Carbohydrate Research 344 (2009) 484–490

Contents lists available at ScienceDirect

Carbohydrate Research

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

Structure of the lipopolysaccharide core of *Vibrio vulnificus* type strain 27562

Evguenii Vinogradov [a], Caroline Wilde [c], Erin M. Anderson [b], Alina Nakhamchik [d], Joseph S. Lam [b], Dean A. Rowe-Magnus [c,d,*]

[a] *Institute for Biological Sciences, National Research Council, 100 Sussex Dr., Ottawa, Ont., Canada K1A 0R6*
[b] *Department of Molecular and Cellular Biology, University of Guelph, 488 Gordon Street, New Science Complex, Guelph, ON, Canada N1G 2W1*
[c] *Division of Clinical Integrative Biology, Sunnybrook Health Sciences Centre, 2075 Bayview Avenue, S1-26A, Toronto, Ontario, Canada M4N 3N5*
[d] *Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Toronto, Canada*

ARTICLE INFO

Article history:
Received 26 September 2008
Received in revised form 5 December 2008
Accepted 17 December 2008
Available online 25 December 2008

Keywords:
Vibrio vulnificus
Lipopolysaccharide
Core
Structure

ABSTRACT

The structure of the lipopolysaccharide core of *Vibrio vulnificus* type strain 27562 is presented. LPS hydrolysis gave two oligosaccharides, OS-1 and OS-2, as well as lipid A. NMR spectroscopic data corresponded to the presence of one Kdo residue, one β-glucopyranose, three heptoses, one glyceric acid, one acetate, three *P*EtN, and one 5,7-diacylamido-3,5,7,9-tetradeoxynonulosonic acid residue (pseudaminic acid, Pse) in OS1. OS2 differed form OS 1 by the absence of glyceric acid, acetate, and Pse residues. Lipid A was analyzed for fatty acid composition and the following fatty acids were found: C14:0, C12:0-3OH, C16:0, C16:1, C14:0-3OH, C18:0, C18:1 in a ratio of 1:3:3:1:2.5:0.6:0.8.

1. Introduction

LPS is the major surface molecule of Gram-negative bacteria.[1,2] LPS consists of three distinct structural domains (the O-antigen, the core, and lipid A). The lipid A (endotoxin) domain of LPS serves as the hydrophobic anchor of LPS and is the bioactive component of the molecule that is associated with Gram-negative septic shock. The core and O-antigen are composed of monosaccharides joined by glycosidic linkages, and an incredibly diverse range of branched and modified LPS molecules are possible. Variations in the composition, sequence, and linkage of the sugars give rise to tremendous structural diversity.

Vibrio vulnificus is a planktonic marine bacterium that is pathogenic to humans and animals.[3–6] Infections may result from ingestion or wound contamination, and the fatality rate of septicemic patients is greater than 50%. It alone is responsible for 95% of all seafood-related deaths in the United States, and it carries the highest death rate of any foodborne disease agent.[7] On the basis of LPS antigens, *V. vulnificus* species can be organized into three biotypes. Biotype 1 is the predominant human pathogen,[8–12] biotype 2 is associated with eels,[13–15] and biotype 3 was recently isolated from fish handlers in Israel.[16,17] Biotype 1 can be divided into at least five antigenic subgroups, one of which (LPS type 1/5) is more prevalent among clinical strains.[18] This suggests either that the presence of this LPS type itself causes increased virulence, or that this LPS type is a marker of more virulent strains. *V. vulnificus* ATCC 27562 belongs to subgroup 1.

The LPS of Enterobacteriaceae is a potent mediator of endotoxic shock. The LPS of *V. vulnificus* biotype 2 strains is a virulence determinant in eels.[19] The role of LPS from biotype 1 strains in *V. vulnificus* pathogenesis is unclear. While one study indicated that the injection of LPS from strain MO6-24/O was lethal,[20] another study showed that the injection of LPS from the same strain into galactosamine-treated or untreated mice was not lethal, and that LPS elicited less of a cytokine response from primary human peripheral blood mononuclear cells than purified capsular polysaccharide (CPS).[21] Furthermore, the injection of LPS from *V. vulnificus* strain C7184 had no effect on mice but was lethal in rats.[22] These conflicting observations and the prevalence of LPS type 1/5 among clinical biotype 1 strains warranted a deeper investigation into the structure of the LPS. Here, we determine the core structure and fatty acid composition of the LPS of *V. vulnificus* 27562.

2. Results and discussion

2.1. Detection of *V. vulnificus* LPS by immunoblotting

LPS from *V. vulnificus* strains 27562, CMCP6, YJ016, and 1003 was isolated from cells grown at 37 °C and was detected by silver

Abbreviations: COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear overhauser enhancement spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond connectivity; LPS, lipopolysaccharide; PS, polysaccharide; GlcN, glucosamine; Hep, L-*glycero*-D-*manno*-heptose; DDHep, D-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-octulosonic acid; Pse, 5,7-diamino-3,5,7,9-tetradeoxynonulosonic acid; Gra, glyceroyl.

* Corresponding author. Tel.: +1 416 480 6100x3318; fax: +1 416 180 5737.
E-mail address: dean.rowe-magnus@sri.utoronto.ca (D.A. Rowe-Magnus).

