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Structure of the O-specific polysaccharide of *Proteus vulgaris* O15 containing a novel regioisomer of *N*-acetylmuramic acid, 2-acetamido-4-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucose

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Abstract

An acidic O-specific polysaccharide was obtained by mild acid degradation of the lipopolysaccharide of *Proteus vulgaris* O15 and studied by sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including 2D COSY, TOCSY, ROESY, and H-detected $^{1}H,^{13}C$ HMQC experiments. The polysaccharide was found to contain an ether of GlcNAc with lactic acid, and the following structure of the repeating unit was established:

→3)-α-D-Glc*p*NAc4(*R*-Lac)6Ac-(1→2)-β-D-Glc*p*A-(1→3)-α-L-6dTal*p*2Ac-(1→3)-β-D-Glc*p*NAc-(1→

where L-6dTal and D-GlcNAc4(*R*-Lac) are 6-deoxy-L-talose and 2-acetamido-4-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucose, respectively. The latter sugar, which to our knowledge has not been hitherto found in nature, was isolated from the polysaccharide by solvolysis with anhydrous triflic acid and identified by comparison with the authentic synthetic compound. Serological studies with the Smith-degraded polysaccharide showed an importance of 2-substituted GlcA for manifesting of the immunospecificity of *P. vulgaris* O15.

Keywords: *Proteus vulgaris*; Lipopolysaccharide; O-antigen; Bacterial polysaccharide structure; 2-Acetamido-4-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucose; Triflic acid solvolysis; 6-Deoxy-L-talose

1. Introduction

Bacteria of the genus *Proteus* are common causative agents of urinary tract infections, which can lead to severe complications, such as acute or chronic pyelonephritis and formation of bladder and kidney stones. Two medically important species, *P. mirabilis* and *P. vulgaris*, are classified into 60 O-serogroups,[1,2] and recently a number of O-serogroups have been proposed for the third species, *P. penneri*.[3,4] The serological O-specificity of *Proteus* is defined by the structure of the O-antigen, which represents the polysaccharide chain of the outer-membrane lipopolysaccharide (LPS). Chemical studies showed that in most *Proteus* O-serogroups the O-specific polysaccharides contain acidic components, including various non-sugar groups, such as phosphate, amide-linked amino acids, sugar acetals with pyruvic acid, and sugar ethers with lactic acid.[3] These groups often play an important role in manifesting of the immunospecificity of *Proteus* strains.[3] Now we report on the structure of another acidic O-specific polysaccharide of *Proteus*, which contains a novel regioisomer of *N*-acetylmuramic acid.

2. Results and discussion

The O-specific polysaccharide was obtained by mild acid degradation of the LPS, which was extracted from

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P. vulgaris O15 cells by the phenol–water procedure,[5] and isolated by GPC on Sephadex G-50 followed by anion-exchange chromatography on DEAE-Trisacryl. Sugar analysis after full acid hydrolysis of the polysaccharide revealed GlcA, GlcN (from GlcNAc), 6-deoxytalose (6dTal), and an acidic amino sugar, which was identified as 2-amino-4-*O*-(1-carboxyethyl)-2-deoxyglucose (GlcN4Lac, see below). GLC analysis of the acetylated glycosides with chiral alcohols showed that GlcN and GlcA have the d configuration, whereas 6dTal has the L configuration.

The ^{13}C NMR spectrum of the polysaccharide contained signals having different intensities, most likely, owing to nonstoichiometric O-acetylation (there was a signal for CH_3 of O-acetyl groups at δ 22.0). The ^1H NMR spectrum of the polysaccharide contained a number of signals for *O*-acetyl and *N*-acetyl groups in the region δ 2.01–2.14.

