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Short communication

Chemical structure of the somatic antigen isolated from *Salmonella* Abortusequi (O4)

Zbigniew Kaczyński[a,*], Jerzy Gajdus[a], Halina Dziadziuszko[b], Piotr Stepnowski[a]

[a] *Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland*
[b] *Department of Molecular Microbiology and Serology, National Salmonella Centre, Medical University of Gdańsk, Do Studzienki 38, 80-227 Gdańsk, Poland*

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ABSTRACT

A neutral O-specific polysaccharide was obtained by mild acid hydrolysis of the lipopolysaccharide (LPS) of *Salmonella* Abortusequi O4 bacterium (previously serogroup B). As determined by compositional analyses and NMR spectroscopy, the O-polysaccharide consists of four or five residues in the repeating subunit. The assigned structures are:

α-Abe*p*
↓3
→2)-α-D-Man*p*-(1→4)-α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→

and

α-Abe*p* α-D-Glc*p*
↓3 ↓4
→2)-α-D-Man*p*-(1→4)-α-L-Rha*p*-(1→3)-α-D-Gal*p*-(1→

A distribution of the repeating units in O-chain was analysed by Western blotting with anti O12 serum and MS spectrometry of oligosaccharides obtained from partial hydrolysis of polysaccharide.

1. Introduction

Salmonella is a genus of Gram-negative enterobacteria, which can cause gastrointestinal and other diseases in both humans and animals. Lipopolysaccharide (LPS), as the major constituent of the cell surface of Gram-negative bacteria, forms the outermost layer and acts in order to protect the cell from the environment. In general, the complete LPS (S-form) consists of three regions: the O-specific polysaccharide (OPS), the core region and the lipid A [1–3]. In pathogenic bacteria, the LPS plays an important role in the interaction between the bacterium and its host, having dramatic effects on the immune system.

Salmonella species are serologically classified into serovars (Kaufmann–White scheme) based on their O-antigenic factors located in the lipopolysaccharide (O), flagellar protein (H), and sometimes the capsular (Vi) antigens. There are more than 2500 known serovars. This classification is regularly updated and revised along with the discovery of new strains [4,5].

For both diagnostic and molecular conformation of a serological classification a structural determination of key parts of the LPS is required. The paper describes the structure of the O-specific polysaccharide isolated from *Salmonella* Abortusequi, belonging to the serogroup O4 (B). The LPS of this serovar is very often used in different clinical studies [6–9], but so far, no structural analyses of O-chain have been done, apart from sugar composition of the whole LPS and immunological tests [10,11].

2. Experimental

2.1. Growth of bacteria, isolation of LPS and preparation of O-polysaccharide

S. Abortusequi (O4) strain was obtained from the National Salmonella Center of Poland, KOS collection Gdańsk, Poland. Bacteria were cultivated and isolated as already described [12]. The LPS was extracted by the hot-phenol/water method [13]. The aqueous phase was dialyzed for 2 days against tap water, next against distilled water overnight, and concentrated under diminished pressure and freeze dried. The sample was precipitated in 40% aq. ethanol at 4 °C and pH 4.5 to remove nucleic acids. After centrifugation (1000 × *g*, −4 °C, 40 min), the supernatant was concentrated

* Corresponding author. Tel.: +48 58 5235397; fax: +48 58 5235472.
E-mail address: zbyszek@chem.univ.gda.pl (Z. Kaczyński).

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under diminished pressure, then the LPS was precipitated in 80% ethanol at 4 °C and pH 7.0. The sample was centrifuged (1000 × *g*, −4 °C, 40 min) and the LPS-containing pellet was suspended in distilled water, dialyzed (cut-off 1000 Da) and freeze dried. The LPS sample (94.2 mg) was hydrolyzed with aq. 1% AcOH at 100 °C for 45 min, and the lipid A precipitate (12.4 mg) was removed by ultracentrifugation (3500 × *g*, 1 h). The supernatant was lyophilized to yield the carbohydrate material (80.2 mg), which was fractionated by gel-permeation chromatography (GPC) on a column (100 cm × 0.9 cm) of BioGel P-10 (200–400 mesh, BioRad, USA) using distilled water as an eluent and monitoring with a differential refractometer (RIDK 101, Czech Republic). The polymeric portion (27.2 mg) was subjected to further analyses.

