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Note

Structure of a colitose-containing O-polysaccharide from the lipopolysaccharide of *Providencia alcalifaciens* O6

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Abstract—The O-polysaccharide was isolated by mild acid degradation of the lipopolysaccharide of *Providencia alcalifaciens* O6 and studied by sugar and methylation analysis, selective hydrolytic removal of 3,6-dideoxy-L-*xylo*-hexose (colitose, Col), ^{1}H and ^{13}C NMR spectroscopy, including 2D ^{1}H,^{1}H COSY, TOCSY, ROESY and H-detected ^{1}H,^{13}C HSQC and HMBC experiments. The polysaccharide was found to have a branched pentasaccharide repeating unit with the following structure:

$$\begin{array}{l} \text{[4)-}\beta\text{-D-Glc}p\text{A-(1}\rightarrow\text{3)-}\beta\text{-D-Glc}p\text{NAc-(1}\rightarrow\text{]}_n \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad 6 \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\qquad\qquad\quad 1 \\ \alpha\text{-Col}p\text{-(1}\rightarrow\text{2)-}\beta\text{-D-Gal}p\text{-(1}\rightarrow\text{3)-}\beta\text{-D-Glc}p\text{NAc} \end{array}$$

Remarkably, the trisaccharide side chain of the O6-polysaccharide represents a colitose ('3-deoxy-L-fucose') analogue of the H type 1 (precursor) antigenic determinant.

Keywords: *Providencia alcalifaciens*; O-antigen; Lipopolysaccharide; Polysaccharide structure; Colitose

Gram-negative bacteria of the genus *Providencia* are facultative pathogens, which under favorable conditions cause enteric diseases as well as wound and urinary-tract infections.[1] These infections are frequently persistent, difficult to treat, and may even result in fatal bacteremia. The genus *Providencia* is divided into six species, including *P. alcalifaciens*, *P. rustigianii*, *P. stuartii*, *P. heimbachae*, *P. rettgerii*, and *P. vermicola*.[1,2] The serological classification scheme of *Providencia* species is based on the lipopolysaccharide (LPS, O-antigen, endotoxin) and flagella (H-antigens). Strains of three species, *P. alcalifaciens*, *P. rustigianii*, and *P. stuartii*, are classified into 63 O-serogroups.[3] Immunochemical studies of *Providencia* O-antigens aim at the creation of the molecular basis for the serological classification and cross-reactivity of *Providencia* strains and related bacteria, including *Proteus*. At present, 28 unique O-polysaccharide structures from 35 O-serogroups have been established (Ref. 4 and references cited therein). In this paper, we report on the structure of a new O-polysaccharide from *P. alcalifaciens* O6.

The LPS was isolated from dry bacterial cells by the phenol–water extraction[5] and degraded under mild acid conditions (2% AcOH, 100 °C, 2.5 h). The subsequent fractionation of the carbohydrate portion by GPC on Sephadex G-50 resulted in a high-molecular-mass polysaccharide (PS-I), which eluted immediately after the

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void volume of the column. Sugar analysis using GLC of the alditol acetates, derived after hydrolysis with 1 M CF_3CO_2H (120 °C, 2 h), showed the presence of 2-amino-2-deoxyglucose, galactose, and a 3,6-dideoxy-*xylo*-hexose (abequose or colitose) in the ratios ~2.6:1.0:0.5. GLC–MS of the acetylated methyl glycosides demonstrated glucuronic acid (GlcA). Determination of the absolute configurations by GLC of the acetylated (+)-2-octyl glycosides showed that GlcN, Gal, and GlcA have the D configuration, whereas the 3,6-dideoxy-*xylo*-hexose has the L configuration and is, thus, colitose (Col).

Linkage analysis by GLC–MS of the partially methylated alditol acetates derived from the methylated PS-I revealed approximately equal amounts of 3,6-disubstituted GlcN, 3-substituted GlcN, 2-substituted Gal, and terminal Gal. In addition to these monosaccharides, similar analysis after carboxyl-reduction of the methylated PS-I demonstrated 4,6-disubstituted Glc, which was evidently derived from 4-substituted GlcA. The absence of methylated Col could be accounted for by its partial removal during mild acid delipidation of the LPS and destruction during acid hydrolysis (1 M CF_3CO_2H, 120 °C, 2 h) of the methylated PS-I.

