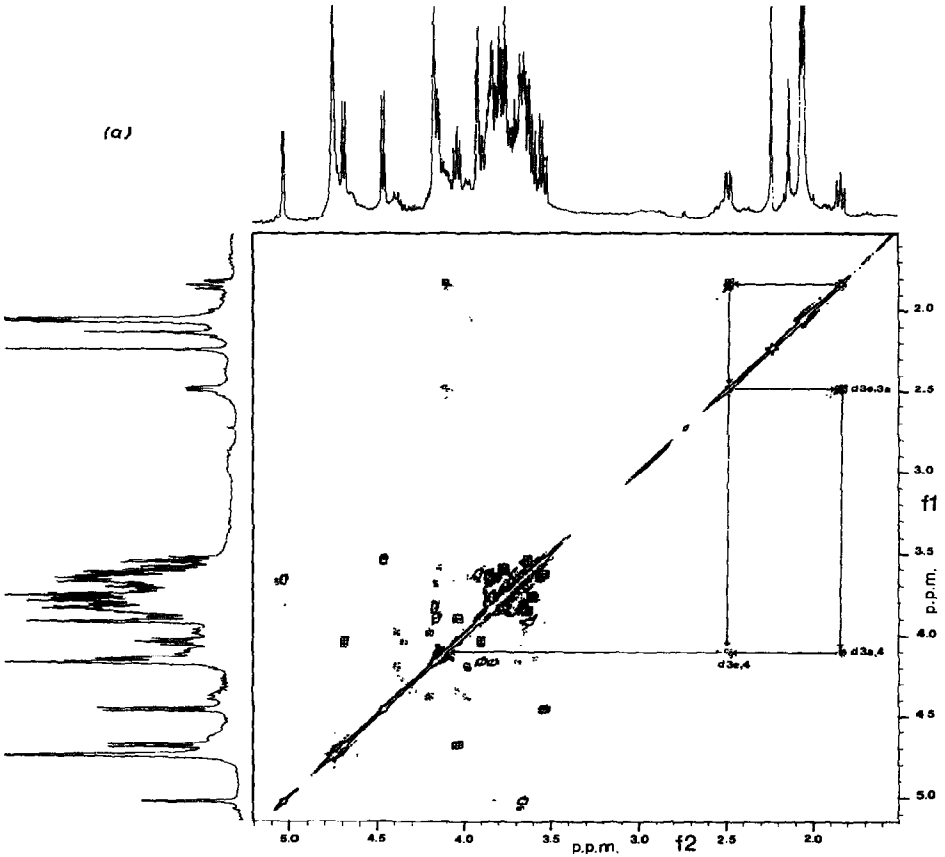
Residue[a]	*Proton, or carbon atom*	*^{1}H-N.m.r. data*			*^{13}C-N.m.r. data*
		δ (p.p.m.)[b]	*J (Hz)*		*δ (p.p.m.)*[b]
a β-Gal-(1→	1	4.45	$J_{1,2}$	7.7	105.56
	2	3.53	$J_{2,3}$	9.9	71.44
	3	3.62	$J_{3,4}$	4.3	73.27
	4	3.91	$J_{4,5}$	<1	69.40
	5				75.80[d]
b →3)-β-GalNAc-(1→	1	4.68	$J_{1,2}$	8.6	103.32
	2	4.03	$J_{2,3}$	10.9	52.30
	3	3.89	$J_{3,4}$	3.2	80.45
	4	4.16	$J_{4,5}$	<1	68.83
	5	~3.69			75.33[d]
	NAc	CH_3 2.06[e]		CH_3	23.00[g]
				C=O	175.71[f]
c →4)-α-Gal-(1→	1	5.03	$J_{1,2}$	3.95	95.94
	2	3.66	$J_{2,3}$	8.15	69.01
	3	3.86			70.08
	4	4.17			77.36
d →4)-Neu5Ac	1				175.02
	2				96.20
	3	*a* 1.83	$J_{3a,3e}$	13.0	36.37
		e 2.48	$J_{3a,4}$	10.7	
	4	4.11	$J_{3e,4}$	4.34	72.67
	5	4.14			50.39
	9				63.99
	NAc	CH_3 2.04[e]		CH_3	23.23[g]
				C=O	175.21[f]

a b c d

[a] β-Gal-(1→3)-β-GalNAc-(1→4)-α-Gal-(1→4)-Neu5Ac. [b] Chemical shift relative to that of acetone (δ 2.23 and 31.07 downfield from the signal for DSS, for ^{1}H and ^{13}C, respectively). [c] →4)-α-Gal-(1→- refers to a 4-linked α-Gal*p*. [d–g] Values may be interchanged.

