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Structural studies of the enteroinvasive *Escherichia coli* (EIEC) O28 O-antigenic polysaccharide

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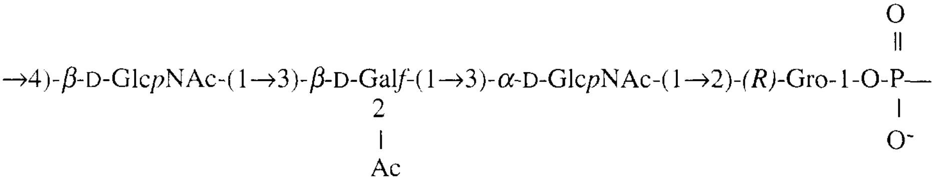
Abstract

The structure of the O-specific side-chain of the lipopolysaccharide from *Escherichia coli* O28 has been investigated. NMR spectroscopy has been the main method used, complemented with sugar and methylation analyses. The polysaccharide contains one equivalent of *O*-acetyl groups per repeating unit. Selective cleavage of the *O*-deacetylated polymer was performed by treatment with aqueous hydrofluoric acid, and resulted in a trisaccharide-glycerol. The polysaccharide thus is of the teichoic acid type and composed of repeating units in which the trisaccharide-glycerol residues are joined by phosphodiester linkages. The O-antigen polysaccharide has the following structure.



$$\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-}\underset{\substack{2\\ |\\ \text{Ac}}}{\text{Gal}f}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow 2)\text{-}(R)\text{-Gro-1-O-}\underset{\substack{|\\ \text{O}^-}}{\overset{\substack{\text{O}\\ \|}}{\text{P}}}\text{—}$$

The absolute configuration of the glycerol moiety as *R*, (i.e., D-glycerol 1-phosphate) was determined by a new method based on TEMPO oxidation of the polysaccharide, followed by GLC analysis of the (+)-2-butyl ester of the resulting glyceric acid. © 1996 Elsevier Science Ltd.

Keywords: *Escherichia coli*; Lipopolysaccharide; Phosphodiester; Teichoic acid type; Enteroinvasive

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1. Introduction

Enteroinvasive *Escherichia coli* (EIEC) are a group of bacteria causing an invasive, dysenteric form of diarrheal illness. The EIEC strains closely resemble *Shigella* in their capacity to invade and proliferate within epithelial cells and cause eventual death of the cell [1]. The invasive capacity of both EIEC and *Shigella* is dependent on the presence of a large (~ 140 MDa) plasmid coding for production of several outer membrane proteins involved in invasiveness [2]. These proteins are closely related antigenically in EIEC and *Shigella*. EIEC is not a taxonomic entity, rather a group represented by isolates restricted to certain serogroups and serotypes. The *E. coli* O28ac belongs to this group. It is worth noting that several EIEC serogroups are identical to, or cross-react with, certain *Shigella* serogroups [3]. O-Antigen identity or cross-reactivity was demonstrated between *E. coli* O124 and *Shigella dysenteriae* 3; between *E. coli* O112ac and *S. dysenteriae* 2, *S. boydii* 1, and *S boydii* 15; between *E. coli* O136 and *S. dysenteriae* 3, and *S. boydii* 1; and between *E. coli* O164 and *S. dysenteriae* 3. No relationship between the O-antigen O28ac and any of the examined *Shigella* was detected [3]. In the present communication we present the complete structure of the *E. coli* O28ac O-antigenic repeating unit. The remaining enteroinvasive *E. coli* O serogroups showed no significant antigenic relationships with any *Shigella* serovar [3].

2. Results and discussion

The lipopolysaccharide (LPS) from *E. coli* O28 was obtained by phenol–water extraction [4] of the killed bacteria and delipidated with acid under mild conditions to give the polysaccharide (O28 PS). Hydrolysis of the O28 PS with aqueous hydrochloric acid yielded glycerol, glucose, galactose, 2-amino-2-deoxyglucose, and heptose in the proportions 2:5:11:39:3. Some of the sugars in the acid hydrolysate are derived from the core of the LPS.

The ^{1}H and ^{13}C NMR spectra of the O28 PS (Figs. 1 and 2) showed, inter alia, the presence of three sugar residues in the repeating unit. The amino sugars are *N*-acetylated since signals for methyl groups of two *N*-acetyl groups were found at δ 23.0 and 23.2 in the ^{13}C NMR spectrum and at δ 2.05 and 2.08 in the ^{1}H NMR spectrum. *O*-Acetyl substitution of the polymer is evident from signals of the methyl group of an *O*-acetyl group at δ_C 21.0 and δ_H 2.14 (3 H). Thus, one equivalent of *O*-acetyl group per repeating unit is present.

