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Note

Structure of the O-polysaccharide of *Providencia alcalifaciens* O8 containing (2*S*,4*R*)-2,4-dihydroxypentanoic acid, a new non-sugar component of bacterial glycans

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ABSTRACT

A glycerol teichoic acid-like O-polysaccharide was obtained by mild acid degradation of the lipopolysaccharide of *Providencia alcalifaciens* O8 and studied by chemical methods and NMR spectroscopy, including 2D ROESY, {^{1}H,^{13}C} HSQC, and HMQC-TOCSY experiments. It was found that the compound contains a new component of bacterial lipopolysaccharides: ether-linked (2*S*,4*R*)-2,4-dihydroxypentanoic acid (Dhpa), which was identified by NMR spectroscopy. The following structure of the repeating unit of the polysaccharide was established:

Dhpa
2
|
4
→3)-β-D-Fuc*p*NAc4N-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→2)-D-Gro-3-P-(O→

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Providencia is a genus of *Enterobacteriaceae*, which, together with genera *Proteus* and *Morganella*, is included in the tribe *Proteae*. Bacteria *Providencia* are facultative pathogens, which under favorable conditions may cause various infections, mainly urinary tract infections, wound infections, and enteric diseases.[1,2] The serological O-specificity of *Providencia* is defined by the structure of the O-polysaccharide chain (O-antigen) of the lipopolysaccharide. Strains of three *Providencia* species, *P. alcalifaciens*, *P. rustigianii*, and *P. stuartii*, are classified into 63 O-serogroups.[3] Immunochemical studies of *Providencia* O-antigens aim at creation of the molecular basis for the serological classification and cross-reactivity of *Providencia* strains and related bacteria, including *Proteus*.

Currently, chemical structures of the O-polysaccharides have been established for about half of *Providencia* O-serogroups.[4] Most of them are acidic due to the presence of uronic acids, ulosonic acids, amides of uronic acids with amino acids, ether-linked lactic acids, acetal-linked pyruvic acid, or phosphate. Now we report on the structure of the O-polysaccharide of *P. alcalifaciens* O8 containing glycerol phosphate and 2,4-dihydroxypentanoic acid.

A high-molecular-mass polysaccharide (PS) was isolated by mild acid degradation of the lipopolysaccharide of *P. alcalifaciens* O8 followed by GPC of the carbohydrate portion on Sephadex G-50. Full acid hydrolysis of the PS gave galactose, which was identified by GLC as the alditol acetate. Further studies showed that the polysaccharide contained also glycerol phosphate and two more monosaccharides, 2-acetamido-4-amino-2,4,6-trideoxygalactose (FucNAc4N) and an ether of GlcNAc with 2,4-dihydroxypentanoic acid (Dhpa). The FucNAc4N was not detected in GLC as it was fully destroyed in the course of acid hydrolysis[5], and GlcNAc-Dhpa owing to too high retention time. The D configuration of Gal and FucNAc4N (after conversion to 2-acetamido-2,6-dideoxyglucose by nitrous acid deamination) was determined by GLC of the acetylated glycosides with (*S*)-2-octanol,[6] and the D configuration of GlcNAc was confirmed by ^{13}C NMR spectroscopy (see below). The absolute configuration of glycerol was established using TEMPO oxidation of the PS followed by hydrolysis and GLC analysis of the acetylated (*S*)-2-octyl L-glycerate.[7] GLC–MS of the partially methylated alditol acetates derived from the methylated N-acetylated PS revealed 3-substituted Gal.

