Carbohydrate Research, 233 (1992) 195–204
Elsevier Science Publishers B.V., Amsterdam

Structural elucidation of the capsular polysaccharide of *E. coli* serotype K47

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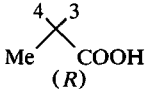
(Received December 11th, 1991; accepted March 12th, 1992)

ABSTRACT

The capsular polysaccharide from *Escherichia coli* K47 was investigated using mainly methylation analysis and ^{1}H and ^{13}C NMR spectroscopy and shown to have the following repeating unit:

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



INTRODUCTION

Pyruvic acid, linked as a cyclic acetal to a pyranosyl residue, is a common component of bacterial polysaccharides[1]. In the *E. coli* series, the pyruvic acid acetal is most often 4,6-linked to a pyranosyl residue, as in the capsular polysaccharides of *E. coli* K29[2], K37[3], and K55[4]. Pyruvate has also been found 2,3-linked to D-glucopyranosyl residues (*E. coli* K33[5]) and 3,4-linked to L-rhamnopyranosyl residues (*E. coli* K26[6]). We now report the structure of the capsular antigen from *E. coli* K47, which contains a pyruvic acid acetal 3,4-linked to a D-galactopyranosyl residue.

RESULTS AND DISCUSSION

Isolation, composition, and linkage analysis.—Bacteria of *E. coli* serotype K47, culture No. A282a, were grown on Luria Bertani agar. The acidic capsular polysaccharide was isolated via its cetyltrimethylammonium complex[7] and further

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purified by chromatography on DEAE-Sepharose CL-6B. The purified capsular polysaccharide (**PS**) had $[\alpha]_D$ +12° (H_2O) and, in gel-permeation chromatography on Sephacryl S-400, showed a broad molecular-weight distribution with a peak maximum at M_r 6.5×10^5.

GLC of the acetylated aldononitriles[8] derived from the products of an acid hydrolysate of **PS** indicated the presence of Man, Gal, and GlcN in the molar ratios 1:2:1. The ratio was unaltered after a methanolysis and reduction, indicating the absence of uronic acid. Each monosaccharide was shown to be D by GLC of the acetylated (−)-2-octyl glycosides[9]. The ^{1}H NMR spectrum of **PS** contained signals for H-1α at δ 5.46 (d, $J_{1,2}$ 3.2 Hz), H-1β at δ 4.91 (d, $J_{1,2}$ 8.0 Hz), 4.85 (unresolved d), and 4.62 (d, $J_{1,2}$ 7.6 Hz). There was, in addition, a signal for NAc at δ 2.09, and *Me*CCOOH at δ 1.68. The ^{1}H-decoupled ^{13}C NMR spectrum contained signals for C-1 at 101.65 (2 C), 100.60, and 100.11 ppm, Me*C*COOH at 107.22 ppm, *C*H_3CON and *Me*CCOOH at 23.45 and 23.15 ppm, and C=O at 175.31 and 174.64 ppm. The results confirmed a tetrasaccharide repeating unit for **PS** with 1 mol of a pyruvic acid acetal.

Methylation analysis of **PS** gave 2,3,6-tri-*O*-methylmannose, 2,3,6-tri-*O*-methylgalactose, 6-*O*-methylgalactose, and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose, which indicated a linear tetrasaccharide repeating unit with the pyruvic acid acetal on the 6-*O*-methylgalactose residue. Treatment of **PS** with bacteriophage-borne enzymes afforded a polysaccharide (**PS-1**) of lower molecular weight which was used in the 2D NMR experiments. The molar ratios of the sugars in **PS** and **PS-1** were identical. The depolymerisation did not produce oligosaccharides corresponding to the repeating unit or multiples thereof, probably due to the low activity of the bacteriophage enzymes.

NMR spectroscopy of PS-1.—The ^{1}H resonances of the four monosaccharides in the repeating unit of **PS-1** were assigned (Table I) on the basis of COSY[10], phase-sensitive NOESY[11], and 2D HOHAHA[12] experiments. The residues in the repeating unit were labelled **a**–**d** in order of decreasing chemical shift of the H-1 resonances. The corresponding H-2 resonances, and that of H-3 for residue **c**, were assigned readily from the COSY spectrum (Fig. 1). These results were confirmed, and the chemical shifts for the resonances H-4 of **c**, H-3,4 of **a** and **d**, and H-3,4,5 of **b** were established from the 2D HOHAHA experiments (Fig. 2). The chemical shift for the H-5 resonance of residue **a** was established via the H-4/H-5 cross-peak in the COSY spectrum.