0008-6215/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2008.12.017

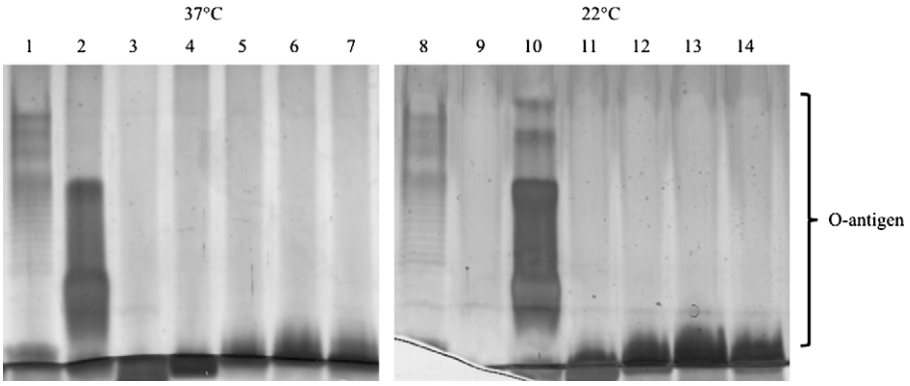


Figure 1. Silver staining of LPS samples. LPS was isolated from the indicated strains at 37 °C or 22 °C, separated by PAGE and detected by silver staining. Lanes 1 and 8, *P. aeruginosa* PA01; lanes 2 and 10, *V. cholereae* N16961; lanes 3 and 9, *S. putrefaciens* 200; lanes 4 and 11, *V. vulnificus* 27562; lanes 5 and 12, *V. vulnificus* CMCP6; lanes 6 and 13, *V. vulnificus* YJ016; and lanes 7 and 14, *V. vulnificus* 1003.

staining following PAGE (Fig. 1). LPS from *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Shewanella putrefaciens* was used as controls. As previously reported, preparations from *P. aeruginosa* and *V. cholerae* had a ladder-like banding pattern that was indicative of smooth LPS,[23–25] while *S. putrefaciens* preparations displayed a typical rough LPS pattern and were devoid of any such material.[26] None of the *V. vulnificus* strains showed a ladder banding pattern, suggesting that they also produced only rough LPS. Furthermore, lower molecular weight elements appeared as diffuse bands that did not clearly resolve into lipid A core and core + one bands. In some bacteria, LPS production is affected by the growth temperature,[27–30] so we also isolated and detected LPS from bacteria grown at 22 °C (Fig. 1). The only change observed was in the banding pattern of *V. cholerae* N16961, where additional high-molecular weight bands appeared (lane 10).

The inability to detect *V. vulnificus* LPS by silver staining has been previously reported;[31–33] however, immunoblotting has been used to visualize LPS in several *Escherichia coli*,[34], *Campylobacter jejuni*,[35] and *V. vulnificus*[31,36] strains. LPS samples from the aforementioned strains were also subjected to immunoblotting with antisera against *V. vulnificus* strain 27562. Immunoblots revealed a ladder-like banding pattern characteristic of smooth LPS for strain 27562, and the ladder was detected in cells that were grown at 37 °C and 22 °C (Fig. 2A). The antisera were specific for strain 27562, as no signal was detected for any of the other strains examined (Fig. 2B). This suggested that strain 27562 indeed produced smooth LPS rather than simply rough LPS, as suggested by silver staining (Fig. 3).

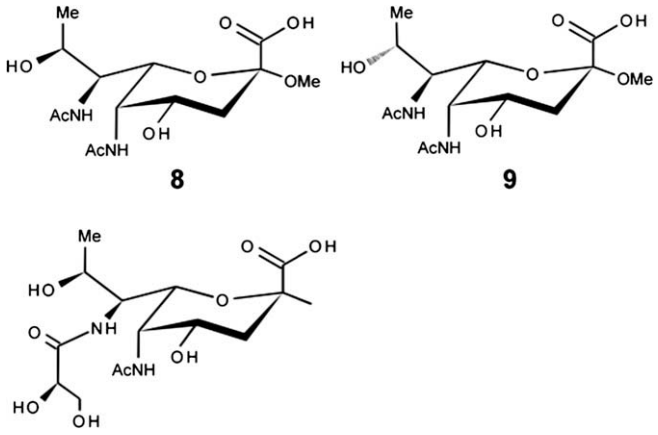


Figure 3. Synthetic methyl glycoside of 3,5,7,9-tetradeoxy-5,7,diacetamido-L- and D-glycero-β-L-manno-nonulosonic acids (8, 9), and Pse residue present in the *V. vulnificus* LPS.

2.2. Structural analysis of the LPS from *V. vulnificus* strain 27562

A previous study on the composition of the polysaccharide portion of LPS from seven *V. vulnificus* serogroups, including strain 27562, failed to identify an unknown amino sugar or to detect any 2-keto-3-deoxyoctulosonic acid[37] (Kdo), a normal constituent

