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STRUCTURAL STUDIES OF THE *Escherichia coli* O-ANTIGEN 25

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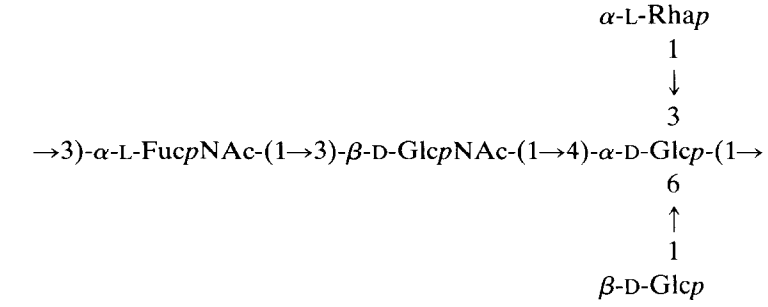
ABSTRACT

The structure of the *Escherichia coli* O-antigen 25 has been investigated using n.m.r. spectroscopy, methylation analysis, and various specific degradations. It is concluded that the O-antigen is composed of pentasaccharide repeating-units having the following structure.



INTRODUCTION

The synthesis and expression of the O-antigenic polysaccharide side-chains of the lipopolysaccharides (LPS) of such Gram-negative enteric bacteria as *Salmonella* and *Shigella* are generally considered to be of importance for the virulence of the bacteria. Conjugal transfer of the *Escherichia coli* O-antigen 25 to virulent *Shigella flexneri* 2a recipients (thereby replacing the 2a polysaccharide chain with the *E coli* O 25 chain) resulted in a hybrid with retained virulence[1]. On similar

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transfer of the *E. coli* O-antigen 8, however, virulence was essentially lost. The *E. coli* O-antigen 8 is a mannan of known structure[2], and its biosynthesis differs from those generally observed for bacterial O-antigens[3]. It was therefore of interest to study the *E. coli* O-antigen 25, and we now report on its structure.

RESULTS AND DISCUSSION

The LPS was obtained from *S. flexneri* O 25 hybrid-strain[1] 542-1-7, by extraction with phenol–water. Extensive immunological tests had demonstrated this hybrid to have *E. coli* O-antigen 25 specificity, and no reactivity characteristic of the *S. flexneri* 2a strain could be demonstrated. The O-antigen from *E. coli* O 25, W 3703, was also prepared and investigated by essentially the same methods as used for the antigen from the hybrid strain. As the results were identical, they will not be reported in detail.

Delipidation of the LPS by treatment with aqueous 1% acetic acid at 100° yielded the polysaccharide (PS), which was purified by gel filtration on a Sephadex G-50 column. The PS had $[\alpha]_{578}^{25}$ −16° and, on hydrolysis, yielded L-rhamnose, D-glucose, 2-amino-2,6-dideoxy-L-galactose, and 2-amino-2-deoxy-D-glucose in the molar proportions ~1:2:1:1. The percentages of galactose and heptose were low, indicating that the major part of the PS consisted of O-specific side-chains. In agreement with this, the signals in the 400-MHz ^{1}H-n.m.r. spectrum, assigned to anomeric protons of the core sugars, were weak compared to the signals given by the sugar residues in the O-specific side-chains (see below). The absolute configurations of the sugars were determined by the method of Leontein *et al.*[4,5]. In the ^{1}H-n.m.r. spectrum of the PS, signals at δ 5.21 (not resolved), 5.00 (J 3.8 Hz), 4.98 (J 3.8 Hz), 4.55 (J 8.3 Hz), and 4.51 (J 7.9 Hz) were assigned to five anomeric protons, signals at δ 2.02 and 1.97 to two *N*-acetyl groups, and signals at δ 1.34 (J 6.2 Hz) and 1.19 (J 6.2 Hz) to the H-6 protons of two 6-deoxyhexoses. In agreement with these assignments, the ^{13}C-n.m.r. spectrum contained signals for five anomeric carbons at δ 103.4, 101.7, 101.5, 101.1, and 99.3, for two *N*-acetyl groups at δ 175.6, 174.7, 23.7, and 23.5, for two C-6 carbons of 6-deoxyhexoses at δ 18.1 and 16.5, and for two C-2 carbons of amino sugars at δ 57.0 and 49.5. These results indicate that the O-antigen is composed of pentasaccharide repeating-units, containing two D-glucopyranosyl residues and one pyranosidic residue each of L-rhamnose, 2-acetamido-2,6-dideoxy-L-galactose, and 2-acetamido-2-deoxy-D-glucose. According to the ^{1}H-n.m.r. spectrum, three residues should be α-linked and two β-linked. The signal at δ 5.21 (not resolved) could be assigned to an α-L-rhamnopyranosyl residue. The signal in the ^{13}C-n.m.r. spectrum at δ 49.5 could be assigned to C-2 of a 2-acetamido-2,6-dideoxy-α-L-galactopyranosyl residue, and the signal at δ 57.0 to C-2 of a 2-acetamido-2-deoxy-β-D-glucopyranosyl residue (*cf.* the signals for the corresponding methyl glycosides in Table II). Consequently, one of the remaining D-glucopyranosyl residues should be α-linked and the other β-linked.

