Carbohydrate Research, 193 (1989) 157–163
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURE OF THE CAPSULAR ANTIGEN OF *Escherichia coli* O8:K8:H4*

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(Received December 28th, 1988; accepted for publication, March 7th, 1989)

ABSTRACT

The structure of the capsular polysaccharide from *Escherichia coli* O8:K8:H4 has been elucidated, using mainly methylation analysis, Smith degradation, and 1D- and 2D-n.m.r. spectroscopy. The polysaccharide, after removal of bound lipid, was found to be composed of repeating units of the linear tetrasaccharide.

$$\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow 2)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow$$
$$\begin{array}{l} 4 \\ | \\ \text{OAc} \end{array}$$

INTRODUCTION

Escherichia coli capsular polysaccharides (K-antigens) have been classified into 74 different serotypes[1]. Over the past two decades, the structures of 47 of these antigens have been elucidated in a continuing programme to understand the implications of chemical structure in serological reactions. In so doing, the reasons for many of the observed serological cross-reactions between bacterial strains and species have become clear. We now report on the structure of the capsular antigen from *E. coli* O8:K8:H4.

RESULTS AND DISCUSSION

Isolation, composition, and linkage analysis of the capsular antigen. — *E. coli* O8:K8:H4 bacteria were grown on a nutrient agar medium as described[2] and the capsular material was extracted with aqueous 1% phenol. The polysaccharide, after

*Presented at the XIVth International Carbohydrate Symposium, Stockholm, Sweden, August 14–19, 1988.
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0008-6215/89/$ 03.50

purification via its cetyltrimethylammonium salt, had $[\alpha]_D$ +68° and, in permeation chromatography on Sephacryl S400, showed a broad distribution of molecular weights with peaks at M_r 53,000 and 500,000. An aqueous solution of the polysaccharide became milky at 90° indicating the presence of lipid. Delipidation of the polysaccharide with aqueous 1% acetic acid at 100° afforded a polymer which, on elution from Sephacryl S400, gave essentially a single broad peak with M_r 55,000.

G.l.c. of the derived alditol acetates showed the polysaccharide to be composed of GlcA, Gal, GlcNAc, and GalNAc. The sugar residues were found to be D by g.l.c. of the acetylated (−)-2-octyl glycosides[3], with and without prior carboxyl reduction. The ^{1}H-n.m.r. spectrum of the purified polysaccharide contained signals for H-1α* at δ 5.086 ($J_{1,2}$ 3.5 Hz), H-1β at δ 4.786 ($J_{1,2}$ 8.5 Hz), 4.581 ($J_{1,2}$ 7.7 Hz), and 4.566 ($J_{1,2}$ 7.9 Hz), two NAc groups at δ 2.018, an OAc group at δ 2.09, and a ring proton at 4.998 (t, ^3J 9.0 and 10.3 Hz). The ^{13}C-n.m.r. data complemented the ^{1}H-n.m.r. results with signals for C-1 at 104.593, 102.531, 100.741, and 98.102 p.p.m., for the methyl carbons of NAc and OAc groups at 23.184 and 21.212 p.p.m., respectively, and for carbonyl carbons at 173.172, 174,750, and 175.417 p.p.m. These results suggested that the polysaccharide consists of a tetrasaccharide repeating-unit.

Methylation analysis of the polysaccharide gave 3,4,6-tri-*O*-methylgalactose, 2-deoxy-4,6-di-*O*-methyl-2-methylacetamidoglucose, 2-deoxy-4,6-di-*O*-methyl-2-methylacetamidogalactose, and 2,4-di-*O*-methylglucose (after carboxyl reduction), which were identified by g.l.c.–m.s. of their derived alditol acetates. These results accord with a tetrasaccharide repeating-unit for the polysaccharide.

