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Structural characterization of the antigenic O-chain of the lipopolysaccharide of *Escherichia coli* serotype O65

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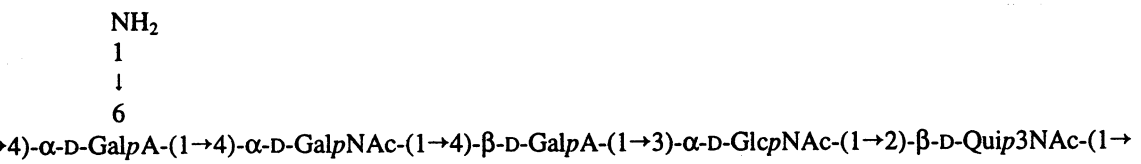
Abstract

The antigenic O-polysaccharide moiety of the lipopolysaccharide produced by *Escherichia coli* serotype O65 was investigated by composition, methylation, base hydrolysis, periodate oxidation, mass spectrometric methods, and by 1D and 2D NMR spectroscopy. The O-polysaccharide had $[\alpha]_D + 108°$ (water) and is a high-molecular-weight unbranched linear polymer of repeating pentasaccharide units composed of 1:1:1:1:1 D-galacturonic acid (D-GalA), D-galacturonamide (D-GalANH_2), 2-acetamido-2-deoxy-D-glucose (D-GlcNAc), 2-acetamido-2-deoxy-D-galactose (D-GalNAc), and 3-acetamido-3,6-dideoxy-D-glucose (D-Qui3NAc), and has the following structure:



Keywords: Lipopolysaccharide; *Escherichia coli*; Polysaccharide structure; NMR spectroscopy

1. Introduction

Lipopolysaccharides (LPSs) of *Escherichia coli* are found on the outer membrane of the bacterium and are, in general, made up of three regions: a hydrophobic lipid A, a core oligosaccharide, and a high-molecular-weight polysaccharide (O-PS) usually composed of repeating oligosaccharide units. The O-antigenic polysaccharide moieties, of which in *E. coli* strains there are some 179 chemically different structures, are related to pathogenicity and to an O-antigen serological typing system [1–3]. Knowledge of the bacterial O-PS structures provides a basis, on the molecular level, for the identification and understanding of the antigenic factors involved in their serological specificities, immunobiological properties, and role in host pathogenesis. In 50 or more cases, the structure of the O-PS of *E. coli* serovars has been determined [3]. In the present study the structure of the serotype O65 antigen of *E. coli* was elucidated and is recorded herein.

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2. Materials and methods

Bacterial cell growth and O-PS production.—*E. coli* O65 (NRCC 4926) was grown on brain–heart infusion (Difco) at 37 °C in a New Brunswick 25-L fermenter (yield 19 g wet weight/L). The saline-washed cells were extracted by stirring with 50% aqueous phenol for 12 min at 65 °C, and after cooling to 4 °C the aqueous phase was dialyzed against running tap water until free from phenol. The dialyzate was lyophilized, and its aqueous solution was incubated (37 °C, 4 h) sequentially with deoxyribonuclease, ribonuclease, and protease K and was then subjected to ultracentrifugation at 105,000*g* for 12 h at 4 °C. The precipitated gel was dissolved in water and lyophilized (yield 4.8 g).

A solution of the LPS (2%) was hydrolyzed with 3% (v/v) acetic acid at 100 °C for 2 h. Precipitated lipid A was removed by low-speed centrifugation, and the lyophilized water-soluble products were dissolved in 0.05 M pyridinium acetate buffer (pH 4.6) and subjected to Sephadex G-50 column (3.5 × 100 cm) chromatography using the same elution buffer. Collected fractions (10 mL) of the eluate were monitored by refractive index and colorimetrically for neutral glycose [4], 2-amino-2-deoxyhexose [5], 3-deoxy-D-*manno*-octulosonic acid [6], and hexuronic acid [7].

Chromatography.—Gas–liquid chromatography (GLC) was done with a Hewlett–Packard 5890A gas chromatograph fitted with a hydrogen flame detector and a DB-17 fused-silica capillary column (0.25 mm × 30 m). Temperature programs (A) 180 (delay 2 min) to 240 °C at 2 °C/min for alditol acetate derivatives, (B) 200 (delay 2 min) to 240 °C at 1 °C/min for methylated alditol acetate derivatives and (C) isothermal at 225 °C for butyl glycoside derivatives were used. Retention times are quoted relative to hexa-*O*-acetyl-D-glucitol (t_{GA}) or 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (t_{GM}). GLC–mass spectrometry (GLC–MS) was carried out under the same conditions using a Hewlett–Packard 5985B GC–MS system and an ionization potential of 70 eV. In all cases reference samples were used to confirm identifications.

Paper chromatography (PC) was done on water-washed Whatman No 1 filter paper using (A) 10:3:3 1-butanol–pyridine–water for neutral sugars, and (B) 4:1:5 1-butanol–ethanol–water (top layer) as the mobile phase for aminoglycoses. Detections were made using periodate–alkaline silver nitrate, *p*-anisidine·HCl, and ninhydrin spray reagents. Mobilities are quoted relative to D-galactose (R_{Gal}).

