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Structure of the O-specific polysaccharide of the O23 antigen (LPS) from *Escherichia coli* O23:K?:H16

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ABSTRACT

The polysaccharide moiety of the O23 antigen (lipopolysaccharide) consists of D-glucose, D-galactose, 2-acetamido-2-deoxy-D-glucose, and 2-acetamido-2-deoxy-D-galactose in the molar ratios 2:1:2:1. Methylation analysis of the polysaccharide as well as one- and two-dimensional ^{1}H and ^{13}C NMR spectroscopy of the polysaccharide and a trisaccharide obtained by Smith degradation showed that the O23 polysaccharide has the primary structure

$$\begin{array}{l} \rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow)\text{-}\alpha\text{-D-Gal}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc}(1\rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad 3 \qquad\qquad\qquad\qquad\qquad\qquad\quad 6 \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \beta\text{-D-Glc}p\text{NAc} \qquad\qquad\qquad\qquad\quad \alpha\text{-D-Glc}p \end{array}$$

INTRODUCTION

Escherichia coli strains have been serologically grouped into more than 150 O-groups, based on the epitope expression of the polysaccharide moieties of their O-antigenic lipopolysaccharides (LPSs)[1,2]. Serological relatedness (cross-reactivity) between O-antigens was found to be due to structural relatedness of the O-specific polysaccharides[1]. Cross-reactivity between the O-antigens (LPSs) of *E. coli* O18 and O23 was described long ago[3] and their differentiation has proved to be difficult. Because of the difficulty in distinguishing O18 and O23 bacteria serologically, SDS-PAGE analysis was used to characterise the respective LPSs[4]. Four O18-specific LPSs (O18A, A1, B, and B1) and four O23-specific LPSs (A–D) could be identified. One O23 LPS with a long polysaccharide chain (O23A) and three different LPSs with much shorter polysaccharide chains (O23 B-D) were described[4]. Cross-reactivity was detected between O18A and O23A. The structures

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of the four O18-specific polysaccharides were reported recently[5]. Here we describe the structural elucidation of the long-chain O23-specific polysaccharide, termed O23A by Pluschke et al.[4].

RESULTS AND DISCUSSION

Isolation and characterisation of the O-specific polysaccharide.—The LPS was obtained by extraction of the bacteria with aqueous 45% phenol at 65°C and subsequent ultracentrifugation of the aqueous phase[6]. The sedimented LPS was subjected to mild acid hydrolysis and the O-specific polysaccharide obtained was purified by gel permeation chromatography on Sephadex G-50[7] (K_d 0.9–0.95). This material did not contain undegraded LPS, as evidenced by ultracentrifugation (no pellet obtained) and by the absence of fatty acids.

The polysaccharide consisted of D-glucose (D-Glc), D-galactose (D-Gal), 2-acetamido-2-deoxy-D-glucose (D-GlcNAc), and 2-acetamido-2-deoxy-D-galactose (D-GalNAc) in the molar ratios 2:1:2:1. Periodate oxidation destroyed both glucose residues and one of the GlcNAc residues.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained six signals in the region for anomeric carbon atoms (δ 98.7, 99.7, 100.3, 103.0, 104.4, and 106.1). These signals and signals characteristic of nitrogen-substituted carbon atoms (δ 49.8, 55.5, and 57.0), as well as signals for the carbonyl groups (δ 175.4–175.7) and methyl groups (δ 23.6–23.8), indicated that the O23 polysaccharide consists of hexasaccharide repeating units containing three *N*-acetylamino sugars. An APT spectrum[8] indicated that two of six hydroxymethyl groups are substituted (signals at δ 66.8 and 69.3)[9].

Methylation analysis.—The polysaccharide was methylated[10] with KH–MeI in Me_2SO. The purified (Sep-Pak C_{18}) product was hydrolysed[11], reduced with sodium borodeuteride, and per-*O*-acetylated, and the resulting partially methylated alditol acetates were characterised by GLC–MS. The results indicated that the O23 polysaccharide contains one terminal and one 6-substituted glucose, one 3,4-disubstituted galactose, one terminal and one 3,6-disubstituted GlcNAc, and one 3-substituted GalNAc. It is therefore a branched polysaccharide with glucose