The polysaccharide was O-deacetylated by treatment with aq ammonia and cleaved with anhydrous triflic acid at -4 °C for 2 h. The products were fractionated by GPC on TSK HW-40 (S) to give an acidic monosaccharide, which was indistinguishable by ^1H and ^{13}C NMR spectra from the authentic synthetic sample of 2-acetamido-4-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucose and different from the corresponding *S*-isomer.[6] Comparison of the specific optical rotation value, $[\alpha]_D^{20}$ $+39.0°$ (water), of the isolated monosaccharide with the values $[\alpha]_D$ $+41.7°$ and $+8.6°$ (water) of the synthetic compounds D-GlcNAc4(*R*-Lac) and D-GlcNAc4(*S*-Lac),[6] respectively, showed that the O-specific polysaccharide of *P. vulgaris* O15 contains D-GlcNAc4(*R*-Lac).

Methylation analysis of the polysaccharide resulted in identification of 6-deoxy-2,4-di-*O*-methyltalose and 2-deoxy-4,6-di-*O*-methyl-2-(*N*-methylacetamido)-glucose. Therefore, the polysaccharide contains 3-substituted 6dTal and 3-substituted GlcNAc.

The ^{13}C NMR spectrum of the O-deacetylated polysaccharide was typical of a regular polymer (Fig. 1). It contained signals for four anomeric carbons at δ 97.0–103.7, two nitrogen-bearing carbons (C-2 of GlcN) at δ 54.4 and 57.3, two $HOCH_2$-C groups (C-6 of GlcN) at δ 62.0 and 62.5, two CH_3-C groups (C-6 of 6dTal and C-3 of lactic acid) at δ 16.7 and 19.5, respectively, the COOH group (C-1) of lactic acid at δ 178.6 (compare published data[6]), two *N*-acetyl groups at δ 23.5 and 23.6 (CH_3), and 15 other carbons in the region δ 69.1–83.5 (sugar ring carbons linked to oxygen and C-2 of lactic acid). Accordingly, the ^1H NMR spectrum of the polysaccharide contained signals for four anomeric protons at δ 4.65–5.35, two *N*-acetyl groups at δ 1.97 and 2.08, two CH_3-C groups (H-6 of 6dTal and H-3 of lactic acid) at δ 1.23 and 1.48, respectively, and other signals at δ 3.41–4.24.

Therefore, the polysaccharide has a tetrasaccharide repeating unit containing one residue each of D-GlcNAc, L-6dTal, D-GlcA, and D-GlcNAc4(*R*-Lac). The four sugar residues were denoted by Roman numerals I–IV according their sequence in the repeating unit (see below).

The ^1H and ^{13}C NMR spectra of the O-deacetylated polysaccharide were assigned using 2D COSY, TOCSY, ROESY, and H-detected ^1H,^{13}C HMQC experiments (Tables 1 and 2). Spin systems for $GlcNAc^I$ and $GlcNAc^{IV}$ were identified by correlations of H-1 with H-2,3,4,5,6 in the TOCSY spectrum and confirmed by correlations of the H-2 signals at δ 4.08 and 3.71 to the signals of nitrogen-bearing carbons (C-2) at δ 54.4 and 57.3 in the ^1H,^{13}C HMQC spectrum. A spin system for 6dTal was revealed by correlations of H-1 with H-2,3,4 and H-4 with H-5,6 in the TOCSY spectrum, and the remaining spin system was assigned to GlcA. The $J_{1,2}$ coupling constant value of 3 Hz indicated that $GlcNAc^I$ is α-linked, whereas the $J_{1,2}$ values of 7–8 Hz showed that GlcA and $GlcNAc^{IV}$ are β-

