ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/236947710

The unique structure of complete lipopolysaccharide isolated from semi-rough Plesiomonas shigelloides O37 (strain CNCTC 39/89) containing (2S)-O-(4-oxopentanoic acid)-α-D-Glcp (α-D...

ARTICLE *in* CARBOHYDRATE RESEARCH · APRIL 2013

Impact Factor: 1.93 · DOI: 10.1016/j.carres.2013.04.015 · Source: PubMed

CITATIONS
3

READS
26

6 AUTHORS, INCLUDING:

Marta Kaszowska
Ludwik Hirszfeld Institute of Immunology a...
10 PUBLICATIONS **52** CITATIONS
SEE PROFILE

Jolanta Lukasiewicz
Polish Academy of Sciences
39 PUBLICATIONS **276** CITATIONS
SEE PROFILE

Czeslaw Lugowski
Instytut Immunologii i Terapii Doświadczal...
90 PUBLICATIONS **1,638** CITATIONS
SEE PROFILE

Available from: Marta Kaszowska
Retrieved on: 24 January 2016

Carbohydrate Research 378 (2013) 98–107

Contents lists available at SciVerse ScienceDirect

Carbohydrate Research

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

The unique structure of complete lipopolysaccharide isolated from semi-rough *Plesiomonas shigelloides* O37 (strain CNCTC 39/89) containing (2S)-O-(4-oxopentanoic acid)-α-D-Glc*p* (α-D-Lenose) ☆

Marta Kaszowska [a,*], Wojciech Jachymek [a], Jolanta Lukasiewicz [a], Tomasz Niedziela [a], Lennart Kenne [b], Czeslaw Lugowski [a,c]

[a] Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla 12, PL-53-114 Wroclaw, Poland
[b] Department of Chemistry, Swedish University of Agricultural Sciences, PO Box 7015, SE-750 07 Uppsala, Sweden
[c] Department of Biotechnology and Molecular Biology, University of Opole, PL-45-035 Opole, Poland

ARTICLE INFO

Article history:
Received 12 March 2013
Accepted 15 April 2013
Available online 30 April 2013

Keywords:
Semi-rough lipopolysaccharide
Oxopentanoic acid
NMR
MALDI-TOF MS
MM2 force field
Plesiomonas shigelloides

ABSTRACT

The complete structure of semi-rough lipopolysaccharide (SR-LPS) of *Plesiomonas shigelloides* CNCTC 39/89 (serotype O37) has been investigated by ^{1}H and ^{13}C NMR spectroscopy, matrix-assisted laser-desorption/ionization time-of-flight MS, and chemical methods. The following structure of the single unit of the O-antigen has been established:

α-D-Len*p*-(1→3)-α-L-Rha*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→4)-β-D-Glc*p*NAc-(1→
2 (OAc) 6 (OAc)

in which α-D-Len*p* is (2*S*)-*O*-(4-oxopentanoic acid)-α-D-Glc*p* residue which has not been found in nature. The absolute configuration of oxopentanoic acid moiety in α-D-Lenose residue was determined by NOESY experiment combined with molecular modeling (MM2 force field). The decasaccharide core is substituted at C-4 of the β-D-Glc*p* residue with a single pentasaccharide unit. Lipid A is built of a β-D-Glc*p*N4P-(1→6)-α-D-Glc*p*N1P disaccharide asymmetrically substituted with fatty acids. It was concluded that the core oligosaccharide and the lipid A are identical with those in *P. shigelloides* CNCTC 113/92 Niedziela et al. (2002)[9] and Lukasiewicz et al. (2006).[10]

© 2013 Published by Elsevier Ltd.

1. Introduction

Lipopolysaccharide (LPS), also known as endotoxin, is a major component of the outer membrane of Gram-negative bacteria. It induces different pathophysiological effects on a wide variety of mammalian cells. These effects can result in sepsis endotoxic shock, and ultimately multiple organ failure and death.[1] Therefore the properties of LPS have been widely investigated.

The amphiphilic LPS isolated from smooth bacterial strains consists of three parts, that is, lipid A, core oligosaccharide, and O-specific polysaccharide.[2] The hydrophobic lipid A is responsible for most of the endotoxic activities of LPS.[3] The core oligosaccharides (OS) have complex structures that are relatively conserved among bacterial species. They are important for biological and physical properties of the entire LPS molecule and play a significant role in the interactions of LPS with the host. The O-specific polysaccharide (O-specific chain, O-antigen) determines bacterial O-serotype, and defines a fingerprint of bacteria. The O-antigen is also a virulence factor in several bacterial species.[4] The O-specific chains are present only in LPS of smooth-type Gram-negative bacteria (S-LPS). When the O-antigen is absent (R-LPS), the colonies are of rough-type. The semi-rough LPS (SR-LPS) contains only a single O-antigen unit.

Abbreviations: SR-LPS, semi-rough lipopolysaccharide; OS, oligosaccharide; MALDI-TOF, matrix-assisted laser-desorption/ionization time-of-flight; MS, mass spectrometry; GC, gas chromatography; NMR, nuclear magnetic resonance; COSY, correlated spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; DEPT, distortionless enhancement by polarization transfer; MM2, molecular mechanics version; CNCTC, Czech National Collection of Type Cultures.

☆ Presented at the 3rd German-Polish-Russian Meeting on Bacterial Carbohydrates, Wroclaw 2004, Poland

* Corresponding author. Tel.: +48 71 370 99 27; fax: +48 71 337 13 82.
E-mail address: Marta.Kaszowska@iitd.pan.wroc.pl (M. Kaszowska).

0008-6215/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.carres.2013.04.015

Plesiomonas shigelloides belongs to the *Enterobacteriaceae* family. It is a Gram-negative, rod shaped, and facultative anaerobic bacterium associated with diarrheal disease in humans. These bacteria are not a part of natural bacterial flora in humans and they cause several types of gastroenteritis.[5] Extraintestinal infections, such as meningitis and bacteremia, are also associated with *P. shigelloides* infection.[6] These bacteria have been isolated from human feces, many wild and domestic animals, and particularly water environment.[7] Two complete structures of *P. shigelloides* S-LPS from CNCTC 113/92 (serotype O54)[8–10] and CNCTC 144/92 (serotype O74)[11,12] have been reported. The core oligosaccharide substituted with a biological repeating unit of strain 302-73 (serotype O1),[13–15] 7-63 and PCM 2231 (serotype O17),[16,17] AM36565,[18] and O-specific polysaccharides of *P. shigelloides* lipopolysaccharides of strain CNCTC 110/92 (serotype O51)[19] and strains 12254, 22074[20] have been investigated. Additionally, the structure of the O-specific polysaccharide isolated of *P. shigelloides* serotype O17 has been reported as identical to that of *Shigella sonnei* phase I.[21]

In the present study we report on complete and unique SR-LPS structure of *P. shigelloides* serotype O37 (CNCTC 39/89) confirming the presence of the novel sugar (2*S*)-*O*-(4-oxopentanoic acid)-α-D-Glc*p* (α-D-Lenose, α-D-Len*p*) residue in the O-antigen part.

2. Results

2.1. Isolation of the core oligosaccharide substituted by single O-antigen unit and unsubstituted core oligosaccharide

LPS of *P. shigelloides* O37 was isolated from both the phenol (LPS_{PhOH}) and the water (LPS_{H2O}) phases and analyzed by SDS–PAGE. The main bands observed on the silver stained gel corresponded to components in LPS isolated from phenol (Fig. 1A) and water (Fig. 1B) phases. The yields of LPS_{H2O} and LPS_{PhOH} were 0.5% and 1.0%, respectively. The mild acid hydrolysis of the LPS_{PhOH} yielded three oligosaccharide fractions: OSI—consisting of a core oligosaccharide substituted by several repeating units (yield <<1.0%), OSII—the core oligosaccharide substituted by a single O-antigen unit (yield 9.7%), and OSIII—the unsubstituted core oligosaccharide (yield 6.3%). From LPS_{H2O} only the core oligosaccharide fraction (OSIII, yield 14.1%) was isolated. The low yield of OSI suggested the semi-rough (SR-LPS) type of *P. shigelloides* O37 LPS. All investigations were carried out on fractions isolated from both LPS_{H2O} and LPS_{PhOH}. The data presented herein concern the LPS_{PhOH}. The OSIII isolated from LPS_{H2O} had the same structure as OSIII isolated from LPS_{PhOH}, thus the data for OSIII (LPS_{H2O}) are not presented herein to avoid unnecessary duplication.