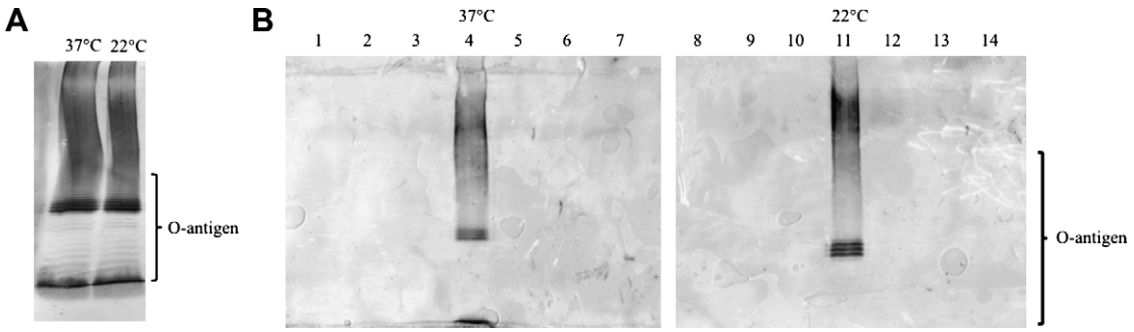


Figure 2. (A) Immunoblotting of *V. vulnificus* 27562 LPS samples. LPS was isolated from *V. vulnificus* strain 27562 at 37 °C or 22 °C, separated by PAGE, and detected by immunoblotting with anntisera to *V. vulnificus* strain 27562. The characteristic ladder pattern of the O-antigen can be clearly seen. (B) Immunoblotting of LPS samples. LPS was isolated from the indicated strains at 37 °C or 22 °C, separated by PAGE, and detected by immunoblotting with anntisera to *V. vulnificus* 27562. Lanes 1 and 8, *P. aeruginosa* PA01; lanes 2 and 9, *V. cholereae* N16961; lanes 3 and 10, *S. putrefaciens* 200; lanes 4 and 11, *V. vulnificus* 27562; lanes 5 and 12, *V. vulnificus* CMCP6; lanes 6 and 13, *V. vulnificus* YJ016; and lanes 7 and 14, *V. vulnificus* 1003.

of the LPS of Enterobacteriaceae.[1,2] Hydrolysis of LPS from strain 27562 with 2% AcOH gave lipid A as well as two oligosaccharides, OS-1 and OS-2, that were separated by ion-exchange chromatography. They were analyzed by NMR spectroscopy and mass spectrometry, as well as by compositional and methylation analysis. Monosaccharide analysis (GC of alditol acetates) showed the presence of Glc, LD-Hep, and DD-Hep. NMR spectra were recorded and completely assigned (Table 1). NMR spectroscopic data corresponded to the presence of one anhydro-Kdo residue, one β-glucopyranose, three heptoses, one glyceric acid, one acetate, three *P*EtN, and one 5,7-diacylamido-3,5,7,9-tetradeoxynonulosonic acid residue (pseudaminic acid, Pse) in OS-1. OS-2 differed from OS-1 by the absence of glyceric acid, acetate, and Pse residues. Composition was confirmed by ESIMS data, which gave a molecular mass of 1692.2 (calcd 1690.3; increased observed mass is due to deuterium exchange in anhydro-Kdo after NMR spectroscopy recording) for OS-1 and 1328.2 (calcd 1328.0) for OS-2. The monosaccharide sequence as presented in the Scheme was deduced from NOE and HMBC data. NOE correlations E1:C5, E1:C7, F1:E3, F1:G5, G1:F2, H1:E4, H1:E6, K3:F2, K3:G1, K3:G2 were observed in OS-1, and