Methylation analysis of the O28 PS revealed 2,3,4,6-tetra-*O*-methylglucose (3%), 2,3,4,6-tetra-*O*-methylgalactose (4%), 2,5,6-tri-*O*-methylgalactose (31%), 2,4,6-tri-*O*-methylglucose (13%), 3,4,6-tri-*O*-methylgalactose (5%), 4,6-di-*O*-methylglucose (11%), 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose (18%), and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)glucose (15%). Determination of the absolute configurations of the sugars was performed by a modification of the methods developed by Leontein et al. [5] and Gerwig et al. [6], using GLC of their acetylated (+)-2-butyl glycosides, and showed D-galactose and 2-acetamido-2-deoxy-D-glucose. From these results together with NMR data (vide infra) it is concluded that the sugar residues in the

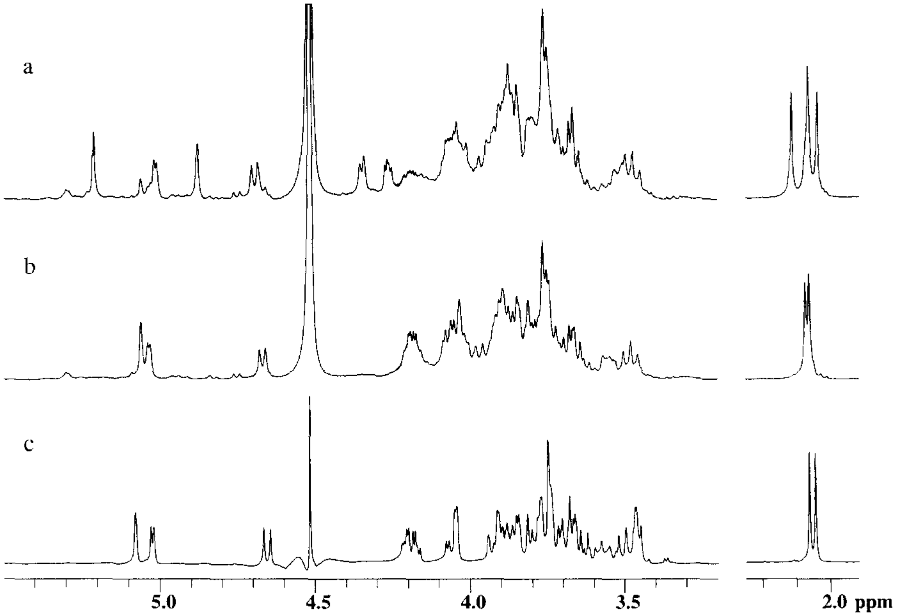


Fig. 1. The ^{1}H NMR spectra at 400 MHz of (a) the *E. coli* O28 O-polysaccharide, (b) the *O*-deacetylated PS, and (c) the trisaccharide-glycerol.

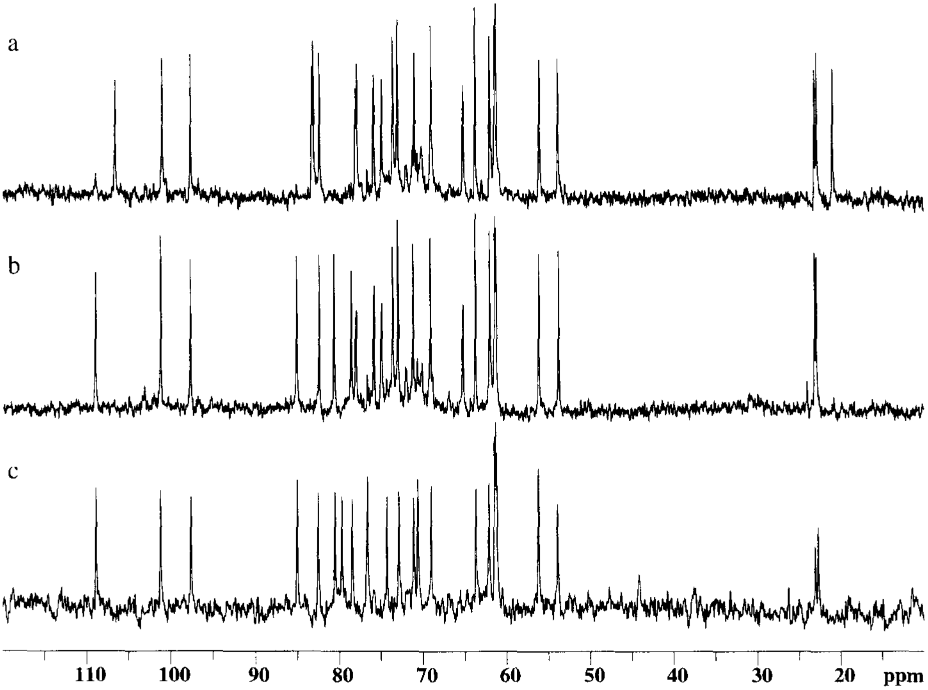


Fig. 2. The ^{13}C NMR spectra at 100 MHz of (a) the *E. coli* O28 O-polysaccharide, (b) the *O*-deacetylated PS, and (c) the trisaccharide-glycerol.