The PS was treated with aq ammonia to cleave a Dhpa lactone and dephosphorylated with aq 48% HF to yield an oligosaccharide (OS1), which was studied using 1D and 2D NMR spectroscopy. The

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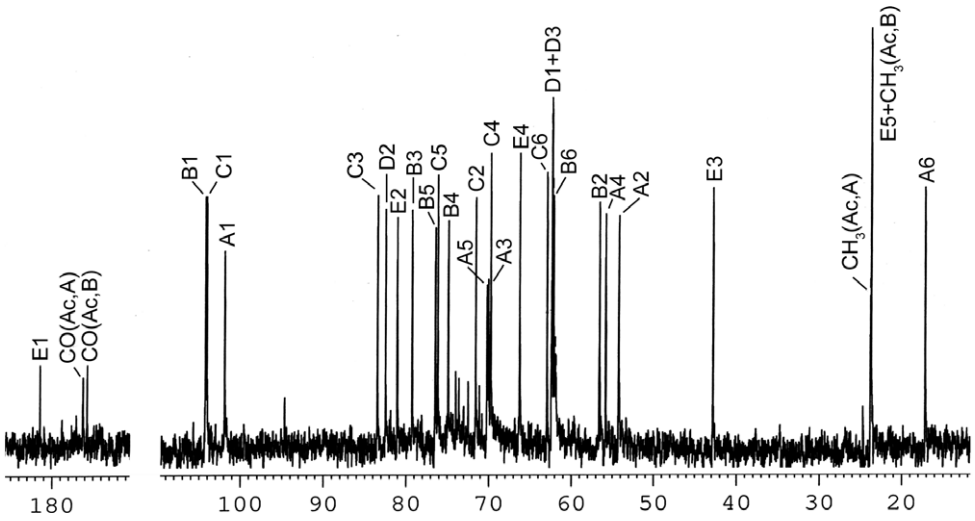

Figure 1. ^{13}C NMR spectrum of OS1.

major subset of the ^{13}C NMR spectrum (Fig. 1) contained signals for three anomeric carbons at δ 104.1, 104.3, and 101.9, three nitrogen-bearing carbons at δ 54.2, 55.8 and 56.6, four C–CH_2OH groups (δ 62.2–63.0), one C–CH_3 group at δ 17.2, two N-acetyl groups at δ 175.7, 176.2 (CO) and δ 23.7, 23.8 (CH_3), one carboxyl group at δ 181.4 and 18 other signals.

The ^{1}H NMR spectrum of the OS1 contained signals for three anomeric protons of β-sugars at δ 4.51, 4.57, 4.65 (d, $^3J_{H1-H2}$ > 7 Hz), two CH–CH_3 groups at δ 1.23 and 1.31, two CO–CH_3 groups at δ 2.01 and 2.04, a multiplet (2H) at δ 1.82, and other protons in the region δ 3.24–4.41.

The ^{1}H and ^{13}C NMR spectra of the OS1 were assigned using ^{1}H,^{1}H COSY, TOCSY, ROESY, ^{1}H,^{13}C HSQC, and ^{1}H,^{13}C HMBC experiments (Tables 1 and 2). The COSY and TOCSY (Fig. 2) spectra revealed the presence of five spin systems (marked as **A**, **B**, **C**, **D**, E in Tables 1 and 2 and as superscript indices below).

*Spin system **A***. Correlations from **A** H-1 to **A** H-4 were found in COSY, and corresponding carbons from **A** C-1 to **A** C-4 were assigned using the HSQC data (Fig. 3). The CH_3 signal at δ 1.31 had a correlation with **A** C-4 in the HMBC spectrum, and thus was assigned to this spin system. **A** C-5 and **A** H-5 were assigned from **A** C-5/**A** H-6 correlation in HMBC (δ 70.2/1.31) and **A** H-5/**A** H-6 (δ 3.80/1.31) correlation in COSY. Chemical shifts of **A** C-2 (54.2) and **A** C-4 (55.8) indicated that these carbons bore nitrogens. The line shape for **A** H-1/**A** H-2, H-3, H-4 signals in the TOCSY spectrum indicated the *galacto*-configuration of this residue, which was confirmed by carbon chemical shifts. Thus, spin system A corresponds to β-Fuc*p*N4N.