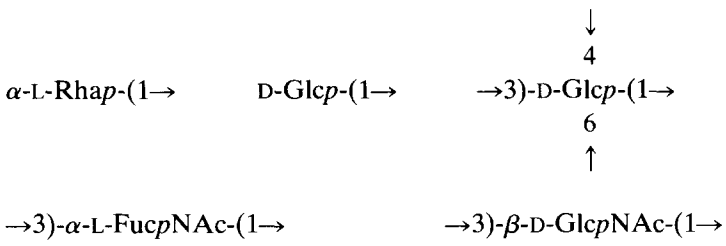
TABLE I

METHYLATION ANALYSES OF THE *E. coli* O-ANTIGEN 25 AND OF DEGRADATION PRODUCTS

Methylated sugar[b]	T[c]	*Relative proportions*[a]			
		A	*B*	*C*	*D*
2,3,4-Rha	0.56	24			
2,3,4,6-Glc	1.00	23	36		
2,3,6-Glc	1.52			48	
2,3-Glc	2.41		35		
2,3,4-FucNAc	2.43				39
2-Glc	3.13	20			
2,4-FucNAc	3.54	15	12	15	
2,4,6-GlcNAc	5.48	19	17	37	61

[a]A, Original PS. B, PS after mild hydrolysis with acid. C, PS after first Smith-degradation. D, Oligosaccharide after second Smith-degradation. The relative proportions were taken from the detector responses. The values for the amino sugars, which have higher response factors than those of the neutral sugars, are consequently too low. [b]3,4,6-Rha = 3,4,6-tri-*O*-methyl-L-rhamnose, *etc.* [c]Retention time of the derived alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an SE-30 W.C.O.T. glass-capillary column at 175°.

Methylation analysis of the PS gave the sugars listed in Table I, column A. These results demonstrate the presence of the following structural elements:



Part of the PS was treated with 0.25M trifluoroacetic acid at 85°, the hydrolysis being monitored by ^{1}H-n.m.r. spectroscopy. After 90 min, when the signal at δ 5.21 had disappeared, the reaction was interrupted and the product fractionated on a Sephadex G-25 column. The polymeric fraction (78%), $[\alpha]_D^{25}$ +6°, was subjected to methylation analysis (Table I, column B). The analysis showed that the L-rhamnosyl residues had been hydrolysed off and that HO-3 in the branching D-glucopyranosyl residue had become exposed. The L-rhamnosyl residue is consequently linked to O-3 of the branching D-glucosyl residue, as in **1**. The ease with which this sugar residue is hydrolysed off is commented on below.

