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Carbohydrate Research 262 (1994) 323–334

CARBOHYDRATE RESEARCH

Structural elucidation of the capsular polysaccharide produced by *Escherichia coli* O20 : K84 : H26 ☆

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Received 3 March 1994; accepted 1 April 1994

Abstract

The primary structure of the acidic capsular antigen produced by *E. coli* K84 was shown by glycose and methylation analysis, and by 1D and 2D ^{1}H and ^{13}C NMR studies of the polysaccharide and an oligosaccharide produced by lithium–ethylenediamine degradation of the polysaccharide, to be comprised of linear hexasaccharide repeating units of the following structure:

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

Key words: Escherichia coli; Capsular polysaccharide; K84 Antigen; Structure; NMR spectroscopy

1. Introduction

Bacteria of the genus *Escherichia coli* have been broadly subdivided into two groups, based primarily on differences in chemical composition, but also in terms of such factors as mode of expression and genetic determination [1]. Group I polysaccharides, which are co-expressed only with O8, O9, and O20, tend to be less structurally diverse than the Group II antigens and have been further sub-divided according to the presence or absence of amino sugars. The *E. coli* K84 capsular antigen may therefore be classified as an amino sugar-containing Group I polysaccharide. It resembles the K83 capsular polysaccharide [2], which is also co-ex-

☆ Presented at the VIIth European Symposium on Carbohydrate Chemistry, Cracow, Poland.

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SSDI 0008-6215(94)00136-4

pressed with O20 and H26, in that it too is a linear polysaccharide. A hexasaccharide repeating unit is, however, unusual as the average number of residues per repeating unit in this genus is four, and larger repeating units are frequently branched.

2. Results and discussion

Isolation, composition, and linkage analysis.—*E. coli* K84 bacteria were grown on Mueller–Hinton agar and the capsular polysaccharide was isolated as previously described using cetyltrimethylammonium bromide (CTAB) [3]. However, the acidic K-antigen precipitated as a gel-like mass only after the crude polysaccharide was treated with aq 1% acetic acid to remove lipid [1]. After dissolution of the CTAB–polysaccharide complex in 3 M NaCl, the material was reprecipitated into ethanol and the precipitated polysaccharide was further purified by dialysis and GPC on Sephacryl S-500. The polysaccharide (**PS**) was resistant to acid hydrolysis and the constituent sugars were not released in stoichiometric proportions. Man, Glc, GalA, GlcN, and ManN were determined to be the constituent monosaccharides by analytical GLC-MS of the derived alditol or *O*-methyloxime acetates following hydrolysis, and methanolysis with carboxyl reduction. GLC analysis of the derived acetylated (−)-2-octyl glycosides showed that all the sugars had the D configuration. **PS** was methylated according to a modified Hakomori [4] procedure, and GLC analysis of the partially methylated alditol acetates derived from an acid hydrolysate of methylated **PS**, with and without carboxyl reduction, revealed the presence of 4-substituted GalA, 3-substituted Man, 3-substituted GlcN, 3-substituted ManN, 2-substituted Man, and 4-substituted Glc. This linkage pattern is consistent with a linear hexasaccharide repeating unit.

NMR spectroscopy.—The proton NMR spectrum of **PS** at 313 K (Fig. 1) contained nine resonances in the anomeric region (δ 4.5–5.5) at δ 5.29, 5.25, 5.17, 4.93, 4.83, 4.75, 4.72, 4.57, and 4.55. In addition, signals for methyl protons of acetyl functions were present at δ 2.10 and 2.04. Coupling constants could only be measured for the signals at δ 5.29 (4.1 Hz) and 5.17 (3.4 Hz), indicative of two α-linked sugars, and for a β-signal at δ 4.57 (8.1 Hz), after acquiring a 1D spectrum at 343 K to improve resolution. The anomeric configurations of the remaining sugars were established from the NOESY [5] spectrum (see below). The ^{13}C spectrum of **PS** showed only six signals for pyranosidically linked hexoses at 97.13, 99.02, 100.34, 101.46, 101.96, and 103.44 ppm, thus proving that three of the nine resonances in the anomeric region of the proton spectrum were non-anomeric. Signals at 50.57 and 53.70 ppm for acetamido-substituted carbons and resonances for carbonyl carbons at 175.80 and 175.29 ppm, and for methyl carbons (2 × C) at 22.94 ppm, were consistent with the presence of two *N*-acetylated amino sugars in the repeating unit. An additional signal for a carbonyl carbon at 173.27 ppm was assigned to C-6 of a uronic acid. The remaining ^{1}H and ^{13}C resonances for the spin systems of the six sugars in the repeating unit were assigned using COSY [6], HOHAHA [7], HMQC [8] (Fig. 2), HMQC-TOCSY [9], and NOESY (Fig. 3)