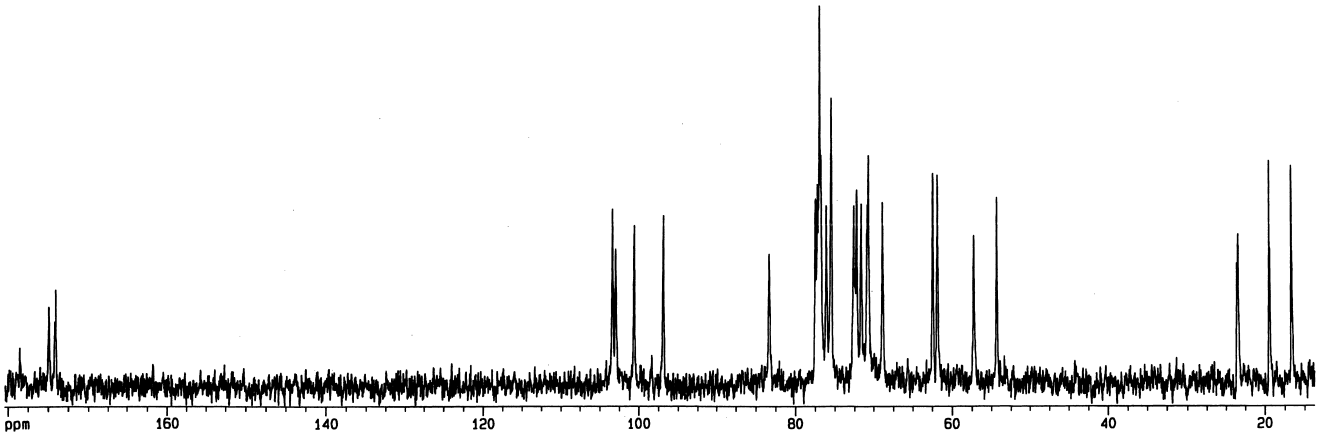

Fig. 1. 125-MHz ^{13}C NMR spectrum of the O-deacetylated polysaccharide.

Table 1
^{1}H NMR data of the O-deacetylated polysaccharide (δ, ppm)[a]

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a, 6b
→2)-β-D-Glc*p*A-(1→	4.81	3.60	3.65	3.63	3.92	
→3)-α-L-6dTal*p*-(1→	4.99	3.85	3.84	3.63	4.21	1.23
→3)-β-D-Glc*p*NAc-(1→	4.65	3.71	3.71	3.41	3.42	3.76, 3.99
→3)-α-D-Glc*p*NAc4(*R*-Lac)-(1→	5.35	4.08	4.24	3.54	4.11	3.87, 3.87

[a] The chemical shifts for NAc are δ 1.97 and 2.08 (Me); for lactic acid residue δ 4.64 (H-2) and 1.48 (H-3).

Table 2
^{13}C NMR data of the O-deacetylated polysaccharide (δ, ppm)[a]

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
→2)-β-D-Glc*p*A-(1→	103.7	77.1	75.6	71.9	75.7	174.2[b]
→3)-α-L-6dTal*p*-(1→	103.2	71.0	76.9	71.9	69.1	16.7
→3)-β-D-Glc*p*NAc-(1→	100.8	57.3	83.5	70.8	77.1	62.5
→3)-α-D-Glc*p*NAc4(*R*-Lac)-(1→	97.0	54.4	76.2	77.3	72.3	62.0

[a] The chemical shifts for NAc are δ 23.5 and 23.6 (both Me), 175.0[b] (2CO); for lactic acid residue δ 178.6 (C-1), 77.7 (C-2), and 19.5 (C-3).
[b] Assignment could be interchanged.

linked. The α configuration of 6dTal was determined by the position of the C-5 signal at δ 69.1 (compare the chemical shifts δ 67.7 and 72.0 for C-5 in α-6dTal and β-6dTal, respectively[7]).