DOC-PAGE was run on separating gels formed with 14% acrylamide and 0.9% sodium deoxycholate [8], and LPS bands were detected by silver staining [9].

Analytical methods.—PS hydrolyses were carried out with 4 M trifluoroacetic acid (105 °C, 4 h) or by treatment with HF (20 °C, 4 h), followed by removal of HF in a nitrogen stream and deionization by passage through Rexyn 101 (H^+) and Amberlite IR-45 (OH^-) ion-exchange resins. Free aminodeoxyglycoses were obtained on further treatment of the monosaccharide products with 2 M HCl (110 °C, 1 h), followed by removal of the HCl in vacuo over NaOH. Aminodeoxyglycoses were recovered from Rexyn 101 (H^+) ion-exchange resin by elution with 0.5 M HCl and concentration.

Methylation analyses were carried out using methylsulfinylmethanide and iodomethane in dimethyl sulfoxide [10]. Products were purified by dialysis against water and then hydrolyzed, reduced ($NaBD_4$), and acetylated with acetic anhydride (105 °C, 3 h).

Periodate oxidation [11] was conducted on O-PS (100 mg) in water (18 mL) containing sodium metaperiodate (300 mg) for 48 h at 20 °C. Excess periodate was destroyed by the addition of ethylene glycol (0.5 mL), and the dialyzed, oxidized product was reduced by the addition of sodium borohydride (200 mg). After 12 h, the excess borohydride was destroyed by neutralization with acetic acid, and the product was dialyzed, then lyophilized.

Specific optical rotations were determined at 20 °C in water using 10-cm microtubes and a Perkin–Elmer 243 polarimeter.

Nuclear magnetic resonance.—NMR spectra were obtained with Bruker AMX 500 and AMX 600 spectrometers using standard Bruker software. All measurements were made

on solutions at 32 °C. Carbohydrate samples were exchanged twice with D_2O and then run in 99.9% D_2O.

The following NMR measurement parameters were recorded. Proton spectra were obtained using a spectral width of 10.6 kHz and a 90° pulse. Broad-band proton-decoupled ^{13}C NMR spectra were obtained using a spectral width of 45.4 kHz, a 90° pulse, and WALTZ decoupling [12]. Acetone was used as the internal standard, and chemical shifts are referenced to the methyl resonances (^1H, 2.225 ppm and ^{13}C, 31.07 ppm, respectively, downfield from Me_4Si). Two-dimensional homonuclear proton experiments (COSY) [13] were measured over a spectral width of 2.5 or 1.4 kHz, using data sets ($t_1 \times t_2$) of 256×2048 or 512×2048 points, and 64 or 32 scans were acquired, respectively. Spectra were processed in the magnitude mode with symmeterization about the diagonal. Two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) experiments [14] were made in a phase-sensitive manner over a spectral width of 2.5 kHz using a data set of 256×2048 points and a mixing time of 150 or 700 ms, and 64 scans for each t_1 value. 2D ROESY [15] had a spin lock value of 300 ms. For 600-MHz 1D z-filtered TOCSY spectra [16], a mixing time of 20–80 ms and a 270° Gaussian pulse with 512 scans collected for each spectrum were used. Heteronuclear 2D $^1H-^{13}C$ chemical shift correlations were measured in the ^1H-detected mode via multiple quantum coherence (HMQC) [17] with proton decoupling in the ^{13}C domain using data sets of 2048×256 points and spectra widths of 5.1 and 15.5 kHz for the ^1H and ^{13}C domains, respectively. For each t_1 value 128 scans were acquired. An HMQC experiment acquired without proton decoupling was employed for determination of one-bond $^1H-^{13}C$ couplings ($^1J_{C,H}$) values.

3. Results and discussion

Fermenter-grown cells of *E. coli* serotype O65 (NRCC 4926) were extracted by a modified aqueous phenol extraction procedure [18], and the LPS (10% yield based on dry weight of cells) was obtained as a precipitated gel on ultracentrifugation of the dialyzed concentrated aqueous phase. DOC-PAGE [8] of the LPS gave a ladder banding separation pattern indicating the major component to be a smooth-type LPS having a polysaccharide O-chain of a high narrow molecular-weight range and spacing indicative of its being composed of repeating pentasaccharide units.

Mild acetic acid hydrolysis of the LPS afforded an insoluble lipid A (19%), and Sephadex G-50 column chromatography of the concentrated water-soluble product gave an O-polysaccharide (O-PS, K_{av} 0.03, 61%), a core oligosaccharide (K_{av} 0.40, 2%), and a fraction (K_{av} 0.93, 16%) containing Kdo and phosphate.

The O-PS fraction from colorimetric analysis monitoring was indicated to be composed of aldose, aminodeoxyglycose and hexuronic acid and had $[\alpha]_D$ +108° (*c* 0.3, water). Anal. Found: C, 40.75; H, 5.53; N, 4.39%, and ash nil.

