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Note

Structure of the O-specific polysaccharide of *Providencia alcalifaciens* O16 containing *N*-acetylmuramic acid

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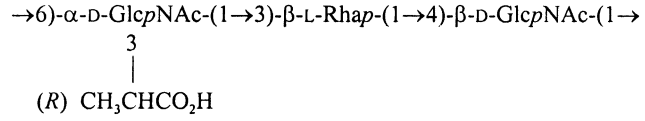
Abstract

The O-specific polysaccharide of *Providencia alcalifaciens* O16 was obtained by mild-acid degradation of the lipopolysaccharide and studied by chemical methods and NMR spectroscopy, including 2D $^{1}H,^{1}H$ COSY, TOCSY, NOESY, and $^{1}H,^{13}C$ HSQC experiments. It was found that the polysaccharide contains *N*-acetylmuramic acid, which was isolated by solvolysis with trifluoromethanesulfonic acid and identified by the specific optical rotation and NMR spectroscopy. The following structure of the trisaccharide repeating-unit of the polysaccharide was established:



Keywords: Lipopolysaccharide; O-Specific polysaccharide; Bacterial polysaccharide structure; *N*-Acetylmuramic acid; *Providencia alcalifaciens*

The genera *Proteus*, *Providencia*, and *Morganella* are a unique group of Gram-negative bacteria in the family Enterobacteriaceae. Due to the ability to oxidatively deaminate a wide range of amino acids, these bacteria play an important role in degradation processes that occur in the natural environment. Under favorable conditions, they cause several types of infections, mainly urinary tract infections, wound infections, and enteric diseases.[1] The genus *Providencia* consists of five species: *P. alcalifaciens*, *P. heimbachae*, *P. rettgeri*, *P. rustiganii*, and *P. stuartii*.[2] Strains of *P. alcalifaciens* are associated with diarrhea in travelers and children but can be found also in non-diarrheic stool specimens.[3,4] Cell invasion has been proposed as the main virulence factor of these bacteria,[2] but the exact mechanism by which *P. alcalifaciens* causes diarrhea is unknown.

Lipopolysacharide (endotoxin, O-antigen) and flagellar H antigen serve as the basis for serotyping of *Providencia*. The combined serological scheme of *P. alcalifaciens* and *P. stuartii* includes 62 O-serogroup.[5,6] Serological relationships were observed between *Providencia* strains, as well as between *Providencia* and *Escherichia coli*, *Proteus*, *Morganella*, *Salmonella*, and *Shigella*.[7] Among enterobacteria, the genus *Providencia* is one of the least studied in respect to the lipopolysaccharide structure. Recently, aiming at the creation of the molecular basis for the serological classification and cross-reactivity of *Providencia*, we have established the structures of the O-specific polysaccharide chains of the lipopolysaccharides of *P. alcalifaciens* O5,[8] O7,[9] and O23.[10] Now we report the new structure of the O-specific polysaccharide of *P. alcalifaciens* O16.

The polysaccharide was obtained by mild-acid degradation of the lipopolysaccharide, isolated from bacterial cells by the phenol–water procedure,[11] followed by GPC on Sephadex G-50. Sugar analysis, including de-

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termination of the absolute configurations of the monosaccharides,[12] revealed L-rhamnose and D-GlcN.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for three sugar residues, including signals for anomeric carbons at δ 95.2, 101.7, and 102.9, one *O*-substituted and one nonsubstituted hydroxymethyl group (C-6 of GlcN) at δ 68.9 and 61.9, respectively, one methyl group (C-6 of Rha) at δ 17.9, two carbons bearing nitrogen at δ 54.9 and 56.7 (C-2 of GlcN), other sugar carbons in the region δ 69.1–79.7, two *N*-acetyl groups (Me at 23.2 and 23.5, CO at δ 175.3 and 176.6), and one *O*-(1-carboxyethyl) group at δ 19.9 (Me) and 182.9 (CO_2H) (compare published data:[13] δ 20.3 and 182.9, respectively). Accordingly, the ^1H NMR spectrum of the polysaccharide contained, inter alia, signals for three anomeric protons at δ 4.51, 4.84, and 5.34, one methyl group (H-6 of Rha) at δ 1.33 (m), two *N*-acetyl groups at δ 2.05 and 2.07 (both s), and one *O*-(1-carboxyethyl) group at δ 1.37 (Me, d, *J* 6.7 Hz) (compare published data:[13] δ 1.31). The 2D $^1H,^1H$ COSY spectrum confirmed the presence of spin systems for three sugar residues and one residue of lactic acid, the latter showing a H-2,H-3 correlation at δ 4.43/1.37.

