Carbohydrate Research 344 (2009) 1054–1057

Contents lists available at ScienceDirect

Carbohydrate Research

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

Note

Structural determination of the O-antigenic polysaccharide from *Salmonella* Mara (O:39)

Jerzy Gajdus, Zbigniew Kaczyński *, Joanna Śmietana, Piotr Stepnowski

Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland

ARTICLE INFO

Article history:
Received 29 September 2008
Received in revised form 8 February 2009
Accepted 19 February 2009
Available online 25 February 2009

Keywords:
O-antigen
Salmonella Mara
Serogroup O:39
3-Acetamido-3,6-dideoxy-L-glucose
NMR

ABSTRACT

The O-antigenic polysaccharide of *Salmonella* Mara O:39 (formerly Q) was investigated by sugar and methylation analyses, absolute configuration assignment, mass spectrometry and NMR spectroscopy. The experiments revealed an O-polysaccharide chain composed of the following linear tetrasaccharide repeating units with the structure:
→2)-α-L-Quip3NAc-(1→3)-α-D-Manp-(1→3)-α-L-Fucp-(1→3)-α-D-GalpNAc-(1→
where α-L-Quip3NAc is the residue of 3-acetamido-3,6-dideoxy-α-L-glucopyranose. This repeating unit is the first published structure of the O-polysaccharide from 27 serotypes of *Salmonella* bacteria belonging to serogroup O:39 in the Kauffmann–White classification system.

Gram-negative bacteria of the genus *Salmonella* belong to the *Enterobacteriaceae* family and cause a variety of food-borne diseases in both humans and animals, commonly referred to as salmonellosis. The surface of bacterial cells is the first line of defence against antimicrobial substances and contains, among other constituents, lipopolysaccharides (LPSs) in the outer membrane. The complete LPS consists of three regions: the O-specific polysaccharide (O-antigen), constructed of oligosaccharide repeating units; the oligosaccharide core part; and the endotoxic, hydrophobic lipid A, anchored in the outer membrane of the cell wall. Structural variations in lipopolysaccharides exist mainly within the O-specific polysaccharide. Usually, variations in the O-antigenic structure of polysaccharides are directly correlated to pathogenicity and therefore also determine the O-antigen (O-PS) serological typing system. Knowledge of new O-antigen structures provides a basis for identifying and understanding the antigenic factors involved in their serological specificities as well as their functioning at the molecular level.

So far, 2579 *Salmonella* serovars in 45 serogroups have been serologically classified based on somatic O-antigens and flagellar H antigens.[1] However, only a few of them have been structurally characterised.[2] One of the first works that investigated the sugar composition of the O-antigenic polysaccharide of *Salmonella* bacteria belonging to serogroup Q (O:39) identified only D-GalN, D-Man and L-Fuc.[3] Later studies additionally identified D-Qui3N (3-amino-3,6-dideoxy-D-glucose) in O-antigens isolated from *Salmonella* Champaign and *Salmonella* Wandsworth, which belong to serogroup Q (O:39).[4] The first fully determined structure of the O-antigen in the *Salmonella* genus, including D-Qui3NAc, was found in *S.* Dakar (O:28).[5] This sugar residue or its derivatives were also found in O-polysaccharides of several species of Gram-negative bacteria, inter alia in *Citrobacter freundii*,[6] *Escherichia coli*, *Proteus penneri*, *Pseudomonas fluorescens*, *Hafnia alvei*, *Vibrio mimicus*[7] and *Providencia stuartii*.[8]