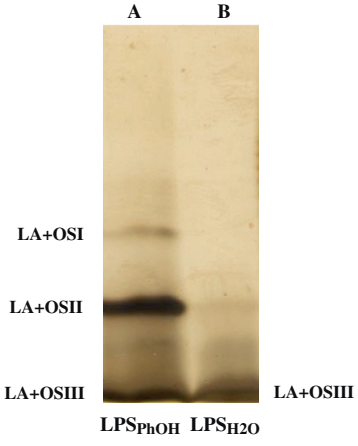

Figure 1. SDS/PAGE analysis of the *P. shigelloides* O37 LPS isolated from phenol (A) and water (B) phases. LA—lipid A, OSI-III—the oligosaccharide fractions labeled as shown in the text.

2.2. Structure analysis of the core oligosaccharide and the single O-antigen unit

The initial NMR investigation of the core oligosaccharide (OSIII) and core oligosaccharide substituted by O-antigen (OSII) indicated the presence of uronic acid, Kdo, and non-N-acetylated GlcN among the constituents. Therefore all subsequent sugar and methylation analyses were performed on N-acetylated and carboxyl-reduced fractions in order to facilitate acid hydrolysis and detection of all monosaccharide components. Composition analysis of the carboxyl-reduced and N-acetylated OSIII fraction together with determination of the absolute configuration revealed the presence of L,D-Hep, D-Gal, D-Glc, D-GlcN, and Kdo. The methyl esters of the permethylated material were reduced with 'Superdeuteride' [$LiB(C_2H_5)_2D$]. Analysis of such derivatives showed additionally the presence of uronic acid in these fractions. The analyses of OSIII showed the presence of 2,3,7-trisubstituted L,D-Hep*p*, 3,4-disubstituted L,D-Hep*p*, terminal L,D-Hep*p*, 4,6-disubstituted D-Glc*p*N, two terminal D-Glc*p*, two terminal D-Gal*p*, 4-substituted D-Gal*p*A, and 5-substituted Kdo*p*. The same procedures were applied for OSII showing the presence of monosaccharides identified for OSIII and 4-substituted D-Glc*p* was identified instead of the one terminal D-Glc*p*. Additionally the 4-substituted D-Glc*p*NAc, 3-substituted D-Glc*p*, two 3-substituted L-Rha*p*, and 2-*O*-(4-oxopentanoic acid)-α-D-Glc*p* (α-D-Lenose) were identified in OSII as residues of the single O-antigen unit.

The OSII and OSIII were analyzed by ^1H, ^{13}C NMR spectroscopy. The ^1H NMR spectrum of the OSIII contained main signals for nine anomeric protons, as well as signals characteristic for the deoxy protons of Kdo*p* residue belongs to core oligosaccharide part (residues **A–K**) (Fig. 2A). The ^1H NMR spectrum of the OSII contained the signals for the core oligosaccharide (residues **A–K**) and as well as those for atoms from five additional sugar residues building to

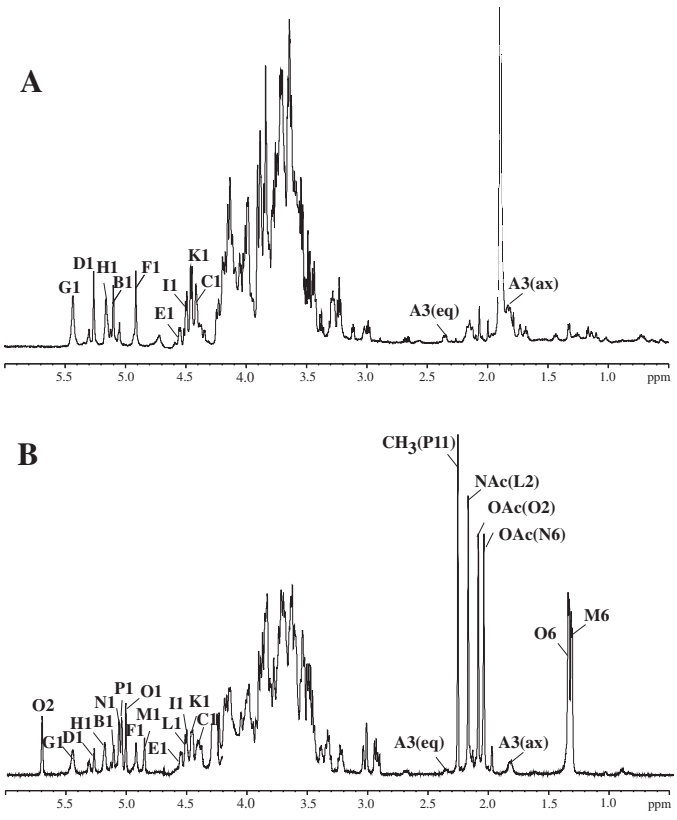

Figure 2. ^1H NMR spectra of the oligosaccharides of *P. shigelloides* O37: (A) OSIII, (B) OSII. The uppercase letters refer to carbohydrate residues as shown in Chart 1, and the numbers refer to protons in the respective residues.

the O-antigen structure (residues **L–P**) (Fig. 2B). The 1D ^{31}P NMR spectra did not indicated a presence of phosphate groups in both fractions. As the core oligosaccharide part of OSII had the same structure as unsubstituted core oligosaccharide fraction (OSIII), the data for OSIII are not presented herein to avoid unnecessary duplication. To avoid repetition only residues identified for O-antigen for *P. shigelloides* O37 were described in details in the text. The COSY spectra allowed for the identification of H-2 signals of the residues and subsequently TOCSY spectra with different mixing times and HSQC-TOCSY spectrum allowed for the assignment of the H-3 to H-6 (or H-7,7′) signals for each residue of the OSII (marked as uppercase letters). The spectra showed the presence of 5-substituted Kdo*p* (residue **A**), 3,4-disubstituted L-*glycero*-α-D-*manno*-Hep*p* (residue **B**), β-D-Gal*p* (residue **C**), 2,3,7-trisubstituted L-*glycero*-α-D-*manno*-Hep*p* (residue **D**), β-D-Glc*p* (residue **E**), L-*glycero*-α-D-*manno*-Hep*p* (residue **F**), 4-substituted α-D-Gal*p*A (residue **G**), 4,6-disubstituted α-D-Glc*p*N (residue **H**), 4-substituted β-D-Glc*p* (residue **I**), and β-D-Gal*p* (residue **K**) as core oligosaccharide residues (Fig. 3, Table 1).

Residue **L** at δ_H/δ_C 4.54/101.8 ppm, $^1J_{C\text{-}1,H\text{-}1}$ ~163 Hz was assigned as the 4-substituted β-D-Glc*p*NAc residue from a low ^{13}C chemical shift of the C-2 signal (δ_C 56.4 ppm), a downfield shift of the C-4 signal (δ_C 77.5 ppm), and the large vicinal couplings between all ring protons. *N*-acyl group at δ_H/δ_C 2.05/22.8 ppm (δ_C 175.4 ppm) was identified.

Residue **M** (δ_H/δ_C 4.85/101.0 ppm, $^1J_{C\text{-}1,H\text{-}1}$ ~163 Hz) was identified as a 3-substituted α-L-Rha*p* from the signals of exocyclic CH_3 groups (δ_H/δ_C 1.32/17.4 ppm) and the small vicinal coupling constants between H-1 and H-2. Relative downfield chemical shifts of the C-3 signal at δ_C 79.1 ppm indicated substitution position for residue.

Residue **N** at δ_H/δ_C 5.06/96.5 ppm, $^1J_{C\text{-}1,H\text{-}1}$ ~170 Hz, was recognized as a 3-substituted α-D-Glc*p*6OAc. Relative downfield chemical shift of the H-6,6′/C-6 signals at δ_H/δ_C 4.28/63.6 ppm identified substitution by *O*-acetyl group (δ_H/δ_C 2.09/21.0, δ_C 174.9 ppm). Significant downfield shift was observed for C-3 of this residue (δ_C 83.0 ppm) as compared with the chemical shift of the corresponding non-substituted monosaccharide, indicating the linkage position for this residue.[22]

Residue **O** at δ_H/δ_C 4.99/99.9 ppm, $^1J_{C\text{-}1,H\text{-}1}$ ~163 Hz was identified as a 3-substituted α-L-Rha*p*2OAc on the basis of the signals of exocyclic CH_3 groups (δ_H/δ_C 1.34/17.5 ppm) and the small vicinal coupling constants between H-1 and H-2. Relative downfield chemical shifts of the C-3 at δ_C 74.9 ppm indicated substitution position. Characteristic chemical shift values of H-2 (δ_H 5.69 ppm) indicated substitution by *O*-acetyl group (δ_H/δ_C 2.17/21.2, δ_C 174.4 ppm).