Further ^{1}H assignments were made from the phase-sensitive NOESY experiment (Fig. 3). The observed inter- and intra-residue NOE contacts are presented in Table II. The α-residue **a** showed the expected NOE contact from H-1 to H-2, whereas the β-residues **b**–**d** each showed characteristic NOEs from H-1 to H-3 and H-5. The latter NOE contacts permitted assignment of the chemical shifts for the H-5 resonance of residues **c** and **d**. The chemical shifts for the H-6a,6b resonances of residues **a**, **b**, and **d** were established from a triple-quantum filter, phase-sensitive COSY experiment.

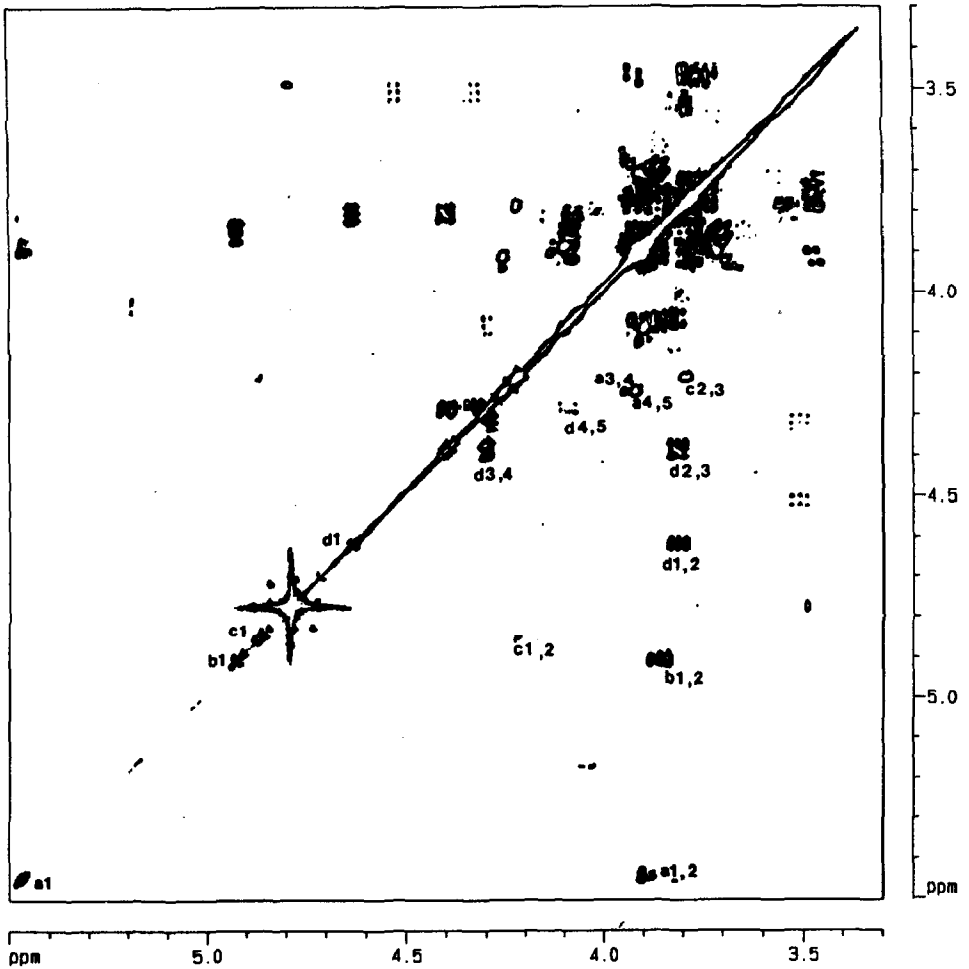


Fig. 1. COSY contour plot for **PS-1** (the sugar residues are labelled **a**–**d**; **a1** connotes H-1 of residue **a**, and **a1, 2** connotes the cross-peak between H-1 and H-2 of residue **a**, etc.).