The LPS of *S.* Mara was isolated by phenol/water extraction and was further purified. After mild acetic acid hydrolysis of the LPS, the insoluble lipid A was removed by centrifugation. Column gel-permeation chromatography (Bio-Gel P 10) of water-soluble substances yielded a high-molecular-weight product—the O-antigen. Sugar analysis revealed the presence of Fuc, Man, GalNAc and 3-acetamido-3,6-dideoxyhexose in the approximate molar ratio of 1:1:1:1. The results of GLC–MS analysis of the partially methylated alditol acetates obtained from O-PS of *S.* Mara identified five products: 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylfucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol, 1,2,5-tri-*O*-acetyl-3,6-dideoxy-4-*O*-methyl-3-(*N*-methyl)acetamidoglucitol, 1,3,5-tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-acetamidogalactitol and 1,3,5-tri-*O*-acetyl-2-deoxy-4,6-di-*O*-methyl-2-(*N*-methyl)acetamidogalactitol (Table 1). The last two of these compounds were derived from the same sugar residue: →3)-GalNAc (**B**). Compositional analysis revealed the following substitution positions of monosaccharide residues: →3)-Fuc (**D**), →3)-Man (**C**), →2)-Qui3NAc (**A**), and the earlier-mentioned →3)-GalNAc (**B**).

The ^{1}H NMR spectrum (Fig. 1) confirmed the sugar analysis results, showing four distinct signals in the anomeric region at δ

* Corresponding author.
E-mail address: zbyszek@chem.univ.gda.pl (Z. Kaczyński).

0008-6215/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2009.02.015

Table 1
Methylation analysis results of *S.* Mara O-PS

Acetyl derivatives	Molar ratio
2,4-Di-*O*-methyl-fucitol	1.0
2,4,6-Tri-*O*-methyl-mannitol	1.0
3,6-Dideoxy-4-*O*-methyl-3-(*N*-methyl)acetamidoglucitol	0.8
2-Deoxy-4,6-di-*O*-methyl-2-acetamidogalactitol 2-Deoxy-4,6-di-*O*-methyl-2-(*N*-methyl)acetamidogalactitol	1.0

4.96 (**D**), 5.13 (**C**), 5.19 (**B**) and 5.58 (**A**) in the approximate ratio of 1:1:1:1. Additionally identified were two signals characteristic of the protons of the methyl groups of 6-deoxysugars (δ 1.19 and δ 1.30), as well as two signals of the *N*-acetyl groups (δ 2.06 and 2.08). The TOCSY spectrum exhibited four different spin systems: this enabled the identification of all the protons of residues **A** and **C**, but only four protons (H-1–H-4) of residues **B** and **D**. Protons H-5 and H-6 of residue **D** (Fuc) were assigned using the correlations H-6/C-4 and H-6/C-5 on the HMBC spectrum. The two remaining signals (δ 3.85 and 3.70–3.90) were identified as H-5 and H-6 of residue **B** (GalNAc), respectively. The proton orders in all spin systems were assigned using the COSY spectrum. The *gluco* configuration of the residue **A** (Qui3NAc), containing the signal characteristic of a methyl group (δ 1.30), was determined on the basis of the large coupling constant values ($^3J_{H\text{-}3,H\text{-}4}$ = 9.9 Hz, $^3J_{H\text{-}4,H\text{-}5}$ = 9.9 Hz) in the ^{1}H NMR spectrum. The *manno* configuration of the residue **C** was assigned on the basis of the $^3J_{H,H}$ coupling constant pattern. The remaining two monosaccharides possessed the *galacto* configuration and were identified as GalNAc (**B**) and Fuc (**D**).

The ^{13}C and ^{1}H,^{13}C HSQC NMR spectra showed four anomeric carbon signals at δ 102.40 (Fuc), 102.88 (Man), 95.10 (GalNAc) and 96.19 (Qui3NAc). The ^{13}C NMR spectrum also contained the remaining sugar carbon resonances (the unresolved cross-peak of C-6 of Man and C-6 of GalNAc at δ 62.04), two signals due to the methyl carbons of the *N*-acetyl groups (at δ 23.48 and 23.68) and two signals due to the carbonyl carbon atoms of the *N*-acetyl groups (at δ 176.07 and 176.12). All ^{1}H and ^{13}C chemical shifts (Table 2) were established using ^{1}H, ^{13}C, correlated ^{1}H,^{1}H (COSY and TOCSY), as well as correlated ^{1}H,^{13}C HSQC experiments.