2.2. Compositional analyses, and partial hydrolysis

The polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 1 h. After reduction with sodium borohydride and acetylation, the samples were analysed by GC and GC–MS. In a two-stage procedure, mild acid hydrolysis (0.2 M TFA, 70 °C, 3 h) was followed by reduction and classical sugar analysis as described above. Absolute configurations of the sugar residues were determined by GC, using acetylated (*S*)-2-butyl glycosides [14,15]. Methylation analyses of the OPS and the OPS after selective cleavage of abequose (0.2 M TFA, 70 °C, 3 h) were performed according to Ciucanu and Kerek [16]. The methylated samples was recovered by use of a Sep-Pak C18 cartridge (Millipore) [17], then hydrolyzed (2 M TFA, 120 °C, 1 h). The compounds were converted into partially methylated alditol acetates and analysed by GLC and GLC–MS.

Partial hydrolysis of the OPS (13.2 mg) was completed during 5 steps using 0.2–0.6 M TFA for 1 h at 4 °C. Obtained products were fractionated using GPC on a column (100 cm × 0.9 cm) of BioGel P-2 (200–400 mesh, BioRad, USA) and remaining polymeric fraction was subjected to next hydrolysis with "stronger" TFA. The pooled products of hydrolysis were fractionated by HPLC on a column Separon SGX NH_2 (30 cm × 0.46 mm, particle size 5 μm) using acetonitryl/water (70:30, v:v) as a mobile phase and monitoring with a differential refractometer. Separated oligosaccharides were analysed by FAB-MS.

2.3. GLC, GLC–MS, and FAB-MS analyses

Alditol acetates, partially methylated alditol acetates and acetylated 2-butyl glycosides were analysed using a CE 8000 TOP instrument equipped with a capillary column DB-23 (60 m × 0.25 mm; 0.15 μm film thickness) and applied temperature gradient of 140–260 °C at 3 °C/min. GLC–MS measurements were performed on VG TRIO-2000 instrument (an electron impact ionization energy of 70 eV) connected to HP-5890 chromatograph equipped with the DB-23 capillary column. FAB measurements were done on VG TRIO 3 mass spectrometer using argon and 5% TFA in glycerol as a matrix.

2.4. NMR spectroscopy

NMR spectra were obtained for the solution of 15 mg OPS in 0.7 ml of ^2H_2O with Varian Mercury 400 MHz spectrometer at 70 °C. Chemical shifts were reported relative to internal acetone (δ_H 2.225; δ_C 31.45). One-dimensional ^1H and ^{13}C NMR, and ^1H,^1H-homonuclear COSY (COrrelation SpectroscopY), TOCSY (TOtal Correlation SpectroscopY), NOESY (Nuclear Overhauser Enhancement SpectroscopY), DQFCOSY (Double-Quantum-Filtered COSY), as well as the ^1H,^{13}C-heteronuclear HSQC (Heteronuclear Single Quantum Correlation) and HMBC (Heteronuclear Multiple-Bond Correlation) experiments were recorded applying of standard Varian software. The mixing times of 100 and 150 ms were applied for TOCSY and NOESY measurement, respectively.

2.5. SDS-PAGE and immunoblotting

The LPS of *S.* Abortusequi (O4) was analysed and the LPS of *Salmonella* Typhi (O9) was used as a reference. The LPS's separation on SDS-PAGE were transblotted from the gel into a nitrocellulose sheet and were incubated with antisera 4 and 9,12. The experiments were performed as described before [18].

3. Results and discussion

S. Abortusequi (4,12: - : e,n,x) belongs to O4 (formerly B) serogroup according to the Kauffmann–White classification [4]. The bacteria were cultivated and harvested in the National Salmonella Center in Gdańsk. The bacterial cell mass was extracted utilizing the hot-phenol/water method. Lipid-free polysaccharide was prepared from the LPS by mild acid hydrolysis and purified by gel-permeation chromatography.