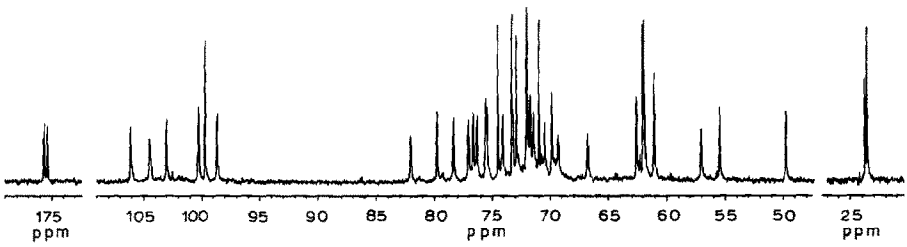


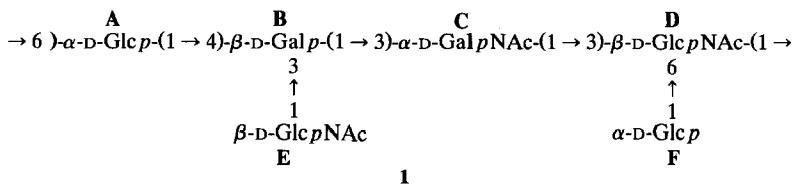
Fig. 1. 75-MHz ^{13}C NMR spectrum (δ 24–27; 48–109; 173–179) of the O23-specific polysaccharide, recorded in D_2O (80°C) with acetone (δ_c 31.45) as internal standard.

and GlcNAc as side-chain substituents. For the differentiation between the GlcNAc and the GalNAc residues, data from NMR analysis were used.

NMR analysis.—The ^{1}H NMR spectrum contained three signals for α-anomeric protons (δ 4.86, $^3J_{1,2}$ 4 Hz; δ 4.95, $^3J_{1,2}$ 3.5 Hz; δ 5.37, $^3J_{1,2}$ 3.5 Hz) and three signals for β-anomeric protons (δ 4.46, $^3J_{1,2}$ 8 Hz; δ 4.56, $^3J_{1,2}$ 8.5 Hz; δ 4.64, $^3J_{1,2}$ 7.5 Hz). Further signals were present indicative of the methyl groups from acetamido substituents (δ 1.96–1.98). Assignments of the signals (Table I) were obtained with 2D COSY and one-, two-, and three-step H-relayed coherence transfer (COSYRCT) 2D spectra[12,13]. The visual multiplicities and coupling constants of the signals were determined using 1D homonuclear double resonance in the difference mode[14]. The results indicated that the O23 repeating unit consists of 6-α-Glc*p*, t-α-Glc*p*, 4-β-Gal*p*, t-β-Glc*p*NAc, 3-α-Gal*p*NAc, 3,4-α-Gal*p*, and 3,6-β-Glc*p*NAc. The linkage positions were verified and the sequence of residues in the polysaccharide was determined using a series of NOE experiments with pre-irradiation of the anomeric protons (Table II). An NOE of H-1 on residue **A** [→ 6)-α-Glc*p*] not only to H-4 of residue **B** [→ 4), → 3)-β-Gal*p*] but also to H-2 of residue **E** (t-β-Glc*p*NAc) indicated the close proximity also of residues **A** and **E**.

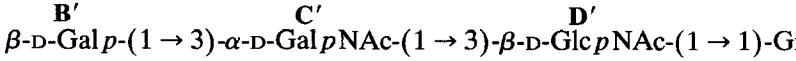
The signals of the ^{13}C NMR spectrum (Fig. 1) were assigned (Table I) with a 2D heteronuclear COSY spectrum (Fig. 2). The absolute configurations of all sugar units were determined as D by calculating the experimental glycosylation effects[15] (Table I), with D-glucose (residues **A** and **F**) as a basis, whose configuration was proved by its reactivity with D-glucose oxidase. The D configuration of residue **B** was also borne out by an NOE of (preirradiated) H-1 of residue **A** with H-6 of residue **B**[16].

The results obtained allow the formulation of the O23 polysaccharide as **1**.



1

This result is in agreement with NMR data (Table III) for a fragment (**2**) obtained from the O23 polysaccharide by Smith degradation[17]. They showed **2** to represent the substructure **B-C-D**, with glycerol at the reducing end.



2

The glycerol constituent of **2** is not included in Table III, in which the residues are termed **B′**, **C**, and **D′** to show their relation to **B**, **C**, and **D**, and to indicate the structural differences due to differences in substitution.