Fig. 3 shows that the H-1 signal at δ 4.68 (J 8.6 Hz) is coupled to an H-2 signal at δ 4.03, and that the H-1 signal at δ 4.45 (J 7.7 Hz) is coupled to an H-2 signal at δ 3.53. The signal at δ 4.68 in the spectrum of P1 can thus be assigned to H-1 of β-GalNAc and that at δ 4.45 to H-1 of β-Gal[19]. The signals at δ 1.83 and 2.48, due to H-3*a* and H-3*e*, respectively, of Neu5Ac, are coupled to a multiplet at δ 4.10, thus allowing this signal to be assigned to H-4 of Neu5Ac. Other chemical shifts which are of interest are those of the signals of H-4 of 4-linked α-Gal (δ 4.17) and H-3 of the 3-linked β-GalNAc (δ 3.89).

2D-Heteronuclear $^{1}H-^{13}C$ shift-correlation spectroscopy allowed the correlation of the signals in the ^{1}H and ^{13}C spectra. Thus, through rigorous assignment of much of the proton spectrum using COSY, almost all of the signals in the ^{13}C spectrum could be assigned. A few signals could not be assigned this way and were assigned by comparison with literature values (C-9 of Neu5Ac[4] and C-5 of the β-Gal and β-GalNAc[20]). A $^{1}H-^{13}C$ shift-correlation map for the spectral regions δ



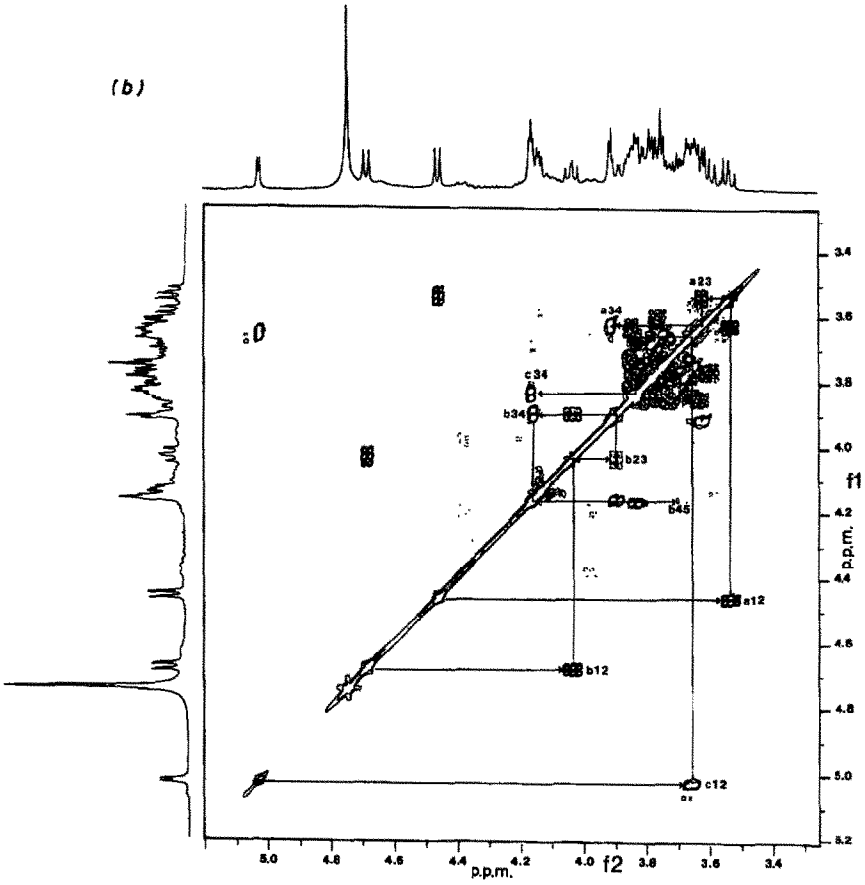


Fig. 3. Contour plots of the 2D spin-correlated spectrum (COSY) of P1 [β-Gal^{a}-(1→3)-β-GalNAc^{b}-(1→4)-α-Gal^{c}-(1→4)-Neu5Ac^{d}] symmetrised about the diagonal: (*a*) full spectrum (d3a,4 refers to the connectivity between H-3*a* and H-4 of residue d, *etc*); (*b*) region δ 3.2–5.2

108.0–20.0 (f2) and δ 5.30–1.95 (f1) is shown in Fig. 4. The chemical shift of the C-4 resonance of the 4-linked Neu5Ac at δ 72.67 is shifted downfield by 3.06 p.p.m., compared to that of unsubstituted Neu5Ac[21]. Concomitant upfield shifts (β-effects) were observed for the C-3 and C-5 resonances, at δ 36.37 and 50.39, respectively. Other resonances in the ^{13}C spectrum that can be identified because they are sites of glycosylation are those at δ 80.45 (C-3 of 3-linked β-GalNAc[20]) and 77.36 (C-4 of 4-linked α-Gal).