*Spin system **B***. The COSY spectrum revealed all H–H correlations within this spin system except H-4/H-5 due to the coincidence of the latter. TOCSY data confirmed these correlations and provided the line shape of **B** H-2 and **B** H-3 (triplets at δ 4.65/3.86 and δ 4.65/4.04 accordingly) typical for *gluco*-configuration. The ^{13}C NMR spectrum of this spin system was assigned using HSQC spectrum (Fig. 3), confirmed β-*gluco*-configuration, and indicated that **B** C-2 bore nitrogen. Thus, spin system **B** is β-Glc*p*N.

Table 1
^{1}H NMR data (δ, ppm)

Residue	Spin system	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
OS1								
β-FucpNAc4N-(1→	**A**	4.57	3.62	3.99	3.24	3.80	1.31	
NAc at C-2		—	2.01					
→3,4)-β-GlcpNAc-(1→	**B**	4.65	3.86	4.04	3.56	3.55	3.80	3.92
NAc at C-2		—	2.04					
→3)-β-Galp-(1→	**C**	4.51	3.59	3.71	4.13	3.69	3.70	3.73
→2)-Gro	**D**	3.71[a]	3.92	3.76[a]				
Dhpa-(2→	**E**	—	4.41	1.82	3.94	1.23		

[a] Signals may be interchanged.

Table 2
^{13}C NMR data (δ, ppm)

Residue	Spin system	C-1	C-2	C-3	C-4	C-5	C-6
OS1							
β-FucpNAc4N-(1→	**A**	101.9	54.2	70.1	55.8	70.2	17.2
NAc at C-2		176.2	23.8				
→3,4)-β-GlcpNAc-(1→	**B**	104.3	56.6	79.3	74.9	76.5	62.2
NAc at C-2		175.7	23.7				
→3)-β-Galp-(1→	**C**	104.1	71.6	83.5	69.8	76.2	63.0
→2)-Gro	**D**	62.4	82.5	62.4			
Dhpa-(2→	**E**	181.4	81.1	42.8	66.3	23.7	

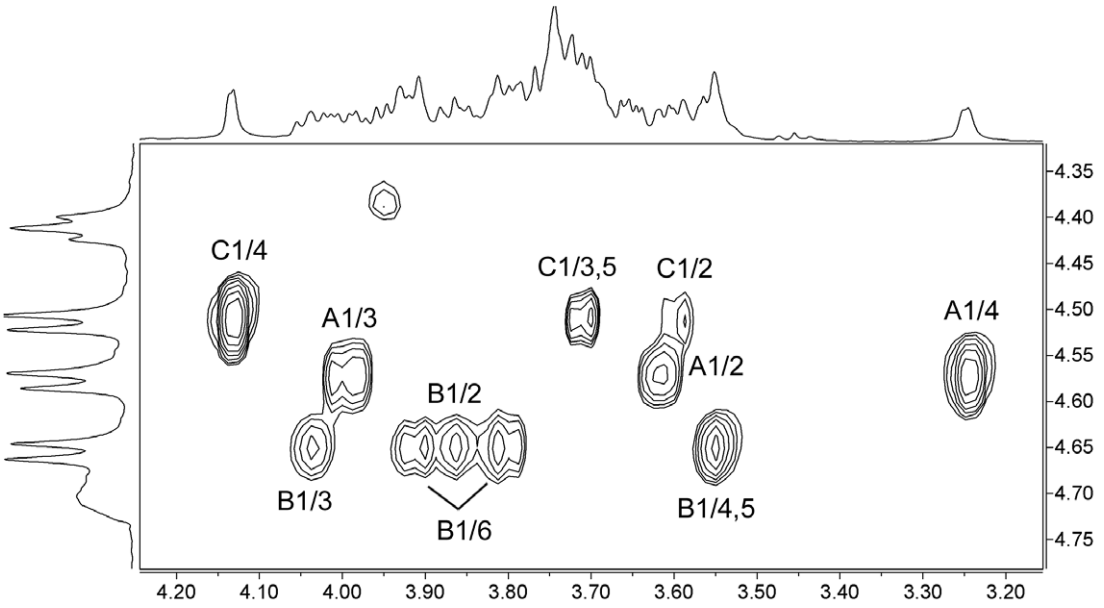

Figure 2. A part of TOCSY spectrum of OS1.

