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Note

Structure of the O-specific polysaccharide of *Proteus mirabilis* O16 containing ethanolamine phosphate and ribitol phosphate

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Dedicated to Professor Joachim Thiem of the occasion of his 60th anniversary

Abstract

The O-specific polysaccharide of *Proteus mirabilis* O16 was studied by ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, NOESY, H-detected $^{1}H,^{13}C$ HMQC, HMQC-TOCSY, and $^{1}H,^{31}P$ HMQC experiments, along with chemical methods. The polysaccharide was found to be a ribitol teichoic acid-like polymer having the following structure

Keywords: *Proteus mirabilis*; O-antigen; O-specific polysaccharide; Lipopolysaccharide; Ribitol phosphate; Ethanolamine phosphate

Bacteria of the genus *Proteus* cause urinary tract infections, which sometimes result in severe complications, such as pyelonephritis and formation of bladder and kidney stones, as well as respiratory and intestinal tract infections. The serological specificity of the bacteria is defined by the structure of the O-specific polysaccharide chain (O-antigen) of the lipopolysaccharide. Based on the O-antigens, two species, *Proteus mirabilis* and *Proteus vulgaris*, have been classified into 60 O-serogroups.[1,2] Recently, more O-serogroups have been proposed for the third species *Proteus penneri*.[3,4]

In all *P. mirabilis* O-serogroups studied so far, the O-specific polysaccharides are acidic or contain both acidic and basic components, such as uronic acids, their amides with amino acids, including lysine, and phosphate groups linking various non-sugar constituents.[3,5–10] We now report on the structure of another

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phosphorylated O-specific polysaccharide of *P. mirabilis*.

LPS was isolated from dried bacterial cells of *P. mirabilis* O16 by the phenol–water procedure[11] and degraded with dilute acetic acid to give a high molecular mass O-specific polysaccharide. Sugar analysis of the polysaccharide revealed GlcN and GalN in the ratio ~1:2, which were identified using an amino acid analyser. GLC of the alditol acetates revealed the presence of ribitol. Determination of the absolute configuration by GLC of acetylated (*S*)-2-butyl glycosides showed that all monosaccharides have the D configuration. The D configuration of ribitol was determined by GLC of the acetylated (*S*)-2-butyl ester of L-glyceric acid derived from 4-substituted ribitol-1-phosphate (see below) by periodate oxidation followed by bromine oxidation. L-Glyceric acid was obtained in a 3.5:1 mixture with D-glyceric acid, which, most likely, derived from 6-substituted D-GalNAc in spite of the short time used for the periodate oxidation (see below).

The regularity of the polysaccharide was masked by non-stoichimetric phosphorylation with 2-aminoethyl phosphate, as shown below. Therefore, to elucidate the carbohydrate backbone structure the polysaccharide was dephosphorylated and depolymerised with aqueous 48% hydrofluoric acid to give a ribitol containing oligosaccharide.

The ^{13}C NMR spectrum of the oligosaccharide contained signals for three anomeric carbons at δ 99.1, 98.8, and 103.5, three carbons bearing nitrogen at δ 51.2, 53.6, and 54.0, five $HOCH_2$–C groups at δ 61.1–64.1 (data of DEPT-135), 12 other carbons bearing oxygen belonging to sugar pyranose rings and ribitol in the region δ 69.15–80.8, and three *N*-acetyl groups at δ 23.3–23.6 (CH_3) and δ 175.3–176.3 (CO). Accordingly, the ^1H NMR spectrum of the oligosaccharide contained, inter alia, signals for three anomeric protons at δ 4.69, 5.11, and 5.36 and three *N*-acetyl groups at δ 2.04–2.06. Therefore, the oligosaccharide contained two GalNAc residues and one residue each of GlcNAc and ribitol.