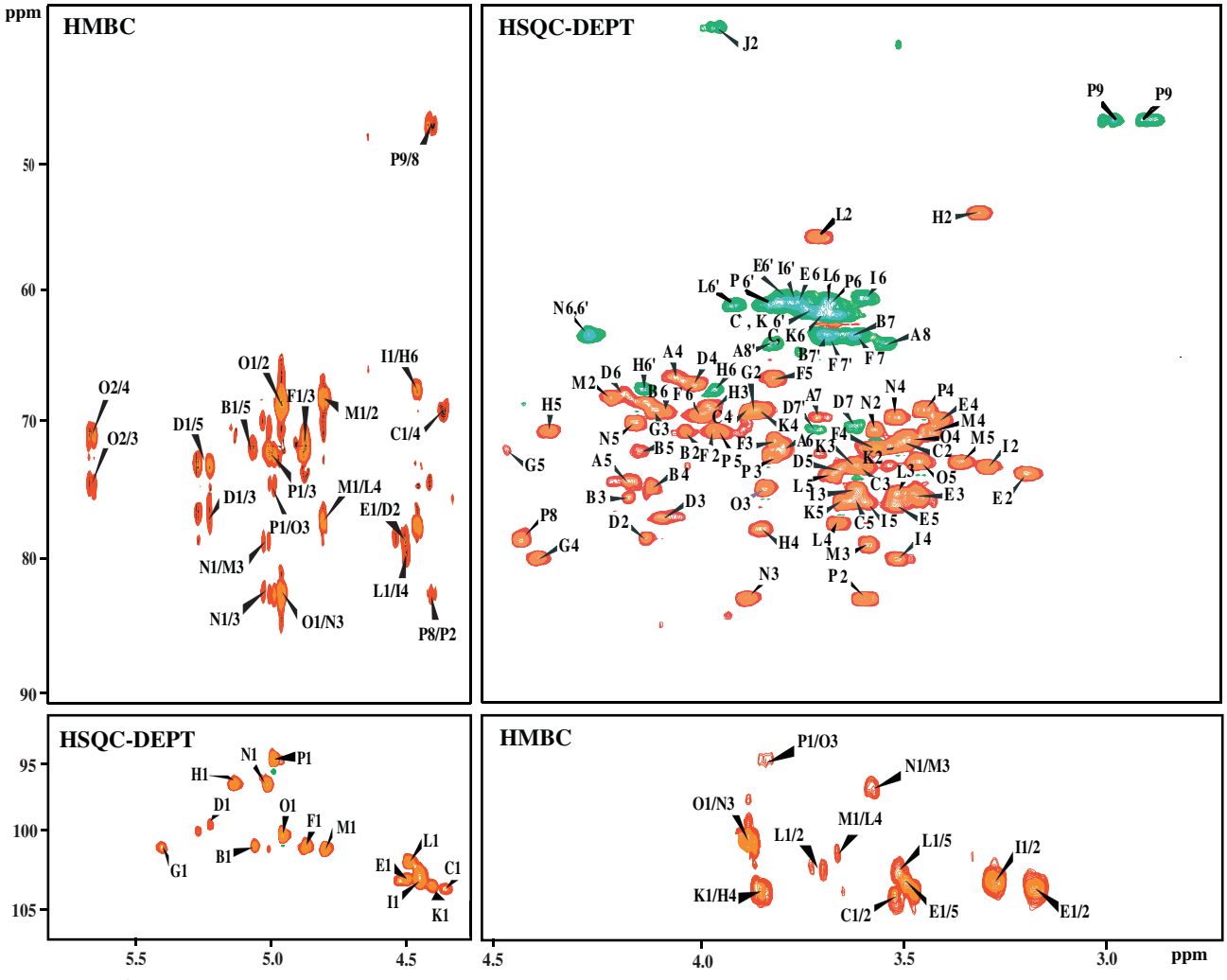

Figure 3. Selected $^1J_{H,C}$- and $^3J_{H,C}$-connectivities in HSQC-DEPT and HMBC spectra of the OSII isolated from *P. shigelloides* O37 LPS constituting of the core oligosaccharide substituted by single O-antigen unit. The cross-peaks are marked as uppercase letter indices in the text.

Table 1
^{1}H and ^{13}C NMR chemical shifts of core oligosaccharide substituted by a single O-antigen unit (OSII) of the *P. shigelloides* O37 (strain CNCTC 39/89)

Residue	Chemical shifts [ppm]										
	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6,6′/C6 (CH_3)	H7,7′/C7 (NAc, OAc) (COOH)	H8,8′/C8	H9/C9	C=O	CH_3
A 5)-α-Kdo*p*			1.82, 2.16	4.07	4.19	3.82	3.72	3.56, 3.85, 64.3			
	174.4	96.4	34.9	66.7	74.4	71.9	69.7				
B →3,4)-L-*glycero*-α-D-*manno*-Hep*p*-(1→	5.10	4.05	4.19	4.13	4.16	4.01	3.64, 3.70				
	100.8	70.7	75.9	74.9	72.2	69.2	63.7				
C β-D-Gal*p*-(1→	4.40	3.54	3.62	3.87	3.65	3.71, 3.74					
	103.7	71.6	73.3	69.1	75.7	61.9					
D →2,3,7)-L-*glycero*-α-D-*manno*-Hep*p*-(1→	5.26	4.14	4.11	4.03	3.69	4.19	3.64, 3.74				
	99.1	78.6	77.1	67.2	73.8	68.0	70.4				
E β-D-Glc*p*-(1→	4.55	3.22	3.49	3.43	3.55	3.79, 3.83					
	103.1	73.8	75.3	69.9	76.0	61.3					
F L-*glycero*-α-D-*manno*-Hep*p*-(1→	4.91	3.98	3.83	3.84	3.59	4.01	3.64, 3.70				
	100.8	70.7	71.5	66.9	71.9	69.5	63.6				
G →4)-α-D-Gal*p*A-(1→	5.43	3.89	4.12	4.41	4.48						
	100.9	69.2	69.1	80.0	72.1	175.0					
H →4,6)-α-D-Glc*p*N-(1→	5.17	3.34	3.99	3.87	4.38	3.98, 4.15					
	96.5	54.6	68.9	77.9	70.7	67.6					
I →4)-α-D-Glc*p*-(1→	4.50	3.32	3.64	3.54	3.61	3.62, 3.78					
	103.0	73.4	75.0	80.0	76.0	60.9					
J Gly		3.97, 3.99									
	168.8	40.9									
K β-D-Gal*p*-(1→	4.45	3.54	3.63	3.87	3.67	3.71, 3.74					
	103.5	71.6	73.3	69.1	76.0	61.9					
L →4)-β-D-Glc*p*NAc-(1→	4.54	3.73	3.71	3.69	3.54	3.70, 3.94	2.05				
	101.8	56.4	74.0	77.5	75.4	61.3	22.8	175.4			
M →3)-α-L-Rha*p*-(1→	4.85	4.23	3.61	3.46	3.38	1.32					
	101.0	68.22	79.1	70.7	72.9	17.4					
N →3)-α-D-Glc*p*6OAc-(1→	5.06	3.60	3.90	3.55	4.17	4.28	2.09				
	96.5	70.6	83.0	69.7	70.1	63.6	21.0	174.9			
O →3)-α-L-Rha*p*2OAc-(1→	4.99	5.69	3.86	3.52	3.48	1.34	2.17	174.4			
	99.9	68.7	74.9	71.3	72.9	17.5	21.2				
P α-D-Len*p*-(1→	5.03	3.63	3.83	3.48	3.93	3.68, 3.84	180.3	4.45	2.93, 3.03	218.8	2.26
	94.7	83.0	72.4	69.1	71.1	62.3		78.7	47.8		30.4

Spectra were recorded for $^{2}H_2O$ solution at 303 K. Acetone (δ_H/δ_C 2.225/31.05 ppm) was used as internal reference.