Taking together sugar analysis and 1D NMR spectroscopic data, it was concluded that the polysaccharide has a trisaccharide repeating unit, which contains one residue of L-rhamnose, D-GlcNAc, and *N*-acetylmuramic acid or an isomer thereof.

For identification of the *O*-(1-carboxyethylated) sugar, the polysaccharide was subjected to solvolysis with anhydrous trifluoromethanesulfonic (triflic) acid at 0 °C for 16 h, and the products were fractionated by GPC on TSK HW-40. NMR spectroscopic studies of the isolated acidic monosaccharide using COSY, NOESY, and $^1H,^{13}C$ HSQC experiments demonstrated GlcNAc substituted with a lactic acid residue at position 3. The ^1H NMR spectrum of the monosaccharide was identical to that of *N*-acetylmuramic acid but different from the spectrum of *N*-acetylisomuramic acid (2-acetamido-3-*O*-[(*R*)- and (*S*)-1-carboxyethyl]-2-deoxy-D-glucose, respectively; see published ^1H NMR data[13]). The specific optical rotation value, $[\alpha]_D^{21}$ + 76.8° (*c* 0.5, water) (compare published data:[14] $[\alpha]_D$ + 48.3°), showed that the sugar isolated from the polysaccharide has the same absolute configuration as *N*-acetylmuramic acid and, hence, is 2-acetamido-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucose (D-GlcNAc3*R*Lac).

Methylation analysis showed that the polysaccharide is linear, rhamnose is 3-substituted, and GlcNAc is 4-substituted. A partially methylated derivative of muramic acid was detected in GLC–MS but its mass spectrum was not interpreted. As could be inferred from the presence in the ^{13}C NMR spectrum of the polysaccharide of an *O*-substituted hydroxymethyl group (see above), the *N*-acetylmuramic acid residue is 6-substituted.

The ^1H and ^{13}C NMR spectra of the polysaccharide were assigned using 2D COSY, TOCSY, NOESY, and H-detected $^1H,^{13}C$ HSQC experiments (Tables 1 and 2). In the TOCSY spectrum, there were cross-peaks for H-1 with H-2,3,4,5,6a,6b of GlcNAc, H-2,3,4,5 of GlcNAc3Lac, and H-2 of Rha. The assignment for Rha was completed by cross-peaks for H-2 with H-3,4,5,6 and H-6 with H-2,3,4,5.

