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STRUCTURAL STUDIES OF THE O-SPECIFIC SIDE-CHAINS OF THE *Escherichia coli* O2 LIPOPOLYSACCHARIDE

PER-ERIK JANSSON, HELENA LENNHOLM, BENGT LINDBERG, ULF LINDQUIST,
Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm (Sweden)

AND STEFAN B. SVENSON
Department of Bacteriology, National Bacteriological Laboratory, S-105 21 Stockholm (Sweden)

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ABSTRACT

The structure of the O-specific side-chains of the *Escherichia coli* O2 lipopolysaccharide has been investigated, different ^{1}H- and ^{13}C-n.m.r. techniques being the main methods used. It is concluded that they are composed of pentasaccharide repeating-units having the following structure, in which D-Fuc3NAc is 3-acetamido-3,6-dideoxy-D-galactose.

$$\begin{array}{l}\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 2 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \alpha\text{-D-Fuc}p\text{3NAc}\end{array}$$

INTRODUCTION

In their studies of sugar components in bacterial lipopolysaccharides, Ørskov *et al.*[1] found several unusual sugars. One of these was 3-amino-3,6-dideoxy-D-galactose (D-Fuc3N) in *E. coli* serotypes 2 and 74. The same sugar has been found in the lipopolysaccharide[2] from *Xanthomonas campestris*, in lipopolysaccharides from other genera[3], and in a cell-wall antigen[4] from *Eubacterium saburreum* strain L13. We now report structural studies of the O-specific side-chains of the *E. coli* O2 lipopolysaccharide. This organism is known to cause upper urinary tract infection and septicaemia.

RESULTS AND DISCUSSION

The lipopolysaccharide was isolated from the bacteria by extraction with phenol–water, and the polysaccharide (PS) was prepared from the lipopolysaccharide by hydrolysis with dilute acetic acid and purified by gel filtration. An acid hydro-

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TABLE I

^{1}H-N.M.R. DATA FOR *E. coli* O-2 POLYSACCHARIDE

Sugar residue	*Chemical shifts (p.p.m.)*[a]						
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*
RhaI	5.16 (0.10)	4.08 (0.21)	3.95 (0.20)	3.46 (0.07)	3.82 (0.02)	1.29[b]	
RhaII	5.05 (−0.01)	4.24 (0.37)	3.97 (0.22)	3.66 (0.27)	3.71 (−0.09)	1.26[b]	
Fuc3NAc	4.96	3.79	4.17	3.77	4.52	1.22	
RhaIII	4.87 (0.07)	4.13 (0.26)	3.61 (0.07)	3.50 (0.18)	3.41 (0.09)	1.24[b]	
GlcNAc	4.79 (0.09)	3.73 (0.02)	~3.67 (~0.1)	3.63 (0.21)	3.52 (0.10)	3.96 (0.05)	3.84 (0.09)

[a]Chemical shift differences relative to the monomers are given in parentheses. [b]These values may be interchanged.

TABLE II

^{13}C-N.M.R. DATA FOR *E. coli* O-2 POLYSACCHARIDE

Sugar residue	*Chemical shifts (p.p.m.)*[a]						
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*NAc*
RhaI	101.6 [173]	78.7 (6.8)	71.0 (−0.1)	73.3 (0)	70.0 (0.6)	17.6[b] —	
RhaII	100.3 [173]	76.3 (4.4)	78.2 (7.1)	72.6 (−0.7)	70.6 (1.2)	17.5[b] —	
Fuc3NAc[c]	97.8 [172]	67.1 (−0.1)	52.0 (0.2)	71.5 (−0.2)	67.9 (0.4)	16.2 (−0.7)	23.1
RhaIII	101.3 [162]	71.4 (−0.8)	80.8 (7.0)	72.4 (−0.4)	73.0 (0.2)	17.3[b] —	
GlcNAc	103.2 [164]	56.9 (−1.0)	75.0 (0.2)	78.4 (7.3)	75.4 (−1.4)	62.1 (0.3)	23.0

[a]Chemical shift differences relative to the monomers are given in parentheses and $J_{C\text{-}1,H\text{-}1}$ values are given in brackets. [b]These values may be interchanged. [c]The values for Fuc3NAc are taken from V. L. L'Vov, N. J. Tochtamysheva, A. S. Shashkov, L. A. Dmitriev, and K. Capec, *Carbohydr. Res.*, 112 (1983) 233–237.