Alternatively, the LPS was degraded by O-deacylation under basic conditions (0.16 M NaOH, 100 °C, 1 h). Linkage analysis of the partially methylated alditol acetates derived by a milder hydrolysis (0.5 M CF_3CO_2H, 100 °C, 30 min) of the methylated carboxyl-reduced O-deacylated LPS (DLPS) demonstrated the presence of 3,6-disubstituted GlcN, 3-substituted GlcN, 2-substituted Gal, 4,6-disubstituted Glc, terminal Col, and only a negligible amount of terminal Gal.

The ^{13}C NMR spectrum of the PS-I showed a structural heterogeneity, which was caused by a partial loss of Col during mild acid hydrolysis of the LPS. The ^{13}C NMR spectrum of the DLPS (Fig. 1) showed the presence of a pentasaccharide repeating unit. In addition to signals of the core and lipid A moieties, the spectrum contained major signals for five anomeric carbons at δ 100.3–104.6, two nitrogen-bearing carbons at δ 55.8 and 56.0, three HO*C*H_2–C groups (C-6 of GlcN and Gal, data of the attached proton test), from which two were nonsubstituted (δ 62.2, 62.5) and the third was substituted (δ 69.2), one C–*C*H_2–C group at δ 34.2 (Col C-3), one *C*H_3–C group (Col C-6) at δ 17.0, one C–*C*O_2H group at δ 174.4 (GlcA C-6), and two *N*-acetyl groups at δ 23.8, 23.9 (both CH_3), 175.3 and 175.8 (both CO). There were no signals in the region δ 82–88, except for one at δ 83.9, which was later assigned to C-3 of disubstituted GlcN; hence, all sugar residues are pyranosidic.[6] The ^1H NMR spectrum of the DLPS contained signals for five anomeric protons at δ 4.43–5.06, one C–CH_2–C group at δ 1.73 and 1.87 (Col H-3), one C–CH_3 group at δ 1.12 (Col C-6), and two *N*-acetyl groups at δ 1.99 and 2.02.

The ^1H and ^{13}C NMR spectra of the DLPS were assigned using $^1H,^1H$ COSY, TOCSY, ROESY, and H-detected $^1H,^{13}C$ HSQC experiments (Tables 1 and 2). The COSY and TOCSY spectra revealed spin-