EXPERIMENTAL

General methods. — Optical rotations were measured in a 1-cm cell at 23–25° with a Perkin-Elmer Model 141 polarimeter. Solutions were concentrated under

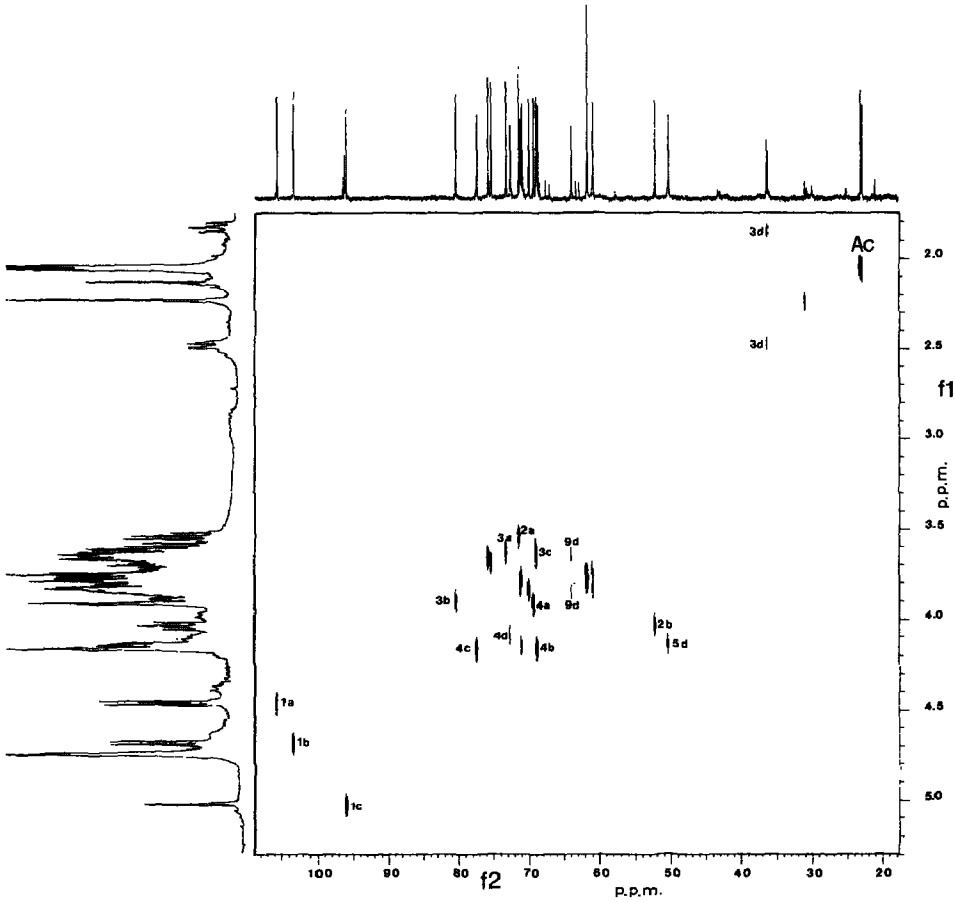


Fig. 4. Heteronuclear ^{1}H–^{13}C shift-correlation map of P1 (see Fig. 3) for the spectral regions of f2 (δ 108.0–20.0) and f1 (δ 5.30–1.95); 1a refers to carbon or proton 1 of residue a, *etc.*

diminished pressure at $\leq 40°$. Carbohydrate samples were dried for 16 h over silica gel at 50° under vacuum. Descending p.c. was conducted on Whatman No. 1 paper with 5:1:5:3 ethyl acetate–acetic acid–pyridine–water and detection with ***A***, periodate–benzidine[22]; and ***B***, alkaline silver nitrate[23]. G.l.c. was performed with a Hewlett-Packard model 5890 gas chromatograph fitted with flame-ionisation detectors, using columns (30 m × 0.25 mm) of OV-1, OV-17, and OV-225 (film thicknesses, 0.25 μm), with helium as the carrier gas. A packed column (1.5 m × 3 mm) containing 3% of ECNSS-M on Gas Chrom Q (100–200 mesh) was also used. Methylated sugars were identified by capillary g.l.c.–m.s., using a VG Micromass 16F spectrometer (ionisation energy, 40 eV) and the appropriate columns. ^{1}H- and ^{13}C-n.m.r. spectra were recorded with a Bruker WM-500 Ft spectrometer at 30°, and in some cases at 95° for ^{1}H-n.m.r. spectroscopy. The Bruker COSY-45 programme was used for 2D homonuclear-shift-correlation spectroscopy on P1. Sam-