The serological cross-reactivity between the O18A and O23A polysaccharides, which was verified by an ELISA test[18], may be due to the branched trisaccharide

TABLE I

^{1}H NMR and ^{13}C NMR data for the O23 polysaccharide [a]

Residue	Proton	δ	Visible multiplet	Coupling $J_{H,H}$	Hz	Carbon	δ	Glycosylation effect	$J_{C\text{-}1,H\text{-}1}$ (Hz)
→6)-α-D-Glc*p*-(1→	H-1	4.86	d	$J_{1,2}$	4	C-1	100.3		170
(A)	H-2	3.39	dd	$J_{2,3}$	9.5	C-2	73.3		
	H-3	3.68	t	$J_{3,4}$	9.5	C-3	74.1		
	H-4	3.40	t	$J_{4,5}$	9.5	C-4	70.5		
	H-5	4.26	bdd	$J_{5,6a}$	< 2	C-5	71.4		
	H-6a	4.09	bd	$J_{6a,6b}$	12	C-6	69.3		
	H-6b	3.91	dd	$J_{5,6b}$	3				
→4)-β-D-Gal*p*-(1→	H-1	4.46	d	$J_{1,2}$	8	C-1	106.1	+8.4	163
3 ↑	H-2	3.57	dd	$J_{2,3}$	10	C-2	71.7		
(B)	H-3	3.69	dd	$J_{3,4}$	3	C-3	82.0		
	H-4	4.18	bd	$J_{4,5}$	< 2	C-4	76.3		
	H-5	3.68	bdd	$J_{5,6a}$	2	C-5	76.6		
	H-6a	3.87	dd	$J_{6a,6b}$	12	C-6	61.0	−1.1	
	H-6b	3.78	dd	$J_{5,6b}$	3				
→3)-α-D-Gal*p*NAc-(1→	H-1	5.37	d	$J_{1,2}$	3.5	C-1	98.7		174
(C)	H-2	4.35	dd	$J_{2,3}$	11	C-2	49.8		
	H-3	3.86	dd	$J_{3,4}$	3.5	C-3	78.3	+9.6	
	H-4	4.14	bd	$J_{4,5}$	< 2	C-4	69.8	−0.1	
	H-5	3.86				C-5	72.0		
	H-6a	3.90 [b]				C-6	62.6		
	H-6b	3.70 [b]							
→3)-β-D-Glc*p*NAc-(1→	H-1	4.56	d	$J_{1,2}$	8.5	C-1	103.0		162
6 ↑	H-2	3.82	dd	$J_{2,3}$	10	C-2	55.5	−1.5	
(D)	H-3	3.73	t	$J_{3,4}$	10	C-3	79.8		
	H-4	3.86	t	$J_{4,5}$	10	C-4	72.0		
	H-5	3.57	m	$J_{5,6a}$	< 2	C-5	75.6		
	H-6a	4.01	bd	$J_{6a,6b}$	12	C-6	66.8	+4.7	
	H-6b	3.74							
β-D-Glc*p*NAc-(1→	H-1	4.64	bd	$J_{1,2}$	7.5	C-1	104.4	+8.2	162
(E)	H-2	3.53	m			C-2	57.0		
	H-3	3.51	m	$J_{3,4}$	9	C-3	75.4		
	H-4	3.34	bt	$J_{4,5}$	9	C-4	72.0		
	H-5	3.38				C-5	77.1		
	H-6a	3.83 [b]				C-6	62.0		
	H-6b	3.72 [b]							
α-D-Glc*p*-(1→	H-1	4.95	d	$J_{1,2}$	3.5	C-1	99.7		171
(F)	H-2	3.52	dd	$J_{2,3}$	9	C-2	72.9		
	H-3	3.71	t	$J_{3,4}$	9	C-3	74.5		
	H-4	3.39	t	$J_{4,5}$	9	C-4	71.0		
	H-5	3.67				C-5	73.3		
	H6a,6b	3.66–3.77 [b]				C-6	61.9		

[a] Recorded in D_2O (80°C), with acetone (δ_H 2.225; δ_C 31.45) as internal standard.
[b] From C/H correlated spectrum.

TABLE II

NOE data [a] for the O23 polysaccharide

NOE observed on		Preirradiated proton					
Residue	Proton	**A**, H-1	**B**, H-1	**C**, H-1	**D**, H-1	**E**, H-1	**F**, H-1
→6)-α-D-Glc*p*-(1→	H-2	+					
(A)	H-3	+ [b]			+ [b]		
	H-6a,6b				+		
→4)-β-D-Gal*p*-(1→	H-2		+				
3 ↑	H-3	+ [b]	+			+	
(B)	H-4	+	+ [b]			+ [b]	
	H-5		+				
	H-6a,6b	+					
→3)-α-D-Gal*p*NAc-(1→	H-2			+			
(C)	H-3		+	+ [b]			
	H-4		+ [b]	+ [b]			
	H-5			+ [b]			
→3)-β-D-Glc*p*NAc-(1→	H-2			+ [b]	+		
6 ↑	H-3			+	+		
(D)	H-5			+ [b]	+		
	H-6a,6b						+
β-D-Glc*p*NAc-(1→	H-2,3	+ [c]				+	
(E)	H-4,5					+	
α-D-Glc*p*-(1→	H-2						+
(F)	H-3						+ [b]