repeating unit of O28 PS comprise a 3-substituted D-galactofuranosyl, a 3-substituted 2-acetamido-2-deoxy-D-glucopyranosyl, and a 4-substituted 2-acetamido-2-deoxy-D-glucopyranosyl residue.

The remaining sugars detected in the sugar and methylation analyses can be attributed to the hexose and heptose regions of the LPS core. The sugar analysis showed both glucose and heptose, which are not part of the O-polysaccharide chain. The heptose component was L-*glycero*-D-*manno*-heptose, as shown by comparison with authentic peracetylated L-*glycero*-D-*manno*-heptitol using GLC. The sugar and methylation analyses suggested an *E. coli* R1 core [7] in the O28 LPS.

The signals in the ^{1}H and ^{13}C NMR spectra of the O28 PS and modified products thereof were assigned using two-dimensional (2D) NMR spectroscopy, and the assignments are given in Tables 1 and 2. The proton-decoupled ^{13}C NMR spectrum of the O28 PS showed 27 signals. Thus, in addition to the above components, there were three additional signals which were derived from a glycerol residue, in agreement with the component analysis. Five ^{13}C signals (Fig. 3) were split due to spin–spin couplings, and since the ^{31}P NMR spectrum of the O28 PS showed a single resonance at δ_P 0.8, it is concluded that these are ^{31}P–^{13}C couplings. The ^{13}C chemical shifts of the anomeric signals indicated one furanoside (δ_C 106.7), one β-linked pyranoside (δ_C 101.1), and one α-linked pyranoside (δ_C 97.8). The ^{1}H NMR spectrum of native O28 PS showed five signals in the low-field region between 4.3–5.3 ppm.

The presence of a galactofuranosyl residue was confirmed by a long-range COSY experiment [8] with a 300-ms delay, from which most of the protons in the Gal*f* unit could be assigned. Three of the five peaks in the low-field region belonged to H-1 (δ_H 5.21), H-2 (δ_H 4.88), and H-3 (δ_H 4.35) in Gal*f*, respectively, from which it was concluded that the *O*-acetyl group substitutes position two in Gal*f*. Resonances H-1 and H-2 in Gal*f* showed small, unresolved vicinal couplings with $\nu_{1/2} < 4$ Hz. The remaining two anomeric signals at δ_H 5.01 ($J_{H-1,H-2}$ 3.8 Hz) and 4.69 ($J_{H-1,H-2}$ 8.0 Hz) were assigned to α-Glc*p*NAc and β-Glc*p*NAc, respectively. Together with the ^{13}C chemical shift assignments it is evident that the 3-substituted GlcNAc residue has the α configuration and the 4-substituted GlcNAc residue the β configuration.

The position of substitution of the *O*-acetyl group was corroborated from spectral changes on treatment of the O28 PS with dilute NaOH (aq). In the *O*-deacetylated O28 PS, H-2 in the Gal*f* residue showed a ^{1}H chemical shift of 4.04 ppm compared to 4.88 ppm in the native material. Large chemical shift changes of the Gal*f* residue upon *O*-deacetylation were also observed in the ^{13}C spectra. The signal for the anomeric carbon in Gal*f* moved 2.3 ppm downfield, C-2 moved 1.7 ppm upfield, and C-3 moved 1.8 ppm downfield. These values confirm that an *O*-acetyl group is located at O-2 in the Gal*f* residue. The ^{13}C chemical shift of C-1 in the galactofuranosyl residue of the *O*-deacetylated O28 PS (δ_C 109.0) demonstrated that it has the β configuration [9].

