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Note

Full structure of the O-specific polysaccharide of *Proteus mirabilis* O24 containing 3,4-*O*-[(*S*)-1-carboxyethylidene]-D-galactose

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Abstract

The structure of a neutral polysaccharide isolated by degradation with dilute acetic acid of the lipopolysaccharide (LPS) of *P. mirabilis* O24 has been determined recently [E. Literacka et al., *FEBS Lett*., 456 (1999) 227–231]. Further studies of this LPS using alkaline degradation and hydrolysis at pH 4.5 showed that the polysaccharide chain includes an acetal-linked pyruvic acid residue, which is removed completely during delipidation with acetic acid. A revision using ^{1}H and ^{13}C NMR spectroscopy and methylation analysis resulted in determination of the following full structure of the repeating unit of the O-specific polysaccharide:

β-D-Gal*p*3,4(*S*-Pyr)
1
↓
3
→3)-β-D-Glc*p*NAc-(1→4)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→

where D-Gal3,4(*S*-Pyr) is 3,4-*O*-[(*S*)-1-carboxyethylidene]-D-galactose.

Keywords: *Proteus mirabilis*; Lipopolysaccharide; O-specific polysaccharide structure; Pyruvic acid acetal

Bacteria of the genus *Proteus* cause mainly urinary tract infections which can lead to severe complications, such as acute or chronic pyelonephritis and formation of bladder and kidney stones. The serological O-specificity of *Proteus* is defined by the structure of the polysaccharide chain of the outer-membrane lipopolysaccharide (O-antigen). Based on the O-antigens, two medically important species, *P. mirabilis* and *P. vulgaris*, have been classified into 60 O-serogroups [1,2]. Chemical studies showed that in most serogroups O-antigens are acidic due to the presence of uronic acids, their amides with amino acids, ether-linked lactic acid, acetal-linked pyruvic

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acid, or phosphate groups [3]. Recently, it has been reported that mild acid hydrolysis of the LPS of *P. mirabilis* O24 results in a neutral polysaccharide (**1**), which has the following structure [4,5].

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

1

Further studies showed that the polysaccharide chain of *P. mirabilis* O24 LPS contains an additional, acidic component, viz. pyruvic acid, which is highly acid-labile and easily eliminated during delipidation of the LPS with dilute acetic acid. Now we report on the full structure of the O-antigen of *P. mirabilis* O24.

The LPS was degraded under alkaline conditions using aqueous ammonia treatment at 37 °C or under mild acidic conditions at pH 4.5 at 100 °C. Comparison of the ^{13}C NMR spectra of both products showed that their polysaccharide parts had the identical repeating unit. Further studies were performed with the polysaccharide **2** obtained at pH 4.5, which gave better-resolved NMR spectra.

The ^{1}H NMR spectrum of the polysaccharide **2** contained signals for four anomeric protons at δ 4.46–4.88, three N-acetyl groups at δ 2.02–2.09, and one methyl group at δ 1.66 (s). The ^{13}C NMR spectrum of the polysaccharide showed signals for four anomeric carbons at δ 102.0–105.3, four $HOCH_2$–C groups (C-6) at δ 61.3–62.1, and three N-acetyl groups at δ 23.5–23.9 (CH_3) and 175.3–175.6 (CO). In the spectrum, there were three more signals, from which one, at δ 24.6, belonged to a methyl group and the two others, at δ 108.7 and 176.9, to quaternary carbons (data of attached-proton test [6]).

These data demonstrated a tetrasaccharide repeating unit containing three 2-acetamido-2-deoxyhexose residues and suggested the presence of an acetal-linked pyruvic acid residue (compare published data [7]). Sugar analysis of the polysaccharide **2** showed that amino sugars are GlcN and GalN (2:1) and revealed Gal as the fourth component of the repeating unit, which has thus the same sugar composition as that of the neutral polysaccharide **1**.

The ^{1}H and ^{13}C NMR spectra of the polysaccharide **2** were assigned using 2D COSY, TOCSY, and H-detected ^{1}H,^{13}C HMQC experiments (Tables 1 and 2). A subsequent NOESY experiment revealed the glycosylation pattern and the sequence in the polysaccharide **2**, which were again the same as in the polysaccharide **1** studied earlier [4,5]. The ^{13}C NMR chemical shift data for the amino sugar residues also fitted well with the published data for the polysaccharide **1** (Table 2) and showed that GalNAc and both GlcNAc residues are substituted in the same manner in both polysaccharides. In contrast, the positions of the signals of Gal were different, the most significant displacements being observed for the C-3 and C-4 signals (downfield

Table 1
^{1}H NMR data (δ, ppm) [a]