The ^{13}C resonances for residues **a**–**d** were assigned by comparing the ^{1}H chemical shift data with the ^{1}H–^{13}C correlation data obtained from the HETCOR[13] experiment (Fig. 4 and Table I). The identification of residue **a** as a 4-linked α-Gal, **b** as a 3-linked β-GlcNAc, and **c** as a 4-linked β-Man followed from a comparison of the ^{1}H and ^{13}C NMR data for these residues with data[14,15] for methyl glycosides. In addition, the *manno* configuration for residue **c** was supported by the intra-residue NOEs observed in the NOESY spectrum. Residue **d** was identified as a 2,3,4-linked β-Gal from the small $J_{4,5}$ value, the intra-residue NOE contacts observed, and the ^{13}C chemical shift data. The significant deshielding of C-4 of **a** and **c**, C-3 of **b**, and C-2,3,4 of **d** accords with the results of methylation analysis.

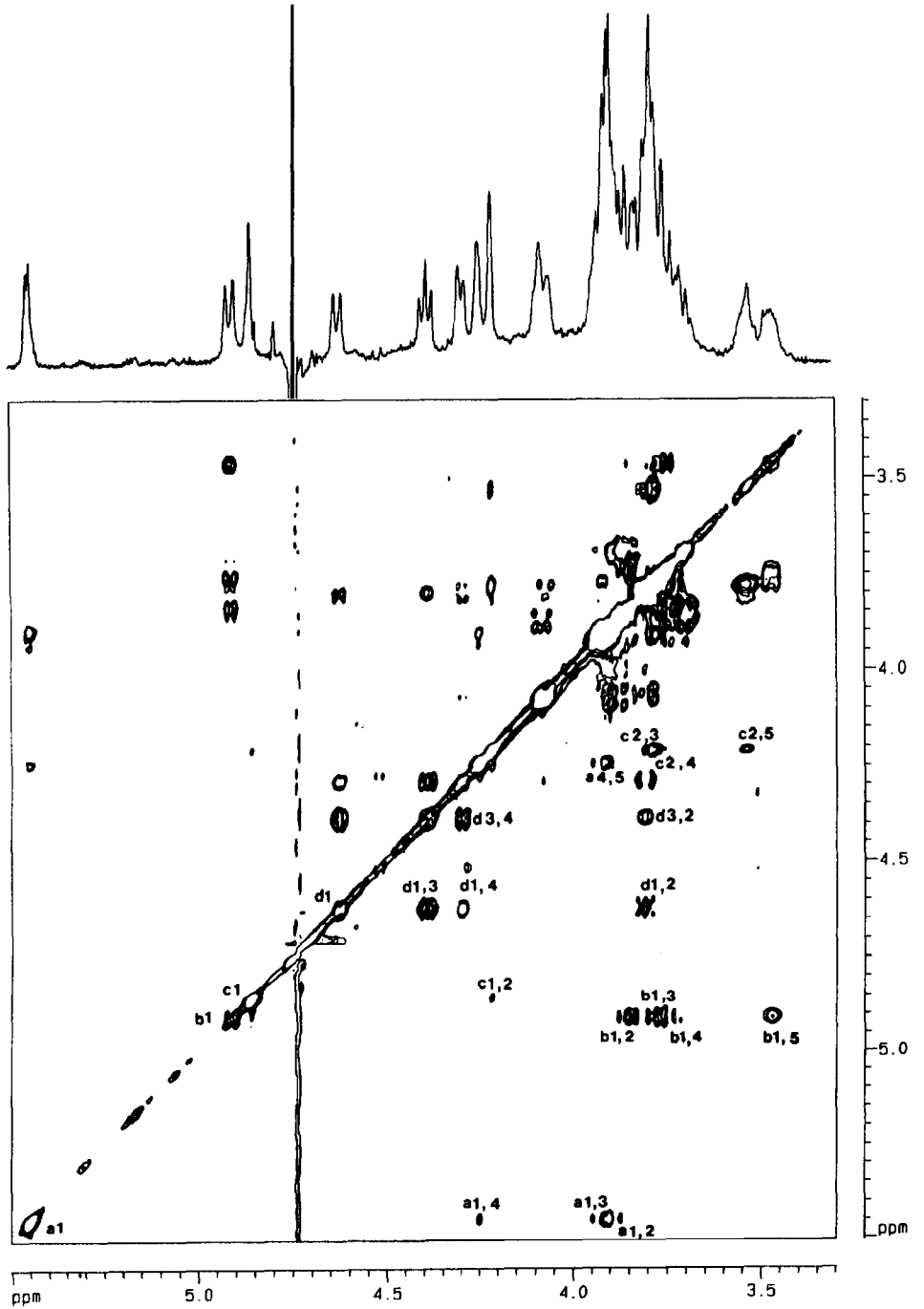


Fig. 2. HOHAHA contour plot for **PS-1** (**a1**, **a1,2**, etc., as for Fig. 1.); the ^{1}H NMR spectrum is projected along the f_2 axis.

