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Structure of the O-specific polysaccharide of the O22-antigen (LPS) from *Escherichia coli* O22:K13

Monika Bartelt [a], Alexander S. Shashkov [b], Helga Kochanowski [a], Barbara Jann [a] and Klaus Jann [a,*]

[a] *Max-Planck-Institut für Immonbiologie, Stübeweg 51, D-7800 Freiburg (Germany)*
[b] *N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow B-334 (Russian Federation)*

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ABSTRACT

The polysaccharide moiety of the O22-antigen (lipopolysaccharide, LPS) consists of 2-acetamido-2-deoxy-D-galactose, D-glucuronic acid, D-glucose, and D-galactose in the molar ratios 2:1:1:1. Methylation analysis as well as 1D and 2D NMR spectroscopy showed that the O22 polysaccharide has the primary structure

$$\rightarrow 6)\text{-}\alpha\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow$$
$$\begin{array}{c} 3 \\ | \\ \text{OAc} \end{array}$$

INTRODUCTION

Escherichia coli may express different surface carbohydrates, such as lipopolysaccharides (LPSs, O-antigens) with a neutral or an acidic O-specific polysaccharide[1,2] as well as acidic capsular polysaccharides (CPSs, K-antigens)[3]. These surface antigens occur in various combinations which determine the serological type of individual *E. coli* strains. Immunoelectrophoresis of saline extracts from *E. coli*[4] revealed characteristic patterns, demonstrating the presence of neutral LPS with or without acidic CPS or the presence of acidic LPS with or without acidic CPS. This classification was corroborated by chemical analyses[1,2]. The *E. coli* O22 antigen, which belongs to one of the 10 most frequent O-groups[5], is an acidic LPS. Generally, LPS preparations contain molecules with different chain length, as can be demonstrated with SDS-PAGE[6]. Probably due to different tendencies of long- and short-chain LPSs to micelle formation, short-chain acidic LPSs are sedimented during ultracentrifugation of bacterial extracts and long-chain acidic LPSs remain in the supernatant solution. Using this separation, the long-

* Corresponding author.

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chain population of the O22 LPS from *E. coli* O22:K13 was isolated, and its structure was elucidated. The results are described in this communication.

RESULTS AND DISCUSSION

Isolation and characterisation of the O-specific polysaccharide.—The acidic LPS was obtained by extraction of the bacteria with aq 45% phenol at 65°C and subsequent ultracentrifugation of the aqueous phase[7]. The sediment contained an LPS fraction (LPS I)[8] which consisted of short-chain LPS and R-LPS. The supernatant solution contained LPS (LPS II) which was isolated by fractional precipitation with cetyltrimethylammonium bromide (CTAB)[7,9]. This LPS II, with its longer O-specific polysaccharide chain, was used for the structural analysis. The O-specific polysaccharide was obtained by mild acid hydrolysis of LPS II and was purified by gel permeation chromatography on Sephadex G-50[10].

The O22 polysaccharide consisted of 2-acetamido-2-deoxy-D-galactose (D-GalNAc), D-glucuronic acid (D-GlcA), D-glucose (D-Glc), and D-galactose (D-Gal) in the molar ratios 2:1:1:1. Periodate oxidation destroyed glucose and glucuronic acid. Carboxyl reduction[11] of the polysaccharide converted GlcA into Glc, the absolute configuration of which was subsequently determined with D-glucose oxidase.

The ^{13}C NMR spectrum of the native O22 polysaccharide (Fig. 1) was typical of a regular polysaccharide with five anomeric carbon signals (δ 96.6, 99.3, 102.9, 103.9, and 104.4) corresponding to five sugar residues in the repeating unit. Two of

