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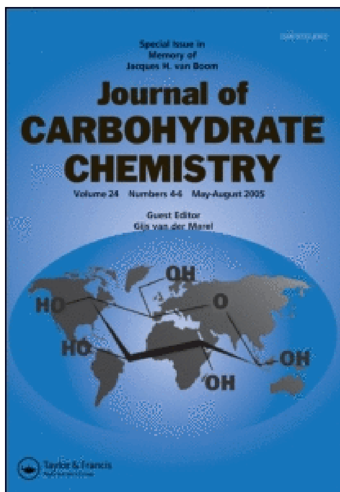

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Structure of the O-polysaccharide of *Escherichia coli* O61, Another *E. coli* O-antigen That Contains 5,7-Diacetamido-3,5,7,9-tetradeoxy-l-*glycero*-D-*galacto*-non-2-ulosonic (Di-*N*-acetyl-8-epilegionaminic) Acid

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Structure of the O-polysaccharide of *Escherichia coli* O61, Another *E. coli* O-antigen That Contains 5,7-Diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic (Di-*N*-acetyl-8-epilegionaminic) Acid

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On mild acid degradation of the lipopolysaccharide of *Escherichia coli* O61, the O-polysaccharide chain was cleaved at a linkage of 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic acid (di-*N*-acetyl-8-epilegionaminic acid, 8eLeg5Ac7Ac). The resultant trisaccharide, an O-deacylated lipopolysaccharide and an O-deacetylated trisaccharide derived from the latter were studied by sugar analyses

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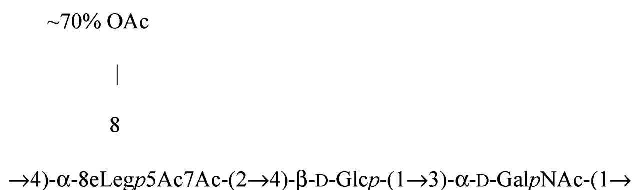
along with ^{1}H and ^{13}C NMR spectroscopy, and the following structure of the O-polysaccharide was established:



Keywords *Escherichia coli*; O-polysaccharide; O-antigen; Lipopolysaccharide; Bacterial polysaccharide structure; Nonulosonic acid; 8-Epilegionaminic acid

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INTRODUCTION

Escherichia coli is a species of enteric bacteria that includes both commensal and pathogenic clones. Based on the serospecificity of O-antigens, *E. coli* strains are divided into 180 O-serogroups, the O-antigen diversity being mainly due to genetic variations in the O-antigen gene cluster. The O-antigen represents the O-polysaccharide chain of the lipopolysaccharide (LPS), which is present on the cell surface of almost all Gram-negative bacteria. O-polysaccharide structures have been established in about half *E. coli* serogroups, and a number of unusual monosaccharides and nonsugar groups have been reported as their components.[1–11, refs. cited in ref. 3] In this work, we identified 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-non-2-ulosonic acid (di-*N*-acetyl-8-epilegionamic acid) as an O-polysaccharide component of *E. coli* O61 and established the structure of the trisaccharide repeating unit of the O-polysaccharide.

MATERIALS AND METHODS

Bacterial Strain and Isolation of Lipopolysaccharide

E. coli O61 strain G1071 was obtained from the Institute of Medical and Veterinary Science, Adelaide, Australia. Bacteria were grown to late log phase in 8 L Luria-Bertani broth using a 10-L fermentor (BIOSTAT C-10, B. Braun Biotech International, Germany) under constant aeration at 37°C and pH 7.0. Bacterial cells were washed and dried as described.[12]

LPS (500 mg) was isolated from dried cells (7.5 g) by extraction with aqueous 45% phenol at 65°C to 68°C (the Westphal procedure[13]), the mixture without separation of the layers was dialyzed for 3 days against distillated water, and the crude LPS was purified by precipitation of nucleic acids and proteins with aqueous 50% trichloroacetic acid at 4°C.[14]

Acid and Alkaline Degradations of Lipopolysaccharide

LPS (100 mg) was heated with aqueous 2% HOAc at 100°C until precipitation of lipid (2.5 h). The precipitate was removed by centrifugation (13,000 × *g*, 20 min), and the supernatant was fractionated by gel-permeation chromatography on a column (56 × 2.6 cm) of Sephadex G-50 Superfine (Amersham Biosciences) in 0.05 M pyridinium acetate buffer, pH 4.5; elution was monitored using a Knauer differential refractometer (Germany). Low-molecular-mass products (74 mg) were further fractionated on a column (80 × 2.5 cm) of TSK HW-40 (Merck) in aqueous 1% HOAc to give a trisaccharide (TS, 13 mg).