TABLE III

OBSERVED n.O.e.-CONTACTS FOR *E. coli* O2 PS TOGETHER WITH ASSIGNMENTS[a]

Anomeric proton	*n.O.e. contact to*
5.16 (RhaI)	5.05 (M, RhaII, H-1), 4.87 (W, RhaIII, H-1), 4.13 (W, RhaIII, H-2), 4.09 (W, RhaI, H-2), ~3.75 (W, RhaII, H-5), 3.62 (M, RhaIII, H-3)
5.05 (RhaII)	5.16 (W, RhaI, H-1), 4.96 (W, Fuc3NAc, H-1), 4.25 (M, RhaII, H-2), 4.09 (S, RhaI, H-2)
4.96 (Fuc3NAc)	5.05 (M, RhaII, H-1), 4.25 (W, RhaII, H-2), 3.80 (W, Fuc3NAc, H-2)
4.87 (RhaIII)	5.16 (M, RhaI, H-1), 4.13 (S, RhaIII, H-2), 3.64 (W, GlcNAc, H-4), 3.62 (S, RhaIII, H-3), 3.41 (M, RhaIII, H-5)
4.49 (GlcNAc)	3.97 (W, RhaII, H-3), ~3.70 (W, GlcNAc, H-3)

[a]The intensities of the signals are indicated by S (strong), M (medium), or W (weak).

lysate of the PS contained L-rhamnose, 2-amino-2-deoxy-D-glucose, and 3-amino-3,6-dideoxy-D-galactose in the relative proportions 3:1:1, together with smaller amounts of D-glucose (0.5), D-galactose (0.1), and a heptose (0.3). The absolute configurations of the sugars were determined by comparison with authentic references, as devised by Gerwig *et al.*[5].

The structure of the PS was determined using n.m.r. spectroscopy. The signals given by protons (Table I) and carbon atoms (Table II) of the individual sugar residues were assigned by means of proton–proton correlated (COSY) and proton–carbon correlated (CHCOR) 2D-spectra. Deviations from the chemical shifts for the corresponding, unsubstituted monomer residues are also given in the Tables. The mutual order of the sugar residues was inferred from a 2D-n.O.e. spectrum (Table III).

It is evident from the chemical shifts and coupling constants of the anomeric protons (Table I) that the repeating unit of the PS contains three L-rhamnosyl residues, one 3-acetamido-3,6-dideoxy-D-galactopyranosyl residue, and one 2-acetamido-2-deoxy-D-glucopyranosyl residue. These residues are designated as RhaI, RhaII, RhaIII, Fuc3NAc, and GlcNAc in the Tables. The H-1 chemical shifts of the L-rhamnosyl residues are recognised by their low coupling constants. The chemical shifts of these residues, and the chemical shifts in the ^{13}C-n.m.r. spectrum, show that they are pyranosidic. The chemical shifts of the anomeric protons also indicate that RhaI and RhaII are α-linked, and that RhaIII is β-linked.

The 3-acetamido-3,6-dideoxy-D-galactosyl residue, on the basis of its H-1 signal at δ 4.96 ($J_{1,2}$ 4 Hz), is α-linked and pyranosidic. The corresponding signal given by the 2-acetamido-2-deoxy-D-glucosyl residue, at δ 4.79 ($J_{1,2}$ 7 Hz), demonstrates that it is β-linked and pyranosidic. The chemical shifts of the proton signals are influenced by the substitution of the sugar residues, but the relations are complicated and it was not possible, from the chemical shift differences, to decide through which positions the different sugar residues are linked.

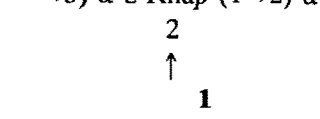
In the coupled ^{13}C-n.m.r. spectrum, signals for two anomeric carbons showed $^1J_{C,H}$ values of ~160 Hz and three showed $^1J_{C,H}$ values of ~170 Hz, indicating two β-linked and three α-linked residues in the repeating unit. From the CHCOR spectrum, these anomeric carbons were correlated with the corresponding anomeric protons. Thus, the carbons giving signals at δ 101.3 and 103.2 belong to β-pyranosyl residues and are correlated with the protons resonating at δ 4.87 and 4.79, respectively. RhaIII is consequently β-linked, and RhaI and RhaII are α-linked.