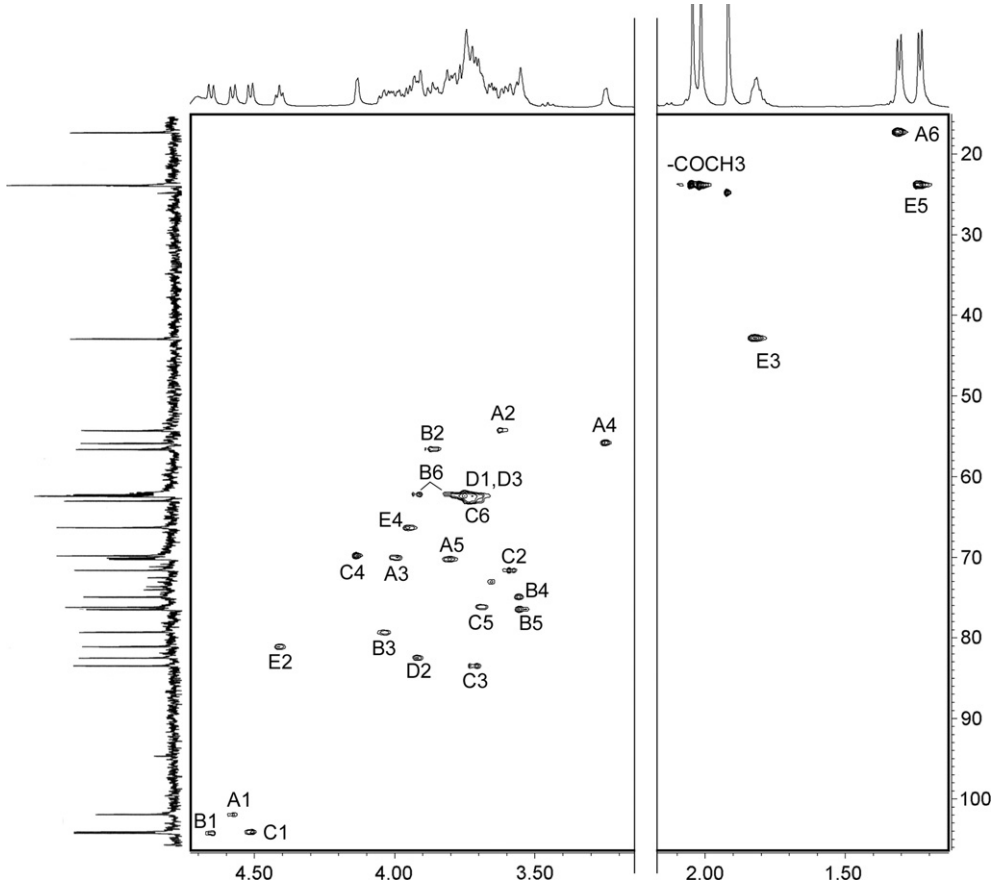

Figure 3. {^{1}H,^{13}C} HSQC spectrum of OS1.

Spin system **C**. Correlations from **C** H-1 to **C** H-4 were found in COSY, and corresponding carbons from **C** C-1 to **C** C-4 were assigned using the HSQC data (Fig. 3). The line shape of **C** H-1/**C** H-2 (δ 4.51/3.59) and **C** H-1/**C** H-3 (δ 4.51/3.71) cross-peaks in the TOCSY spectrum (Fig. 2) indicated the β-*galacto*-configuration. The HMBC spectrum contained a correlation δ 104.1/3.69, which was assigned as **C** C-1/**C** H-5 and indicated the coincidence of **C** H-3 and **C** H-5. The **C** C-6 was assigned from **C** C-6/**C** H-5 correlation in HMBC (δ 63.0/3.69). These data together identified spin system **C** as β-Gal*p*.

