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STRUCTURAL STUDIES OF THE O-ANTIGENS FROM *Salmonella greenside* AND *Salmonella adelaide*

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ABSTRACT

The structures of the O-specific side-chains in the lipopolysaccharides of *Salmonella greenside*, group Z, and *Salmonella adelaide*, group O, have been investigated. The former proved to be identical with that of *Escherichia coli* O 55. The latter, which was more extensively studied, was composed of repeating units having the structure

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

in which Col is colitose (3,6-dideoxy-L-*xylo*-hexose). This was also shown to be the biological repeating-unit. The same structure has been proposed for the O-antigen of *E. coli* O 111. The biological repeating-unit for the *S. greenside* O-antigen was also defined. The structural studies also confirmed that both lipopolysaccharides contain the hexose region typical for the *Salmonella* core.

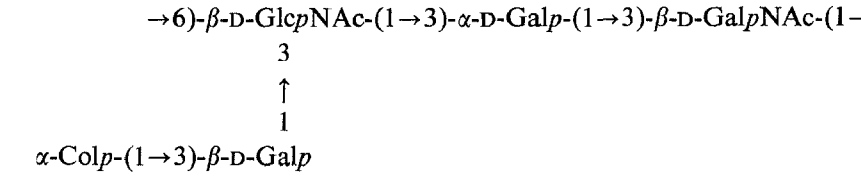
INTRODUCTION

Colitose (3,6-dideoxy-L-*xylo*-hexose) is a component of the O-antigen of some bacteria belonging to the Enterobacteriaceae[1]. It is present in chemotype X, which includes *Salmonella* group O, *E. coli* O 111, and *Arizona* groups 9 and 20, and also in chemotype XI, including *Salmonella* group Z and *E. coli* O 55. It has further been

found in the lipopolysaccharide (LPS) from *Yersinia pseudotuberculosis*[2], serogroup VI. The structure of the O-antigen of *E. coli* O 55 has been determined[3] and that of *E. coli* O 111 briefly reported[4]. We now report on structural studies of the O-antigens of *S. greenside*, group Z, and *S. adelaide*, group O.

RESULTS AND DISCUSSION

Preliminary studies of the LPS from *S. greenside* indicated that its O-specific side-chains were similar to, or identical with, those of *E. coli* O 55 (**1**)[3]. Sugar and methylation analyses gave the same components and linkages, whereas the ^{1}H- and ^{13}C-n.m.r. spectra of the polysaccharides, prepared from the LPS by hydrolysis with acid under mild conditions followed by gel-permeation chromatography, demonstrated that the O-antigens were actually identical.



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In the fraction of low molecular weight obtained on gel filtration, the core part of the LPS is enriched. Methylation analysis of this fraction from *S. greenside* gave the partially methylated hexoses[5] typical for the *Salmonella* (Ra) core[6], thus showing, by chemical evidence, that the LPS investigated is actually derived from a *Salmonella* species.

In this analysis, the relative proportions of 2-deoxy-3,4,6-tri-*O*-methyl-2-methylacetamido-D-glucose (from the core), 2-deoxy-4,6-di-*O*-methyl-2-methylacetamido-D-glucose, 2-deoxy-4,6-di-*O*-methyl-2-methylacetamido-D-galactose, and 2-deoxy-4-*O*-methyl-2-methylacetamido-D-glucose were ~1:1:2:1. The results indicate that the LPS fraction studied contains, on average, two repeating-units and that the branching 2-acetamido-2-deoxy-D-glucopyranosyl residue terminates the biological repeating-unit (**1**).

Sugar analysis of the LPS from *S. adelaide* gave colitose, D-glucose, D-galactose, 2-amino-2-deoxy-D-glucose, and a heptose in the relative proportions 1.3:1.0:1.0:0.5:0.1. That the 3,6-dideoxyhexose was colitose and not its enantiomer, abequose, was confirmed by g.l.c. of its acetylated glycosides with chiral 2-octanol[7]. The colitosyl linkages are sensitive to hydrolysis with acid, and the liberated colitose was degraded on prolonged treatment with acid. The hydrolysis was therefore performed in two steps, with a reduction with sodium borohydride after the first step, which substantially increased the yield of colitose. That the 2-amino-2-deoxy-D-glucose is *N*-acetylated was evident from the ^{1}H- and ^{13}C-n.m.r. spectra, as discussed below.

