Note

Structure of the O-polysaccharide of Providencia alcalifaciens O35 containing an N-[(S)-1-carboxyethyl]-l-alanine (alanopine) derivative of 4-amino-4,6-dideoxyglucose

Olga G. Ovchinnikova a,⇑, Olga A. Valueva a, Nina A. Kocharova a, Nikolay P. Arbatsky a, Agnieszka Maszewska b, Agnieszka Zablotnic, Alexander S. Shashkov a, Antoni Rozalski b, Yuriy A. Knirel a

a N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 119991 Moscow, Russian Federation
b Department of Immunobiology of Bacteria, Institute of Microbiology, Biotechnology and Immunology, University of Lodz, PL 90-237 Lodz, Poland
c Department of General Microbiology, Institute of Microbiology, Biotechnology and Immunology, University of Lodz, PL 90-237 Lodz, Poland

ABSTRACT

The O-polysaccharide of Providencia alcalifaciens O35 was studied by sugar and methylation analyses along with 1H, 13C NMR spectroscopy, including 2D 1H, 13C HMBC, and NOESY experiments in D2O and, to detect correlations for NH protons, in a 9:1 H2O/D2O mixture. A unique N-[(1-carboxyethyl)]alanine (alanopine, Alo) derivative of 4-amino-4,6-dideoxyglucose (Qui4N) was identified as the polysaccharide component. Alanopine was isolated by solvolysis of the polysaccharide with triflic acid followed by acid hydrolysis, and its (2S,4S)-configuration was determined by the specific optical rotation. The following structure of the O-polysaccharide was established (the D configuration of Qui4N was ascribed tentatively):

\[
\rightarrow 4)-\alpha-D-GalpNAc-(1\rightarrow 6)-\alpha-D-GlcP-(1\rightarrow 4)-\beta-D-GlcP\alpha-(1\rightarrow 3)-\beta-D-GalpNAc-(1\rightarrow 6
\]

\[
\uparrow
\]

\[
\beta-D-\text{Qui}p4N(2S,4S-Alo)
\]

Providencia is a genus of Gram-negative bacteria within the family Enterobacteriaceae. These bacteria are opportunistic pathogens, which under favorable conditions may cause wound and urinary tract infections and enteric diseases. They can also be found in non-diarrheic stool specimens. The genus Providencia is subdivided into eight species, including Providencia alcalifaciens, Providencia stuartii, Providencia rustigianii, Providencia rettgerii, Providencia heimbachae, Providencia vermicola, Providencia sneebia, and Providencia hurhodogranariea. Based on lipopolysaccharide (LPS) O-antigens (O-polysaccharides) and flagella H-antigens, the first three species are classified into 63 O-serogroups and 30 H-serogroups. At present, chemical structures have been established for the O-polysaccharides of more than half of the O-serogroups and a number of amino acids and their derivatives have been identified as their components, including l-alanine, l-serine, l- and l-aspartic acid, and N-[(R)- and (S)-1-carboxyethyl]-l-lysine. Recently, N-[(1-carboxyethyl)]alanine (alanopine, Alo) has been found as a component of the O-polysaccharides of P. alcalifaciens O35 and Proteus vulgaris O76 as well as the LPS core of Proteus mirabilis O6 and O57 but its absolute configuration remained unknown. Now, we report on the structure of the O-polysaccharide of P. alcalifaciens O35 including determination of the absolute configuration of alanopine.

LPS was isolated from dry bacterial cells by the phenol–water procedure and degraded under mild acid conditions. Fractionation of the carbohydrate portion by GPC on Sephadex G-50 resulted in a high-molecular-mass O-polysaccharide and two oligosaccharide fractions. Sugar analysis using GLC of the alditol acetates derived after acid hydrolysis of the polysaccharide revealed Glc and GalN in the ratio of 1:1.4 as well as a trace amount of 4-amino-4,6-dideoxyglucose (Qui4N). In addition, glucuronic acid (GlcA) was
identified by anion-exchange chromatography using a sugar analyzer. NMR spectroscopic studies showed the presence of an amino acid identified as alanopine (see below).