The 1D ^1H NMR (Fig. 1(A)) and ^{13}C NMR (Fig. 2(A)) spectra of the O-PS, considered in conjunction with the composition data, gave an initial indication that the O-antigen was formed from repeating pentasaccharide units. The spectra revealed five distinct anomeric proton signals (5.73–4.43 ppm) having $J_{1,2}$ values consistent with the presence of three α and two β anomeric linkages and five anomeric carbon signals (104.43–95.50) having $J_{C\text{-}1\text{–}H\text{-}1}$ coupling constants also indicative of three α and two β glycosidic linkages. In addition there were five carbonyl signals (171.48–75.45 ppm) consistent with the occurrence of three $NHCOCH_3$ and two uronic acid CO_2H functions, three methyl proton signals (2.05–2.00 ppm, 9 H) and three methyl carbon signals (23.18–22.97 ppm) characteristic of three *N*-acetyl functions, one proton signal (1.27 ppm, 3 H), and a single carbon signal (17.90 ppm) characteristic of a methyl resonance arising from a 6-deoxyhexose residue, and three carbon signals (56.44–50.50 ppm) arising from the acetamidodeoxy-substituted carbon atoms of the three aminodeoxyglycose O-PS components. During the NMR analyses of the O-PS under a change of pD conditions, one hexuronic acid residue ap-

peared to be present as a subsequently identified amide derivative, since the chemical shift of its carbonyl resonance was not influenced, whereas the H-5 proton signal and carbonyl signal of the second hexuronic acid residue showed characteristic shifts (H-5**e**, 4.46–4.16 ppm and C-6**e** 173.58–171.48 ppm with change of pD 2.8–7.0) indicative of an unsubstituted free C-6 carboxyl function [19].

GLC–MS (program A) analysis of the reduced ($NaBD_4$) and acetylated hydrolysis products of O-PS gave derivatives identified as 1,2,4,5-tetra-*O*-acetyl-3-acetamido-3,6-dideoxyglucitol-1*d* (t_{GA} 0.88) 1,3,4,5,6-penta-*O*-acetyl-2-acetamido-2-deoxyglucitol-1*d* (t_{GA} 1.28), and 1,3,4,5,6-penta-*O*-acetyl-2-acetamido-2-deoxygalactitol-1*d* (t_{GA} 1.32) in approximately equal molar ratios. A similar analysis was made on the reduced ($NaBD_4$) methanolysis product of O-PS that was subsequently subjected to acid hydrolysis and further reduction ($NaBD_4$) and acetylation. GLC–MS analysis revealed the above previously identified aminoglycitol and, in addition, a peak identified as hexa-*O*-acetylgalactitol-1*d*,6d_2 (t_{GA} 1.01). The latter galactitol derivative was tentatively considered to arise from a galacturonic acid component of the O-PS.

Following hydrolysis of the O-PS (60 mg) with TFA, the products were separated by preparative paper chromatography (solvent B) and collected into two ninhydrin-positive fractions (R_{Gal} 0.76 and 1.22). The fast-moving fraction gave an *N*-acetyl derivative (R_{Gal} 2.44, solvent A), which had $[\alpha]_D$ +26° (*c* 0.12, water) (lit. $[\alpha]_D$ +26° (water) [20]) and was characterized as 3-acetamido-3,6-dideoxy-D-glucose, since it gave a ^{1}H NMR spectrum indistinguishable from that of an authentic derivative [20] and the alditol acetate derivative characterized by GLC–MS (see above). The slower-mobility PC fraction that had $[\alpha]_D$ +75° (*c* 0.6, water) was shown by GLC–MS (program C) of its acetylated (+)-2-butyl glycoside derivatives to be composed of 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose (1:1). The neutral glycose obtained on hydrolysis (TFA) of the reduction ($NaBH_4$) products from the methanolysis of O-PS was isolated by PC (R_{Gal} 1.00, solvent A) and characterized as D-galactose since it (1) had $[\alpha]_D$ +78° (*c* 0.5, water); (2) on reduction and acetylation gave hexa-*O*-acetylgalactitol having mp and mmp = 166 °C, and (3) gave acetylated (+)-2-butyl glycosides with GLC–MS (program C) behavior identical to those afforded by a reference sample. It can thus be

