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The structure of the carbohydrate backbone of the core–lipid A region of the lipopolysaccharide from *Proteus vulgaris* serotype O25

Evgeny Vinogradov [a,*], Maciej Cedzynski [b], Antoni Rozalski [c], Andrzej Ziolkowski [b], Anna Swierzko [b]

[a] *Institute for Biological Sciences, National Research Council, 100 Sussex Drive, Ottawa, Ont., Canada K1A 0R6*
[b] *Microbiology and Virology Centre, Polish Academy of Science, Lodowa 106, PL-93232 Lodz, Poland*
[c] *Institute of Microbiology and Immunology, University of Lodz, Banacha 12/16, PL-90234 Lodz, Poland*

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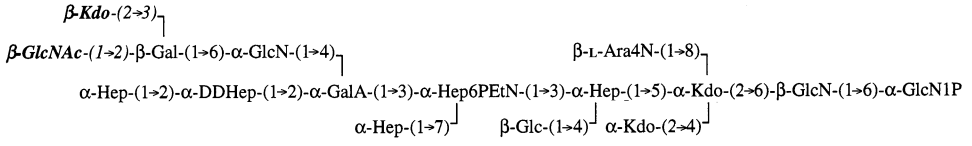
Abstract

The following structure of the lipid A–core region of the lipopolysaccharide (LPS) from *Proteus vulgaris* serotype O25 was determined by using NMR and chemical analysis of the core oligosaccharide, obtained by mild acid hydrolysis of LPS, of the products of alkaline deacylation of the LPS, and of the products of LPS deamination:



Terminal residues of β-GlcNAc and β-Kdo (indicated by bold italics) are present alternatively in approximately 3:2 amount, leaving no unsubstituted β-Gal. All sugars are in the pyranose form, α-Hep is the residue of L-*glycero*-α-D-*manno*-Hep, α-DDHep is the residue of D-*glycero*-α-D-*manno*-Hep.

Keywords: Lipopolysaccharide; *Proteus*; Core

1. Introduction

Lipopolysaccharides (LPS) are components of the outer membrane of Gram negative bacteria. LPS is responsible for a number of biological properties of bacteria, particularly endotoxicity, immunogenicity and virulence. Although the lipid part of LPS is responsible for its toxic properties, a contribution from the polysaccharide part of LPS to the toxicity is also postulated [1–3]. *Proteus vulgaris* O25 LPS was shown to have unusual biological properties, which are thought to be dependent on the polysaccharide structure: an induction of anaphylactoid reaction in muramyldipeptide-primed mice causing lymph node enlargement and strong anticomplementary and adjuvant activity [4]. Similar features have

Abbreviations: LPS, lipopolysaccharide; Hep, L-*glycero*-D-*manno*-heptose; DD-Hep, D-*glycero*-D-*manno*-heptose; GalA, galacturonic acid; Kdo, 3-deoxy-D-*manno*-octulosonic acid; P, phosphate; PEtN, 2-aminoethylphosphate; Ara4N, 4-amino-4-deoxy-L-arabinose; anh-Man, 2,5-anhydromannose.

* Corresponding author. Tel.: +1-613-9900397; fax: +1-613-9529092.

E-mail address: evguenii.vinogradov@nrc.ca (E. Vinogradov).