N.m.r. studies. — The ^{1}H resonances of the four sugar residues in the repeating unit were established (Table I) mainly from ^{1}H–^{1}H spin-correlated experiments (COSY[4] and RELAY COSY[5]). The H-1 resonances of the residues in the repeating unit were labelled arbitrarily **a**–**d** in order of decreasing chemical shift. All the ^{1}H resonances of **a** and **d** were readily assigned from the COSY experiments, but only H-1/4 of residues **b** and **c**. It was necessary to perform the COSY experiments at 40°, 60°, and 75° in order to distinguish between H-2 and H-3 of **c** and between H-2 of **c**, H-5 of **b**, and H-4 of **a**. Since cross-peaks between the resonances for H-4 and H-5 of **c** and **b** were not observed, assignments for H-5, H-6, and H-6′ for these residues were established as follows: H-5 of **b** was assigned from the intra-residue n.O.e. contacts established for this unit in the NOESY[6] experiment (see below), and H-6 and H-6′ were then traced from the COSY spectrum. The H-6 and H-6′ resonances for **c** could now be identified readily from the ^{1}H–^{13}C shift-correlated (HETCOR[7]) experiment (see below), with the H-5 resonance following from the COSY experiment. The ^{1}H coupling constants were obtained from a 1D spectrum and a 2D *J*-resolved spectrum of the polysaccharide. The ^{13}C resonances for **a**–**d** were determined by comparing the ^{1}H resonance data, established above, with the ^{1}H–^{13}C correlation data obtained from the HETCOR experiment.

*H-1α implies the anomeric proton of an α-linked residue.

TABLE I

^{1}H- AND ^{13}C-N.M.R. DATA[a] FOR THE K8 POLYSACCHARIDE

Residue		*Proton or carbon*						
		1	*2*	*3*	*4*	*5*	*6*	*6'*
→3)-β-GlcA (**d**)	*H*	4.566	3.511	3.892	4.998	3.790		
	^3J (Hz)	7.9 (1,2)	10.4 (2,3)	9.0 (3,4)	10.3 (4,5)			
	C	104.593	72.381	78.508 (+2.0)[b]	73.866	74.019		
→2)-β-Gal (**c**)	*H*	4.581	3.665	3.702	3.885	3.625	3.834	3.753
	J	7.7 (1,2)		3.2 (3,4)	1.7 (4,5)			
	C	100.741	78.994 (+7.2)[b]	74.505	69.621	75.596	61.978	
→3)-β-GalNAc (**b**)	*H*	4.786	4.039	3.812	4.141	3.651	3.746	3.746
	J	8.5 (1,2)	10.7 (2,3)	4.5 (3,4)	~0.5 (4,5)			
	C	102.531	51.999	81.602 (+9.7)[b]	68.711	75.718	61.523	
→3)-α-GlcNAc (**a**)	*H*	5.086	4.053	3.991	3.673	4.171	3.836	3.810
	J	3.5 (1,2)	10.7 (2,3)	9.0 (3,4)	10.1 (4,5)			
	C	98.102	52.939	75.509 (+3.6)[b]	68.287	72.108	60.977	

[a]Chemical shift in p.p.m. downfield from the signal for acetone at δ 2.230 and 31.070 for ^{1}H and ^{13}C, respectively. Spectra recorded at 40° and at 400 MHz (^{1}H) and 100.6 MHz (^{13}C). [b]Glycosylation shifts[8,10,11] in p.p.m.

Based on the monosaccharide composition, the ^{1}H and ^{13}C chemical shift data, and the $^3J_{H,H}$ values, residue **a** was identified as α-GlcNAc, **b** as β-GalNAc, **c** as β-Gal, and **d** as α-GlcA. The observed coupling constants do not differ substantially from the values reported for methyl glycosides[8] and indicate that the residues have the $^4C_1$ conformation. Each tetrasaccharide repeating-unit in the K8 polysaccharide is *O*-acetylated. The appearance of the signal for H-4 of **d** in the anomeric region of the n.m.r. spectrum instead of its more usual position of resonance at δ ~3.5 indicates that the acetyl group is located at position 4 of **d**. Downfield shifts of 1.0–1.4 p.p.m. have been reported[9] for protons attached to carbon atoms bearing OAc groups.