TABLE I

^1H and ^{13}C NMR data [a] (30°) for the tetrasacharide repeating unit of the *E. coli* K47 polysaccharide

Residue		Proton or carbon						
		1	2	3	4	5	6a	6b
a	H	5.46	3.89	3.94	4.26	3.91	~ 3.78	~ 3.70
→ 4)-α-Gal		($J_{1,2}$ 3.2)			($J_{4,5}$ 1.2)			
	C	100.11	69.30	70.10	76.97	71.18	60.88	
b	H	4.91	3.86	3.77	3.74	3.45	~ 3.76	~ 3.76
→ 3)-β-GlcNAc		($J_{1,2}$ 8.0)						
	C	100.60	55.21	80.49	71.46	76.45	61.28	
c	H	4.85	4.22	3.79	3.77	3.52		
→ 4)-β-Man		($J_{1,2}$ 1.0)						
	C	101.65	70.83	72.39	78.25	75.71		
d	H	4.62	3.81	4.39	4.29	4.07	~ 3.91	~ 3.84
→ 2)-β-Gal		($J_{1,2}$ 7.6)	($J_{2,3}$ 6.1)	($J_{3,4}$ 6.1)	($J_{4,5}$ ~ 0.8)			
3,4	C	101.65	79.16	79.96	76.21	73.76		
HOOC Me								

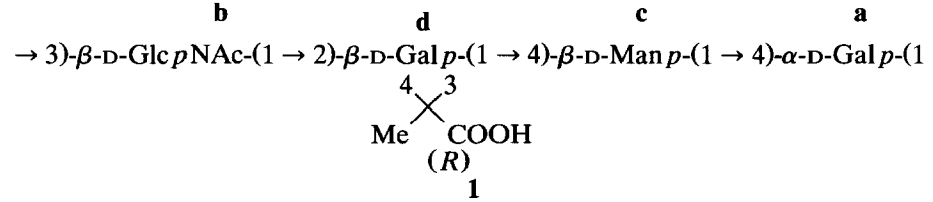
[a] Chemical shifts in ppm relative to that for acetone (δ 2.23 for ^1H and 31.07 ppm for ^{13}C), *J* in Hz.

The sequence of the residues in the repeating unit was established from the inter-residue NOE contacts observed in the NOESY spectrum (Fig. 3 and Table II) between each H-1 and the relevant protons of the adjacently linked residues as

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

The pyruvic acid acetal group can now be located at positions 3 and 4 of residue **d**. The occurrence of the ^{13}C resonance for the asymmetric acetal carbon atom at 107.22 ppm indicates[16] that it is present in a dioxolane ring. The orientation (*R*) of the methyl group in the 3,4-acetal was established from the dipolar interaction observed for the methyl group and H-2 of residue **d** in the NOESY spectrum (Table II).

The foregoing data permit the tetrasaccharide repeating unit of **PS** to be written as **1**.



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The capsular polysaccharide of *E. coli* K47 is the second example in the *E. coli* series where the acidity is due to a pyruvic acid acetal, the other being *E. coli* K37[3].

Molecular models show that, in forming a 3,4-acetal, the $^4C_1$ conformation of the β-D-galactopyranose moiety is distorted and approaches that of a skew