Spin system ***E***. The proton spin system from **E** H-2 to **E** H-5 was clearly distinguished from the COSY data. Chemical shifts (δ 4.41,

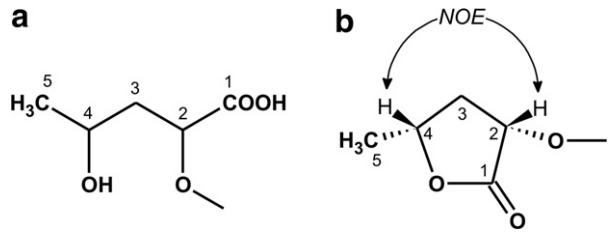

Figure 4. (a) (2*S*,4*R*)-2,4-Dihydroxypentanoic acid residue, (b) (2*S*,4*R*)-2,4-dihydroxypentanoic acid lactone residue and observed *intra*-residue NOE.

1.82, 3.94, 1.23 accordingly) and integral intensities indicated that **E** H-3 was a C–CH_2–C group, **E** H-5 was a C–CH_3 group, while **E** H-2 and **E** H-4 were single protons at carbons bearing hydroxyl groups. The corresponding carbons from **E** C-2 to **E** C-5 were assigned using the HSQC data (Fig. 3). The correlation of the CO group signal at δ 181.4 with **E** H-2 signal at δ 1.82 in the HMBC spectrum revealed that this carbon is **E** C-1 and bears no hydrogen. Thus, the order of groups in spin system E was HOOC–CH(OH)–CH_2–CH(OH)–CH_3. Proton chemical shifts of **E** H-2 (δ 4.41) and **E** H-4 (δ 3.94) indicated that this residues was in the open form, which is 2,4-dihydroxypentanoic acid (Dhpa, Fig. 4a). The chemical shift of **E** C-2 (δ 81.1) indicated that Dhpa forms a bond at this position.

*Spin system **D***. Three remaining signals in the ^{13}C NMR spectrum have their corresponding protons combined into this spin system. Their chemical shifts fit the glycerol residue, substituted at **D** C-2 (δ 81.1).

Two N-acetyl groups were assigned to three nitrogen bearing carbons using HMBC data. Two CO signals at δ 175.7 and 176.2 (which were assigned to the acetyl groups from δ 2.04/175.7 and δ 2.01/176.2 correlations in HMBC) had cross-peaks with **B** H-2 (δ 3.86) and **A** H-2 (δ 3.62) accordingly, but not with **A** H-4. Taking these data into account, it was concluded that the monomeric composition of OS1 was β-Fuc*p*NAc4N (**A**), β-Glc*p*NAc (**B**), β-Gal*p* (**C**), Gro (**D**), Dhpa (**E**).

The ^{13}C chemical shift values for C-3 (δ 79.3) and C-4 (δ 74.9) of β-Glc*p*NAc, C-3 (δ 83.5) of β-Gal*p*, and C-2 (δ 82.5) of Gro as compared to the data for unsubstituted residues[8,9] indicated substitution at these positions. The *inter*-residue correlations in the

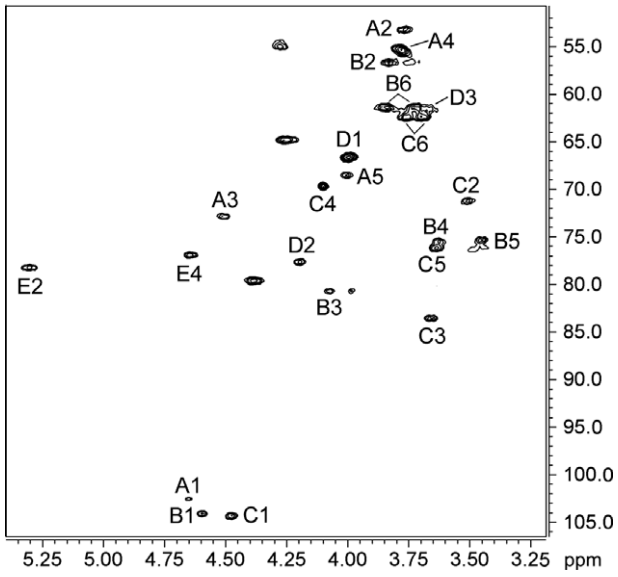

Figure 5. Part of {^{1}H,^{13}C} HSQC spectrum of PS.