Comparison of the ^{1}H and ^{13}C resonances for **a**–**d** with published data[8,10,11] for methyl glycosides indicates that **a**, **b**, and **d** are 3-linked and that **d** is 2-linked. The glycosylation shift experienced by the resonance of C-3 of **d** is much smaller than those observed for the linkage carbons of the other residues. This situation is because the downfield glycosylation shift of the resonance for C-3 in **d** is opposed by the β-effect of the OAc group on C-4. The linkage sites for **a**–**d**, determined by n.m.r. spectroscopy, accord with the results of methylation analysis.

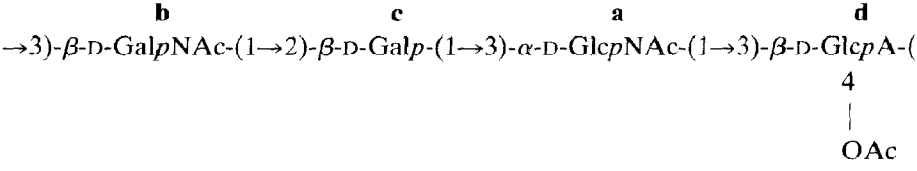
The sequence of the residues **a**–**d** in the repeating unit was established by a NOESY[6] experiment. The observed inter- and intra-residue n.O.e. contacts are presented in Table II. The α-residue **a** showed the expected intramolecular n.O.e.

TABLE II

N.O.E. CONTACTS FOR THE K8 POLYSACCHARIDE

Anomeric proton	*N.O.e. contacts*
a	3.892 (**d**, H-3), 4.053 (**a**, H-2)
b	3.812 (**b**, H-3), 3.651 (**b**, H-5)
	3.665 (**c**, H-2)
c	3.991 (**a**, H-3)
d	3.812 (**b**, H-3), 3.892 (**d**, H-3)
	3.790 (**d**, H-5)

from H-1 to H-2, whereas β-residues **b** and **d** showed characteristic n.O.e. contacts to H-3 and H-5. Surprisingly, no intramolecular n.O.es. were observed for **c**. Inter-residue n.O.e. contacts between anomeric protons and the relevant protons of the adjacent glycosidically linked residues were clearly observed (Table II). The combined n.m.r. and methylation analysis data permit the structure of the tetrasaccharide repeating-unit of the capsular polysaccharide of *E. coli* K8 to be written as **1**.



1

Smith degradation. — In order to confirm the sequence of the residues in the tetrasaccharide repeating-unit, deacetylated K8 polysaccharide was subjected to a Smith degradation. The product, SDI, a tetrasaccharide-alditol, was separated by chromatography on Bio-Gel P-2. SDI, on further Smith degradation, afforded a trisaccharide-alditol, SDII.

Methylation analysis of SDII gave 2,3,4-tri-*O*-methylglucose (after carboxyl reduction), 2-deoxy-4,6-di-*O*-methyl-2-methylacetamidogalactose, and 1,3-di-*O*-methylglycerol, and SDI gave, in addition to the last two methylated sugar residues, 2-deoxy-3,4,6-tri-*O*-methyl-2-methylacetamidoglucose and 2,4-di-*O*-methylglucose (after carboxyl reduction). The ^{1}H-n.m.r. spectrum of SDI contained one less resonance for H-1β than the polysaccharide, whereas SDII had no resonances for H-1α. The ^{1}H-n.m.r. and methylation results for SDI and SDII, when considered in conjunction with the corresponding data for the polysaccharide, clearly establish the structures **2** and **3** and confirm the structure **1**.