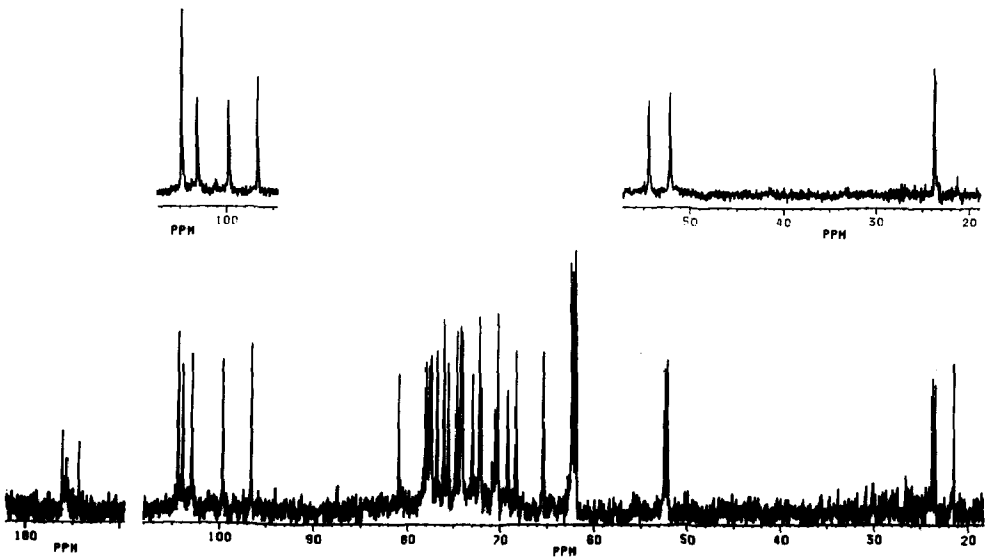


Fig. 1. 75-MHz ^{13}C NMR spectrum (δ 18–118; δ 170–182) of the native O22 polysaccharide with parts of the ^{13}C NMR spectrum (δ 19–57; δ 95–107) of the *O*-deacetylated O22 polysaccharide as insets.

the sugar residues were *N*-acetylamino sugars with signals characteristic of two nitrogen-substituted carbon atoms (δ 52.1 and 52.4), two methyl groups (δ 23.4 and 23.7), and two carbonyl groups (δ 175.6 and 176.0). Four hydroxymethyl groups (C-6; δ 61.9, 62.1, 62.3, and 69.1) were present, one of which (δ 69.1) was substituted, as shown in an APT (attached proton test) experiment[12,13]. This, together with the signal of a carbonyl group at δ 174.3, is in agreement with the presence of a uronic acid in the pentasaccharide repeating unit. Correspondingly, one of the carbonyl signals was shifted to higher field at low pH (δ 174.3 at pH 7.0; δ 172.7 and pH 2.0). A signal at δ 21.4 indicated the presence of an *O*-acetyl group in the O22 repeated unit. The ^{13}C NMR spectrum of the *O*-deacetylated polysaccharide showed characteristic changes, as compared to that of the native polysaccharide (Fig. 1, insets). One of the signals due to nitrogen-substituted carbon atoms was shifted from δ 52.1 to 54.4 and, in the anomeric region, the signal at δ 103.9 had also shifted and now overlapped with that at δ 104.4. Concomitantly, the signal at δ 21.4 (due to the methyl group of an *O*-acetyl substituent) had disappeared. These changes indicated that C-3 of one of the GalNAc residues was *O*-acetylated in the native polysaccharide.

Methylation analysis.—The native as well as the carboxyl-reduced polysaccharides were methylated[14] with KH–MeI in Me_2SO. The respective purified (Sep-Pak C_{18}) products were hydrolysed[15], reduced with sodium borodeuteride, and *O*-acetylated, and the resulting partially methylated alditol acetates were characterised by GLC–MS. The results (Table I) indicated that the native O22 polysaccharide is a linear polysaccharide, containing one 3-substituted Gal, one 4-substituted GlcA, one 6-substituted Glc, and two GalNAc residues, one 3- and one 4-substituted. This interpretation was based on the pyranose ring form of the sugars, as borne out by NMR spectroscopy.

NMR analysis.—The ^1H NMR spectrum of the native O22 polysaccharide showed five signals in the region of anomeric protons, two of them characteristic of α-anomeric protons of the *gluco / galacto* configuration (δ 5.39, $J_{1,2}$ 4.0 Hz, and δ 5.06, $J_{1,2}$ 4.0 Hz) and three of β-anomeric protons of the *gluco/galacto* configura-

TABLE I

Products of methylation analysis of the O22 polysaccharide before (I) and after carboxyl reduction (II) as identified by GLC-MS

Partially methylated alditol acetates	Present in		Interpretation
	I	II	
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol	+	+	1,3-Gal
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol	–	+	1,4-GlcA
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol	+	+	1,6-Glc
1,4,5-Tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)galactitol	+	+	1,4-GalNAc
1,3,5-Tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)galactitol	+	+	1,3-GalNAc