Anomeric configurations of monosaccharides were defined on the basis of $^3J_{H\text{-}1,H\text{-}2}$ and $^1J_{C\text{-}1,H\text{-}1}$ coupling constants derived from ^{1}H and ^{1}H,^{13}C HSQC (recorded without decoupling; spectrum not shown) NMR experiments, respectively (Table 2). The values of $^1J_{C\text{-}1,H\text{-}1}$ were >170 Hz: this revealed the α anomeric configuration of all residues. The pyranose ring of all monosaccharide residues was inferred from GLC–MS analysis of partially methylated alditol acetates. The six-membered rings of all monosaccharides were also confirmed by the lack of carbon atom signals in the δ ~83–88 region of the ^{13}C NMR spectrum.[9,10]

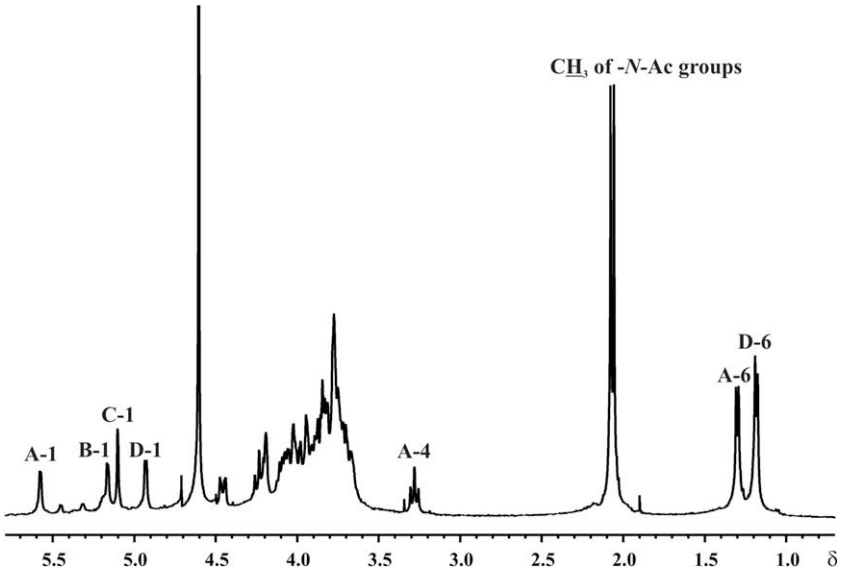


Figure 1. The ^{1}H NMR spectrum of the O-PS isolated from *S.* Mara. The letters refer to the carbohydrate residues as defined in Table 2, and the Arabic numerals refer to the protons in the respective residues.

Table 2
^{1}H and ^{13}C NMR data of the OPS isolated from *Salmonella* Mara

Residue	Coupling constants (Hz)	^{1}H, ^{13}C Chemical shifts (δ)					
	$^1J_{H\text{-}1,C\text{-}1}$ $^3J_{H\text{-}1,H\text{-}2}$	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6
→2)-α-L-Qui*p*3NAc-(1→	172	5.58	3.70	4.26	3.30	3.94	1.30
A	3.3	96.19	73.28	53.21	73.81	69.90	17.90
→3)-α-D-Gal*p*NAc-(1→	179	5.19	4.48	3.75	4.05	3.85	3.70–3.90[a]
B	3.5	95.10	49.40	76.91	69.91	72.74	62.04
→3)-α-D-Man*p*-(1→	177	5.13	4.22	4.09	3.79	3.81	3.70–3.90[a]
C	<2	102.88	71.08	76.21	68.68	74.59	62.04
→3)-α-L-Fuc*p*-(1→	172	4.96	3.86	4.01	3.97	4.12	1.19
D	4.3	102.40	68.45	78.35	72.71	68.26	16.50