[a] The test was performed using standard Bruker software NOEMULT. [b] Small signal due to spin diffusion. [c] Long-range interaction.

substructure **A-B-E**, possibly with the side-chain β-D-Glc*p*NAc (residue **E**) as the dominant part. Whereas the branch point D-Gal*p* (residue **B**) is β in the main chain of the O23 polysaccharide, the corresponding sugar is α in the O18 polysaccharide[5]. This should, however, not impede the steric availability of the side-chain β-D-Glc*p*NAc.

EXPERIMENTAL

Bacteria and cultivation.—E. coli A184 (O23:K?:H16) was obtained from M. Achtman (Max-Planck-Institut für Molekulargenetik, Berlin, Germany). The bacteria were grown to the late exponential phase (5–7 h) in 14-L batch cultures at 37°C in a medium containing, per L, tryptone (7.5 g), yeast (10 g), D-glucose (10 g), NaCl (3 g), $Na_2HPO_4 \cdot 12H_2O$ (8 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), and poly(ethylene

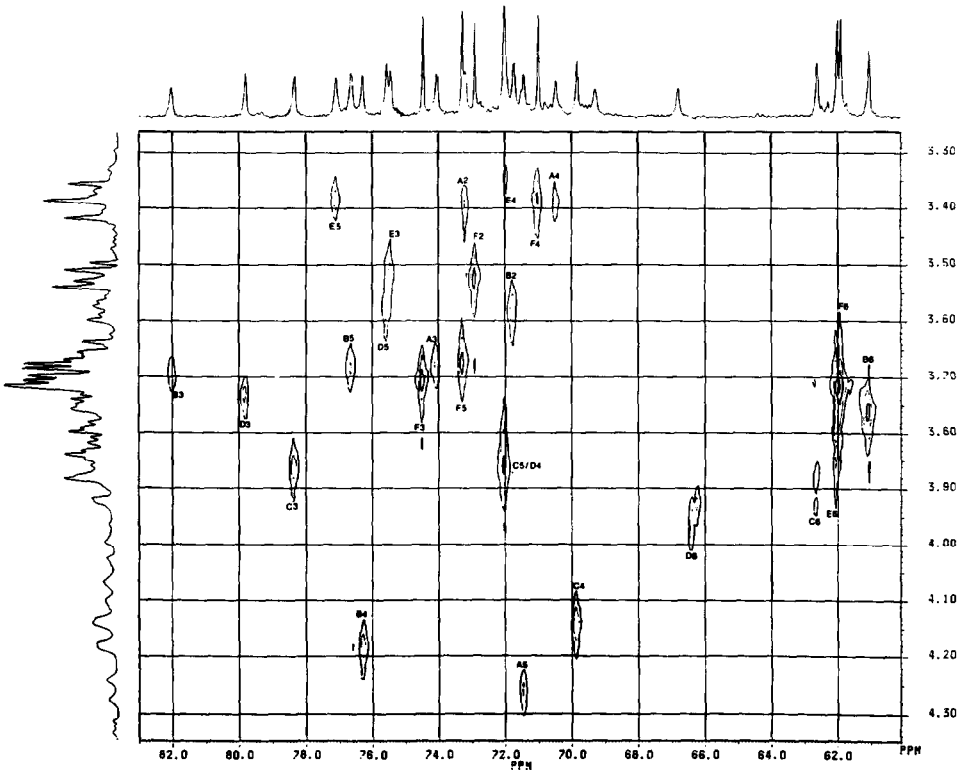


Fig. 2. 2D 300-MHz heteronuclear $^{13}C/^{1}H$ COSY spectrum of the O23-specific polysaccharide with the signals assigned analogously to the sugar residues in Table I. The A6 correlation (signal at δ 69.3/δ 4.09) could be detected when the plane of measurement was lowered. Since, in that case, the signal of the neighboring C-4 correlation (δ 69.8/δ 4.2) was very broad, an illustration was not possible.

glycol) (0.3 g). D-Glucose and magnesium sulfate were sterilised separately. At the end of the cultivation, the bacteria were killed with phenol (1% final concentration) and harvested by centrifugation.