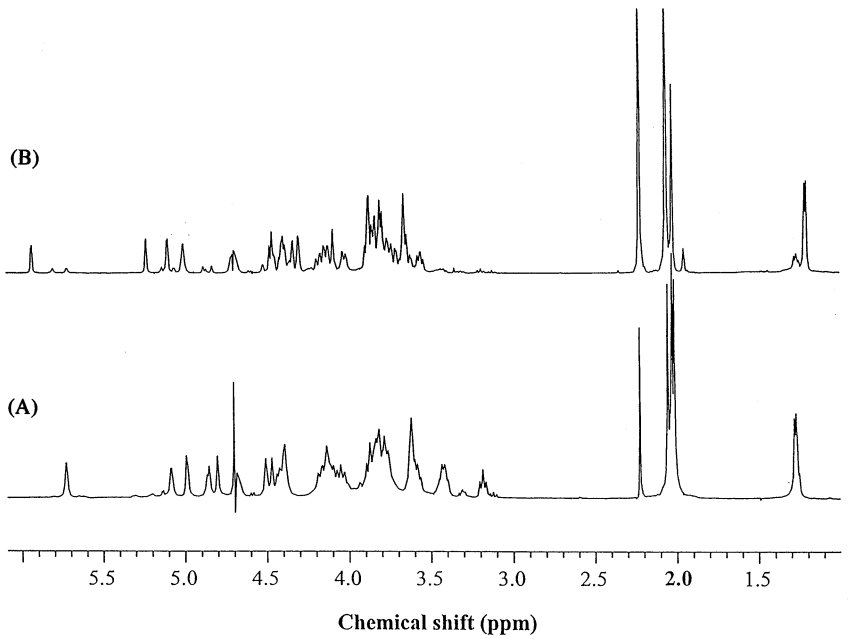

Fig. 1. ^{1}H NMR spectra of (A) native *E. coli* O65 O-PS and (B) oligosaccharide alkaline degradation product from *E. coli* O65 O-PS.

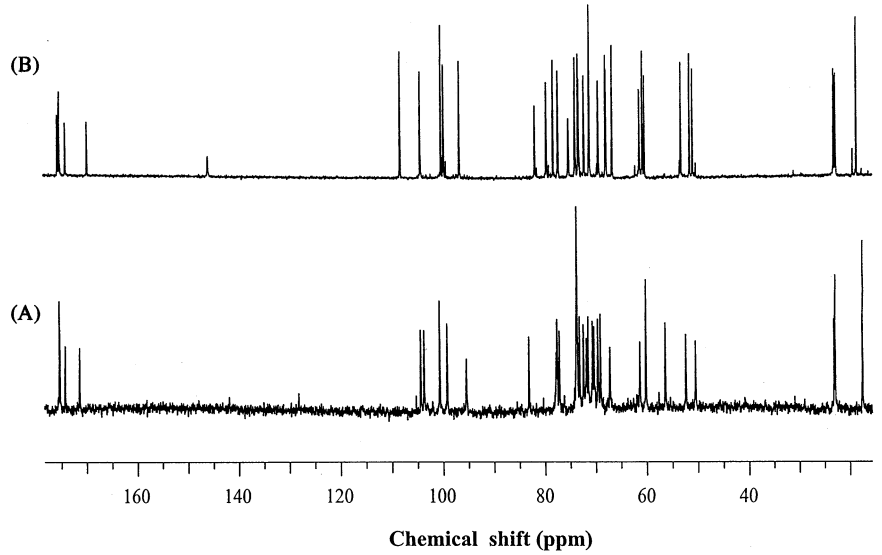

Fig. 2. ^{1}C NMR spectra of (A) native *E. coli* O65 O-PS and (B) oligosaccharide alkaline degradation product from *E. coli* O65 O-PS.

concluded that the D-galactose is derived from D-galacturonic acid. The accumulated composition and NMR evidence is consistent with the O-PS being composed of repeating pentasaccharide units composed of 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, 3-acetamido-3,6-dideoxy-D-glucose, and D-galacturonic acid in the molar ratio of 1:1:1:2.

The methylation analysis of the O-PS involving the GLC–MS (program B) of the derived alditol acetates of carboxyl reduced ($NaBD_4$) hydrolysis products showed four peaks identified as 1,2,5-tri-*O*-acetyl-3,6-dideoxy-4-*O*-methyl-3-(*N*-methylacetamido)-D-glucitol-1*d* (t_{GM} 2.17), 1,4,5-tri-*O*-acetyl-2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)-D-galactitol-1*d* (t_{GM} 3.11), 1,3,5,tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido) - D - glucitol - 1*d* (t_{GM} 3.25), and 1.4,5,6 - tetra - *O* - acetyl - 2,3 - di - *O* - methyl - D-galactitol-1*d*,6d_2 (t_{GM} 1.62). The analysis indicated that the interglycosidic residues →2)-D-Qui*p*3NAc-(1→, →4)-D-Gal*p*NAc-(1→, →3) - D - Glc*p*NAc - (1→, and →4)-Gal*p*A-(1→ are present in the O-PS and that it is a linear, unbranched polymer.