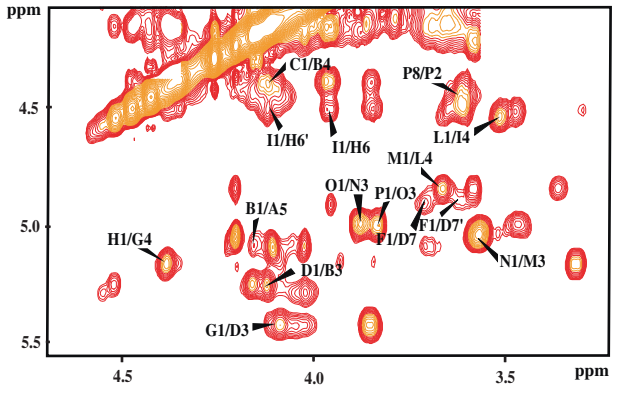

Figure 4. Selected part of the NOESY spectrum of the OSII isolated from *P. shigelloides* O37 LPS. The cross-peaks are labeled as shown in the text and Chart 1. Only inter-residue NOE connectivities from the anomeric protons of the OSII were assigned.

Residue **P** at δ_H/δ_C 5.03/94.7 ppm, $^{1}J_{C\text{-}1,H\text{-}1} \sim 170$ Hz, was recognized as a 2-*O*-(4-oxopentanoic acid)-α-D-Glc*p* (α-D-lenose, α-D-Len*p*). The downfield ^{13}C chemical shift of the C-2 signal at δ_C 83.0 ppm shows the position of ether bound substitution. The additional positive signal at δ_H/δ_C 4.45/78.7 ppm (H-8), negative signals at δ_H/δ_C at 2.93, 3.03/47.8 ppm of the CH_2 group (H-9,9′), and a signal from CH_3 group at δ_H/δ_C 2.26/30.4 ppm (H-11) showed in the HMBC spectrum correlations to carboxyl group resonance at δ_C 180.3 ppm (C-7), and a keto group resonance at δ_C 218.8 ppm (C-10).

In the HSQC-DEPT spectrum additional negative CH_2 signals at δ_H/δ_C 3.97, 3.99/40.9 ppm were detected. The resonance at δ_H/δ_C 3.97/40.9 ppm showed correlation with a carbonyl carbon signal at δ_C 168.8 ppm in the HMBC spectrum, suggesting the presence of glycine (residue **J**). This residue was also confirmed by amino acid analysis and mass spectrometry, however the substitution position was not determined.

Each disaccharide element in the OSII was identified by NOESY and HMBC experiments. NOESY spectra showed strong inter-residue cross-peaks between the following transglycosidic protons: H-1 of **B**/H-5 of **A**, H-1 of **D**/H-3 of **B**, H-1 of **C**/H-4 of **B**, H-1of **G**/H-3 of **D**, H-1 of **E**/H-2 of **D**, H-1 of **F**/H-7,7′ of **D**, H-1 of **H**/H-4 of **G**, H-1 of **K**/H-4 of **H**, H-1 of **I**/H-6,6′ of **H**, H-1 of **L**/H-4 of **I**, H-1 of **M**/H-4 of **L**, H-1 of **N**/H-3 of **M**, H-1 of **O**/H-3 of **N**, and H-1 of **P**/H-3 of **O** (Fig. 4, Table 2). The HMBC spectrum showed cross-peaks between signals from the anomeric proton and the carbon at the linkage position and between signal from the anomeric carbon and the proton at the linkage position, which confirmed the structure of the OSII in the LPS of *P. shigelloides* O37 (Fig. 3, Table 2). These data confirmed substitution positions of the monosaccharide residues and demonstrated their sequence in the OSII.

The NMR spectra of the OSII fraction provided data on the linkage between the O-antigen unit and the core oligosaccharide part. The →4)-β-D-Glc*p*NAc-(1→ (residue **L**) of the O-antigen unit is linked to C-4 (δ_C 80.1 ppm) of β-D-Glc*p*-(1→ (residue **I**) corresponding to the terminal form of this residue in the unsubstituted core oligosaccharide (Figs. 3 and 4). Thus the combined results suggest the following structure of the OSII of the *P. shigelloides* O37 with core oligosaccharide part identical with that of *P. shigelloides* O54 LPS[9] (Chart 1).

Table 2
Selected inter-residue NOE and $^3J_{H,C}$ connectivities from the anomeric atoms of the O-antigen unit observed for the OSII fraction isolated from *P. shigelloides* O37 (strain CNCTC 39/89)

Residue	H1/C1	Connectivities to		Inter-residue atoms/residues
	δ_H/δ_C	δ_H	δ_H	
	ppm			
B →3,4)-L-*glycero*-α-D-*manno*-Hep*p*-(1→	5.10 100.8	4.19	74.4	H-5, C-5 of **A**
C β-D-Gal*p*-(1→	4.40 103.7	4.19	75.6	H-4, C-4 of **B**
D →2,3,7)-L-*glycero*-α-d-*manno*-Hep*p*-(1→	5.26 99.1	4.13	74.9	H-3, C-3 of **B**
E β-D-Glc*p*-(1→	4.55 103.1	4.14	78.6	H-2, C-2 of **D**
F L-*glycero*-α-D-*manno*-Hep*p*-(1→	4.91 100.8	3.64, 3.74	70.4	H-7, 7' C-7 of **D**
G →4)-α-D-Gal*p*A-(1→	5.43 100.9	4.11	77.1	H-3, C-3 of **D**
H →4,6)-α-D-Glc*p*N-(1→	5.17 96.5	4.41	80.0	H-4, C-4 of **G**
I →4)-β-D-Glc*p*-(1→	4.50 103.0	3.98, 4.15	67.6	H-6, 6' C-6 of **H**
L →4)-β-D-Glc*p*NAc-(1→	4.54 101.8	3.54[a]	80.0	H-4, C-4 of **I**[b]
M →3)-α-L-Rha*p*-(1→	4.85 101.0	3.69	77.5	H-4, C-4 of **L**
N →3)-α-D-Glc*p*6OAc-(1→	5.06 96.5	3.61	79.1	H-3, C-3 of **M**
O →3)-α-L-Rha*p*2OAc-(1→	4.99 99.9	3.90	83.0	H-3, C-3 of **N**
P α-D-Len*p*-(1→	5.03 94.7	3.86	74.9	H-3, C-3 of **O**

[a] This value represents NOE connectivities only.
[b] The linkage between the O-antigen unit and the core oligosaccharide part.

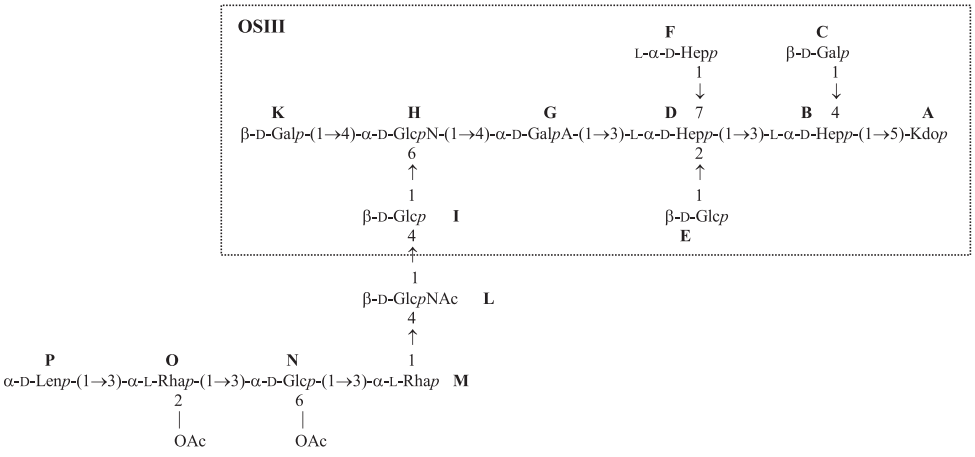

Chart 1.

The NOE connectivities of the selected protons of α-D-Lenose residue combined with MM2 calculations allowed for determination of the absolute configuration of the 2-hydroxy-4-oxopentanoic acid part of residue P. The conformation maps were obtained for the (2*S*)- and (2*R*)-isomers of the acid part of residue P in the P→O disaccharide fragment. Proximity between H-8 and H-2 of P protons could be observed for both (2*S*)- and (2*R*)-isomers (distances of 2.4 and 2.6 Å, respectively). However, the proximity between H-8 and H-1 of P could only be observed for the (2*R*)-isomer (2.4 Å), whereas in the (2*S*)-isomer the distance between these protons was too long to provide NOE contact (4.3 Å). The NOESY experiment confirmed the (2*S*)-configuration as no correlation between H-8 and H-1 of P could be observed (Fig. 5).