Methylation analysis of the LPS gave the sugars listed in Table I, indicating that the O-specific side-chains are composed of pentasaccharide repeating-units

TABLE I

METHYLATION ANALYSES OF THE LIPOPOLYSACCHARIDE AND THREE POLYSACCHARIDE FRACTIONS FROM *Salmonella adelaide*

Methylated sugar[a]	T_A[b]	T_B[b]	*Detector response (%)*			
			LPS	*PSI*	*PSII*	*PSIII*
2,4-Col[c]	0.44	0.38	11	22	26	13
2,3,4,6-Glc	1.00	1.00	11	7	7	11
2,3,4,6-Gal	1.14	1.19	6	1	4	8
2,3,6-Gal	1.86	2.22	29	33	24	21
3,4,6-Gal	1.96	2.15	5	3	7	15
2,3,4-Glc	2.00	2.22	–	–	–	+[d]
3,6-Glc	2.94	3.73	3	1	5	8
2,4-Glc	3.50	4.21	9	2	9	15
2,3-Glc	3.50	4.50	–	14	7	5
2-Glc	5.15	6.6	26	17	11	4
4,6-GlcNAc[e]			+	+	+	+

[a]2,4-Col = 2,4-di-*O*-methylcolitose, *etc.* [b]Retention time of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an SP-1000 (T_A) and OV-225 (T_B) column, respectively. [c]This derivative is volatile and was partially lost during concentrations. [d]Incompletely separated from 3,4,6-Gal, but identified from its mass spectrum. [e]The analysis of this component, which was *N*-methylated, is qualitative only.

containing two terminal colitopyranosyl groups, a 3-linked 2-acetamido-2-deoxy-D-glucopyranosyl residue, a 4-linked D-galactopyranosyl residue, and a branching D-glucopyranosyl residue, linked through O-3, O-4, and O-6. That the two latter residues are pyranoid and linked through O-4, and not furanoid and linked through O-5, is evident from their resistance to acid hydrolysis and also from the signals given by the anomeric proton and carbon atoms in the n.m.r. spectra.

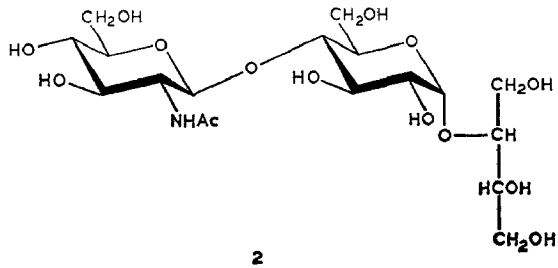
For further studies, the polysaccharide was prepared from the LPS by treatment with 1% acetic acid at 100°, followed by gel filtration on Sephadex G-50. Three polysaccharide fractions (PSI, PSII, and PSIII) were obtained. In fraction PSI, the O-antigen was considerably enriched. A fraction eluted after PSIII contained free colitose that had been released during the delipidation. In the methylation analysis of PSI (Table I), comparable amounts of 2-*O*-methyl- and 2,3-di-*O*-methyl-D-glucose were obtained, demonstrating that one of the colitosyl groups is linked to O-3 of the D-glucopyranosyl residue and that ~50% of this group had been released during delipidation.