The complete structural analysis of the O-PS was made through the application of 1D and 2D ^{1}H and ^{13}C NMR spectroscopic methods. A 2D homonuclear proton correlation (COSY, Fig. 3) and a TOCSY experiment allowed complete assignment of all the proton signals (Table 1), and 1D *z*-filtered TOCSY experiments allowed complete $J_{H,H}$ coupling constants to be determined. The anomeric proton signals were arbitrarily labeled **a**–**e** in order of decreasing chemical shifts. Residues **b**, **c**, and **e** were assigned to glycoses having the D-*galacto* configuration since they showed characteristically large $J_{2,3}$ ($\sim$10 Hz) and small $J_{3,4}$ ($\sim$3–4 Hz) coupling constants. Residue **c** was identified as an α-D-Gal*p*NAc residue from consideration of its small $J_{1,2}$ (2.7 Hz) and corresponding $J_{C\text{-}1\text{–}H\text{-}1}$ (175 Hz) coupling constant and direct correlation of H-2**c** (4.18 ppm) to the C-2**c** (50.55 ppm) via HMQC (Fig. 4, Table 2), which had the characteristic chemical shift of an acetamido substituent. Residue **b** was identified as α-D-Gal*p*ANH_2 from its small $J_{1,2}$ (3.2 Hz), with a downfield shift of H-5**b** (4.80 ppm) resulting from deshielding by the amide-substituted carboxyl C-6 atom. ^{1}H NMR experiments in water (90%) confirmed the presence of a hexuronamide function giving a characteristic NH_2 resonance at 8.47 ppm. Correlation of the H-5**b** resonance (4.802 ppm) to the C-6**b** resonance (174.40 ppm) in an HMBC experiment (not shown) confirmed the identity of the D-Gal*p*ANH_2 residue. From its characteristic large $J_{1,2}$ (7.6 Hz) proton and $J_{C\text{-}1\text{–}H\text{-}1}$ coupling constants (162 Hz), residue **e** was

identified as β-D-Gal*p*A with C-6**e** (171.48 ppm) correlated to the H-5**e** (4.460 ppm) in an HMBC experiment. A change of pD of the solution of the O-PS from pD 3 to 7 showed a shift of H-5 of the free hexuronic acid residue **e** from 4.46 to 4.16 ppm, thus confirming the free carboxylic acid function present in residue **e**. In contrast, the resonance of the H-5 of the D-galacturonic acid (**b**) residue remained at 4.802 ppm under the same change of pD, a result consistent with the presence of a uronic acid amide derivative. Residues **a** and **d** were assigned the D-*gluco* configuration from consideration of their characteristic large $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ (9–10 Hz) coupling constants. Residue **d** was identified from its large $J_{1,2}$ (6.6 Hz) and corresponding $J_{\text{C-1–H-1}}$ coupling (166 Hz), and by direct correlation of the H-3**d** (3.869 ppm) to the acetamido carbon resonance C-3**d** (56.45 ppm) in a HMQC experiment, thus establishing **d** as a 3-acetamido-3,6-dideoxy-β-D-glucopyranose (D-Qui*p*3NAc) residue. Since H-4**a** and H-5**a** exhibited the same chemical shift (3.620 ppm), only the $J_{3,4}$ is reported from 1D NOE and TOSCY experiments. A HMQC experiment revealed a direct correlation between H-2**a** (4.11 ppm) and the corresponding C-2**a** (52.44 ppm) resonance, having a chemical shift characteristic of a carbon atom bearing a acetamidodeoxy function, and the magnitude of the $J_{1,2}$ (2.3 Hz) and corresponding $J_{\text{C-1–H-1}}$ (175 Hz) coupling constants, characterized it as a 2-acetamido-2-deoxy-α-D-glucopyranosyl residue.

The sequence and linkage positions of the glycose residues in the O-PS were established from observed transglycoside NOE connectivities relating the anomeric and aglyconic protons in adjacent glycosyl residues. A strong NOE between H-1**a** (α-D-Glc*p*NAc) to H-2**d** (D-Qui*p*3NAc) and to its own H-2**a** confirmed