The chemical shift differences in the ^{13}C-n.m.r. spectrum clearly demonstrate the positions through which the different sugar residues are linked. Thus, RhaI is linked through O-2, RhaII through O-2 and O-3, RhaIII through O-3, and 2-acetamido-2-deoxy-D-glucose through O-4, and 3-acetamido-3,6-dideoxy-D-galactose is terminal.

A methylation analysis of the PS also gave the expected sugars, namely, 3,4-di-*O*-methyl-L-rhamnose, 2,4-di-*O*-methyl-L-rhamnose, 4-*O*-methyl-L-rhamnose, 3,6-dideoxy-2,4-di-*O*-methyl-3-*N*-methylacetamido-D-galactose, and 2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamido-D-glucose, although the stoichiometry of the analysis was not very good.

In the 2D-n.O.e. experiment (Table III), several n.O.e. contacts were established. Due to the short distance between the anomeric proton of a glycosyl residue and the proton (the α-proton) on the carbon atom to which it is linked, an n.O.e. relaxation is expected and may be used to establish a linkage between the two residues. In addition, n.O.e. contacts were established between an equatorial H-1 and an internal, axial H-2, between an axial H-1 and an internal, equatorial H-2, and between an axial H-1 and internal, axial H-3 and H-5. Furthermore, if substituents vicinal to the linkage position are axial, n.O.e. contact with the corresponding equatorial protons may sometimes be obtained. This is true both for the glycosyl and the glycosylated residue.

Starting with the anomeric proton of RhaII, four protons with n.O.e. contact are observed. Two of these, at δ 5.16 and 4.96, are with anomeric protons. A third, at δ 4.25, is an internal contact with H-2. The fourth, at δ 4.09, is with H-2 of RhaI, as no other signals occur in this vicinity. This establishes the structural element **1**.



The H-1 of the 3-acetamido-3,6-dideoxy-L-galactopyranosyl group has four n.O.e. contacts. Those at δ 5.16 and 5.05 are with other anomeric protons and that at δ 3.80 is an internal contact with H-2. The remaining contact, at δ 4.25, is with H-2 of RhaII. This, in conjunction with previous results, establishes partial structure **2**.

→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→
2
↑
1
α-L-Fuc*p*3NAc

2

The anomeric proton of the β-L-rhamnopyranosyl residue (RhaIII) has five n.O.e. contacts, including H-1 of RhaI and internal contacts with H-2, H-3, and H-5. The remaining contact is with H-4 of the 2-acetamido-2-deoxy-D-glucopyranosyl residue. This, together with results discussed above, demonstrates the partial structure **3**.

→3)-β-L-Rha*p*-(1→4)-β-D-Glc*p*NAc-(1→

3

From these results, the structure of the complete pentasaccharide repeating-unit (**4**) can be constructed. In agreement with this structure, the anomeric proton of the 2-acetamido-2-deoxy-D-glucopyranosyl residue has, in addition to an internal contact with H-3, a contact with H-3 of the branching α-L-rhamnopyranosyl residue (RhaII), at δ 3.97. The absence of an n.O.e. contact to H-5 may be due to the effective relaxation of this proton against other protons and to broadening of the signal.

→3)-α-L-Rha*p*II-(1→2)-α-L-Rha*p*I-(1→3)-β-L-Rha*p*III-(1→4)-β-D-Glc*p*NAc-(1→
2
↑
1
α-L-Fuc*p*3NAc

4

The anomeric proton of RhaI has six n.O.e. contacts, including that to H-3 of RhaIII, as required by structure **4**. Even if the structure of the pentasaccharide repeating-unit is defined by the experiments discussed above, there are some unexplained n.O.e. contacts, *e.g.*, between anomeric protons. In order to study possible n.O.e. contacts, a computer-drawn model (Fig. 1) of the PS was constructed, using the HSEA-programme[6]. In this figure, the inter-residue n.O.e. relaxation network is indicated by dashed lines.