The fractions of core oligosaccharide (OSIII) and core oligosaccharide substituted by the O-antigen unit (OSII) were analyzed by MALDI-TOF MS. Ten sugar residues: two Gal, two Glc, three Hep, GalA, GlcN, and Kdo, give together a monoisotopic mass of 1799.571 Da (M_{OSIII}). The MALDI-TOF mass spectrum of OSIII showed main ions at *m*/*z* 1804.505 $[M_{OSIII}-H_2O+Na]^+$, *m*/*z* 1822.505 $[M_{OSIII}+Na]^+$ for the fraction of the complete, unsubstituted core oligosaccharide (Fig. 6A). The ions at *m*/*z* 1642.454 $[M_{OSIII}-Hex-H_2O+Na]^+$ and *m*/*z* 1660.455 $[M_{OSIII}-Hex+Na]^+$corresponded to the core oligosaccharide devoid of one hexose residue (terminal Glc). The presence of glycine in OSIII was confirmed by ions at *m*/*z* 1699.470 $[M_{OSIII}-Hex-H_2O+Gly+Na]^+$ and *m*/*z* 1861.515 $[M_{OSIII}-H_2O+Gly+Na]^+$. The mass spectrum of OSII

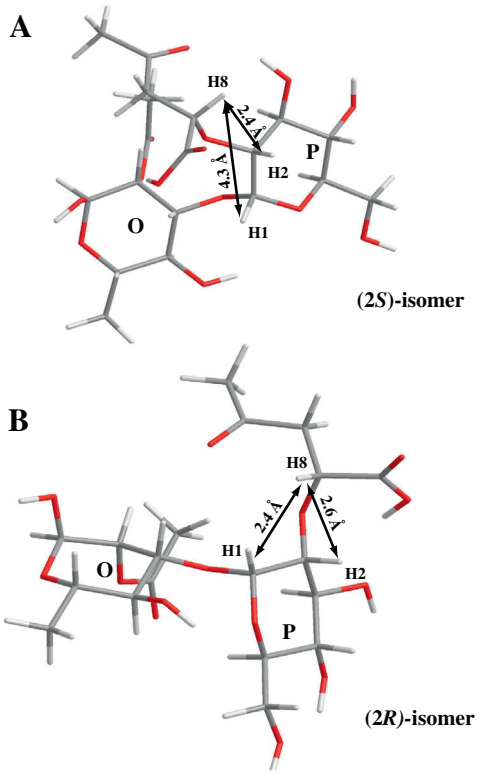

Figure 5. Calculated preferred conformations of the P→O fragment of the O-antigen unit. (A): the major conformation of the fragment with the (2*S*)-isomer (bond torsions between H-2 and H-8 of P residue: 140°, 160°, MM2 energy 56 kcal/mol); (B) the conformation with (2*R*)-isomer (bond torsions between H-2 and H-8 of residue P: −170°, 90°, MM2 energy 40 kcal/mol). Designated are proton pairs with distinguishing NOEs and distances between them (Å).

showed main ions at *m*/*z* 2822.293 $[M_{OSII}-H_2O+Na]^+$, *m*/*z* 2840.296 $[M_{OSII}+Na]^+$, and *m*/*z* 2780.257 $[M_{OSII}-Ac-H_2O+Na]^+$ (Fig. 6B). The five sugar residues, two acetyl groups give together a monoisotopic mass of 1035.364 Da. The mass difference between OSII and OSIII fractions resulted from the presence of a single unit of the O-antigen substituting the core oligosaccharide fragment in OSII. The remaining ions corresponded to different sodium and potassium adducts.

2.3. Structural analysis of lipid A

The structure of the *P. shigelloides* O37 lipid A has been investigated by chemical methods combined with ESI-MS^n.

The ESI mass spectrum recorded in the negative ion mode showed that lipid A isolated from *P. shigelloides* O37 LPS_{PhOH} is heterogeneous and is represented by two forms differing in their acylation patterns (LA_I and LA_{II}) (Fig. 7A, outlined structures). Both forms of lipid A contained the β-D-Glc*p*N4P-(1→6)-α-D-Glc*p*N1P disaccharide backbone. The carbohydrate backbone is substituted with four primary and two secondary fatty acids. The qualitative analyses of the acyl components of lipid A were carried out separately for amide-[(*R*)-3-hydroxytetradodecanoic acid 14:0(3-OH)] and ester-bound [(*R*)-3-hydroxydodecanoic (12:0(3-OH), tetradecanoic (14:0), dodecanoic (12:0)] fatty acids. The absolute configuration of GlcN residues in lipid A was determined as D.

2.3.1. ESI-MS^n analysis of the fatty acid distribution in lipid A

The most abundant ion at *m*/*z* 1660.4 represented the main population of the *P. shigelloides* O37 lipid A (LA_I), that is the monophosphorylated and hexaacylated molecule. The ion at *m*/*z* 1740.3 with lower intensity represented the bisphosphorylated form of this main structure (LA_I). The second form of lipid A (LA_{II}) represented by ion at *m*/*z* 1632.4 showed significantly lower abundance. This ion could be explained by the monophosphorylated, hexaacylated form of lipid A. The mass difference between the LA_I and LA_{II} forms is 28 Da (C_2H_4 fragment) suggesting the presence of fatty acids with a different chain length—dodecanoic acid (12:0) in LA_{II} and tetradecanoic acid (14:0) in LA_I.

2.3.2. ESI-MS^n analysis of the main form of lipid A (LA_I)

The ion at *m*/*z* 1660.4 represented the monophosphorylated, hexaacylated lipid A substituted with [14:0(3-OH)] at N-2 and N-2′, [12:0(3-OH)] at O-3 and O-3′, and (12:0) and (14:0) are secondary acids (Fig. 7A). This ion was isolated and subjected to MS^2. The obtained MS^2 spectrum exhibited signals for ions at *m*/*z* 1460.1, 1243.8, and 1063.7 which mainly reflected the neutral loss of fatty acids (Fig. 7B, outlined structures). The most abundant ion at *m*/*z* 1243.8 was selected for the MS^3 fragmentation (Fig. 7C, outlined structures). The recorded fragment ions after the MS^2 and MS^3 fragmentations confirmed that the main form of the *P. shigelloides* O37 lipid A is identical with the lipid A structure reported for the *P. shigelloides* CNCTC 144/92.[12]

3. Discussion

As lipopolysaccharides of *P. shigelloides* cells play an important role in the enteropathogenicity, the elucidation of chemical structures of these molecules became even more important.[23]

In structural studies of *P. shigelloides* LPS, serotypes: O1 (strain 302-73), O17 (strains PCM 2231 and 7-63), O51 (CNCTC 110/92), O54 (CNCTC 113/92), O74 (CNCTC 144/92), and O37 (CNCTC 39/89) established features which are characteristic for this bacterium, such as, the lack of phosphate groups, the presence of galacturonic acids in the core oligosaccharide, and the unusual hydrophobicity due to the O-antigen structure.[8–17]

The presence of a carbohydrate backbone substituted by asymmetrically distributed fatty acids (2+4) in lipid A structure is common for *P. shigelloides* strains. The lipid A obtained from phenol-soluble SR-LPS of *P. shigelloides* O37 was heterogeneous. Two forms of lipid A present in serotype O37 have also been identified in serotypes O54 and O74.[10,12]

The similarity of the core oligosaccharide and the lipid A backbone in two serotypes of *P. shigelloides* O37 and O54 allows for assumption that the linkage between them could be identical: →5)-α-Kdo*p*-(2→6)-β-D-Glc*p*N4P-(1→6)-α-D-Glc*p*N1P.

The herein presented LPS of *P. shigelloides* O37 is the first known semi-rough form of *P. shigelloides* phenol-soluble LPS. Isolation of the R-LPS from water and SR-LPS from phenol phases suggests that the O-antigen part is responsible for the hydrophobic properties of this LPS.