HMBC spectrum confirmed these data and revealed the sequence of residues: H-1 Fuc*p*NAc4N/C-3 Glc*p*NAc (δ 4.57/79.3) and C-1 Fuc*p*NAc4N/H-3 Glc*p*NAc (δ 101.9/4.04); H-1 Glc*p*NAc/C-3 Gal*p* (δ 4.65/83.5) and C-1 Glc*p*NAc/H-3 Gal*p* (δ 104.3/3.71); H-1 Gal*p*/C-2 Gro (δ 4.51/82.5) and C-1 Gal*p*/H-2 Gro (δ 104.1/3.92); H-2 Dhpa/C-4 Glc*p*NAc (δ 4.41/74.9).

Therefore, the OS1 has the structure shown in Chart 1.

The spectra of the PS were assigned analogously using COSY, TOCSY, HSQC (Fig. 5), and HSQC-TOCSY experiments (Tables 3 and 4). They showed the following differences from the spectra of the OS1:

The signals for C-3 Gro (**D** C-3) and for C-3 Fuc*p*NAc4N (**A** C-3) were displaced to δ 66.7 and δ 72.9 accordingly, and **A** H-3 demonstrated abnormally low-field chemical shift (δ 4.51), which allowed to suggest phosphorylation at these positions. The {^{1}H,^{31}P} HSQC spectrum revealed two correlations (δ −0.63/4.00 and δ −0.63/4.51), confirming this suggestion.

2S,4R-Dhpa
2
|
4
β-Fuc*p*NAc4N-(1→3)-β-Glc*p*NAc-(1→3)-β-Gal*p*-(1→2)-Gro

2S,4R-Dhpa lactone
2
|
4
→3)-β-D-Fuc*p*NAc4N-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→2)-D-Gro-3-P-(O→

Chart 1. The structures of the OS1 and PS.

Table 3
^{1}H NMR data (δ, ppm)

Residue	Spin system	H-1a,b	H-2	H-3a,b	H-4	H-5	H-6a,b
PS							
→3)β-FucpNAc4N-(1→	**A**	4.66	3.78	4.51	3.79	4.01	1.32
→3,4)-β-GlcpNAc-(1→	**B**	4.60	3.84	4.08	3.62	3.46	3.74, 3.85
→3)-β-Galp-(1→	**C**	4.47	3.52	3.67	4.11	3.63	3.70, 3.77
→2)-Gro-(3-P→	**D**	3.70, 3.82	4.20	4.00	–	–	–
Dhpa lactone(2→	**E**	–	5.32	1.82, 2.94	4.67	1.44	–

2 CH_3CO at δ_H 2.02.

Table 4
^{13}C NMR data (δ, ppm)

Residue	Spin system	C-1	C-2	C-3	C-4	C-5	C-6
PS							
→3)β-FucpNAc4N-(1→	**A**	102.6	53.2	72.9	55.3	68.6	17.2
→3,4)-β-GlcpNAc-(1→	**B**	104.2 +8.0[a] DD +7.8 DL +3.4	56.8	80.7 +5.6[a] DD +7.5 DL +9.5	75.6	75.4	61.4
→3)-β-Galp-(1→	**C**	104.5	71.2	83.7	69.8	76.3	62.5
→2)-Gro-(3-P→	**D**	61.8	77.7	66.7	–	–	–
Dhpa lactone(2→	**E**	~176	78.3	38.4	77.0	21.3	–

2 CH_3CO at δ_C 23.5, 24.0 and 176.0.
For **B** C-1 and **B** C-3 the experimental and expected glycosylation effects are shown.
[a] The experimentally observed glycosylation effect, as compared to the chemical shift of signals of the free residue. DD indicates the expected value for the same absolute configurations of the linked residues, DL is the expected effect for different absolute configurations.