Determination of the absolute configuration by GLC of the acetylated (+)-2-octyl glycosides showed that Glc and GlcA have the α configuration. The β configuration of GalN was established by analysis of chemical shifts in the 13C NMR spectrum of the polysaccharide using known regularities in glycosylation effects. The absolute configuration of Qui4N was not established and was assigned tentatively as α as in all other cases of identification of this monosaccharide in bacterial polysaccharides (see Bacterial Carbohydrate Structure Database at http://csdb.glycoscience.ru/bacterial/).

Alanopine was isolated by HPLC after solvolysis of the polysaccharide with triflic acid followed by hydrolysis with CF3CO2H. Its structure as N-(1-carboxyethyl)-alanine has been established earlier by NMR spectroscopy (for the assigned 1H and 13C chemical shifts see Table 1). GLC analysis of the acetylated (S)-2-octyl ester showed that the isolated alanopine stereoisomer is different from the meso-form but did not allow distinguishing between the (2S,4S) and (2S,4R) isomers. The specific optical rotation [α]D20 +13.4° (water) showed that alanopine has the (2S,4S) configuration (compare published data [α]D20 +12.1° (water) for (2S,4S)-Alo and [α]D20 –11° (water) for (2S,4R)-Alo).

Linkage analysis by GLC–MS of the partially methylated alditol acetates derived from the methylated polysaccharide revealed 6-acetates of two GalN residues at δ 75.2, 72.0, 71.2, and 81.8, as compared with their positions in the spectra of the corresponding non-substituted monosaccharides at δ 69.56, 62.11, 61.84, 72.69, and 72.01, respectively. The 13C NMR chemical shifts for C-2–C-6 of Qui4N were close to those of the unsubstituted monosaccharide. These findings confirmed the methylation analysis data and showed that the polysaccharide is branched with a lateral Qui4N residue and a 4,6-disubstituted GalN residue at the branching point.

Sequence analysis of the polysaccharide was performed using a NOESY experiment, which showed interresidue cross-peaks between the following anomeric protons and protons at the linkage carbons: α-GalN H-1/α-Glc C-6, β-GlcA B-3 at δ 5.49/3.68, 3.96; α-Glc C-1/β-GlcA B-4 at δ 5.46/3.79; β-GlcB B-1/β-GalNA A-3 at δ 5.36/3.84; β-GalNA A-1/α-GalNA D-4 at δ 4.66/4.18; and β-Qui4N E-1/α-GalNA D-6 at δ 4.49/3.89. The monosaccharide sequence was confirmed independently by correlations between anomeric protons and linkage carbons and vice versa demonstrated by an 1H,13C HMBC spectrum (Fig. 3).

The HMBC experiment was also used for determination of the position of alanopine (Fig. 3). Qui4N E H-4/Alo F C-1 at δ 3.62/171.8 cross-peak showed that alanopine is attached to N-4 of Qui4N E. Correlations between H-2 of GalNA and GalNA A and

<table>
<thead>
<tr>
<th>Table 1</th>
<th>1H and 13C NMR data (δ, ppm) of the O-polysaccharide and isolated alanopine from P. alcalifaciens O35</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residue</td>
<td>C-1 H-1</td>
</tr>
<tr>
<td>O-Polysaccharide</td>
<td></td>
</tr>
<tr>
<td>→3)-β-β-GalpN(1→</td>
<td>A</td>
</tr>
<tr>
<td>→4)-β-β-GlcpA(1→</td>
<td>B</td>
</tr>
<tr>
<td>→5)-α-β-Glcp(1→</td>
<td>C</td>
</tr>
<tr>
<td>→4,6)-α-β-GalpN(1→</td>
<td>D</td>
</tr>
<tr>
<td>β-β-Qui4N(1→</td>
<td>E</td>
</tr>
<tr>
<td>(2S,4S)-Alo</td>
<td>F</td>
</tr>
<tr>
<td>(2S,4S)-Alo</td>
<td>C-1</td>
</tr>
</tbody>
</table>

1H NMR chemical shifts are given in italics. Additional chemical shifts for the N-acetyl groups are αC 23.4 (Me) and 176.4 (CO), δN 2.02; δC 23.8 (Me) and 176.0 (CO), δN 2.03. Assignment of NAc groups to particular sugar residues was made using the 1H,13C HMBC spectrum (Fig. 3).
C-1 of two N-acetyl groups at δ 4.16/176.0 and 3.99/176.4 indicated that both GalN residues are N-acetylated. A NOESY experiment with a polysaccharide solution in a 9:1 H₂O/D₂O mixture revealed a correlation between Alo CH₂-2 and Qui4N NH-4 at δ 1.57/8.62, thus confirming the location of alanopine on Qui4N E.