The presence of deoxysugars, aminosugar, new unique α-D-Lenose residue, *N*-acetyl, and *O*-acetyl groups could contribute to the overall hydrophobic nature of the SR-LPS molecule. However it has been also suggested that the main solubility factor may be conformational rather than compositional.[24] As we have demonstrated, the oligosaccharides in LPS from water and phenol phases of *P. shigelloides* O37 are built up of the same core oligosaccharide structure and differ only by substitution with a single O-antigen unit. The occurrence of the R-type LPS in the water phase and SR-type LPS in the phenol phase was only reported for serotype O37. However, similar LPS properties were observed in S-LPS of serotypes O51,[19] O74,[11,12] and O1[13–15] where LPS isolated from the phenol phase had a higher number of repeating units in the O-specific polysaccharide compared to that isolated from the water phase. Therefore the composition of the O-antigen unit seems to be

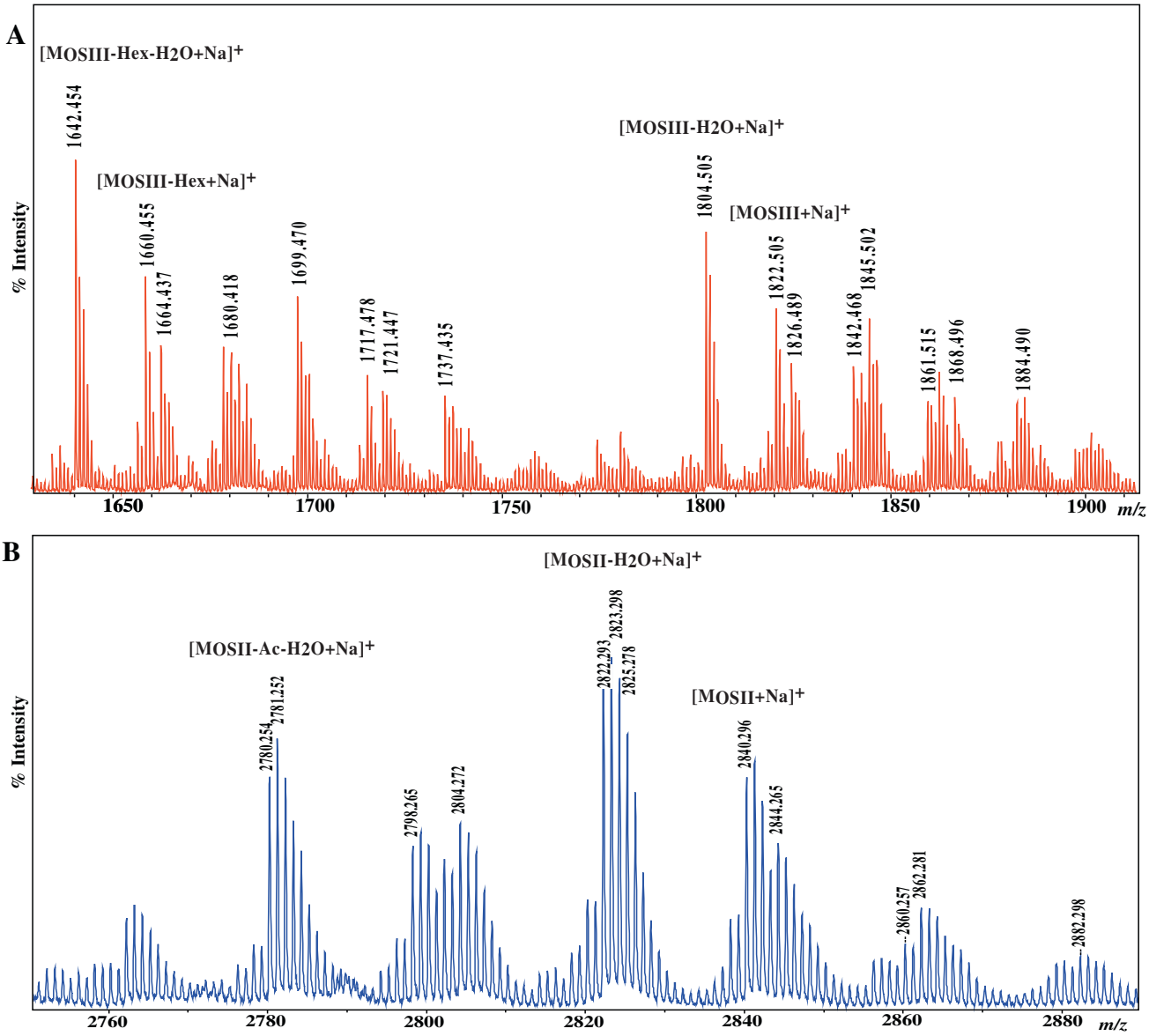

Figure 6. MALDI-TOF mass spectra of OSIII (A) and OSII (B) isolated from *P. shigelloides* O37 LPS. The spectra were obtained in the positive ion reflectron mode with 2,5-dihydroxybenzoic acid (DHB) as a matrix.

the main factor influencing the physicochemical properties, and could affect the immunogenicity and pathogenicity of the endotoxin. The possible role of the LPS—associated high hydrophobicity in the pathogenicity of *P. shigelloides* has not been investigated so far.

A few unusual residues have been found in structures of *P. shigelloides* LPS, including: 2-*O*-Ac-6d-β-D-*manno*-Hep*p*, D-*glycero*-β-D-*manno*-Hep*p*, and β-D-Gal*f*,[8] β-D-Qui*p*3NAc, α-D-Fuc*p*NAc,[11] β-D-Glc*p*NAc3NA, α-L-Fuc*p*Am3OAc, α-D-Qui*p*NAc,[19] α-L-PneNAc4*O*Ac, α-L-Fuc*p*NAc, and β-D-QuiNAc4NHb*p*[13] and this appears to be a recurring feature of O-antigen structures of *Plesiomonas* species.

Recently, ether-linked diastereomeric (2*R*,4*R*)-2,4-dihydroxypentanoic acid (Dhpa) (or its 1,4-lactone) with D-mannose and an ether of (2*S*,4*R*)-2,4-dihydroxypentanoic acid (Dhpa) with D-Glc*p*NAc*p* were identified in the O-polysaccharides of *Providencia alcalifaciens* O31 and O8.[25,26,57]

Antigens present on the surface of bacteria are able to elicit specific antibodies during immunization with bacterial cells. Most of these antibodies are directed against the O-polysaccharide part of endotoxin. There is an evidence for association between O-antigen chain length and pathogenicity of several bacterial species like *Salmonella* Montevideo, *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, *Yersinia enterocolitica*, *Shigella flexneri*, and *Pseudomonas aeruginosa*.[27–38] It has been suggested that the defined chain length range and composition of the O-antigen constitute for the virulence of bacteria.

4. Experimental

4.1. Growth conditions and isolation of the lipopolysaccharide and the polysaccharide

P. shigelloides strain CNCTC 39/89 was classified as serotype O37:HNM. This strain was obtained from the Institute of Hygiene and Epidemiology, Prague, Czech Republic. The bacteria were grown and harvested as described previously.[39]

The LPS was extracted from bacterial cells of *P. shigelloides* O37 by the hot phenol/water method.[40] LPS was analyzed by SDS–PAGE