Dephosphorylation of *O*-deacetylated O28 PS with aqueous HF, followed by gel permeation chromatography, yielded a single product in the oligosaccharide region. Hydrolysis of the oligosaccharide with 4 M HCl gave glycerol, galactose, and 2-acetamido-2-deoxyglucose in the proportions 1:1.1:3.5. Methylation analysis of the oligosaccharide showed the presence of 2,5,6-tri-*O*-methylgalactose, 2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)glucose, and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methyl-

Table 1
^{1}H NMR chemical shifts (δ) of the *E. coli* O28 PS and modified products thereof

Compound	Unit	H-1	H-2	H-3	H-4	H-5	H-6	CH_3
Native PS	→ 4)-*β*-D-Glc*p*NAc-(1 →	4.69 (8.0) [a]	3.72	3.76	3.97	3.51	3.78, 3.89	2.08
O-Deacetylated PS		4.68 (7.8)	3.73	3.76	3.97	3.56	3.77, 3.92	2.10 [c]
Oligosaccharide	*β*-D-Glc*p*NAc-(1 →	4.65 (8.5)	3.68	3.58	3.46	3.47	3.76, 3.93	2.08 [c]
Native PS	→ 3)-*β*-D-Gal*f*2Ac-(1 →	5.21 [3.5] [b]	4.88 [4.0]	4.35	4.26	3.92	~ 3.64, ~ 3.68	2.14
O-Deacetylated PS	→ 3)-*β*-D-Gal*f*-(1 →	5.07 [4.0]	4.04	4.21	4.18	3.91	~ 3.65, ~ 3.69	
Oligosaccharide		5.08 (1.5)	4.05	4.21	4.18	3.90	~ 3.65, ~ 3.67	
Native PS	→ 3)-*α*-D-Glc*p*NAc-(1 →	5.01 (3.8)	4.06	3.86	3.47	3.84	3.80, 3.86	2.05
O-Deacetylated PS		5.05 (3.7)	4.08	3.86	3.48	3.89	3.77, 3.82	2.09 [c]
Oligosaccharide		5.03 (3.8)	4.06	3.84	3.49	3.88	3.80, 3.87	2.06 [c]
Native PS	→ 2)-(*R*)-Gro-(1 →	~ 4.02, ~ 4.06	3.89	~ 3.76, ~ 3.76				
O-Deacetylated PS		~ 4.04, ~ 4.08	3.93	~ 3.79, ~ 3.79				
Oligosaccharide	→ 2)-Gro	~ 3.71, ~ 3.74	3.79	~ 3.75, ~ 3.75				

[a] $J_{H\text{-}1,H\text{-}2}$ values in parentheses.
[b] $\nu_{1/2}$ in square brackets.
[c] Tentative assignment.

Table 2
^{13}C and ^{31}P NMR chemical shifts (ppm) of the *E. coli* O28 PS and modified products thereof

Compound	Unit	C-1	C-2	C-3	C-4	C-5	C-6	CH_3	C=O	P
Native PS	→ 4)-*β*-D-Glc*p*NAc-(1 →	101.1	56.1	73.6 (2.4) [a]	75.0 (6.2)	75.9 (5.5)	61.3	23.2	175.3	
O-Deacetylated PS		101.3	56.2	73.7 (2.7)	75.0 (6.5)	75.9 (5.2)	61.3 [b]	23.1 [b]	175.3 [b]	
Oligosaccharide	*β*-D-Glc*p*NAc-(1 →	101.3	56.2	74.4	70.7	76.7	61.5	23.1 [b]	175.3 [b]	
Native PS	→ 3)-*β*-D-Gal*f*2Ac-(1 →	106.7	82.4	83.3	83.2	71.0	63.8	21.0	173.3	
O-Deacetylated PS	→ 3)-*β*-D-Gal*f*-(1 →	109.0	80.7	85.1	82.5	71.2	63.8			
Oligosaccharide		109.0	80.6	85.1	82.6	71.2	63.7			
Native PS	→ 3)-*α*-D-Glc*p*NAc-(1 →	97.8	53.9	77.9	69.1	73.1	61.4	23.0	174.8	
O-Deacetylated PS		97.7	53.8	78.6	69.2	73.0	61.4 [b]	23.0 [b]	175.0 [b]	
Oligosaccharide		97.7	54.0	78.5	69.1	73.0	61.4	22.7 [b]	174.9 [b]	
Native PS	→ 2)-(*R*)-Gro-(1 →	65.2 (5.8)	78.1 (7.4)	62.1						0.8
O-Deacetylated PS		65.3 (5.9)	78.1 (7.1)	62.1						0.9
Oligosaccharide	→ 2)-Gro	61.2 [b]	79.8	62.2 [b]						

[a] $J_{C,P}$ ±0.5 Hz in parentheses.
[b] Tentative assignment.