Table 1
NMR spectroscopy data for the isolated oligosaccharides

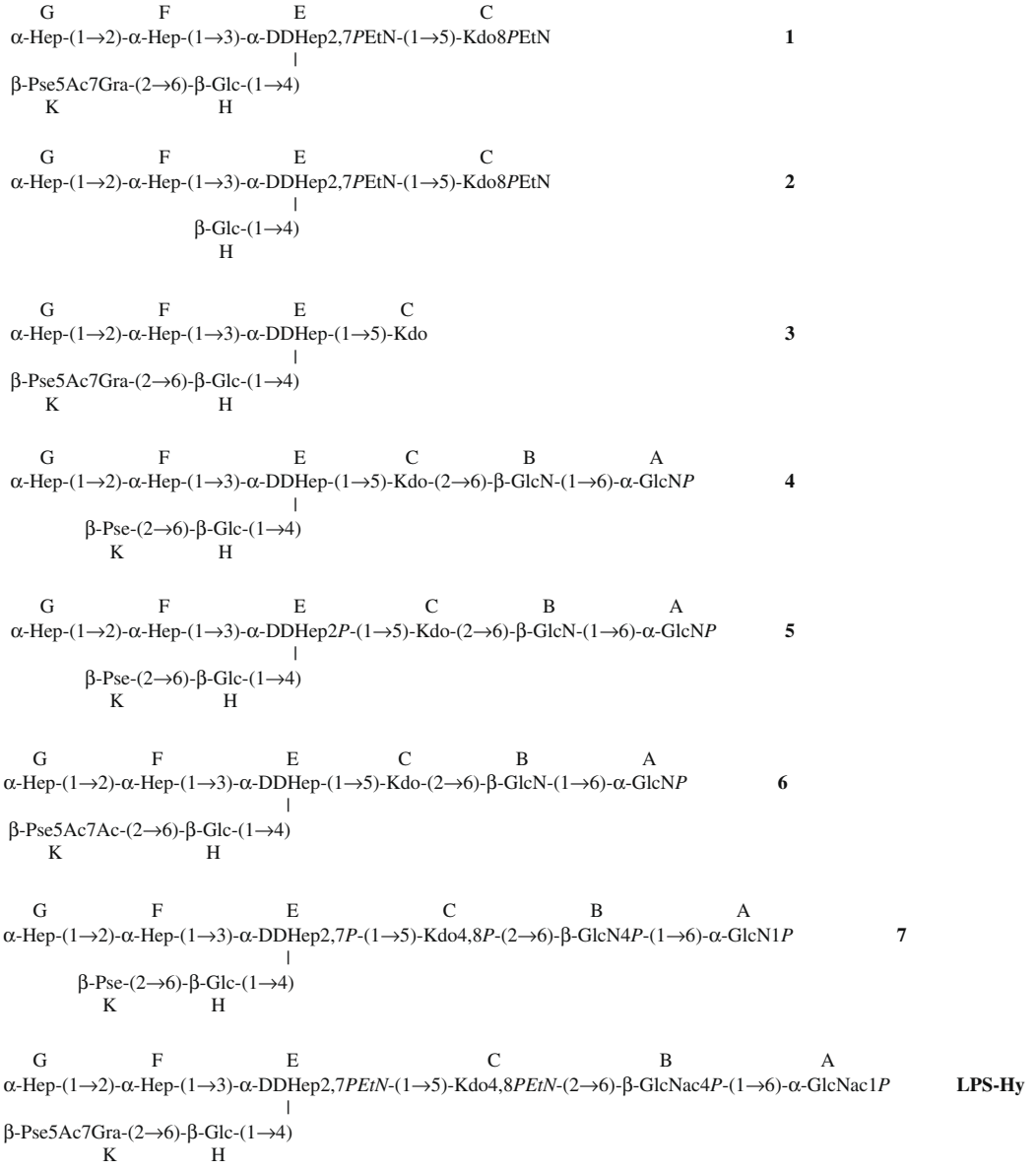
Unit, compound	H/C 1	H/C 2/3eq $J_{3a,e}$	H/C 3ax $J_{3a,4}$	H/C 4 $J_{3e,4}$	H/C 5 $J_{4,5}$	H/C 6 $J_{5,6}$	H/C 7 $J_{6,7}$	H/C 8 $J_{7,8}$	H/C 9 $J_{8,9}$
α-GlcN*P*, A, 4	5.69	3.40	3.93	3.44	4.17	4.25	3.89		
	92.3	55.4	70.9	71.2	74.1	69.9			
β-GlcN B, 4	4.99	3.03	3.67	3.49	3.65	3.60	3.63		
	100.5	56.9	73.3	71.3	75.7	62.8			
β-GlcN4*P* B, 7	4.90	4.15	3.89	3.90	3.76	3.63	3.78		
	100.7	57.6	73.7	76.3	75.9	64.6			
Kdo C, 1			3.47	4.49	4.20	4.31	4.11	3.99	3.99
			42.0	78.3	85.9	76.7	82.9	66.2	
Kdo C', 1			3.17	4.65	4.36	4.24	3.96	3.95	4.05
			42.0	76.8	81.8	76.5	84.3	67.0	
α-Kdo C, 4		1.89	2.18	4.19	4.17	3.73	3.82	3.97	3.66
		36.3	36.3	67.2	76.9	72.9	70.6	64.8	
α-Kdo4,8*P* C, 7		2.12	2.38	4.63	4.37	3.97	3.88	4.11	4.19
			36.0	71.9	74.2	73.3	69.9	68.8	
Hep2,7-*P*EtN E, 1	5.23	4.46	3.94	4.17	3.91	4.31	3.99	4.20	
	98.8	76.2	73.1	75.8	76.3	69.2	66.3		
Hep2,7-*P*EtN E', 1	5.15	4.42	3.88	4.14	3.80	4.31	3.99	4.20	
	97.2	76.2	73.5	75.8	76.1	69.2	66.3		
Hep E, 3	5.08	3.99	3.90	4.12	3.89	4.06	3.74	3.97	
	99.3	71.6	74.3	76.0	76.1	70.5	62.2		
Hep E', 3	5.03	3.96	3.88	4.12	3.89	4.06	3.74	3.97	
	97.6	71.6	74.6	76.0	76.1	70.5	62.2		
α-Hep E, 4	5.05	4.12	4.00	4.12	4.29	4.12	3.87	3.74	
	102.3	71.2	75.0	76.0	75.4	71.2	62.7		
α-Hep E, 5	5.22	4.49	4.05	4.18	4.31	4.13	3.94	3.75	
	101.1	75.4	73.9	75.9	75.1	71.1	62.5		
Hep F, 1	5.61	4.30	3.92	3.92	3.64	4.05	3.66	3.78	
	100.4	80.4	70.9	66.7	73.3	70.7	65.0		
α-Hep F, 4	5.62	4.30	3.99	3.95	3.64	4.09	3.75	3.64	
	100.4	80.7	71.1	66.9	72.9	69.7	64.7		
Hep G, 1	5.21	3.97	3.88	3.81	3.81	4.06	3.74	3.74	
	102.8	71.7	71.7	67.3	72.6	70.5	64.6		
α-Hep G, 4	5.19	4.03	3.90	3.81	3.81	4.07	3.75	3.75	
	102.9	71.9	71.8	67.5	72.8	70.6	64.7		
Glc H, 1	4.53	3.51	3.49	3.61	3.56	3.46	4.22		
	104.2	74.7	77.4	70.3	75.1	63.1			
Glc H, 2	4.56	3.43	3.47	3.38	3.47	3.79	3.92		
	103.8	74.9	77.3	71.3	77.3	62.0			
β-Glc H, 4	4.47	3.49	3.58	3.53	4.19	3.73	4.19		
	104.1	74.8	77.4	70.7	75.3	63.7			
Pse K, 1		2.56	1.59	3.88	4.21	3.97	4.03	4.11	1.21
	174.0	101.6	37.3	67.5	49.4	74.3	54.6	70.3	18.4
Pse K, 3		2.59	1.61	3.89	4.22	4.04	4.03	4.11	1.23
	174.0	101.6	37.2	67.5	49.5	74.4	54.5	70.5	18.6
β-Pse K, 4		2.78	1.66	4.15	3.69	4.28	3.55	4.32	1.43
		102.0	36.5	65.5	51.6	70.6	57.3	65.6	18.9
β-PseNAc K, 6		2.58	1.61	3.89	4.20	3.94	4.04	4.11	1.22
J, Hz		12.3	12.5	4.9	3.8	2	9.5	3.9	6.8
		102.0	37.3	67.4	49.5	74.2	54.8	69.9	17.8
L-β-L-Pse **8**		2.48	1.62	4.08	4.29	3.96	4.15	4.18	1.12
J, Hz		13.0	12.9	4.7	3.6	2.4	10.5	3.4	
			36.7	67.3	49.9	74.3	53.8	68.2	16.7
D-β-L-Pse **9**		2.50	1.64	4.10	4.22	4.14	3.86	4.25	1.10
J, Hz		13.2	12.8	4.9	4.5	2.1	10.4	1.6	6.4
			36.8	68.0	49.1	73.0	54.4	67.4	20.0
Glyceric acid, 1		4.13	3.79						
	175.1	73.6	64.4						
EtN, 1	4.03/4.13	3.29/3.34							
	63.0	41.2							
Ac, 1		2.02							
	175.9	23.3							