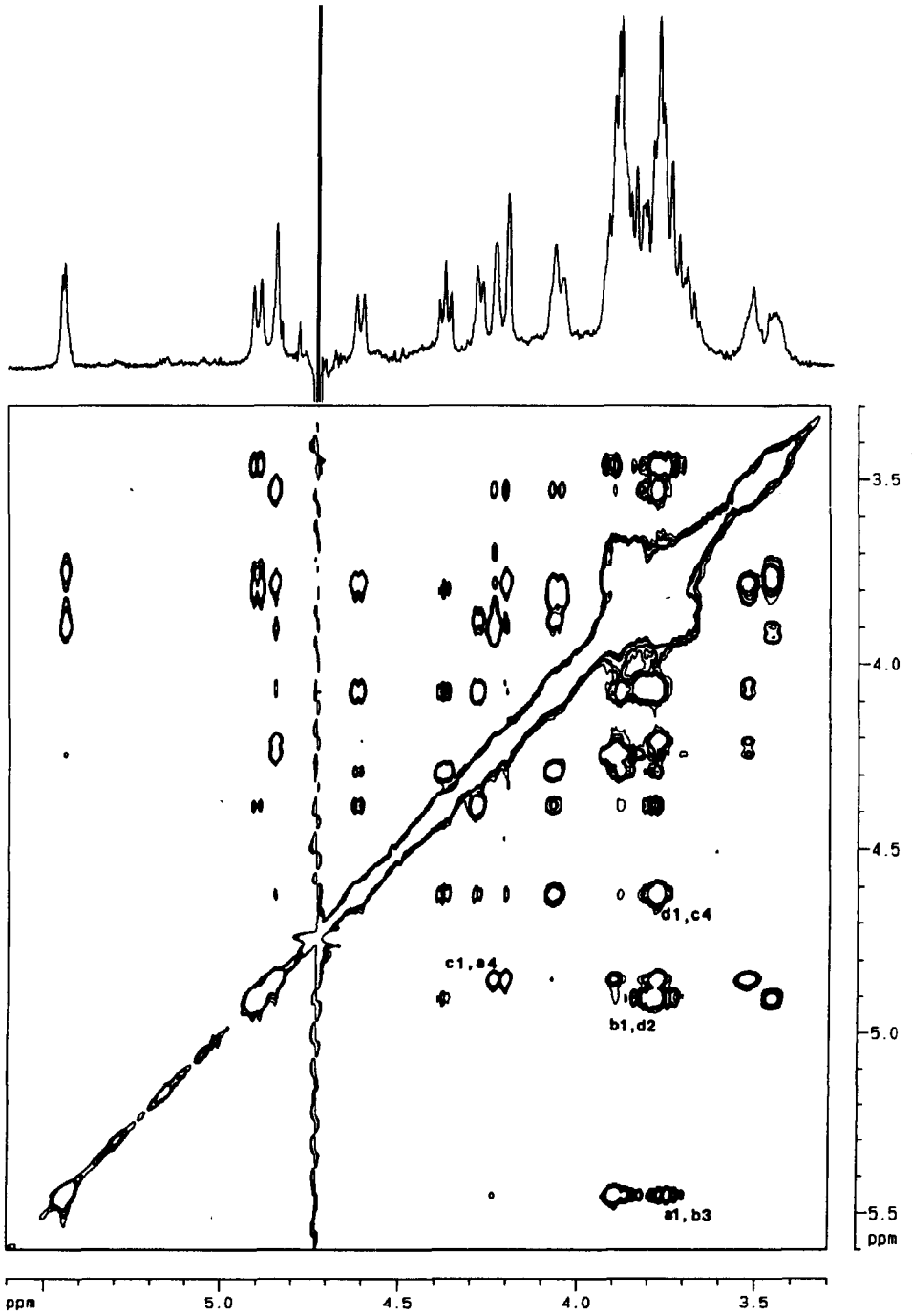

Fig. 3. NOESY contour plot for **PS-1**, showing the inter-residue NOEs, (**a1,b3** connotes the cross-peak between H-1 of residue **a** and H-3 of residue **b**, etc.); the ^{1}H NMR spectrum is projected along the f_2 axis.

TABLE II

Inter- and intra-residue NOE contacts for the *E. coli* K47 polysaccharide

Residue	Proton	NOE contact to
a	H-1	3.89 (**a**, H-2), 3.77 (**b**, H-3)
b	H-1	3.81 (**d**, H-2), 3.77 (**b**, H-3)
		3.45 (**b**, H-5)
c	H-1	4.26 (**a**, H-4), 4.22 (**c**, H-2)
		3.79 (**c**, H-3), 3.52 (**c**, H-5)
	H-2	3.79 (**c**, H-3)
	H-3	3.52 (**c**, H-5)
d	H-1	4.39 (**d**, H-3), 4.07 (**d**, H-5)
		3.77 (**c**, H-4)
	H-3	4.29 (**d**, H-4), 4.07 (**d**, H-5)
	H-4	4.07 (**d**, H-5)
	Me of pyruvate	3.81 (**d**, H-2)