The sugar analysis of the OPS identified abequose, rhamnose, mannose, galactose and glucose in the approximate molar ratio of 0.3:1.0:1.0:1.0:0.3. Since abequose is a very labile monosaccharide, classic sugar analysis was preceded with mild hydrolysis (0.2 M TFA) and reduction. The two steps sugar analysis of the polysaccharide revealed abequose, rhamnose, mannose, galactose and glucose in the approximate molar ratio of 0.9:1.0:1.0:1.0:0.3. The absolute configurations assignment identified the monosaccharides to be D-mannose, D-galactose, D-glucose, and L-rhamnose. The methylation analysis of O-antigen revealed several compounds: 1,5-di-*O*-acetyl-2,4-di-*O*-methyl-abequitol, 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-rhamnitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol, 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-mannitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol, 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl-mannitol, 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-sgalactitol in the approximate molar ratio of 0.15:1.00:0.25:0.05:0.80:1.00:0.30. These data indicated the OPS is composed of terminal abequose and glucose, 3- and 3,4-substituted galactose, 4-substituted rhamnose, 2- and 2,3-substituted mannose. A small amount of terminal abequose can be explained by its decomposition during hydrolysis of the methylated polysaccharide. A trace of 2-substituted mannose was explained by a cleavage of small quantity of abequose during the LPS isolation and the OPS preparation. To confirm this assumption, the methylation analysis of O-chain, after selective hydrolysis of abequose residues, was done. In a result, the same derivatives were obtained, but with the reverse molar ratio of two compounds: 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-mannitol and 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl-mannitol (1.00:0.10, respectively). It confirmed the previous assumption, and unambiguously identified a position of abequose residue attachment to be 3-mannose.

The ^1H NMR spectrum of the O-specific polysaccharide contained four main and five minor signals in the anomeric region (Fig. 1, and Table 1). Comparison of integration values of anomeric protons (the approximate ratio 1.0:0.4) suggested presence of two different repeating units, what will be proofed later. Additionally, the signal of $-CH_2-$ group, characteristic for 3,6-di-deoxy-hexose at δ 1.99, and two signals of CH_3 groups at δ 1.18 and 1.33 (6-deoxyhexose) were identified. Based on compositional analyses results and NMR data, the anomeric signals were assigned to: (**A**) →2,3)-α-D-Man*p*-(1→(δ 5.29), (**a**) →2,3)-α-D-Man*p*-(1→(δ 5.26), (**b**) →3,4)-α-D-Gal*p*-(1→(δ 5.22), (**B**) →3)-α-D-Gal*p*-(1→(δ 5.19), (**d**) →4)-α-L-Rha*p*-(1→(δ 5.17), (**c**) α-Abe*p*-(1→(δ 5.13), (**C**)

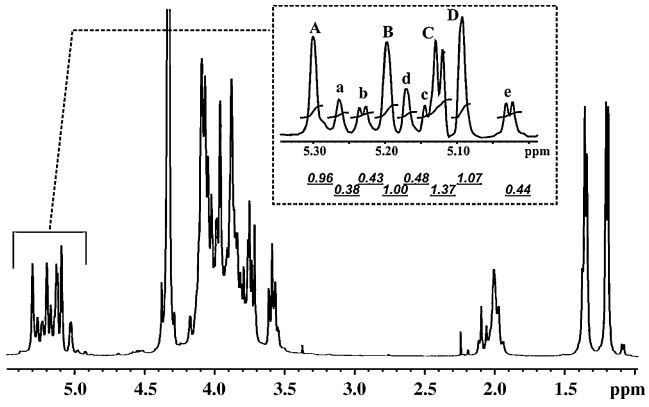

Fig. 1. The ^{1}H NMR spectrum of the OPS isolated from *Salmonella* Abortusequi. The spectrum was recorded at 400 MHz and 70 °C. The sugar residues were labeled **A**–**D**, according to the decreasing chemical shifts of the anomeric protons of the residues in the tetrasaccharidic repeating unit; **a**–**e**: anomeric protons in the pentasaccharidic repeating unit. The *letters* refer to the carbohydrate residues as shown in Fig. 3, and Table 1.