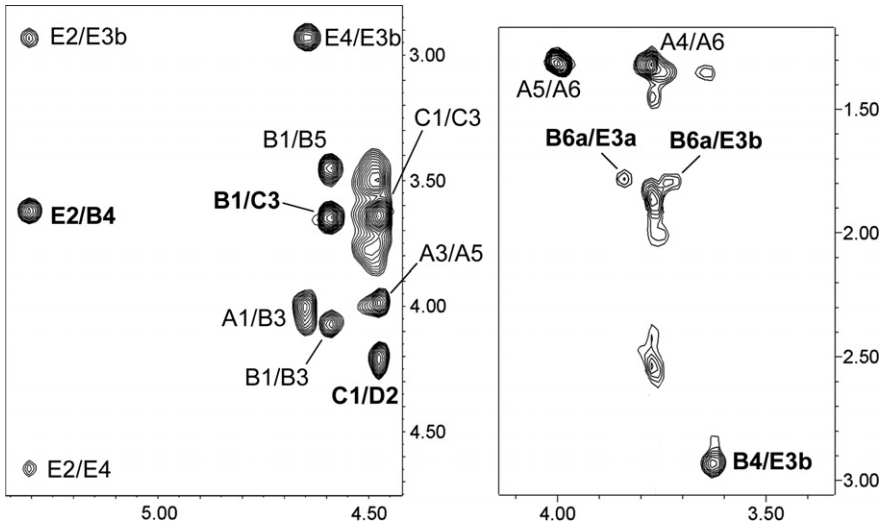

Figure 6. A part of ROESY spectrum of PS.

Spin system **E** possessed different set of chemical shifts, particularly **E** H-2, **E** H-4, and **E** C-4 were strongly displaced downfield as compared to their positions in the spectra of OS1. This allowed to suggest that the Dhpa residue was in the lactone form (Fig. 4b). The ROESY (Fig. 6) cross-peak at δ 5.32/4.67 proved that the configuration of **E** H-2 and **E** H-4 protons is either 2*R*,4*S* or 2*S*,4*R*. Other cross-peaks observed in the ROESY spectrum confirmed the structure of the residue (**E** H-3b/**E** H-2 at δ 2.94/5.32 and **E** H-3b/**E** H-4 at δ 2.94/4.67) and its linkage by **E** H-2 to **B** H-4 (δ 5.32/3.62).

The glycosylation effect value at C3 β-Glc*p*NAc (δ +5.6) confirmed that β-Fuc*p*NAc4N and β-Glc*p*NAc have the same absolute configuration. The expected effect for the same absolute configuration is δ +7.5;[8] the difference with the experimental value can be explained by β-effect of substitution at C4 β-Glc*p*NAc. The expected value for different absolute configurations is δ +9.5; the difference from the experimental value is too high to be explained by β-effect of substitution at C4 β-Glc*p*NAc. The glycosylation effect value at C1 β-Glc*p*NAc (δ +8.0) corresponds to the same absolute configurations of the linked GlcNAc and Gal residues (expected value is δ +7.8, as compared to δ +3.4 for different absolute configurations[8]). Together with GLC data (see above) these data show that all three monosaccharides have D configuration.

The ROESY spectrum showed that **E** H-3a is close to H-6a and H-6b of β-d-Glc*p*NAc (cross-peaks at δ 1.82/3.74 and δ 1.82/3.85), which is possible only in the case that Dhpa lactone has the 2*S*,4*R*-configuration, in accordance with the conformational search in the MM2 force field that was carried out for both variants of absolute configurations of Dhpa lactone (results will be published elsewhere).

Therefore, the O-polysaccharide of *P. alcalifaciens* O8 has the structure depicted in Chart 1. A peculiar feature of this polysaccharide is the presence of an ether-linked (2*S*,4*R*)-2,4-dihydroxypentanoic acid, which has not been found hitherto in natural carbohydrates. A stereoisomer of this acid is a component of the O-polysaccharide of *P. alcalifaciens* O31 (the structure of the latter will be published in *Natural Product Communications*, 2008).