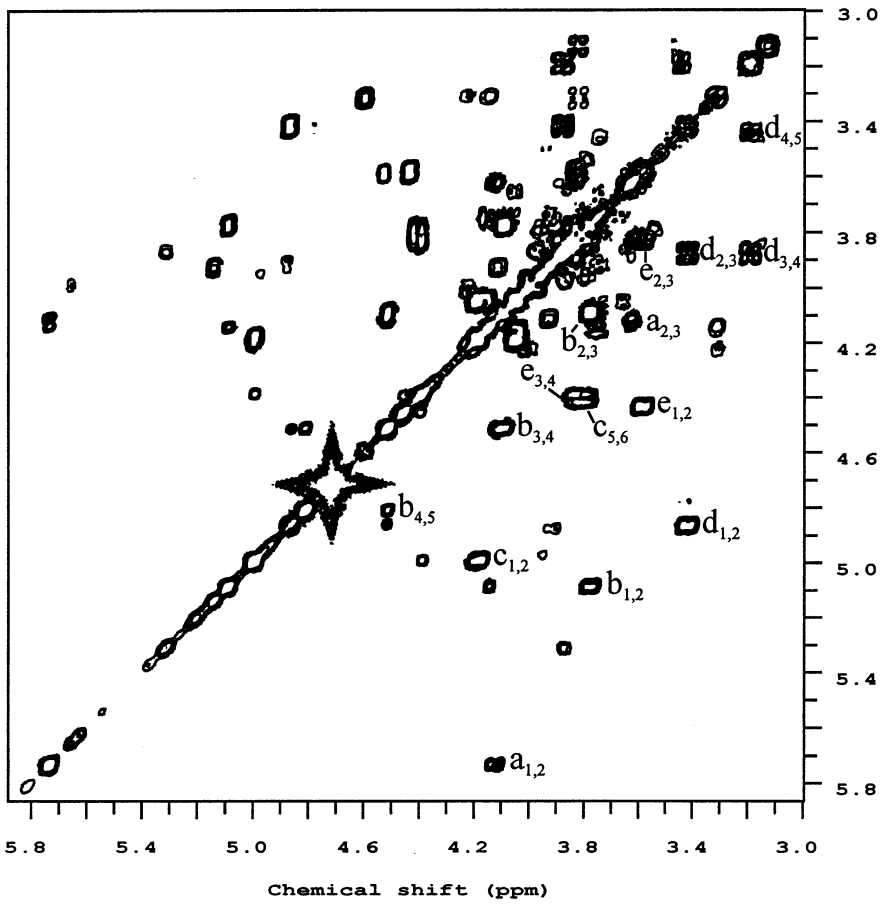

Fig. 3. Contour plot obtained by gradient COSY, using an Inova 500 spectrometer and Varian software, to give a spectrum (5.9–3.0 ppm) of *E. coli* O65 O-PS showing cross-peak assignments. Cross-peaks relating H-5**a** and H-6**a** are not seen at this level.

Table 1
^{1}H NMR chemical shifts (ppm) and coupling constants (Hz) for the O-PS of *E. coli* O65 LPS [a]

Residue	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5	H-6 ($J_{5,6}$)	H-6′ ($J_{5,6'}$)
a →3)-α-D-Glc*p*NAc-(1→	5.724 (2.3)	4.111(10.2)	3.618(~9)	3.620	3.620	3.920	3.851
b →4)-α-D-Gal*p*ANH$_2$-(1→	5.083(3.2)	3.767(10.2)	4.081(3.8)	4.508(~1)	4.802	-	-
c →4)-α-D-Gal*p*NAc-(1→	4.990(2.7)	4.180(10.0)	4.040(3.2)	4.150(~1)	4.395	3.842	3.822
d →2)-β-D-Qui*p*3NAc-(1→	4.850(6.6)	3.409(9.8)	3.869(8.9)	3.183(10.2)	3.438	1.269(5.6)	
e →4)-β-D-Gal*p*A-(1→	4.431(7.6)	3.579(10.2)	3.825(3.4)	4.410(~1)	4.460	-	-

[a] Measured at 32 °C and pD 2.8.

an O-PS structural unit α-D-Glc*p*NAc-(1→2)-β-D-Qui*p*3NAc-(1→. A strong NOE between H-1**d** and H-4**b** and the intraresidue enhancements to its own H-3**d**, H-4**d**, and H-5**d** proton signals established a β-D-(1→4)-linkage to **b**, thus identifying a trisaccharide α-D-Glc*p*-NAc-(1→2)-β-D-Qui*p*3NAc-(1→4)-α-D-Gal*p*ANH$_2$-(1→ as an O-PS structural component. NOEs between H-1**b** (α-D-Gal*p*ANH$_2$) and H-4**c** (α-D-Gal*p*NAc), and to its own H-2**b** confirmed an α-D-(1→4)-linkage of residues **b**–**c**, thus characterizing an O-PS tetrasaccharide component having the structure α-D-Glc*p*NAc-(1→2)-β-D-Qui*p*3NAc-(1→4)-α-D-Gal*p*ANH$_2$-(1→4)-α-D-Gal*p*NAc-(1→. Residue **c** (α-D-Gal*p*NAc) showed a strong NOE to its own H-2**c** and an inter-residue NOE to H-4**e** (β-D-Gal*p*A). A strong inter-residue NOE between H-1**e** across the ring to H-3**a** and within the ring to H-3**e**, H-4**e** and H-5**e** confirmed the β-D-(1→3)-linkage of **e** to the α-D-Glc*p*NAc residue **a**. The observed connectivities considered in conjunction with the HMQC–NOESY and HMQC–TOCSY experiments (not shown) establish an unbranched linear sequence of the glycose residues in the O-PS as –**b**–**c**–**e**–**a**–**d**, as shown:

→4)-α-D-G$^{(b)}$al*p*ANH$_2$-(1→4)-α-D-G$^{(c)}$al*p*-NAc-(1→4)-β-D-G$^{(e)}$al*p*A-(1→3)-α-D-Gl$^{(a)}$c*p*-NAc-(1→2)-β-D-Qui$^{(d)}$*p*3NAc-(1→