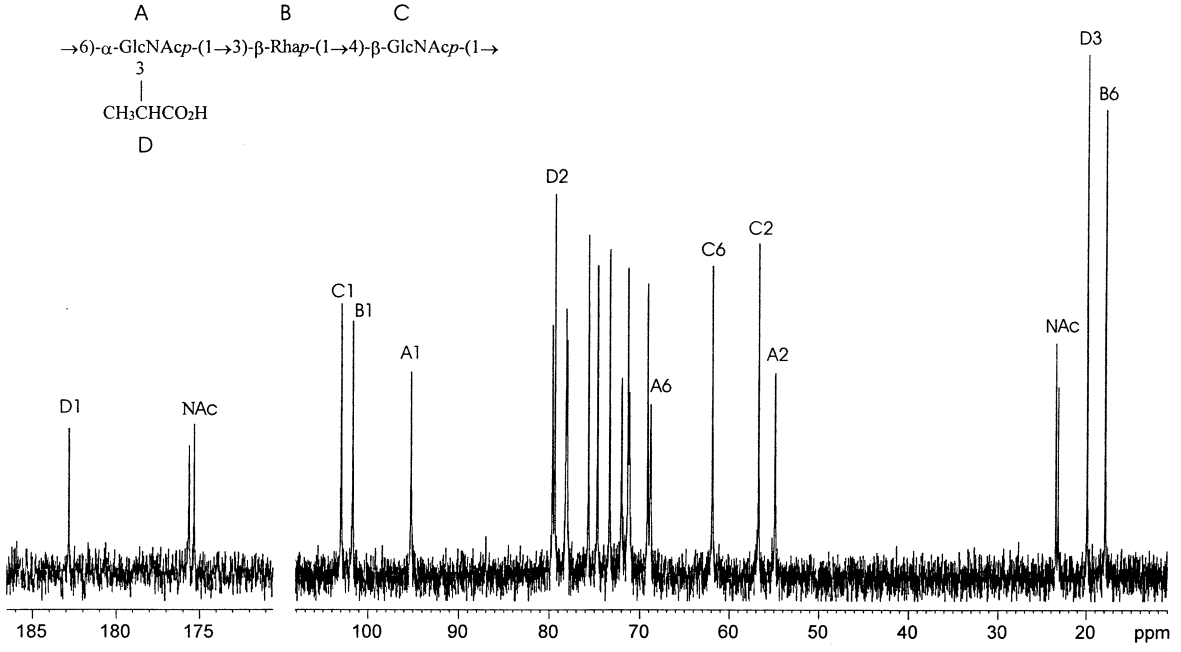


Fig. 1. 125-MHz ^{13}C NMR spectrum of the O-specific polysaccharide.

Table 1
500-MHz ^{1}H NMR chemical shifts of the O-specific polysaccharide (δ, ppm) [a]

Sugar residue		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
→6)-α-D-Glc*p*NAc3*R*Lac-(1→	(**A**)	5.34	3.63	3.75	4.03	3.61	3.83	4.04
→3)-β-L-Rha*p*-(1→	(**B**)	4.84	4.07	3.57	3.41	3.40	1.33	
→4)-β-D-Glc*p*NAc-(1→	(**C**)	4.51	3.73	3.71	3.68	3.51	3.84	3.93
(*R*)-Lactic acid	(**D**)		4.43	1.37				

[a] Signals for NAc are at δ 2.05 and 2.07.

Table 2
125-MHz ^{13}C NMR chemical shifts of the O-specific polysaccharide (δ, ppm) [a]

		C-1	C-2	C-3	C-4	C-5	C-6
→6)-α-D-Glc*p*NAc3*R*Lac-(1→	(**A**)	95.2	54.9	78.2	72.0	71.2	68.9
→3)-β-L-Rha*p*-(1→	(**B**)	101.7	69.1	79.7	71.3	73.3	17.9
→4)-β-D-Glc*p*NAc-(1→	(**C**)	102.9	56.7	74.7	78.0	75.7	61.9
(*R*)-Lactic acid	(**D**)	182.9	79.4	19.9			

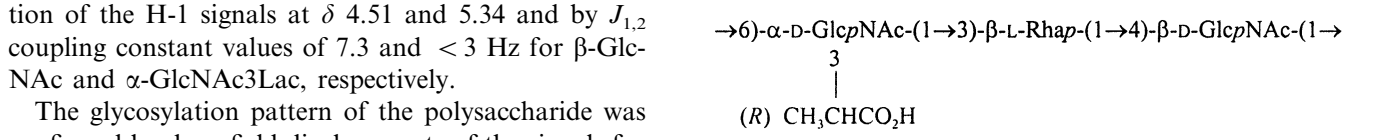
[a] Signals for NAc are at δ 23.2, 23.5 (both Me), 175.3, and 176.6 (both CO).

The presence in the NOESY spectrum (Fig. 2) of strong intraresidue H-1,H-3,5 cross-peaks for Rha and GlcNAc indicated the β configuration of the glycosidic linkages, whereas the presence of a strong H-1,H-2 cross-peak with no H-1,H-3,5 cross-peaks showed the α configuration of GlcNAc3Lac. The anomeric configurations of the amino sugars were confirmed by the position of the H-1 signals at δ 4.51 and 5.34 and by $J_{1,2}$ coupling constant values of 7.3 and < 3 Hz for β-GlcNAc and α-GlcNAc3Lac, respectively.