The ^1H NMR spectrum of the oligosaccharide was assigned using 2D COSY and TOCSY experiments (Table 1). Based on the $^3J_{H,H}$ coupling constant values in conjunction with GLC data, three sugar spin systems were assigned to α-Glc*p*NAc, α-Gal*p*NAc and β-Gal*p*NAc, the $J_{3,4}$ and $J_{4,5}$ values being used to differentiate between sugars with the gluco and galacto configuration and the $J_{1,2}$ values to determine the anomeric configuration. The remaining spin system was assigned to ribitol. The configurations of the glycosidic linkages was confirmed by a ROESY experiment, which revealed intra-residue H-1, H-3 and H-1, H-5 correlations for one β-linked Gal*p*NAc.

Table 1
^1H NMR data (δ ppm)

Sugar residue	H-1	H-2	H-3	H-4	H-5(5a)	H-6a(5b)	H-6b	CH_3CON
Oligosaccharide								
β-Gal*p*NAc-(1→	4.69	3.92	3.77	3.93	3.67	3.80	3.80	2.04 [a]
→4)-α-Gal*p*NAc-(1→	5.36	4.14	3.92	4.18	3.87	3.79	3.92	2.05 [a]
→3)-α-Glc*p*NAc-(1→	5.11	4.05	3.89	3.69	3.80	3.71	3.85	2.06 [a]
→4)-Rib-ol	3.64, 3.81 [b]	3.75	3.91	3.96	3.83	3.90		
Polysaccharide [c]								
→6)-β-Gal*p*NAc-(1→	4.71	3.92	3.77	3.98	3.83	4.05	4.06	2.04 [a]
→4)-α-Gal*p*NAc-(1→	5.34 (5.35)	4.13	3.92	4.19	3.87	3.81	3.90	2.06 [a]
→3)-α-Glc*p*NAc-(1→	5.10 (5.09)	4.06 (4.04)	3.93 (3.92)	3.75 (3.69)	4.14 (3.81)	4.12 (3.72)	4.18 (3.86)	2.07 [a]
→4)-Rib-ol-1-	3.97, 4.06 [b]	3.83	3.93	3.98	3.82	3.90		
Etn-	4.14	3.29						

[a] Assignment could be interchanged.
[b] H-1a and H-1b.
[c] When different, data of the repeating units lacking Etn-*P* are given in parentheses.

Table 2
^{13}C NMR data (δ, ppm; $J_{H,P}$, Hz)

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	CH_3CON	CH_3CON
Oligosaccharide								
β-Gal*p*NAc-(1→	103.5	54.0	72.3	69.2	76.2	62.3	23.3 [a]	175.3 [b]
→4)-α-Gal*p*NAc-(1→	99.1	51.2	69.1	76.5	71.6	61.3	23.4 [a]	175.5 [b]
→3)-α-Glc*p*NAc-(1→	98.8	53.6	78.0	72.2	73.7	61.6	23.6 [a]	176.3 [b]
→4)-Rib-ol	64.1	72.7	73.5	80.8	61.1			
Polysaccharide [c]								
→6)-β-Gal*p*NAc-(1→	103.5	54.0	72.2	68.8	74.8	65.6	23.3 [a]	175.3 [b]
					$J_{5,P}$ 8.2	$J_{6,P}$ 5.1		
→4)-α-Gal*p*NAc-(1→	99.1	51.2	69.0	76.6	71.6	61.6	23.4 [a]	175.5 [b]
	(99.0)			(76.5)				
→3)-α-Glc*p*NAc-(1→	99.3	53.6	77.9	71.5	72.5	65.3	23.6 [a]	176.2 [b]
	(99.2)		(78.0)	(72.1)	(73.6)	(61.5)		
					$J_{5,P}$ 6.8	$J_{6,P}$ 5.0		
→4)-Rib-ol-1-	68.2	71.6	73.0	81.4	61.2			
	(68.1)		(72.9)	(81.2)	(61.1)			
	$J_{1,P}$ 5.6	$J_{2,P}$ 6.7						
Etn-	63.1	41.3						
	$J_{1,P}$ 5.1	$J_{2,P}$ 7.3						

[a] Assignment could be interchanged.
[b] Assignment could be interchanged.
[c] When different, data of the repeating units lacking Etn-*P* are given in parentheses.