TABLE II

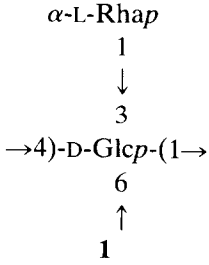
TENTATIVE ASSIGNMENTS OF ^{13}C-N.M.R. SIGNALS GIVEN BY THE POLYSACCHARIDE AND OLIGOSACCHARIDE OBTAINED AFTER CONSECUTIVE DEGRADATIONS OF THE *E. coli* O-ANTIGEN 25

Substance	α-L-*Fuc*p*NAc*					
	1	*2*	*3*	*4*	*5*	*6*
α-L-FucNAc-(1→3)-β-D-GlcNAc-(1→2)-D-Erythritol	99.3	50.9	69.4	72.1	68.4	16.8
→3)-α-L-FucNAc-(1→3)-β-D-GlcNAc-(1→4)-α-D-Glc-(1→	99.6	49.8	77.8	72.4	68.5	16.8
Me α-L-Fuc*p*NAc[7]	99.3	50.8	69.1	72.2	67.6	16.6
Me β-D-Glc*p*NAc[8]						
Me α-D-Glc*p*[9]						

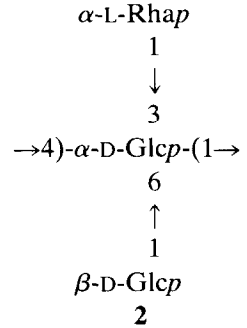
Substance	β-D-*Glc*p*NAc*					
	1	*2*	*3*	*4*	*5*	*6*
α-L-FucNAc-(1→3)-β-D-GlcNAc-(1→2)-D-Erythritol	103.2	57.0	80.1	70.1	77.1	62.2
→3)-α-L-FucNAc-(1→3)-β-D-GlcNAc-(1→4)-α-D-Glc-(1→	102.6	57.1	80.3	70.2	77.4	62.4
Me α-L-Fuc*p*NAc[7]						
Me β-D-Glc*p*NAc[8]	103.2	56.7	75.2	71.2	77.1	62.0
Me α-D-Glc*p*[9]						

Substance	α-D-*Glc*p *or* D-*Erythritol*					
	1	*2*	*3*	*4*	*5*	*6*
α-L-FucNAc-(1→3)-β-D-GlcNAc-(1→2)-D-Erythritol	62.7	83.4	72.7	63.8		
→3)-α-L-FucNAc-(1→3)-β-D-GlcNAc-(1→4)-α-D-Glc-(1→	101.8	72.7	73.1	81.0	72.7	61.8
Me α-L-Fuc*p*NAc[7]						
Me β-D-Glc*p*NAc[8]						
Me α-D-Glc*p*[9]	100.3	72.5	74.2	70.6	72.7	61.7

Substance	O ‖ *C*	O ‖ *C*	*CH*$_3$	*CH*$_3$	*OCH*$_3$
α-L-FucNAc-(1→3)-β-D-GlcNAc-(1→2)-D-Erythritol	175.8	175.3	23.7	23.7	
→3)-α-L-FucNAc-(1→3)-β-D-GlcNAc-(1→4)-α-D-Glc-(1→	176.0	175.3	24.0	23.7	
Me α-L-Fuc*p*NAc[7]	175.7		23.1		56.4
Me β-D-Glc*p*NAc[8]	176.1		23.3		58.3
Me α-D-Glc*p*[9]					56.2