Therefore, the O-polysaccharide of *P. alcalifaciens* O35 has the structure shown in Chart 1. It was confirmed independently by high-resolution ESI mass spectrometry analysis of two oligosaccharides obtained, in addition to the polysaccharide, by mild acid hydrolysis of the LPS. The difference of 1032.38 Da between the molecular masses of the oligosaccharides, which were derived from the R- and SR-forms of the LPS, was in full agreement with the repeating unit structure established.

Most likely, the structure shown in Chart 1 represents the biological repeating unit with β-GalNAc A being the first monosaccharide and the most uncommon component, the alanopine derivative of Qui4N, occupying the terminal non-reducing end of the polysaccharide.

Rabbit polyclonal antiserum against heat-killed bacteria of *P. alcalifaciens* O35 was tested in enzyme-immunosorbent assay (EIA) and Western blot with the LPS of various *Providencia* and *Proteus* strains with structurally similar O-antigens, including those containing alanopine. A strong reaction was observed with the homologous LPS and a weak cross-reactivity with the LPSs of *Proteus vulgaris* O34, O76, *Proteus mirabilis* O6, O16, O29, O57, and *P. alcalifaciens* O40 (Table 2). In Western blot (Fig. 4), anti-*P. alcalifaciens* O35 serum recognized both slow and fast migrating bands of the homologous LPS, which correspond to high- and low-molecular mass LPS species with and without the O-specific polysaccharide chain, respectively. In addition, it cross-reacted with high-molecu-
lar mass LPS species of Proteus serogroups O16, O34, 57, and O76 as well as *P. alcalifaciens* O40, thus indicating that a cross-reactive epitope(s) resides on the O-specific part of the LPSs. Western blot also revealed a weak cross-reactivity of anti- *P. alcalifaciens* O35 serum with low-molecular mass LPS species of *P. mirabilis* O6, O29, and O57, and hence, these strains share a common epitope(s) located in the LPS core region. 

The O-polysaccharides of *P. alcalifaciens* O35 and *P. vulgaris* O76 as well as the LPS core oligosaccharides of *P. mirabilis* O6 and O57 all contain an alanopine derivative of Qui4N7 that may be responsible for the observed cross-reactivity of the LPSs. In addition, the O-polysaccharides of *P. alcalifaciens* O35 and *P. vulgaris* O76 share the β-D-GalpNAc-(1→4)-D-GalpNAc disaccharide, and the same disaccharide is also present in the O-polysaccharides of *P. mirabilis* O16 and *P. vulgaris* O34.17 Another common disaccharide, β-D-GlcPA-(1→3)-D-GalpNAc, occurs in the O-antigens of *P. alcalifaciens* O35 and *P. alcalifaciens* O40.17 It is most likely that the cross-reactive epitopes within the O-polysaccharides of these strains are associated with the common disaccharide fragments. Elucidation of the molecular basis of the serological relatedness of *P. alcalifaciens* O35 LPS and *P. mirabilis* O29 LPS requires determination of the chemical structure of *P. mirabilis* O29 LPS core region.

### 1. Experimental

#### 1.1. Bacterial strain, isolation, and degradation of the lipopolysaccharide

*Providencia alcalifaciens* O35:H18, strain 901/49 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 ~5% of dry bacterial weight by the phenol–water extraction followed by dialysis of the extract without layer separation and freed from insoluble contaminations by centrifugation. The resultant solution was treated with cold (4 °C) aq 50% CCl₃CO₂H; after centrifugation the supernatant was dialyzed against distilled water and freeze-dried.