The ^{13}C NMR spectrum of the oligosaccharide was assigned using H-detected ^{1}H, ^{13}C HMQC and HMQC-TOCSY experiments (Table 2). Significant downfield displacements by 6.0–7.3 ppm of the signals for C-3 of GlcNAc, C-4 of α-GalNAc, and C-4 of Rib-ol to δ 76.5–80.8, as compared with their positions in the spectra of the corresponding non-substituted monomers,[12,13] defined the linkage positions. The ROESY experiment showed the following interresidue correlations between the transglycosidic protons: β-GalNAc H-1, α-GalNAc H-4 at δ 4.69/4.18, α-GalNAc H-1, GlcNAc H-3 at δ 5.36/3.89, and GlcNAc H-1, Rib-ol H-4 at δ 5.11/3.96. These data confirmed the substitution pattern and revealed the sequence of residues in the oligosaccharide, which, thus, has the following structure:

β-D-Gal*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→3)-

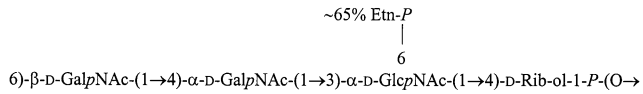
α-D-Glc*p*NAc-(1→4)-Rib-ol

The ^{31}P NMR spectrum of the initial polysaccharide contained signals for two phosphate groups at δ 2.71 and 2.96. The ^{1}H and ^{13}C NMR spectra that were assigned as described above (Figs. 1 and 2, Tables 1 and 2), showed two series of signals with the intensity ratio ~1:2, the minor series closely resembling the spectrum of the oligosaccharide. The major series was distinguished by the presence of the characteristic signals for ethanolamine phosphate (Etn-*P*) at δ_H 3.29 and 4.14, δ_C 41.3 and 63.1 for CH_2N and CH_2O groups, respectively. In the ^{1}H, ^{31}P HMQC spectrum, the CH_2O signal correlated with the ^{31}P signal at δ 4.14/2.71. These data suggested that the major repeating units contain Etn-*P*, which is absent from the minor repeating units.

The most significant differences between the ^{13}C NMR spectra of the polysaccharide (major series) and the oligosaccharide were observed for the signals for C-6 of GlcNAc (δ 65.3 vs. 61.6), C-6 of β-GalNAc (δ 65.6 vs. 61.6), and C-1 of ribitol (δ 68.1 vs. 64.1, respectively). All these and the neighbouring carbon signals in the polysaccharide were split due to coupling to phosphorus (Table 2). The ^{1}H, ^{31}P HMQC spectrum showed cross-peaks for the ^{31}P signal of Etn-*P* at δ 2.71 with GlcNAc H-6a, 6b at δ 4.12 and 4.18. The ^{31}P

signal at δ 2.96 gave cross-peaks with Rib-ol H-1a, H-1b at δ 3.97 and 4.06, respectively; the latter cross-peak evidently coincided with cross-peaks for β-GalNAc H-6a, H-6b. These data suggested that Etn-*P* is attached to C-6 of GlcNAc and that Rib-ol and β-GalNAc are (1→6)-interlinked by the phosphate group.

On the basis of the data obtained, it was concluded that the O-specific polysaccharide of *P. mirabilis* 16 is a ribitol teichoic acid-like polymer having the following structure:



Although both ethanolamine phosphate[3,9,10,14–16] and ribitol phosphate[9,10,14,15] have been frequently found in *Proteus* O-antigens, the structure of the polysaccharide is unique, which is in agreement with classification of the strain studied in a separate *Proteus* O-serogroup.