are in agreement with the presented structure. All corresponding interglycoside HMBC correlations (E1:C5, F1:E3, G1:F2, H1:E4, K3:F2, K2:H6) were also observed. Attachment of Pse to Glc H O-6 followed from the HMBC correlation between the Pse C-2 and the H6b proton. Also, C-6 signals of the Glc H were shifted downfield due to this substitution, which was clearly visible in comparison between OS-1 and OS-2, where Pse is absent. The substitution of N-5 of Pse with acetate and of N-7 with glycerate was determined from the observation of HMBC correlations from C-1 of the acyl residues to H-5 or H-7 of Pse.

Kdo in both oligosaccharides was completely converted into two stereoisomers of 4,7-anhydro-Kdo. Formation of a five-membered ring led to characteristic low-field signals of C-4–C-7 (Table 1). As a result of the presence of two Kdo isomers, other monosaccharides in OS-1 and OS-2 gave two series of signals (Table 1).

OS-1 and OS-2 contained three *P*EtN residues, which gave ^{31}P NMR spectroscopy signals at −2.1 (E2), −1.0, and −1.1 ppm (C8 and E8). Their position was determined from ^{1}H–31P HMQC and ^{1}H-^{31}P HMQC-TOCSY spectra, where correlations between phosphates and protons C-8, E-2, and E-7 were observed, indicating the position of phosphorylation.

Monosaccharide analysis of the LPS showed the presence of LD- and DD-heptoses. However, it was not possible to determine the configuration at C-6 for any of the heptose residues from NMR spectroscopic data. It seemed that this problem originated in part from the phosphorylation of the Hep E, which gave unpredictable chemical shifts. To resolve this problem, OS-1 was dephosphorylated to give OS-3. However, the spectra (Table 1) did not aid in determining the configuration of heptoses. The C-6 signals of all residues had similar chemical shifts (C-6 of DD-Hep is expected to have an ∼3 ppm lower shift than C-6 of LD-Hep; this was not observed, probably due to the influence of substituents). OS-3 was methylated by the Ciucanu–Kerek procedure,[38] and partially methylated alditol acetates were analyzed by GC–MS. This led to the identification of the derivatives of 6-substituted Glc, terminal and 2-substituted LD-Hep, and of 3,4-disubstituted DD-Hep. This result showed that Hep E had a D-*glycero*-D-*manno*-configuration, while the two other residues had a L-*glycero*-D-*manno*-configuration.



Scheme 1. Structures of the isolated oligosaccharides derived from the LPS of *V. vulnificus* 27562. In the LPS-Hy structure, 'ac' indicates fatty acid residue.

Determination of the configuration of Pse was based on the comparison of NMR spectroscopy chemical shifts and coupling constants with model compounds. The value of $J_{3a,4}$ (Table 1; J values are given for compound **6** where it was easier to measure) was 12.5 Hz, which corresponds to an axial orientation of H-4. $J_{4,5}$ and $J_{5,6}$ had small values of 3.8 and 2 Hz, respectively, due to the equatorial orientation of H-5. The large $J_{6,7}$ coupling constant of 9.5 Hz limits the possible configurations to L/D-*glycero*-L-*manno*; no other described nonulosonic acid possesses large $J_{6,7}$ and $J_{3a,4}$ coupling constants at the same time. The large distance between proton signals H-3a and H-3e indicates an axial orientation for the carboxyl group, which corresponds to a β-configuration in this case. The configuration at C-8 remained unclear; it could be determined from chemical shifts of C-8 and C-9, but the observed values lay between the values described for both isomers. Also the position of C-7 signals was too far from those in both model compounds. This could be the result of the influence of the bulk substituent, glycerate, at N-7.