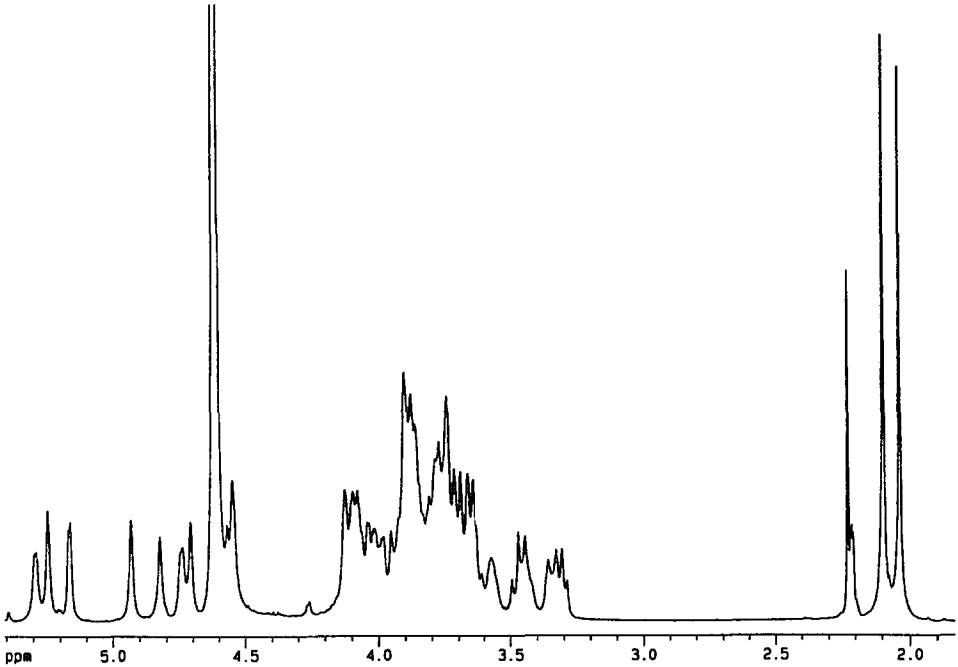

Fig. 1. ^{1}H NMR spectrum of K84 **PS** at 313 K.

experiments, and these data are presented in Table 1. Sugars are labelled **a**–**f** in decreasing order of the chemical shifts of their H-1 resonances.

Residue **a** *[→4)-α-GalA].*—The ^{1}H resonances for residue **a** were readily established via their cross-peaks in the COSY spectrum and further confirmation followed from cross-peaks observed in the HOHAHA spectrum. The chemical shifts of the corresponding directly coupled ^{13}C nuclei were then assigned by comparing the ^{1}H resonances obtained above with the $^{1}H-^{13}C$ correlation data obtained from an HMQC spectrum of **PS** (Fig. 2). H-4 (δ 4.55) and H-5 (δ 4.72) of this residue account for two of the three non-anomeric signals present in the anomeric region. Such low-field chemical shifts for H-4 and H-5 are a characteristic of →4)-α-GalA residues.