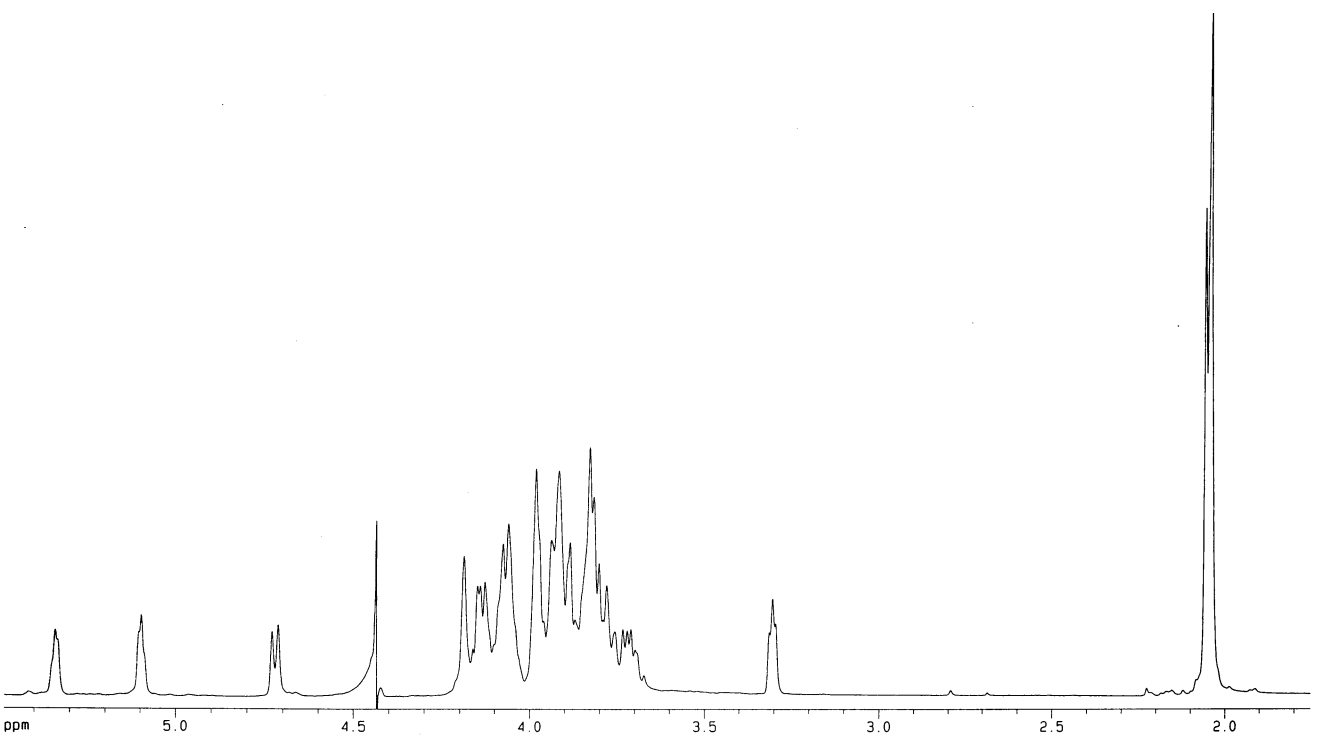


Fig. 1. ^{1}H NMR spectrum of the O-specific polysaccharide.