A relatively low-field position of the signals for C-3 of GlcNAc^{IV}, GlcNAc^{I} and 6dTal at δ 83.5, 76.2 and 76.9, respectively, as well as that for C-2 of GlcA at δ 77.1, as compared with their positions in the corresponding non-substituted monosaccharide,[6–9] demonstrated the modes of glycosylation of the monosaccharides. A 2D ROESY experiment revealed strong interresidue cross-peaks between the anomeric protons and protons at the linkage carbons at δ 5.35/3.60, 4.81/3.84, 4.99/3.71, 4.65/4.24, which were assigned to GlcNAc^{I} H-1,GlcA H-2, GlcA H-1,6dTal H-3, 6dTal H-1,GlcNAc^{IV} H-3, and GlcNAc^{IV} H-1,GlcNAc^{I} H-3 correlations, respectively. These data established the sequence of the monosaccharide residues in the repeating unit. A cross-peak between H-2 of the lactic acid residue and H-4 of GlcNAc^{I} at δ 4.64/3.54 showed the location of the ether linkage at position 4 and thus confirmed GlcNAc^{I}4Lac.

Positions of the *O*-acetyl groups were determined using 2D ^{1}H,^{13}C HMQC spectroscopy. Most of GlcNAc^{I} H-6/C-6 and 6dTal H-2/C-2 cross-peaks in the spectrum of the O-specific polysaccharide laid in a lower field in the *F1* (^{1}H) dimension at δ 4.43/64.3 and 4.94/70.0 as compared with their positions in the spectrum of the O-deacetylated polysaccharide at δ 3.87/62.0 and 3.85/71.0, respectively. These displacements were due to a deshielding effect of the *O*-acetyl groups and indicated O-acetylation of GlcNAc^{I} at position 6 and 6dTal at position 2. The degree of O-acetylation was estimated as 80–90% in both positions.

On the basis of the data obtained, it was concluded that the O-specific polysaccharide of *P. vulgaris* O15 has the following structure:

→3)-α-D-Glc*p*NAc4(*R*-Lac)6Ac-(1→2)-β-D-Glc*p*A-(1→3)-α-L-6dTal*p*2Ac-(1→3)-β-D-Glc*p*NAc-(1→

I II III IV

This polysaccharide is distinguished by the presence of an unusual component of bacterial polysaccharides, D-GlcNAc4(*R*-Lac), which represents a new regioisomer of *N*-acetylmuramic acid and a stereoisomer at the lactic acid residue of D-GlcNAc4(*S*-Lac) reported earlier as a component of the O-specific polysaccharide of *P. penneri* 41 (serogroup O62).[6,10] Another unusual constituent monosaccharide, L-6dTal, has been previously found in the O-specific polysaccharide of *P. penneri* 2 (serogroup O66).[11] Remarkably, both 6dTal-containing *Proteus* polysaccharides share also an α-L-6dTal*p*2Ac-(1→3)-β-D-Glc*p*NAc disaccharide fragment.

Rabbit polyclonal anti-*P. vulgaris* O15 serum reacted with the homologous LPS in a passive hemolysis test

and enzyme immunosorbent assay (EIA) at high titres of 1:51,200 and 1:256,000, respectively. The reaction in EIA was strongly inhibited by both alkali-treated LPS and O-specific polysaccharide (minimal inhibitory dose 19.6 and 4.8 ng, respectively), whereas the Smith-degraded polysaccharide with destroyed GlcA residues showed no inhibiting activity at a dose of up to 100 μg. This finding indicated an important role of 2-substituted GlcA in manifesting of the immunospecificity of *P. vulgaris* O15.