In order to obtain better agreement between the chemical shifts of the *V. vulnificus* Pse and the shifts in model compounds, the glycerate at N-7 of Pse was replaced by acetate using the following procedure. LPS was dephosphorylated by 48% HF and deacylated by KOH treatment. Dephosphorylation was performed to avoid phosphate migration, which would lead to an inseparable mixture of similar structures. Two main compounds, **4** and **5**, were obtained. Both represented the products of incomplete dephosphorylation due to insolubility of the LPS in HF. They were purified by HPAEC. Their NMR spectra were interpreted, giving the presented structures. OS-4 and OS-5 differed by the presence of a phosphate group at O-2 of Hep E in product 5. OS-4 was then N-acetylated by treatment with Ac_2O in satd aq $NaHCO_3$ to give compound **6**. Comparison of the chemical shifts of C-7–C-9 of Pse in OS-6 with the values for synthetic products **8** and **9** gave better agreement with compound **8**, which had the L-*glycero*-L-*manno*-configuration. Thus, we concluded that Pse in *V.vulnificus* also has this configuration.

The next experiment was performed to determine the nature of the substituent at C-4 of Kdo, which was likely lost in all the compounds described above. LPS was treated with 4 M KOH for complete deacylation, and the products were partially purified by HPAEC. Due to phosphate migration, a mixture of closely related isomers was produced that eluted as one broad peak from the Carbopac column. This product (structure **7** in Scheme 1, given tentatively in regard to the phosphate positions) was analyzed by NMR spectroscopy and MS. The chemical shifts of the H/C-4 of the Kdo residue (Table 1) corresponded to the presence of a phosphate substituent. Also a phosphorylation was detected at O-4 of the lipid glucosamine residue B, which led to a downfield shift of the C-4 signal. The ^{31}P NMR spectrum was of low quality and presented one broad signal between +3 and −1 ppm. No ^{1}H–^{31}P correlation spectra were obtained. This was a result of phosphate migration between hydroxyl groups C-7–C-8 and E-6–E-7, as well as of partial loss of EtN substituents. Mass spectrum contained peaks corresponding to the structure with the composition $Hex_1Hep_3Pse_1Kdo_1GlcN_2P_6EtN_1$, M = 2055, and a minor peak of the structure with one phosphate group less with M = 1975, as well as a number of small unidentified peaks. Both structures contained one EtN group, but possibly at different positions, which contributed to the signal split in the NMR spectra.

On the basis of the above data, we propose the structure of the O-deacylated LPS as shown in Scheme 1 (LPS-Hy, ac = C12:0-3OH or C14:0-3OH).

The absolute configuration of the L-glyceric acid was determined by GC–MS of its (*S*)-2-butyl ester acetate in comparison with the standards, prepared from commercial L-glyceric acid and (*R*) and (*S*)-2-butanol.

Lipid A, obtained after AcOH hydrolysis of the LPS, was analyzed for fatty acid composition using GC–MS of methyl ester acetates. The following fatty acids were found: C14:0, C12:0-3OH, C16:0, C16:1, C14:0-3OH, C18:0, C18:1 in a ratio of 1:3:3:1:2.5:0.6:0.8. The same acids were found in the LPS.

Attempts to analyze lipid A by NMR spectroscopy were not successful. Lipid A was soluble in chloroform, but its spectra contained only signals of fatty acids. This is not unusual; sugar signals in the lipids from LPS are usually not visible in pure chloroform, but appear after addition of methanol and D_2O, usually in a ratio of 2:3:1. However, on addition of even a small amount of methanol, all material precipitated. Equally unsuccessful were attempts to obtain MALDI mass spectra of the lipid. The reasons for this are unclear. One could propose that lipid A is mixed with some irrelevant lipid, but this explanation contradicts the observation of a characteristic fatty acid profile.

3. Experimental

3.1. Bacterial strains and media

P. aeruginosa PA01, *V. cholerae* N16961, and *V. vulnificus* clinical strain 27562 were obtained from the Collection de l'Institut Pasteur (Paris, France). *S. putrefaciens* 200 was a gift from Dr. Flynn Picardal (Indiana University). Dr. Anita Wright (University of Florida) kindly provided the *V. vulnificus* CMCP6 and YJ016 strains, and we thank Brenda Grau (Louisiana State University) for providing the *V. vulnificus* 1003 strain. All bacteria were grown aerobically in LB medium at the indicated temperatures to an OD_{600} of 1.0.

3.2. Small-scale LPS isolation and immunodetection

LPS was prepared using the method of Hitchcock and Brown.[39] LPS was separated using precast 4–12% bis-Tris NuPage gels (Invitrogen) as recommended by the manufacturer. LPS was visualized by using the ultrafast silver staining method of Fomsgaard et al.[40] LPS was visualized by immunostaining using rabbit antisera against formalin-killed whole cells of *V. vulnificus* 27562. The secondary antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG at a dilution of 1:2000 (Jackson ImmunoResearch). Blots were developed using the 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt (BCIP) and *p*-nitro blue tetrazolium chloride (NBT) alkaline phosphatase color development reagents (Bio-Rad).