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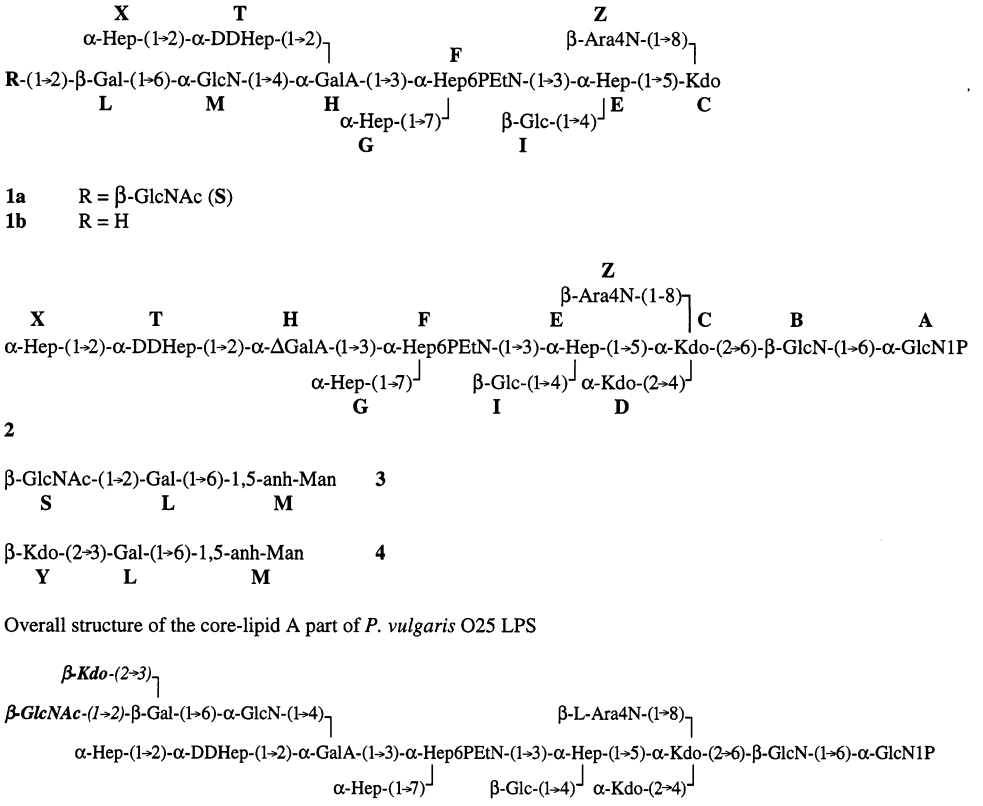

Scheme 1. Structures of the isolated oligosaccharides and proposed structure of the core–lipid A carbohydrate backbone of the LPS from *P. vulgaris* O25. All sugars are in the pyranose form except Kdo in **1** and anh-Man in **3** and **4**. Bold italics indicate non-stoichometric substituents.

been demonstrated so far for only a few LPSs, particularly for LPS from *Klebsiella pneumoniae* O3 [4–6]. Here we present the structure of the carbohydrate backbone of the core–lipid A region of *P. vulgaris* O25 LPS. The structure of the repeating unit of the O-specific polysaccharide from *P. vulgaris* O25 consisting of glucosamine, galactosamine, rhamnose, ribose and 3-[(*R*)-1-carboxyethyl]-D-glucose was determined previously [7].

2. Experimental

Bacterial strains and lipopolysaccharide isolation.—*P. vulgaris* O25 (PrK 48/57) originated from The Czech National Type Culture Collection (Prague, Czech Republic). Bacteria were cultivated (18 h, 37 °C) in normal broth (BTL, Lodz, Poland). Lipopolysaccharide was obtained by the procedure of Westphal [8] and purified by DNase, RNase (Sigma Chemical Co., St. Louis, MO, USA) and proteinase K (Boehringer Mannheim GmbH, Germany) treatment and repeated runs in a preparative ultracentrifuge.

NMR spectroscopy, general methods, and preparation of oligosaccharides **1** *and* **2**.—These were performed as described in Ref. [9].

Preparation of the oligosaccharides **3** *and* **4**.—LPS (200 mg) was dissolved in water (20 mL), 100 mg of $NaNO_2$ and 1 mL of AcOH were added, after 3 h at 25 °C the lipid-containing part of the LPS was removed by ultracentrifugation (55,000 rpm), and the supernatant was separated by gel chromatography on Sephadex G50S. Target oligosaccharides partially coeluted with salt and were additionally desalted on a Sephadex G15 column, yield 10 mg.

3. Results and discussion

Mild acid hydrolysis of *P. vulgaris* O25 LPS with 2% AcOH, followed by the separation of products by gel chromatography gave the core oligosaccharide **1**. Complete O,N-deacylation

of LPS by treatment with 4 M NaOH and separation of the products by gel chromatography and HPAEC gave one major oligosaccharide **2**. Deamination of the LPS with $NaNO_2$–AcOH in water gave, after desalting, a mixture of oligosaccharides **3** and **4**, which were analyzed without further separation.

The monosaccharide composition of all oligosaccharides was determined by conventional monosaccharide analysis (GLC of alditol acetates or methyl glycoside acetates). Absolute configurations (GLC of the acetates of 2-butylglycosides) were determined for D-Gal and D-GlcNAc in oligosaccharides **3** and **4** and for heptose residues in oligosaccharide **1**. Methylation analysis using the Ciucanu method [10] was performed for $NaBH_4$ reduced oligosaccharides **1**, **3**, and **4** and gave results consistent with the structures presented in Scheme 1.

