Carbohydrate Research 344 (2009) 683–686

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Note

Structure of the O-polysaccharide from the lipopolysaccharide of *Providencia alcalifaciens* O31 containing an ether of D-mannose with (2*R*,4*R*)-2,4-dihydroxypentanoic acid

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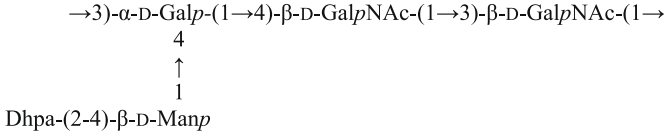
ARTICLE INFO

Article history:
Received 7 December 2008
Received in revised form 6 January 2009
Accepted 6 January 2009
Available online 16 January 2009

Keywords:
Providencia alcalifaciens
O-antigen
Lipopolysaccharide
Bacterial polysaccharide structure
2,4-Dihydroxypentanoic acid
NMR analysis

ABSTRACT

Recently, ether-linked diastereomeric 2,4-dihydroxypentanoic acids have been reported as new components of bacterial glycans [Shashkov, A. S. et al. *Nat. Prod. Commun.* **2008**, *3*, 1625–1630]. In this work, an ether of (*2R,4R*)-2,4-dihydroxypentanoic acid (Dhpa) with D-mannose was identified in the O-polysaccharide of *Providencia alcalifaciens* O31, and the polysaccharide structure was elucidated. Studies by NMR spectroscopy confirmed the ether linkage between O-2 of Dhpa and O-4 of Man, and the absolute configuration of Man was determined after ether cleavage with boron trichloride. In the polysaccharide, Dhpa was found to exist partially in the form of 1,4-lactone. Using sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including 2D $^{1}H,^{1}H$ COSY, TOCSY, ROESY, H-detected $^{1}H,^{13}C$ HSQC, and gHMBC experiments, the following structure of the tetrasaccharide repeating unit of the polysaccharide was established:



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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 subdivided into six species, including *P. alcalifaciens*, *P. rustigianii*, *P. stuartii*, *P. heimbachae*, *P. rettgeri,* and *P. vermicola.*[1,2] The existing serological scheme of three *Providencia* species used in serotyping of clinical isolates is based on the lipopolysaccharide (LPS) O-antigens and flagella H-antigens, and includes 63 O-serogroups and 30 H-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, chemical structures of the O-polysaccharides have been established for about half *Providencia* O-serogroups and a number of uncommon sugar derivatives identified as their components.[4] Recently, ether-linked diastereomeric 2,4-dihydroxypentanoic acids have been reported as new components of bacterial glycans.[5] In this work, we identified an ether of (*2R,4R*)-2,4-dihydroxypentanoic acid (Dhpa) with D-mannose as a component of one of the glycans, namely, the O-polysaccharide of *Providencia alcalifaciens* O31. The polysaccharide structure was established as well.

The LPS was isolated from dry bacterial cells by the phenol–water[6] extraction and degraded under mild acid conditions. The subsequent fractionation of the carbohydrate portion by GPC on Sephadex G-50 gave a high-molecular-mass polysaccharide. Sugar analysis of the polysaccharide using GLC–MS of the alditol acetates derived after acid hydrolysis showed the presence of Gal, GalNAc, and an unknown low-volatile derivative with retention times 18.90, 21.89, and 27.91 in the ratios 1.0:1.3:0.8 (detector response), respectively. Based on the fragmentation pattern in the electron impact mass spectrum, the last compound was assigned the structure of an ether-interlinked hexitol and 1,2,4-pentanetriol (**1**) (Chart 1). Further studies clarified the origin of **1** as a product of

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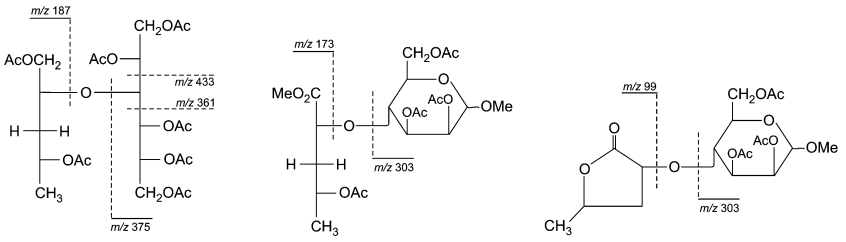
doi:10.1016/j.carres.2009.01.003



1 2 3

Chart 1. Structures of Dhpa-Man ether derivatives obtained in sugar analyses of acetylated alditols (**1**) and acetylated methyl glycosides (**2** and **3**).

reduction of an ether of Man with Dhpa, evidently via Dhpa 1,4-lactone (Dhpl) formation. The assignment of **1** was confirmed by GLC–MS analysis of the acetylated methyl glycosides, which revealed two derivatives from the same Man ether, one containing Dhpa methyl ester (**2**, minor) and the other 1,4-lactone (**3**, major) (Chart 1).