Spectra were recorded at 40 °C in ^2H_2O relative to internal acetone (δ_H 2.225; δ_C 31.45).
The values of ^{13}C chemical shifts of glycosidically linked carbons are underlined.
[a] Overlapped signals

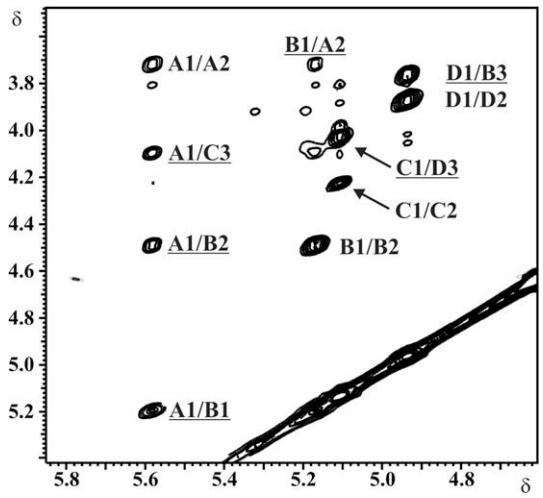
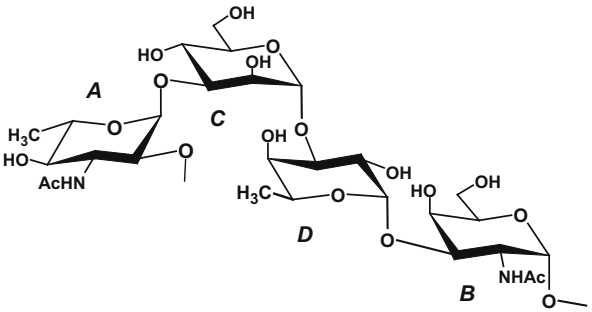


Figure 2. The section of NOESY spectrum of O-PS isolated from *S.* Mara. The letters refer to the carbohydrate residues as defined in Table 2, and the Arabic numerals refer to the protons in the respective residues. *Inter*-residual NOE contacts are underlined.

The absolute configuration of sugar constituents, except that of Qui3NAc, was established by GLC of (*S*,*R*)- and (*S*)-but-2-yl glycosides. This experiment demonstrated the D configuration of the Man*p* and Gal*p*NAc residues, and the L configuration of the Fuc*p* residue. The L configuration of Qui*p*3NAc was based on the analysis of the glycosylation effect on ^{13}C chemical shifts, which used published rules and NMR data.[11–13] The calculation performed for the disaccharide **A**→**C** **[**→2)-α-Qui*p*3NAc-(1→3)-α-D-Man*p*-(1→**]** gave +4.7 ppm effect for C-3 of Man, and +3.5 ppm effect for C-1 of Qui3NAc, which clearly revealed the different absolute configurations of both the constituent monosaccharides (D-Man*p* and L-Qui*p*3NAc). In the case of the same absolute configuration of both the residues, the values should be ~+7.5 ppm and ~+8.3 ppm, respectively.

The sugar sequence within the tetrasaccharide repeating unit of the O-antigen was shown in the NOESY spectrum (Fig. 2) that exhibited *inter*-residual NOE contacts between the following anomeric protons and corresponding glycosidically linked protons: **A**1/**C**3, **A**1/**B**1, **A**1/**B**2, **B**1/**A**2, **C**1/**D**3 and **D**1/**B**3. Compositional analyses and NMR data revealed the structure of the repeating unit of the O-antigen as shown below.