LPS (100 mg) was O-deacylated with aqueous 12.5% ammonia (37°C, 16 h), the precipitate was removed by centrifugation (13,000 × *g*, 20 min), and the supernatant was fractionated by gel-permeation chromatography on TSK HW-40 as described above to yield an O-deacylated LPS (60 mg).

The O-deacylated LPS (60 mg) was heated with aqueous 3% HOAc at 100°C (5 h). The precipitate was removed by centrifugation (13,000 × *g*, 20 min), and the supernatant was fractionated by gel-permeation chromatography on TSK HW-40 to give an O-deacetylated TS (15 mg).

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Chemical Analyses

TS was hydrolyzed with 2 M CF_3CO_2H (120°C, 2 h), and monosaccharides were reduced with 0.25 M $NaBH_4$ in aqueous 1 M ammonia (20°C, 1 h), acetylated with a 1:1 (v/v) mixture of pyridine and acetanhydride (120°C, 30 min), and analyzed by GLC on a Hewlett-Packard 5890 chromatograph (USA) equipped with an Ultra-1 column using a temperature gradient of 150 to 290°C at 5°C min^{-1}. The absolute configuration of the monosaccharides were determined by GLC of the acetylated (*S*)-(+)-2-octyl glycosides[15] under the same conditions as in sugar analysis.

NMR Spectroscopy

Samples were deuterium exchanged by freeze-drying from 99.9% D_2O and then examined as solutions in 99.95% D_2O at 30°C and 40°C for trisaccharides and O-deacylated LPS, respectively. NMR spectra were recorded on a Bruker DRX-500 spectrometer (Germany) using internal TSP (δ_H 0) and acetone (δ_C 31.45) as references. 2D NMR spectra were obtained using standard Bruker software, and the data were acquired and processed using the XWINNMR 2.6 program. Mixing times of 200 and 100 ms were used in TOCSY and ROESY experiments, respectively. Other NMR parameters were set essentially as described.[16]

RESULTS AND DISCUSSION

LPS was isolated from dried cells of *E. coli* O61 by the phenol-water procedure. On degradation with dilute acetic acid aiming at splitting off lipid A from the carbohydrate moiety, LPS afforded no polysaccharide but a TS owing to cleavage of the O-polysaccharide chain at an acid-labile glycosidic linkage of 8-epilegionamic acid (8eLeg, see below). Therefore, LPS was treated with ammonia under mild conditions to afford an O-deacylated LPS. Mild acid degradation of the latter resulted in an O-deacetylated TS. All products were isolated by gel-permeation chromatography on TSK HW-40.

Sugar analysis of the O-deacylated LPS using GLC of the alditol acetates derived after full acid hydrolysis revealed Glc and GalN. GLC of the acetylated glycosides with (*S*)-2-octanol indicated that both monosaccharides have the D configuration.

The ^{13}C NMR spectra (Fig. 1) showed that all isolated compounds consist of three different monosaccharides. The spectrum of the O-deacylated LPS (Fig. 1A; Table 1) contained major signals for three anomeric carbons at δ 94.6, 101.9 (quaternary carbon as shown by a DEPT experiment; C-2 of 8eLeg), and 105.8; three nitrogen-bearing carbons at δ 49.4–55.0; one C-CH$_2$-C group (C-3 of 8eLeg) at δ 37.6; one CH$_3$-C group (C-9 of 8eLeg) at δ 20.2; two HOCH$_2$-C groups (C-6 of Glc and GalN) at δ 61.3 and 62.2; 10 oxygen-bearing sugar carbons in the region δ 69.5–79.2; three *N*-acetyl groups at δ 23.3–23.7 (Me); and a number of CO groups at δ 175.0–175.9. The ^{1}H NMR spectrum of the O-deacylated LPS showed, inter alia, signals for two anomeric protons at δ 4.45–5.01, one C-CH$_2$-C group (H-3 of 8eLeg) at δ 1.63 and 2.78, one CH$_3$-C group (H-9 of 8eLeg) at δ 1.22, and three *N*-acetyl groups at δ 1.95–2.03. Therefore, all amino groups of the amino sugars are *N*-acetylated.