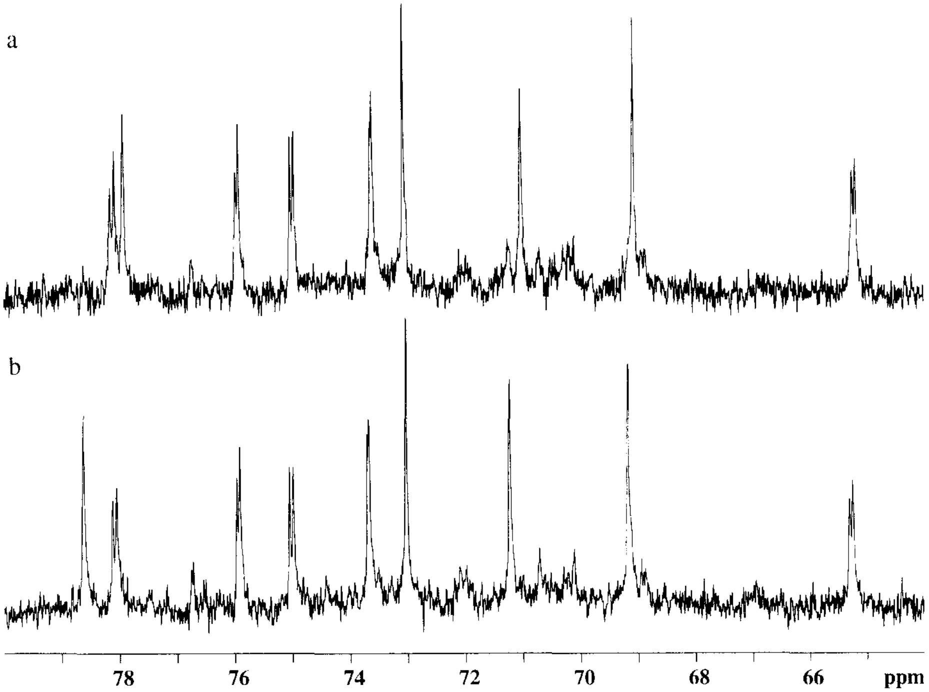
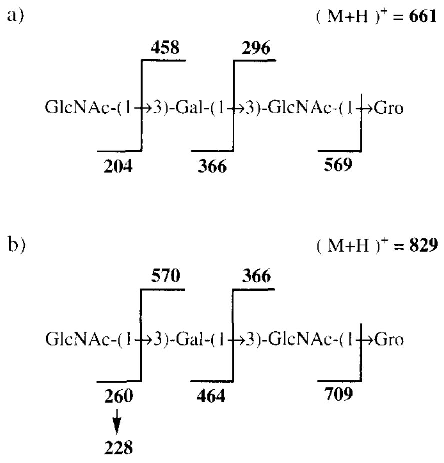


Fig. 3. Expansion of the ^{13}C NMR spectra of (a) native *E. coli* O28 PS and (b) *O*-deacetylated PS. Five peaks in each spectrum are split due to spin–spin couplings to phosphorus. The spectra were processed using 0.5-Hz exponential line broadening.

acetamido)glucose in the ratios 1:0.6:0.3. The oligosaccharide should consequently contain a terminal 2-acetamido-2-deoxy-D-glucopyranosyl group, a 3-substituted D-galactofuranosyl residue, a 3-substituted 2-acetamido-2-deoxy-D-glucopyranosyl residue, and a glycerol moiety.

The FABMS spectrum of the underivatised oligosaccharide in the positive ion mode showed a peak at *m/z* 661 attributed to $[M + H]^+$. A linked scan with a constant B/E ratio FABMS experiment, by which daughter ions of a selected parent ion can be monitored, gave in the positive ion mode on *m/z* 661, inter alia, the fragments with *m/z* 569, 458, 366, 296, and 204. FABMS, also in the positive ion mode, of the permethylated oligosaccharide showed a peak at *m/z* 829 attributed to $[M + H]^+$. The FABMS experiment using linked scan with a constant B/E ratio on *m/z* 829 gave in the positive ion mode the fragments with *m/z* 709, 570, 464, 366, 260, and 228. The peak at *m/z* 228 arises from elimination of methanol from the *m/z* 260 fragment. No such loss was observed from the other fragments in the permethylated oligosaccharide. The results from FABMS of the underivatised and methylated oligosaccharide together with hydrolysis and methylation analysis data resulted in the sequence GlcNAc-(1 → 3)-Gal-(1 → 3)-GlcNAc-(1 → Gro, as shown in Scheme 1.