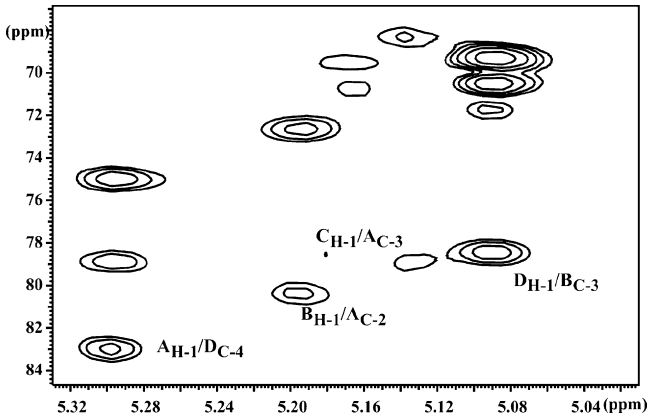

Fig. 2. The section of the HMBC spectrum of the OPS isolated from *Salmonella* Abortusequi. The spectrum was recorded at 400 MHz and 70 °C. The *letter* refer to the carbohydrate residues as defined in Fig. 3, and Table 1.

α-Abe*p*-(1→(δ 5.12), (**D**) →4)-α-L-Rha*p*-(1→(δ 5.09), (**e**) α-D-Glc*p*-(1→(δ 5.02). The ^{13}C NMR spectrum showed nine signals in anomeric region, which confirmed the presence of nine anomeric protons in the ^{1}H NMR spectrum and nine spin system in the TOCSY experiment. COSY, TOCSY, and HSQC spectra allowed the complete assignment of all ^{1}H and ^{13}C chemical shifts (Table 1). The anomeric configurations of all residues were assigned by the coupling constants $^{1}J_{H1,C1}$, which were identified in the HSQC experiment recorded without decoupling. All values were >170 Hz, what revealed α anomeric configuration of all monosaccharides (Table 1).

The ROESY and HMBC experiments revealed the sequence of the sugar residues in the repeating unit. Based on the HMBC experiment (Fig. 2) the inter-residual proton–carbon correlations has been assigned: **A**,H-1/**D**,C-4; **B**,H-1/**A**,C-2; **C**,H-1/**A**,C-3 and **D**,H-1/**B**,C-3 that identified the sequence of the sugar residues in the tetrasaccharidic repeating unit (Fig. 3). The sequence was confirmed by the NOESY experiment. The strong inter-residual NOE contacts were observed between protons: **A**1/**D**4, **B**1/**A2**, **C**1/**A**3, and **D**1/**B3**. Additionally, the strong NOE contact was observed between

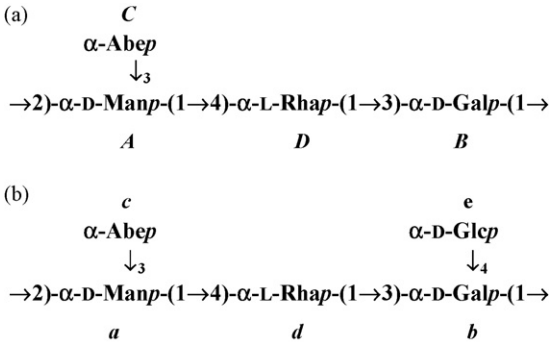

Fig. 3. The structure of the repeating units of the OPS isolated from *Salmonella* Abortusequi (a) the tetrasaccharidic repeating unit and (b) the pentasaccharidic repeating unit.

protons **e1** and **b4**, which confirmed the substitution of galactose residue at C-4 by glucose residue in the pentasaccharidic repeating unit.

The data of compositional analyses, and NMR spectroscopy indicated, that the OPS consisted of the tetrasaccharidic and pentasaccharidic repeating units (Fig. 3).

Table 1
^{1}H and ^{13}C NMR data of the OPS from the LPS of *Salmonella* Abortusequi. The residues of the tetrasaccharidic and pentasaccharidic repeating units are labelled with upper case and lower case, respectively. The spectra were recorded in $^{2}H_2O$ at 70 °C relative to internal acetone (δ_H 2.225; δ_C 31.45). Underlined chemical shifts indicate substituted positions.