Interpretation of NMR spectra of all oligosaccharides (Tables 1 and 2, Fig. 1) led to the structures presented in Scheme 1. NMR data for the oligosaccharide **2** are not presented because **2** was shown to be identical to the previously described product from *P. penneri* 16 (compound **2a** in Ref. [11]) by NMR, MS, and HPAEC. Product **1** had different Kdo variants at the reducing end, and contained two structures, **1a** and **1b**, differing by the presence of the terminal residue of β-GlcNAc S. The identity of monosaccharides was determined on the basis of their vicinal coupling constants (which were in agreement with the expected values), the intraresidual NOEs, and carbon-13 chemical shifts. The discrimination between L-*glycero*-D-*manno*- and D-*glycero*-D-*manno*-heptoses was achieved on the basis of the position of the C-6 signal [12,13]. The β-anomeric configuration of the Kdo residue in **4** follows from the low-field position of the signal of its H-3a proton at 2.46 ppm, whereas for α anomer this signal should be at about 2.1 ppm; all other proton signals have chemical shifts close to that of Kdo methyl β-glycoside and not of the α

Table 1
^{1}H NMR data

Unit, compound	H-1	H-2 (3a)	H-3 (3e)	H-4	H-5	H-6 (a)	H-7a (6b)	H-8a (7b)	H-8b
C, **1**		1.82	2.20	4.11	4.18			3.63	
E, **1**	5.08	4.07	4.13	4.29	4.13	4.13	3.72	3.72	
	5.17		3.98						
	5.27		4.01						
F, **1**	5.32	4.13	4.02	4.02	3.76	4.73	3.70	3.90	
	5.35	4.15	4.01			4.71	3.86	4.00	
G, **1**	4.91	3.98	3.87	3.87	3.66	4.02	3.71	3.71	
	4.94	3.97	3.81	3.81	3.60	4.05			
H, **1**	5.47	4.06	4.23	4.44	4.48				
I, **1**	4.56	3.30	3.50	3.38	3.38	3.74	3.87		
	4.60	3.30	3.52	3.38	3.40	3.77	3.88		
L, **1a**	4.47	3.71	3.69	3.85	3.69	3.73	3.77		
L, **1b**	4.43	3.54	3.66	3.92					
L, **3**	4.49	3.67	3.68	3.86	3.65	3.71	3.75		
L, **4**	4.47	3.57	3.79	4.13	3.69	3.73	3.73		
M, **1**	5.17	3.30	3.90	3.89	4.40	3.97	4.13		
M, **3**	5.07	3.75	4.17	4.18	4.03	3.83	4.08		
M, **4**	5.07	3.75	4.17	4.18	4.03	3.77	4.08		
S, **1a**	4.83	3.75	3.56	3.43	3.43	3.77	3.93		
S, **3**	4.81	3.81	3.54	3.42	3.43	3.78	3.92		
T, **1**	5.27	3.94	3.98	3.77	3.94	4.01	3.68	3.82	
X, **1**	5.05	4.04	3.84	3.84	3.68	4.00	3.74	3.74	
Y, **4**		1.87	2.46	3.76	3.95	3.58	3.88	3.71	3.74
Z, **1**	5.00	3.74	4.16	3.79	3.76	4.11			
EtN	4.18	3.30							
Nac		2.03							

Table 2
^{13}C NMR data (ppm)

Unit, compound	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
C, **1**			35.6	67.0	75.9			69.8
E, **1**	101.1	70.8	75.9	74.8	72.2	70.7	63.7	
	101.2		74.8					
	99.5							
F, **1**	102.1	70.4	79.9	66.3	73.2	74.3	68.4	
						73.0	66.8	
G, **1**	101.1	71.0	71.6	67.3	72.5	70.0	64.0	
	100.4		71.9					
H, **1**	99.2	72.7	67.7	80.2	72.4	176.0		
L, **1a**	103.0	78.8	74.7	70.0	76.2	62.1		
L, **1b**	104.4	71.9	73.7	69.8				
L, **3**	102.9	79.5	74.4	70.1	76.0	62.0		
L, **4**	104.0	70.4	78.8	69.7	75.7	62.0		
M, **1**	97.1	55.1	70.4	69.7	72.4	68.9		
M, **3**	90.6	85.3	78.4	78.4	77.5	82.6	70.1	
M, **4**	90.6	85.3	78.4	78.4	77.5	82.6	70.6	
I, **1**	103.6	74.9	76.4	70.7	77.1	62.3		
S, **1a**	102.5	57.0	74.8	71.0	77.0	62.1		
S, **3**	102.7	57.2	74.9	71.0	77.1	62.0		
T, **1**	96.2	80.7	70.9	68.3	74.6	72.7	63.1	
X, **1**	103.2	71.1	71.5	67.3	73.5	70.6	64.3	
Y, **4**	175.1	103.8	35.9	68.5	66.4	74.8	70.1	65.4
Z, **1**	99.3	69.0	66.6	53.0	59.1			
EtN	63.2	41.2						
Nac	175.9	23.2						