The substitution positions were confirmed by NOE and HMBC experiments as shown in Tables 3 and 4. For the native O28 PS, interglycosidic three-bond connectivities were



Scheme 1. Ions found in B/E-linked-scan FABMS experiments of the trisaccharide-glycerol obtained from partial hydrolysis of the O28 PS with aqueous HF: (a) underivatised material and (b) permethylated material. The A and B fragments [10] are given below and above the formulas, respectively.

found from C-1 in β-GlcNAc (δ_C 101.1) to H-3 in β-Gal (δ_H 4.35), from H-1 in β-GlcNAc (δ_H 4.69) to C-3 in β-Gal (δ_C 83.3), from C-1 in β-Gal (δ_C 106.7) to H-3 in α-GlcNAc (δ_H 3.86), and from H-1 in β-Gal (δ_H 5.21) to C-3 in α-GlcNAc (δ_C 77.9). A corresponding NOE was observed between H-1 in β-Gal (δ_H 5.21) and H-3 in α-GlcNAc (δ_H 3.86). An interglycosidic NOE was also observed between H-1 in β-GlcNAc (δ_H 4.69) and H-2 in β-Gal (δ_H 4.88 ppm), which is not the site of attachment, and could be the result of a conformational change due to the *O*-acetyl group substitution of C-2 in β-Gal*f*. The results from the HMBC experiments agree with the substitution positions determined by methylation analyses.

The glycerol residue in the O28 PS showed resonances at δ_C 65.2, 78.1, and 62.1 for C-1, C-2, and C-3, respectively. The resonances of C-1 and C-2 were split due to spin–spin coupling to phosphorus. The large downfield chemical shift of C-2 compared to C-2 in unsubstituted glycerol ($\Delta\delta$ 5.2 ppm) indicated the glycosylation position. This was further confirmed by the HMBC experiments, which showed correlations from H-1 in α-GlcNAc (δ_H 5.01) to C-2 in glycerol (δ_C 78.1) and from C-1 in α-GlcNAc (δ_C 97.8) to H-2 in glycerol (δ_H 3.89). In addition, an NOE was found between H-1 in α-GlcNAc (δ_H 5.01) and H-2 in glycerol (δ_H 3.89). The linkage position of the phosphodiester was indicated by a downfield ^{13}C shift of C-1 (δ_C 65.2), compared to C-3 (δ_C 62.1) and the splitting of the C-1 signal due to phosphorus–carbon coupling. A DEPT experiment with the Θ pulse set to 135° was performed on the native material in order to verify all the methylene carbons. Five signals with opposite phase compared to methine and methyl carbons were found at δ_C 61.3, 61.4, 62.1, 63.8, and 65.2 in accordance with the NMR chemical shift assignments.

The ^{31}P NMR spectra at pD 6 (not shown), using δ_P 0.00 for 85% phosphoric acid, showed resonances at δ_P 0.8 and 0.9 for native and *O*-deacetylated O28 PS, respectively (Table 2). These ^{31}P chemical shifts at ~ 1 ppm indicate a phosphodiester

Table 3
NOE from anomeric protons of native *E. coli* O28 PS and of the oligosaccharide obtained from partial hydrolysis

Compound	Unit	Anomeric proton	NOE to proton	Atom, residue
		δ_H	δ_H	
Native PS	→ 4)-β-D-Glc*p*NAc-(1 →	4.69	4.88	H-2, Gal
			3.51	H-5, β-GlcNAc
	→ 3)-β-D-Gal*f*2Ac-(1 →	5.21	4.88	H-2, Gal
			3.86	H-3, α-GlcNAc
	→ 3)-α-D-Glc*p*NAc-(1 →	5.01	4.06	H-2, α-GlcNAc
			3.89	H-2, Gro
Oligosaccharide	β-D-Glc*p*NAc-(1 →	4.65	4.21	H-3, Gal
			4.05	H-2, Gal
			3.47	H-5, β-GlcNAc
	→ 3)-β-D-Gal*f*-(1 →	5.08	3.84	H-3, α-GlcNAc
	→ 3)-α-D-Glc*p*NAc-(1 →	5.03	4.06	H-2, α-GlcNAc
			3.79	H-2, Gro

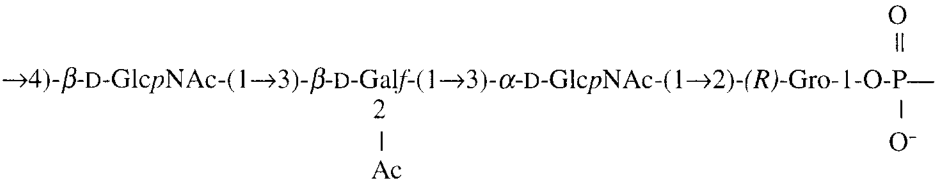
linkage. Anionic phosphomonoesters are reported to have $\delta_P < 0$ and dianionic phosphomonoesters $\delta_P > 2$ [11], whereas pyrophosphates have $\delta_P > 10$ [12]. For comparison, other glycerol phosphodiesters are reported to have similar δ_P values (0.8–1.6 ppm) [13–16]. The location of the phosphorus was further corroborated by the splitting of the ^{13}C NMR signals for C-3 ($^3J_{C,P}$ 2.4 Hz), C-4 ($^2J_{C,P}$ 6.2 Hz), and C-5 ($^3J_{C,P}$ 5.5 Hz) of the β-GlcNAc residue, and for C-1 ($^2J_{C,P}$ 5.8 Hz) and C-2 ($^3J_{C,P}$ 7.4 Hz) of the glycerol moiety in the O28 PS. The magnitudes of the ^{13}C,^{31}P two- or three-bond coupling constants were almost identical in native and *O*-deacetylated O28 PS. The two-dimensional inverse-detected ^{1}H,^{31}P-correlated NMR experiment [17] of native and