α-D-GlcNAc-(1→3)-β-D-GlcA-(1→3)-β-D-GalNAc-(1→2)-glycerol

2 SDI

β-D-GlcA-(1→3)-β-D-GalNAc-(1→2)-glycerol

3 SDII

EXPERIMENTAL

General methods. — G.l.c. was performed using a Hewlett–Packard 5890A gas chromatograph fitted with flame-ionisation detectors and a 3392A recording integrator. A J & W Scientific fused-silica DB-17 bonded-phase capillary column (30 m × 0.25 mm, 0.25-μm film) was used with helium as the carrier gas and operated using the temperature programmes: *A*, 180° for 1 min, then 2°/min to 250° (for methylated alditol acetates); *B*, 165° for 5 min, then 3°/min to 240° (for acetylated octyl glycosides); and *C*, 180° for 2 min, then 5°/min to 240° (for alditol acetates). The identities of each derivative were confirmed by g.l.c.–m.s. on a Delsi Nermag R10-10C quadrupole mass spectrometer coupled to a Varian Vista 6000 series gas chromatograph, with an ionisation energy of 70 eV and an ion-source temperature of 160°. Gel-permeation chromatography of the K8 polysaccharide was performed on a column (1.6 × 65 cm) of dextran-calibrated Sephacryl S400 with 0.1M pyridinium acetate as eluent, and of the Smith-degradation products was done on a column (1.6 × 63 cm) of Bio-Gel P-2 with water as eluent. Fractions were analysed either by the phenol–sulphuric acid method[12] or by differential refractometry.

Oligo- and poly-saccharide samples were hydrolysed[13] with 4M trifluoroacetic acid at 125° for 1 h. Acid hydrolysates were co-concentrated with water and solutions were concentrated under reduced pressure at ≤40° (bath). Alditol acetates were prepared conventionally by reduction of aqueous solutions of hydrolysates with sodium borohydride followed by acetylation with 1:1 acetic anhydride–pyridine. Samples were carboxyl-reduced by treatment with refluxing methanolic 3% hydrogen chloride for 16 h and the methyl esters were treated with sodium borohydride in anhydrous methanol. Methylations were carried out on the acid form of samples, using potassium dimsyl[14] and methyl iodide in methyl sulphoxide.

Preparation of the polysaccharide. — An authentic culture of *E. coli* O8:K8:H4 was obtained from Dr. I. Ørskov (Copenhagen), and the bacteria were propagated[2] on Mueller–Hinton agar. The acidic polysaccharide was separated from the cells by ultracentrifugation and purified using cetyltrimethylammonium bromide. The polysaccharide (840 mg) was delipidated by treatment with aqueous 1% acetic acid at 100° for 1.5 h and the polysaccharide was recovered by freeze-drying the supernatant solution after centrifugation.

N.m.r. spectroscopy. — Samples were deuterium-exchanged by freeze-drying solutions in D_2O and were then dissolved in 99.99% D_2O (0.45 mL) containing a trace of acetone as internal reference (δ 2.23 for ^1H and 31.07 p.p.m. for ^{13}C). Spectra were recorded at 40°, 60°, or 75° on a Bruker WH-400 or AM-400 spectrometer, equipped with an Aspect 3000 computer and an array processor, and using standard Bruker software.

^1H-Homonuclear shift-correlated experiments[4] (COSY) were performed at 40°, 60°, and 75°, 1-step RELAY COSY[5] was performed at 40°, and homonuclear dipolar-correlated[6] (NOESY) experiments were performed at 40° and 75° using a

spectral width of 1320 Hz. Data matrices of 256 × 1024 data points were collected for 32 or 96 transients for each t_1 delay. The matrices were zero-filled in the t_1 dimension, transformed in the magnitude mode by use of a non-shifted sine-bell window function in both dimensions, and symmetrised. Digital resolution in the resulting 512 × 1024 matrices was 2.6 Hz per point. Relaxation delays of 1.2–1.5 s were used. For the RELAY COSY experiment, a fixed delay of 0.036 s was used. The mixing delay in the NOESY experiments was varied between 0.1 and 0.3 s. *J*-Resolved spectra were obtained using a data matrix of 128 × 1024 points and spectral widths of 1024 Hz and ±16.0 Hz in the chemical shift (t_2) and the coupling constant (t_1) dimensions, respectively. The data matrix was zero-filled to 256 × 2048 points and transformed using a non-shifted sine-bell window function. The transformed matrix was tilted 45° and symmetrised.