TABLE II

^{1}H and ^{13}C NMR data [a] of the O22 polysaccharide

Residue		Proton	δ (ppm)	Visible multiplicity	$J_{H,H}$	Hz	Carbon	δ (ppm)	Glyco-sylation effects	$J_{C\text{-}1,H\text{-}1}$ (Hz)
→6)-α-D-Glc*p*-(1→	(A)	H-1	5.39	d	$J_{1,2}$	4.0	C-1	99.3		174.5
		H-2	3.43	dd	$J_{2,3}$	9.5	C-2	73.0		
		H-3	3.64	t	$J_{3,4}$	9.5	C-3	74.1		
		H-4	3.37	t	$J_{4,5}$	9.5	C-4	70.5		
		H-5	3.76	ddd	$J_{5,6a}$	< 2	C-5	72.0		
		H-6a	4.02	dd	$J_{6a,6b}$	12.0	C-6	69.1		
		H-6b	3.82		$J_{5,6b}$	3.0				
→4)-β-D-Glc*p*A-(1→	(B)	H-1	4.43	d	$J_{1,2}$	8.0	C-1	104.4		164.5
		H-2	3.42	dd	$J_{2,3}$	9.0	C-2	74.7		
		H-3	3.70	t	$J_{3,4}$	9.0	C-3	77.4	+0.4	
		H-4	3.75	t	$J_{4,5}$	9.0	C-4	78.0		
		H-5	3.78	d			C-5	77.6		
							C-6	174.3		

→4)-β-D-Gal*p*NAc-(1→	(C)	H-1	4.75	d	$J_{1,2}$	9.0	C-1	103.9	+7.3	164.5
3		H-2	4.17	dd	$J_{2,3}$	11.0	C-2	52.4		
OAc		H-3	5.03	dd	$J_{3,4}$	3.0	C-3	74.2		
		H-4	4.21	d	$J_{4,5}$	< 2	C-4	74.3		
		H-5	3.71	dd			C-5	75.5		
		H-6	3.75 [b]				C-6	61.9	±0	
→3)-α-D-Gal*p*-(1→	(D)	H-1	5.06	d	$J_{1,2}$	4.0	C-1	96.6	+3.1	170.0
		H-2	3.87	dd	$J_{2,3}$	10.0	C-2	68.3		
		H-3	3.78	dd	$J_{3,4}$	3.0	C-3	80.9	+10.3	
		H-4	4.15	d	$J_{4,5}$	< 2	C-4	70.2	−0.4	
		H-5	3.82 [b]				C-5	72.2		
		H-6	3.72 [b]				C-6	62.3		
→3)-β-D-Gal*p*NAc-(1→	(E)	H-1	4.53	d	$J_{1,2}$	8.5	C-1	102.9		161.0
		H-2	4.03	dd	$J_{2,3}$	10.5	C-2	52.1		
		H-3	3.80	dd	$J_{3,4}$	4.0	C-3	76.8	+4.4	
		H-4	4.12	d	$J_{4,5}$	< 2	C-4	65.4	−3.7	
		H-5	3.63	dd			C-5	76.0		
		H-6	3.70 [b]				C-6	62.1		

[a] Recorded in D_2O (80°C), with acetone (δ ^1H 2.225; δ ^{13}C 31.45) as internal standard. [b] From C/H correlated spectrum.

TABLE III

NOE data [a] for the O22 polysaccharide

NOE observed on			Pre-irradiated proton				
Residue		Proton	A, H-1	B, H-1	C, H-1	D, H-1/C, H-3	E, H-1
→ 6)-α-D-Glc*p*-(1 →	(A)	H-2	+				
		H-3	+ [b]				
		H-6b					+
→ 4)-β-D-Glc*p*A-(1 →	(B)	H-2		+			
		H-3		+			
		H-4	+	+ [b]			
		H-5	+ [b]	+			
→ 4)-β-D-Gal*p*NAc-(1 →	(C)	H-2			+	+	
3		H-3			+		
OAc		H-4		+		+	
		H-5			+		
→ 3)-α-D-Gal*p*-(1 →	(D)	H-2				+	
		H-3			+	+ [b]	
→ 3)-β-D-Gal*p*NAc-(1 →	(E)	H-2					+
		H-3				+	+
		H-4				+	
		H-5					+

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

tion (δ 4.75, $J_{1,2}$ 9.0 Hz; δ 4.53, $J_{1,2}$ 8.5 Hz, and δ 4.43, $J_{1,2}$ 8.0 Hz). Further signals were indicative of the methyl groups from acetamido substituents (δ 1.93–2.08). Assignments of the proton signals (Table II) were obtained with 2D homonuclear COSY in combination with one-, two-, and three-step H-relayed coherence transfer (COSYSTRCT) 2D spectra[16,17]. The results showed that the O22 repeating unit consists of α-Glc*p* (**A**), β-Glc*p*A (**B**), two β-Gal*p*NAc (**C** and **E**), and α-Gal (**D**). The visual multiplicities, which are characteristic of the protons from individual sugar units, as well as the coupling constants were obtained with 1D homonuclear double resonance in the difference mode[18]. The data obtained which corroborate the assignments are included in Table II.