In the ^{1}H-n.m.r. spectra of PSI, signals in the region for anomeric protons were observed at δ 5.40 (0.5 H, not resolved), 5.36 (0.5 H, not resolved), 5.28 (0.5 H, *J* 3.4 Hz), 4.97 (0.5 H, *J* 3.4 Hz), 4.95 (0.5 H, *J* 3.4 Hz), 4.87 (0.5 H, *J* 3.4 Hz), 4.83 (0.5 H, *J* 3.6 Hz), and 4.71 (1 H, broad). The multitude of signals is caused by direct and remote substituent effects of the colitosyl residue present in only 50% of the repeating units. The signal at δ 5.28 is due to this residue, as it decreased on

treatment of PSI with 0.2M trifluoroacetic acid at 70° and disappeared after 30 min. For the resulting, modified polysaccharide, the signals for anomeric protons were observed at δ 5.40 (1 H, not resolved), 4.97 (1 H, J 3.2 Hz), 4.83 (1 H, J 3.4 Hz), and 4.70 (1 H, J 7.1 Hz). On prolonged hydrolysis (3 h), a colitose-free polysaccharide was obtained. The absence of the signal at δ 4.83 in the ^{1}H-n.m.r. spectrum of this material demonstrates that this signal is due to the colitosyl group linked to O-6 of the D-glucopyranosyl residue. The chemical shifts and coupling constants indicate that both colitopyranosyl groups are α-linked and that two of the three hexopyranosyl residues are also α-linked, and the third β-linked. It was further shown, both by ^{1}H- (δ 2.00, 3 H, s) and ^{13}C-n.m.r. data (δ 24.0 and 175.6), that the 2-amino-2-deoxy-D-glucopyranosyl residue is *N*-acetylated. Methylation analysis of the partially hydrolysed material, isolated by gel filtration, showed it to be a linear polysaccharide in which the original, branching D-glycopyranosyl residue had been converted into a chain residue linked through O-4.

The fully acetylated PSI was treated with chromium trioxide in acetic anhydride[8]. Sugar analysis showed that the 2-acetamido-2-deoxy-D-glucopyranosyl residues had been completely oxidised, but not the D-glucopyranosyl or D-galactopyranosyl residues. Consequently, the former are β-linked and the latter pair are α-linked. The colitopyranosyl groups are also oxidised; this oxidation should be independent of their anomeric configuration, as the free-energy difference between the two possible chair conformations is relatively small.

The LPS was subjected to a Smith degradation[9], *i.e.*, periodate oxidation, borohydride reduction, and hydrolysis with acid under mild conditions. These conditions, however, were chosen so that the colitosyl groups, which are resistant to periodate oxidation, were also hydrolysed off. A disaccharide glycoside (**2**), composed of 2-acetamido-2-deoxy-D-glucose, D-glucose, and threitol, was obtained, and its structure was determined by sugar and methylation analyses and by n.m.r. spectroscopy. There were two signals in the anomeric region of the ^{1}H-n.m.r. spectrum, at δ 5.07 (H, J 3.6 Hz) and 4.61 (H, J 7.9 Hz), in agreement with the findings, discussed above, that the D-glucopyranosyl residue is α-linked and that the 2-acetamido-2-deoxy-D-glucopyranosyl group is β-linked. In the ^{13}C-n.m.r. spectrum, C-1 and C-2 of the latter gave signals at δ 102.5 and δ 56.8, as expected for a β-linked group. The values for the corresponding, α-linked group should be[10] approximately 99.1 and 54.5.



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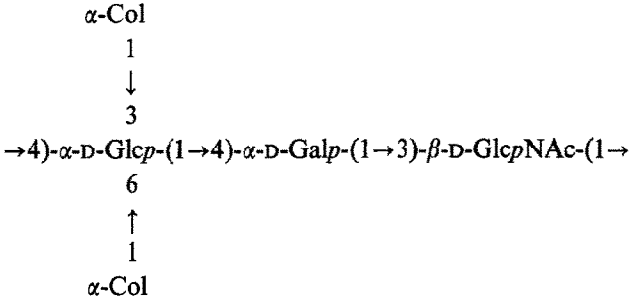
From the combined evidence presented above, structure **3** is proposed for the pentasaccharide repeating-unit of the *S. adelaide* O-antigen. This is identical with the structure proposed for the *E. coli* O 111 O-antigen[4], but does not agree with the results of Edstrom and Heath[11], who have also investigated the *E. coli* O 111 O-antigen. The dicolitosyl-D-glucose, representing the immunochemically significant branching-point in this antigen, has been synthesised[12].