1. Experimental

1.1. Bacterial strain, isolation and degradation of the lipopolysaccharide

P. alcalifaciens O8:H23, strain 1598/50, 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 lipopolysaccharide was isolated, in a yield of 2.0% of dry bacte-

rial weight, by phenol–water extraction and purified by ultracentrifugation.

A portion of the lipopolysaccharide (145 mg) was heated with 2% acetic acid (5 mL) for 1 h at 100 °C and the carbohydrate-containing supernatant was fractionated on a column of Sephadex G-50 (60 × 2.5 cm) in 0.05 M pyridinium acetate buffer. The yield of the polysaccharide (PS) was 14% of the lipopolysaccharide weight.

1.2. Isolation of the oligosaccharide

A PS sample was treated with aqueous 12% ammonia at 37 °C for 16 h, the solution was freeze-dried and the product was dephosphorylated with aqueous 48% HF for 48 h at 4 °C. HF was removed in a stream of nitrogen, and an oligosaccharide (OS1) was isolated by gel-permeation chromatography on a column (80 × 1.5 cm) of TSK HW-40 in 1% AcOH in a yield of 25% of the PS weight.

1.3. Monosaccharide analysis

The PS was hydrolyzed with 2 M CF_3CO_2H for 2 h at 120 °C. Alditol acetates were prepared by reduction with an excess of $NaBH_4$ (20 °C, 2 h) followed by acetylation (0.2 mL Ac_2O, 0.2 mL pyridine, 100 °C, 1 h), and analyzed by GLC–MS on a Hewlett-Packard 5971A instrument equipped with an HP-1 glass capillary column (0.2 mm × 12 m) using a temperature program of 150→270 °C at 8 °C/min.

For determination of the absolute configuration of Gal, the O-polysaccharide (0.4 mg) was hydrolyzed with 2 M CF_3CO_2 H for 2 h at 120 °C, subjected to 2-octanolysis[6] [100 μL (*S*)-2-octanol, 15 μL CF_3CO_2H, 120 °C, 16 h], acetylated, and analyzed by GLC–MS as mentioned above.

For determination of the absolute configuration of FucNAc4N, the oligosaccharide (2 mg) was deaminated with a solution of $NaNO_2$ (15 mg) in aq 10% HOAc (0.5 mL) for 1 h at 20 °C, and the product was applied to a 2-mL column of Dowex 50 × 2 (H^+-form). The desired neutral compound eluted with water was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h), a portion of the hydrolysate after preparation of alditol acetates was analyzed using GLC–MS to reveal quinovosamine. Another portion was N-acetylated (400 μL $NaHCO_3$, 60 μL Ac_2O, 0 °C, 1 h) to give *N*-acetylquinovosamine, which was converted into acetylated (*R*)-2-octyl glycoside[6] and analyzed by GLC–MS using reference samples of the corresponding (*R*)- and (*RS*)-2-octyl glycosides.

The absolute configuration of glycerol 1-phosphate was determined as described.[6,7]

1.4. Methylation analysis

Methylation of the polysaccharide was performed according to the Hakomori procedure,[10] the products were recovered by Sep-Pak. Partially methylated monosaccharides were obtained by hydrolysis with 2 M CF_3CO_2H as mentioned above, converted into the alditol acetates, and analyzed by GLC–MS as mentioned above.

1.5. NMR spectroscopy

Samples were freeze-dried twice from a ^2H_2O solution and dissolved in 99.96% ^2H_2O with internal TSP (δ_H 0) and external acetone (δ_C 31.45) as references. ^1H and ^{13}C NMR spectra were recorded at 30 °C using a Bruker DRX-500 NMR spectrometer and XwinNMR Bruker software on SGI Indy/Irix 5.3 workstation. Mixing time of 300 ms and spin-lock time of 300 ms were used in ROESY and TOCSY experiments, respectively. Glycosylation effect calculations for different combinations of sugar absolute configurations were performed using ^{13}C NMR spectra prediction tool of Bacterial Carbohydrate Structure Database.[11]

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