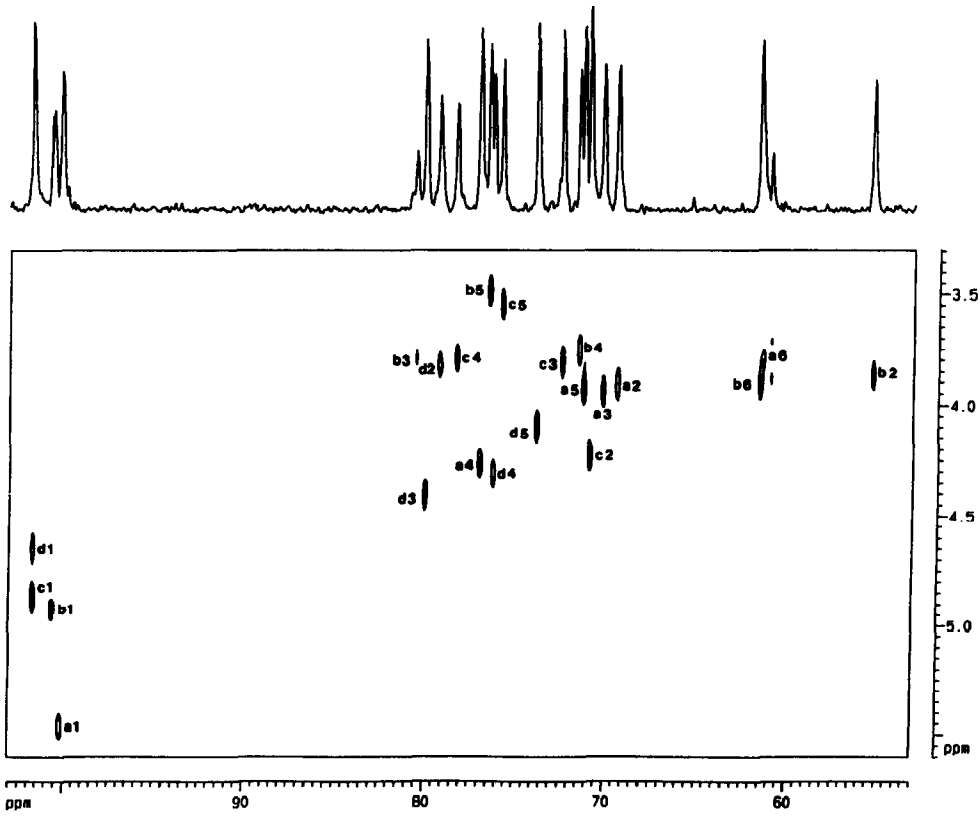

Fig. 4. $^{1}H-^{13}C$ correlation map of the spectral region f_2 103–53 ppm (^{13}C) and f_1 3.3–5.6 ppm (^{1}H) for **PS-1** (**a1**, **b1**, etc., as for Fig. 1).

half-chair. This conformational change is accompanied by a lessening of the H-2,3 and H-3,4 dihedral angles which, according to the Karplus relationship, should result in $J_{2,3}$ being smaller and $J_{3,4}$ being larger than for β-D-galactopyranose. Thus, for residue **d**, $J_{2,3} = J_{3,4} = 6.1$ Hz (Table I), whereas the values for β-D-galactopyranose were 8–10 and 3–4 Hz, respectively.

The K47 capsular polysaccharide appears to be the first example which contains a 3,4-*O*-(1-carboxyethylidene)-β-D-galactopyranosyl residue other than at a terminal position in side chains as in the capsular polysaccharides of *Klebsiella* K13[17], K30[18], and K33[19], and the M-antigen[20] elaborated by Enterobacteriaceae species. The methyl group of the pyruvic acetal in the K47 polysaccharide is *endo* in the *cis*-fused dioxolane system as reported for the other polysaccharides[16]. However, whereas the absolute configuration of the acetalic carbon atom in the latter polysaccharides[16] is *S*, it is *R* in the K47 polysaccharide because the 3,4-*O*-(1-carboxyethylidene)-β-D-galactopyranosyl residue is 2-linked and as a consequence of the priority rules.

3,4-*O*-Carboxyethylidene groups have also been found on terminal α-L-rhamnopyranosyl residues in the capsular polysaccharides of *Klebsiella* K72[21] and *E. coli* K26[6], and on 2-linked α-L-rhamnopyranosyl residues in the capsular polysaccharide of *Klebsiella* K32[22], but the absolute configurations have not been reported.