A number of *Proteus* LPS with the known structure of the O-specific polysaccharide were tested in passive hemolysis, and only those of *P. mirabilis* O30 and *P. penneri* 25 were found to cross-react with anti-*P. vulgaris* O15 serum (titre 1:1,600 for both). In a Western blot, anti-*P. vulgaris* O15 serum recognised slow migrating bands of high-molecular-mass LPS species from *P. mirabilis* O30 (faint staining), thus showing that the cross-reactive epitope resides in the O-specific polysaccharide. Most likely, this epitope is associated with a β-D-Glc*p*NAc-(1→3)-D-Glc*p*NAc disaccharide fragment present in both *P. vulgaris* O15 and *P. mirabilis* O30[12] antigens (Fig. 2). Earlier, it has been suggested that a single β-D-Glc*p*NAc residue common to the O-specific polysaccharides is sufficient to provide serological relatedness of the LPS of *P. mirabilis* O6 and O23.[13] In case of *P. penneri* 25, a marked binding of anti-*P. vulgaris* O15 serum was observed to fast-migrating bands corresponding to low-molecular-mass LPS species devoid of the polysaccharide chain. Hence, the epitope shared by *P. vulgaris* O15 and *P. penneri* 25 is located in the LPS core of the latter strain, but it cannot be assigned to any particular fragment since the core structure of *P. penneri* 25 LPS is unknown.

Despite the presence of a common α-L-6dTal*p*2Ac-(1→3)-β-D-Glc*p*NAc disaccharide fragment in the O-specific polysaccharides (Fig. 3), anti-*P. vulgaris* O15 serum did not react with the LPS of *P. penneri* 2 (O66), thus suggesting that the corresponding potential epitope is not accessible for antibodies. The absence of any cross-reactivity with the LPS of *P. penneri* 41 could be expected taking into account different configurations of