Table 4
Transglycosidic heteronuclear correlations in HMBC experiments for anomeric atoms of native *E. coli* O28 PS

Residue	Anomeric atom		Connectivity to		Atom, residue
	δ_H	δ_C	δ_H	δ_C	
→ 4)-β-D-Glc*p*NAc-(1 →	4.69			83.3	C-3, Gal
		101.1	4.35		H-3, Gal
→ 3)-β-D-Gal*f*2Ac-(1 →	5.21			77.9	C-3, α-GlcNAc
		106.7	3.86		H-3, α-GlcNAc
→ 3)-α-D-Glc*p*NAc-(1 →	5.01			78.1	C-2, Gro
		97.8	3.89		H-2, Gro

O-deacetylated O28 PS showed correlations from phosphorus to H-1[a] and H-1[b] in glycerol and to H-4 in *β*-GlcNAc. After dephosphorylation with aqueous HF all five signals, which were split in the ^{13}C NMR spectrum of the native O28 PS, became singlets. The chemical shifts of these carbon signals also moved upon dephosphorylation, with the largest differences, ~ 4 ppm upfield, for C-4 in *β*-GlcNAc and C-1 in glycerol. These data show that phosphorus is a part of the polysaccharide backbone, situated between glycerol and *β*-GlcNAc as phosphodiester linkages.

The absolute configuration of the glycerol was determined by oxidation of primary hydroxyl groups in the native O28 PS with TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy radical) in alkaline NaOCl solution, followed by hydrolysis, esterification with (+)-2-BuOH, and acetylation (T. Rundlöf and G. Widmalm, submitted). The resulting glyceric acid ester was compared with acetylated (+)-2-butyl esters of D- and L-glyceric acid by GLC. The retention times relative to tri-*O*-acetylglycerol were 1.59 for the oxidised glycerol derivative of O28 PS, and 1.57 and 1.59 for the D- and L-glyceric acid derivatives, respectively. The glyceric acid obtained on oxidation of the O28 PS thus has the L configuration, and the glycerol moiety in this polysaccharide is consequently derived from a D-glycerol 1-phosphate residue [using IUPAC recommendations, an (*R*)-glycerol phosphate]. The repeating unit of the O-polysaccharide from *E. coli* O28 thus has the structure:



$$\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-}\underset{\underset{\text{Ac}}{|}}{\underset{2}{\text{Gal}f}}\text{-}(1\rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow 2)\text{-}(R)\text{-Gro-1-O-}\overset{\overset{\text{O}}{\|}}{\underset{\underset{\text{O}^-}{|}}{\text{P}}}\text{—}$$

Polysaccharides containing a 2-substituted glycerol moiety with a phosphodiester linkage between a primary position in the glycerol and a sugar residue in the polysaccharide have been reported [13,14,18–21], but the chirality at C-2 was not determined. The 2-substituted glycerol phosphate moiety in the capsular polysaccharide from *Escherichia coli* K2, which is of a teichoic acid type, has also been shown to have the *R* configuration, as determined by enzymic analysis of the released glycerol phosphate [22]. The D-glycerol 1-phosphate (*sn*-glycero-3-phosphate) component in the O-antigen polysaccharide from *E. coli* O28 should, in accordance with biosynthetic considerations, be derived from CDP-glycerol [23,24].

3. Experimental

General methods.—Evaporations were performed under diminished pressure at < 40 °C (bath) or by flushing with air or N_2. For GLC, a Hewlett–Packard 5890 series II instrument, fitted with a flame-ionisation detector, was used. Separations were performed on an HP-5 capillary column, using isothermic runs at 130 °C or a temperature program 170 °C (1 min) → 180 °C at 1 °C/min (1 min), 180 → 210 °C at 4 °C/min. GLC–MS was performed on a Hewlett–Packard 5970 MSD instrument, using a HP-5 MS capillary column. Hydrolysis was performed with 4 M HCl at 100 °C for 1 h. The sugars were converted into alditol acetates. Methylation analyses of native material were performed as previously described [25,26]. Methylation of dephosphorylated material was performed using a modification of the NaOH method [27]. The permethylated products were solvolysed with anhydrous HF at 25 °C for 3 h followed by hydrolysis with 2 M CF_3CO_2H at 100 °C for 30 min. The absolute configurations of galactose and 2-acetamido-2-deoxyglucose were determined, essentially as described by Leontein et al. [5] and by Gerwig et al. [6], by GLC of their acetylated (+)-2-butyl glycosides. Gel permeation chromatography was performed on a Bio-Gel P-2 column, using 1% 1-butanol in water as eluent. A differential refractometer was used for monitoring the gel chromatography effluents. FABMS spectra in the positive mode were performed on a JEOL SX102 instrument, using Xe atoms (6 keV) and a matrix of thioglycerol or glycerol/thioglycerol, at a resolution of 3000. In the B/E-linked-scan experiment He was used as collision gas.