EXPERIMENTAL

General methods.—Optical rotations were measured in a 1-cm cell at 23 ±2° using a Perkin–Elmer model 141 polarimeter. Gel-permeation chromatography was performed on columns of Biogel P-4 and Sephacryl S-400 coupled to a R401 Waters differential refractometer, by elution with 0.1 M NaOAc buffer (pH 5). Ion-exchange chromatography was performed on a column of DEAE-Sepharose Cl-6B by elution with a gradient from 0 → 1.0 M NaCl in 0.01 M tris(hydroxymethyl)aminomethane at 0.3 mL/min. Fractions were analysed by the phenol–H_2SO_4 method[23]. Analytical GLC was performed using a Hewlett–Packard 5890A gas chromatograph, fitted with a flame-ionisation detector and a 3392A recording integrator. DB-225 and DB-17 bonded-phase capillary columns (J. & W. Scientific) were used and operated at 205° or at 180° for 2 min, then 2°/min to 250°, respectively. GLC–MS was conducted with a Hewlett–Packard 5988A system. The EI-mass spectra were recorded at 70 eV and an ion-source temperature of 200°.

Preparation of the E. coli *K47 polysaccharide* (**PS**).—An authentic culture of *E. coli* K47 (No. A282a, obtained from Dr. I. Ørskov, Copenhagen) was propagated on Luria-Bertani agar. The acidic **PS** was separated from the cells by ultracentrifugation and purified by precipitation with cetyltrimethylammonium bromide[7].

Sugar composition.—Purified **PS** (3 mg) was hydrolysed[8] with 4 M trifluoroacetic acid for 1 h at 125°, and the products were converted into acetylated aldononitriles[24] and examined by GLC. **PS** was treated also with methanolic 3%

HCl for 16 h at 80°, the methanolysate was neutralised, and the products were reduced with $NaBH_4$ in anhyd MeOH, hydrolysed with 4 M trifluoroacetic acid (1 h, 125°), converted into acetylated aldononitriles, and analysed by GLC.

Methylation analysis.—**PS** (acid form, 15 mg) was methylated once by the modified[25] Hakomori method[26] and then by the Kuhn method[27]. Methylated **PS** was hydrolysed (4 M trifluoroacetic acid, 1 h, 125°), and the products were reduced ($NaBH_4$), acetylated, and analysed by GLC–MS. Methylated **PS** was methanolysed (methanolic 3% HCl, 16 h, 80°), and the products were reduced, hydrolysed, reduced, acetylated, and analysed by GLC–MS.

Bacteriophage-mediated depolymerisation of **PS**.—A bacteriophage for *E. coli* K47, isolated from Grahamstown sewage water, was used to depolymerise **PS** (150 mg). Chromatography of the products on Biogel P-4 by elution with 0.1 M NaOAc (pH 5) gave one major fraction (**PS-1**) that was hydrolysed (4 M trifluoroacetic acid, 1 h, 125°); the products were converted into acetylated aldononitriles, and examined by GLC.

NMR spectroscopy.—The ^1H and ^{13}C NMR spectra were recorded at 30° with a Bruker AMX 400 spectrometer. Samples (~ 20 mg) were deuterium-exchanged and then dissolved in 99.99% D_2O (~ 0.5 mL) containing a trace of acetone as internal standard (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). The parameters used for 2D experiments were as follows: COSY, 512 × 2048 data matrix, zero-filled to 1024 data points in t_1, 48 scans per t_1 value, 1.4-s recycle delay, spectral width 2037 Hz, unshifted sine-bell filtering in t_1 and t_2; HOHAHA, data matrix and spectral width as above, 0.7-s recycle delay, 128 scans per t_1 value, shifted sine-squared filtering in t_1 and t_2, mixing times of 87.5 and 28.1 ms; phase-sensitive NOESY, data matrix and spectral width as above, 112 scans per t_1 value, 0.7-s recycle delay, shifted sine-squared filtering in t_1 and t_2, mixing time of 0.3 s; HETCOR, 128 × 2048 data matrix, zero-filled to 1024 points in t_1, spectral width 1640 Hz in t_1 and 10204 Hz in t_2, 300 scans per t_1 value, 0.7-s recycle delay and a shifted sine-bell filter.

ACKNOWLEDGMENTS

We thank Dr. Ida Ørskov (Copenhagen) for the test strain of *E. coli* K47, and the Foundation for Research Development for financial support (to H.P.) and an M.Sc. bursary (to A.H. de B.)

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