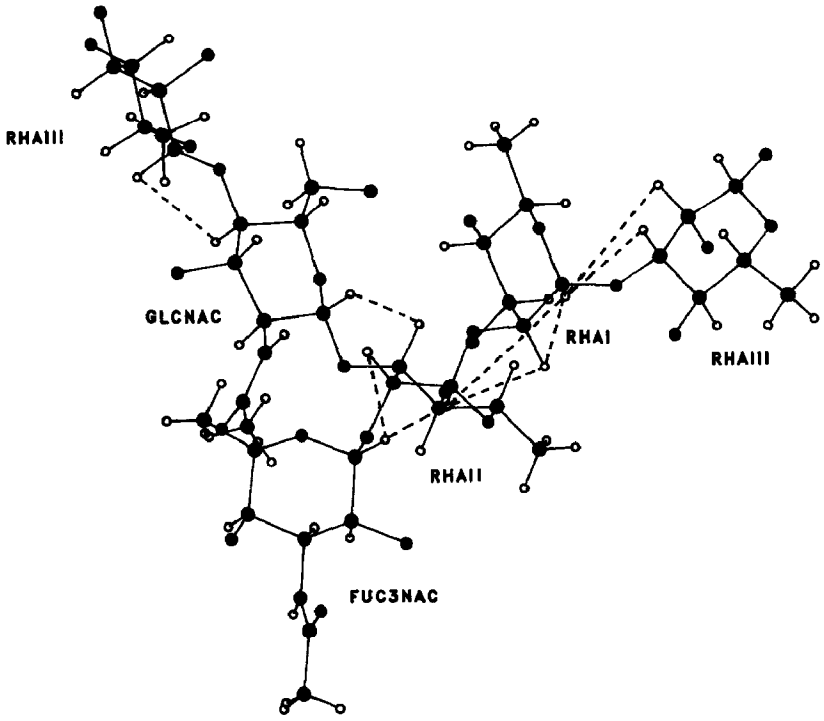


Fig. 1. HSEA-Calculated model of the O-polysaccharide of *E. coli* O-2. Dashed lines indicate interresidue n.O.e.-contacts.

The hard-sphere calculations for the polysaccharide employed crystal co-ordinates for α-L-rhamnose and 2-acetamido-2-deoxy-β-D-glucose. β-L-Rhamnose was obtained from β-D-mannose by inversion of co-ordinates and atom modification. 3-Acetamido-3,6-dideoxy-D-galactose was obtained by modification of D-galactose. For the *N*-acetyl group, dihedral angles of 0° were used. The calculations suggested that most glycosidic bond angles were equal or similar to those in the disaccharide elements. All the calculated proton–proton distances of anomeric protons less than 3 Å were observed in the 2D-n.O.e. experiment. In addition, two weak n.O.e. contacts were observed for H-1 and RhaI to H-2 in RhaIII and for H-1 in RhaI to H-1 in RhaII at a calculated distance of ~4 Å. An observed n.O.e. contact between H-1 in RhaI and H-1 in RhaIII could not be explained, but spin diffusion is the likely reason for this signal.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°. For g.l.c., a Hewlett–Packard 5830 A instrument, fitted with a flame-ionisation detector, was used. Separations

were performed on an Ultra 2 (cross-linked 5% phenyl methyl silicone) fused-silica capillary column, using a temperature programme (150° for 2 min; 150→220°, 2°/min). G.l.c.–m.s. was performed with a Hewlett–Packard 5790–5970 system, using the same g.l.c. phase. A differential refractometer was used for monitoring the gel-filtration effluents. Absolute configurations were determined by the procedure of Gerwig *et al.*[5]. Methylation analyses were performed essentially as previously described[7,8]. Methylated products were purified on Sep-Pak C_{18}-cartridges as described by Waeghe *et al.*[9]. All hydrolyses were performed[10] by solvolysis with anhydrous hydrogen fluoride for 3 h at room temperature followed by hydrolysis with 2M trifluoroacetic acid for 2 h at 100°.

Preparation of polysaccharide. — Isolation of the polysaccharide from the lipopolysaccharide was achieved by mild hydrolysis with aqueous 2% acetic acid at 100° for 1.75 h, and subsequent work-up was performed as described previously[11].

N.m.r. spectroscopy. — N.m.r. spectroscopy was performed on a JEOL GX-400 instrument with 2% D_2O-solutions at 400 MHz, using TSP (^1H) or 1,4-dioxane, δ 67.4 (^{13}C) as internal references. All samples were heated to 100° and ultrasonicated. All spectra were recorded at 70°. Two-dimensional n.m.r. spectroscopy was performed with standard COSY and NOESY pulse sequences. For the NOESY experiments, only one mixing time (800 ms) was used.

After a trapezoid multiplication of the FID matrix, the physical presentation was given in the power mode. The analysis of spectra was done by a combination of COSY-spectra, NOESY-spectra, and NOESY-cross-sections.

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