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

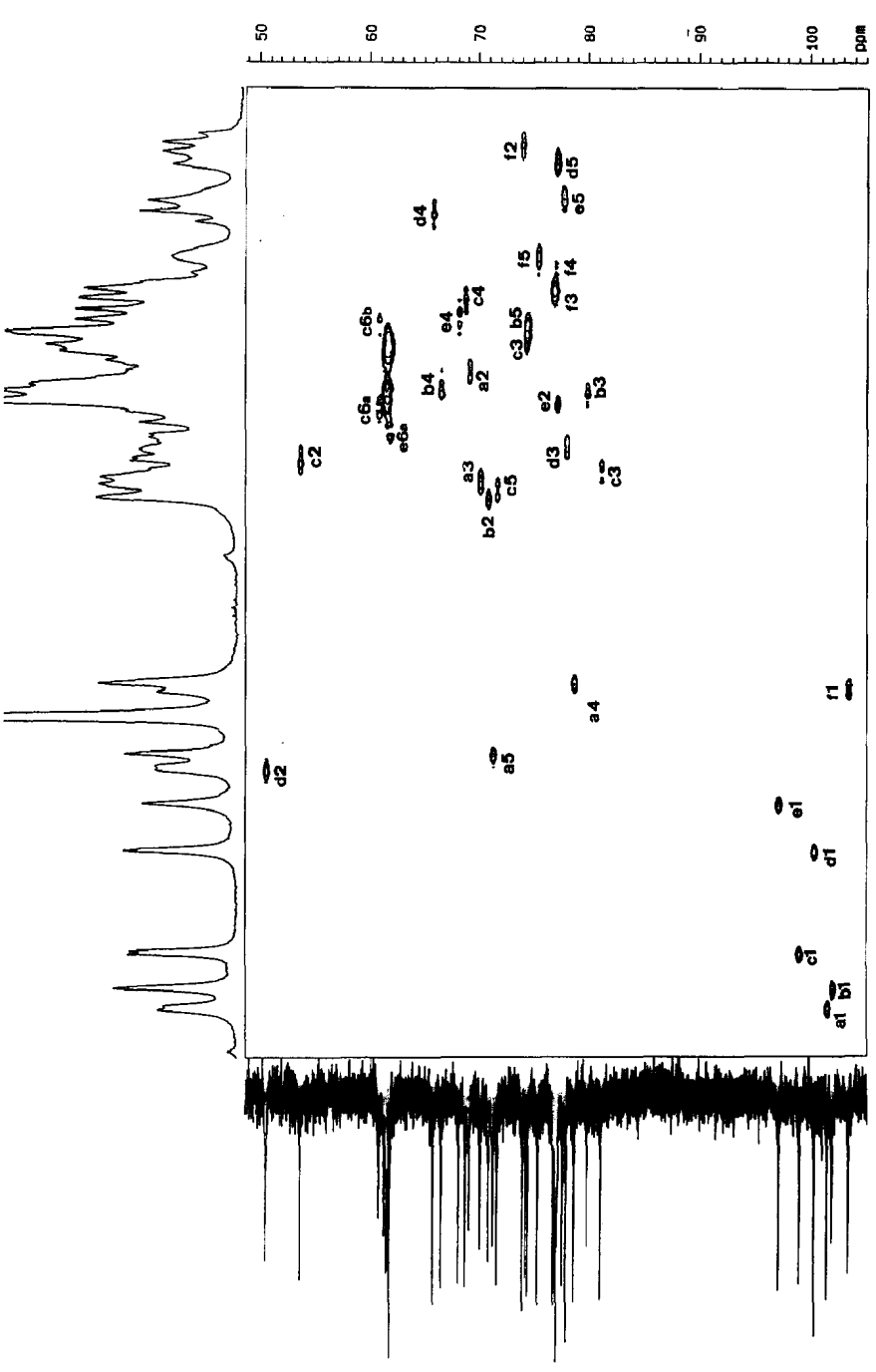

Fig. 2. HMQC $^{1}H-^{13}C$ shift correlation map of the regions f_1 110–48 ppm and f_2 δ 5.4–3.2 for K84 PS at 313 K. Correlated resonances are labelled **a–f**.