Treatment of the O-PS with dilute sodium hydroxide (0.1 M, 37 °C, 12 h) or with aqueous barium hydroxide (1.6 M, 50 °C, 40 min) caused specific cleavage of the 1→4 glycosidic linkage to the β-D-galactouronamide residue **b** via a β-elimination reaction [21]. Following reduction ($NaBH_4$) of the alkali reaction product and Sephadex G-10 column chromatography, it afforded an essentially quantitative yield of a pentasaccharide (K_{av} 0.17) having $[\alpha]_D$ +132° (*c* 0.7, water) in which the degraded uronic acid residue **b** was converted to a L-*threo*-hex-4-enuronopyranosyl terminal end group and the oligosaccharide reducing terminal glycose (D-Qui*p*3NAc) present, after reduction ($NaBH_4$), was a 3-acetamido-3,6-dideoxy-D-glucitol end group. Complete assignments of ^{1}H NMR signals (Fig. 1(B), Table 3) were made, and assignments of ^{13}C NMR carbon resonances (Fig. 2(B), Table 4) were determined from direct correlations in an HMQC experiment and confirmed by 1D *z*-filtered TOCSY experiments. A change of pD of the ^{1}H NMR sample from pD 8.0 to 3.0 resulted in a chemical shift of the uronic acid H-5 signal from 4.09 to 4.32 ppm, confirming that the β-elimination product arises from the D-Gal*p*ANH$_2$ **b** residue. A ^{1}H NMR spectrum of the oligosaccharide in water (90%) showed the absence of an NH_2 proton chemical shift as seen from the uronic acid amide function in the native O-PS, but did show three NH proton signals (7.6–8.4 ppm) arising from the acetamido functions of the three residual glycoses in the oligosaccharide. Complementary NOESY and long-range heteronuclear NMR experiments confirmed the structure of the oligosaccharide as: β-L-*threo*-hex-enuronopyranosyl-(1→4)-α-D-Gal*p*NAc-(1→4)-β-D-Gal*p*A-(1→3)-α-D-Glc*p*NAc-(1→2)-D-Qui3NAcol. MALDI-TOF-MS in the positive-ion mode confirmed an expected mass ion *m*/*z* 948, consistent with the deduced structure of the oligosaccharide product.

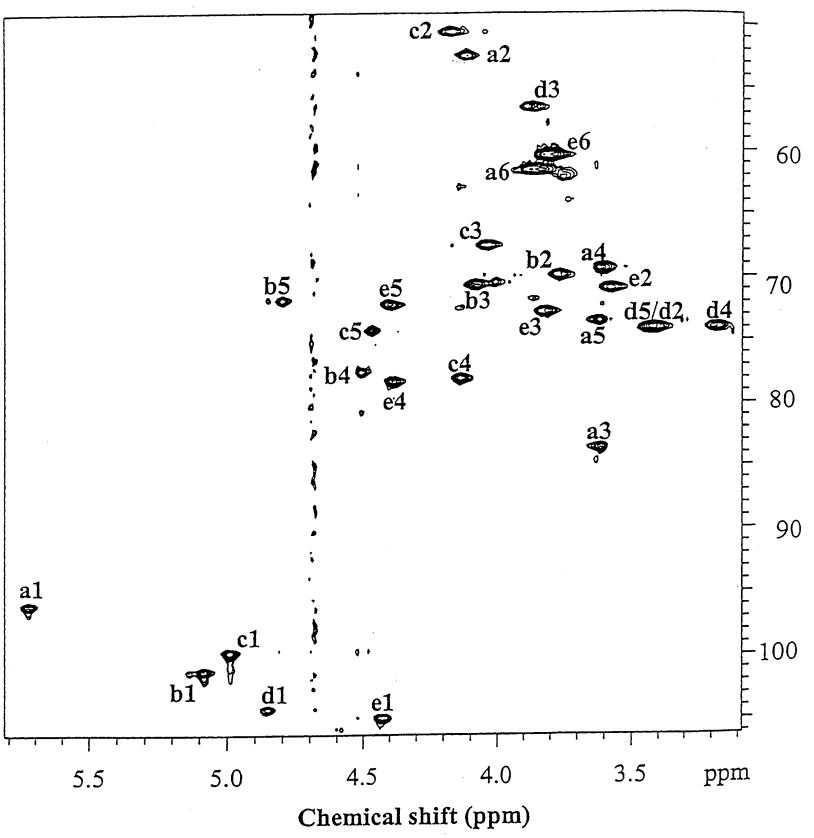

Fig. 4. Heteronuclear 2D $^{1}H-^{13}C$ NMR chemical shift correlation of the 5.8–3.1 ppm ^{1}H and 106–49 ppm ^{13}C region resonances of the *E. coli* O65 O-PS.

Table 2
^{13}C NMR chemical shifts (ppm) and coupling constants (Hz) for the O-PS of *E. coli* O65 LPS [a]

Residue	C-1	C-2	C-3	C-4	C-5	C-6
a →3)-α-D-Glc*p*NAc-(1→	95.52(175)	52.43	83.21	69.14	73.22	61.42
b →4)-α-D-Gal*p*ANH_2-(1→	100.71(175)	69.62	70.38	77.20	71.55	174.40
c →4)-α-D-Gal*p*NAc-(1→	99.24(175)	50.55	67.28	77.67	71.88	60.27
d →2)-β-D-Qui*p*3NAc-(1→	103.76(166)	73.73	56.44	73.72	73.73	17.55
e →4)-β-D-Gal*p*A-(1→	104.43(162)	70.67	72.47	77.86	74.22	171.48

[a] Measured at 32 °C and pD 2.8. Coupling constants ($J_{C\text{-}1\text{–}H\text{-}1}$) in Hz are in parentheses.