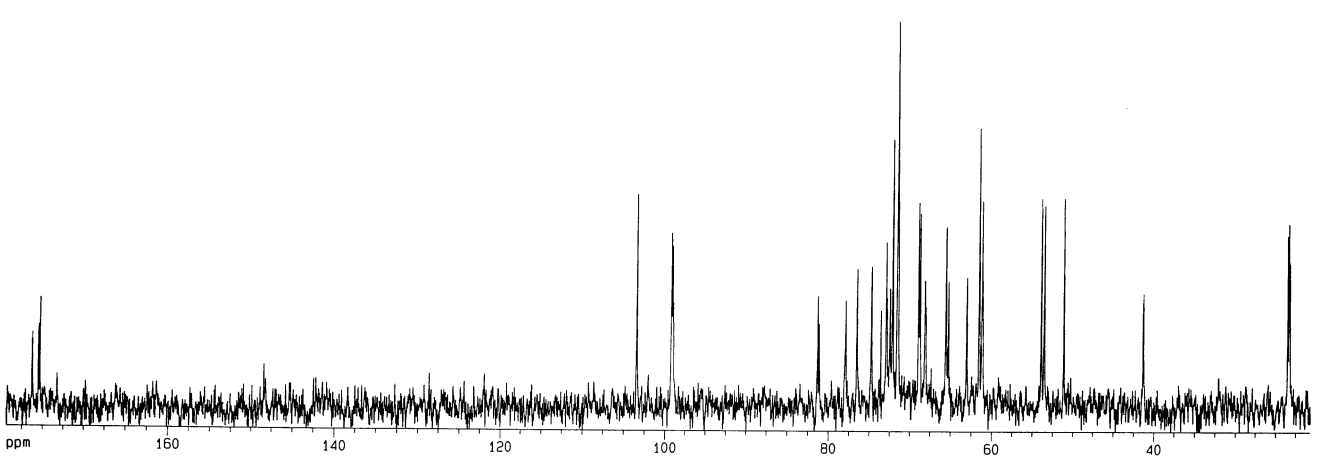


Fig. 2. ^{13}C NMR spectrum of the O-specific polysaccharide.

1. Experimental

Isolation and degradation of the lipopolysaccharide.—*P. mirabilis* O16 (strain PrK 31/57) from the Czech National Collection of Type Cultures (CNCTC; Institute of Epidemiology and Microbiology, Prague) was grown as described.[17] The lipopolysaccharide was isolated from dried bacterial cells of *P. mirabilis* by extraction with hot aq phenol[11] and purified by treatment with cold aq 50% CCl_3CO_2H followed by dialysis of the supernatant.

Acid degradation of the lipopolysaccharide was performed with aq 1% HOAc at 100 °C for 1.5 h. The O-specific polysaccharide was isolated by GPC on a column (3 × 65 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer (pH 4.5).

The polysaccharide (30 mg) was dephosphorylated with aq 48% HF (0.2 mL, 5 °C, 24 h), and the dephosphorylated oligosaccharide (9.5 mg) was isolated by GPC on a column (3 × 65 cm) of Sephadex G-25 in water.

Sugar and alditol analysis.—The polysaccharide was hydrolysed with 3 M CF_3CO_2H (100 °C, 4 h), amino sugars were identified using a Biotronik LC-2000 amino acid analyser. Alditols were identified by GLC of the alditol acetates on a Hewlett–Packard 5890 chromatograph equipped with an Ultra-2 column using a temperature gradient of 3 °C/min starting from 180 °C.

For determination of the absolute configurations, 8 mg polysaccharide was partially oxidized by $NaIO_4$ (5 mg/0.5 mL, 20 °C, 15 min), desalted on a column of (3 × 65 cm) of Sephadex G-15, and the oligosaccharide fraction (2.5 mg) was separated on a column (3 × 65 cm) of Sephadex G-25. This was dissolved in 0.3 mL water and treated with 50 μL bromine at ambient temperature for 3 h, the solution was evaporated, and the product was hydrolysed with 4 M HCl (105 °C, 4 h). Amino sugars were N-acetylated, converted into acetylated (*S*)-2-butyl glycosides,[18,19] and analysed by GLC as above using a temperature gradient of 2 °C/min starting from 200 °C. Glyceric acid derived from ribitol was analysed by GLC in the same sample as the acetylated (*S*)-2-butyl ester using a temperature gradient of 2 °C/min starting from 120 °C.

NMR spectroscopy.—NMR spectra were recorded with a Bruker DRX-500 spectrometer in D_2O at 45 °C using internal acetone (δ_H 2.225, δ_c 31.45) as reference. 2D NMR experiments were performed using standard Bruker software, and XWINNMR 2.1 program (Bruker) was used to acquire and maintain NMR data. Mixing times of 200 and 300 ms were used in TOCSY and ROESY experiments, respectively.

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