Sugar residue	Proton						
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
*Polysaccharide **1*** [5]							
→3)-β-D-Glc*p*NAc-(1→	4.91	3.77	3.83	3.50	3.39	3.74	3.91
→3,4)-β-D-Gal*p*NAc-(1→	4.58	3.90	3.90	4.31	3.69	3.74	3.77
→4)-β-D-Glc*p*NAc-(1→	4.61	3.70	3.77	3.58	3.54	3.65	3.85
β-D-Gal*p*-(1→	4.44	3.58	3.63	3.96	3.66	3.77	
*Polysaccharide **2***							
→3)-β-D-Glc*p*NAc-(1→	4.88	3.77	3.79	3.51	3.42	3.78	3.91
→3,4)-β-D-Gal*p*NAc-(1→	4.55	3.93	3.89	4.33	3.70	3.77	3.90
→4)-β-D-Glc*p*NAc-(1→	4.57	3.73	3.73	3.59	3.53	3.65	3.85
β-D-Gal*p*3,4(*S*-Pyr)-(1→	4.46	3.48	4.23	4.16	4.02	3.83	3.90

[a] The chemical shifts for NAc are δ 2.02 (6 H) and 2.09 (3 H); that for the pyruvic acid residue is δ 1.66 (all s).

Table 2
^{13}C NMR data (δ, ppm) [a]

Sugar residue	Carbon					
	C-1	C-2	C-3	C-4	C-5	C-6
Polysaccharide ***1*** [5]						
→3)-β-D-Glc*p*NAc-(1→	102.5	56.0	82.8	69.9	76.1	62.4
→3,4)-β-D-Gal*p*NAc-(1→	103.0	53.0	81.8	75.6	75.6	62.0
→4)-β-D-Glc*p*NAc-(1→	102.5	56.2	73.4	81.0	75.6	61.4
β-D-Gal*p*-(1→	106.6	72.2	74.2	69.9	76.1	62.4
Polysaccharide ***2***						
→3)-β-D-Glc*p*NAc-(1→	102.0	55.9	82.9	70.1	76.3	62.0 [b]
→3,4)-β-D-Gal*p*NAc-(1→	102.8	52.9	81.4	75.2	75.6	62.1 [b]
→4)-β-D-Glc*p*NAc-(1→	102.4	56.2	73.3	80.8	75.6	61.3
β-D-Gal*p*3,4(*S*-Pyr)-(1→	105.3	74.6	80.2	76.3	74.3	62.1 [b]

[a] The chemical shifts for NAc are δ 23.5, 23.6, 23.9 (CH_3), 175.6 (2 C), and 175.3 (CO); those for C-1, C-2, and C-3 of the pyruvic acid residue are δ 176.9, 108.7, and 24.6.
[b] Assignment could be interchanged.

by 6 and 6.4 ppm, respectively). Accordingly, in the ^{1}H NMR spectra of the two polysaccharides the chemical shifts were similar for the amino sugar residues but different for the galactose residue (Table 1). These data suggested that pyruvic acid is acetal-linked to O-3 and O-4 of Gal in the polysaccharide **2**. The ^{1}H and ^{13}C NMR chemical shifts for the pyruvic acid residue were in agreement with published data for 3,4-*O*-(1-carboxyethylidene)-β-D-galactopyranose (Ref. [7] and refs. cited therein), that for C-1 (δ 108.7) being typical of a 1,3-dioxolane ring.

The NMR data were also confirmed by methylation analysis of the polysaccharide **2**, which showed the presence of 3-substituted, 4-substituted and 3,4-disubstituted 2-amino-2-deoxyhexose residues, as well as a 3,4-disubstituted galactose residue. A small amount of a terminal galactose residue was revealed as well, which may result from partial removal of the pyruvic acid acetal during degradation of the LPS at pH 4.5.

The ^{1}H and ^{13}C NMR chemical shifts of δ_H 1.66 and δ_C 24.6 for the acetal methyl group indicated the S configuration at the pyruvic acid acetal carbon [7]. This was confirmed by a 1D NOE experiment with pre-saturation of the acetal methyl group, which resulted in a sole response (about 7% of the presaturated signal intensity) on H-2 of Gal (Fig. 1). Consideration of Dreiding stereomodels and molecular mechanic calculations using the MM$^+$ field showed that such connectivity is only possible in the case of the S configuration (**3**), whereas a spatial proximity of the methyl group and H-4 of Gal is predicted for the *R* isomer (**4**) (Table 3).