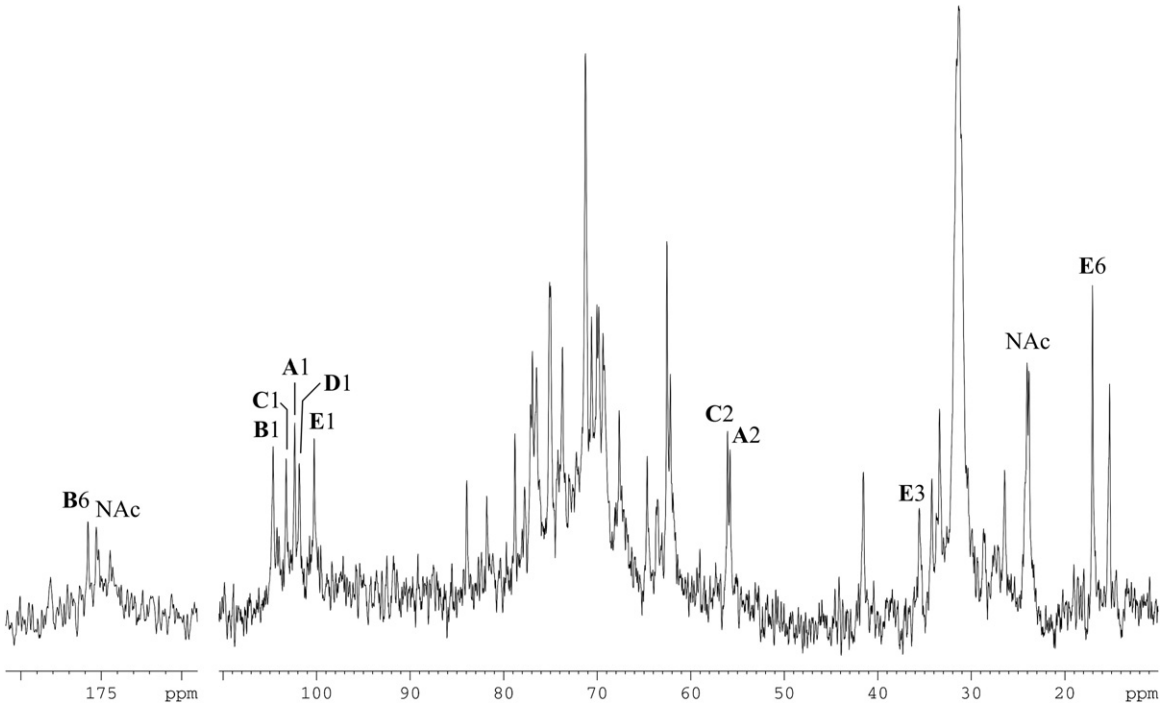

Figure 1. ^{13}C NMR spectrum of the DLPS from *P. alcalifaciens* O6. Arabic numerals refer to carbons in sugar residues denoted by letters as shown in Tables 1 and 2.

Table 1. ^{1}H NMR data (δ, ppm)

Sugar residue		H-1	H-2	H-3 (3ax)	H-3eq	H-4	H-5	H-6 (6a)	H-6b
DLPS									
→3,6)-β-D-Glc*p*NAc-(1→	**A**	4.52	3.79	3.71		3.46	3.43	3.82	4.14
→4)-β-D-Glc*p*A-(1→	**B**	4.44	3.30	3.53		3.70	3.57		
→3)-β-D-Glc*p*NAc-(1→	**C**	4.43	3.66	3.92		3.50	3.69	3.73	3.86
→2)-β-D-Gal*p*-(1→	**D**	4.58	3.58	3.76		3.84	3.63	3.70	3.75
α-Col*p*-(1→	**E**	5.06	3.95	1.73	1.87	3.74	4.12	1.12	
PS-II									
→3,6)-β-D-Glc*p*NAc-(1→	**A**	4.55	3.85	3.77		3.55	3.58	3.86	4.17
→4)-β-D-Glc*p*A-(1→	**B**	4.50	3.36	3.60		3.72	3.79		
→3)-β-D-Glc*p*NAc-(1→	**C**	4.56	3.78	3.74		3.53	3.47	3.78	3.92
β-D-Gal*p*-(1→	**D**	4.40	3.52	3.63		3.90	3.70	3.75	3.75
Colitose									
α-Col*p*		5.14[a]	4.01	1.97		3.86	4.11	1.15	
β-Col*p*		4.55[b]	3.63	1.72	2.21	3.80	3.83	1.20	

The chemical shifts for the *N*-acetyl groups are δ_H 1.99 and 2.02 in the DLPS, δ_H 2.02 and 2.04 in the PS-II.
[a] $J_{1,2}$ 3.5 Hz.
[b] $J_{1,2}$ 8.0 Hz.

systems for three sugar residues having the *gluco* configuration (GlcNAc **A** and **C**, GlcA **B**), one residue with the *galacto* configuration (Gal **D**), and colitose (**E**). In agreement with the *xylo* configuration, colitose has an axial proton H-2 and an equatorial proton H-4 and showed in the ROESY spectrum H-3ax/H-5 and H-3eq/H-2 correlations at δ 1.73/4.12 and 1.87/3.95, respectively. As judged by the $J_{1,2}$ coupling constants ~7 Hz, Gal **D** and GlcNAc **A** are β-linked, whereas Col **E** is α-linked ($J_{1,2} < 3$ Hz). As the H-1 signals of GlcA **B** and GlcNAc **C** were overlapped and poorly resolved, the β configuration of their glycosidic linkages was inferred based on the chemical shift data $\delta_{H\text{-}1}$ 4.44 and 4.43, $\delta_{C\text{-}5}$ 75.1 and 77.1, respectively (compare published data).[7,8]