The sequence of the sugar residues was determined using a series of 1D NOE experiments with pre-irradiation of the anomeric protons (Table III), a technique which uses NOE interactions between proximal protons through space. For instance, pre-irradiation of H-1 of residue **E** (Gal*p*NAc) resulted in an NOE of H-2, H-3, and H-5 of this residue, indicative of a β-linkage; an additional NOE at H-6b of residue **A** (Glc*p*) was characteristic of a 6-linkage to residue **A**. Thus, the sequence β-Gal*p*NAc-(1 → 6)-Glc*p* is part of the repeating unit. This analysis was applied to all NOE responses. The **A** → **B**, **B** → **C**, and **C** → **D** linkages could be defined in this way as α-Glc*p*-(1 → 4)-Glc*p*A, β-Glc*p*A-(1 → 4)-Gal*p*NAc, and β-Gal*p*NAc-(1 → 3)-Gal*p*, respectively.

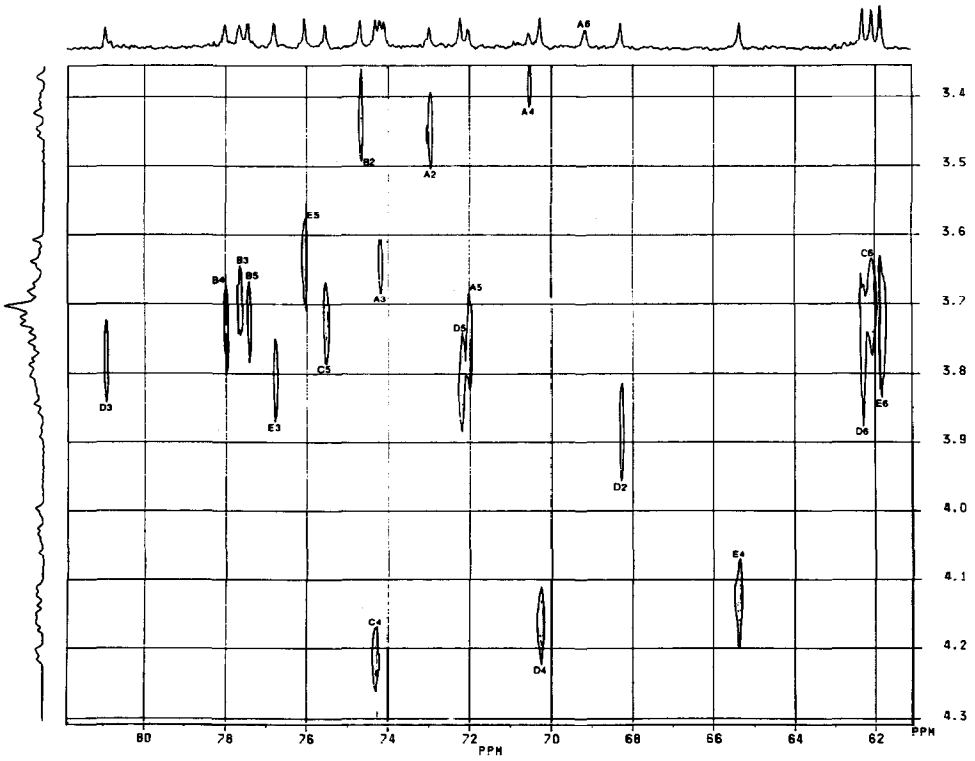


Fig. 2. 2D 300-MHz heteronuclear $^{13}C-^{1}H$ COSY spectrum of the O22-specific polysaccharide with the signals assigned analogously to the sugar residues in Table II.

Irradiation of H-1 of residue **D** (α-Gal*p*) also irradiated H-3 of 3-*O*-acetylated residue **C** (β-Gal*p*NAc), due to the close chemical shifts of these protons (δ 5.06 and 5.03). The response was therefore twofold: an intraresidue response of H-2 and H-4 from residue **C** (induced from irradiated H-3) and an interresidue response of H-3 and H-4 from residue **E** (induced from irradiation of H-1 of residue **D**). The latter response was more pronounced for H-4 than for H-3. This phenomenon is generally observed for sugars in the *galacto* configuration and substituted at C-3 with an α-sugar X. It can be explained by a close apposition of $H\text{-}1_x$ and $H\text{-}4_{Gal}$ in such a constellation[19]. The 3-linkage of residue **E** was apparent from the chemical shift of **E**-C-3 in the ^{13}C NMR spectrum. This was corroborated by the results of the methylation analysis (Table I).