ples in D_2O were deuterium-exchanged by freeze-drying 3 or 4 times from 99.8% D_2O. Acetone (δ 2.23 for ^1H and 31.07 p.p.m. for ^{13}C, measured against aqueous sodium 4,4-dimethyl-4-silapentane-1-sulphonate) was used as the internal standard.

Isolation and purification of the polysaccharide. — A culture of *E. coli* O9:K9:H12 bacteria was obtained from Drs. F. and I. Ørskov (Copenhagen) and propagated using Mueller-Hinton broth and agar containing 2% of added NaCl. The polysaccharide was isolated from the bacteria as described previously[24] and purified *via* its cetyltrimethylammonium salt to yield 1.56 g of acidic polysaccharide (3 batches of 5 × 1.5 L of medium, 207 mg of polysaccharide/L of medium). The polysaccharide had $[\alpha]_D$ + 19° (*c* 0.3, water), and gel-permeation chromatography on Sepharose 4B (M NaCl as eluent) showed a single symmetrical peak (M_r 360,000; calibration with dextrans).

Analysis of component sugars. — Dried polysaccharide (15 mg) was treated with methanolic 0.5M hydrogen chloride (5 mL) for 24 h at 85°. The solution was neutralised with Ag_2CO_3, acetic anhydride (1 mL) was added, the mixture was stirred for 6 h at room temperature and then centrifuged, and the supernatant solution was concentrated to dryness. The residue was shaken vigorously with pyridine (1 mL), hexamethyldisilazane (0.2 mL), and chlorotrimethylsilane (0.1 mL) for 5 min. The mixture was stored for 1 h at room temperature and then concentrated to dryness. The residue was suspended in $CHCl_3$ (20 μL) and a 1-μL sample was injected on to a capillary column of OV-1 (temperature programme: 200° for 10 min, 2°/min to 240°). The results revealed Gal, GalNAc, and Neu5Ac in the ratios 2.0:0.7:0.7. The sugars were identified by co-chromatography with authentic samples.

Methylation analysis. — The acid form of the polysaccharide (100 mg) was methylated [Hakomori[7] as modified by Narui *et al.*[8] (equal volumes of methyl sulphoxide and 1,1,3,3-tetramethylurea)]. A portion (14 mg) was methanolysed (methanolic 3% HCl, 16 h, 85°) and neutralised (Ag_2CO_3), and the products were acetylated (0.5 mL of pyridine, 1 mL of acetic anhydride, 1 h, 100°). G.l.c. (capillary column, OV-17, temperature programme: 170° for 10 min, 3°/min to 240°) gave the results in Table I, column I. Another portion (19 mg) of methylated polymer was depolymerised by acetolysis/hydrolysis[9]. The dried sample was treated with 5 mL of M H_2SO_4 in aqueous 95% acetic acid for 16 h at 80°. Water (5 mL) was then added, heating was continued for 5 h at 80°, and the hydrolysate was cooled and eluted from a column of Amberlite IR-45 (AcO) resin with methanol. The eluate was concentrated to dryness, the residue was dissolved in water (4 mL), and $NaBH_4$ (50 mg) was added. After 2 h, the mixture was worked-up conventionally, and the products were acetylated and subjected to g.l.c. on a column of 3% ECNSS-M (190°). The results are shown in Table I, column II.

Periodate oxidation and Smith degradation[14]. — To a solution of a portion (43.0 mg) of polysaccharide *A* in distilled water (10 mL) was added 0.03M $NaIO_4$ (10 mL). The mixture was stored at room temperature in the dark and the uptake of periodate was monitored spectrophotometrically[14]. A similar experiment was per-

formed on polysaccharide *B*.

To a solution of polysaccharide *B* (60 mg) in water (15 mL) was added 0.03M $NaIO_4$ (15 mL). The mixture was stored for 72 h in the dark, ethylene glycol (15 mL) was then added, and the solution was dialysed and freeze-dried. The residue was dissolved in water (10 mL) and $NaBH_4$ (50 mg) was added. After 4 h, the excess of borohydride was decomposed with acetic acid, and the solution was dialysed and freeze-dried. A solution of the product in 0.05M trifluoroacetic acid was stored for 24 h at room temperature and then concentrated. A portion (10 mg) of the residue was methylated (Hakomori) and methanolysed, and the products were acetylated. G.l.c.–m.s. on OV-17 then gave the results shown in Table I, column III. P.c. of the remainder of SD (detection with reagent *A*) gave SD1 (35 mg) which was studied by ^1H- and ^{13}C-n.m.r. spectroscopy (Table II).