Isolation of the polysaccharide.—The LPS was isolated from the bacteria with aq 45% phenol at 65°C (10 min) and the material obtained from the aqueous phase was purified by repeated ultracentrifugation as described[6]. The polysaccharide was obtained from the LPS by hydrolysis in aq 1% acetic acid (100°C, 90 min) and purified by chromatography on Sephadex G-50.

Preparation of **2**.—The periodate-oxidised and borohydride-reduced polysaccharide was hydrolysed (25°C, 48 h) in 0.25 M CF_3CO_2H. After lyophilisation, the material was dissolved in a minimum amount of water and chromatographed on a column of Sephadex G-25 with water as eluant.

Methylation analysis of the polysaccharide.—The polysaccharide was methylated[10] with dimethyl Me_2SO–KH–MeI. The methylated product was purified[19] with a Sep-Pak C_{18} cartridge, hydrolysed with 90% formic acid and subsequently with

TABLE III

^{1}H NMR and ^{13}C NMR data for the O23 oligosaccharide [a]

Residue	Proton	δ	Visible multiplet	Coupling		Carbon	δ	$J_{C\text{-}1,H\text{-}1}$ (Hz)
				$J_{H,H}$	Hz			
β-D-Gal*p*-(1→ (**B′**)	H-1	4.43	d	$J_{1,2}$	8	C-1	105.9	163
	H-2	3.50	dd	$J_{2,3}$	10	C-2	71.8	
	H-3	3.60	dd	$J_{3,4}$	3.5	C-3	73.9	
	H-4	3.90	d	$J_{4,5}$	< 2	C-4	69.9	
						C-5	76.2	
						C-6	62.0	
→3)-α-D-Gal*p*NAc-(1→ (**C**)	H-1	5.40	d	$J_{1,2}$	4.0	C-1	98.6	174
	H-2	4.35	dd	$J_{2,3}$	10.5	C-2	49.7	
	H-3	3.85	dd	$J_{3,4}$	4.5	C-3	78.4	
	H-4	4.20	d	$J_{4,5}$	3.5	C-4	69.8	
	H-5	3.86	m			C-5	71.9	
						C-6	62.2	
→3)-β-D-Glc*p*NAc-(1→ (**D′**)	H-1	4.55	d	$J_{1,2}$	8.0	C-1	102.5	162
	H-2	3.78	dd	$J_{2,3}$	10.5	C-2	55.5	
	H-3	3.71	t	$J_{3,4}$	10.5	C-3	79.4	
	H-4	3.69	t	$J_{4,5}$	10.5	C-4	72.1	
	H-5	3.42	ddd	$J_{5,6a}$	< 2	C-5	77.0	
	H-6a	3.88	dd			C-6	61.9	
	H-6b	3.74						

[a] Recorded in D_2O (80°C), with acetone (δ_H 2.225; δ_C 31.45) as internal standard.

0.25 M H_2SO_4, and neutralised with $Ba(OH)_2$ (ref. 11). After reduction with $NaBD_4$, the sample was subjected to GLC–MS.

Analytical methods.—The sugar residues were determined as their alditol acetates by GLC using an ECNSS-M column at 170°C (neutral sugars) and Poly-A-103 at 230°C (differentiation of amino sugars)[20]. The Elson–Morgan reaction[21] was used for the quantitation of amino sugars. The absolute configuration of glucose was determined with D-glucose oxidase. GLC–MS was carried out on a Hewlett–Packard HP5988A instrument with an ionising energy of 70 eV in combination with an HP5890 gas chromatograph, using a DB1 capillary column (0.25 mm × 30 m), with He as carrier gas and a temperature program of 100 → 180°C at 50°C/min, 180 → 200°C at 2°C/min, and 200 → 240°C at 10°C/min. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AM-300 spectrometer at 80°C in D_2O, using acetone (δ_H 2.225; δ_c 31.45) as the internal standard. Standard Bruker software was used for homonuclear H,H COSY (COSYHG); one-, two-, and three-step H-relayed homonuclear 2D H,H COSY (COSYRCT, COSYRCT2, and COSYRCT3); and heteronuclear 2D C,H COSY (XHCORRD); as well as for the determination of the T_1 relaxation time with a ^{13}C NMR inversion recovery experiment (INVREC). 1D NOE experiments were performed in the truncated driven (TOE) mode[22] with the Bruker NOEMULT program. The relaxation delay D1 was 1 s, the irradiation

time of every component of multiplets (D2) was 0.1 s, and the total preirradiation time for whole multiplets was 1.0–1.2 s.

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