A ^{13}C–^{1}H shift-correlated[7] (HETCOR) experiment was recorded at 40° with proton decoupling in the F_1 domain. The initial matrix of 128 × 1024 data points was zero-filled to 512 × 2048 points and processed with Gaussian functions and a magnitude calculation to give a final resolution of 9.76 Hz/point in the F_2 domain and 5.86 Hz/point in the F_1 domain.

Smith degradation of the K8 polysaccharide. — The polysaccharide (145 mg) was treated with 0.1M sodium hydroxide (15 mL) at 37° for 4 h and the *O*-deacetylated product was isolated by dialysis and freeze-drying. To a solution of the *O*-deacetylated polysaccharide (133 mg) in water (5 mL) was added a solution of sodium metaperiodate (180 mg) in water (20 mL), and the mixture was kept at 6° in the dark for 48 h, then treated with ethylene glycol (1.5 mL), followed by sodium borohydride (200 mg) for 16 h. After destruction of the excess of sodium borohydride with aqueous 10% acetic acid, the mixture was dialysed and freeze-dried. The product was heated at ~100° (steam bath) in aqueous 2% acetic acid for 2 h, the solution was freeze-dried, and the residue was reduced conventionally with sodium borohydride and eluted from Bio-Gel P-2 with water to give SDI (40 mg). SDI was subjected to a further Smith-degradation and yielded SDII (15 mg) after g.p.c.

ACKNOWLEDGMENTS

We thank Dr. Ida Ørskov (Copenhagen) for the serological test strain for *E. coli* K8, the Foundation for Research and Development (Pretoria) for financial support (to H.P.), the Natural Sciences and Engineering Research Council of Canada for financial support (to G.G.S.D.), and Dr. P. R. Phillips for recording the HETCOR experiment.

REFERENCES

1 I. Ørskov, F. Ørskov, B. Jann, and K. Jann, *Bacteriol. Rev.*, 41 (1977) 667–710.
2 H. Parolis, L. A. S. Parolis, and S. M. R. Stanley, *Carbohydr. Res.*, 175 (1988) 77–83.
3 K. Leontein, B. Lindberg, and J. Lönngren, *Carbohydr. Res.*, 62 (1978) 359–362.

4 A. BAX AND R. FREEMAN, *J. Magn. Reson.*, 44 (1981) 542–561.
5 A. BAX AND G. DROBNY, *J. Magn. Reson.*, 61 (1985) 306–320.
6 R. BAUMANN, G. WIDER, R. R. ERNST, AND K. WÜTHRICH, *J. Magn. Reson.*, 44 (1981) 402–406.
7 A. BAX AND G. MORRIS, *J. Magn. Reson.*, 42 (1981) 501–505.
8 K. BOCK AND H. THØRGERSEN, *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–57.
9 P.-E. JANSSON, L. KENNE, AND E. SCHWEDA, *J. Chem. Soc., Perkin Trans. 1*, (1987) 377–383.
10 P. A. J. GORIN AND M. MAZUREK, *Can. J. Chem.*, 53 (1975) 1212–1223.
11 K. IZUMI, *Carbohydr. Res.*, 170 (1987) 1–17.
12 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
13 J.-R. NEESER AND T. F. SCHWEIZER, *Anal. Biochem.*, 142 (1984) 58–67.
14 L. R. PHILLIPS AND B. A. FRASER, *Carbohydr. Res.*, 90 (1981) 149–152.