A portion of the LPS (450 mg) was heated with 2% acetic acid for 2.5 h at 100 °C, a lipid precipitate was removed by centrifugation, and the carbohydrate-supernatant was fractionated on a column (60 × 2.5 cm) of Sephadex G-50 Superfine in 0.05 M pyridinium acetate buffer pH 4.5. The yield of the O-polysaccharide was ~13% of the LPS mass.

1.2. Monosaccharide analyses

For sugar analysis, a polysaccharide sample was hydrolyzed with 10 M HCl (80 °C, 30 min), the monosaccharides were reduced with an excess of NaBH₄ (20 °C, 2 h), acetylated with a 1:1 Ac₂O/pyridine mixture (100 °C, 1 h) and analyzed by GLC on a Hewlett-Packard 5890 chromatograph (USA) equipped with an Ultra-1 column using a temperature gradient of 150–290 °C at 5 °C min⁻¹. Uronic acids were analyzed on a Biotronik LC-2000 sugar analyzer as described.¹⁰ For determination of the absolute configurations of the monosaccharides,¹⁹ a polysaccharide sample was subjected to methanolysis (1 M HCl/MeOH, 80 °C, 2 h), acetylated with a 1:1 Ac₂O/pyridine mixture (100 °C, 1 h) and analyzed by GLC as above.

1.3. Isolation and identification of alanopine

A polysaccharide sample (40 mg) was treated with triflic acid for 2 h at 7 °C; after neutralization with 10 M NaOH at 0 °C, the products were fractionated on a Sephadex G-25 column (100 × 4 cm) in 0.1% HOAc. The fraction of approximately disaccharide size (8 mg) was hydrolyzed with 4 M CF₃CO₂H (120 °C, 2 h), and the products were fractionated by HPLC (BioRad, USA) on a DE-Silasorb column (250 × 4 mm) in 2% HOAc (1 mL min⁻¹) to give alanopine (0.85 mg), having the same elution time as the authentic alanopine sample synthesized as described.²⁰ Its structure was confirmed by NMR spectroscopy and GLC of the acetylated (S)-2-octyl ester obtained by methanolysis with 1 M HCl in MeOH (85 °C, 2 h) followed by N-acetylation with a 1:1 Ac₂O/pyridine mixture (85 °C, 1 h) and treatment with (S)-2-octanol (100 µL) in the presence of CF₃CO₂H (15 µL) (100 °C, 1 h). The authentic alanopine samples for comparison were derived from synthetic (2S,4S)-, (2S,4R)-, and (2R,4R)-alanopine. The specific optical rotation of isolated alanopine, [α]D²² +13.4° (c 0.08, water), was measured on a Jasco DIP-360 digital polarimeter (Japan).

1.4. Methylation analysis

A polysaccharide sample was methylated according to the Hakomori procedure,²¹ the products were recovered using a Sep-Pak cartridge and divided into two parts, one of which was reduced with LiBH₄ in aq 70% 2-propanol (20 °C, 2 h). Partially methylated monosaccharides were derived by hydrolysis with 10 M HCl (80 °C, 30 min), converted into the alditol acetates, and analyzed by GLC–MS on a Hewlett-Packard HP 5989A instrument equipped with a 30-m HP-5 ms column using a temperature gradient of 150 °C (3 min) to 320 °C at 5 °C min⁻¹.

1.5. NMR spectroscopy

Samples were freeze-dried twice from a 99.9% D₂O soln and dissolved in 99.96% D₂O. H and 13C NMR spectra were recorded at 30 °C on a Bruker Avance II 600 MHz spectrometer (Germany). Internal sodium 3-(trimethylsilyl)propanoate-2,2,3,3-d₄ (δH 0) and acetone (δC 31.45) were used as references. 2D NMR spectra were obtained using standard Bruker software, and Bruker TopSpin 2.1 program was used to acquire and process the NMR data. A mixing time of 200 and 150 ms was used in NOESY and TOCSY experiments, respectively. Other NMR experimental parameters were set essentially as described.²²

1.6. Serological techniques

Preparation of rabbit O-antiserum,²³ ELISA with LPS as antigen,²⁴ sodium deoxycholate polyacrylamide gel electrophoresis, and Western blot²⁴ were performed as described.

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References