Determination of the absolute configurations by GLC of the acetylated (+)-2-octyl glycosides[7] showed that Gal and GalN have the D configuration. The D configuration of Man was determined by a similar analysis after cleavage of the Dhpa ether with boron trichloride.[8] The (2*R*,4*R*) configuration of Dhpa has been established earlier[5] by studies of the Dhpl ether using NOE spectroscopy (2D ROESY) combined with molecular modeling.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) showed a structural heterogeneity, which was caused by the presence of either Dhpa or Dhpl on Man. The spectrum contained signals for four sugar residues, including those for 4 anomeric carbons at δ 101.7, 102.1, and 104.6 (2C), four HO*C*H$_2$–C groups at δ 61.5–62.5, 2 nitrogen-bearing carbons at δ 52.7 and 53.8, 18 oxygen-bearing carbons in the region δ 67.2–80.8, two C–*C*H$_2$–C groups at δ 37.4 (major) and 43.7 (minor) (data of attached-proton test), two *C*H$_3$–CH groups at δ 21.6 (major) and 23.4 (minor), two *C*H$_3$–CO groups at δ 23.8 and 23.9 as well as three CO groups at δ 175.4, 176.2, and 179.0.

The ^{1}H NMR spectrum of the polysaccharide (Fig. 2) contained six signals in the low-field region, including those for four anomeric protons at δ 4.59, 4.67, 4.95, and 5.03, and two multiplets at δ 4.74 and 4.91. In the high-field region, there were signals for two *CH*$_3$–CH groups at δ 1.25 (minor) and 1.39 (major), two *CH*$_3$–CO groups at δ 2.03 and 2.06, and two CH–*CH*$_2$–CH groups at δ 1.79, 1.89 (both minor), 2.40, and 2.52 (both major).

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using ^{1}H, ^{1}H COSY, TOCSY, ^{1}H, ^{13}C HSQC, and gHMBC experiments (Table 1). The COSY and TOCSY spectra revealed six spin systems, including those for three sugar residues having the *galacto* configuration (Gal **A**, GalN **B**, and GalN **C**), one monosaccharide with the *manno* configuration (Man **D**), 2,4-dihydroxypentanoic acid, and its 1,4-lactone (Dhpa **E** and Dhpl **E′**, respectively). As judged by $J_{1,2}$ coupling constants, both GalN residues are β-linked ($J_{1,2}$ 8 Hz), and Gal is α-linked ($J_{1,2}$ <3 Hz, signal not resolved). The β configuration of Man was inferred by the H-5 and C-5 chemical shifts at δ_H 3.46 and δ_C 76.1 (compare published data[9] δ_H 3.82 and δ_C 73.34 for α-mannopyranose, δ_H 3.38, and δ_C 77.00 for β-mannopyranose).

Significant downfield displacements of the signals for α-Gal **A** C-3 and C-4, β-GalN **B** C-4, β-GalN **C** C-3 and β-Man **D** C-4 to δ 78.9, 77.5, 78.0, 80.8, and 78.0, respectively, compared with their positions in the corresponding non-substituted monosaccharides[9] at δ 70.13, 70.28, 68.85, 72.01, and 67.69, respectively, revealed the substitution pattern of the monosaccharides in the repeating unit.

The ROESY spectrum of the polysaccharide (Fig. 3) showed *inter*-residue cross-peaks between the anomeric protons and protons at the linkage carbons, which, taking into account the positions of