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Methylation analysis of PSII and PSIII, which have molecular weights lower than that of PSI, gave, *inter alia*, 2,3,4,6-tetra-*O*-methyl-D-galactose, 3,4,6-tri-*O*-methyl-D-galactose, and 2,4-di-*O*-methyl-D-glucose, which are typical products of the methylation of a LPS having a *Salmonella* (Ra) core and should not be obtained on similar analyses of LPS containing other Enterobacteriaceae cores.

Methylation analyses of PSI, PSII, and PSIII showed, as expected, that the proportion of O-specific side-chains relative to core increased with increasing molecular weight. In the methylation analyses of PSII and PSIII, 2-*O*-methyl- and 2,3-di-*O*-methyl-D-glucose are partially replaced by 2,4-di- and 2,3,4-tri-*O*-methyl-D-glucose. These results indicate that the O-specific side-chains are terminated by the branching D-glucopyranosyl residue, and that **3** is the biological repeating-unit of the *S. adelaide* O-antigen.

A high percentage of 3,6-di-*O*-methyl-D-glucose was obtained in the methylation analysis of PSII and PSIII from *S. adelaide* (Table I) and also from similar fractions of *S. greenside*. This sugar derives from the penultimate α-D-glucopyranosyl residue of the *Salmonella* core. The results demonstrate that both antigens are linked to O-4 of this residue, as has previously been demonstrated for the *S. typhimurium* O-antigen. In the latter, it is a β-D-galactopyranosyl residue that is linked to this position; in the former two, it is a 2-acetamido-2-deoxy-D-glucopyranosyl or -D-galactopyranosyl residue, respectively, of unknown anomeric configuration.

Rather high percentages of 2,3,4,6-tetra-*O*-methyl-D-glucose were observed in the methylation analysis of the LPS and the polysaccharide fractions from *S. adelaide*. This sugar should not be derived from the O-antigen or the hexose region of the Ra core, and its structural significance is obscure.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°. G.l.c. was performed on SE-30 (*N*-acetyl derivatives) and SP-1000 W.C.O.T. glass-capillary columns (25 m × 0.25 mm) at 210°, and on glass columns (190 × 0.15 cm) containing 3% of OV-225 on Gas Chrom Q (100–120 mesh) at 170° and 3% of OV-17 on Gas Chrom Q at 190°. G.l.c.–m.s. was performed with a Varian MAT 311-SS 100 instrument. The n.m.r. spectra were recorded for solutions in D_2O with JEOL FX-100 and FX-200 spectrometers, using external tetramethylsilane (^{13}C-n.m.r.) and internal sodium tetradeuterio-3-trimethylsilylpropionate (^{1}H-n.m.r.) as references. Gel filtrations were monitored by differential refractrometry.

Bacterial strains. — *Salmonella* strains S_{II} *greenside* and *S. adelaide* were obtained from the International Salmonella Centre, Institut Pasteur, Paris.

Preparation of the antigen. — *S. adelaide* was grown in 20-litre fermenters, using Evans medium[14]. Eighteen-hour cultures were phenol-killed and extracted by the phenol–water method[15]. The aqueous extract was dialysed and then lyophilised, to yield 5 g of crude LPS. This material was dissolved in Tris-HCl buffer (0.02M, pH 7.0) and incubated at 37% for 4 h with ribonuclease and deoxyribonuclease (Worthington Biochemicals). Dialysis against de-ionised water, concentration to 200 mL, and ultracentrifugation at 100,000*g* for 18 h gave pure LPS as a gelatinous pellet. A solution of this material in water was centrifuged, and the pellet was lyophilised, to yield 2.5 g of LPS containing <0.5% of nucleic acid. Delipidation was performed by treatment of the LPS (0.8 g) with 1% aqueous acetic acid (80 mL) at 100° for 1 h. Centrifugation and lyophilisation yielded water-soluble material, which was fractionated by gel filtration on a Sephadex G-50 column. Three fractions were obtained: PSI (120 mg), PSII (53 mg), and PSIII (100 mg); PSI had $[\alpha]_D^{22}$ +61° (*c* 0.5, water).