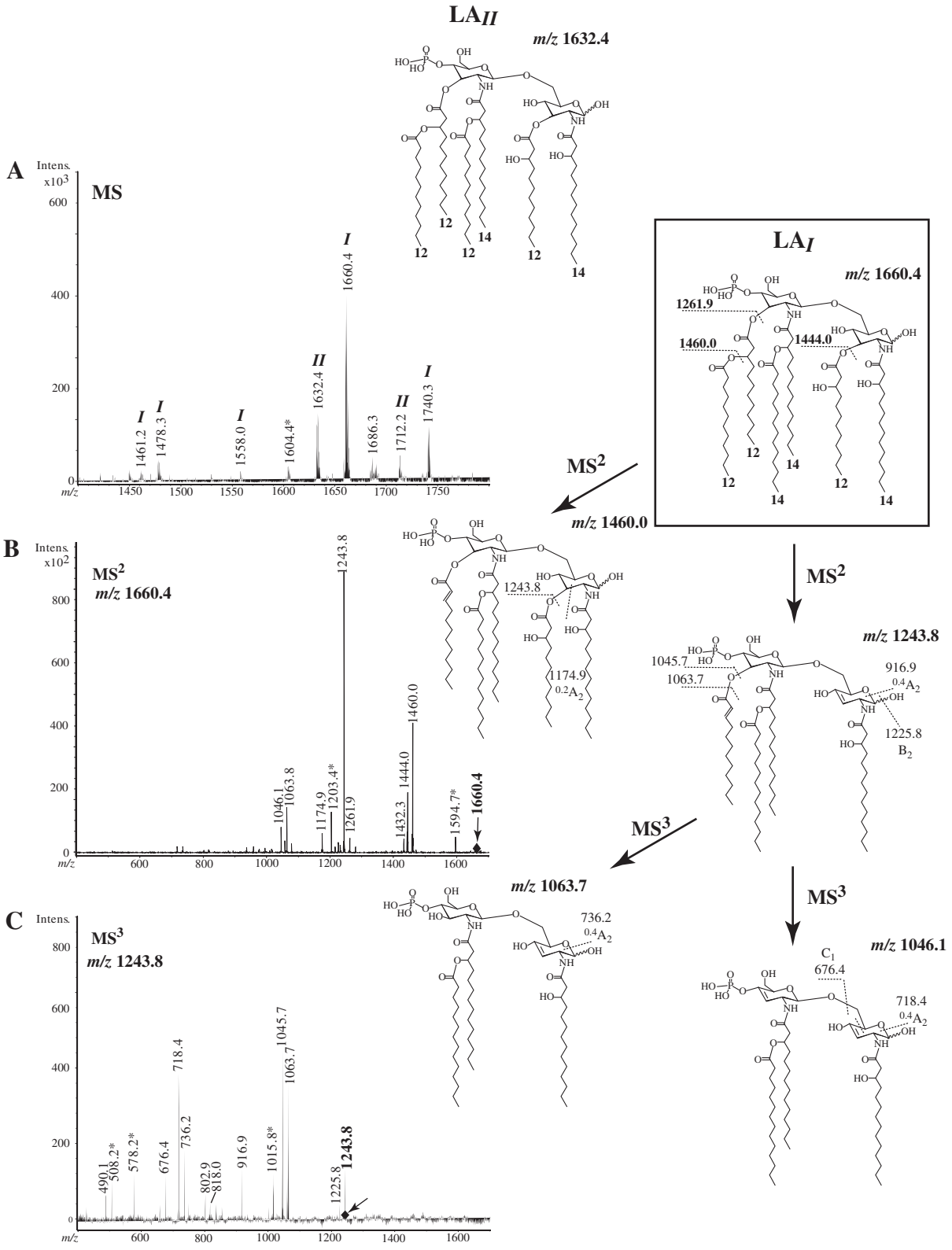

Figure 7. (A) Negative ion mode ESI-MS^n of the two lipid A forms from *P. shigelloides* O37 represented by ions at *m*/*z* 1660.4 (LA_I) and 1632.4 (LA_{II}). (B) MS^2 of the ion at *m*/*z* 1660.4. (C) MS^3 of the ion at *m*/*z* 1243.8. The interpretation of observed fragment ions is presented in inset structures. Ions marked with asterisk were not interpreted. The number of carbons in fatty acids is indicated by 12, 14.

according to the method of Laemmli[41] with modifications described previously,[42] and visualized by the silver staining method.[43] LPS (200 mg) was degraded by treatment with 1.5% acetic acid at 100 °C for 45 min. The supernatant was fractionated on a column (1.6 × 100 cm) of Bio-Gel P-10, equilibrated with 0.05 M pyridine/acetic acid buffer, pH 5.4. Eluates were monitored with a Knauer differential refractometer and all fractions were checked by NMR spectroscopy and MALDI-TOF MS.

4.2. Chemical methods

The sugars were converted into alditol acetates by conventional methods and analyzed by GC–MS.[39] The absolute configurations of the monosaccharides were determined as described by Gerwig et al. using (−)-2-butanol (Fluka, Buchs, Switzerland) for the formation of 2-butyl glycosides.[44,45] The trimethylsilylated butyl glycosides were identified by comparison with authentic samples using GC–MS. Carboxyl reduction of the native core oligosaccharide was carried out according to the method of Taylor et al.[46] The N-acetylation of the OSII was performed as described by Niedziela et al.[9] Methylation of OSIII and OSII was performed both on the N-acetylated/carboxyl-reduced oligosaccharides and N-acetylated oligosaccharides according to the method of Hakomori.[47] Reduction of ester groups with 'Superdeuteride' [$LiB(C_2H_5)_3D$] after methylation was carried out according to Bhat et al.[48] The methylated sugars were analyzed as partially methylated alditol acetates by GC–MS with a Hewlett-Packard 5971A system using an HP-1 fused-silica capillary column (0.2 mm × 12 m) and a temperature program 150→270 °C at 8 °C/min.

Amino acid analysis was carried out as described by MacKenzie et al.[49,50] The *N*-heptafluorobutyryl *n*-butyl ester derivative of amino acid was analyzed by GC–MS on the same system as described above, but a different temperature program was used: 100→270 °C at 5 °C/min.

The absolute configuration of Glc*p*N residues in lipid A was determined on the deacylated and dephosphorylated lipid A fraction, using (−)-2-butanol as previously described.[44,45]

Amide- and ester-bound fatty acids from lipid A were analyzed separately by GC–MS[51] with a Hewlett–Packard 5971A system, using an HP-1 fused-silica capillary column (0.2 mm × 12 m) and the temperature program 100→270 °C at 8 °C/min. In order to determine the absolute configuration of 3-hydroxy fatty acids, they were converted into (*S*)-3-methoxy-phenylethylamide derivatives as described by Gradowska et al.[52] These derivatives were analyzed by GC–MS using the HP-5 column (0.25 mm × 30 m) with a temperature program, 100→270 °C at 8 °C/min.

4.3. Instrumental methods

All NMR spectra were recorded on a Bruker DRX-600 Avance spectrometer equipped with a 2.5 mm microprobe, incorporating gradients along the *z* axis. The measurements were performed at 303 K without sample spinning and using the acetone signal (δ_H/δ_C 2.225/31.05 ppm) as an internal reference. The signals were assigned by one and two-dimensional experiments: ^1H–^1H-COSY, TOCSY, NOESY, ^1H–^{13}C HSQC-DEPT, HSQC-TOCSY, and HMBC. In the TOCSY experiments the mixing times were 30, 60, and 100 ms. NOESY experiment was performed with the mixing time of 200 ms, and HMBC experiment with a delay of 80 ms. For observation of phosphorus atoms one dimensional ^{31}P NMR spectra were recorded. The data were acquired and processed using standard Bruker software. The processed spectra were assigned with the help of the SPARKY program.[53]

4.4. Mass spectrometry

All fractions were analyzed using MALDI TOF BRUKER ULTRAFLEXTREME instrument. The MALDI-TOF MS spectra were obtained in positive ion modes. 2,5-dihydroxybenzoic acid [10 mg/mL in 1:1 AcN/0.1% formic acid mixture (v/v)] was used as matrix for analyses of oligosaccharides.

Negative-ion electrospray ionization mass spectra (ESI-MS^n) were recorded using an ESQUIRE-LC (Bruker Daltonics, Bremen, Germany) ion trap mass spectrometer. Lipid A samples were desalted by extraction with a chloroform/water mixture (1:1, v/v) and dissolved in a methanol/chloroform solution (1:1, v/v, mg/mL). The samples were continuously infused through the capillary head at 4 kV into the ion source, using a linear syringe pump at a rate of 2 μL/min. Spectra were scanned in the *m*/*z* 200–2000 range. The mass isolation window for the precursor ion selection was set to 4 Da in all the MS^n analyses. The fragmentation rules described in the published ESI-MS studies on glycolipids, phospholipids, and lipid A were applied during interpretation of mass spectra.[54–56]

4.5. Molecular modeling

(*MM2 force field*). Determination of the absolute configuration of the oxopentanoic acid moiety in α-D-Lenose (α-D-Len) residue from the O-antigen was carried out by NOE spectroscopy and molecular modeling (MM2 force field) as described by Shashkov et al.[57] The dihedral driver MM2 calculations were carried out using Chem-Bio3D version 11.0.

Acknowledgments

The research was supported by the Wroclaw Research Centre EIT+ under the project, 'Biotechnologies and advanced medical technologies'—BioMed (POIG.01.01.02-02-003/08) financed from the European Regional Development Fund (Operational Programme Innovative Economy, 1.1.2).