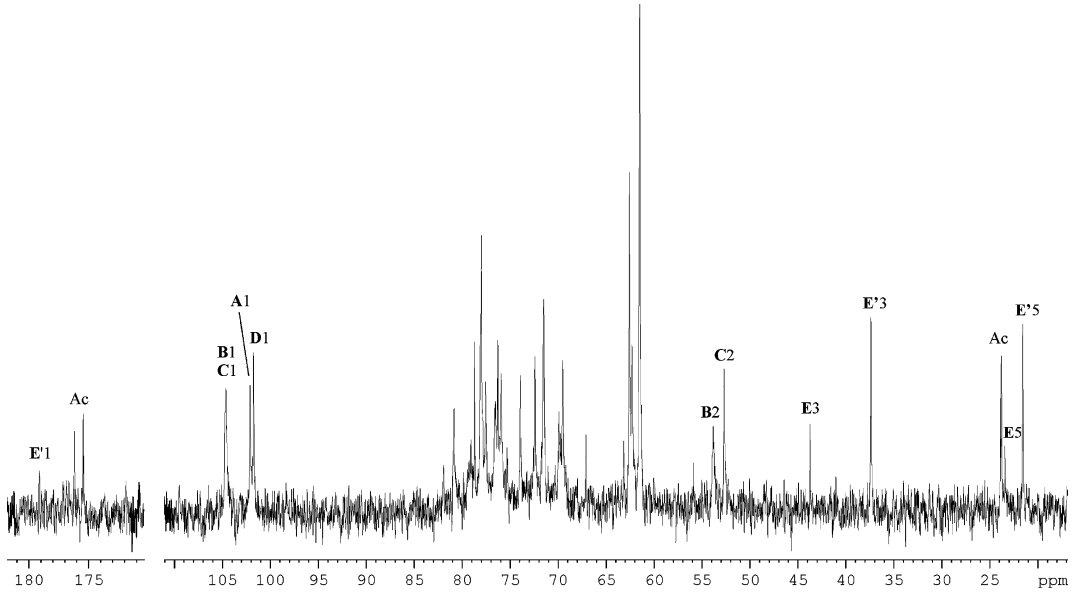


Figure 1. ^{13}C NMR spectrum of the O-polysaccharide of *P. alcalifaciens* O31. Arabic numerals refer to carbons in sugar and Dhpa residues denoted by letters as shown in Table 1.

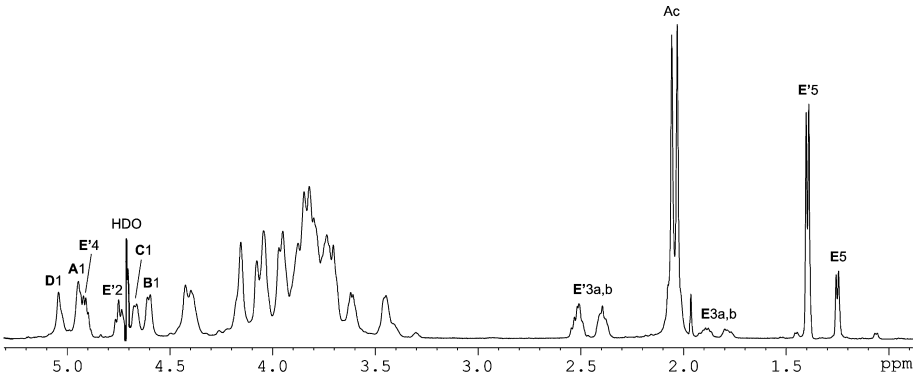


Figure 2. [1]H NMR spectrum of the O-polysaccharide of *P. alcalifaciens* O31. Arabic numerals refer to protons in sugar and Dhpa residues denoted by letters as shown in Table 1.

Table 1
[1]H and [13]C NMR data (δ, ppm) of the O-polysaccharide of *P. alcalifaciens* O31

Residue		H-1	H-2	H-3a,b	H-4	H-5	H-6a	H-6b
→3,4)-α-D-Gal*p*-(1→	**A**	4.95	3.96	4.16	4.43	4.40	3.62	3.75
→4)-β-D-Gal*p*NAc-(1→	**B**	4.59	3.95	3.84	4.04	3.74	3.82	3.87
→3)-β-D-Gal*p*NAc-(1→	**C**	4.67	4.05	3.86	4.16	3.71	3.79	3.79
β-D-Man*p*-(1→	**D**	5.03	4.07	3.84	3.71	3.46	3.79	3.96
2,4-Dhpa-(2-[a]	**E**		4.37	1.79, 1.89	4.05	1.25		
2,4-Dhpl-(2-[b]	**E′**		4.74	2.40, 2.52	4.91	1.39		
		C-1	C-2	C-3	C-4	C-5	C-6	
→3,4)-α-D-Gal*p*-(1→	**A**	102.1	69.9	78.9	77.5	71.5	61.5	
→4)-β-D-Gal*p*NAc-(1→	**B**	104.6	53.8	71.5	78.0	76.3	61.5	
→3)-β-D-Gal*p*NAc-(1→	**C**	104.6	52.7	80.8	69.5	75.9	62.5	
β-D-Man*p*-(1→	**D**	101.7	72.5	73.9	78.0	76.1	62.3	
2,4-Dhpa-(2-[a]	**E**	n.d.	71.6	43.7	67.2	23.4		
2,4-Dhpl-(2-[b]	**E′**	179.0	78.0	37.4	78.7	21.6		