Residue	$^{3}J_{H1,H2}$ [Hz]/$^{1}J_{H1,C1}$ [Hz]	^{1}H,^{13}C chemical shifts [δ]					
		H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
→2,3)-α-D-Man*p*-(1→	<1.5	5.29	4.03	4.08	3.88	3.97	3.84
A	175	101.0	80.3	78.8	69.6	74.9	61.9
→3)-α-D-Gal*p*-(1→	3.5	5.19	3.95	3.97	4.08	4.08	3.75
B	172	102.4	69.3	78.3	70.4	71.7	62.2
α-Abe*p*-(1→	4	5.12	4.05	1.99	3.87	4.10	1.18
C	175	101.7	64.8	34.4	70.6	68.1	16.9
→4)-α-L-Rha*p*-(1→	<1.5	5.09	4.10	4.00	3.58	3.95	1.33
D	173	102.9	68.2	70.5	82.9	69.3	18.4
→2,3)-α-D-Man*p*-(1→	<1.5	5.26	4.03*	4.08*	4.06	3.95	3.84*
a	174	101.6	80.3	78.8	67.9	74.6	61.9
→3,4)-α-D-Gal*p*-(1→	3.5	5.22	4.10	4.08	4.17	4.06	3.75
b	174	102.5	73.21	76.8	78.4	67.8	62.2
α-Abe*p*-(1→	4	5.13	4.05*	1.99*	3.87*	4.10	1.19
c	175	101.8	64.8	34.4	70.6	68.1	16.9
→4)-α-L-Rha*p*-(1→	<1.5	5.17	4.10*	3.87	3.58	3.95*	1.34
d	173	102.8	68.2	70.7	82.9	69.3	18.4
α-D-Glc*p*-(1→	3.5	5.02	3.55	3.79	3.58	3.90	3.95
e	175	101.1	73.3	73.9	70.5	73.3	61.9

The signals overlapped with the signals of the tetrasaccharidic repeating unit are with an asterisk.

The way of distribution of the both oligosaccharides in O-chain was characterised by electrophoretic separation of LPS, then Western blotting with antisera O4 (Abe → 3Man) and O12 (Glc → 4Gal). Incubation with antisera O4 showed the presence of Abe → 3Man factor in both short and long LPS, while staining with O12 revealed Glc → 4Gal factor in long LPS chains. The Western blotting results clearly showed that glucose is attached to galactose at some considerable distance from the core part, and that this attachment occurs in the higher molecular-weight LPS fractions. The way of distribution of glucose residues in the chain was confirmed by a partial hydrolysis of rhamnosidic bonds. The mixture of the products was fractionated using GPC and HPLC. A degree of polymerization of the obtained fractions was identified by comparison to the retention time of standards, and by the molecular mass using FAB-MS spectra. This consideration did not concern abequose residue, which was cleaved during hydrolysis. Two main fractions were heksasaccharide (Hex_4dHex_2: two tetrasaccharidic repeats without abequose [959 u]) and oktasaccharide (Hex_6dHex_2: two pentasaccharidic repeats without abequose [1283 u]). Moreover, minor fraction was assigned to heptasaccharide (Hex_5dHex_2: one tetrasaccharidic and one pentasaccharidic repeats without abequose residues [1121 u]). A very small amount of the last fraction confirmed the ordered arrangement of the pentasaccharidic and tetrasaccharidic repeating units in the chain. The hexasaccharide originated from the beginning of OPS (close to the core part of LPS), the octasaccharides from the end of O-chain, while the heptasaccharide exactly from "the border", between tetra-, and pentasaccharidic repeating units.

The chemical structure of the repeating unit of the somatic O antigen (OPS) isolated from *S.* Abortusequi was identified as a tetrasaccharide with the additional substitution of galactose residue by glucose residue in the ~30% of repeating units at the end of O-chain. The nonstoichiometric distribution of the glucose residues in the OPS structure was reported in other *Salmonella* serovars, which posses serological factor of 12 [α-D-Glc*p*-(1 → 4)-α-D-Gal*p*-(1→], namely *Salmonella* Typhi (9,12), *Salmonella* Enteritidis (1,9,12) [19], and *Salmonella* Agona (1,4,12) [20].

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