Significant downfield displacements of the signals for β-GlcNAc **A** C-3 and C-6, β-GlcA **B** C-4, β-GlcNAc **C** C-3, and β-Gal **D** C-2 to δ 83.9, 69.2, 81.8, 78.8, and 77.8, respectively, from their positions in the corresponding non-substituted monosaccharides at δ 74.8, 61.9, 72.7, 74.8, and 73.0[8] confirmed the modes of glycosylation of the monosaccharides determined by methylation analysis. The Col **E** C-2–C-6 signals were characteristic for an unsubstituted residue,[6] thus confirming that Col occupies the terminal position in the side chain. The ROESY spectrum (Fig. 2) showed clear interresidue Gal **D** H-1,GlcNAc **C** H-3 and Col **E** H-1,Gal **D** H-2 cross-peaks at δ 4.58/3.92 and 5.06/3.58, respectively, whereas those between the other anomeric protons and protons at the linkage carbons could not be unambiguously assigned owing to overlapping with intraresidue cross-peaks and appearance of additional unwanted cross-peaks due to spin diffusion (e.g., a GlcNAc **C** H-1,H-4 cross-peak at δ 4.43/3.50).

Table 2. ^{13}C NMR data (δ, ppm)

Sugar residue		C-1	C-2	C-3	C-4	C-5	C-6
DLPS							
→3,6)-β-D-Glc*p*NAc-(1→	**A**	102.3	55.8	83.9	70.0	76.9	69.2
→4)-β-D-Glc*p*A-(1→	**B**	104.6	73.7	75.1	81.8	75.1	174.4
→3)-β-D-Glc*p*NAc-(1→	**C**	103.2	56.0	78.8	69.4	77.1	62.2
→2)-β-D-Gal*p*-(1→	**D**	101.8	77.8	75.0	70.6	76.5	62.5
α-Col*p*-(1→	**E**	100.3	64.6	34.2	69.8	67.6	17.0
PS-II							
→3,6)-β-D-Glc*p*NAc-(1→	**A**	102.5	55.9	83.8	69.4	75.3	69.7
→4)-β-D-Glc*p*A-(1→	**B**	104.5	73.8	75.1	82.0	77.0	[a]
→3)-β-D-Glc*p*NAc-(1→	**C**	102.9	55.8	83.8	70.0	76.8	62.1
β-D-Gal*p*-(1→	**D**	105.0	72.1	73.9	70.0	76.7	62.4
Colitose							
α-Col*p*		93.0	64.6	33.8	69.8	67.5	17.0
β-Col*p*		99.5	67.9	38.5	69.9	75.7	17.2

The chemical shifts for the *N*-acetyl groups are δ_C 23.8, 23.9 (both CH_3), 175.3 and 175.8 (both CO) in the DLPS, δ_C 23.8, 23.9 (both CH_3), 176.1 and 176.3 (both CO) in the PS-II.
[a] Not found.

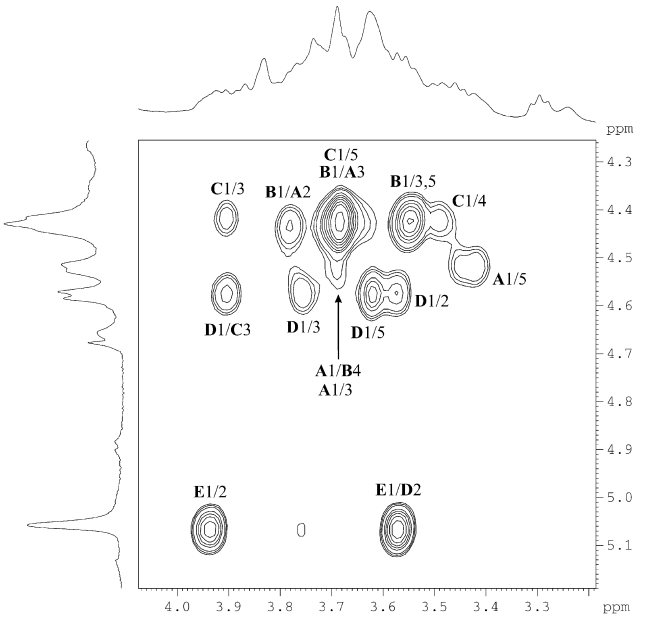

Figure 2. Part of a 2D ROESY spectrum of the DLPS of *P. alcalifaciens* O6. The corresponding parts of the ^{1}H NMR spectrum are shown along the axes. Arabic numerals refer to protons in sugar residues denoted by letters as shown in Tables 1 and 2.