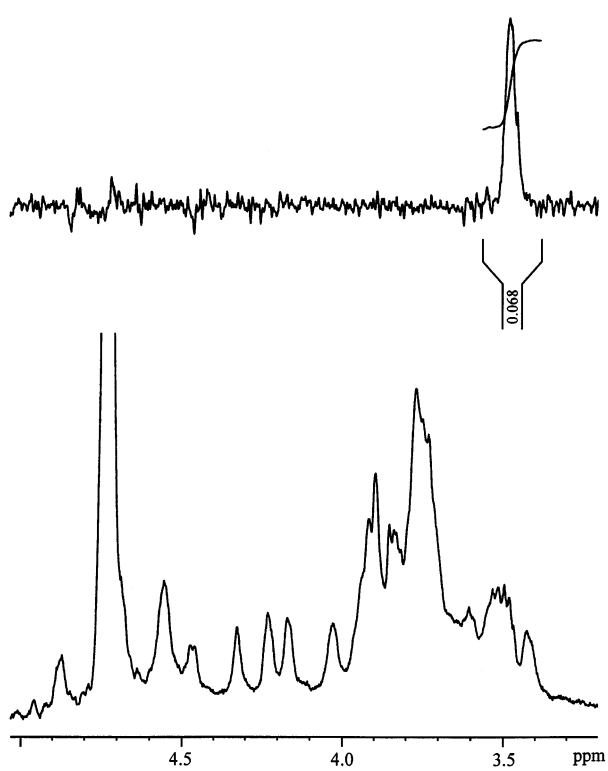

Fig. 1. A part of a ^{1}H NMR spectrum of the polysaccharide **2** (bottom) and a 1D NOE difference spectrum with pre-saturation of the acetal methyl group at δ 1.66 (top). The integral intensity of the pre-saturated signal was taken as 1.

Table 3
Distances between protons in methyl 3,4-*O*-(1-carboxyethylidene)-β-D-galactopyranosides (Å, according to MM^+ calculations for the global minimum)

Configuration at the acetal carbon	Distance between the acetal methyl group and galactose protons		
	H-2	H-3	H-4
S	3.23	4.37	4.07
R	4.37	4.02	3.01

Therefore, the tetrasaccharide repeating unit of the O-specific polysaccharide of *P. mirabilis* O24 has the following structure:

β-D-Gal*p*3,4(*S*-Pyr)
1
↓
3
→3)-β-D-Glc*p*NAc-(1→4)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→
2

where D-Gal3,4(*S*-Pyr) is 3,4-*O*-[(*S*)-1-carboxyethylidene]-D-galactose.

As with all other *P. mirabilis* O-antigens studied so far [1], this polysaccharide is acidic. Pyruvic acid is a new component of *P. mirabilis* polysaccharides. Previously, D-Gal4,6(*R*-Pyr) has been found in the O-specific polysaccharide of *Proteus vulgaris* ATCC 49990 [8] and *Proteus penneri* 15 [9], which have the identical structure. Although 3,4-linked acetals are less common than 4,6-linked acetals [10], D-Gal3,4(*S*-Pyr) has been found in several other bacterial polysaccharides (Ref. [7] and refs. cited therein), where it occupies a terminal position. The methyl group in this derivative, as well as in the 2-substituted D-Gal3,4(*R*-Pyr) from the capsular polysaccharide of *Escherichia coli* K47 [11], has the *endo* configuration in the *cis*-fused dioxolane system.

1. Experimental

Growth of the bacterium, isolation and degradation of the lipopolysaccharide.—*P. mirabilis* O24, strain PrK 47/57, was cultivated as described [12], and LPS was isolated from dried bacterial cells by hot phenol–water extraction [13].

The LPS (70 mg) was degraded with 0.1 M AcONa (pH 4.5) at 100 °C for 1 h. The precipitate was removed by centrifugation (13,000*g*, 20 min), and the supernatant was fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer (pH 4.5), with monitoring using a Knauer differential refractometer (Germany). Fractions containing a high-molecular-mass polysaccharide were pooled, concentrated, centrifuged, and freeze-dried; the polysaccharide yield was 53% of the LPS weight.

The LPS (50 mg) was incubated in aq 12.5% NH_3 at 37 °C for 16 h, the reaction mixture was worked up as above, and an O-deacylated LPS was obtained in a yield of 60% of the LPS weight.

Methylation analysis.—Methylation of the polysaccharide was performed with CH_3I in Me_2SO in the presence of sodium methylsulfinylmethanide [14]. Partially methylated monosaccharides were derived by hydrolysis with 2 M CF_3CO_2H (120 °C, 2 h), converted into alditol acetates, and analysed by GLC–MS on a Hewlett–Packard 5890 chromatograph equipped with a DB-5 fused-silica capillary column and a NERMAG R10-10L mass spectrometer, using a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min.

NMR spectroscopy.—NMR spectra were recorded with a Bruker DRX-500 spectrometer for solutions in D_2O at 50 °C, using acetone (δ_H 2.225, δ_C 31.45) as an internal reference. Standard Bruker software (XWIN-NMR 1.2) was used to acquire and process the NMR data. A 1D NOE experiment was performed in the difference mode. A mixing time of 200 ms was used in 1D NOE, 2D TOCSY and NOESY experiments.

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