NMR spectroscopy.—NMR spectra of solutions in D_2O were recorded at 50 °C using a JEOL GSX-270, JEOL alpha-400, or Varian unity + 600 instrument. Samples containing phosphorus were run at pD 6. Chemical shifts are reported in ppm relative to acetone (δ_H 2.225, δ_C 31.00) as internal reference and 85% H_3PO_4 (δ_P 0.00) as external reference. $^1H,^1H$ COSY, 300-ms long-range $^1H,^1H$ COSY, relayed $^1H,^1H$ COSY, double-relayed $^1H,^1H$ COSY, and TOCSY experiments were used to assign ^1H NMR signals. The TOCSY experiments were recorded with mixing times of 60, 90, 120, and 150 ms, and the NOESY experiments were recorded using a mixing time of 300 ms. The ^{13}C NMR signals were assigned by 2D $^1H,^{13}C$ heteronuclear correlation spectroscopy (inverse-detected HSQC and ^{13}C-detected HETCOR) with or without decoupling. Long-range $^1H,^{13}C$-correlated spectra (HMBC) were recorded with delay times of 45 and 90 ms. The methylene carbon signals were identified from a DEPT experiment with Θ pulse set to 135°. The $^1H,^{31}P$-correlated spectra were recorded on a Varian unity 500 spectrometer using a coupled inverse-detected experiment [17].

Bacterial strain.—*E. coli* O28ac:H^- was obtained from the International *Escherichia* and *Klebsiella* Centre at Statens Seruminstitut, Copenhagen, Denmark.

Isolation and purification of the O-polysaccharide.—*E. coli* O28 bacteria were grown in Ty medium (30-L culture). Bacteria were killed by the addition of formaldehyde (1% final concentration) and harvested by centrifugation. The LPS was extracted by the hot phenol–water method [4], and delipidated by treatment with 1% AcOH (aq) at 100 °C for 1 h. Liberated lipid A was centrifuged off, and the supernatant lyophilised. The product was purified by gel permeation chromatography on a column of Bio-Gel P-2.

O-*Deacetylation of O28 PS.*—O28 PS was treated with 0.1 M NaOH (aq) at 25 °C for 15 h. The solution was stirred with Dowex 50 H^+ resin for 1 h and lyophilised.

Dephosphorylation of O-*deacetylated O28 PS.*—The polysaccharide (16 mg) was treated with 40% HF (aq) (1 mL) at 8 °C for 48 h. After evaporation of solvent and neutralisation with aq NH_4OH (1 M), the sample was freeze-dried. Gel permeation chromatography of the sample yielded 5 mg of an oligosaccharide.

Determination of absolute configuration of the glycerol.—TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy radical, 0.2 mg), NaBr (2.8 mg), and aq NaOCl (13%, 0.06 mL) were dissolved in H_2O (1.5 mL). The polysaccharide (2 mg) dissolved in H_2O (0.5 mL) was added at 0 °C. The mixture was stirred and kept at 0 °C and pH 10.8 for ca. 1 h. Aq 0.1 M NaOH was added to keep the desired pH. The reaction was quenched with EtOH (0.2 mL), the alkali neutralised with HCl (aq), and the solution concentrated to a volume of ~0.5 mL. The oxidised polysaccharide was precipitated with EtOH, recovered by centrifugation, dissolved in water, lyophilised, and hydrolysed with 2 M CF_3CO_2H (aq) at 100 °C for 30 min. The solvent was evaporated and after drying over P_2O_5 the residue was treated with (+)-2-butanol (0.2 mL) and AcCl (15 mL) at 80 °C for 8 h. The solvent was evaporated and the product acetylated with 1:1 Ac_2O–pyridine at 120 °C for 3 h. After evaporation of the solvent the acetylated product was extracted into EtOAc, the solution filtered, and the product compared with the authentic acetylated (+)-2-butyl esters of D- and L-glyceric acid by GLC.

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