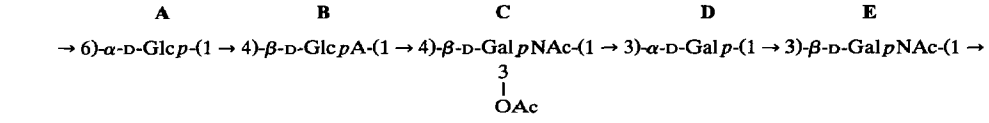
Complete signal assignments of the ^{13}C NMR spectrum (Fig. 1, Table II) was achieved with a 2D heteronuclear COSY spectrum (Fig. 2). The assignments of the ^{1}H and ^{13}C NMR spectra were fully supported by the results of the methylation analysis.

The absolute configurations of all sugars were determined as D by calculating the experimental glycosylation effects[20] (Table II) with D-glucose and D-glucuronic

acid as a basis, as derived from their reactivity with D-glucose oxidase before and after carboxyl reduction, respectively.

The linkage of the *O*-acetyl group to C-3 of residue **C** (4 → β-GalNAc) was indicated by a large α-shift of H-3 of residue **C** in the ^1H NMR spectrum (Table II) and by a shift of C-2 of residue **C** from δ 52.4 to 54.7 in the ^{13}C NMR spectrum after *O*-deacetylation (Fig. 1).

The results obtained allow the formulation of the O22 polysaccharide as



EXPERIMENTAL

Bacteria and cultivation.—*E. coli* O22:K13 (Freiburg collection number 21749) was obtained from Drs. I. and F. Ørskov (Statens Seruminstitut, Copenhagen, Denmark). The bacteria were grown to the late logarithmic phase (5–7 h) in 14-L batch cultures at 37°C with aeration (1 L/min) 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 polyethylene glycol (0.3 g). 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 LPSs were 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[7]. The O22 LPS II was obtained from the supernatant solution by fractional precipitation with CTAB, and the precipitated complex was converted into the sodium salt as described[7,8]. The O22 polysaccharide was obtained from LPS II by hydrolysis in aq 1% acetic acid (100°C, 90 min) and purified by chromatography on Sephadex G-50.

O-Deacetylation.—A solution of the O22 polysaccharide (60 mg) in NaOH (3 mL) was kept at 56–60°C for 1 h, neutralised with HCl, dialysed for 24 h, and lyophilised, to give *O*-deacetylated O22 polysaccharide (51 mg).

Carboxyl reduction.—The O22 polysaccharide (60 mg) was treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide at pH 4.7 and the resulting esters were reduced with $NaBH_4$ (ref. 11). After dialysis and lyophilisation, carboxyl-reduced polysaccharide (45 mg) was obtained. This treatment converted ~90% of the glucuronic acid into glucose.

Methylation analysis of the polysaccharide.—The polysaccharide was methylated[14] with Me_2SO–KH–MeI. The methylated product was purified[21] with a Sep-Pak C_{18} cartridge, hydrolysed with aq 90% formic acid and subsequently with 0.25 M H_2SO_4, and neutralised with $Ba(OH)_2$ (ref. 15). 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). Identification of the amino sugars was carried out by the ninhydrin reaction and by GLC using a Poly-A-103 column at 230°C. The Elson–Morgan reaction[22] was used for the quantitation of amino sugars. Glucuronic acid was determined with the carbazole reagent. The absolute configuration of glucose was determined with D-glucose oxidase, as was that of D-glucuronic acid after reduction with $NaBH_4$. 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. ^1H and ^{13}C NMR spectra were recorded with a Bruker AM-300 spectrometer at 80°C in D_2O, using acetone (δ ^1H 2.225; δ ^{13}C 31.45) as the internal standard. Standard Bruker software was used for homonuclear H, H COSY (COSYHG), for one-, two-, and three-step H-relayed homonuclear 2D, H, H COSY (COSYRCT, COSYRCT2, and COSYRCT3), as well as for heteronuclear 2D, C, H COSY (XHCORRD). 1D NOE experiments were performed in the truncated driven (TOE) mode[23] 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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