3.3. Large-scale LPS extraction

Large-scale LPS preparations were obtained by the phenol–water method as described.[41]

3.4. NMR spectroscopy

NMR spectra were recorded at 25 °C in D_2O on a Varian UNITY INOVA 600 instrument, using acetone as reference for ^{1}H (2.225 ppm) and ^{13}C (31.5 ppm) NMR spectra. Varian standard programs COSY, NOESY (mixing time of 400 ms), TOSY (spinlock time 120 ms), HSQC, and gHMBC (long-range transfer delay 80 ms) were used.

3.5. Mass spectrometry

ESIMS spectra were obtained using a micromass Quattro spectrometer in 50% MeCN with 0.2% HCOOH at a flow rate of 15 μL/min with direct injection.

3.6. Monosaccharide analysis

Hydrolysis was performed with 4 M TFA (110 °C, 3 h). Monosaccharides were conventionally converted into alditol acetates and were analyzed by GC on an Agilent 6850 chromatograph equipped with DB-17 (30 m × 0.25 mm)-fused silica column using a temperature gradient of 180 °C (2 min)→240 °C at 2 °C/min.

3.7. Fatty acid analysis

LPS, lipid, or LPS-Hy sample (1 mg) was treated with 1 M HCl in MeOH (100°, 3 h), dried, and extracted with $CHCl_3$–water (1 mL × 1 mL). The $CHCl_3$ layer was dried, acetylated, and analyzed by GC–MS.

3.8. Methylation analysis

A sample of the oligosaccharide (0.5 mg) was dissolved in Me_2SO (0.5 mL). Powdered NaOH was added (~20 mg), and the mixture was stirred for 30 min. Then 0.2 mL of MeI was added, the mixture was stirred for 30 min, and excess MeI was removed by an air stream. Water (5 mL) was added, and the methylated product was extracted with $CHCl_3$. The extract was washed with water three times and dried. It was then hydrolyzed (3 M TFA, 120 °C, 3 h), reduced with $NaBD_4$, acetylated, and analyzed on a Varian Saturn 2000 ion-trap GS–MS instrument.

3.9. Mild hydrolysis of the LPS

The LPS (30 mg) was treated with 2% HOAc at 100 °C for 3 h. The precipitate was removed by centrifugation, and soluble products were separated by gel chromatography on a Sephadex G50 column (2.5 × 80 cm) in pyridine–HOAc buffer (4 mL pyridine and 10 mL HOAc in 1 L of water) to give one oligosaccharide product. It was further purified by anion-exchange chromatography on a Hitrap Q column (Amersham) in water to 1 M NaCl gradient (water for the first 20 min, then linear gradient to 1 M NaCl over 1 h) with UV detection at 220 nm.

3.10. Partial deacylation of the LPS

LPS (30 mg) was treated with anhyd hydrazine (3 mL) for 1 h at 50 °C. The solution was poured into acetone (200 mL), and the precipitate of LPS-Hy was dissolved in water and freeze-dried.

3.11. LPS Dephosphorylation

LPS (20 mg) was vortexed with 2 mL of 48% HF and was then kept overnight at 5 °C. The sample was then diluted with water (100 mL), dialyzed and lyophilized.

3.12. Alkaline deacylation

LPS or dephosphorylated LPS (15 mg) was heated at 120 °C for 4 h in 4 M KOH (2 mL) containing a small amount of $NaBH_4$, cooled, and treated with 2 M HCl. The precipitate was removed by centrifugation, and the products were separated on a Sephadex G50 SF gel with monitoring by a Waters differential refractometer. The products were further separated on a Carbopac PA1 (250 × 9 mm) column using a gradient from 10% to 100% of 1 M AcONa in 0.1 M NaOH at 3 mL/min over 1 h. Oligosaccharides were desalted by gel chromatography on a Sephadex G-15 column (1.6 × 50 cm).

3.13. N-Acetylation

OS 4 was dissolved in water (2 mL), solid $NaHCO_3$ was added until saturation, and then 0.05 mL of Ac_2O was added with fast stirring. After 30 min, the product was desalted by cationite Dowex 50WX8-200 (H^+ form) and freeze-dried.

3.14. Determination of the absolute configuration of the monosaccharides and glyceric acid

(*S*)-2-Butanol (0.5 mL) was added to OS 3, and the mixture was cooled in dry ice. Then 0.05 mL of AcCl was added, and the mixture was heated in a closed tube with occasional shaking for 3 h at 100 °C. The solvent was removed under an air stream; the products were acetylated by Ac_2O–Py, and then analyzed by GC–MS.

Acknowledgments

This work was supported by funding from the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council of Canada (NSERC) to DRM. The authors thank the National Research Council (NRC) mass spectrometry group, and in particular Dr. Jianjun Li for recording the mass spectra.