The assigned structure of the repeating unit and further immunochemical study will enable the coagglutination processes of *Salmonella* Mara O:39 with antisera against *Salmonella* bacteria belonging to other serogroups to be explained.[14]

1. Experimental

1.1. Bacterial culture; lipopolysaccharide isolation

Salmonella Mara (O:39) No. KOS 1492 was obtained from the National Salmonella Centre, Gdańsk, Poland. Bacteria were cultivated, as previously described,[15] on an enriched agar medium at 37 °C for 24 h, washed from the agar with 0.85% NaCl, killed with acetone and dried. The lipopolysaccharide was obtained from bacteria with the hot phenol/water extraction procedure.[16] The aqueous phase was dialysed against running tap water, and then with distilled water, then until free of phenol. The dialysate was lyophilised. Nucleic acids were precipitated with 40% ethanol (pH 4.5). After centrifugation the supernatant was dialysed and freeze-dried, after which the LPS was precipitated with 80% ethanol at pH 7. The LPS was dissolved, dialysed against distilled water and lyophilised.

1.2. Preparation and isolation of O-antigen of *Salmonella* Mara

The LPS (71.1 mg) was hydrolysed with 1% acetic acid at 100 °C for 3 h. The precipitated lipid A was removed by centrifugation. Freeze-dried water-soluble products (57.9 mg) were fractionated by gel-permeation chromatography on a 100 × 0.9 cm column (Bio-Gel P 10, 200–400 mesh; BioRad, Richmond, USA) with water as eluent. The collected fractions were monitored by refractive index (differential refractometric detector RIDK 101, Czech Republic). The highest molecular mass polymeric compound—the O-antigen (26.7 mg)—was subjected to further structural analysis.

1.3. Chemical methods

The sugar components of O-PS were determined as follows: hydrolysis (2 M trifluoroacetic acid—TFA; 120 °C, 3 h), reduction ($NaBH_4$), acetylation (Ac_2O, AcONa; 120 °C, 2 h). O-PS was methylated according to Hakomori (DIMSYL in DMSO and CH_3I)[17] and then the product was purified by dialysis against distilled water, then using a Sep Pak C_{18} cartridge.[18] The permethylated product was hydrolysed in aq 2 M TFA (at 120 °C for 3 h), reduced ($NaBH_4$) and acetylated as described above. All alditol acetates and partially methylated alditol acetates were analysed on a Perkin–Elmer Clarus 500 gas chromatograph with a flame-ionisation detector (FID) and Rtx-5 fused-silica capillary column (30 m, 0.25 mm I.D., 0.25 μm film thickness, Restek Co., USA). The temperature programme was 100–260 °C at 2 °C/min.

To determine the D and L configurations of the sugar components the O-PS of *S.* Mara was hydrolysed (2 M TFA, 120 °C, 3 h), and was then N-reacetylated (1% aq $NaHCO_3$, Ac_2O, 25 °C, 1 h). The hydrolysate of O-PS was treated with (*S*)- or (*R*,*S*)-2-butanol in the presence of TFA (105 °C, 6 h) and acetylated.[19,20] The acetylated (*S*)- and (*R*,*S*)-2-butyl glycosides were analysed on a TOP GC 8000 (CE Instruments) gas chromatograph with an FID. Separations were carried out on a DB-23 fused-silica capillary column (60 m, 0.3 mm I.D., 0.15 μm film thickness, J&W SCIENTIFIC, USA). A temperature programme of 120–260 °C at 2 °C/min, then 30 min at 260 °C was applied. The GLC peak of these diastereomeric derivatives was identified by GLC co-injection with standards and analysis of MS spectra.

GLC–MS analysis of all the above-mentioned derivatives was performed on a Hewlett Packard 5890 gas chromatograph, equipped with a DB-23 capillary column (as described above), and coupled to a TRIO-2000 mass spectrometer (VG Biotech, UK) with an electron impact ionisation energy of 70 eV.

1.4. NMR spectroscopy

NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer for 15 mg of the O-antigen dissolved in 0.7 mL of D_2O. Chemical shifts were reported relative to internal acetone (δ_H 2.225; δ_C 31.45). All NMR spectra—^1H, ^{13}C, ^1H,^1H-correlated NMR experiments (COSY—correlation spectroscopy, TOCSY—total correlation spectroscopy, NOESY—nuclear Overhauser enhancement spectroscopy)—as well as the ^1H,^{13}C-correlated experiment

(HSQC—heteronuclear single quantum correlation) were recorded at 40 °C. The TOCSY and NOESY experiments were measured in the phase-sensitive mode with mixing times of 100 ms and 300 ms, respectively.