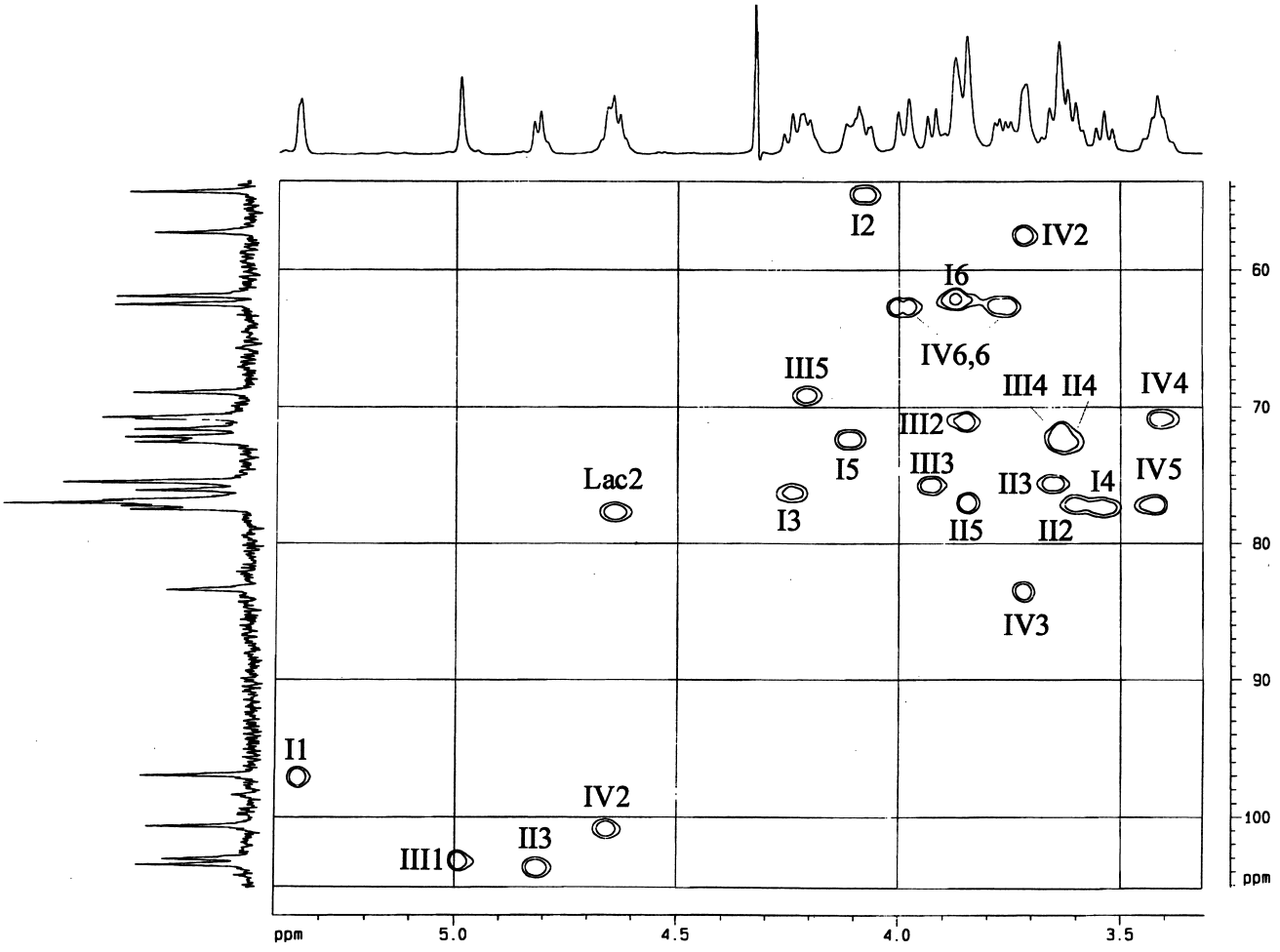

Fig. 2. Part of a 2D $^{1}H,^{13}C$ HMQC spectrum of the O-deacetylated polysaccharide. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively. Arabic numerals refer to the atoms in the lactic acid and sugar residues denoted as follows: Lac, lactic acid; I, α-GlcNAc; II, GlcA; III, 6dTal; IV, β-GlcNAc.

Proteus vulgaris O15 (this work)

→2)-β-D-Glc*p*A-(1→3)-α-L-6dTal*p*2Ac-(1→3)-β-D-Glc*p*NAc-(1→3)-α-D-Glc*p*NAc4(*R*-Lac)6Ac-(1→

Proteus mirabilis O30[12]

→4)-β-D-Glc*p*A-(1→6)-α-D-Gal*p*NAc-(1→6)-β-D-Glc*p*NAc-(1→3)-β-D-Glc*p*NAc4Ac-(1→

Proteus penneri 2 (O66)[11]

β-L-Rha*p*NAc3NAc
1
↓
3
→3)-α-L-6dTal*p*2Ac-(1→3)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*-(1→

Proteus penneri 41 (O62)[10]

β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc4(*S*-Lac)
1
↓
2
→2)-α-D-Gal*p*6Ac-(1→3)-β-D-Glc*p*NAc-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→

Fig. 3. Structures of the O-specific polysaccharide of *P. vulgaris* O15 and structurally related polysaccharides.

the sugar anomeric centre and the lactic acid residue of GlcNAc4Lac in the O-specific polysaccharides of *P. vulgaris* O15 and *P. penneri* 41.

3. Experimental

Bacterial strain, cultivation, and isolation of the lipopolysaccharide.—*P. vulgaris* O15, strain PrK 30/57, came from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague). The bacteria were cultivated under aerobic conditions in nutrient broth (BTL, Lodz, Poland). The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with water, and lyophilised.

The LPS was isolated from dried bacterial cells by hot phenol–water extraction[5] and purified by treatment with DNAse and RNAse and ultracentrifugation as described.[12]

Isolation and modifications of the O-specific polysaccharide.—Delipidation of the LPS (200 mg) was performed with aq 2% HOAc at 100 °C until lipid precipitation. The precipitate was removed by centrifugation (13,000*g*, 20 min), and the supernatant was fractionated on a column (56 × 2.6 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer (pH 4.5) monitored with a Knauer differential refractometer (Germany). A high-molecular-mass polysaccharide was obtained in a yield of 25% of the LPS weight. For further purification, the crude polysaccharide was applied to a column (0.7 × 14 cm) of DEAE-Trisacryl equilibrated with 0.005 M sodium phosphate buffer (pH 6.3), neutral contaminants were eluted with the same buffer, and the target acidic polysaccharide with 0.1 M sodium phosphate buffer (pH 6.3); monitoring was performed using a Technicon sugar analyser (USA).