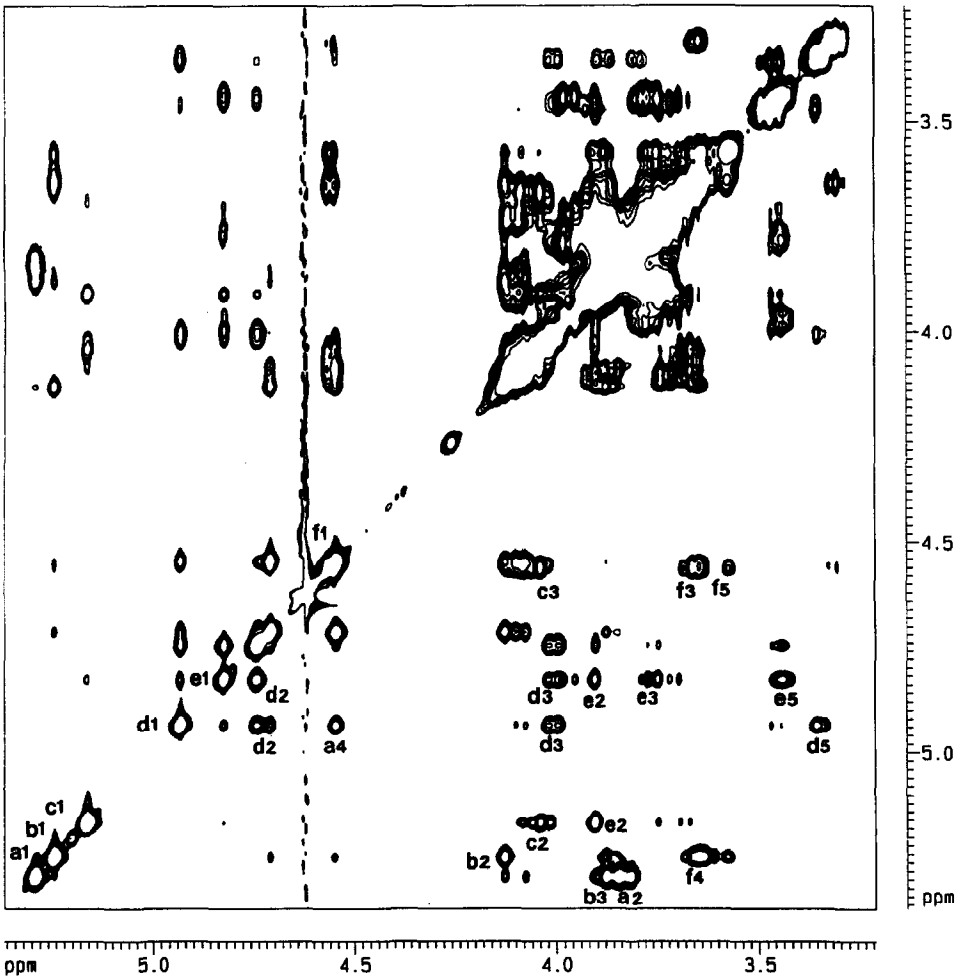

Fig. 3. 2D NOESY spectrum of K84 PS at 313 K. Relevant intra- and inter-residue NOEs are denoted on the spectrum and are recorded in Table 3.

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

Residue **d** *[→3)-β-ManNAc].*—The entire spin system for this residue was readily established by following the cross-peaks in the COSY and HOHAHA spectra, and the ^{13}C data for C-1 to C-5 were then assigned from the ^{1}H–^{13}C correlation data obtained from the HMQC spectrum. H-2 (δ 4.75) of this residue could clearly be identified as the third non-anomeric signal between δ 4.5 and 5.5. Due to excessive overlap in the HMQC spectrum and the absence of an H-5/C-6

Table 1
^{1}H and ^{13}C chemical shift data for K84 PS at 313 K

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

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

relay in the HMQC-TOCSY spectrum, the chemical shift for C-6 could not be assigned with certainty.

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

Residue **f** *[→ 4)-β-Glc].*—β-Glc residues are normally easy to assign, as their stereochemistry is conducive to strong magnetisation transfer. However, in this

instance, almost complete resonance overlap between H-3 and H-4 and poor magnetisation transfer between H-5 and H-6a/6b precluded the unambiguous assignment of the entire spin system from the COSY spectrum. Examination of a 1D slice through the cross-peaks relaying to H-1 in the HOHAHA spectrum revealed overlap of H-3 and H-4 and permitted elucidation of the proton spin system. Correlation of these resonances with their corresponding carbon nuclei, via the correlation data in the HMQC spectrum, permitted assignment of C-1 through C-5. It is interesting to note that the ^{13}C chemical shifts for C-3 and C-4 are also very close together. Surprisingly, C-6 could not be assigned with certainty because of the absence of an H-5/C-6 relay in the HMQC-TOCSY spectrum.