The glycosylation pattern of the polysaccharide was confirmed by downfield displacements of the signals for C-3 of β-Rha, C-4 of β-GlcNAc, and C-6 of α-GlcNAc3Lac to δ 79.7, 78.0, and 68.9, respectively, as compared with their position at δ 74.0, 71.2, and 61.9 in the corresponding nonsubstituted monosaccharides.[15]

In accordance with *O*-alkylation by the 1-carboxyethyl group, the position of the C-3 signal of α-GlcNAc3Lac shifted downfield by 6.2 ppm as compared with its position in α-GlcNAc.[15] The site of attachment of the lactic acid residue was further confirmed by a correlation between Lac H-2 and α-GlcNAc H-3, which was revealed in a NOESY experiment with the polysaccharide (Fig. 2). The D configuration of the sugar residue in GlcNAc3Lac was confirmed by a relatively small effect on C-1 of α-GlcNAc ($+3.1$ ppm) indicating different absolute configurations of the monosaccharides in the α-Glc*p*NAc-(1→3)-β-L-Rha*p* disaccharide fragment (an effect of > 7 ppm would be observed in case of the same absolute configuration of the constituent monosaccharides[16]).

The NOESY spectrum of the polysaccharide (Fig. 2) showed strong interresidue cross-peaks between the following transglycosidic protons: α-GlcNAc3Lac H-1,β-Rha H-2,3 at δ 5.34/4.07, 3.57; β-Rha H-1,β-GlcNAc H-4 at δ 4.84/3.68; and β-GlcNAc H-1,α-GlcNAc3Lac H-6a,6b at δ 4.51/3.83, 4.04, respectively. These data defined the monosaccharide sequence in the repeating unit and showed that the O-specific polysaccharide of *P. alcalifaciens* O16 has the following structure:



Previously, *N*-acetylmuramic acid, which is an important common constituent of bacterial peptidoglycan, has been found in the O-specific polysaccharide of *Yersinia ruckerii*[17] and the capsular polysaccharide of *Vibrio vulnificus* ATCC 27562.[18] Remarkably, all other known isomeric *O*-(1-carboxyethyl) derivatives of GlcNAc, including 2-acetamido-3-*O*-[(*S*)-1-carboxyethyl]-2-deoxy-D-glucose (*N*-acetylisomuramic acid),[13,19] 2-acetamido-4-*O*-[(*S*)-1-carboxyethyl]-2-deoxy-D-glucose,[20,21] and 2-acetamido-4-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucose (authors' unpublished data), have been found in the O-specific polysaccharides of various O-serogroups of *Proteus*. Further studies will show whether the O-antigen of *P. alcalifaciens* O16 is serologically related to the *Proteus* O-antigens.

1. Experimental

Bacterial strain and growth.—*Providencia alcalifaciens* O16 (strain 19394) from the Hungarian National