References

1. Morrison, D. C.; Ryan, J. L. *Annu. Rev. Med.* **1987**, *38*, 417–432.
2. Alexander, C.; Rietschel, E. T. *J. Endotoxin Res.* **2001**, *7*, 167–202.
3. Zähringer, U.; Lindner, B.; Rietschel, E. T. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 211–276.
4. Caroff, M.; Karabian, D. *Carbohydr. Res.* **2003**, *338*, 2431–2447.
5. Brenden, R. A.; Miller, M. A.; Janda, J. M. *Rev. Infect. Dis.* **1988**, *10*, 303–316.
6. Billiet, J.; Kuypers, S.; Van Lierde, S.; Verhaegen, J. *J. Infect.* **1989**, *19*, 267–271.
7. Sakazaki, R.; Balows, A. The genus *Plesiomonas* In *The Prokaryotes a Handbook on Habitats, Isolation, and Identification of Bacteria*; Starr, M. P., Stolp, H. G., Truper, H. G., Balows, A., Schlegel, H. G., Eds.; Springer-Verlag: Berlin, 1981; Vol. 2, pp 1285–1679.
8. Czaja, J.; Jachymek, W.; Niedziela, T.; Lugowski, C.; Aldova, E.; Kenne, L. *Eur. J. Biochem.* **2000**, *267*, 1672–1679.
9. Niedziela, T.; Lukasiewicz, J.; Jachymek, W.; Dzieciatkowska, M.; Lugowski, C.; Kenne, L. *J. Biol. Chem.* **2002**, *227*, 11657–11663.
10. Lukasiewicz, J.; Niedziela, T.; Jachymek, W.; Kenne, L.; Lugowski, C. *Glycobiology* **2006**, *16*, 538–550.
11. Niedziela, T.; Dag, S.; Lukasiewicz, J.; Dzieciatkowska, M.; Jachymek, W.; Lugowski, C.; Kenne, L. *Biochemistry* **2006**, *45*, 10422–10433.
12. Lukasiewicz, J.; Dzieciatkowska, M.; Niedziela, T.; Jachymek, W.; Augustyniuk, A.; Kenne, L.; Lugowski, C. *Biochemistry* **2006**, *45*, 10434–10447.
13. Pieretti, G.; Corsaro, M.; Lanzetta, R.; Parrilli, M.; Canals, R.; Merino, S.; Tomás, J. M. *Eur. J. Org. Chem.* **2008**, 3149–3155.
14. Pieretti, G.; Corsaro, M.; Lanzetta, R.; Parrilli, M.; Vilches, S.; Merino, S.; Tomás, J. M. *Eur. J. Org. Chem.* **2009**, 1365–1371.
15. Pieretti, G.; Carillo, S.; Lindner, B.; Lanzetta, R.; Parrilli, M.; Jimenez, N.; Reguè, M.; Tomás, J. M.; Corsaro, M. M. *Carbohydr. Res.* **2010**, *345*, 2523–2528.
16. Kübler-Kielb, J.; Schneerson, R.; Mocca, C.; Vinogradov, E. *Carbohydr. Res.* **2008**, *343*, 3123–3127.
17. Maciejewska, A.; Lukasiewicz, J.; Kaszowska, M.; Jachymek, W.; Man-Kapusinska, A.; Lugowski, C. *Marine Drugs* **2013**, *11*, 440–454.
18. Sävèn, E.; Östervall, J.; Landersjö, C.; Edblad, M.; Weintraub, A.; Ansaruzzaman, M.; Widmalm, G. *Carbohydr. Res.* **2012**, *348*, 99–103.
19. Maciejewska, A.; Lukasiewicz, J.; Niedziela, T.; Szewczuk, Z.; Lugowski, C. *Carbohydr. Res.* **2009**, *344*, 894–900.
20. Kenne, L.; Lindberg, B.; Petersson, K.; Katzenellenbogen, E.; Romanowska, E. *Carbohydr. Res.* **1980**, *78*, 119–126.
21. Aldova, E. *J. Hyg. Epidemiol. Microbiol. Immunol.* **1985**, *29*, 201–210.
22. Jansson, P. E.; Kenne, L.; Widmalm, G. *Carbohydr. Res.* **1989**, *193*, 322–325.
23. Okawa, Y.; Ohtomo, Y.; Tsugawa, H.; Matsuda, Y.; Kobayashi, H.; Tsukamoto, T. *FEMS Microbiol. Lett.* **2004**, *239*, 125–130.
24. Haseley, S. R.; Holst, O.; Brade, H. *Eur. J. Biochem.* **1998**, *251*, 189–194.
25. Ovchinnikova, O. G.; Kocharova, N. A.; Shashkov, A. S.; Bialczak-Kokot, M.; Knirel, Y. A.; Rozalski, A. *Carbohydr. Res.* **2009**, *344*, 683–686.
26. Toukach, F. V.; Kocharova, N. A.; Maszewska, A.; Shashkov, A. S.; Knirel, Y. A.; Rozalski, A. *Carbohydr. Res.* **2008**, *343*, 2706–2711.
27. Grossman, N.; Schmetz, M. A.; Foulds, J.; Klima, E. N.; Jiminez, V.; Leive, L.; Joiner, K. A. *J. Bacteriol.* **1987**, *169*, 856–863.

28. Bravo, D.; Silva, C.; Carter, J. A.; Hoare, A.; Álvarez, S. A.; Blondel, C. J.; Zaldívar, M.; Valvano, M. A.; Contreras, I. *J. Med. Microbiol.* **2008**, *57*, 938–946.
29. Murray, G. L.; Attridge, S. R.; Morona, R. *J. Bacteriol.* **2006**, *188*, 2735–2739.
30. Porat, R.; Johns, M. A.; McCabe, W. R. *Infect. Immun.* **1987**, *55*, 320–328.
31. Shepherd, J. G.; Wang, L.; Reeves, P. R. *Infect. Immun.* **2000**, *68*, 6056–6061.
32. Clarke, B. R.; Cuthbertson, L.; Whitfield, C. *J. Biol. Chem.* **2004**, *279*, 35709–35718.
33. Najdenski, H.; Golkocheva, E.; Vesselinova, A.; Bengoechea, J. A.; Skurnik, M. *FEMS Immunol. Med. Microbiol.* **2003**, *38*, 97–106.
34. Bengoechea, J. A.; Najdenski, H.; Skurnik, M. *Mol. Microbiol.* **2004**, *52*, 451–469.
35. Morona, R.; Daniels, C.; Van Den Bosch, L. *Microbiology* **2003**, *149*, 925–939.
36. Carter, J. A.; Blondel, C. J.; Zaldívar, M.; Álvarez, S. A.; Morolda, C. L.; Valvano, M. A.; Contreras, I. *Microbiology* **2007**, *153*, 3499–3507.
37. Sandlin, R. C.; Goldberg, M. B.; Maurelli, A. T. *Mol. Microbiol.* **1997**, *22*, 63–73.
38. Engels, W.; Endert, J.; Kamps, M. A. F.; Van Boven, C. P. A. *Infect. Immun.* **1985**, *49*, 182–189.
39. Petersson, C.; Niedziela, T.; Jachymek, W.; Kenne, L.; Zarzecki, P.; Lugowski, C. *Eur. J. Biochem.* **1997**, *244*, 580–586.
40. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–89.
41. Laemmili, U. K. *Nature* **1970**, *227*, 680–685.
42. Niedziela, T.; Petersson, C.; Helander, A.; Jachymek, W.; Kenne, L.; Lugowski, C. *Eur. J. Biochem.* **1996**, *237*, 635–641.
43. Tsai, C. M.; Frasch, C. E. *Anal. Biochem.* **1982**, *119*, 115–119.
44. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1978**, *62*, 349–357.
45. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1979**, *77*, 1–7.
46. Taylor, R. L.; Shively, J. E.; Conrad, H. E. Methods *Carbohydr. Chem.* **1976**, *7*, 149–151.
47. Hakomori, S. *J. Biochem.* **1964**, *55*, 205–208.
48. Bhat, U. R.; Krishnaiah, B. S.; Carlson, R. W. *Carbohydr. Res.* **1991**, *220*, 219–227.
49. MacKenzie, S. L.; Tenaschuk, D. *J. Chromatogr.* **1974**, *97*, 19–24.
50. MacKenzie, S. L.; Hogge, L. R. *J. Chromatogr.* **1977**, *132*, 485–493.
51. Wollenweber, H. W.; Schlecht, S.; Lüderitz, O.; Rietschel, E. T. *J. Biochem.* **1983**, *130*, 167–171.
52. Gradowska, W.; Larsson, L. *J. Microbiol. Methods* **1994**, *20*, 55–67.
53. Goddard, T. D.; Kneller, D. G. *SPARKY*, 3rd ed.; University of California: San Francisco, 2001.
54. Kussak, A.; Wientraub, A. *Anal. Biochem.* **2002**, *307*, 131–137.
55. Sforza, S.; Silipo, A. *J. Mass Spectrom.* **2004**, *39*, 378–383.
56. Vadovic, P.; Fodorova, M.; Toman, R. *Acta Virol.* **2007**, *51*, 249–259.
57. Shashkov, A. S.; Kocharova, N. A.; Toukach, F. V.; Kachala, V. V.; Knirel, Y. A. *Nat. Prod. Commun.* **2008**, *3*, 1625–1630.