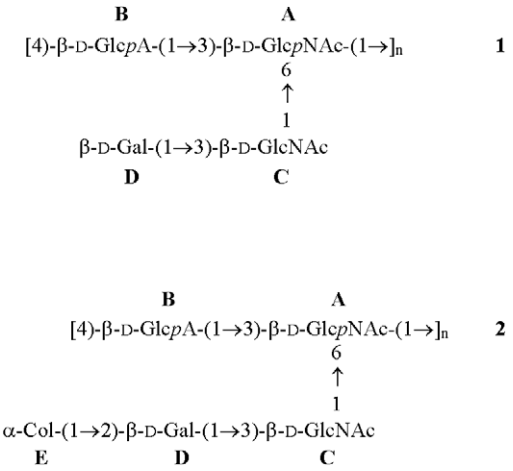

Chart 1. Structures of the PS-II (**1**) and the O-polysaccharide of *P. alcalifaciens* O6 (**2**).

To overcome the problem the LPS was degraded for a longer time (2% AcOH, 100 °C, 17 h) to give colitose and a colitose-free polysaccharide (PS-II) isolated by GPC on TSK HW-40. The PS-II was studied by NMR spectroscopy as described above for the DLPS. The assigned ^{1}H and ^{13}C NMR chemical shifts (Tables 1 and 2) showed the expected changes in unit **D** as well as in the next unit **C** caused by the loss of (1→2)-linked colitose residue (unit **E**) from the sterically hindered **E**-(1→2)-**D**-(1→3)-**C** fragment. In addition, a downfield displacement was observed for the H-5 signal of unit **B** (GlcA) and accounted for by a lower pD of the PS-II solution as compared to that of DLPS.

Sequence analysis of PS-II was performed using ROESY and ^{1}H,^{13}C HMBC experiments. The ROESY spectrum demonstrated the following interresidue cross-peaks: GlcA **B** H-1,GlcNAc **A** H-3 at δ 4.50/3.77; GlcNAc **C** H-1,GlcNAc **A** H-6a at δ 4.56/3.86; and Gal **D** H-1,GlcNAc **C** H-3 at δ 4.40/3.74. The HMBC spectrum confirmed the **D**→**C** and **B**→**A** fragments and the overall **B**→(**E**→**D**→**C**)**A** sequence. The **A**→**B** linkage could not be unambiguously proved from either ROESY or HMBC spectra owing to strong overlapping but was inferred taking into account the substitution of GlcA **B** at position 4 (see above).

Therefore, the PS-II has structure **1** and, hence, the O-polysaccharide of *P. alcalifaciens* O6 has structure **2** shown in Chart 1. Remarkably, the α-Col*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc trisaccharide side chain of the O-polysaccharide represents a colitose ('3-deoxy-L-fucose') analogue of the H type 1 α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc antigenic determinant, a precursor of the Lewis b antigenic determinant, which is expressed by normal and tumor tissues and is especially abundant in the epithelial cells of gastric mucosa. Such resemblance between a bacterial antigen and a host antigen is considered as a molecular mimicry, which may be beneficial for the persistence of infection.[9]

1. Experimental

1.1. Bacterial strain and isolation of the LPS

Providencia alcalifaciens O6:H6, strain 2634 obtained from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest) was cultivated under aerobic conditions in tryptic soy broth supplemented with 0.6% yeast extract. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water and lyophilized. The LPS was isolated in a yield of 2.8% of dry bacterial weight by the phenol–water extraction[5] followed by dialysis of the extract without layer separation and freed from insoluble contaminations by centrifugation. The resultant crude LPS soln was treated with cold aq 50% CCl_3CO_2H; after centrifugation the supernatant was dialyzed and freeze-dried.