anomer [14]. From ^{13}C NMR data the position of the C-6 signal of Kdo Y at 74.8 ppm also indicates the β configuration [15].

The monosaccharide sequence was assigned on the basis of the interresidual NOEs and long range C–H correlations (HMBC spectra) and confirmed by ^{13}C NMR spectral data (Table 2). All aldoses gave NOE and HMBC correlations from H-1 over glycoside bonds, and intraresidual HMBC correlations to C-3 and C-5. Kdo residues gave HMBC correlations from H-3 to C-1 and C-2, but not over glycoside bonds. The position of the Kdo residue in oligosaccharide **4** was determined on the basis of the high chemical shift of C-3 of β-Gal L (78.8 ppm) and confirmed by methylation analysis. The ~3:2 ratio of residues S:Y follows from integral intensities of the signals in the ^1H NMR spectrum of the mixture of **3** and **4**.

Oligosaccharide **1** contained an ethanolamine phosphate substituent at O-6 of heptose residue F (^{31}P signals at 0.3 ppm).

The structure of oligosaccharides **1a**,**b** was confirmed by negative mode ESMS (Fig. 2). Compounds **1a** and **1b** gave $[M-2]^{2-}$ and $[M-3]^{3-}$ peaks of compound **1a**, corresponding to molecular masses of 2317.8 and 2299.4 (calculated mass $C_{82}H_{141}N_4O_{69}P_1 =$ 2318.0 for intact Kdo and $C_{82}H_{139}N_4O_{68}P_1$, 2300.0 for anhydro Kdo), and peaks of compound **1b** corresponding to molecular masses of 2113.8 and 2096.0 (calculated mass $C_{74}H_{128}O_{64}N_3P_1$ 2114.8 for intact Kdo and $C_{74}H_{126}N_3O_{63}P_1$ 2096.8 for anhydro Kdo), as well as signals of the sodium salts of the corresponding compounds.

ESMS of the oligosaccharide mixture after alkaline deacylation of the LPS showed the presence of one major compound with a mass of 2395.3 (calculated mass for **2**: $C_{82}H_{140}N_4O_{72}P_2$ 2395.9); the same peak gave isolated compound **2**.

Negative mode ESMS of the mixture of **3** and **4** contained $[M-1]^-$ ions of both compounds at m/z 526.2 and 561.1 (calculated