The unusually low chemical shift observed for the anomeric proton of the α-D-Glc*p*NAc residue **a** (5.724 ppm) in the ^{1}H NMR spectrum of the O-PS is tentatively ascribed to a deshielding effect due to its close relation with the C-3 acetamido function of the β-D-Qui*p*3NAc **d** residue brought about by the spatial conformation resulting from the 1,2-glycosidic linkage of **a**–**d**. This conclusion is supported by the fact that the anomeric proton signal of the α-D-Glc*p*NAc (5.097 ppm) residue is restored to its expected region of the spectrum of the O-PS oligosaccharide degradation product, in which the original residue **d** is present in its reduced alditol form.

Periodate oxidation of the O-PS followed by reduction ($NaBH_4$) afforded a polymeric product, which on hydrolysis and GLC analysis of derived alditol acetates showed GalNol, GlcNol, Qui3Nol, and threitol. The result is consistent with the proposed structure, indicating that, as expected, the 4-substituted D-Gal*p*A residues (**b** and **e**) were the only glycoses susceptible to periodate oxidative cleavage.

Table 3
^{1}H NMR chemical shifts (ppm) and coupling constants (Hz) for the oligosaccharide product from the alkaline depolymerization of *E. coli* O65 O-PS [a]

Residue	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5	H-6 ($J_{5,6}$)	H-6′ ($J_{5,6'}$)
β-L-*threo*-Hex-4-enurono*p*-(1→	5.229(2.1)	3.884(7.6)	4.448(3.3)	5.935(3.1)			
→3)-α-D-Glc*p*NAc-(1→	5.097(3.2)	4.131(9.4)	3.831(9.4)	3.635(9.4)	3.747	3.880	3.880
→4)-α-D-Gal*p*NAc-(1→	5.005(3.3)	4.176(8.0)	4.018(3.0)	4.331(2.7)	4.394(∼1)	4.124	
→4)-β-D-Gal*p*A-(1→	4.465(7.8)	3.552(9.8)	3.766(2.3)	4.297(2.0)	4.088	3.880(6.5)	
→2)-D-QuiNAcol	3.704(3.9)	3.837(6.5)	4.385(7.1)	3.652(1.2)	3.651(∼1)	1.215(5.7)	

[a] Measured at 32 °C and pD 8.0. Coupling constants ($J_{H,H}$) in Hz are in parentheses.

Table 4
^{13}C NMR chemical shifts (ppm) and coupling constants (Hz) for the oligosaccharide product from the alkaline depolymerization of *E. coli* O65 O-PS [a]

Residue	C-1	C-2	C-3	C-4	C-5	C-6
β-L-*threo*-Hex-4-enurono*p*-(1→	100.72(174)	70.93	66.34	109.22	143.47	167.20
→3)-α-D-Glc*p*NAc-(1→	96.61(174)	53.25	81.83	69.37	73.31	61.30
→4)-α-D-Gal*p*NAc-(1→	99.78(175)	50.94	67.85	77.54	75.55	60.53
→4)-β-D-Gal*p*A-(1→	104.26(164)	71.13	73.16	79.67	75.50	174.42
→2)-D-QuiNAcol	59.88	78.14	51.54	69.37	67.94	16.36

[a] Measured at 32 °C and pD 8.0.

A combination of methylation, periodate oxidation, and characterization of alkaline hydrolysis products afforded complementary evidence confirming the structure of the *E. coli* serotype O65 O-PS determined by 1D and 2D ^{1}H and ^{13}C NMR analyses.

Serological cross-reactions of *E. coli* serotypes O65, 05, 70, and O71 antisera and the respective O-antigens of the same serotypes [22] have been recorded. The identification of the epitopes responsible for the cross-reactivities has so far not been established. It is interesting to note that the recently characterized O-PS of *E. coli* O5 [23] shares a common D-Qui*p*3NAc residue with the O65 antigen. However, in the O65 O-PS this residue is glycosidically 2-substituted, in contrast to that in the O5 antigen in which the residue is 4-substituted, both residues having a β-D configuration. Initial studies on *E. coli* O70 and O71 LPSs indicated that the O-antigens contained 4-substituted β-D-Qui*p*3NAc residues (unpublished results), and these residues probably form part of a common epitope responsible for the above-reported serological serological cross-reactivities. The occurrence of the 3-acetamido-3,6-dideoxyhexose derivatives is not restricted to some *E. coli* serotypes but these glycoses have been identified in the O-antigens of the LPSs of *Escherichia*, *Pseudomonas*, *Proteus*, *Vibrio*, *Citrobacter*, and *Salmonella* species [3,24]. Further studies are required to define the role of the 3-aminohexoses in the epitopes responsible for serological reactivities between related Gram-negative bacteria.

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