The chemical shifts for the *N*-acetyl groups are δ_H 2.03, 2.06, δ_C 23.8, 23.9 (both CH_3), 175.4, and 176.2 (both CO).
[a] (*2R,4R*)-2,4-Dihydroxypentanoic acid.
[b] (*2R,4R*)-2,4-Dihydroxypentanoic acid, 1,4-lactone.

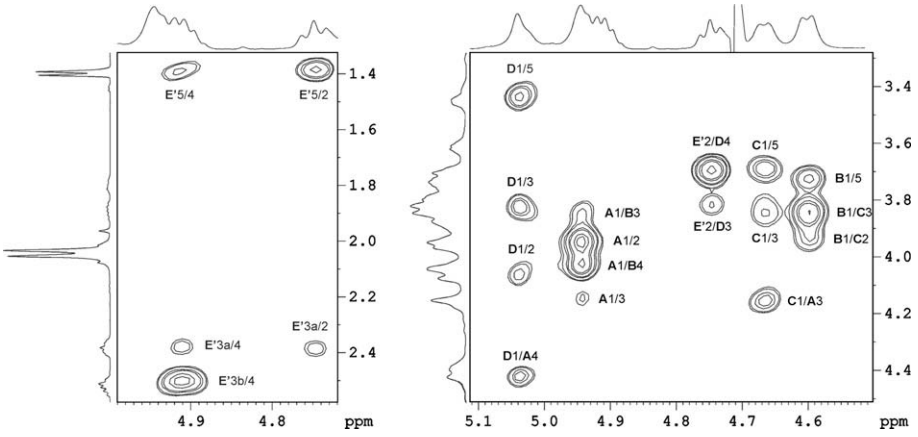


Figure 3. Parts of a 2D ROESY spectrum of the O-polysaccharide of *P. alcalifaciens* O31. The corresponding parts of the [1]H NMR spectrum are shown along the axes. Arabic numerals refer to protons in sugar and Dhpa residues denoted by letters as shown in Table 1.

glycosylation of the monosaccharides, could be interpreted as follows: **B** H-1,**C** H-3 at δ 4.59/3.86; **C** H-1,**A** H-3 at δ 4.67/4.16; **A** H-1,**B** H-4 at δ 4.95/4.04, and **D** H-1,**A** H-4 at δ 5.03/4.43. These data confirmed the glycosylation pattern and established the monosaccharide sequence in the repeating unit. Correlations between **E′** H-2 and **D** H-4 at δ 4.74/3.71 in the ROESY spectrum and between E′ H-2 and **D** C-4 at δ 4.74/78.0 in the gHMBC spectrum confirmed the location of Dhpl at position 4 of Man.

The sugar substitution pattern was confirmed by linkage analysis using GLC–MS of the partially methylated alditol acetates derived from the methylated polysaccharide. In addition to the expected 3,4-disubstituted Hex (from Gal), 3-substituted HexN, and 4-substituted HexN (both from GalN), analysis revealed terminal Hex and 4-substituted Hex in the ratios 1.0:0.4:0.7:0.6:1.3 (detector response), respectively. The derivatives of the last two could result from methylated Dhpa-(2-4)-Man by partial replacement of Dhpa with Me in

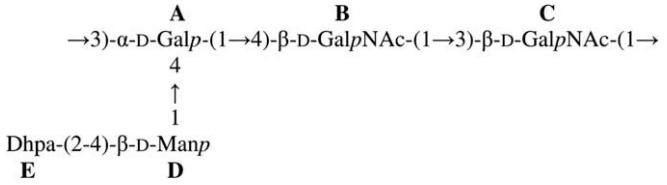


Chart 2. Structure of the O-polysaccharide of *P. alcalifaciens* O31. Dhpa stands for (2*R*,4*R*)-2,4-dihydroxypentanoic acid partially present as 1,4-lactone.

the course of methylation and partial cleavage of the ether bond during acid hydrolysis, respectively. Underestimation of the Gal and GalN derivatives could be accounted for by their poor release from the GalN→GalN→Gal trisaccharide derivative on acid hydrolysis of the methylated polysaccharide.