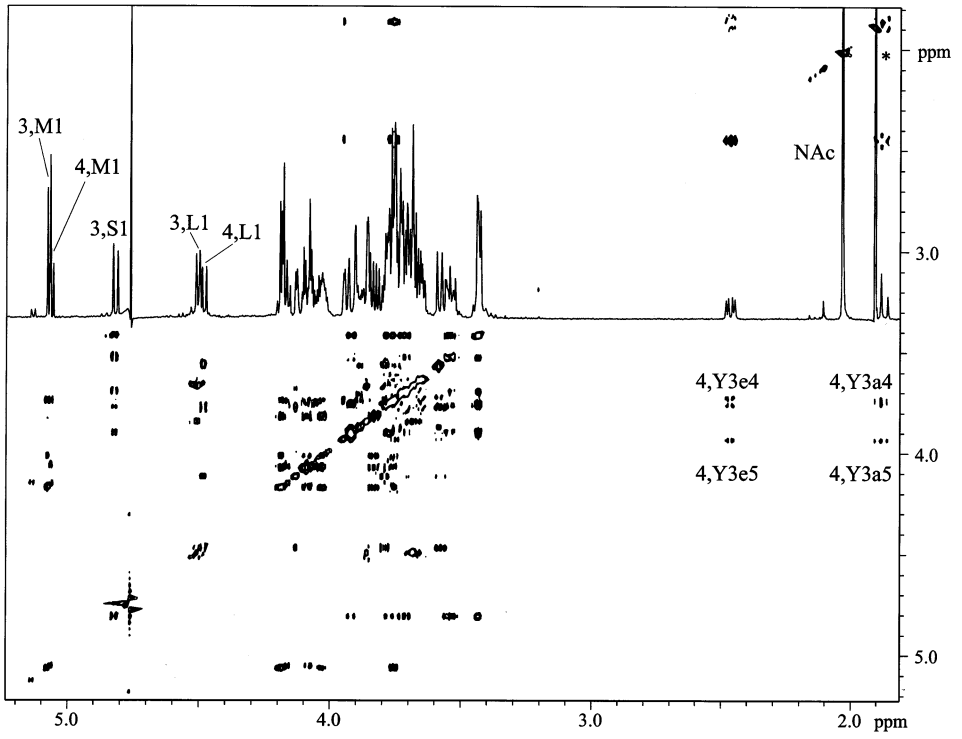

Fig. 1. ^{1}H and TOCSY spectra of the mixture of oligosaccharides **3** and **4**. Signals are described by product number, residue letter, and proton number. * peak belongs to the acetic acid from the gel chromatography buffer.

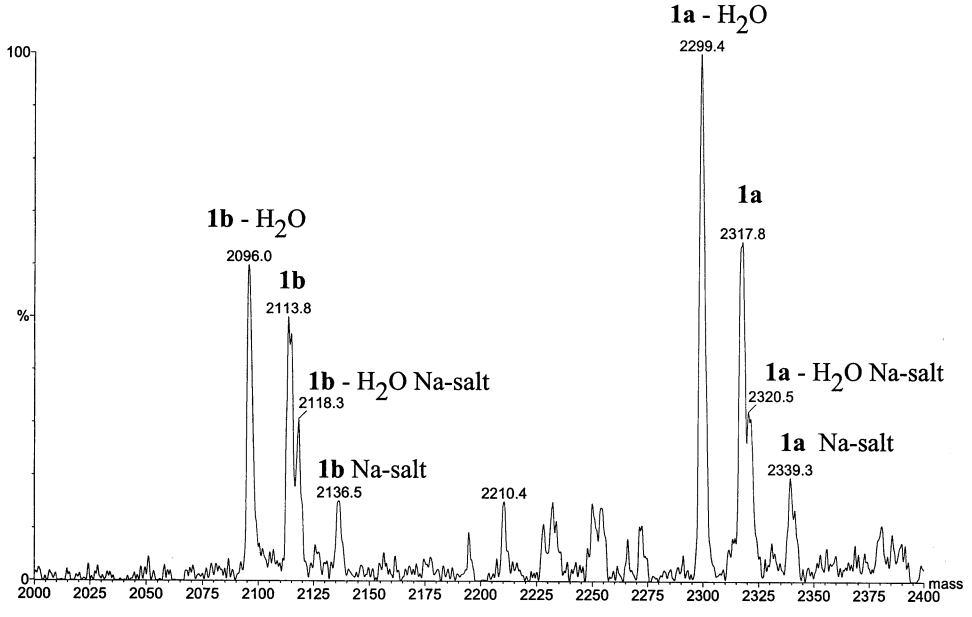

Fig. 2. Transformed negative mode ESMS spectrum of the core fraction from *P. vulgaris* O25 LPS.

exact mass for non-hydrated **3**: $C_{20}H_{33}NO_{15}$ 527.2; for hydrated **4**: $C_{20}H_{34}O_{18}$ 562.2). NMR spectra showed the presence of only the hydrated aldehyde group of anhydro mannose in both oligosaccharides, but in mass spectra signals of both forms are sometimes observed, with different intensity.

The absence of a phosphate group at O-4 of glucosamine B in the LPS deacylation products may indicate that this position in the LPS was substituted by glycosyl phosphate, which eliminates under the conditions of alkaline deacylation, as found in *Proteus* R45/1959 strain [16].

The structure of the inner region of the core part of *P. vulgaris* O25 LPS, including a α-Hep-(1→2)-α-DD-Hep- substituent at O2 of α-GalA, is nearly identical (differing by various degree of the presence of Ara4N) to the structures from *P. vulgaris* O2 and *P. penneri* 16 [11,13]. *P. vulgaris* O25 LPS does not contain the spermidine or putrescine found in the strains having a β-GalA residue on Hep G [9,17]. The presence of a Kdo residue at the nonreducing end of the core is unique for *P. vulgaris* O25 LPS. The question of whether this terminal structure influences biological activity of the whole LPS in the induction of anaphylactoid reaction needs further investigation.

Acknowledgements

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