The ^{13}C NMR spectra of TS and O-deacetylated TS (Fig. 1B, C) contained signals for the same sugars and *N*-acetyl groups (Table 1), the major difference being observed between the chemical shifts for C-2 of 8eLeg (δ 96.9–97.1 for the reducing 8eLeg residue in the trisaccharides vs. δ 101.9 for the linked 8eLeg residue in the O-deacylated LPS). The ^{13}C NMR spectrum of TS showed a signal for an *O*-acetyl group at δ 21.9 (Me), which was absent from the spectra of the O-deacylated LPS and O-deacetylated TS.

The ^{1}H and ^{13}C NMR signals of the isolated compounds were assigned using 2D ^{1}H,^{1}H COSY, TOCSY, ROESY, ^{1}H,^{13}C HSQC, and HMBC experiments (Table 1). Tracing connectivities in the COSY and TOCSY spectra and estimation of $^{3}J_{\mathrm{H,H}}$ coupling constants from the 2D NMR spectra enabled identification of spin systems for β-Glc*p*, α-Gal*p*N, and α-8eLeg*p*. The last sugar was identified based on the following data. The ^{13}C NMR spectrum contained

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Table 1: ^{1}H and ^{13}C NMR data of the O-polysaccharide in the O-deacylated LPS, O-deacetylated TS, and TS from *E. coli* O61 (δ, ppm; *J*, Hz).

Sugar residue	Nucleus	1	2	3 ($J_{3ax,4}$; $J_{3eq,3ax}$)	4 ($J_{3eq,4}$)	5 ($J_{4,5}$)	6 ($J_{5,6}$)	7 ($J_{6,7}$)	8 ($J_{7,8}$)	9 ($J_{8,9}$)
O-Polysaccharide[a]										
→4)-β-D-Glc*p*-(1→	^{1}H	4.45	3.27	3.51	3.98	3.41	3.76; 3.84			
	^{13}C	105.8	74.3	76.2	73.2	76.2	61.3			
→3)-α-D-Gal*p*NAc-(1→	^{1}H	5.01	3.34	3.70	4.17	3.72	3.69; 3.71			
	^{13}C	94.6	49.4	79.2	70.0	73.2	62.2			
→4)-α-8eLeg*p*5Ac7Ac-(2→	^{1}H			1.63ax(~ 10) 2.78eq(~ 10)	3.72 (<5)	3.72 (~10)	3.86 (~10)	3.91 (<2)	3.98 (5.0)	1.22 (5.5)
	^{13}C	n.d.	101.9	37.6	72.4	51.7	75.2	55.0	69.5	20.2
O-Deacylated TS[b]										
β-D-Glc*p*-(1→	^{1}H	4.44	3.27	3.48	3.41	3.43	3.74; 3.86			
	^{13}C	106.0	74.2	77.0	70.8	77.2	61.9			
→3)-α-D-Gal*p*NAc-(1→	^{1}H	4.99	4.34	3.72	4.21	3.78	3.75; 3.77			
	^{13}C	94.4	49.4	79.2	70.1	72.5	62.3			
→4)-β-8eLeg*p*5Ac7Ac	^{1}H			1.71ax(13.0) 2.41eq(11.6)	3.98 (4.5)	3.82 (10.2)	4.18 (10.5)	3.92 (2.1)	3.92 (6.9)	1.17 (6.1)
	^{13}C	175.5	97.1	37.1	72.0	52.0	72.9	54.9	69.5	20.0
TS[c] (data for the O-acetylated trisaccharide)										
β-D-Glc*p*-(1→	^{1}H	4.44	3.27	3.47	3.40	3.43	3.74; 3.86			
	^{13}C	105.9	74.3	77.0	70.8	77.2	61.9			
→3)-α-D-Gal*p*NAc-(1→	^{1}H	4.98	4.34	3.72	4.21	3.79	3.75; 3.78			
	^{13}C	94.5	49.4	79.1	70.0	72.5	62.3			
→4)-β-8eLeg*p*5Ac7Ac8Ac	^{1}H			1.73ax(13.0) 2.40eq(12.5)	3.99 (5.0)	3.84 (11.0)	4.18 (11.0)	4.12 (<2)	5.09 (7.5)	1.24 (6.0)
	^{13}C	*n.d.*	96.9	37.2	71.7	51.9	72.0	53.4	72.8	17.8

The signals for NAc are at [a]δ_H 1.95–2.03; δ_C 23.3–23.7 (Me) and 175.0–175.9 (CO); [b]δ_H 1.98–2.02; δ_C 23.3–23.7 (Me) and 175.1–176.0 (CO); [c]δ_H 1.99 (Me); δ_C 23.0–23.6 (Me) and 175.1–176.0 (CO). The signals for OAc are at δ_H 2.00 and δ_C 21.9 (Me). n.d., not determined.