Bacteriophage degradations. — The bacteriophage acting on *E. coli* K9, $\phi 9$, was obtained from Professor S. Stirm (Justus-Liebig University, Giessen) and propagated on its host bacteria in nutrient broth (Difco) until a solution (500 mL) having a total of 1.4×10^{12} plaque-forming units (PFU) was obtained (phage assayed by the plaque assay technique). This solution was dialysed against running water, concentrated to 430 mL, and added to polysaccharide *B* (300 mg). After 96 h at 32° in the presence of $CHCl_3$ (2 mL), the solution was freeze-dried, a solution of the residue in water was dialysed against distilled water (6 × 500 mL), and the diffusates were collected and freeze-dried. A solution of the residue (1.430 g) in water (40 mL) was passed down a column of Amberlite IR-120 (H^+) resin several times until a clear solution was obtained. The solution was then freeze-dried. P.c. (detection with reagent *B*) of the product (218 mg) revealed only one major reducing compound (PA, $R_{\text{CELLOBIOSE}}$ 0.34). Preparative p.c. gave 27.3 mg of PA, the ^1H- and ^{13}C-n.m.r. spectra of which are shown in Table II. A portion of PA was reduced with $NaBH_4$ (40 mg) and then methylated. The methylated product was methanolysed and the products were acetylated. G.l.c.–m.s. analysis on OV-17 gave the results in Table I, column IV.

A second bacteriophage-degradation of polysaccharide *B* (300 mg) was carried out under modified conditions. The polysaccharide was depolymerised with a solution (500 mL) containing 5.25×10^{12} PFU for 96 h at 30°. The material obtained after freeze-drying was dissolved in water (40 mL), the solution was dialysed against distilled water (mol. wt. cut-off, 3500), and the diffusate was collected every 12 h (4 ×) and freeze-dried. An aqueous solution of the product (1.127 g) was then passed down an ice-jacketed column of Amberlite IR-120 (H^+) resin three times to remove protein and nucleic acids. Analytical gel-permeation chromatography of the product (422 mg) on a column (1.6 × 67 cm) of Bio-Gel P-4, using 0.1M pyridinium acetate buffer (pH 5.2) as eluent (13 mL/h), gave three well-separated peaks corresponding to a tetrasaccharide (P1), an octasaccharide (P2), and material of higher molecular weight. The phage digest was then applied in two portions to a column (2.6 × 73 cm), and the components were separated and isolated to yield P1 (45.6 mg) and P2 (22.3 mg). Pyridine was removed from the

samples by passage through the ice-jacketed cation-exchange resin, and acetic acid was removed by freeze-drying. N.m.r. spectroscopy showed that P1, $[\alpha]_D$ +12° (*c* 1.1, water), corresponded to the chemical repeating-unit of the polymer, and that P2 was the dimer thereof. P1 was studied by ^{1}H- and ^{13}C-n.m.r. spectroscopy; COSY-45 and heteronuclear shift-correlation experiments were also performed (see Table III, and Figs. 3 and 4). P1 (10 mg) was reduced with $NaBH_4$ (6 h, water), and the product methylated. P1 and P1-alditol were methylated and methanolysed, and the products were acetylated. G.l.c. on OV-17 gave the results in Table I, columns V and VI.

Determination of absolute configuration of monosaccharides. — Gal (9.5 mg) and GalNAc (5.1 mg) were isolated after p.c. (detection reagent *B*) of an acid hydrolysate of the polysaccharide (40 mg in 10 mL of 2M trifluoroacetic acid, 1.5 h, 100°). The Gal was shown to be D by g.l.c. of the acetylated (−)-2-octyl glycosides[5] and the GalNAc was shown to be D by oxidation with D-galactose oxidase[6].

ACKNOWLEDGMENTS

We thank N.S.E.R.C. (Ottawa) for financial support, and the C.S.I.R. (Pretoria) for financial support of H.P. and for a research bursary (to L.A.S.P.), Drs. F. and I. Ørskov (Copenhagen) for the culture of *E. coli* K9, Professor S. Stirm (Giessen) for the bacteriophage, Dr. S. C. Churms (University of Cape Town) for the molecular weight measurement, and Mr. I. Antonowitz of the National Chemical Research Laboratory (Pretoria) for performing the n.m.r. experiments.

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