Comparison of the ^{1}H and ^{13}C chemical shift data for residues **a–f** with literature values for methyl glycosides [10–13] permitted identification of the six sugar residues and, in agreement with the linkage pattern obtained from methylation analysis, C-4 of **a**, C-3 of **b**, C-3 of **c**, C-3 of **d**, C-2 of **e**, and C-4 of **f** experienced significant deshielding. It is interesting to note that the proton chemical shifts, particularly those for residues **b** and **e** (the two mannose residues), do not reliably indicate the linkage positions; for example, H-2 of residue **b** experiences greater deshielding than H-3, the linkage site. This underlines the necessity for obtaining full ^{13}C chemical shift information in complex carbohydrate structural studies, especially in the absence of methylation data.

Lithium–ethylenediamine degradation of **PS**.—In order to generate an oligosaccharide suitable for further confirmatory NMR studies, **PS** was degraded using lithium dissolved in ethylenediamine. Certain modifications to the published procedure [14] were found to be necessary. Neither dialysis nor passage down a cation-exchange resin column was appropriate for removing the large quantity of lithium acetate which forms after the addition of acetic acid to the basic mixture, due to the presence of *N*-deacetylated amino functions in the degraded product and its low molecular weight. The acidified mixture was therefore desalted on a Bio-Gel P-2 column, and the carbohydrate fraction was isolated and refraction-

Table 2
^{1}H chemical shift data [a] for oligosaccharide **1**

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

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

ated. A single oligosaccharide-alditol (oligosaccharide **1**) was isolated, re-*N*-acetylated, and further purified using semipreparative HPLC prior to NMR analysis.

^{1}H NMR studies of oligosaccharide **1**.—The 1D ^{1}H NMR spectrum of oligosaccharide **1** contained four signals in the anomeric region at δ 5.29, 5.26, 4.81, and 4.57. $^3J_{1,2}$ Coupling constants could be measured for the signals at δ 5.26 (2.9 Hz) and 4.57 (7.9 Hz), consistent with an α- and a β-linked residue, respectively. Assignment of the chemical shifts of the remaining protons in the *J*-coupled spin systems of the four sugar residues and the terminal alditol was accomplished using COSY, HOHAHA, and ROESY [15] experiments (Table 2). The five constituent residues are labelled **a'** to **d'** in decreasing order of the chemical shifts of their H-1 resonances, and the alditol is labelled **e'**. The anomeric configuration for residue **a'** was established as α from its chemical shift while that for **c'** was established as β from the intraresidue NOEs observed between H-1 and H-3 and H-1 and H-5 in the ROESY spectrum of oligosaccharide **1**.

Sequencing the repeating unit.—The sequence of the residues in the repeating unit was established from interresidue NOEs obtained from the NOESY spectrum

Table 3
Observed NOEs for K84 **PS**

Residue	Proton	NOE at
a		
→ 4)-α-D-Gal*p*A	H-1 (5.29)	3.83 (**a**, H-2)
		3.88 (**b**, H-3)
b		
→ 3)-α-D-Man*p*	H-1 (5.25)	4.13 (**b**, H-2)
		3.63 (**f**, H-4)
c		
→ 3)-α-D-Glc*p*NAc	H-1 (5.17)	4.03 (**c**, H-2)
		3.91 (**e**, H-2)
d		
→ 3)-β-D-Man*p*NAc	H-1 (4.93)	4.75 (**d**, H-2)
		4.55 (**a**, H-4)
		4.01 (**d**, H-3)
		3.35 (**d**, H-5)
e		
→ 2)-β-D-Man*p*	H-1 (4.83)	4.75 (**d**, H-2)
		4.01 (**d**, H-3)
		3.91 (**e**, H-2)
		3.77 (**e**, H-3)
		3.43 (**e**, H-5)
f		
→ 4)-β-D-Glc*p*	H-1 (4.57)	4.07 (**c**, H-3)
		3.65 (**f**, H-3)
		3.57 (**f**, H-5)

of PS and a ROESY spectrum of oligosaccharide **1**. The observed NOEs for PS are detailed in Table 3. Interresidue NOEs for oligosaccharide **1** were observed between H-1 of **a'** and H-4 of **d'**, H-1 of **d'** and H-3 of **b'**, H-1 of **b'** and H-2 of **c'**, and between H-1 of **c'** and H-3 of **e'**, thus enabling the structure of the lithium-degraded product to be written as:

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

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

This structure, together with the NOEs obtained for **PS**, are supportive of the following sequence for the repeating unit:

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

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

The linkage NOE between H-1 of **e** and H-3 of **d** in **PS** was noted to be weaker than the NOE observed to H-2 of **d**. A stronger NOE to H-2 is frequently observed in sugars with the *manno* configuration which are substituted at O-3 by a β-D-(or α-L-)glycopyranosyl) residue. The anomeric configurations for residues **b**, **d**, and **e**, which could not be assigned from $^3J_{1,2}$ coupling constant values being of the *manno* type, were established from intraresidue NOEs present in the NOESY spectrum of **PS**. Residues **d** and **e** were clearly β-linked as both residues showed H-1/H-2, H-1/H-3, and H-1/H-5 NOEs, whilst residue **b**, which showed only an H-1/H-2 intraresidue NOE, was assigned the α configuration.

3. Conclusion

The combined data obtained from chemical and NMR analysis of the polysaccharide and oligosaccharide **1** show that the *E. coli* K84 polysaccharide is comprised of linear hexasaccharide repeating units having the structure shown in the Abstract. It is only the second *E. coli* capsular antigen found to contain ManNAc. The *E. coli* K50 polysaccharide [16], the structure of which was established in this laboratory shortly before this study, also contains ManNAc, although it is α-linked in this instance.

4. Experimental

General methods.—Analytical GLC was performed with a Hewlett–Packard 5890A gas chromatograph, fitted with flame-ionisation detectors and a 3392A recording integrator, with He as carrier gas. A J&W Scientific fused-silica bonded-phase DB-17 capillary column (30 m × 0.25 mm, 0.25-μm film) was used to separate alditol acetates and partially methylated alditol acetates, operated on a temperature programme of 180°C for 2 min then 2°C min^{-1} to 240°C; head pressure, 100 kPa. A Hewlett–Packard 5988A GLC–MS instrument was used to

confirm the identities of partially methylated derivatives, using the appropriate column, and with an ionisation energy of 70 eV and an ion-source temperature of 200°C. Hydrolysis of samples with CF_3CO_2H, carboxyl reduction of methyl esters resulting from methanolyses, determination of the absolute configuration of the sugars, preparation of alditol acetates, and methylation of the polysaccharide were carried out as previously described [17]. Where necessary, samples of **PS** were converted into the acid form by passage down an Amberlite IR-120 (H^+) resin column and freeze-dried prior to analysis. *O*-Methyloxime derivatives were prepared as described by Neeser and Schweizer [18], and were analysed by GLC on a DB-Wax capillary column operated at 180°C for 2 min then 3°C min^{-1} to 240°C; column head pressure, 140 kPa.

Isolation and purification.—An authentic culture of *E. coli* O20:K84:H26 bacteria (Culture No. CDC-2292-55) was obtained from Dr. I. Ørskov (Copenhagen). The bacteria were propagated on Mueller–Hinton agar at 37°C and the capsular polysaccharide was isolated as previously described [3], giving a total yield of 474 mg. An additional step to remove attached lipid (aq 1% AcOH, 60°C, 1 h) was required prior to precipitation with CTAB, as its presence was found to impair CTAB complex formation. Further purification of the isolated capsular polysaccharide was achieved by GPC on Sephacryl S-500 (100 × 2.5 cm), using aq NaOAc buffer (0.1 M) as eluent. Material was detected by refractive index.