The O-antigen from *S. greenside* was isolated and fractionated as described above.

Sugar analysis. — Samples (~2 mg) were hydrolysed with M trifluoroacetic acid (2 mL) at 100° for 1 h. Each hydrolysate was concentrated to dryness, the residue was treated with sodium borohydride (10 mg) in water (2 mL) for 2 h, the solution was neutralised with acetic acid and concentrated to dryness, and boric acid was removed by distillation of methanol (3 × 2 mL) from the residue. The material was then subjected to a second hydrolysis with M trifluoroacetic acid at 100° for 16 h, worked-up as described above, and acetylated.

Methylation analyses. — These were performed essentially as described by Jansson *et al.*[16], but the partially methylated amino sugars were eluted from the Dowex 50 (H^+) resin (used to deionise the product after the borohydride reduction) with 0.5M hydrochloric acid, and analysed separately on the SE-30 glass-capillary column.

Determination of absolute configuration. — The absolute configurations of the

sugars from PSI were determined by the method of Leontein *et al.*[7], using (+)-2-octanol. The pattern given by the acetylated 3,6-dideoxyhexosides in g.l.c., using the SP-1000 column, was the same as that obtained from the acetylated abequosides of (−)-2-octanol.

Partial hydrolysis of the PS. — PS (10 mg) was treated with 0.2M trifluoroacetic acid in deuterium oxide in an n.m.r. tube at 70°. The hydrolysis was followed by ^{1}H-n.m.r. spectroscopy. When the signals for the anomeric protons of the colitopyranosyl groups had disappeared (3 h), the solution was diluted with water and freeze-dried. Fractionation on a Biogel P-2 column (65 × 1.8 cm) gave a polymeric fraction and colitose.

Smith degradation of the LPS[9]. — A solution of the LPS (100 mg) in 0.14M acetate buffer (pH 6, 13 mL) was treated in the dark with sodium metaperiodate (210 mg) at 5° for 56 h. The excess of periodate was reduced with ethylene glycol (0.5 mL), and the solution was dialysed overnight and freeze-dried. The product was reduced with sodium borohydride (30 mg) in water (5 mL) for 4 h, and the solution was neutralised with acetic acid, dialysed and freeze-dried. Part (2 mg) of the product (76 mg) was used for sugar analysis, which showed that almost all of the D-galactose had been oxidised. The product was hydrolysed with 0.5M trifluoroacetic acid (10 mL) at room temperature for 16 h, and the hydrolysate was diluted with water and freeze-dried. The material was suspended in water, and the non-soluble, lipid part (24 mg) was removed by centrifugation. The aqueous solution was added to a column (90 × 3 cm) of Sephadex G-25 that was irrigated with water. ^{1}H-N.m.r. spectroscopy of the main fraction showed it to be heterogeneous, because some of the colitosyl groups had been hydrolysed off. The main fraction was therefore subjected to a second hydrolysis with 0.2M trifluoroacetic acid in deuterium oxide at 70°. The hydrolysis was monitored by ^{1}H-n.m.r. spectroscopy, which showed that all of the colitosyl groups had been hydrolysed off after 2 h. The solution was then diluted with water and freeze-dried. The product was fractionated on a column (35 × 2.5 cm) of Biogel P-2 by irrigation with water, yielding **2** as the main component.

Chromic acid oxidation. — Peracetylated PSI (20 mg) was treated with chromium trioxide (60 mg) in acetic anhydride (0.6 mL) for 1 h, with peracetylated *myo*-inositol as the internal standard, essentially as described by Hoffman and Lindberg[8]. Sugar analyses were performed on unoxidised and oxidised material.

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