The data obtained indicated that the O-polysaccharide of *P. alcalifaciens* O31 contains an ether of D-mannose with (2*R*,4*R*)-2,4-dihydroxypentanoic acid (or its 1,4-lactone), and has the structure as shown in Chart 2. Recently, an ether of diastereomeric (2*S*,4*R*)-2,4-dihydroxypentanoic acid with D-GlcNAc was found in *P. alcalifaciens* O8.[5,10] Interestingly, in O-polysaccharides of various *Providencia* O-serogroups some other uncommon components also occur in two stereoisomeric forms, for example, N-linked D- and L-aspartic acid, ether-linked (*S*)- and (*R*)-lactic acid, and carboxyl-linked N^{ε}-[(*S*)- and (*R*)-1-carboxyethyl]-L-lysine (for polysaccharide structures see Bacterial Carbohydrate Structure Database at http://www.glyco.ac.ru/bcsdb).

1. Experimental

1.1. Bacterial strain, isolation, and degradation of the LPS

P. alcalifaciens O31:H^-, strain 5867 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 by the phenol–water extraction[6] followed by dialysis of the extract without layer separation. Insoluble contaminations were removed by centrifugation, and the crude LPS soln was acidified with cold aq 50% CCl_3CO_2H to pH 2; after centrifugation the supernatant was dialyzed and freeze-dried to give purified LPS in a yield of 5.4% of dry bacterial cells mass.

A LPS sample (150 mg) was heated with 2% AcOH for 140 min at 100 °C, a lipid A precipitate was removed by centrifugation, and the supernatant fractionated by GPC on a column (60 × 2.5 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer, pH 4.5. The yield of the polysaccharide was 9% of the LPS mass.

1.2. Chemical methods

For sugar analysis, a polysaccharide sample was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h) and reduced with an excess of $NaBH_4$ in water (20 °C, 2 h) or subjected to methanolysis (0.5 M HCl in methanol, 85 °C, 1 h). The products were acetylated with a 1:1 Ac_2O–pyridine mixture (100 °C, 1 h) and analyzed by GLC–MS using an Agilent MSD 5975 instrument equipped with a HP-5ms column using a temperature gradient from 150 °C (3 min) to 320 °C at 5 °C min^{-1}.

For determination of the absolute configuration of Gal and GalN, a polysaccharide sample was hydrolyzed as mentioned above, N-acetylated (60 μL Ac_2O in 400 μL aq saturated $NaHCO_3$, 0 °C, 1 h), heated with (+)-2-octanol[7] (100 μL) in the presence of CF_3CO_2H (15 μL) (120 °C, 16 h), acetylated, and analyzed by GLC on a Hewlett–Packard HP 5890 chromatograph equipped with an Ultra-1 capillary column (Hewlett–Packard) using a temperature gradient from 160 to 290 °C at 7 °C min^{-1}. For determination of the absolute configuration of Man, a polysaccharide sample (2 mg) was suspended in dry CH_2Cl_2 (1 mL) and treated[8] with boron trichloride (2 g). The product was hydrolyzed with 2 M CF_3CO_2H, subjected to (+)-2-octanolysis and studied by GLC as above.

Methylation of the polysaccharide was performed by the procedure of Ciucanu and Kerek[11] with CH_3I in dimethylsulfoxide in the presence of powdered NaOH. Partially methylated monosaccharides were obtained by hydrolysis with 2 M CF_3CO_2H, converted into the alditol acetates, and analyzed by GLC–MS as mentioned above.

1.3. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying twice from D_2O and then examined as solns in 99.96% D_2O. NMR spectra were recorded on a Bruker DRX-500 spectrometer at 30 °C using internal TSP (δ_H 0.0) and acetone (δ_C 31.45) as references. Bruker standard pulse sequences COSY, TOCSY (spin-lock time 200 ms), ROESY (mixing time 100 ms), HSQC, and gHMBC (long-range transfer delay 60 ms) were used. Other NMR experimental parameters were set essentially as described.[12]

Acknowledgments

Authors thank Mr. H. Moll (Research Center Borstel, Germany) for help with GLC–MS. This work was supported by the Russian Foundation for Basic Research (Project 05-04-48439).

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