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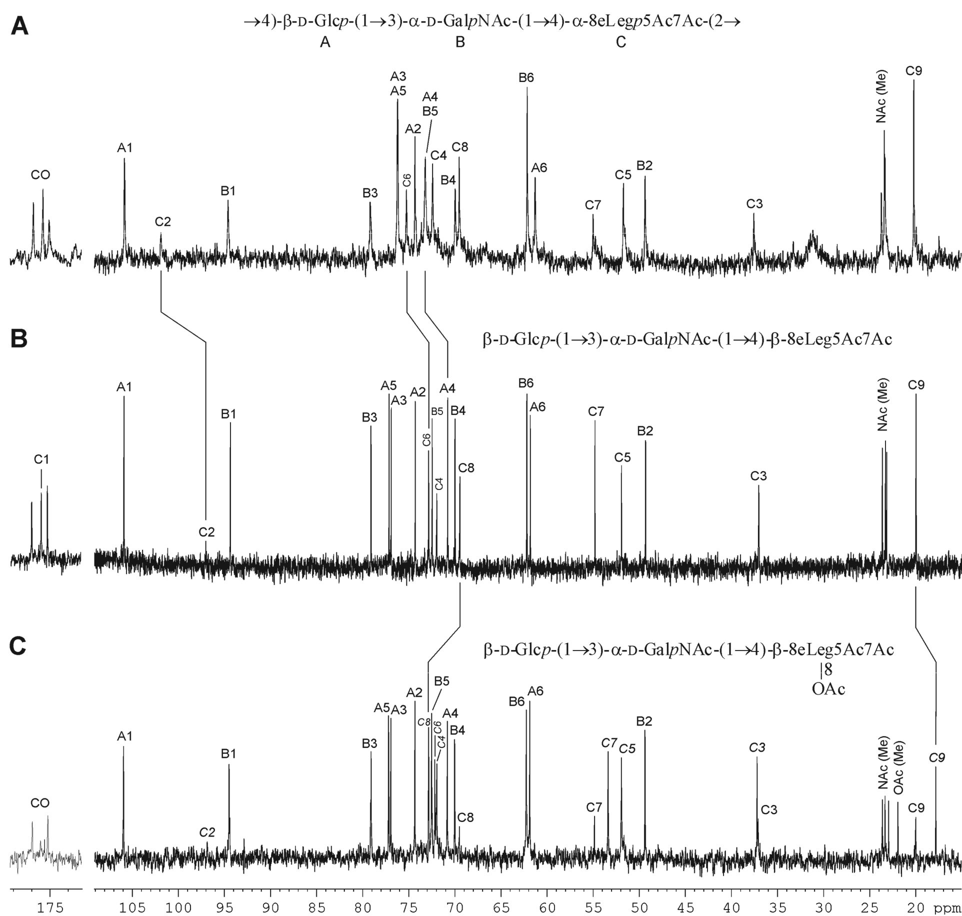

Figure 1: ^{13}C NMR spectra of the O-deacylated LPS (**A**), O-deacetylated TS (**B**), and TS (**C**) from *E. coli* O61. Arabic numerals refer to carbons in sugar residues denoted as follows: A, Glc; B, GalNAc; C, 8eLeg5Ac7Ac. Peak annotations for 8-O-acetylated 8eLeg5Ac7Ac are shown in italics. Structures of the compounds are shown in the insets.

characteristic signals for C-1–C-9 of a 5,7-diamino-3,5,7,9-tetradeoxynon-2-ulosonic acid, including those for keto group (C-2), deoxy units (C-3 and C-9), nitrogen-bearing carbons (C-5 and C-7), and oxygen-bearing carbons (C-4, C-6, and C-8). Relatively large $J_{3ax,4}$, $J_{4,5}$, and $J_{5,6}$ coupling constants of 10 to 11 Hz demonstrated the axial orientation of H-4, H-5, and H-6 and, hence, the *arabino* configuration of the C-4–C-6 fragment. Relatively small values of $J_{6,7}$ ~2 Hz and $J_{7,8}$ 5 to 7 Hz showed the *threo* configuration of each of the C-6–C-7 and C-7–C-8 fragments. Therefore, the nonulosonic acid has the L-*glycero*-D-*galacto* or D-*glycero*-L-*galacto* configuration (compare published data[17] of various isomers of 5,7-diamino-3,5,7,9-tetradeoxynon-2-ulosonic acids).