The polysaccharide (35 mg) was treated with aq 12.5% ammonia at 37 °C for 16 h, and the O-deacetylated polysaccharide (27 mg) was isolated by GPC on a column (90 × 2.5 cm) of TSK HW-40 (S) (Supelco) in aq 1% HOAc. Smith degradation of the polysaccharide (10 mg) was performed with 0.1 M $NaIO_4$ (1 mL) at 20 °C in dark for 48 h, followed by reduction with an excess of $NaBH_4$ and desalting by dialysis against distilled water.

Sugar analysis.—The polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h). Amino sugars were identified using a Biotronik LC-2000 amino acid analyser (Germany) equipped with a column (0.4 × 22 cm) of Ostion LG AN B cation-exchange resin at 55 °C using 0.35 M sodium citrate buffer (pH 5.28). Neutral sugars and uronic acids were identified using a Biotronik LC-2000 sugar analyser on a column (0.4 × 15 cm) of Dionex A × 8 anion-exchange resin at 70 °C using 0.5 M sodium borate buffer (pH 8.0) or 0.04 M sodium phosphate buffer (pH 2.4),[14] respectively. The absolute configurations of the monosaccharides were determined by GLC of the acetylated (*S*)-2-butyl glycosides (for GlcN and GlcA) or (*R*)-2-octyl glycosides (for 6dTal) according to the published method,[15,16] modified as

described.[17] GLC was performed on a DB-5 column (25 m × 0.25 mm) using a Hewlett–Packard 5880 instrument (USA) and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min.

Methylation analysis.—Methylation of the polysaccharide was performed with CH_3I in dimethyl sulfoxide in the presence of sodium methylsulfinylmethanide.[18] Partially methylated monosaccharides were derived by hydrolysis under the same conditions as in sugar analysis, reduced with $NaBD_4$, acetatylated and analysed by GLC–MS on a Hewlett–Packard 5890 chromatograph equipped with a NERMAG R10-10L mass spectrometer (France) under the same chromatographic conditions as in sugar analysis.

Triflic acid solvolysis.—The O-deacetylated polysaccharide (27 mg) was treated with anhydrous CF_3SO_3H at −4 °C for 2 h. After neutralisation with aq 5% ammonia at 0 °C, products were fractionated by GPC on TSK HW-40 (S) in aq 1% AcOH, followed by rechromatography on the same column in water to give D-GlcNAc4(*R*-Lac) (2.8 mg). The specific optical rotation of D-GlcNAc4(*R*-Lac), $[\alpha]_D$ +39.0° (*c* 0.1, water), was measured on a Jasko DIP-360 polarimeter at 20 °C.

NMR spectroscopy.—NMR spectra were recorded with a Bruker DRX-500 spectrometer for a solution in D_2O at 57 °C for the polysaccharide and 30 °C for the monosaccharide, using internal acetone (δ_H 2.225, δ_C 31.45) as reference. Bruker software XWINNMR 1.2 was used to acquire and process the NMR data. A mixing time of 200 and 100 ms was used in 2D TOCSY and ROESY experiments, respectively.

Serological technique.—Rabbit polyclonal anti-*P. vulgaris* O15 serum was obtained by immunisation of New Zealand white rabbits with heat-killed bacteria as described.[19] Alkali-treated LPS was prepared by saponification of the LPS with 0.25 M NaOMe in abs MeOH at 37 °C for 15 h. The passive hemolysis test with alkali-treated LPS and EIA with LPS as antigen, inhibition of the reaction in EIA, and Western blot after sodium deoxycholate polyacrylamide gel electrophoresis, were performed as described previously.[20]

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