In the ^{1}H-n.m.r. spectrum, signals at δ 5.02 (J 3.9 Hz), 4.99 (J 3.7 Hz), 4.58 (J 8.3 Hz), and 4.45 (J 7.8 Hz) were assigned to the four remaining anomeric protons, signals at δ 2.02 and 1.99 to two *N*-acetyl groups, and a signal at δ 1.18 (J 6.6 Hz) to the H-6 protons of the 2-acetamido-2,6-dideoxy-α-L-galactopyranosyl residue. The chemical-shift differences of the anomeric protons, relative to the original PS, are caused by direct and remote substituent effects of the α-L-rhamnopyranosyl group. The PS was subjected to a Smith degradation[6] (periodate oxidation, borohydride reduction, and acid hydrolysis under mild conditions), during which the acetalic linkages of modified sugar residues should be hydrolysed but the glycosidic linkages should be resistant. Methylation analysis of the polymeric product (Table I, column C) showed that the two terminal sugar residues in the PS had been eliminated and that HO-3 and HO-6 in the branching D-glucopyranosyl residue had become exposed. The D-glucopyranosyl group is consequently linked to O-6 of the branching D-glucopyranosyl residue. The ^{13}C-n.m.r. spectrum of the modified PS (Table II) confirmed that the 2-acetamido-2,6-dideoxy-L-galactopyranosyl and 2-acetamido-2-deoxy-D-glucopyranosyl residues are α- and β-linked, respectively, and further demonstrated that the (originally branching) D-glucopyranosyl residue is α-linked. The terminal D-glucopyranosyl group must therefore be β-linked. The ^{1}H-n.m.r. spectrum of the Smith-degraded product contained signals at δ 5.02 (J 3.7 Hz), 4.98 (J 4.1 Hz), and 4.55 (J 8.3 Hz) for anomeric protons, 2.02 and 1.98 for *N*-acetyl protons, and 1.17 (J 6.6 Hz) for H-6 protons of a 6-deoxyhexose. The results therefore demonstrate the partial structure **2** in the original PS.



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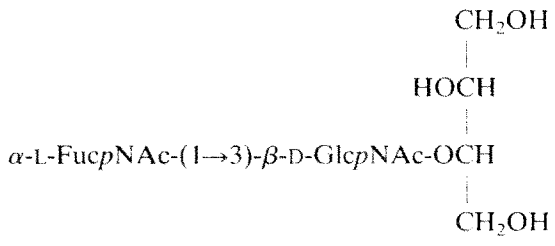
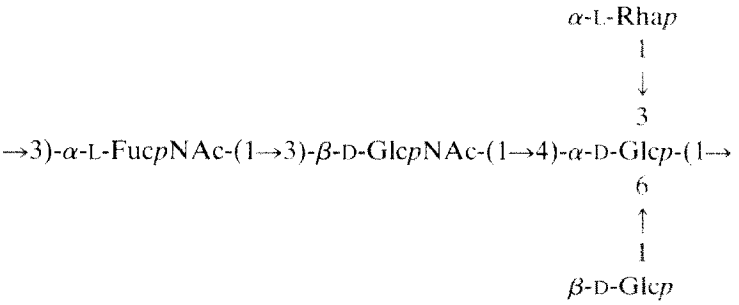


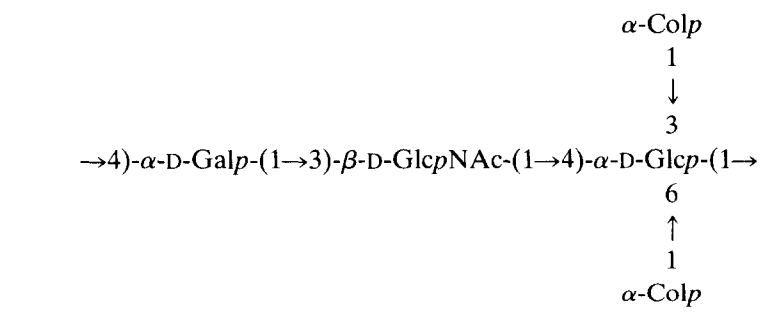
3

In order to demonstrate the sequence of the sugar residues in the PS chain, the Smith-degraded material was subjected to a second Smith-degradation, which, as expected, yielded a disaccharide-glycoside of erythritol. This, according to methylation analysis (Table I, column D) had structure **3**. In agreement with this structure, the ^{1}H-n.m.r. spectrum contained signals at δ 5.0 (J 3.9 Hz), 4.57 (J 8.1 Hz), 2.05, 2.00, and 1.19 (J 6.6 Hz). By comparison with reference substances, it was possible to assign tentatively all signals in the ^{13}C-n.m.r. spectra of the products from the first and the second Smith-degradation (Table II).