A relatively large difference (~1.15 ppm) between the chemical shifts of H-3ax and H-3eq of Leg in the ^{1}H NMR spectrum of the O-deacylated LPS is typical of 3-deoxyald-2-ulosonic acids with the axial position of the carboxyl group and thus indicated the α configuration of 8eLeg*p*. As other nonlinked nonulosonic acids of this class,[17] 8eLeg at the reducing end of the trisaccharides exists in the β-anomeric form with the equatorial carboxyl group, which is characterized by a smaller difference between the H-3ax and H-3eq chemical shifts (0.67–0.70 ppm). Both relative and anomeric configurations of 8eLeg were confirmed by the C-6 chemical shift of δ 75.2 for α-8eLeg in the O-deacylated LPS and δ 72.9 for β-8eLeg in the O-deacetylated TS (Fig. 1A, B; compare published data[17]).

The modes of substitution of sugar residues were inferred by downfield displacements of the signals for C-4 of 8eLeg, C-4 of Glc, and C-3 of GalN by 3.5, 3.2, and 10.5 ppm, respectively, in the ^{13}C NMR spectrum of the O-deacylated LPS, as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides.[17–19] A 2D ROESY experiment with the O-deacylated LPS revealed strong interresidue cross-peaks at δ 2.78/3.98, 4.45/3.70, and 5.01/3.72, which were assigned to 8eLeg H-3eq/Glc H-4, Glc H-1/GalN H-3, and GalN H-1/8eLeg H-4 correlations, respectively. The sequence of the monosaccharides thus established was confirmed by 8eLeg C-2/Glc H-4, Glc H-1/GalN C-3, and GalN H-1/8eLeg C-4 correlations at δ 101.9/3.98, 4.45/79.2, and 5.01/72.4, respectively, which were observed in the ^{1}H,^{13}C HMBC spectrum of the O-deacylated LPS.

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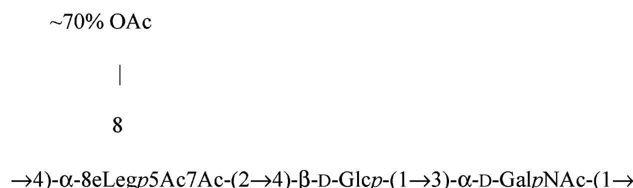
A comparison of the ^{13}C NMR chemical shifts of the O-deacetylated TS (Table 1) and free 8eLeg5Ac7Ac[17] revealed a relatively small α-effect on C-4 (3.7 ppm) and a relatively large negative β-effect on C-3 (–3.3 ppm) caused by glycosylation of 8eLeg5Ac7Ac with α-D-Gal*p*NAc at position 4 in the O-deacetylated TS. These values are characteristic for the L configuration of C-6[17] and, hence, the L-*glycero*-D-*galacto* configuration of 8eLeg. Essentially the same effects on 8eLeg5Ac7Ac caused by glycosylation with α-D-Glc*p*NAc at position 4 were observed in an oligosaccharide from LPS of *E. coli* O108.[11]

The ^{13}C NMR spectrum of TS (Fig. 1C) contained two series of signals, which indicated a nonstoichiometric O-acetylation. The minor series resembled much the spectrum of the O-deacetylated TS, whereas in the major series the signal for C-9 of 8eLeg was shifted upfield by 2.2 ppm (Fig. 1B, C), evidently as a result of O-acetylation of this sugar at position 8 (β-effect of O-acetylation[20]). The position of the *O*-acetyl group was confirmed by an ^{1}H,^{13}C HSQC experiment, which showed a downfield displacement in both dimensions of ~70% 8eLeg H-8/C-8 cross-peak from δ 3.92/69.5 in the O-deacetylated TS to 5.09/72.8 in TS.

Therefore, the isolated products have the structures shown in the insets of Figure 1 and, hence, the O-polysaccharide repeating unit of *E. coli* O61 has the following structure:



After the O-polysaccharide of *E. coli* O108,[11] this is the second *E. coli* O-polysaccharide that contains di-*N*-acetyl-8-epilegionaminic acid. One more monosaccharide of this class, namely, di-*N*-acetylpseudaminic acid with the L-*glycero*-L-*manno* configuration, has been identified as a component of the O-polysaccharide of *E. coli* O136.[21]

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