1.2. Degradation of the LPS

A LPS sample (100 mg) was heated with 2% AcOH for 2.5 h at 100 °C, a lipid precipitate was removed by centrifugation, and the carbohydrate-containing supernatant fractionated by GPC on a column (60 × 2.5 cm) of Sephadex G-50 in 0.05 M pyridinium acetate buffer, pH 4.5 to give PS-I (70 mg).

A portion of PS-I (11 mg) was hydrolyzed with 2% AcOH for 17 h at 100 °C, and the products were fractionated by GPC on a column (80 × 1.5 cm) of TSK HW-40 in 1% AcOH to give PS-II (6 mg) and colitose (1.4 mg).

For O-deacylation, a LPS sample (100 mg) was heated with 0.16 M NaOH for 1 h at 100 °C, neutralized with 1 M HCl and fractionated by GPC on Sephadex G-50 as above. The yield of the DLPS was 61 mg.

1.3. NMR spectroscopy

Samples were freeze-dried twice from a ^2H_2O soln and dissolved in 99.96% ^2H_2O with internal TSP (δ_H 0) and acetone (δ_C 31.45) as references. ^1H and ^{13}C NMR spectra were recorded at 30 °C on a Bruker DRX-500 spectrometer, using a SGI Indy/Irix 5.3 workstation and XWINNMR software. Mixing time of 300 ms and spin-lock time of 30 ms were used in ROESY and TOCSY experiments, respectively. Other NMR experimental parameters were essentially as described previously.[10]

1.4. GLC and GLC–MS

Analyses were performed on a Hewlett-Packard HP 5890 chromatograph equipped with a Ultra-2 capillary column (Hewlett-Packard) using a temperature gradient from 160 to 290 °C at 7 °C min^{-1} (A); a Hewlett-Packard 5971A GLC–MS system with a HP-1 capillary column using a temperature program of 150–270 °C at 8 °C min^{-1} (B); and a ThermoQuest Finnigan model Trace series GC 2000 GLC–MS instrument equipped with an EC-1 column using a temperature gradient from 150 °C (2 min) to 250 °C at 10 °C min^{-1} (C).

1.5. Chemical methods

For sugar analysis, a PS-I sample was hydrolyzed with 1 M CF_3CO_2H (120 °C, 2 h) and reduced with an excess of $NaBH_4$ (20 °C, 2 h) (for neutral and amino sugars) or subjected to methanolysis (1 M HCl/MeOH, 85 °C, 24 h) (for uronic acid). The products were acetylated with a 1:1 Ac_2O–pyridine mixture (100 °C, 1 h) and analyzed by GLC (A).

For determination of the absolute configurations of the monosaccharides, a PS-I sample was subjected to methanolysis (1 M HCl/MeOH, 100 °C, 16 h). The products were heated with (+)-2-octanol[11] (100 μL) in the presence of CF_3CO_2H (15 μL) at 120 °C for 16 h, acetylated and analyzed by GLC–MS (B). For determination of the absolute configuration of colitose, a DLPS sample was hydrolyzed with 0.5 M CF_3CO_2H (100 °C, 30 min), then subjected to (+)-2-octanolysis as above, acetylated and analyzed by GLC (A).

A PS-I sample was methylated according to the Hakomori procedure,[12] the products were recovered using a Sep-Pak cartridge and divided into two parts, one of which was reduced with $LiBH_4$ in aq 70% 2-propanol (20 °C, 2 h). Partially methylated monosaccharides were derived by hydrolysis with 1 M CF_3CO_2H (120 °C, 2 h), converted into the alditol acetates and analyzed by GLC–MS (B). A DLPS sample was treated prior to methylation with an Amberlite IR-120 (H^+) cation-exchange resin to remove cations. Partially methylated monosaccharides were obtained by hydrolysis of the permethylated carboxyl-reduced DLPS with 0.5 M CF_3CO_2H (100 °C, 30 min), converted into the alditol acetates and analyzed by GLC–MS (C).

Acknowledgments

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