4

From the combined evidence, structure **4** is proposed for the pentasaccharide repeating-unit of the *E. coli* O-antigen 25. The presence of a doubly branched sugar residue in this structure is unusual, but not unique. Thus, a similar substitution of an α-D-glucopyranosyl residue occurs in the pentasaccharide repeating-unit (**5**) of the antigen that is common to *E. coli* O 111 and *Salmonella adelaide*[10]. In both of these O-antigens, the glycosidic linkage of the sugar linked to O-3 of the α-D-glucopyranosyl residue, an α-L-rhamnopyranosyl group in **4** and a 3,6-dideoxy-α-L-*xylo*-hexopyranosyl (colitosyl) group in **5**, is unexpectedly sensitive to hydrolysis with acid. This may be due to the crowded environment around the doubly branched α-D-glucopyranosyl residue.



5

EXPERIMENTAL

General methods. — These were the same as previously reported[10]. N.m.r. spectra were recorded for solutions in D_2O with JEOL FX-100 and GX-400 spectrometers, using external tetramethylsilane (^{13}C) and internal sodium tetradeuterio-3-trimethylsilylpropionate (^1H) as references.

Material. — The LPS was isolated from *E. coli*, W 3703, and *Shigella flexneri* O 25 hybrid-strain 542-1-7 as described[1]. Delipidation was performed by treating the LPS with aqueous 1% acetic acid for 2 h at 100°. The product was centrifuged, the supernatant solution was freeze-dried, and the residue was fractionated on a Sephadex G-50 column with water as irrigant. The PS, eluted in the void volume, was isolated by freeze-drying and had $[\alpha]_{578}^{25}$ −16° (*c* 0.2, water).

Sugar analysis. — The PS (10 mg) was treated with 0.5M trifluoroacetic acid (3 mL) for 19 h at 100°, and the solution was concentrated. For quantitative analysis, the residue was reduced with sodium borohydride and acetylated, and the products were analysed by g.l.c. For determination of the absolute configuration, the neutral sugars and the amino sugars were first separated on a column of Dowex 50 (H^+) resin (elution with water followed by 0.5M hydrochloric acid), and then transformed into acetylated glycosides of (−)-2-octanol which were analysed by g.l.c.[4,5].

Methylation analysis. — This was performed essentially as described by Jansson *et al.*[11].

Partial hydrolysis of the PS. — PS (5 mg) was treated with 0.25M trifluoroacetic acid in deuterium oxide (0.4 mL) in an n.m.r. tube at 85°. The hydrolysis was monitored by ^1H-n.m.r. spectroscopy. When the signals (δ 5.21) for the anomeric proton of the α-L-rhamnopyranosyl groups had disappeared, the solution was concentrated to dryness and the residue fractionated on a column of Sephadex G-25. The polymeric fraction (3.8 mg), eluted in the void volume, had $[\alpha]_{578}^{25}$ +6° (*c* 0.2, water).

Smith degradations. — A solution of the PS (108 mg) and sodium metaperiodate (962 mg) in 0.1M sodium acetate buffer (pH 5.0, 100 mL) was kept for 2 days in the dark at 5°. Excess of periodate was reduced with ethylene glycol,

and the solution was dialysed and freeze-dried. The residue was dissolved in water (20 mL), sodium borohydride (40 mg) was added, and the solution was kept overnight, neutralised with 50% aqueous acetic acid, dialysed, and freeze-dried. A solution of the product (56 mg) in 0.5M trifluoroacetic acid (10 mL) was kept at 25° for 48 h, diluted with water (20 mL), and freeze-dried. The polymeric reaction-product (32 mg) was isolated by chromatography on a column of Sephadex G-50.

For the second Smith-degradation, the product from the first oxidation (30 mg) was oxidised with periodate, reduced with borohydride, and hydrolysed as above. The product was fractionated on a column of Sephadex G-15, to give pure **3** (13.2 mg).

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