Acknowledgements

We thank Professor Dr. R. Głośnicka and Dr. H. Dziadziuszko (National Salmonella Centre, Medical University of Gdańsk) for the bacterial cells culture of *S.* Mara. Financial support was provided by the University of Gdańsk under grant BW/8000-5-0115-8. The grant of the Polish Ministry of Research and Higher Education under DS 8200-4-0085-9 is greatly acknowledged.

References

1. Grimont, P. A. D.; Weill, F.-X. Antigenic Formulae of the *Salmonella* Serovars, 9th ed., WHO Collaborating Centre for Reference and Research on *Salmonella*, Institut Pasteur, Paris, 2007.
2. Gajdus, J.; Głośnicka, R.; Szafranek, J. *Wiad. Chem.* **2006**, *60*, 621.
3. Lüderitz, O.; Staub, A. M.; Westphal, O. *Bacteriol. Rev.* **1966**, *30*, 192.
4. Lüderitz, O.; Ruschmann, E.; Westphal, O.; Raff, R.; Wheat, R. *J. Bacteriol.* **1967**, *93*, 1681.
5. Kumirska, J.; Szafranek, J.; Czerwicka, M.; Paszkiewicz, M.; Dziadziuszko, H.; Kunikowska, D.; Stepnowski, P. *Carbohydr. Res.* **2007**, *342*, 2138.
6. Raff, R. A.; Wheat, R. W. *J. Bacteriol.* **1968**, *95*, 2035.
7. Kochetkov, N. K. *Russ. Chem. Rev.* **1996**, *65*, 735.
8. Kocharova, N. A.; Błaszczyk, A.; Zatonsky, G. V.; Torzewska, A.; Bystrowa, O. V.; Shashkov, A. S.; Knirel, Y. A.; Rozalski, A. *Carbohydr. Res.* **2004**, *339*, 409.
9. Duus, J. Ø.; Gotfredsen, C. H.; Bock, K. *Chem. Rev.* **2000**, *100*, 4589.
10. Bock, K.; Pedersen, C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27.
11. L'vov, V. L.; Tochtamysheva, N. V.; Shashkov, A. S.; Dmitriev, B. A.; Capek, K. *Carbohydr. Res.* **1983**, *112*, 233.
12. Shashkov, A. S.; Lipkind, G. M.; Knirel, Y. A.; Kochetkov, N. K. *Magn. Reson. Chem.* **1988**, *26*, 735.
13. Lipkind, G. M.; Shashkov, A. S.; Knirel, Y. A.; Vinogradov, E. V.; Kochetkov, N. K. *Carbohydr. Res.* **1988**, *175*, 59.
14. Popoff, M. Y. Guidelines for the preparation of *Salmonella* antisera, 6th ed., WHO Collaborating Center for Reference and Research on *Salmonella*, Institut Pasteur, Paris, 2001.
15. Szafranek, J.; Gajdus, J.; Kaczyński, Z.; Dziadziuszko, H.; Kunikowska, D.; Głośnicka, R.; Yoshida, T.; Vihanto, J.; Pihlaja, K. *FEMS Immunol. Med. Microbiol.* **1998**, *21*, 243.
16. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83.
17. Hakomori, S.-I. *J. Biochem. (Tokyo)* **1964**, *55*, 205.
18. Waeghe, T. J.; Darvill, A. G.; McNeil, M.; Albersheim, P. *Carbohydr. Res.* **1983**, *123*, 281.
19. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1978**, *62*, 349.
20. Leontein, K.; Lindberg, B.; Lönngren, J. *Carbohydr. Res.* **1978**, *62*, 359.