Lithium–ethylenediamine degradation.—A sample of **PS** (25 mg, acid form) was dried in vacuo (60°C, 12 h) and dissolved in dry ethylenediamine (15 mL). Lithium wire (8–12 × 3 mm lengths) was added at intervals to the solution to maintain a deep blue colour and the contents of the flask were gently swirled for a period of 1 h. After cooling (ice–water bath), the reaction was quenched by the addition of water (25 mL), followed by removal of the water and ethylenediamine as the toluene azeotrope. The powdery residue of LiOH and carbohydrate which remained was cooled (ice–water bath), dissolved in the minimum quantity of water, and titrated to pH 4.5 with glacial acetic acid. The resulting acidic solution was concentrated under reduced pressure and the viscous solution was desalted on a Bio-Gel P-2 column (70 × 1.6 cm) in two stages using water as eluent. The carbohydrate fractions were isolated, freeze-dried, re-*N*-acetylated [(2 mL H_2O–0.1 mL MeOH–0.1 mL Ac_2O, 3 h, 20°C) followed by the addition of 0.1 mL 25% (aq) NH_3)], and rechromatographed on an identical column. Further purification was carried out using semi-preparative HPLC (Progel TSK-oligo-PW column, 10^{-4} M NaOH as eluent). Prior to NMR analysis, a final reduction step ($NaBH_4$) was also performed in order to ensure complete reduction of the product to the corresponding alditol (yield, 4.6 mg).

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying solutions in D_2O, and then dissolved in 99.99% D_2O (0.5 mL) containing a trace of acetone as internal reference (δ 2.23 for ^1H and 31.07 ppm for ^{13}C). Spectra were recorded on a Bruker AMX-400 NMR spectrometer equipped with an X32 computer using UXNMR software. Experiments on **PS** were performed at 313 K and the parameters used for the 2D experiments were as follows: COSY [256 × 2048 data matrix, zero-filled to 1024 data points in t_1; 1.0-s recycle delay; 112 scans per

t_1 value; spectral width, 2008 Hz; unshifted sine-bell filtering in t_1 and t_2 prior to transformation and symmetrisation]; HOHAHA [512 × 2048 data matrix, zero-filled to 1024 data points in t_1; 1.0-s recycle delay; 64 scans per t_1 value; mixing time, 84 ms; spectral width, 2008 Hz; shifted sine-squared filtering in t_1 and t_2]; NOESY [512 × 2048 data matrix, zero-filled to 1024 data points in t_1; 60 scans per t_1 value; 0.3-s mixing delay; a phase-shifted sine-squared window function was applied during transformation]; HMQC [512 × 4096 data matrix, zero-filled to 1024 data points in t_1; 52 scans per t_1 value; spectral width, 14085 Hz in t_1 and 2008 Hz in t_2; 1.0-s recycle delay]; HMQC-TOCSY [512 × 4096 data matrix, zero-filled to 1024 data points in t_1; 48 scans per t_1 value; MLEV-17 mixing time, 25 ms; spectral width, 14085 Hz in t_1 and 2008 Hz in t_2; 1.0-s recycle delay]. All experiments on oligosaccharide **1** were carried out at 303 K and the parameters used for the 2D experiments were as follows: COSY-90 and COSY-PR [256 × 2048 data matrix, zero-filled to 1024 data points in t_1; 1.0-s recycle delay; 112 scans per t_1 value; spectral width, 2604 Hz; unshifted sine-bell filtering in t_1 and t_2 prior to transformation and symmetrisation]; HOHAHA [512 × 2048 data matrix, zero-filled to 1024 data points in t_1; 1.0-s recycle delay; 136 scans per t_1 value; mixing time, 84 ms; spectral width, 2008 Hz; shifted sine-squared filtering in t_1 and t_2]; ROESY [256 × 2048 data matrix, zero-filled to 1024 data points in t_1; 112 scans per t_1 value; spectral width, 3601 Hz; recycle delay, 2 s; shifted sine-squared filtering in t_1 and t_2; carrier frequency placed at far left-hand side of spectrum to minimise COSY and HOHAHA cross-peaks].

Acknowledgments

We thank Dr. I Ørskov (Copenhagen) for the test strain of *E. coli* K84, the Foundation for Research Development (Pretoria) for financial support (to H.P.) and a special merit doctoral bursary (to D.W.), and the Loewenstein Educational Trust for financial assistance (to D.W.).

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