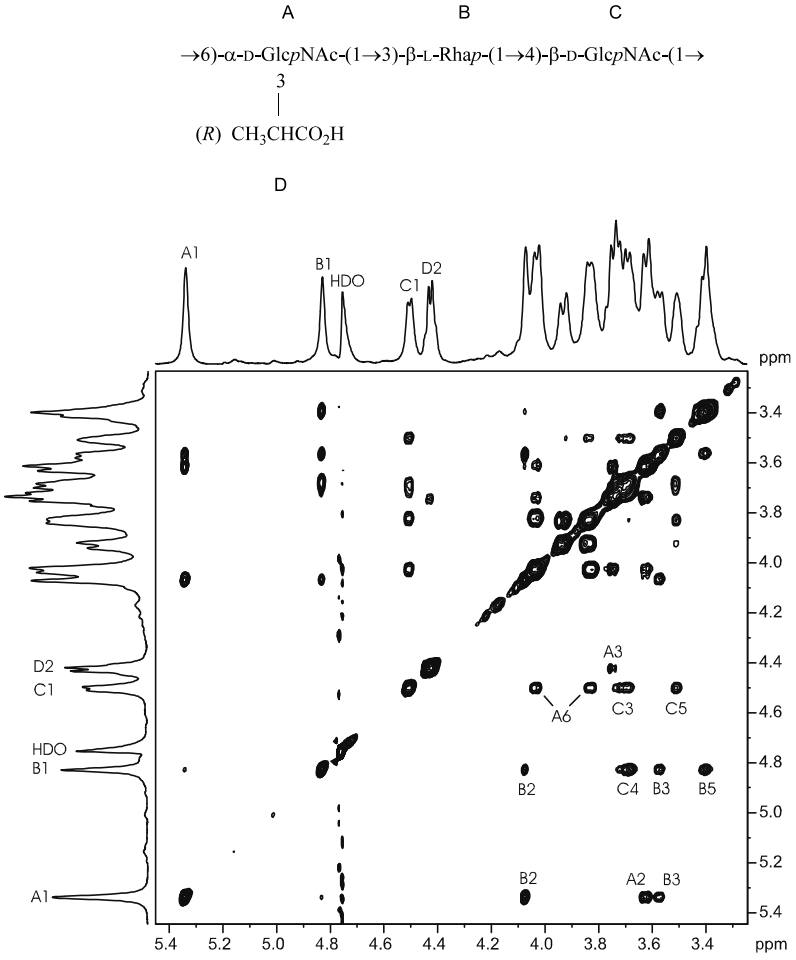


Fig. 2. Part of a 500-MHz NOESY spectrum of the O-specific polysaccharide. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes.

Collection of Medical Bacteria (National Institute of Hygiene, Budapest) was cultivated under aerobic conditions in nutrient broth supplemented with 1% glucose. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water and lyophilised.

Isolation of the lipopolysaccharide and the polysaccharide.—Lipopolysaccharide was isolated from bacterial cells by phenol–water extraction[11] and purified by treatment with cold aq 50% CCl_3CO_2H at 4 °C;[22] the supernatant was dialysed against distilled water and lyophilised. A high-molecular-mass polysaccharide was prepared by degradation of the lipopolysaccharide with aq 2% HOAc at 100 °C for 9 h followed by GPC of the water-soluble portion on a column (60 × 3.0 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer, pH 4.5, with monitoring the elution using a Knauer differential refractometer (Germany). The yield of the polysaccharide was 29% of the lipopolysaccharide weight.

Sugar analysis.—The polysaccharide was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h), the monosaccharides were converted into the alditol acetates[23] and analysed by GLC using a Hewlett–Packard 5880 instrument with a DB-5 capillary column and a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min. The absolute configurations of rhamnose and GlcNAc were determined by GLC of the acetylated glycosides with (+)-2-octanol[12,24] under the same chromatographic conditions as above.

Methylation analysis.—Methylation was performed as described.[25] After hydrolysis with 2 M CF_3CO_2H (120 °C, 2 h), the partially methylated monosaccharides were reduced with $NaBH_4$, acetylated 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.

Isolation of N-*acetylmuramic acid.*—The polysaccharide (17 mg) was treated with anhyd trifluoromethanesulfonic acid[26] (0.5 mL) at 0 °C for 16 h, the reaction mixture was neutralised with aq 5% ammonia at 0 °C and applied to a column (80 × 1.6 cm) of TSK HW-40

in aq 1% HOAc. For further purification, crude *N*-acetylmuramic acid (8 mg) was applied to a column (5×0.8 cm) of a Dowex 1×8 anion-exchange resin (CO_2^--form) in water and eluted with aq 10% HOAc.

NMR spectroscopy.—Samples were deuterium-exchanged by freeze-drying three times from D_2O and then examined in a solution of 99.96% D_2O. Spectra were recorded using a Bruker DRX-500 spectrometer at 25 °C (1D ^{13}C NMR) or 57 °C (1D ^{1}H NMR and 2D NMR spectra). A mixing time of 150 and 200 ms was used in 2D TOCSY and NOESY experiments, respectively. Chemical shifts are reported related to internal acetone (δ_H 2.225; δ_C 31.45).

Acknowledgements

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