References

1. Raetz, C. R. H.; Whitfield, C. *Ann. Rev. Biochem.* **2002**, *71*, 635–700.
2. Whitfield, C. *Trends Microbiol.* **1995**, *3*, 178–185.
3. Linkous, D. A.; Oliver, J. D. *FEMS Microbiol. Lett.* **1999**, *174*, 207–214.
4. Strom, M. S.; Paranjpye, R. N. *Microbiol. Infect.* **2000**, *2*, 177–188.
5. Gulig, P. A.; Bourdage, K. L.; Starks, A. M. *J. Microbiol.* **2005**, *43 Spec No*, 118–131.
6. Chakraborty, S.; Nair, G. B.; Shinoda, S. *Rev. Environ. Health* **1997**, *12*, 63–80.
7. Cdcp *J. Am. Med. Assoc.* **1996**, *276*, 937–938.
8. Chan, W. L.; Chan, C. H.; Chan, T. Y. *Trans. R. Soc. Trop. Med. Hyg.* **1999**, *93*, 174.
9. Horre, R.; Becker, S.; Marklein, G.; Shimada, T.; Stephan, R.; Steuer, K.; Bierhoff, E.; Schaal, K. P. *Infection* **1998**, *26*, 399–401.
10. Kim, S. J.; Kim, B. C.; Kim, D. C.; Kim, M. K.; Cho, K. H.; Seo, J. J.; Shin, J. H. *Clin. Microbiol. Infect.* **2003**, *9*, 568–571.
11. Serrano-Jaen, L.; Vega-Lopez, F. *Br. J. Dermatol.* **2000**, *142*, 386–387.
12. Wise, K. A.; Newton, P. J. *Pathology* **1992**, *24*, 121–122.
13. Biosca, E. G.; Llorens, H.; Garay, E.; Amaro, C. *Infect. Immun.* **1993**, *61*, 1611–1618.
14. Collado, R.; Fouz, B.; Sanjuan, E.; Amaro, C. *Dis. Aquat. Organ.* **2000**, *43*, 91–101.
15. Marco-Noales, E.; Milan, M.; Fouz, B.; Sanjuan, E.; Amaro, C. *Appl. Environ. Microbiol.* **2001**, *67*, 4717–4725.
16. Bisharat, N.; Amaro, C.; Fouz, B.; Llorens, A.; Cohen, D. I. *Microbiology* **2007**, *153*, 847–856.
17. Colodner, R.; Raz, R.; Meir, I.; Lazarovich, T.; Lerner, L.; Kopelowitz, J.; Keness, Y.; Sakran, W.; Ken-Dror, S.; Bisharat, N. *J. Clin. Microbiol.* **2004**, *42*, 4137–4140.
18. Zuppardo, A. B.; Depaola, A.; Bowers, J. C.; Schully, K. L.; Gooch, J. A.; Siebeling, R. J. *J. Food Prot.* **2001**, *64*, 1172–1177.
19. Amaro, C.; Fouz, B.; Biosca, E. G.; Marco-Noales, E.; Collado, R. *Infect. Immun.* **1997**, *65*, 2475–2479.
20. Chae, M. R.; Park, B. H.; Kim, J. S.; Rho, H. W.; Park, J. W.; Kim, H. R. *Microbiol. Immunol.* **2000**, *44*, 335–340.
21. Powell, J. L.; Wright, A. C.; Wasserman, S. S.; Hone, D. M.; Morris, J. G., Jr. *Infect. Immun.* **1997**, *65*, 3713–3718.
22. Mcpherson, V. L.; Watts, J. A.; Simpson, L. M.; Oliver, J. D. *Microbios* **1991**, *67*, 141–149.
23. Waldor, M. K.; Colwell, R.; Mekalanos, J. J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11388–11392.
24. Abeyrathne, P. D.; Daniels, C.; Poon, K. K.; Matewish, M. J.; Lam, J. S. *J. Bacteriol.* **2005**, *187*, 3002–3012.
25. Westman, E. L.; Preston, A.; Field, R. A.; Lam, J. S. *J. Bacteriol.* **2008**, *190*, 6060–6069.
26. Korenevsky, A. A.; Vinogradov, E.; Gorby, Y.; Beveridge, T. J. *Appl. Environ. Microbiol.* **2002**, *68*, 4653–4657.
27. Kovach, M. E.; Shaffer, M. D.; Peterson, K. M. *Microbiology* **1996**, *142*, 2165–2174.
28. Kropinski, A. M.; Lewis, V.; Berry, D. *J. Bacteriol.* **1987**, *169*, 1960–1966.
29. Poole, K.; Braun, V. *J. Bacteriol.* **1988**, *170*, 5146–5152.
30. Tso, M. D.; Dooley, J. S. *J. Med. Microbiol.* **1995**, *42*, 32–38.
31. Amaro, C.; Biosca, E. G.; Fouz, B.; Garay, E. *Curr. Microbiol.* **1992**, *25*, 99–104.
32. Bahrani, K.; Oliver, J. D. *Biochem. Cell Biol.* **1990**, *68*, 547–551.
33. Bahrani, K. F.; Oliver, J. D. *Microbios* **1991**, *66*, 83–93.
34. Karch, H.; Leying, H.; Opferkuch, W. *FEMS Microbiol. Lett.* **1984**, *22*, 193–196.
35. Preston, M. A.; Penner, J. L. *Infect. Immun.* **1987**, *55*, 1806–1812.

36. Biosca, E. G.; Amaro, C.; Larsen, J. L.; Pedersen, K. *Appl. Environ. Microbiol.* **1997**, *63*, 1460–1466.
37. Iguchi, T.; Kondo, S.; Hisatsune, K. *Microbiol. Immunol.* **1989**, *33*, 833–841.
38. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
39. Hitchcock, P. J.; Brown, T. M. *J. Bacteriol.* **1983**, *154*, 269–277.
40. Fomsgaard, A.; Freudenberg, M. A.; Galanos, C. *J. Clin. Microbiol.* **1990**, *28*, 2627–2631.
41. Westphal, O.; Jann, K. *Methods Carbohydr. Chem.* **1965**, *5*, 83–91.